Novel Cellulases through culture-dependent and metagenomics based approaches: Discovery and Characterization

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Ph.D. Thesis under the Faculty of Environmental Studies

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Certificate

*This is to certify that research work presented in the thesis entitled "**Novel Cellulases through culture - dependent and metagenomics based approaches: Discovery and Characterization**" is based on the original work done by Ms. Asha P. under my guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the doctoral committee have been incorporated in the thesis.*

Cochin-682016
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Declaration

I hereby do declare that the work presented in this thesis entitled "**Novel Cellulases through culture-dependent and metagenomics based approaches: Discovery and Characterization"** is based on the original work done by me under the guidance of Dr. I. S. Bright Singh, UGC BSR Faculty, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Cochin-682016 **Asha P.**
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Asha P.

Contents

Chapter 1

GENERAL INTRODUCTION

Contents

1.1 Lignocellulosic-Biofuel: Bio-sustainable alternative to conventional-petroleum derived fuels

Current global energy demand is mostly fulfilled by the use of fossil fuel resources such as coal, oil, petroleum and natural gases, which account for about 79% of total energy use (European Commission, 2006). Long term exploitation of these resources significantly contributed to the emission of greenhouse gases (GHG) to biosphere. Moreover, exhaustion of fossil fuel resources and the rising demand for energy have instigated the scientific

community to explore the possibility of utilizing sustainable and alternative forms of renewable energy such as bio- based energy from biomass resources. These offer solutions to GHG emissions, energy security issues reinforcing rural, agricultural and industrial economy.

The term "bio-refinery" was primarily established by National Renewable Energy Laboratory (NREL) during 1990, for the integrated production of fuels, chemicals, power, food and feed products from biomass resources. The same principle of petroleum refineries which generate crude oil based products can be applied to bio-refineries as well for sustainable and eco-friendly energy production (Kamm and Kamm, 2004). The future of bio-refinery is reliant on the production of second generation biofuels from waste biomass feedstock, whereas first generation biofuel has been from food and feed resources. This has not been a successful venture as the production cost has been prohibitive (Banerjee et al., 2010). It is anticipated that this situation can be overcome by using less expensive substrates such as lignocellulosic wastes and by applying hyper cellulase producing microbes for an overall cost effective bioprocess (Asha et al., 2016). The enzymes produced through such bioconversion processes would serve efficient bio-catalytic enzyme cocktails as it was particularly induced by the diverse cellulose components present in the native lignocellulosic biomass (Kovacs et al., 2009).

Hydrolytic enzymes like cellulases and xylanases convert cellulosic biomass to sugars that can be further fermented by various microbes to biofuels and other value-added products. The high cost of the biomass-conversion enzymes remains a major caveat to their commercial applications in biofuel industry and the problem could be addressed by improving the efficiency of already known enzymes, identifying new and more competitive

enzymes and formulating tailored enzyme blends for complete lignocellulose degradation (Merino and Cherry, 2007).

1.1.1 Lignocellulosic biomass

Lignocellulosic materials consist of cellulose (40–60%), hemicelluloses (20–30%) and lignin (15–30%) and a low amount of other polymeric materials such as starch, pectin, proteins, minerals, ash etc (Kuhad et al., 1997) and are being considered as organic carbon rich feed stock material for bio fuel production. Chemical structure of different components of lignocellulose is shown in Fig.1.

Lignin is a highly complex, aromatic biopolymer with a molecular weight of 10,000 Da giving rigidity to the plant cell wall (Mielens, 2001). It originates from the free-radical polymerization of three precursor molecules such as p-hydroxycinnamyl alcohol, 4-hydroxy-3-methoxycinnamyl alcohol and 3, 5-dimethoxy-4-hydroxycinnamyl alcohol (Ramos, 2003; Lee et al., 2008). Classically, lignin is characterized by its aromatic groups (p-hydroxybenzyl, guaiacyl, or syringyl) which differ in their degree of methoxylation (Ramos, 2003). Lignin's resistance to chemicals and enzymes, coupled with its ability to crosslink with structural polysaccharides such as cellulose and hemicelluloses. Similarly, hydrogen bonding between cellulose chains makes it more compact, inhibiting the bonding of the molecules that must occur in the hydrolytic breaking of the glycosidic linkages. In addition, lignin hindrance to cellulose hydrolysis necessitates a higher enzyme loading rate because this lignin-adsorption to cellulose resulted in non-productive enzyme attachment and confines the accessibility of cellulose to cellulase (Chen et al., 2006) Moreover the polyphenolic compounds generated during lignin degradation deactivate the cellulolytic enzyme performance.

Hemicellulose is a low polymerized-heterogeneous glucan with a short side chain of two or more monosaccharides. It contains the sugar units of mainly xylose, glucose, mannose, arabinose, galactose and their various derivatives. It forms covalent bonds with lignin and an ester linkage with acetyl units and hydroxylcinnamic acids.

Cellulose is a linear polymer of approximately 12,000 residues of D-glucose units linked through β-1, 4-linkages with a degree of polymerization ranging from 2,000 to 25,000 (Kuhad et al., 1997). The intra and intermolecular hydrogen bonds confer cellulose molecule a more rigid, insoluble, crystalline structure and natural cellulose molecules are structurally heterogeneous with amorphous and highly ordered crystalline structures (Iqbal et al., 2011; Taherzadeh and Karimi, 2008). The crystalline regions are highly resistant to enzymic attack and to most organic solvents. Native cellulose molecules exist as bundles which aggregated together in the form of micro-fibrillar structure. In addition to the crystalline and amorphous regions, cellulose fibers contain various types of irregularities, such as kinks or twists of the microfibrils, or voids such as surface micropores, large pits, and capillaries (Blouin et al., 1970; Cowling et al., 1975; Fan et al., 1980)

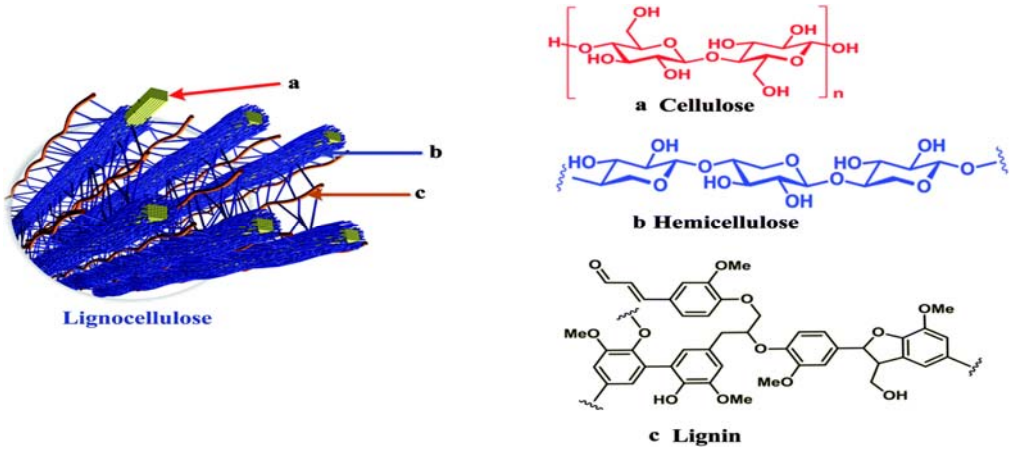

Fig.1. The structure and composition of lignocellulosic biomass (adapted from Sebayang et al., 2016)

Efficiency of cellulose hydrolysis towards cellulose is attributed to pretreatment of lignocelluloses, which has usually been related to the formation of surface openings or internal slits, voids, or spaces by the removal of other cell wall components, enhancing the direct physical contact between the enzymes and the substrate (Hammel et al., 2002). For instance, an acid pretreatment that removes hemicellulose to permit more cellulase-cellulose interaction, coupled with a way to physically disrupt the crystallinity of the cellulose structure, would greatly improve the enzyme digestibility of the biomass. Accordingly, Bertran and Dale (1985) showed that the lower the initial crystallinity of cellulose, the higher the extent of conversion to soluble sugars. Recent studies evaluating the selective lignin removal from wood cell walls by ionic liquids clearly demonstrated a direct correlation among the percent of lignin removal from the cell walls and the increased digestibility of the remaining material by cellulases along with reduced amounts of crystalline cellulose (Lee et al., 2009). This fact is in close agreement with previous studies by Ramos et al. (1992), where an 80% of reduction in lignin helped to a six fold lower enzyme loading to obtain the same degree of cellulose conversion. According to Silverstein et al. (2007), alkaline pretreatment of lingo-cellulosic materials can increase the cell wall porosity through delignification, breaking down some ester bonds cross-linking lignin and xylan, and causing swelling of microfibrils. The characterization of the cell wall pores (i.e., size and size distribution) has been proposed as a means to predict the reactivity of the substrate to enzymatic hydrolysis (Mooney et al., 1998; Sanjuán et al., 2001; Jeoh et al., 2008). Past seminal work performed by Grethlein (1985), Ishizawa et al. (2007) and Walker and Wilson (1991) showed that dilute acid pretreatment of lignocellulosic materials provided increased porosity as well as surface area of the substrate which in turn resulted in increased biomass conversion; however the results also indicated that porosity may not be the unique variable.

The pre-treatment should: (1) maximize the enzymatic digestibility of the pre-treated material, (2) minimize the loss of sugars, (3) optimize the production of by-products, (4) minimize capital and operating costs, and (5) be effective on multiple feed-stocks (Holtzapple and Humphrey, 1984). As the plant cell wall is naturally impermeable to cellulases, pretreatment with acids and bases was required to solubilize the hemicellulose and/or the lignin and, thus, increase the porosity of the polymer (Gould, 1984; Schell et al., 1991). Physical pre-treatments and biological pretreatments with fungi and bacteria that promote selective degradation of the lignin and hemicellulose represent another option, but the biodegradation rates are usually very slow (Eriksson et al. 1980). Therefore, pre-treatments that combine physical and chemical principles usually represent the best options to fractionate the lignocellulosic biomass (Ramos, 2003).

1.1.2 Structure of cellulose

Cellulose produced by plants usually exists within a matrix of other polymers primarily hemicellulose, lignin, pectin and other substances, forming the so-called lignocellulosic biomass, while microbial cellulose is quite pure, has a higher water content, and consists of long chains. It is a carbohydrate polymer with formula $(C_6H_{10}O_5)_n$, consisting of a linear chain of several hundred to over ten thousand 1,4-β-D-glucose units linked through acetal functions between the equatorial -OH group of C_4 and the C_1 carbon atom (Jagtap and Rao, 2005). Molecular chains arrange regularly in the cell wall structure to form protofibrils, which further form microfibrils, and then the microfibrils form fiber fines. Hemicellulose fills between protofibrils. Lignin and hemicellulose wrapped around the microfibrils, and a chemical connection exists between lignin and hemicellulose. Thus, in the cell wall, cellulose constitutes a cellulose skeleton in the form of microfibrils. Lignin and hemicelluloses are cross-linked by a covalent bond to form a three-

dimensional structure, enwrapping microfibrils inside. Microfibril regions of compact and dense interchain hydrogen and van der walls bonding form insoluble crystalline cellulose, whereas the hydrolysable amorphous region shows a lesser degree of crystallinity and therefore more prone to microbial degradation. In short, crystallinity and surface area play an important role in cellulose hydrolysis.

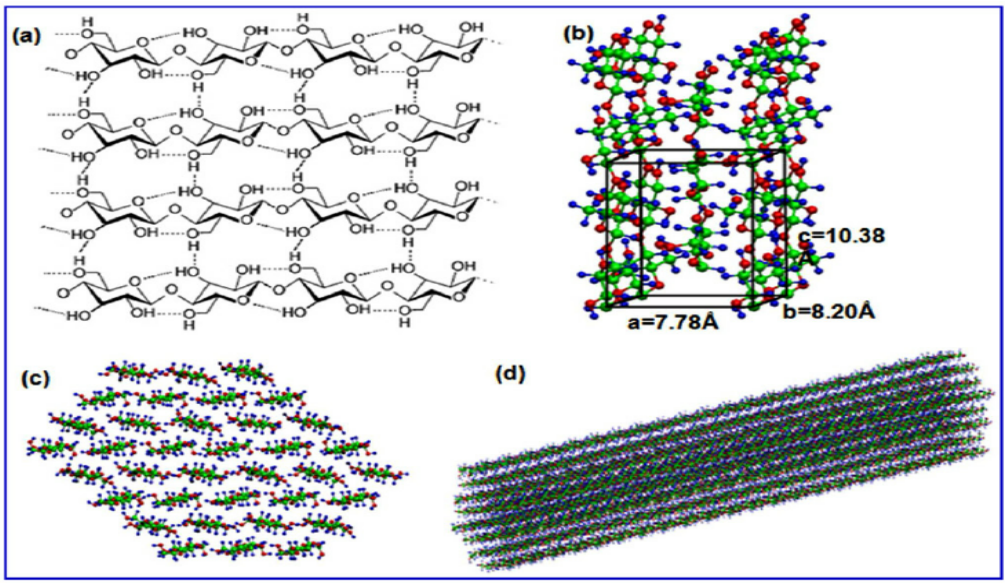

Fig.2. (a) The network arrangement of hydrogen bonds in cellulose microfibrils; (b) Crystalline structure of a unit cell of cellulose; (c) Cross-sectional view and (d) 3D view of a cellulose microfibril bundle (Adapted from Zhang et al., 2015).

1.2 Hyrolytic enzymes for cellulose hydrolysis

1.2.1 The cellulase system: catalytic players for complete cellulose hydrolysis

Cellulases are the major catalytic players for cellulose hydrolysis and are produced mainly by fungi, bacteria and actinomycetes as well as other organisms like plants and animals and even some protozoa, mollusks, and

nematodes (Watanabe and Tokuda, 2001). A variety of different kinds of cellulose-degrading enzymes are known, with different structure and mode of action. Classically, the cellulolytic enzyme complex breaks down cellulose polymer into glucose units and involves the following types of enzymes such as endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.74), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Li et al., 2010).

Endoglucanase or endo-β-1,4-glucan-4-glucanohydrolases (EC 3.2.1.4) is the cellulolytic enzyme responsible for initiating the cellulose hydrolysis. It randomly hydrolyze internal β-1,4-glycosidic linkages in soluble and amorphous regions of the cellulose and produce new chain ends and ultimately resulted in reduction of degree of polymerization (DP) along with increased reducing sugar liberation. It is also known as Cx-enzyme due to the differentiation of cellulose components in different strains and "CMCase", since carboxy methyl cellulose (CMC) is used as the substrate for enzyme activity assays. Fungal endoglucanases generally have a catalytic module with or without a carbohydrate-binding module (CBM), while bacterial endoglucanases possess multiple catalytic modules, CBMs and other domains.

Exoglucanases (EC 3.2.1.74) hydrolyze cellulose chains by processively removing cellobiose units either from the non-reducing and reducing chain ends. Exoglucanases consists of cellobiohydrolase (CBH) and cellodextrinases. The CBH or β-1,4-D-glucan cellobiohydrolase (EC 3.2.1.91), known to hydrolyze only the terminal non-reducing ends and oligosaccharides with degree of polymerization (DP) > 3 releasing cellobiose as the major end product, although there are few reports available on its hydrolysis at reducing ends. Cellodextrinases capable of hydrolysing water soluble cellodextrins is little reported. The cellobiohydrolase or CBH is responsible for "amorphogenesis", a phenomenon not yet fully elucidated, but known to involve in the physical

breakdown and segmentation of the cellulose substrate, resulting in increased rate of cellulose hydrolysis and thereby making amorphous regions of crystalline cellulose polymer more exposed to cellulase action. The CBH also can be divided into two types: CBH I, which hydrolyzes terminal reducing ends (R), whereas CBH II hydrolyzes terminal non-reducing ends (NR) and both are inhibited by its hydrolysis product, cellobiose (Zhang and Lynd, 2004). Fungal cellobiohydrolases possess a catalytic module with or without CBM, while bacterial cellobiohydrolases may possess more than one catalytic module, more than one CBM of different families, and other functionally known or unknown domains.

β-Glucosidases (EC 3.2.1.21) or β-glucoside glucohydrolase convert cellobiose and other soluble cellodextrins with DP <7 into glucose. It fastens the cellulose hydrolysis by preventing the accumulation of cellobiose, which otherwise may act as feedback inhibitor (Castro and Pereira , 2010; Leite et al., 2007and 2008). The β-glucosidase secretion accounts for only 1%, compared to other cellulase enzyme components such as endoglucanases and exoglucanases of complete cellulase enzyme system.

Majority of the glucan chains in cellulose-microfibrils are inaccessible for enzymatic attack and glycosidic bonds hydrolysed through the endoglucanase action can readily be reformed due to the stable orientation of the glucan chains. This confirmed that the synergistic action of endo and exoglucanase are the primary requirement for the hydrolysis of crystalline cellulose. Successive or in concert action (processive hydrolysis) of exoglucanase quickly removes cellobiose units from the newly generated ends through endoglucanase action, thus preventing the reformation of glucosidic bonds. Endocellulases can be processive or non-processive types. Both exo- and endoglucanases are repressed by cellobiose and the efficiency of

processive cellulases contributes to the rate-limiting step of cellulose degradation (Lee, 1997). A schematic representation of a typical cellulose hydrolysis process with its different component cellulase enzymes are shown in Fig. 3.

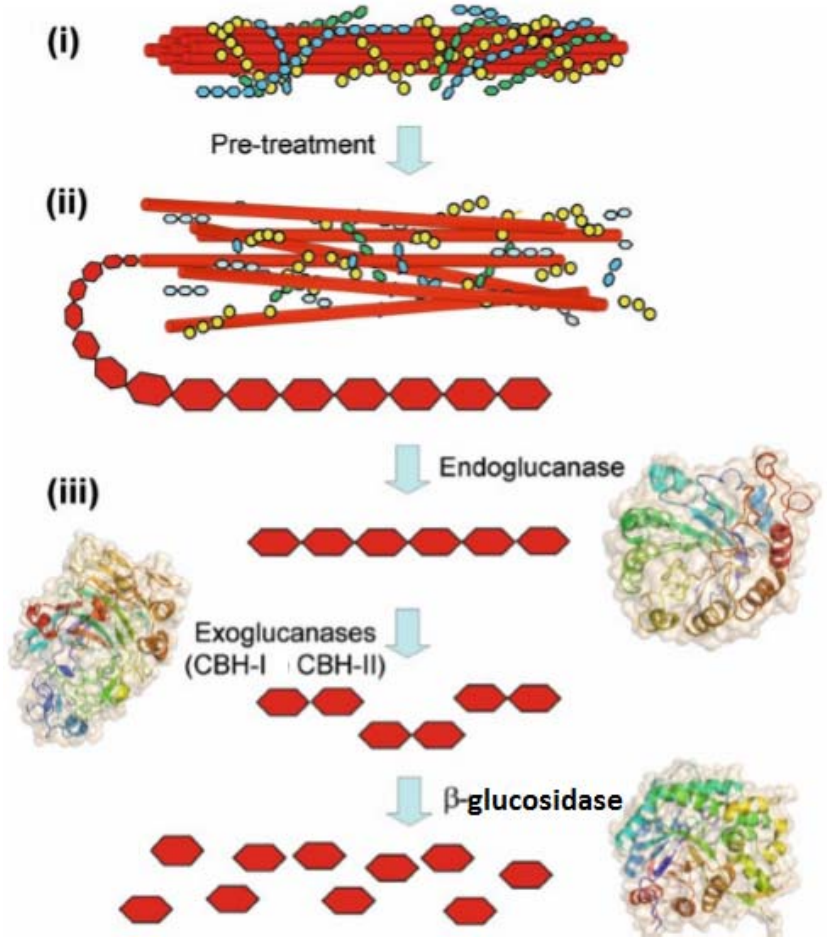

Fig.3. Schematic representation of a typical cellulose hydrolysis process. The 3D structures shown here are the catalytic domain (CCD) of endocellulase 'Cel5' of *Thermobifida fusca* (PDB code 2CKS) (Berglund et al., 2007), the exocellulase 'Cel7A' cellobiohydrolase (CBHI) from *Hypocrea jecorina* (PDB code 5CEL) (Divne et al., 1998) and the 'Cel1'β -glucosidase A (BglA) from *Thermotoga maritima* (PDB code 1OD0) (Zechel et al., 2003). Picture adapted from Ward (2011).

Cellulase is an inducible enzyme complex with cellulose, cellobiose, lactose, sophrose etc act as visible inducers and its production is mainly regulated by activation and repression mechanism (Bisaria and Mishra, 1989). Most of the earlier studies in *Trichoderma reseei* showed that sophrose is required at very low concentrations whereas other inducers needed at high concentrations. Cellulase expression is also subjected to catabolite repression by glucose or cellobiose assumed to take place at translation levels. Exoglucanases are primarily inhibited by cellobiose, whereas glucose inhibits mainly β-glucosidase. Other factors that may also be contributing to the decrease in the hydrolysis rate include enzyme adsorptive loss to lignin, thermal, mechanical, and /or chemical enzyme deactivation.

1.2.2 Cellulase producing microorganisms- A few of the many

Cellulases are preferentially produced from wood decaying basidiomycetes as *Phanerochaete chrysosporium* (Igarashi et al., 2008), *Trametes versicolor* (Lahjouji et al., 2007), *T. hirsute* (Nozaki et al., 2007), *Ceriporiopsis subvermispora* (Magalhães et al., 2006), *Agaricus bisporus* (De Groot et al., 1998), *Volvariell volvacea* (Ding et al., 2006) and *Schizophyllum commune* (Henrissat et al., 1989). However, as reported by Henrissat et al. (1989), the bacteria *Clostridium thermocellum*, *Erwinia chrysanthemi*, *Bacillus* spp., *Cellulomonas fimi*, *Streptomyces* spp., *Pseudomonas fluorescens* and *Cryptococcus albidus* also produce cellulases in high levels. Filamentous fungi as *Hypocrea jecorina* (*Trichoderma reesei*) (Schmoll and Kubicek, 2003) and *Aspergillus* spp. (Dedavid and Silva, 2009) and *Penicillium* (Camassola and Dillon, 2007) are also reported as cellulase producers.

Phanerochaete chysosporium produces oxidative enzymes that also play an important function in the cellulose degradation. The flavoprotein

cellobiose:quinine oxidoreductase (EC 1.1.5.1) reduces the quinines and phenoxy radicals which were produced during lignin degradation, in the presence of cellobiose, which is oxidized to cellobio-α-lactone. The flavoheme protein, cellobiose oxidase (EC 1.1.3.25) is another enzyme which oxidizes cellobiose and higher cellodextrins into their corresponding onic acids using molecular oxygen. Both enzymes have been shown to bind at microcrystalline cellulose while retaining enzyme activity. Wood and Wood (1992) have suggested that cellobiose:quinine oxidoreductase is not an independent enzyme, but a breakdown product of cellulose oxidase instead, which acts as a link within the lignin–cellulose degradation processes.

1.2.3 Cellulase system- Other minor components

Besides to endo, exo and β-glucosidases, other enzymes involved in cellulose degradation are cellobiose dehydrogenase (CDH), cellobiose quinone oxidoreductase (CBQ), phosphorylase and cellulosome. Cellobiose dehydrogenase also known as cellobiose oxidase (CBO) (EC 1.1.99.18) is a heme flavoprotein and is mainly synthesized by filamentous fungi that can hydrolyze lignocellullose. It can oxidize cellobiose and cellooligosaccharide to corresponding lactone. Flavin adenine dinucleotide (FAD) and heme are its prosthetic group. CDH has a broad substrate range, including cellotriose, cellooligosaccharide with DP of 5, lactose and cellulose. CDH is also known as multi-dehydrogenase involved in a fenton reaction to generate a hydroxyl radical (• OH), thus plays an oxidation and degradation role in the cellulose degradation process (Fang et al., 2010). Cellobiose quinone oxidoreductase (CBQ) is a flavoprotein, containing only FAD, and its other physicochemical properties are similar to CDH except that CBQ cannot reduce cytochrome C. CDH and CBQ were isolated from *Phanerochaete chrysosporium* first, and later were found in *Sporotrichum thermophile*, *Aspergillus niger*, *Sclerotium*

rolfsii, *Fomes annosus*, and *Moniliaceae* spp. Another component enzyme such as phosphorylase make cellobiose or cellulose phosphorylated and finally metabolized. Cellulosome is the major enzyme complex present in anaerobic bacterium such as *Clostridium thermocellum*, which synergistically hydrolyse different forms of cellulose.

In addition, the expansins found in growing tissues of plant and plant-parasitic roundworm *Globodera rostochiensis* (Cosgrove, 2000), the swollenin produced by *T. reesei* (Saloheimo et al., 2002), and the Gt factor produced by *Gloeophyllum trabeum* (Wang and Gao, 2003) were to improve the hydrolysis efficiency when used as additives for hydrolysis of cellulose. Moreover, Han and Chen (2007) found that the crude cell wall extracts of corn stover could influence the cellulase activity due to the presence of a "Zea h" corn stover protein.

1.3 Diversity of cellulase system

Hild´en and Johansson (2004) have given much attention in reporting the diversity observed among the cellulase systems produced from both bacterial and fungal organisms. Aerobic bacteria and fungi secrete their cellulases as non-complexed systems in free form as most of the filamentous cellulolytic fungi have hyphal extensions which penetrate the cellulosic substrate and often present their confined activities in the cellulose polymer .This contain cellulose binding domains (CBD) which transport the catalytic domain in direct binding with the insoluble cellulose (Zhang and Lynd, 2004; Carvalho et al., 2004). Cellulases deficient in CBD had confirmed reduced performance against crystalline cellulose though retaining the capability to depolymerise soluble cellulosic substrates (Bolam et al., 1998 ; Klyosov, 1990) Most of the anaerobic microorganisms structurally organize their cellulases in multi-enzyme complex form, recognized as cellulosome, which is

linked to the cell surface (Bayer et al., 1998; Schwarz, 2001). A cellulosome consists of different cellulases arranged on a non-catalytic scaffolding protein which helps in strongly binding to the surface of cellulose-substrate (Schwarz , 2001), while showing enough flexibility to bind crystalline cellulose as well, thus the diffusional flow of the formed sugar is not allowed (Shoham et al ., 1999). Cellulases from fungi have a two-domain structure with one catalytic domain, and one cellulose binding domain, that are connected by a flexible linker containing 6-59 amino acid residues, rich in proline and glycosylated hydroxyamino acid residues (Gilkes et al., 1988; Bhat and Bhat, 1997). CBM is usually present at the carboxy terminus or at the amino terminus of the catalytic module (Hill et al., 2006; Bhat and Bhat, 1997). However, there are also cellulases that lack cellulose binding domain. CBMs are responsible for increasing the binding affinity between cellulase and cellulose substrate, which further helps in higher cellulose conversion (Linder et al., 1995; Takashima et al., 2007). Soluble forms of cellulose such as carboxymethyl cellulose and amorphous cellulose do not require CBM whereas the crystalline cellulose requires CBM for hydrolysis (Rabinovich, 2002) and they are particularly important for the initiation and processivity of exoglucanases (Teeri et al., 1998).While researching the cellulose degradation model proposed by Reese et al. (1950), a probable additional non-catalytic role for CBMs in cellulose hydrolysis was suggested i.e., the “sloughing off” of cellulosic fragments from surface of cellulose (for example , cotton fibers), thereby enhancing cellulose hydrolysis (Din et al.,1994). The anaerobic cellulosomal cellulases consist of a dockerin domain that binds to cohesion modules of the scaffolding subunit. The dockerin–cohesin complex binds to enzyme by a flexible linker peptide to display catalytic domain. The

scaffolding also has a CBM to recognize and bind to cellulosic substrates (Bayer et al., 2004).

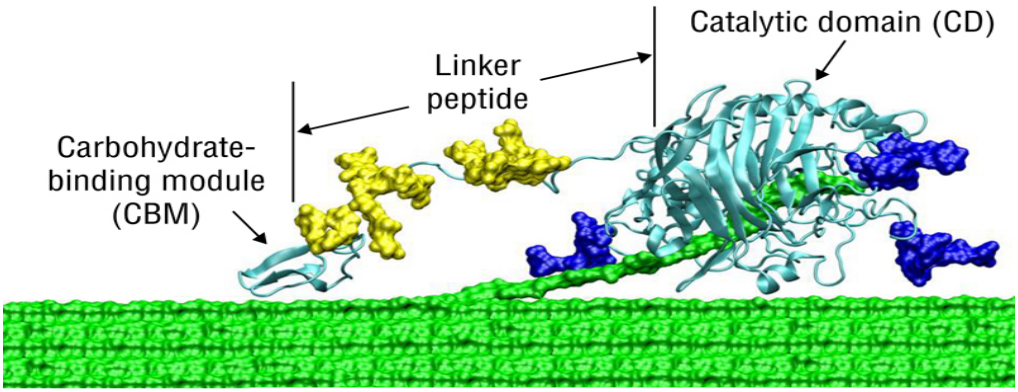

Fig.4. Model schematic representation of catalytic module, carbohydrate binding module (CBM) and a linker peptide in a cellulase enzyme. Picture credit: National Renewable Energy Laboratory.

1.4 Cellulase synergy

Cellulase systems exhibit a unique phenomenon of synergism in which the combined activities of the component enzyme subunits are greater than the sum of their individual activities. Previously, four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases, acting in the amorphous regions of cellulose polymer, provides reducing and non-reducing end for the action of CBH I and CBH II, respectively. (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains. (iii) synergy between exoglucanases and β-glucosidases that remove cellobiose and cellodextrins as end products thereby relieving catabolite repression and (iv) intra- synergy between catalytic domains(CDs) and CBMs (Din et al., 1994). A synergy model for cellulose hydrolysis is shown in Fig.5.

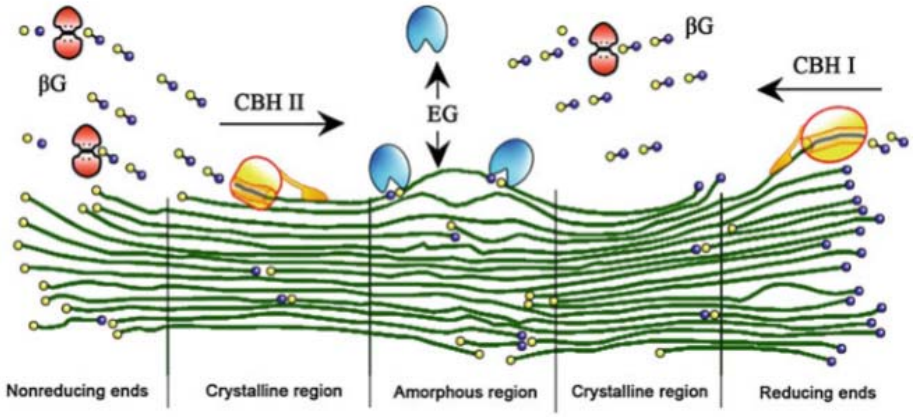

Fig.5. The synergy model for cellulose hydrolysis. CBH is cellobiohydrolases or exo-β-glucanases , EG is endo-β-glucanases and BG is β-glucosidases (adapted from Wang et al., 2012)

The presence of both exo and endoglucanase enzyme systems is a primary requirement for enhanced hydrolysis (synergism) of cellulose polymers to glucose monomers (Suga et al., 1975) even if the degradation rate for the endoenzyme was much higher. Medve et al (1994) investigated the adsorption and synergism during the hydrolysis of microcrystalline cellulose (Avicel) by CBH I and CBH II from *Trichoderma reesei*. When Avicel was hydrolyzed by increasing amounts of CBH I and /or CBH II, either alone or in formulated equimolar enzyme blends, a linear correlation was found between substrate conversion and the amount of adsorbed enzyme. The enzyme adsorption process however, was slow (30-90 min) to reach 95% of the equilibrium binding. Although more CBH I was adsorbed than CBH II, CBH I had a lower specific activity. Synergism between these cellobiohydrolases during the degradation of amorphous Avicel was at a maximum as a function of the total enzyme concentration. The two enzymes were shown to compete for the adsorption sites with stronger competition shown by CBH I (Medve et al., 1994). Fujii and Shimizu (1986) proposed a kinetic model for the hydrolysis of water-soluble cellulose derivatives by mixed endo and exo

enzyme systems. The authors supposed that at an early stage of the reaction, endo-enzymes split the substrate molecule in order to supply the newly formed non-reducing ends to exoenzymes until the molecular weight of the substrates reaches a low value. After that point, the reaction kinetics obeyed only the rate equation of the exo-enzymes reaction in which the reaction parameters change linearly with a decrease in the molecular weight of the substrates. In studies with soluble cellulose derivatives such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC), this critical molecular weight of the substrate, after which the endo-enzyme action can be ignored, was estimated to be 4000 Da. Synergism by these enzymes enhanced the early rate of the reaction. Visual evidence of the synergism between endo- and exo-glucanase was offered by White and Brown (1981), who used cellulose from *Acetobacter xylinum* as a model substrate for visualizing the action of cellulase enzymes from the fungus *Trichoderma reesei*. Scanning electron microscopy revealed that the enzymes initially bound to the cellulose ribbon produced by bacteria. Within 10 min, the ribbon was split along its long axis into bundles of microfibrils which were subsequently thinned until they were completely dissolved. Purified CBH I produced no visible change in the cellulose structure. Purified endoglucanase IV produced some splaying of cellulose ribbons into microfibril bundles. In both cases, whole ribbons were present even after 60 min of incubation, visually confirming the synergistic mode of action of these enzymes. In another study, the action of an exoglucanase, CBH I, and an endoglucanase, EG II- alone and in combination on cotton fibers were visualized using atomic force microscopy (Lee et al., 2000). The action of CBH I resulted in the appearance of distinct physical pathways or tracks along the length of the macrofibril, whereas the action of EG II resulted in shedding and smoothing of the fiber surface. In combination, their effect was

observed to be greatest when both enzymes were present simultaneously, again affirming their synergistic action on cellulose.

1.5 Complexed and Non-complexed cellulase systems

The cellulase hydrolytic machinery capable for cellulose degradation is widely distributed among fungi and bacteria (Klass, 1983). These multiple cellulase enzymes that are either exist as cell-free or cell-associated, known as Cellulase Systems (Bhat and Bhat, 1997; Lynd et al., 2002) and are classified into two major systems such as;

i) Non-complex systems are mainly found in filamentous fungi and actinomycetes, having the ability to penetrate complex cellulosic substrate through hyphal extensions and do not produce stable high molecular weight complex (Eriksson et al., 2002; Lynd et al., 2002). Such type of cellulase systems are present in filamentous fungi *Phanerocheate chrysosporium* (Broda et al., 1996; Lynd et al., 2002), *Trichoderma reesei* (Bhat and Bhat, 1997; Lynd et al., 2002), *Aspergillus niger* (De Vries and Visser, 2001), *Humicola insolens* (Schulein, 1997; Lynd et al., 2002), *Fusarium oxysporum* (Sulzenbacher et al., 1996; Bhat and Bhat, 1997) etc.

ii) Complex systems are stable high molecular weight enzyme complexes well anchored into the bacterial cell wall and are flexible enough to bind to the cellulose surface for efficient adsorption of products of cellulose hydrolysis (Bayer et al., 1998). The 'Complex Cellulase Enzyme System', also known as 'Cellulosomes', are mainly observed in anaerobic clostridia and many ruminal bacteria. In addition, cellulosome also has the ability to increase the uptake efficacy of these cellulose components by the host organisms (Bayer et al., 1998;

Schwarz, 2001; Eriksson et al., 2002; Lynd et al., 2002). The major differences between complexed and non-complexed cellulolytic systems are presented in the Fig.6.

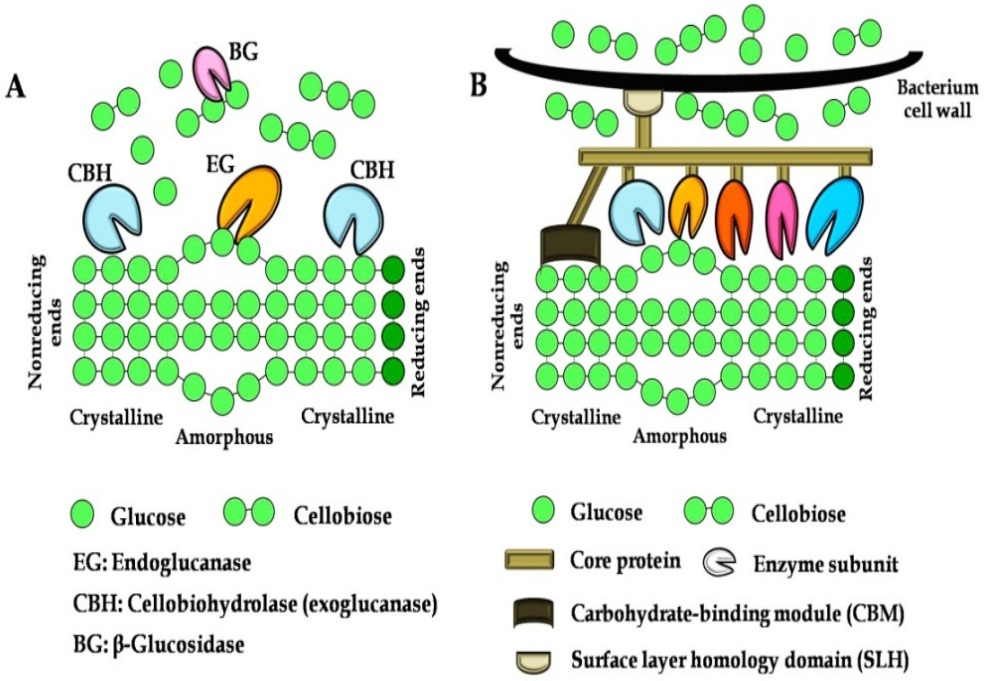

Fig.6. The difference between Non-complexed and complexed cellulase systems. Picture credit: Ratanakhanokchai et al., 2013.

The most widely studied cellulosome of the thermophilic anaerobic bacterium, *C. thermocellum* (Klass, 1983; Bhat and Bhat, 1997; Lynd et al., 2002) is composed of 14–18 complexed polypeptides containing several endoglucanases along with exoglucanases and xylanases. Additionally, it may contain polypeptides with no enzymatic activity, which helps in the cellulosome attachment to the cell surface and/or to the substrate (Klass, 1983). Cellulosomes are usually produced during the exponential phase of bacterial growth and secreted when the culture enters into stationary phase (Klass, 1983; Lynd et al., 2002) and are more capable for hydrolysing crystalline cellulose than amorphous cellulose. The higher potential of the

cellulosome has been attributed to the following factors such as the presence of heterogenous enzymatic activity, the maintenance of optimum synergism ratio between their catalytic domains and the proper spacing between the individual modules for enzyme-substrate binding. The cellulosome contains large non catalytic multimodular scaffoldin protein Cip A and includes nine cohesive domain, four hydrophilic X- modules, and a family III CBD. The Cip A scaffoldin is anchored to the bacterial cell surface through type II cohesion domains. Cip A consists of nine polypeptide domains, each with 146 residues which is separated by Pro- and Thr- rich peptide segments of 17 to 19 residues. These domains identify a catalytic module (22 residues) each of which display one of the activity as endoglucanases, cellobiohydrolases, β-glucosidases and xylanases of the cellulosome. Out of these 22 , at least 9 of this module exhibit endoglucanase activity and are referred as Cel A, Cel B, Cel D, Cel E, Cel F, Cel G, Cel H, Cel N, and Cel P, four of which exhibits exoglucanases activity such as Cbh A, Cel K, Cel O and Cel S), five of them exhibit hemicellulase activity; Xyn A, Xyn B, Xyn V, Xyn Y, and Xyn Z, 1 chitinase (Man A), and one lichenase activity (Lic B). These catalytic modules possess dockerin moieties that can bind with Cip A protein to assemble the cellulosome. Cip A and certain catalytic components of the cellulosome also contain CBM that bind the cellulosome to the surface of micro-crystalline cellulose (Lytle and Wu, 1998). The exoglucanase Cel S is a processive cellulase particularly active on microcrystalline or amorphous cellulose excluding carboxy methyl cellulose (CMC) (Lynd et al., 2002). Cellulosome are widely glycosylated on the scaffoldin moiety, which evidently protect the cellulosome against proteases and help the scaffoldin to recognize the cohesion- dockerin region (Bayer et al., 1998). The schematic representation of cellulosome is given in Fig.7. In addition, anaerobic fungi (almost six

genera and eighteen species) flourishing in gastrointestinal tracts of ruminants and non-ruminant herbivores also possess cellulosome or non-free cellulases (Wilson and wood, 1992; Bayer et al., 2004). Cellulose degraded by fungal cellulosome produces glucose as the end product, while bacterial cellulosome produces cellobiose as the major hydrolytic product that requires the supplementation of β-glucosidase to complete cellulose hydrolysis (Dijkerman et al., 1997).

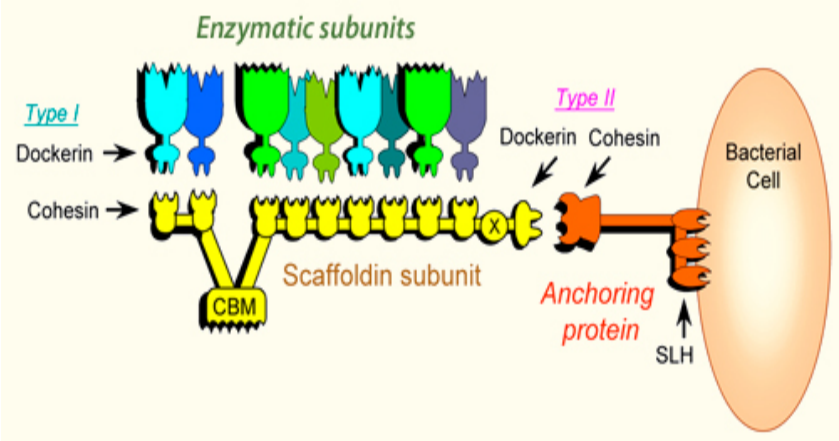

Fig.7. Cellulosome for complexed enzyme systems. Picture Credit: Cellusome from CAZYpedia

1.6 Cellulase classification – The CAZy family

Cellulases are glycoside hydrolases constantly updated in the Carbohydrate-Active Enzymes database (CAZy; http://www.cazy.org) (Cantarel et al., 2009) which cleave the bond between two carbohydrates or a carbohydrate moiety and another molecule. The Enzyme Classification System (EC) classifies glycoside hydrolases (EC 3.2.1.--) based on the substrate specificity. It is not based on the structural or evolutionary information and substrate preferences. Therefore, Henrissat et al. (1998) has pioneered the glycoside hydrolase family classification based on amino acid sequences, hydrophobicity plots, and reaction mechanisms (Henrissat, 1991; Henrissat

and Bairoch, 1993; Henrissat and Bairoch, 1996). For instance, β-glucosidases grouped in two glycoside hydrolase families (GHFs), 1 and 3, and the β-galactosidase (EC 3.2.1.23) in four families, 1, 2, 35, and 42. Even though this system explains enzyme relationships, it does not designate the natural substrate or function of the enzyme. Some glycoside hydrolase families contain enzymes with different substrate specificities; for example, family 5 contains cellulases, xylanases, and mannanases. This suggests divergent evolution of a basic fold at the active site to hold different substrates. Moreover, cellulases from several families showing convergent evolution, and thus from different folds with either an inverting or retaining mechanism, are found in the same microorganism. Moreover, Henrissat et al. (1998) have suggested a new type of nomenclature for glycoside hydrolases in which the first three letters assign the favoured substrate, the number represents the glycoside hydrolase family, and the following capital letter shows the order in which the enzymes were first reported. For instance, the cellulases such as CBHI, CBHII, and EGI of *Trichoderma reesei* are designated as Cel7A (CBHI), Cel6A (CBHII), and Cel6B (EGI). Based on the latest update (13 July 2010), glycoside hydrolases were categorized into 118 families, though 876 glycoside hydrolases have assigned to a "Non Classified-GHF", since some of them display distant homology to existing GH families to allocate a reliable GHF classification.

1.7 Mode of action / Reaction mechanism of cellulases

The mode of stereochemical hydrolysis of β-1,4- glycosidic bonds of cellulose by cellulases proceeds via either an retention (double-displacement catalytic mechanism) or an inversion (single-displacement catalytic mechanism) of anomeric configuration (Bhat and Bhat, 1997; Teeri, 1997; Coutinho and Henrissat, 1999). The Fig.8 depicts a representation of the two types of mechanisms hypothesized for cellulase enzymes.

a) Retaining Mechanism

The retaining mechanism or double displacement mechanism consists of two steps such as glycosylation and deglycosylation steps, which aid in retaining the β- configuration at the anomeric carbon C1 after hydrolysis. In the first step of glycosylation, one of the two carboxylic groups catalyse the substrate through an acid hydrolysis mechanism, while the second carboxylic group gives a nucleophilic attack on the anomeric carbon(C1) to form a covalently unstable enzyme - substrate intermediate and in the second deglycosylation step, the first carboxyl group deprotonates the water molecule for an incoming nucleophile, and a new glycosyl hydrolase group in case of transglycosylation, by yielding a proton from it. This activated nucleophile then hydrolyses the substrate-enzyme complex (Sandgren et al., 2004). During deglycosylation the substrate experiences a ring distortion within the enzyme active site so as to achieve a 'twisted boat' conformation (Mai et al., 2004). The retaining mechanism is similar to the reaction of lysozyme (Kelly et al., 1979).

b) Inverting Mechanism

The inverting enzymes proceeds through a single nucleophilic displacement mechanism, where the hydrolysis of a β-1, 4- glycosidic bond of the cellulose at anomeric carbon resulted in a product with an α- configuration. Inverting enzymes give a protonic assistance (from an Asp residue) to a glycosidic hydroxyl group and then a catalytic base deprotonates a water molecule for nucleophilic substitution at the anionic carbon (Beguin and Aubert, 1994; Davies and Henrissat, 1995; Sandgren et al., 2004), which in turn facilitates formation of glycosyl enzyme intermediate such as oxocarbonium ion-like transition states (Gilkes et al., 1988; Varrot et al., 2000). This single nucleophilic substitution give way to a product with opposite stereochemistry to the substrate, with the same mechanism as

observed in ß-amylase also. A detailed review of glycoside hydrolase - catalytic mechanism can also be found in several previous studies (Vasella, et al., 2002; Zechel and Withers, 2000; Zechel and Withers, 2001).

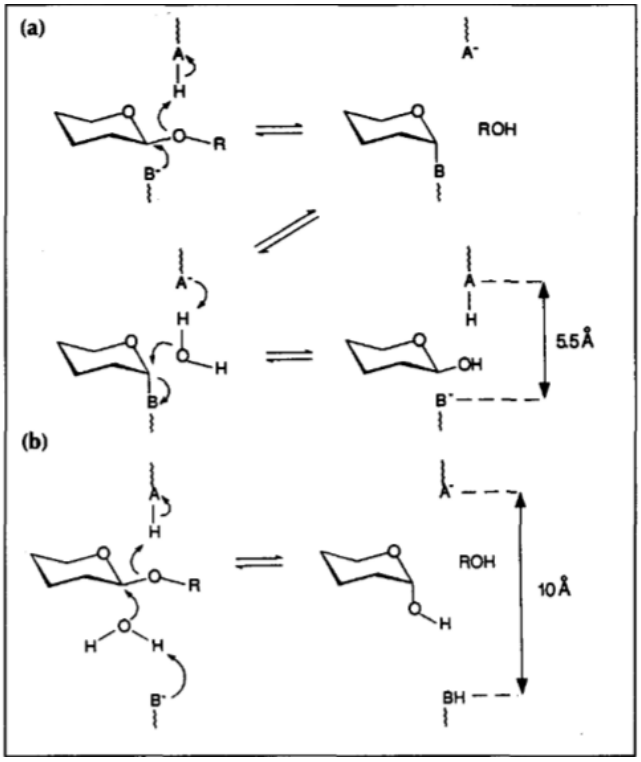

Fig.8. Retaining and inverting mechanism of Cellulase enzyme catalysis (a) The retaining mechanism in which the glycosidic oxygen is protonated by the AH, acid catalyst, and a nucleophilic substitution to a glycon departure is given by the base (B). The resulting glycosyl enzyme is cleaved by a water molecule and this second nucleophilic substitution at the anomeric carbon (C1) yields a product with the same stereochemistry as the substrate. (b) The inverting mechanism in which protonation of the glycosidic oxygen and the subsequent glycon removal is accompanied by a associated attack of a water molecule that is activated by the base residue (B). This single nucleophilic substitution yields a product with opposite stereochemistry to the substrate (Davies and Henrissat, 1995).

Cellulases are glycoside hydrolase (GH) enzymes that utilize the acid-base catalysis mechanism of hydolysis, with inversion or retention of the glucose anomeric configuration. There are three common types of cellulase

active sites. GHs with open (groove, cleft) active sites that typically exhibit endocellulolysis (endocellulases), binding anywhere along the length of the cellulose molecule and hydrolysing the β-1,4 glycosidic linkage. The other types are those with tunnel like active sites exhibiting exocellulolytic activity (cellobiohydrolses) binding ends of the cellulose molecule and producing short-chain oligosaccharides (Gray et al., 2006; Prasad et al., 2007; Balat et al., 2008; Sukharnikov et al., 2011). Another type of topology observed is pocket or crater. This topology is optimal for the binding of a saccharide non-reducing ends and is exhibited in monosaccharidases such as β-galactosidase, β-glucosidase, sialidase, neuraminidase, β-amylase etc (Davies and Henrissat, 1995).

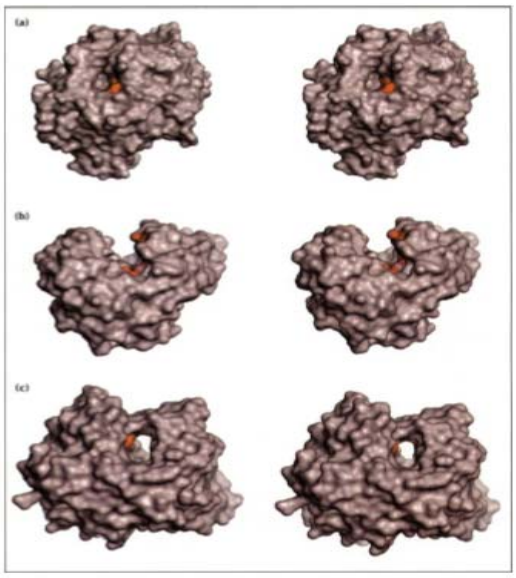

Fig.9. The three types of active site found in glycosyl hydrolases. (a) The pocket (glucoamylase from *A. awamori*). (b) The cleft (endoglucanase E2 from *T. fusca*). (c) The tunnel (cellobiohydrolase II from *T. reesei*). The proposed catalytic residues are shaded in red. Picture adapted from (Davies and Henrissat, 1995).

1.8 Structure of glycoside hydrolases

Present computational predictions of the proteins depends on the protein conserved regions to perform full-length sequence homology searches

or they should possess specific markers, such as distinctive protein domains and domain combinations, motifs and accessory proteins to obtain reliable predictions (Wuichet and Zhulin, 2010). Cellulase proteins that hydrolyse the β-1,4 glycoside bond using the mechanism of acid–base catalysis belong to at least eight distinct protein folds (Fig.10), which further classifieds into even more protein families (Cantarel et al., 2009), for example, cellulase Cel5E from *P. fluorescens* has an $(\beta/\alpha)_8$ fold belongs to GH 5 (Hall et al., 1995), Egl-257 from *Bacillus circulans* has an $(\alpha/\alpha)_6$ barrel fold of GH 8 (Hakamada et al., 2002) and *C. thermocellum* cel44a has a TIM-like barrel and β-sandwich domain fold, which belongs to GH 44 (Kitago et al., 2007). According to the updated version of CAZy database, cellulases are allocated into 11 or 13 CAZy families (Fontes and Gilbert, 2010; Cantarel et al., 2009), considered as representatives of a large class of nonhomologous isofunctional enzymes, which means that cellulases independent proteins catalyse the same biochemical reaction, but are unrelated in sequence and structure.

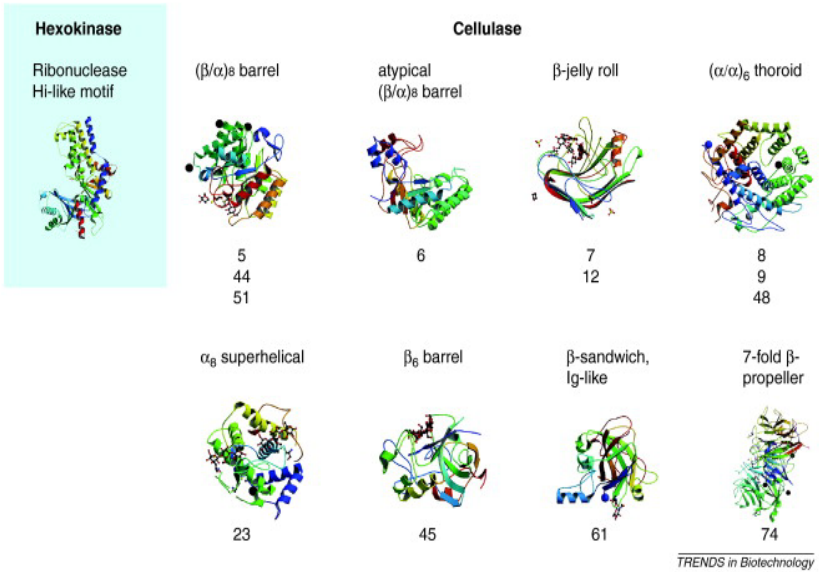

Fig.10. Structural conservation and diversity among cellulase glycoside hydrolases, their CAZy family classification are showed below structures Images from the RCSB PDB (www.pdb.org). Picture adapted from Sukharnikov et al., 2011.

The highly rich information provided by the GH structure analysis demonstrates that the substrate specificity and the mode of action of these enzymes are governed by fine and particular details of their three-dimensional structures rather than by their global architecture (Davies and Henrissat, 1995). Development of sampling in structure–functional space for cellulases and glycoside hydrolases is very important for rational protein engineering of exo- and endoglucanases, which depends on the accessibility of 3D structure of the enzyme and homologous proteins and relies on our understanding of their structure, dynamics, and structural elements which determine the enzymatic action techniques (Zhang et al., 2006). At present, only limited data on high-resolution structures is available for entire two-domain GH, mostly obtained for enzymes with short linker peptides (Fujimoto et al., 2000; Pell et al., 2004). In addition, no high-resolution structure of a single intact multidomain cellulase, consisting of the catalytic core, linker region, and CBM, has been determined, most probably hampered by the intrinsic flexibility and disorder of their long and extended linker polypeptides. Naturally, CBM and CCD cellulase domains function in a determined way, full-length structures of cellulases are essential for understanding its function. To overcome the problem, low-resolution structural analysis in solution using small-angle X-ray scattering or electron microscopy might be useful in providing important insights into cellulase tertiary and quaternary organization. Pioneering studies of intact *T. reesei* CBHI and CBHII by SAXS, which have been conducted at the end of 1980s, provided important insight into the tertiary organization of these two cellulase domains (Abuja et al. 1988a). The extended “tadpole” architecture of the exoglucanases has been described, but these early results were imperfect because of the lack of known

three-dimensional structure for the isolated cellulase domains, as well as the limitations of equipment and software at that time. Recently, modern SAXS studies of *H. insolens* cellulase Cel45 and chimera fusion enzyme Cel6A–Cel6B using synchrotron radiation have been done, revealing important, and much more precise, information on the tertiary structure of the enzymes and the conformational flexibility of the connecting linker region (Receveur et al., 2002; von Ossowski et al., 2003).

1.9 Cellulase market

The potential application studies for cellulases started in early 1980's, first in animal feed followed by food applications, textile, laundry as well as in the pulp and paper industries (Bhat, 2000). During the last three decades, the use of cellulases and hemicellulases has increased considerably, especially in textile, food, brewing and wine as well as in pulp and paper industries and bioethanol production (Godfrey and West, 1996, Saddler, 1993, Uhlig, 1998). Cellulases are currently the third largest industrial enzyme worldwide, by dollar volume and these enzymes account for approximately 20% of the world enzyme market (Mantyla et al., 1998).The world market for enzymes increased by nearly 1.45 billion dollars in 1995 to almost 3.7 million dollars in 2004 with growth forecast global demand of 6.5% per year until 2010. Currently, the technical industries, such as detergent, starch, textile, fuel alcohol, account for the majority of the total enzyme market, alongside those of food and feed industry, totalling only about 35% of the market. Nevertheless, cellulases will turn out to be the largest volume industrial enzyme, if ethanol, butanol, or some other fermentation product of sugars, produced from biomass by enzymes, becomes a major transportation fuel (Wilson, 2009).

The cost of enzyme accounts for a significant part of cellulosic ethanol production costs. β-glucosidase supplementation is essential since commercial cellulases often lack this enzyme. β-glucosidase reduces cellobiose to glucose thus minimizing the accumulation of cellobiose which in aqueous phase, inhibits endogluconase and cellobiohydrolase enzymes. This subsequently increases the costs of enzyme. Nevertheless, the cost of enzyme has gone down by nearly 20-fold in recent years. Recent US DOE grants to two major enzyme producers–Genencor and Novozyme was a major push toward reducing the cost of enzymes. In recent years, enzyme costs have gone down from $0.50 per gallon of ethanol to $0.10-$0.30 per gallon of ethanol. For the economic viability of cellulosic ethanol, the enzyme cost should be $0.045-0.09/gal. For corn-based ethanol plants, the enzyme cost is just around $0.03-0.04/gal. Thus, further cost reductions are still needed for the economic viability of lignocellulose-ethanol plants. Enzyme recycling may be adopted to hydrolyze multiple batches of substrate and may reduce the overall enzyme cost. One way to achieve this is by immobilizing enzymes in or onto an inert carrier. Consolidated bioprocessing (CBP) is another approach of lowering the enzyme cost. One study projects an enzyme cost of $0.042/gal for CBP compared to $0.189/gal for simultaneous saccharification and co-fermentation (Zhang and Lynd, 2008).

1.10 Metagenomics: Hunting for novel cellulase-bioprospects

For a long time the industries depend upon the very small percent (1%) of cultivable organisms present in nature. But, the biosphere is dominated by microorganisms that have not been studied yet (Amann et al., 1995; Handelsman et al., 1998). Recently, metagenomics has emerged as a new meadow of research which can explicate the genomes of the uncultured

microbes with the target to better comprehend global microbial ecology as well as to meet the biotechnological demands for novel enzymes and biomolecules (Rees et al., 2003; Schmeisser et al., 2007). Though the classical approach of screening a wide variety of microbes has yielded a few novel cellulolytic activities since a large proportion of microorganisms are not yet cultivable, the new approach of metagenomics provide better chance to identify and clone most efficient genes with novel properties from the environment and to express them in an appropriate secretory-expression system. Soil metagenome has been successful to study single genes (e.g., Cellulases), pathways (e.g., antibiotic synthesis), organisms (e.g., Archaea), and communities (e.g., acid mine drainage biofilm) as they contain several thousand species of microorganisms (Torsvik et al., 1990; Chatzinotas et al., 1998).

Mining the metagenomes of such microorganisms allows for the discovery of metabolites such as therapeutic compounds (MacNeil et al., 2001; Courtois et al., 2003; Seow et al., 1997), enzymes (Ferrer et al., 2005; Entcheva et al., 2001; Elend et al., 2006) but more specific to this study, cellulases (Healy et al., 1995; Voget et al., 2006; Pang et al., 2009). Therefore, this study will lead to innovations in the field paving the way for industrial development in cellulase production which will solve the issue of bio-fuel generation from agro-waste.

'Metagenomics' approach emerged in 1990's as a new field of research which can elucidate the genomes of the uncultured microbes to meet the biotechnological demands for novel enzymes and biomolecules. It describes both sequence based and functional analysis of the communal microbial genomes contained in an environmental sample (Handelsman, 1998). Other terms have been used to describe the metagenomics include "zoolibraries", "soil DNA libraries", "cDNA libraries", "recombinant environmental libraries",

"whole genome treasures" , "community genome" , "whole genome shotgun sequencing" etc. Metagenomic approach helps to uncover the hidden potentials of non-culturable microbes, and to better understand the global microbial ecology as well as to screen the industrial biotechnological demands for novel enzymes and biomolecules. Only 0.001–0.1% of the total microbes in sea water, 0.25% in freshwater, 0.25% in sediments and only 0.3% soil microorganisms were approximated to be cultivable under laboratory conditions (Amann, 1995). Recently reported reviews (Ward, 2006 and Xu, 2006) on the metagenomic studies have mostly improved due to the construction of efficient cloning vectors (e.g. bacterial artificial chromosomes or BACs, and yeast artificial chromosomes orYACs etc.), which allow cloning and expression of larger DNA fragments or genes. The idea of cloning DNA directly from environmental samples was first hypothesised by Pace (Pace et al., 1986), and in 1991, the first attempt was done in a phage vector (Schmidt et al., 1981). The next advance was the metagenomic library construction with total DNA extracted from the community organisms enriched on dried grasses. Surprisingly the first report was on the clones expressing cellulolytic activity were designated as zoolibraries, a term that has not been used commonly in the field (Healy et al., 1995). The present status to find novel cellulase genes for biomass refining industries derive from the importance of utilizing cellulose, most potent source for second-generation biofuel production. Metagenomics will enable the discovery of novel cellulosic enzymes with unique biocatalytic activities through metabolic engineering of enzyme complexes and novel pathways for cellulose hydrolysis, which ultimately resulted in the production of biofuels from cellulosic materials.

1.11 Sourcing and analysing microbial metagenome

The current research strategies employed in metagenomic gene discovery are reviewed here. This includes approaches to environmental nucleic acid extraction and purification, sequence independent gene discovery strategies *i.e.* environmental library construction and activity based screening as well as sequence dependent approaches such as gene-specific PCR.

Metagenome can be accessed from a broad range of environments to exploit the genetic potential of the microbial communities present in it. The studies included soil (Brennerova et al., 2009; Fan et al., 2011; Jiang et al., 2011; Lämmle et al 2007; Van Elsas et al 2008), sediment (Jeon et al. 2009; Parsley et al. 2010; Zanaroli et al. 2010), freshwater (Wexler et al. 2005), marine habitats (Breitbart et al., 2002; Martin-Cuadrado et al. 2007; Venter et al. 2004), and the guts of animals (Bao et al. 2011; Li et al. 2008; Wang et al. 2011). Moreover, extreme environments such as the Arctic (Jeon et al. 2009), glacial ice (Simon et al. 2009), acidic (Tyson et al. 2004), and hypersaline environments (Ferrer et al. 2005) as well as a hyperthermal ponds (Rhee et al. 2005) have been met by metagenomics-based studies. Some scientists look forward for a pre-cultivation step to improve environmental DNA quality. This reduces the overall diversity of the native microbial community but enhances the microorganisms capable of carrying out the target trait (Entcheva et al., 2001; Rhee et al., 2005; Elend et al., 2006). A general Schematic representation of strategies/methods involved in mining novel genes is depicted in Fig.11.

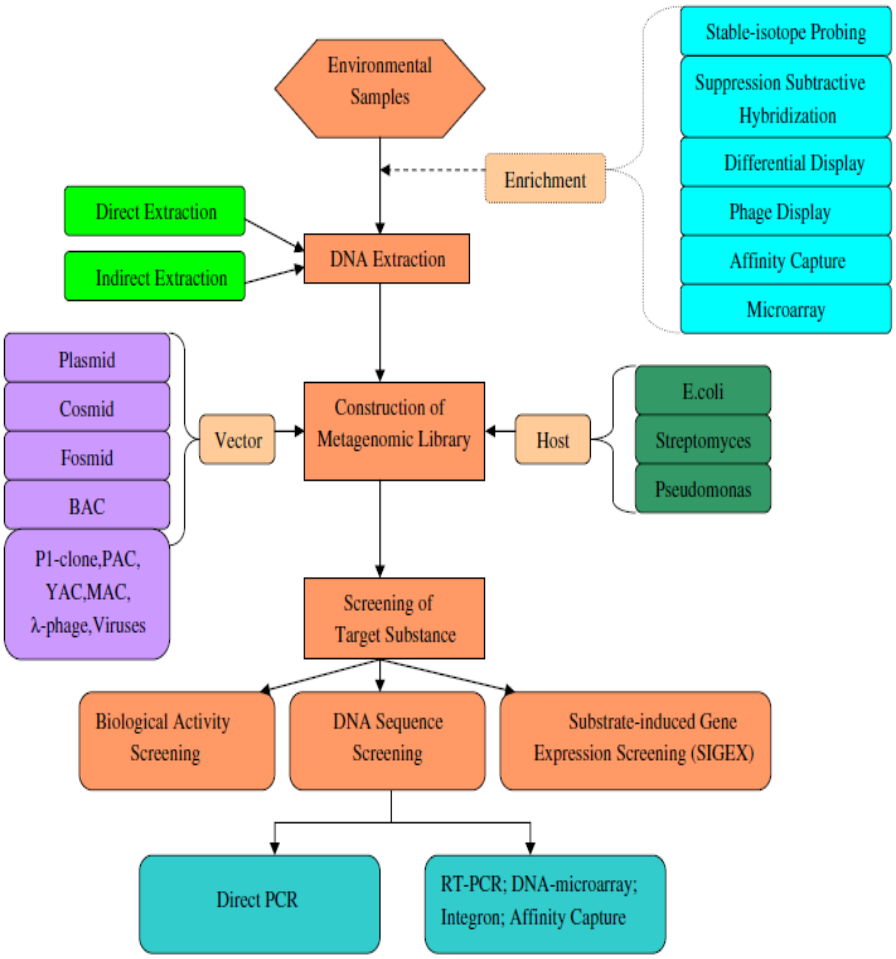

Fig.11. Schematic representation of general strategies involved in mining novel genes. Picture adapted from Xing et al., 2012.

Some of the previous pioneering work on 'ecological enhancement' or 'habitat biasing' increased the chances of finding useful target functions *in situ* in the total extracted metagenome. In a matter-of-fact, an environmental sample is biased towards specific groups of organisms by supplementing selective substrates or modifying its physico-chemical characteristics (Van Elsas et al., 2007) and the selection pressure for enrichment can be nutritional, physical and chemical (Entcheva et al., 2001). According to the report of Healy et al. (1995), a fourfold enrichment of cellulase genes were achieved

with a culture enriched on carboxymethylcellulose, suggesting the fact that culture enrichment will inevitably reduce some extent of microbial diversity by selecting fast growing specific culturable organisms. Alternatively, a mild selection pressure can be introduced so that presence of non enriched cultures is not lost totally. Different types of enrichment techniques such as stable isotope probing (SIP) (Radajewski et al., 2002), and 5-Bromo–2-deoxyuridine labelling, suppressive subtractive hybridization (SSH) (Galbraith e al., 2004), differential expression analysis (DEA) (Liang, 2002), phage display (Crameri and Suter, 1993) and affinity capture (Demidov et al., 2000) have been developed to increase the possibility of hitting the target gene (Cowan, 2005).

Stable isotope probing (SIP) provides the means whereby the genomes of specific metabolically active organisms in an environmental sample can be labelled and selectively isolated through density gradient centrifugation. This technology is based on the utilization of a specific labelled 'enrichment' substrate that causes the incorporation of heavier isotopes into the nucleic acid of the target microorganisms. The method thus enables the establishment of a direct link between identity and function (Chen and Murrell, 2010; Cupples, 2011; Dumont and Murrell, 2005; Radajewski et al., 2003; Uhlik et al., 2009, 2003). The major disadvantages of this technique include cross-feeding, high cost and recycling of the labelled substrate and the requirement for unnaturally high concentrations of labelled substrate. Another technical disadvantage is the difficulty in differentiation between labelled and unlabeled DNA i.e., unlabeled high G–C% DNA may have a density profile that come close to that of labelled low G–C% DNA (Buckley et al., 2007). But Chen and Murrell (2010) identified that in spite of such limitations; SIP is a very valuable tool to reduce sample complexity and enhances the hit rates of particular target genes (Chen and Murrell, 2010).

BrdUTP labelling provides an important tool to access the metabolically active organisms but it is less specific than SIP. Suppressive Subtraction Hybridisation (SSH) can identify genetic differences between closely related microorganisms and is therefore a powerful technique for specific gene enrichment. The method is carried out by ligating adaptors to the DNA populations to select DNA fragments unique to each DNA sample. Recently, it has been used to identify differences between two complex DNA samples isolated from bovine rumens (Galbraith et al., 2004). A more practical approach of differential expression technologies which rely on the isolation of mRNA to identify transcriptional differences in gene expression, suggested the possibility of comparing the expression profile of a metagenomic sample before and after exposure to a specific substrate or xenobiotic. Several other methods have been developed which include phage-display expression libraries provides a better practical approach of isolating a given DNA sequence by affinity selection of the surface-displayed protein to an immobilised ligand. 'Biopanning' involve repeated cycles of binding that will successively enrich the pool and individual clones are identified by DNA sequencing (Crameri and Sater, 1993). This method is efficient and agreeable to high-throughput screening, offering the potential to enrich even rare DNA sequences in the metagenome, however recent phage technology limits expression of proteins upto 50kDa. In the Affinity capture technique, oligonucleotides are covalently immobilised to a solid support can be used to affinity purify target genes, which further improved by using metagenomic mRNA or single-stranded DNA (Demidov et al., 2000). Recently microarrays used for high-throughput robotic screening for targeting multiple gene products (Wu et al., 2001). The economic availability of microarray technology is fastly decreasing, making this an increasingly captive choice.

1.11.1 Soil sampling and Community nucleic acid isolation

Sample processing is the preliminary and most crucial step in any metagenomics studies. Soils represent more or less structured environments contain mineral, organic and biotic fractions. Specifically, soil microbial communities are composed of bacteria, archaea and protists showing diversity in the cell wall characteristics and differing in their susceptibility to cell lysis (Kauffmann et al., 2004). The extracted DNA should be representative of all cells present in the sample and adequate amounts of high-purity DNA must be obtained for subsequent library production and sequencing. Sample processing requires specific protocols for each sample type as they vary in their physical and chemical characteristics. Although, various commercial kits are available for DNA isolation from environmental samples and many laboratories have developed their own optimised extraction methods depending on the purpose of metagenomic analysis (Frostegard et al., 1999; Krsek & Wellington, 1999; Miller et al., 1999). Soil is a particularly complex matrix containing many substances, such as humic acids, which can be co-extracted during DNA isolation. Physical separation and isolation of cells from the samples might be an important step to maximize the DNA recovery and avoid the contamination of co-extracted humic acids. Community nucleic acid isolation for metagenomic library construction can be roughly divided into two strategies: (1) “direct extraction”- microbial community DNA is directly isolated from the sample matrix and (2) “indirect extraction”-the microbial cells are first separated from the sample prior to cell lysis (Robe et al., 2003; Van Elsas et al., 2008). Both methods have their own advantages and disadvantages. Selection of an appropriate DNA extraction method depends upon the key

parameters such as yield, purity, fragment size, and representativeness of the microbial community. But practically it has been observed that the enhancement of one parameter will often have a negative impact on other factors. These extraction methods may result in either low yield DNA containing large DNA fragment sizes versus high yield small-fragment DNA. However the extraction protocol should have optimized for each sample depending on the parameters needed.

After a series of publications regarding the extraction methods based on the direct lysis strategy suggests that this does not require the isolation of microorganisms prior to cell lysis. The most important disadvantage of direct lysis methods is the co-recovery of humic and fulvic acids with the environmental DNA, as a result of its similar physicochemical properties (Tebbe and Vahjen, 1993; Ogram et al., 1987). These contaminants will act as inhibitors in both restriction endonuclease and polymerase chain reactions (Tebbe and Vahjen, 1993; Jackson et al., 1997; Miller et al., 1999). Direct lysis isolation procedure involves the disruption of the soil matrix and the lysis of the bacterial cells. A combination of physical, chemical and enzymatic methods has been developed by many scientists (Miller et al., 1999). The most widely used mechanical lysis methods are thermocycling (Hugenholtz et al., 1998) and bead mill homogenization (Kuske et al., 1998; Miller et al., 1999), although ultrasonication (Picard et al., 1992), microwave heating (Dijkmans et al., 1993) and grinding under liquid nitrogen have also been reported (Zhou et al., 1996). All of the above methods have proven to be both necessary and effective for optimum DNA isolation (Miller et al., 1999). The mechanical disruption of the sample matrix causes a more effective dislodging and

separation of the micoorganisms from the soil matrix, allowing an easier access to the lysis buffer and therefore it will result in increased cell lysis. A greater disadvantage of this method is the degradation and fragmentation or smearing of the isolated DNA. A potential risk when employing PCR based techniques on sheared DNA is the possibility of chimera formation (Field et al., 1997). Miller and co-workers (1999) have established that by altering the duration and speed of the bead mill homogenization step, larger fragments can be selectively produced. Chemical and enzymatic lysis methods are comparatively more gentle procedures that minimize DNA damage. However, these methods tend to be less effective at disrupting the soil matrix and exposing the cells to the lysis buffer. In most of the chemical lysis procedures a detergent such as sodium dodecyl sulfate (Zhou et al., 1996) or sarkosyl (Holben et al., 1988) is used to aid cell membrane lysis (Berthelet et al., 1996). In addition buffers are complemented by the addition of other compounds such as chelating agents (EDTA, Chelex 100) to inhibit nuclease activity and disperse the soil matrix (Miller et al., 1999). Humic acid complexing compounds such as PVPP (Gray and Herwig, 1996) and CTAB (Zhou et al., 1996) are also used in an effort to increase DNA purity. Chemical lysis methods are also accompanied by the addition of enzymes to promote cell lysis. Commonly used enzymes include lysozyme (Rochelle et al., 1992; Tebbe and Vahjen, 1993), Proteinase K (Zhou et al., 1996), a chromopeptidase (Liu et al., 1997) and pronase E (Jacobsen and Rasmussen, 1992). Phenol and/or chloroform extraction steps are also included in extraction protocols to increase the recovery of DNA (Tebbe and Vahjen, 1993; Zhou et al., 1996). The use of saturated salt solutions such as sodium chloride, potassium chloride, ammonium acetate, sodium acetate and potassium acetate to separate

the DNA from contaminating proteins has also been investigated (Selenska and Klingmuller, 1991; Frostegard et al., 1999; Miller et al., 1999). After these steps the DNA can be concentrated via ethanol, isopropanol or PEG precipitation. It has been recommended that alcoholic precipitation promotes the co-extraction of humic acids whereas PEG acts as a PCR inhibitor and must also be removed, resulting in a further loss of DNA and isopropanol is considered as a better precipitation method since it produces a good yield without compromising DNA purity (Roose- Amsaleg et al., 2001).

Various purification methods were described in many of the previous studies regardless of the DNA extraction method applied. Recent reports revealed that phenol and chloroform extraction, and/or treatment with CTAB (Cetyl trimethyl ammonium bromide) or PVPP (Polyvinyl polypyrrolidone) coupled with CsCl density gradient centrifugation or purification using hydroxyapatite column chromatography (Holben et al., 1988; Selenska and Klingmuller, 1991; Knaebel and Crawford, 1995; Roose- Amsaleg et al., 2001; Lee et al., 2004) yielded a low DNA yield (Steffan et al., 1988). In a study conducted by Xia et al in 1995, had shown that washing step prior to cell lysis in indirect DNA extraction removed soluble inhibitors and extracellular small fragments of DNA (Xia et al., 1995; Harry et al., 1999). Conversely, many soil samples require a combination of these purification steps, which significantly increases processing time and can lead to an even greater loss of DNA. This is in line with the study which compared DNA extracted from five different soils with varying organic matter contents and found that the samples with the highest organic matter content required five steps of purification to yield sufficiently high purity DNA (Van Elsas et al., 1997). Sephadex G-200

spin columns have been proven to be one of the best ways to remove contaminants from soil DNA (Miller et al., 1999). Recently, a pulse field electrophoresis method using a two phase agarose gel, with one phase containing polyvinyl polypyrrolidone (PVPP), was developed for removal of humics (Quaiser et al., 2002). Metagenomic DNA purification with Q-Sepharose beads has been proven to be another prospective method for purifying large molecular weight metagenomic DNA (Sharma et al., 2007). An interesting new method for separating DNA from highly contaminated samples, called synchronous coefficient of drag alteration, applies a rotating dipole and quadruple electric field in an aqueous gel by which DNA is concentrated at a focal point while contaminants are pushed outwards (Pel et al., 2009). The above purification methods confirmed the potential of purification prior to size fractionation and cloning .Thus the DNA may be physically sheared or restriction digested and then size selected by gel extracting the DNA in the appropriate size range from an agarose or low melting point agarose gel (Riesenfeld et al., 2004a; Lammle et al., 2007). The size selected DNA can be column-purified, the gel slices may be digested with GELase enzyme (Epicentre), or the DNA may be electroeluted from the gel prior to cloning (Osoegawa et al., 1998).

1.12 Construction of a small insert or large insert metagenomic library

Riesenfeld et al., (2004b) studied about the construction and analysis of small-insert metagenomic libraries (less than 10 kb average insert size). This study explained a potential approach to identify gene product(s) encoded by a relatively smaller gene fragment, such as most enzymes, or antibiotic

resistance genes (Riesenfeld et al., 2004a; Parsley et al., 2010). Vectors used for the construction of small-insert expression libraries required a promoter for transcription of the cloned gene inserts and should be compatible with the host system selected for screening. A vector with two promoter sites flanking the multiple cloning site facilitates gene expression that is independent of gene orientation and the promoters associated with inserts (Lammle et al., 2007). From the literatures it is also evident that the selected vector should have the ability to replicate in multiple hosts to enable heterologous expression of specific gene of interest. However, the advantage of using *E. coli* as a heterologous host for metagenomic library construction has been well-established in recent reports (Rondon et al., 2000; Pfeifer and Khosla, 2001; Gillespie et al., 2002; Liles et al., 2004).To circumvent the limitation of small insert vectors, researchers have been employing large insert libraries that can accommodate intact biosynthetic pathways involved in the synthesis of antimicrobial compounds, enzymes with multiple catabolic activity, or operons responsible for complex metabolic functions. In addition to its potential advantages, large-insert cloning presents many technical challenges as the selection of an appropriate cloning vector is essential to the maintenance and expression of the cloned pathways. Several vector choices exist for cloning HMW DNA from environmental samples, such as cosmid DNA libraries with insert size ranging from 25-35 kb (Entcheva et al., 2001) or bacterial artificial chromosome (BAC) libraries with insert size up to 200 kb (Beja et al., 2000; Rondon et al., 2000). BAC vectors are based on the F-factor replicon, conferring long term stability and have the capacity to maintain 100 kb of insert DNA (Shizuya et al., 1992), along with these a modified BAC vector containing an origin of replication (RK2) is capable of inducible copy

number, alternating between single-copy and high-copy BAC maintenance (Wild et al., 2002). Additionally, the construction of fosmid with inserts of 40 kb of foreign DNA has been reported (Beja et al., 2012). The low copy number of fosmid vectors offers higher stability than high-copy number cosmids. Aakvik et al. (2009) recently reported about broad-host-range screenings in which fosmid and BAC vectors were successfully transferred into the host strains, *Pseudomonas fluorescens* and *Xanthomonas campestris*. The main highlighting aspect is the inducible copy number for controlled gene. However fosmid vectors are limited in insert size compared to BAC vectors, their significantly higher cloning efficiency facilitate construction of metagenomic libraries with many thousands of transductants. Conversely, BAC vectors are capable of accommodating higher insert sizes, though exhibiting lower cloning efficiency than that of fosmid vectors. Another additional feature of BAC vectors is that it can stably maintain cloned inserts hundreds of kilobases in size and provide a greater chance of isolating intact metabolic pathways or of linking phylogenetic and functional genetic information (Stein et al., 1996). Thus the studies have shown that the predicted size of the pathway of interest, its natural level of activity, and its relative abundance within the community must be taken into account when selecting an appropriate cloning system for large-insert metagenomic library construction. *S. lividans* has been used as another heterologous host for library screening, and it has more strong promoter recognition signals and regulation properties when compared to *E. coli* (Martinez et al., 2005). Bacterial strains from genera like *Burkholderia, Bacillus, Sphingomonas, Streptomyces,* and *Pseudomonas* have also been studied as alternative hosts (Courtois et al., 2003; Eyers et al., 2004; Martinez et al., 2004; Van Elsas et al., 2008).

Metagenomic library can be expressed in a single host or multi host system. In recent years muti-host strategy is used in order to sequentially express the substantial part of the transformed genes. Vector or host organism must also contain co-factors, post-translation modification enzymes, inducers, chaperones for successful expression of the cloned pathways.

1.13 Metagenomic approaches: Function driven Vs Sequence driven

Once the source metagenome is identified and metagenomic DNA had been isolated, the goal shifts to isolation of gene of interest from the metagenomic library. There can be two approaches, function based and Sequence driven approaches (Kakirde et al., 2010; Schloss and Handelsman, 2003). In the first approach, screening is based on the activity of a target gene cloned in an expression host. In the second one, the target gene sequences can be detected by using hybridization probes or PCR specific primers. According to Uchiyama et al. (2005), function-based screening is a simple way to obtain novel genes having desired functions, but is often problematic due to the biased and insufficient expression of unknown genomic fragments in *E. coli*. Despite the potential for mining of genetic novelty, it can also successfully amplify a set of unknown genes of unknown origin in a foreign host in high throughput. Another disadvantage is the repeated rediscovery of previously known functions, which limits the success of the metagenomics approach (Binga et al., 2008). The first two disadvantages appear to exacerbate the apparent inaccessibility of the existing genetic diversity through functional metagenomics approach (Li et al., 2007). By contrast, the gene sequences obtained through sequence-based approach are limited to those having

homologies to the probe sequence and may not allow us to obtain novel genes. A lot of attempts have been made to answer these problems in the screening of useful genes from the metagenomes.

Simon and Daniel (2009) described three general detection strategies for functional screening of a specific gene. First strategy is the phenotypic insert detection (PID), where the cultivation of metagenomic clones on indicator plates allowing analysis of defined enzyme activities through the biocatalytic conversion of an indicator substrate that leads to the formation of a clear or coloured halo surrounding the positive colony. However the frequency of active clones is usually quite low and strongly depends on the sensitivity of the used assay system as well as on the gene expression capability. Modulated detection (MD), a strategy that relies on the production of a gene product that is necessary for growth under selective conditions and the third strategy, substrate induction (SIGEX), based on the induced expression of cloned genes via a specific substrate. Another productive approach for functional screening is to construct reporter fusions that act in response to expression of the targeted gene. If clones expressing the reporter can be identified rapidly by antibiotic resistance selection or fluorescence-activated cell sorting method, then libraries of sufficient size to represent the diversity of a given environment could be screened. In function driven approach, metagenomic libraries are screened for expressed traits and once identified the clones are characterized by biochemical and sequence analysis. Screening by heterologous gene expression is a potent yet demanding approach to metagenomic analysis. Successful transcription and translation of the genes, protein folding and secretion from the host are required for a positive hit during functional screening and the positive clones thus

obtained with no sequence information studied to date. There are many activity based assays that do not produce metabolites and can be easily detected by a change in reaction colour. Many high-throughput methods were also developed to screen such kind of libraries (Ferrer et al., 2009).Thus the above information clearly confirmed that how future efforts in this line may be extremely helpful for describing the functions to generate sequence based information. A schematic representation of general steps involved in a function-driven metagenomic approach is shown in Fig.12.

Furthermore, sequence driven approach includes the designing of primers or hybridization probes based on a known conserved sequence through PCR amplification or hybridization, to identify a target clone. Tyson et al. (2004) had reported the complete genome of a symbiotic microbial community by this sequence-based method. However, gene-specific PCR has two major drawbacks; such as the first one, the design of primers is completely dependent on existing sequence information which limited the homology search in favour of known sequence types. Genes derived from convergent evolution having similar biological functions are not likely to be detected by a single gene/family-specific set of PCR primers. Secondly, only a small fragment of a structural gene will usually be amplified by gene-specific PCR and requires further steps to obtain the full-length genes. The resulting amplicons can be labelled as probes to identify the putative full-length genes in large metagenomic library constructs. Alternatively, PCR-based strategies for the recovery of either upstream or downstream flanking regions including universal fast walking (Mishra, 2002; Myrick and Gilbert.,2002), panhandle PCR (Megonigal, 2000), random primed PCR (Liu and whittier, 1995), inverse PCR and adaptor ligation

PCR (Ochman, 1993) can be used to access the full-length gene/s. These methods are technically not easy to apply at a metagenomic level on account of the increased complexity of a metagenomic DNA. Both functional and sequence based approach has its own advantages and disadvantages, and together these approaches have nourished our understanding of unexplorable microbial world. Random sequencing can also be performed to identify new microbial taxa or gene/s of interest. All the distribution and redundancy of functions in a community, linkage of traits, genomic organization and horizontal gene transfer can be inferred from whole-genome sequence based analysis. The recent efforts of sequencing technologies include reconstruction of the genomes of uncultivated communities in acid mine drainage (Tyson et al., 2004) and the Sargasso Sea (Venter and Reminton, 2004) illustrate the power of large scale sequencing effort to support our understanding of uncultivable microflora (Tringe et al, 2005).Other DNA sequence screening methods published in literature are reverse transcription PCR (RT-PCR) (Wilson et al., 1999), DNA microarrays (Park et al., 2008, Park et al., 2010 and Wu et al., 2001), integron (Rowe-Magnus and Mazel, 2001), affinity capture (Stull and Pisano, 2001) and subtractive hybridization magnetic bead capture (Meiring et al., 2010 and Meyer et al., 2007) etc. In addition, compound configuration screening is another method of screening novel structural compounds based on the identification of chromatographic peaks relative to the host cell proteins. Accordingly, novel compounds from the transformants were identified using rapid HPLC-ESIMS screening method and downstream database analysis (Wang et al., 2000).

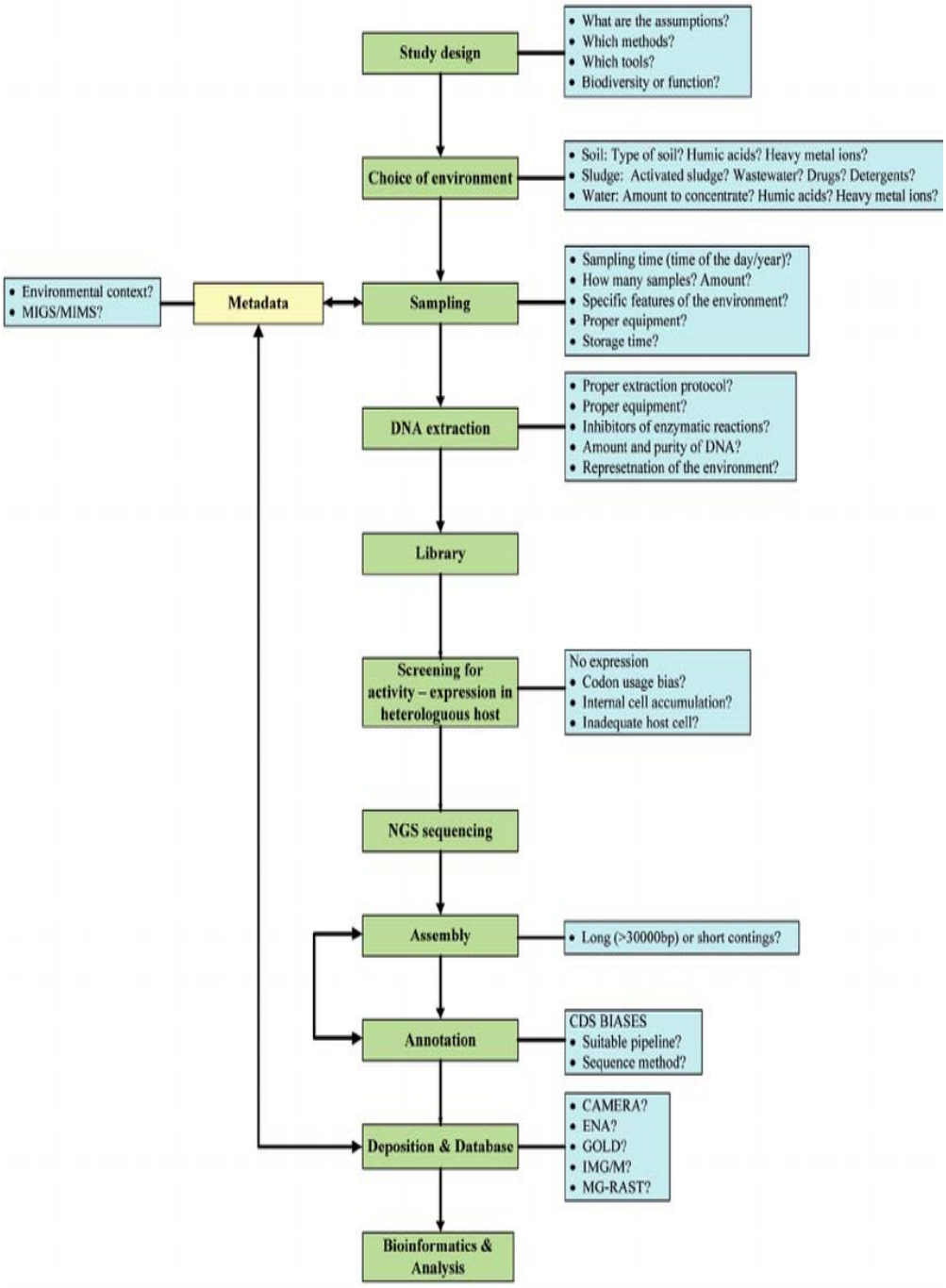

Fig.12. The schematic diagram showing steps involved in a function-driven metagenomic approach, general steps are shaded in green, blue boxes indicate common problems associated with each step. Figure adapted from Felczykowska et al., 2015.

