"Purification and Characterization of an Alkaline Protease of Biotechnological Significance"

A thesis submitted to Goa University for the Award of the Degree of

Doctor of Philosophy

in

Biotechnology

By

Akmadevi alias Asha S. Nadurmath

Goa University,

Taleigao Goa

April, 2016

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Research Guide

Prof. Usha D. Muraleedharan

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CERTIFICATE

This is to certify that the research work presented in this thesis entitled ***"Purification and Characterization of an Alkaline Protease of Biotechnological Significance"***, submitted by Mrs. Akmadevi alias Asha S. Nadurmath for the award of the degree of Doctor of Philosophy in Biotechnology is based on original studies carried out by her under my guidance and supervision at the Department of Biotechnology, Goa University , in partial fulfillment of the requirements and that no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles or recognition.

Place: Goa University

Date: 11/04/2016

Dr. Usha D. Muraleedharan

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Professor & Head, Department of Biotechnology

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Statement

As required under the University ordinance OB-9.9(ii), I state that the present thesis entitled "***Purification and Characterizationof an Alkaline Protease of Biotechnological significance",*** is my original contribution and that the same has not been submitted on any previous occasion for any degree. To the best of my knowledge, the present study is the first comprehensive work of its from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Place: Goa, India

Date: 11/04/2016

Akmadevi S. Nadurmath

DEDICATED TO MY FAMILY.....

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Introduction and Review of Literature

Introduction and Review of Literature

Enzymes are nature's catalysts as they speed up the rates of reactions without themselves undergoing any permanent change. Civilizations have been using enzymes for thousands of years without understanding what they were or how they worked. Over the past several generations, science has opened up the mystery of enzymes and has applied this knowledge to better exploit these miraculous substances in a spiralling number of applications. Microorganisms represent the most common source of enzymes which are relatively more stable and active than those derived from plant or animal sources. Microbes serve as a preferred source of enzymes owing to their rapid growth, requirement of limited space for their multiplication and the ease for manipulating them genetically to generate new enzymes with altered properties that are desirable for their various applications. In fact microbial proteases do account for approximately 60% of the total enzyme sales in the world (Singh *et al*., 2001; Banik and Prakash, 2004).

A protease is any enzyme that starts protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. They perform both degradative and synthetic functions. With respect to their physiological roles as well as their commercial applications proteolytic enzymes are considered one of the most useful of enzyme groups (Gupta *et al*., 2002; Fulzele *et al*., 2011).

Since proteases are physiologically necessary for living organisms, they are ubiquitously found in a wide diversity of sources such as plants, animals, and microorganisms.

1.1 Sources of Commercial Proteolytic enzymes

a) Plant Proteases

The use of plants as a source of proteases is governed by several factors such as land availability for cultivation and climatic conditions for suitable growth. Commercial production of proteases from plants takes long time. Papain, bromelain, keratinases and ficin, however, are some of the well-known plant proteases. Plant proteases are employed as milk-clotting enzymes in the cheese industry (Shas *et al.*, 2013).

b) Animal Proteases

The animal origin proteases are pancreatic trypsin, chymotrypsin, pepsin, and rennins (Boyer, 1971). Production of animal proteases depends on the availability of livestock for slaughter. And hence not feasible from an industrial point of view.

c) Microbial proteases

As mentioned earlier, microbes are indeed an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation. They elaborate a large array of proteases, which may be intracellular and/or extracellular.

Intracellular proteases are important for different cellular and metabolic processes such as protein turnover, sporulation and differentiation, maturation of hormones and enzymes and maintenance of the cellular protein pool. Extracellular proteases are vital for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz, 1988). They have also been commercially exploited to help protein

degradation in industrial processes (Kumar and Takagi, 1999; Outtrup and Boyce, 1990).

(i) Bacterial Proteases

Bacteria ubiquitously inhabit soil, air, water and animal tissue. Marine bacteria are found throughout the oceans, from the seabed to the insides of stomach of fishes. The oceanic home contains many distinct habitats such as underwater mountains, deep-sea sediments, seamounts and even the outside of an algal cell. These microbes have developed unique mechanisms to survive diverse conditions, including salty to merely brackish water, and temperatures varying from 33°C in tropical waters to –5°C in the polar and deep-sea waters. It is these unique survival mechanisms that make marine bacteria very promising as a source of novel biologically active substances, with industrial potential in medicines and foods.

(ii) Fungal Proteases

More than the bacteria, fungi elaborate a wider variety of enzymes. For example, *Aspergillus oryzae y*ields acid, neutral and alkaline proteases. Fungal proteases are generally active over a wider pH range (pH 4 to 11) and exhibit broad substrate specificity. However, they have a lower reaction rate and poorer heat tolerance than do the bacterial enzymes. Production of fungal enzymes can be commercially done in a solid-state fermentation process. Optimal pH values of fungal acid proteases are between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheese making industry due to their narrow pH and temperature specificities. Fungal neutral proteases are active at pH 7.0 and are inhibited by chelating agents. They are bestowed with peptidase

activity and specific ability to hydrolyse hydrophobic amino acid bonds. They could therefore supplement the action of proteases from other sources in reducing the bitterness of food protein hydrolysates. (Rao *et. al.*, 1998).

(iii) Viral Proteases

Viral proteases are functionally involved in the processing of proteins of viruses that cause certain fatal diseases such as AIDS and cancer. Serine, aspartic and cysteine peptidases are found in various viruses. All of the virus-encoded peptidases are endopeptidases; there are no metallopeptidases. Vast literature is available on the purification, expression and enzymatic analysis of retroviral aspartic protease and its mutants (Kuo and Shafer, 1994). Extensive research has focused on the three-dimensional structure of viral proteases and their interaction with synthetic inhibitors for designing potent inhibitors that can combat the relentlessly spreading and devastating epidemic of AIDS.

1.2 Classification of Proteases

1.2.1 Based on site of action.

Proteases are grouped into exopeptidases and endopeptidases based on their action at or away from the termini, respectively.

(i) Exopeptidases

The exopeptidases act only at the ends of polypeptide chains at the N or C terminus. Those acting at a free N terminus (aminopeptidases) liberate a single amino acid residue, a dipetide (dipeptidyl-peptidases) or a tripeptide (tripeptidyl-peptidases) while those that act at a free C terminus (carboxypeptidases) liberate a single amino acid or a

dipeptide (peptidyl-dipeptidases). Some exopeptidases are specific for dipeptides (dipeptidases) or remove terminal residues that are substituted, linked or cyclized by isopeptide bonds. Depending on the nature of the amino acid residues at the active site of the enzymes carboxypeptidases are mainly of three types, (a) serine carboxypeptidases, (b) metallocarboxypeptidases and (c) cysteine carboxypeptidases (Rao *et al*., 1998).

(ii) Endopeptidases

Preferentially endopeptidases act in the inner regions of peptide chains away from the C and N termini. The presence of free amino or carboxyl groups has a negative effect on the enzyme activity. These endopeptidases can be ordered further, according to the reactive groups at the active site involving catalysis, into serine- (EC 3.4.21), cysteine- (EC 3.4.22), aspartic-proteinases (EC.3.4.23) and metalloendopeptidases or metalloproteinases (EC 3.4 24). Enzymes whose reaction mechanisms have not been completely elucidated are classed under subgroup EC. 3.4. 99.

1.2.2. Based on Mechanism of Action. Six broad groups have been identified :

- Serine proteases
- Cysteine proteases
- Aspartate proteases
- Metalloproteases
- Threonine proteases
- Glutamic acid proteases

The threonine and glutamic-acid proteases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involved is in making an

amino acid residue that has the threonine and cysteine or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine or threonine as a nucleophile. The MEROPS database presents an up-to-date classification of proteases into families (Rawlings *et. al.*, 2010).

- **Serine proteases**

Serine proteases are the most widely distributed group of proteolytic enzymes of both animal and microbial origin. They are characterized by the presence of a serine group at their active site and are generally inhibited by diisopropyl fluorophosphates (DFP) and phenylmethyl sulphonyl fluoride (PMSF). Many of the proteases are also inhibited by some thiol reagents, such as *p*-chloromercuric benzoate (*p*CMB), probably due to the presence of cysteine residue near the active site, but which does not participate in the catalytic mechanism of the enzyme. Serine proteases are generally active at neutral and alkaline pH, with an optimum pH between 7-11 (Ellaiah *et.al.* 2002). They are numerous and widespread among viruses, bacteria and eukaryotes, suggesting that they are vital to these organisms. Serine proteases are subdivided into four classes: chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC) and *Escherichia* D-Ala–D-Ala peptidase A (SE). Their molecular masses range between 18 and 35kDa.

- **Metalloproteases**

Metalloproteases are the most diverse of the catalytic types of proteases (Manni *et al.*, 2008; Sorror *et al.*, 2009). They are characterized by the requirement of a divalent metal ion for their activity. Most of the fungal and bacterial metalloproteases are zinc-containing enzymes (Ellaiah, *et. al.*, 2002). These are sensitive to metal chelators such

as EDTA and are unaffected by sulphydryl agents. Based on their specificity of action, metalloproteases can be classified into four groups: (i) neutral (ii) alkaline (iii) *Myxobacter* I and (iv) *Myxobacter* II proteases.

- **Aspartic proteases**

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. These are widely distributed in fungi but rarely found in bacteria. They are inhibited by epoxy- and diazo-ketone compounds in the presence of copper ions and also by pepstatin or Streptomyces pepsin inhibitor. Most aspartic proteases show maximal activity at pH 3 to 4, have isoelectric points in the range of pH 3 - 4.5 and molecular weight in the range of 30 – 45 kDa.

- **Cysteine/ Thiol proteases**

The activity of all cysteine proteases depends on a catalytic site consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents. They are sensitive to sulphydryl agents such as *p*CMB, iodoaceticacid, iodoacetamide and heavy metals and are activated by reducing agents such as DTT, EDTA and potassium cyanide or cysteine. Based on their side chain specificity, they are broadly classified 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'. These enzymes are active at pH 5-8.

1.2.3. Based on optimal pH

Yet another classification of proteases is in relation to the optimal pH at which they are active:

- *Acid proteases*
- *Neutral proteases*
- *Alkaline proteases*

1.3. Biotechnological Applications of Proteases

Proteases are hard enzymes with a sizeable industrial potential as detergent additives, in leather processing, silver recovery, medical purposes, food processing, meat tenderization, cheese making, dehairing, waste treatment, feeds and chemical industries. These enzymes facilitate the development of high value-added applications or products by using enzyme-aided (partial) digestion. Probably the largest application of proteases is in laundry detergents, wherein protein-based stains are removed from clothing (Banerjee *et al*., 1999; Sharma *et al.*, 1980). For the use of an enzyme as a detergent additive, stability and activity in the presence of typical detergent ingredients such as builders, bleaching agents, bleach activators, fillers, surfactants, fabric softners and various other formulation aids are most essential. In the textile industry also, proteases may be used to remove the stiff and dull gum layers of sericine from the raw silk fiber to achieve improved softness and lustre. Protease treatments can modify the surface of wool and silk fibers to provide a new look and unique finish.

1.3.1. As Detergent Additives

Enzymes used in detergents include proteases, amylases and lipases, of which alkaline proteases hold 60-65% of the global industrial enzyme market (Amoozegara *et al*., 2007). Over the past 20 years, the development of subtilisins as typical detergent proteases has employed all the tools of enzyme technology, bringing in a constant flow of new and improved enzymes. The idea of using proteases in detergents dates back to the use of pancreatic extracts by Roehm in 1913 Sandhya *et al.*, 2006). Only since 1960's with the availability of enzymes from bacteria, however, their use becomes efficient in the technical as well as in the economic sense. From early times these enzymes were produced using *Bacillus* species, starting with *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. The alkaline proteases from *Bacillus* species represent the lead molecules for subtilisins (Maurer, 2004). The increased usage of proteases as additives is mainly due to the cleaning capabilities of these enzymes in environmentally acceptable, non-phosphate detergents. In addition to improved washing efficiency, the use of these enzymes allows lower wash temperatures and shorter periods of agitations, often after a preliminary period of soaking. Ideally, proteases as well as other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzymes used should be active at low levels and should also be compatible with various detergent components, besides oxidizing and sequestering agents.

1.3.2. In Silver Recovery

Alkaline proteases are potentially exploited for silver recovery in the bioprocessing of used X-ray films containing silver in its gelatin layers. The conventional practice of silver recovery by burning film poses a major environmental pollution problem. The enzymatic hydrolysis of gelatin layers on the X-ray films enables silver recovery as well as recycling of the polyester film base (Ishikawa *et al*., 1993) by an ecofriendly process.

1.3.3. Medical Uses

Proteases are used to treat blood clots, clean wounds and to enhance the effectiveness of some antibiotics. One type of protease obtained from pineapple stems is used as an anti-inflammatory agent while other protein-degrading enzymes are used to treat severe sepsis. Collagenases with alkaline protease activity are used for therapeutic applications (Ellaih *et. al.,* 2002*)*. Proteases from *Bacillus* species have been recognized as being safe to humans and an alkaline protease with fibrinolytic activity has been used as a thrombolytic agent (Ellaiah *et. al.,* 2002). Proteases are also useful in biopharmaceutical products such as contact-lens enzyme cleaners and enzymic debridement (Anwar and Saleemuddin, 2000). They offer a gentle and selective debridement thereby supporting the natural healing process in the successful local management of skin ulcerations by efficient removal of necrotic material (Sjodahl *et al*., 2002).

1.3.4. Food Industry

Proteases have been used in foods for centuries. A protease (rennet) obtained from the stomachs of unweaned calves, has traditionally been used in the production of cheese. Alkaline proteases can hydrolyze proteins from plants, fishes or animals to produce

hydrolysates of well-defined peptide profile. A commercial alkaline protease also known as alcalase has broad specificity, with some preference for terminal hydrophobic amino acids. Neutral proteases have been reported to be used to produce soy sauce and soy products which are less bitter; they are used in the brewing industry as they are not sensitive to natural plant proteinase inhibitors (Rao *et al.*, 1998). Proteases play an active part in the production of sausages, luncheon meat and flour. They are also used to recover protein from parts of animals (and fish) which would otherwise go to waste after butchering. Proteases are used in the baking industry too. Where appropriate, dough may be prepared more quickly if its gluten is partially hydrolysed. A heat-labile fungal protease is used so that it is inactivated at an early stage in the baking.

1.3.5. Tannery, Leather and Wool industries

The leather industry consumes a significant proportion of the world's enzyme production by the use of an alkaline proteases to remove hair from hides (Khan, 2013). This process is more pleasant and far safer than the traditional methods involving sodium sulfide. Alkaline proteases possessing keratinolytic and elastolytic activity offer an effective biotreatment of leather, especially in the dehairing and bating of skins and hides. The alkaline conditions basically enable the swelling of root hairs and then the attack of proteases on the hair follicle protein allow for easy removal of the hair. The bating followed by dehairing process involves the degradation of elastin and keratin, removal of hair residues and the deswelling of collagen, all of which produce a good, soft leather mainly used for making leather clothes and goods.

Proteases have been used in the past to 'shrinkproof' wool. This method involved the partial hydrolysis of the scale tips with the protease papain that gave the wool a silky lustre and boosted its value (Doshi and Shelke, 2001).

1.3.6. For Waste Treatment

Alkaline proteases provide biopotential application for waste management generated from food industries and household activities (Ellaiah *et. al.* 2002) by solubilizing proteins in wastes. The enzymes from *Bacillus subtilis* were reported to be used for treatment of waste feathers (Dalev, 1994).

1.3.7 Protein Engineering

With the increasing demand for alkaline proteases, much research has been initiated relating to DNA shuffling and cloning. The existing knowledge on structure is utilized to couple with gene-shuffling techniques. Advances in biotechnology have created a favourable niche to develop proteases which would be helpful to provide a sustainable environment. Jaouadi *et al* (2014) have described the advantageous features of proteases and the resulting promising opportunities for the improvement of various industrial and consumer products (2014). *Serratia marcescens* protease gene was cloned and expressed in *E. coli* that caused specific excretion of the protease into the extracellular medium through the outer membrane of *E. coli* host cells (Kim *et al.*, 1992; Ohnishi and Horinouchi, 1996). More than 50% of the industrially important enzymes were reported to be produced by genetically engineered microorganisms (Hodgson, 1994).

1.4. Round up of significant reports on microbial proteases

Almost 65% of the total enzyme market is dominated by alkaline proteases (Rao *et al.,* 1998) and the preference of microbial proteases over plant and animal sources has been highlighted (Devi *et al.*, 2008). More recently, Jisha *et al.* (2013) have described the versatility of microbial proteases. The industrial demand for enzymes and specifically alkaline proteases is escalating by the year, drawing increasing attention to the use of marine microorganisms for biotechnological purposes. *Bacillus* sp. have emerged as key producers of extracellular proteases with potential applications in food, pharmaceutical, leather, detergent and chemical industries (Patel *et al.*, 2005, 2006; Dodia *et al.,* 2008 ; Sorror *et al.,* 2009; Singh *et al.,* 2009). Among the several *Bacillus* species involved in protease production are *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* (Ammar *et al*., 1991; Banik and Prakash, 2004; Beg and Gupta, 2003; Greze *et al*., 2005 and Soares *et al*., 2005). Isolation, production and characterization of thermostable alkaline protease from *Bacillus licheniformis* MIR 29 was reported by Ferrero *et al.* (1996). Lee *et al.* (1991) and Siddalingeshwar *et al.* (2010) have also reported alkaline protease production and characterization from other *Bacillus* species. Moon and Parulekar (1991) had carried out a parametric study of protease production in batch and fed- batch cultures of *Bacillus firmus.*

Halophilic bacteria are good sources for protease production because of their tolerance to high salt conditions (Foophow and Tangjitjaroenkun, 2012). *Bacillus* strains isolated from the saltern pond of Kakinada were screened and identified with high alkaline protease activity (Laxmi *et.al*., 2014). These are known to have significant proteolytic activity and stability at high pH and temperature (Shumi *et al.,* 2004). The production of

alkaline enzymes by alkalophilic microorganisms has been reported by Horikoshi (1971) and Gessesse and Gashe (1997). An alkaline protease from alkaliphilic *Bacillus licheniformis* obtained from Lonar Soda Lake in Maharashtra, India showed high stability and compatibility with detergents (Pathak and Deshmukh, 2012).

Cheong *et al.*(1993) have reported on the production and properties of an alkaline protease from *Pseudomonas* sp. SJ-320 (1993). Biochemical properties and potential application of a solvent stable protease obtained from *Pseudomonas aeruginosa* PT 121 were studied by Tang *et al.* (2010). Production of thermostable alkaline protease by an alkaline resistant *Streptomyces* isolate was reported by Ahmed (2011). A protease was extracted from *Pseudomonas aeruginosa* PD100 and its potential applications were studied (Najafi *et al.,* 2005). Madan *et al.* (2002) have purified and characterized alkaline protease from a mutant of *Bacillus polymyxa*. Mukhtar and Haq (2012) have described the production, purification and characterization of alkaline proteases from a mutant strain of *Bacillus subtilis* obtained from a culture bank. Isolation, partial purification and characterization of alkaline protease from the seeds of *Cucumis melo var agrestis* has been reported (Devi and Latha, 2014). Son and Kim (2002) have characterized caseinolytic extracellular protease from *Bacillus amyloliquefaciens* S94. Optimization of conditions for production of extracellular protease from a newly isolated *Bacillus cereus* strain CA 15 has also been reported (Uyar *et al.*, 2011).

Alkaline protease had been produced in a low cost medium by alkalophilic B*acillus* sp. and its enzymatic properties studied (Fujiwara and Yamamoto, 1987). Chicken feather was used as a substrate to grow alkalophilic bacteria, resulting in the production of a novel alkaline protease (Gessesse *et al.,* 2003). Agro waste was found to be helpful and

economical in protease production (Paranthaman *et al.,* 2009; Muhammad *et al.,* 2008) and was utilized as substrate in the medium to extract alkaline protease from a soil *S. marcescens* (Joseph and Palaniyandi , 2011).

Production of purified protease is invariably influenced by different factors (Kezia *et al.,* 2011).

Purification of proteases has been described as performed using precipitation methods (Dupon, 1994 and Arulmani *et al.,* 2007), chromatographic methods (Thumar and Singh, 2007; Gupta et al., 2005) and electrophoretic methods (El-shanshoury *et al.,* 1995). Thermostable alkaline protease from alkalophilic *Bacillus pumilus* (Kumar, 2002) and an oxidation stable and thiol-dependent serine alkaline protease from *Bacillus mojavensis (*Gupta and Beg, 2003) were purified and characterized. Purification and characterization of a 56 kDa cold-active protease has been carried out from *Serratia marcescens* (Tariq *et al.*, 2011). Agarwal *et al.* (2012) have reported on the purification and characterization of extracellular alkaline protease from *Bacillus* strains obtained from a gene pool. *Bacillus cohnii APTS* isolated from a soil sample from Turkey was studied for production and properties of alkaline protease (Tekin *et al*., 2012). More recently, a *Bacillus* strain identified as *Bacillus aryabhattai* has been used for alkaline protease production and purification (Sharma *et al.,* 2014).

Purification and characterization of protease from *Aspergillus flavus* produced under solid state fermentation has been studied by Muthulakshmi *et al.* (2011) while Aruna *et al.* (2014) have documented the production and partial characterization of alkaline protease from *Bacillus tequilensis* isolated from spoilt cottage cheese. An extracellular alkaline protease was obtained from a novel haloalkaliphilic bacterium of *Bacillus*

species and the role of extracellular proteases in hydrolysis of large proteins and intracellular in the regulation of metabolism has been explained (Kalisz 1988; Rao *et. al,*. 1998).

Solid substrate fermentation method was employed for production of a thermostable alkaline protease by a new *Pseudomonas* sp. (Chakraborty and Srinivasan, 1993). Similarly, protease production from *Bacillus amyloliquefaciens* was successfully carried out by solid state fermentation method and its application in the dehairing of hides and skin tested (George *et al.*, 1995).

The bacterium *Serratia marcescens* has been widely described as a good protease producer (Henriette *et al.*, 1993; Byun *et al.*, 1995; Romero *et al.*, 2001) and appeared a better protease producer than *B.subtilis* in terms of absolute production (Longo *et al.*, 1999*)*. A metalloprotease was successfully characterized and purified from *S.* marcescens S3-R1 inhabiting Korean ginseng rhizosphere (Myoung *et al.*, 2013). *S.marcescens* isolated from soil sample of dairy waste could produce thermostable alkaline protease that was stable and active under alkaline conditions, projecting its potential in detergent and leather industries and silver recovery process (Annapurna *et* al., 2012).

Utilization of dairy waste for production and characterization of a novel cysteine protease has also been reported (Gul *et al.*, 2012). Whey served as a substrate to extract and produce two extracellular proteases from *S. marcescens* ATCC 25419 (Fredy *et al.*, 2001). Production of extracellular proteases by *Mucor circinelloides* using glucose as substrate was reported by Andrade *et al.* (2002). *Bacillus subtilis* isolated from soils of

side roads of Chennai was studied for the production, optimization and partial purification of protease (Pant *et al.,* 2015). Keratinolytic alkaline proteases have been used in feed technology for the production of peptides to degrade keratinous waste materials in bathtub and drains in public places (Takami *et al*., 1992).

Bhosle *et. al.* (2004) isolated obligate alkaliphilic bacteria from various estuarine ecosystems of Goa and reported enzyme activities of lipase, amylase and protease under alkaline conditions. More recently, *Bacillus* strains isolated from the saltern pond (Kakinada) have been screened and identified for high alkaline protease activity (Lakshmi *et al.*, 2014). Garg and Singh (2015) have compiled the scattered information on some extremophiles and their alkali-stable proteases. An earlier review on microbial alkaline proteases by Ellaiah *et al*. (2002) describes the resistance of proteases in extreme alkaline environments.

Aspergillus species have been the organisms of choice for large scale production of bulk industrial enzymes, as they can be grown on relatively inexpensive media and can secrete bulk quantities of enzymes (Bergquist *et al*., 2002). Various proteases are useful in the detergent industries (Maurer, 2004). A detailed study of alkaline protease from *Aspergillus niger* and its compatibility with commercial detergents was carried out by Dubey *et al.* (2010). Enzymes have been widely used in feed, brewing and laundry industry (Horikoshi 1999; Baredo 2004; Ahmed *et al.,* 2011), for which strains of *B. licheniformis, B. subtilis, B amyloliquifaciens*, and *B. mojavensis* (George *et al.*,1995; Rao *et al., 1998;* Kumar and Takagi, 1999; Gupta *et al.,* 2002; Adinarayana *et al.,* 2005; Haq and Mukhtar 2006; Shaheen *et al.*, 2008), *B.megaterium (Joo*, 2005), *B.stearothermophilus and B. thermoproteolyticus* (Rehman *et al., 2005*) are prominent

sources. Purification and characterization of an alkaline protease from *Bacillus licheniformis* UV-9 was carried out and tested for detergent formulations (Nadeem *et al.,* 2013). Alkaline protease was tested as a depilating agent in leather processing technology (Mukhtar and Haq, 2008). In the textile industry protease treatment is performed to remove the stiff and dull gum layer of sericin from the raw silk to achieve good lustre and softness (Doshi and Shelke, 2001). Various applications of microbial proteases have been tested in the pharmaceutical industry (Chanalia *et al.,* 2011). Shankar et *al,* (2010) reported silver recovery from used X-ray film with the help of alkaline protease from C*onidiobolus coronatus.*

Comprehensive research has been carried out on alkaline protease activity from eukaryotic marine protists thraustochytrids obtained from the mangrove ecosystem of Goa and with potential for use in the detergent and leather industry (Kanchana, *et. al.,* 2004). Matondkar *et. al* (1981) reported heterotrophic bacteria exhibiting cellulolytic, pectinolytic, amylolytic and proteolytic activity in Goa's mangrove areas. Purification, characterization and applications of thermostable alkaline protease from marine S*treptomyces* sp. obtained from the marine sediment of west coast of India has also been reported (Mane *et al.,* 2013). Hoffman and Decho (2000) examined the activities of cellular and extracellular proteolytic enzymes associated with the marine bacterium *Pseudoalteromaonas atlantica* in response to physiological state and changing environmental variables, under laboratory conditions.

Fungi from deep-sea sediments are very efficient sources of proteases. Deep-sea isolates of fungi from 5000 m depth in the Central Indian Ocean Basin have been screened for proteolytic activity (Damare, *et. al.,* 2004). These have been extensively studied as a

source of alkaline and cold-tolerant proteases (Damare *et al.,* 2009). An *Aspergillus ustus* isolate specifically produced alkaline, cold-tolerant protease at 30^{o}C and atmospheric pressure and the enzyme activity has been tested successfully to be stable in the presence of several commercial detergents (Patent EP1692296A1 - A process for producing alkaline proteases).

The lack of reports on systematic and comprehensive studies on proteolytic activity from various distinct and specific ecological niches in the coastal state of Goa as well as an interest in enzymes with novel properties from deep-sea sediments paved the way for the present research, which was hence initiated with the following primary objectives:

- Screening of samples from different niches, with a view to elicit high proteolytic activity (of bacterial/fungal origin) of industrial relevance.
- Extraction and preliminary characterization of a few enzyme extracts.
- Testing of selected enzyme extracts for potential biotechnological application in the detergent/oil/leather/textile industry.
- Purification and characterization of the most promising proteolytic activity.

In addition to the above, with the progress of the planned research, it was of interest to delve into characterization of a promising deep-sea protease-producing isolate and touch upon its pigment-producing capabilities. These data provided the basis of an additional Chapter to the thesis.

