PROTEASES FROM AN ENVIRONMENTAL ISOLATE OF *PSEUDOMONAS AERUGINOSA* MCCB 123 AND THEIR APPLICATIONS

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by

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Certificate

This is to certify that the research work presented in the thesis entitled **"PROTEASES FROM AN ENVIRONMENTAL ISOLATE OF *PSEUDOMONAS AERUGINOSA* MCCB 123 AND THEIR APPLICATIONS**" is based on the original work done by Ms. Divya Jose (Reg. No. 3065) under the guidance of Dr. A Mohandas, Professor Emeritus, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi -682016 and co-guidance of Dr. I.S Bright Singh, Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi- 682016, in partial fulfilment of the requirements for the degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the thesis entitled **"PROTEASES FROM AN ENVIRONMENTAL ISOLATE OF *PSEUDOMONAS AERUGINOSA* MCCB 123 AND THEIR APPLICATIONS**" is a genuine record of research work done by me under the guidance of Dr. A Mohandas, Professor Emeritus, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi-682016 and co-guidance of Dr. I.S Bright Singh, Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi – 682016, and that no part of this work has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Abbreviations

°C	degree celsius
µg	microgram
µl	microlitre
ANOVA	Analysis of variance
BLAST	Basic local Alignment Search Tool
bp	Base pair
Caco-2	Human colon carcinoma cells
CFU	Colony Forming Units
DEAE	Diethyl amino ethyl
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicilic acid
DTT	Dithiothreitol
EDTA	Ethylene-diamine tetra acetic acid
EGTA	Ethylene glycol tetraacetic acid
FBS	Fetal Bovine Serum
g	Gram
h	hours
HeLa	Henritta Lacks, human cervical carcinoma cell lines
Hep-2	Human larynx epithelial cell lines
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Dalton
l	Litre
LB	Luria–Bertani
LD_{50}	Lethal dose, 50%
M	molar
MDCK	Madin-Darby canine kidne
MEM	Minimal essential media
mg	milligram
ml	Milli litre
mM	Milli Molar
O D	Optical Density
PBD	Plackett-Burman Design
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PVP	Poly vinlyl pyrrolidone
rpm	revolutions per minute
SOC	Super Optimal Broth with Catabolite repression
TCA	Trichloroacetic acid
TLCK	Tosyllysine Chloromethyl Ketone (hydrochloride)
X-gal	(5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)

Chapter 1

GENERAL INTRODUCTION

Contents

1.1 Introduction

Proteases or peptidyl-peptide hydrolases (EC 3.4.21-24 and 99) are degradative enzymes capable of cleaving proteins into peptides and amino acids (Beg et al., 2003a; Reddy et al., 2008; Bayoumi and Bahobil, 2011) and occupy a provital position in both commercial and physiological applications (Rao et al., 1998). A protease is an enzyme that carries out proteolysis or begins with protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain.

1.2 Microbial proteases

Microorganisms represent an excellent source of enzymes thanks to their rapid growth, ease of cultivation, requirement of low cost nutrients, diversity, rapid doubling time, easiness in scaling up, susceptibility to genetic manipulation, and ease in the generation of recombinant enzymes with desired properties, compared to their plant and animal counter parts (Chand and Mishra, 2003; Laxman et al., 2005; Chu, 2007; Potumarthi et al., 2007; Shikha et al., 2007; Ramesh et al., 2009; Rao et al., 2009; Bajaj and Sharma, 2011). Among the intracellular and extracellular microbial proteases, the latter has been commercially exploited to assist protein degradation in various industrial processes (Kumar and Takagi, 1999; Gupta et al., 2002a; Shivanand and Jayaraman, 2011), and are important for various

cellular and metabolic processes such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool, hydrolysis of proteins in the cell free environments enabling the cells to absorb and utilize the hydrolytic products. They also perform a variety of functions in organisms from cellular level to organ level, to produce cascade systems such as haemostasis and inflammation, involved in physiological functions of cells, and in abnormal pathophysiological conditions (Rao et al., 1998).

1.2.1 Classification of proteases

According to the International union of Biochemistry and Molecular Biology, the classification and naming of the enzyme is based on the chemical name of the substrate and type of reaction that undergoes. Proteases are included in subgroup 4 of group 3 (hydrolases) (114a) and are classified into groups according to the types of reactions catalysed, chemical nature of catalytic site and evolutionary relationship with respect to its structure. Peptidases which catalyses the hydrolysis of larger peptide fragments are subdivided into exopeptidases and endopeptidases based on their site of action and functional group present on the active site (Barrett, 1994; Gonzales and Robert-Baudouy, 1996; Rao et al., 1998; Sandhya et al., 2005a).

1.2.1.1 Exopeptidases

The exopeptidases act only near the ends or extremities of polypeptide chains. They cleave the peptide bond proximal to the amino or carboxy termni of the substrate. Either a free C or N terminus is essential for an exopeptidase. Based on their site of action at the N or C terminus, they are classified as aminopeptidases and carboxypeptidases, respectively (Barrett, 1994; Gonzales and Robert-Baudouy, 1996; Rao et al., 1998; Mahajan and Badgujar, 2010).

1.2.1.1.1 Aminopeptidases

Aminopeptidases selectively hydrolyse an amino acid residue from N-terminal position of peptides of proteins (Gonzales and Robert-Baudouy, 1996; Lin et al., 2010).They hydrolyse the first peptide bond in polypeptide chain and release a single amino acid residue or may remove dipeptides or tripeptides from polypeptide substrates. They are important in protein maturation, protein turnover, hormone level regulation, hydrolysis of regulatory peptides, nitrogen nutrition, modulation of gene expression, generation of metabolic energy , recycling of reduced cofactors and also involved in cellular functions such as processing of newly synthesized proteins (Gonzales and Robert-Baudouy, 1996; Christensen et al., 1999; Fernandez-Espla and Rul, 1999; Fundoiano-Hershcovitz et al., 2004; Arima et al., 2006; Sanz, 2007; Mahajan and Badgujar, 2010; Mucha et al., 2010; Jankiewicz and Wnuk, 2011). Based on the mechanism of catalysis, bacterial aminopeptidases are classified as metallo aminopeptidases, serine aminopeptidases and cysteine aminopeptidases (Jankiewicz and Bielawski, 2002). Metalloaminopeptidases are inhibited by metal chelators such as EDTA and 1, 10 phenanthroline. Zn^{+} is the most commonly associated cation in the metallopeptidase family. The family is divided into two subgroups. The first consists of PepN aminopeptidases from *E.coli* and lactic acid bacteria. They contain one Zn^{+} per molecule and have the characteristic HEXXH motif. The second subgroup has two closely related Zn^{+} per molecule and the enzyme includes PepA from *E.coli* (Gonzales and Robert-Baudouy, 1996). Cysteine or serine aminopeptidases require a highly reactive cysteine or serine residue. Serine aminopeptidases are inhibited by phenylmethylsulfonyl fluoride and cysteine aminopeptidases are inhibited by iodoacetamide and p-chloromercuribenzoate. The serine aminopeptidase activity is reported in the D-aminopeptidase of *Ochrobactrum anthropi*, the proline imino peptidase family and X-proline dipeptidyl aminopeptidase family. The

cysteine peptidases of broad specificity include PepC type whose active site consists of amino acids residues such as Gln, Cys, His, Asn/Asp which are important for catalysis. Aminopeptidases are further subdivided into four groups: (i) Aminoacyl and iminoacyl peptidases which strictly hydrolyse first bond in a polypeptide chain and release a single aminoacid residue, (ii) Dipeptidyl and tripeptidyl peptidases which hydrolyses dipeptides or tripeptides from polypeptide chains, (iii) Tripeptidases which hydrolyse only tripeptides, and (iv) Dipeptidases which hydrolyse only dipeptides (Sanz, 2007).

1.2.1.1.1.1 Specificity of aminopeptidase

Microbial aminopeptidases are classified into following groups based on their specificities: (i) general zinc-dependent metallo aminopeptidases showing broad specificities which include PepN aminopeptidase or lysyl aminopeptidase found in *Pseudomonas*, *Escherichia coli*, lactic acid bacteria, PepL aminopeptidase which are serine peptidases found in *Lactobacillus* species, PepC and bleomycin hydrolases that are cysteine aminopeptidases of broad specificity and found in lactic acid bacteria and yeasts. (ii) Aminopeptidases with narrow specificity which specifically hydrolyse acid residues (PepA) or glutamyl aminopeptidase found in *Lactococcus lactis* and having specificity towards Glu , Asp and to a lesser extent Ser residues from the N-terminus of oligopeptides, and Methionine residues (MAP) that are found in *E.coli*, *Salmonella typhimrium* and *Saccharomyces cerevisia*e playing important biological roles since their inactivation results in lethal phenotypes, (iii) Proline specific aminopeptidases that hydrolyse proline - containing peptide bonds and are found in lactic acid bacteria. This group includes aminopeptidases, PepI and PepP and dipeptidases PepQ and PepR and a dipeptidyl-peptidase PepX which is found in *Streptococcus gordonii* and *S. agalactiae* (Sanz, 2007).

A methionine aminopeptidase has specificity towards N-terminal methionine and removes methionine from amino-terminus of newly synthesized proteins (Mucha et al., 2010; Olaleye et al., 2010) with the action of key active site residues and a dinuclear metal centre (Lowther and Matthews, 2000). The enzyme carries out the co-translational N-terminal methionine excision and is essential for bacterial survival and was purified from *Mycobacterium tuberculosis* (Lu and Ye, 2010). There are two subtypes of methionine aminopeptidases (MetAP), type 1 and 2. Most bacteria and archeae have either type 1 or type 2, while ekaryotes harbour both types (Zhang et al., 2009; Yuan et al., 2011). Leucine aminopeptidases are zinc requiring metallopeptidases that metabolizes L-leucylglycine and other peptides with N-terminal leucine (Nagy et al., 2008; Lin et al., 2010). Bacterial sources of leucine aminopeptidase include *Escherichia coli* (Vogt, 1970), *Pseudomonas* (Merkel et al., 1981), *Pseudomonas putida* (Kale et al., 2010), *Streptomyces griseus* (Spungin and Blumberg, 1989) *Streptomyces mobaraensis* NRRL B-3729, *Streptomyces gedanensis* IFO 13427 and *Streptomyces platensis* NRRL 2364 (Nagy et al., 2008). The fungal source includes *Aspergillus oryzae* strains LL1 and LL2 (Lin et al., 2010), *Aspergillus sojae* (Chien et al., 2002). PepS, a monomeric metallopeptidase from *Streptococcus thermophilus* having specificity towards peptides possessing arginine or aromatic aminoacids at the N-terminus (Fernandez-Espla and Rul, 1999).SGAP, an aminopeptidase from *Streptomyces griseus* (a double-zinc aminopeptidase) that has preference for large hydrophobic amino-terminus residues in the substrate and having Glu131 and Try246 as the catalytic residues (Fundoiano-Hershcovitz et al., 2004). A prolyl aminopeptidase shows preference for a particular amino acid residue in P1 or P1´position.The term prolyl is used when prolyl bond is cleaved. Prolyl aminopeptidase was reported from *Penicillium camemberti* (Fuke and Matsuoka, 1993). A proline aminopeptidase hydrolyses the bond on the imino side of proline (Barrett, 1994). A novel 54.3 kDa leucine aminopeptidase was

purified from *Lactococcus lactis*. The enzyme differed in its optimum temperature and pH from other bacterial aminopeptidases (Ye and Ng, 2011). An X-prolyl-dipeptidyl aminopeptidase which releases X-pro dipeptides from amino-terminus of the peptide chains has been purified from *Lactococcus lactis* (Chich et al., 1992). β-peptidyl aminopeptidase has specificity for N-terminal cleavage of β-aminoacids from oligopeptides and hydrolyses β-peptides into their constituting aminoacids. The L-aminopeptidase D-Ala-esterase/amidase from *Ochrobactrum anthropi* (DmpA) was the first purified enzyme in this class (Geueke and Kohler, 2007). An aminopeptidase from *Streptomyces sp.* KK565 degrades beta amyloid monomers, oligomers and fibrils and has the potential in Alzheimer disease therapeutic strategies (Yoo et al., 2010). A thermostable aminopeptidase, which was found to have aminopeptidase activity on N-blocked and non-blocked substrates with optimum activity at 90°C, was purified from *Pyrococcus horikoshii* (Ando et al., 1999).

1.2.1.1.2 Carboxypeptidases

The carboxypeptidase hydrolyses peptide bonds from free C-terminus of peptides or proteins and liberates a single residue or a dipeptide and has physiological roles in processing of precursor proteins (Barrett, 1994; Cheng et al., 1999; Arndt et al., 2002; Liu et al., 2004). Carboxypeptidases are classified into serine carboxypeptidases, metallo carboxypeptidase and cysteine carboxypeptidases based on the catalytic type (Barrett, 1994). Metallocarboxypeptidases are the largest class of carboxypeptidases and have a single zinc ion bound to the active site. Based on the substrate specificities, metallocarboxypeptidases are divided into carboxypeptidase A, which has preference to neutral hydrophobic amino acids, carboxypeptidase B which prefers basic amino acids, and carboxypeptidase T, which has specificity for basic and hydrophobic amino acids and has four binding sites for Ca^{2+} ions which are essential for temperature stability. Carboxypeptidase T from *Thermoactinomyces vulgaris* is a metallo

carboxypeptidase of single polypeptide chain of 326 aminoacids and has one zinc ion (Teplyakov et al., 1992; Arndt et al., 2002). Serine carboxypeptidases release amino acids from the carboxyl end of peptides and proteins. The active site of these enzymes contains a catalytic triad consisting of Ser, His, and Asp (Ramírez-Zavala et al., 2004). It consists of two groups of enzymes: (i) Single chain enzymes containing approximately 420 amino acid residues which includes carboxypeptidase Y from yeast (Svendsen et al., 1995). Carboxypeptidase Y, which showed hydrolytic activity against some hydrophobic peptides of the tryptic digests of αs1- and β-caseins was reported from *Kluyveromyces fragilis* (Transfiguracion et al., 1998) and, (ii) Enzymes that are processed into two chains of approximately 260 and 160 amino acid residues (Svendsen et al., 1993).

1.2.1.1.2.1 Specificity of carboxypeptidases

Carboxypeptidase from *Sulfolobus solfatavicus* showed broad substrate specificity by its ability to release basic, acidic and aromatic amino acids from the respective benzoylglycylated and benzyloxycarbonylated amino acids (Colombo et al., 1992). Carboxypeptidase of class G hydrolyses the C-terminal glutamate moiety from folic acid and analogs such as methotrexate (MTX), polyglutamate derivatives of folic acid, sub fragments such as p-aminobenzoylglutamate, and specific small peptides with C-terminal glutamate residues (Minton et al., 1983; Rowsell et al., 1997). Carboxypeptidase G_2, a zinc metallopeptidase that catalyses hydrolytic cleavage of reduced and non-reduced folates to pteroates and glutamate was isolated from *Pseudomonas sp*. (Lloyd et al., 1991).The zinc dependent carboxypeptidase G_2, hydrolyses C-terminal glutamate moiety from folic acid and its analogs such as methotrexate (Rowsell et al., 1997). The D-alanyl-D-alanine carboxypeptidases (D-D carboxypeptidases) hydrolyse carbonyl carbon of the $_D$Ala-$_D$Ala amide bonds of $_L$Xaa- $_D$Ala-$_D$Ala terminated peptides, where Xaa is a diamino residue (Leyh-Bouille et al., 1981).

1.2.1.2 Endopeptidases

Endopeptidases act on peptide bonds inside the polypeptides (Gonzales and Robert-Baudouy, 1996).They act in the inner region of peptide chains away from the C and N termni. The binding site of an endopeptidase will not accommodate at free C or N terminus and the presence of a free amino or carboxyl groups has a negative effect on enzyme activity. Based on catalytic mechanism, endopeptidases are classified into serine type proteases, cysteine type proteases, aspartic type proteases and metallo type proteases (Barrett, 1994).

Serine and metalloproteases include alkaline proteases of commercial importance, hence more emphasis is given on the review of serine and metalloproteases.

1.2.1.2.1 Serine protease

Serine proteases (EC. 3.4.21) are characterised by the presence of a serine residue in the active site of the enzyme and are dependent on a serine residue for catalytic activity. The catalytic triad consists of serine, histidine and aspartic acid residues. Serine proteases have the conservation of glycine residues in the vicinity of the catalytic serine residue to form the motif Gly-Xaa-Ser-Yaa-Gly (Barrett, 1994; Rao et al., 1998). They are widely distributed in both prokaryotes and eukaryotes and include exopeptidase, endopeptidase, oligopeptidase and omegapeptidase. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11.The isoelectric points are generally between pH 4 and 6 (Rao et al., 1998; Singh et al., 2001a; Gupta et al., 2002a).

Bacterial serine proteases are divided into two superfamilies, chymotrypsin superfamily which includes proteases from *Streptomyces erythreus*, *S. fradiae*, *S. griseus* etc. which share sequence homology with mammalian enzymes (Gupta et al., 2002a), and subtilisins family that has

no homology to mammalian enzymes (Clark et al., 2000). Subtilisins or serine alkaline protease (SAP, EC 3.4.21.14) has specificity towards aromatic or hydrophobic residues such as tyrosine, phenylalanine, and leucine at P1 position. They belong to the S8 family of peptidases and are synthesized as pre-pro-subtilisin precursors. The various types of subtilisins reported are subtilisin E from *Bacillus subtilis* (Takagi et al., 1997), subtilisin Carlsberg from *Bacillus licheniformis* (Jacobs et al.,1985), subtilisin BPN´ or Bacterial protease Novo type from *Bacillus amyloliqufaciens* (Miyazawa et al., 2003; Maurer,2004), subtilisin NAT from *Bacillus subtilis* (*natto*) (Yamagata et al., 1995a), subtilisin Sendai from *Bacillus* sp. (Yamagata et al., 1995b), and subtilisin- like serine protease TK1689 from *Thermococcus kodakaraensis* (Rasool et al., 2010) which exerts a toxic effect to host cells when secreted in pro-protein form. Subtilisin Sendai consists of 269 amino acids (Yamagata et al., 1995b), Subtilisin AprB also consists of 269 amino acid residues and has a high content of Glu and Asp residues and a low content of Arg and Lys residues on the surface, a unique feature from other subtilisins (Deng et al., 2011). The mature subtilisins E and BPN' display 275 residues (274 for Carlsberg) with a catalytic triad involving His64 (63 for Carlsberg), Asp32 and Ser221 (220 for Carlsberg) residues and two calcium binding sites (three for Carlsberg) which stabilize the three dimensional (3D) structure (Jaouadi et al., 2010b). Both subtilisin Carlsberg and subtilisin BPN´ have similar catalytic triad consisting of Ser221, His64 and Asp32 and similar optimum pH and temperature of 10 and 60°C , respectively (Gupta et al., 2002a).The size of subtilisins varies from 18 kDa to 90 kDa (Maurer, 2004).

The catalytic mechanism of serine proteases follows a two step reaction. The nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the substrate is catalyzed by a histidine imidazole group as a general base. This leads to formation of a tetrahedral

intermediate and an imidazolium ion (addition reaction). The tetrahedral intermediate breaks down by general acid catalysis to an acyl-enzyme, an imidazole base and an amine product (elimination reaction). During the acylation step, the imidazole group transfers the proton of the serine hydroxyl to the amine leaving group. The acyl-enzyme is then deacylated through the reverse reaction pathway of acylation, but in the second addition-elimination reaction a water molecule instead of the serine residue is the attacking nucleophile (Rao et al., 1998; Polgár, 2005).

1.2.1.2.1.1 Families of serine proteases

Serine proteases are grouped into 20 families based on their structural similarities which have been further subdivided into 6 clans (the term clan is used to describe group of families that share a common ancestor) with common ancestors. The structures of the clans chymotrypsin (SA), subtilisin (SB), carboxypeptidases C (SC), and Escherichia D-Ala–D-Ala peptidase A (SE) are totally unrelated, suggesting that there are at least four separate evolutionary origins for serine proteases (Barrett, 1994; Rao et al., 1998).

(a) Clan SA: Chymotrypsin

This clan consists of 10 families belonging to endopeptidases which differ widely in specificity.

1) **Chymotrypsin family (S1)** includes microbial proteases in the chymotrypsin family and consists of trypsin- like enzymes from *Streptomyces, Saccharopolyspora* and *Fusarium oxysporum* (Rawlings and Barett, 1993; Barrett, 1994). The catalytic unit of these proteases is a polypeptide chain consisting of 220 amino acid residues. The catalytic unit is always the C-terminal domain with the exception of acrosin and complement component C2 which have C-terminal extensions beyond the peptidase domain. Proteolytic cleavage

at the N terminus of the catalytic domain of a proenzyme in the chymotrypsin family forms a new N-terminal amino acid residue with a hydrophobic side chain. The new terminal or amino group forms a salt bridge with Asp-194, which leads to the assembly of the functional catalytic site. Asp-194 is crucial in maintaining the activity of members of S1 family. A surprising feature of the genes for peptidases of the chymotrypsin family is the diversity of codons used for the active site serine residue. The six available codons for serine fall into two groups (TCA, TCG, TCT, TCC vs. AGC, AGT) such that inter conversion between groups requires two base changes. All six codons are used for active site serine residues in the chymotrypsin family.

2) **α-lytic endopeptidase family** (S2) consists of endopeptidases that show specificity for P1 residues that is basic and hydrophobic. The family contains enzymes having cleavage action on glutamyl bonds. The active site serine residues are encoded by TCX codons with the exception of *Bacillus* enzymes which use AGX codons.

3) **Sindbis Virus core endopeptidase family** (S3), in which the viral genome encodes two polyproteins, p130 which is a serine endopeptidase and p270 which is a cysteine endopeptidase. The active serine site is encoded by AGX codons.

4) **Lysyl endopeptidase family** (S5) in which the disulfide bridge Cys-Cys maintains the structure of active site. There is a C-terminal domain to the potential active site residues. This domain along with the long N-terminal peptide is removed during proteolytic activation of lysyl endopeptidase.

5) **IgA- specific serine endopeptidase family** (S6) consists of serine endopeptidases, having specificity towards prolyl bonds at the hinge region. The enzyme secretion takes place by a precursor mosaic protein of more than 1500 amino acids. The proprotein contains N-terminal leader peptide that directs the protein to the periplasmic space and is removed by the leader peptidase. There is a peptidase domain and C- terminal helper domain to create a pore in the outer membrane of bacterium to permit the secretion of active peptidase.

6) **Tobacco etch virus 35-kDa endopeptidase family**, in which the viral polyprotein contains three proteinases, two of which are cysteine proteinases (families C4 and C6) and one serine proteinase. The residues His-214, Asp-223 and Ser-256 are essential for activity. The presence of His/Asp/Ser order of catalytic residues and the sequence surrounding the catalytic Ser residues are consistent with the chymotrypsin (S1) family.

7) **Yellow fever virus NS3 endopeptidase family** (S7), in which the RNA encodes a single polyprotein which is processed by viral endopeptidase and cellular enzymes. The viral core protein is a nonstructural protein and occurs inside the polyprotein. The endopeptidase cleaves on the C-terminal side of paired basic amino acids and excises all non structural proteins from polyprotein. His-53, Asp-77 and Ser-138 form the catalytic triad.

8) **Hepatitis C virus NS3 endopeptidase family** (S29), in which the viral RNA encodes a nonstructural polyprotein. The catalytic triad consists of His/Asp/Ser.

9) **Cattle diarrhea virus p80 endopeptidase family** (S31) consists of single stranded RNA virus which encodes a large polyprotein. The catalytic triad includes His-217 and Asp-

254. The p80 protein, which is approximately in the middle of the polyprotein, has been identified as the peptidase responsible for processing all nonstructural viral proteins.

10) **Equine arteritis virus putative endopeptidase** (S32) consists of viral genome that encodes helicase and polymerase polyproteins. The catalytic triad consists of His-1103, Asp-1129 and Ser-1184 (Barrett, 1994).

(b) Clan SB: Subtilisin

This clan consists of subtilisin family. The family includes endopeptidases and tripeptidyl-peptidases and are widespread among eubacteria, archeaebacteria, eukaryoteas and viruses. The catalytic triad consisted of Asp, His, Ser residues. They hydrolyse peptide bonds of large hydrophobic residues which have tyrosine, phenylalanine or leucine at the C-terminal side of the splitting bond. Subtilisins produced by *Bacillus* spp. are the best known and were first discovered in *Bacillus subtilis*. The 56 kDa protease from herpes virus I forms the sole viral member of family. The role of SB family peptidases is in nutrition and protein processing (Page and Di Cera, 2008).

(c) Clan SC: Carboxypeptidase

The clan SC consisting of 4 families and has a common catalytic triad consisting of the three amino acids, serine (nucleophile), aspartate (electrophile), and histidine (base).The active site consists of linear arrangement of catalytic triad: Ser, Asp, and His. Clan SC peptidases are α/β hydrolase-fold enzymes consisting of parallel β - strands surrounded by α helices which enables catalytic function/platform (Page and Di Cera, 2008). The families are: (1) **Prolyl oligopeptidase family** (S9), in which the catalytic residues consist of Ser/Asp/His arrangement. Members included enzymes from prokaryotic and eukaryotic organisms and include both membrane bond and soluble peptidases. The enzymes of this family

lack proteolytic processing and are synthesized in the active form. (2) **Carboxypeptidase C family** (S10). The catalytic triad consists of Ser/His/Asp residues. The carboxypeptidases in this family have acidic pH optimum and this is because the residue preceding the catalytic Ser is a Glu residue. The enzymes are classified as carboxypeptidase C which has preference for a hydrophobic amino acid in P1' and carboxypeptidase D, which release C-terminal arginine or lysine. This family is unique among clan SC due to their ability of catalytic action in acidic environment (Page and Di Cera, 2008). (3) **Lysosomal Pro-X carboxypeptidase family** (S28), the sole member of this family is a carboxypeptidase having specificity towards prolyl bonds. It has an acidic pH optimum and the glutamate residue preceding the active site serine is responsible for this. The enzyme is synthesized with a signal peptide and propeptide. The active site residues consist of Ser-134, Asp-333 and His-411. (4) **Lactococccus X-pro-peptidase family** (S15), which contains enzyme from *Lactococcus lactis* known as X-prolyl dipeptidyl-peptidase IV cleaving Xaa-Pro-peptide bonds to release N-terminal peptides. The enzyme does not have a proenzyme and exists as a homodimer. The catalytic site consists of Asp and His residues. (5) **Neisseria prolyl aminopeptidase family** (S33), consists of a 35 kDa peptidase from *Neisseria gonorrhoea* that is specific towards the hydrolysis of N-terminal Pro residues. The peptidase has serine at the active site (Barrett, 1994).

(d) Clan SE: Serine-Type D-AIa-D-Ala Peptidases

Clan SE peptides has catalytic dyad mechanism consisting of pairing of Ser and Lys separated by two residues and the third residue which is Ser or Tyr residue may also involve in the abstraction of proton from nucleophilic Ser (Page and Di Cera, 2008).The clan consists of following families: (1) **Family S11** contains D-Ala-D-Ala peptidases and strict transpeptidase of *Streptomyces* K15, (2) **Family S12**, consists of D-Ala-D-peptidase from *streptomyces*, a D-aminopeptidase from *Ochrobactrum*,

lipolytic esterase from *Pseudomonas* and proteins from *Bacteriodes nodosus* that may be involved in the assembly of fimbriae. The active site motif of the protein is Ser-Xaa-Xaa-Lys, and (3) **Family S13** consists of D- Ala-D-Ala peptidases from *Actinomadura* and a pencillin binding protein 4 from *E.coli* which has both carboxypeptidase and endopeptidase activities (Barrett, 1994).

(e) Clan SF

Members of clan SF use Ser, Lys dyad in prokaryotes and Ser, His dyad in eukaryotes and have endoproteolytic catalytic action. Most peptidases in the clan are self activating and have strong specificity towards aliphatic side chains such as Ala, particularly with Ala-Xaa-Ala, where Xaa is a large hydrophobic aminoacid (Page and Di Cera, 2008).The clan consists of three families:

1) **Repressor LexA family** (S24). The LexA protein has cleavage action on Ala-Gly bond. The protein represses about 20 genes of the SOS regulon that are involved in the DNA repair in *E.coli.* The LexA molecules and related represses consisted of 200 aminoacid residues of which the N-terminal 90 forms the binding domain. The C- terminal forms the catalytic site for autolytic peptide bond cleavage.

2) **Bacterial leader peptidase I family** (S26).This family belongs to eubacteria and consists of three leader peptidases namely (i) murein prelipoprotein peptidase, which is an aspartic endopeptidase that removes leader peptide from one of component of bacterial outer membrane, (ii) type IV prepilin leader peptidase which is a cysteine peptidase, and (iii) serine type leader peptidase which helps in removing the leader peptides from other secreted proteins and proteins that are targeted to the periplasm and periplasmic membrane.

3) **Eukaryote signal peptidase family** (S27). This family consists of peptide complex that is responsible for removing signal peptides from secretory proteins as they are transported into the lumen of endoplasmic reticulum. The signal peptide depends up on Ser/ His dyad for catalytic activity (Barrett, 1994).

(f) Clan SG: ATP-Dependent Endopeptidases

This clan consists of three families:

1) **ClpP endopeptidase subunit family** (S14). Clp endopeptidase or endopeptidase Ti is a bacterial, ATP dependent enzyme. This endopeptidase complex consists of ClpP and ClpA subunits of composition ClpP12ClpA6. The full endopeptidase activity is the result of combined action of Clp in complex with ClpA. The catalytic diad consists of Ser-111 and His-136 residues (in *E.coli*).

2) **Endopeptidase La family** (S16). The endopeptidase La is a single-chain mosaic protein, containing an ATP-binding domain and a peptidase domain and the ATP binding domain is homologous to ClpA and ClpB subunits of the Clp endopeptidase. The catalytic residue consists of Ser-679.This endopeptidase is found in yeasts.

3) **Multicatalytic endopeptidase complex family** (S25).The multicatalytic endopeptidase complex (MEC) is a 700-kDa endopeptidase containing about 24-28 similarly sized, homologous subunits and occurs in cytoplasm and nuclei of eukaryotic cells and of archeabacterium *Thermoplasma acidophylum*. The archeabacterium endopeptidase complex consists of α and β subunits and are divided into A and B groups, while in eukaryotes there are more variant subunits of α and β type of structure. The peptidase activities belonging to this

family are trypsin -like activity (cleavage of Arg bonds), chymotrypsin- like (cleavage of Leu-Try-and Phe- bonds) and glutamyl peptidase (cleavage of Glu bonds). The eukaryotic endopeptidase shows all the three activities. The catalytic activity is contributed by both A and B subunits and each contains some active site residues, and residues Gly-80, Asp-84 and Gly-166 are conserved in both sequences. The catalytic residues conserved in subunit A are Ser-16, Tyr-26, Asp-84 and Arg-130, while in subunit B, Asp-184 is the only conserved catalytic residues (Barrett, 1994).

1.2.1.2.2 Metalloprotease

Metalloprotease (EC 3.4.24) depends on the presence of bound divalent cations for catalytic activity. Metalloproteases are divided into neutral proteases which show specificity towards hydrophobic amino acids, alkaline proteases which possesses a very broad specificity. *Myxobacter* I & II proteases are specific for very small amino acid residues on either sides of the cleavage bond. Metalloproteases are enzymes containing the His-Glu-Xaa-Xaa-His (HEXXH) motif to form a part of the site for binding of the metal. Glu143 assists the nucleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond, which is polarized by the Zn21 ion (Rao et al., 1998).

1.2.1.2.2.1 Families of Metalloproteases

(a) HEXXH + E Metallopeptidase Clan (MA)

This clan consists of five families and a glutamic acid residue completes the metal binding site. The following are the families which come under the clan: (1) **Thermolysin family** (M4) containing eubacterial endopeptidases of moderate size (35 kDa) from Gram- positive and Gram-negative bacteria. The tertiary structure of thermolysin shows zinc ligands to be His-142, His-146 and Glu-166, and the catalytic residue Glu-143.

The enzymes have substrate specificity for aromatic residue in P1' which includes pseudolysin (*pseudomonas* elastase). The broader substrate specifity of pseudolysin on elastin and collagen may be attributable to the wider active site cleft. (2) **Mycolysin family** (M5) exhibits the same substrate specificity, and residues involved in catalytic activity are the same as those of thermolysin family. (3) **Neprilysin Family** (M13) contains eukaryotic and prokaryotic oligopeptidases of larger molecular weight (90 kDa). They act on oligopeptides and polypeptides. In neprilysin, zinc ligands are analogous to thermolysin. (4) **Alanyl aminopeptidase family** (M1) contains aminopepidases from *Lactococcus, Escherichia* and *Caulobacter* which differ widely in specificity in hydrolyzing acid, basic and neutral amino acid residues. (5) **Peptidyl-dipeptidase A family** (M2) consists of mammalian peptidase (Barrett, 1995).

(b) HEXXH + H Metallopeptidase Clan (MB)

The peptidases of this clan are termed as "metzincins". They have the characteristic HEXXH motif and a Met residue is conserved throughout this clan which serves vital function in the structure of the active site of the enzymes. This clan consists of two families and all enzymes of this clan belong to endopeptidases. The conserved Met-143 residue serves a vital role in the structure of the active site of the enzymes. The Gly residue (Gly-95) is totally conserved in the extended active-site motif, HEXXHXXGXXH contributing to an important hairpin loop that permits the third His to bind the zinc atom. The members of clan MB (except M10,M11and M12) possess a cysteine-switch mechanism in which a cysteine residue occurs in a conserved motif in the propeptide and interacts with the essential atom to prevent it from binding the catalytic water molecule, maintaining the proenzyme in an inactive state.

The important families belonging to this clan are: (1) **Interstitial collagenase family** (M1O), the members of which are classified into: (i) *Serralysin subfamily*: Serralysins are secreted as proproteins that require proteolytic activation. Serralysins contain six glycine rich repeats (Gly-Gly-Xaa-Gly-Asn-Asp) in the C- terminal domain. The members belonging to this subfamily are serralysins from *Pseudomonas* and *Erwinia.* (ii) *Matrixin subfamily*: containing eukaryotic matrix metalloproteinases. The catalytic domains contain two zinc atoms, of which one is catalytic and other contributes to protein structure. The non catalytic zinc atom is bound by one Asp and three His residues. The catalytic domain contains one or two calcium atoms. (2) ***Streptomyces* extracellular neutral proteinase family** (M7), enzyme from *Streptomyces* sp. is the only member of the family. The deduced sequence of the proenzyme shows a signal peptide and an HEXXH motif with a Leu-Gly-Leu sequence nearby. Met-143 is the only Met residue C-terminal to the HEXXH motif. The enzyme is stabilised by calcium and contains a putative disulfide bridge.

(c) Other HEXXH Metallopeptidase Families

They contain the characteristic HEXXH motif as part of zinc-binding site and additional metal ligands are not identified in this set of families. (1) **Thimet oligopeptidase family** (M3) contains eubacterial peptidyl-dipeptidase and has two conserved Glu residues C-terminal to the HEXXH motif, Glu-172 and Glu-179. The conserved His-149 seems too close to the HEXXH motif to be a ligand. The oligopeptidase are thiol dependent due to the presence of cysteine residue close to HEXXH motif. (2) **Immune inhibitor A family** (M6) contains an endopeptidase from *Bacillus thuringiensis* as the sole member. (3) **Vibrio collagenase Family** (M9) consists of collagenase from *Vibrio* which is synthesized as a precursor with a signal peptide and apparently a propeptide. The enzyme cleaves Xaa-Gly-Pro-Xaa bonds in helical region of collagen. (4)

Clostridium collagenase Family (M31) contains *clostridium perfringens* collagenase. The enzyme shares a C-terminal domain to the peptidase domain. The C-terminus contains two repeats of a 90-residue domain. (5) **Immunoglobulin A-specific metalloendopeptidase family (M26)**, consists of immunoglobulin A specific endopeptidase from *Streptococcus sanguis* which cleaves Pro-Thr bond in the hinge region of heavy chain of immunoglobulin A and contains an HEXXH motif. (6) **Tetanus toxin family** (M27) contains two toxin proteins namely botulinum toxin and tetanus toxin. The catalytic domain of the toxins, a zinc binding motif and botulinum toxin, contain one zinc atom per molecule. (7) ***Staphylococcus hyicus* extracellular metalloendopeptidase family** (M30) contains a 38.5 kDa zinc endopeptidase which is synthesized with a signal peptide and a 75 residue propeptide. The sequence includes an HEXXH motif and Glu-269 may be a third ligand. (8) **Carboxypeptidase Taq family** (M32) consists of carboxypeptidase Taq as an enzyme from the thermophilic eubacterium *Thermus aquaticus* that is synthesized with a 28-residue prepropeptide. The carboxypeptidase contains one atom of zinc per molecule. It contains an HEXXH potential zinc-binding site and carboxypeptidase Taq is the first carboxypeptidase to be discovered that binds zinc at such a motif.

(d) Families of Metallopeptidases with Metal Ligands Other than HEXXH

The families include: (1) **Pitrilysin family** (M16) in which the enzymes bind zinc at an HXXEH sequence. Pitrilysin is secreted as a large protein of 950 residues including a 23 residue signal peptide. His-88, His-92 and Glu-169 are the zinc ligands and Glu-91 as catalytic residue. (2) **Carboxypeptidase A family** (M14) includes enzymes from actinomycetes and is synthesized as inactive precursors with propeptides. Three dimensional structure of the procarboxypeptidase consists of a propeptide with a globular domain followed by a α-helical segment which shields the catalytic site without making contact with it, whereas the

substrate-binding site is blocked by specific contacts. (3) **Zinc D-Ala-D-Ala carboxypeptidase family** (M15) consists of metallopeptidase which is synthesized with an N-terminal extension of 45 residues including a propeptide. Enzyme from *Streptomyes* sp. of this family consists of structure with His -153, His-194 and His-196 as the zinc ligands. The enzyme consists of two domains of which the N-terminal contains 80 residues and is homologous to C-terminal third of *Bacillus* N-acetylmuramoyl-L-alanine amidase. (4) **Leucyl aminopeptidase family** (M17) includes those from *E.coli*. The enzyme is a homohexamer, with two zinc atoms bound per subunit by residues clustered in the relatively short segment at residues 250-334. For one atom, the ligands are Asp-255, Asp-332, and Glu-334, and for the other, Lys-250, Asp-255, Asp-273, and Glu-334. (5) **Methionyl aminopeptidase family** (M24) consists of aminopeptidases containing "co-catalytic" metal atoms. The enzymes included in this family are bacterial methionyl aminopeptidase, which removes N-terminal Met-residues in association with ribosomes acting co-translationally.

(e) Metallopeptidase Families with Unknown Metal Ligands

This group consists of nine families with members of unknown metal ligands. (1) **Yeast aminopeptidase I family** (M18) contains a leucyl aminopeptidase, which is a glycoprotein from *Saccharomyces cervisiae*. The enzyme is synthesized as a precursor with a 45 residue prepropeptide and is activated by the cleavage of a Leu-Glu bond to yield a 424 residue mature enzyme. (2) **Membrane dipeptidase family** (M19) shows broad specificity toward dipeptides. It is synthesized with a signal and C-terminal hydrophobic domain; both are removed in post-translational processing. The family includes enzymes *Klebsiella* and *Acinetobacter*, bacterial haemolysin activators of *Serratia marcescens.* (3) **Glutamate Carboxypeptidase family** (M20) consists of glutamate carboxypeptidase, a homodimer, in which each subunit binds two zinc atoms. The family contains two peptidases, succinyldiaminopimelate and

acetylornithine deacetylase involved in biosynthesis of arginine lysine respectively. *Lactococcus* peptidase V hydrolyses β-Ala-His. (4) **O-sialoglycoprotein endopeptidase family** (M22) contains enzyme from *Pasteurella haemolytica* which cleaves proteins that are heavily *O*-sialoglycosilated. (5) **β-lytic endopeptidase family** (M23) includes enzymes having specificity towards Gly bonds, especially in Gly-Gly-Xaa sequence. These are *β*-lytic endopeptidases of *Lysobacter* and *Achromobacter*, LasA protease of *Pseudomonas aeruginosa* and fibrinolytic enzyme from *Aeromonas* which lyse the cell wall of Gram- positive bacteria in which peptidoglycan cross links contains multiple Gly- residues. *β*-lytic endopeptidases of *Lysobacter* is synthesized with a 171 residue propeptide at the N-terminus. LasA of *Pseudomonas aeruginosa* acts on elastin by the cleavage of glycyl bonds, the fibrinolytic activity of enzyme from *Aeromonas* is attributed by the cleavage of Gly-Gly-Ala bonds located near the cross link site in fibrin. (6) **X-His dipeptidase family** (M25) consists of a cytoplasmic exopeptidase from *E.coli* that cleaves β-Ala-His, and is the only member of the family. (7) **Vibrio leucyl aminopeptidase family** (M28) contains an aminopeptidase secreted by *Vibrio* and has a preference towards leucyl bonds. During maturation of enzyme, 99 residue C-terminal domains as well as 85- residue N-terminal propeptide are being removed and the enzyme is secreted as a 30 kDa protein. (8) **Aminopeptidase T family** (M29) includes oligomeric, cobalt dependent aminopeptidase having high temperature stability from *Bacillus stearothermophilus, Thermus aquaticus* and *Thermus thermophilus.* (9) **Aminopeptidase Y family** (M33) consists of glycosylated, vacoular enzyme containing zinc, but no HEXXH motif. It is synthesized as a 56 kDa propeptide. The mature aminopeptidase Y consists of four segments. The second of these resembles a sequence inserted between the active site His and Ser residues of several bacterial members of the subtilisin family of serine peptidases (S8), including the cell-wall associated endopeptidases of

Lactococcus and *Lactobacillus*, the C5a peptidase from *Streptococcus*, and an intracellular endopeptidase from *Bacillus*. The third segment of aminopeptidase Y is similar to one found in a metal-dependent leucyl aminopeptidase from *Vibrio* (family M29) and an *E. coli* arginyl aminopeptidase of unknown catalytic mechanism (family U2). The catalytic residues of aminopeptidase Y seem likely to be located in the first or fourth segments of the enzyme, which are not detectably related to other proteins (Barrett, 1995).

1.2.1.2.3 Aspartic proteases

Aspartic proteases (EC 3.4.23) depend on aspartic acid residues for their catalytic activity and are characterised by their acidic pH optima and are specifically inhibited by pepstatin (Singh et al., 2001a). Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from parretroviruses (A3) (13), and have been placed in clan AA. Most of them show optimum activity at low pH (3 to 4), and isoelectric point between 3 and 4.5. The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr. Microbial aspartic proteases are divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* spp. (Rao et al., 1998).

1.2.1.2.4 Cysteine proteases

Cysteine proteases EC (3.4.22) depend on catalytic dyad consisting of cysteine and histidine and catalyse the hydrolysis of peptide, amide, ester, thiol ester and thiono ester bonds (Singh et al., 2001b; Grzonka et al., 2007).Cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid,

and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. About 20 families of cysteine paptidases are identified (Barrett, 1994).

1.2.2 Specificity of proteases

An enzyme exhibits a high degree of specificity like substrate specificity (which allows them to discriminate between different substrates), regiospecificity (allows discrimination of similar parts of molecules) and stereospecificity (allows discrimination between optical isomers). These specificities result in the production of only necessary end products and the elimination of unnecessary ones (Krajewska, 2004).

Keratinases (EC 3.4.99.11) are serine or metalloproteases capable of hydrolysing insoluble keratinous proteins and have specificity towards aromatic or hydrophobic aminoacid residues (Gupta and Ramani, 2006; Radha and Gunasekkaran, 2009; Tiwary and Gupta, 2010; Shrinivas and Naik, 2011) and have action on soluble proteins such as casein, gelatin, bovine serum albumin, haemoglobin and on insoluble substrates such as wool, silk, collagen, elastin, horn, stratum corneum, hair, azokeratin, nail etc. (Gupta and Ramani, 2006). Keratin degradation in *Stenotrophomonas* sp. is the cooperative action of a serine protease and a disulfide reductase which on mixing results in tremendous increase in keratinase activity (Yamamura et al., 1992). Other keratinolytic proteases reported are from *Streptomyces pactum* DSM 40530 (Bockle et al., 1995), *Thermoactinomyces* sp (Gousterova et al., 2005; Gupta and Ramani, 2006), *Bacillus halodurans* (Takami et al., 1999), *Stenotrophomanas* (Cao et al., 2009; Jeong et al., 2010), *Chrysobacterium* (Riffel et al., 2003), *Microbacterium arborescens* (Thys et al., 2004); *Kocuria rosea* (Bernal et al., 2006), *Clostridium sporogenes* bv. *pennavorans* bv. nov. (Lonata et al., 2008). keratinases from

Bacillus includes those reported from *Bacillus pseudofirmis* (Gessesse et al., 2003), *Bacillus halodurans* AH-101 (Takami et al., 1999), *Bacillus subtilis* KS-1 (Suh and Lee, 2001). Keratinolytic fungal genera belong to fungi imperfectii consisting of genera such as *Chrysosporium*, *Aspergillus*, *Cladosporium*, *Fusarium, Pencillium* etc. (Gupta and Ramani, 2006). A keratinolytic enzyme with broad specificity with capability of degrading globular and fibrillar proteins was reported from the fungus *Doratomyces microspores* (Friedrich and Kern, 2003).

Collagenolytic proteases mainly belong to metalloprotease and serine protease of which metallo collagenolytic proteases cleavage takes place between the peptide bond of residue X and Gly-Pro (VanWart and Steinbrink, 1981; Harrington, 1996; Watanabe, 2004), while serine collagenolytic protease shows a broader range of specificity and their products are hydrolyzed at various but specific peptide bonds (Okamoto et al., 2001; Watanabe, 2004). Collagenolytic enzymes are divided into collagenases (E.C.3.4.24.3) which have specific site for cleavage of collagen and are produced by *Clostridium histolyticum* (Toyoshima et al., 2001) and *Vibrio vulnificus* CYK279H (Kang et al., 2005), and collagenolytic proteases (E.C.3.4.21.62) degrade various protein substrates including collagen and are produced by *Bacillus* sp. strain MO-1 (Okamoto et al., 2001), *Alicyclobacillus sendaiensis* strain NTAP-1 (Tsuruoka et al., 2003) and *Alkalimonas collagenimarina* AC40^T (Kurata et al., 2010).

Nattokinase, a potent fibrinolytic enzyme with high specificity towards fibrin was purified from *Bacillus subtilis* TKU007 (Wang et al., 2011). A novel fibrinolytic enzyme active over a wide pH range was also reported from *Bacillus subtilis* A26 (Agrebi et al., 2009). Fibrinolytic protease from *Streptomyces omiyaensis* has high hydrolytic activity towards mimic peptides of fibrin and plasminogen (Uesugi et al., 2011). Fibrinolytic alkaline proteases are also reported from *Fusarium* sp. (Ueda

et al., 2007; Wu et al., 2009). A haemoglobin degrading protease was reported from *Bacillus pumilus* NJM4 (Yao et al., 2011).

1.2.3 Protease production

Microbial proteases are produced either by solid-state fermentation or submerged fermentation (Fernández-Lahore et al., 1998).

1.2.3.1 Solid state fermentation

Solid state fermentation (SSF) is defined as the fermentation process that takes place in the absence or near absence of water (Pandey et al., 2000). The important parameters affecting the protease production include accumulation density, bed height, agitation etc. (Sandhya et al., 2005a). Quantity of water should not exceed the saturation capacity of bed (Aguilar et al., 2008). Water and nutrients absorbed on the substrate support the growth of microbes. SSF has exhibited advantages of obtaining higher enzyme concentration, lower capital and operational cost, lesser wastewater output and is convenient at small-scale operations (Chand and Mishra, 2003).

The success of solid state fermentation depends upon selection of suitable substrate, physico-chemical and biochemical process parameters. The metabolic process of microbes depends on pH, temperature, substrate, water content and inoculum. Solid state fermentation offers several advantages when compared to submerged fermentation (Elibol and Moreira, 2005), which include the usage of waste materials as substrates, minimal water requirement which allows the production of metabolites in concentrated form and thus makes the downstream processing less expensive, retaining stability of the products due to less dilution in the media, saving water and energy, and the enzyme produced are less sensitive to catabolic repression or induction (Fernández-Lahore et al., 1998; Germano et al., 2003; Chellappan et al., 2006; Aguilar et al., 2008; Rai et al., 2009). Solid state fermentation results in high productivity,

low production cost and energy requirement, better product recovery, superior volumetric productivity, and involves usage of cheaper substrates, less effluent generation and requires simple equipment for enzyme production when compared to submerged fermentation (Fernández-Lahore et al., 1998; Pandey et al., 1999; Uyar and Baysal, 2004; Chutmanop et al., 2008; Paranthaman et al., 2009; Chen et al., 2011).

Determination of suitable environmental conditions of microorganisms is very important in order to achieve maximum enzyme production. The major factors affecting the microbial enzyme production include selection of suitable microorganism, pre-treatment of substrate, particle size, water content, relative humidity, temperature control, rate of oxygen consumption and carbon-dioxide evolution (Pandey et al., 1999; Pandey et al., 2000; Ellaiah et al., 2002; Germano et al., 2003; Uyar and Baysal, 2004; Elibol and Moreira, 2005). Solid state fermentation is mainly preferred for fungal enzyme production, due to the low moisture requirements of fungus when compared to bacteria (Chutmanop et al., 2008), especially for filamentous fungi that have the ability to grow on solid substrates such as wood pieces, and other natural organic materials and produce a wide range of extracellular enzymes (Fernández-Lahore et al., 1998; Pandey et al., 2000; Germano et al., 2003; Dahiya et al., 2005; Chellappan et al., 2006). Various fungal genera used for alkaline protease production under solid state fermentation include *Pencillium, Trichoderma, Rhizopus, Aspergillus* (Malathy and Chakraborty, 1991; Tunga et al., 2003; Agarwal et al., 2004; Paranthaman et al., 2009).Wheat bran was found to be the most suitable substrate for alkaline protease production in solid state fermentation by fungal species such as *Aspergillus flavus, Aspergillus niger* (Malathy and Chakraborty, 1991; Chakraborty et al., 1995). Sandhya et al. (2005a) reported 3.5-fold increase in protease production by *Aspergillus oryzae* in solid state fermentation when compared to submerged fermentation. Solid state fermentation of

bacterial protease is limited to the genus *Bacillus* (Uyar and Baysal, 2004). A strain of *Bacillus subtilis* gave 10 fold higher protease yields when cultivated under solid state fermentation using soycake as substrate (Soares et al., 2005).

1.2.3.2 Submerged fermentation

Submerged fermentation consists of stirred or nonstirred process, where microbial biomass is surrounded in liquid culture medium (Sandhya et al., 2005b). Approximately 90% of industrial enzymes are produced by submerged fermentation. Industrial production of microbial metabolites are carried out by submerged fermentation due to the availability of proper agitation and mixing of substrate, allowing proper monitoring of dissolved oxygen, pH and aeration, reduced microbial contamination due to shorter period of growth, requiring less processes monitoring and easiness in the scale up methods (Joo and Chang, 2006; Mukherjee and Rai, 2011). Submerged fermentation is reported for production of proteases from *Bacillus* sp. (Joo and Chang, 2006; Potumarthi et al., 2007). The production of protease from *Aspergillus tamarii* increased 6 times upon the supplementation of glucose in submerged fermentation (Boer and Peralta, 2000).

1.2.4 Optimization of protease production

Media optimization is usually done to maintain a specific ratio between various medium components so that it will be completely utilised by microorganism and thus prevents the wastage of medium components and hence obtain a cost-effective metabolite yield at the end of fermentation. Each microorganism has specific medium requirements for optimum enzyme production and therefore it is necessary to optimize each specific nutrient requirement for growth and enzyme secretion (Kumar and Takagi, 1999; Gupta et al., 2002b; Hajji et al., 2008; Oskouie et al., 2008; Bhunia et al., 2010; Bayoumi and Bahobil, 2011). Medium

optimization has a significant role in industrial enzyme production, since 30 to 40 % costs accounts for growth medium (Joo et al., 2002; Laxman et al., 2005; Hajji et al., 2008). Cost-effectiveness is an important criterion taken in account while developing a production medium which can be achieved by the inclusion of cheap agricultural residues (Lazim et al., 2009).

Conventional optimization studies was based on ´one at a time´ strategy which is time consuming, requires more experimental data sets and cannot predict the interaction between the components, while statistical optimization methodologies like Response Surface Methodology (RSM) include full factorial central composite design that predicts the interaction effects between the components, limits the number of experiments and select optimum conditions of variables for a desired response and therefore showed satisfactory results in many fermentation processes. Statistical medium optimization is carried out by selection of significant parameters affecting protease production by Plackett-Burman design followed by optimization of significant parameters and studying their interaction effects by multi-factorial response surface methodology (RSM) approach (Beg et al., 2003b; Chauhan and Gupta, 2004) and have a very significant role in yield improvement in enzyme production (Puri et al., 2002; Beg et al., 2003b; Dutta et al., 2004; Bas and Boyaci, 2007; Oskouie et al., 2008; Haddar et al., 2010a; Liu et al., 2010; Rai and Mukherjee, 2010; Karan et al., 2011). Plackett-Burman design allows the selection of significant factors affecting the enzyme production and elimination of unwanted variables (Reddy et al., 2008). It is a two level fractional factorial saturated design that uses only k +1 treatment combinations to estimate the main effects of k factors independently (Plackett and Burman, 1946; Singh and Chhatpar, 2010). It is useful in the first step screening of significant medium components affecting protease production and decreases the number of variables in further optimization step

(Fakhfakh-Zouari et al., 2010; Liu et al., 2010; Rai and Mukherjee, 2010; Tiwary and Gupta, 2010). Many reports are available on the use of Plackett-Burman design for screening of significant factors (Yu et al., 1997; Chauhan and Gupta, 2004; Singh and Chhatpar, 2010).

1.2.5 Downstream processing of protease

The initial step in downstream processing involves the removal of cells, solids and colloids from the fermentation broth and is important for the recovery of protease (Kumar and Takagi, 1999). Addition of coagulating or flocculating agents helps in the removal of solids that are colloidal in nature (Boyer and Byng, 1996).Flocculating agents are generally employed to effect the formation of larger flocs or agglomerates, which in turn accelerate the solid-liquid separation (Kumar and Takagi, 1999). The use of polyelectrolyte Sedipur TF5 as a flocculating agent at 150 ppm, pH 7.0 to 9.0 gave 74% yield of alkaline protease activity (Sitkey et al., 1992). After separating the bacterial cell materials from fermentation broth, the culture supernatant is concentrated (Gupta et al., 2002b).Concentration is done for the removal of water from the culture filtrate in order to recover the enzyme which is present in low amount (Kumar and Takagi, 1999).The process is pressure driven and is inexpensive and results in little loss of enzyme activity and offers purification, concentration and diaflitration (Sullivan et al., 1984; Peek et al., 1992; Boyer and Byng, 1996).

1.2.5.1 Precipitation

After the separation of cell from fermentation broth, culture supernatant is concentrated by ultrafiltration and the enzyme precipitated by salting out by solid ammonium sulphate or solvent precipitation using acetone. The addition of salts or organic solvents lowers the solubility of proteins in aqueous solutions thereby it tends to precipitate out of the solution. Precipitation is used as both purification and a concentration step and is generally effected

by the addition of a salt or organic solvent which lowers the solubility of the desired proteins in an aqueous solution (Kumar and Takagi, 1999). Many reports are available on the use of different concentrations of ammonium sulphate for precipitation. A 70% concentration of ammonium sulphate is reported for the precipitation of alkaline protease from *Bacillus proteolyticus* CFR3001 (Bhaskar et al., 2007), 20-90% for the precipitation of alkaline protease from fungus *Engyodontium album* BTMFS10 (Chellappan et al., 2006), 75% for precipitation of alkaline serine protease from a haloalkalophilic *Bacillus* sp. (Dodia et al., 2008), 40-70% saturation for fibrinolytic subtilisin-like serine protease from *Bacillus subtilis* TP-6 (Kim et al., 2006), 50-70% for alkaline protease from *Bacillus* sp.Y (Mala and Srividya, 2010), 60% saturation for obtaining active protease from *Bacillus subtilis*-150 (Normurodova et al., 2010), 50-80 % for alkaline protease from *Bacillus licheniformis* RSP - 09-37 (Sareen and Mishra, 2008), 60% for alkaline protease from *Bacillus cereus* (Shah et al., 2010), 20-80% saturation for an alkaline serine protease from *Bacillus* sp.PN51 (Tanskul et al., 2009). Acetone fractionation is another concentration of alkaline protease. Concentrations from 50-80% was used for the alkaline protease from *Salinivibrio* sp. strain AF-2004 (Karbalaei-Heidari et al., 2007b), 20-80% for *Bacillus mojavensis* A21 (Haddar et al., 2009 a,b), 80% for *Halobacillus karajensis* (Karbalaei-Heidari et al., 2009), 60% saturation for bleach tolerant alkaline protease from a gamma-proteobacterium (Sana et al., 2006).

1.2.5.2 Ultrafiltration

Ultrafiltration technique is used for the concentration of proteolytic enzymes (Bohdziewicz, 1994).A higher concentration of protease was obtained from *Streptomyces thermovulgaris*, when the cell free supernatant was concentrated by ultrafilration (Yeoman and Edwards, 1997). Ultrafiltration was employed for the recovery of alkaline protease from *Bacillus licheniformis* with an optimum transmembrane pressure of 90 kPa

and feed flux of 714 L/h/m^2 which gave highest protease recovery of 83% (Bezawada et al., 2011). A hollow fibre ultrafiltration system was reported for the concentration of alkaline protease from *Haloferax lucentensis* VKMM 007 (Manikandan et al., 2009). A temperature sensitive hydrogel ultrafiltration was employed for the concentration of alkaline protease (Han et al., 1995).Ultrafiltration is also reported in the purification of alkaline protease from *Virgibacillus* sp. SK33 (Sinsuwan et al., 2010), *Bacillus sphaericus* (Singh et al., 2001b), *Arthrobacter nicotianae* (Smacchi et al., 1999), *Oligotropha carboxydovorans* (Kang et al., 1999), *Bacillus* sp. B001 (Deng et al., 2010), *Bacillus licheniformis* MIR29 (Ferrero et al., 1996), *Bacillus cereus* BG1 (Ghorbel-Frikha et al., 2005), *Bacillus licheniformis* MP1 (Jellouli et al., 2011), *Halobacillus karajensis* (Karbalaei-Heidari et al., 2009), *Aspergillus nidulans* (Peña-Montes et al., 2008), *Pseudomonas* sp. (Shastry and Prasad, 2002).Diafiltration technique has been used for the removal of salt removal or changing the salt concentration (Banik and Prakash, 2006).

Further purification is carried out by one or more chromatographic techniques such as ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography etc.

1.2.5.3 Ion exchange chromatography

Generally, alkaline proteases are positively charged and they are not bound to anion exchangers and therefore cation exchangers are used. The different matrices used for anion exchange chromatography contains functional groups such as diethyl amino ethyl (DEAE) and carboxy methyl (CM) which gets associated with the charged protein molecules, thereby adsorbing the protein into the matrices and the adsorbed molecule is eluted by a gradient change in the pH or ionic strength of the eluting buffer or solution (Kumar and Takagi, 1999; Gupta et al., 2002b). Ion-exchange chromatography on DEAE-cellulose was performed for

purification of alkaline protease from *Bacillus altitudans* (Vijay Kumar et al., 2011), *Engyodontium album* (Chellappan et al., 2006), gamma-proteobacterium (Sana et al., 2006), *Bacillus cereus* (Shah et al., 2010), *Beauveria* sp (Shankar et al., 2011), *Bacillus* sp. PN51 (Tanskul et al., 2009), *Streptomyces clavuligerus* (Thumar and Singh, 2007). Ion- exchange chromatography using DEAE-Toyopearl column was used for collagenolytic subtilisin like protease from *Alkalimonas collagenimarina* AC40^T (Kurata et al., 2010), Mono Q-sepharose column for alkaline protease from *Bacillus licheniformis* MP1 (Jellouli et al., 2011), Q-Sepharose HP column for alkaline protease from *Salinivibrio* sp. strain AF-2004 (Karbalaei-Heidari et al., 2007 a,b), *Bacillus sphaericus* (Singh et al., 2001b), for *Halobacillus karajensis* (Karbalaei-Heidari et al., 2009), DEAE-Sepharose and phenyl sepharose chromatography for an alkaline keratinolytic protease from *Chryseobacterium indologenes* TKU014 (Wang et al., 2008b), DEAE-Sepharose CL-6B and phenyl sepharose for purification of nattokinase from *Bacillus subtilis* TKU007 (Wang et al., 2011), DEAE-sepharose for serine protease from *Thermoascus aurantiacus* var *levisporus* (Li et al., 2011), CM-Sephadex C50-F for *Bacillus subtilis*-150 (Normurodova et al., 2010), sephadex G-50 for alkaline protease from *Pseudomonas aeruginosa* MCM B-327 (Zambare et al., 2011), DEAE-sephacel column was used for the purification of alkaline proteinases from *Arthrobacter nicotianae* 9458 (Smacchi et al., 1999),CM-cellulose chromatography for alkaline protease from *Bacillus halodurans* JB99 (Shrinivas and Naik, 2011), Poros-HQ ion exchange column for *Streptomyces* sp. CS684 (Simkhada et al., 2010), Protease from *Aeromonas veronii* PG01 was purified by ion exchange chromatography followed by gel permeation with 53 fold purification and a yield of 32% (Divakar et al., 2010). Gel filtration and cation exchange chromatography were employed for purification of alkaline serine protease from *Bacillus pumilus* TMS55 (Ibrahim et al., 2011). A single step purification of alkaline protease from fungus *Conidiobolus coronatus* on

hydrophobic ligands resulted in 20 fold purification with 40% yield (Adikane et al., 2002). Ammonium sulphate precipitation followed by ion exchange chromatography was employed for the purification of protease from *Bacillus subtilis* -150 (Normurodova et al., 2010). A combination of ultrafiltration, bacitracin–Sepharose affinity chromatography and Sephadex G-100 gel filtration was employed for protease purification from *Haloferax lucentensis* VKMM 007 (Manikandan et al., 2009). A two step purification method involving ammonium sulphate precipitation and affinity precipitation with α-casein agarose resin was used for protease purification from *Bacillus licheniformis* RSP-09-37 (Sareen and Mishra, 2008).

1.2.5.4 Hydrophobic interaction chromatography

It uses the variability of external hydrophobic residues on different proteins leading to protein interaction by virtue of the fact that in aqueous solvents, hydrophobic patches on proteins preferentially seek out other hydrophobic surfaces. These hydrophobic interactions are strengthened by high salt concentrations and higher temperatures, and are weakened by the presence of detergents or miscible organic solvents. The extent of binding of a hydrophobic protein depends on the type and density of substitution of the matrix, as well as on the nature of buffer conditions. Hydrophobic interactions are much more variable in behaviour than ion exchangers, and because of this, resolution is generally poorer than ion exchange chromatography (Gupta et al., 2002b). Hydrophobic interaction chromatography on phenyl sepharose 6 fast flow column was used for purification of alkaline proteases from *Oceanobacillus iheyensis* O.M.$A_1$8 and Haloalkaliphilic bacterium O.M.$E_1$2 (Purohit and Singh, 2011), haloalkalophilic bacterium sp. AH-6 (Dodia et al., 2008), phenyl-Sepharose column for *Virgibacillus* sp. SK33 (Sinsuwan et al., 2010), phenyl agarose column for alkaline protease from *Bacillus sphaericus* (Singh et al., 2001b).

1.2.5.5 Affinity chromatography

In this technique, a ligand polymer is added to the enzyme solution under the conditions favoring binding the protein of interest. The ligand polymer is then precipitated and the supernatant is removed. The protein of interest is then eluted from the polymer under suitable conditions and the polymer can be recycled (Gupta et al., 2002b). Affinity chromatography with α-casein agarose resin was used for the purification of alkaline protease from *Bacillus licheniformis* RSP-09-37 with 85-fold purification (Sareen and Mishra, 2008), maxatase from *Bacillus licheniformis* (Pecs et al., 1991).

1.2.5.6 Gel Filtration chromatography

Gel filtration is used for the rapid separation of macromolecules based on size and are used in early-to-middle stage of purification (Gupta et al., 1996; Gupta et al., 2002b). Sephacryl S-200 gel filration chromatography was used for purification of alkaline protease from *Bacillus cereus* BG1 (Ghorbel-Frikha et al., 2005), keratinase from *Streptomyces* sp. strain 16 (Xie et al., 2010), sephadex G-75 for alkaline serine proteases from *Bacillus mojavensis* A21 (Haddar et al., 2009 a,b), BIO-GEL P-100 gel column for protease from *Pseudomonas* sp. CL1457 (Shastry and Prasad, 2002), sephadex G-100 for alkaline protease from *Bacillus halodurans* JB99 (Shrinivas and Naik, 2011), sephadex G-75 for protease produced by *Pseudomonas putida* (Singh et al., 2011), sephadex G-50 column for alkaline protease from *Bacillus cereus* VITSN04 (Sundararajan et al., 2011).

1.2.5.7 Aqueous two phase extraction

Aqueous two phase extraction is another alternative and is widely used for the recovery of biomolecules (Banik et al., 2003). It consists of two polymers or a polymer and a salt and is considered as environmental benign due to the replacement of volatile organic solvents, involvement in phase forming polymers that rely on water for the formation of phase systems and for solubilising the insoluble

hydrophobic solutes and the system allows the recycling of phase forming components. Aqueous two phase extraction system depends up on the nature and concentration of the polymer (molecular weight and hydrophobicity of the polymer and its concentration), temperature (polymer-polymer systems separates into phases at lower polymer concentration and at lower temperatures, while polymer-salt system requires high polymer concentrations at lower temperatures), salt type (whether multivalent or polyvalent) and its concentration (Raghavararao et al., 2003). It is economical in terms of less energy consumption, cell separation and product concentration can be achieved in a single step and thus replaces the conventional methods and is easy to scale up. It has a high capacity for handling of solids and has high partition coefficients and is thus a good alternative for large scale operation (Chand and Mishra, 2003; Chouyyok et al., 2005; Porto et al., 2007).

Partioning of alkaline protease from *Bacillus* sp. was carried out using aqueous two phase system consisting of 5% (w/v) polyethylene glycol (PEG-6000) and 35% (w/v) K_2HPO_4 (Sinha et al., 1996). Extraction of alkaline protease from cell free fermentation broth of *Bacillus subtilis* TISTR25 was carried out using polyethylene glycol (PEG 1000) and potassium phosphate (Chouyyok et al., 2005). An aqueous two phase system consisting of PEG1000 and potassium phosphate at 18.0 and 13.0, respectively at pH 9.5 was found to be efficient in the extraction of alkaline protease from *Bacillus subtilis* NS99 (Wongmongkol and Prichanont, 2006). Removal of proteases from *Clostridium perfringens* fermentation broth was achieved by PEG-citrate aqueous two phase system PEG (8000) and citrate of 24 and 15%, respectively, at pH 8.0 (Porto et al., 2007). Aqueous two phase system consisting of polyethylene glycol 550/potassium phosphate and polyethylene glycol 8000/potassium phosphate with a protein yield of 11 and 4%, respectively, was found to be efficient in the isolation and purification of protease from *Norcadiopsis* sp. (Porto et al., 2005).

1.2.6 Properties of protease

1.2.6.1 pH and Temperature kinetics

The optimum pH and temperature for alkaline protease is in the range between 9 to 11 (Kumar and Takagi, 1999; Beg and Gupta, 2003a; Denizci et al., 2004; Gupta et al., 2005 a,b ; Banik and Prakash, 2006; Bhaskar et al., 2007; Dodia et al., 2008; Deng et al., 2010; Xie et al., 2010; Jellouli et al., 2011; Purohit and Singh, 2011; Shrinivas and Naik, 2011; Singh et al., 2011; VijayKumar et al., 2011). Exceptional cases of extreme pH stability were reported in the case of proteases with an optimum pH of 12.0 (Ferrero et al., 1996; Kumar et al., 1999; Mei and Jiang, 2005; Mala and Srividya, 2010), optimum pH of 11.5 (Tobe et al., 1975; Takami et al., 1990; Kumar, 2002) pH 12.3 (Kobayashi et al., 1995), pH 12-13 (Fujiwara et al., 1993). Alkaline proteases also have high isoelectric points (Gupta et al., 2002b). The optimum temperature for thermostable protease ranges from 50-70°C (Kumar, 2002; Beg and Gupta, 2003a ; Najafi et al., 2006; Jaouadi et al., 2008; Haddar et al., 2009 b ; Manikandan et al., 2009; Deng et al., 2010; Haddar et al., 2010 b; Rachadech et al., 2010; Shah et al., 2010; Tang et al., 2010).Extreme thermostable proteases that are stable up to 80°C (Nilegaonkar et al., 2002; Fu et al., 2003) are reported. Protease from haloalkaliphilic bacterium O.M.$A_1$8 optimally catalyzed the reaction over a wide range of temperature from 50-90°C, with a half life of 36h at 90°C (Purohit and Singh, 2011).

1.2.6.2 Effect of metal ions and inhibitors

The ion Ca^{2+} plays a major role in enzyme stabilisation by increasing the activity and thermal stability of protease at high temperatures. Addition of Ca^{2+} ion is reported to have an effect in increasing the activity of alkaline protease (DeAzeredo et al., 2003; Sana et al., 2006; Zhu et al., 2007; Dodia et al., 2008; Tatineni et al., 2008; Simkhada et al., 2010; Sinsuwan et al.,

2010;VijayKumar et al., 2011).There are reports of significant enhancement of thermostability of alkaline protease in presence of Ca^{2+} ion (Gupta et al., 2005 a,b; Doddapaneni et al., 2006; Sana et al., 2006; Hajji et al., 2007). At 60°C, 2mM Ca^{2+} increased the protease activity of protease produced by *Bacillus cereus* BG1 by 500% (Ghorbel et al., 2003). Bivalent metal ions such as Mg^{2+}, Mn^{2+},$Cu^{2+,}$ Ba^{2+}, Co^{2+}, Fe^{3+}, Zn^{2+} and Mn^{2+} are also reported to have enhancement effect of the activity of alkaline protease (Beg and Gupta, 2003a; Ghorbel et al., 2003; Doddapaneni et al., 2006; Sana et al., 2006; Hajji et al., 2007; Zhu et al., 2007; Sinsuwan et al., 2010; VijayKumar et al., 2011). These cations protects enzyme against thermal denaturation and play a vital role in maintaining the active conformation of enzyme at high tempreatures (Kumar and Takagi, 1999; Gupta et al., 2002a).

Enzyme inhibition studies primarily give an insight into the nature of the enzyme, its cofactor requirements and the nature of the active centre (Sigma and Mooser, 1975). Alkaline proteases are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) as PMSF inhibits the essential serine residue in the active site and results in the complete loss of activity. The metal dependent alkaline proteases, due to their sensitivity to metal chelating agents and are inhibited by metal chelators such as EDTA (Kumar and Takagi, 1999).

1.2.7 Application of proteases

Microbial enzymes are biological catalysts which can replace chemical catalysts in industries such as leather, food, paper, pharmaceuticals, textiles, meat tenderisation, detergency, dairy, diagnostics, recovery of silver from X-ray films etc. (Rao et al., 1998; Kumar and Takagi, 1999; Kocabıyık and Erdem, 2002; Sellami-Kamoun et al., 2008; Ramesh et al., 2009). Alkaline proteases are preferred in industries due to their high catalytic activity, inherent stability at harsh and extreme conditions and hence are extensively

used in detergency, tannery, pharmaceuticals, food industry, silver recovery, waste treatment etc., (Rao et al., 1998; Gupta et al., 2002a; Doddapaneni et al., 2007; Rao et al., 2009; Shrinivas and Naik, 2011).

1.2.7.1 Detergent industry

Proteases have wide spread applications in house hold detergent formulations for the removal of proteinaceous stains such as keratin, blood, egg, grass, milk, sauces (Maurer, 2004; Ribitsch et al., 2010). Protease based detergents have better cleansing properties and alleviate pollution when compared to synthetic detergents which make them a key ingredient in detergent formulation (Rai and Mukherjee, 2009). Stability in presence of ionic and non ionic detergents, oxidising and sequestering agents, tolerance to high pH (9-11) and temperatures between 30-60°C are the pre-requisites of enzyme to be used as detergent additive (Bakhtiar et al., 2002; Gupta et al., 2002a; Mei and Jiang, 2005; Venugopal and Saramma, 2006; Jaouadi et al., 2008; Sellami-Kamoun et al., 2008; Ramesh et al., 2009).

Subtilisins (serine alkaline protease) from *Bacillus* sp. that belong to the family A of the subtilases superfamily are the major source of proteases in laundry and dish washing detergents due to their stability in presence of alkali and surfactants (Saeki et al., 2007; Shikha et al., 2007; Gupta et al., 2008; Jellouli et al., 2011) of which subtilisin Carlsberg produced by *Bacillus licheniformis* and subtilisin novo produced by *Bacillus amyloliqufaciens* are the important enzymes in detergency (Putten et al.,1996; Saeki et al., 2007; Sellami-Kamoun et al., 2008). Most subtilisins are of low molecular weight of around 28 kDa (Yamagata et al., 1995b). But there is also a report of high molecular mass subtilisin of 72 kDa from *Bacillus* sp. (Ogawa et al., 2003).

