"Isolation and screening of xylanolytic bacteria from Cochin estuary and optimisation of xylanase production by *Streptomyces* sp."

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By

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Declaration

I hereby declare that the thesis entitled "Isolation and screening of xylanolytic bacteria from Cochin estuary and optimisation of xylanase production by Streptomyces sp." is a genuine record of research work done by me under the supervision and guidance of Dr. A.V. Saramma, Department of Marine Biology, Microbiology and Biochemistry, School of Marine sciences, Cochin University of Science and Technology and that no part of this work has been presented for the award of any degree, diploma, associateship in any University or Institution earlier.

Cochin,
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Emilda Rosmine

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Dedicated to

My Lord Almighty ....

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ABBREVIATIONS

˚C	Degree Celsius
%	Percentage
ANOVA	Analysis of variance
bp	Base pair
BLAST	Basic Local Alignment Search Tool
CCD	Central Composite Design
DEAE	Diethyl amino ethyl
DNA	Deoxyribonucleic acid
g	Gram
h	Hours
ISP	International Streptomyces Project
L	Litre
M	Molar
m	Metre
m^2	Square metre
mg	Milligram
min	Minute
mL	Milli litre
mM	MilliMolar
mm	Millimetre
OFAT	One-Factor-at-a-Time
PAGE	Poly Acrylamide Gel Electrophoresis

PBD	Plackett Burman Design
PCR	Polymerase chain reaction
ppm	parts per thousand
rpm	Rotation per minute
RSM	Response surface methodology
SDS	Sodium dodecyl sulphate
SmF	Submerged fermentation
Sq.km	Square kilometre
SSF	Solid state fermentation
s	Seconds
µL	Microlitre

Chapter - 1

GENERAL INTRODUCTION

Contents

Among the three major habitats of the biosphere, the marine realm covers 70% of the earth's surface and provides the largest inhabitable space for living organisms, particularly microbes (Qasim, 1999). As marine ecosystem is characterised by very special conditions, involving high salinity, high pressure, low temperature and special lighting conditions that differ from those found in other habitats, it is surmised that marine microorganisms accumulate structurally unique bioactive primary and secondary metabolites different from those of terrestrial counterparts (Bhakuni and Rawat, 2005; Zhang and Kim, 2010). Owing to its huge

genetic, biochemical diversity and unique characteristics, marine microorganisms are a promising source of new enzymes and other secondary metabolites with hyperthermostability, salt tolerance and cold adaptation properties for various industrial applications (Sivaperumal *et al.,* 2017).

In the period of evolution of multidrug-resistant bacteria and viral strains leading to life-threatening infections and growing demand on enzymes with unique properties, research in novel regions of marine ecosystem for unique natural compounds became indispensable as known compounds from already overexploited terrestrial ecosystems have been exhausted (Basha and Rao, 2017). Estuaries and mangrove forests are unique ecosystems in semi sheltered areas near the ocean coastline and are the most productive ecosystems ("Estuaries, Salt Marshes, and Mangroves - MarineBio.org", 2017).

Cochin backwater system is the longest estuarine system on the southwest coast of India, which forms more or less a northward extension of Vembanadu Lake. This positive tropical estuary is well connected to the rivers and lagoons on one side and to the Arabian Sea on the other (Varma *et al.,* 1981). Mangroves are the tidal forests of coastal wetlands, existing in the intertidal zones of sheltered shores, estuaries, tidal creeks, backwaters, lagoons, marshes and mud-flats of the tropical and sub tropical regions of the world (Balasubramaniyan and Ajmal, 2002). Mangrove ecosystems are found in isolated patches along the banks of Cochin estuary. They cover an area of 2.6 km^2 (Narayanan, 2006). The microbial source of the Cochin estuary is still untapped and not exploited for production of industrially important enzymes and other bioactive secondary metabolites.

For decades, microbial natural products have been one of the major resources for discovery of novel drugs and enzymes. Among the microorganisms, actinomycetes have been proven to be a prolific source for the vast majority of compounds discovered (Augustine, 2014).

1.1 Actinomycetes

Actinomycetes are a separate taxonomic group within the domain bacteria and belong to the order Actinomycetales. They are gram-positive, aerobic and spore forming bacteria, with high G+C content (69-78%) in DNA. Actinomycetes contain about 40 families, over 170 genera and about 2000 species (Harwani, 2013). They are ubiquitous in nature, found both in terrestrial and aquatic habitats, including mangroves, water and sediments of estuary and sea (Veiga *et al.,* 1983; Chaudhary *et al.,* 2013; Das *et al.,* 2014).

Actinomycetes are physiologically varied as evidenced by their production of about half of the discovered bioactive secondary metabolites, particularly antibiotics, antitumor agents, immunosuppressive agents and enzymes (Deshpande and Kale, 2013). Biodegradation by actinomycetes plays an essential role in recycling of materials in nature. They are reservoirs of many enzymes, including cellulases, xylanases, amylases, lipases, collagenases, proteases, chitinases, ligninases etc. They also produce and excrete pigments viz., melanins, carotenoids, phenazines etc., with pharmacological activities particularly antitumour, antimicrobial and antioxidant activities (Augustine, 2014).

Streptomycetes are an economically significant group of actinomycetes and are the pivotal source for a wide range of biologically active compounds with properties, like antibacterial, antifungal, antiparasitic, antitumor, immunosupression etc. and many commercially

significant enzymes (Avilala *et al.,* 2014; Sathya and Ushadevi, 2014). About three-quarters of all the known commercially and medicinally useful antibiotics and several agriculturally vital compounds were obtained from Streptomycetes (Cundlife, 1989). Thus, they are widely recognised as industrially important microorganisms (Higginbotham and Murphy, 2010).

Aquatic actinomycetes are referred to those widely distributed in aquatic habitats, like estuary, mangroves etc. Some of these are actually the wash from surrounding terrestrial habitats (Suryavanshi, 2007). In aquatic habitats, actinomycetes play a great role in carbon cycle due to their ability to grow at low concentration of carbonaceous substances and to degrade recalcitrant organic matter (Kuznetsov, 1970). They make up large part of microbial population of aquatic system and a rich source for the production of industrially important enzymes (Adinarayana and Ellaiah, 2002; Radhika *et al.,* 2011). Bioactivity of mangrove actinomycetes is manifold ranging from production of industrial enzymes, antimicrobial activity, antitumor activity to enzyme inhibitors and other notable bioactivities (Basha and Rao, 2017).

1.2 Microbial Enzymes-Xylanase

Microbial enzymes gained significant attention when a search began for better, less expensive and more readily available sources of enzymes (Underkofler, 1957). They are more active and stable than plant and animal enzymes, particularly for applications in industries on commercial scales. Moreover, the microorganisms embody an alternative source of enzymes owing to their biochemical diversity and susceptibility to gene manipulation. Besides, they can be cultured in large quantities in a short time by fermentation (Perisamy *et al*., 2013). Amongst the various enzymes produced by microorganisms, xylanases occupy an important platform, as

they are extensively used in biotechnological applications mainly in paper and pulp industries, food, animal feed as well as for bioconversion of lignocellulosic waste into value-added products, covering all the sectors of industrial enzymes market. Xylanases are glycosidases which catalyse the endo hydrolysis of 1, 4-β-D-xylosidic linkages in xylan (Collins *et al.,* 2005).

1.3 Xylan: occurrence, structure and composition

The substrate of xylanases, xylan, is a major structural polysaccharide linked to lignin and cellulose in plant cell wall. It is the second most abundant polysaccharide in nature, accounting for around one-third of all renewable organic carbon on earth (Prade, 1995). The main chain of xylan consists of a homopolymeric backbone of endo-β-1, 4-xylopyranosyl residues having side-group substitution to changing degree with glucuronopyranosyl, 4 - O - methyl - D - glucuronopyranosyl, α – L - arabinofuranosyl, acetyl, feruloyl and p-coumaroyl groups (Li *et al.,* 2000). Consistent with their structural chemistry and side-group substitutions, the xylans seem to be interspersed, intertwined, and covalently linked at lignin by an ester linkage in 4-O-methyl-D-glucuronic acid residues present as substituent in xylan backbone, while producing a coat around underlying strands of cellulose via hydrogen bonding (Biely, 1985; Joseleau *et al.,* 1992; Roberts, 1996). It varies in structure between different plant species and in several types of tissues and cells. Xylan is found in huge quantities in hardwoods from angiosperms (15–30% of the cell wall content) and softwoods from gymnosperms (7–10%), as well as in annual plants (< 30%) (Singh *et al.,* 2003). It is characteristically located in the secondary cell wall of plants but is also found in the primary cell wall, particularly in monocots (Wong *et al.,* 1988).

1.4 Xylanases

The xylanolytic enzyme system chiefly comprises of two types- main chain acting enzymes and the accessory enzymes. The main chain acting enzymes are endo-1, 4-β-D-xylanases and 1, 4-β-D-xylosidases, which hydrolyse the main chain to liberate xylooligosaccharides and xylose. The substituent groups are cleaved by accessory enzymes, such as α-L-arabinofuranosidase, α-D-glucuronidase, acetyl xylan esterases, ferulic acid esterases and p-coumaric acid esterases (Subramaniyan and Prema, 2002).

1.5 Endoxylanase (β-1, 4-D-xylanohydrolase, E.C. 3.2.1.8)

Endoxylanases are the hemicellulases that depolymerise xylan to short chain xylooligosaccharides of varying chain length (Kuhad *et al.*, 1997). They are principally of two types- non-debranching and debranching (Dekker and Richards, 1976). The non-debranching type is the non-arabinose-liberating endoxylanases which fall into two categories, one releasing xylose and xylobiose as the end products and the other releasing xylooligosaccharides as end products. The former cannot act on L-arabinofuranosyl initiated branch points at β (1-4) linkages and the latter cannot cleave branch points at α (1-2) and α (1-3). These endoxylanases do not act on xylotriose and xylobiose. The debranching or arabinose-liberating endoxylanases can be categorised into two groups. One group hydrolyse branch points and produce xylooligosaccharides and arabinose as the end products and the other that can cleave the xylan chain and the branch points, releasing mainly xylobiose, xylose and arabinose. The occurrence of debranching activities in xylanases appears to be inconsistent (Bajpai, 1997a).

1.6 Regulation of xylanase biosynthesis

Xylanolytic enzymes from both bacteria and fungi are inducible in nature (Kulkarni *et al.,* 1999). In general, the xylanase induction is a complex phenomenon and the level of response to an individual inducer varies with organisms. An inducer producing greatest xylanase activity in one species may be an inhibitor for the other species (Haltrich *et al.,* 1996; Beg *et al.,* 2001). The substrate derivatives and the enzymatic end products may often play a key role in the induction of xylanases. They can also be active as the end product inhibitors, possibly at a much higher concentration. Xylan, being a high molecular mass polymer, cannot penetrate the cell wall. The low molecular mass fragments of xylan which play a key role in the regulation of xylanase biosynthesis are xylose, xylobiose, xylooligosaccharides, heteropolysaccharides of xylose and glucose and their positional isomers (Kulkarni *et al.,* 1999).

A basal level of the hydrolytic enzymes is always detectable in the cell. This leads to the generation of small signalling molecules that are generated by the degradation of substrate molecules. These signalling molecules are able to enter the cell and induce the synthesis of the corresponding enzyme (Kuhad *et al.,* 1997). It was proposed that xylan or xylan-containing carbon sources are necessary for the xylanase production as their hydrolytic products serve as both inducers and carbon source for the organism. However, preference for lignocellulosic substrate depends upon the strain (Anthony *et al.,* 2003).

Enzyme synthesis was also found to be repressed when easily metabolisable carbon sources, like glucose were present in the growth medium, suggesting that the synthesis of the enzyme is controlled by transition state regulators and catabolite repression (Kulkarni *et al.,* 1999).

1.7 Sources of xylanase

Xylanases are produced by bacteria, fungi and actinomycetes. Microbial xylanases are the preferred catalysts for xylan hydrolysis owing to their high specificity, mild reaction conditions, negligible substrate loss and no side product generation (Beg *et al.,* 2001; Haki and Rakshit, 2003). The presence of multicomponent xylanolytic enzyme system is quite widespread amongst bacteria and fungi (Bailey *et al.,* 1992; Coughlan and Hazlewood, 1993). Though high xylanase-producing cultures are reported from the world of fungi, there are major problems associated with the use of fungal xylanases, like low pH and temperature optima. Cellulase contamination poses an additional problem for application of the fungal enzymes in pulp industries as the former reduces the extent of cellulose recovery in the pulp (Subramaniyan, 2000). Among bacteria, actinomycetes are a potential source of xylanase. Streptomycetes have been found to be the most abundant hemicellulases producers among actinomycetes (Saini *et al.,* 2015). Xylanase production has also been seen in *Thermomonospora curvata*, *Thermomonospora alba*, *Micromonospora* sp., *Microbispora bispora*, *Nocardia*, *Saccharomonospora viridis* and *Thermoactinomyces* sp. (Ball and Mc Carthy, 1988).

1.8 Xylanase production

Large scale cultivation of microorganisms for enzyme production remains an economical challenge. The cost of the enzyme is one of the main factors determining the economics of any process. Xylanase production depends upon the culture conditions of microorganism, yield, strain and end-use of the enzymes. There are largely two possibilities for the cultivation of microbial enzymes producing strains, solid state fermentation and submerged fermentation (Gawande and Kamat, 1999).

1.9 Solid state fermentation

Solid state fermentation (SSF) is characterised by the growth and fermentation of microorganisms on moist, water insoluble and solid substrates, in the absence of free flowing water, however, the substrate must possess enough moisture to support growth and metabolism of microorganisms. SSF requires no fermentation controls, lesser water volumes, greater volumetric productivity and is considered as self-controlled process (Beg *et al.,* 2001; Pandey, 2003; Shah and Madamwar, 2005). A wide range of low-cost agro-industrial residues, such as sugarcane bagasse, wheat bran, wheat straw, corn cob, rice straw etc., are generally considered as the best substrates for SSF. Several factors, such as selection of suitable microorganisms, substrate, particle size, water content and water activity of the substrate, relative humidity, type and size of inoculum, temperature etc., are to be considered in SSF. SSF cannot control physical parameters, such as pH, dissolved oxygen, nutrients etc., which can be controlled during submerged fermentation (Pandey *et al.,* 1999, 2000).

1.10 Submerged fermentation

Submerged fermentation (SmF) involves the growth and fermentation of microorganisms in the presence of sufficient water so as to dissolve the whole medium components in it and is well characterised with homogenous conditions maintained throughout the processing. The physical parameters, for instance pH, temperature and dissolved oxygen can be effectively controlled and scaling up of the process is feasible due to defined conditions. Lower production yield, higher risk of contamination, high energy input etc., are some of the disadvantages (Satyanarayana and Adhikari, 2006). However, most xylanase manufacturers produce these enzymes using SmF (nearly for 90% of the total xylanase sales worldwide)

(Polizeli *et al.,* 2005). SmF allows better control of the fermentation conditions and it is the preferred method of production when the preparations require more purified enzymes (Frost and Moss, 1987; Corral and Ortega, 2006). Xylanase production is extensively reported by both the fermentation processes (Jones *et al.,* 2012).

1.11 Optimisation of xylanase production

Production of xylanase depends on various process parameters, such as carbon source, nitrogen source, pH, temperature, substrate concentration, inducer sources and its concentration and inoculum level (Lin *et al*., 2006). Thus the improvement of xylanase production could be achieved through manipulation of nutritional and fermentation parameters.

There are several strategies used for modelling and optimisation of fermentation parameters using conventional and statistical experimental designs. Conventionally, medium optimisation using 'one-factor-at-a-time' approach was used for enhancing xylanase production. This method is considered to be cumbersome, time-consuming and limiting considering the interaction effects of media components. During the fermentation process, the operating conditions interact and its influence is reflected in the yield of enzymes. Thus advanced statistical methods, such as Response Surface Methodology (RSM) which analyse the interaction effects and evaluate a set of process conditions are considered to be superior for media optimisation (Salihua *et al.,* 2011). RSM is a collection of statistical techniques that are used for modelling and analysis, wherein a response of interest is influenced by several variables and the objective is to optimise this response (Myers and Montgomery, 2002).

1.12 Use of agrowaste as substrates for xylanase production

Agrowastes are rich in cellulose, hemicellulose, and lignin. Xylan constitutes about 15-30% of hemicellulose which is good as an inducer for xylanase production (Svarachorn, 1999). Lignocellulosic materials are utilised in culture medium formulation because they are plenteous in nature, low in cost and possess a high level of carbohydrate content which is suitable to generate fermentable sugars (Ling, 2014).

Lignocellulosic substrates, like the husk of barley, corn cob, straw and bran of wheat are some of the best substrates of xylan along with leaf litter, cow dung, rotten vegetable products, agricultural wastes, food processing wastes, forestry wastes etc. (Corral and Ortega, 2006). In India, approximately 625 million tonnes of agricultural wastes are generated annually, including groundnut cake, rice bran, rice straw, wheat bran, sugarcane bagasse, cotton leaf scraps, fruits and vegetable wastes etc. The management of these wastes effectively and economically must be given a prime priority not only for reducing the detrimental impact of the wastes to environment, but also for the transformation of these wastes into useful raw materials for the production of value-added products of industrial and commercial potential at low cost (Kumar *et al.,* 2003).

1.13 Purification and applications of xylanase

Purification of xylanases to homogeneity is necessary for detailed biochemical and molecular studies for the successful determination of their primary amino-acid sequences and their three-dimensional structures (Sa-Pereira *et al.,* 2003). Protein purification varies from a simple one-step purification procedure to large-scale purification processes. Purification of xylanases is usually based on the multi-step series of nonspecific

techniques, such as ammonium sulphate precipitation, gel filtration, ion exchange, affinity chromatography etc. (Subramaniyan and Prema, 2002).

One of the most significant biotechnological applications of xylanase is its use in pulp bleaching and deinking of waste paper (Viikari *et al.,* 1994; Bajpai, 1999). The other potential applications of xylanases include the bioconversion of lignocellulosic material and agrowastes into fermentative products, the clarification of juices, the improvement of the consistency of beer and the digestibility of animal feedstock (Wong *et al.,* 1988). They may also be applied in the production of rayon, cellophane and several chemicals such as cellulose esters (acetates, nitrates, propionates and butyrate) and cellulose ethers, which are all produced by dissolving pulp and purifying fibres from other carbohydrates (Subramaniyan and Prema, 2002). Xylanase, together with other hydrolytic enzymes, can be used for the generation of biological fuels, such as ethanol, from lignocellulosic biomass (Lundgren *et al.,* 1994). They may also improve the extraction of coffee, plant oils, and starch and also the quality of bread, by increasing the bread's specific volume (Maat *et al.,* 1992; Wong and Saddler, 1993). The xylose resulting from xylan depolymerisation may also be converted to xylitol, a valuable sweetener that can be used in both the pharmaceutical and food industries (Sirisansaneeyakul, 1995; Parajó *et al.,* 1998; Soleimani *et al.,* 2006). The enzymatic hydrolysis of xylan may result in xylooligosaccharides, which may be used in pharmaceutical, agriculture and feed products (Vázquez *et al.,* 2000).

1.14 Relevance of the study

On account of huge industrial applications, a significant research effort has been devoted towards mining and characterisation of xylanases. There have been many models for solving the problems related to pulp and

paper industry effluents, stretching from purely chemical methods to biotechnology-aided processes including application of fungal xylanases and even a combination of the above methods. Initial focus had been on the fungal xylanases due to their high activity, but constraints faced during mass production and industrial applications, like the cellulase contamination, low pH, low temperature optima, low stability at the required pH values and temperatures, placed aquatic actinomycete xylanases as a tough competitor in the industrial arena. In the meantime, utilisation of microorganisms producing higher levels of xylanase is a good alternative in agrowaste treatment, silage treatment, in solving food industry related problems etc. Naturally high productive microorganisms have many advantages over the genetically manipulated ones because of their genetic stability and the more predictable biological nature. The manipulation of cultural and nutritional conditions alone can result in the over production of the metabolite of interest. All kind of applications of xylanases or enzymes in general, need characterisation studies of crude and purified proteins.

The present study is undertaken at Cochin estuary and its associated mangroves. Mangrove and estuarine environment harbours a variety of microorganisms wherein actinomycetes are one among them. At the same time, they are one among the least explored ecosystems on earth, which has immense potential as a source of potent microorganisms that produce valuable compounds particularly, enzymes such as xylanases. There is meagre knowledge regarding the distribution of actinomycetes and their potential for producing bioactive metabolites, microbial composition, particularly the xylanase producers of the study area. Thus, it is crucial that a broad spectrum activity of actinomycetes from hitherto unexplored habitats be considered as sources of novel bioactive secondary metabolites and enzymes.

Recognising the significance of aquatic actinomycetes as a source of xylanase with industrially important characteristics, in the present study, an effort has been made to isolate actinomycetes from Cochin estuary and to screen their ability to produce antimicrobial compounds and industrially important enzymes. A high xylanase-yielding strain was subjected to detailed analysis with a view to developing an indigenous strain capable of producing xylanase using agricultural wastes which further makes the process cost-effective and ecofriendly.

1.15 Objectives of the study:

- To isolate actinomycetes from sediment samples of Cochin estuary and mangroves
- To screen for antimicrobial activity and various hydrolytic enzyme production from isolated actinomycetes.
- To optimise the xylanase production by one-factor-at-a-time and statistical methods.
- To purify and characterise the xylanase
- To study the use of agrowastes for xylanase production.
- To study the application of xylanase in paper deinking and fruit juice clarification.

The thesis is presented in 6 chapters. The first chapter comprises of a general introduction including the significance of the work. The second chapter deals with isolation and screening of actinomycetes from Cochin estuary and its associated mangroves for antimicrobial activity and various hydrolytic enzymes production. Among the various isolates obtained, isolate ER1 which produced the maximum xylanase was identified as *Streptomyces* sp. and selected for the current study. The third chapter gives an account of the optimisation of xylanase production from *Streptomyces* sp.

ER1 using conventional one-factor-at-a-time method and statistical methods- Plackett- Burman and Central Composite Design. The fourth chapter deals with the purification and characterisation of xylanase from *Streptomyces* sp. strain ER1. The fifth chapter projects the xylanase production using various agrowastes, the optimisation of xylanase production from selected agrowastes and applications of xylanase in paper deinking and fruit juice clarification.

Chapter -2

ISOLATION AND SCREENING OF ACTINOMYCETES FOR BIOACTIVE COMPOUNDS

Contents

2.1 Introduction

The Cochin estuary, is the longest estuarine system with an area of about 157 sq.km on the southwest coast of India, extending between 9°40'12" and 10°10'46"N and 76°09'52" and 76°23'57"E with its northern boundary at Azheekodu and southern boundary at Thannermukham bund. It stretches parallel to the coastline and is connected with the Arabian Sea at the Cochin harbour by a Barmouth of 450 m width and an average depth of 8-10 m. It possesses all the characteristics of a tropical positive estuary and comprises a system of interconnected lagoons, bays and swamps penetrating the mainland and enclosing many islands in between. The estuary receives runoff throughout the southwest monsoon and also to some extent during the northeast monsoon season.

The salinity gradient in the Cochin estuary supports diverse species of flora and fauna, based on their tolerance for the saline environment. Mangrove vegetation is seen along with the backwater channels and along

the banks of Cochin estuary, in the form of patches or narrow continuous belt. Among the existing mangrove ecosystems, Puthuvyppu and Valanthakadu are pristine mangrove systems and hence sampling was done mainly from these sites. Puthuvyppu mangroves are protected by the Fisheries Research Unit of Kerala University of Fisheries and Ocean Studies, about 100 m away from the estuarine front and are free from sewage inputs. Valanthakadu Island is one of the untouched patches of mangrove vegetation with rich biodiversity. Sampling was also done from mangroves at Thevara and Aroor of the Cochin estuary. However, the area of mangrove vegetation at Thevara has been eroded to one tenth of the earlier size and Aroor is a dwindling mangrove site with low plant density.

Interest in enzymes from marine actinomycetes increased because of its high stability than enzymes derived from terrestrial microbes, plants and animals. In concomitant with this information and emerging problems of multi-drug resistance and new phytopathogens, actinomycetes became the centre of research interest and isolation of actinomycetes from the relatively unexplored region was popularised (Nilar *et al*., 2002; Becerril-Espinosa *et al*., 2013). The concept was proved completely relevant with the recent worldwide exploration, especially in China and Japan (Zheng *et al*., 2000; Boudemagh *et al*., 2005; Li *et al*., 2010). India with its enormous geographical diversity offers unique environmental dynamics supporting a diverse group of microorganisms especially different kinds of actinomycetes. Actinomycete diversity and their biological activities is a potential field of research in the Indian context as several new species of this phylum have been reported (Arumugam *et al*., 2011). Many reports described that the east coast area is a major source of actinomycetes in India (Danasekaran *et al.,* 2005; Vijayakumar *et al.,* 2007). However, only a few reports are available on actinomycete diversity on the west coast and

mangrove soil of India (Gulve and Deshmukh, 2007). So far, there are no reports on actinomycetes from Cochin estuary. Keeping the above in view, the present study is an attempt to isolate actinomycetes from Cochin estuary and its associated mangroves and to study the antimicrobial activity and various hydrolytic enzymes production from the actinomycete isolates.

2.2 Review of literature

Actinomycetes are widely distributed in various terrestrial and aquatic habitats but soil remains the most important habitat with the largest population found in the surface layer (Hayakawa, 2008). This is also supported by few reports from the east coast of India (Sivakumar *et al*., 2005; Vijayakumar *et al*., 2007; Dhanasekaran *et al*., 2008). They are also found in the lower horizons to substantial depths of soil. In natural soil habitat, Streptomycetes exist as a major component of actinomycetes population.

There are many studies on isolation of actinomycetes from terrestrial soil (Jain and Jain, 2007; Kavita and Vijayalakshmi, 2007). Isolation of actinomycetes from the marine environment has been less studied when compared to that from the terrestrial environment. However, research has gained momentum in the last three decades and several attempts have been made to isolate the actinomycetes from the marine environment (Manivasagan *et al.,* 2013).

Among the various marine ecosystems, studies on actinomycete diversity of estuarine and mangrove sediment, which is a bountiful resource, have not been extensively investigated, although their ubiquitous presence in the marine sediments has been well documented (Jensen *et al.,* 1991; Takizawa *et al.,* 1993; Moran *et al.,* 1995). Estuaries are complex ecosystems, characterised by changing physical and chemical conditions

both laterally and with depth. Microorganisms may be transported into estuaries from riverine flow, surface runoff, groundwater seepage and tidal currents, resulting in a mixed estuarine community of soil and marine derived species adapted to the estuarine environment. The mixing of freshwater and seawater results in physical and chemical gradients in salinity, nutrient input and organic matter composition (Crump *et al*., 2004). As the estuarine environment is characterised by different conditions from those found in terrestrial habitats, estuarine actinomycetes may produce unique bioactive compounds and enzymes so as to adapt and survive in the estuary. Mangrove forests consist of woody trees and shrubs that plentifully thrive in the saline sediments of tropical and subtropical coastline. Due to the presence of high salinity, high temperature, extreme tides, high sedimentation and high evaporation, the muddy anaerobic mangrove sediment differs from the terrestrial one in terms of the microbial diversity (Giri *et al*., 2011). Among the actinomycetes, species of *Streptomyces* constitute second highest percentage after *Nocardia* in terms of distribution in the mangrove ecosystem (George *et al*., 2012).

2.2.1 Use of selective media and antibiotics for the isolation of actinomycetes

The use of amino acids was suggested in the past literature, particularly L-arginine for the isolation of actinomycetes (Lindenbein, 1952). A method of combining an antifungal antibiotic with glycerol-arginine medium was described, which selectively encouraged the development of aerobic, soil inhabiting actinomycetes (Porter *et al*., 1959). Use of several different media was suggested for isolation of actinomycetes from soil. Most of them contained carbon and nitrogen sources which are utilised by bacteria and moulds as well as by actinomycetes and therefore are not selective for the latter (Lingappa and Lockwood, 1961). Different

media were investigated for selective isolation of *Streptomyces* sp. from the soil. The most frequently used carbon and nitrogen sources were glucose and asparagine, respectively. Glycerol, potassium nitrate, peptone, casein and starch were employed with moderate frequency. The best media, allowing good development of Streptomycetes while suppressing bacterial growth, were those containing starch or glycerol as the carbon source with casein, arginine or nitrate as nitrogen source (Kuster and Williams, 1964).

Use of antibiotics, like Nystatin (50 µg/mL), Actidione (50 µg/mL), Polymyxin B sulphate (5.0 µg/mL) and Penicillin (1.0 µg/mL) resulted in the preferential isolation of actinomycetes (Williams and Davies, 1964). However, antibiotics like Novobiocin (100 µg/mL), Gentamicin (2-5 µg/mL), Rifampicin (25 µg/mL) have also been used (Cross, 1968; Chormonova, 1978; Shearer, 1987).

Selective media were also useful to measure the optimal conditions for the isolation of rare actinomycetes from soil and macromolecules, such as casein, chitin, hair hydrolysate, and humic acid were chosen as carbon and nitrogen sources (Naikpatil and Rathod, 2011). Colloidal chitin agar has been anticipated as a suitable medium for intensive isolation of actinomycetes (Hsu and Lockwood, 1975). Humic acid vitamin agar medium was proposed for the selective isolation of *Micromonospora, Microbispora, Streptosporangium, Dactylosporangium and Thermomonospora* species (Hayakawa and Nonomura, 1987). The bovine bone powder was used for enriching the population of actinomycetes (Yokoyama and Mino, 1990). Gellan gum media was suggested for the isolation of rare actinomycetes along with *Streptomyces* sp. (Suzuki, 2001). Hair hydrolysate vitamin agar and Guaze I medium were also proposed as the selective media for the isolation of actinomycetes (Seong *et al*., 2001;

Zakharova *et al.,* 2003). Various selective methods for the isolation of rare actinomycete genera was reviewed (Hayakawa, 2008).

2.2.2 Diversity of actinomycete population in estuary and mangroves

Estuarine and mangrove sediment is a prolific source of actinomycetes and many reports from all over the world have proved it. Actinomycetes were isolated from the salt marsh and mangrove areas of Georgia, Athens, using 16S rRNA and found that *Streptomyces* sp. were the indigenous components (Moran *et al*., 1995). About 100 strains were isolated from a mangrove sand of Morib, Malaysia, in an earlier study (Vikineswary *et al*., 1997).

A rare actinomycete, *Agromyces* sp., was isolated from the rhizosphere of mangroves in the estuary of the River Shiira, Japan (Takeuchi and Hatano, 2001). Investigation in two tropical estuaries of Santos-São Vicente, Brazil, revealed the presence of high actinobacterial communities in the sediment samples (Piza *et al.*, 2004). Different aquatic actinomycetes were isolated and identified in aqueous environments (Fiedler *et al*., 2005). Many different actinomycete species belonging to three different genera and three species and an actinobacteria, *Gordonia lacunae,* were isolated from the estuarine sediments of Plettenberg Bay, South Africa (Roes *et al*., 2008).

A few reports from globally different geographical locations of different mangroves have described the diversity and isolation of novel rare actinobacteria. Occurrence of *Microsmonospora* was reported from the Sunshine Coast, Australia (Eccleston *et al*., 2008). Diverse genera, such as *Brevibacterium, Dermabacter, Kytococcus, Microbacterium, Nesterenkonia* and *Rothia* were isolated from mangrove sediments in Brazil (Dias *et al*., 2009). There were many reports on isolation of rare

actinobacteria from mangrove soil of Thailand, Taiwan, Japan and China (Suriyachadkun *et al*., 2011; Tseng *et al.,* 2011; Hamada *et al*., 2012; Xu *et al*., 2012).

Streptomyces qinlanensis, a novel species, was isolated from mangrove soil in Wenchang, China. The strain was characterised by white aerial mycelium and long spore chains (Hu *et al.,* 2012). In all, 11 *Streptomyces* pure cultures were obtained from sediment samples of a hypersaline estuary, the Laguna Madre, Texas (Luis *et al.,* 2013).

Many different genera of rare actinobacteria were identified with the predominant genus *Micromonospora* in both mangrove and medicinal plant rhizosphere soil samples in Dhaka, Bangladesh (Ara *et al*., 2013). Similar results were observed from a Malaysian mangrove forest. Many other genera of rare actinobacteria, such as *Leifsonia, Streptacidiphilus, Sinomonas* and *Terrabacter*, were discovered which were not commonly found in mangrove environment (Lee *et al*., 2014).

The diversity of actinomycetes from a mangrove forest of Pahang, Malaysia, was studied and a total of 4850 actinomycete isolates were recovered using several isolation media (Nurfathiah *et al.*, 2014). Different aquatic actinomycetes were isolated from the mangrove forest sediments in the south of Iran (Kafilzadeh and Dehdari, 2015).

2.2.3 Diversity of actinomycetes from the Indian marine environment

The study on the diversity of actinomycetes from the Indian marine environment was initiated in 1966 (Baam *et al*., 1966). A total of 518 *Streptomyces* strains were isolated from the sediments of estuarine, backwater, marine, freshwater and mangrove environment of Porto Novo, using Grein and Meyer's agar, Kuster's agar and glucose asparagine agar (Lakshmanaperumalsamy, 1978).

Many *Streptomyces* species were isolated from the sediments of the Veli estuarine lake (Dhevendaran and Annie, 1999). Two new species of actinomycetes, *Streptomyces kathirae* and *Actinopolyspora indiensis* were isolated using different isolation media from the Pitchavaram mangrove ecosystem, Tamil Nadu (Sivakumar 2001a, 2001b).

In all, 160 strains of actinomycetes were isolated from the sediments of mangrove, estuary, sand dune and industrially polluted marine environment of Cuddalore. Of these, mangrove sediments were the rich sources of actinobacteria (Kathiresan *et al*., 2005). Different actinomycetes were isolated from water and sediment samples of the Vellar estuary, Tamil Nadu and found that the sediments harbour higher population density of actinomycetes as compared to the water samples (Sahu *et al*., 2005; Senthilkumar *et al*., 2005).

The occurrence of actinobacteria belonging to the genus *Streptomyces, Streptoverticillium* and *Micromonospora* were studied from the mangrove sediments of the River Gaderu of the Gautami-Godavari estuarine system in Andhra Pradesh (Raghavendrudu and Kondalarao, 2007). Isolation of actinobacteria from Annagkoil estuarine soils of Tamilnadu was also studied (Dhanasekaran *et al*., 2009).

Studies on the marine soil and sediment samples collected from different locations of Muthupet mangrove ecosystem, Tamilnadu, revealed different marine actinomycete isolates, using selective isolation media, like starch casein agar and Kuster agar (Vijayakumar *et al*., 2010; Sathiyaseelan and Stella, 2011). Totally, 107 actinomycete isolates were screened from mangrove sediment samples of Thondi and Karankadu of Palk Strait region, situated along the southeast coast of India. More number of isolates was

found in Karankadu mangrove region followed by Thondi region particularly in monsoon season (Ravikumar and Suganthi, 2011).

The soil samples collected from the mangrove forests of Karwar was studied and 53 rare actinomycete strains were isolated using selective isolation approaches. The isolates belonged to genera, such as *Micromonospora, Microbispora, Actinoplanes and Actinomadura* (Naikpatil and Rathod, 2011). Similarly, many actinomycete strains from coastal, estuarine and mangrove ecosystems of Karwar, Karnataka, were isolated using starch casein agar medium (Sanjotha, 2016).

The actinomycete diversity of the marine sediments from Pulicat estuary and Ennore estuaries, Tamil Nadu, were studied. Totally, 227 isolates obtained were morphologically distinct on the basis of spore mass colour, aerial and substrate mycelia formation and production of diffusible pigments. The majority were assigned to genera *Streptomyces*, *Actinopolyspora* and *Nocardioides* (Sharma and David, 2012).

Actinomycete strains from the marine sediments and water samples of Royapuram, Muttukadu, Mahabalipuram Sea shore and Adyar estuary, were isolated using actinomycete isolation agar (Valli *et al*., 2012). A novel actinomycete, *Streptomyces barkulensis* sp. nov. was isolated from an estuarine sediment of Chilka Lake, Odisha (Ray *et al.*, 2014).

Totally, 50 actinomycete isolates were obtained from the estuarine region of Puducherry (Priya *et al.*, 2015). There were many reports on isolation of actinomycetes from mangrove sediments of Koringa (Andhra Pradesh), Sunderbans and Dumas-Bhimpore mangrove region (Surat) (Duddu and Guntuku, 2015; Sengupta *et al.,* 2015; Shah and Soni, 2016). A novel actinomycete, *Streptomyces himastatinicus,* was isolated from the

mangrove sediment of Bhitarkanika, along Odisha coast (Priyadarshini *et al.,* 2016).

2.2.4 Bioactive potential of actinomycetes from Indian mangroves and estuary

2.2.4.1 *Antimicrobial screening*

More recently, advances in technology have sparked resurgence in the discovery of natural product antibiotics from bacterial sources. Particularly, efforts have been refocused on finding new antibiotics from old sources (Streptomycetes) and new sources (rare and marine actinomycetes). Screening remains an outstanding programme for the selection of potent antibiotic producing microorganisms. This has resulted in some newly discovered antibiotics with unique scaffolds and novel mechanisms of action, with the potential to form a basis for new antibiotic classes.

The adequate exploration of the versatility of marine actinomycetes is yet to be done (Stach *et al*., 2003; Magarvey *et al*., 2004). The adaptability of actinomycetes with respect to secondary metabolite production has been evidenced by their quantum of metabolite production that, out of nearly 23,000 metabolites yielded by varied microbes, 10,000 were reported from actinomycetes summing up to 45% of entire production (Berdy, 2005). The genus *Streptomyces* alone contributed nearly 7,600 metabolites out of 10,000 (Berdy, 2005). The majority of reported secondary metabolites were active antibiotics (Sharma, 2014). The root point for versatility of actinomycetes metabolite production especially from estuarine and mangrove actinomycetes are due to its large genome, regular environmental changes in mangrove and estuarine ecosystem by way of variations in salinity and tidal gradient, presence of NRPS – Non Ribosomal Polyketide Synthase and PKS – Polyketide Synthase Pathways and huge genome with

adequate transcription factors which regulates the expression of genes acting in tune with specific requirements (Li and Piel, 2002; Salmon *et al.*, 2003; Salami, 2004; Bonjar *et al*., 2005; Long *et al*., 2005; Hong *et al*., 2009; Padmadhas and Raghunathan, 2010; Santos *et al.,* 2012).

The Sundarbans mangrove forest, characterised by moderate to highly saline water is a potent source for the isolation of novel antibiotic-producing actinomycetes (Kala and Chandrika, 1993). An actinomycete isolated from the Sundarbans region showed potent antimicrobial activity against Gram-negative and positive bacteria including multi-drug resistant bacteria, like MRSA, molds and yeast (Saha *et al.,* 2005). The relationship between the distribution of actinomycetes and antagonistic behaviour with the physicochemical characteristics of the Sundarbans, off the Bay of Bengal was examined. In all, 350 actinomycetes from southern, northern and eastern regions of Sundarbans were isolated and tested for antibacterial activity against Gram-positive bacteria. About 50% of isolates were active against at least one of the test bacteria and 58.33% of the isolates showed higher antibacterial activity when cultured in natural seawater (Mitra *et al.*, 2008). A total of 55 actinomycetes isolates from the soil sample of Karanjal region in Sundarbans were characterised for morphological identification and antimicrobial activity. The tested isolates had a broad spectrum antibacterial activity and hence suggested as potential sources of antibiotics for pharmaceutical interest (Arifuzzaman *et al.,* 2010). Totally, 54 actinomycetes were isolated from unexplored heritage sites of Sunderbans mangroves and analysed for antimicrobial activity against 15 test organisms including 3 phytopathogens. Among the 54 isolates, 3 specific strains had higher degree of antimicrobial potential, effective in a broad range including phytopathogenic fungus (Sengupta *et al.,* 2015).

Actinomycetes isolated from sediment samples of Adyar estuary were subjected to preliminary screening by using cross-streak method against 2 Gram-positive and 8 Gram-negative bacteria. All the isolates were active against atleast one of the test organisms. The antibacterial substances were extracted from most potent strains of *Streptomyces* sp. The antibacterial assay of the extracts was performed using Kirby-Bauer disc diffusion method (Valli *et al.,* 2012).

The potency of mangrove actinomycetes to produce antimicrobial substances had been studied with 38 strains isolated from different samples of Pichavaram mangrove (Sweetline *et al.*, 2012).

The isolates obtained from the sediments of Pulicat estuary, Muttukadu estuary and Ennore estuary, TamilNadu, showed both antibacterial and antifungal activities. The isolates, namely *Streptomyces* sp. RM17 and *Streptomyces* sp. RM42, *Actinopolyspora* sp., *Halophila*, *Actinopolyspora mortivallis* and *Actinopolyspora* sp. *Iraqiensis* had broad spectrum antimicrobial activity, but Nocardiodes showed a low profile (Sharma *et al.,* 2012).

A total of 40 strains of actinobacteria isolated from the sediments of Vellar estuary were studied for antagonistic activity against *B. subtilis, P. vulgaris, S. flexneri, K. pneumoniae, V. cholerae* and *S. aureus.* Of which 22.5% were found to be very active (Sahu *et al.*, 2005). Some of the very capable antibiotics-producing actinomycetes isolated from Vellar estuary, strongly inhibited the growth of both Gram-positive and Gram-negative bacteria and also yeast like fungi (Dhanasekaran *et al.*, 2009).

The *in vitro* antimicrobial activity of 25 alkaliphilic and halotolerant actinomycetes isolated from the sediment samples of Valapattanam mangrove swamp in Kerala was studied. Antimicrobial activity of 4 selected

actinomycete isolates was determined against bacterial and fungal pathogens of nosocomial origin by agar well diffusion method (Das *et al.,* 2014).

Marine actinomycetes isolated from the Manakudi Estuary of Arabian Sea, Tamilnadu, exhibited higher antagonistic activity against the Gram-positive bacteria including methicillin resistant and susceptible *Staphylococcus aureus* (Santhi *et al.,* 2010). About 50% of the actinomycetes isolates from the similar sites showed activity against *S. aureus* followed by *V. cholerae, B. subtilis, P. vulgaris, E. coli, K. pneumoniae* and *S. typhi* (Balagurunathan *et al.,* 2010). Antibacterial activity of actinobacterial strains isolated from Parangipettai mangrove ecosystem was tested by agar plug method. Among 20 isolates, 12 exhibited antibacterial activity, in which 1 strain showed promising activity against 12 pathogenic Gram-negative bacterial strains (Radhakrishnan and Mohana, 2014). Antimicrobial activity of 3 dominant species of wild and UV mutated actinomycetes isolated from mangrove soil region of Maravakkadu Reserve forest, Tamil Nadu, showed activity against 5 human bacterial and fungal pathogens (Ashok *et al.*, 2014). Amongst 57 different actinobacterial strains isolated from the Muthupet mangrove sediment samples, 6 showed inhibitory activity against the fungal pathogens, *Rhizoctonia solani* and *Fusarium oxysporum* (Priya *et al.,* 2014).

In all, 15 actinomycete strains were isolated from the mangrove ecosystem of Bhitarkanika, Odisha and 1 strain among the 15 showed good antimicrobial activity against pathogenic strains of *Pseudomonas*, *E.coli, Klebsiella, Bacillus,* and *Proteus* (Kishore, 2011). It is stated that *Streptomyces* strains isolated from mangrove sediment produced potential antibacterial, antifungal and broad spectrum antibiotic compounds (Rao *et al.*, 2012).

S. spectabilis VITJS10 was isolated from marine soil samples of southeast coast of Tamil Nadu, Kanyakumari – Chinnamuttom and found that it produced antimicrobial activity. The antibacterial potential revealed the effective activity against *Shigella flexneri* (MTCC No: 1457), *Salmonella typhi* (MTCC No: 1167), *Escherichia coli* (MTCC No: 1588), *Pseudomonas aeruginosa* (MTCC No: 4676) at 20 mg/mL concentration (Selvakumar *et al*., 2015).

Amongst, 125 actinobacterial isolates, obtained from mangrove sediments of Nagapattinum, 29 isolates showed antagonistic activity in the preliminary process. The antimicrobial and cytotoxic activity of *Streptomyces* sp. PA-32 isolated, was studied in detail (Kiruthika and Bhuminathan, 2015). Broad spectrum antifungal activity and antibacterial activity were confirmed by the actinomycete isolates from the mangrove soil samples of the backwater area, Ariyankuppam, Puducherry (Janaki *et al.,* 2016).

2.2.4.2 *Hydrolytic Enzymes Production*

Actinomycetes have long been credited as an important resource of enzymes and bioactive compounds. They have exhibited degradation of starch and casein besides the production of antimicrobial agents (Barcina *et al*., 1987; Zheng *et al*., 2000). The diverse range of enzymes produced by actinomycetes was capable of catalysing various simple to complex biochemical reactions. There were many reports on the production and characterisation of enzymes from *Streptomyces* and *Nocardiopsis*, mostly terrestrial, although few reports were available from marine isolates (Stamford *et al*., 2001; Tsujibo *et al*., 2003; Goshev *et al*., 2005; Kavitha and Vijayalakshmi, 2011; Chakraborty *et al.,* 2012). In aquatic environments, microbial extracellular hydrolytic enzyme production was the

major biological mechanism for the decomposition of sedimentary particulate organic carbon and nitrogen (Brunnegard *et al*., 2004; Dang *et al*., 2009). There were reports on the multi-enzyme activity of actinomycetes from marine sediments (Leon *et al*., 2007; Ramesh and Mathivanan, 2009).

Aquatic actinomycetes were isolated from Bengal Gulf and the results showed that the aquatic actinomycetes could be a source for production of bioactive compounds and industrial enzymes (Sharma and Pant, 2001). Another study also revealed that Bengal Gulf was the potential source with a range of aquatic actinomycetes that had the ability to produce industrial enzymes, such as lipase, amylase, cellulase, caseinase and gelatinase (Ramesh and Mathivanan, 2009). In all, 60 strains of amylase producing actinomycetes were isolated from sediment samples of Machilipatnam and Kakinada coast located near Bay of Bengal (Ellaiah *et al.,* 2002, 2004).

Actinomycetes are important for the production of enzymes, such as chitinases (*Streptomyces viridificans*), cellulases (*Thermonospora* spp.), xylanases (*Microbispora* spp.), ligninases (*Nocardia autotrophica*), sugar isomerases (*Actinoplanes missouriensis*), pectinases, hemicellulases and keratinases (Solans and Vobis, 2003).

Streptomyces sp. isolated from Andaman Islands coast had the ability to produce industrial enzymes (Peela *et al*., 2005). Actinomycetes recovered from molluscan and sediment samples of the Vellar estuary, southeast coast of India, showed the production of extracellular enzymes (amylase, cellulase protease, lipase and chitinase) (Sahu *et al.,* 2005). Marine actinomycetes isolated from Tamil Nadu coast of Bay of Bengal, were reported to secrete extracellular enzymes, such as amylases, cellulase,

gelatinase etc. (Ramesh *et al.,* 2006). Actinomycetes were isolated with multi-enzymatic activities from the central coast of Peru (Leon *et al*., 2007).

Marine samples were collected from Pulicat Lake to Kanyakumari and 208 actinomycetes were isolated. Among them, many produced lipase, gelatinase, caseinase, cellulase and amylase (Ramesh and Mathivanan, 2009). Three types of actinomycetes were isolated from the southwest coast of India which had the ability to produce industrial enzymes, such as amylase, cellulase and lipase (Selvin *et al*., 2009). In the report on Kodiyakarai coastal sediments of Bay of Bengal, a strain, GK-22, tentatively identified as *Streptomyces alboniger,* was found to exhibit multiple enzyme activity (Manivasagan *et al.,* 2010).

In all, 56 actinomycete strains were reported from marine sediments collected from different sites in Chennai, Tuticorin, Kerala and Pooombukar in south India. Among them, 9 isolates showed the production of amylase, cellulase and lipase enzymes as detected by agar plate screening assays (Selvam *et al.,* 2011). Majority of the mangrove actinobacteria isolated from sediments of Nizampatnam and Coringa, Andhra Pradesh, produced commercially important enzymes, such as L-asparaginase, cellulase and amylase (Mangamuri *et al.*, 2014). Totally, 35 morphologically distinct *Streptomyces* species were isolated from Muthupet mangrove soil for screening economically valuable enzymes and most of the isolates produced amylase, protease and gelatinase followed by esterase and lipase (Sathya and Ushadevi, 2014). Actinomycetes isolated from mangrove soil of Dumas – Bhimpore mangrove region, Surat showed great potential in producing industrially important enzymes, like amylase, cellulase, L-asparaginase and protease (Shah and Soni, 2016).

2.2.5 Identification and characterisation of actinomycetes

Actinomycetes classification and characterisation, include morphological, physiological and biochemical characterisation in phenotypic approach; whereas, molecular methods are in phylogenetic characterisation part.

2.2.5.1 *Cultural and morphological observation of actinomycetes*

Based on methods for *Streptomyces* spp. characterisation, the basic observation involved cultivation of cultures on various media like yeast extract – malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts – starch agar (ISP4) and glycerol – asparagine agar (ISP5) (Shirling and Gottlieb, 1966). Growth and morphology of *Streptomyces* spp. were observed when cultures grew matured with heavy spore mass in order to verify aerial spore mass colour, substrate mycelium colour and diffusible pigments colour. Preliminary differentiation of a large number of different isolates could be done by colour grouping. However, this study only involved *Streptomyces* spp.; which fell into the same group with similar morphological and physiological properties based on their colour grouping (Williams *et al*., 1993). Sporophore morphology is considered a stable and clearly defined feature for actinomycetes classification. However, this is only applicable without the occurrence of strain degeneration due to the subculture or improper maintenance (Pridham *et al*., 1957). Based on different spore chain morphology, the authors divided *Streptomyces* spp. into seven groups of "morphological sections" and every section was further categorised into another six "series" according to the spore mass colour: white, olive-buff (buff to tan to olive-buff), yellow, blue (blue to blue-green to green), red (pink to red to lavender to lavender-grey), grey (light grey to mouse-grey to brown-grey to grey-brown). The spores then could be

subdivided into distinct ornamentation: smooth, rugose, spiny, hairy, warty, knobby, tuberculate or verrucose (Vobis, 1997).

2.2.5.2 *Physiological characterisation of actinomycetes*

Physiological characterisation of actinomycetes involved a study of their growth and nutrition, their metabolic processes and their reaction to environmental conditions (Waksman, 1959). Only melanoid production and carbon sources utilisation were characterised for *Streptomyces* spp. physiology (Shirling and Gottlieb, 1966). But, depending upon number of isolates and objective of the study, there were some other physiological characteristics that had been considered to categorise actinomycetes, especially *Streptomyces* spp. They were optimum temperature range, nitrate reduction test, NaCl tolerance, production of hydrogen sulfide (H_2S), starch hydrolysis, liquefaction of gelatin, nitrogen source utilisation, pH sensitivity and sensitivity to some antibiotics (Gottlieb, 1960; Williams *et al*., 1989).

2.2.5.3 *16S rRNA gene sequence analysis of Streptomyces spp.*

16S rRNA gene sequences used in phylogenetic relationship inference among microorganisms besides to characterise unknown isolates has been generally accepted (Stackebrandt *et al*., 1992). Relationships measurement amongst bacteria could be done due to the nature of universality of this gene in bacteria (Clarridge, 2004). The 16S rRNA gene sequence is about 1,550 base pairs (bp) long and is basically composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide sufficient sequence information that permits statistically significant comparisons. Universal primers are complementary to the conserved regions at the start of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1,550-bp region), and the sequence of the variable region in between is

used for the comparative taxonomy. Even though 500 and 1,500-bp were the common length to sequence and compare, sequences in databases could be of diverse lengths (Chen *et al*., 1989). 16S rRNA gene sequences comparisons permitted organisms' differentiation at the genus level. This included strain classification at species and subspecies level (Clarridge, 2004). A comparison of partial nucleotide sequences (120-bp) spanning the variable α region of 16S rRNA in 89 *Streptomyces* strains demonstrated that these short nucleotide sequences were useful for *Streptomyces* species rapid identification. At the intra-species level, partial 16S rRNA gene sequence deduced the *Streptomyces* strains phylogenetic relationships (Kataoka *et al*., 1997). However, the 16S rRNA phylogenetic marker usage was criticised because of its heterogeneity of the same genome or due to its lack of resolution at the species level (Acinas *et al*., 2004; Pontes *et al*., 2007). Opportunely, it is still used as a unique and valuable standard for bacterial identification.

Little is known about the diversity of actinomycetes in sediments of Cochin estuary and its associated mangroves which still remains under-explored. The present study was aimed at finding out the major actinomycetes in the sediments of Cochin estuary and their bioactive potential in terms of antibacterial and hydrolytic property.

2.3 Materials and Methods

2.3.1 Sampling sites

An extensive field survey was conducted along the study area and based on the survey, 12 sampling stations (Fig 2.1) were fixed along the Cochin estuary. The selection of sampling stations was done on the basis of habitat type and presence of mangroves. The details of the sampling sites are presented in the Table 2.1.