Chapter 2

Isolation of Protease Producers And their Ecological Relevance

Introduction

Microorganisms represent a very good commercial source of enzymes by way of their biochemical diversity and susceptibility to genetic manipulation. While they could be obtained in the pure form from culture collections, the more challenging and perhaps rewarding first step in developing producer strains would be their isolation from natural habitats. Proteases, being physiologically necessary for living organisms, may be obtained from widely diverse sources such as plants, animals and microorganisms. They play an important role in many biological processes in the release of protein, utilization of proteinaceous nutrient, autolysis and spore germination, besides other physiological phenomena.

The marine environment that covers about 70% of the earth's surface harbours a rich diversity of microbes including archaea, bacteria, cyanobacteria, eubacteria, actinomycetes, yeasts, filamentous fungi, micro- and macro- algae as well as protozoans (Chandrasekaran & Kumar, 1997). Primary screening procedures would allow the detection and isolation of microorganisms that possess potentially interesting industrial applications.

As a source of diverse enzyme classes, marine microorganisms hold a very important industrial potential in applications such as pharmaceuticals, nutritional supplements, agrochemicals, cosmetics and the like. The main isolation methods used routinely from marine sediment samples are serial dilution and spread plating. Primary screening is time-consuming and labour intensive since a large number of isolates have to be screened to identify a few ones of good potential. Marine microorganisms have already

been proven to have a great number of beneficial bioactivities such as production of industrially relevant enzymes.

Proteases constitute 59% of the global market of industrial enzymes (Deng *et.al.*, 2010). Insufficient information and increasing demands trigger the necessity to search for enzymes with novel properties. A primary objective of the present investigation was to identify a microorganism with strong protease-producing ability.

Protease producing microorganisms are found almost everywhere on the earth. Most important and active are the ones in sediments as they play an active role in remineralization (Xhang *et. al.,* 2015). Alkaline proteases produced by such microorganisms have been reported to have potential in industries pertaining to detergents, food, leather, textile, silver recovery, *etc.* (Ellaiah *et. al.,* 2002). To quote a few recent studies, *Bacillus* strains screened and identified for high alkaline protease activity have been isolated from the saltern pond of Kakinada (Laxmi *et. al.* 2014). Pathak and Deshmukh (2012) studied *Bacillus licheniformis*, an alkaline protease producing bacterial isolate from Lodhana Soda lake, Maharashtra. Soil, sediment and water samples from Tithal beach located at Valsad in South Gujarat coastal area were screened for protease producing micobes by Naik *et. al.* (2014). Proteases were reported from bacterial strains isolated from the sandy beaches of the south Baltic Sea (Mudryk & Podgórska, 2006). Marine sediment samples from the east coastal region of Thondi, Palk Bay, India were also screened for alkaline protease activity (Ibrahim *et. al.*,2005).

Microbes from the mangrove environment play a very critical role in creating and maintaining this ecosystem and also serve as a source of biotechnologically important

and valuable products (Thatoi *et. al.*, 2013). Many researchers have reported on alkaline protease producing microbes from mangrove ecosystems (Kanchana & Muraleedharan, 2010; Kathiresan & Bhingam, 2001; Sahoo & Dhal, 2008). The participation of marine microorganisms in degradation of organic compounds mark their potential as a highly rich source of hydrolytic enzymes of industrial relevance (Chandrasekharan & Kumar, 1997). The deep-sea environment is yet another unique source of microorganisms, having great potential for biotechnological exploitation (Smith *et. al,* 1992; Banse, 1990; Yuan *et. al.* 2009). Besides, mangroves, rivers, estuaries, lakes, beaches and salterns are also some of the possible nutrient-rich aquatic zones favouring flourishing of microbial communities that produce hydrolytic enzymes such as proteases. Deep sea sediments have been documented as a useful source in the search for alkaline proteases (Damare *et. al.*, 2006).

Microbes have a great role in the mineral cycle in the marine environment, degrading complex organic matter such as cellulose and hemicellulose into simple sugars which are then used by the organisms as a source of carbon, energy and nutrients. Those among them habituated to extreme environments are referred to as extremophilic. Such bacteria are considered a source of highly stable enzymes. For instance, enzymes derived from halophilic bacteria, which exhibit high activity and stability over a wide range of salinities, are often industrially useful enzymes. Organisms that thrive in natural to saline environments (0.5% to saturated NaCl), can be divided into three groups: slight, moderate and extreme halophiles, based on their requirements for NaCl (Larsen, 2001). Marine microorganisms secrete different enzymes based on their habitat and their ecological functions. They have been proven to have useful bioactivities such as the production of industrially promising enzymes (Chatellier et al., 2011).

Microbial degradation of protein in the environment can be regulated by nutrient availability. In marine environments several bacterial groups play a key role in the degradation of organic matter whereas some others are responsible for a significant contribution to the cycling of C, N and P (Deming, 2002). As seawater percolates through the beach sands, it can both deposit microbes, as well as mobilize existing microbes from the beach sands (Alexandria *et al.,* 2014). Specific or extreme environments are valuable natural reservoirs of extracellular hydrolases with unusual properties, worth exploring for biotechnological and bioremediation applications. Each microbial species carries specific combinations of genes for extracellular enzymes and is adapted to degrade specific substrates. Both substrate uptake and bacterial growth may be coupled with extracellular hydrolysis mediated by enzymes. The varied chemical structure of organic matter would require a suite of extracellular enzymes to access the carbon and nutrients embedded in detritus. The role of bacterial extracellular enzymes in the degradation of organic matter and their broad range of substrates make them suitable candidates for remediation of pollutants from contaminated environments. Based on this property of extracellular hydrolysis, sampling areas from diverse ecosystems were selected for the present study. Sediment samples from environs as varied as the deep-sea, mangroves, salterns, estuaries and a freshwater lake of Goa were sourced to study the diversity of alkaline protease-producing microorganisms.

Proteases are categorized as acidic, neutral and alkaline. Sodium carbonate is generally the major source of alkalinity in natural environments (Ellaiah *et al.,* 2002). Alkaline proteases produced by alkaliphilic strains show better resistance to alkali and some other denaturing chemicals in the reaction mixture, besides having higher affinity towards proteinaceous substrates. Such properties are very important for application in

the detergent and leather tanning industries (Kalisz, 1988). With the increasing demand for alkaline proteases there is a dire need to isolate, identify and utilize newer alkaline protease-producing microorganisms of industrial importance. Therefore unexplored niches are constantly being delved into, to identify potential producers of alkaline proteases.

This Chapter of the thesis has thus been dedicated to the search for protease producers from environmentally distinct locations and a discussion of the relationship of the alkaline proteases obtained, with respect to the ecological niches from which the individual microbes were sourced.

Types of Ecosystems Explored

a. Salterns

Extremely and moderately halophilic bacteria dominate saline environments (0.5% to saturated NaCl). There are a large number of recognized species of the genus *Bacillus* and related genera that could be described as moderately halophilic or halotolerant, some of which are also alkaliphilic (Surve *et al.*, 2012). Halophilic bacteria use different strategies for preserving their cell structure under high salt conditions. In the bargain they may produce compounds of industrial interest, such as extracellular hydrolytic enzymes with diverse potential applications in industries.

b. Coastal / Estuarine/Riverine

Most of the coastal regions are characterized as sensitive fragile ecosystems because they represent the interface between the land and the sea. These coastal areas are being subjected to high human pressures. Mass movement of people has led to urbanization, industrial and agricultural pollution, recreation and tourism, overexploitation of natural

resources, waste disposal, shipping, fishery industries, aquaculture, mining, oil spills, *etc*. Estuaries are where rivers discharge into the sea. They are semi-enclosed bodies of water, connected to the open sea, but where the seawater is diluted by freshwater from the land and within which there is a measurable variation of salinity due to the mixture of sea water with freshwater derived from land drainage. Estuaries from the tropics represent one of the most exploited ecosystems in the world (Blaber, 2000). They are rich in biodiversity and may have the highest economic value per hectare relative to any other aquatic environment (Costanza, 1997).

The Cumbharjua canal is an important waterway connecting the Zuari and Mandovi rivers of Goa. It is about 15 km long, forming the eastern boundary of the largest island in Goa that is delimited by the Mandovi in the north, the Zuari in the South and the Arabian Sea in the west. The Cumbharjua canal is an interesting example of two rivers interacting dynamically through a common channel within the estuarine region. The REIA study conducted in the year 2005 by Terra Firma Consultancy has reported that the Cumbharjua canal, being estuarine in nature, shows high salinity levels and pH values for sediment samples varying in a narrow range of 7.31 to 8.10.

c. Beach

Tides and waves flush large volumes of seawater through the beach daily. As waves pass through the beach, organic material and nutrients in seawater are remineralized and cycled. The permeable nature of sandy beach sediments allows for large quantities of seawater to pass through them over relatively short time scales. As a result, dissolved and particulate organic materials get mineralized. Microorganisms are responsible for

most of the biogeochemical cycling in the beach but there are only limited studies that characterized their diversity in intertidal sands.

d. Mangrove

Mangrove regions are unique swampy regions with water region being alkaline in nature and sediment or soil region having a neutral to slightly acidic pH, thus forming a unique ecological site to different microbes. Microbes help in recycling and transformation of various nutrients in the mangrove ecosystem thus making it more productive. This ecosystem shows a diversity of microbes that include bacteria, fungi, actinomycetes and the marine protists thraustochytrids. Microbial flora plays a significant role in the degradation of mangrove litter. The mangrove vegetation of Goa is plentifully distributed along the Mandovi and the Zuari rivers and the Cumbharjua canal that connects them. Shanbhag *et al.* (1994) have reported that mangrove environments harbour 50 bacterial strains, mostly gram positive.

e. Wetland

Wetlands are among the earth's most productive ecosystems. The Carambolim lake in Goa is a freshwater wetland. Wetlands play an important role in water storage and flood control. They contain significant floral and faunal habitats. Due to their relatively high rates of primary productivity and accumulation of organic matter in the sediments they also act as carbon sinks. Wetlands also contribute significantly to the fluxes of nitrogen, sulfur and phosphorus and possibly some pollutants such as heavy metals (Sarkar, 1998). Microorganisms present in wetlands play a vital role in the demineralization process.

f. Deep Sea

The deep sea is a unique and extreme environment characterized by high pressure, low temperature, lack of light and variable salinity and oxygen concentrations. Deep sea marine microorganisms take an active part in mineralization of complex organic matter through degradation. Deep sea bacteria were first recovered by Certes as a result of the Travaillier and Talisman Expeditions of 1882–83 (Das *et. al.,* 2006). Two types of fungi, the obligate and facultative marine fungi, are reported to occur in the marine environment (Kohlmeyer & Kohlmeyer 1979). The former grow and complete their life cycle only in the sea, whereas the 'facultative' ones are terrestrial or freshwater aquatic fungi which may also grow and reproduce in the marine environment. Spores and hyphal fragments of terrestrial fungi may get deposited in deep-sea sediments in close proximity to the coast through terrestrial runoff, sedimentation, currents and air blown from the land (Raghukumar *et al.,* 1998*).*

2.1 Materials and Methods

Site locations were selected to elicit most productive sample screening from diverse niches such as the mangrove, estuarine, riverine and lake ecosystems as well as sandy beaches, besides the more extreme environments including salt pans and the deep sea (Table 2.1).

All chemicals used in this study were of analytical grade and obtained from Hi-media Laboratories, Merck, Sisco Research Laboratories or S. D. Fine Chemical Laboratories.

Table 2.1. Sampling sites and ecosystem types

Serial No.	Sampling Site	Type of Ecosystem	Latitude / Longitude
1.	Kakra Beach	Sandy beach	15°.45' N / 73°.84' E
2.	Dona Paula Jetty	Estuarine	15°.45' N / 73°.80' E
3.	Diaz Beach	Sandy beach	15°.45' N / 73°.80' E
4.	Patto Jetty	Estuarine (river)	15°.50' N / 73°.84' E
5.	Cumbharjua canal	Estuarine (river)	15°.49' N / 73°.96' E
6.	Carambolim lake	Freshwater	15°.49 ' N / 73°.93' E
7.	Ribandar saltern	Hypersaline	15°.52' N / 73°.88' E
8.	Curca saltern	Hypersaline	15°.46' N / 73°.87' E
9.	Mangrove area (off Panaji)	Mangrove	15°.49' N / 73°.83' E
10	Divar	Mangrove	15°.53' N / 73°.91' E
11	Indian Ocean	Deep sea	9-16^{o} S / 73-76^{o} E

2.1.1 Sample Collection

Sediment samples were collected from Diaz Beach, Kakra (*aka* Cacra) beach, Dona Paula jetty, Panjim jetty, salterns of Ribandar, Carambolim lake, Cumbharjua canal and mangrove areas around Panaji in Goa. Sediment samples were collected by surface scooping into sterile polythene bags using a sterile scoop.

2.1.2 Isolation of Microbes

The collected sediment samples were serially diluted using sterile distilled water. From the suitable dilutions of the order of 10^{-6}, 100 µl suspensions were drawn and spread-plated on enrichment medium-N (Appendix A) for obtaining bacterial isolates. Fungi were isolated by surface-plating on Malt Extract Agar fortified with streptomycin and penicillin to inhibit bacterial growth. The isolated cultures of bacteria and fungi were subcultured at room temperature (RT) on Nutrient Agar (NA) and Malt Extract Agar (MEA), respectively. Ambient temperature was around 28 - 30^{o}C.

2.1.3 Screening for Proteolytic Activity

The isolated colonies were screened for protease production using skimmed-milk-agar medium, pH 7.0. Plates were incubated for 24h at 37°C for eliciting bacterial growth and 72h at RT for fungal growth. A clear zone of skimmed milk hydrolysis around the colonies was taken as indication of protease production by the organisms.

2.1.4 Enzyme Extraction from Bacterial Isolates

For protease production studies bacteria were inoculated in Nutrient Broth prepared in seawater and maintained at room temperature for 24 h on an orbital shaker (140 rpm), at the end of each which period, it was centrifuged at 10,000 rpm and 4°C for 15 min. The clear supernatant was used as the crude enzyme preparation.

2.1.5 Enzyme Extraction from Fungal isolates

Fungal isolates was inoculated in Czapek Dox medium and the flasks were kept static at room temperature. After 72 h enzyme extraction was carried out at room temperature by filtration through GF/F filters using a vacuum filtration unit. The filtrate was stored at 4°C for use as enzyme source.

2.1.6 Assay of Protease Activity

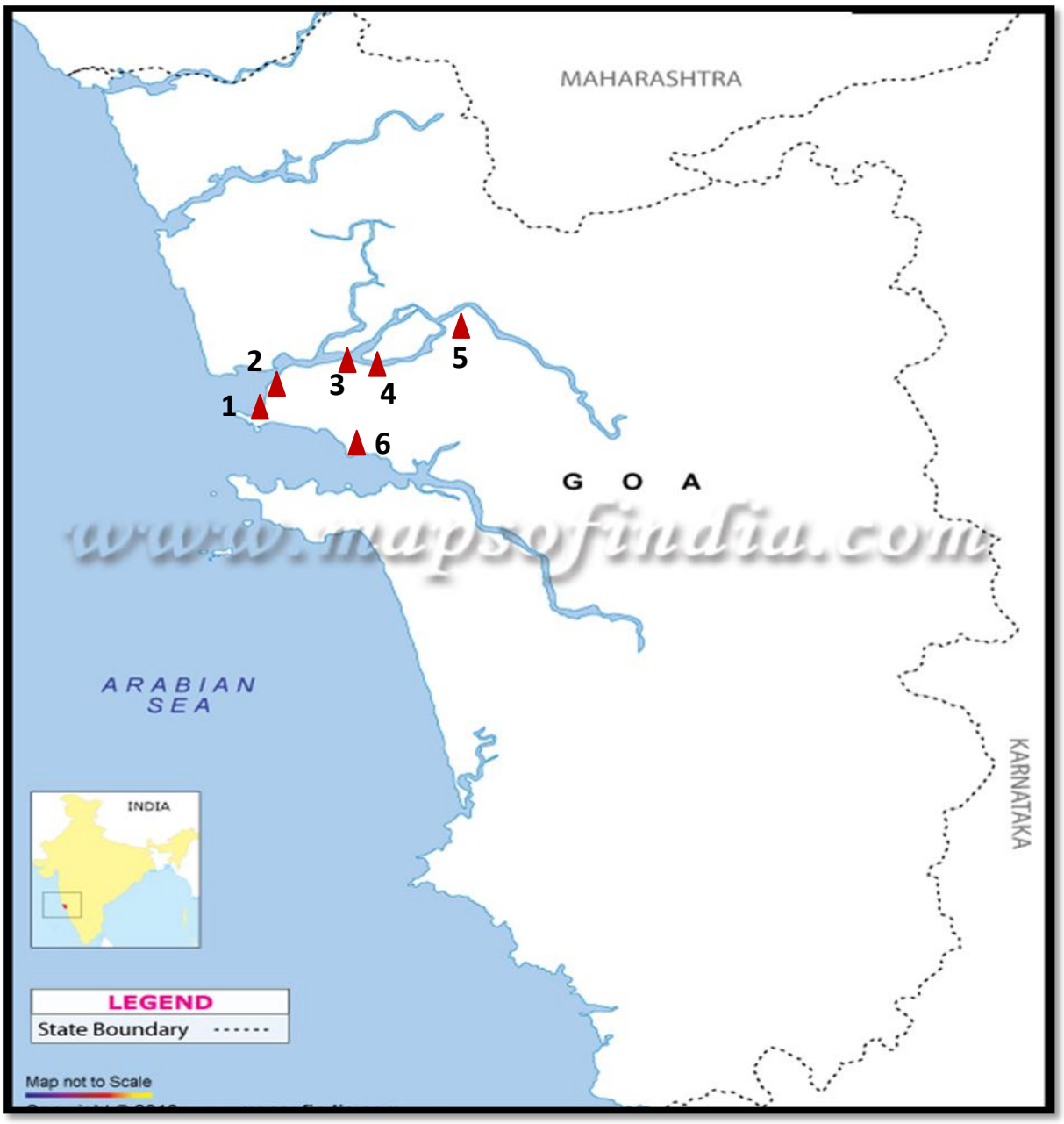
Protease activity in the culture supernatant/filtrate was determined according to Tsuchida et *al.* (1986), using casein as substrate. A mixture of 0.5% casein in borate buffer (50 mM, pH 9) and appropriate volume of crude enzyme extract were incubated (in a final volume of 2 ml) for 30 min in a water bath at 37°C. The enzymatic reaction was terminated by addition of 2 ml of 20% TCA and the tubes kept in ice for 15 min. The reaction mixture was centrifuged at 10,000 rpm for 15 min to separate the precipitated proteins. The supernatant was assayed for liberated tyrosine by the method of Lowry *et al.* (1951) using appropriate enzyme blanks and substrate blanks in the reaction. One unit of protease activity (1U) is defined as the amount of enzyme that releases 1 µg of tyrosine per minute under the assay conditions.

Protein was estimated by the method of Lowry *et al.* (1951) based on Bovine Serum albumin (BSA) as the standard.

2.2 Results and Discussion

2.2.1 Screening of Protease Producers

During the course of this survey 43 bacterial and 20 fungal cultures were isolated in all, from diverse ecosystems spread along the banks of the Mandovi and the Zuari rivers (Fig. 2.1). In addition, 12 saltern isolates (laboratory collection, courtesy Dr. S. Kerkar) and two deep sea isolates (courtesy, Dr. C. Raghukumar) were also tested for proteolytic activity. The different bacterial and fungal colonies obtained and their respective clearance zones are listed out in Tables 2.2 and 2.3.



Site 1: Dona Paula Bay; Site 2: Diaz Beach; Site 3: Panjim Jetty; Site 4: Ribandar; Site 5: Cumbarjua; Site 6: Cacra Beach

Fig. 2.1 Sampling locations in Goa

Table 2.2 Bacterial isolates showing zone of clearance on agar plates containing 1% skimmed milk.

Sr.no.	Sampling Site	Bacterial Isolate	Zone of clearance
1	Dona Paula jetty	BAU_1	++
2	Dona Paula jetty	BAU_2	+++
3	Dona Paula jetty	BAU_3	-
4	Dona Paula jetty	BAU_4	+++
5	Dona Paula jetty	BAU_5	+++
6	Dona Paula jetty	BAU_6	+
7	Panjim jetty	BAU_7	+++
8	Panjim jetty	BAU_8	++
9	Panjim jetty	BAU_9	++
10	Panjim jetty	BAU_{10}	+
11	Panjim jetty	BAU_{11}	+
12	Panjim jetty	BAU_{12}	++
13	Diaz beach	BAU_{13}	+++
14	Diaz beach	BAU_{14}	+++
15	Diaz beach	BAU_{15}	+++
16	Kakra beach	BAU_{16}	++
17	Kakra beach	BAU_{17}	+++
18	Cumbharjua canal	BAU_{18}	+++
19	Mangrove area (off Panaji)	BAU_{19}	+++
20	Mangrove area (off Panaji)	BAU_{20}	+++
21	Ribandar saltern	BAU_{21}	+
22	Ribandar saltern	BAU_{22}	+

23	Ribandar saltern	BAU_{23}	+
24	Ribandar saltern	BAU_{24}	+
25	Ribandar saltern	BAU_{25}	-
26	Ribandar saltern	BAU_{26}	-
27	Ribandar saltern	BAU_{27}	-
28	Carambolim lake	BAU_{28}	++
29	Carambolim lake	BAU_{29}	++
30	Carambolim lake	BAU_{30}	+++
31	Carambolim lake	BAU_{31}	-
32	Deep sea	DCU_1	+++
33	Deep sea	DCU_2	+++
34	Cumbharjua canal (Mangrove)	BAU_{32}	++
35	Cumbharjua canal (Mangrove)	BAU_{33}	+
36	Cumbharjua canal (Mangrove)	BAU_{34}	+
37	Cumbharjua canal (Mangrove)	BAU_{35}	++
38	Divar (Mangrove)	BAU_{36}	-
39	Divar (Mangrove)	BAU_{37}	+
40	Divar (Mangrove)	BAU_{38}	+
41	Divar (Mangrove)	BAU_{39}	+
42	Kamurli (Mangrove)	BAU_{40}	-
43	Kamurli (Mangrove)	BAU_{41}	+
44	Kamurli (Mangrove)	BAU_{42}	+
45	Kamurli (Mangrove)	BAU_{43}	+
46	Ribandar (Saltern)	968	+
47	Curca (Saltern)	971	+

48	Ribandar (Saltern)	972	+
49	Curca (Saltern)	975	+
50	Ribandar (Saltern)	976	+
51	Curca (Saltern)	981	+
52	Ribandar (Saltern)	983	+
53	Ribandar (Saltern)	984	+
54	Curca (Saltern)	985	+
55	Ribandar (Saltern)	986	+
56	Ribandar (Saltern)	991	+
57	Ribandar (Saltern)	987	-

Note: + indicates extent of protease production

- indicates non-proteolytic

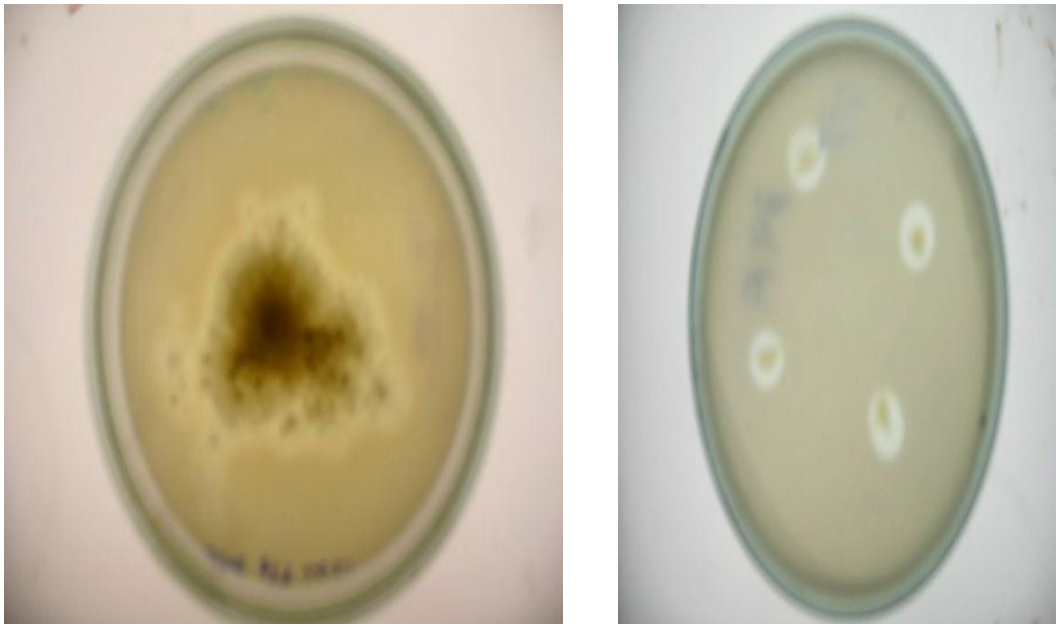
Table 2.3. Fungal isolates showing zone of clearance on Czapek Dox media plates containing 1% skimmed milk.

Sr.no.	Sampling Site	Fungal Isolate	Zone of clearance
1	Diaz beach	FAU_1	-
2	Diaz beach	FAU_2	-
3	Diaz beach	FAU_3	+
4	Diaz beach	FAU_4	++
5.	Patto jetty	FAU_5	-
6	Patto jetty	FAU_6	+++
7	Patto jetty	FAU_7	+
8	Patto jetty	FAU_8	+++
9	Patto jetty	FAU_9	+
10	Patto jetty	FAU_{10}	+
11	Patto jetty	FAU_{11}	+
12	Carambolim lake	FAU_{12}	-
13	Carambolim lake	FAU_{13}	-
14	Carambolim lake	FAU_{14}	-
15	Carambolim lake	FAU_{15}	-
16	Dona Paula jetty	FAU_{16}	+
17	Dona Paula jetty	FAU_{17}	++
18	Dona Paula jetty	FAU_{18}	-
19	Mangrove area (off Panaji)	FAU_{19}	-
20	Mangrove area (off Panaji)	FAU_{20}	+

Note: + indicates extent of protease production

- indicates non-proteolytic

A total of 57 bacterial strains from different niches were thus screened for protease producing ability by inoculating and testing for qualitative production of protease on NA plates containing 1% skimmed milk (Fig 2.2a), of which 48 isolates showed proteolytic activity with a zone of clearance on the plates. The 20 fungal colonies isolated were tested for qualitative production of protease on Czapek Dox media plates containing 1% skimmed milk (Fig 2.2b), of which 11 isolates showed proteolytic activity. All the sampling sites thus clearly harboured protease producers. This was not surprising as proteolytic bacteria are known to be widespread in nature, being able to grow under various conditions (Prabhakaran *et al*. 2015).



a **b**

Fig. 2.2 Casein hydrolysis by typical (a) fungal and (b) bacterial isolates

2.2.2 Protease Activity in Ecological Niches

In an ecosystem, extracellular enzyme activity is mostly controlled by enzyme-substrate interactions such as inhibition, adsorption, stabilization and humification. The bacterial population in marine samples holds a very important and diversified enzymatic potential. Coastal areas are getting increasingly polluted by domestic, commercial, agricultural and industrial pollutants. The metal contamination of sea water is mainly due to discharge of the chemical load from various industries into the rivers and from the rivers to the sea. The metal accumulating (Cu, Cd, Ni, Pb) property of some marine bacteria makes them potential sources for bioremediation (De *et al*., 2006; Miranda and Rojas, 2006).

Systematic screening of moderately halophilic bacteria producing extracellular hydrolytic enzymes of diverse types carried out by Sanchez-Porro *et al.* (2003) and Rohban *et al.* (2009) have revealed that such bacteria are potential sources of extracellular hydrolytic enzymes such as amylases, DNases, lipases and proteases.