Commercial producers of detergent proteases include *Bacillus amyloliquefaciens*, *B. licheniformis*, *B. clausii*, *B. lentus*, *B. alkalophilus*,

and *B. halodurans* (Maurer, 2004). Alkaline protease from *Bacillus licheniformis* MP1 showed higher stability and compatibility in presence of surfactants and detergents and helps in the removal of blood stain (Jellouli et al., 2011). Wash performance analysis of alkaline protease from *Bacillus* sp. RGR-14 showed that protease alone resulted in increase in reflectance of 14% with grass stains and 25% with blood stains on cotton fabrics, while enzyme combined with detergents showed increase in reflectance of 46% and 34% for grass and blood stain removal, respectively, at 45 °C (Oberoi et al., 2001). The protease produced by *Bacillus licheniformis* RP1 had optimum pH between 10 to 11 and temperature of 65-70°C and showed stability under various commercial detergent formulations and surfactants suggesting its application in detergent formulations (Sellami-Kamoun et al., 2008). Stability of alkaline protease from *Bacillus* sp. in presence of EDTA indicates its suitability as a detergent additive (Genckal and Tari, 2006).

Thermostable alkaline protease from *Streptomyces fungicidicus* MML1614 is capable of removing blood stain efficiently in a combination of detergents rather than when applied with a single detergent (Ramesh et al., 2009). Alkaline protease from *Botrytis cinerea* has high storage and thermostability and has compatibility in detergents and wash conditioners and thus proved useful in detergency (Abidi et al., 2008). The activity of protease from *Nocardiopsis* showed significant enhancement of activity in presence of K^+, Na^+, and Mg^{++} ions which makes it a potential candidate for detergent applications (Moreira et al., 2002). The protease of *Vibrio fluvialis* was found to be stable in presence of H_2O_2 and retains 100% activity in presence of 1% H_2O_2 and activity enhanced to 132 % with 4% H_2O_2, and was also found to be stable in presence of ionic and non-ionic detergents suggesting it as a suitable enzyme in detergent applications (Venugopal and Saramma, 2006). Cold

active alkaline proteases found application in detergency, where synthetic fabrics are sensitive to high temperature wash (Chauhan and Gupta, 2004).

Keratinases are useful in detergency, since they have action on the proteinaceous stains attached to the solid surfaces and thus useful as additives in hard surface cleaners. They are used in detergent formulations used in the cleaning of drains clogged with keratinous wastes (Farag and Hassan, 2004; Gupta and Ramani, 2006). An alkaline β-keratinse produced by *Bacillus subtilis* RM-01 strain exhibited significant stability in presence of surfactants and commercial detergents indicating its feasibility as a detergent additive (Rai et al., 2009).

1.2.7.2 Food industry

Proteases find applications in food industry for cheese manufacturing, baking, preparation of soy hydrolysates, meat tenderization and hydrolysis of wheat gluten for the preparation of protein hydrolysates (Kong et al., 2007). In cheese making, the primary function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-k-casein and macropeptides (Rao et al., 1998; Thys et al., 2006). Neutral proteases have role in improving taste and functional properties (Ramírez-Zavala et al., 2004) and neutral proteases from *Bacillus* are preferred in food industry as they generate less bitterness in hydrolysed food proteins due to their intermediate rate of reaction and are insensitive to natural plant inhibitors (Sandhya et al., 2005a; Alvarez et al., 2006; Thys et al., 2006).

The hydrophobic amino acid in the protein hydrolysates and presence of proline residue in the centre of the peptide cause bitterness to protein hydrolysate and hence proteases that can cleave hydrophobic amino acids and proline are useful in removing bitterness (Rao et al., 1998). Carboxypeptidases are used to reduce the bitterness of protein products (Ramírez-Zavala et al., 2004). Wheat carboxypeptidase is

employed for the debittering of peptides from milk casein (Umetsu et al., 1983). Proline specific exopeptidases are widely used in debittering due to the ability to hydrolyse imino bond. There was significant reduction in the bitterness of casein hydrolysate by hydrolysing with post-proline dipeptidyl aminopeptidase (PPDA), which releases amino acyl proline residues from the N-terminus, in conjunction with a general aminopeptidase activity. Proline specific endopeptidase which on hydrolysis releases C-terminal prolines which help in reducing bitterness of food protein hydrolysates (FitzGerald and O'Cuinn, 2006). Hydrolysis of soy protein with commercial proteases yielded low molecular weight positively charged peptides which can stimulate immunomodulating activity. Soy protein hydrolysates are important in human nutrition because of better intestinal absorption property due to the increase in solubility and peptide content (Kong et al., 2008). Kojizyme^{TM} and Flavourzyme^{TM} are the commercial proteases used to produce fish protein hydrolysate (FPH) from fish soluble concentrate (FSC) and can minimize the bitterness of the fish protein hydrolysate (Nilsang et al., 2005).Immobilised alkaline protease is reported in cheese making (Ohmiya et al., 1979). Alcalase, an alkaline protease, was used for the preparation of whey protein hydrolysate from cheese whey (Perea et al., 1993; Gupta et al., 2002a).

1.2.7.3 Leather industry

The use of protease as alternative to chemicals considerably reduces the rate of environmental pollution (Rao et al., 1998). Conventional liming procedure carried out for the purpose of dehairing involves the usage of lime and sodium sulphide which results in environmental pollution in terms of increase in BOD and COD. Meanwhile, enzymatic dehairing is considered as a reliable alternative to the polluting conventional lime-sulphide process (Varela et al., 1997; Macedo et al., 2005; Giongo et al., 2007; Zambare et al., 2007; Sundararajan et al., 2011). The advantages of enzymatic dehairing are: (i) total elimination of lime and sulphide from effluent, (ii)

recovery of hair as a by- product which may be used for production of synthetic fibres, biogas and foaming agent for fire extinguishers. Hydrolyzed hair is used as agricultural fertilizer, soil conditioner, compost and for the production of cosmetics and pharmaceuticals (Kumar et al., 2011).

Enzymatic dehairing reduces COD and total solids by 45% and 20%, respectively, when compared with conventional leather processing (Thanikaivelan et al., 2004; Macedo et al., 2005; Pillai et al., 2011) and helps in bringing down 80 to 90% of pollution load, as a whole (Huang et al., 2003).

Alkaline proteases are preferred in dehairing due to their activity and stability under alkaline pH, stability in presence of surfactants, chelating reagents, and bleaching agents and having high activity over a wide temperature range and long shelf-life (Zambare et al., 2007; Sivasubramanian et al., 2008; Ibrahim et al., 2011). The alkaline condition enables the swelling of hair roots, and the subsequent attack of protease on the hair follicle protein allowing easy removal of the hair (Gupta et al., 2002a; Wang et al., 2007b; Prakash et al., 2010).The combined proteolytic activity of *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. velesensis* showed remarkable dehairing activity on bovine pelts (Giongo et al., 2007). Alkaline protease produced by *Bacillus circulans* showed stability in high pH and is capable of efficient dehairing goat skin within 12h without the addition of sodium sulphide (Rao et al., 2009). The crude alkaline protease preparation from *Bacillus cereus* VITSN04 was found to be effective in dehairing of goat skins with the hair being removed along with epidermis with much efficiency compared to chemical treatment (Sundararajan et al., 2011). Alkaline protease from *Bacillus* sp. was found to be very efficient in dehairing without disturbing collagen and hair integrity (Wang et al., 2007b; Kumar et al., 2011). The

crude enzyme of *Bacillus* sp. PPKS-2 could be used for dehairing goat hide with in 16 h at pH 11.0 and at an ambient temperature of 28°C (Prakash et al., 2010).

The proteases that selectively hydrolyze proteins such as albumins and globulins without affecting the collagen components are more preferred (Rao et al.,1998).There is the report of novel enzyme that specifically attacks the epidermis without degrading the collagen (Cantera, 2000). Proteases with non-keratinase and non-collagenolytic activities are more important for dehairing application, since collagen, the major leather forming protein if attacked will reduce the tensile strength of the finished pelt (Gupta et al., 2002a ; Dayanandan et al., 2003; Macedo et al., 2005; Gupta and Ramani, 2006; Wang et al., 2007b; Pillai and Archana, 2008; Rao et al., 2009; Prakash et al., 2010; Shah et al., 2010).The thermoalkalophilic protease from *Bacillus halodurans* proved to be very efficient in the degradation of buffalo and goat hide without damaging collagen layer (Shrinivas and Naik, 2011). Protease from *Pseudomonas aeruginosa* MCM B-327 is reported to be efficient in dehairing of buffalo hide and the enzymatic dehairing resulted in the lowering of COD and has reduced the pollution load of the tannery effluent (Zambare et al., 2011).

The use of keratinases in dehairing eliminates the use and release of hazardous chemicals and also results in the recovery of good quality hair or wool and high quality pelt (Pillai and Archana, 2008; Ibrahim et al., 2011). Chemical based “hair- destroying dehairing” could be replaced by keratinase-based cleaner “hair-saving dehairing” technology (Macedo et al., 2005; Gupta and Ramani, 2006). Enzymatic dehairing assisted by keratinases in pre-tanning operations can avoid the obnoxious odour and pollution and prevent the rise of BOD and COD caused by the chemicals (Dayanandan et al., 2003; Thanikavelan et al., 2004; Macedo et al., 2005;

Pillai and Archana, 2008). Keratinolytic proteases isolated from *Bacillus subtilis, Bacillus amyloliqufaciens* and *Bacillus velesensis* showed remarkable dehairing activity on bovine pelts, and the epidermis along with the hair follicles was completely removed (Giongo et al., 2007). A subtilisin- like keratinase from *Bacillus subtilis* exhibited remarkable dehairing property at pH 8 and found to be an alternative for the sodium sulphide, which is a major pollutant of tannery effluent (Macedo et al., 2005). A novel dimeric 58 kDa keratinase is reported from *Bacillus licheniformis* ER-15 capable of dehairing buffalo hide within 16 h in presence of 3% $Ca(OH)_2$ (Tiwary and Gupta, 2010).

1.2.7.4 Waste treatment

Deproteinization of shrimp shells with proteases can replace the usage of strong bases (Yang et al., 2000a) and yields chitin which has many commercial applications (Singh and Chhatpar, 2010). Alkaline protease from *Bacillus licheniformis* MP1 was found to be efficient in shrimp waste deproteinization with efficiency of 75% (Jellouli et al., 2011). Keratinases found significant applications in the bioconversion of keratin containing waste materials such as nails, hair, feathers, horns and other hard-to-degrade proteinaceous materials from poultry and cattle slaughterhouses which mainly consist of keratin into useful value added products (Li et al., 2007; Pillai et al., 2011). They are used in the production of proteinaceous fodder from waste feathers (Suzuki et al., 2006; Pillai and Archana, 2008). Chemical treatment of keratinous wastes may destroy certain amino acids and also prevent environmental pollution (Riffel et al., 2007; Jeong et al., 2010), while the waste on hydrolysis with keratinases can be converted into to feed-stuffs, fertilizer, glues and films or selected amino acids such as serine, cysteine and proline (Radha and Gunasekaran, 2007; Riffel et al., 2007; Pillai and Archana, 2008). Biodegradation of keratinous waste in to feather meal by hydrolysing with keratinases forms a valuable source of proteins and amino acids

(Jaouadi et al., 2010a). The production of feather meal by treatment of feather at high temperature results in the destruction of heat sensitive amino acids like methionine, lysine and tryptophan and also leads to the release of non-nutritive amino acids such as lysinoalanine and lanthionine which will results in poor digestibility (Brandelli and Riffel, 2005; Prakash et al., 2010; Tiwary and Gupta, 2010), while enzymatic hydrolysis of keratin wastes from leather and poultry industries to yield valuable products such as feedstuffs, fertilizers, polymers and in the production of amino acids such as serine, cysteine and proline (Grazziotin et al., 2006; Brandelli, 2008; Radha and Gunasekkaran, 2009; Fakhfakh-Zouari et al., 2010; Prakash et al., 2010).Capability of protease in reducing sulfhydryl group is important in feather degradation (Ghosh et al., 2008). Feather degrading enzymes have been reported from *Bacillus pseudofirmus* FA30-01(Kojima et al., 2006). Crude keratinase produced by *Bacillus licheniformis* significantly increased the total amino acid digestibility of raw feathers and commercial feather meal (Lee et al., 1991; Grazziotin et al., 2007). Feather degrading protease from *Bacillus cereus* DCUW has found application in the bio treatment of feather wastes and utilisation of degraded products in feed and foodstuffs (Ghosh et al., 2008). An alkaline keratinase produced by *Streptomyces* sp. proved to be useful in improving the nutritional quality of feather meal by increasing the aminoacid content by keratin hydrolysis (Tatineni et al., 2008).

Enzymatic hydrolysis of marine waste materials is used for the recovery of fish protein hydrolysates with high protein content and can be a source of peptides or nitrogenous substrates for fermentation media. Hydrolysis of tuna waste was carried out using a commercial protease preparation Umamizyme at pH 7 and 45°C with a degree of hydrolysis up to 22.5%, obtained with an enzyme/substrate ratio of 1.5%, after 4 h of hydrolysis (Guerard et al., 2002).

1.2.7.5 Peptide synthesis

Organic solvent stable proteases are useful for synthetic reactions. Catalytic action of protease in water restricted media leads to the synthesis of esters and peptides. Proteases that can mediate catalytic reactions in non-aqueous conditions offers advantages such as shifting of thermodynamic equilibrium in favour of synthesis, increasing the solubility of hydrophobic substrates and products, facilitating product recovery and improving thermal stability of the enzymes (Gupta et al., 2002a ; Shah et al., 2010). Proteases for peptide synthesis are selected on the basis of their specificity against amino acid residues on either side of the splitting point (Kumar and Bhalla, 2005). Enzymatic peptide synthesis has several advantageous over chemical synthesis such as increased solubility of hydrophobic substrates, minimum diffusional barrier, curtailed water-induced side reactions and reduced microbial contamination. Reactions can be performed stereospecifically and reactants do not require side-chain protection. There is also less need for expensive protecting-groups, organic solvents or hazardous chemicals, thus resulting in production costs competitive with those of chemical methods (Gupta et al., 2002a ; Miyazawa et al., 2003; Kumar and Bhalla, 2005; Ruiz and DeCastro, 2007; Patil and Chaudhari, 2009).

Proteases from genus *Pseudomonas* and *Bacillus* are reported to produce solvent stable proteases (Ogino et al., 1999 a,b; Ogino et al., 2000 a,b; Ghorbel et al., 2003; Gupta et al., 2005b; Rahman et al., 2005; Rahman et al., 2006; Wang and Yeh, 2006; Rahman et al., 2007; Sareen and Mishra, 2008; Patil and Chaudhari, 2009; Ritthitham et al., 2009; Shah et al., 2010; Tang et al., 2010; Singh et al., 2011). The enzymatic activity of an organic solvent stable protease from *Aeromonas veronii* PG01 was activated 1.3- and 1.5-fold by n-hexane and n-dodecane, respectively (Divakar et al., 2010). Protease from *Salinivibrio* sp. strain AF-2004 was found to be more stable in organic solvents than in aqueous

solutions with a half life period longer than 24 h and can be used as a biocatalyst for peptide synthesis in the presence of hydrophilic organic solvents (Karbalaei-Heidari,2007b).

1.2.7.6 Other applications

Fibinolytic enzymes can be used to dissolve blood clots and have the potential to be developed into thrombolytic agents (Ashipala and He, 2008). Nattokinase, a strong fibrinolytic enzyme isolated from *Bacillus natto* was found to have potential application in oral thrombolytic therapy (Peng et al., 2003). A novel metallo fibrinolytic protease which has application in thrombolytic therapy was reported from *Streptomyces* sp. CS684 (Simkhada et al., 2010). Alkaline protease of *Bacillus* sp. B21-2 finds application in the enzymatic hydrolysis of gelatine layers of X-ray films to release silver particles (Ishikawa et al., 1993). Alkaline protease from *Conidiobolus* has been used as a substitute of trypsin for cell dissociation in animal cell culture (Chiplonkar et al., 1985). Bacterial collagenolytic proteases has therapeutic applications in wound healing, treatment of sciatica in herniated intervertebral discs, treatment of retained placenta, pre-treatment for enhancing adenovirus-mediated cancer gene therapy (Watanabe, 2004). Alkaline proteases produced by *Penicillium* sp. and *Beauveria felina* found application in soy protein hydrolysis (Agarwal et al., 2004; Agarwal et al., 2005). Crude protease produced by *Bacillus amyloliqufaciens* is used in the production of protein hydrolysate using chick pea as protein source (George et al., 1997). Alcalase finds application in the production of protein hydrolysate which forms a good nutrient medium for microbial growth (Guérard et al., 2001). Alkaline protease from *Bacillus* sp. was found to be useful in ultra filtration membrane cleaning (Kumar and Tiwari, 1999). Serine protease produced by *Vibrio parahaemolyticus* is reported as an efficient vaccine candidate for the disease control caused by this bacterium (Liu et al., 2011).

1.2.8 Microbial proteases in market

Industrial enzyme market is divided into technical enzymes, food enzymes and animal feed enzymes among which technical enzymes have the major share of 52%, and the largest market share is for alkaline proteases (Chellappan et al., 2011). Proteases from *Bacillus* strains predominates the industrial market due to the robust nature of organism, capability of secreting substantial amount of protease with high activity, high pH and temperature stability of enzymes, high catalytic activity and high degree of substrate specificity (Joo et al., 2002; Joo and Chang, 2005; Laxman et al., 2005; Ramani et al., 2005; Tari et al., 2006; Saran et al., 2007; Sellami-Kamoun et al., 2008; Haddar et al., 2010 a,b; Infante et al., 2010; Mala and Srividya,2010; Jellouli et al., 2011; Pillai et al., 2011; Sundrarajan et al., 2011). *Bacillus* derived alkaline serine proteases or subtilisins represent one of the largest and important group of industrial enzymes and account for about 60% of the total worldwide sale of enzymes (Rao et al., 1998; Chouyyok et al., 2005; Joo and Chang, 2006; Amoozegar et al., 2007; Tanskul et al., 2009; Mahajan and Badgujar, 2010; Bayoumi and Bahobil, 2011; Chellappan et al., 2011; Shrinivas and Naik, 2011; Singh et al., 2011). Subtilisins produced by *Bacillus amyloliqufaciens*, *B.licheniformis* and *B.subtilis* dominate the industrial market (Çalık et al., 1998; Rao et al., 1998; Çalık and Ozdamar, 2001; Gupta et al., 2002a). *Bacillus* derived commercial proteases includes Alcalase® , Savinase®, Esperase®, Purafect®, Properase®, Maxatase, Maxacal, Opticlean, Optimase ,and Proleather (Chauhan and Gupta, 2004; Joo and Chang, 2005).

1.2.9 Objectives of the present study

From the review of literature, it is obvious that the importance and relevance of proteolytic enzymes in industry are not fully explored. *Bacillus* species are viewed as promising agents for many industrial

applications and proteases from *Bacillus* sp. dominate the industrial market. *Pseudomonas* proteases have also been considered for such uses as they are either similar or more active than those produced by the former. Among the genus *Pseudomonas*, *Pseudomonas aeruginosa* is the species capable of producing a variety of proteases with novel properties. Furthermore, *Pseudomonas aeruginosa* can grow in alkaline conditions and in water-soluble oil and its enzymes are adapted to extreme conditions (Karadzic et al., 2004). A few reports exist on the proteolytic enzymes from *Pseudomonas aeruginosa* (Bayoudh et al., 2000). The predominant proteases produced by this bacterium are LasA protease, LasB protease, protease IV and alkaline protease (Caballero et al., 2001; Tingpeg et al., 2007), and these enzymes are not yet utilized in industrial enzyme market.

Therefore, the present study was undertaken, and focused on the industrial relevance of these proteolytic enzymes from *Pseudomonas aeruginosa*. The study has the following objectives:

- Screening and identification of the protease producing bacterial strain (*Pseudomonas aeruginosa*) and assessment of pathogenicity
- Optimization of protease production from *Pseudomonas aeruginosa* MCCB 123 by Response Surface Methodology (RSM)
- Purification and characterization of LasB protease from *Pseudomonas aeruginosa* MCCB 123
- Purification and characterization of LasA protease from *Pseudomonas aeruginosa* MCCB 123 and its application in bacterial DNA extraction
- Purification and characterization of β-1,3 glucanase from *Pseudomonas aeruginosa* MCCB 123 and its application in fungal DNA extraction

Chapter 2

SCREENING AND IDENTIFICATION OF PROTEASE PRODUCING BACTERIAL STRAIN (*PSEUDOMONAS AERUGINOSA*) AND ASSESSMENT OF ITS PATHOGENICITY

Contents

2.1 Introduction

Screening of potent protease producing microbial strains and selection of the best candidate organism are the most important steps for industrial production of enzymes. Industries require enzymes which are able to perform at varying physico-chemical conditions and therefore selection of suitable enzymes having optimal performance at desired conditions is of prime importance (Karbalaei-Heidari et al., 2009; Moradian et al., 2009). The requirement of proteolytic enzymes with higher activity and stability at extreme pH and temperatures has been the prime driving force in the search for novel sources of enzymes (Doddapaneni et al., 2009).

Thermophiles from extreme aquatic ecosystems form a unique source of thermostable enzymes which offer many advantages compared to their mesophilic counter parts. Thermophilic bacteria from hydrothermal vents have been one of the major sources of thermostable enzymes advantageous enough by virtue of their high temperature stability, preventing mesophilic microbial contamination. Invariably, thermozymes eliminate the risk of undesirable bacterial growth

(Kublanov et al., 2009). High temperature enzymatic reactions allow higher reaction rates due to decrease in viscosity of the medium, increase in diffusion coefficient and solubility of substrates, higher process yield and favourable equilibrium displacement in endothermic reactions (Haki and Rakshit, 2003). *Anoxybacillus* isolated from hydrothermal vent of Lake Baikal showed optimum proteolytic activity at pH 10 to 10.5 and had a wide thermal optima from 55-70°C (Lavrenteva et al., 2009). Protease from *Staphylothermus marinus* has an optimum of 92°C and a broad pH tolerance from 3 to 12.7 (Mayr et al., 1996).

Halophiles, which inhabit saline and hypersaline environments, are the source of halophilic enzymes. Enzymes from haloalkalophilic bacteria are not well explored (Patel et al., 2005; Karbalaei-Heidari et al., 2009), and the utilization of these enzymes reduce the cost of industrial processes (Manikandan et al., 2009).The high salt tolerance of halophilic microorganisms enables the industries to adapt to non-sterile cultural conditions there by reduce the cost of cultivation (Vidyasagar et al., 2007). The surfaces of halophiles are highly charged to tolerate high salt concentrations in the surrounding medium which makes them stable in nonaqueous system also. This is because of their less requirement of water of hydration. Hence halophilic enzymes are important biocatalysts in aqueous and non aqueous media (Karan et al., 2011). A haloalkalophilic potent strain of *Geomicrobium* sp. produces protease which showed remarkable stability in presence of organic solvents, salts and detergents (Karan et al., 2011). An extracellular alkaline protease produced by *Salinivibrio* sp. strain AF-2004 supported maximum protease production in the medium containing 1% NaCl, and the protease was industrially important due to its stability over a wide pH range and its high salt tolerance, up to 10% NaCl (Amoozegar et al., 2007).

Majority of the commercial enzymes are derived from terrestrial sources and the marine microorganisms which are the sources of many novel enzymes are yet to be explored fully (Chellappan et al., 2010; Ibrahim et al., 2011). Marine microbial enzymes have unique catalytic properties due to their distinct physiological and metabolic characteristics, efficient nutrient utilization in oligotrophic waters etc. and are also the source of novel biocatalysts like cold adapted enzymes which are economical in terms energy savings (Estrada-Badillo and Márquez-Rocha, 2003; Sana et al., 2006; Shanmugapriya et al., 2008; Ramesh et al., 2009; Cristóbal et al., 2011). Protease produced by gamma proteobacterium isolated from marine environment of Sunderbans was found to be salt, solvent, detergent and bleach stable with an alkaline pH optimum of 9.0 (Sana et al., 2006). The alkaline protease of *Bacillus mojavensis* isolated from marine waters was found to be active at a broad alkaline pH range with its optimum at 8.5 and temperature optimum of 60°C and has stability in presence of detergents and oxidising agents (Haddar et al., 2009a).

2.1.1 The genus *Pseudomonas*

The genus *Pseudomonas* includes many species of ecological, economical and health related importance (Palleroni, 1984; Tanase et al., 2009) and currently the genus is comprised of 100 species that are genotypically and phenoypically well defined (Bennasar et al., 2010). *Pseudomonas aeruginosa* is the type species of the genus *Pseudomonas* (Dawson et al., 2002) and is a ubiquitously distributed gammaproteobacterium capable of thriving dissimilar ecological niches (Kiewitz and Tummler, 2000; Khan et al., 2008). The ubiquitous environmental occurrence of the organism is due to its ability to colonise multiple environmental niches and to utilize many environmental compounds as energy source (Lyczak et al., 2000). They are metabolically versatile, making them attractive candidates in bioremediation (Tanase et al., 2009).

2.1.2 Identification of the protease producing strain

2.1.2.1 Phenotypic identification

Growth on Kings A medium and growth at 4°C and 42°C are described as the standardized procedures for the routine conformation of *P. aeruginosa* when applying ISO 16266:2006 standards (Casanovas-Massana et al., 2010). Lysenko (1961) evaluated phenotypic characteristics such as cell and colony morphologies, carbohydrate utilisation tests, hydrolytic activities, denitrification tests, production of pyocyanin and fluroscein pigments, H_2S production, antibiotic resistance tests, resistance to bile, effect of growth temperatures, catalase and oxidase production for the general classification of *Pseudomonas*. According to Doudoroff and Palleroni (1974) *Pseudomonas* species are distributed into two groups, based on their physiological characteristics: group I: the species that do not require growth factors, and group II: those which require growth factors. Group I was further subdivided into two subgroups, depending on the accumulation or absence poly-β-hydroxy-butyrate. The production of fluorescent pigments, arginine dihydrolase, denitrification and the use of DL-arginine or betatine are the criteria used for the grouping of species within these subgroups. Palleroni (2005) performed taxonomical classification of *Pseudomonas* based on cell morphology and structure, cell wall composition, pigment types, nutritional and metabolic characteristics, susceptibility to different compounds, antibiotic production, and pathogenicity for other organisms, antigenic structure, genetic and ecological characteristics. Many commercial test kits are available for the performance of phenotypic tests that specifically identify a given bacterial group. API 20NE (Biomerieux) has been designed mainly for *Pseudomonas* (Barr et al., 1989), which is adequate for the identification of clinical strains of *P. aeruginosa* and a few strains of *P. fluorescens,* while it is not suitable for the identification of environmental isolates (Behrendt et al., 1999; Peix et al., 2003). The

SDS-PAGE profiles also have proven to be useful in fingerprinting *Pseudomonas* isolates from divergent phylogenetic groups at species level (Vancanneyt et al., 1996). Auling et al. (1991) differentiated *Pseudomonas* species based on polyamine composition. Meyer et al. (2002) performed taxonomic grouping of *Pseudomonas* based on the isoelectrophoretic characterization of the main siderophores and determination of pyoverdine mediated iron uptake specificity of the strains which led to the characterization of several *Pseudomonas* strains into species level through the finding of species specific pyoverdins. Fluorescent spectroscopy finger printing has been reported to be useful to discriminate *Pseudomonas* at genus and species level (Tourkya et al., 2009). MALDI-TOF-MS (matrix-assisted laser desorption ionization-time of flight- mass spectrometry) has proved to be an efficient method for the identification of clinical and marine strains of *Pseudomonas* through the analysis of ribosomal proteins (Dieckmann et al., 2005; Degand et al., 2008).

Even though, phenotypic and chemotaxonomic studies have relevance in taxonomic studies, gene sequencing has proved great advances in the taxonomy of *Pseudomonas* (Peix et al., 2009).

2.1.2.2 Genotypic identification

The Subcommittee on Standards of taxonomy of *Pseudomonas* and other related genera has kept standards for the description of new species in the genus *Pseudomonas* and it is mandatory to include 16S rRNA sequencing, DNA-DNA hybridization, fatty acid analysis and phenotypic characterization for the description of a new species or subspecies description (Stackebrandt et al., 2002). Phylogenetic relationships of bacteria could be determined by comparing stable part of genetic codes which includes the genes that code for 5S, 16S and the 23S rRNA genes (Clarridge III, 2004).The use of stable low molecular weight RNA (5S

rRNA and tRNA) in the identification of *Pseudomonas* showed that the reference strains of this genus had a very specific pattern both at genus and species level (Hofle, 1992). However, only 16S rRNA gene molecule has a high evolutionary rate enough to find variability among different species and with a high degree of conservation sufficient to assure that differences corresponded to stable taxonomic categories as genus and species. 23S rRNA is excessively conserved and 5S rRNA is too small. Thus, 16S rRNA gene is the key molecule in the classification of prokaryotes including *Pseudomonas* (Palleroni, 2005; Anzai et al., 2009).16S rRNA sequence analysis has established the major role of relationships among nucleotide sequences in the definition of bacterial species (Stackebrandt et al., 2002).The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the conserved regions at the beginning 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 (Clarridge III, 2004), which has promoted the use of 16S rRNA as an identification tool. 16S rDNA gene sequences is widely used as a universal marker in order to delineate the phylogenetic status of species in *Pseudomonas*, because it permits description of a strain to the genus and allows comparisons between very divergent bacteria (Moore et al., 1996; Anzai et al., 2000; Santos and Ochman, 2004; Mulet et al., 2010; Parkinson and Elphinstone, 2010). Based on16S rDNA sequence data, rapid PCR assays were developed that allowed the differentiation of *P. aeruginosa* from other *Pseudomonas* cultures (Spilker et al., 2004). The 16S-23S rRNA intergenic spacer (ITS) which presents high variability in both size and sequence is used as a phylogenetic marker

discriminating *Pseudomonas* species (Guasp et al., 2000). Phylogenetic analysis has divided the genus *Pseudomonas* into five groups (rRNA groups 1-V) based on RNA-DNA relatedness (Palleroni, 1984). But later studies have restricted *Pseudomonas* to rRNA group I, and the genus is restricted to rRNA group I, and the genus belongs to the γ subclass of the Proteobacteria (Anzai et al., 2000; Peix et al., 2009). 16S rDNA gene sequencing has distributed *Pseudomonas* species into *alpha, beta* and *gamma* subclasses of proteobacteria (Kersters et al., 1996).

For sufficient discrimination at the inter-species level, the analysis of housekeeping genes provides better resolution (Mulet et al., 2010) than 16S rRNA gene. Taxonomic studies through the analysis of "housekeeping genes" such as *recA, atpD, carA, gyrB, rpoB* and *rpoD* have proved to be useful in the species differentiation of *Pseudomonas* and help in the understanding the phylogenetic relationships within the genus (Hilario et al., 2004; Mulet et al., 2010). *rpoB* gene sequences have been used for identification and to study the phylogenetic relation within the genus *Pseudomonas* (Tayeb et al., 2005) which has been postulated as a discriminative gene that can be used for routine identification of laboratory and clinical isolates of *Pseudomonas* (Tayeb et al., 2005; Adékambi et al., 2009).The use of *rpoB* gene in discriminating closely related *Pseudomonas* with a phylogenetic resolution of the *rpoB* tree is 3 times higher than 16S rRNA gene tree (Tayeb et al., 2005). *gyrB* gene has been used for the accurate detection and identification of *Pseudomonas aeruginosa* because the gene is distributed ubiquitously and is rarely transmitted horizontally and its molecular evolution rate is higher than that of 16SrRNA (Anzai et al., 2000; Motoshima et al., 2007). A duplex real time PCR assay targeting *ecfX* and *gyrB* genes has been employed for the identification of *Pseudomonas aeruginosa* (Anuj et al., 2009). Several other genes such as *ampC, citS, flicC, oriC, oprI*, and *pilA* have been analyzed from several clinical and environmental isolates in order to

determine the relationship between genera or species (Kiewitz and Tummler, 2000; Bennasar et al., 2010). An improved resolution in the phylogenetic relationships among *Pseudomonas* species is developed by the combined analysis of genes such as *atpD, carA, recA*, and 16S rDNA (Hilario et al., 2004; Bennasar et al., 2010).

DNA based typing techniques and phenotypic finger printing has been used for assessing the inter- and- intraspecific- diversity of fluorescent pseudomonads (Dawson et al., 2002). RAPD-PCR (random amplified polymorphic) was used for the characterization of *Pseudomonas aeruginosa* strains obtained from cystic fibrosis patients (Ortiz-Herrera et al., 2004). ARDRA patterns obtained from nine different nucleotides were used for the differentiation of *Pseudomonas* strains isolated from soil contaminated oil (Tansae et al., 2009). Other superior molecular techniques such as real-time PCR and fluorescence in situ hybridization (FISH) have been used in the identification of clinical pathogens of *Pseudomonas aeruginosa* (Wellinghausen et al., 2005). Atzel et al. (2008) employed PCR protocols that uses primers targeting 16S rDNA variable regions V2 and V8 (PA-SS PCR) and detection of exotoxin-A coding gene (ETA) for the detection of environmental isolates of *Pseudomonas aeruginosa*. A soybean peroxidase-labeled peptide nucleic acid (PNA) probes targeted to a species-specific sequence in *P. aeruginosa* rRNA has been used in the rapid detection and identification of *P. aeruginosa* (Stendera et al., 2000). Multilocus Sequence Typing (MLST) through the analysis of seven housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA*, and *trpE*) were used for the phylogenetic analysis of *P. aeruginosa* isolates from ocean (Khan et al., 2008). The PseudoMLSA Database server provides cumulative and reliable information to facilitate MultiLocus Sequence Analysis for studies of *Pseudomonas* taxonomy, phylogeny, and evolution. Furthermore, it serves as a reference repository for MLST, an unambiguous procedure for

characterizing isolates of bacterial species using the sequences of internal fragments of usually seven housekeeping genes. This method assigns as distinct alleles the different sequences present within a bacterial species and, for each isolate, the alleles at each loci define the allelic profile or sequence type. PseudoMLSA database could play two essential roles in the field of *Pseudomonas* research, it fulfil the need for the integration of information about the genus *Pseudomonas* that is currently widely dispersed across existing databases; and it serves as a platform for a consistent identification procedure based on the analysis of sets of multiple gene sequences to settle the difficulties in assigning new isolates to already existing *Pseudomonas* species, and for defining novel species (Bennasar et al., 2010).

2.1.3 Assessment of pathogenicity

Assessment of pathogenicity of the protease producer strain is important for its industrial survival. *P.aeruginosa* is ubiquitously distributed in aquatic habitats and soil and is a normal bacterial flora of intestine, mouth and skin. The colonization is normally harmless and infection occurs only when general or local defence mechanism is reduced (Kiewitz and Tummler, 2000) i.e., it is an opportunistic human pathogen (Lyczak et al., 2000; Ortiz-Herrera et al., 2004). It is frequently isolated from hospital environments, clinical specimens and soil and water environments (Palleroni, 1992). As several members of *P. aeruginosa* are known human pathogens, it is pertinent to differentiate between pathogenic and non-pathogenic strains. Environmental isolates of *P.aeruginosa* can be found in soils, surface and ground water and the number of living cells in the soil does not reach the level of infection risk (Atzel et al., 2008). Clinical strains of *P. aeruginosa* are believed to be pathogenic when compared to environmental counterparts. The major reason for its emergence as a pathogen is due to its intrinsic resistance to antibiotics and disinfectants (Senthil et al., 2011). The pathogenic strains

of *P. aeruginosa* are known to produce a multitude of virulence factors. The following are some of the factors which accounts for the pathogenicity in *P. aeruginosa.*

2.1.3.1 Motility

Bacterial motility is used for initiating contact with an abiotic surface and in the formation and development of biofilm. However, the role of motility in infection process is not clear. *P. aeruginosa* is capable of three types of motility: twitching motility, swimming motility and swarming motility. Twitching motility is mediated by type IV pili on solid substrates. This type of motility is a form of surface translocation that is mediated by type IV pili, which are involved in biofilm architecture and are responsible for the formation of micro colonies in biofilm. Swimming and swarming motilities are mediated by flagellum in aqueous environments. Flagella mediated motility serves to bring the cells into close proximity with surface thereby overcoming repulsive forces between bacterium and the surface to which it will attach (Deligianni et al., 2010).

2.1.3.2 Biofilm formation

The ability to form biofilm is one of the properties of the bacterium for colonization into the host and is also associated with numerous chronic and recurrent bacterial infections and diseases. Biofilm formation in *P. aeruginosa* involves surface attachment or adhesion, micro colony formation and differentiation into a mature antibiotic resistant population encased in an extracellular polymeric matrix (Head and Yu, 2004; Ramsey and Whiteley, 2004). Several surface associated factors such as flagella and type IV pili are found to be essential for adhesion and micro colony formation. After the adhesion of single bacteria into the formation of a monolayer, twitching motility is used to initiate the grouping of cells into micro colonies. Further development of biofilms leads to the emergence of mature biofilms, which

are characterized by alginate-encased bacterial aggregates (Head and Yu, 2004). Biofilm formation initially requires flagella-dependent association and binding to a surface to allow formation of a single cell monolayer. Individual cells of this monolayer then conglomerate into a number of micro colonies through twitching motility via type IV pili. Once attached and manifested twitching motility, *P. aeruginosa* could then form fully mature biofilm structures (O'Toole and Kotler, 1998; Deligianni et al., 2010). Biofilms exhibit increased resistance to antimicrobial agents due to the production of extracellular polymeric substances, presence of high concentration of β-lactamases, slower metabolic rates of the cells due to nutrient limitation and the presence of persistent cells (Deligianni et al., 2010).

2.1.3.3 Type III secretory toxin genes

One of the virulence determinants is type III secretion system (Zhu et al., 2006).To promote severe illness, *P. aeruginosa* uses a type III secretion system to inject toxic effector proteins into the cytoplasm of eukaryotic cells. There are mainly four types of effector proteins, *exoU, exoS, exoT* and *exoY*. *exoU* is a potent cytotoxin with phospholipase A_2 activity, *exoS* and *exoT* are ADP ribosylating enzymes with 75 % amino acid identity and *exoY* is an adenylate cyclase (Shaver and Hauser, 2004; Zhu et al., 2006). *exoU* is known as cytotoxic protein, which causes rapid lysis of a variety of mammalian cell types (*invitro*) including macrophages, epithelial cells and fibroblasts and causes cytotoxicity. *exoS* has role in modulating bacterial phagocytosis, in invasion into non pathogenic cells and induces apotosis of epithelial cells, fibroblasts and lymphocytes (Zhu et al., 2006). The clinical strains of *P. aeruginosa* are known to be heterogenous, harbouring either *exoU* or *exoS* genes and there are strains that neither harbour both of these genes. Those that harbour *exoU* gene are referred as cytotoxic phenotype, that harbour *exoS* are referred to as invasive phenotype and those that doesn't harbour any of these genes are considered neither as

cytotoxic nor invasive. Therefore, there exists three phenotypes of *P. aeruginosa*, cytotoxic, invasive and neither cytotoxic nor invasive (Fleiszig et al., 1997; Zhu et al., 2006; Choy et al., 2008). Some clinical isolates of *P. aeruginosa* don't secrete type III effector proteins *invitro* despite harbouring the genes (Roy-Burman et al., 2001; Hauser et al., 2002; Zhu et al., 2006). Deletion of *exoU* gene significantly reduces the overall virulence and prevents the overall development of severe pathology of the lung (Finck-Barbancon et al., 1997; Hauser et al., 1998; Shaver and Hauser, 2004). In mammalian cells, the direct injection of *exoU* causes irreversible damage to cellular membranes and rapid necrotic death (Sato and Frank, 2004). Therefore, it can be concluded that strains that harbour *exoU* gene are potentially cytotoxic to mammalian cells. However, a specific deletion of *exoS* and *exoT* genes from a mutant strain of *P. aeruginosa* failed to detect the role of these genes in the virulence and thus the role of these proteins is unclear. *exoU* has the greatest effect on the virulence of the type III secreted proteins, while *exoT* has only small effect (Shaver and Hauser, 2004).

2.1.3.4 Adhesion and invasion

Pathogenic strains of *P. aeruginosa* are capable of adhering to epithelial cells and is considered as an important step in the bacterial colonisation in the lung with patients of cystic fibrosis (Deligianni et al., 2010). Fleiszig et al. (1996) examined the relationship between cell invasion and cytotoxicity and concluded that *P. aeruginosa* can display invasive phenotype and invasion is inversely correlated with cytotoxicity. Strains that harbour *exoS* gene are considered as invasive phenotype and those harbouring *exoU* are considered as cytotoxic phenotype (Fleiszig et al., 1997; Zhu et al., 2006; Choy et al., 2008). The determination of whether a strain of *P. aeruginosa* is invasive in epithelial cells is related

to its ability to kill eukaryotic cells, with strains of low cytotoxicity showing high invasion and vice versa (Fleiszig et al., 1996).

2.1.3.5 Antibiotic resistance

Virulent strains of *P. aeruginosa* exhibit resistance to antibiotics. Enzymes such as β-lactamases exhibit antibiotic resistance in *P.aeruginosa*. Emerging resistance of ocular isolates of *P. aeruginosa* or *Pseudomonas* spp. to antibiotics including fluoroquinolones has been reported (Zhu et al., 2006). The development of antibiotic resistance has been associated with certain serogroups and environmental sources (Tassios et al., 1998; Bouza et al., 1999). The antibiotic resistance in *P. aeruginosa* is due to the combination of several factors such as its low permeability of the cell wall, genetic capacity to express a wide repertoire of resistance mechanisms, ability to become resistant through mutation in chromosomal genes which regulate resistance genes, and ability to acquire additional genes from other organisms via plasmids, transposons and bacteriophages (Lambert, 2002).

2.2 Materials and methods

2.2.1 Screening of heterotrophic bacteria for protease production

Heterotrophic bacteria isolated from coir retting grounds of Kerala, which formed part of the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kerala, India was used in this study. The isolates were purified by repeated streaking and screened for proteolytic activity on casein agar plates composed of (gl^{-1}): Peptone, 5; Beef extract, 5; Yeast extract, 1; NaCl, 15; agar, 20; and casein, 10. Plates were spot inoculated and incubated at 28°C for 24 to 48 h. After incubation the positive cultures were detected by the presence of clear zones around the colonies. From 500 isolates screened, 10 protease producing bacterial isolates were selected based on the halo zones produced around the colonies, and the

segregated ones were maintained at – 80°C as glycerol stocks in nutrient broth supplemented with 60 % (v/v) glycerol. Further screening of the proteolytic cultures was done based on the unit enzyme activity following the method of Khembavi et al. (1993). An aliquot of 0.5 ml suitably diluted enzyme solution was mixed with 0.5 ml substrate (1% Hammerstein casein in 50 mM Tris-Cl buffer, pH 9) and incubated at 60ºC for 30 min. The reaction was stopped by the addition of 0.5 ml 20 % TCA and kept for 10 min at 25ºC in order to complete the reaction, centrifuged at 8260*g* at 4ºC for 15 min and the absorbance measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to liberate 1 µg tyrosine per ml per min. The experiments were carried out in triplicates and the mean value expressed as unit protease activity. The isolate which showed the highest activity among the group was coded as MCCB 123 and subjected for further characterization for its identity.

2.2.2 Phenotypic characterization

Identification of the potent protease producer was done on the basis of morphological, biochemical and molecular characteristic of the strain as per Baumann and Schubert (1984).

2.2.2.1 Motility assay

2.2.2.1.1 Soft agar method

Motility was tested in soft agar medium having the following composition:

Beef extract	5.0g
Peptone	5.0g
Agar	3.0g
Distilled water	1000 ml
pH	7.2±0.1

Medium was melted and poured into tubes in 3 ml aliquots and autoclaved at 15 lbs for 15 minutes. Stab inoculated the medium and incubated at 28°±0.5°C for 24 to 48 h. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility. A thick growth along the line of inoculation was considered as negative.

2.2.2.1.2 Hanging drop method

The organisms were grown in nutrient broth of the above composition. A loopful of the 18-24 h old culture was placed at the centre of the cover slip. Vaseline was then kept at the four corners of the cover slip. Then the cavity slide was kept over the drop in such a way that the drop should come within the cavity. The Vaseline helped to adhere cover slip to the slide and the whole preparation was inverted quickly so that the drop of the culture was seen hanging from the cover slip. The slide was placed under oil immersion objective and observed for actual displacement of the cells that could very well be differentiated from Brownian movement.

2.2.2.2 Kovac's oxidase test (Cytochrome oxidase activity)

According to the method recommended by Kovac's (1956) the organisms were freshly grown on nutrient agar slants. A platinum loop was used to pick a bit of inoculum and made a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned violet within 10 seconds, indicating the formation of indophenol.

2.2.2.3 Citrate utilization

Simmon's citrate agar (Simmon's, 1926) used had the following composition:

Sodium citrate	0.2g
$MgSO_4.7H_2O$	0.02 g
NaCl	15.0 g
$(NH_4)_2HPO_4$	1.0 g
K_2HPO_4	1.0 g
Bromothymol blue	0.02 g
Agar	20.0 g
pH	6.9
Distilled water	1000ml

Simmon's citrate agar medium was prepared in the form of agar slopes in tubes. The medium was sterilized and the slope was inoculated by streaking over the surface with a loopful of culture and was incubated for 3-4 days. Colour change from green to bright blue indicated that the culture was positive.

2.2.2.4 Denitrification test

The test is used to check the presence of enzyme nitrate reductase which causes the reduction of nitrate, in the presence of suitable electron donor, to nitrite and further to nitrogen which can be tested by an appropriate colorimetric reagent. Composition of the nutrient broth is as follows:

Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
KNO3	1.0g
pH	7.5
Distilled water	1000ml

Autoclaved at 15 lbs for 15 minutes in 5 ml aliquots, inoculated with the test culture and incubated at 28±0.4°C for 48 h.

Preparation of reagents:

Solution A	
Sulphanilic acid	1.0 g
5N glacial acetic acid	100 ml
Solution B	
Dimethyl α-napthylamine	0.6 g
5N glacial acetic acid	100 ml

The presence of nitrate could be determined by adding to 5 ml of the culture, 0.5 ml of solution A, followed by 0.5 ml of solution B. The development of a red colour indicated that the nitrite had been reduced to nitrate.

2.2.2.5 Arginine Dihydrolase

Thronley's medium (Thornley, 1960) is used and has the following composition:

Peptone	1.0g
NaCl	5.0g
K_2HPO_4	0.3g
Agar	3.0g
L (+)-arginine hydrochloride	10.0g
pH	7.0
Distilled water	1000ml

The solids were dissolved in distilled water and pH adjusted to 7.0, added the indicator solution. The medium was sterilized at 15 lbs for 15 min and amino acid was added. The medium was dispensed in 2 ml aliquots and overlaid with liquid paraffin and the medium was sterilized at 10 lbs for 10 min. The test organism was stab inoculated into the medium through the liquid paraffin layer. Colour changes were recorded after incubation at 28±0.5°C for upto 7 days. The colour change from yellowish orange to red was considered positive.

2.2.2.6 Acid production from sugars

Hugh and Leifson's basal medium (Hugh and Leifson, 1953) with the following composition:

Peptone	2.0g
NaCl	5.0g
K_2HPO_4	0.3g
Phenol red (1% aqueous solution)	30 ml
pH	7.3±0.2
Distilled water	1000ml

The carbohydrates [D(-) Ribose, D(-) Xylose, L(+) Arabinose, D(-)Sorbitol, Mannose, Rhamnose, Lactose, Sucrose, Trehalose and Adonitol] were added to a final concentration of 0.1% (w/v). Acid production was readily observed by incorporating an appropriate pH indicator, phenol red. The basal medium was first autoclaved at 15 lbs for 15 min along with the plugged tubes. All the carbohydrates were added to the sterile basal medium to a final concentration of 0.1 % (w/v). The medium was dispensed into the sterile tubes aseptically and was autoclaved at 10 lbs for 10 min. The tubes were inoculated with an inoculation needle and incubated at 28±0.5°C for 3 days and the results

were recorded. The production of acid induced a change in the phenol red indicator, which changed from pink to yellow under acidic conditions.

2.2.2.7 Starch hydrolysis

Nutrient agar with the following composition was used as basal medium for demonstrating amylase production:

Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Soluble starch	10.0g
pH	7.5
Distilled water	1000 ml

The test medium was prepared according to the above composition, autoclaved at 15 lbs/ 15 min and poured into plates. The plates containing the medium were spot inoculated with the test organism and incubated until good growth was obtained at 28°C for 24-72 h. The plates were then flooded with Gram's iodine solution prepared in the following manner

Iodine	1.0g
K1	2.0g
Distilled Water	100 ml

Amylase producing or starch utilizing organisms showed a halo around and beneath them. The colour of the zones dependent up on the degree of hydrolysis of the starch, when it was hydrolysed to the stage of

dextrin, then the zones were reddish brown, and when the break down had gone further, they were colourless.

2.2.2.8 Production of Lecithinase

Bacterial phospholipases (lecithinase) decompose phospholipid complexes that occur as emulsifying agents in serum and egg yolk (Holding and Collee, 1971). The enzymatic activity breaks the emulsion and liberates free fats so that turbidity is produced. The basal medium has the following composition:

Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Agar	20.0g
pH	7.2±0.2
Distilled Water	1000ml

An aliquot of 4% of sterile fresh egg yolk was added to the sterile nutrient broth medium at 55°C just before the plates were poured. The test organisms were spot inoculated heavily and incubated at 27°C for 24-48 h. Phospholipase production was characterized by a zone of turbidity in the medium surrounding the colony.

2.2.2.9 Gelatin hydrolysis

When proteolytic organisms are grown on a plate of nutrient medium, into which gelatin (2%) is incorporated, zone of gelatinase activity around the colonies are demonstrated, when the plates are flooded with acid mercuric chloride solution. The solution reacts with gelatin in the medium to produce opacity, and where ever gelatin is hydrolysed around the colonies, the medium remains clear (Frazier, 1926).

The medium has the following composition:

Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Gelatin	20.0g
Agar	20.0g
pH	7.3±0.2
Distilled Water	1000ml

The prepared medium was autoclaved at 15 lbs for 15 min and poured into plates. The test organisms were spot inoculated and the plates were incubated at 28°C for 24-72 h. Gelatinase production was tested by flooding the plates with mercuric chloride solution of the following composition

$HgCl_2$	5g
Con.HCl	20 ml
Distilled water	100 ml

2.2.2.10 Casein hydrolysis

Casein agar has the following composition:

Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Agar	20.0g
Distilled water	750 ml
pH	7.5±0.3

The medium was autoclaved at 15 lbs for 15 min. A quantity of 30g of casein in 250 ml of distilled water was sterilized separately and mixed with the above medium before pouring into the plates. The test organism was heavily spot inoculated on the plates and incubated at 27°C for 24-48 h. Caseinase production was detected by the presence of clear zones around the colonies.

2.2.2.11 Pigment production

To test the production of pigment, the medium developed by King et al. (1954) of the following composition:

Medium A (for pyocyanin)

Peptone	20.0g
Glycerol	10.0g
Sodium sulphate	10.0g
KCl	20.0g
$MgCl_2.6H_2O$	1.4g
Agar	20.0g
Distilled water	1000 ml
pH	7.2

Medium B (for fluroscein)

Peptone	20.0g
Glycerol	10.0g
Sodium sulphate	10.0g
KCl	20.0g
NaCl	17.0g
$MgSO_4.7H_2O$	1.5g
Agar	20.0g
Distilled water	1000 ml
pH	7.2

The medium was poured in to tubes in 4 ml aliquots to get a generous butt, sterilized at 15 lbs for 15 min. The media for pyocyanin and fluroscein were inoculated, incubated at 27°C for 7 days and observed under UV light. The production of pyocyanin resulted in greenish fluorescence, while yellowish fluorescence indicated the production of fluroscein.

2.2.2.12 Utilization of carbon sources

To test the utilization of carbon sources, basal medium developed by Baumann et al. (1972) of the following composition:

Medium A	
NH_4Cl	5.0g
NH_4NO_3	1.0g
Na_2SO_4	2.0g
K_2HPO_4	3.0g
KH_2PO_4	1.0g
NaCl	10.0g
Distilled water	1000ml
Medium B	
$MgSO_4.7H_2O$	0.1g
$MgCl_2.6H_2O$	4.0g
Yeast extract	0.1g
Distilled Water	1000ml

The pH of the medium was adjusted to 7.0. The media A and B were sterilized separately, cooled and mixed. The carbon sources (Glucose, Sucrose and Trehalose) were added to a final concentration of 0.2 % (w/v). The media were dispensed into tubes in 1 ml aliquots and sterilized at 15 lbs for 15 min. The organism was inoculated and

incubated at 27°C for 3-7 days. Positive result was indicated by the presence of turbidity in tubes.

2.2.3 Molecular characterization

2.2.3.1 Sequencing 16S rRNA gene

The 16S rRNA gene sequencing involved the following steps:

2.2.3.1.1 DNA extraction

To extract the DNA, a sample of 2 ml of bacterial cell suspension (18 h old bacterial cell suspension grown in Luria–Bertani broth) was centrifuged at 15,000 *g* for 10 min at 4°C. The pellet was collected and resuspended in 500µl of TNE buffer (10mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.15 mM NaCl) and centrifuged again at 15,000 *g* for 10 min at 4°C. Subsequently, the pellets were resuspended in 500 µl lysis buffer (0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS, 0.2 % PVP and 0.1% mercaptoethanol) (Lee et al., 2003) and 10µl of proteinase K (20mg/ml) was added and incubated initially for 1 h at 37°C and then for 2 h at 55°C . Further extraction was carried out by phenol-chloroform method (Sambrook and Russell, 2001). The sample was deproteinated by adding equal volume of phenol (Tris- equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and the aqueous layers were separated by centrifugation at 15,000 *g* for 15 min at 4°C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once more. Following this, an equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15,000 *g* for 15 min at 4°C to separate the aqueous phase which was then transferred to a fresh tube. Then the DNA was precipitated by incubation at -20°C overnight after adding equal volume of chilled absolute ethanol. The precipitated DNA was collected by centrifugation at 15,000 *g* for 15 min at 4°C and the pellet washed with 70% ice cold ethanol. Centrifugation was repeated

once more and the supernatant decanted and the tubes left open until the pellet got dried. The DNA pellet was dissolved in 100µl MilliQ (Millipore) grade water. The isolated DNA was quantified spectrophotometrically (Abs_{260}) and the purity of DNA assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs_{260}/Abs_{280}). Electrophoresis was done using 1% agarose gel.

Concentration of DNA ($\mu g\ \mu l^{-1}$) = $Abs_{260} \times 50 \times$dilution factor

2.2.3.1.2 PCR amplification of the extracted DNA

Amplification of 16S rRNA gene was performed according to Reddy et al. (2000) using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16 S2 (ACG GCT ACC TTG TTA CGA CTT). The amplification was performed using DNA Thermal cycler (Eppendorf). Reaction mixture (final volume 25 µl) contained 2.5 µl 10 X buffer, 1 µl 10 pmol each of oligonucleotide primer, 1.5 µl DNA template, 2.5 µl 2.5 mM each deoxynucleoside triphosphate, 1 µl Taq polymerase, and the remaining volume made up with sterile Milli Q water. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20s, annealing at 58°C for 30s and extension at 68°C for 2 min followed by a final extension at 68°C for 10 min. The PCR product was separated on 1 % agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

2.2.3.1.3 Cloning into pGEM-T Easy vector

The amplified PCR product of 16S rRNA was purified using QIAEX II gel purification kit (Qiagen) and was used for cloning into pGEM-T Easy vector (Promega, USA). The ligation mix (10µl) consisted of 5 µl of ligation buffer (2X), 0.5 µl of vector (50 ng μl^{-1}), 3.5 µl of the PCR product and 1 µl of T4 DNA ligase (3U μl^{-1}). The ligation mix was incubated overnight at 4°C. The entire ligated mix was used to transform *Escherichia coli* JM 109 competent cells prepared using

calcium chloride method. The ligation mix was added to 10 ml glass tube previously placed on ice to which 50 µl of competent cells were added and incubated on ice for 20 min, a heat shock at 41.5°C was given for 90s and immediately the tubes were placed on ice for 2 min and then a 600 µl sample of SOC media was added and incubated for 2 h at 37°C in an incubator shaker at 250 rpm. The transformation mixture (200 µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampcillin (100 µg ml^{-1}), IPTG (100mM) and X-gal (80µg ml^{-1}). The plates were incubated at 37°C overnight. The colonies were selected using the blue/white screening. The white colonies were selected and streaked to purify on LB-Amp+X-gal+IPTG plates and incubated overnight at 37°C. To confirm the insert, colony PCR of the white colonies were carried out using the vector primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-GATTTAGGTGACACTATAG-3'). White colonies (template) picked up from the plate were dispensed into the PCR reaction mix (25 µl) containing 2.5 µl 10X PCR buffer, 2.0 µl of 2.5 mM dNTP's, 1 µl of 10 pmol μl^{-1} of T7 and SP6 primers, 0.5 U of taq polymerase and the remaining volume was made up with sterile MilliQ water. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 15s, annealing of primers at 57°C for 20s, primer extension at 72°C for 60s followed by a final extension at 72°C for 10 min and the products were then analyzed on 1 % agarose gel.

2.2.3.1.4 Plasmid extraction and purification

Plasmids from positive clones were extracted using the 'Gen Elute HP' plasmid miniprep kit (Sigma). An overnight recombinant *E.coli* was harvested by centrifugation and subjected to a modified alkaline –SDS lysis procedure followed by adsorption of the plasmid DNA onto silica (column) in the presence of high salts. Contaminants were removed by spin wash step. Finally, the bound plasmid DNA was eluted in 5 mM Tris-Cl, pH 8.0.

2.2.3.1.5 Sequencing and analysis

Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Microsynth AG, Switzerland. The primers used were T7 and SP6. Sequenced DNA data were compiled and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI). After removing the contaminating vector regions, the sequences were matched with homologous sequence obtained from the GenBank database using the BLAST algorithm (Altschul et al., 1990) available from the NCBI website (http://www.ncbi.nlm.nih.gov).

2.2.4 Assessment of pathogenicity

2.2.4.1 Detection of type III toxin genes (*exoU* and *exoS)* by PCR

Genomic DNA of the bacterium was extracted by phenol-chlorofom method (Sambrook and Russell, 2001) as described above. PCR amplification of *exoU* and *exoS* gene was carried out using the primers as described by Zhu et al. (2006). A 428-bp fragment of the *exoU* gene was amplified using primers *exoU* (F) (5′-GGG AAT ACT TTC CGG GAA GTT-3′) and *exoU* (R) (5′ -CGA TCT CGC TGC TAA TGT GTT-3′). A 1352-bp fragment of the *exoS* gene was amplified using primers *exoS* (F) (5′-ATC GCT TCA GCA GAG TCC GTC-3′) and *exoS* (R) (5′ -CAG GCC AGA TCA AGG CCG CGC-3′). Reaction mixture (final volume 25 µl) contained 2.5 µl 10 X buffer, 1 µl 10 pmol each of oligonucleotide primer, 1µl DNA template, 2.5 µl 2.5 mM each deoxynucleoside triphosphate, 1 µl Taq polymerase, and the remaining volume made up with sterile Milli Q water. The amplification profile consisted of initial denaturation at 94°C for 2 min followed by 30 cycles of annealing of primers for 30s at 59°C for *exoU* and 68°C for *exoS* and primer extension at 72°C for 1.5 min.The PCR product was separated on 1 % agarose gel.

2.2.4.2 Swimming motility

Swimming motility was done according to the method of Deligianni et al. (2010). Swim plates were prepared by using 1% tryptone, 0.5 NaCl, 0.3 % (w/v) agar. The plates were inoculated with the bacterium using sterile tooth pick and incubated over night at 37°C. The ability to swim was assessed by the radius of the colony. The swimming zones were measured after 48 h incubation at 37°C. The experiments were conducted in triplicates.

2.2.4.3 Swarming motility

Swarming motility was done according to Deligianni et al. (2010). The medium used consisted of 0.5% nutrient broth, 0.5 % glucose and 0.5 % agar. Plates were inoculated with a 5 µl aliquot from an overnight culture of the bacterium in LB broth on the top of the agar and incubated at 37°C for 48 h.

2.2.4.4 Twitching motility

Twitching motility was done according to Head and Yu (2004). Freshly prepared and briefly dried twitch plates (Tryptic soy broth solidified with 1% (w/v) Difco granulated agar) were stab inoculated with a sharp toothpick into the bottom of the Petri dish. After incubation at 37°C for 24 h, the agar was removed from the twitching activity plate and the plate was stained with 0.25% (w/v) commassie blue for 30 minutes. Stain was removed and the twitching activity was measured in centimetres.

2.2.4.5 Ability to form Biofilm

Biofilm assay was performed according to Head and Yu (2004). Overnight culture of the bacterium was diluted 1:100 in fresh LB medium, dispensed 125 µl to the wells of a 96-well micro titre plate and grown for 15 h at 37°C without aeration. After incubation, wells were stained with 100 µl of 0.25 % crystal violet for 30 min at 25ºC. Stain

was discarded and the plate was rinsed three to five times in standing water and allowed to dry. Stained biofilm was solubilised with 200 µl of 95 % ethanol for 10 min and optical density was read at 570 nm. Assays were done triplicates.A suitable control (LB medium without inoculation) was also kept. Biofilms were classified according to the method of Stepanovic et al. (2000). When there was no increase of optical density over control, it was considered as a non biofilm producer. Meanwhile, up to a 2 fold increase in optical density was considered as a weak producer, up to 4 fold increase in optical density as moderate producer and greater than 4 fold increase in optical density as strong producer.

2.2.4.6 Adhesion and invasion assay on Hep-2 and HeLa monolayers

Adherence and invasion assays were carried out by the modified method of Prasad et al. (1996). An 18 h old culture of the bacterium was centrifuged at 10,000*g* for 15 min at 4°C. The bacterial pellet was re-suspended in MEM with 10% FBS and the inoculum was adjusted to 10^7 CFU/ml. Hep-2 and HeLa cells were grown to confluence in 24-well plate using Minimal Essential Medium (MEM) containing 10% FBS at 37ºC in 95% air and 5% CO_2. The monolayers were inoculated with 200µl bacterial suspension in triplicate wells. In to the other set of triplicate wells, gentamicin (200µg ml^{-1}) was added. The plate was incubated for 3 h at 37ºC in 95% air and 5% CO_2. In to the first set of triplicate wells antibiotic was not added in order to enumerate the number of bacteria that have adhered and invaded the cell lines. All the wells were washed with sterile MEM with 10% FBS to remove un-adhered bacterial cells. The monolayers were lysed with 0.01% Triton X-100. The lysed monolayer suspensions were then serially diluted (10^{-1} to 10^{-6}) and 100 µl of each suspension were plated on LB agar and incubated at 37°C for 24 h. The viable count was determined as colony forming units (CFU) on the plates multiplied by its dilution

factor. Viable bacteria recovered from the wells with gentamicin were considered intracellular (those which invaded the cells) and bacteria recovered from the wells without gentamicin were considered as the extracellular + intracellular. The adherence was calculated by the formulae: (CFU/ml from well without gentamicin at particular dilution - CFU/ml from the well with gentamicin at the same dilution) × dilution factor). The assays were carried out in triplicates.

2.2.4.7 Antibiotic susceptibility testing

Antimicrobial susceptibility of the bacterium was determined according to the modified disk diffusion method of Zhu et al. (2006). The bacterial culture was adjusted to 10^7 CFU/ml (equivalent to a 0.5 McFarland standard) and was plated on to Muller-Hinton agar. The plates were allowed to dry for 5 min and not more than 6 antibiotics discs (Himedia) were applied on each agar plate. The plates were then incubated at 37°C for 18 h. The zone of bacterial inhibition was examined by measuring the annular radius after incubation. The organism was considered as susceptible, reduced susceptible or resistant to a particular antibiotic on the basis of the diameters of the inhibitory zones that matched the criteria of manufacturer's interpretive table, which followed the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2007).

2.3 Results

2.3.1 Identification of the bacterial isolate

2.3.1.1 Phenotypic identification

The cells appeared as Gram negative short rods. The isolate was motile, oxidase positive, produced diffusible greenish pyocyanin pigment, have denitrification activity and capable of growing at 41°C. Moreover, *Pseudomonas* MCCB 123 produced acid from xylose and mannose and was positive for utilization of glucose and sucrose as a

sole carbon source for its growth. It was positive for gelatin, starch, casein hydrolysis. The characteristics considered have been detailed in Table 2.1. The phenotypic characteristics were suggestive of the bacterium as *Pseudomonas aeruginosa,* according to Baumann and Schubert (1984).

2.3.1.2 Molecular identification by 16S rRNA gene sequencing

DNA was isolated (Fig.2.1) and amplified using universal primers of 16S rRNA gene yielding a PCR product of 1.5 kb length (Fig.2.2). Cloned in to pGEM – T easy Vector and transformed *Escherichia coli* (JM109) and confirmed of the insert by colony PCR using T7 and SP6 vector primers which produced a product of 1.7 kb (Fig. 2.3). Plasmid (2.5 kb) from the transformed organisms was extracted (Fig.2.4) and partially sequenced using T7 and SP6 vector primers. When the sequence was compared with GenBank data base using BLAST algorithm available from NCBI (www.ncbi.nlm.nih.gov), it showed 100% query coverage with 16SrRNA gene sequence of *Pseudomonas aeruginosa* strain IRMD-2010 (JF708942), *Pseudomonas aeruginosa* strain DQ8 (GU269267), *Pseudomonas aeruginosa* strain AS2 (GU447238), *Pseudomonas aeruginosa* strain MML2212 (EU344794), *Pseudomonas aeruginosa* strain NK 2.1B-1 (EU352760), *Pseudomonas aeruginosa* isolate PAL106 (DQ464061), *Pseudomonas aeruginosa* (AB117953), *Pseudomonas aeruginosa* strain AL98 (AJ249451). Thus the identity of the isolate was confirmed as *Pseudomonas aeruginosa*. The BLAST result obtained is shown in Table 2.2. The sequence is deposited in the GenBank with the accession number FJ665510 and is given in Fig 2.5.

2.3.2 Assessment of pathogenicity of *P.aeruginosa* MCCB 123

2.3.2.1 Analysis of type III secretion toxin genes

Analysis of the presence of type III secretion toxin encoding genes revealed that *P. aeruginosa* MCCB 123 did not harbour *exoU* gene, while

a positive amplification with an amplicon of 1352 bp was obtained for *exoS* gene (Fig.2.6).

2.3.2.2 Motility assays

P. aeruginosa MCCB 123 exhibited swimming and swarming motilities with distance of migration of 1.46 ±0.05 cm and 1.53 ±0.11 cm radius, respectively, from the inoculation point. The twitching motility was distinguished by the presence of twitch zone formed by colony expansion with a zone diameter of 3.4 ±0.17cm. The results are shown in Table 2.3.

2.3.2.3 Biofilm formation

P. aeruginosa MCCB 123 is considered as a moderate biofilm producer with 3.10±0.52 fold increase in the optical density when compared to control (LB medium without inoculation) at Abs_{570} (Table 2.4).

2.3.2.4 Adhesion and Invasion assay on epithelial cell lines

The capability of *P. aeruginosa* MCCB 123 to invade and adhere human epithelial cell lines (Hep-2 and HeLa) was assessed by gentamicin survival assays and the strain *P. aeruginosa* MCCB 123 was found to be non-adherent and also is not capable of invading into the human epithelial cell lines (Table 2.5).

2.3.2.5 Antibiogram

The susceptibility of *P. aeruginosa* MCCB 123 to 16 antibiotics belonging to six categories such as pencillins, cephems, carbapenems, lipopeptides, aminoglycosides and fluroquinolones was examined and listed in Table 2.6. Out of the 16 antibiotics belonging to six classes, the strain was sensitive to 13 antibiotics, resistant to one antibiotic (piperacillin) and intermediately sensitive to two antibiotics (cefotaxime and ceftizoxime).

Table 2.1 Biochemical tests for identification of protease producing isolate

Tests	Results
Motility	motile
Grams stain	gram negative rods
Oxidase	positive
Citrate utilization	positive
Agrinine dihydrolase	positive
Denitrification	negative
Starch hydrolysis	positive
Gelatin hydrolysis	Positive
Casein hydrolysis	positive
Lecithinase	positive
Production of pyocyanin	positive
Growth at 41°C	positive
Utilisation of:	
Glucose	positive
Trehalose	negative
Sucrose	positive
Acid production from:	
D(-) Ribose	negative
D(-) Xylose	positive
L(+) Arabinose	negative
D(-)Sorbitol	negative
Mannose	positive
Rhamnose	negative
Lactose	negative
Sucrose	negative
Trehalose	negative
Adonitol	negative

Table 2.2 BLAST result for 16S rRNA gene sequence for *Pseudomonas aeruginosa* MCCB 123

Accession No.	Description	Query coverage	Max. identity
JF708942	*Pseudomonas aeruginosa* strain IRMD 2010 16S ribosomal RNA gene, partial sequence	100%	99%
GU269267	*Pseudomonas aeruginosa* strain DQ8 16S ribosomal RNA gene, partial sequence	100%	99%
GU566322	*Pseudomonas* sp. NR2(2010) 16S ribosomal RNA gene, partial sequence	100%	99%
GU447238	*Pseudomonas aeruginosa* strain AS2 16S ribosomal RNA gene, partial sequence	100%	99%
FJ665510	*Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence	100%	99%
GU447238	*Pseudomonas aeruginosa* strain MML2212 16S ribosomal RNA gene, partial sequence	100%	99%
EU352760	*Pseudomonas aeruginosa* strain NK2.1B1 16S ribosomal RNA gene,	100%	99%
EU236261	Uncultured bacterium clone16S ribosomal RNA gene, partial sequence	100%	99%
DQ464061	*Pseudomonas aeruginosa* isolate PAL106 16S ribosomal RNA gene, partial sequence	100%	99%
AF125317	*Pseudomonas* sp. pDL01 16S ribosomal RNA gene, partial sequence	100%	99%
AB117953	*Pseudomonas aeruginosa* gene for 16SrRNA, Strain:watG	100%	99%
AJ249451	*Pseudomonas aeruginosa* gene for 16S rRNA, Strain:AL98	100%	99%
JN003625	*Pseudomonas aeruginosa* strain BS8 16S ribosomal RNA gene, partial sequence	100%	99%
HQ840718	*Pseudomonas aeruginosa* strain AK1 16S ribosomal RNA gene, partial sequence	100%	99%
JF708186	*Pseudomonas aeruginosa* strain MGP 16S ribosomal RNA gene, partial sequence	100%	99%
GQ180118	*Pseudomonas aeruginosa* strain MW3AC 16S ribosomal RNA gene, partial sequence	100%	99%
GQ180117	*Pseudomonas aeruginosa* strain MW3A 16S ribosomal RNA gene, partial sequence	100%	99%

Table 2.3 Motility assays of *Pseudomonas aeruginosa* MCCB 123

Type of motility	Distance migrated (cm)
Swimming motility	1.46±0.05
Swarming motility	1.53±0.11
Twitching motility	3.4±0.17

Table 2.4 Biofilm assay of *Pseudomonas aeruginosa* MCCB 123

Test Abs_{570}	Control Abs_{570}	Test Abs_{570}/ Control Abs_{570}
1.72±0.28	0.55±0.003	3.10±0.52

Table 2.5 Adherence and Invasion assay of *Pseudomonas aeruginosa* MCCB 123

Adherence		Invasion	
Hep-2	HeLa	Hep-2	HeLa
0 (CFU/ml)	0 (CFU/ml)	0 (CFU/ml)	0(CFU/ml)

Table 2.6 Antibiogram of *Pseudomonas aeruginosa* MCCB 123

Antibiotics	Disk content	Zone diameter	Inference
Pencillins			
Carbenicillin	100µg	23	S
Piperacillin	100µg	15	R
Ticarcillin	75 µg	16	S
Cephalosporins			
Ceftazidime	30 µg	21	S
Cefoperazone	75 µg	21	S
Cefotaxime	30 µg	21	I
Ceftizoxime	30 µg	15	I
Carbapenems			
Imipenem	10 µg	32	S
Polymyxin			
Colistin	10 µg	11	S
Aminoglycosides			
Gentamicin	10 µg	21	S
Amikacin	10 µg	17	S
Tobramycin	10µg	22	S
Netilmicin	30 µg	16	S
Fluroquinolones			
Lomefloxacin	10µg	28	S
Ofloxacin	5µg	28	S
Norfloxacin	10 µg	24	S

S: Sensitive R: Resistant I: Intermediate

Fig. 2.1 DNA extracted from *Pseudomonas aeruginosa* MCCB 123

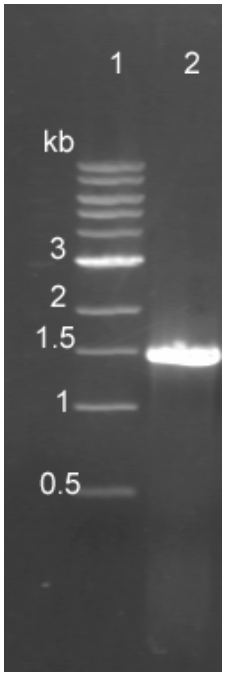


Fig.2.2 PCR amplification of 16S rRNA gene of *Pseudomonas aeruginosa* MCCB 123.Lane 1, molecular marker (1 kb), lane2, 1.5 kb amplification product of the gene.