1.14 Metagenome derived cellulases – To name a few

Following the revolutionary advances in sequencing and screening techniques, metagenomics has been used to obtain a surplus of cellulolytic enzymes with special characteristics, with increasing potential for the discovery of new enzymes with novel properties for industrial use. Because cellulose is a valuable biopolymer for the production of biofuels, a handful of publications have been reported on the isolation of metagenome – derived cellulases , since the first publication about the isolation of a cellulase gene from lignocellulosic-metagenome maintained in a thermophilic, anaerobic digester (Healy et al., 1995). In their study, 12 clones exhibiting carboxy methyl cellulase activity and 11 clones displaying 4-methylumbelliferyl- β-D cellobioside (MUC) hydrolase activity were identified in the functional metagenomic library. Four of them were further characterized and they exhibited temperature optima (60–65^{0}C) and pH optima (pH 6–7) in accordance with conditions of the enrichment followed by other studies (Grant et al., 2004; Rees et al., 2003; Voget et al., 2003& 2006), hindgut contents of higher termite (Warnecke et al., 2007). Thereafter, metagenomic approaches have been widely used to isolate cellulases from various environmental samples, where plant cell wall materials were degraded intensively, including soil (Jiang et al., 2009; Kim et al., 2007 & 2008), compost (Pang et al., 2009), contents of rabbit cecum (Feng et al., 2007), sludge from a biogas reactor (Jiang et al., 2010), and functional screening of a soil metagenomic library for cellulases has shown a total of eight cellulolytic clones, one of which was over-expressed and functionally characterised (Voget et al., 2006). Even though this library had been generated from a non-extremophilic environment, the cellulase displayed a high level of stability up to pH 9, stable at 40°C for up to 11 h and was highly halotolerant and stable in 3M NaCl. Many of the

metagenomic studies in extreme environments such as soda-lakes in Africa and Egypt identified more than a dozen cellulases, some of which displayed habitat associated halotolerant properties (Rees et al., 2003; Grant et al., 2004). Rees et al. (2003) had sequenced two endo-β-1, 4-glucanase genes from functional DNA libraries constructed from their enrichment cultures of lake water samples. One endo-β-1, 4-glucanase gene was cloned from the microbial consortia in the soil through enrichment (Voget et al., 2003). Nine endo-β-1,4-glucanase genes and one β-glucosidase gene were screened from rumen metagenome (Ferrer et al., 2005). Walter et al (2005) had cloned one β-glucosidase gene from the large-bowel microbiota of mouse. Wang et al. (2009) have reported the isolation and identification of seven independent clones specifying cellulase activities out of which five are endo-β-1,4-glucanases and two are β-glucosidases. Upon sequence analysis only two of the genes indicated any solid affiliation to already known sequence, whereas the rest formed deep-branched lineages with no close relatives thereby underlining the great diversity of cellulases in natural environment. However, cellulases with carbohydrate binding motif including cellobiohydrolases or multidomain cellulases were not retrieved in any of the previous studies. This might be due to their inability either to fold appropriately or by the insufficient and biased expression in *E. coli*. Voget et al. (2006) have reported the biochemical characterization of a novel, soil metagenome-derived cellulase (endoglucanase), Cel5A. According to their studies, the Cel5A was unable to hydrolyse crystalline form of cellulose and also unable to hydrolyse substrates that lack β-1, 4- linkages, such as laminarin and substrates such as β-1, 4 linked xylose.

The biochemical assays have revealed that these cellulases are with high stability in salt and show pH tolerant characteristics. The rumen

microbiome represents the most potent fibre fermentation system and remained as potentially important supply of unique cellulases and several metagenomic studies were also targeted on this environment (Duan et al., 2009; Ferrer et al., 2005; Liu et al., 2009; Palackal et al., 2007; Shedova et al., 2009; Wang et al., 2009). Ilmberger et al. (2012) constructed metagenomic libraries from a biogas plant sample, elephant faeces and from an enrichment culture inoculated with crude protein extract of the shipworm *Teredo navalis* and enzymes were isolated with high activities and remarkable stabilities in the presence of elevated concentrations of different ionic liquids. Hence, it could be an ideal candidate for industrial applications. Apart from activity-based functional screening, the sequence based screening methods began to rapidly develop based on high-throughput analysis. However, only a few reports clearly attempted to identify genes encoding cellulases directly from environmental DNA by sequence-based metagenomic approach. Ohtoko et al. (2000) identified GH45 cellulase homologs from the symbiotic protists living in the hindgut of the termite *Reticulitermes speratus* using consensus PCR and cDNA library screening. Edwards et al. (2008) designed primers for fungal cellobiohydrolase I (CBHI) and used this to clone CBHI homologs from forest soil by sequence-based PCR. Izquierdo et al. (2010) have analyzed the diversity of GH48 cellulases in cellulolytic consortia enriched from thermophilic biocompost. But the cellulase genes obtained by sequence-based method showed high similarity with already known genes. So it was found very difficult to get novel cellulases with desirable properties from the metagenome using the sequence based approach. But it can overcome the problems associated with insufficient biased expression in *E. coli* in the function-based approach.

1.15 Development of bioinformatic tools in metagenomic studies

Traditionally, DNA sequencing has almost exclusively been carried out using the Sanger method (Sanger et al., 1977). Over the past few years, alternative sequencing platforms have become widely available which allow faster sequencing with a reduction in costs by over two orders of magnitude (Shendure, 2008). The "second generation sequencing technologies" for example, the 454 pyrosequencing, avoid the requirement for traditional library construction or designing of primers homologous to the template DNA. These new platforms are diverse at many levels, but their work flows are conceptually similar. Summarized concept consists in sequencing of a dense array of amplified DNA fragments through repeated cycles of enzymatic manipulation and imaging based data collection (Shendure, 2008). Relative to other post-Sanger sequencing platforms, the main advantage of the 454 platform is the read-length (Fig.13). Even more modern sequencing technologies are therefore called as " third generation sequencing platforms ", which comprise sequencing of individual molecules (Xu et al., 2009), mainly based on fluorescence detection have already been introduced and others are being developed by different companies (Metzker, 2010; Xu et al., 2009). The launching of next-generation sequencing platforms, such as 454 pyrosequencing (Margulies, 2005b) from Roche Applied Science, Illumina Genome Analyzer from Illumina and Solexa technology, AB SOLiD systems from Applied Biosystems had a big impact on metagenomic research. The rapid advances in high throughput and cost reduction have dramatically increased the number and size of the metagenome sequencing projects. Sequencing technologies has now been accelerating, with a multitude of novel biocatalyst discovery using high-throughput and low-cost genetic screening.

Platform	Read-length [bp]	Giga bases pr run	Chemistry
Roche/454's GS FLX Titanium[a]	400-500	0,45 (10h)	PS
Illumina/Solexa's Genome Analyzer *IIx*[b]	100	18 (9,5 days)	RTs
Life/APG's SOLiD 4 *hq*[c]	75	Up to 300 (3-14 days[f])	SBL
Helicos BioSciences[d]	25-55	21-35 Gigabases (8 days)	RTs
Pacific Biosciences[e]	Average: 1000	N/A	Real-time

[a]-[c]: Second generation sequencing technologies: [a] www.454.com, [b] www.illumina.com, [c] www.appliedbiosystems.com. [d]-[e]: Third generation sequencing technology: [d] www.helicosbio.com, [e] www.pacificbiosciences.com. [f] depending on individual read lengths. N/A: Not available, PS: pyrosequencing, RT: reverse terminator, SBL: sequencing by ligation.

Fig.13. The table enlisting second and third generation sequencing technologies (adapted from Xu et al. (2009) and Metzker (2010), the corresponding references are indicated in the bottom of table.

Name	Web site	Short description
AMPHORA	http://bobcat.genomecenter.ucdavis.edu/AMPHORA	Software for phylogenetic analysis of single gene or whole genomes
ARB	http://www.arb-home.de	Interacting software tools for sequence database, maintenance and analysis
CAMERA	http://camera.calit2.net	Metagenomic database of marine and oceanic sequences
CARMA	http://www.cebitec.uni-bielefeld.de/brf/carma/carma.html	Algorithm characterizing the genetic diversity of short-read metagenomes
COG	http://www.ncbi.nlm.nih.gov/COG	Database for phylogenetic classification of proteins encoded in complete genomes
DDBJ	http://www.ddbj.nig.ac.jp	Database tools for collection and analysis of nucleotide sequences
DOTUR	http://www.plantpath.wisc.edu/fac/joh/dotur.html	Sequence assignment to OTUs and richness estimation
EMBL	http://www.ebi.ac.uk/embl	Database tools for collection and analysis of nucleotide sequences
INSDC	http://www.insdc.org	International synchronized collaboration between DDBJ (Japan),GenBank(USA) and the EMBL (Europe) databases
KEGG	http://www.genome.jp/kegg	Database tools for computational prediction of cellular metabolic processes
GenBank	http://ncbi.nlm.nih.gov/Genbank/metagenome.html	An annotated collection database of all publicly available DNA sequences
GOLD	http://genomesonline.org	It provides metadata information related to genome and metagenome projects worldwide
GSC	http://gensc.org/gc_wiki/index.php/Main_Page	A consortium which provides genomic standards and methods for harmonization of metadata collections
MEGAN	http://www-ab.informatik.uni-tuebingen.de/software/megan	Algorithm which supports comparison of multiple and large datasets
Megx.net	http://www.megx.net	Database tools for analysis of Marine Metagenomics
MetaGene	http://metagene.cb.k.u-tokyo.ac.jp	Algorithm for prokaryotic gene-finding from environmental genome shotgun sequences
NAST	http://greengenes.lbl.gov/NAST	Algorithm for creating multiple sequence alignments
MG-RAST	http://metagenomics.nmpdr.org	Bioinformatic tool for phylogenetic and functional analysis of metagenomes
PHACCS	http://biome.sdsu.edu/phaccs	Bioinformatic tool for the analysis of viral metagenomic data
RefSeq	http://ncbi.nlm.nih.gov/RefSeq	Collection of sequences representing genomic data and proteins from 2400 organisms
SILVA	http://www.arb-silva.de	Database for analysis and alignment of high-quality ribosomal RNA sequence data
SINA	http://www.arb-silva.de/aligner	Bioinformatic tool for sequence alignment based on SEED
StrainInfo.net	http://www.straininfo.net	A bioportal of information integration services for the microbial community
TETRA	http://www.megx.net/tetra	A tetranucleotide-based tool correlating large DNA sequences
UniFrac	http://bmf2.colorado.edu/unifrac/index.psp	Comparison of microbial communities using phylogenetic information
XplorSeq	http://vent.colorado.edu/phloware	Compilation, management and phylogenetic analysis of DNA sequences

Fig.14. Bioinformatic tools and databases used in metagenomic analysis, Mocali and Benedetti., 2010

The next generation sequencing technology has allowed us to perform massive parallel ultra-deep sequencing and transformed the traditional microbial genomics through their capability to produce hundreds of megabases in a single run. However, the use of these instruments require the uniform processing of different metagenomic DNA samples for library preparation, since these are based on different sequencing technologies. The library preparation requires random fragmentation of the metagenomic DNA based on Adaptive Focused Acoustics (AFA) technology or the transposome facilitated fragmentation technique or nebulized or enzymatically fragmented. The DNA fragments are then modified with the ligation of an adaptor or barcode and amplified, the double stranded DNA is then denatured and the nucleotide composition on the single stranded DNA molecules is identified by the sequencers. These procedures are dependent upon the sequencing platform/instrument used for sequencing (Head et al., 2014). Moreover, the commercial DNA library preparation kits (Illumina, Nextera XT DNA sample preparation kit and other similar kits) have eliminated the requirement of high concentration of input DNA required for library preparation. Due to the still shorter read lengths and/or the higher error rates of these technologies compared to the Sanger procedure, a higher coverage is needed to obtain data of high quality. Nevertheless, this transition to the next-generation sequencing technologies opens the possibility for Gb-scale metagenomic projects, including sequencing of complex communities nearly to saturation (Chistoserdova, 2010). However, successful implementations of such projects are also dependent on improved sampling, DNA extraction, bioinformatical analysis tools, and data storage infrastructures.

These advancements in the sequencing research using GS20, the first instrument based on the 454 pyrosequencing technology have the capacity to

sequence up to 25 million bases of a bacterial genome in a 4 h run (Margulies et al., 2005a). A recent model 454 GS-FLX sequencer using Titanium chemistry can achieve read lengths of up to 500 bp, with more expected future improvements in read length. Illumina Solexa platform based on fluorescently labeled sequencing by synthesis generates 35-76 bp on average. The most modern version of the short read sequencer from Applied Biosystems, called the SOLiD4, generates 100 Gb per run with read length of 50 bp. All of these considerations mentioned above suggested that NGS technologies provide an overall coverage for single genomes, the short read lengths can be a serious limitation for efficient assembly of metagenomic sequences. The 454 sequencing is highly economically accessible at approximately $10 per megabase followed by Solexa and SOLiD at about $5 and $2, respectively (Rothberg and Leamon, 2008). The cost and read estimates will drop as the NGS technology advances and it has enabled the individual academic researchers to access the modern sequencing technologies. By this huge amount of data generated by the recent sequencing efforts made bioinformatics tools for such high-throughput sequence pipelines. Contig assembly can also be practised from the edited sequences by eliminating ambiguous base pairs, then deposited in the sequence databases (e.g., GenBank env) and searched against other environmental metagenomic datasets. A functional tool for accessing metagenomic information is CAMERA (Community cyber infrastructure for Advanced Marine Microbial Ecology Research and Analysis), developed to advance the community ecology research by generating a data repository and a bioinformatics resource to provide metagenomic sequence data storage, access, analysis, and synthesis (Smarr, 2006). In addition, Metagenomics RAST server (MG-RAST) compares nucleotide and protein databases for functional annotation of sequences in the metagenome along with a phylogenetic summary

(Meyer et al., 2008) and gene prediction tools, such as MEGAN (MEtaGenome ANalyzer), a program that compares a set of DNA reads/contigs against known sequence databases using BLAST algorithms. MEGAN can then be used to compute and interactively reconnoitre the taxonomical content of the dataset to summarize the outputs (Huson et al., 2007). Once the metagenomic sequences with significant GenBank hits has been assembled, they were further categorized by SEED- a subsystem approach to consolidated and fast annotation of predicted gene functions according to related biological function (Overbeek et al., 1998). The predicted genes may also be allocated a phylogenetic classification using Treephyler for rapid taxonomic profiling of metagenomic sequences (Schreiber et al., 2010). After a series of publications dedicated to bioinformatics tools and approaches, it should be acknowledged that the prognostic power of the sequence analysis is restricted by the previously described gene functions and that many putative functions may be inaccurately annotated. Though this potential source of bias affects the efficacy of a sequence-based approach to metagenomics, such demanding sequence-driven surveys of natural environments have extremely affected our collective view of prokaryotic diversity and the extent of functional genetic diversity that has yet to be understood in terms of biological functionality (Venter et al., 2004).

1.16 Application of sequencing technology in Cellulose degrading habitats

The introduction of metagenomic sequencing technologies help to solve the problem of low hit rate of the positive clones due to insufficient biased expression in the functional metagenomic approach. Metagenomic sequencing projects are ongoing in several ecosystems related to cellulose degradation, including the rumen microbiomes (Brulc et al. 2009) and hindgut microflora of

higher termites (Warnecke et al., 2007), the metagenomic sequence data from those microbiome inhabitants had shown 100 glycoside hydrolase gene modules relevant to cellulose hydrolysis in termite hindgut, showing similarity to the catalytic domains of GH5, GH94, and GH51. The positive hit rate was about 1 cellulase gene/0.4 Mb metagenomic DNA, but the GH6, GH7, and GH48 endoglucanases and cellobiohydrolase microbial cellulase systems of *Trichoderma reesei* and the *Cellulomonas* were absent (Warnecke et al., 2007). However, the cow rumen microbiome contains fewer representatives of glycoside hydrolase modules involved in cellulose degradation (GH5, 9, 44, 74 and 94) with the positive hit rate was about 1cellulase gene/1.55 Mb. A single GH48 homolog encoding an exoglucanase was screened from one of the fiber-adherent microbiome metagenomic library (Brulc et al. 2009) .Conversely, certain limitations and difficulties were identified by some authors in the application of metagenomic sequencing for cellulase enzyme discovery. The first difficulty was genome assembly due to the metagenome complexity and the short reads resulting from pyrosequencing, which can be overcome by the implementation of third-generation sequencing technology (Eid et al., 2009). The second difficulty is the classification of GH families based on amino acid sequence similarity, since novel cellulase families could not be identified from the known sequence database and might be annotated as hypothetical proteins. Warnecke et al. (2007) suggested a combination of metagenomic sequencing and function-based screening for discovering novel cellulases.

1.17 Industrial applications of metagenome-characterized cellulases

The ideal cellulases derived through metagenomic approaches show potential industrial/biotechnological applications. Generally, enzymes retrieved from certain environments might be dependable with source environmental conditions. Feng et al. (2007) described about cellulase clones

from rabbit cecum exhibited highest activities at pH 5.5–7.0 and at temperatures of 40–55^{0}C, conditions unique to those in the rabbit cecum. Jiang et al. (2009) identified an alkaline β-glucosidase from alkaline soil. However, this is inconsistent with the result of Pang et al. (2009), who identified cellulase positive clones with properties of the cold-adapted enzymes, which are different from those of source habitats such as high-temperature compost and a halo-tolerant environment (Voget et al., 2006) and these are primarily characterised through crude lysates of the recombinant clones expressing cellulase activities (Duan et al., 2009; Feng et al., 2007; Ferrer et al., 2005). Another alternative approach is to determine the activity of an active clone under the specific assay conditions with which the enzyme will be used. The soil metagenome-derived endoglucanase Cel5A is highly stable over a wide temperature and pH range, and in the presence of high salt concentrations. It is also functional in the presence of a broad range of divalent cations, chelating agents and detergents, which are commonly in industrial use. Those results demonstrate that Cel5A is a suitable candidate for industrial applications (Voget et al., 2006). Palackal et al. (2007), characterised multifunctional hybrid glycosyl hydrolase of GH6248, contained independent catalytic modules of GH5 and GH26, showing enzyme activity of both glucanase and mannanase respectively. Such multifunctional glycoside hydrolases have many prospective industrial applications, such as in biomass saccharification, wine making, animal feed nutritional enhancement, textile, and pulp and paper industries. The acidic and mesophilic cellulases retrieved from the cow rumen microbiome were suitable in yeast fermentation for ethanol production. These enzymes have possible applications in cellodextrin hydrolysis as well as in the simultaneous saccharification and co-fermentation of lignocellulose (Duan et al., 2009; Liu et al., 2009). Pottka¨mper et al. (2009) identified three novel

metagenome-derived cellulases that were applicable for cellulose degradation in the presence of high ionic concentrations which can dissolve cellulose.

Though novel cellulase genes have been identified from various habitats through metagenomics based approaches, culture-dependent approach also has advantages to find ideal candidate strains to fulfill certain bioprocess conditions. Both approaches have its own advantages in cellulase identification and they complement each other, to attain novel cellulase discovery.

Having this insight in vision the following objectives were undertaken to take forward the endeavor in the field paving the way for industrial development in cellulase production.

A) Culture-dependent approach:

1) Screening and identification of potent hydrolytic organisms from MTCC culture collection for saccharification of delignified coir pith and development of enhanced cellulose hydrolysis
2) Purification and characterisation of a processive-type endoglucanase and β-glucosidase from *Aspergillus ochraceus* MTCC 1810 through bioconversion of delignified coir pith into glucose
3) Proteomics of the purified cellulases of *Aspergillus ochraceus* MTCC 1810 and their application in bioconversion of delignified coir pith

B) Functional Metagenomic approach:

4) Construction of Metagenomic lambda library from Valanthacaud Mangrove sediment
5) Screening of metagenomic lambda library for Cellulase encoding clones and Sequence analysis of one of the potent clones
6) Sub-cloning, Over-expression, Purification and Characterisation of recombinant cellulase

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Chapter 2

SCREENING AND IDENTIFICATION OF POTENT HYDROLYTIC ORGANISMS FROM MTCC CULTURE COLLECTION FOR SACCHARIFICATION OF DELIGNIFIED COIR PITH AND DEVELOPMENT OF ENHANCED CELLULOSE HYDROLYSIS

Contents

2.1 Introduction

Current fossil fuel consumption profile has forced scientific efforts to explore alternative energy sources such as lignocellulosic biomass due to its exceptional chemical composition (Asha et al., 2016). Lignocellulosic materials consist of cellulose (40–60%), hemicellulose (20–30%) and lignin (15–30%) (Kuhad et al., 1997; Saha and Cotta, 2006) and are being considered as complex feed stock material for enzymatic saccharification. Lignin recalcitrance makes the substrate more difficult for enzymatic infiltration and hydrolysis. Pre-treatment which removes lignin from cross linking into the cellulose-hemicellulose partners and subsequently uncover the cellulose polymer for bioconversion into fermentable sugars, is an essential step towards bioenergy generation. Numerous pre-treatment methods such as dilute acid and alkaline

pre-treatment, steam explosion, supercritical CO_2 explosion etc., were studied on a broad range of biomass feed stocks. This resulted in efficient cellulose hydrolysis due to reduced biomass recalcitrance, crystallinity and increased surface area (Ibbett et al., 2013), but released toxic compounds that may inhibit subsequent fermentation. Therefore, an efficient pre-treatment is still yet to be developed. In this study we used alkaline peroxide treated coir pith for the production of cellulases (Rojith and Singh, 2012).

Cellulosic materials have been considered as the most promising feedstock for bioenergy generation and some value-added by-products; their holistic utilization could answer modern waste disposal problems and also weaken the dependence on fossil fuels by providing a suitable and renewable source of energy in the form of glucose (Kapdan and Kaegi, 2006). Cellulases are the major catalytic players of enzymatic hydrolysis and exist as multiple enzyme system, comprising endoglucanase (carboxy methyl cellulose, EC3.2.1.4), exocellobiohydrolase (avicelase, EC3.2.1.91), and β-1,4-glucosidase (EC3.2.1.21) (Yi et al., 1999) that synergistically hydrolyse cellulose into monomeric glucose units. Fermentation industry requires high yield and stable enzymes versatile to extreme bioprocess conditions which are yet obstructed by its production cost (Banerjee et al., 2010). This can be overcome by using less expensive substrates such as lignocellulosic wastes and the use of hyper cellulase producing microbes for an overall cost effective bioprocess (Asha et al., 2016). The enzymes produced through such bioconversion process would serve as efficient biocatalytic enzyme cocktails as it is particularly induced by

the diverse cellulose components present in the native lignocellulosic biomass (Kovacs et al., 2009).

Cellulases are mainly produced by bacteria, actinomycetes and fungi (Kuhad et al., 1997), but only a few microorganisms produce substantial amounts of cell-free enzymes which are able to completely hydrolyse crystalline cellulose under *in vitro* conditions. *Trichoderma reesei* is the most efficient cellulase producer such as endo- and exo- glucanases (Miettinen-Oinnonen and Suominen, 2002), while it does not secrete sufficient amounts of β-glucosidase i.e., (≤0.1 IU/mL) (Kovacs et al., 2008; Wen et al., 2005). But those of the genus *Aspergillus* secrete relatively large quantities of endo-β-1,4-glucanase and β-glucosidase with very low levels of exo-β- glucanase production. Other major cellulase producing fungi belonged to *Fusarium* sp., *Alternaria* sp., *Pencillium* sp. , *Phanerochaete chrysosporium* and *Rhizopus* sp. , cellulolytic bacteria belonging to the genus of *Cellulomonas*, *Clostridium*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can also produce cellulases effectively (Saratale et al., 2008). However, the cellulolytic microorganisms commercially exploited for industrial cellulase production are mostly limited to *T. reesei* (*Hypocrea jecorina*), *Humicola insolens, A. niger, Thermomonospora fusca* and *Bacillus* sp. (Nakari-Seta La and Penttila, 1995; Okada, 1988; Davies et al., 2000) and a few other organisms reported in Table 1.

Table 1: Microorganisms exploited in industrial –level cellulase production [Source: Sukumaran et al., 2005]

Microorganisms used for commercial cellulose production	References
A. niger	Ong et al., 2004
A. nidulans	Kwon et al., 1992
A. orynze	Takashima et al., 1998
Fusarium solani	Wood and McCrae, 1977
F. oxysporum	Ortega, 1990
Humicola insolens	Schulein, 1997
H. grisea	Takashima et al., 1996
Melanocarpus albomyces	Oinonen et al., 2004
Penicillium brasilianum	Jorgensen et al., 2003
P. occitanis	Chaabouni et al., 1995
P. decumbans	Mo et al., 2004
Trichoderma reesei	Schulein, 1988
T. longibrachiatum	Fowler et al., 1999
T. harzianum	Galante et al., 1998
Acidothermus cellulolyticus	Tucker et al., 1989
Bacillus sp.	Mawadza et al., 2000
Bacillus subtilis	Heck et al., 2002
C. acetobutylicum	Lopez-Contreras et al., 2004
C. thremocellum	Nochure et al., 1993
Pseudomonas cellulosa	Yamane et al., 1970
Rhodothemus marinus	Hreggvidsson et al., 1996
Cellulomonas fimi	Shen et al., 1996
C. bioazotea	Rajoka and Malik, 1997
C. uda	Nakamura and Kitamura, 1983
Streptomyces drozdowiczii	Grigorevvski de-Limaa et al., 2005
Streptomyces sp.	Okeke and Paterson, 1992
S. lividans	Theberge et al., 1992
Thermononospora fusca	Wilson, 1988
T. curvata	Fennington et al., 1982

Coconut pith or coir pith, a natural renewable resource, produced as a by-product during the defibering of coconut husks from the coir industries of tropical and subtropical Nations. This agro-industrial waste product is highly resistant to natural degradation and usual solid waste disposal methods are

discovered unfit due to its high lignin and polyphenol content. This may cause serious pollution problems to nearby aquatic as well as terrestrial environments (Muniswaran and Charyulu, 1994; Jabasingh, 2011; Rojith and Singh, 2012). Several research efforts are being progressed aiming at sustainable utilization of this highly polluting agri-waste into useful products as it contains about 25% cellulose content. Annually, coir industries in India generate up to 7.5 million tons of coir pith, making this lignocellulosic waste material a massive organic carbon source and therefore could be utilized as cheap and easily available substrate for second generation bioethanol production. Several studies have been reported about the solid state fermentation (SSF) for the production of cellulases from coir pith (Muniswaran and Charyulu, 1994; Jabasingh, 2011).Though cellulase production from pre-treated coir pith through submerged fermentation (SmF) has not been reported till now. This chapter describes various qualitative as well as quantitative cellulase screening methods for the selection of a potent cellulase producing microorganism for saccharification of pre-treated coir pith to glucose. Subsequently, the fermentation conditions such as inoculum size, pH, temperature, carbon and nitrogen sources, metal ions and surfactants were optimized with conventional approach to attain maximum cellulase production and development of a suitable medium for on-site hyper cellulase production using cheapest lignocellulosic waste biomass, the coir pith.

2.2. Materials and Methods

2.2.1 Agrowaste material

Coir pith was collected from coir retting unit, Cherthala district, Kerala, India. It was washed, air dried, and sieved to remove long fibers, stored in air

tight containers and pre-treated with 2% H_2O_2 at pH 11.5 for 10 h (Rojith and Singh, 2012) for removing the lignin fraction.

2.2.2 Procurement of microorganisms

All isolates were procured from Microbial Type Culture Collection, IMTECH, Chandigarh. The cultures were routinely maintained on Potato dextrose agar (PDA) slants and stored at 4^oC.

2.2.3 Primary screening for cellulolytic enzyme activity

2.2.3.1 Screening using PASC (phosphoric acid swollen cellulose) agar medium

All isolates were subjected for a plate screening test. The screening medium containing Mandel's mineral salt solution ; Urea 0.3 g/L, $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2.0 g/L, $CaCl_2$ 0.3 g/L, $MgSO_4$ 0.3 g/L, yeast extract 0.25 g/L and protease peptone 0.75 g/L (Mandels et al., 1974) with the addition of 17.5 g/L agar. Phosphoric acid–swollen cellulose (PASC), 10 g/L was used for the primary isolation of cellulolytic bacteria and fungi. This was prepared according to the procedure recommended by Stewart et al. (1982). Briefly, swelling of cellulose was carried out by adding 20 g of hydroxyl ethyl cellulose powder (HEC) to 800 g of cold (0°C) phosphoric acid with rapid stirring bath. At that time, each cellulose was diluted with 2 litres of cold water, thoroughly mixed, and allowed to sediment, after which the overlying liquid was removed by siphoning and this step was repeated several times to reduce the acid content. The cellulose slurries were then neutralized with solid $NaHCO_3$, decanted and then kept inside bags formed from nylon-reinforced paper towelling. These bags were filled with 1 litre of sterile distilled water, and then excess liquid was removed and the process was repeated for 20 times. The bags were then dialysed ten times against 5 litres of cold deionized water

(until the phosphate content of the dialyzate reached <1 μg/L) and tightly hand squeezed to allow the removal of the equilibrated solutions, and then the celluloses were removed from the bags, lyophilized and then readily used as a supplement into the Mandel's mineral salt agar medium. The plates were spot inoculated with bacterial and fungal strains such as a known volume of inoculum (OD adjusted to 0.1) in the case of bacteria and actinomycetes and mycelial discs of 3-5 dm from 5-10 day-old cultures of fungal strains and incubated at 25^{o}C for 4-7 days, followed by 24 h incubation at 50^{o}C, after which clear zones could be observed only around the colonies of active cellulolytic strains. The positive cellulolytic strains were selected based on the enzyme index (EI) value i.e., the ratio between the clearing zone diameter and colony zone diameter.

2.2.3.2 Screening by turbidity method

The turbidity test was carried out in a medium with the following composition; solution A containing NH_4Cl (5.0g/L), NH_4NO_3 (1.0 g/L), Na_2SO_4 (2.0 g/L), K_2HPO_4 (3.0 g/L), KH_2PO_4 (1.0 g/L), NaCl (10.0 g/L) and solution B containing $MgSO_4.7H_2O$ (0.1 g/L), $MgCl_2.6H_2O$ (4.0 g/L), yeast extract (0.01 g/L) at pH 7.0. Both solutions were autoclaved separately, mixed together and then added to the sterile culture tubes containing whatman No. 1 filter paper strips (50 mg) as sole carbon source. Test organisms were inoculated into the medium and incubated at 27°C for 3-7 days. Turbidity or growth of the organism in the medium indicated active cellulolytic strains.

2.2.4 Secondary screening with a mixture of chromogenic substrates

All positive isolates from the primary screening test were subjected for the secondary screening. For screening cellulase enzyme activity against insoluble cellulose, hydroxy ethyl cellulose was covalently coupled with

Cibacron Brilliant Red 3B-A (Lee and Ten, 2005). To the hydroxyl ethyl cellulose suspension (2g in 30 mL distilled water), 10 mL of 2 M NaOH, 1.9 g of Cibacron Brilliant Red 3B-A and 1.2 mL of 1,4-butanediol diglycidyl ether were added, stirred for 5 min and left standing at room temperature. After 48 h, the mixture was solidified into a gel. The gel was mixed with 100 mL distilled water and was ground by a blender for 15s.To remove the unbound dye, the ground particles were washed with boiling water and filtered (Whatman, type I) repeatedly until the filtrate was colourless. The wet product was used as a supplement to the agar medium .The content of the chromogenic substrate in the wet product was 0.91%. Microorganisms selected after primary screening tests were streaked onto the chromogenic agar plates and incubated at 30^{o}C for 1-5 days. Any colony that showed solubilization of one or more substrates and a corresponding distinct halo that exceeded the colony diameter by a factor of two or more was considered as cellulose degrading enzyme producer and was consequently selected.

2.2.5. Rapid tube test method

Screening was also carried out using a Rapid tube test method as recommended by Smith (1977) with slight modifications. This was carried out in soft agar medium without any carbon source. Cellulose-azure (2%) was layered on the top of the semisolid agar medium and surface inoculated with mycelium spores and bacterial suspensions i.e., a known volume of inoculum (OD adjusted to 0.1) in the case of bacteria and mycelial discs of 3-5 dm from 5-10 day-old cultures of fungal strains and then incubated at 30^{o}C for 3-4 days. Positive tubes were inspected periodically for the evidence of dye release by examining basal layers for blue colouration. The microorganisms showed cellulase activity in more than one screening tests were consequently selected for further quantitative enzymatic assays.

2.2.6 Production of extracellular enzymes through submerged fermentation (SmF) - Quantitative screening of cellulase activity

Microorganisms for the production of extracellular cellulase were chosen according to the primary and secondary screening methods. It was carried out in the Reese and Mandel's medium containing 0.4g/L $(NH_4)_2SO_4$, 2.2g/L KH_2PO_4, 0.3g/L $MgSO_4.7H_2O$, 4mg/L $FeSO_4.7H_2O$, 0.04g/L $CaCl_2.2H_2O$, 1.4 mg/L $ZnSO_4.7H_2O$, 0.6g/L peptone supplemented with 1% chemically delignified coir pith as cellulose substrate. A known volume of inoculum (OD adjusted to 0.01) in the case of bacteria and actinomycetes and mycelia discs of 3-5 diameter from 5 day old cultures of cellulolytic fungal strains were used to inoculate 250 ml Erlenmeyer flask containing 100 ml of fermentation medium sterilized at 121^0C for 15 minutes. Three replicate flasks were incubated at 30°C in a rotary shaker at 100 rev/min for 10 days. After that samples were withdrawn daily and centrifuged at 10,000*g* and supernatants were analysed for reducing sugar liberation and cellulase activity was determined as described below in the section 2.2.6.1.

2.2.6.1 Analytical methods

The total cellulase (filter paper cellulase, FPase), carboxy methyl cellulase (CMCase) and β-glucosidase activities were determined according to the International Union of Pure and Applied Chemistry procedures (IUPAC) as reported by Ghose (1987). A micro-plate based filter paper assay (FPA), 25-fold scale down of the IUPAC protocol (Xiao et al., 2004) was carried out to determine total cellulase activity in terms of filter paper units. Briefly, the reaction mixture was prepared by adding 20 µL of the cell-free culture supernatant into 40 µL of 50 mM citrate buffer (pH 4.8) for a final volume of 60 µL. The suitably diluted samples were added into the micro-titer well plates

containing whatman No.1 filter paper (6mm, 3 mg) cut using an office hole punch. Substrate blank containing only the filterpaper disc and 50 mM citrate buffer (60 µL); Reagent blank containing only 50 mM citrate buffer (60 µL) were also run along with the samples. An enzyme blank containing 20 µL of enzyme in 40 µL of 50 mM citrate buffer without adding filter paper substrate was also included to reduce the background reducing sugar absorbance. The assay mixture contained in the micro-titer plates were sealed with parafilm, covered with aluminium foil and incubated at 50°C in a water bath for 60 min. After incubation, 140µL of 3, 5-dinitrosalicylic acid (DNS) reagent was added according to the method of Miller (1959), and then plate was resealed with parafilm, boiled at 100^{o}C to develop the colour and to stop the reaction. The microplate was cooled at room temperature using a cooling water bath and the reducing sugar absorbance at 540nm was measured using a microplate reader (Tecan Infinite 200 Pro, Switzerland). The sample values were corrected for reagent blank, substrate blank and enzyme blank values. A glucose standard curve with a concentration range of 0 to 2 mg/mL was also run. All of the samples and the standards were run in triplicate, while the blanks were run in duplicate. Filter Paper Activity (FPA) was expressed as FPU/ml and was calculated using the concept that 0.37 FPU of enzyme will release 2 mg of glucose under standard assay conditions.

Unit Calculation

Concentration = 1/dilution (= Volume of enzyme in dilution / Total Volume of Dilution) . Estimated concentration of the enzyme which would release exactly 2 mg of glucose.

$$\text{FPU} = \frac{0.37}{\text{Enzyme concentration to release 2mg glucose}} \text{ Units ml}^{-1}$$

Endoglucanase (CMCase) activity was carried out by incubating the total reaction mixture containing 20 µl of culture supernatant diluted in 20 µl of 50 mM citrate buffer (pH 4.8) with the substrate such as 20 µl of 1% (w/v) carboxy methyl cellulose (CMC) dissolved in citrate buffer (50 mM, pH 4.8). The final reaction mixture (60 µl) was then incubated at 50°C for 60 min. After incubation, 140 µL of 3,5-dinitrosalicylic acid (DNS reagent) was added according to the method of Miller (1959), and then plate was resealed with parafilm, boiled at 100^{o}C to develop the colour and to stop the reaction. Substrate blank containing only 20 µl of 1% (w/v) carboxy methyl cellulose (CMC) in 40 µl of 50 mM citrate buffer; Reagent blank containing only 50 mM citrate buffer (60 µL) were also run along with samples. An enzyme blank containing 20 µL of enzyme supernatant, 40 µL of 50mM citrate buffer without CMC substrate was also included to reduce the background reducing sugar absorbance. The microplate was cooled at room temperature using a cooling water bath and the reducing sugar absorbance at 540nm was measured using a microplate reader (Tecan Infinite 200 Pro, Switzerland). The sample values were corrected for reagent blank, substrate blank and enzyme blank values. A glucose standard curve with a concentration range of 0 to 2 mg/mL was also run. All of the samples and the standards were run in triplicate, while the blanks were run in duplicate. Endoglucanase (CMCase) activity was calculated following the concept that 0.185 CMC of enzyme will release 0.5mg of glucose under standard assay conditions. One unit of CMC activity is defined as the amount of enzyme needed to liberate 1 µmol of reducing sugars from 1 mL of culture broth in one minute under standard assay conditions.

Unit Calculation

Concentration = 1/dilution (= Volume of enzyme in dilution / Total Volume of Dilution). Estimated concentration of the enzyme which would release exactly 0.5 mg of glucose.

$$\text{CMC} = \frac{0.185}{\text{Enzyme concentration to release 0.5mg glucose}} \text{ Units ml}^{-1}$$

Cellobiase (β-glucosidase) activity was measured by incubating the total reaction mixture containing 20 µl of culture supernatant diluted in 20 µl of 50mM citrate buffer (pH 4.8) with 20 µl of 15 mM cellobiose (freshly prepared in 50 mM citrate buffer, pH 4.8). The final reaction mixture was then incubated at 50°C for 60 min. After incubation, 140 µL of 3,5-dinitrosalicylic acid (DNS reagent) was added according to the method of Miller (1959), and then plate was resealed with parafilm, and boiled at 100°C to develop colour and to stop the reaction. Substrate blank containing only the 20µl of 15 mM cellobiose solution in 40 µl of 50 mM citrate buffer; Reagent blank containing only 50 mM citrate buffer (60 µL) were also run along with samples. An enzyme blank containing 20 µL of enzyme supernatant plus 40 µL of 50mM citrate buffer without cellobiose substrate was also included to reduce the background reducing sugar absorbance. The micro-plate was cooled at room temperature using a cooling water bath and the reducing sugar absorbance at 540nm was measured using a micro-plate reader (Tecan Infinite 200 Pro, Switzerland). The sample values were corrected for reagent blank, substrate blank and enzyme blank values. A glucose standard curve with a concentration range of 0 to 2 mg/mL was also run. All the samples and the standards were run in triplicate, while the blanks were run in duplicate. Cellobiase activity (CB) was calculated following the concept that 0.0926

units of cellobiase will release 1.0 mg of glucose under standard assay conditions.

Unit Calculation

Concentration = 1/dilution (= Volume of enzyme in dilution / Total Volume of Dilution). Estimated concentration of the enzyme which would release exactly 1.0 mg of glucose.

$$\text{CMC} = \frac{0.0926}{\text{Enzyme concentration to release 1.0 mg glucose}} \text{ Units ml}^{-1}$$

2.2.7 Optimization of cellulase production under SmF using conventional (OFAT) approach

The cellulase production by the most potent cellulolytic organisms screened through both qualitative as well as quantitative screening tests, were further optimized following the conventional method of one factor at a time (OFAT) approach. The procedure was to optimize each variable independently and subsequently optimum conditions were used in each experimental run. The effect of various factors such as pH (3.0–10.0), incubation temperature (25–60^0C), inoculum size (4-16%), metal ions, surfactants and different carbon and nitrogen suppliments to enhance cellulase production was analysed. The experiments were conducted with the appropriate organism selected i.e., *Aspergillus ochraceus* (MTCC 1810).Three replicate experiments were run in parallel and cellulase assays were performed using DNS method (IUPAC) as described in the section 2.2.6.1 and the values were recorded as the mean of the three replicate (±) standard deviation.

2.2.7.1 Effect of pH

The pH of the fermentation medium was optimized by using various buffers of pH in the range of 3-10; citrate buffer (pH 3 to 6), phosphate buffer (pH 7 to 8) and glycine – NaOH buffer (pH 9 to 10). Inoculum size of 4% (v/v) *A. ochraceus* mycelial spores were inoculated into 250 ml-Erlenmeyer flasks containing 100 ml of delignified coir pith fermenting medium as described in the section 2.2.6. The flasks were placed in an orbital shaker (180 rpm) at 30^0C for an optimum incubation period of 7days. Then the medium was centrifuged at 12,000 rpm for 5 min to get mycelium-free supernatant. The supernatant thus obtained was saved as enzyme source to determine the cellulase activity (CMCase, FPase and β-glucosidase) using IUPAC protocol as described in section 2.2.6.1.

2.2.7.2 Effect of fermentation temperature

The fermentation medium was adjusted at the obtained optimum pH value and inoculated with 4% (v/v) mycelia spores. The inoculated flasks were incubated in an orbital shaker at 180 rpm at different temperatures (25, 30, 35, 40, 45, 50, 55 and 60^{o}C) for seven days. The cell-free supernatant obtained after centrifugation at 12,000 rpm for 5 min was stored for assaying individual cellulase activity such as CMCase, FPase and β-glucosidase using IUPAC protocol as described in section 2.2.6.1.

2.2.7.3 Effect of Inoculum size

The pure culture of *A. ochraceus* MTCC 1810 (3days old) maintained on the potato dextrose agar (PDA) plate was used for inoculum preparation. Two to three numbers of approximately 2cm^2 sections of mycelial mat was used to inoculate Reese and Mandel's Mineral based medium with composition similar to the production medium and incubated at 180rpm/30^{o}C

for 5 days in an orbital shaker to obtain a homogenous fungal spore suspension. This pre-inoculum was added at different inoculums sizes (4, 6, 8, 10, 12, 14 and 16% v/v) into Reese and Mandel's fermentation media supplemented with 1% chemically delignified coir pith and incubated under 35^0C at optimum pH 6.0. At the end of incubation period, cellulase activity was assayed as previously described in the section 2.2.6.1.

2.2.7.4 Effect of additional carbon sources on cellulase production

Different carbon sources such as sucrose, cellobiose, maltose, glucose, fructose, lactose, carboxy methyl cellulose and starch at (0.5% w/v) were separately added into the fermentation medium along with 1% chemically delignified coir pith to study their effects on cellulase production. Carbon sources were autoclaved separately at 10 lb pressure for 10 min and aseptically added into the medium. It was then inoculated with 10% (v/v) of optimized inoculum size and incubated at 35^0C /pH6.0 in an orbital shaker at 180 rpm. The medium without any additional carbon sources was used as the control. At the end of incubation period, the individual cellulase enzyme activity in cell-free culture supernatant was assayed using the IUPAC protocol as described in the section 2.2.6.1.

2.2.7.5 Effect of different concentration of the carbon sources

The carbon source which produced highest amount of reducing sugar was selected for further study. The concentration of this carbon source was varied from 0.25 to 1.5% (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5%).The medium was inoculated with 10% (v/v) of *A. ochraceus* spores and incubated under the optimum conditions. The culture supernatant was obtained by centrifugation at 12,000 rpm for 5 min and it was used for assaying cellulase activity as mentioned in section 2.2.6.1.

2.2.7.6 Effect of nitrogen source on cellulase production

The effect of different organic and inorganic nitrogen sources on cellulase enzyme production was determined by replacing the prescribed nitrogen source of the fermentation medium i.e., $(NH_4)_2SO_4$ and peptone. The nitrogen sources such as yeast extract, tryptone, beef extract, casein, ammonium sulphate, sodium nitrate, urea and peptone were added at 0.25% (w/v) and fermentation was carried out at 35°C, 180 rpm and pH 6.0.The crude enzyme was prepared as described above and cellulase activity was determined as in the section 2.2.6.1.

2.2.7.7 Effect of various concentrations of nitrogen source

The nitrogen source which supported maximum cellulase activity was selected for further optimization. Accordingly, the concentration of this nitrogen source was varied from 0.25% to 1.5% (0.25 %, 0.5%, 0.75 %, 1.0%, 1.25% and 1.5%) and supplemented into the 1% (w/v) delignified coir pith fermenting medium, which was then incubated under optimal conditions. The cellulase activities such as CMCase, FPase and β-glucosidase were determined from the cell-free culture supernatant according to the IUPAC protocol (section 2.2.6.1).

2.2.7.8 Effect of various metal ions on cellulase production

The effect of various metal ions on cellulase activity was determined by the addition of 5 mM of each metal ion such as calcium chloride (Ca^{2+}), cupric sulphate (Cu^{2+}) , ferric chloride (Fe^{3+}), magnesium chloride (Mg^{2+}), manganese chloride (Mn^{2+}), sodium chloride (Na^{+}), mercuric chloride (Hg^{2+}), Nickel sulphate (Ni^{2+}), lead sulphide (Pb^{2+}) and zinc sulphate (Zn^{2+}) into the fermentation medium by replacing the fixed metal ions of the fermentation medium. It was then incubated under optimum conditions of 35^{o}C at pH 6.0. for 7 days. Medium without the addition of any of the metal ions specified

above was kept as control. Samples were separated for enzyme assay under standard assay conditions as described in section 2.2.6.1.

2.2.7.9 Effect of surfactants on cellulase production

Different surfactants such as Tween 20, Tween 60, Tween 80, Triton X-100 and PEG 6000 at 0.4% (w/v) were added at the 3rd day of fermentation period (for eliminating its growth inhibition on the initial days of fermentation) to the fermentation medium for the of selection of a suitable surfactant for enhanced cellulase production. Medium without the addition of surfactants was kept as control medium. Individual cellulase activities such as CMCase , FPase and β-glucosidase were determined (section 2.2.6.1) from the culture supernatant stored as enzyme source.

2.2.7.10 Effect of various concentrations of surfactant on cellulase production

The surfactant which produced maximum cellulase activity was selected for further studies. Consequently, the surfactant concentration was varied from 0.2% to 1.2% (0.2 %, 0.4%, 0.6 %, 0.8%, 1.0% and 1.2%) and then added into the 1% (w/v) delignified coir pith fermenting medium. After incubation at 35^{o}C /pH 6.0 for 7 days, enzyme samples were analysed for CMCase, FPase and β-glucosidase according to the IUPAC protocol as described in section 2.2.6.1.

2.2.7.11 Statistical Analysis

All experimental points are the average of three independent experiments. Average and standard error of the mean were calculated from Microsoft Excel spreadsheet. Further, data were analyzed using one-way ANOVA followed by Brown-Forsythe post test of Graph pad prism 7.0. (Graph Pad software Inc., San Diego, CA). P value < 0.05 was considered as significant.

2.3 Results and Discussion

Qualitative plate screening methods are a pre-requirement, particularly when one have to screen out an appropriate microorganism from a large collection of potent microorganisms. Another important factor is that screening methods should not be expensive, laborious and inconvenient as reported by Leonid et al. (2004). Generally, plate-screening methods are based on the complex formation between polysaccharides and dyes (Teather and Wood, 1982) as soluble and insoluble dye-bound polysaccharides to produce detectable clear halo zone around the microbe-derived enzyme (Castro et al., 1995). The chromogenic plate screening methods offered comparatively simple tools for accurate detection of polysaccharide-degrading fungi without producing any artefacts.

2.3.1 Primary screening for cellulolytic enzyme activity

2.3.1.1 Screening on Phosphoric acid swollen cellulose (PASC) agar plates

Qualitative screening of cellulose-degrading activity was determined by measuring the ratio of hydrolysis zone diameter to the colony zone diameter i.e., zone size (ZS)/colony size (CS) (Teather and Wood, 1982; Peciulyte, 2007). The results indicated in Table 2, showed that the enzyme index value, i.e., ratio of degradation zone size to colony zone diameter ranged from 1.45 cm to 2.4cm on phosphoric acid swollen cellulose (PASC) agar plates, demonstrating the capability of cellulolytic microorganisms to degrade amorphous–type of cellulose or extracellular carboxy methyl cellulase (CMCase or endoglucanase) activity. Enzyme index value greater than one also indicated that their degradation zone diameter (cm) was larger than the size of its colony diameter (cm), representing a

greater hydrolytic effeciency. Highest enzyme index (EI) value was shown by *Pleurotus sapidus* (2.4 cm) followed by *Bacillus* sp. (2 cm) > *Fusarium oxysporum* (1.8 cm) > *Aspergillus ochraceus* (1.75cm) > *Paenibacillus macerans* (1.6cm) > *Chaetomium globosum* (1.45cm) > *Brevibacillus parabrevis* (1.33 cm) > *Cellulomonas cellulans* (1.1cm). The enzyme index value obtained in the present study is lower than those reported by Lu et al. (2004) and Gupta et al. (2012) who observed maximum EI value of 4.85-13.11cm and 9 to 9.8 cm respectively. In phosphoric acid swollen cellulose agar medium, the zone diameter linearly correlated well with the colony diameter of all the positive isolates and among them, *P. sapidus* and *Bacillus* sp. produced double the size of degradation zone with respect to its colony size, indicating its higher extracellular cellulolytic potential than other strains (Fig.1a & 1b).

2.3.1.2 Screening by turbidity method

The filter paper degradation assay was carried out in a mineral based medium with whatman No.1 filter paper as the only carbon source. Turbidity was observed due to the degradation of both amorphous and crystalline parts of filter paper substrate and the organisms were consequently selected as FPase or total cellulase producers. Highest amount of turbidity was observed in the isolate *Aspergillus ochraceus* MTCC 1810 (++++) followed by *Pleurotus ostreatus* (++), *Aspergillus ochraceus* MTCC 1877(++) and *Phanerochaete chrysosporium* (++) as shown in Table 2. The filter paper in the medium was fully ground or degraded by cellulolytic microorganisms and thus it turned into a pulpy or cloudy in appearance. However, the isolates such as *Cellulomonas cellulans* (+), *Aspergillus fumigatus* (+) , *Paenibacillus macerans* (+) , *Pleurotus sapidus* (+) and *Bacillus* sp. (+) produced slight turbidity in the filter

paper based cellulose medium. A positive turbidity test with respect to its control is shown in Fig.2. Till now, only a few micro-organisms have been reported for crystalline cellulose degradation *in-vitro*. The filter paper degrading capacity of the selected microorganisms indicated its ability to produce more than one type of cellulase, and thus it could be called as the crystalline cellulose degraders. Thus the organisms could be considered as more potent-cellulase producers as most of the model cellulolytic organisms studied till date are lacking in crystalline cellulose degradation.

2.3.2 Secondary screening with a mixture of chromogenic substrates

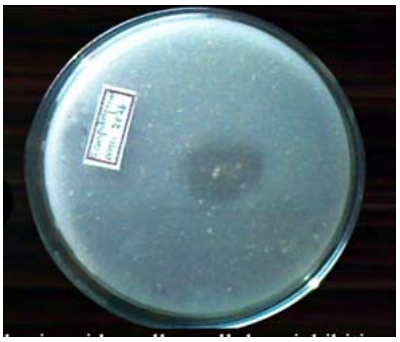
In secondary screening with insoluble chromogenic substrates, cellulolytic microorganisms were selected according to visible parameters such as solubilisation of dye and hydrolysis zones around the colonies. The highest enzyme activity index (EI) i.e., the solubilisation of cellulose incorporated with the dye, cibacron brilliant red 3BA, was produced by *Aspergillus ochraceus* (3.5 cm) followed by *Bacillus* sp.(2.1 cm) > *Fusarium oxysporum* (1.9cm) > *Paenibacillus macerans* (1.8cm) > *Chaetomium globosum* (1.75cm). Among the PASC-positive organisms, *C. cellulans*, *P. sapidus* and *B. parabrevis* could not solubilise chromogenic dye bound cellulose, which showed that they are incapable for insoluble cellulose degradation. Any of the PASC- negative isolates could not also hydrolyse the chromogenic insoluble cellulose. Therefore, this method could be considered as suitable screening method for the artefacts–free detection of cellulose degrading microorganisms. Furthermore, the degradation zone diameter was found to be greater than colony diameter which yielded hydrolysis enzyme activity index value greater than one as represented in Table 2 and Fig.3.

2.3.3 Rapid tube test

Rapid tube test is a simple and effective method for rapid detection of cellulolytic microorganims. It is based on the release of the dye from the dye bound cellulose by decolourizing it into bottom layers by the action of cellulolytic micro-organisms. The fungi such as *A. ochraceus* and *P. sapidus*, uncoupled the blue dye from dye-bound cellulose and subsequently decolourised within three days of incubation when compared to the other fungal and bacterial species. The speed of release and the diffusion of the dye into the basal layers of the medium revealed the efficiency of cellulolytic activity by these microorganisms. In addition, decolourization of the azure dye could be correlated with lignin modifying enzymes present in the organisms, which provided a simultaneous evaluation of both lignin and cellulose degrading activity i.e., *P. sapidus* and *A. ochraceus* were found to produce both cellulase and ligninase. *Bacillus* sp. produced only a partial decolourization or dissolution of the blue dye into the basal layers of the medium on its 3rd day of incubation, whereas *C. globosum* showed only one-fourth dissolution of the dye from the dye-bound cellulose. Cellulolytic species such as *P. macerans* and *F. oxysporym* showed no indication of dye release, and appeared same as the un-inoculated control tube (Fig.4). This method provides clear-cut data compared to other screening methods adopted here because it gives a rapid visual detection of cellulolytic organisms without any artefacts.

Table 2: List of microorganisms used in the qualitative screening for cellulolytic activity

Cat. No. (MTCC No:)	Species	Phosphoric acid-swollen cellulose zone of clearance EI	Turbidity method	Hydroxy ethyl cellulose Red EI	Rapid tube test with cellulose-azure dye
3025	*Streptomyces badius*	-	-	-	-
3027	*Streptomyces viridosporus*	-	-	-	-
1764	*Streptomyces albaduncus*	-	-	-	-
2826	*Sterptomyces cuspidovorus*	-	-	-	-
23	*Cellulomonas cellulans*	1.1	+	-	-
24	*Cellulomonas fimi*	-	-	-	-
2501	*Aspergillus flavus*	-	-	-	-
2483	*Aspergillus fumigatus*	-	+	-	-
1800	*Pleurotus fossulatus*	-	-	-	-
1810	***Aspergillus ochraceus***	**1.75**	++++	**3.5**	+
1877	*Aspergillus ochraceus*	-	++	-	-
2193	***Chaetomium globosum***	**1.45**	-	**1.75**	-
138	*Coriolus versicolor*	-	-	-	-
1755	***Fusarium oxysporum***	**1.8**	-	**1.9**	-
1756	*Fusarium solani*	-	-	-	-
2553	*Penicillium citrinum*	-	-	-	-
1805	*Pleurotus pulmonarius*	-	-	-	-
1804	*Pleurotus ostreatus*	-	-	-	-
1803	*Pleurotus ostreatus*	-	++	-	-
1807	***Pleurotus sapidus***	**2.4**	+	-	+
1806	*Pleurotus sajor-caju*	-	-	-	-
787	*Phanerochaete chrysosporium*	-	++	-	-
141	*Pleurotus sajor-caju*	-	-	-	-
142	*Pleurotus ostreatus*	-	-	-	-
145	*Daedalea flavida*	-	-	-	-
1370	*Scopulariopsis acremonium*	-	-	-	-
1469	*Scopulariopsis acremonium*	-	-	-	-
146	*Heterobasidion annosum*	-	-	-	-
1356	*Oidodendron echinulatum*	-	-	-	-
1334	*Phialophora hoffmannii*	-	-	-	-
136	*Trametes hirsuta*	-	-	-	-
137	*Pycnosporus sanguineus*	-	-	-	-
297	***Bacillus*** sp.	**2**	+	**2.1**	+
2708	***Brevibacillus parabrevis***	**1.33**	-	-	-
2294	***Paenibacillus macerans***	**1.6**	+	**1.8**	+
122	***Paenibacillus polymyxa***	-	-	-	-



(Fig.1a) **(Fig.1b)**

Fig.1a and lb. Inhibiton Zone on phosphoric acid swollen cellulose agar by cellulase producers

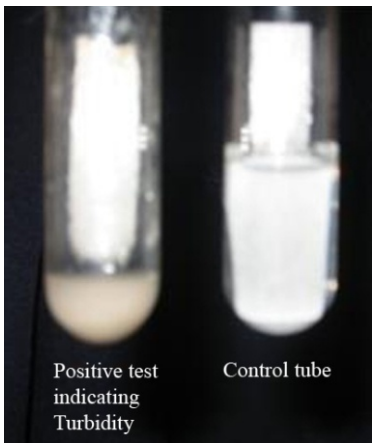
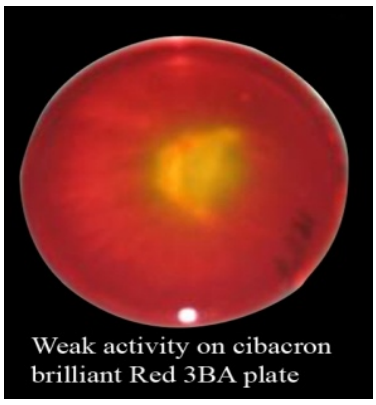


Fig.2. Degradation of the Filter paper by Cellulolytic organisms



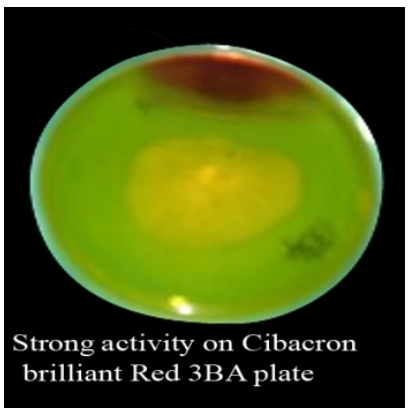


Fig.3. Insoluble chromogenic substrate such as Cibacron brilliant red 3BA-cellulose degradtion by cellulolytic strains

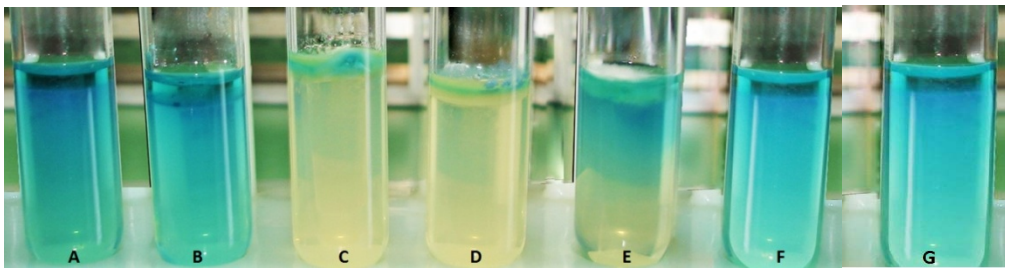


Fig.4. Decolorisation of Cellulose-azure dye in a Rapid tube test method, Tube A (control), Tube B (*Chaetomium globosum*) , Tube C (*Pleurotus sapidus*), Tube D (*Aspergillus ochraceus*) ,Tube E (*Bacillus* sp.), Tube F (*Paenibacillus macerans*) , Tube G (*Fusarium oxysporum*)

2.3.4 Production of extracellular enzymes through Submerged fermentation (SmF) - Quantitative screening of cellulase activity

The production of cellulase is a key factor in the hydrolysis of cellulosic biomass and it is very essential to make the process economically viable in the present energy scenario. Since the cost of the substrate plays a crucial role in the economics of an enzyme production, the cheap and easily available lignocellulosic material such as coir pith was used in this study aiming towards reduction in the cost of cellulase enzyme production. It is also found to be a value–added process since valuable biofuels are produced from agricultural industrial waste.

2.3.4.1 Proximate composition and Pre-treatment of coir pith

The proximate composition of the coir pith was analysed at Central Coir Research Institute (CCRI), Alappuzha and it is summarized in Table 3. From the results it was clear that coir pith contains cellulose, hemicellulose, nitrogen, phosphorous and organic carbon in reasonable quantities and the amount of lignin is 38.87%, which should be removed for better production of cellulases. The presence of lignin in significant amounts could overlap the cellulosic fraction making it unavailable for the fungus to act upon (Oberoi et

al., 2008). The optimized pre-treatment procedure with alkaline peroxide, 2% H_2O_2 at pH 11.5 for 10 hours (Rojith and Singh, 2012) was found suitable for further saccharification. The oxidative delignification was found to be practical for removing lignin (53%) and thus soften the material by reducing the crystallinity of the substrate, which enhance the accessibility for cellulase enzymes. Sterilization also found to be a suitable process for the pre-treatment of the substrate as it provides nutrients available for the fungus and bacterial strains for its growth and metabolism and these pre-treatments are aimed at loosening the highly crystalline structure of cellulose and extending the amorphous state. Since coir pith is not utilised for any commercial use but contains readily available sugars like glucose, xylose, arabinose and also cellulose, it could be an ideal substrate for cellulase production as reported by Muniswaran and charayalu (1994), Jabasingh and Nachiyar (2011). An important observation which could also be drawn from the results in Table 3 is that the essential elements like nitrogen, phosphorous, pottassium and organic carbon present in the coir pith could be beneficial for initiating growth of the microorganisms and subsequent enzyme production.

Fig.5. Pre-treatement of raw coir pith with 2% H_2O_2 at pH 11.5 to remove lignin (53%) (Rojith and Singh, 2012)- served as the substrate for saccharification in this study

Table 3: The proximate composition of delignified coir pith

Proximate composition	Content in %
Nitrogen	0.125
Phosphorous	0.003
Potassium	0.56
Organic Carbon	28.7
Lignin	38.87
Cellulose	21.85
Hemicellulose	1.89
Pectin	0.25

2.3.4.2 Screening of microorganisms using quantitative cellulase assay

From among thirty six isolates, four fungal strains and two bacterial strains showed degradation in more than one screening tests were selected for quantitative enzyme screening. The cellulolytic isolates selected were *Bacillus* sp. (MTCC 297), *Paenibacillus macerans* (MTCC 2294), *Fusarium oxysporum* (MTCC 1755), *Aspergillus ochraceus* (MTCC 1810), *Chaetomium globosum* (MTCC 2193) and *Pleurotus sapidus* (MTCC 1807). Although the plate screening, turbidity method, chromogenic dye solubilisation and rapid tube test method were sensitive enough for preliminary isolation and screening of cellulose degrading bacteria, but the clear zone width was not an accurate measure indicating the efficiency of cellulase activity. Most of the strains had shown good cellulolytic activity while their clear zones were smaller. This is in accordance with the report of Krootdilaganandh (2000), who showed that among 77 thermotolerant bacterial isolates grown on CMC agar, only one isolate CMU4-4 exhibited the highest enzyme activity whereas its clear zone was smaller than other isolates. The cellulolytic strains which produced no clearance zone on plate based screening tests and turbidity detection were eliminated from the further quantitative screening assays.

The selected microorganisms were subjected for cellulase activity assays such as exoglucanase (EC 3.2.1.91), β-1,4- endoglucanase (EC 3.2.1.4) and β-1,4-glucosidase (EC 3.2.1.21) by measuring the reducing sugar contents released from the substrates such as Whatman No.1 filter paper, Carboxy methyl cellulose and Cellobiose respectively.

2.3.4.2.1 Assaying filter paper activity for total cellulase production

The production pattern of total cellulase by both bacterial and fungal strains using delignified coir pith was shown in Fig.6. Fermentation experiments were run for ten days and the findings showed that total cellulase activity increased parallel with incubation time. Among the selected isolates, *Aspergillus ochraceus* produced highest amount of filter paper activity (8.317±0.026 FPU/ml) on the 7th day of fermentation period followed by *P. sapidus* (2.618±0.015 FPU/ml) on the 8th day, *Bacillus* sp. (0.883±0.007 FPU/ml) on the 6thday, *C. globosum* (0.835±0.009 FPU/ml) on the 9th fermentation day, *P. macerans* (0.056±0.006 FPU/ml) on the 7th day and *F. oxysporum* (0.049±0.006 FPU/ml) on the 8th day. The lowest FPase production by *F. oxysporum* was in agreement with the reports of Christakopoulos et al. (1994).The activity decrease after the optimum incubation period, might be due to the changes in the culture medium conditions during metabolic growth of the fungus coupled with depleted nutrient concentration which could result in cell death and subsequent denaturation of the enzymatic activity (Muniswaran and Charyalu, 1994). The results also suggested that fungal enzymes were more suitable for cellulose degradation than the bacterial enzymes. Moreover, highest FPase activity of *A. ochraceus* on coir pith residues, reported in our study was significantly higher than those reported from other *Aspergillus* strains studied so far. Cellulase production from various *Aspergillus* strains has been studied on solid or submerged conditions by Coral et al. (2002). Recently, an FPase activity of 1.02 U/ml and 3.2 U/ml

was reported from *A. fumigates* (Sarkar and Aikat, 2014) and *A. heteromorphus* (Singh et al., 2009) using rice straw and wheat straw biomass as growth substrates, respectively. Conversely, higher activities had been reported from the mixed fungal liquid culture studies using *T. reesei* and *Aspergillus phoenicis* (12.3 IU/l/hr) by Duff (1986) and also from *T. reesei* and *Aspergillus wentii* (9.7 IU/l/hr) grown on baggase residue by Panda et al.(1987). Xu et al. (2006) have reported filter paper activity of 171.12 U FPA/g dry koji on the third day of fermentation from a high-cellulase producing strain *Aspergillus glaucus* XC9. Ojumu et al. (2003) produced cellulase from *Aspergillus flavus linn* isolate using 1% NaOH treated baggase and highest enzyme production was recorded within twelfth hour of fermentation period. Several studies were also carried out to produce cellulolytic enzymes from lignocellulosic wastes by many microorganisms including fungi such as *Trichoderma, Penicillium, Aspergillus* sp. etc. by Mandels and Reese (1985), Hoffman and Wood (1985), Brown et al. (1987), Lakshmikant and Mathur (1990) etc. Similarly celluloytic property of bacterial species like *Pseudomonas*, *Cellulomonas*, *Bacillus, Micrococcus*, *Cellovibrio* and *Sporosphytophaga* sp. were also reported previously by Nakamura and Kappamura (1982) and Immanuel et al. (2006).

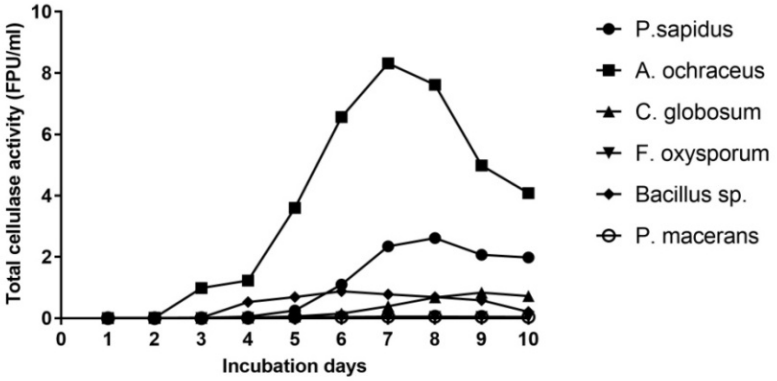


Fig.6. Quantitative screening of total cellulase activity (FPU/ml) by different cellulolytic microorganisms

2.3.4.2.2 Assaying carboxy methyl cellulase activity (β-1, 4- endoglucanase)

Similar to FPase production profile, CMCase production increased linearly with incubation time, peaked at its highest, and then decreased as shown in Fig.7. All isolates had shown higher CMCase activity compared to their filter paper activity, during the experimental time investigated and this observation was in line with the previous studies on different cellulolytic fungus with various substrates (Singh et al., 2009). Among the selected strains, *Aspergillus ochraceus* produced significantly higher amount of CMCase (11.75±0.015 U/ml) on the 7th day of fermentation period followed by *P. sapidus* (6.37±0.031 U/ml) on the 8th day, *F. oxysporum* (4.93±0.019 U/ml) on the 7th day, *Bacillus* sp. (2.85±0.014 U/ml) on the 6th day, *C. globosum* (2.19±0.012 U/ml) on the 9th day, and *P. macerans* (0.98±0.006 U/ml) on the 7th day. Comparable peak level of cellulase activity such as 7.5 U/ml, 6.88U/ml and 4.79 U/ml of CMCase had been obtained on the 7th and 8th day of fermentation of *A. candidus* for rice, millet and sawdust substrates respectively (Milala et al., 2009). CMCase produced by *Fusarium oxysporum* and *P. macerans* were approximately ten times higher than its FPase, whereas endoglucanase activity of *Bacillus* sp. and *C. globosum* were seventy percent higher than its FPase activity. Among the bacterial strains studied, *Bacillus* sp. produced highest CMCase followed by *P. macerans*. In general, fungal strains produced higher CMCase than bacterial strains and its cellulase activity was increased from 4th day to 7th or 8th day of fermentation period. The different enzyme production profile exhibited by the organisms might be due to their different genomic composition or physico-chemical and environmental conditions used during growth of the organism (Sudan and Bajaj, 2007).The enzyme activities obtained are comparable with those obtained by Shamala and Srikantaiah, 1986 by cultivating *Trichoderma viridae* on rice straw and *T.*

harzianum (6.35 IU /ml). However, the CMCase value reported in the present study was in line with Muniswaran and Charyulu (1994), who observed maximum CMCase of 12.05 U/g in seven days fermentation of *T. viride* on H_2O_2 treated coir pith under SSF. Findings of their study also revealed that the decrease in enzymatic activity after its optimum value, have been due to the deactivation of the enzymes, probably resulting from pH variation during fermentation, indicating acidic conditions prevailed in the fermentation medium. Jabasingh et al. (2014) had reported an endoglucanase activity of 28.64 U/g on 8g of coir pith from *A. nidulans* under solid-substrate optimized culture conditions, which is also comparable with the present observation.

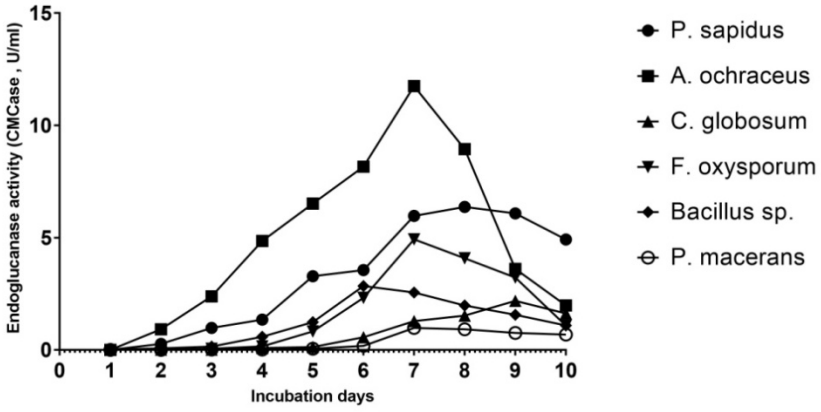


Fig.7. Quantitative screening of endoglucanase activity (CMCase) by different cellulolytic microorganisms

2.3.4.2.3 Assaying Cellobiase activity (β-glucosidase)

The cellobiase (β-glucosidase) activity produced by the segregated organisms is shown in Fig.8. Among the selected strains, *A. ochraceus* produced significantly higher cellobiase activity (9.86±0.023 U/ml) on the 7th day of fermentation period followed by *F. oxysporum* (3.582±0.027 U/ml) on its 9th fermentation day, *C. globosum* (2.357±0.024 U/ml) on 7th day, *Bacillus* sp. (1.816±0.025 U/ml) on 9th day, *P. sapidus* (0.912 ±0.008 U/ml) on 8th day and

P. macerans (0.853±0.049 U/ml) on 8th day of fermentation. In comparison to CMCase and FPase production, *P. sapidus* produced very low levels of β-glucosidase, whereas *A. ochraceus* and *F. oxysporum* produced an approximately equal amount of CMCase and β-glucosidase, which could be much useful for complete cellulose bioconversion. In addition, the high β-glucosidase to FPase ratio is important in simultaneous saccharification and fermentation to circumvent accumulation of the cellobiose as cellobiohydrolase inhibitor in the culture medium (Maeda et al., 2011; Ryu and Mandel, 1980). As a result, the rate and extent of cellulose hydrolysis were greater in *A. ochraceus* than other organisms used in this study. This observation found correlations with the findings of Lakshmikant (1990) and Persson et al. (1991). However, recent reports by Sarkar and Aikat (2014) ; Ahmady and Naggar (2015) have shown that *A. fumigatus*, produced highly active β-glucosidase (80.1 U/ml) and a new strain of *A. terreus* EMOO 6-4 showed a maximum β-glucosidase of 4457.162 U/g using rice straw as substrate under statistically optimized culture conditions. The cellulase activity produced by *A. ochraceus* was comparable to other *Aspergillus* strains studied till date; even though a direct comparison would be very difficult, since the physico-chemical and nutritional factors for enzyme production and nature of substrate greatly influence the enzyme activity (Sharma and Sreekantiah, 1986). Similar observations had been made by Coral et al. (2002), Onsori et al. (2005), Vanwyk (1998) and Lakshmikant (1990), on various natural lignocellulosic substrates.

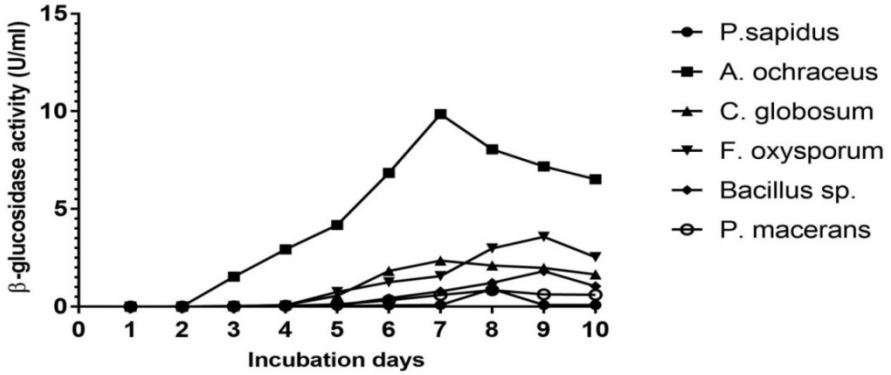


Fig.8. Quantitative screening of cellobiase activity (β-glucosidase) by different cellulolytic microorganisms

The results obtained from the quantitative screening of cellulase activities using coir pith as major substrate, indicated that it could be utilized as a cheap raw material for glucose production, with simultaneous cellulase production. The maxium CMCase (11.75±0.015 U/ml), FPase (8.317±0.026 U/ml) and β-glucosidase (9.86±0.023 U/ml) from the industrially important filamentous fungus *A. ochraceus* MTCC 1810, provides a complete cellulase for the bioconversion of delignified coir pith. On the basis of these results, *A. ochraceus* could be considered as the appropriate microorganism for utilizing pre-treated coir pith under submerged fermentation (SmF).The cellulase activity shown by this organism was comparable with that of *T. reesei* and other potent cellulolytic fungi and this could be scaled up by optimization of different fermentation parameters.

2.3.5 Optimization of cellulase production under SmF using conventional (OFAT) approach

The cellulase production by microorganisms can be influenced by a number of fermentation factors, including physical (pH and temperature) and chemical factors (macro and micro nutrients). Therefore, for obtaining high

titer of cellulase production, all major influencing factors were optimized in order to make the upstream production process more economical.

2.3.5.1. Effect of pH

pH is an important factor affecting cellulase production (Pardo and Forchiassin, 1999). Production of extracellular-cellulases by *A. ochraceus* through shake flask fermentation was investigated at varying pH levels ranging from 3-10, since pH has been considered as the most essential physiological parameter for high yield enzyme production as well as nutrient and enzyme transfer across the cell membrane (Kapoor et al., 2008) .The optimum CMCase (20.48 U/ml), total cellulase (13.67FPU/ml) and β-glucosidase (11.64 U/ml) were observed at pH 6.0. Beyond the optimum pH value, there was a progressive diminishing in enzyme activity, whereas decreasing pH values from 6.0 to 3.0, resulted in significant reduction in CMCase, FPase and β-glucosidase activities. Fifty percent of CMCase activity was reduced when the pH of the fermentation medium was increased from 6.0 to 10.0 (acidic to alkaline), whereas ninety percent reduction in enzyme activity could be observed on account of FPase and β-glucosidase. At next higher level of pH than its optimum i.e., when the pH was raised from 6.0 to 7.0, the production of CMCase remained more or less steady while FPase and β-glucosidase sharply decreased by more than 20%.Comparatively lower FPase and β-glucosidase synthesis with that of CMCase was probably due to the different cellular location of the enzymes which in turn brought about various cell-porousness and enzyme production (D'Souza and Volfova, 1982). The outcomes demonstrated in the Fig.9, shown that the fungus synthesised all the three individual cellulases in different proportions produced at same optimum pH could be sourced as a suitable enzymatic cocktail in heterogenous biomass conversion.

Many of the fungal strains exhibited a slightly acidic pH in the fermentation medium for its metabolism and enzyme biosynthesis (Haltrich et al., 2006), which is well correlated with the present study. Besides, Bansal et al. (2012) reported that optimum cellulase production by *A. niger* was acquired at pH 6.0 and 7.0 respectively. But, this is different from the previous reports by Shrivastava et al. (1984) who found an optimum pH of 5.5 for cellulase production by *A. sydowii*. Along these lines, it could be concluded that optimum pH for maximum fungal cellulase production is variable and is basically relies upon the type of carbon substrate and strain used for cultivation (Niranjane et al., 2007). The other prominent reason for influence of pH on cellulase production may be the hydrogen ion effect and the stability (Kalra and Sandhu, 1986).

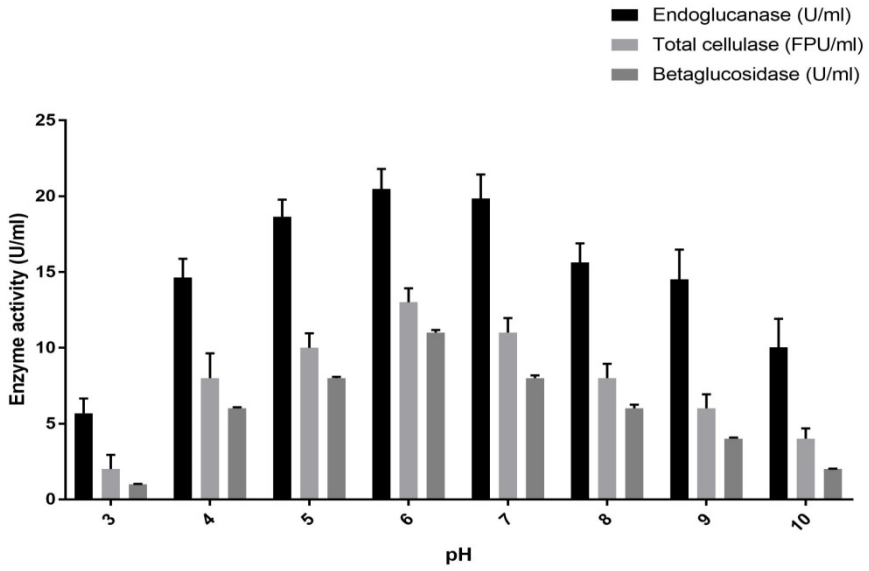


Fig.9. Effect of pH on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

2.3.5.2 Effect of Temperature

Temperature has a noticeable effect on lignocellulosic bioconversion. The temperature for assaying cellulase activities are usually ranged between 50–65°C for various microbial strains (Menon et al., 1994; Steiner et al., 1993), whereas growth temperature and enzyme production of these microbial strains was found to be 25–30°C and 30-65^{0}C respectively (Macris et al., 1989). Enzymes have an optimum temperature at which their maximum activity is displayed and at higher or lower temperatures, enzyme deactivation occurs. The optimization of fermentation temperature for cellulase production through submerged fermentation at different temperatures (25, 30, 35, 40, 45, 50, 55 & 60^{o}C) indicated that endoglucanase and total cellulase production gradually increased from 25 to 40^{0}C and thereafter a gradual decrease in enzyme activity was observed upto 60^{0}C (Fig.10). The optimum enzyme production of endoglucanase (25.57 U/ml) and total cellulase (12.98 FPU/ml) was observed at 35^{o}C, whereas β-glucosidase (15.83 U/ml) production was highest at 40^{o}C. Of all the enzyme activities tested, CMCase activity was drastically reduced above 40^{o}C, whereas FPase and β-glucosidase activities remained more or less stable from 40 to 60^{o}C. Hanif et al. (2004) also reported an increase in cellulase production from *Aspergillus niger* up to 30^{o}C and thereafter the production declined. There was 30% reduction in activity at 45^{0}C which further reduced by 20% at 50^{0}C. However, Bastawade (1992) have reported a similar temperature optimization profile at 40^{0}C for highest FPA, endoglucanase and β-glucosidase production. In addition, previous studies have shown that temperature affects the cellulose-cellulase adsorption behaviour. There was a direct relationship observed between adsorption and saccharification of cellulosic substrates at temperatures below 60°C. But at temperatures greater than 60^{o}C, the adsorption characteristic was possibly

decreased due to the loss of enzyme configuration leading to denaturation of the enzyme activity (Van-Wyk, 1997).

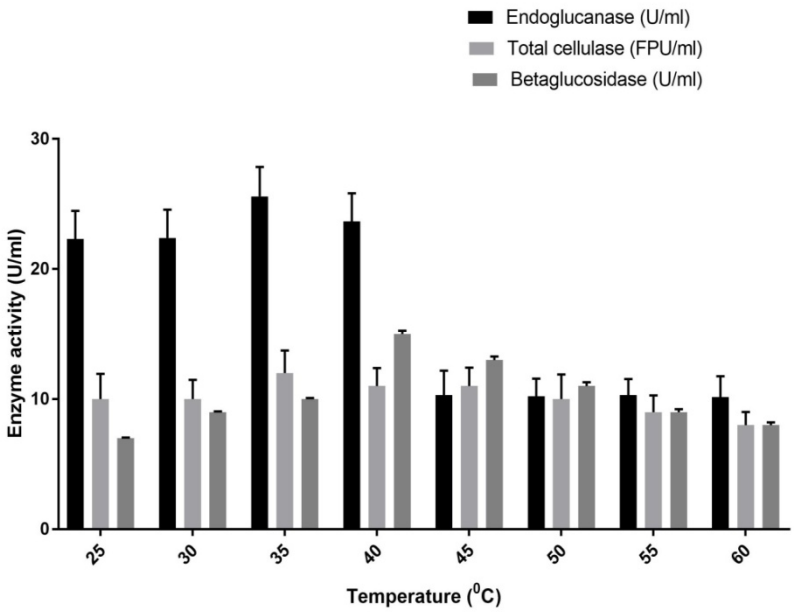


Fig.10. Effect of temperature on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

2.3.5.3 Effect of Inoculum size

Inoculum size plays a significant role in liquid state cultivation and subsequent enzyme production. An optimum level of inoculum is needed for initiating fungal sporulation and its metabolic activities. A lower inoculum size than its optimum resulted in longer lag phase in fungal multiplication and insufficient substrate utilization for production of desired enzymes where as higher inoculum size may lead to competitive inhibition and thereby enzyme inactivation and nutrient depletion (Sabu et al., 2005).This could overcome by maintaining the balance between proliferating fungal biomass and substrate utilization as reported by Ramachandran et al. (2004). In the present study maximum cellulase activity was observed at an inoculums size of 10% (v/v)

such as CMCase (33.92±1.68 U/ml), FPase (20.17±1.38 U/ml) and β-glucosidase (15.68 ± 1.05 U/ml) and is represented in Fig.11. Significant increase in enzyme production was observed when the inoculum size was increased from 4 to 10% (v/v). However, it decreased gradually when the inoculum size was further increased from 10% (v/v) to 16% (v/v), thus showing its optimum inoculum size was at 10% (v/v). Of all the three enzyme activities tested, β-glucosidase activity was found to be less dependent upon inoculum size. Similar results have also been reported from *Aspergillus niger* and *Trichoderma harzianum* on pretreated wheat straw under SSF (Sherief, 2010). Accordingly, Fadel (2000) reported maximum cellulase activity (216.2 IU/g) at 10% inoculum size with wheat straw as substrate. Omojasola and Jilani (2008) reported optimum cellulase activity with 8% inoculum size.

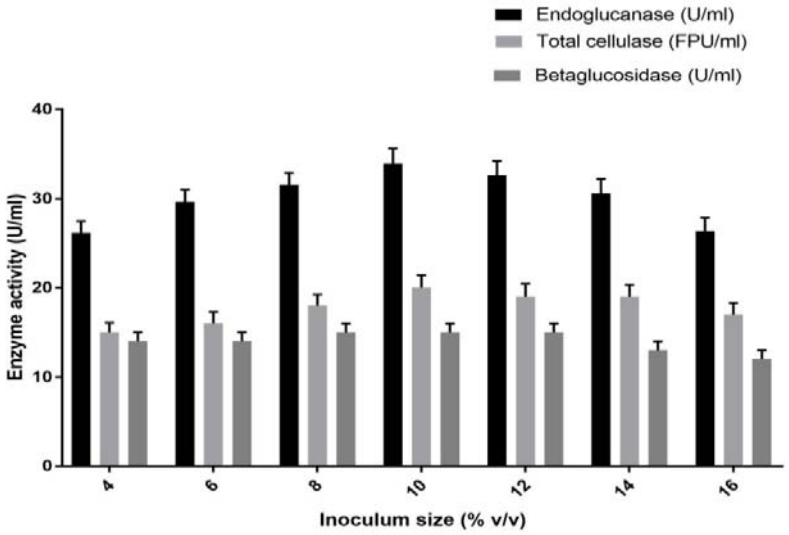


Fig.11. Effect of inoculum size on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

2.3.5.4 Effect of additional carbon sources on cellulase production

The effect of carbon sources on cellulase production was investigated by adding different sugars in the fermentation medium. Sugars may act as inducers

or repressors for enzyme biosynthesis. The effect of different sugars on cellulase production by *A. ochraceus* is illustrated in Fig.12. The media supplemented with 1% chemically delignified coir pith served as the control medium at 35 ^{o}C/pH 6.0 and the medium additionally supplied with different sugars such as sucrose, cellobiose, maltose, glucose, fructose, lactose, cellulose and starch at 0.5% (w/v) were analysed for individual cellulase activity. Among the sugars tested, carboxy methyl cellulose (CMC) was found to induce maximum production of CMCase (35.42 U/ml), FPase (22.51 U/ml) and β-glucosidase (15.58 U/ml). CMC had been found as the good inducer for CMCase activity followed by cellobiose, lactose, maltose, sucrose and fructose. Approximately 40% and 56% of CMCase activity was repressed by glucose and starch respectively. A different induction pattern was observed in the case of FPase activity, in which CMC gave better induction followed by sucrose , maltose, lactose, cellobiose and fructose, whereas the activity was repressed by starch (23%) and glucose (30%) at 0.5% (w/v). However, β-glucosidase activity was neither stimulated nor repressed in the presence of CMC, but slightly repressed by cellobiose (13%), maltose (16%), sucrose (23%), lactose (24%) and fructose (28%) and strongly inhibited in the presence of starch (63%) and glucose (66%). The results proposed that CMC was found to be the strongest inducer for all the three cellulase activities whereas addition of starch and glucose significantly reduced the enzyme activity. Similar results were obtained in the case of a consortium of *A. niger* and *T. viride* (Ikram-ul-Hag et al., 2006) and *A. niger* (Narasimha et al., 2006). However, contrary observations by Rabinovich et al. (2002) and Rao et al. (1983) reported that addition of CMC in the cultivation medium showed no effect on the enzyme yield.

It was also observed that most of the externally added sugar sources are fully utilized as evidenced by background reducing sugars present in the

substrate and enzyme blanks. Except CMC, maltose, lactose and sucrose, all other sugars were completely exhausted after 3rd day of incubation. Only a partial utilization of the CMC was observed during the whole experiment, but it induced more cellulase than other tested sugars, which suggested the negligible requirement of this sugar for proper enzyme induction. Of all the sugar suppliments, glucose was utilized faster from the medium over 2days followed by cellobiose and fructose (3days). Similar observations have been made by Mandels and Reese (1965), who attributed low yields from glucose and cellobiose to its faster and complete utilization, while in the present study CMC gave more yield since its slow consumption by the fungus.

Based on the above experiment, various concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5%) of CMC were added to select the best induction concentration for *A. ochraceus* cellulases along with 1% chemically delignified coir pith and the results are depicted in Fig.13. CMCase activity of *A. ochraceus* MTCC 1810 was gradually increased starting at 0.25%, peaked at about 0.75%, and repressed when the concentration was increased upto 1.5%, whereas FPase and β-glucosidase activity were peaked at about 0.5% and then repressed gradually when the CMC concentration was increased upto 1.5%. Chellapandi and Himanshu (2008) have also observed a similar behaviour due to the reduced oxygen supply to the cells in the high viscosity CMC-supplimented medium which in turn resulted in reduced cell growth and enzyme-catabolite repression. Talboys (1958) have also reported similar induction and repression of cellulase by various sugars in different types of fungi.

