ALKALINE PROTEASES FROM BACTERIA ISOLATED FROM COCHIN ESTUARY

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CERTIFICATE

This is to certify that the thesis entitled ***ALKALINE PROTEASES FROM BACTERIA ISOLATED FROM COCHIN ESTUARY*** *is an authentic record of the research work carried out by Ms. Meera Venugopal, under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of science and Technology, in partial fulfilment of the requirements for the degree of* ***Doctor of Philosophy*** *in Microbiology of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

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DECLARATION

I hereby declare that the thesis entitled ***ALKALINE PROTEASES FROM BACTERIA ISOLATED FROM COCHIN ESTUARY*** *is an authentic record of the research work carried out by me under the supervision and guidance of Dr. A.V. Saramma, Reader in Microbiology, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, for the degree of Doctor of Philosophy in Microbiology of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

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CONTENTS

CHAPTER 1 Introduction **1- 6**

CHAPTER 2 Isolation and Screening of Protease Producing Bacteria **7- 26**

2.1 Review of literature 8

2.2 Materials and Methods 11

2.2.1 Collection of samples and isolation of bacteria 11

2.2.2 Sampling sites 12

2.2.3 Identification of cultures 13

2.2.4 Primary screening for protease production 14

2.2.5 Secondary screening for potent strains producing alkaline protease 15

2.3 Results 16

2.4 Discussion 21

2.4.1 Selected strains 23

2.4.2 Mangroves- a unique ecological niche to be conserved 24

CHAPTER 3 Effect of Culture Conditions on Growth and Production of Alkaline Proteases **27- 78**

3.1 Review of literature 28

3.2 Materials and Methods 30

3.2.1 Selected strains 30

3.2.2 Growth media 30

3.2.3 Inoculum preparation 31

3.2.4 Measurement of growth 31

3.2.5 Preparation of crude enzyme 31

3.2.6 Assay of alkaline protease 31

3.2.7 Effect of different physico chemical parameters on growth and alkaline protease production 32

3.3 Statistical analysis 34

3.4 Results 34

3.4.1 Effect of shaking speed on growth and protease production 34

3.4.2 Effect of period of incubation on growth and protease production 37

3.4.3 Effect of pH on growth and protease production 40

3.4.4 Effect of temperature on growth and protease production 43

3.4.5 Effect of NaCl concentration on growth and protease production 46

3.4.6 Effect of metallic salts on growth and protease production 49

3.4.7 Effect of carbon sources on growth and protease production 54

3.4.8 Effect of Nitrogen sources on growth and protease production 60
3.5 Discussion 65
3.5.1 Effect of shaking speed on growth and protease production 65
3.5.2 Effect of period of incubation on growth and protease production 67
3.5.3 Effect of pH on growth and protease production 69
3.5.4 Effect of temperature on growth and protease production 69
3.5.5 Effect of NaCl on growth and protease production 70
3.5.6 Effect of metallic salts on growth and protease production 71
3.5.7 Effect of carbon sources on growth and protease production 72
3.5.8 Effect of Nitrogen sources on growth and protease production 74
CHAPTER 4 Purification and Characterisation of Proteases 77-122
4.1 Review of literature 77
4.2 Materials and Methods 80
4.2.1 Determination of protein 81
4.2.2 Determination of molecular weight by SDS PAGE 84
4.2.3 Zymography (Activity Gel) 84
4.2.4 Assay of alkaline protease 86
4.2.5 The enzyme kinetics 86
4.3 Statistical Analysis 87
4.4 Results 88
4.4.1 Purification of the alkaline proteases 88
4.4.2 Molecular weight determination 89
4.4.3 Effect of pH on protease activity 89
4.4.4 Effect of pH on the stability of alkaline protease 91
4.4.5 Effect of temperature on enzyme activity 94
4.4.6 Effect of temperature on stability of the enzymes 96
4.4.7 Effect of substrate concentration on protease activity 98
4.4.8 Effect of various metallic salts on protease activity 100
4.4.9 Effect of inhibitors on protease activity 103
4.5 Discussion 107
4.5.1 Purification of the alkaline proteases 107
4.5.2 SDS PAGE and molecular weight 108
4.5.3 Zymogram analysis 109
4.5.4 Effect of pH on protease activity and stability 111

4.5.5 effect on temperature on protease activity and stability 113
4.5.6 Effect of substrate concentrations on enzyme activity- Kinetic constants 117
4.5.7 Effect of metal ions on the activity and stability of proteases 115
4.5.8 The mechanistic classes of proteases based on inhibitor studies 118
CHAPTER 5 Application of the Alkaline Proteases as Detergent Additives 123- 147
5.1 Review of literature 126
5.2 materials and methods 128
5.2.1 Effect of pH on the activity and stability of proteases 128
5.2.2 Effect of temperature on the activity and stability of proteases 128
5.2.3 Activity of the proteases on different protein substrates 128
5.2.4 Stability of the proteases in different commercial detergents 129
5.2.5 Stability of the proteases in SDS, Triton X- 100 and H_2O_2 129
5.3 Results 130
5.3.1 Effect of pH on the activity and stability of proteases 130
5.3.2 Effect of temperature on the activity and stability of proteases 130
5.3.3 Activity of the proteases on different protein substrates 131
5.3.4 Stability of the proteases in different commercial detergents 131
5.3.5 Effect of H_2O_2 on the activity and stability of proteases 135
5.3.6 Effect of SDS on the activity and stability of proteases 136
5.3.7 Effect of Triton X- 100 on the activity and stability of proteases 138
5.4 Discussion 140
5.4.1 Effect of pH on the activity and stability of proteases 140
5.4.2 Effect of temperature on the activity and stability of proteases 141
5.4.3 Activity of the proteases on different protein substrates 143
5.4.4 Stability of the proteases in different commercial detergents 144
5.4.5 Effect of H_2O_2 on the activity and stability of proteases 146
5.4.6 Effect of SDS and Triton X- 100 on the activity and stability of proteases 147
CHAPTER 6 Summary 150- 153
APPENDIX 154- 167
REFERENCES 168-197

ABBREVIATIONS

Abs.	Absorbance
rpm	revolutions per minute
mg	Milligram
mM	Milli moles
µg	Microgram
µl	Microlitre
hr.	Hour
Km	Michelis Menten Constant.
g	Gram
nm	nanometer
%	Percentage
g l^{-1}	gram per litre
Fig.	Figure
et al.	Co-authors
ASW	Artificial Sea Water
°C	Degree Celsius
TEMED	NNN'N' Tetramethyl ethylenediamine.
APS	Ammonium persulphate
SDS	Sodium Dodecyl Sulphate.
BSA	Bovine Serum Albumin
PEG	Poly ethylene glycol
EDTA	Ethylenediaminetetraaceticacid
EGTA	Ethyleneglycol-O, O' bis, (2 amino ethyl) NNN'N' tetraaceticacid
PMSF	Phenyl methane sulphonyl fluoride
IAA	Iodo Acetic Acid
DTT	Dithiothreitol
ANOVA	Analysis of variance
LSD	Least Significant Difference
DFP	Diisopropyl fluorophosphates
E64	L-trans- epoxysuccinyl- leucylamido (4-guanidino butane)
ONPG	O- Nitrophenyl-β-D Galactopyranoside
HPLC	High Performance Liquid Chromatography
IEC	Ion Exchange Chromatography
O/129	2,4 – diamino-6,7 diisopropyl pteridine

CHAPTER 1
INTRODUCTION

Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are characterized by their extraordinary specificity and reactivity. They occur in all living systems where they activate and regulate chemical reactions essential to the continued existence of the individual organism. Many microorganisms produce extracellular enzymes, which are chiefly hydrolases and are involved in the degradation of macromolecules to simpler units like peptides and amino acids. Over the past two decades considerable research has been undertaken to discover industrially useful enzymes and to increase their yield by environmental and genetic manipulations. Enzymes have now become very much a part of new industrial processes and appear to offer a great potential in a wide variety of as yet undeveloped applications.

The current estimated value of the worldwide sales of industrial enzymes is $ 1 billion (Godfrey and West, 1996). Of the industrial enzymes 75% are hydrolytic. Proteases represent one of the three largest groups of industrial enzymes.

Proteases belong to a class of enzymes, which occupies a pivotal position with respect to their application in both physiological and commercial fields. Proteolytic enzymes catalyse the cleavage of peptide bonds in proteins. Proteases are degradative enzymes, which catalyse the total hydrolysis of proteins. Advances in analytical techniques have demonstrated that proteases conduct highly specific and selective modifications of proteins such as, activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots and processing and transport of secretory proteins across the membranes. Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level to produce cascade systems such as haemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. The vast diversity of proteases, in contrast to the specificity of their action has

attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Poldermans, 1990; Fox *et al.*, 1991).

Since proteases are physiologically necessary for living organisms they are ubiquitous, being found in a wide diversity of sources such as plants, animals and microorganisms. Important plant proteases are papain, obtained from the latex of *Carica papaya* fruits, bromelain, prepared from the stem and juice of pineapples, some keratinases and ficin. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennins. The production and use of plant and animal proteases depend on several factors such as availability of land for cultivation, the suitability of climatic conditions for growth, availability of livestock for slaughter which in turn, is governed by political and agricultural policies.

The inability of plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms elaborate a large variety of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes. Extracellular proteases are significant for the hydrolysis of proteins in cell-free environments and they enable the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases are of commercial value and exploited in various industrial sectors. Microorganisms serve as excellent sources of commercial enzymes as they can be cultured in large quantities, in a relatively short time by established methods of fermentation and they produce abundant regular supply of the desired product and also, owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbes account for a two third share of commercial protease production in the world (Kumar and Takagi, 1999). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess all the characteristics desired for their biotechnological applications.

Proteases are grossly subdivided into two major groups viz. exopeptidases and endopeptidases, depending on their site of action.

Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate whereas, endopeptidases cleave peptide bonds distant from the termini of the substrates. Microbial proteases are classified into three groups based on their pH optima of activity. They are acidic proteases, neutral proteases and alkaline proteases (Keay, 1971). A more rational system is now based on the comparison of active sites, mechanism of action and three-dimensional structure (Neurath, 1989). Four mechanistic classes of proteases are recognized by the International Union of Biochemistry, and within these classes, six families of proteases are recognized to date. The four classes are, serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Table 1.1).

Table1.1 Families of proteolytic enzymes

Family	Representative protease	Characteristic active site residues*
Serine protease I	**Chymotrypsin** Trypsin Elastase Pancreatic kallikrein	Asp^{102}, Ser^{195}, His^{57}
Serine protease II	**Subtilisin**	Asp^{32}, Ser^{221}, His^{64}
Cysteine proteases	**Papain** Actinidin Rat liver cathepsins B and H	Cys^{25}, His^{159}, Asp^{158}
Aspartic proteases	**Penicillopepsin** *Rhizopus chineses* and *Endothia parasitica* acid proteases Renin	Asp^{33}, Asp^{213}
Metallo-protease I	**Bovine carboxy peptidase A**	Zn, Glu^{270}, Try^{248}
Metallo-protease II	**Thermolysin**	Zn, Glu^{143}, His^{231}

*The amino acid residues correspond to the amino acid sequence of the enzymes listed in **bold** in column no 2

Many other proteolytic enzymes have been identified and isolated that do not fit into this classification(Lorand, 1976; Colowick and Lorand, 1980).

Proteases have a large variety of applications mainly in the detergent and food industries. In view of the recent trend of developing environment friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. The worldwide requirement of enzymes for industrial applications varies considerably. Proteases are used extensively in pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations whereas, those that are used in medicines are produced in small amounts but require extensive purification before they can be used. Today proteases account for approximately 40% of the total enzyme sales in various industrial market sectors such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. This dominance in the industrial market is expected to increase further by the year 2005 (Godfrey and West, 1996). However, until today the largest share of the enzyme market has been held by detergent alkaline proteases that are active and stable in the alkaline pH range.

Alkaline proteases

Alkaline proteases are defined as those proteases, which are active in a neutral to alkaline pH range (Gupta *et al.*, 2002a). They are either serine proteases or metalloproteases, and the alkaline serine proteases are the most important group of enzymes exploited commercially.

Alkaline proteases are of considerable interest in view of their activity and stability at alkaline pH. Alkaline proteases belong to a physiologically and commercially important group of enzymes used primarily as detergent additives. They play a specific catalytic role in the hydrolysis of proteins. The use of alkaline proteases in laundry industry accounts for approximately 25% of the total worldwide sale of enzymes. An upward trend in the use of alkaline protease is expected by the turn of the decade.

Although many microbial species are known to produce alkaline proteases, only a few are recognized as commercial producers. Most of them belong to the genus *Bacillus* (Rao *et al.*, 1998). Bacterial alkaline proteases are characterized by their high activity at alkaline pH, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

Looking into the vast diversity of microbial population, there is always a chance of finding microorganisms producing novel enzymes with better properties that are suitable for commercial applications. The multitude of physicochemically diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products and processes. Microbes have developed a wealth of physiological and molecular adaptations that enabled their survival in virtually every environmental niche, some so extreme and inhospitable that no other life form could coexist. The versatility and adaptive power of the prokaryotic design was such that with their evolutionary headstart, the bacterial and archaeal kingdom produced a degree of, organism and molecular diversity unparalleled in nature. Microbial biodiversity is our planet's greatest, but least developed resource for biotechnological innovations. The biosphere is dominated by microorganisms, yet todate majority of microbes in nature have not been studied.

Microorganisms distributed in the marine and brackish environments play an important role in the decomposition of organic matter and mineralisation in the system (Seshadri and Ignacimuthu, 2002). Estuary is one of the most productive ecosystems, at the same time one among the least explored ecosystems on earth, which has immense potential as a source of potent microorganisms that produce valuable compounds particularly, enzymes such as proteases. In this scenario, it is very appropriate to embark on finding novel alkaline protease producers from the estuarine system. The area where the present investigation was carried out,

is a part of the extensive estuarine system of South India viz. Cochin Estuary. There is meagre knowledge regarding the microbial composition, particularly the protease producers of Cochin Estuary. Hence, the present study has been undertaken with the objective of finding novel alkaline protease producing bacteria from Cochin Estuary.

Objectives of the study

The major objectives of the present study are: -

- Isolation and identification of alkaline protease producing bacteria from Cochin Estuary.
- Selection of the most potent strains, capable of producing alkaline proteases.
- Purification and characterization of the selected proteases.
- Application of the selected proteases in detergent formulations.

The thesis is presented in six chapters. The first chapter gives a brief introduction to the subject. The second chapter deals with the isolation, identification and screening of bacteria for alkaline protease production. The third chapter describes the effect of culture conditions on the growth and enzyme production of the selected strains. The fourth chapter deals with the purification and characterisation of the selected alkaline proteases. The use of the selected proteases in the detergent formulations is dealt with in the fifth chapter. The major findings of the thesis are summarized in the sixth chapter.

CHAPTER 2

ISOLATION AND SCREENING OF PROTEASE PRODUCING BACTERIA

Aquatic environment is an ideal habitat for the growth of microorganisms. Bacteria are widely distributed in aquatic habitats, which are represented by almost all taxonomic groups. A sharp separation of them into soil bacteria and aquatic bacteria is not possible as the inland waters are constantly exposed to the flora of the soil. Interesting bacteriological conversions occur at the edges of huge aquatic ecosystems like the sea, the estuaries, marshlands, salt marshes etc. The particulate organic matter reaching the estuary finally finds its way into the sediment where they undergo transformation. Except 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, and lipids are found in small quantities. Ubiquitous nature of phosphates reaching the estuaries probably makes it the main cause of eutrophication. The bacteria harboured in the sediment water transitional zone play a key role in the degradation of organic matter reaching the sediment and thus the replenishment of the essential nutrients in the aquatic system. Bacteria have highly efficient extracellular enzyme systems such as proteases, amylases, lipases and phosphatases, in order to catabolize complex materials into simpler fractions. Hence bacteria and other microorganisms play a significant role in the ocean as they do in terrestrial environments but only meagre information is available about their occurrence and activities.

Estuaries are considered as the most productive ecosystems on earth. The backwaters of Kerala support as much biological productivity as the tropical rain forests. Cochin backwaters is one among them, which has been subjected to intense biological research.

Cochin estuary, a part of the extensive estuarine system of backwaters on the South West Coast of India, is a tropical positive estuarine system which is situated at the tip of the northern Vembanad Lake, and is the largest estuary in the State of Kerala, extending between 9°40' and 10° 12' N and 76° 10' and 76° 30' E with its Northern boundary at Azheekode and Southern boundary at Thannirmukkam bund. It has permanent connections with the Arabian Sea,

and water from the two major rivers Periyar and Muvattupuzha drain into this estuary. The major hydrological variable in Cochin estuary is salinity similar to the situations encountered in estuaries, with a gradual declension of salinity from 30 ppt at the entrance of the estuary to 0.2 ppt at the point entry of the rivers. Low lying swamps and tidal creeks, dominated by sparse patches of mangroves with their nutrient rich physical environment, support diverse species of flora and fauna (Menon *et al.*, 2000).

2.1 Review of literature

Though extensive studies have been conducted on the physicochemical and biological aspects of Cochin estuary, there is still far more to be explored regarding the microbiological aspects of the estuary. Only limited works are available on the microbiological composition of the estuary.

A few researchers had studied the proteolytic bacteria from Cochin estuary. Philip (1987) studied the distribution of bacteria in Cochin estuary and assessed the seasonal variation in species composition and the production of proteolytic enzymes by these bacteria. Chitinoclastic and proteolytic bacteria from Cochin backwaters were isolated and studied by Amar (2001). A study on benthic bacteria of Cochin estuary, producing extracellular enzymes including proteases was carried out by Krishnan (2003).

Reports are numerous regarding protease production in bacteria from a variety of habitats. Despite the long list of protease producing microorganisms only a few are considered as appropriate producers for commercial exploitation being 'generally regarded as safe' (GRAS), non toxic and non pathogenic. Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environment have been explored for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis*, *B. subtilis, B. amyloliquifaciens, and B. mojavensis*. (Kalisz, 1988; Rao *et al.*, 1998; Kumar and Takagi, 1999; Gupta *et al.*, 2002b;). Production of thermostable alkaline

proteases by a halo tolerant *Bacillus licheniformis*, in seawater was reported by Manachini and Fortina (1998).

Proteolytic bacteria from diverse habitats had been isolated and characterized by many more researchers. The proteolytic bacteria of Long Island Sound water column were studied by Murchelano and Brown (1970). Reinheimer (1972) noted that most of the isolates from Arabian Sea of Northern Gulf of Oman were strongly proteolytic. Sizemore *et al.* (1973) studied the distribution and activity of the proteolytic bacteria in the sediments from the North Inlet Estuary near Georgetown. Boeyo *et al.* (1975) reported that proteolytic forms were widely encountered in North Sea sediments. Observations on proteolytic and lipolytic bacterial counts in the sediments of marine environment of New York Bight Ape, Sandy Hook Bay and Great Bay of New Jersey showed that proteolytic counts were 2-4 times higher in the polluted areas (Nitkowski *et al.*, 1977). Distribution of heterotrophic bacteria and the production of several extracellular enzymes including proteases in marine sediments were studied by Nair *et al.* (1978). Little *et al.* (1979) observed that the microbial proteolysis in Lake Champlain was very much dependent upon water temperature. Studies have been conducted on the protease from a marine *Pseudomonas* sp. by Makino *et al.* (1983). Proteolytic activity of a ruminal bacterium *Butyrivibrio fibrisolvens* was reported by Cotta and Hespell (1986). Protease production in an extreme halophile *Halobacterium halobium* was reported by Ryu *et al.* (1994). Protease production from a thermophilic *Bacillus* sp. isolated from an alkaline hot spring at Ethiopia was studied (Atalo and Gashe, 1993). Santos *et al* (1996) reported protease production in a dairy strain of *Aeromonas hydrophila.* The protease production aspects of a new alkalophilic strain of *Microbacterium* isolated from alkaline soda lake were reported (Gessesse and Gashe, 1997). Extracellular protease from the fish pathogen, *Yersinia ruckeri* was reported (Secades and Guijarro, 1999). Protease production in a *Streptomyces cyaneus* isolated from Brazilian cerrado soil was reported by Petinate *et al.* (1999). Protease producers from Antartic soil were isolated by Wery *et al.* (2003).

The production of other extracellular enzymes by aquatic bacteria was also studied by some scientists. The presence of extracellular amylase in three *Vibrio* species isolated from Cochin estuary and its characterization were studied by Saramma (1992). Gopinath (2002) studied the generic composition of bacteria in Cochin estuary and Arabian Sea and tested their efficacy to produce extracellular, acid and alkaline phosphatase. Another study regarding the phosphate solubilising bacteria from the South East Coast of India was carried out by Seshadri and Ignacimuthu (2002).

A number of works are available on the microbial species composition of Cochin estuary and on different specialized groups of bacteria. Thiobacilli from Cochin backwaters were studied by Gore and Unnithan (1977). Ramamoorthy and Jayabalan (1982) have given an account of the microbial species composition of Cochin estuary. The occurrence and seasonal distribution of aerobic heterotrophic bacteria, in water and sediment of Cochin backwaters and their ecophysiology and biochemical characters were studied, by Chandrika and Ramachandran (1994).

Some early works give information on the bacterial distribution in estuarine and freshwater sediments (Zobell and Felthem, 1942; Rodina 1963; Stevenson *et al.*, 1974). Distribution of *Vibrio parahaemolyticus* and allied *Vibrios* in backwater and mangrove biotope at Port Novo was studied by Natarajan *et al.* (1979). Kannapiran *et* al., (1999) reported magneto bacteria from estuarine mangrove and coral reefs environs in Gulf of Mannar. Veljo *et al.* (2002) studied the microbial composition of Northern Baltic Sea.

Studies on photosynthetic bacteria isolated from an estuarine beach of Goa were carried out by Karanth *et al.* (1977). A study on the nitrifying bacteria in Vellar estuary was conducted by Rajendran and Venugopalan (1977). Nitrogen fixing *Azetobacter* sp. in aquatic sediments has been studied by Lakshmanaperumalswamy (1987). Marine biological studies along the Indian coasts mainly on occurrence, species/generic composition and abundance of viable bacteria have been helpful in realizing the importance of hetrotrophic prokaryotes in coastal biological processes (Ramaiah, 1989,

Nair *et al.*, 1994). Bacterial abundance communities and heterotrophic activities in the coastal waters off Tamilnadu were studied by Ramaiah *et al.* (1996). Studies on marine photosynthetic bacteria from South East coast of India were carried out by Kalaichelvan and Rajkumar (1990). Distribution and ecology of methanogenic bacteria in mangrove sediments of Pitchavaram East Coast of India, were studied by Ramamurthy *et al.* (1990).

In the present study, an attempt has been made, to isolate and identify the bacteria in the sediment samples from different stations in Cochin estuary, to find the proteolytic bacteria and to select the most potent producers of alkaline protease, for further study.

2.2 Materials and methods

2.2.1 Collection of samples and isolation of bacteria

Sediment samples were collected from ten stations in Cochin estuary, using a Petersons grab. Serial dilutions of samples were prepared using 50% sterile seawater and pour plated with Zobell's agar 2216e medium. The plates were incubated at 28° C for 24 hr. The colonies that developed were subcultured onto nutrient agar slants. These cultures were then streaked on nutrient agar plates and the separated colonies were isolated in pure culture and maintained in nutrient agar slants with periodic sub culturing.

Zobell's agar 2216e medium

Peptone	5g
Yeast Extract	1.0g
Ferric phosphate	0. 02g
50 % seawater	1000 ml
pH	7.2

2.2.2 Sampling sites

Bacteria were isolated from sediment samples from 10 stations in Cochin estuary including a mangrove station (Table 2.1, Fig.2.1).

Table 2.1 Sampling sites in Cochin estuary

Station Number	Station	Location
1	Barmouth	9° 58' 26" 76° 14' 39"
2	Mattanchery	9° 56' 47" 76° 15' 52"
3	Thevara	9° 55' 35" 76° 17' 53"
4	Aroor	9° 54' 33" 76° 17' 35"
5	Bolgatti	9° 58' 52"N 76° 15' 50"E
6	Vaduthala	10° 12' 13"N 76° 15' 09"E
7	Varapuzha	10° 04' 30"N 76° 16' 48"E
8	Eloor	10° 05' 23"N 76° 15' 49"E
9	Kannamali	9° 52'07. 5"N 76° 15'47. 9"E
10	Puduvaippu (Mangrove)	9° 59' 26.1"N 76° 14' 08.4"E

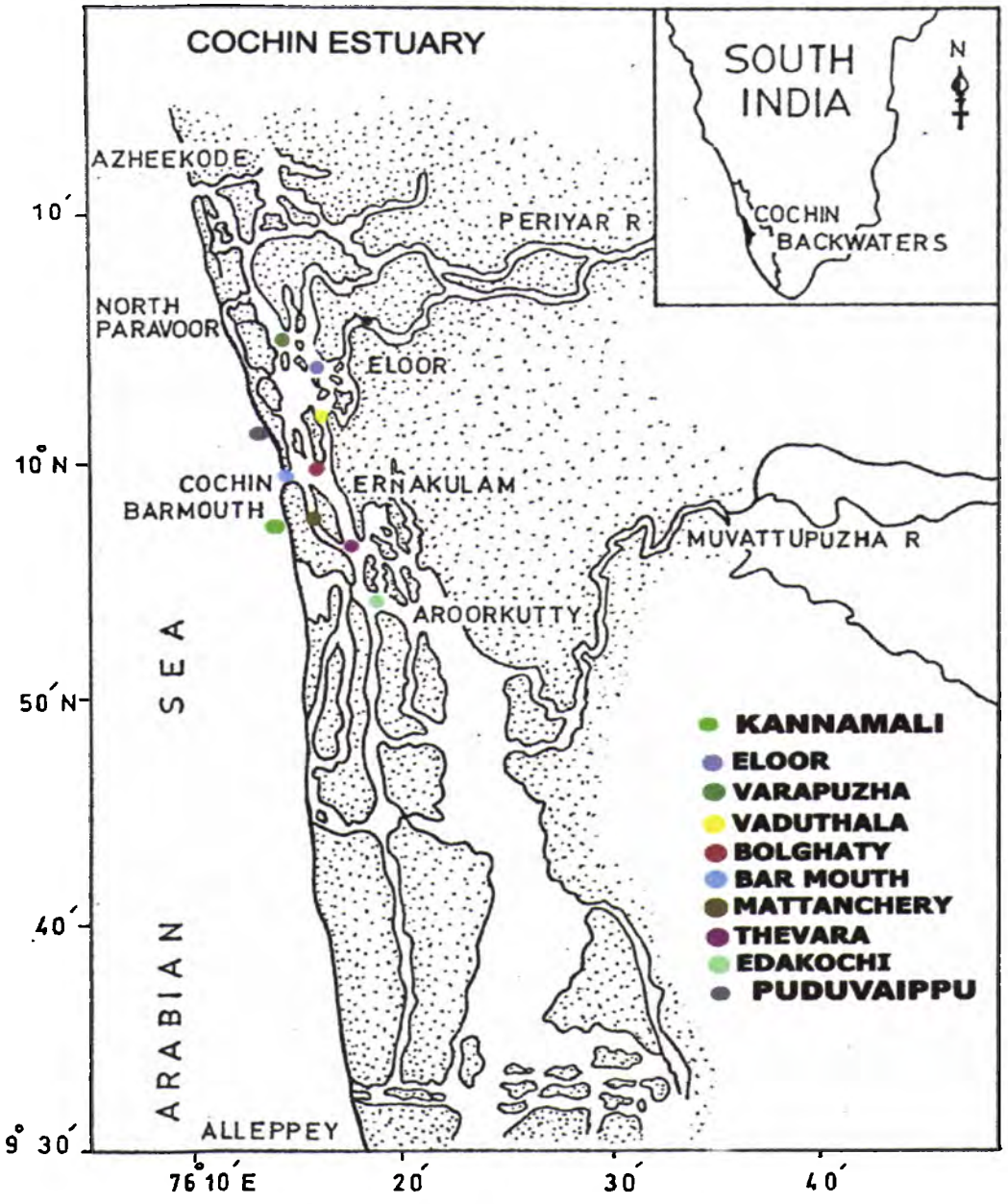


Fig.2.1 Map showing sampling locations in Cochin estuary

2.2.3 Identification of cultures

Identification of cultures was done, based on morphological, and biochemical characteristics, employing standard schemes based on Bergey's Manual of Determinative Bacteriology and Systematic Bacteriology. (Gordon *et al.*, 1973; Breed *et al.*, 1973; Kreig and Holt, 1984; Alsina and Blanch, 1994).

2.2.4 Primary screening for protease production

Screening for protease production was done using two substrates gelatin and casein. Thus, the bacteria were tested for their ability to produce both gelatinase and caseinase.

2.2.4.1 Gelatinase production

Frazier's gelatin agar medium (modified) of Harrigane and McCane (1972) of the following composition was used.

Peptone	5g
Beef extract	3g
NaCl	5g
Gelatin	10g
Agar	20g
50% Aged seawater	1000 ml
pH	7.2

Gelatin agar plates were spot inoculated with the cultures to be tested and incubated at 28°C for 24 hr. After incubation, the plates were overlaid with 15% $HgCl_2$ in Con. HCl. ($HgCl_2$-15 g, Con. HCl – 20 ml, distilled water-100 ml). Clear transparent zones around the colony indicated gelatinase production.

2.2.4.2 Caseinase production

Casein agar medium of Harrigane and McCane (1972) of the following composition was used.

Basal medium

Peptone	10g
Beef extract	3g
Agar	20g
50% Aged seawater	750 ml
pH	7.2

10g casein in 250 ml distilled water was sterilized separately and mixed with the basal medium just before pouring into plates. After incubation at 28°C for 24 hr. plates were overlaid with 15% $HgCl_2$ in Con. HCl. Clear transparent zones around the colony indicated caseinase production.

2.2.5 Secondary screening for potent strains producing alkaline protease

Thirty isolates which had shown the potential to produce both gelatinase and caseinase in the primary screening were selected based on the diameter of the zone of clearance, and further screened to select the most potent strains from among them. This was done by the quantitative assay in liquid medium.

2.2.5.1 Inoculum preparation

The selected cultures were inoculated onto nutrient agar slants and incubated for 24 hr., and after incubation, the cells were harvested into sterile saline. A volume adequate to obtain an absorbance of 0.02 at 600 nm for the total medium was added to 50 ml nutrient broth in 250 ml conical flask and incubated at 28°C on a rotary shaker at 100 rpm. This was treated as the absorbance at 0 hr of incubation.

2.2.5.2 Measurement of growth

Bacterial growth was determined by measuring the absorbance of the culture spectrophotometrically at 600nm and was expressed in units of absorbance (Abs).

2.2.5.3 Preparation of crude enzyme

After incubation, the samples were drawn from the 50 ml culture to determine growth, and the remaining culture broth was centrifuged at 10000 rpm for 15 minutes at 4°C to remove the cells. The cell free supernatant obtained contained the enzyme. This was assayed for protease activity.

2.2.5.4 Assay of alkaline protease

All the thirty potent strains selected after the primary screening were used for quantitative assay. The assay was performed at alkaline pH, to detect the alkaline proteases. Protease production was assayed in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Protease assay was done by a modification of the casein digestion method of Kunitz (1947).

To 3ml of 0.6% casein in an appropriate buffer of alkaline pH (phosphate buffer for pH 7-8, Glycine-NaOH buffer for pH 9-10), 0.5 ml of suitably diluted crude enzyme was added and incubated for 30 minutes at 37°C after which 3ml of 5% trichloroacetic acid was added to stop the reaction and allowed to stand for 15 minutes at room temperature and the resultant mixture was filtered through Whatman no.1 filter paper. The absorbance of this filtrate was measured at 280 nm in a Hitachi 2000-20 UV-Visible spectrophotometer. A suitable control was run simultaneously, in which TCA was added prior to the addition of enzyme solution. One unit of proteolytic activity was defined as that amount of enzyme, which liberated 1 µg of tyrosine per ml per minute under the specific conditions of assay. The absorbance at 280 nm (test – control) indicated the tyrosine content of the filtrate, which has been released by the hydrolysis of the protein substrate by the enzyme. The tyrosine content of the sample was read from the standard calibration curve prepared with pure tyrosine.