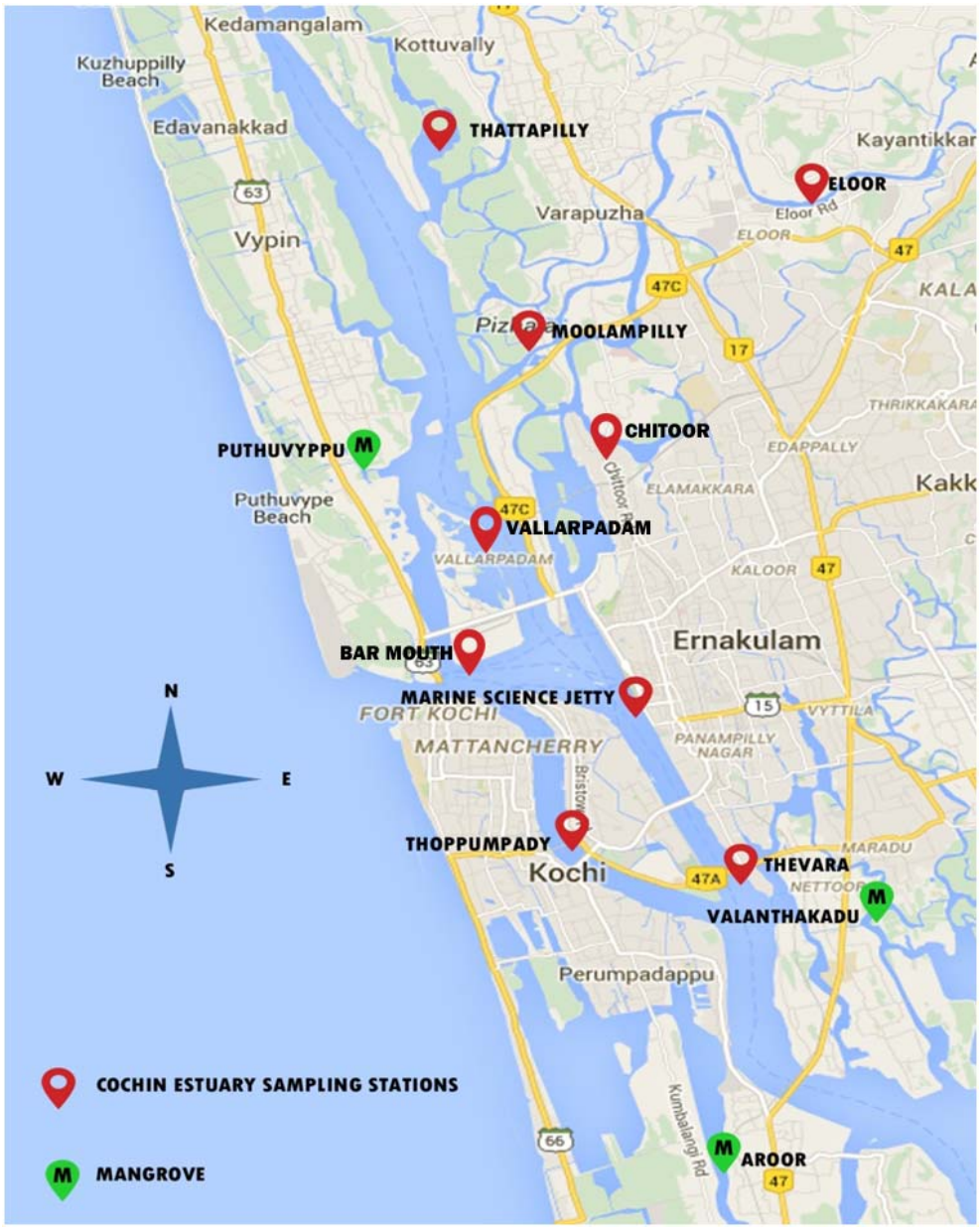


Fig 2.1. Map showing the sampling stations of Cochin estuary

Table 2.1. Details of the sampling site

Sl. No.	Stations	Position		Description
		Latitude	Longitude	
1	Thevara	9°55'35"N	76°17'53"E	Highly influenced by the tidal action and the currents from the Arabian Sea. The zone exhibits sparse patches of mangroves.
2	Thoppumpady	9°56'8.14"N	76°15'39.41"E	Influenced by the tidal influx and currents emerging from the Arabian Sea.
3	Barmouth	9°58'26"N	76°14'39"E	Cochin harbour entance
4	Marine Science Jetty	9°57'37"N	6°16'54"E	Site of domestic pollution and oil spills.
5	Moolampilly	10°2'21.94"N	76°15'49.83"E	Effects from activities associated with Vallarpadam container terminal.
6	Thattapilly	10°7'37.88"N	76°15'55.97"E	Sand mining
7	Eloor	10°5'23"N	76°16'49"E	Industrial belt
8	Vallarpadam	10°0'37.93"N	76°16'31.53"E	Effects from construction activities and industrial activities and it has scattered patches of mangroves
9	Chitoor	10°1'46.11"N	76°17'13.24"E	Influenced by fishing and duck culture
10	Aroor	9°52'23.71"N	76°18'10.57"E	Highly influenced by the tidal action, currents and saline water from the Arabian Sea. The sampling site has an extensive stretch of mangroves
11	Valanthakadu	9° 55'. 24"N	76° 20'1. 23"E	Isolated island with rich mangroves
12	Puthuvyppu	9°59'26.1"N	76°4'8.4"E	Mangrove area

2.3.2 Collection of soil samples

Marine sediment samples were collected from different stations using a van Veen grab (0.04 m^2) and were placed in small pre-labelled plastic bags

using a sterile spatula and tightly sealed. The samples were preserved in an ice box and transported to the lab.

2.3.3 Pre-treatment of the sediment samples

The samples were subjected to pre-treatment in order to facilitate isolation of actinomycetes. 10 g of the sediment from each sampling station was weighed and suspended in 90 mL sterile saline water. It was then kept in an orbital shaker at 200 rpm for 30 min to dislodge the actinomycetes from the sediment. The samples were then incubated at 65°C for 6 min to stimulate the isolation of actinomycetes by eliminating most unwanted Gram-negative bacteria.

2.3.4 Isolation and maintenance of actinomycetes from sediment samples

Actinomycetes were isolated by pour plate and spread plate technique by using starch casein agar, Kusters agar, glycerol-arginine agar and actinomycete isolation agar and incubated at 28°C for 2 to 3 weeks. The media were supplemented with gentamicin sulphate (25 µg/mL) and nystatin (50 µg/mL) to minimize bacterial and fungal contamination, respectively. The actinomycete colonies that appeared on the petri plates were counted from the 7^{th} day of incubation, up to the 28^{th} day. All isolates were identified as actinomycetes based on colony morphology and color of mycelium (Williams and Cross, 1971). Morphologically, diverse actinomycete colonies were sub-cultured, purified, maintained in nutrient agar (prepared in 50% seawater) slants and then stored, at 4^{o}C for further use.

2.3.5 Screening of antimicrobial activity of crude extracts of actinomycetes against test microorganisms

2.3.5.1 *Preparation of crude extract*

All the actinomycete isolates were tested for their ability to produce antimicrobial activity. Spores of actinomycete cultures were inoculated into 50 mL of marine actinomycete broth prepared in 50% seawater, in a 250 mL conical flask. The inoculated broth was placed on an incubator shaker, set at 150 rpm, 28°C for 14 days. The culture broth was withdrawn at 5,7,10 and 14 days of incubation and centrifuged at 10,000 rpm at 4°C for 10 min to obtain cell-free supernatant. Cell-free supernatant of the culture broth was used as crude extract for antimicrobial assay.

2.3.5.2 *Antimicrobial Assay*

The cell-free supernatant of the actinomycete culture broth was tested against human and fish pathogens. Kirby Bauer disc diffusion method was employed for testing antimicrobial activity (Kirby *et al.,* 1966). Muller Hinton agar plates for bacteria and Sabouraud Dextrose Agar for yeast/fungi were prepared and swab inoculation of the pathogens was made on the surface to produce a lawn culture. Sterile filter paper discs impregnated with cell free supernatant (20 µL) of the culture broth was placed on the surface of inoculated agar. The plates were incubated at 28°C for 24-48 h and inhibition was observed as zone of clearance, which was measured and recorded.

2.3.5.3 *Test microorganisms*

The antimicrobial activity of the isolates was tested against bacterial and yeast pathogens associated with human diseases, *viz. Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Enterobacter* sp. and *Candida* sp. (yeast pathogen), Gram-positive bacteria, *viz. Bacillus* sp., *Planococcus* sp. and

those associated with fish diseases, *viz. Vibrio parahaemolyticus* MCCB 141, *Vibrio harveyi* MCCB 151, *Vibrio alginolyticus* MCCB 142 and *Aeromonas* sp. MCCB 152.The bacterial and yeast pathogens were bought from Doctors Diagnostic Centre International, Ernakulam, Kerala and the Gram-positive bacteria and fish pathogens were obtained from the National Centre for Aquatic Animal Health, CUSAT, Kerala.

2.3.6 Solvent extraction

Solvents of differing polarity were screened for extraction of the bioactive compounds to determine the suitable solvent. The fermentation broth was centrifuged at 10,000 rpm for 15 min in a cooling centrifuge at 4°C to separate the mycelia from the supernatant. The supernatant was extracted with 4 solvents with increasing polarity, like n-hexane, ethyl acetate, chloroform and butanol-methanol.

Culture supernatants were mixed with equal volume of the solvents, sealed using parafilm and kept overnight on a shaker for thorough agitation. The solvent layer was separated using a separating funnel. The solvent extracts were concentrated in a rotary vacuum evaporator and tested for antimicrobial activity by Kirby-Bauer disc diffusion method as described under section 2.3.5.2.

2.3.7 Screening for hydrolytic enzymes

The actinomycete isolates were screened for the production of various hydrolytic enzymes, viz. amylase, lipase, cellulase, xylanase, lecithinase, L-asparaginase and L-glutaminase.

2.3.7.1 *Cellulase and Amylase*

Nutrient agar medium supplemented with starch (1%) and caboxy methyl cellulose (1%) was prepared separately. Plates were spot inoculated

and incubated at room temperature (28 ± 2°C) for 3 to 5 days and observations were made. Starch and cellulose agar plates were flooded with Gram's iodine solution. Appearance of clearance zone around the colonies was noted as positive and the diameter of the zone was recorded (González *et al.,* 1978; Kasana *et al.,* 2008).

2.3.7.2 *L-asparaginase*

Modified M9 medium supplemented with L-asparagine (0.5%) and phenol red indicator (0.3 mL/100 mL) was prepared. Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 10 days and observations were made. Appearance of pink zone around the colonies was noted as positive (Gulati *et al.,* 1997).

2.3.7.3 *L-glutaminase*

Modified nutrient agar medium supplemented with L- glutamine (0.5%) and phenol red indicator (0.3 mL/100mL) was prepared. Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 10 days and observations were made. Appearance of pink zone around the colonies was noted as positive (Krishnakumar *et al.,* 2011)

2.3.7.4 *Lipase*

Rhodamine-olive oil-agar medium was used for lipase assay. The medium was autoclaved and cooled to about 60ºC. Then, 31.25 mL of olive oil and 10 mL of rhodamine B solution (1.0 mg/mL distilled water and sterilised by filtration) was added with vigorous stirring. It was then poured into petri plates under aseptic conditions and allowed to solidify. Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 3 days and observations were made. The hydrolysis of substrate causes the

formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation (Ozcan *et al*., 2009).

2.3.7.5 *Lecithinase*

Egg yolk agar supplemented with egg yolk emulsion (11.111 mL/100mL) was prepared. Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 5 days and observations were made. Appearance of opaque zones was measured as indicators of lecithinase production (McClung and Toabe, 1947).

2.3.7.6 *Xylanase*

Nutrient agar medium supplemented with xylan (0.25%) were prepared. Plates were spot inoculated and incubated at room temperature (28 ± 2°C) for 5 days and observations were made. After incubation culture plates were flooded with 0.1% Congo red solution for 15 min and poured off. The plates were further washed by flooding with 1M NaCl for 10 min. A clear zone formation around the microbial colonies indicates the hydrolysis of xylan and the diameter of the zone was recorded (Teather and Wood, 1982).

2.3.8 Morphological and Cultural Characterisation

Morphological, physiological and biochemical properties of the strains were studied as per International Streptomyces Project (ISP) and Bergey's manual of systematic bacteriology (Shirling and Gottlieb, 1966; Williams *et al.,* 1989). The isolates were streaked on to starch casein agar, yeast extract malt extract agar (ISP 2), glycerol asparagine agar (ISP 5) and nutrient agar and the colony characteristics were noted; colour of mature sporulating aerial mycelium, substrate mycelium, macromorphology, diffusible

pigment, colony reverse colour, colony texture etc. were recorded after observing the plates under a Stereomicroscope (Tresner and Backus, 1963).

2.3.8.1 *Cover slip Culture technique*

The isolates were inoculated into marine actinomycete broth and incubated at 28°C for 1-2 days. Plates containing nutrient agar medium were prepared. Sterile cover slips (3-4) were inserted at an angle of 45°C into the agar medium. A loopful of spore suspension of actinomycete was dispensed at the intersection of the medium and cover slip. The plates were incubated at 28°C for 4-8 days. The cover slips were removed at intervals of 2- 4 days and were observed under high power and oil immersion objectives. Morphology of aerial mycelium, substrate mycelium, arrangement of sporogenous hyphae, their morphology (straight, flexuous, spiral shaped) were recorded according to ISP (Nonomura, 1974; Shirling and Gottlieb, 1966).

2.3.8.2 *Biochemical and Physiological characterisation*

The physiological and biochemical tests for characterisation of aerobic sporogenous actinomycetes were done according to protocol by Berd, with slight modifications (Berd, 1973).

2.3.8.2.1 Decomposition of organic substrates

Decomposition of tyrosine, xanthine, hypoxanthine and casein was considered as a basic taxonomic criterion in the characterisation of actinomycetes.

a) **Tyrosine**: Tyrosine (0.5 g) was added to 10 mL of distilled water and autoclaved. This suspension was mixed with 100 mL of sterile nutrient agar media at 50°C, thoroughly mixed and poured into petri

plates. Clearing zone around the actinomycete colony was scored as positive.

b) **Hypoxanthine**: Hypoxanthine (0.5 g) was added to 10 mL of distilled water and autoclaved. This suspension was mixed with 100 mL of sterile basal media (nutrient agar) at 50°C, mixed well and poured into petri plates. Clearing zone around the colony was scored as positive.

c) **Xanthine**: To check xanthine decomposition, 0.4 g xanthine was mixed with 10 mL of distilled water and autoclaved and this suspension was added to sterile nutrient agar media (100 mL) and poured into petriplates as above. Spot inoculation of isolates was done in the respective media and the plates were incubated at 28°C for 5-7 days. Clearing zone around the colony was scored as positive.

d) **Casein**: Decomposition of casein was done in skim milk agar prepared by autoclaving 10 g of skim milk in 100 mL distilled water. Two grams of agar was added to 100 mL of seawater and were autoclaved separately. Sterile skim milk and agar were mixed thoroughly and poured into petri dishes. Spot inoculation of isolates was done and the plates were incubated at 28°C for 5-7 days. Clearing of skim milk agar around the colony was recorded as positive.

2.3.8.2.2 Urea hydrolysis

Christensen's urea agar medium was used to test the production of urease. The media ingredients except urea were dissolved in 950 mL of distilled seawater and autoclaved at 15 lbs for 15 min. Urea was sterilised using diethyl ether and dissolved in 50 mL sterile distilled water. This urea was added to the basal medium, dispensed into test tubes (3 mL each) and slants were prepared. Cultures were inoculated and after incubation a change of colour in the medium to pink was noted as urea hydrolysis. The

actinomycetes utilise urea and liberate ammonia, making the medium alkaline, which is indicated by a pink colour in the medium.

2.3.8.2.3 Melanin production

Melanin production ability of actinomycetes was tested using peptone yeast extract iron agar (ISP6) and tyrosine agar (ISP 7). Loopful of spores were inoculated on to the agar slants and incubated at 28°C for 5-7 days. Brown to back diffusible pigment in the medium was recorded as positive. Absence of brown to black colour, or total absence of diffusible pigment, was considered as negative for melanin production.

2.3.8.2.4 Carbohydrate utilisation test

The ability of strains to utilise and produce acid from various carbon sources was studied by the method recommended in the ISP. Utilisation of carbon sources, like glucose, fructose, mannitol, sucrose, arabinose, rhamnose, inositol, raffinose, and cellulose were tested on basal carbohydrate utilization agar (ISP 9). Carbohydrate solution (1%) of each carbon source sterilised by seitz filtration was added to sterile basal media and the media was dispensed into test tubes (3 mL) as agar slants (Nonomura, 1974). Basal media without supplemented carbon source was used as the control tube. The isolates were inoculated into respective carbohydrate agar slants and incubated at 28°C for 5-7 days. Growths were observed by comparing them with control.

2.3.8.2.5 Gram Staining

Smears were prepared from young cultures and allowed to air dry and heat fixed. Primary stain (Crystal violet) was added to these smears and allowed to react for 1 min. Slides were then washed with distilled water. Gram's iodine was added to smears and again allowed to react for 1 min.

Smears were rinsed with distilled water and decolourising agent (acetone) was added drop by drop until crystal violet fails to wash off from the smear. The smears were then washed with distilled water and counterstained with safranin for 30 s. Slides were washed again with distilled water and air dried. After drying, slides were observed under oil immersion objective of the light microscope.

2.3.9 Selection of actinomycete isolates

The actinomycete isolates identified up to generic level based on morphological and biochemical characterisation were subjected to molecular characterisation for species level identity.

2.3.10 Genomic DNA extraction from actinomycete isolates

Total genomic DNA from the selected actinomycete strains was extracted as per standard protocol (Sambrook *et al*., 1989). Spore suspension of the isolate was suspended in nutrient broth and incubated in an orbital shaker at 30°C, 120 rpm for 48-72 h to form a pellet of vegetative cells (pre-sporulation). The cells were harvested by centrifugation at 10000 rpm for 10 min and then suspended in TE buffer having 1% sodium dodecyl sulphate (SDS). Proteinase-K (20 mg/mL) was then added to a final concentration of 100 µg/mL and mixed gently. The suspension of lysed cells was cooled to room temperature and an equal volume of phenol equilibrated with 0.5 M Tris-HCl (pH 8.0) was added and gently mixed. A third extraction with a 24:1 mixture of chloroform and iso amyl alcohol was carried out and the aqueous phase was collected carefully. DNA dissolved in solution was precipitated after the addition of 0.1 volume of 3 M sodium acetate (pH 7.5) and 0.6 volume of isopropanol. Incubating at -20°C for 12 h, precipitated the DNA. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 min at room temperature. The excess

salt was removed by washing the DNA pellets three times in 70% ethanol. The DNA samples were dried under vacuum and dissolved in appropriate volume of TE buffer and stored at –20°C.

Agarose gel electrophoresis was done to check the purity of DNA. DNA concentration and purity was assessed spectrophotometrically by comparing absorbance at 260 and 280nm followed by 0.8% agarose gel electrophoresis. Concentration of DNA was found out from the following formula.

$$Conc.\ of\ DNA\ (\mu g/ml) = OD\ at\ 260\ nm \times 50 \times dilution\ factor$$

Where OD is the Optical Density

2.3.11 Amplification of 16S rRNA gene

The 16S rRNA gene was amplified using the universal eubacterial primers 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). The primers were used to amplify nearly full length 16S rDNA sequences. PCR was performed in a 25 µL reaction volume containing 1x standard Taq buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The thermal profile applied was an initial denaturation of 94°C for 5 min, 30 cycles of denaturation (94°C for 40 s), annealing (55°C for 40 s), extension (72°C for 1 min 30s), and a final extension (72°C for 10 min). The PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide (10 µg/mL) to ensure that a fragment of the correct size had been amplified. The gel was visualised on a Gel Documentation System (BIO-RAD).

2.3.12 Gene sequence analysis and species identification

The PCR products were sequenced using the universal 27F and 1492R primers with an ABI prism model 3700 Big Dye Sequencer (Applied

Biosystems, USA) at SciGenom, Kochi, India. The nucleotide sequences obtained were assembled using Gene Tool software and the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm at the National Centre for Biotechnology information (NCBI), USA (www.ncbi.nlm.nih.gov). 16S rRNA gene sequences were aligned using the CLUSTAL W and the evolutionary history of the isolate was inferred using the using the Neighbor-Joining (NJ) algorithm in Molecular Evolution 98 Genetic Analysis (MEGA) software version 6.0.

2.3.13 Nucleotide Sequence Accession Numbers

The sequences were deposited in the GenBank database using the web based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt).

2.4 Results

2.4.1 Sampling and isolation of actinomycetes

In the course of screening for novel bioactive compounds and enzymes, 90 actinomycete isolates were obtained from estuarine and mangrove sediments, collected from Cochin estuary and its associated mangroves (Table 2.2). The isolates were designated as ER1 to ER90 based on their colony morphology observed on the master plate. All the 90 isolates exhibited dark grey, grey, dark brown, brownish, orange, whitish and yellowish white colours. The isolates were small to medium size, round and powdery. Most of the isolates were obtained from the sediment collected from mangroves. Sediment samples from Puthuvyppu mangroves gave the highest number of actinomycete isolates.

Table 2.2. Isolates obtained from each sampling station

Sl. No.	Stations	Number of isolates
1	Thevara	10
2	Thopumpady	7
3	Barmouth	6
4	Marine Science Jetty	7
5	Moolampilly	4
6	Thattapilly	5
7	Eloor	6
8	Vallarpadam	4
9	Chitoor	4
10	Aroor	10
11	Valanthakadu	12
12	Puthuvypu	15

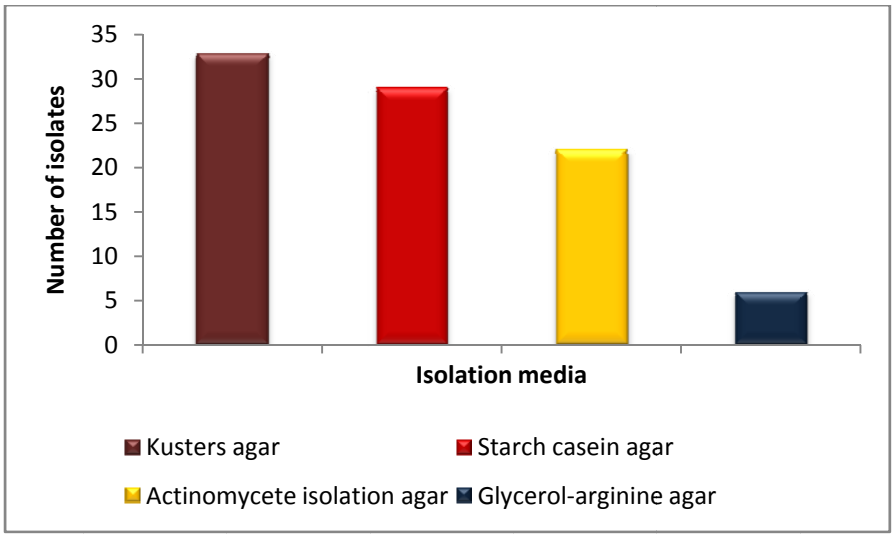


Fig 2.2. Effect of different isolation media on isolation of actinomycetes

Among the 4 different isolation media used, Kusters agar was the most effective in isolation of actinomycetes followed by starch casein agar, actinomycete isolation agar and glycerol-arginine agar (Fig 2.2).

2.4.2 Screening of antimicrobial activity of crude extracts of actinomycetes against test microorganisms

Out of 90 isolates, only 2 isolates (ER7 and ER10) showed antibacterial activity against human and fish pathogens tested. The ER7 isolate showed higher antimicrobial activity against Gram-positive bacteria, human and fish pathogens tested when compared with that of the ER10 isolate (Table 2.3). The antimicrobial activity of ER7 and ER10 isolates are shown in Fig 2.3.

Table 2.3. Antimicrobial activity of the crude extract (cell-free supernatant) of the isolates ER7 and ER10.

Tested microorganism	Diameter of zone of inhibition (mm)	
	ER7	ER10
Bacillus sp.	16.7	11.0
Planococcus sp.	14.0	10.0
Proteus sp.	11.0	-
Klebsiella sp.	12.0	9.0
Aeromonas sp.	9.7	-
E. coli	10.0	8.0
Vibrio alginolyticus	15.0	-
V. parahaemolyticus	-	-
V. harveyi	10.0	-
Candida sp.	-	-

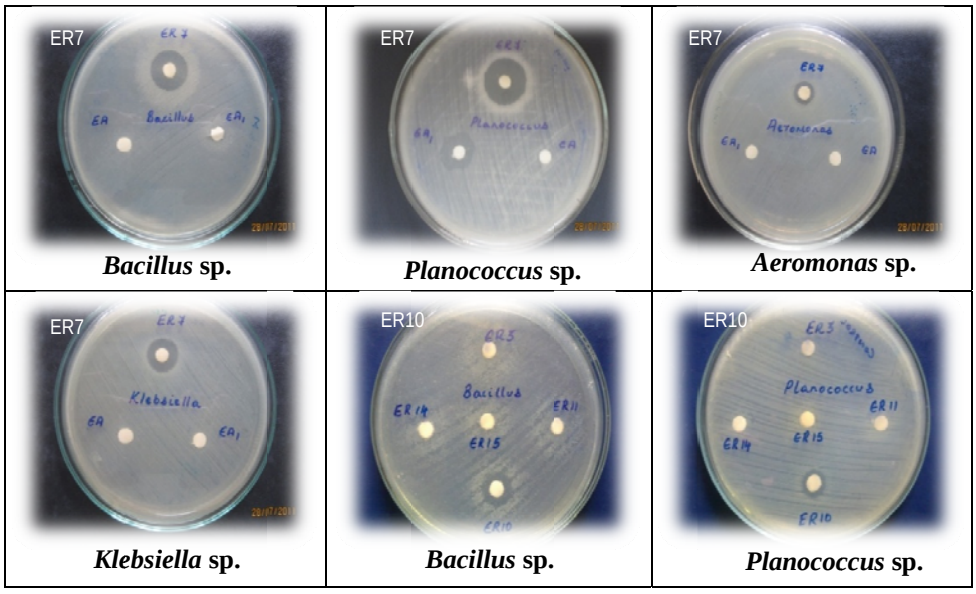


Fig 2.3. Antimicrobial activity of crude extract of ER7 and ER10 isolates against test microorganisms

2.4.3 Solvent extraction

The isolate ER7 produced the maximum zone of growth inhibition against the tested pathogens compared to ER10. Hence, ER7 isolate was subjected to solvent extraction of its bioactive compounds. The antimicrobial activity of all the solvent extracts, (hexane, ethyl acetate, chloroform, butanol and methanol) of the isolate ER7 was determined. The results are shown in Table 2.4. In this study, only the methanol extract showed antimicrobial activity against the human and fish pathogens tested (Fig 2.4).

Table 2.4. Antimicrobial activity of the solvent extracts of the isolate ER7

Tested microorganisms	Diameter of zone of inhibition (mm) of solvent extracts of ER7				
	Methanol	**Butanol**	**Chloroform**	**Ethyl acetate**	**Hexane**
Bacillus sp.	21.0	-	-	-	-
Planococcus sp.	20.0	-	-	-	-
Proteus sp.	12.0	-	-	-	-
Klebsiella sp.	13.3	-	-	-	-
Aeromonas sp.	12.0	-	-	-	-
E. coli	13.0	-	-	-	-
Vibrio alginolyticus	18.0	-	-	-	-
V. harveyi	12.0	-	-	-	-
V. parahaemolyticus	-	-	-	-	-
Candida sp.	-	-	-	-	-

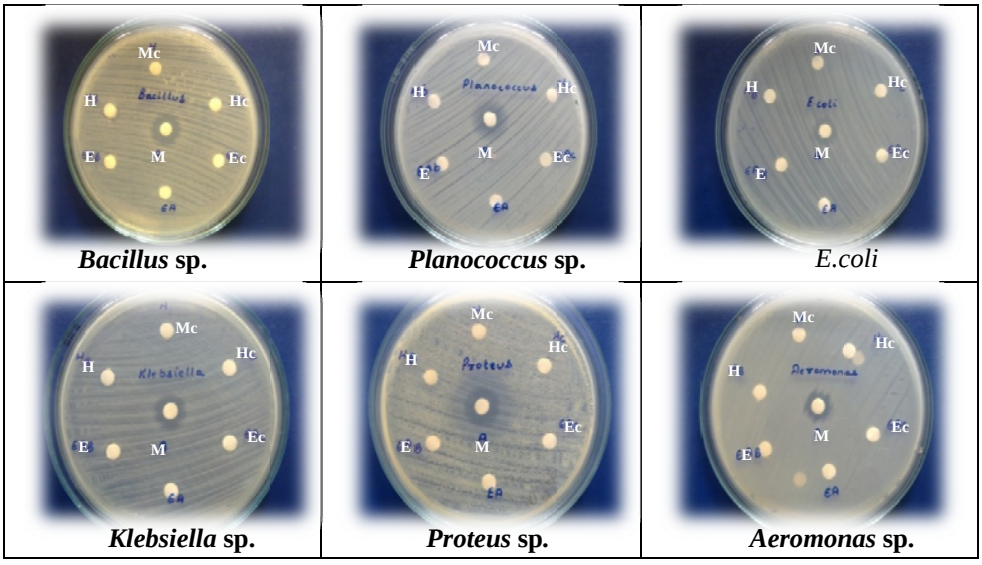


Fig 2.4. Antimicrobial activity of methanol extract of ER7 isolate M: Methanol extract; M_c: Methanol solvent (Control); H: Hexane extract; H_c: Hexane solvent (control); E: Ethyl acetate extract; E_c: Ethyl acetate solvent (control)

2.4.4 Screening for hydrolytic enzymes

The actinomycetes isolated from the sediments of Cochin estuary and its associated mangroves invariably had the potential to elaborate a wide array of enzymes. The isolates showed the ability to secrete extracellular enzymes- cellulase, amylase, xylanase, lipase and lecithinase (Fig 2.5). None of the isolates produced L-asparaginase and L-glutaminase. Most of the isolates produced cellulase (48), followed by amylase (37), lipase (33), xylanase (10), and lecithinase (6) (Table 2.5). Fig 2.6 shows hydrolysis of cellulose, starch, xylan and lecithin on respective agar plates.

Table 2.5. Zone of hydrolysis and enzyme production by the isolates

	Diameter of zone hydrolysis (mm)						
Isolate	**Cellulase**	**Amylase**	**Xylanase**	**Lipase**	**Lecithinase**	**L-asparaginase**	**L-glutaminase**
ER1	-	-	45.0	-	-	-	-
ER2	35.0	11.0	-	-	-	-	-
ER3	-	20.0	-	-	-	-	-
ER4	-	20.0	-	-	-	-	-
ER5	24.0	-	-	+	-	-	-
ER6	21.0	-	-	-	-	-	-
ER7	15.0	-	-	-	+	-	-
ER8	13.0	-	35.0	-	+	-	-
ER9	-	-	-	-	-	-	-
ER10	16.0	-	15.0	-	+	-	-
ER11	25.0	-	-	+	-	-	-
ER12	-	12.0	-	+	-	-	-
ER13	-	14.0	-	-	-	-	-
ER14	-	13.0	-	-	-	-	-
ER15	28.0	-	-	-	+	-	-
ER16	-	-	-	+	-	-	-
ER17	-	-	-	-	-	-	-
ER18	35.0	20.0	-	+	-	-	-
ER19	-	15.0	-	-	-	-	-
ER20	31.0	17.0	-	-	-	-	-
ER21	-	21.0	-	-	-	-	-

ER22	-	-	-	-	-	-	-
ER23	20.0	-		+	-	-	-
ER24	-	-	-	-	-	-	-
ER25	-	11.0	-	-	-	-	-
ER26	-	-	-	-	-	-	-
ER27	28.0	10.0	-	+	-	-	-
ER28	-	-	-	+	-	-	-
ER29	-	-	-	+	-	-	-
ER30	20.0	10.0	-	-	-	-	-
ER31	17.0	-	-	-	+	-	-
ER32	-	-	15.0	-	-	-	-
ER33	-	-	-	+	-	-	-
ER34	-	-	-	+	-	-	-
ER35	-	23.0	-	-	-	-	-
ER36	-	-	16.0	-	-	-	-
ER37	-	-	-	+	-	-	-
ER38	-	-	-	+	-	-	-
ER39	15.0	-	-	+	+	-	-
ER40	-	-	23.0	-	-	-	-
ER41	-	-	-	+	-	-	-
ER42	-	16.0	-	-	-	-	-
ER43	-	-	-	+	-	-	-
ER44	-	-	-	+	-	-	-
ER45	27.0	-	-	-	-	-	-
ER46	-	20.0	-	-	-	-	-
ER47	25.0	14.0	25.0	-	-	-	-
ER48	30.0	-	-	-	-	-	-
ER49	15.0	-	-	+	-	-	-
ER50	-	12.0	-	-	-	-	-
ER51	-	-	-	+	-	-	-
ER52	-	15.0	-	-	-	-	-
ER53	-	-	-	+	-	-	-
ER54	25.0	-	35.0	-	-	-	-
ER55	17.0	-	-	+	-	-	-
ER56	10.0	-	-	-	-	-	-
ER57	33.0	16.0	26.0	-	-	-	-
ER58	28.0	13.0	16.0	-	-	-	-

ER59	25.0	-	-	+	-	-	-
ER60	17.0	16.0	-	+	-	-	-
ER61	25.0	13.0	-	-	-	-	-
ER62	-	-	-	+	-	-	-
ER63	17.0	10.0	-	-	-	-	-
ER64	-	-	-	+	-	-	-
ER65	-	-	-	+	-	-	-
ER66	17.0	25.0	-	-	-	-	-
ER67	19.0	-	-	-	-	-	-
ER68	15.0	10.0	-	-	-	-	-
ER69	-	11.0	-	-	-	-	-
ER70	23.0	-	-	+	-	-	-
ER71	13.0	-	-	-	-	-	-
ER72	20.0	12.0	-	-	-	-	-
ER73	23.0	-	-	-	-	-	-
ER74	20.0	-	-	-	-	-	-
ER75	-	16.0	-	+	-	-	-
ER76	-	11.0	-	-	-	-	-
ER77	28.0	10.0	-	-	-	-	-
ER78	11.0	-	-	+	-	-	-
ER79	-	-	-	+	-	-	-
ER80	17.0	12.0	-	-	-	-	-
ER81	12.0	13.0	-	-	-	-	-
ER82	13.0	-	-	-	-	-	-
ER83	14.0	-	-	+	-	-	-
ER84	16.0	13.0	-	+	-	-	-
ER85	17.0	-	-	+	-	-	-
ER86	18.0	-	-	-	-	-	-
ER87	10.0	15.0	-	-	-	-	-
ER88	20.0	17.0	-	-	-	-	-
ER89	21.0	-	-	+	-	-	-
ER90	-	19.0	-	-	-	-	-

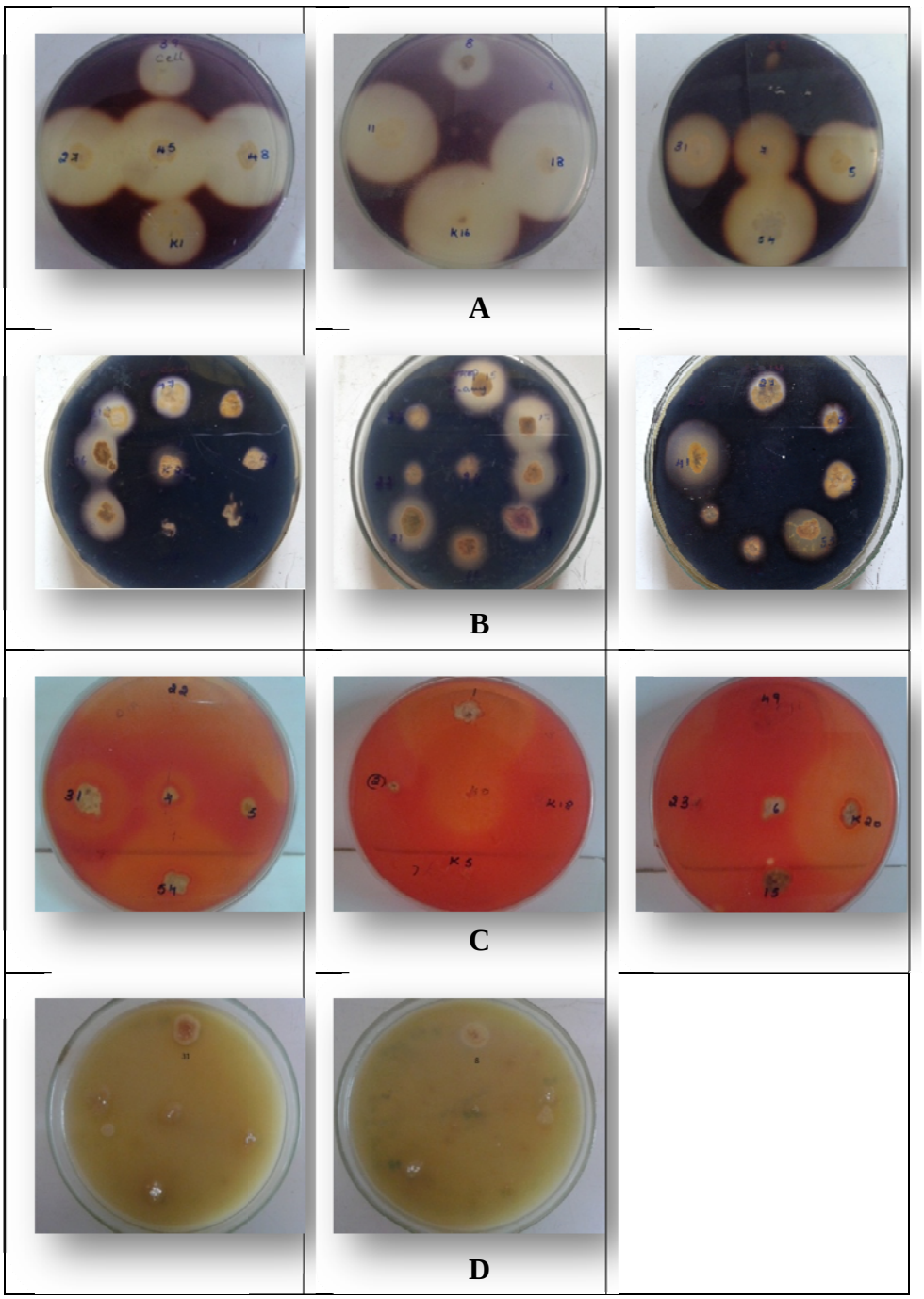


Fig 2.6. Hydrolytic enzyme activity of actinomycetes on respective agar plates.
A: Cellulose hydrolysis; B: Amylase hydrolysis;
C: Xylan hydrolysis; D: Lecithin hydrolysis.

2.4.5 Characterisation and identification of selected *Streptomyces* spp.

The two isolates ER7 and ER10 with antimicrobial activities and ER1 isolate with maximum xylanase activity were selected for further characterisation on the basis of morphological, physiological and biochemical characteristics.

2.4.5.1 Morphological and cultural characterisation

The ER1 isolate exhibited good growth on all the ISP media used. The ER7 and ER10 actinomycete isolates showed excellent growth and abundant aerial mycelial formation on ISP medium No. 4 (inorganic salts-starch agar). The ER7 isolate showed moderate growth on ISP media No. 2, 3 and 5. The ER10 isolate showed good growth on ISP medium No. 2 (yeast extract-malt extract agar) and ISP medium No. 3 (oat meal agar) and moderate growth on ISP medium No. 5 (glycerol asparagine agar base).

The spore mass colour of actinomycetes is considered as a taxonomic criterion for grouping actinomycetes. The aerial and substrate mycelium were media dependent. Among the 3 actinomycete isolates, ER1 isolate exhibited ash/grey series and ER7 and ER10 isolates produced white/off white spore mass (Table 2.6). No diffusible pigment was produced by the isolates in any of the culture media tested. The colour of the substrate mycelium in ISP2 to ISP5 media were observed and varied from off white to yellow. Morphological characteristics of the isolates were also studied using Gram staining. Gram staining revealed that the isolates were Gram-positive.

Table 2.6. Morphological and biochemical characteristics of ER1, ER7 and ER10 on different cultural media.

Medium	Isolate	Day of observation	Colour of aerial mycelium	Colour of substrate mycelium
ISP2	ER1 ER7 ER10 ER10	7,17 7,10,14 7 10,14	Grey - White Flesh	White Yellow Yellow Yellow
ISP 3	ER1 ER7 ER10	7,10,14 7,10,14 7,10,14	Grey - Flesh	White Yellow Yellow
ISP 4	ER1 ER7 ER7 ER10	7,10,14 7,10 14 7,10,14	Grey - Flesh Flesh	White Yellow Yellow Yellow
ISP 5	ER1 ER7 ER10	7,10,14 7,10,14 7,10,14	Grey - -	White Yellow White

2.4.5.2 Microscopic observation of selected isolates

The spore chain morphology of actinomycetes grown in coverslip observed under high power and oil immersion objectives revealed three types of spore chain morphology. The spore chain of ER7 is simple and straight (rectus type) and that of ER10 is simple and spiral (spira type). The ER1 isolate showed the retinaculum spore chain. By comparing the morphology of spore-bearing hyphae of the isolates with the entire spore chain and structure of the spore chain with the actinomycetes morphologies, ERl, ER7 and ER10 were found to belong to the genus *Streptomyces*.

2.4.5.3 Biochemical and physiological characterisation

Decomposition of various substrates such as xanthine, hypoxanthine and tyrosine carried out in respective agar media revealed that among the 3 isolates characterised, only ER10 had the ability to decompose xanthine (Fig 2.7A); ER1 and ER10 decomposed tyrosine (Fig 2.7B) and none of the 3 isolates decomposed hypoxanthine. Decomposition of casein by actinomycete isolates were tested on skim milk agar. All the three isolates, ER1, ER7 and ER10 were able to decompose casein which was indicated by a clearing zone around the colonies (Fig 2.7C).

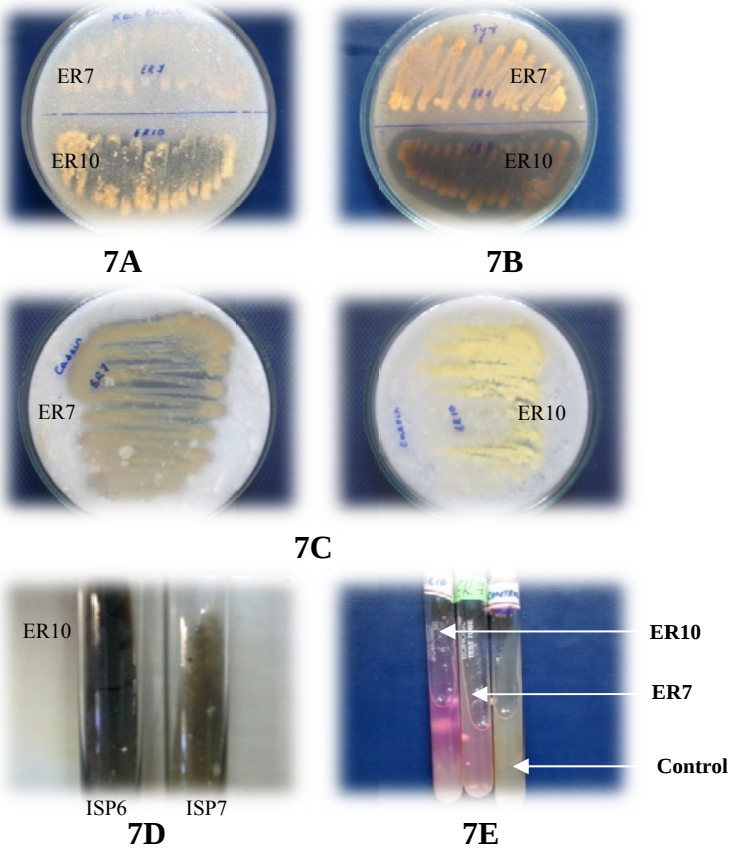


Fig 2.7. A: Xanthine hydrolysis by ER7 and E10 isolates; B: Tyrosine hydrolysis by ER7 and ER10 isolates; C: Casein hydrolysis by ER7 and ER10 isolates; D: Melanin production by ER10 isolate; E: Urea hydrolysis by ER7 and ER10 isolates

Melanin production ability was exhibited by only ER10 isolate on peptone yeast extract iron agar and tyrosine agar (Fig 2.7D). ER1, ER7 and ER10 isolates were able to hydrolyse urea with the production of urease enzyme (Fig 2.7E).

2.4.5.4 Carbohydrate utilisation test

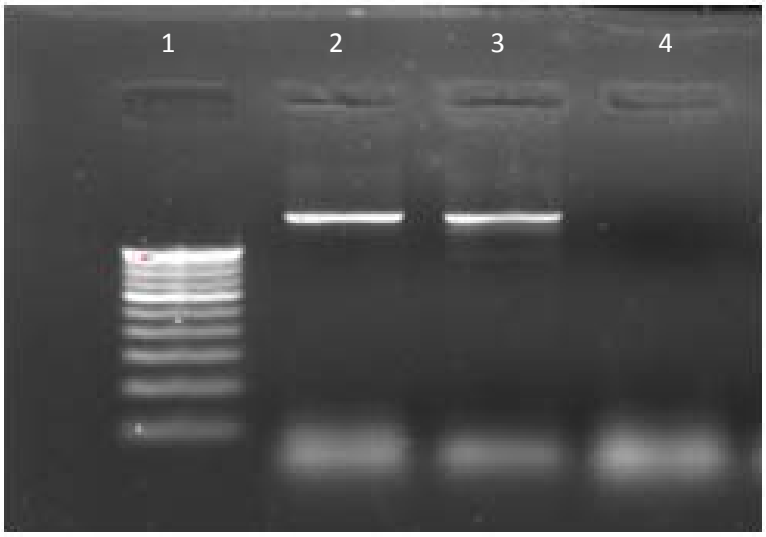
All carbohydrates tested, ranging from monosaccharides, disaccharides to sugar alcohols were utilised by the actinomycete isolates. However, ER1 isolate could not utilise cellulose and L-rhamnose. Heavy growth and sporulation were observed for all 3 isolates in media supplemented with D-glucose, L-arabinose and D-mannitol. The biochemical characteristics of ER1, ER7 and ER10 are presented in Table 2.7.

Table 2.7. Biochemical characteristics of ER1, ER7 and ER10

Biochemical tests	ER1	ER7	ER10
Grams stain	+	+	+
Aerial mycelium	Grey	Flesh	Flesh
Colony colour	Grey	Flesh	Flesh
Melanin	-	-	+
Carbon sources (1% w/v)			
D-glucose	+	+	+
Sucrose	+	+	+
D-fructose	+	+	+
Raffinose	+	+	+
Cellulose	-	+	+
L-rhamnose	-	+	+
Mannitol	+	+	+
Inositol	+	+	+
L-arabinose	+	+	+
Casein	+	+	+
Xanthine	-	-	+
Hypoxanthine	-	-	-
Tyrosine	+	-	+
Urea	+	+	+

2.4.6 Amplification of 16S rRNA gene of ER1 isolate:

The isolate ER1, was further identified using 16S rRNA sequencing, as it was only used for further study in this thesis. The molecular characteristics of *Streptomyces* sp. ER1 was carried out by PCR amplification of 16S rRNA gene and their sequencing. 16S rRNA gene amplification using a set of universal eubacterial primers: 27F and 1492R yielded a single amplicon of approximately 1500-bp for actinomycete isolate ER1 (Fig 2.8). Following BLAST analysis of the sequence of ER1, it was identified as *Streptomyces* sp. The sequence of *Streptomyces* sp. ER1 16S rRNA genes were deposited in genbank (http://www.ncbi.nlm.nih. gov/ genbank) and received the accession number KY449279. Fig 2.9 shows phylogenetic relationships of ER1 isolates with its nearest neighbours.



Lane 1: 100 bp DNA ladder

Lane 2 and 3: PCR product amplified

Lane 4: Negative control

Fig 2.8. PCR amplification of 16S rRNA gene of *Streptomyces* ER1 isolate

The sequences showed a homology ranging between 94 to 99% to the closest neighbours. An alignment of the 16S rDNA gene sequence of the isolate ER1 (GenBank, accession number KY449279) with the appropriate sequences available in the GenBank database, demonstrated that the isolate ER1 should be classified as a *Streptomyces* sp. and that its closest relative is *Streptomyces* sp. S J-IJOM-37-09 (99% identity, 100% query coverage).

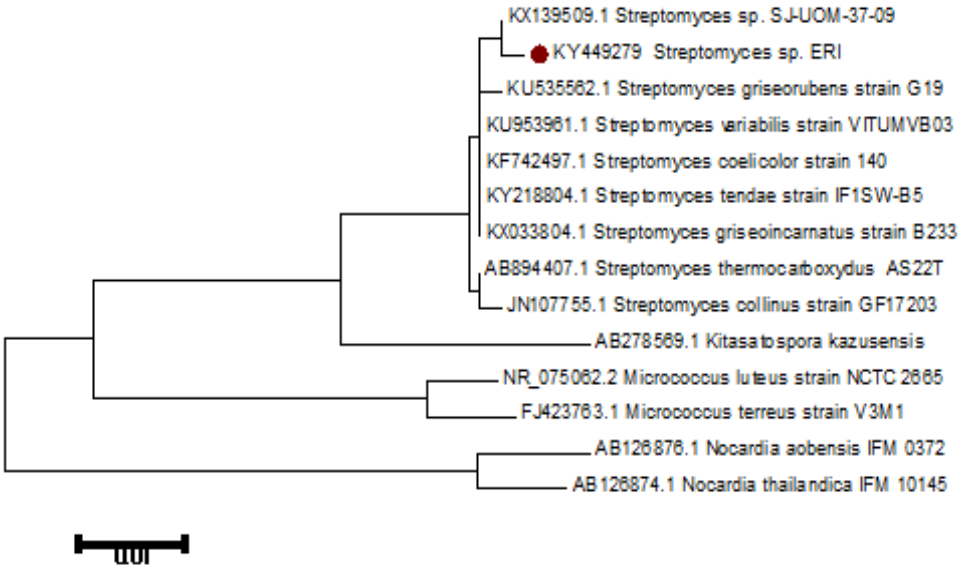


Fig 2.9. The neighbor-joining tree based on 16S rRNA gene sequences of *Streptomyces* ER1 isolate

2.5 Discussion

Microbial systematics, physiology and natural product chemistry are underpinning disciplines in unraveling biodiscovery of marine natural products (Bull and Stach, 2007). Maximising the novel bio-discovery process lies in the fact that understanding of the actinomycete systematics from the vast ocean realm is indispensable (Augustine, 2014). This study was undertaken with a view to isolating novel antibiotic and hydrolytic enzyme producers from unexplored ecosystem and selecting the potent

strains. It seems timely to extend this approach to another hitherto unexplored area such as the Cochin estuary and its associated mangroves.

In all, 90 different actinomycete isolates were obtained from estuarine and mangrove sediments of Cochin estuary. In the present study, maximum number of isolates was obtained from Kusters agar (33 isolates). Early reports also stated that Kusters agar was the most suitable medium for the isolation of actinomycetes from the water, sediment, seaweed and molluscs samples since it contained glycerol that most actinomycetes used as a carbon source (Sahu *et al.,* 2007; Radhakrishnan and Mohana, 2014). Kusters agar medium exhibited good growth of actinomycetes as compared to the growth on other media, like Bennet's agar, starch casein agar, actinomycetes isolation agar, glucose asparagine agar and chitin agar (Jayashree *et al.,* 1991).

In the present study, 2 isolates, namely, ER7 and ER10 were active against Gram-positive bacteria (*Bacillus* sp. and *Planococcus* sp.) and Gram-negative human and fish pathogens tested. ER7 and ER10 isolates were obtained from Puthuvyppu mangrove sediment. Puthuvyppu mangrove is a pristine mangrove ecosystem, which is rich in traditional, medicinal and ornamental plants and where conditions are conducive for microbial growth and is free from sewage input. The conditions prevailing in the mangrove ecosystem like the presence of high salinity, high temperature, extreme tides, high sedimentation and high evaporation, the muddy anaerobic sediment, high degree of moisture content and abundance of organic matter produced from the autolysis and microbial decomposition of fallen leaves, twigs, flowers and fruits differs from that of the terrestrial ecosystem. Hence it is assumed that mangrove actinomycetes might be more potent and bioactive compared to terrestrial actinomycetes (Giri *et al.,* 2011; Nag *et al.,* 2012). It was also hypothesised that antibiotic production confers a

competitive advantage to microbes and thus may contribute significantly to microbial fitness in the soil, likewise survival advantages in the marine environment (Williams *et al*., 1989; Maplestone *et al*., 1992). Previous studies also showed that mangrove actinomycetes were potent antimicrobial producers in nature. *Streptomyces* sp. isolated from mangrove soil in the eastern coast of Surabaya, Indonesia and Thai mangroves were capable of producing a series of antibiotics against Gram-positive and Gram-negative bacterial pathogens (Retnowati, 2010; Phongsopitanun *et al.,* 2014). Several other researchers had already reported similar antimicrobial activity of actinomycetes against various human pathogens (Krishnakumari *et al*., 2006; Thangapandian and Ponmurugan, 2007). Many actinomycetes do not show antifungal activity (Hunadanamra *et al.*, 2013; Phongsopitanun *et al.,* 2014). In the present study also, the isolates did not show antifungal activity when tested against the human pathogen *Candida* sp.

According to Sattler *et al*. (1998), most of the metabolites extracted from actinomycetes inhibited the growth of Gram-positive bacteria, but are ineffective against Gram-negative bacteria due to alteration of peptidoglycan and its building blocks in the cell wall. In the current study, on screening for antibacterial activity, ER7 and ER10, showed better antagonistic activity against Gram-positive than Gram-negative bacteria which agreed with the earlier findings of actinomycetes from other marine environments (Sacramento *et al*., 2004; Arasu *et al*., 2008; Nithya *et al*., 2012; Valli *et al*., 2012).

In the solvent extraction, the methanol extract of ER7 was found to contain the antimicrobial compound as the methanol extract was active against all the pathogens tested. This also revealed that the antimicrobial compound was polar in nature. The methanol extract of *Streptomyces* isolated from Parangipettai mangrove rhizosphere sediment showed

antibacterial activity against Gram-negative bacterial pathogens (Radhakrishnan and Mohana, 2014). Similarly, the secondary metabolites extracted with methanol exhibited antibacterial and antifungal activities (Khan and Patel, 2011; Arasu *et al.,* 2014).

Very few reports are available regarding the enzyme profile of marine actinomycetes. The results regarding enzymatic activities of actinomycetes are extremely interesting for biocatalytic exploitation (Torres *et al*., 2008). Screening of the actinomycetes for hydrolytic enzyme production showed that most of the isolates were cellulase producers (48 isolates). In a similar study, actinomycetes isolated from Sangumugham Vizhinjam and Veli coast, Thiruvananthapuram, were tested for their cellulase, lipase and protease activities and found that cellulase producing actinomycetes were more (Lekshmi *et al.,* 2014). Previous reports also revealed that actinomycetes were one of the known cellulase producers (Jang and Chenks, 2003).

In the present study, more than 90% of the isolates showed at least one of the extracellular enzymatic activities, and among the 90 isolates tested, 40 isolates produced more than one extracellular enzyme. Similarly, it was observed that in marine bacterial strains, when the presence of one extracellular hydrolytic activity was detected, production of other hydrolytic enzymes was frequently associated (Tropeano *et al*., 2012). In the present study, the isolates showed a potential to produce a wide range of enzymes which may be due to the result of natural selection of microorganisms in order to survive in the competing environment (Arjit *et al*., 2012). Earlier studies have also proved that marine bacteria including marine actinomycetes were exhibiting diverse pattern in secreting extracellular enzymes (Jayaprakashvel *et al.*, 2008; Ramesh and Mathivanan, 2009). The enzyme activity of the actinomycetes varied from isolate to isolate

depending upon the growth and physiological condition of the isolates. This difference may also be due to the nature of the sample from which they were isolated. It was observed that the biological functions of actinomycetes mainly depended on the sources from which the bacteria were isolated (Sharma, 2014).

Among the various hydrolytic enzymes screened, like cellulase, amylase, lipase, xylanase, lecithinase, L-asparaginase and L-glutaminase, very high potential for xylanase production (zone of hydrolysis 45 mm) was shown by the isolate ER1 on xylan agar plate followed by ER18 isolate (35 mm) on cellulose agar plate and ER66 (25 mm) on starch agar plate. Even though, more number of isolates produced cellulose (48), amylase (37) and lipase (33) than xylanase (10), the highest zone of hydrolysis was produced by the xylanolytic isolate ER1. Taking into account, the very high potential of ER1 for xylanase production and also the importance of xylanase in many industries, especially in paper and pulp industries, ER1 was selected for further studies.

ER1 isolate with hyper-xylanolytic ability and ER7 and ER10 isolates with antimicrobial activity were selected for morphological and biochemical characterisation. Chromogenicity of aerial mycelium is considered as an important character for grouping of actinomycetes (Pridham and Tresner, 1974). It was found that the actinomycete cultures- ER1, ER7 and ER10, isolated from sediments of Cochin estuary and its associated mangroves were morphologically distinct on the basis of spore mass colour, aerial, substrate mycelium, spore chain morphology etc. ER7 and ER10 had whitish spore mass, ER1 had grey spore mass.