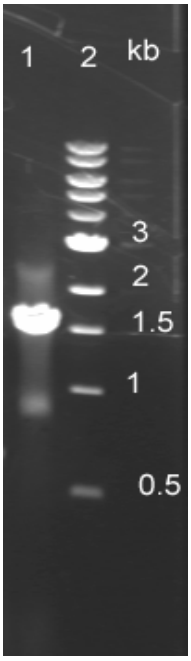


Fig. 2.3 Colony PCR of the clones carrying 16S rRNA gene insert. Lane 1, 1.7 kb insert; lane 2, molecular marker (1 kb)

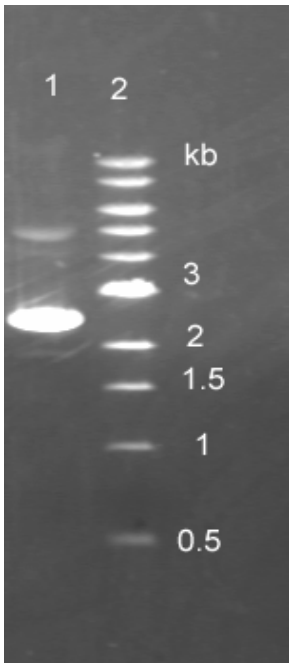


Fig. 2.4 Purified plasmid. Lane 1, 2.5 kb plasmid with gene of insert; lane 2, 1 kb DNA ladder

1	gagtttgatc	ctggctcaga	ttgaacgctg	gcggcaggcc	taacacatgc	aagtcgagcg
61	gatgaaggga	gcttgctcct	ggattcagcg	gcggacgggt	gagtaatgcc	taggaatctg
121	cctggtagtg	ggggataacg	tccggaaacg	ggcgctaata	ccgcatacgt	cctgagggag
181	aaagtggggg	atcttcggac	ctcacgctat	cagatgagcc	taggtcggat	tagctagttg
241	gtggggtaaa	ggcctaccaa	ggcgacgatc	cgtaactggt	ctgagaggat	gatcagtcac
301	actggaactg	agacacggtc	cagactccta	cgggaggcag	cagtggggaa	tattggacaa
361	tgggcgaaag	cctgatccag	ccatgccgcg	tgtgtgaaga	aggtcttcgg	attgtaaagc
421	actttaagtt	gggaggaagg	gcagtaagtt	aataccttgc	tgttttgacg	ttaccaacag
481	aataagcacc	ggctaacttc	gtgccagcag	ccgcggtaat	acgaagggtg	caagcgttaa
541	tcggaattac	tgggcgtaaa	gcgcgcgtag	gtggttcagc	aagttggatg	tgaaatcccc
601	gggctcaacc	tgggaactgc	atccaaaact	actgagctag	agtacggtag	agggtggtgg
661	aatttcctgt	gtagcggtga	aatgcgtaga	tataggaagg	aacaccagtg	gcgaaggcga
721	ccacctggac	tgatactgac	actgaggtgc	gaaagcgtgg	ggagcaaaca	ggattagata
781	ccctggtagt	ccacgccgta	aacgatgtcg	actagccgtt	gggatccttg	agatcttagt
841	ggcgcagcta	acgcgataag	tcgaccgcct	ggggagtacg	gccgcaaggt	taaaactcaa
901	atgaattgac	gggggcccgc	acaagcggtg	gagcatgtgg	tttaattcga	agcaacgcga
961	agaaccttac	ctggccttga	catgctgaga	actttccaga	gatggattgg	tgccttcggg
1021	aactcagaca	caggtgctgc	atggctgtcg	tcagctcgtg	tcgtgagatg	ttgggttaag
1081	tcccgtaacg	agcgcaaccc	ttgtccttag	ttaccagcac	ctcgggtggg	cactctaagg
1141	agactgccgg	tgacaaaccg	gaggaaggtg	gggatgacgt	caagtcatca	tggcccttac
1201	ggccagggct	acacacgtgc	tacaatggtc	ggtacaaagg	gttgccaagc	cgcgaggtgg
1261	agctaatccc	ataaaaccga	tcgtagtccg	gatcgcagtc	tgcaactcga	ctgcgtgaag
1321	tcggaatcgc	tagtaatcgt	gaatcagaat	gtcacggtga	atacgttccc	gggccttgta
1381	cacaccgccc	gtcacaccat	gggagtgggt	tgctccagaa	gtagctagtc	taaccgcaag
1441	ggggacggtt	accacggagt	gattcatgac	tggggtgaag	tcgtaacaag	gtagccgt

Fig. 2.5 Partial nucleotide sequence of 16S rRNA gene of *Pseudomonas aeruginosa* MCCB 123. The sequence is deposited in Genbank with the Accession No. FJ665510

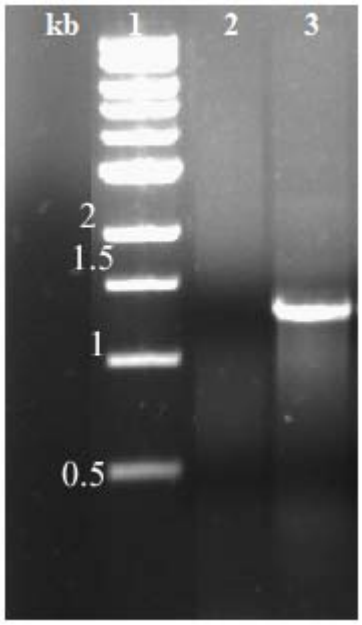


Fig. 2.6 Analysis of type III secretory toxin genes of *P. aeruginosa* MCCB 123. Lane1, 1 kb DNA ladder; lane 2, shows the absence of *exoU* gene of the *P. aeruginosa* MCCB 123, lane 3, amplification of 1352 bp of *exoS* gene of *P. aeruginosa* MCCB 123.

2.4 Discussion

Isolation, screening and identification of potential protease producing microbial strains are the prime steps in the development of any industrial fermentation process. Conventional identification of any novel bacterial isolate is mostly performed on the basis of biochemical and serological differentiation and other conventional biochemical tests (Tanase et al., 2009). However, standard phenotypic techniques are time consuming and have inherent limitations (Cattoir et al., 2010) and do not always correlate with molecular taxonomy (Dawson et al., 2002). The back bone of bacterial systematic has been derived from the 16S rRNA gene sequence based phylogeny (Ludwig and Klenk, 2001) and is the most broad spectrum method used for bacterial classification and identification (Singh et al., 2011). 16S rRNA gene sequence analysis is proposed as a species-specific identification tool which can provide fine sensitivity down to single cell detection (Singh et al., 2011).16S rRNA gene

sequence analysis has established the major role of relationships among nucleotide sequences in the definition of bacterial species (Stackebrandt et al., 2002).They are stable molecular markers and are ubiquitous, functionally constant and conserved and are less subjected to lateral gene transfer, and therefore 16S rRNA-based phylogeny of higher taxa is in good agreement with analyses retrieved from genomic approaches (Schleifer, 2009).16S rDNA gene sequences are widely used as universal marker in order to delineate the phylogenetic status of species in *Pseudomonas* because it permits description of a strain to the genus and allows comparisons between very divergent bacteria (Laguerre et al., 1994; Moore et al., 1996; Anzai et al., 2000; Godfrey et al., 2001; Santos and Ochman, 2004; Tanase et al., 2009; Mulet et al., 2010; Parkinson and Elphinstone, 2010). Other advanced tools in DNA technology used in the phylogenetic description of *Pseudomonas* include DNA-DNA hybridization (Stackebrandt et al., 2002; Parkinson and Elphinstone, 2010).

P. aeruginosa MCCB 123 strain used in this study was initially characterized by phenotypic traits. The strain MCCB 123 was Gram-negative rods and produced pyocyanin and was capable of growing at 41ºC. Moreover, it was positive to Kovacs oxidase and exhibited dentirification. It showed the capability to hydrolyze gelatin, starch and lecithin. These characteristics were those of *P. aeruginosa* as per Baumann and Schubert (1984).The characteristics of the isolate were in agreement with those described by the previous workers (Karadzic et al., 2004; Cheng et al., 2009). Further, molecular characterization based on 16S rRNA gene sequencing confirmed its identity to *P. aeruginosa* and was deposited in GenBank with the accession no. FJ665510.

Confirmation of pathogenicity of the producer isolate is important for its industrial acceptance. Pathogenicity of *P. aeruginosa* MCCB 123 was assessed by a panel of virulence assays like the presence of type III secretion

toxin genes, motility assays, ability to form biofilm, adhesion and invasion on human epithelial cell lines and antibiotic resistance profiles.

One of the virulence determinants of *P. aeruginosa* is the presence of type III secretory toxin genes such as *exoU, exoS, exo T* and *exoY* (Zhu et al., 2006). Analysis of the important type III toxin encoding genes, *exoU* and *exoS* can confirm the cytotoxic and invasiveness of *P. aeruginosa* (Choy et al., 2008).Those strains that harbour *exoU* gene are considered as cytotoxic phenotype and those that harbour *exoS* are considered as invasive phenotype, and strains that neither harbour *exoU* and *exo*S genes are considered as neither cytotoxic nor invasive (Zhu et al., 2006). The cytotoxicity of the non-invasive strains of *P. aeruginosa* is mainly due to the action of *exoU* gene (Hauser et al., 2002). Secretion of *exoU* has been regarded as a marker for high virulent strains of *P. aeruginosa* isolated from infection, but *exoS* was not consistently associated with increased virulence (Schulert et al., 2003). The reason for *exoS* positive strains being associated with lower virulence may be attributed to the poor expression of *exoS* phenotypes in these isolates and such isolates are phenotypically classified as "neither invasive or nor cytotoxic" (Zhu et al., 2002). Analysis of type III toxin genes in *P. aeruginosa* MCCB 123 showed the presence of *exoS* gene, while *exoU* gene, a major contributor to the potential pathogenesis of *P. aeruginosa* (Lin et al., 2006) was not detected. *exoU* has been previously shown to play a major role in mediating a cytotoxic phenotype of *P. aeruginosa* against lung epithelial cells and HeLa cells (Zaborina et al., 2006).The absence of *exoU* gene in *P. aeruginosa* MCCB 123 confirms the non cytotoxic phenotype of the strain, while the presence of *exoS* gene shows the invasive phenotype. Even though, *P. aeruginosa* MCCB 123 carry the gene for invasive phenotype (*exoS* gene), it is neither capable of adhesion nor invasion on both the epithelial cell lines tested. This may be due to the absence of effector protein (*exoS*) responsible for invasion. The presence

of the *exoS* gene and the absence of *exoU* gene in *P. aeruginosa* MCCB 123 suggest the genetic differences between the environmental isolate and the other clinical isolates (Kaszab et al., 2011).Zaborina et al. (2006) demonstrated that most of the multi drug resistant clinical isolates of *P. aeruginosa* with barrier disruptive phenotypes harboured *exoU* gene and displayed cytotoxicity against Caco-2 monolayers. However, clinical isolates that harboured *exoS* gene were not cytotoxic to Caco-2 cells. Fleiszig et al. (1996) screened clinical isolates of *P. aeruginosa* for their ability to invade into corneal epithelial cells of mice. After 1 h of invasion assay, there were no significant differences in the invasion among the isolates, but following a 3 h of infection, *P. aeruginosa* could be differentiated into invasive and cytotoxic strains and suggested that invasion was inversely correlated with cytotoxicity. Fleiszig et al. (1997) tested the invasion of *P. aeruginosa* strains on polarized MDCK cells and found low levels of invasion for both cytotoxic and invasive strains at 1h. However, at 3 h of infection, the percentage of associated bacteria invaded had increased approximately 4 to 9 fold for the invasive isolates but decreased 13- to 15-fold for the cytotoxic isolates. In addition, the total number of associated bacteria for the cytotoxic isolates increased 6 to 8 fold, while there was little to no increase for invasive isolates. However, in case of *P. aeruginosa* MCCB 123 used in the present study, even after 3 hrs of incubation on both the monolayers (Hep-2 and HeLa), the strain was unable to adhere to or invade the cell line and thus the organism could be considered as a phenotype which was neither adhesive (cytotoxic) nor invasive agreeing with Zhu et al. (2006) who stated that there were phenotypes of *P. aeruginosa* that could be considered as neither cytotoxic nor invasive.

Common features of *P. aeruginosa* with high destructive capability on intestinal cell lines include high swimming motility, increased adhesiveness and the presence of *exoU gene* (Zaborina et al., 2006).The non adhesive nature and the absence of *exoU* gene indicated the non pathogenic

nature of *P. aeruginosa* MCCB 123. However, the strain exhibited three types of motilities such as swimming, swarming and twitching motilities and proved to be a moderate biofilm producer with a 3.10±0.52 fold increase in the optical density when compared to control (medium without inoculation) at Abs_{570}. Biofilms exhibits increased resistance to antimicrobial agents due to the production of extracellular polymeric substances, presence of high concentration of β-lactamases, slower metabolic rates of the cells due to nutrient limitation and the presence of persistent cells (Deligianni et al., 2010). The characteristic property of bacterial biofilm is their remarkable resistance to antibiotics. The overall resistance depends upon the entire population of cells and therapy needs to be directed against a multicellular community (Stewart and Costerton, 2001).

Environmental isolates of *P.aeruginosa* are more susceptible to antibiotics when compared to their clinical counterparts (Ruiz et al., 2004). The antibiogram of *P. aeruginosa* MCCB 123 shows that the strain is sensitive to most of the antibiotics tested belonging to various classes, pencillins (carbenicillin, ticarcillin), cephems (ceftazidime, cefoperazone), carbapenems (imipenem), lipopeptides (colistin), aminoglycosides (gentamicin, amikacin, tobramycin, netilmicin), fluroquinolones (lomefloxacin, ofloxacin, norfloxacin), showed intermediate sensitivity to cefotaxime and ceftizoxime which belong to the class of cephems and is resistant to piperacillin which belongs to the class of penicillin. Antibiotics commonly used in the treatment of *P. aeruginosa* infection belong to the classes such as pencillins, cephalosporins, aminoglycoside, fluroquinolones, polymixin and carbapenems (Hancock and Speert, 2000). The results of antibiogram of *P. aeruginosa* MCCB 123 suggested the possibility of antibiotic treatment as an effective method for eradication of its biofilm. The sensitivity of *P.aeruginosa* MCCB 123 to both ceftazidime and cefoperazone suggests that these can be used as a single agent against *P. aeruginosa* (Hancock and Speert, 2000).

Gentamicin is usually effective against non-clinical environmental isolates of *P. aeruginosa* (Trypathy et al., 2007), while the pathogenic strains from clinical environment from cystic fibrosis patients were found to be resistant to this antibiotic (Deredjian et al., 2011). *P. aeruginosa* MCCB 123 showed sensitivity to gentamicin. Broad spectrum fluoroquinolones are used for the treatment of *Pseudomonas* keratitis (O'Brien et al., 1995; Bower et al., 1996), fluoroquinolones and aminoglycosides for the treatment of endophthalmitis (Elder and Morlet, 2002). Fluroquinolones were also reported to be effective drugs for non-clinical (environmental isolates) of *P. aeruginosa* (Silva et al., 2008; Kaszab et al., 2011). *P. aeruginosa* MCCB 123, being an environmental isolate, was sensitive to all the fluroquinolones tested .The organism also showed intermediate sensitivity to third generation cephalosporins such as cefotaxime and ceftazidime. Environmental isolates of *P. aeruginosa* from compost is also reported to be resistant to third generation cephalosporins such as cefotaxime and ceftazidime (Kaszab et al., 2011). Further, the pathogenic clinical strains of *P. aeruginosa* were reported to be resistant to various classes of antibiotics belonging to the classes such as aminoglycosides, carbapenems, cephalosporins (Brown and Izundu, 2004). However, *P. aeruginosa* MCCB 123 is sensitive to the tested antibiotics belonging to the above said classes indicating that the organism is controllable by antibiotics.

In the present study, a potent protease producing bacterial isolate was identified as *Pseudomonas aeruginosa* based on phenotypic and molecular characterization. The isolate was found to exhibit non cytotoxic characteristics based on invasion and adhesion assays on human epithelial monolayers. Its sensitivity to antibiotics indicated that it could be controllable by antibiotic therapy. The high level of proteolytic activity of the bacterium suggested its suitability in industrial applications.

Chapter 3

OPTIMIZATION OF PROTEASE PRODUCTION OF *PSEUDOMONAS AERUGINOSA* MCCB 123 BY RESPONSE SURFACE METHODOLOGY

Contents

3.1 Introduction

Protease production is an inherent capacity of microorganisms and only those microbes which could produce substantial amount of protease are suited for industrial applications (Gupta et al., 2002a). The secretion of extracellular protease depends upon production media and type of strain used and therefore it is important to design fermentation medium according to the requirements (Sandhya et al., 2005b; Shikha et al., 2007; Kezia et al., 2011; Pillai et al., 2011). Media components and their interactions were found to have a significant effect on enzyme synthesis and production in microorganisms (Joo and Chang, 2005; Patel et al., 2005; Tari et al., 2006; Reddy et al., 2008; Wang et al., 2008a ; Liu et al,. 2010) and even minor improvements on production process have great commercial success, since 30-40 % costs accounts for growth medium (Joo et al., 2002; Laxman et al., 2005; Hajji et al., 2008; Reddy et al., 2008; Paranthaman et al., 2009; Bhunia et al., 2010).

3.1.1 Optimization of protease production

Optimization is usually done to maintain a specific ratio between various media components so that those will be completely utilized by

microorganism, and thus preventing the wastage of media components and hence obtain a cost-effective metabolite yield at the end of fermentation. Each microorganism has specific medium requirements for optimum enzyme production and therefore it is necessary to optimize each specific nutrient requirement for growth and enzyme secretion (Kumar and Takagi, 1999; Gupta et al., 2002b; Hajji et al., 2008; Oskouie et al., 2008; Bhunia et al., 2010; Bayoumi and Bahobil, 2011). Cost-effectiveness is an important criterion taken in to account while developing a production medium which can be achieved by the inclusion of cheap agricultural residues (Lazim et al., 2009).

Conventional optimization studies were based on ´One-variable–at-a-time´ technique, which is laborious and time consuming, requiring more experimental data sets, unable to predict the interaction between the components without depicting the complete effects of the parameters on the process (Weruster-Botz, 2000; Wang and Lu, 2004; Bas and Boyaci, 2007; Li et al., 2008; Liu et al., 2010). At present, this approach has been replaced by statistical optimization methodologies. Statistical experimental design for optimization of fermentation media involves selection of the most significant media components (screening), identification of the significant ranges of the selected variables (narrowing), optimum identification of the variables (optimum search), and experimental verification of the identified optimum (Weruster-Botz, 2000).

Screening experiments are useful to identify the independent parameters for which factorial designs are generally used. After identification of important parameters, next step is to determine the level of parameters, which is important for a successful optimization. All the parameters should be normalized before regression analysis. Each coded variable ranges from -1 to 1, so that they all affect the

response more evenly. Commonly used equation for coding is represented as follows:

$$X = \frac{x - [x_{max} + x_{min}] / 2}{x_{max} - x_{min} / 2} \quad \text{---------- (1)}$$

Where, x is the natural variable, X is the coded variable and x_{max} and x_{min} are the maximum and minimum values of the natural variable (Bas and Boyaci, 2007). A Plackett-Burman design is widely used for the screening and identification of the most important media components. The design was originally developed by Plackett and Burman (1946) and is a two level factorial design which identifies the critical physico-chemical parameters required for elevated enzyme production by screening N variables in N+ 1 experiments (Plackett and Burman, 1946; Jian and Nian-fa, 2007; Anisha et al., 2008; Hajji et al., 2008; Singh and Chhatpar, 2010). Each variable is represented at two levels, high and low, denoted by (+) and (-), respectively. The number of positive and negative signs per experiment or trial are $(n + 1)/2$ and $(n- 1)/2$, respectively. Each column should contain equal number of positive and negative signs (Singh and Satyanarayana, 2006). The plackett- Burman design was based on the following first order model:

$$Y = \beta_0 + \sum \beta_i x_i \quad \text{---------- (2)}$$

Where, Y represents the yield (protease activity), β_0 is the model intercept, β_i is the linear coefficient, xi is the level of the independent variable (Liu et al., 2010; Mukherjee and Rai, 2011).

Plackett-Burman design allows the selection of significant factors affecting the enzyme production and elimination of unwanted variables (Reddy et al., 2008). It is useful in the first step in the screening of significant medium components affecting protease production and is useful in decreasing the number of variables and number of experiments

in further optimization step (He et al., 2009; Fakhfakh-Zouari et al., 2010; Liu et al. 2010; Tiwary and Gupta, 2010; Rai and Mukherjee, 2011) when compared to full factorial designs which require 2N (N denotes the number of factors) experiments (Xu et al., 2008). Many reports are available on the use of Plackett-Burman design for screening of significant factors (Yu et al., 1997; Chauhan and Gupta, 2004; Singh and Chhaptar, 2010).

Response Surface Methodology (RSM) is used for the optimization of significant parameters and studying their interaction effects (Beg et al., 2003b; Chauhan and Gupta, 2004) and has a very significant role in yield improvement in enzyme production. It is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously, and first described by Box and Wilson (1951). It is a collection of statistical and mathematical technique used for the optimization of a particular response that is influenced by several variables. It also defines the effect of individual variables and interaction effects between the variables (Thys et al., 2006; Bas and Boyaci, 2007; Hajji et al., 2008; Wang et al., 2008a). The first step is to find a suitable approximation for relationship between responses and independent variables. Low-order polynomial in some region of the independent variables is employed for modelling. If the response is well modelled by a linear function of the independent variables, then the approximating function is the first-order. If there is curvature in the system, then a polynomial of higher degree must be used, such as the second-order model. A central composite design (CCD) is used to estimate coefficients of a quadratic models and consists of three groups of design points: fractional factorial, axial and central points (Oskouie et al., 2008). In the optimization of media components using this approach, the concentrations of different media components forms the variables and some base value is

assigned to them and varies in a certain pattern in order to yield information by a minimum number of experiments (Chen et al., 2002). This method has been successfully applied for the optimization of multiple variables in many fermentation process and showed satisfactory results (Liu et al., 2010). The model used in RSM is generally a full quadratic equation. The second order model can be written as follows:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\ \beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j, \quad \text{---------- (3)}$$

Where Y represents the response variable, β_0 represents the interception coefficients, β_i is the coefficient of linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of the interaction effect (Bas and Boyaci, 2007; Jian and Nian-fa, 2007; Venil et al., 2009).

In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model which describes the chemical or biochemical processes (Myers and Montgomery, 1995; Bas and Boyaci, 2007).

The relationship between the response and the input is give in equation

$$\eta = f(x1, x2 \ldots\ldots x_n) + \varepsilon \quad \text{---------- (4)}$$

Where η is the response, f is the unknown factor of response, x1, x2.....xn denotes independent variable, $_n$ is the number of independent variables and finally ε is the statistical error that represents other source of variability not accounted for by f. These sources include the effects such as measurements error. It is generally assumed that ε has a normal distribution with mean zero and variance (Bas and Boyaci, 2007).

The central composite design (CCD) is the most frequently used RSM design and has three groups of design points:

a) Two-level factorial or fractional design points - all possible combinations of the +1 and -1 levels of the factors (2^k).

b) Axial points or star points - all of the factors set to 0, the midpoint, except one factor, which has the value +/- alpha. However, in the case of axial points of face centered central composite design, all the factors are set to 0 (midpoint), except one factor, which is at the +1/-1 value (i.e., the star points is set at the face of the cube portion on the design)

c) Center points - points with all levels set to coded level 0 (midpoint).

RSM also involves the graphical representation of the model equation and determination of optimal operating conditions. The visualization of the predicted model equation can be obtained by the response surface plot or contour plot. The response surface plot is the theoretical three dimensional plots showing the relationship between the response and the independent variables. The two dimensional display of the surface plot is called contour plot and in the contour plot, lines of constant responses are drawn in the plane of the independent variables. The contour plot helps to visualize the shape of a response surface. When the contour plot displays ellipses or circles, the centre of the system refers to a point of maximum or minimum responses. Sometimes, contour plot may display hyperbolic or parabolic system of the contours. In this case, the stationary point is called the saddle point and is neither a maximum nor a minimum point. These plots give useful information about the model fitted but they may not represent the true behavior of the system (Myers and Montogomery, 1995).

There are many reports of employing RSM which resulted in significant role in yield improvement in enzyme production (Puri et al., 2002; Beg et al., 2003b; Dutta et al., 2004; Dutta et al., 2005; Bas and

Boyaci, 2007; Oskouie et al., 2008; Haddar et al., 2010a; Liu et al., 2010; Karan et al., 2011; Rai and Mukherjee, 2010).

Extracellular protease production is influenced by carbon and nitrogen supplements, glucose, metal ions, aeration and agitation, dissolved oxygen, inoculums density, pH, temperature and incubation time (Johnvesly and Naik, 2001; Gupta et al., 2002b; Puri et al., 2002; Beg et al., 2003b; Patel et al., 2005; Thys et al., 2006; Poutmarthi et al., 2007; Abidi et al., 2008; Hajji et al., 2008; Anbu et al., 2009; Ducros et al., 2009; Nadeem et al., 2009; Chudasama et al., 2010; Haddar et al., 2010a) and therefore it is important to maintain the level of the above said components to the desired level in order to obtain a good yield for which media optimization using above mentioned statistical techniques plays a significant role.

3.1.1.1 Carbon and Nitrogen source

The protease production greatly depends upon the availability of carbon and nitrogen sources in the medium (Beg et al., 2002; Joshi et al., 2008), which exerts a regulatory effects on enzyme synthesis (Chu et al., 1992; Reddy et al., 2008) .The ability to utilize carbon and nitrogen source varies from species to species (Liu et al., 2010). It is necessary to maintain an optimum level of carbon source, since high level may have an inhibitory effect on protease production (Sandhya et al., 2005). Protease production was reported to be favoured in presence of organic nitrogen sources (Kumar and Takagi, 1999; Joshi et al., 2008; Anbu et al., 2009). Extracellular alkaline protease production in *Bacillus* sp. is influenced by carbon and nitrogen sources such as starch, casaminoacids and soybean meal which resulted in 12.85 fold increase in protease production (Chauhan and Gupta, 2004). The use of agricultural residues such as wheat bran, rice bran, casein and soy meals as carbon and nitrogen sources significantly reduces the cost of protease production (Naidu and

Devi, 2005). Wheat bran was found to be an ideal crude carbon source for protease production in *Bacillus* sp. P-2, *Bacillus* sp. S4, *Pseudomonas* sp. S22 and *Aspergillus oryzae* (Kaur et al., 2001). Supplementation of wheat bran along with soy protein increase protease production to about 50% in *Penicillium* sp. under solid state fermentation (Agarwal et al., 2004). A 5:5 ratio of wheat bran and chopped date peptones proved to be an efficient mixture for protease production by *Streptomyces* sp.CN902 (Lazim et al., 2009). Mustard cake is reported as an ideal nitrogen source for extracellular protease production by a newly isolated *Streptomyces* sp.DP2 (Bajaj and Sharma, 2011). *Bacillus mojavensis* gave high yield of extracellular protease when sardinella peptone and hulled grain of wheat was supplemented as nitrogen source (Haddar et al., 2010a). *Mirabilis jalapa* tuber powder, a complex carbon source increased the protease production in *Aspergillus clavatus* (Hajji et al., 2008). Soybean meal appeared to have a strong positive effect on keratinolytic protease production from *Bacillus subtilis* P13 (Pillai et al., 2011). Defatted soybean meal induced protease production by *Halobacterium* sp. Js1 (Vijay Anand et al., 2010). In their studies on protease production by *Conidiobolous coronatus* , Laxman et al., (2005) found that 2 to 3 % soybean meal was found to be the best inducer for protease production. The combination of tryptone and soybean meal (1:1 ratio) was found to have a synergistic effect on protease production by *P. aeruginosa* MCM B-327 (Zambare et al., 2011). Animal fleshing (ANFL), the major proteinaceous solid waste discharged from leather manufacturing industries was used as the substrate for the production of alkaline protease by *P. aeruginosa* (Kumar et al., 2008). Alkaliphilic bacterial strains, *Nesternkonia* sp. AL-20 and *Bacillus pseudofirmus* AL-89 were capable of producing alkaline protease utilizing chicken feather as sole carbon and nitrogen source. Potato peel and *Imperata cylindrica* grass in a ratio of 1:1 significantly increased the protease production in *Bacillus subtilis* (Mukherjee et al., 2008). Prakash

et al. (2010) included raw chicken feather, horn meal with 0.1% feather hydrolysate supplements as a low cost alternative carbon and nitrogen sources for alkaline keratinase production by *Bacillus* sp. PPKS-2. Molasses was proved to be an optimal substrate for alkaline protease production by *Bacillus pantotheneticus* (Shikha et al., 2007).

The nitrogen sources function as inducers on enzyme production (Chauhan and Gupta, 2004; Reddy et al., 2008). A combination of yeast extract and peptone strongly induced protease production in *Botrytis cinerea*. Absence of nitrogen source significantly lowers protease production (Abidi et al., 2008). Casein and soybean meal was found to enhance protease production by *Bacillus* sp., *Bacillus sphaericus*, *Bacillus licheniformis, Bacillus horikoshi, Pseudomonas aeruginosa* etc. (Ferrero et al., 1996; Joo et al., 2002; Gupta and Khare, 2007; Liu et al., 2010; Mukherjee and Rai, 2011). Casein increased protease production by *Aspergillus niger* var. *tieghem* (Chakraborty et al., 1995). A combination of yeast extract and peptone was found to be the best for protease production in *Bacillus aquimaris* strain VITP4 (Shivanand and Jayaraman, 2009).

Organic nitrogen sources were reported to have a positive effect on protease production (Kumar and Takagi, 1999; Joshi et al., 2008; Anbu et al., 2009). In contrary, casein and peptone reduced protease production in *Streptomyces* sp.CN902 (Lazim et al., 2009).Casein and gelatin decreased protease production in *Aspergillus flavus* (Malathy and Chakraborty, 1991). Protease production was also found to be suppressed by rapidly metabolizable nitrogen sources such as amino acids, ammonium ions, small sugars and minerals in the culture medium (Kole et al., 1988a; Ferrero et al., 1996; Kumar and Takagi, 1999; Mehta et al., 2006).

Protease production was suppressed by inorganic nitrogen sources (Joshi et al., 2008; Liu et al., 2010). Inorganic nitrogen sources such as

NH_4Cl, $(NH_4)_2SO_4$ inhibited protease production in *Bacillus* sp. P-2 (Kaur et al., 2001) and in *Bacillus mojavensis* (Haddar et al., 2010a). Ammonium nitrate and sodium nitrate greatly reduced protease production in *Streptomyces* sp.CN902 (Lazim et al., 2009). On the contrary, there are reports on the positive effects of inorganic nitrogen sources on protease production. Protease production was found to be induced in the presence of inorganic nitrogen sources like ammonium and nitrate in *Bacillus licheniformis* (Ferrero et al., 1996). Sodium nitrate is reported to enhance protease production in *Bacillus subtilis* Y-108 (Yang et al., 2000a). The use of sodium nitrate as a sole carbon source increased protease production in *Pseudomonas aeruginosa* strain K, while ammonium nitrate inhibited the production (Rahman et al., 2005). Ammonium sulphate was found to enhance the fibrionlytic protease production by *Bacillus* sp. strain AS-S20-1 in submerged fermentation (Mukherjee and Rai, 2011). Ammonium phosphate was fond to be the best nitrogen source for protease production by *Bacillus licheniformis* ATCC 21415 (Mabrouk et al., 1999). Diammonium hydrogen phosphate supported protease production in *Conidiobolous coronatus* (Laxman et al., 2005). Potassium nitrate (KNO_3) serves as a good inorganic nitrogen source for protease production in *Bacillus clausii* (Oskouie et al., 2008).Sodium nitrate ($NaNO_3$) has a positive effect on production of alkaline β-keratinase from *Bacillus subtilis* RM-01 strain (Rai et al., 2009).

Carbohydrates were reported to have catabolic repression on protease production. The catabolite repression by carbohydrates is a common regulatory mechanism for biosynthesis of microbial proteases (Grazziotin et al., 2007; Prakash et al., 2010). Protease production was lowered by the addition of readily available carbon sources due to catabolic repression (Shivanand and Jayaraman, 2009).Catabolite repression was observed in alkaline protease production by *Roseobacter*

sp. (MMD040) when the medium was supplemented with readily available carbon sources (Shanmughapriya et al., 2008). Carbohydrates showed an inhibitory action on alkaline keratinase production by *Bacillus* sp. PPKS-2 (Prakash et al., 2010). The inclusion of glucose resulted in a strong catabolic repression on protease production by *Bacillus* sp. (Patel et al., 2005). Glucose was found to inhibit protease production in *Bacillus* sp. (Puri et al., 2002), *Bacillus mojavensis* (Beg et al., 2003a), *Aeromonas hydrophila* (O´Reilly and Day, 1983), *Bacillus horikoshii* (Joo et al., 1992), inhibited keratin synthesis in *Chryseobacterium* sp. kr6 (Brandelli and Riffel, 2005), *Nesternkonia* sp. AL-20 (Gessesse et al., 2003). Other carbon sources such as galactose, lactose, fructose, sucrose and starch showed an inhibitory effect on production of alkaline β-keratinase production by *Bacillus subtilis* RM-01 (Rai et al., 2009).

Contrary reports are also available on the enhancing effect of carbohydrates on protease production. Alkaline protease production in *Bacillus mojavensis* is reported to be enhanced by the de-repression and induction using intermittent feeding of glucose and casamino acids (Beg et al., 2002; Beg et al., 2003). The supplementation of maltose and glucose in the production medium significantly increased the alkaline β-keratinase production by *Bacillus subtilis* RM-01 (Rai et al., 2009).

3.1.1.2 Metal ions

Protease production in microbes is influenced by the presence of metal ions (Valera et al., 1997; VijayAnand et al., 2010). Cations were found to have an inductive effect on enzyme activity and stability (Anwar and Saleemuddin, 1998; Joshi et al., 2008).The metalloprotease production in *Bacillus cereus* SV1 was found to be calcium dependent (Secades et al., 2001; Manni et al., 2008). $CaCl_2$ significantly induced the extracellular protease production in *Botrytis cinerea* (Abidi et al., 2008), *Bacillus* sp. (Saran et al., 2007), *Bacillus mojavensis* (Haddar et al., 2010a).

$MgCl_2$ supported protease production in *Geomicrobium* sp. EMB2 (Karan et al., 2011). Other metal ions such as, $MgSO_4$, K_2HPO_4 and KH_2PO_4 had significant effects on extracellular protease production by *Bacillus mojavensis* (Haddar et al., 2010a).The supplementation of $MgSO_4$ increased the keratinase production in *Bacillus pumilus* A1 (Fakhfakh-Zouari et al., 2010). $FeSO_4$ was found to have an inducing effect on protease production in *Botrytis cinerea* (Abidi et al., 2008).

Some other metal ions were reported to have an inhibitory effect on protease production. $CuSO_4$, $ZnSO_4$, $MnSO_4$ and $BaCl_2$ decreased the keratinase production in *Bacillus pumilus* A1 (Fakhfakh-Zouari et al., 2010). $MgCl_2$ inhibited protease production in *Streptomyces fungicidius* MML1614. (Ramesh et al., 2009). $HgCl_2$ is reported to have an inhibitory action on alkaline protease produced by *Streptomyces fungicidius* MML1614 (Ramesh et al., 2009). $CaCl_2$ inhibited protease production by *Streptomyces fungicidius* MML1614. (Ramesh et al., 2009), *Bacillus pumilus* A1 (Fakhfakh-Zouari et al., 2010). Others such as Zn, Fe and CO strongly inhibited enzyme production in by an extremophile *Halobacterium* sp. Js1 (Vijay Anand et al., 2010).

Protease production in halophilic bacterium, *Bacillus* MBIC3303 was enhanced by mono-valent cation (KCl) (Joshi et al., 2008). NaCl supported extracellular protease production by *Bacillus mojavensis* (Haddar et al., 2010a), *Bacillus* sp. (Patel et al., 2005) and *Bacillus pumilus* A1 (Fakhfakh-Zouari et al., 2010), while it is found to have an inhibitory effect on alkaline protease production by *Streptomyces fungicidius* MML1614. (Ramesh et al., 2009). Divalent metal ions were found to have inducing effect on extracellular protease production. The supplementation of Ca^{2+} enhanced protease production by *Bacillus cereus* BG1 strain (Sellami-Kamoun et al., 2011), *Bacillus* sp. (Gupta et al., 2005a), *Alcaligenes faecalis* (Thangam and Rajkumar, 2000) and,

Halogeometricum sp. TSS101 (Vidyasagar et al., 2006).Mg^{2+} enhanced the enzyme production by *Alcaligenes faecalis* (Thangam and Rajkumar, 2000). Supplementation of Mn^{2+} was found to have an inducing effect on protease production by *Bacillus cereus* Strain 146 (Shafee et al., 2005).The positive effects of divalent metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+} are attributed to their role in the maintenance of active enzyme conformation and also to the stabilization of enzyme–substrate complexes (Haaland, 1989; Pillai et al., 2011). On the contrary, divalent metal ions such as Mg^{2+}, Na^{+}, Zn^{2+} and Cu^{2+} interfered with protease production by *Bacillus* sp. SSR1 (Singh et al., 2001a).

3.1.1.3 pH and Temperature

Extracellular protease production in microorganisms is greatly influenced by pH and temperature (Dutta et al., 2004; Dutta et al., 2005; 2008; Hajji et al., 2008; Haddar et al., 2010a) and is important for growth and metabolite production in microorganisms. pH affects the microbial growth indirectly by affecting the nutrient availability or affects directly by action on the cell surfaces. The metabolic activities of the microorganisms are sensitive to the pH changes and the pH of the culture media has marked effect on the type and amount of enzyme produced. Bacterial protease production is greatly influenced by extracellular pH by affecting transport of various components across the cell membranes, which in turn sustain the cell growth and the product formation (Naidu and Devi, 2005; Sandhya et al., 2005b; Kumar et al., 2008). Alkaline pH supported the maximum protease production in bacterial species (Anbu et al., 2009). Protease production was found to be more favorable in uncontrolled pH operations than in controlled pH operations in *Bacillus licheniformis* and *Bacillus clausii* (Calik et al., 2002; Joo and Chang, 2006), while contrary results were obtained by Putten et al. (1996) which states that controlled pH operation was favorable for the production of subtilisin Carlsberg by *Bacillus licheniformis*.

Temperature is another significant environmental factor affecting protease production, which has a major influence on the energetics of the cell (Nikerel et al., 2008). The temperature affects the protease production by changing the physical properties of the cell membrane (Vijay Anand et al., 2010). Higher temperature has destructive effects on protease production (Hofman-Bang 1999; Ikram-Ul-Haq et al., 2006). The protease production in *Penicillum* sp. was sensitive to temperature, where as protease production drastically decreased when the temperature raised above 28°C under solid state fermentation (Agarwal et al., 2004).

3.1.1.4 Aeration and Agitation

The extracellular enzyme production in aerobic microorganism is influenced by the availability of dissolved oxygen in the medium (Nadeem et al., 2009). Oxygen transfer is essential for alkaline protease production and is provided by aeration and agitation (Çalık et al., 1998; Potumarthi et al., 2007; Nadeem et al., 2009). Oxygen has diverse effects on the product formation through aerobic fermentation by influencing the metabolic pathway and changing metabolic fluxes of organisms (Çalık et al., 1998; Ducros et al., 2009; Nadeem et al., 2009). There is a link between enzyme synthesis and energy metabolism in *Bacillus* sp. which is controlled by temperature and oxygen uptake (Naidu and Devi, 2005). Mixing is another important factor for the production of microbial proteases which affects the nutrient availability to microorganisms and is also provided by proper agitation and aeration for better product formation (Çalık et al., 1998; Çalık et al., 2000; Potumarthi et al., 2007; Saran et al., 2007).

Aeration and agitation were found influence the protease production in *Staphylococcus aureus* mutant RC128, with maximum protease production obtained in bioreactor at an agitation of 300 rpm and aeration of 2vvm (Ducros et al., 2009). A combination of 3 vvm aeration rate and

200 rpm of agitation rate supported maximum protease production in *Bacillus licheniformis* NCIM-2042 (Potumarthi et al., 2007). The biomass and protease production increased when a higher dissolved oxygen set point (DO) is given (Kole et al., 1988b). There was a decrease in the protease activity with decrease in aeration rate (Moon and Parulekar, 1991). Agitation was found to have a positive effect on protease production by *Bacillus mojavensis*, *Bacillus licheniformis* ATCC 21415, and *Bacillus* sp. (Mabrouk et al., 1999; Beg et al., 2003b; Saran et al., 2007; Haddar et al., 2010a). The protease yield of *Bacillus licheniformis* mutant UV-9 was highly dependent on aeration and agitation rates, protease production increases with increase in aeration and agitation rates (Nadeem et al., 2009). Protease production in *Botrytis cinerea* was highest at aeration 150 rpm (Abidi et al., 2008).

From the above reports, it is understood that a large volume of literature is available on optimization of protease production from different bacterial species, especially from *Bacillus*. However, only very limited studies exist on protease production from *Pseudomonas aeruginosa* (Dutta et al., 2004; Dutta et al., 2005; Rahman et al., 2005; Khan et al., 2006; Zambare et al., 2011). Therefore, in the present study, an attempt has been made to ascertain if protease production can be enhanced from a potent protease producing strain of *Pseudomonas aeruginosa* MCCB 123 by media optimization through Response Surface Methodology and the results of the study are included in this chapter.

3.2 Materials and Methods

3.2.1 Optimization of the protease production

3.2.1.1 Culture medium

Optimization of protease production of *P. aeruginosa* MCCB 123 was carried out in synthetic medium reported by Morihara (1964) which was considered to be the best for protease production by most strains of *P.*

aeruginosa. The modified medium composed of (g l^{-1}): glucose, 10; yeast extract, 2.0; $NH_4H_2PO_4$, 10; Na_2HPO_4, 10; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.001; $ZnCl_2$, 0.001; casein, 10; pH, 7.0.

3.2.1.2 First step optimization

Initial screening of factors was carried out by one-variable-at-a-time approach in shake flasks in order to find out ranges of the medium components that have to be used for further optimization. The experiments were carried out in 250 ml Erlenmeyer flasks with a working volume of 50 ml. Concentration ranged from (in g l^{-1}): glucose, 2.5–20; casein, 2.0-20 ; yeast extract, 2.5-20.0 ; $NH_4H_2PO_4$, 0.5-10 ; Na_2HPO_4, 0.5-10; KH_2PO_4, 0.5-2.0; $MgSO_4.7H_2O$, 0.2-1.0 ; $CaCl_2$, 0.000625-1.28 ; $ZnCl_2$, 0.01-1.28 ; pH, 5-10, temperature, 20-40°C. The flasks were inoculated with 1 % v/v of 18 h old culture (10^7CFU/ml cells) and incubated at 25°C in a temperature controlled shaker (Orbitek, Scigenics Biotech. (Pvt.) Ltd., India) at 120 rev.min^{-1} for 48h. Cultures were centrifuged at 8260*g* for 15 min at 4°C and supernatant was used for the estimation of protease activity. Protease assay was carried out following the method of Khembavi et al. (1993) as detailed in section 2.2.1 (Chapter 2).

Data generated from the experiments were analyzed using One-way Analysis of Variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD. Mean of the results was compared using SPSS 17.0 package for Windows at a significance level of $p<0.05$. Data are presented as mean± standard deviation.

3.2.1.3 Selection of significant components using Plackett and Burman design

A Plackett- Burman design (Plackett and Burman, 1946) was employed to find out the significant medium components affecting protease production. A total of 11 parameters was included for selection with each variable represented at two levels, high (+1) and low (-1). The

design consisting of a set of 12 experiments was used to determine the relative importance of 11 factors that influenced the enzyme production by *P. aeruginosa* MCCB 123. Response value was measured in terms of protease activity. The design was developed using Design Expert version (6.0.9), Stat-Ease Inc, Minneapolis, MN. All the trials were carried out in triplicates and the average protease activity of each trial was used as the response variable. Regression analysis determined the variables that had significant ($p<0.05$) effect on protease activity and these variables were evaluated in the further optimization experiments.

3.2.1.4 Optimization of protease production by Response surface Methodology

Response surface approach using Face Centred Central Composite Design (FCCCD) was applied to find out the optimum levels of significant variables and the effects of their interaction on enzyme production. A 2^3 FCCCD of 3 factors with an alpha value of 1, including six centre points with a set of 20 experiments were carried out. Each independent variable was studied at three different levels (low, medium and high, coded as -1, 0 and +1, respectively. The centre point of the design was replicated eight times for the estimation of errors. Each run was performed in triplicate and the average protease activity was taken as the experimental value of the dependent variable or response (Y), while predicted values of the response were obtained from quadratic model fitting. A multiple regression analysis of the data was carried out to define the response in terms of independent variables. The response surface graphs were obtained to understand the effects of the variables individually and in combination and to determine their optimum levels for maximum enzyme production. The data on protease production was subjected to analysis of variance (ANOVA). The soft ware, Design Expert (version 6.0.9, Stat-Ease Inc, Minneapolis, MN) was used for the experimental design, data analysis and quadratic model building.

3.2.1.5 Validation of the model

The statistical model was validated with respect to protease production under the conditions predicted by the model. Samples were withdrawn at desired intervals and protease assay was carried out as described earlier. The experiments were carried out in triplicates and results were expressed as mean ± standard deviation. The experimental values were subsequently compared with the predicted values.

3.2.2 Scale up of protease production in 5-l Fermenter

P. aeruginosa MCCB 123 was grown for 18h at 25 °C under shaking conditions (120 rev min^{-1}). The optical density of this culture was adjusted to 0.1 Abs_{600} (10^7CFU/ml) and 1% of the inoculum was transferred into 5-l fermenter (Biostat-B-Lite bench top fermenter, Sartorius, Germany) containing 3 l optimized medium. Fermentation was carried out at pH 7.0, 25°C and 300 rev min^{-1} and sterile air supplied at the rate 2.5 l min^{-1} and the pH maintained at 7.0 ± 0.05. Protease activity was monitored at regular intervals of 6 h for 54 h.

3.3 Results

3.3.1 First step optimization of protease production

One - dimensional screening of 11 factors (9 nutritional factors and 2 physical parameters) was carried out to find out the range of each parameter to be used for further optimization experiments. The effect of different physical and chemical parameters on protease production is represented in Fig.3.1(a-k). The following are the significant (p<0.05) ranges selected for the various media components in g l^{-1}: glucose (2.5 -10), casein (1-5), yeast extract (2.5-5), $NH_4H_2PO_4$ (1-10), Na_2HPO_4 (1-10), KH_2PO_4 (0-2), $MgSO_4.7H_2O$ (0-1), $ZnCl_2$ (0-0.32), $CaCl_2$ (0- 0.01), pH (7-8), temperature (25 to 30°C). The results of statistical analysis are given in Appendix 1.

3.3.2 Selection of parameters using Plackett-Burman design

Further selection of significant medium components was carried out using Plackett-Burman design. The Plackett-Burman design was used for screening 11 selected variables along with their corresponding experimental and predicted values is shown in Table 3.1. The effect of each variable along with the coefficient estimate, *p* value and *F* level is represented in Table 3.2. When the value of the coefficient of the tested variable was positive, the variable has positive influence on response, and when the coefficient value is negative, the variable has a negative influence on the response. The *p* value was less than 0.05 indicating that the model terms were significant. Therefore, considering the *p* value and coefficient estimate, factors such as casein, $NH_4H_2PO_4$ and KH_2PO_4 were found to be significant factors affecting protease production. The magnitude of coefficient of each variable indicated the intensity of its effect on the studied response. The greater the magnitude, the higher the significance of the variable. Thus, KH_2PO_4 was found to be most influential on protease production followed by $NH_4H_2PO_4$ and casein. Even though, when considering *p* value, yeast extract, $CaCl_2$ and temperature were significant, they had a negative coefficient estimate value and hence found to have a negative impact on protease production and therefore these factors were held at a minimum level in the basal medium in further experiments.

3.3.3 Optimization by Face Centered Central Composite Design (FCCD)

The significant variables affecting protease production selected by Plackett-Burman design were further optimized by Response Surface Methodology using Face Centered Central Composite Design (FCCCD). The FCCD design matrix of the variables (casein: A, $NH_4H_2PO_4$: B, and KH_2PO_4: C) along with the experimental (n=3) and predicted values for protease production and the actual level of factors is given in Table 3.3 and 3.4, respectively. The FCCCD matrix was statistically analyzed by

the analysis of variance (ANOVA) and the results are shown in Table 3.5. The quality of the model can be checked using various criteria. The ANOVA of the quadratic regression model demonstrated that the model was highly significant (p<0.0001) as indicated by the high Fisher's F-value of 22.85. The 'lack of fit value' was insignificant (p<0.9762). (R^2) had a value of 0.9536 indicating that the model could explain up to 95.36 % of the variability of the response and the model did not explain 4.64 % of the total variation. The "adequate precision value" is an index of the signal to noise ratio and a ratio greater than 4 is desirable. The adequate precision for the present model was 18.32 indicating a good fit. The value of lack of fit F and P>F were found to be 0.14 and 0.97, respectively, implying that the lack of fit was not significant. Non significant lack of fit made the model fit. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The adjusted R^2 value (0.9119) is high indicating the significance of the model .The "Pred R-Squared" value of 0.9033 (correlation coefficient) is in reasonable agreement with Adj R-Squared value of 0.9119. A relatively lower value of coefficient of variation (CV=1.65) was observed. For protease production, A,C,A^2,B^2,C^2, AB, AC and BC are the significant model terms. Linear coefficients such as A and C, quadratic coefficients, A^2 (F-value 48.91), B^2 (F-value 9.95), C^2 (F-value 12.05) and the interaction coefficients such as AB, AC and BC were also found to be significant model terms for protease activity suggesting that casein (A), $NH_4H_2PO_4$ (B) and KH_2PO_4 (C) had a significant effect on protease production by *P. aeruginosa* MCCB 123. The interaction effects between casein and $NH_4H_2PO_4$ (AB), casein and KH_2PO_4 (AC) and $NH_4H_2PO_4$ and KH_2PO_4 (BC) were found to be significant. However, the linear coefficient B was found to be insignificant.

The mathematical relationship of the independent variable and the response (protease activity) was calculated by second-order polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB, \beta_{13} AC, \beta_{23} BC \text{---- (5)}$$

Where Y is the predicted response, β_0 is the intercept, β_1, β_2, and β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are the squared coefficients and β_{12}, β_{13} and β_{23} are the interaction coefficients and A,B,C,A^2,B^2,C^2,AB,AC and BC are independent variables.

The calculated regression equation for the optimization of medium components showed protease activity (Y) as a function of these variables. The RSM gave the following regression equation for protease activity as a function of casein (A), $NH_4H_2PO_4$ (B) and KH_2PO_4 (C). By applying multiple regression analysis on the experimental data, the following equation was found to explain protease production:

$$Y = +22412.35 + 681.25A - 237.49B - 379.17C + 1560.80\ A^2 - 703.82B^2 - 774.60\ C^2 + 1158.34AB - 361.45AC + 577.08BC, \text{--------} \quad (6)$$

Where Y represents the response i.e. protease activity and A, B, C, A^2, B^2, C^2, AB, AC and BC are the variables.

The two dimensional contour plots and their respective three dimensional contour response surface plots showing significant interaction effects of casein and $NH_4H_2PO_4$, casein and KH_2PO_4 and $NH_4H_2PO_4$ and KH_2PO_4 on protease production when all the other parameters were kept at their optimum level are represented in Figs. 3.2a to 3.2c. Fig. 3.2a shows the response surface and contour plots, respectively, for the interactive factors; $NH_4H_2PO_4$ and casein, when KH_2PO_4 was kept at 3g l^{-1}. Fig. 3.2b shows the response surface and contour plots, respectively, for interaction effects of KH_2PO_4 and casein, when $NH_4H_2PO_4$ was kept at 10.04 g l^{-1}. Fig. 3.2c shows the response

surface and contour plots respectively for interaction effects between $NH_4H_2PO_4$ and KH_2PO_4, when concentration of casein was kept at 10g l^{-1}. The optimal values obtained from the contour plots were almost equal to the results obtained from the regression equation (6).

3.3.4 Validation of the experimental model

The model was validated with triplicate experiments as predicted by the numerical optimization solution by the model. The solution obtained from the model is given in Table 3.6. The maximum predicted value for protease activity was 24805.52 Uml^{-1} with the optimum values of the tested variables as casein, 10gl^{-1}, $NH_4H_2PO_4$, 10.4 gl^{-1} and KH_2PO_4, 3 gl^{-1}. The model was validated by repeating the experiment under optimum conditions under shake flask conditions which resulted in 24479±83.22 Uml^{-1}, which indicated a good correlation between predicted and experimental values and thus proving the validity of the model.

3.3.5 Fermentation curve in shake flask

The time course of protease production by *P. aeruginosa* MCCB 123 in the optimized medium in shake flask is shown in Fig.3.3a. Protease production reached maximum at 48th h with an activity of 24329.17±40.18 Uml^{-1}

3.3.6 Scale up study in 5-l fermenter

The time course of protease production by *P. aeruginosa* MCCB 123 in the optimized medium is shown in (Fig.3.3b). Protease production reached maximum at 36th h with an activity of 24754.17±7.21 Uml^{-1} in fermenter and there was 1.84 fold higher than that in the un optimized medium (13481.3±43.89 Uml^{-1}).The production of protease in 5-l fermenter is shown in Fig.3.4.

Table 3.1 Plackett-Burman matrix for screening of significant factors influencing protease activity

Run	A	B	C	D	E	F	G	H	J	K	L	Protease activity (Uml^{-1})	
												Observed	Predicted
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	2100	3182
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	23012.5	22847.05
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	7750	6667.88
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	24050	23230.03
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	21950	22115.45
6	-1	1	1	-1	-1	1	-1	1	-1	-1	-1	25037.5	25857.47
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	20608.3	21035.94
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	18231.3	17803.65
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	17956.3	18776.22
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	19000	17917.88
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	20837.5	20672.05
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	17743.8	18171.35

A: Glucose, B:casein, C:yeast extract, D:$NH_4H_2PO_4$,E:Na_2HPO_4, F:KH_2PO_4, G:$MgSO_4.7H_2O$, H:$ZnCl_2$, J:$CaCl_2$, K:pH, L:Tempreature.

[a]The observed values of protease activity were the mean values of three replicates.

Table 3.2 Results of regression analysis for the Plackett-Burman design

Code	Variables	Low level (-1)	High level (+1)	Coefficient estimate	*F*-value	*p*-value
A	Glucose	2.5	10	1001.91	5.87	0.0940[a]
B	Casein	1	5	1676.22	16.42	0.0271[b]
C	Yeast extract	2.5	5	-1893.58	20.95	0.0196[c]
D	$NH_4H_2PO_4$	1	10	2778.30	45.11	0.0067[b]
E	Na_2HPO_4	1	10	n	n	n
F	KH_2PO_4	0	2	3058.51	54.66	0.0051[b]
G	MgSO4.$7H_2O$	0	1	-1068.58	6.67	0.0816[a]
H	$ZnCl_2$	0	0.32	n	n	n
J	$CaCl_2$	0	0.01	-3118.58	56.83	0.0048[c]
K	pH	7	8	n	n	n
L	Temperature	25	30	-2415.80	34.10	0.0100[c]

Coefficient of determination (R^2) = 0.9823
Adjusted R^2 = 0.9548
Predicted R^2 = 0.8030

[a] Non significant (p>0.05)

[b] Significant (p<0.05) with positive effect

[c] Significant (p<0.05), but negative effect

n Terms not included in the model

Table 3.3 Experimental design and results of Face Centred Central Composite Design (FCCD)

Run	Casein (gl^{-1})	$NH_4H_2PO_4$ (gl^{-1})	KH_2PO_4 (gl^{-1})	Protease activity (Uml^{-1})	
				Observed[a]	Predicted
1	-1	-1	-1	23800	23804.10
2	1	-1	-1	23633.3	23572.84
3	-1	1	-1	19745.8	19858.30
4	1	1	-1	24291.7	24260.38
5	-1	-1	1	22570.8	22614.51
6	1	-1	1	21037.5	20937.43
7	-1	1	1	20904.2	20977.01
8	1	1	1	23925	23933.28
9	-1	0	0	23525	23291.89
10	1	0	0	24470.8	24654.40
11	0	-1	0	21833.3	21946.02
12	0	1	0	21633.3	21471.04
13	0	0	-1	22041.7	22016.92
14	0	0	1	21283.3	21258.57
15	0	0	0	22733.3	22412.35
16	0	0	0	22200	22412.35
17	0	0	0	22254.2	22412.35
18	0	0	0	22962.5	22412.35
19	0	0	0	21587.5	22412.35
20	0	0	0	22637.5	22412.35

[a]The observed values of protease activity were the mean values of three replicates.

Table 3.4 Range of variables used for Face centered Central Composite Design (FCCD)

Variables	Levels (gl^{-1})		
	-1	0	+1
Casein	1.0	5.5	10
$NH_4H_2PO_4$	1.0	8.0	15
KH_2PO_4	0.5	2.8	5

Table 3.5 Analysis of variance (ANOVA) for Face Centered Central Composite Design

Source	Sum of square	*df*	Mean square	*F* value	*P* value	
Model	28170000	9	3130000	22.85	<0.0001	Significant
A	4641000	1	4641000	33.88	0.0002	
B	564000	1	564000	4.12	0.0699	
C	1438000	1	1438000	10.5	0.0089	
A^2	6669000	1	6669000	48.91	<0.0001	
B^2	1362000	1	1362000	9.94	0.0103	
C^2	1650000	1	1650000	12.05	0.006	
AB	10730000	1	10730000	78.36	<0.0001	
AC	1045000	1	1045000	7,.63	0.0200	
BC	2664000	1	2664000	19.45	0.0013	

Coefficient of determination (R^2) 0.9536

Correlation coefficient (Adj. R^2) 0.9119

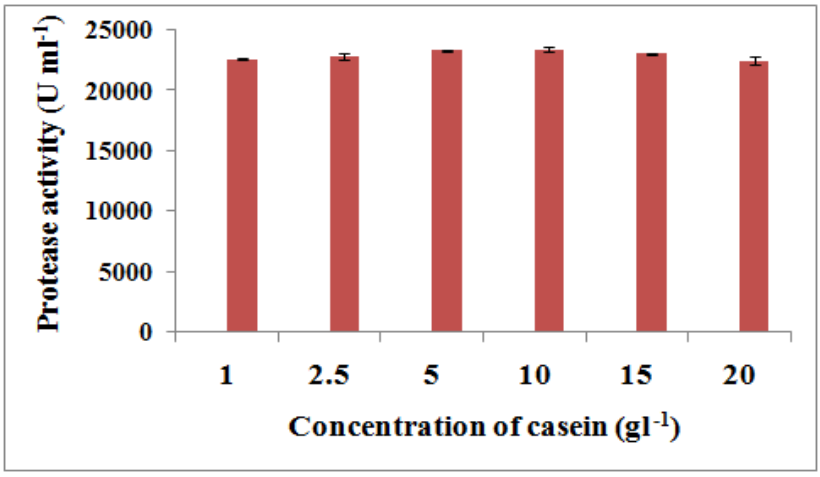
Pred.R^2 0.9033

CV=1.65

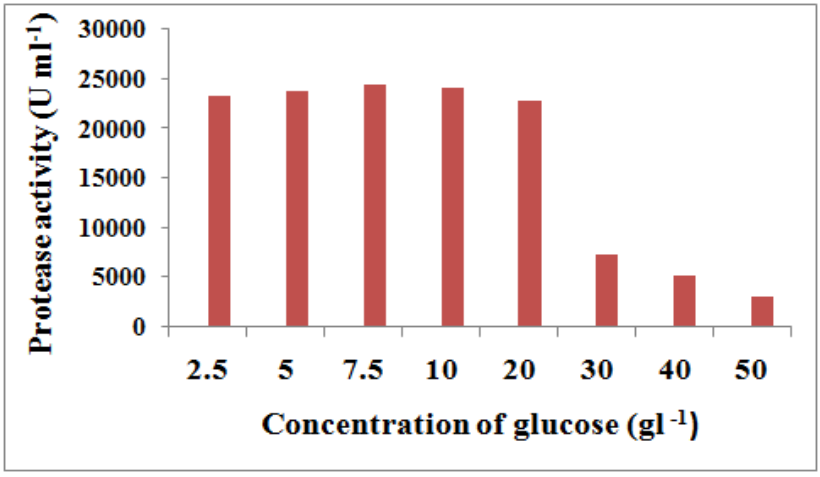
Table 3.6 Solution predicted by the model for maximum protease activity

Casein (gl^{-1})	$NH_4H_2PO_4$ (gl^{-1})	KH_2PO_4 (gl^{-1})	Protease activity (Uml^{-1})	Desirability
10	10.04	3	24805.52	1

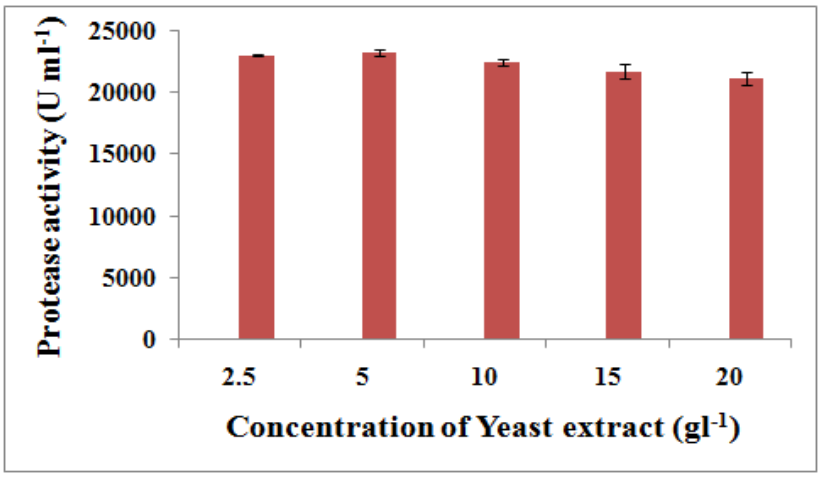
Fig. 3.1 (a-k) One dimensional screening of physico-chemical factors affecting protease production



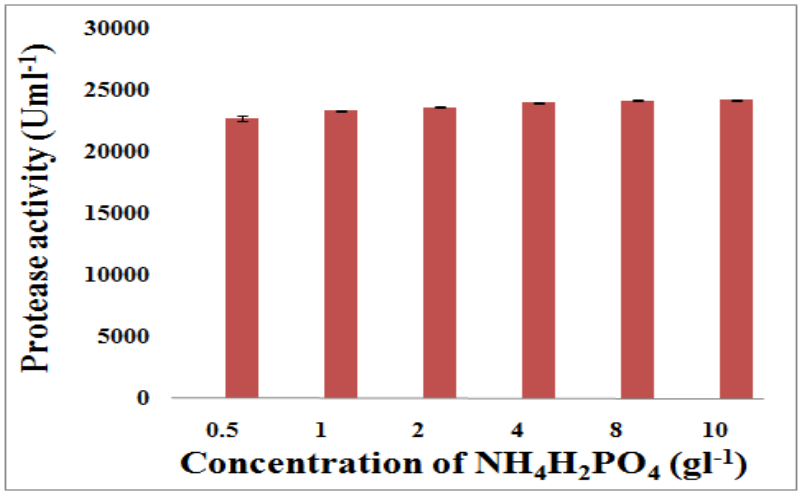
(a)



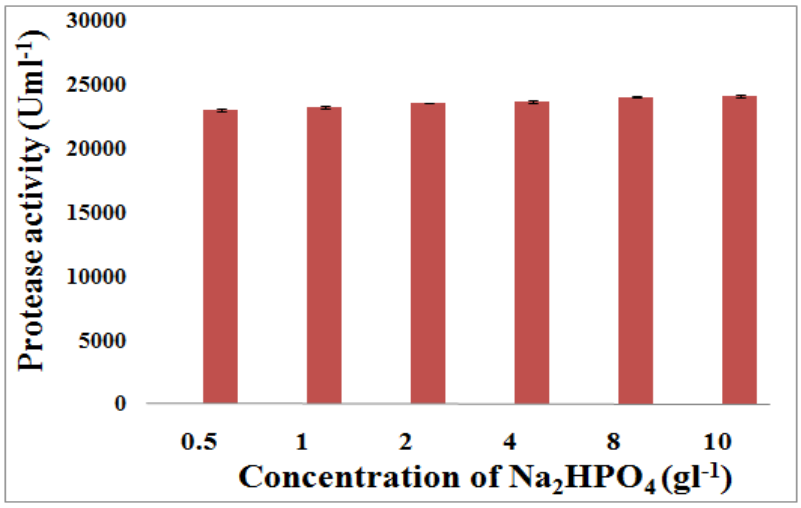
(b)



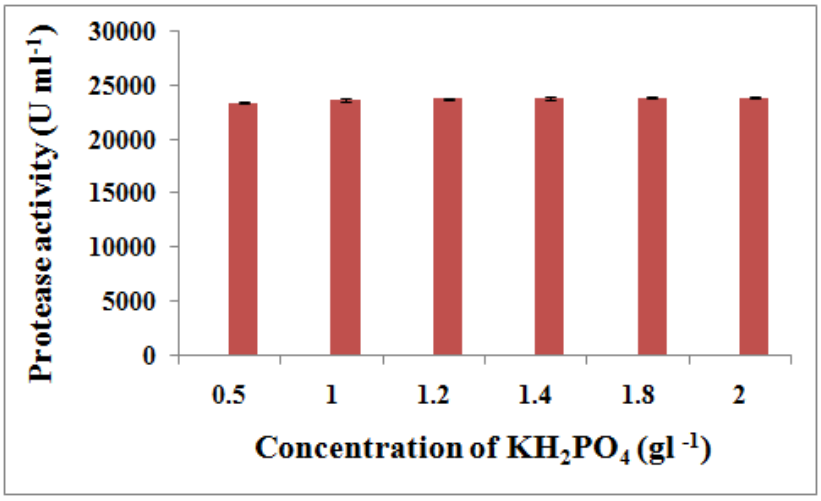
(c)


Protease activity (Uml-1)
30000
25000
20000
15000
10000
5000
0
0.5
1
2
4
8
10
Concentration of NH4H2PO4 (gl-1)

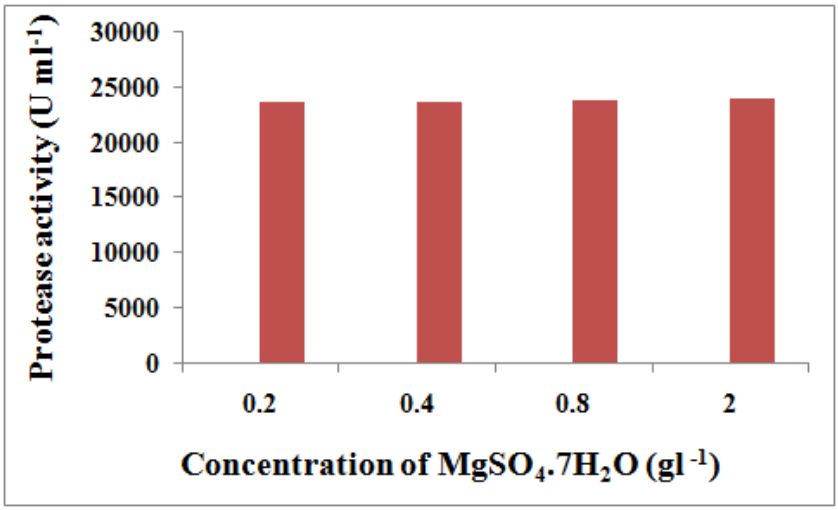
(d)


Protease activity (Uml-1)
30000
25000
20000
15000
10000
5000
0
0.5
1
2
4
8
10
Concentration of Na2HPO4 (gl-1)

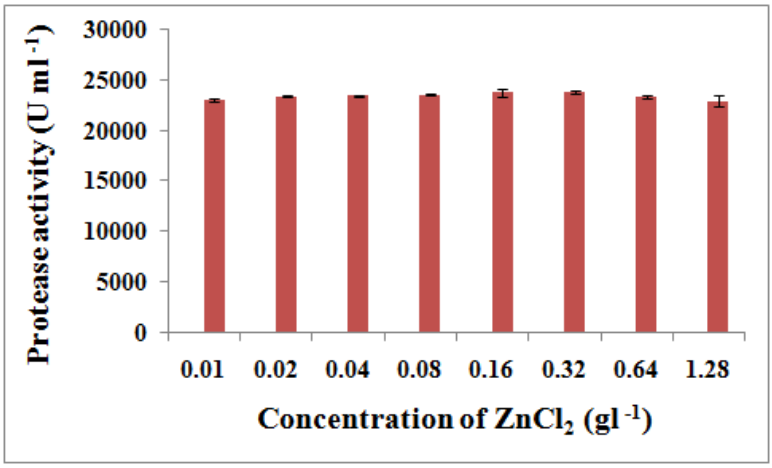
(e)


Protease activity (U ml-1)
30000
25000
20000
15000
10000
5000
0
0.5
1
1.2
1.4
1.8
2
Concentration of KH2PO4 (gl -1)

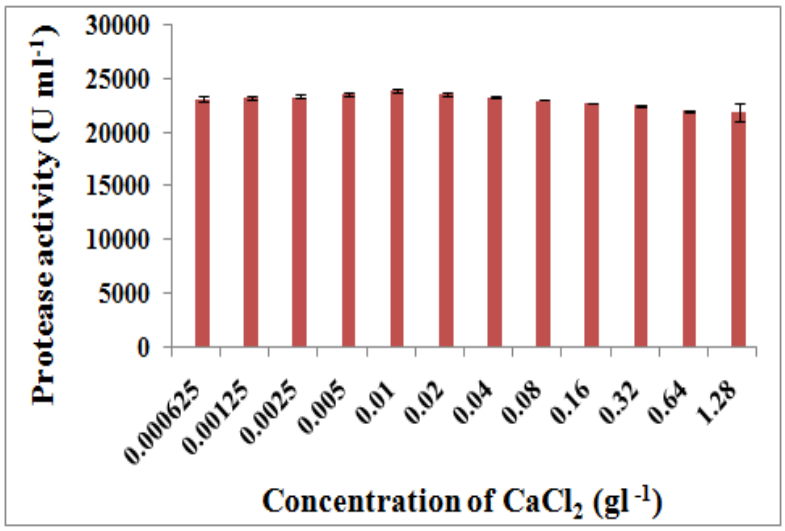
(f)


Protease activity (U ml-1)
30000
25000
20000
15000
10000
5000
0
0.2
0.4
0.8
2
Concentration of MgSO4.7H2O (gl-1)

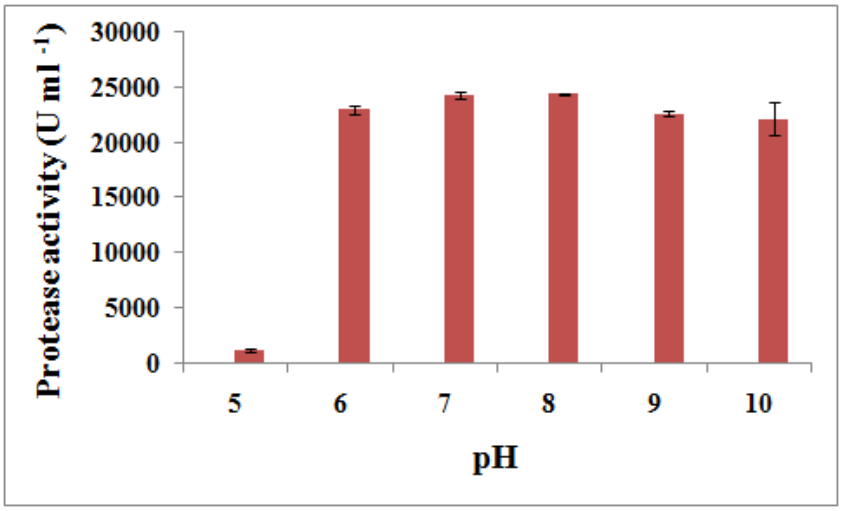
(g)


Protease activity (U ml-1)
30000
25000
20000
15000
10000
5000
0
0.01
0.02
0.04
0.08
0.16
0.32
0.64
1.28
Concentration of ZnCl2 (gl-1)

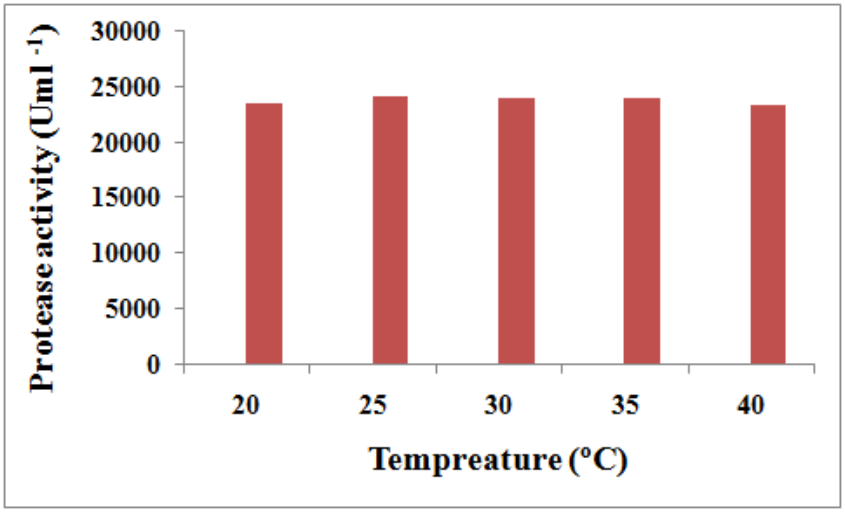
(h)


Protease activity (U ml-1)
30000
25000
20000
15000
10000
5000
0
0.000625
0.00125
0.0025
0.005
0.01
0.02
0.04
0.08
0.16
0.32
0.64
1.28
Concentration of CaCl2 (gl-1)