2.3 Results

A total of 251 bacteria were isolated from the sediment samples collected from Cochin estuary. Of this, 130 were gram-negative rods, 105 isolates were gram-positive rods and 16 gram positive cocci. *Bacillus* was clearly the single largest genus, which comprised of 70 isolates (28% of the total). The next abundant genus was *Vibrio* (18%) followed by Enterobacteriaceae group (11%)(Appendix1.1).

In most of the stations an abundance of *Bacillus* sp. was noted, followed by *Vibrio* sp. In Eloor, *Bacillus* predominated with 55% of the total isolates, followed by *Vibrio*. Other genera were *Pseudomonas, Arthrobacter, Acinetobacter, Micrococcus* and *Staphylococcus*. Samples from Kannamali station showed abundance of *Bacillus* (35%) and *Vibrio. Pseudomonas, Micrococcus, Brevibacterium, Arthrobacter* and *Flavobacterium* were the other genera isolated. *Bacillus* comprised of 40% of the total number of bacteria isolated from Varappuzha station. *Vibrio*, Enterobacteriacea group, *Aeromonas, Bordetella* and *Acinetobacter, Flavobacterium, Alcalignes and Achromobacter* were isolated from this station. Vaduthala had 55% of its population as *Bacillus. Vibrio, Aeromonas, Pseudomonas, Flavobacterium, Chromobacterium and Bordetella* were the other genera found. Samples from Bolgatty station had *Bacillus* (31%) and *Pseudomonas* as the predominant genera. *Vibrio, Enterobacteriaceae, Staphylococcus* and *Aerobacter* were also found here. *Vibrio* was the abundant genus with 35% of the totally isolated strains from the Barmouth station. *Bacillus,* Enterobacteriaceae, *Pseudomonas, Aerococcus, Bordetella* and *Alcaligenes* were the other genera found here. Mattanchery station had 55% *Bacillus;* Enterobactreiaceae group, *Pseudomonas, Acinetobacter, Chromobacterium, Flavobacterium* and *Arthrobacter* were the other genera found. In the samples from Thevara, *Bacillus, Vibrio,* Enterobacteriaceae, *Pseudomonas, Alcaligenes,* and *Aerobacter* were isolated. The predominant genus in Edakochi station was *Vibrio. Bacillus, Aeromonas, Pseudomonas, Staphylococcus, Arthrobacter, Aerococcus and Achromobacter* were the other genera found. The mangrove station, Puduvaippu, had an abundance of *Bacillus* (25%). Other genera were *Vibrio, Pseudomonas, Brevibacterium, Alcaligenes, Chromobacterium, Bordetella, Achromobacter, Staphylococcus*, Enterobacteriaceae group and *Micrococcus*.

Of the 250 isolates obtained, 182 (73%) were found to be gelatinolytic and 73 (35%) caseinolytic. The percentage of proteolytic forms varied among

the genera. The generic composition and the relative abundance of gelatinase and caseinase producing strains are given in Fig. 2.2 and Fig.2.3.

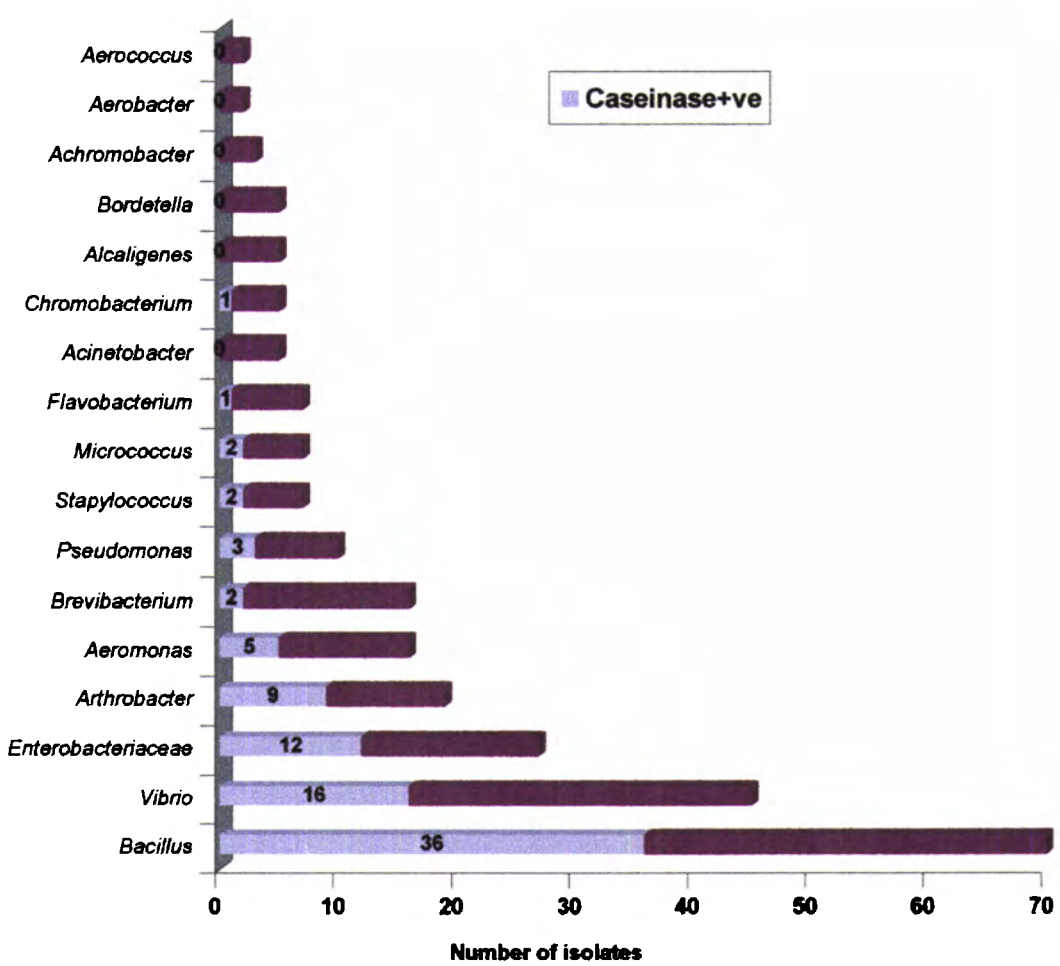


Fig. 2.2 Generic distribution of isolates and caseinase producing forms

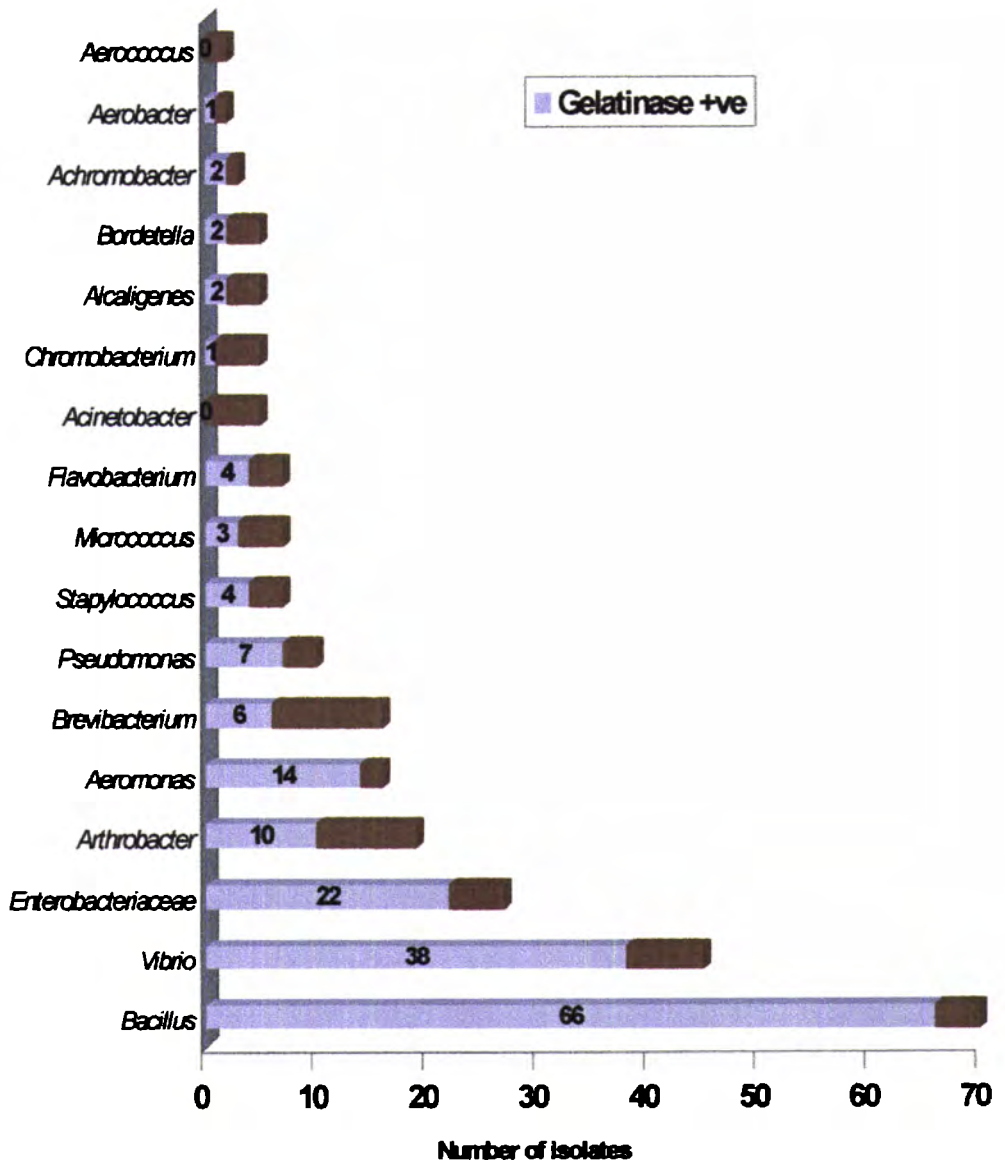
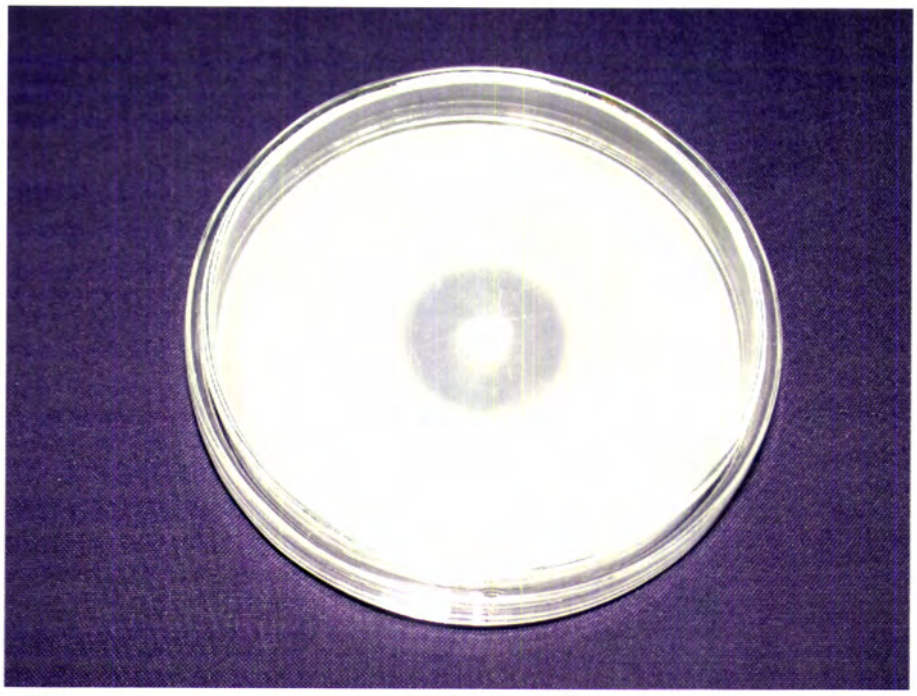


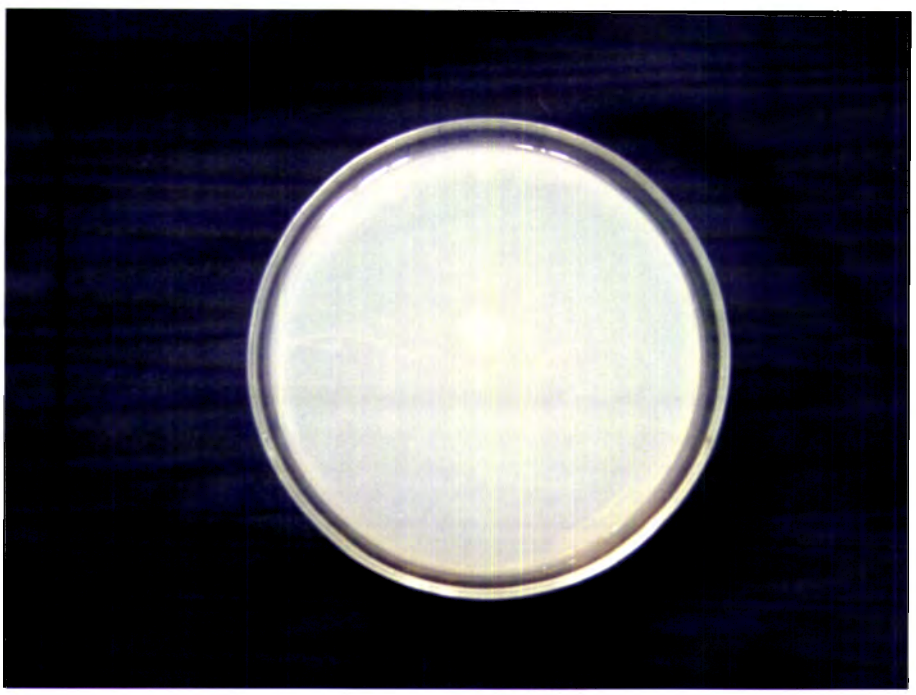
Fig. 2.3 Generic distribution of isolates and gelatinase producing forms

After the primary screening, 30 strains were selected for secondary screening which were positive for both gelatinase and caseinase production. Finally three strains producing alkaline protease were selected after secondary screening, and were subjected to further study. Gelatin and casein hydrolysis are shown in Plates 1 and 2.

Out of the three strains selected one belonged to the genus *Bacillus* and two to the genus *Vibrio*. The selected strains were identified up to species level by further biochemical tests.



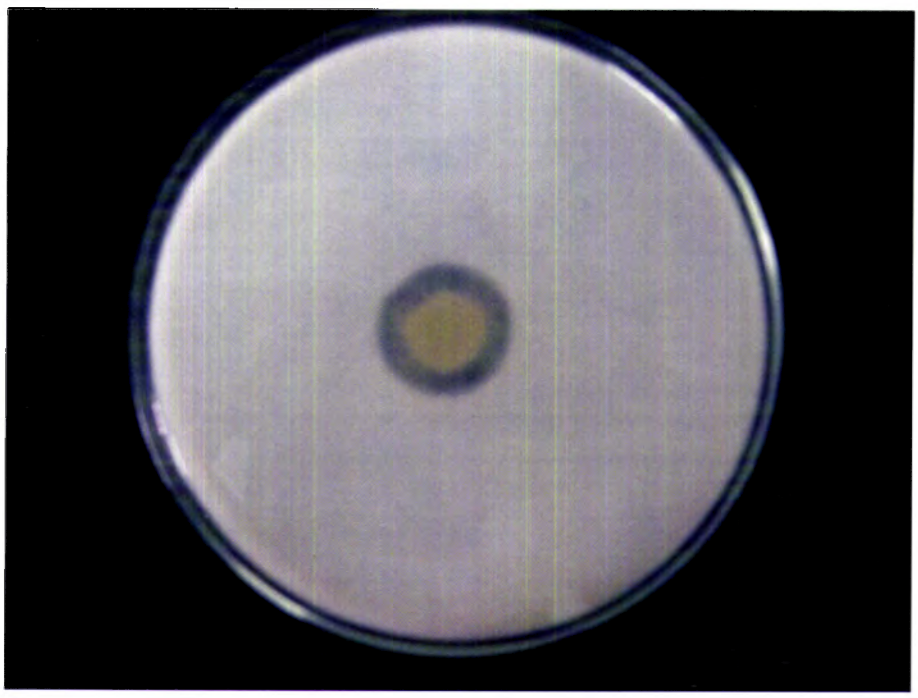
Gelatinase Positive Culture



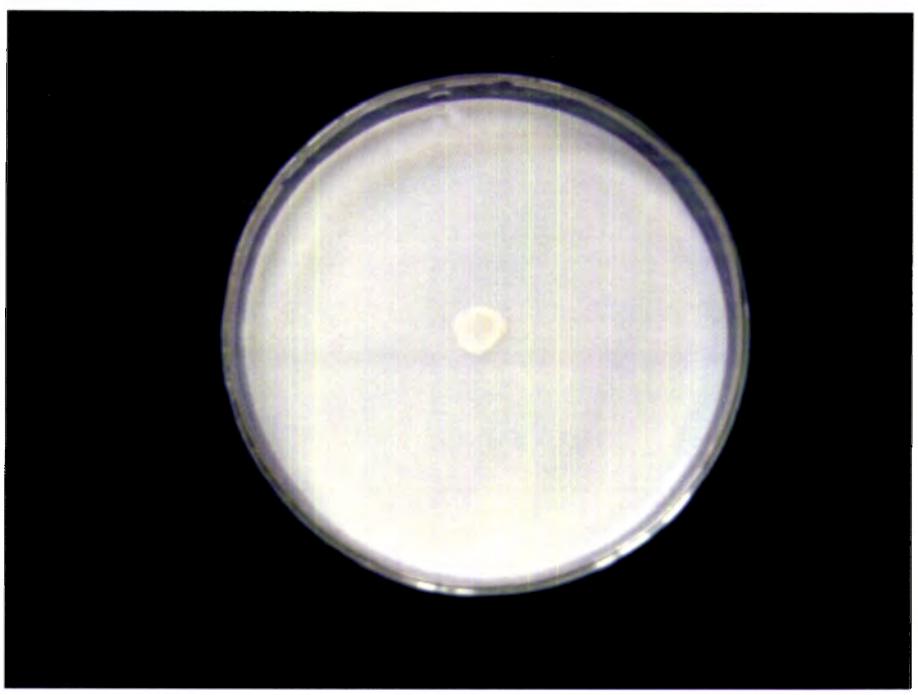
Gelatinase Negative Culture

Screening for gelatinase producing bacteria

PLATE 1



Caseinase Positive Culture



Caseinase Negative Culture

Screening for caseinase producing bacteria

PLATE 2

They were identified as

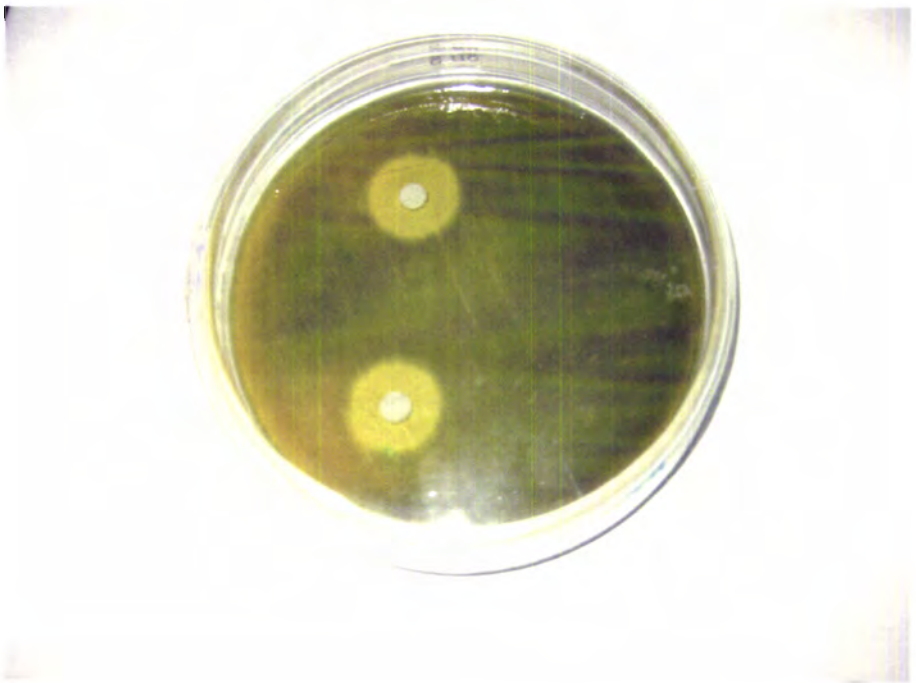
1. *Bacillus circulans* (B15)
2. *Vibrio fluvialis* (V10)
3. *Vibrio* sp. (V26)

The gram stained preparations of the selected strains are shown in plate 3. The biochemical characters of the selected strains are given in table 2.2 and 2.3. The sensitivity to Vibriostatic compound O/129 is shown in plate 4.

Table 2.2. Morphological and Biochemical characteristics of B15

Culture	B15
Gram reaction	+
Spore	+, Oval
Motility	+
Catalase	+
O/F	+/-
MR	-
VP	-
pH in VP broth	5.9
Indole	-
Citrate	-
Amylase	+
Phosphatase	+
Mannitol utilization	-
Arabinose utilization	-
Gas from glucose	-
Growth in 0% NaCl	+
Growth in 5 % NaCl	+
Growth in 6% NaCl	+
Growth in 7% NaCl	-
Growth in 8% NaCl	-
Growth in 10% NaCl	-
Growth at 65°C	-
Growth at 0°C	-
NO_3 reduction	+
Oxidase	+
Phenylalanine deaminase	-
Identified as	*Bacillus circulans*

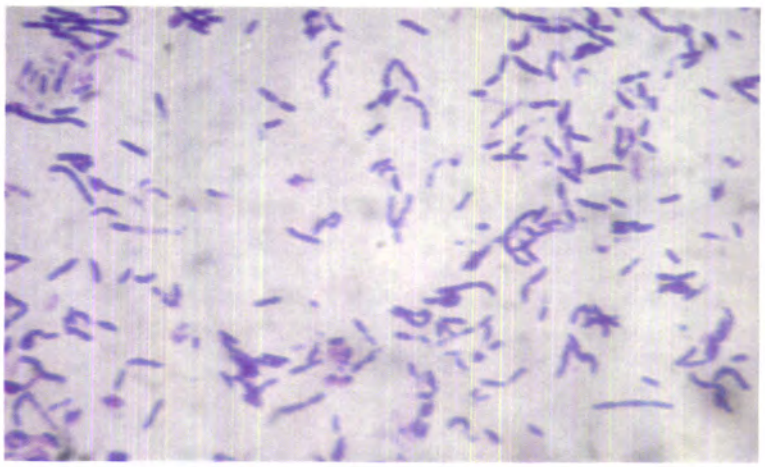
Vibrio fluvialis



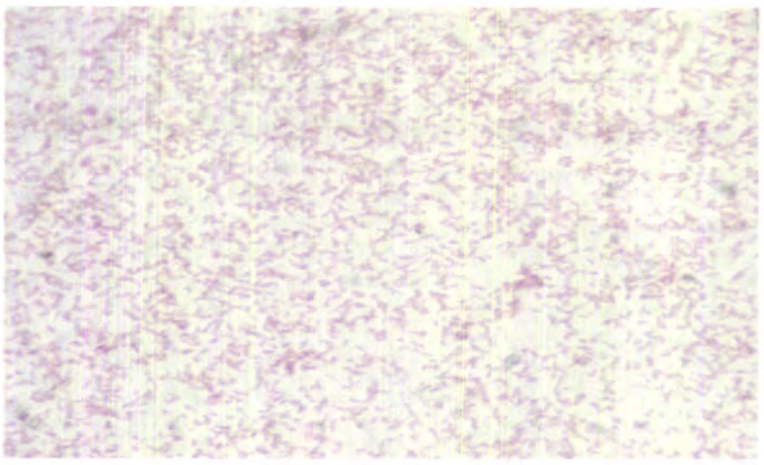
Vibrio sp.

Sensitivity of two strains of *Vibrio* to Vibriostatic compound O/129, on TCBS plates

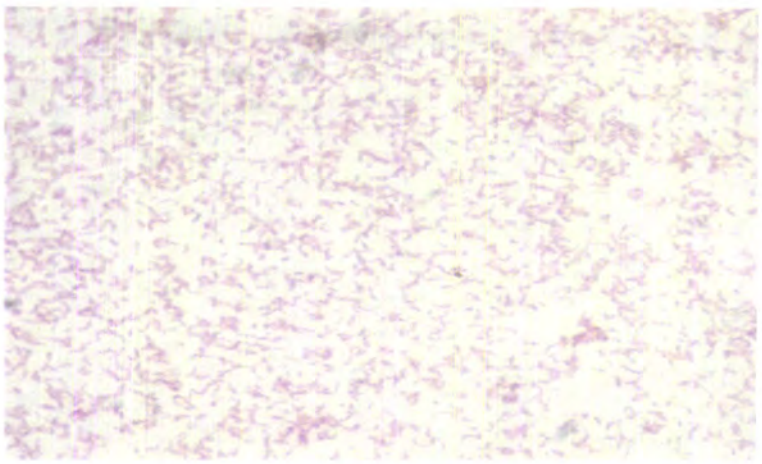
PLATE 4



Bacillus circulans



Vibrio fluvialis



Vibrio sp.

Gram stained preparations of selected bacterial strains

PLATE 3

Table 2.3 Morphological and Biochemical characteristics of V10 and V26

Cultures	V 10	V 26
Gram reaction	-	-
Motility	+	+
Oxidase	+	+
O/F	+/+	+/+
Growth on TCBS Agar	+ Yellow colony	+ Yellow colony, become green afterwards
Arginine dihydrolase	+	-
Lysine decarboxylase	-	+
Ornithine decarboxylse	-	+
Indole	-	+
Citrate	+	+
MR	+	+
VP	-	+
Amylase	+	-
Phosphatase	+	+
Mannitol utilization	+	+
Arabinose utilization	+	-
Gas from glucose	-	-
Growth in 0% NaCl	+	+
Growth in 6% NaCl	+	+
Growth at 0°C	-	-
NO_3 reduction	+	+
ONPG	-	+
Resistance to O/129 (150 µg)	+	
Catalase	+	+
Phenylalanine deaminase	-	-
Identified as	*Vibrio fluvialis*	*Vibrio* sp.*

* Could not be assigned to any known *Vibrio* sp. based on these tests.

2.4 Discussion

Biogeochemical turnover in the aquatic environment is mainly due to the metabolism of microbial population and this is performed through aerobic and anaerobic decomposition by which microbial cells are supplied with energy (Rajendran and Venugopalan, 1977). The sediment microflora play an important role in biodegradation of organic compounds and maintaining the ecological balance of the system. In the present study, microorganisms

are found distributed widely in aquatic environments. Both gram-negative bacteria and gram-positive bacteria were found in almost equal proportions. i.e. 52% and 48% respectively. But in some of the earlier works on the bacterial population of water and sediment samples from Cochin estuary, gram-negative forms were reported to show predominance over gram-positive forms (Philip, 1987; Chandrika and Ramachandran, 1994; Gopinath, 2002). The variation in the present observation could be due to the fact that in the present study only the sediment samples were analysed, which accounts for the occurrence of almost equal proportion of gram positive and gram-negative bacteria. As per the observation by Ramamoorthy and Jayabalan (1982) the most important heterotrophic genera present in estuaries were *Alcaligenes, Vibrio, Pseudomonas, Aeromonas, Flavobacterium* and *Micrococcus. Bacillus* was found mainly in the surface sediments. Philip (1987) found *Vibrio* and *Pseudomonas* to be the predominant genera in Cochin estuary. The abundance of *Bacillus* was also cited. According to Gopinath (2002), the predominant genera were *Pseudomonas, Vibrio, Bacillus* and *Staphylococcus.* The predominant genus in the present observation was *Bacillus*, followed by *Vibrio. Bacillus* being a spore former can survive better in the sediment sample, when compared to non-spore forming bacteria. Amar (2001) obtained similar result, while studying the composition of microflora from Cochin estuary, where *Bacillus* was the dominant species, followed by *Coryneformes, Vibrios, Streptococcus, Pseudomonas* and *Acinetobacter.*

Veljo *et al.* (2002) reported that the same group of bacterial species dominated independently of the season investigated, based on his study conducted in Northern Baltic Sea. But according to Philip (1987), there was seasonal variation in the generic distribution as well as the occurrence of proteolytic forms in Cochin estuary.

In the present study regarding the proteolytic potential of the strains, 73% of the total isolates were gelatinase positive and 35% were caseinase

positive. It was to be noted that 94% of *Bacillus* strains isolated were gelatinolytic and 51% caseinolytic. Of the 30 strains selected after primary screening 11 were *Bacillus*, 10 *Vibrio*, 3 *Pseudomonas*, 3 *Micrococci*, and 1 belonging to Enterobacteriaceae group. In an attempt to understand the ecological implication of the extracellular protein degradation promoted by estuarine bacteria, Sizemore *et al.* (1973) studied the distribution and activity of the proteolytic bacteria in the sediments from the North Inlet Estuary near Georgetown, S. Carolina as well as the effects of various parameters on protein hydrolysis by bacteria and observed that 56% of the isolates obtained from the sediment exhibited caseinolytic activity. While studying the physiological characteristics of 649 bacteria isolated from Long Island Sound water column, Murchelano and Brown (1970) observed that 63.9% were proteolytic. They indicated that although the generic composition of the bacterial flora changed with season there was no significant variation in the number of proteolytic, lipolytic and amylolytic flora. Reinheimer (1972) noted that most of the isolates from Arabian Sea of Northern Gulf of Oman were strongly proteolytic. Boeyo *et al.* (1975) reported that proteolytic forms were widely encountered in North Sea sediments. Observations on the microbial proteolysis in Lake Champlain revealed that the proteolysis was very much dependent upon water temperature. *Pseudomonas* and *Flavobacterium* were found to be the predominant proteolytic flora (Little *et al.*, 1979). Observations on proteolytic and lipolytic bacterial counts in the sediments of marine environment of New York Bight Ape, Sandy Hook Bay and, Great Bay of New Jersy showed that proteolytic counts were 2-4 times higher in the polluted areas (Nitkowski *et al.*, 1977).

2.4.1 The selected strains

The strains selected for detailed study are *Bacillus circulans, Vibrio fluvialis* and *Vibrio* sp. As widely reported, *Bacillus* is the most commercially exploited bacterial species for protease production (Gupta *et al.*, 2002b) and

they are considered as appropriate producers for commercial exploitation, being non toxic and non pathogenic and thus safe to use.

The genus *Vibrio* contains a number of species of marine origin (Baumann *et al.*, 1980). The production of extracellular enzymes is common among marine members of this genus. But as yet enzyme secretion has been studied in detail in only very few marine species such as *V. alginolyticus* (Reid *et al.*, 1978, 1980) and *V.gazogenes* (Ratcliffe *et al.*, 1982). Although it is known that many *Vibrio* sp. in particular *Vibrio cholerae* O1 can be responsible for disease state in humans, only a few *Vibrio* spp. have been investigated in clinical and food microbiology. These species include *Vibrio paramaemolyticus, Vibrio cholerae and Vibrio vulnificus*, which are known to be aetiological agents in several gastrointestinal pathologies linked to consumption of contaminated water and sea food products (Piersimoni *et al.*, 1991; Chakraborthy *et al.*, 1997; Jackson *et al.*, 1997; Arias *et al.*, 1999; Wright *et al.* 1999; Strom and Paranjpye 2000). However, in recent years the interest of microbiologists has turned to other halophilic *Vibrios* also. *Vibrio fluvialis*, which is one among the autochthonous flora of aquatic ecosystems, has only rarely been associated with pathologies in humans and even then have not been identified as the cause of these pathologies (Baffone *et al.*, 2001).