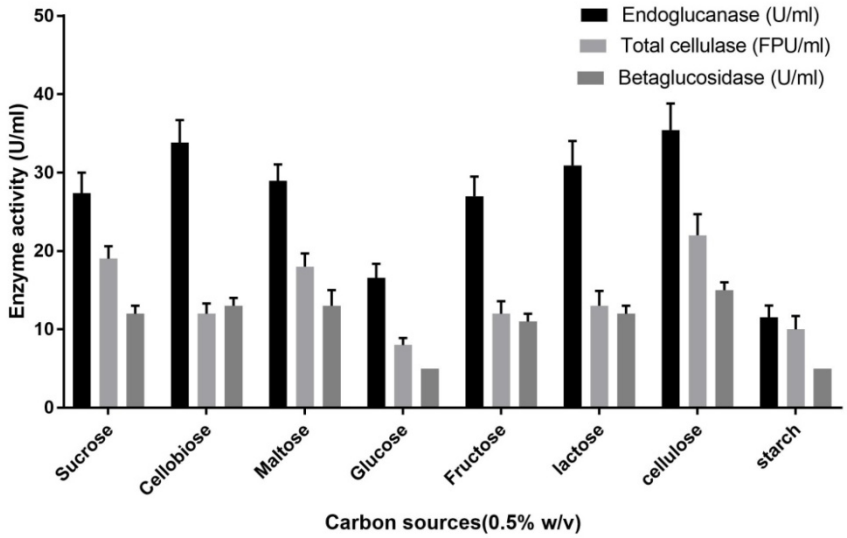


Fig.12. Effect of additional carbon sources on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

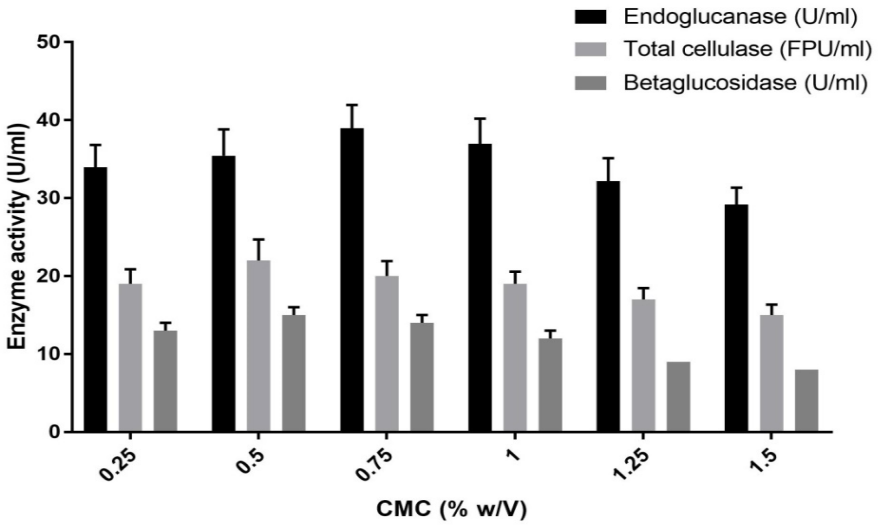


Fig.13. Effect of different concentration of CMC (%w/v) on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

2.3.5.5 Effect of nitrogen sources on cellulase production

The effect of nitrogen sources on cellulase production was variable with respect to the fungi and nitrogen sources supplemented (Kachlishvili et al., 2006). A significant effect on enzyme production was observed with different concentrations of nitrogen sources (Panagiotou et al., 2003). The effect of different organic and inorganic nitrogen sources (2.5g/L) on cellulase production was studied and is depicted in Fig.14. Different organic nitrogen sources such as yeast extract, tryptone, beef extract, casein, urea and peptone and inorganic nitrogen sources such as ammonium sulphate and sodium nitrate were incorporated in Rees and Mandels mineral salt medium with 1% chemically delignified coir pith to select a suitable nitrogen source for optimum production of cellulases. Among various organic nitrogen sources, urea caused highest stimulation of CMCase (42.59 U/ml) and FPase (24.97 U/ml), whereas β-glucosidase was maintained at the same level as that of control medium. Peptone and urea were found to exhibit an equal effect on β-glucosidase (15.96 U/ml and 15.17 U/ml respectively) when compared to control medium. In the case of inorganic nitrogen source supplementation, ammonium sulphate would provide apparently very low or inhibited production of CMCase (30.61U/ml), FPase (19.36 U/ml) and β-glucosidase (13.98 U/ml), which recommended very lower consumption of inorganic nitrogen source by *A. ochraceus* MTCC 1810 for cellulase enzyme secretion. Supplementation of other organic nitrogen sources such as tryptone, casein, yeast extract and beef extract could not produce any increase in CMCase, FPase and β-glucosidase yield as compared to control. Considering individual cellulase activities, CMCase and FPase were found to be dependent on the

nitrogen source whereas β-glucosidase production was appeared to be independent of nitrogen supplementation. The results obtained in the present study was in agreement with the observations of Deswal et al. (2011) who found that urea is the best nitrogen source for maximum CMCase production and inorganic nitrogen sources did not provide any significant increase in enzyme production. Contrary observations had been made by Sasi et al. (2012) who observed that inorganic nitrogen source such as ammonium sulphate stimulated cellulase production in *Aspergillus flavus* than organic nitrogen sources. Similarly, Rajoka et al. (2003) reported KNO_3 and NH_4NO_3 as the best nitrogen sources for cellulase production in *Cellulomonas flavigena* and *Thermomonospora fusca* (Spiridonov and Wilson, 1988). $(NH_4)_2SO_4$ was observed to be the best nitrogen source for endoglucanse and exoglucanase production by Kocher (2008) and Vyas and Vyas (2005). Conversely, Menon et al. (1994) observed a significant reduction in cellulase activity in the presence of ammonium salts as the nitrogen source.

According to the results obtained in the above experiment, different concentrations of urea (0.25% to 1.5% w/v) were supplied into the fermentation medium for maximum cellulase production. This showed a remarkable difference in enzyme activity which proved that high cellulase yield is concentration dependent. The optimum CMCase (46.83±1.69 U/ml), FPase (28.95±1.93 FPU/ml) and β-glucosidase (19.76±0.86 U/ml) were observed at 0.75% urea and a gradual decrease in enzyme production was observed beyond 0.75% to 1.5% of urea concentration (Fig. 15).

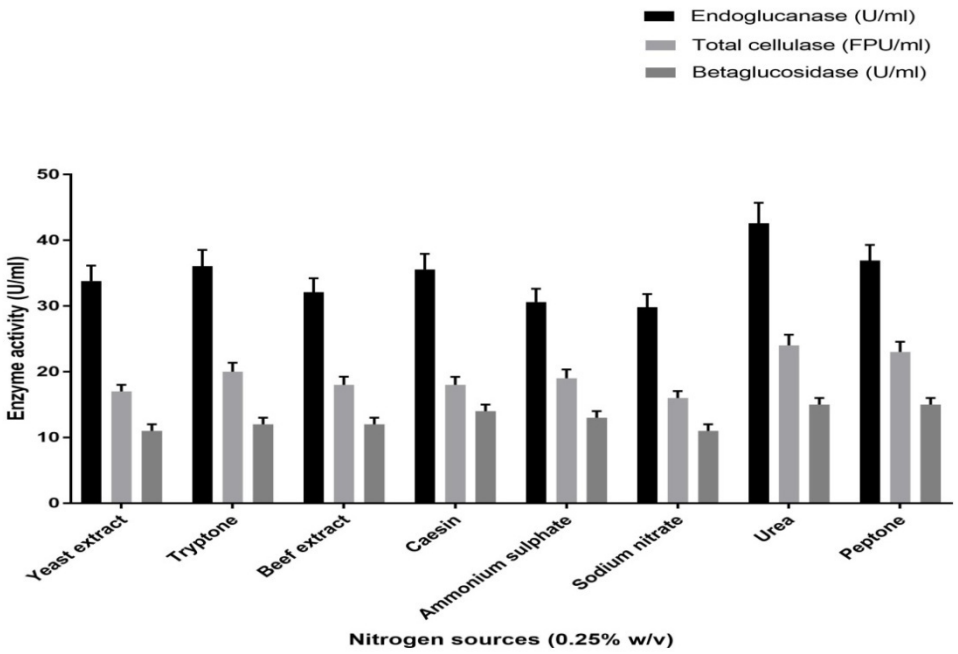


Fig.14. Effect of different organic and inorganic nitrogen sources on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

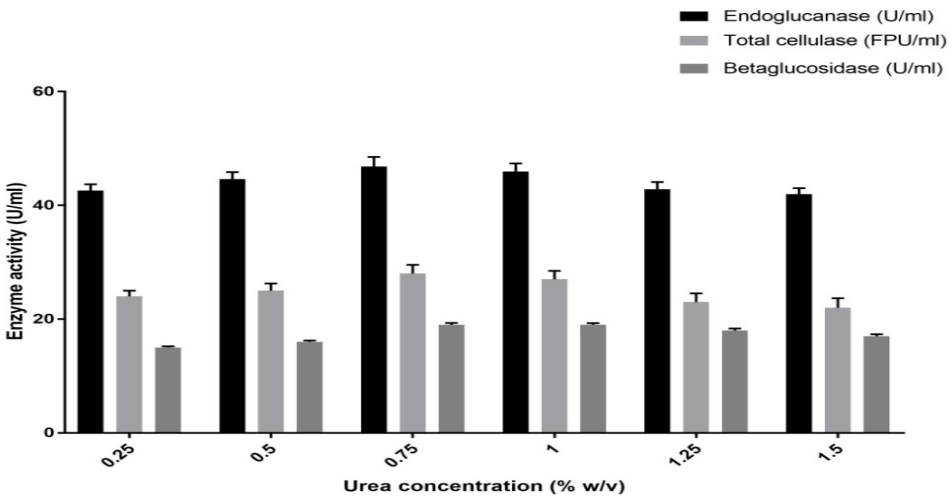


Fig.15. Effect of different concentrations of urea (% w/v) on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

2.3.5.6 Effect of metal ions on cellulase production

Metal ions play a vital role in enzyme catalysis by binding directly to enzyme active site or indirectly by maintaining the enzyme structure in a poised conformation. Presence of metal ions in the fermentation media had shown stimulatory or inhibitory effect on cellulase production. In the present study various metal ions (5mM) were added to the mineral based medium to determine their effects on cellulose saccharification (Fig.16). Of the various metal ions tested, ferric ions (52.95±2.01 U/ml), magnesium (51.63±1.83 U/ml) and nickel ions (50.27±1.49 U/ml) supported maximum stimulation of CMCase production, while sodium (31.67±1.35 U/ml), calcium (30.61±1.15 U/ml) and cupric ions (30.56± 1.22 U/ml) exhibited maximum FPase production. The addition of cupric ions (20.74±1.06 U/ml), magnesium ions (20.13±1.26 U/ml) and nickel ions (19.97 ± 1.46) had a slight stimulatory effect on β-glucosidase production where as other metal ions inhibited its production. The addition of Hg^{2+} to the fungal culture medium was found to exert a strong inhibition on CMCase (60%), FPase (63%) and β-glucosidase (69%). According to the results obtained in this study, there was no common effect of metal ions on the three enzyme activities but a varied level of effect on enzyme production by different metal ions were observed. The heavy metal ion, Hg^{2+} known as a "fatal enzyme inhibitor" (Tejirian and Xu et al., 2010; Lehninger, 1982), had additionally confirmed the results of our observations. Most of the stimulatory metal ions stabilize enzyme-substrate binding site as well as the catalytic site confirmation whereas heavy metals bind with the enzyme thiol (-SH) groups thereby inhibiting enzyme activity (Mandels and Reese., 1965; Jellison et al., 1997). Comparable stimulatory and inhibitory effect of metal ions was reported by Kim et al. (2001), Tao (2001) and Kalra and Sandhu, (1986). On the contrary, cellulase production by *Trichoderma*

harzianum C-4 was neither stimulated nor inhibited in the presence of Mg^{2+}, Co^{2+}, Ca^{2+} and Zn^{2+} (Yun et al., 2001), while Sindhu et al. (1986) had reported about strong inhibitory effect of Cu^{2+} on endoglucanse, exoglucanase and β-glucosidase production.

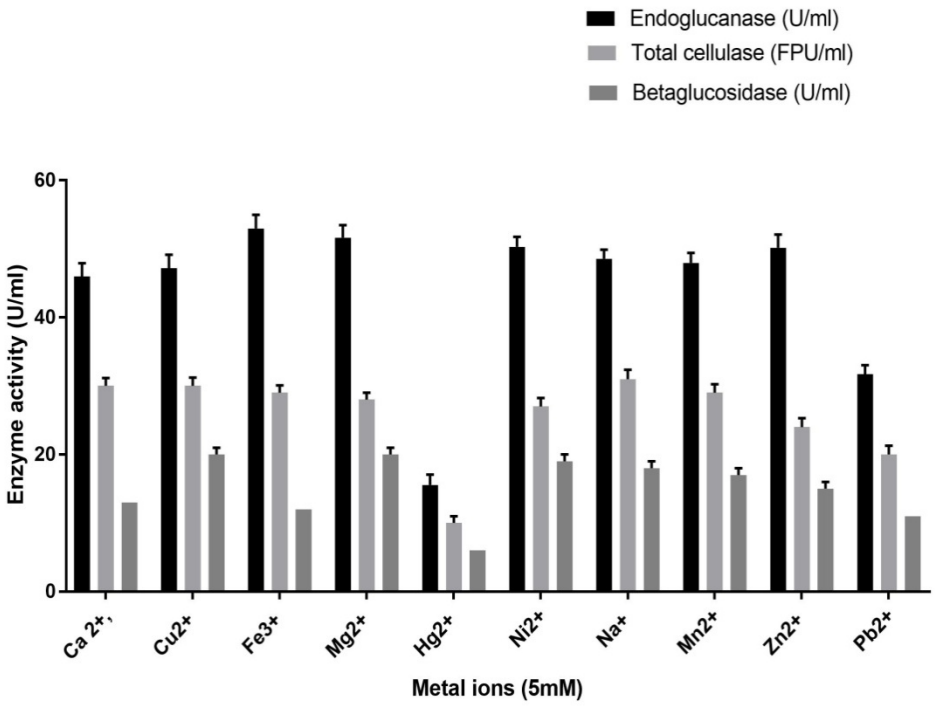


Fig.16. Effect of different metal ions on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

2.3.5.7 Effect of surfactants on cellulase production

Surfactants stand out from other physiological parameters because of its high enzyme induction property (Kapoor et al., 2008). It increases the bacterial cell membrane permeability and thereby helps in membrane transport as well as enzyme secretion into the production media (Okeke and Obi, 1993). Different surfactants at 0.4% w/v such as Tween 20, Tween 60, Tween 80, Triton X-100 and PEG 6000 were used to study their effects on cellulase

production. These were added at the third day of incubation since it slightly affected the growth of the fungus in the production medium (Reese and Manguire, 1969). Among the surfactants used, Tween 80 caused maximum production of CMCase (62.35±1.97 U/ml), FPase (36.17±1.36 U/ml) and β-glucosidase (21.58±1.21 U/ml) followed by Tween 60, PEG 6000,Tween 20 and Triton x-100 (Fig.17). In the present experiment, the control medium produced only 53.67 U/ml of CMCase, 30.96 U/ml of FPase and 19.83 U/ml of β-glucosidase, whereas Tween 80 supplemented medium enhanced the cellulase activities by 13%, 14% and 8% respectively on endoglucanase, total cellulase and β-glucosidase production. All surfactants had increased CMCase and FPase, whereas β-glucosidase was found slightly inhibited by Tween 20 as compared to control. Different surfactants showed varying cellulase activities as their secretion is more depend upon the cellular location of different enzymes (Reese and Manguire, 1969). The presence of surfactant also minimizes the irreversible binding of cellulases to the substrate and promotes the release of cell bound enzymes to the fermentation medium (Chandra et al., 2007). Enhancement of cellulase production through surfactant addition has been reported previously by Kuhad et al. (1994); Menon et al. (1994) and Pushalkar et al. (1995). Reese and Manguire (1969) also reported that the competence of Tween 80 in increasing cellulase yield would vary from organism to organism and also for different enzymes of the same organism.

The effect of different concentrations of Tween 80 on cellulase production by *A. ochraceus* MTCC 1810 was illustrated in Fig.18. Cellulase production increased significantly when the surfactant concentration was raised from 0.2% to 0.8%, peaked at about 0.8%, and decreased gradually from 0.8% to 1.2%. Maximal yield of CMCase (65.35±1.77 U/ml), FPase (38.40 ±1.24 U/ml) and β-glucosidase (23.16±1.26 U/ml) was achieved with

same concentration (0.8%) of Tween 80. Moreover, decreased cellulase activity at higher concentrations of Tween 80, revealed the secretion of other extracellular proteins which might co-repressed or conceal the targeted cellulase activity. This observation proposed that higher concentration of surfactants could increase cell membrane permeability which intensified liberation of extracellular and cell-bound cellulase proteins in to the culture medium (Pardo, 1996; Reese and Manguire, 1969). In contrast to the present study, Wen et al. (2005) have reported that 0.2% of Tween 80 was optimum for cellulase and β-glucosidase production by mixed culture of *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure and Hari Krishna et al. (2000) recorded about 1% and 0.2% (v/v) of Tween 80 for optimum cellulase production by *Trichoderma reesei* strain QM-9414 and *Streptomyces flavogriseus*. These findings again intensified the fact that surfactant stimulation varied from organism to organism and also from different proteins of the same organism.

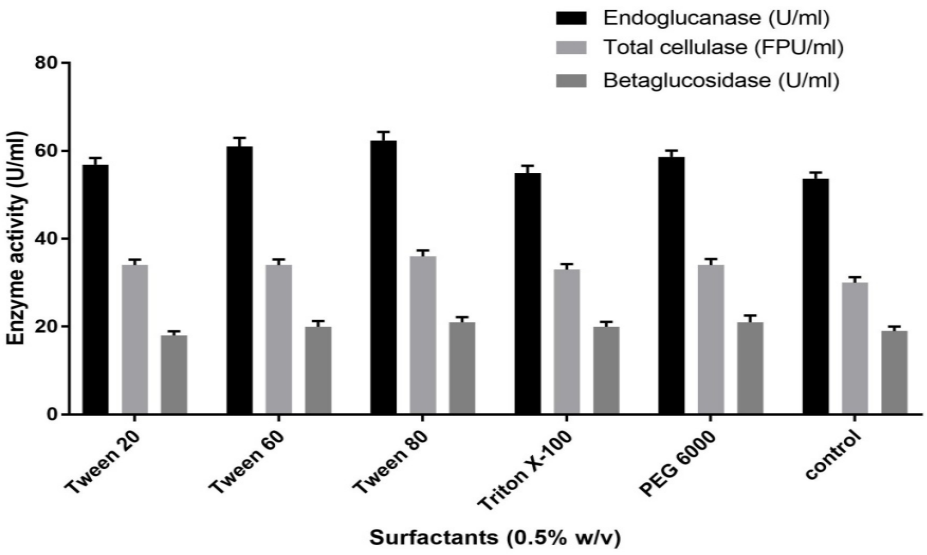


Fig.17. Effect of different surfactants on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

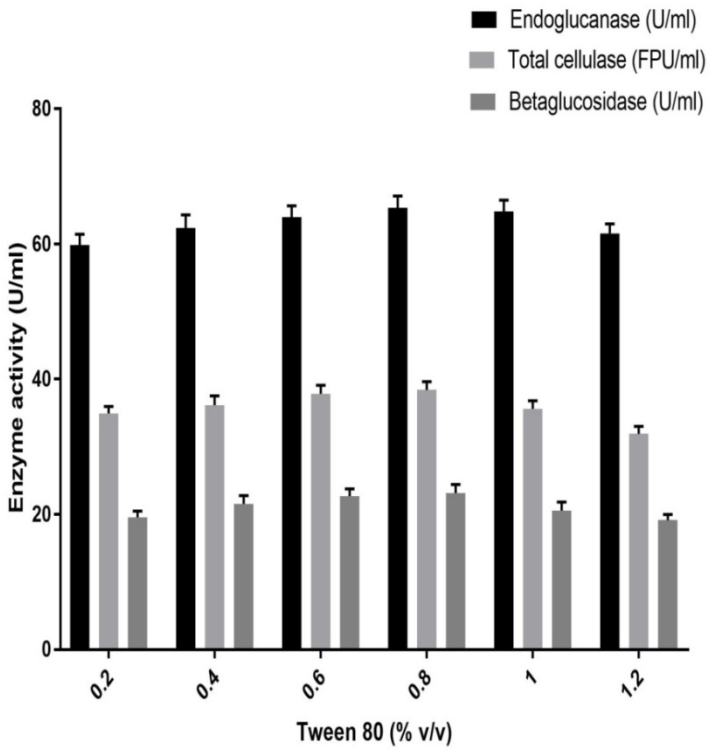


Fig.18. Effect of different concentrations of Tween 80 on cellulase activity by *A. ochraceus* MTCC 1810 using pre-treated coir pith as substrate

2.3.6 Statistical analysis

Statistical analysis by one-way ANOVA test indicated that, the effect of various fermentation parameters on cellulase enzyme production was found to be statistically highly significant (P value $\leq$ 0.05) using Graph pad prism 7.0., (Graph Pad software Inc., San Diego, CA).

2.3.7 Conclusion

Preliminary qualitative cellulase screening tests showed that only four fungal and two bacterial strains could produce detectable cellulase activity from among thirty six cellulolytic isolates procured from Microbial Type Culture Collection , IMTECH, Chandigarh and the following organisms *viz*.,

Bacillus sp. MTCC 297, *Paenibacillus macerans* MTCC 2294, *Fusarium oxysporum* MTCC 1755, *Aspergillus ochraceus* MTCC 1810, *Chaetomium globosum* MTCC 2193 and *Pleurotus sapidus* MTCC 1807 were selected after preliminary screening and subjected for quantitative enzymatic screening using delignified coir pith as cellulose substrate .Among them, *A.ochraceus* MTCC 1810 was selected for cellulase production optimization based on its highest yield of CMCase (11.75±0.015 U/ml), FPase (8.317±0.026 U/ml) and β-glucosidase (9.86±0.023 U/ml) . After optimization of culture conditions, the initial activity was raised to 65.35±1.77 U/ml, 38.40 ±1.24 U/ml and 23.16±1.26 U/ml respectively through conventional OFAT approach. The optimum cellulase activity was obtained at pH 6.0 with an optimum temperature of 35^{o}C for endoglucanase and total cellulase, whereas β-glucosidase was at its optimum at 40^{o}C with an optimum inoculum size of 10% (v/v). Other nutritional parameters such as 0.75% urea (nitrogen source), 0.5-0.75% carboxy methyl cellulose (sugar supplimentation), 5 mM of metal ions such as Fe^{3+}, Mg^{2+} , Ni^{2+} and Zn^{2+} and 0.8% Tween 80 (surfactants) were required for optimum cellulase secretion from *Aspergillus ochraceus* MTCC 1810. The highest fold of enzyme activity after production optimization than its primary activity has evidently supported the effect of fermentation process variables on the yield of cellulases.

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Chapter 3

PURIFICATION AND CHARACTERISATION OF A PROCESSIVE-TYPE ENDOGLUCANASE AND β-GLUCOSIDASE FROM *Aspergillus Ochraceus* MTCC 1810 THROUGH BIOCONVERSION OF DELIGNIFIED COIR PITH INTO GLUCOSE

Contents

3.1 Introduction

The prime significance in using fungi for industrial cellulase production comes from their capability to secrete large amounts of cellulases into the fermentation medium with the target of easy isolation and purification. Microorganisms of the filamentous fungal genera *Trichoderma* and *Aspergillus* spp. have been considered as the hypercellulolytic model microorganisms for cellulose degradation (Gusakov, 2007; Zhang et al., 2015). The crude enzymes produced by these microorganisms are commercially available in the world enzyme market (Mantyla et al., 1998; Godfrey and West, 1996; Uhlig, 1998). The genome database of *T. reesei* revealed two exoglucanases and eight endoglucanases (Ouyang et al., 2006; Foreman et al., 2003), but lacking in β-glucosidase activity, and majority of them are observed to be intracellular with low expression levels (Ouyang et al., 2006). In contrary, *Aspergillus* genome sequence has uncovered the vicinity of large

number of cellulase and hemicellulase genes as well as high amounts of extra cellular β-glucosidase. As per reports, *Aspergillus* contains genes encoding 5 exoglucanases, 22 endoglucanases, 18 β-glucosidases , 7 xylanases and other putative cellulose degrading genes with conserved domains and this undeniably recommends the occurrence of multiple cellulase genes in *Aspergillus* species (De Vries et al., 2001 ; Kumar and Parikh , 2015). For example, *A. terreus* DSM 826 produced significant amounts of varied cellulases (viz., endoglucanase and cellobiohydrolase), when grown on media containing corn cobs, corn stalks, rice straw or sugar cane bagasse as carbon substrates (Hassan et al., 2008). Another purified endoglucanase (31 KDa) from *Aspergillus glaucus* XC9, grown on 0.3% sugar cane bagasse as a carbon source, showed wide pH stability and optimum temperature at 50^0C (Tao et al., 2010). It also shares some common properties with that of industrial cellulase producing fungi, such as *A. niger* and *T. reesei* signifying its possible industrial application.

The most widely accepted synergism is a sequential action, it is presumed that endoglucanase initiates attack on cellulose to form new chain ends, which then serve as attack points for processive hydrolysis by cellobiohydrolase. Endoglucanase and cellobiohydrolase hydrolyze insoluble cellulose on the cellulose surface to soluble cellodextrin, followed by β-glucosidase-mediated hydrolysis of cellobiose to glucose (Lynd et al., 2002; Zhang and Lynd, 2004).The accessibility of cellulose to the reactive sites on the surface of native cellulose is a rate-limiting factor in the enzymatic hydrolysis (Sánchez et al., 2004). The adsorption capacity of the individual cellulase is reflected by their modular structure, most of which consist of at least a catalytic domain and a CBM. Different endoglucanases have different preference for cellulose regions to bind or the site of glycosidic bond to cleave

due to the difference in CBM specificity, multiplicity, linkage to the catalytic module. There is no significant synergism on the purified homogeneous cellulose among different endoglucanases, whereas the endoglucanases are beneficial to degradation on polymorphous and heterogeneous cellulose (Wilson, 2008). For instance, endoglucanase Cel12 without CBM acts first on amorphous in the outer part of cellulose, and endoglucanase with CBM then acts on crystalline in the inner part of cellulose (Rabinovich et al., 2002).

Another important property of cellulases is the "processivity", that is, the ability to remain attached to the substrate in between subsequent hydrolytic reactions (Davies and Henrissat, 1995; Robyt and French, 1967; Rouvinen et al., 1990; Teeri, 1997). Such a mechanism is considered to be favourable for the hydrolysis of crystalline cellulose since the enzyme remains closely bound with the detached single polymer chain in between subsequent hydrolytic steps (Harjunpää et al., 1996; Teeri, 1997; von Ossowski et al., 2003), which resulted in formation of disaccharides due to the 180^0 rotation between consecutive sugar units (Davies and Henrissat, 1995; Rouvinen et al., 1990). Processive cellulases from *Trichoderma reesei* have been widely investigated previously by Divne et al., 1998; Harjunpää et al., 1996; Igarashi et al., 2008, 2011; Imai et al., 1998; Kipper et al., 2005; Koivula et al., 1998; von Ossowski et al., 2003 and so have processive cellulases from *Humicola insolens* (Varrot et al., 2005), *Thermobifida fusca* (Li et al., 2007; Vuong and Wilson, 2009; Zhou et al., 2004), and *Clostridium cellulolyticum* (Mandelman et al., 2003). The molecular mechanism behind processivity on biomass conversion efficiencies are not fully understood till now. The binding of a processive enzyme, either an endo-binding or an exo-binding enzyme, to a highly polymeric complex substrate leads to liberation of one product with an odd number of sugars (usually a trimer or a monomer), whereas all other products resulting from the same initial

enzyme-substrate binding will be dimers. This is the underlying principle in processivity assays for quantification purpose. All products released after processive cellulose hydrolysis had soluble reducing ends, except for the first product where initial binding is endo (internal), mainly generate insoluble reducing ends. In addition , processive endoglucanases are usually belong to the GH9 bacterial cellulolytic systems (Zhang et al., 2010), but processive cellulases belonging to the GH5 family have been recently identified in a marine bacterium, *Saccharophagus degradans* and a fungus *Volvariella volvacea* (Watson et al. 2009; Zheng and Ding 2013).

This chapter reveals a new type of processive endoglucanase from *A.ochraceus* MTCC 1810 demonstrating sequential synergy with its own β-glucosidase and thus provide a complete hydrolytic machinery for the bioconversion of pre-treated coir pith into glucose.

3.2 Materials and Methods

Aspergillus ochraceus MTCC 1810 was cultured in submerged conditions at 35°C (CMCase and FPase) and 40^0C (β-glucosidase) at pH 6.0 with 180 rpm with all other optimized parameters as described in the section 2.3.7. After 7 days (optimized fermentation period) of submerged fermentation, crude enzyme extract was produced and was used for purification using AKTA FPLC. Following steps were performed for purification of cellulases.

3.2.1 Separation of fungal mycelium from Crude Extract

The crude culture supernatant was filtered through Whatman No.1 filter paper and then centrifuged at 10,000 rpm for 15 min at 4°C to separate the crude enzyme from the cells. The crude enzyme (culture supernatant) was stored in 300 µl aliquots at -20°C for further purification and characterization as described below.

3.2.2 Purification of cellulases

Purification of cellulase was carried out using standard protein purification protocols, consisting of ammonium sulphate precipitation, dialysis using Amicon UF stirred cells, ion exchange chromatography, lyophilisation and concentration. All the experiments were done at 4ºC unless otherwise specified.

3.2.2.1 Ammonium sulphate precipitation

Ammonium sulphate required to precipitate cellulase was optimized by its addition at varying levels of concentrations (20, 30, 40, 50, 60, 70, 80 and 90% saturation), to the crude extract in a stepwise manner (Englard and Seifter, 1990).To precipitate the protein, ammonium sulphate was gradually added at 20% saturation to the crude extract while keeping in ice with gentle agitation. After complete dissolution of ammonium sulphate, the solution was kept at 4ºC for overnight. The precipitated protein was then centrifuged at 10,000 rpm for 15 min at 4ºC and the precipitate was resuspended in minimum quantity of 0.25 M sodium citrate buffer at pH 4.5. To the supernatant collected, ammonium sulphate required for next level of saturation was added, and the procedure was repeated. This precipitation process was continued up to 80% saturation, precipitates were removed by centrifugation at 10,000 rpm for 15 min at 4°C and discarded, collected the precipitate the same way and resuspended.

3.2.2.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against the sodium citrate buffer 0.25 M (pH 4.5) in order to remove the remnants of ammonium sulphate from the precipitate. Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cutoff

membrane (Omega, 25MM, 10K, Pall life sciences) was used for dialyzing the enzyme. Yield and fold of purification were calculated as described in section 3.2.5.4.

3.2.3 Ion exchange chromatography

The product obtained after ammonium sulphate concentration (80% fraction) was further subjected to ion exchange chromatography using diethyl aminoethyl (DEAE) cellulose as column packing material.

3.2.3.1 Activation of DEAE-Sepharose

A quantity of 5 g DEAE Sepharose (Sigma-Aldrich Co.) was soaked in 0.1 M sodium citrate buffer (pH 4.5), stirred properly and kept overnight for swelling. The swollen DEAE cellulose was then filtered on a Buchner funnel using vacuum filtration and incubated with 1 M NaCl solution for 1 hr. The filtration process was repeated until the washings attain pH 4.5.

3.2.3.2 Standardization of pH binding of cellulase to DEAE Sepharose

The optimum pH at which cellulase binds to the anion exchanger was standardized by eluting the enzyme solution from the DEAE Sepharose column. It was activated by following the method described in section 3.2.3.1, suspended in distilled water and equilibrated to pH using 0.01 M buffers of glycine-HCl (2.5-3.5), sodium acetate (4.0-7.5), Tris-Cl (8.0-9.0) and Carbonate-bicarbonate buffer (pH 9.5-10.5). An aliquot of 1 ml of diluted sample of 80% ammonium sulphate precipitated fraction was mixed with 2 ml slurry of DEAE Sepharose equilibrated to each pH, incubated at 4°C for overnight, and the supernatant was decanted carefully and assayed for cellulase as described in Chapter 2 (section 2.2.6.1) . The protein content was analysed as detailed in below section 3.2.4.2.Yield and fold of purification was calculated as described in section 3.2.3.4.

3.2.3.3 DEAE-Sepharose chromatography

The crude enzyme was loaded on AKTA Prime protein purification system equipped with a DEAE Sepharose C10/20 column [(10mm×20cm) (GE Healthcare Biosciences, Uppsala)] equilibrated in 10 mM Tris-HCl buffer (pH 8.0). The column was washed with the same buffer to separate the unbound proteins and the enzyme was eluted by applying a linear gradient of NaCl from 0-1000 mM at a flow rate of 0.5 ml min^{-1} and fractions of 2 ml were collected. Active fractions were pooled and dialysed against 20 mM Tris-Cl buffer at pH 8 for 24 h at 4^{o}C using Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cut off membrane (Omega, 25MM, 10K, Pall life sciences) and then concentrated by lyophilisation.

3.2.3.4 Calculation of Yield of Protein, Yield of Enzyme Activity and Fold of Purification

Yield of protein and enzyme activity of each fraction obtained during purification is the percentage activity calculated by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract.

$$\text{Yield of Protein} = \frac{\text{Total Protein content of the fraction x 100}}{\text{Total Protein content of the crude extract}}$$

$$\text{Yield of activity} = \frac{\text{Total activity of the fraction x 100}}{\text{Total activity of the crude extract}}$$

$$\text{Fold of Purification} = \frac{\text{Specific activity of the fraction}}{\text{Specific activity of the crude extract}}$$

3.2.4 Characterization of cellulase

The enzyme obtained after purification viz., ammonium sulfate precipitation, dialysis and ion exchange chromatographic technique were

assayed for cellulase activity and protein analysis. The purified enzyme was subjected to electrophoresis for the determination of molecular weight. Effect of pH and temperature for enzyme activity and stability, inhibition and activation of enzyme activity by different metal ions, surfactants, chemical agents and organic solvent stability and other unusual features such as multiple substrate specificity, synergy, processivity were studied in detail.

3.2.4.1 Cellulase assay (CMCase, FPase and β-glucosidase)

Peak fractions from the column were pooled and assayed for individual cellulase activity such as endoglucanase (CMCase), total cellulase (FPase) and β-glucosidase as described in the section 2.2.6.1 of Chapter 2.

3.2.4.2 Protein assay

Quantification of protein was carried out following the Bicinchoninic acid (BCA) method (Invitrogen) and bovine serum albumin was used as the protein standard (Smith et al., 1986; Walker et al., 1994). The reagents for the assay were: Reagent A: 0.1 g sodium bicinchoninate, 0.16 g sodium tartrate (dihydrate), 2.0 g $Na_2CO_3.H_2O$, 0.4g NaOH, and 0.95 g NaH_2CO_3 dissolved in 100 ml distilled water (adjusted to pH 11.25 with 10 M NaOH). Reagent B: 0.4 g $CuSO_4.5H_2O$ dissolved in 10 ml of distilled water. The standard working reagent was then prepared from mixing 50 volumes of Reagent A and 1 volume Reagent B. The standard assay was carried out by mixing 0.5 ml of the sample or protein standard with 0.5 ml of the BCA standard working reagent. The mixtures were incubated at 37°C for 30 min, cooled to room temperature and then absorbance was read at 562 nm.

3.2.4.3 Specific activity

Specific activity was calculated by dividing the enzyme units with the protein content

$$\text{Specific activity (U/mg)} = \frac{\text{Total unit activity } \left(\text{U ml}^{-1}\right)}{\text{Total protein content } \left(\text{mg ml}^{-1}\right)}$$

3.2.4.4 Relative activity

It is the percentage enzyme activity of the sample with respect to the sample for which maximum activity was obtained

$$\text{Relative activity} = \frac{\text{Total activity } \left(\text{U ml}^{-1}\right) \text{x} 100}{\text{Maximum activity } \left(\text{U ml}^{-1}\right)}$$

3.2.4.5 Residual activity

Residual activity is the percentage enzyme activity of the sample with respect to the activity of the control (untreated sample)

$$\text{Relative activity} = \frac{\text{Activity of sample } \left(\text{U}\right) \text{ x } 100}{\text{Activity of control } \left(\text{U}\right)}$$

3.2.5 Determination of molecular mass

The relative molecular mass of enzyme was measured by performing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 5% stacking gel and 12% resolving gel electrophoresis (Laemmli, 1970). Molecular weight of unknown protein was determined by comparing with that of standards (PMWM- Genei, India). The resolved protein bands were visualized by staining with 0.025% Coomassie brilliant blue stain R-250 and de-stained with 5% methanol and 7% acetic acid.

3.2.6 Zymogram analysis

The CMCase, exoglucanase and β-glucosidase activities were visualised by zymogram analysis, which was carried out using 0.5% CMC for

endoglucanase, 35 mM 4-methyl umbelliferyl-β-D-Cellobioside (4-MUC) for exoglucanase and 0.2% esculin hydrate and 0.01% ferric chloride for β-glucosidase activity. After electrophoresis, SDS was removed by washing the gel four times for 30 min in 50 mM disodium hydrogen phosphate and 12.5 mM citric acid, pH 6.3 and 25% isopropanol were included in the first two washes. For zymogram analysis of individual cellulase activity, respective substrates as mentioned above were prepared in 50 mM disodium hydrogen phosphate, and 12.5 mM citric acid, pH 6.0 .The agar was then allowed to gel at 4°C. The polyacrylamide gel was washed, dried and was laid on top of the agar sheet. Transfer of the desired protein band to the substrate gel and subsequent hydrolysis was allowed to proceed for 1 to 3 h at 40°C. The sandwich was then left to cool for at least 15 min at 4°C. The CMC agar replica was dipped into 0.1% Congo red and washed with 1 M sodium chloride until excess stain was totally removed from the active bands. The clearance zone could be visualized as yellow zone against a red background. Exoglucanase (cellobiohydrolase) activity of the 4-MUC replica agar could be detected under UV-fluorescence due to the hydrolysis of 4-MUC into 4-methylumbelliferone which is a fluorescent compound. The β-glucosidase activity can be detected on esculin hydrate-ferric chloride agar replica as a black precipitate.

3.2.7 Effect of pH on enzyme stability and activity

The optimum pH of the purified cellulases was determined by incubating the mixture in the presence of various buffers of pH in the range of 3-10 ; citrate buffer (pH 3 to 6) , Phosphate buffer (pH 7 to 8) and Glycine - NaOH buffer (pH 9 to 10) for 60 min at 40^{o}C. The substrates for each enzyme activity were prepared in the respective buffers and assayed under standard assay conditions. The pH stability was measured by pre-incubating the enzyme

in different buffers for 10 h at 40°C and the residual enzyme activity was determined under standard assay conditions.

3.2.8 Effect of temperature on enzyme stability and activity

The optimum temperature of the purified cellulases was measured by incubating the enzymes in 0.05 M citrate buffer pH 6.0 along with the substrates; 0.5% CMC for endoglucanse, 50 mg whatman No.1 filter paper for total cellulase, and 0.5 mM cellobiose for β-glucosidase at different temperatures ranging from 4 to 70^{o} C and relative activity was assayed after 60 mins of incubation. Temperature stability was measured by pre-incubating the enzyme at different temperatures (4 to 70^{o} C) for 10 h at pH 6.0 and the residual enzyme activity was determined under standard assay conditions.

3.2.9 Effect of various metal ions on enzyme activity

The effect of various metal ions on the purified enzyme activity was evaluated by pre-incubating the enzymes with 5 mM concentrations of different metal ions at 40^{o}C (pH 6.0) for 60 min followed by measuring the residual enzyme activity. The metal ions used in the study were calcium chloride (Ca^{2+}), cadmium sulphate (Cd^{2+}), cupric sulphate (Cu^{2+}), ferric chloride (Fe^{3+}) , magnesium chloride (Mg^{2+}), manganese chloride (Mn^{2+}) , sodium sulphate (Na^{+}), mercury chloride (Hg^{2+}) , potassium chloride (K^{+}), silver sulphate (Ag^{2+}) and zinc sulphate (Zn^{2+}) .The residual enzyme activity were assayed under standard assay conditions and the activity of control, in the absence of metal ions, was taken as 100%.

3.2.10 Effect of various additives on enzyme activity

The purified enzyme preparations were incubated with 1% concentration of Tween 20, Tween 80, Triton X-100, β-mercaptoethanol (2-

ME), dimethylsulfoxide (DMSO), sodium dodecyl sulphate (SDS), ethylenediamine tetra acetic acid (EDTA) and glycerol at 40^0C for 60 min and the residual activity was estimated.

3.2.11 Effect of organic solvents on the enzyme activity

The effect of various organic solvents viz., methanol, ethanol and acetone on enzyme activity was evaluated by pre-incubating the enzymes with 20% v/v organic solvent at 40^0C for 60 min and thereafter the residual activity was assayed under standard assay conditions considering control as 100%.

3.2.12 Determination of Kinetic parameters

The effect of substrate concentration on the reaction velocity of the purified cellulases was determined with carboxy methyl cellulose (0.5 to 2.5 mg/ml) for endoglucanase, avicel (0.2 to 1.5 mg/ml) for total cellulase activity, 4-methyl umbelliferyl-β-D-glucopyranoside (0.5 to 2.5 mg/ml) for β-glucosidase activity. The purified enzymes (50 μg/ml) were incubated with the respective substrates at 40^oC for 60 min in sodium citrate buffer, pH 6.0. The apparent enzyme kinetic parameters such as the Michaelis constant (K_m) , maximum velocity (V_{max}), enzyme turn over number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) were determined from the Lineweaver–Burk plot using nonlinear regression with GraphPad Prism, version 6.0 (http://www. graphpad.com/ scientific-software/prism/).

3.2.13 Substrate specificity assay

The substrate specificity of the purified cellulases from *A.ochraceus* MTCC 1810 was determined by performing assay with different cellulosic substrates such as Whatman no. 1 filter paper, microcrystalline cellulose (Avicel , Sigma), birch wood xylan (Sigma), 4-methyl-β-umbelliferyl-β-D-cellobioside

(MUC, Sigma), 4-methyl-beta-umbelliferyl-β-D-glucopyranoside (MUG, Sigma), carboxy methyl cellulose (CMC , Sigma), phosphoric acid swollen-avicel (PASC), hydroxy ethyl cellulose (HEC, Sigma), cellobiose (Sigma), esculin (Sigma), delignified coir pith and rice hull. The assay was carried out at 0.5 M sodium citrate buffer, pH 6.0 at 40^0C for 10 h. Determination of reducing sugar in the supernatant was done by dinitrosalicylic acid (DNS) method. Specific activity was expressed in U/mg. Enzyme specificity against the fluorogenic substrates such as 4-MUC and 4-MUG were measured by hydrolysis of 200 μM 4-MUG and 4-MUC into 4-methyl umbelliferone (MUF). The reaction assay prepared in 50 mM citrate buffer (pH 6.0) was incubated for 10 h at 40^oC and the reaction was stopped by adding bicarbonate buffer (pH 10.3) in order to measure MUF fluorescence. The fluorescence was measured at 465 nm using a spectrofluorometer at an excitation wavelength of 360 nm. Enzyme activity was calculated using a standard curve representing 1 to 100 μmol of 4-methyl umbelliferone (MUF) and unit enzyme activity was represented as amount of enzyme required to liberate 1μmol of MUF per minute under standard assay conditions.

3.2.14 Substrate binding assay

The cellulose substrate binding assay was performed by incubating the purified processive endoglucanase (50 μg/ml) with the insoluble substrates such as avicel (1%) and filter paper discs (1%) in 10 ml of 0.5 M sodium citrate buffer, pH 6 after 4 hrs of gentle shaking at 25^0C. The reaction mixture was centrifuged at low speed, (1000×*g*) for 10 min and the supernatant was analysed for examining the remaining cellulase activity. Reduction in the cellulase activity in the supernatant was expressed as substrate binding property of the purified enzyme.

3.2.15 Processivity assay

The processivity of the endoglucanase (AS-HT-Celuz A) was determined by estimating the ratio of soluble to insoluble reducing sugars by using a modified procedure of Zhang et al. (2010). The assay was run on filter paper substrate with 50 ug/ml of enzyme by measuring the reducing sugars present in the soluble (supernatant) as well as in the insoluble (FP) fraction after 3, 6 and 9 h of incubation. Initially the reducing sugar in the supernatant was measured using the dinitrosalicylic acid method and the corresponding cellobiose concentration was represented as 15% low of the reducing sugar liberated .The residual FP discs were rinsed with 6 M guanidine hydrochloride to eliminate the bound protein and again washed four times with sodium citrate assay buffer (pH 6.0) and sterile water. The washed FP discs were re-suspened in 400 µl of assay buffer and the reducing sugar was measured using microBCA reagent kit (Invitrogen) as described in the section 3.2.4.2.

3.2.16 Synergy between purified cellulases

Synergy between the purified cellulase, AS-HT-Celuz A (processive endoglucanase) and AS-HT-Celuz B (β-glucosidase activity) was investigated with CMC:Cellobiose (1:1) as substrate in 0.05 M citrate buffer at pH 6.0 in a final volume of 10 ml and allowed to hydrolyse at 40^0C for 4 h . The reaction mixtures contained AS-HT-Celuz A and AS-HT-Celuz B were each diluted to equal unit activities (25 U/ml) and synergy experiment was carried out with each enzyme alone and with simultaneous and sequential addition of the enzymes. Sequential addition of the enzymes was performed with a single enzyme for 2 h and then heat inactivated the enzyme mixture for 20 min. After cooling of the hydrolysate, the second enzyme was added and incubated for further 2 h. After incubation the reaction mixture was heat inactivated by

boiling for 20 min. All samples were assayed for glucose production using GOD-POD glucose assay kit (Biolab Diagnostic Pvt. Ltd.). Briefly, working solution was prepared by transferring the contents of one vial of glucose reagent 1 (Glucose oxidase, peroxidase, aminoantipyrine, buffer, stabilizers) to an amber coloured plastic bottle (provided with the kit) and reconstituted the content of each bottle with reagent 2 (phenol diluent). An aliquot of 150 µl of the supernatant sample was mixed with 150 µl of working glucose oxidase reagent, mixed well and incubated at 37^{o}C for 15 min. Then absorbance was measured at 505 nm against reagent blank. The absorbance recorded is directly proportional to the glucose concentration. The degree of synergy with respect to the saccharification activity was determined by dividing the amount of glucose units produced by the cellulase mixture by the sum of the amounts of the glucose units produced by the individual cellulases (Hoshino and Ito et al., 1997).

3.2.17 Two-dimensional electrophoresis of AS-HT-Celuz A and AS-HT-Celuz B

The idea of separation of proteins using two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D-PAGE) was first described in 1975 (O'Farrell, 1975; Pandey and Mann, 2000; Sechi, 2002). In 2D-PAGE, the proteins are separated with respect to two independent properties, isoelectric point (pI) and molecular weight. The individual proteins will appear as single spots, due to the differences in pI and molecular weight. Different isomeric forms of same protein often appear as separate spots on the gel, as the pI is influenced by post- translational and glycosidic modifications. This is also a simple way to detect homogenous nature of the protein samples.

3.2.17.1 Sample preparation for 2D PAGE electrophoresis

The sample preparation for isoelectic focussing (IEF) was done using the 2-D Clean-Up Kit (GE Healthcare) by following the procedure supplied by the manufacturer. Briefly, 400µg of FPLC purified fractions of AS-HT-Celuz A and AS-HT-Celuz B were mixed with 300µL of precipitant by vortexing and incubated on ice for 15min.Then, 300 µL of co-precipitant was added and vortexed briefly. The mixture was then centrifuged at 12,000*g* at 4°C for 5min and the resulting supernatant was completely removed immediately without causing any disturbance to the pellet. An aliquot of 40 µL of co-precipitant was layered on top of the pellet and incubated in ice for 5 min. It was then centrifuged at 12,000g at 4°C for 5min and the supernatant was removed. Sterile distilled water (25 µL) was added to pellet and vortexed for 5-10s. Following the addition of 1mL wash buffer and 5 µL of wash additive, the pellet was fully dispersed by vortexing. The samples were then incubated at -20°C for 30 min and vortexed in every 10 min, centrifuged at 12,000*g* at 4°C for 5 min and the supernatant was discarded and the pellet was air dried. The pellet was then re-suspended in 100 µL of Destreak Rehydration buffer (equilibrated at room temperature for 30 min), vortexed for 30 s and incubated overnight at room temperature to allow complete solubilisation. The tubes were then centrifuged at 12,000*g* at 4°C for 5 min to remove any insoluble material and to reduce any foam. The supernatant was then transferred into another tube. Immobilized pH gradient (IPG) buffer of appropriate pH (3-10) was added to Destreak Rehydration buffer at 0.5% and the total volume was made up to 125 µL for Immobiline^{TM} Drystrip of 13cm length. The samples were again incubated for 1.5 hrs at 25°C and centrifuged at 13,000*g* at 20°C for 5 min and the supernatant was used for strip rehydration.

3.2.17.2 Rehydration of Immobiline Dry strip

Rehydration of Immobiline Drystrip (GE Healthcare) or the IPG strip was done in ceramic strip holders (13cm).The samples prepared in Destreak Rehydration solution (300 µL) was evenly distributed into the strip holders and the Immobiline Drystrip was carefully placed on to the sample with the positive end towards the anode of the strip holder. The strips were then overlaid with the DryStrip cover fluid, covered and subjected to overnight rehydration.

3.2.17.3 Isoelectric focusing

The isoelectric focusing of the samples was done on Ettan^{TM} IPGphor^{TM}3 Isoelectric Focusing system (GE Healthcare).The two ceramic strip holders with the rehydrated Immobiline Drystrip were placed on the platform of the system with the pointed end on anode and blunt end over the cathode. After closing the safety lid, the strips were subjected to an IPGphor software controlled rehydration at 20°C for 2 hrs.The automatic IEF was performed at 20°C using the following voltage focusing protocol: the first step of 500V for 30 mins at linear voltage ramp, second step of 5000V for 2.5 hrs for another linear voltage ramp and final step of 5000V for 20,000 Volt hours with rapid ramp. Changing voltage or linear ramping would help to stabilize the resistance developed during the sample run. IPG strips after isoelectric focussing was stored at -80^{o}C.

3.2.17.4 Equillibration of Immobiline Drystrips

Prior to the second dimension of proteins, it is necessary to equilibrate the separated proteins contained in the IPG strip for further solubilization and binding of proteins to SDS in second dimension gel. After the completion of first-dimension electrophoresis, the strips were removed from the strip holder

and excess oil and protein solution were absorbed by filter paper. The gel strips were equilibrated in 5 ml of SDS equilibration buffer (6 M Urea, 50 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) containing 60 mM DTT (Dithiothreitol) for 15 mins. The IPG strips were then subjected to a second equilibration with equilibration buffer containing 50mM Iodoacetamide for further 15mins.

3.2.17.5 Second Dimensional SDS-PAGE and Visualization

The first-dimension separated proteins in the IPG strips were then subjected for second-dimension SDS-PAGE consisted of 12% acrylamide, pH 8.8 for resolving gel and 4% acrylamide, pH-6.8, for the stacking gel. The IPG strip marker was then sealed with sealing solution (0.5% agarose, 0.002% Bromophenol blue). Electrophoresis was carried out on a Hoefer^{TM} miniVE vertical electrophoresis system (Amershm Biosciences, Sweden) at 15mA per gel at room temperature until the dye front reached the bottom of the gel. Silver staining or coomassie blue staining was employed to visualise the protein spots. The gels were then scanned using Image scanner III with the software Labscan 6.0 (GE Healthcare) and the gel images were analyzed.

3.3. Results & Discussion

This chapter elucidated the purification and characterisation of an extracellular enzyme system comprising a processive-type endoglucanase designated as AS-HT-Celuz A and β-glucosidase designated as AS-HT-Celuz B from the strain *Aspergillus ochraceus* MTCC 1810 when fermented on delignified coir pith based medium. Interestingly the uniqueness of the present study is the occurrence of all the synergistic components of the cellulase system from the fungus *Aspergillus* which is not reported in earlier studies published till date.

3.3.1 Purification of multi-component cellulases from *Aspergillus ochraceus* MTCC 1810 through bioconversion of delignified coir pith

The extracellular cellulolytic enzyme system of *A. ochraceus* MTCC 1810 was partially purified from the culture supernatants of Reese and Mandel's fermentation medium by 80% ammonium sulphate precipitation followed by desalting through dialysis and ultra filtration. Further, the dialysed and concentrated protein was subjected to ion exchange chromatography on DEAE sepharose column equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The protein concentration and the activity of individual cellulolytic enzymes were compared with the crude culture filtrate and the steps used for purifying processive endoglucanase and β-glucosidase are presented in the Table1. Recovery of 42.17% total protein was achieved after 80% ammonium sulphate precipitation whereas DEAE Sepharose column chromatography yielded only 22.44% of the total protein from the culture supernatant. Further, the specific activities of partially purified endoglucanase, exoglucanase and β-D-glucosidase activities were incremented at each purification step and it was found to be higher as compared to the crude filtrate specific activities. The three enzyme activities were purified at different purification folds such as endoglucanase activity resulted in 2.39 fold purification with yield of activity 53.84%, total cellulase resulted in 3.26 fold purification with 73.3% yield of activity and β-glucosidase fraction resulted in 2.92 fold purification with an activity yield of 65.58% (Table 1).

Table 1: Summary of purification of cellulase from *Aspergillus ochraceus* MTCC 1810

Purification Step	Total Protein	Enzyme activity (Units/ml)			Specific activity Units/mg protein			Yield activity %			Purification fold		
	(mg)	EnG[a]	FPU[b]	BG[c]	EnG	FPU	BG	EnG	FPU	BG	EnG	FPU	BG
Crude	14.7	65.35	38.4	23.16	4.42	2.61	1.57	100	100	100	1	1	1
Ammonium sulfate precipitation	6.2	42.17	30.96	18.93	6.77	4.99	3.05	64.61	80.62	81.73	1.53	1.91	1.94
DEAE Sepharose chromatography	3.3	35.63	28.15	15.19	10.6	8.53	4.60	53.84	73.30	65.58	2.39	3.26	2.92

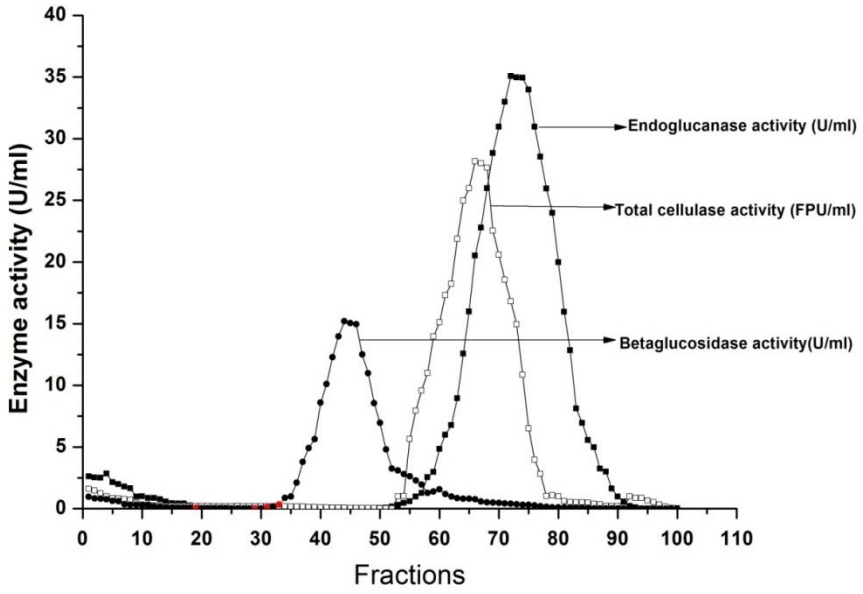
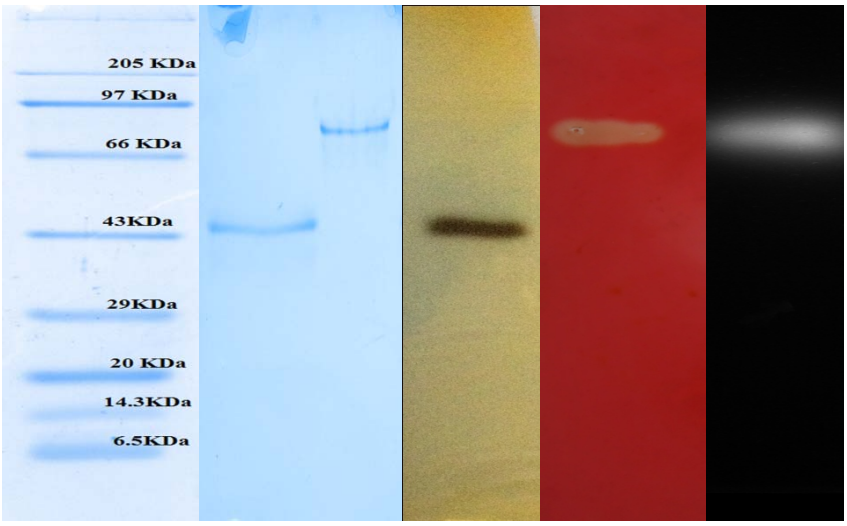


Fig.1. Protein purification profile of cellulases from *A.ochraceus* using DEAE-Sepharose ion exchange chromatography

The DEAE-Sepharose ion exchange chromatography profile (Fig.1) of the cellulases isolated were observed to contain endoglucanase, total cellulase and β-glucosidase activities which appeared as single prominent peak for individual cellulase activities (Fig.1) . To analyse the purity of cellulase enzymes, the eluted fractions were analysed on 12% SDS-PAGE (Fig.2a). Most of the endoglucanase as well as the total cellulase activity were contained in the fractions of 53-89 and

it showed a homogenous enzyme profile with molecular mass of approximately 78 KDa (Fig.2a, lane 2). This is similar to the endoglucanase purified from *A. terreus* with M_r of 78 K Da (Nazir et al., 2009) and a processive endoglucanase with M_r of 90 KDa from *Clostridium phytofermentans* (Zhang et al., 2010). But it is highly different from the previously reported specific activities of 10.6 U/mg and 8.53 U/mg with CMC and filter paper respectively. The overlapped enzyme activities indicate the presence of cellulase enzyme capable for degradation of both amorphous and crystalline cellulose. This finding could also imply that both the enzyme activities were residing in different active sites of the same protein indicating the processive nature of the purified cellulase. Therefore it can be classified as processive endoglucanase appeared at first from *Aspergillus ochraceus* MTCC 1810. Similar bifunctionally active cellulases from various bacteria and fungi had also been reported before (Han et al., 1995). Zymogram analysis also agreed with the protein profile which revealed the presence of endoglucanase and exoglucanase activity on CMC-congo red gel and 4-MUC gel respectively (Fig.2b, lane2 &3).While most of the β-glucosidase activity was concentrated on the fractions of 36-61 and showed an apparent molecular mass of 43 KDa (Fig.2a , lane 1) with a specific activity of 4.6 U/mg protein and its zymogram profile showed a black precipitate on Esculin hydrate-ferric chloride agarose gel due to the hydrolysis of the esculin to esculetin (Fig.2b, lane1) by the β-glucosidase enzyme action. Thus the purified β- glucosidase from *A. ochraceus* have a distinct molecular mass of 43 KDa which is different from the 93 KDa and 138 KDa β-glucosidases of *A. oryzae* HML366 (He et al., 2013). Further purification methods were not followed in this study because the proteins purified were in homogenous form with single peak of enzyme activities.



(Fig.2a) **(Fig.2b)**

Fig.2a. 12% SDS-PAGE analysis of processive-type endoglucanase(AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B) purified from *Aspergillus ochraceus* MTCC 1810 ; Lane 1: Broad range protein molecular weight marker (Genei) with molecular mass in KDa, Lane 2 : Purified β-glucosidase (43 KDa) , Lane 3 : Purified processive endoglucanase (≈78KDa).

Fig.2b. Zymogram analysis of purified cellulases ; Lane 1: Detection of β-glucosidase activity in 0.02% $FeCl_2$ and 0.1% esculin hydrate agarose gel, Lane2: Activity staining for endoglucanase in CMC-congo red agarose gel , Lane 3: Exoglucanase activity detection in 50 mM 4-MUC supplemented agarose gel.

3.3.2 Bio-chemical Characterisation of cellulase activities

3.3.2.1 Optimum pH and pH stability

The effect of various pH ranging from pH 2.0 to 10.0 on both processive-type endoglucanase and β-glucosidase were examined using different buffers and the results are shown in Fig.3a and 3b. Endoglucanase, total cellulase and β-glucosidase showed a similar pH stability profile and wide pH tolerance from pH 3 - 8. Based on the optimum enzyme activity at pH 6.0, it decreased upto 50% at above pH 9.0 and at below pH 3.0 (Fig.3b). All enzyme activities of the two purified cellulases showed 90% of its total

activity under the pH 5.0, 6.0 and 7.0 and 50% loss of enzyme activity was observed under pH 9.0 and 10.0. When the residual activity of the both the enzymes were tested, it has been found that enzymes were rather stable after 10 h incubation at a pH range of 3-9 and at pH 10, only 30% of the residual activity was maintained in the case of endoglucanase and total cellulase activity whereas only 15% of the β-glucosidase activity was maintained at the same pH. Of the three enzymes assayed, β-glucosidase retained only 43.98 % activity at pH 2.0, whereas the endoglucanase and total cellulase showed 60% stability (Fig.3b). These results agreed with the cellulases produced by other fungi having active temperature range of 40–80^{o}C and pH of 3–6 (Gao et al., 2008; Jatinder et al., 2007).

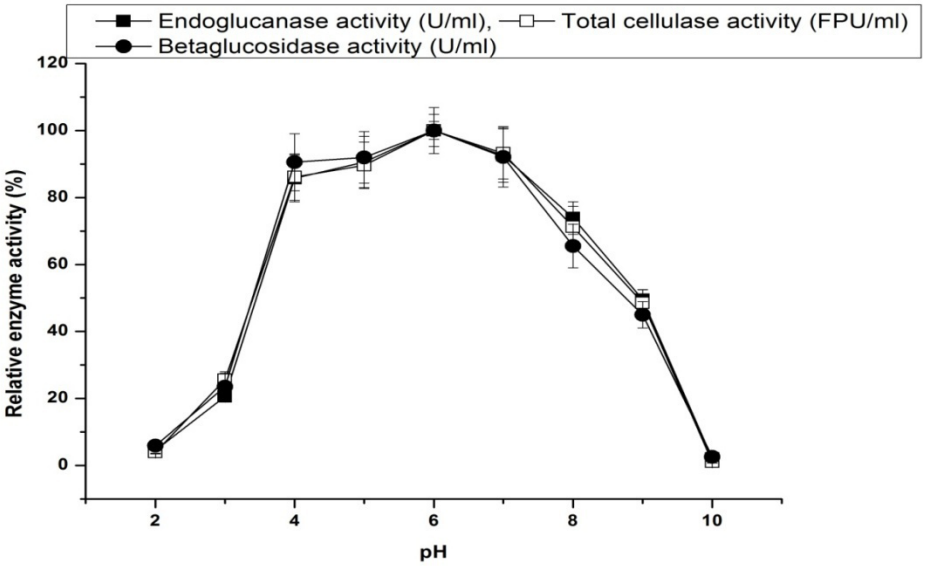


Fig.3a. Effect of pH on the activity of cellulase enzymes from *A. ochraceus* MTCC 1810. The enzymes were incubated with various buffers of pH ranging from 2-10 at room temperature for 1h for determining (–■–) endoglucanase activity from AS-HT-Celuz A, (–□–) total cellulase activity from AS-HT-Celuz A and (–●–) β-glucosidase activity from AS-HT-Celuz B.

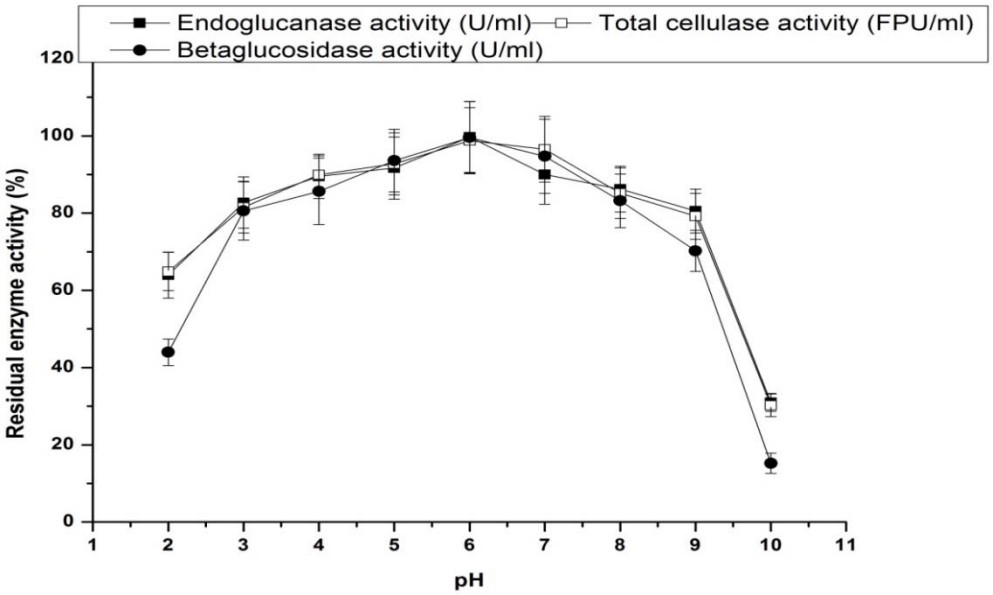


Fig. 3b. The pH stability on the activity of cellulase enzymes from *A. ochraceus* MTCC 1810. The enzymes were pre-incubated with various buffers of pH range from 2-10 at room temperature for 10h for determining (–■–) endoglucanase activity from AS-HT-Celuz A , (–□–) total cellulaseactivity from AS-HT-Celuz A and (–●–) β-glucosidase activity from AS-HT-Celuz B.

3.3.2.2 Optimum temperature and temperature stability

Both processive–type endoglucanase (AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B) exhibited similar temperature optima (Fig.4a) and stability profile (Fig.4b) with significant enzyme activities in the temperature range of 4 to 60^{o}C, at which optimum enzyme activity was displayed at 40^{0}C (pH 6.0). Approximately 90-100% of residual enzyme activity was retained and decreased to below 10% at 70^{o}C. Specifically, processive endoglucanase enzyme lost about 5% of its activity, while β-glucosidase lost approximately 10% of its activity when incubated at a temperature of 50^{o}C. About 2-3% of initial endoglucanase and total cellulase activity was maintained in the case of processive endoglucanase, while β-glucosidase showed complete

inactivation profile at 70°C. At 4°C and 10°C, both enzymes exhibited no loss of activity. Interestingly the enzymes produced from the mesophilic fungus *A. ochraceus* (which displayed no temperature tolerance above 35°C), but showed a wide temperature stability and optimum activity at 40°C. This is in accordance with the cellulases purified from fungi with optimal temperature ranging from 50 and 70°C (Lee et al., 2008; Saha, 2004). These two purified cellulases are highly significant in terms of its compatible temperature and pH optima and stability profile and prospectively demonstrate the industrial potency of these enzymes.

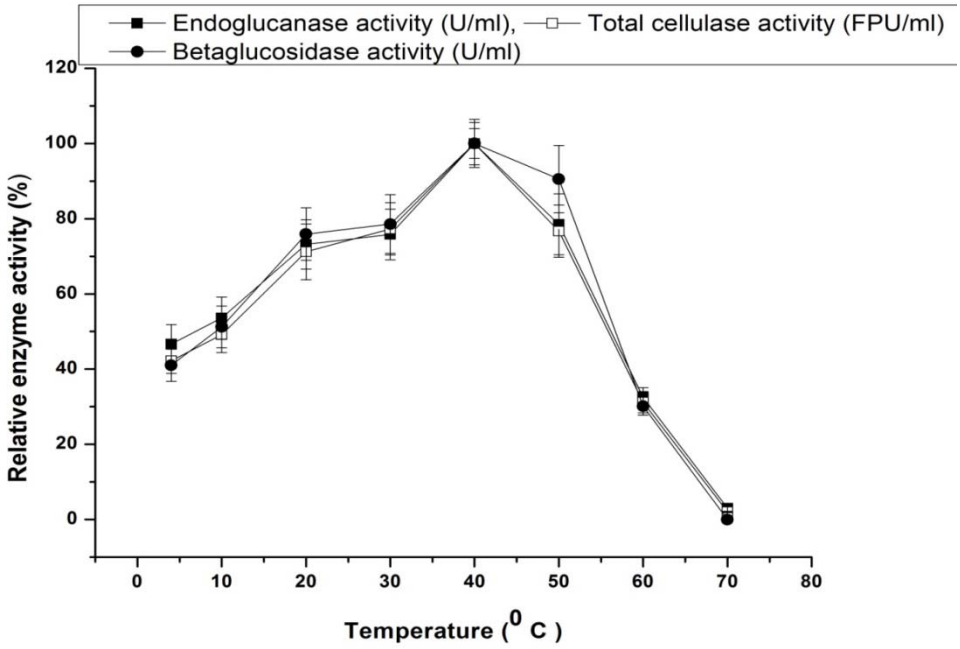


Fig.4a. Effect of temperature on the activity of cellulase enzymes from *A. ochraceus* MTCC 1810. The enzymes were incubated at various temperature range from 4-70°C (pH6.0) for determining (–■–) endoglucanase activity from AS-HT-Celuz A, (–□–) total cellulase activity from AS-HT-Celuz A and (–●–) β-glucosidase activity from AS-HT-Celuz B.

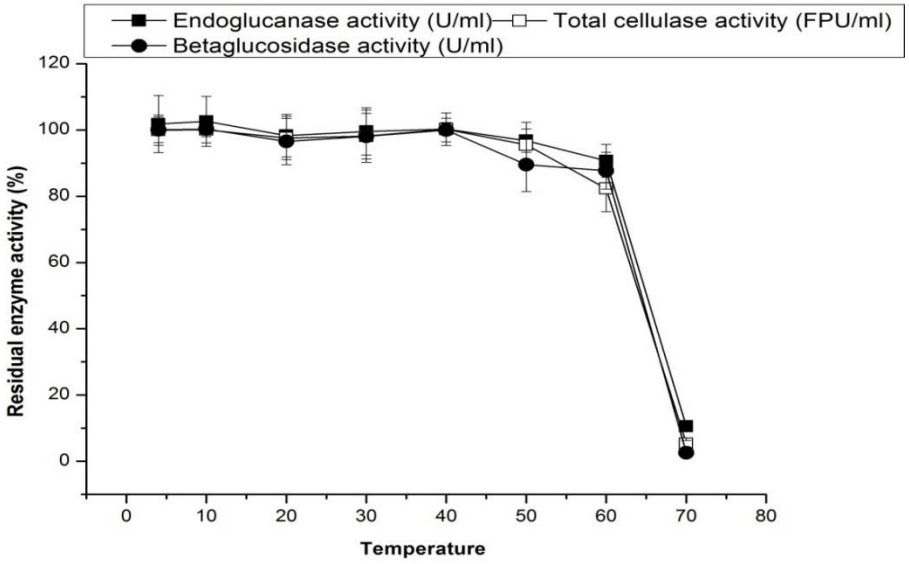


Fig.4b. Thermal stability of cellulase enzymes from *A. ochraceus* MTCC 1810. The enzymes were incubated at various temperature ranges from 4-70°C (pH 6.0) for determining (–■–) Endoglucanase activity from AS-HT-Celuz A, (–□–) Total cellulase activity from AS-HT-Celuz A and (–●–) β-glucosidase activity from AS-HT-Celuz B

3.3.2.3 Effects of Metal Ions on enzyme stability

The enhancement and deactivation of cellulase activity by various metal ions (5mM) are shown in Table 2. Both the processive endoglucanase and β-glucosidase were enhanced by Na^{+}, K^{+}, Mg^{2+}, Cu^{2+} and Fe^{3+}, but inhibited to a lesser extent by Mn^{2+}, Ca^{2+}, Zn^{2+} and Cd^{2+}. Complete inhibition of all cellulase activities were observed when treated with Hg^{2+}. Results shown in the Table 2 suggest that maximum relative activities exhibited by processive endoglucanase in the presence of Na^{+}, K^{+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} were 198.63% , 187.27% , 179.52% , 176.15% 170.19% , 94.41% , 90.17% , 60.33% , 52.97% and 5.4% respectively, whereas total cellulase activities were 186.24%, 174.39% , 168.87% , 153.36% , 138.44% , 73.39% , 67.71 % , 76.19 % , 73.39% , 45.17% and 2.97% respectively. Similarly, the relative activities exhibited by the β-glucosidase enzyme were 160.82%, 155.91% , 154.29%,

160.36% , 166.11% , 41.18% , 50.93% , 42.38% , 27.35% and 2.97% respectively in the presence of above mentioned metal ions. Among the various metal ions studied, endoglucanase activity (70-98%), total cellulase activity (67-86%) were enhanced by the metal ions in the order of $Na^+ > K^+ > Mg^{2+} > Cu^{2+} > Fe^{3+} > Co^{2+}$. Enhancement of bacterial cellulase activity by Ca^{2+}, Mg^{2+}, and Na^+ had also been previously reported by Yoon et al. (1994) and Bakare et al. (2005). Zn^{2+} had greater enhancing effect on the total cellulase activity compared to endoglucanase. Mg^{2+} and Cu^{2+} showed an improved activity on the β-glucosidase than its total cellulase activity ; Ca^{2+} showed slight inhibition, $Cd^{2+}$and Zn^{2+} showed moderate inhibition and Hg^{2+} showed strong inhibition on the endoglucanase activity. Comparable results were also reported for other *Aspergillus* strains (Begum and Absar et al., 2009; Jabasingh et al., 2014; Narra et al., 2012; Nazir et al., 2009). The presence of metal ions such as Ca^{2+}, Zn^{2+} and Mn^{2+}, reduced the β-glucosidase activity to 50% and Cd^{2+} showed weak inhibition (27%). Hg^{2+} completely inhibited the enzyme activities to nearly 2% when incubating with its respective substrates. Similar effects had been reported by some of the earlier authors that the major inhibition by the Hg^{2+} might be due to its binding with thiol groups or with the hydrophobic aminoacids such as tryptophan or tyrosine residue present in the cellulase enzyme (Li et al., 2006; Lamed et al., 1994) Tryptophan residues predominantly involved in the substrate binding of enzymes through cellulose binding domains (Lamed et al., 1994). It was also observed from the results that univalent metal ions had a stronger influence on the enzyme activity than divalent and trivalent metal ions. This showed similarity to the findings of Wang et al. (2012) who had also reported a strong inhibition on the cellulase activity by the higher ionic valence metal ions than the lower valence metal ions.

Table 2. Effect of metal ions on enzyme stability

Metal ion [b]	Residual activity (%)		
	Endoglucanase Activity	Total cellulase activity	Betaglucosidase Activity
Control	100±3.56[a]	100±4.49	100±2.56
Ca^{2+}	90.17±7.92	76.19±6.51	50.93±3.54
Cu^{2+}	176.15±10.39	153.36±9.34	160.36±4.96
Fe^{3+}	170.19±9.85	168.87±8.81	166.11±8.02
Mg^{2+}	179.52±9.18	138.44±7.26	154.29±6.99
Hg^{2+}	5.4±0.92	2.97±0.65	2.97±0.51
K^{+}	187.27±8.6	174.39±7.51	155.91±6.33
Na^{+}	198.63±9.38	186.24±8.11	160.82±7.82
Mn^{2+}	94.41±6.55	67.71±4.29	41.18±2.38
Zn^{2+}	60.33±4.29	73.39±6	42.38±2.57
Cd^{2+}	52.97±3.81	45.17±3.94	27.35±1.99

[a] Standard deviation from triplicate measurements
[b] Metal ions were used at 5mM concentration

3.3.2.4 Effect of various additives on enzyme stability

The effect of different chemical agents *viz.*, ethylenediamine tetra acetic acid (EDTA), β-mercapto ethanol, dimethyl sulphoxide (DMSO) and glycerol at 1% concentration were examined by incubating with purified enzymes under optimized conditions. The endoglucanase activity was retained at 85.37%, 51.33%, 86.15% and 110.59% respectively of its original activity and total cellulase activity showed relative activities such as 89.55%, 45.96%, 87.18% and 98.73% respectively for the chemical agents mentioned above (Table 3). The results suggested that the purified processive endoglucanase retained complete activity in the presence of 1% glycerol, where as only partial activity was retained in the presence of 1% β-mercaptoethanol suggested that histidine residues and sulfhydryl groups were involved in its enzyme action. Accordingly, β-glucosidase retained 100% activity towards glycerol and 90.55% activity towards DMSO and lost about 37.27% activity in the presence of β-mercaptoethanol. A 30% reduction in activity was observed

in the presence of 1% EDTA indicating that it is a metalloenzyme and requires divalent cations for its enzyme activity. Subsequently, the effects of surfactants such as 1% sodium dodecyl sulphate (SDS), 1% of Tween 20, Tween 80 and Triton X-100 on cellulase activity were also investigated. Kim et al. (1982) had reported that surfactants could increase the digestability of the enzymes as well as they adsorb on to the air-liquid interface and thus prevent enzyme denaturation. In the presence of non-ionic surfactants such as Tween 20 and Tween 80, the enzyme activity of both processive endoglucanase and β-glucosidase were slightly increased by 2-5% whereas in the case of triton X-100, the relative activities were decreased by 15-20%. Although, both enzymes were partially inhibited in the presence of SDS. Conversely, a highly thermo-stable and alkaline cellulase which showed 95% stability after 1 h incubation with SDS (10 mM) was reported by Yin et al. (2010). Another study carried out by Sadhu et al. (2013) and Wang et al. (2009) have confirmed that SDS and tween-80 partially inhibited cellulase activity by 59-71% and 50-59%, respectively.

Table 3: Stability of purified enzymes in presence of various chemical agents (1%)

Chemical agents [f]	Residual activity (%)		
	Endoglucanase Activity	Total cellulase Activity	Betaglucosidase Activity
Tween20	102.3±3.56[e]	96.59±6.91	98.53±5.66
Tween80	105.8±3.98	107.3±9.54	100.29±4.92
TritonX100	85.19±4.67	86.17±7.19	80.53±6.94
2-ME[a]	51.33±2.97	45.96±2.18	62.83±8.54
EDTA[b]	85.37±4.65	89.55±3.56	70.56±4.73
SDS[c]	53.91±3.11	55.64±3.15	48.92±2.35
DMSO[d]	86.15±6.72	87.18±4.74	90.55±6.91
Glycerol	110.59±8.64	98.73±4.41	102.21±7.39
Control	100±5.79	100±4.99	100±6.52

[a] 2-ME: β-mercapto ethanol; [b] EDTA: Ethylene diamine tetra acetic acid; [c] SDS: Sodium dodecyl sulphate; [d] DMSO: Dimethyl sulphoxide ;
[e] Standard deviation from triplicate measurements
[f] Chemical agents were used at 1% concentration

3.3.2.5 Effect of organic solvents on cellulase stability

The effect of various organic solvents (20%, v/v) on cellulase stability was examined and the results are shown in Table 4. It is clear from the results that both enzymes were highly stable in the presence of xylene i.e., 30% stimulating effect on the total cellulase activity whereas its endoglucanase activity remained same and β-glucosidase increased by 10% of its initial activity . The higher total cellulase activity in the presence of xylene might be due to binding to the solid substrate such as filter paper used in assaying total cellulase activity.A similar enhanced effect on the hydrolysis of solid cellulose in the presence of xylene by cellulase has been reported by Kumakura (1997). In the presence of ethanol (20% w/v), processive endoglucanase maintained 63.95% of endoglucanase activity, 73.87% of total cellulase activity and β-glucosidase maintained 70.05% of enzyme activity. These results suggested that the ethanol tolerance property shown by the purified cellulase from *A. ochraceus* will have prospective applications in biofuel industry. Annamalai et al. (2013) had also reported an organic solvent (25%, v/v) stable alkaline cellulase from *Bacillus halodurans* CAS 1 strain. Besides, almost all enzyme activities such as 110.59% for endoglucanase, 98.73% for total cellulase and 102.21% for β-glucosidase were maintained in the presence of glycerol. The enhancement of enzyme activity by organic solvents might be due to the open active site conformation of enzymes acquired through hydrophobic non-polar interface of the organic solvent in action (Zaks and Klibanov, 1988). However, the presence of acetone reduced all the enzyme activities to a larger extent (75%). Therefore, acetone precipitation was not recommended for the precipitation and purification of the cellulases in the present study. Only a partial stability was observed when the enzymes were treated with 20% methanol for 60 min. To the best of our knowledge, this is the first report on

the *Aspergillus* derived cellulase with higher stability in (20%) ethanol and therefore owing to its usefulness in industrial bio-ethanol production.

Table 4: Stability of enzymes in presence of various organic solvents (20% v/v)

Organic solvents [b]	Residual activity (%)		
	Endoglucanase Activity	Total cellulase Activity	Betaglucosidase Activity
Control	100±2.85[a]	100±3.96	100±2.18
Methanol	48.21±3.56	42.01±3.65	38.97±3.61
Ethanol	63.95±5.98	73.87±5.91	70.05±4.99
Acetone	23.97±2.97	21.99±3.71	28.63±8.63

[a] Standard deviation from triplicate measurements
[b] Organic solvents were used at a final concentration of 20% (v/v)

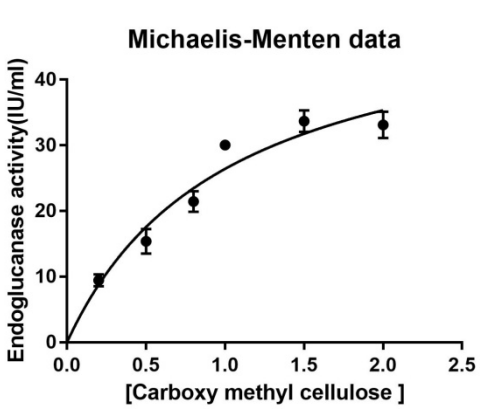
3.3.3 Kinetic parameters; Km, Vmax and Kcat

Kinetic paramaters for both processive endoglucanase and β-glucosidase are shown in Table 5. The kinetic studies revealed that the apparent Km, Vmax and Kcat values of purified processive endoglucanase (AS-HT-Celuz A) were 0.8175mg/ml,45.72 (μmol/ml/min) and $6.8x10^3$ s^{-1} respectively for CMC and the enzyme displayed a Km value of 1.020 mg/ml, Vmax value of 53.36 μmol/ml/min and a Kcat value of $6.3x10^3S^{-1}$ against avicel under the optimized assay conditions. The lowest Km value of the enzyme indicated that the AS-HT-Celuz A had greater affinity towards CMC than avicel. In addition , the Km, Vmax and Kcat values of purified β-glucosidase were estimated from the Lineweaver-Burk plot and representd as 3.535 mg/ml , 37.28 μmol/ml/min and $7.8x10^3$ s^{-1} respectively for 4-MUG. The Km value reported for the AS-HT-Celuz B was very higher than the Km values obtained from the β-glucosidase of other previously reported *Aspergillus* species (0.2mM to 1.6 mM), Bgl1D (0.54 mM) and Bgl1E (2.11 mM) from uncultured soil microorganisms (Jiang et al., 2011). It indicated that the enzyme had only least affinity towards the artificial substrate 4-MUG used in this present study. Of the three enzyme activities

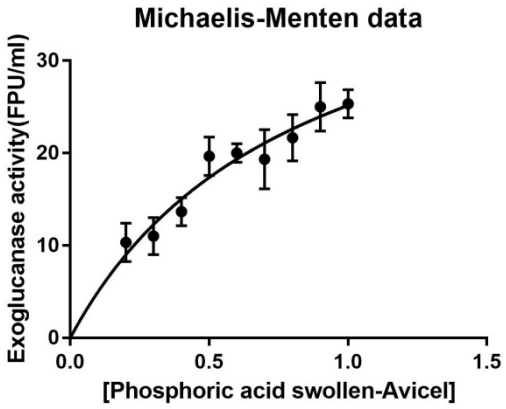
tested, highest catalytic efficiency was reported for AS-HT-Celuz A i.e., $8.3x10^3$ $S^{-1}mg^{-1}ml$ when incubated with the CMC, while it showed an efficiency of $6.1x10^3$ $S^{-1}mg^{-1}ml$ against avicel indicating its higher catalytic efficiency towards CMC and AS-HT-Celuz B has displayed an efficiency of $2.2x10^3$ $S^{-1}mg^{-1}ml$ against 4-MUG.

Table 5: Kinetic parameters of AS-HT-Celuz A (processive-type endoglucanase) and AS-HT-Celuz B (β-glucosidase)

Enzyme activity	Substrate	V_{max} (μmol/ml/min)	K_m (mg/ml)	K_{cat} (S^{-1})	K_{cat}/K_m ($S^{-1}mg^{-1}ml$)
Endoglucanase activity AS-HT-CEluz A (50μg/ml)	CMC	45.72	0.817	$6.8x10^3$	$8.3x10^3$
Total cellulase activity AS-HT-Celuz A (50μg/ml)	Avicel	53.36	1.020	$6.3x10^3$	$6.1x10^3$
β-glucosidase activity AS-HT-Celuz B (50μg/ml)	4-MUG	37.28	3.535	$7.8x10^3$	$2.2x10^3$



(Fig.5a)



(Fig.5b)

Fig.5a. Michaelis-Menten graph showing the Km and Vmax of AS-HT-Celuz A against carboxy methyl cellulose; Fig.5b. Michaelis-Menten graph showing the Km and Vmax of AS-HT-Celuz A against phosphoric acid swollen avicel

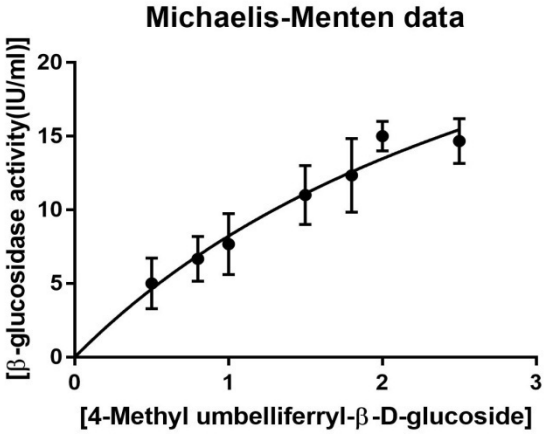


Fig.6. Michaelis Menten graph showing the Km and Vmax of AS-HT-Celuz B against the fluorogenic substrate 4-methyl umbelliferyl-β-D-glucoside

3.3.4 Substrate specificity and binding assays

Substrate specificity was determined by performing assay with various natural, synthetic and fluorogenic cellulosic substrates. The results depicted in the Table 6 revealed that AS-HT-Celuz A had high substrate specificity against rice hull followed by delignified coir pith indicating its higher catalytic efficiency towards natural cellulosic substrates than synthetic polysaccharides. It might also be ascribed to the potency of the enzyme, which is imparted by the induction of different cellulolytic enzymes due to the heterogeneity of cellulosic substrate used to produce hydrolytic enzymes (Feng et al., 2011). Specifically, it could also be argued that enzymes produced from a natural carbon substrate could perform hydrolysis of that particular substrate at a faster rate than with other cellulose substrates (Ilmen et al., 1997). The rate of synthetic cellulose degradation by purified AS-HT-Celuz A was in the order of CMC > phosphoric acid swollen avicel > hydroxyl ethyl cellulose > avicel > filter paper discs. The enzyme showed higher activity towards phosphoric acid swollen avicel than the microcrystalline avicel suggested its higher affinity towards soluble cellulose than the insoluble one. The outcomes also indicated that the endoglucanase purified from *A.ochraceus* could be confirmed as

processive endoglucanase as it hydrolysed both amorphous and crystalline cellulose even though the hydrolysis efficiency towards crystalline cellulose was less than that of soluble amorphous cellulose. Another uniqueness exhibited by processive endoglucanase was its activity against birch wood xylan. Similarly, EG purified from *A. terreus* showed high activity against xyloglucan substartes (Nazir et al., 2009). This property could be contributed towards the industrial heterogenous biomass conversion and also in the wine production industries, where it could be supplemented as an additive in potential enzyme cocktail formulation. However AS-HT-Celuz B could not hydrolyse any of the complex polysaccharides indicated that it is a pure β-glucosidase and showed activity only towards cellobiose, esculin and 4-methyl umbelliferryl β-D-glucopyranoside (4-MUG).

Table 6: Substrate specificity of purified cellulases against various cellulose substrates

Substrates	Specific activity (U/mg)	
	AS-HT-Celuz A	AS-HT-Celuz B
CMC[a] (50mM)	39.17	ND[g]
HEC[b] (50mM)	26.35	ND
Avicel (50mM)	11.63	ND
PASC[c] (50mM)	28.95	ND
FP [d] (1%)	9.56	ND
Delignified coir pith (1%)	42.17	2.96
Rice hull (1%)	46.39	0.56
Birchwood Xylan (50mM)	8.67	ND
Cellobiose (50mM)	3.97	12.97
Esculin (50mM)	0.68	20.87
4-MUC[e] (10mM)	18.98	ND
4-MUG[f] (10mM)	ND	26.15

[a] Carboxy methyl cellulose ; [b] Hydroxy ethyl cellulose ; [c] Phosphoric acid swollen cellulose ; [d] Filter paper ; [e] 4-Methyl umbelliferryl-β-D-cellobioside ; [f] 4-Methyl umbelliferryl-β-D-glucopyranoside ; [g] Not detected

The substrate binding property of the processive endoglucanase was determined on insoluble avicel and whatman No.1 filter paper. The unbound fraction was analysed for the avicelase activity and FPase activity and was found that the enzyme showed 30% and 43% binding to the insoluble avicel and filter paper discs respectively as determined from its initial activity before binding with the substrate. The binding property clearly demonstrated the presence of carbohydrate binding modules (CBD) in the processive endoglucanase, though their molecular mechanisms are still not studied in detail. Furthermore, the purified AS-HT-celuz B was not subjected for substrate binding assay since it didn't show hydrolytic activity towards avicel and filter paper and behaved as a pure β-glucosidase enzyme. Reinikainen et al. (1995) have reported the catalytic role of CBDs in the crystalline cellulose degradation which increases the efficiency of endoglucanses to strongly adhere into the cellulose polymer without detaching from it and progressed the enzyme action with higher processivity (Carrard et al., 2000).

3.3.5 Processivity determination

To determine the processivity of endoglucanse, the ratio of the soluble reducing ends to insoluble reducing ends (Irwin et al., 1993; Reverbel-Leroy et al., 1997) was calculated (Table 7). As shown in Fig.7, the processivity ratio increased from 1.79 to 3.12 when the incubation time was increased from 3 to 9 hrs. The similar effect have been observed by Zhang et al. (2010), who reported an increase in the processive endoglucanase activity of *Clostridium* cellulase when the incubation time was prolonged. The outcome undoubtedly suggested that AS-HT-Celuz A could be classified as a processive endoglucanase with respect to its activity in both amorphous and crystalline cellulose. Therefore, it could be considered as a better substitute for endoglucanase and exocellobiohydrolase representing complete hydrolytic

machinery for the synergistic degradation of coir pith cellulose into glucose with the help of its own β-glucosidase.

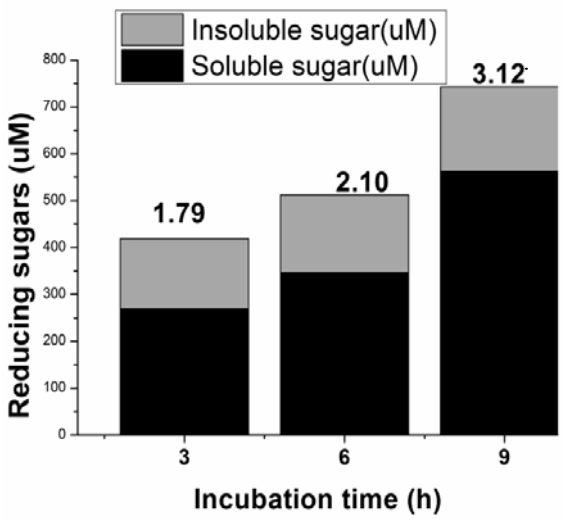


Fig.7. Reducing sugar liberated from soluble and insoluble fractions of filter paper discs by AS-HT-Celuz A (50 ug/ml) after 3, 6 and 9 h of incubations. The processivity ratio as determined by the ratio of soluble fraction to insoluble fraction was represented above the column.