An attempt is made in the present study to source for proteases of possible industrial importance from moderately halophilic bacteria. Forty eight bacterial isolates and 11 fungal isolates (which during primary screening had shown potential to produce protease) were selected based on the zone of clearance and further screened to select the most potent strains from amongst them.

Hobbie *et. al.* (1977) had reported that in higher salinity sediments, more energy is required for the production of osmolites and less is used for the release of extracellular enzymes, resulting in decreased extracellular enzymatic activity. In our study on bacterial isolates from salterns we could detect protease activity in the range of 2.6 to

11.26 U/ml, except from the Curca saltern isolate which produced enzyme of activity 19.19 U/ml. Kamat *et al.* (2011) had reported that 21% of bacteria isolated from salt pans of Goa tested positive for amylase production while only 6% exhibited protease activity.

Surve *et al.* (2012) isolated protease producing bacteria from Ribandar salterns and reported protease activity of 14.1 U/ml and 12.76 U/ml for strains A1 and V1, respectively. A halophilic thermostable alkaline protease producing haloarchaea was isolated from marine solar salterns of Mulund, Mumbai (Pathak and Sardar, 2014). Similarly, protease-producing halophilic microorganisms *Bacillus subtilis* and *Aspergillus flavus* isolated from Sambhar lake and Mumbai seashore were reported by Annapurna *et al.* (2012). Very little is documented in literature about the diversity of alkaline protease producing microbes, specifically with respect to protease producers from various saltpans of Goa.

The deep sea sediment is rich in detritic material which helps microorganisms to grow and take active part in mineralization of organic matter. Both our bacterial isolates obtained from the deep-sea habitat of the Indian Ocean exhibited proteolytic activity. Protease activities from isolate DCU_1 were, respectively, 34.70 and 45.52 U/ml when grown in 0% and 0.5% skimmed milk medium, whereas isolate DCU_2 had protease activities of 2.02 and 8.31U/ml, respectively, thus making isolate DCU_1 a better option for further characterization.

As per Cunha *et. al.*, (2010) freshwater lakes and estuarine waters, being less buffered than the oceans, exhibit daily seasonal changes in pH. Furthermore, coastal and estuarine environments also show high spatial variability in pH over short time scales. The fertility of the estuary depends on the flow of nutrients from the river and on tidal

currents. While drainage from the land is the major source of nutrient inputs into the estuary, industrial effluents and city waters also find their way into the estuary (Verlencar, 1994). Thus the Mandovi-Zuari rivers are rich in nutrients, especially nitrates and phosphates. Earlier studies by Bharathi *et al.* (1991) have reported occurrence of delta proteobacteria in mangrove swamps of the Zuari estuarine system in Goa. The Dona Paula and Panjim jetty areas are affected by tourism activities (motor boating).

Eleven bacteria and 8 fungi capable of producing proteases were isolated from the jetty areas covered in our studies. The bacterial isolate from Dona Paula jetty showed very high protease activity of 159.09 U/ml whereas an activity of 67.3U/ml was the highest from among the bacterial isolates from Panjim jetty. The Cumbharjua canal is affected by industrial effluents and boat activities. In fact two major industrial estates at Corlim and Kundaim are located very close to our sampling site in this canal area. Isolate BAU_{33} from the Cumbharjua canal showed the highest protease activity of 231 U/ml among all.

The mangrove ecosystem plays a vital role in nutrient and metal cycling (Harbison, 1981; Acerda and Abrao *et al.*, 1984). To a great extent, the organic content of the sediment determines the abundance of heterotrophic bacteria (Kuznetsov, 1968). Microbial organisms such as yeast, bacteria and fungi play a very important and dominant role in the decomposition of mangrove foliage, regeneration of nutrients and mineralization. The decomposition of mangrove litter fall produces detritus, which in turn is colonized by heterotrophic microorganisms, thereby enhancing its nutritive value. The Divar mangrove ecosystem lies along the Mandovi estuary. This estuary receives a considerable supply of inorganic nitrogenous nutrients from various sources

such as mining wastes (De Souza, 1999), land runoff (Sardessai and Sundar, 2007) and sewage effluents (Ansari *et al.*, 1986). The literature regarding alkaline proteolytic bacteria from mangroves areas of Goa is scanty. Significantly, in the present study eight out of ten bacterial isolates and one of the two fungal isolates tested from mangrove samples were positive for protease production.

Proteases were synthesized very intensively by bacterial strains isolated from the sand of the Sopot beach of Poland (Mudryk & Podgórska, 2005). Corals on rocky beaches produce mucus which plays an important role in reef metabolism as a significant source of organic material and supports high bacterial activity. Coral mucus consists mainly of polysaccharides and protein and the mucus released from the corals serves as good growth substrates for bacteria (Das *et. al.*, 2006). In our study, beach sands were collected from Diaz Beach and Kakra Beach which were exposed to recreational and fishing boat activities. Among the three bacterial isolates from Diaz beach, strain BAU_{14} produced highest protease activity of 86.4 U/ml and among the two from Kakra beach; strain BAU_{16} produced highest protease activity of 83.9 U/ml (Table 2.4).

Agro-industrial residues are proven to be a good source of substrate for alkaline proteases (Akcan & Uyar, 2011). Wetland plays a crucial role in the regional ecosystem, such as the regulation of climate, cleansing of environment and balancing of regional water (Deka *et al.*, 2011). The increased input of organic nutrient into the lakes due to surface runoff, washing of cattle and night soil generated by floating population seems to facilitate the luxuriant growth of the bacterial population in the Carambolim lake. Water pH is considered to be the reflection of many chemical and

biological processes taking place in natural waters. The pH of waters of the Carambolim lake ranges from 6.10 to 6.63 (Shanbagh *et. al.*, 1994). Due to the rich ecology, this wetland is blessed with many species of migratory birds as well as waterbirds. One would therefore expect significant proteolytic activity in these wetlands, facilitating degradation of the bird feathers and droppings. In our study, among the four bacterial isolates screened from the Carambolim lake, three were protease producing, of which strain BAU_{30} showed highest protease activity of 10.92 U/ml (Table 2.4).

Table 2.4 Alkaline Protease activity from bacterial isolates grown in medium with or without skimmed milk

Sr. No.	Area of collection	Isolate	Activity with 0% milk in medium (U/ml)	Activity with 0.5% milk in medium (U/ml)
1	Dona Paula jetty	BAU_1	24.3	39.7
2	Dona Paula jetty	BAU_2	77.9	159.1
3	Dona Paula jetty	BAU_4	7.8	19.1
4	Dona Paula jetty	BAU_5	30.9	1.5
5	Dona Paula jetty	BAU_6	10.2	9.0
6	Panjim jetty	BAU_7	3.5	67.4
7	Panjim jetty	BAU_8	2.2	3.0
8	Panjim jetty	BAU_9	1.1	39.3
9	Panjim jetty	BAU_{10}	2.4	0
10	Panjim jetty	BAU_{11}	0.9	64.2
11	Panjim jetty	BAU_{12}	1.4	8.3
12	Diaz beach	BAU_{13}	36.6	23.9
13	Diaz beach	BAU_{14}	86.4	1.7
14	Diaz beach	BAU_{15}	35.2	65.9
15	Kakra beach	BAU_{16}	4.5	83.9
16	Kakra beach	BAU_{17}	12.6	69.2

17	Ribandar saltern	BAU_{20}	4.8	0
18	Ribandar saltern	BAU_{21}	2.6	0
19	Ribandar saltern	BAU_{22}	2.5	0.7
20	Ribandar saltern	BAU_{23}	6.1	11.3
21	Ribandar saltern	BAU_{24}	3.2	0
22	Carambolim lake	BAU_{28}	4.6	0
23	Carambolim lake	BAU_{29}	2.1	4.0
24	Carambolim lake	BAU_{30}	1.6	10.9
25	Panjim	BAU_{19}	3.2	0.1
26	Cumbharjua canal	BAU_{18}	4.0	117.5
27	Cumbharjua canal	BAU_{32}	20.4	215.6
28	Cumbharjua canal	BAU_{33}	18.6	231.0
29	Cumbharjua canal	BAU_{34}	42.63	214.2
30	Divar	BAU_{37}	9.45	1.56
31	Divar	BAU_{38}	4.73	38.60
32	Divar	BAU_{39}	5.55	3.81
33	Kamurli	BAU_{41}	3.29	7.28
34	Kamurli	BAU_{42}	4.51	22.18
35	Kamurli	BAU_{43}	45.49	195.50
36	Ribandar saltern	968	3.64	2.43
37	Curca saltern	971	11.79	4.16
38	Ribandar saltern	972	3.34	2.43
39	Curca saltern	975	2.77	5.72
40	Ribandar saltern	976	3.47	2.91
41	Curca saltern	981	6.59	19.19

42	Ribandar saltern	983	5.37	3.29
43	Ribandar saltern	984	1.74	5.20
44	Curca saltern	985	4.16	5.72
45	Ribandar saltern	986	2.60	5.20
46	Ribandar saltern	991	2.95	6.67
47	Deep Sea	DCU1	34.70	45.52
48	Deep Sea	DCU2	2.02	8.31

Commercial bacterial proteases are produced from high yielding strains including species of *Bacillus,* of which *Bacillus subtilis* and *Bacillus licheniformis* form the most important group (Prabhakaran *et al*., 2015).

Fungi have a potential to grow under varying environmental conditions such as pH and temperature, utilizing a wide variety of substrates as nutrients (Haq *et al.*, 2006). Several strains of fungi (*Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratinophilum, Fusarium graminarum, Penicillium griseofulvin and Scedosporium apiosermum*) have been reported to produce proteases (Chinnassamy *et al.,* 2011). In our study, nine fungal strains isolated from various niches showed alkaline protease activity (Table 2.5). Isolates from Panjim jetty and Diaz beach, when induced with 3% skimmed milk, showed activity of 20.55 U/ml and 9.41 U/ml, respectively.

Table 2.5 Alkaline protease activity from fungal isolates grown in medium with or without skimmed milk

Sr. No.	Area of collection	Isolate	Activity with 0% milk in medium (U/ml)	Activity with 3% milk in medium (U/ml)
1	Diaz beach	FAU_3	1.26	0
2	Diaz beach	FAU_4	2.78	9.41
3	Panjim jetty	FAU_6	2.08	20.55
4	Panjim jetty	FAU_7	1.91	0
5	Panjim jetty	FAU_8	2.21	15.27
6	Panjim jetty	FAU_9	1.73	0
7	Panjim jetty	FAU_{10}	1.26	0
8	Panjim jetty	FAU_{11}	0.87	0
9	Dona Paula jetty	FAU_{16}	0	0
10	Dona Paula jetty	FAU_{17}	2.43	11.61
11	Panjim Mangrove	FAU_{20}	0	0

In summary, the bacterial and fungal strains (48 bacterial and 11 fungal) isolated in our study were also shown to significantly produce proteolytic enzymes. The strains from Dona Paula jetty, Carambolim lake, Cumbharjua canal and Panjim jetty were the dominant protease producers, probably supported by the presence of a variety of substrates (mangrove litter, shells, dead fishes, human activity, *etc*.).

Microorganisms thus not only maintain the pristine nature of the environment but also serve as biological mediators through their involvement in the biogeochemical processes. Decomposition of protein takes place by proteolytic bacteria, e.g. *Pseudomonas* and other eubacteria (Das *et al.,* 2006). Recent anthropogenic interventions in the marine environment have menaced all lives, including microorganisms. The existing scientific data is hence still insufficient to predict consequences of global change on extracellular enzymatic activity, the marine environment and on the subsequent processes of carbon cycle. Thus study of marine microbial biodiversity is of crucial importance to the understanding of the different processes of the ocean, which may present novel microorganisms for screening of bioactive compounds and useful enzymes.

Chapter 3

Studies on Proteases from selected isolates and Potential biotechnological applications

Introduction

Proteases have established themselves as substitutes for chemicals in many industries**.** Those with good activity at alkaline pH and high temperature are often of preferred interest in industrial units. Approximately 60% of the total enzyme sales in the world is accounted for by microbial proteases (Gupta R., 2002; Ellaiah *et al.*, 2003). In fact, alkaline proteases alone account for 20% of the world enzyme market, with their gaining prominence in leather processing and in the detergent industry (Oberoi *et al.*2001). During the screening and search for proteases with novel properties that could prove of industrial significance, optimizing assay conditions for eliciting maximum enzyme activity is a first step. This would also serve to elucidate the nature of the enzyme activity, *i.e.,* whether acidic, alkaline or neutral and suggest probable and specific biotechnological applications that could be explored.

According to the latest report published by *market and market.com* (2015), the industrial enzymes market was valued at USD 4.2 billion in the year 2014. This is projected to reach USD 6.2 Billion by 2020, growing at a Compound Annual Growth Rate (CAGR) of 7.0% from 2015 to 2020. It is estimated that the World enzyme demand would rise 6.3% annually to $7 billion in 2017. Rising *per capita* income in large countries such as China and India will support consumer demand for higher value goods such as detergents and food products, wherein enzymes would inevitably find a place. Advances in biotechnology will further boost speciality enzyme demand.

Microbial proteases are preferred over the enzymes from plant and animal sources as they possess almost all the desired characteristics favouring industrial applications. Alkaline proteases, in particular, are of considerable interest in diverse industrial

applications where their activity and stability at alkaline pH serve as plus points. Microbial alkaline proteases have enormous applications in several industries such as leather processing, detergents, silver recovery, medical purposes, food processing, feeds and chemical industries, as well as in waste treatment where their potential is much greater. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds. Ecological biodiversity plays an important role in the search for improved strains of microorganisms which serve as an invaluable resource for biotechnological innovations. For instance, proteases isolated from extremophilic organisms are likely to possess special properties desirable for commercial applications.

Industrial applications of proteases have posed several problems and challenges for further improvements (Sawant and Nagendran 2014). To curb pollution and to provide a sustainable environment for improving the quality of human life, advancement in biotechnology for the development of proteases should continue. The world has an increasing demand for eco-friendly processes and technologies in order to reduce negative environmental impact.

Today, the major producers of detergent enzymes, supplying up to 95% of the global market of proteases are Novozyme and Gencor International. BIO-40 introduced in 1956 was the first bacterial enzyme containing detergent (Rao et al., 1998). All major subtilisins incorporated in detergents are produced by *Bacillus* spp because these species secrete large amounts of extracellular enzymes (Gupta *et al*., 2002). Most of the protease enzymes originate from *B. amyloliquefaciens*, *B. licheniformis*, *Bacillus clausii*, *Bacillus lentus*, *Bacillus alkalophilus* and *Bacillus halodurans* (Khan, 2013).

Ishikawa *et al.* (1993) and Masui *et al.* (1999) developed an interesting application for alkaline proteases in reporting the use of one such enzyme to decompose the gelatinous coating of X-ray films, from which silver could be recovered. Proteases are also useful in biopharmaceutical products such as contact-lens enzyme cleaners and enzymic debriders (Anwar and Saleemuddin, 2000). Regulatory, safety and environmental issues have prompted the development of aqueous enzymatic extraction (AEE) for obtaining components from oil-bearing materials. In general, enzyme-assisted oil extraction is known to yield oil having highly favourable characteristics (Mat *et.al.,* 2014). The use of enzyme has the potential to increase quality, productivity and efficiency of the oil obtained from seeds (Kalia *et. al.,* 2001).

In Chapter 2 the results of protease activity quantified from bacterial and fungal isolates obtained from different niches have been reported. The strains from the Cumbharjua canal, Carambolim lake, Diaz beach, Panjim jetty and deep-sea showed remarkable activity and have hence been considered for preliminary characterization, the results of which are discussed in this Chapter. Such isolates were then tested for their potential applications in the detergent industry, in oil extraction and in silver recovery from X-ray films. Other studies requiring more purified enzyme preparations are reported in Chapter 6.

3.1 Materials and Methods

The microbial media components were products of Hi-Media Laboratories, India. All other chemicals used were of analytical grade.

All estimates have been carried out in triplicate, and the values presented are representative of two to three independent experiments. Statistical analysis of data has also been carried out.

3.1.1 Enzyme assay of the selected bacterial and fungal isolates

The selected bacterial and fungal strains were grown as described under Sections 2.1.4 and 2.1.5 respectively. By way of the first round of detailed studies on quantification, crude culture filtrates were used as enzyme source for carrying out assays at 28°C (ambient temperature) and at pH 9.0, since the microbial samples screened from different niches grew naturally and could produce enzymes under similar conditions.

3.1.2 Assay temperature studies

Alkaline protease activity of the enzyme extracts was determined at pH 9.0 and temperatures ranging from 5°C to 90°C, maintaining all other conditions as routine.

3.1.3 Assay pH studies

The effect of pH on protease activity in the crude enzyme extracts was tested by carrying out assays at the optimum temperature determined for the respective isolates. Buffers used were at pH values ranging from 3.6 to 10.0, maintaining all other conditions as routine.

3.1.4 Stability of enzyme in presence of commercial detergents

To study the effect of various commercial detergents and SDS, the protease assay was carried out at 45ºC after pre-incubating the enzyme preparation with detergent for 30 min at RT.

3.1.5 Blood/Egg stain removal

Equal size fabric pieces were stained with the same quantity of human blood and air-dried. The stained fabric was incubated for 30 min at RT with detergent with or without added enzyme, and using appropriate controls. The medium containing any washed out protein was collected. Each piece was then individually rinsed with tap water and dried.

The same protocol was followed for egg stain tests also.

After the swatch test for blood stains, the absorbance of the supernatant fluid was read at 416 nm.

3.1.6 Decomposition of gelatinous coating of X-ray film

X-ray film pieces (1x2 cm) were incubated with 1.5 ml of enzyme samples from selected bacterial and fungal isolates at room temperature (28 - 30°C) for 1h. The protein that eluted from gelatin layers was estimated by the Folin-Ciocalteau method (Lowry *et. al.,* 1951) using BSA as standard.

3.1.7 Aqueous extraction of coconut oil

Method of enzyme assisted aqueous extraction of coconut oil was followed from Debrah and Ohta (1997), with slight modifications**.** Finely milled copra meal samples were mixed with distilled water and the enzyme extract (100µl) in 3ml Eppendorf centrifuge tubes and incubated at 45°C for 19 h. Thereafter the mixture was centrifuged and floating oil was observed. Quantity of oil extracted was measured. Extraction of oil in the absence of added enzyme served as control.

3.2 Results and Discussion

3.2.1 Assay Temperature and pH Studies

The crude enzyme extracts from selected fungal and bacterial isolates were subjected to preliminary characterization to evaluate assay conditions such as optimal temperature and pH as well as low temperature activity. Of the two fungal and eight bacterial isolates selected for this first round of evaluation of enzyme properties, bacterial isolates BAU_{33} and BAU_{34} showed highest protease activity of 214.55 U/ml and 277.72 U/ml, respectively, when assayed at RT and pH 9.0 (Table 3.1).

Strains BAU_{33} and BAU_{34} were isolated from the Cumbharjua canal which has an estuarine environment. Desai *et. al.* (2004) reported 25 strains of obligate alkaliphilic bacteria isolated from various estuarine ecosystems of Goa exhibiting an optimum growth pH of 10.5. Among those isolates 50% showed protease activity, 39% showed amylase activity and all showed lipase activity under alkaline conditions. This diversity of enzyme production in such estuarine ecosystems indicates that such organisms have an important role to play in recycling organic matter. The Cumbharjua canal has mining ore transport activities which might add metals to the sediment. Some alkaline proteases require metal ions in the form of salts in the production medium. It has been reported by Nigam *et. al.* (2014) that $FeSO_4.7H_2O$ and $MgSO_4.7H_2O$ enhanced the protease production by *Bacillus subtilis* RSKK 96. Nehra *et.al.* (2004) reported Mg^{2+} as an activator of the alkaline protease produced by *Aspergillus* sp. The addition of Fe^{2+} and Mg^{2+} further increased alkaline protease production whereas Zn^{2+} and Cu^{2+} repressed protease production by *B. subtilis* RSKK96 (Akcan & Uyar, 2011). Positive effects of

metal cations on protease production have also been observed by other researchers (Wang *et al.,* 2005; Haddar *et al.,* 2009; Uyar *et al.,* 2011).

The Cumbharjua canal also has abundant mangrove vegetation that possibly adds litter as substrate for the growth of microorganisms. As mentioned earlier, Matondkar *et.al.* (1981) reported cellulolytic, amylolytic, pectinolytic and proteolytic activity from heterotrophic bacteria isolated from the mangrove areas of Goa. The detritus produced by the mangrove is the base of an extensive food web that supports numerous organisms of ecological and commercial importance (Holguin *et.al.,* 2001).

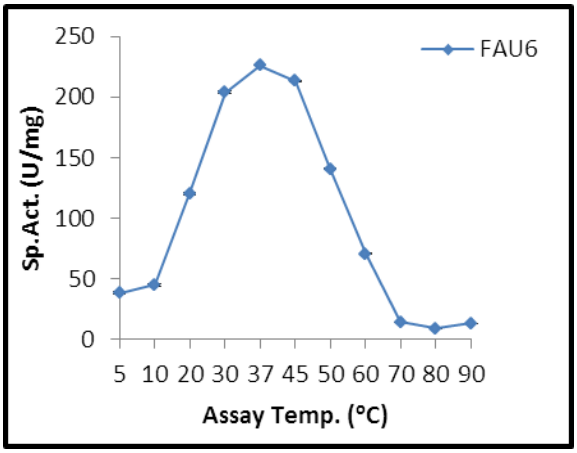
Table 3.1. Enzyme activity from selected bacterial and fungal isolates at RT and pH 9.0

Sr.no.	Enzyme source (Isolate) (Isolate) (Sample	Activity(U/ml) (U/ml)
1	BAU_{17}	1.72
2	BAU_{30}	23.05
3	BAU_{32}	3.29
4	BAU_{33}	214.55
5	BAU_{34}	277.72
6	BAU_{42}	30.75
7	DCU_{1}	24.96
8	DCU_{2}	15.26
9	FAU_{4}	5.33
10	FAU_{6}	44.51

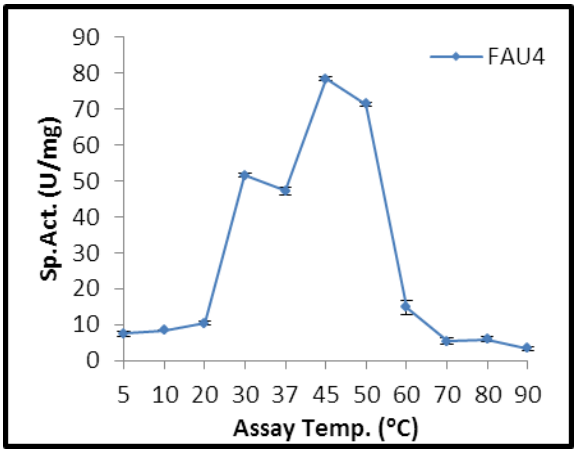
While investigating the effect of assay temperature on alkaline protease activity from the different isolates, in most cases the optimum temperature was found to be in the range of 45-50 ^{o}C, beyond which the enzyme activity sharply declined, as depicted in Fig.3.1 (a-j). Even at 60^{o}C, isolate BAU_{34} retained 81.23% of its highest activity (which was at 45^{o}C). Isolate BAU_{30} which was obtained from the Carambolim lake had an even higher optimal activity temperature of 60-70 ^{o}C.

Two distinct temperature optima were evident for the alkaline protease activity from isolates BAU_{7} *viz.,* at 37 ^{o}C and 50^{o}C. No specific reason could be attributed to this but for the possibility that the crude culture supernatant contained more than one alkaline protease.

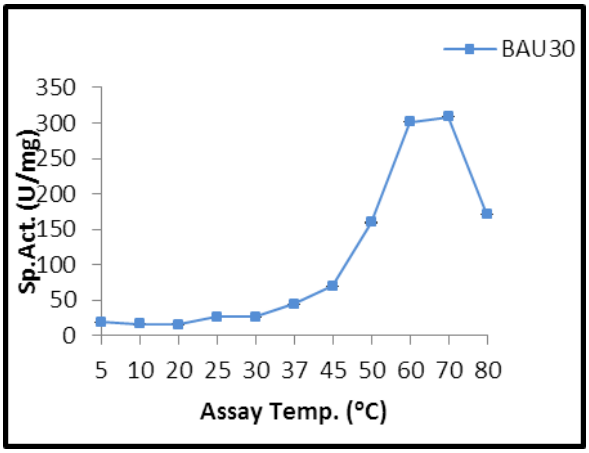
Of the two deep-sea isolates, although DCU_{2} had a distinct assay temperature peak at 50^{o}C, DCU_{1} had a 5.27 fold higher activity at its optimal temperature (40 - 45^{o}C), making the latter the deep-sea isolate of choice for further studies.