2.4.2 Mangroves - a unique ecological niche to be conserved

One thing worth mentioning based on the present observation is the abundance of proteolytic forms obtained from the mangrove samples. 90% of the bacterial isolates from mangrove samples were producing gelatinase and 70% were positive for caseinase production. The abundance of *Bacillus* sp. also was noticed among the mangrove isolates. Also, there was a predominance of gram-positive forms over gram-negative forms. Of the 30 strains selected after primary screening 50% were mangrove isolates. The three strains finally selected after the two screening steps for the maximum production of alkaline protease were also from the mangroves. This shows

that there is definitely some enhancing factor for the proteolytic forms, in the sediment samples of mangroves. The distribution of bacteria in sediments depends on several factors. The organic content of the sediment determines the abundance of heterotrophic bacteria, to a great extent. (Kuznetsov, 1968). Nevertheless, the role of other physical and chemical characteristics of the sediment as well as the surrounding environment influencing the bacterial proliferation and characterization of physiological groups cannot be underestimated (Sugahara *et al.*, 1974). In this case we can see that all these factors are influential, as the mangrove sediment is rich in organic matter with the contribution from its indigenous flora and fauna. The literature regarding the proteolytic bacteria from mangroves is scanty.

Mangrove ecosystem plays a key role in the nutrient and metal cycling (Harbison, 1981; Lacerda and Abrao *et al.*, 1984) and it acts as a buffer between transitional near shore and lagoonal / estuarine environments with respect to their influence on freshwater discharge, salinity regime and the adjacent aquatic systems in general. The unique flora of mangrove with their specialized ecological characters afford suitable atmosphere for a set of peculiar flora and fauna- both living in their own highly specialized ecosystem. Man in his craving for supremacy over nature, has failed to understand the intricate 'ecological niche' of mangroves and started replacing the natural vegetation in the name of agriculture and industrial developments. The result is almost a total annihilation of the vast extent of mangrove vegetation of the Kerala coasts.

The mangrove where the present study was conducted is Puduvaippu, which is the biggest mangrove area on the Kerala coast. This is at the southern tip of Vypeen Island, located at the North -Western bank of Cochin bar-mouth facing the Arabian Sea. The land is regularly inundated by a semi diurnal tidal rhythm of Cochin bar mouth. This is a mangrove ecosystem, which has escaped the destruction from outside anthropic factors, and if left

undisturbed, the whole area will undoubtedly develop into a good mangrove forest (Basha, 1991).

The present study showed that proteolytic bacteria are widely distributed in the sediment samples of Cochin estuary, and *Bacillus* clearly showed abundance both in distribution and protease production, followed by *Vibrio* sp. The three strains selected for further study also belonged to *Bacillus* and *Vibrio* sp. The mangrove area was a potential habitat that is worth further exploration.

CHAPTER 3

EFFECT OF CULTURE CONDITIONS ON GROWTH AND PRODUCTION OF ALKALINE PROTEASES

Microorganisms show considerable variation with respect to their nutritional requirements for optimum growth and enzyme production. Very often the optimal conditions for growth may not be optimal for product formation. Their growth rate and metabolism depend very much on the composition of the medium and the prevalent physical and chemical factors. By understanding the specific requirements of a microbial species, it is possible to establish conditions *in vitro* to support the optimal growth and enzyme production of that organism.

In commercial practice, medium composition is optimized continuously over the years so that the balance between the various components is maintained, thus maximizing productivity of microorganisms and minimizing the amount of unutilized components at the end of fermentations. This balance is highly dependent on the interaction between the particular strain of microorganism, the type of medium components and the process conditions. 30-40% of the production cost of industrial enzymes is estimated to be accounted for by the cost of growth medium (Joo *et al*, 2002).

Alkaline proteases are generally produced by submerged fermentation. In addition, solid-state fermentation process has been exploited to a lesser extent for production of these enzymes (Malathy and Chakraborthy, 1991;Chakraborthy and Srinivasan, 1993; George *et al*., 1995). With a view to developing an economically feasible technology, research efforts are mainly focused on the improvement in the yield of alkaline proteases and optimization of the fermentation medium and production conditions. For this, the parameters commonly standardized include (i) evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes. (ii) requirement of divalent metal ions in the medium; and (iii) optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation (Kumar and Takagi, 1999).

No defined medium has been established for the best production of alkaline protease from different microbial sources. Each organism has its own special conditions for the optimum production. In the present study, an

effort has been made to optimize the culture conditions for the maximum production of alkaline proteases from the three strains isolated from a mangrove area in Cochin estuary.

3.1 Review of literature

Numerous reports are available regarding protease production in *Bacillus*. Despite the long list of protease producing microorganisms only a few are considered as appropriate producers for commercial exploitation being 'generally regarded as safe' (GRAS), non toxic and non pathogenic. Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environments have been explored for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. megaterium, B. licheniformis, B. subtilis, B. amyloliquifaciens and B. mojavensis*. (Millet *et al.*, 1969; Kalisz, 1988; Manachini and Fortina, 1998; Rao *et al.*, 1998; Kumar and Takagi, 1999; Yang *et al.*, 2000; Gupta *et al.*, 2002b). Effect of glucose and amino acids on protease synthesis in *Bacillus megaterium* was reported by Votruba *et al.*, (1987). Aderibigbe *et al.* (1990) studied the variability in extracellular protease production among the strains of *B.subtilis* group. The culture conditions were optimized for protease production by *Bacillus acidophilus* strain (Giesecke *et al*, 1991). Protease production in continuous suspension cultures of *Bacillus firmus* was reported by Moon and Parulekar (1993). The protease production conditions were optimized for a *Bacillus* sp. by Atalo and Gashe (1993). Kembhavi *et al.* (1993) reported the production of a thermostable alkaline protease from a *Bacillus subtilis* strain. Production of a thermo stable alkaline protease by a halotolerant strain of *Bacillus licheniformis* was reported by Manachini and Fortina (1998). Optimal production of *Bacillus* alkaline protease using a cheese whey medium was reported by Kumar *et al.* (1999). Sumandeep *et al.*, (1999) isolated an alkalophilic *Bacillus* sp. from soil, which produced a thermostable alkaline protease. Madan *et al* (2000) optimized the culture conditions for protease production by a UV- mutant of *Bacillus polymyxa*.

Optimization of the production of protease from *Bacillus horikoshii* was reported by Joo *et al.* (2002).

Studies on the effects of cations on protease production of marine bacteria were conducted by Sakata *et al.*, (1977). Another study of proteases of marine origin was by Sashihara *et al.* (1975) Protease of a *Clostridium botulinum* strain was studied by Nakane (1978). The effects of culture conditions were optimized for protease production were studied in *Pseudomonas maltophila* strain by Kobayashi *et al.* (1985). Effect of different culture conditions on protease production by *Butyrivibrio fibrisolvens* was studied by Cotta and Hespell (1986). The role of casein on protease production by lactobacilli was studied by Sakellaris and Gikas (1991). The effect of glucose on *Thermoactinomyces* sp. was reported by Sunitha *et al.* (1999). The effect of divalent cations on *Pseudomonas fluorescens* protease production was studied by Liao and Mc Callus (1998). Another bacterial source known as potential producer is *Pseudomonas* sp. (Ogino *et al.,* 1999; Bayoudh *et al.*, 2000).

Though scarce, reports on protease production in *Vibrio* species are also available. Hare *et al.* (1981) studied the role of temperature and oxygen in the regulation of exoprotease production in *Vibrio alginolyticus.* Another study in *V. alginolyticus* was by Long *et al.* (1981) regarding the regulation of production of extracellular alkaline protease. The effect of growth media and temperature on a dairy strain of *Aeromonas hydrophila* was reported by Santos *et al.*, (1996). The protease production aspects of an alklophilic bacteria isolated from an alkaline soda lake were reported (Gessesse and Gashe, 1997). Effect of culture conditions on production of an extracellular protease from the fish pathogen, *Yersinis ruckeri* was studied by Secades and Guijarro (1999).

There are a number of reports on alkaline proteases from fungi. The production of an alkaline serine protease from a mold, *Monascus*, which is a close relative of *Aspergillus* and *Penicillium,* was reported (Aso *et al.*, 1989). A comparison between two *Arthrobotrys* sp. regarding the nematode

degrading ability on the basis of protease production has been studied (Bedelu *et al.*, 1998). Direct determination of the proteolytic activity and the inhibition profiles from the culture itself without the need of extraction or purification had been reported in a plant pathogenic fungus, *Fusarium sp.* (Girard and Michaud, 2002).

Ordas *et al.* (2001) studied proteolytic activity of a protozoan *Pseudoperkinsus tapetis*. The protease from an oyster protozoan parasite *Perkinsus marinus* was also reported (MacIntyre *et al.*, 2003).

3.2 Materials And Methods

3.2.1 Selected strains

Three bacterial strains isolated from a mangrove area in Cochin estuary, which were potent producers of alkaline proteases were selected for further study. They were

B15	*Bacillus circulans*
V10	*Vibrio fluvialis*
V26	*Vibrio* sp.

3.2.2 Growth media

Nutrient broth supplemented with 0.2% gelatin and a mineral medium containing artificial seawater base (ASW) of Mac Leod (1968) and 0.2% sucrose as carbon source were used for the optimization studies.

a. Nutrient broth supplemented with gelatin

Peptone	5 g
Beef extract	3 g
NaCl	15 g
Water	1000 ml
pH	7.2

b. Mineral Medium

Composition of Artificial Seawater Base (ASW)

NaCl	23.4 g l^{-1}
$MgSO_4.7H_2O$	24 g l^{-1}
KCl	1.5 g l^{-1}
$CaCl_2.2H_2O$	2.9 g l^{-1}

Salts were dissolved separately and combined

Basal medium

Tris hydroxymethyl-aminomethane	
(Adjusted to pH 7.5 with HCl)	6.1 g l^{-1}
NH_4Cl	1.0 g l^{-1}
$K_2HPO_4.3H_2O$	75 mg l^{-1}
$FeSO_4.7H_2O$	28 mg l^{-1}
Sucrose	2 g l^{-1}
Yeast extract	10 g l^{-1}

Basal medium was mixed with half strength ASW

3.2.3 Inoculum preparation

Inoculum preparation was done as described in section 2.2.5.1

3.2.4 Measurement of growth

Growth was measured as described in section 2.2.5.2

3.2.5 Preparation of crude enzyme

Crude enzyme was prepared as described in section 2.2.5.3

3.2.6 Assay of alkaline protease

Alkaline protease was assayed as described in section 2.2.5.4

3.2.7 Effect of different physico chemical parameters on growth and alkaline protease production

Optimal conditions required for maximum protease production by the three test strains were determined by subjecting them to different levels of agitation, various incubation temperatures, periods of incubation, pH, NaCl concentrations, salts, carbon sources and nitrogen sources.

3.2.7.1 Effect of agitation speed on growth and protease production

The effect of aeration on the growth and protease production was determined by growing the strains, in nutrient broth supplemented with gelatin and incubating them at different rpm (0, 50,100,150 and 200) in a rotary shaker. Growth and enzyme production were determined after 24 hr. of incubation.

3.2.7.2 Effect of period of incubation on growth and protease production

The optimum period of incubation required for maximum growth and enzyme production was determined by inoculating the cultures in nutrient broth supplemented with gelatin. Growth and enzyme production were monitored at different time intervals. (2, 4, 6, 8,10,12,14, 24, 36, 60 and 72 hr).

3.2.7.3 Effect of pH on growth and protease production

The effect of pH on growth and enzyme production was studied by inoculating the organisms in nutrient broth supplemented with gelatin, having varying pH (pH 4,5,6,7,8,9 and 10). The cultures were incubated at 28°C for 24 hr. and growth and enzyme production were measured.

3.2.7.4 Effect of temperature on growth and protease production

Effect of temperature on growth and enzyme production was determined by inoculating the cultures in nutrient broth supplemented with gelatin and incubating them at different temperatures (15°C, 20°C, 25°C,

30°C, 35°C and 40°C). Growth and enzyme production were determined after 24 hr. of incubation.

3.2.7.5 Effect of NaCl concentration on growth and protease production

The effect of NaCl concentration on growth and enzyme production was studied by inoculating the organisms in nutrient broth supplemented with gelatin having varying NaCl concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5%). The cultures were incubated at 28°C for 24 hr. and growth and enzyme production were measured.

3.2.7.6 Effect of metallic salts on growth and protease production

The effect of various ions on growth and enzyme production was studied by adding individually varying concentrations of the salts, $CoCl_2$, $CaCl_2$, $MnSO_4$, $MgSO_4$, KH_2PO_4, and $Fe(PO4)_3$ as source of ions to nutrient broth and inoculating the media with the cultures. Growth and enzyme production were detected after 24 hr. of incubation at 28°C.

3.2.7.7 Effect of carbon sources on growth and protease production

Effect of carbon sources on growth and enzyme production was determined by inoculating the cultures in mineral medium containing different concentrations (0.1, 0.2, 0.4,and 0.6%) of glucose, mannose, maltose and sucrose, and different concentrations (0.05, 0.1, 0.2 and 0.4%) of molasses, as carbon sources. Growth and enzyme production were determined after incubation of 24 hr. at 28°C.

3.2.7.8 Effect of nitrogen sources on growth and protease production

Effect of nitrogen sources on growth and enzyme production was determined by inoculating the cultures in mineral medium containing one of the nitrogen sources at 1% concentration. The organic nitrogen sources used were beef extract, yeast extract, peptone, gelatin, casein, and tryptone, and the inorganic nitrogen sources were NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$ and urea. Growth and enzyme production were determined after incubation of 24 hr. at 28°C.

3.3 Statistical analysis

The data obtained were subjected to two factor ANOVA (Analysis of Variance) with replication (for the effect of carbon sources), and Single factor ANOVA (for all the other parameters and the Least Significant Difference (LSD) was calculated in each case.

3.4 Results

Results of the study are shown as graphs and tables. The data are given as Appendix 2.

3.4.1 Effect of shaking speed on growth and protease production

B15

B. circulans showed considerable increase in enzyme production when the culture was agitated than when it was kept stationary. The production was maximal at a range of 50 to 150 rpm. At 200 rpm, production was significantly lowered. Growth was not much affected by shaking speed. However, there was decrease in growth rate in stationary condition (Fig. 3.1, Table 3.1).

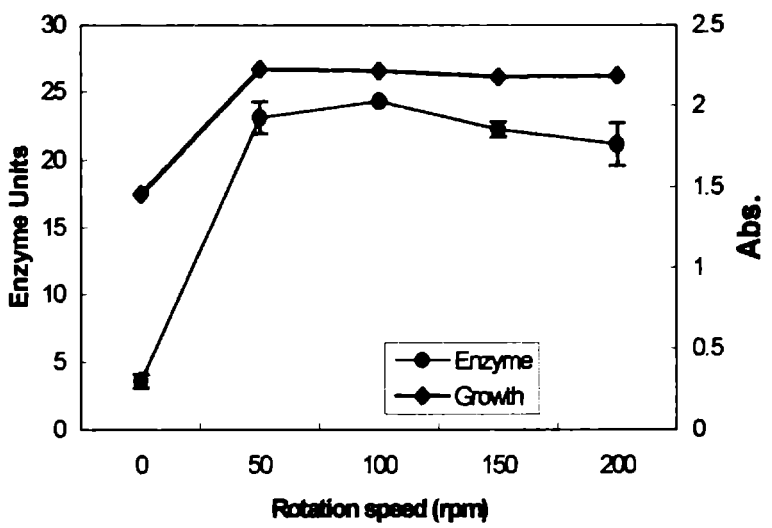


Fig. 3.1 Effect of rotation speed on the growth and protease production by *B. circulans*

Table 3.1 ANOVA for the effect of rotation speed on protease production of *B. circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	Significance
Between Groups	891.1351	4	222.7838	252.026	<0.001
Within Groups	8.839711	10	0.883971		
Total	899.9748	14			

LSD= 2.285

Analysis of Variance showed that shaking speed significantly influenced protease production. At shaking speeds between 50, 100 and 150 rpm there was no significant difference in protease production and production was maximum.

V10

V. fluvialis exhibited a significant increase in enzyme production when the culture was agitated than when it was kept stationary without shaking. The production was maximal in a range of 50 to 150 rpm. In this case also at 200 rpm enzyme production was significantly lowered. Growth was not much affected by shaking speed. But, there was decrease in growth rate under stationary condition (Fig. 3.2, Table 3.2).

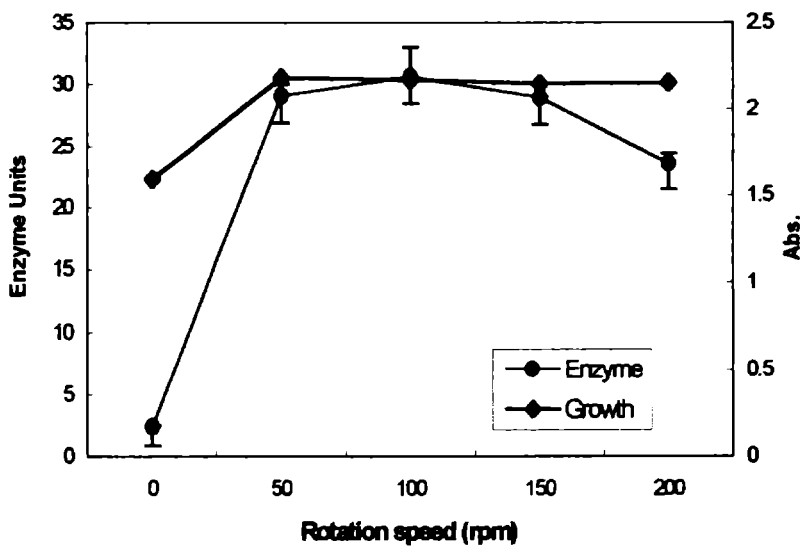


Fig. 3.2 Effect of rotation speed on the growth and protease production by *V. fluvialis*

Table 3.2 ANOVA for the effect of rotation speed on protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	1655.006	4	413.7516	292.415	<0.001
Within Groups	14.14946	10	1.414946		
Total	1669.156	14			

LSD = 2.891

Analysis of Variance showed that the shaking speed significantly influenced protease production by V10. At shaking speeds between 50, 100 and 150 rpm there was no significant difference in protease production and production was maximum.

V26

In the case of V26 also there was significant increase in enzyme production when the culture was agitated than when it was kept stationary without shaking. The production was maximal at a range of 50 to 150 rpm. At 200 rpm production was significantly lowered. Growth was not much affected by shaking speed. Nevertheless, growth rate was low in stationary condition (Fig. 3.3, Table 3.3).

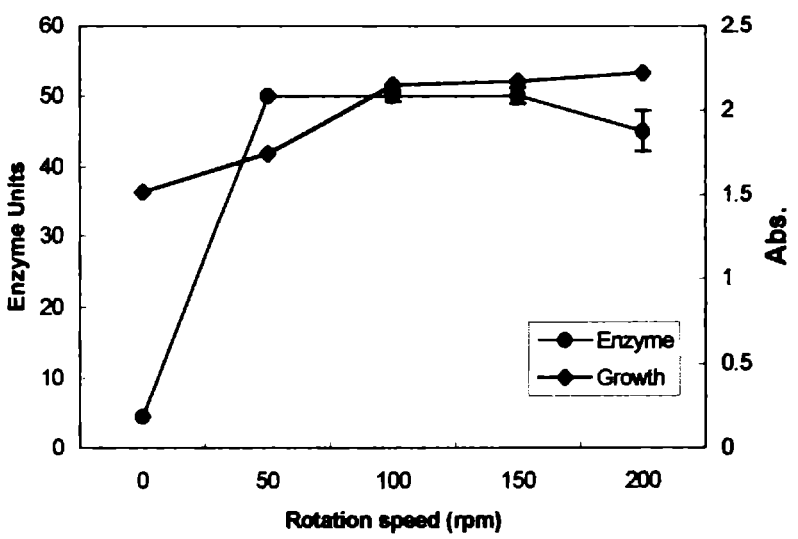


Fig. 3.3 Effect of rotation speed on the growth and protease production by *Vibrio* sp.

Table 3.3 ANOVA for the effect of rotation speed on protease production of *Vibrio* sp.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	4764.075	4	1191.019	575.847	<0.001
Within Groups	20.68288	10	2.068288		
Total	4784.758	14			

LSD =3.4954

Analysis of Variance showed that shaking speed significantly influenced protease production in V26. Between 50, 100 and 150 rpm there was no significant difference in protease production and production was maximum at these speeds.

3.4.2 Effect of period of incubation on growth and protease production B15

Maximum growth of *Bacillus circulans* was obtained after 36 hr. of incubation whereas maximum enzyme production was obtained after 60 hr. Enzyme production commenced when the culture was in the logarithmic phase of growth and continued till the culture reached late stationary phase of growth. There was a steep decrease in the activity afterwards (Fig. 3.4, Table 3.4).

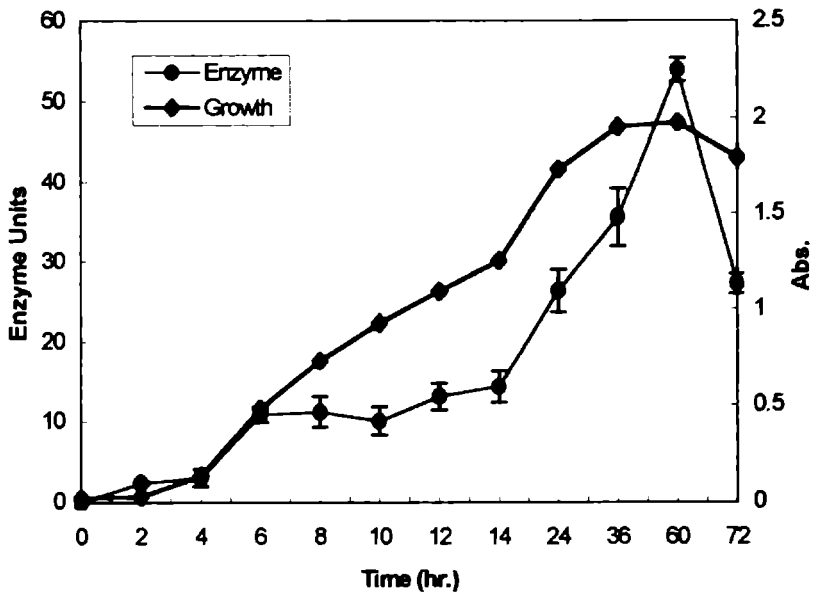


Fig. 3.4 Effect of period of incubation on the growth and protease production by *B. circulans*

Table 3.4 ANOVA for the effect of period of incubation on protease production *B. circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	11172.6	11	1015.691	256.546	<0.001
Within Groups	142.5276	36	3.9591		
Total	11315.13	47			

LSD = 3.798

ANOVA showed that there was considerable difference in enzyme production with respect to period of incubation.

V10

For the culture V10 maximum growth was reached at 24 hrs and the cells remained in the stationary phase till 36 hrs. of incubation. The maximum production of protease was obtained at 36hr. The enzyme production was found to follow the same pattern as that of growth. (Fig. 3.5; Table 3.5)

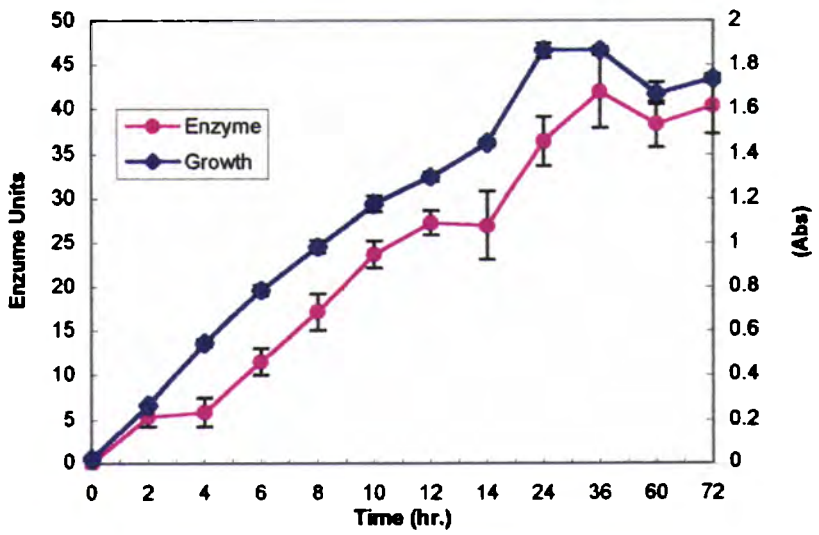


Fig 3.5 Effect of period of incubation on the growth and protease production on *V. fluvialis*

Table 3.5 ANOVA for the effect of period of incubation on the protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	7475.617	11	679.6016	113.892	<0.001
Within Groups	214.8137	36	5.967047		
Total	7690.431	47			

LSD = 4.664

Statistical analysis showed that the incubation period has significant effect on the growth and protease production by *V. fluvialis*.

V 26

V 26 showed maximum growth after 36 hr. and maximum enzyme production was at 60 hr. Growth steadily increased from 2 to 24 hours. The enzyme production also showed a steady increase along with the increase in growth up to 36 hrs and then showed a decrease in 60 hr. (Fig. 3.6, Table 3.6)

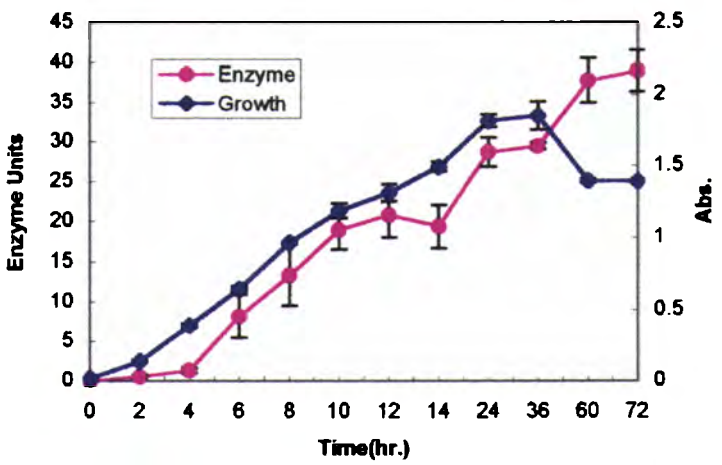


Fig. 3.6 Effect of period of incubation on the growth and protease production by *Vibrio* sp.

Table 3.6 ANOVA for the effect of rotation speed on the protease production of *Vibrio* sp.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	6833.805	11	621.255	66.51777	<0.001
Within Groups	336.2287	36	9.339685		
Total	7170.034	47			

LSD = 5.835

Statistical analysis showed that the incubation period has significant effect on the growth and protease production.

3.4.3 Effect of pH on growth and protease production

pH was found to influence the growth and protease production by these strains. They prefer neutral to alkaline pH for protease production.

B15

B. circulans exhibited no growth at pH 4 and 5. Growth reached the maximum level at pH 6 and was almost steady up to pH 10. Enzyme production started at pH 6 and it reached its maximum value at pH 7 and decreased thereafter. Still there was enzyme production up to pH10 (Fig. 3.7; Table 3.7).

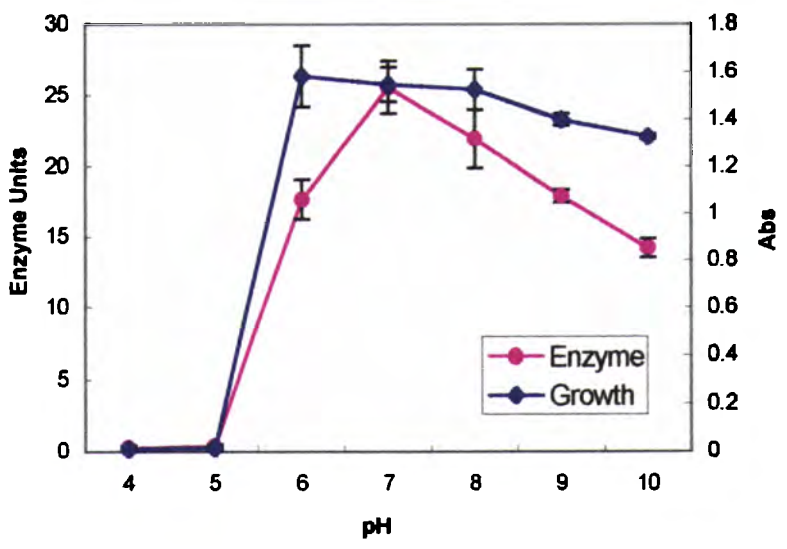


Fig. 3.7 Effect of pH on the growth and protease production by *B. circulans*

Table 3.7 ANOVA for the effect of pH on the protease production of *B. circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	Significance
Between Groups	3608.263	6	601.3772	403.29	<0.001
Within Groups	52.19088	35	1.491168		
Total	3660.454	41			

LSD = 1.9106

Statistical analysis showed that pH had a significant effect on the enzyme production.

V10

V10 at pH 4 did not show any growth whereas, it showed growth in the pH range of 5 to 10. The enzyme production was maximal at pH 7. Though protease was produced at pH 5 and 6 it reached maximum at pH 7. Even though enzyme production was maximal at pH 7, the culture could produced considerably good quantity of protease in the pH range 5 to 10 (Fig. 3.8; Table 3.8).

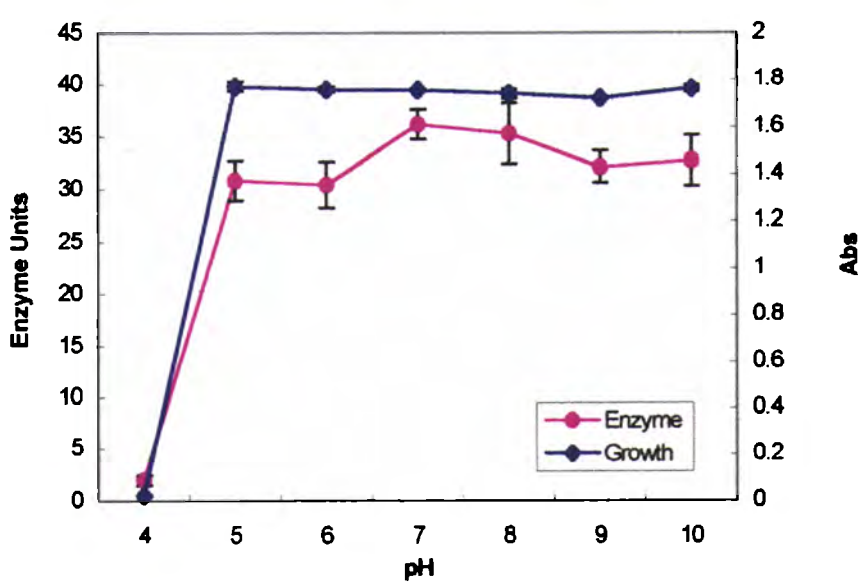


Fig. 3.8 Effect of pH on the growth and protease production by *V. fluvialis*

Table 3.8 ANOVA for the effect of pH on protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	5146.054	6	857.6757	220.0521	<0.001
Within Groups	136.4161	35	3.897603		
Total	5282.47	41			

LSD = 3.088

ANOVA results showed that the protease production was significantly influenced by pH change. There was no significant difference in enzyme production between pH 5 and 6; pH 7 and 8; and between pH 9 and 10.

V26

V26 did not show growth at pH 4 and 5. At pH 6 growth was maximum and enzyme production also was considerably high. Optimum pH for protease production was in the range pH 7 to 8 and there was good production at pH 9 and 10 though lesser than the maximum value (Fig. 3.9; Table 3.9).

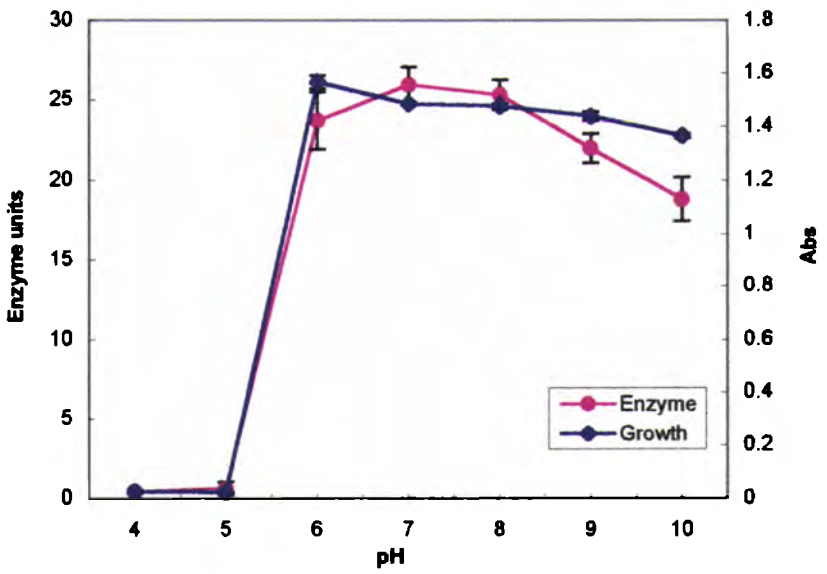


Fig. 3.9 Effect of pH on the growth and protease production of *Vibrio* sp.