The isolation and characterisation of microbial species are important in understanding their existence in natural ecosystems. The isolation of

diverse and novel actinobacteria provided a theoretical guide for the exploitation and utilisation of actinobacterial resources (Li *et al*., 1996). Initially, actinobacteria were characterised on the basis of morphological features so as to have a preliminary determination of the genus. Actinobacteria were observed under the light microscope using coverslip culture and slide culture techniques to study several characters, such as presence or absence of aerial and substrate mycelia, fragmentation or non fragmentation of substrate and aerial mycelium, spore chain morphology and colour of aerial spore mass (Kavitha and Vijayalakshmi, 2007, 2011; Vijayakumar *et al*., 2007; Khan, 2008; Arifuzzaman *et al.,* 2010; Vaijayanthi *et al.*, 2013). Especially, spore chain morphology was considered as one of the important characteristics in the identification of *Streptomyces* and it greatly varied among the species (Tresner and Backus, 1961). It has already been reported that the majority of the marine Streptomycete isolates produced aerial mycelia with coiled spiral spore chains followed by rectiflexibiles spore morphology(Das *et al*., 2008; Mukherjee and Sen, 2004; Peela *et al*., 2005; Roes and Meyer, 2005; Chacko *et al*., 2012). In the present study, the spore chain morphology of ER1, ER7 and ER10 were retinaculum, rectus and spira, respectively. The microscopical studies of the 3 isolates undoubtedly placed these isolates under the genus *Streptomyces*.

It has been reported by many workers that, the Streptomycetes are the predominant soil actinobacteria in both terrestrial and marine environments (Ehrlich and Anderson, 1954; Vijayakumar, 2006; Vijayakumar *et al*., 2007, 2009; Remya and Vijayakumar, 2008; Cholarajan and Vijayakumar, 2012, 2013; Thirumurugan and Vijayakumar, 2012, 2013). It is indicated that several morphological, physiological and reproductive characteristics of *Streptomyces* (filamentous growth, formation of hyphae and the production

of spores) would allow its species to occupy extreme environments (Schutze and Kothe, 2012). The actinomycetes from marine sediments were ahead of the terrestrial counterparts in decomposition of tyrosine, hypoxanthine, xanthine and urease. In the present study, it was observed that the degradation of the substrates, casein, tyrosine and xanthine was variable among the different isolates. Similar observations were made in previous reports (Taddei *et al*., 2006; Augustine, 2014). Actinomycetes from marine habitats have evolved to tide over the harsh environmental conditions and have altered their biochemical and metabolic machinery. Outstanding and diverse physiological traits of microbial population could be attributed to their underlying genetic diversity and also their mechanisms of generating genetic variation (Li *et al*., 2013).

Only very few actinomycete isolates from various ecological niches exhibited the ability to produce melanin. Out of the 3 isolates (ER1, ER7, ER10) screened in the present study, only 1 isolate (ER10) had the ability to produce melanin in the melanin production medium. The results correlated with previous findings where Streptomycetes from various sources were screened and only less than 10% were found to produce melanin pigments (Mathew, 1995; Devan, 1999; Dhevendaran and Annie, 1999). It was noticed that out of 30 isolates from Egyptian soil, only a single strain had the ability to produce melanin (Shaaban *et al.,* 2013). Nine strains among 180 (5%) *Streptomyces* isolates from soil samples of Gulbarga region produced a diffusible dark brown pigment on both peptone-yeast extract iron agar and synthetic tyrosine agar (Dastager *et al.,* 2006).

Carbohydrate utilisation test played a prominent role in the taxonomic characterisation of actinomycetes. The physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physiological conditions (Baskaran *et al*., 2011). The ability to

utilise a wide range of substrates suggested better survival in different environments (Williams *et al*., 1989). Actinomycetes have a reputation for marked nutritional versatility which was supported by the results of the present analysis. In the present study, all the three isolates (ER1, ER7, ER10) were able to utilize and grow in a variety of carbon sources. Earlier study on nutritional characterisation of actinomycetes found that actinomycetes utilised variety of sugars for their growth. The difference in carbon utilisation may be as a result of availability of the carbon source and adaptation of isolates to different niches in the marine environment (Proser and Palleroni, 1978). The actinomycetes formed abundant mycelium on the media with D-glucose, L-arabinose and D-mannitol. A similar observation was reported by Lyons *et al*. (1969).

Since the isolates ER7 and ER10 lost the property of antimicrobial activity on storage, only isolate ER1 was used for further study and was subjected to molecular characterisation and identified as *Streptomyces* sp. ER1 with accession number KY449279.

The study unveils the metabolic potentialities of actinomycetes from the estuarine habitat which would be promising as a source of bioactive molecules and many industrially important enzymes. With growing awareness on environmental protection, chemical catalysts are being replaced by microbial enzymes in various pharmaceutical, food, textile and agricultural industries. It is revealed from the present study that the Cochin estuary is a potential source for a wide spectrum of antimicrobial and industrial enzyme-producing actinomycetes.

Chapter -3

OPTIMISATION OF XYLANASE PRODUCTIONFROM *STREPTOMYCES* SP. ER 1 USING STATISTICAL AND NON STATISTICAL METHODS

Content

3.1 Introduction

Enzymes found their way in several new industrial processes after being discovered in the latter half of the 19th century as an effective biocatalyst (Hoondal *et al.,* 2002). Among enzymes, xylanases are one of the leading industrial enzymes with tremendous applications. The biotechnological applications of xylanases started in 1980's. One of the most significant applications of xylanase is its use in paper pulp bleaching. The other potential applications include the bioconversion of lignocellulosic material and agrowastes into fermentative products, the clarification of fruit juices, the improvement of the consistency of beer, raising bread dough and the digestibility of animal feedstock (Viikari *et al.*, 1994; Bhat, 2000). Xylanases are also applicable in production of rayon, cellophane and several chemicals, such as cellulose esters and cellulose ethers (Subramaniyan and Prema, 2002).

The high demand and cost of available commercial enzymes urge for bioprospecting and manipulation of microbes for higher productivity and enhancement of existing commercial enzymes (Kumar *et al.,* 2014; Khusro *et al.,* 2016). Enzyme productivity from microorganisms could be increased by optimising the production processes, like manipulation of the cultural (pH, temperature, agitation, aeration and inoculum concentration) and nutritional conditions (Jose and Jebakumar, 2014). The maximum production of xylanase with cost-effective way in less time period could be achieved by employing alternate strategies of culture medium optimisation (Khusro *et al.,* 2016).

In conventional 'one-factor-at-a-time' (OFAT) approach, the nutritional/cultural parameters are optimised by changing one factor at a time, and keeping other parameters constant. This approach is simplest to execute, and primarily helps in selection of significant parameters affecting the enzyme yield. However, this method is time restrictive and also ignores the combined interaction(s) among various physical and nutritional parameters (Vishwanatha *et al.,* 2010).

Consequently, screening of medium components for their significant result on product formation was done using statistical factorial designs like Plackett Burman Design (PBD). The PBD is a well recognized and extensively used statistical design technique for the screening of the medium components in shake flask (Feng *et al.,* 2011; Singh and Rai, 2012). The significant media components could then be optimised using RSM (Adinarayana *et al.,* 2003; Wang *et al.,* 2011).

RSM is an alternative statistical strategy to get better enzyme yield by designing least number of experiments for large number of parameters (Bocchini *et al.,* 2002). It explains the combined effects of all the

independent variables in a fermentation process and explores an approximate interaction between a response variable and a set of design independent variables (Khusro *et al.,* 2016)

The choice of the substrate is of great significance for the successful production of xylanases. In this context, purified xylans could be excellent substrates, as the low molecular weight compounds derived from them are the best xylanase inducers and also lead to increased yields of xylanase production and selective induction of xylanases with concomitant low cellulase activity in various microorganisms (Corral, 2006).

This chapter deals with optimisation of xylanase production by conventional OFAT and statistical approaches, PBD and RSM from *Streptomyces* sp. ER1 which produced maximum xylanase on xylan agar plate from previous isolation and screening studies, described in Chapter 2. The purpose of this study is to characterise the xylanase produced by *Streptomyces* sp. ER1 and to gain a better understanding of optimal conditions for xylanase production. This knowledge has potential application in industrial processes, such as increasing the digestibility of feedstock, bleaching of pulp and paper, obtaining fermentable sugars for use in biofuels and decreasing the variability in the nutritional quality of baked goods.

3.2 Review of literature

The main chain of xylan consists of a homopolymeric backbone of endo-β-1, 4-xylopyranosyl residues having side-group substitution to changing degree with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and p-coumaroyl groups (Li *et al.,* 2000). For the complete hydrolysis of the xylan complex, a number of xylanolytic enzymes were required. These enzymes worked

synergistically and co-operatively to convert xylan to its simplest sugar. The hydrolysis of xylan was carried out by main chain enzymes including endo-1, 4-β-D-xylanases (β-1,4-D-xylanxylanohydrolase; EC 3.2.1.8), 1, 4-β-D-xylosidases (β-1,4-D-xyloside xylohydrolase; EC 3.2.1.37) and the side chain enzymes including α-L-arabinofuranosidases and α-D-glucuronodases as well as the esterases liberating acetyl, coumaroyl and feruloyl substituents (Coughlan and Hazlewood, 1993). Microbial xylan-degrading enzyme systems differed markedly with respect to whether they exist as free entities or as components of complexes (Gilbert and Hazlewood, 1993). Some microbial hemicellulases were secreted freely and not associated with either the cell envelope or with other secreted proteins (Hazlewood *et al.,* 1992). Microbial endo-1, 4-β-D- xylanases were the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss and side product generations (Sharma and Kumar, 2013).

3.2.1 Xylanase from actinomycetes

Among microorganisms, actinomycetes are a potential source of xylanase. Earlier studies have shown the production of xylanase from actinomycetes, like *Thermomonospora fusca*, *Saccharomonospora viridis*, *Microtetraspora flexuosa* and *Thermoactinomyces thalophilus* (McCarthy *et al.,* 1985; Ball and McCarthy, 1988, Roberts *et al.,* 1990; Berrens *et al.,* 1996; Kohli *et al.,* 2001). *Thermomyces bifida* produced β-1, 4-endoxylanases (xyl10A, xyl10B, and xil11A), xylosidases, α-L-arabinofuranosidases, xyloglucanases, β-1, 3-glucanases (GH81) and α-N-arabinofuranosidases (xil43) (Lykidis *et al.,* 2007; Del-Pulgar and Saadeddin, 2014).

Streptomycetes have been reported to be the most copious hemicellulases producers among actinomycetes. Different species of *Streptomyces* produced xylanases, such as *Streptomyces lividans*, *S. lividans* 66, *Streptomyces roseiscleroticus, Streptomyces chattanoogensis* UAH 23, *Streptomyces chattanoogensis* CECT 3336, *S. olivaceoviridis* E-86, *Streptomyces violaceoruber*, *Streptomyces thermocyanaeviolaceus* and *Streptomyces* sp. RCK-2010 (Kluepfel *et al.,* 1986, 1990; Grabski and Jeffries, 1991; Grabski *et al.,* 1993; Fernández *et al.,* 1995; López-Fernández *et al.,* 1998; Ding *et al.,* 2004; Khurana *et al.,* 2007; Shin *et al.,* 2009; Kumar *et al.,* 2012). Other studies have indicated production of β-xylosidases by *Streptomyces albogriseolus*, *S. nitrosporeus*, and *Micromonospora melanosporea* (van Zyl, 1985). Xylanase enzyme has also been formed from other strains of *Streptomyces* sp., such as *Streptomyces* sp. CD3, *Streptomyces* sp. strain C1-3, *Streptomyces* sp. 7b, (Sharma and Bajaj, 2005; Maryandani, 2007; Bajaj and Singh, 2010).

Among the aquatic actinomycetes, the hydrolytic enzyme xylanase was reported from marine actinobacteria, namely *Thermomonospora fusca* and *Streptomyces* sp. Ab106 (Bachmann and McCarthy, 1989; Elegir *et al.,* 1995). Likewise, *S. thermoviolaceus* OpC-520, *S. viridosporus* T7A and *Thermomonspora curvata* also produced alkaline and thermostable xylanase (Tsujibo *et al.,* 1992; Magnuson and Crawford, 1997; Stutzenberger and Bodine, 2008). Xylanase producing *Streptomyces albus* MAC6 was isolated from marine sediment samples of the south coast of India (Sathyapriya *et al.*, 2012). Similarly, the xylanase producing marine *Streptomyces* sp. was isolated from mangrove sediment samples of Pitchavaram and these isolates synthesised the maximum amount of xylanase (Umadevi and Raghunathan, 2013). The xylanolytic properties of an actinobacterium, *Streptomyces*

olivaceus (MSU3), isolated from mangroves was also evaluated (Sanjivkumar *et al.,* 2017).

3.2.2 Production of xylanase

Microbial xylanase production is carried out in both SmF and SSF. Compared to SSF, SmF has been widely employed for the production of enzymes and to study physiological aspects of the synthesis of enzymes (Patil and Dayanand, 2006). Production of enzymes in SmF at industrial level, provided several advantages over the other fermentation modes, including decrease of contamination due to relatively short growth period, lower capital investment when compared to continuous processes for same bioreactor volume, higher conversion levels of the raw medium substrates owing to the controlled growth period, less production monitoring labour and well developed scale-up methods. In submerged cultures, environmental control was comparatively simple due to homogeneity of the cell microbial suspension, nutrient solution and the products at the liquid stage (Holker *et al*., 2004; Kar *et al.*, 2013).

There are many reports on the production of xylanases in SmF (Purkarthofer *et al.,* 1993; Polizeli *et al.,* 2005; Kuancha and Apiraksakorn, 2012; Pathania *et al.,* 2012; Sugumaran *et al.,* 2013; Terrasan *et al.,* 2013).

A comparative study on the production of xylanases by *Thermomyces lanuginosus* in SmF and SSF exhibited 2172 U/mL of xylanase yield after 7 days and 20220 U/g dry solid within 9 days respectively (Purkaithofer *et al.,* 1993). Several reports on xylanase production from *Streptomyces* sp. were in SmF, such as *S. roseiscleroticus* (NRl-B-11019), *Thermomyces thalophilus* and *Streptomyces* sp. 106 (Grabski and Jefferies, 1991; Kohli *et al.,* 2001; Techapun *et al.,* 2002).

A novel Streptomycete strain from mangrove soil of Kadalundi, Kerala, was isolated and production of its unique xylanase was studied in SmF (Thomas *et al.,* 2013). The enzyme activity in the liquid medium supplemented with oat spelt xylan (0.5%) was investigated using *Streptomyces* spp. CA24 (Porsuk *et al.,* 2013). Many *Streptomyces* sp. were isolated from marine ecosystems and studied for xylanase production in SmF (Fatokun *et al.,* 2016; Sivakumar and Kumar, 2016). Production of bacterial xylanases was further optimised by controlling, various physical and nutritional parameters.

3.2.3 Optimisation of xylanase production

The economic viability of the microbial enzyme applications usually depended on the cost of their production processes. As various nutritional and fermentation parameters affected enzyme production to obtain high and commercially viable yields of microbial enzymes, it was vital to optimise the fermentation medium used for microbial growth and enzymes production. Optimal parameters of the enzymes biosynthesis from microbial origin, varied greatly with the disparity of the producing strain, environmental, and nutritional conditions (Frost and Moss, 1987).

3.2.3.1 Inoculum age and size

Optimum concentration of inoculum was necessary for maintaining equilibrium between proliferating biomass and available nutrients for maximum enzyme production. An inoculum level higher than the optimum could effect in faster nutrient consumption and hence lower enzyme yield. Furthermore, a large inoculum size was undesirable during scale up of the fermentation process and hence not preferred in industrial fermentation (Kumar *et al.,* 2014).

Several researchers have reported the use of 1.0–5.0% (v/v) inoculum for higher production of xylanase (Subramaniyan *et al.,* 2001; Kar *et al.,* 2006; Battan *et al.,* 2007). Among actinomycetes, enhanced xylanase production was observed from *Streptomyces* sp., when 0.5 mL spore suspension (10^7 CFU/mL) was used to inoculate the production medium (Rifaat *et al*., 2005). Optimum inoculum age and size varied with microorganisms. *S. violaceoruber* and *S. viridosporus* T7A (ATCC 39115) produced maximum xylanase with 1% (v/v) of 36 h old and 12% (v/v) of 48 h old respective seed cultures (Khurana *et al.,* 2007; Albertan *et al*., 2009). *S. chartreusis* of 5days old was used to inoculate the production medium for higher xylanase production (Li *et al.,* 2011). 1 mL of heavy spore suspension of *S. rameus* and 1.0% (v/v) of *Streptomyces* RCK-2010 were used for maximum production of xylanase (Bhosale *et al.,* 2011; Kumar *et al.,* 2012). Maximum xylanase was produced from a mangrove *Streptomyces* sp. and *Streptomyces* sp. K37 with 1 mL of spore suspension ($1X10^6$ spores/mL) of 48 h old culture (Thomas *et al.,* 2013; Nour El-Dein *et al.,* 2014). On the other hand, 2.5 mL of 72 h old *Streptomyces* sp. P12-137 was used for enhanced production of xylanase (Coman and Bahrim, 2011). The optimum inoculum size for a novel mangrove actinobacterium, *S. olivaceus,* was also found to be 2.5% (Sanjivkumar *et al.,* 2017).

3.2.3.2 Incubation period

Cultivation period for the enzyme production is the time taken by the microorganism for utilising the accessible nutrients from the culture medium for the synthesis of desired product. The incubation period depended upon the type of fermentation, growth and its pattern of microorganism and enzyme production (Nagar *et al.,* 2012). The duration of incubation period also varied with the microorganism, being smaller for

bacterial cultures as compared to fungi because the growth rate of the former is faster than the latter (Ho and Heng, 2015).

Different incubation periods have been reported by several workers to produce xylanolytic enzymes, such as 18 h, 24 h, 48 h, 72 h, 96 h and 120 h (Paul and Verma, 1990; Bataillon *et al.,* 1998; Breccia *et al.,* 1998; Damaso *et al.,* 2000; Ghanem *et al.,* 2000; Kohli *et al.,* 2001; Subramaniyan *et al.,* 2001; Cordeiro *et al.,,* 2002; Pandey and Pandey 2002; Han *et al.,* 2004; Simoes and Tornisielo, 2005; Kar *et al.,* 2006; Sharma *et al.,* 2007; Okafor *et al.,* 2007; Cui *et al.,* 2008; Sanghi *et al.,* 2009; Wahyuntri *et al.,* 2009; Prakash *et al.,* 2011; Bajaj and Manhas, 2012; Ncube *et al.,* 2012).

Among actinomycetes, the optimum incubation period was found to be 5 days for xylanase production from *Streptomyces* sp. Ab106 (Techapun *et al.,* 2002). Similar results were obtained from *S. albus, S. chromofuscus S. rochei* and *Thermomyces lanuginosus* SK (Rifaat *et al.*, 2005; Nadia *et al.,* 2005; Kumar *et al.,* 2009). *Streptomyces* sp. RCK-2010 exhibited higher xylanase production after 48 h of incubation (Kumar *et al.,* 2012).

An alkaline tolerant marine derived *S. viridochromogenes* strain produced maximum xylanase after 8 days of incubation (Liu *et al.,* 2013). The optimum incubation period of *Streptomyces* sp., isolated from mangrove soil of Kerala was found to be 48 h (Thomas *et al.,* 2013). However, *Streptomyces* Mcasbt1 isolated from marine mangrove sediment samples produced extracellular xylanase after 10 days of incubation (Umadevi and Ragunathan, 2013). Optimum incubation period of 72 h and 48 h was reported from a marine *Streptomyces* sp. isolated from marine sediment of Nahoon beach and a novel mangrove associated actinobacterium, *S. olivaceus*, respectively (Fatokun *et al.,* 2016; Sanjivkumar *et al.*, 2016).

3.2.3.3 Incubation temperature and pH

Incubation temperature and pH are the most important physical factors affecting the microbial growth and enzyme production. Each bacterial strain showed higher enzyme yield at its unique optimum growth temperature and pH of the production medium. The pH of the production medium may alter during fermentation due to metabolic activities of the microorganism. Different types of microorganisms, species and genus required inimitable cultivation temperature and pH for enzyme production. The pH of the medium controlled the enzyme production in two ways: first, it can affect the growth of the organism, and therefore, the enzyme production would be effected; second, the produced enzyme may be subjected to inactivation or denaturation by unfavourable ionic environment caused by pH of the medium.

Xylanase production by various bacteria and fungi has been shown to be obviously dependent on pH (Smith and Wood, 1991; Wong *et al.,* 1988; Yoshida *et al.,* 1989). Acidic pH (4 to 6) usually favoured fungal xylanases whereas higher pH favoured bacterial xylanases (Bisaria and Ghose, 1981; Bajpai, 1997). Several workers have reported the highest production of xylanase at pH 7 (Roy, 2004; Kuhad *et al.,* 2006; Battan *et al.,* 2007; Sanghi *et al.,* 2009; Wahyuntri *et al.,* 2009; Ghoshal *et al.,* 2012). Maximum xylanase production was also reported between pH of 6.5 and 7.0; at pH 7.5 and pH 7.72 (Cordeiro *et al.,* 2002; Cui *et al.,* 2008). Different actinomycete species produced xylanase with its optimum at alkaline pH values, such as *Thermoactinomyces thalophilus* at pH 8.5 and *Thermomonospora* sp. at pH 9 (Kohli *et al.,* 2001; Sudeep *et al.,* 2001).

Most of the xylanases from actinomycetes showed pH optima near to neutrality or towards slightly acidic, although some possessed best activity

under mild alkaline conditions (from pH 5-6, 5-7 or 5.5 -8). Similarly most of the xylanases showed best activity at 50-65^{o}C. The pH optimum of the enzyme determines its applications. Higher temperature optima and stability is a key requirement for most of the industrial applications of xylanases (Thomas *et al.*, 2013).

Among various incubation temperatures studied, the maximum xylanase production by a newly isolated *Streptomyces* sp. RCK-2010 was achieved at 40^{o}C (Kumar *et al.,* 2012). Many thermophilic actinomycetes, like, *Thermoactinomyces thalophilus, Thermomonospora* sp. and *Thermomyces lanuginosus* produced xylanase at optimum temperature 45^{o}C and 50^{o}C (Kohli *et al.,* 2001; Sudeep *et al.,* 2001; Gaffney *et al.,* 2009).

Many Streptomycetes, like, *Streptomyces* sp. Ab106, *S. rochei, S. hygroscopicus*, *S. albus* and *Streptomyces* sp. CA24, produced xylanase at optimum pH 7 and 50^{o}C, pH 6.0 and 40^{o}C; pH 9 and 60^{o}C; from pH range of 9 to11 and 50^{o}C; pH 7 and 60^{o}C; pH 7.5 and 40 ^{o}C and pH 7 and 55^{o}C, respectively (Techapun *et al.*, 2001; Nadia *et al.,* 2010; Priya *et al.,* 2012; Porsuk *et al.*, 2013).

Among marine Streptomycetes, pH 9 and 30^{o}C was observed as optimum pH and temperature for xylanase production from a mangrove *Streptomyces* sp. (Thomas *et al.,* 2013). Xylanase production was optimum at pH 8 and incubation temperature 35^{o}C for a marine *Streptomyces* sp. isolated from Nahoon beach sediment (Fatokun *et al.,* 2016). Similarly, the optimum pH and incubation temperature observed for xylanase production by *Streptomyces olivaceus,* were 7 and 30^{o}C, respectively (Sanjivkumar *et al*., 2016).

3.2.3.4 Carbon and Nitrogen sources

Carbon and nitrogen sources are the essential components of the production medium required for the growth of microorganism. The choice of substrate was important for the enzyme production. Xylanases were typically inducible enzymes secreted in media containing pure xylan or xylan-rich residues (Balakrishnan *et al.,* 1997). Nevertheless, constitutive production of xylanase has also been reported (Khasin *et al.,* 1993). The vast majority of xylanases were released into the extracellular environment since the large size of the substrate prevented its penetration into the cell. In fact, the present idea was that xylanase production was induced by means of the products of their own action (Biely, 1985).

Various kinds of xylans, such as birchwood xylan, larchwood xylan, beechwood xylan and oatspelt xylan have been used as carbon sources for xylanase production in several reports. Some of the carbon sources reported for the xylanase production were glucose, xylose, arabinose, xylan, and carboxymethyl cellulose (CMC) (Rawashdeh *et al,* 2005).

Use of commercial xylan increased the activity of xylanase in many microorganisms (Biely *et al.,* 1980; Dubeau *et al.,* 1987; Gessesse and Gashe, 1997; Marques *et al.,* 1998; Damaso *et al.,* 2000; Rani and Nand, 2000; Bakir *et al.,* 2001; Chivero *et al.,* 2001; Nascimento *et al.,* 2002; Subramaniyan and Prema, 2002; Coelho and Carmona, 2003; Medeiros *et al.,* 2003; Krogh *et al.,* 2004; Rawashdesh *et al.,* 2005; Kumar *et al.,* 2009; Lee *et al.,* 2009; Giridhar and Chandra, 2010; Nawel *et al.,* 2011; Ahmed *et al.,* 2012; Moses and Leelavathy, 2012; Terrasan *et al.,* 2013; Tork *et al.,* 2013).

In *Saccharomonospora viridis*, a thermophilic actinomycete which degraded xylan but not cellulose, both extracellular xylanase and cell-bound

β-xylosidase were induced by the growth on xylan but not on glucose or xylose (Roberts *et al.,* 1990). *Streptomyces* sp. AMT-3 strain and *Streptomyces* strain Ib 24D gave 70 and 1447 U/mL xylanase, respectively using larchwood xylan (Nascimento *et al.,* 2002). The highest xylanase production was obtained with 20.0 g xylan as sole carbon source for *Streptomyces* spp. (Rifaat *et al.,* 2005). Beechwood xylan was optimum for xylanase production by a marine *Streptomyces* sp.(Fatokn *et al.,* 2016).

Nitrogen source could be provided in either inorganic (potassium nitrate, ammonium chloride, ammonium dihydrogen phosphate etc.) or organic form (peptone, yeast extract, beef extract etc.), the later being more effective in stimulating xylanase production. Nitrogen makes up about 14% of total dry weight of microbial cells. It was often a growth limiting nutrient for many organisms; because their anabolism stopped as they did not have sufficient nitrogen to build proteins and nucleotides (Bauman, 2006). Examples of nitrogen sources used for xylanase production were peptone, urea, sodium nitrate, yeast extract, ammonium nitrate and ammonium sulphate (Kheng and Omar, 2005). Literature states that peptone had been successfully utilised for xylanase production by many researchers (Singh *et al.,* 1995; Kuhad *et al.,* 1998; Ghanem *et al.,* 2000; Schneider *et al.,* 2001; Subramaniyan *et al.,* 2001; Qinnghe *et al.,* 2004; Kachlishvili, 2005; Kuhad *et al.,* 2006; Norazlina *et al.,* 2012; Sakthiselvan *et al.,* 2012). The use of yeast extract also proved in maximum xylanase production (Laxmi *et al.,* 2008; Bakri *et al.,* 2013; Sugumaran *et al.,* 2013).

Among actinomycetes, soybean bran for *Streptomyces viridosporus* T7A, sodium nitrate for *Thermomyces lanuginosus* A72 and ammonium nitrate for *Thermomyces lanuginosus* YMN72 were found to be optimum for xylanase production (Alberton *et al.,* 2009; Ali *et al.,* 2013). Concerning the inorganic nitrogen sources used, a slight increase in xylanase production

was observed with potassium nitrate in *Streptomyces* species. In case of organic nitrogen sources, a maximum increase was observed with yeast extract rather than with peptone and casein. In addition, yeast extract showed a maximum production comparable with different inorganic nitrogen sources (Rifaat *et al.,* 2005). Potassium nitrate was used as the optimum nitrogen source for maximum xylanase production from *Streptomyces* sp. P12-137 (Coman and Bahrim, 2011). The xylanase excretion was enhanced when the medium was supplemented with oat spelt xylan (0.5%) and yeast extract (0.5%) from *Streptomyces* sp. CA24 (Porsuk *et al.,* 2013). Yeast extract was found to be the optimum nitrogen source for xylanase production from marine *Streptomyces* sp. (Fatkun *et al.,* 2016; Sajivkumar *et al.,* 2016).

3.2.3.5 Agitation and aeration

Agitation and aeration are used to meet the oxygen demand and uniform mixing of nutrients during fermentation. The lower enzyme production under stationary conditions might be due to decrease in oxygen level in the medium which, in turn, unfavourably affected the enzyme yield. Various microorganisms required different agitation speed for optimum production of enzymes. A long range of agitation speed has been reported by several workers for the highest production of xylanase such as 100 rpm, 120 rpm, 140 rpm, 150 rpm, 160 rpm, 170 rpm, 180 rpm, 200 rpm, 250 rpm and 400 rpm (Dey *et al.,* 1992; Okazaki *et al.,* 1985; Yoshida *et al.,* 1994; Dhillon and Khanna, 2000; Kohli *et al.,* 2001; Rizzati *et al.,* 2001; Kaur *et al.,* 2004; Kar *et al.,* 2006; Sudan and Bajaj, 2007; Kapoor *et al.,* 2008; Bokhari *et al.,* 2009; Azeri *et al.,* 2010; Arevalo-Villena *et al.,* 2011; Irfan *et al.,* 2012). Among actinomycetes, optimum xylanase production in *Streptomyces violaceoruber* under SmF was found at 200 rpm (Khurana *et al.,* 2007). However, *Streptomyces* sp. CA24 gave the maximum xylanase

production at 100 rpm (Porsuk *et al.,* 2013). In another study of a marine Streptomycete, the optimum agitation speed was found to be 150 rpm (Fatakun *et al.,* 2016).

3.2.3.6 Metal ions, surfactants and other additives

There are many reports related to the effect of various metal ions, chelators, detergents and surfactants for xylanase production. The effect of concentration of metal ion and mechanism of induction on xylanase production varied from species to species (Saxena *et al.,* 1994). It has been reported that stimulatory effect of these additives resulted from efficient spore dispersion, rheological properties of the medium, availability of nutrients and oxygen and physiological functions of the cells (Chen, 1996).

Xylanases from *Streptomyces* sp. AMT-3 were strongly inhibited by the addition of Fe^{2+}, Cu^{2+}, Mg^{2+} (Nascimento, 2002). Supplementation of $CaCl_2$ (5 mM) as a metal additive considerably induced the whole enzyme system from *Streptomyces* sp. MDS (Saratale *et al.*, 2012). Supplementation of Fe^{3+}, Ca^{2+} and Mn^{2+} as metal additives slightly induced the xylanase enzyme system from a strain of *Streptomyces* CD3 while Hg^{2+} strongly inhibited the xylanase production (Sharma and Bajaj, 2005).

Xylanase production was improved by addition of Tween 20, Tween 80, olive oil and oleic acid (Kuhad *et al.,* 1998). SDS also repressed the xylanase production, when used in the production media (Gomes *et al.,* 1994; Zhang *et al.,* 2013). Rhamnolipid and Tween 80 had a marked stimulatory effect on xylanase production during the peak enzyme production phase (Liu *et al.,* 2011). A concentration of 0.2% (v/v) of olive oil as an additive for enzyme production and such compounds probably increased the permeability of the cell membrane and caused rapid secretion of the enzymes (Battan *et al.,* 2007).

3.2.4 Optimisation of xylanase production by non-statistical and statistical methods

Culture medium optimisation by the conventional OFAT technique required a substantial amount of work and time. Furthermore, it was very difficult to determine the cumulative effect of more than 2 factors by using this approach. However, optimisation using statistical approach, such as PBD and RSM could be used to examine the interactive effects of various factors and to optimise biotechnological processes (Bocchini *et al.,* 2002; Nagar *et al.,* 2011). RSM is an experimental strategy in search of the optimum conditions for a multivariable system (Chen *et al.,* 2002). It is a powerful mathematical model with a collection of statistical techniques in which interactions between multiple process variables can be identified with fewer experimental trials. There are various advantages in using statistical methodologies in terms of rapid and consistent short listing of process conditions. Thus, RSM experimental design is an efficient move to deal with a large number of variables and there are several reports on application of RSM for the production of primary and secondary metabolites through microbial fermentation (Balusu *et al.,* 2005; Jarghalsaikhan *et al.,* 2009).

Reports were available in literature regarding the production of xylanase using RSM. Xylanase production from *Schizophyllum commune* and *Thermomyces langinosus* was enhanced by Central Composite Rotator Design (CCRD) to 5.47 IU/mL and 2.7 IU/mL, respectively (Haltrich *et al.,* 1993, Purkarthofer *et al.,* 1993).

Among actinomycetes, RSM using CCD was used to optimise fermentation medium for the production of alkaline xylanase from *Streptomyces violaceoruber* under SmF. 3 fold increase in xylanase production (1500 IU/mL) was seen, as compared to its initial level (500

IU/mL). Analysis of variance (ANOVA) showed a high coefficient of determination (R^2) value of 0.9718, ensuring a satisfactory adjustment of the quadratic model with the experimental data (Khurana *et al.*, 2007). RSM and CCD were used to optimise a biosynthesis medium for the production of xylanases by *Streptomyces* sp. P12-137 in SmF culture. It resulted in 3-fold increase in the level of the xylanase (27.77 U/mL) production compared to the initial level (8.30 UA/mL) after 120 h of fermentation, while the value predicted by the quadratic model was 26.45 UA/mL (Coman and Bahrim, 2011).

The xylanase production from *Streptomyces* sp. RCK-2010, was optimised for varying culture conditions employing OFAT and RSM approaches. The RSM showed that the optimum level of these factors resulted in almost 3 fold improvement in xylanase production (Kumar *et al.*, 2012).

The xylanase production was optimised from a novel *Streptomyces* sp. in SmF using OFAT (Thomas *et al.*, 2013). The use of optimised conditions (pH 9, temperature 30ºC, 48 h incubation period) resulted in maximum xylanase production (204 IU/mL). Xylanase was produced from *Streptomyces* sp. CA24 in SmF and obtained maximum enzyme yield in basal medium supplemented with oat spelt xylan (0.5%) and yeast extract (0.5%) at pH 10. After optimisation, the enzyme yield increased by 5-fold (255 U/mL) (Porsuk *et al.*, 2013).

Optimisation of culture conditions resulted in 205.3 IU/mL of alkali-thermostable xylanase production. Magnesium sulphate as metal salt and xylan as additive increased the xylanase productivity. CCD and RSM were employed to optimise these significant process parameters and for evaluation of interactive factors (Shanti and Roymon, 2015).

The isolate used in the present study, *Streptomyces* sp. ER1 obtained from the estuarine sediment of Cochin is a potent hyperxylanolytic strain. Literature pertaining to estuarine xylanase producing actinomycetes is scarce and so far no studies have been done on xylanase producing actinomycetes from Cochin estuary. Aquatic actinomycetes are well known for their unique enzyme characteristics. Keeping the above in view, this chapter deals with the optimisation of xylanase production from *Strpetomyces* sp. ER1 using OFAT and statistical methods.

3.3 Materials and methods

3.3.1 Experimental organism

Streptomyces sp. ER1 used in the present study was isolated following an extensive screening for xylanase producers as described in section 2.3.4 and 2.3.7.6. The culture was periodically sub cultured and stored in nutrient agar xylan slants.

3.3.2 Selection of production medium for xylanase production:

In all, 11 different culture media (production media A-K) were tested in order to select a suitable medium, for optimising xylanase production. The media used for the study are presented in Table 3.1. Enzyme production was carried out with 100 mL, in each of the 11 production media in 250 mL Erlenmeyer flasks (pH 7), inoculated with 5% (v/v) inoculum and incubated at 30 ± 2°C under shaking condition (100 rpm) for 188 h. After incubation, culture broth was centrifuged at 10,000 rpm for 15 min at 4°C, and the cell-free supernatant was used as crude enzyme to measure xylanase activity. Comparing xylanase activity (U/mL) obtained with the 11 different production media; the medium which showed maximum xylanase activity (U/mL) was selected as the production medium.

Thereafter, the best medium was used to investigate the effect of different media components on the extracellular xylanase production by *Streptomyces* sp. ER1.

Table 3.1. Different media used for selecting the best xylanase production medium by *Streptomyces* sp. ER1

Production medium	Composition (g/L)
A (Techapun *et al.,* 2001)	KH_2PO_4 1.5, K_2HPO4 2, $(NH_4)_2SO_4$ 4.5, yeast extract 0.075, peptone 0.075, Tween 80 0.075, $ZnSO_4.7H_2O$ 140mg, $MnSO_4.H_2O$ 160mg, $FeSO_4.7H_2O$ 500mg, $COCl_2.2H_2O$ 200mg, xylan 2.5
B (M9 medium), (Roy, 2004)	Yeast extract 2, NaCl 2.5, NH_4Cl 5, KH_2PO_4 15, Na_2HPO_4 30, $MgSO_4$ 0.25, xylan 2.5
C (Mandel's and Sternburg's medium) (Mandels and Sternburg, 1976)	Urea 10, yeast extract 0.75, peptone 0.3, $(NH_4)_2$ SO_4 0.25, KH_2PO_4 1.4, $ZnSO_4.7H_2O$ 1.4, Tween 80 0.1%, $CaCl_2$ 2, $MgSO_4.7H_2O$ 0.3, $NaNO_3$ 3, KCl 0.5, $MnSO_4.4H_2O$ 1.6, $COCl_2.6H_2O$ 20, xylan 2.5
D (Raghunathan and Padma, 2013)	Yeast extract 6, $NaHPO_4$ 5.03, KH_2PO_4 1.98, NaCl 0.2, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.05, trace element solution 1mL ($FeSO_4.7H_2O$ 1, $MnSO_4.7H_2O$ 0.2, $ZnSO_4.7H_2O$ 0.9), xylan 2.5
E (Kim *et al.,* 1985)	Peptone 0.5, urea 0.3, K_2HPO_4 0.2, $CaCl_2$ 0.3, Tween 80 0.2, $(NH_4)_2$ SO_4 1.4, $MgSO_4$ 0.3, xylan 2.5
F (Suneetha *et al.,* 2011)	Urea 0.3, $MgSO_4$ 0.1, K_2HPO_4 0.2, $CaCl_2$ 0.3, Tween 80 0.2, $(NH_4)_2SO_4$ 1.4, $MgSO_4.7H_2O$ 0.3, xylan 2.5
G (Liu *et al.,* 2013)	$K_2HPO_4.3H_2O$ 1, $KH_2PO_4.3H_2O$ 3.5, $(NH_4)NO_3$ 3, $MgSO_4.7H_2O$ 2, $CaCl_2$ 0.3, glucose 1.5, peptone 2.5, beef extract 3, xylan 2.5
H (M medium) (Obi and Odibo, 1984)	K_2HPO_4 0.1, $(NH_4)_2SO_4$ 0.1, NaCl 0.1, $MgSO_4.7H_2O$ 0.1, xylan 2.5
I (Ramakrishnan and Narayanan, 2013)	Starch 20, glucose 15, yeast extract 5, $CaCO_3$ 3, $MgSO_4$ 2.5, xylan 2.5
J (Tryptic soy broth) (Liu *et al.,* 2013)	Casein 17, K_2HPO_4 2.5, glucose 2.5, NaCl 5, soya peptone 3, xylan 2.5
K (Nutrient broth with xylan) (Hiremath and Patil, 2011)	Peptone 5 , NaCl 5 , yeast extact 2, beef extract 1 , xylan 2.5

3.3.3 Culture medium used for optimisation studies

The organism was grown in the selected xylanase production medium A (Techapun *et al.,* 2002).

3.3.4 Inoculum

The inoculum was raised in the xylanase production medium A. The culture was grown in Erlenmeyer flask containing 100 mL of the xylanase production medium on an incubator shaker (100 rpm) at ambient room temperatures (30 ± 2°C). Unless otherwise specified, the inoculum of age 24 h was used at 5% v/v ($452X10^1$ CFU/mL).

3.3.5 Production of xylanase

Production of xylanase was studied in correlation with the growth profile of the culture. SmF was carried out using xylanase production medium A in 250 mL Erlenmeyer flasks with a working volume ratio of 0.4 (100 mL medium in 250 mL flasks) at 100 rpm on an incubator shaker. The incubation was carried out for 120 h at ambient room temperature (30 ± 2°C). After the incubation period, cells were separated by centrifugation (10000 rpm, 15 min, 4°C) and the supernatant served as the extracellular crude xylanase.

3.3.6 Biomass estimation

10 mL of fermented production medium A broth was collected in a pre-weighed centrifuge tube and centrifuged at 8000 rpm for 15 min. Supernatant was discarded and the pellet was washed thrice with sterile distilled water, followed by drying the pellet at 95°C, till attaining constant weight. The biomass was expressed in dry cell weight (mg/mL) (Khusro *et al.,* 2016).

3.3.7 Xylanase assay

Endo-xylanase [l, 4-β-D-xylan xylanohydrolase (E.C.3.2.l.8)] was assayed following the method of Bailey with some modifications using 1% beechwood xylan (Sigma Chemicals Co.) (Bailey *et al.,* 1992). The substrate (xylan) was dissolved by heating in 0.1 M phosphate buffer. The reaction mixture contained 280 µL of preincubated beechwood xylan suspension and 20 µL of suitably diluted crude enzyme, which was then incubated for 30 min at 55^{o}C. Enzyme blank was prepared by adding dinitrosalicylic acid (DNS) reagent prior to the enzyme addition so that only the reducing sugars present in the enzyme preparations would be answering. The reagent blank was prepared in the same manner but 300 µL phosphate buffer (0.1 M, pH 7) was used instead of crude enzyme. The reaction in the mixture and blanks were terminated by adding 300 µL of DNS reagent, which was then kept in boiling water bath for 15 min. The concentration of reducing sugars released was estimated against xylose standard by noting the absorbance at 540 nm (Miller, 1959). The stock solution for xylose standard (Xylose, HiMedia) was prepared in 15 mg/10mL concentration and appropriate dilutions were used as the standard. One unit of endo-xylanase activity was defined as µmol of xylose liberated per min per mL of enzyme preparation.

3.3.8 Optimisation of nutritional and physical parameters by One-Factor-at-a-Time (OFAT) method

In the conventional OFAT method, various nutritional and physical parameters were optimised by maintaining all parameters at a constant level in the production medium A, except the one under study. Each subsequent parameter was examined after taking into account the previously optimised

parameter(s). In this study, the biomass of *Streptomyces* sp. ER1 was correlated with its xylanase production for each parameter optimised.

3.3.8.1 *Effect of incubation period on growth and xylanase production by Streptomyces sp. ER1*

Xylanase fermentation experiments were carried out up to 14 days in xylanase production medium A and biomass and xylanase activity measured at 24 h intervals.

3.3.8.2 *Effect of temperature on growth and xylanase production by Streptomyces sp. ER1*

Streptomyces sp**.** ER1 was grown in xylanase production medium A at varying temperatures ranging from 20-40°C (20, 25, 30, 35, and 40). The temperatures were set at different incubators without shaking. The culture supernatant was used for biomass estimation and xylanase assay.

3.3.8.3 *Effect of pH on growth and xylanase production by Streptomyces sp. ER1*

In order to study the effect of initial pH of the culture medium on the production of extracellular endo-xylanase, *Streptomyces* sp. ER1 was grown in xylanase production medium A with the initial media pH adjusted within a range of 3 to 11 at 1 unit increment. In all cases, the initial pH values have been uncontrolled throughout the fermentation. The culture supernatant was used for biomass estimation and xylanase assay.

3.3.8.4 *Effect of age of inoculum and inoculum concentration on growth and xylanase production by Streptomyces sp. ER1*

To monitor the effect of inoculum age on xylanase production, culture was incubated for 6, 24 and 30 h separately on an incubator shaker. The culture was harvested at respective (6, 24 and 30 h) time intervals and

used to inoculate the production medium A with an inoculum size of 5% (v/v). In order to minimize the time lag in fermentation process, inoculum was raised in the medium with a composition similar to that of the fermentation medium (Lincoln, 1960).

The effect of inoculum concentration on xylanase production was studied by using different concentrations of optimised inoculum age of *Streptomyces* sp**.** ER1, such as 1%, 3%, 5%, 7%, 9 % and 11%. The culture supernatant was taken for biomass estimation and xylanase assay.

3.3.8.5 *Effect of agitation on growth and xylanase production by Streptomyces sp. ER1*

Submerged fermentation was carried out at different agitation rates ranging from 50, 100 and 200 rpm on an incubator shaker and xylanase production was studied. The xylanase production by the unagitated culture was also determined. The culture supernatant was used for analyses as described earlier.

3.3.8.6 *Effect of salinity on growth and xylanase production by Streptomyces sp. ER1*

The production medium was prepared using distilled water and seawater of different salinities (0, 5, 10, 15, 20 ppt). The fermentation was carried out and the effect of salinity on xylanase production and biomass of the culture was studied.

3.3.9 Optimisation of nutritional parameters

Optimisation studies were conducted to evaluate the higher production environment using three different strategies i.e. studies on the optimisation of carbon sources, nitrogen sources and mineral ions for the enhanced production of xylanase from *Streptomyces* sp. ER1.

3.3.9.1 *Effect of carbon source on xylanase production*

Optimisation experiments were carried out in xylanase production medium A as mentioned earlier. Different carbon sources were added to the medium replacing xylan. In the first set of experiments, production of xylanase by *Streptomyces* sp. ER1 was monitored using various carbon sources. The carbon sources studied included: monosaccharides, (xylose and glucose) disaccharide, (sucrose), polysaccharides, (cellulose, xylan, and starch) and a polyol compound (glycerol). The medium supplemented with xylan as carbon source was used as control.

To ascertain the effect of various carbon sources on xylanase production, 100 mL of production medium A (pH 7) was prepared in 250 mL Erlenmeyer flasks. The medium was supplemented with one of the above said carbon sources at 2.5 g/L. After fermentation, the culture supernatant was used for xylanase assay and the medium without carbon source was used as control.

3.3.9.2 *Effect of surfactants and other additives on growth and xylanase production by Streptomyces sp. ER1*

The effect of different surfactants (Tween 60, Tween 80, SDS and Triton X-100) and other additives (olive oil, EDTA, polyethylene glycol and β-mercaptoethanol) on xylanase production was investigated by individually supplementing these in the production medium A. The fermentation was carried out and the culture supernatant was analysed for biomass and xylanase assay. The production medium without the additive was taken as control.

3.3.9.3 *Effect of metal ions on growth and xylanase production by Streptomyces sp. ER1*

Studies on the influence of different metal ions were carried out using Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, $Zn^{2+,}$ Mn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+} and Hg^{2+} in chloride and phosphate salts at 10 mM concentration in the production medium A (Bagewadi *et al.,* 2015). All other parameters were kept constant. The culture supernatant was analysed for biomass and xylanase assay. The production medium without the metal ion source was taken as control.

3.3.9.4 *Effect of nitrogen sources on growth and xylanase production by Streptomyces sp. ER1*

The production of xylanase using different nitrogen sources was analysed. The nitrogen source giving highest xylanase production in comparison with the control was treated as the most potent one and used for further studies.

The nitrogen sources used were organic nitrogen sources, tryptone, beef extract, yeast extract, peptone, albumin, gelatin, casein, soya bean meal, phenyl alanine and urea and inorganic nitrogen sources, ammonium nitrate, ammonium chloride, di ammonium phosphate, sodium nitrate, ammonium sulphate and potassium nitrate.

To analyse the effect of various nitrogen sources on xylanase production, 100 mL of the xylanase production medium A was supplemented with one of the above said inorganic and organic nitrogen sources respectively. Biomass estimation and xylanase assay were performed. The production medium without the nitrogen source was taken as control.

3.3.9.5 *Effect of different levels of xylan on growth and xylanase production by Streptomyces sp. ER1*

The selected carbon source was tried at different concentrations and the subsequent effect on xylanase production by *Streptomyces* sp. ER1 was monitored. The different concentrations tried were (%w/v): 0.1 to 0.5 with 0.1 unit difference. The culture supernatant was taken and used for analysing biomass and xylanase assay.

3.3.10 Screening of significant medium components and fermentation conditions using Plackett-Burman Design (PBD)

To determine the significant variables that affected the xylanase production by *Streptomyces* sp. ER1, PBD using statistical software package Design-Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA), was used. The variables screened in the current study included incubation period, xylan concentration, pH, inoculum age, inoculum concentration, agitation speed, salinity, incubation temperature, concentration of selected additive, carbon, inorganic and organic nitrogen sources and metal ions (Table 3.2). The selected surfactant, inorganic and nitrogen sources and metal ions from OFAT method were olive oil, potassium nitrate, peptone, Mg^{2+} and Mn^{2+}, respectively. Each variable was tested at 2 levels. Low and high levels of each of these variables were used to prepare a combination of 20 experimental runs of the production medium (Table 3.3). The impact of each variable on xylanase production was estimated based on comparison of the difference in the mean in xylanase activity between the high level (+) and the low level (−). Insignificant ones were eliminated in order to obtain a smaller, manageable set of variables. A linear approach was considered sufficient for screening the effect of these variables.

$R_1 = \beta_0 + \Sigma\beta_i X_i (i=1,....., k)$ (Equation 1)

Where R_1 is the estimated xylanase activity and β_i is the regression coefficient.

Inoculation was carried out using 5% (v/v) of 24 h old *Streptomyces* sp. ER1 and the day of inoculation was considered as day 0. The positive variables influencing xylanase production (xylanase activity) was selected from Pareto Chart analysis. The experiments were carried out in triplicates and the average xylanase activity was recorded as the response. F value and p values and the proportion of variance (R^2) determined by the model were significant at p<5% level.

Table 3.2. Variables and their levels employed in PBD for screening fermentation and nutrition conditions affecting xylanase production by *Streptomyces* sp. ER1

Variables	Name	Units	Minimum (-1)	Maximum (+ 1)
A	Incubation period	days	5	8
B	Xylan concentration	%	0.2	0.4
C	pH		6	9
D	Inoculum age	h	20	30
E	Inoculum concentration	%	3	9
F	Agitation speed	rpm	60	150
G	Salinity	ppt	7	18
H	Incubation temperature	^oC	30	40
I	Concentration of olive oil (additive)	mg/L	100	200
J	Concentration of ammonium chloride (inorganic nitrogen source)	g	1	10
K	Concentration of peptone (organic nitrogen source)	g	0.1	1
L	Concentration of Mn^{2+} (metal ion)	mg	100	200
M	Concentration of Ca^{2+} (metal ion)	g	1	10

Table 3.3. PBD for 20 trials to identify the significant variables affecting the xylanase production by *Streptomyces* sp. ER1

Rows	Factors												
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
1	+	+	+	+	-	+	-	+	-	-	-	-	-
2	+	+	-	+	-	+	-	_	-	-	+	+	+
3	-	+	-	-	-	-	+	+	-	+	+	-	+
4	-	-	+	+	-	+	+	-	-	+	+	+	-
5	+	-	+	-	-	-	-	+	+	-	+	+	+
6	+	-	-	-	-	-	-	-	-	-	-	-	+
7	+	+	-	+	+	-	-	+	+	+	+	-	-
8	-	-	+	+	-	-	+	+	+	+	-	+	-
9	+	-	-	+	+	+	+	-	+	-	+	-	-
10	-	+	+	-	+	+	-	-	+	+	+	+	+
11	-	-	-	-	-	+	+	-	+	+	-	-	-
12	-	-	-	+	+	-	-	+	-	-	+	+	-
13	+	+	+	-	-	+	+	+	+	-	+	-	-
14	+	+	+	+	+	-	+	-	+	-	-	-	+
15	-	+	-	-	+	+	+	+	-	+	-	+	+
16	+	-	+	-	+	-	-	-	-	+	+	-	+
17	-	+	-	+	-	-	-	-	+	+	-	+	-
18	+	+	+	-	+	-	+	-	-	-	-	+	+
19	-	-	+	+	+	+	-	+	-	+	-	-	-
20	-	-	-	-	+	+	-	+	+	-	-	+	+

3.3.11 Optimisation of medium components using Central Composite Design (CCD)

RSM was used to optimise the significant fermentation parameters for enhancing extracellular xylanase production from *Streptomyces* sp. ER1. A CCD was employed for the optimisation process (Coman and Bahrim, 2011). Based on the results from the Pareto Chart analysis, 4 factors that significantly affected the xylanase production were identified. They were xylan concentration, inoculum age, agitation speed and concentration of olive oil.

Each independent variable in CCD matrix was studied at 5 different levels ($-\alpha$, -1, 0, +1, $+\alpha$) as shown in Table 3.4. The design involved 6 centre points with an alpha value being ± 2. The 5 coded levels of alpha, studied in the present study were -2, -1, 0, +1 and +2. According to the present experimental design, the total number of experimental runs is $2k + 2k + n$, where 'k' is the number of independent variables and 'n' is the number of repetition of experimental runs at the central point. The experimental design consisted of 30 experimental runs of 4 variables in order to optimise the xylanase production. Xylanase activity corresponding to combined effects of 4 components was studied with respect to, xylan concentration (0.35 — 0.45%), inoculum age (16.5---- 22.5 h), agitation speed (42----72 rpm) and olive oil concentration (5 — 65 mg/L).

The experimental plan of independent significant variables with respect to their coded values is shown in Table 3.5. All the experiments were performed in triplicates and average xylanase activity (R_1) was taken as dependent variable. The effects of variables to the response were analysed by using a second-order polynomial equation [Equation 2]:

$R_1 = \beta0 + \beta1A + \beta2B + \beta3C + \beta4D + \beta11A^2 + \beta22B^2 + \beta33C^2 + \beta44D^2$

$+ \beta12AB + \beta13AC + \beta14AD + \beta23BC + \beta24BD + \beta34CD + \beta34CE + \beta34DE$

Where R_1 = xylanase activity; β0 = intercept; β1, β2, β3 and β4 = linear coefficients; β11, β22, β33 and β44 = squared coefficients; β12, β13, β14, β23, β24, and β34 = interaction coefficients; A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD= levels of independent variables.

Table 3.4. Selected variables and their levels employed in CCD for optimisation of culture conditions affecting xylanase production by *Streptomyces* sp. ER1

Variable	Name	Units	Minimum (- 1)	0	Maximum (+ 1)	-α	+ α
A	Olive oil Concentration	mg/L	5	35	65	2.5	95
B	Xylan concentration	%	0.35	0.40	0.45	0.30	0.50
C	Agitation speed	rpm	42.5	57.5	72.5	27.50	87.50
D	Inoculum age	h	16.5	19.5	22.5	13.50	25.50

Table 3.5. CCD matrix for optimisation of variables influencing xylanase production by *Streptomyces* sp. ER1

Run	Variables			
Order	Olive oil Concentration (mg/L)	Xylan concentration (%)	Agitation speed (rpm)	Inoculum age (h)
1	-1	+1	-1	-1
2	0	0	0	0
3	0	0	0	0
4	+1	-1	-1	+1
5	+1	+1	-1	-1

6	+1	+1	+1	+1
7	-1	+1	+1	-1
8	-1	-1	+1	+1
9	+1	-1	+1	+1
10	+1	-1	-1	-1
11	-1	-1	-1	+1
12	-1	+1	+1	+1
13	-1	+1	-1	+1
14	+1	+1	+1	-1
15	-1	-1	+1	-1
16	+1	+1	-1	+1
17	0	0	0	0
18	0	0	0	0
19	+1	-1	+1	-1
20	-1	-1	-1	-1
21	0	- α	0	0
22	0	0	0	0
23	0	0	- α	0
24	- α	0	0	0
25	0	+ α	0	0
26	0	0	0	0
27	0	0	0	- α
28	0	0	0	+ α
29	0	0	+ α	0
30	+ α	0	0	0

The goodness of fit of the polynomial equation was expressed by R^2 and its statistical significance level was checked by F test. The desirability was kept at maximum. Statistical analysis of the data was performed by design package, Design Expert 7.0.0 to evaluate ANOVA, to determine the significance of each term in the equation fitted and to estimate the goodness

of fit in each case. The fitted polynomial equation was then expressed in the form of three dimensional (3D) response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. The combination of different optimised variables, which yielded the maximum response, was determined to verify the validity of the model. In order to verify the accuracy of the predicted model an experiment was conducted with initial and optimised media. The optimal concentrations of the critical variables were obtained by analysing 3D response surface plots

3.3.12 Validation of model

The design was validated by running the experiments at optimum values of the variables suggested by the software for maximum xylanase production to confirm the experimental value and predicted value of xylanase production.