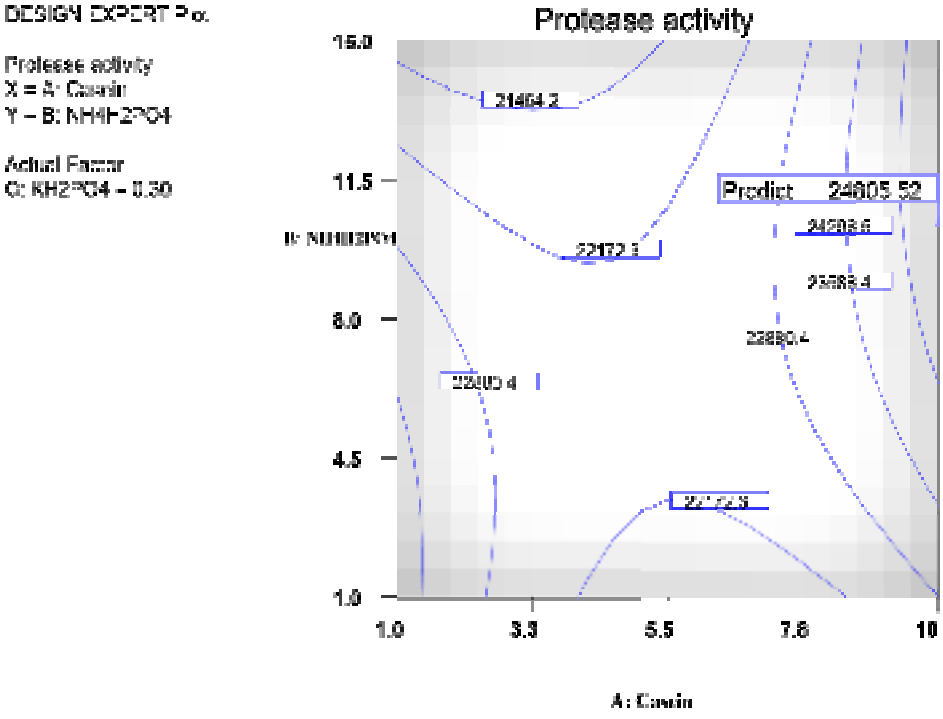
(i)


Protease activity (U ml -1)
30000
25000
20000
15000
10000
5000
0
5
6
7
8
9
10
pH

(j)


Protease activity (Uml -1)
30000
25000
20000
15000
10000
5000
0
20
25
30
35
40
Tempreature (°C)

(k)



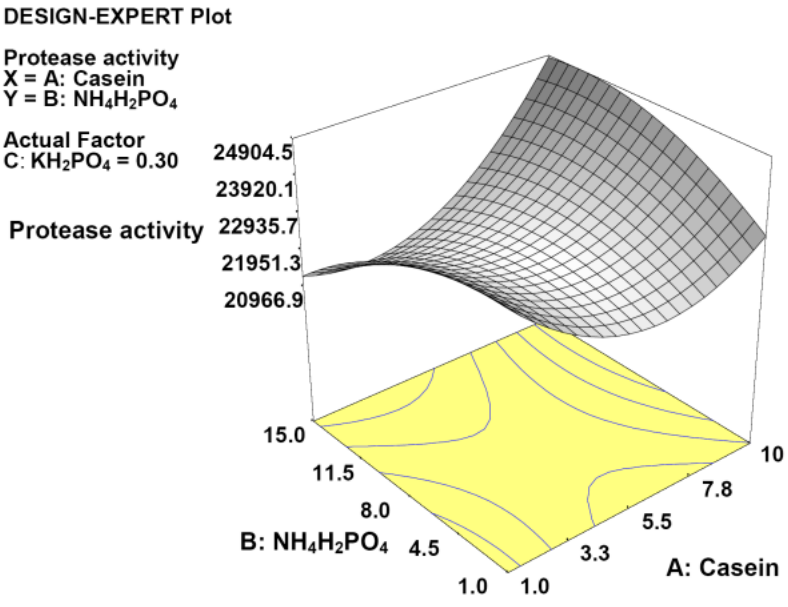
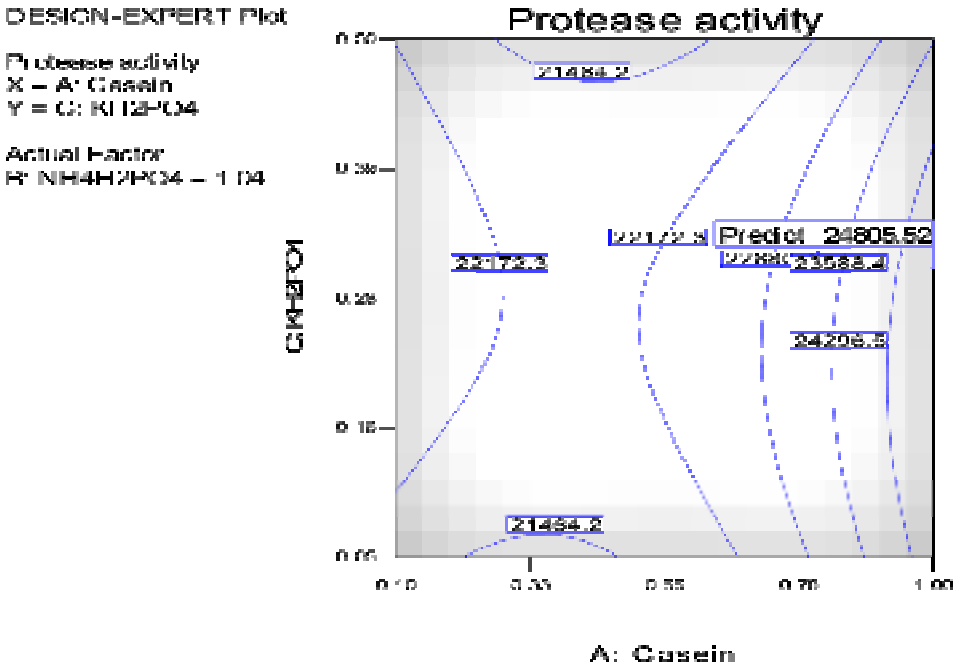


Fig. 3.2 a Contour plots and three dimensional plots respectievely showing the interaction between $NH_4H_2PO_4$ casein



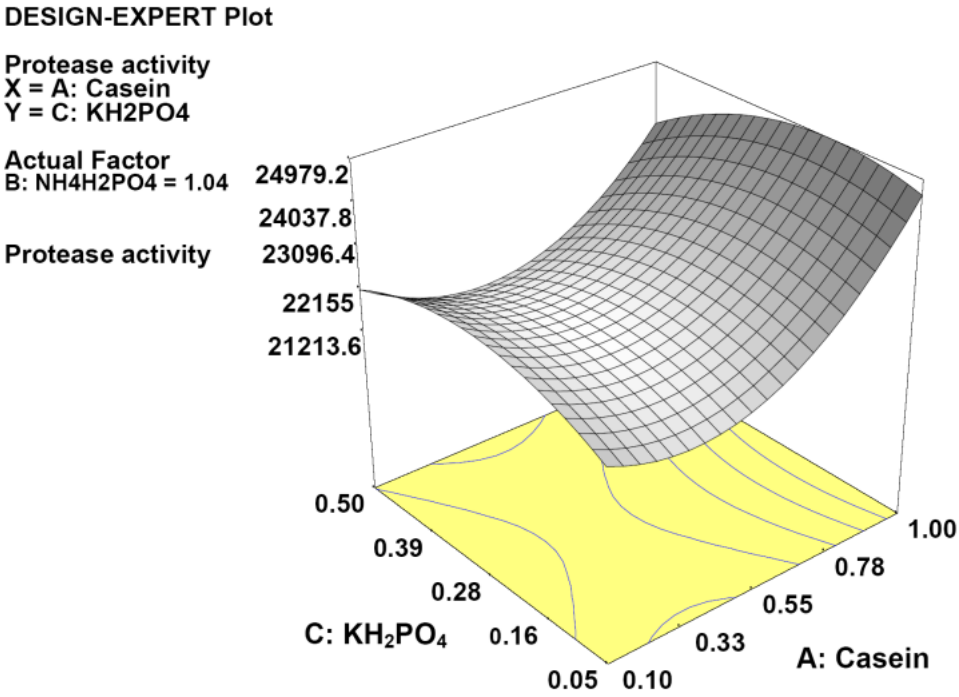
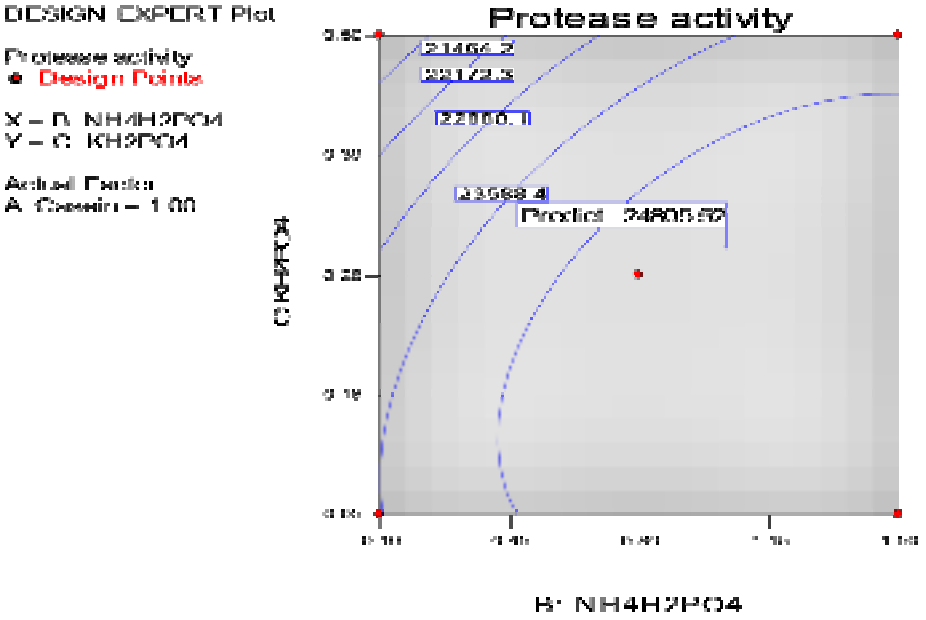


Fig. 3.2b Contour plots and three dimensional plots respectievely showing the interaction between KH_2PO_4 and casein



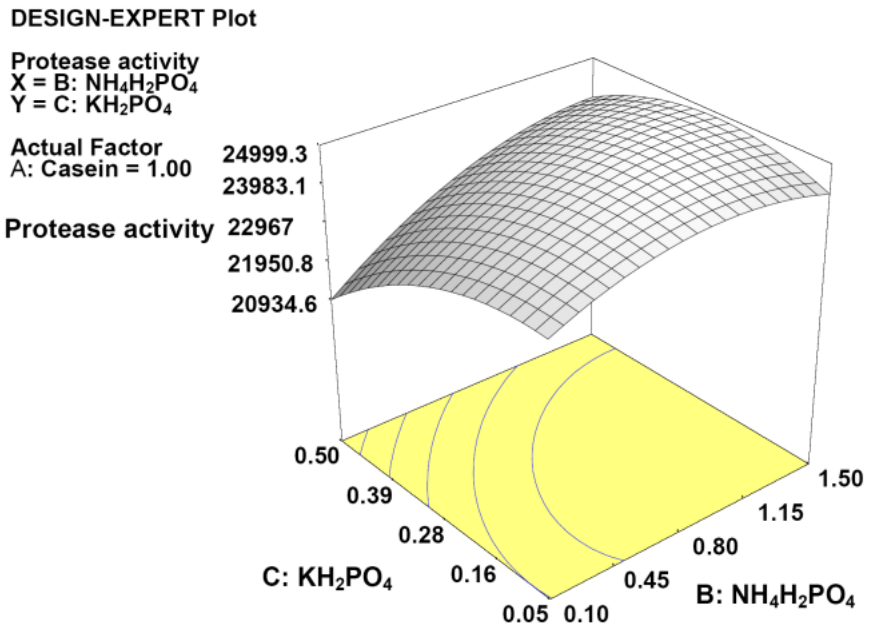
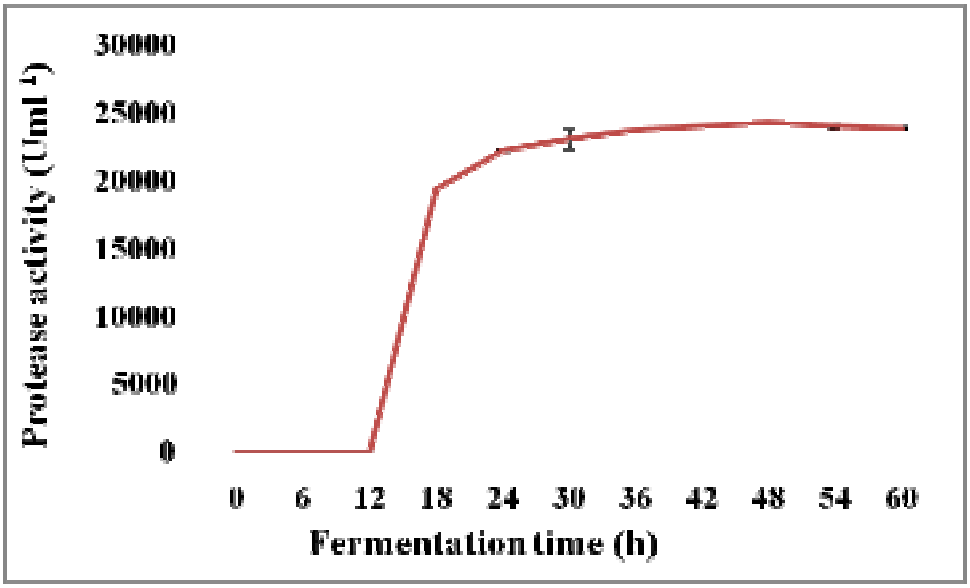
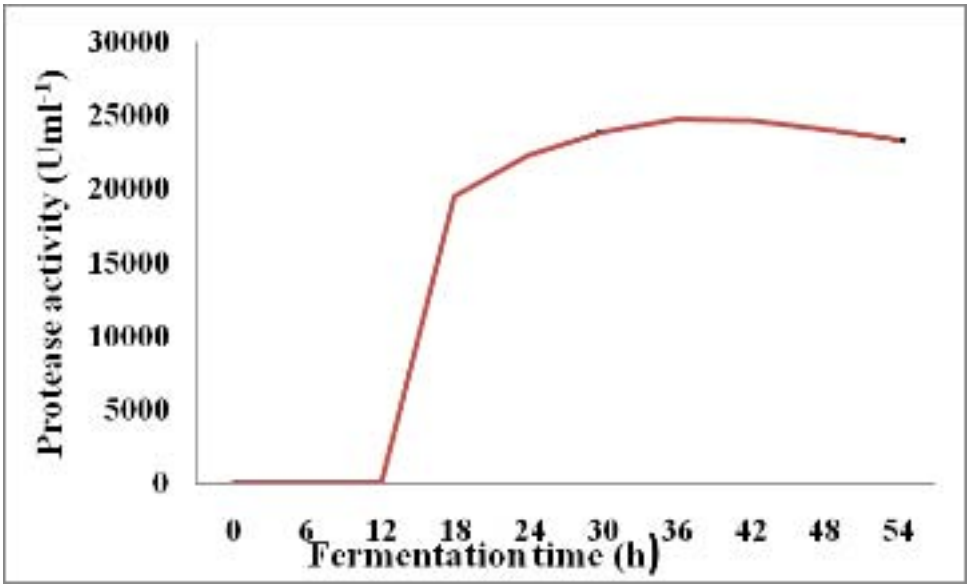


Fig. 3.2 c Contour plots and three dimensional plot respectievely showing the interaction between KH_2PO_4 and $NH_4H_2PO_4$



(a)



(b)

Fig. 3.3 (a) Time course of protease production in shake flask, (b) Time course of protease production in 5-l fermenter

Fig 3.4 Protease production in 5-l fermenter

3.4 Discussion

Medium optimization has a significant role in industrial enzyme production (Joo et al., 2002; Laxman et al., 2005; Hajji et al., 2008). Designing an appropriate fermentation medium is of critical importance in improvising the product yield (Rai and Mukherjee, 2010), since the secretion of proteases is strongly dependent upon the growth media and is therefore important to determine the medium and culture parameters in order to maximize the protease production (Pillai et al., 2011). Every organism is unique in its nutrient requirements for maximum enzyme production and there is no defined medium for

microbial protease production (Beg et al., 2003b; Chauhan and Gupta, 2004; Tari et al., 2006) which necessities the media optimization in order to maximize the protease production.

The conventional one-variable at a time approach is time consuming and requires more experimental data sets and cannot predict the interaction between the components and it does not depict the complete effects of the parameters on the process (Weruster-Botz, 2000; Wang and Lu, 2004; Bas and Boyaci, 2007; Li et al., 2008; Liu et al., 2010), while, the use of statistical techniques for the optimization of media components take in to account of the interaction among the variables and is less time consuming due to the limited number of experiments. There has been a tremendous trend in the use and acceptance of statistical experimental designs in biotechnology. Statistical experimental designs such as factorial designs, and design of experiments investigate defined input factors to a converting system from which mostly common and well defined output factors or responses such as product yield and productivity are generated. Response surface methodology (RSM) is the most accepted statistical technique for bioprocess optimization which can be used to examine the relationship between a set of controllable experimental factors and observed results (Rai and Mukherjee, 2010).

Most of the reports available on protease production are from *Bacillus* (Ferrero et al., 1996; Putten et al., 1996; Çalık et al., 1998; Mabrouk et al., 1999; Çalık et al., 2000; Çalık and Ozdamar, 2001; Beg et al., 2003b; Çalık et al., 2003; Chauhan and Gupta, 2004; Joo and Chang, 2005; Joo and Chang, 2006; Potumarthi et al., 2007; Saran et al., 2007; Shikha et al., 2007; Oskouie et al., 2008; Nadeem et al., 2009; Rai et al., 2009; Shivanand and Jayaraman, 2009; Fakhfakh-Zouari et al., 2010; Haddar et al., 2010a; Liu et al., 2010;Prakash et al., 2010; Mukherjee and

Rai, 2011; Sellami-Kamoun et al., 2011), and *Bacillus* species are viewed as promising agents for industrial applications and their proteases are extensively studied (Karadzic et al., 2004). However, only a limited number of reports exists on the proteolytic enzymes from *Pseudomonas aeruginosa* (Baoudh et al., 2000; Dutta et al., 2004; Dutta et al., 2005; Rahman et al., 2005; Khan et al., 2006; Zambare et al., 2011). *Pseudomonas* species also produce extracellular alkaline proteases similar to those produced by *Bacillus* species and furthermore, the species *P. aeruginosa* is capable of producing stable enzymes (Karadzic et al., 2004) and hence the enzymes from this species are of great industrial relevance.

In the present study, the attempt made to optimize the protease production in *P. aeruginosa* MCCB 123 has been a success as this species proved to be a good protease producer. One dimensional screening (initial screening experiment) of various media components was carried out in order to find the significant ranges of medium components affecting the protease production. The factors were further screened by Plackett-Burman design in order to find the significant factors affecting the protease production. When more than five independent variables are to be screened, the Plackett-Burman design is used to find out the most significant variables influencing the response (Stanbury et al., 1997). Plackett-Burman design was found to be critical in bringing down the number of parameters for further analysis and in the selection of significant factors affecting the protease production and provides indications of how each factor tends to affect the enzyme production (Mukherjee and Rai, 2011).

Among the various media components screened using Plackett-Burman design, casein, $NH_4H_2PO_4$ and KH_2PO_4 were found to be most influential in protease production. Factors such as glucose, yeast extract, $CaCl_2$, $ZnCl_2$ and $MgSO_4.7H_2O$ were found to have a negative effect on

protease activity as evident from their negative coefficient estimate. The negative effect of glucose on protease production of *P. aeruginosa* MCCB 123 may be due to catabolic repression by carbohydrates which is a common regulatory mechanism for biosynthesis of microbial proteases as indicated by Shivanand and Jayaraman (2009) and Grazziotin et al. (2007). The inclusion of glucose in the production media resulted in a strong catabolic repression on protease production by *Bacillus* sp. (Patel et al., 2005). Glucose was reported to have an inhibitory effect on protease production by *Bacillus* sp. (Puri et al., 2002; Pillai et al., 2011), *Bacillus mojavensis* (Beg et al., 2003b), *Bacillus horikoshii* (Joo et al., 1992) and, *Aeromonas hydrophila* (O´Reilly and Day, 1983). The addition of glucose markedly inhibited keratin synthesis in *Chryseobacterium* sp. kr6 (Brandelli and Riffel, 2005). The organic nitrogen source, yeast extract was also found to have a negative effect on protease production by *P. aeruginosa* MCCB 123.

Metal ions such as $CaCl_2$, $ZnCl_2$ and $MgSO_4.7H_2O$ had negative influence on protease production in *Pseudomonas aeruginosa* MCCB 123. Reports are available on the negative influence of $CaCl_2$ on protease production on different bacterial species.$CaCl_2$ decreased keratinase production in *Bacillus pumilus* A1 (Fakhfakh-Zouari et al., 2010), and *Streptomyces fungicidius* MML1614. (Ramesh et al., 2009). Zn^{2+} ions interfered with protease production by *Bacillus* sp. SSR1 (Singh et al., 2001).

Significant factors that affected protease production by *P. aeruginosa* MCCB 123 were further optimized by Face centered Central Composite Design (FCCCD) of response surface methodology in order to maximize the protease production. Subsequently, the quadratic regression model was found to be highly significant for protease activity. Coefficient of determination (R^2) is defined as the ratio of the explained variation to the total variation and is a measure of fit (Haber and Runyon, 1977). It

gave a measure of how much variability in the observed response value could be explained by the experimental factors and their interactions. When the R^2 is large, then, the regression has accounted for a large proportion of the total variability in the observed value of *Y* which favored the regression model equation (Rai and Mukherjee, 2010).The R^2 value should be between 0 and 1. A value > 0.75 indicates the aptness of the model. The closer the R2 value to 1, the stronger the model and better it predicts the response (Khuri and Cornell, 1987; Haaland, 1989). The analysis of variance (ANOVA) for the selected quadratic model showed that the model is adequate with no significant lack of fit (prob>F = 0.9762) .The observed R^2 value 0.9536 explains that the fitted model could explain 95.36% of the total variation and the model did not explain 4.64 % of the total variation, thus indicating the good response prediction by the model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model (Rai and Mukherjee, 2010). The adjusted R^2 value of 0.9119 in the present study indicates the high significance of the model (Cochran and Cox, 1957; Box et al., 1978). An 'adequate precision value' is used to measure the ratio of signal (controllable) to noise (uncontrollable), and a value of greater than 4 is generally desirable for a model to be a good fit (Wang and Lu, 2004; Oskouie et al., 2008). Here, the value of ratio (18.326) suggests that the polynomial quadratic model is of adequate signal, and can be used to navigate the design space. A relatively lower value of coefficient of variation (CV=1.65 %) indicated a good precision and reliability of the experiment (Khuri and Cornell, 1987). The lack 'of fit'value is a measure of how well the model fits the data. Ifthe model has a significant lack of fit, then it is not a good predictor of the response and should not be used. In this model, the 'lack of fit' (*p* value = 0.9762) was found to be' not significant'.

Based on results of ANOVA on optimization of protease production by FCCD, casein was found to be the most significant factor affecting protease production by *Pseudomonas aeruginosa* MCCB 123 with an optimum value of 10g l^{-1}. Casein was reported to be a favorable media component in the protease production by *Pseudomonas aeruginosa* PseA (Gupta and Khare, 2007), *Bacillus sphaericus* (Liu et al., 2010), *Bacillus horikoshi* (Joo et al., 2002) and *Aspergillus niger* var. *tieghem* (Chakraborty et al., 1995). As a substrate for protease production, casein may act as an inducer for protease production which in turn favoured protease production. KH_2PO_4 was found to be the next influential factor followed by casein. Protease production in microbes is influenced by the presence of metal ions (Valera et al., 1997; VijayAnand et al., 2010). KH_2PO_4 is reported to have positive effects on extracellular protease production by *Bacillus subtilis* P13 (Pillai et al., 2011) and *Bacillus mojavensis* (Haddar et al., 2010a).

Even though, $NH_4H_2PO_4$ is found to be insignificant as an individual factor, its interaction effect (quadratic effect) is found to be significant for protease production by *P. aeruginosa* MCCB 123. If the factor is significant at the quadratic level, it can act as a limiting factor (Elibol, 2004). The optimal concentration of $NH_4H_2PO_4$ suggested by the model is 10g l^{-1}. The influence of $NH_4H_2PO_4$ is due to the requirement of high concentration of orthophosphate for protease production by *P. aeruginosa* MCCB 123 and the results were in agreement with Nigam et al. (1981), who reported the requirement of high concentration of orthophosphate concentration for protease production by *P. aeruginosa* with 2-fold increase in protease production at high phosphate ion concentration.

The 3D response surface plots and the 2D contour plots are the graphical representations of the regression equations (Elibol, 2004).

These plots help to understand the interaction of factors in the model and the optimum values of each factor required for maximum protease production. Response surface and contour plots provide a method to visualize the relation between the response and experimental levels of each variable, and the type of interactions between the test variables (Sen and Swaminathan, 1997). Shape of the contour plot, circular or elliptical indicates whether the mutual interactions between the corresponding variables are significant or not. A circular contour plot indicates that the interactions between the corresponding variables are negligible. An elliptical nature of contour plot indicates that the interactions between the corresponding variables are significant (Dutta et al., 2004; Wang and Lu, 2004). The elliptical nature of the contour plots in the case of protease activity of *P. aeruginosa* MCCB 123 indicates that the interactions effects between casein and $NH_4H_2PO_4$, casein and KH_2PO_4 and $NH_4H_2PO_4$ and KH_2PO_4 were found to be most significant for protease production.

Scale up study in 5-l fermenter showed that the optimization of fermentation medium resulted in 1.84 fold increase in protease activity. The primary component in the total protease secretion was observed within 24 h, reached maximum at 36^{th} h with slight increment and after that it showed a decline in the protease activity. Similar observation was made by Rao et al. (2006).The results indicate that the protease production was related to exponential growth phase of the bacteria. The majority of alkaline protease was secreted within the first 24 h (Reddy et al., 2008). There noted a decline in the protease activity after 36^{th} h of fermentation. This decline may be due to autoproteolysis (Jang et al., 2001), autodegradation (Hajji et al., 2008), denaturation or decomposition of protease as a result of the interactions with other compounds in the fermented medium (Uyar and Baysal, 2004), proteolytic activity of other proteases under starvation conditions (Chauhan and Gupta, 2004), and

protease degradation by some proteolytic activity on the cell surface of nitrogen starved cells in the cultivation broth (Chu et al., 1992). An earlier peak in the protease activity was obtained in 5-l fermenter (36^{th} h) when compared to shake flask (48^{th} h). Rao et al. (2006) also made similar observation that extracellular protease production in 5-l tank peaked earlier (in 6 days) when compared to shake flask (7^{th} Day). Rai and Mukherjee (2010) observed that the maximum time for protease production in a process controlled fermenter was 60h, while it was 72 h under shake flask study and explained that this may be due to slight variation in the experimental condition in fermenter and Erlenmeyer flasks, because the concentration of dissolved oxygen and pH of the medium could be maintained at desired level in a bioreactor that favors higher protease production in less time.

In order to formulate cost-effective and economical media, it is necessary to include cheap media components for the production of microbial products (Singh and Satyanarayana, 2006). Protease production by *P. aeruginosa* MCCB 123 was carried out in a synthetic mineral medium with minimal supplementation of organic nitrogen sources such as yeast extract ($2g\ l^{-1}$) and casein ($10g\ l^{-1}$) and thus proved to be an economical medium for large scale protease production. *P. aeruginosa* MCCB 123 secreted almost similar maximum protease titers in optimized medium in shake flask conditions ($24329.17\pm40.18\ Uml^{-1}$) and in fermenter ($24754.17\pm7.21\ Uml^{-1}$), suggesting a good scope for the scale up of enzyme production.

Chapter 4

PURIFICATION AND CHARACTERIZATION OF LasB PROTEASE FROM *PSEUDOMONAS AERUGINOSA* MCCB 123

Contents

4.1 Introduction

Microorganisms elaborate an array of intracellular and extracellular proteases of which the latter are made use of in various industrial processes. Among microbial proteases, bacterial alkaline proteases account for a major share of the enzyme market (Kumar and Takagi,1999), and are supplemented as ingredients in detergents, used in food processing, leather industry, waste treatment, dairy, food and feed manufacturing (Horikoshi, 1996; Anwar and Saleemuddin,1998; Rao et al., 1998; Gupta et al., 2002a) and peptide synthesis (Kumar and Bhalla, 2005).

Protease production is an inherent capacity of microorganisms and only those microbes which could produce substantial amount of protease are suited for industrial applications (Gupta et al., 2002a). A few reports exist on the proteolytic enzymes from *Pseudomonas aeruginosa* (Bayoudh et al., 2004). Even though, *Bacillus* spp. have been viewed as a promising group of organisms for protease production towards industrial applications, *Pseudomonas* proteases have also been considered for such

uses as they are either similar or more active than those produced by the former. Furthermore, *P. aeruginosa* can grow in alkaline conditions and in water-soluble oil and its enzymes are adapted to extreme conditions (Karadzic et al., 2004). The *Pseudomonas* proteases do have applications in deproteinization of shrimp and shell waste (Wang and Chio, 1998; Oh et al., 2000), peptide synthesis (Ogino et al., 1999 a,b; Ogino et al., 2000 a,b), and also as biocatalysts for enzymatic synthesis in presence of organic solvents (Geok et al., 2003; Tang et al., 2008). *P. aeruginosa* produces several proteolytic enzymes and the predominant proteases secreted by the bacterium are alkaline protease and LasB protease /elastase (Jellouli et al., 2008).

4.1.1 LasB protease (Pseudolysin)

LasB protease (elastase) is one of the several extracellular enzymes produced by *P. aeruginosa* (Bever and Iglewski, 1998). The protease belonging to thermolysin family of zinc dependent neutral metalloendopeptidase (Morihara, 1995; Kessler et al., 1998). It is a metal chelator sensitive neutral protease which requires Zn^{2+} for enzymatic activity (Morihara and Tsuzuki, 1977). The name elastase relates to its ability to degrade elastin, thus distinguishing it from alkaline protease (Morihara, 1965; Galloway, 1991). On the other hand, as a proteolytic enzyme, elastase displays four times the activity towards casein as opposed to trypsin and about 10 times the proteolytic activity of the alkaline proteinase from *Pseudomonas* (Morihara, 1965). The proteolytic activity of elastase far exceeds its elastolytic activity. However, the specific elastolytic activity of *Pseudomonas* elastase is not very efficient as it displays only one quarter of that of *Bacillus* thermolysin and one seventh of the elastolytic activity of purified human neutrophil elastase (Peters and Galloway, 1990).Therefore, *Pseudomonas* elastase is a very efficient proteolytic enzyme capable of degrading elastin as a special case (Galloway, 1991). The LasA protease enhances the activity of *P. aeruginosa* elastase/LasB protease (Goldberg and Ohman, 1987; Peters et al., 1992; Kessler et al.,

1997) by nicking the elastin structure rendering it more susceptible to the proteolytic action of elastase (Peters and Galloway, 1990). Olson and Ohman (1992) suggest that LasA facilitates the elastinolytic activity of elastase/LasB protease by allowing normally inaccessible sites within the elastin to be exposed to the elastolytic activity of elastase. Peters and Galloway (1992) reported that LasA protein interacts with elastin substrate rather than modifying elastase.

4.1.2 Secretion of LasB protease

Encoded by the LasB gene, the protease is synthesized as a preproenzyme of 53.6 kDa (Gustin et al., 1996; Braun et al., 1998) of 498 aminoacids with three distinct domains: a signal peptide of 23 aminoacids, a propeptide of 174 amino acids, and a mature elastase of 301 aminoacids (Bever and Iglewski, 1988). LasB protease is synthesized as a much larger precursor called preproelastase with a molecular mass of approximately 60 kDa (Schad and Iglewski, 1988). The translocation of preproelastase through the cytoplasmic membrane and processing of the signal peptide occurs co-translationally, which indicates how the polypeptide chain folds in to the native protein with a unique three dimensional structure, or possibly shortly afterwards. Removal of signal sequences from preproelastase yields another short lived precursor, proelastase I, with a molecular mass of approximately 50 kDa found in the periplasmic space. Proelastase I in combination with components of outer membrane is rapidly processed to a smaller proenzyme form, proelastase II (is slightly larger than the mature elastase with molecular mass of approximately 33.5 kDa) and a 17 kDa component. The proelastase II is reported to be inactive (Jensen et al., 1980; Fecycz and Campbell, 1985; Kessler and Safrin, 1988a, b). The 17 kDa component remains non-covalently associated with proelastase II, resulting in the inhibition of its proteolytic activity. In some unknown manner, proelastase II passes through the outer membrane and is

converted to the 33 kDa active form of elastase in the process (Galloway, 1991). LasB gene is referred as the elastase structural gene (Bever and Igelwski, 1988). LasB protease is produced late in the logarithmic phase of growth and the quantity produced is inversely proportional to the concentration of iron in the growth medium. The production decreases if the cells are cultured in the presence of ammonium chloride, glucose or sub-inhibitory concentrations of certain antibiotics (Kessler and Safrin, 1983; Whooley et al., 1983). Several environmental factors such as iron, ammonium chloride, glucose and zinc have been implicated in regulating elastase production (Igelwski et al., 1990; Brumlik and Storey, 1992).

4.1.3 Structure of LasB protease

P. aeruginosa LasB protease is a zinc metalloprotease with 301 aminoacids (Thayer et al., 1991). Structural gene analysis of LasB protease suggests that a preproelastase of 498 aminoacids is eventually processed and secreted as a mature protein of 301 amino acids (Galloway, 1991; Thayer et al., 1991). The native structure of the protein is as follows: The amino terminal domain of the protein is constructed primarily of antiparallel β strands, while the carboxyl-terminal domain is α helical. The two helices span the active site and provide ligands for zinc (active site helices) and the residues 135-178 connect the loops between them. The zinc ligands are His-140, His-144 and Glu-164 associated with zinc binding. Glu-141 is the active centre, and Tyr-155, His-223 and Asp-221 are associated with substrate binding. The active site glutamic acid is considered as essential for catalysis. The two residues, Tyr-155 and His-223 are implicated in interactions with substrate carbonyl oxygen atoms. The carboxyl group of Asp-221 is in close proximity to the side chain imidazole of His-223. There is a four-helix bundle with in the final 70 residues of the carboxy-terminal domain. Neither the length of the helices nor the number of residues in the loops connecting them is conserved

precisely (Thayer et al., 1991). The mature polypeptide contains four cysteines which together form two disulfide bonds or bridges in the folded enzyme and the bonds are not localized in close proximity of the active centre of the protein. One disulfide bridge, between Cys-30 and Cys-57, is located in the N-terminal part of the mature enzyme and connects two β-strands. The other disulfide bond, between Cys-270 and Cys-297 is located close to the C-terminus and connects two α-helices. One disulfide bond is formed in the proenzyme and is essential for subsequent autoproteolytic processing to occur. The other disulfide bond is formed only after autocatalytic processing and appears to be required for the full proteolytic activity of the enzyme and contributes to its stability. The Disulfide oxidoreductase (DsbA) protein catalyzes the formation of both of these disulfide bonds (Braun et al., 2001). The relative orientation of the two domains may be governed by the packing of residues within the three loops: residues 109-111 prior to the final two antiparallel β strands, residues 125-134 between the final β-strands of the amino-terminal domain and the first active site α helix of the carboxyl-terminal domain and the residues 190-195 following the second active-site helix of the carbonyl-terminal domain. The side chains of both Tyr-63 and Lys-64 are on the exterior of the elastase molecule and are remote from the active site cleft. There is a single bound calcium ion in *P. aeruginosa* elastase. The presence of Tyr at position 188 and the residues 180-182 in the loop segment precludes *P. aeruginosa* elastase binding a second calcium ion (Thayer et al., 1991).

4.1.4 Specificity of LasB protease

P. aeruginosa LasB protease appears to have broader substrate specificity than many other metalloproteases (Galloway, 1991). *Pseudomonas* elastase is less specific than pancreatic elastase and yet capable of more complete degradation of elastin (Saulnier et al., 1989). It requires only four sub-sites in order to demonstrate activity and therefore it is easier to find cleavage sites within the structure of elastin. The

protease preferentially cleaves peptide bonds on the amino side of the hydrophobic residues (Morihara, 1965). It has a high level activity towards broad range of substrates (Gustin et al., 1996) hydrolyzing internal peptide bonds of proteins and peptides on the amino side of hydrophobic residue with phenylalanine as the preferred residue in the position P1′ (Morihara, 1995; Kessler et al., 1998). Substrate specificity studies showed that the substrate carbobenzoxy-Gly-X-NH_2 is most susceptible to digestion when X is an aromatic or bulky aminoacid residue such as Phe, Leu or Tyr (Galloway, 1991) and the order of specificity is Phe>Tyr>Leu suggesting that *P. aeruginosa* LasB protease (elastase) can accommodate larger hydrophobic side chains at the P1′ position (Thayer et al., 1991). The protease can cleave X-Tyr sequences, where X is any amino acid (Saulnier et al., 1990) and this substrate specificity makes elastase an interesting candidate for peptide synthesis. The elastase activity of the protease is not related to side-chain specificity, but is based upon their ability to absorb to the elastin structure (Morihara and Tsuzuki, 1967).

LasB protease of *P. aeruginosa* can be considered as an alkaline protease as most of the reported ones from *P. aeruginosa* exhibited optimum pH values ranging from 8.0 to 9.0 (Morihara et al., 1965; Gupta et al., 2005b; Jellouli et al., 2008). The recombinant elastase from *P. aeruginosa* in *Pichia pastoris* has its optimum pH at 7.4 (Lin et al., 2009). Alkaline proteases are of considerable biotechnological interest due to their activity and stability at alkaline pH (Kumar and Takagi, 1999).

4.2 Materials and methods

4.2.1 Enzyme production

LasB protease was purified from the optimized medium composed of (in g l^{-1} Distilled water) glucose,7.5; yeast extract,2.5; $NH_4H_2PO_4$,10.04; Na_2HPO_4, 0.5; KH_2PO_4, 3.0;$MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.000625; $ZnCl_2$, 0.01; casein, 10.0; pH, 7.0 in a 5-l fermenter (Biostat-B-Lite , Sartorius,

Germany) . Fermentation was carried out at 25°C, pH 7.0 ± 0.05, 300 rpm and supplied with sterile air at the rate 2.5 l min^{-1}. For enzyme extraction the culture was centrifuged at 8260 *g* for 15 min at 4°C and the supernatant stored in 300 ml aliquots at -20°C, and used for further purification and characterization.

4.2.2 Purification of LasB protease

4.2.2.1 Ammonium sulphate precipitation

Ammonium sulphate was added to cell-free culture supernatant up to 30 % saturation, precipitates removed by centrifugation at 8260 *g* for 15 min at 4°C and discarded, and continued up to 80 % saturation, collected the precipitate the same way and resuspended in 20 mM Tris-Cl buffer, at pH 8.5. The enzyme was dialyzed against the same buffer using Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cutoff membrane (Omega, 25MM, 10K, Pall life sciences) and used for further purification.

4.2.2.2 DEAE-cellulose chromatography

The enzyme was loaded on AKTA Prime protein purification system equipped with a C10/20 ((10mm×20cm) (GE Healthcare Biosciences, Uppsala)) DEAE cellulose (Sigma – Aldrich Co.) column equilibrated with 20 mM Tris-Cl buffer, at pH 8.5. The column was washed with the same buffer to remove the unbound proteins and the enzyme was eluted by applying a linear gradient of NaCl from 0 – 1000 mM at a flow rate of 0.5 ml min^{-1} and fractions of 2 ml were collected. Active fractions were pooled and concentrated by lyophilization.

4.2.3 Characterization of LasB protease

4.2.3.1 Protease assay

Protease assay was carried out following the method of Khembavi et al. (2003). An aliquot of 0.5 ml suitably diluted enzyme solution was mixed

with 0.5 ml substrate (1% Hammerstein casein in 50 mM Tris-Cl buffer, at pH 9) and incubated at 60ºC for 30 min. The reaction was stopped by the addition of 0.5 ml 20 % TCA and kept for 10 min at 25ºC in order to complete the reaction, centrifuged at 8260*g* at 4ºC for 15 min and the absorbance measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to liberate 1 µg tyrosine per ml per min. The experiments were carried out in triplicates and the mean value expressed as unit protease activity.

4.2.3.2 Elastase assay

Elastase activity was determined using elastin – Congo red (Sigma – Aldrich Co.) as the substrate according to the modified method of Kessler et al. (1997). The reaction mixture consisting of 1 ml enzyme solution, 5 mg elastin Congo red, and 1 ml 50 mM Tris-Cl buffer (pH 8) was incubated at 40ºC for 3 h. Reaction was stopped by the addition of 0.1 ml 100 mM EDTA, centrifuged at 8260*g* for 15 min, and absorbance measured at 495 nm. Assays were carried out in triplicates and the mean value was expressed as elastase activity.

4.2.3.3 Protein assay

Quantification of protein was carried out following the method of Hartree (1972) using Bovine Serum Albumin as standard.

4.2.3.4 Specific activity

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

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

4.2.3.5 Relative activity

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

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

4.2.3.6 Residual activity

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

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

4.2.3.7 Electrophoresis and Zymography

The lyophilized active fractions of the enzyme were subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS –PAGE) and native PAGE following the method of Laemmli (1970) using 5% stacking gel and 15 % resolving gel at a constant current of 12mA. After electrophoresis, gels were stained with 0.025 % Coomassie brilliant blue stain R-250 and then destained in a solution of 5% methanol and 7% acetic acid. Molecular weight of the unknown protein band was determined by comparing with the molecular weight standards from Bangalore Genei.

Zymography (substrate gel electrophoresis) was performed with 4 % stacking gel and 10 % resolving gel copolymerized with 0.1 % gelatin. Electrophoresis was done at a constant current of 12 mA. After electrophoresis, the gel was incubated for 1 h in 100 ml of 50 mM Tris-Cl, at pH 9.0 containing 2.5 % (v/v) Triton X-100 to remove SDS. The gel was then washed with distilled water to remove Triton X-100 and then incubated in the same buffer for 1 h and stained with

0.025% Coomassie brilliant blue R-250, destained as mentioned earlier. Protease activity was detected as clear zones against dark background.

4.2.3.8 Effect of pH on LasB protease and elastase activity

Effect of pH on the protease activity was determined over a pH range of 6 -12 using the buffers of 50 mM concentrations: sodium – phosphate (6,7), Tris-Cl (8,9), glycine-NaOH (9 , 10, 11,12) for 30 min at 60°C, and on elastase activity over the same range using the same buffers for 3h at 40°C. The substrates (casein and elastin – Congo red) were prepared in the respective buffers and assayed under standard conditions as detailed in section 4.2.3.1 (Protease assay) and 4.2.3.2 (Elastase assay) and the relative activities were measured as described in section 4.2.3.5.

4.2.3.9 Effect of pH on LasB protease and elastase stability

For the measurement of pH stability, the enzyme was incubated in different buffers mentioned above for 1 h at 25°C and the residual protease and elastase activities were determined as detailed in section (4.2.3.6). Untreated enzyme was taken as control (100% activity).

4.2.3.10 Effect of temperature on LasB protease and elastase activity

Effect of temperature on protease activity was tested by carrying out the assay at temperature ranges of 30, 40, 50,60,70 and 80°C for 30 min in 50 mM Tris-Cl buffer (pH 9.0), and on elastase activity at the same range for 3 h in 50 mM Tris-Cl buffer (pH 8.0). The relative activities were measured as detailed in section 4.2.3.5.

4.2.3.11 Effect of temperature on LasB protease and elastase stability

Effect of temperature on protease and elastase stability was examined by incubating the enzyme at temperatures ranging from 30, 40, 50, 60, 70 and 80°C for 1 h in 50 mM Tris-Cl buffer at pH 9.0 and 8.0,

respectively, and the residual protease and elastase activities were measured as detailed in section 4.2.3.6. The non-heated enzyme was considered as the control (100% activity).

4.2.3.12 Effect of metal ions, inhibitors and oxidizing agents on LasB protease and elastase activity.

Effects of various metal ions (5 mM CaCl2, $ZnCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $BaCl_2$, and Pb (NO3) $_{2,}$ final concentration), inhibitors (5 mM phenyl methyl sulphonyl fluoride (PMSF), EDTA, 1, 10 phenanthroline and 1 mM iodoacetic acid, final concentration), surfactants (1% v/v Triton X-100, 0.5 % w/v SDS, final concentration), and an oxidizing agent (0.1% v/v H_2O_2, final concentration) on protease and elastase activities were studied by including them in the assay mixture, and the relative activities were as detailed in section 4.2.3.5. Untreated enzyme was taken as the control (100% activity).

4.2.3.13 Comparison of LasB protease activity with commercial proteases Savinase®

Specific Las B protease activity of the partially purified protease was compared with that of the commercial protease Savinase® from *Bacillus* sp. (Sigma – Aldrich Co., Product no P3111) under the assay conditions at pH 9.0 and temperature 60°C.

4.2.3.14 Cytotoxicity analysis of LasB protease

Hep-2 cells were seeded in 96 well plates (Greiner Bio-One) containing 82mM glutamine, 1.5g l^{-1} sodium bicarbonate and 10% fetal bovine serum. Purified enzyme in concentrations of 0, 1, 3, 5, 10, 25, 50,100, 250 and 500µg ml^{-1} (v/v) was added to the wells in triplicates. A control was kept without the added enzyme. After 14 h incubation MTT assay was performed and the percentage of inhibited cells at each concentration of the protease was calculated using SPSS software (SPSS 17.0 package for Windows).

4.2.3.14.1 MTT assay

After replacing the medium, 50µl MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-Aldrich Co.) having a strength of 5 mg ml^{-1} in PBS (720mOsm) was added to each well and incubated for 5 h in dark. MTT was added to the control wells with the medium alone. The medium was removed and MTT-formazan crystals were dissolved in 200µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in micro plate reader (TECAN Infinite Tm, Austria).

4.2.3.15 PCR amplification, purification and sequencing of LasB protease gene

Genomic DNA was purified using the phenol-chlorofom method as described by Sambrook and Russell (2001).The LasB protease primers were synthesized based on Jellouli et al. (2008).Amplification was performed using the primers (AAGCGTCGGCCGAGTACTTCG forward) and (GACCGGCATTCCTTCCTGGAG, reverse). The amplification was performed using DNA Thermal cycler (Eppendorf). Reaction mixture (final volume 25 µl) contained 2.5 µl 10 X buffer, 1 µl 10 pmol each of oligonucleotide primer, 1 µl DNA template, 2.5 µl 2.5 mM each deoxynucleoside triphosphate, 1 µl Taq polymerase, and the remaining volume was made up with sterile Milli Q water. Amplification profile consisted of 1 cycle of 94°C for 2 min, 66°C for 45 s, and 72°C for 1 min 30 s; 30 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 1 min 30 s. Amplified PCR products were analyzed on an agarose gel and purified using gel purification kit of Sigma and sequenced using the service of Xcelris, Ahmadabad. Alignment of the protein sequence was done by CLUSTAL W programme (Thompson et al., 1994).

4.2.4 Statistical analysis

Data generated from the experiments were analyzed using One-way Analysis of Variance (ANOVA) with post-hoc multiple comparison analysis

performed using Tukey's HSD using SPSS 17.0 package for Windows at a significance level of $p<0.05$. Data are presented as mean± standard deviation.

4.3 Results

4.3.1 Purification of LasB protease

In the present study, LasB protease was purified from an environmental isolate of *P. aeruginosa* MCCB 123. The enzyme was purified by a two step procedure, by precipitation with ammonium sulphate followed by DEAE-cellulose chromatography. Ammonium sulphate fractions from 30-80 % showed protease and elastase activities. Active fractions were pooled and concentrated by ultrafiltration using a 10 kDa membrane. The pooled fractions were then loaded into DEAE-cellulose chromatography column. The results of purification procedure are summarized in Table 4.1. The enzyme was purified with 4.85 fold increase in specific protease activity and 5.59 fold increase in specific elastase activity. The elution profile of the LasB protease on DEAE-cellulose column is shown in Fig.4.1. The LasB protease got eluted between 0.44 M to 0.68 M NaCl (fractions, 44 to 68).

4.3.2 Characterization of LasB protease

4.3.2.1 Determination of molecular weight

The purified MCCB 123 LasB protease was homogenous on SDS-PAGE and its molecular weight estimated has a 33 kDa by reducing SDS-PAGE (Fig. 4.2a). The gelatin zymogram showed a single clearance at ~100 kDa (Fig.4.2b).

4.3.2.2 Effect of pH on LasB protease and elastase activity

The effect of pH on protease and elastase activities was determined using buffers in the pH range of 6 to 12 at 60 and 40°C, respectively. The percentage relative protease and elastase activities at different pH is shown in Fig. 4.3. The enzyme was found to exhibit protease activity from pH 6 to10

with its optimum at 9.0. The enzyme showed elastase activity from pH 7 to 9 with its optimum at 8.0.The optimum pH of LasB protease was 9.0 and that of elastase 8.0. The statistical analysis revealed there was a significant (p<0.05) difference in the protease activity between pH values from 6 to 10 (Appendix 2, Tables 2.1a to 2.1c) significant (p<0.05) difference in elastase activity between pH 7 and 8 (Appendix 2, Tables 2.2a to 2.2c).

4.3.2.3 Effect of pH on LasB protease and elastase stability

Effect of pH on LasB protease stability showed that the enzyme was stable from pH 7 to10 for one hour and retained more than 90 % of its residual activity from pH 7 to 9 and 48 % activity at pH 10. At pH 11 and 12 there was significant decline in the activity. Effect of pH on elastase stability showed that the residual activities at pH 7, 8 and 9 were about 71, 93 and 38%, respectively, when compared to the control (Fig.4.4). The statistical analysis revealed that there was a significant (p<0.05) difference in protease stability between pH values from 6 to 12 (Appendix 2, Tables 2.3a to 2.3c) and elastase stability from pH 7 to 9 (Appendix 2, Tables 2.4a to 2.4 c).

4.3.2.4 Effect of temperature on LasB protease and elastase activity

The enzyme was found to exhibit protease activity from 40 to 70°C with its optimum at 60°C. There was a significant loss in the activity at 80°C. The enzyme exhibited elastase activity from 30 to 60°C with its optimum at 40°C. There was a significant loss in the activity at 70°C. (Fig.4.5). Statistical analysis showed that there was significant (p<0.05) difference in protease activity between temperature ranges from 30 to 80°C (Appendix 2, Tables 2.5a to 2.5c) and significant (p<0.05) difference in the elastase activity between temperature ranges of 30 to 60°C (Appendix 2, Tables 2.6a to 2.6c).

4.3.2.5 Effect of temperature on LasB protease and elastase stability

Effect of temperature on LasB protease and elastase stability was determined by incubating the enzyme from 30 to 80°C for 1 hour and the residual activities measured under standard assay conditions. The enzyme retained more than 80 % protease activity and 60% elastase activity from 30 to 50°C. From 70°C onwards, there was significant loss in protease and elastase activities (Fig.4.6). There was significant (p<0.05) difference in the protease a stability between temperature ranges from 30 to 70°C (Appendix 2.7a to 2.7c) and significant (p<0.05) difference in the elastase stability between temperature ranges of 30 to 60°C (Appendix 2, Tables 2.8a to 2.8c).

4.3.2.6 Effect of metal ions, inhibitors and oxidizing agents on LasB protease and elastase activity

The effects of various metal ions on protease and elastase activities were investigated by including them at a final concentration of 5 mM in the reaction mixture. The results are shown in Table 4.2. The addition of Mg^{2+}, $Ba^{2+}$and Ca^{2+} resulted in a slight activation in protease activity by 2, 9 and 15 %, respectively, while $Mn^{2+}$and CO^{2+} lowered the activity by 58 and 71%, respectively. The enzyme activity was totally inhibited by Zn^{2+} and Pb $(NO_3)_2$. None of the metal ions seemed to have activation on elastase activity. The elastase activity was totally inhibited by Zn^{2+}, Pb $(NO_3)_2$ and CO^{2+}.

The effect of various active site inhibitors, detergents and oxidizing agents on protease and elastase activity is summarized in Table 4.3. The protease activity was totally inhibited by 5mM of 1, 10 phenanthroline and partially by 5 mM EDTA while elastase activity was completely inhibited by these chemicals. The enzyme retained 93 and 99 % protease activity in presence of 5mM of PMSF and 1 mM Iodo acetic acid, respectively, and 90 % elastase activity in presence of 1 mM Iodo acetic acid confirming that the enzyme did not belong to the class of serine and

cysteine protease, respectively. The enzyme retained 95 and 59 % protease activity in presence of 0.1 % H_2O_2 and 1%Triton X – 100, respectively.

4.3.2.7 Comparison of LasB protease activity with commercial Protease Savinase®

Activity of MCCB 123 (LasB) protease was compared with commercial proteases Savinase® from *Bacillus* sp. It was found that the specific protease activity of purified MCCB 123 LasB protease (14048.91±26.55U/mg) was 3.96 fold greater when compared to Savinase® (3546.40±35.21 U/mg) under similar conditions (Table 4.4).

4.3.2.8 Cytotoxicity analysis of purified LasB protease

Cytotoxic effects on Hep-2 (Human lung carcinoma) cells were studied at different concentrations of enzyme in the range of 0, 1, 3, 5, 10, 25, 50,100, 250 and 500µg ml^{-1} enzyme. At concentrations from 1 – 25 µg ml^{-1}, it did not cause any significant change in the cell morphology, (Fig.4.7) and 47.28±1.28 µg ml^{-1} was the LD_{50} dose (50 % inhibition) (Fig.4.8).

4.3.2.9 Sequencing of LasB protease gene

Primers were designed to amplify the zinc binding conserved region of the LasA protease gene. A PCR product of amplicon size of 1500 bp was obtained (Fig.4.9). The PCR product obtained was sequenced. The nucleotide sequence submitted to Gen Bank data base and was assigned the Accession No. JN118955. The nucleotide sequence of MCCB 123 LasB protease gene was compared with other LasB protease sequences available in the GenBank. BLAST analysis (www.ncbi.nlm.nih.gov) of the nucleotide sequence revealed that it shared 98% similarity to LasB gene/elastase gene of *P. aeruginosa* (Accession Nos.M19472, AB029328, M24531, EU021222, DQ350610, DQ153386, DQ150629) and 97% similarity to LasB gene of *P.aeruginosa* (Accession No. EU265777) and prepro elastase gene of *P.aeruginosa* (Accession No. JF502075) (Table 4.5).

The translation of nucleotide sequence was performed with ExPASy Molecular Biology server (http://www.expasy.com).The nucleotide sequence along with the deduced aminoacid sequence of the LasB protease gene of *P. aeruginosa* MCCB 123 is given in Fig.4.10. The aminoacid sequence shows the presence of HEXXH motif (HEVSH), active site residues Glu (E) and His (H). The putative residues of prepeptide, propeptide and mature LasB protease are identified in the sequence. The aminoacids representing calcium ligands, zinc ligands and active centre are also identified in the sequence (Fig.4.10). Sequence homology study of the deduced aminoacid revealed that the LasB protease of *P. aeruginosa* MCCB 123 showed homology to LasB protease/elastase gene of *P. aeruginosa* PAO1 (NP252413), elastase precursor of *P. aeruginosa* (AAA25811), organic solvent tolerant elastase of *Escherichia-Pseudomonas* shuttle vector pCon2 (3)-Zeo (AEA29834) and elastase LasB of *P. aeruginosa* 2192 (ZP04935737). It also exhibited sequence homology hypothetical protein of *P. aeruginosa* PACS2 (ZP01367156), elastase LasB precursor of *Stenotrophomonas maltophilia* (ADM89079), keratinase KP2 of *P. aeruginosa* (ADP00718), class 4 metalloprotease of *Chromobacterium violaceum* ATCC 12472 (NP899727) and *C. violaceum* (AAN78225), neutral protease of *Collimonas fungivorans* Ter331 (YP004752195), elastase of *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (YP855393), *Aeromonas caviae* Ae398 (ZP08521632), metalloprotease of *Aeromonas sobria* (ABG89854), protease of *Aeromonas veronii* B565 (YP004394066), pro-aminopeptidase protease of *Aeromonas punctata* (BAA82875), zinc metalloprotease (elastase) of *Reinekea* sp. MED297 (ZP01112982), neutral protease precursor of *Vibrio cholerae* TMA 21 (ZP04403750), hemagglutinin/proteinases of *V. cholerae* HE39 (EGR01640), *V. cholerae* 623-39 (ZP01980763), *V. cholerae* AM-19226 (ZP04959463), *V. cholerae* HE48 (EGR10267), *V. cholerae* V51 (ZP04918414), vibriolysin of *V. cholerae* O1 (ACX48920), *V. cholerae*

RC385 (ZP06942058), *V. cholerae* HE-09 (EGS58583) and *V. cholerae* HC-49A2 (EGR05582.The BLAST results are represented in Table 4.6.The sequence homology of MCCB 123 LasB protease which shares with other proteases has been indicated in Fig.4.11.

Table 4.1 Purification profile of LasB protease produced by *Pseudomonas aeruginosa* MCCB 123

Purification step	PA (Uml^{-1})	EA ($U\ ml^{-1}$)	Total Protein (mg)	Specific PA (U/mg)	Specific EA (U/mg)	Purification fold (PA)	Purification fold (EA)
Culture filtrate	24754.17	654.14	8.55	2895.22	76.50	0	0
$(NH4)_2SO_4$ Precipitation	22737.5	574.14	4.09	5559.29	140.37	1.92	1.83
DEAE-cellulose chromatography	9693.75	295.23	0.69	14048.91	427.86	4.85	5.59

PA: Protease activity EA: Elastase activity

Table 4.2 Effect of metal ions on Protease and Elastase activity

Reagents	% relative activity	
	Protease	Elastase
Control	100	100
$CaCl_2$	115	75
$BaCl_2$	109	76
$MgCl_2$	102	48
$MnCl_2$	42	68
$CoCl_2$	29	4
Pb $(NO3)_2$	7	0
$ZnCl_2$	2	0

Table 4.3 Effect of inhibitors and oxidizing agents on protease and elastase activity of LasB protease

Reagents	Concentration	% relative activity	
		Protease	Elastase
Control		100	100
PMSF (mM)	5	93	*
EDTA (mM)	5	45	0
1, 10 Phenanthroline (mM)	5	0	0
Iodo acetic acid (mM)	1	99	90
H_2O_2 (%)	0.1	95	0
Triton X-100 (%)	1	59	0
SDS (%)	0.5	30	0

*unable to measure

Table 4.4 Comparison of specific protease activity of MCCB 123 LasB protease with commercial protease Savinase® from *Bacillus* sp.

Specific protease activity	
MCCB 123 LasB protease from *Pseudomonas aeruginosa*	14048.91±26.55U/mg
Savinase® from *Bacillus* sp. (Sigma – Aldrich Co., Product no P3111)	3546.40±35.21U/mg

Table 4.5 BLAST results for LasB protease gene sequence of *P.aeruginosa* MCCB 123 obtained by BLAST analysis (www. ncbi.nlm.nih.gov)

Accession	Description	Max score	Total score	Query coverage	Max. identity
JN118955.1	Pseudomonas aeruginosa strain MCCB 123 elastase LasB precursor (lasB) gene, complete cds	2802	2802	99%	100%
AE004091.2	Pseudomonas aeruginosa PAO1, complete genome	2772	2772	98%	99%
M19472.1	P.aeruginosa elastase precursor (lasB) gene, complete cds	2772	2772	98%	99%
AB029328.1	Pseudomonas aeruginosa lasB gene for PST-01 protease (pseudolysin) precursor, complete cds	2761	2761	98%	99%
JF502075.1	Pseudomonas aeruginosa strain ATCC 27853 preproelastase gene, complete cds	2756	2756	97%	100%
M24531.1	P.aeruginosa elastase gene, complete cds	2745	2745	98%	99%
FM209186.1	Pseudomonas aeruginosa LESB58 complete genome sequence	2739	2739	98%	99%
EU265777.1	Pseudomonas aeruginosa strain ACCC I0647 elastase precursor (lasB) gene, complete cds	2739	2739	97%	99%
JF276397.1	Escherichia-Pseudomonas shuttle vector pCon2(3)-Kan, complete sequence	2728	5435	97%	99%
JF276396.1	Escherichia-Pseudomonas shuttle vector pCon2(3), complete sequence	2728	5435	97%	99%
JF276393.1	Escherichia-Pseudomonas shuttle vector pCon2(3)-Zeo, complete sequence	2728	5435	97%	99%
EU021222.1	Pseudomonas aeruginosa strain K organic solvent tolerant elastase gene, complete cds	2723	2723	98%	99%
JF276395.1	Expression vector pCon5, complete sequence	2706	2706	97%	99%
JF276394.1	Expression vector pCon4, complete sequence	2706	2706	97%	99%
FJ715960.1	Pseudomonas aeruginosa strain MTCC 7837 elastase precursor-like (lasB) gene, partial sequence	2656	2656	98%	98%
DQ350610.1	Pseudomonas aeruginosa PseA protease precursor (lasB) gene, complete cds	2639	2639	98%	98%
CP000438.1	Pseudomonas aeruginosa UCBPP-PA14, complete genome	2639	2639	98%	98%
DQ153386.1	Pseudomonas aeruginosa strain MN1 elastase LasB precursor (lasB) gene, complete cds	2639	2639	98%	98%

Table 4.6 Protein Sequences producing significant alignment to LasB protease of *Pseudomonas aeruginosa* MCCB 123 obtained by BLAST analysis (www. ncbi.nlm.nih.gov)

Accession	Description	Max. score	Total score	Query coverage
NP252413.1	elastase LasB [Pseudomonas aeruginosa PAO1]	1019	1019	100%
AAA25811.1	elastase precursor (EC 3.4.24.4) [Pseudomonas aeruginosa]	1018	1018	100%
AEA29834.1	organic solvent tolerant elastase [Escherichia-Pseudomonas shuttle vector pCon2 (3)-Zeo	1017	1017	100%
ZP04935737.1	elastase LasB [Pseudomonas aeruginosa 2192]	1014	1014	100%
ADM89079.1	elastase LasB precursor [Stenotrophomonas maltophilia]	1011	1011	100%
ABS59783.1	organic solvent tolerant elastase (Pseudomonas aeruginosa)	1010	1010	100%
YP789440.1	LasB [Pseudomonas aeruginosa UCBPP-PA14]	1009	1009	100%
ZP01367156.1	hypothetical protein PaerPA_01004307 [Pseudomonas aeruginosa PACS2]	991	991	97%
YP001346781.1	elastase LasB [Pseudomonas aeruginosa PA7]	978	978	100%
ADP00718.1	keratinase KP2 [Pseudomonas aeruginosa]	973	973	95%
NP899727.1	class 4 metalloprotease [Chromobacterium violaceum ATCC 12472]	614	614	98%
AAN78225.1	class 4 metalloprotease [Chromobacterium violaceum]	610	610	98%
YP004752195.1	Neutral protease [Collimonas fungivorans Ter331]	550	550	95%
BAA95457.1	protease [Aeromonas punctata]	521	521	98%
YP855393.1	elastase [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	516	516	98%
ZP08521632.1	elastase [Aeromonas caviae Ae398]	516	516	98%
ABG89854.1	metalloprotease [Aeromonas sobria]	516	516	98%
YP004394066.1	protease [Aeromonas veronii B565]	514	514	98%
BAA82875.1	pro-aminopeptidase protease [Aeromonas punctata]	502	502	98%
BAD22597.1	protease [Aeromonas veronii bv. Sobria]	493	493	84%
ZP01112982.1	Zinc metalloprotease (elastase) [Reinekea sp. MED297]	499	499	99%
ZP04403750.1	neutral protease precursor [Vibrio cholerae TMA 21]	493	493	89%
NP762896.1	Neutral protease [Vibrio vulnificus CMCP6]	491	491	98%
ZP04411813.1	neutral protease precursor [Vibrio cholerae TM 11079-80]	491	491	89%
EGR01640.1	hemagglutinin/proteinase [Vibrio cholerae HE39]	491	491	89%
ZP01980763.1	hemagglutinin/protease [Vibrio cholerae 623-39]	490	490	89%
ZP06048800.1	hemagglutinin/proteinase precursor (HA/protease) (Vibriolysin)	490	490	89%
ZP04959463.1	hemagglutinin/protease [Vibrio cholerae AM-19226]	490	490	89%
EGR10267.1	hemagglutinin/proteinase [Vibrio cholerae HE48]	490	490	89%
ZP04918414.1	hemagglutinin/protease [Vibrio cholerae V51]	490	490	89%
ACX48920.1	vibriolysin [Vibrio cholerae O1]	488	488	89%
BAI66359.1	metalloprotease [Vibrio vulnificus]	490	490	98%
ZP04416044.1	neutral protease precursor [Vibrio cholerae bv. albensis VL426]	489	489	89%
EGS56236.1	hemagglutinin/proteinase [Vibrio cholerae HC-02A1]	489	489	89%
YP004191592.1	vibriolysin extracellular zinc protease/pseudolysin extracellular zinc protease [Vibrio vulnificus MO6-24/O]	489	489	98%
ZP06942058.1	hemagglutinin/protease [Vibrio cholerae RC385]	489	489	89%
EGS58583.1	hemagglutinin/proteinase [Vibrio cholerae HE-09]	489	489	100%
EGR05582.1	hemagglutinin/proteinase [Vibrio cholerae HC-49A2]	489	489	89%

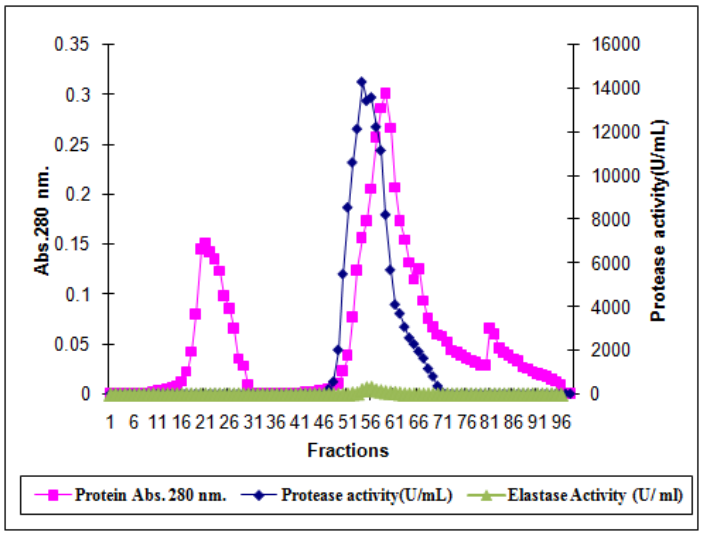


Fig.4.1 Elution profile of LasB protease through DEAE –cellulose C 16/40 column

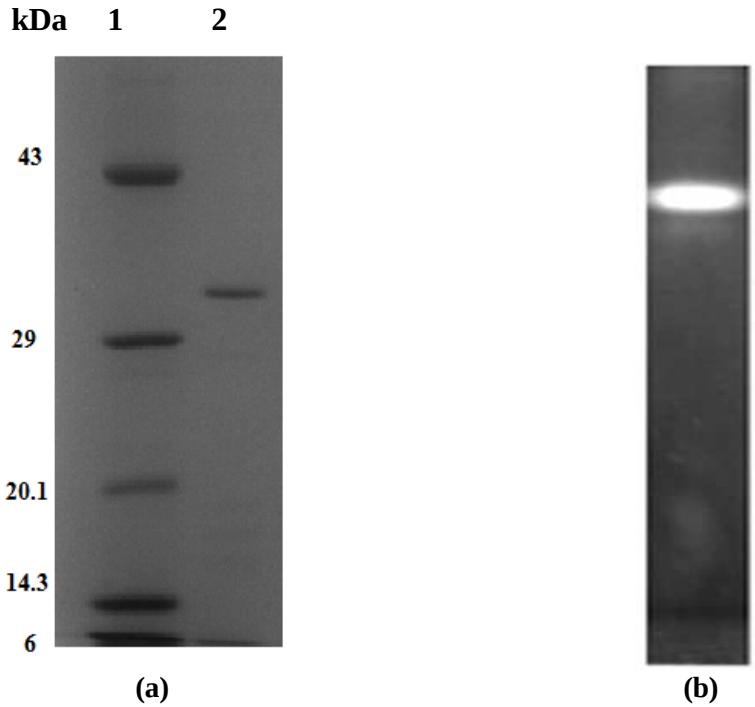


Fig.4.2 a) SDS-PAGE profile of purified LasB protease. Lane 1, molecular weight markers; (Ovalbumin, 43kDa; carbonic anhydrase, 29 kDa; soyabean trypsin inhibitor, 20.1 kDa; Lysozyme, 14.3 kDa; Aprotinin, 6.5 kDa; Insulin, 3kDa,) lane 2, 33 kDa LasB protease. b) Zymogram of LasB protease