Table 3.9 ANOVA for the effect of pH on the protease production of *Vibrio* sp.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	Significance
Between Groups	4586.138	6	764.3563	664.078	<0.001
Within Groups	40.28512	35	1.151003		
Total	4626.423	41			

LSD = 1.678

ANOVA showed that there was significant variation in enzyme production with respect to different pH. There was no significant difference in enzyme production between pH 7 and 8.

3.4.4 Effect of temperature on growth and protease production

All the tested strains showed almost similar pattern with respect to their temperature requirement for their optimum growth and enzyme production.

B15

B15 showed wide temperature range for growth. Almost the same rate of growth was shown at temperatures from 20 to 45°C. Enzyme production was maximum at 25°C to 30°C followed by 35°C. However enzyme production was very little at 20°C and 40°C (Fig. 3.10, Table 3.10)

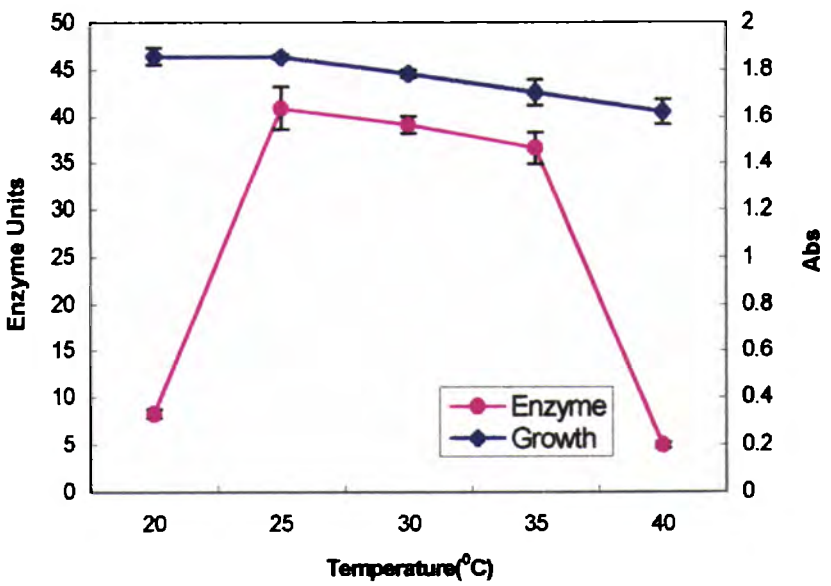


Fig. 3.10 Effect of temperature on the growth and protease production of *B. circulans*

Table 3.10 ANOVA for the effect of temperature on the protease production of *B. circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	7590.555	4	1897.639	1031.85	<0.001
Within Groups	45.97663	25	1.839065		
Total	7636.532	29			

LSD = 2.182

Statistical analysis showed that temperature had significant effect on the enzyme production of B15. There was no significant difference in production between 25°C and 30°C.

V10

V10 showed good growth in the temperature range 20 to 40°C. Even though the growth was maximum at 20°C enzyme production was negligibly low at this temperature. Enzyme production was maximum at 25°C followed by 30°C and 35°C. Enzyme production declined sharply when the temperature of incubation was higher (Fig. 3.11; Table 3.11).

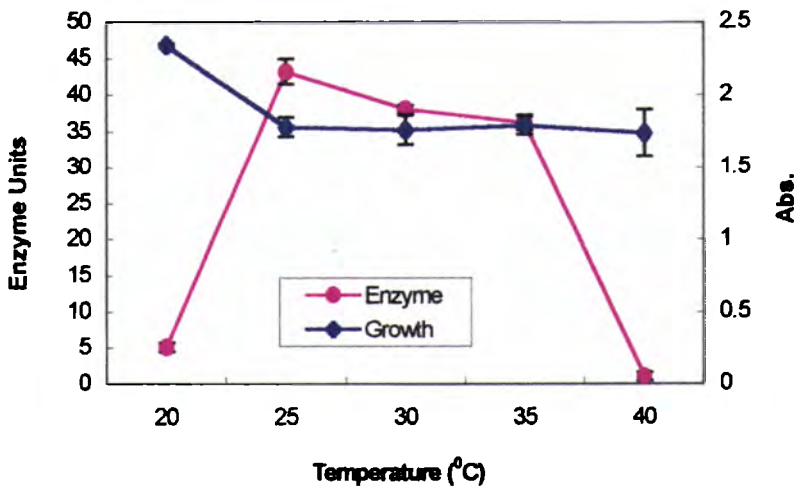


Fig. 3.11 Effect of temperature on the growth and protease production of *V. fluvialis*

Table 3.11 ANOVA for the effect of temperature on the protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	9609.724	4	2402.431	2342.58	<0.001
Within Groups	25.63866	25	1.025546		
Total	9635.362	29			

LSD= 1.6294

Statistical analysis showed that temperature had significant effect on the enzyme production of V10. There was no significant difference in production between 25°C to 35°C.

V26

For V26, there was very low enzyme production at 20°C even though there was very good growth at this temperature. At 25°C, 30°C, 35°C and 40°C growth rate was similar. Enzyme production was maximum at 25°C followed by 30°C and 35°C and at 40°C the production was minimum (Fig. 3.12; Table 3.12).

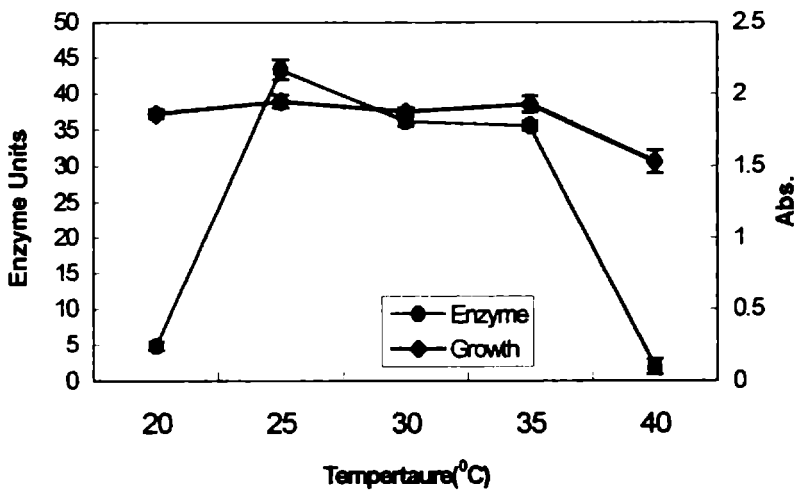


Fig. 3.12 Effect of temperature on the growth and protease production of *Vibrio* sp.

Table 3.12 ANOVA for the effect of temperature on the protease production of *Vibrio* sp.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	9038.407	4	2259.602	2721.88	<0.001
Within Groups	20.75405	25	0.830162		
Total	9059.161	29			

LSD = 1.466

ANOVA shows that there is significant effect of temperature on the protease production in V26. There was no significant difference in production between 30°C and 35°C.

3.4.5 Effect of NaCl concentration on growth and protease production

All the three strains selected showed considerable variation with respect to the sodium chloride requirement for growth and protease production.

B15

The isolate B15 was found to be a freshwater form showing no requirement for sodium chloride. The culture showed maximum growth and enzyme production at 0 salinity. Growth was maintaining the same rate at higher concentrations of NaCl, but enzyme production was significantly lesser at higher concentrations as compared to that at 0% NaCl. There was no significant difference in enzyme production at concentrations ranging from 0.5% to 5% (Fig. 3.13; Table 3.13).

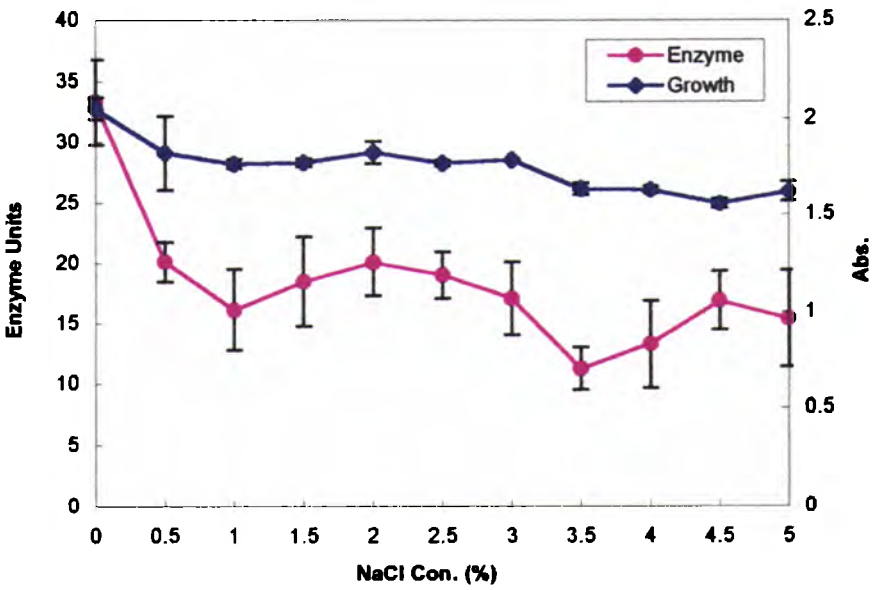


Fig 3.13 Effect of NaCl concentration on the growth and protease production of *B.circulans*

Table 3.13 ANOVA for the effect of NaCl concentrations on the protease production of *B.circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	1942.202	10	194.2202	21.46972	1.44E-15
Within Groups	497.5431	55	9.046238		
Total	2439.745	65			

LSD = 4.61908

ANOVA results showed that there was significant effect for the NaCl concentration on the growth and enzyme production of B15.

V10

V10 was found to require sodium chloride for growth. At 0 salinity there was no growth and enzyme production. At 0.5%NaCl there was good growth and maximum enzyme production. V10 showed maximum growth when the NaCl concentration was in the range 0.5 to 5% in the medium, whereas enzyme production started declining above 0.5% NaCl level (Fig. 3.14; Table 3.14).

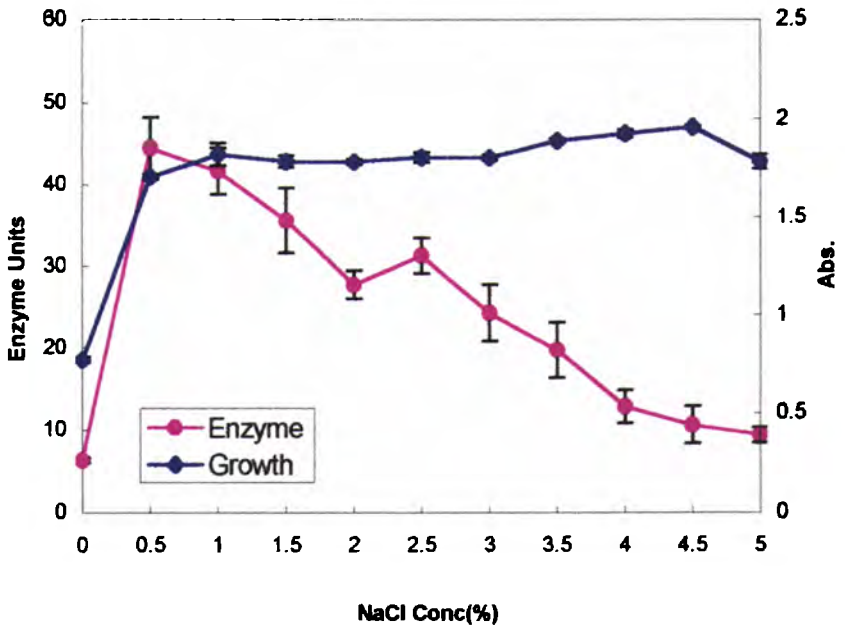


Fig 3.14. Effect of NaCl concentration on the growth and protease production of *V. fluvialis*

Table 3.14 ANOVA for the effect of NaCl concentrations on the protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	10738.37	10	1073.837	149.902	<0.001
Within Groups	393.9954	55	7.163553		
Total	11132.37	65			

LSD = 4.11

ANOVA results showed that there was significant effect for the NaCl concentration on the growth and enzyme production of V10.

V26

Growth was maximal at 1% to 3% NaCl concentrations, in the case of V 26. At higher concentrations growth was decreased. Enzyme production was highest at 0 salinity then decreased gradually up to 1% and then maintained a steady state up to 3%. Enzyme production decreased considerably when the NaCl concentration was above 3% (Fig. 3.15, Table 3.15).

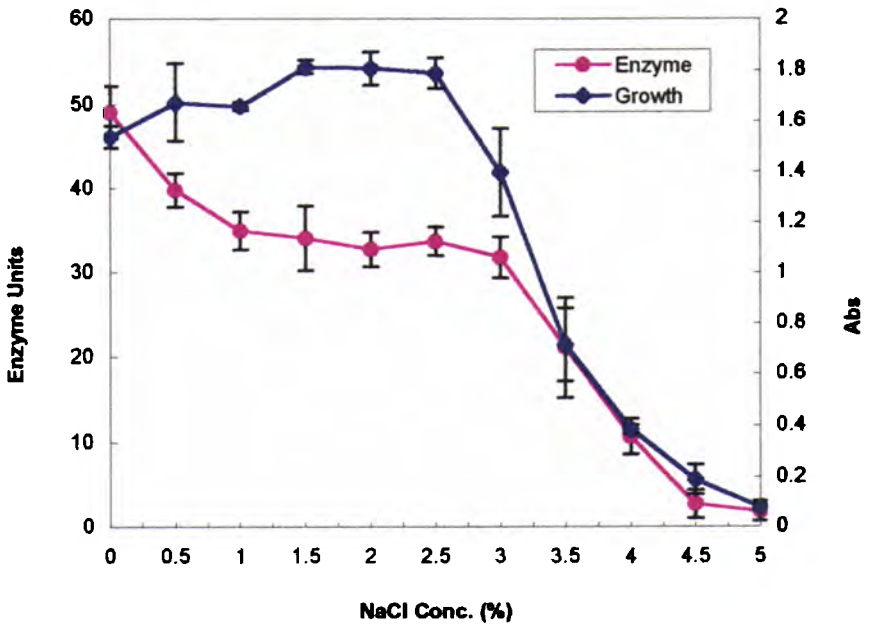


Fig. 3.15 Effect of NaCl concentration on the growth and protease production of *Vibrio* sp.

Table 3.15 ANOVA for the effect of NaCl concentrations on the protease production of *Vibrio* sp.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	14309.23	10	1430.923	176.3625	<0.001
Within Groups	446.2444	55	8.113535		
Total	14755.47	65			

LSD = 4.3744

Statistical analysis showed that there was significant effect for the NaCl concentration on the growth and enzyme production of V10. There was no significant difference in enzyme production between 1 to 3% concentrations of NaCl.

3.4.6 Effect of metallic salts on growth and protease production

The various metal ions tested in the present study showed considerable variation with respect to their effect on growth and enzyme production.

B15

In the case of B15, $CoCl_2$ at all concentrations tested and 0.02 and 0.05 mM concentrations of $MnSO_4$ were completely inhibiting growth and enzyme production. Growth rate was not affected by the presence of any other salt. However all the salts tested, at different concentrations had significant effect on the enzyme production. A few salts were enhancing enzyme production significantly the best being $CaCl_2$ followed by KH_2PO_4 and $MgSO_4$. $(FePO_4)_3$ was found to reduce enzyme production (Fig.3.16, 3.17; Table 3.16).

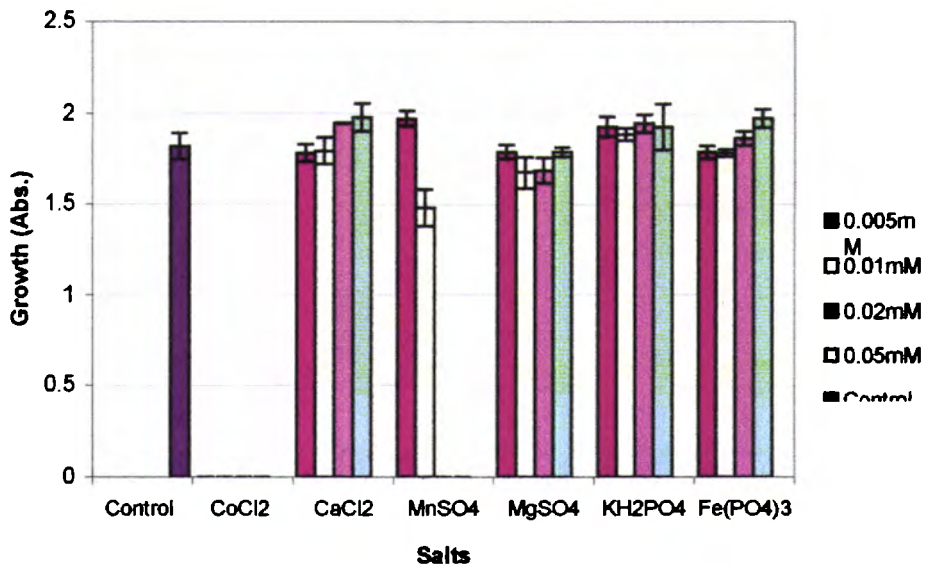


Fig. 3.16 Effect of metallic salts on the growth of *B. circulans*

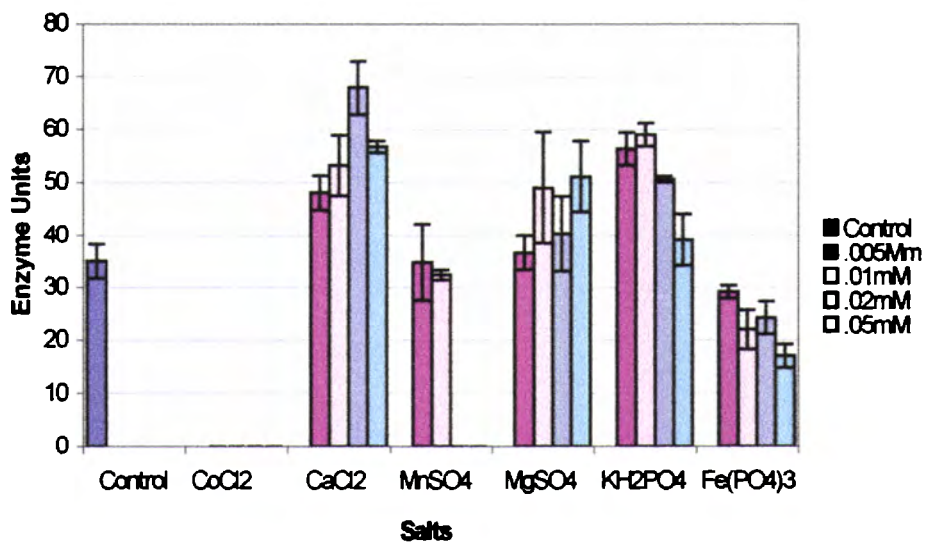


Fig. 3.17 Effect of metallic salts on the protease production of *B. circulans*

Table 3.16 ANOVA for the effect of metallic salts on the protease production of *B. circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F- value	P-value
Sample	19557.48	4	4889.371	49.4637	<0.001
Columns	1270.166	3	423.3887	4.2832	<0.001
Within	7117.023	72	98.8475		
Total	27944.67	79			

LSD for salts = 9.139

LSD for concentrations = 8.1744

ANOVA showed that the different metallic salts as well as their different concentrations had significant effect on the protease production by B15. There is no significant difference in the effect of KH_2PO_4 and $MgSO_4$ and between $CaCl_2$ and KH_2PO_4, on protease production by B15.

V10

$CoCl_2$ was completely inhibiting growth and enzyme production of V10 at all concentrations tested. $MnSO_4$ at 0.05 concentration was also inhibitory. Growth was not affected much by the presence of other salts in the medium, whereas an enhancing effect was noticed on the protease production. KH_2PO_4 was having the maximum effect, followed by $MgSO_4$, $Fe(PO4)_2$, and $CaCl_2$ (Fig. 3.18, 3.19; Table 3.17).

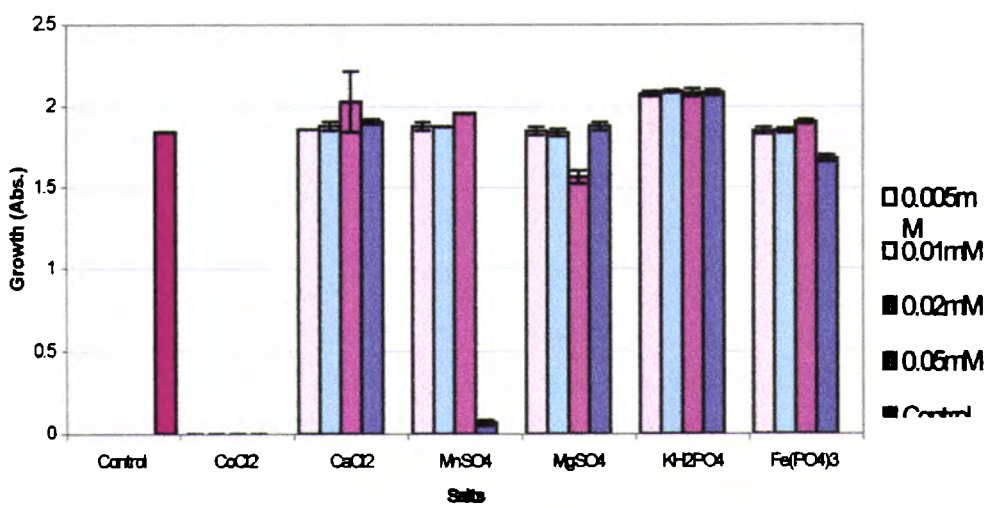


Fig 3.18 Effect of metallic salts on the growth of *V. fluvialis*

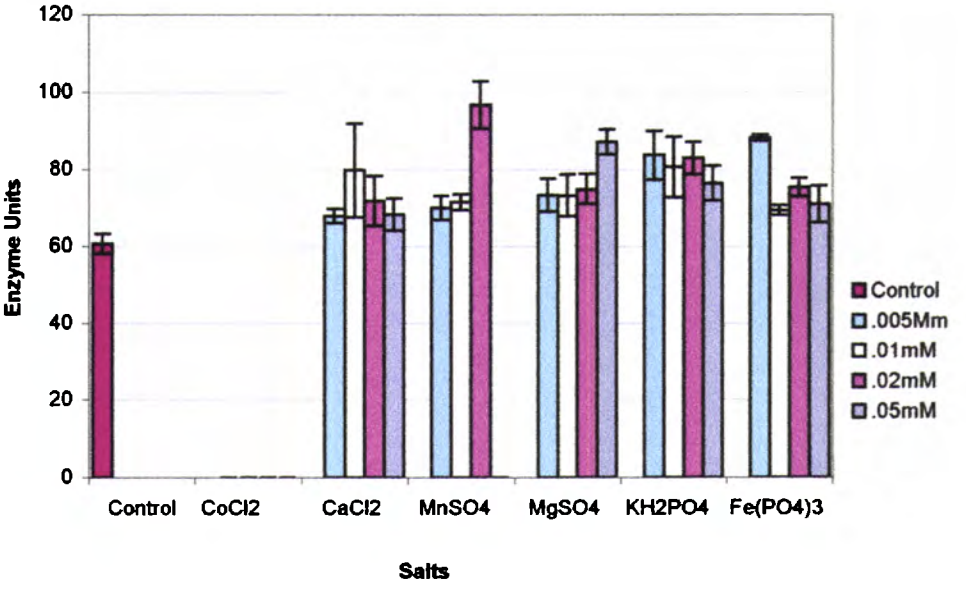


Fig. 3.19 Effect of metallic salts on the protease production of *V. fluvialis*

Table 3.17 ANOVA for the effect of metallic salts on the protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	1485.835	4	371.4588	4.38	<0.001
Columns	467.8696	3	155.9565	1.841	NS
Within	6098.825	72	84.705		
Total	8052.53	79			

LSD for metallic salts = 8.46

Concentrations - Not significant

ANOVA showed that the different metallic salts influenced the protease production by V10 significantly. Different concentrations did not have significant difference in their effects. There was no significant difference between the effects of $CaCl_2$ and $Fe(PO_4)_3$; $Mn(SO_4)_2$ and $MgSO_4$; $MgSO_4$ and KH_2PO_4; KH_2PO_4 and $Fe(PO_4)_2$; $Fe(PO_4)_2$ and $MgSO_4$.

V26

In the case of V26 also $CoCl_2$ at all concentrations and $MnSO_4$ at two higher concentrations were inhibitory to growth and enzyme production. All

the others were giving good growth and enhanced production of protease (Fig. 3.20, 3.21; Table 3.18)

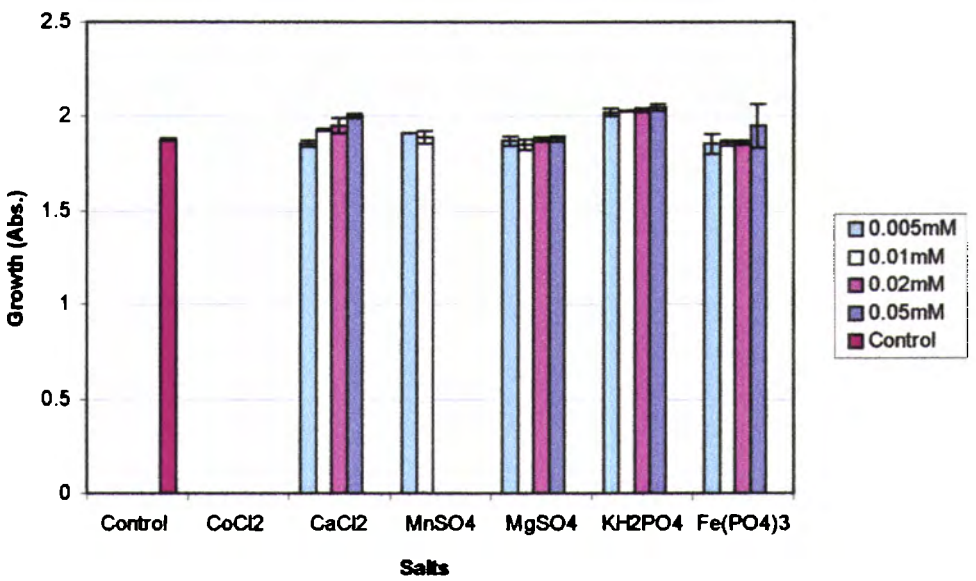


Fig. 3.20 Effect of metallic salts on the protease production of *Vibrio* sp.

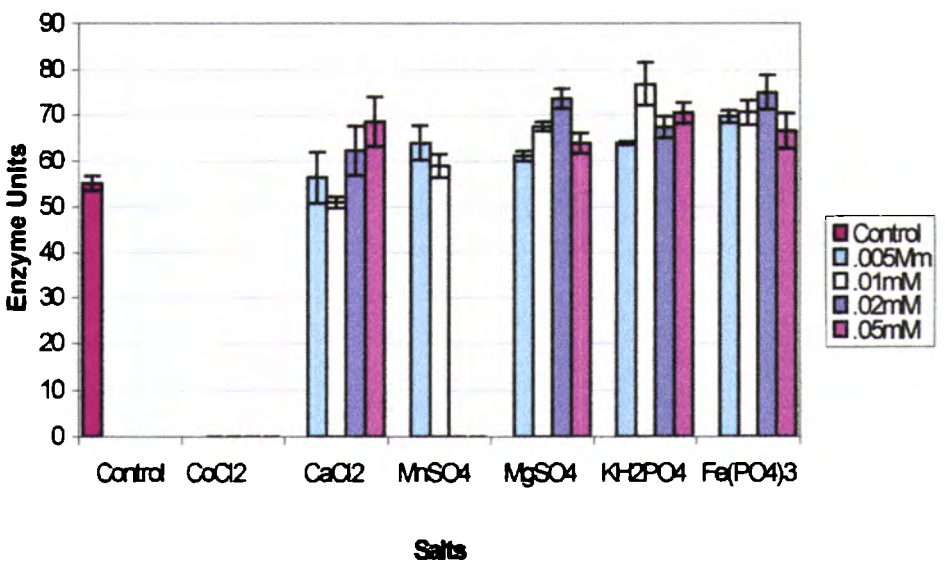


Fig. 3.21 Effect of metallic salts on the protease production of *Vibrio* sp.

Table 3.18 ANOVA for the effect of metallic salts on the protease production of *Vibrio* sp.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	17611.9	4	4402.975	20.212	<0.001
Columns	1768.439	3	589.4797	2.7060	NS
Within	15684.3252	72	217.8378		
Total	35064.66	79			

LSD for salts = 12.135

ANOVA shows that the different metallic salts influenced the protease production in V26 significantly. Different concentrations did not have significant difference in their effects. There was no significant difference between the effects of $CaCl_2$, $MgSO_4$ and KH_2PO_4; $MgSO_4$ and $Fe(PO_4)_3$; KH_2PO_4 and $Fe(PO_4)_3$.

3.4.7 Effect of carbon sources on growth and protease production

Using two way ANOVA with replication, a comparison has been made between glucose, mannose, maltose and sucrose, and also between these four carbon sources and molasses, as different concentrations (0.05, 0.1, 0.2, 0.4%) had been used for molasses from that used for the other four (0.1, 0.2, 0.4, 0.6%).

B15

The growth of B15 was not much affected by the different sources of carbon tested. It showed good growth in presence of all the carbon sources, except at high concentrations of glucose. But the different carbon sources had significant effects on the protease production. Maltose was found to be the best source, sucrose being almost as effective as maltose. Molasses was found to be a good source in inducing protease production, still giving lower production than maltose and sucrose. Glucose at the three lower concentrations tested was as effective as maltose and sucrose, but was completely repressing enzyme production at the highest concentration tested. Mannose was inhibiting protease production at the two higher

concentrations tested. However, growth was not much affected by these concentrations of mannose. (Fig.3.22, 3.23; Table 3.19, 3.20).

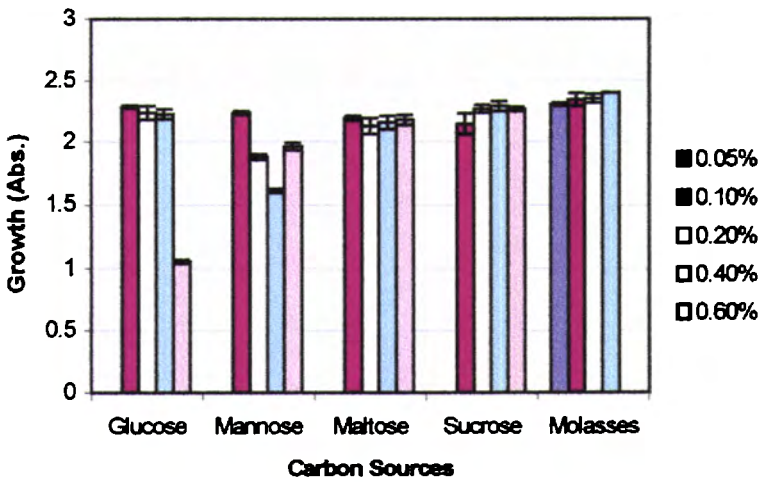


Fig. 3.22 Effect of carbon sources on the growth of *B. circulans*

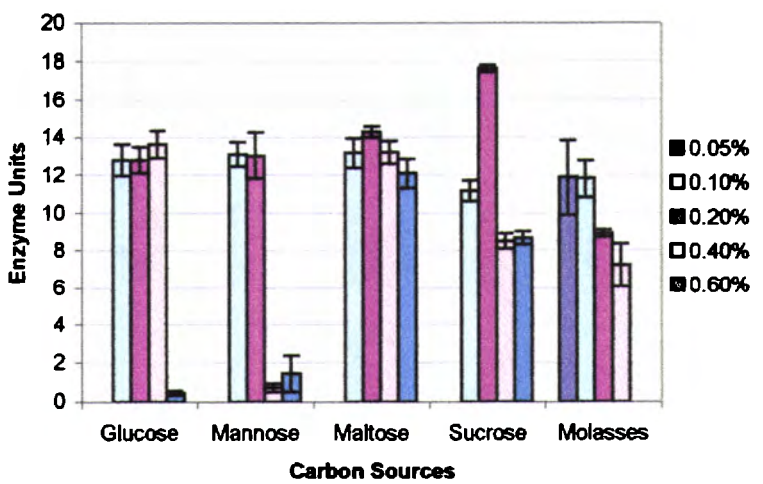


Fig 3.23 Effect of carbon sources on the protease production of *B. circulans*

Table 3.19 ANOVA for the effect of carbon sources (glucose, mannose, maltose and sucrose) on the protease production of *B. circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	323.7323	3	107.9108	10.6525	<0.001
Columns	723.2334	3	241.0778	23.7984	<0.001
Within	577.813	57	10.13		
Total	1624.779	63			

LSD for carbon sources = 2.993

LSD for concentrations = 2.993

ANOVA results showed that there was significant effect for the carbon sources on protease production. There was no significant difference between the effects of maltose and sucrose; and glucose and mannose.