Table 7: Amount of reducing sugar liberated from soluble and insoluble fractions of filter paper discs by AS-HT-Celuz A (50 ug/ml) after 3, 6 and 9 h of incubations and the corresponding processivity ratio as determined by the ratio of soluble fraction to insoluble fraction.

Incubation time (h)	Soluble sugars(uM)	Insoluble sugars(uM)	Processivity
3	269	150	1.79
6	347	165	2.10
9	563	180	3.12

3.3.6 Synergistic hydrolysis by purified cellulase components

The synergistic effect of purified cellulase enzymes is shown in Table 8. Increased rate of hydrolysis and degree of synergy were observed with sequential addition of enzymes than the simultaneous addition. The activity produced by the enzyme mixture blend was extensively higher than single enzyme treatment signifying its synergistic communication. The calculated

degree of synergy was 0.82 for the sequential addition and 0.68 for the simultaneous hydrolysis. For single enzyme treatment, the degree of synergy was 0.60 for AS-HT-Celuz A and 0.39 for AS-HT-Celuz B. An increment of 20% glucose production was clearly apparent in the sequential incubation than with simultaneous addition of equivalent unit of enzymes. As well, the rate of glucose production was less in the combined enzyme incubations than the theoretical sum of individual activities of AS-HT-Celuz A and AS-HT-Celuz B. Approximately 30% increase in glucose yield was obtained when β-glucosidase was added to the processive endoglucanase and this value is higher than those reported before (Zhang et al., 2010) In addition, an earlier report on the external addition of a metagenomic β-glucosidase into commercial *Trichoderma reesei* cellulase enzyme brought about a net increase of 20% glucose from corn stover at 50^0C (Del Pozo et al., 2012).

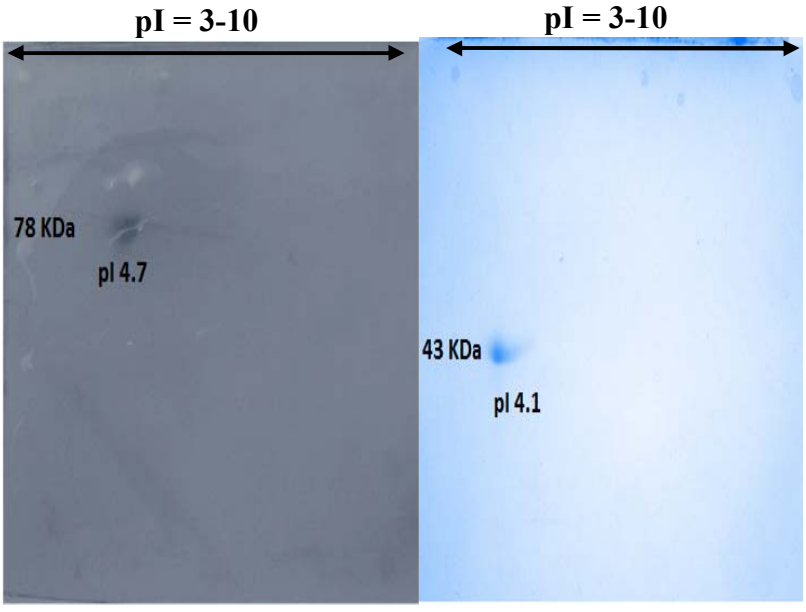
Table 8: The synergism of purified cellulases in the hydrolysis of 1:1 of CMC and cellobiose

Enzymes	Glucose Production (mM)	Degree of synergy
AS-HT-CeluzA (Processive endoglucanase)	1.4	0.60
AS-HT-CeluzB (β-glucosidase)	0.97	0.39
AS-HT-CeluzA + AS-HT-CeluzB (sequential addition)	2.02	0.82
AS-HT-CeluzA + AS-HT-CeluzB (simultaneous addition)	1.67	0.68

3.3.7 Two-Dimensional gel Analysis of AS-HT-Celuz A and AS-HT-Celuz B

The 2-Dimensional gel analysis of the purified AS-HT-Celuz A (processive-type endoglucanase) and AS-HT-Celuz B (β-glucosidase) had shown two distinctly separated spots (Fig.8a and Fig.8b respectively). The pI of AS-HT-Celuz A was found to be ≈ 4.7 (acidic pI), which is similar to its theoretical pI of 4.7. Comparatively a closer pI value of 4.1 was found for AS-HT-Celuz B. Different forms of *Aspergillus aculeatus* endoglucanase (I,

II, III, IV) were reported to have pI of 4.8 , 4.0, 3.5 and 3.4 respectively (Murao et al., 1988) whereas *A. niger* and *A. terreus* endoglucanase (II) with an acidic pI of 3.67 and 3.5 respectively (Okada, 1988 ; Nazir et al., 2009) and *A. niger* β-glucosidase had an acidic pI value of 4.55 as reported by Lima et al. (2013). Similarly, Bukhtojarove et al. (2004) reported that most of the cellulase proteins from *Aspergillus* spp. had an acidic to neutral pI values ranging from 3.8-6.7. The single spots obtained for even higher concentrations (400 µg) indicated that proteins were in homogenous form, and also it did not contain any subunit proteins. Moreover , the single spot obtained for AS-HT-Celuz A, is highly tempting to speculate that a single identical protein is responsible for both endo- and exo-glucanase activities, as previously detected by its zymogram analysis (section 3.3.1), and therefore it can be confirmed as a "processive-type cellulase"



(Fig.8a) **(Fig.8b)**

Fig. 8a. Two-dimensional analysis of AS-HT-Celuz A (78 KDa) with pI ≈ 4.7 (Coomassie stained 12% SDS-PAGE gel); Fig .8b. AS-HT-Celuz B (43 KDa) with pI≈4.1 (Silver stained 12%SDS-PAGE gel)

3.4 Conclusion

The study concluded that *Aspergillus ochraceus* MTCC 1810 had the potential to produce complete cellulase system comprising a processive endoglucanase (AS-HT-Celuz A, 78 KDa) and β-glucosidase (AS-HT-Celuz B , 43 KDa) capable for hydrolysing the cellulosic components of the coir pith into fermentable sugars. Unique properties like thermo stability, pH stability, ethanol tolerance, insoluble substrate binding affinity, multiple substrate specificity, sequential synergy and processivity showed that the purified enzymes could find wide applications in fermentation industry.

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Chapter 4

PROTEOMICS OF THE PURIFIED CELLULASES OF *Aspergillus ochraceus* MTCC 1810 AND THEIR APPLICATION IN BIOCONVERSION OF DELIGNIFIED COIR PITH

Contents

4.1 Introduction

Developments in the late 1980s, such as electrospray ionization or ESI (Fenn et al., 1989) and matrix assisted laser desorption/ionization or MALDI (Karas and Hillenkamp, 1988) connected to Time Of Flight (TOF) analyzers, drastically changed the scenario of proteomics and made polypeptides highly accessible to rapid mass spectrometric analysis. Proteomics is now being dominated by peptide-driven approaches, which allows sensitive and highthroughput detection of complex proteins with excellent mass accuracy and high resolution. The technique is also referred to as peptide-mass mapping or peptide-mass fingerprinting. MALDI-TOF MS is usually used in conjunction with prior protein fractionation methods such as one dimensional or two dimensional electrophoresis, since a profoundly homogenous protein is necessary for MS analysis. The general workflow for proteome based mass spectrometric analysis consists of three stages. In the first stage, proteins to be analysed is digested with specific enzymes and the resulting peptide is

fractionated. In the next stage, the fractionated peptides are subjected to qualitative as well as quantitative mass spectrometric analysis and in the final phase, the large data sets generated are identified by comparing the list of experimentally obtained peptide masses with the theoretical peptide masses through a database search and a subsequent statistical analysis to ensure confidence in the identification (Domon and Aebersold, 2006)

One of the important steps in proteomics is obtaining and processing the protein sample of interest. This approach is based on the digestion of gel separated proteins into peptides by trypsin (trypsinization). The advantage is that the peptides are very easily eluted from gels and even a small set of peptides would provide huge information for MS. In the 'peptide-mass mapping' approach as suggested by Henzel et al. (1993), the mass spectrum of the purified peptide mixture is acquired which results in 'peptide-mass finger printing' of the targeted protein (Henzel et al., 1993; Pandey and Mann, 2000). The peptide masses can be measured by matrix assisted laser desorption ionisation (MALDI), in which a co-precipitate of light absorbing matrix such as α-cyano-4-hydroxycinnamic acid or dihydroxy benzoic acid and the peptide mixture is irradiated by a short pulse of UV light in a vacuum. The discharged peptides are then getting ionised by attachment of protons and are accelerated due to a strong electric field. They are finally detected on a channeltron detector by being twisted around by an energy correcting ion mirror. As a result of this measurement, the time of flight (TOF-MS) distribution of the peptides in the trypsin digested protein is made known. After calibrating the mass spectrum through peak centroid values, a set of highly accurate peptide masses with a peak resolution of about 10,000 and a mass accuracy of a few parts per million (ppm) can be acquired, with the advantage of being versatile. A large number of peptide samples can be put on a single probe holder during a single run.

The main objective of this chapter is to characterize the purified proteins such as processive-type endoglucanase (AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B) from *A. ochraceus* MTCC 1810 through MALDI-TOF MS analysis. The proteomic identification of AS-HT-Celuz A was found difficult, since there were no reports found on processive cellulase activity and thus no database reports were available from an aerobic filamentous fungus, *Aspergillus* till now, while peptide analysis of AS-HT-Celuz B was done through usual MASCOT (www.matrixscience.com) searches, with all entries specified. The initial proteomics approaches such as 1-DE, 2-DE and zymography prior to the MS analysis have given precise data on its cellulase particular action. Based on these facts, a general approach of comparing experimentally obtained MS data of AS-HT-Celuz A with theoretical peptide masses of endoglucanases from other *Aspergillus* strains as well as processive endoglucanase from other glycoside hydrolase family was done by applying suitable cleavage rule to the entries in the sequence database. Subsequently, proteins are identified based on statistically significant mass values by peptide matches in the database.

Production of stable cellulase preparations with elevated specific activity on solid substrates (Zhang et al., 2006), high tolerance to extreme conditions such as high temperatures, low or high pH values, organic solvents, common protein denaturing agents and multiple substrate specificity makes these biocatalysts a powerful tool in depolymerization of lignocellulosic biomass. The economic production of cellulases can be achieved through the application of a proficient enzyme blend containing all component of cellulases required for complete hydrolysis of the cellulose polymer into its constituent glucose monomers. In addition to the proteomic identification of the complete cellulase system produced by *A. ochraceus,* this chapter also reports the applicability of partially purified

enzymes for bioconversion of delignified coir pith into glucose. The structural modifications of the pre-treated coir pith after enzymatic hydrolysis were also investigated through FTIR (Fourier Transform Infrared Spectroscopy), XRD (X-ray Diffraction) and SEM (Scanning electron microscopy).

4.2 Materials and Methods

4.2.1 Protein identification using molecular mass fingerprinting of peptide fragments - (MALDI-TOF MS)

4.2.1.1 In-gel tryptic digestion of protein samples

Two-dimensional electrophoresis (2-DE) of proteins such as AS-HT-Celuz A and AS-HT-Celuz B were performed as described in the section 3.2.17 of Chapter 3. The proteins of interest were excised from the 2D gel and subjected to an in-gel digestion protocol of Shevchenko et al. (1996). Each spots were cut using pipet cones and 500 ml acetonitrile per spot was added for 15 min to reduce the size the gel pieces and kept for a short spin. The supernatant above the gel pieces were discarded after a brief spin. Then the gel pieces were subjected for a reduction step using 50 µl of freshly prepared DTT solution (25 mM DTT in 25 mM ammonium bicarbonate) and incubated at 56^{o}C for 30 min in a water bath and cooled at room temperature (RT). Further, 500 µl of acetonitrile was added to each tube and incubated for 10 min and the supernatant was removed after a short spin. The gel pieces were then subjected for an alkylation step using 50 µl of freshly prepared iodoacetamide solution (55 mM iodoacetamide in 25 mM ammonium bicarbonate) and incubated in dark at RT for 30 min. The gel pieces were finally dehydrated with 500 µl of acetonitrile and all the liquid was removed. The gel pieces were covered with 50 µl of trypsin buffer (25 mM ammonium bicarbonate containing 15% (v/v) acetonitrile and incubated at 4^{o}C for 30 min. Freshly prepared, 20 µl of trypsin

stock solution (20 µg/ml in trypsin buffer prepared in 1 mM HCl) was added to the gel pieces containing proteins and incubated for 14 hrs at 37^{o}C. After incubation, the supernatant above the gel pieces were transferred into a sterile tube and peptide extraction buffer containing one volume of formic acid (5%) and two volumes of acetonitrile was added to the gel pieces and incubated at 37^{o}C for 30 min. The peptide extraction solution was removed and mixed with the supernatant obtained in the previous step.

4.2.1.2 Mass spectrometry of digested peptides

MALDI-TOF MS analysis was carried out by spotting 1µL of sample aliquot onto a metal target plate with 1 µL of matrix (α-cyano-4-hydroxycinnamic acid, 4 mg/mL in 80% v/v acetonitrile , 0.05% v/v Trifluoro acetic acid (TFA) and allowed to air dry. Matrix assisted laser desorption ionisation (MALDI) mass spectrometry was done with an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in MS mode. The metal target plate was appropriately aligned and calibrated using calibration mixture (4700 proteomics analyzer calibration mix, AB SCIEX, USA). The resultant mass spectra were in reflectrom mode and the instrument was then switched over MS/MS (TOF/TOF) mode where the highly intense peptides from the MS scan were identified and fragmented, then re-accelerated to quantify their mass (m/z) and intensities. The peak list generated was exported into a suitable format for database search program Mascot (Matrix Science Ltd, London UK). The MALDI-TOF MS analysis was accessed from the proteomic facility of Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India.

4.2.1.3 Protein identification using FindPept and MASCOT

Multiple amino acid sequence alignment (msa) of cellulase genes from different *Aspergillus* strains were carried out with ClustalX version 2.1 (Larkin

et al., 2007). *A. niger* endoglucanase and β-glucosidase unreviewed protein sequences were retrieved from UniProtKB database and kept as a query sequence in NCBI protein BLAST. Cellulase protein sequence from different *Aspergillus* spp. having more than 70% sequence homology with the query were taken for multiple sequence alignment with AS-HT-Celuz A and AS-HT-Celuz B. Similarly, processive endoglucanase domains of different fungal species were also taken for analysis of carbohydrate binding module (CBM) and exoglucanase protein domains. Experimentally obtained mass spectrum (MS peaks) of both AS-HT-Celuz A and AS-HT-Celuz B were matched with the theoretically obtained mass peaks of reference organisms using FindPept (http://au.expasy.org/tools/findpept.html) and FindMod (http://au.expasy.org/ tools/findmod/) programme for determining post-translational modifications. The input search parameters used for FindPept tool included, 1 missed cleavage; 2 and 0.5 Da mass accuracy allowed for parent and the fragment ions, respectively, fixed modification- carbamidomethyl, variable modifications- oxidized methionine. Similarly, glycosyl hyrolase family classification was also done by retrieving similar proteins from CAZy database (http://afmb.cnrs-mrs.fr/CAZY/). Peptide fragments of AS-HT-Celuz A and AS-HT-Celuz B were analysed with the MASCOT program (www.matrixscience.com) using following input parameters: Swiss-Prot sequence database, taxonomy – all entries, enzyme – trypsin, allowed missed cleavages – 1, fixed modifications – carbamidomethyl (C), variable modifications – none and Oxidation (M), peptide tolerance – 0.2Da and 0.4Da, MS/MS tolerance – 0.5Da, peptide charge – 1, instrument – MALDI-TOF/TOF. Thus the protein will be successfully identified if it had hit with a random match probability (p value) < 0.05. All database searches were carried out using a generic database at UniProtKB/ Swiss-Prot (http://au.expasy. org/databases/), with no species specified.

4.2.2 Production and application of AS-HT-Celuz A and AS-HT-Celuz B in the saccharification of pre-treated coir pith

Cellulases were produced under optimal conditions as described in Chapter 2. The modified Reese and Mandel's fermentation medium supplemented with 1% chemically delignified coir pith, 10% inoculum, 0.75% urea and 0.8% Tween 80 with other optimized conditions was incubated at 35±5^{0}C and pH 6.0 for 7 days. Subsequently, fermentation medium was scaled up to 2000 ml for mass production of cellulases. The enzymes were extracted using 80% ammonium sulphate precipitation, desalted for 12 hours against 0.05 M citrate buffer (pH 6.0) at 4^{0}C. The resultant enzyme preparation was concentrated using Amicon ultra centrifugal filters (Millipore, USA), according to the manufacturer's instructions. The procedure adopted for ammonium sulphate precipitation and concentration was already mentioned in Chapter 3 section 3.2.2. The enzyme activities were determined as described in the section 2.2.6.1 of Chapter 2 and stored at -20^{0}C until use.

Hydrolysis of pre-treated coir pith was carried out at 12% (w/v) of substrate concentration with sequential enzyme loading of AS-HT-Celuz A (25 FPU/g of cellulose) for initial 12 h and AS-HT-Celuz B (10 U/g of cellulose) for further 12 h in a final volume of 100 ml of sodium citrate buffer (pH 6.0). The second hydrolysis experiment was set up with a simultaneous enzyme loading of AS-HT-Celuz A (25 FPU/g of cellulose) and AS-HT-Celuz B (10 U/g of cellulose) for a total of 24 hours. The fermentation flasks were supplemented with sodium azide (0.02%) to prevent any microbial contamination. The reaction mixture in screw-capped conical flasks was then incubated at 40^{o}C for 24 hrs at 100 rpm. Control experiments were also run with single enzyme alone and

without enzyme treatment. After enzymatic saccharification, the reaction mixtures were centrifuged at 10,000 rpm for 20 min and the supernatant was assayed for reducing sugars by dinitrosalicylic acid method as described in Chapter 2 (section 2.2.6.1). The glucose liberated was estimated using GOD-POD glucose assay kit (Biolab Diagnostics Pvt. Ltd.). Briefly, working solution was prepared by transferring the contents of one vial of glucose reagent 1 (Glucose oxidase, peroxidase, aminoantipyrine, buffer, stabilizers) to an amber coloured plastic bottle (provided with the kit) and reconstituted the content of each bottle with reagent 2 (phenol diluent) and an aliquot of 150 µl of the supernatant sample was mixed with 150 µl of working glucose oxidase reagent, mixed well and incubated at 37^{o}C for 15 min. Then absorbance was measured at 505 nm against reagent blank.The absorbance recorded is directly proportional to the glucose concentration. Saccharification efficiency was also calculated using the following equation (Taniguchi et al., 2005).

$$\text{Saccharification efficiency (\%)} = \frac{C \times 0.89 \times 100}{M}$$

C=Concentration of glucose in mg/ml, M= amount of cellulose waste in mg/ml, the multiplication factor 0.89 converts monosaccharides to polysaccharides due to water uptake during hydrolysis.

4.2.2.1 End product analysis using thin layer chromatography

Residues from the all the hydrolysis experiments (single enzyme treatments, sequential treatment and simultaneous treatment) of pre-treated coir pith with partially purified cellulases were spotted on silica gel plates (Silica gel 60 F254, Merck) and the chromatography was developed in a mobile phase of n-butanol: glacial acetic acid: water (2:1:1) for 2 h. Sugars were detected by

spraying with 5% sulfuric acid in methanol followed by heating at 100^{o}C for 10 min . The standard used was 20 mg/ml of glucose as it is the only predicted end product.

4.2.2.2 End product analysis using gas chromatography

The hydrolytic residue after 24 h incubation (sequential hydrolysis) of the pre-treated coir pith with the partially purified enzymes from *A. ochraceus* were collected after terminating the reaction by heating at 100^{0}C . Samples were then filtered through a 0.2 μm syringe filter to remove solid particles and 5 ml of the reaction was lyophilized. The end product was identified after silylation with hexamethyldisilazane (HMDS) and pyridine and separation by gas chromatographic method of Fabbri et al.(2002).The derivatized sample was analysed by gas chromatography (Agilent Technologies-7890 GC System, 5975C inert MSD) using the instrument facility available at Department of Applied Chemistry , CUSAT. The resultant mass spectra were compared against a library of known carbohydrate standards.

4.2.3 Micro-Structural modification of the pretreated coir pith after enzymatic saccharification

It is necessary to characterize the chemically pretreated coir pith (CTC) and enzyme hydrolysed coir-pith (ETC) for understanding the structural modifications induced during the enzymatic hydrolysis .The instrumental tools such as FTIR (Fourier Transform Infrared Spectroscopy), XRD (X-ray Diffraction) and SEM (Scanning electron microscopy) were adopted in the present study to analyse the structural changes occurred in the substrate.

4.2.3.1 FTIR analysis

The comparison spectra of the structural modification of the coir pith before and after enzymatic hydrolysis was obtained by fourier transform infrared (FTIR) analysis. FTIR spectrum of the enzymatically treated coir pith (ETC) along with delignified coir pith (CTC), was taken on an FTIR spectrophotometer using KBr disc containing 1% finely ground samples. Thirty two scans were taken for each sample with a resolution of 4 cm^{-1} and the corresponding absorbance spectra were recorded at wave numbers from 500 – 4000 cm^{-1}.

4.2.3.2 XRD analysis

X-ray diffraction study was carried out to analyse the changes in the crystalline nature of the chemically pre-treated (CTC) and enzyme hydrolysed coir pith (ETC) by using Rigaku X – Ray diffractometer. The radiation used was of CuKα radiation at a wavelength of 1.5418 A^o. The samples were scanned at a scan rate of 1^o and a step time of 60 sec with scan angle (2θ) from 7^o to 40^o and the step size of $0.02^o(2\theta)$. Cellulose crystallinity index (CI) from the XRD pattern was calculated using the following equation (Segal et al., 1959).

$CI_{XRD}(\%) = (I_{002} - I_{AM})/I_{002} \times 100$; where CI indicates relative degree of crystallinity, I_{002}, maximum intensity (in arbitrary units) of the 002 lattice diffraction and I_{AM}, intensity of diffraction in the same units at 2θ = 18°.

4.2.3.3 Scanning electron microscopy (SEM)

Scanning electron microscope analysis was performed on both chemically treated (CTC) and enzyme treated coir pith (ETC) to analyze the morphological changes induced after enzymatic saccharification. SEM images

of pre-treated and enzyme hydrolysed coir pith were taken at 10 μm range with magnification level of 1500X.

4.3 Results and Discussion

4.3.1 Peptide mass fingerprinting and protein identification

Mass spectrometry is a proteome based approach to identify protein structural information such as amino acid sequences through peptide mass analysis (Perkins et al., 1999; Pandey and Mann, 2000). The individual spots for AS-HT-Celuz A and AS-HT-Celuz B were excised from 2D gel and analysed by mass spectrometry. The corresponding peak list (m/z values) generated from AS-HT-Celuz A and AS-HT-Celuz B was manually uploaded on the FindPept online tool of ExPASy. The experimentally obtained m/z values were matched with the theoretical mass list generated from the tryptic digest of query amino acid sequence of endoglucanase, processive cellulase, endo-exo glucanases (bifunctional domains), fungal carbohydrate binding modules (CBM) and β-glucosidase proteins from multiple aligned sequences of similar protein sequences from other filamentous fungi and *Aspergillus* strains available in UniProtKB and CAZy database as input parameters. The output result page showed user mass, database mass (theoretical mass), delta mass (difference in theoretical and user mass), peptide sequence consistent with mass and corresponding amino acid position in query sequence.

The summary of MALDI-TOF mass spectrum analysis of the trypsin digested proteins such as AS-HT-Celuz A and AS-HT-Celuz B were shown in Fig.1 and Fig.4 respectively, and the corresponding peptide sequences that matched to the experimental mass, glycoside hydrolase family classification

and biological function are given in Tables 1 &2 (AS-HT-Celuz A) , Table 3 (AS-HT-Celuz B). Experimentally obtained peptide masses showed unspecific cleavage with the enzyme and thus showed no match with any of the theoretically digested peptide masses (not included in the Tables), but it did not prevent the program from identification of the protein. The deduced peptide sequence from AS-HT-Celuz A mass spectrum (Fig.1a) showed highest similarities to endoglucanase of *Clostridium cellobioparum* (44%) and *Clostridium termitidis* (44%), exoglucanase of *Clostridium* sp.(40%), endoglucanase of bacterium JKG (40%), exoglucanase of *Lachnoclostridium phytofermentans* (40%), endoglucanase of *Herbinix* sp. (37%) through NCBI BlastP analysis (Fig.2). The peptide sequence was further analysed for detecting the presence of novel processive endoglucanase domains from *Clostridium* sp., endo-and exo- bifunctional domains and fungal specific carbohydrate binding domains through Find Pept analysis (Table 1 & 2). The basis of obtaining reference protein sequences from the databases was limited to its experimental molecular mass, enzyme activity, pI and type of the organism. However the functional annotation of the hypothetical processive endoglucanase from AS-HT-Celuz A through FindPept analysis was found to be more effective than MASCOT searches, since the processive activity had not been reported till and thus no sequence information being available from *Aspergillus* through database searches. Cellulase processivity assays (section 3.3.5), zymogram (section 3.3.1) and 1-DE (3.3.1) and 2-DE analysis (section 3.3.7) supported the mass spectrum data to categorise novel AS-HT-Celuz A in the processive cellulase family. Further, AS-HT-Celuz B was identified as β-glucosidase of glycoside hydrolase family 3 and its peptide sequence deduced from its corresponding peptide masses are shown in Fig.4a. The

aminoacid sequences showed significant similarities to β-glucosidases of *A. niger* (56%), *A. kawachii* (54%), *A. luchuensis* (54%), *A. terreus* (53%), A. *saccharolyticus* (51%), *A. aculeatus* (51%), *A. nidulans* (49%) , *A. calidoustus* (47%), *A. clavatus* (47%), *A. flavus* (51%), *A. oryzae* (51%) , *A. fumigatus* (49%), *A. rambellii* (48%) and *A. ochraceoroseus* (48%). Multiple sequence alignment of AS-HT-Celuz B with its related sequences are shown in Fig.5.

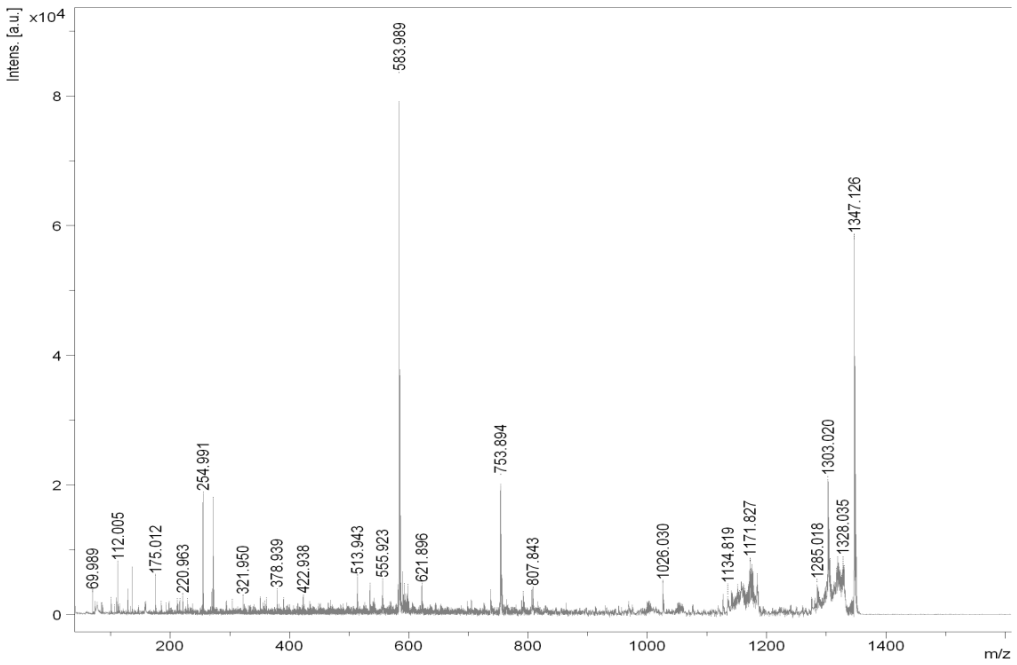
All the identified proteins are glycoside hydrolytic enzymes involved in carbohydrate metabolism (Davies and Henrissat, 1995; Coutinho and Henrissat, 1999), such as processive endoglucanase and β-glucosidase capable for complete cellulose saccharification into glucose monomers. The several glycoside hydrolase family domains (1, 3, 4, 9 and 48) present in AS-HT-Celuz A (Table 1& 2) might be because of the multiple-substrate specificity shown by the protein, which confirmed the hypothesis of divergent evolution of a basic fold at its catalytic site to accommodate different substrates (Lynd et al., 2002).

Homology models for both AS-HT-Celuz A and AS-HT-Celuz B were predicted using SWISS-MODELwork space (http://swissmodel.expasy.org) and Model-Template alignment of AS-HT-Celuz A with the template,GH48 cellobiohydrolase from *Caldicellulosiruptor bescii* showed a similarity of 51.15% (Fig. 3) and AS-HT-Celuz B with the reference model, *Aspergillus* sp. Family GH3 beta-D-glucosidases showed similarity of 63.24% (Fig. 6). The sequence similarity obtained for both proteins with their templates are significantly higher (>50%), so the protein structural models could be further validated for downstream-proteomic analysis.

Table 1: Summary of matching peptides of AS-HT-Celuz A (Processive-type endoglucanase) as predicted by FindPept program (http://au.expasy.org/tools/findpept.html). Peptide masses were analysed for endoglucanase and carbohydrate binding module (CBM) of cellulase proteins from multiple sequence alignhnment files (msa) of different *Aspergillus* strains.

Exp. Mass	Theo. Mass	Δmass (daltons)	Peptide sequence consistent with mass	Peptide position	GH family	Known activity(CAZy)
175.012	175.119	0.106	(K)/R/(A)	27-27	1	Enoglucanase
475.918	476.246	0.328	(K)/TNNK/(K)	257-260	1	Enoglucanase
628.048	628.414	0.366	(K)/IQVLR/(D)	63-67	1	Enoglucanase
887.303	887.447	0.143	(K)/ALTDPQDK/(L)	211-218	1	Enoglucanase
1361.024	1360.722	-0.302	(K)/ERVTEATQWLK/(D)	223-243	9	Enoglucanase
1375.157	1374.738	-0.419	(K)/ERLQTATEWLK/(T)	246-256	9	Enoglucanase
1391.998	1391.669	-0.329	(R)/YSGSIISSTSDFK/(T)	124-136	9	Enoglucanase
1474.092	1473.836	-0.256	(R)/VAFRMERLIPNK/(M)	54-65	9	Enoglucanase
1504.369	1504.775	0.406	(R)/VTSATQWLKDNNK/(V)	249-261	9	Enoglucanase
1703.074	1702.923	-0.154	(K)/ERVSAATKWLKDNGK/(V)	222-236	9	Enoglucanase
1768.256	1767.903	-0.352	(R)/SAGMNIFRVPFLMER/(L)	69-83	9	Enoglucanase
1991.204	1990.954	-0.249	(R)/FNGEIISTASDFQTFWK/(N)	125-141	9	Enoglucanase
2035.079	2034.927	-0.19	(R)/FNGEIMSTPSDFQTFWK/(N)	125-141	9	Enoglucanase
2107.676	2108.008	0.331	(K)/IGIIGEFDGGDNDQCRTAVK/(G)	238-257	9	Enoglucanase
415.991	416.262	0.27	(R)/QIR/(D)	264-266	1	Fungal CBD
621.866	622.356	0.489	(R)/SKPYK/(L)	124-128	1	Fungal CBD
753.852	753.362	-0.489	(R)/SSETTTK/(T)	60-66	1	Fungal CBD
755.296	755.372	0.076	(K)/FISQFN	324-329	1	Fungal CBD

Fig.1. (A)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1285.018
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378.939
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513.943
555.923
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800
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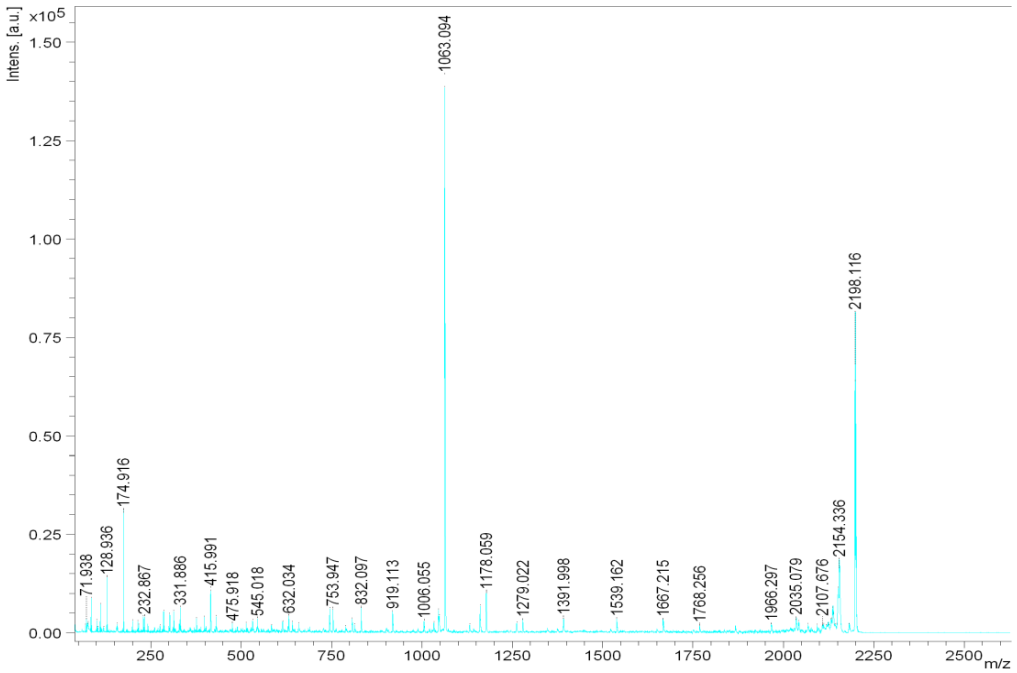
m/z

Fig.1.(B)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753.947
832.097
919.113
1006.055
1178.059
1279.022
1391.998
1539.162
1667.215
1768.256
1966.297
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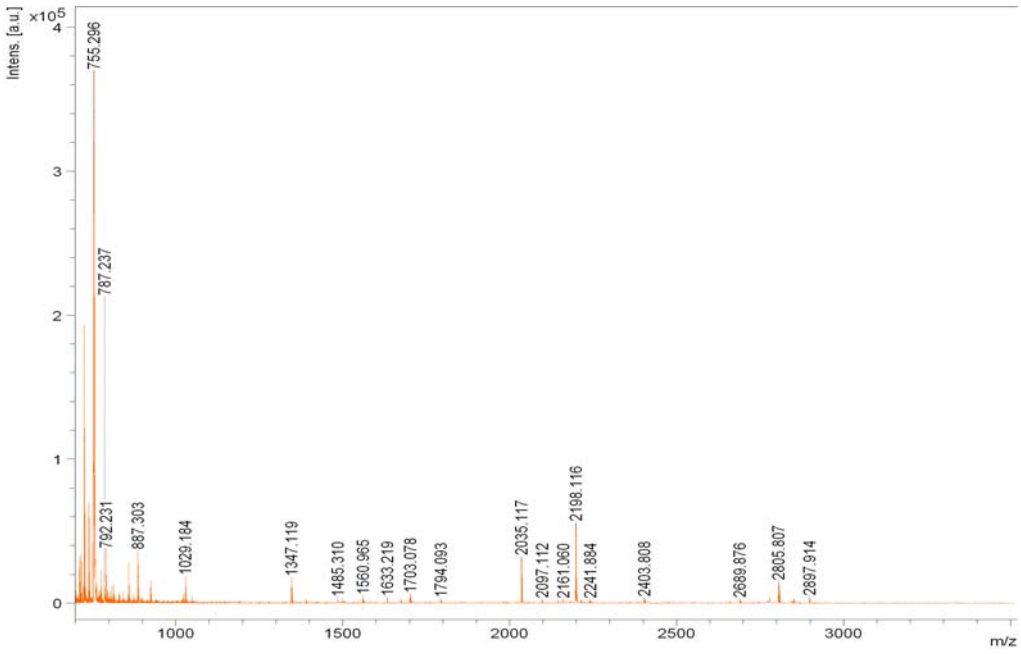
m/z

Fig.1. (C)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1703.078
1794.093
2035.117
2097.112
2161.060
2198.116
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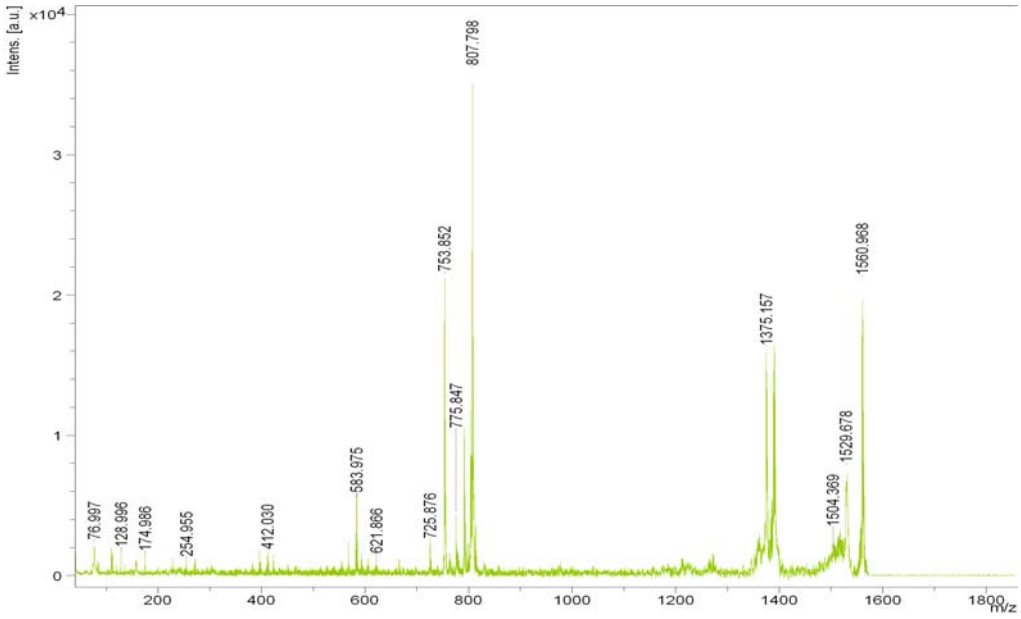
2500
3000
m/z

Fig.1. (D)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128.996
174.986
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m/z

Fig.1. (E)

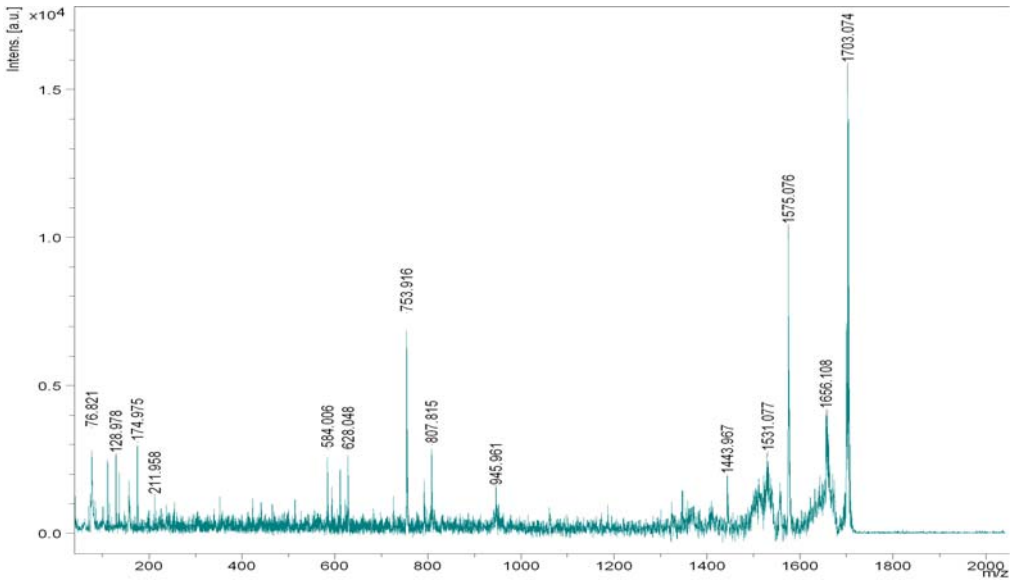


Fig.1. (F)

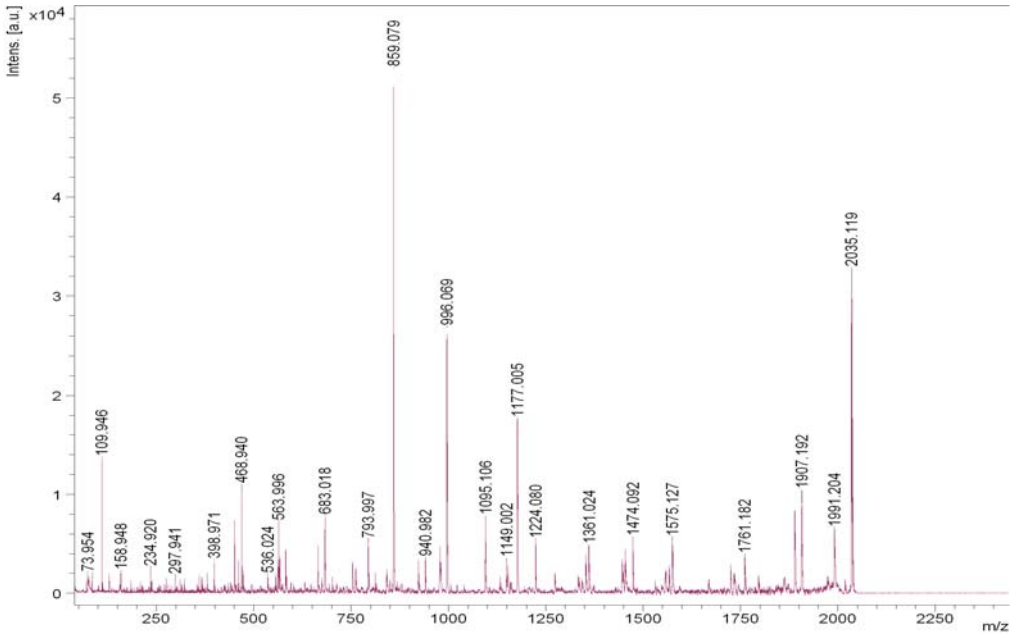


Fig.1. MALDI-TOF mass spectra of AS-HT-Celuz A (Processive-type endoglucanase)

Table 2: Summary of matching peptides of AS-HT-Celuz A (processive-type endoglucanase) using FindPept program (http://au.expasy.org/tools/findpept.html). Peptide masses were analysed for processive endoglucanase, endo-exoglucanase bifunctional domains and multiple glucoside hydrolase family domains retrieved from Swiss-Prot and TrEMBL databases.

Experimental Mass	Theo. Mass	Δmass (daltons)	Peptide sequence consistent with mass	Peptide position	GH family	Known activity(CAZy)
475.918	476.283	0.364	(K)/SLTR/(Q)	347-350	4	endoglucanase
683.018	683.299	0.281	(R)/EDYTR/(F)	563-567	4	processive endoglucanse
725.876	726.305	0.429	(K)/YDDASR/(V)	513-518	4	processive endoglucanse
753.894	754.355	0.461	(K)/MGDYLR/(Y)	256-261	4	processive endoglucanse
753.916	754.355	0.439	(K)/MGDYLR/(Y)	256-261	4	processive endoglucanse
753.947	754.355	0.408	(K)/MGDYLR/(Y)	256-261	3	exoglucanse
775.847	775.431	-0.416	(K)/TNDVKAK/(Q)	456-462	3	exoglucanse
832.097	832.472	0.374	(K)/WVTWIK/(S)	451-456	3	exoglucanse
919.113	919.463	0.35	(K)/SGQVPEFR/(Y)	595-602	4	processive endoglucanse
945.961	945.515	-0.445	(K)/VWAKEQGK/(F)	232-239	48	glycoside hydrolase
945.961	945.515	-0.445	(K)/VWAKEQGK/(F)	232-239	48	Exoglucanase
1026.03	1026.525	0.495	(K)/VDFFVNGTK/(V)	675-683	48	Exoglucanase
1026.03	1026.474	0.443	(R)/VSAADDGTYK/(A)	22-31	48	Exoglucanase
1063.094	1063.48	0.386	(K)/SQNGATDWGK/(S)	337-346	48	Exoglucanase
1177.005	1176.568	-0.436	(K)/YKQDPDWPK/(L)	580-588	48	Exoglucanase
1177.005	1176.568	-0.436	(K)/YKQDPDWPK/(L)	580-588	3	Exoglucanase
1224.08	1224.576	0.495	(K)/DQNYSKQWR/(Y)	204-212	3	Exoglucanase
1224.08	1224.576	0.495	(K)/DQNYSKQWR/(Y)	204-212	3	processive endoglucanse
1347.119	1347.606	0.487	(K)/DYGVNVDTYSSK/(A)	275-286	3	processive endoglucanse
1347.126	1347.606	0.48	(K)/DYGVNVDTYSSK/(A)	275-286	3	processive endoglucanse
1361.024	1360.686	-0.338	(K)/SPNGASDWAKSLK/(R)	335-347	3	processive endoglucanse
1375.157	1374.701	-0.455	(K)/SPTGANDWAKSLK/(R)	335-347	48	processive endoglucanse
1391.998	1391.695	-0.302	(R)/SKYKQDPDWPK/(L)	578-588	48	processive endoglucanse
1391.998	1391.695	-0.302	(R)/SKYKQDPDWPK/(L)	578-588	48	processive endoglucanse
1443.967	1443.663	-0.304	(R)/WFGFQAWSMQR/(V)	431-441	48	processive endoglucanse
1485.31	1484.858	-0.451	(K)/NLAKELLDRVWK/(L)	544-555	48	Exoglucanase
1485.31	1485.733	0.423	(K)/QFKYTIASDADAR/(A)	242-254	48	Exoglucanase
1531.077	1530.802	-0.274	(K)/SPTGANDWAKSLKR/(Q)	335-348	48	Exoglucanase
1560.965	1560.813	-0.152	(K)/LYRDDKGVAAPEAR/(A)	556-569	48	glycoside hydrolase
1560.965	1560.769	-0.195	(K)/QDPDWPKLEAAYK/(S)	582-594	48	glycoside hydrolase
1560.965	1560.769	-0.195	(K)/QDPDWPKLEAAYK/(S)	582-594	3	glycoside hydrolase
1560.968	1560.813	-0.155	(K)/LYRDDKGVAAPEAR/(A)	556-569	3	glycoside hydrolase
1560.968	1560.769	-0.198	(K)/QDPDWPKLEAAYK/(S)	582-594	3	glycoside hydrolase
1560.968	1560.769	-0.198	(K)/QDPDWPKLEAAYK/(S)	582-594	3	Exoglucanase
1761.182	1760.897	-0.285	VTGVYQGRFNELYSK/(L)	13-28	3	Exoglucanase
1794.093	1793.889	-0.203	(R)/ASKLGDYLRYSMFDK/(Y)	280-294	3	Exoglucanase
2097.112	2097.258	0.146	(K)/QILDKWVTWIKSVVILR/(A)	446-462	3	Exoglucanase

V S A A D D G T Y K - V A F R M E R L I P N K - S S E T T T K - I Q V L R -
SAGMNIFRVPFLMER-YSGSIISSTSDFK-SKPYK-FNGEIMSTPSDFQTFWK-
DQNYSKQWR-ALTDPQDK-ERVSAATKWLKDNGK-ERVTEATQWLK-
V W A K E Q G K - I G I I G E F D G G D N D Q C R T A V K - Q F K Y T I A S D A D A R -
E R L Q T A T E W L K - V T S A T Q W L K D N N K - M G D Y L R - T N N K - Q I R -
DYGVNVDTYSSK-ASKLGDYLRYSMFDK-FISQFN-SPTGANDWAKSLKR-
SLTR-WFGFQAWSMQR-QILDKWVTWIKSVVILR-WVTWIK-TNDVKAK-
Y D D A S R - N L A K E L L D R V W K - L Y R D D K G V A A P E A R - E D Y T R -
SKYKQDPDWPK-YKQDPDWPK-QDPDWPKLEAAYK-SGQVPEFR-
VDFFVNGTK

Fig.1a. AS-HT-Celuz A protein sequence deduced from its corresponding peptide masses

```
AS-HT-CeluzA            ---------VKAK-YDDASR-NLAKELLDRVWK-LYRDDKGVAAPEAR-EDYTR-SKY-K
endogluca.              ----------------DTQARDMAKSLLDRMWA-LYRDDKGLAAPETRSD-YERF-DDPI
exogluc.                NALLYYSKAA-----NDVAAKNLAKELLDRVWK-LYRDDKGVAAPEARAD-YKRFFEQTV
exogluc.[L.             NTLLYYSKAA-----NDVAAKNLAKELLDRVWN-LYRDDKGVAAPELRTD-YKRFFEQVI
GH[Caldicellulosiruptor NALLYYSAGTKKYGVFDEEAKNLAKELLDRMWK-LYRDNKGLSAPEKRAD-YKRFFEQEV
exogluca.[Herbinix      NALLYYAKAS-----GDDEARELAKELLDRIWT-LYRDDKGVAAPESRED-YKRFFEQEV
exoglucan.[Clostridium  NTLLYYSKAS-----GDDEARVLAKELLDRMWT-LYRDDKGLAIPEKRAD-YKR------
endogluc.[Clostridium   NTLAYYAAAS-----GDTTSQNNAKKLLDSMWN-NYQDSKGISVPESRSD-YHRLLDQEV
endogluc.[Herbinix      NALAYHGKAT-----GNETSKNNAKKLLDCMWN-NYQDDKGIVSEEVRED-YFRFLEQEV
                                       :      **.*** :* * *:*.**:   * * : * *

AS-HT-CeluzA            -QDPDWPK----------YKQDPDWPK-QDPDWPKLEAAYK-SGQVPEFR-VDFFVNG
endogluca.              YIPSGWSGRMPNGDPINSSSTFLSIRSKYRQDPQWSKVQAYLN-GGPVPTFTYHRF----
exogluc.                YVPSTFNGKMPNGDVIKSGIKFLDIRSKYLQDPSYPKLLAAYQ-SNKSPEF---------
exogluc.[L.             YVPSTFSGKMPNGDVIKSGVKFLDIRSKYLQDPSYQKLLAAYQ-NNKTPEFTYHRF----
GH[Caldicellulosiruptor YIPAGWTGKMPNGDVIKSGVKFIDIRSKYKQDPDWPKLEAAYK-SGQVPEFRYHRF----
exogluca.[Herbinix      YVPEGWTGTMPNGDKIEHGITFLDIRSKYLDDPDYPKLLEAYE-KGEAPEFTYHRF----
exoglucan.[Clostridium  ----------------------------------------------------------
endogluc.[Clostridium   YVPQGWSGTMANGDKIQPGIKFIDIRSKYKQDPDWQRVEAD------------------
endogluc.[Herbinix      YVPEGWSGTMPNGDEIKPGIKFIDIRSKYRQDPDWARVEADLL-AGRAPTMKYHRF----

AS-HT-CeluzA            ---------VKAK-YDDASR-NLAKELLDRVWK-LYRDDKGVAAPEAR-EDYTR-SKY-K
endogluca.              ----------------DTQARDMAKSLLDRMWA-LYRDDKGLAAPETRSD-YERF-DDPI
exogluc.                NALLYYSKAA-----NDVAAKNLAKELLDRVWK-LYRDDKGVAAPEARAD-YKRFFEQTV
exogluc.[L.             NTLLYYSKAA-----NDVAAKNLAKELLDRVWN-LYRDDKGVAAPELRTD-YKRFFEQVI
GH[Caldicellulosiruptor NALLYYSAGTKKYGVFDEEAKNLAKELLDRMWK-LYRDNKGLSAPEKRAD-YKRFFEQEV
exogluca.[Herbinix      NALLYYAKAS-----GDDEARELAKELLDRIWT-LYRDDKGVAAPESRED-YKRFFEQEV
exoglucan.[Clostridium  NTLLYYSKAS-----GDDEARVLAKELLDRMWT-LYRDDKGLAIPEKRAD-YKR------
endogluc.[Clostridium   NTLAYYAAAS-----GDTTSQNNAKKLLDSMWN-NYQDSKGISVPESRSD-YHRLLDQEV
endogluc.[Herbinix      NALAYHGKAT-----GNETSKNNAKKLLDCMWN-NYQDDKGIVSEEVRED-YFRFLEQEV
                                       :      **.*** :* * *:*.**:   * * : * *

AS-HT-CeluzA            -QDPDWPK----------YKQDPDWPK-QDPDWPKLEAAYK-SGQVPEFR-VDFFVNG
endogluca.              YIPSGWSGRMPNGDPINSSSTFLSIRSKYRQDPQWSKVQAYLN-GGPVPTFTYHRF----
exogluc.                YVPSTFNGKMPNGDVIKSGIKFLDIRSKYLQDPSYPKLLAAYQ-SNKSPEF---------
exogluc.[L.             YVPSTFSGKMPNGDVIKSGVKFLDIRSKYLQDPSYQKLLAAYQ-NNKTPEFTYHRF----
GH[Caldicellulosiruptor YIPAGWTGKMPNGDVIKSGVKFIDIRSKYKQDPDWPKLEAAYK-SGQVPEFRYHRF----
exogluca.[Herbinix      YVPEGWTGTMPNGDKIEHGITFLDIRSKYLDDPDYPKLLEAYE-KGEAPEFTYHRF----
exoglucan.[Clostridium  ----------------------------------------------------------
endogluc.[Clostridium   YVPQGWSGTMANGDKIQPGIKFIDIRSKYKQDPDWQRVEAD------------------
endogluc.[Herbinix      YVPEGWSGTMPNGDEIKPGIKFIDIRSKYRQDPDWARVEADLL-AGRAPTMKYHRF----
```

Fig.2. Multiple Sequence Alignment (Clustal X) of AS-HT-Celuz A from *Aspergillus ochraceus* MTCC 1810 with some of its neighbouring sequences, endoglucanase of *Clostridium cellobioparum* (44%) and *Clostridium termitidis* (44%), exoglucanase of *Clostridium* sp.(40%), endoglucanase of bacterium JKG (40%), exoglucanase of *Lachnoclostridium phytofermentans* (40%), endoglucanase of *Herbinix* sp. (37%) , conserved sequences are shown in astriques (*) .

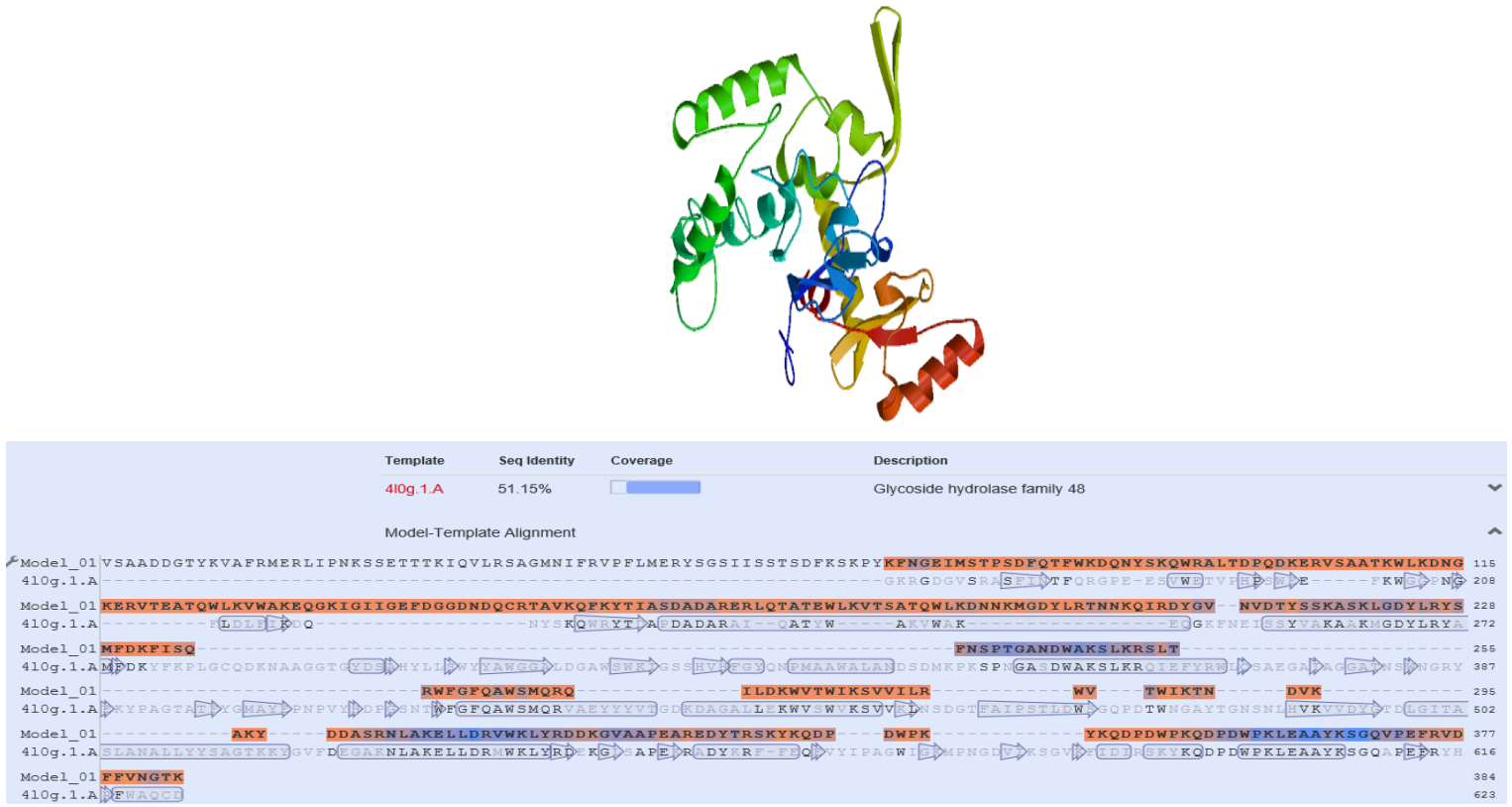
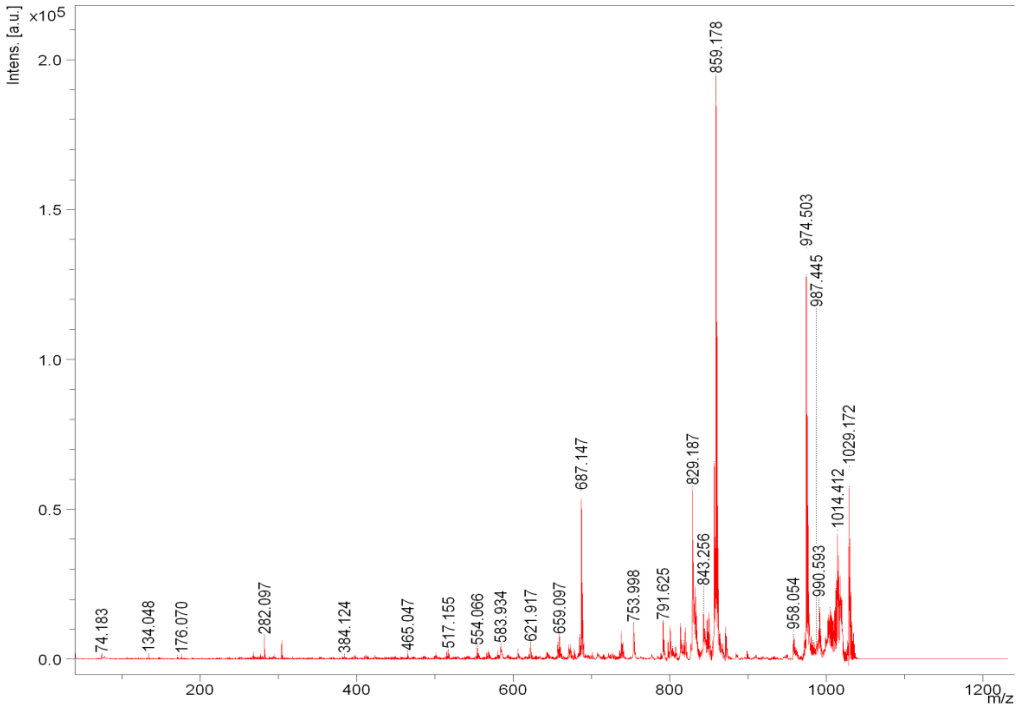


Fig. 3. The 3D-structural model of AS-HT-Celuz A (processive-type endoglucanase) as predicted through SWISS-MODELwork space (http://swissmodel.expasy.org) and Model-Template alignment with GH48 cellobiohydrolase from *Caldicellulosiruptor bescii* (51.15%)

Fig.4. (A)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1.0
0.5
0.0
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400
600
800
1000
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m/z
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134.048
176.070
282.097
384.124
465.047
517.155
554.066
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621.917
659.097
687.147
753.998
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990.593
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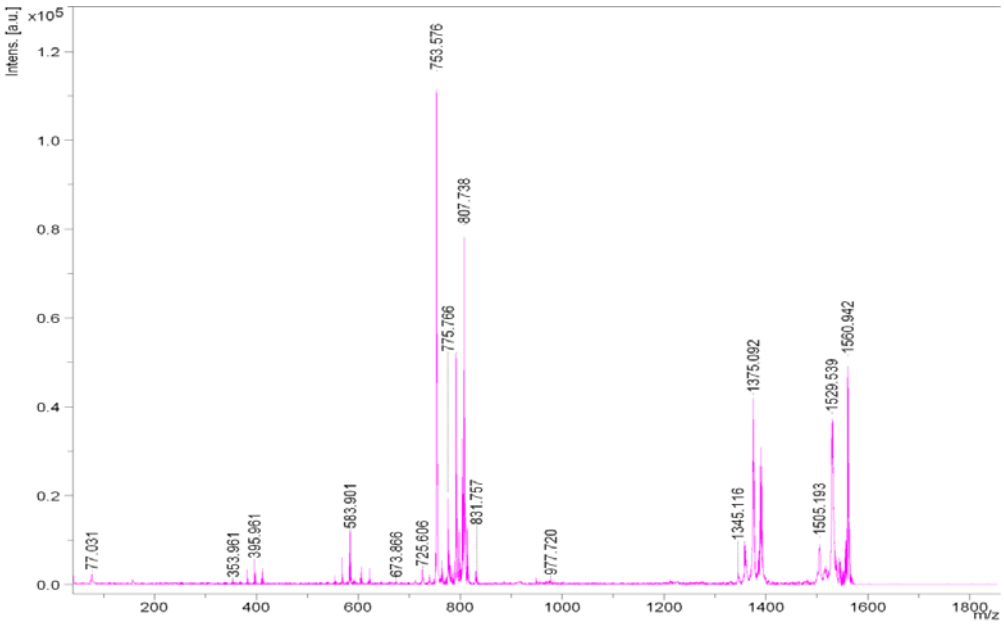
Fig.4. (B)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600
800
1000
1200
1400
1600
1800
m/z
77.031
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395.961
583.901
673.866
725.606
753.576
775.766
807.738
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1375.092
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1560.942

Fig.4. (C)

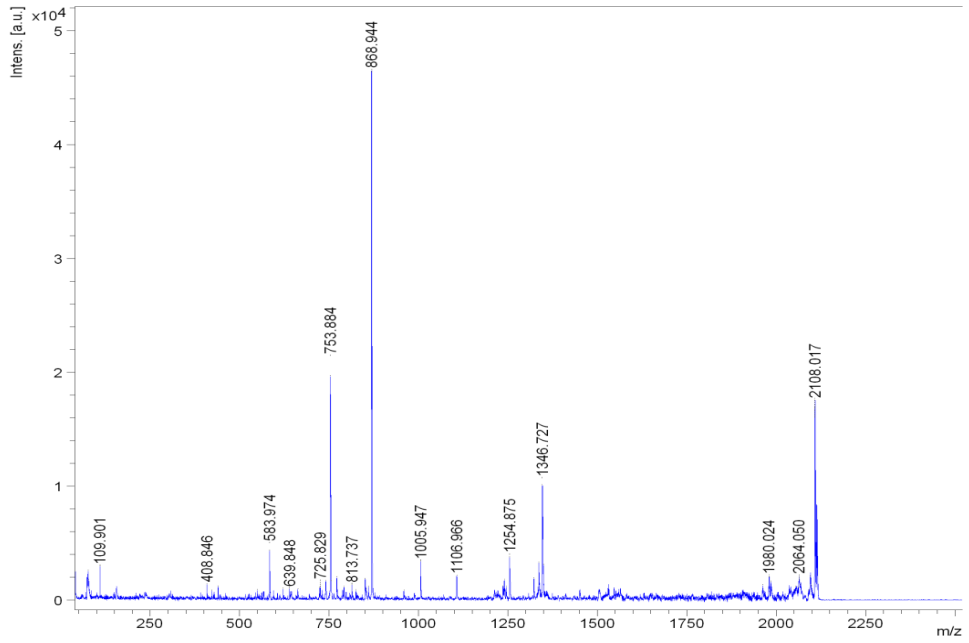

Fig.4. (D)

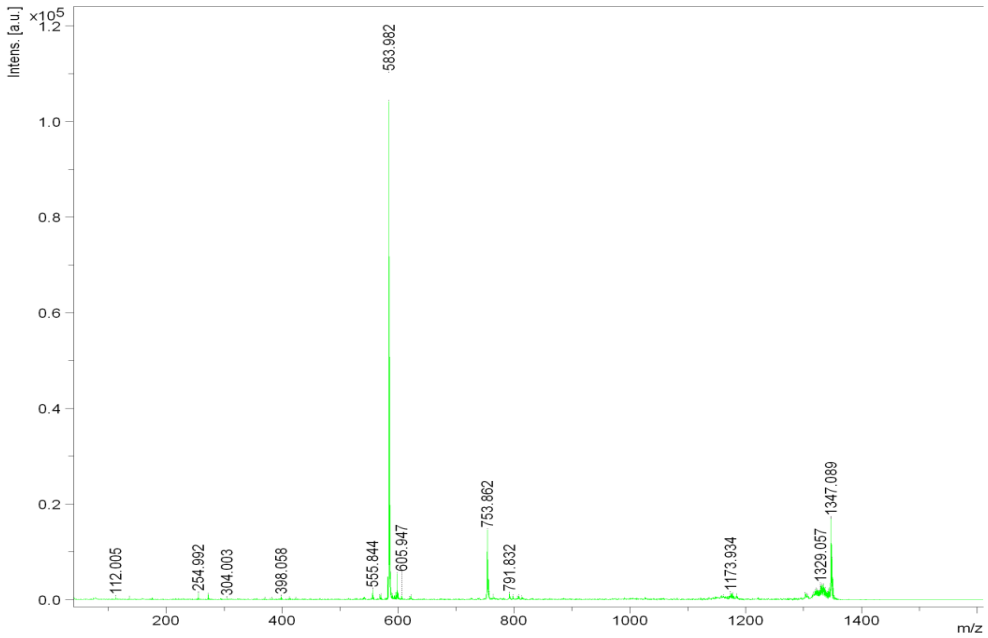

Fig.4. MALDI-TOF mass spectra of AS-HT-Celuz B (β-glucosidase)

Table 3: Summary of matching peptides of AS-HT-Celuz B (β-glucosidase) as predicted by FindPept program (http://au.expasy.org/tools/findpept.html).

User mass	DB mass	Δmass (daltons)	Peptide sequence consistent with mass	Peptide position	GH family	Known activity(CAZy)	Biological function
304.003	304.162	0.158	(K)/ER/(L)	415-416	3	Beta-glucosidase	carbohydrate metabolism
408.846	409.208	0.362	(R)/FDK/(Y)	638-640	3	Beta-glucosidase	carbohydrate metabolism
517.155	517.346	0.19	(K)/RITK/(F)	705-708	3	Beta-glucosidase	carbohydrate metabolism
621.917	622.331	0.413	(R)/GFDKR/(N)	617-621	3	Beta-glucosidase	carbohydrate metabolism
753.998	754.493	0.495	(R)/KLPLAGR/(L)	850-856	3	Beta-glucosidase	carbohydrate metabolism
754.794	754.493	-0.3	(R)/KLPLAGR/(L)	850-856	3	Beta-glucosidase	carbohydrate metabolism
787.289	787.431	0.141	(K)/NGENLIK/(A)	510-516	3	Beta-glucosidase	carbohydrate metabolism
829.187	829.428	0.24	(R)/VMAAFYK/(V)	318-324	3	Beta-glucosidase	carbohydrate metabolism
859.178	859.438	0.26	(R)/IMAAYYK/(V)	318-324	3	Beta-glucosidase	carbohydrate metabolism
868.944	868.464	-0.48	(R)/NHSELIR/(R)	365-371	3	Beta-glucosidase	carbohydrate metabolism
977.72	977.541	-0.178	(K)/VWTTTLTR/(R)	819-826	3	Beta-glucosidase	carbohydrate metabolism
990.593	990.537	-0.056	(K)/VWTTTLNR/(R)	825-832	3	Beta-glucosidase	carbohydrate metabolism
1014.412	1014.619	0.207	(R)/VGADSIVLLK/(N)	376-385	3	Beta-glucosidase	carbohydrate metabolism
1029.165	1029.63	0.458	(R)/IGAQSTVLLK/(N)	376-385	3	Beta-glucosidase	carbohydrate metabolism
1190.171	1190.595	0.424	(R)/VPPNFSSWTR/(D)	358-367	3	Beta-glucosidase	carbohydrate metabolism
1254.875	1254.716	-0.158	(K)/NVGALPLTGKER/(K)	412-423	3	Beta-glucosidase	carbohydrate metabolism
1345.116	1344.738	-0.377	(K)/YPKKVHVGSSSR/(K)	850-861	3	Beta-glucosidase	carbohydrate metabolism
1560.938	1560.868	-0.074	(K)/MVFVGSSSRKPPLR/(A)	840-853	3	Beta-glucosidase	carbohydrate metabolism
1703.028	1702.996	-0.031	(K)/TIFVGTSSRKLPLAGR/(L)	841-856	3	Beta-glucosidase	carbohydrate metabolism
1763.229	1762.866	-0.363	(R)/DRLWTPPNFSSWTR/(D)	348-361	3	Beta-glucosidase	carbohydrate metabolism
1980.024	1980.124	0.099	(R)/NHSEVIRKLGADSTVLLK/(N)	368-385	3	Beta-glucosidase	carbohydrate metabolism

VMAAFYKI-MAAYYK-DRLWTPPNFSSWTR-VPPNFSSWTR-NHSELIR-
VGADSIVLLK-IGAQSTVLLK-NVGALPLTGKER-ER-NGENLIK-GFDKR-
FDK-RITK-VWTTTLTR-VWTTTLNR-MVFVGSSSRKPPLR-
TIFVGTSSRKLPLAGR-KLPLAGR-YPKKVHVGSSSR