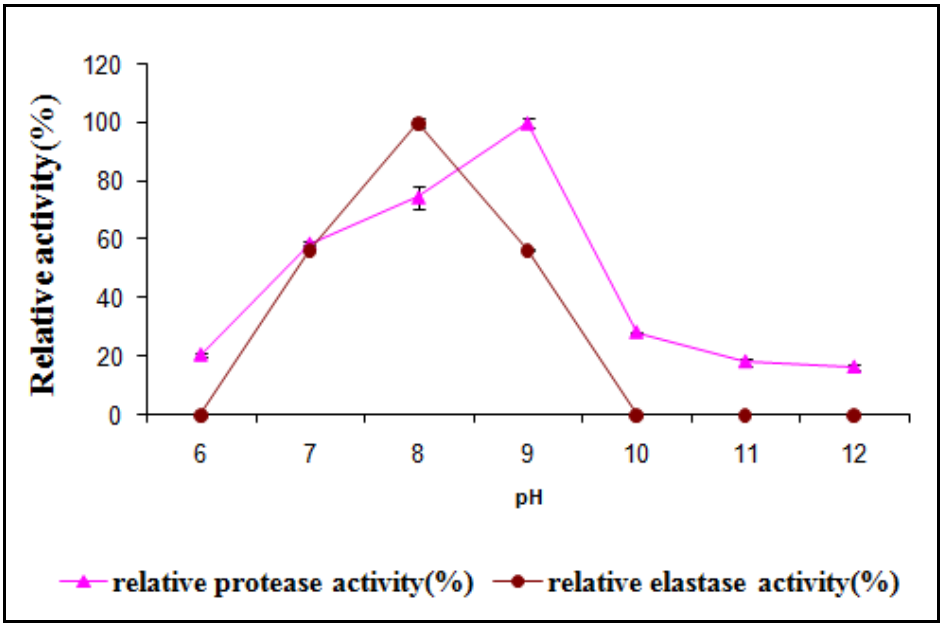


Fig. 4.3 Effect of pH on LasB protease and elastase activity

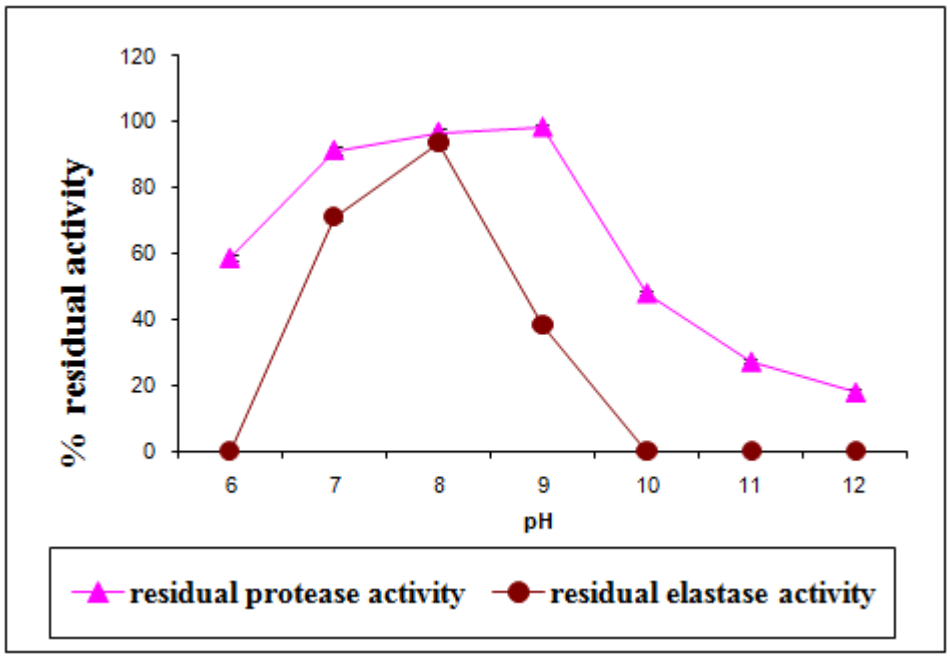


Fig. 4.4 Effect of pH on LasB protease and elastase stability

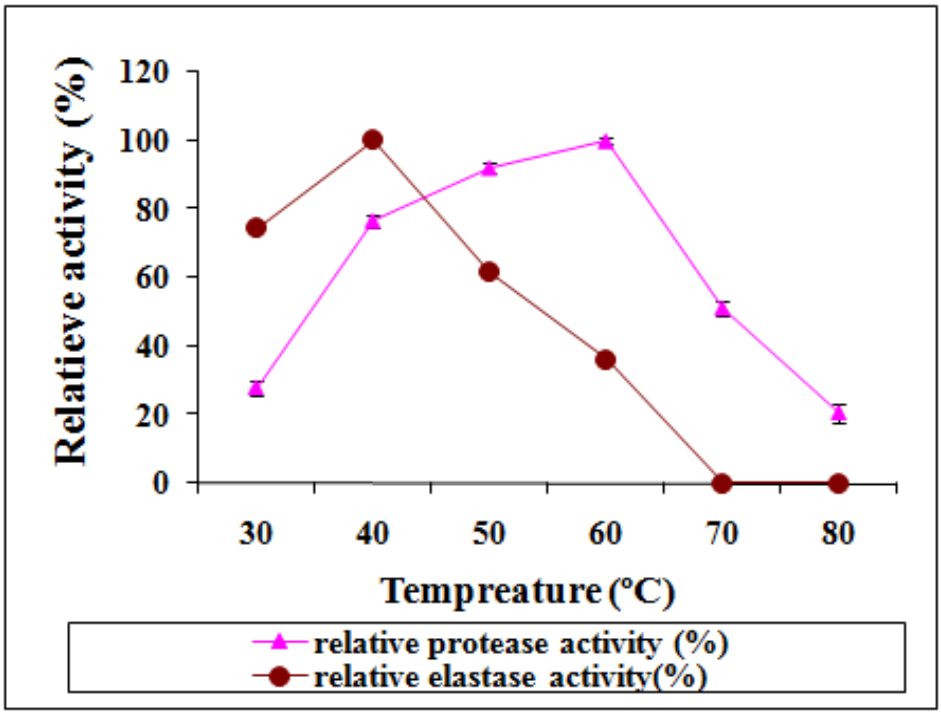


Fig. 4.5 Effect of temperature on LasB protease and elastase activity

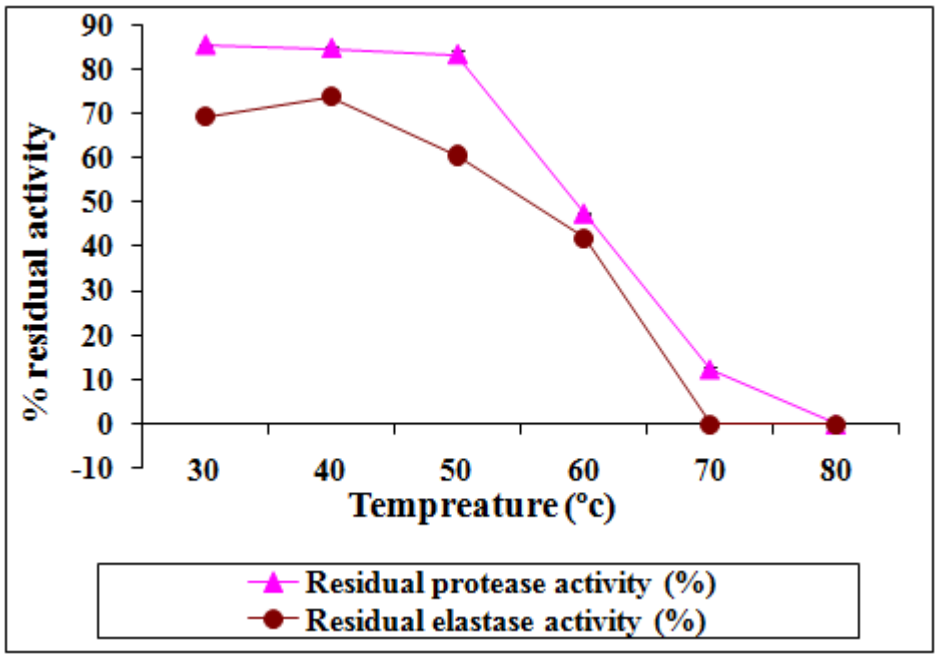
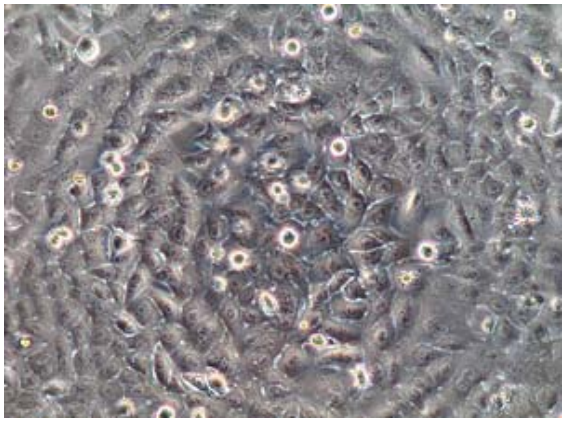
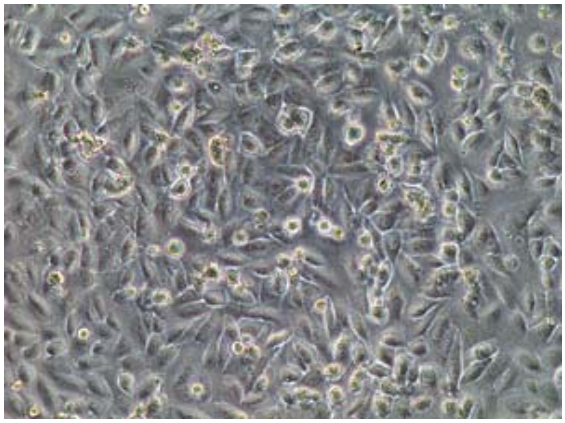


Fig. 4.6 Effect of temperature on LasB protease and elastase stability



(a)



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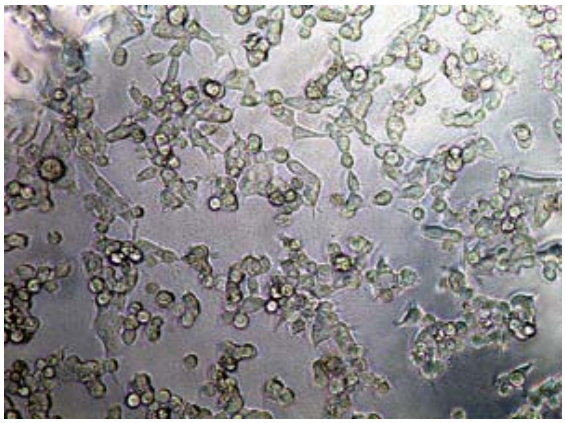

(c)

Fig. 4.7 Cytotoxic effects of purified *Pseudomonas aeruginosa* MCCB 123 LasB protease on Hep-2 cells. a) Control showing Hep2 monolayer, b) 25µg ml^{-1} protease, c) 50µg ml^{-1} protease showing cell rounding

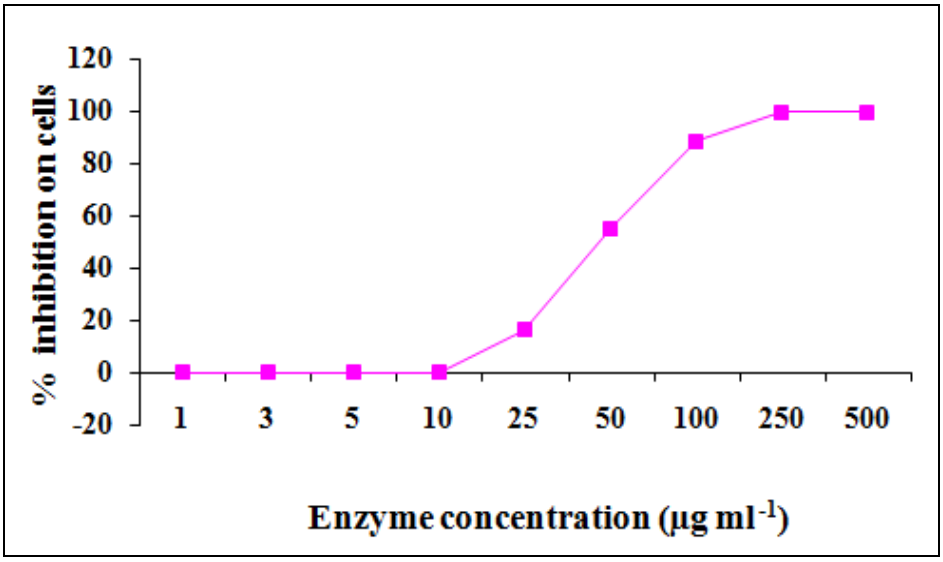

Fig.4.8 Sigmoid curve for cytotoxicity analysis of *Pseudomonas aeruginosa* MCCB 123 LasB protease

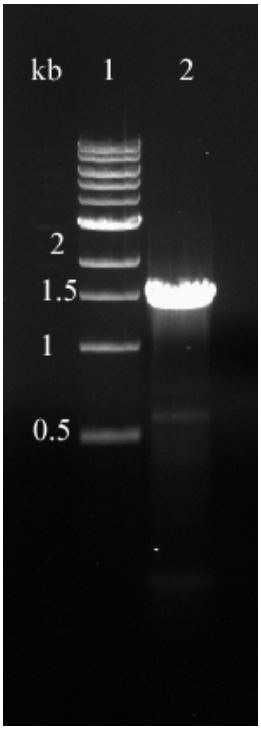

Fig.4.9 PCR amplification of LasB protease gene of *Pseudomonas aeruginosa* MCCB 123. Lane 1, 1 kb DNA ladder, lane 2, 1500 bp LasB protease gene

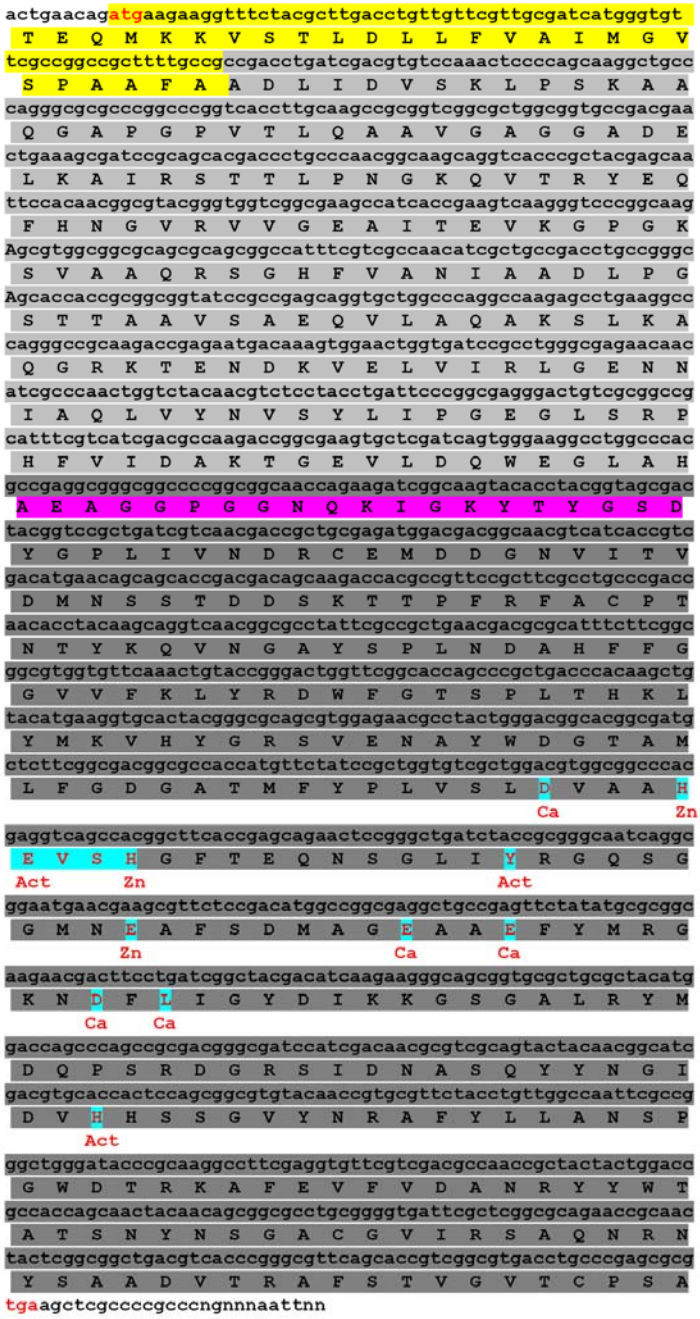

Fig. 4.10 Nucleotide sequence along with deduced aminoacid sequence of LasB protease gene of *Pseudomonas aeruginosa* MCCB 123. The putative starting residues of prepeptide (yellow shaded), propeptide (gray 25% shaded) and mature LasB protease (gray 50% shaded)are indicated. The active site residues Glu (E) and His (H) are represented. The HEXXH motif (H E V S H) which is the characteristics of M4 metallopeptidase family is also represented. The initation codon (atg) and the stop codon (tga) are also represented. The N-terminal sequence of the mature elastase is represented in pink shaded region. The underlined aminoacids with 'Ca', 'Zn' and 'Act' represents residues of calcium ligands, zinc ligands and active centre respectively.

Fig. 4.11 Comparison of deduced aminoacid sequence of MCCB 123 LasB with other proteases. The signature pattern of metalloprotease represented by HEVSH motif. The sequences were aligned using Bioedit and multiple alignment was done by CLUSTAL W. Numbers at right hand side correspond to the positions of the individual proteins. For pair wise comparison, similar aminoacids are shaded. Dashes indicate gaps introduced to optimize alignment. a) LasB protease precursor of *Pseudomonas aeruginosa* MCCB 123, b) elastase LasB of *Pseudomonas aeruginosa* PAO1(NP252413), c) hypothetical protein PaerPA [*Pseudomonas aeruginosa* PACS2] (ZP 01367156), d) elastase LasB precursor of *Stenotrophomonas maltophilia* (ADM89079), e) keratinase KP2 of *Pseudomonas aeruginosa* (ADP00718), f) class 4 metalloprotease of *Chromobacterium violaceum* ATCC 12472 (NP899727), g) Neutral protease of *Collimonas fungivorans* Ter331 (YP004752195), h) elastase of *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (YP855393), i) metalloprotease of *Aeromonas sobria* (ABG89854), j) Zinc metalloprotease (elastase) of *Reinekea* sp. MED297 (ZP01112982), k) neutral protease precursor of *Vibrio cholerae* TMA 21 (ZP04403750), l) hemagglutinin/proteinase of *Vibrio cholerae* HE39 (EGR01640), m) vibriolysin of *Vibrio cholerae* O1 (ACX48920).

```
a ---MKKVSTL DLLFVAIMGV SPAAFAADLI DVSKLP---S KAAQGAPGPV TLQAA----- 49
b ---MKKVSTL DLLFVAIMGV SPAAFAADLI DVSKLP---S KAAQGAPGPV TLQAA----- 49
c SPAAFAADLI DVSKLP---S KAAQGAPGPV TLQAA----- ---------- -------MGV 35
d ---MKKVSTL DLLFVAIMGV SPAAFAADLI DVSKLP---S KAAQGAPGPV TLQAA----- 48
e ---------- ---------- ------ADLI DVSKLP---S KAAQGAPGPV TLQAA----- 26
f MRKEQLMLRG -LVLSALAVF SSATMAAERI DLE------- --KQGKAQAN SASAF----- 45
g MKPVLRHVEG PLFLSLLLAA SSSAIAADRI DLESYTPQAA AAKQAGPGPI TAQAF----- 55
h -------MNK VYLAVVLACW GSAALAAEQV DVHQVA-GIQ GAP--SGAAG VS-------- 42
i -------MNK IYLAVALACW GSAALAAQEV EINVTANGIR AMTGLSAASG TL-------- 45
j --MHQRPSQN QNLLMTSDNY DNKDIPMKKL QYTLLVSGCL LASSALMAAD RVPATEAHLT 58
k --MKMIQRPL NWLVLAGAAT GFPLYAAQMV TIDDASMVEQ ALA----QQQ YS-------- 46
l --MKMIQRPL NWLVLAGAAT GFPLYAAQMV TIDDASMVEQ ALA----QQQ YS-------- 46
m ---------- ---------- ---------- ---------- -------QQQ YS--------  5

a --VGAGGADE LKAIRSTTLP NGKQVTRYEQ FHNGVRVVGE AITEVKGPG- -KSVAAQRSG 105
b --VGAGGADE LKAIRSTTLP NGKQVTRYEQ FHNGVRVVGE AITEVKGPG- -KSVAAQRSG 105
c --VGAGGADE LKAIRSTTLP NGKQVTRYEQ FHNGVRVVGE AITEVKGPG- -KSVAAQRSG  91
d --VGAGGADE LKAIRSTTLP NGKQVTRYEQ FHNGVRVVGE AITEVKGPG- -KSVAAQRSG 105
e --VGAGGADE LKAIRSTTLP NGKQVTRYEQ FHNGVRVVGE AITEVKGPG- -KSVAAQRSG  82
f --TGVS-QGD LKALRSTQFA SGKVVTRYQQ YYQGVPVWGE GVVEEKPAAA LKSVQGKLSG 109
g --LGLT-ADE LKPLRSQNYA NGKVVTRYQQ YFQGVPLWDQ AIVEQRSAS- --QAQPAMSG 109
h ---ALAGDGE FRQVRAVKLP NGQQRVRYEQ TWHGIPVWGQ VLVAEQSLG- --GQISQVSG  96
i ---QLE-DGE FRQVRVVKLP NGQQRIRYEQ VWNGIPVMGQ VVVADKSLN- --GQLQQASG  98
j QGVMSGNATS VQTIKSRTLA SGVTVEKYQQ TYQGVPVWGE SITRR-SGG- --TFAPTVSG 114
k ---MMPAASG FKAVNTVQLP NGKVKVRYQQ MYNGVPVYGT AVVATESSK- --G-ISQVYG  99
l ---MMPAASG FKAVNTVQLP NGKVKVRYQQ MYNGVPVYGT AVVATESSK- --G-ISQVYG  99
m ---MMPAASG FKAVNTVQLP NGKVKVRYQQ MYNGVPVYGT VVVATESSK- --G-ISQVYG  99

a HFVANIAADL PGSTTAA--- ------VSAE QVLAQAKSLK AQGRKTE--- --NDKVELVI 151
b HFVANIAADL PGSTTAA--- ------VSAE QVLAQAKSLK AQGRKTE--- --NDKVELVI 151
c HFVANIAADL PGSTTAA--- ------VSAE QVLAQAKSLK AQGRKTE--- --NDKVELVI 157
d HFVANIAADL PGSTTAA--- ------VSAE QVLAQAKSLK AQGRKTE--- --NDKVELVI 128
e HFVANIAADL PGSTTAA--- ------VSAE QVLAQAKSLK AQGRKTE--- --NDKVELVI 147
f HYIAGIQADL A-SAKPT--- ------LSSA QVLSQAKALK ANGNPTY--- --NDKAELVV 153
g SLIRNIENDL P-SAKPV--- ------YAAT DVLLQAKSIA R-AAVTE--- --NEQAKLYV 137
h QILRQIDADV ASPTA----- -----ALSPA DAASKAR--A GAKGS----- --NERVKLFV 139
i RMLRQIDQDV ASPAA----- -----TLSPQ DAASKAK--A GSKGS----- --NEQVKLYV 174
j EFVTEIESDL PQATPRFDGK AILNLAMEQH TLMTQGAIPS KEALQKMLLS AENKQQKLWV 149
k QMAQQLEADL PTVTP----- -----DIESQ QAIALAVSHF GEQHAGESLP VENESVQLMV 149
l QMAQQLEADL PTVTP----- -----DIESQ QAIALAVSHF GEQHAGESLP VENESVQLMV 149
m QMAQQLEADL PTVTP----- -----DIESQ QAIALAVSHF GEQHAGESLP VENESVQLMV 145
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a RLGENNIAQL VYNVSYLIP- GEGLSRPHFV IDAKTGEVLD QWEGLAHAEA G—GGPGGNQK  208
b RLGENNIAQL VYNVSYLIP- GEGLSRPHFV IDAKTGEVLD QWEGLAHAEA G—GGPGGNQK  208
c RLGENNIAQL VYNVSYLIP- GEGLSRPHFV IDAKTGEVLD QWEGLAHAEA G—GGPGGNQK  194
d RLGENNIAQL VYNVSYLIP- GEGLSRPHFV IDAKTGEVLD QWEGLAHAEA G—GGPGGNQK  208
e RLGENNIAQL VYNVSYLIP- GEGLSRPHFV IDAKTGEVLD QWEGLAHAEA G—GGPGGNQK  185
f RLNERNVAQL VYLVSFVVD- GKEPSRPHLI IDANSGQVLK QWEGLNHAEA N—GGPGGNAK  204
g QLGSNNVAQL IYVVSFVNKS AAKPSRPYFI IDANTGAILK KWEGITHYEA S—GGPGGNAK  211
h MQDEAGQARL VYLVSWLAA- SDEPSRPFVV IDAQNGSELK RWEGINHKDA T—GGPGGNVK  194
i MQDENGLARL VYQVSFIAE- GDKPSRPFVI LDAQSGEELK RWEGINHKDA T—GGPGGNIK  196
j RLNDNNEAQL VYLVSWVDY- GVEPTRPHYF IDAMTGEVLD HWDGLAHQDA T—GGPGGNEK  231
k RLDDNQQAQL VYLVDFFVA- SETPSRPFYF ISAETGEVLD QWDGINHAQA TGTGPGGNQK  208
l RLDDNQQAQL VYLVDFFVA- SETPSRPFYF ISAETGEVLD QWDGINHAQA TGTGPGGNQK  208
m RLDDNQQAQL VYLVDFFVA- SETPSRPFYF ISAETGEVLD QWDGINHAQA TGTGPGGNQK  167

a IGKYTY--GS DYGP-LIVND R---CEMDDG NVITVDMNSS TDDSKTTPFR FACPT----N 258
b IGKYTY--GS DYGP-LIVND R---CEMDDG NVITVDMNSS TDDSKTTPFR FACPT----N 258
c IGKYTY--GS DYGP-LIVND R---CEMDDG NVITVDMNSS TDDSKTTPFR FACPT----N 244
d IGKYTY--GS DYGP-LIVND R---CEMDDG NVITVDMNGS TNDSKTTPFR FACPT----N 258
e IGKYTY--GS DYGP-LIVND R---CEMDDG NVITVDMNSS TDDSKTTPFR FACPT----N 235
f TGQYYY--GK DYGP-LIVTS D---CKMDSG NVATVNLNGG T--SGSTPYK FACPT----N 252
g TGQYEYQPGG KYGP-LIVDS N---CNMITT NVITVDLQNG T--SGTTPFH FTCPR----N 261
h TGKYFY--GA DFGP-LLVDD N---CRMTSP NVDTLNMNHA T--TGGAIHQ FTCPE----N 242
i TGKYFY--GT DFGP-LIVDD N---CRMTSP NVDTINLNHA T--SGGAIHQ FTCPE----N 244
j TGQYIY--GQ DFPA-LQVDS N---CRMNTS NVETVDMQNR T--SGGSVFQ FTCPE----N 279
k TGRYEY--GS NGLPGFTIDK TGTTCTMNNS AVKTVNLNSG T--SGSTAFS YACNNSTNYN 264
l TGRYEY--GS NGLPGFTIDK TGTTCTMNNS AVKTVNLNGG T--SGSTAFS YACNNSTNYN 264
m TGRYEY--GS NGLPGFTIDK TGTTCTMNNS AVKTVNLNGG T--SGSTAFS YACNNSTNYN 223

a TYKQVNGAYS PLNDAHFFGG VVFKLYRDWF GTSPLTHKLY MKVHYGRSVE NAYWDGTAML 318
b TYKQVNGAYS PLNDAHFFGG VVFKLYRDWF GTSPLTHKLY MKVHYGRSVE NAYWDGTAML 318
c TYKQVNGAYS PLNDAHFFGG VVFKLYRDWF GTSPLTHKLY MKVHYGRSVE NAYWDGTAML 304
d TYKQVNGAYS PLNDAHFFGG VVFNLYRDWF GTSPLTHKLY MKVHYGRSVE NAYWDGTAML 318
e TYKQVNGAYS PLNDAHFFGG VVFKLYRDWF GTSPLTHKLY MKVHYGRSVE NAYWDGTAML 295
f TYKAINGAYS PLNDAHYFGN VVFNLYKDWF NLKPITQKLL MKVHYSRNYE NAFWDGTAMT 312
g TYKAVNGAFS PLNDAHYFGN VVFNMYRDWL SLRPISQTLY MKVHYGNSYE NAFWDGSAMN 321
h TVKEINGAYS PLNDAHYFGN VVFDMYRNWY NTAPLTFKLK MRVHYSRNYE NAFWDGSQMT 302
i RVKEINGAYS PLNDAHYFGN VVFDMYRNWY NTAPLTFKLK MRVHYSKNYE NAFWDGSQMT 304
j TNRYTNGAYS PLNDAHFFGN VVFNMFDDWY NTAPISQKLR MRVHYGNNYE NAFWDGSQMT 339
k SVKTVNGAYS PLNDAHFFGK VVFDMYQQWL NTSPLTFQLT MRVHYGNNYE NAFWDGRAMT 324
l SVKTVNGAYS PLNDAHFFGK VVFDMYQQWL NTSPLTFQLT MRVHYGNNYE NAFWDGRAMT 324
m SVKTVNGAYS PLNDAHFFGK VVFDMYQQWL NTSPLTFQLT MRVHYGNNYE NAFWDGRAMT 283

a FGDGATMFYP LVSLDVAAHE VSHGFTEQNS GLIYRGQSGG MNEAFSDMAG EAAEFYMRGK 378
b FGDGATMFYP LVSLDVAAHE VSHGFTEQNS GLIYRGQSGG MNEAFSDMAG EAAEFYMRGK 478
c FGDGATMFYP LVSLDVAAHE VSHGFTEQNS GLIYRGQSGG MNEAFSDMAG EAAEFYMRGK 364
d FGDGATMFYP LVSLDVAAHE VSHGFTEQNS GLIYRGQSGG MNEAFSDMAG EAAEFYMRGK 378
e FGDGATMFYP LVSLDVAAHE VSHGFTEQNS GLIYRGQSGG MNEAFSDMAG EAAEFYMRGK 355
f FGDGYNTFYP LVSLDVSAHE VSHGFTEQNS GLVYSGQSGG INEAFSDMAG EAAEYYMKGK 372
g FGDGASTFYP LVALDVSGHE VSHGFTEQNS GLVYSGMSGG MNEAFSDMAG EASENYMKGS 381
h FGDGATTFYP LVSLDVAAHE VSHGFTEQNS GLVYSGQSGG INEAFSDMAG EAAENFMKGS 362
i FGDGATTFHP LVSLDVAAHE VSHGFTEQNS GLIYSGQSGG INEAFSDMAG EAAEFYMKGS 354
j FGDGASRFYP LVSLDVSAHE VSHGFTEQNS GLVYQNQSGG INEAFSDMSG EAAEFYMKGS 399
k FGDGYTRFYP LVDINVSAHE VSHGFTEQNS GLVYRDMSGG INEAFSDIAG EAAEYFMRGN 384
l FGDGYTRFYP LVDINVSAHE VSHGFTEQNS GLVYRDMSGG INEAFSDIAG EAAEYFMRGN 384
m FGDGYTRFYP LVDINVSAHE VSHGFTEQNS GLVYRDMSGG INEAFSDIAG EAAEYFMRGN 343

a NDFLIGYDIK KGSGALRYMD QPSRDGRSID NASQYYNGID VHHSSGVYNR AFYLLANSPG 438
b NDFLIGYDIK KGSGALRYMD QPSRDGRSID NASQYYNGID VHHSSGVYNR AFYLLANSPG 438
c NDFLIGYDIK KGSGALRYMD QPSRDGRSID NASQYYNGID VHHSSGVYNR AFYLLANSPG 424
d NDFLIGYDIK KGSGALRYMD QPSRDGRSID NASQYYNGID VHHSSGVYNR AFYLLANSPG 438
e NDFLIGYDIK KGSGALRYMD QPSRDGRSID NASQYYNGID VHHSSGVYNR AFYLLANSPG 415
f NDFLVGAEIF KKTGALRYFA DPTKDGQSIG NAKDYYNGLD VHYSSGVYNK AFYLIATSPN 432
g NDFLVGTEIF KATGALRYMA NPTQDGRSID NAKNYTSSLD VHYSSGVYNK AFYLLATTAG 441
h NDWLVGAQIF KGNGSLRYFE DPTRDGSSIG HASDYYDGID VHHSSGVYNR AFYLLANTSG 422
i NDWLVGAQIF KGNGSLRYFE DPTRDGRSIG HAADYQEGMD VHHSSGVYNR AFYLLATSSG 424
j NDWKVGADIF KGSGSLRYMD DPTKDGRSIG HASDYTSGMD VHYSSGVFNR AFYLIATASG 459
k VDWIVGADIF KSSGGLRYFD QPSRDGRSID HASQYYSGID VHHSSGVFNR AFYLLANKSG 444
l VDWIVGADIF KSSGGLRYFD QPSRDGRSID HASQYYSGID VHHSSGVFNR AFYLLANKSG 444
m VDWIVGADIF KSSGGLRYFD QPSRDGRSID HASQYYSGID VHHSSGVFNR AFYLLANKSG 403
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a WDTRKAFEVF VDANRYYWTA TSNYNSGACG VIRSAQNRNY SAADVTRAFS TVGVT---CP 495
b WDTRKAFEVF VDANRYYWTA TSNYNSGACG VIRSAQNRNY SAADVTRAFS TVGVT---CP 495
c WDTRKAFEVF VDANRYYWTA TSNYNSGACG VIRSAQNRNY SAADVTRAFS TVGVT---CP 481
d WDTRKAFEVF VDANRYYWTA TSNYNSGACG VISSAQNRNY SAADVTRAFS TVGVT---CP 495
e WDTRKAFEVF VDANRYYWTA TSNYNSGACG VIRSAQNRNY SAADVTRAFS TVGVT---CP 472
f WNTRKAFEVF VDANRLYWNA NATYNSAACG VVKAADARGY NSADVTKAFA AVGVT---CQ 489
g WSTRKAFEVM ADANHLYWTA NSTFNQGACG VEKAATSRGY VVADVTAAFK AVGVS---CS 498
h WNTRKAFEVF VLANRLYWGA NTTFDQGACG VTKAATDLGY SVTDVAAAFT TVGVN-ASCG 481
i WNTRKAFEVF LLANRLYWGA NTNYQQGACG VSRATADLGY SQADVARAFQ TVGVD-ASCG 483
j WDTRKAFDVF VLANQVYWNQ NTNYIDGACG ALSAAGDLNY DTNVVVSAFN TVGVSTSSCS 519
k WNVRKGFEVF AVANQLYWTP NSTFDQGGCG VVKAAQDLNY NTADVVAAFN TVGVN-ASCG 503
l WNVRKGFEVF AVANQLYWTP NSTFDQGGCG VVKAAQDLNY NTADVVAAFN TVGVN-ASCG 503
m WNVRKGFEVF AVANQLYWTP NSTFDQGGCG VVKAAQDLNY NTADVVAAFN TVGVN-ASCG 462

a SA-------- ---------- ---------- ---------- ---------- ---------- 497
b SAL------- ---------- ---------- ---------- ---------- ---------- 498
c SAL------- ---------- ---------- ---------- ---------- ---------- 484
d SAL------- ---------- ---------- ---------- ---------- ---------- 498
e SAL------- ---------- ---------- ---------- ---------- ---------- 475
f ---------- ---------- ---------- ---------- ---------- ----------
g TSSGNV---L VKGVPVTGIS LATGASNVYS ITVPAGARNL SFQLSGGSGD GDIYVKWNAT 489
h GTTPQPGSVL QNGVPVSGLS AAKGGKLNFT IDVPAGKSQL VIASSGGTGD ADLYVKFGSV 555
i TTPPPSDNVL QNGVPVSNLA ASKGGKLNYT LNLPAGTRSL QISSSGGTGD ADLYVRFGSA 541
j GGGGNTVTEL DNGVPVTGLS GPSGSEAMFK LDVLAGASSV SVVMNGGSGD ADLYVRFGAQ 543
k TTPPPVGKVL EKGKPITGLS GSRGGEDFYT FTVTN-SGSV VVSISGGTGD ADLYVKAGSK 579
l TTPPPVGKVL EKGKPITGLS GSRGGEDFYT FTVTN-SGSV VVSISGGTGD ADLYVKAGSK 562
m TTPPPVGKVL EKGKPITGLS GSRGGEDFYT FTVTN-SGSV VVSISGGTGD ADLYVKAGSK 562
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4.4 Discussion

A potent LasB protease (elastase) with very high protease and elastase activity was purified from *P. aeruginosa* MCCB123. The enzyme was purified with 4.85 fold increase in specific protease activity and 5.59 fold increase in specific elastase activity. The molecular mass of the MCCB 123 LasB protease was found to be 33 kDa and is in close agreement with the previous reports (Fukushima et al., 1989; Olson and Ohman, 1992; Shastry and Prasad, 2002; Gupta et al., 2005a; Jellouli et al., 2008; Lin et al., 2009). When the molecular mass of the MCCB 123 LasB protease was analyzed using zymogram, approximately 96 kDa was obtained, while in SDS-PAGE it showed a protein of 33kDa, a considerable variation. There was the report of a complex elastase from P. *aeruginosa* which had 103 kDa in native conformation, but in SDS-PAGE it had a molecular weight of 33 kDa indicating that it was a complex protein (Kamath et al., 1998). Accordingly, based on the above literature it could be concluded that MCCB 123 elastase was a complex of three monomers. Schmidtchen et al. (2003) reported an elastase that appeared

as approximately 150 kDa component on zymogram, which when boiled on SDS-PAGE sample buffer migrated as approximately 35 kDa protein, which indicated that the native elastase migrated as a multimeric of approximately 150 kDa on zymogram, and that on denturation by boiling generated monomeric elasatase.

The MCCB 123 LasB protease recorded maximum protease activity at pH 9.0 which entitles it to be classified under the category of alkaline protease. Mean while, most of the LasB protease/elastase of *P.aeruginosa* were reported to have their optimum hydrolysis of casein at pH 8 (Shastry and Prasad, 2002; Gupta et al., 2005a; Jellouli et al., 2008). Thus, it appears to be different from other reported elastases with respect to its pH optimum of its protease activity. The optimum pH for elastase activity was found to be pH 8.0 and is in agreement with Morihara et al. (1965). The MCCB 123 LasB protease showed optimal activity at 60°C and showed stability up to 60°C retaining 47% of protease activity. A similar optimum temperature for the action of LasB protease/elastase has been reported from other strains of *P.aeruginosa* (Shastry and Prasad, 2002; Gupta et al., 2005a; Jellouli et al., 2008), as well as from genus of *Bacillus* (Adinarayana et al., 2003; Almas et al., 2009; Tanskul et al., 2009; Deng et al., 2010).Exceptionally high temperature optima of 75°C has been reported for alkaline protease from *Bacillus laterosporus*-AK1 (Arulmani et al., 2007).

The optimum activity of MCCB 123 LasB protease in the alkaline pH is a very important characteristic for its use as a laundry ingredient, in leather processing, and other industrial processes that are carried out in the alkaline pH range. The protease exhibited good stability up to pH 10. Alkaline proteases are particularly important, because they are active and stable under harsh conditions such as high temperatures (50-60°C), high pH and in the presence of surfactants or oxidizing agents (Chen et al., 2006).

MCCB 123 LasB protease was active over a wide range of temperature from 30 to 80°C with its optimum at 60°C and at 30°C it was found to be well active. This property could be of great advantage in detergent industry, which is now looking for alkaline proteases that works well under ambient temperature (Maurer, 2004), and is a pre-requisite to maintain fabric quality and also for reducing the energy demand (Venugopal and Saramma, 2006).

The temperature stability profile of protease activity revealed that the enzyme was highly stable from 30 to 50 °C retaining greater than 80% of its residual activity. This high degree of temperature stability of enzyme can be utilized in the areas where proteases is exposed to these temperature conditions for long durations such as in the recovery of silver from x-ray films, and in detergency. However, it was found that MCCB 123 LasB protease was found to be less stable (retained only 46% protease activity) when preincubated at its optimum temperature for a longer period of 1 h. The proteases from *Salinivibrio* sp., *V. flivilis* and *Bacillus* strain SAL1 too have been found to be unstable at their optimum temperatures for action (Karbalaei-Heidari et al., 2007 a,b; Wang et al., 2007a, Almas et al., 2009). Even alkaline protease used in commercial detergents tends to get inactivated on extended exposures to temperature of 60°C or more. The denaturation, followed by inactivation of the enzyme due to prolonged exposure at high temperature is responsible for this drop in activity.

Enzyme inhibition studies primarily give an insight into the nature of the enzyme, its cofactor requirements and the nature of the active centre (Sigma and Mooser, 1975). In the present study, both protease and elastase activities of MCCB 123 LasB protease were completely inhibited by 1,10Phenanthroline (5mM), the zinc specific chelator and partially inhibited (55 % inhibition) by EDTA (5 mM). Both are known inhibitors

of metallo proteases suggesting the enzyme to be a metalloprotease. The elastase activity of LasB protease *P.aeruginosa* is reported to be inhibited by these chemicals (Morihara et al., 1965).

The metal ions such as Ba^{2+}, Ca^{2+} and Mg^{2+} had a slight increasing effect in the MCCB 123 LasB protease activity, while these ions were found to have an inhibitory effect on the protease activity in other strains of *P. aeruginosa* (Shastry and Prasad, 2002; Gupta et al., 2005b; Jellouli et al., 2008). Metal ions such as Co^{2+}, Pb^{2+} and Zn^{2+} had an inhibitory effect on both protease and elastase activities. The inhibitory effect of metal ions on alkaline protease activity is well documented (Johnvesly et al., 2002; Mei and Jiang, 2005; Venugopal and Saramma, 2006). The inhibitory potential of Zn^{2+} indicated that the protease is most likely a zinc metalloprotease. High concentrations of zinc inhibits metalloprotease (Teo et al., 2003) by the formation of zinc monohydroxide that bridges the catalytic zinc ion to side chain of the active site of the enzyme (Larsen and Auld, 1991). The enzyme exhibited a reasonable degree of stability in protease activity in presence of strong ionic surfactant SDS (retained 30% activity in 0.5% SDS). Stability of the protease in presence of SDS is advantageous, since most other proteases generally are inhibited by SDS (Tremacoldi et al., 2007). Combined effects such as reduction in the hydrophobic interactions and the direct interactions with the protein molecule are believed to be the cause for the inhibition by SDS (Creighton, 1989). The MCCB 123 LasB protease exhibited good degree of stability towardsH_2O_2, an oxidizing agent retaining 95% activity in presence of 0.1 % H_2O_2.

Stability of protease activity in presence of alkaline pH, detergents, oxidizing agents and enhancement of activity in presence of metal ions like calcium and magnesium make it an ideal choice in detergents for hard water washes. The major application of alkaline

protease is in detergency, and it is always desirable for the enzyme to be stable in presence of various detergent ingredients such as surfactants, bleaches. Alkaline proteases useful for detergent applications are mostly active in the pH range from 8-12 and temperatures between 50 and 70°C (Gupta et al., 2002b). The optimum pH of MCCB 123 LasB protease for protease activity was found to be alkaline 9.0 and the protease was found to be thermostable with an optimum of 60°C. Thus protease was found to be alkaline and thermostable and could find application in detergency. Activity of MCCB 123 LasB protease was compared with commercial proteases Savinase® from *Bacillus* sp. It was found that the specific protease activity of purified MCCB 123 LasB protease was 3.96 fold greater when compared to Savinase® suggesting its superior quality in industrial applications and the possible economic viability.

The cytotoxicity of MCCB 123 LasB protease on Hep-2 cells was found to increase with the concentration of protease indicating that the degree of toxicity was dose dependent. Cytotoxic effect such as cell rounding and cell death at higher concentrations was observed. Elastase of *P. aeruginosa* has shown to be relatively non-toxic with an LD_{50} for mice in the range of 60-400µg depending upon the root of inoculation (Galloway, 1991). Alkaline protease and elastase of *P. aeruginosa* at 50 µg ml^{-1} and 25µg ml^{-1} respectively is reported to cause toxicity in NK cell (Human Natural killer Cell) and the neutrophil studies indicated that proteases acts on the cell surface by proteolytic cleavage of the receptors involved in the cell functions (Pedersen and Kharazmi, 1987). However, Stepińska et al. (2010) noted that there was no direct influence of protease with elastase activity on the cytotoxic or invasive phenotypes of *P. aeruginosa* upon infection of both RAW 264.7 and PME cells in vitro.

The 1500 bp LasB protease gene of *P. aeruginosa* MCCB 123 was sequenced. The nucleotide sequence of MCCB 123 LasB protease gene

was compared with other LasB protease sequences available in the GenBank. BLAST analysis (www.ncbi.nlm.nih.gov) of the nucleotide sequence revealed that it shared 98% similarity to LasB gene/elastase gene of *P. aeruginosa* (Accession nos.M19472, AB029328, M24531, EU021222, DQ350610, DQ153386, DQ150629) and 97% similarity to LasB gene of *P. aeruginosa* (Accession no. EU265777) and prepro elastase gene of *P. aeruginosa* (Accession no. JF502075).The BLAST analysis (www.ncbi.nlm.nih.gov) indicated that the enzyme belonged to M4 neutral protease GluZincin superfamily. The deduced amino acid sequence showed that MCCB 123 LasB protease is synthesized as preproenzyme of 497 amino acids. The N-terminal signal peptide consists of 23 residues bordered with the signal peptidase recognition site Ala-Phe-Ala-Ala, a group of strongly hydrophobic amino acids (Watson, 1984). Belonging to the signal sequence, the pro-sequence consisting of 174 amino acids has to be cleaved by autoproteolytic processing in the periplasm (Kessler et al., 1992; Jellouli et al., 2008). The propeptide sequence of MCCB 123 LasB protease also consists of 174 aminoacids. The mature LasB protease consists of 300 aminoacids. The typical metalloendopeptidases consensus zinc-binding sequence HEXXH and the catalytic residues in the active site are conserved in the MCCB 123 LasB gene. The aminoacid sequence of the MCCB 123 LasB protease agrees well with that reported by Jellouli et al. (2008).The N-terminal sequence (A E A G G P G G N Q K I G K Y T Y G S D) of the mature LasB protease of *Pseudomonas aeruginosa* MCCB 123 is the same as that described by previous workers (Bever and Iglewski, 1988; Ogino et al., 2000 b; Jellouli et al., 2008). The aminoacid representing the calcium ligands, zinc ligands and active centre is identified in the sequence . The deduced aminoacid sequence of MCCB 123 LasB protease showed homology to LasB protease gene of *P. aeruginosa.* It also exhibited sequence homology hypothetical protein of *P. aeruginosa,* elastase LasB

precursor of *Stenotrophomonas maltophilia*, keratinase of *P. aeruginosa,* class 4 metalloprotease of *Chromobacterium violaceum,* Neutral protease of *Collimonas fungivorans* Ter331 (YP004752195), elastase of *Aeromonas hydrophila* subsp. *hydrophila*, metalloprotease of *Aeromonas sobria*, Zinc metalloprotease (elastase) of *Reinekea* sp. MED29, neutral protease precursor of *Vibrio cholerae* TMA 21 , hemagglutinin/proteinase of *Vibrio cholera* and vibriolysin of *Vibrio cholera*.

Metallopeptidases are often recognized by the presence of short conserved signature sequences containing histidine and glutamate residues. Gluzincins, have the HEXXH motif and a glutamic acid as the third zinc ligand. The metzincins are characterized by HEXXH motif, a histidine as the third zinc ligand .The inverzincin is characterized by HXXEH motif; the HXXE defines the carboxypeptidase family (Hooper, 1994) and HXH motif is the characteristic of β-lytic endopeptidase family (Hooper, 1994; Rawlings and Barrett, 1995; Gustin et al., 1996; Spencer et al., 2010). The zinc dependent metalloproteinase has the characteristic HEXXH zinc binding motif (Barett, 1995; Kessler et al., 1997).

Metalloproteases are enzymes containing the His-Glu-Xaa-Xaa-His (HEXXH) motif to form a part of the site for binding of the metal (Rao et al., 1998). The deduced amino acid sequence of MCCB 123 LasB protease includes a zinc metalloprotease HEXXH consensus motif, which is HEVSH (His-Glu-Tyr-Thr-His), which suggests it as a zinc dependent metalloproteinase. The critical role of the amino acid structure HEXXH with bound zinc in catalysis was previously described in mammalian and bacterial zinc metalloprotease family (Vallee and Auld, 1990).This motif is important to transfer electron with zinc, for the hydrolysis of peptide bonds (Li et al., 1998). In general classification of zinc proteases, five groups are present, three out of these five groups have the HEXXH motif (Lipscomb and Strater, 1996).The HEXXH motif and the surrounding

sequences of MCCB 123 LasB agree well with that reported by previous workers (Bever and Iglewski, 1988; Ogino et al., 2000b; Jaoudai et al., 2008). The enzyme was classified as a metalloprotease based on its inhibitory action on its protease and elastase activities by metal chelators. Furthermore, the inhibitory action of Zn^{2+} also proved the protease as a zinc metalloprotease. High concentrations of zinc inhibits metalloprotease (Teo et al., 2003) by the formation of zinc monohydroxide that bridges the catalytic zinc ion to side chain of the active site of the enzyme (Larsen and Auld, 1991). Therefore, the classification based on the deduced amino acid sequence demonstrated that the MCCB 123 LasB protease is a zinc dependent metalloendopeptidase belonging to M4 neutral protease GluZincin superfamily. The M4 peptidase family (metalloprotease) is also known as thermolysin family. Pseudolysin, Aerolysin and Bacillolysin are also included in this family. The family contains secreted eubacterial endopeptidases from Gram -positive and Gram- negative sources. All the members of this compressive family are produced as pre-pro-proteins. The mature enzymes are of moderate size around 35 kDa. These proteases contain the typical HEXXH motif, which requires Zn^{2+} ions for their activity and contains multiple Ca^{2+} ions for stability. All enzymes are optimally active under neutral pH. The findings in this study seem to agree with all characteristic of M4 peptidase family expect with regard to optimum pH. Here in this study, the enzyme was identified as an alkaline protease and not a neutral one.

Elastase of *P. aeruginosa* is reported as a neutral metalloprotease requiring zinc for enzymatic activity and calcium for stability (Morihara and Tsuzuki, 1975; Morihara and Homma, 1985). Even though, the BLAST analysis of deduced amino acid sequence of MCCB 123 LasB protease showed as neutral protease, it should be considered as an alkaline protease with respect to its alkaline pH optimum (pH 9) for protease activity and pH 8.0 for elastase activity. Other elastase of *P.*

aeruginosa with an alkaline pH optimum of 8.0 was reported (Morihara et al., 1965; Gupta et al., 2005b; Jellouli et al., 2008).

Savinase, a product of Novozyme Corp., is an endoprotease of serine type. It is a protease derived from *Bacillus* sp. and has very broad substrate specificity. It is active through the pH range of interest for most detergent applications, namely pH 8-12. It functions between 30-60°C. The activity of MCCB 123 LasB protease was compared to this commercial enzyme. The comparative study indicated that MCCB 123 LasB protease was 3.96 fold more active which indicates the commercial viability of this protease in the industry. So far, proteases from *Bacillus* sp. have been used for various industrial applications. *Pseudomonas* species also produces extracellular proteases similar to those produced by *Bacillus* species (Karadzic et al., 2004). The 3.96 fold higher protease unit activity in comparison to commercial protease from *Bacillus* sp. suggests its importance for several industrial applications. Some of the important features of MCCB 123 LasB protease are high activity and stability at high pH and temperature, in presence of surfactants, oxidizing agents, bleaches and metal ions. The very high protease and elastase activity of LasB protease and it's persistence in presence of calcium and magnesium ions make it an ideal choice for detergency for hard water washes.

Elastolytic enzymes have applications in medicine in curing hyperlipidemia and arteriosclerosis (Robert et al., 1998), production of soluble elastin for use in cosmetic industry (Xu et al., 2005) and as a meat tenderizer in food industry (Takaj et al., 1992; Considine and Hely, 1999). MCCB 123 LasB protease, the elastase of *P. aeruginosa* having elastase activity, also suggests its application in the medical area too. LasB protease of *P. aeruginosa* is also identified as a depilating protease and thus found its potential application as a depilating protease in leather

industry for the enzymatic depilation of animal hide and can be considered as a viable alternative to the chemical depilation process (Pandeeti et al., 2011). This suggests the possible application of MCCB 123 LasB protease in tannery industry too.

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Chapter 5

PURIFICATION AND CHARACTERIZATION OF LasA PROTEASE FROM *PSEUDOMONAS AERUGINOSA* MCCB 123, AND ITS APPLICATION IN BACTERIAL DNA EXTRACTION

Contents

5.1 Introduction

Pseudomonas is a very large and heterogeneous group of Gram-negative bacteria with various degradative abilities. *Pseudomonas aeruginosa* is the species type of the genus *Pseudomonas* (Dawson et al., 2002) and is ubiquitously distributed gammaproteobacterium capable of thriving dissimilar ecological niches (Kiewitz and Tummler, 2000; Khan et al., 2008). The ubiquitous environmental occurrence of the organism is due to its ability to colonize multiple environmental niches and to utilize many environmental compounds as energy source (Lyczak et al., 2000). Some species are metabolically versatile, making them attractive candidates in bioremediation (Tanase et al., 2009). Some strains of *P. aeruginosa* have unique protease with different substrate specificity whereas some strains produce two or three proteases (Morihara, 1964; Najafi et al., 2006).The bacterium secretes many proteins into extracellular medium. The secreted proteins are synthesized in the cytoplasm and have to pass both the membranes of the cell envelope.

Mainly, four pathways are identified in protein secretion in *P. aeruginosa* referred to as type 1, II, III and auto transporter pathway (Braun et al., 2001).

5.1.1 LasA protease (staphylolysin)

LasA protease of *Pseudomonas aeruginosa* is reported to have lytic action on heat- killed cells of *Staphylococcus aureus* (Kessler et al., 1993; Kessler et al., 1997; Caballero et al., 2001). It is a 20-kDa staphylolytic endopeptidase that cleaves peptide bonds following Gly-Gly bonds that hydrolyze the penta-glycine bridge required for the peptidoglycan stabilization in the cell wall of staphylococci which enable the lysis of the cells of *staphylococcus aureus* in vitro by cleaving peptidoglycan layer (Barequet et al., 2004; Barequet et al., 2009), and is the reason for its high level of staphylolytic activity of this protease (Kessler et al., 1993; Gustin et al., 1996; Kessler and Ohman, 1998).

LasA protease belongs to the M23 β-lytic endopeptidase family (Hooper, 1994; Kessler, 1995; Rawlings and Barett, 1995; Gustin et al., 1996; Kessler et al., 1998; Kessler et al., 2004; Spencer et al., 2010). The protease is placed in β-lytic endopeptidase family of bacteriolytic proteases due to its sequence homology to lytic peptidases of *Lysobacter enzymogenes* and *Achromobacter lyticus* (Kessler et al., 1993; Kessler, 1995). β-lytic proteases are members of endopeptidases which hydrolyze specific peptide bonds within the cell wall of peptidoglycan of either their own (autolysins) or other bacteria leading to the solubilization of peptidoglycan and other cell lysis (Strominger and Ghuysen, 1967).The amino acid sequence of LasA shows about 40% identity with those of β-lytic proteases from *Lysobacter enzymogenes* (Schad and Iglewski, 1988) and *Achromobacter lyticus* (Li et al., 1990). LasA protease belongs to M23A subclass, while majority of the other members of β-lytic endopeptidase family belongs to the subclass M23B (Spencer et al., 2010).

5.1.2 Specificity of LasA protease

The protease has very narrow specificity, hydrolyzing internal peptide bonds subsequent to Glycine-Glycine pairs, in particular, those followed by alanine or phenylalanine (Kessler et al., 1997). The protease is recognized primarily for their glycyl-glycine endopeptidase activity leading to the lysis of Gram-positive bacterial cell walls through the cleavage of the pentaglycine interpeptides that cross-link adjacent peptidoglycan chains (Kessler et al., 1993; Kessler, 1995; Spencer et al., 2010). The protease has far more restricted specificity acting predominantly at glycine-glycine peptide bonds and at relatively small number of sites in the elastin polypeptide (Spencer et al., 2010). LasA protease acts on glycine rich synthetic peptides such as Gly_5, Gly_6 and Leu-Gly-Gly-Gly-Ala, sequence found in elastin, which is cleaved after the second glycine bond (Vessilier et al., 2001). The protease cleaves elastin at Gly-Gly-Ala sequences and Gly-Gly-Phe sequences (Kessler et al., 1997). Such sites are scarce in elastin, which is the reason for the limited elastinolytic power of LasA protease (Kessler et al., 2004). LasA was shown to enhance the elastolytic activity of LasB protease (elastase B) of *P. aeruginosa* (Goldberg and Ohman, 1987; Schad and Iglewski, 1988; Peters and Galloway, 1990; Peters et al., 1992; Coin et al., 1997; Kessler et al., 1997).). The enhancement of elastolysis is by cleaving Gly-Gly peptide bonds abundant in elastin (Kessler et al., 1993). Peters and Galloway (1990) reported that LasA protease was shown to enhance the elastase activity of mature elastase, but not the proteolytic activity and they concluded that LasA interacts with elastin substrate rather than modifying elastase. LasA appears to be a second elastase that nicks the elastin substrate, providing the opportunity for LasB protease (elastase) to degrade the modified, and unfolded elastin rapidly by proteolytic digestion (Galloway, 1991).

Strains of *P. aeruginosa* in which LasB gene was inactivated was found to exhibit some elastase activity, and therefore, it has been suggested that, LasA was found to be the second elastase which by nicking elastin increases its susceptibility to elastase and other proteases (Darzins et al., 1990; Peters and Galloway, 1990). There was a synergistic effect reported between LasA protease and other *Pseudomonas aeruginosa* proteases in that LasA protease when assayed in presence of LasB protease (1 µg) and alkaline protease (10 µg), the staphlolytic activity of LasA increased 2.5 and 1.4 fold respectively (Gustin et al., 1996).

5.1.3 Structure of LasA protease

The LasA structure is a three-layered sandwich of antiparallel β-sheets in which a central sheet of eight strands (β16, β1, β2, β10, β6, β5, β4 and β8) is flanked by two shorter sheets of three (β3, β7 and β9) and four (β12 – β15, forming a distinct C terminal sub domain) strands each. A single zinc ion lies at the bottom of a prominent deep groove running across one face of the protein. The floor of this groove is formed by the β-core of the protein and its walls by four connecting loops: loop 1 (Pro19–Ser34, linking β1 and β2); loop 2 (Ser63–Cys65, β4–β5); loop 3 (Ala103–Pro119, β9–β10); and loop 4 (Gly129–Phe131, β10–β11). Two disulphide bridges, Cys65 – Cys111 and Cys155 – Cys170, anchor the base of loop 3 to the central β core and link strands β14 and β15 in the C-terminal sub domain. Loop 3 and the β14–β15 loop (Asn162–Thr167) occupy slightly different positions in the two, independently refined, molecules of the tartrate complex structure. The conformation of loop 3 varies between the native and tartrate complex structures. In the native (uncomplexed) structure, loop 3 is held in position by a hydrogen bond between the Tyr80 side chain hydroxyl and the backbone carbonyl oxygen of Gly-113. In the tartrate complex this contact is lost and the active site groove

widens, suggesting that changes in the conformation of loop 3 might be associated with substrate binding (Spencer et al., 2010).

The LasA gene encodes a precursor known as pre-proLasA of 45,582 Da. The preproenzyme domain structure can be divided into three domains, the signal peptide (31 aminoacids; 3298 Da), the propeptide (205 aminoacids; 22,319 Da) and the mature LasA protease (182 aminoacids; 19,965 Da) (Gustin et al., 1996). The LasA protease has the characteristic His-X-His (HXH) motif of β-lytic endopeptidase family (Hooper, 1994; Rawlings and Barett, 1995; Gustin et al., 1996; Spencer et al., 2010). The HXH motif of the protease consists of His120-X-His 122 from positions 120-122 and was considered as potential zinc ligand (Kessler et al., 1997). The presence of His120 is proved to be essential for enzymatic activity of LasA (Gustin et al., 1996).

5.1.4 Secretion of LasA protease

Mainly, four pathways are identified in protein secretion in *P. aeruginosa* referred to as type 1, II, III and auto transporter pathway. LasA protease is secreted by type II pathway, otherwise called as general secretory pathway which follows a two step mechanism. The first step involves the translocation across the inner membrane which is mediated by the Sec machinery which facilitates translocation of proteins across and in to biological membranes. In the periplasm containing chaperones and folding catalysts, the exoproteins fold into a near native conformation. This folding in the periplasm is essential for subsequent translocation across the outer membrane to occur. (Braun et al., 2001).

The protease is synthesized as preproenzyme with an unusually long amino terminal propeptide which is larger than the mature domain and is exported in its unprocessed proenzyme form. The propeptide is degraded extracellularly by the action of elastase and other secreted proteases and the processing of the LasA propeptide proceeds via a

distinct 28- kDa intermediate. A corresponding 14- kDa propeptide fragment is also detected (Kessler et al., 1998). The mature LasA protease is processed into a protein of 20 kDa (Gustin et al., 1996).

5.1.5 Bacteriolytic enzymes

Enzymes that hydrolyze the bacterial peptidoglycan are known as murein hydrolases. Based on their bond specificities, they are classified into (i) glycosidases, which split polysaccharide chains (lysozymes or muramidases and glucosaminidases), (ii) endopeptidases, which split polypeptide chains, and (iii) amidases, which cleave the junction between polysaccharides and peptides (Salzar and Asenjo, 2007). Autolysins are enzymes that can digest cell wall peptidoglycan from cells that produce them and of some other related bacteria and are characterized from *Bacillus subtilis* (Smith et al., 2000), *Staphylococcus aureus* (Foster, 1995) and *Streptococcus pneumonia* (Lopez et al., 2000). Endolysins or lysins are either β-*N*-acetylglucosaminidase or *N*-acetylmuramidase, endopeptidases or amidases that are phage encoded enzymes and they digest bacterial peptodioglycan at the terminal stage of the phage reproduction cycle, allowing the release of viral progeny out of the cell (Fischetti, 2005). The action of endolysins is controlled by holins, an enzyme produced by the same phage lytic system which disrupts the membrane and permits the access of the lytic enzyme to the peptidoglycan (Wang et al., 2000). The activity spectrum of lysins is mainly restricted to the host bacterial species of the phage from which the endolysin is derived, and also depends on the unique linkages to be cleaved in the cell wall, the specific enzyme activation by components present exclusively in the cell wall, and on the specificity in substrate recognition and cell wall binding (Salzar and Asenjo, 2007). Hen Egg White Lysozyme (HEWL) is the most widely used enzyme for bacterial cell disruption, and in the case of Gram-positive bacteria which are resistant to lysozyme, autolysins and endolysins are applied (Salzar and Asenjo, 2007). Other lytic enzymes include achromopeptidase from

Achromobacter lyticus M497-1, a broad spectrum bacteriolytic enzyme lysing *Staphylococcus aureus* and *Micrococcus luteus* (Li et al., 1998), lysyl-endopeptidase produced by *Lysobacter* sp. IB-9374 (Chohnan et al., 2002; Ahmed et al., 2003), labiase, secreted by *Streptomyces fulvissimus*, used for bacterial DNA/RNA extraction from *Lactobacillus*, *Aerococcus* and *Streptococcus* (Niwa et al., 2005).

5.1.6 Application of Bacteriolytic enzymes in DNA extraction

Bacteriolytic enzymes have potential applications in the field of molecular biology in which bacterial cell lysis is the first step towards isolation of genomic and plasmid DNA. Many Gram-positive bacteria are resistant to lysozyme and other enzymes used for lysis due to the thick cell wall consisting of peptidoglycans. Therefore, in DNA extraction mechanical disruption coupled with enzymatic lysis is employed in order to break bacterial cell wall (Johnson, 1994). Such, harsher extraction methods may destroy DNA from Gram-positive and negative cells (Schneegurt et al., 2003). Therefore, identification of an appropriate lytic enzyme with a broader spectrum of activity has been considered as a desirable proposition (Niwa et al., 2005). The lytic enzyme labiase from *Streptomyces fulvissimus*, which has both β- N-acetyl-□-glucosamidase and muramidase activities, was used in DNA/RNA extraction and lysis of many bacterial strains were observed with labiase followed by SDS treatment. The enzyme showed high lytic activity against bacterial strains with A1α chemotype and A3 α chemotype (Niwa et al., 2005). Achromopeptidase was used for the cell lysis of Gram-positive bacteria in order to determine the DNA base composition (Ezaki et al., 1990). Lysostaphin is commonly used for DNA extraction from *Staphylococcus* sp. (Johnson and Tyler, 1993). The lytic enzyme lysozyme along with detergent and chelating agent is used for DNA extraction from pathogenic bacteria (Kalia et al., 1999). Lysozyme in combination with multiple detergents and chelating agents is used for

DNA extraction from *Salmonella typhi* (Nair et al., 2004). Mutanolysin, a *N*-acetyl muramidase like lysozyme derived from *Streptomyces globisporus* along with the detergent Triton X-100 was used for the DNA extraction from Gram- positive microbes (Veyrat et al., 1999). Proteinase K along with SDS (Wilson, 1990) and a combination of lysozyme and proteinase K and SDS (Neumann et al., 1992) is used for DNA extraction from Gram-positive and Gram-negative bacteria.

5.1.7 Application of bacteriolytic enzymes as therapeutics

Lysostaphin, a 27 kDa staphylolytic endopeptidase that degrades the pentaglycine bridges of peptidoglycan of *Staphylococcus aureus* is proposed as an alternative approach to antibiotic treatment of *S. aureus* infections (Barequet et al., 2004). It also kills multiple human pathogens and is useful as multipathogen targeting antimicrobial agent (Salzar and Asenjo, 2007). Lysostaphin is also shown to be effective in eradication of oxacillin resistant and vancomycin-intermediate-susceptible *S.aureus* strains in a rabbit model of endocarditis (Climo et al., 1998; Patron et al., 1999) and methicillin resistant *S. aureus* (MRSA) in rabbit models of both keratitis (Dajcs et al., 2000; Dajcs et al., 2002) and endophthalmitis (Dajcs et al., 2001; Dajcs et al., 2002). LasA protease (staphylolysin) of *P. aeruginosa* is a 20 kDa staphylolytic endopeptidase and has lytic action on a wide range of *S.aureus* strains and other Staphylococci (Brito et al., 1989). It also inhibits the growth of *S.aureus* cells in vitro (Perestelo et al., 1985; Mansito et al., 1987). Thus it forms a valuable therapeutic agent against *S. aureus* (Kessler et al., 2004).Topical treatment with staphylolysin of *P. aeruginosa* is shown to be effective in eradicating methicillin sensitive *S. aureus* as well as methicillin resistant *S. aureus* in rabbit model of keratitis (Barequet et al., 2004) and in the treatment of methicillin resistant *S. aureus* in rat model (Barequet et al., 2009).

5.1.8 Other applications of bacteriolytic enzymes

Endolysins have high activity and specificity and are used for various *invitro* and *invivo* applications in food science, microbial diagnostics and for the treatment of experimental infections (Loessner, 2005; Borysowski et al., 2006). They are used as antimicrobial agents for the elimination of the opportunistic pathogen *Listeria monocytogenes* from food and food grade ingredients (Loessner, 2005). Temperature sensitive lytic system is used for efficient recovery of recombinant proteins from *Escherichia coli* (Yang et al., 2000b). Lytic enzymes were used to accelerate the production of hyaluronidase by recombinant *Clostridium perfringens* in the course of batch cultivation (Zukaite and Biziulevicius, 2000). Bacteriolytic enzymes are used for the production of transgenic cattle resistant to microbial infections (Kerr and Wellnitz, 2003; Donovan et al., 2005).

In the present study, LasA protease was purified from *P. aeruginosa* MCCB 123 and found to be a potent bacteriolytic enzyme with lytic activity on a broad range of Gram-positive and Gram-negative bacterial cells and thereby its application as a lytic enzyme in bacterial DNA extraction was evaluated.

5.2 Materials and Methods

5.2.1 Enzyme production

LasA protease was purified from the optimized synthetic medium (Morihara, 1964) composed of (in g l^{-1}) glucose,7.5; yeast extract,2.5; $NH_4H_2PO_4$,10.04; Na_2HPO_4, 0.5; KH_2PO_4, 3.0;$MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.000625; $ZnCl_2$, 0.01; casein, 10.0; pH, 7.0 in a 5-l fermenter (Biostat-B-Lite , Sartorius, Germany). Fermentation was carried out at 25°C, pH 7.0 ± 0.05, 300 rpm and supplied with sterile air at the rate 2.5 l min^{-1}. For enzyme extraction the culture was centrifuged at 8260 *g* for 15 min at 4°C and the supernatant stored in 300 ml aliquots at -20°C, and used for further purification and characterization.

5.2.2 Purification of LasA protease

5.2.2.1 Ammonium sulphate precipitation

Partial purification of protease was carried out by precipitation of the cell-free culture supernatant with ammonium sulphate between 30 and 80% saturation. The precipitates were collected by centrifugation at 8260 *g* for 15 min at 4℃ and the active fractions were pooled and resuspended in 20 mM Tris-Cl buffer, at pH 8.5. The partially purified enzyme was dialyzed against 20 mM Tris-Cl buffer, at pH 8.5 using Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cutoff membrane (Omega, 25MM, 10K, Pall life sciences) and used for further purification.

5.2.2.2 DEAE-cellulose chromatography

The enzyme was then loaded on an AKTA Prime protein purification system equipped with a C16/40 (16mm×40cm) (GE Healthcare Biosciences, Uppsala) DEAE cellulose (Sigma – Aldrich Co.) column equilibrated with 20 mM Tris-Cl buffer, at pH 8.5. The column was washed with the same buffer to remove the unbound proteins, and the enzyme was eluted by applying a linear gradient of NaCl from 0 – 1000 mM at a flow rate of 0.5 ml min^{-1}., and fractions of 2 ml were collected. Active fractions were pooled and concentrated by lyophilization.

5.2.3 Characterization of LasA protease

5.2.3.1 Determination of molecular weight

The lyophilized active fractions of the enzyme were subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli (1970) using 4% stacking gel and 15 % resolving gel at a constant current of 12mA. After electrophoresis, gels were stained with 0.025 % Coomassie brilliant blue stain R-250 and then destained in a solution of 5% methanol and 7% acetic acid. Molecular weight of the unknown protein band was determined by comparing with the molecular weight standards from Bangalore Genei.

5.2.3.2 Staphylolytic assay

Staphylolytic activity was determined by the modified method of Kessler et al. (1997). The activity was determined by monitoring the decrease in absorbance of a live cell suspension of *Staphylococcus aureus* subsp.*aureus* MTCC 737. The cells were harvested by centrifugation at 15,000*g* for 15 min at 4°C and the optical densities was adjusted to 0.8 at Abs_{600} and were suspended in1 ml protease (1mg ml^{-1}) , pH 8.5 and incubated at 25°C for 30 min. One unit enzyme is defined as the amount that causes a decrease in the optical density by 1 unit min^{-1} at Abs_{600}. A control without enzyme was also kept.

5.2.3.3 Effect of inhibitors on staphylolytic activity

LasA protease was pre incubated for 30 min at 25°C with the specified inhibitors as the final concentration in the assay mixture. The inhibitors were 1,10 phenanthroline,1mM; $ZnCl_2$, 0.01 and 0.1mM; dithiothreitol, 5 and 10 mM; EDTA, 10 and 25 mM; EGTA, 25 mM; PMSF, 0.4 and 2 mM; TLCK,5 mM; phosphoramidon,1 mM; leupeptin,10µg ml^{-1} and soybean trypsin inhibitor, 50 and 10µg ml^{-1}, and the assay was carried out as described above. Untreated enzyme was taken as the control (100% activity).

5.2.3.4 Muramidase assay

Muramidase activity was determined according to the method of Hoijer et al. (1995). The LasA protease (1mg ml^{-1}) was mixed with 1 mg ml^{-1} N-acetylmuramic acid (Sigma) and mixture was incubated with 50 µl of 1 M NaOH at 36°C for 30 min. After the addition of 1 ml of 18.8 M H_2SO_4 (concentrated), samples were heated for 3.5 min at 100°C, rapidly cooled in ice, and then mixed with 10µl of 160 mM $CuSO_4$ $5H_2O$ in Milli Q water and 20µl of 90 mM p-hydroxydiphenyl (Himedia) in ethanol. The blue color developed to a maximum within 30 min at 30°C at Abs_{570} was measured.

5.2.3.5 β-casein assay

β–casein assay was performed according to the method of Kessler et al. (1997). LasA protease, (50 ng in 25 µl of 20 mM Tris-HCl, pH 8.5) was mixed with β–casein (7µg in 5µl of 20 mM Tris-HCl, pH 8.5), and the solutions were incubated for 3 h at 37°C. The reaction was terminated by heating (100°C, 2 min) in SDS sample buffer, and the products were identified by SDS-PAGE.

5.2.3.6 Effect of inhibitors on β–casein activity

For inhibition studies of protease on β–casein digestion, enzyme samples were pre incubated for 1 h at 25ºC with the specified inhibitors (1mM phosphoramidon,0.4mM PMSF, 5mM TLCK, 2mM 1, 10 phenanthroline,) and the reaction was initiated by adding LasA protease (50 ng in 25 µl 0.02M Tris-Cl buffer, pH 8.5) to β–casein (7µg in 5µl of 20 mM Tris-HCl, pH 8.5) and the solutions were incubated for 3h at 37°C. The reaction was terminated by heating (100°C, 2 min) in SDS sample buffer, and the products were identified by SDS-PAGE.

5.2.3.7 Protein assay

Quantification of protein was carried out according to the method of Hatree et al. (1972) using Bovine Serum Albumin (BSA) as standard.

5.2.3.8 Specific activity

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

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

5.2.3.9 PCR amplification, cloning and sequencing of LasA protease gene

Genomic DNA was purified using the phenol-chlorofom method as described by Sambrook and Russell (2001). Primers were designed based on

the DNA sequence of the LasA protease gene obtained from the Pseudomonas genome project database (www.pseudomonas.com) and was used for the designing of LasA protease gene sequence. Amplification of LasA protease gene was performed using the primers GCT CGC CGT TCC TCT TCG TCT TGC (forward) and GGA CGG AAA GCG CAA TGG GTG GTG (reverse). The amplification was performed using DNA Thermal cycler (Eppendorf). Reaction mixture (final volume 25 µl) contained 2.5 µl 10 X buffer, 1 µl 10 pmol each of oligonucleotide primer, 1 µl DNA template, 2.5 µl 2.5 mM each deoxynucleoside triphosphate, 1 µl Taq polymerase, and the remaining volume was made up with sterile Milli Q water. Amplification profile consisted of initial denaturation for 5 minutes at 94°C followed by 29 cycles of denaturation at 94°C for 1 minute, annealing of primers at 57°C for 1 minute, primer extension at 72°C for 1.5 minutes followed by a final extension at 72°C for 10 minutes. The PCR product was separated on 1 % agarose gel and cloned into pGEM-T Easy vector (Promega, USA). The cloning, plasmid extraction and purification were carried out by the procedure as described in Chapter 1 (Sections 2.2.3.1.3 and 2.2.3.1.4) . The product was sequenced using the primer walking service of Xcelris, Ahmadabad. The primes used were T7 and SP6. Sequenced DNA data were complied and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI). After removing the contaminating vector regions, the sequences were matched with homologous sequence obtained from the GenBank database using the BLAST algorithm (Altschul et al., 1990) available from the NCBI website (http://www.ncbi.nlm.nih.gov).

5.2.3.10 Cytotoxicity analysis of LasA protease

HeLa cells were seeded in 96 well plates (Greiner Bio-One) containing 82mM glutamine, 1.5g l^{-1} sodium bicarbonate and 10% fetal bovine serum. Purified enzyme in concentrations of 0, 1, 3, 5, 10, 25, 50,100 and 250µg ml^{-1} (v/v) was added to the wells in triplicates. A

control was kept without the enzyme addition. After 14 h incubation MTT assay was performed and the percentage of inhibited cells at each concentration was calculated using probit analysis using SPSS software (SPSS 17.0 package for Windows).

5.2.3.10.1 MTT assay

After replacing the medium, 50µl MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-Aldrich Co.) having a strength of 5 mg ml^{-1} in PBS (720mOsm) was added to each well and incubated for 5 h in dark. MTT was added to the control wells with the medium alone. The medium was removed and MTT-formazan crystals were dissolved in 200µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in micro plate reader (TECAN Infinite Tm, Austria).

5.2.4 Application of LasA protease in bacterial DNA extraction

5.2.4.1 Standardization of *p*H, temperature and incubation time for bacterial cell lysis using *Staphylococcus aureus* subsp.*aureus* MTCC 737 as the reference strain

Optimization of DNA extraction was carried out according to the modified method of Niwa et al. (2005). Optimization of pH was carried out as follows: An 18 h of *Staphylococcus aureus* subsp.*aureus* MTCC 737 was harvested and the absorbance of cell suspension was adjusted to 1.0 at Abs_{600}. The cells were suspended in 1ml of LasA protease (10 mg enzyme suspended in 1 ml 50mM sodium acetate buffer at pH 5 and 6; 10 mg enzyme suspended in 1 ml 50 mM Tris-Cl buffer at pH 7 to 10) and incubated for 30 min at 25°C for pH optimization. For temperature optimization, the cells were suspended in 1ml of LasA protease (10 mg enzyme suspended in 1 ml 50mM Tris-Cl at pH 7) and incubated for 30 min at various temperatures ranging from 25 to 75°C. To determine optimum incubation time for cell lysis, the cells were suspended in 1ml of

LasA protease (10 mg enzyme suspended in 1 ml 50mM Tris-Cl at pH 7) at 35°C and incubated up to 60 min drawing samples for DNA extraction at every 10 min interval.

After each experiment, unlysed cells were removed by centrifugation at 15,000*g* for 15 min at 4°C. Into the supernatant equal volume of absolute ethanol was added and the DNA was recovered by centrifugation at 15,000*g* for 15 min at 4°C and dissolved in 100 µl sterile Milli Q water and the presence of DNA was confirmed on 1% agarose gel and the DNA yield was determined from triplicate measurements at Abs_{260}. Reactions without the enzyme were included as controls. Optimum was determined based on DNA yield. The band intensity was calculated using Quantity one software, BioRad, USA.

5.2.4.2 DNA extraction from Gram-positive and Gram-negative bacteria and bacterial consortia

The bacterial cultures were centrifuged at 15,000*g* at 4°C for 15 min and the absorbance of each suspension was adjusted to 1.0 at $Abs_{600,}$ and the pellets were suspended in 1 ml of LasA protease (10 mg enzyme suspended in 1 ml 50mM Tris-Cl, pH 7) at 35°C for 60 min. In the case of *Streptomyces* sp., cells were recovered from 1 ml of culture straightaway and DNA extraction was carried out as described above. For DNA extraction from ammonia oxidizing and nitrite oxidizing bacterial consortia (Achuthan et al., 2006) 1ml of the consortium was taken, centrifuged the cells at 15,000 *g* at 4°C for 15 min, and the DNA extraction and yield determinations were carried out as described above.

5.2.4.3 Nucleic acid yield and purity

Nucleic acid extracted from bacterial isolates was quantified using UV-visible spectrophotometer (UV-1601, Shaimadzu).The absorbance at 260 nm (A_{260}) was measured for each sample and used to calculate the average total nucleic acid ($Abs_{260}\times50\times$dilution factor) yield for each set of

triplicate samples. To estimate the purity of extracted nucleic acid, the absorbance at 280 nm (A_{280}) was measured and the average ratio between the Abs_{260} and Abs_{280} (Abs_{260}/Abs_{280}) was calculated for each set of triplicate samples. Samples with mean Abs_{260}/Abs_{280} ratios between 1.8 and 2.0 were presumed to be free of contamination (Manchester, 1995; Sambrook and Russell, 2001). Samples with mean Abs_{260}/Abs_{280} ratios below 1.8 were presumed to contain protein or other contaminants, whereas samples with ratios above 2.0 were presumed to be due to the presence of RNA (Sambrook and Russell, 2001). However, nucleic acid preparations free of phenol should have Abs $_{260}/_{280}$ ratios near to 1.2 (Sambrook and Russell, 2001; Lemarchand et al., 2005).

5.2.4.4 PCR amplification of genes

5.2.4.4.1 16S rRNA gene amplification of bacteria

PCR amplification of bacterial 16S rRNA gene was performed according to Reddy et al. (2000) using universal primers as given in Table 5.1. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20s, annealing at 58°C for 30s and extension at 68°C for 2 min. The amplification was performed using DNA Thermal cycler (Eppendorf) and the PCR products were separated on 1 % agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

5.2.4.4.2 16S rDNA amplification of β-ammonia oxidizing proteobacteria

The primers used for PCR amplification of functional genes are described in Table 5.1. PCR amplification of 16S rRNA gene of β-proteo-bacterial ammonia oxidizers was carried out according to Voytex and Ward (1995).The amplification profile consisted of initial denaturation of 95°C for 3 min followed by 35 cycles at 82 for 2.30 min, annealing at 52.3°C for 1 min and extension of 72°C for 2.5 min, 94°C for 1 min, 52.3°C for 1 min, 72°C for 2.30 min and the PCR products were separated

on 1 % agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

5.2.4.4.3 Amplification of *amoA* gene of ammonia oxidizing consortia

PCR amplification of *amoA* functional gene was carried out according to Rotthauwe et al. (1997).The primer sequence is give in Table5.1.The amplification profile consisted of initial denaturation at 94°C for 5 min followed by 42 cycles of denaturation at 94°C for 1 min, annealing at 56.8°C for 90s, extension at 72°C for 90s. The products were separated on 1% agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

5.2.4.4.4 Amplification of *nirS* gene of nitrifying consortia

The PCR amplification of *nirS* (nitrate reductase) functional gene of nitrate oxidizing bacteria was carried out according to Barker et al. (1998).The primer sequence is give in Table 5.1.The amplification profile consisted of initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°Cfor 30s, annealing at 45°C for 40s, 72°C for 1 min and 43°C for 40s. The PCR products were separated on 1 % agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

5.2.4.5 Analysis of bacterial cell rupture on treatment with LasA protease

To test the effect of LasA protease on bacterial cells, samples lysed with LasA protease were observed under phase contrast microscope (Olympus fluroscent Microscope, BX51) and compared with that of control (untreated cells).

5.2.5 Statistical analysis

Data generated from the experiments were analyzed using one-way Analysis of Variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD. Mean of the results was compared using

SPSS 17.0 package for Windows at a significance level of $p<0.05$. Data are presented as mean± standard deviation.

5.3 Results

5.3.1 Purification of LasA protease

In the present study, LasA protease was purified from an environmental isolate of *P. aeruginosa* MCCB 123. The enzyme was purified by a two step procedure, by precipitation with ammonium sulphate followed by DEAE-cellulose chromatography. Ammonium sulphate fractions from 30-80 % showed staphylolytic activity. Fractions containing staphylolytic activity were pooled and concentrated by ultrafiltration using a 10 kDa membrane. The pooled fractions were then loaded into DEAE-cellulose chromatography column. The results of purification procedure are summarized in Table 5.2. The enzyme was purified 27.51 fold with a specific staphylolytic activity of 728.86 U/mg protein. The elution profile of the LasA protease on DEAE-cellulose column is shown in Fig.5.1. The LasA protease eluted between 0.13 M to 0.21 M NaCl (fractions, 13 to21).

5.3.2 Characterization of LasA protease

5.3.2.1 Determination of molecular weight

The purified MCCB 123 LasA protease was homogenous on SDS-PAGE and its molecular weight was estimated to be 20.5 kDa by reducing SDS-PAGE (Fig. 5.2).

5.3.2.2 Staphylolytic activity and effect of inhibitors

Purified LasA protease showed 218.66 U ml^{-1} staphylolytic activity. The effects of various inhibitors on staphylolytic activity of LasA protease are shown in Table 5.3. The staphylolytic activity was markedly inhibited by metalloprotease inhibitors such as 2 mM 1, 10 phenanthroline (97.33% inhibition), 25mM EDTA (86.16% inhibition) and 25mM EGTA (87.72%

inhibition), while other class of inhibitors such as PMSF, leupeptin and trypsin soybean inhibitor, phosphoramidon did not have a significant effect on the staphylolytic activity. The reducing agent DTT (10mM) and metal ion $ZnCl_2$ (0.1 mM) also inhibited the enzyme activity at 97.77 % and 81%, respectively.

5.3.2.3 β-casein assay and effect of inhibitors

MCCB 123 LasA protease was found to have hydrolytic action on β-casein substrate and the cleavage action resulted in the disappearance of the β-casein protein band and leads to its breakage into different fragments (Fig.5.3).The study on the effect of inhibitors on β-casein digestion activity showed that the serine protease inhibitor TLCK (5mM) inhibited the β-casein activity and absence of TLCK resulted in the degradation of β-casein by LasA protease. The presence of metalloprotease inhibitors such as 1, 10 phenanthroline and phosphoramidon did not inhibit the hydrolysis of β-casein by LasA protease.

5.3.2.4 Muramidase activity

The purified LasA protease exhibited 8.40±0.05 U ml^{-1} muramidase activity (Table 5.4).

5.3.2.5 Sequencing of LasA protease gene

Primers were designed to amplify the LasA protease gene. A PCR product of amplicon size of 1200 bp was obtained (Fig.5.4a). The restriction digestion of the plasmid with LasA protease gene is shown in Fig 5.4b. The PCR product obtained was sequenced. The nucleotide sequence was submitted to Gen Bank data base and assigned the accession no HQ896829. The translation of nucleotide sequence was performed with ExPASy Molecular Biology server (http://www.expasy.com).The nucleotide sequence along with the deduced aminoacid sequence of the LasA protease gene of *Pseudomonas aeruginosa* MCCB 123 is given in Fig.5.5. The nucleotide sequence of MCCB 123 LasA protease gene was

compared with other protease sequences available in the GenBank. BLAST analysis (www.ncbi.nlm.nih.gov) of the nucleotide sequence revealed that it shared 98% similarity to LasA gene of *Pseudomonas aeruginosa* (Accession Nos.U68175 and X55904) and 97% similarity to LasA gene of *Pseudomonas aeruginosa* (Accession No.M20982) (Table 5.5). The deduced amino acid sequence of mature LasA protease is shown in Fig.5.6. It shows the presence of conserved sequences of metal ligands, residues with potential mechanistic roles, cysteine residues involved in LasA disulphide bonds and the zinc binding motif (HXH motif).The identical amino acids it (LasA protease) shares with other proteases have been indicated in Fig.5.7.Sequence homology study of the deduced aminoacid revealed that the LasA protease of *P. aeruginosa* MCCB 123 showed homology to LasA protease precursor of *P. aeruginosa* (Accession Nos. ZP 04933587, YP 002441038, ZP 04928288, EG M13284, YP 791368, NP 250562, YP001348777). It also exhibited sequence homology to peptidase M23B of *Shewanella denitrificans* (Accession no.YP 563624) and *Shewanella baltica* (Accession No.YP 001052575) and β-lytic protease of *Lysobacter* sp. IB93 (BAB86844) (Table 5.6).

5.3.2.6 Cytotoxicity analysis of LasA protease

Cytotoxic effects of LasA protease on HeLa cells were studied at different concentrations of the enzyme in the range of 0, 1, 3, 5, 10, 25, 50,100 and 250 μg ml^{-1} enzyme. At concentrations from 1 – 50 μg ml^{-1}, it did not cause any significant change in the cell morphology (Fig.5.8), and 89.43±3.11μg ml^{-1} was the LD_{50} dose (50 % inhibition) (Fig.5.9). Cytopathic effects were observed as cell rounding with the concentrations such as 100 and 250 μg ml^{-1}.

5.3.3 Application of LasA protease in Bacterial DNA extraction

Optimization of pH for bacterial DNA extraction was determined over a pH range of 5 to 10 using cells of *Staphylococcus aureus subsp. aureus*. The purified LasA protease exhibited good lytic activity on cells of *S.aureus* subsp.*aureus* from pH 5 to 10 with its optimum at 7.0 as indicated by the peaking with a DNA yield of 286.66±9.46 μg μl^{-1} (Fig. 5.10). The gel image of the effect of pH on DNA extraction from *S. aureus subsp*. is represented in Fig. 5.13a. The optimum pH for enzymatic cell lysis is in agreement with the report of Park and Galloway (1995). Statistical analysis using One-way ANOVA revealed that there existed significant (p<0.05) influence of pH between 6 to 8 on DNA yield (Appendix 3, Tables 3.1a to 3.1c).The effect of temperature on lytic activity of LasA protease was studied in a range from 25 to 75°C. The protease was found to have lytic action or cell lysis from 25 to 65°C. The optimum temperature for cell lysis was found to be 35°C with a DNA yield of 286.66±9.46 μg μl^{-1} (Fig 5.11). There was no significant (p>0.05) difference in the DNA yield between 25 to 55°C. (Appendix 3, Tables 3.2a to 3.2c).The gel image on the effect of temperature on DNA extraction is represented in Fig. 5.13b. From 45 to 75°C, there was a decreasing trend in DNA yield.

The influence of incubation time on cell lysis with LasA protease was investigated from 10 to 60 min. The DNA yield reached almost steady after 30 min incubation (286.66±9.46 μg μl^{-1}) after which it became stable, indicating that the cells were lysed within the first 30 min incubation (Fig.5.12). Incubation time was found to have a significant (p<0.05) influence on DNA yield up to 30min and after 30 min the DNA yield reached stable and henceforth it lacked significance (p>0.05, Appendix 3, Tables 3.3a to 3.3c). The gel image is presented in Fig.5.13c.The results are in agreement with Caballero et al. (2001) and Park and Galloway (1995)

who reported that, with LasA protease of *Pseudomonas aeruginosa* could complete the cell lysis of *Staphylococcus aureus* in the first 30 min.

5.3.3.1 DNA extraction and determination of yield and purity

DNA was extracted from 37 bacterial species belonging to 22 genera. The DNA yield and quality is given in Table 5.7. The quality of DNA was assessed by the ratio of $Abs_{260}/_{280}$. This ratio was found to be in the range of 1.1 to 1.3. The image of extracted DNA from various bacterial species is given in Fig.5.14.

5.3.3.2 PCR amplification of 16S rRNA gene of Gram-positive and Gram-negative bacterial DNA

Representative DNA samples were subjected to 16S rRNA gene amplification and the expected product size of 1500 bp could be obtained (Fig.5.15a)

5.3.3.3 DNA extraction of ammonia oxidizing and nitrite oxidizing bacterial consortia and analysis of functional genes

DNA was extracted from ammonia oxidizing bacterial consortia (Fig.5.15b) and nitrite oxidizing bacterial consortia (Fig. 5.15c) by the action of LasA protease attaining DNA yield of 137.5±5 $\mu g\ \mu l^{-1}$ and 172.5±5 $\mu g\ \mu l^{-1}$ respectively (Table 5.7).The PCR amplification of 16S rRNA gene specific for β-proteo-bacterial ammonia oxidizers from the DNA extracted from ammonia oxidizing consortia gave an amplicon of 1080 bp and that of *amoA* gene an amplicon of 490 bp (Fig.5.15b), and of nitrate reductase gene (*nirS*) an amplicon of 940 bp (Fig. 5.15c).

5.3.3.4 Bacterial cell rupture on treatment with LasA protease

For the examination of cell rupture on treatment with LasA protease, lysed cells were observed under phase contrast microscope and compared with that of control (untreated cells).The effect of LasA

protease on bacterial cells is presented in Fig.5.16. The cells lost their normal appearance on treatment with LasA protease compared to control

Table 5.1 Primer sequences and cycle profiles used for PCR amplification

Primer	Sequence (5'-3')	Target gene	Target gene
16S1 (f)	GAG TTT GAT CCT GGC TCA	16S rRNA gene	Reddy et al., 2000
16S2 (r)	ACG GCT ACC TTG TTA CGA CTT		
NITA (f)	CTT AAG TGG GGA ATA ACG CAT CG	16S rRNA gene of	Voytex and Ward,
NIT B (r)	TTA CGT GTG AAG CCC TAC CCA	β-proteo-bacterial ammonia oxidizers	1995
nirS 1(f)	CGA (C/T) TGG CCG CC(A/G)CAC (A/G)T	nirS functional gene	Baker et al., 1998
nirS-6-(r)	CGT TGA AAC TT(A/G) CCG GT		
amoA 1(f)	GGG GTT TCT ACT GGT GGT	amoA functional gene	Rotthauwe et al.,
amoA 2(r)	CCC CTC (G/T) G (G/C) AAA GCC TTC TTC		1997

f: forward primer , r: reverse primer

Table 5.2 Purification profile of LasA protease from *Pseudomonas aeruginosa* MCCB 123

Purification step	Staphylolytic activity (Uml^{-1})	Total Protein (mg)	Specific activity (U/mg)	Purification fold
Culture filtrate	225.2	8.5	26.49	0
$(NH4)_2SO_4$ Precipitation	221.46	4.09	54.14	2.04
DEAE-cellulose chromatography	218.66	0.3	728.86	27.51

Table 5.3 Effect of inhibitors on staphylolytic activity

Inhibitors	Concentration (mM)	Inhibition (%)
Control		0
1,10 phenanthroline	2	97.33
ZnCl2	0.01	25.44
	0.1	81
DTT	5	94
	10	97.77
EDTA	10	84.05
	25	86.16
EGTA	25	87.72
TLCK	5	17.22
Phosphoramidon	1	18.61
PMSF	0.4	27.50
	2	27.7
Leupeptin	10 $ugml^{-1}$	17.66
Trypsin soybean inhibitor	50 $ugml^{-1}$	21.11

Table 5.4 Muramidase activity of purified MCCB 123 LasA protease

Muramidase activity	8.40 ± 0.05 U ml^{-1}

Table 5.5 BLAST analysis of the nucleotide sequence of LasA protease gene of *Pseudomonas aeruginosa* MCCB 123

Sequences producing significant alignments

Accession	Description	Max score	Total score	Query coverage	Max identity
FM209186	*Pseudomonas aeruginosa* LESB58 complete genome sequence	2047	2047	96%	98%
U68175	*Pseudomonas aeruginosa* staphylolytic protease preproenzyme LasA (lasA) gene, complete cds	2047	2047	96%	98%
AE004091	*Pseudomonas aeruginosa* PAO1, complete genome	2008	2008	96%	98%
X55904	*Pseudomonas aeruginosa* lasA gene for LasA protein (elastocytic enhancer of elastase)	2008	2008	96%	98%
M20982	*P.aeruginosa* structural elastase protein (lasA) gene, complete cds	1960	1960	96%	97%
CP000438	*Pseudomonas aeruginosa* UCBPP-PA14, complete genome	1936	1936	95%	96%
CP000744	*Pseudomonas aeruginosa* PA7, complete genome	1184	1184	93%	85%

Sequence homologies producing significant alignments obtained by BLAST analysis (www.ncbi.nlm.nih.gov)

Table 5.6 Protein sequences producing significant alignment to LasA protease of *Pseudomonas aeruginosa* MCCB 123 obtained by BLAST analysis (www. ncbi.nlm.nih.gov)

Accession No.	Description	Max. Score	Total score	Query coverage
ZP04933587	LasA protease precursor [*Pseudomonas aeruginosa* 2192]	508	508	92%
YP002441038	LasA protease precursor [*Pseudomonas aeruginosa* LESB58]	508	508	92%
ZP04928288	LasA protease precursor [*Pseudomonas aeruginosa* C3719]	508	508	92%
EG M13284	LasA protease precursor [*Pseudomonas aeruginosa* 138244]	506	506	92%
YP791368	LasA protease precursor [*Pseudomonas aeruginosa* UCBPP-PA14]	506	506	92%
CA A39397	precursor protein [*Pseudomonas aeruginosa* PAO1]	504	504	92%
NP 250562	LasA protease precursor [*Pseudomonas aeruginosa* PAO1]	505	505	92%
ZP 07796462	staphylolytic protease, preproenzyme LasA [*Pseudomonas aeruginosa* 39016]	505	505	92%
ZP 01365223	hypothetical protein PaerPA_01002339 [*Pseudomonas aeruginosa* PACS2]	502	502	92%
ZP 06879207	LasA protease precursor [*Pseudomonas aeruginosa* PAb1]	502	502	92%
YP 001348777	LasA protease precursor [*Pseudomonas aeruginosa* PA7]	446	446	92%
AAA25873	Structural elastase [*Pseudomonas aeruginosa*]	410	410	92%
YP563624	Peptidase M23B of *Shewanella denitrificans* OS	296	296	92%
YP 0010552575	Peptidase M23B of *Shewanella baltica* OS155	296	296	92%
ADU33224	Elastinolytic metalloproteinase (*Pseudoalteromonas* sp. CF6-2)	177	177	92%
BAB86844	Beta lytic protease (*Lysobacter* sp. IB-93)	122	122	92%

Table 5.7 Bacterial strains used for DNA extraction along with the DNA yield and purity

Species	code	Culture Conditions	Temp	DNA yield (μg μl^{-1})	DNA purity (OD260/280)
Gram positive bacteria					
Bacillus cereus	MTCC 1272	Nutrient broth	28°C	154.16±1.44	1.10±0.01
Bacillus subtilis	MTCC 2274	Nutrient broth	28°C	266.66±3.81	1.11±0.04
Bacillus licheniformis	MCCB 145	Nutrient broth	28°C	207.5±4.33	1.27±0.07
Paenibacillus polymyxa	MTCC 122	Nutrient broth	28°C	156.66±2.88	1.11±0.04
Lactobacillus gasseri	ATCC 4963	MRS broth	37°C	163.33±2.88	1.12±0.03
Lactobacillus sp.	ATCC 8001	MRS broth	37°C	132.5±5	1.10±0.02
Streptococcus pyogens	MTCC 1924	BHI broth	37°C	148.33±2.88	1.18±0.06
Enterococcus faecalis	NCTC 775	BHI broth	37°C	120±4.33	1.18±0.03
Micrococcus lysodetikus	ATCC 4698	Nutrient broth	37°C	140±0	1.36±0.03
Staphylococcus aeures subsp.*aureus*	MTCC 737	Nutrient broth	37°C	307.5±5	1.25±0.05
Listeria monocytogens	MTCC 1143	BHI broth	37°C	206.66±6.29	1.16±0.07
Arthrobacter nicotianae	MCCB 104	Nutrient broth	28°C	183.33±3.81	1.15±0.05
Streptomyces rochi	MCCB148	Zobell's broth	28°C	136.66±1.44	1.18±0.01
Streptomyces tendae	MCCB 149	Zobell's broth	28°C	164.16±3.81	1.18±0.07
Streptomyces murablis	MCCB 150	Zobell's broth	28°C	149.16±5.20	1.17±0.02
Microbacterium sp.	MCCB146	Zobell's broth	28°C	211.66±2.88	1.15±0.06
Gram negative bacteria					
Marinobacter sp.	MCCB 147	Zobells broth	28°C	307.5±5	1.27±0.03
Agrobacterium sp.	ATCC 31750	Nutrient broth	28°C	318.33±3.81	1.12±0.03
Shigella flexnerii	MTCC 1457	Nutrient broth	28°C	285±2.5	1.18±0.005
Yersenia enterocolytica	MTCC 859	Nutrient broth	28°C	188.33±6.29	1.15±0.01
Vibrio chlorea	MTCC 3906	Nutrient broth	28°C	264.16±7.63	1.22±0.05
Escherichia coli	MTCC 77	Nutrient broth	28°C	312.5±10	1.20±0.03
Pseudomonas aeruginosa	MTCC 1934	Nutrient broth	37°C	260.83 ±7.63	1.27±0.01
Aeromonas hydrophila	ATCC 7966	Nutrient broth	28°C	326.66±2.88	1.19±0.005
Aeromonas salmonicida	ATCC 27013	Nutrient broth	28°C	334.16±5.77	1.12±0.02
Aeromonas caviae	ATCC 15468	Nutrient broth	28°C	295.83±7.63	1.25±0.04
Edwardsiella tarda	MTCC 2400	Nutrient broth	28°C	132.5±5	1.14±0.06
Vibrio harveyi	LMG 4044	Zobell's broth	28°C	282.5±6.61	1.23±0.04
Vibrio vulnificus	LMG 13545	Zobell's broth	28°C	231.66±6.29	1.23±0.003
Vibrio parahaemolyticus	LMG 2850	Zobell's broth	28°C	185.83±5.20	1.27±0.02
Vibrio mediterrenaei	LMG 11258	Zobell's broth	28°C	190.83±2.88	1.21±0.01
Vibrio proteolyticus	LMG 3722	Zobell's broth	28°C	286.66±6.29	1.19±0.007
Vibrio fluvialis	LMG 11654	Zobell's broth	28°C	369.16±6.29	1.26±0.02
Vibrio alginolyticus	LMG 4409	Zobell's broth	28°C	310.83±7.63	1.26±0.02
Plesiomonas shigelloides	ATCC 14029	Nutrient broth	28°C	142.5±4.33	1.11±0.02
Photobacterium phospherum	ATCC 11040	Photobacterium broth	28°C	158.33±6.29	1.20±0.0
Photobacterium leognathi	ATCC 25521	Photobacterium broth	28°C	205±4.33	1.19±0.04
Bacterial consortia					
Ammonia oxidizing consortia	AMONPCU	Zobell's broth	28°C	137.5±5	1.24±0.0
Nitrite oxidizing consortia	NIOPCU	Zobell's broth	28°C	172.5±5	1.27±0.03

Abbreviations: MTCC : Microbial Type Culture Collection ,Institute of Microbial Tecbnology, Chandriagh, **MCCB**: Microbial Culture Collection of Bacteria, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, **LMG**: Belgian Coordinated Collections of Microorganisms, **ATCC:** American Type Culture Collection

Datas are represented as mean±standard deviation

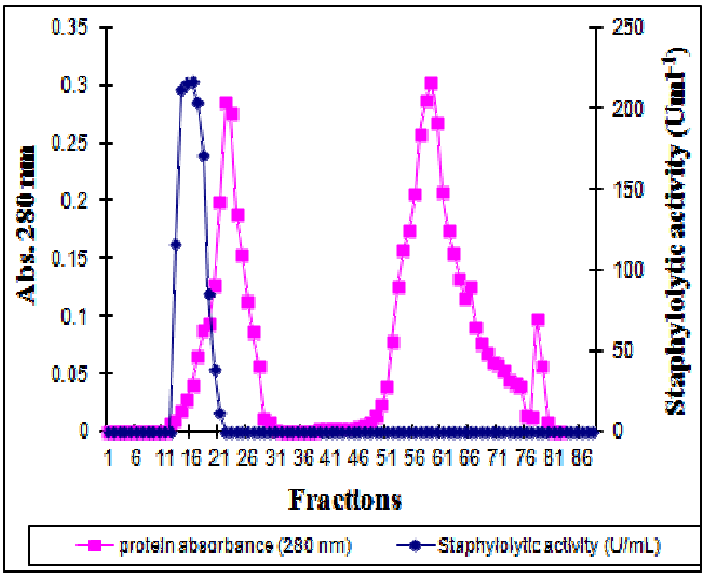


Fig. 5.1 Elution profile of LasA protease on DEAE-cellulose C16/40 column

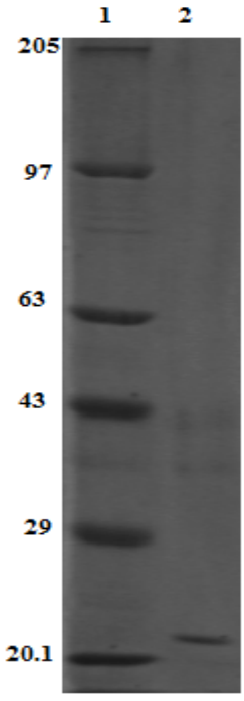


Fig. 5.2 SDS-PAGE profile of purified LasA protease. Lane 1, Molecular weight markers (3 kDa, insulin; 6.5 kDa, aprotinin; 14.3 kDa, lysozyme; 20.1 kDa, trypsin soybean inhibitor; 29 kDa,carbonic anhydrase; 43 kDa,

ovalbumin; 66 kDa, bovine serum albumin; 97.4 kDa, phosphorylase b; 205 kDa, myosin), lane 2, 20.5 kDa LasA protease

1, 10 P	-	-	-	+	-	+	+	+	+
TLCK	-	-	-	+	+	-	+	+	-
PMSF	-	-	-	+	+	+	-	+	-
PHSN	-	-	-	+	+	+	+	-	+

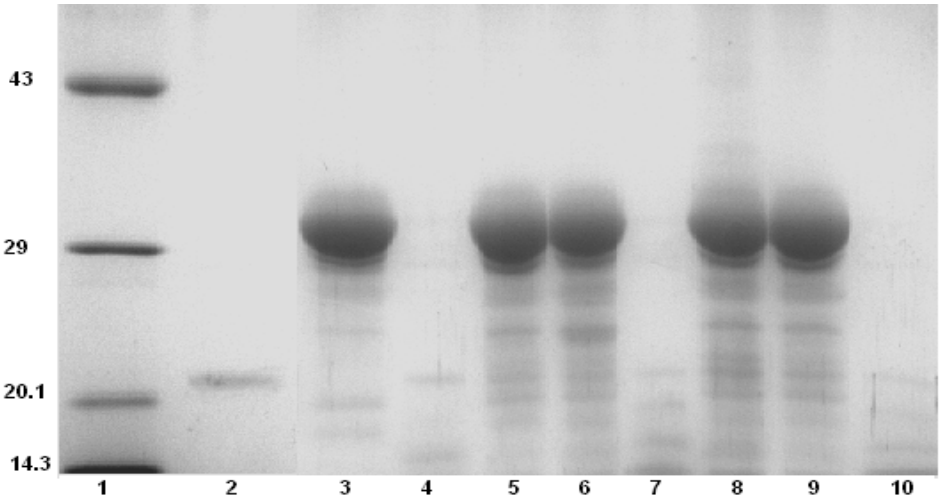
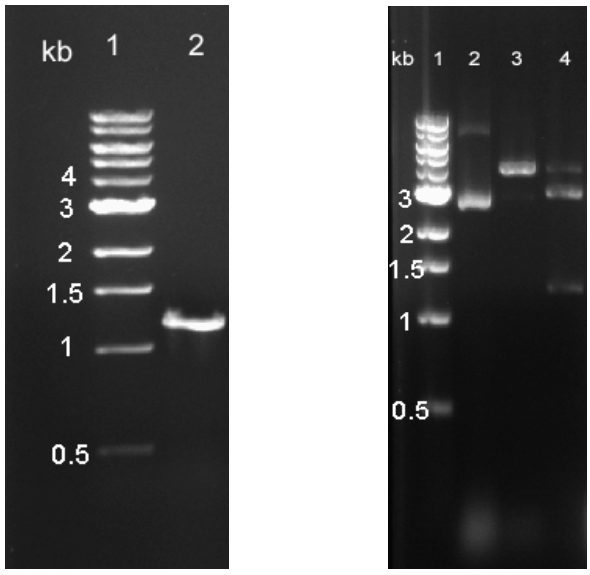


Fig.5.3 β-casein digestion patterns and effect of inhibitors on digestion by purified LasA protease. Lane 1, molecular weight markers; lane 2, LasA protease alone; lane 3, β-casein alone; lanes 4-10, β-casein with specified inhibitors



(a) **(b)**

Fig. 5.4 a) PCR amplification of LasA protease gene. Lane 1, 1kb DNA ladder, lane 2, 1.2 kb LasA protease gene. b) Restriction digestion of the plasmid with LasA protease gene insert. Lane 1, 1 kb DNA ladder; lane 2, plasmid extracted (3kb); lane 3, restriction digestion

of the plasmid with Sal1 (4.2 kb product); lane 4, restriction digestion of the plasmid with EcoR1 (3kb plasmid and 1.2 kb LasA protease gene product)