Table 3.20 ANOVA for the effect of carbon sources (glucose, mannose, maltose and sucrose and molasses at 0.1%, 0.2% and 0.4% concentrations) on the protease production of *B. circulans*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	226.033	4	56.50824	7.3578	<0.001
Columns	244.4301	2	122.2151	15.9134	<0.001
Within	407.32	53	7.68		
Total	877.7832	59			

LSD for carbon sources = 3.009

LSD for concentrations = 2.33

Since we have taken into consideration only the three concentrations in this case, (0.1%, 0.2% and 0.4%) the results showed that glucose, maltose and sucrose did not vary significantly in their effect on protease production. Next better source was molasses and lastly mannose.

V10

In the case of V10 growth was not significantly influenced by the addition of various carbon sources. But enzyme production was significantly affected as shown from the ANOVA results. Glucose, mannose and sucrose at 0.6% concentrations were found to be depressing enzyme production drastically, though good growth was obtained at these concentrations. Maltose was found to be the best carbon source for enzyme production. Molasses was a good source in inducing enzyme production and it was not inhibitory at any concentration tested (Fig. 3.24, 3.25; Table 3.21, 3.22).

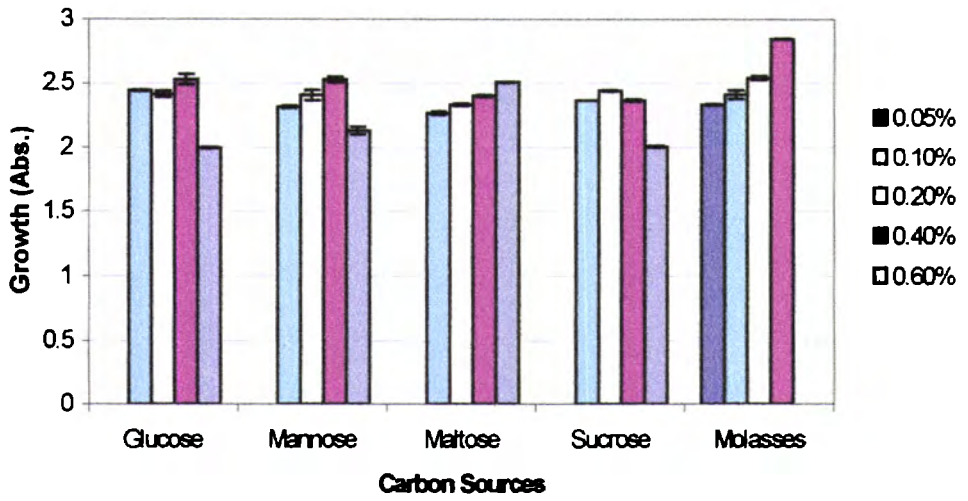


Fig. 3.24 Effect of carbon sources on the growth *V. fluvialis*

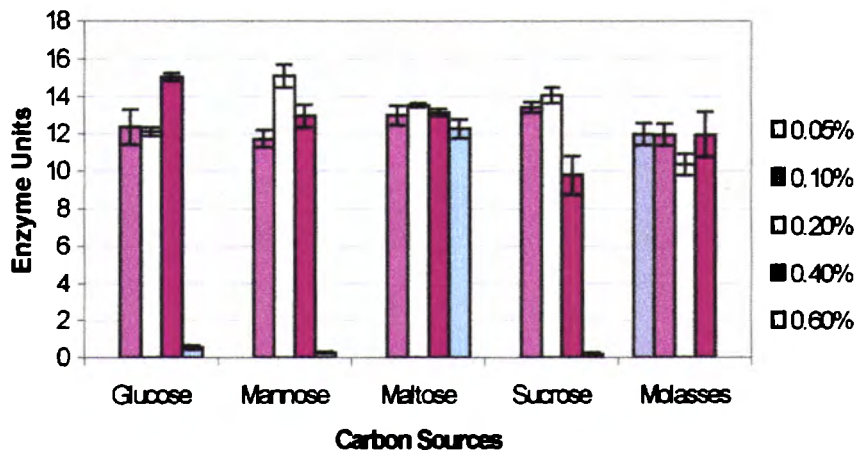


Fig. 3.25 Effect of carbon sources on the protease production of *V. fluvialis*

Table 3. 21 ANOVA for the effect of carbon sources (glucose, mannose, maltose and sucrose) on the protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F	P-value
Sample	126.5535	3	42.18452	6.0784	<0.001
Columns	1146.386	3	382.1288	54.7933	<0.001
Within	397.51912	57	6.974		
Total	1670.459	63			

LSD for carbon sources = 2.48

LSD for concentrations =2.48

ANOVA results showed that the different carbon sources at the different concentrations tested had significant effect on the enzyme production. Maltose was the best source giving significantly higher enzyme production than all the other carbon sources tested. There was no significant difference between the effects of glucose, mannose and sucrose. 0.6% concentration of the sugars was giving significantly lower enzyme production than all the other concentrations tested.

Table 3.22 ANOVA for the effect of carbon sources (glucose, mannose, maltose and sucrose and molasses) on the protease production of *V. fluvialis*

Source of Variation	Sum o Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	30.51896	4	7.629739	3.73	<0.001
Columns	3.398527	2	1.699263	0.83	NS
Within	108.41	53	2.045		
Total	142.3279	59			

LSD for carbon sources = 1.55

ANOVA showed that there was no significant effect of different concentrations of carbon sources tested, as we have taken into consideration only the 0.1%, 0.2% and 0.4% concentrations. There was no significant difference between the effects of maltose and mannose; maltose and sucrose; and between sucrose and molasses.

V 26

All the carbon sources tested had the same effect on growth of V26. Protease production was found to be much impeded by glucose. Maltose was found to give maximum production irrespective of the different concentrations tested (Fig. 3.26, 3.27; Table 3.23, 3.24).

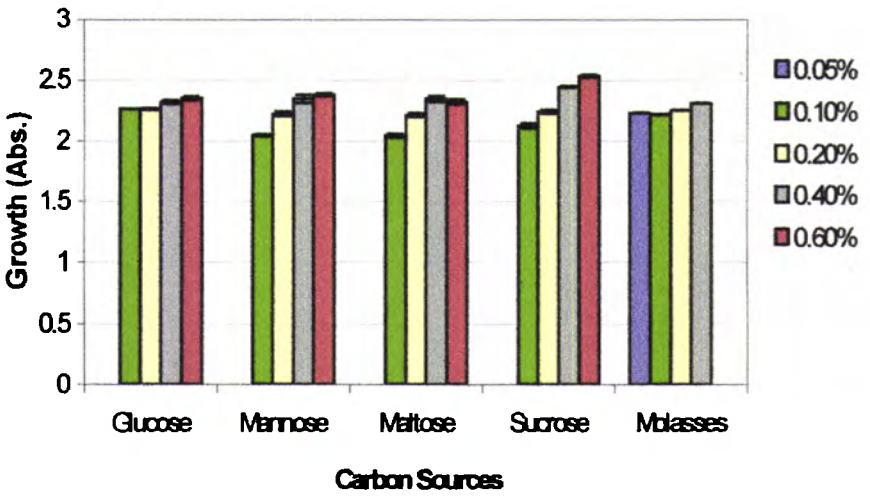


Fig 3.26 Effect of carbon sources on the growth of *Vibrio* sp.

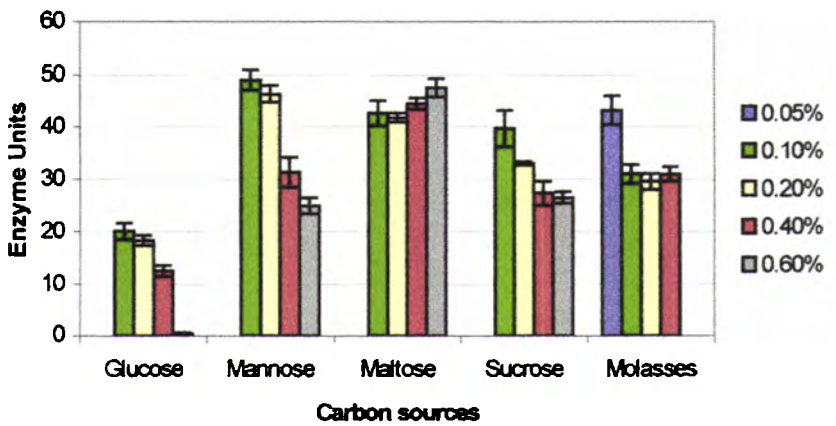


Fig. 3.27 Effect of carbon sources on the protease production of *Vibrio* sp

Table 3.23 ANOVA for the effect of carbon sources (glucose, mannose, maltose and sucrose) on the protease production of *Vibrio* sp

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	8763.984	3	2921.328	103.0632	<0.001
Columns	1632.036	3	544.012	19.19252	<0.001
Within	1615.7089	57	28.345		
Total	12011.73	63			

LSD for carbon sources = 3.688

LSD for concentrations = 3.688

ANOVA showed that maltose was giving significantly higher production than all the other carbon sources, followed by mannose and sucrose. Glucose was giving significantly lower production.

Table 3.24 ANOVA for the effect of carbon sources (glucose, mannose, maltose, sucrose and molasses) on the protease production of *Vibrio* sp

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	5360.167	4	1340.042	970.20	<0.001
Columns	522.3137	2	261.1569	16.978	<0.001
Within	815.2073	53	15.3812		
Total	6697.688	59			

LSD for carbon sources = 4.25

LSD for concentrations = 3.298

In this case, where only the three concentrations have been taken into consideration (0.1%, 0.2% and 0.4%) maltose and mannose as well as sucrose and molasses, were not having significant difference in their effects. Glucose was giving significantly lower production.

3.4.8 Effect of nitrogen sources on growth and protease production

Both organic and inorganic nitrogen sources were tested for studying their effects on growth and protease production. Organic compounds were found to be more suitable for enzyme production.

B15

In the case of B15, organic nitrogen sources were found to be more suitable for growth and enzyme production. Growth and enzyme production was low with inorganic nitrogen sources. Casein supported very good growth and enzyme production followed by peptone and tryptone. Even though growth was maximum in the presence of yeast extract enzyme production was considerably lower. The organism showed considerable growth in presence of inorganic nitrogen sources but enzyme production was negligible. (Fig 3.28, 3.29; Table 3.25)

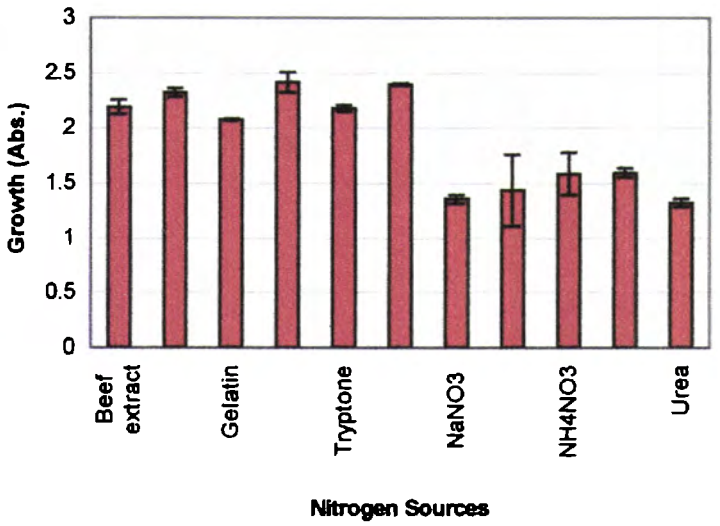


Fig. 3.28 Effect of nitrogen sources on growth of *B. circulans*

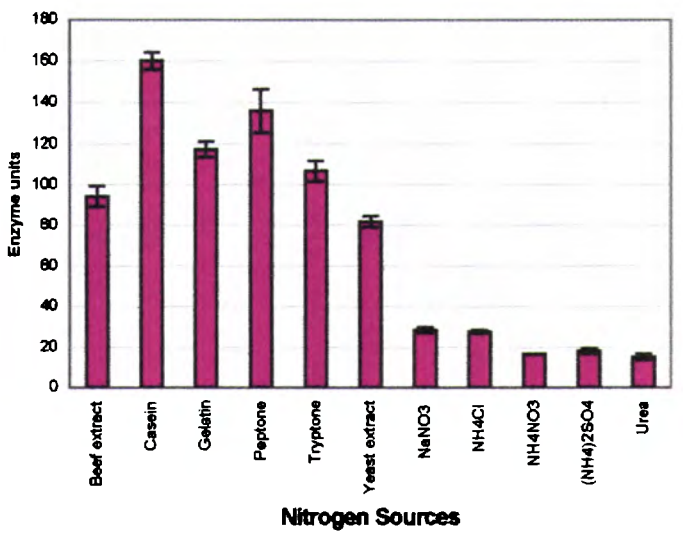


Fig. 3.29 Effect of nitrogen sources on enzyme production of *B. circulans*

Table 3.25 of ANOVA for the effect of nitrogen sources on the protease production of *B. circulans*

Source of Variation	Sum of squares	Degrees of freedom	Mean of squares	F- value.	P-value
Between Groups	114880.3	10	11488.03	613.500	<0.001
Within Groups	617.9371	33	18.72537		
Total	115498.2	43			

LSD = 8.32279

Statistical analysis showed that the protease production was significantly influenced by the different nitrogen sources tested. Casein was giving significantly higher protease production than all other nitrogen sources.

V10

In the case of V10 also organic nitrogen sources were found to be more suitable for growth and enzyme production. Casein and yeast extract were found to support maximum growth and protease production followed by beef extract and peptone. Inorganic nitrogen sources when used as the sole source of nitrogen were found to be ineffective for protease synthesis (Fig. 3.30, 3.31; table 3. 26).

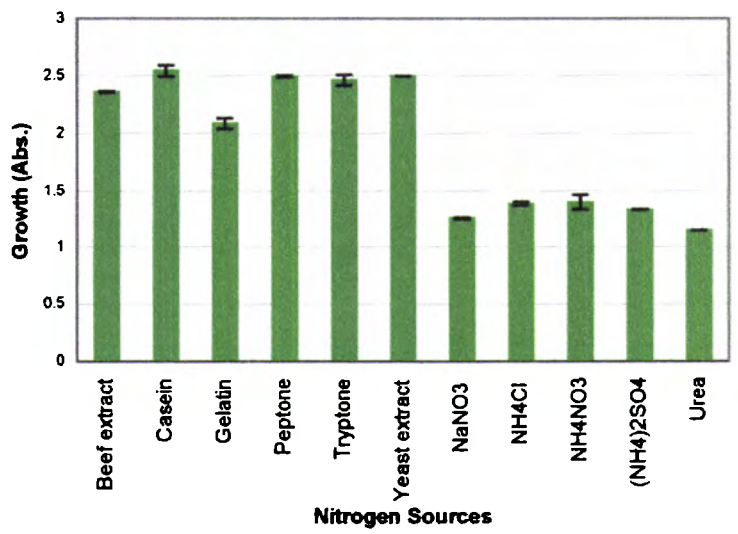


Fig. 3.30 Effect of nitrogen sources on growth of *V. fluvialis*

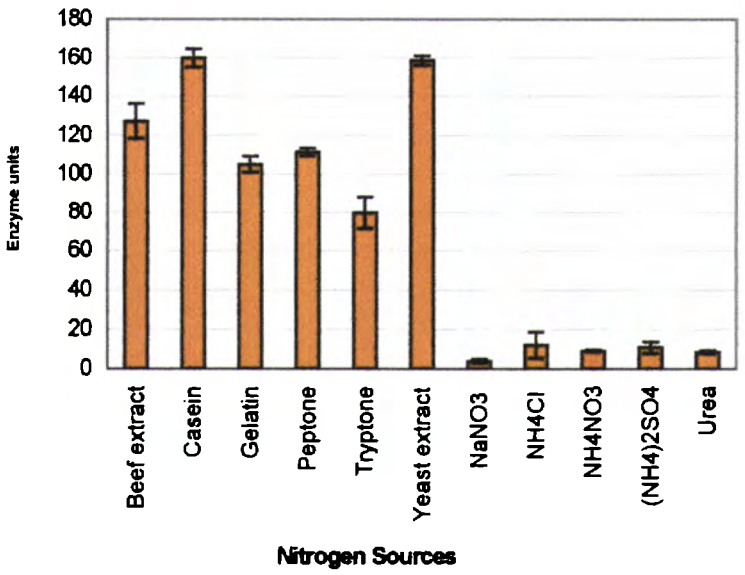


Fig. 3.31 Effect of nitrogen sources on protease production of *V. fluvialis*

Table 3.26 ANOVA for the effect of nitrogen sources on the protease production of *V. fluvialis*

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F	P-value
Between Groups	163381.5	10	16338.15	705.7394	<0.001
Within Groups	763.963	33	23.15039		
Total	164145.4	43			

LSD = 9.25407

Statistical analysis showed that the protease production was significantly influenced by the different nitrogen sources tested. Casein was giving significantly higher protease production than all other nitrogen sources. There was no significant difference between the effects of gelatin and peptone.

V26

V26 showed good growth in presence of both organic and inorganic nitrogen sources, but enzyme production was negligible in presence of inorganic nitrogen sources. Maximum enzyme production was obtained with beef extract and casein, followed by tryptone and yeast extract. Inorganic nitrogen sources were ineffective in protease production (Fig. 3.32, 3.33; Table 3.27)

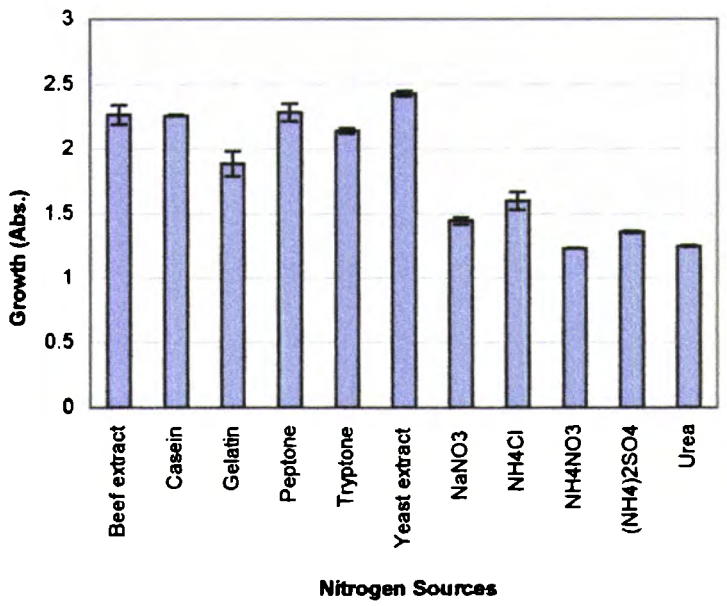


Fig. 3.32 Effect of nitrogen sources on growth of *Vibrio* sp.

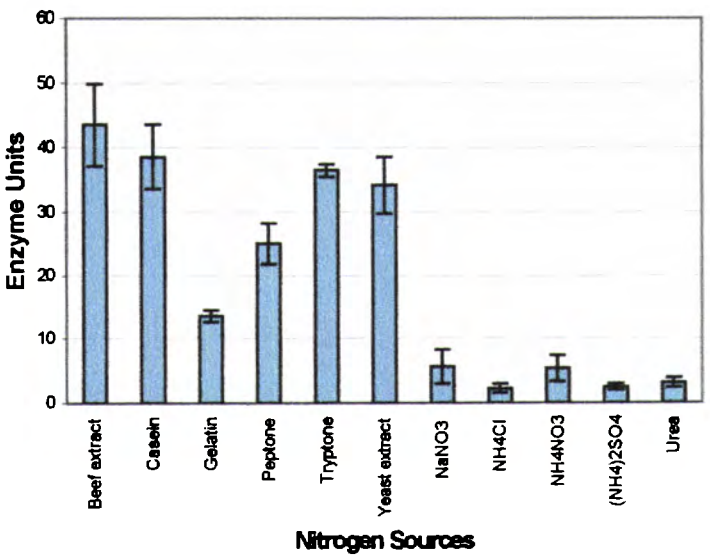


Fig. 3.33 Effect of nitrogen sources on protease production of *Vibrio* sp.

Table 3. 27 ANOVA for the effect of nitrogen sources on the protease production of *Vibrio* sp.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	10985.31	10	1098.531	111.239	<0.001
Within Groups	325.8868	33	9.875358		
Total	11311.2	43			

LSD = 6.04384

Statistical analysis showed that protease production was significantly influenced by the different nitrogen sources tested. Casein was giving significantly higher protease production than all other nitrogen sources tested. There was no significant difference between the effects of tryptone and yeast extract on protease production.

3.5 Discussion

Generally proteases produced by microorganisms are constitutive; however, at times they are partially inducible (Kalisz, 1988; Beg *et al.*, 2002a) in nature. Extracellular protease production in microorganisms is strongly influenced by media components such as, variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose and metal ions. Protease synthesis is also affected by the rapidly metabolizable nitrogen sources viz. amino acids in the medium. Besides, several physical factors such as aeration, inoculum density, pH, temperature and period of incubation also affect the amount of protease produced (Hameed *et al.*, 1999; Puri *et al.*, 2002).

Though bacteria capable of producing proteases are many, interest is limited only to those that produce substantial quantities of the enzyme. It is essential that these organisms be provided with optimal growth conditions for maximum enzyme production. Often the enzyme production was found to be growth associated. At least in some instances the culture conditions that promoted enzyme production were found significantly different from the culture conditions that promoted cell growth (Moon and Parulekar, 1991). In the present study also, culture conditions have been found to influence growth and quantity of protease produced by the selected strains.

3.5.1 Effect of shaking speed on growth and protease production

In the present study, agitation of the medium was found to enhance enzyme production considerably, as it greatly influences the availability of nutrients as well as dissolved oxygen to the organism. For all the three strains tested, after 24 hr. of incubation, the enzyme production was very low

when the culture was kept stationary. The enzyme production was high between 50 to 150 rpm. Though the enzyme production was negligibly low in the still culture, growth was not impeded much. However, growth was lower than that incubated with agitation.

Similar observations on protease production had been made by several workers. In *Vibrio alginolyticus* (Hare *et al.*, 1981) the protease production was markedly influenced by agitation. *Bacillus* sp. B-21-2 produced increased enzyme yields at 600 rpm (Fujiwara and Yamamoto, 1987). Donham *et al.* (1988) reported 17-fold increase in growth and 7-fold increase in exoprotein production in aerated cultures of *Staphylococcus stimulans* compared to stationary culture. A high alkaline protease production was reported in a *Bacillus* sp. at 300 rpm (Takami *et al.*, 1989). Similarly *Bacillus firmus* exhibited maximum production at agitation rate of 360 rpm and aeration rate of 7.0 l min^{-1} However, lowering the aeration rate to 0.1 l min^{-1} caused a drastic reduction in the protease yields (Moon and Parulekar, 1991). Optimum yields of alkaline proteases were produced at 200 rpm for *Bacillus subtilis* ATCC 14416 (Chu *et al.*, 1992) and *Bacillus licheniformis* (Sen and Satyanarayana, 1993). Matta *et al.* (1994) reported better protease production by *Pseudomonas* sp. AFT-36 under continuous agitation (180 rpm) than that under intermittent or no agitation. In standing cultures protease levels were very low compared to the shaking cultures. The positive effect of aeration was reported by Madan *et al.* (2000), as aerated cultures gave better protease production than that by stationary culture.

The variation in the agitation speed influences the extent of mixing in the shake flask and will also affect the nutrient availability. This is also one of the means to obtain different dissolved oxygen profiles. During fermentation the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Therefore the reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis.

3.5.2 Effect of period of incubation on growth and protease production

Bacteria when grown in batch cultures exhibit a characteristic growth curve involving four phases, the lag phase, logarithmic phase, stationary phase and a phase of decline. The phase at which protease production begins in the cell is determined by measuring the growth and enzyme production at different time intervals, after inoculating the culture in the optimal medium. In the present study, enzyme production was found to begin at the logarithmic phase of growth and reached the maximal level at the stationary phase of growth. For *Bacillus circulans* maximum production was at 60 hr. and it drastically reduced at 72 hr. For *V. fluvialis* (V10) maximum yield was obtained at 36 hr. of incubation and *Vibrio* sp. (V26) gave maximum production at 60 hr maintaining it even at 72 hr.

The production of enzymes exhibits a characteristic relationship with regard to the growth phase of that organism. In general, the synthesis of protease in *Bacillus* sp. is controlled by numerous complex mechanisms operative during the transition state between exponential phase and the stationary phase (Priest, 1977; Strauch and Hoch, 1993). The production of extracellular protease during the stationary phase of growth is characteristic of many bacterial species (Priest, 1977), which is marked by the transition from vegetative growth to sporulation stage in spore formers. At early stationary phase, two or more proteases are secreted and the ratio of the amount of individual proteases produced, also varied with the *Bacillus* strains (Uehara *et al.*, 1974; Priest, 1977). In several cases, the function of the enzyme is not very clear, but its synthesis is correlated with the onset of a high rate of protein turnover and often sporulation (Power and Adams, 1986; Chu *et al.*, 1992). Therefore protease production is often related to the sporulation stage in many bacilli such as *Bacillus licheniforms* (Hanlon and Hodges, 1981) and *Bacillus subtilis* (O' Hara and Hageman, 1990). On the contrary a few reports also suggests that sporulation and protease production- although co occurring- are not related, as the spore deficient strains of *Bacillus licheniformis* were not protease deficient (Fleming *et al.*,

1995). Khan (2000) established that sporulation and protease production are two independent events in the stationary phase. A similar observation was made in *B. licheniformis* (Bierbaum *et al.*, 1991) by analysis of GTP and ATP in the cells. In this study it was conclusively suggested that extracellular protease production is a manifestation of nutrient limitation at the onset of stationary phase. However final protease yield during this phase is also determined by the biomass produced during exponential phase. Therefore, medium manipulation is needed to maximize growth and hence protease yields. Guntelberg (1954) reported that protease production started towards the end of logarithmic growth phase, and also referred to the absence of spores in the culture. The protease production in *Pseudomonas* I-6 was reported (Sakata *et al.,* 1977a) to be very high in the logarithmic phase and then decreased.

In some cases, there is little or no enzyme production during the exponential growth phase (Frankena *et al.*, 1985). However, in the case of *B. subtilis* ATCC 14416 (Chu *et al.*, 1992) and *B. sphericus* BSE18 (Dumosois and Priest, 1993) enzyme production was growth associated, and occurs maximally in the mid exponential phase, and often a rapid auto deactivation was observed after the culture reached the maximum enzyme activity (Chu *et al.*, 1992). In the present study also this phenomenon was observed with *B. circulans*, where it produced the maximum amount of protease in the stationary phase at 60 hr. of incubation and a steep fall in the enzyme production was observed at 72 hr.

In a number of cases the synthesis and secretion of proteases was initiated during the exponential growth phase with a substantial increase near the end of this growth phase and with maximum amounts of protease produced in the stationary growth phase (Durham *et al.*, 1987; Tsai *et al.*, 1988, Taki *et al.*, 1990; Moon and Parulekar, 1991; Ferrero *et al.*, 1996; Manachini *et al.*, 1998) which was exactly what was observed in the present study for all the three strains tested.

3.5.3 Effect of pH on growth and protease production

The culture pH strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). pH has been implicated as a major factor influencing the release of cell bound enzymes. It is one of the key factors, which influences the production of extracellular proteases by bacteria, especially by alkalophiles. For increased protease yields from these alkalophiles the pH of the medium must be maintained above 7.5 through out the fermentation period (Aunstrup, 1980). In the present study, the organism selected were not obligate alkalophiles as it was observed that they had good growth at pH 6 and *V. fluvialis* showed growth even at pH 5. Protease production also started at these pH values. However, maximum enzyme production was obtained at pH 7 for all the three strains and they maintained almost the same rate up to pH 10.

Comparable values of pH optima were reported for other proteolytic strains. The optimum pH for protease production in a *Flavobacterium* sp. It was 7.4 (Morita *et al.*, 1998). Protease production was best at pH 9 in *B. polymyxa* (Madan *et al.*, 2000). Thangam and Rajkumar (2002) reported the optimum pH for protease production by *Alcaligenes faecalis* as 8. pH optima for protease production in various *Bacillus* sp. were in the range 7 and 10 (Purva *et al.*, 1998; Banerjee *et al.*, 1999; Hameed *et al.*, 1999; Longo *et al.*, 1999; Johnvesly and Naik, 2001; Kaur *et al.*, 2001; Mabrouk *et al.*, 1999; Oberoi *et al.*, 2001; Singh *et al.*, 2001a; Singh *et al.*, 2001b; Puri *et al.*, 2002; Beg *et al.*, 2002a; Kumar, 2002)

3.5.4 Effect of temperature on growth and protease production

Every organism possesses a minimal temperature, optimum temperature and maximum temperature for the growth and metabolism. Temperature below optimum is bacteriostatic and above optimum is bactericidal. So temperature is a critical parameter that has to be controlled in any fermentation. The actual mechanism by which temperature controls

enzyme production is not well understood (Chaloupka, 1985). However, studies by Frankena *et al.* (1986) showed that a link existed between enzyme synthesis and energy metabolism in Bacilli, which was controlled by temperature and oxygen uptake. Wide variation occurs in the optimum temperature of enzyme production from organism to organism. Alkaline protease production in *V. alginolyticus,* as studied by Hare *et al.* (1981), was maximum at 30°C and protease yields decreased above this temperature. In a *Flavobacterium* sp. optimum temperature was found to be as low as 10°C. (Morita *et al.*, 1998) and 30°C and 36°C for two *Serratia marcescens* strains (Longo *et al.*, 1999;Romero *et al.*, 2001). For different *Bacillus* sp. the optimum temperature ranged from 30°C to 50°C (Purva *et al.*, 1998; Banerjee *et al.*, 1999; Hameed *et al.*, 1999; Longo *et al.*, 1999; Johnvesly and Naik, 2001; Kaur *et al.*, 2001; Mabrouk *et al.*, 1999; Oberoi *et al.*, 2001; Singh *et al.*, 2001a; Singh *et al.,* 2001b; Puri *et al.*, 2002; Beg *et al.*, 2002a; Kumar, 2002). However temperature was not a limiting factor for protease production by a dairy strain of *A.hydrophila* (Santos *et al.*, 1996).

In the present study temperature was found to have a profound influence on protease production. At 20°C and 40°C there was little production of protease though growth rate was not much affected at this temperatures. The protease production was maximal at temperatures ranging from 25°C to 35°C. There was no significant difference between the production rates among these strains. For the *Bacillus* and the two *Vibrio* sp. the effect of temperature on the enzyme production was similar.