Fig.4a. AS-HT-Celuz B (β-glucosidase) protein sequence as deduced from peptide masses

```
AS-HT-CeluzB              -VMAAFYKI-MAAYYK-DRLWTPPNFSSWTR-VPP------NFSSWT--------R-NHS
BG[A.nidulans]            -VMAAFYKV------GRDRLATPPNFSSWTRAEKGYEHASIDGGAYGTVNEFVDVQQDHA
BG[A.calidoustus]         RIMAAYYKV------GRDRLHTPPNFSSWTRDEYGFAHFAVSEGEYGRVNEFVDVQRDHA
BG[A.rambellii]           RIMAAYYKV------GRDRLQHPPNFSSWTRDEFGYAYIAVSEGPYERVNEFVEVQRDHA
BG[A.ochraceoroseus]      RIMAAYYKV------GRDRLQHPPNFSSWTRDEFGYAYIAVSEGPYERVNEFVEVQRDHA
BG[A.clavatus]            RIMTAYYKV------GRDRLRVPPNFSSWTRDEYGYEHAAVSEGAWKKVNDFVNVQRDHA
BG[A.fumigatus]           RIMTAYYKV------GRDRLRIPPNFSSWTRDEYGWEHSAVSEGAWTKVNDFVNVQRSHS
BG[A.flavus]              RIMAAYYKV------GRDTKYTPPNFSSWTRDEYGFAHNHVSEGAYERVNEFVDVQRDHA
BG[A.oryzae]              RIMAAYYKV------GRDTKYTPPNFSSWTRDEYGFAHNHVSEGAYERVNEFVDVQRDHA
BG[A.terreus]             RIMAAYYKV------GRDRLWTPPNFSSWTRDEYGFAHFFPSEGAYERVNEFVNVQRDHA
BGII[A.niger]             RIMAAYYKV------GRDRLWTPPNFSSWTRDEYGFKYYYVSEGPYEKVNQFVNVQRNHS
BG[A.kawachii]            RIMAAYYKV------GRDRLWTPPNFSSWTRDEYGYKYYYVSEGPYEKVNHYVNVQRNHS
BG[A.luchuensis]          RIMAAYYKV------GRDRLWTPPNFSSWTRDEYGYKYYYVSEGPYEKVNHYVNVQRNHS
BG[A.saccharolyticus]     RIMAAYYKV------GRDRLYQPPNFSSWTRDEYGFKYYYSQEGPYEKVNQYVNVQRNHS
BG[A.aculeatus]           RIMAAYYKV------GRDRLYQPPNFSSWTRDEYGFKYFYPQEGPYEKVNHFVNVQRNHS
                           :*:*:**:*       *    *********        . . :         : .*:

AS-HT-CeluzB              ELIR-VGADSIVLLK-IGAQSTVLLK-NVGALPLTGKER-ER-NGENLIK-GFDKR-FDK
BG[A.nidulans]            SLIRRVGADSIVLLK----------NEGSLPLTGKER--------------------
BG[A.calidoustus]         SIIRRVASNSVVLLK----------NAGSLPLSGKER--------------------
BG[A.rambellii]           SVAHRVAADSVVLLK----------NHGSLPLTGKER--------------------
BG[A.ochraceoroseus]      SVAHRVAADSVVLLK----------NHGSLPLTGKER--------------------
BG[A.clavatus]            QLIREVGSASTVLLK----------NVGALPLTGKERK-------------------
BG[A.fumigatus]           QIIREIGAASTVLLK----------NTGALPLTGKE--------------------
BG[A.flavus]              DLIRRIGAQSTVLLK----------NKGALPLSRKEK-------------------
BG[A.oryzae]              DLIRRIGAQSTVLLK----------NKGALPLSRKEK-------------------
BG[A.terreus]             QVIRRIGADSVVLLK----------NDGALPLTGQEK-------------------
BGII[A.niger]             ELIRRIGADSTVLLK----------NDGALPLTGKER--------------------
BG[A.kawachii]            ELIRRIGADSTVLLK----------NDGALPLTGKER--------------------
BG[A.luchuensis]          ELIRRIGADSTVLLK----------NDGALPLTGKER--------------------
BG[A.saccharolyticus]     EVIRKVGADSTVLLK----------NNNALPLTGKERK-------------------
BG[A.aculeatus]           EVIRKLGADSTVLLK----------NNNALPLTGKERK-------------------
                          .: : :.: * *****          **  :***: :*    *       *     *
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Fig.5. Multiple Sequence Alignment (Clustal X) of AS-HT-Celuz B (β-glucosidase) from *Aspergillus ochraceus* with some of its neighbouring sequences, *A. niger* (56%), *A. kawachii* (54%), *A. luchuensis* (54%) , *A. terreus* (53%), *A. saccharolyticus* (51%), *A. aculeatus* (51%) , *A. nidulans* (49%), *A. calidoustus* (47%), *A. clavatus* (47%), *A. flavus* (51%) , *A. oryzae* (51%) , *A. fumigatus* (49%) , *A. rambellii* (48%) and *A. ochraceoroseus* (48%), conserved sequences are shown in astriques (*) .

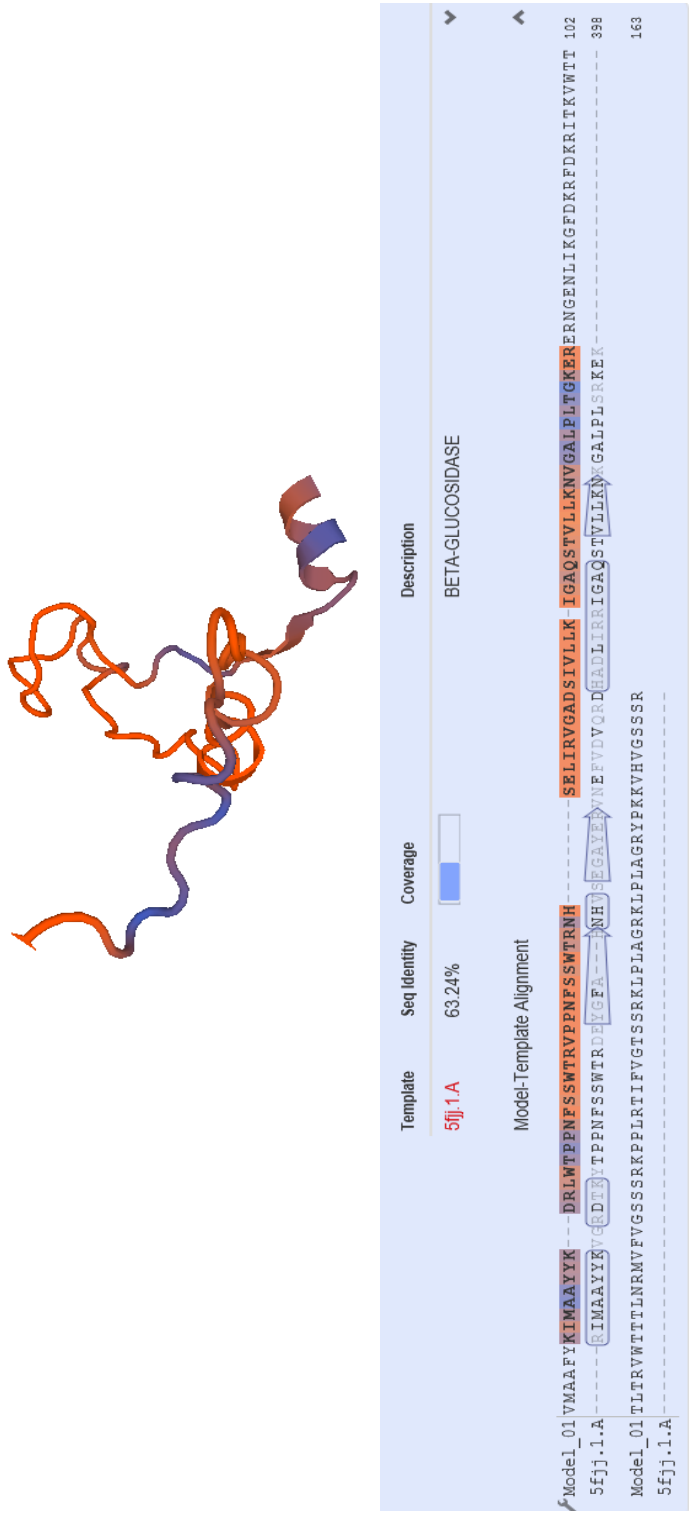


Fig.6. The 3D-structural model of AS-HT-Celuz B (β-glucosidase) as predicted through SWISS-MODELwork space (http://swissmodel.expasy.org) and Model-Template alignment with *Aspergillus* sp. Family GH3 beta-D-glucosidases(63.24%)

4.3.2 Saccharification yield of partially purified cellulases on pretreated coir pith and analysis of end-products

Sequential hydrolysis with AS-HT-Celuz A (25 FPU/g of cellulose) and AS-HT-Celuz B (10 U/g of cellulose) on pretreated coir pith biomass for 24 h, yielded 8.01g glucose/L with an efficiency of 72.11%, whereas simultaneous hydrolysis released a glucose yield of 6.165 g/L, with only a partial sacchrification efficiency of 55.48%. The obtained glucose yield was comparable to that of the previous reports by Narra and Dixit et al. (2012), they got a sugar yield of 676 mg/g substrate with an enzyme load of 9 FPU/g on alkali treated rice straw. The glucose yield obtained in this study was very higher than the glucose yield (2.23 g/L) obtained by Zhang and Cai (2008) for alkali pretreated rice straw with *Trichoderma reesei* ZM4-F3 cellulase produced through submerged fermentation. Moreover, the percent saccharification rate of sequential hydrolysis experiment was a little higher than the earlier reported values of dilute acid pretreated cardoon with 70% saccharifcation yield (Fernandes et al., 2015), yet 10% lower than those obtained by Ballesteros et al. (2008) and Martinez et al. (1990). Meanwhile, no monomeric glucose residues are formed when treated with the single enzyme alone specifying the homogenous nature and behavioural properties of the purified enzymes. The hydrolysis experiments also recommended that sequential synergistic interaction existing between the purified enzymes resulted in the formation of glucose as the only one end-product. With these results it could be concluded that delignified coir pith is an excellent substrate for both enzyme production and subsequent hydrolysis into fermentable sugars. Thus the cellulases produced through hydrolysis of pretreated coir pith by *A.ochraceus* MTCC 1810 could be considered as a prospective source for industrial cellulose fermentation

The extent of hydrolysis were analysed by thin-layer chromatography (Fig. 7) and the profile revealed that glucose was the only one end product

released through the hydrolysis of cellulosic fraction of the pretreated coir pith. Sequential saccharification yielded higher amount of sugar moieties as obvious from the larger and darker spots (Fig.7, spot no.3) than the simultaneous addition of enzymes produced comparatively lighter spots (Fig. 7, spot no. 2). Soluble monosaccharide units could hardly be observed after incubation with single enzyme treatment (Fig.7, spot no 4&5). In addition, gas chromatographic mass spectrum analysis of the hydrolytic residue (sequential incubation with AS-HT-Celuz A and AS-HT-Celuz B), showed the presence of glucose as methyl silyl oxime derivative of glucose (retention time, 6.005 min) with defragmentation mass spectra at m/z of 368 (Fig.8). Finally the results demonstrated that sequential as well as simultaneous addition of partially purified enzymes, AS-HT-Celuz A and AS-HT-Celuz B, allowed the complete bioconversion of the polymeric carbohydrates into fermentable monomeric glucose units which in this manner could be fermented into ethanol.

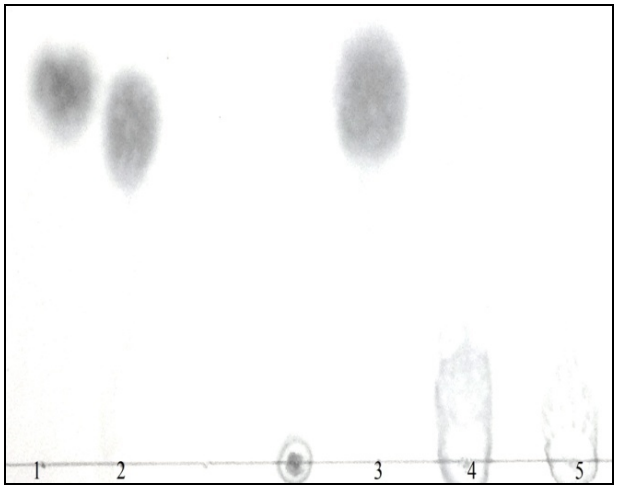


Fig.7. End-product formed after enzymatic hydrolysis of delignified coir pith with AS-HT-Celuz A and AS-HT-Celuz B, as demonstrated by thin layer chromatography. Lane1 , glucose standard ; lane2 , end-product formed after simultaneous hydrolysis with AS-HT-Celuz A and AS-HT-Celuz B; lane 3, end-product formed after sequential hydrolysis with AS-HT-Celuz A and AS-HT-Celuz B; lane 4, end-product formed after hydrolysis with AS-HT-Celuz A alone ; lane 5, end-product formed after hydrolysis with AS-HT-Celuz B alone. All the hydrolysis experiments were done at 40^{o}C at pH6.0 for 24 h.

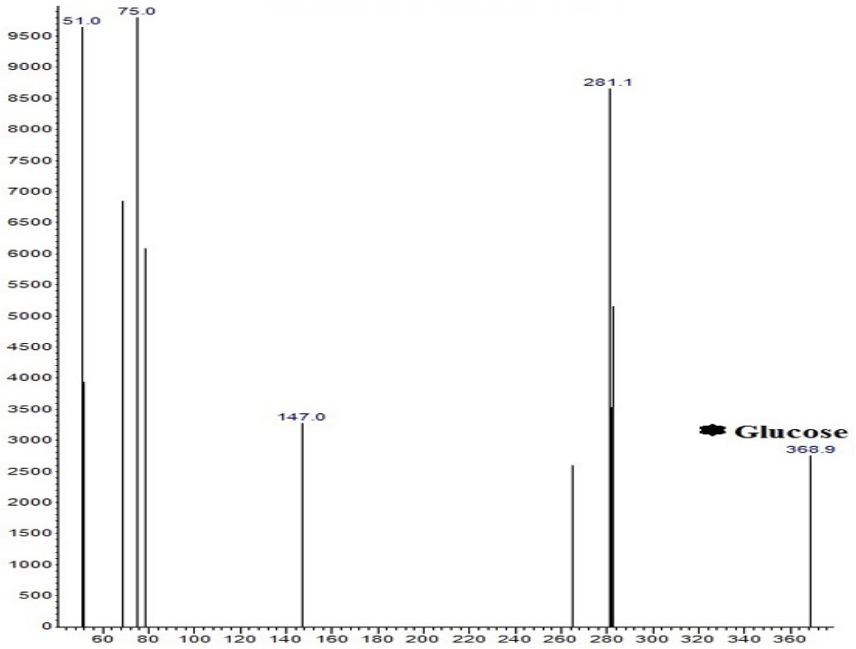


Fig. 8. Analysis of hydrolysis product through gas chromatography. The chromatogram represent the typical defragmentation mass spectra (m/z=368) of methylsilyl oxime derivative of glucose compared against known glucose standards.

4.3.3 Proximate composition of the coir pith and Structural modifications after enzymatic treatment

Characterization of chemically delignified and enzymatically hydrolysed coir pith were done with the help of Central Coir Research Institute, Kalavoor, Alleppey and the results are summarised in the Table 4. It is obvious from the results that raw coir pith contained cellulose, hemicelluloses, nitrogen, phosphorous and organic carbon in reasonable quantities and the amount of lignin (38.87%) was successfully removed to 18.27% after chemical pre-treatment for better production of cellulases. The presence of lignin in significant amounts would overlap the cellulosic fraction making it unavailable for the fungus to act upon. Therefore the optimized pre-treatment procedure with 2% alkaline peroxide at pH 11.5 for 10 hrs was

found suitable for further saccharification (Rojith and Singh, 2012). The chemical delignification (53%) was found to be practical for removing lignin so as to soften the material by reducing its crystallinity and made the substrate more accessible for the enzymatic attack. An important observation which could be drawn from the results in Table 4 was that organic carbon forms the predominant element in the coir pith followed by potassium, nitrogen and phosphorous. However, the elemental components remained more or less same which could be beneficial for initiating enzyme production. An increase in the cellulose as well as hemicellulose content of the coir pith was observed after chemical delignification owing to the dissolution of lignin fraction and thus exposing the cellulose-hemicellulose fraction of the coir pith for effective enzyme penetration. Although enzymatic hydrolysis instigated a reduction of cellulose content from 24.85% to 3.27% showing effectiveness of the hydrolysis. A theoretical hydrolysis efficiency of 86.84% was accomplished after enzymatic treatment with AS-HT-Celuz A and AS-HT-Celuz B.

Table 4: Chemical composition of enzyme hydrolysed coir pith in comparison to chemically delignified and raw coir pith

Sl.No.	Parameter Tested	Raw coir pith (% w/w)	After chemical delignification (% w/w)	After enzymatic saccharification (% w/w)
1	Nitrogen	0.125	0.109	0.008
2	Phosphorous	0.003	0.002	0.005
3	Potassium	0.56	0.36	0.18
4	Organic Carbon	28.7	23.7	22.6
5	Lignin	38.87	18.27	16.79
6	Cellulose	21.85	24.85	3.27
7	Hemicellulose	1.89	2.22	0.61
8	Pectin	0.25	0.21	0.18

4.3.3 .1 FTIR analysis

The Fourier transform infrared spectroscopy was used for assessing the functional group modifications imposed to the cellulose microstructure during hydrolysis with purified enzymes. All the peak assignments were summarized in the Table 5. As can be seen from the Fig. 9, the FTIR spectral profiles and the peak intensities of most of the bands are similar to the lignin (Buta and Galetti, 1989), cellulose and hemicelluloses (Cao and Tan, 2004).The observation suggested that there is no significant changes in the macromolecular structure of cellulose or hemicelluloses whereas the spectral intensity of the enzyme treated coir pith (ETC) showed lower peak values than alkaline peroxide treated coir pith. From the Fig. 9, three major peaks spanning from 800-900 , 1000-1100 , 1300-1400 cm^{-1} were lower than the spectra of chemically treated coir pith elucidating the degraded nature of cellulose and hemicelluloses subunits. The chemically treated coir pith (CTC) showed higher absorbance values than the enzymatically treated coir pith (ETC) proved that the cellulosic and hemicellulosic components had utilized for glucose production. A higher peak at 1620 cm^{-1} represents C=C stretching associated with aromatic skeletal vibrations such as C=O, C-O, OH groups and water associated with lignin. This is due to the fact that during enzymatic saccharification, the water holding capacity of the delignified coirpith increases and it becomes hydrated and smoothened for the proper attachment of cellulases on the surface of coir pith. The spectral band at 2930 cm^{-1}, assigned to C-H stretching vibration in the aromatic methoxy groups was an indication of the polysaccharide content of the coir pith. The characteristic band at 1450 cm^{-1} is typical for the methoxy group ($O\text{-}CH_3$) of lignin structure, C-O stretch and C-H, OH or CH_2 bendings in

hemicelluloses units. The 1730 cm^{-1} band is described to characterise the carbonyl stretching group (C=O) in the hemicelluloses of the coir pith showed intensity decrease after enzymatic treatment (Rojith and Singh, 2012). The broad absorption band at 3400-4000 cm^{-1} was representative of microcrystalline cellulose due to the stretching of H bonded OH groups and it exposed more after chemical pre-treatment (Jabasingh et al., 2014).The characteristic peak located at 1510 and 1550 cm^{-1} necessarily showed the defragmentation of lignin due to the presence of C-C linkages, conjugated hydrogen bonded carboxyl groups and other aromatic skeletal vibrations of the benzene ring in the lignin structures. There was no disappearance of peaks rather a considerable decrease in its peak intensity was observed. This could find no correlations with the studies of Jabasingh et al., (2014), who observed disappearance of peaks and peak shifts after enzymatic hydrolysis of coir pith; but similar peak profiles were obtained. Rojith and Singh (2012) also reported several characteristic peak modifications after the oxidative delignification of coir pith. Conversely, any peak intensification or peak shifts after the enzyme treatment was observed in the present study. Thus FTIR spectroscopy provides a suitable model to assess the chemical and enzymatic degradation of lignocellulosic biomass. In addition, the lower peak intensities of enzymatically treated coir pith (ETC) compared to the alkaline peroxide treated coir pith (CTC) revealed the hydrolytic potential of the cellulases purified from the *A.ochraceus* MTCC 1810 and their efficiency in bioconversion of crystalline cellulose.

Table 5: FTIR peak assignments of enzyme treated coir pith in comparison to chemically treated coir pith

Peaks in cm^1	Functional Groups	Assignment
3300-3400	Hydroxyl (O-H) groups present in the cellulose, water and lignin structures	Cellulose , lignin
2930	C-H stretching in aromatic methoxyl groups, aliphatic C-H stretching group; C-H stretching vibration present in the cellulose and hemicelluloses components	Cellulose , hemicelluloses
2300	Carbondioxide in the atmosphere contributed the peak; taken as a background absorption peak	Co_2
1730	Carbonyl stretching group (C=O) in the hemicellulose	Hemicellulose
1620	C=C stretching associated with aromatic skeletal vibrations, C=O, C-O, OH groups, water associated with lignin.	Water
1550	Conjugated hydrogen bonded carboxyl group	Lignin
1510	C=C stretching, aromatic skeletal vibrations of the benzene ring in the lignin	Lignin
1450	Methoxy group (0-CH_3) of lignin structure, C-O stretch and C-H , OH or CH_2 bendings in hemicelluloses	Lignin , hemicelluloses
1300-1400	Bending vibrations of C-H and C-O groups of the aromatic rings in polysaccharides	Cellulose
1250	O-H deformation arising from phenolic group, C-OH stretching of guaiacyl units	Lignin
1060	C-O stretching in the secondary alcohol, C-C, stretching and C-OH bending in polysaccharides in cellulose	Cellulose
890-900	Presence of β-glycosidic linkages between the monosaccharides, C-H deformation vibration of cellulose	Cellulose

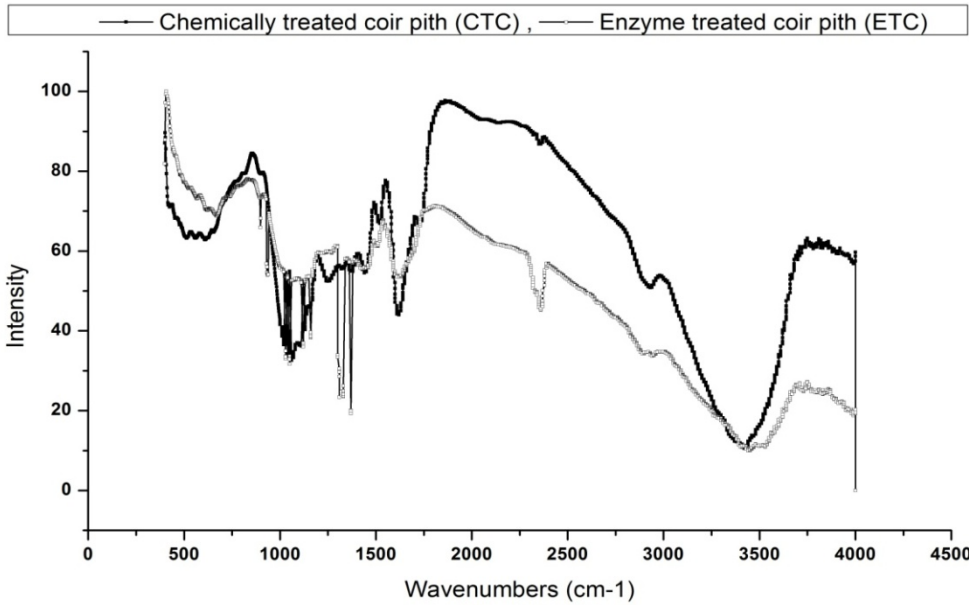


Fig. 9. FTIR spectra of enzymatically treated coir pith (ETC) in comparison to the chemically treated coir pith (CTC).

4.3.3.2 XRD

Quantitative estimation of cellulose crystallinity was calculated from the XRD peak profiles as shown in Fig.10. It was obvious from the results that the X-Ray diffraction spectral pattern of chemically treated coir pith (CTC) and enzyme hydrolysed coir pith (ETC) showed similar peak profiles with a higher primary peak at the diffraction angle $2\theta = 22^0$ followed by two broad secondary peaks at $2\theta = 12^0$ and 18^0.An overall crystallinity reduction of 13% could be observed from the comparison of CTC (CI = 27.5) and ETC (CI= 14.37). The results showed in Table 6, led to the conclusion that the enzymatic degradation predominantly contributed to the change of its crystalline state into amorphous form. This is in contrast with many of the earlier studies by Fackler et al. (2011) and Salaita et al. (2008), who showed an increase in crystallinity of the substrate, due to the faster degradation of amorphous cellulose followed by re-crystallisation resulting in the increase of crystallinity. The noticeable decrease in cellulose crystallinity in this study,

contributed due to the enhanced enzymatic penetration on the substrate and thereby it reformed as a gifted source for biofuel production.

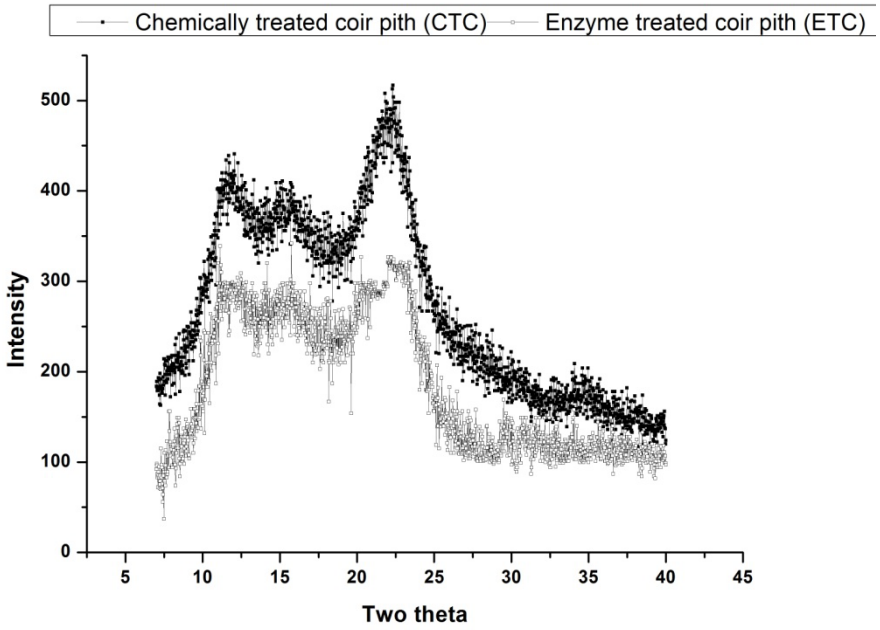


Fig.10. XRD Chromatogram of enzymatically treated coir pith (ETC) in comparison to chemically treated coir pith (CTC) for determination of the crystalline cellulose contents

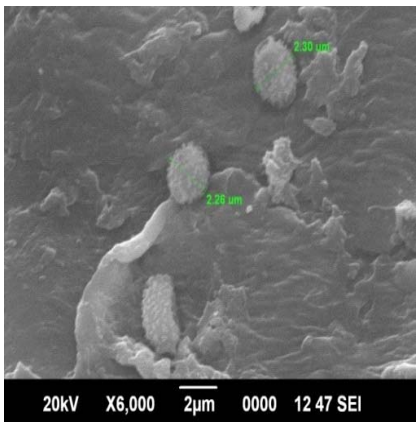
Table 6: Crystallinity index of biologically treated and chemically treated coir pith

Sample	I_{002} (22.02)	I_{am} (18.02)	CrI
Pretreated coir Pith	480	348	27.5
Enzyme treated coirpith	327	280	14.37

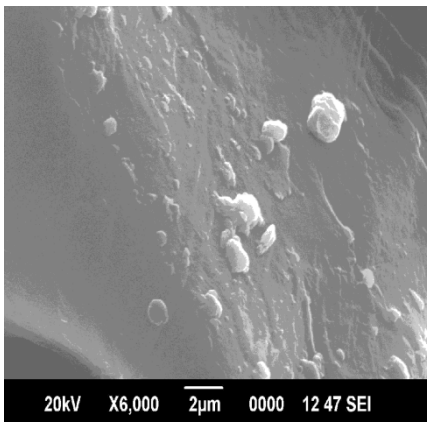
4.3.3.3 SEM analysis

Scanning electron microscopy (SEM) images of raw coir pith, chemically treated coir pith and enzyme treated coirpith at 1500X magnification level were illustrated in Figure 11(a) , 11(b) and 11(c) respectively. Morphology of the fibre was varied with the different treatment process which might be due to the defibrillation of the complex cellulose polymers, re-localisation of lignin derivatives and increased porosity on the enzyme treated coir pith. Simply the fact is demonstrated that enzymatic

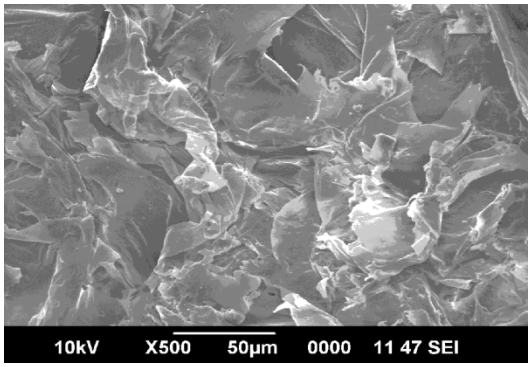
degradation converted the compressed and regularly arranged cellulosic polymers in the coir pith into a more relaxed, pleated and loosened state of cellulose .The formation of a rough surface after enzymatic treatment was in accordance with the previous study of Jabasingh et al., (2014). SEM results provided a methodical verification on the enzymatic infiltration of the purified enzymes and also provide a confirmation on the total degradation of cellulose components present in the coir pith.



(Fig. 11a)



(Fig. 11b)



(Fig. 11c)

Fig. 11a . SEM micrographs of raw coir pith; **Fig. 11b.** Chemically treated coir pith (CTC) ; **Fig.11c.** CTC after enzymatic hydrolysis (ETC).

4.4 Conclusion

This chapter concluded that *Aspergillus ochraceus* MTCC 1810 had the potential to produce complete cellulase system comprising a processive endoglucanase (AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B), capable for hydrolysing the cellulosic components of the coir pith into fermentable sugars. Peptide mass finger printing and FindPept analysis of AS-HT-Celuz A showed the presence of fungal specific carbohydrate binding domains, processivity domains, endo-exo bifunctional domains and multiple–substrate specific glycoside hydrolase domains, which indicated that AS-HT-Celuz A could be classified as novel processive cellulase and the peptide mass spectrum of AS-HT-Celuz B showed strong evidence for β-glucosidase. The 3D Swiss-model of both proteins showed more than 50% similarity to known cellulase templates and suggested that the model could be used for downstream proteomic analysis. In addition, the structural modifications investigated through XRD patterns, FTIR spectra and SEM images, revealed that the enzymes purified were suitable for the saccharification of pre-treated coir pith with 72.11% efficiency offering an innovative solution towards the management of 'coir pith'.

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Chapter 5

CONSTRUCTION OF METAGENOMIC LAMBDA LIBRARY FROM VALANTHACAUD MANGROVE SEDIMENT

Contents

5.1 Introduction

Metagenomics is a culture-independent approach which involves genomic analysis of DNA extracted from its natural environment, and is accordingly a more comprehensive strategy to exploit microbial genetic reservoirs compared to traditional, culture-dependent approaches (Handelsman et al., 1998). Metagenome can be accessed from any type of environment such as highly diverse environments (e.g. soil, sediment and seawater), naturally enriched environments suitable for target gene isolation or extreme environments. There is no universal method available for the community nucleic acid extraction, because of the physicochemical heterogeneity present in environmental samples. The parameters such as yield, purity, fragment size and representativeness of the sample community are the key factors involved in the selection of a suitable DNA extraction method in metagenomic analysis. The DNA extraction protocol should be optimized for each sample site depending on the purpose of metagenomic analysis.

The community nucleic acid extraction protocol often distinguishes between direct extraction methods where the cells are lysed within the environmental sample matrix, and indirect extraction methods where the cells are isolated before lysis. The direct lysis method may provide 10 to 100-fold more DNA, but the DNA purity will be less (Gabor et al., 2007). Lysis is mainly done using chemical or enzymatic methods, physical/mechanical treatments (thermal shocks, bead-beading, ultra-sonication, microwave heating), or a combination of these procedures. The major disadvantage of most mechanical treatments is the subsequent DNA shearing (Burgmann et al., 2001; van Elsas et al., 1997). Several direct extraction methods are available which provide DNA of significant purity for small-insert metagenomic library construction or PCR-based screening or pyrosequencing applications (Ogram et al., 1987; Tsai and Olson, 1991; Zhou et al., 1996), and commercial kits are also available. It is fundamental to utilize more gentle DNA extraction methods that avoid shearing for obtaining high molecular weight DNA. Immobilization of the cells into agarose plugs prior to lysis is a frequently used procedure to preserve intact high molecular weight DNA (Liles et al., 2008). After cell lysis, deproteinisation and subsequent DNA precipitation is carried out most frequently using organic solvents such as isopropanol or ethanol, respectively. Another major problem with environmental samples are co-extraction of humic acids, fulvic acids and polyphenolic compounds which inhibit subsequent enzymatic modification reactions such as restriction digestion, ligation, PCR and bacterial transformation. Purification strategies include pre-prossessing of the sample (e.g. through addition of hexadecyl trimethylammonium bromide, CTAB), agarose gel purification and various chromatographical separations, or a combination of these have been described by Liles et al. (2008) and Rajendhran and Gunasekaran (2008).

The metagenomic libraries constructed from DNA directly extracted from environmental samples provide access to the entire gene content of a habitat (Steele et al., 2009). This method have been demonstrated to be a powerful tool for the discovery of novel biomolecules (Handelsman, 2004 ; Steele et al., 2009).The construction of metagenomic libraries involves similar steps as that of the genomic DNA cloning derived from individual microorganisms. The necessary steps include fragmentation of environmental DNA by restriction digestion or shearing, insertion into an appropriate expression vector, and transformation into a suitable host system (Daniel, 2005). The development of metagenomic library is practically simple, though the vast sizes of most metagenomes such as those derived from soil and sediment samples and, likewise, the large number of clones required for a significant coverage of the metagenome are great technological constraints (Riesenfeld, 2004a ; Daniel, 2004).Two types of libraries concerning average insert size of metagenome can be generated: small-insert libraries in plasmid vectors (less than 10 kb) and large-insert libraries in cosmid and fosmid vectors (up to 40 kb) or BAC vectors (more than 40 kb). The choice of a vector system for library construction depends on the purity of the isolated environmental DNA, the desired average insert size of the library, the copy number, the host, and the screening strategy that will be used (Daniel, 2004 and 2005). Small-insert metagenomic libraries, particularly those made in the lambda phage vectors, are valuable for the identification and expression of single gene or small operons encoding novel biomolecules. The small insert expression libraries will be under the control of strong gene expression promoter signals due to the appropriate gene orientation of smaller fragments, and therefore have a good chance of being expressed and detected by activity based screens. However, large-insert libraries are the appropriate system for

identifying complex metabolic pathways encoded by large gene clusters (Bertrand et al., 2005). It reduces the number of clones required to represent a given habitat or sample community, but provide a better chance to discover full length open reading frames (Handelsman, 2004).

Primary activity-based identification of a gene or gene cluster in a functional- expression library is followed by identification of the particular gene in the large-insert library through genome walking and further sub-cloning and over expression of the gene for maximizing the production of recombinant proteins, which comprise a powerful means of discovering novel biomolecules through functional metagenomics approach. *Escherichia coli* is the most favored host strain for library confirmation but recently *Streptomyces* species and *Bacillus* species have also been used as suitable host systems (Nakashima et al., 2002). Besides, several trans factors need to be provided by the host cell such as transcription factors, cofactors, inducers, precursors, chaperones and post-translationally acting factors, rare codon usage etc. Sometimes, the potential toxicity of the heterologous product to the host cell can be overcome by using vectors that can be transferred to and maintained in various host-systems. This Chapter describes construction of a lambda phage library from the sediment sample collected from Valanthacaud mangroves distributed along the banks of Cochin backwaters in Vembanad Lake.

5.2 Materials and Methods

5.2.1 Sample collection from Valanthacaud mangroves

Sample was collected from Valanthacaud mangroves (9o 55’ 10.24’’N, 76^{o} 20’ 01.23’’E)**.** The area is hardly 5-6 km from the heart of Cochin City. This mangrove exists as fringing vegetation distributed along the sides of the Cochin backwaters in Vembanad Lake. Among the existing mangroves

ecosystems of Cochin backwaters, Valanthacaud Island is one of the untouched patches of mangrove vegetation with rich biodiversity. The uncultivable biota present in this mangrove sediment has yet not been explored. In the present study an attempt has been made to construct a metagenomic lambda library from the sediment sample collected from the region. About 100 g of wetland sediment containing organic matter from decaying vegetation was collected from the sediment-water interface. The sample was immediately placed on ice and transported to the laboratory and was preserved at -80^{o}C.

Fig.1. Valanthacaud mangrove station 1

Fig.2. Valanthacaud mangrove station 2

5.2.2 Metagenomic DNA extraction-Direct lysis method

Direct lysis method of DNA extraction was followed in this study because of the better DNA recovery than the indirect lysis method. The sediment sample was sieved through a sterile mesh of 250µm in size to remove plant roots and other particulate matter and subjected for four different DNA extraction methods such as harsh lysis using liquid N_2 grinding, soft lysis using

proteinase K , agarose plug DNA extraction , polyvinylpolypyrrolidone and polyethylene glycol method (PVPP-PEG)

5.2.2.1 Extraction using Proteinase K (Soft lysis)

Metagenomic DNA extraction was carried out as described by Zhou et al. (1996) with slight modifications. A quantity of 5 g each of soil samples were mixed with 13.5 mL of DNA extraction buffer containing 100 mM Tris-HCl (pH 8), 100 mM EDTA (pH 8), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% (w/v) CTAB and 100 µL of proteinase K (10 mg/mL). The mixture was horizontally shaken at 225 rpm for 30 minutes at 37°C. Then 1.5 mL of 20% SDS was added to the sample and incubated at 65°C in a water bath for 2 hours with gentle end-over-end inversions for every 15 to 20 minutes (Zhang et al., 2003). The supernatant sample was collected by centrifugation at 10,000 g for 10 minutes at room temperature. It was then transferred to 50 mL sterile Oak ridge tubes. The remaining soil extract was extracted twice more with 4.5 mL of the extraction buffer having the same composition as described above and 0.5 mL of 20% SDS was added, vortexed briefly for 10 seconds, further incubated at 65°C for 60 minutes and centrifuged as before. All the supernatants were pooled together and an equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added, centrifuged at 10,000 *g* for 10 minutes and the aqueous phase transferred to a new Oak ridge tube using a wide bore pipette tip. The brown coloured DNA was precipitated by adding 0.6 volume of 100% isopropanol and incubated at room temperature for 1 hour. The precipitate was pelleted by centrifugation at 16,000*g* for 20 minutes at room temperature and the DNA was washed with cold 70% ethanol, air-dried and resuspended in1 ml of 10mM Tris–HCl pH 8.5.

5.2.2.2 Liquid N_2 grinding (Harsh lysis)

The protocol adopted was similar to proteinase K lysis method except that collected sample (5 g) was first ground in the presence of liquid N_2 (Zhou et al., 1996) using a hand mortar and pestle. This step was repeated for 3-4 times, which provide a freeze-thaw condition for lysis of cells in soil matrix. The grinded suspension was again macerated by adding 9 ml of DNA extraction buffer (100mM Tris-HCl pH 8.0, 100mM EDTA, 100mM sodium phosphate, 1.5M NaCl, 1% CTAB and 2% SDS). The suspension was collected in sterile falcon tubes and incubated at 65^0C for 2 h with gentle horizontal shaking for every 15–20 min. The supernatant liquid was collected by centrifugation at 6000*g* for 10 min. The remaining pellet was re-extracted as above and the combined supernatants were extracted with equal volume of chloroform–isoamylalcohol (24:1, v/v and centrifuged at 10,000 *g* for 10 minutes. The aqueous phase was transferred to a new Oak ridge tube using a wide bore pipette tip. The brown coloured DNA was precipitated by adding 0.6 volume of 100% isopropanol and incubated at room temperature for 1 hour. The precipitate was pelleted by centrifugation at 16,000 *g* for 20 minutes at room temperature and the DNA was washed with cold 70% ethanol, air-dried and resuspended in1 ml of 10mM Tris-HCl pH 8.5.

5.2.2.3 Agarose plug embedded DNA extraction

The method described by Bakken and Lindahl (1995) for indirect DNA extraction coupled with agarose plug lysis was modified here for direct extraction of high molecular weight DNA. Briefly, 1g of sediment sample was mixed with 1mL of suspension buffer (10 mM Tris-HCl, 1M NaCl, 50 m M EDTA) and 1mL of 1% low melting point agarose. Slurry agarose mixture was kept at 40^oC and pipetted into molds. After setting at 4^oC, these plugs were

transferred into an Eppendorf tube containing 1.5 mL lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 0.2 % (w/v) sodium deoxycholate, 0.5 % (w/v) cetyl ether, 0.5 % (w/v) lauroyl sarcosine sodium salt, 5 mg/ml lysozyme) and incubated at 37°C for 24 h with gentle horizontal agitation. Lysis buffer was then replaced with 1.5 ml digestion buffer (100 mM Tris-HCl , 50 mM EDTA , 100 mM Sodium phosphate , 1.5 M Nacl , 1.5 mg/ml Proteinase K , 0.5 % (w/v) SDS) and the tubes were incubated at 50°C for 24 hrs with a gentle horizontal agitation. The plugs were then collected and washed with 50µl wash buffer (10 mM Tris-HCl, 100 mM EDTA) and then several times with ice cold 0.5X TBE buffer and then stored in 1X TBE buffer for further analysis.

5.2.2.4 Polyvinylpolypyrrolidone and Polyethylene glycol method (PVPP-PEG method)

This method was performed as described by Clegg et al. (1997) with some modifications. A quantity of 5 g sediment sample was weighed and 1g of acid-washed Polyvinylpolypyrrolidone (PVPP) was added and mixed well. An aliquot of 15 ml lysis solution (0.5 mol/l NaCl, 0.1 mol/l Na_2EDTA; pH 7.0 and 30 mg of lysozyme) was added, homogenized thoroughly and incubated at 37^0C for 1-2 hrs. After incubation, 1 ml 20% SDS was added to the soil solution and again incubated at 50°C for 30 min before freezing at -80°C. Homogenized samples were then thawed immediately by incubating at 50°C for 10-15 min. The freeze-thaw cycle was repeated for three times, suspensions were centrifuged at 12,000 *g* (15min, 4°C). Supernatant liquid was pooled out to another sterile tube. The pellet separated after centrifugation was re-suspended in 10 ml of lysis solution and mixed well, kept for incubation at 37^0C for 1 hour and centrifuged at 12,000*g* for 15 min. The supernatant fluids were then pooled together and extracted with equal volume of chloroform-isoamyl alcohol (24:1). Mixed thoroughly by vortexing and centrifuged at

12,000 *g* for 10 min. aqueous phase was removed and precipitated with 10 % Polyethylene glycol and the crude DNA was washed twice with cold 70% (v/v) ethanol and air-dried. The crude DNA pellets were resuspended in 1 ml of 10mM Tris-HCl , pH 8.5.

5.2.3 Determination of yield and purity of the metagenomic DNA

Co-extracted humic compounds are the major contaminant when DNA is extracted from soil. These compounds absorb at 230 nm whereas DNA absorbs at 260 nm and protein at 280 nm. To evaluate the purity of the extracted DNA, absorbance ratios at 260 nm/230 nm (DNA / humic acids) and 260 nm/280 nm (DNA / protein) were determined.

DNA concentration was measured with a Qubit fluorometer using a Quant-iT dsDNA BR Assay kit (Invitrogen) using manufacture's protocol. Fluorescence based methods are very sensitive and are generally used to quantitate extremely low amounts of DNA. An enhancement of fluorescence is produced upon binding with the dsDNA and the amount of fluorescent signal is directly proportional to the concentration of DNA. The dsDNA BR assay is very sensitive and accurately measure for 100pg/µL-1000ng/µL of initial sample concentrations. Before quantification of DNA, the Qubit 2.0 fluorometer was calibrated using the standards supplied with the kit. After calibration, working solution was prepared by diluting 1µl of dsDNA BR reagent in 200µl of dsDNA BR buffer. Standard reactions required 190 µL: 10µl of working solution and standard, whereas sample assay was made by mixing 1µL of sample to 199µL of working solution. The final volume was made as 200 µL in thin-walled, 0.5ml qubit assay tubes, followed by vortexing and incubating for 2 mins at room temperature. The intensity of the sample after binding with the fluorescent dye was read by Quibt 2.0 Fluorometer

(Invitrogen). The Qubit fluorometer show values of DNA in μg ml^{-1}. The sample concentration was calculated using the following equation: Concentration of sample = QF value x (200/X); where QF value = the reading given by the Qubit 2.0 Fluorometer, X = sample volume (μl) added to the assay tube.

5.2.4 Metagenome DNA size determination by Pulsed Field Gel Electrophoresis (CHEF DR II Bio-Rad)

The CHEF-DR II system (Bio-Rad), most advanced PFGE used by laboratories today, separates large and small DNA fragments of DNA (100 bp to over 5 mb) with better resolution, speed and accuracy compared to initial pulsed field methods. The system provides a homogenous electric field using a hexagonal array of 24 electrodes clamped to a particular voltage, which avoids lane distortion, sense changes in buffer conductivity due to buffer breakdown, change in buffer type, gel thickness or temperature. The electrodes allow the electric field to switch through a fixed orientation angle of 120° between pulses. The voltage is proportional to the migration rate and the size range of fragments to be separated (Fig. 3).

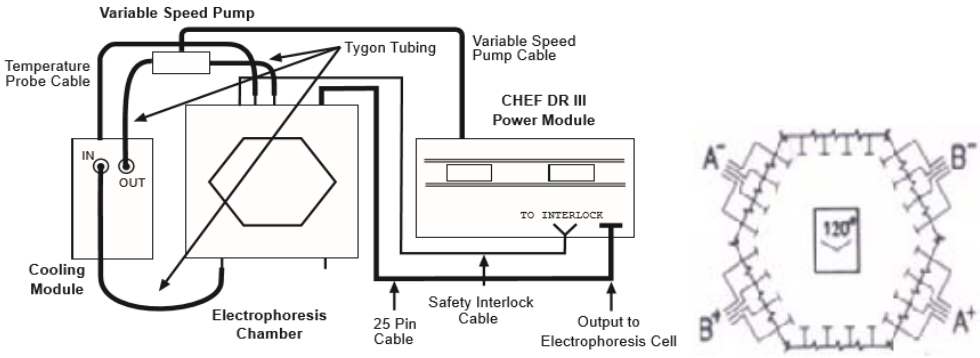


Fig.3. Interconnections between different modules of the CHEF-DR II system with a representation of hexagonal array of 24 electrodes to a particular voltage provides a homogenous electric field

The procedure adopted for size-determination of DNA was as follows: The metagenomic DNA samples extracted using different lysis methods were mixed with equal volume of molten 2% (w/v) Low Melting Point (LMP) agarose. The mixture was stirred gently but thoroughly and carefully transferred in to the plug molds, while keeping the DNA-agarose mixture at 50°C. The mixture was then allowed to solidify at 4°C for 10–15 minutes. After that , plugs were washed four times with TE buffer and cut into approximately 2.5 mm slices using a sterile scalpel and stored in 0.5X Tris-Acetate-EDTA (TAE) at 4°C until loading into the PFGE. The gel casting stand on PFGE platform was positioned with end gates and placed the combs into the comb holder. The comb was placed at least 2mm above the surface of platform. Pulse field agarose (1%) was prepared in 0.5X Tris-Acetate-EDTA (TAE) and gently poured into the casting stand in order to get a thickness of 5–6 mm. The comb and comb holder were carefully removed after the solidification of the agar and appropriately sized agarose plugs were carefully introduced in to the wells on the gel. After sample introduction, the wells were sealed with 1% pulse field agarose solution for filling the empty spaces between agarose plugs and the gel. After that screws were loosened slowly and gently slided towards platform into the slot.

The PFGE chamber (CHEF-DR II, Variable Angle System, BioRad) was filled with 2.5litres of 0.5X TBE running buffer and pre-cooled to 14°C for 1 hour. Subsequently, electrophoresis was performed using two different programs, the first programme used the following conditions : 6 V/cm , ramped pulse times from 1to 16 seconds for 22 hrs by maintaining current in the range 230-300 mA and second programme with 6 V/cm , ramped pulse times from 1to 14 seconds for 17 hrs by maintaining current in the range 230-300 mA. Lambda PFG Marker and Low Range PFG Marker (NEB Biolabs) were used as reference points to check the size of the extracted DNA. After electrophoresis the gel was stained for 20 min in ethidium bromide

solution (0.5µg/mL) and destained for 20 min in sterile, double-distilled water. The gel was documented under gel imaging system (Bio-Rad).

5.2.5 PCR efficacy analysis

In order to determine the PCR inhibition by humic substances, the soil metagenomic DNA extracted using Proteinase K-based method (optimized method) was serially diluted at 1:5 , 1:10, 1:20, 1:30, 1:40, 1:50 and 1:60 for PCR amplification of universal 16S rRNA gene. The PCR mixture (25 µl) was consisted of 1µl DNA template, 2.5 µl dNTP, and 2.5 µl 10 X buffer, 1µl Taq DNA polymerase and 1µl each of forward and reverse primer. PCR conditions were as follows; 5 min at 94°C followed by 35 cycles of 20 sec at 94°C, 30 sec at 58°C and 20 sec at 68 °C followed by a 10 min extension step at 68°C. Primers used were 16S1: GAG TTT GAT CCT GGC TCA, 16S2: ACG GCT ACC TTG TTA CGA CTT. PCR was performed using an Eppendorf Mastercycler personal.The samples were electrophoretically separated in a 0.7% agarose gel in 0.5X Tris-Acetate-EDTA buffer and visualized using ethidium bromide under ultraviolet illumination.

5.2.6 Q-Sepharose Purification of Metagenomic DNA

Purification of metagenomic DNA was performed by using Q-Sepharose (Sigma) as described by the method of Sharma et al. (2007). Initially, Q-sepharose was washed and equilibrated with 10 mM potassium phosphate buffer (pH 7.2). Approximately 500µl of solid washed beads were resuspended in 1.5 ml of 10 mM potassium phosphate buffer. After proper mixing, 300 µl aliquots of Q-Sepharose suspended in potassium phosphate buffer were transferred in 5 Eppendorf tubes and centrifuged at 1000 *g* for 1 min to remove the overlaying buffer. Different volumes (5-400µl) of metagenomic DNA preparation (125µg) in TE buffer were added to the pre-washed and equilibrated Q-Sepharose beads. It was mixed thoroughly by inverting the tubes up and down slowly for 15 min and kept at room

temperature for 15 min to settle the contaminant proteins on to the beads. The brown coloured humic acids were bound immediately to the Q-Sepharose. Then the preparations were centrifuged at 1000 *g* for 5 min. The supernatant containing purified DNA was stored for quantitative analysis as well as restriction digestion analysis with Sau3A1.

5.2.7 Metagenomic lambda library construction

Small insert expression libraries, particularly those constructed in the lambda phage vectors are generally used for functional or activity based screening; however, in contrast with cosmid or fosmid vectors, the ZAP Express vector (Fig.4) allows cloning of up to 12 Kb with 12 unique cloning sites: Apa I, BamH I, EcoR I, Hind III, Kpn I, Not I, Sac I, Sal I, Sma I, Spe I, Xba I, and Xho I. One of the major advantages of lambda phagemid vectors is that it combines high transfection efficiency of recombinant lambda phage vectors with the rapid in vivo excision ability of M13-based helper phagemids, which permits both eukaryotic and prokaryotic expression.

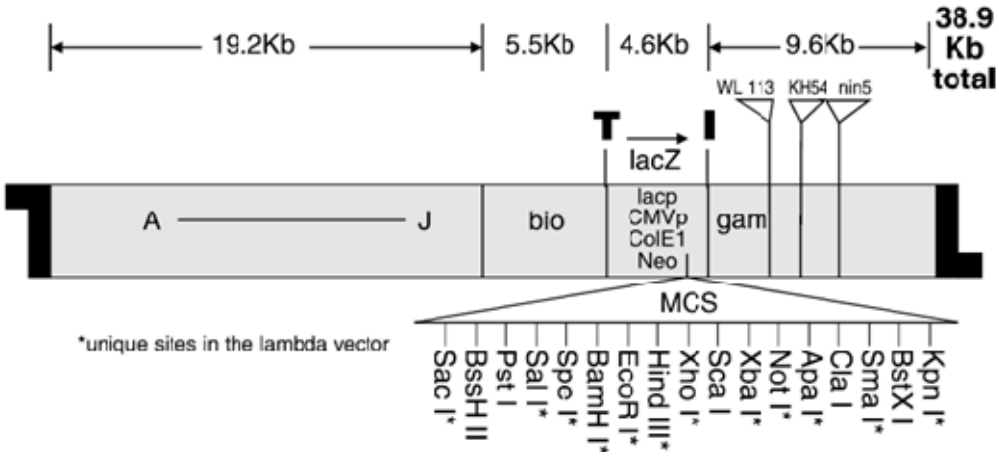


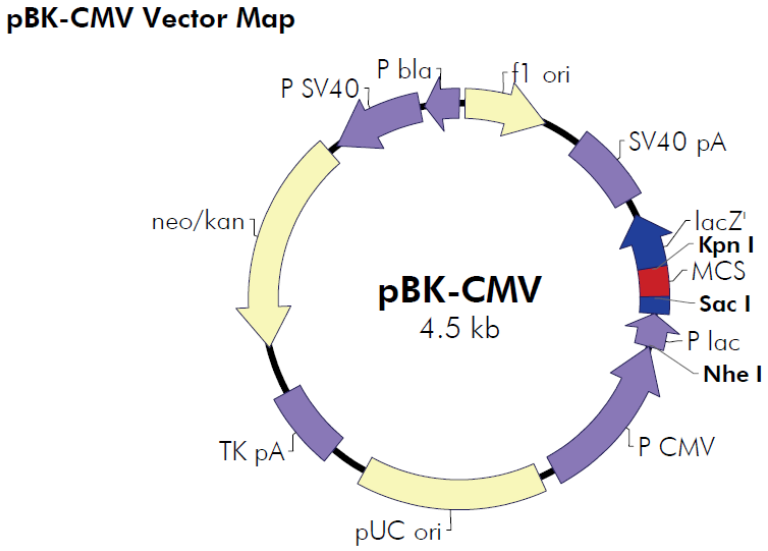
Fig.4. Map of Lambda ZAP Express vector

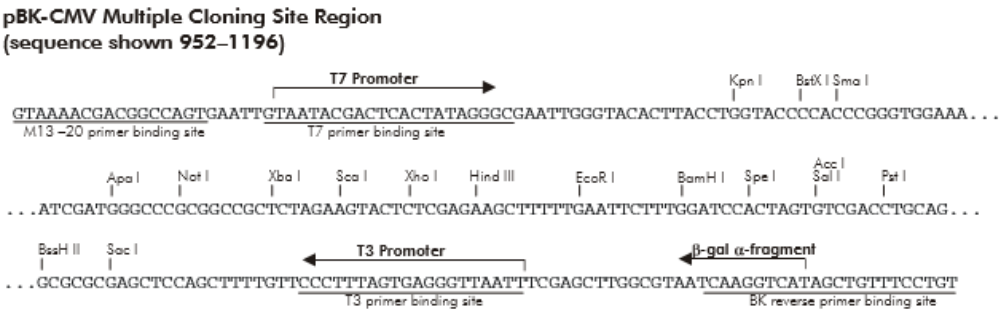
The λ ZAP Express vector is designed for simple, efficient and rapid in vivo excision and recircularization of any cloned insert contained within the linear lambda vector to form a circular phagemid containing the cloned insert.

The in vivo excision depends on the presence of a variety filamentous (e.g., M13) bacteriophage-derived proteins, which recognize the f1 bacteriophage "origin of replication" with an initiation and termination site for DNA synthesis, which are subcloned separately into the ZAP Express vector. The inserted DNA within the lambda phage can be excised with the help of M13 helper phage proteins by simultaneously infecting a non-suppressing *E. coli* strain with both the lambda vector and the M13 helper phage.

Inside the *E. coli* strain, M13- helper phage proteins recognize the initiation site for DNA synthesis, and duplicate the insert DNA just downstream of the nicked site and continue synthesis until a termination signal is captured. Then newly synthesized ssDNA molecule is circularized by the gene II product from the M13 phage, resulting in the conversion or sub-cloning of a linear lambda vector into circular pBK-CMV phagemid vector with the cloned insert (Fig.5). All other sequences in the lambda vectors are positioned outside of the initiator and terminator signals and are not incorporated in the phagemid.

In addition, the "packaging signals" are linked to phagemid f1 origin sequence, which helps the circular ssDNA to be packaged inside the phagemid particles through a packaging reaction and secreted outside while infection with *E. coli*. The phagemids can then be excised with the help of helper phage proteins. After excision, the lambda phage particles can be lysed by heat treatment at 70°C .The phagemid is then infected to *E. coli* and can be plated on selective media to form kanamycin resistant positive phagemids. Phagemid DNA from excised colonies can be isolated for insert characterization such as DNA sequencing, subcloning, and mapping.





Feature	Nucleotide Position
f1 origin of ss-DNA replication	24–330
SV40 polyA signal	469–750
β-galactosidase α-fragment coding sequence (lacZ')	812–1183
multiple cloning site	1015–1122
lac promoter	1184–1305
CMV promoter	1306–1895
pUC origin of replication	1954–2621
HSV-thymidine kinase (TK) polyA signal	2760–3031
neomycin/kanamycin resistance ORF	3209–4000
SV40 promoter	4035–4373
bla promoter	4392–4518

Fig.5. Map of pBK-CMV phagemid vector and its multiple cloning site described in detail

5.2.7.1 Preparing insert DNA for cloning with Lambda ZAP express vector system

5.2.7.1.1 Restriction digestion of insert DNA

In order to obtain Sau 3a1digested DNA fragments of about 6-10 kb, a series of reactions were carried out as follows:

- **Reaction 1 : 0.05 U (0.005 U/ul)**

  6 µl DNA (75.4 µg/µl), 1µl NE buffer (10X), 1 µl BSA buffer (10X), 1 µl H_20, 1 µl Sau 3a1 (0.5 U/10µl)

- **Reaction 2 : 0.1 U (0.01 Uµ/l)**

  6 µl DNA (75.4 µg/µl), 1µl NE buffer (10X), 1 µl BSA buffer (10X), 1 µl H_20, 1µl Sau 3a1 (1 U/10µl)

- **Reaction 3 : 0.2 U (0.02 U/µl)**

  6 µl DNA (75.4 µg/µl), 1µl NE buffer (10X), 1 µl BSA buffer (10X), 1 µl H_20, 1 µl Sau 3a1 (2 U/10 µl)

All the reaction mixtures were incubated for 30 min at 37^0C and the reaction was stopped by heating the samples at 65^0 C for 15 min.

5.2.7.1.2 Gel extraction of digested DNA using GELase (Epicentre)

The best restriction digestion condition was selected and scaled up to obtain a minimum concentration of 2µg DNA (Sau3a1digested) and the samples were loaded on a combined well made in a low melting agarose gel (Bio-Rad). Cut out the desired gel region (6-10 Kb) and trimed excess agarose without exposing to UV light to minimize the formation of pyrimidine dimers. The DNA was then eluted from the agarose gel using GELase (Epicentre) protocol with the following steps. The excised gel slices were weighed in a

tared tube and 1× GELase buffer was added (3 mL of 1× GELase buffer per mg of gel). It was incubated at room temperature for 1 h and then removed the buffer, melted the gel slice by incubating at 70^0C for 3 min for each 200mg of gel. The melted agarose solution was transferred immediately to 45^0C and equilibrated for 2 min for each 200 mg of gel slice. Then the agarose was digested with 1U of GELase for each 600 mg of gel for 120 min at 45^0C and then reaction mixture was heat inactivated at 70^0C for 10 min, chilled the tube on ice for 10 min. The digested agarose mixture was centrifuged at 15000 rpm for 20 min at 4^0C to pellet any insoluble oligosaccharides. The upper portion containing DNA was carefully removed into a sterile 1.5 ml tube, precipitated by adding one tenth volume of 3 M sodium acetate and 2.5 volume of absolute ethanol and mixed gently. The DNA pellets were washed with 70% ethanol, air-dried and re-suspended in TE buffer.

5.2.7.1.3 Concentration of DNA using Amicon Ultra 0.5ml (Millipore) filter devices

The DNA was concentrated in Amicon Ultra 0.5ml (Millipore) concentrator membrane (100 KDa cut off) at 4^0C to a final volume of 10 µl. Briefly, Amicon Ultra-0.5 device was inserted into one of the provided microcentrifuge tubes and approximately 500 µL of gel extracted DNA was added into it. The assembled filter device with the DNA sample to be concentrated was placed in the centrifuge rotor in such a way that the cap strap was faced towards the centre of the rotor, counterbalanced with a similar device and centrifuged the device at 14,000*g* for approximately 40 min. After centrifugation, the flow through collected in the microcentrifuge tube was removed and the Amicon ultra filter device was placed upside down in a fresh sterile centrifuge tube, immediately centrifuged for 2 minutes at 1,000*g* to transfer concentrated ultrafiltrate from the device to the tube. The ultrafiltrate

(approximately 10µl) was stored in a sterile tube. The concentration of DNA was determined using Qubit fluorometer 2.0 as described in section 5.2.3.

5.2.7.2 Ligation in to BamH1 digested lambda ZAP Express vector

Sau3A1 digested DNA and BamH1 digested ZAP Express vector (Agilent technologies) were ligated overnight at 22^0C using the following ligation conditions such as 1µl BamH1pre-digested Zap Express vector (1 µg/µl) , 0.5 µl T4 ligase buffer (Fermentas), 3.2 µl (0.17µg/µl) concentrated insert, 0.6 µl T4 DNA ligase (Fermentas), 0.2 µl rATP in a final volume of 5.5 µl. This will result in the ligation of lambda ZAP arms with the insert (6-10 Kb) to form a lambda phage-metagenome strand. The recombinant lambda vector was then packaged with the phage coat protein supplied in the Gigapack III Gold Packaging Extract. The phage packaging kit is expected to produce 1×10^6 recombinant plaques.

5.2.7.3 Packaging with Gigapack III Gold Packaging Extract; Construction of the Primary Phage library from Valanthacaud mangrove sediment

In vitro packaging of the ligated mixtrure into the phage particles was performed using Gold Pack III packaging system. Gigapack III Gold packaging extracts are restriction minus (HsdR– McrA– McrBC– McrF– Mrr–) to optimize packaging efficiency (more with concatameric DNA) and a better library representation from highly methylated DNA.

The packaging extract was removed from $^{-}80^0$C deep freezer and thawed quickly. An aliquot of 4 µL ligated DNA was added to the packaging extract and mixed gently. The tube was given a quick spin to make sure that all the contents were settled at the bottom and incubated the reaction tube at 22^0C for 2 hrs. Packaging efficiency may decrease when the incubation time was increased more than two hours. After the incubation, 200 µL of SM buffer

(5.8 g NaCl, 2 g $MgSO_4$, 50 ml of 1 M Tris-HCl, 5 ml of 2% gelatin per liter) was added to stop the reaction followed by an addition of 20 µL of chloroform to prevent any bacterial contamination. The tubes were then stored at 4^0C until determining titer of packaged reaction.

5.2.7.4 Titering the packaged phage for determining the strength of the primary Phage library

The RecA– *E. coli* host strain XL1-Blue MRF´supplied with the ZAP Express vector kit was used for determining the efficiency of primary phage library. pBK-CMV phagemid vector grows very efficiently with this strain, since the F´ episome present in the XL1-Blue MRF´ strain helps in many functions, mainly the ΔM15 lacZ gene present on the F´ episome is necessary for the β-galactosidase-based blue/white recombinant selection strategy i.e., when the foreign DNA is present in the multiple cloning site, the lacZ gene expression is hindered and resulted in white plaques, whereas if no insert is present in the MCS, the amino terminus of β-galactosidase is expressed and non-recombinant clones can be visualized as blue plaques. Secondly, the in vivo excision of pBK-CMV phagemid vector from ZAP express vector requires superinfection with a filamentous helper phage, as the F´episome contains genes for F´pili formation which in-turn helps in infection with f1 filamentous phage. Another important factor is that lac repressor (lacIq gene) present in the F´ episome regulates expression of fusion proteins which may be toxic to the *E. coli* host strain. These factors potentially increase the efficiency of XL1-Blue MRF´ for screening the amplified library.

The strength of the primary phage library was determined as follows: firstly, XL1-Blue MRF´ *E. coli* supplied with the ZAP Express vector kit, was freshly streaked onto LB agar plates containing the tetracycline (10mg/ml) and

incubated overnight at 37°C and inoculated into 50 ml LB broth supplemented with 500 µl of 1M $MgSO_4$ and 500µl of 20%(w/v) maltose. Tetracycline was not added into the medium as this may affect phage infection onto bacterial cell wall. The inoculated flask was then incubated overnight at 30°C/200 rpm, till it reached OD_{600} of 1. The bacterial cells were pelleted at 1000 *g* for 10 minutes and re-suspended in 25 ml of filter sterilized 10 mM $MgSO_4$. The suspension of XL1-Blue MRF′ cells was diluted to an OD_{600} of 0.5 with freshly sterile 10mM $MgSO_4$.The titer of the packaged mixture was determined by mixing 1µl of the final packaged reaction product with 200µl of XL1-Blue MRF′ cells at an OD_{600} of 0.5 and 1µl of a 1:10 dilution of the final packaged reaction with 200 µl of XL1-Blue MRF′ cells at an OD_{600} of 0.5. Both reaction mixtures were incubated at 37°C for 15 minutes to allow the phage to attach to the bacterial cells. An aliquot of 3 ml NZY top agar (NZY broth per liter: 5g NaCl, 2 g $MgSO_4.7H_2O$, 5 g yeast extract 10 g NZ amine. Adjust pH to 7.5 with NaOH and then 1.5% agar was added , sterilized) was prepared, melted and cooled to ~ 50°C, added into the tubes containing infection mixture and immediately plated onto dry, pre-warmed NZY agar plates. The plaques were visible after 8-10 hours of incubation at 37°C. The titer value in plaque forming units (pfu/µl) was determined using the following equation.

$$\text{Plaque forming units/microliter (pfu/ µl)} = \frac{\text{[Number of plaques (pfu) x dilution factor]}}{\text{Volume plated (µl)}}$$

5.2.7.5 Determination of recombination efficiency through blue-white screening

The reaction mixture containing 1 µl of the final packaged reaction product with 200 µl of XL1-Blue MRF′ cells at an OD_{600} of 0.5 and 1 µl of a 1:10 dilution of the final packaged reaction with 200 µl of XL1-Blue MRF′ cells at an OD_{600} of 0.5 were incubated at 37°C for 15 minutes to allow the phage to attach to the bacterial cells. An aliquot of 3 ml of NZY top agar was

prepared as described above and 15 µl of 0.5 M IPTG (in water) and 50 µl of X-gal [250 mg/ml (in DMF)] was added separately to the 3ml of melted NZY agar with proper mixing in between the additions. IPTG and X-gal at higher concentrations may cause precipitation in the media. The cooled NZY top agar media with the infection mixture was immediately plated onto dry, pre-warmed NZY agar plates. The plaques were visible after 10-12 hours of incubation at 37°C.The recombinant plaques were appeared as white and are about 10-100 fold above the ground, while blue background plaques represented non-recombinants.

5.2.7.6 Amplification of the Primary Phage library (Secondary Phage library)

Primary phage library was amplified to a stable secondary library prior to the excision of pBK-CMV phagemid vector in order to make a large, stable and statistically significant titer of the primary phage library. This avoids the problem of under-representation of slowly growing clones. The procedure adopted for phage library amplification was as follows: The library suspension containing ~2 × 10^4 pfu/ml of bacteriophage was mixed with 600 µl of XL1-Blue MRF′ cells having an OD_{600} of 0.5, in BD Falcon polypropylene tubes. A total of 20 such reactions (each with 2×10^4 pfu/ml) were used to amplify into 2×10^6 plaques. The volume of primary phage library should not be more than 300µl per 600µl of *E. coli* cells. The reaction mixture containing phage and host cells were incubated for 15 minutes at 37°C to allow phage attachment to the cells. The phage infected bacterial mixture was then added with 6.5 ml of melted and cooled NZY top agar and spread evenly onto a freshly poured 150-mm NZY agar plate, allowed to set for 10 minutes. Plates were inverted and incubated at 37°C for 7-8 hours, plaques should not get larger than 1–2 mm; in the meantime the plaques were touching each other. Approximately 8–10 ml of SM buffer was overlaid onto the surface of the plates and kept at 4°C

overnight (in a gel rocker) for the diffusion of the phage into the SM buffer. The amplified mixture of bacteriophage suspensions were recovered from each plate and pooled into a sterile 30 ml falcon tube. An additional 2ml of SM buffer was added to rinse the plates and pooled them together. Further, chloroform was added at 5% (v/v) final concentration, stirred well and incubated for 15 minutes at room temperature. The cell debris was removed by centrifugation for 10 minutes at 500*g* and repeated the step till the supernatant was clear. Chloroform was added to the clear supernatant at 0.3 % (v/v) final concentration and stored at 4°C. The highly stable and amplified secondary library was stored by adding 7% (v/v) DMSO at ⁻80°C.The titer of the amplified library was further checked using the host cells and different dilutions of the library as described in the previous section 5.2.7.4.

5.2.7.7 In Vivo excision of pBK-CMV phagemid vector using Ex Assist helper phage with XLOLR strain

Mass excision of pBK-CMV phagemid vector from λ ZAP express vector was done using ExAssist helper phage with *E. coli* XLOLR strain. The excision process is devoid of helper phage co-infection as it cannot replicate in XLOLR strain having amber mutation. Thus the non-suppressing *E. coli* strain such as XLOLR prevents replication of the helper phage genome and allows only the excised pBK-CMV phagemid to replicate in the host. In addition, single-stranded rescue cannot be performed in this strain as XLOLR cells are resistant to lambda infection and prevents phage DNA contamination after in vivo excision. A simple cartoon representation of the mechanism of foreign gene transfection into Lambda ZAP vector and in vivo excision of pBK-CMV phagemid vector from lambda ZAP vector is shown in Fig.6.

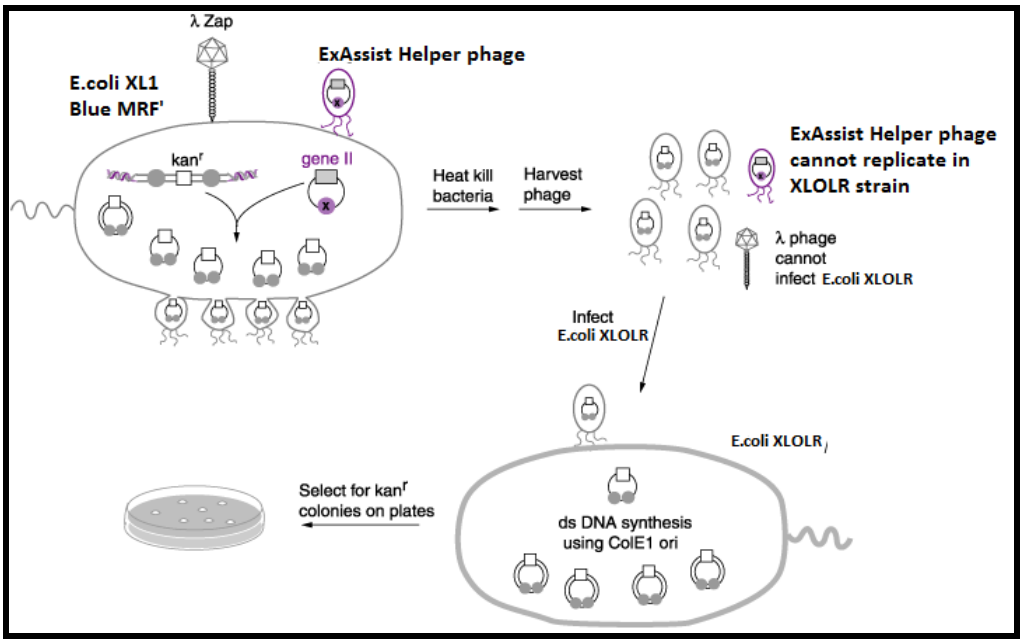


Fig.6. Cartoon showing the mechanism of foreign gene transfection into Lambda ZAP vector and in vivo excision of pBK-CMV phagemid vector from lambda ZAP vector in the form of Kan^r *E. coli* clones (AMG Science Clip Art is from "Applied Molecular Genetics" (R.L. Miesfeld, J. Wiley Inc. © 1999, ISBN-0471156760).

5.2.7.7.1 Determining titer value of Ex Assist helper phage prior to mass-excision

The ExAssist helper phage stored in dimethylsulfoxide (DMSO, 7%) was removed from ¯80°C and it is very important to titer the helper phage prior to mass-excision, titer value can be determined by infection with *E. coli* XL1-Blue MRF´. The strain was freshly streaked onto LB agar plates containing the tetracycline (10mg/ml) and incubated overnight at 37°C and inoculated into 10 ml LB broth supplemented with 100µl of 1M $MgSO_4$ and 100µl of 20%(w/v) maltose. Tetracycline was not added into the medium as this may affect phage infection onto bacterial cell wall. The inoculated flask was then incubated overnight at 30^o C/200 rpm, till it reached OD_{600} of 1.

Diluted the phage (10^{-4} to 10^{-7}) in SM buffer and mixed 1µl of each dilution with 200 µl of XL1-Blue MRF′ cells (OD_{600} = 1.0). Infection mixture containing helper phage and the XL1-Blue MRF′cells was incubated at 37°C for 15 minutes to allow the phage to adsorb on the cells. An aliquot of 3 ml of NZY top agar was prepared, melted and cooled to ~ 50°C and added in to the infection mixture. Then it was immediately plated onto dry, pre-warmed NZY agar plates. The cloudier plaques were visible after 8-10 hours of incubation at 37°C. The titer value in plaque-forming units (pfu/µl) was determined as described in the section 5.2.7.4.

5.2.7.7 .2 Amplification of Ex Assist helper phage

After determining the titer value in pfu/µl, the helper phage was added to the *E. coli* XLI-Blue MRF'cells at a multiplicity of infection (MOI) of 20:1(phage-to-cells ratio). Infection was achieved by incubating the tubes at 37°C for 15 minutes to allow the phage attachment to the cells and again incubated with shaking at 37°C for 10 hours, heated the tube at 65°C for 15 minutes followed by centrifugation at 12,000 rpm for 10 min to pellet the heat killed *E.coli* cells. The supernatant was transferred to a fresh conical tube and the titer value was determined as described above. The amplified helper phage was stored in dimethyl sulfoxide (DMSO) to a final concentration of 7% (v/v) and stored at ⁻80°C until mass-excision.

5.2.7.8 Mass Excision of pBK-CMV phagemid containing insert DNA from λ ZAP Express vector

The *E.coli* strains, XL1-Blue MRF′ and XLOLR cells in 50 ml LB broth with supplements were grown at 30°C and spun down the cells (1000*g*) for 10 min and resuspended each of the cell pellets in 25 ml of 10 mM $MgSO_4$. Growth was measured by taking OD_{600} of the cell suspensions and then

adjusted the concentration of the cells with 10 mM $MgSO_4$ to an OD_{600} of 1.0, which gives 8×10^8 cells/ml.

In a 50-ml conical tube, 100µl of amplified lambda bacteriophage library ($2x10^7$ pfu/ml) was mixed with 250 µl of XL1-Blue MRF′ cells ($2x10^8$ cells/ml) at a multiplicity of infection (MOI) of 1:10 lambda phage-to-cell ratio. Titer value of 10- to 100-fold more lambda phage than the size of the primary library was necessary to ensure statistical representation of the excised clones. Therefore, 50 µl Ex Assist helper phage ($2x10^9$) was added i.e., at 10:1 helper phage-to-cells ratio, which represent the maximum possibility that every cell was transfected with lambda phage and helper phage. The final infection mixture contained 1:10:100 (lambda phage: *E.coli* cells: helper phage) was incubated at 37°C for 15 minutes to allow both the phages to attach to the cells. LB broth (20ml) supplemented with 200 µl each of 1M Maltose and 1M $MgSO_4$ was added to the conical tube and incubated for 2.5–3 hours at 37°C with shaking. After incubation, the tube was heated at 65-70°C for 20 minutes to lyse the XL1-Blue MRF′ cells to release the excised phagemid from the cells. The tube was again centrifuged at 1000*g* for 10 minutes to pellet the cell debris and lysed phages. The clear supernatant containing excised phagemids was transferred into a sterile conical tube.

5.2.7.9 Determination of titer value for pBK-CMV phagemid containing insert DNA

The titer value of excised phagemids was determined by combining 1 µl of this supernatant with 200µl of XLOLR cells (fresh cells were prepared as described previously).The mixture was incubated at 37°C for 15 minutes and 40µl of 5xNZY broth (final concentration of 1x) was added and incubated the tube at 37°C for 45 minutes to allow sufficient expression of the kanamycin-

resistance phagemid clones prior to plating on selective medium. The phage infected cell mixture (approximately 100µl) was plated onto LB–kanamycin agar plates (50µg/ml) and incubated overnight at 37°C. After incubation, recombinant clones with inserted genome were appeared on selective LB-kanamycin plates. This represented the metagenomic lambda library from Valanthacaud mangrove islands and subjected for cellulase screening as described in next Chapter.

5.3 Results and Discussion

5.3.1 Metagenomic DNA extraction

Direct lysis method of DNA extraction was followed in this study because of the better DNA recovery than the indirect lysis method. Moreover, the extracted DNA seems to be more representative of the microbial community of the sample, as larger number of microorganisms had undergone lysis, particularly those adsorbed onto soil organo mineral aggregates (Bakken, 1985; Steffan et al., 1988).

An efficient DNA extraction method is an essential prerequisite for a successful metagenomic library construction. Four different types of direct DNA extraction methods from mangrove sediment were compared with respect to yield, purity and fragmentation. In addition, metagenomic DNA isolation from complex soil samples should accomplish the following requirements such as recovery of high molecular weight DNA without severe shearing, the extracted DNA should be free from inhibitors and complete lysis of microorganisms within the sample matrix. In this Chapter, efforts were put on to experiment with different direct DNA extraction methods such as Proteinase K lysis (soft enzymatic lysis), liquid nitrogen grinding (freeze grinding or harsh

lysis), PVPP-PEG method and agarose plug DNA extraction method for getting high purity DNA for metagenomic lambda library construction.

Here we adopted a spectrophotometric method for determining quality and quantity of metagenomic DNA, though gel electrophoresis of extracted DNA provided information about only DNA fragmentation. Humic acid contamination is the major problem associated with DNA purity. These contaminants inhibit PCR, restriction digestion and cloning as well as degrade the DNA during long term storage (Stevenson, 1976; Opel et al., 2010). Humic acids possessing similar size, charge and physico-chemical characteristics to DNA resulting in their co-precipitation with nucleic acids (Opel et al., 2010), as visualized by the brown colouration in the extracted DNA. Crude DNA extracts can be contaminated by approximately 0.7–3.3mg/mL of humic acid depending upon various soil types (Tebbe and Vahjen, 1993). Humic materials also interfere with DNA quantification by exhibiting absorbance at both 230 and 260nm, the parameter used to quantitate DNA. A high 260/230 ratio (>2) is indicative of pure DNA, while a lower ratio indicate humic acid contamination. Similarly, a high 260/280 ratio (>1.7) indicated DNA purity, whereas a lower ratio represented protein contamination. Accurate and sensitive measurement of high molecular weight DNA is an essential step before going into any metagenomic analysis, as UV spectrophotometric method did not distinguish between DNA, RNA and single stranded nucleic acids, which resulted in a cumulative reading at 260nm. Qubit fluorescence based DNA measurement provides a rapid and highly accurate method for assessing DNA quantity in terms of picograms of DNA, well acceptable method for downstream metagenomic analysis.

When different DNA extraction methods were compared (Table1), Proteinase K based lysis method gave highest yield (276µg DNA per gram of

dry soil) followed by liquid nitrogen grinding method (175µg DNA per gram of dry soil) and PVPP-PEG method yielded only 86µg DNA per gram of dry soil. The DNA yield in µg per gram of dry soil was calculated using the following formula, Yield (µg/g soil) = [concentration of DNA (µg/µL) /weight of soil (g)]×[final suspension volume of extracted DNA (µl)]. The results are given in Table 1. The highest DNA yield obtained in Proteinase K lysis might be due to the effective break down of the cell wall of microorganism by peptidoglycan targeted proteases such as proteinase K, which released DNA easily than other methods (Zhang et al., 2003). In addition, proteinase K coupled with SDS hot lysis provided better lysis of cells aggregated in the complex sediment matrix. When purity of extracted DNA was compared, PVPP –PEG method (A_{260}/A_{230} = 1.91) had only met the absorbance criteria i.e., absorbance value very close to two. The compounds such as PVPP and PEG were very effective in the removal of co- extracted humic acids. Similar results were obtained by Miller et al. (1999) and Frostegard et al. (1999). Conversely, very low yield obtained through this method was due to the very close binding of these materials to DNA, resulted in poor DNA yield with very less co-precipitated humic acids (Zhou et al., 1996). According to La Montagne (2002), the use of PEG instead of isopropanol resulted in a fourfold reduction in humic substances. DNA yield was intermediate (175µg/gram dry soil) with liquid nitrogen freeze grinding method. Lysozyme and SDS were added sequentially rather than in one single step, since the lysozyme was inhibited in the presence of SDS (Smith and Stoker, 1949). In theory ice crystal formation during freeze-thaw resulted in cell rupture, but its effect on microbial cells aggregated in to the soil matrix appeared to be moderate. Moreover, this method produced DNA without any severe shearing unlike the previous studies of Kabir et al. (2003) and Ogram et al. (1987), who

experienced severe shearing by a similar freeze-grinding method for extracting DNA from various culturable organisms. However, Zhou et al. (1996) observed the combination of freeze-grinding and SDS-hot lysis methods in giving much higher DNA yields without any severe fragmentation of high molecular weight DNA. Most of the previous studies showed that a combination of chemical-enzymatic and mechanical lysis can yield 2-fold higher amount of DNA than by any single step of extraction (More et al., 1994). Agarose plug embedded DNA extraction was found very effective in the recovery of high molecular weight DNA (>267 Kb), while other methods yielded DNA fragments in the range of 73 Kb. Agarose plug extracted DNA was not selected for library construction since it gave very low DNA yield (visualized through PFGE), than other enzymatic and mechanical lysis methods. To our knowledge, the first report on the isolation of high molecular weight DNA from soil through a combination of direct cell extraction and lysis of biomass in agarose plugs yielded a size of approximately 1 Mb. This method resulted in the removal of humic contaminants and preserved genomic DNA intact (Bakken and Lindahl, 1995). Since our aim was to select a DNA extraction method which would give higher DNA yield rather than size and purity, Proteinase K-lysis method was found suitable for further cloning into small capacity lambda ZAP express vector. The purity and yield of DNA extracted through different in-situ lysis methods are described in Table 1.

Table 1: Spectrophotometric assessment of purity and yield of total DNA extracted by different direct lysis methods

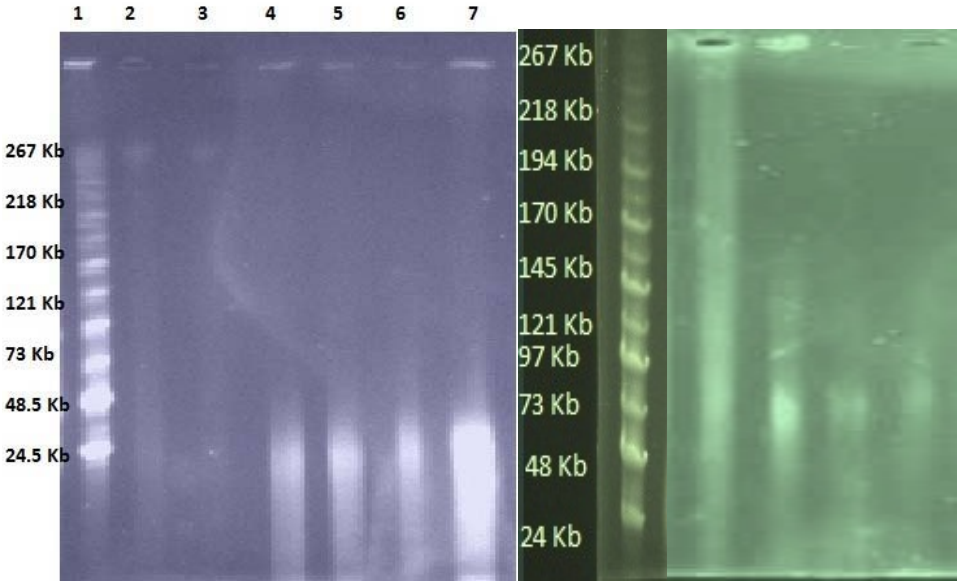
S.No	Method	Qualiative and quantitative analysis of metagenomic DNA		
		A_{260}/A_{230}	A_{260}/A_{280}	Concentration (ug/gram dry soil) using Qubit fluorometer
1	Harsh method (Liq N_2 grinding)	1.32	1.31	175
2	Soft lysis (Proteinase K & lysozyme)	1.67	1.42	276
3	PVPP + PEG method	1.91	1.63	86

5.3.2 Metagenome DNA size determination by Pulsed Field Gel Electrophoresis (CHEF DR II Bio-Rad)

PFGE analysis of the DNA extracted through different direct lysis methods provided better size determination of DNA, which could not be resolved through conventional stable- field gel electrophoresis. A unidirectional flow of current is maintained in conventional systems, whereas the orientation of electric field across the PFGE gel is regularly shifted. This variability in electric field direction allowed PFGE to resolve very large fragments of DNA. There are a variety of PFGE conditions that differ in pulse time of electrophoretic current delivered to the low melting agarose gel, which ultimately result in reorientation and resolution of high molecular weight DNA. Therefore, PFGE conditions such as pulse time, voltage and running time should be standardized for individual runs.

In this experiment, we have used two different programmes, the first one used a pulse time of 1-16 seconds for 22 hours (running time) which could not resolve the DNA properly. A high smearing pattern of DNA were observed for all extractions, except the agarose plug embedded DNA which was resolved better (>267 Kb) using this programme (Fig.7b). The second programme with a pulse time of 1-14 seconds for 17 hours running time was able to resolve differently extracted DNA. The DNA extracted using Proteinase K lysis, liquid nitrogen-freeze grinding and PVPP-PEG methods yielded a size range of 73 Kb (Fig.7b , lane 3-5), whereas the agarose plug extracted DNA yielded a size range of greater than 267 Kb (Fig.7b, lane 2). The pulse time is the most important factor involved in size separation and re-orientation of DNA in PFGE. The first

programme used a longer linear pulse time (1-16 seconds) for 22 hours which is more than the needed pulse time. Then lowering of linear pulse time and running time in the second programme i.e., a linear switching from 1 to 14 seconds for 17 hours was found to be sufficient for HMW DNA reorientation than conventional uniform electric field gel electrophoresis. Longer run time decreased the band sharpness and a greater possibility for degradation due to nuclease action. PFGE analysis of agarose plug embedded DNA showed an upper band (near the well) with size >267 Kb and smeared towards the lowest band (approximately 24 Kb), suggesting that pulse time used in both programmes were shorter than the pulse time needed for high molecular weight DNA, which caused the PFGE system to work under the average of applied electric fields, resulted in migration of DNA independent of size. Of all the four direct lysis methods examined quantitatively as well as qualitatively, proteinase K-lysis method was selected for further metagenomic lambda library construction because of satisfactory yield and purity of DNA. This modified enzymatic lysis method of Zhou et al. (1996) was gentler and able to yield 73 Kb DNA in comparison with Yeates et al. (1998) (<20 Kb), Zhou et al. (1996) and Miller et al. (1999) (<23 Kb).



(Fig.7a) **(Fig.7b)**

Fig.7a. Size separation of DNA extracted using different direct lysis methods (electrophoretic condition 1-16 S pulse time, 22 hrs). Lane1: lambda digested with *Hin*dIII, Lane 2 &3: agarose plug DNA extraction, Lane 4&5 : crude metagenomic DNA extracted by Liq N_2 method, Lane 6 : PEG +PVPP method , Lane 7 : Proteinase K lysis method

Fig.7b. Size separation of DNA extracted using different direct lysis methods (electrophoretic condition 1-14 S pulse time, 17 hrs). Lane1: lambda digested with *Hin*dIII, Lane 2: crude metagenomic DNA extracted by agarose plug DNA extraction, Lane 3: Proteinase K lysis method; Lane 4: Liq N_2 method, Lane 5: PEG +PVPP method.

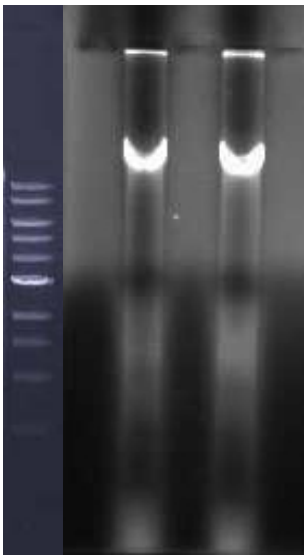


Fig.8. DNA extracted from valanthacaud mangrove sediment sample analysed through conventional horizontal electrophoresis system with 1Kb DNA ladder in lane 1, lane2-3: DNA extracted through Proteinase K lysis method (optimized protocol)

5.3.3 PCR efficacy analysis

An effective PCR amplification is generally considered as an indicator of DNA purity for cloning and subsequent genomic library construction (Cullen and Hirsch, 1998; Moreira, 1998; Burgmann et al., 2001). Humic acids interfere with the PCR through sequence specific binding to DNA, reducing the concentration of template DNA for amplification (Tsai and Olson, 1991). Therefore, PCR efficacy was carried out with different serial dilution of metagenomic DNA to determine the inhibitory effect of humic substances (Fig.9). The results obtained clearly showed that metagenomic DNA isolated from valanthakaud mangrove sediment contained relatively low concentration of PCR inhibitory substances as evidenced by successful amplification of bacterial 16S rRNA gene from 100- fold dilution of the metagenomic soil DNA (lane 2 in the Fig. 9). Crude DNA (0-fold dilution) appeared on the 1st lane of the gel did not show any amplification due to PCR-inhibitory substances present in the crude soil, while 100-fold dilution of crude

DNA resulted in increased specificity of PCR primers by reducing the concentration of inhibitory substances through dilution. However, crude DNA diluted to higher volumes such as 1:200 (lane 3), 1:300 (lane 4), 1:400 (lane 5), 1:500 (lane 6) and 1:600 (lane 7), could not amplify 16S rRNA, which might be due to the insufficient concentration of DNA required for PCR amplification. It could also be found that amplification was possible with 100-fold diluted metagenomic DNA without the addition of any PCR enhancers such as bovine serum albumin or dimethyl sulphoxide, which also revealed the efficiency of DNA extraction method chosen. Since the lambda library required higher concentrations of DNA (0.2µg of insert DNA per µg of vector), it was decided to purify the crude DNA using Q-Sepharose, as only 100-fold dilution of DNA yielded 16S r RNA gene without prior purification. Earlier experiments of Pang et al. (2008) also showed that diluted DNA (10^{-3} – 10^{-4}) was needed for successful amplification of the 16S rRNA gene from soil DNA, as it might relieve humic acid inhibition.

Fig.9. Evaluation of PCR efficacy from isolated metagenomic DNA by using 16S rDNA universal primers. Lane 1: crude metagenomic DNA (0-fold dilution) , lane 2: 100-fold dilution of metagenomic DNA, lane 3: 200-fold dilution of metagenomic DNA, lane 4: 300-fold dilution of metagenomic DNA, lane 5: 400-fold dilution of metagenomic DNA, lane 6: 500-fold dilution of metagenomic DNA, lane 7: 600-fold dilution of metagenomic DNA , lane 8 : 1Kb marker

5.3.4 Purification of Metagenomic DNA from humic acids using Q-Sepharose

The extracted metagenomic DNA was brown in colour indicating the presence of co-extracted humic acids and polyphenolic compounds, which interfere with cloning (Miller et al., 1999; Tebbe & Vahjen, 1993). Therefore, humic acids should be removed from the metagenomic DNA before cloning. Q-sepharose based single step purification of DNA was found to be an improved method for getting high molecular weight un-fragmented DNA, which can be subsequently digested with restriction enzymes for metagenomic library construction. Therefore, the method was optimized with different volumes of metagenomic DNA (50-400 µl; final concentration 125µg/ml) and values for percentage recovery of DNA and loss of humic acids are presented in the Table 2. From the results, it has been made clear that for lower volumes of DNA (50-150 µl), the loss of DNA was nearly 20-28 % of the initial DNA concentration used for Q-Sepharose purification, while at increased volume (300 µl) of DNA to Q-Sepharose (300µl) resulted in enhanced recovery of DNA (96%) i.e., 1:1 ratio of metagenomic DNA with Q-Sepharose was found optimum for purification, as it revealed maximum recovery of DNA with highest removal of humic acids (96%). At the optimum value, the concentration of DNA before and after purification was 125µg/ml and 120 µg/ml respectively. At higher DNA concentrations, most of the polyphenols, humic acid and small fragments of DNA were bound to Q-Sepharose immediately, whereas high molecular weight DNA remained unbound (Sharma et al., 2007). For higher volumes of DNA (400 µl), it was observed that the removal of humic acids was low as calculated spectrophotometrically. It is a quick and acceptable method of purification to get intact and good quality metagenomic DNA.

Previously many purification methods were reported, such as mini-column purification and gel-plus-concentrator methods (Zhou et al., 1996), Sephadex G-200 spin column purification (Miller et al., 1999), Caesium chloride (CsCl) density gradient purification (Bertrand et al., 2005) etc. Such multi-step purification methods were found to be time consuming, expensive and laborious with significant reduction in DNA yield. Contrary observations had been made by Harry et al. (1999) and Roose-Amsaleg et al. (2001), who observed that only a combination of purification methods can yield DNA of sufficient purity for successful molecular applications. However, in the present study, single step purification of metagenomic DNA using Q-Sepharose was seen to be a better method in terms of yield and purity of DNA obtained and therefore optimized for further analysis.

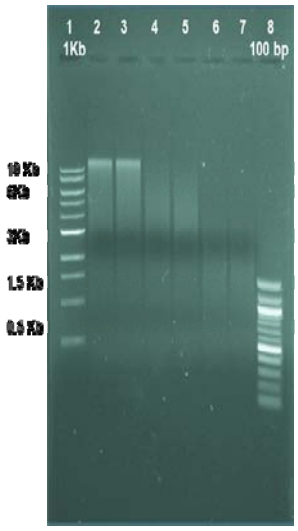
Table 2: Standardisation of Q-Sepharose based purification as evaluated by percent loss of DNA and percent reduction in humic acid. All the values are mean±SD of three measurements

Volume of mDNA (Final con. 125 µg/ml)	Con. of DNA after treating with 300 µl Q-Sepharose (µg/ml)	Percent loss of DNA	Percent removal of humic acids as determined by A_{260}/A_{230}
50 µl	89.6 ±0.2	28.32%	90 %
100 µl	96 ±0.5	23.2 %	92 %
150 µl	98±0.6	21.6 %	96 %
200 µl	105±0.6	16 %	89 %
250 µl	119±0.05	4.8 %	89 %
300 µl	120±0.2	4%	96 %
400 µl	118±0.3	5.6 %	70 %

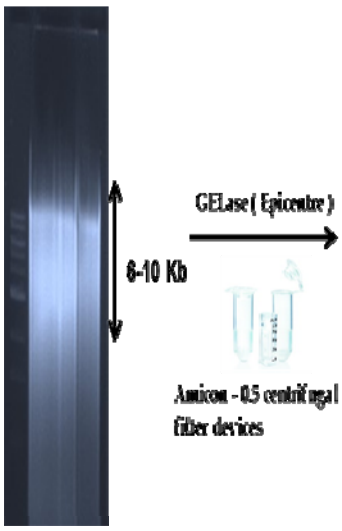
5.3.5 Preparing insert DNA for cloning with Lambda ZAP express vector system

The purified metagenomic DNA adjusted at a concentration of 75.4 µg/µl was partially digested with sau3a1 restriction enzyme with varied digestion conditions to identify the optimum ratio of enzyme to DNA for getting 6-10 Kb

fragments of metagenomic DNA. Digestion pattern was analysed by running an aliquot of digested DNA on 0.8% agarose gel and the result is shown in Fig.10. Based on the results, it could be assessed that the reaction mixture containing 0.1U Sau3a1enzyme provided better digestion of approximately 75.4 µg/µl of metagenomic DNA (Fig.10, lane 2&3). Subsequently, the optimized reaction was scaled up to 500µl volume for mass extraction of insert DNA for library preparation. The digested bands of 6-10 Kb was excised carefully from the preparative gel as there was little smearing up to 4Kb due to pipette mixing during gel loading. The excised gel slices were extracted using GELase digestion (Epicentre) and visualized on 0.8% agarose gel (Fig.11).The pooled DNA was membrane concentrated using Amicon 10KDa (Merck Millipore , USA) (Fig.12) and the digested DNA(6-10 Kb) having a concentration of 0.2µg/µl was used for ligation and subsequent library preparation.



(Fig.10)



(Fig.11)

(Fig.12)

Fig.10. Preparation of insert DNA using restriction digestion with Sau3a1 for cloning into ZAP Express vector; Lane 1: 1 Kb marker, Lane 2&3: reaction 1, Lane 4 &5: reaction 2, Lane 6&7: reaction 3, Lane 8: 100bp ladder; Fig. 11. Scale up volume of reaction 2 in a combined well for gel elution; Fig.12. DNA (6-10Kb) recovered DNA after GELase extraction

5.3.6 Construction of Lambda ZAP metagenomic library (PG-Val-Mg-1)

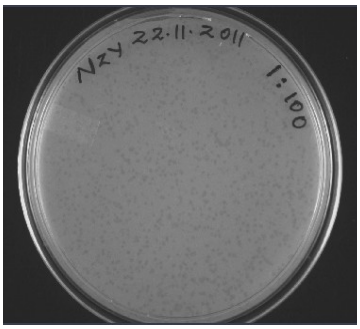
The Sau3a1 digested DNA (6-10 Kb, 0.17µg/µl) with a final concentration of 0.544µg was ligated with λ ZAP Express vector (1µg/µl). It was also suggested in the manufacturer's instructions that, an efficient ligation of a 4 kb insert to the λ arms, needed 0.1 µg of insert per every 1 µg of vector, and it should be free from contaminants and should contain a high percentage of ligatable ends. As per the instructions, ligation efficiency of the vector was checked with the control insert, pBub test insert (0.4 µg) supplied along with the kit and after packaging with Gigapack III Gold Packaging Extract, it was found that the titer value reached the expected titer value of 1×10^6–1.5×10^7, showing the high efficiency of ligation and packaging reaction. It was also inferred that ligations carried out at DNA concentrations of 0.2 µg/µl or greater with volume less than 5µl, favoured concatamers and not circular DNA molecules containing only one cos site. Subsequently, the ligated DNA for metagenomic library construction was packaged with Gigapack III Gold packaging extract. These are restriction minus (HsdR– McrA– McrBC– McrF– Mrr–) designed to optimize packaging efficiency and library representation particularly from highly methylated DNA. The reaction mixture after packaging with Gigapack III Gold packaging extract was termed as Primary phage library. The strength of primary phage library or the efficiency of packaging reaction (plaque forming units/ml) was determined by titration of the samples and was found to be $22x10^3$ pfu/ml,which represented a highly efficient packaging reaction.

Recombination efficiency was determined by blue-white screening, based on the principle of loss of β-galactosidase activity in recombinant phages. In the absence of insert DNA, lambda arms were ligated to each other, which encode the β-galactosidase enzyme in the ligation site, while the presence of an

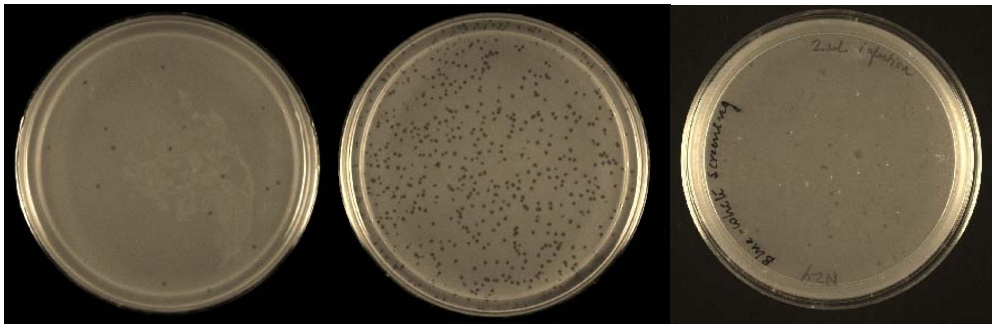
insert disrupts the β-galactosidase gene. The host bacteria, *E. coli* XLI-Blue MRF', was infected with the packaging reaction and plated in the presence of 15 µl of 0.5M IPTG and 50µl of X-gal (250 mg/ml) (in DMF) to determine the recombination efficiency. Approximately 10% of the total plaques remained as non-recombinants (i.e., 2 blue plaques out of total 22 plaques/µl) , due to β-galactosidase activity, implicated that greater than 90% of the clones were white and represented a recombination efficiency of greater than 90%. Thus the strength of library containing positive inserts was found to be 20 x 10^3 pfu/ml or 2 X10^4 pfu/ml. Similar results have been obtained for a lambda expression library having titer of 1x10^7 PFU/µg DNA with 90% recombination efficiency (Pevow et al., 2012). Since phages that are packaged in vitro are not very stable, half the library was immediately amplified to a stable secondary library. The secondary library overcomes the problem of under-representation of clones. Thus the stable, high-titer secondary library had strength of 4x10^7 pfu/ml and preserved as a metagenomic library repository at -80^{o}C for future use. The library was named as PG-Val-Mg-1; where 'PG' represents 'phagemid', 'Val' represents 'Valanthacaud' and 'Mg' represents 'Mangrove'. The pattern of plaque formation in control library (Fig.13), primary metagenomic library (Fig.14), secondary metagenomic library (Fig.15) and blue-white screening of primary library (Fig.16) are represented accordingly.

Conversion of the λ ZAP primary library into a pBK-CMV phagemid library was carried out by in vivo mass excision using Ex Assist helper phage with XLOLR strain, which reduced time for library analysis and insert characterization. In order to get a maximum representation of clones present in the primary phage library, only a partial amplified library (~2x10^7pfu) was used for mass excision. A successful excision of pBK-CMV phagemid from lambda ZAP required at least 1X10^2 lambda phage particles. The titer value of mass-excised

pBK-CMV phagemid was estimated to be 5.74x10^2cfu/µl (colony forming units in microliter).The recovered phagemid library was used for phagemid isolation and subsequent screening purposes, which will be discussed in Chapter 6.



(Fig.13)



(Fig.14) **(Fig.15)** **(Fig.16)**

Fig.13. Control library in lambda ZAP express vector with pBub test insert; **Fig.14.** Primary phage library (2×10^4 pfu/ml), **Fig.15.** Secondary library after amplification (4×10^7 pfu/ml), **Fig.16.** Blue white screening of primary library

5.3.7 Statistical validation of the metagenomic library PG-Val-Mg-1

The metagenome is composed of total community DNA from organisms present in a given habitat and the average genome size is difficult to be predicted as there are a large number of uncultured microorganisms that have not yet been studied. The number of phagemid clones in a metagenomic library required to give a statistical representation of the metagenome can be estimated by the

equation N = ln (1-P) / ln (1- f), where N is number of phagemid clones required; P is the desired probability of a given sequence present in a genomic library (0.99); and f is the proportion of the genome contained in a single recombination (Sambrook & Russell, 2001). If there are approximately 6,000 genomes present (Torsvik et al., 1990; Ovreas and Torsvik, 1998) and the average genome size is assumed as 4 Mb (Raes et al., 2007), then that would be equal to 2.4 $x10^{10}$ bases. Thus the number of phagemid clones required to represent 99% of probability that the DNA sequences to be contained within a lambda library made with maximum size of 10kb is: N = ln (1-0.99) ÷ ln (1 – [1 x 10^4 bases/2.4 x 10^{10} bases]) = 4605 plaques. Based on this result, it could be concluded that the metagenomic library constructed (PG-Val-Mg-1) ensure a good representation of uncultured biota present in the environment, as we could achieve a total of 10,000 plaques (2×500 , i.e., Phage titer of primary library in microlitre volume × total volume of packaged library in microlitre) from the entire metagenome. In addition, the library represented double strength than the expected value, which clearly indicated a complete representation of all the clones, without any significant loss of the function to be screened. Similarly, to obtain a 95% probability of having a given DNA sequence represented in a phage library (f = average insert size/lamda genome size) was also calculated using the above equation, N= ln[1-0.99] ÷ ln [1-(8X10^3bases/38,900 bases)]=19.59 plaques in a single recombinantion, which was found statistically significant as it was approximately equal to the experimental value (20 plaques/microliter). The number of plaques is then multiplied by three for reading frame representation and then multiplied by two for gene orientation. Thus the total number of plaques to be screened to have a 95% probability for representing any given DNA sequence in the phagemid library is 60,000 phagemid clones [20(Pfu/µl) × 500 (total volume of phage library in microlitres) × 3 (reading frame correction) ×2 (gene orientation)].

The PG-Val-Mg-l metagenomic library contained 6-10 Kb fragments as inserts, therefore it represented about a minimum of 60 Mb of environmental genome to a maximum of 100 Mb (insert size × total number of clones). According to the view of Dr. Jo Handelsman, (pioneer of Metagenomics), who has been estimated that "106 Bacterial Artificial Chromosomes (BAC) clones, with an insert size of 100 kb, must be screened for coverage of the all the distinct prokaryotic species present in one gram of soil" (Handelsman et al., 1998).

5.4 Conclusion

The soil sampling for the construction of metagenomic library was conducted from Valanthacaud mangrove islands. Total community DNA extraction from the sample was standardized by taking DNA size and yield as desired parameters. Out of the four direct DNA extraction methods, the one based on Proteinase K was found as the best method in terms of quantity. Humic acid was efficiently removed using Q-Sepharose beads. After restriction digestion and concentration, 6-10 KbDNA was used as the insert for ligating into small insert expression system such as Bam H1 digested lambda ZAP express vector. In vitro packaging was performed using Gold Pack III packaging system and strength of primary phage library was found to be $2x10^4$ pfu/ml. Recombination efficiency (90%) was determined by plating on X-gal and IPTG plate. Primary phage library was amplified to a stable secondary library ($4x\ 10^7$pfu/ml). pBK- CMV phagemid clones were excised using Ex Assist helper phage with XLOLR strain available with the kit. The library is maintained at -80^oC as Metagenomic Repository and renamed as PG-Val-Mg-1. The library has been statistically validated for significant representation of clones.

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Chapter 6

SCREENING OF METAGENOMIC LAMBDA LIBRARY FOR CELLULASE ENCODING CLONES AND SEQUENCE ANALYSIS OF ONE OF THE POTENT CLONES

Contents

6.1 Introduction

Metagenomic screening approaches have emerged as a promising tool in bridging the cultivation gap to explore novel biomass-conversion enzymes (Gottschalk et al., 2010). Since cellulose is a valuable biopolymer for the production of biofuels (i.e., ethanol) and other bio-based products, a notable number of publications have been reported on the isolation of metagenome-derived cellulases (Feng et al., 2007 ; Jiang et al., 2009 ; Pang et al., 2009 ; Voget et al., 2006 ; Duan et al., 2009; Pottka¨mper et al., 2009 etc).The first reported research paper on metagenome-derived biocatalysts presented the detection of cellulases from a thermophilic, anaerobic digester maintained by lignocellulosic fuel (Healy et al.,1995) in which 12 clones showing CMCase activity and 11 clones encoding 4-methyl umbelliferyl-β-D-cellobioside (MUC) hydrolase were reported from the functional metagenomic library and in another study, a total of more than 12 habitat related-cellulases were detected from extreme environments such as soda lakes in Africa and Egypt

(Grant et al., 2004 ; Rees et al., 2003). While most of the metagenomic bioprospecting studies were aimed at extreme or enriched environments, there is also enough data existing on highly stable and versatile cellulases from non-extreme and highly diverse environments (voget et al., 2006; Pottkämper et al., 2009). In addition, various examples of targeted isolation of metagenome-derived cellulases have been reported from various environmental samples including soil (Jiang et al., 2009; Kim et al., 2008), hindgut contents of higher termite (Warnecke et al., 2007), rabbit cecum (Feng et al., 2007), compost (Pang et al., 2009), biogas reactor sludges (Jiang et al., 2010), enrichment cultures (Grant et al., 2004; Rees et al., 2003; Voget et al., 2003, 2006) as well as high cellulase hit rates from natural enrichment systems such as cow rumen (Duan et al., 2009; Ferrer et al., 2005; Liu et al., 2009; Palackal et al., 2007; Shedova et al., 2009; Wang et al., 2009).

Cellulases stand out from other glycoside hydrolases by their capability to hydrolyze β-1,4-glycosidic bonds between glucose units. The enzymatic breakdown of the β-1, 4-glycosidic linkages in cellulose continue through a process of acid hydrolysis by utilizing a proton donor and nucleophile or base. The hydrolysis can either bring about the inversion or retention (double replacement mechanism) of the anomeric arrangement of C-1 at the reducing end (Beguin and Aubert., 1994; Birsan et al., 1998; Hilden and Johansson., 2004). Three major types of enzymatic activity are fundamental for complete degradation of cellulose: endoglucanases or CMCase (1,4-β-D-glucan-4-glucanohydrolases ; EC 3.2.1.4), exoglucanases or cellodextrinases (1,4-β-D-glucan glucanohydrolases ; EC 3.2.1.74), exo-cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases ; EC 3.2.1.91) and β-1,4-glucosidases or cellobiases (EC 3.2.1.21) (Bayer et al., 1998 ; Kumar et al., 2008). The recently updated

version of CAZy server (http:// afmb.cnrs-mrs.fr/CAZY/) listed out more than 100 families of glycosyl hydrolases, which are categorised into 14 clans.

Functional metagenomic screening includes the phenotypic detection (based on enzyme activity "clearance zone"), heterologous complementation approaches, and reporter gene based modulated detection (Simon and Daniel, 2009). This approach does not depend on previous sequence information in publicly available databses to identify enzymes and it therefore offers tremendous potential to discover gene novelty. The possible caveats identified with bioactivity detection using functional metagenomic approaches are; a) gene expression conditions for metabolite production in a particular heterologous host should be suitable to the insert DNA requirements, b) production of functional protein also depends on improper codon usage, promoter recognition, formation of insoluble inclusion bodies and gene product toxicity, c) gene regulation or a functional metabolite production factors such as co-factors, precursors, sigma factors and rare tRNAs also determine inability of the host to induce gene expression (Riesenfeld , 2004a), therefore the rate of positive clones obtained may be very poor (Ekkers et al., 2012). Out of these, the most important challenge is the selection of a suitable host system for gene expression, for example, Martinez (2004) compared the gene expression frequency of antibiotic biosynthesis gene clusters constructed on BAC vectors in three different hosts such as *Streptomyces lividans, E. coli* DH10B, and *Pseudomonas putida* found that expression pattern of the same antibiotic clusters differed among the three host systems. However, *E. coli* has proven to be a suitable host system for functional metagenomic screens, when one who looking for enzymes or compounds derived from Proteobacteria. As suggested by Allen et al. (2009), the choice of an optimal expression host depends greatly on the source of DNA, for e.g., when screening for biosynthesis gene clusters from a soil-derived

metagenomic library, actinomycete rich in natural products may be the suitable expression system, as they are considered to be the most prominent members of the soil microbial community.

6.2 Materials and Methods

6.2.1 Screening libraries with cellulosic substrates (Phenotypic screening)

The metagenomic lambda library (PG-Val-Mg-1) was screened using a functional screening approach, through which clones encoding novel cellulase activities could be discovered. Three different screening approaches were adopted to obtain an efficient metagenome derived cellulase clone from the metagenomic library of size ($2x10^4$ pfu/ml). To detect cellulases by functional screening, three alternative screening methods were followed; a) Phenotypic detection in substrate incorporated agar plates that can be visually scored by clearing zones, b) detection of enzyme activity in crude cell extracts of positive clones selected from substrate screening plates, c) sequence based screens of functional metagenomic library using a conserved domain (glycoside hydrolase) based PCR amplification, d) Final screening using a proteome-based zymography.

Primary screening on solid agar media containing different cellulosic substrates such as carboxymethyl cellulose (CMC) for endoglucanase activity, esculin hydrate and ferric ammonium citrate for β-glucosidase activity and 4-methyl umbelliferyl β-D-cellobioside (4-MUC) for cellobiohydrolase activity (exoglucanase) were used for screening individual cellulase activity .

6.2.1.1 Phenotypic screening of endoglucanase positive clones

Metagenomic libraries were screened for endoglucanase activity on CMC agar plates with quick and sensitive assay system using the dye congo

red for detection. The following protocol was used for screening cellulases. The metagenomic clones stored at 4°C were patched into 0.5% (w/v) CMC (Sigma-Aldrich) supplemented LB agar plates (50 µg/mL kanamycin and 0.5 mM isopropyl thio-β-D-galactoside (IPTG) and incubated for 24 h at 37°C, followed by further incubation at room temperature for 3 days. After incubation, colonies were washed off with distilled water to allow uniform diffusion of the congo red dye into the agar medium. The plates were then flooded with an aqueous solution of congo red (0.2% w/v) for 10 min. Cellulase positive clones were indicated by a yellow halo zone against a red background due to degradation of cellulose. The agar plates were then destained twice with 1M NaCl for 30 min. The hydrolysis zone can be stabilized for at least 2 weeks by additional flooding with 1 M HCl for 15 min, which changes the dye colour to blue and inhibits further enzymatic activity.

6.2.1.2 Phenotypic screening of β-glucosidase positive clones

For screening β-glucosidase activities from the metagenomic library, the clones stored at 4°C were separately patched into LB agar plates (50 µg/mL kanamycin and 0.5 mM isopropyl thio-β-D-galactoside, IPTG) supplemented with esculin hydrate (0.1% w/v) and ferric ammonium citrate (0.25% w/v) as screening substrates for β-glucosidase as described in the method of Eberhart et al. (1964), therefore it is also known as esculinase. The streaked plates were incubated for 24 h at 37°C. Positive clones were detected by the formation of black halos around the colonies due to the oxidation of ferric ammonium citrate to ferrous ammonium citrate.

6.2.1.3 Phenotypic screening of exoglucanase positive clones

Cellobiohydrolase (exoglucanase) activity was screened from the pool of lambda clones by incorporating 100 µl of 4-Methylumbelliferyl-β-D-

cellobioside (5 mg/ml) as a cellulosic substrate into the LB medium supplemented with 50 µg/mL kanamycin and 0.5 mM IPTG. The lambda clones were patched onto LB agar plate and incubated at 37°C for 24 h. The presences of exoglucanases hydrolyse 4-MUC into 4-methyl umbelliferrone (4-MU), which could be detected as UV fluorescence at 365 nm.

6.2.2 Quantitative screening of cellulase positive clones

6.2.2.1 Quantitative screening of endoglucanase positive clones

The efficiency of endoglucanase positive clones to utilize both soluble and insoluble cellulose substrate was quantitatively determined using CMCase assay and total cellulase (FPase) assay. Initially, the crude protein extracts from recombinant *E. coli* bearing phagemids was done by inoculating endoglucanase positive clones into 200 mL LB-kanamycin (50 µg/ml).The cells were grown at 37°C to an OD_{600} of 0.6 and at this OD, 0.5mM IPTG was added to the culture broth to induce the cells. These were further incubated at 37°C for 12 h and cells were harvested at 16,000*g* at 4°C for 10 min. The pelleted cells (5ml) were resuspended in 200 µl of lysis buffer containing 50 mM KH_2PO_4 (pH 7.8), 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5 % triton X-100 and 10 mM imidazole. A sonication cycle of 0.5 for 5 min at 50% amplitude was repeated for five times. The cells were pelletized at 12,000 rpm for 20 min at 4°C. The supernatant containing recombinant proteins were collected and stored at -20°C until the assay was done. Endoglucanase and total cellulase activity were assayed by measuring the amount of reducing sugar released from CMC (Sigma) and Whatman No.1 filter paper respectively, using 3,5-dinitrosalicylic acid method and the assay was carried out with 20 µl of crude protein samples having concentrations of 10 ng/µl. Reducing sugars liberated during cellulose hydrolysis react with DNS (yellow

colour) to form 3-amino-5-nitrosalicylic acid (orange red), with an absorption maximum at 540 nm (Miller, 1959). All the cellulase assays were carried out according to the IUPAC protocol (Ghose, 1987) as described earlier in the section 2.2.6.1 of Chapter 2. The method was scaled down to microtiter volumes for ease in screening.

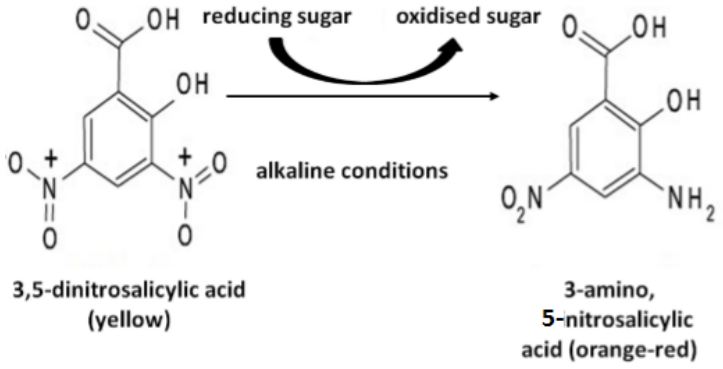


Fig.1. Endoglucanase (CMCase) detection through DNS assay

6.2.2.2 Quantitative screening of MUC-positive clones

4-Methyl umbelliferyl-β-D-Cellobioside (4-MUC) assay is specific for detecting exoglucanases. For the quantitative determination of exoglucanase activity, protein extracts from exoglucanase positive clones were prepared as described in the section 6.2.2.1 and the ability of the protein extracts to hydrolyze the fluorogenic substrate analogue 4-MUC (Sigma) was determined with 50 µM substrate concentrations. Briefly, the assay was performed in amber coloured 96 well micro-titer plates (flat-bottomed, polystyrene plates, black, Corning 96-Well Microplates**,** Sigma-Aldrich). Approximately 20 µl samples having concentrations of 10 ng/µl were mixed with 100 µl of 0.05 M citrate buffer (pH 4.8) containing 4-MUC as substrate. Then the plates were sealed with parafilm and incubated at 37^{o}C for 12 h. The assay was stopped by adding bicarbonate buffer (pH 10.3) in order to measure MUF fluorescence at 465 nm using spectrofluorometer (Shimadzu RF-5301 PC) at an excitation wavelength of 360 nm. After

substracting the background fluorescence contributed by the substrate blank, enzyme blank and uninduced control, activity of each sample was calculated using a standard curve representing 4-Methyl umbelliferone standard solution (0.2-1.8 µM) in potassium phosphate buffer (0.2 M, pH 7.2). Enzyme activity was expressed in U/ml, which corresponded to 1µmol of 4-MU released per minute under standard assay conditions.

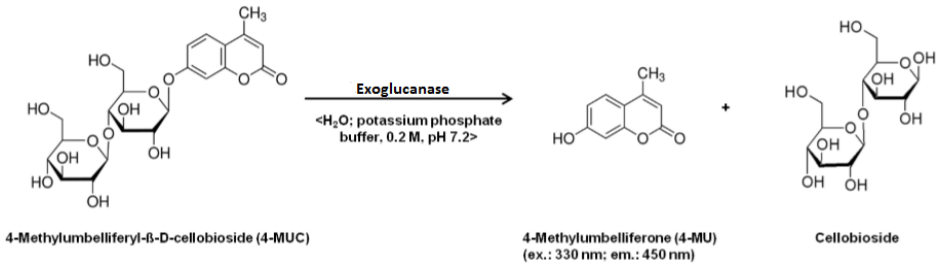


Fig.2. Detection of exoglucanase activity through 4-Methylumbelliferyl-ß-D-cellobioside (4-MUC) assay system (Lehmann et al., 2012)

6.2.2.3 Quantitative screening of β-glucosidase positive clones

Protein extracts from β-glucosidase positive clones were prepared as described in the section 6.2.2.1 and enzyme activity was measured by incubating the total reaction mixture containing 20 µl of crude protein extract diluted in 50 mM citrate buffer (pH 4.8) with 20 µl of 15 mM cellobiose solution (freshly prepared) in citrate buffer (50 mM, pH 4.8). The assay was carried out as described in the section 2.2.6.1 of chapter 2. One unit of cellobiase activity is defined as the amount of enzyme needed to liberate 2 µmoL of glucose/min under standard assay conditions and was expressed in U/ml.

6.2.3 Sequence-based screening of endoglucanase positive clones

6.2.3.1 Phagemid DNA isolation and gene level screening

The phagemid DNA was extracted and purified from all the endoglucanase positive lambda clones using AxyPrep plasmid midiprep kit

(Axygen). An aliquot of 50 ml of overnight LB culture was centrifuged at 3,000*g* for 8 min to pellet the *E. coli* XLOLR cells containing phagemids. The pellet was then resuspended in 4.5 ml resuspension solution containing RNase A and lysed by adding 4.5 ml lysis buffer and mixed gently. An aliquot of 4.5 ml pre-chilled neutralization buffer was then added, mixed gently and incubated at room temperature for 5 min followed by the addition of 4.5ml of pre-chilled DNA binding buffer, mixed gently and centrifuged at 6,000*g* at 4°C for 10 min. The lysate was loaded onto Midiprep syringe filter fixed on vacuum connected Sigma Vacuum Manifold (Sigma-VM20). The plunger was carefully inserted into the syringe filter and pushed slowly with a steady motion to discharge the filtrate into Midiprep plasmid column and switched on the vacuum source till the complete lysate passed through the column. Added 7 ml of first wash buffer and then 8 ml of desalting buffer, and drew the solution through the Midiprep plasmid column by vacuum. Column was removed from the assembly and placed into a 1.5 ml micro-centrifuge tube, added 300 µl desalting buffer and centrifuged at 12,000*g* for 2 min. To elute the plasmid DNA, the column was transferred to another fresh collection tube, added 100 µl eluent (2.5 mM Tris-HCl, pH 8.5) to the centre of the membrane, incubated at room temperature for 1 min, centrifuged at 12,000*g* for 1 min and stored at -20ºC. The plasmids were resuspended in MilliQ or TE buffer. Purity and yield of the plasmid DNA obtained were analysed through 0.8% agarose gel electrophoresis.

Sequence-based screening of endoglucanase positive clones were done using GH9-multi primer set designed from conserved domains present in cellulases of glycoside hydrolase family 9. These conserved domain primers were designed from a metagenomic fosmid library containing cellulase positive clones rather than known sequence based degenerate primers. In this approach, 3 sets of multi-primers (as listed below in Table1) were used to amplify cellulase partial sequence (Xiong et al., 2012)

Table1: List of multi-primer used to amplify cellulase partial sequence (Xiong et al., 2012)

Name	Sequence (5′ → 3′)	Tm (°C)
GHF9-1f	CGGTTGGTATGACGCCGGTGAC	62.9
GHF9-2f	GGCAAGTATGTGGTCAATGGCGG	62.0
GHF9-3f	TCCACGGCTGCCACGTTGAAC	62.6
GHF9-1r	GACCGCCGCCCAATAAAATTCAT	61.7
GHF9-2r	TGCATCTGCATTGGAGCCCCA	63.0
GHF9-3r	AAGCCAGGGGTGCGTTCCAGTT	62.6

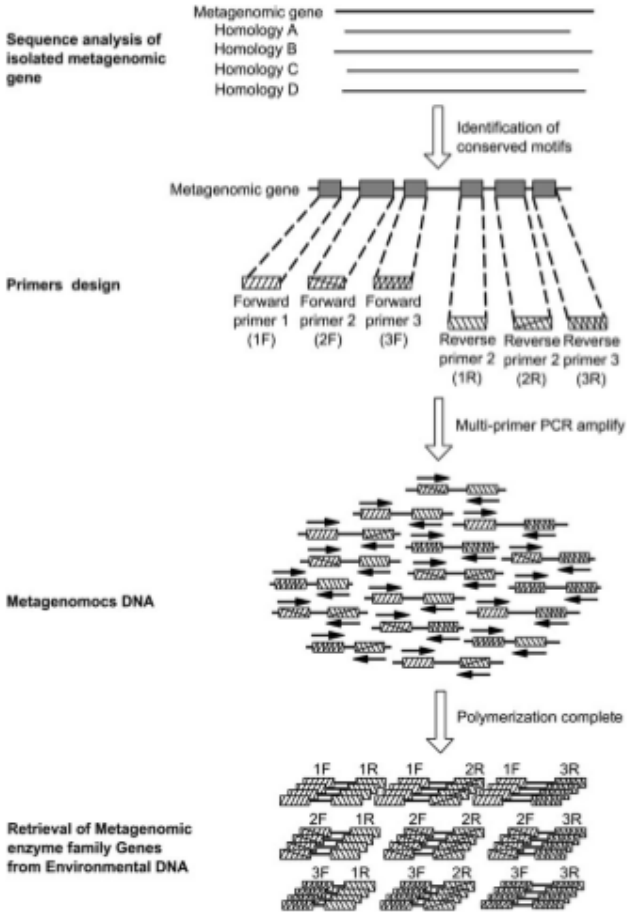


Fig.3. Pictorial representation of designing of multiple glycoside hydrolase family cellulase gene primers from the metagenomic library derived cellulase gene sequence (Figure adapted from Xiong et al., 2012).

Sequence-based screening or PCR screening was carried out only with endoglucanase encoding lambda clones, as the multi-primer set was based only on endoglucanase gene sequence. The reaction mixture contained 2.5 µl 10X buffer, 2.5 µl dNTP (2.5 mM), 1µl Taq polymerase (0.5 U/µl), 20 ng purified lambda DNA template, 6.7 pmol of each primer and the mixture was made up to 25 µl with MilliQ. The reaction cycles were as follows; denaturation at 94°C for 30 s and annealing at 58°C for 30 s followed by an extension step at 72°C for 1 min 20 s. This cycle was repeated for 35 times followed by a 10 min final extension step at 72°C, according to Xiong et al. (2012). PCR products were analyzed by electrophoresis using 1% (w/v) agarose gel.

6.2.3.2 Cloning of PCR amplified product in pGEM®-T Easy vector for sequence analysis

The pGEM®-T Easy vectors (Fig.4) are linearized vectors with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (eg. Taq DNA polymerase).

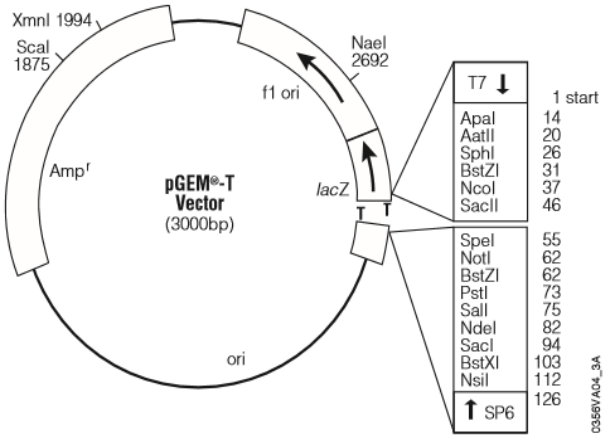


Fig.4. The pGEM®-T Easy Vector map with MCS (www.promega.com)

These vectors are high-copy-number vectors, containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region (MCS) within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide causes the formation of white clones which allows identification of recombinants by blue/white screening on indicator plates.

The A-tailed PCR product of partial cellulase gene from one of the selected positive phagemid clone (Vmg-Eg-3) was ligated into pGEM-T easy vector (Promega, USA) by following the manufacturer's instructions. Briefly, 10 µl ligation mixture containing 0.5 µl pGEM-T vector (50 ng μl^{-1}), 3.5 µl PCR product, 1 µl ligation buffer (10X), 1 µl ligase enzyme and 4 µl MilliQ was incubated at 4°C overnight. This allowed the ligation of PCR products with pGEM-T easy vector and subjected to transformation with *E. coli* DH5α. Briefly, 10µl of the ligation mixture was added to a sterile 15 ml culture tube already placed on ice and 50-100 µl of competent cells were transferred to the ligation mix on ice. The tubes were gently flicked to mix and placed on ice for further 20 min and immediately transferred to 42°C water bath for 90 sec in order to give a heat shock and immediately returned to ice for 2 min, 600 µl of super optimal broth with catabolite repression (SOC- Composition for 10 ml: 0.2 g tryptone; 0.05 g yeast extract; 0.005g NaCl, 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1M glucose) was added to the transformed cells. $MgCl_2$ and glucose were added just before transformation and incubated for 1-1.5 h at 37°C with shaking at 220-230 rpm. An aliquot of 100 µl of each transformation culture was plated onto duplicate/triplicate LB/ampicillin (100µg ml^{-1}) /X-gal (80 µg ml^{-1}) /IPTG (100 mM) and incubated the plates overnight (12-16 h) at 37°C.

Transformed clones were selected and patched on LB/ampicillin (100 µg/ml)/X-gal(80µgml^{-1})/IPTG(100mM) plates to reconfirm the transformation.

All individually streaked colonies were subjected to colony PCR using vector primers designed from either side of the multiple cloning site of the vector. Accordingly, PCR mixture (25 µl) contained 2.5 µl 10X buffer, 2.5 µl dNTP (2.5 mM), 1µl Taq polymerase (0.5 U μl^{-1}), pinch of colony, 1 µl of T7 forward and SP6 reverse primers each (10 pmol μl^{-1}) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete gene was 95°C for 5 min followed by holding at 80°C for Taq polymerase addition, cycles of denaturation at 94°C for 15 sec, annealing at 58°C for 45 sec, extension at 72 °C for 1 min, followed by final extension at 72°C for 10 min was repeated for 35 times. An aliquot of 10µl PCR product was analyzed by 1% agarose gel electrophoresis, stained using ethidium bromide (EtBr), visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

Recombinant plasmids in *E. coli* DH5α was grown in LB-ampicillin media (100 µg/µl) and incubated at 37°C with shaking at 220 rpm (12 hrs).The plasmid containing insert DNA was extracted using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences, USA) following manufacturer's specifications. Briefly, an aliquot of 2 ml culture after overnight incubation at 37°C was pelletised at 12,000*g* for 1 min. The pellet was resuspended in 200µl resuspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350 µl neutralization solution was added and centrifuged at 12,000*g* for 10 min to remove the cell debris. The cleared lysate was loaded into GenElute HP Miniprep binding column inserted into a 1.5 ml microcentrifuge tube and centrifuged at 12,000 *g* for 1 min. Plasmid DNA bound to the column was washed twice with a wash solution to remove the endotoxins, salt and other contaminants. The column was transferred to a fresh collection tube to elute the plasmid DNA, 100 µl of 10 mM Tris-Cl (pH 8.0.)

was added and centrifuged at 12,000*g* for 1 min. The plasmid DNA was stored at -20ºC. The cloned plasmid was sequenced at SciGenome Labs Pvt. Ltd, Cochin, India.

The obtained sequence was submitted for homology search using NCBI BLASTn algorithm using its sequence match and the corresponding amino acid sequence was deduced using ExPASy Proteomics server (http://us.expasy.org/tools/pi_tool.html) and searched against BLASTp suite of NCBI.

6.2.4 Proteomic Screening of the positive clone using zymography

The phagemid clone showing high endoglucanase activity was selected for a final proteomic screening (zymogram analysis). Recombinant protein cell extract was prepared as described in the section 6.2.2.1. An aliquot of 30 µl of lysed cell preparation was heated at 60^{o}C in 10 µl sample buffer (62.5 mM Tris-Cl; pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.2% bromophenol blue) for 5 min. The sample was given a short spin and supernatant was subjected to 12.5% reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis following the method of Laemmli (1970). The protein was separated and analyzed using 5% stacking gel and 12% resolving gel prepared into 10×10.5 cm vertical gel plate of mini vertical electrophoresis unit (Hoefer-Amersham, USA). Electrophoresis was performed in 1XTris- glycine SDS (pH8.3) buffer at a voltage of 12 mA (EPS 301, Amersham, USA). After electrophoretic separation, gel was stained in coomassie brilliant blue stain R-250 (0.025 % coomassie brilliant blue R-250, 40% methanol and 7% acetic acid in distilled water), de-stained in de-staining solution 1(40% methanol and 7% acetic acid in distilled water) for 20 min and further in de-staining solution II (5% methanol and 7% acetic acid in distilled

water) for 10 min and photographed using Gel-DOC^{TM} XR+ imaging system (BioRad, USA).

After SDS-PAGE electrophoresis was completed, SDS was removed by washing the gel four times for 30 min in 50 mM disodium hydrogen phosphate and 12.5 mM citric acid, pH 6.3 and 1% Triton X-100. The first two washes contained 25% isopropanol. CMC agar replica (0.5% w/v) was prepared in 50 mM disodium hydrogen phosphate, and 12.5 mM citric acid, pH 6.3.The agar was then allowed to solidify at 4°C. The polyacrylamide gel was washed, partially dried and was laid on top of the agar sheet for the transfer of renatured or refolded cellulase into the CMC-supplemented agarose gel and subsequent hydrolysis of CMC was allowed to proceed for overnight at 40°C. The sandwich was then left to cool for at least 15 min at 4°C. The bottom glass plate with the agar replica was dipped into 0.1% congo red .Then it was poured off and the gel was washed with 1 M sodium chloride until excess stain was totally removed from the active bands. The clearance zone could be visualized as yellow zone against a red background. After a final rinse in 5% acetic acid, the background turned dark blue and the positive hydrolysis zone could be seen as a light halo against a dark blue background.

6.2.5 Confirmation of the positive clone by retransformation

The potential cellulase activity of the clone, Vmg-Eg-3 was reconfirmed by transforming the phagemid DNA into another expression host, *E. coli* BL21. After transformation as described in the earlier section 6.2.2.2, the cells were spread on LB agar (50 µg/ml kanamycin and 100 µg/ml ampicillin) supplemented with 0.5% (w/v) CMC. After incubation for 3 h at 37^0C, the plates were flooded with an aqueous solution of congo red (0.1 % w/v) for 10 min. Cellulase positive clones were indicated by a yellow halo

zone against a red background due to degradation of cellulose. The corresponding clones showed positive activity with both hosts *E. coli* strains were confirmed as positive clones and stored for further work.

6.2.6 Insert size characterization of the positive clone using long PCR approach

The size of the insert contained in the positive endoglucanase clone, Vmg-Eg-3 was confirmed by a long PCR screening approach using Phusion™ High- Fidelity PCR kit (Finnzymes, Finland). PCR was performed in 50 µl using the following mixture: 20 ng purified lambda clone DNA, 6.7pmol of pBK-CMV phagemid (Vector) specific T7 forward (TAATACGACTCAC TATAGGG) and T3 reverse (ATTAACCCTCACTAAAGGGA) primers and 25 µl of *Pfu* master mix. The reaction cycles were performed as follows: denaturation at 98ºC for 30 s, second denaturation at 98°C for 10 s and annealing at 58°C for 30 s followed by an extension step at 72°C for 3 min. This sequence was repeated 35 times followed by a 10 min final extension step at 72°C. PCR products were analyzed by electrophoresis using a 0.8% (w/v) agarose gel.

6.2.7 Designing primers with restriction sites

Based on the partial sequence obtained from the sequence-based method, a total of six different oligonucleotide primer sets were designed, with reference to the similar published sequences in the data repository of CAZy and NCBI. The primers designed using PrimerIdent software (http://primerident.up.pt, University of Porto, Portugal), have the sequence as given in Table 3. Primers were designed with restriction sites such as *Eco*R I sequence "GAATTC" was added to the 5′ end and *Xho* I sequence "CTCGAG" was added to the 3′end of both the primers.

T**able 3:** Endoglucanase primers designed with restriction sites

Primer	Primer sequence(5'-3')	Tm	Product Size
Endo-1	F-AAT**GAATTC**AATTTGAAGGTTGAATTCTACAAC R-GG**ACTCGA**GGGTTCTTTACCCCATACAAGAAC	60	1340
Endo-2	F-AAT**GAATTC**CACGGCCTGATAGAAGACC R-ACT**CTCGA**TACTGCCCGCACTCAGTCCC	60	1500
Endo-3	F-CATAT**GAATTC**TCCGCATTGCTTG- R-GGAT**CTCGA**CTATTTCTTATTTTCACG	58	1460
Endo-4	F-GGGAA**GAATTC**GGCCCCGGTCATCTGCGAGAA R-CGAAA**CTCGA**GCTCGCCGTGCAGGTGAAGC	61	1380
Endo-5	F-AACT**GAATTC**ATGATGCGAAGGAGGAAAAG R-ACGT**CTCGA**CTAATTTGGTTCTGTTCCCC	60	1400
Endo-6	F-CCA**GAATTC** AAGAATGGCCAGC R-GGATA**CTCGA**CGCCGCTTTGTGC	58	1228

6.2.8 PCR amplification of full-length putative endoglucanase gene

The full-length endoglucanase gene was amplified from the Vmg-Eg-3 lamda DNA template using the primers with restriction sites as listed in Table 3. Briefly, PCR was conducted in a 25 µl reaction volume containing 2.5 µl of 10X *Pfu* buffer with 20 mM $MgSO_4$, 2.5 mM dNTP (2.5 µl), 1µl of 10 pmol/µl of forward and reverse primer and 0.3µl of 2.5 U/µl Taq DNA polymerase and 1µl lambda DNA template. The following gradient PCR programme was used, with initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, gradient temperatures from 50-65°C for 45 sec, extension at 72°C for 2 min with a final extension at 72°C for 10min. An aliquot of 10µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained in ethidium bromide (EtBr) , visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

6.2.9 TA vector construction of putative endoglucanase gene in pGEM-T Easy vector for sequence analysis

The A-tailed PCR product from a successful putative endoglucanase gene amplification reaction was selected and proceeded for ligation with pGEM-T easy vector (Promega, USA). Briefly, 10 µl ligation mixture containing 0.5 µl pGEM-T easy vector (50 ng/µl), 3.5 µl PCR product, 1 µl of 10X ligation buffer, 1 µl ligase (3Weiss units/µl) and 4 µl MilliQ were incubated at 4°C overnight. This allowed the ligation of PCR products with pGEM-T easy vector.

6.2.9.1 Transformation into *E. coli* DH5α

The *E. coli* DH5α competent cells were thawed by placing on ice for 5-10 min. Approximately 10µl of the ligation mixture was added to a sterile 15 ml culture tube already placed on ice and 50-100 µl of competent cells were transferred to the ligation mix on ice. The tubes were gently flicked to mix and placed on ice for further 20 min and immediately transferred to 42°C water bath for 90 sec in order to give a heat shock and immediately returned to ice for 2 min, 600 µl of super optimal broth with catabolite repression (SOC-Composition for 10 ml: 0.2 g tryptone; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2M $MgCl_2$; 200 µl 1M glucose) was added to the transformed cells. $MgCl_2$ and glucose were added just before transformation and incubated for 1-1.5 hrs at 37°C with shaking at 220-230 rpm. An aliquot of 100 µl of each transformation culture was plated onto duplicate/triplicate LB/ampicillin (100 µg ml^{-1})/X-gal (80 µg ml^{-1}) /IPTG (100 mM) and incubated the plates overnight (12-16 hrs) at 37°C.

6.2.9.2 PCR confirmation of inserted gene in the selected clones

Transformed clones were selected and patched on LB/ampicillin (100 μgml^{-1})/X-gal (80 μgml^{-1})/IPTG (100 mM) plates to reconfirm the transformation. All individually streaked colonies were subjected to colony PCR using vector primers designed from either side of the multiple cloning site of the vector. Accordingly, PCR mixture (25 µl) contained 2.5 µl 10X buffer, 2.5µl dNTP (2.5 mM), 1µl Taq DNA polymerase (0.5 U/µl), pinch of colony, 1 µl of T7 forward and SP6 reverse primers each (10 pmol/µl) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete gene was 95°C for 5 min followed by holding at 80°C for Taq polymerase addition. The cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min, followed by final extension at 72°C for 10 min was repeated for 35 times. An aliquot of 10 µl PCR product was analyzed by 1% agarose gel electrophoresis, stained using ethidium bromide (EtBr), visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

6.2.9.3 Propagation of pGEM-T easy vector construct and plasmid extraction

Recombinant plasmids in *E. coli* DH5α was grown in LB-ampicillin media (100 µg/µl) and incubated at 37°C with shaking at 220 rpm (12 hrs). Plasmid was extracted using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences, USA) following manufacturer's instructions. Briefly, an aliquot of 2 ml culture after overnight incubation was pelletized at 12,000*g* for 1 min. The pellet was re-suspended in 200 µl re-suspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350µl neutralization solution was added and centrifuged at 12,000*g* for 10 min to remove the cell

debris. The lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centrifuged at 12,000*g* for 1 min. Plasmid DNA bound to the column was washed twice with a wash solution to remove the endotoxins, salt and other contaminants. The column was transferred to a fresh collection tube to elute the plasmid DNA, 100µl of 10 mM Tris-Cl was added and centrifuged at 12,000 *g* for 1 min. The plasmid DNA was stored at -20ºC. Purity of the plasmid DNA obtained was analyzed by agarose gel electrophoresis and by determining the ratio of the absorbance at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® fluorometer (invitrogen™, USA).

6.2.10 Sequencing of putative endoglucanase clone

The positive plasmid was sequenced at SciGenome Labs Pvt. Ltd, Cochin, India. The sequence was subjected to BLAST search (Nucleotide) in NCBI database for confirmation of the gene sequenced.

6.2.11 Sequence analysis and homology modeling

The nucleotide sequence obtained from the positive clone was assembled using the Gene Tool software. The obtained sequence was submitted for homology search using NCBI BLASTn algorithm (http://www. ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). The ORF translation and calculation of the theoretical MW of the deduced protein was performed using ExPASy Proteomics server (http://us.expasy.org/ tools/pi_tool.html).The protein domain search was done using CDD databases (Marchler-Bauer et al. 2007). Multiple alignment of deduced protein sequence was performed with Clustal X, version 2.0.3 using its default values (Thompson et al., 1997). SignalP 4.0 software (Bendtsen et al., 2004) was used for the prediction of classical N-terminal secretion of signal peptides.The subcellular localization of

the identified protein was predicted using the PSORTb v4.0 program. Protein transmembrane helices were predicted using TMHMM 2.0. All of this software is publicly available from the Centre for Biological Sequence Analysis at the Technical University of Denmark.

Secondary structure of the protein was predicted using PSIPRED VIEW (http:// bioinf2.cs.ucl.ac.uk). Comparative modeling was performed by mapping of the translated ORF with unknown structure against known homologous proteins. The protein sequence was submitted to SWISS-MODEL (http://www.expasy.org/swissmod/) to obtain the 3D structure of the recombinant protein. The model was viewed using Swiss-PDB Viewer and the 3D structure of the protein was further modified by PyMOL (version 1.4.1, http://www.pymol.org/).

6.3 Results and Discussion

6.3.1 Functional Screening of Metagenomic lambda library for cellulase-encoding clones (Phenotypic Screening)

In this study, a functional metagenomic approach was employed for the discovery of novel cellulolytic genes from a mangrove metagenome. Mangrove ecosystems represent a unique microbiome for the exploration of biomass degrading enzymes due to the environmental conditions such as salinity, oxygen contents and nutrients prevailed in this ecosystem (Gao et al., 2010). These habitats possess both terrestrial and marine environmental conditions, which in turn helps in the identification of novel cellulases capable for cellulose degradation under special conditions of hyper-saline, anaerobic or organic-rich circumstances (Hyde and Lee, 1995; Jiang et al., 2006).

Functional screening for novel genes in metagenomic libraries explore the genetic potential of a given habitat by detecting products or enzymatic

activity of the metagenomic clones generated. The important things to be considered prior to the selection of positive clones is that all the recombinant clones should be cultivated under same growth conditions after which phenotypic differences could be visually scored as "halo zone" on substrate specific plates or by enzyme activity assaying techniques. Another important factor is the "plating density" of the clones, which depend upon the nature of the activity to be screened. It is desirable to plate at a density of 100 to 500 CFU/plate, such that enzyme activity in the form of coloured halo zone can be observed clearly. In this study, we used a plating density of 574 CFU/plate for visualizing cellulase hydrolysis zone and it was found to be sufficient for phenotypic detection of cellulase clones after 4-5 days (maximum) of growth on cellulose-specific substrates.

The metagenomic lambda library (PG-Val-Mg-1) constructed from valanthacaud mangrove sediment DNA contained approximately 10,000 clones with an average insert size of 8Kb (6-10 Kb), represented about 80 Mb of the environmental genome. In order to screen for cellulase-encoding clones, a total of 7000 clones were functionally screened from an amplified lambda library ($4x10^7$ pfu/ml). Cellulase activities such as endoglucanase, exoglucanase and β-glucosidase were screened on different cellulosic substrates specific for each enzyme. The principle behind phenotypic screening is that the cellulosic substrates can be readily internalized by the host *E. coli* and subsequently hydrolysed by intracellularly located cellulases. A more layered approach of functional screening method includes selection of clones using the common substrate followed by using a more specific substrate for confirmation of activity. Here we adopted a preliminary phenotypic screening using different cellulosic substrates followed by a more specific quantitative enzyme screening

and further confirmation of the positive clones using a gene level and protein level approach seems desirable.

6.3.1.1 Phenotypic screening of endoglucanase positive clones

Congo red staining coupled with CMC provides a rapid, sensitive and efficient screening of cellulolytic microorganisms as the positive clones are surrounded by a yellow halo zone against a red background. Congo red strongly binds with β-1,4-glycosidic linkages in polysaccharides and resulted in degradation zone only if the polymeric substrate is hydrolyzed into oligomers with less than 5 sugar units (Neil et al., 1984). The method is also readily adaptable for high throughput screening of cellulase clones, more specifically endoglucanases. Sixteen numbers of positive endoglucanase clones were screened from a pool of 7000 clones after congo red staining on LB-CMC agar plates and were named as Vmg-Eg-1 to Vmg-Eg-16, where 'Vmg' denotes for Valanthacaud mangrove from where the sample was collected for metagenomic library construction 'Eg' for endoglucanase and the number represents the clone number. The hydrolysis zone produced by positive lambda clones could be visualized in the Fig.5.

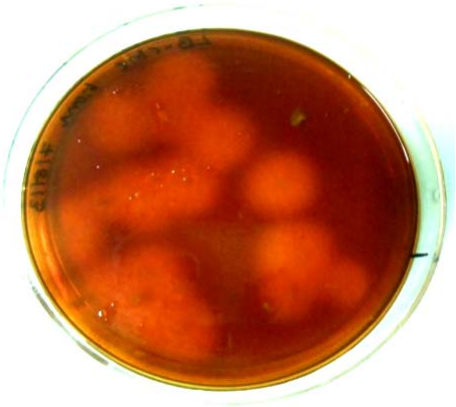


Fig.5. Effect of congo red on cellulolytic zone in CMC agar plate has shown as yellow clearance zone

6.3.1.2 Phenotypic screening of β-glucosidase positive clones

For functional screening of clones with β-glucosidase activity, LB agar was supplemented with esculin hydrate (0.1%) and ferric ammonium citrate (0.25%). Positive clones were then detected by the formation of a black halo around the clones after incubation at 37°C for 20-24 hrs. The esculin present in the medium was hydrolysed into esculetin by the action of β-glucosidase, which further oxidise ferric ammonium citrate to ferrous ammonium citrate resulting in a black halo around β-glucosidase positive clones. As shown in Fig.6, seven β-glucosidase positive clones were detected and they were renamed as Vmg-Bg-1 to Vmg-Bg-7, where 'Vmg' denotes for Valanthacaud mangrove from where the sample was collected for library construction, 'Bg' for β-glucosidase and the number represents the clone number. Efficiency of clones was primarily measured by comparing the size of each black halo.

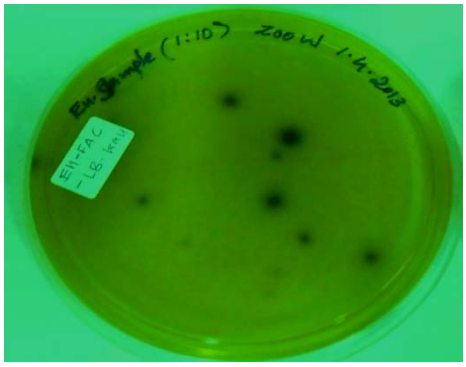


Fig.6. Betaglucosidase Positive clones showing cellulolytic activity on esculinhydrate-ferric ammonium citrate agar plate

6.3.1.3 Phenotypic screening of exoglucanase positive clones

Cellobiohydrolase (exoglucanase) activity was screened from the pool of lambda clones by incorporating 4-Methylumbelliferyl-β-D-cellobioside (4-MUC) as a cellulosic substrate into the LB medium. The compound had been

hydrolysed into 4-methylumbelliferone (4-MU) which is a fluorescent compound and could be detected by UV fluorescence. Only 3 numbers of clones could produce the fluorescence and they were considered as positive exoglucanase-encoding clones (Fig.7).The clones were subsequently renamed as Vmg-Ex-1, Vmg-Ex-2 and Vmg-Ex-3, where 'Vmg' denotes for Valanthacaud mangrove from where the sample was collected for library construction, 'Ex' indicates exoglucanase and the number represents the clone number.

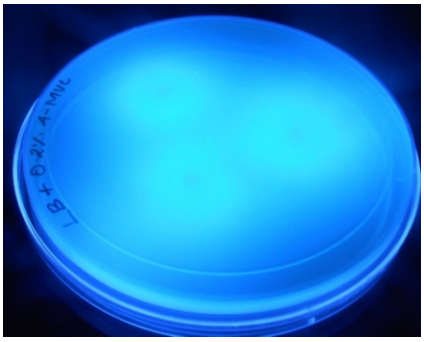


Fig.7. Exoglucanase positive clones showing fluorescence on 4-methyl umbelliferyl-β-D-cellobioside plate

The positive hit rate of the cellulase-encoding clones from the library PG-Val-Mg-1 was approximately 1/333, which was significantly higher than the positive selection rates of cellulase-positive clones from other previously reported libraries, i.e., approximately 1/25,000 (Pang et al., 2009) and 1/70,000 respectively (Kim et al., 2008). The high content of cellulosic materials from the decaying vegetation might have served as a natural enrichment condition for cellulolytic genes and that could be one of the reasons for the higher positive hit rate of cellulase-encoding clones in the present study. Almost all of the bacterial genome in a mangrove ecosystem possesses cellulase genes. The higher abundance of different glycoside hydrolase families (GH) previously identified from the mangrove ecosystems also indicates its capability to degrade a wide array of cellulosic substrates. Therefore small insert metagenomic lambda library constructed in the present

study (represented about 80 Mb of the environmental genome) was sufficient for screening cellulase activity from a cellulase-rich mangrove environment. For studying less abundant enzyme activity, it is recommended to construct metagenomic libraries of greater than 1 Gb strength. For example, Allen et al. (2009) have identified one β-lactamase per Gb of metagenomic DNA from a 12Gb soil metagenomic library since the activity was considered to be less abundant in the natural soil environment.

6.3.2 Quantitative screening of positive clones

6.3.2.1 Quantitative screening of endoglucanase positive clones

Both CMCase and FPase assays were carried out using the crude protein lysate prepared from the positive endoglucanase-encoding clones (Vmg-Eg-1 to Vmg-Eg-16) to determine its ability to degrade both soluble and insoluble cellulose respectively and to select a suitable clone for further subcloning and over-expression. Reducing sugar was estimated using DNS assay.

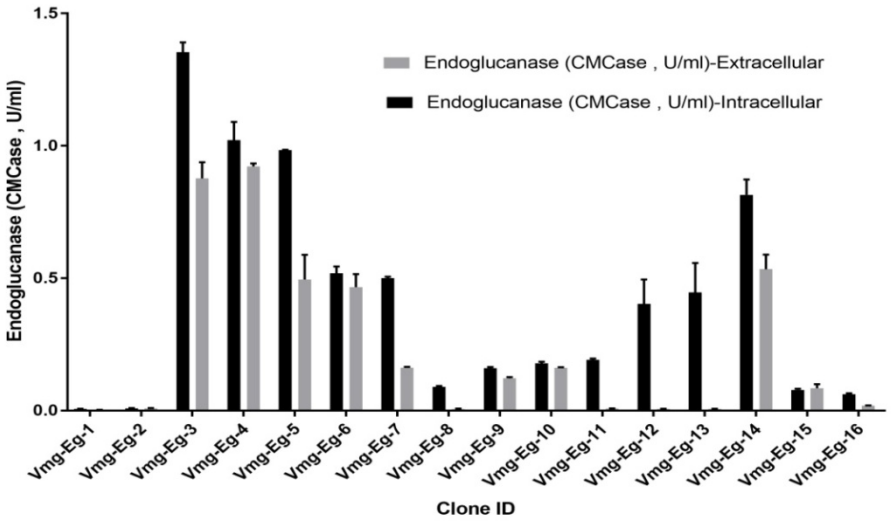


Fig.8. Endoglucanase activity (CMCase, U/ml) from extracellular (culture supernatant) and intracellular (crude lysate) fractions of endoglucanase positive clones (Vmg-Eg-1 to Vmg-Eg-16). Values are represented as mean of the three replicate measurements.

The results in Fig.8, illustrated that the clone Vmg-Eg-3 produced highest CMCase (1.354 U/ml) followed by Vmg-Eg-4 (1.02 U/ml), Vmg-Eg-5 (0.984 U/ml) and Vmg-Eg-14 (0.81 U/ml).The extracellular activities produced by these clones were higher than their intracellular activity whereas the clones such as Vmg-Eg-12 (0.402 U/ml) and Vmg-Eg-13 (0.44 U/ml) showed endoglucanase activity in intracellular protein fractions only. The clones, Vmg-Eg-10 and Vmg-Eg-15 showed comparatively equal extracellular and intracellular endoglucanases. None of the clones produced higher extracellular CMCase when compared to its intracellular CMCase activity. Most of the clones produced endoglucanase activity in both protein fractions, which further suggested that the *E. coli* host machinery of the clones was found suitable to secrete functional cellulases.

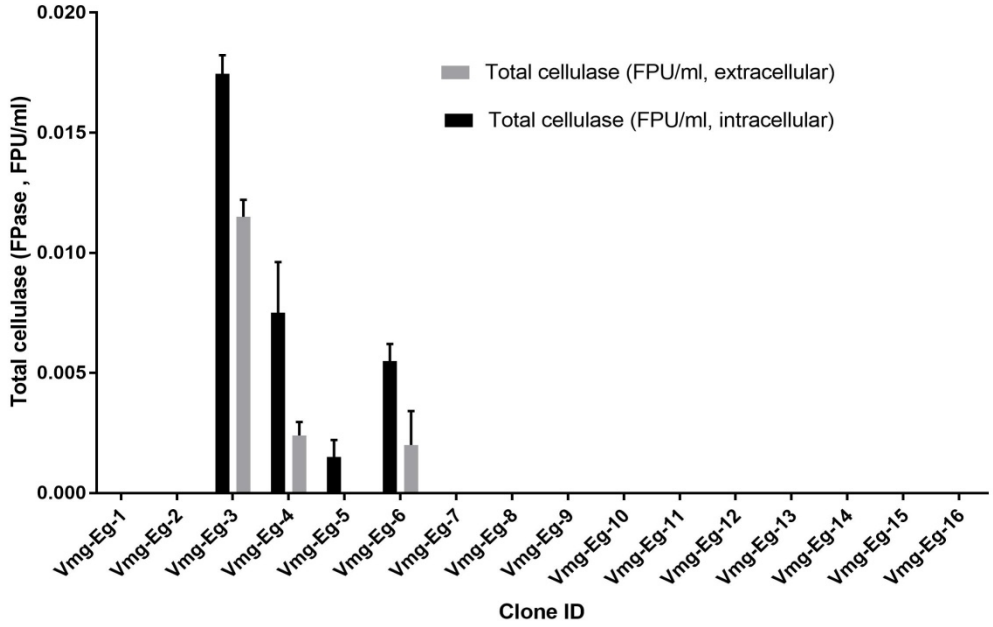


Fig.9. Total cellulase activity (FPase, FPU/ml) from extracellular (culture supernatant) and intracellular (crude lysate) fractions of endoglucanase positive clones (Vmg-Eg-1 to Vmg-Eg-16). Values are represented as mean of the three replicate measurements

The results depicted in the Fig.9, showed that highest FPase activity (0.0174 FPU/ml) was produced by the clone Vmg-Eg-3 followed by Vmg-Eg-4 (0.0075 FPU/ml), Vmg-Eg-5 (0.0015 FPU/ml) and Vmg-Eg-6 (0.005 U/ml). All the clones produced very negligible FPase activity compared to its CMCase activity. Similar to CMCase activity, FPase activity was also higher in the intracellular fractions than extracellular culture supernatant. The clone Vmg-Eg-5 showed intracellular FPase activity only. The clones, Vmg-Eg-1, Vmg-Eg-2 and Vmg-Eg-7 to 16, could not produce any intracellular and extracellular FPase compared to its endoglucanase activity and the corresponding clones were considered to be incapable for crystalline cellulose degradation. The results obtained from the quantitative enzymatic screening provided a fruitful data for the selection of a potent clone from the pool of positive endoglucanase encoding clones. Among all the positive clones tested, the clone Vmg-Eg-3 produced highest CMCase as well as FPase activity, suggested that the clone was capable of both amorphous and crystalline cellulose degradation, which could be further processed for over-expression of cellulase gene and mass production for industrial applications.

6.3.2.2 Quantitative Screening of exoglucanase positive clones

A fluorescence-based quantitative assay was used for screening exoglucanase encoding clones (Vmg-Ex-1, Vmg-Ex-2 and Vmg-Ex-3), which offered a highly sensitive assay system similar to high throughput screening platforms. The basic principle behind 4-MUC detection system for cellulase quantification was first explained by Chernoglazov et al. (1988). Exoglucanases hydrolyse 4-MUC into water soluble fluorophore 4-Methylumbelliferone (4-MU) and the corresponding activity can be easily recorded in a continuous mode using 96-well microtiter plates (Fig.10). The intensity of methyl umbelliferone fluorescence is pH-dependent with its

maximum at a pH value of 10 (Mead et al., 1955). Three clones such as Vmg-Ex-1, Vmg-Ex-2 and Vmg-Ex-3 were subjected for fluorescence assay and its activity was measured at 465 nm using a spectrofluorometer at an excitation wavelength of 360 nm. After substracting the background fluorescence contributed by the substrate blank, enzyme blank and uninduced control, activity of each sample was calculated using a standard curve representing 1 to 100 pmol of 4-methyl umbelliferone. The enzyme activity was expressed in U/ml i.e., enzyme concentration to release 1µmol of 4-MU per minute under standard assay conditions.

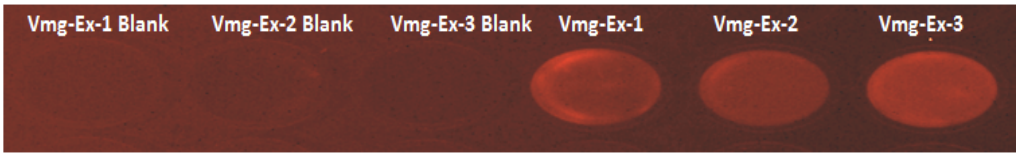


Fig.10. Exoglucanase activity detection using 4-MU detection system in a 96-well microtiter plate

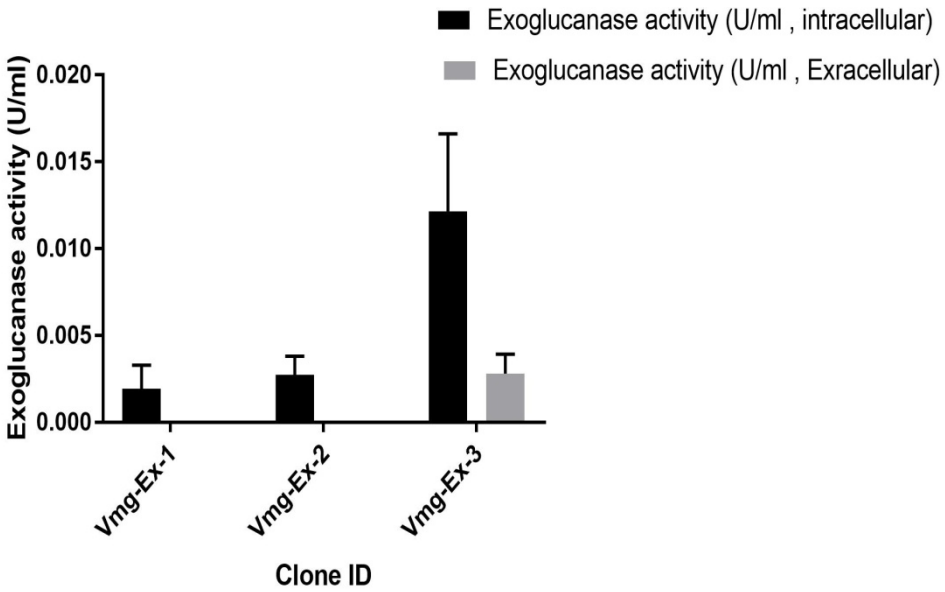


Fig.11. Exoglucanase activity (U/ml) from extracellular (culture supernatant) and intracellular (crude lysate) fractions of exoglucanase positive clones (Vmg-Ex-1 to Vmg-Ex-3). Values are represented as mean of the three replicate measurements

The results from the Fig.11 showed that only one of the clones, Vmg-Ex-3 (0.0121 U/ml intracellular and 0.0028 U/ml extracellular) could effectively contribute to the quantitative exoglucanase production. The clones, Vmg-Ex-1 (0.0019 U/ml) and Vmg-Ex-2 (0.0027 U/ml) could display only an intracellular expression of exoglucanases, though the plate screening of all the three clones produced highly amplified fluorescence but only after 24 h of incubation with the substrate.

The results obtained in this study are highly significant because only a few numbers of exoglucanases were identified from metagenomic libraries constructed so far. Some of the previously characterized novel exoglucanase encoding clones belonged to the GH6 (bacteria and fungi), GH 7 (fungi), and GH 48 (bacteria) catalytic domains were only capable of producing an MUC fluorescence in plate screening (Edwards et al., 2008 ; Berger et al., 2007), but further sequencing of these positive clones showed the presence of either endoglucanases (Healy et al., 1995; Liu et al., 2009) or cellodextrinases (Duan et al., 2009).This becomes a major drawback in over-expression and purification of exoglucanases. Moreover, exoglucanases from fungal origin could not be successfully over-expressed in *E. coli* host system due to the lack of promoters and other intron sequences. Considering these facts, it was decided to proceed with the endoglucanase clone, Vmg-Eg-3 since it showed both soluble and insoluble cellulose degradation.

6.3.2.3 Quantitative screening of β-glucosidase positive clones

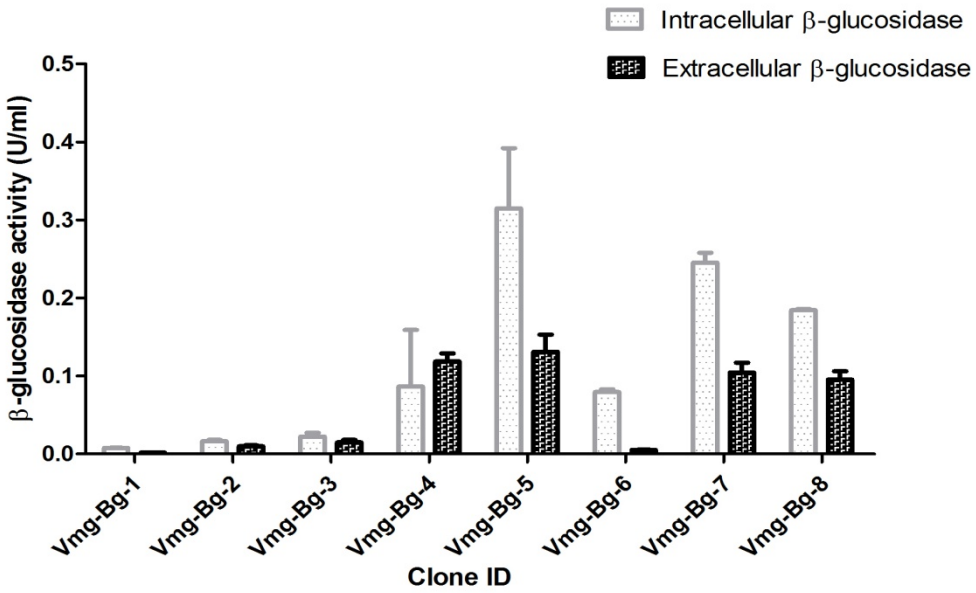


Fig.12. Determination of β-glucosidase activity (U/ml) from extracellular (culture supernatant) and intracellular (crude lysate) fractions of β-glucosidase positive clones (Vmg-Bg-1 to Vmg-Bg-8).Values are represented as mean of the three replicate measurements

The results depicted in the Fig.12, showed that highest β-glucosidase activity (0.315 U/ml) was produced by the clone Vmg-Bg-5 followed by Vmg-Bg-7 (0.2455 U/ml), Vmg-Bg-8 (0.1845 U/ml), Vmg-Bg-4 (0.0865 U/ml) and Vmg-Bg-6 (0.0795 U/ml). All the clones produced a higher intracellular activity compared to its extracellular β-glucosidase except the clone Vmg-Bg-4. The clone Vmg-Bg-6, produced only intracellular β-glucosidase activity. The clones Vmg-Bg-1, Vmg-Bg-2 and Vmg-Bg-3 had produced very negligible β-glucosidase activities, but the degradation zone produced by these positive clones could not linearly be correlated with their quantitative enzyme production.

6.3.3 Sequence-based screening of endoglucanase positive clones

Sequence-based screening methods are not much highly efficient and reliable as functional screening methods to retrieve novel enzyme gene families. However the need to develop PCR-based screening strategy for obtaining novel genes encoding in a functional library is urgent as the sequencing of a complete insert contained in a large metagenomic library is cumbersome, time-consuming and expensive. This method is highly beneficial for one who looking for a particular gene of interest from a large metagenome. In addition the partial genes obtained through amplification using conserved-domain primers could be used for genome walking or DNA shuffling to retrieve full-length functional genes (Boubakri et al., 2006; Kotik, 2009; Wang et al., 2010).Conventional degenerate primer based amplification are also difficult in obtaining novel genes as the primer degeneracy increases to capture distantly related sequences resulting in low amplification or non-specific amplification of target genes (Rose et al. 2003). Designing degenerate primers for unknown genes is difficult and often this trial and error method of PCR, intensified the non-specific amplification due to the low copy number of target genes represented in the metagenomic pool of diverse genomes (Contreras-Moreira et al., 2009).

The multiple glycoside hydrolase family specific primers designed by Xiong et al. (2012) from a fosmid library-derived cellulase gene sequence was found as a reliable and pragmatic method for PCR-based retrieval of unknown cellulase genes from functionally potent cellulase clones generated in the present study. Similarly, a total of 127 glycoside hydrolase family 9 cellulase genes had been retrieved from soil-derived metagenomic library and also from environmental soil samples using the same multi-primer set (Xiong et al., 2012).

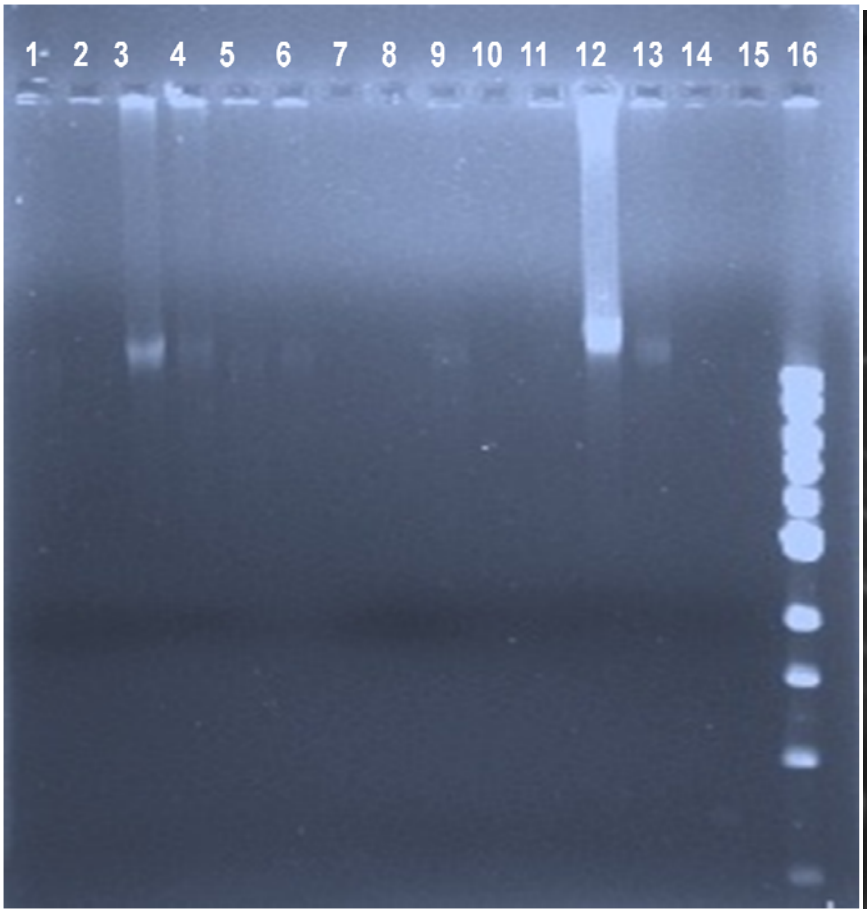
The different combination of all the conserved domain primer sets such as GHF9-1f/1r, GHF9-1f/2r, GHF9-1f/3r, GHF9-2f/1r, GHF9-2f/2r, GHF9-2f/3r, GHF9-3f/1r, GHF9-3f/2r and GHF9-3f/3r were tried for PCR screening of endoglucanase positive clones (Vmg-Eg-1 to Vmg-Eg-16). Of all the primer combinations used, only 3 combinations such as GHF9-1f/1r, 1f/2r and 2f/2r had yielded a specific amplification of 800bp from the endoglucanase clone derived DNA. The clones such as Vmg-Eg-3 and Vmg-Eg-4 yielded specific amplification (800 bp) for all the three combinations of PCR (GHF9-1f/1r, 1f/2r and 2f/2r) as shown in Fig.15, Fig.16 and Fig.17 respectively, whereas Vmg-Eg-6 gave amplification only with the primer set 1f/2r. In addition Vmg-Eg-7 (1f/1r), Vmg-Eg-8 (1f/1r and 2f/2r), Vmg-Eg-9 (1f/1r), Vmg-Eg-11and Vmg-Eg-12 (1f/1r,2f/2r), Vmg-Eg-13 (1f/2r and 2f/2r),Vmg-Eg-15 and Vmg-Eg-16 (1f/1r) successfully amplified a partial cellulase gene of 800 bp and was represented in Fig.15 (amplification using 1f/1r) , Fig.16 (1f/2r) and Fig.17 (2f/2r). In order to identify the partial cellulase gene amplification, only one endoglucanase clone i.e., Vmg-Eg-3 was selected for sequencing due to its positive amplification in all the three primer combinations (1f/1r, 1f/2r and 2f/2r) as well as its highest enzyme activity when compared to other endoglucanase-positive clones. The cellulase subclone of Vmg-Eg-3 in the pGEMT-easy vector was submitted for sequencing (Fig.18 and 19).

The obtained sequence was trimmed, aligned using Gene tool software and homology was searched using BLASTn algorithm of NCBI database. The FASTA format of sequence was translated to amino acid output using the Translate tool of ExPASy (SIB Bioinformatics Resource Portal) and shown in Fig.20. The translated protein sequence was analysed in the NCBI BLASTp

suite (Fig.21). The nucleotide sequences of the partially amplified PCR product (800 bp) showed 100% homology at nucleotide level with Glycoside hydrolase family 9 cellulase from uncultured organism AEH57901.1 (99%), AF143956.1 (98%), AEH57899.1 (89%), AEH57902.1 (88%), AFI43974.1 (90%), AEH57873.1 (88%), AEH57900.1 (88%), AEH57887.1 (87%), AFI43965.1 (88%), AFI43958.1 (90%) and AEH 57927.1 (77%) with a query coverage of 100%. The sequence was deposited in the GenBank database under the accession number AIX 96883.1. Further validation of the amino acid sequence deduced from its nucleotide sequence through ExPASy translate tool had shown significant homology with the published sequences of cellulase genes with catalytic conserved domain of GH9, such as [GKYVVNGGIS]. The amino acid similarities of the cellulase partial sequence (AIX 96883.1) with other published sequences are depicted in Table 4, with the highest percent of similarity of 62% and query coverage of 100% with glycoside hydrolase 5 catalytic domains. These results represented that they belonged to novel cellulase-glycoside hydrolase domains. Similar conserved catalytic sites of cellulases had been previously identified by Xiong et al. (2012) and Khademi et al. (2002). Significantly similar sequences containing the conserved catalytic residues of cellulases were retrieved from the protein database (UniProt KB) and analysed by multiple sequence alignment using Clustal X and was shown in Fig.22. These results demonstrated that this metagenome-specific PCR screening approach was successful in identifying the sequence diversity of partial cellulase gene encoded in the functional cellulose-degrading clones.

Table 4: Aminoacid sequence similarity of cellulase subclone from Vmg-Eg-3 (accession number AIX 96883.1.) to other neighbouring cellulase sequences from protein database

Description	Query coverage	Identity	Accession Number
cellulose 1,4-beta-cellobiosidase [*Treponema caldarium*]	100%	62%	WP_013970084.1
glycosyl hydrolase family 5 [*Streptomyces atroolivaceus*]	99%	58%	WP_033298656.1
endoglucanase [*Streptomyces sp.* MUSC164]	100%	56%	WP_046087965.1
glycosyl hydrolase family 5 [*Nocardiopsis halotolerans*]	99%	59%	WP_026122572.1
glycosyl hydrolase family 5 [*Streptomonospora alba*]	100%	56%	WP_040275461.1
cellulase [*Clostridium cellulolyticum*]	99%	57%	WP_015924278.1
cellulose 1,4-beta-cellobiosidase CelK [*Clostridium saccharoperbutylacetonicum*]	100%	55%	WP_015391950.1
glycosyl hydrolase family 5 [*Glycomyces tenuis*]	99%	57%	WP_051325799.1
hypothetical protein [*Nocardiopsis alkaliphila*]	99%	58%	WP_017604267.1
endoglucanase [*Sorangium cellulosum*]	99%	60%	KYF93147.1
cellulase [*Clostridium cellobioparum*]	99%	57%	WP_027630842.1
glycoside hydrolase family 9 [*Clostridium termitidis*]	99%	57%	WP_004623852.1
endoglucanase [*Hahella chejuensis*]	99%	61%	WP_011394083.1
endoglucanase [*Sorangium cellulosum*]	99%	59%	WP_061608903.1
glycosyl hydrolase family 5 [*Thermobifida cellulosilytica* TB100]	99%	62%	KUP98620.1
hypothetical protein [*Gynuella sunshinyii*]	100%	57%	WP_052830268.1
glycosyl hydrolase family 5 [*Streptomyces pratensis*]	99%	58%	WP_014152324.1
MULTISPECIES: glycosyl hydrolase family 5 [*Streptomyces*]	99%	58%	WP_033249742.1
glycosyl hydrolase family 5 [*Kitasatospora papulosa*]	99%	58%	WP_030125083.1
glycosyl hydrolase family 5 [*Streptomyces sp.* NRRL S-325]	99%	58%	WP_037829312



(Fig.13) **(Fig.14)**

Fig.13. Phagemid DNA isolated through alkaline plasmid extraction method , Lane 1: phagemid DNA isolated from Vmg-Eg-1 , lane 2 : Vmg-Eg-2 , lane 3: Vmg-Eg- 3 , lane 4 : Vmg-Eg-4 , lane 5: Vmg-Eg-5 , lane 6: Vmg-Eg- 6, lane 7 : Vmg-Eg- 7 , lane 8: Vmg-Eg- 8 , lane 9 : Vmg-Eg- 9, lane 10 : Vmg-Eg-10 , lane 11: Vmg-Eg-11 , lane 12: Vmg-Eg-12 , lane 13: Vmg-Eg- 13 , lane 14: Vmg-Eg- 14, lane 15 : Vmg-Eg-15 , lane 16: 1 Kb marker

Fig.14. Phagemid DNA isolation from one of the clone using Axyprep ™ plasmid midi preparation kit

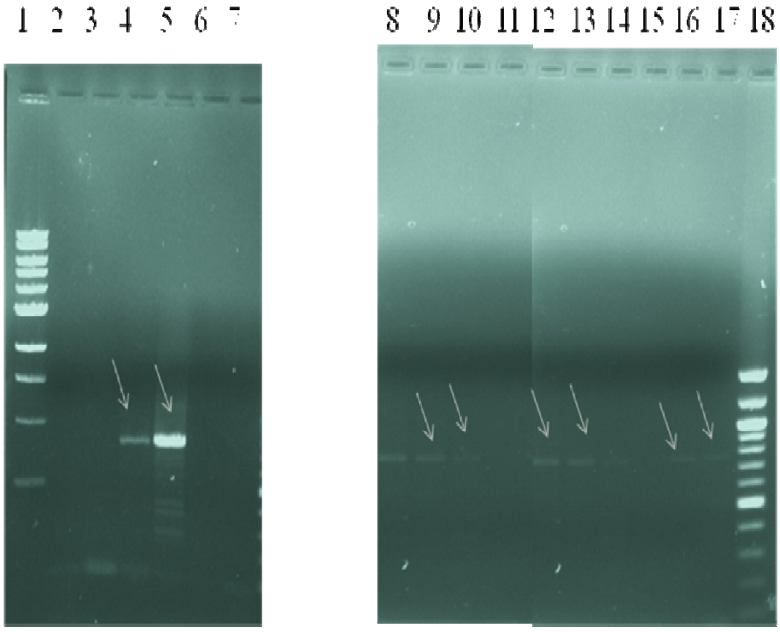
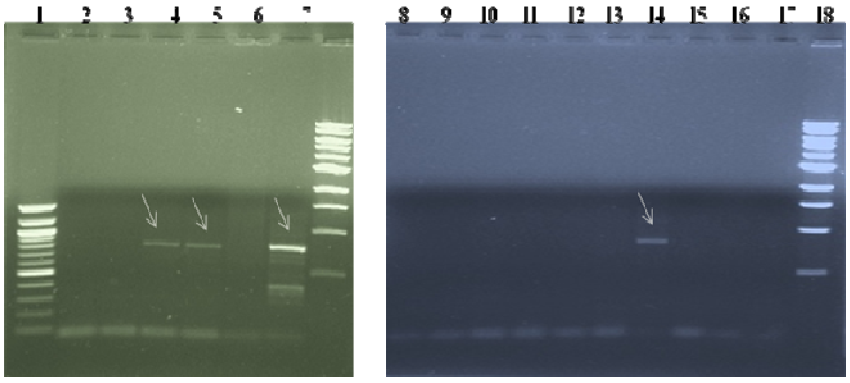


Fig.15. Amplification of cellulase genes from the positive clones using GH9 1f/1r : Lane 1: 1 Kb marker , lane 2 : Eg-1 (amplification from Vmg-Eg-1) , lane 3: Eg-2 (amplification from Vmg-Eg-2) , lane 4 : Eg-3 (Vmg-Eg-3) , lane 5: Eg-4 (Vmg-Eg-4) , lane 6: Eg-5 (Vmg-Eg-5), lane 7 : Eg-6 (Vmg-Eg-6) , lane 8: Eg-7 (Vmg-Eg-7) , lane 9 : Eg-8 (Vmg-Eg-8), lane 10 : Eg-9 (Vmg-Eg-9) , lane 11: Eg-10 (Vmg-Eg-10) , lane 12: Eg-11(Vmg-Eg-11) , lane 13: Eg-12 (Vmg-Eg-12), lane 14: Eg-13 (Vmg-Eg-13), lane 15 : Eg-14 (Vmg-Eg-14) , lane 16: Eg-15 (Vmg-Eg-15) , lane 17 : Eg-16 (Vmg-Eg-16), lane 18 : 100 bp ladder



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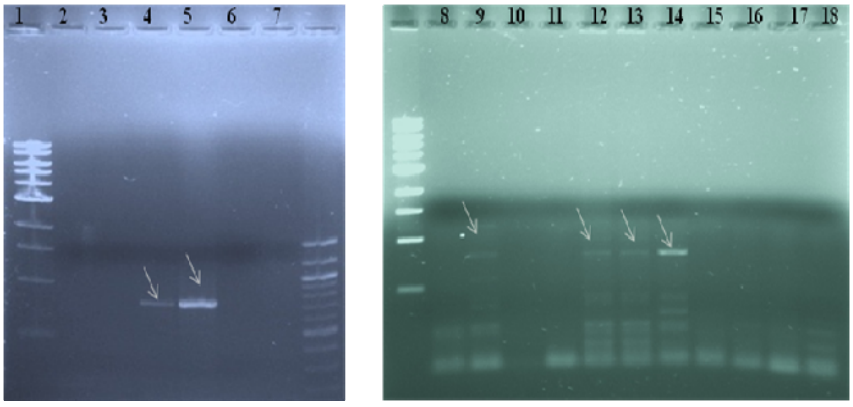
(Fig.16)



(Fig.17)

Fig.16. Amplification of cellulase genes from the positive clones using GH9 1f/2r

Fig.17. Amplification of cellulase genes from the positive clones using 2f/2r
Lane descriptions - Lane 1: 1 Kb marker , lane 2 : Eg-1 (amplification from Vmg-Eg-1) , lane 3: Eg-2 (Vmg-Eg-2) , lane 4 : Eg-3 (Vmg-Eg-3),lane 5: Eg-4 (Vmg-Eg-4) , lane 6: Eg-5 (Vmg-Eg-5), lane 7 : Eg-6 (Vmg-Eg-6) , lane 8: Eg-7 (Vmg-Eg-7) , lane 9 : Eg-8 (Vmg-Eg-8), lane 10 : Eg-9 (Vmg-Eg-9) , lane 11: Eg-10 (Vmg-Eg-10), lane 12: Eg-11(Vmg-Eg-11) , lane 13: Eg-12 (Vmg-Eg-12), lane 14: Eg-13 (Vmg-Eg-13), lane 15 : Eg-14 (Vmg-Eg-14) , lane 16: Eg-15 (Vmg-Eg-15) , lane 17 : Eg-16 (Vmg-Eg-16), lane 18 : 100 bp ladder

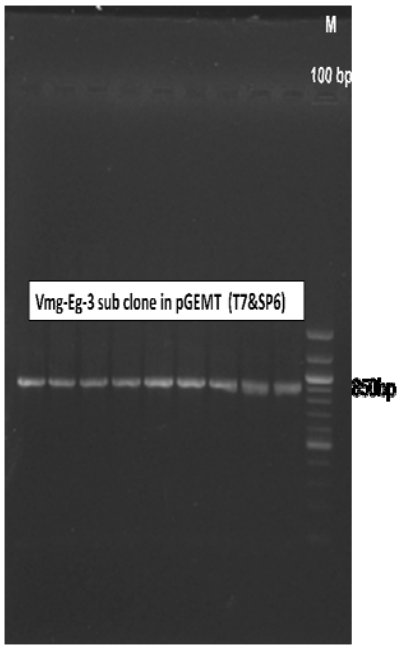


Fig. 18

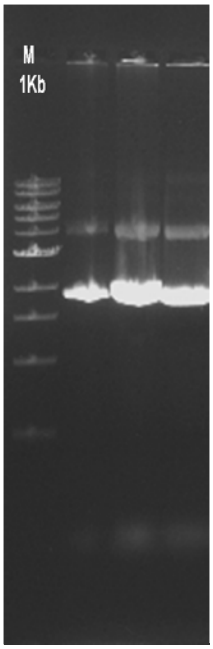


Fig.19

Fig.18. Colony PCR of Vmg-Eg-3 cellulase subclone in pGEMT using T7&SP6 primers;

Fig.19. Plasmids extracted using GenElute HP Plasmid Miniprep kit