```
tacgcctgttacctacttcgttctttgcaaaaaaaggtcnccttgngcggtacggccnat
 Y  A  C  Y  L  L  R  S  L  Q  K  K  V  X  L  X  G  T  A  X
gcccatgaccatctgcctgccggccttccgctattcggcggagttactcggccagttgca
 A   H  D  H  L  P  A  G  L  P  L  F  G  G  V  T  R  P  V  A
actgcccagcgtggccctgccgctgactgacgacctgttcctctacggtcgcgacgccga
 T  A  Q  R  G  P  A  A  D  -  R  P  V  P  L  R  S  R  R  R
ggcgttcgacctcgaggcctacctggcggtgaatgccccggcgctgcgcgacaagagcta
 G  V  R  P  R  G  L  P  G  G  E  C  P  G  A  A  R  Q  E  L
Atacctggagcactggaccggctactacagcatcaacccgaaagtgttgctggccctgat
 I  P  G  A  L  D  R  L  L  Q  H  Q  P  E  S  V  A  G  P  D
ggtcatgcaatccgggccnttgggggcgccggacgagcgcgccttggcggcgccgctggg
 G  H  A  I  R  A  X  G  G  A  G  R  A  R  L  G  G  A  A  G
acggctgtcggcgaaacgcggcttcnatgcccaggtccgcgacgtgttgcagagttgtcn
 T  A  V  G  E  T  R  L  X  C  P  G  P  R  R  V  A  E  L  X
cggcgctactacggtttcgaggaataccagttgcgccaggcggctgcgcgcaaggctgtc
 R  R  Y  Y  G  F  E  E  Y  Q  L  R  Q  A  A  A  R  K  A  V
ggcgaggacggcctgaacgcggcatcggccgcgctgctcggtctgttgcgagaggggcg
 G  E  D  G  L  N  A  A  S  A  A  L  L  G  L  L  R  E  G  A
aaggcctccgccgtgcaaggcggcaatccgctcggcgcctacgcgcagaccttccagcgc
 K  A  S  A  V  Q  G  G  N  P  L  G  A  Y  A  Q  T  F  Q  R
ctgttcggcaccccggccgcggaactcctgcagccgcgcaaccgcgtggcccggcaactc
 L  F  G  T  P  A  A  E  L  L  Q  P  R  N  R  V  A  R  Q  L
                Mature cleavage
Caggcgaaggccgcgctggcgccgccatccaacctgatgcaattgccctggcgccagggc      ↓
 Q  A  K  A  A  L  ↑ A  P  P  S  N  L  M  Q  L  P  W  R  Q  G
tattcctggcagcccaacggagcgcattcgaacaccggctcgggctatccgtactcgtcc
 Y  S  W  Q  P  N  G  A  H  S  N  T  G  S  G  Y  P  Y  S  S
ttcgatgcgtcctacgactggccgcgctggggcagtgcgacctacagcgtggtcgccgcc
 F  D  A  S  Y  D  W  P  R  W  G  S  A  T  Y  S  V  V  A  A
cacgccggtacggtacgggtgctgtcgcgctgccaggtacgggtgacccaccccagcggc
 H  A  G  T  V  R  V  L  S  R  C  Q  V  R  V  T  H  P  S  G
Tgggcgaccaactactaccatatggaccagatccaggtgagcaacggccagcaggtcag   Mature LasA
 w  A  T  N  Y  Y  H  M  D  Q  I  Q  V  S  N  G  Q  Q  V  S
Gccgacaccaagctcggcgtctatgccggcaacatcaacaccgcgctctgcgagggtggc
 A  D  T  K  L  G  V  Y  A  G  N  I  N  T  A  L  C  E  G  G
agctccaccggaccgcacctgcatttctcgctgctctacaacggcgccttcgtttccctg
 S  S  T  G  P  H  L  H  F  S  L  L  Y  N  G  A  F  V  S  L
cagggcgccagcttcggtccgtaccggatcaacgtcggcaccagcaactacgacaacgac
 Q  G  A  S  F  G  P  Y  R  I  N  V  G  T  S  N  Y  D  N  D
tgtcgccgctactatttctacaaccagagcgccggcaccacccattgcgctttccagtcg
 C  R  R  Y  Y  F  Y  N  Q  S  A  G  T  T  H  C  A  F  Q  S      ↑
```

Fig. 5.5 Nucleotide sequence along with deduced aminoacid sequence of the matureLasA protease gene of MCCB 123 (Acession no. HQ896829 Gray shaded portion represents the mature LasA protease of 175 aminoacids). H L H represents the characteristic HXH motif of M23 β-lytic peptidase family

```
A P P S N L M Q L P W R Q G Y S W Q P N G A H S N T G S G Y
P Y S S F D A S Y D W P R W G S A T Y S V V A A H A G  V R
V L S R C Q V R V T H P S G W A T N Y Y H M D Q I Q V S N G
Q Q V S A D T K L G V Y A G N I N T A L C E G G S S T G P
H L H F S L L Y N G A F V S L Q G A S F G P Y R I N V G T S
N Y D N D C R R Y Y F Y N Q S A G T T H C A F Q S
```

Fig. 5.6 Deduced aminoacid sequence of the mature LasA protease of *Pseudomonas aeruginosa* MCCB 123. Pink shade denotes the conserved

sequence in M23A family, Cyan shade denotes metal ligands, Green shade denotes residues with potential mechanistic roles, Yellow shade denotes cysteine residues involved in LasA disulphide bonds, H L H denotes the signature pattern of M23 family

```
a  ------RPVP LRSRRR---- --RGLPGGEC PGAARQELIP G--ALDRLLQ HQPESVAGP- 45
b  --MQHKRSRA MASSRSPFLF VLLALAVGGT ANAHDDGLPA FRYSAELLGQ LQLPSVALPL 58
c  MNISKIRCTA LWIGRTLSMS VVMILPTMAS DVPVNQYHIE P----SEINL MAQSSILLPF 56
d  ---------- ---------- -------MKK ISKAGLGLAL MCALATIGGN ASAQGHGLSG 33
e  ---------- --MNKHLLTL AVTTGLGFSS IAFAGVHNHE TFEFSDQAVE QLNLNSLLIM 48

a ---------- ----DGHAIR AX-GGAGRAR G--------- ---------- ------GAGT 65
b NDDLFLYGRD AEAFDLEAYL ALNAPALRDK SEYLEHWSGY YSINPKVLLT LMAMQSGPLG 118
c NNRMFLYGTD ASQFDLTDFI YVNAPDLTDK KETISHWAGY YSINPKVVLT LMEMQSQLIS 116
d EDLVYSYD-E MFDFDIDAYL AKHAPHLRKH SEEISHWAGY SGISPKVLIA LMEQQS-GAV 91
e DDQTFVFNND LLNEDWDNYF ASYAPELQSK QAFILHWAGY YSINPKVILA LIEQQSEGLS 108

a AVGETRLXCP G----PRR-- -------VAE LXRRYYGFEE YQLRQAAARK AVGEDGLNAA 112
b APDERALAAP LGRLSAKRGF DAQVRDVLQQ LSRRYYGFEE YQLRQAAARK AVGEDGLNAA 178
c FPTAAALNRP LGALSGKQGF EEQLQDVLSQ LSQRFYAYEE LQLK---GQR QQSTEAINAS 173
d SA-KRATNRP FGKLARADGF GAQTREVALA LRESLYERDP DGAKGPVTLA R--------- 141
e DP-SVELESV FKNISDKQGF EEQVKDVVFK LSQRFYAFKH WQEQAVKHDK N--------- 158

a SAALLGLLR- ---EGAKASA VQGGNPLG-- AYAQTFQRLF GTPAAELLQ- --PRNRVARQ 163
b SAALLGLLR- ---EGAKASA VQGGNPLG-- AYAQTFQRLF GTPAAELLQ- --PRNRVARQ 229
c SFALLDLLSS NPLEQKNQAA MASDEVLGLD QFLEQFRLLF GNSDSELLMS PAPKNGVALD 233
d ANPLQALFE- ---RSGDNEP AAALRG--DG EFQLVYGRLF N--------- ---EPRQAKA 183
e SNSIKHLIR- ---PSQVSTA ATAALASMMS KQHNLHGQAN DSLTRFLDIF EQLSPEQSLI 214

a LQAKAALAPP SNLMQLPWRQ GAPPSNLMQL PWRQGYSWQP NGAHSNTGSG -YPYSSFDAS 221
b LQAKAALAPP S--------- -----NLMQL PWRQGYSWQP NGAHSNTGSG -YPYSSFDAS 274
c STQSMQNVTP MANVTIN--- SLPPSNMLQM PWRQGYSWQA NGAHSHTGSG -YPLSSIDVS 289
d ASDRFAKAGP DVQPLSP--- ----NGLLQF PFPRGASWHV GGAHTNTGSG NYPMSSLDMS 236
e LNTDQVTFSG EEQSVQA--- ----TFTMNL PWSQGYYWYS GGAHSNTGSG -YPYSSLDFN 266

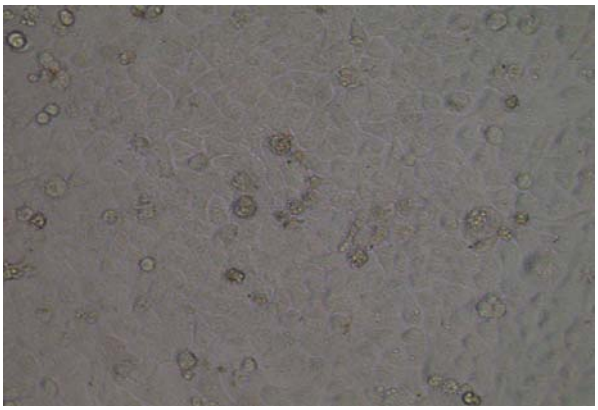
a YDWPRWGSAT YS--VVAAHA G-VRVLSRCQ VRVTHPSGWA TNYYHMDQIQ VSNGQQVSAD 279
b YDWPRWGSAT YS--VVAAHA GTVRVLSRCQ VRVTHPSGWA TNYYHMDQIQ VSNGQQVSAD 332
c YDWPQWGSST YS--VTAAHG GTVNVLSRCQ VRVTNTNGWA TNYYHMDQIT VRNGQYVNPN 347
d RG-GGWGSNQ NGNWVSASAA GSFKRHSSCF AEIVHTGGWS TTYYHLMNIQ YNTGANVSMN 295
e NGSGGWGSNT P--WVQAAHG GVITRFSSCN IRVTHSSGFA TNYYHMSNLQ YNNGDTVQPG 324

a TKLGVYAGNI NTALCEGGSS TGPHLHFSLL YNGAFVSLQG ASFGPYRINV GTSNYDNDCR 339
b TKLGVYAGNI NTALCEGGSS TGPHLHFSLL YNGAFVSLQG ASFGPYRINV GTSNYDNDCR 392
c TVMGIYANNR NTALCEGGSS TGPHLHFSLL KDGRHMSLQG VNFGQYRINI GSYNYDNNCN 407
d TAIANPANTQ AQALCNGGQS TGPHEHWSLK QNGSFYHLNG TYLSGYRITA TGSSYDTNCS 355
e TLLGRYANSY NQALCEGGQS SGPHVHFTLL QNGQQVSLHN RYISNYRIDV GNSNYDSNCN 384