3.5.5 Effect of NaCl on growth and protease production

The requirement of NaCl was found to vary in the three strains tested. B15 was found to be a freshwater form showing no requirement for sodium chloride. The culture showed maximum growth and enzyme production at 0 salinity. Growth was maintaining the same rate at higher concentrations of NaCl, but enzyme production was significantly lesser at higher concentrations. V10 was found to require sodium chloride for growth. At 0

salinity there was no growth and enzyme production. At 0.5% NaCl there was good growth and maximum enzyme production. In the case of V 26, Growth was maximal at 1% to 3% NaCl concentrations. However protease production was highest at 0 salinity. The selected strains are subjected to high salinity variations in their natural habitat. The salinity- nutrient changes in the Cochin estuary is evidently influenced by the influx of freshwater and intrusion of seawater (Anirudhan *et al.*, 1987). During monsoon, the entire estuary attains near freshwater conditions, except at bottom near the barmouth. In October, a gradual increase in salinity to the range from 10 to 20 ppt occurs (Lakshmanan *et al.*, 1982; Anirudhan *et al.*, 1987). This may account for the moderate requirement of NaCl by V26 strain, and non-halophilic nature of B15 and V10 strains.

3.5.6 Effect of metallic salts on growth and protease production

Divalent metal ions such as Calcium, Cobalt, Copper, Boron Iron, Magnesium, Manganese and Molybdenum are required in the fermentation medium for growth and optimum production of alkaline proteases. However, the requirement for specific metal ions depends on the source of enzyme.

Potassium phosphate has been used as a source of phosphate in most studies (Fujiwara *et al.*, 1991; Moon and Parulekar, 1991; Mao *et al.*, 1992; Hubner *et al.*, 1993). This was shown to be responsible for buffering the medium. Phosphate at the concentration of 2 g/l was found to be optimal for protease production. However, higher concentrations showed an inhibition in cell growth and repression on protease production (Moon and Parulekar, 1991). But in some cases salts did not have any effect on protease yield (Phadatare *et al.*, 1993). In some strains like *Bacillus* sp. AR-009 and *Bacillus licheniformis* ATCC 21415 (Gessesse, 1997; Mehrotra *et al.*, 1999) NaCl (0.2% w/v) and $NaNO_3$ (0.5% w/v) caused reduction in protease production and potassium phosphate (0.5%w/v) induced production.

In the present study, some ions like Co^{2+} were inhibitory to growth and enzyme production for all the three strains at all concentrations tested. Mn^{2+}

at 0.05 mM concentration was found to inhibit the growth of *V. fluvialis*. For *B. circulans* and the *Vibrio* sp. Mn^{2+} at 0.02mM and 0.05mM concentrations were inhibitory. $CaCl_2$ and KH_2PO_4 were found to enhance the enzyme production considerably in *Bacillus circulans*. The enhancing effect of Ca^{2+} ion on protease production in a marine *Pseudomonas* sp. also was reported by Sakata *et al.* (1977). Na^+ and K^+ ions are expected to maintain cell integrity and stimulate cell growth. Mg^{2+} is intimately involved in the maintenance of the structural integrity of the cell envelope and affects the resistance of the cells to stress. It also prevents cell disruption and stimulates cell growth. Ca^{2+} ions are thought to stimulate the production and release of protease from the cells.

3.5.7 Effect of carbon sources on growth and protease production

Extracellular enzymes are usually susceptible to catabolite repression by rapidly metabolized carbon sources. Earlier studies have indicated a reduction in protease production due to catabolite repression by glucose (Hanlon *et al.*, 1982; Frankena *et al.*, 1985; Frankena *et al.*, 1986; Kole *et al.*, 1988; Secades and Guijarro, 1999). Similar results were obtained in the present study, where protease production was very low in *V.fluvialis* when 0.6% glucose was added to the medium, though growth was not affected. The same was true with mannose and sucrose at this concentration. For *B. circulans* glucose at 0.4% and0.6% and mannose at 0.6% concentrations inhibited protease production. On the other hand Zamost *et al.* (1990) correlated the low yields of protease production with the lowering of pH by the rapid growth of the organism. In commercial practice, high carbohydrate concentrations were found to repress enzyme production. Therefore, carbohydrate was added either continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited and thereby reducing the power requirements (Aunstrup, 1980). However 1.5-fold increase in production of protease by a *Pseudomonas maltophila* strain was reported by the use of a glucose medium over peptone medium (Kobayashi *et al.*, 1985).

Increased yields of alkaline proteases were reported by several workers who used different sugars such as lactose (Malathi and Chakraborthy, 1991), maltose (Tsuchiya *et al.*, 1991), sucrose (Phadatare *et al.*, 1993) and fructose (Sen and Satyanarayana, 1993) as sources of carbon. However, a repression in enzyme synthesis was observed with these ingredients at higher concentrations. Whey, a waste byproduct of dairy industry containing mainly lactose and salts has been demonstrated as a potential substrate for alkaline protease production (Donaghy and McKay, 1993; McKay, 1992) Similarly maximum alkaline protease secretion was observed in *Thermomonospora fusca* YX when pure cellulose was used as the principal carbon source (Gusek *et al.*, 1988).

In the present study the use of molasses, a byproduct in the beverage industry was found as a good source of carbon for protease production. It gave good enzyme yield comparable with the other carbon sources. Therefore, molasses could be suggested as a cheap raw material for the production of the alkaline proteases.

Madan *et al.*, (2000) reported the production of alkaline protease by a mutant of *Bacillus polymyxa* and the carbon source, which induced better production of the protease, was glucose when compared to starch, maltose mannitol or sucrose. Starch was reported to be the best carbon source for different *Bacillus* sp. (Sinha and Satyanarayana, 1991; Kembhavi *et al.* (1993; Gajju *et al.*, 1996).

Joo *et al.* (2002) optimized the production medium for *Bacillus* serine protease and found tryptic soy broth as the most suitable medium. Addition of 1% glucose and lactose to the medium reduced enzyme production to 45% and 25% respectively. But in some strains like *Bacillus* sp. AR-009 and *Bacillus licheniformis* ATCC 21415 (Gessesse, 1997; Mehrotra *et al.*, 1999) glucose and lactose were found to be effective carbon sources. For the alkalophilic *Bacillus* sp. reported by Sumandeep *et al.* (1999), the production was highest in a medium containing glucose, peptone and yeast extract. The addition of glucose to the medium was found to be reducing the protease

production in *Vibrio alginolyticus* strain (Long *et al.*, 1981), in *Pseudomonas maltophila* (Boethling, 1975) and in *Staphylococcus aureus* (Yoshikawa *et al.*, 1974), suggesting that in the absence of sugars (e.g. glucose), proteases play a role in supplying, peptides and amino acids, as carbon and energy sources in addition to being the nitrogen source. Thus, protease synthesis may be repressed when the energy status of the cells is high. This kind of regulatory mechanisms has been reported for proteases of other bacteria like *Vibrio* strain SA1 (Wiersma *et al.*, 1978 a), *Pseudomonas aeruginosa* (Whooley *et al.*, 1983).

3.5.8 Effect of nitrogen sources on growth and protease production

In most microorganisms both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. Alkaline protease comprises 15.6% nitrogen and its production is dependent on the availability of both carbon and nitrogen in the medium (Kole *et al.*, 1988). Although complex nitrogen sources are usually used for alkaline protease production, the requirement for a specific nitrogen supplement differs from organism to organism.

In the present study, low levels of protease production were observed with the use of inorganic nitrogen sources. Organic nitrogen sources were giving better production with all the three strains tested, casein being a very good source for all the three strains. For B15, protease production was significantly higher when casein was used as the sole source of nitrogen followed by peptone and tryptone. For V10, casein and yeast extract were found to be most suitable for protease production, followed by beef extract and peptone. For V26, maximum enzyme production was obtained with beef extract and casein followed by tryptone and yeast extract. Similar observations were made by Ong and Gaucher (1976) and Phadatare *et al.* (1993). They suggested tryptone (2%) and casein (1- 2%) as excellent nitrogen sources for protease production. The use of soy meal and casein for protease production has been reported by several workers (Chandrasekaran and Dhar, 1983; Tsai *et al.*, 1988; Fujiwara and Yamamoto 1987; Gomaa *et*

al., 1990; Taki *et al.*, 1990; Sen and Satyanarayana, 1993; Cheng *et al.*, 1995). The inductive role of casein in protease production has been reported by Sakellaris and Gikas (1991). Casein supported protease production compared to other nitrogen sources in *B. polymyxa* and *Psudomonas* sp. (Madan *et al.*, 2000). Joo *et al.*, (2002) reported addition of 1% casein enhanced enzyme production by approximately 30%.

Among the organic nitrogen sources gelatin, was found to give a slightly decreased growth compared to the other organic nitrogen sources, in all the three strains, and also less protease production in V26. A similar observation was reported in *Y.ruckeri* that did not grow in the presence of sodium caseinate or gelatin, after 24 hr. of incubation (Secades and Guijarro, 1999). This suggested that the basal level of protease was insufficient, during early exponential growth, to release enough peptides and therefore, there was a lack of induction of enzyme production and hence a lack of growth. Alternatively, low levels of particular amino acids in those proteins could explain the inability of bacteria to grow on these substrates.

Corn steep liquor (CSL) was found to be a cheap and suitable source of nitrogen by some workers (Fujiwara and Yamamoto, 1987; Malathi and Chakraborthy, 1991; Sen and Satyanarayana, 1993). Apart from serving as a nitrogen source CSL also provided several micronutrients, vitamins and growth promoting factors. However, their use is limited by its seasonal, and inter- batch variability. Suitable nitrogen sources as substitutes for CSL are still being evaluated.

Similar to the present study, low levels of alkaline protease production were reported with the use of inorganic nitrogen sources on the production medium (Chandrasekaran and Dhar, 1983; Sen and Satyanrayana 1993; Chaphalkar and Dey, 1994). Addition of certain amino compounds was shown to be effective in the production of extracellular enzymes by alkalophilic *Bacillus* sp. (Ikura and Horikoshi, 1987). In some studies use of oil cakes tried as nitrogen source did not favour enzyme production (Sinha and Satyanarayana 1991; Sen and Satyanarayana 1993). The production of

a 47-kDa protease in *Fusarium* sp. was influenced by the composition of the culture medium (Secades and Guijarro, 1999). Growth and protease production was optimum in peptone medium whereas there was no activity in a medium containing casaminoacids, suggesting that the intact peptides were necessary in the induction process. A similar behaviour has been observed for *Vibrio* sp. (Dreisbach and Merkel, 1978), *Serratia marcescens* (Braun and Schmitz, 1980) and *Yersinia chrysanthemi* (Stevenson, 1997).

Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources like amino acids, or ammonium ion concentrations in the medium (Cruegar and Cruegar, 1984; Giesecke *et al.*, 1991). Glycine appeared to have inhibitory effects on protease production in a *Bacillus* sp. (Ikura and Horikoshi, 1987). Casamino acids were also found to inhibit protease production (Ong and Gaucher, 1976). The presence of ammonium significantly reduced protease production in *A.salmonicida* (Liu and Hsieh, 1969), *Vibrio* strain SA1 (Wiersma *et al.*, 1978 a), *A. hydrophila* (O'Reilly and Day, 1983), and the fish pathogen *Yersinia ruckerii* (Secades and Guijarro, 1999). Ammonium specific repression is likely to be the explanation. However no repression in the protease activity was reported with the use of ammonium salts in a strain of *Bacillus licheniformis* (Nehete *et al.*, 1986). An increase in protease production by the addition of ammonium sulphate and Potassium nitrate was also observed by Sinha and Satyanarayana (1991). Similarly sodium nitrate (0.25%) was found to be stimulatory for alkaline protease production (Banerjee and Bhattacharya, 1992). The replacement of soyabean flour with ammonium sulphate in a fed batch process proved cost-effective and resulted in the elimination of unpleasant odours as well (Mao *et al.*, 1992).

It has been well demonstrated that the various physicochemical factors had profound effect on the alkaline protease production in the three strains of interest, and maximum production of protease could be obtained by the manipulation of the culture medium in accordance with the above findings.

CHAPTER 4
PURIFICATION AND CHARACTERISATION OF PROTEASES

Crude preparations of alkaline proteases are usually employed for commercial use. Nevertheless, purification of the enzyme is important from the perspective of developing better understanding of the functioning of the enzyme (Tsai *et al.*, 1988; Takagi 1993). There are no set rules for the purification of the proteases. After separating the cells from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by means of ultra filtration (Kang *et al.*, 1999; Smacchi *et al.,* 1999), salting out by solid ammonium sulphate (Hutadilok-Towatana *et al.,* 1999; Kumar 2002), or solvent extraction methods using acetone (Kwon *et al.*, 1994; Kumar *et al.*, 1999; Thangam and Rajkumar 2002) and ethanol (El Shanshouri *et al.*, 1995). In addition, other methods, such as the use of PEG-35 000(Larcher *et al.*, 1996) activated charcoal (Aikat *et al.*, 2001), heat treatment of enzyme (Rahman *et al.*, 1994) and lyophilization (Manonmani and Joseph 1993) are also used for concentration of alkaline proteases.

To further purify the enzyme, a combination of one or more techniques is applied, viz. affinity chromatography, ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and gel filtration chromatography. Other methods of choice, including aqueous two phase systems (Sinha *et al.*, 1996), dye ligand chromatography (Cowan and Daniel 1996), and foam fractionation (Banerjee *et al.*, 1993), have also been employed on a small scale and still await commercial exploitation.

To have a better understanding of the various properties of the selected proteases and to find the conditions that are most suitable for their optimum activity, an attempt has been made to characterize these enzymes.

4.1 Review of literature

Extensive studies have been conducted for the characterization of alkaline proteases from *Bacillus* sp. such as *B. amyloliquefaciens* (Matsubara *et al.*, 1965; Markland *et al.*, 1967; Wells *et al.*, 1983), *B.subtilis* (Tsuru *et al*, 1967) and *B. licheniformis* (Smith *et al.*, 1968; Jacobs *et al.*, 1985). A thermostable alkaline protease has been characterized by Manachini *et al*

(1988). Takami *et al.* (1989, 1990) described an extremely thermostable alkaline protease from *Bacillus* sp. no. AH- 101, which was elastolytic as well as keratinolytic. Takii *et al.* (1990) described the crystallization and characterization of an alkaline serine protease from *B. alcalophilus* sub sp.*halodurans* KP 1239. Sumandeep *et al* (1999) characterized a thermo stable protease of an alkalophilic *Bacillus* sp. isolated from soil. Purification and characterization of a *Bacillus polymyxa* alkaline protease has been reported by Madan *et al.* (2000). Production and purification of protease from a *Bacillus subtilis* strain was reported by Yang *et al* (2000). Characterization of an SDS stable protease of *Bacillus* sp. was reported by Oberoi *et al.*(2001). A *Bacillus horikoshii* protease was characterized by Joo *et al.* (2002) Huang *et al.* (2003) reported alkaline protease production by *Bacillus pumilus* and its dehairing function. Ghorbel *et al.* (2003) conducted stability studies on the protease from *B.cereus*.

Several extracellular proteases have been obtained from actinomycetes and characterized as serine- and metallo-proteases. Chandrasekaran *et al* (1986) described characteristics of a protease from *Streptomyces moderatus*. A *Streptomyces* sp. protease was purified by Sampath *et al.* (1997). A *Streptomyces albidoflavus* protease was characterized by Bressollier *et al.* (1999). Serine protease of *Streptomyces cyaneus* was purified and characterized (Petinate *et al.*, 1999). Purification and characterization studies of a *Streptomyces tendae* protease were conducted by Seong *et al.* (2004).

Jobin and Grenier (2003) described the identification and characterization of four proteases produced by *Streptococcus suis*. Alkaline proteases from gram-positive bacteria especially *Bacillus* sp. have been well studied, and the information about the expression, processing and industrial application have been markedly accumulated. However, in gram-negative bacteria only limited studies have been carried out. The toxicity and poor ability to secrete proteins have limited their study mainly to the genetic level and comparatively little is known of them. Still, a number of reports are available on protease production and purification in gram-negative bacteria. Purification of a

protease from a marine *Vibrio* sp. was described (Merkel and Sipos, 1971). Wiersma *et al.* (1978 b) reported the purification and properties of two extracellular proteases by *Vibrio* SA1). Kwon *et al.* (1994) described alkaline proteases production by *Vibrio metschnikovii* strain RH 530 isolated from wastewater. Protease of a marine *Pseudomonas* had been studied by Makino *et al.*, (1983). Purification of an alkaline protease produced by *Pseudomonas maltophila* has been described by Kobayashi *et al.* (1985). Purification and properties of a marine *Pseudomonas* was studied by Sakata *et al.* (1977). A metalloprotease from a moderately halophilic marine *Pseudomonas* had been described (Qua *et al.*, 1981). Kwon *et al.* (1994) purified and characterized one major protease and one minor protease from a *Pseudomonas* sp. Extracellular protease from *Pseudomonas fluorescens* CY 091 was purified and characterized (Liao and Mc Callus, 1998).

Protease from a strain of *Aeromonas proteolytica* has been characterized by Griffin and Prescott (1970). *A.hydrophila* protease was characterized by Pansare *et al.* (1986). A rapid purification strategy has been adopted for *Aeromonas proteolytica* aminopeptidase and its crystallization has been carried out (Schalk *et al.*, 1992). Proteolytic activity of a ruminal bacterium *Butyrivibrio fibrisolvens* was reported by Cotta and Hespel (1986). An *Achromobacter lyticus* protease was purified and characterized by Masaki *et al.* (1986). A cysteine protease of a gram-negative anaerobe, *Porphyromonas gingivalis* was characterized by Kontani *et al.* (1996). Characteristics of a new alkalophilic *Microbacterium* were described by Gessesse and Gashe (1997). Purification and characterization of protease from fish pathogen *Yersinia ruckeri* was done by Secades and Guijarro (1999). Purification and characterization of an extracellular serine protease from *Xenorhabdus nematophila*, a bacterium pathogenic to insects has been reported (Caldas *et al.*, 2002). Thangam and Rajkumar (2002) purified and characterized an alkaline protease from *Alcaligenes faecalis.* Secades *et al.* (2003) purified and characterized a protease from a fish pathogen *Flavobacterium psychrophilum.*

Production and characterization of thermophilic extracellular protease by an extreme thermophile *Thermus aquaticus* has been reported by Matsuzawa *et al.* (1983). A protease from a mutant strain of an extreme halophile *H.halobium* protease was studied by Izotova *et al.* (1983). Another *Halobacterium halobium* protease was characterized by Ryu *et al.* (1994). Proteases from Antarctic soil bacteria were characterized by Wery *et al.* (2003).

Lopez-Llorca (1990) reported the purification and properties of a nematophagous fungus *Verticillium suchlasporium.* Purification and characterization of a novel protease from a white rot fungus, *Pleurotus ostratus* were described by Palmieri *et al.* (2001). Bararta (2002) reported purification and characterization of a trypsin like protease of *Fusarium oxysporum.* In an *Aspergillus parasiticus* protease, purification and characterization studies were conducted by Tunga *et al.* (2003). Purification and characterization of a protease from a fungus *Paecilomyces lilacinus* was described by Khan *et al.* (2003).

Purification studies on proteases of non-microbial origin also are available, which resemble microbial proteases in several respects. Purification of a ginger protease was reported (Ohtsuki *et al.*, 1995). Isolation, purification and preliminary characterization of cryophilic proteases of Antarctic krill (*Euphasia superba*) were described by Salamanca *et al.* (2002). Jonsdottir *et al.* (2003) reported the purification of a recombinant trypsin from the Atlantic code.

4.2 Materials and methods

Purification of the alkaline proteases from the three selected cultures was done by ammonium sulphate precipitation followed by dialysis. To the chilled cell free culture fluid, solid ammonium sulphate was added with gentle stirring, up to 80% saturation (561g/l) and kept overnight at 4°C. The precipitate was collected by centrifugation and was dissolved in a minimum quantity of 0.2M phosphate buffer at pH 7.5. The solution was dialyzed

overnight against the same buffer at 4°C. This dialyzed enzyme was used for further studies.

4.2.1 Determination of protein

Protein was determined by the method of Lowry *et al.* (1951) with Bovine Serum Albumin (BSA) as standard. The concentration of protein during purification studies was calculated from the standard curve.

4.2.2 Determination of molecular weight by SDS PAGE

The homogeneity of the dialysate was checked by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE). SDS PAGE was performed as described by Laemmli, (1970). 10% acrylamide gel was prepared according to standard procedure and run the gel at 60 Volts till the dye crossed the stacking gel margin and then increased to 140 Volts till the end. The unit was switched off when the dye front reached the bottom of the running gel and then transferred the gel to distilled water and then to the Coomassie Brilliant Blue staining solution. Kept in stain for 1½ hr. Then the gel was transferred to the destaining solution until the bands became clear and the background completely destained.

Molecular weights of the enzymes were determined by interpolation from a linear semi-logarithmic plot of relative molecular weight versus the Rf value (relative mobility) using standard molecular weight markers (3.5, 15.9, 35.5, 89.1, 141.3 and 205 kDa).

Composition of reagents used for SDS PAGE

Resolving Gel mixture (10%)

Acrylamide stock	10 ml
4X separating gel buffer	7.5 ml
10% SDS	0.3 ml
Distilled water	12.1ml
10% APS	150 µl
TEMED	10 µl

Acrylamide stock solution

Acrylamide	30 g
Bis- acrylamide	0.8 g
Distilled Water	100 ml

Stacking gel

Acrylamide stock	1.33 ml
4Xseparating gel buffer	2.5 ml
10% SDS solution	0.1 ml
Distilled water	6 ml
10% APS	50 µl
TEMED	5 µl

Resolving Gel buffer (1.5 M Tris HCl pH 8.8)

Tris	18.15 g
Distilled water	75 ml

HCl was added to adjust the pH and made up to 100 ml.

Stacking gel buffer (0.5M Tris-HCl pH 6.8)

Tris	3 g
Distilled water	40 ml

HCl was added to adjust the pH and made up to 100 ml

Tank Buffer (Gel Running Buffer)

Tris	3.028 g
Glycine	14.41 g
SDS	1 g
Distilled water	1000 ml

Sample buffer

4X stacking gel buffer	2.5 ml
10% SDS solution	4 ml

Glycerol	2 ml
Bromophenol blue	2 mg
β-Mercaptoethanol	1ml

Added distilled water up to the final volume of 10 ml, made 0.5 ml aliquots and stored.

Running gel overlay solution

4X Running Gel Buffer	25 ml
10% SDS	1ml

Added distilled water to a final volume of 100 ml.

Water saturated n-butanol

N-butanol	50 ml
Distilled Water	5ml

Staining solution

Methanol	500 ml
Coomassie Brilliant Blue R	0. 5g
Acetic acid	100 ml
Distilled water	400 ml

Coomassie Brilliant Blue was dissolved in methanol completely and water was then added to it.

Destaining solution

Distilled water	880ml
Methanol	50 ml
Acetic acid	70 ml

Sample preparation for SDS PAGE

Equal volumes of sample to be electrophoresed and 2X sample buffer were mixed and kept in boiling water bath for 90 seconds and then it was placed in ice till loading.

4.2.3 Zymography (Activity Gel)

SDS-PAGE zymograms were performed as described by Schmidt *et al.* (1988). 10% running gel was prepared according to the standard procedure. While preparing the running gel, casein (according to Hammersten-10 mg/ml in H_2O) was added to get the substrate concentration of 0.1%(1mg/ml). One part of the sample was mixed with one part denaturing sample buffer (2X) and let stand for 10 minutes at room temperature. The sample was not heated. Samples were applied and the gel was run with running buffer according to the standard running conditions. The run was complete when the bromophenol blue tracking dye reached the bottom. After running, the gel was gently transferred to zymogram renaturing buffer (10X) diluted 1: 9 and gently washed in this buffer for 3 x 15 minutes at room temperature so as to remove SDS from the gel. Zymogram renaturing buffer was decanted and replaced with 1X zymogram developing buffer and incubated at 37° C for at least four hours. The optimal result was obtained by varying the sample load and incubation time. Then the gel was stained with Coomassie Brilliant blue R-250 for 30 minutes. For maximum contrast a stain concentration of 0.5% (w/v) instead of the usual 0.15 was used. Gels were destained with the destaining solution (Methanol: Acetic acid: water = 50:10:40). Areas of protease activity appeared as clear bands in a dark blue background.

Composition of reagents used for zymography

Sample buffer (2X)

0.5M Tris-HCl pH 6.8	2.5 ml
Glycerol	2 ml
10%(w/v) SDS	4 ml
0.1% Bromophenol Blue	0.5 ml
Distilled water	10 ml

Running buffer

	10X	1X
Tris base	29 g	2.9 g
Glycine	144 g	14.4 g
SDS	10 g	1 g

pH was adjusted to 8

Made up to 1 litre with distilled water.

Sample buffer	2X
0.5M Tris HCl, pH 6.8	2.5 ml
Glycerol	2 ml
10% SDS	4 ml
0.1% Bromophenol Blue	0.5 ml

Made up to 10 ml with distilled water.

Renaturing buffer

Triton X 100, 25%(v/v) water

Developing buffer (Incubation buffer)

$CaCl_2$	1.47g
Tris base	6.06g
$CaCl_2$	1.47g
NaCl	2.92g
Brij-35	0.5g

Made up to 800 ml with distilled water, pH-adjusted to7.6 with HCl. Added water to final volume of 1 litre.

4.2.4 Assay of alkaline protease

The measurement of the enzyme activity was done and the enzyme unit was defined as described in section 2.2.5.4

4.2.5 The enzyme kinetics

The enzyme kinetics was studied by determining the effect of substrate concentration and various physico chemical parameters on the enzyme activity.

4.2.5.1 Effect of substrate concentration on the enzyme activity

Varying concentrations of substrate (casein according to Hammersten) were used (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/ml) in the reaction mixture. The assay was conducted at standard conditions and enzyme activity was measured. Line Weaver-Burk plot was constructed by plotting the reciprocals of substrate concentrations on X-axis, and reciprocals of enzyme velocity (enzyme activity in units) on Y-axis. From the plot, the Km value and Vmax of the enzyme were determined.

4.2.5.2 Effect of various physico chemical factors on enzyme activity and stability

The influence of various factors such as the effect of shaking speed, pH, temperature, various metallic salts, various chemical inhibitors of different classes of proteases, on protease activity and stability were studied.

4.2.5.2.1 Effect of pH on the enzyme activity and stability

The effect of pH on protease activity was tested using different buffers ranging from pH 7 to pH 13 in the reaction mixture. Buffers used were phosphate buffer (pH 7-8), Glycine-NaOH buffer (pH 9-10) and dilute NaOH for pH 12 and 13. The enzyme activity was measured using the standard assay procedure. Stability of the enzyme at various pH values was tested by incubating the enzyme in buffers of different pH for 1hr. After incubation, the residual enzyme activity was measured at the optimum pH of the enzyme.

4.2.5.2.2 Effect of temperature on the enzyme activity and stability

Effect of temperature on enzyme activity was determined by incubating the reaction mixture at different temperatures ranging from 20°C to 85°C and measuring the enzyme activity. To study the effect of temperature on enzyme stability, the dialyzed enzyme was pre incubated at different temperatures ranging from 20°C to 85°C for one hour and the residual activity was assayed at the optimum temperature of enzyme activity.

4.2.5.2.3 Effect of various metallic salts on the enzyme activity and stability

Effect of various ions on the enzyme activity and stability was determined by pre incubating the enzyme in various concentrations (1mM, 2.5mM, 5mM) of different metallic salts ($HgCl_2$, $ZnCl_2$, $CoCl_2$, $CuSO_4$, $CaCl_2$, $MnSO_4$, $MgSO_4$ NaCl, KCl and $Fe(PO4)_3$) for 1 hr. and then assaying under standard conditions.

4.2.5.2.4 Effect of various protease inhibitors on enzyme activity

The enzymes were pre-incubated with varying concentrations (5mM, 2.5mM, and 1mM) of various protease inhibitors like EDTA, PMSF (Phenyl methyl sulphonyl fluoride), Iodo Acetic Acid (IAA), Urea, Dithiothreitol, Mercapto ethanol, SDS and 1,10 Phenanthroline for 1hr. and then assaying the residual activity at standard conditions of assay. From this the inhibitory effect of various compounds on the enzyme and the nature of the alkaline proteases were determined.

4.3 Statistical analysis

For analyzing the data, Analysis of Variance was done. Single factor ANOVA was done for all the parameters except for the effect of metallic salts, for which two way ANOVA without replication was used, and for different inhibitors on enzyme activity two way ANOVA with replication was used.

4.4 Results

Results are presented in graphs, and the ANOVA tables are also given with the Least Significant Difference (LSD). The results of the ANOVA are given along with the general explanation of the results. The values are given as Appendix 3.

4.4.1 Purification of the alkaline proteases

Proteases were purified by ammonium sulphate precipitation followed by dialysis. For all the three proteases 500 ml of the crude enzyme was purified. After $(NH_4)_2SO_4$ precipitation the volumes recovered were 16.25 ml, 15.5 ml and 20.2 ml for B15, V10 and V26 proteases respectively. The protein content, enzyme activity and specific activity are presented in table 4.1- 4.3

Table 4.1 Purification of alkaline proteases of *B. circulans*

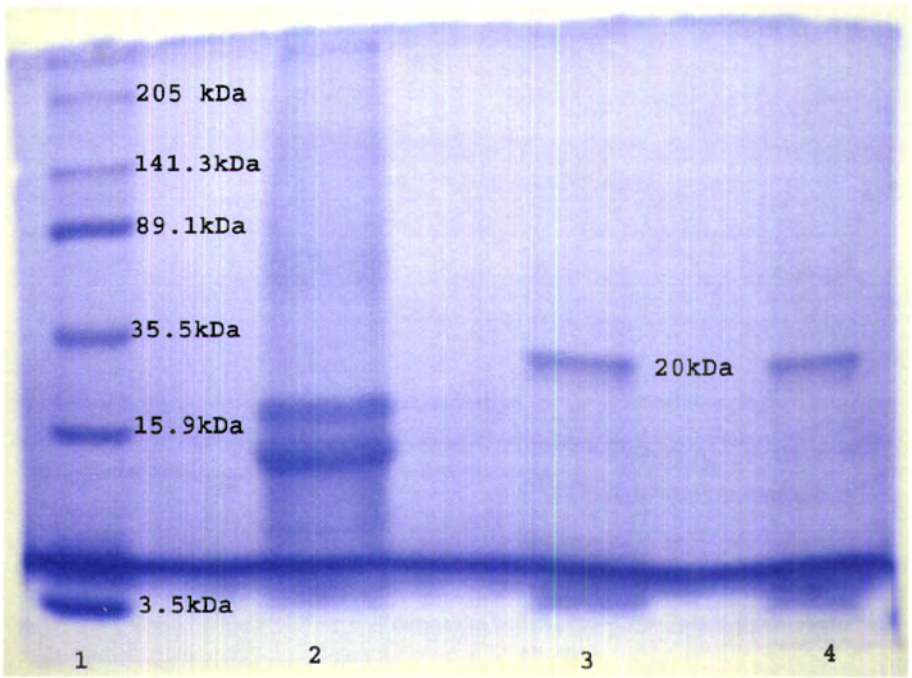
Purification step	Volume (ml)	Total Activity (Enz.units)	Total Protein (mg)	Specific Activity (Enz.units/mg of protein)
Culture Filtrate	500	12155.39	2330.769	5.221
$(NH_4)_2SO_4$ precipitation	16.25	11166.34	2006.095	5.5662

Table 4.2 Purification of alkaline proteases of *V. fluvialis*

Purification step	Volume (ml)	Total Activity (Enz.units)	Total Protein (mg)	Specific Activity (Enz.units/mg of protein)
Culture Filtrate	500	15311.54	1867.31	8.199
$(NH_4)_2SO_4$ precipitation	15.5	14617.75	1431.331	10.212

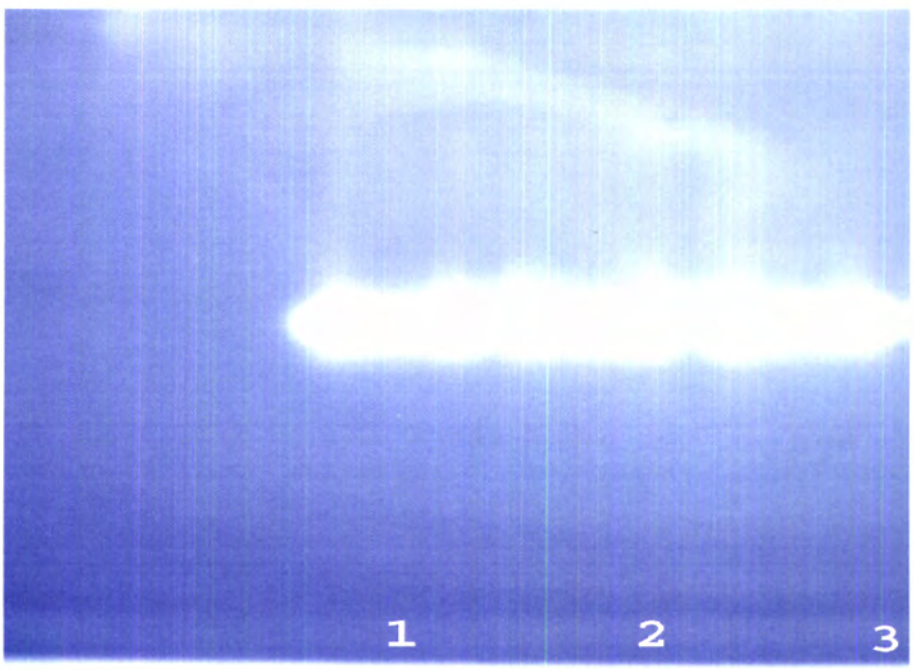
Table 4.3 Purification of alkaline protease of *Vibrio* sp.