3.3.13 Software used

The independent variables of the experimental design were optimised and interpreted using Design Expert Version 7.0.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA). ANOVA was used to validate statistical parameters. Fisher's F-test for ANOVA was performed on experimental data to evaluate the statistical significance of the model. The statistical significance of regression coefficients was evaluated using Students t-test. 3D response surface plots were drawn to illustrate the main and interactive effects of the independent variables on xylanase production. The optimum values of the selected variables were obtained by solving the regression equation and by analysing the response surface contour plots (Myers and Montgomery, 2002).

3.4 Results

3.4.1 Selection of production medium for xylanase production

In all, 11 different production media were examined to study their effect on xylanase production by *Streptomyces* sp. ER1. The production medium A was selected as the best xylanase production medium as it gave the highest xylanase activity (U/mL) compared to other media (Table 3.6).

Table 3.6 Xylanase production by *Streptomyces* sp. ER1 in different media

Production medium	Xylanase activity (U/mL)	Standard deviation
A (Techapun *et al.,* 1991)	**1277.33**	6.78
B (M9 medium), (Roy, 2004)	568.5	1.99
C (Mandel's and Sternburg's medium) (Mandels and Sternburg, 1976)	59.91	3.77
D (Raghunathan and Padma, 2013)	624.5	6.97
E (Kim *et al.,* 1985)	360.3	4.15
F (Suneetha *et al.,* 2011)	135.73	4.02
G (Liu *et al.,* 2012)	782.6	2.13
H (M medium) (Obi and Odibo, 1984)	50.49	3.21
I (Jayapradha and Mahesh, 2013)	539.7	1.92
J (Tryptic soy broth) (Liu *et al.,* 2012)	258.1	2.43
K (Nutrient broth with xylan) (Hiremath and Patil, 2011)	522.4	2.11

Hence medium A was selected for further optimisation for maximum xylanase production.

3.4.2 Effect of incubation period on biomass and xylanase production by *Streptomyces* sp. ER1

The biomass and rate of xylanase production increased with the increase in the fermentation period and reached its maximum at 120 h incubation (Fig 3.1). The total biomass declined and remained stationary after 120 h of incubation. Xylanase production also decreased after 120 h of incubation.

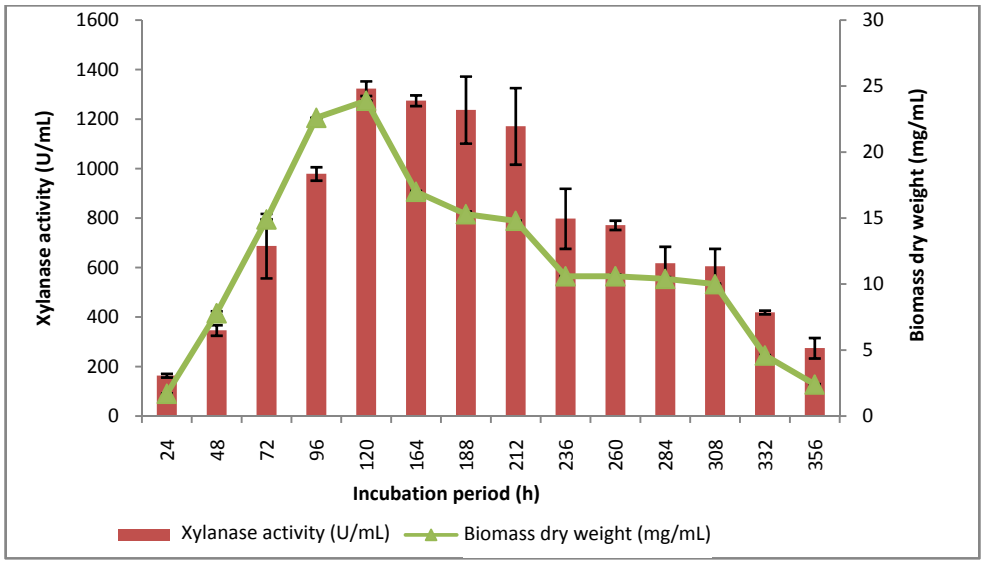


Fig 3.1. Effect of incubation period on biomass and xylanase production

3.4.3 Effect of temperature on biomass and xylanase production

Streptomyces sp. ER1 was grown in xylanase production medium A at temperatures ranging from 20-40°C with 5°C unit difference for 120 h. Optimal growth and xylanase production was detected at 35°C.

At growth temperatures of 20°C, 25°C and 30°C xylanase production was 9.14%, 29.15% and 36.56% respectively of that produced at 35°C (Fig 3.2). There was a decrease of 23.68% in xylanase production at 40°C when compared to that produced at 35°C. Maximum growth was also observed at 35°C.

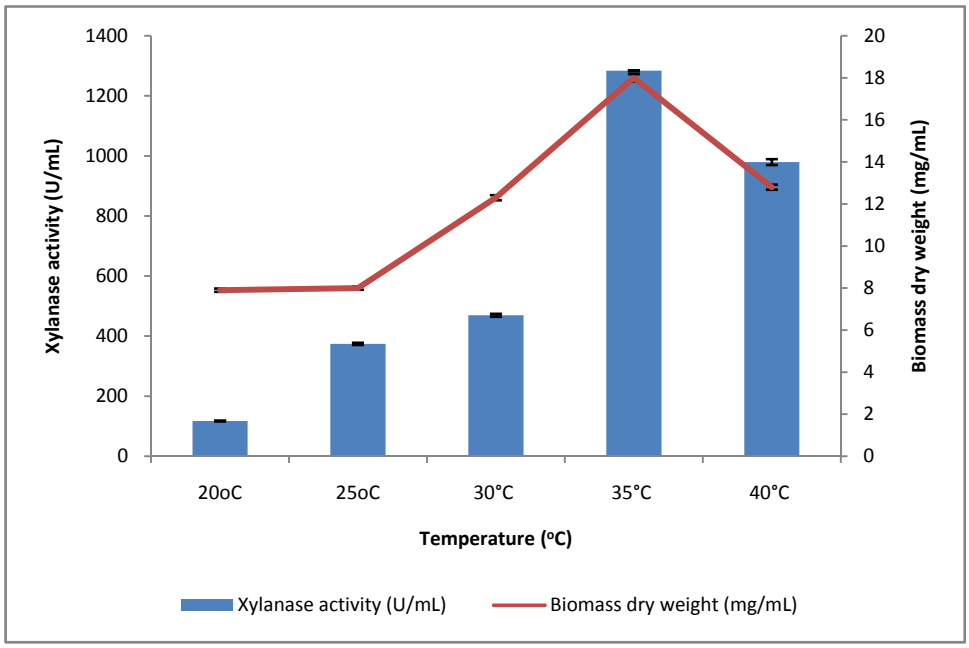


Fig 3.2 Effect of temperature (^{o}C) on biomass and xylanase production

3.4.4 Effect of pH on biomass and xylanase production

In order to study the effect of initial pH of culture (pH being uncontrolled during the fermentation process) on the production of xylanase, *Streptomyces* sp. ER 1 was grown in xylanase production medium A with different pH, ranging from pH 3 to 11 for 120 hours. The culture showed maximum xylanase production and growth at pH 7.0 (Fig 3.3).

The xylanase production at pH 6, pH 8 and pH 9 was 56.06%, 86.55% and 73.41% respectively of that produced at pH 7. At pH 10, there was a

decrease of 71.85% of xylanase production when compared with that produced at pH 7. However no growth and xylanase production was observed at pH 3 and pH 11 and very low growth and enzyme production was observed at pH below 5.

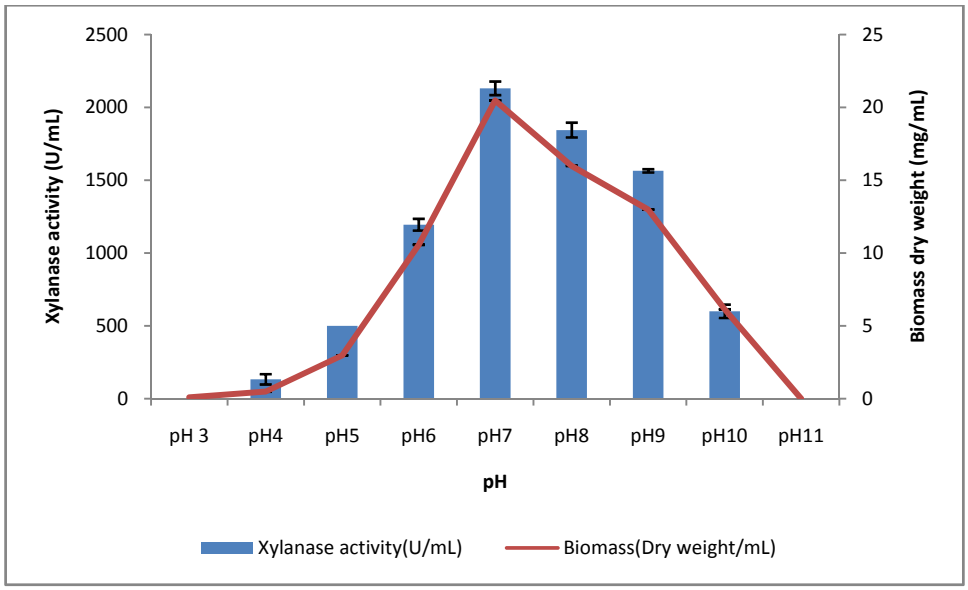


Fig 3.3 Effect of pH on biomass and xylanase production

3.4.5 Effect of age of inoculum and its concentration on biomass and xylanase production

Impact of age of inoculum on xylanase production was evaluated and the results are presented in Fig 3.4. It was found that inoculum age of 24 h supported maximal xylanase production. When inoculum of 6 h age was used, there was a decrease of 44.61% in xylanase production compared with that produced using 24 h old inoculum. Similarly, inoculum of age above 24 h did not support enhanced levels of xylanase production. Xylanase production of 1640 U/mL only could be obtained when 30 h inoculum was used.

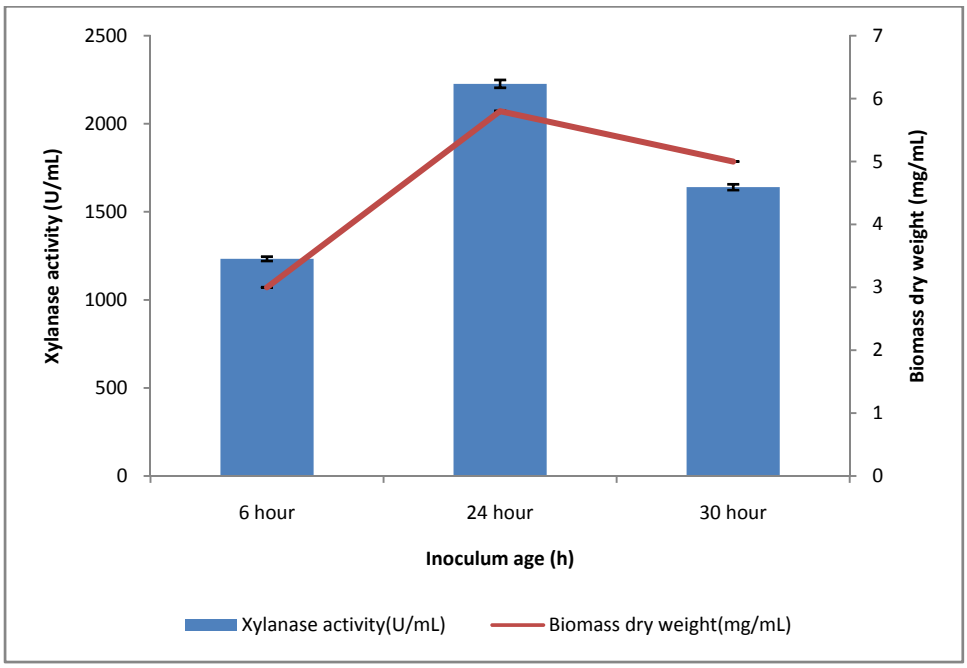


Fig 3.4. Effect of age of inoculum on biomass and xylanase production

It was observed that biomass production also showed exactly the same trend which was observed for the xylanase production with respect to different inoculum age.

The effect of inoculum concentration on the production of xylanase by *Streptomyces* sp. ER1 was studied at different levels (%) of 1,3,5,7,9,11 respectively. The cell count of each inoculum size is represented as colony forming units (CFU) (Table 3.7).

Biomass production increased from 1% to 9% but remained more or less similar at 9% and 11% concentration (Fig 3.5). At 1% inoculum concentration, lower levels of xylanase production resulted. Highest xylanase production was effected by 5% inoculum concentration which was closely followed by 7% (with 68.25% of xylanase production of that with 5% inoculum concentration) while 3% and 9% of inoculum concentration produced xylanase at 67.1% and 63% respectively of that produced with

5% inoculum. However at 11% of inoculum concentration, there was a decrease of 58.18% in xylanase production when compared to that with 5% of inoculum size.

Table 3.7 Cell count (CFU) in each inoculum size

Inoculum size (%)	CFU/ 0.1 mL
1	101
3	297
5	390
7	452
9	480
11	485

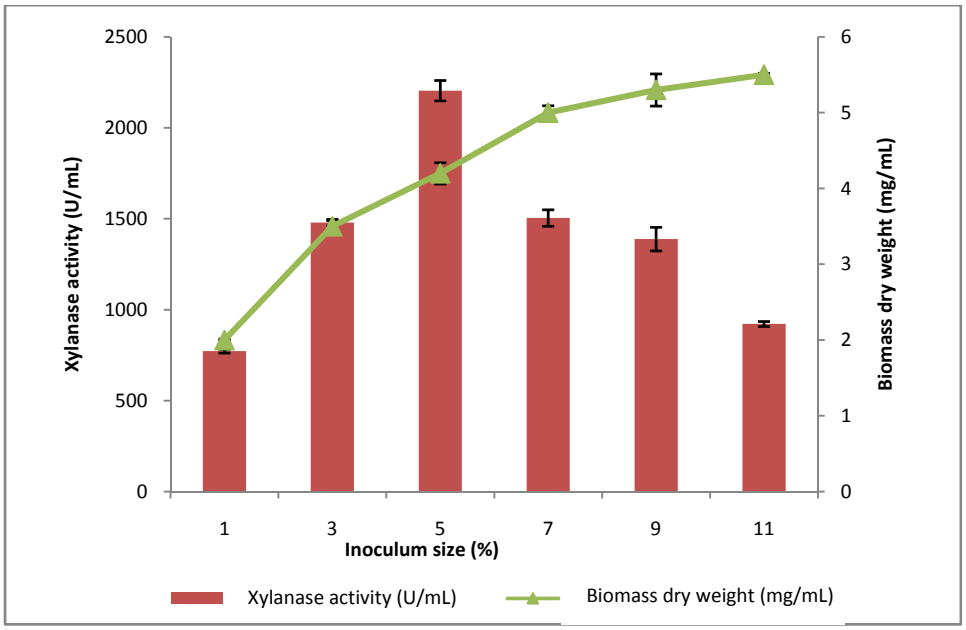


Fig 3.5 Effect of inoculum concentration (%) on biomass and xylanase production

3.4.6 Effect of agitation on biomass and xylanase production

Enzyme production by *Streptomyces* sp. ER1 was studied for growth under static and agitation (50,100 and 150 rpm) conditions. The results clearly indicated higher xylanase production under agitation (50 rpm) (Fig 3.6).

Considerable decrease in xylanase production was observed when culture was allowed to grow under stationary conditions as compared to the one kept on shaker at 50 rpm (Figure 3.6). However there was a decrease in xylanase production with increase in the agitation speed. At 100 and 150 rpm, there was a decrease of 9.27% and 14.18% in xylanase production, respectively when compared to that produced at 50 rpm. Biomass production was also high at 50 rpm and it reduced at higher agitation speed.

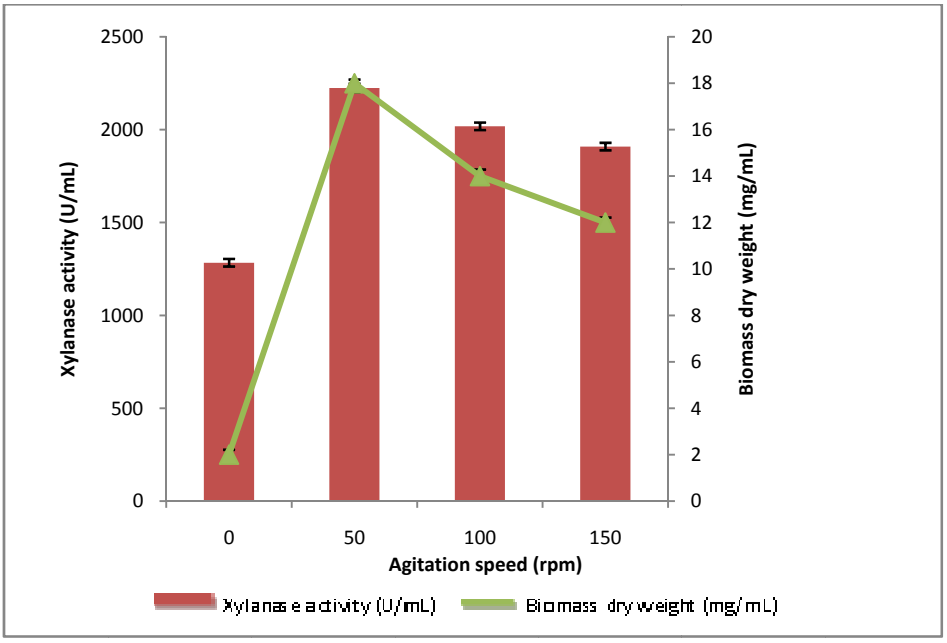


Fig 3.6 Effect of agitation on biomass and xylanase production

3.4.7 Effect of salinity on biomass and xylanase production

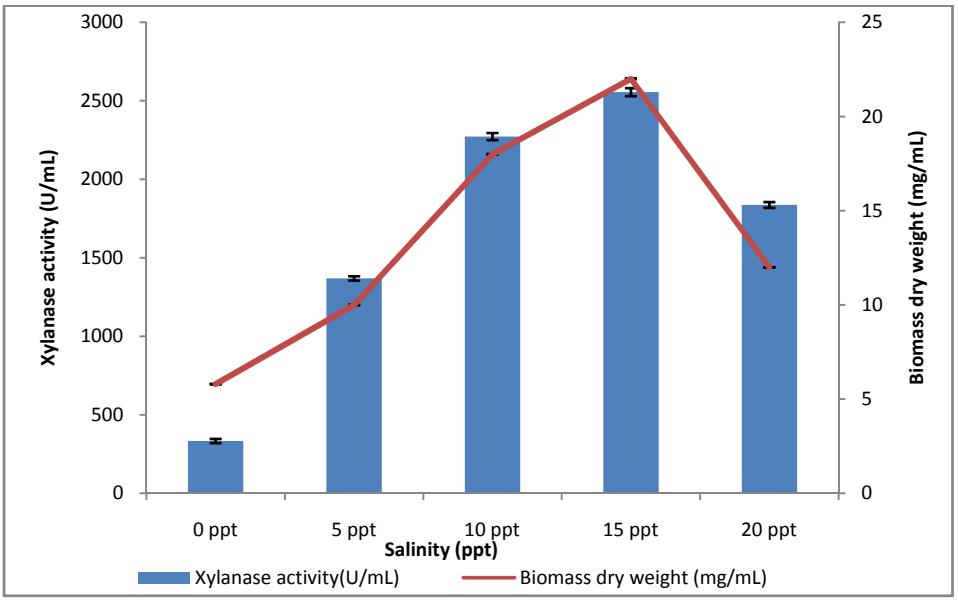


Fig 3.7 Effect of salinity (ppt) on biomass and xylanase production

From the data presented in Fig 3.7, it was found that *Streptomyces* sp. ER1 required 15 ppt salinity for maximum xylanase production. Nevertheless, 10ppt also supported considerable level of xylanase production of 88.91% compared to that at 15 ppt. However at 20 ppt, xylanase production reduced by 28.13%. Biomass production also showed exactly the same trend which was observed for that of xylanase with respect to different salinity.

3.4.8 Optimisation of Nutritional Parameters

3.4.8.1 Effect of Carbon source on Xylanase Production

The culture was grown with different carbon sources and the maximum xylanase production occurred in each case was compared with that of xylan, the most effective carbon source. Each carbon source had varying effects on the xylanase production by *Streptomyces* sp ER1. Results

shown in the Fig 3.8 indicated that xylan was the optimum carbon source inducing the highest level of xylanase production. The xylanase production with starch (4.05%) was much lower when compared to the xylan-containing medium. But glycerol and cellulose did not support the production of xylanase.

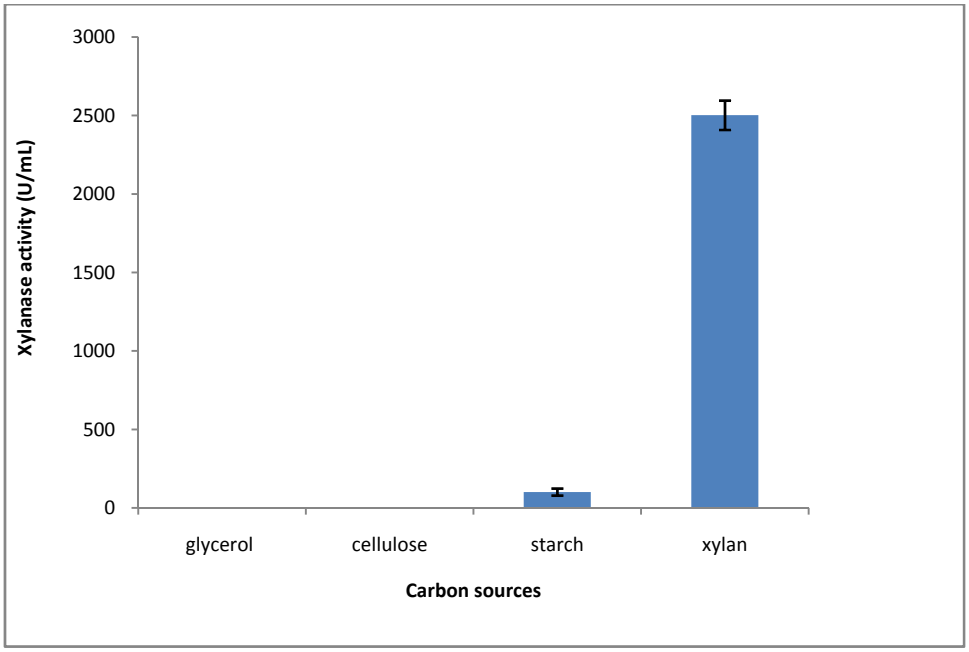


Fig 3.8 Effect of different carbon sources on xylanase production by *Streptomyces* sp. ER1

3.4.8.2 Effect of different surfactants and other additives on biomass and xylanase production

Impact of different surfactants and other additives in the fermentation medium on xylanase production was evaluated. From the results presented in Fig 3.9, it was found that among the 9 surfactants tested, addition of Triton X 100, β-mercatoethanol, EDTA and SDS to the production media resulted in inhibition of growth and xylanase production by *Streptomyces* sp. ER1. Incorporation of olive oil led to maximum xylanase production.

However, polyethylene glycol, Tween 60 and Tween 80 resulted in an increase of 19.87%, 14.31% and 2.69%, respectively in xylanase production compared with that of control. It was observed that biomass production showed the same trend with respect to different surfactants.

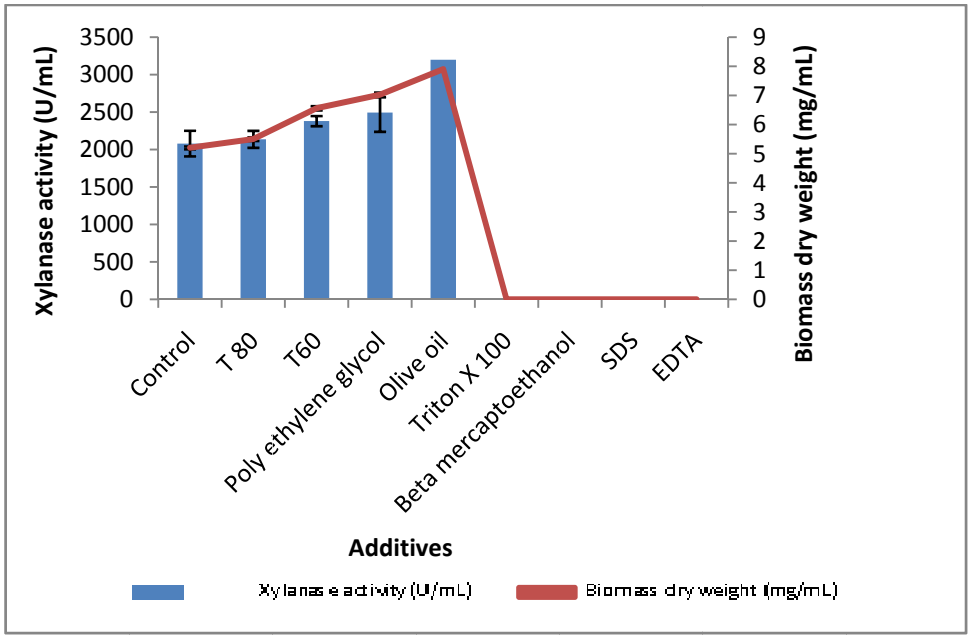


Fig 3.9 Effect of different surfactants and other additives on biomass and xylanase production

3.4.8.3 Effect of different metal ions on biomass and xylanase production

Highest xylanase production occurred with Ca^{2+} which was closely followed by Mn^{2+} ions (Fig 3.10). However, Cd^{2+} slightly reduced xylanase production by 2.76% compared to that of control. Hg^{2+} and Cu^{2+} ions drastically reduced xylanase production by 97.4% and 86.27% respectively. Biomass production recorded with different metal ions also showed exactly the same trend which was observed for the enzyme activity with respect to different metal ion sources.

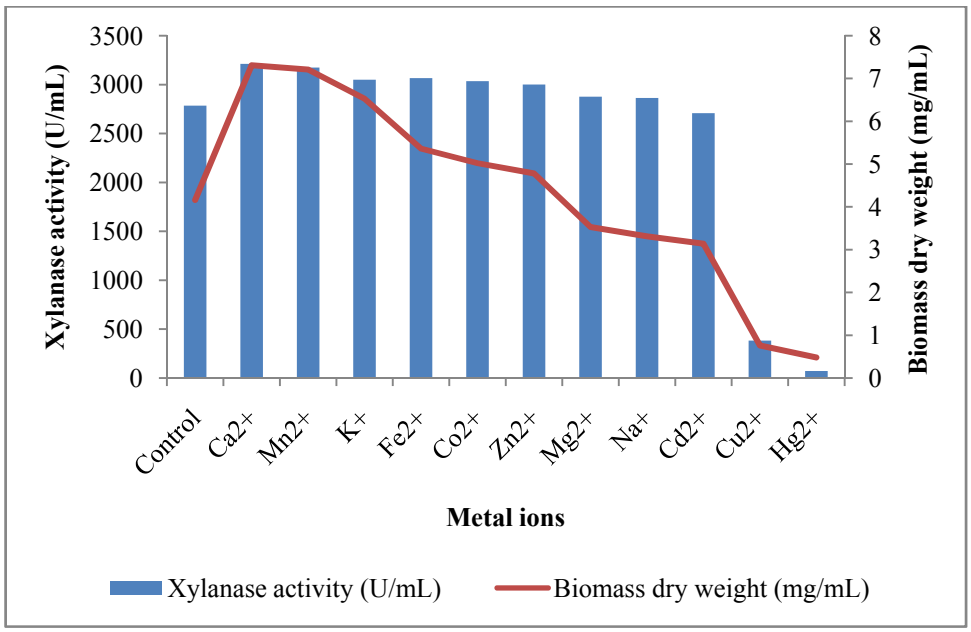


Fig 3.10 Effect of different metal ions on biomass and xylanase production

3.4.8.4 *Effect of different nitrogen sources on biomass and xylanase production*

3.4.8.4.1 Effect of inorganic nitrogen sources

Studies on the effect of inorganic nitrogen sources on xylanase production were carried out with different inorganic sources (Section 3.3.8.7.4). The results are given in Fig 3.11. The highest xylanase production was observed in medium with potassium nitrate followed by di ammonium phosphate and ammonium chloride. Other nitrogen sources, like ammonium sulphate, sodium nitrate and ammonium nitrate decreased xylanase production by 7.88%, 7.97% and 10.47%, respectively when compared to that of control (Fig 3.11). Biomass production recorded with different inorganic nitrogen sources also showed exactly the same trend which was observed for the enzyme production with respect to different inorganic nitrogen sources.

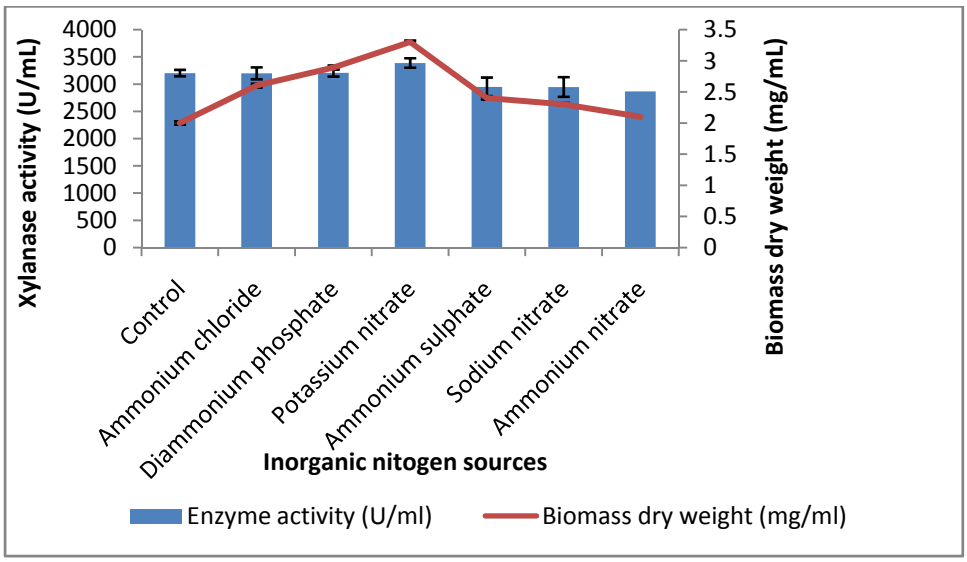


Fig 3.11 Effect of different inorganic nitrogen sources on biomass and xylanase production

3.4.8.4.2 Effect of organic nitrogen sources

Studies on the effect of organic nitrogen sources on xylanase production were carried out using various organic nitrogen sources, mentioned under section 3.3.8.7.4. The results are shown in Fig 3.12.

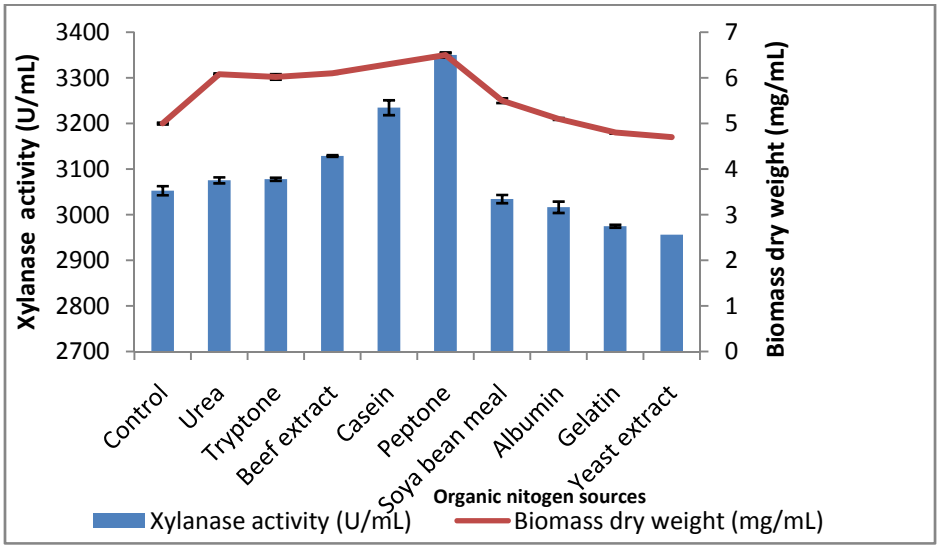


Fig 3.12 Effect of different organic nitrogen sources on biomass and xylanase production

The highest xylanase production was with peptone followed by casein, beef extract, tryptone and urea. However, xylanase production slightly reduced by 0.6% and 0.19% with soya bean meal and albumin, respectively, when compared to that of control. Xylanase production reduced further by 2.55% and 3.17% with gelatin and yeast extract respectively. Biomass production recorded with different organic nitrogen sources also showed exactly the same trend which was observed for the xylanase production with respect to different organic nitrogen sources.

3.4.8.5 Effect of different levels of xylan on biomass and xylanase production

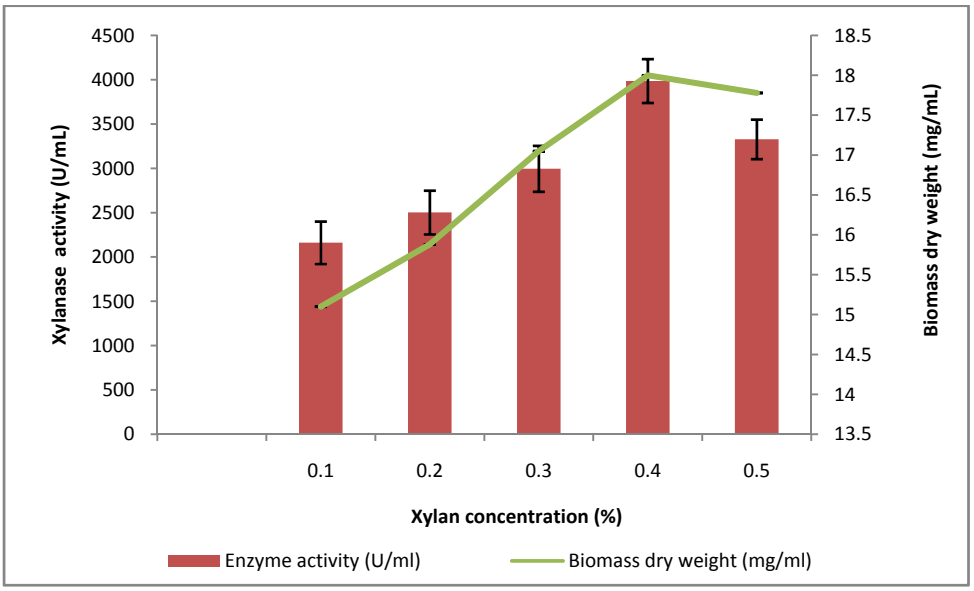


Fig 3.13 Effect of xylan concentration (%) on biomass and xylanase production

Different levels of xylan were clearly affecting the xylanase production from *Streptomyces* sp. ER1 (Fig 3.13). 0.4% xylan showed highest xylanase production. The xylanase production increased with increase in the xylan concentration. However, 0.5% xylan declined xylanase production by 16.51%. Biomass production also showed exactly

the same trend which was observed for the xylanase production with respect to different xylan concentration.

3.4.9 Effect of fermentation and nutritional parameters for xylanase production by *Streptomyces* sp. ER1 by PBD

PBD is a complete factorial design for screening experiments. It was carried out by using 20 experimental runs to identify the significant parameters affecting xylanase production.

Table 3.8 Plackett-Burman experimental design matrix for screening of factors affecting xylanase production by *Streptomyces* sp. ER1

Run	Factors														
	A	B	C	D	E	F	G	H	I	J	K	L	M	Actual value	Predicted value
1	+	+	+	+	-	+	-	+	-	-	-	-	-	1466.66	1987.46
2	+	+	-	+	-	+	-	_	-	-	+	+	+	6204.75	5958.86
3	-	+	-	-	-	-	+	+	-	+	+	-	+	1900.1	1987.46
4	-	-	+	+	-	+	+	-	-	+	+	+	-	421	4.2235
5	+	-	+	-	-	-	-	+	+	-	+	+	+	2601	3024.96
6	+	-	-	-	-	-	-	-	-	-	-	-	+	3040	3614.31
7	+	+	-	+	+	-	-	+	+	+	+	-	-	3440	3614.31
8	-	-	+	+	-	-	+	+	+	+	-	+	-	5893.33	5369.51
9	+	-	-	+	+	+	+	-	+	-	+	-	-	1813.33	1398.11
10	-	+	+	-	+	+	-	-	+	+	+	+	+	4506.66	4418.84
12	-	-	-	+	+	-	-	+	-	+	+	-	+	4700	3614.31
13	+	+	+	-	-	+	+	+	+	+	_	-	+	6440	5958.86
14	+	+	+	+	+	-	+	-	+	-	-	-	-	1173.33	1398.1
15	-	+	-	-	+	+	+	+	-	-	+	+	+	1904	1544.24
16	+	-	+	-	+	-	-	-	-	+	-	+	-	2493.33	2938.12
17	-	+	-	+	-	-	-	-	+	-	+	+	-	4893.33	5369.5
18	+	+	+	-	+	-	+	-	-	-	+	-	+	1086.63	954.88
19	-	-	+	+	+	+	-	+	-	-	-	+	+	1173.33	1544.24
20	-	-	-	-	+	+	-	+	+	-	+	-	+	3200	3975.62

The factors screened for its effect on xylanase production were A (incubation period), B (xylan concentration), C (pH), D (inoculum age), E (inoculum concentration), F (agitation speed), G (salinity), H (incubation temperature), I (concentration of olive oil), J (concentration of potassium nitrate), K (concentration of peptone), L (concentration of Mn^{2+}) and M (concentration of Ca^{2+}). The xylanase production varied between 421 to 6440 U/mL within the tested conditions as given by PBD. The PBD matrix along with the experimental results of predicted responses for the xylanase production is shown in Table 3.8. The experimental runs were performed in triplicates.

From the Table 3.9, the F value of 52.68 implied that the model is significant with variables concentration of xylan (B), inoculum age (D), agitation speed (F) and concentration of olive oil (I) as significant model terms. The model is significant as the p value of model is (0.0000000118) is less than 0.05 (5%). Further the p values of B, D, F, I variables were 0.0001, 0.027712, 0.001332, 0.00000000249, 0.0000373 respectively and were considered to have a significant effect on xylanase production. These 4 variables (B, D, F, I) were selected for further optimization using RSM. Remaining components had their p values greater than 0.5 and were considered insignificant in the present study. The R^2 for xylanase production was calculated as 0.93. The predicted R^2 value and the adjusted R^2 value were 0.88 and 0.91, respectively. Adequate Precision ratio of the model was 22.028 which indicated an adequate signal. This model was applicable to the design space. Table 3.9 represents the ANOVA results for PBD.

The corresponding response of the xylanase production was expressed in terms of the following regression equation (Equation1):

$$R_{1(\text{Xylanase activity})} = 10089.19 + 2946.765B - 95.0665D - 33.5637F - 13.9388I,$$

where, B is xylan concentration, D is inoculum age, F is agitation speed and I is olive oil concentration.

Table 3.9 Statistical analysis of components by PBD for xylanase production by *Streptomyces sp.* ER1

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	61594193	4	15398548	52.68084	0.0000000118	significant
B-xylan concentration	1736685	1	1736685	5.941471	0.027712	
D-inoculum age	4518820	1	4518820	15.45959	0.001332	
F-agitation speed	45624139	1	45624139	156.0873	0.00000000249	
I-olive oil concentration	9714549	1	9714549	33.23499	0.0000373	

The Pareto Chart is an important tool for analysing all the parameters and then to focus on the most significant parameters. It uses to separate the less significant parameters by identifying that parameter having the greatest cumulative effect on the system. It is shown by a series of bars in which impact of parameters is represented by heights. In the present analysis, agitation speed, inoculum age, xylan concentration and concentration of olive oil showed as the most significant factors affecting the xylanase production (Fig 3.14).

Fig 3.16-3.18 explained the negative influence of agitation speed, olive oil concentration and inoculum age on xylanase production and Fig 3.15 explained the positive influence of xylan concentration on xylanase production.

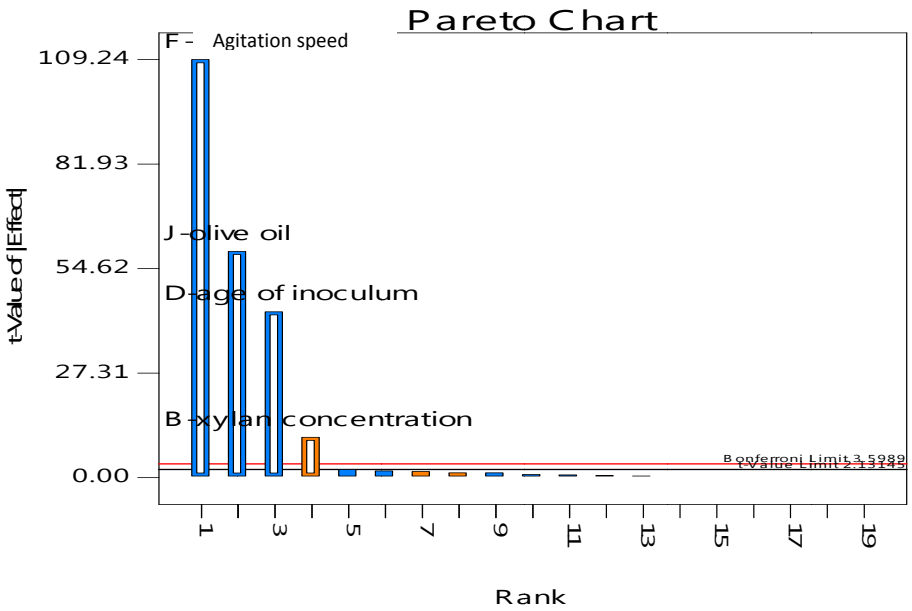


Fig 3.14. Pareto Chart showing the effect of media components on xylanase production

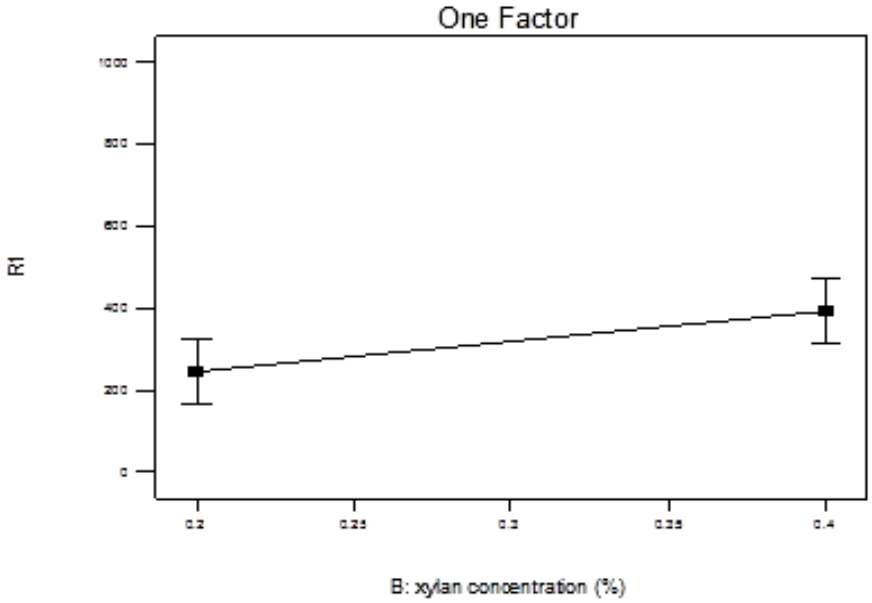


Fig 3.15. Graph showing interaction of xylan concentration (%) on xylanase production by *Streptomyces* sp. ER1.

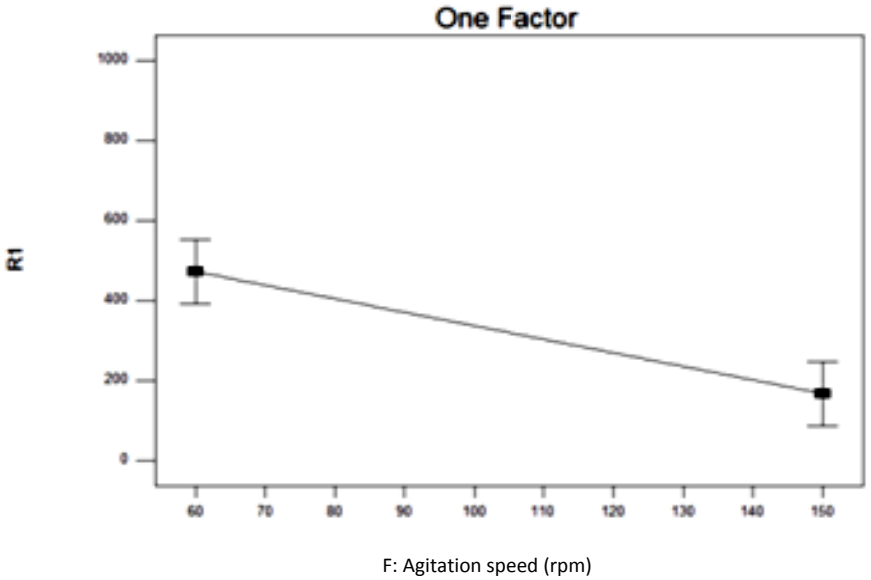


Fig 3.16. Graph showing interaction of agitation speed (rpm) on xylanase production by *Streptomyces* sp. ER1.

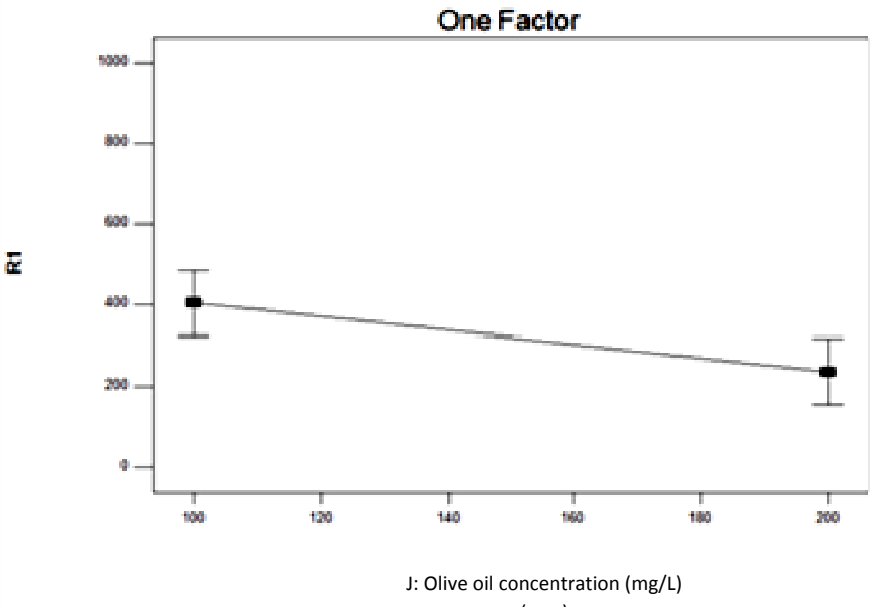


Fig 3.17. Graph showing interaction of olive oil concentration (mg/L) on xylanase production by *Streptomyces* sp. ER1.

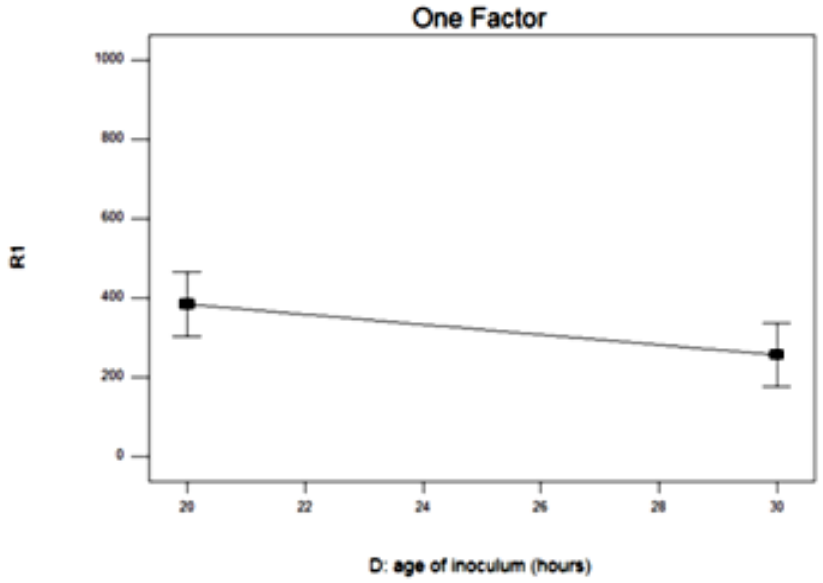


Fig 3.18. Graph showing interaction of inoculum age (h) on xylanase production.

3.4.10 Optimization using CCD

The experimental design performed using RSM is based on mathematical techniques that enabled us to investigate the interactions between variables of the medium components. The CCD was used to determine the optimal concentration (level) of the medium components. A total of 30 experimental run with 4 variables (significant factors as determined by PBD) and 5 coded levels (5 different concentrations) were performed. Based on the results obtained from the PBD, 4 variables were selected, viz. agitation speed, inoculum age, concentrations of xylan and olive oil. The other components of the production medium were found to be insignificant, and their concentrations were set as follows: KH_2PO_4 1.5 g/L, $CaCl_2$ 2 g/L, KNO_3 4.5 g/L, peptone 0.075 g/L, casein 0.075 g/L, manganese chloride 160 mg/L, inoculum size 5%, and temperature 35^oC, pH 7, salinity 15ppt and fermentation hours 120 h.

The CCD matrix along with the experimental results of predicted responses for the xylanase production is shown in Table 3.10. The experimental runs were performed in triplicates.

Table 3.10 CCD matrix for screening of variables affecting xylanase production by *Streptomyces* sp. ER1

Run	Variables				Actual Value	Predicted Value
Order	Olive oil concentration (mg/L)	Xylan concentration (%)	Agitation speed (rpm)	Inoculum age (h)		
1	-1	+1	-1	-1	5986.8	5005.87
2	0	0	0	0	7693.33	8583.14
3	0	0	0	0	7266.17	4267.14
4	+1	-1	-1	+1	8693.33	8583.14
5	+1	+1	-1	-1	8728.53	7848.03
6	+1	+1	+1	+1	2644.11	1622.54
7	-1	+1	+1	-1	8693.33	8583.14
8	-1	-1	+1	+1	8861.87	8771.09
9	+1	-1	+1	+1	5750.13	7826.98
10	+1	-1	-1	-1	4053.33	9081.39
11	-1	-1	-1	+1	9340	9399.71
12	-1	+1	+1	+1	3258.35	3319.80
13	-1	+1	-1	+1	3198.99	3277.85
14	+1	+1	+1	-1	9396.53	9438.78
15	-1	-1	+1	-1	6111.87	4624.54
16	+1	+1	-1	+1	4895.5	10531.30

17	0	0	0	0	7693.33	8583.14
18	0	0	0	0	7504.53	9524.24
19	+1	-1	+1	-1	3418.99	2354.79
20	-1	-1	-1	-1	3811.87	3874.27
21	0	- α	0	0	10546.7	9538.10
22	0	0	0	0	11226.7	11432.40
23	0	0	- α	0	12000	9898.37
24	- α	0	0	0	8106.67	8030.91
25	0	+ α	0	0	9426.7	10351.28
26	0	0	0	0	10120	10085.56
27	0	0	0	- α	7197.3	11432.40
28	0	0	0	+ α	6731.48	3411.75
29	0	0	+ α	0	12120.9	14430.43
30	+ α	0	0	0	7466.67	7431.86

3.4.11 Analysis of variance

The experimental values for the regression coefficient were obtained by quadratic polynomial equation, where only significant coefficients ($p < 0.05$) were considered. The smaller p-values indicated higher significance of the corresponding coefficient. Concentration of olive oil, xylan concentration and agitation speed were with a very low p value. The insignificant coefficients were not omitted from the equations, since it was a hierarchical model. The predicted response, R_1, the xylanase activity was obtained as follows (Equation 2):

R_1 (Xylanase activity) = -1.53810E+005+0.77808 (olive oil concentration) +2.98031E+005(xylan concentration) +60.91500 (agitation speed) +10581.17684 (inoculum age) +447.37625 (olive oil concentration)(xylan concentration) -1.35398(olive oil concentration) (agitation speed) - 2.54837(olive oil concentration) (inoculum age) +244.84750 (xylan concentration) (agitation speed) - 14190.62917 (xylan concentration) (inoculum age) - 14.79660 (agitation speed) (inoculum age) -0.80423 (olive oil)^2 -69521.04167 (xylan concentration)^2 + 190.46249 (agitation speed)^2 -103.26404 (inoculum age)^2

The statistical significance of the quadratic model for the experimental responses was evaluated by the ANOVA. According to the ANOVA results (Table 3.11), the model was significant with a F-test of a very low probability value < 0.0001. The goodness of fit for the model was expressed by the R^2 and the values were found to be 0.9924. The 'Adjusted R^2 of 0.9849 supported the model was significant. The Predicted R^2 was 0.9757. The lack of fit F-value (0.17) for the xylanase production was not significant relative to pure error. In the present study, the value of adequate precision ratio was higher (46.667) than desire value for xylanase production. The significance of each coefficient was determined by p-value, which is listed in Table 3.11.

Table 3.11. Statistical analysis of components by CCD for xylanase production by *Streptomyces* sp. ER 1

Source	Sum of		Mean	F	p-value	
	Squares	df	Square	Value	Prob > F	
Block	6.591E+007	1	6.591E+007			
Model	1.387E+008	14	9.908E+006	131.26	< 0.0001	Significant
A-olive oil concentration	3.657E+005	1	3.657E+005	4.84	0.0450	
B-xylan concentration	1.251E+006	1	1.251E+006	16.57	0.0011	
C- agitation speed	4575.25	1	4575.25	0.061	0.8091	
D-inoculum age	8.402E+005	1	8.402E+005	11.13	0.0049	
AB	7.205E+006	1	7.205E+006	95.45	< 0.0001	
AC	5.940E+006	1	5.940E+006	78.68	< 0.0001	
AD	8.416E+005	1	8.416E+005	11.15	0.0049	
BC	5.396E+005	1	5.396E+005	7.15	0.0182	
BD	7.249E+007	1	7.249E+007	960.34	< 0.0001	
CD	7.094E+006		7.094E+006	93.97	< 0.0001	
A^2	1.437E+007	1	1.437E+007	190.36	< 0.0001	
B^2	8.285E+005	1	8.285E+005	10.98	0.0051	
C^2	3.258E+006	1	3.258E+006	43.16	< 0.0001	
D^2	2.369E+007	1	2.369E+007	313.84	< 0.0001	
Residual	1.057E+006	14	75488.51			
Lack of Fit	3.212E+005	10	32118.72	0.17	0.9884	Not Significant
Pure Error	7.357E+005	4	1.839E+005			
Cor total	2.057E+008	29				

A lower value of the coefficient of variation (CV=3.71%) showed that the conducted experiments were precise and reliable.