```
ggcaagtatgtggtcaatggcggcatttcaacctggacactggtgaacctgtatgaacac
G  K  Y  V  V  N  G  G  I  S  T  W  T  L  V  N  L  Y  E  H
agcccgaaagatttcctagacggatcgctcaatatacccgaaagccagaatgggctgccg
S  P  K  D  F  L  D  G  S  L  N  I  P  E  S  Q  N  G  L  P
gatatattagatgaagcccgctgggaaatggattttatgctgcggatgcaagtgccggaa
D  I  L  D  E  A  R  W  E  M  D  F  M  L  R  M  Q  V  P  E
gggcaaccgctggcgggcatggcgcaccataagctgcatgacctgcattggagcggtgta
G  Q  P  L  A  G  M  A  H  H  K  L  H  D  L  H  W  S  G  V
ccgatcaagctgcctaccgaatttgacaacgacagcccgaccaatggccgttatctgatg
P  I  K  L  P  T  E  F  D  N  D  S  P  T  N  G  R  Y  L  M
ccgcccagcaccgccgcgacactcaatctggcggcgacagcagctcaatgcgcccgcgtg
P  P  S  T  A  A  T  L  N  L  A  A  T  A  A  Q  C  A  R  V
tggaaaatgctggacgccgatttcgccgcacgctgttgaaagccgctgaaactgcctggg
W  K  M  L  D  A  D  F  A  A  R  C  T  K  P  L  K  L  P  G
aggcggccaacgcgcatcctgatatgttgtatgggcgtatccccggcgatggcggcgggg
R  R  P  T  R  I  L  I  C  C  M  G  V  S  P  A  M  A  A  G
attatggcaatagcggcgtgcaggatgaattttattgggcggcggtca
I  M  A  I  A  A  C  R  M  N  F  I  G  R  R  S
```

Fig.20. Nucleotide and ExPASy translated aminoacid sequence of Vmg-Eg-3 subclone , submitted at GenBank as Uncultured organism clone SED-CELL-510 glycoside hydrolase family 9 cellulase (GH9) gene, partial CDS under the accession number AIX96883.1

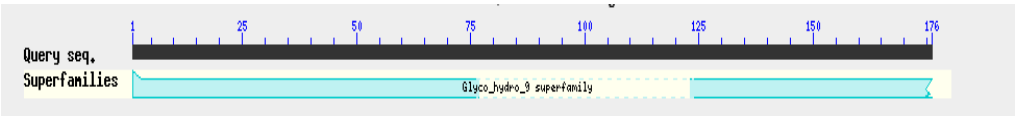


Fig.21. Nucleotide and protein sequence of GH9 cellulase partial gene **AIX96883.1**

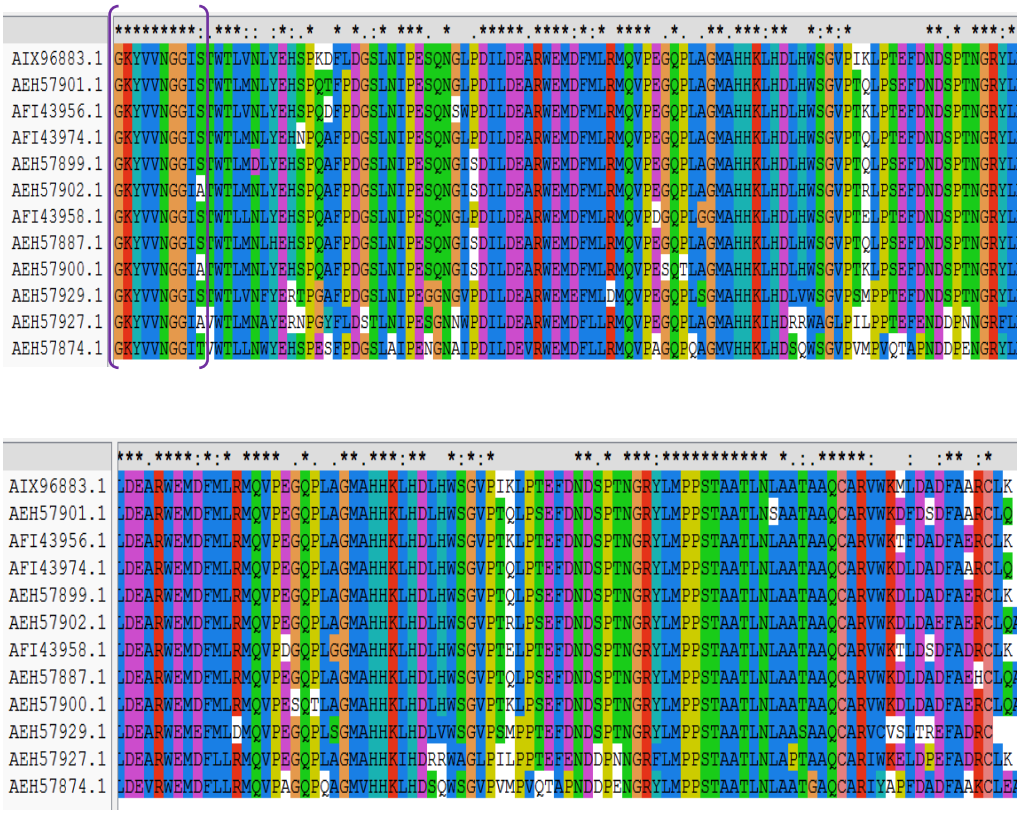


Fig.22. Multiple sequence alignment of partial endoglucanase gene from Vmg-Eg-3 with its closest neighbouring sequences (glycoside hydrolase 5 domains) from the protein database. Conserved domains of catalytic residues are shown in purple square brackets.

6.3.4 Proteomic screening of endoglucanase clone using zymogram analysis

Zymogram analysis or activity staining after SDS-PAGE appears to be a suitable confirmatory screening of endoglucanase encoding clone, Vmg-Eg-3. This method couples the advantage of enzyme identification along with its molecular weight dependence. Recombinant proteins were first resolved using 12.5% SDS-PAGE. After electrophoretic separation, the SDS present in the gel was removed by washing with refolding buffer containing isopropanol and Triton X-100. Because SDS may denature the enzymes and a proper functional activity could not be resolved in the gel even if the true activity was present. Some of the proteins may also denature during incubation with sample buffer and such activity reduction could be minimized by reducing the heating temperature of sample buffer from 100 to 60^{o}C. In addition, the proper diffusion of enzyme bands into the CMC agar replica was made practical by incubating overnight at 40^{o}C, which is the optimum working temperature instructed for most of the cellulase assays. After optimizing all these conditions, the zymogram of the Vmg-Eg-3 protein lysate could produce a yellow clearance zone against a red background (Fig.23b) and the background turned dark blue after rinsing with 5% acetic acid (Fig.23c). The positive hydrolysis zone corresponding to the cellulase protein in the SDS-PAGE gel was found at approximately 55 KDa (Fig.23a). These results suggested that the protein expressed in the endoglucanase clone, Vmg-Eg-3 is functional, accurately refolded and found compatible for expression machinery present in the host *E. coli* system.

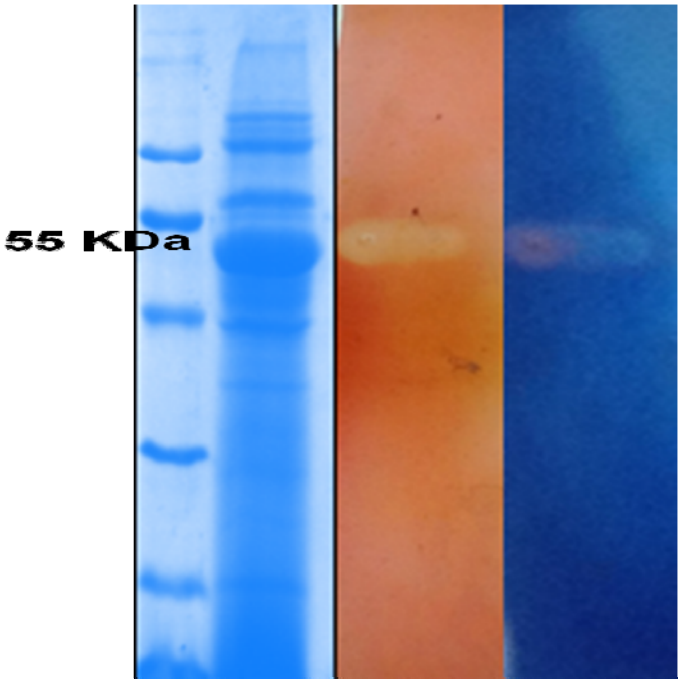


Fig.23: (a) Recombinant protein expression of lambda ZAP expression construct ; (b) : Cellulase activity staining of the CMC-agarose gel in congo red dye ; (c) : Destaining and stabilization of clearance zone of cellulase activity gel with 5% acetic acid

6.3.5 Confirmation of the putative endoglucanase clone by retransformation

The potential cellulase activity produced by the clone, Vmg-Eg-3 was re-confirmed by transforming the phagemid DNA into another expression host, *E. coli* BL21. After transformation and incubation on CMC agar plate coupled with congo red staining, the positive clones were selected on the basis of a yellow halo zone against a red background due to degradation of cellulose. The corresponding clone enough to reproducibly confer cellulolytic activity upon retransformation with *E. coli* BL21 was subsequently confirmed as the positive clone (Fig.24). The results also showed that the observed

cellulase catalytic activity was produced from the cloned insert only and not from any contaminants or the host *E. coli* host strain used in the experiment.

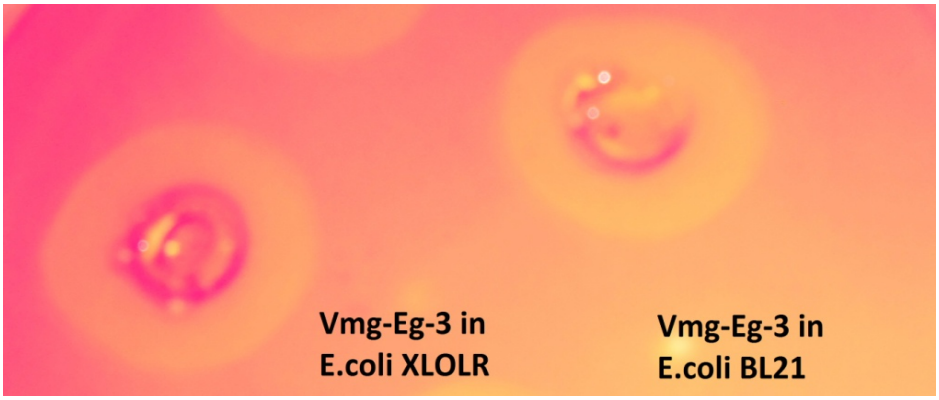


Fig.24. Retransformation of Vmg-Eg-3 phagemid DNA into another host, *E. coli* BL21to reconfirm cellulase activity on CMC-congo red agar plate

6.3.6 Insert size characterization in the lambda clone Vmg-Eg-3 using long PCR approach

The determination of insert DNA size in the positive lambda clone Vmg-Eg-3 was confirmed by a long PCR screening (with lambda ZAP vector specific T7 forward and T3 reverse primers) using Phusion^{TM} High- Fidelity PCR kit. In addition, the use of *Pfu* DNA polymerase would speed up the specificity of a PCR and yield optimal amplification products for longer sized DNA. The resultant PCR product was then analyzed by electrophoresis using 1% (w/v) agarose gel and the insert size was found to be approximately 6 Kb (Fig.25). Specific amplification of the inserted fragment (6 Kb) with any nonspecific amplification products suggested that the protocol was specific for the amplification of the target DNA. The size of the amplified insert DNA was found to be near to the predicted size range of average insert size (8Kb) of the metagenomic lambda library.

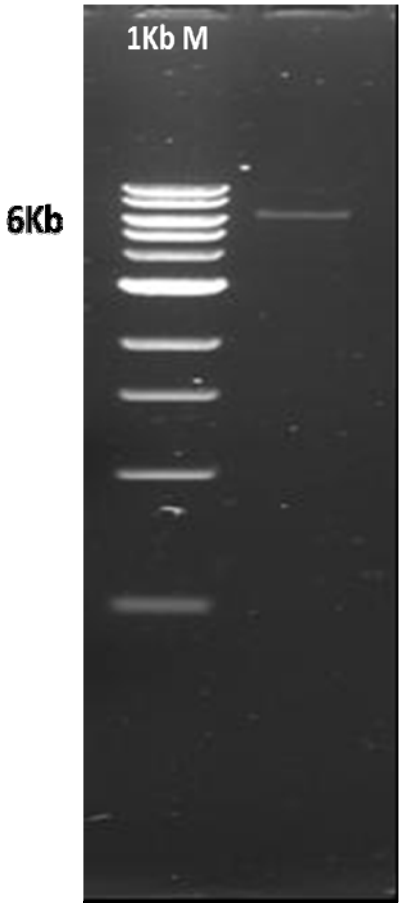


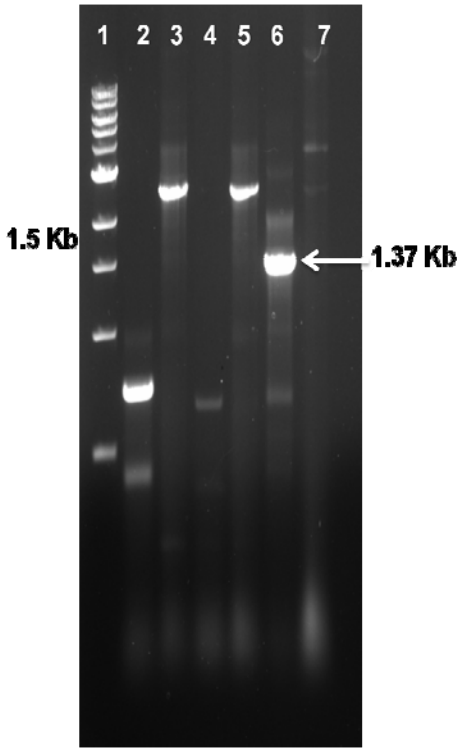
Fig.25. Insert size characterisation of the clone Vmg-Eg-3 using long PCR, lane1 ; 1 Kb Marker , lane 2; Amplified insert of 6 Kb using the T3 forward and T7 reverse specific primers for pBK-CMV phagemid

6.3.7 Cloning of putative endoglucanase gene and sequence analysis

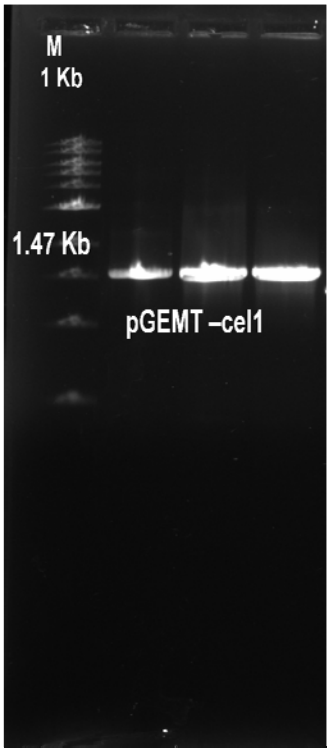
The designed primers (with restriction sites) based on the sequence information from the cellulase partial sequence (Fig.20) was used for the amplification of full-length endoglucanase gene from the Vmg-Eg-3 phagemid DNA template using conventional PCR. Of all the restriction primer sets used, the successful amplification of full-length endoglucanase gene (≈ 1.37 Kb) was achieved with the primer set, Endo-5 (Fig.26, lane 6) and the resulting PCR product was subsequently cloned into the pGEM-T easy vector. The

colony PCR to check for the presence of insert orientation yielded an approximate product size of 1.47 Kb (Fig.27). The sequencing output (FASTA format) of the cloned insert was translated into amino acid output using the Translate tool of ExPASy (SIB Bioinformatics Resource Portal), indicated an open reading frame (ORF) which started with an ATG start codon and terminated with a TAG stop codon (Fig. 28) . The ORF of endoglucanase gene consisted of 1368 nucleotides encoding a protein of 456 amino acid residues with a predicted molecular weight of approximately 50 kDa (Fig.30). Further sequence analysis and protein domain search (http://www.ncbi.nlm.nih. gov/Structure/cdd/cdd.shtml) showed that the amino acid sequence consisted of a glycosyl hydrolase 1 super family, however the multi-domain analysis of the amino acid sequence revealed the presence of GHF5 catalytic domain from position 75 to 453 with an E-value of 4.52e-18 , GH9 domain from amino acid position 259 to 281 with an E-value of 7.09e-04 and a BglC domain from position 28 to 182 with an E-value of 8.45e-08 (Fig.29) . This also supported the sequence information obtained from gene-level screening using GH9 multi-primer sets. Homology searches using BlastX analysis showed that the amino acid sequence of Cel1 was most closely related to endoglucanase from an uncultured bacterium (AFX88671.1) and endoglucanases of *Bacillus* spp. Aminoacid sequence similarity of putative cellulase gene (Cel1) to other neighbouring cellulase sequences of protein database is shown in Table 5. The NCBI Blast hit analysis showed that, along with the glycoside hydrolase 1 superfamily catalytic domain, Cel1 also harboured a family 9 and 5 cellulase protein domains. None of the endoglucanases (endo-acting cellulases) has not been previously identified from the glycoside hydrolase family 1 domain (which is remaining as a major dominant source of β-glucosidases) to date. This is the first report of a metagenome-derived endoglucanase belonging to

the glycoside hydrolase super family 1 (GH1) therefore, it was renamed as 'Cel1'. Most of the metagenome-derived cellulases identified through activity-based screening belonged to glycoside hydrolase families (GHF) 5 and 9 (Duan and Feng, 2010). Metagenome-derived GHF9 cellulases were isolated from an aquatic community (Pottkämper et al., 2009), compost soil (Pang et al., 2009), elephant dung (Wang et al., 2009), and alkaline lake enrichment culture (Grant et al., 2004) etc.



(Fig. 26)



(Fig. 27)

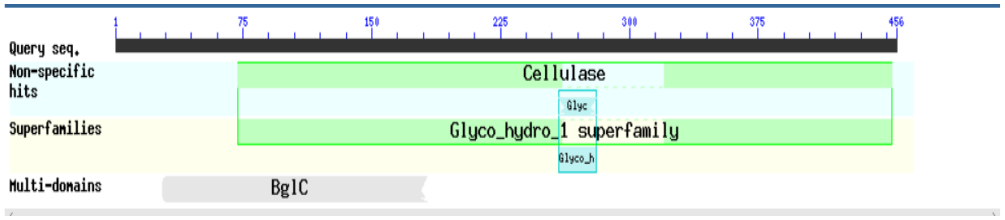
Fig.26. Endoglucanase gene (Cel1) amplification from Vmg-Eg-3 lambda DNA template using different restriction primer sets , lane 1; 1 Kb marker , lane 2; amplification of cel1 using Endo-1 primer set , lane 3; amplification of cel1 using Endo-2 primer set , lane 4; amplification of cel1 using Endo-3 primer set , lane 5; amplification of cel1 using Endo-4 primer set , lane 6; amplification of cel1 using Endo-5 primer set , lane 7; amplification of cel1 using Endo-6 primer set.

Fig.27. Colony PCR of Cel 1 in pGEM-T with T7 and Sp6 primers

>Vmg-Eg-3 _Cel1