FAU6
Sp.Act. (U/mg)
250
200
150
100
50
0
5 10 20 30 37 45 50 60 70 80 90
Assay Temp. (°C)

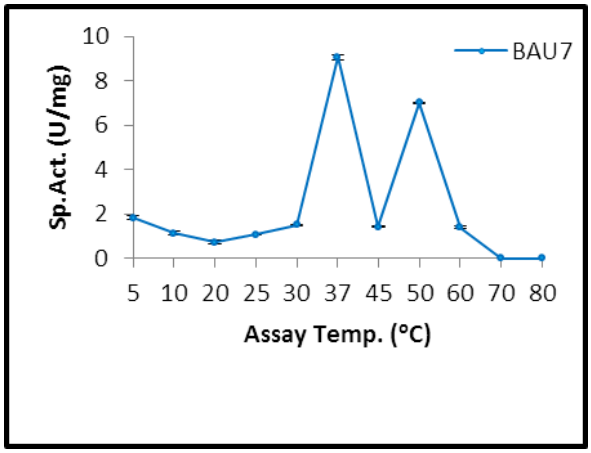
a


FAU4
Sp.Act. (U/mg)
90
80
70
60
50
40
30
20
10
0
5 10 20 30 37 45 50 60 70 80 90
Assay Temp. (°C)

b


BAU30
Sp.Act. (U/mg)
350
300
250
200
150
100
50
0
5 10 20 25 30 37 45 50 60 70 80
Assay Temp. (°C)

c


BAU7
Sp.Act. (U/mg)
10
8
6
4
2
0
5 10 20 25 30 37 45 50 60 70 80
Assay Temp. (°C)

d

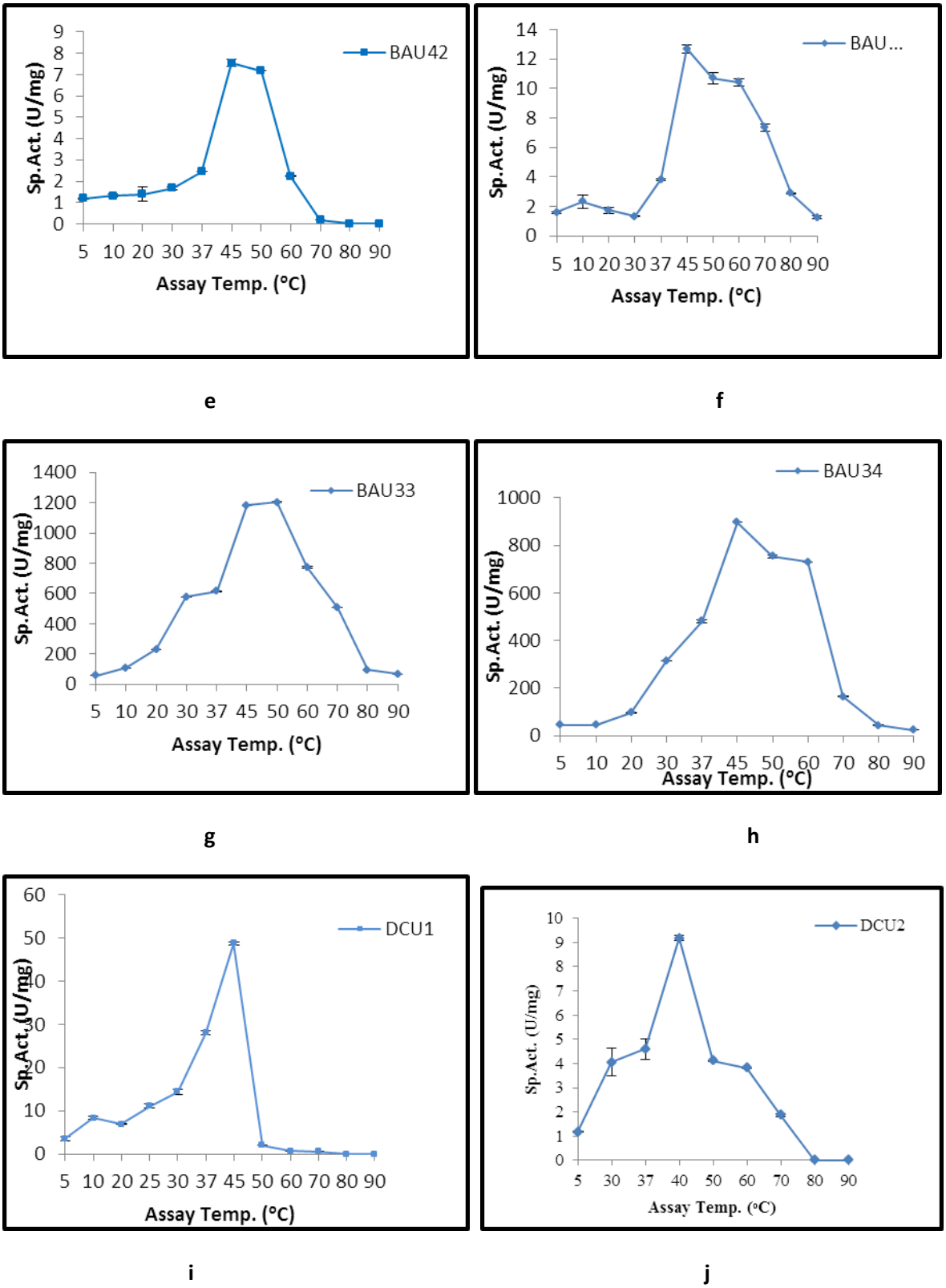


Fig. 3.1 Effect of assay temperature variation on alkaline protease activity from selected isolates (a-j; FAU_6, FAU_4, BAU_{30}, BAU_7, BAU_{42}, BAU_{32}, BAU_{33}, BAU_{44}, DCU_1, DCU_2)

Enzymes endowed with low temperature activities are used in the food, cosmetics, pharmaceutical and biofuel industries; they are also applied to substances for molecular biology, in nanotechnology, in the manufacturing of household detergents, in the cleaning of animal wastes, with peptidases for cleaning contact lenses and with pectinases for extraction and clarification of fruit juices (Dalmaso *et.al.*, 2014; Huston, 2008). The application of cold active enzymes enables lowering of the temperature and shortening of processing times without loss of efficiency, resulting in reduced energy consumption. Such enzymes are thus of potential use in some industrial applications such as food processing, detergent additives and biotransformation of chemicals (Ramteke, 2011). With this in view, assays at low temperatures ranging from 5 to 25°C were also carried out on our enzyme samples. Some of the bacterial isolates such as BAU_7, $BAU_{30,}$ $BAU_{33,}$ BAU_{34}, DCU_1 and fungal isolate FAU_6 produced low-temperature active alkaline proteases active at pH 9.0 (Fig. 3.1).

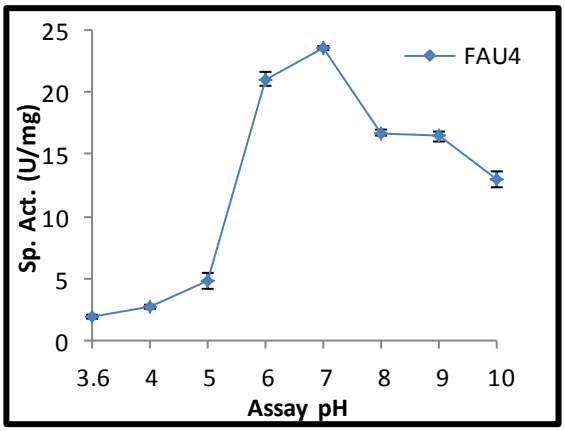
The appreciable activity of the alkaline proteases at low, ambient and higher temperatures highlights their capacity to be used at any wash temperature when added to laundry detergents (Niyonzima, 2015).

Factors such as pH, temperature, aeration, inoculum density, *etc.*, also have a profound effect on protease production (Puri *et al*., 2002). Based on assay temperature results, pH studies were carried out for those selected isolates exhibiting enzyme activity above 10 U/mg.

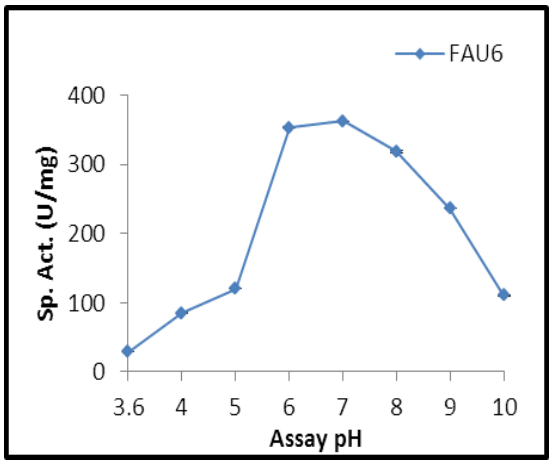
The results of our assay pH studies indicated that both the fungal isolates produced proteases optimally active at neutral pH (Fig. 3.2 a & b). Likewise the enzymes from bacterial isolates BAU_{33} and BAU_{34} which had shown highest proteolytic activity, as well as that from the deep-sea isolate DCU_2 could be better classed as neutral proteases

(Fig.3.2 g). Neutral proteases have been exploited for food industries and are reported to be used to produce soy sauce and soy products which are less bitter and also in brewing industry (Rao *et al*., 1998).

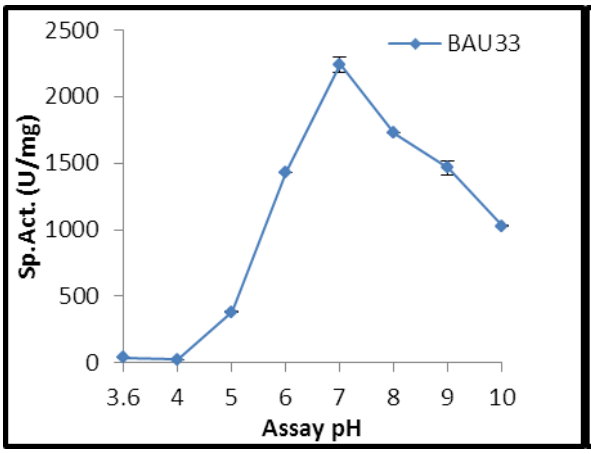
Alkalinity of the Carambolim lake is around 30 m.eq.$l^{-1}$and pH is 7.32, as reported by RIEA studies conducted by Terra Firma Environmental Consultancy. Takami *et. al*. (1990) have commented that high protease activity in the alkaline region could be attributed to the better binding of enzyme to the substrate since pH is a strong deciding factor in the binding of enzyme to substrate. The protease from isolate BAU_{30} isolated from the Carambolim lake had a rather broad pH optimum ranging from pH 6.0 and peaking at pH 9.0 (Fig.3.2 e). Agro-industrial residues found due to paddy fields near the lake might be acting as a good carbon source for alkaline enzyme producers as reported by Prakasham *et.al*. (2006). Metal ions such as $Fe^{3+,}$ $Ca^{2+,}$ Mg^{2+} and Zn^{2+} also affect protease production in microorganisms (Singh *et al*., 2001; Adinarayana *et al*., 2003). The calcium, manganese and iron content in the Carambolim lake might be responsible for the enhanced alkaline protease activity. Mazollo *et al*. (2011) demonstrated that feather waste could be used as a cheap and ecofriendly substrate for enzyme production. As migratory and other common birds visit this lake, it would have a natural availability of keratin as substrate favouring alkaline protease production. The unique temperature and pH requirements for the alkaline protease activity from the Carambolim lake isolate BAU_{30} thus appeared to fall in line with the ambient environmental conditions.


FAU4
Sp. Act. (U/mg)
Assay pH
0
5
10
15
20
25
3.6
4
5
6
7
8
9
10

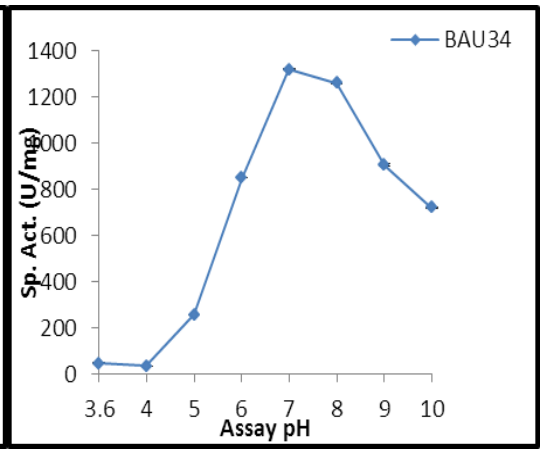
a


FAU6
Sp. Act. (U/mg)
Assay pH
0
100
200
300
400
3.6
4
5
6
7
8
9
10

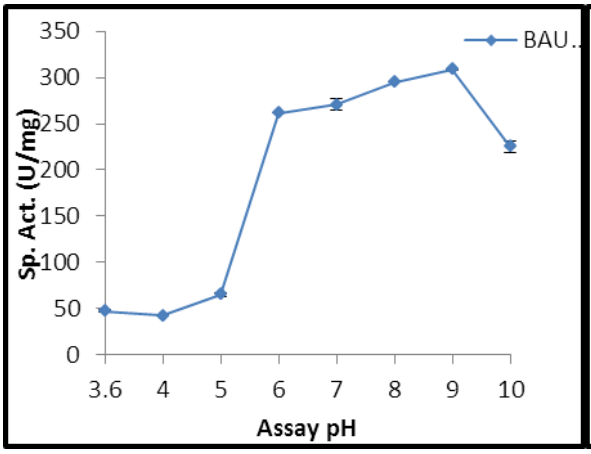
b


BAU33
Sp.Act. (U/mg)
Assay pH
0
500
1000
1500
2000
2500
3.6
4
5
6
7
8
9
10

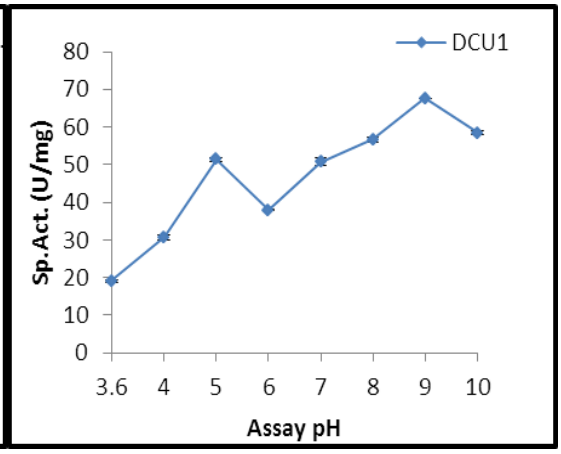
c


BAU34
Sp. Act. (U/mg)
Assay pH
0
200
400
600
800
1000
1200
1400
3.6
4
5
6
7
8
9
10

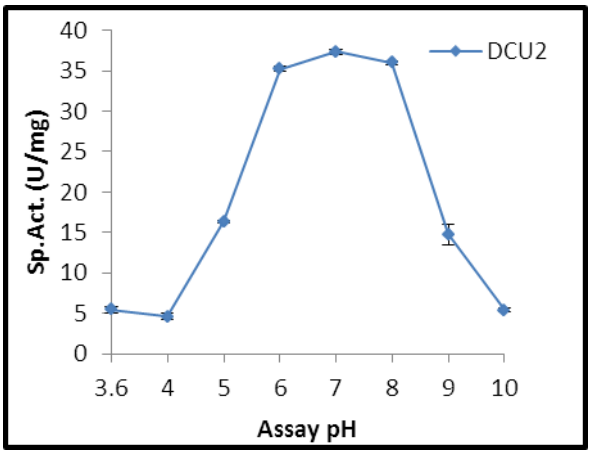
d


BAU..
Sp. Act. (U/mg)
Assay pH
0
50
100
150
200
250
300
350
3.6
4
5
6
7
8
9
10

e


DCU1
Sp.Act. (U/mg)
Assay pH
0
10
20
30
40
50
60
70
80
3.6
4
5
6
7
8
9
10

f



g

Fig.3.2 Effect of assay pH variation on alkaline protease activity from selected isolates (a-g; FAU_4, FAU_6, BAU_{33}, BAU_{34}, BAU_{30}, DCU_1, DCU_2)

At 37^{o}C, there appears to be broad pH specificity over the neutral and alkaline range for the enzyme from the deep sea isolate DCU_1, in addition to a lower but distinct activity peak at pH 5.

3.2.2 Application in the Detergent Industry

Laundering is an uncontested worldwide common need and proteases are increasingly being incorporated into laundry detergents. To meet the demand for new, improved and modified products the alkaline protease market requires continuous consideration of new materials. The suitability of the alkaline protease from isolate DCU_1 as a detergent additive was evaluated by testing its stability in the presence of several commercial detergents.

As shown in Fig. 3.3, the enzyme was stable in the presence of most commercial detergents at the recommended detergent concentrations normally used for washing, thus rendering it a suitable candidate for commercial use. These observations concur with those from other similar studies (Sellami-Kamoun *et al.*, 2008; Devi *et al.,* 2012; Anwar and Saleemuddin, 1997) that reported the stability of bacterial protease enzymes with a wide range of commercial solid detergents. In the case of 'A' and 'F' (Fig. 3.3) the enzyme activity was lower, probably because these detergent preparations have inherent proteases which could act on the enzyme in our samples. The alkaline protease from the deep-sea bacterial strain DCU_1 showed most promising results since upon addition to detergent, it was able to effectively remove egg as well as blood stains from fabric (Figs. 3.4 & 3.5).

While monitoring the extent of solubilisation of blood into the wash medium, the absorbance (colour that eluted) was higher in all cases where detergent was supplemented with enzyme as compared to detergent alone, except for 'F' which is marketed as an enzyme- supplemented product (Fig. 3.6). The active protease from strain DCU_1 was found to be effective in protein stain removal from clothes even at room temperature, without damaging the texture of the cloth. This protease could thus have potential as a detergent additive.

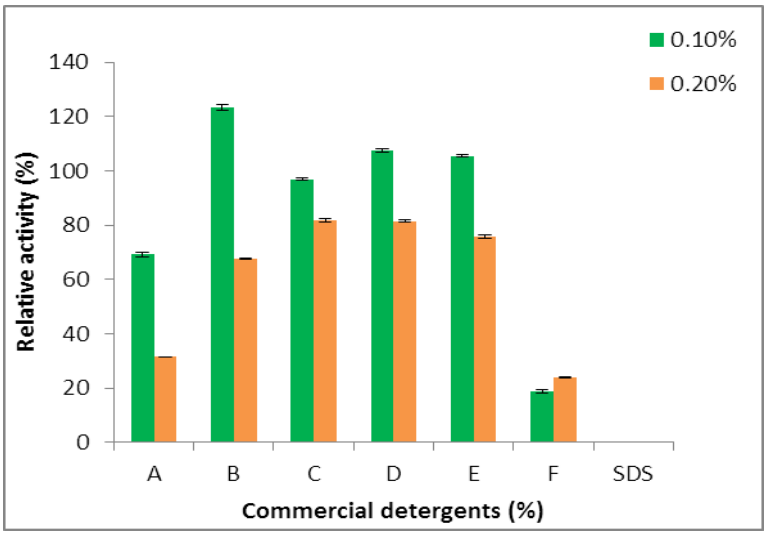
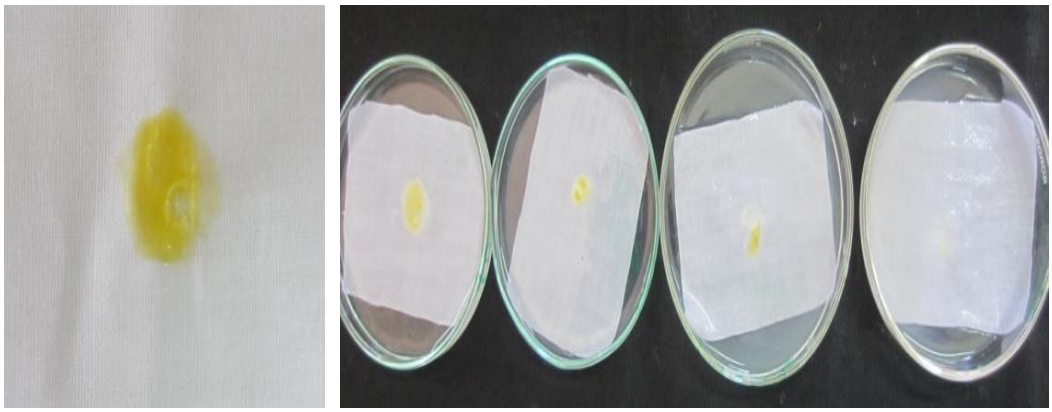


Fig.3.3 Effect of detergents on enzyme activity



Before wash Tap water Detergent Enzyme Detergent +Enzyme

Fig.3.4 Clearing of egg stain from fabric

Before wash Tap water Detergent Enzyme Detergent +Enzyme

Fig.3.5 Removal of blood stain from fabric

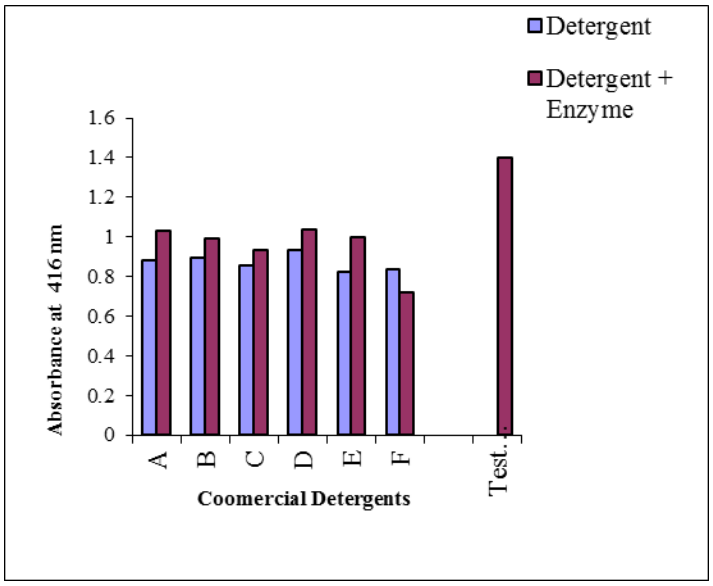
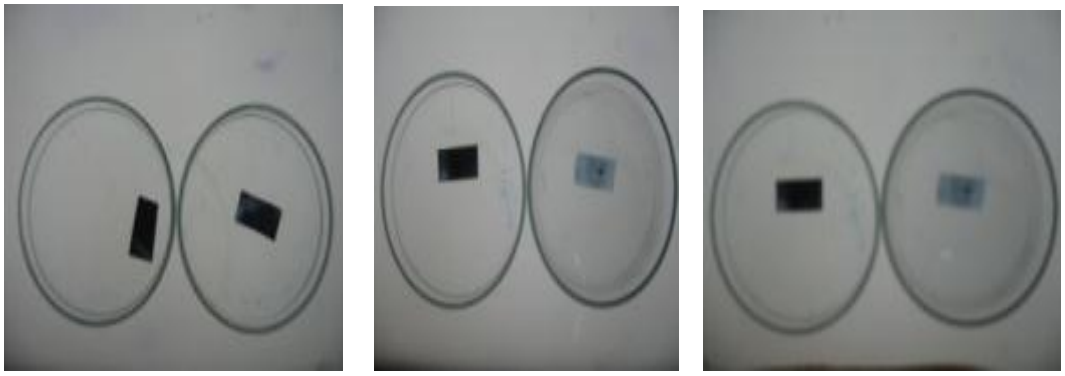


Fig.3.6 Solubilisation of blood in swatch assay

3.2.3 Application in silver recovery from used X-ray Films

Since the emulsion layer on X-ray film contains silver and gelatin, it is possible to break down the gelatin layer using proteases and release the silver (Nakiboglu *et al*., 2001). The waste X-ray/ photographic films contain 1.5 - 2 % (w/w) black metallic silver which is recovered and reused. Around 18-20% of the world's silver needs are supplied by recycling photographic waste (Laxman *et al.* 2010). Alkaline protease from our isolate DCU_1 was tested for enzymatic hydrolysis of gelatin from used X-ray films. Gelatin degradation was observed most clearly in the case of the deep-sea bacterial isolate DCU_1 and fungal isolates PJ8 & DB7, releasing the silver and leaving the polyester film clean and clear for re-use (Fig. 3.7).



Control BAU_{30} BAU_{7}

FAU_{4} FAU_{6} DCU_{1}

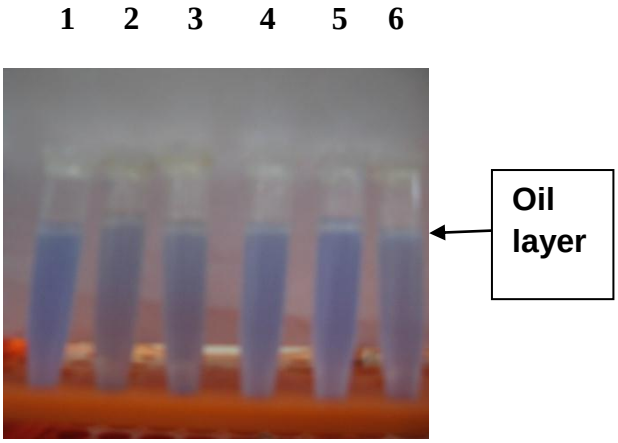
Fig 3.7 Clearance of gelatinous coating on X-ray film after incubation of 2h with microbial enzymes from different sources.

3.2.4 Application in Enzyme Assisted Aqueous Extraction of Coconut Oil

Preliminary experiments have indicated that enzymatic hydrolysis by our different protease samples significantly increased extraction yield of oil from desiccated coconut. Increase in yield was observed in all the tested samples relative to experimental controls (Table 3.2**)**, suggesting the possibility of using enzyme-assisted oil extraction methods to improve upon traditional copra processing. The blue colour seen in tubes was due to the addition of a drop of Coomassie Brilliant Blue stain to get a clearer image of the floating oil layer in the tube (Fig. 3.8). This is an environment friendly process which could be studied in more detail to further improve upon the extraction procedure and possible utilization in the oil industry.

In summary, out of the eight bacterial and two fungal strains tested, the deep-sea isolate DCU_1 producing alkaline protease optimally active at 45-50°C was preferentially selected for further characterization, since most of the industrial operations prefer enzymes that are active at higher temperatures and alkaline conditions.

Table 3.2 Enzyme-assisted oil Extraction



Tube No.	Isolate used as enzyme source	Oil extracted (µl)
1	BAU_{30}	115
2	BAU_{7}	100
3	DCU_{1}	210
4	FAU_{4}	150
5	FAU_{6}	110
6	Control	100

Fig.3.8 Oil extracted by adding Protease enzyme from the isolate:

1. BAU30 2. BAU7 3. $DCU_1$4. FAU4 5. FAU6 6. Control with D/W.

Chapter 4

Characterization of Alkaline protease from Isolate DCU_1

Introduction

Enzymes exhibit many interesting properties which provide a basis for industrial operations. In fact the abiding commercial interest in enzyme systems has resulted from an exigency to apply their vast catalytic potential, high specificity and high catalytic activity under mild environmental conditions of temperature, pH and pressure. Since proteases form a complex group of enzymes they differ widely in properties such as substrate specificity, active site and catalytic mechanism (Rao *et. al.* 1998). Alkaline proteases are industrially important enzymes with a wide range of applications. Most of the industrial processes being carried out under specific physical and chemical conditions such as alkaline pH and high temperatures, it thus becomes crucial to gain an understanding of the optimal pH and temperature, thermal and pH stability as well as other functional aspects such as enzyme kinetics and substrate specificity. Some industrial applications of proteases also call for stability in the presence of organic solvents. Certain industries such as those related to food or pharmaceuticals understandably demand enzymes in the pure form. Preliminary characterization of the enzyme becomes advantageous both to gauge the industrial potential as well as to develop strategies for the purification process.

4. 1 Materials and Methods

All chemicals used were of analytical grade from Hi-media Laboratories, Merck, Sisco Reasearch Laboratories and S.D. Fine Chemicals.

4.1.1 Growth, Enzyme and Pigment Production

The deep sea bacterial strain DCU_1 was inoculated at different timings with 2h interval, in NB medium containing NaCl (0-4%) to study the growth pattern, salt requirement and enzyme activity. Absorbance was read at 660nm as an indicator of turbidity and at 535 nm for pigment production.

Strain DCU_1 was plated on NA media plates and incubated at different temperatures to monitor the pigment production pattern.

4.1.2 Effect of Temperature on Enzyme Stability

Thermostability of the alkaline protease was examined by pre-incubating the enzyme preparation at pH 9.0 and different temperatures (40°C - 90°C) for a 10 min time period. The supernatant obtained after centrifugation was assayed for the residual protease activity at 45^{o}C and pH 9.0, which was then expressed as percentage of the initial activity (taken as 100%).

For the time course study, enzyme sample was pre-incubated at 45°C for varying time periods up to 4h. The supernatant obtained after centrifugation was used for estimation of the residual alkaline protease activity as above.

4.1.3 Effect of pH on Enzyme Stability

The effect of pH on alkaline protease stability was studied by pre-incubating the enzyme in borate buffer (pH 9.0) for 20 min at RT. The residual activities were determined at 45°C for a 45 min reaction time and then expressed as percentage of the initial activity (taken as 100%).

4. 1.4 Kinetic Constants of the Enzyme

The enzyme was assayed at pH 9 and 45°C using various concentrations of casein (0.25-3.0 mg/ml). The K_M and V_{max} values were calculated by linear regression analysis using the Lineweaver-Burk plot.

4. 1.5 Studies on Effectors of Enzyme Activity

The enzyme sample was pre-incubated at RT with EDTA, iodoacetamide or phenyl methylsulphonyl fluoride (PMSF) (10 min), metal ions (30 min) or reducing agents (15 min). The residual activity was then measured under the routine assay conditions.

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4.1.6 Dialysis

The crude enzyme was subjected to dialysis at 4°C, providing three changes of the buffer (25 mM borate, pH 9.0). The dialysate was used for further study.

4.1.6 Substrate Specificity Studies

Various substrates such as casein, BSA, gelatin, hemoglobin and keratin were used at a concentration of 0.5% (w/v) in the enzyme assay, with the activity towards casein serving as control.

4.2 Results and Discussion

4.2.1 Growth Kinetics and Protease Production

Marine organisms generally require salt for growth and enzyme production. The growth pattern of the deep sea isolate DCU_1 in NB medium containing 0-4% NaCl was hence monitored. After the initial lag period, growth was exponential up to 15h, followed by the

stationary phase (Fig. 4.1). The pattern was similar at the various salt concentrations, slowing down marginally only at the highest concentration tested (4% NaCl).