3.4.12 Graphical interpretation of the response surface model

To investigate the interaction between 2 factors on xylanase production, RSM was used and 3D response plots were drawn between 2 factors keeping third factor at fixed level. The circular shape of the curve indicated no interaction while elliptical shape indicated good variation of two variables.

Fig 19 to Fig 23 represented 3D response surface graph showing the interactive effects of medium components on xylanase production. Each figure presented the effect of 2 variables on the production of xylanase, while other 2 variables were held at constant level. The 3D response surface plots and their respective contour plots illustrated the response over a region of interesting factor levels, the relationship between the response and experimental levels of each variable and the type of interactions between the test variables in order to deduce the optimal composition of the culture medium. In contrast to the circular shape contour plots, the elliptical nature of the curves indicated significant mutual interactions between variables.

Fig 19 described the behaviour of xylanase production, main effect, interaction effect, and squared effect (nonlinear) of olive oil and xylan at different concentrations with agitation speed and age of inoculum fixed at 57.50 rpm and 19.50 h respectively.

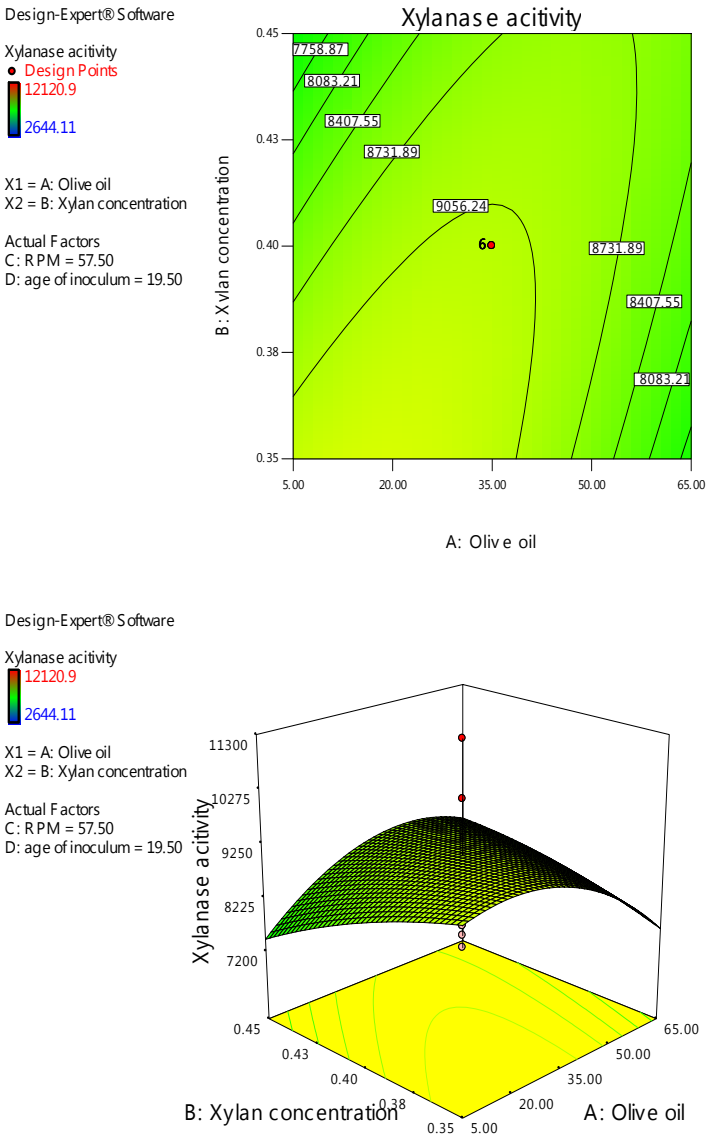


Fig 19. Contour and 3D graph representing the interaction between concentration of olive oil (mg/L) and xylan (%) on xylanase production by *Streptomyces* sp. ER1.

The 3D curve and contour plots of the calculated response surface from the interaction between olive oil concentration and agitation speed (rpm) while keeping fixed concentration of xylan and inoculum age at 0.4% and 19.5 h, respectively, are shown in Fig 20.

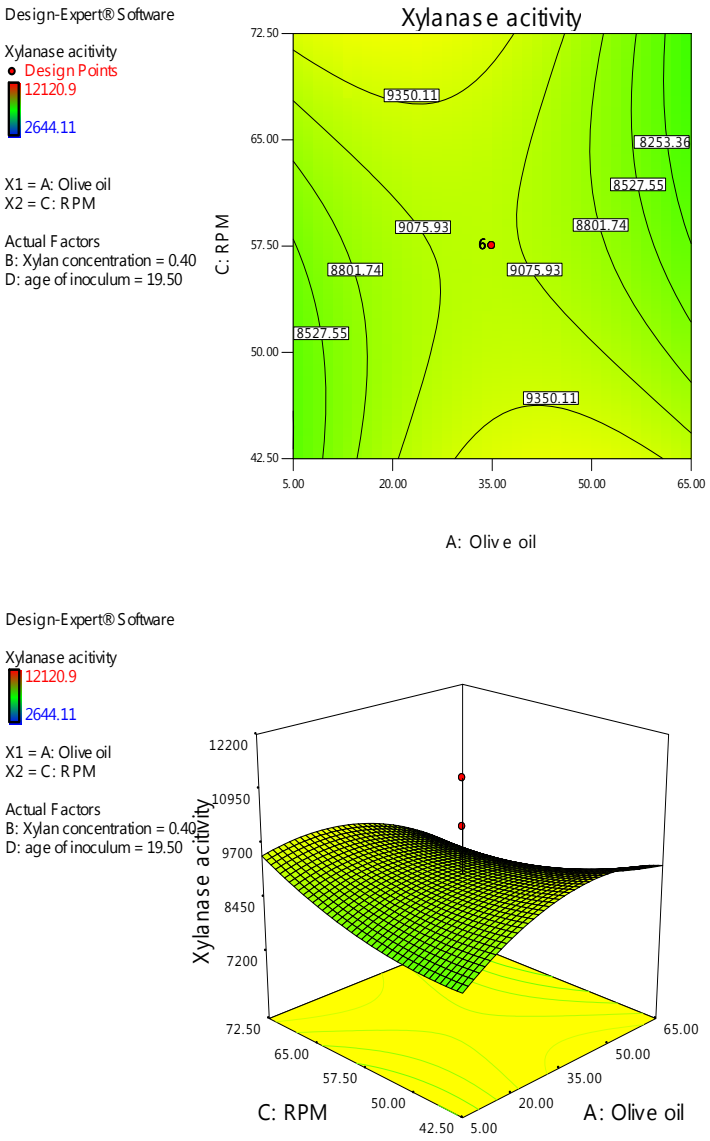


Fig 20. Contour and 3D graph representing the interaction between concentration of olive oil (mg/L) and agitation speed (rpm) on xylanase production by *Streptomyces* sp. ER1

The interaction plot between concentration of olive oil and inoculum age is shown in Fig 21, where the shape of the response surface indicated the interaction of olive oil concentration and inoculum age with the xylan concentration and agitation speed fixed at 0.4% and 57.5 rpm.

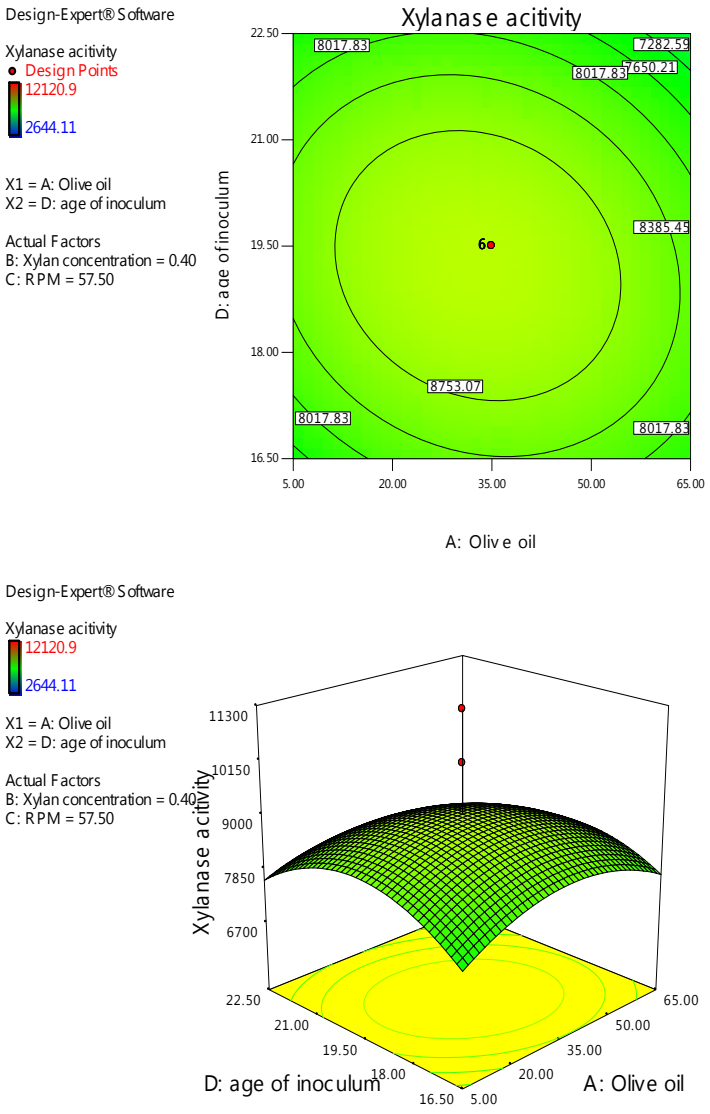


Fig 21. Contour and 3D graph representing the interaction between concentration of olive oil (mg/L) and inoculum age (h) on xylanase production by *Streptomyces* sp. ER1

The 3D curve and contour plots of the calculated response surface from the interaction between xylan concentration and agitation speed (rpm) while keeping fixed concentration of inoculum age and olive oil at 19.5 h and 35.0 mg are shown in Fig 22.

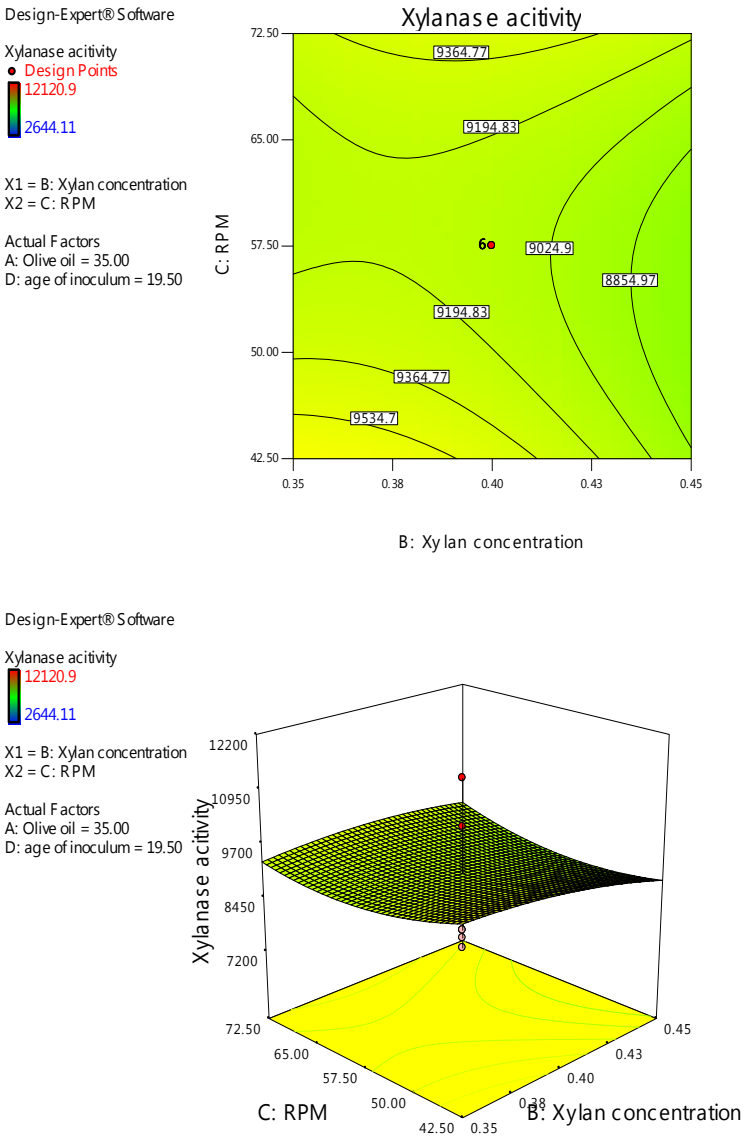


Fig 22. Contour and 3D graph representing the interaction between xylan concentration (%) and agitation speed (rpm) on xylanase production by *Streptomyces* sp. ER1

3D response plot shown in Fig 23 described the behaviour of xylanase production, main effect, interaction effect, and squared effect (nonlinear) of inoculum age and xylan at different concentrations.

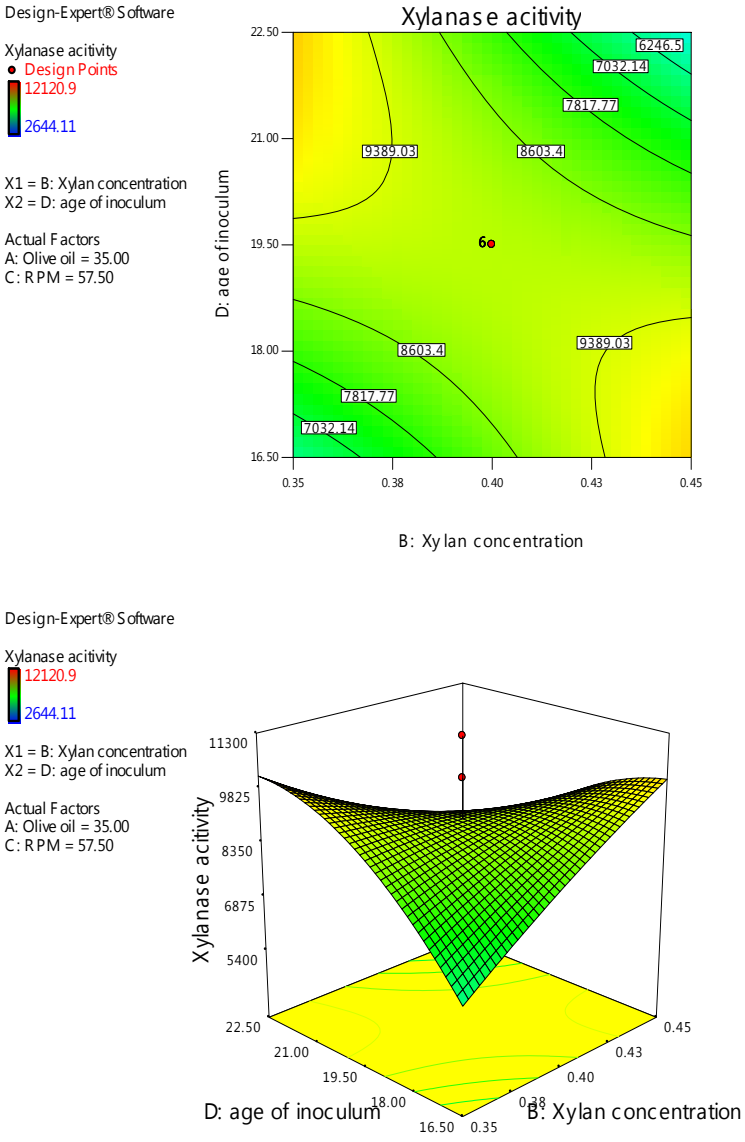


Fig 23. Contour and 3D graph representing the interaction between xylan concentration (%) and inoculum age (h) on xylanase production by *Streptomyces* sp. ER1.

The 3D curve and contour plots of the calculated response surface from the interaction between inoculum age and agitation speed (rpm) while keeping fixed concentration of xylan concentration and olive oil concentration at 0.4% and 35.0 mg are shown in Fig 24.

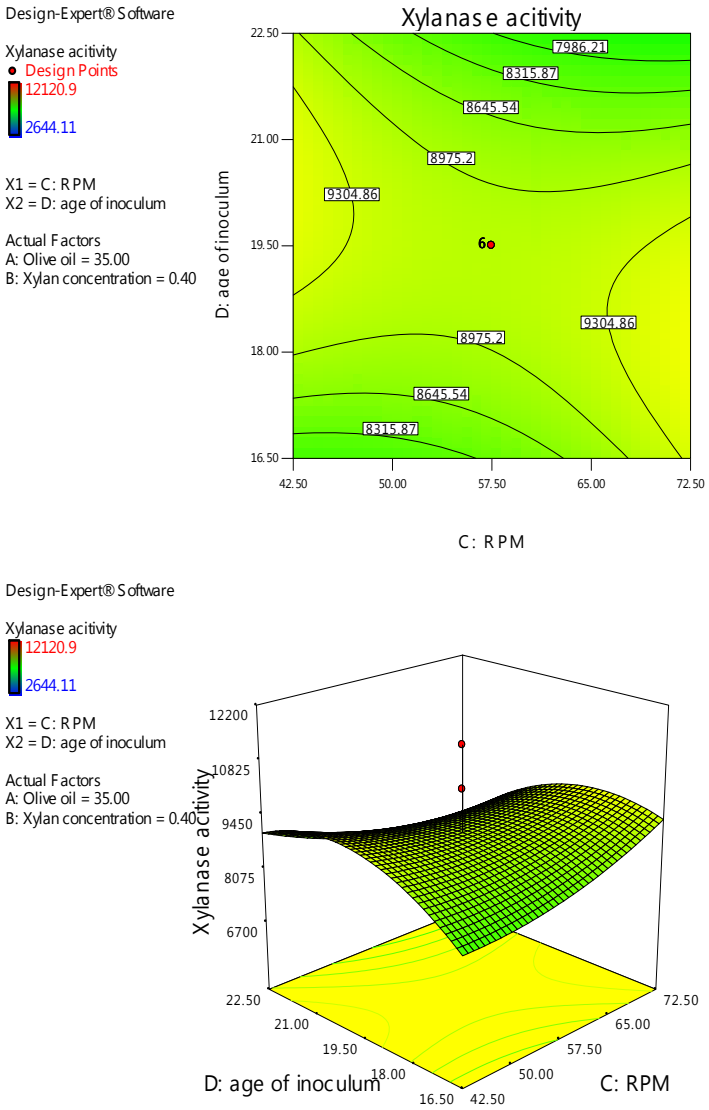


Fig 24. Contour and 3D graph representing the interaction between agitation speed (rpm) and inoculum age (h) on xylanase production by *Streptomyces* sp. ER1

3.4.13 Comparison of observed and predicted xylanase activity

Using the second-order regression equation, the model predicted the response (xylanase activity) corresponding to particular values of the regressor variables. The plot for the observed xylanase activity (the response) versus model predicted xylanase activity shows that these are very close with each other. The parity plot showed a satisfactory correlation between experimental values and predicted values (Fig 25), wherein the points cluster around the diagonal, which indicated the good fit of the model, since the deviation between the experimental and predicted values were small. We can then conclude that the second order model is adequate to describe the response surface and can be used as a prediction equation in the studied domain.

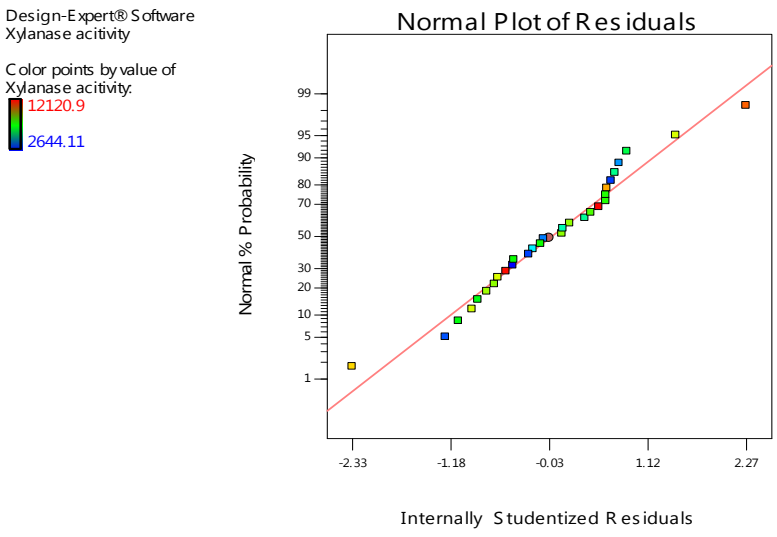


Fig 25. Parity plot showing the distribution of experimental vs predicted values of xylanase production by *Streptomyces* sp. ER1

In general, it is necessary to compare the fitted model with the real system and confirm that the fitted model provided an adequate approximation. Unless the model showed an adequate fit, proceeding further

with a fitted response surface may end in misleading results. The residuals from the least squares fit played an important role in judging the model adequacy. Fig 26 presents a plot of residuals versus the predicted response.

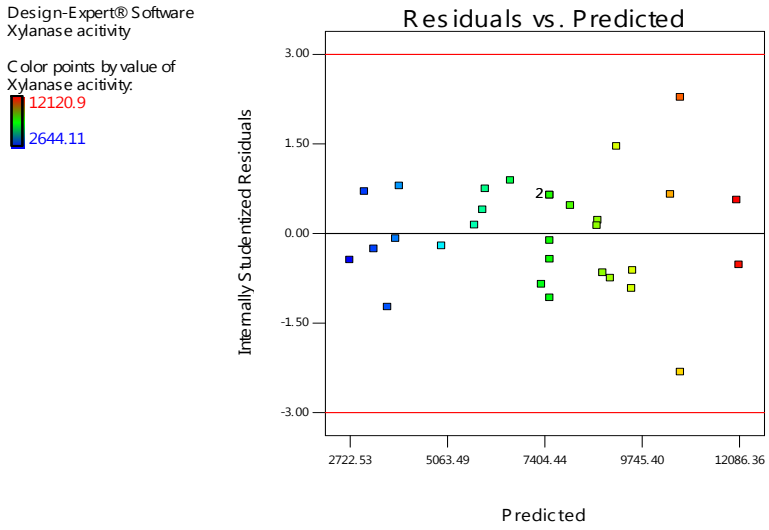


Fig 26. Plot showing the interaction between the actual and predicted values.

The plot showed that the residuals scattered randomly on the display, suggesting that the variance of the original observation was constant for all values of xylanase enzyme activity (R_1). Because the plot of xylanase production was satisfactory, the conclusion could be drawn that the empirical model was adequate to describe the xylanase activity by response surface.

The model predicted the optimum concentrations of olive oil, xylan, agitation speed and inoculum age were 33.10 mg/L, 0.37%, 43 rpm and 21.5 h, respectively, and maximum response 10292 U/ml. Validation studies were performed in triplicates for further confirmation as suggested by the model. The condition led to xylanase production of 10547 U/ml., which was

comparable to the model predicted data (10292 U/ml). It was clear from these results that the model was useful to predict the xylanase yield as well as the optimisation of experimental fermentation conditions. Hence, the CCD based RSM models were considered to be accurate and reliable for predicting the production of xylanase by *Streptomyces* sp. ER1.

The optimised conditions revealed the response values close to the predicted one, indicating the validation strategy towards xylanase production. The reasonable accepted values of R^2 defined the true behaviour of the statistical system that can be used for interpolation in the experimental domain.

3.5 Discussion

Improved industrial fermentation could be ascribed to the development of superior strains by genetic manipulation. However, other parameters like nutritional and physical environments to which an organism was exposed were also known to influence significantly the yield (Greasham and Inamine, 1986). There has been lot of emphasis given to purification, characterisation and elucidation of the xylanase genes. However, not much importance has been given for the optimisation of cultural and nutritional conditions for the production of xylanase from bacteria including *Streptomyces* sp. which could have influenced the production (Nakamura *et al.,* 1993). The physical and cultural parameters studied included incubation period, pH, temperature, inoculum age and concentration, agitation and salinity, carbon and nitrogen sources, metal ions and additives which have affected xylanase production by *Streptomyces* sp. ER1.

3.5.1 Effect of incubation period on biomass and xylanase production

The culture was grown in production medium A containing xylan as the carbon source. The xylanase production was studied in association with growth. The xylanase production was initiated after 24 h of incubation and during the logarithmic phase of growth, the extracellular xylanases production was high and reached the peak at 120 h. However, the culture growth attained stationary phase by 164 h, there was still an increase in enzyme production to the end of stationary phase. After this period, the xylanase production decreased. The observed peaking and decrease of the production of xylanase production could be attributed to: (1) The differences in the timing of induction of separate components of the xylanase system; (2) The products of action of one component inducing the synthesis of another; (3) Differential reticence by products of substrate hydrolysis; (4) Degree of difference in inactivation by proteases, or variation in the pH during cultivation conditions (Tuohy and Coughlan, 1992; Wang *et al.,* 1993). Presence of xylanase activity in the early hours of growth was presumed to be due to the xylanases present in considerable amounts in the inoculum causing the hydrolysis of xylan in the medium. The production of xylanase increased during the growth phase of the cultures after 24 h. The higher xylanase activity of *Streptomyces* sp. ER 1 during later stages of growth (from 164 h) could be the reflection of small amounts of xylanase liberated from cells undergoing autolysis as well as the non availability of insoluble particles in the medium which if present might have bound the xylanases (Femandez-Espinar *et al.,* 1992; Subramaniyan *et al.,* 1997; Connerton *et al.,* 1999). After 212 h of growth there was a gradual decline in xylanase activity suggesting the possible action of intracellular proteases from the autolysed cells. It may also be due to the depletion of nutrients in the medium which stressed the actinomycete physiology resulting in the

inactivation of secretary machinery of the enzymes or due to denaturation or decomposition of xylanase by interaction with other components in the medium (Nochure *et al.,* 1993; Kamble and Jadhav, 2012a). These results indicated that the production of extracellular enzymes by *Streptomyces* sp. ER1 was growth associated and this was in agreement with other actinomycetes (Ball and McCarthy, 1989; Trigo and Ball, 1994; Tuncer, 1999).

Incubation time depended on the characteristics of the culture, growth rate and enzyme production. *Streptomyces* sp. ER1 with maximum xylanase production of 1323.47 U/mL at 120 h of growth was found to be a potent producer of xylanases than most of the reported cases from *Streptomyces* sp. The highest xylanase production obtained was 1653 U/mL from *Streptomyces* sp. followed by 255 U/mL from *Streptomyces* sp. CA24 (Ding *et al.,* 2004; Porsuk *et al.,* 2013).

Optimum xylanase production at 120 h of incubation was also reported earlier (Kansoh *et al.,* 2001; Bakri *et al.,* 2008; Sarkar and Aikat, 2014). Thus the production of xylanases by *Streptomyces* sp. ER1 was comparable with the earlier reports. The production could be further increased by the optimisation of fermentation conditions.

3.5.2 Effect of incubation temperature on xylanase and biomass production

Streptomyces sp. ER1 was grown in production medium A at temperatures ranging from 20-40°C. Optimal production of xylanase and biomass was detected at 35°C. A rise in growth temperature to 40°C lowered xylanase production by 23.68% when compared to that occurred at optimal temperature. *Streptomyces* sp. ER 1 could grow at temperatures near

40° C and it indicated that the organism could be classified under thermo-tolerant bacteria (Dubeau *et al.,* 1987).

In concordance with our study, optimal xylanase production at 35^{o}C by *Streptomyces* sp. F2621 has been documented, while another study reported 32^{o}C as optimum with significant reduction of over 50% xylanase productivity by *S. halstedii* at 45^{o}C (Tuncer *et al.,* 2004; Abdelwahed *et al.,* 2011). Likewise, *Streptomyces chartreusis* strain L1105 produced xylanase which increased with increasing temperature from 30 to 40°C and decreased from 40°C (Li *et al*., 2011).

Incubation temperature is supreme for optimal enzyme production owing to alterations in microbial protein structure and properties with temperature variations (Juturu and Wu, 2014). At temperatures below or above the optimum, metabolic activities were abridged, with consequential inhibition in growth and enzymes synthesis (Ray *et al.,* 2007). While some studies proposed higher xylanase production with increased temperature, others have suggested otherwise (Abdelwahed *et al.,* 2011; Thanongsak *et al.,* 2011). The variations between the present study finding and the previous report may be because of the source of the isolation and kind of bacterial strain.

3.5.3 Effect of pH on xylanase and biomass production

The pH of the fermentation medium influenced the growth of microbial strains and consequent metabolic product formation (Okaiyeto *et al.,* 2016). Many enzymatic processes and transfer of various components across the cell membrane are strongly affected by the pH of medium (Kapoor *et al.,* 2008). An optimum pH was required to maintain the 3D shape of the active site of and the change in pH resulted in a loss of

functional shape of the enzyme due to alterations in the ionic bonding of the enzyme (Kaseke *et al.,* 2016).

In order to study the effect of initial pH of medium on the formation of xylanase, *Streptomyces* sp. ER1 was grown in production medium A with pH ranges - pH 3 to 11.The culture showed maximum xylanase production and growth at pH 7 (Fig 3.3). Higher production of xylanase was noticed when the pH of the medium was in the range of 7-9. Similar findings were reported earlier (Bajaj *et al.,* 2010 and Bhosale *et al.,* 2011). However, *Streptomyces* sp. CA24 produced xylanase over a broad pH range of 6–10 with its optimum at pH 10 (Porsuk *et al.,* 2013).

In the present study, *Streptomyces* sp. ER1 was found to be alkali tolerant and could grow at high alkaline conditions and produced xylanase. Xylanase production retarded below pH 5 and above pH 10 of the medium. Alteration in pH tolerance observed during xylanase production might be due to different enzyme mixtures secreted and/or the post-translational alterations in the xylanase secretion procedure; for example, glycosylation enhancing reliability in extreme pH and temperature conditions (Park *et al.,* 2002; Sa- Pereira *et al.*, 2002).

In the present study, there was no growth and xylanase production at pH 3 and 11. Cultivation of the organism at an unfavourable pH may limit the growth, xylanase production and substrate accessibility (Bajpai, 1997).

3.5.4 Effect of age of inoculum and its concentration on biomass and xylanase production

The effect of inoculum age was studied by measuring the xylanase production after inoculating 100 mL of the production medium with 5 % (v/v) of 6 h, 24 h and 30 h old inoculum. The highest xylanase production was recorded when 24 h old inoculum was used. This could be due to the

logarithmic phase of *Streptomyces* sp. ER1, used as inoculum. However, only 55.4% of xylanase was produced with 6 h old inoculum when compared to that with 24 h old inoculum. With increase in the inoculum age to 30 h, the xylanase production reduced by 26.35% of xylanase production obtained with that of 24 h old inoculum.

For maximum growth and xylanase production, the inoculum should be from the logarithmic stage of growth. This is because the age of inoculum of a sporulating organism like *Streptomyces* sp. ER1 is an important factor as it would have resulted in the transfer of high quantities of spores if transferred after metabolically active stages (i.e. during the stationary phase or death phase). Presence of higher percentage of spores in the inoculum has been reported to result in the long lag phase of the fermentation profile (Meyarth and Suchanek, 1972).

Inoculum size played an important role in achieving maximum biomass and xylanase production by *Streptomyces* sp. ER1 in a shake flask system. Generally, inoculum size affected the morphology of the bacteria as well as their series through growth phases. Therefore, it is important to select a suitable inoculum size with respect to the type and age of microorganism to obtain a high yield of the desired product (El-Enshasy *et al*., 2000). The fermentation profile of an organism is usually affected by the size of the inoculum and its physiological conditions (Meyrath and Suchanek, 1972). In the present study, in order to minimise the time lag in fermentation process, inoculum was raised in the same medium with a composition similar to that of fermentation medium. However, it resulted in the transfer of small quantities of xylanases synthesised in the xylan containing seed culture causing an initial increase in reducing sugar

concentration of the fermentation medium. Similar observation was made by Subramaniyan *et al.* (1997).

The effect of inoculum concentration on the production of xylanases by *Streptomyces* sp. ER1 was studied at levels of 1%, 3%, 5%, 7% 9% and 11% respectively.

The biomass production increased with the concentrations of the inoculum. In the present study, it was noticed that 1% and 3% of inoculum concentration could not support higher xylanase production which could be due to low cell density. An optimum inoculum level was necessary for maintaining the balance between proliferating biomass and accessible nutrients to obtain maximum enzyme yield. A lower xylanase yield at higher inoculum level of 7%, 9% and 11% was observed. With subsequent increase in inoculum size, competition for carbon source increased and resulted in rapid depletion of macro and micro nutrients, inhibiting their growth and enzyme production (Omojasola *et al.,* 2008). Increased level of inoculum mostly condensed xylanase production in industrial fermentation process (Battan *et al.*, 2007). Further, a large inoculum size could lead to formation of thick suspension and hence improper mixing of substrates (Kuhad *et al.,* 1998).

Higher enzyme production at inoculum size of 5% was related to the rapid growth of *Streptomyces* sp. ER1, which resulted in higher degradation of substrates and increased availability of nutrients (Kavya and Padmavathi, 2009). The optimum inoculum size in a fermentation process should be identified because lower inoculum density might result in inadequate biomass. However, higher inoculum density might produce too much biomass, which would deplete the nutrients necessary for product formation (Sangeetha *et al*., 2004). This might be the reason for lesser

xylanase production, in the present study, at inoculum concentration above 5% even though the biomass production increased from 1% to 11%.

Several researchers have reported the use of 1.0–5.0% (v/v) inoculum for hyperproduction of xylanase (Subramaniyan *et al.,* 2001; Kar *et al.,* 2006; Battan *et al.,* 2007). In contrast to the present results, xylanase production by a newly isolated *Streptomyces* sp. RCK-2010 was found to be highest with inoculum size of 1.0% (v/v) (Kumar *et al.*, 2012).

3.5.5 Effect of agitation on biomass and xylanase production

The process of agitation and aeration was generally used to meet the demand of oxygen during fermentation process. An important aspect of aeration in the fermentation process was the resistance offered to the transfer of dissolved oxygen through the medium into the microbial cell, which was generally overcome by increased agitation (Rani, 2013). The transfer of oxygen from air to solution formed the second factor; which could be solved by modelling the working volume ratios. Providing large area of gas-liquid inter-phase could increase the rate of formation of dissolved oxygen in the fermentation broth. A number of means for obtaining large surface area have been tried which included: (i) thin layer cultures (maintenance of restricted volume of liquid in the fermentation vessel), (ii) agitation of the liquid by shaking (reciprocal or rotary shakers) (iii) forced aeration of a liquid with air under pressure through a gas distributor. Of the above, former two could be utilised for a batch experiment in shake flasks. Agitation has been reported to affect the level of aeration and mixing of the nutrients, mass and heat transfer in the fermentation medium (Ibrahim *et al.,* 2015). Agitation was crucial for maximum production in microbial enzyme fermentation (Jang and Chang, 2005).

In the present study, the maximum xylanase production was obtained at 50 rpm. By further increasing the agitation speed to 150 rpm, the biomass remained dispersed as small pellets. An important advantage of the pelleted morphology was a significant decrease in viscous appearance of the fermentation broth, which could result in improved mixing and mass transfer characteristics (Biswas *et al.,* 2010). But there was a slight reduction in the xylanase production at 150 rpm. This was probably because of the deleterious effect of high shear stress on the actinomycete, which caused cell lyses and release of proteases that could inhibit xylanase production (Fang *et al.,* 2007). The culture morphology was critical for product formation (Papagianni, 2004). A close relationship between a particular morphological form and augmented process productivity has been found to be the characteristic of a number of industrially important fermentations. The appropriate size of the pellet of the actinomycete was also significant for the productivity of the process. The combination of an active biomass with a favourable pellet size played an important role in higher product formation. Pellets with a more compact surface structure displayed a larger diffusion barrier for a substrate than pellets with a rather open peripheral structure (Li *et al.,* 2007).

In support to the present results, the hairy surface of the pellets of *M. albomyces* IITD3A at 600 rpm seemed to support the diffusion of the substrate to the core of the pellet resulting in higher synthesis of xylanase. On increasing the agitation speed, there was a decrease in product formation due to breakage of exposed hyphae under mechanical stress. (Biswas *et al.,* 2010) Such a phenomenon was also observed in *Streptomyces* sp. Ab106, where xylanase production was unfavourably affected when agitation rate was increased beyond 250 rpm as it led to shearing of cells (Techapun *et al.,* 2002).

3.5.6 Effect of salinity on biomass and xylanase production

Salinity plays an important role in the growth and metabolism of microorganisms. In the present study, maximum xylanase production and biomass was obtained with the production medium of 15 ppt showing the halophillic character of *Streptomyces* sp. ER1. However, xylanase production decreased at 20 ppt, compared to that at 15 ppt. This might be due to the osmotic stress caused by NaCl (Kaur *et al.,* 2015). Similar results were observed with *B. subtilis* BS04 with maximum xylanase production at 0.2% NaCl concentration and further increased concentration resulted decline in enzyme production (Irfan *et al.,* 2016).

3.5.7 Effect of carbon sources on xylanase production

The rate of assimilation of a carbon source could often influence the formation of biomass and production of primary or secondary metabolites. Rapid growth resulted from high concentrations of readily metabolised sugars were often associated with low productivity of metabolites (NPCS Board of Consultants & Engineers, 2011). In the present study, various carbon sources were screened for their suitability to promote culture growth and high xylanase production. They had varying effects on the xylanase production by *Streptomyces* sp. ER1.

Results indicated that xylan was the optimum carbon source inducing the highest level of xylanase production. Similar results were obtained in earlier studies (Li *et al.,* 2011; Nour El Dhein, 2014; Ho and Heng, 2015). In the present study, starch produced 4.05% of xylanase indicating it is a poor inducer of xylanase. Similar results were obtained with *Fusarium solani* and *Streptomyces* sp. CA24 (Gupta *et al.,* 2009; Porsuk *et al.,* 2013). Cellulose and glycerol also failed to induce xylanase

production by *Streptomyces* sp. ER1. Similar findings were observed by several workers (Joshi and Kumar, 2012; Ho and Hood, 2014).

3.5.8 Effect of different surfactants and other additives on xylanase and biomass production

The presence of an appropriate additive was of great importance for the enhanced production of xylanases. The additive not only served as carbon and energy source, but also provided the necessary inducing compounds for the organisms, preferably for an extended period of time, which could result in an increased overall productivity of the fermentation process (Peng *et al.,* 2006; Xu *et al.,* 2008; Bajaj and Singh, 2010). The stimulatory effect of these additives resulted from efficient spore dispersion, rheological properties of the medium, availability of nutrients and oxygen and physiological functions of the cells (Chen, 1996).

In the present study, addition of olive oil and polyethylene glycol, Tween 60 and Tween 80 individually to the basal medium stimulated the xylanase production. These results were in line with earlier reports in which Tween 80 was found to enhance xylanase production from *S. chartreusis* L1105 and alkaliphilic *Streptomyces* sp. (Li *et al.,* 2011; Sharma and Bajaj, 2005).

Improved xylanase production by supplementing the production medium with olive oil was also observed in earlier reports (Kuhad *et al.*, 1998); Battan *et al.*, 2007; Nagar *et al.,* 2010; Kumar *et al.,* 2014). Polyethylene glycol also increased xylanase production in *Streptomyces* sp. (El-Gendy and El-Bondkly, 2014). Such compounds presumably increase the permeability of the cell membrane and cause rapid secretion of the xylanase and also (i) increase enzyme stability and prevention of enzyme denaturation; (ii) affecting the substrate structure positively and making it

more available for enzymatic hydrolysis; (iii) affecting enzyme-substrate interactions positively, leading to a more effective conversion of substrate. Addition of surface-active agents provided superior permeability of oxygen and extracellular enzyme transport through the cell membranes of microorganism (El-Batal *et al.,* 2015). Thus, the addition of surfactants to the medium not only helped in secretion of the extracellular enzyme, but could increase the bioavailability of less-soluble substrates for the organism and also stimulated its growth (Patel and Gupte, 2015).

The addition of surfactants like SDS, Triton X-100 and EDTA completely inhibited the growth of *Streptomyces* sp. ER1, hence no xylanase production was observed. These surfactants might have solubilised the cell membrane, resulting in no mycelial growth. Inhibition of enzyme synthesis with SDS might be due to cell membrane lysis or due to multiple reasons such as conformational changes in the secondary and tertiary structure of the protein, binding of surfactants to the active site of the enzyme, or varying in the nature of the substrate by decreasing the availability of reaction sites (Kapoor *et al.,* 2008). Inhibition by Triton X 100 might be ascribed to the change in the nature of the substrate by decreasing the availability of reaction sites (Eriksson *et al.,* 2002). Reduction of xylanase secretion in the presence of SDS was observed in *Aspergillus tubingensis* and Triton X 100 inhibited xylanase production in *Trichoderma* sp. (Adhyaru *et al.,* 2015; Balakrishnan *et al.,* 2013).

3.5.9 Effect of different metal ions on xylanase and biomass production

Metal salts and their concentration played very important role in microbial growth and enzyme production by providing ideal osmotic pressure. The influence of different metal ions on endo-1, 4-β-xylanase

production from *Streptomyces sp. ER1* was examined. Metal ions have been classified as hard and soft acids and bases and Class A, Class B and border line ions (Pearson, 1963; Nieboer and Richardson, 1980). Some class A ions (eg. Mg, Ca, Na) were essential for the growth of microorganisms. Some border line ions (eg. Cu,Fe, Ni, Zn) were required as micronutrients and some class B ions (eg. Hg, Pb) were toxic and not necessary for biological function (Collins and Stotzky, 1996).

Ca^{2+} induced maximum xylanase production by *Streptomyces* sp. ER1. Similar results were found in earlier reports (Murugan *et al.,* 2011; Bibi *et al.,* 2014; Adhyaru *et al.,* 2015). K^{+} and Na^{+} also induced xylanase production in the present study. The presence of Na^{+} in culture media mostly stimulated enzyme production and this might develop a particular membrane potential that favoured enzyme release from the cell (Naveen and Siddalingeshwara, 2015).

In the present study, Mn^{2+}, Fe^{2+}, Co^{2+} and Zn^{2+} enhanced the xylanase production compared to that of control. This might be owing to the alteration of structural conformation of the enzyme (Khandeparkar and Bhosale, 2006).

However, Cu^{2+} and Hg^{2+} inhibited xylanase production by *Streptomyces* sp. ER1. It has been reported that the xylanase production was inhibited by Hg^{2+} ion, which might be due to its interaction with sulfhydryl groups of cysteine residue in or close to the active site of the enzyme (Bastawde, 1992). The inhibition of xylanase by Cu^{2+} ions could be due to competition between the exogenous and the protein-associated cations, resulting in decreased metalloenzyme activity or may be because Cu^{2+} ions were known to catalyse the auto-oxidation of cysteines to form intra molecular disulphide bridges or the formation of sulphenic acid (Vieille and

Zelkus, 2001). Decrease in xylanase production in presence of Hg^{2+} ion may also be due to nonspecific binding or aggregation of this ion with some essential enzymes. They might also cause a decrease in catalytic activity due to partial denaturation of enzyme (Tunga *et al.,* 1999). Similar results were obtained with *Streptomyces* sp. S38, *Bacillus subtilis*, *S. rameus*, *Simplicillium obclavatum***,** *Bacillus vallismortis* and *C. Oxysporum* (Collins *et al.,* 2002; Sanghi *et al.,* 2010; Bhosalae *et al.,* 2011; Roy *et al.,* 2013; Gaur *et al.,* 2015; Guan *et al.,* 2016).

Metal ions such as Co^{2+}, Ca^{2+}, Zn^{+2}, and Fe^{2+} were found strongly stimulating xylanase production. In agreement with the present study, supplementation of Zn^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Ca^{2+} were also positive for xylanase production in *S. rameus* and *C. oxysporum* (Bhosalae *et al.,* 2011; Guan *et al.,* 2016).

3.5.10 Effect of different nitrogen sources on xylanase and biomass production

In the present study, potassium nitrate was found to be the optimum inorganic nitrogen source for xylanase production. A high xylanase production with Streptomycetes using potassium nitrate as an inorganic nitrogen source was also reported previously (Rifaat *et al.*, 2005; Bakri *et al.,* 2008; Bajaj *et al.*, 2010; Coman and Bahrim, 2011; Porsuk *et al.*, 2013). Ammonium sulphate, sodium nitrate and ammonium nitrate seemingly had less influence on xylanase production when compared to that of potassium nitrate. Similar results were obtained with *Streptomyces albus*, *S. chromofuscus* and *Aspergillus brasiliensis* (Rifaat *et al.,* 2005; Ho and Hood, 2014).

Among organic nitrogen sources, peptone was found to be the optimum nitrogen source. Similar reports were observed earlier (Sanghi *et*

al., 2009; Bhushan *et al.,* 2012; Pal and Kaushik, 2012; Ho and Hood, 2014*;* Shanthi and Roymon, 2015). The observation of high xylanase production with peptone could be due to the presence of enormous amount of amino acids, peptides, vitamins, trace elements and mineral salts (Pham *et al.,* 1998). Casein was the next optimum nitrogen source obtained for maximum xylanase production by *Streptomyces* sp. ER1. Similar observation was made in the case of xylanase production by *Cellulosimicrobium* sp. (Kamble and Jadhav, 2012).

3.5.11 Effect of different levels of xylanase on biomass and xylanase production

0.4% xylan in the production medium A showed -the highest xylanase production followed by 0.3%. Higher concentration of xylan (0.5%) gradually declined xylanase production. Reduced synthesis of the enzymes in high concentrations of xylan might be due to the accumulation of high concentration of reducing-sugars (Gomes *et al.,* 1994). Increased viscosity of fermentation medium eventually caused negative effect on the uniform circulation of nutrient and oxygen, reduction of microbial growth and ultimately declined endo-1, 4-β-xylanase production (Karim *et al.,* 2014).

Growth pattern of *Streptomyces* sp. ER1 at different concentration of xylan showed the maximum growth at 0.4% xylan. Similar results were reported from *Streptomyces* sp.F2621 which produced highest endoxylanase production at 0.4% of oats spelt xylan (Tuncer *et al.,* 2004). The endo-1, 4-β-xylanase production increased with the increase of xylan concentration and maximum production was obtained at 0.5% xylan from *Thermomyces lanuginosus* TISTR 3465. Further increase of xylan concentration beyond 0.5% declined the enzyme production and more than 50% reduction was observed when 3.0% xylan was supplemented in production medium (Bibi

et al., 2014). Similar findings were reported with *Streptomyces thermocarboxydus* MW8 strain (Chi *et al*., 2013). In contrast to the present results, *Thermomyces lanuginosus* TISTR 3465 secreted maximum xylanase with 1.5% xylan concentration (Khucharoenphaisan *et al.,* 2010).

3.5.12 Effect of fermentation and nutritional variables for xylanase production by *Streptomyces* sp. ER1 using Plackett- Burman Design (PBD)

12 variables (A (incubation period), B (xylan concentration), C (pH), D (inoculum age), E (inoculum concentration), F (agitation speed), G (salinity), H (incubation temperature), I (concentration of olive oil), J (concentration of potassium nitrate), K (concentration of peptone), L (concentration of Mn^{2+}) and M (concentration of Ca^{2+}) were analysed for their effects on xylanase production using PBD. The components were screened at a confidence level of 95%. When components showed significance at or above 95% confidence level and its effect is negative, it was considered effective for production but the amount required may be lower than the indicated (−1) concentration in PBD. If the effect was found positive, a higher concentration than the indicated (+) concentration was required. The magnitude and direction of the factor coefficient in the equation explained the influence of the twelve variables on the xylanase production by *Streptomyces* sp. ER1. The greater magnitude of the coefficient indicated a large effect on the response.

The probability value (p value) is a tool for evaluating the significance and contribution of each of the parameters to the statistical polynomial model equation. The pattern of interactions between the variables was indicated by these coefficients. Smaller p-value was an indication of high significance of the corresponding coefficient (Karthikeyan e*t al.,* 1996).

Variables with very low probability levels (close to 0.00) contributed to the model, while others could be neglected and eliminated from the model. In the present study, 4 variables namely, agitation speed, inoculum age, concentration of xylan and olive oil were found to be significant as their p values were less than 0.05 and close to 0.00. Agitation speed, inoculum age and concentration of olive oil had a negative effect on the xylanase production by *Streptomyces* sp. ER1 within the design range, as evident from the minus symbols in the coefficient of the linear regression equation. The decrease in their values increased the xylanase production. But xylan concentration had a positive effect on the xylanase production, as evident from the positive symbol in the coefficient of the linear regression equation. Its increase in concentration increased xylanase production. Similar results were obtained with *Aspergillus flavus* where agitation at its lowest level resulted in higher xylanase production and increase in carbon concentration would increase the xylanase production in *Trichoderma reesei* based on PBD (Pal and Kanum, 2010; Jampala *et al.,* 2015). Substrate concentration and agitation speed were reported to be the significant factors in *Penicillum citrinum* for xylanase production (Goshall *et al.*, 2011).

From the regression equation, it was evident that among the four significant variables, the greatest magnitude of the coefficient is that of xylan concentration. Hence it has large effect on the response.

To examine the fitting quality of the model, the proximate correlation coefficient (R^2) close to 1 indicated better fitting of the predicted values from the equation to the experimental values. In the present study, the coefficient of determination (R^2) for xylanase production was calculated as 0.93 that is close to 1 and it explained to 93.35% variability of the response. The predicted R^2 of 0.97 was in reasonable agreement with the adjusted R^2 of 0.98. Adequate precision measured the signal to noise ratio. A ratio

greater than 4 was desirable. The ratio in the present study was 22.028 which indicated an adequate signal and thus this model was applicable to the design space.

3.5.13 Optimisation using Central Composite Design (CCD)

The four significant variables, namely, inoculum age, agitation speed, xylan and olive oil concentration were further optimised using CCD. The statistical significance of the second-order polynomial equation was evaluated by F-test ANOVA which revealed that this regression was statistically highly significant for xylanase production. The model F-value of 131.26 implied that the model was significant. There was only a 0.001% chance that a large 'model F-value' could occur due to noise. The goodness of fit for the model was expressed by R^2 and the values were found to be 0.99. The values of R^2 indicated that the experimental values were significantly in agreement with the predicted values and also suggested that the model was suitable and practicable. The lack of fit F-value (0.17) for the xylanase production was not significant relative to pure error. Non-significant lack of fit was good for the model to fit. There was a 98.84% chance that a "Lack of Fit F-value" this large could occur due to noise.

The purpose of statistical analysis was to determine the experimental variables that generated signals, which were large in comparison to noise. The adequate precision value measured signal to noise ratio and ratio greater than 4.0 was desirable. In the present study, the value of adequate precision ratio was higher (46.667) than desired value for xylanase production and suggested that the polynomial quadratic model could be used to navigate the design space and further optimisation.

The coefficient of variation (CV) indicated the degree of precision with which the experiments were compared. The lower reliability of the

experiment was usually indicated by high value of CV. In the present case, a low CV (3.71) denoted that the experiments performed were reliable.

R^2 value was 0.99 which indicated that only 1% of the total variations were not explained by this model. This implied that experimental data was quite satisfactory. The predicted R^2 of 0.97 was in reasonable agreement with the adjusted R^2 of 0.98.

3.5.14 Graphical interpretation of the response surface model

The 3D response surface plots were employed to determine the interaction of the basal medium components and the optimum levels that had the most significant effect on xylanase production. From the 3D plots, it was evident that the interaction between concentration of olive oil (additive) and xylan; olive oil concentration and agitation speed; inoculum age and olive oil concentration; xylan concentration and agitation speed; inoculum age and xylan concentration were significant. In support to the present study, significant interaction between the xylan source and additive was observed from *Aspergillus flavus* (Pal and Kanum, 2010).

The parity plot existing in straight line showed the better accuracy of the model. In the present context, the statistical optimisation had been applied efficiently to SmF that had overcome the limitations of OFAT.

Optimum condition is the one at which the maximum xylanase production was attained. Such an optimum condition for xylanase production could be obtained by solving the second order polynomial equation using RSM. The central point is the point at which the slope of the contour is zero in all directions. The coordinates of the central point within the highest contour levels in each of the contour plots would correspond to the optimum values of the respective constituents. The optimum values drawn from these plots were in close agreement with those obtained by

optimising the regression model Equation 2. The sequential quadratic programming in Design expert 7 was used to solve the second-degree polynomial regression Equation 2. The optimum values for maximum xylanase production were: xylan concentration -0.37%, olive oil concentration – 33.10 mg/L, agitation speed- 43 rpm and inoculum age – 21.5 h. The optimal values for the variables as predicted were found to be within the design region. This showed that the model correctly explained the influence of the chosen variables on the xylanase production

3.5.15 Comparison of OFAT and RSM on optimisation of xylanase production

Compared with the OFAT approach which was unable to detect the frequent interactions occurring between two or more factors although they often do occur, RSM has immeasurable effects and tremendous advantages. The xylanase production was 3986 U/mL after optimisation by OFAT approach. However the xylanase production increased to 10547 U/mL after optimisation with RSM. The substantial increase in xylanase production by RSM optimisation could be due to significant interaction between the independent variables, concentration of xylan and olive oil, inoculum age and agitation speed.

Thus, the production of xylanase by *Streptomyces* sp. ER1 was optimised using OFAT and CCD. They appeared to be valuable tools for optimisation as xylanase production increased by about 1.65 fold. The CCD also increased xylanase production by 1.5 fold in *Bacillus tequilensis* compared to OFAT (Khusro *et al.,* 2016). Compared to the unoptimised production medium A (1277.33 U/mL), statistical optimisation increased xylanase production by 7.26 fold by *Streptomyces* sp. ER1. Similarly, optimisation of growth conditions of *Schizophyllum*

commune and *Bacillus circulans* through statistical methods resulted in improvement of xylanase yields by 3 folds over unoptimized conditions and yields obtained were close to predicted values (Haltrich *et al*., 1993; Heck *et al.,* 2005). CCD based RSM models appeared to be accurate and reliable for predicting the production of xylanase by *Fusarium* sp. BVKT R2 (Ramanjaneyulu and Reddy, 2016).

From the present study, it was found that that PBD and CCD could be used effectively, one after the other, to determine the significant variables and their optimum conditions for enhanced xylanase production. Using PBD, xylan concentration, agitation speed, inoculum age and olive oil concentration were identified as the most influencing parameters affecting xylanase production, and CCD helped to study their interactive effects on xylanase yield. The medium designed using a limited number of experiments, minimum efforts and time resulted in 1.65 fold increase in xylanase production compared with that by OFAT. This systematic and statistical optimization carried out using PBD and CCD has many advantages over conventional method of optimisation which was incapable of reaching the true optimum conditions. The traditional method, OFAT, assumed that various fermentation parameters did not interact and that the process response was a direct function of the single varied parameter. In contrast, it was observed from the present study that the observed behaviour of fermentation resulted from the interactive influences of various variables. The statistical strategy as a whole proved to be adequate for the design and optimisation of the bioprocess for enhanced xylanase production by *Streptomyces* sp. ER1.