```
ctggaatttttagcaaaattaccggcaccatgatgggccgccagaaacgcagcgatatg
L  E  F  F  S  K  I  T  G  T  M  M  G  R  Q  K  R  S  D  M
accgcagcattagcatttttattacctgcgcgctgattagcgtgctgaccatgggcctg
T  R  S  I  S  I  F  I  T  C  A  L  I  S  V  L  T  M  G  L
ctgctgccgagcccggcgaccgcgaccggcaccaaaccgaacgtgctgaaaggcggccag
L  L  P  S  P  A  T  A  T  G  T  K  P  N  V  L  K  G  G  Q
ctgagcattaaagatacccagctggtgctgcgcgatggcaaagcggtgcagattcagggc
L  S  I  K  D  T  Q  L  V  L  R  D  G  K  A  V  Q  I  Q  G
attagcagccatagcctgcaggattatggcagcagcgtgctgtatgatattctgcagtgg
I  S  S  H  S  L  Q  D  Y  G  S  S  V  L  Y  D  I  L  Q  W
ctgcgcgatgcgtggggcattaccgtgtttcgcgcggcgatgtataccgcggatggctgg
L  R  D  A  W  G  I  T  V  F  R  A  A  M  Y  T  A  D  G  W
tatattgataacccgagcgtggcgaacagcgtgaaagaagcggtggcgtatgcgcaggaa
Y  I  D  N  P  S  V  A  N  S  V  K  E  A  V  A  Y  A  Q  E
ctgggcatttgggtgattgatgatgcgcatattgcgaacaaaggcaacggcaaatatgtg
L  G  I  W  V  I  D  D  A  H  I  A  N  K  G  N  G  K  Y  V
gtgaacggcggcattagcacctggaccctggtgaacccgaaccagaacaaaaaagatttt
V  N  G  G  I  S  T  W  T  L  V  N  P  N  Q  N  K  K  D  F
ctggatggcagcctgaacattccggaaagcgaaaaaggcaaactgttttttaaagaaatg
L  D  G  S  L  N  I  P  E  S  E  K  G  K  L  F  F  K  E  M
agcccgctgaacaaaggcaacacccatgatctgcattggagcggcgtgccgattaaactg
S  P  L  N  K  G  N  T  H  D  L  H  W  S  G  V  P  I  K  L
ccgctgaacgtgatttatgaaattgcgaccgaaccgtttggcgatgcgggctggtggcgc
P  L  N  V  I  Y  E  I  A  T  E  P  F  G  D  A  G  W  W  R
gatattaaaccgaacagcagcggccgctatctgatgccgccgagcaccgcggcgaccgaa
D  I  K  P  N  S  S  G  R  Y  L  M  P  P  S  T  A  A  T  E
gatatattagatgaagcccgctgggaaatggattttatgctgcggatgcaagtgccggaa
D  I  L  D  E  A  R  W  E  M  D  F  M  L  R  M  Q  V  P  E
gggcaaccgctggcgggcatggcgcaccataagctgcatgacctgcattggagcggtgta
G  Q  P  L  A  G  M  A  H  H  K  L  H  D  L  H  W  S  G  V
ccgatcaagctgcctaccgaatttgacaacgacagcccgaccaatggccgttatctgatg
P  I  K  L  P  T  E  F  D  N  D  S  P  T  N  G  R  Y  L  M
gtgattagcgtgattcgcaaaaacgatccggataacattattgatgtgggcaccggcacc
V  I  S  V  I  R  K  N  D  P  D  N  I  I  D  V  G  T  G  T
atgctgcgcatgcaggtgccggaatggagccagtttgtgaacgatgcggcggatgatctg
M  L  R  M  Q  V  P  E  W  S  Q  F  V  N  D  A  A  D  D  L
gatgcggattttgcggcgcgctgcctgcagctgaacgatgcgaacgtgatgtatgcgctg
D  A  D  F  A  A  R  C  L  Q  L  N  D  A  N  V  M  Y  A  L
cattttatgcgggcacccatctgaacagcggcgtgcaggatgaatttattggcagagc
H  F  Y  A  G  T  H  L  N  S  G  V  Q  D  E  F  Y  W  Q  S
ctgaccgataaaaccaactataactttggcaaaggcgcgccgatttttgtgaccacctgg
L  T  D  K  T  N  Y  N  F  G  K  G  A  P  I  F  V  T  T  W
ggcgcgaaagatccgagcggcaacggcggcgtgtttctggatcagagccgcgaatggagc
G  A  K  D  P  S  G  N  G  G  V  F  L  D  Q  S  R  E  W  S
ccgtatctggcgcagaaaaactttccgtggcagaactggaaaagctttagc
P  Y  L  A  Q  K  N  F  P  W  Q  N  W  K  S  F  Stop
```

Fig.28. Nucleotide sequence and corresponding amino acid residues identified from pGEMT-Cel 1 through ExPASy translate tool



List of domain hits

Name	Accession	Description	Interval	E-value
Cellulase	pfam00150	Cellulase (glycosyl hydrolase family 5);	72-453	4.52e-18
Glyco_hydro_9	pfam00759	Glycosyl hydrolase family 9;	259-281	7.09e-04
BglC	COG2730	Aryl-phospho-beta-D-glucosidase BglC, GH1 family [Carbohydrate transport and metabolism];	28-182	8.45e-08

Fig.29. Protein domain analysis of Cel1

```
        10         20         30         40         50         60
LEFFSKITGT MMGRQKRSDM TRSISIFITC ALISVLTMGL LLPSPATATG TKPNVLKGGQ

        70         80         90        100        110        120
LSIKDTQLVL RDGKAVQIQG ISHSLQDYGS SVLYDILQWL RDAWGITVFR AAMYTADGWY

       130        140        150        160        170        180
IDNPSVANSV KEAVAYAQEL GIWVIDDAHI ANKGNGKYVV NGGISTWTLV NPNQNKKDFL

       190        200        210        220        230        240
DGSLNIPESE KGKLFFKEMS PLNKGNTHDL HWSGVPIKLP LNVIYEIATE PFGDAGWWRD

       250        260        270        280        290        300
IKPNSSGRYL MPPSTAATED ILDEARWEMD FMLRMQVPEG QPLAGMAHHK LHDLHWSGVP

       310        320        330        340        350        360
IKLPTEFDND SPTNGRYLMV ISVIRKNDPD NIIDVGTGTM LRMQVPEWSQ FVNDAADDLD

       370        380        390        400        410        420
ADFAARCLQL NDANVMYALH FYAGTHLNSG VQDEFYWQSL TDKTNYNFGK GAPIFVTTWG