Purification step	Volume (ml)	Total Activity (Enz.units)	Total Protein (mg)	Specific Activity (Enz.units/mg of protein)
Culture Filtrate	500	25001.17	2519.165	9.9243
$(NH_4)_2SO_4$ precipitation	20.2	12251.62	1123.71	10.9



Lane 1 Standard molecular weight markers
Lane 2 Negative control
Lane 3 and 4 : *Bacillus circulans* protease

SDS PAGE

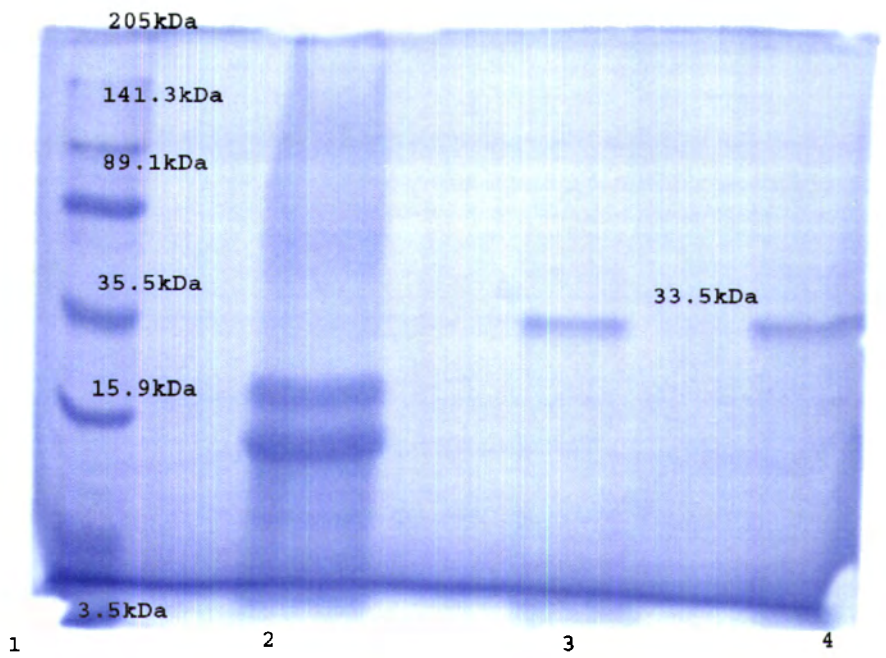


Lanes 1,2 and 3: *Bacillus circulans* Protease

ZYMOGRAM

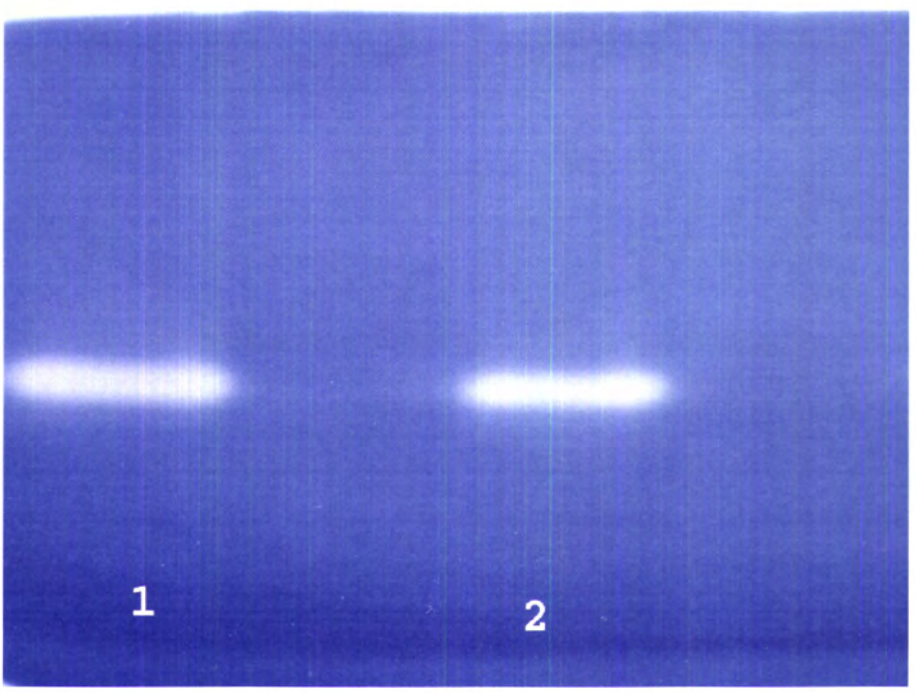
SDS PAGE and Zymogram analysis of *Bacillus circulans* protease

PLATE 5



Lane 1 Standard molecular weight marker
Lane 2 Negative control
Lane 3 and 4: *Vibrio fluvialis* protease

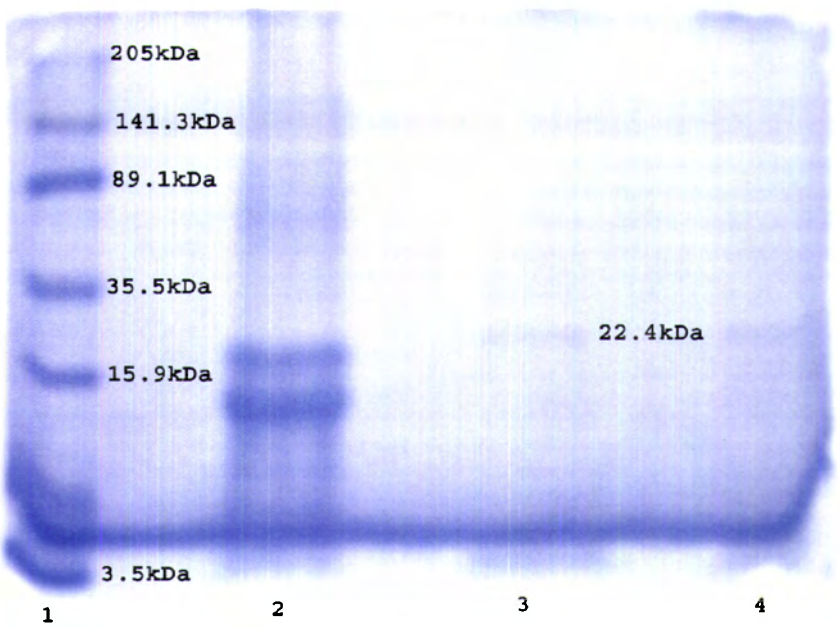
SDS PAGE



Lanes 1and 2: *Vibrio fluvialis* protease

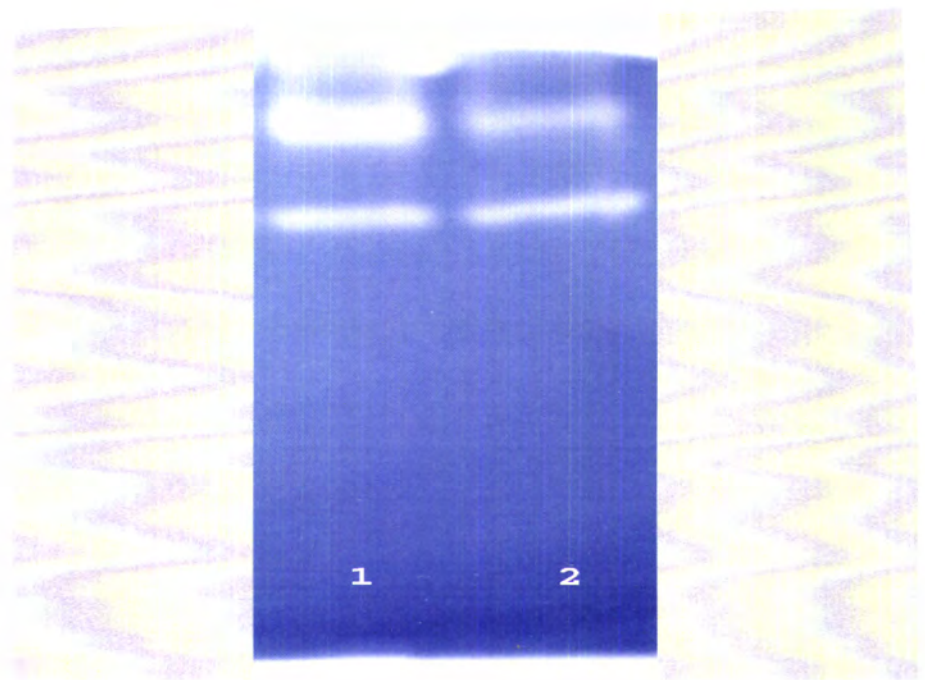
ZYMOGRAM

SDS PAGE and Zymogram analysis of *Vibrio fluvialis* protease



Lane 1 Standard molecular weight markers
Lane 2 Negative control
Lane 3 and 4: *Vibrio* sp. protease

SDS PAGE



Lanes 1 and 2: *Vibrio* sp. protease

ZYMOGRAM

SDS PAGE and Zymogram analysis of *Vibrio* sp. protease

PLATE 7

4.4.2 Molecular weight determination

Molecular weights of the three alkaline proteases were determined by SDS PAGE analysis. (Plates 5, 6 and 7). They are as follows

B. circulans (B15)	20 kDa
V. fluvialis (V10)	33.5 kDa
Vibrio sp. (V26)	22.4 kDa

4.4.3 Effect of pH on protease activity

B15

Though the optimum pH for protease from B15 was 7, it was active over pH values 7 to 10. It had nearly 75% of the maximum activity at pH 8, 9 and 10 but retained only 25% activity at pH 11 (Fig. 4.1, Table 4.4).

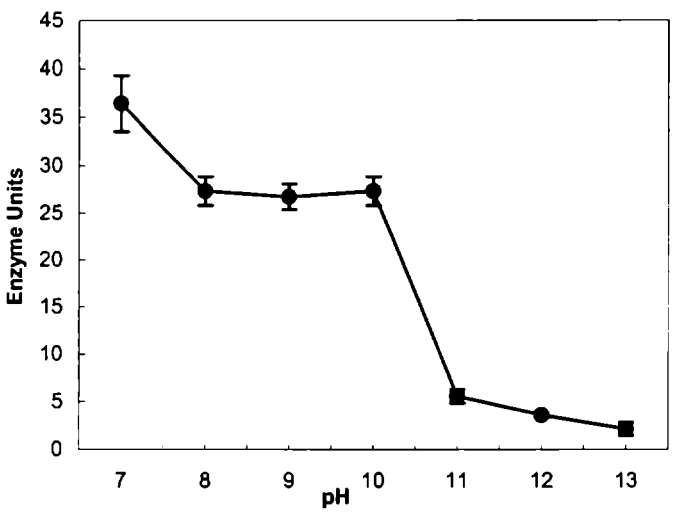


Fig.4.1. Effect of pH on activity of *B. circulans* protease

Table 4.4 ANOVA for the effect of pH on the activity of *B. circulans* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	3609.088	6	601.5147	270.9624	<0.001
Within Groups	31.07887	14	2.21992		
Total	3640.167	20			

LSD=3.6216

The statistical analysis showed that pH had significant influence on enzyme activity.

V10

Protease from V10 showed activity in the range of pH 7 to 11, the optimum was at pH 8. At pH 7 and 9 it had 98% of the maximum activity but only 46% and 31% at pH 10 and 11 respectively. At pH 12 it had negligible activity (Fig. 4.2; Table 4.5).

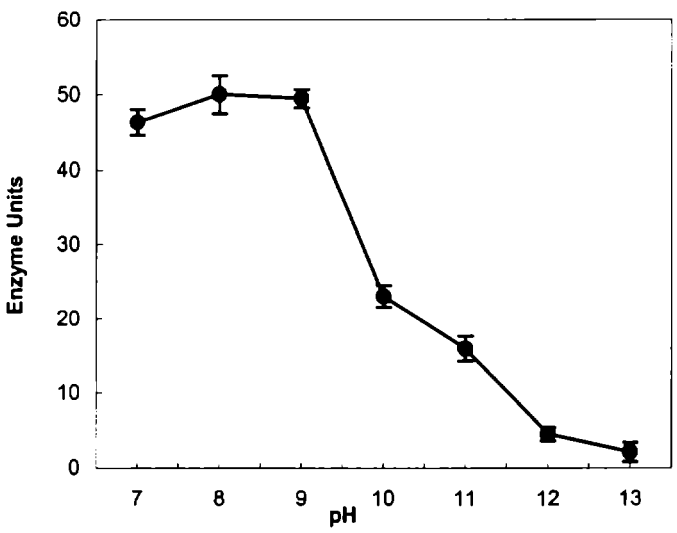


Fig.4.2. Effect of pH on activity of *V. fluvialis* protease

Table 4.5. ANOVA for the effect of pH on the activity of *V. fluvialis* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	8028.872	6	1338.145	524.379	<0.001
Within Groups	35.72607	14	2.551862		
Total	8064.598	20			

LSD = 3.8829

As seen in ANOVA, pH had significant effect on the activity of V10 protease.

V26

V26 protease showed activity in the range of pH 7 to 11(76% and 65%respectively), the maximum being at pH 9. At pH 7,8,10 and 11 it had 76%, 83%, 76%, 65% of activities respectively (Fig. 4.3, Table 4.6)

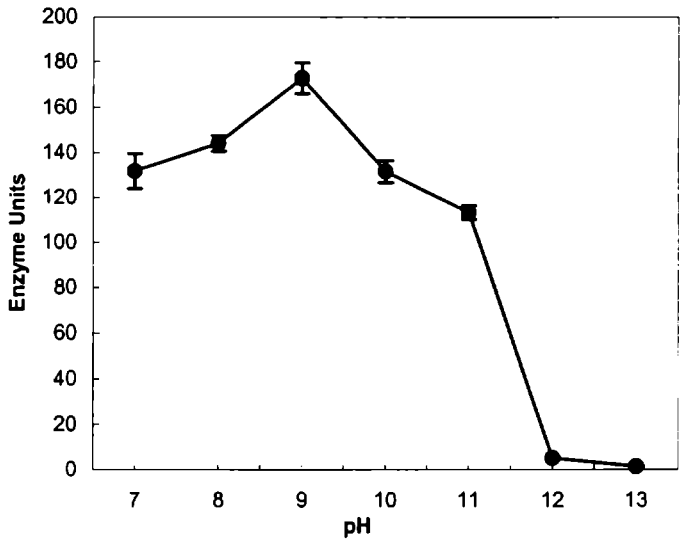


Fig.4.3. Effect of pH on activity of *Vibrio* sp. protease

Table 4.6. ANOVA for the effect of pH on the activity of *Vibrio* sp. protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	84627.16	6	14104.53	650.687	<0.001
Within Groups	303.4686	14	21.67633		
Total	84930.63	20			

LSD=11.31676

V26 protease activity was significantly influenced by change in pH, as seen from ANOVA.

4.4.4 Effect of pH on the stability of alkaline protease

B15

Regarding the pH stability, protease from B15 was stable between pH 7 and 11. Maximum stability was at pH 8. At pH 11, 52% of the activity was retained. At pH 7 and 10 the enzyme retained almost the same activity (Fig. 4.4, Table 4.7)

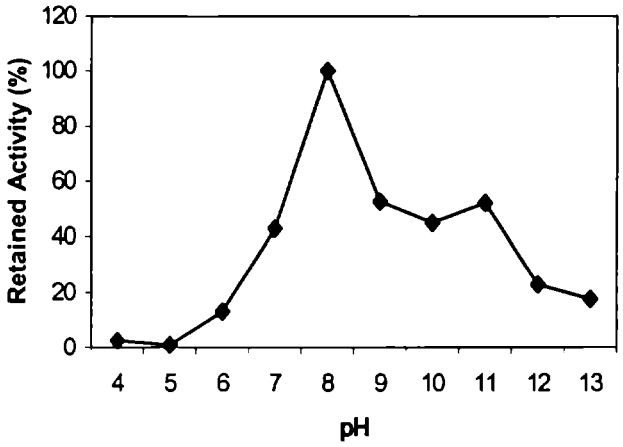


Fig. 4.4 Effect of pH on the stability of *B. circulans* protease

Table 4.7. ANOVA for the effect of pH on the stability of *B. circulans* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	64657.4	9	7184.155	650.5363	<0.001
Within Groups	552.1717	50	11.04343		
Total	65209.57	59			

LSD = 5.1803

The stability of B15 protease was significantly influenced by change in pH.

V10

The V10 protease showed a broader range of pH stability i.e. between pH 5 and 13. It had maximum stability at pH 8. From pH 5 the stability gradually increased and reached the maximum at pH 8, and then showed slight decrease, gradually up to pH 13 (Fig. 4.5, Table 4.8).

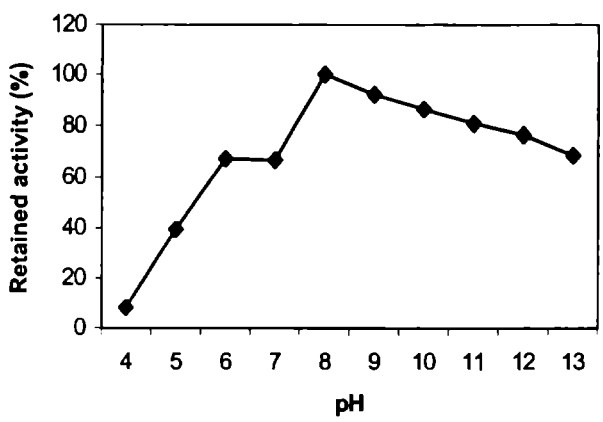


Fig. 4.5 Effect of pH on the stability of *V. fluvialis* protease

Table 4.8. ANOVA for the effect of pH on the stability of *V. fluvialis* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	7857.826	9	873.0918	78.70579	<0.001
Within Groups	554.6554	50	11.09311		
Total	8412.482	59			

LSD = 1.6298

There was significant variation in the stability of V10 protease with respect to pH.

V26

For V26 the pH stability range was between pH 6 and 13. Maximum stability was obtained at pH values 9, 10 and 11, the difference in values being not significant. From these observations we can see that the maximum pH stability was shown by V10 protease, followed by V26 and B15 (Fig. 4.6, Table 4.9).

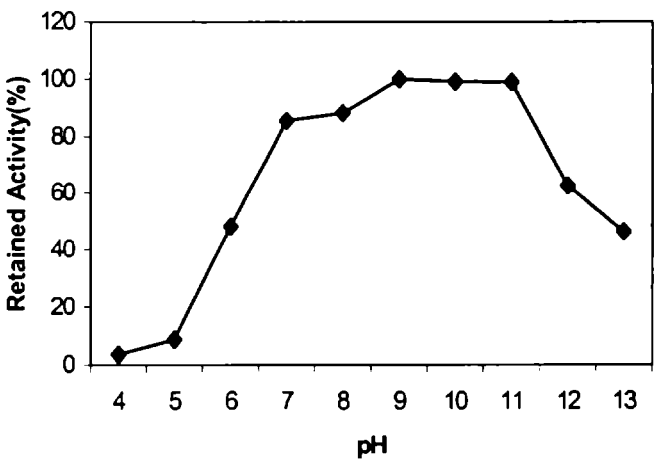


Fig. 4.6 Effect of pH on the stability of *Vibrio* sp. protease

Table 4.9. ANOVA for the effect of pH on the stability of *Vibrio* sp protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	23497.87	9	2610.874	278.078	<0.001
Within Groups	469.4499	50	9.388999		
Total	23967.32	59			

LSD = 4.7765

There was significant effect for pH on the activity of V26 protease.

4.4.5 Effect of temperature on enzyme activity

B15

For B15 the optimum temperature of activity was 40°C, and it was active over a wide range of temperatures from 15°C to 75°C having 29% and 49% of the maximum activity, respectively. It had more than 50% activity at a range of 30°C to 70°C (Fig. 4.7, Table 4.10).

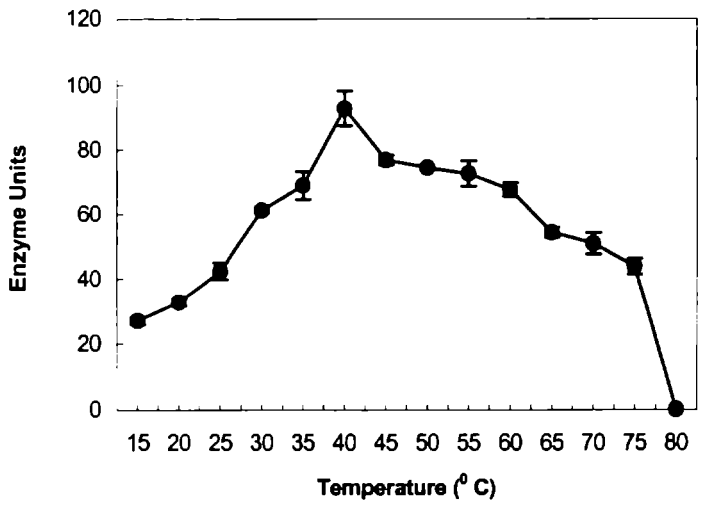


Fig. 4.7. Effect of temperature on the activity of *B. circulans* protease

Table 4.10. ANOVA for the effect of temperature on the activity of *B. circulans* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	22788.97	13	1752.998	245.9026	<0.001
Within Groups	199.6072	28	7.128828		
Total	22988.58	41			

LSD = 6.0234

Temperature significantly affected the activity of the B15 protease.

V10

V10 protease was also active over a wide range of temperatures, 25°C to 75°C. Optimum activity was at 55°C. At 60°C also it was equally active having no significant difference from that at 55°C. It had more than 85% of the maximum activity between 45°C and 75°C and more than 60% between 30°C and 40°C (Fig. 4.8, Table 4.11).

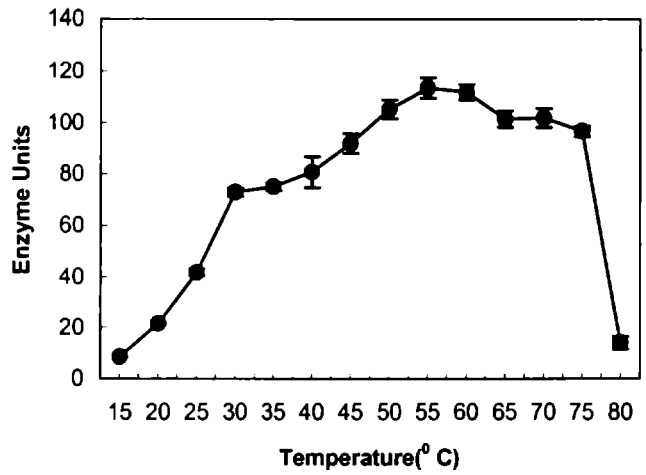


Fig. 4.8 Effect of temperature on the activity of *V. fluvialis* protease

Table 4.11. ANOVA for the effect of temperature on the activity of *V. fluvialis* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	64651.52	14	4617.966	525.6768	<0.001
Within Groups	263.544	30	8.784801		
Total	64915.07	44			

LSD = 6.655

V10 protease was significantly affected by temperature change.

V26

For V26 protease the maximum activity was obtained at 75°C, and then steeply decreased to 49 % of activity at 80°C. It was active over a wide range. (30°C to 80°C) (Fig. 4.9, Table 4.12).

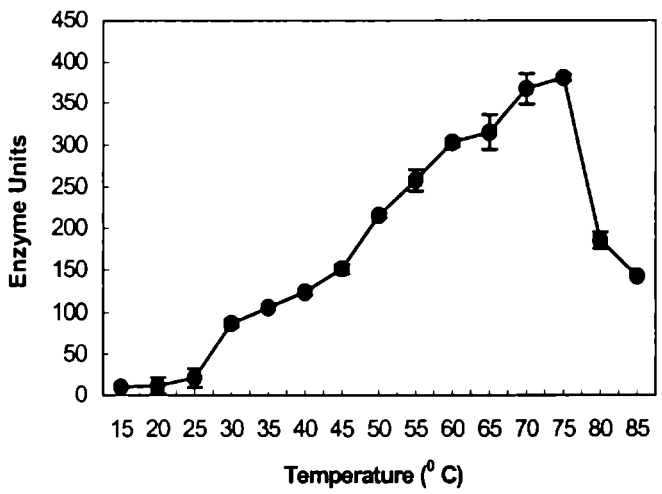


Fig. 4.9. Effect of temperature on the activity of *Vibrio* sp. protease

Table 4.12 ANOVA for the effect of temperature on the activity of *Vibrio* sp. protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	563825.5	13	43371.19	434.19	<0.001
Within Groups	2796.89	28	99.88893		
Total	566622.4	41			

LSD= 22.54726

Temperature had significant influence on V26 protease activity.

4.4.6 Effect of temperature on stability of the enzyme B15

B15 protease was stable up to 50°C. It retained almost 100% activity up to 30°C, i.e. most stable within a range of 0°C and 30°C. Then it was slightly decreasing and reached a residual activity of 55% at 50 °C and 26% at 55 °C (Fig. 4.10, Table 4.13).

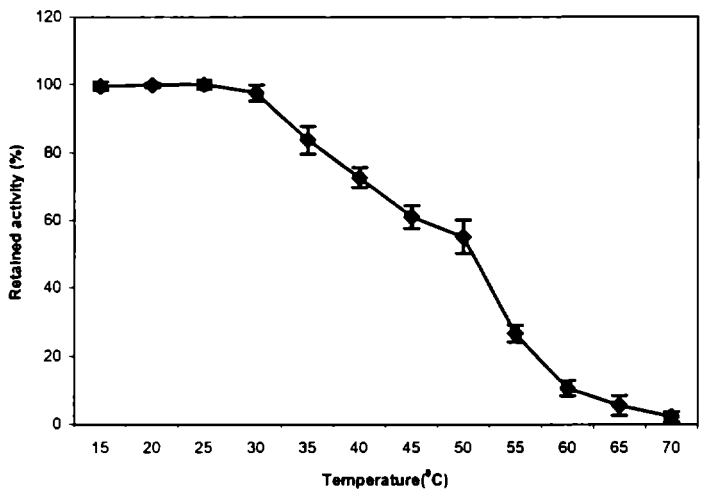


Fig. 4.10 Effect of temperature on the stability of *B. circulans* protease

Table 4.13. ANOVA for the effect of temperature on the stability of *B. circulans* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	52752.3	11	4795.664	626.857	<0.001
Within Groups	183.608	24	7.650332		
Total	52935.91	35			

LSD= 6.3166

B15 protease stability was significantly influenced by temperature change.

V10

V10 protease was absolutely stable up to a temperature of 50°C, and showed a decrease in stability and reached 42% at 55°C and 29% of residual activity at 60°C (Fig. 4.11, Table 4.14).

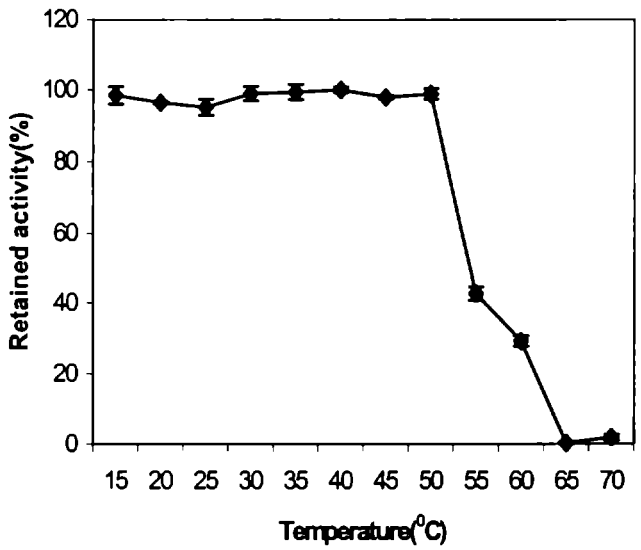


Fig. 4.11. Effect of temperature on the stability of *V. fluvialis* protease

Table 4.14. ANOVA for the effect of temperature on the stability of *V. fluvialis* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F	P-value
Between Groups	52690.2	11	4790.018	1919.545	<0.001
Within Groups	59.88941	24	2.495392		
Total	52750.09	35			

LSD= 1.33

Temperature significantly influenced the stability of V10.

V26

V26 protease showed considerable stability, over a wide range of temperature (0°C to 55°C). The stability studies of this enzyme revealed that it was absolutely stable up to 50°C. At 55°C it retained 71%, and 42% at 60°C and 17% at 65°C (Fig. 4.12, table 4.15).

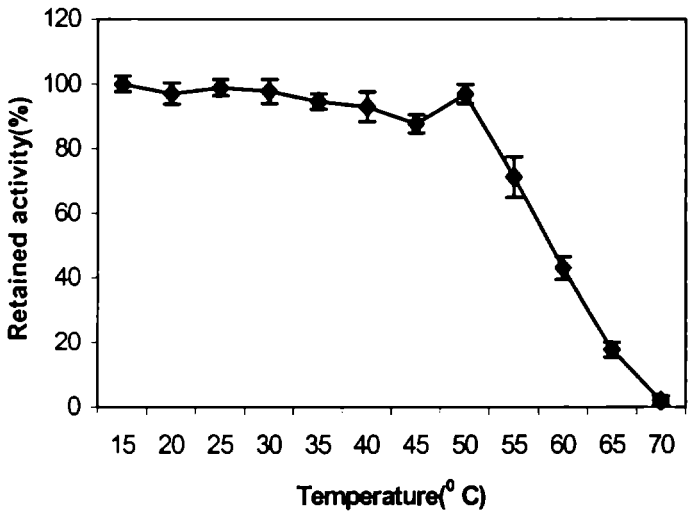


Fig. 4.12 Effect of temperature on the stability of *Vibrio* sp. protease

Table 4.15. ANOVA for the effect of temperature on the stability of *Vibrio* sp. protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Between Groups	360046.3	11	32731.48	460.3414	<0.001
Within Groups	1706.463	24	71.10262		
Total	361752.7	35			

LSD= 19.257

Temperature had significant effect on the stability of V26 protease, as indicated by ANOVA results.

4.4.7 Effect of substrate concentration on protease activity

B 15

For B15 protease of the various substrate concentrations tested, optimum concentration for maximum activity was found to be 9mg/ml. From the lowest concentration tested the activity was gradually increased and reached the maximum value at 9mg/ml and then decreased on further increase of concentration. By the construction of Lineweaver - Burk plot, Km value was determined to be 0.101mg/ml and Vmax was 203.9308 U (Fig. 4.13).