Chapter -4

PURIFICATION AND CHARACTERISATION OF XYLANASE

Contents

4.1 Introduction

Purification of an enzyme is of prime importance in learning about its structural and functional properties and to predict its applications. The major aim behind deciding the strategy for purification is to obtain a maximum possible yield of the desired enzyme with highest catalytic activity and maximum possible purity. Enzyme purification varies from a simple one-step purification procedure to large-scale purification processes. The key for successful and efficient purification strategies is the selection of appropriate techniques that maximise yield and purity with a minimum number of steps (Palmer, 2001).

Xylanases were purified and characterised from different microbial sources. The degree of purification of xylanase depends upon the kind of commercial application intended. Most of the industrial applications do not demand the need for homogenous preparation of enzymes. However, a certain degree of purity is necessary for industries, such as fine chemicals, cosmetics and pharmaceuticals. Industrial purification processes are

designed in such way that they are economically viable, rapid, high-yielding and amenable to large-scale operations (Saxena *et al.,* 2003). Purified enzymes are often subjected to characterisation studies including molecular mass analysis. Characterisation of xylanase is necessary for proper application of enzymes in industries and the stability of enzymes is often revealed by characterisation analysis.

Although a number of xylanases-producing microbes were used for industrial productions, only a few aquatic actinomycetes were exploited for the production of xylanase. Enzymes from aquatic microorganisms are unique with properties that are different from those of its terrestrial counterparts. In this context, studies on xylanase purification and characterisation from *Streptomyces* sp. ER1 isolated from Cochin estuary would help develop its use for various industrial applications.

4.2 Review of literature

Most of the purification methods which are used in laboratory research can be scaled to industrial processes. Such methods are filtration, centrifugation, ultrafiltration, diafiltration, precipitation and chromatography. Purification of xylanases is usually based on the non-specific techniques, such as ammonium sulphate precipitation, gel filtration, ion exchange, affinity chromatography etc. (Subramanyian and Prema, 2002).

One of the most vital steps in the purification of extracellular xylanases is clarification. This step is mainly used to remove polyphenols, pigments, nucleic acids etc., which may be a contaminant during further purification steps. Clarification can be done by precipitation, centrifugation or filtration. This method is hardly ever mentioned in the literature for

xylanases, but there are certain reports which have utilised it for purification of xylanase (Nakamura *et al.,* 1993; Dhillon and Khanna, 2000).

Lower yields were generally obtained when a greater number of purification steps were used. Less number of steps, generally, has higher recovery yields. Precipitation with ammonium sulphate and ethanol was used in the purification studies as the initial step. It also removed low molecular proteins (Nakamura *et al.,* 1993). An alternative to precipitation was ultrafiltration. The membranes used were with 5-30 kDa molecular-weight cut-off value (Sa-Pereira *et al.,* 2003). Extracellular xylanase from *Streptomyces viridosporus* T7A and *Streptomyces* sp. K37 was purified by ultrafiltration (Timothy *et al.,* 1997; Mansour *et al.,* 2003). Large number of purification protocols for xylanases from Streptomycetes mentioned, ammonium sulphate precipitation as the preliminary step in purification, like in *Streptomyces roseiscleroticus* and *Streptomyces cyaneus* SN32 (Grabski and Jeffries, 1991; Ninawe *et al.,* 2008).

Microbial xylanases are mainly purified by chromatographic methods, using two to five purification steps and providing recovery yields ranging from 0.2 to 78%. Ion exchange and gel permeation chromatography or the combinations of both, are the most common methods. Both cation and anion exchange chromatography are used for the purification of xylanases. The use of matrices in the ion exchange chromatography differed with type of enzyme and microorganisms. Anion exchange columns, like Sepharose-Q and DEAE- Sepharose were used for purification of xylanase (Dhillon and Khanna, 2000; Lappalainen *et al.,* 2000). Use of cation exchange chromatography was also reported using Carboxymethyl Sepharose and DEAE-Sephadex (Matte and Forsberg, 1992; Lappalainen *et al.,* 2000; Carmona *et al.,* 2005; Fengxia *et al.,* 2008). DEAE-Cellulose column chromatography was also used for the purification of xylanase and colour

removal from the extract of alkali-tolerant *Aspergillus fischeri Fxn*1 (Chandra and Chandra, 1996).

Gel permeation chromatography was the second most employed purification method for xylanases after ion exchange chromatography. Xylanases were purified from *Aspergillus caespitosus* and *Ceriporiopsis subvermispora* using gel permeation chromatography with a matrix of Sephadex G-100 and Sephadex G-50, respectively (Sandrim *et al.,* 2004; Milagres *et al.,* 2005). In maximum cases, the gel filtration chromatography has been used in combination of other chromatography for purification of xylanase. However, single use of gel permeation chromatography was also reported (Lee *et al.,* 1993; Chapla *et al.,* 2011).

Apart from ion exchange and gel permeation chromatography, other techniques, such as affinity chromatography, hydroxyapatite chromatography, HPLC etc., have been done in special cases. Classical affinity chromatography has not been proved so amenable to large scale work due to its high expense and short life of the matrices. However, there were reports available, which used such techniques to purify xylanase (Lee *et al.,* 1993).

The chromatographic methods were sometimes coupled with HPLC columns for the better resolution than the standard conventional column. A reverse-phase HPLC column was used for purification of xylanase from *Chainia* sp. (Rao *et al.,* 1996). A minor form of xylanase from *Aspergillus versicolor* have been purified using combination of various chromatographic techniques and finally eluted from HPLC GF-510 system for complete purification (Carmona *et al.,* 2005). With the advancement in the instrumentation facilities, another chromatography, such as Fast Protein Liquid Chromatography (FPLC), had emerged as the efficient tool for

protein purification. Ion exchange and hydrophobic interaction chromatography were used on FPLC system for the purification of xylanase from *Penicillium brasilianum* IBT20888 (Jorgensen *et al.,* 2003). Cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 was purified with the help of Sepharose-Q fast flow column (Li *et al.,* 2005).

There were also reports of purification of xylanase with hydrophobic interaction column chromatography (Wong and Saddler, 1992). Since considerable number of bacteria and fungi produced multiple xylanases, at least three-step purification was necessary for the separation of these isozymes. In the purification of xylanases to electrophoretic homogeneity, however, the yield and purification fold varied in different cases.

4.2.1 Purification of xylanase from *Streptomyces* sp.

The purification fold and yield of xylanase produced by different *Streptomyces* sp. varied between 3.9-48 and 6.7% to 33% respectively (Marui *et al.,* 1985; Keskar *et al.,* 1989; Nakanishi *et al.,* 1992).

Xylanase from *Streptomyces roseiscleroticus* was purified by cation exchange FPLC. The supernatant was applied to a Pharmacia C 10/10 column packed with carboxymethyl Bio-Gel A cation exchange resin coupled to a Pharmacia FPLC. Bound protein was eluted by using a linear 50 mL gradient of 0 to 0.4 M NaCl in the buffer. Elution was monitored for protein at 280 nm. Fractions containing the xylanase activity were dialysed against buffer for approximately 5 h. The dialysed sample was applied to a Pharmacia Mono-S cation exchange column and chromatographed again for purity (Grabskit and Jeffries, 1991).

Two endoxylanases were isolated from the xylanolytic enzyme system of the thermophilic actinomycete *Microtetraspora flexuosa* SIIX, and purified by ammonium sulphate fractionation, DEAE-Sepharose

chromatography, gel filtration on Sephacryl S-200 and FPLC on Q-Sepharose (Berens *et al.,* 1996). Purification of xylanase from *Streptomyces viridosporus* T7A was achieved by treatment of the culture supernatant with Q-Sepharose, concentration of the unbound proteins by ultrafiltration, fractionation of these proteins by Sephadex G-75 gel filtration chromatography, followed by Rotofor Preparative Isoelectric Focusing (Timothy *et al.,* 1997).

Extracellular xylanase from *Streptomyces* sp. K37 was purified to 33.53% by ultrafiltration and cation exchange chromatography followed by gel filtration chromatography (Mansour *et al*., 2003). The xylanase from *Streptomyces cyaneus* SN32 was purified to homogeneity by ammonium sulphate precipitation followed by anion exchange chromatography using DEAE–Sepharose column, with 43% yield (Ninawe *et al.,* 2008).

Streptomyces sp. 7b produced xylanase which was purified by ammonium sulphate precipitation and Carboxymethyl Sephadex chromatography. The enzyme was purified to the extent of 5.68 fold by salt precipitation and ion exchange chromatography (Bajaj and Singh, 2010). Xylanases from *Nonomuraea flexuosa* and *Thermoascus aurantiacus* were purified by heat treatment and gel permeation chromatography (Zhang *et al*., 2011).

Xylanase of *Streptomyces* sp. ESRAA-301097 was purified by 70% (w/v) saturated ammonium sulphate precipitation, DEAE-Cellulose chromatography and gel permeation chromatogahy using Sehadex G-200 and Sehadex G-100 columns **(**El-Gendy and El-Bondkly, 2014**).**

The xylanase produced by *Streptomyces althioticus* LMZM was purified 12.65 fold through ammonium sulphate precipitation, Sephadex G-25, DEAE-Cellulose chromatography, followed by gel filtration through a

Sephadex G–100 column (Luo *et al.,* 2015). The purification of xylanase from an actinobacterium, *Streptomyces olivaceus* (MSU3), isolated from the sediment sample of mangrove, resulted in 4.27 fold increase with the yield of 15.57% at the final step using Sephadex G-75 chromatography (Sanjivkumar *et al.,* 2017).

4.2.2 Characterisation of xylanase

Most of the earlier studies on the characterisation of xylanase from *Streptomyces* sp. revealed that xylanase was stable at 50^{o}C to 55^{o}C and at 5-6.5 pH (Marui *et al.,* 1985; Keskar *et al.,* 1989; Nakanishi *et al.,* 1992). Extracellular xylanase by Streptomyces sp. CH-M-1035 was characterised and found that the optimum pH and temperature for xylanase activity was 5 and 60^{o}C, respectively (Flores *et al.,* 1997).

Xylanase produced by *Streptomyces* sp. AMT-3 was characterised in terms of temperature, and thermostability. Greatest enzyme activity was observed at the temperature ranging from 55 to 65°C and at pH 6. The enzyme retained 50% of its activity after 20 h at 55°C. By itself, this xylanase could be considered as a thermotolerant biocatalyst, being interesting for biotechnological applications (Nascimento *et al.,* 2002). Extracellular xylanase from *Streptomyces* sp. K37 was purified and the optimum pH and temperature were found to be pH 6 and 60^{o}C, resectively (Mansour *et al.,* 2003).

In a study carried out to characterise the xylanase from *Streptomyces* sp. strain C1-3, it was found that the enzyme was highly thermostable (90^{o}C) and had a low acidic pH optimum (pH 3). The enzyme had preferential activity towards birchwood and arabinoxylans (Meryandini, 2007). The optimum temperature and pH for maximum xylanase activity

were 50°C and 6, respectively from *Streptomyces* sp. 7b (Bajaj and Singh, 2010).

A xylanase produced by *Streptomyces* sp. SWU10 showed very broad pH optima, ranging from low acidic pH (3) to alkaline pH (8). The enzyme was optimally active at 60^{o}C but showed partial activity retention at 80^{o}C as well (Deesukon *et al.,* 2011).

Xylanase from *S. chartreusis* was more stable under alkaline conditions and retained more than 80% activity after 30 min incubation at pH 6-10. It also showed specific activity towards oat-spelt xylan and corncob xylan, hydrolysing them to xylobiose and xylotriose as principle products without the formation of xylose. These properties revealed that the purified xylanase could potentially be used in biotechnological applications, such as xylooligosaccharide preparation (Zhu *et al.,* 2012).

A xylanase produced by a strain of *Streptomyces* sp. AMT-3 showed the best activity between the temperature ranging from 55-65^{o}C and pH 6. The enzyme retained 50% of its activity after 20 h at 55^{o}C. Hence, this xylanase could be considered as a thermotolerant enzyme suitable for the biotechnological applications (Nascimento *et al.,* 2002). A strain of *S. chartreusis,* which produced a novel xylanase, showed pH and temperature optima at 9 and 70^{o}C, respectively. The enzyme was substantially active at pH 12 and 70^{o}C with a good stability between pH 8-12 (Thomas *et al.,* 2013).

Xylanase from *Streptomyces* sp. AOA40 was partially purified by ion exchange chromatography and gel filtration chromatography.. The optimum pH for the activity of partially purified xylanase was at pH 6 in phosphate buffer, while the optimum temperature was 60°C. The relative xylanase activity in the pH ranges of 4–9 remained between 59.93 and

54.43% of that at pH 6 (100%). The xylanase activity exhibited a half-life of 172 min at 70°C, which was reduced to 75 min at 80°C. The enzyme was highly inhibited by 10–100 mM of Hg^{+2}, EDTA, Mg^{+2}, SDS and 100 mM Cu^{+2} (Adiguzel and Tuncer, 2016).

The maximum activity of purified xylanase obtained from an actinobacterium, *Streptomyces olivaceus* (MSU3), isolated from the mangrove sediment sample was observed at pH 8 and temperature 40°C (Sanjivkumar *et al.,* 2017).

Very little studies have been done on the characterisation of xylanase from estuarine actinomycetes. The present chapter deals with the purification and characterisation of xylanase from *Streptomyces* sp. ER1 isolated from Cochin estuary.

4.3 Materials and Methods

4.3.1 Production of xylanase

The selected isolate of high xylanase producing *Streptomyces* sp. ER1 was used for the study.

4.3.2 Medium used

Modified production medium A (Table 4.1) was used based on the optimum conditions obtained by OFAT and CCD, as described in Chapter 3. Modified production medium A (200 mL) was prepared in 500 mL screw capped flasks, pH adjusted to 7, and sterilised at 121°C for 20 min in an autoclave.

Table 4.1. Chemical composition of modified production medium A

Ingredients	Composition (per L)
KH_2PO_4	1.5 g
$CaCl_2$	2g
KNO_3	4.5 g
Peptone	0.075 g
Casein	0.075 g
$MnCl_2$	160 mg
Olive oil	33.10 mg
Xylan	0.37% (w/v)

4.3.3 Inoculum preparation

The inoculum was raised in the modified xylanase production medium A. The culture was grown in Erlenmeyer flask containing 100 mL of the xylanase production medium on an incubator shaker (100 rpm) at ambient room temperatures (35 ± 2°C). The inoculum of age 21.5 h was used at 5% v/v ($452X10^1$ CFU/mL).

4.3.4 Inoculation and xylanase production

The production media was inoculated with 21.5 h old inoculum of *Streptomyces* sp. ER1 and then incubated at 35°C for 120 h on an incubator shaker at 50 rpm. After completion of the incubation period, the medium was centrifuged in a cooling centrifuge (Eppendorf, Germany) at 10000 rpm for 15 min at 4°C. Xylanase activity and specific activity was checked to confirm xylanase production. The cell free supernatant was used as the crude enzyme.

4.3.5 Xylanase Assay

Xylanase activity was measured following the assay as described under the section 3.3.7. Specific activity was expressed by the relation between enzyme activity and protein content.

4.3.6 Estimation of protein

Estimation of total extracellular protein was performed through Bradford test (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard. Protein content per millilitre of test samples was determined against the standard curve. During the chromatographic steps, proteins were detected by reading absorbance at 280 nm.

4.3.7 Purification of xylanase

The cell-free culture supernatant was subjected to purification which involved two steps, such as precipitation by organic solvent and ion exchange chromatography.

4.3.7.1 *Cold acetone precipitation*

The crude enzyme from the cell-free culture supernatant was concentrated by cold acetone precipitation. Ice cold acetone was gradually added to culture supernatant (2:1 V), vortexed and allowed to stand for 2 h in -20 °C followed by centrifugation at 14,000 rpm at 4°C for 30 min. The supernatant was discarded and the pellets were dissolved in 0.1 M phosphate buffer (pH 7) and subjected to DEAE-Cellulose ion exchange chromatography.

4.3.7.2 *DEAE-Cellulose ion exchange chromatography*

The DEAE-cellulose resin was purchased from Sigma (U.S.A) and activated as per manufacturer's instructions. The resin was packed into the column (1.5 X20 cm) and care was taken in such a way that air bubbles

were not trapped. All the buffers were filtered before each run and the column was pre-equilibrated with 0.1 M phosphate buffer, pH 7. Precipitated enzyme (1 mL) was loaded into the pre-equilibrated column. Following this, the column was washed with the same buffer (0.1 M phosphate buffer, pH 7) to remove the unbound proteins. The bound protein was eluted by applying a linear gradient of NaCl (0.05 to 1.0 M) at a flow rate of 1 mL/ min. Eluted fractions (1.0 mL) were collected in micro centrifuge tubes and analysed for xylanase activity and protein concentration. Fractions exhibiting highest xylanase activity were pooled together and used for further studies.

4.3.7.3 *Determination of molecular weight*

Determination of molecular weight of purified xylanase was carried out using one dimensional SDS-PAGE. Stacking gel of 4% was prepared with 0.5 M Tris-HCl at pH 6.8 and resolving gel of 12% was prepared with 1.5 M Tris-HCl at pH 8.8 (Laemmli, 1970). Enzyme samples collected from each stage such as crude enzyme extract, acetone precipitated sample and purified samples were mixed with sampling buffer and subjected to SDS¬PAGE.

Sampling buffer

Distilled water	3.55 mL
Glycerol	2.5 mL
0.5% bromophenol blue	0.2 mL
0.5 M Tris-HCl (pH 8)	1.25 mL
10% SDS	2.0 mL
β-mercaptoethanol	50 µL

Electrophoresis was carried out at a constant current (150 V) using vertical electrophoresis running unit (BioRad, USA). A molecular weight marker (16-98 KDa) (Merck, U.S.A) was also added. Run was allowed to continue until the dye front reached the bottom of the gel. After the completion of electrophoresis, glass plates were carefully removed from the unit. The gel was then scooped carefully from the glass plates and stained with 0.025% Coomassie brilliant blue R250. After the staining procedure, decolourisation was performed with destaining solution. The gel was then visualised under the UV transilluminator (GelDoc EZ imager, BioRad).

4.3.7.4 Native-PAGE and Zymogram analysis

For the native-PAGE, the protein samples were mixed with native sample buffer. The samples without heating were loaded onto 4% stacking gel and 10% separating gel. The electrophoresis was performed at 4°C. The native gel was then soaked in 1% (w/v) beechwood xylan/0.1 M sodium phosphate buffer (pH 7) at 60^{o}C for 3 h prior to staining with Congo red solution (0.1%-10 min). The gel was destained with 1 M NaCl until the clear zone was observed. After 20 min, the gel was destained in a 5% (v/v) acetic acid solution to create a contrast (Beguin, 1983).

4.3.8 Characterisation of enzyme

4.3.8.1 Effect of pH on enzyme activity and stability

The optimum pH for xylanase activity was determined in a pH range of 4 to 11 with 1 pH unit increment. Stability was checked using different buffers, such as acetate buffer (pH 4-6), sodium phosphate (pH 7), Tris-Cl (pH 8, 9) and Glycine-NaOH (pH 11) in the reaction mixture. The activity of the enzyme was expressed in terms of relative activity. The pH stability of the enzyme at the optimum pH was tested by pre-incubating the enzyme

in the respective buffer for 6 h and the relative activity (%) was determined in every 1 h.

4.3.8.2 Effect of temperature on the enzyme activity and stability

The optimum temperature for enzyme activity was assessed by carrying out the xylanase assay at different temperatures from 20 to 100°C. The difference in activity was calculated as percentage relative activity. The temperature stability of the enzyme was assayed by pre-incubating the enzyme at the optimum temperature for 6 h followed by assay of the relative xylanase activity under standard assay conditions.

4.3.8.3 Effect of metal ions on enzyme activity

The influence of different metal ions on the xylanase activity was tested as follows. Different metal ions (Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, K^{+}, Ca^{2+}, Mg^{2+}) in their salt form were prepared at a final concentration of 5mM and purified enzyme was added to the metal ion solution. This mixture was incubated for 30 min at 60°C. The percentage residual activity was calculated by comparing the activity of the treated enzyme with that of the untreated enzyme (in the absence of metal ions) which served as a control and the value was taken as 100%.

4.3.8.4 Effect of surfactants on enzyme activity

To determine the stability of the enzyme in the presence of surfactants, the purified enzyme was pre-incubated with the surfactants such as Tween 60, 80, Polyethylene glycol, olive oil, Triton X 100, β-mercaptoethanol and SDS at a concentration of 0.1% for 30 min at 60°C. After the incubation period, activity was calculated in terms of residual activity.

4.3.8.5 Effect of organic solvents on enzyme activity

The stability of xylanase in different organic solvents such as butanol, chloroform, ethyl acetate, and hexane was checked by pre-incubating the purified enzyme in the solvents for 30 min at 60°C. Residual activity was calculated and purified enzyme without organic solvent was kept as a control of the experiment.

4.3.8.6 Effect of NaCl on enzyme activity

To determine the stability of the enzyme in the presence of NaCl, the purified enzyme was pre-incubated with NaCl in the concentration of 10, 15 and 20% and the mixture was incubated for 30 min at 60°C. After the incubation period, activity was calculated in terms of residual activity. The purified enzyme without NaCl was kept as a control of the experiment.

4.3.8.7 Substrate specificity of xylanase and determination of kinetic constant

The substrate specificity of the purified enzyme was determined by using various substrates in the reaction mixture for xylanase assay. The various substrates used were 1% (w/v) of cellulose, starch, glycerol and xylan. The difference in activity was calculated as percentage relative activity. Km and Vmax values of the xylanase were determined by measuring enzyme activity at various concentrations of xylan (2-6 mg/mL) and were calculated using Lineweaver-Burk equation method.

4.4 Results

4.4.1 Purification of xylanase

Xylanase produced from *Streptomyces* sp. ER1 under SmF was purified by cold acetone precipitation and anion-exchange chromatography on DEAE-Cellulose. The xylanase activity in the crude sample was 10547

U/mL. The total protein content of the sample decreased from 200 mg in the crude sample to 2.5 mg in the final step. The specific activity increased from 52.73 U/mg in the crude sample to 293.97 U/mg in the chromatographic step. The yield of the enzyme was 6.97%. The results of the purification steps are summarized in Table 4.2.

Table 4.2. Summary of the purification of xylanase from *Streptomyces* sp. ER1

Sample	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	200	10547	52.73	100	1
DEAE-Cellulose 52	2.5	734.93	293.97	6.97	5.57

4.4.2 Determination of molecular weight of the xylanase produced by *Streptomyces* sp. ER1

The active fractions obtained from DEAE-cellulose chromatography were pooled and subjected to native and SDS PAGE. Protein purification was successfully achieved to homogeneity as evidenced by a single sharp band observed at approximately 23 kDa on SDS-PAGE (Fig 4.1).

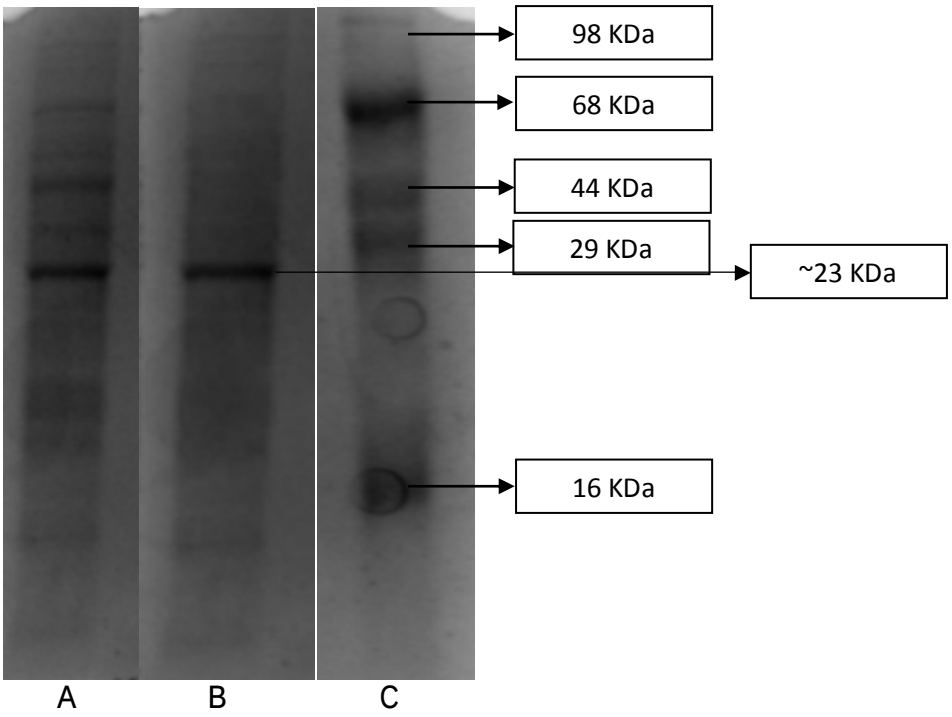


Fig 4.1. SDS PAGE analysis of crude enzyme and purified enzyme. Lane A: Crude enzyme; Lane B: pure enzyme; Lane C: Marker

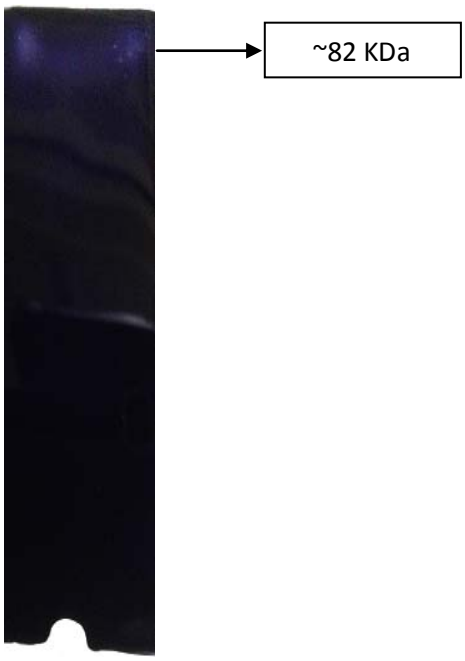


Fig 4.2. Zymogram analysis of the xylanase from *Streptomyces* sp.ER1

The zymogram exhibited a single significant activity band of xylanase suggesting that the enzyme had only one isoform (Fig.4.2).

4.4.3 Characterisation of xylanase

4.4.3.1 Effect of pH on enzyme activity

The effect of pH on the xylanase activity was determined in the pH range 4 to 11. Xylanase activity increased with increase in pH and reached its maximum at pH 7. Above this pH, the enzyme was active up to pH 8 and then there was a decrease in activity when pH was further increased to11. The results are presented in Fig 4.3.

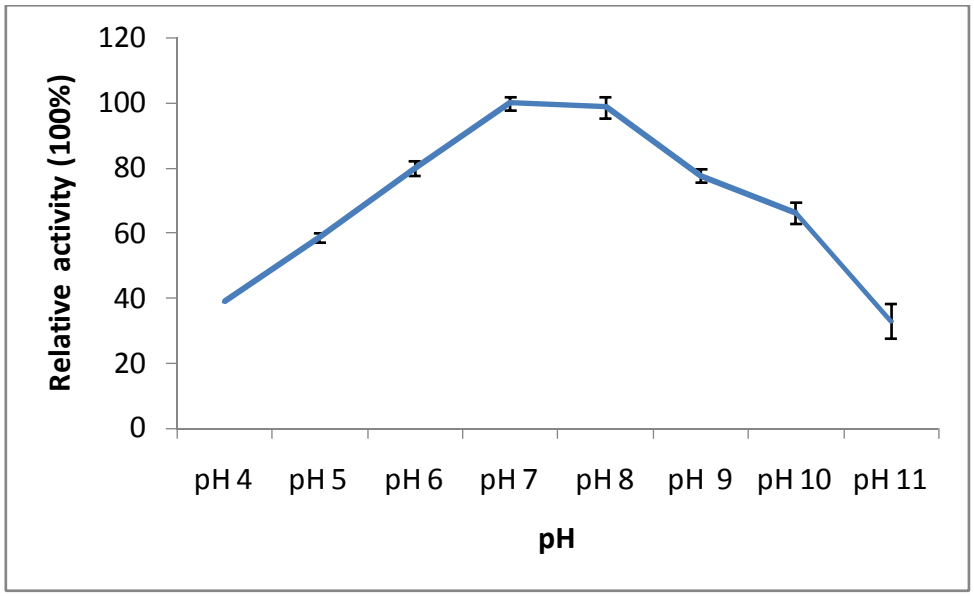


Fig 4.3. Effect of pH on xylanase activity

4.4.3.2 Effect of pH on stability of the enzyme

The pH stability was examined by incubating the enzyme in phosphate buffer at pH 7 for 6 h and the xylanase activity was assayed at one hour interval under standard conditions. As indicated in Fig 4.4, the enzyme was stable for 3 h at pH 7 and then the activity reduced on further incubation.

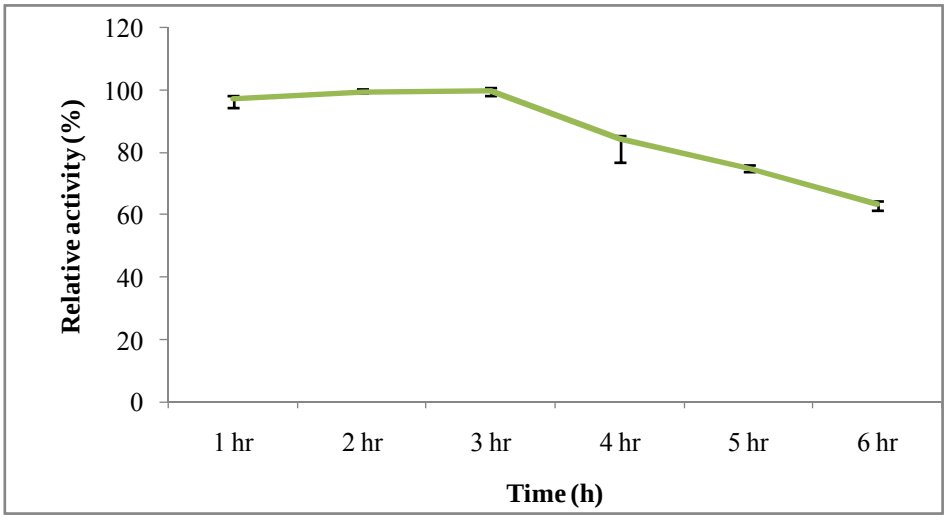


Fig 4.4. Effect of pH on stability of the xylanase

4.4.3.3 Effect of temperature on xylanase activity

Effect of temperature on xylanase activity was studied in the temperature range 20 to 100°C. The enzyme showed maximum activity (100%) at 60°C. There was very little enzyme activity below 40°C. At 40°C and 50°C, there was 77.48% and 86.15% of relative activity, respectively. A rapid drop in enzyme activity was observed from 70°C. Above 80°C, the enzyme got denatured (Fig 4.5).

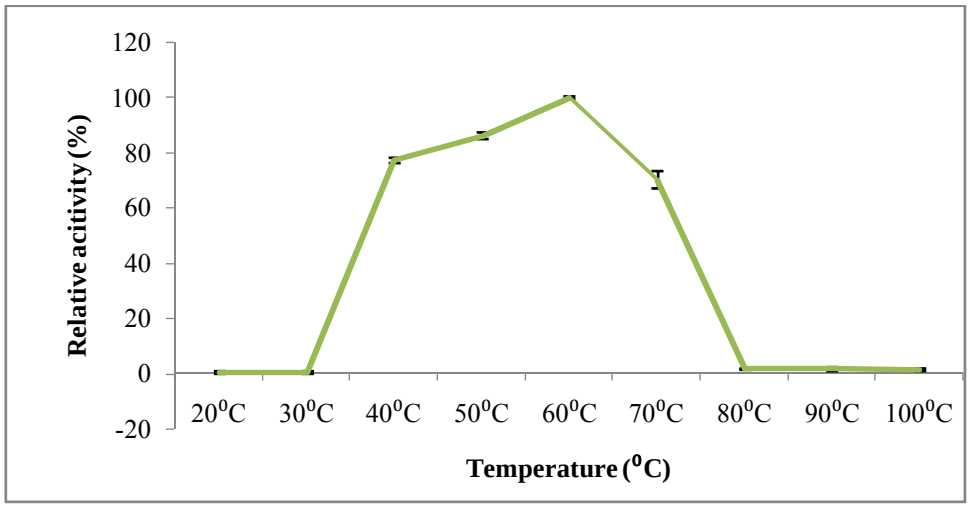


Fig 4.5. Effect of temperature on xylanase activity

4.4.3.4 Effect of temperature on stability of the enzyme

The stability of the enzyme at the optimum temperature was studied by incubating the enzyme at 60°C for 6 h and the enzyme activity was assayed under standard conditions every hour. As Fig 4.6 depicts, the enzyme was stable for 3 h at temperature 60°C. Nevertheless, the enzyme activity dropped and showed 84.82% of relative activity at 6th h of incubation, whereas, the enzyme at 4th and 5th h of incubation displayed more stability with 98.23 and 96.06% of relative activity, respectively.

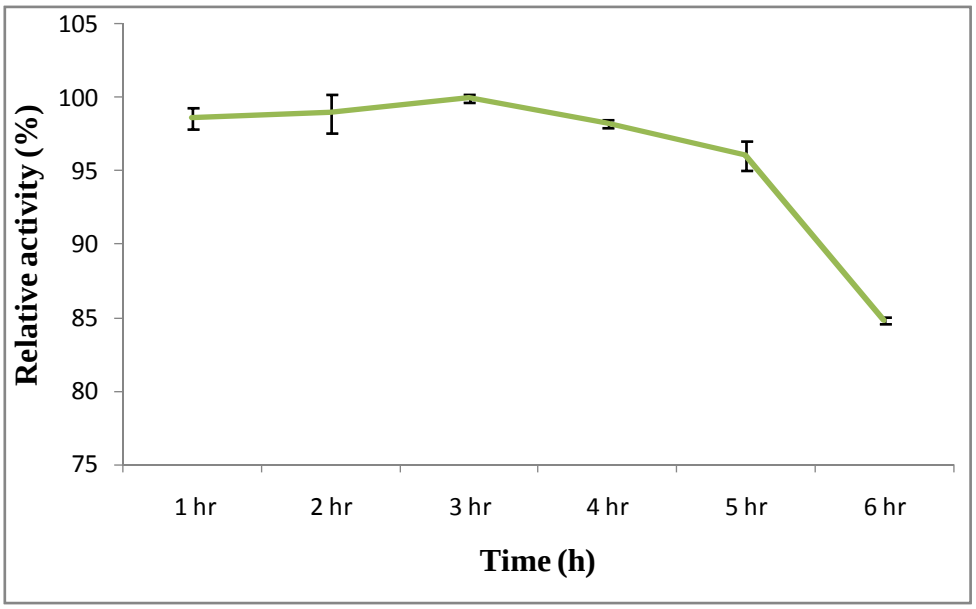


Fig 4.6. Thermo stability of xylanase

4.4.3.5 Effect of organic solvents on enzyme activity

The effect of various organic solvents on the xylanase activity is shown in Fig 4.7. Hexane enhanced the enzyme activity whereas, all other solvents tested reduced the activity.

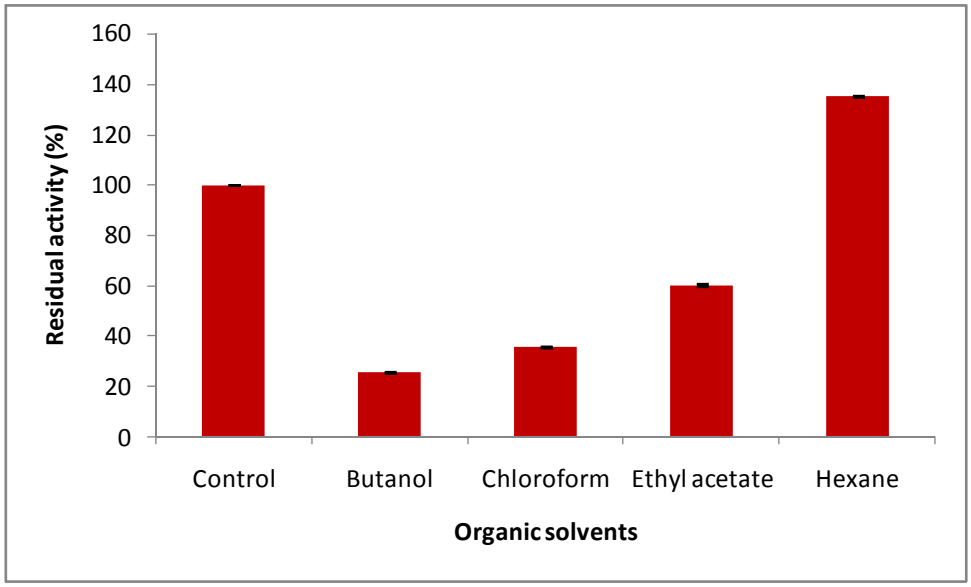


Fig 4.7. Effect of organic solvents on xylanase activity

4.4.3.6 Effect of metal ions on enzyme activity

The result of the effect of metal ions on xylanase activity is presented in Fig 4.8. The metal ions such as Co^{2+}, Mn^{2+}, K^{+}, Ca^{2+} and Mg^{2+} enhanced activity since residual xylanase activity was greater than 100%, compared to that of control. The metal ions such as Cu^{2+}, Fe^{2+}, Zn^{2+} and Hg^{2+} significantly reduced the xylanase activity when compared to that of control. Hg^{2+} was found to be the most potent inhibitor, as the residual enzyme activity was only 2%, compared to that of control.

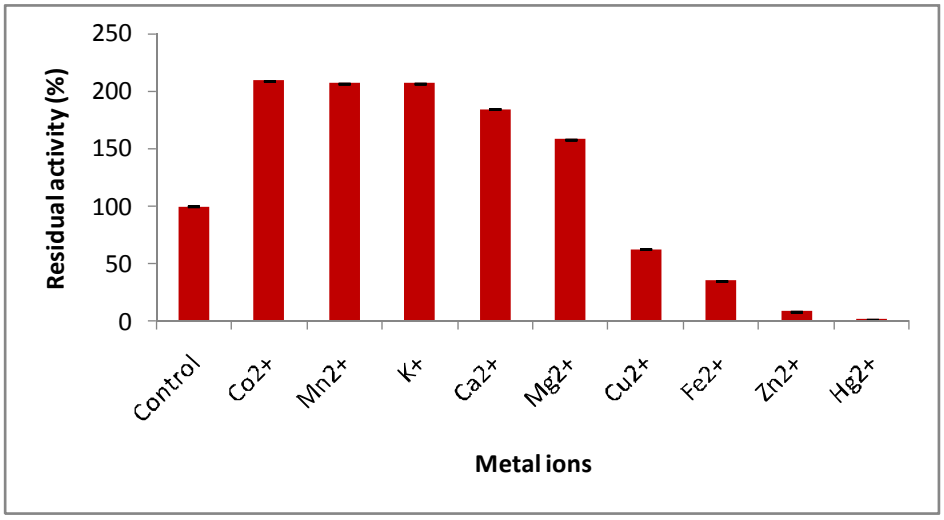


Fig 4.8. Effect of metal ions on xylanase activity

4.4.3.7 Effect of surfactants on enzyme activity

Polyethylene glycol and olive oil enhanced xylanase activity and the enzyme was more or less stable in the presence of Triton X 100, Tween 80, Tween 60. However, SDS and β-mercatoethanol were found to be inhibitory and the residual activity was reduced to 41.61% and 5.01%, respectively. β-mercaptoethanol was found to be the most potent inhibitor (Fig 4.9).

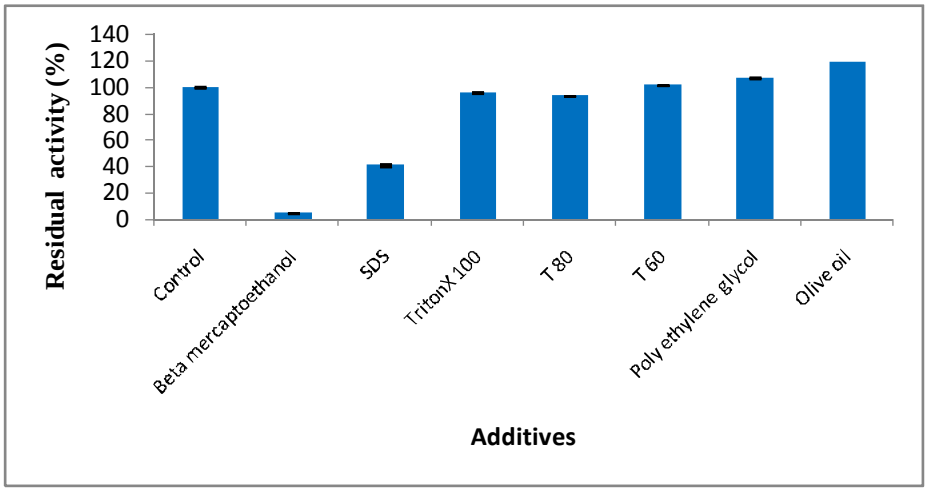


Fig 4.9. Effect of surfactants on xylanase activity

4.4.3.8 Effect of NaCl on enzyme activity

The influence of NaCl was estimated by incubating the enzyme at different concentrations of NaCl for 30 min before the xylanase assay. 10 and 15% of NaCl enhanced the xylanase activity, as the residual activity was greater than 100%, compared to that of control. However, there was a slight decrease in the residual activity with 20% of NaCl (91.5%) (Fig 4.10).

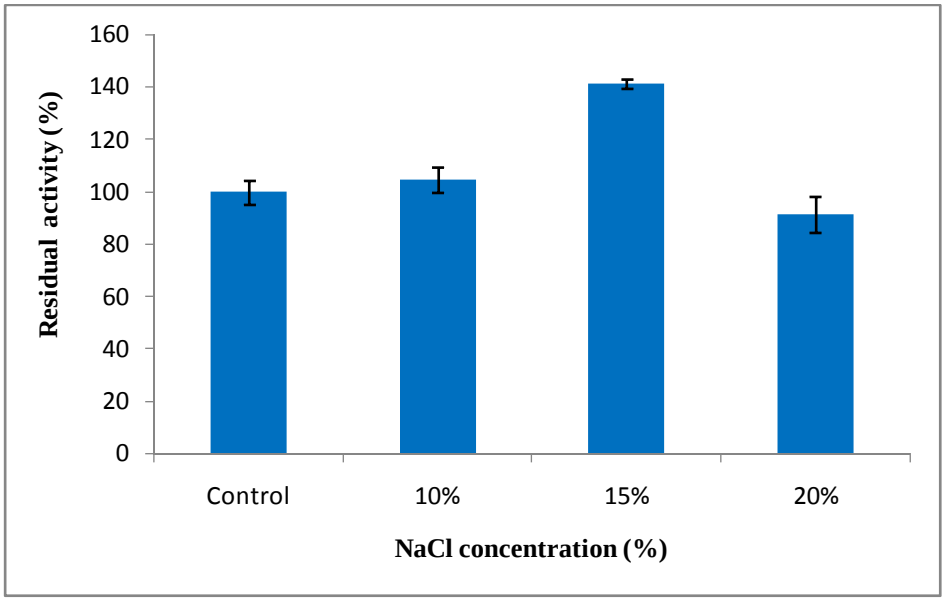


Fig 4.10. Effect of NaCl on xylanase activity

4.4.3.9 Substrate specificity

To obtain a better understanding of the functions and properties of xylanase from *Streptomyces* sp. ER1, the specificity and kinetic parameters were investigated using several substrates, namely, beechwood xylan, starch, cellulose and glycerol. The enzyme was specific for beechwood xylan. No activity was shown in presence of other substrates.

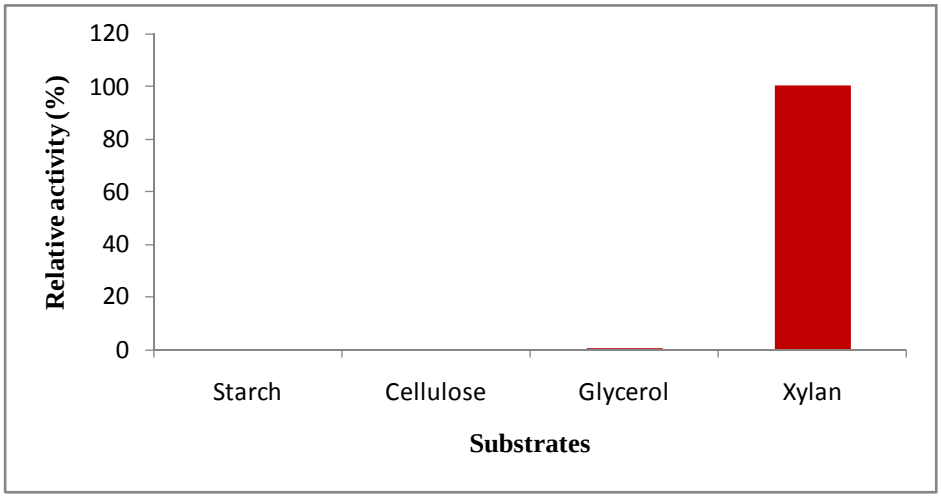


Fig 4.11. Substrate specificity of xylanase

4.4.3.10 Determination of kinetic parameters of purified xylanase

To determine the Vmax and Km of the xylanase enzyme, its activity was assayed using varying concentration of beechwood xylan (2-6 mg/mL). From the Lineweaver-Burk plot (Fig 4.12), the K_m value obtained was 0.018 µmol and the V_{max} was 11.72 µmol/min.

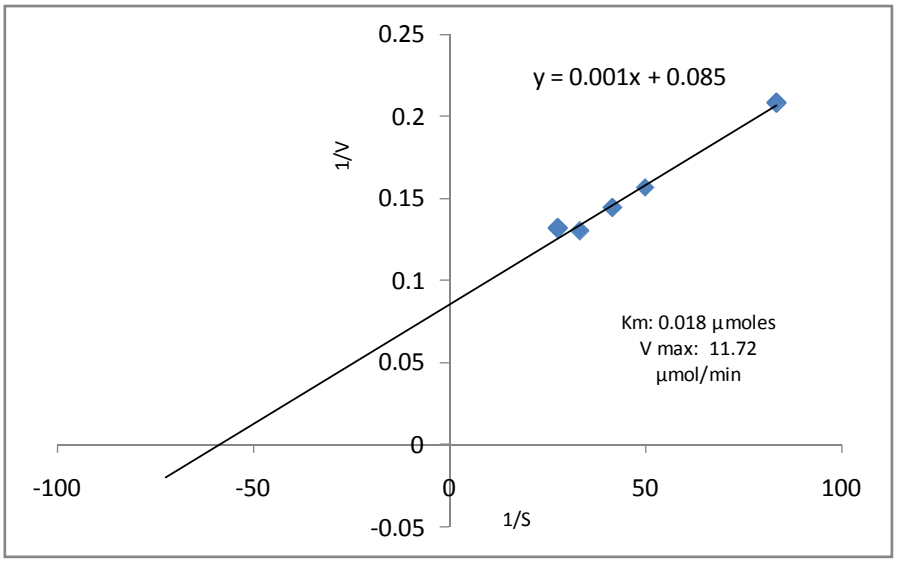


Fig 4.12. Lineweaver-Burk plot

4.5 Discussion

Purification and characterisation of the enzymes are essential pre-requisites for its successful biotechnological application. Purification of xylanases from microorganisms generally requires precipitation of the active protein followed by two to three chromatographic steps (Sunna and Antranikian, 1997; Ninawe *et al.,* 2008). In the present study, xylanase from *Streptomyces* sp. ER1 was purified by a two-step process; cold acetone precipitation and DEAE-Cellulose ion-exchange chromatography.

4.5.1 Purification of xylanase from *Streptomyces* sp. ER1

Purification of an enzyme is essential for studying the properties and biochemical characterisation of any enzyme. Precipitation of enzyme protein using cold acetone is used for initial purification step in major enzyme purification strategies (Kurrataayun *et al.,* 2015; Nema *et al*., 2016; Tangtua *et al*., 2017). Crude xylanase produced by *Streptomyces* sp. ER1, under SmF was subjected to cold acetone (100%) precipitation. Further purification was carried out using DEAE-Cellulose column chromatography. After this step, 5.57 fold purification with 6.97% of xylanase recovery was attained.

The level of purification obtained varies depending upon the strain and the methods adopted for purification. Purification index reported for xylanase from *Bacillus pumilus* GESF-1 and *Bacillus licheniformis* was 7.83 and 7.9 fold and 7.9% and 6.2% yield, respectively, after subjecting to DEAE-Cellulose ion-exchange chromatograhy (Menon *et al.,* 2010; Chaturdevi and Khurana, 2016). Xylanase from *Pseudomonas* sp. XPB-6 and a halobacterium-OKH was purified to 2.15 and 4.02 fold, respectively, using DEAE-Cellulose ion-exchange chromatography (Sharma and Chand, 2012a; Sanghvi *et al.,* 2014).

In the present study, the purified sample obtained by DEAE-Cellulose chromatography along with crude enzyme extract were analysed by native and SDS-PAGE. The single xylanase of approximately 23 kDa molecular weight was purified from the crude extract. Similarly, most of the *Streptomyces* studied were shown to consist of one xylanase (Iizuka and Kawaminami, 1965; Kusakabe *et al*., 1977; Nakajima *et al.,* 1984). However, there was one report on the enzyme system of *Streptomyces ostreogriseus,* which consisted of many xylanases (Park and Toma, 1982). The molecular weight of xylanase of the other xylanolytic microorganisms such as *S. viridisporus* T7A, *S. thermoviolaceus* OPC-520, *Streptomyces* sp B-12-2, *Streptomyces* sp. QG-11-3, *Streptomyces* sp. SKK1-8, *S. cyaneus* SN32 and *S. chartreusis* L1105 were 15-36, 59, 33-54, 23.8-40.5, 20.5, 31.6 and 20 kDa, respectively (Magnuson and Crawford, 1997; Tsujibo *et al*., 1992; Elegir *et al*., 1995; Beg *et al.,* 2000; Meryandini *et al.,* 2006; Ninawe *et al*., 2008; Li *et al*., 2011). A xylanase with a low molecular mass would be highly valued in pulp bleaching, because smaller enzymes can access the fibre wall structure more easily and alter the pulp properties more readily (Camacho and Aguilar, 2003) . Thus, in the present study, the purified xylanase enzyme, with the molecular weight of approximately 23 kDa, could be employed in pulp bleaching.

4.5.2 Effect of temperature and thermal stability on xylanase activity from *Streptomyces* sp. ER1

Temperature can influence reaction rate of an enzyme. Higher temperature will lead to higher activity. But as protein, it will be limited by its thermal denaturation. Temperature plays a crucial role in the activity of enzymes. In the present study, the optimum temperature for xylanase activity was 60^{o}C. Many investigators reported optimum reaction temperature of 55 to 75°C for xylanase activity from *Streptomyces*

xylophagus, *Streptomyces* sp., *Streptomyces* sp. QG-11-3, *S. viridisporus* T7A, *S. thermoviolaceus* OPC-520, *Streptomyces* sp. B-12-2, S. *chartreusis* L1105, *Streptomyces* sp. S38, *S. cyaneus* SN 32 and *Streptomyces sp.* Ab106 (Iizuka and Kawaminami, 1964; Marui *et al*., 1985; Tsujibo *et al*.,1992; Elegir *et al*.,1995; Magnuson and Crawford, 1997; Beg *et al*., 2000; Li *et al*.,2011; Ninawe *et al*., 2008; Gosami and Pathak, 2013). Biobleaching with xylanase at high temperature improves the degree of delignification and the brightness of pulp (Bajpai, 1997). Xylanase enzymes to be used for biobleaching of pulps must be active at high temperatures (Polizeli *et al*., 2005).

Utilisation of enzymes in industrial processes often encounters the problem of thermal inactivation of the enzyme. In the present study, the enzyme retained 84.82% of xylanase activity after 6 h of incubation at 60^{o}C. Thus, this study reveals that the enzyme is robust and maintains its properties for long duration for their applicability, especially in paper and pulp bleaching.

4.5.3 Effect of pH on xylanase activity and stability from *Streptomyces* sp. ER1

Xylanase activity is significantly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution of both substrate and enzyme molecules. pH can exert its effect in different ways; on the ionisation of groups in the enzyme's active site, either on the ionisation of groups in the substrate, or by affecting the conformation of either the enzyme or the substrate. These effects are manifested in the changes in kinetic constants, in K_m and V_{max} (Dennison, 2003).

In the present study, the optimum pH for xylanase activity was found to be 7. Xylanase was quite steady in a wide range of pH from 5 to 9.

The decline in enzyme activity above 9 and below 5 may be due to alteration of substrate binding and catalysis, which is often affected by charge distribution on both the substrate and particularly enzyme molecules (Shah and Madamwar, 2005). Characterisation of several actinomycete xylanases showed the neutral nature of xylanase. The optimum pH for xylanolytic activity of *Streptomyces xylophagus*, *Streptomyces* sp. QG-11-3, *S. viridisporus* T7A, *S. thermoviolaceus* OPC-520, *Streptomyces* sp. B-12-2, *S. chartreusis* L1105, *S. cyaneus* SN 32, *Streptomyces* sp. S38 and *Streptomyces* sp. ESRAA-301097 were 6.2, 6.7 - 7.7; 6 – 7; 7 -8; 8.6; 6; 6.8 - 7.8 and 6 – 8, respectively (Iizuka and Kawaminami, 1964; Tsujibo *et al*., 1992; Elegir *et al*., 1995; Magnuson and Crawford, 1997; Beg *et al.,* 2000; Ninawe *et al*., 2008; Li *et al*., 2011; ElGendy and ElBondkly, 2014). On the other hand, the optimum pH for xylanase activity from *Streptomyces* sp. Ab106, *S. actuosus* A-151 and *S. olivaceoviridis* A1 were found to be 9 (Techapun et *al*., 2002; Wang, *et al.,* 2003, 2007).

In the present study, the relative xylanase activity at pH 8 was 98.69% with that at pH 7. Therefore, this xylanase could be described as an alkaline enzyme and suitable for pre bleaching process.

4.5.4 Effect of metal ions on xylanase activity from *Streptomyces* sp. ER1

Usually, enzymes need some activators to express their catalytic power. Cofactor-mediated activation is used in industry to increase catalytic efficiency. In the present study, all the metal ions were tested at a concentration of 5 mM. Hg^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+}, significantly reduced the xylanase activity compared to that of control. Xylanase activity was strongly inhibited by Hg^{2+}, which was possibly due to combination with sulfhydryl groups, indicating that there was an important cysteine residue in or close to

the active site of the enzyme (Nakamura *et al.,* 1984). The decrease in the xylanase activity by Cu^{2+} ions might be due to the auto oxidation of cysteine molecules which lead to the formation of intra and intermolecular disulphide bridges or to the formation of sulfenic acid (Vieille and Ziekus, 2001). Similar findings on the inhibitory effect of metal ions on xylanase activity have been reported in previous studies (Wejse *et al.,* 2003; Menon *et al*., 2010). Inhibitory action of Cu^{2+} and Fe^{3+} on xylanase activity from *Streptomyces* sp. AMT 3 was also reported (Nascimento *et al.,* 2002).

In the present study, the metal ions such as Co^{2+}, Mn^{2+}, K^{+}, Ca^{2+} and Mg^{2+} were found to stimulate the xylanase activity. Similar findings were reported in previous studies (Menon *et al*., 2010; Yin *et al*., 2010). Significant increases in the activity of xylanase in the presence of Mn^{2+} were in concurrence with that of *Bacillus* sp. GRE 7 (Kiddinamoorthy *et al.,* 2008). However, in contrast to the present findings, Mn^{2+} has been reported to inhibit xylanase activity of *Bacillus* sp. K-1 and *Bacillus halodurans* S7 (Ratanakhanokchai *et al.,* 1999).