       430        440        450
AKDPSGNGGV FLDQSREWSP YLAQKNFPWQ NWKSFS
```

Fig.30. Prediction of theoretical pI (5.50) and MW (50955.74) using ExPASy Compute pI/Mw.

Table 5: Aminoacid sequence similarity of putative cellulase gene (Cel1) to some of the neighbouring cellulase sequences of protein database

Description	Query coverage	Identity	Accession Number
endo-1,4-beta-glucanase [uncultured bacterium]	80%	61%	AFX88671.1
cellulose hydrolase [*Bacillus pumilus*]	82%	61%	ACY72384.1
endo-beta-1,4-glucanase [*Bacillus subtilis*]	82%	61%	AAA22496.1
BglC protein [*Bacillus velezensis* FZB42]	82%	60%	CAE11243.1
cellulase [*Bacillus subtilis*]	82%	60%	ABK63475.1
endo-1,4-beta-glucanase [uncultured bacterium]	80%	60%	AGW99975.1
endo-1,4-beta-glucanase [uncultured bacterium]	80%	60%	AFX88667.1
endo-1,4-beta-glucanase [uncultured bacterium]	80%	60%	AGW99969.1
endo-1,4-beta-glucanase [uncultured bacterium]	80%	60%	AFX88671.1
endo-1,4-beta-glucanase [*Bacillus subtilis*]	82%	60%	AAK94871.1
MULTISPECIES: endoglucanase [*Bacillus*]	80%	60%	WP_007410148.1
endo-1,4-beta-glucanase [uncultured bacterium]	80%	60%	AFX88672.1
bifunctional cellulase precursor [*Bacillus* sp.]	80%	59%	AAC43478.1
endo-1,4-beta-glucanase [uncultured bacterium]	80%	59%	AGW99960.1

The translated sequence was further analyzed for the prediction of classical N-terminal secretion signal peptides using the SignalP 4.0 server, based on a combination of several artificial neural networks trained on the identification of signal peptides from Gram-positive eubacteria. The signal peptide cleavage site was identified between the aminoacid position 38 and 39 in Cel1 (Fig.31).The sub-cellular localization of the Cel1 was predicted using the PSORTb v4.0 program and the results showed that the protein was localized to the extracellular compartment (Fig.33). Amino acid sequence analysis of Cel1 on the TMHMM Server, predicted the existence of a putative transmembrane helical domain at the N terminus of the protein (amino acids 9–38), and its catalytic domain (amino acids 39–456) to be facing outward from the membrane (Fig.32). Thus Cel1 was predicted to be a membrane-

bound enzyme containing an N-terminal trans-membrane domain, which helps in the insertion into the cytoplasmic membrane (Netzer et al., 1998)

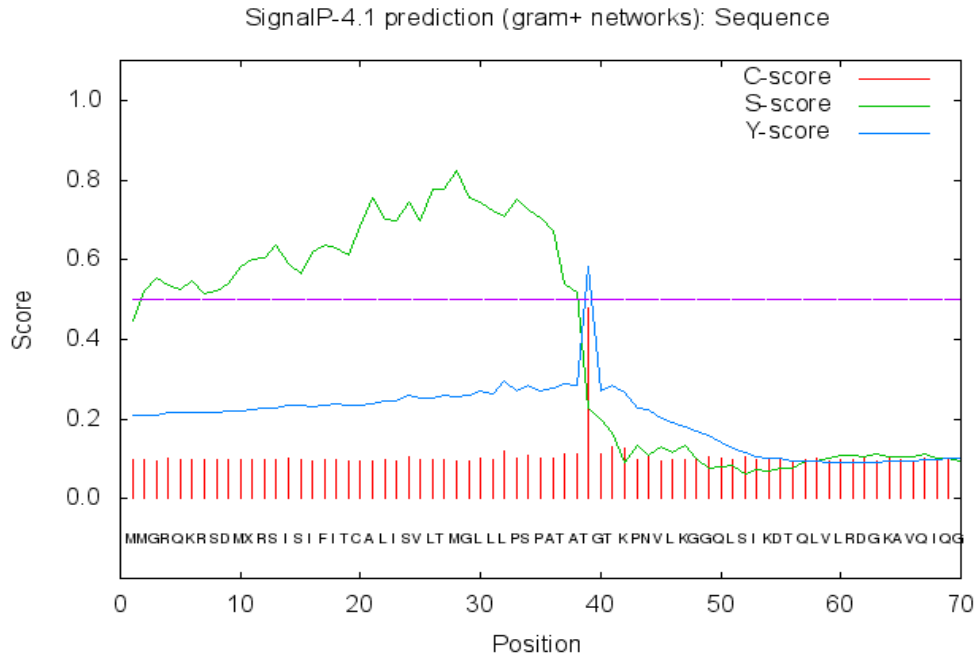


Fig.31. Signal Prediction through SignalP.4-TM , shows Cleavage site between amino acid positions 38 and 39: ATA-TG D=0.605 D-cutoff=0.450

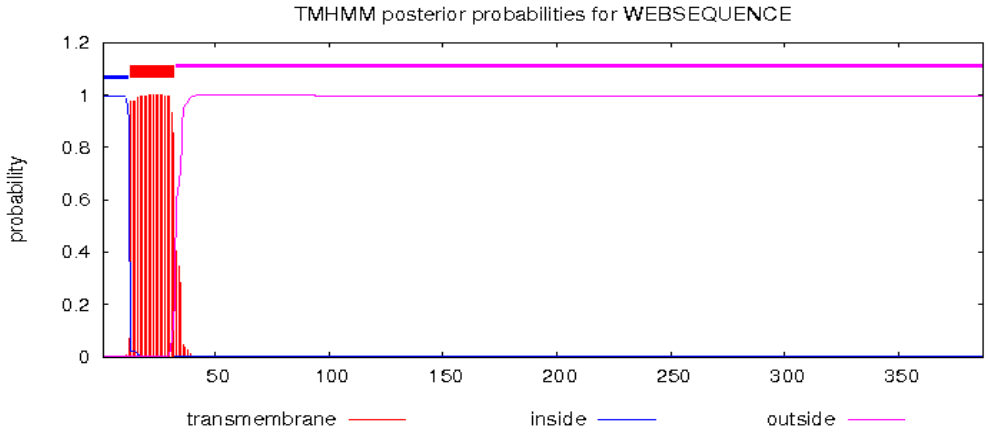


Fig.32. Presence of transmembrane helix as predicted using the program TMHMM 2.0

Signal+ Non-cytoplasmic	[Signal peptide detected]
Localization Scores:	
Cytoplasmic	0.0
CytoplasmicMembrane	0.0
Cellwall	0.2
Extracellular	9.98
Final Prediction:	
Extracellular	9.98

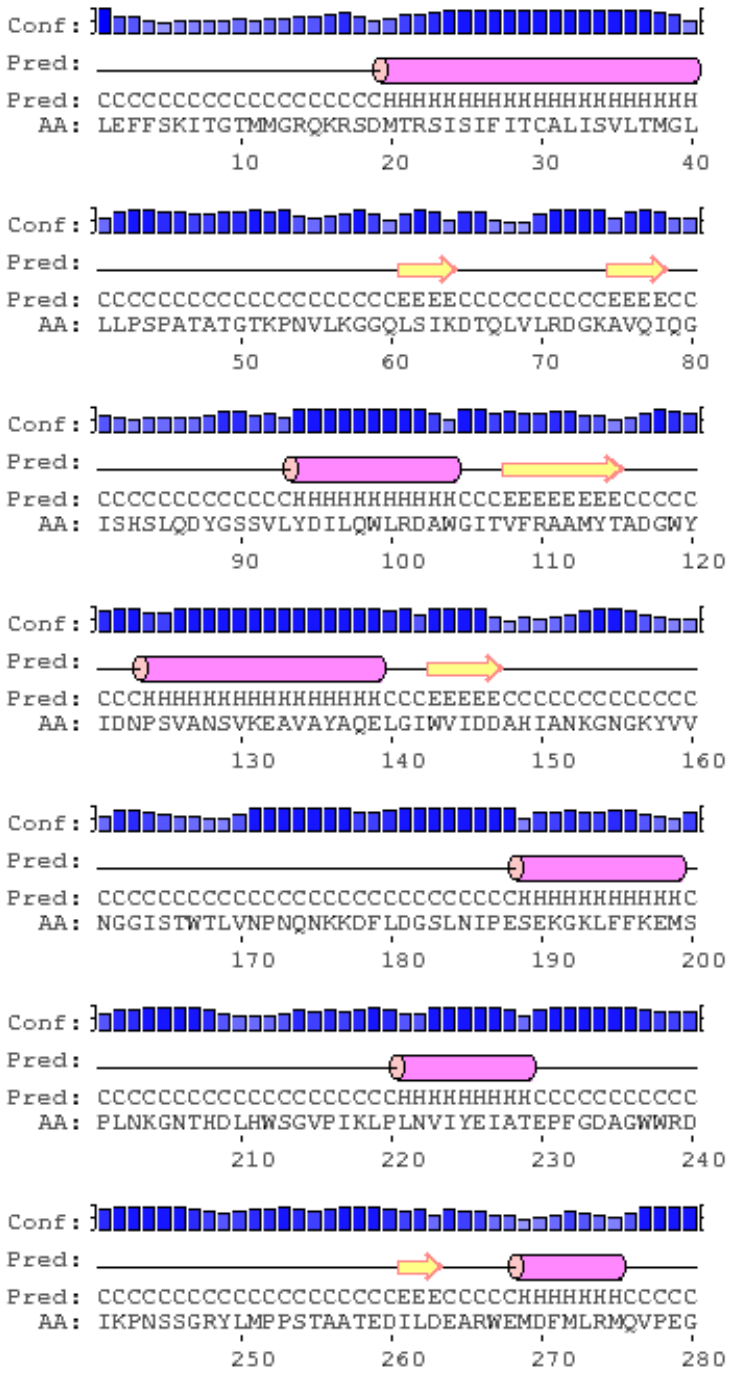
Fig.33. Localization of recombinant protein, Cel1 as predicted by PSORTb v4.0

6.3.8 Homology modeling of Cel1

Homology modeling is currently restricted to protein sequences that share more than 30% sequence identity to an experimentally solved target protein template (Baker and Sali, 2001). If the sequence similarity between the unknown protein and the template sequence are sufficiently high (>50%), the procedure can be automated with reasonable results. Homology modeling consists of the following steps such as selection of a suitable model template, alignment of the target sequence with the template structure, 3D-model building, energy minimization and/or refinement and model quality assessment. The prediction of secondary structure of proteins provides information about various turns, coils and helices, regularly repeated at particular positions and stabilized by hydrogen bonds. Protein secondary structure prediction of Cel1 from its amino acid sequence using PSIPRED VIEW is depicted in Fig.34. It revealed the presence of many alpha helices, beta sheets and coiled regions at various positions in the protein structure along with their confidence level for such occurrence.

The tertiary structure of protein was built by packing of its secondary structure elements to form discrete domains or autonomous folding units. Comparative modeling predicted the 3D structure of Cel 1, based primarily on its alignment to 3pzt.1 (endoglucanase of *Bacillus subtilis*) and the BLAST search of Cel1 with the PDB revealed 72.18% similarity to the model template 3pzt.1, endo-1,4-β-glucanase from *Bacillus subtilis* (Fig.35).The 3D structure

model of Cel1 was modified by PyMOL (version1.4.1, http://www.pymol.org/) as shown in Fig.36.



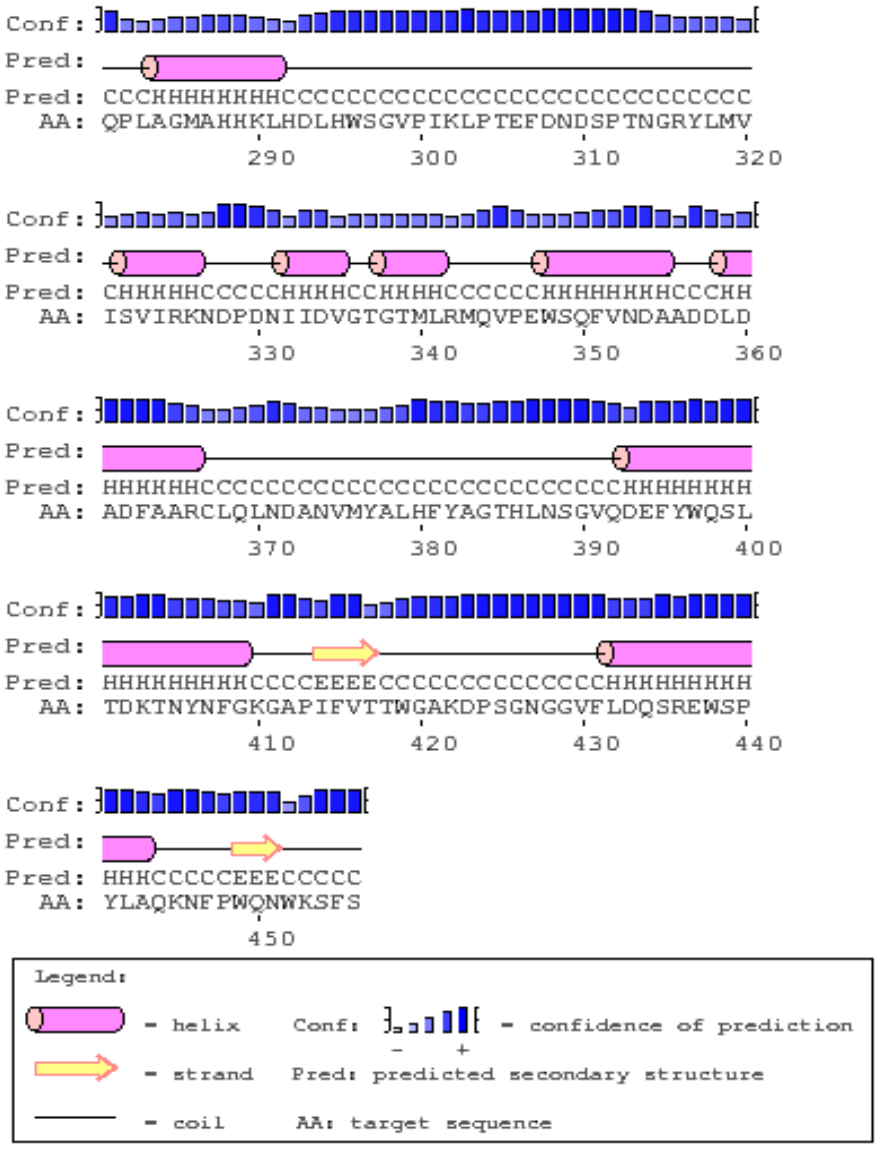


Fig.34. Secondary structure of Cel1 predicted using PSIPRED VIEW software indicated helix (shown as cylinders in pink colour) and beta strands (shown as arrow region in yellow colour) and coils (as continuous line) with confidence level of prediction (Jones, 1999; McGuffin et al., 2000)

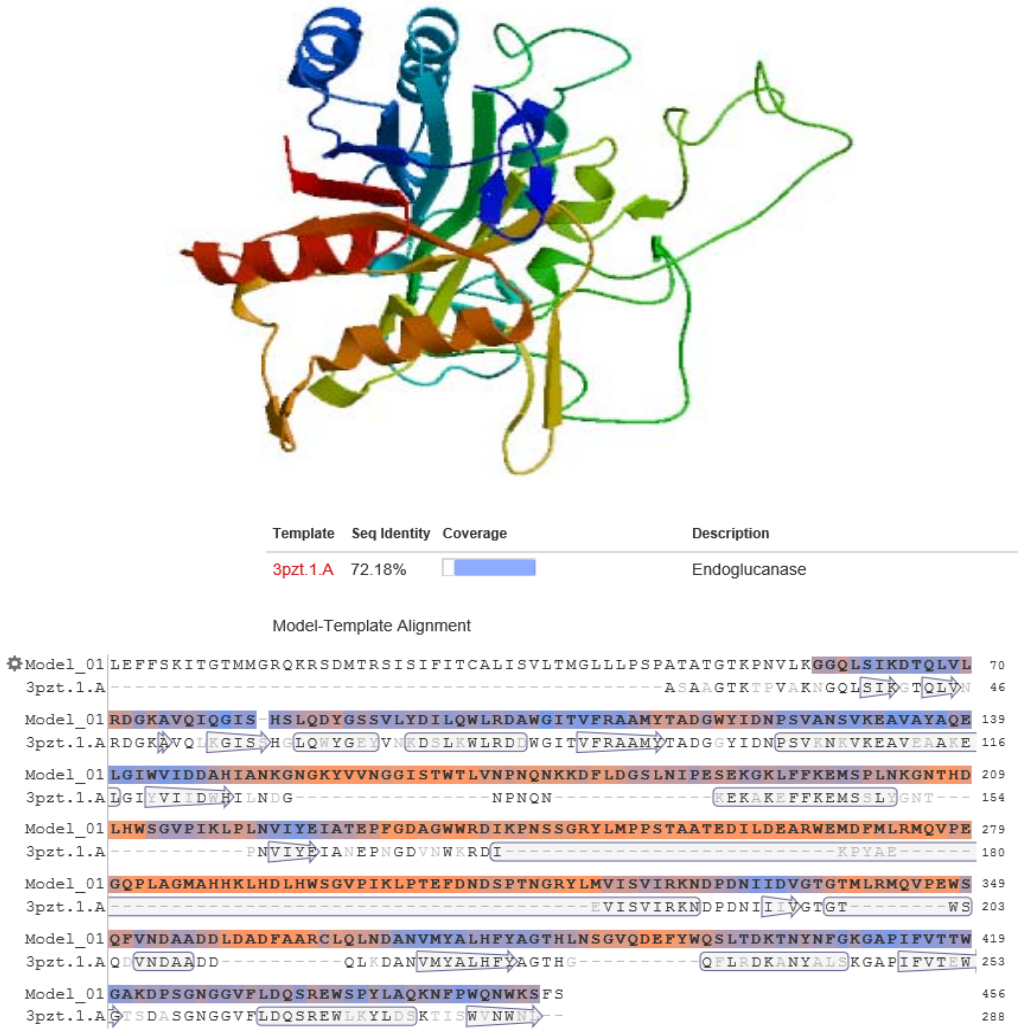


Fig.35. The 3D-structural model of Cel1 as predicted through SWISS-MODEL work space (http://swissmodel.expasy.org) and Model-Template alignment with 3pzt.1, endo-1,4-beta-glucanase from *Bacillus subtilis* (72.18%) .

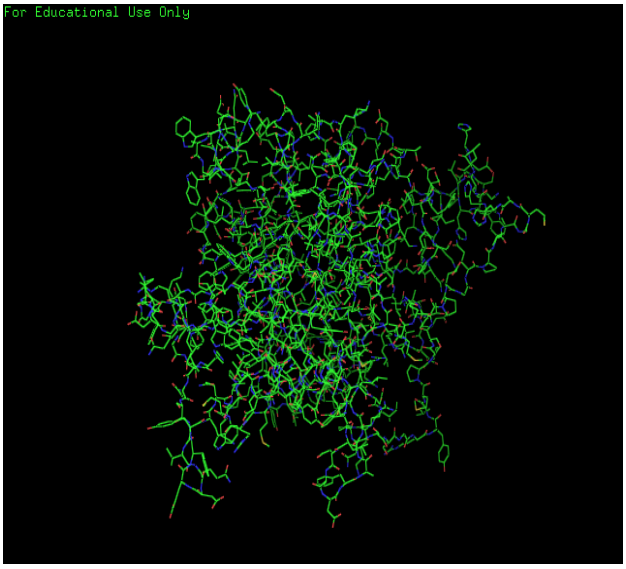


Fig.36. The 3D-structural model of Cel1 modified by PyMOL (version1.4.1, http://www.pymol.org/)

6.4 Conclusion

This chapter describes phenotypic as well as quantitative screening methods for obtaining a potential and novel cellulase-encoding clone from the mangrove metagenomic library (PG-Val-Mg-1) for further subcloning and over-expression of the cellulase gene. The screening methods include phenotypic detection with specific cellulose substrates, quantitative enzyme assay method, gene level screening with cellulase conserved domain primers and a proteome-based screening. The hit rate was about 1/333 with 16 numbers of endoglucanase encoding clones (Vmg-Eg-1 to Vmg-Eg-16) , eight numbers of β-glucosidase positive clones (Vmg-Bg-1 to Vmg-Bg-8) and three numbers of exoglucanase clones (Vmg-Ex-1 to Vmg-Ex-3), which represented a highly diverse and abundant cellulase activity from the Valanthacaud mangrove metagenome (80 Mb). Further confirmation of the positive clone (Vmg-Eg-3) was performed by zymogram analysis, Long PCR and retransformation into another expression host. Retrieval of full-length cellulase

gene was done on the basis of already known partial cellulase gene sequence and accordingly amplification with restriction primers yielded a full-length cellulase gene (1. 37Kb) and cloned into pGEM-T easy vector for sequencing. Further aminoacid sequence analysis showed 60% identity with an uncultured clone endoglucanase gene. The secondary structure of putative endoglucanase (Cel1) showed different helical and coiled structures, predicted with a high level of confidence. The 3D structure of Cel1 was predicted through SWISS-MODEL work space and showed 72.18 % similarity with 3pzt.1, endo-1,4-beta-glucanase from *Bacillus subtilis* (72.18%) .

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Chapter 7

SUB-CLONING, OVER EXPRESSION, PURIFICATION AND CHARACTERISATION OF RECOMBINANT CELLULASE

Contents

7.1 Introduction

Metagenomic exploration of environments where lignocellulosic biomass is being effectively degraded is the most hopeful pathway towards novel cellulase discovery. Novel cellulases identified from environments with extreme temperatures, pH and ionic conditions are highly adaptable to the industrial processes (Ilmberger and Streit, 2010). Metagenomic (functional) screening, detection and functional characterisation of biocatalysts must be improved to provide novel options for cellulose degradation. The present study was aimed at the functional characterisation of mangrove metagenome-derived cellulases in terms of factors which affect their stability and activity under a wide range of conditions and making them suitable candidate for industrial applications.

Metagenomic libraries are typically constructed in lambda vectors because of the ease in activity-based screening as well as such vectors provide the most efficient means of introducing recombinant DNA molecules into bacterial host systems for subsequent propagation to yield most complete library

possible. Plasmid vectors represent a more stable and high copy number vector system containing SP6, T7 or T3 promoters upstream from the multiple cloning sites and can be amplified up to 300-fold by plasmid propagation in *E. coli* . Thus it is often preferable to generate an initial library in a lambda vector and to screen that library to identify an individual clone of interest and then to transfer the clone into a plasmid vector for subsequent propagation and manipulation. Thus 'subcloning' involves the liberation and recovery of an insert DNA fragment from one vector and ligation of that fragment into a different vector to facilitate further propagation of that particular clone. In this chapter, the 'Cel1' gene derived from the lambda clone Vmg-Eg-3 was initially subcloned into pJET 1.2 blunt vector system and then overexpressed in the translation vector pET32a (+) for further propagation and protein purification.

The pET system is the most potent bacterial expression system designed for cloning and expression of recombinant proteins in *E. coli*. Derivatives of pET vectors have been designed with enhanced features to allow easier subcloning, detection and purification of target proteins. For protein production, the recombinant plasmid is transferred into host *E. coli* strains containing T7 RNA polymerase. These *E. coli* host systems are lysogens of bacteriophage DE3, a λ derivative that contains the 'phage 21' immunity region and carries a DNA fragment containing the LacI repressor gene (*lacI*) , the *lacUV5* promoter and the gene for T7 RNA polymerase. The *lacUV5* promoter is inducible by the non-metabolizable lactose analogue, isopropyl-β-D-thiogalactopyranoside (IPTG). T7 RNA polymerase transcribes eight times faster than *E. coli* RNA polymerase, allowing high-level protein expression. The selection of pLysS as the host system increases the tolerance of λDE3 lysogens for plasmids with toxic inserts. Unstable plasmids with toxic inserts become stable and plasmids that would not otherwise be propagated

can be maintained and expressed. Purification of recombinant fusion protein utilizes immobilized metal-ion affinity chromatography (IMAC), which takes the advantage of the adsorption of polyhistidine (6x His) containing proteins and peptides to a nickel-chelate-nitrilotriacetic acid (Ni-NTA) column. This highly selective interaction allows purification of tagged proteins or protein complexes from <1% to >95% homogeneity in a single step. The use of this purification procedure at large scale would clearly be economic in all aspects.

In the present chapter, the putative cellulase gene derived from the positive lambda clone , Vmg-Eg-3 was subcloned into a blunt end vector, pJET1.2 and further over-expressed into recombinant translation expression vector (pET32a+) for recombinant/fusion protein expression. The recombinant/fusion protein of Cel1 was Ni-NTA purified, detected through functional assays (CMC-Congo red) and biochemically characterised for exploring its industrial potential.

7.2 Materials and Methods

7.2.1 Cloning and expression vectors used in the present study

7.2.1.1 pJET1.2/blunt cloning vector

The pJET1.2/blunt cloning vector (Fig.1) is a positive selection vector from Clone JET ™ PCR Cloning kit (MBI Fermantas/Thermo Scientific). Either a blunt or sticky-end can be successfully cloned using this vector. The pJET1.2/blunt is a linearized cloning vector, which accepts inserts from 6 bp to 10 kb. The 5'-ends of the vector cloning site contain phosphoryl groups, therefore, phosphorylation of the PCR primers is not required. Blunt-end PCR products generated by proofreading DNA polymerases (*Pfu*) can be directly ligated in just 5 min with the pJET1.2/blunt cloning vector. All common laboratory *E. coli* strains can be directly transformed with the ligation product.

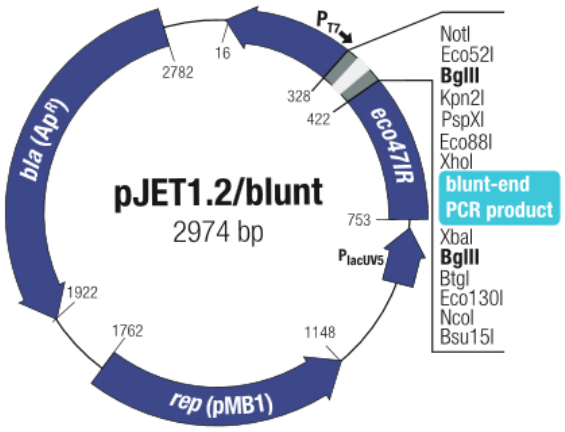


Fig.1. Map of pJET1.2/blunt cloning vector with MCS region (Picture available with the Clone JET™ PCR Cloning Kit)

Recircularized pJET1.2/blunt vector lacking an inserted fragment expresses a lethal restriction enzyme gene, which kills the *E. coli* host cell after transformation and is not propagated. This positive selection method speeds up the colony screening process and eliminates expensive blue/white screening method. The vector contains an expanded multiple cloning site (MCS), as well as a T7 promoter for in vitro transcription.

7.2.1.2 pET 32a (+) Translation vector

pET series as a whole is a powerful system designed for enhanced features to permit easier subcloning, detection and purification of target proteins in *E. coli*. Target genes are cloned in pET plasmids under the strong control of bacteriophage T7 transcription and translation signals. Expression is induced by providing a selective source of T7 RNA polymerase in the host cell, and when it is fully induced, almost all of the cell's resources are converted to target gene expression and the desired protein product can comprise more than 50% of the total cell protein a few hours after induction with IPTG. Another important advantage of this system is its ability to maintain target genes transcriptionally silent in the uninduced state. The vector

map of pET32a(+) translation vector with cloning and expression region is shown in Fig. 2.

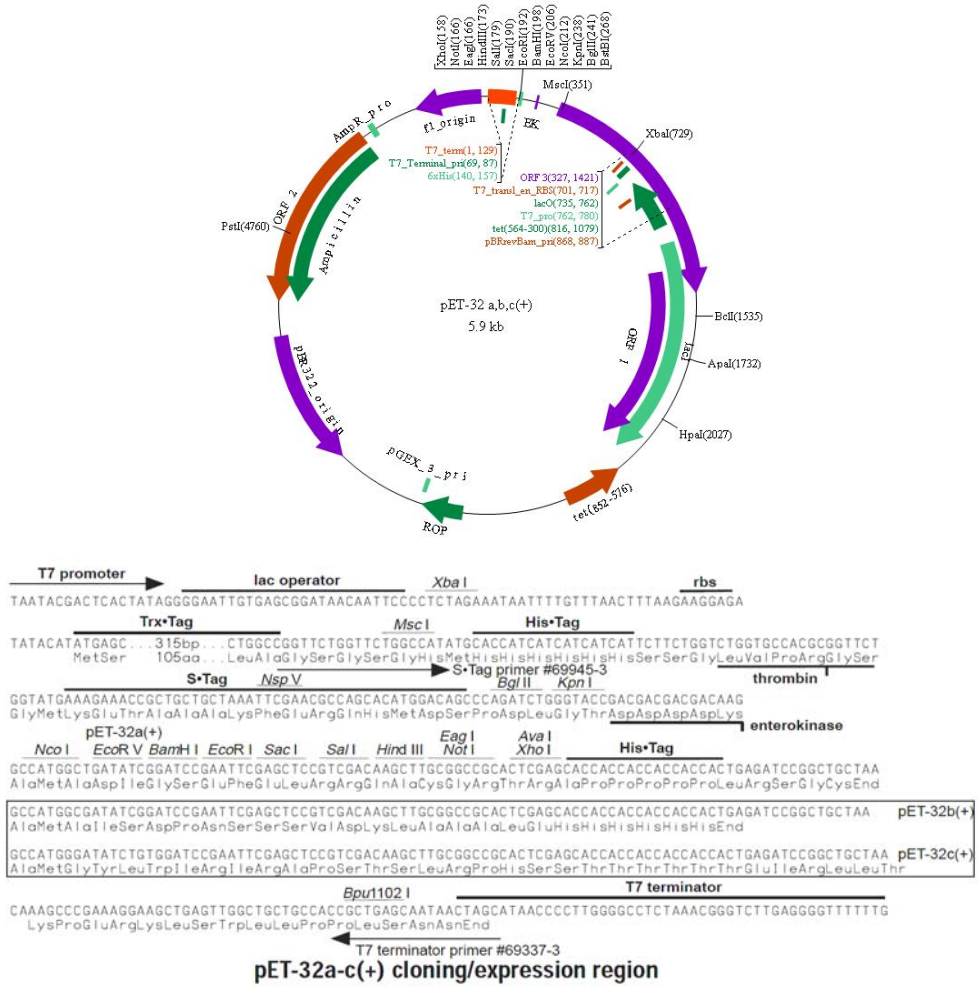


Fig.2. The vector map of pET32a(+) Translation vector with cloning and expression region in detail (http://www.biovisualtech.com/bvplasmid/ pET-32_a_b_c (+).htm)

Target genes are initially cloned using hosts lacking T7 RNA polymerase gene, thus avoid the plasmid instability due to protein toxicity. Once transferred into a non-expression host, target protein expression may be initiated either by infecting the host cell with λCE6 or by transferring the plasmid into an expression host containing T7 RNA polymerase gene under

lacUV5 control. Expression is also induced by the addition of IPTG to the bacterial culture containing the target protein.

The pET-32 series is developed for cloning and high-level protein expression of peptide sequences fused with the 109aa Trx•Tag™ thioredoxin protein. Multiple cloning sites (MCS) are available for producing fusion proteins also containing cleavable His•Tag® and S•Tag™ sequences for detection and purification of the fusion protein. The pET translation vector names are distinguished from the transcription vector names by the addition of a letter suffix following the name, e.g., pET-32a(+), which denotes the reading frame relative to the *Bam*H I cloning site recognition sequence, GGATCC. All vectors with the suffix "a" express from the "GGA" triplet, vectors with the suffix "b" express from the "GAT" triplet, and vectors with the suffix "c" express from the "ATC" triplet of the *Bam*H I recognition sequence. Vectors with a "d" suffix also express from the "c" frame but contain an upstream *Nco* I cloning site in place of the *Nde* I site in that series for insertion of target genes directly into the ATG start codon.

7.2.2 Plasmid Extraction of pJET 1.2 blunt end vector and pET32a (+) translation vector

pJET 1.2 vector and pET32a(+) vector were propagated in *E. coli* DH5α in LB medium with ampicillin (100 µg/µl) at 37°C with shaking at 230 rpm. Plasmid extraction was carried out as detailed earlier in the section 6.2.11.3 of Chapter 6.

7.2.3 Construction of pJET-Cel1: Subcloning of cellulase gene (Cel1) into pJET 1.2

The PCR product from a successful putative endoglucanase gene (1.37Kb) amplification reaction was selected as described in the previous

Chapter (section 6.2.10 **)** and preceded for ligation with pJET 1.2 blunt vector (Fermentas, GmBH, Germany) by following the manufacture's protocol. Briefly, the ligation mixture contained 0.5µl pJET vector (50 ng/µl), 3.4µl PCR Product, 1µl of ligation buffer (10X), 1µl of ligase (1U/µl) and the final volume was made upto 10µl with MilliQ. The ligation mixture was incubated at 22°C for 14 hours, which resulted in the ligation of PCR products with pJET 1.2 blunt ended vector, thus termed as pJET-cel1 construct.

7.2.3.1 Transformation into *E. coli* DH5α

The transformation was carried out as described earlier in section 6.2.11.1.

7.2.3.2 PCR confirmation of insert DNA in the selected clones

A random number of clones were selected and patched on LB/ampicillin plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using pJET vector primers. PCR mixture was prepared in a final volume of 25µl, which contained 2.5µl of 10X buffer, 2.5µl dNTP (2.5 mM), 1µl Taq DNA polymerase (0.5 U/µl), pinch of colony, 1µl of pJET1.2 Forward (5'CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2 Reverse (5'AAGAACATCGATTTTCCATG GCAG-3') primers each and 17µl MilliQ. The hot start PCR programme used for the amplification of full length endoglucanase gene was as followed; 95°C for 5 min followed by holding at 80°C, 35 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 45sec, extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. An aliquot of 5µl of PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide (EtBr), visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

7.2.3.3 Confirmation of insert orientation by PCR

Further confirmation of insert orientation in the positive clones was done by PCR using pJET1.2 Forward (5'-CGACTCACTATAGGGAGAGCG GC-3') and gene specific reverse primer of endoglucanase gene (R-ACGTCTCGACTAATTTGGTTCTGTTCCCC). PCR mixture was prepared in a final volume of 25µl, which contained 2.5µl of 10X buffer, 2.5µl of dNTP (2.5 mM), 1µl of Taq DNA polymerase (0.5 U/µl), pinch of colony, 1µl of pJET1.2 forward and gene specific reverse primers each and 17µl of MilliQ. The PCR programme (hot start) used for the amplification of complete gene was 95°C for 5 min followed by holding at 80°C for Taq polymerase addition, 35 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min, followed by final extension step at 72°C for 10 min. An aliquot of PCR product (5µl) was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

7.2.3.4 Propagation of confirmed colony and plasmid extraction

After confirmation of insert orientation, the transformed *E. coli* DH5α containing recombinant plasmids were propagated in 10 ml LB ampicillin (100 µg/µl) medium at 37°C with shaking at 230 rpm. Plasmid extraction was carried out as described previously in section 6.2.11.3 of Chapter 6.

7.2.4 Sequence analysis of full length putative cellulase gene (Cel1)

One of the positive plasmid was sequenced (at SciGenome Labs Pvt. Ltd, Cochin, India) and the sequence was submitted for BLAST (Nucleotide) analysis in NCBI database for detection of any mutational errors and in-frame of the gene.

7.2.5 Restriction digestion of pJET-cel1 construct to release the cel1 gene

The purified plasmid, pJET-cel1 was restriction digested with *Eco*R I and Xho I (New England Biolabs, UK) to release the cloned endoglucanase gene. The reaction mixture (20 µl) containing 5 µl of plasmid (pJET-cel1), 0.5 µl of *Eco*R I and *Xho* I (10,000 Units/ml), 2 µl of reaction buffer, 0.4µl of 100X BSA and 12.1µl MilliQ water, was incubated for 1 h at 37°C and the reaction was terminated by heat inactivation at 65°C for 20 min. The digested fragments were confirmed by 1% agarose gel electrophoresis.

The restriction digested and thus the released cel1 gene was gel extracted and purified using GenElute™ Gel extraction kit (Sigma, USA) following manufacturer's protocols. Briefly, the DNA fragment of appropriate size was excised from the agarose gel using GenElute™ Gel Extraction kit (Sigma, USA). The excised gel pieces were taken in a 2.0 ml tared tube, weighed and approximately 3 gel volumes of gel solubilisation solution was added, incubated at 60ºC for 10 min and gently vortexed the mixture in every 2 min. Approximately one gel volume of 100 % isopropanol was added to the incubated mixture and mixed well until it became a homogenous solution. The column preparation solution was added to the binding column, centrifuged at 12,000*g* for 1 min. Then the solubilised gel solution was loaded onto the already prepared binding column and was centrifuged at 12,000 *g* for 1min. An aliquot of wash solution (700 µl) was added and centrifuged for 1 min at 12,000 *g* .The washing step was repeated twice and residual wash solution was removed. The binding column was transferred to a fresh 2 ml collection tube and 50 µl preheated (65ºC) solution of 10 mM Tris-HCl (pH 9.0) was carefully added to the centre of the binding column and centrifuged at 12,000*g* for 1 min. The collected solution (purified preparation of Cel1) was stored at -20ºC.

7.2.6 Recombinant protein expression in pET32a(+) translation vector

The pET32a+ translation vector (Novagen, UK) was restriction digested with *Eco*R I and *Xho* I as explained in the previous section 7.2.5. Restriction digested plasmid was treated with CIP (Calf Intestinal Phosphatase) to remove the phosphate groups and thus to prevent self-ligation of the plasmid ends. The reaction mixture containing 20µl of digested plasmid (pET32a+), 0.1µl CIP enzyme and 5µl buffer was incubated at 37ºC for 60 min followed by heat inactivation at 65ºC for 20 min. Restriction digested and CIP treated vector was gel purified using GenElute™ Gel Extraction kit (Sigma, USA) as explained earlier in this chapter.

The gel purified and digested 'cel1' was ligated with digested pET32a+ translation vector (Novagen, UK) by following the manufacture's protocols. Briefly, 10 µl ligation mixture containing 1 µl of digested pET32a+ vector (50 ng/µl), 4.0 µl of digested PCR product (cel1), 1 µl ligation buffer (10X), 1 µl T4 DNA ligase (1U/µl) and 3.0 µl MilliQ was incubated overnight at 22°C. This yielded the ligation of PCR products with pET32a+ translation vector.

7.2.6.1 Transformation into *E. coli* DH5α

The transformation was performed as discussed earlier in the section 6.2.11.1.

7.2.6.2 PCR confirmation of insert DNA in the selected clones

Positively transformed clones (white) were randomly selected and separately patched onto LB-ampicillin (100 µg/ul) plates to reconfirm the transformation reaction. All individually streaked colonies were subjected for colony PCR using vector specific primers. The PCR mixture (25 µl)

contained, 2.5 µl 10X buffer, 2.5 µl dNTPs (2.5 mM), 1 µl Taq DNA polymerase (0.5 U/µl), pinch of colony, 1 µl each of T7-Forward (5'-TAATACGACTCA CTATAGGG-3') and T7-Reverse (5'-CTAGTTATTGCTCAGCGGTG-3') primers, was made up to 25 µl with sterile MilliQ. The hot start PCR programme used for the amplification of targeted gene consist of an initial denaturation step at 95°C for 5 min, followed by a hold at 80°C for adding Taq DNA polymerase and a sequence of steps such as denaturation at 94°C for 15 sec, annealing at 52°C for 20 sec, extension at 72°C for 1 min was repeated for 35 times, followed by a final extension step at 72°C for 10 min. An aliquot (5 µl) of final PCR products were analyzed by running an agarose gel (1%) electrophoresis and the gel was visualized by staining with ethidium bromide (EtBr) and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

7.2.6.3 Confirmation of insert DNA orientation by PCR

The orientation of inserted gene in the positive clones was confirmed by PCR using T7-Forward (5'-TAATACGACTCACTATAGGG-3') and gene specific reverse primer of endoglucanase gene (5'-ACGTCTCGACTAATT TGGTTCTGTTCCCC-3'). PCR mixture was prepared in a final volume of 25 µl, which contained 2.5 µl 10X buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq DNA polymerase (0.5 U/µl), a pinch of colony, 1 µl each of T7- Forward and gene specific reverse primer and 17 µl of MilliQ. The PCR programme (hot start) used for the amplification of complete gene was 95°C for 5 min followed by holding at 80°C for Taq DNA polymerase addition, 35 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min, followed by final extension step at 72°C for 10 min. PCR products (5 µl) were analyzed by 1% agarose gel electrophoresis, gel was stained with ethidium

bromide and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

7.2.6.4 Propagation of recombinant expression plasmid, pET-cel1 in *E. coli* DH5α and plasmid extraction for sequencing

The recombinant plasmid, pET-cel1 in *E. coli* DH5α was grown in 10 ml LB-ampicillin (100 µg/µl) medium at 37°C, with shaking at 230 rpm for 12 hours. Plasmid extraction was carried out as described earlier in this chapter. Plasmid (6 µl) was transformed into an expression host, *E. coli* Rosettagami (DE3) pLysS for further screening of recombinant endoglucanase (rCel1*)* expression.

7.2.6.5 Sequence analysis of pET-Cel1

The cloned plasmid was sequenced (at SciGenom Labs Pvt.Ltd, Cochin, India) and the sequence was subjected to BLAST search (Nucleotide) in NCBI database for confirmation of any kind of mutational errors and in-frame of the gene with the ATG of the pET32a(+) system.

7.2.7 Competent cell preparation using *E. coli* Rosettagami (DE3) pLysS

E. coli Rosettagami (DE3) pLysS cells were streaked on LB agar plate for obtaining single colonies. A single colony was inoculated in 10 ml LB medium and grown overnight at 37°C with shaking at 150 rpm. An aliquot of 5 ml of overnight culture was inoculated into 50 ml LB and incubated at 37°C for 2 hrs at 150 rpm. The re-inoculation helps to get *E. coli* cells in their log phase. The cells (50 ml) were centrifuged at 6000 rpm for 20 min at 4°C. All the steps were carried out at 4°C. The supernatant was decanted and cells were re-suspended by gentle vortexing with 0.1 M $CaCl_2$ ($1/4^{th}$ original culture volume). The re-suspended cells were placed on ice for 45 min with intermittent stirring and mixing. The cells were centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was decanted and cell pellet was re-suspended in 1 ml of 0.1M

$CaCl_2$. The competent cells formed were stored at ⁻80°C with addition of 10-12 % glycerol.

7.2.8 Transformation into *E. coli* Rosettagami (DE3) pLysS

The *E. coli* Rosettagami (DE3) pLysS competent cells were thawed by placing on ice for 5 min. An aliquot (5 µl) of positive plasmid construct, pET-cel1 was added into a sterile 15 ml culture tube already placed on ice. Competent cells (100 µl) were added to the 15 ml tubes (containing pET-cel1constructs) on ice. The tube was gently flicked to mix and placed on ice for further 20 min and a heat shock was given at 42°C for 90 sec in a water bath. The tube was immediately returned to ice for 2 min. An aliquot of 600 µl super optimal broth with catabolite repression (SOC - 0.2 g tryptone; 0.05 g yeast extract; 0.005 g NaCl ; 100 µl 1M KCl ; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose) was added to the tube .The tube was incubated for 90 minutes at 37°C with shaking at 230 rpm in an orbital shaker . After the transformation reaction was completed, 100 µl of cells were plated in duplicate/triplicate on to LB (ampicillin 100 µg/µl, kanamycin 15 µg/ml, tetracyclin 12.5 µg/ml, chloramphenicol 34 µg/ml) plates. The plates were incubated overnight (14 hrs) at 37°C.

7.2.9 Expression of fusion protein (rCel1) in *E. coli* Rosettagami (DE3) pLysS

Recombinant pET-cel1 constructs in *E. coli* Rosettagami (DE3) pLysS was inoculated into 3 ml LB/ampicillin (100 µg/µl) medium and incubated at 37°C at 250 rpm until the OD_{600} of 0.5 was obtained. *E. coli* Rosettagami (DE3) pLysS without vector and with pET32a+ vector were also processed as a control. The whole culture (3 ml) was added to 100ml LB (ampicillin 100 µg/µl, kanamycin 15 µg/ml, tetracyclin 12.5 µg/ml , chloramphenicol 34 µg/ml) medium and further incubated at 37°C for 2-3 hrs until OD_{600} of 0.6

(Optimum OD for protein expression) was reached. An aliquot of 5 ml culture was aseptically transferred into another culture tube just before the induction and labelled as un-induced control. Then, the expression of recombinant fusion protein was induced by adding IPTG to a final concentration of 0.2 mM, 0.5 mM , 0.8 mM and 1mM . After an additional 12 h of growth at 30°C with shaking at 220 rpm, the bacterial cells were separated by centrifugation at 12,000 rpm for 5 min, washed with ice-cold PBS and stored at -80°C until further processing. Over expression pattern of the fusion protein (rCel1) was confirmed through 12% SDS-PAGE analysis.

7.2.10 Cellular localization of rCel1 fusion-protein

Cellular localization of the fusion protein rCel1 was carried out by analysing the extracellular fraction (medium fraction), intracellular periplasmic fraction and cytoplasmic fractions (soluble and insoluble fractions). Extracellular protein was obtained by harvesting the IPTG-induced (1mM) culture by centrifugation at 10,000*g* for 10 min at 4°C. After centrifugation, the supernatant solution was transferred into a new tube and stored at 4°C till analysis. The remaining pellets were used for intracellular periplasmic protein preparation.

The pelleted cells were re-suspended in periplasming buffer (200 mM Tris-HCl, pH 7.5; 20% sucrose; 1 mM EDTA; 2.5 million units of lysozyme (0.00625 g/5 ml) and incubated at 37^0C for 30 min followed by an addition of ice-cold H_2O with a volume corresponding to 6 ml/g (wet weight) of the initial cell pellet. The re-suspended cells were then incubated on ice for 10 min, centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant solution represented the periplasmic fraction was transferred into a sterile tube and stored at 4^0C for cellulase assays. The remaining pellet was used for cytoplasmic protein preparation with two lysis buffers for the release of the cytoplasmically-

expressed recombinant protein. Lysis buffer 1(50mM KH_2PO4 - pH 7.8, 400mM NaCl, 100mM KCl, 10% Glycerol, 0.5% Triton X-100, 10mM Imidazole) was added for a volume calculation of 200µl for 4ml pelletized culture. A sonication cycle of 20 pulses with 1min gap was repeated for 5 times. The cells were pelletized at 6000 rpm for 10 min at 4°C. The supernatant was collected and stored at 4°C for cellulase activity assays. To the lysed pellet was added lysis buffer 2 (31.25 mM Tris buffer pH 6.8, 25% glycerol, 10% SDS). The sonication cycle was repeated as mentioned for lysis buffer 1. The cells were pelletized at 6000 rpm for 10 min at 4°C. The supernatant was separated and stored at 4°C for cellulase activity assays. The resulting supernatants from the two lysis buffers (lysis buffer 1 and lysis buffer 2) represented cytoplasmic soluble and insoluble fraction respectively. The cellular localization of recombinant fusion protein (rCel1) was determined by assaying cellulase activity (Ghose, 1987) as described earlier in the section 2.2.6.1 of Chapter 2.

The activity fraction showing highest cellulase activity was further analyzed for recombinant protein expression by Coomasie stained 12 % SDS-PAGE. An aliquot of 10 µl of lysis 1 and lysis 2 supernatants (cytoplasmic proteins) of the induced and uninduced samples were heated at 65^0C in 10 µl sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.2% bromophenol blue) for 5 min. The samples were given a short spin and supernatant was subjected to 12.5% reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis following the method of Laemmli (1970). The protein was separated and analyzed using 5% stacking gel and 12% resolving gel prepared into 10×10.5cm vertical gel plate of mini vertical electrophoresis unit (Hoefer-Amersham, USA). Electrophoresis was performed in 1X Tris-glycine SDS (pH8.3) buffer at a voltage of 12 mA (EPS 301, Amersham, USA). After electrophoretic separation, gel was stained in coomassie brilliant blue

stain R-250 (0.025 % coomassie brilliant blue R-250, 40% methanol and 7% acetic acid in distilled water), de-stained in de- staining solution 1(40% methanol and 7% acetic acid in distilled water) and de-staining solution II (5% methanol and 7% acetic acid in distilled water), and photographed using Gel-DOCTM XR+ imaging system (BioRad, USA). Recombinant protein expression was determined by comparing the presence and absence of protein profile with that of uninduced cells. Molecular weight of protein was determined by comparing with that of standards (PMWM- Genei, India).

7.2.11 Screening of pH based rCel1protein solubility

The solubility of recombinant protein was optimized by varying the pH conditions (pH 5.0 - pH 9.0) of the modified lysis buffer containing 50 mM KH_2PO_4 (pH 7.8), 400 mM NaCl, 100 mM KCl, 10% Glycerol, 0.5% Triton X-100, 10 mM Imidazole, lysozyme (1mg/ml), Phenylmethylsulfonyl fluoride (PMSF, 20 μg/ml). The pH at which the protein preparation showing increased solubility was taken as the appropriate lysis buffer pH for extraction of recombinant fusion protein (rCel1) from *E. coli* Rosettagami (DE3) pLysS. Solubilized protein yield was calculated with respect to fully solubilised protein by boiling at 100^0C.

7.2.12 Purification of rCel1 using Metal affinity Chromatography (IMAC)

The Ni-NTA Purification System is designed for purification of recombinant fusion proteins and it shows high affinity and selectivity for proteins that are tagged with six tandem histidine residues. Ni-NTA superflow cartridges (Qiagen) pre-filled with Ni-NTA Agarose, which uses nitrilotriacetic acid (NTA), a tetradentate chelating ligand, in a highly cross-linked 6% agarose matrix.

The decision whether to purify 6xHis-tagged proteins under native, denaturing or hybrid conditions totally depends on the solubility, cellular location of the protein and the maintenance of biological function for downstream applications. Instead of directly selecting a purification method, all the three methods such as purification of rCel1 under native conditions (varying imidazole concentration), denaturing conditions (presence of 8M Urea with varying pH of buffers) and hybrid conditions were carried out in order to achieve an optimum purification of targeted protein (rCel1).

7.2.12.1 Manual purification of 6xHis-tagged recombinant protein, rCel1 under native conditions

The cell pellet was resuspended in native lysis buffer containing 50mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole (pH 8.0), 1 mg/ml lysozyme and 1mM PMSF and lysed by sonication on ice to avoid over-heating of the sample. DNase I (5 µg/ml) and RNase A (10 µg/ml) were added to the sample to reduce viscosity. The lysate was then centrifuged at 10,000*g* for 25 min at 4^0C to recover the supernatant containing His-tagged proteins. Then the Ni-NTA column was equilibrated with 10 column volumes of native lysis buffer at a flow rate of 1 ml/min. The cleared lysate was then applied to the pre-equilibrated column containing Ni-NTA agarose (Qiagen Inc.). The column was then allowed to drain by gravity flow and target protein binding was maximized by reapplying the flow through to the column at a flow rate of 1 ml/min. The cartridge was washed with 20 ml of native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 50 mM Imidazole (pH 8.0) to remove the contaminating proteins. The His-tagged protein was then eluted with 10 ml of native elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 50 - 250 mM Imidazole (pH 8.0). All fractions were collected for SDS-PAGE analysis.

7.2.12.2 Manual purification of 6xHis-tagged recombinant protein, rCel1 under denaturing conditions

The cell pellet was resuspended in denaturing lysis buffer B (8 M Urea, 100 mM NaH_2PO_4, pH 8.0) and 1mM PMSF and lysed by sonication on ice to avoid overheating of the sample. DNase I (5 μg/ml) and RNase A (10 μg/ml) were added to the sample to reduce viscosity. The lysate was then centrifuged at 10,000*g* for 25 min at 4^0C to recover the supernatant containing His-tagged proteins. Then the Ni-NTA column was equilibrated with 10 column volumes of denaturing lysis buffer B at a flow rate of 1ml/min. The cleared lysate was then applied to the pre-equilibrated column containing Ni-NTA agarose (Qiagen Inc.). The column was then allowed to drain by gravity flow and target protein binding was maximized by reapplying the flow through to the column at a flow rate of 1ml/min. The cartridge was washed with 20 ml of buffer C (wash buffer-8 M Urea, 100 mM NaH_2PO_4, pH 6.3) at a flow rate of 1 ml/min to remove contaminating proteins. The His-tagged protein was then eluted with 10 column volumes of buffer E (elution buffer- 8 M urea, 100 mM NaH_2PO_4, and pH 4.5). All fractions were collected for SDS-PAGE analysis.

7.2.12.3 Manual purification of 6xHis-tagged recombinant protein, rCel1 under hybrid conditions

The cell pellet was re-suspended in denaturing binding buffer with 8M urea (100 mM NaH_2PO_4, 100 mM Tris, 200 mM NaCl with 8 M urea; pH 8), 1 mg/ml lysozyme and 1 mM PMSF and lysed by sonication on ice to avoid over-heating of the sample. DNase I (5 μg/ml) and RNase A (10 μg/ml) were added to the sample to reduce viscosity. The lysate was then centrifuged at 10,000 *g* for 25 min at 4^0C to recover the supernatant containing His-tagged proteins. Then the Ni-NTA column was equilibrated with 10 column volumes of denaturing binding buffer at a flow rate of 1ml/min. The cleared lysate was then applied to the pre-equilibrated column containing Ni-NTA agarose (Qiagen

Inc.). The column was then allowed to drain by gravity flow and target protein binding was maximized by reapplying the flow through to the column at a flow rate of 1ml/min. The cartridge was washed with 20 ml of native wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 - 100 mM Imidazole (pH 8.0) to remove the contaminating proteins. The His-tagged protein was then eluted with 10 ml of native elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 - 500 mM Imidazole (pH 8.0). All fractions were collected for SDS-PAGE analysis.

7.2.13 Concentration and refolding of recombinant fusion protein (rCel1)

The Ni-NTA purified fractions of rCel1 was concentrated using Amicon Ultra -15 (Millipore, USA) desalting column. Aliquots of 4 ml samples were loaded onto the columns and centrifuged at 4000*g* for approximately 10 min. The step was repeated to concentrate the 10-15 ml of the purified recombinant proteins to 0.5 ml-1ml. The concentrated solute was recovered by inserting a gel loading pipette tip into the bottom of the concentrating filter device. The sample was then analyzed on 12% SDS-PAGE to check the purity of the purified protein.

The concentrated sample was reconstituted to the original volume using the refolding buffer (50 mM Tris-Cl pH 8, 0.1 mM EDTA and 0.15 M NaCl) and centrifuged at 3000-4000*g* maximum for approximately 20 min. The concentrated sample was given 5-6 washes with the refolding buffer to remove denaturing salts and thus to refold the protein to its biologically active form.

7.2.14 Qubit analysis of the recombinant protein

The concentration of recombinant protein (rCel1) was quantified using the Quant-iT^{TM} protein assay kit using Qubit fluorometer (Invitrogen, UK). It makes the protein quantification easy and precise with high sensitivity in detecting sample concentrations from 12.5 μg ml^{-1} to 5 mg ml^{-1} and displays

low protein-to-protein variation. The assays were carried out at room temperature, and the signal was stable for 3 hrs.

The Quant-iT working solution was prepared by diluting Quant-iT protein reagent in Quant-iT protein buffer in 1:200 ratio. The Quant-iT working solution was mixed well without trapping any air bubbles and an aliquot of 199 µl was added to 0.5 ml tubes. An aliquot of 1 µL of sample (rCel1) was added to 199 µL of working solution. The final volume was made as 200 µL in thin-walled, 0.5ml qubit assay tubes, followed by vortexing and incubating for 15 min at room temperature. The intensity of the sample after binding with the fluorescent dye was read by Quibt 2.0 Fluorometer (Invitrogen). It showed values of protein in µg/ml. The sample concentration was calculated using the following equation: Concentration of sample = QF value x (200/X) where QF value = the reading given by the Qubit 2.0 Fluorometer, X = sample volume (µl) added to the assay tube.

7.2.15 Functional assay for expression of rCel1

To detect functional expression of the recombinant Cel1, cell lysates were prepared from control *E. coli* cells (without pET-Cel1), uninduced pET-cel1 and induced (1mM IPTG) pET-cel1 as described in the section 7.2.10. Further, the cell lysates were assayed for the protein concentration using Quibt fluorometer method as described in the section 7.2.14. Endoglucanase (CMCase) activity assays were carried out as mentioned in the section 2.2.6.1 of Chapter 2. In addition, a quick and sensitive assay system using CMC-Congored detection was followed for the phenotypic identification of the cell lysates. Briefly, an aliquot of 25µl (0.8 µg/µl) cell lysates were spotted into 0.5% (w/v) CMC supplemented LB agar plates followed by incubation for 30 min at 37^0C. After incubation, the plate was flooded with an aqueous solution of congo red (0.2% w/v) for 10 min. Cellulase activity was indicated by an yellow halo zone against a red background.

7.2.16 Bio-chemical characterisation of rCel1

7.2.16.1 Effect of pH on stability and activity of rCel1

The optimum pH of the recombinant cel1was determined by incubating 25 µl of rCel1 (0.8 µg/µl) with 1% (w/v) CMC in the presence of various buffers (100 mM) of pH in the range of 3-10; citrate buffer (pH 3 to 6), phosphate buffer (pH 7 to 8) and glycine – NaOH buffer (pH 9 to 10) for 60 min at 25^{o} C. Further, pH stability was evaluated by pre-incubating the recombinant protein in the presence of various pH (3-10) buffers for 6 h at 25°C followed by measuring the residual enzyme activity as described in the section 3.2.6.5.

7.2.16.2 Effect of temperature on enzyme stability and activity

The optimum temperature of the rCel1 was measured by incubating 25 µl of rCel1 (0.8 µg/µl) with 1% (w/v) CMC in 0.05 M citrate buffer (pH 5) at different temperatures ranging from 25 to 65^{o} C for 1h. Temperature stability was determined by pre-incubating rCel1 in 0.05 M citrate buffer (pH 5) at various temperatures (25 to 65^{o}C) for 6h. The residual enzyme activity was determined as described in the section 3.2.6.5. One international unit (U) corresponds to the release of reducing sugar at 1µmol/min.

7.2.16.3 Effect of various metal ions on the stability of rCel1

The effect of various metal ions on rCel1 stability was evaluated by pre-incubating 25 µl of rCel1 (0.8 µg/µl) in 0.05 M citrate buffer (pH 5) in the presence of 5 mM concentrations of different metal ions at 45^{o}C for 6 h followed by measuring the endoglucanase activity with 1% (w/v) CMC under standard assay conditions (2.2.6.1). The metal ions used in the study were calcium chloride (Ca^{2+}), cadmium sulphate (Cd^{2+}), cupric sulphate (Cu^{2+}) , ferric chloride (Fe^{3+}), magnesium chloride (Mg^{2+}), manganese chloride

(Mn^{2+}), sodium sulphate (Na^{+}), mercury chloride (Hg^{2+}) , potassium chloride (K^{+}), and zinc sulphate (Zn^{2+}). The residual enzyme activity was calculated as described in the section 3.2.6.5 and the activity of control, in the absence of metal ions was taken as 100%.

7.2.16.4 Effect of various additives on rCel1 stability

An aliquot of 25 µl rCel1 (0.8 µg/µl) in 0.05 M citrate buffer (pH 5) was pre-incubated in the presence of 1% Tween 20, Tween 80,Triton X-100, β-mercaptoethanol (2-ME), Sodium dodecyl sulphate (SDS), Ethylenediamine tetra acetic acid (EDTA) and PEG 6000 at 45^{0}C for 6 hrs. Thereafter endoglucanase (CMCase) activity of rCel1 was determined with 1% (w/v) CMC under standard assay conditions. The residual enzyme activity was calculated as mentioned in the section 3.2.6.5 and the activity of control, in the absence of metal ions was taken as 100%.

7.2.16.5 Effect of NaCl concentration on rCel1 stability

An aliquot of 25 µl rCel1 (0.8 µg/µl) in 0.05 M citrate buffer (pH 5) was pre-incubated in the presence of various NaCl concentrations at 45^{0}C for 6 hrs. Thereafter endoglucanase (CMCase) activity of rCel1 was determined with 1% (w/v) CMC under standard assay conditions. The residual enzyme activity was calculated as given in the section 3.2.6.5 and the activity of control, in the absence of metal ions was taken as 100%.

7.3 Results and Discussion

7.3.1 Blunt-end vector construction of Cel1 gene in pJET1.2 and Sequence analysis

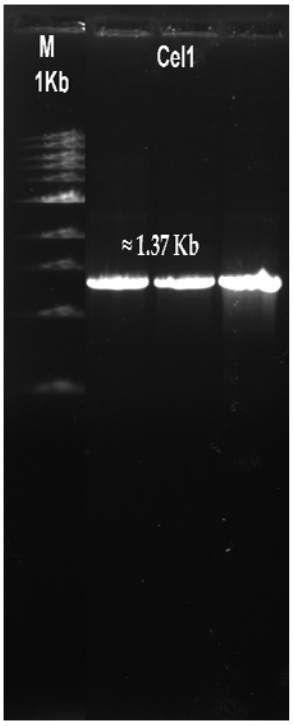
The full-length cellulase gene (≈1.37Kb) was amplified from the phagemid DNA template of Vmg-Eg-3 clone using *Pfu* DNA polymerase using

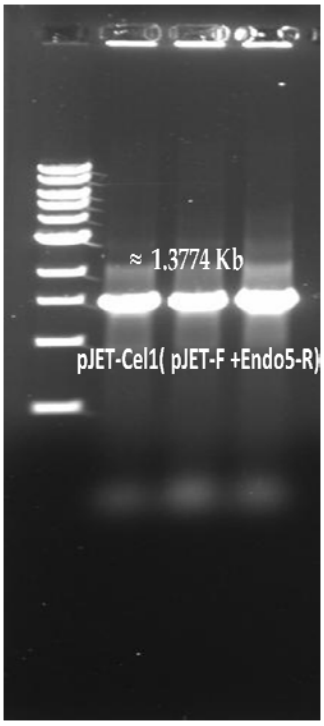
the primer set (Endo-5) as described in the previous Chapter (section 6.2.10). An aliquot of 5µl of PCR product was separated by 0.8% agarose gel electrophoresis and visualized by staining with ethidium bromide (Fig.3). The predicted 1.37 Kb gene fragment with added restriction sites was cloned into blunt end pJET1.2 vector (2.974 Kb) for constructing blunt end vector constructs. The construct was named based on the classification of cellulase gene in the glycoside hydrolase family. Accordingly, recombinant pJET plasmid with cellulase gene of glycoside hydrolase family 1 was designated as pJET-Cel1 (Fig.6). *Pfu* DNA polymerase works as a proofreading enzyme and thus there will not be any insertion or deletion of bases which will change the frame of the inserted gene. After ligation and transformation with *E. coli* DH5α, the orientation of gene in the recombinant clones was confirmed by colony PCR with pJET-forward primer and gene specific reverse primer (Fig.4) and the product size was found to be approximately 1.3774 Kb (vector region spans approximately 62 bp). The positive plasmids were then propagated overnight at 37ºC in LB broth supplemented with ampicillin (100 µg/ml) for plasmid DNA isolation (Fig.5). The plasmid DNA was then digested using *EcoR* I and *Xho* I and the products were analyzed on 0.8% agarose gel (Fig.8). One of the positive plasmids was sequenced (at SciGenome Labs Pvt .Ltd, Cochin, India) and the sequence was subjected to BLAST search (Nucleotide) in NCBI database for confirmation of any kind mutation errors and in-frame of the genes and is represented in Fig.7. The FASTA format of sequence of Cel1 was translated into amino acid output using the Translate tool of ExPASy (SIB Bioinformatics Resource Portal). The translated protein sequence was analysed in the NCBI Blastp suite and confirmed the identity of the genes in comparison with published sequences resulted in up to 60% aminoacid similarity with an uncultured clone endoglucanase gene. The nucleotide sequence, ExPASy

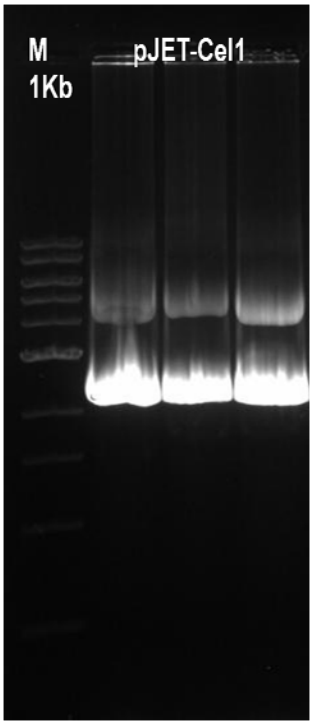
translated protein sequence and the 3D SWISS-MODEL were given in the Chapter (6) in Figures 62, 63 and 64.

7.3.2 Construction of recombinant translation vector system for recombinant protein expression

Sequenced plasmid (pJET-Cel1) with a size of approximately 4.3452 Kb was digested using the restriction enzymes *EcoR*1 and *Xho*I and sequence analysis showed that there was no addition of any vector sequence, thus preserving the frame of the insert. The expression vector pET32a(+) was also digested using the same enzymes (*Eco*RI/*Xho*I) as that included in the primers of the insert. The digested product was separated by 0.8 % agarose gel electrophoresis and visualized using ethidium bromide gel staining (Fig.9). The gel purified fragments of digested Cel1(Fig.10) was then expression-cloned into *Eco*RI/*Xho*I digested pET32a(+) vector for the construction of translation expression system, which was named as pET-Cel1 and it was transformed into *E. coli* DH5α .The positive clones were screened through blue/white colour selection and further confirmed using the vector primers (T7-F and T7-R) as shown in Fig.11 and the orientation of the inserts in the expression vector constructs were confirmed by colony PCR with T7-Forward primer and gene specific reverse primer (Fig.12). The positive plasmids were then propagated overnight at 37ºC in LB broth supplemented with ampicillin (100 μg/ml) for plasmid DNA isolation. Further, the in-frame of the insert in the expression vector construct (pET-Cel1) was confirmed by sequencing the positive plasmid isolated (Fig.15). The expression vector construct of pET-Cel1 (7.271Kb) was shown in Fig.14.







(Fig. 3) **(Fig. 4)** **(Fig. 5)**

Fig.3. Pfu amplified putative endoglucanase gene (≈ 1.37 Kb)
Fig.4. Colony PCR – Insert orientation confirmation by pJET-F+ Endo5-R
Fig.5. Plasmid extracted from the positive clones of pJET blunt end vector constructs (pJET-Cel1)

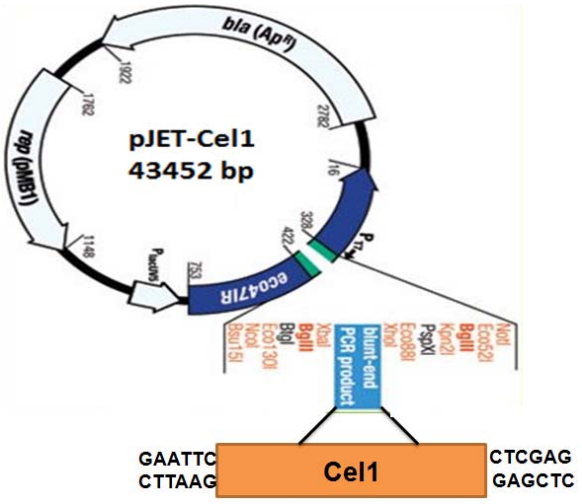


Fig.6. pJET-Cel1 vector (4.3452 Kb)

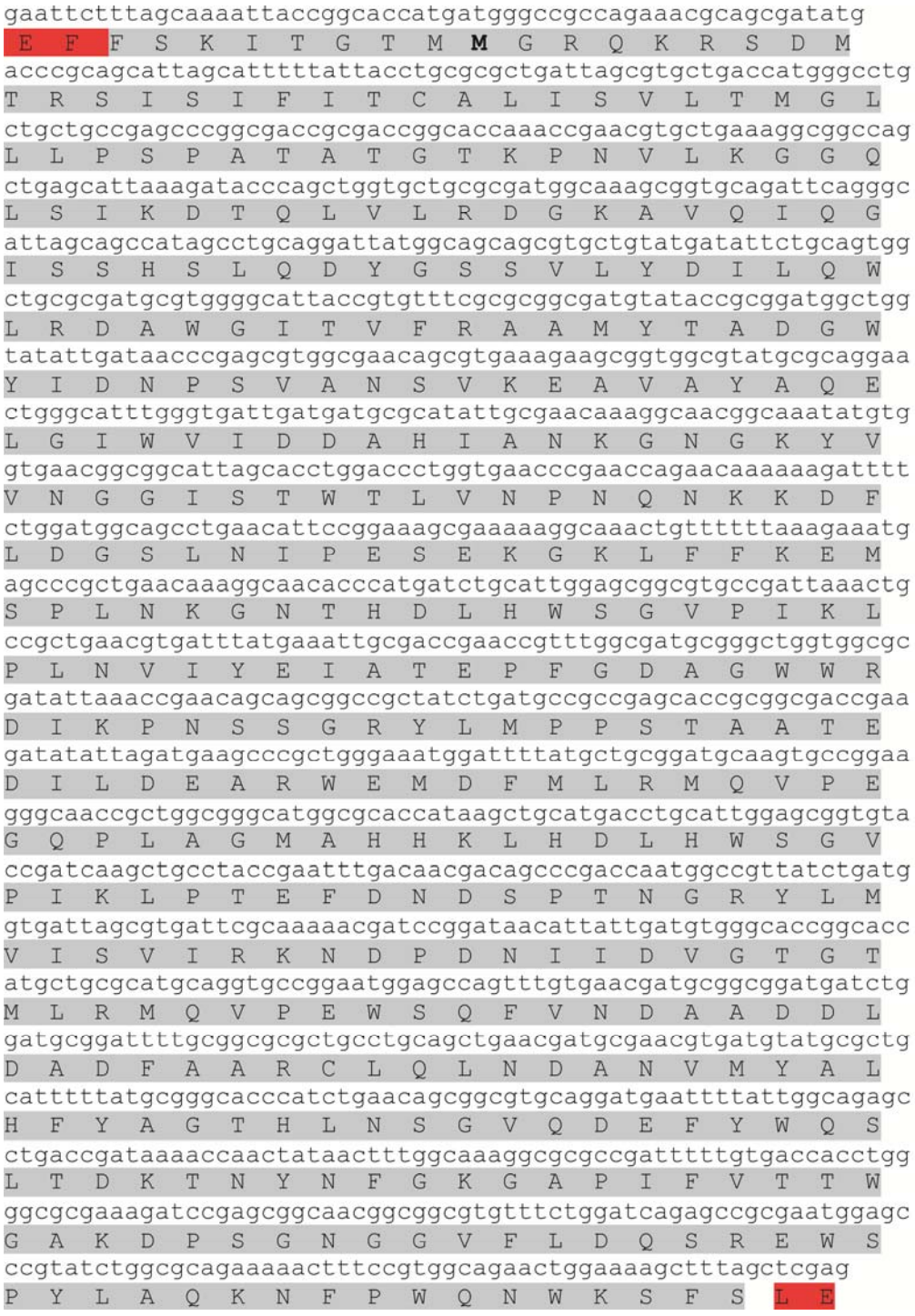
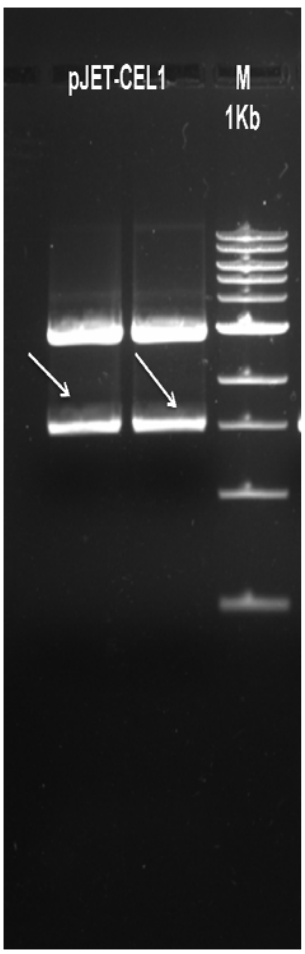
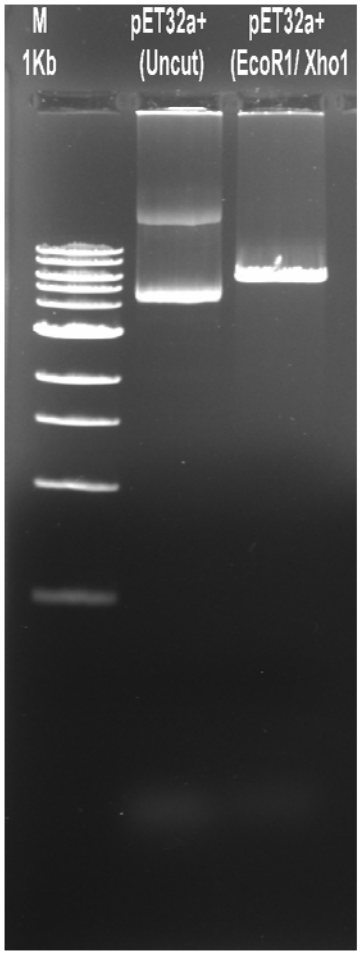
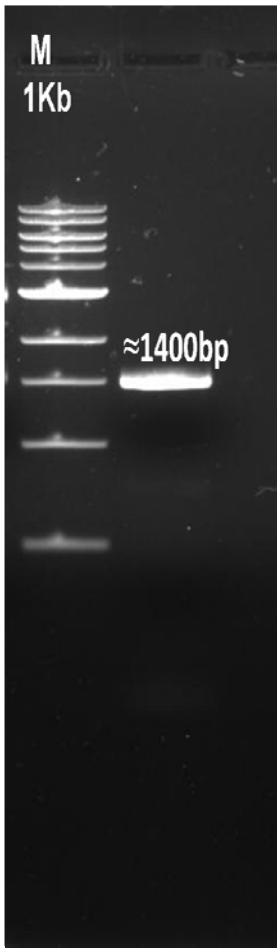


Fig.7. In-frame sequence of Cel1 in pJET-Cel1 vector construct with 5' *Eco* R1 and 3' *Xho1*

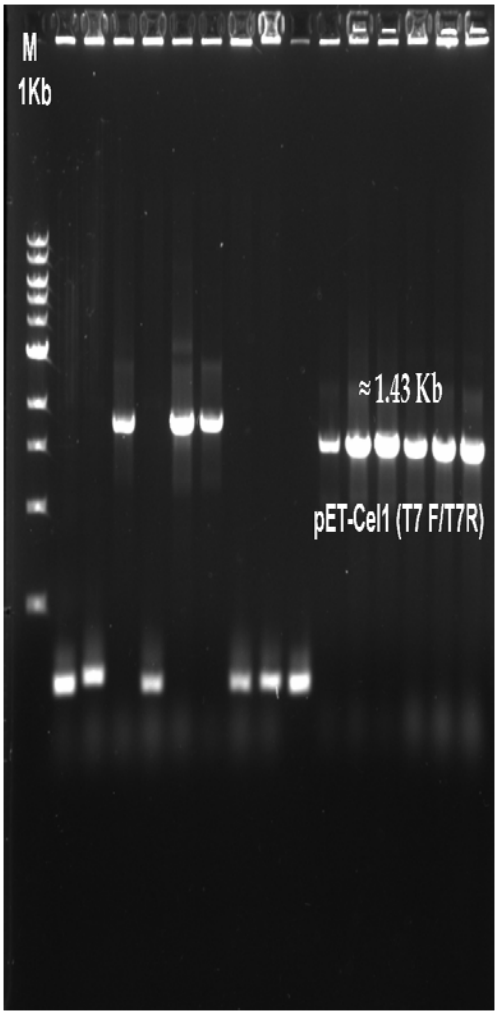


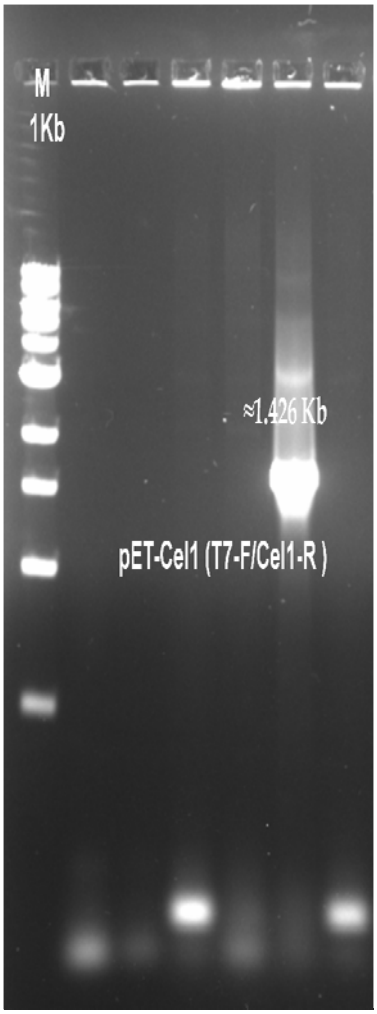




(Fig. 8) **(Fig. 9)** **(Fig.10)**

Fig.8. EcoR I-Xho I restriction of Cel1from pJET-Cel1
Fig.9. EcoR I-Xho I restriction of pET32a(+)
Fig.10. Gel purified Cel1gene





(Fig. 11) **(Fig.12)**

Fig.11. Colony PCR of Cel1 in pET32a(+) with T7-F and T7-R primers (vector specific primers)

Fig.12. Orientation of insert Cel1 in pET32a(+) with T7-F and gene specific reverse primer

7.3.3 Expression of the recombinant fusion protein (rCel1) using IPTG induction

To characterise the functional expression of recombinant Cel1, it was expressed as an N-terminal His-tagged fusion protein using pET32a(+) translation expression system under the control of T7 lac promoter in *E. coli* Rosettagami DE3 pLysS. Before induction with IPTG, the over-expression of Cel1 was optimized with two different incubation temperatures at 30^0C and 37^0C, since the expression characteristics of each targeted gene product may vary. The growth of *E. coli* containing rCel1 at 37^0C resulted in a high-level protein expression and therefore accumulated as protein-inclusion bodies, whereas growth at 30^0C resulted in highly soluble and active protein (Schein and Noteborn, 1989) and also , it was found to be the optimal condition since the target gene (Cel1) contained leader signal sequences.

To examine the functional expression pattern of the rCel1, the recombinant clones were propagated in the LB medium followed by induction with different concentrations of IPTG (0.2, 0.5, 0.8 and 1mM) for overnight induction at 30°C for the optimal yield of soluble protein. The IPTG concentration required for an optimum recombinant protein expression was found to be 1mM and was subsequently selected for further analysis. The SDS PAGE protein expression profile of IPTG induction is shown in Fig.18. Cellular location of the rCel1 was determined by assaying CMCase activity in the extracellular (medium fraction), periplasmic fraction and cytoplasmic soluble as well as insoluble fractions. Highest CMCase activity was observed in cytoplasmic soluble fraction (3.841 U/ml) followed by periplasmic fraction (1.351U/ml) and negligible activity was observed in the extracellular medium fraction (0.085 U/ml). In addition, no inclusion bodies were formed in *E. coli*

cell lysates carrying recombinant Cel1 plasmids, suggested that the target protein was expressed only as cytoplasmic soluble form.

7.3.4 Optimizing the solubility of rCel1 based on pH of modified lysis buffer

Screening of different pH (5 - 9) was done to identify the optimum pH favourable for solubilising recombinant fusion protein (rCel1) and the results were represented in Table 1. The optimum solubilisation of the target protein was attained with modified lysis buffer at pH 8.0, with a total protein yield of 82%, whereas at a higher pH (pH 9.0) than its optimum, could not proportionately able to yield a higher protein concentration. Modified lysis buffer with lower pH (pH<8.0) showed poor solubility pattern when compared to the completely solubilised protein (complete solubilisation by boiling at 100^0C). The highly acidic pH (pH<7) perhaps worked the worst, when compared to all other pH. From these results, it could also be concluded that pH had a greater effect on protein solubility. Lindwall et al. (2000) had also studied the effect of different pH on protein solubility. Therefore, the modified lysis buffer at pH 8.0 was selected as the suitable pH for maintaining the solubility of recombinant fusion protein (rCel1) after cell lysis.

Table1. Optimizing solubility of rCel1 at different pH

Tris Buffer pH	Protein yield Micro BCA Assay (%)
5	15
6	35
7	70
8	82
9	85

Further recombinant clones containing Cel1plasmids were induced at different IPTG concentrations (0.2, 0.5, 0.8 and 1mM) for overnight induction at

30°C and cytoplasmic soluble fractions were extracted using modified lysis buffer (pH 8.0) from both induced and uninduced *E. coli* strains carrying rCel1. Prolonged induction time (12 hrs) was found to be suitable to maximize the expression of His-tagged protein compared to very less induction times of 3-4 hours. The protein expression pattern was further verified by SDS–PAGE as shown in Fig.18. The analysis of the crude protein extracts from all the induced cells containing pET-Cel1 (0.2, 0.5, 0.8 and 1mM), showed a high-level expression of 6xHis tagged proteins, as noticed by the presence of a protein band migrating at about 68 KDa when compared to uninduced *E. coli* carrying pET-Cel1 and negative control of *E. coli* with empty vector (Fig.18). The recombinant fusion protein molecular weight was found to be corresponding to the predicted molecular mass of Cel1 gene (50 KDa) plus molecular weight of fusion protein from the vector (20 KDa). However, no positive band was observed in the negative control *E. coli* Rosetta DE3 pLysS cells with an empty vector and a very low abundant expression of rCel1in the uninduced *E. coli* with pET-Cel1 (Fig.18, lane 1and 2) These results helped us to conclude that the expected recombinant Cel1 gene had the correct reading frame with histidine tag at the N-terminus.

In conclusion, the factors that had shown to enhance protein solubility were expression at very low temperatures, IPTG concentration, induction time, buffer pH and additives such as salts, ionic compounds, glycerol and detergents (Bondos and Bicknell, 2003). However, the solubility of recombinant Cel1was found more influenced by incubation temperature (30^{o}C) and induction time period (12 h) compared to other parameters mentioned above.

7.3.5 Purification of rCel1 using Ni-NTA Metal affinity Chromatography

Purification of recombinant fusion protein (rCel1) was performed using native, denaturing and hybrid conditions by IMAC (Immobilized Metal ion-

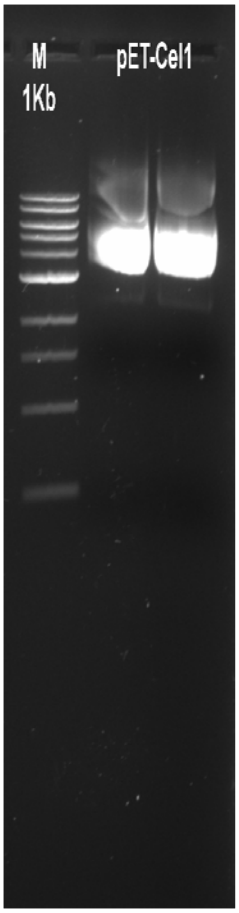
Affinity Chromatography). The use of mild buffer conditions and imidazole as the eluant often yields biologically active purification products. Proteins that remain soluble in the cytoplasm, or that are secreted into the periplasmic space can be purified using native conditions. If the protein is in insoluble form or aggregated as inclusion bodies, it must be solubilized under denaturing conditions before purification was performed. But in some exceptional cases, i.e., if the protein is soluble and the His-tag is occluded by protein tertiary structure thus the soluble protein required a denaturation step prior to binding to the Ni-NTA resin. Proteins usually purified under denaturing conditions (6 M guanidinium hydrochloride or 8 M urea) can then be refolded into their biologically active states by desalting the denaturants present in the eluted fractions (Wingfield, 1995).

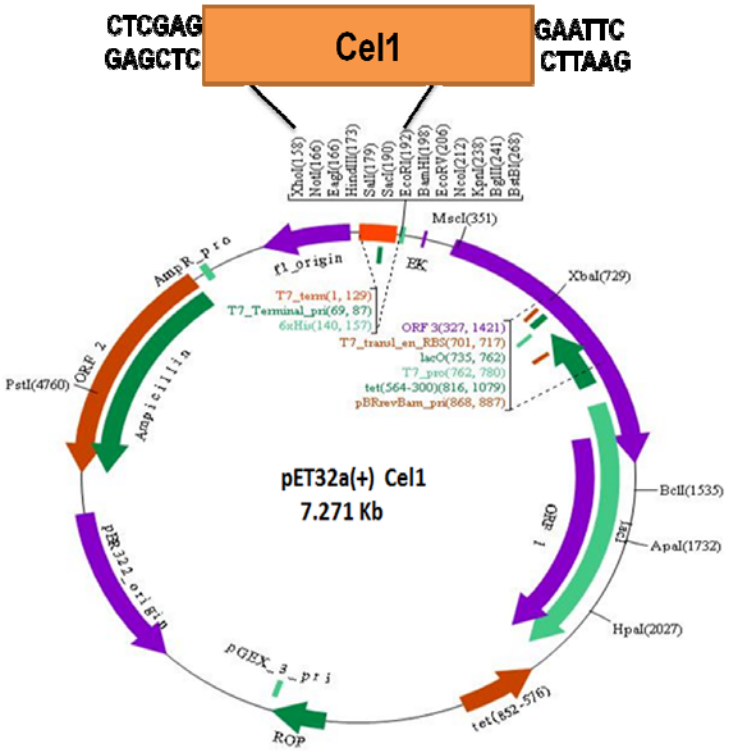
Following optimization of soluble His-tagged Cel1expression, it was decided to initially purify through Ni-NTA affinity column under native conditions since the expressed protein was found to be soluble. Cell pellet lysed with native lysis buffer was further processed to remove other contaminating (non-specific) *E. coli* native proteins from binding into the Ni-NTA agarose matrix before elution of His-tagged rCel1.The concentration of imidazole at 20mM in the wash buffer was not found satisfactory for removing non-specific binding proteins. SDS-PAGE analysis of the eluted fractions at 50mM, 100 mM and 250mM imidazole concentrations revealed that only a few contaminating proteins had been removed from the Ni-NTA column, while all other non-target proteins were also eluted from the column upon elution with higher concentrations of imidazole (Fig.19). There were no significant changes observed in the expression profile of purified protein fractions with increasing imidazole concentrations in the washing and elution buffer (50-250mM).

Purification under denaturing condition was attempted with the aim of purifying the target protein (rCel1) from 1mM IPTG-induced *E. coli* rosettagami DE3 pLysS propagated in LB broth. Initially, the cell pellets were lysed with denaturing lysis buffer containing 8M Urea and eluted with lower imidazole concentrations of 50mM, 100mM and 200 mM. These elution conditions could not subsequently remove non-specifically bound *E. coli* native proteins. In order to remove non-target proteins from the Ni-NTA agarose resin, whilst still retaining His-tagged Cel1, increasing concentrations of imidazole (300mM, 400mM and 500 mM) were used in the elution buffer. SDS-PAGE analysis (Fig.20) revealed that all the elution conditions followed a similar pattern in the purification of His-tagged rCel1, except that increasing target protein concentration was achieved with higher imidazole eluting concentrations. Since the eluted protein fractions under denaturing conditions contained the urea (denaturant), refolding and desalting of the sample by ultrafiltration was performed after purification in order to retain its biological activity. Background contamination with non-specific bound proteins usually arose from the protein with neighbouring histidine residues and thus showed affinity for the Ni-NTA resin. Most of the contaminating proteins can be removed either by lowering the pH of elution buffer to protonate the histidine residues or by increasing the imidazole concentrations, which compete with the His-tagged proteins for binding sites. Here the purification was optimized with increasing imidazole concentrations rather than pH lowering since protein structural confirmation might not change at higher imidazole concentrations.

Further, purification of the target protein i.e., His-tagged rCel1 was carried out under hybrid conditions as the native and denaturing conditions could not specifically elute the His-tagged protein from other contaminating *E. coli* native proteins. Here the cell pellet was first lysed under denaturing

conditions (which completely exposes 6x His-tag) and the urea present in the Ni-NTA resin was washed with native wash buffer containing increasing concentrations of imidazole (50 - 80 mM) and some of the Ni-NTA resin bound His-tagged proteins were found to come out at above 80 mM imidazole concentrations in the washing buffer (data not shown).The specifically bound His-tagged rCel1 to Ni-NTA matrix was then eluted at 50-500 mM of increasing imidazole concentrations. All the eluted fractions were collected and analysed through SDS-PAGE (Fig.21). Eluted fractions at 50 - 200 mM of imidazole, showed a few non-specific *E. coli* protein bands other than the expressed His-tagged rCel1 (≈ 68Kda) (Fig.21, lane 2- 4). But at higher eluting concentrations (200 mM - 500 mM imidazole), it had yielded specific target band of His-tagged rCel1 with a molecular mass of approximately 68 KDa. Higher target protein (rCel1) concentration was achieved at highest imidazole concentrations (500 mM) contained in the elution buffer. While considering all the three methods of Ni-NTA purification (native, denaturing or hybrid conditions), purification under hybrid conditions was found to be the best method as the His-tagged rCel1was highly accessible for the Ni-NTA resin under denaturing conditions and subsequently the protein activity was restored by refolding of the protein using native washing and elution conditions. The fusion protein, rCel1, was purified to homogeneity (approximately 68 KDa) from the cytoplasmic cellular extracts using Ni-NTA agarose slurry under hybrid condition. The SDS-PAGE analysis of a crude extract of control *E. coli* with empty pET vector, the crude extract of uninduced *E. coli* with pET-Cel1, the crude extract of induced *E. coli* containing pET-Cel1 and purified rCel1 using Ni-NTA chromatography under hybrid conditions was given in Fig.22. Further, the purified recombinant Cel1 was quantified using Qubit fluorometer method and subsequently used for functional assays and biochemical characterisation.





(Fig. 13) **(Fig. 14)**

Fig.13. Plasmid from positive vector constructs in pET-Cel1

Fig.14. Vector construct of pET-Cel1 (7.271Kb)

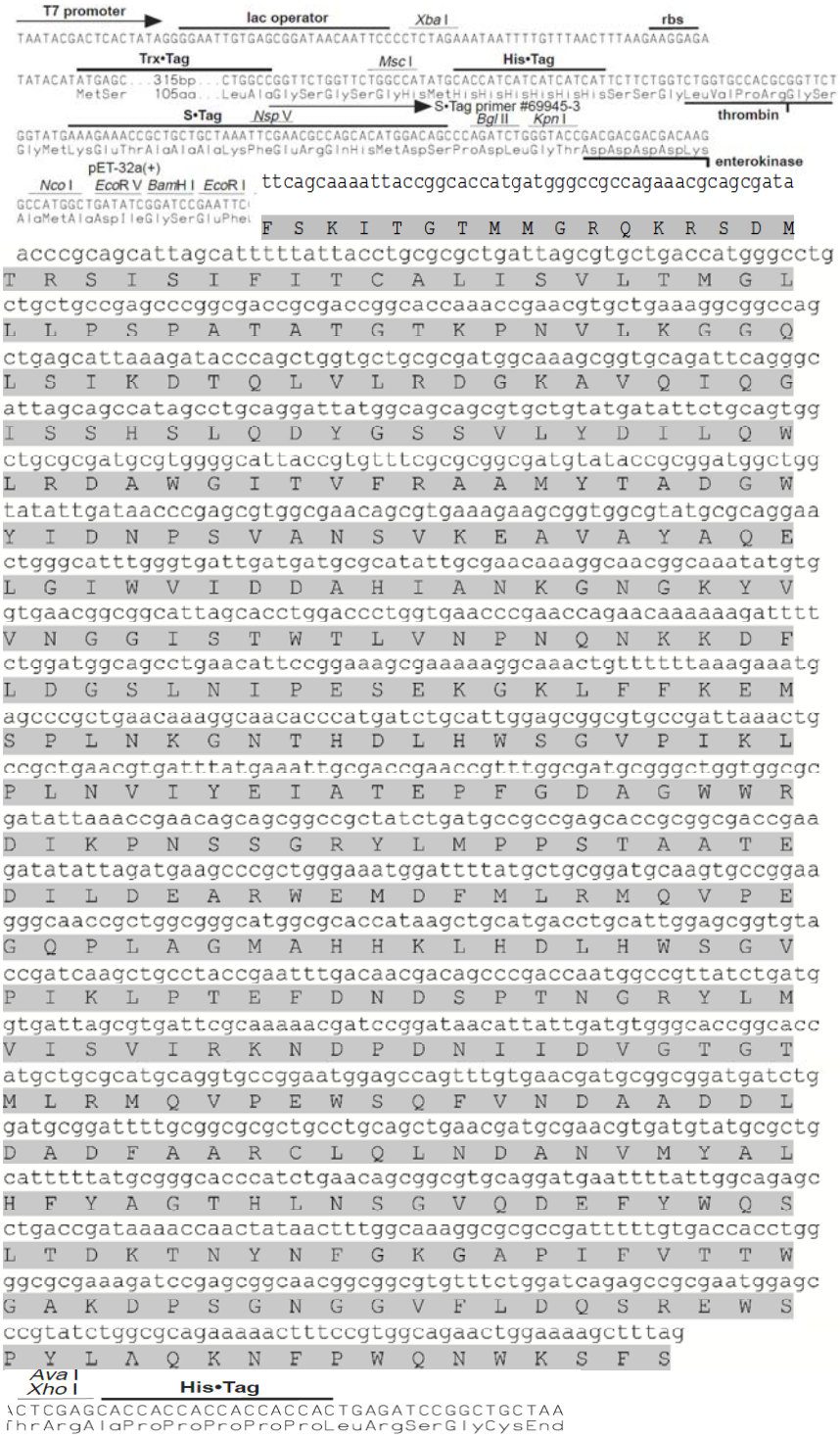


Fig.15. pET32a (+) Cel1 recombinant protein theoretical pI (5.50) and MW (50955.74)

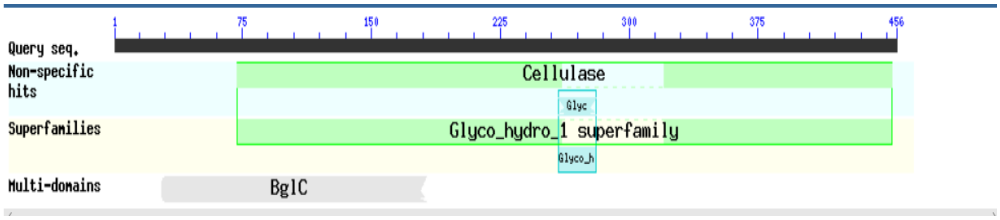
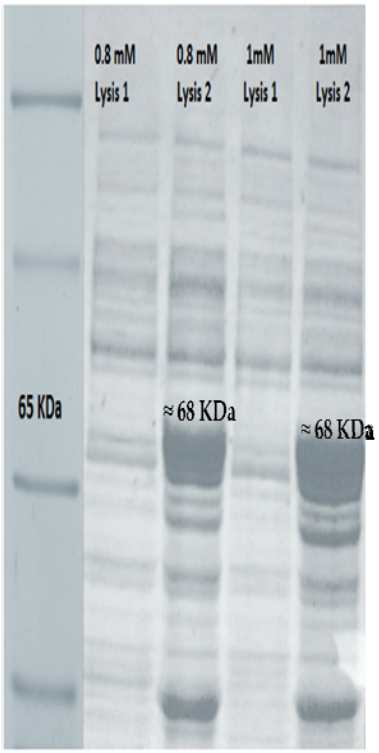
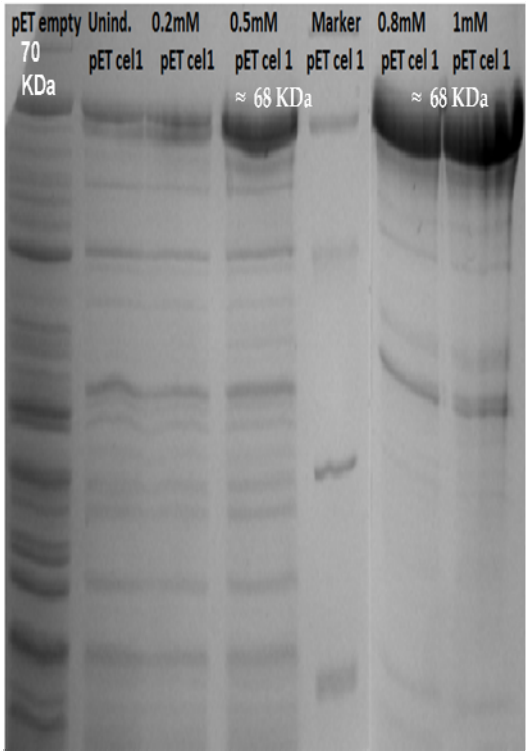


Fig.16. Cel1 recombinant protein BLAST result

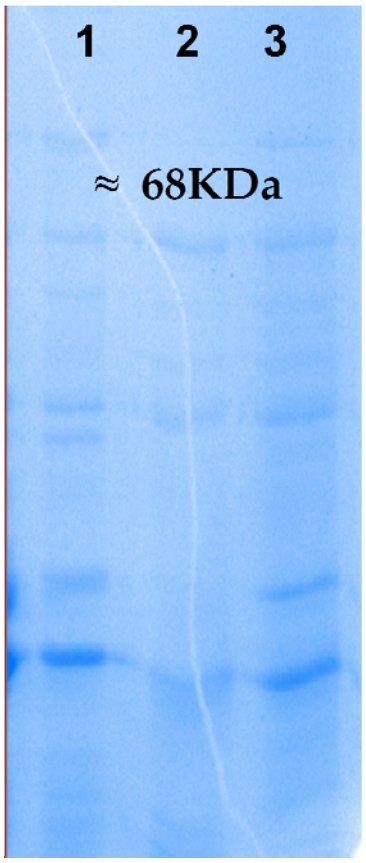


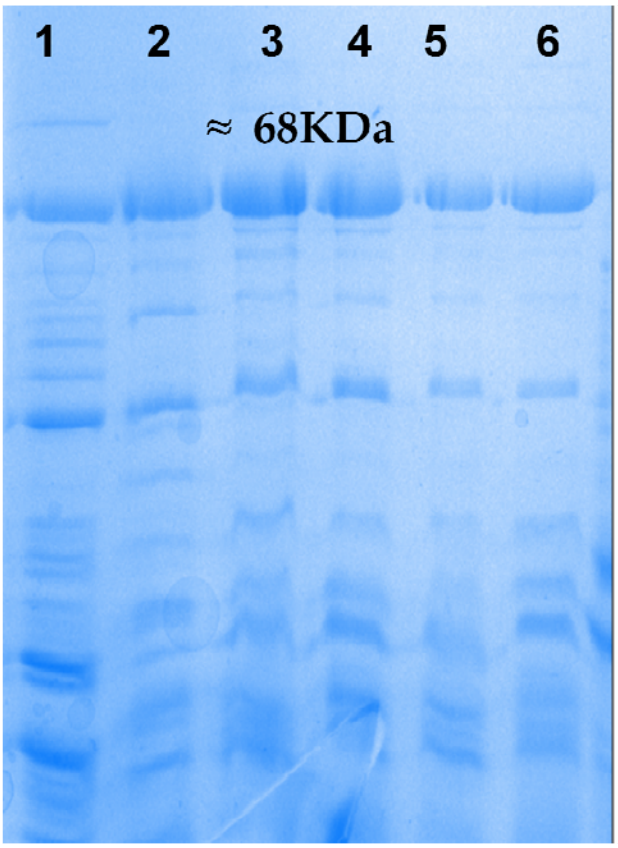


(Fig.17) **(Fig.18)**

Fig.17. Recombinant protein extraction of pET-Cel1 using lysis buffer 1 & 2 for cytoplasmic insoluble and soluble fractions respectively with pET-Cel 1 induced at IPTG concentration, 0.8 mM and 1mM ; lane 1: broad range protein marker , lane 2 : 0.8 mM IPTG induced pET-Cel1 lysis using lysis buffer 1 , lane 3 : 0.8 mM IPTG induced pET-Cel1 lysis using lysis buffer 2 , lane 4 : 1 mM IPTG induced pET-Cel1 lysis using lysis buffer 1 , lane 5 : 1mM IPTG induced pET-Cel1 lysis using lysis buffer 2.

Fig.18. Optimization of recombinant protein expression at different IPTG concentrations (0.2mM , 0.5 mm , 0.8mM and 1mM) using modified lysis buffer with optimum pH 8.0.

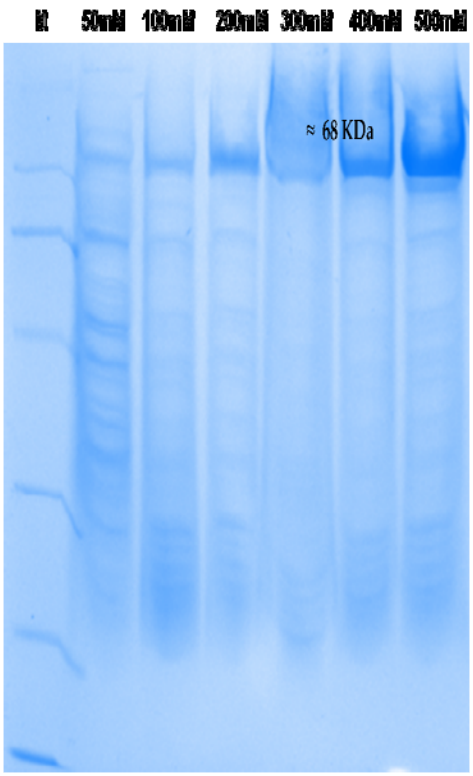


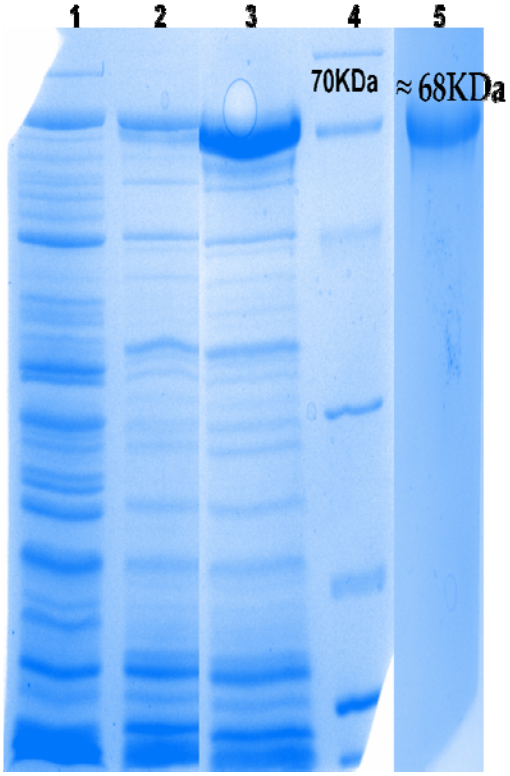


(Fig.19) **(Fig.20)**

Fig.19. Ni-NTA purification of rCel1 protein under native conditions; lane 1: rCel1 eluted at 50 mM Imidazole, lane 2: rCel1 eluted at 100 mM Imidazole ; rCel1 eluted at 250 mM Imidazole

Fig.20. Ni-NTA purification of rCel1 protein under denaturing conditions; lane 1: rCel1 eluted at 50 mM Imidazole, lane 2 : rCel1 eluted at 100 mM Imidazole, lane 3: rCel1 eluted at 200 mM Imidazole, lane 4 : rCel1 eluted at 300 mM Imidazole, lane 5: rCel1 eluted at 400mM Imidazole, lane 6 : rCel1 eluted at 500 mM Imidazole concentrations





(Fig. 21) **(Fig. 22)**

Fig.21. Ni-NTA purification of rCel1 protein under hybrid conditions. lane 1: rCel1 eluted at 50 mM Imidazole, lane 2 : rCel1 eluted at 100 mM Imidazole, lane 3 : rCel1 eluted at 200 mM Imidazole, lane 4 : rCel1 eluted at 300 mM Imidazole, lane 5: rCel1 eluted at 400mM Imidazole, lane 6 : rCel1 eluted at 500 mM Imidazole concentrations.

Fig.22. SDS-PAGE analysis of recombinant cel1 Lane 1: crude extract of control E.coli without rCel1, Lane 2: crude extract of *E. coli* containing rCel1 without IPTG induction; lane 3 : crude extract of 1 mM IPTG-induced *E. coli* with rCel1; lane 4 : protein molecular weight marker; Lane 5: Purified rCel1 using nickel nitrilotriacetic acid (Ni-NTA) chromatography under hybrid conditions

7.3.6 Functional assay of rCel1

The cell lysate prepared from induced and un-induced *E. coli* carrying rCel1, 50 mM cellobiose treated rCel1 (to determine cellobiose resistance), cell lysate from the control *E. coli* with an empty pET vector were tested against CMC (soluble substrate) and Whatman No.1 filter paper (insoluble substrate) and the liberated reducing sugar was estimated using the DNS method. Both induced and uninduced fractions showed activity only towards soluble CMC and were unable to hydrolyse the insoluble cellulose substrate (filter paper). CMCase activity produced by cytoplasmic-cell lysis preparations of transformed *E. coli* strains (induced and uninduced) and empty-vector negative control strain were shown in Table 2. An aliquot of 25µl of protein lysate (0.8 µg/µl) from the above mentioned protein samples were loaded separately on the CMC supplemented LB agar. The plates were incubated for 1h at 37°C and CMCase activity was visualized by flooding with 0.1% Congo red as described under the section Materials and Methods. As shown in Fig.23, the crude cytoplasmic preparations (induced and uninduced) of the cel1 transformants produced cellulase activity as indicated by the appearance of yellow halo zone against the red background. In addition, 25µl of rCel1- lysate (0.8 µg/µl) treated in the presence of 50mM cellobiose for 5 hours, had also showed a yellow hydrolysis zone on CMC-Congo red agar plate with its degradation zone diameter was same as that produced by both induced and un-induced cells, indicated its resistance towards cellobiose-feedback inhibition. Furthermore, no CMCase activity was detected in the negative control *E. coli* protein lysate. These results further confirmed the successful expression of Cel1 in *E. coli* and cellulase activity of the cloned Cel1product. Thus 'rCel' could be referred to as an endo-acting enzyme (endo-1, 4-β-glucanase) based on its activity only towards CMC, even though its parent phagemid clone (Vmg-Eg-3) showed activity towards

both amorphous and crystalline type of cellulose. This might be due to the loss of some of the carbohydrate binding domains (CBM) from the Vmg-Eg-3 phagemid clone during sub-cloning or over-expression. The CBMs are mainly involved in the hydrolysis of insoluble cellulose and cellulases lacking CBD are able to hydrolyse only the soluble forms of cellulose (Bolam et al., 1998; Coutinho et al., 1993; Fontes et al., 1997)

Table 2: Endoglucanase activity (CMCase) produced by recombinant *E. coli* containing pET-Cel1

Protein Sample (25 µl, 0.8 µg/µl)	CMCase (U/ml)
Control *E. coli* with empty pET	0.005
Uninduced pET-Cel1	3.927
Induced pET-Cel1	4.538
Cellobiose treated pET-Cel1	4.287

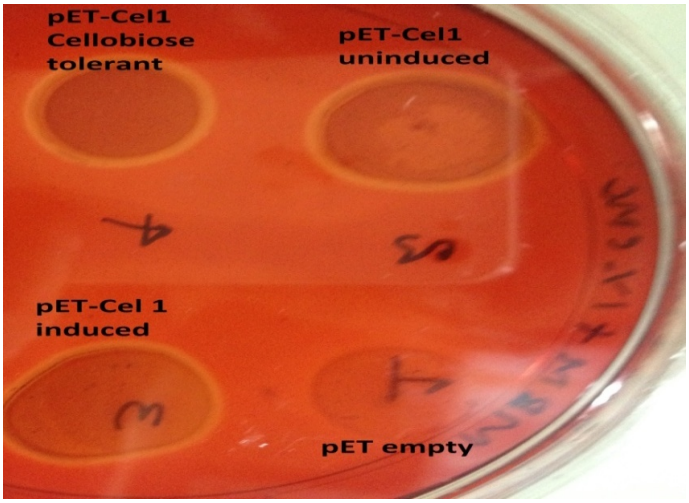


Fig.23. CMC agar for the detection of cellulase activity of pET-Cel1; spot 1 : negative control *E. coli* with an empty vector, spot 2 : Uninduced *E. coli* with pET-Cel1 gene, spot 3 : Induced (1mM) *E. coli* with pET-Cel1 gene, spot 4 : Induced (1mM) *E. coli* with pET-Cel1 gene treated with 50 mM cellobiose for 5 hrs (to determine cellobiose tolerance). The detected yellow halo against the red background indicates the cellulase activity of the cloned gene products.

7.3.7 Bio-chemical characterisation of rCel1

7.3.7.1 Effect of pH on stability and activity of rCel1

The purified rCel1 was stable over a broad range of pH from 3.0 to 10 when pre-incubated for 1 h at 25^0C in various buffers (Fig. 25). The optimal activity was observed at pH 5.0 (Fig.24) with significantly a wide range of pH activity between the pH 3.0 to 8.0.The purified rCel1still retaining greater than 90% of enzyme activity in the pH range of 3 to 8. Approximately 82% of CMCase activity was maintained at pH 9.0 for 1 h, whereas only 30% of enzyme activity loss was observed when the pH was increased to 9.0. The broad pH stability displayed by rCel1 was consistent with the properties of other metagenome-derived endoglucanases (Healy et al., 1995). Similar lower optimum pH (5.0) for cellulase activity with broad ranges of pH stability was also previously reported from many of the cultivable organisms such as *Thermomonospora* (George et al., 2001), *Bacillus* strain M-9 (Bajaj et al., 2009) etc. The purified rCel1 had greater significance because of its high level of pH stability and optimum activity against a wide range of pH from 3-9 when compared to most of the previously characterised endoglucanases from uncultured organisms, such as endoglucanase Cel5A with a pH optimum at 6.5 (Voget et al., 2006), endoglucanase Umcel5G with optimum pH 6.5 (Feng et al., 2007), endoglucanase celM2 with optimum activity at pH 4.0 (Kim et al., 2008), endoglucanase C67-1with pH optimum at 4.5 (Duan et al., 2009), endoglucanase Umcel5 with an optimum pH at 5.5 (Liu et al., 2009) and endoglucanase Umcel9B with its optimum CMCase activity at pH 7.0 (Pang et al., 2009). Similarly a highly pH stable endoglucanase C67-1was isolated from the buffalo rumen, which showed high stability under both acidic (pH 3.5) and alkaline (up to pH 10.5) conditions (Duan et al., 2009). The enzyme stability towards variable pH conditions might be due to the unique conditions prevailed

in the endangered "mangrove ecosystem" from which the metagenome was constructed. The higher pH stability also demonstrated its broad applications in biofuel, textile and food processing industries.

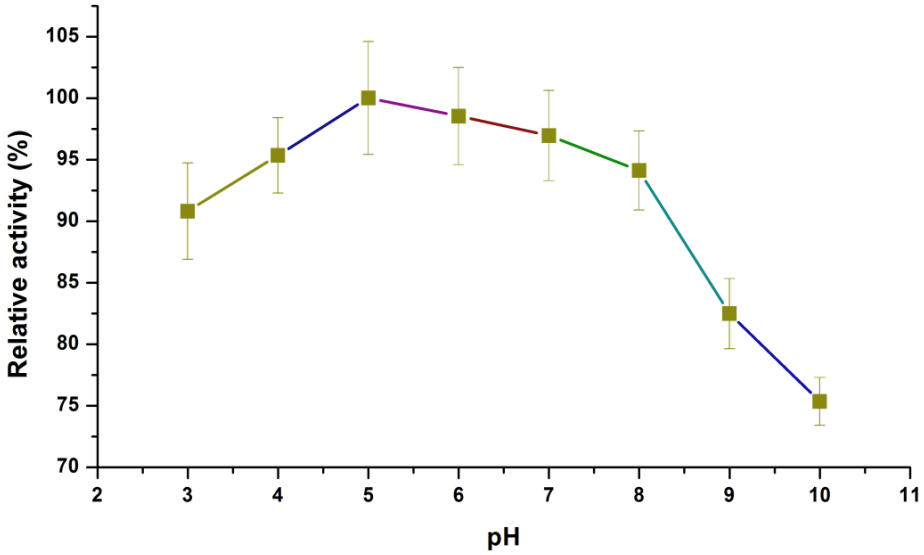


Fig.24. Effect of pH on the activity of rCel1. The error bars represent the standard deviations in the triplicate experiments

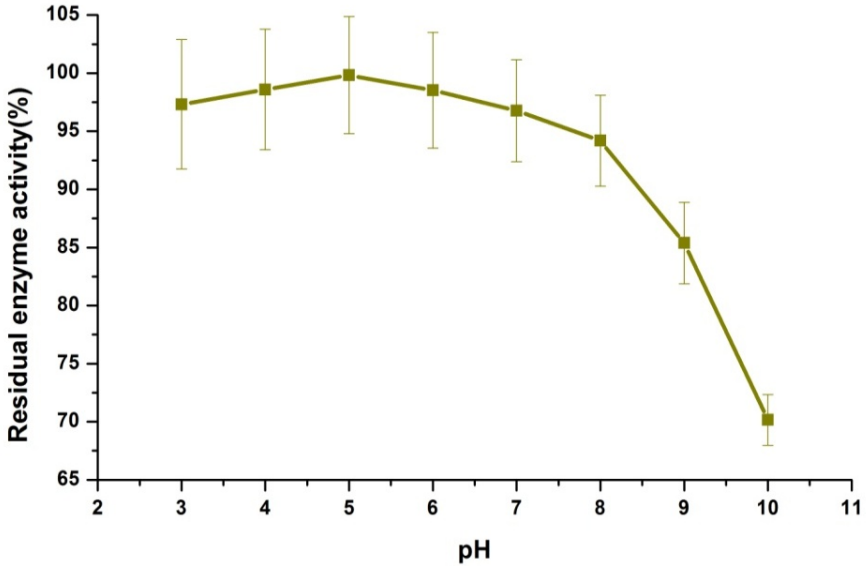


Fig.25. Effect of pH on the stability of rCel1. The error bars represent the standard deviation in the triplicate experiments

7.3.7.2 Effect of temperature on stability and activity of rCel1

Effect of temperature on purified rCel1 was determined over a broad range of temperature (25 - 65°C) with its optimal activity observed when it was incubated at 45°C and declined thereafter (Fig.27). The rCel1 was completely stable in the broad temperature range of 25-50°C by retaining its activity to greater than 90% after 1 h incubation at different temperatures (Fig.26). However, when the temperature was raised to 65^0C with an increment of 5°C, there was a gradual decrease in enzyme stability ranging between 11-18% up to 65°C. Approximately 90-100% of relative activity was maintained between the temperatures of 35 to 45^0C. Such type of temperature stability shown by the enzyme might fulfill the industrial bio-process requirements. Similarly, Voget et al. (2006) had isolated a halotolerant endoglucanase from soil metagenome, showed temperature stability at 40^oC for up to 11 h. The temperature optima reported for other metagenome-derived endoglucanases investigated so far are Umcel5G with its optimum activity at 55^0C (Feng et al., 2007), endoglucanase celM2 at 45^0C (Kim et al., 2008), endoglucanase C67-1 at 45^oC (Duan et al., 2009), endoglucanase Umcel5N with an optimum temperature at 55^0C (Liu et al., 2009) and endoglucanase Umcel9B with its optimum CMCase activity at a temperature of 25^0C (Pang et al., 2009). In addition, rCel1 maintained more than 80% of enzyme activity at 25-65^0C, indicated the advantage of using this enzyme at lower temperature reaction conditions i.e., in food processing (Siddiqui and Cavicchioli, 2006) and wine production (Todaro et al., 2008).

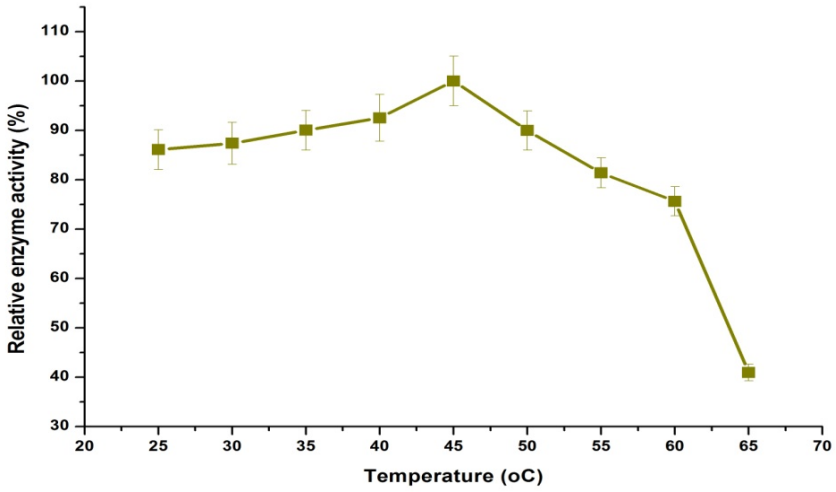


Fig.26. Effect of temperature on the stability of rCel1. The error bars represent the standard deviation in the triplicate experiments

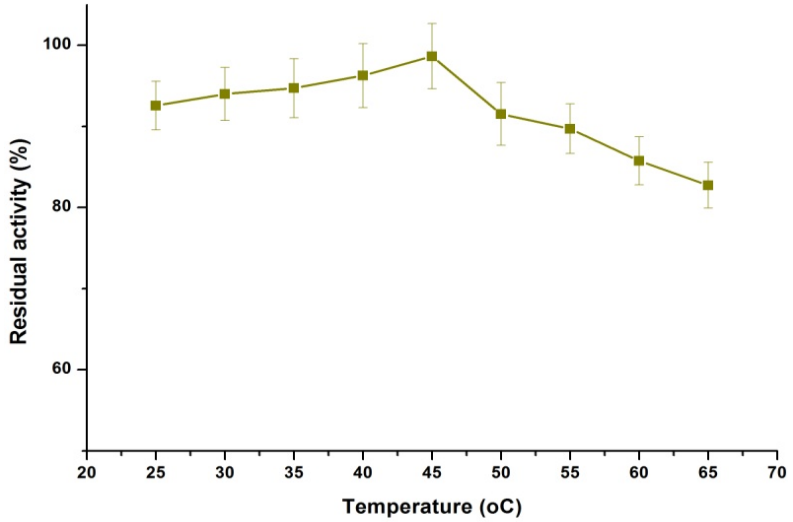


Fig.27. Effect of temperature on the activity of rCel1. The error bars represent the standard deviation in the triplicate experiments

7.3.7.3 Effect of various metal ions on the stability of rCel1

The effect of different metal ions (5mM) on rCel1 stability was determined under optimal conditions (Fig. 28). The presence of metal ions such as

Cu^{2+}, Mg^{2+}, K^{+} and Ca^{2+} enhanced the enzyme activity to 150%, 134%, 135% and 110% respectively, whereas Mn^{2+}, Fe^{3+} and Na^{+} had slightly inhibited the enzymatic activity to 92%, 90% and 80% respectively. The addition of Zn^{2+} had partially inhibited the enzyme activity to 61% and Cd^{2+} had reduced the enzyme activity to 35%. Hg^{2+} known as a "fatal inhibitor" resulted in almost complete loss (80%) of residual activity of rCel1and this might be due to the binding of Hg^{2+} ion with thiol groups, tryptophan residue, or the carboxyl group of amino acid residues in rCel1. The metal ion, Ca^{2+} had an enhancing effect on some of the few metagenome derived endoglucanases such as Umcel5G (Feng et al., 2007), Umcel5N (Liu et al., 2009) and Umcel9B (Pang et al., 2009) etc. The metal ion, Mn^{2+} had a repressing effect on rCel1 activity which was in agreement with the study of Mai et al. (2014), who reported a similar inhibition of cellulase activity in a mangrove metagenome derived endoglucanase 'MgCel44'. But contrary observations had been made by Voget et al. (2006) and Pang et al (2009), who observed a significant stimulation of cellulase activity in the presence of Mn^{2+}. The same level of inhibition by Zn^{2+} and Fe^{3+} on rCel1 stability, was also observed in a soil metagenome derived endoglucanases (Voget et al., 2006; Feng et al., 2007; Liu et al., 2009).

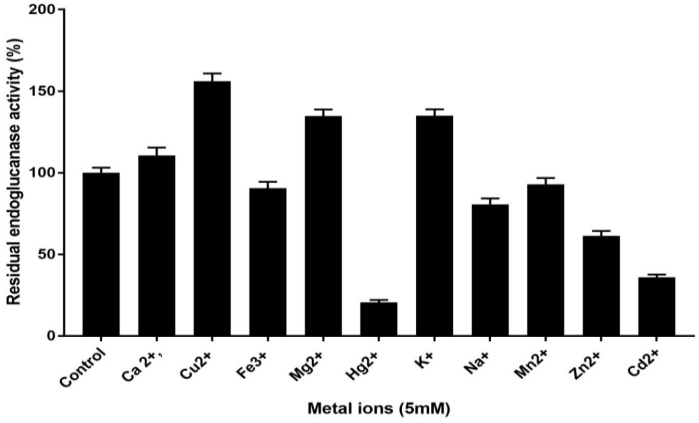


Fig.28. Effect of different metal ions (5mM) on the stability of rCel1. The error bars represent the standard deviation of the triplicate experiments

7.3.7.4 Effect of various additives on rCel1 activity

The stability of rCel1 in the presence of a range of non-ionic surfactants, detergents and chelating agents were tested in order to determine their potential in the detergent industry. As shown in Fig.29, rCel1 was significantly stable in the presence of non-ionic surfactants such as Tween-20, Tween-80 and Triton X-100 after 6 hrs of incubation. The enzyme had highly stimulated in the presence of Triton X-100 (35%), whereas little enhancement of enzyme activity had exhibited in the presence of Tween-80 (8%) and Tween-20 (5%). The hypothesis behind the enhancement of cellulase activity by non-ionic surfactants was thoroughly described by Zheng et al. (2008), who reported that the addition of non-ionic surfactants stabilize the enzyme by reducing thermal or mechanical shear forces, enhances the substrate accessibility by changing the substrate confirmation, enhances the enzyme-substrate interaction by preventing non-productive enzyme adsorption into the substrate surface. Cel1 activity had greatly inhibited in the presence of anionic surfactant such as 1%SDS, displaying only 30% of residual activity in comparison to the control, which further indicated its unsuitability as effective detergent additives. Similar inhibition of SDS in cellulase activity was noticed from GHF9 endoglucanase Umcel9B (Pang et al., 2009). The presence of chelating agent, EDTA slightly reduced the residual enzyme activity to 89 %, indicated that there is no absolute requirement of divalent cations for the enzyme to degrade soluble cellulose. The addition of the reducing agent β -mercaptoethanol (β-ME) retained its catalytic activity to approximately 82% , which might be due to the protective effect of β-ME against the oxidation of methionine residues present in rCel1 (Caldwell et al., 1978).

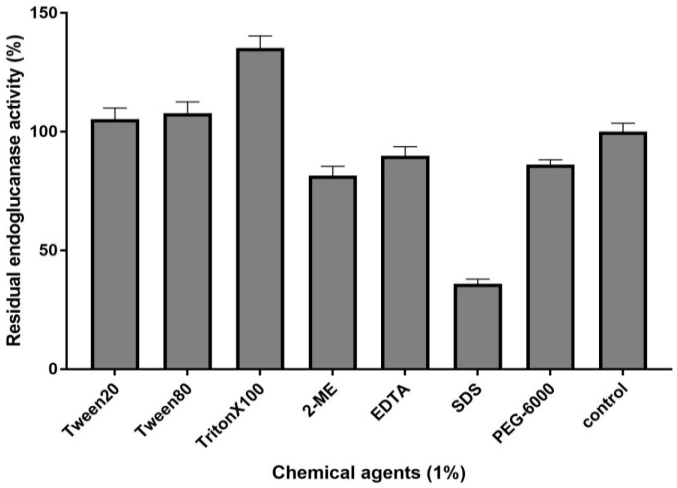


Fig.29. Effect of different chemical agents (1%) on the stability of rCel1. The error bars represent the standard deviation of the triplicate experiments.

7.3.7.5 Effect of NaCl on rCel1 stability

The effect of various NaCl concentrations (1M - 5M) on rCel1 stability was represented in Fig.30. Cel1 was found highly active in the presence of very high salt concentrations by retaining greater than 80% of its enzyme activity after 6 hrs of pre-incubation in the presence of 1M to 4M NaCl and it retained approximately 60 % of its enzyme activity with 5 M NaCl. This type of halostability observed in rCel1 might be due to the unique salinity characteristics, which prevailed in the mangrove ecosystems. This remarkable salt tolerance is highly significant since the demand for salt tolerant enzymes are still limited (Oren, 2010). There are also reports on commercially available cellulases such as accellerase-1500, which performs crystalline cellulose degradation in optimal reaction systems containing 1X - 4X concentration of seawater (Grande et al., 2012). A halotolerant cellulase designated as Cel7482 was isolated through functional screening of metagenomic libraries and

showed extreme tolerance towards 2 M NaCl and was found stable after 20 hrs of incubation in the presence of 5 M NaCl (Voget et al., 2006). But the halotolerance displayed by the metagenome-derived Exo2b was slightly different i.e., high salt concentration changed the structure of Exo2b without any denaturation of the enzyme and reduced its activity to about 65% (Voget et al., 2006; Wang et al., 2009). An interesting type of halotolerant cellulase was isolated from *Thermotoga maritime,* which was stabilised at high temperatures by the addition of up to 5 M NaCl (Bronnenmeier et al., 1995; Liebl et al., 1996). In addition, Mai et al. (2014) had isolated a salt tolerant endoglucanase 'MgCel44' from a mangrove environment, its activity was stimulated to 1.6-fold in 0.5 M NaCl with more than 50% activity retained in 1.5 M NaCl after incubation for 24 h. Another interesting cellulase isolated from a non-saline environment, CelDZ1 showed a dual property of halostability and halotolerance in the presence of high salt concentrations after incubation for several days at 3 M NaCl or KCl (Zarafeta et al., 2016). This property is highly applicable for biomass-processing industries, where the lignocellulosic biomass were subjected for strong alkaline pre-treatment processes followed by acid neutralization which resulted in higher amounts of salt formation (Klinke et al., 2004)

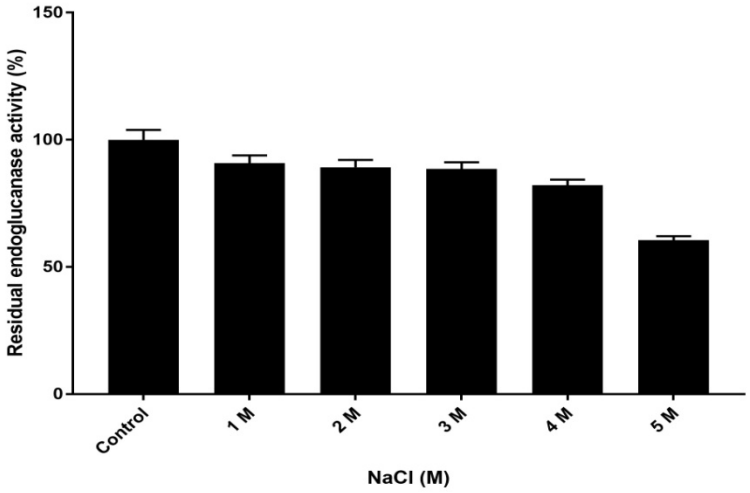


Fig.30. Effect of different NaCl concentrations on the stability of rCel1. The error bars represent the standard deviation of the triplicate experiments

7.4 Conclusion

In conclusion, a novel putative endoglucanase gene (Cel1) of glycoside hydrolase family1 was identified from a metagenomic lambda library with the endoglucanase clone Vmg-Eg-3 and the full-length endoglucanase gene (1.37 Kb) was sub-cloned into blunt-end vector pJET1.2 and overexpressed in to pET32a(+) translation vector. The recombinant Cel1 was Ni-NTA purified and functionally characterised. This enzyme was active only towards soluble cellulose substrate and remarkably stable over a wide pH range with moderate thermostability. The recombinant protein was stable at 1- 4 M NaCl and 35% of enzyme activity was enhanced with Triton X-100. These properties formulated the enzyme as an efficient catalytic resource for the biofuel industry.

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Chapter 8

SUMMARY AND SCOPE FOR FUTURE RESEARCH

Contents

8.1 Introduction

Cellulase refers to the glycoside hydrolase enzymes mainly produced by fungi, bacteria, and protozoans (culturable organisms) and also from uncultivable organisms that catalyze the cellulose hydrolysis. The immense potential that cellulases have in industrial biotechnology is the motivating force for continuous basic and applied research on these biocatalyst discovery through both culture dependent and culture independent approaches. Cellulases have many advantages such as in animal feeding, brewing and wine, paper and pulp, food, textile and laundry etc. But their most remarkable application towards the conversion of lignocellulosic biomass into fermentable sugars has provided an additional request for stable cellulases .This PhD thesis presents research in developing novel cellulases with improved biocatalytic properties through culture-dependent and metagenomics based approaches, including application of fungal-derived cellulases using the agro-industrial by-product, coir pith as raw material for cellulase production and metagenome-derived cellulase 'Cel1' for their implementation at industrial level. The discovery of high yield and stable hydrolytic enzymes versatile to extreme bioprocess conditions is necessary for a commercially viable bioethanol production.

Research on cellulase has advanced very rapidly in the past few decades, with emphasis on enzymatic hydrolysis of cellulose to hexose sugars. The complete enzymatic hydrolysis of cellulose requires the use of cellulase, a multiple enzyme system consisting of endo-1,4,-β-Dglucanase (CMCase, EC 3.2.1.4), exo-1,4,-β-D-glucanases (1,4-β-D glucan cellobiohydrolase, EC 3.2.1.91) and β–glucosidase (cellobiase or β-D-glucoside glucanohydrolase, EC 3.2.1.21). The major hurdles to exploiting the industrial potential of cellulases are the yield, stability, specificity, and the production economics. Endoglucanases are proposed to attack initially at multiple internal glycosidic linkages in the amorphous regions of the cellulose polymer to open up binding sites for subsequent attack of cellobiohydrolases. Exoglucanase comprise about 40-70% of the total cellulase proteins and can depolymerise crystalline cellulose by removing mono-and dimers from the end of the glucose chain. β-glucosidase hydrolyses cellobiose units to release glucose monomers. Generally, the endo- and exoglucanase work synergistically in cellulose hydrolysis but the basic mechanism is still ambiguous. Microorganisms generally appear to have multiple forms of endo- , exoglucanases and β-glucosidases and a wide array of cellulolytic microorganisms have been identified and characterised over the years and this list still continues to grow. However, only a few microorganisms have been reported so far with all the necessary component enzymes in sufficient quantities for complete cellulose hydrolysis. With rising energy demands and diminishing energy resources, the utilization of lignocellulosic biomass for biofuel production offers a renewable alternative. But a number of technological lacunas are existing with the theoretically possible looking system such as the substrate complexity and crystallinity. Another major drawback is the end product inhibition which reduces the rate of cellulase production resulting in incomplete utilization of lignocellulosic biomass. In nut shell, the

major goals for future cellulase research would be: (1) Reduction in the cost of cellulase production and (2) Improving the performance/activity of cellulases to reduce the enzyme input. More strategic research is needed to make designer cellulase enzymes (synthetic cellulases) suited for specific applications.

Ever since the emergence of metagenomics in 1990's, the research on identifying new and varied biocatalysts has picked up tremendous momentum worldwide. During this time there have been methodological advances that have enabled the sequencing of natural populations as well as experiments that have used these techniques for the study of non-cultured organisms. The barriers to genome sequencing ranging from lack of sufficient material for the construction of sequencing libraries to the cost of sequencing have begun to pave way for improved cloning and sequencing technologies. An important potential of metagenomics lies in the direction of biofuel production from lignocellulose. Though the classical approach (culture-dependent) of screening a wide variety of microbes has yielded a few novel cellulolytic activities, a metagenomic approach has been conceptualized here to clone most efficient cellulolytic genes from the environment and to express them in an appropriate secretory-expression system. Therefore, this study will lead to innovations in the field paving the way for industrial development in cellulase production which will solve the issue of bio-fuel generation from agro-waste. These developments together with improved scientific knowledge are expected to pave the way for a remarkable success in the novel cellulase discovery in the 21st century.

The aim of the first chapter was to present up-to-date broad information from all fields related to culture-dependent and independent strategy to obtain "ideal" and "unique" cellulase. Efforts have been made to give an insight into the lignocellulosic ethanol production, components of lignocellulosic biomass, different types of cellulase systems, its classification into glycoside hydrolase

family, diversity of cellulase system (complexed and non-complexed), synergy existing between cellulses, its mode of action, structure of glycoside hydrolases etc. Different types of metagenomic approaches (functional and sequence based approaches) for hunting novel cellulases are also discussed, addressing their particular advantages and disadvantages. Furthermore, advancements in sourcing and analysing metagenomes are discussed with a special emphasis on functional screening and expression of metagenome-derived cellulases, underlining the necessity of functional characterisation for their industrial application has also been discussed.

Conclusively the subject matter of the thesis has been divided under the following heads:

Culture-dependent approach:

1. Screening and identification of potent hydrolytic organisms from MTCC culture collection for saccharification of delignified coir pith and development of enhanced cellulose hydrolysis.
2. Purification and characterisation of a processive-type endoglucanase and β-glucosidase from *Aspergillus ochraceus* MTCC 1810 through bioconversion of delignified coir pith into glucose.
3. Proteomics of the purified cellulases of *Aspergillus ochraceus* MTCC 1810 and their application in bioconversion of delignified coir pith.

Functional Metagenomic approach:

4. Construction of Metagenomic lambda library from Valanthacaud Mangrove sediment
5. Screening of metagenomic lambda library for Cellulase encoding clones and sequence analysis of one of the potent clones

6. Sub-cloning, Over-expression, Purification and Characterisation of recombinant cellulase

8.2 The concise description of the overall achievements

8.2.1 Screening and identification of potent hydrolytic organisms from MTCC culture collection for saccharification of delignified coir pith and development of enhanced cellulose hydrolysis

- Preliminary qualitative cellulase screening tests showed that only four fungal and two bacterial strains could produce detectable cellulase activity from among the thirty six cellulolytic isolates procured from Microbial Type Culture Collection, IMTECH, Chandigarh.
- The following organisms *viz*., *Bacillus sp*. MTCC 297, *Paenibacillus macerans* MTCC 2294, *Fusarium oxysporum* MTCC 1755, *Aspergillus ochraceus* MTCC 1810, *Chaetomium globosum* MTCC 2193 and *Pleurotus sapidus* MTCC 1807 were selected and subjected for quantitative enzymatic screening using delignified coir pith as cellulose substrate in Reese and Mandel's submerged fermentation medium.
- *A. ochraceus* MTCC 1810 was selected for cellulase production optimization based on its highest yield of CMCase (11.75±0.015 U/ml), FPase (8.317±0.026 U/ml) and β-glucosidase (9.86±0.023 U/ml) using delignified coir pith as cellulose substrate.
- The optimum cellulase activity such as CMCase (65.35 U/ml), total cellulase (38.4 FPU/ml) and βG (23.16 U/ml) were obtained at pH 6.0 with an optimum temperature of 35^{o}C for endoglucanase and total cellulose, whereas β-glucosidase was found to be optimum at 40^{o}C with an optimum inoculum size of 10% (v/v).

- In addition , other nutritional parameters such as 0.75% urea (nitrogen source), 0.75% carboxy methyl cellulose (sugar supplimentation), 5 mM of metal ions such as Fe^{3+}, Mg^{2+}, Ni^{2+} and Zn^{2+} and 0.8% Tween 80 (surfactants) were required for optimum cellulase secretion from *Aspergillus ochraceus* MTCC 1810.
- The highest fold of enzyme activity after production optimization than its primary activity has evidently supported the effect of fermentation process variables on the yield of cellulases.

8.2.2 Purification and characterisation of a processive-type endoglucanase and β-glucosidase from *Aspergillus ochraceus* MTCC 1810 through bioconversion of delignified coir pith into glucose

- Purification of cellulases from *Aspergillus ochraceus* MTCC 1810 was standardized with 80% ammonium sulphate precipitation, which yielded 42.17% total protein and further purification using DEAE Sepharose column chromatography yielded only 22.44% of total protein.
- Endoglucanse was purified at 2.39 fold with yield activity of 53.84%, total cellulase was purified at 3.26 fold (73.3%) and β-glucosidase fraction resulted in 2.92 fold purification with an activity yield of 65.58%.
- Purified proteins appeared as single prominent peaks and most of the endoglucanase and total cellulase activity (overlapped) corresponded to the molecular mass of ≈78 kDa , suggested that both enzyme active sites were resided in the single protein, observed for the first time in *A. ochraceus* MTCC 1810.

- Further zymogram analysis also agreed with the protein profile of endoglucanase and exoglucanase on CMC-Congo red and 4-MUC gel respectively.
- Zymogram of the β-glucosidase (43 kDa), showed a black precipitate due to the hydrolysis of esculin to esculitin.
- Biochemical characterisation of processive-type endoglucanase (AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B) showed a similar pH stability profile from pH 3 to 8 at an optimum pH 6.0.
- Optimum temperature of cellulase activity was at 40^0C with significant enzyme activity in the range of 4–60^oC and a complete enzyme inactivation at 70^0 C.
- Endoglucanase and total cellulase activity were profoundly stimulated in the presence of $Na^+ > K^+ > Mg^{2+} > Cu^{2+} > Fe^{3+} > Co^{2+}$, with a strong inhibition of enzyme activity in the presence of Hg^{2+}, SDS and EDTA. β-mercapto ethanol partially inhibited the same.
- Both enzymes showed higher organic solvent stability in 20% v/v ethanol, much useful in industrial bio-ethanol production.
- The kinetic parameters such as Km, Vmax and Kcat values of processive endoglucanase (AS-HT-Celuz A) were 0.8175mg/ml, 45.72 (μmol/ml/min) and $6.8x10^3$ s^{-1} respectively for CMC and Km of 1.020 mg/ml, Vmax of 53.36 μmol/ml/min and Kcat of $6.3x10^3S^{-1}$ against avicel and a lowest Km value of AS-HT-Celuz A indicated its greatest affinity towards CMC than avicel.
- Km, Vmax and Kcat values of purified β-glucosidase were representd as 3.535 mg/ml , 37.28 μmol/ml/min and $7.8x10^3$ s^{-1} respectively against 4-MUG

- AS-HT-Celuz A showed higher catalytic efficiency ($8.3x10^3$ $S^{-1}mg^{-1}ml$) against CMC and $6.1x10^3$ $S^{-1}mg^{-1}ml$ against avicel, AS-HT-Celuz B had displayed $2.2x10^3$ $S^{-1}mg^{-1}ml$ against 4-MUG.
- AS-HT-Celuz A had high substrate specificity against rice hull followed by delignified coir pith indicated its higher catalytic efficiency towards natural cellulosic substrates than synthetic polysaccharides.
- The rate of synthetic cellulose degradation by AS-HT-Celuz A was in the order of CMC > phosphoric acid swollen avicel > hydroxy ethyl cellulose > avicel > filter paper discs.
- Another unusual feature by processive endoglucanase was its activity against birch wood xylan much useful in heterogenous biomass conversion.
- The substrate binding property of AS-HT-Celuz A was 30 and 43% towards insoluble avicel and filter paper discs respectively, indicated a physical evidence of carbohydrate binding modules (CBD) in the processive endoglucanase.
- The processivity ratio of AS-HT-Celuz A was increased from 1.79 to 3.12 when the incubation time was increased from 3 to 9 hrs, which further confirmed its processive activity.
- Increased rate of hydrolysis and degree of synergy were observed with sequential addition of enzymes than with the simultaneous addition
- The 2D gel analysis of AS-HT-Celuz A and AS-HT-Celuz B had shown two distinctly separated spots at ≈ 4.7 and 4.1 respectively.
- Therefore, the two industrially potent cellulases purified in the present study will have remarkable applications in biofuel industry.

8.2.3 Proteomics of the purified cellulases of *Aspergillus ochraceus* MTCC 1810 and their application in bioconversion of delignified coir pith

- The MALDI-TOF/MS analysis of the purified protein, AS-HT-Celuz A showed highest similarities to endoglucanase of *Clostridium cellobioparum* (44%) and *Clostridium termitidis* (44%), exoglucanase of *Clostridium* sp.(40%), endoglucanase of bacterium JKG (40%), exoglucanase of *Lachnoclostridium phytofermentans* (40%), endoglucanase of *Herbinix* sp. (37%) through NCBI Blast P analysis.
- Further peptide sequence analysis of AS-HT-Celuz A through FindPept analysis supported the mass spectrum data to categorise novel AS-HT-Celuz A in the processive cellulase family.
- AS-HT-Celuz B was identified as β-glucosidase of glycoside hydrolase family 3 and its peptide sequence showed significant similarities to β-glucosidases of *A. niger* (56%), *A. kawachii* (54%), *A. luchuensis* (54%), *A. terreus* (53%), *A. saccharolyticus* (51%), *A. aculeatus* (51%), *A. nidulans* (49%) , *A. calidoustus* (47%) , *A. clavatus* (47%), *A. flavus* (51%) , *A. oryzae* (51%) , *A. fumigatus* (49%) , *A. rambellii* (48%) and *A. ochraceoroseus* (48%).
- All the identified proteins were glycoside hydrolytic enzymes involved in carbohydrate metabolism. The several glycoside hydrolase family domains (1, 3, 4, 9 and 48) present in AS-HT-Celuz A indicated the property of multiple-substrate specificity.
- The 3D Swiss-model of AS-HT-Celuz A with the template, GH48 cellobiohydrolase from *Caldicellulosiruptor bescii* showed a similarity of 51.15% and AS-HT-Celuz B with the reference model, *Aspergillus*

sp. Family GH3 beta-D-glucosidases (63.24%). The sequence similarity of (>50%) to known cellulase templates suggested the suitability of the model for downstream proteomic analysis.

- Sequential hydrolysis with AS-HT-Celuz A and AS-HT-Celuz B on pre-treated coir pith released 8.01g glucose/L with an efficiency of 72.11%, whereas simultaneous hydrolysis with only partial sacchrification efficiency of 55.48%.
- Thin-layer chromatography and gas chromatography of the hydrolysis residue revealed glucose as the only end product.
- FTIR, XRD (13% crystallinity reduction) and SEM analysis of enzymatically treated coir pith strongly elucidated the degraded nature of cellulose and hemicellulose subunits.
- The results conclude that sequential addition of partially purified enzymes, AS-HT-Celuz A and AS-HT-Celuz B, allowed the complete bioconversion of the polymeric carbohydrates into fermentable glucose units, thus offering an innovative solution towards the management of 'coir pith'.

8.2.4 Construction of Metagenomic lambda library from Valanthacaud Mangrove sediment

- The soil sampling for the construction of Metagenomic lambda library was conducted from Valanthacaud mangrove islands.
- Out of the four direct DNA extraction methods, the one based on proteinase K was found as the best method in terms of quantity (276µg DNA per gram of dry soil) followed by liquid nitrogen grinding

method (175µg DNA per gram of dry soil) and PVPP-PEG method (86µg DNA per gram of dry soil).

- PVPP–PEG method was optimum for obtaining higher purity DNA whereas agarose plug DNA extraction yielded high molecular weight DNA (>267 Kb) compared to other methods (73 Kb).
- Evaluation of PCR efficacy from isolated metagenomic DNA (Proteinase K based method) using 16S rDNA universal primers showed that the metagenomic DNA was devoid of humic acid contamination and can proceed with any sequence-based analysis.
- Humic acid was observed as the major accumulate in the extracted DNA for functional metagenomic library construction and was efficiently removed using Q-sepharose beads. Standardisation of purification has shown that increased volume such as 300 µl of DNA (125 µg/ml) to 300 µl of Q-Sepharose (1:1) resulted in enhanced recovery of DNA with 96% removal of humic acids.
- DNA sample was restriction digested with Sau3a1and 6-10 Kb of DNA fragments were used as the insert for ligating into small insert expression system such as, *Bam* H1 digested lambda ZAP express vector.
- In vitro packaging was performed using Gold Pack III packaging system and strength of primary phage library was found to be $2x10^4$pfu/ml.
- Recombination efficiency (90%) was determined by plating on X-gal and IPTG plate.
- Primary phage library was amplified to a stable secondary library ($4x10^7$pfu/ml).

- pBK-CMV phagemid clones were excised using ExAssist helper phage with XLOLR strain with a titer value of 574 cfu/µl.
- The library was maintained at $^{-}80^{o}C$ as metagenomic repository (PG-Val-Mg-1).The library represented 60-100 Mb of environmental genome and was statistically validated for significant representation of clones.

8.2.5 Screening of metagenomic lambda library for Cellulase encoding clones and sequence analysis of one of the potent clones

- Phenotypic as well as quantitative screening methods were adopted for obtaining a potential and novel cellulose degrading clone from the metagenomic lambda library.
- Functional screening on carboxy methyl cellulose agar plates has shown sixteen numbers of endoglucanase positive clones and renamed as Vmg-Eg-1 to Vmg-Eg-16
- β-glucosidase specific screening on esculin hydrate –ferric ammonium citrate agar plate produced 8 numbers of positive clones (Vmg-Bg-1 to Vmg-Bg-8).
- Exoglucanase encoding clones were screened on 4-MUC detection system and the positive clones showing fluorescence were designated as Vmg-Ex-1 to Vmg-Ex-3.
- The positive hit rate of the cellulase encoding clones from the library PG-Val-Mg-1 was estimated as approximately 1/333.
- One of the positive endoglucanase encoding clone (Vmg-Eg-3) was selected for downstream metagenomic analysis based on its highest cellulase yield (CMCase; 1.354U/ml, FPase; 0.0174 FPU/ml).

- Gene level screening of positive endoglucanase encoding clones using multi-primer sets for cellulase conserved domain, yielded a specific amplification of 800bp from the endoglucanase clone derived DNA.
- The translated protein sequence of cellulase subclone from Vmg-Eg-3, showed similarity of 62% with GH5 catalytic domains and submitted under the accession number AIX 96883.1.
- Proteomic screening of whole-cell lysate of Vmg-Eg-3 using zymogram indicated clear evidence for cellulase activity along with its retransformation into another expression host for confirming the desired enzyme activity.
- Insert size characterisation (6kb) in the Vmg-Eg-3 was done by long PCR.
- Accordingly, the restriction primers were designed based on the existing sequence information from known partial cellulase gene sequence (800bp) and used to amplify full length cellulase gene (1.37Kb) from its 5' and 3' ends. Its corresponding protein sequence showed 60% homology to an uncultured clone endoglucanase gene and renamed as 'Cel1'.
- The secondary structure of Cel1 showed different helical and coiled structures, predicted with high level of confidence.
- The 3D structure of Cel1 was predicted through SWISS-MODEL work space and showed 72.18 % similarity with 3pzt.1, endo-1,4-beta-glucanase from *Bacillus subtilis* and found that the model is stable for any future proteomic studies.

8.2.6 Sub-cloning, Over expression, Purification and Characterisation of recombinant cellulase

- The putative endoglucanase gene (1.37 Kb) with added restriction sites (*Eco*R1 and *Xho*1) was cloned into blunt end pJET1.2 vector for constructing pJET-Cel1 (4.3452 Kb).
- pJET-Cel1 was restricted with *Eco*R1 and *Xho*1, confirmed by in-frame sequence analysis and over expressed in to *Eco*R1-*Xho*1 digested pET32a(+) translation vector, which was fused with 6x His tag and is expressed in *E. coli* DE3 pLyS rosettagami. The resulting recombinant plasmid was renamed as pET-Cel1 (7.271Kb).
- Cellular location of the rCel1 was determined by assaying CMCase activity in the extracellular (medium fraction), periplasmic fraction, and cytoplasmic soluble and insoluble fractions. Highest CMCase activity was observed in cytoplasmic soluble fraction (3.841 U/ml) followed by periplasmic fraction (1.351U/ml) and negligible activity in the extracellular medium fraction (0.085 U/ml).
- The optimum pH suitable for maintaining the solubility of recombinant fusion protein (rCel1) was obtained using modified lysis buffer at pH 8.0, with a total protein yield of 82% and found that pH had a greater effect on protein solubility.
- The recombinant fusion protein molecular weight was found to be corresponding to the predicted molecular mass of Cel1 gene (50KDa) plus molecular weight of fusion protein from the vector (20 KDa) and sequence analysis showed correct reading frame with Histidine tag at the N-terminus.

- The fusion protein (≈ 68 KDa) containing His tag was purified by Ni-NTA affinity chromatography under hybrid conditions and subjected to amicon-dialysis for removing the salts and then concentrated to the homogeneity.
- The purified rCel1 displayed a wide range of pH activity from 3-8, with maximum at pH 5.0. This acidic cellulase was highly stable at 45^{o}C, with a wide range of temperature stability from 25 to 65^{o} C, which offered potential applications in biofuel industry.
- The presence of metal ions such as Cu^{2+}, Mg^{2+}, K^{+} and Ca^{2+} enhanced rCel1 activity by 50%, 34% , 35% and 10% respectively, whereas Hg^{2+} completely inhibited the enzyme activity by 80%.
- The recombinant protein was highly stable at 1-4 M NaCl and 30% of enzyme activity was enhanced with Triton X-100, 60% of activity was inhibited in the presence of 0.5% SDS. These properties formulated the enzyme as an efficient catalytic resource for biofuel-industries.

8.3 Future Prospects

Immediate step:

- A bioprocess can be developed with the cellulases generated by *Aspergillus ochraceus* for the production of glucose from delignified coir pith, and using a desirable yeast strain, simultaneous fermentation of ethanol also can be accomplished.

"A potential direction for Cellulase Research"

- Development of a more effective cellulase cocktail from both culture-dependent and metagenomic sources with desirable catalytic properties

for cost effective-complete cellulose hydrolysis – "Cellulase blends of the future"

- By identifying the molecular and structural architecture of the *Aspergillus ochraceus* cellulases (with varied substrate specificity), a process strategy has to be implemented for expressing the "whole cellulases" in a single organism such as *Zymomonas mobilis* . The availability of recombinant cellulase system in *Zymomonas mobilis*, surely holds the capability to reveal improved cellulases to carry out simultaneous saccharification and fermentation, demanded by the industry- "a model platform for biofuel generation".
- The metagenomic lambda library constructed in this study (PG-Val-Mg-1) provides a resource for the potential discovery of other novel genes also, as 0.5% of the library has only been utilized in the present work.
- Designing of directed evolution experiments for the construction of random mutagenesis libraries and high-throughput screening for mutants with improved properties meeting all criteria required for the successful running of a biofuel industry.

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Annexure

1

RESEARCH PAPERS BASED ON THE CURRENT INVESTIGATION

A. Research Paper in the published journal

Asha, P., Divya, J., Bright Singh, I.S., 2016. Purification and characterisation of processive-type endoglucanase and β-glucosidase from Aspergillus ochraceus MTCC 1810 through saccharification of delignified coir pith to glucose. Bioresour. Technol. 213, 245–248. **IMAPCT FACTOR 4.49**

B. Papers Presented in Conferences

P.Asha and I.S. Bright Singh,"Purification and characterisation of multi-substrate specific cellulases from Aspergillus ochraceus MTCC 1810 through saccharification of delignified coir pith to glucose " in the NHBT conference , BRSI and NIIST , Trivandrum, 2015 .

P.Asha and I.S. Bright Singh, "Bioprospecting coastal mangrove metagenome for potential Cellulase genes" in the 3 rd International symposia on Marine biology (MECOS) , Cochin, 2014.

P.Asha and I.S. Bright Singh,"Cellulases-Is pure culture study sufficient for Cellulose Saccharification" in the International conference on Biosciences organized by SESR at Lakesong Resort, Kumarakom, Kerala , India, during 11,12 september 2014(won " **BEST PAPER PRESENTATION AWARD**")

P. Asha , G. Rojith and I.S. Bright Singh,"Screening and selection of appropriate microorganisms for the bioconversion of Coir pith and its utilization for cellulase production" in the International Conference on Genomic Sciences , MKU , Madurai , November 2010.

P. Asha , G. Rojith and I.S. Bright Singh, presented poster entitled "The feasibility of using coirpith for cellulase enzyme production" in the 98 th Indian Science congress held at SRM University , Chennai .

P. Asha , G. Rojith and I.S. Bright Singh,"Screening of microorganisms for the bioconversion of chemically delignified coir pith" in the 3 rd International Science congress held at Karunya University , Coimbatore .

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Annexure

2

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Short Communication

Purification and characterisation of processive-type endoglucanase and β-glucosidase from *Aspergillus ochraceus* MTCC 1810 through saccharification of delignified coir pith to glucose

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HIGHLIGHTS

- Purification of a new processive-type endoglucanase and β-glucosidase from *A. ochraceus*.
- Cellulases displayed multiple-substrate specificity, processivity and sequential synergy.
- Offers a novel and cost effective bioprocess for bioconversion of delignified coir pith to glucose.

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ABSTRACT

The study describes purification and characterisation of processive-type endoglucanase and β-glucosidase from *Aspergillus ochraceus* MTCC 1810 through bioconversion of delignified coir pith to fermentable glucose. The purified processive endoglucanase (AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B) were found to have molecular mass of ≈78-kDa and 43-kDa respectively with optimum endoglucanase (35.63 U/ml), total cellulase (28.15 FPU/ml) and β-glucosidase (15.19 U/ml) activities at 40 °C/pH 6. The unique feature of AS-HT-Celuz A is the multiple substrate specificity and processivity towards both amorphous and crystalline cellulose. Zymogram indicated both endo and exoglucanase activities residing in different binding sites of a single protein exhibiting sequential synergy with its own β-glucosidase. Accordingly, the identified enzymes could be implemented as synergistic cellulases for complete cellulose saccharification which still considered an unresolved issue in bio-refineries.

1. Introduction

Current fossil fuel consumption profile has forced scientific efforts to explore alternative energy sources, and one among them is lignocellulosic biomass having composed of cellulose (40–60%), hemicelluloses (20–30%) and lignin (15–30%) (Kuhad et al., 1997), an organic carbon rich feed stock for enzymatic saccharification. Cellulases are the main catalytic players of enzymatic hydrolysis and exist as multiple enzyme system comprising endoglucanase (EC3.2.1.4), cellobiohydrolase (avicelase, EC3.2.1.91), and β-D-glucosidase (EC3.2.1.21) that synergistically hydrolyse cellulose into monomeric glucose units (Yi et al., 1999). Their most remarkable applications are in the fermentation industry which demands high yield and stability under extreme bioprocess conditions. However, to make it economically viable, production cost has to be minimised (Banerjee et al., 2010); one way of attaining would be to use less expensive substrates such as lignocellulosic wastes, besides utilising efficient cellulase producers.

Coconut pith or coir pith, a natural renewable resource, is a by product of coir industry having 25% cellulose. Easy availability of coir pith and its possible utility as substrate for saccharification, and the capability of *Aspergillus ochraceus* MTCC 1810 in hyper-cellulase on-site enzyme production would make the saccharification economically feasible. In the present study, we describe the presence of a new type of processive endoglucanase in *A. ochraceus* MTCC 1810 showing sequential synergy with its own β-glucosidase and thus provide complete hydrolytic machinery for the bioconversion of pre-treated biomass to glucose for subsequent bio-ethanol production. Thus the purified industrially potent enzymes, renamed as AS-HT-Celuz A (processive endoglucanase) and AS-HT-Celuz B (β-glucosidase) with compatible biochemical profile

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were employed for studying the micro-structural modifications occurred in the coir pith after enzymatic saccharification.

2. Methods

2.1. Microorganism and culture conditions

The filamentous fungus, *A. ochraceus* MTCC 1810 was selected as the appropriate microorganism from among thirty-seven cellulolytic isolates procured from Microbial Type Culture Collection, IMTECH, Chandigarh (unpublished data). It was inoculated into the shake flasks containing Reese and Mandel's submerged fermentation medium at pH 6 (0.4 g/L $(NH_4)_2SO_4$, 2.2 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 4 mg/L $FeSO_4{\cdot}7H_2O$, 0.04 g/L $CaCl_2{\cdot}2H_2O$, 1.4 mg/L $ZnSO_4{\cdot}7H_2O$, 2% NaCl, 0.8% Tween 80 and supplemented with 1% pre-treated coir pith, which was chemically delignified according to the optimised conditions (Rojith and Bright Singh, 2012) and grown at 35 °C, 100 rev/min for 5 days. Endoglucanase (CMCase), total cellulase (FPase) and β-glucosidase assay were done according to Ghose (1987).

2.2. Purification of extracellular cellulases from A. ochraceus MTCC 1810

Extracellular proteins were ammonium sulphate precipitated and dialysed using Amicon UF stirred cell (10 kDa, Millipore, USA). Crude protein was then applied on ÄKTA Prime Plus system (GE Healthcare, Uppsala) equipped with C16/40 DEAE Sepharose column (Sigma) equilibrated with 20 mM Tris–HCl buffer (pH 8). The enzyme was eluted by applying linear gradient of NaCl (0–1 M) at a flow rate of 0.5 ml min^{-1}. Active fractions showing cellulase activity were pooled, concentrated by lyophilisation, dialysed and protein was estimated through BCA assay reagent kit (Invitrogen). SDS–PAGE was carried out according to Laemmli (1970) and individual enzyme activity was determined by zymogram analysis (Schwarz et al., 1987)

2.3. Biochemical characterisation of purified enzymes

Optimum pH and temperature was determined in the presence of various buffers with pH range of 2–10 and at temperature range of 4–70 °C for 60 min. Enzymes were pre-incubated with different metal ions (5 mM), 1% of various chemical additives and organic solvents (20% v/v) at room temperature for 60 min. The analysed parameters were shown in Supplementary Tables 1–3. Enzyme stability in terms of residual activity was determined under standard assay conditions.

2.4. Substrate specificity and substrate binding assay

Substrate specificity was measured against various natural and synthetic cellulosic substrates. The assay was prepared in 0.5 M sodium citrate buffer (pH 6.0) at 40 °C for 10 h. Substrate binding assay was performed by incubating the enzymes (50 μg/ml) with 1% insoluble substrates such as avicel and filter paper discs.

2.5. Processivity assay and determination of synergy between purified cellulases

Processivity was determined using whatman No. 1 filter paper as substrate with 50 μg/ml of AS-HT-Celuz A (Zhang et al., 2010). Synergy between the purified cellulases (25 U/ml); AS-HT-Celuz A and AS-HT-Celuz B, were investigated using CMC: cellobiose (1:1) as substrate in 0.05 M citrate buffer at pH 6.0/40 °C for 4 h with single enzyme alone as well as simultaneous and sequential additions of enzymes (Amano, 1997).

2.6. Application of purified enzymes in the saccharification of pre-treated coir pith and end product analysis

Hydrolysis of 12% (w/v) pre-treated coir pith was carried out with sequential enzyme loading of 25 FPU/g – cellulose of AS-HT-Celuz A for initial 12 h and 10 U/g – cellulose of AS-HT-Celuz B for further 12 h, enzyme loading rate selected according to our previous studies (unpublished). Control experiments were also run with single enzyme alone and without enzyme treatment. Saccharification efficiency was calculated (Taniguchi et al., 2005). Residues from the hydrolysis were subjected for thin-layer chromatography and gas chromatography. FT-IR, XRD and SEM were adopted to analyse the micro-structural changes after the enzymatic saccharification.

3. Results and discussion

3.1. Purification of multi-component cellulase from A. ochraceus MTCC 1810 through bioconversion of delignified coir pith

Purification steps of cellulase from *A. ochraceus* are summarised in the Table 1. Purified proteins appeared as single prominent peaks (Supplementary Fig. S1) and most of the endoglucanase and total cellulase activity (fractions 53–89) corresponded to the molecular mass of ≈78 kDa (Fig. 1a, lane 2). The overlapped enzyme activities indicated the presence of processive cellulase capable for degradation of amorphous and crystalline cellulose, implying that both enzyme active sites were residing in the single protein, noticed for the first time in *A. ochraceus* MTCC 1810. Meanwhile, similar bifunctional cellulases from various bacteria and fungi have been reported (Han et al., 1995). Zymogram analysis also agreed with the protein profile of endoglucanase and exoglucanase on CMC-congo red and 4-MUC gel respectively (Fig. 1b, lane 2 and 3). While most of the β-glucosidase activity was concentrated on the fractions 36–61 with an apparent molecular mass of 43 kDa (Fig. 1, lane 2), its zymogram profile showed a black precipitate due to the hydrolysis of esculin to esculitin (Fig. 1b, lane 1).

3.2. Biochemical characterisation of purified cellulases

Both enzymes showed a similar pH stability profile from pH 3 to 8 after 10 h of incubation (Supplementary Fig. S2a and b). Based on the optimum activity at pH 6.0, it showed a decreasing profile up to 50% at above pH 9.0 and at below pH 3.0. Optimum temperature was observed at 40 °C with significant enzyme activity in the range of 4–60 °C and a complete inactivation profile at 70 °C (Supplementary Fig. S2c and d). Thus the two purified cellulases demonstrated the industrial potency in terms of its compatible temperature and pH stability. Endoglucanase (70–98%) and total cellulase (67–86%) activities were found enhanced by the metal ions in the order of $Na^+ > K^+ > Mg^{2+} > Cu^{2+} > Fe^{3+} > Co^{2+}$ (Supplementary Table 1), while Hg^{2+}completely inhibited the enzyme activities. Non-ionic surfactants such as tween 20, tween 80 and triton X-100 were found to enhance the enzyme activities, whereas SDS, EDTA and β-mercapto ethanol partially inhibited the same (Supplementary Table 2). Both enzymes showed higher organic solvent stability in the presence of 20% v/v ethanol (Supplementary Table 3). Annamalai et al. (2013) reported an organic solvent (25%, v/v ethanol) stable alkaline cellulase from *Bacillus halodurans* CAS 1 strain as well. This is the first report on *Aspergillus* derived cellulases with higher stability in (20%v/v) ethanol much useful in industrial bio-ethanol production.

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Table 1
Summary of purification of cellulases from *Aspergillus ochraceus* MTCC 1810.

Purification step	Total protein (mg)	Enzyme activity (units/ml)			Specific activity (units/mg protein)			Yield activity (%)			Purification fold		
		EnG[a]	FPU[b]	BG[c]	EnG	FPU	BG	EnG	FPU	BG	EnG	FPU	BG
Crude	14.7	65.35	38.4	23.16	4.42	2.61	1.57	100	100	100	1	1	1
Ammonium sulphate precipitation	6.2	42.17	30.96	18.93	6.77	4.99	3.05	64.61	80.62	81.73	1.53	1.91	1.94
DEAE Sepharose chromatography	3.3	35.63	28.15	15.19	10.6	8.53	4.60	53.84	73.30	65.58	2.39	3.26	2.92

[a] Endoglucanase activity (U/ml).
[b] Total cellulase activity (FPU/ml).
[c] Betaglucosidase activity (U/ml).

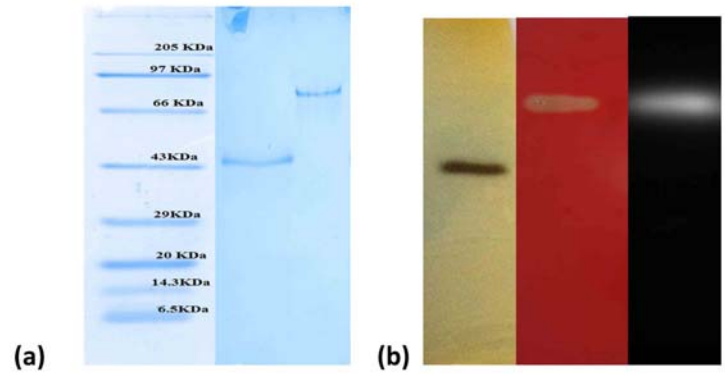

Fig. 1. (a) 12% SDS–PAGE analysis of processive-type endoglucanase (AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B) purified from *Aspergillus ochraceus* MTCC 1810. *Lane 1:* broad range protein molecular weight marker (Genei) with molecular mass in kDa. *Lane 2:* purified β-glucosidase (43 kDa). *Lane 3:* purified processive endoglucanase (≈78 kDa). (b) Zymogram analysis of purified cellulases. *Lane 1:* detection of β-glucosidase activity in 0.02% $FeCl_2$ and 0.1% esculin hydrate agarose gel. *Lane 2:* activity staining for endoglucanase in CMC-congo red agarose gel. *Lane 3:* exoglucanase activity detection in 50 mM 4-MUC supplemented agarose gel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Substrate specificity and binding assays

AS-HT-Celuz A had high substrate specificity against rice hull followed by delignified coir pith indicating its higher affinity towards natural cellulosic substrates than synthetic polysaccharides (Table 2). Specifically, enzymes produced from a natural carbon substrate could induce the hydrolysis of that particular substrate at a faster rate than with other cellulose substrates (Ilmen et al., 1997). Another unusual feature of the processive endoglucanase was its activity towards birch wood xylan, and it showed about 30% and 43% binding to the insoluble avicel and filter paper discs respectively, demonstrating the presence of carbohydrate binding modules, even though their molecular mechanisms were still not studied.

Table 2
Substrate specificity of purified cellulases against various cellulose substrates.

Substrates	Specific activity (U/mg)	
	AS-HT-Celuz A	AS-HT-Celuz B
CMC[a] (50 mM)	39.17	ND[g]
HEC[b] (50 mM)	26.35	ND
Avicel (50 mM)	11.63	ND
PASC[c] (50 mM)	28.95	ND
FP[d] (1%)	9.56	ND
Delignified coir pith (1%)	42.17	2.96
Rice hull (1%)	46.39	0.56
Birchwood xylan (50 mM)	8.67	ND
Cellobiose (50 mM)	3.97	12.97
Esculin (50 mM)	0.68	20.87
4-MUC[e] (10 mM)	18.98	ND
4-MUG[f] (10 mM)	ND	26.15

[a] Carboxy methyl cellulose.
[b] Hydroxy ethyl cellulose.
[c] Phosphoric acid swollen cellulose.
[d] Filter paper.
[e] 4-Methyl umbelliferryl-β-D-cellobioside.
[f] 4-Methyl umbelliferryl-β-D-glucopyranoside.
[g] Not detected.

3.4. Processivity and synergistic hydrolysis using purified cellulase

The processivity ratio increased from 1.79 to 3.12 when the incubation time was prolonged from 3 to 9 h (Fig. 2). Similar increase in the processivity of *Clostridium* cellulase had been reported by Zhang et al. (2010). The observed degree of synergy was more with sequential addition of enzymes than the simultaneous addition. The activity of enzyme mixture was extensively higher than the single enzyme treatment signifying its synergistic communication. The degree of synergy was 0.82 for sequential addition and 0.68 for simultaneous hydrolysis. For single enzyme treatment, the degree of synergy was 0.60 for AS-HT-Celuz A and 0.39 for AS-HT-Celuz B. An increment of 20% glucose production was observed in the sequential incubation than simultaneous addition of equivalent unit of enzymes. As well, the rate of glucose production was less in the combined enzyme incubation than the theoretical sum of individual activities of AS-HT-Celuz A and AS-HT-Celuz B.

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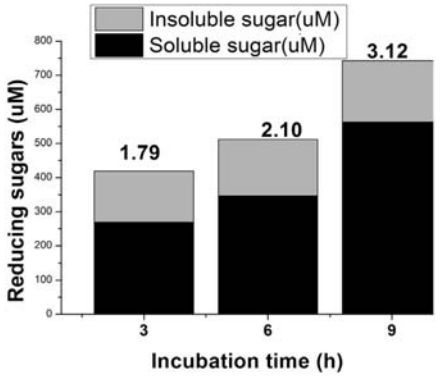

Fig. 2. Reducing sugar liberated from soluble and insoluble fractions of filter paper discs by AS-HT-Celuz A (50 μg/ml) after 3, 6 and 9 h of incubations. The processivity ratio as determined by the ratio of soluble fraction to insoluble fraction was represented above the column.

3.5. Saccharification efficiency of purified enzymes on pre-treated coir pith and analysis of end-products

Sequential hydrolysis with AS-HT-Celuz A and AS-HT-Celuz B on pre-treated coir pith biomass yielded 801.25 mg glucose/g substrate with an efficiency of 72.11% whereas simultaneous hydrolysis released 616.5 mg glucose/g substrate with partial saccharification efficiency of 55.48%. Thin-layer chromatography of the hydrolysis residue revealed glucose as the only end product. Gas chromatographic mass spectrum analysis also showed the presence of glucose as methyl silyl oxime derivative with defragmentation mass spectra at m/z of 368.

3.6. Micro-structural modifications after enzymatic hydrolysis

FTIR spectral profiles of both chemically and enzymatically treated coir pith (CTC and ETC) could be linearly correlated with that of the lignin, cellulose and hemicelluloses (Cao and Tan, 2004). Three major peaks spanning from 800–900, 1000–1100, 1300–1400 cm^{-1} were lower for enzymatically treated coir pith (ETC) than the spectra of chemically treated coir pith (CTC) elucidating the degraded nature of cellulose and hemicellulose subunits. Rojith and Bright Singh (2012) had reported several characteristic peak modifications after the chemical delignification of coir pith. Conversely, in the present study no peak intensification or shifts and only peak decrease after the enzymatic hydrolysis were observed. Quantitative estimation of cellulose crystallinity (Segal et al., 1959) showed an overall crystallinity reduction of 13% from the crystallinity index (CI) comparison of CTC (CI = 27.5) and ETC (CI = 14.37). Scanning electron microscopy showed that enzymatic degradation had converted the compressed and regularly arranged cellulosic polymers in the coir pith to a more relaxed, pleated and loosened state of cellulose, as evaluated based on the study of Jabasingh and Valli Nachiyar (2011).

4. Conclusion

A. ochraceus MTCC 1810 has the potential to produce complete cellulase system comprising processive endoglucanase and β-glucosidase capable for hydrolysing cellulosic components of coir pith to fermentable sugars. Unique properties such as thermo and pH stability, multiple substrate specificity, sequential synergy and processivity showed that the purified enzyme could find application in fermentation industry. Micro-structural characterisation of treated substrate also revealed that, the enzymes purified were suitable for saccharification of pretreated coir pith with 72.11% efficiency offering an innovative solution to the management of coir pith.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.03.013.

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