Enzyme synthesis is related to cell growth and therefore there is a correlation between incubation period and enzyme production (Kaur, *et al*., 1998). Protease production by strain DCU_1 in relation to its growth is shown in Fig 4.2. While low amounts of alkaline protease were produced from the early exponential phase of growth, the peak of enzyme production was well into the stationary phase. Thereafter a decline in activity was observed which might be due to cessation of enzyme synthesis coupled with autoproteolysis. Similar findings were reported by Nisha & Divakaran (2014) and Olajuiyigbe *et al*. (2005) for protease production by *Bacillus* sp.

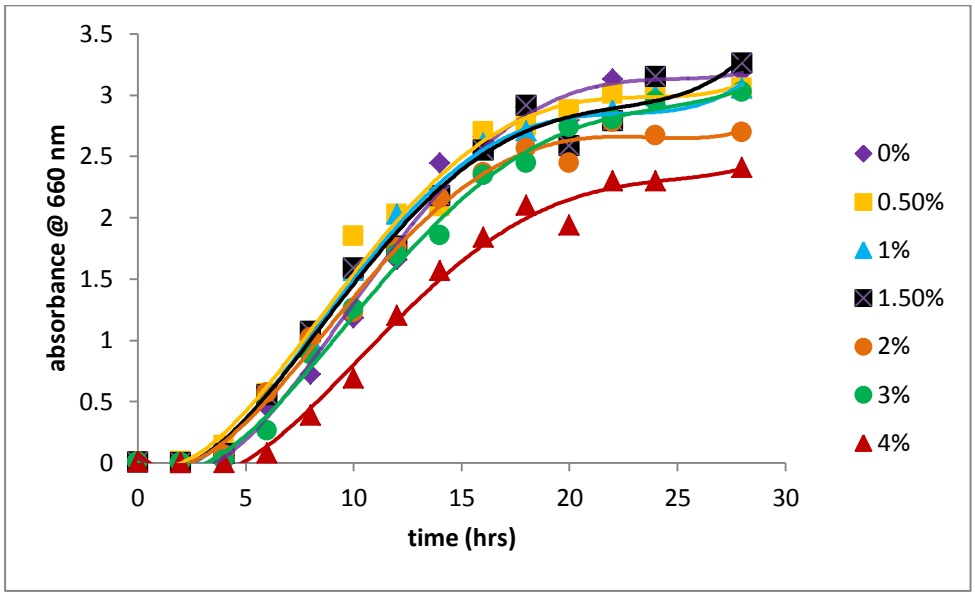


Fig 4.1. Growth of DCU_1 in NB medium

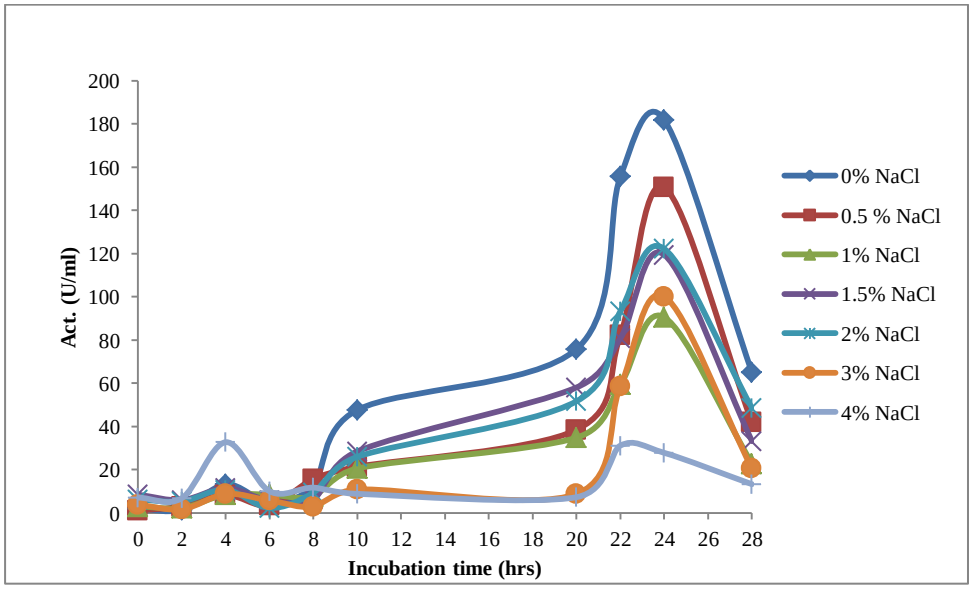


Fig.4. 2 Enzyme production at different stages of growth

As in the case of growth of the isolate in culture, the trend of enzyme activity was similar at the various salt concentrations, although slowing down at the higher concentrations. When 24 h grown cultures were analyzed for the effect of salt concentration on enzyme production, the alkaline protease activity decreased with increase in NaCl concentration although production was significant even at 6% NaCl (Fig. 4.3).

4.2.2 Enzyme Concentration Studies

When assayed using different concentrations of enzyme protein, typical saturation kinetics was observed correlating the enzyme activity with protein concentration (Fig. 4.4). For all further assays, protein in the range of 0.05 to 0.1mg was used.

4.2.3 Time Course of Assay

Enzyme assay was carried out for different reaction times at pH 9.0 and at 45°C to find the optimum reaction time for enzyme assay (Fig 4.5). The reaction time thus selected for all further experiments was 45 min.

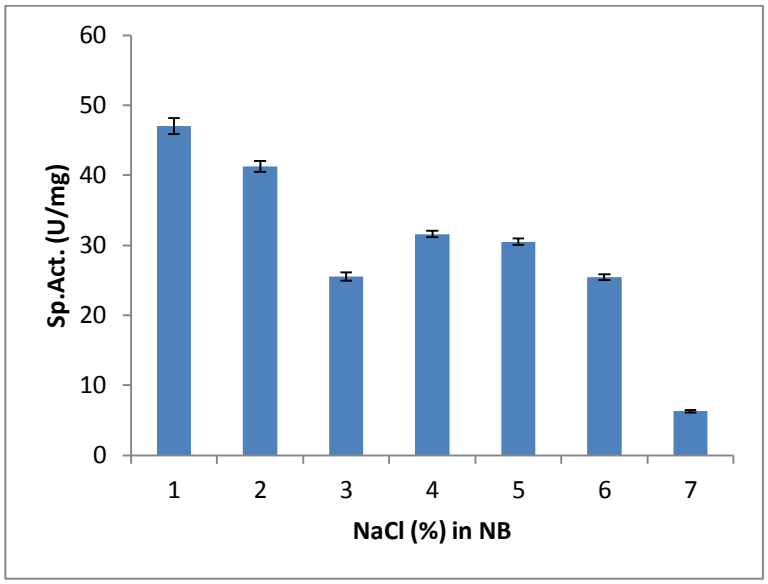


Fig. 4.3 Effect of NaCl on enzyme production when cells harvested after 24h.

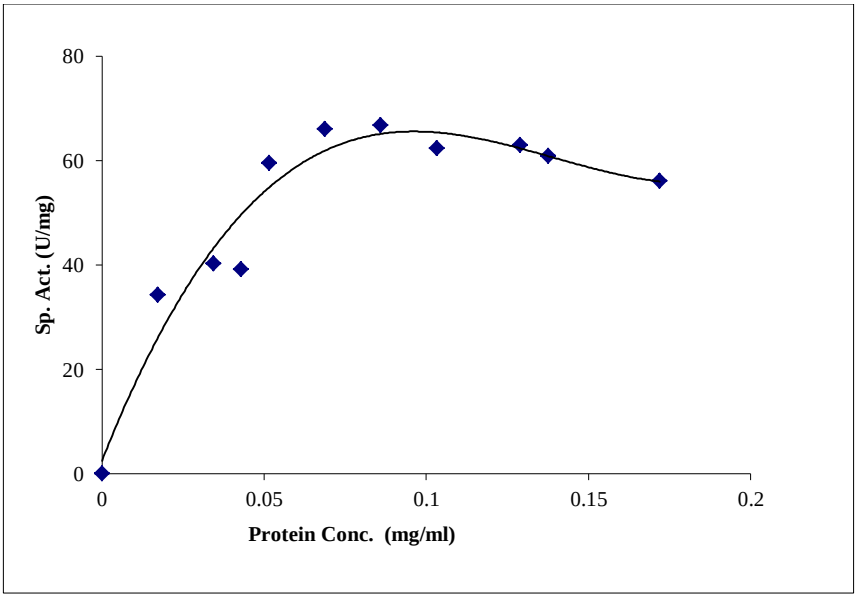


Fig. 4.4 Enzyme concentration curve

4.2.4 Thermal Stability

Thermal stability of the enzymes is either due to the presence of some metal ions or for efficiency to carry out their biological activity at higher temperature (Al-Shehri, 2004; Haq *et al.*, 2006). When tested for stability at different temperatures for 10 min, maximum residual activity for the enzyme preparation from isolate DCU_1 had been obtained at 40°C (Fig 4.6).

The results of a time course study of residual activities after incubation at 45°C and 40°C are given in Figs 4.7 and 4.8, respectively. These studies indicated retention of about 95% of activity even after 4 h incubation at 40°C (specific activity of control being 54.89 U/mg) and about 76% of the activity after 2h incubation at 45°C. In fact, incubation for 1 h at 40°C apparently stimulated the activity by about 30% **(**Fig. 4.9**).** Such events are not uncommon in situations where a thermolabile inhibitor is present in the enzyme extract. Most alkaline proteases have been reported to remain stable at high temperature in the presence of Ca^{2+} ions (Rahman *et al.*, 1994; Kumar *et al.*, 1999).

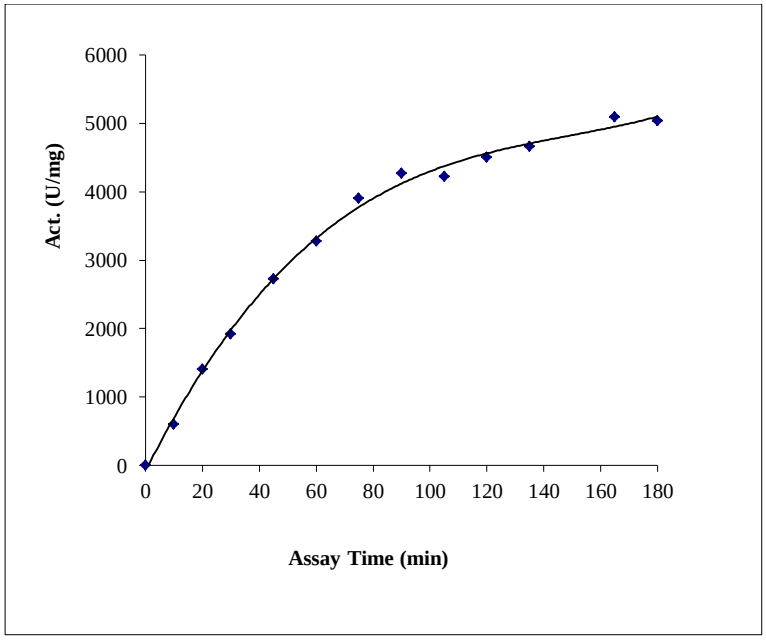


Fig.4.5 Enzyme activity at different reaction times

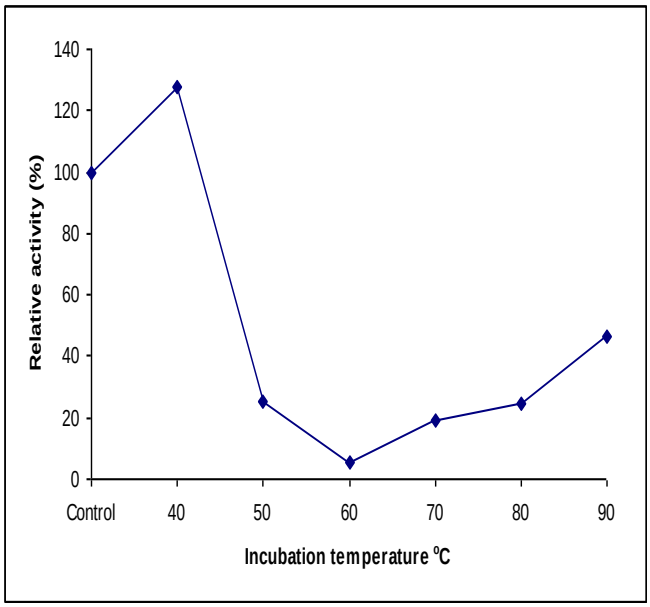


Fig.4.6 Thermal stability for 10min at various temperatures

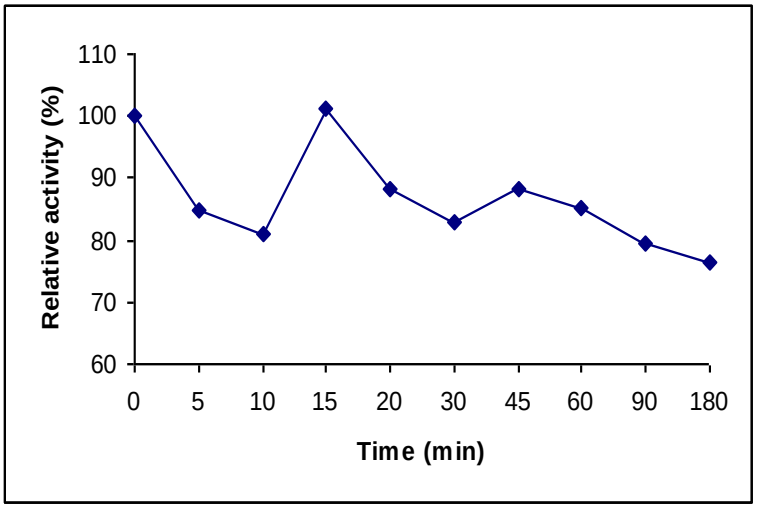


Fig.4.7 Thermal stability at 45°C

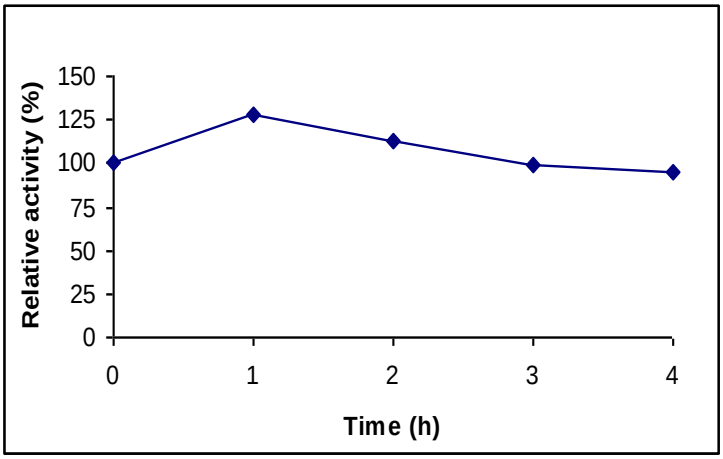


Fig. 4.8 Thermal stability at 40°C

Abusham *et. al.* (2009) have reported an alkaline protease from *B. subtilis* with 100% stability in the temperature range of 35–55 °C. The thermostability shown by the alkaline protease from our isolate was comparable with or better than that reported for alkaline proteases from different *Bacillus* species (Laxmi, 2015; Aruna, 2014; Saranya, 2014).

4.2.5 Effect of pH on Enzyme stability

The pH is a key factor which determines the effectiveness of an enzyme as a detergent additive. Generally all detergent-compatible enzymes have their pH optima values in the range of 9-12 (Gupta et al., 1999). Stability at high temperature and alkaline pH enhance the possibility of using the protease in the pre-tanning processes of leather and in other biotechnological applications that would require higher working temperatures (Agrawal *et. al.* 2012). The alkaline protease enzyme from isolate DCU_1 was stable between pH 5 and 10 (**Table 4.1)**, while even at pH 4, 58.66% of the activity was retained.

Table 4.1 Effect of pH on enzyme stability

pH	Relative Activity (%)
4	58.66
5	77.17
6	87.73
7	72.11
8	78.46
9	84.94
10	85.12

4. 2.6 Kinetic Constants of the Enzyme

Enzyme assay was carried out at different concentrations of substrate and as shown in Fig. 4.9 the enzyme followed typical Michaelis-Menten kinetics.

Lineweaver-Burk analysis of the substrate concentration data had shown that the enzyme had a K_M value of 1.25 mg/ml for casein and a V_{max} of about 100 µg tyr/min/ mg (Fig. 4.10), which is higher than most reports in literature.

4.2.7 Effect of Salt Concentrations

Salt affects the stability of the enzyme in a cooperative manner (Ryu *et al.,* 1994). *B. licheniformis* protease from marine sediment was strongly activated by 1 to 1.5 M NaCl, with a three-fold increase in activity (Manachini and Fortina, 1998). In the present study when varying concentrations of NaCl (0- 1M) were added in the routine enzyme assay and the residual activity calculated, alkaline protease activity was found to be stimulated by concentrations up to 0.25 M NaCl (Table 4.2).

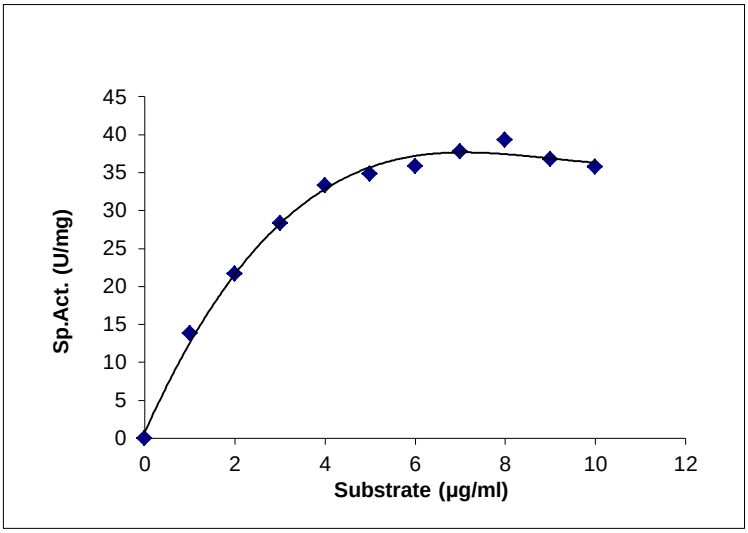


Fig. 4.9 Substrate concentration curve

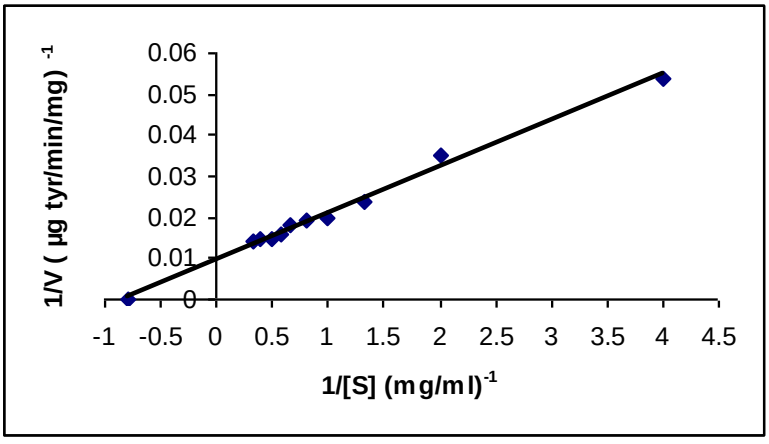


Fig.4.10 Lineweaver-Burk analysis

4.2.8 Effect of Modulators on Enzyme Activity

a. Nature of the Alkaline Protease

The effect of various known protease inhibitors was studied on the alkaline protease activity from isolate DCU_1. As shown in Table 4.3, the protease activity appeared to be almost totally inhibited by both iodoacetamide and EDTA. There is thus the indication that the enzyme might be a cysteine- or a metallo- protease. The apparent inhibitory effect of PMSF was investigated further.

Solutions of PMSF had been prepared in isopropanol. Since isopropanol in itself would be inhibitory at the concentrations at which it was used as a solvent for these studies, further experiments were carried out. It was evident that the observed PMSF effects (Table 4.4) were purely due to isopropanol (Table 4.5), ruling out any possibility of the enzyme being a serine protease.

Table 4.2 Effect of Salt concentrations on enzyme activity

NaCl (M)	Residual activity (%)
0	100
0.10	118.92
0.25	131.35
0.5	81.08
1.0	43.78

Table 4.3 Effect of inhibitors on enzyme activity

Enzyme Inhibitor	Concentration (mM)	Relative Activity (%)
PMSF	2.0	2.06
EDTA	2.0	3.34
Iodoacetamide	1.25	0

Table 4.4 Effect of PMSF on enzyme activity.

Inhibitor	Concentration (mM)	Enzyme activity (% control)
PMSF	0.4	1.317
	0.8	0.953
	1.6	0.139
	2.4	0.052
	4.0	0.139
Isopropanol	0.016	1.577
	0.065	0.901
	0.260	0.017
	0.585	0.208
	1.630	0.173

b. Effect of EDTA and Divalent Metal Ions

When pre-incubated with EDTA for 10 min and then assayed for enzyme activity, only 26.96 % activity was retained at 0.5 mM EDTA and concentrations higher than 2mM totally inhibited the enzyme (Fig.4.11). This suggests a possible divalent metal ion requirement for the active enzyme. Alkaline proteases have been reported to require a divalent cation such as Ca^{+2}, Mg^{+2} or Mn^{+2} or a combination of these cations, for maximum activity (Steele *et. al.*, 1992). Cations such as Mg^{2+} and Mn^{2+} stimulated enzyme activity and stabilized the protease enzyme (Sharma *et al*., 2006; Anandan et al., 2007; Kalpana *et al.,* 2008; Dubey *et al*., 2010). Such cations were also found to enhance the thermal stability of a *Bacillus* alkaline protease (Paliwal *et al*. 1994). It is believed that the cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures. In addition, specific Ca^{2+} binding sites apart from the catalytic site that influence the protein activity and stability had been described for protease K (Bajorath *et al.*, 1988).

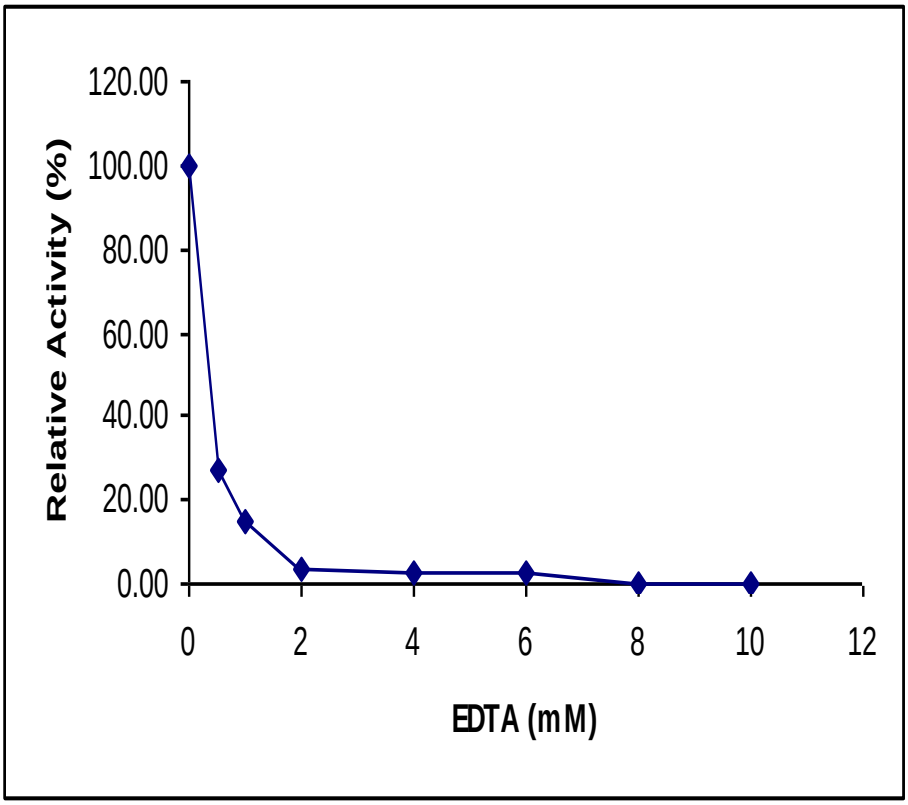


Fig. 4.11 Effect of EDTA on the enzyme activity

While Mg^{2+} had no effect on the enzyme activity, Zn and Fe^{2+} were inhibitory but only at a high concentration of 10 mM (Table 4.6). Zn^{2+} has been reported as a cysteine protease inhibitor (Damare *et. al* 2006; Gaur and Wadhwa, 2008). Inhibition of our alkaline protease by Zn^{2+} at 10mM could be an added indication of the enzyme being a cysteine protease. In this study copper and mercury ions showed a more graded concentration-dependent inhibition. Francois (2014) has recently reported 25% loss in enzyme activity in the presence of Hg^{2+} . The alkaline proteases of *Aspergillus* species were also shown to be inhibited by Hg^{2+} (Sharma *et al*., 2006; Anandan *et al*., 2007). Interestingly, the presence of Mn^{2+} caused a concentration-dependent stimulation of the alkaline protease activity obtained from strain DCU_1. The metal ions Mg^{2+} or Fe^{3+} also led to a stimulation of the protease activity (Table 4.6). The latter have been reported to protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Sharma *et al*, 2014). An increase in protease activity was also reported in the presence of 200 mM Ca^{2+} and Mg^{2} (Sehar and Hameed, 2011). Patel *et. al.* (2006) in his study on mutant *Bacillus sp.* observed that Ca^{2+} and Mn^{2+} had a slight enhancing effect on the activity of the alkaline protease while Zn^{2+} showed highest inhibition. The enzyme from strain DCU_1 thus probably has a metal ion requirement in the form of Mg^{2+} Fe^{3+} or Mn^{2+}. The effect of Ca^{2+} needed to be analyzed further.

Table 4.6 Effect of metal ions on enzyme activity

Metal ion	Concentration (mM)	Relative activity (%)
Cupric	0.1	97.16
	1.0	22.87
	10.0	13.35
Mercuric	0.1	52.41
	1.0	7.54
	10.0	5.69
Magnesium	0.1	104.82
	1.0	115.33
	10.0	97.16
Zinc	0.1	98.15
	1.0	90.48
	10.0	7.54
Ferric	0.1	112.36
	1.0	107.66
	10.0	5.86
Manganese	0.1	122.87
	1.0	141.90
	10.0	330.53

c. Effect of $CaCl_2$ and EDTA:

Enzyme assays were carried out on both the crude as well as the dialyzed enzyme extracts, with or without pre-incubation with $CaCl_2$ or EDTA and the results have been summarized in Table 4.7.

A minor increase in enzyme activity was observed upon addition of $CaCl_2$ to the crude enzyme and the effect was the same whether or not incubated for a longer time. Upon addition of $CaCl_2$ to the dialyzed and concentrated enzyme, however, there was an approximately 3.5-fold stimulation of activity. It was thus possible that the enzyme indeed had a requirement of Ca^{2+} for its activity. After a 10 min incubation of the crude enzyme with EDTA about 60% activity was lost, while there was minimal loss of activity when the dialyzed enzyme was used. These observations imply the presence of calcium ions in the crude enzyme preparation, which were sequestered in the presence of EDTA. The requirement of calcium ions for enhanced enzyme activity was further confirmed by the results on the dialyzed enzyme. Joshi *et al.* (2007) reported that enzyme activity was accelerated by the addition of $CaCl_2$. Gehan *et al.* (2006) also reported such activation and inhibition of halophilic protease by $CaCl_2$ and $HgCl_2$, respectively. As per some earlier reports also, calcium ions were found increase the activity of several proteases (Kobayashi *et al.*, 1985; Takami *et al*, 1989; Secades and Guijarro 1999; Ghorbel *et. al.*, 2002).

The enzyme from our isolate DCU_1 thus does appear to have some metal ion requirements although it cannot be claimed as a metalloprotease.