4.5.5 Effect of organic solvents on xylanase activity from *Streptomyces* sp. ER1

Stability of xylanase in various organic solvents, hexane, ethyl acetate, chloroform and butanol, was studied. After incubation with n-hexane, the residual xylanase activity increased to 135.37% and the presence of ethyl acetate reduced the residual activity to 60%. Similar reports were found in the previous studies (El-Gendy and El-Bondkly, 2014 and Gaur *et al*., 2015). However, the presence of butanol and chloroform had shown to affect adversely the enzyme, in the present study, since enzyme activity dropped to < 60 % of residual activity.

The increase in xylanase activity with non-polar solvents (hexane) might be due to their hydrophobic properties and decrease in activity with ethyl acetate, butanol and chloroform could be attributed to the high polarity of these solvents that stripped the water layer surrounding the enzyme causing enzyme inactivation (Woldesenbet *et al.,* 2012). Stability of enzyme in organic solvents is considered to be significant in industries, such as fine chemical synthesis (Gargouri *et al.,* 1984).

4.5.6 Effect of surfactants and other additives on xylanase activity from *Streptomyces* sp. ER1

Various additives and surfactants were tested to study their influence on xylanase activity under standard assay conditions. In the present study, surfactants, such as Triton X 100, Tween 80, Tween 60, polyethylene glycol and olive oil increased xylanase activity. However, SDS and β-mercaptoethanol reduced xylanase activity. Similar results were observed by El-Gendy and El-Bondkly (2014). It is believed that the addition of surfactants, like Tween 80 and polyethylene glycol played an important role in preventing nonspecific binding of the enzyme to substrate, allowing more enzymes to be available for the conversion of substrate and results in a higher conversion rate (Palmer, 2000).The decrease in xylanase activity in the presence of β-mercaptoethanol indicated the relationship between the reduced form of the cysteine residue and the reduced xylanase activity (Ximenes *et al.,* 1999; Bataillon *et al.,* 2000). The severe reduction in enzyme yield by SDS might be due to conformational changes in the tertiary secondary structure of the protein, binding of SDS to the active site of the enzyme or by changing the substrate nature through decreasing the availability of reaction sites. Previously xylanase production by *Streptomyces chartreusis* L1105 and alkalophilic *Streptomyces* species greatly enhanced when the medium was supplemented with Tween 80

(Sharma and Bajaj, 2005; Li *et al.,* 2011). Surfactant stable enzymes are used in industries such as detergents and dishwashers (Joseph et *al.,* 2007).

The present study established a simple strategy for the purification of xylanase from *Streptomyces* sp. ER1 under SmF. The biochemical properties of the purified xylanase indicated its possible applications in processes operated at high temperatures and alkaline pH. Studies on characterisation of xylanase from *Streptomyces* sp. ER1 showed that the enzyme could be potent in many industrial applications, like the bio-bleaching of kraft pulp, detergent industrial applications and saccharification of agro-residues to produce fermentable sugars for bioethanol production.

Chapter -5

XYLANASE PRODUCTION USING AGROWASTES AND WATER HYACINTH AND APPLICATIONS OF XYLANASE IN PAPER DEINKING AND FRUIT JUICE CLARIFICATION

Contents

5.1 Introduction

White biotechnology, which can be defined as the use of biotechnology for industrial production, remains a challenge as new biocatalytic processes have to try to win economically with well established chemical processes that have been optimised for years (Otten and Quax, 2005). In this context, agricultural waste utilisation for industrial development has emerged as a significant area in industrial biotechnology (Dashtban *et al.,* 2009).

The conversion of lignocellulosic biomass into valuable enzymes is of tactical importance for the sustainability and advancement of several industries. These materials may be used by microorganisms as a carbon source, which results in the production of cellular proteins, enzymes, organic acids, biologically important secondary metabolites and prebiotic

oligosaccharides (Chandel *et al.,* 2012; Anwar *et al.,* 2014). Successful biotechnological applications need low-cost enzymes. Lignocellulosic residues not only provide as cost–effective substrates but also offer environmental advantages.

Xylanase is one of the most important industrial enzymes applied in paper and pulp industry, food industry, textiles, feed, pharmaceuticals etc. Because of their biotechnological importance, major progress has been made in the use of agro-residues for microbial xylanase production. The use of purified xylan as an inducer for improved xylanase production is not viable and needs alternative sources.

In India, the applications of xylanolytic enzymes in the bioconversion of agrowastes are still incomplete. Lack of the basic knowledge for the application of xylanolytic enzymes in the bioconversion process is one of the known factors. Studies on the use of agricultural wastes as the carbon source for the industrial xylanase production by *Streptomyces* sp. is scarce and not methodically studied compared and reported in SmF and SSF, although these agricultural residuals are simple and cost-effective to yield xylanase. By reusing and recycling the organic agricultural wastes and converting them into resources, it becomes better alternative as the carbon source for industrial xylanase production.

Keeping the above in view, the present study is an attempt to enhance xylanase production by *Streptomyces* sp. ER1 through optimisation of nutritional/physical parameters by conventional OFAT from selected agrowastes and a novel substrate- water hyacinth (*Eichhornia crassipes*) and its application in newspaper deinking and fruit juice clarification.

5.2 Review of Literature

The choice of the substrate is of great significance for the selection of the fermentation process and the successful production of xylanases. The use of agrowastes as substrates has led to higher yields of xylanase and a selective induction of xylanase, with concomitantly low cellulase activity in a number of microorganisms (Motta *et al.,* 2013). The market demand for xylanases has considerably increased during the past few decades. Since biotechnological applications require large quantities of low-cost enzymes, agrowastes as substrates promise as an alternative that could substantially reduce enzyme costs and increase productivity (Knob *et al.,* 2014).

The choice of suitable agrowaste for the fermentation process has indicated the type of a lignocellulosic material that favours both microbial growth and product formation (Kumar and Kanwar, 2012). The assessment of this parameter and other process conditions, such as temperature, pH, and aeration, has encouraged the screening of several agrowatses for the production of xylanases (Singhania *et al*., 2009). However, because the growth of microorganisms in different carbon sources is associated with a differential expression of functionally separate xylanases, much exploration still needs to be carried out to identify sustainable substrates that allow production of xylanases with desirable characteristics (Badhan *et al*., 2007). Since compounds derived from xylan are necessary for xylanase induction, several reports evaluated the xylanase production using isolated xylans as substrate. However, the high cost of xylans has limited their applications for large-scale production processes (Knob *et al.,* 2014).

In this context, some lignocellulosic substrates, like sawdust, sugarcane bagasse, soy flour, maize straw, or wheat bran, have been compared in relation to purified xylans (Okafor *et al*., 2007; Goyal *et al*.,

2008 ; Jhosi and Khare 2012; Guimaraes *et al*., 2013) . Many of these have performed considerably better than isolated xylans with respect to the yields of xylanase in large-scale production processes. However, the winning application of enzymes depends not only on the substrate choice but on the optimisation of production, which may directly result in cost reduction (Knob *et al.,* 2014).

Natural xylan sources, such as sugarcane bagasse, wheat bran, rice bran and corn cob that are copiously available in many countries are the potential raw materials used as the carbon source for xylanase production (Gupta and Kar 2009). Since, the present chapter deals with optimisation of xylanase by *Streptomyces* sp. ER1 using selected substrates, like sugarcane bagasse, corn cob, coconut oil cake and water hyacinth, literature pertaining to the selected substrates is only discussed.

Sugarcane bagasse

Sugarcane is one of the main crops cultivated in countries, such as Brazil, India, and China (Chandel *et al.,* 2012). The remnants after the juice extraction from the sugarcane stem are called sugarcane bagasse (Pandey *et al.,* 2000). Sugarcane bagasse represents a large portion of annually collected agro-residues.

Thermoascus aurantiacus ATCC 204492 produced a higher level of thermostable xylanase when sugar cane bagasse was used as a substrate (Milagres *et al.,* 2004). The use of SSF for enzymes production is an attractive alternative for the valorisation of sugarcane bagasse. Higher xylanase yields under SSF using sugarcane bagasse as a substrate were obtained with *Aspergillus niger, T. aurantiacus, Streptomyces viridosporus, Thermomyces lanuginosus* SSBP and *Penicillium citrinum* MTCC 2553

(Gutierrez- Correa and Tengerdy, 1998; Milagres *et al.,* 2004; Alberton *et al.*, 2009; Manimaran *et al.,* 2009; Ghoshal *et al.,* 2012).

Many investigations have also been carried out to assess xylanase production when employing sugarcane bagasse under SmF. Optimisation of growth conditions for xylanase production by *T. lanuginosus* under SmF resulted in substantial xylanase activity of 946 U/mL (Ali *et al* ., 2013). Higher xylanase production using sugarcane bagasse as substrate under SmF was reported with *Thermomonospora curvata, Trichodema harzianum* and *Streptomyces rameus* (Stutzenberger, 1994; Rezende *et al.*, 2002; Bhosale *et al.,* 2011).

Corn cob

Corn cob represents large, renewable sources of lignocellulosic biomass. It is a significant by-product of the sweet corn processing industry (Kumar *et al.,* 2008). Because of its rich nutritional content, corn cob can be used as a medium to cultivate microorganisms. Moreover, the elevated xylan content in corn cob (40%), the highest among all agricultural waste, makes it a potential substrate for xylanase production (Ashour *et al.,* 2013).

Remarkable xylanase production by *Aspergillus foetidus*, using corn cob under optimised growth conditions in SSF, taking into account the parameters of temperature, pH, moistening agents, moisture level, and nitrogen sources was observed (Shah and Madamwar, 2005). The xylanase production process by *A. niger* CECT 2700 was successfully established in a laboratory- scale horizontal tube bioreactor, achieving the highest xylanase activity of corn cob under SSF (Perez-Rodriguez *et al.,* 2014).

Corn cob has a good potential as a substrate for xylanase production under SmF. *T. lanuginosus, Streptomyces cyaneus, T. lanuginosus* MC 134, and *Streptomyces* sp. CS628 has been reported to be one of the best

producers of xylanase when grown on corn cob under SmF (Singh *et al.,* 2000; Ninawe and Kuhad, 2005; Kumar *et al.,* 2009; Rahman *et al.,* 2014).

Oil Cakes

Oil cakes are by-products obtained after oil is extracted from seeds. These materials have been explored by several researchers for their use as substrates or as supplements for production media in enzyme production (Ramachandran *et al.,* 2007). Oil cakes, such as soya oil cakes and deoiled jatropha seed cake have occasionally been used for xylanase production by *P. Canescens* and *Scytalidium thermophilum,* respectively (Assamoi *et al.,* 2008; Joshi and Khare, 2011). Literature pertaining to xylanase production from coconut oil cake is almost nil.

Water hyacinth (*Eichhornia crassipes*)

The water hyacinth is a native plant of Brazil but has been naturalised in many tropical/temperate countries. It is a free-floating aquatic plant and has spread to more than 50 countries on five continents (Cavallo, 2002). The plant sustains extremes in water level fluctuations, seasonal variations in flow velocity, nutrient availability, pH, temperature and toxic substances (Eusuf *et al.,* 1985). It can even grow at salinity levels up to 0.24% as was reported in Indonesia. The coverage of waterways by water hyacinth has created various problems, such as destruction of ecosystems, irrigation problems and an increase in mosquito populations (Zhuan, 2006). It is a noxious weed which rapidly depletes the nutrient and oxygen content of the water, thereby affecting the flora and fauna of the ecosystem and also interferes with water transportation and fishing. One of the major rivers in Pune, India i.e. the River Mula is also facing a problem due to the weed infestation and an enormous amount is being spent for the control of these weeds that are causing problems environmentally, economically and

socially (Patel, 2012). In spite of the attempts, they still continue to be a hazard. Therefore, attempts have been geared towards the use of biological, chemical and mechanical approaches for preventing the spread of, or eradication of, water hyacinth (Masami *et al*., 2007; Kurup *et al.,* 2005). Large availability of water hyacinth makes it an attractive raw material. The use of this weed as a suitable substrate for xylanase is being considered as they do not compete for land, have a negligible cost and grow at a fast rate (Fileto-Perez *et al.,* 2013).

5.2.1 Pre-treatment of Substrates for Xylanase Production

Pre-treatment of lignocellulosic biomass is a crucial step before enzymatic hydrolysis. It enhances enzymatic hydrolysis of lignocellulosic substrates. Steaming with or without explosion, is an accepted method of pre-treatment. Steam explosion is initiated at a temperature of 160-260^{o}C, from several seconds to few min before the material is open to atmospheric pressure. Steam explosion is a basic pre-treatment method of lignocellulosic materials because the process has relatively moderate energy cost (Sun and Cheng, 2002).

Another method of pre-treatment is the use of dilute alkali. Higher yields of reducing sugars were achieved when substrates, such as wheat straw, rice straw and corncobs were pre-treated with dilute NaOH as compared to untreated substrates. After an initial phase of rapid sugar formation, the rate of hydrolysis decreased. This may be due to enzyme inactivation or depletion of an easily hydrolysable fraction of hemicelluloses. Untreated substrates were found fewer available towards enzymatic hydrolysis as evidenced by very low amount of reducing sugar. Detection of sugar from the hydrolysate by thin layer chromatography exhibited remarkable presence of xylose with very less amount of glucose in

all the pre-treated substrates. Hence methods, dilute NaOH was proved to be beneficial in bioconversion of agro residues (Chapla *et al.,* 2010). Similar reports were obtained with dilute NaOH treated jute fibre, corn cob powder and bleached bagasse pulp (Gokhale *et al.,* 1998).

5.2.2 Optimisation of process parameters

In most cases, agro-residues used to produce enzymes by SSF or SmF either do not contain all the necessary nutrients for this function or they may be available but in sub-optimal concentrations. In these conditions, the substrate must be supplemented to stimulate or improve enzyme production by the addition of extra carbon or nitrogen sources (Galiotou-Panayotou and Kapantai, 1993; Patil and Dayanand, 2006; Ritter *et al*., 2013). Supplementation can also be carried out by using a mineral salt solution to adjust the initial moisture content of the residue (Khandeparkar and Bhosle, 2006; Papinutti and Forchiassin, 2007). Optimisation of fermentation conditions, like pH, incubation temperature, agitation speed, substrate concentration, inoculum age and size also influence enzyme production (Frost and Moss, 1987).

5.2.3 Application of xylanase

5.2.3.1 Deinking

The raw material for the paper production, cellulosic pulp, is obtained from the softwood and hardwood collected from forests. The scarcity of the green plants and the policy of conservation of forests have provoked the paper industry to look for alternative sources for cellulosic pulp. Recycled waste paper materials generated from different sources, such as school waste papers, office waste papers, newspapers, xeroxed papers and laser/inkjet printed papers offer cheaper and eco-friendly source of pulp. Old newsprint is the major material reused for papermaking (Virk *et al.,*

2013). Deinking is an important step in the recycling process and involves the dislodgement of ink particles from fibre surface and then removal of the detached ink particles by flotation, washing etc. (Ibarra *et al.,* 2012). The developments in the deinking process have hugely helped the utilisation of secondary fibre. It is easier to remove impact ink in old newsprint as compared to the non-impact ink present in xeroxed or laser/inkjet printed papers (Virk *et al.,* 2013).

Utilising chemicals, such as sodium hydroxide, sodium silicate, hydrogen peroxide, chlorine based chemicals and other chelating agents in the conventional methods of deinking, is environmentally hazardous (Maity *et al.,* 2012). Enzymatic deinking has involved enormous attention by reducing the consumption of chemicals thereby lowering the process cost and making it eco-friendly (Ibarra *et al.,* 2012). Enzymes can act either directly on the fibre or on the ink (Call and Stettmatter, 1992). The major factors affecting the deinking process included the consistency of pulp, pulp pH and operating temperature (Pathak *et al.,* 2011).

In case of newspapers, enzymatic deinking using xylanase often resulted in pulp having enhanced brightness, when compared to chemical deinking (Sykes *et al.,* 1996). Cellulose, being the basic composition of paper, is covalently bound by hydrogen bonding with xylan and lignin. Xylanases act on xylan molecule causing the detachment of ink particles (Michelin *et al.,* 2010). Hence, there is need for xylanase which degrades only the xylan part of newsprints and by this means removes lignin without adversely affecting pulp strength. The above objective could be fulfilled by the use of suitable cellulase-free xylanase (Masui *et al.,* 2012). For deinking purpose, purified xylanase is not essential; as the purification adds to the cost of the process (Anthony *et al.,* 2003). Crude xylanase can capably serve as deinking agent (Mohandass and Raghukumar, 2005). The deinking

efficiency of paper pulp can be measured by either determining the brightness of the pulp or estimating the visible ink specks on paper made from the deinked pulp (Borchardt, 1997; Li *et al.,* 2011a). Many workers have studied xylanase as an efficient deinking agent (Zhang *et al.,* 2008; Maity *et al.,* 2012; Virk *et al.,* 2013; Pathak *et al.,* 2014).

5.2.3.2 Fruit juice clarification

The traditional method of juice extraction is through the use of mechanical presses viz., traditional rack and cloth press, screw presses, Bucher-Guyer horizontal press, and the belt press. Juice extraction can also be done by using screw type juice extractor, fruit pulper, diffusion extraction, decanter centrifuge (Lotha *et al.,* 1994; Beveridge and Rao, 1997). The yield of juice using such juice extraction methods can be increased by combining them with enzymatic extraction (Chadha *et al.,* 2003).

The enzymatic process has more advantages over mechanical-thermal comminution of several fruit pulps. In particular, the use of xylanases has been an essential part of the modern fruit processing technology. The enzyme treatment not only facilitates easy pressing and increase in juice recovery but also ensures the highest possible quality of the end products (Kilara, 1982; Roumbouts and Pilnik, 1978). These enzymes help in softening the plant tissue and release of cell contents that may be recovered with high yield (Sreenath *et al.,* 1984). Clarification is a process by which the semi-stable emulsion of colloidal plant carbohydrates of a freshly pressed juice is "broken". During this process the viscosity of the juice is reduced and the opacity of the cloudy juice is changed to an open splotchy look. This can also be accomplished enzymatically (Kilara and Van Buren, 1989). Fruit contains polysaccharides which may lead to fouling

during filtration through membrane. Enzymatic treatment causes degradation of polysaccharides, like hemicelluloses. Enzymatically clarified juice results in viscosity reduction and cluster formation, which facilitates separation through centrifugation or filtration. As a result, the juice presents higher clarity, more concentrated flavour and colour (Abdullah *et al.,* 2007).

Enzymatic hydrolysis of the cell walls increased the extraction yield, reducing sugars, soluble dry matter content, galacturonic acid content and titrable acidity of the products (Joshi *et al.,* 1991). The resultant pulp had a lower viscosity and the quantity of waste pomace is reduced (Dorreich, 1996). Enzymatic degradation of the biomaterial is depended upon the type of enzyme, incubation time, incubation temperature, enzyme concentration, agitation, pH and use of different enzyme combinations (Bauman, 1981).

Xylanase produced by *Pseudomonas* sp., *Bacillus pumilus* SV-85S and *Bacillus megaterium* were used for clarification of musambi orange, apples, pineapples and tomato juices (Sharma and Chand, 2012b; Nagar *et al.,* 2012; Padke and Momin, 2015).

So far, no work has been done on optimisation of xylanase production using water hyacinth and coconut oil cake. Moreover, literature pertaining to application of xylanase from Cochin estuarine *Streptomyces* sp. in newspaper deinking and fruit juice clarification is scarce.

5.3 Materials and methods

5.3.1 Substrates

Wheat bran, rice bran, corn cob, groundnut oil cake, coconut oil cake, sugarcane bagasse, corn flour, potato peel, banana peel, soya bean meal, neem oil cake and water hyacinth were used as substrates. Wheat bran, rice bran, corn cob, groundnut oil cake, coconut oil cake, sugarcane bagasse

waste, corn flour, soya bean meal, neem oil cake was procured from a local market. Corn cob was prepared by stripping corn of all the kernels. Raw banana and potato, purchased at a local store, were washed three times with water in order to remove unwanted dust particles, peeled and chopped into small pieces. Water Hyacinth was collected from Vembanad Lake, Kochi, Kerala, was washed three times with water in order to remove unwanted dust particles and the shoots and the leaves were chopped into small pieces. All the agrowastes were dried in an oven at 110^{o}C till constant weight was achieved and stored for further use at 4^{o}C.

5.3.2 Pre-treatment of substrates

The samples were autoclaved for 1 h at 121ºC with 5% (w/v) NaOH (20 mL per g of substrate) in separate conical flasks for delignification. They were then neutralized with 1M HCl.

5.3.3 The selected microorganism used for the optimisation studies.

The selected isolate of high xylanase producing, *Streptomyces* sp. ER1 was used in the present study.

5.3.4 Inoculum

The inoculum was raised in the xylanase production medium A described under Table 3.1. The cultures were grown in an Erlenmeyer flask containing 100 mL of the xylanase production medium A on a rotary shaker (100 rpm) at ambient room temperatures (35+/- 2°C). Unless otherwise specified, the inoculum of age 24 hrs was used at 5% v/v (452X10^{1} CFU/mL).

5.3.5 Xylanase production by SSF

The medium for SSF contained 10 g of each of twelve substrates and 6 mL of the xylanase production medium A in separate Erlenmeyer flasks (250 mL). Each flask was inoculated and incubated at 35+/- 2°C for 120 h.

5.3.6 Extraction of enzyme

After 120 h of incubation, the enzyme was extracted from the SSF media). 50 mL of cold sodium phosphate buffer (0.1 M) was added to the fermentation flasks and agitated at 100 rpm on an incubator shaker at 25°C for 2 h. Contents of the flasks were then filtered with autoclaved cheese cloth and centrifuged at 4000 rpm for 10 min. Supernatant was collected and filtered 3 times with Whatman No.1 filter paper and the filtrate was centrifuged at 10,000 rpm at 4°C (Eppendorf, Germany) for 20 min (Alva *et al.*, 2007). The clear supernatant was used as crude enzyme and used for xylanase assay.

5.3.7 Xylanase production by SmF

SmF was carried out using xylanase production medium A in 250 mL Erlenmeyer flasks with a working volume ratio of 0.4 (100mL medium in 250 mL flasks). The carbon source of the production medium A was replaced with 2% (w/v) of each of the twelve substrates, in separate Erlenmeyer flasks (250 mL). Each flask was then inoculated and incubated at temperature 35+/- 2°C for 120 h on a rotary shaker.

5.3.8 Extraction of enzyme

After 120 h of incubation, each sample was then centrifuged at 10,000 rpm and at 4°C for 20 min, and the clear supernatant was used as crude enzyme and assayed for xylanase activity.

5.3.9 Xylanase assay

Xylanase activity was measured following the xylanase assay as described under the section 3.3.7.

5.3.10 Selection of production media for xylanase production

Three xylanase production media were tested in order to select suitable medium, for optimising xylanase production from selected substrates. The media used were Medium A (Techapun *et al.,* 2001), Medium B (Roy, 2004) and Medium C (Mandels and Sternburg, 1976). Enzyme production was carried out in 250 mL Erlenmeyer flasks with 100 mL of the 3 production media (pH 7), separately, inoculated with 5% (v/v) inoculum and incubated at 35±2°C under shaking condition (100 rpm) for 120 h. After incubation, culture broth was centrifuged at 10000 rpm for 15 min at 4°C, and the cell free supernatant was used as crude enzyme to measure xylanase activity. Comparing xylanase activity (U/mL) obtained with the 3 different production media; the best medium was selected based on the highest value for xylanase activity (U/mL). Thereafter, the selected medium was used to investigate the effect of different concentrations of the medium components and other fermentation conditions on the extracellular xylanase production by *Streptomyces* sp. ER1 from the selected substrates.

5.3.11 Optimisation of nutritional and physical parameters by OFAT method

In the conventional scaling-up approach, various nutritional and physical parameters were optimised by maintaining all factors at a constant level in the basal medium, except the one under study. Each subsequent factor was examined after taking into account the previously optimised factor(s). The optimisation was done for selected agrowastes as substrates under SmF.

5.3.11.1 *Effect of incubation period on the xylanase production with the selected substrates*

Xylanase fermentation experiments were carried out up to 120 h in selected production medium and xylanase activity measured at 24 h intervals.

5.3.11.2 *Effect of age of inoculum on the xylanase production with the selected substrates*

To monitor the effect of inoculum age on xylanase production, culture was incubated for 16, 20 and 24 h separately. The culture was harvested at respective time intervals of 6, 20 and 24 h and used to inoculate the production medium with an inoculum size of 5% (v/v). In order to minimize the time lag in fermentation process, inoculum was raised in the medium with a composition similar to that of the fermentation medium (Lincoln, 1960). The fermentation was carried out and the culture supernatants were used for xylanase assay.

5.3.11.3 *Effect of salinity on the xylanase production with the selected substrates*

The production medium was prepared using distilled water and seawater of different salinities (0, 5, 10, 15, 20 ppt). The fermentation was carried out and xylanase activity was measured at different saline conditions.

5.3.11.4 *Effect of pH on the xylanase production with the selected substrates*

In order to study the effect of initial pH of the culture medium on the production of xylanases by *Streptomyces* sp. ER1, xylanase production medium was prepared with the initial media pH adjusted within a range of 6

to 9 at 1 unit increment. In all cases the initial pH values have been uncontrolled throughout the fermentation. The fermentation was carried out and the culture supernatants were used for xylanase assay.

5.3.11.5 *Effect of temperature on the xylanase production with the selected substrates*

Streptomyces sp**.** ER1 was grown in xylanase production medium at varying temperatures ranging from 30-40°C (30, 35 and 40°C). The temperatures were set at different incubators without shaking. The fermentation was carried out and the culture supernatants were used for xylanase assay.

5.3.11.6 *Effect of agitation on the xylanase production with the selected substrates*

SmF was carried out at different agitation rates ranging from 50, 100 and 200 rpm in a shaker incubator and xylanase production was studied.

5.3.12 Optimisation of Nutritional Parameters

The optimisation studies were conducted to evaluate the higher production environment using three different strategies i.e. studies on the optimisation of carbon sources, nitrogen sources and mineral ions for the enhanced production of xylanases by *Streptomyces* sp. ER1 with selected substrates**.**

5.3.12.1 *Effect of different levels of selected substrates on Xylanase production*

The selected substrate was tried at different concentrations and the subsequent effect on the fermentation profile of *Streptomyces* sp. ER1 was monitored. The different concentrations tried were (% w / v): 0.5 to 3.0 with

0.5 unit difference. The culture supernatants were taken for estimating xylanase assay.

5.3.12.2 *Effect of nitrogen source on xylanase production with selected substrates*

The production of xylanase was analysed in different nitrogen sources. The nitrogen source giving highest enzyme yield in comparison with the control was treated as the potent one and used for further studies.

5.3.12.3 *Nitrogen sources studied for the enhancement of xylanase production.*

The nitrogen sources used were organic nitrogen sources like tryptone, beef extract, yeast extract, peptone, albumin, casein, soya bean meal, urea and inorganic nitrogen sources like ammonium chloride, di ammonium phosphate, ammonium sulphate and potassium nitrate.

To define the effect of various nitrogen-sources on xylanase production, 100 mL of selected xylanase production medium was prepared in 250 mL Erlenmeyer flasks and supplemented with each of the above said inorganic and organic nitrogen sources separately. Xylanase assays were performed and the production medium without the nitrogen source was taken as control.

5.3.12.4 *Effect of surfactants and other additives on xylanase production*

The effect of different surfactants (Tween 60, Tween 80) and other additives (olive oil, poly ethylene glycol) on xylanase production was investigated by individually supplementing these in the selected production medium. The fermentation was carried out and the culture supernatants were analysed for xylanase assay. The production medium without the additive source was taken as control.

5.3.13 Application of crude xylanase in deinking of newspaper

5.3.13.1 *Preparation of paper pulp*

Newspapers were pulped by soaking in hot water for 2 h and macerated in a domestic mixer after adding 0.1% Tween 80, a non-ionic surfactant. The pulp was oven dried at 50°C and stored in sterile container at 4°C until further use (Mohandass and Raghukumar, 2005).

5.3.13.2 *Deinking of paper pulp using cell-free bacterial culture supernatants*

Streptomyces sp. ER1 was grown in nutrient broth supplemented with Tween 80 for 120 h at 35^{o}C. The 120 h old cultures were centrifuged (8000 rpm for 15 min) under sterile conditions and the clear cell-free culture supernatant was used. Immediately before use, the pulp was soaked in water for 30 min. Pulp at a consistency of 3-6% was sterilised by autoclaving. After cooling it to room temperature it was incubated with 50 mL of the cell-free culture supernatant for 120 h at 60^{o}C. After 120 h, the pulp was washed thoroughly with tap water. The washed pulp was filtered over a Buchner funnel under suction to obtain the pulp in a form of hand sheets. The hand sheets thus obtained were pressed flat between two stainless steel plates and oven-dried at 50^{o}C for 5 days. Newspaper pulp without treatment with bacterial culture supernatant was used as control (Mohandass and Raghukumar, 2005).

5.3.13.3 *Analysis of Collected Filtrate*

The colour removal from the pulp was determined with a spectrophotometer from 200 nm to 800 nm. The phenolic compounds generated were estimated by measuring the absorbance at 237 nm and

release of hydrophobic compounds were measured from the absorbance at 465 nm (Patel *et al.,* 1993; Gupta *et al.,* 2000).

5.3.13.4 ***Clarification of fruit juices***

Xylanase enzyme purified as described under section 4.3.7 was used for fruit juice clarification.

Orange (*Citrus sinensis*), Pineapple (*Ananas comosus*) and Musambi (*Citrus limetta*) were purchased from local markets, washed, peeled and macerated using a blender to form a smooth textured pulp. The purified xylanase (20 U/g of xylanase) and pulp were incubated at 60^{o}C. After incubation, the suspension was heated in boiling water bath for 5 min; cooled, filtered and centrifuged at 10,000 rpm for 15 min. The supernatant (juice) was used for determining juice clarity by recording transmittance at 650 nm taking distilled water as the blank. % Clarification was calculated as follows:

% clarification = Tt – Tc/Tc x 100;

Where, Tt = Transmittance of test; Tc = Transmittance of control.

Effect of clarification time for all the juices was studied for 4 h by taking 2 mL of the sample at every 30 min and transmittance was recorded at 650 nm. The flow rate of the juice was determined by filtering 2 ml of juice clarified by Whatman filter paper 1 and comparing its value by flow rate of control (Sharma and Chand, 2012). Total titrable acidity (% citric acid) of the juice was determined as described by Nagar (Nagar *et al.,* 2012). All the experiments were performed in triplicates and their mean values are given. The untreated pulp for each of the fruit was kept as control.

5.4 Results

5.4.1 Comparison of xylanase production under SSF with SmF using different agrowastes

In all, 12 different substrates were subjected to SSF and SmF by *Streptomyces* sp. ER1. Fermentation was carried out in triplicates. It was observed that SmF was better than SSF as the former showed more enzyme production compared to the latter (Table 5.1). Further studies on optimisation of culture conditions were carried out under SmF.

Table 5.1. Effect of agrowastes on xylanase production under Submerged (SmF) and Solid State Fermentation (SSF)

Substrate	Xylanase activity (U/mL)	
	SmF	SSF
Corn cob	7394.4	479.33
Sugarcane bagasse	6965.07	1421.33
Water hyacinth	5984	1001.79
Coconut oil cake	4608.13	3069.33
Wheat bran	4494.22	1661.33
Rice bran	4298.67	1119.11
Soya bean meal	3718.22	497.77
Neem oil cake	3248.89	1248
Corn flour	2673.78	1384.89
Ground nut oil cake	2564.44	1996.44
Banana peel	2420	1439.67
Potato peel	1874.67	1407.33

5.4.2 Selection of substrates for xylanase production

In order to identify the capability of various hemicelluloses- rich substrates for xylanase production by *Streptomyces sp.* ER1, 12 substrates (Fig 5.1) were used as the carbon source. *Streptomyces* sp. ER1 was able to produce xylanase using all the 12 substrates as alternative cost-effective carbon sources. Among these substrates, maximum xylanase was produced with corn cob followed by sugarcane bagasse, water hyacinth and coconut oil cake under SmF, suggesting their importance in selecting them as substrates for xylanase production. Thus, the 4 substrates- corn cob, sugarcane bagasse, water hyacinth and coconut oil cake were selected for further optimisation studies under SmF. Fig 5.2 shows the image of growth of *Streptomyces* sp. ER1 on the selected substrates.

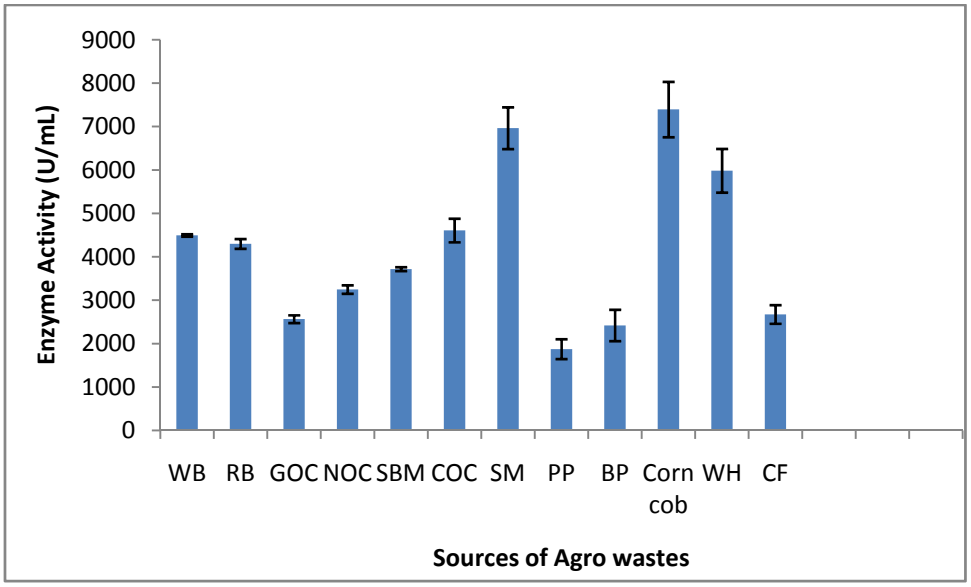


Fig 5.1. Selection of substrates for xylanase production

WB: Wheat bran**; RB:** Rice bran**; GOC:** Ground nut oil cake**; NOC:** Neem oil cake**; SBM:** Soya bean meal**; COC:** Coconut oil cake**; SM:** Sugarcane bagasse**; PP:** Potato peel**; BP:** Banana peel**; WH:** Water hyacinth**; CF:** Cornflour

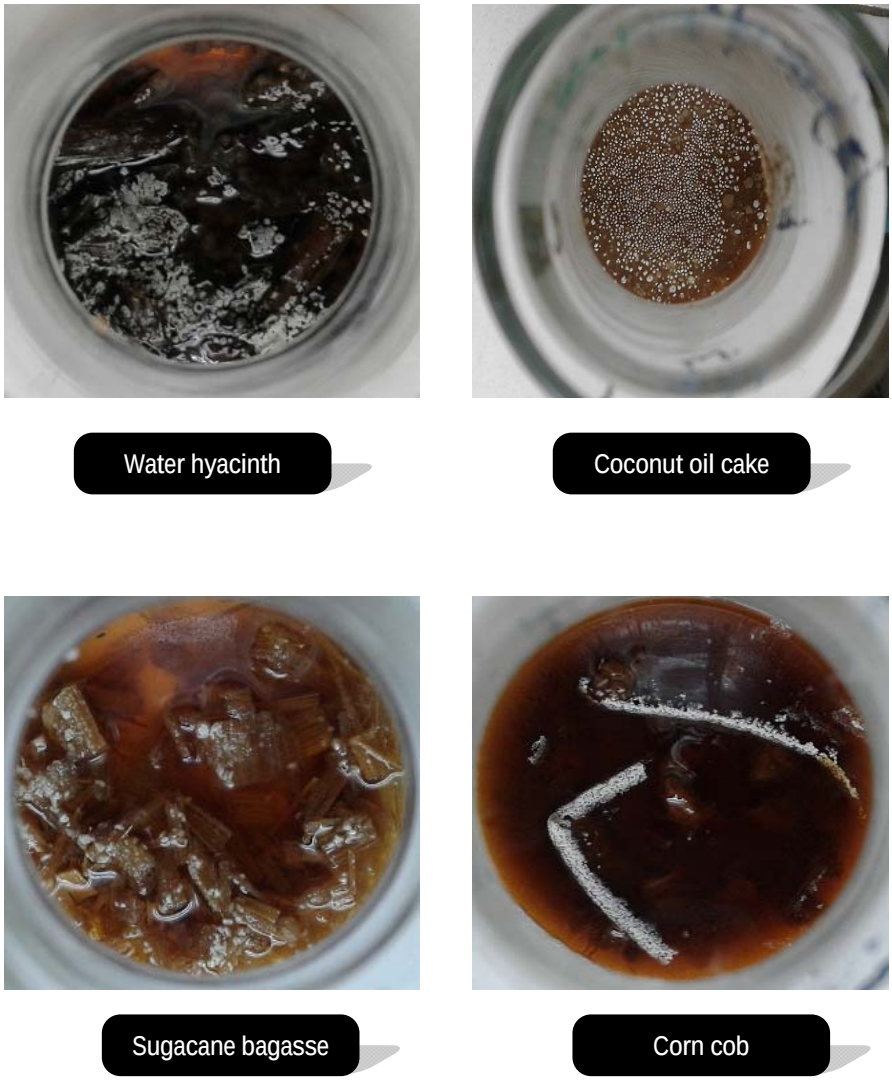


Fig 5.2. Growth of *Streptomyces* sp. ER1 on water hyacinth, coconut oil cake, sugarcane bagasse and corn cob.

5.4.3 Selection of production media for xylanase production using the selected substrates

Xylanase production in the 3 different production media with the selected substrates is given in Table 5.2. The production medium A was

selected as the suitable xylanase production medium for coconut oil cake and water hyacinth and production medium B was found to be suitable for sugarcane bagasse and corn cob, as they gave the highest xylanase activity with the respective substrates. Hence, xylanase production media, A and B, were selected for further optimisation of xylanase production with the selected substrates.

Table 5.2. Xylanase production in different production media with the selected substrates

Production medium	Substrate	Enzyme activity (U/mL)
A (Techapun *et al.*, 2001)	Coconut oil cake	4608.13
	Corn cob	5255.73
	Sugarcane baggase	1628.67
	Water hyacinth	5138.67
B (M9 medium) (Roy, 2004)	Coconut oil cake	301.86
	Corn cob	7491.87
	Sugarcane bagasse	6997.1
	Water hyacinth	2226.67
C (Mandels and Sternburg, 1976)	Coconut oil cake	3861.47
	Corn cob	4805.33
	Sugarcane bagasse	6289.33
	Water hyacinth	3413.33

5.4.4 Effect of incubation period on xylanase production using the selected substrates

It was found that the rate of enzyme production increased with the increase in the fermentation period and reached its maximum after 72 h of incubation with all the selected substrates (Fig 5.3). A prolonged incubation time beyond 72 h did not increase the enzyme production.

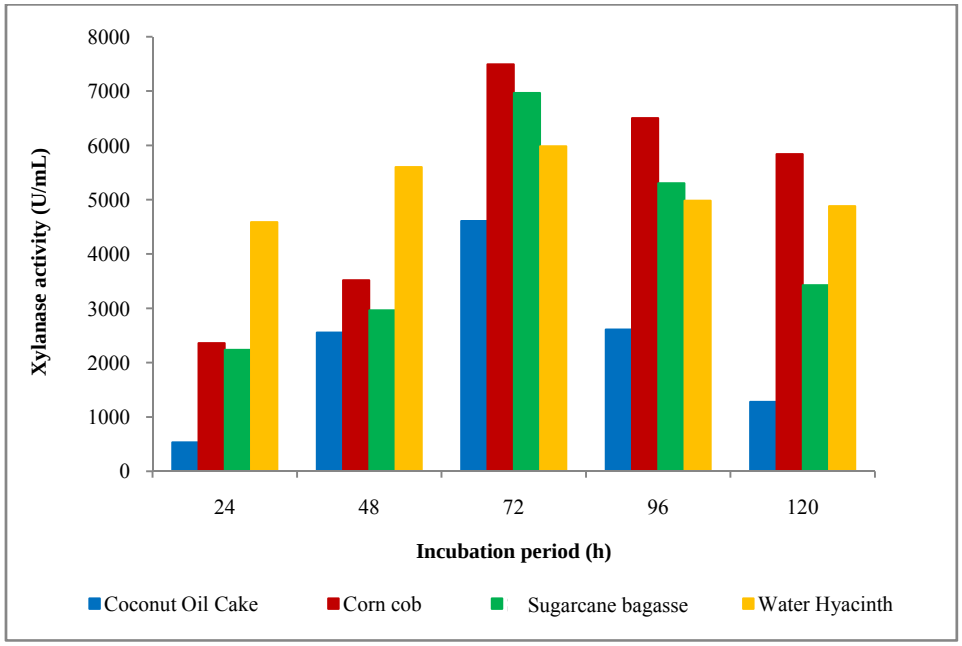


Fig 5.3. Effect of incubation period for xylanase production using selected substrates

5.4.5 Effect of age of inoculum and its concentration on xylanase production using the selected substrates

Impact of age of inoculum on xylanase production by *Streptomyces* sp. ER1 was evaluated and the results are presented in Fig 5.4. It was found that inoculation of production media with inoculum of age 20 h, supported maximum xylanase production with all the 4 selected substrates. Inoculum of age above and below 20 h did not support enhanced levels of xylanase production.

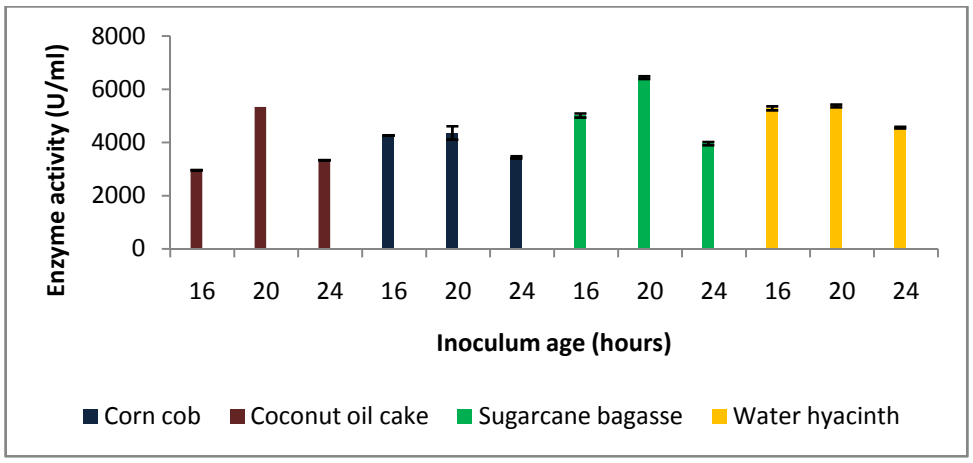


Fig 5.4. Effect of inoculum age on xylanase production using selected substrates

5.4.6 Effect of salinity on xylanase production using selected substrates

Effect of salinity on xylanase production was studied by varying the salinity of the production media from 0 ppt to 20 ppt. Results are presented in Fig 5.5.

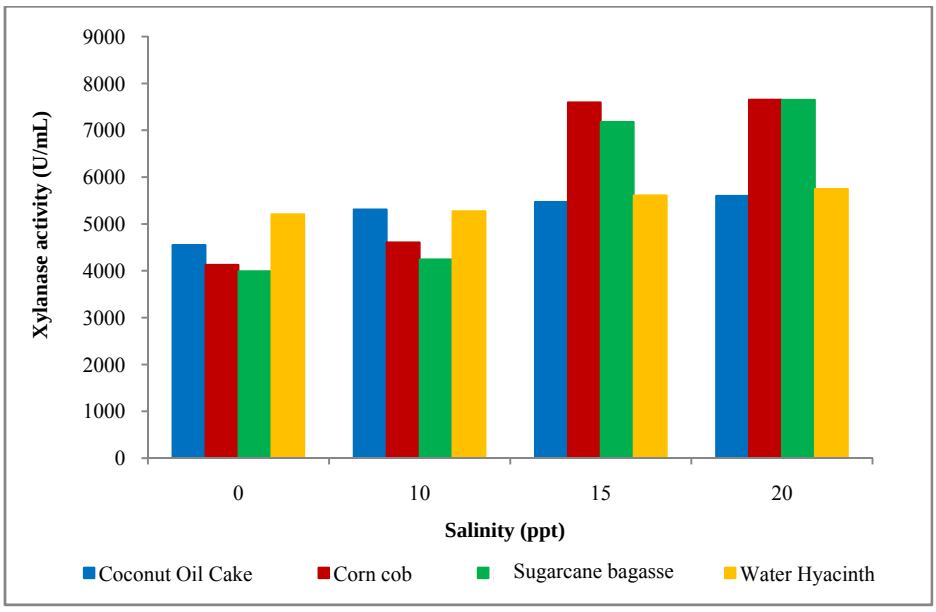


Fig 5.5. Effect of salinity on xylanase production using selected substrates

From the data presented in Fig 5.5, it was found that *Streptomyces* sp. ER1 required 20 ppt salinity for effecting maximum xylanase production with all the 4 selected substrates. Nevertheless 15ppt also supported almost similar level of xylanase production (> 90%) with all the 4 selected substrates compared to that at 20 ppt.

5.4.7 Effect of pH on xylanase production using the selected substrates

Effect of pH was studied by varying the pH of the production medium from pH 6 to pH 9 for the production of xylanases by *Streptomyces* sp. ER1. The culture showed maximum xylanase production at pH 7 with coconut oil cake, corn cob and sugarcane bagasse and at pH 8 with water hyacinth (Fig 5.6).

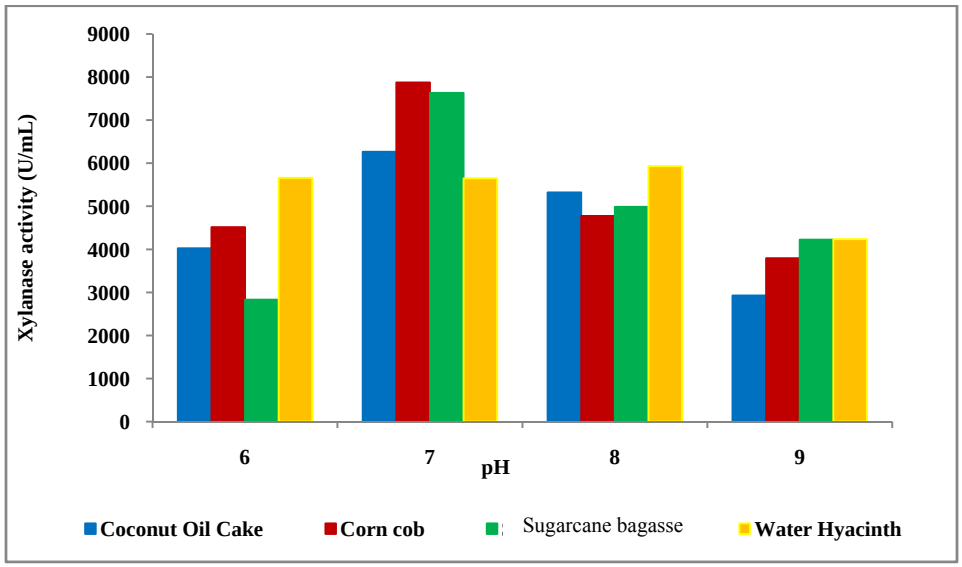


Fig 5.6. Effect of initial pH on xylanase production using selected substrates

The xylanase production at pH 6 was 64.26% with coconut oil cake; 57.32% with corn cob and 37.1% with sugarcane bagasse compared to that produced at pH 7. At pH 9, there was a decrease of 53.27% with coconut oil cake; 51.8% with corn cob and 44.6% with sugarcane bagasse in enzyme

activity of that produced at pH 7. However, the xylanase activity with water hyacinth at pH 6 and pH 7 was 95.41% and 95.22% respectively and there was a decrease of 28.56% in enzyme activity compared to that produced at pH 8.

5.4.8 Effect of temperature on xylanase production using the selected substrates

Streptomyces sp. ER1 was grown in xylanase production media A and M9 at temperatures ranging from 30-40°C with 5°C unit difference. Optimum production of xylanases was detected at 35°C with corn cob and water hyacinth.

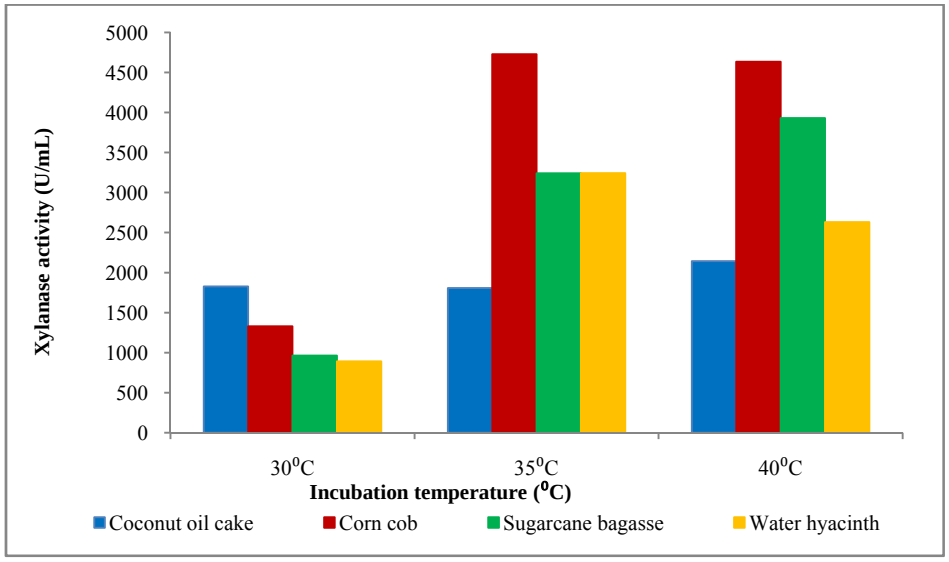


Fig 5.7. Effect of incubation temperature on xylanase production using selected substrates

Optimum temperature for xylanase production using corn cob and water hyacinth was 35°C whereas with sugarcane bagasse and coconut oil cake optimum production was at 40°C. Enzyme production was comparatively lesser at 30°C. At this temperature, xylanase production with corn cob and water hyacinth was 28.12% and 27.5%, respectively,

compared to that produced at 35°C. There was a decrease of 1.97% and 18.9% in xylanase activity at 40°C with corn cob and water hyacinth, respectively, compared to that produced at 35°C. At growth temperatures of 30°C and 35°C, xylanase production was 85.24% and 84.34% with coconut oil cake and 24.5% and 82.5% with sugarcane bagasse respectively of that produced at 40°C (Fig 5.7).

5.4.9 Effect of agitation on xylanase production using selected substrates

Enzyme production by *Streptomyces* sp. ER1 with the selected substrates was studied under static conditions and with agitation (50,100 and 150 rpm). The results clearly indicated superior xylanase production under agitation (Fig 5.8). For all further experiments, *Streptomyces* sp. ER1 was grown under agitation for xylanase production.

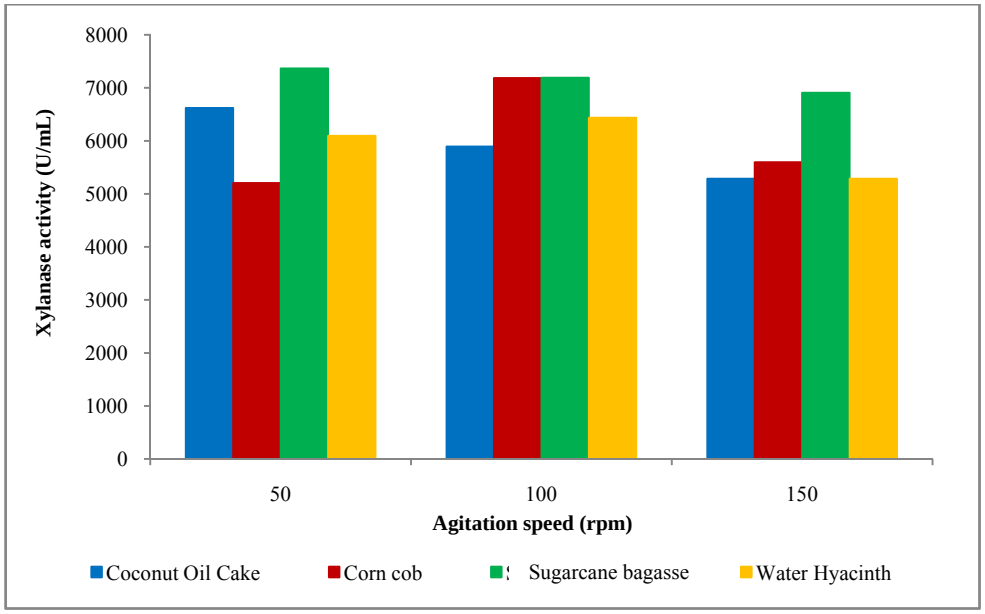


Fig 5.8. Effect of agitation on xylanase production using selected substrates

The optimum agitation speed for xylanase production with coconut oil cake and sugarcane bagasse was 50 rpm. However, there was decrease in xylanase activity with increase in the agitation speed. At 100 and 150 rpm, there was a decrease of 11% and 20.14% in xylanase activity with coconut oil cake and by 2.37% and 6.22% with sugarcane bagasse, respectively, compared to that produced at 50 rpm. However, optimum agitation speed for xylanase production with corn cob and water hyacinth was 100 rpm. At 150 rpm, the xylanase activity reduced by 22.1% with corn cob and 17.87% with water hyacinth, compared to that produced at 100 rpm.

5.4.10 Optimisation of Nutritional Parameters

5.4.10.1 *Effect of different levels of selected substrates on xylanase production*

In order to study the effect of different levels of the selected substrates on xylanase production, the following concentrations (%) were tried. (i) 0.5, (ii) 1, (iii) 1.5, (iv) 2.0, (v) 2.5, (vi) 3.0.

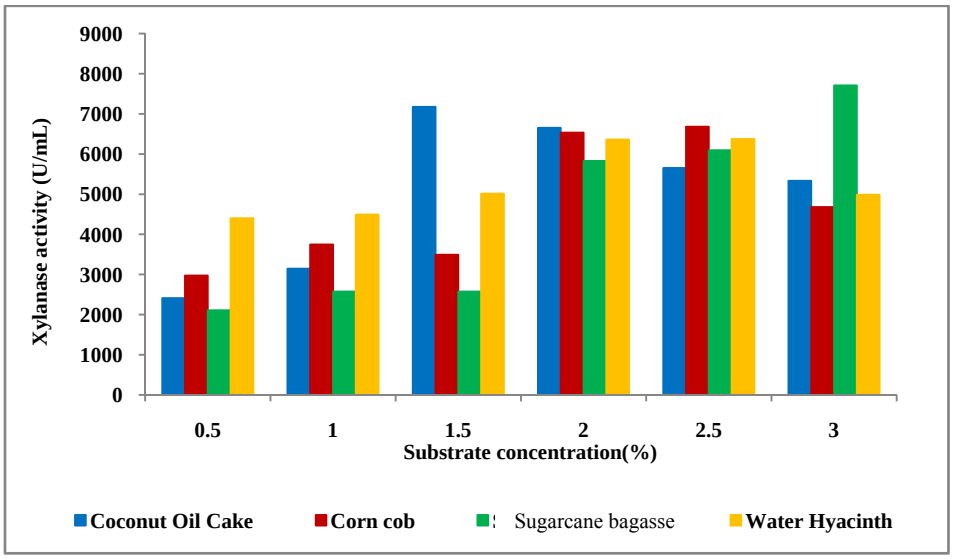


Fig 5.9. Effect of substrate concentration on xylanase production using selected substrates

Different levels of the selected substrates were clearly affecting the xylanase production by *Streptomyces* sp. ER1 (Fig 5.9). 2% of coconut oil cake; 2.5% of corn cob and water hyacinth and 3% of sugarcane bagasse showed highest enzyme production.