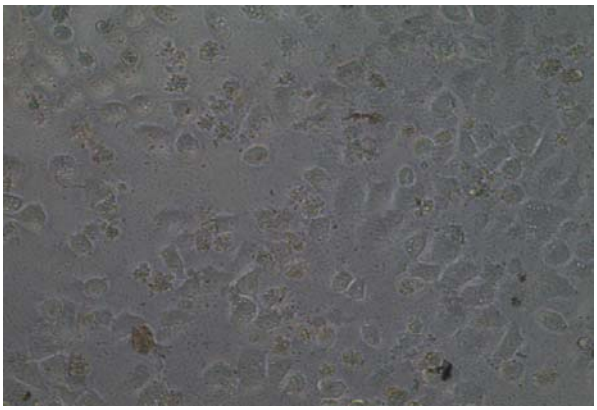
a RYYFYNQSAG TTHCAFQS-- ------                                      402
b RYYFYNQSAG TTHCAFRPLY NPGLAL                                     418
c RFNLFDLNNN RTLCAWTPLY NAGSI-                                     432
d RFYLT--KNG QNYCYGYYVN PGPN--                                     377
e NFYFE--RNG RRTCAWRPLY R-----                                     403
```

Fig. 5.7 Comparision of deduced aminoacid sequence of MCCB 123 LasA with other proteases. The identical aminocid sequence it shares with other proteases is shaded in gray. The signature pattern of M23 family is represented in cyan shade. (a) LasA protease of *Pseudomonas aeruginosa* MCCB 123 (b) LasA protease precursor of *Pseudomonas aeruginosa* ZP04933587 (c) Peptidase M23B of *Shewanella dentirificans* YP563624 (d) Beta-lytic protease of *Lysobacter* sp BAB86844 (e) Elastinolytic metalloproteinase of *Pseudoalteromonas* sp. ADU33224. The multiple alignment of sequences was done by CLUSTAL W. Numbers at right hand

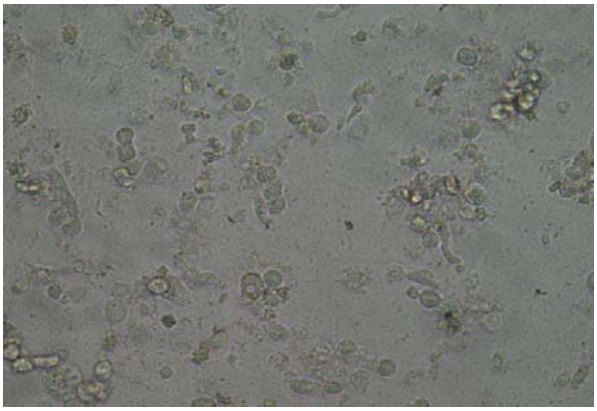
side correspond to the positions of the individual proteins. Dashes indicate gaps introduced to optimize alignment.



(a)



(b)



(c)

Fig. 5.8 Cytotoxic effects of purified MCCB 123 LasA protease on HeLa cell line. a) control showing HeLa monolayer. b) cells treated with

50µg ml^{-1} purified LasA protease. c) cells treated with 100 µg ml^{-1} LasA protease showing cell rounding

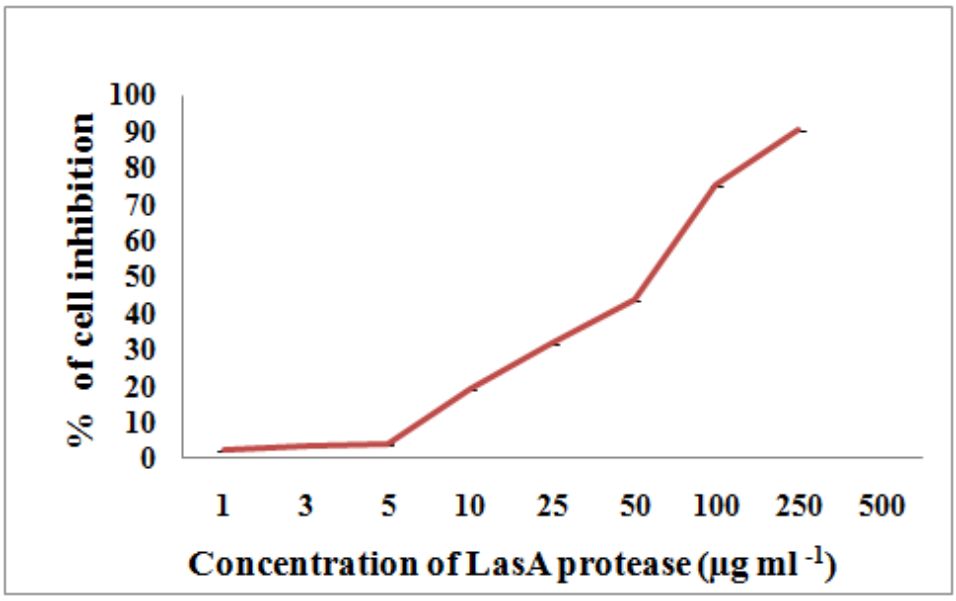


Fig.5.9 Sigmoid curve for cytotoxicity of LasA protease

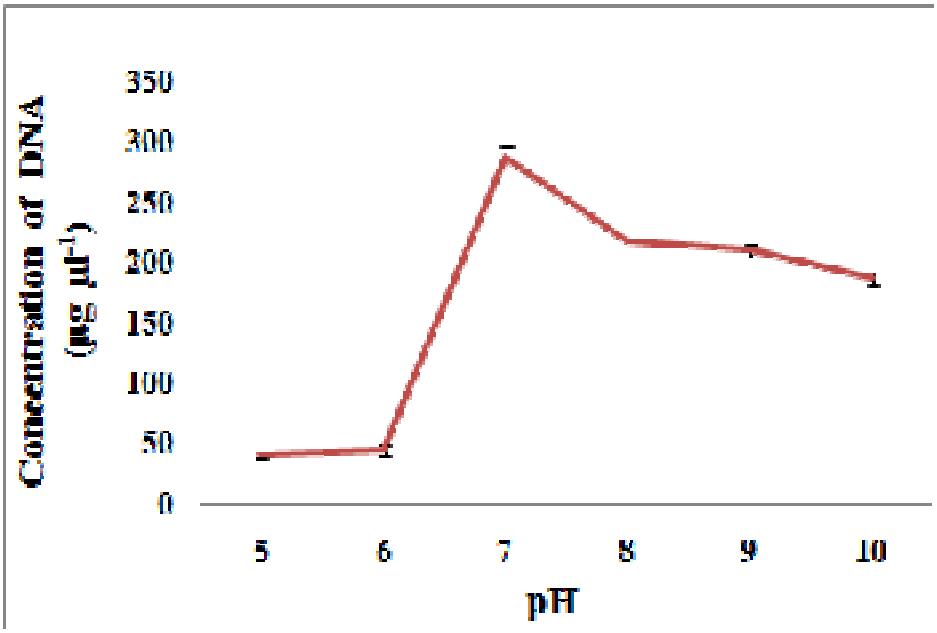


Fig.5.10 Determination of optimum pH of LasA protease for bacterial DNA extraction using *Staphylococcus aureus* subsp.*aureus* MTCC 737

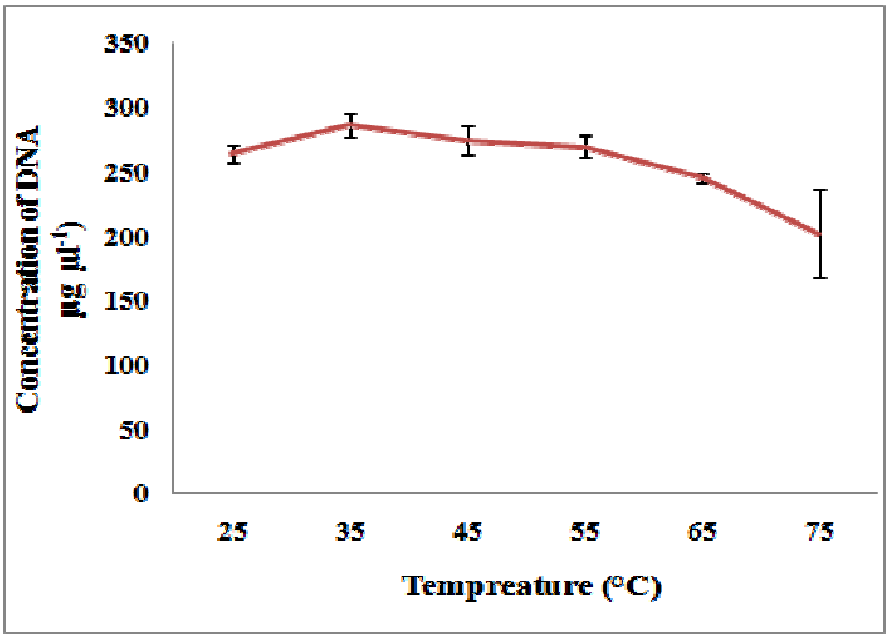


Fig. 5.11 Determination of optimum temperature of LasA protease for bacterial DNA extraction using *Staphylococcus aureus* subsp.*aureus* MTCC 737

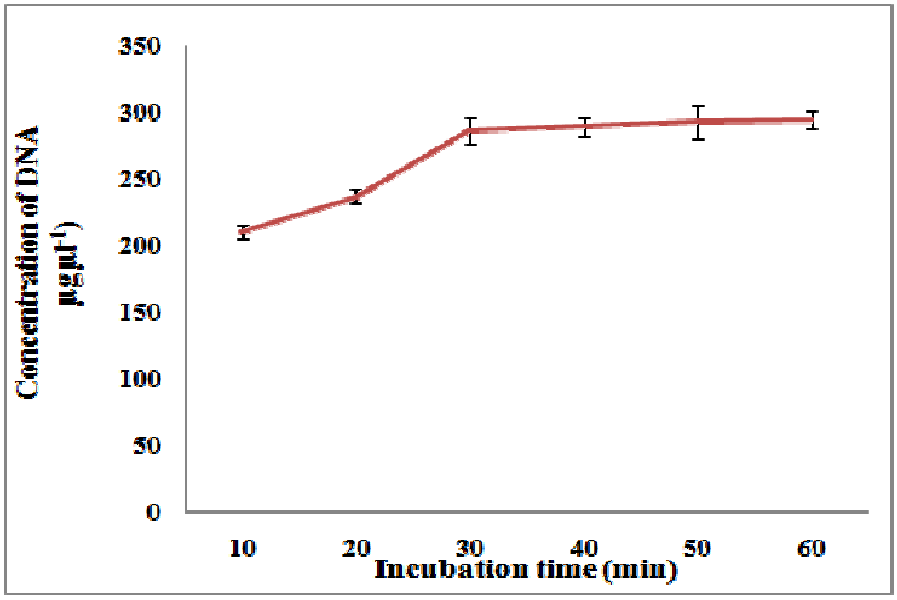


Fig. 5.12 Determination of optimum incubation time of LasA protease for bacterial DNA extraction using *Staphylococcus aureus* subsp.*aureus* MTCC 737

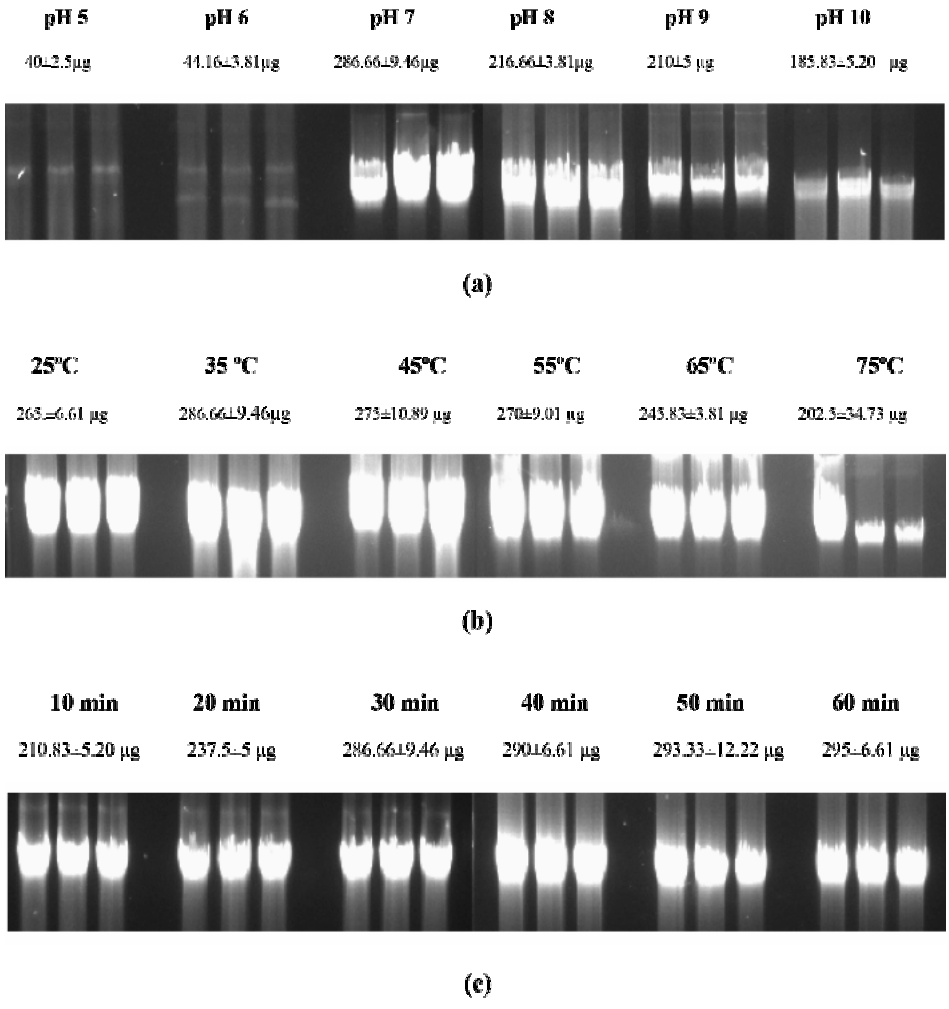
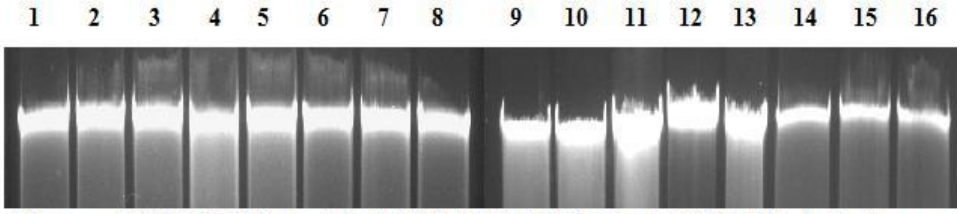
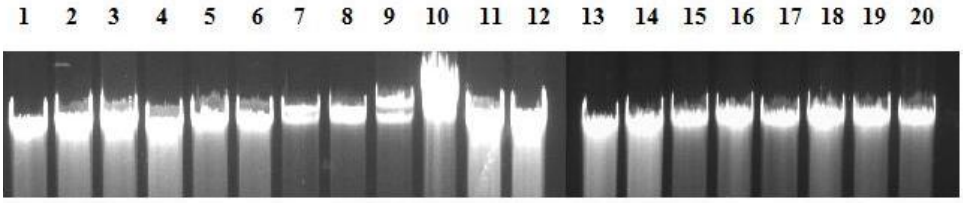


Fig. 5.13 Optimization of pH (a), temperature (b) and incubation time (c) for cell lysis using *Staphylococcus aureus* subsp. *aureus* MTCC 737 as reference strain. Concentration of DNA in µg μl^{-1} (average± standard deviation) is represented



(a)

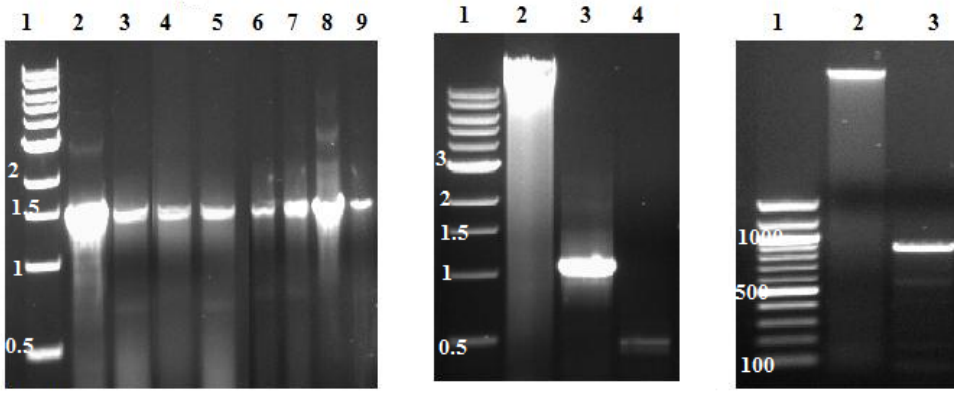
Lane 1, *Bacillus cereus* MTCC 1272; lane 2, *Bacillus subtilis* MTCC 2274; lane 3, *Bacillus licheniformis* MCCB 145; lane 4, *Paenibcillus polymyxa* MTCC 122; lane 5, *Lactobacillus gasseri* ATCC 4963; lane 6, *Lactobacillus* sp. ATCC 8001; lane 7, *Micrococcus lysodetikus* ATCC 4698; lane 8, *Enterococcus faecalis* NCTC 775; lane 9, *Streptococcus pyogens* MTCC 1924; lane 10, *Streptococcus cremoris* MCCB 147; lane 11, *Listeria monocytogens* MTCC 1143; lane 12, *Microbacterium* sp. MCCB 146; lane 13, *Arthrobacter nicotianae* MCCB 104; lane 14, *Streptomyces murabilis* MCCB 150; lane 15, *Streptomyces rochi* MCCB 148; lane 16, *Streptomyces tendae* MCCB 149



(b)

Lane 1, *Escherichia coli* MTCC 77; lane 2, *Pseudomonas aeruginosa* MTCC 1934; lane 3, *Shigella flexneri* MTCC 1457; lane 4, *Yersenia enterocolytica* MTCC 859; lane 5, *Agrobacterium sp.* ATCC 31750; lane 6, *Vibrio chlorea* MTCC 3906; lane 7, *Vibrio parahaemolyticus* LMG 2850; lane 8, *Vibrio proteolyticus* LMG 3722; lane 9, *Vibrio harveyi* LMG 4044; lane 10, *Vibrio fluvialis* LMG 11654; lane 11, *Vibrio alginolyticus* LMG 4409; lane 12, *Vibrio mediterrenaei*LMG 11258; lane 13, *Aeromonas hydrophila* ATCC 7966; lane 14, *Aeromonas caviae* ATCC 15468; lane 15, *Aeromonas salmonicida* ATCC 27013; lane 16, *Photobacterium phospherum*ATCC 11040; lane 17, *Photobacterium leognathi* ATCC 25521; lane 18, *Plesiomonas shigelloides*ATCC 14029; lane 19, *Edwardsiella tarda* MTCC 2400; lane 20, *Marinobacter sp.* MCCB 147

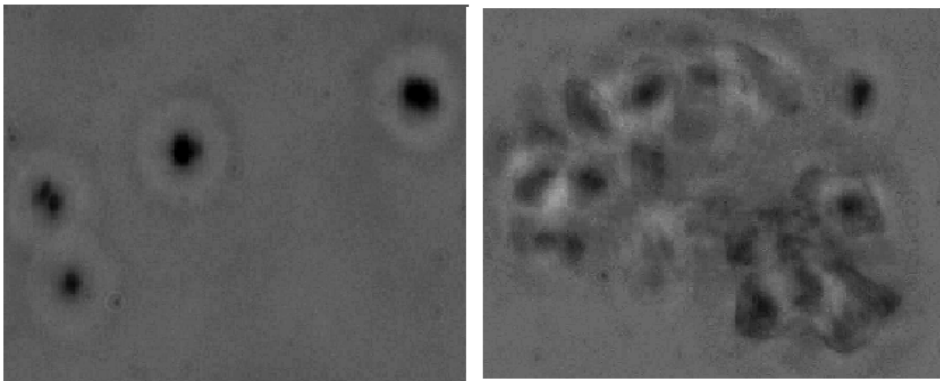
Fig.5.14 DNA extracted from Gram positive bacteria (a) and Gram negative bacteria (b) by LasA protease



(a) (b) (c)

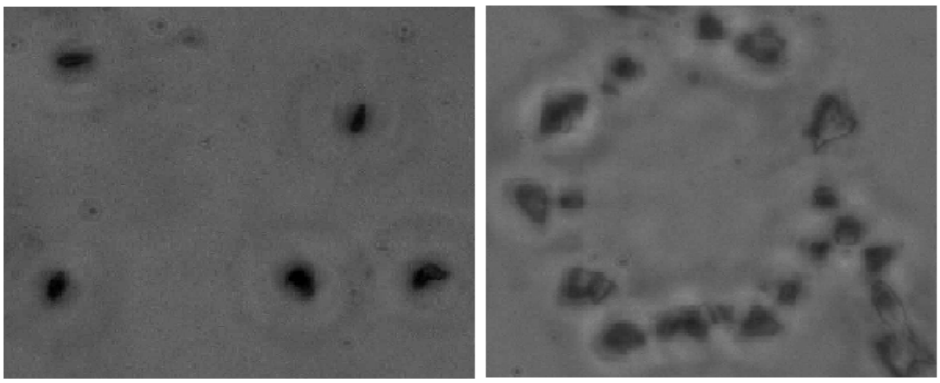
Fig. 5.15 (a) PCR amplification of DNA isolated by LasA protease with universal primers for 16S rRNA gene yielding amplicons of 1500 bp. Lane 1, 1 kb DNA ladder; lane 2, *E.coli* MTCC 77; lane 3, *P.aeruginosa* MTCC 1934; lane 4, *S.flexneri* MTCC 1457; lane 5, *Y.enterocolytica* MTCC 859; lane 6, *B.cereus* MTCC 1272; lane 7, *L.gasseri* ATCC 4963; lane 8, *M.lysodetikus* ATCC 4698; lane 9, *E.faecalis* NCTC 775. (b) DNA extraction from ammonia oxidizing bacterial consortia by LasA protease and detection of functional gene. Lane 1, 1 kb DNA ladder; lane 2, total DNA of the consortia; lane 3, PCR amplification of 16S rRNA gene of β-proteo-bacterial ammonia oxidizers yielding amplicon of 1080 bp; lane 4, 490 bp amplicon of amoA functional gene of the consortia. (c) DNA extraction from nitrite oxidizing bacterial consortia by LasA protease and detection of functional gene. Lane 1, 100 bp DNA ladder; lane 2, total DNA of the consortia; lane 3, 940 bp amplicon of nirS functional gene

Fig.5.16 (a-n). Rupture of bacterial cells on treatment with LasA protease. Control represents untreated bacterial cells. Test represents the bacterial cells treated with LasA protease (10 mg ml^{-1}). As a result on treatment with LasA protease bulging and rupturing of bacterial cells could be seen



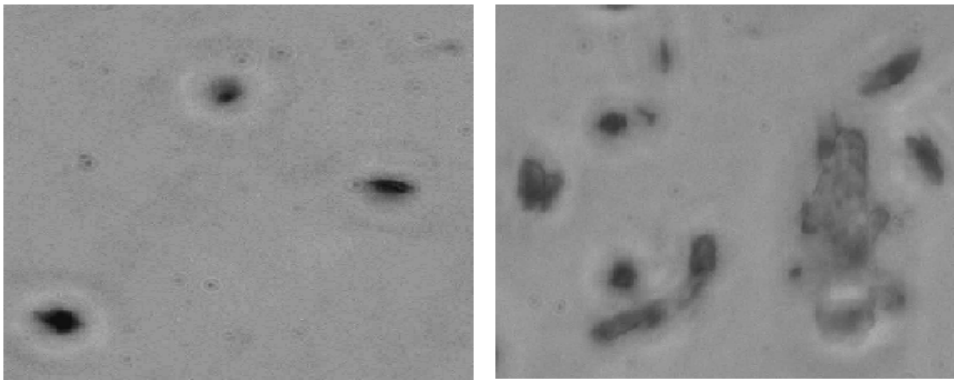
Bacillus cereus **MTCC 1272 (Control)** ***Bacillus cereus*** **MTCC 1272 (Test)**
Magnification 100X

(a)



Bacillu subtilis **MTCC 2272 (Control)** ***Bacillus cereus*** **MTCC 2272 (Test)**
Magnification 100X

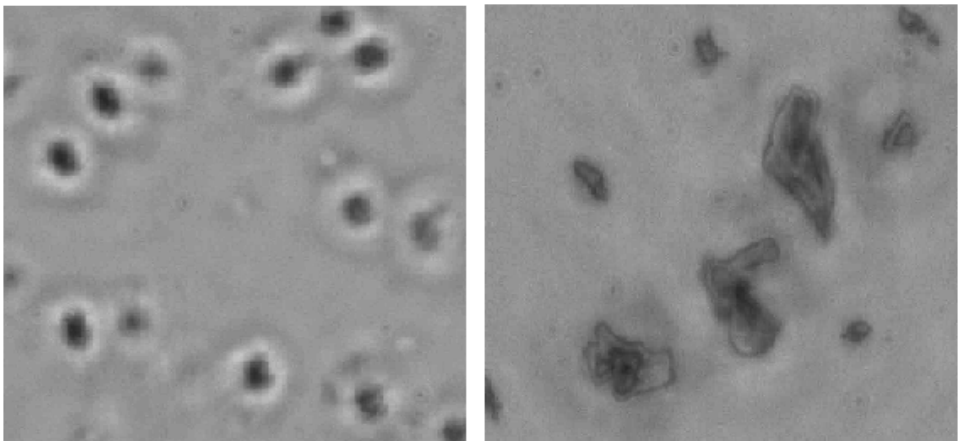
(b)



***Enterococcus faecalis* NCTC 775 (Control)** **Magnification 100X**

***Enterococcus faecalis* NCTC 775 (Test)** **Magnification 100X**

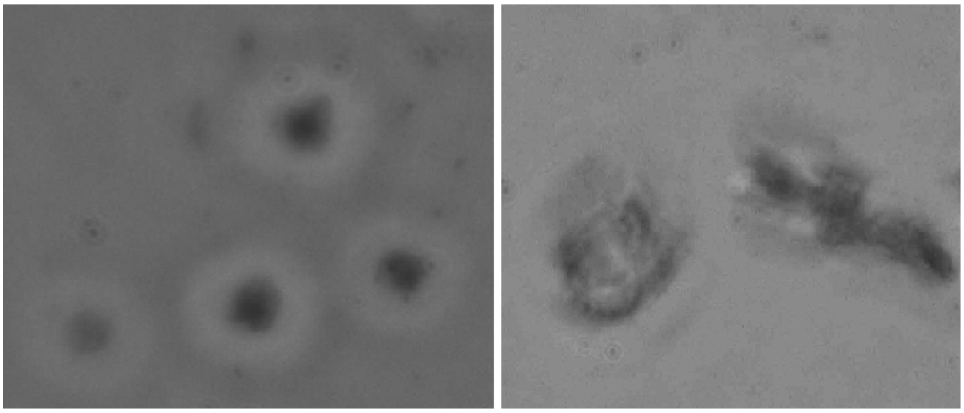
(c)



***L. gasseri* ATCC 4963 (Control)** **Magnification 100X**

***L. gasseri* ATCC 4963 (Test)** **Magnification 100X**

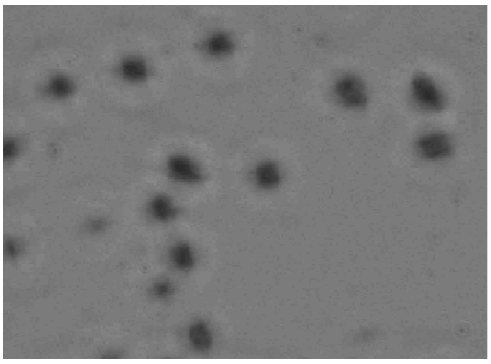
(d)

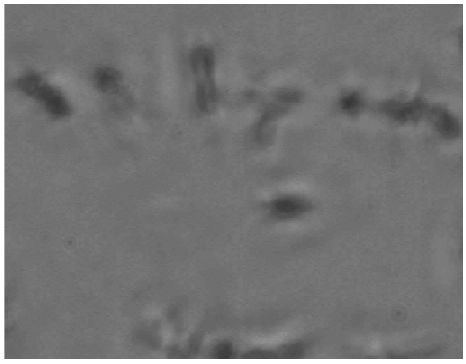


***M. lysodetikus* ATCC 4698 (Control)** **Magnification 100X**

***M. lysodetikus* ATCC 4698 (Test)** **Magnification 100X**

(e)

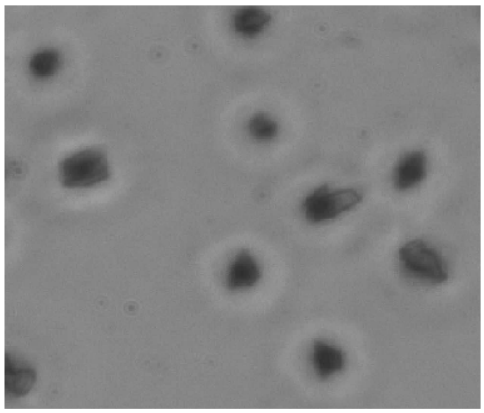


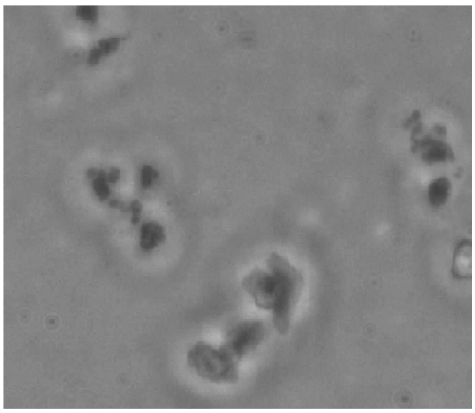


***Microbacterium* sp. MCCB 146 (Control)** ***Microbacterium* sp. MCCB 146 (Test)**

Magnification 100X

(f)

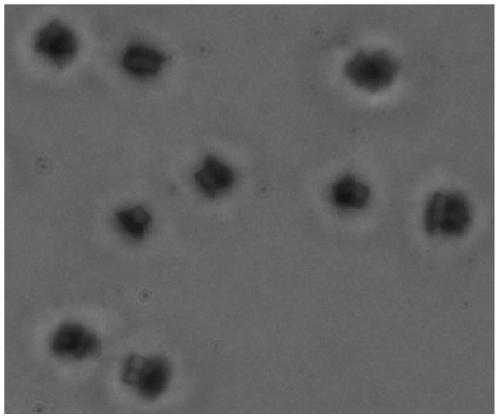


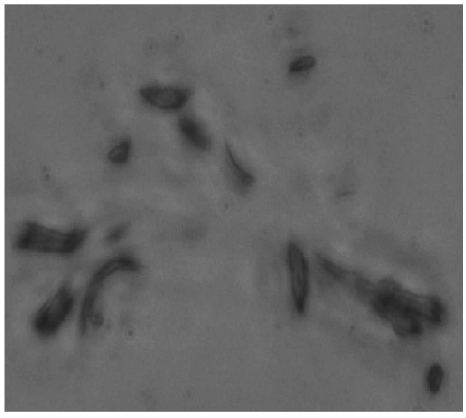


***P. polymyxa* MTCC 122 (Control)** ***P. polymyxa* MTCC 122 (Test)**

Magnification 100X

(g)

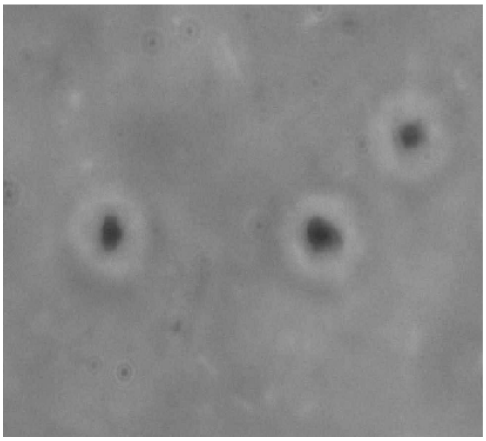


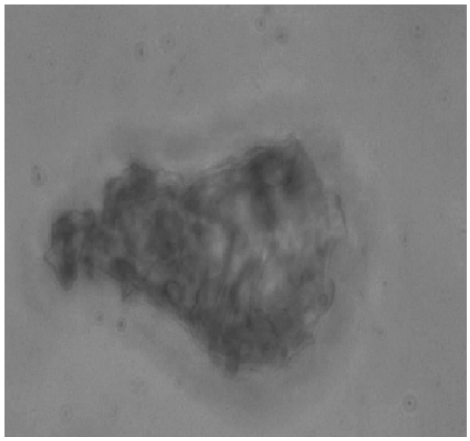


***S. aureus* susp. *aureus* MTCC 737 (Control)** ***S. aureus* subsp. *aureus* (Test)**

Magnification 100X

(h)

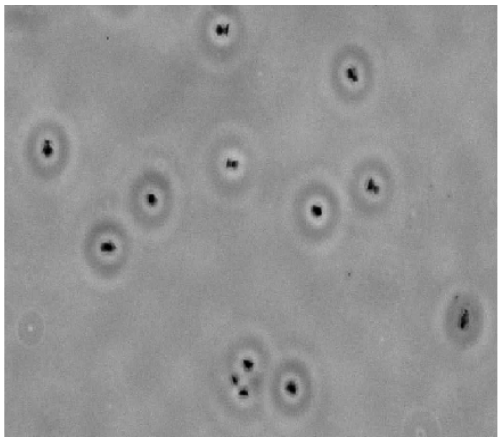


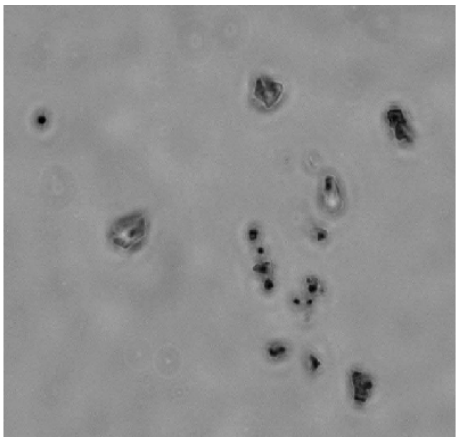


S. pyogens **MTCC 1924 (Control)** ***S. Pyogens*** **MTCC1924 (Test)**

Magnification 100X

(i)

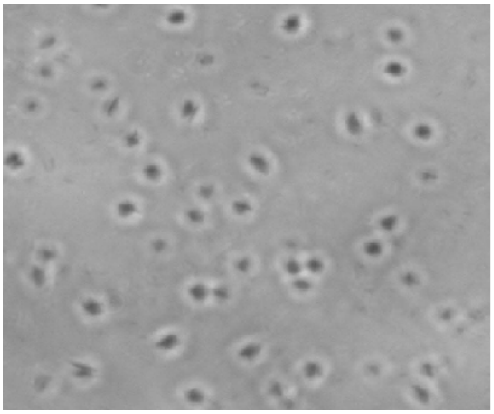


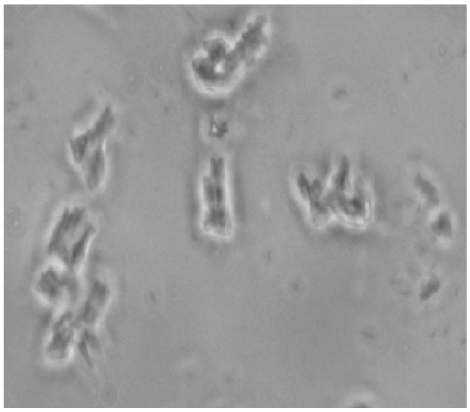


E. coli **MTCC 77 (Control)** ***E.coli*** **MTCC 77 (Test)**

Magnification 60X

(j)

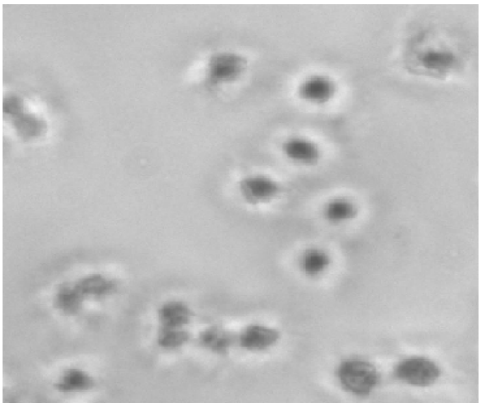


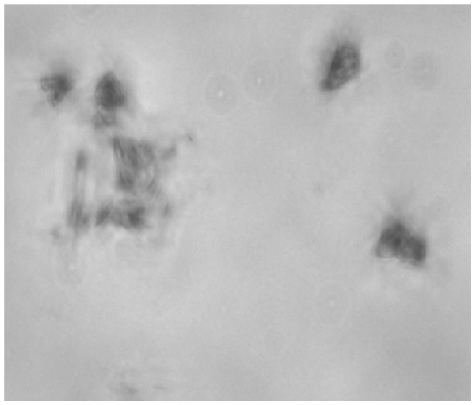


P. aeruginosa **MTCC 1934 (Control)** ***P. aeruginosa*** **MTCC 1934 (Test)**

Magnification 100 X

(k)

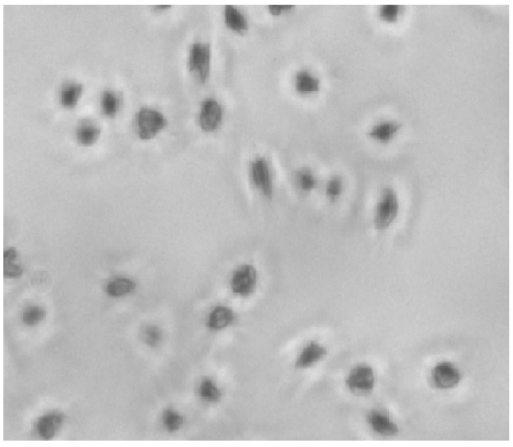


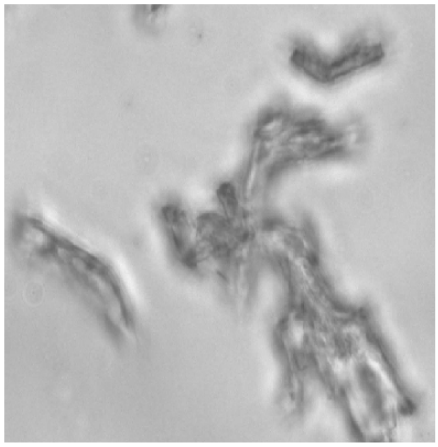


***P. phosphoreum* ATCC 11040 (Control)** ***P. phosphoreum* ATCC 11040 (Test)**

Magnification 100X

(l)

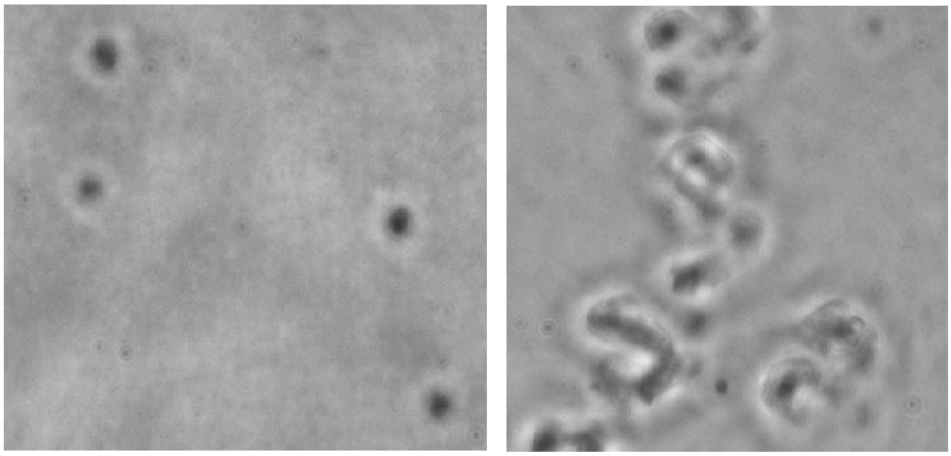




***V.Chlorea* MTCC 3906 (Control)** ***V.Chlorea* MTCC 3906 (Test)**

Magnification 100X

(m)



Y.enterocolytica **MTCC 859 (Control)** ***Y.enterocolytica*** **MTCC 859 (Test)**

Magnification 100X

(n)

5.4 Discussion

A LasA protease with a broad range of bacteriolytic activity was purified from an environmental isolate of *P. aeruginosa* MCCB 123. The enzyme was purified with 27.51 fold increase in specific staphylolytic activity. The molecular mass of the protease was found to be 20.5 kDa by SDS-PAGE which is in close agreement with those described by others (Darzins et al., 1990; Peters and Galloway, 1990; Kessler et al., 1993; Park and Galloway, 1995; Gustin et al., 1996; Kessler et al., 1997& 1998). However, Caballero et al. (2001) reported another LasA protease of 24 kDa. Staphylolytic assay is specific in order to designate the enzymatic activity as that of LasA protease of *P. aeruginosa* (Kessler et al., 1997). Accordingly, based on the lytic action on *Staphylococcus aureus*, and its molecular weight, the fraction of the protease produced by *P. aeruginosa* MCCB 123 has been identified as LasA protease.

Enzyme inhibition studies primarily give an insight into the nature of the enzyme, its cofactor requirements and the nature of the active centre (Sigma and Mooser, 1975).

In the present study, staphylolytic activity of MCCB 123 LasA protease was markedly inhibited by the strong chelators such as 1, 10 phenanthroline, EDTA and EGTA. However, the staphylolytic activity was less sensitive to PMSF (serine protease inhibitor), TLCK (inhibitor of lysine specific serine protease), leupeptin, trypsin soybean inhibitor and phosphoramidon (specific inhibitor of *Pseudomonas aeruginosa* elastase). Since the staphylolytic activity was inhibited by the zinc chelators EDTA, EGTA and 1, 10 phenenthroline and not by any other specific class of inhibitor, the MCCB 123 LasA can be designated as a metalloendopeptidase in agreement with Kessler et al. (1993 &1998). The reducing agent dithiothreitol (DTT) also inhibited the enzyme activity which may be due to the reduction of critical disulphide bonds (Kessler et al., 1997). $ZnCl_2$ was found to have an inhibitory effect only at 0.1 mM level (81% inhibition), while at 0.01 mM it resulted in only 25.44% inhibition. The requirement of relatively higher concentration of zinc for inhibition is the characteristic of zinc-dependent peptidases. The results suggest that MCCB 123 LasA protease is a zinc-dependent metlloendopeptidase, which is in agreement with the findings of Kessler et al. (1997).

LasA protease of *Pseudomonas aeruginosa* has been reported to cleave β-casein into two distinct fragments by cleaving at Lys^{29}-Ile^{30} peptide bond (Peters et al., 1992; Park and Galloway, 1995). MCCB 123 LasA protease also was found to have a hydrolytic action on β-casein substrate. However, the inhibition pattern observed for this β-casein cleavage was different with that found for staphylolytic activity. The inhibitor of β–casein digestion activity (5mM TLCK) had no effect on the staphylolytic activity of LasA, and 1, 10-phenanthroline which exhibited inhibitory action on LasA staphylolytic activity did not block β-casein degradation. The inhibitory action of TLCK on β-casein cleavage agrees with the reports of previous workers (Peters et al., 1992; Park and Galloway, 1995; Kessler et al., 1997). The inhibitory action of TLCK on

β-casein cleavage indicates that LasA may be a modified serine protease containing a critical His residue in the active site (Elliot and Cohen, 1986). The active site of MCCB 123 LasA protease also consists of the characteristic HXH (His-X-His) motif. The HXH motif of MCCB 123 LasA protease consists of His120-X-His 122 from positions 120-122 and is considered as potential zinc ligand (Kessler et al., 1997). The presence of His120 is proved to be essential for LasA activity (Gustin et al., 1996) and this may be the critical His residue of the active site proposed by Elliot and Cohen (1986). Hence, *Pseudomonas aeruginosa* LasA was proposed as a 'modified' serine protease with a key histidine residue involved in the enzymatic mechanism (Galloway, 1991; Peters et al., 1992; Park and Galloway, 1995) with respect to its β-casein cleavage. However, contradictory results have been obtained by Kessler et al. (1997) stating that β-casein is resistant to LasA and the observed β-casein cleavage is due to the result of a contaminant 28 kDa lysine specific serine protease that eluted along with the LasA during purification. However, along with MCCB 123 LasA protease, no such contaminant protein could be observed.

The 1200 bp LasA protease gene of *P. aeruginosa* MCCB 123 was sequenced and compared with the other LasA protease sequences available in the GenBank. BLAST analysis (www.ncbi.nlm.nih.gov) of the nucleotide sequence revealed that it shared 98% similarity to LasA gene of *Pseudomonas aeruginosa* (Accession Nos.U68175 and X55904) and 97% similarity to LasA gene of *Pseudomonas aeruginosa* (Accession No.M20982). The LasA protease of the GenBank submission with Accession No.U68175 was the one reported by Gustin et al. (1996), to which the aminoacid sequence of the mature MCCB 123 LasA protease had agreed well. The sequence homology study of the deduced amino acid revealed that the LasA protease of *P. aeruginosa* MCCB 123 showed homology to LasA protease precursor of *Pseudomonas*

aeruginosa (Accession Nos. ZP04933587, YP 002441038, ZP 04928288, EG M13284, YP 791368, NP 250562, ZP 07796462, ZP 06879207, YP001348777). Metallopeptidases are often recognized by the presence of short conserved signature sequences containing histidine and glutamate residues. Gluzincins, have the HEXXH motif and a glutamic acid as the third zinc ligand. The metzincins are characterized by HEXXH motif, a histidine as the third zinc ligand and a Met-turn. The inverzincin is characterized by HXXEH motif and the HXXE motif defines the carboxypeptidase family (Hooper, 1994). The HXH motif is the characteristic of β-lytic endopeptidase family (Hooper, 1994; Rawlings and Barrett, 1995; Gustin et al., 1996; Spencer et al., 2010).

β-lytic endopeptidase family (M23) is classified as metallopeptidase family with unknown metal ligands (Barrett, 1995). LasA protease of *Lysobacter* and *Achromobacter* are β-lytic metalloendopeptidase that does not contain the HEXXH zinc binding motif which is typical of zinc dependent metalloproteinase (Kessler et al., 1997), instead it has the HXH motif (Li et al., 1990; Gustin et al., 1996). The HXH motif of β-lytic endopeptidase family was proposed to serve as a zinc ligand for Zn^{2+} and thus could play a role in catalysis (Gustin et al., 1996).

The HXH motif and the sequences surrounding it are identified in the aminoacid sequence of mature LasA protease of *P. aeruginosa* MCCB 123 (Figs. 5.5 and 5.6) and agrees well with that reported by previous workers (Schad and Iglewski, 1988; Gustin et al., 1996; Spencer et al., 2010). The enzyme has been classified as a metalloprotease based on its inhibitory action on its staphylolytic activity by metal chelators. Furthermore, the inhibitory action of Zn^{2+} is predominant at higher concentration which indicates that the LasA protease *P. aeruginosa* MCCB 123 is a zinc dependent metalloprotease. High concentrations of Zinc inhibits metalloprotease (Teo et al., 2003) by the formation of zinc

monohydroxide that bridges the catalytic zinc ion to side chain of the active site of the enzyme (Larsen and Auld, 1991).

The inhibitory action of DTT on MCCB 123 LasA protease is due to the reduction of disulphide bonds (Kessler et al., 1997), indicating that the disulphide bridges is essential for the activity of LasA protease.

The deduced amin oacid sequence of the mature LasA protease shows the presence of conserved sequences of M23A family, metal ligands, residues with potential mechanistic roles, cysteine residues involved in LasA disulphide bonds and the putative zinc binding motif (HXH motif) and agrees with that described by Spencer et al. (2010). The BLAST analysis (www.ncbi.nlm.nih.gov) of amino acid sequence of MCCB 123 LasA protease also indicates that the enzyme belonged to M23 peptidase superfamily. Therefore, analysis of the deduced amino acid sequence demonstrated that the mature MCCB 123 LasA protease is a zinc metalloproteinase of M23 peptidase family. M23 metallopeptidases are part of larger metallozyme superfamily termed LAS enzymes that act primarily as peptidoglycan hydrolases (Bochtler et al., 2004). LasA protease belongs to M23A subclass, while majority of the other members belongs to the subclass M23B (Spencer et al., 2010). Spencer et al. (2010) described the conserved amino acid sequences of the members of M23A family. The amino acid sequence of MCCB 123 LasA agrees well with that described by Spencer et al. (2010). Thus, the findings in the study agree well with all the characteristics of M23A peptidase family and thus it has been concluded that MCCB 123 LasA protease in the present study belongs to M23A class of metallopeptidase family.

LasA protease encodes a precursor with a preproenzyme domain structure that can be divided into three domains: the signal peptide (consisting of 31amino acids), propeptide (consisting of 205 amino acids) and the mature LasA protease (182 amino acids) (Gustin et al., 1996). The

first 172 amino acids of the mature MCCB 123 LasA protease agrees well with the other reported LasA proteases (Schad and Iglewski, 1988; Gustin et al., 1996; Spencer et al., 2010). The N-terminal sequence (A P P S N L M Q L P) of the mature LasA protease of *P.aeruginosa* MCCB 123 is the same as that described by previous workers (Gustin et al., 1996; Spencer et al., 2010).

The BLAST analysis (www.ncbi.nlm.nih.gov) of aminoacid sequence of MCCB 123 LasA protease also indicated that the enzyme belonged to M23 peptidase super family. The enzymes of this family are recognized by their glycyl-glycine endopeptidase activity leading to the lysis of Gram- positive cell walls through the cleavage of pentaglycine interpeptides that cross-link adjacent peptidoglycan chains (Spencer et al., 2010).The LasA protease of this study was found to have a lytic activity on a broad range of Gram- positive cell wall including *Staphylococcus aureus*. The deduced amino acid sequence of MCCB 123 LasA protease on BLAST analysis showed sequence homologies to β -lytic protease of *Lysobacter* sp. IB-93 (BAB86844). The β-lytic protease of *Lysobacter enzymogenes* belongs to the M23A metallopeptidase family (Spencer et al., 2010). LasA and β-lytic endopeptidase of *Lysobacter enzymogenes* are distinguished from other members of M23 metallopeptidase family due to their ability to hydrolyze additional peptide substrates (Kessler, 1995).The translated amino acid sequence of MCCB 123 LasA showed sequence homologies to peptidase of *Shewanella dentirificans* (YP563624) and *Shewanella baltica* (YP0010552575), both belonging to M23B family. Sequence alignment of MCCB 123 LasA protease with the above members of M23A and M23B metallopeptidase family showed significant sequence homologies. An HXH motif is the characteristic of M23 β-lytic endopeptidase family (Spencer et al., 2010). Members of M23 metallpeptidase family have the common HXH motif. H represents Histidine. The middle X can be any aminoacid and varies with enzyme. The HXH motif of MCCB 123 LasA protease, LasA protease precursor of

P. aeruginosa (ZP04933587), peptidase M23B of *S. denitrificans* (YP0010552575) consists of HLH sequence, while that of β-lytic protease of *Lysobacter* sp BAB86844 consists of HEH and elastinolytic metalloproteinase of *Pseudoalteromonas* sp. ADU33224 has an HVH sequence (Fig.5.7). The HLH (HXH motif) sequence of the active site as well as conserved sequences, residues with potential mechanistic roles, cysteine residues involved in LasA disulphide bridge of MCCB 123 LasA agrees well with the other reported LasA proteases (Gustin et al., 1996; Spencer et al., 2010). The above findings also support that the *P. aeruginosa* MCCB 123 LasA protease described in the present study belongs to M23A class of peptidase superfamily.

The cytotoxicity of MCCB 123 LasA protease on HeLa cells was found to increase with the concentration of protease indicating that the cytotoxicity is dose dependent. Cytotoxic effects such as cell rounding and cell death at higher concentration was observed and the LD_{50} value on HeLa cell line was found to be 89.43±3.11µg ml^{-1}.

LasA protease of *P. aeruginosa* is reported to have lytic activity on heat- killed cells of *staphylococcus aureus* (Kessler et al., 1993; Park and Galloway, 1995; Kessler et al., 1998). However, the action of this protease on other bacterial cell walls was hitherto unknown. Interestingly, the purified LasA protease of *P. aeruginosa* MCCB 123 could lyse the cell wall of a broad range of Gram- positive and Gram- negative bacteria. This is the first report on the lytic action of LasA protease of *P. aeruginosa* on bacteria other than on *Staphylococcus aureus*.

Enzymes capable of digesting peptidoglycan layer of bacteria are known as murein hydrolases. Lysins can either be β-N-acetylglucosaminidase or N-acetylmuramidase, endopeptidase or amidase (Wang et al., 2000; Salzar and Asenjo, 2007). LasA protease of *P. aeruginosa* MCCB 123 is shown to hydrolyze the *N*-acetyl-muramic acid component of the bacterial

peptidoglycan and thus proved to be an *N*-acetylmuramidase. The *N*-acetylmuramidase activity MCCB 123 LasA protease is the reason for its lytic activity on a wide variety of Gram-positive and Gram-negative cells. So far, no reports are available on the *N*-acetylmuramidase activity on LasA protease of *P. aeruginosa*. Peptidoglycan is classified based on the mode of cross-linkage and the proposed paths of biosynthesis. There are two main groups of cross-linkage called A and B, depending on the anchoring point of the cross-linkage to the peptide subunit. (Schleifer and Kandler, 1972). MCCB 123 LasA protease has high lytic activity for A1γ chemotype as evident by the hydrolysis of *Escherchicha coli, Listeria monocytogens, Bacillus subtilis, Bacillus cereus, Bacillus licheniformis*, A2 chemotype as evident by the hydrolysis of *Micrococcus lysodetikus*, A3α as evident by the hydrolysis of *Streptococcus pyogens, Staphylococcus aureus, Enterococcus faecalis* and A4 α chemotype, and as evident by its of hydrolysis of *Arthrobacter nicotianae* (Schleifer and Kandler, 1972).

LasA protease of *P. aeruginosa* belongs to M23 metallopeptidases , which are part of larger metallozyme superfamily termed LAS enzymes that act primarily as peptidoglycan hydolases (Bochtler et al., 2004), which supports the broad range of lytic activity of the MCCB 123 LasA protease. Other reported bacteriolytic enzymes capable of lysing Gram-positive cell wall includes achromopeptidase from *Achromobacter lyticus* M497-1, which is capable of lysing *Staphylococcus aureus* and *Micrococcus luteus* (Li et al., 1998), lysyl-enopeptidase from *Lysobacter* sp. IB-9374 (Chohnan et al., 2002; Ahmed et al., 2003), labiase from *Streptomyces fluvissimus* used for bacterial DNA/RNA extraction from *Lactobacillus*, *Aerococcus* and *Streptococcus* (Niwa et al., 2005), mutanolysin from *Streptomyces globisporus* capable to hydrolyze peptidoglycan from *Listeria*, *Lactococcus*, *Lactobacillus*, *Pneumococcus* and other streptococci and lysostaphin, which specifically cleaves the

cross-linking pentaglycine bridges in the cell wall of staphylococci strains (Recsei et al.,1987).

Bacteriolytic enzymes are reported to have many applications. They have been used in the DNA extraction from Gram- positive bacteria (Ezaki et al., 1990; Niwa et al., 2005), production of transgenic cattle resistant to microbial infections (Kerr and Wellnitz, 2003; Donovan et al., 2005), as antimicrobial for medical and food applications (Sava ,1996; Loeffler et al., 2001; Fischetti , 2003; Masschalck and Michiels , 2003) and for the release of recombinant proteins (Ruyter et al., 1997; Zhang et al., 1999; Zukaite and Biziulevicius, 2000). Bacteriolytic enzymes as potential agents for DNA extraction has contributed to biotechnology industry for the extraction of nucleic acids from bacteria and for cell transformation (Salazar and Asenjo, 2007). Pretreatment with a detergent or a chelating agent is usually necessary to remove the outer membrane of Gram-negative cells (Salzar and Asenjo, 2007). The bacterial peptidoglycan is a polymer made up of alternating β-1,4-linked *N*-acetyl D-glucosamine and *N*-acetyl muramic acid residues (Schleifer and Kandler, 1972; Salazar and Asenjo, 2007). Gram-positive bacteria have multiple layers of peptidoglycan forming glycan tetrapeptides which are repeated many times through the cell, and pentaglycine bridges connect tetrapeptides of adjacent polymers. Lytic enzymes itself can cause the lysis of Gram-positive cells, whereas in Gram-negative bacteria, the outer membrane precludes the access to lytic enzymes and so pretreatment with a detergent or chelating agent is often necessary to remove the outer membrane of Gram negative cells (Salzar andAsenjo, 2007). Bacteria such as *Staphylococcus aureus* are resistant to lysozyme and others such as *Mycobacterium tuberculosis* and *Streptococcus pyogenes* show reduced susceptibility due to their complex nature of their cell wall (Lachica et al., 1971). Hence, DNA extraction from Gram-positive and

Gram-negative bacteria demands distinct protocols (Schneegurt et al., 2003).

Different types of DNA extraction methodologies are developed based on the nature of the bacterial cell wall. Lysostaphin is commonly used for DNA extraction from *Staphylococcus* sp. (Johnson and Tyler, 1993), hyaluronidase and mutanolysin for the lysis of streptococcal cells (Nelson et al., 1991). DNA extraction from pathogenic bacteria was carried out by treatment with 70% ethanol which increased the susceptibility to subsequent lysis. The method involves the use of detergent, lytic enzyme (lysozyme) and chelating agent EDTA for DNA extraction followed by freezing and thawing at 68°C, followed by the use of reagents such as SDS and CTAB, followed by the extraction of DNA by phenol-cholorofom method (Kalia et al., 1999) and the entire process is time consuming. A combination of different physical and chemical extraction methods such as chemical lysis combined with phenol-chlorofom isoamyl alcohol extraction, sonication combined with phenol-chlorofom isoamylalcohol extraction, freeze thaw and lysozyme SDS-lysis procedure, bead beating separation and phenol-chloroform isoamylalcohol extraction, combination of lytic enzymes, detergents and chelating agents, freezing and grinding in liquid N_2 followed by phenol-chloroform extraction etc., were reported for the extraction of bacterial DNA (Flamm et al., 1984; Wilson, 1990; Neumann et al., 1992; Kalia et al., 1999; Nair et al., 2004; Veyrat et al. 1999; Syn and Swarup, 2000; Lee et al., 2003; Chui et al., 2004; Tilsala-Timisjärvi et al., 2004; Lemarchand et al., 2005; Ruiz-Barba et al., 2005; Cheng and Jiang, 2006; Estarda et al., 2007; Martin-Platero et al., 2007; Parayre et al., 2007; Jara et al., 2008; Dauphin et al., 2009; Mamlouk et al., 2011).

Even though the above mentioned methods proved to be suitable for bacterial DNA extraction, they have drawbacks such as laborious manipulations, and prolonged incubation time, and involve elution,

washing and drying steps. In addition, all the reported methods involve the usage of detergent such as SDS to lyse the cell wall which often remains in DNA solution and inhibits further manipulations (Cheng and Jiang, 2006). The phenol-chloroform extraction is time consuming and are prone to sample cross contamination and PCR inhibition from phenol/chloroform carryover (Yang et al., 2011). Mechanical disruption method results in the shearing of DNA, which limits its applications relying on intact chromosomes (Lipthay et al., 2004). Sonication results in the disruption of DNA molecules and leads to the degradation of DNA (Picard et al., 1992).

Ideally, DNA extraction methods should be simple, quick and efficient. Choosing an extraction method often involves a trade-off between cost (materials and labor), the optimal yield of DNA and removal of substances that could influence the PCR reaction (Cankar et al., 2006).The simplest method for DNA extraction for PCR involves the introduction of bacterial colony directly into the reaction mix and disruption of cells by raising the temperature to 95°C for 4-5min in the initial denaturation step (Güssow et al., 1989). However, these methods are not equally suitable with all the strains and the results cannot be easily reproducible, and when complex samples such as microbial communities are used in DNA extraction, the quality and quantity of DNA used to be low (Martin-Platero et al., 2007).

MCCB 123 LasA protease has a broad range of lytic action on Gram-positive and Gram-negative bacteria, which facilitates its application in bacterial DNA extraction. On-contrary to the above mentioned reports, the data generated from the present study demonstrated that the LasA protease from *P. aeruginosa* MCCB 123 can independently lyse both Gram-negative and Gram-positive cells releasing DNA without any additives. DNA extraction from both Gram-positive and Gram-negative bacteria could be achieved by the lytic action of this

enzyme in the absence of detergents and hence the use of this lytic enzyme in bacterial DNA extraction can avoid the use of distinct protocols that have been employed in bacterial DNA extraction. Through the present investigation, it is demonstrated that purified MCCB 123 LasA protease alone could lyse the cell wall of a variety of Gram-positive and Gram- negative bacteria, and DNA could be extracted without the addition of detergents or chelating agents which makes this method unique over the existing methods reported in bacterial DNA extraction. MCCB 123 LasA protease is shown to hydrolysis *N*-acetylmuramic acid component of the bacterial cell wall, thereby it has shown to exhibit muramidase activity. The hydrolytic action on a wide variety of bacterial cell wall may be due to the hydrolysis of glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramate in the bacterial cell wall. Lysozyme is reported to have such an effect on bacterial cell wall (Stryer, 1995). The extracted DNA using MCCB 123 LasA protease could be directly used for PCR amplification which indicates the absence of any PCR inhibitors. This method of DNA extraction is simple, rapid and cheap and it neither requires specialized equipment, nor complicated extractions protocols with organic solvents such as phenol or chloroform or denaturing agents such as guanidium isocynate. The quality of the extracted nucleic acid samples is important for further processing. Samples with mean A_{260}/A_{280} ratios below 1.8 were presumed to contain protein or other contaminants, whereas samples with ratios above 2.0 were presumed to be due to the presence of RNA (Sambrook and Russell, 2001). However, nucleic acids preparations free of phenol should have Abs $_{260/280}$ ratios near 1.2 (Sambrook and Russell, 2001; Lemarchand et al., 2005).In case of DNA extracted with LasA protease from various Gram-positive and Gram-negative bacterial strains, this ratio was found to be in this range of 1.1 to 1.3 and phenol is not used in the DNA extraction.

DNA extraction using MCCB 123 LasA protease has several advantages. First, the number of steps in DNA extraction procedure was minimized by replacing phenol chloroform extraction method and it also does not involves the addition of any detergents or other lytic agents and several samples can be processed within a short time period of 30 min. The other method of DNA extraction needed at least 2-48 h (Nair et al., 1999; Chui et al., 2004).Secondly, this method seems to be very cost-effective since it use only the lytic enzyme LasA protease as the sole reagent for DNA extraction, and it does not involve phenol-chlorofom extraction step or any additional use of lysozyme, proteinase K, SDS or other lytic agents. Thirdly, the extraction method using LasA protease is applicable to a broad range of Gram-positive and Gram-negative bacteria and thus avoids demand of distinct protocols. This is the first report of a lytic enzyme being employed in DNA extraction without the addition of detergents such as sodium dodecyl sulphate (SDS).

Since LasA protease has lytic action on the cells of *Staphylococcus aureus*, it has therapeutic potential in treatment of *Staphylococcus aureus* infections. The therapeutic potential of staphylolytic protease, LasA secreted by *Pseudomonas aeruginosa* in eradicating *Staphylococcus aureus* was evaluated in a rabbit model of experimental keratitis and the results highlighted the potential of the LasA protease (staphylolysin) as a therapeutic tool in the management of *S.aureus* corneal infections (Barequet et al., 2004). Barequet et al. (2009) evaluated the versatility of LasA protease in an experimental rat model of methicillin resistant *Staphylococcus aureus* in order to investigate the bactericidal activity of staphylolysin (LasA protease) in the vitreous humor and found that the treatment with LasA protease was effective in the treatment of methicillin resistant *Staphylococcus aureus* endophthalmitis and causes no morphological adverse effects to ocular tissues. Thus, it could be concluded that staphylolysin (LasA protease) might be beneficial in the treatment of *S.*

aureus endophthalmitis in humans (Barequet et al., 2009). Since peptidoglycan hydrolase kills multiple human pathogens, it may have application as a selective, multipathogen-targeting antimicrobial agent that could potentially reduce the use of broad range antibiotics in fighting clinical infections (Salzar and Asenjo, 2007). This is very evident in the case of MCCB 123 LasA protease, which is a peptidoglycan hydrolase and is capable of lysing the cell wall of a broad range of Gram-positive and Gram-negative bacteria including many pathogenic bacterial strains, thus indicating the therapeutic potential of this potent lytic protease against different human pathogens, and therefore it can be used as a multipathogen targeting antimicrobial agent also.

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Chapter 6

PURIFICATION AND CHARACTERIZATION OF β-1, 3 GLUCANASE FROM *PSEUDOMONAS AERUGINOSA* MCCB 123 AND ITS APPLICATION IN FUNGAL DNA EXTRACTION

Contents

6.1 Introduction

β -1, 3 glucanases represent a well-known class of enzymes widespread in bacteria and fungi, and are hydrolases specific to O-glycoside bonds between 1,3-linked glucopyranose residues found in a variety of β-glucans (Zhu et al., 2008). In fungi, several of these enzymes have roles during cell separation in unicellular organisms, and the development of cell wall architecture in yeasts and filamentous fungi (Adams, 2004). In bacteria, these enzymes take part in the degradation of polysaccharides that can be present in their natural environment and be used as an energy source (Planas, 2000). Exo - β-1, 3 glucanases cleave glucose residues from non - reducing ends, while, endo - β-1, 3 glucanases cleave β-linkages at random sites along polysaccharide chain releasing short oligosaccharides (Garciduenas et al., 1998). Exo-glucanases degrade the polysaccharides completely and release monosaccharide residues, where as endo-glucanases produce oligosaccharides by partial degradation of the exopolysaccharides (Vijayendra and Kashiwagi, 2009).

Bacterial and fungal β-1, 3 glucanases are involved in the degradation of polysaccharides that can be present in their natural environment and used as an energy source (Planas, 2000).

6.1.1 Structure of microbial β-1, 3 glucanase

β-1, 3 -1, 4 glucanases are classified as members of family 16 with a jellyroll β- sandwich structure. The three dimensional structure of family 16 1,3-1,4-β-glucanase can be explained by the structure of a hybrid H(A16-M) 1,3-1,4-β-glucanase of mature *Bacillus amyloliqufaciens* and *Bacillus macerans*. The core of the protein is formed by two β-sheets stacking atop each other in a sandwich like manner, which consists of seven anti-parallel strands each that are bent and create a cleft crossing one side of the protein where the substrate is found. Loops between the β-strands are mostly stabilised by β-turns and there is only one turn with an α-helical geometry. A major surface loop (residues 20-36 in *B. macerans* numbering) covers partially the binding site cleft created by the bending of the β-sheets, where the disulfide bond between Cys30 and Cys59 links this loop to the β-strand 56-64 of the protein core. On the convex side of the molecule, remote from the active site, a calcium ion is bound which plays a role in stabilising the native protein structure. Cation binding has been analysed by comparing the crystal structures and stabilities of the hybrid H (A16-M) with bound Ca2 or Na^+ ions. Calcium is bound with nearly perfect octahedral geometry coordinating to the backbone carbonyl oxygens of Pro7, Gly43 and Asp205, a carboxylate oxygen of Asp205 (*B. macerans* numbering), and two water molecules, whereas sodium is trigonal-bipyramidally coordinated and provides lower thermal stability to the folded protein. The cleft on the concave side of the molecule defines the oligosaccharide substrate binding site. It is lined with mainly aromatic residues on its walls and with acidic residues at the bottom. The catalytic residues are located in the same β-strand where there is a strict alternation of polar (acidic) and non-polar side chains, the first pointing toward the

surface of the protein where they are able to interact with the substrate, the latter toward the hydrophobic interior (Planas, 2000).

6.1.2 β -1, 3 glucanases as a mycolytic enzyme

Several strains of bacteria are able to lyse and grow on viable yeast and fungal cells by producing a variety of cell wall degrading enzymes such as endo-β-1, 3 glucanases, β-1, 6 glucanases, mannanases and chitinases (Ferrer, 2006). Mycolytic enzymes produced by antagonistic microorganisms are very important in biocontrol technology (Diby et al., 2005). β-1,3 glucanases hydrolyse hydrolyzes β-1,3 glucans which is an important structural component of the cell walls in many agronomically important pests and have received considerable attention as they play a role in plant growth-promoting systems against plant pathogens. The enzymatic digestion or deformation of cell wall components of these organisms by the enzyme could present an effective method for their biological control (Lim and Kim, 1995). The structure of yeast cell wall composed of complex polymers such as β-1,3 and β-1,6 glucans, mannoproteins and smaller amount of chitin (Ferrer, 2006; Salazar and Asenjo, 2007) implies that synergistic action of these enzymes is necessary to hydrolyse its components. Enzyme systems for yeast cell lysis are usually a mixture of different enzymes such as β-1,3 glucanase, β-1,6 glucanase, protease, mannanase and chitinase, which acts synergistically for the lysis of cell wall (Ferrer, 2006; Salzar and Asenjo, 2007). A β-1,3 glucanase from *Chaetomium* sp. was found to degrade the cell wall of plant pathogens like *Rhizoctonia solani*, *Gibberella zeae*, *Fusarium* sp, *Colletotrichum gloeosporioides* and *Phoma* sp (Sun et al., 2006). An antifungal β-1, 3 glucanase enzyme was reported from the biocontrol fungus *Trichoderma atroviride* (O'Kennedy et al., 2011). An endo-α-D-(1→3) glucanase capable of hydrolyzing various α (1→3) glucans has been isolated from the fungus *Trichoderma viride* (Hasegawa and Nordin, 1969). The complementary action of GluA, GluB and GluC

gene products of β-1, 3 glucanase from *Lysobacter enzymogenes* Strain N4-7results in the hydrolysis of β-1,3 glucans from fungal cell walls (Palumbo et al., 2003). The β-glucanase produced by *Bacillus subtilis* NSRS 89-24 plays a crucial role in the degradation of the fungal cell walls (Leelasuphakul et al., 2006).

6.1.3 β -1, 3 glucanases of *Pseudomonas*

β -1, 3 glucanase produced by *Pseudomonas stutzeri* YPL-1 was identified as one of the key enzymes in decomposition of fungal cell walls and the enzyme inhibited 53% mycelial growth of the fungus *Fusarium solani*. The optimum pH and temperature for β -1, 3 glucanase activity was found to be 5.5 and 40°C, respectively (Lim and Kim, 1995). β-1, 3(4) glucanase A from *Pseudomonas* sp. PE2 was found to be an essential enzyme for the degradation of *Pythium porphyrae* cell walls (Kitamura and Kamei, 2006). Mycolytic enzyme such as β -1, 3 glucanases and β -1, 4 glucanases produced by *Pseudomonas fluorescens* was found to be efficient in the lysis of *Phytophthora capsici* mycelium (Diby et al., 2005). β -1, 3 glucanase enzyme produced by fluorescent pseudomonad isolates GRC_3 and GRC_4 was found to have involvement in the growth inhibition/ suppression of the phytopathogenic fungi *Rhizoctonia solani* (Arora et al., 2007). A β-1, 3 glucanase producing *Pseudomonas cepacia* was found to have an efficient role in the biocontrol of soilborne plant pathogen such as *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* and decreased disease incidence caused by them by 85, 48 and 71%, respectively (Fridlender et al., 1993). A glucanase producing strain of *Pseudomonas aeruginosa* was used in the biological control of cyst forming nematode *Heterodera cajani* on sesame (Kumar et al., 2009). A *Pseudomonas fluorescens* strain producing enzymes such as β-1,3 and β-1,6 glucanases was used in the biocontrol of soil borne fungal plant pathogen *Rhiozoctonia solani* (Dev and Dawande, 2010). β-1, 3 and β-1,4 glucanases produced by *Pseudomonas fluorescences* strains NRC1 and

NRC3 were found to have strong lytic effect on tomato root-rot causing fungal pathogens such as *Rhizoctonia solani* and *Phytophthora capsici* (Saad ,2006). The production of lytic enzyme β-1, 3 glucanase correlated with the antifungal activity of *Pseudomonas fluorescences* against the fungal pathogen *Rhizoctonia solani* (Nandakumar et al., 2002). β-1, 3 glucanase produced by *Pseudomonas aeruginosa* PN1 is reported to cause mycelial lysis, vacuolation and granulation of cytoplasm, hyphal deformities and branching in polyphagous fungus *Macrophomina phaseolina* (Singh et al., 2010). Chan et al. (2003) also had a similar observation. A significant relationship was observed between the level of β-1, 3 glucanase production of *Pseudomonas fluorescences* strains and its antagonistic potential towards the rice sheath blight fungus *Rhizoctonia solani* (Nagarajkumar et al., 2004). The accumulation of β-1, 3 glucanase by *Pseudomonas chlororaphis* PA-23 in canola leaf tissue was found to be responsible for the reduction of *Sclerotinia sclerotiorum* infection in pathogen inoculated plants (Fernando et al., 2007). In pea, seed treatment with *Pseudomonas fluorescens* strain 63-28 has produced chitinases and β-1,3- glucanases which accumulate at the site of penetration of the fungus, *Fusarium oxysporum* resulting in the degradation of fungal cell wall (Benhamou et al., 1996). β-1, 3 glucanase has been found to increase the resistance in Berangan banana plantlets against the fungal pathogen *Fusarium oxysporum* when treated with *Pseudomonas* sp. UPMP3 (Fishal et al., 2010).

6.1.4 Application of microbial β -1, 3 glucanases

Yeast lysing glucanases have enormous applications (Salazar and Asenjo, 2007). The various applications include the preparation of protoplasts, cell fusion and transformation of yeast (Kitamura, 1982), production of intracellular enzymes (Zomer et al., 1987), pre-treatment to increase yeast digestibility (Kobayashi et al., 1982), preparation of soluble glucan polysaccharides (Jamas et al., 1986), alkali extraction of yeast proteins (Kobayashi et al., 1982), production of yeast extracts

(Conway et al., 2001), food preservation (Scott et al., 1987), and release of recombinant proteins from *Saccharomyces cerevisiae* (Asenjo et al., 1993; Ferrer et al., 1996). Yeast lysing glucanases are used for modulating the cell wall permeability as a first step in a downstream process for protein recovery from yeast. Specific activity of the lytic enzymes is a key factor that has to be considered when analyzing the possibility of using lytic enzymes for cell disruption and product release. It has to be sufficiently high to obtain fast cell breakage without allowing endogenous intracellular proteases degrading the product (Salazar and Asenjo, 2007). Purified, protease free glucanase has been used for the controlled cell lysis of the yeast *Saccharomyces cerevisiae,* which results in the selective release of cloned intracellular protein particles (Asenjo et al., 1993).

The bacterial β-1,3 glucanases have potential applications in brewing. During malting, due to the heat inactivation of endogenous 1,3-1,4- β-glucanases, large amount of high molecular weight β-glucans may cause problems such as reduced yields of extracts and lower filtration rates as well as the appearance of gelatinous precipitates in the finished beer. Thus, the level of activity of glucan hydrolases achieved during germination and the amount of their substrates are important factors for good quality brewers malt, and thermostable bacterial 1,3-1,4-β- glucanases are often added to reduce viscosity during mashing. In animal feedstuff, especially for broiler chickens and piglets, addition of enzymatic preparations containing bacterial L-glucanases improves digestibility of barley-based diets, and reduces sanitary problems such as sticky droppings (Planas, 2000).

Pseudomonas aeruginosa MCCB 123 was found to be a potential producer of β-1,3 glucanase having lytic action on a wide range of fungus and therefore an evaluation have been made in the application of this enzyme in fungal DNA extraction.

6.2 Materials and Methods

6.2.1 Enzyme production

β-1,3 glucanase was purified from the optimized synthetic medium composed of (in g l^{-1} Distilled water): glucose,7.5; yeast extract,2.5; $NH_4H_2PO_4$,10.04; Na_2HPO_4, 0.5; KH_2PO_4, 3.0;$MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.000625; $ZnCl_2$, 0.01; casein, 10.0; pH, 7.0 in a 5-l fermenter (Biostat-B-Lite , Sartorius, Germany). Fermentation was carried out at 25°C, pH 7.0 ± 0.05, 300 rpm and supplied with sterile air at the rate 2.5 l min^{-1}. For enzyme extraction the culture was centrifuged at 8260 *g* for 15 min at 4°C and the supernatant stored in 300 ml aliquots at -20°C, and used for further purification and characterization.

6.2.2 Purification of β -1, 3 glucanase

6.2.2.1 Ammonium sulphate precipitation

Partial purification of enzyme was carried out by precipitation of the cell-free culture supernatant with ammonium sulphate between 30 and 80% saturation. The precipitates were collected by centrifugation at 8260 *g* for 15 min at 4°C and the active fractions were pooled and resuspended in 20 mM Tris-Cl buffer, at pH 8.5. The partially purified enzyme was dialyzed against 20 mM Tris-Cl buffer, at pH 8.5 using Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cut off membrane (Omega, 25MM, 10K, Pall life sciences) and used for further purification.

6.2.2.2 DEAE-cellulose chromatography

The enzyme was then loaded on an AKTA Prime protein purification system equipped with a C16/40 (16mm×40cm) (GE Healthcare Biosciences, Uppsala) DEAE cellulose (Sigma – Aldrich Co.) column equilibrated with 20 mM Tris-Cl buffer, at *p*H 8.5. The column was washed with the same buffer to remove the unbound proteins, and the enzyme was eluted by applying a linear gradient of NaCl from 0 – 1000 mM at a flow rate of 0.5

ml / min., and fractions of 2 mL were collected. Active fractions were pooled and concentrated by lyophilization.

6.2.3 Characterization of β -1, 3 glucanase

6.2.3.1 Determination of molecular weight

The lyophilized active fractions of the enzyme were subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS –PAGE) following the method of Laemmli (1970) using 4% stacking gel and 15 % resolving gel at a constant current of 12mA. After electrophoresis, gels were stained with 0.025 % Coomassie brilliant blue stain R-250 and then destained in a solution of 5% methanol and 7% acetic acid. Molecular weight of the unknown protein band was determined by comparing with the molecular weight standards from Bangalore Genei.

6.2.3.2 β-1, 3- glucanase assay

β-1, 3 glucanase activity was measured by using laminarin from *Laminaria digita* (Sigma –Aldrich Co.) as substrate according to the modified method of Zhu et al. (2008). The laminarase activity was based on the measurement of reducing sugar cleaved from laminarin. The reaction mixture consisted of 0.5 ml of 5 mg ml^{-1} laminarase (dissolved in 50 mM sodium-phosphate buffer, pH 6.0) and incubated at 50°C for 30 min. After incubation 1 ml DNS reagent was added and tubes were placed in boiling water for 10 min, cooled and 4 ml of distilled water was added and the amount of reducing sugar liberated was measured at 540 nm. Assays were carried out in triplicates. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1 µg of D-glucose per minute under standard assay conditions.

6.2.3.3 Protein assay

Quantification of protein was carried out according to the method of Hatree (1972) using Bovine Serum Albumin as standard.

6.2.3.4 Specific activity

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

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

6.2.3.5 Effect of pH on β-1, 3 glucanase activity

Effect of pH on β-1, 3 glucanase activity was determined over a pH range of 3 to 10 using the buffers of 50 mM concentrations: sodium – phosphate (6,7), Tris-Cl (8 and 9), glycine-NaOH (9 , 10, 11 and 12) for 30 min at 37°C.

6.2.3.6 Effect of temperature on β-1, 3 glucanase activity

Effect of temperature on laminarase activity was tested by carrying out the assay at temperature ranges of 30, 40, 50, 60, 70 and 80°C for 30 min in 50 mM Tris-Cl buffer (pH 9.0).

6.2.3.7 Effect of inhibitors on β-1, 3 glucanase activity

Various inhibitors (5 mM phenyl methyl sulphonyl fluoride (PMSF), EDTA, 1, 10 phenanthroline, leupeptin, pepstatin, phosphoramidon and TLCK) were studied by including them in the assay mixture, and the relative activity was measured under standard assay conditions. Untreated enzyme was taken as the control (100% activity).

6.2.3.8 Cytotoxicity analysis of MCCB 123 β-1, 3 glucanase

HeLa cells were seeded in 96 well plates (Greiner Bio-One) containing 82mM glutamine, 1.5g l^{-1} sodium bicarbonate and 10% fetal bovine serum. Purified enzyme in concentrations of 0, 1, 3, 5, 10, 25, 50,100, 250 µg ml^{-1} (v/v) was added to the wells in triplicates. A control was kept without the enzyme addition. After 14 h incubation MTT assay was performed and the percentage of inhibited cells at each

concentration of the protease was calculated using SPSS software (SPSS package for Windows).

6.2.3.8.1 MTT assay

After replacing the medium, 50µl MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-Aldrich Co.) having a strength of 5 mg ml^{-1} in PBS (720mOsm) was added to each well and incubated for 5 h in dark. MTT was added to the control wells with the medium alone. The medium was removed and MTT-formazan crystals were dissolved in 200µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in micro plate reader (TECAN Infinite Tm, Austria). Probit analysis for percentage cell inhibition was done with SPSS software package (version 17).

6.2.4 Application of MCCB 123 β-1, 3 glucanase in fungal DNA extraction

6.2.4.1 Standardization of pH, temperature and incubation time for cell lysis of β-1,3glucanase on fungal cells using *Saccharomyces cervisiae* MTCC 1766 as the reference strain

Lytic activity was carried out according to the modified method of Niwa et al. (2005). *Saccharomyces cervisiae* MTCC 1722 was grown for 48 h at 28°C.The absorbance of cell suspension was adjusted to 1.0 Abs_{600}, centrifuged the cells at 15,000 *g* at 4°C for 15 min and the pellets were recovered. For pH optimization, cells were suspended in 1 ml of β-1,3 glucanase enzyme (10 mg enzyme suspended in 50mM sodium acetate for pH 5 to 6, 50 mM Tris-Cl from pH 7 to 10) and incubated for 30 min at 25°C. For temperature optimization, cells were suspended in 1 ml of purified β-1,3 glucanase enzyme (10 mg enzyme suspended in 1 ml of 50 mM Tris-Cl, pH 7.0) and incubated for 30 min at various temperatures ranging from 25 to 75°C. To determine optimum incubation time for cell lysis, cells were suspended in 1 ml of β-1,3 glucanase enzyme (10 mg enzyme suspended 1ml of 50 mM Tris-Cl, pH 7.0) at

35°C and incubated up to 60 min drawing samples for DNA extraction at every 10 min interval.

After each experiment, un-lysed cells were removed by centrifugation at 15,000*g* for 15 min at 4°C. Into the supernatant equal volume of absolute ethanol was added, kept for 30 min and the pellet was recovered by centrifugation at 15,000*g* for 15 min at 4°C and dissolved in 100 µl sterile Milli Q and the presence of DNA was confirmed on 1% agarose gel and the DNA yield was determined by triplicate measurements at 260nm. Reactions without enzyme were included as controls. Optimum was determined based on band intensity and DNA yield. The band intensity was calculated using Quantity one software, BioRad, USA.

6.2.4.2 DNA extraction from fungal cultures

Fungal cultures used for DNA extraction and their culture conditions are listed in Table 3.Fungal cultures were grown until enough fungal mycelia have grown, 1ml of the culture centrifuged at 15,000*g* at 4°C for 15 min and mycelia were treated with 1 ml of 10 mg ml^{-1} of the purified glucanase enzyme resuspended in 50 mM Tris-Cl, pH 7.0 and incubated at 65°C for 60 min. DNA extraction and yield determination were carried out by the method as described earlier.

6.2.4.3 Nucleic acid yield and purity

Nucleic acid extracted from fungal isolates was quantified using UV-visible spectrophotometer (UV-1601, Shaimadzu).The absorbance at 260 nm (Abs_{260}) was measured for each sample and used to calculate the average total nucleic acid yield for each set of triplicate samples. To estimate the purity of extracted nucleic acid, the absorbance at 280 nm (Abs_{280}) was measured and the average ratio between the Abs_{260} nm and Abs_{280} nm (A_{260}/Abs_{280}) was calculated for each set of triplicate samples. Samples with

mean Abs_{260}/Abs_{280} ratios between 1.8 and 2.0 were presumed to be free of contamination (Manchester, 1995; Sambrook and Russell, 2001). Samples with mean Abs_{260}/Abs_{280} ratios below 1.8 were presumed to contain protein or other contaminants, whereas samples with ratios above 2.0 were presumed to be due to the presence of RNA (Sambrook and Russell, 2001). Nucleic acids preparations free of phenol should have Abs $_{260/280}$ ratios of 1.2 (Sambrook and Russell, 2001; Lemarchand et al., 2005).

6.2.4.4 PCR amplification of ITS region

PCR amplification of ITS region consisting of ITS 1 and ITS 2 was performed according to White et al. (1990) using primers ITS1 (5' TCC GTA GGT GAA CCT GCGG-3') and ITS4 (TCC TCC GCT TAT TGA TAT GC-3').The amplification was performed using DNA Thermal cycler (Eppendorf). Reaction mixture (final volume 25 µl) contained 2.5 µl 10 X buffer, 1 µl 10 pmol each of oligonucleotide primer, 1.5 µl DNA template, 2.5 µl 2.5 mM each deoxynucleoside triphosphate, 1 µl Taq polymerase, and the remaining volume made up with sterile Milli Q water. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 45 s and extension at 72°C for 1 min followed by final extension at 72°C for 10 min and the PCR products were separated on 1% agarose gel.

6.2.4.5 Microscopic examination of β-1,3 glucanase treated fungal hyphae and yeast cells under phase contrast microscope and comparison with untreated cells (control)

For the examination of cell rupture, lysed cells were observed under phase contrast microscope (Olympus) and compared with those of control (untreated cells).

6.2.5 Statistical analysis

Data generated from the experiments were analyzed using one-way Analysis of Variance (ANOVA) with post-hoc multiple comparison analysis

performed using Tukey's HSD. Mean of the results was compared using SPSS 17.0 package for Windows at a significance level of $p<0.05$. Data are presented as mean± standard deviation.

6.3 Results

6.3.1 Purification of β-1, 3 glucanase

In the present study, β-1, 3 glucanase having lytic action on a broad range of fungal cell walls was purified from an environmental isolate of *Pseudomonas aeruginosa* MCCB 123. The enzyme was purified by a two step procedure, by precipitation with ammonium sulphate followed by DEAE-cellulose chromatography. Ammonium sulphate fractions from 30-80 % showed β-1, 3 glucanase activity. Active fractions were pooled and concentrated by ultrafiltration using a 10 kDa membrane. The pooled fractions were then loaded into DEAE-cellulose chromatography column. The results of purification procedure are summarized in Table 6.1. The enzyme was purified 9.52 fold increase in specific activity. The elution profile of the β-1, 3 glucanase on DEAE-cellulose column is shown in Fig.6.1. The protease eluted between 0.70 M to 0.81 M NaCl (fractions, 70 to 81) contained β-1, 3 glucanase.

6.3.2 Characterization of β-1, 3 glucanase

The purified MCCB 123 β-1, 3 glucanase was homogenous on SDS-PAGE and its molecular weight was estimated to be 45 kDa by reducing SDS-PAGE (Fig. 6.2).

6.3.3 Effect of pH on activity of β-1,3 glucanase

The effect of pH on β-1, 3 glucanase activity was determined using buffers in the pH range of 6 to 12 at 50°C, respectively. The relative activity of the enzyme at different pH are given in Fig.6.3.The enzyme was found to exhibit activity from pH 3 to10 with its optimum at pH 7.0. Statistical analysis by One-way ANOVA indicated that there was a

significant (p<0.05) difference in the β-1, 3 glucanase activity between pH values from 3 to 7 (Appendix 4, Tables 4.1a to 4.1c).

6.3.4 Effect of temperature on activity of β-1, 3 glucanase

The enzyme was found to exhibit activity from 30 to 80°C with its optimum at 50°C (Fig. 6.4). There was a significant (p<0.05) difference in the β-1,3 glucanase activity in temperature ranges between 30 to 50°C (Appenedix 4, Tables 4.2a to 4.2c).

6.3.5 Effect of inhibitors on activity of β-1, 3 glucanase

There was a partial inhibition (42.98%) of enzyme activity by metalloprotease inhibitor EDTA thus proving to be metalloprotease. The enzyme retained 83.34, 84.22, 81.51 and 95.62 % activity in presence of 5mM 1, 10 phenanthroline, 50μM leupeptin, 10 μM pepstatin and 0.1 mM phosphoramidon, respectively, confirming that the enzyme did not belong to the class of serine and cysteine protease, respectively (Table 6.2).

6.3.6 Cytotoxicity analysis of purified β-1,3 glucanase

Cytotoxic effects on HeLa cells were studied at different concentrations of enzyme in the range of 0, 1, 3, 5, 10, 25, 50,100 and 250 μg ml^{-1} enzyme. There were no significant visible cytopathic effects at any of the concentrations tested (Fig.6.6) and 236.87±1.89μg ml^{-1} was the LD_{50} dose (50 % inhibition) (Fig.6.5).The addition of 1 to 3 μg ml^{-1} enzyme resulted in the increase in cell number (Fig.6.6).

6.3.7 Application of β-1, 3 glucanase in fungal DNA extraction