Table 4.7 Effect of $CaCl_2$ and EDTA

Enzyme sample	$CaCl_2$ (1mm)/ EDTA (2mm)	Specific activity (U/mg)	
		Crude sample	Dialyzed sample
Without incubation	-	11.72	140.99
	$CaCl_2$	13.74	523.17
With incubation (30 min)	-	11.46	128.46
	$CaCl_2$	14.69	417.41
Sample with incubation (10 min)	EDTA	4.84	126.83

d. Effect of Reducing Agents

Sulphydryl agents which are abundantly generated in the washing process, appear to stabilize the enzyme activity, The proteases intended to serves as additives in detergents should be stable in the presence of this H_2O_2 (Venugopal, 2004). In the present study carried out to check the effect of reducing agents, DTT, glutathione, sodium thioglycolate and β-mercaptoethanol had a stimulating effect on enzyme activity (Fig. 4.12). The results showed that alkaline protease activity was stimulated in presence of 0.1% of H_2O_2, whereas Oberoi *et al.* (2001) had reported that 60% of the maximum activity was retained in presence of 5% H_2O_2.

Stabilization of the enzyme activity in presence of sulphydryl agents is one more indication of its likelihood of being a cysteine protease.

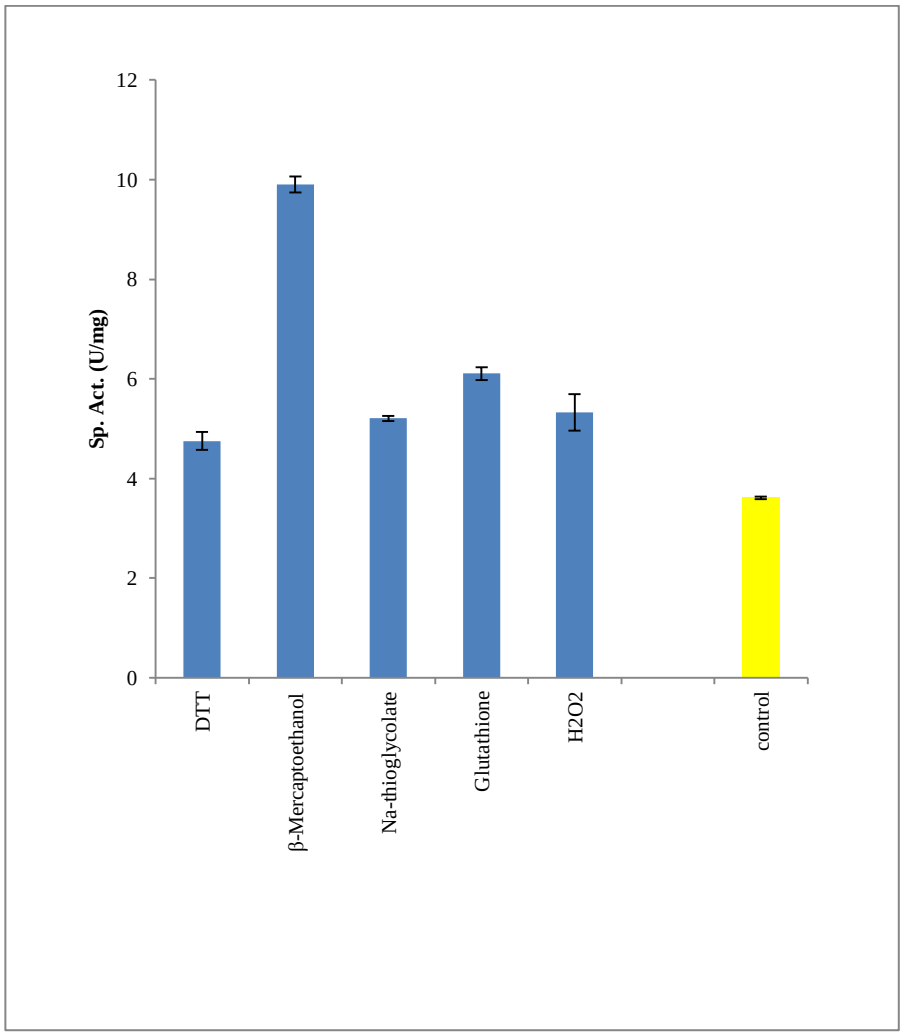


Fig.4.12 Effect of reducing agents

4.2.9 Substrate Specificity Studies

When assayed in the presence of other substrates besides casein, the alkaline protease exhibited broad substrate specificity (Table 4.8) and some of these results supported the possibility of exploitation for industrial uses such as those dealt with in Chapter 2.

In summary, the potential of this crude alkaline protease preparation for different biotechnological applications could be explored, for which the broad substrate specificity would be an asset. Results of preliminary laboratory-level experiments discussed in Chapter 2 indicate a potential extendable to the detergent industry, oil extraction as well as to aid in textile industry processes, for which thermal stability at higher temperature and pH stability at alkaline pH (9.0) reported above prove favorable as such industrial processes are likely to be carried out at higher temperatures and at alkaline pH. Due to these interesting properties the deep sea isolate DCU_1 was chosen for characterization and purification of its extracellularly produced protease.

Table 4.8 Substrate specificity on the enzyme

Substrate	**Specific Activity(U/mg) (U/mg)**
Casein	7.23
Gelatin	7.47
Haemoglobin	8.57
BSA	1.77
Egg Albumin	2.49
Keratin	3.31

Chapter 5

Identification of the Deep Sea Isolate DCU_1

Introduction

Identification and classification of microorganisms are of utmost importance in the fields of environmental and industrial microbiology as well as microbial ecology. Nature has placed bacteria in every habitat on earth: soil, rock, oceans, arctic snow and even in animal and plant tissue (Hauser, 1986). Our bacterial strain DCU_1 isolated from a deep-sea ecosystem appears to have now joined this class, especially in view of some of its special features which includes its potential as a valuable alkaline protease producer.

Methods for identification of microbial species include investigating the morphology, biochemical analyses as well as by analysis of the 16S or 18S rDNA sequences:

1. The Bergey's Manual of Determinative Bacteriology (1994) is a standard reference for laboratory identification of bacteria.
2. Phenotypic analysis measures biological characteristics of organisms.
3. Morphological characteristics are useful in identifying microorganisms, especially when aided by differential staining techniques such as Gram's staining that is based on the structural characteristics of bacterial cell walls. By combining morphology and Gram-staining, most bacteria can be classified into one of four groups, *viz.*, Gram-positive cocci, Gram-positive bacilli, Gram-negative cocci and Gram-negative bacilli (https://en.wikipedia.org/wiki/Bacteria).
4. Biochemical testing determines the presence of various enzymes, which helps in identifying microorganisms to a certain extent.

5. Serological tests, involving the reactions of microorganisms with specific antibodies, are useful in determining the identity of strains and species. Slide agglutination, ELISA and Western blotting are examples of serological tests.
6. Fatty acid profiles can be used to identify some organisms. Examination of esterification patterns (ester *vs* ether linkages in lipids) can also indicate phylogenetic relatedness on a broad scale.
7. While the DNA and RNA based methods are used primarily for classification rather than identification, 16S rRNA gene sequencing has emerged a faster and more accurate method to identify a wide variety of aerobic and anaerobic bacteria.

a. The polymerase chain reaction **(**PCR**)** can be used to detect small amounts of microbial DNA in a sample. Amplification by PCR may be used for DNA fingerprinting, sequencing and detection of specific nucleotide sequences characteristic of specific organisms.
b. rRNA sequencing**:** The sequence of bases in ribosomal RNA can be used in the classification of organisms.

Molecular epidemiology is a genotypic analysis targeting genomic or plasmid DNA. Species, strain or type-specific DNA sequences are the sources of genotype information. Molecular-based methods offer sensitive and direct detection of microorganisms. Due to the high sensitivity and specificity, proper quality control would thus be critical for molecular testing. PCR and other amplification methods are extremely sensitive and very specific. For an accurate test interpretation using proper controls, a universal target for bacterial identification is 16S rDNA sequencing. It

provides reliability, reproducibility and high accuracy for identification of any bacterial isolate.

Most of these methods are, however, time consuming and complex. A more advanced method of identification of microorganisms by MALDI-TOF MS has of late attracted attention as a supplement to these methods. MALDI-TOF MS offers an overall measurement of microorganisms. Such type of overall measurement is a new analytical method that captures the molecules in the microorganism by observing protein, peptide and lipid ions.

In the present study, morphological analysis, Gram staining, biochemical testing, FAME as well as 16S rDNA sequencing protocols have been adopted towards identification of the bacterial isolate DCU_1.

5.1 Materials and Methods

5.1.1 Identification of Organism using Phenotypic Criteria

Phenotypic properties are the expressed properties of the organism, such as shape, size, staining properties and reactions in biochemical tests. Phenotypic criteria are based on observable physical or metabolic characteristics.

a) Gram Staining

As a first step in the identification of isolate DCU_1 Gram staining was performed as per the standard protocol (Hauser, 1986).

b) KOH String Test

A loopful of growth from a colony was emulsified into a drop of 3% KOH on a glass slide. The suspension was mixed for 1 min and the loop then lifted slowly to observe the formation of a string (Arthi *et. al.,* 2003).

c) Scanning Electron Microscopy

Freshly grown culture was used to prepare a thin smear on slide to discern the morphology of the isolate by Scanning Electron Microscopy (SEM). The samples were subjected to gold spluttering using JEOL JFC1600 Autofine Coater for SEM (model JEOL JSM 6360LV SEM).

d) Biochemical Tests

Biochemical tests such as those for anaerobicity, production of oxidase, catalase, H_2S and acid, citrate and arginine utilization as well as nitrate reduction were carried out employing standard schemes based on Bergey's Manual of Determinative Bacteriology and Systematic Bacteriology (Holt *et. al.,* 1994). Carbohydrate utilization tests were also carried out, using Hicarbohydrate Utilization Kit (KB009) obtained from Hi-media Laboratories.

e) Gelatin Liquefication Test

Nutrient Agar gelatin plates were prepared and sterilized. Test organisms were then inoculated and incubated at 37℃ for 24h. Following incubation, the plates were placed at 4℃ for 30 min. Culture media which remained liquefied after 30 min were considered positive for gelatin liquefaction.

5.1.2 Molecular Characterization of the Strain

Phylogenetic studies by 16S rDNA analysis were carried out at Bioaxis DNA Research Centre Pvt., Ltd. (BDRC), Hyderabad.

5.1.3 BLAST Analysis of 16S rRNA Gene Sequence

Database searches were conducted with the BLAST programs available at the NCBI (Bethesda, USA) to determine the genetic similarities of the isolate. For phylogenetic tree construction, the sequence was compared with 16S rRNA gene sequences in GenBank database using the alignment tool of the MEGA software package. A phylogram was constructed with MEGA 7.0 software programs using neighbor-joining procedure.

5.1.4 Fatty Acid Methyl Ester (FAME) Analysis

FAME analysis of strain DCU_1 was carried out at the Regional Centre of the National Institute of Oceanography, Kochi.

5.1.5 Pigment Extraction

The bacterial cells were first grown for 24 h followed by centrifugation at 10,000 rpm. Both the supernatant and bacterial cell pellets were extracted using 95% (v/v) methanol in the ratio 1:5 (supernatant) or until the pellet was colorless, *i.e.*, complete pigment extraction has been achieved. The bacterial cell pellet was then discarded while the coloured supernatant obtained was used as pigment source.

The pH of crude culture filtrate (pigmented) was adjusted using 0.1 N HCl or 0.1 N NaOH to study the effect of neutral, acidic and alkaline conditions on the pigment. Pigment extracted using absolute methanol was used for studying UV–vis spectral characteristics.

5.2 Results and Discussion

5.2.1 Phenotypic and Biochemical Tests

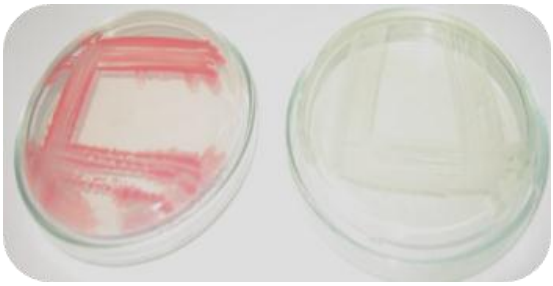
The basic cellular morphological features of the deep sea isolate DCU_1 are evident from Fig 5.1a, depicting the cells as circular and small. Gram staining caused the bacterial cells to stain pink, indicating the strain to be Gram negative (Fig 5.1b).

KOH string test was used as a confirmatory test for the Gram staining results (Powers, 1995; Arthi *et al*., 2003): The formation of a string (DNA) in 3% KOH further supported that the isolate had Gram-negative characteristics.

Pink pigmentation was observed when strain DCU_1 was grown at 28°C but no such pigment formation was seen when grown at 37°C (Fig.5.2, a & b). Scanning Electron Microscopy has been widely used in identifying bacterial morphology by characterizing their surface structure to gauge cell attachment and morphological changes (Kenzata and Tani, 2012). In the present study, the bacterial cells appeared as short rods in the SEM utput of isolate DCU_1 (Fig. 5.3).

a **b**

Fig. 5.1. a. Cells of isolate DCU_1 visualized under light microscopy
b. Gram staining of isolate DCU_1



a **b**

Fig. 5.2 Strain DCU_1 growing on Nutrient Agar:

a. at 28°C and b. at 37°C

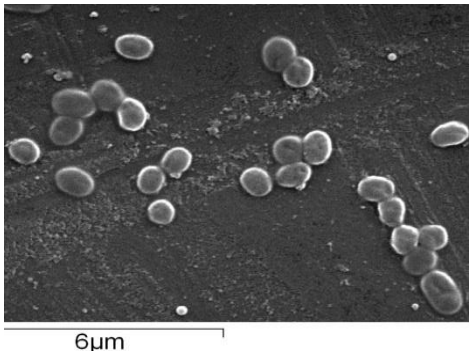


Fig. 5.3. Scanning electron micrograph of strain DCU_1

The colony morphology featured pink to red pigmentation and was elevated and transparent in nature (Table 5.1). The results of biochemical tests are also tabulated therein.

Gelatin is an incomplete protein lacking the essential amino acid tryptophan and it acts as nutrient source for many microorganisms. When gelatin is enzymatically hydrolyzed it loses its stability for gel formation even at low temperature. Upon inoculation with strain DCU_1 the gelatin in the region of the spot failed to solidify (Fig. 5.4).

The results of carbohydrate utilization tests are reported in Table 5.2 while characteristics derived from biochemical reactions using the Hi-media kit (KB002) are described in Table 5.3.

Table 5.1. Morphological characteristics and biochemical tests of strain DCU_1

Colony characteristic	Observation
Motility	motile
Shape	circular
Size	small
Pigment	pink/red
Margin	entire
Elevation	elevated
Opacity	transparent
Anaerobicity	+ve
Oxidase	+ve
Catalse	+ve
H_2S production	-ve
Citrate utilisation	+ve
Arginine utilization	-ve
Gelatin liquefaction	+ve
Nitrate reduction	+ve

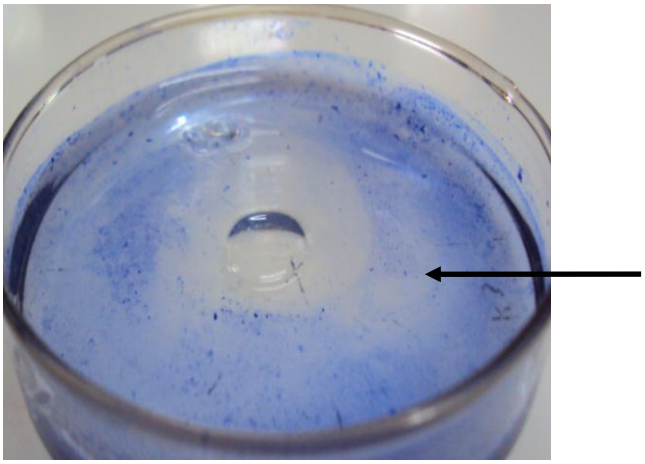


Fig. 5.4 Gelatin hydrolysis in plate

Table 5.2 Carbohydrate utilization studies using Hi-media kit (KB009)

Test	Reaction
Lactose	-
Xylose	-
Maltose	+
Fructose	+
Dextrose	-
Galactose	+
Raffinose	-
Trehalose	ND
Melibiose	-
Sucrose	+
L-arabinose	-
Mannose	+
Inulin	-
Sodium gluconate	+
Glycerol	+
Salicin	+
Glucosamine	+
Dulcitol	-
Inositol	+
Sorbitol	-
Mannitol	+
Adonitol	-
α-Methyl-D-glucoside	-
Ribose	+
Rhamnose	-
Cellobiose	-
Melezitose	-
α-Methyl-D-mannoside	-
Xylitol	partially +
ONPG	+
Esculin hydrolysis	+
D-Arabinose	-
Citrate utilization	+
Malonate utilization	+
Sorbose	-

*ND = not detectable

Table 5.3 Biochemical tests using Hi-media kit KB002

Test	Reaction
Citrate utilization	-
Lysine utilization	-
Ornithine utilization	-
Urease	-
Phenylanine deamination	-
Nitrate reduction	+
H_2S production	-
Glucose	+
Adonitol	-
Lactose	-
Arabinose	-
Sorbitol	-

5.2.2 FAME Analysis

Fatty acid profile was obtained using gas chromatography and is depicted in Table 5.4.

Table 5.4. Fatty acid profile

E125184.57A [3608] 1 Page 1

Volume: DATA File: E125184.57A Samp Ctr: 4 ID Number: 3608
Type: Samp Bottle: 2 Method: TSBA6
Created: 5/18/2012 12:11:49 PM Created By: administrator
Sample ID: 1

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.896	4.327E+8	0.028	----	7.024	SOLVENT PEAK	----	< min rt	
2.804	507	0.031	----	8.814		----	< min rt	
3.406	440	0.033	1.232	9.997	10:0	0.27	ECL deviates -0.003	Reference -0.007
4.052	726	0.028	1.164	10.921	Sum In Feature 2	0.42	ECL deviates -0.007	unknown 10.928
4.497	8363	0.032	1.131	11.421	10:0 3OH	4.74	ECL deviates -0.001	
5.039	2318	0.033	1.095	12.005	12:0	1.27	ECL deviates 0.005	Reference 0.003
6.440	1828	0.040	1.030	13.175	12:0 2OH	0.94	ECL deviates -0.002	
6.588	3335	0.040	1.025	13.282	12:1 3OH	1.71	ECL deviates -0.006	
6.821	3648	0.038	1.017	13.450	12:0 3OH	1.86	ECL deviates -0.004	
7.584	10247	0.046	0.992	14.001	14:0	5.10	ECL deviates 0.001	Reference 0.001
8.362	2866	0.045	0.971	14.501	unknown 14.502	----	ECL deviates -0.001	
9.141	1454	0.039	0.953	15.001	15:0	----	ECL deviates 0.001	
9.475	7045	0.047	0.946	15.198	14:0 2OH	3.34	ECL deviates -0.005	
9.951	17193	0.046	0.937	15.481	Sum In Feature 2	8.08	ECL deviates 0.001	16:1 iso I/14:0 3OH
10.511	14406	0.047	0.927	15.813	Sum In Feature 3	6.70	ECL deviates -0.009	16:1 w7c/16:1 w6c
10.823	66185	0.045	0.922	15.998	16:0	30.62	ECL deviates -0.002	Reference 0.001
10.978	3071	0.064	----	16.087		----		
12.372	30248	0.048	0.902	16.884	17:0 cyclo	13.68	ECL deviates -0.004	
12.572	2197	0.052	0.900	16.998	17:0	0.99	ECL deviates -0.002	Reference 0.001
13.479	513	0.040	----	17.507		----		
13.905	1870	0.045	----	17.746		----		
14.029	31620	0.048	0.887	17.815	Sum In Feature 8	14.07	ECL deviates -0.008	18:1 w7c
14.348	1610	0.046	0.885	17.994	18:0	0.71	ECL deviates -0.006	Reference -0.002
15.117	2767	0.053	----	18.429		----		
15.491	1908	0.048	0.880	18.641	19:0 iso	0.84	ECL deviates 0.007	Reference 0.011
15.939	10499	0.051	0.879	18.894	19:0 cyclo w8c	4.63	ECL deviates -0.008	
16.047	1067	0.045	----	18.955		----		
16.215	2783	0.049	----	19.051		----		
----	17919	---	----	----	Summed Feature 2	8.51	12:0 aldehyde ?	unknown 10.928
----	----	---	----	----		----	16:1 iso I/14:0 3OH	14:0 3OH/16:1 iso I
----	14406	---	----	----	Summed Feature 3	6.70	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	31620	---	----	----	Summed Feature 8	14.07	18:1 w7c	18:1 w6c

ECL Deviation: 0.005 Reference ECL Shift: 0.005 Number Reference Peaks: 7
Total Response: 225888 Total Named: 213817
Percent Named: 94.66% Total Amount: 203550

Matches:

Library	Sim Index	Entry Name
TSBA6 6.00	0.355	Escherichia-fergusonii-GC subgroup A
	0.321	Serratia-marcescens-GC subgroup A
	0.315	Salmonella-typhi-GC subgroup A (confirm with other tests)
	0.290	Enterobacter-aerogenes-GC subgroup B
	0.288	Klebsiella-pneumoniae-pneumoniae-GC subgroup A
	0.274	Escherichia-coli-GC subgroup D (DNA homology with Shigella)
	0.261	Klebsiella-pneumoniae-pneumoniae-GC subgroup C
	0.253	Serratia-rubidaea
	0.251	Klebsiella-pneumoniae-pneumoniae-GC subgroup B
	0.222	Enterobacter-gergoviae

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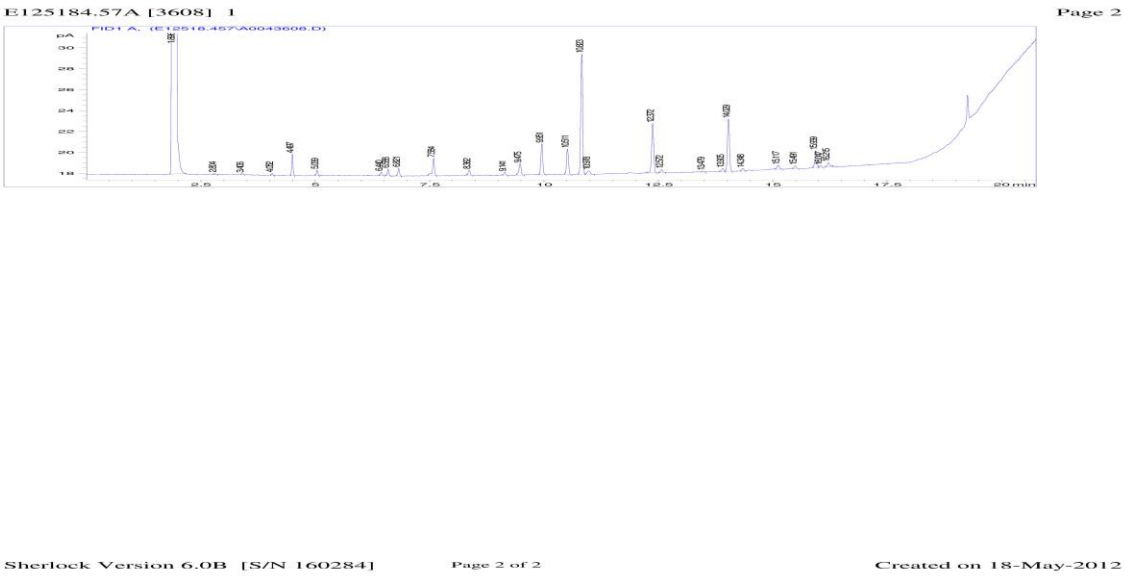


Fig 5.5. GC profile of FAME analysis

5.2.3 Phylogentic Analysis

These results matched with those of FAME analysis wherein a similarity index of 0.321 was indicated with *Serratia-marcescens*-GC subgroup as per the report (Table 5.5).

According to the report on phylogenetic studies by 16S rDNA analysis from BDRC, Hyderabad our bacterial isolate DCU_1 had been identified as *Serratia marcescens* strain RPS 9 16S ribosomal RNA gene partial sequence Length of 1481, Score of 2370 bits (1283) and Strand=Plus/Plus.

Further, phylogenetic analysis of DCU_1 strain carried out using 16S rRNA gene sequencing and the phylogenetic tree constructed by the neighbor-joining method (Fig.5.6) indicated that the deep sea bacterial isolate was part of a cluster within the genus *Serratia*. Comparative 16S rRNA gene sequence analysis indicated that the strain was a member of the genus *Serratia*, sharing highest sequence similarities with *Serratia nematadophila* (99.8%) and *S.marcescens* subsp. *marcescens* DSM 30121T (99.5%). *Serratia nematadophila* is a novel red-pigmented, Gram-negative, motile, fluorescent, rod-shaped strain DZ0503SBS1T, with a single lateral flagellum and was isolated from the intestine of the nematode *Heterorhabditidoides chongmingensis* (Zhang *et. al.*, 2009).

Table 5.5. Biochemical characteristics of the isolate DCU_1 examined in this study and comparative data for *Serratia marcescens* and *Serratia nematadophila.*

Biochemical characteristic	Observation for Isolate DCU_1	Literature report for *S. nematadophila*	Literature report for *S. marcescens*
D-Arabinose	-	+	-
D-Melibiose	-	+	-
D-Xylose	-	-	-
L-Rhamnose	-	+	-
Sorbitol	-	+	+
Mannitol	+	+	**ND**
Adonitol	-	+	+
Raffinose	-	+	-
Lactose	-	+	-
Xylitol	V	+	**ND**
Glycerol	+	ND	+
Starch	-	+	
L-arabinose	-	+	-
Sucrose	+	+	+
Salicin	+	+	+
Citrate utilisation	+	+	
Lysine decarboxylase	+	+	+
H_2S production	-	-	
Nitrtate reduction	+	+	

Gelatin-hydrolysis	+	**ND**	+
Arginine utilization	+	+	-
Catalase	+	+	+
Oxidase	+	-	-
Malonate utilization	+	**ND**	
Ribose	+	+	

+ Positive; - Negative; V = Variable reactions; ND = Not determined

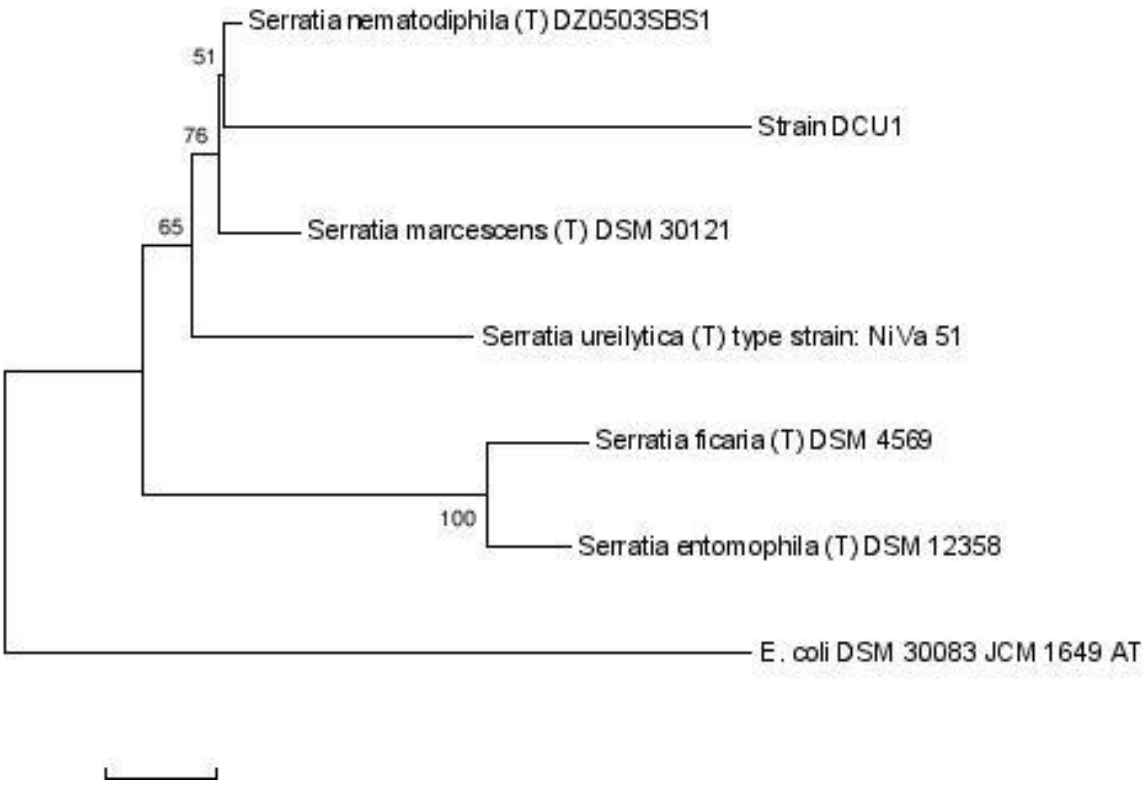


Fig.5.6. Phylogenetic tree of bacterial strain DCU_1 and related bacteria based on 16S rDNA gene sequences constructed using neighbour-joining method.