5.4.10.2 *Effect of different nitrogen sources on biomass and xylanase production*

5.4.10.2.1 Effect of inorganic nitrogen sources

Studies on the effect of inorganic nitrogen sources on xylanase production were carried out with different inorganic sources. The results are given in Fig 5.10.

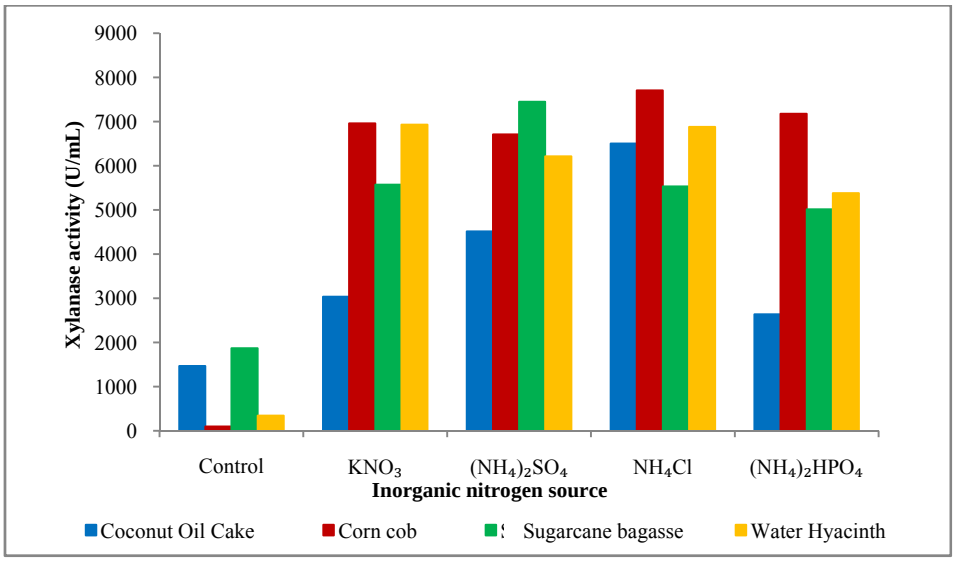


Fig 5.10. Effect of inorganic nitrogen sources on xylanase production using selected substrates

The optimum inorganic nitrogen source for xylanase production with coconut oil cake and corn cob was ammonium chloride and that with sugarcane bagasse and water hyacinth were, ammonium sulphate and potassium nitrate, respectively. The inorganic nitrogen sources other than the optimum also enhanced the xylanase production with the selected substrates, compared to that of control (Fig 5.10).

5.4.10.2.2 Effect of organic nitrogen sources

Studies on the effect of organic nitrogen sources on xylanase production were carried out with organic nitrogen sources.

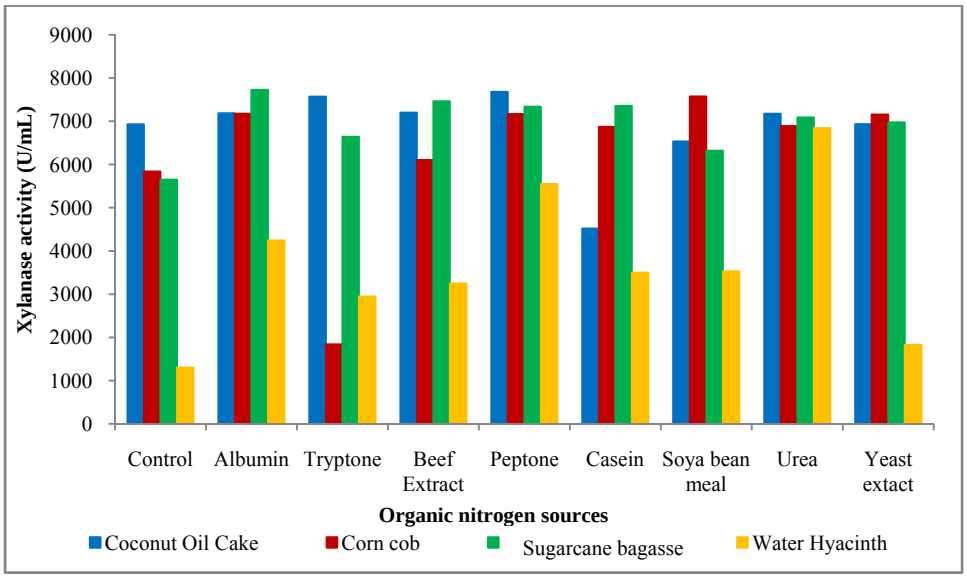


Fig 5.11. Effect of organic nitrogen sources on xylanase production using selected substrates

The optimum organic nitrogen source for xylanase production with coconut oil cake, corn cob, sugarcane bagasse and water hyacinth were peptone, soya bean meal, albumin and urea, respectively. The organic nitrogen sources other than the optimum also enhanced the xylanase production with the selected substrates, compared to that of control. However, there was a decrease of 68.46% and 34.81% in xylanase activity with tryptone and corn cob and casein and coconut oil cake, respectively, when compared to that of control.

5.4.10.3 *Effect of different surfactants and other additives on xylanase production using selected substrates*

Impact of different surfactants and other additives in the fermentation medium on xylanase production was evaluated.

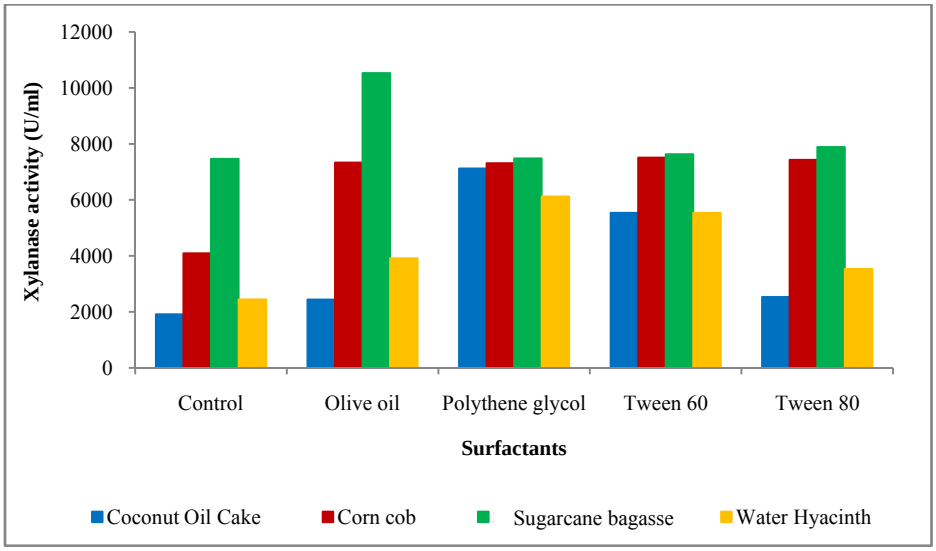


Fig 5.12. Effect of different surfactants on xylanase production using selected substrates

The optimum surfactant for xylanase production with coconut oil cake and water hyacinth was polyethylene glycol and that with corn cob and sugarcane bagasse were Tween 60 and olive oil, respectively. It was found that other surfactants also enhanced xylanase production with the selected substrates compared to that of control.

5.5 Application of xylanase in paper deinking and clarification of fruit juice

5.5.1 Analysis of phenolic compounds and hydrophobic compounds in effluent released from pulp before and after xylanase treatment

The results of the present study clearly indicated that the cell-free culture supernatant of *Streptomyces* sp. ER1 had tremendous potential for environment friendly technique of biological deinking. Treatment with cell-free culture supernatant containing xylanase resulted in several fold increase in brightness of pulp (Fig 5.13).

Fig 5.13. Manually pressed pulp before and after treatment with xylanase

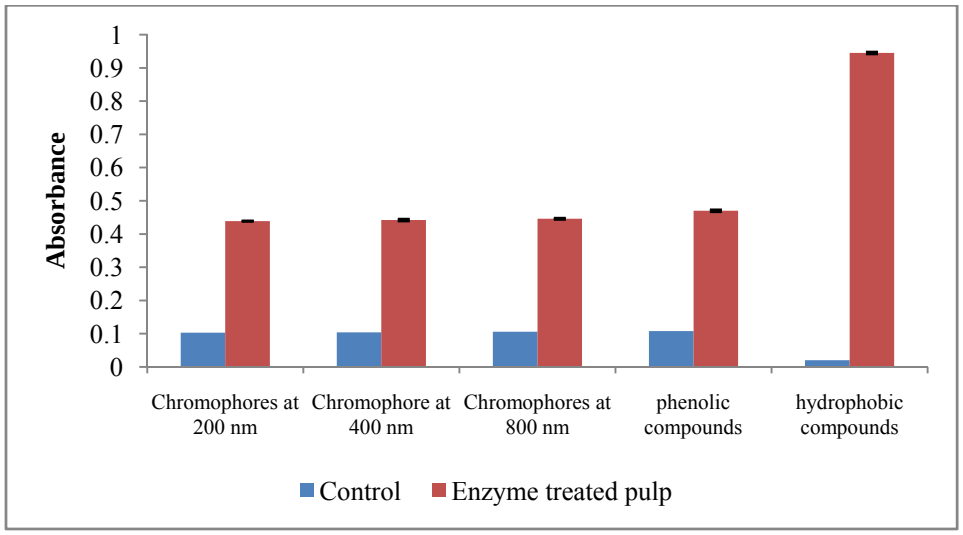


Fig 5.14. Analysis of phenolic compounds and hydrophobic compounds in effluents released from the paper pulp before and after the enzyme treatment

From the graph (Fig. 5.14), it is revealed that chromophore compounds are removed from the newspaper pulp samples as there was an increase in absorbance from 200 to 800 nm, compared to that of control. Increase in absorbance at 237 and 465 nm also revealed that higher levels of

phenolic and hydrophobic compounds were released from newspaper pulp samples compared to that of control (untreated pulp).

5.5.2 Clarification of fruit juice

The enzyme was found suitable for clarification of fruit juices. The optimum incubation period for clarification of orange, musambi and pineapple juice was at 150, 120 and 90 min respectively. Enzyme treated juice gave 20.87%, 23.64% and 27.89% increase in clarity of orange, musambi and pineapple juice, respectively, compared to that of control. The results are shown in Fig 5.15. The titrable acid was found to be higher in pineapple and musambi juices obtained from xylanase- treated fruit pulps as compared to control (untreated pulps). However, there was no significant variation observed in the acidic neutrality value of the control and enzyme treated orange juice (Table 5.3). Based on the present results, an increase in rate of filtration of enzyme-treated juice was observed compared to that of control (Table 5.4).

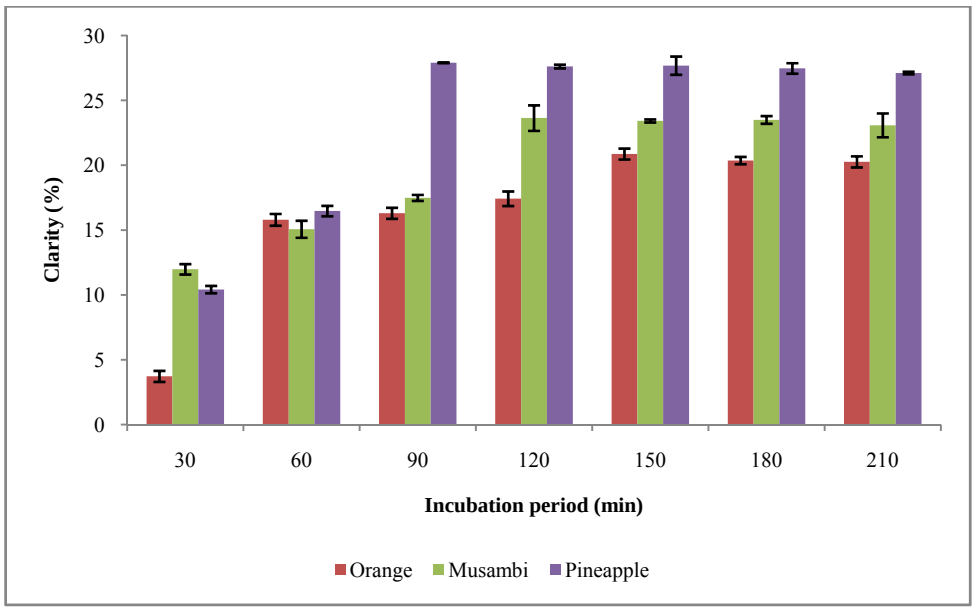


Fig 5.15. Effect of incubation period on clarification of fruit juices

Table 5.3. Titrable acid (%) of fruit juice before and after xylanase treatment

Fruit juice	Titrable acid (%)			
	Control	Standard deviation	Test	Standard deviation
Orange	0.03	0.05	0.034	0.06
Musambi	0.049	0.07	0.077	0.05
Pineapple	0.019	0.06	0.038	0.04

Table 5.4. Rate of filtration (s) of fruit juice before and after xylanase treatment

Fruit juice	Filtration rate (s)			
Orange	20	0.09	12	0.05
Musambi	22	0.07	15	0.04
Pineapple	17	0.08	9	0.03

5.6 Discussion

Biomass is a significant contributor to the world economy. Agriculture and forest produce- based industries offer food, feed, and a wide range of necessary products and are also a source of chemicals, materials, electricity and fuels (Chum and Overend, 2001). Considering the present scenario, it would be appropriate to exploit concurrently and use the enormous potential of already available agro-industrial biomass waste resources. Production of xylanase from agricultural and biodegradable wastes provides a feasible solution to multiple environmental problems and at the same time creating a sink for waste and renewable energy production.

5.6.1 Pre-treatment of substrates

Lignocellulose is intractable due to a spatial network that prevents degradation. Intractable properties of lignocellulose include surface area accessible to enzymes, cell wall pore size, particle size, and site-specific surface area (Zhao *et al.,* 2012). Hence pre-treatment of biomass is essential to disorganise the micro- and macro- fibrils, in order to release the polymer chains of hemicelluloses and cellulose and modify the pores in them to allow the enzymes to penetrate into the fibrils for enzymatic hydrolysis. But, the pre-treatment methods should not cause any degradation, loss of carbohydrates and formation of inhibitory by-product during pre-treatment (Galbe and Zacchi, 2002).

In the present study, the substrates were pre-treated by steaming in an auto clave and alkali treatment (NaOH). Steaming removes the major part of hemicelluloses from the material and makes it more vulnerable to enzymatic degradation. Alkali pre-treatment of substrate reduces the lignin content of the agro residues (Chapla *et al.,* 2010). Dilute NaOH treatment of lignocellulose may cause swelling; leading to increase in internal surface areas, decrease in the degree of polymerisation and crystallinity, separation of structural linkage between lignin and carbohydrate and disruption of lignin structure and easy accessibility of substrate for enzymatic hydrolysis (Fan *et al.,* 1987; Soccol *et al.,* 2011). Mild NaOH pre-treatment has been improved the susceptibility of hemicelluloses to enzymatic hydrolysis (Okeke and Obi, 1995; Gokhale *et al.,* 1998).

5.6.2 Comparison of SSF with SmF using various Agrowastes for xylanase production

In all, 12 different substrates were chosen for xylanase production. They were: wheat bran (WB), rice bran (RB), ground nut oil cake (GOC),

neem oil cake (NOC), soya bean meal (SBM), coconut oil cake (COC), sugarcane bagasse (SB), potato peel (PP), banana peel (BP), corn cob (CC), WH (water hyacinth) and corn flour (CF). To select the best fermentation condition for xylanase production by *Streptomyces* sp. ER1, all the substrates were subjected to SmF and SSF. *Streptomyces sp.* ER 1 managed to produce xylanase using 12 substrates as the alternative cost-effective carbon sources under SmF and SSF. It was observed that the former was better than the latter. Many reports are available on xylanase production by SmF and SSF (Sanghi *et al.,* 2009; Irfan *et al.,* 2012; Kamble and Jadhav 2012a; Jones *et al.,* 2012). Currently, 80-90% of xylanases are produced in submerged culture because; the microbial biomass and the substrates are homogeneously distributed in a liquid medium under SmF. Besides that, SmF has a higher degree of intensification and higher level of automation (Gaanappriya *et al.,* 2011). Due to the better understanding of bacterial metabolism, characteristics and their response, there is an increasing demand on the development of xylanase production under SmF.

Lignocellulose wastes are commonly substituted as the carbon and energy source for enzymes production. On account of its ease of access and cost-effective advantages, sugars from lignocellulose residuals are frequently utilised and compared to expensive limited resources, such as xylan, in industry to manufacture enzymes. Although the xylanase production from beechwood xylan exerted high enzymatic activity in the present study, the cost of production would be tremendously overwhelming and unbearable. Agricultural wastes used as the prime carbon source for xylanase production by *Streptomyces* sp. ER1 under SmF and SSF proved to be possible with better prospect of scaling up. There are reports on Streptomyces sp., producing xylanase on various feed stuffs like sugarcane

bagasse, oat spelt xylan, rice bran, wheat bran and saw dust under SmF (Kalpana and Rajeshwari, 2015).

Based on the present study, SmF showed more enzyme production as compared to SSF. Hence, further optimisation studies were carried out under SmF.

5.6.3 Selection of substrates for xylanase production by *Streptomyces* sp. ER1

Corn cob, sugarcane bagasse, coconut oil cake and water hyacinth which gave the highest xylanase production under SmF compared to other substrates, were selected for further optimisation studies.

Xylanase production with different agro-residues has been reported by several researchers (Lee *et al.,* 2011; Hideno *et al.,* 2011). Cost, availability and physicochemical characteristics of substrates are the major factors during their selection as substrates for enzyme production (Delabona *et al.,* 2012). Enzyme production is dependent on the nature of carbon source, favourable degradability, physical associations and accessibility of substrate and presence of some nutrients (Gao *et al.,* 2008).

Corn cob has elevated tendency to produce xylanase which is used to develop low-cost media for the mass-production of xylanase (Fang *et al.*, 2008). In addition, the elevated xylan content in corn cob (40%), the highest among all agricultural waste, makes it a potential substrate for xylanase production (Knob *et al.,* 2014). Damaso *et al.*, (2000) obtained maximum yield of xylanase using corn cob as a substrate in SmF. Higher xylanase production using corn cob was reported in *Streptomyces cyancus* SN32, *Streptomyces chartreusis* and *Streptomyces thermovulgaris* TISTR1948 (Ninawe and Kuhad, 2005; Li *et al.,* 2011; Boonchuay *et al.,* 2016).

In the present study, the xylanase was found to be capable of hydrolysing natural xylan, which is a desirable property for industrial applications like bleaching of pulps, where it results in better porosity, swelling up and separation of pulp microfibrils and pulp fibres (Beg *et al.,* 2000).

Sugarcane bagasse was also a suitable substrate for xylanase production by *Streptomyces* sp. ER1.Therefore, this finding, justifies the selection of this isolate and proves its ability to ferment and produce sufficient amounts of xylanase in the presence of various lignocelluloses. Previous studies also support the use of lignocellulosic wastes for enzyme production. (Bocchini *et al.,* 2005; Kapoor *et al.,* 2008). Sugarcane bagasse is a by-product of sugarcane stalks which is in the form of fibrous residues after the production of sugarcane juice and sugar. Cellulose and hemicellulose are the two main polysaccharides (70%) of the sugarcane bagasse (Brienzo *et al.,* 2009). Sugarcane bagasse with its high water retention capacity is a good inducer for xylanase production (Albertan *et al.,* 2009). It has been found to be a suitable substrate for xylanase production (Rajaram and Varma, 1990; Techapun *et al.*, 2001; Kumar *et al.,* 2009; Sepahy *et al.,* 2011; Irfan *et al.,* 2012; Guha *et al.,* 2013; Raj *et al.,* 2013; Kalpana and Rajeshwari, 2015).

India is one of the leading countries in the production of coconut oil cake. Edible oil cakes have a rich nutritional value, especially having protein content ranging from 15% to 50% (http://www.seaofindia.com/). The composition of oil cake differs depending on their variety, growing condition, and extraction methods (Ramachandran *et al.,* 2007). Several different enzymes have been produced that utilise coconut oil cake as a raw material (Pandey *et al.,* 1995; Selvakumar and Pandey, 1999; Sabu *et al.,*

2002; Ramachandran *et al.,* 2004; Ramachandran *et al.,* 2005). The studies on xylanase production using coconut oil cake as the substrate are scarce.

Water hyacinth is a freshwater aquatic plant found commonly in India. The biomass of water hyacinth has about 48% hemicelluloses, 18% cellulose and 3.5% lignin (Nigam, 2002). Besides, being an aquatic plant, it has an added advantage of not being a competition to food crops for arable land resources (Mishima *et al.,* 2008). It is known to contain a high level of carbohydrates but sufficient study has not been conducted on them (Fileto-Perez *et al.,* 2013). Xylanase was produced using water hyacinth by *T. reesei* and *F. oxysporum* under SmF (Pothiraj *et al.,* 2014). *Trichoderma reesei* NRRL-3652 was used to produce xylanase under SSF, using acid pre-treated water hyacinth biomass as a substrate (Manivannan and Narendhirakannan, 2015).

Thus, agrowastes were able to provide improved uses as the carbon and energy sources in the industrial production of xylanase instead of self-decomposition that resulted in relatively severe water and environmental pollution. In order to ensure a good fermentation, various parameters should be optimised and maintained according to the limitation of process, such as temperature, pH, substrate concentration, size of inoculum, cultivation time and aeration.

5.6.4 Selection of production media for xylanase production using selected substrates

Every microorganism requires appropriate and sufficient nutrients for growth and other cellular processes. Three types of media were used in the present study to determine the optimum medium formulation for the maximum xylanase production under SmF by *Streptomyces* sp. ER1 with coconut oil cake, sugarcane bagasse, corn cob and water hyacinth as

substrates. Significant difference in xylanase activity was observed from the different types of media in the study. Highest xylanase activity was found in the production medium A for substrates, coconut oilcake and water hyacinth while M9 medium (medium B) was found to be optimum for corn cob and sugarcane bagasse. Medium A was proved to be optimum for xylanase production from agricultural wastes (Techapun *et al.*, 2002). M9 medium was also found to be optimum for xylanase production using sugarcane bagasse (Song and Wei, 2010).

The presence of yeast extract and peptone in production medium A might have positively affected the xylanase production using coconut oil cake and water hyacinth. This was supported by previous reports that organic nitrogen including yeast extract and peptone produced higher xylanase activity (Battan *et al.,* 2007; Nagar *et al.,* 2010). Additionally, the release of ammonium ion from peptone also stimulated the growth of *microorganism*, thus producing higher xylanase activity (Sanghi *et al.,* 2009). In these regards, the optimum medium formulation with essential growth-limiting nutrients is an important operation mode to optimise and increase the productivity of xylanase.

Lower xylanase activity observed from medium B was probably due to the different composition of the medium that were less favourable to *Streptomyces* sp.ER1.

5.6.5 Effect of incubation period on xylanase production using selected substrates

The culture was grown in xylanase production media (A and M9) containing the respective selected substrates as the carbon source to study the optimum period for maximum xylanase production under SmF. Xylanase production with the selected substrates was found to increase up to 72 h of fermentation period. The impact of time of fermentation on the

xylanase production revealed that at the start of experiment the organism was in acclimatizing stage, and so could not produce sufficient quantities of the enzyme. At 72 h, the culture was most dynamic and secreted maximum xylanase, while after 72 h, the xylanase production decreased due to both depletion of the nutrients from the culture medium and loss of xylanase by the proteolytic enzymes present in the culture medium.

Optimum incubation period of 72 h was also reported for xylanase production using agrowastes, by several other researchers (Camacho and Aguillar, 2003; Senthilkumar *et al.,* 2005a; Gupta and Kar, 2009); Ahmad *et al.,* 2012). However, corncob xylan supported the highest xylanase activity after 7 days of cultivation by *S. chartreusis* L1105 (Li *et al.*, 2011).

In contrast to the present study, there were several reports on optimum incubation period of 48 h and 120 h for xylanase production using agrowastes (Pothiraj *et al.,* 2014; Irfan *et al.,* 2016).

Shorter fermentation time (72 h) offers greater and cost-effective production of xylanase which is always favorable in the industrial production. In a nutshell, xylanase production by *Streptomyces* sp. ER1 using agrowastes is more economical in terms of capital investment and operational cost.

5.6.6 Effect of inoculum age and size on xylanase production using selected substrates

The effect of inoculum age was studied by measuring the xylanase activity after inoculating the production medium with 5 % (v/v) of 16 h, 20 h and 24 h old inoculum of *Streptomyces* sp. ER1. Maximum xylanase production was obtained with 5% (v/v) of 20 h old inoculum with sugarcane bagasse, water hyacinth and coconut oil cake whereas 24 h old inoculum yielded maximum xylanase with corn cob. However, less xylanase

production with 16 h old inoculum, could be because of the fact that *Streptomyces* sp, ER1 might not have entered into log phase of growth. The age of inoculum of a sporulating organism like *Streptomyces* sp. is an important factor as it would have resulted in the transfer of high quantities of spores if transferred after metabolically active stages (i.e. during the stationary phase or death phase). Presence of higher percentage of spores in the inoculum has been reported to result in the long lag phase of the fermentation profile (Meyarth and Suchanek, 1972).

5.6.7 Effect of salinity on xylanase production using selected substrates

The effect of salinity on xylanase production was studied by preparing the production media with different salinity ranging from 0ppt to 20 ppt. The study showed that 20 ppt salinity was optimum for xylanase production with all the selected substrates. It exhibits the halophilic nature of *Streptomyces* sp. ER1.

B. subtilis BS04 showed maximum xylanase production with sugarcane bagasse at 0.2% NaCl concentration (Irfan *et al.,* 2016). Xylanase production was maximum under the optimized conditions of 4% NaCl with alkali pre-treated sugarcane bagasse by *Achromobacter xylosoxidans* (Mahalakshmi and Jayalakshmi, 2016).

5.6.8 Effect of initial pH on xylanase production using selected substrates

Each microorganism possesses a precise pH range for its growth and activity. The extracellular pH has a strong influence on the metabolic pathways and product formation by microorganism. If the cultivation of the organisms is carried out at an undesirable pH, it may limit their growth as well as enzyme production. Furthermore, xylanase production by various

bacteria and fungi has been reported to be markedly dependent on pH (Battan *et al.,* 2007).

To optimize the xylanase production further, *Streptomyces* sp. ER1 was grown in media with initial pH ranging from 6 to 9. The pH showed a profound influence on xylanase production. The actinomycete showed maximum production in a neutral pH of 7 with coconut oil cake, corn cob and sugarcane bagasse as substrates. However, pH 8 was found to be optimum for xylanase production with water hyacinth as the substrate.

Earlier studies also indicated neutral pH values between 6 and 7 for optimum xylanase production by *Streptomyces* strains, such as *Streptomyces* sp. 594 and *S. chartreusis* L1105 (Li *et al.,* 2011). Several other researchers have reported optimum pH 7 for xylanase production using sugarcane bagasse and corn cob (Techapun *et al.,* 2002; Monisha *et al.*, 2009; Bhosale *et al.,* 2011; Ahmad *et al.*, 2012; Rahmani *et al.,* 2014). In contrast, bacterial xylanase production at its maximum activity was reported at alkaline condition (pH 8) (Battan *et al.*, 2007). *S. cyaneus* SN32, *S. tendae* SN77 and *S. caelestis* SN83 exhibited maximum production of xylanase at pH 9 (Ninawe and Kuhad, 2005).

Decrease in enzyme production at pH other than the optimum, might be due to the fact that the variation of external pH directly affects cytoplasmic pH of microbial cells which decreases the microbial growth or enzyme production by disrupting the plasma membrane or inhibiting the activity of different metabolic enzymes. Alteration of pH may also change the ionization state of nutrient molecules and reduce their availability to organisms (Bibi *et al.,* 2014).

5.6.9 Effect of incubation temperature on xylanase production using selected substrates

Streptomycetes, generally, are mesophilic in nature with a growth temperature range of 15-45°C, very low temperature may not activate the metabolism of the organism while very high temperature (over 45°C) results in the denaturation of the metabolic enzymes (El-Gendy and El-Bondkly, 2014). The usual temperature set in SmF systems is in the range of 25-32°C, depending on the growth kinetics of microorganism working for fermentation purposes (Lonsane *et al.,* 1985). Microorganisms grow slowly at a temperature below or above the normal temperature due to reduced rate of cellular production. At lower temperature, substrate transport across the cells is suppressed and lowest product yields are obtained (Rajoka, 2004).

To optimise the incubation temperature for xylanase production *Streptomyces* sp. ER1 was gown in production media at different temperatures ranging from 30^{o}C to 40^{o}C. The actinomycete showed maximum xylanase production at 40^{o}C using coconut oil cake and sugarcane bagasse as substrates. However, 35^{o}C was found to be optimum for xylanase production with water hyacinth and corn cob as substrates.

Significant xylanase production was observed at a temperature range of 30ºC to 50ºC in *Streptomyces rameus* with sugarcane bagasse (Bhosalae *et al.,* 2011). The optimum temperature range for *S. cyaneus* SN32, *S. tendae* SN77, *S. caelestis* SN83*S. chartreusis* L1105 was 30ºC to 50ºC (Ninawe and Kuhad, 2005; Li *et al.,* 2011). Similar to the present study, xylanase was produced optimally at 35^{o}C with water hyacinth by *T. reesei* and *F. Oxysporum* (Pothiraj *et al.,* 2014).

The preference of higher temperature by *Streptomyces* sp. ER1 for xylanase production qualified it as a thermotolerant organism. Thermotolerant microorganisms have great role in industrial applications and have attracted the attention of researchers engaged in different fields (Immanuel *et al.,* 2006). At higher temperature, the maintenance energy requirement for cellular growth is high due to thermal denaturation of metabolic enzymes resulting in highest production (Aiba *et al.,* 1973).

5.6.10 Effect of substrate concentration on xylanase production

Carbon sources and its concentration in the medium formulation exerted a profound effect on the xylanase production of bacteria as it is one of the vital elements of the microbial fermentation medium with the major role on the overall growth and metabolism. Thus, optimum carbon source and concentration are of great importance to achieve the maximum quantity of xylanase in fermentation process. Hence, in the present study, the production media was manipulated with respect to concentration of the lignocellulosics substrates (0.5 to 3%). With increasing concentrations of substrates, in production media, substantial increase in enzyme production was recorded. 2% of coconut oil cake, 2.5% of corn cob and water hyacinth; and 3% of sugarcane bagasse was found to be optimum for xylanase production by *Streptomyces* sp. ER1. Higher concentration of lignocellulosic substrates was not perfect according to the enzyme economical protocols (Kapoor *et al.,* 2008). Several other workers reported 0.5%, 1.5%, 2% and 2.5% of sugarcane bagasse for optimum xylanase production (Saleem *et al.*, 2002); Bhosalae *et al.,* 2011; Rahmani *et al.,* 2014). Maximum xylanase yield was obtained with (w/v) 0.87%, 2% and 3.5% of corn cob (Ninawe and Kuhad, 2005; Suleman *et al.,* 2016; Irfan *et al.,* 2016).

5.6.11 Effect of agitation on xylanase production using selected substrates

Mechanical agitation is vital, because of its effectiveness in mixing the contents of the medium, uniform air distribution and prevention of cell clumping (Gusek *et al.,* 1991). It also affects the dissolved oxygen and mass transfer of the microorganisms (Dietmar *et al.,* 1996; Purwanto *et al.,* 2009). The shaking rate also seemed to have a profound effect on enzyme production which may be due to reduction in oxygen levels in the medium that adversely affected enzyme production (Palma *et al.,* 1996).

In the present study, optimum xylanase production was at 50 rpm using coconut oil cake and sugarcane bagasse as substrates while 100 rpm was optimum for corn cob and water hyacinth. As agitation speed increased, the xylanase production decreased. The higher shear force at high agitation speed might have caused lower xylanase production. Similarly, high agitation speed of 200 rpm led to the shear stress on *A. brasiliensis* and as a result, it caused damage to the mycelium of fungi (Ho and Lau, 2014). Similar observations were made by Purwanto *et al.* (2009) and Palma *et al.* (1996).

In agreement with the present study, Nasr *et al.* (2013) and Ho (2014) also reported maximum xylanase enzyme production at 60 rpm and 90 rpm respectively. In the present study, low xylanase activity was obtained in non-agitated flasks, most probably due to oxygen or mass transfer limitations, while on agitation; high xylanase was produced, probably due to good oxygen supply. Therefore, in SmF, aeration and agitation are very important to ensure availability of oxygen, nutrients and other essential substances to the growing cells. Similar observations were reported previously (Kavya and Padmavathi, 2009; Irfan *et al.,* 2012).

5.6.12 Effect of nitrogen sources on xylanase production using selected substrates

Nitrogen source can considerably affect the pH of the medium during the course of fermentation which in turn may influence enzyme activity and stability. The mechanisms that govern the formation of enzymes are subjected to the availability and type of nitrogenous precursors for protein synthesis. Different nitrogen sources were studied for their effect on xylanase production by *Streptomyces* sp. ER1. Among all the organic sources tested, peptone, soya bean meal, albumin and urea were found to be inducers for xylanase production using coconut oil cake, corn cob, sugarcane bagasse and water hyacinth respectively. Peptone was also found to be a good inducer for xylanase production using agrowastes by several other *Streptomyces* sp. (Ninawe and Kuhad, 2005; Kuhad *et al.,* 2006; Bhosale *et al.,* 2011). Complex nitrogen sources like peptone release $NH^{+}{}_{4}$ ions, which stimulates growth and at the same time increases enzyme yield because of its protease inhibiting nature at its low concentration (Forage *et al.,* 1985). In the present study also peptone is found to be beneficial for xylanase production. Soybean meal is a complex and conditioned nitrogen source that does not cause catabolite repression and almost certainly contains approximately all kinds of amino acids (Bajaj and Abbass, 2011). Thus, it could be readily absorbed by *Streptomyces* sp. ER1 mycelium. Similar to the present study, soybean meal was observed to be the best nitrogen source for xylanase production by alkalophilic *Streptomyces* species CD3 and *Streptomyces* sp. ESRAA-301097 (Sharma and Bajaj, 2005; Nasr *et al.,* 2013**).** Urea also increased xylanase production by *Bacillus safensis* P20 using sugarcane bagasse (Rahmani *et al.,* 2014).

Among the inorganic sources, ammonium chloride for coconut oil cake and corn cob; ammonium sulphate for sugarcane bagasse and

potassium nitrate for water hyacinth were found to be optimum for xylanase production. There were reports that addition of ammomium chloride, potassium nitrate and ammonium sulphate favoured better xylanase production using agrowastes (Bakri *et al.,* 2008).

Thus, the optimum medium formulation with essential growth limiting nutrients is an important operation mode to optimise and increase the productivity especially xylanase.

5.6.13 Effect of different surfactants on xylanase production using selected substrates

Detergent effects on xylanase production by *Streptomyces* sp. ER1 varied with the substrates. The optimum surfactant for xylanase production was Tween 60 with corn cob and coconut oil cake, olive oil with sugarcane bagasse and polyethylene glycol with water hyacinth. This result was similar to the previous study (El-Gendy and El-Bondkly, 2014). Stimulatory effect of Tween 60 on xylanase production could be attributed to its effect on cell membrane permeability or by disrupting nonspecific binding of enzymes to substrates and thus exerting a positive effect on desorption and recycling of xylanase (El-Gendy and El-Bondkly, 2014).

The other possible reasons could be (i) increased enzyme stability and prevention of enzyme denaturation; (ii) affecting the substrate structure positively and making it more accessible for enzymatic hydrolysis; (iii) affecting enzyme-substrate interactions positively, leading to a more effective conversion of substrate. The influence of olive oil on enzyme activity revealed that the olive oil enhanced xylanase secretion via increasing cell membrane permeability significantly (Sepahy *et al.,* 2011).

5.6.14 Deinking of paper pulp

The results of the present study clearly indicated that the cell-free culture supernatants of *Streptomyces* sp. ER1 showed tremendous potential for environment friendly technique of biological deinking. Treatment with cell-free culture supernatants containing xylanase activity resulted in several fold increase in brightness of paper pulp. Xylanase treatment may have caused either decolourization only or both decolourization and dislodging of ink particles from pulp fibre when compared to that of control. This was further supported by the fact that on comparing the absorbance of effluents (200 and 800 nm) to detect the release of colour compounds, xylanase treated pulp effluent showed high absorbance whereas untreated (control) pulp effluents were colourless. Several authors have used highly purified or concentrated enzymes for deinking purpose (Marques *et al.,* 2003). However, in the present study, the crude culture supernatant alone could bring about deinking and decolourisation of newspaper used at 3-6% pulp consistency within 5 days of incubation.

5.6.15 Clarification of fruit juice

Enzyme treated juice gave 20.87%, 23.64% and 27.89% increase in clarity of orange, musambi and pineapple juices, respectively. The clarification is due to disruption of hemicellulosic material and hence polymer xylan is hydrolysed. There is better %clarification in case of the pineapple juice than in musambi and orange juice. Similar observation was made by Kumar *et al.* (2014). The maximum amount of juice clarification in case of orange, musambi and pineapple juice was observed at 150, 120 and 90 min respectively. Prolonged incubation time could result in the formation of haze particles consisting of protein, carbohydrate and protein-protein complexes (Shama and Chand, 2012). Other researchers have documented

30–90 min as the optimum time for fruit juice clarification with xylanase (Kumar *et al.,* 2014). Titratable acidity determines the quality of fruit juice and an increase in titratable acidity would increase the shelf life of juice (Baker and Bruemmer, 1972). Titrable acidity was found to be higher in enzyme-treated pineapple and musambi juice pulp as compared to untreated pulps. However, there was no significant variation observed in the acidic neutrality value of the control and enzyme treated juice of the orange. Similar observations were made by Nagar *et al.* (2012). In the present study, an increase in filterability of juice after treatment with xylanase was observed which could be the result of xylan hydrolysis by the enzyme. Similar results were reported by Dhiman *et al.* (2011).

The present study showed that *Streptomyces* sp. ER1 could produce xylanase using several agrowastes as substrates. Among the 12 substrates screened, corn cob, sugarcane bagasse, water hyacinth and coconut oil cake gave the highest xylanase production under SmF. Hence, *Streptomyces* sp. ER1 could be considered as a promising agent for xylanase production using agrowastes which help in converting waste materials into commercially important valuable products. Moreover, xylanase produced by *Streptomyces* sp. ER1 could bring about deinking of newspaper and clarification of musambi, orange and pineapple juices.

Chapter -6

SUMMARY AND CONCLUSION

Actinomycetes are well- known as enzyme and antibiotic producers and, therefore, have been the subject of interest to scientific and industrial community. Two third of today's antibiotics are produced by actinomycetes. Besides antibiotics, actinomycetes are found to be good producers of several enzymes of industrial, pharmaceutical and medical importance. They are found to have applications in agriculture, transformation of antibiotics and decomposition of complex organic matter. Because of this high economic significance actinomycetes from terrestrial habitat have been extensively studied but relatively few efforts with aquatic actinomycetes have been made.

The thesis presents isolation and screening of actinomycetes isolated from Cochin estuary and its associated mangroves and emphasis has been given to xylanase production, optimisation and characterization. The study is divided into two parts; the first part includes isolation of actinomycetes from sediment samples, screening for antimicrobial activity and hydrolytic enzyme production. One of the isolates, *Streptomyces* sp. ER1 was found to be highly potent in xylanase production and this strain was subjected to detailed studies. The second part focuses on xylanase production. The sediment isolate ER 1 with maximum xylanase production was selected for

detailed study and the strain was identified as *Streptomyces* sp. KY449279. The optimisation of xylanase production by *Streptomyces* sp. ER 1 was performed using one-factor-at-a-time method and statistical tools such as Plackett- Burman Design (PBD) and Central Composite Design (CCD) for various fermentation and nutritional factors. The purification of xylanase was carried out by DEAE anion exchange chromatography and molecular weight was determined by SDS-PAGE. The scope of using different agro-industrial wastes for xylanase production, the stability of xylanase in organic solvent and surfactants for application in various industries was also studied.

The important findings of the study can be summarized as follows:

- A total of 90 different actinomycetes were isolated from sediment samples from the various sampling stations of Cochin estuary. Of the four different isolation media used, Kusters agar and Starch casein agar were most effective in isolation of actinomycetes.
- In the antimicrobial screening of the isolates, only two isolates, *Streptomyces* spp. (ER7 and ER10) showed antimicrobial activity against the tested pathogens. Among the various industrial enzymes screened, 48 isolates were cellulase producers followed by amylase (37); lipase producers (34); xylanase producers (10); and lecithanase (6). None of the isolates produced L-asparaginase or L-glutaminase.
- Considering the industrial importance of xylanase, the strain ER1 showing maximum xylanase production was selected for further studies .
- ER1, ER7 and ER10 were subjected to phenotypic and biochemical characterisation and identified as *Streptomyces* spp. Since further study

was carried out with the strain ER 1, it was selected for molecular identification using 16S rDNA sequencing.

- The sequencing of 16S rDNA gene from ER1 strain was carried out by BLAST analysis and the sequence homology ranged between 97.7 to 100 % to the closest neighbors. BLAST analysis revea led that the strain belonged to *Streptomyces* sp. KY449279.
- Optimization of xylanase production by 'one factor at a time' method was employed and the optimum conditions for xylanase production wer e found to be temperature 35°C; pH7, agitation 50 rpm; salinity 15 ppt; inoculum age 24 h; inoculum size 5%; carbon source beechwood xylan; xylan concentration 0.4 %; surfactant (olive oil), metal ions (Calcium ion), nitrogen source (potassium nitrate and - peptone) and incubation period (120 h).
- PBD was used to screen the medium and physical components for their influence on xylanase production by *Streptomyces* sp. ER1. Using the PBD, 12 independent variables (pH, incubation temperature, agitation speed, inoculum age, inoculum size, salinity, substrate concentration, concentration of organic nitrogen source (peptone) concentration of inorganic nitrogen source (potassium nitrate), concentration of additive (olive oil), concentration of metal ion salts (calcium chloride and manganese chloride) were screened by representing them at two levels, low (-) and high (+) in 20 trials. The experiments were carried out in triplicates and the average xylanase activity was measured. F value and P values and the proportion of variance R^2 determined which showed that the model was significant at P = 5% level. In the present analysis, the Student's t values for inoculum age, agitation speed, the concentration of olive oil and xylan shows that these were the most significant factors affecting the enzyme production.

- Parameters such as inoculum age, agitation speed, concentration of olive oil and xylan were optimised using Response Surface Methodology by the software, Design Expert (Version 7, Stat-Ease. U.S.A.). The Central Composite Design was employed for designing 30 experiments with 6 central points as suggested by the software for optimisation.
- The model suggested by the software was Quadratic Regression Model and ANOVA of the model was found to be significant with an F value of 134.10. The multiple correlation coefficients of xylanase production R 2 was 0.9924 which suggested that the model can explain 99.24% variation in response. The ‘‘Predicted RSquared” of 0.9757 is in reasonable agreement with the ‘‘Adjusted R-Squared” of 0.9849 which depicted adequacy of the model to predict response.
- The ‘Lack of fit’ for the model was found to be 0.9884 which was not si gnificant and it also indicated the prosperity of the model. The ANOVA table also indicated that the quadratic terms olive oil and xylan concentration, inoculum age, A^2 (olive oil concentration), B^2 (xylan concentration) and C^2 (agitation speed), D^2 (inoculum age) were significant. Interactions between optimised factors were also observed to be significant in enhancing xylanase production based on 3D graphs. The interaction between the independent variables such as the concentration of olive oil (additive) and xylan; olive oil concentration and agitation speed; inoculum age and olive oil concentration; xylan concentration and agitation speed; inoculum age and xylan concentration showed a significant effect on xylanase production by *Streptomyces* sp. ER1.
- The optimized conditions for xylanase production were identified using the model as agitation 43 rpm, inoculum age 21.05 h, olive oil

concentration 33.10 mg/L and xylan concentration 0.37%. Validation of the model was carried out by experimental analysis. Experimental value was 10,547 U/mL which was close to the predicted value of 10,292 U/mL.

- Purification of xylanase was done by acetone precipitation followed by DEAEcellulose ion exchange chromatography and a purification fold of 5.57 was achieved.
- Determination of molecular weight of xylanase was performed using S DSPAGE analysis which was found to be approximately 23 KDa.
- Purified extracellular xylanase showed optimum temperature of 60ºC. Its thermostability profile revealed that it was fully stable at 60ºC followed by a continuous decline up to 100ºC.
- The xylanase was found to be active at a wide range of pH from 4 to 11 and optimum activity was detected at pH 7. However, stability of the en zyme was found to reduce drastically as pH increased from 9 to 11.
- Xylanase from *Streptomyces* sp. ER1 was found to be highly stable in the presence of solvents such as hexane and ethyl acetate and only two organic solvents such as chloroform and butanol reduced the activity to > 50%.
- Out of the nine different metal ions tested, mercury, copper, iron and zinc were found to have inhibitory effect on xylanase activity. Cobalt, manganese, potassium, calcium and magnesium were noted to have stimulatory effect on xylanase activity.
- The enzyme was stable in the presence of surfactants such as Triton X - 100, Tween 80, Tween 60, polythene glycol and olive oil. SDS and beta-mercaptoethanol was found to have inhibitory effect on xylanase activity.

- *Streptomyces* sp. ER1 could produce xylanase using selected agro wastes and water hyacinth. Among the twelve substrates, maximum xylanase yield was observed for corn cob followed by sugarcane molasses, water hyacinth and coconut oil cake in submerged fermentation suggesting the application of these agro residues for xylanase production. This is the first attempt to optimise xylanase production from coconut oil.
- For all the substrates optimal temperature for enzyme production was detected as 35°C to 40°C, optimal incubation period as 72 h, optimum pH as 7-8, agitation speed 50-100 rpm, salinity 20ppt and substrate concentration 1.5%-3% . The optimum nitrogen sources detected were different with each substrate like soya bean meal and ammonium chloride for corn cob; albumin and ammonium sulphate for sugarcane bagasse; peptone and ammonium chloride for coconut oil cake and urea and potassium nitrate for water hyacinth. The optimum surfactant for xylanase production from corn cob was Tween 60; olive oil for sugarcane bagasse and polyethylene glycol for coconut oil cake and water hyacinth.
- The xylanase from *Streptomyces* sp. ER1 successfully deinked newspaper and also could bring about clarification of orange, musambi and pineapple juices.
- The medium designed by CCD using a limited number of experiments, less efforts and time resulted in approximately 1.65-fold increase in xylanase production compared to the conventional method of OFAT which is incapable of reaching the true optimum conditions.

The current investigation revealed the the existence of antimicrobial activity against human and fish pathogens among these actinomycetes. Antibiotic resistance is a growing concern around the globe and

antimicrobial activity from actinomycetes of Cochin estuary may be a promising source for new antimicrobial drugs. Enzyme profiling of actinomycetes revealed their potential to produce various commercially valuable enzymes. The demand for enzymes is increasing every year and developed countries are gaining momentum in enzyme market which involves billions of dollars. Novel sources of enzymes with unique properties from environments such as Cochin estuary and its associated mangroves may provide unexplored potential enzyme producers and thus warrant further studies in this area. Studies on xylanase from *Streptomyces* sp. ER1 indicated its possible applications in agro--industrial waste management, in paper deinking and fruit juice clarification. However, a detailed study on the feasibility of large scale production of the enzyme in fermentors is essential for considering its industrial applications.

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APPENDIX

S.N.	Medium	Ingredients	Quantity (g/L)	pH
1	Starch casein agar	Soluble starch Casein (Vitamin Free) KNO_3 $MgSO_4.7H_2O$ K_2HPO_4 $MgSO_4.7H_2O$ $CaCO_3$ $FeSO_4.7H_2O$ Agar	10.0 0.3 2.0 2.0 2.0 0.05 0.02 0.01 20.0	7.0
2	Kusters agar	Glycerol Casein KNO_3 NaCl K_2HPO_4 $MgSO_4.7H_2O$ $CaCO_3$ Agar	10.0 0.3 2.0 2.0 2.0 0.5 2.0 16.0	7.2
3	Glycerol-arginine agar	Glycerol Arginine monochloride $MgSO_4.7H_2O$ Yeast extract Sucrose Agar	12.5 1 0.5 3 10.3 20	7.5
4	Actinomycete isolation agar	Sodium caseinate L-Asparagine Sodium	2.0 0.1 4.0	7.5

		propionate Dipotassium phosphate Magnesium sulphate Ferrous sulphate Agar	 0.5 0.1 0.001 15.0	
5	Nutrient agar	Peptone Beef extract Yeast extract Agar	5.0 1.5 1.5 15.0	7.5
6	Marine actinomycete broth	Starch Yeast extract Peptone Agar	1.0 0.4 0.2 2.0	7
7	Mueller Hinton agar	Meat, infusion solids from 300g Casein acid hydrolysate Starch Agar	2.0 17.5 1.5 17.0	7.3
8	Sabouraud dextrose agar	Dextrose Mycological peptone Agar	40.0 10.0 15.0	5.6
9	Modified M9 medium	$Na_2HPO_4.2H_2O$ KH_2PO_4 L-asparagine $MgSO_4.7H_2O$ $CaCl_2.2H_2O$ Glucose Agar Phenol red	6.0 3.0 5.0 0.5 0.014 2 %(w/v) 20.0 3 mL/L	6.2
10	Modified nutrient agar	Glucose Peptone Beef extract NaCl L-glutamine Agar	5.0 5.0 3.0 5.0 10.0 18.0	6.2

		Phenol red	3 mL/L	
11	Rhodamine olive oil agar	Nutrient broth NaCl Agar Olive oil Rhodamine B solution:	8.0 4.0 10.0 31.25 mL/L 0.001 % (w/v)	7
12	Egg yolk agar	Proteose peptone Na2HO4 KH2PO4 NaCl MgSO4 Glucose Hemin: 0.005 Egg yolk emulsion Agar	40.0 5.0 1.0 2.0 0.1 2.0 0.005 111.11 mL/L 25.0	7.6
13	ISP 2	Peptic digest of animal tissue Yeast extract Malt extract Dextrose Agar	5.0 3.0 3.0 10.0 20.0	6.2
14	ISP 3	Oat Meal Agar Ferric sulphate heptahydrate Trace salts, - Manganese chloride tetrahydrate Zinc sulphate heptahydrate	20.0 18.0 0.001 0.001 0.001	7.3
15	ISP 4	Starch Dipotassium phosphate	10.0 1.0	7.2

		Magnesium sulphate heptahydrate	1.0	
		Sodium chloride	1.0	
		Ammonium sulphate	2.0	
		Calcium carbonate	2.0	
		Ferrous sulphate heptahydrate	0.001	
		Manganous chloride heptahydrate	0.001	
		Zinc sulphate heptahydrate	0.001	
		Agar	20.0	
16	ISP 5	L-Asparagine	1.0	7.4
		Dipotassium phosphate	1.0	
		Trace salt solution	1.0 mL	
		Agar	20.0	
		1ml of Trace salt solution contains -		
		Ferrous sulphate heptahydrate	0.001	
		Manganese chloride tetrahydrate	0.001	
		Zinc sulphate heptahydrate	0.001	
17	ISP 6	Peptic digest of animal tissue	15.0	6.7
		Proteose peptone	5.0	
		Yeast extract	1.0	
		Ferric ammonium	0.5	

		citrate Dipotassium phosphate Sodium thiosulphate Agar	 1.0 0.08 15.0	
18	ISP 7	L-Asparagine L-Tyrosine Dipotassium phosphate Magnesium sulphate heptahydrate Sodium chloride Trace salt solution Agar 20.000 Trace salt solution contains - Ferrous sulphate heptahydrate Copper chloride dihydrate Cobalt chloride hexahydrate Sodium molybdate dihydrtae Zinc chloride Boric acid Manganese chloride tetrahydrate Sodium tartarate	1.0 0.5 0.5 0.5 0.5 1.0 mL 20.0 1.360 mg 0.027 mg 0.04 mg 0.025 mg 0.02 mg 2.85 mg 1.8 mg 1.77 mg	7.3
19	ISP 9	$(NH_4)_2 SO_4$ KH_2PO_4	2.64 2.38	7.0

		K_2HPO_4. $3H_2O$ $MgSO_4$. $7H_2O$ Pridham and Gottlieb trace salts (B) : $CuSO_4$. $5H_2O$ $FeSO_4$.$7H_2O$ $MnCl_2$.$4H_2O$ $ZnSO_4$. $7H_2O$	5.65 1.0 1.0 mL 0.64 0.11 0.79 0.15	
20	Urea Agar Base (Christensen)	Peptic digest of animal tissue Dextrose Sodium chloride Disodium phosphate Monopotassium phosphate Phenol red Agar	1.0 1.0 5.0 1.2 0.8 0.012 15.0	6.8
	Reagent and Buffers	**Ingredients**	**Quantity (g/L)**	**pH**
21	Gram's iodine	Iodine Potassium iodide Distilled water	1.0 2.0 300.00 mL	
22	TE Buffer	1M Tris pH 8 0.5M EDTA Distilled water	5 mL 1 mL 496 mL	8
24	30% acrylamide (pH 7.0)	Acrylamide N, N'-bisacrylamide Distilled water	15 0.4 50 mL	
25	1.0M tris	Tris base Distilled water	121.1 1000 mL	6.8
26	1.5M tris	Tris Distilled water	18.15 100 mL	8.8

27	5X Tris Glycine buffer	Tris base Glycine Distilled water	15.1 94 1000 mL	8.3
28	Staining solution	Methanol Glacial acetic acid Distilled water Coomassie brilliant blue	45 mL 10 mL 45 mL 0.1 g	
29	De-staining solution	Isopropanol Acetic acid Distilled water	25 mL 10 mL 65 mL	
30	Acetate buffer	0.2 M acetic acid 0.2 M sodium acetate Distilled water	11.55 mL 27.2 g 1000 mL	4-6
31	0.1 M Sodium phosphate buffer	Sodium phosphate monobasic Sodium phosphate dibasic Distilled water	27.8 g 53.65 g 1000 mL	7
32	Tris-HCl	Tris HCl Distilled water	24.2 g 0.2 M 1000 mL	8-9
33	Glycine-NaOH	Glycine NaOH Distilled water	15.01 g 8 g 1000 mL	10-11

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- Emilda Rosmine, Neethu Changanedassery Sainjan, Reshma Silvester, Aneesa Alikkunju, Saramma Aikkarakunnath Varghese. 2017. Statistical optimisation of xylanase production by estuarine *Streptomyces* sp. and its application in clarification of fruit juice. Journal of Genetic Engineering and Biotechnology. In Press. http://doi.org/10.1016/j.jgeb.2017.06.001.

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