Optimization of pH for DNA extraction was accomplished over a pH range of 5-10 using 50mM sodium acetate for pH 5 to 6, 50 mM Tris-Cl for pH 7 to 10 at 25°C for 30 min. The enzyme exhibited good lytic activity on cells of *Saccharomyces cervisiae* from pH 5 to 10 with

its optimum at 7.0 with a DNA yield of 231.66±5.20 µg μl^{-1} (Fig. 6.7). The statistical analysis revealed that pH had a significant (p<0.05) difference in the DNA yield between pH 8 and 9. However, there was no significant (p>0.05) difference in the DNA yield between pH 5 and 6, 7 and 8, 9 and 10 (Appendix 4, Tables 4.3a to 4.3c). The gel image of DNA extracted from at various pH is give in Fig.6.10a.

The enzyme was found to have good cell lysis from 25 to 75°C with its optimum at 65°C with a DNA yield of 310±2.5 µg μl^{-1} (Fig.6.8). There was a significant (p<0.05) difference in the DNA yield only from temperature range from 45 to 65°C (Appendix 4, Tables 4.4a to 4.4c). The gel image of DNA extracted at various temperatures is represented in Fig. 6.10b.

The DNA yield reached maximum after 60 min incubation (321.66±5.2 µg μl^{-1}) (Fig.6.9). The DNA yield was found to be significantly (p<0.05) different from 20 to 30 min incubation (Appendix 4, Tables 4.5a to 4.5c). The gel image of DNA extracted at various time intervals from 10 to 60 min is represented in Fig. 6.10c.

6.3.8 DNA extraction from different fungal species

The DNA extracted from various fungal (Fig.6.11) species along with their DNA yield and quality is described in Table 6.3.

6.3.9 PCR amplification of ITS region

ITS amplification for fungi yielded an expected product size of 590 bp (Fig.6.12).

6.3.10 Microscopic examination of β-1, 3 glucanase treated ruptured fungal hyphae and yeast cells under phase contrast microscope and comparison with untreated cells (control)

For the examination of fungal cell rupture on treatment with β-1,3 glucanase lysed cells were observed under phase contrast microscope and

compared with that of control (untreated cells).The effect of β-1,3 glucanase enzyme on fungal cells is represented in Fig.6.13. The cells lost their normal appearance on treatment with enzyme when compared to that of the control.

Table 6.1 Purification profile of β-1,3 glucanase of *Pseudomonas aeruginosa* MCCB 123

Purification step	β-1,3 glucanase activity(UmI^{-1})	Total Protein (mg)	Specific activity (U/mg)	Purification fold
Culture filtrate	359.71	8.5	42.31	0
$(NH4)_2SO_4$ Precipitation	188.08	4.09	45.98	1.08
DEAE-cellulose chromatography	98.74	0.245	403.02	9.52

Table 6.2 Effect of inhibitors on β-1,3 glucanase activity

Inhibitors	Concentration	Relative activity (%)
Control		100
EDTA	10 mM	57.02
PMSF	2 mM	100
1,10 Phenanthroline	5 mM	83.34
TLCK	0.1 mM	100
Leupeptin	50 µM	84.22
Pepstatin	10 µM	81.51
Phosphoramidon	0.1 mM	95.62

Table 6.3 Fungal species used for DNA extraction along with their DNA yield and quality

Fungal species	code	Culture Conditions	Temp	DNA yield ($\mu g\ \mu l^{-1}$)	DNA purity (OD260/280)
Saccharomyces cervisiae	MTCC 1766	YEPD	30°C	280 ± 7.5	1.10 ± 0.03
Candida albicans	MTCC 854	MYA	37°C	266.66 ± 5.20	1.14 ± 0.03
Aspergillus flavus	MTCC 277	CYA	30°C	164.16 ± 8.77	1.07 ± 0.04
Aspergillus foetidus	MTCC 151	CYA	30°C	188.33 ± 8.77	1.10 ± 0.07
Aspergillus ochracens	MTCC 1810	CYA	35°C	154.16 ± 3.81	1.16 ± 0.02
Phanerochaete chrysogenum	MTCC 787	MEA	25°C	156.66 ± 7.63	1.11 ± 0.07
Fusarium solani	MTCC 350	PSA	30°C	170.83 ± 10.10	1.13 ± 0.05
Acremonium diospyri	MTCC 1316	PDA	25°C	311.66 ± 8.77	1.19 ± 0.003
Heterobasidion annosum	MTCC 146	YGA	25°C	183.33 ± 5.20	1.12 ± 0.01
Trichoderma reesei	MTCC 164	MEA	25°C	134.16 ± 3.81	1.10 ± 0.02
Pencillium citrinum	MTCC 2553	CYA	30°C	146.66 ± 3.81	1.17 ± 0.05
Pleurotus sajor-caju	MTCC 141	PDA	25°C	218.33 ± 5.20	1.17 ± 0.04
Pleurotus sajor-caju	MTCC 1806	PDA	25°C	227.5 ± 5	1.08 ± 0.02
Daedalea flavida	MTCC 145	YGA	25°C	247.5 ± 5	1.07 ± 0.01
Pleurotus ostreatus	MTCC 142	YGA	30°C	226.66 ± 3.81	1.11 ± 0.03
Pleurotus sapidus	MTCC 1807	PDA	25°C	224.16 ± 3.81	1.11 ± 0.02
Pleurotus ostreatus	MTCC 1803	PDA	25°C	255.83 ± 5.77	1.23 ± 0.02
Pleurotus fossulatus	MTCC 1800	PDA	25°C	226.66 ± 5.20	1.10 ± 0.006
Coriolus versicolor	MTCC 138	YGA	25°C	219.16 ± 5.20	1.10 ± 0.02
Trametes hirsuta	MTCC 136	YGA	25°C	240.83 ± 6.29	1.06 ± 0.02
Pycnoporus sanguineus	MTCC 137	YGA	25°C	219.16 ± 6.29	1.11 ± 0.05

CYA Czapek Yeast Extract Agar PSA Potato Sucrose agar MYA Malt extract agar
PDA Potato Dextrose agar YGA Yeast glucose agar

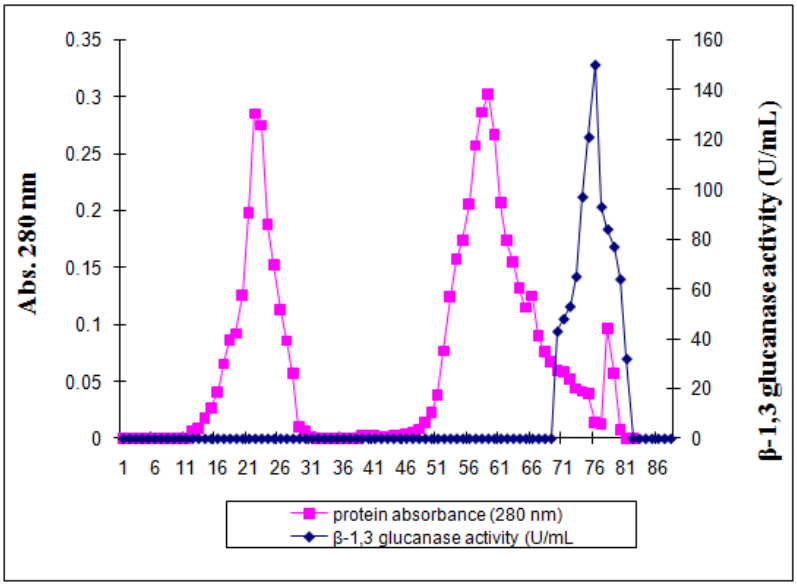


Fig .6.1 Elution profile of β-1,3 glucanase on DEAE-cellulose C16/40 column

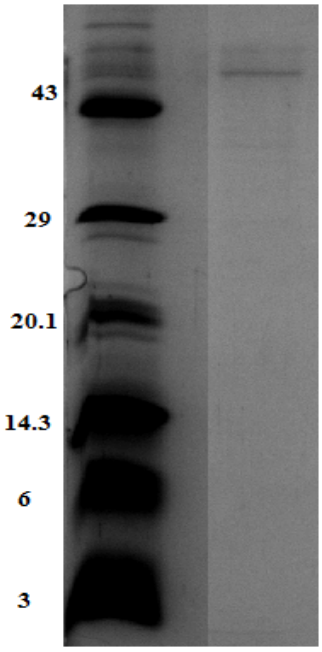


Fig. 6.2 SDS-PAGE profile of purified β-1,3 glucanase. Lane 1, Molecular weight marker; lane 2, 45 kDa β-1,3 glucanase enzyme

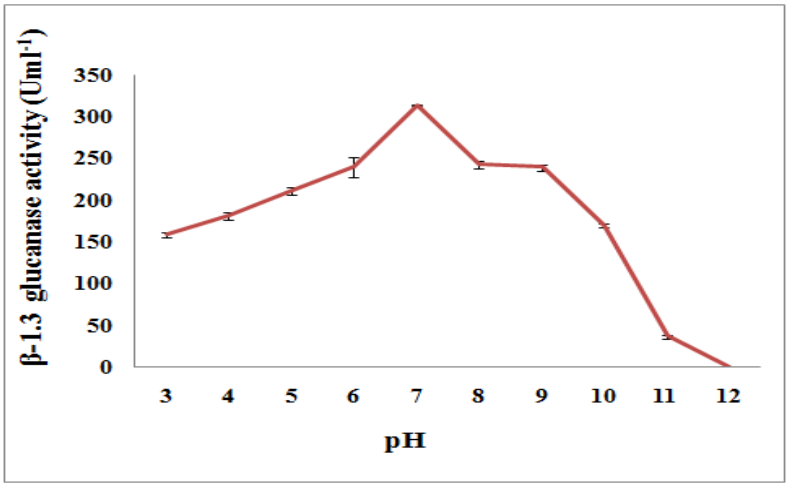


Fig.6.3 Optimization of pH for β-1,3 glucanase activity

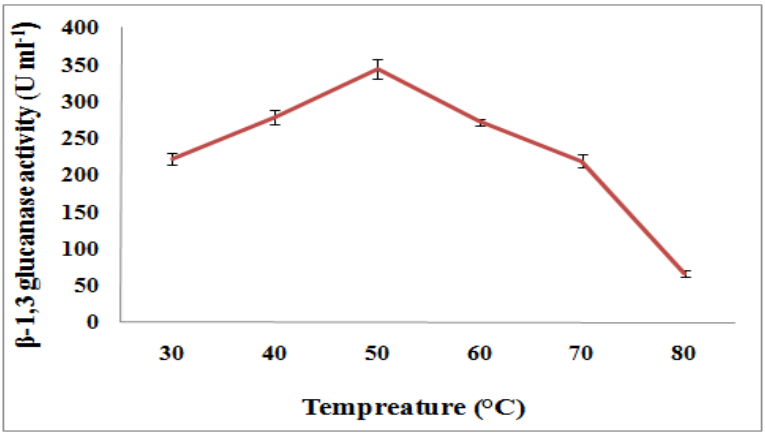


Fig. 6.4 Optimization of temperature for β-1,3 glucanase activity

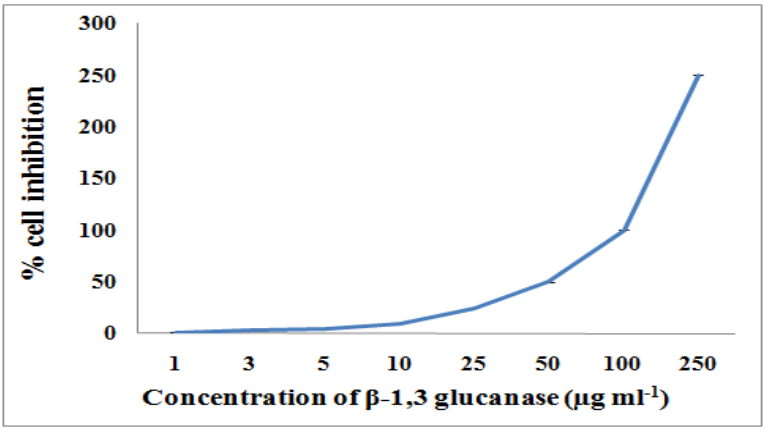
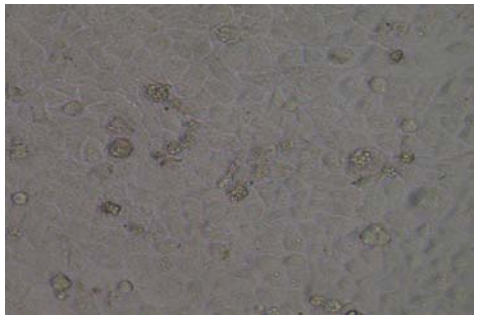
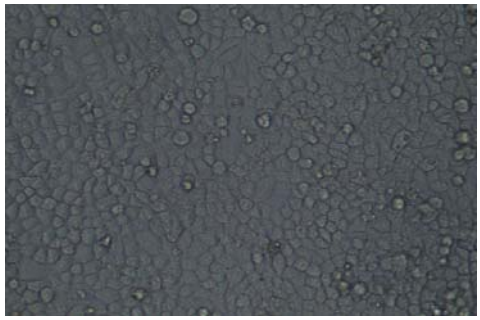


Fig. 6.5 Sigmoid curve for cytotoxicity of β-1,3 glucanase



(a)

(b)



(c)

(d)

Fig. 6.6 Cytopathic effects of β-1,3 glucanase on HeLa cell line. a) control cells without β-1,3 glucanase enzyme,b) 1µg ml^{-1} enzyme, c) 3 µg ml^{-1} enzyme, d) 250 µg ml^{-1} enzyme.The addition of 1 and 3 µg ml^{-1} enzyme resulted in the increase in the number of cells when compared to control

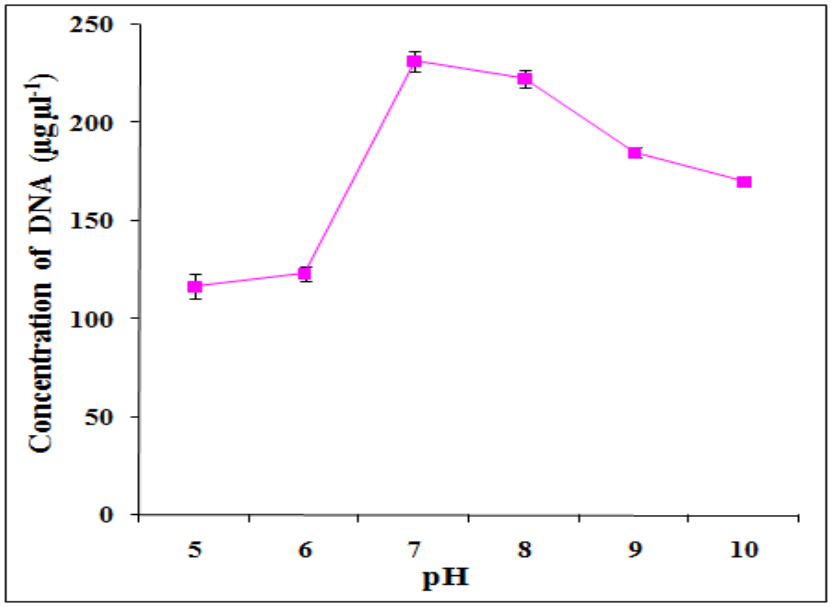


Fig.6.7 Determination of optimum pH of β-1,3 glucanase for fungal DNA extraction using *Sacchromyces cervisiae* MTCC 1766 as reference strain

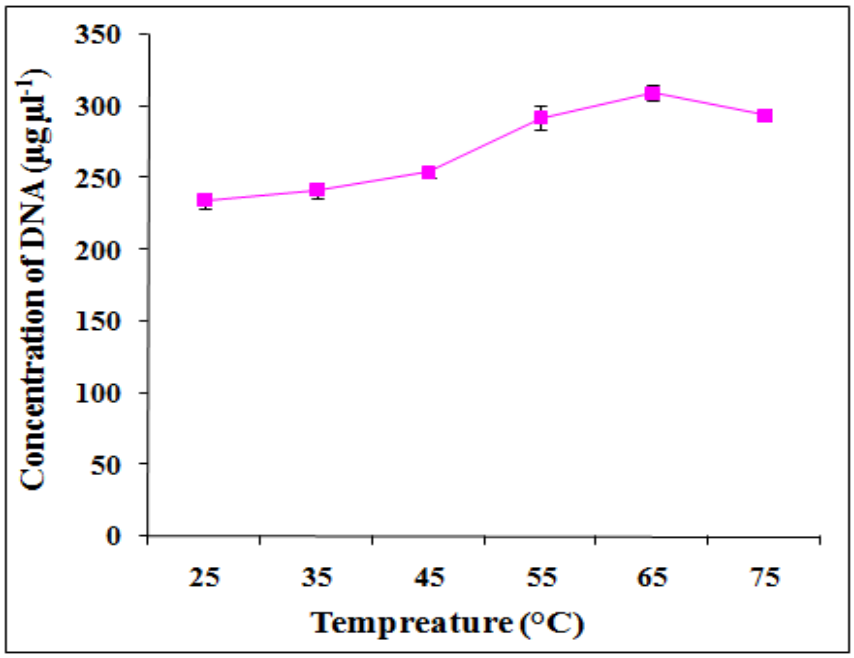


Fig.6.8 Determination of optimum temperature of β-1,3 glucanase for fungal DNA extraction using *Sacchromyces cervisiae* MTCC 1766 as reference strain

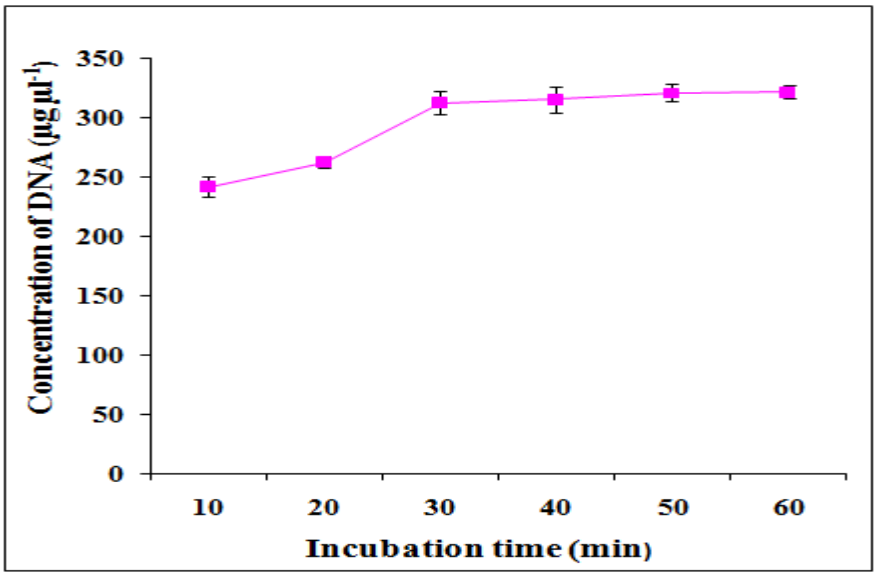


Fig. 6.9 Determination of optimum incubation time of β-1,3 glucanase for fungal DNA extraction using *Sacchromyces cervisiae* MTCC 176 as reference strain

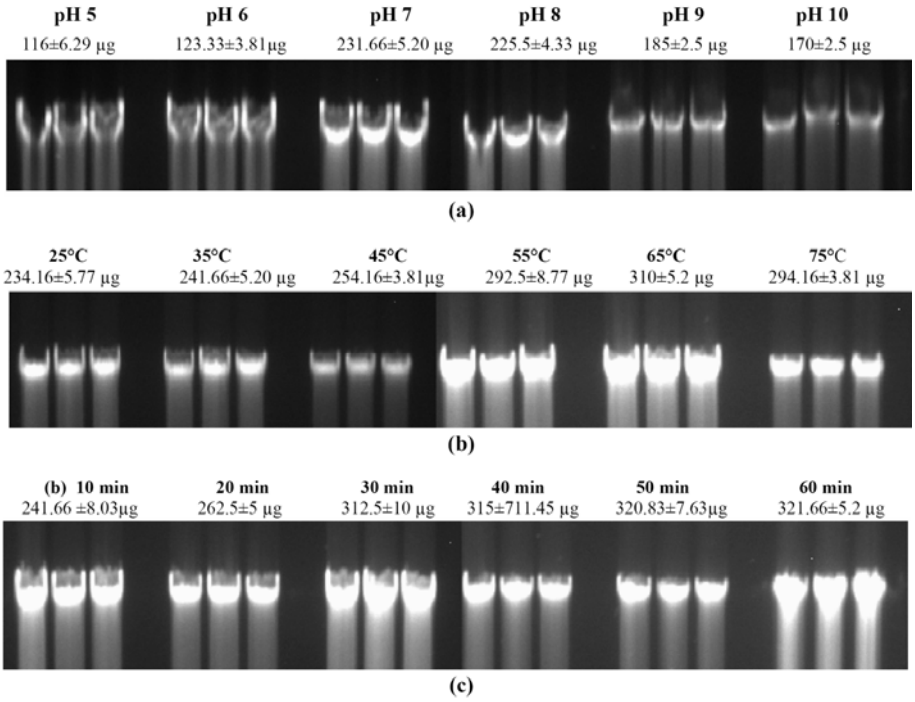
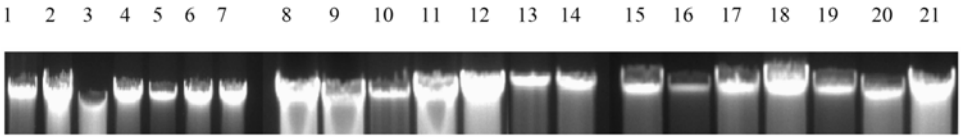


Fig. 6.10 Optimization of pH (a), temperature (b) and incubation time (c) for cell lysis for fungal DNA extraction using *Sacchromyces cervisiae* MTCC 1766 as reference strain. Concentration of DNA in µg μl^{-1} (average ±standard deviation is represented



Lane 1, *Aspergillus flavus* MTCC 277; lane 2, *Aspergillus foetidus* MTCC 151; lane 3, *Fusarium solani* MTCC 350; lane 4, *Acremonium diospyri* MTCC 1316; lane 5, *Phanerochaete chrysosporium* MTCC 787; lane 6, *Candida albicans* MTCC 854; lane 7, *Saccharomyces cerevisiae* MTCC 1766; lane 8, *Pleurotus sajor-caju* MTCC 141; lane 9, *Pleurotus sajor- caju* MTCC 1806; lane 10, *Pleurotus sapidus* MTCC 1807; lane 11, *Pleurotus fossulatus* MTCC 1800; lane 12, *Pleurotus ostreatus* MTCC 1803; lane 13, *Trametes hirsute* MTCC 136; lane 14, *Coriolus versicolor* MTCC 138; lane 15, *Aspergillus ochracens* MTCC 1810; lane 16, *Pencillium citrinum* MTCC 2553; lane 17, *Trichoderma reesei* MTCC 164; lane 18, *Heterobasidion annosum* MTCC 146; lane 19, *Pycnoporus sanguineus* MTCC 137; lane 20, *Pleurotus osterus* MTCC 142; lane 21, *Daedalea flavida* MTCC 145.

Fig. 6.11 DNA extracted from fungal strains by the lytic action of β-1,3 glucanase enzyme

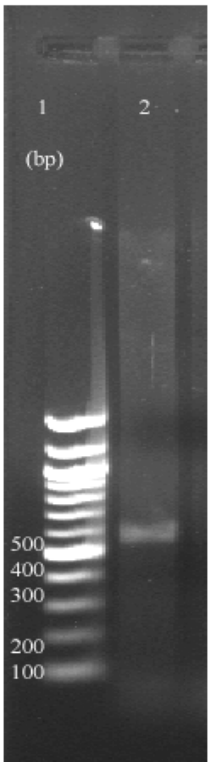
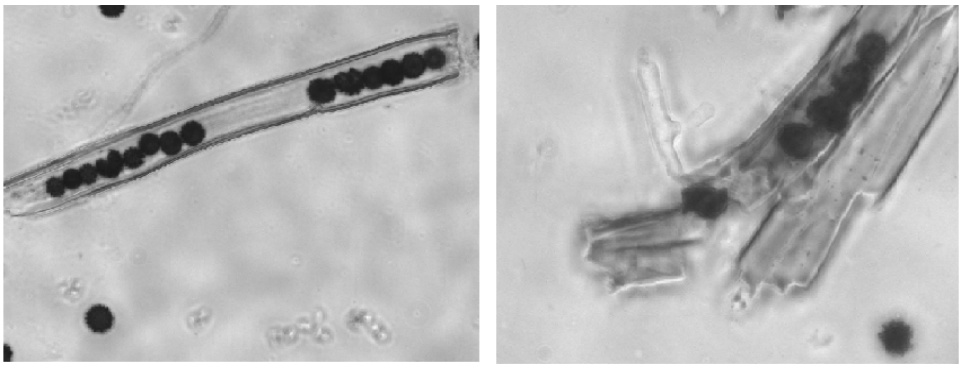


Fig. 6.12 PCR amplification of ITS region. Lane 1, 100 bp DNA ladder; lane 2, 540 bp amplicon of ITS region of *Saccharomyces cerevisiae* MTCC 1766

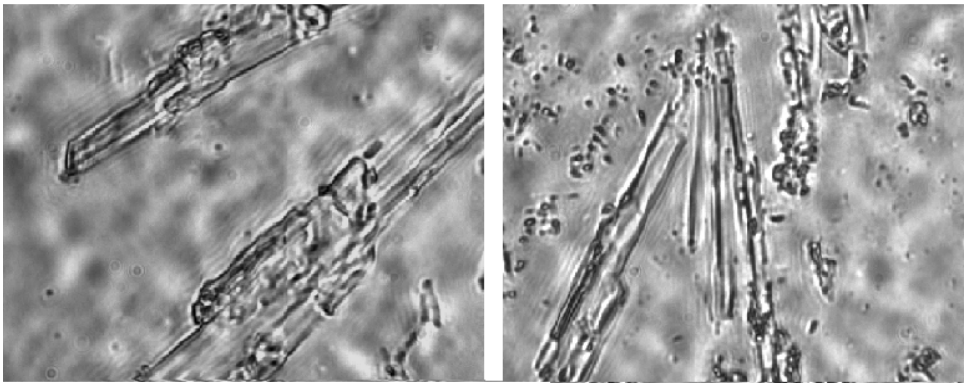
Fig.6.13 (a-k). Rupture of fungal hyphe on treatment with β-1,3 glucanase of *Pseudomonas aeruginosa* MCCB 123. Control represents untreated fungal hyphae and test represents the changes in fungal hyphae on treatment with 10 mg ml^{-1} of purified β-1,3 glucanase



Control **Test**

***Aspergillus foetidus* MTCC 151 (Magnification 60X)**

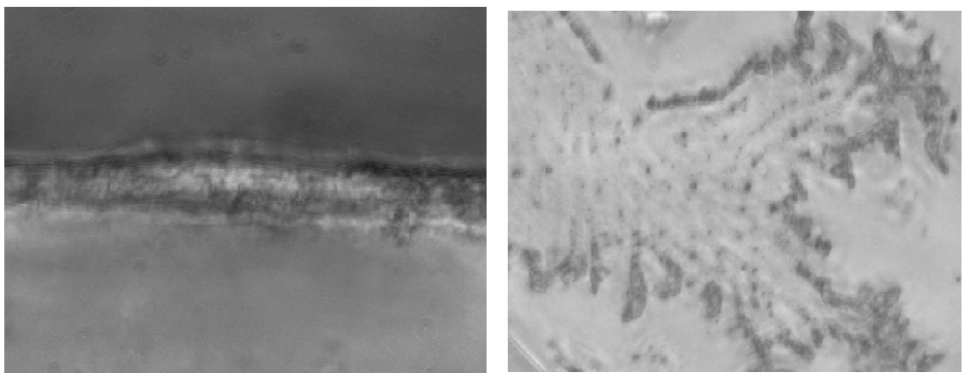
(a)



Control **Test**

***Aspergillus ochracens* MTCC 1810 (60X)**

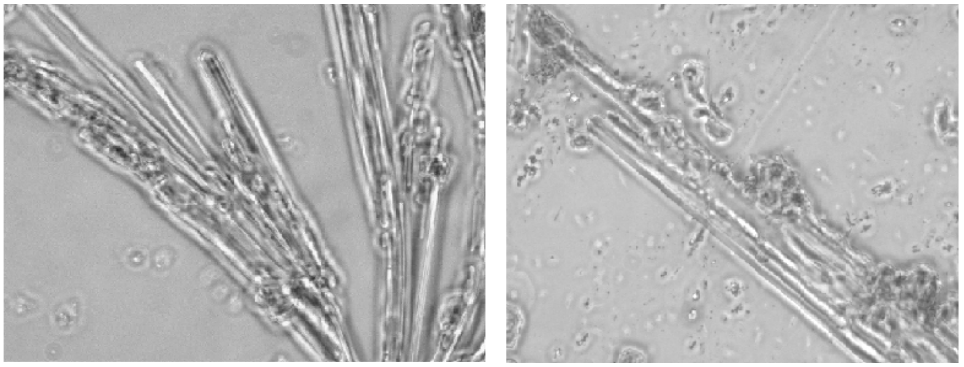
(b)



Control **Test**

***Aspergillus flavus* MTCC 277 (Magnification 60X)**

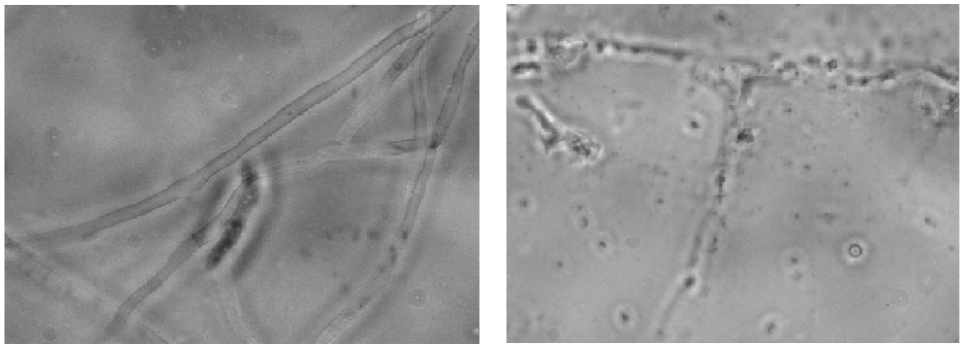
(c)



Control **Test**

***Acremonium diospyri* MTCC 1316 (Magnification 60X)**

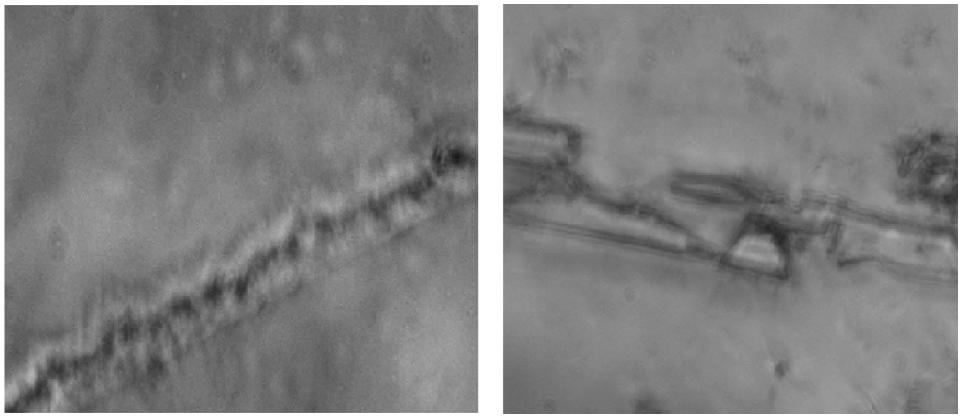
(d)



Control **Test**

***Fusarium solani* MTCC 350 (Magnification 60X)**

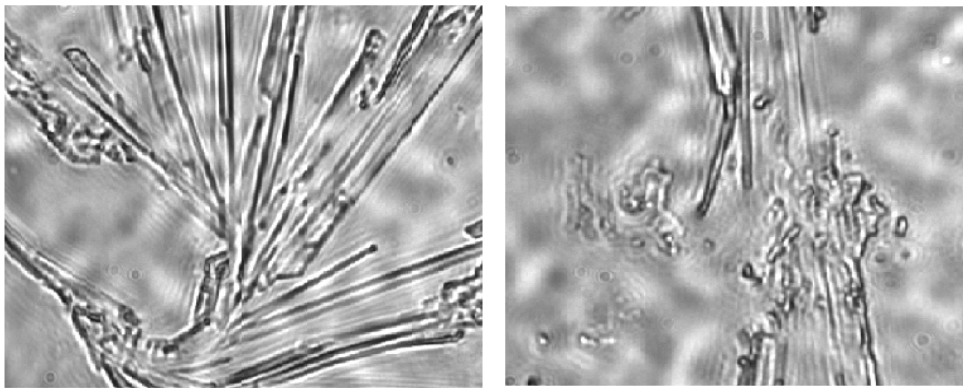
(e)



Control **Test**

***Phanerocheate chrysogenum* MTCC 787 (Magnification 60X)**

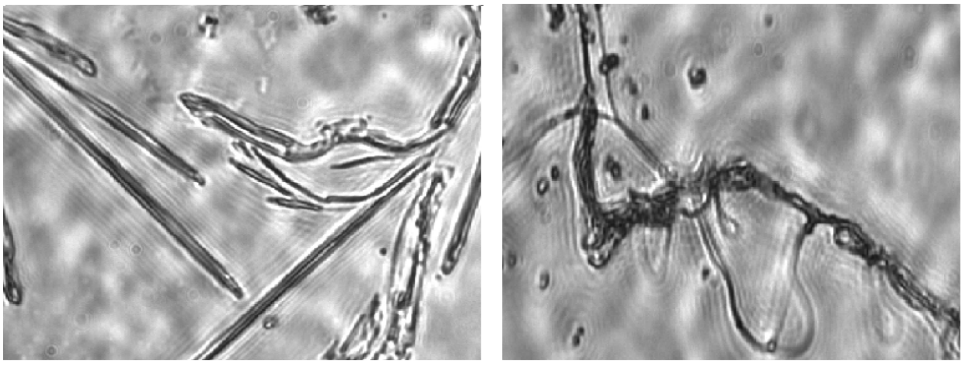
(f)



Control **Test**

Heterobasidion annosum **MTCC 146 (Magnification 60X)**

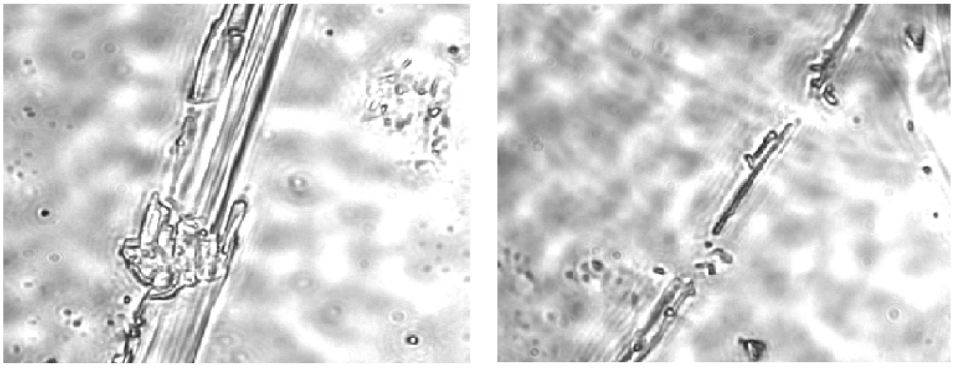
(g)



Control **Test**

Pencillium citrinum **MTCC 2553 (Magnification 60X)**

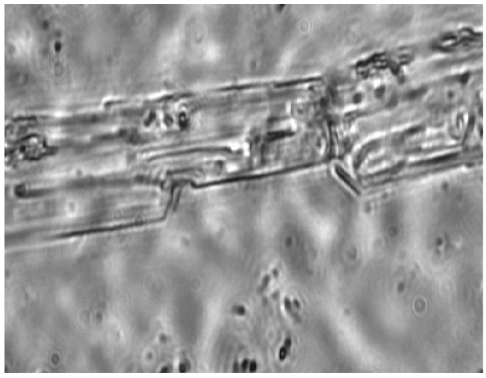
(h)

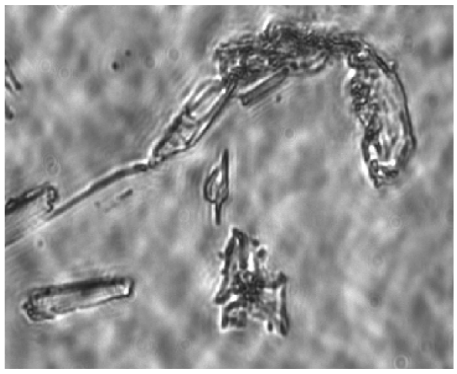


Control **Test**

Pleurotus fossulatus **MTCC 1800 (Magnification 60X)**

(i)

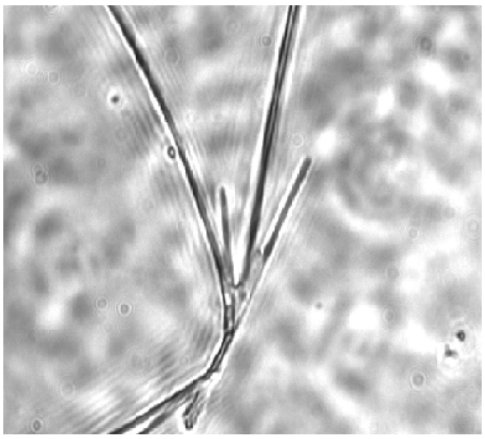


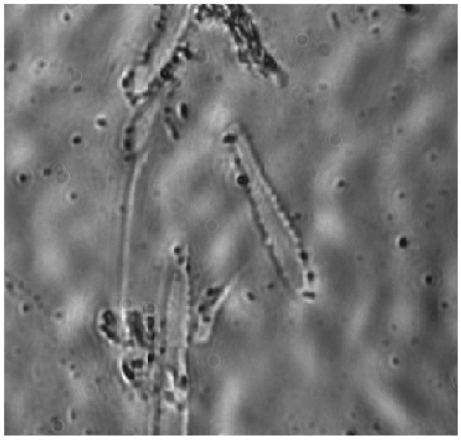


Control **Test**

***Pleurotus sajor-caju* MTCC 141 (Magnification 60X)**

(j)





Control **Test**

***Trichoderma reesei* MTCC 164 (Magnification 60X)**

(k)

6.4 Discussion

A β-1, 3 glucanase enzyme with a broad range of lytic activity on a broad range of fungal cell walls was purified from *Pseudomonas aeruginosa* MCCB 123. The enzyme was purified with 9.52 -fold increase in specific activity. The molecular mass of the enzyme was found to be 45 kDa by SDS-PAGE.

Enzyme inhibition studies primarily give an insight into the nature of the enzyme, its cofactor requirements and the nature of the active

centre (Sigma and Mooser, 1975). In the present study, β-1, 3 glucanase activity was only partially inhibited by the metal chelator EDTA. However, the activity was less sensitive to PMSF (serine protease inhibitor), TLCK (inhibitor of lysine specific serine protease), leupeptin, trypsin soybean inhibitor and phosphoramidon (specific inhibitor of *Pseudomonas aeruginosa* elastase). Since the β-1, 3 glucanase activity was inhibited by the zinc chelator, EDTA, and not by any other specific class of inhibitor, it can be concluded that MCCB 123 β-1,3 glucanase belongs to the class of metalloenzyme. An exo- β-1, 3 glucanase of *Rhizoctonia solani* also showed 29% loss in activity in the presence of EDTA (Vijayendra and Kashiwagi, 2009), while the activity of an endo-β-1, 3-glucanase from *Agaricus brasiliensis* is not significantly affected by EDTA (Shu et al., 2006).

The optimum pH for β-1, 3 glucanase activity was found to be at pH 7.0. This pH optima is different from the acidic pH optimum reported for *Pseudomonas stutzeri* YPL-1 (Lim and Kim, 1995). Other reported microbial endo and exo β-1, 3 glucanases also have an acidic pH optimum. Ferrer et al. (1996) observed an acidic pH optimum of 4.0 for β-1, 3 glucanase activity of *Oerskovia xanthineolytica* LL G109 (*Cellulomonas cellulans*), 5.2 for β-1, 3 glucanase from *Ascochyta rabiei* (Hanselle and Barz, 2001); 5.0 for exo-β-1,3-glucanase of *Rhizoctonia solani* (Vijayendra and Kashiwagi, 2009); 4.5 for GluC β-1,3 glucanase gene product and 4.5 and 5.0 for GluB β-1,3 glucanase gene product of *Lysobacter enzymogenes* Strain N4-7 (Palumbo et al., 2003).Thus, MCCB 123 β-1,3 glucanase differs from other reported glucanases with respect to its pH optimum. However, the pH optimum observed for β-1, 3 glucanase described in the present study was within the range of optimal pH of 6.5-9.5 for β-1, 3 glucanase activity from *Bacillus subtilis* NSRS 89-24 (Leelasuphakul et al., 2006) and close to that from *Chaetomium* sp which was reported to have an optimum pH of 6.0 (Sun et al., 2006) and that reported from *Pichia pastoris* (Xu et al., 2006).

The optimum temperature for the activity of MCCB 123 β-1, 3 glucanase was found to be 50°C. Similar temperature optima were reported for the activity for β-1, 3 glucanase from *Bacillus subtilis* NSRS 89-24 (Leelasuphakul et al., 2006). The temperature optima for GluC and GluB β-1, 3 glucanases produced by *Lysobacter enzymogenes* strain N4-7 was found to be 45°C and 41°C respectively (Palumbo et al., 2003). A temperature optimum of 40°C is reported for β-1,3 glucanase from *Pseudomonas stutzeri* YPL-1 (Lim and Kim, 1995), *Rhizoctonia solani* (Vijayendra and Kashiwagi, 2009), a temperature optimum of 45°C is reported for *Agaricus brasiliensis* ATCC 76739 (Shu et al., 2006) and a lower temperature optimum of 30°C was observed for β-1,3-glucanase from *Chaetomium* sp (Sun et al., 2006).

The cytotoxicity of MCCB 123 β-1, 3 glucanase on HeLa cells was found to increase with concentration indicating that the cytotoxicity is dose dependent. LD_{50} value on HeLa cell line was found to be 236.87±1.89 μg ml^{-1}. It is also noted that supplementation of β-1, 3 glucanase from 1-3μg ml^{-1} was found to have an inducing effect on the growth of HeLa cells suggesting its possible application in cell culture.

Only a few reports are available on the production and lytic action of β-1, 3 glucanase from *Pseudomonas aeruginosa* (Kumar et al., 2009; Singh et al., 2010) and the reported studies have not given emphasis to the characterisation of the enzyme. In this context, characterization studies of MCCB 123 β-1, 3 glucanase and understanding its novel properties are very relevant. β-1, 3 glucanase of *Pseudomonas aeruginosa* MCCB 123 was found to have lytic action on a broad range of fungal and yeast strains. Since MCCB 123 glucanase is able to hydrolyse the *Saccharomyces cerevisiae*, it may be through the cleaving action of β (1→3)-$_D$-glucan of the cell wall. In the yeast *Saccharomyces cerevisiae*, the cell wall contains β (1→3)-$_D$-glucan, β (1→6)-$_D$-glucan, chitin and mannoprotein (Kollar et al., 1997). Salazar and Asenjo (2007) reported

that the lysis of yeast cell wall begins with binding of lytic protease to the outer mannoprotein layer of the wall. The protease opens up the protein structure releasing the wall proteins and mannans and exposing the glucan surface below. The glucanase then attacks the inner wall and solubilise the glucan. In vitro, this enzyme cannot lyze yeast in the absence of reducing agents, such as dithiothreitol or b-mercaptoethanol, because the breakage of disulphide bridges between mannose residues and wall proteins is necessary for appropriate exposition of the inner glucan layer. When the combined action of the protease and glucanase has opened a sufficiently large hole in the cell wall, the plasma membrane and its content are extruded as a protoplast.

However, β-1,3 glucanase of *P. aeruginosa* MCCB 123 alone is sufficient for the lysis of yeast cell lysis as evident from its lytic action on different yeast strains such as *Saccharomyces cerevisiae* and *Candida albicans*. Type 1 β-1,3 glucanases are capable of readily solubilising yeast glucan and inducing complete lysis of viable yeast cells, while members of type II β-1,3 glucanases has limited capacity to solubilise glucan (Doi and Doi, 1986). Thus, MCCB 123 β-1,3 glucanase belongs to type I β-1,3 glucanase since it exhibited good hydrolytic activity on yeasts such as *Saccharomyces cerevisiae* and *Candida albicans*.

β-1, 3 glucanase is reported to have lytic action on fungal cell walls (Jones et al., 1974; Reiss, 1977; Lim et al., 1991; Lim and Kim, 1995; Lahsen et al., 2001; Sun et al., 2006). The structure of fungal cell wall is highly complex which consists of thick layers of chitin, (1-3)-β –D-glucans, 1-6 β-glucans, lipids and peptides (Karakousis et al., 2006) and the yeast cell wall is composed of complex polymers such as β-1,3 and β-1,6 glucans, mannoproteins and smaller amounts of chitin (Ferrer, 2006) which implies that synergetic action of several enzymes is necessary to hydrolyse these components. The lytic action of *P. aeruginosa* MCCB

123 enzyme on fungal cell walls is supported by its β-1, 3 glucanase activity thereby it is shown to hydrolyze β-1, 3 glucan component of fungal cell wall causing subsequent cell rupture. β-1, 3 glucanase is reported to have action on fungal cell wall resulting in the degradation and loss of inner contents of cells (Benhamou et al., 1996). Exo and endo glucanases were found to have hydrolytic action on cell walls of plant fungal pathogens. Yang et al. (1993) reported the degradation of cell walls of *Rhizoctonia solani, Gibberella*, *zeae, Fusarium sp., Colletotrichum gloeosporioides* and *Phoma sp.* by the action of β-1, 3 glucanase. The cooperative action of endo and exo β-1,3 glucanases from parasitic fungi *Coniothyrium minitans* and *Trichoderma viride* was reported to degrade cell wall glucans of *Sclerotinia sclerotiorum* (Jones et al., 1974). Reiss (1977) reported the serial enzymatic hydrolysis of cell walls of the yeast *Histoplasma capsulatum* with α (1→3)-Glucanase and β-(1→3)-Glucanase. Lahsen et al. (2001) reported the lytic activity and antifungal activity of exo-type α -1, 3-glucanase against fungal plant pathogens.

As the structure of fungal cell wall is highly complex consisting thick layers of chitin, (1-3)-β –D-glucans, 1-6 β-glucans, lipids and peptides and tough surface layer of melanin on which most enzymes fail to lyse, development of a single universal fungal DNA extraction method has significance and has not been accomplished so far. This has paved the way for developing DNA extraction methods using a combination of different disruption methods. Karakousis et al. (2006) employed the use of digestive enzymes, mechanical disruption methods like freezing in liquid nitrogen, grinding with mortar and pestle, sonication, glass bead milling and microwaving and non mechanical disruption methods such as treatment with alkaline chemicals, detergents and other chemicals for DNA extraction from medically important fungi. DNA extraction from ectomycorrhizal basidiomycete *Tylospora fibrillose* Donk was carried out by freezing in liquid nitrogen and three cycles of thawing at 65°C to break

the cell walls, grinding in sterile sand followed by phenol-chlorofom extraction (Erland et al., 1994). DNA extraction from white rot fungi involves the use of CTAB, mercaptoethanol in the lysis buffer followed by chloroform-3-methyl-1-butanol extraction and ethanol precipitation (Kuhad et al., 2004). DNA extraction from mycorrhizal fungi was carried out by crushing in pestle followed by three alternate cycles of freezing in liquid nitrogen and incubation at 100°C for 1 min followed by freezing and final incubation at 100° for 10 min and purification using DNA columns (Manian et al., 2001). A bead beating methods involving the use of glass beads for crushing the cells followed by phenol-chlorofom extraction was used for DNA extraction from filamentous fungi (Plaza et al., 2004). Enzymatic cell lysis using lyticase followed by bead beating was employed for DNA extraction from filamentous fungi in biofilms (Saad et al., 2004). Accordingly, there exists no single method for fungal DNA extraction. Most of the lysis buffers for DNA extraction include SDS. (Dean et al., 1994; Erland et al., 1994; Haugland et al., 1999; Plaza et al., 2004).

From the above literature, it is understood that no single protocol appropriate for cell lysis for all fungi does exist and each species requires a specific method for efficient DNA extraction (Manian et al., 2001; Karakousis et al., 2006) and many of the fungal DNA extraction methods are often laborious, expensive and time consuming (Plaza et al., 2004). Existing methods for genomic DNA preparation from fungi take several hours to complete (Muller et al., 1998; Sambrook and Russell et al., 2001).

Therefore, in this context developing a single extraction method for fungal DNA is a desirable preposition. DNA could be extracted from 21 fungal species by the lytic action of the purified β-1, 3 glucanase from *P. aeruginosa* MCCB 123 without the addition of other chemicals and

mechanical treatments which makes this method unique among the methods reported. The extracted DNA could be directly used for PCR amplification without further purification. The above properties make this enzyme unique among the lytic enzymes used in fungal DNA extraction. Moreover, the method is inexpensive since it employs only β-1,3 glucanase as the sole reagent, the quality and quantity of DNA obtained is suitable for molecular assays and it doesn't require the use of expensive and specialised equipment or hazardous reagents.

The quality of the extracted nucleic acid is important for further processing. Samples with mean A_{260}/A_{280} ratios below 1.8 were presumed to contain protein or other contaminants, whereas samples with ratios above 2.0 were presumed to be due to the presence of RNA (Sambrook and Russell, 2001). However, nucleic acids preparations free of phenol should have Abs $_{260/280}$ ratios near 1.2 (Sambrook and Russell, 2001; Lemarchand et al., 2005). In the case of DNA extracted with β-1,3 glucanase from various fungal species, $Abs_{260/280}$ ratio was found to be in the range of 1.0 to 1.1, and phenol had not been used in the process and thus it could be concluded that the DNA extracted using this method was free of any contamination and was suitable for PCR amplification. The amplicon obtained from the PCR amplification of the ITS region was in agreement with previous workers (Dean et al., 2004).

Fungal DNA extraction using MCCB 123 β-1,3 glucanase has several advantages. First of all, the number of steps in DNA extraction procedure was minimized by replacing phenol chloroform extraction method and it also doesn't involves the addition of any detergents or other lytic agents and other mechanical lytic methods such as grinding with sand, repeated freeze thaw cycles in liquid nitrogen. Secondly, several samples can be processed within a short time period of 30 min. Thirdly, the method yielded high quality DNA compared with standard phenol-chloroform protocol. Fourthly, this method

seems to be very cost-effective since β-1, 3 glucanase alone is used as the sole reagent. Finally, this extraction method is applicable to a broad range of fungal species. This makes this enzyme unique over all other lytic enzymes. This is the first report of a lytic enzyme alone being employed in fungal DNA extraction without the addition of detergents such as sodium dodecyl sulphate (SDS) and incorporating other mechanical lytic steps. In this context, broad range of lytic activity of glucanase on a wide range of fungal cells has immense benefits in DNA extraction.

Chapter 7

SUMMARY AND CONCLUSIONS

The study focuses on the hydrolytic extracellular enzymes from an environmental isolate of *Pseudomonas aeruginosa* having bio-medical and industrial applications. In this regard a detailed account of two proteases such as LasA and LasB, and a β-1,3 glucanase produced by the organism *P. aeruginosa* MCCB 123 is given. The producer strain was identified on the basis of biochemical characteristics and 16S rRNA gene sequencing. Process optimization, purification, and characterization of these enzymes have been carried out and their possible applications have been either experimented with or highlighted.

Following were the objectives of the investigation:

1. Screening and identification of the protease producing bacterial strain, *Pseudomonas aeruginosa* MCCB 123, and assessment of its pathogenicity.
2. Optimization of protease production from *Pseudomonas aeruginosa* MCCB 123,
3. Purification and characterization of LasB protease from *Pseudomonas aeruginosa* MCCB 123
4. Purification and characterization of LasA protease from *Pseudomonas aeruginosa* MCCB 123 and its application in bacterial DNA extraction
5. Purification and characterization of β-1,3 glucanase from *Pseudomonas aeruginosa* MCCB 123 and its application in fungal DNA extractions

Major findings are summarised as given below:

- The potent protease producing bacterial isolate was identified based on phenotypic and molecular characterisation. The 16S rRNA gene sequence on comparison with GenBank database showed 100% similarity to 16S rRNA gene of *Pseudomonas aeruginosa* and thus the identity of the isolate was confirmed as *Pseudomonas aeruginosa*.
- Analysis of type III secretion toxin genes revealed the presence of gene of invasive phenotype (*exoS* gene), while cytotoxic gene, *exoU,* was absent. However, it was neither capable of invasion nor adhesion to human epithelial cell lines as evidenced by the invasion and adherence assays on Hep-2 and HeLa monolayers. The possible reason for the non invasiveness is the non-secretion of the effector proteins responsible for invasion by this bacterium. The presence of the virulence gene *exoS* and the absence of *exoU* gene in *Pseudomonas aeruginosa* MCCB 123 suggested the genetic differences between the environmental isolate and the other reported clinical isolates.
- Even though, *P. aeruginosa* MCCB 123 exhibited motility and the capability to produce biofilm it was sensitive to majority of antibiotics like pencillins, cephalosporins, aminoglycoside, fluroquinolones, polymixin and carbapenems suggesting that the biofilm could be either controlled or eliminated by the antibiotic therapy.
- Optimization of total protease production could be accomplished by employing Plackett-Burman and Face Centred Central Composite Design of Response Surface Methodology.
- The analysis of variance (ANOVA) of the quadratic model showed that the model was adequate with no significant lack of fit (prob> F = 0.9762).

- The observed R^2 value 0.9536 explains that the fitted model could explain 95.36% of the total variation and the model did not explain 4.64 % of the same, thus indicating the good response prediction.
- An adequate precision value of 18.326 suggested that the polynomial quadratic model was of an adequate signal, and could be used to navigate the design space.
- A relatively lower value of coefficient of variation (CV=1.65 %) indicated a good precision and reliability of the experiment.
- In this study, the linear terms, A (casein), C (KH_2PO_4) and quadratic terms of factors, (AB: casein and $NH_4H_2PO_4$, AC: casein and KH_2PO_4 and BC: $NH_4H_2PO_4$ and KH_2PO_4) significantly influenced the protease production in *Pseudomonas aeruginosa* MCCB 123.
- The elliptical nature of the contour plots in the case of protease activity of *P. aeruginosa* MCCB 123 indicated that the interaction effects between casein and $NH_4H_2PO_4$ (p>F value is <0.0001), casein and KH_2PO_4 (p>F value is 0.0200) and $NH_4H_2PO_4$ and KH_2PO_4 (p>F value is 0.0013) were found to be most significant for protease activity.
- The optimization of fermentation medium resulted in 1.84 fold increase in protease activity.
- Statistical techniques such as Plackett-Burman design and Response Surface Methodology helped in reducing the cost of production and thus made the process more economical. *P. aeruginosa* MCCB 123 secreted almost similar protease titres in optimized medium in shake flask conditions (24479±83.22 Uml^{-1}) and in fermenter (24754.17±7.21 Uml^{-1}) suggesting a good scope for the scale up of enzyme production.
- A potent LasB protease/elastase with very high protease and elastase activity was purified from *P. aeruginosa* MCCB123. The

enzyme was classified as a zinc metalloprotease based on its inhibitory profile on protease and elastase activity.

- The molecular mass of the protease was found to be 33 kDa in reducing state, while in native conformation approximately 96 kDa was obtained indicating that it was a complex of three monomers.
- The optimum pH and temperature for protease activity were found to be 9.0 and 60°C, respectively, and that of elastase activity 8.0 and 40°C. Effect of pH on LasB protease stability showed that the enzyme was stable from pH 7 to10 for one hour and retained more than 90 % of its residual activity from pH 7 to 9. The enzyme exhibited more than 80% protease activity from 30 to 50°C.
- The cytotoxicity of MCCB 123 LasB protease on Hep-2 cells was found to be dose dependent and the LD_{50} value was 47.28±1.28 $\mu g\ ml^{-1}$. Cytotoxic effect such as cell rounding and cell death at higher concentrations was observed.
- The sequencing of 1500 bp LasB protease gene of *P. aeruginosa* MCCB 123 shared 98% similarity to LasB gene/elastase gene of *Pseudomonas aeruginosa* (Accession nos.M19472, AB029328, M24531, EU021222, DQ350610, DQ153386, DQ150629) and 97% similarity to LasB gene of *Pseudomonas aeruginosa* (Accession no. EU265777) and preproelastase gene of *Pseudomonas aeruginosa* (Accession no. JF502075).
- The enzyme belonged to M4 neutral protease GluZincin superfamily.
- The deduced amin ocid sequence of LasB protease gene exhibited sequence homology to hypothetical protein of *Pseudomonas aeruginosa*, elastase LasB precursor of *Stenotrophomonas maltophilia*, keratinase of *Pseudomonas aeruginosa,* class 4 metalloprotease of *Chromobacterium violaceum,* Neutral protease of *Collimonas*

fungivorans Ter331 (YP004752195), elastase of *Aeromonas hydrophila subsp. hydrophila*, metalloprotease of *Aeromonas sobria*, Zinc metalloprotease (elastase) of *Reinekea* sp. MED29, neutral protease precursor of *Vibrio cholerae* TMA 21, hemagglutininproteinase of *Vibrio cholerae* and vibriolysin of *Vibrio cholera.*

- The deduced amino acid sequence showed that the preproenzyme consisted of 497 aminoacids, the propeptide sequence of 174 amino acids and the mature LasB protease of 300 amino acids.
- The typical metalloendopeptidases consensus zinc-binding sequence HEXXH and the catalytic residues in the active site were conserved in the MCCB 123 LasB gene. The amino acid representing the calcium ligands, zinc ligands and active centre are identified in the MCCB123 LasB protease sequence.
- Comparison of MCCB 123 LasB protease activity with that of the commercial protease Savinase® from *Bacillus* sp. showed that the specific protease activity of purified MCCB 123 LasB was 3.96 fold greater suggesting its superior quality in industrial applications and the possible economic viability.
- The optimum activity of MCCB 123 LasB protease in the alkaline pH is a very important characteristic for its use as a laundry ingredient, in leather processing, and other industrial processes that are carried out in the alkaline pH range.
- MCCB 123 LasB protease was active over a wide range of temperatures from 30 to 80°C with its optimum at 60°C, and at 30°C also it was found to be well active. This property could be of great advantage in detergent industry, which is now looking for alkaline proteases that works well under room temperature conditions, as this would facilitate washing under ambient

temperatures, a pre-requisite to maintain fabric quality and also for reducing the energy demand.

- The metal ions such as Ba^{2+}, Ca^{2+} and Mg^{2+} imparted a slight increase in the protease activity and the enzyme also showed reasonable stability in the presence SDS and H_2O_2. Stability of protease activity in presence of alkaline pH, detergents, oxidizing agents and enhancement of activity in presence of metal ions like calcium and magnesium make it an ideal choice in detergents for hard water washes.
- A 20.5 kDa LasA protease with a broad range of bacteriolytic activity was purified from *P. aeruginosa* MCCB 123 with 27.51 fold increase in specific staphylolytic activity. The protease was classified as a zinc-dependent metalloprotease based on its inhibition profile on staphylolytic activity.
- The sequencing of 1200 bp LasA protease gene of *P.aeruginosa* MCCB 123 showed 98% similarity to LasA gene of *Pseudomonas aeruginosa* (Accession nos.U68175 and X55904) and 97% similarity to LasA gene of *Pseudomonas aeruginosa* (Accession no.M20982).
- Sequence homology study of the deduced amino acid revealed that the LasA protease of *P. aeruginosa* MCCB 123 showed homology to LasA protease precursor of *Pseudomonas aeruginosa* (Accession nos. ZP04933587, YP 002441038, ZP 04928288, EG M13284, YP 791368, NP 250562, ZP 07796462, ZP 06879207, YP001348777). It also exhibited sequence homology to peptidase M23B of *Shewanella denitrificans* (Accession no.YP 563624) and *Shewanella baltica* (accession no.YP 001052575) and β-lytic protease of *Lysobacter* sp. IB93 (BAB86844).
- Classification analysis based on deduced amino acid sequence demonstrated that the mature protease is a zinc metalloproteinase of M23A class of metallopeptidase family

- The HXH motif, which is the putative zinc binding motif is identified in the amino acid sequence of mature LasA protease of *P.aeruginosa* MCCB 123. The amino acid sequence of the mature LasA protease showed the presence of conserved sequences in M23A family, metal ligands, residues with potential mechanistic roles, and cysteine residues involved in LasA disulphide bonds.
- The cytotoxicity of MCCB 123 LasA protease on HeLa cells was found to be dose dependent with an LD_{50} value of 89.43±3.11µg ml^{-1}. Cytotoxic effects such as cell rounding and cell death at higher concentration were observed.
- The purified LasA protease of *P aeruginosa* MCCB 123 could lyse the cell wall of a broad range of Gram-positive and Gram-negative bacteria and is the first report on the lytic action of LasA protease of *P. aeruginosa* on bacteria other than on *Staphylococcus aureus*.
- Its wide range of bacteriolytic activity on a broad range of Gram-negative and Gram-positive bacterial cells can be exploited in DNA extraction without the addition of detergents or other chemical agents. The extracted DNA could be directly used for PCR amplification. The optimum pH and temperature for bacterial DNA extraction was found to be 7 and 35°C respectively.
- The advantages of bacterial DNA extraction using MCCB 123 LasA protease include minimizing the number of steps in extraction procedure by replacing phenol chloroform extraction method and with out the involvement of detergents or other lytic agents. Accordingly, several samples could be processed within a short time period of 30 min.
- The method seems to be very cost-effective since it only uses the lytic enzyme LasA protease as the sole reagent for DNA extraction.

- A β-1, 3 glucanase enzyme with a broad range of lytic activity on fungal cell walls was purified from *P. aeruginosa* MCCB 123. The enzyme was identified as metallozyme based on its inhibitory profile.
- The optimum pH for β-1, 3 glucanase activity was found to be 7.0 and was different from the other reported β-1, 3 glucanases which were with an acidic pH optimum. The optimum temperature for the activity of MCCB 123 β-1, 3 glucanase was found to be 50°C.
- The cytotoxicity analysis of MCCB 123 β-1, 3 glucanase on HeLa cells was found to be dose dependent with an LD_{50} value of 236.87±1.89µg ml^{-1}. It is also noted that supplementation of β-1, 3 glucanase from 1-3µg ml^{-1} was found to have an inducing effect on the growth of HeLa cells suggesting its possible application as a supplement in cell culture medium.
- MCCB 123 β-1, 3 glucanase was found to have lytic action on a broad range of fungal and yeast strains. This could be exploited in DNA extraction from 21 fungal species without the addition of other chemicals, and by having any mechanical treatments. This makes the method unique among the ones reported so far. The extracted DNA could be directly used for PCR amplification without further purification.

In short, through the present study, heterotrophic protease producing bacterial isolates were screened for protease activity and a potent protease producing bacterial isolate was selected, identified and coded as *Pseudomonas aeruginosa* MCCB 123. The organism was capable of producing three different types of enzymes each having potential industrial applications. The non-toxic nature of the bacterial strain and the relatively non-toxic nature of the three enzymes suggested their potential application in various industries. Application of LasA protease and β-1, 3 glucanase in DNA extraction is a

promising area for commercial utilization. LasB protease can find its potential application in detergent and tanning industries. As on today *Bacillus* sp. has been the source of commercial proteases, and the ones produced from *P.aeruginosa* 123 can pave way for making the industrial and biomedical processes more cost effective and refined.

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APPENDIX

Appendix -1

Table (1.1a): Effect of casein on protease production

Casein (g l^{-1})	Protease activity (Uml^{-1})
1	22583.33± 68.84^{c}
2.5	22845.83±224.07^{bc}
5	23345.83±76.37^{ab}
10	23450±208.04^{a}
15	23066.66±40.18^{abc}
20	22500±393.50^{c}

Values with same superscripts did not vary significantly

Table (1.1b) ANOVA for the effect of casein on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2300069.444	5	460013.889	10.594	.000
Within Groups	521041.667	12	43420.139		
Total	2821111.111	17			

Table (1.1c) Homogenous subsets for the effect of casein on protease production

Tukey HSD^{a}

Casein (g l^{-1})	N	Subset for alpha = 0.05		
		1	2	3
20	3	22500.00		
1	3	22583.33		
2.5	3	22845.83	22845.83	
15	3	23066.66	23066.66	23066.66
5	3		23345.83	23345.83
10	3			23450.00
Sig.		.052	.100	.283

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.2a) Effect of glucose on protease production

Glucose (gl^{-1})	Protease activity (U ml^{-1})
2.5	23383.33 ± 125.20^{c}
5	23808.33 ± 206.28^{b}
7.5	24383.33 ± 231.95^{a}
10	24083.33 ± 7.21^{ab}
20	22775 ± 12.5^{d}
30	7316.66 ± 7.21^{e}
40	5166.66 ± 7.21^{f}
50	3050 ± 12.5^{g}

Values with same superscripts did not vary significantly

Table (1.2b) ANOVA for the effect of glucose on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.959E9	7	2.799E8	19901.520	.000
Within Groups	225000.000	16	14062.500		
Total	1.959E9	23			

Table (1.2c) Homogenous subsets for the effect of glucose on protease production

Tukey HSD[a]

Glucose (gl^{-1})	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
50.0	3	3050.00						
40.0	3		5166.66					
30.0	3			7316.66				
20.0	3				22775.00			
2.5	3					23383.33		
5.0	3						23808.33	
10.0	3						24083.33	24083.33
7.5	3							24383.33
Sig.		1.000	1.000	1.000	1.000	1.000	.152	.097

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.3a) Effect of Yeast extract on protease production

Yeast extract (gl^{-1})	Protease activity (U ml^{-1})
2.5	23079.16± 85.08[a]
5	23283.33±298.25[a]
10	22500±234.18[ab]
15	21758.33±626.04[bc]
20	21179.16±473.40[c]

Values with same superscripts did not vary significantly

Table (1.3b) ANOVA for the effect of Yeast extract on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9437145.833	4	2359286.458	15.378	.000
Within Groups	1534166.667	10	153416.667		
Total	1.097E7	14			

Table (1.3c) Homogenous subsets for the effect of Yeast extract on protease production

Tukey HSD[a]

Yeast extract (gl^{-1})	N	Subset for alpha = 0.05		
		1	2	3
20.0	3	21179.16		
15.0	3	21758.33	21758.33	
10.0	3		22500.00	22500.00
2.5	3			23079.16
5.0	3			23283.33
Sig.		.418	.216	.179

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.4a) Effect of $NH_4H_2PO_4$ on protease production

$NH_4H_2PO_4$ (g l^{-1})	Protease activity (Uml^{-1})
0.5	22716.66±245.69[c]
1	23333.33±72.16[b]
2	23625±45.06[b]
4	23970.83±38.18[a]
8	24170.83±68.84[a]
10	24195.83±57.73[a]

Values with same superscripts did not vary significantly

Table (1.4b) ANOVA for the effect of $NH_4H_2PO_4$ on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4926119.792	5	985223.958	76.636	.000
Within Groups	154270.833	12	12855.903		
Total	5080390.625	17			

Table (1.4c) Homogenous subsets for the effect of $NH_4H_2PO_4$ on protease production

Tukey HSD[a]

$NH_4H_2PO_4$ (gl^{-1})		Subset for alpha = 0.05		
	N	1	2	3
0.5	3	22716.6667		
1.0	3		23333.3333	
2.0	3		23625.0000	
4.0	3			23970.8333
8.0	3			24170.8333
10.0	3			24195.8333
Sig.		1.000	.071	.220

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.5a) Effect of Na_2HPO_4 on protease production

Concentration of Na_2HPO_4 (g l^{-1})	Protease activity (Uml^{-1})
0.5	23016.66± 75.34[c]
1	23241.66±148.07[c]
2	23570.83±43.89[b]
4	23675±69.59[b]
8	24029.16±75.34[a]
10	24091.66±81.33[a]

Values with same superscripts did not vary significantly

Table (1.5b) ANOVA for the effect of Na_2HPO_4 on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2702916.667	5	540583.333	69.504	.000
Within Groups	93333.333	12	7777.778		
Total	2796250.000	17			

Table (1.5c) Homogenous subsets for the effect of Na_2HPO_4 on protease production

Tukey HSD[a]

Na_2HPO_4 (gl^{-1})	N	Subset for alpha = 0.05		
		1	2	3
0.5	3	23016.6667		
1.0	3	23241.6667		
2.0	3		23570.8333	
4.0	3		23675.0000	
8.0	3			24029.1667
10.0	3			24091.6667
Sig.		.074	.701	.947

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.6a) Effect of KH_2PO_4 on protease production

Concentration of KH_2PO_4 (g l^{-1})	Protease activity (U ml^{-1})
0.5	23383.33± 83.22[b]
1	23683.33±158.27[a]
1.2	23758.33±68.84[a]
1.4	23870.83±125.20[a]
1.8	23891.66±38.18[a]
2.0	23895.83±81.33[a]

Values with same superscript did not vary significantly

Table (1.6b) ANOVA for the effect of KH_2PO_4 on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	584548.611	5	116909.722	11.600	.000
Within Groups	120937.500	12	10078.125		
Total	705486.111	17			

Table (1.6c) Homogenous subsets for the effect of KH_2PO_4 on protease production

Tukey HSD[a]

KH_2PO_4 (gl^{-1})	N	Subset for alpha = 0.05	
		1	2
0.5	3	23383.3333	
1.0	3		23683.3333
1.2	3		23758.3333
1.4	3		23870.8333
1.8	3		23891.6667
2.0	3		23895.8333
Sig.		1.000	.173

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.7a) Effect of $MgSO_4.7H_2O$ on protease production

Concentration of $MgSO_4.7H_2O$ (g l^{-1})	Protease activity (U/ml^{-1})
0.2	23654.16±31.45[b]
0.4	23716.66±57.73[b]
0.8	23879.16±40.18[a]
1.0	23950±62.5[a]

Values with same superscript did not vary significantly

Table (1.7b) ANOVA for the effect of $MgSO_4.7H_2O$ on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	178593.750	3	59531.250	23.689	.000
Within Groups	20104.167	8	2513.021		
Total	198697.917	11			

Table (1.7c) Homogenous subsets for the effect of $MgSO_4.7H_2O$ on protease production

Tukey HSD[a]

$MgSO_4.7H_2O$	N	Subset for alpha = 0.05	
		1	2
0.2	3	23654.1667	
0.4	3	23716.6667	
0.8	3		23879.1667
1.0	3		23950.0000
Sig.		.466	.287

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.8a) Effect of $ZnCl_2$ on protease production

Concentration of $ZnCl_2$ (gl^{-1})	Protease activity (Uml^{-1})
0.01	23062.5 ± 188.74^{bc}
0.02	23391.66 ± 71.07^{abc}
0.04	23475 ± 100^{abc}
0.08	23600 ± 94.37^{abc}
0.16	23766.66 ± 356.70^{ab}
0.32	23883.33 ± 177.3^{a}
0.64	23337.5 ± 156.12^{abc}
1.28	22920.83 ± 535.65^{c}

Values with same superscripts did not vary significantly

Table (1.8b) ANOVA for the effect of $ZnCl_2$ on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2262337.240	7	323191.034	4.882	.004
Within Groups	1059166.667	16	66197.917		
Total	3321503.906	23			

Table (1.8c) Homogenous subsets for the effect of $ZnCl_2$ on protease production

Tukey HSD[a]

$ZnCl_2$ (gl^{-1})	N	Subset for alpha = 0.05		
		1	2	3
1.28	3	22920.8333		
0.01	3	23062.5000	23062.5000	
0.64	3	23337.5000	23337.5000	23337.5000
0.02	3	23391.6667	23391.6667	23391.6667
0.04	3	23475.0000	23475.0000	23475.0000
0.08	3	23600.0000	23600.0000	23600.0000
0.16	3		23766.6667	23766.6667
0.32	3			23883.3333
Sig.		.076	.061	.226

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.9a) Effect of $CaCl_2$ on protease production

Concentration of $CaCl_2$ (gl^{-1})	Protease activity (U ml^{-1})
0.000625	23054.16± 264.08^{bc}
0.00125	23204.16±109.21^{abc}
0.00250	23325±152.06^{abc}
0.00500	23604.16±170.17^{bc}
0.01	23925±184.13^{a}
0.02	23566.66±160.72^{ab}
0.04	23287.5±119.24^{abc}
0.08	23004.16±28.86^{bc}
0.16	22687.5±54.48^{cd}
0.32	22520.83±97.09^{cd}
0.64	22012.5±81.96^{d}
1.28	21916.66±863.52^{d}

Values with same superscripts did not vary significantly

Table (1.9 b) ANOVA for the effect of $CaCl_2$ on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.275E7	11	1159013.573	14.289	.000
Within Groups	1946666.667	24	81111.111		
Total	1.470E7	35			

Table (1.9c) Homogenous subsets for the effect of $CaCl_2$ on protease production

$CaCl_2$ (gl^{-1})	N	Subset for alpha = 0.05			
		1	2	3	4
1.28	3	21916.6667			
0.64	3	22012.5000			
0.32	3	22520.8333	22520.8333		
0.16	3	22687.5000	22687.5000		
0.08	3		23004.1667	23004.1667	
0.000625	3		23054.1667	23054.1667	
0.00125	3		23204.1667	23204.1667	23204.1667
0.04	3		23287.5000	23287.5000	23287.5000
0.00250	3		23325.0000	23325.0000	23325.0000
0.02	3			23566.6667	23566.6667
0.00500	3			23604.1667	23604.1667
0.01	3				23925.0000
Sig.		.092	.068	.342	.140

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.10a) Effect of pH on protease production

pH	Protease activity (Uml^{-1})
5	1162.5 ± 203.22^{c}
6	22979.16 ± 426.52^{ab}
7	24316.66 ± 292.97^{a}
8	24437.5 ± 62.5^{a}
9	22650 ± 305.41^{ab}
10	22162.5 ± 1492.21^{b}

Values with same superscripts did not vary significantly

Table (1.10b) ANOVA for the effect of pH on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.247E9	5	2.495E8	568.518	.000
Within Groups	5265937.500	12	438828.125		
Total	1.253E9	17			

Table (1.10c) Homogenous subsets for the effect of pH on protease production

Tukey HSD[a]

pH	N	Subset for alpha = 0.05		
		1	2	3
5.00	3	1162.5000		
10.00	3		22162.5000	
9.00	3		22650.0000	22650.0000
6.00	3		22979.1667	22979.1667
7.00	3			24316.6667
8.00	3			24437.5000
Sig.		1.000	.665	.055

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (1.11a) Effect of temperature on protease production

Temperature (°C)	Protease activity (Uml^{-1})
20	23583.33±107.77[a]
25	24195.83±47.32[a]
30	24004.16±163.13[a]
35	23937.5±414.38[a]
40	23420.83±460.69[a]

Values with same superscripts did not vary significantly

Table (1.11b) ANOVA for the effect of temperature on protease production

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1211916.667	4	302979.167	3.569	.047
Within Groups	848854.167	10	84885.417		
Total	2060770.833	14			

Table (1.11c) Homogenous subsets for the effect of temperature on protease production

Tukey HSD[a]

Temperature (°C)	N	Subset for alpha = 0.05
		1
40.00	3	23420.8333
20.00	3	23583.3333
35.00	3	23937.5000
30.00	3	24004.1667
25.00	3	24195.8333
Sig.		.053

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix -2

Table (2.1a) Effect of pH LasB on protease activity

pH	Protease activity (U ml^{-1})	Relative activity (%)
6	1983.33±9.54 [e]	20.58
7	5654.16±30.83[c]	58.68
8	7183.33±270.29 [b]	74.55
9	9635.41±164.21[a]	100
10	2725.00±8.26 [d]	28.28
11	1758.33±22.60 [ef]	18.24
12	1578.12±13.62[f]	16.37

Values with same superscripts did not vary significantly

Table (2.1b) ANOVA for the effect of pH on protease activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	180916098.400	6	630152683.067	2072.702	.000
Within Groups	203665.365	14	14547.526		
Total	181119763.765	20			

Table (2.1c) Homogenous subset for the effect of pH on protease activity

Tukey HSD

		Subset for alpha = .05					
PH	**N**	**1**	**2**	**3**	**4**	**5**	**6**
12.00	3	1578.12					
11.00	3	1758.33	1758.33				
6.00	3		1983.33				
10.00	3			2725.00			
7.00	3				5654.16		
8.00	3					7183.33	
9.00	3						9635.41
Sig.		.551	.315	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table (2.2a) Effect of pH on LasB elastase activity

pH	Elastase activity (Uml^{-1})	Relative activity (%)
7	166.41 ± 0.38^{b}	56.68
8	293.57 ± 5.20^{a}	100
9	165.57 ± 0.80^{b}	56.39

Values with same superscripts did not vary significantly

Table (2.2b) ANOVA for the effect of pH on elastase activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32553.451	2	16276.726	1751.895	.000
Within Groups	55.746	6	9.291		
Total	32609.197	8			

Table (2.2c) Homogenous subsets for the effect of pH on elastase activity

Tukey HSD

	N	Subset for alpha = .05	
PH		**1**	**2**
9.00	3	165.5767	
7.00	3	166.4100	
8.00	3		293.5716
Sig.		.941	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table (2.3a) Effect of pH on LasB protease stability

pH	Protease activity (Uml^{-1})	Residual activity (%)
6	4470.56 ± 6.65^{d}	58.48
7	7000 ± 19.09^{c}	91.57
8	7418.75 ± 9.73^{b}	97.05
9	7625 ± 22.53^{a}	99.75
10	3681.25 ± 10.82^{e}	48.16
11	2093.75 ± 60.05^{f}	27.39
12	1381.25 ± 7.21^{g}	18.07

Values with same superscripts did not vary significantly

Table (2.3b) ANOVA for the effect of pH on protease stability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.193E8	6	1.988E7	29059.801	.000
Within Groups	9575.147	14	683.939		
Total	1.193E8	20			

Table (2.3c) Homogenous subsets for the effect of pH on protease stability

Tukey HSD[a]

pH	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
12.00	3	1381.2567						
11.00	3		2093.7521					
10.00	3			3681.2500				
6.00	3				4470.5625			
7.00	3					7000.0000		
8.00	3						7418.7523	
9.00	3							7625.0000
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table (2.4a) Effect of pH on LasB elastase stability

pH	Elastase activity (Uml^{-1})	Residual activity (%)
7	163.24 ± 0.76^{b}	71.05
8	214.99 ± 0.52^{a}	93.57
9	88.24 ± 0.62^{c}	38.41

Values with same superscript did not vary significantly

Table (2.4b) ANOVA for the effect of pH on elastase stability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24534.412	2	12267.206	29443.650	.000
Within Groups	2.500	6	.417		
Total	24536.912	8			

Table (2.4c) Homogenous subsets for the effect of pH on elastase stability

Tukey HSD[a]

pH	N	Subset for alpha = 0.05		
		1	2	3
9.00	3	88.2498		
7.00	3		163.2468	
8.00	3			214.9947
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (2.5a) Effect of temperature on LasB protease activity

Temperature (°C)	Protease activity (Uml^{-1})	Relative activity (%)
30	2168.75±49.60[e]	22.50
40	5985.41±113.93[c]	62.11
50	7106.25±124.84[b]	73.51
60	9635.41±164.21[a]	100
70	4000.00±84.08[d]	41.51
80	1600.00±45.06[f]	16.60

Values with same superscript did not vary significantly

Table (2.5b) ANOVA for the effect of temperature on protease activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	142288272.569	5	28457654.514	2544.787	.000
Within Groups	134192.708	12	11182.726		
Total	142422465.278	17			

Table (2.5c) Homogenous subsets for the effect of temperature on protease activity

Tukey HSD

Temperature (°C)	N	Subset for alpha = .05					
		1	2	3	4	5	6
80.00	3	1600.0000					
30.00	3		2168.7500				
70.00	3			4000.0000			
40.00	3				5985.4167		
50.00	3					7106.2500	
60.00	3						9635.4167
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table (2.6a): Effect of temperature on LasB elastase activity

Temperature (°C)	Elastase activity (Uml^{-1})	Relative activity (%)
30	200.90 ± 0.62^{b}	68.43
40	293.57 ± 5.20^{a}	100
50	165.91 ± 0.62^{c}	56.51
60	96.66 ± 0.38^{d}	32.92

Values with same superscripts did not vary significantly

Table (2.6b) ANOVA for the effect of temperature on elastase activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	60408.183	3	20136.061	2874.671	.000
Within Groups	56.037	8	7.005		
Total	60464.220	11			

Table (2.6c) Homogenous subsets for the effect of temperature on elastase activity

Tukey HSD

Temperature (ºC)	N	Subset for alpha = .05			
		1	2	3	4
60.00	3	96.6628			
50.00	3		165.9100		
30.00	3			200.9086	
40.00	3				293.5716
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table (2.7a) Effect of temperature on LasB protease stability

Temperature (°C)	Protease activity (Uml^{-1})	Residual activity (%)
30	6564.54± 9.54[a]	85.04
40	6545.80±6.69[b]	84.80
50	6393.75±4.36[c]	82.83
60	3610.18±3.60[d]	46.77
70	843.75±9.54[e]	10.93

Values with same superscript did not vary significantly

Table (2.7b) ANOVA for the effect of temperature on protease stability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.728E7	4	1.932E7	372850.883	.000
Within Groups	518.147	10	51.815		
Total	7.728E7	14			

Table (2.7c) Homogenous subsets for the effect of temperature on protease stability

Tukey HSD[a]

Temperature (°C)	N	Subset for alpha = 0.05				
		1	2	3	4	5
70.00	3	843.75				
60.00	3		3610.18			
50.00	3			6393.75		
40.00	3				6545.80	
30.00	3					6564.54
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (2.8a) Effect of temperature on LasB elastase stability

Temperature (°C)	Elastase activity (Uml^{-1})	Residual activity (%)
30	204.49± 0.62[b]	69.26
40	217.74 ±0.52[a]	73.75
50	178.74±0.76[c]	60.54
60	122.74±0.99[d]	41.91

Values with same superscript did not vary significantly

Table (2.8b) ANOVA for the effect of temperature on elastase stability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15959.655	3	5319.885	9458.330	.000
Within Groups	4.500	8	.562		
Total	15964.155	11			

Table (2.8c) Homogenous subsets for the effect of temperature on elastase stability

Tukey HSD[a]

Tempreature (ºC)	N	Subset for alpha = 0.05			
		1	2	3	4
60.00	3	122.74			
50.00	3		178.74		
30.00	3			217.74	
40.00	3				204.49
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix -3

Table (3.1a) Effect of pH on DNA yield from *S. aureus* subsp. *aureus*

pH	DNA yield (µg µl^{-1})
5	40±2.5^{d}
6	44.16±3.81^{d}
7	286.66±9.46^{a}
8	216.66±3.81^{b}
9	210±5^{b}
10	185.83±5.20^{c}

Values with same superscript did not vary significantly

Table (3.1b) ANOVA for the effect of pH on DNA yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	150448.611	5	30089.722	1019.511	.000
Within Groups	354.167	12	29.514		
Total	150802.778	17			

Table (3.1c) Homogenous effect for the effect of pH on DNA yield

Tukey HSD

		Subset for alpha = .05			
pH	N	1	2	3	4
5.00	3	40.0000			
6.00	3	44.1667			
10.00	3		185.8333		
9.00	3			210.0000	
8.00	3			216.6667	
7.00	3				286.6667
Sig.		.928	1.000	.669	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table (3.2a) Effect of temperature on DNA yield from *S. aureus* subsp. *aureus*

Temperature (°C)	DNA yield (µg μl^{-1})
25	265±6.61[a]
35	286.66±9.46[a]
45	275±10.89[a]
55	270±9.01[a]
65	245.83±3.81[ab]
75	202.5±34.73[b]

Values with the same superscripts did not vary significantly

Table (3.2b) ANOVA for the effect of temperature on DNA yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13591.667	5	2718.333	10.494	.000
Within Groups	3108.333	12	259.028		
Total	16700.000	17			

Table (3.2c) Homogenous effect for the effect of temperature on DNA yield

Tukey HSD[a]

temp (°C)	N	Subset for alpha = 0.05	
		1	2
75.00	3	202.5000	
65.00	3	245.8333	245.8333
25.00	3		265.0000
55.00	3		270.0000
45.00	3		275.0000
35.00	3		286.6667
Sig.		.055	.076

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (3.3a) Effect of incubation time on DNA yield from *S. aureus* subsp. *aureus*

Incubation time (min)	DNA yield (µg µl^{-1})
10	210.83±5.20^{c}
20	237.5±5^{b}
30	286.66±9.46^{a}
40	290±6.61^{a}
50	293.33±12.22^{a}
60	295±6.61^{a}

Values with same superscripts did not vary significantly

Table (3.3 b) ANOVA for the effect of incubation time on DNA yield

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	35268.403	5	7053.681	152.741	.000
Within Groups	554.167	12	46.181		
Total	35822.569	17			

Table (3.3c) Homogenous subsets for the effect of incubation time on DNA yield

Tukey HSD^{a}

Incubation time	N	Subset for alpha = 0.05		
		1	2	3
10.00	3	210.8333		
20.00	3		237.5000	
30.00	3			286.66
40.00	3			290
50.00	3			293.33
60.00	3			295
Sig.		1.000	1.000	.670

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix -4

Table (4.1a): Effect of pH on β-1,3 glucanase activity

pH	β-1,3 glucanase activity (Uml^{-1})
3	158.42± 3.52[e]
4	180.83±4.41[d]
5	211.39±4.41[c]
6	239.41±11.67[b]
7	313.02±0.44[a]
8	241.96±4.4[b]
9	238.90±3.52[b]
10	169.37±2.20[de]
11	36.93±2.20[f]

Values with same superscripts did not vary significantly

Table (4.1b) ANOVA for the effect of pH on β-1,3 glucanase activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	142041.324	8	17755.165	696.441	.000
Within Groups	458.895	18	25.494		
Total	142500.218	26			

Table (4.1c) Homogenous subsets for the effect of pH on β-1, 3 glucanase activity

Tukey HSD[a]

pH	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
11.00	3	36.9311					
3.00	3		158.4216				
10.00	3		169.3736	169.3736			
4.00	3			180.8349			
5.00	3				211.3986		
9.00	3					238.9059	
6.00	3					239.4153	
8.00	3					241.9622	
7.00	3						313.0227
Sig.		1.000	.231	.188	1.000	.997	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (4.2a) Effect of temperature on β-1,3 glucanase activity

Temperature (°C)	β-1, 3 glucanase activity (Uml^{-1})
30	221.58 ± 7.64^{c}
40	278.89 ± 10.10^{b}
50	343.84 ± 13.23^{a}
60	272.52 ± 4.411^{b}
70	219.03 ± 8.82^{c}
80	66.22 ± 4.41^{d}

Values with same superscripts did not vary significantly

Table (4.2b) ANOVA for the effect of temperature on β-1,3 glucanase activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	132275.217	5	26455.043	350.806	.000
Within Groups	904.945	12	75.412		
Total	133180.161	17			

Table (4.2 c) Homogenous subsets for the effect of temperature on β-1,3 glucanase activity

Tukey HSD[a]

Temperature		Subset for alpha = 0.05			
(°C)	N	1	2	3	4
80.00	3	66.2212			
70.00	3		219.0395		
30.00	3		221.5865		
60.00	3			272.5259	
40.00	3			278.8933	
50.00	3				343.8411
Sig.		1.000	.999	.940	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean sample size = 3.00

Table (4.3 a) Effect of pH on DNA yield from *Saccharomyces cervisiae* MTCC 1766

pH	DNA yield (µg µl^{-1})
5	116±6.29^{c}
6	123.33±3.81^{c}
7	231.66±5.20^{a}
8	222.5±4.33^{a}
9	185±2.5^{b}
10	170±2.5^{b}

Values with same superscripts did not vary significantly

Table (4.3b) ANOVA for the effect of pH on DNA yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35227.292	5	7045.458	225.856	.000
Within Groups	374.333	12	31.194		
Total	35601.625	17			

Table (4.3c) Homogenous subsets for the effect of pH on DNA yield

pH	N	Subset for alpha = 0.05		
		1	2	3
5.00	3	116.0000		
6.00	3	123.3333		
10.00	3		170.0000	
9.00	3		185.0000	
8.00	3			222.5000
7.00	3			231.6667
Sig.		.609	.056	.391

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (4.4a) Effect of temperature on DNA yield from *Saccharomyces cervisiae* MTCC 1766

Temperature (°C)	DNA yield (µg µl^{-1})
25	234.16± 5.77^{d}
35	241.66±5.20^{cd}
45	254.16±7.63^{c}
55	292.5±5^{b}
65	310±2.5^{a}
75	294.16±3.81^{b}

Values with same superscript did not vary significantly

Table (4.4b) ANOVA for the effect of temperature on DNA yield

Yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15061.111	5	3012.222	109.813	.000
Within Groups	329.167	12	27.431		
Total	15390.278	17			

Table (4.4 c) Homogenous subsets for the effect of temperature on DNA yield

Temperature (°C)	N	Subset for alpha = 0.05			
		1	2	3	4
25.00	3	234.1667			
35.00	3	241.6667	241.6667		
45.00	3		254.1667		
55.00	3			292.5000	
75.00	3			294.1667	
65.00	3				310.0000
Sig.		.526	.103	.999	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table (4.5 a) Effect of incubation time on DNA yield from *Saccharomyces cervisiae* MTCC 1766

Incubation time (min)	DNA yield (µg µl^{-1})
10	241.66±8.03[b]
20	262.5±5[b]
30	312.5±10[a]
40	315±11.45[a]
50	320.83±7.63[a]
60	321.66±5.2[a]

Values with same superscripts did not vary significantly

Table (4.5 b) ANOVA for the effect of incubation time on DNA yield

yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17947.569	5	3589.514	53.014	.000
Within Groups	812.500	12	67.708		
Total	18760.069	17			

Table (4.5 c) Homogenous subsets for the effect of incubation time on DNA yield

Tukey HSD[a]

Incubation time (min)	N	Subset for alpha = 0.05	
		1	2
10.00	3	241.66	
20.00	3	262.5	
30.00	3		312.5000
40.00	3		315.0000
50.00	3		320.8333
60.00	3		321.6667
Sig.		.077	.746

Means for groups in homogeneous subsets are displayed.