While the revelation of the identity of the deep sea isolate as belonging to genus *Serratia* and a certain degree of closeness to *S. marcescens* of the Enterobacteriaceae family came as a surprise in the first instance, it does find support from well-established studies on such other microbes of terrestrial origin, hardy enough to have adapted to and survived the drastic environmental change. For instance, Raghukumar and Raghukumar (1998) have indicated the possibility of spores and hyphal fragments of terrestrial fungi getting deposited in deep-sea sediments through terrestrial runoff, wind or water movements. Similarly, deep-sea isolates of fungi from 5,000m in the Central Indian Ocean Basin were screened and successfully grown at 5°C and 30°C (1bar pressure) for the production of alkaline proteases in simulated conditions in the laboratory (Damare *et. al.*, 2006).

Serratia sp. have occupied different habitats such as water, surface of plants, animals and insects, soil and hospitalized patients. They are both chromogenic and non-chromogenic. Chromogenic species are usually isolated from the environment from water, soil, plants or insects. The non-chromogenic ones are more of a hospital threat, being human pathogens (Kumar & Aparna, 2014).

The genus *Serratia* belongs to the family Enterobacteriaceae under the class Gammaproteobacteria. Some members of this genus have clinical importance (Grimont & Grimont, 1992). *Serratia marcescens* is a motile, short rod-shaped, Gram-negative, facultative anaerobic bacterium, classified as an opportunistic pathogen. It grows at temperatures ranging from 5–40°C and at pH levels ranging from 5 to 9 (Begam *et. al.*, 2012). It is distinguished from other Gram-negative bacteria by the ability to perform casein hydrolysis, which allows it to produce extracellular metallo-proteinases (www.Biology-online.org/dictionary/*Serratia marcescens)*. A primary

characteristic of *Serratia marcescens* is its ability to produce the red pigment prodigiosin (Khanafari *et al.*, 2006). Due to the ability for pigment production, it was first used in 1906 as a marker to trace bacterial activity or transmission and was initially thought to be harmless (non-pathogenic)

Serratia marcescens has a wide range of applications, its pigment prodigiosin being a major anticancer agent. It has already been proven that the pigmented strain of *Serratia marcescens* is less virulent than non-pigmented strains (Kamble & Hiwarale, 2012). Grimont & Grimont (2006) reported that the non-pigmented strains of *Serratia marcescens* are more resistant to antibiotics than the pigmented strains. The pigment Prodigiosin is produced under the genus *Serratia* by strains of *S.marcescens, S.rubide* and *S.plymuthica*. *Serratia* strains belonging to the biotype A4 can synthesize a pink diffusible pigment called pyrimine (Grimont & Grimont, 2006). *S. marcescens* strain of biotype A8a produces a yellow pigment due to loss of ability to grow on aromatic compounds.

Serratia spp are also known to produce various types of enzymes such as lipase, protease, chitinase, laccase, nuclease and gelatinase. Using chitin as nutrient, marine microorganisms push on the cycle of carbon sources and nitrogen sources through producing chitinase. Chitinase-producing microorganisms of the genera *Bacillus, Serratia* and *Streptomyces* have been already reported (Liu *et. al.*,2015).

Five species of *Serratia* are described as gelatinolytic as per Bergey's Manual (1994) and *S. marcescens* was reported to produce extracellular protease (Miyata *et.al.*, 1970). Romero *et.al.* (2001) also reported protease production from *S. marcescens* using whey as substrate. Solvent-tolerant bacteria have also been isolated from the deep sea, coastal sediment, soil, *etc.* Wan *et. al.* (2010) described a novel solvent-tolerant characteristic

of protease from a hydrophilic organic-solvent-tolerant microorganism, *S. marcescens* MH6, obtained from oil contaminated soil from China. Cellulose and chitinase activity was studied by Zeng *et. al*. (2010) from the bacterial strain WP02-1 retrieved from deep-sea sediment, which had a close (99%) resemblance to *Serratia marcescens*. Tariq *et. al*. (2011) screened *S. marcescens* TS1 from the soil of dense apple garden around Badran Magam in Kashmir at an altitude of 1630 meters above the sea level. Femi-Ola *et. al*. (2015) isolated *S. marcescens* from the gut of termite. *Serratia marcescens* biovar A2/A6, was isolated from an Indonesian freshwater source (Hardjito *et. al.,* 2002**).** The bacterium *S. marcescens* P3 was isolated from soil of the Brazilian Atlantic Forest (Bach *et. al.*, 2012). Darah (2014) obtained Prodigiosin as an antibacterial red pigment produced by *Serratia marcescens* IBRL USM 84 associated with a marine sponge *Xestospongia testudinaria*..

5.2.4. Pigment Production Studies

Maximum pigment production was observed at the beginning of the stationary phase (Fig 5.7). The deep sea strain DCU_1 could thus grow in the range 0 to 4 % NaCl although growth, pigment production as well as alkaline protease activity were reduced at higher concentrations of *i.e.*, by 4% NaCl.

The pigmentation pattern was distinctly related to growth temperature. When inoculated at RT on NA plates, obvious signs of growth were observed after 19 h and the colonies were pinkish orange in appearance. Growth was slower at 5°C and no pink pigmentation was observed at this temperature or at the higher temperature of 37° C (Fig 5.7 a to c). The bacterial strain DCU_1 could thus grow at different temperatures but could not produce pigment at 5°C or at 37°C (Table 5.6). Ideal pink pigment production was at 25-32°C. Similar pigment production patterns have been reported for *S.*

marcescens by Williams *et al*. (1971), Giri *et al*. (2004) as well as Keneni and Gupta (2011).

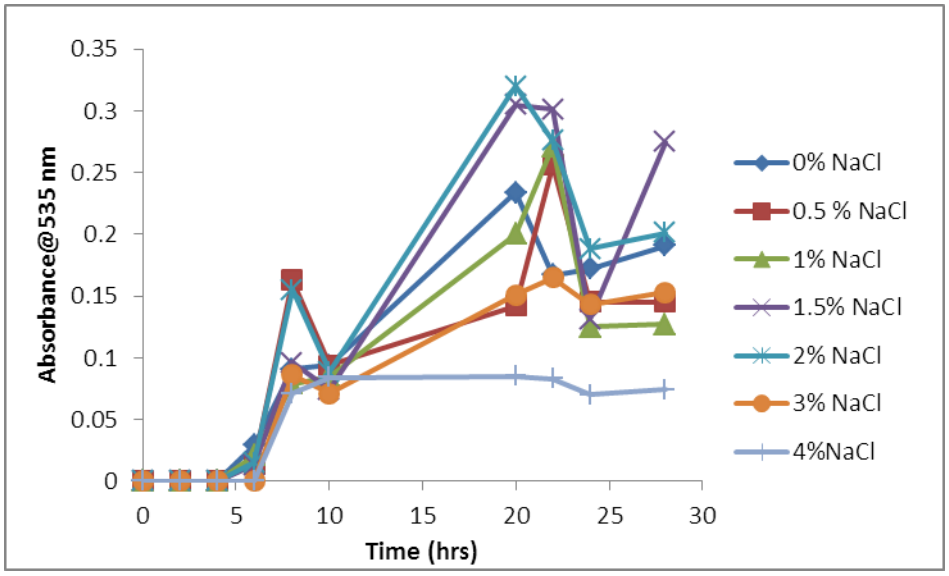


Fig. 5.7. Effect of pigment production with NaCl

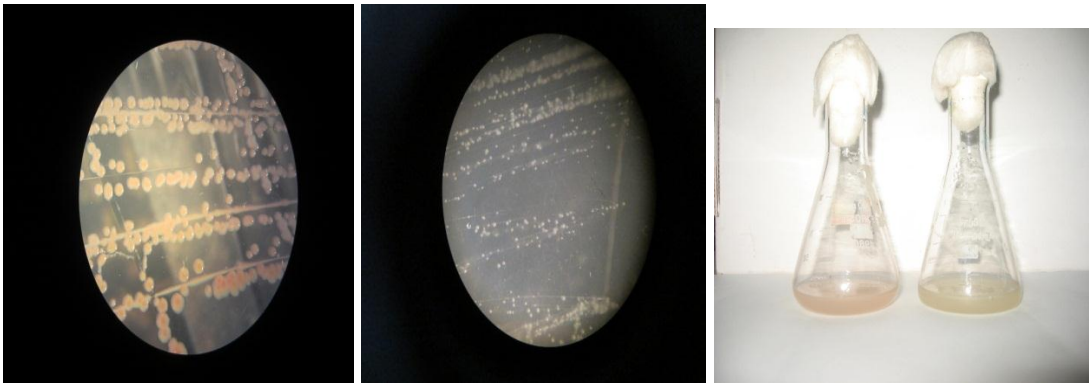


Fig. 5.8. a) Growth at 27°C b) Growth at 37°C c) Broth culture at 37 and 32°C

Table 5.6 Pigment production at different temperatures

Temperature (^{o}C)	**Pigment colouration**	**Growth evident at (h)**
25	Pink	24
28	Pinkish orange	19
37	Cream	24
5	Cream	120

When pH of the crude culture filtrate (generally at 8.5) was adjusted using 0.1 N HCl or 0.1N NaOH, the colour changed to pink at pH 4.5 and orange at pH 8 to 10, while retaining its pink colour at pH 7 (Table 5.7). Similar observations have been previously reported by Min-jung *et al.* (2006) for pigment colour change with respect to pH.

The methanolic pigment extract obtained from cultures grown at 28^{o}C (Fig. 5.9a) was analyzed for its spectral characteristics. There was an absorbance peak at 534 nm (Fig. 5.9b).

Table 5.7 Variation of pigment colour with acidic, alkaline and neutral pH.

pH	**Colour**
4.5	Pink
7.0	Pink
8.0	Orange
8.5	Orange
10.0	Orange

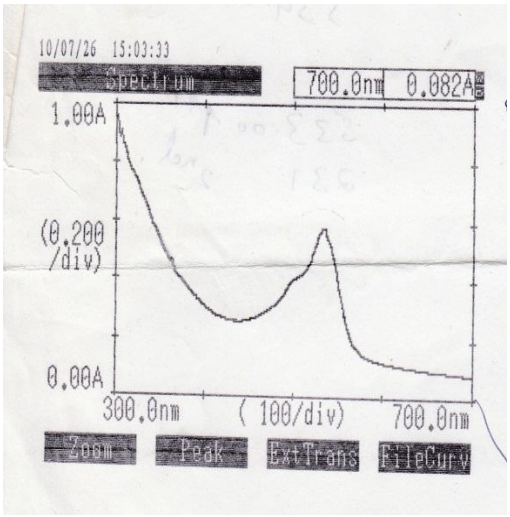


Fig. 5.9 a. Methanol extract of pigment b. Visible spectrum of the extract

5.2.5 Use of Bacterial Pigment for Dyeing

Our deep-sea isolate being a pigment producer under controlled conditions, application of its red pigment in the dyeing of cloth has been attempted. Lab scale dyeing experiments were carried out on white cotton fabric using the produced dye without mordanting. Pigment was extracted into the culture supernatant by centrifuging the culture broth at 4°C and 10,000 rpm. A piece of white cotton fabric was soaked overnight in the extracted pigment and then washed with tap water and air dried. A similar experiment was performed on polyethylene centrifuge tubes and petriplates.

5.2.6 Dyeing Potential of the Bacterial Pigment

Environmental concerns regarding continuous use of synthetic dyes saw a revival in the demand for natural dyes as these generally exhibit better biodegradability and compatibility with the environment. One of the major limitations for the use of natural dyes or pigments is due to low extraction yield factors. The exploitation of other biological sources would therefore offer interesting alternatives. Microbial pigments of bacterial origin offer an advantage in terms of production compared to pigments extracted from vegetable or animal sources. Our deep-sea isolate being a pigment producer under controlled conditions, application of its red pigment was tested in the dyeing of cloth and it was observed to easily and brightly colour the cotton fabric used (Fig.5.10a). The pigment could even stain the polyethylene tubes and petriplates permanently (Fig.5.10, b & c). It may be noted that the red pigment produced by *Serratia* sp. and recognized as prodigiosin or prodigiosin-like pigment has been

reported only for pharmaceutical applications (Kamble & Hiwarale, 2012) whereas the pigment obtained from DCU_1 could be evaluated for use as a dyeing agent for textile, rubber, paper and plastic. Natural dyes from marine microorganisms could provide the much needed alternative to the complex and polluting world of chemical dyes conventionally employed in the textile industry. Being a natural pigment it would likely be harmless and ecofriendly.

The brief literature review above thus consolidates reports of *Serratia marcescens* as found to be present in soil, animals and also in the deep-sea. Our pigment-producing strain DCU_1 isolated from the deep-sea, while confirmed by phylogenetic tree analysis as *Serratia* sp showed morphological, biochemical as well as pigment-producing characteristics that draw its identity closer to non-pathogenic strains of *Serratia marcescens* (Table 5.5). This is the first report of its kind of such a pigmented protease producing *Serratia* sp. from deep-sea sources.

Before After

a

Centrifuge tubes

b

Petriplates

c

Fig. 5.10. Dyeing potential of pigment produced by DCU_1

Chapter 6

Purification and Properties of Alkaline Protease from Isolate DCU_1

Introduction

Protein purification involves a series of processes intended to isolate one or a few proteins of specific interest from a complex mixture. Purification of an enzyme is imperative for studying its properties and structure-function relationship. Separation steps usually exploit differences in protein size, physicochemical properties, binding affinity and biological activity. A favoured first step commonly involves concentration of the crude extract through procedures such as salting out (Kumar, 2002), solvent precipitation (Kumar *et al.* 1999), ultrafiltration (Smacchi *et al.* 1999) or lyophilization (Manonmani and Joseph 1993).

Effective purification generally utilizes three properties to separate proteins. Accordingly, proteins may be purified according to their isoelectric points by running them through a pH graded gel or an ion exchange column. Secondly, proteins can be separated according to their size or molecular mass, *via* size exclusion chromatography or by electrophoretic techniques. Their separation by preparative 2D-polyacrylamide gel electrophoresis (PAGE) followed by analysis through peptide mass fingerprinting to establish the protein identity is also adopted at times (Arthur, 2008; Xiao *et. al.*, 2014). Such protocols are of special significance when the protein content is very low since only nanogram quantities would be required for the analysis. Thirdly, proteins may be separated based on polarity/hydrophobicity *via* high performance liquid chromatography or reversed-phase chromatography.

The key to successful and efficient protein purification lies in selecting the most appropriate techniques, optimizing their performance to suit the requirements and

combining them in a logical way to maximize yield and minimize the number of steps required. Chromatography has become an essential tool for protein purification. The availability of various chromatographic techniques with different selectivities provides a powerful combination for the purification of any biomolecule. Recombinant DNA developments over the past decade have revolutionized the production of proteins in large quantities. Presence of contaminants and problems related to solubility, structural integrity and biological activity still exist. Most purification protocols require more than one step to achieve the desired level of product purity. This would cause some loss of product in each step. One thus needs to limit the number of steps in a purification procedure and minimize sample handling at every stage. Contaminants which degrade or inactivate the protein or interfere with analyses should be removed as early as possible. Assays which are fast and reliable need to be selected. Additives should be used only if essential for stabilization of product or improved extraction and only those which are easily removed should be considered.

Alkaline proteases from *Bacillus* sp. (Agarwal *et al.*,2012 ; Mukhtar and Haq, 2012), *Aspergillus niger* (Dubey *et al.*,2010; Devi *et al.*,2008; Asker *et al.*,2015), *Rhizopus oryzea* (*Musht*aq *et al.*, 2015), *Pseudomonas aeruginosa* (Najafi *et al.*, 2005), *Salinivibrio* sp. (Shahbazi and Karbalaei-Heidari, 2012), S*erratia marcescens* (Fredy *et al.*, 2001) and cold-tolerant alkaline protease from deep sea fungi (Damare *et al.*, 2006) are some of the documented sources of purified proteases.

Besides the academic interest in characterization of enzymes with novel and useful features, certain commercial usages of enzymes specifically call for purified products.

This Chapter thus focuses on a major objective of the present study, *viz.*, to purify and characterize a promising alkaline protease.

6.1 Materials and Methods

Unless otherwise specified, all steps in the purification procedures were routinely carried out at 0-4°C. Analytical grade reagents have been used throughout.

6.1.2 Ammonium Sulphate Fractionation

The crude enzyme was brought to 85% saturation by gradual addition of solid ammonium sulphate while stirring at 4^0C and the precipitated protein then obtained by centrifugation for 15 min at 10,000 rpm and 4^0C. The pellet was re-suspended in a minimum quantity of 50mM borate buffer (pH 9.0). Where required, this suspension was dialyzed against the same buffer and used as a source of "partially purified enzyme".

6.1.3 Gel Filtration Chromatography

The enzyme obtained after ammonium sulphate precipitation (0-85% fraction) was loaded on to a Sepharose CL-6B column pre-equilibrated with 25mM borate buffer (pH 9.0). Fractions of 3ml were collected at a flow rate of 20ml/h and the absorbance was read at 280nm. Selected fractions were assayed and those possessing protease activity were pooled and concentrated.

6.1.4 Native PAGE

Non-denaturing PAGE was carried out on a 10% gel at 50 mV until the dye front crossed the stacking gel and then raised to 100 mV, following the procedure of

Laemmli (1970). Coomassie Brilliant Blue R-250 was used to stain the separated proteins.

6.1.5 In Situ Detection of Enzyme Activity

After the electrophoretic run, the native polyacrylamide gel was blotted over a casein gel (1% agarose gel containing 1.0% casein prepared in buffer of pH 9.0 and incubated for 1 h at room temperature in a moist chamber. The casein-agarose gel was then stained with Coomassie Brilliant Blue R-250. Appearance of a clear band against the blue background of the stained gel was indicative of alkaline protease activity.

6.1.6 Determination of Molecular Weight by SDS-PAGE

Procedures adopted for SDS-PAGE were as described by Laemmli (1970). Molecular weight of the enzyme was calculated from a linear semi-logarithmic plot of relative molecular weight versus the R_f value (relative mobility) prepared using a standard molecular weight marker kit (Sigma, 14-66 kDa).

6.1.7 Study of Enzyme Characteristics

Protocols for studying effect of temperature, pH, substrate concentration, additives such as reducing agents, etc., on the purified enzyme were as described in earlier Chapters for the crude enzyme preparation.

The enzyme was pre-incubated with various protease inhibitors / detergents such EDTA, PMSF (Phenyl methylsulphonyl fluoride), iodoacetic acid (IAA), 1,10-phenanthroline, Triton X-100 or Tween 80 for 10 min and the residual activity then assayed under the standard assay conditions.

6.2 Results and Discussion

Analysis of the characteristics of the alkaline proteases (Chapter 3) revealed that the enzyme from the deep sea bacterial isolate DCU_1 was superior to those from other selected isolates, with respect to specific activity, thermal stability, pH stability and affinity for the substrate. It was thus chosen for a more detailed study on the characteristics and applications of the purified enzyme. While crude preparations of alkaline proteases are usually employed for commercial use, purification of the enzyme is important from the expectation of developing better understanding of the functioning of the enzyme (Tsai et al., 1988; Takagi 1993) or for cases that demand the pure enzyme for industrial purposes.

6.2.1 Purification

Purification of the alkaline protease was carried out by a simple two-step procedure which involved concentration by salting out, followed by gel filtration on a Sepharose CL-6B matrix. Such a procedure was enabled by the careful optimization of the culture conditions (as described earlier) to specifically enhance production of the enzyme of interest. The elution profile from the gel filtration column showed one major protein peak and a single sharp peak of alkaline protease activity (Fig 6.1). Fractions with high enzyme activity were pooled and concentrated using sucrose. Table 6.1 summarizes the results of the purification of the alkaline protease from the bacterial isolate DCU_1. The alkaline protease was purified 35.52-fold with a yield of about 49%.

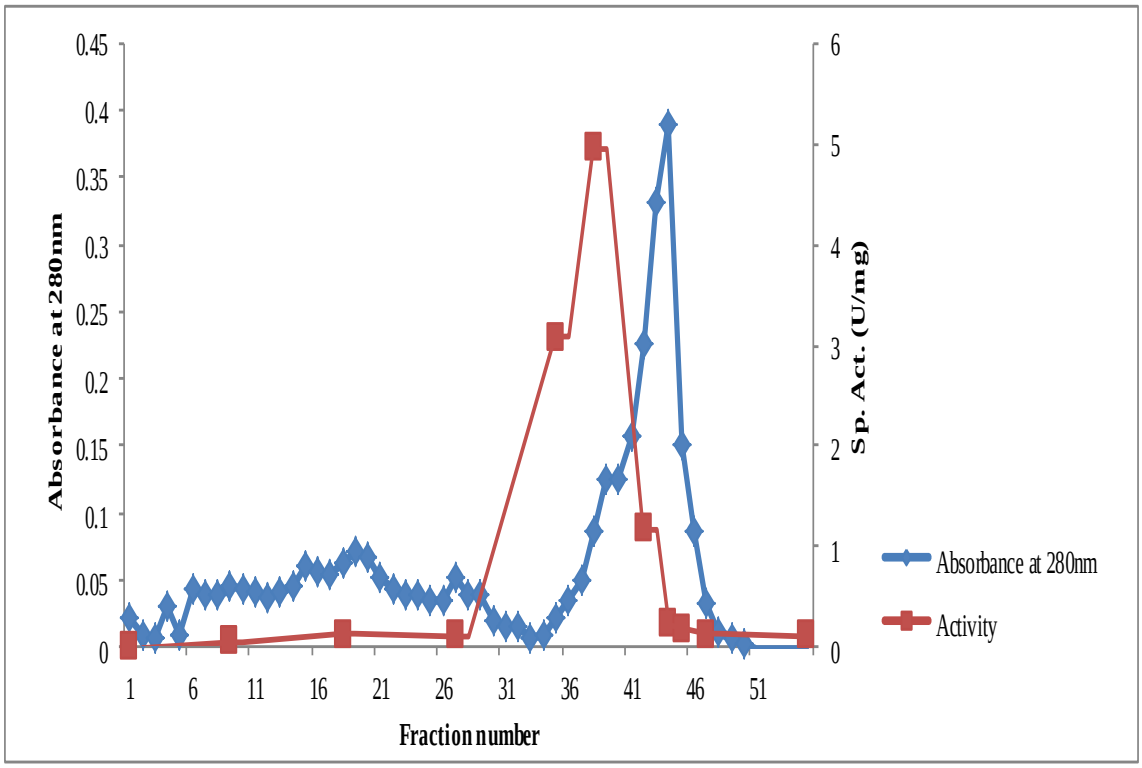


Fig 6.1 Elution profile of the enzyme on Sepharose CL-6B column

Table 6.1 Protein purification table

Fraction	Volume (ml)	Total protein (mg)	Total activity	Sp. Activity (U/mg)	Yield (%)	Fold purification
Crude culture supernatant	150	505.5	7107.33	14.06	100	1
Ammonium sulphate precipitate	5.5	31.03	4214.49	135.82	59.30	9.66
Sepharose CL-6B eluate	3.0	6.98	3485.53	499.36	49.04	35.52

6.2.1.1 Native PAGE and Zymogram

A single protein band was observed on the native gel when the sucrose-concentrated column eluate sample was subjected to non-denaturing PAGE (Fig 6.2 a). One corresponding activity band appeared on the zymogram (Fig 6.2 b), confirming the homogeneity of the preparation.

6.2.1.2 Molecular Weight Determination

The Sepharose CL-6B column was calibrated using proteins of known molecular weight and a standard plot prepared, from which the molecular weight of the purified native protein was deduced as 34.67 kDa (Fig. 6.3).

When SDS-PAGE analysis was carried out with standard molecular weight markers run in parallel lanes, the band of purified alkaline protease corresponded to a molecular weight of 33.11 kDa (Fig 6.4 a & b). The purified protease from isolate DCU_1 is thus monomeric in nature, which also to a certain extent supports its observed thermotolerance.

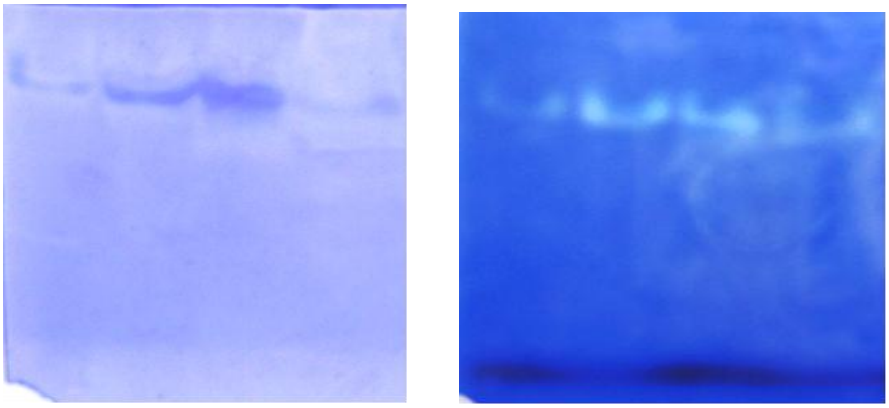


Fig. 6.2 a) Native PAGE. Lane 1 : crude enzyme (grown in 0% milk medium), Lane 2 : crude enzyme (grown in 0.5% milk medium), Lane 3 : 0-85% ammonium sulphate fraction (dialyzed), Lane 4 : Purified protein.

b) Zymogram Lane 1 : crude enzyme (grown in 0% milk medium), Lane 2 : crude enzyme (grown in 0.5% milk medium), Lane 3 : 0-85% ammonium sulphate fraction (dialyzed), Lane 4 : purified protein.

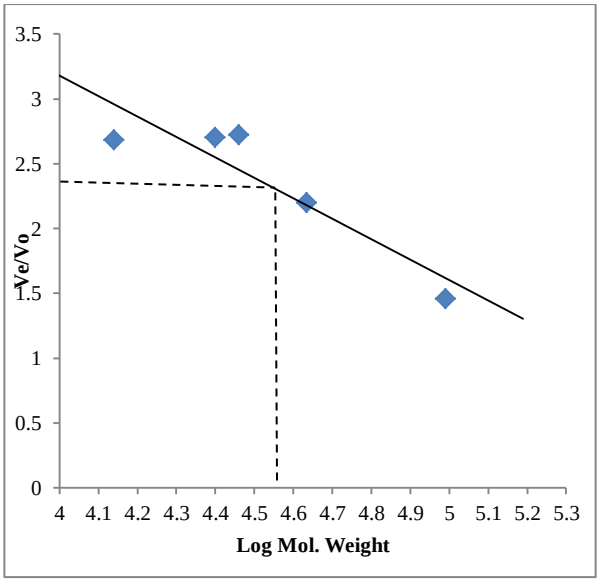


Fig. 6.3. Molecular weight of purified native protein

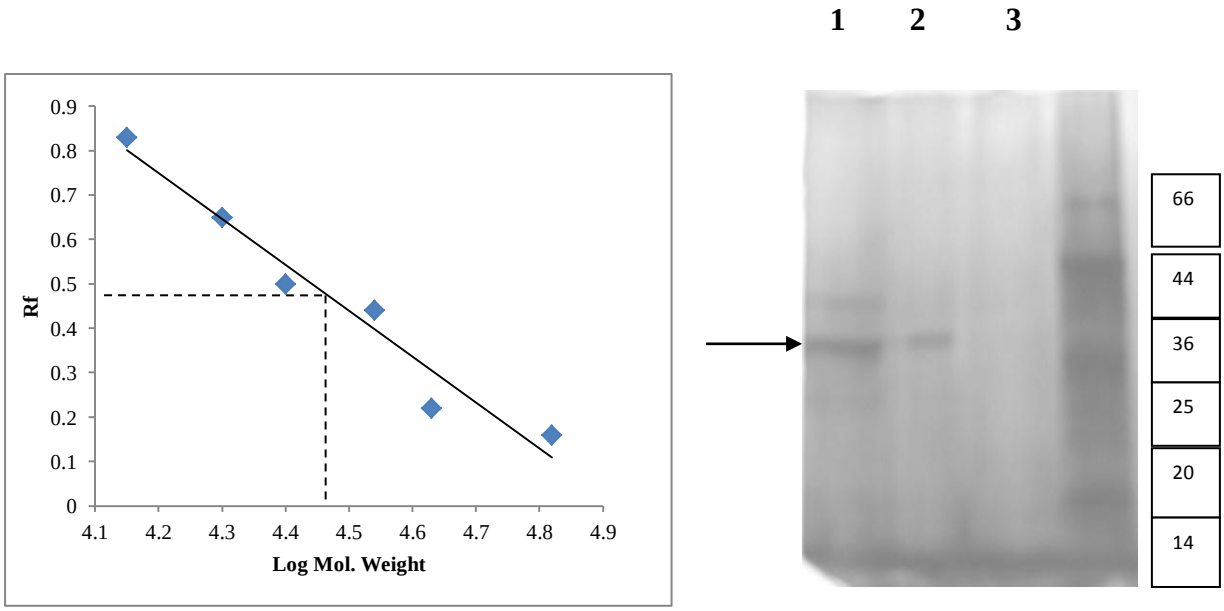


Fig. 6.4 SDS-PAGE of purified enzyme and molecular weight markers (14-66 kDa)

Lane 1: crude protein, Lane 2: purified protein,

Lane 3: Standard molecular weight markers

6.3.2 Properties of the Purified Protein

6.3.2.1 Effect of pH

During repeated experiments, the pH-activity profile of the purified protein consistently showed two peaks of activity, one at pH 5.0 and a second broader range from pH 7-9 as shown in Fig. 6.5. This pattern was similar to that obtained for the crude preparation. Reports of similar studies on proteases from other sources indicated that the pH range for optimal activity of most alkaline proteases was generally between pH 6 and 9 (Cowan and Daniel, 1982) or between pH 9 and 11 (Kumar and Takagi, 1999), while some had higher pH optima of 11.5 (Tobe *et al*., 1975; Takami *et al*., 1990; Takii *et al.*, 1990; Yum *et al.*, 1994).

6.3.2.2 Effect of Temperature

The optimum temperature for alkaline protease activity often ranges from 50 to 70°C. For instance, a northern Taiwan *Bacillus subtilis* strain was reported to be active at 50°C (Yang et al., 2000) and *B. licheniformis* from marine sediments, at 70°C (Manachini and Fortina, 1998). Optimal activity at pH 9.0 for the purified enzyme from our isolate $DCU_1$was observed at 37-45^{o}C, after which the activity steadily fell, as shown in Fig. 6.6. As reported earlier, the crude preparation was optimally active at 45 ^{o}C.

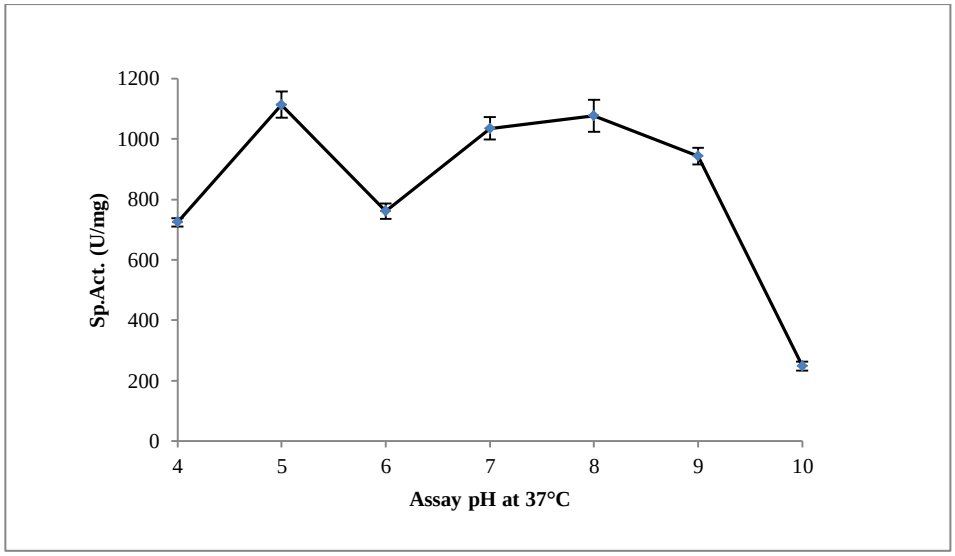


Fig 6.5. Effect of assay pH on activity of purified enzyme from strain DCU_1 at 37°C.

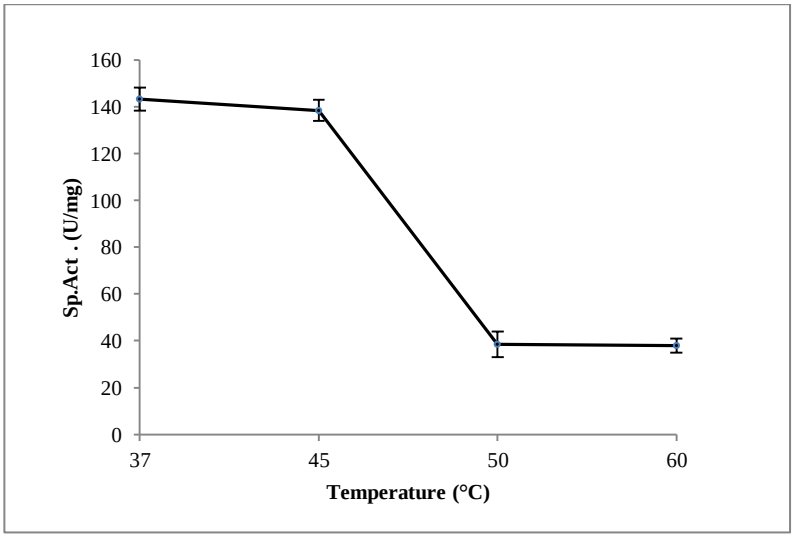
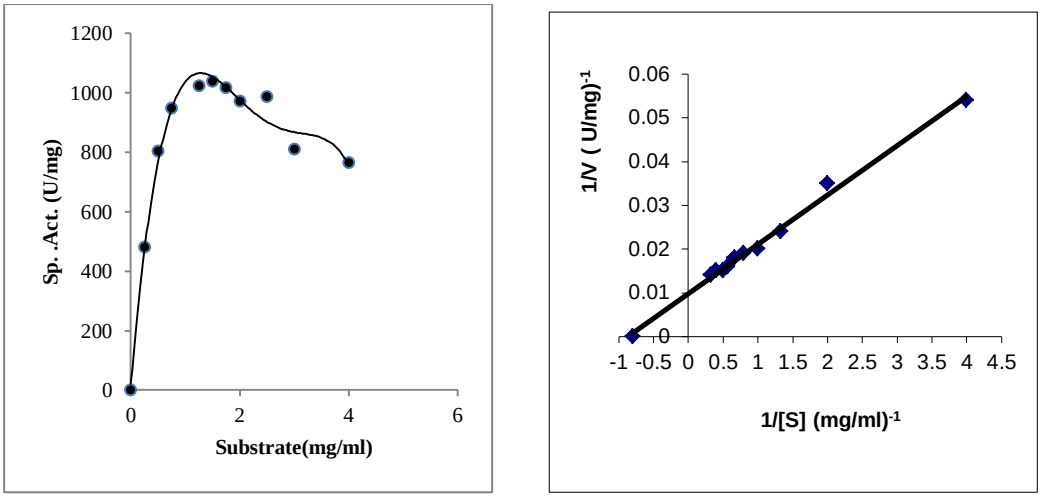


Fig. 6.6 Effect of Assay temperature on activity of purified enzyme from strain DCU_1 at pH 9.

6.3.2.3 Kinetic Constants

The purified enzyme followed typical Michaelis-Menten kinetics (Fig. 6.7 A) as also earlier reported for the crude preparation. The K_M for casein as substrate as estimated by Lineweaver-Burk analysis was found to be 0.4 mg/ml for casein (Fig. 6.7 B), as against 1.25 mg/ml for the crude preparation. The K_M value often parallels the dissociation constant of the enzyme-substrate complex, lower values being generally indicative of improved enzyme-substrate affinity. A comparison of the kinetics data on similar purified enzymes from other sources appears in Table 6.2.



A **B**

Fig. 6.7 Substrate concentration studies on the purified enzyme.

A: Michaelis-Menten kinetics; B: Lineweaver-Burk analysis.

Table 6.2 Comparison of K_M values for alkaline proteases from different isolates

Reference	Isolate	K_M (mg/ml)
Nadeem *et al.* (2013)	*B. licheniformes* UV-9	5.0
Kaur *et al.* (1998)	*B. polymyxa*	3.7
Thangam and Rajkumar (2002)	*Alcaligenes faecalis*	1.66
Jaswal and Kocher (2007)	*B.circulans*.	5.0
Muttulakshmi *et al.* (2011)	*Aspergillus flavus*	0.6
Madan *et al.* (2002)	*Bacillus polymyxa*	2.9
Mane and Bapat (2001)	*B.subtilis* NCIM 2713	2.5
Mabrouk *et al.* (1999)	*B. licheniformis*	9.1
Present study	DCU_1 (crude extract)	1.25
Present study	DCU_1 (purified enzyme)	0.4

6.3.2.4 Effect of $CaCl_2$

Alkaline proteases preferably require divalent cations such as Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations, for maximum activity. It is purported that these cations protect the enzyme against thermal denaturation and play an important role in maintaining the active conformation of the enzyme at high temperatures (Kumar, 2002; Lee *et. al*., 1996; Manni *et al.,* 2008). Increased activity of an alkaline serine protease was reported from *Bacillus subtilis* in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+} (Bayoudh *et al.,* 2000; Adinarayana *et al.,* 2003; Gessesse *et al.,* 2003). In an earlier report, James *et. al.* (1999) had suggested that Ca^{2+} stabilises the protein through specific or non-specific binding sites, also allowing for additional bonding within the enzyme molecule and thereby preventing unfolding at higher temperatures. In the present study addition of $CaCl_2$ in the assay sharply stimulated the enzyme activity up to 1mM (Fig. 6.8). This tallies with the conclusions drawn from data derived from the crude enzyme (Table 4.3) Substrate diversity is an important criterion to analyze the potency of a protease. As reported in Chapter 4, the crude enzyme had shown broad substrate specificity, highlighting its potential in varied biotechnological applications. As a substrate, gelatin was shown to be equally effective as casein. When tested with the purified enzyme, the activity on gelatin improved further, to almost 1.8 times that elicited in the presence of casein as standard.

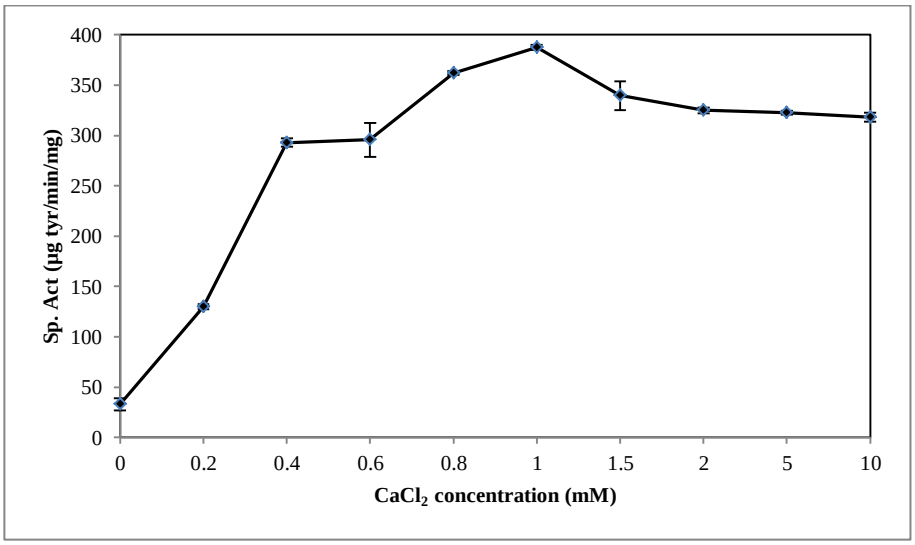


Fig. 6.8. Effect of $CaCl_2$

6.3.2.5 Various Effectors of the Enzyme Activity.

The effect of some additives which were not tested for on the crude enzyme (Chapter 4) was looked into (Table 6.3). The detergents triton X-100 and tween 80 caused a marginal decrease in activity compared to the control. It was observed that 1,10-phenanthroline, a strong chelator of most metal ions, brought about a concentration dependent inhibition of the enzyme activity. The divalent metal-ion requirement suggested by the results of the studies on the crude enzyme is thus strengthened by these results.

The stimulation of activity in the presence of sulphydryl agents (Table 6.4) confirms the cysteine protease nature identified in Chapter 4. The observed stability in the presence of H_2O_2 enhances the potential for use in detergent formulations. In the case of the enzyme from our deep-sea isolate, this potential application has already been noted (Chapter 2).

Table 6.3: Effect of various additives on the enzyme activity

Additive	Concentration (mM)	Sp. activity (U/mg)
1-10 phenanthroline	50	90.07
	100	50.91
	200	31.33
Triton X-100	10	101.82
Tween 80	10	113.57
Control	-	138.37

Table 6.4 Effect of reducing agents on the enzyme activity

Reducing agent	Concentration (%)	Specific activity (U/mg)
ß-mercaptoethanol	0.1	1507.8
	0.5	1206.0
Na- thioglycolate	0.1	1499.7
	0.5	1801.5
H_2O_2	1.0	1190.4
	2.0	1378.4
Glutathione	0.1	1370.6
	0.5	1918.7
Control	-	669.1

Summary, Conclusions

And Future Prospects

Several bacterial and fungal groups significantly contribute to the degradation of organic matter. The enzymes produced by such organisms participate in these processes, highlighting the importance of their isolation and study.

Proteases, prominent as being among the three largest groups of industrially applied enzymes, account for about 60% of the total global enzyme sales. Yet very few specific enzymes have actually contributed to this success. There is thus an ever growing need for isolation of enzymes with improved/novel features favoring industrial requirements. The search for organisms producing these enzymes would be benefitted by screening from specialized niches such as extremophilic environments, which might trigger off the production of enzymes with special characteristics.

Bacteria play an important role in the ecology of deep-sea sediments. Ever since the report by Zobell and Morita (1957) on isolation of 'barophilic' bacteria specifically adapted to grow under high pressures, the presence and ecological importance of deep-sea bacteria has gained increasing recognition. Interesting bacteriological conversions occur at the edges of huge aquatic ecosystems such as the sea, estuaries, marshlands, salt marshes, *etc*.. The particulate organic matter reaching the estuary finally finds its way into the sediment where it undergoes transformation. Except for a few millimeters in the upper layer the sediment hosts an anaerobic environment. The organic matter accumulating in the sediment as detritus contains mainly protein and carbohydrates, with lipids in small quantities. The bacteria harboured in the sediment water transitional zone play a key role in the degradation of this detritus and thus replenishment of the essential nutrients in the aquatic system.

With the increasing urge for development of environmentally friendly technologies, the marked potential of alkaline proteases for application in the leather as well as the detergent industries besides the food, pharmaceutical and waste processing industries can not be underplayed. In the detergent industry, proteases added to laundry detergent would enable release of protein-based stains from fabric (Masse and Tilburg, 1983), improve cleansing efficiency at shorter agitation periods, with perhaps just a prior soaking period (Demidyuk *et al*., 2008). Purification and characterization would thus be a basic need to elucidate such precise and specific properties of a newly isolated enzyme that would favor its applications

in industry (Kumar, 2002; Adinarayana *et al.*, 2003), although depending upon the industry, it may at times prove more cost effective to use the enzyme in its crude extracted form.

By the use of standardized fermentation methods microorganisms can be cultured in significant quantities in a comparatively short time to facilitate an abundant, consistent supply of the desired product. They would thus undoubtedly serve as an attractive source of proteases.

The study at hand was initiated with the following primary objectives in view :

1. Screening of samples from different niches, with a view to elicit high proteolytic activity (of bacterial/fungal origin) of industrial relevance.
2. Extraction and preliminary characterization of a few enzyme extracts.
3. Testing of selected enzyme extracts for potential biotechnological application in the detergent/oil/leather/textile industry.
4. Purification and characterization of the most promising proteolytic activity.

Accordingly, microbial isolates have been obtained from diverse ecosystems covering estuaries, salterns, rivers, lake, mangroves and the deep-sea; qualitative as well as quantitative techniques have been employed in screening for their alkaline protease producing ability.

Since proteases play an important role in many biological processes in the release of protein, utilization of proteinaceous nutrient, autolysis and spore germination, besides other physiological phenomena, one chapter of the thesis has been dedicated to the discussion of the relationship of the alkaline proteases obtained, with respect to the ecological niches from which the individual microbes were sourced.

The crude enzymes from selected fungal and bacterial isolates have been subjected to preliminary characterization to evaluate assay conditions such as optimal temperature and pH. Isolates producing proteases optimally active at 45-50°C were preferentially selected for further characterization. Some isolates also exuded cold active enzymes which could be

a fact of potential significance, as such enzymes are known to find use especially in the detergent industry.

The potential of some of the crude alkaline protease preparations in different biotechnological applications was explored, for which the broad substrate specificity proved an asset. Results of preliminary laboratory-level experiments indicate a potential extendable to the detergent industry as well as to aid in oil extraction processes. Enzyme-assisted degradation of the gelatinous coating of X-ray films has also been carried out with the aim of silver recovery for reuse.

Natural dyes from marine microorganisms could provide the much needed alternative to the complex and polluting world of chemical dyes conventionally employed in the textile industry. One of our deep-sea isolates, being a pigment producer under controlled conditions, application of its reddish pigment in the dyeing of cloth has been looked into.

The alkaline protease produced by the deep-sea isolate DCU_1 was selected for further characterization with respect to enzyme concentration, time course of activity, optimal temperature and pH of assay, thermal stability, pH and salt tolerance, effect of different detergents and metal ions, *etc.* and these studies contribute to yet another chapter. The properties are indicative of cysteine protease characteristics and a possible divalent metal-ion requirement.

The selected deep-sea isolate DCU_1 that exuded an alkaline protease with special features signifying its biotechnological potential was identified and characterized. In addition to the basic biochemical tests, the isolate was characterized through its fatty acid profile as well as by phylogenetic studies. Identity of the strain was established as *Serratia* sp of the Enterobacteriaceae family and some of the biochemical studies were suggestive of a closeness to *S. marcescens.* While this came as a surprise in the first instance, it does find support from well established studies on such other microbes of terrestrial origin, hardy enough to have adapted to and survived the drastic environmental change. For instance, Raghukumar and Raghukumar (1998) have indicated the possibility of spores and hyphal fragments of terrestrial fungi getting deposited in deep-sea sediments through terrestrial runoff, wind or water movements.

The enzyme from the deep-sea strain DCU_1 has been purified to homogeneity by employing ammonium sulphate precipitation followed by single step column chromatography. The initial rigorous efforts on culture medium optimization to specifically and rather selectively induce alkaline proteases contributed in a large way to the simplicity of the purification procedures. Properties of the purified enzyme including its basic kinetic features are being reported. Molecular weight determination by size exclusion chromatography as well as by SDS-PAGE confirm the protein to be a monomeric cysteine protease of about 33-34 kDa.

This study is thus a unique documentation of quantified alkaline protease activity from microbes from the diverse ecosystems of Goa, besides throwing light on a deep-sea bacterial *Serratia* sp. as a pigment as well as alkaline protease producer of potential industrial significance.

FUTURE PROSPECTS

- Industrial potential of the alkaline protease from the deep–sea isolate as well as from the other isolates could be explored further.
- Other applications such as in leather industry and contact lens cleansing could be looked into.
- A more detailed study on the pigment producing ability of the deep- sea isolate could be carried out with a view to understand its potential in textile dyeing.
- The possibility of the pigment possessing antibiotic properties could also be investigated.
- Identification of the alkaline protease by mass-based analysis could be attempted.

Posters Presented at Conferences

1. **Akmadevi S. Nadurmath** and Usha D. Muraleedharan**.** *Thermotolerant Alkaline Protease Activity from a Deep-sea Bacterial Isolate*. National Seminar on Frontiers in Biotechnology: Proteomics, Genomics & Nanobiotechnology; Mumbai (2009).

   (Awarded **second** place under the **Best Poster** category).

2. **Akmadevi S. Nadurmath** and Usha D. Muraleedharan**.** *Characterization and Biotechnological Application of Thermotolerant Alkaline Protease Activity from a Deep-sea Bacterial Isolate.* International Conference on Frontiers in Biological Sciences; Orissa (2010**).**

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Appendix

Appendix A

1. Medim N for Bacteria

Skimmed milk *	1.0 g
Yeast extract	0.1g
$MgSo_4$	0.005
$FeSo_4$	0.001g
NaCl	0.005 g
pH	9.5
s/w	100 ml
Agar	1.5g

*No Skimmed milk for initial screening.

2. Malt extract Agar for Fungi

Malt Extract Agar	0.5%
Antibiotics	1 %
(Penicillin & Streptomycin)	
D/W	100ml

3. Czapec Dox Medium (for 1 L)

D- Glucose	10 %
$NaNO_3$	3%
$MgSO_4$	0.5 %
$FeSO_4.H_2O$	0.01 %
K_2HPO_4	1%
KCl	0.5%
Agar	1.5 %
s/w	900ml

Autoclave 100 ml of 3% skimmed milk separately and add to the above 900 ml.

Appendix-B

Reagents for protein estimation

1. Folin Lowry reagents (Stock solutions)

Solution A (100ml)

1. $CuSO_4 5H_2O$ 1.0 g
2. Sodium potassium tartarate 2.0g

Before experiment, mix 500µl each of 1 and 2 of above to get 1ml of solution A.

Solution B

Na_2CO_3 20g

NaOH 4g

D/W 100ml

Alkaline reagent

To prepare this alkaline regent mix 1ml of Solution A and 49 ml Solution B.

Folin CioCalteu's phenol reagent

1:1 dilution with D/W

2. Borate buffer ($Na_2B_4O_7.10H_2O$) (50 mM)

19.05 g was dissolved in 500ml D/W, the pH was adjusted to 8 and 9 with 0.1N HCl and the volume was made up to 1000ml with D/W. to make buffer of pH 10 and 11, it was adjusted with 0.1N NaOH and made up to 1000ml with D/W.

3. Sodium Acetate buffer (200mM)

Appropriate volumes of 0.2 M acetic acid and 0.2M Sodium acetate were mixed the of desired pH.

4. Reagents for PAGE

Tank Buffer

Glycine 14.4g

Tris 3.0g

Make up volume to 1000 ml with D/W.

For SDS-PAGE, add SDS to a final concentration of 0.1%.

Separating gel buffer (4X)

Tris base 36.3 g

pH (adjusted with HCl) 8.8

Make up volume to 200ml with D/W.

Stacking gel buffer (4X)

Tris base	3.0g
pH (adjusted with HCl)	6.8

Make up volume to 50ml with D/W.

Ammonium persulphate (APS), 10% (To be prepared freshly before use)

APS	0.1 g
D/W	1.0 ml

Gel loading buffer for native PAGE (4X)

Glycerol	2.0ml
4X Separating gel buffer	2.5 ml
Bromophenol blue (1%)	1.0 ml

Make up volume to 10ml with D/W.

Gel loading buffer for SDS-PAGE (2X)

Glycerol	2.0ml
4X Separating gel buffer	2.5 ml
SDS (10% stock)	4.0 ml
Bromophenol blue (1%)	1.0 ml

Make up volume to 10ml with D/W.

Acrylamide stock solution (30%)

Acrylamide	29.2g
N,n-methylene bis Acrylamide	0.8 g

Make up volume to 100ml with D/W. Store at 4°C in dark.

Staining solution

Comassie Brilliant blue R-250	0.25 g
Methanol	45 ml
D/W	45ml
Acetic Acid	10ml

Filter through a whatman No.1 filter to remove any particulate matter.

Destaining Solution

Methanol	45 ml
D/W	45 ml
Acetic Acid	10ml

Reagents for silver staining

Fixing solution

Acetic Acid	10%

Keep the gel in fixing solution for 30min.

Sodium thiosulphate	0.02%

Add, shake for 1 min and wash the gel with D/W for 20 sec

$AgNO_3$	0.2%
Formaldehyde	0.075 ml
D/W	100 ml

Incubate for 20 min in dark and wash the gel with D/W.

Developing solution

Na_2CO_3	6 g
Sodium thiosulphate	0.0004g
Formaldehyde	0.05 ml
D/W	100 ml

Abbreviations

APS	-	Ammonium persulfate
BLAST	-	Basic Local Alignment Search Tool
BSA	-	Bovine Serum Albumin
DNA	-	Deoxyribonucleic acid
DW	-	Distilled Water
EC	-	Enzyme Commission
EDTA	-	Ethylenediaminetetraacetic acid
EGTA	-	Ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'- tetraacetic acid
HPLC	-	High Performance Liquid Chromatography
kDa	-	KiloDalton
Km	-	Michaelis Menton constant
MW	-	Molecular Weight
NCBI	-	National Centre for BiotechnologyInformation
OD	-	Optical Density
PAGE	-	Poly Acrylamide Gel Electrophoresis
PCR	-	Polymerase Chain Reaction
PSU	-	Practical salinity units
RNA	-	Ribonucleic Acid
Rpm	-	rotations per minute
SDS	-	Sodium Dodecyl Sulphate
TCA	-	Trichloroacetic acid
TEMED	-	N-N-N'-N'-Tetramethylethylenediamine
Vmax	-	Maximal Velocity

NA	-	Nutrient Agar
NB	-	Nutrient Broth
MEA	-	Malt extract Agar
RT	-	Room Temperature
PMSF	-	Poly methyl sulphonyl fluoride
DTT	-	Dithiothreitol
Sp.act	-	Specific Activity
A_{nm}	-	Absorbance in nm, at the given wavelength
GF	-	Glass microfiber filters
MALDI-TOF MS	-	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
(pCMB)	-	*p*- chloromercuric benzoate
SEM	-	Scanning Electron Microscope