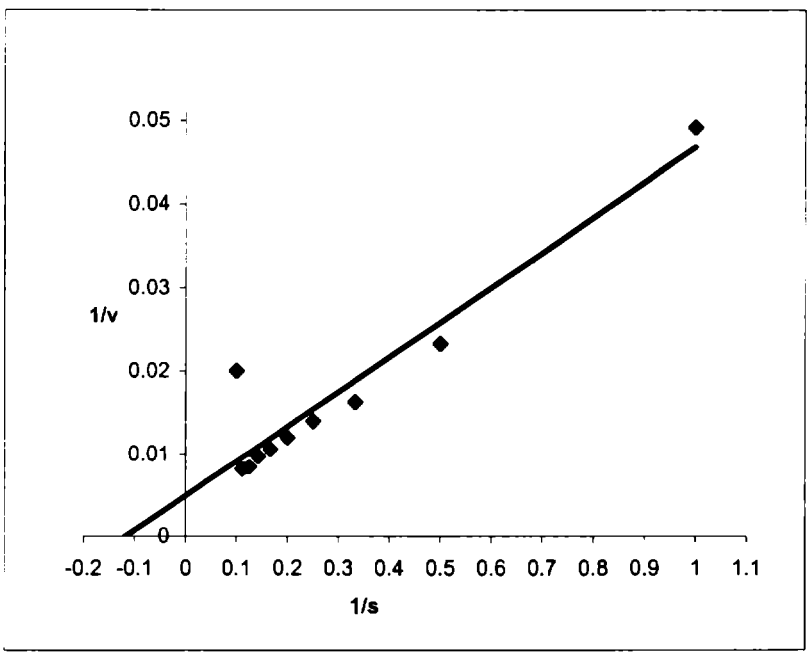


Fig. 4.13 Line Weaver –Burk plot for *B. circulans* protease.

V 10

For V10 protease also the optimum substrate concentration was 9 mg/ml and Km value was 0.168.mg/ml and Vmax was 231.816 U (Fig. 4.14).

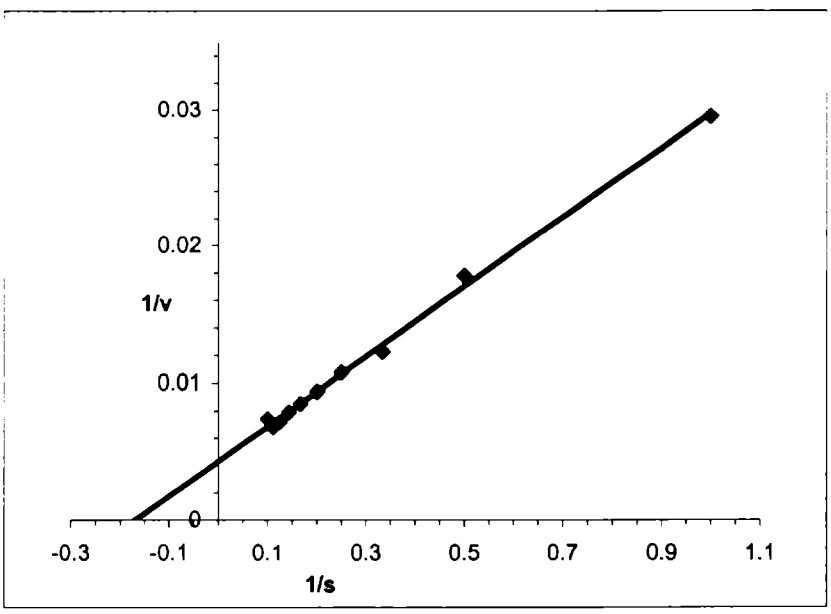


Fig. 4.14. Line Weaver –Burk plot for *V. fluvialis* protease.

V 26

For V26 protease the optimum substrate concentration was 8mg/ml. and Km value was calculated as 0.656 mg/ml and Vmax as 344.05U (Fig. 4.15).

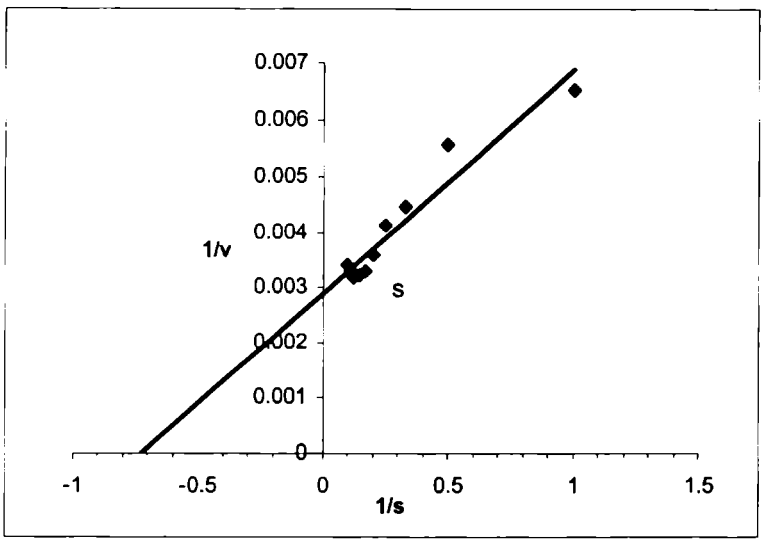


Fig. 4.15. Line Weaver –Burk plot for the protease of Vibrio sp.

4.4.8 Effect of various metallic salts on protease activity.

Effect of various ions on protease activity in three different concentrations (5mM, 2.5mM, 1mM) was studied. It was found that these ions had significant influence on the activity of the three proteases tested and the various ions were imparting their effects differently in proteases of different strains.

B15

B15 protease was profoundly influenced by the presence of various metallic salts in the incubation mixture. Here it was seen that Hg^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+}, completely and Mn^{2+} to some extent were inhibiting the enzyme activity. NaCl was having neither enhancing nor inhibitory effect. All the other salts tested were enhancing the activity significantly; the best enhancer being Ca^{2+} whose stimulatory effect was significantly higher than the other ions tested. $Mg^{2+}$and K^{+} were having almost similar effects of

enhancement on the activity. Fe^{2+} was stimulating the activity slightly (Fig. 4.16, Table 4.16).

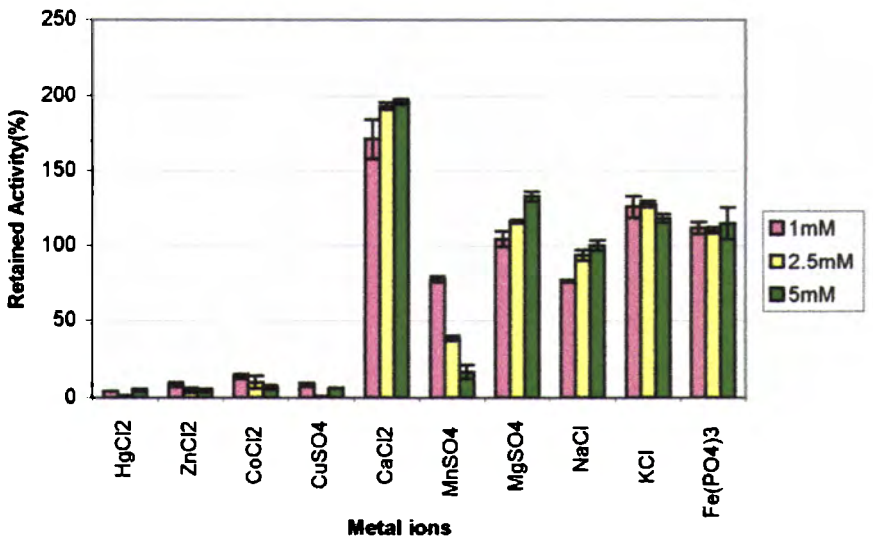


Fig. 4.16 The effect of metallic salts on the activity of *B. circulans* protease

Table 4.16. ANOVA for the effect of metallic salts on the activity of *B. circulans* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F- value	P-value
Concentrations	1.57424	2	0.78712	0.004532	NS
Metallic salts	114305.2	9	12700.58	73.12514	<0.001
Error	3126.292	18	173.6829		
Total	117433.1	29			

LSD for the different metallic salts = 30.9687

ANOVA showed that the different metal ions had significant effect on enzyme activity, but the different concentrations tested were not having significant impact on the activity.

V10

In the case of V10 protease Hg^{2+} and Cu^{2+} were completely inhibitory for activity and Zn^{2+} also caused reduction in activity. In contrast to the effect on B15 protease Co^{2+} was having an enhancing effect that too more

than all the other ions tested. Ca^{2+} was also a very good enhancer of V10 protease not differing significantly from Co^{2+} in its effect. Fe^{2+} also was enhancing the activity slightly; where as all the other ions tested were showing almost similar effects, giving slightly lesser activity than the control (Fig. 4.17, table 4.17).

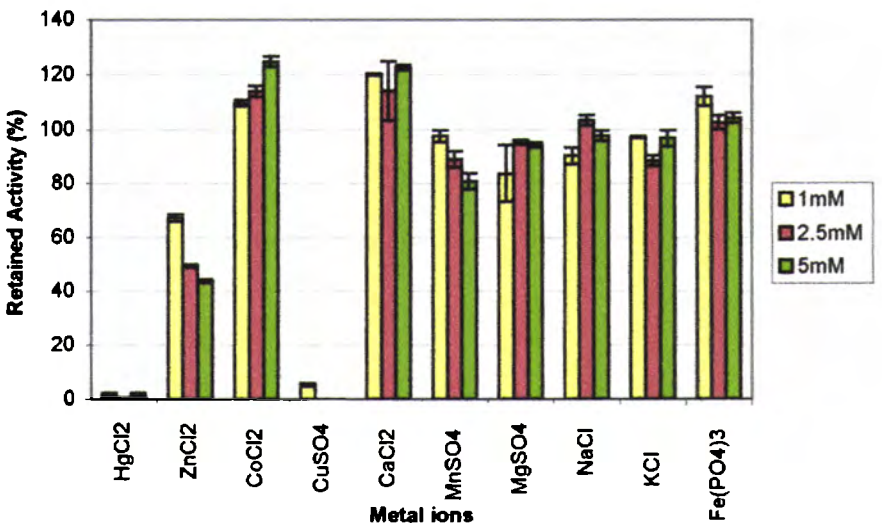


Fig. 4.17 The effect of metallic salts on the activity of *V. fluvialis* protease.

Table 4.17. ANOVA for the effect of metallic salts on the activity of *V. fluvialis* protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Concentrations	36.17754	2	18.08877	0.387198	NS
Metallic salts	51552.42	9	5728.046	122.6115	<0.001
Error	840.907	18	46.71706		
Total	52429.5	29			

LSD samples (metallic salts)=16.06138

Results of ANOVA indicated that different metallic salts had significant influence on the protease activity of V10, but the different concentrations tested were not influencing the activity significantly.

V26

For V26 protease Hg^{2+} and Cu^{2+} were completely inhibitory and Mn^{2+}, Fe^{3+} and Ca^{2+} were not having much effect, either positive or negative. The enhancers of the activity were Co^{2+}, Mg^{2+}, Na^{+} and K^{+} (Fig. 4.18, Table 4.18).

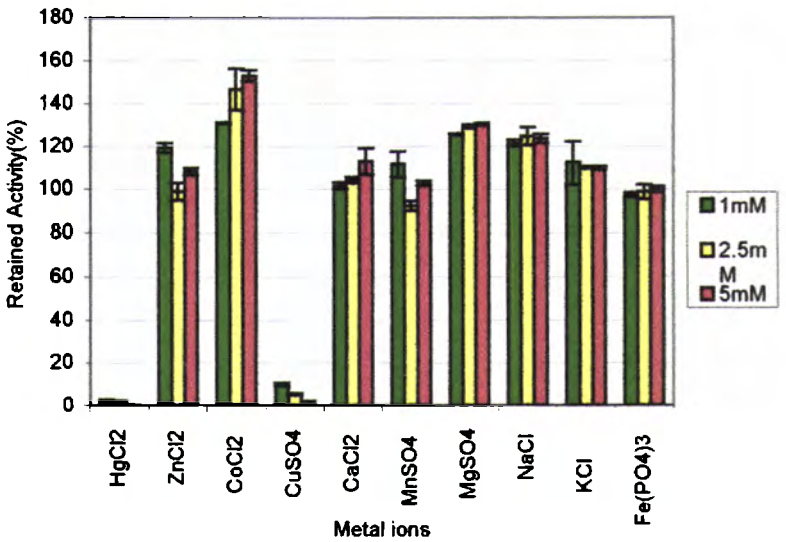


Fig. 4.18 The effect of metallic salts on the activity of *Vibrio* sp. protease.

Table 4.18. ANOVA for the effect of metallic salts on the activity of *Vibrio* sp. protease.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-Value	P-value
Rows	48.55423	2	24.27712	0.595678	NS
Columns	64846.23	9	7205.137	176.7896	<0.001
Error	733.5976	18	40.75542		
Total	65628.38	29			

LSD for metallic salts = 15.001

As inferred from ANOVA, there was much significance for the effect of various metallic salts on enzyme activity. The various concentrations tested did not influence the activity.

4.4.9 Effect of inhibitors on enzyme activity

The effect of various chemical compounds viz. EDTA, PMSF, IAA, urea, SDS, dithiothreitol, mercaptoethanol, and phenanthroline, which are known

inhibitors of different classes of proteases, on enzyme activity, was determined to study the properties of the enzymes.

B15

In the case of B15, EDTA was found to inhibit the enzyme activity completely. PMSF has shown almost 85% inhibition. IAA at 2.5mM and 5mM concentrations inhibited the activity, and phenanthroline showed complete inhibition only at 5mM concentration and at 1mM it reduced the activity only slightly. Dithiothreitol was non inhibitory, and the difference shown by the three different concentrations is not significant as shown by the one-way ANOVA done for each compound separately. Urea, SDS and mercaptoethanol were found to affect the activity to some extent. (Fig. 4.19, table 4.19).

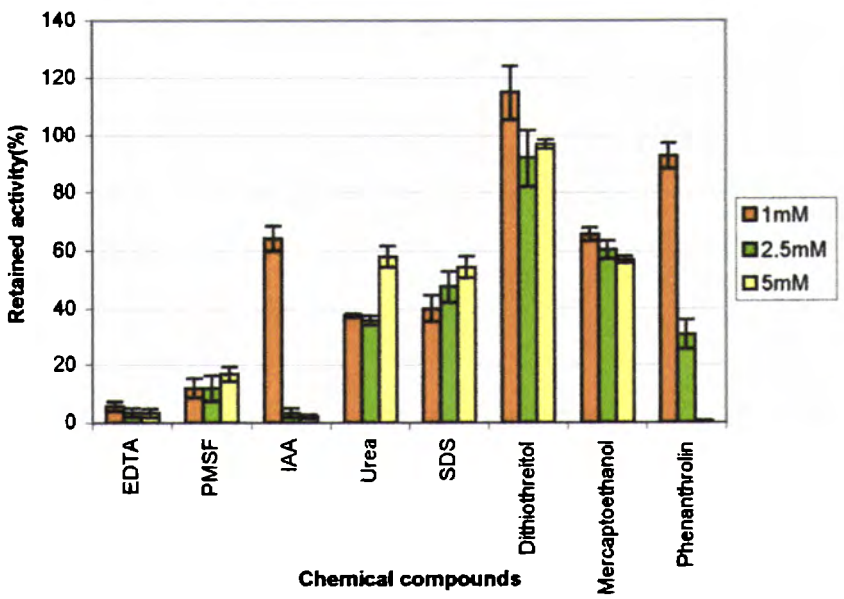


Fig. 4.19. The effect of inhibitors on the activity of *B. circulans* protease.

Table 4.19. ANOVA for the effect of inhibitors on the activity of *B. circulans* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-Value	P-value
Sample	58463.58	7	8351.941	37.999	<0.001
Columns	5296.624	2	2648.312	10.161	<0.001
Within	18812.741	72	261		
Total	82572.95	71			

LSD for compounds =19.8

LSD for concentrations = 12.12

ANOVA results showed that the various compounds, at different concentrations tested were having significant inhibitory effects on the enzyme activity.

V10

For V10 protease complete inhibition was shown by 5mM concentrations of EDTA and 2.5 and 5mM concentration of IAA. The inhibitory effect of PMSF was between 80% and 60%. 1mM phenanthroline inhibited the protease activity only about 40%. All the others decreased the activity slightly. There was no significant difference between the effects of urea, SDS, mercaptoethanol and dithiothreitol. Likewise, there was no significant difference between the inhibitory effects of EDTA and PMSF and IAA, but there was significant difference between the inhibition of phenanthroline with these three compounds. i.e. phenanthroline was not as inhibitory as these compounds (Fig. 4.20, Table 4.20).

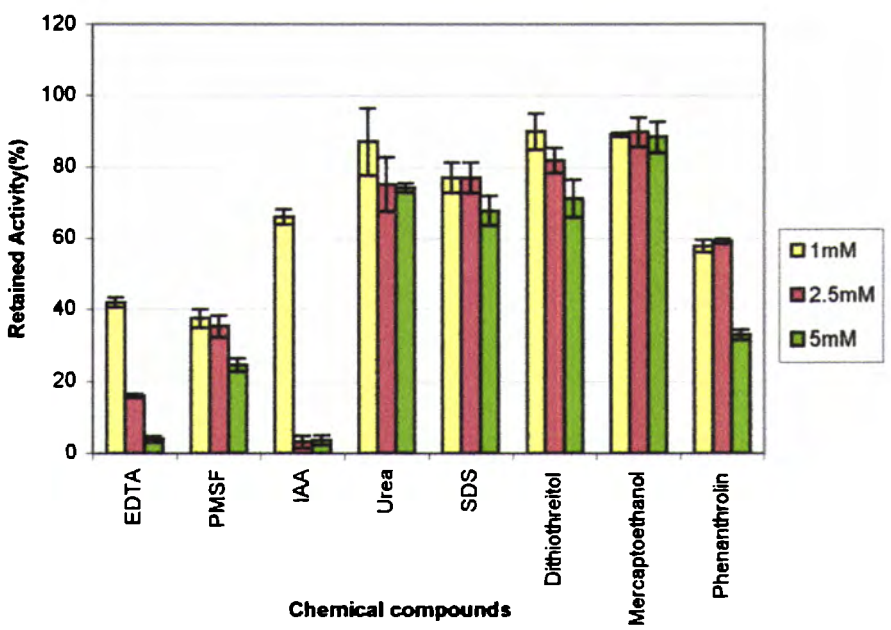


Fig. 4.20. The effects of inhibitors on the activity of *V. fluvialis* protease.

Table 4.20. ANOVA for the effect of chemical compounds on the activity of *V. fluvialis* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	48888.22	7	6984.032	60.829	<0.001
Columns	6293.796	2	3146.898	27.4087	<0.001
Within	71118.4391	62	114.8135		
Total	62300.46	71			

LSD for compounds = 13.1329

LSD for concentrations = 8.0422

Statistical analysis showed that the various compounds, at different concentration tested were having significant inhibitory effects on the enzyme activity.

V26

EDTA at all concentrations was inhibiting the V26 protease up to about 90%. IAA and phenanthroline at 2.5 and 5 mm concentrations were inhibitory. DTT at 2.5mM and 5mM concentrations was more than 50% inhibitory. PMSF, urea, SDS, and mercaptoethanol were not having considerable inhibitory effect on this protease and they did not vary significantly from each other (Fig. 4.21, Table 4.21).

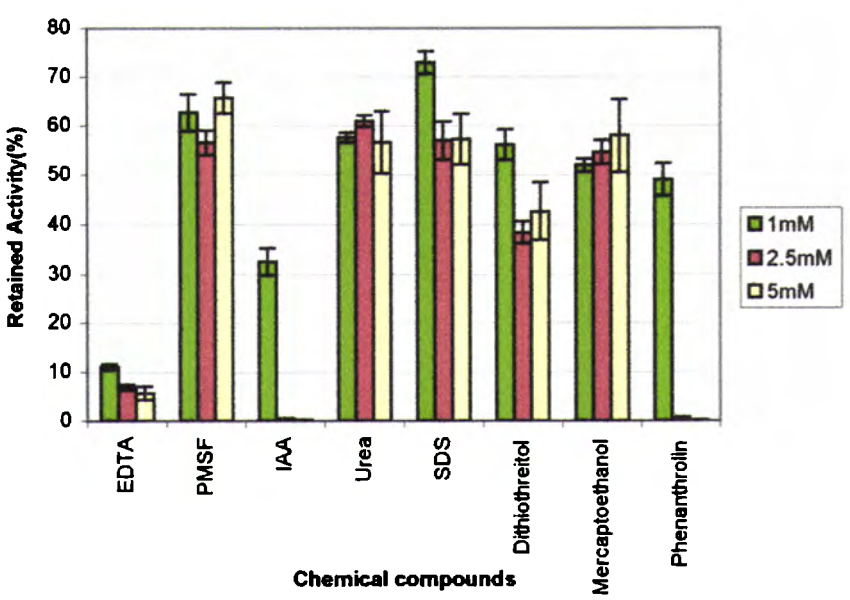


Fig. 4.21.The effects of inhibitors on the activity of *Vibrio* sp. protease.

Table 4.21. ANOVA for the effect of inhibitors on the activity of *Vibrio* sp. protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Sample	35742.29	7	5106.041	59.02	<0.001
Columns	3205.881	2	1602.94	18.524	<0.001
Within	5364	62	86.516		
Total	44312.17	71			

LSD for compounds = 11.40

LSD for concentrations = 6.9812

As indicated by ANOVA there was significant variation in the inhibitor profile of various compounds on the V26 protease and the different concentrations tested also influenced this property.

4.5 Discussion

4.5.1 Purification of the alkaline proteases

Precipitation is the most commonly used method for the isolation and recovery of proteins from crude biological mixtures (Bell *et al.*, 1983). It performs both purification and concentration steps. It is generally effected by the addition of reagents such as salt or organic solvent, which lowers the solubility of the desired proteins in the aqueous solution. In the present study, ammonium sulphate up to 80% saturation has been used with good results. Protease purification was monitored at various stages by enzymatic assay, total protein determination, and SDS PAGE analysis and zymography (activity gel) for demonstrating the protease activity of the purified sample.

A number of alkaline proteases from different sources have been purified and characterized by $(NH_4)_2SO_4$ precipitation, e.g. *Bacillus* sp. (Hutadilok-Towatana *et al.*, 1999; Kumar *et al.*, 1999; *Bacillus subtilis* (Laeur *et al.*, 2000. Singh *et al.*, 2001a), *B. pumilus* (Kumar 2002) and *B. sphaericus* (Singh *et al.*, 2001b), *Pimelobacter* (Oyama *et al.*, 1997) *Pseudomonas auuiginosa* strains (Ogino *et al.*, 1999; Bayoudh *et al.*, 2000) and *Serratia marcescens* (Romero *et al.*, 2001) *Aeromonas proteolytica* (Schalk *et al.*, 1992) and a fish pathogen *Yersinia ruckeri* (Secades and Guijarro, 1999). The 75kDa subtilisin like

protease from *Pleurotus ostreatus* was purified by ammonium sulphate precipitation up to 80% saturation, followed by dialysis and further purified by chromatography (Palmieri *et al.*, 2001).

The results from the electrophoretic analysis of the ammonium sulphate precipitated fraction of the three selected alkaline proteases in the present study, showed that this single purification step could lead to apparent purity of the alkaline protease, as indicated by the single bands obtained in the SDS PAGE. After purification the protease from *B. circulans* (B15) had specific activity of 5.666 and 1.07 fold purification. For *V. fluvialis (V10)* protease the specific activity was 10.212 with 1.25 fold purification and for *Vibrio* sp. (V26) it was 10.9 and 1.1 respectively. Comparable results were reported for the *Streptomyces* sp. G 15, where the specific activity after the first step of purification was 11(Sampath *et al.*, 1997). *Aeromonas hydrophila* protease had specific activity 3 and 2 fold purification was obtained after ammonium sulphate precipitation (Pansare *et al.*, 1986). Aderibigbe *et al* (1990) reported proteases from strains of *B.subtilis*, which obtained 0.514, fold purification by ammonium sulphate precipitation. Another strain of *B. subtilis* had specific activity of 3.88U/mg and 4-fold purification after $(NH_4)_2SO_4$ precipitation. (Yang *et al.*, 2000). However, in an *Aeromonas proteolytica* protease, the specific activity of the culture filtrate was 15 and that of ammonium sulphate precipitated fraction was 43 with a yield of 76% (Griffin and Prescott, 1970).

4.5.2 Molecular weight determination by SDS PAGE

SDS- PAGE was successfully used for the determination of molecular mass in most of the works on protease purification (Kwon *et al.*, 1994; Liao and Mc Callus, 1998). In the present study, single bands were obtained in the SDS- PAGE analysis for the dialysate of the ammonium sulphate precipitated fraction, which indicated the homogeneity of the sample.

The molecular weight of the B15 protease was a 20kDa, V10 protease 33.5 kDa, and V26 protease 22.4 kDa. These values are well in agreement

with the earlier works, where the molecular masses of alkaline proteases ranged from 15 to 30 kDa (Fogarty *et al.*, 1974) with few reports of higher molecular masses of 31.6 kDa (Freeman *et al.*, 1993), 33 kDa (Larcher *et al.*, 1996; Samal *et al.*, 1991); 34.8 kDa (Griffin and Prescott 1970), 36 kDa (Tsujibo *et al.*, 1990) and 44 kDa (Yang *et al.*, 2000), 45 kDa(Kwon *et al.*, 1994), 46 kDa (Kobayashi *et al.*, 1985), 40 kDa and 48 kDa (Pansare *et al.*, 1986). Thangam and Rajkumar (2002) have reported a 68kDa serine protease from *Alcaligenes faecalis*. Some proteolytic enzymes from certain microbial sources were reported to have molecular weights of 20 kDa to 77 kDa (Morihara 1957; Prescott and Wilkes 1966; Nakadai *et al.*, 1972,1973). The halophilic protease from *Pseudomonas* sp. A-14 had a molecular weight of 120 kDa (Qua *et al.*, 1981).

A protease from *Kurthia spiriforme* had an extremely low molecular weight of 8kDa (Steele *et al.*, 1992).

In some *Bacillus* sp., multiple electrophoretic forms of alkaline proteases were observed (Zuidweg *et al.*, 1972; Kobayashi *et al.*, 1996; Kumar 1997), which might be the result of non enzymatic, irreversible deamination of glutamine or asparagines residues in the protein molecules or of auto proteolysis (Kobayashi *et al.*, 1996).

4.5.3 Zymogram analysis

Since the paper by Heussen and Dowdle (1980) was published, the electrophoretic procedure for the direct visualization of plasminogen activators on substrate containing SDS-PAGE has been successfully used. The major advantage of this method is the action of the SDS in semi denaturation of the enzyme molecules with the result of preventing substrate digestion during the run. After staining and destaining, proteolytic enzyme is visualized as a white band in a completely dark blue background. Since large molecular weight protein substrates co polymerize with the polyacrylamide and after the electrophoresis under semi denaturing conditions, many proteolytic enzymes (Lacks and Springhorn, 1980) are easily renatured once

the denaturant is washed away, the method has been applied to many proteolytic enzymes with different specificity (Kleiner and Stevenson 1994; Hummel *et al.*, 1996). Activity staining of protease inhibitors was done by Felicioli *et al.* (1997). Choi *et al.* (2002) described analysis of protease from *Bacillus* sp. DJ-4, by zymography. Real time zymography and reverse zymography for detecting matrix metalloproteases (MMP) and their inhibitors respectively were reported (Hatori *et al.*, 2002).

In the present study, single band of proteolytic activity has been obtained for B15 protease and V10 protease, when appropriate dilution of the enzyme was used. But for the alkaline protease of V26, the activity zone was not obtained as a single band, but as two bands. A similar phenomenon has been observed with the zymogram analysis of extracellular protease from nematophagous fungus *Verticellium suchlasporum* (Lopez-Llorca, 1990), where zones of clearance, instead of bands on the activity gel were obtained, when the culture filtrate was used as enzyme source. While the purified protease produced only a single band of a 32 kDa protein, in SDS PAGE an extra band with faint activity was observed in the zymogram. A keratinolytic *Streptomyces albidoflavus* (Bressollier *et al.*, 1999) produced six extracellular proteases as revealed by six different bands in zymogram analysis. Likewise, when the alkaline serine protease of different species of a fungus *Monascus* was studied by zymography, the culture filtrates of these strains showed at least three different bands each, of proteolytic activity on the zymogram (Aso *et al.*, 1989). A similar case was reported by Salamanca *et al.* (2002) in a cryophilic protease of Antartic krill. But here in case of V26 protease the single band obtained in SDS- PAGE excludes the possibility of the occurrence of different proteases. In a report on improved staining method for quantification of matrix metallo-proteases (MMP) using zymography (Leber and Balkwill, 1997) it was suggested that the proteases were released from cells in a proteolytically inactive pro- form (zymogen), which was about 10kDa larger than the active form. Since the proform became activated during the process of renaturation, after gel electrophoresis both forms could be detected on zymograms (Kleiner and Settler, 1994; Birkedal *et al.*, 1982).

Therefore the possibility of activated zymogens could be one explanation for the multiple bands detected on the zymogram of V26 protease. Activity gels were used by some researchers, to determine the molecular weight of the protease, by comparing the position of the clear band in the gel with the standard molecular weight markers. In such a study, of the protease of *Streptomyces cyaneus* the proteolytic activity on activity gel corresponded to a position of 30 kDa, but when it was subjected to HPLC gel filtration analysis, a single peak corresponding to a molecular weight of 120 kDa was observed (Petinate *et al.*, 1999), which showed that the enzyme existed in a tetrameric form. From these observations we could assume that zymography was not an absolute method to find the molecular weight or to find the number of proteases produced; but a method very much appropriate to confirm the proteolytic activity of the sample in question.

Use of zymography has been reported by some other authors also; gelatin was used as substrate for the zymography of proteases of *Vibrio Metschnikovii* (Kwon *et al.*, 1994), *Pseudoperkinsus tapetis* (Ordas *et al.*, 2001), *Alcaligenes faecalis* (Thangam and Rajkumar, 2002) and proteases of a protozoan parasite *Perkinsus marinus* (MacIntyre *et al.*, 2002). Casein gel was used for zymogram analysis of *Streptococcus suis* protease (Jobin and Grenier, 2003).

4.5.4 Effect of pH on protease activity and stability

The proteases employed in the present study showed very good activity and stability over a wide range of pH. They showed variation with regard to their pH optima for maximum activity. For B15 protease the optimum pH was 7, and it was active over pH values up to 10. It had nearly 75% of the maximum activity at pH 8, 9 and 10. V10 protease was active in the pH range 7 to 11 the optimum being at pH 8.At pH 7 and 9 it had 98% of the maximum activity. For V26 protease the activity was in the range 7 to 11, the maximum being at pH 9. At pH 7,8,10 and 11 it had 76%, 83%, 76% and 65% of activities respectively. Of the three proteases, V26 protease showed highest pH optimum for maximum activity.

Regarding the pH stability, B 15 was stable between pH 7 and 11. Maximum stability was at pH 8. At pH 11, 52% of the maximum activity was retained. The V10 protease showed a broader range of pH stability i.e. between pH 5 and 13. From pH 5 the stability gradually increased and reached the maximum at pH 8, as in the case of B15, and then showed slight decrease, gradually up to pH 13. For V26 protease the pH stability range was between pH 6 and 13. Maximum stability was obtained at pH values 9, 10 and 11, the difference in values being not significant. From these observations we can see that the maximum pH stability was shown by V10 protease, followed by V26 and B15.

Similar studies on proteases from other bacteria showed that the pH range of most alkaline proteases was generally between pH 6-9 (Cowan and Daniel, 1982), and between 9 and 11 (Kumar and Takagi, 1999), with a few exceptions of higher pH optima of 11.5 (Tobe *et al.*, 1975; Takami *et al.*, 1990; Takii *et al.*, 1990; Yum *et al.*, 1994), pH 11-12 (Kumar, 1997), pH 12.3 (Nakanishi *et al.*, 1974; Kobayashi *et al.*, 1995) and pH 12-13 (Fujiwara *et al.*, 1993; Takami *et al.*, 1989). They are also generally stable between pH 6 and 12 (Horikoshi and Akiba, 1982). *Bacillus* sp. P-001A had good activity over a wide range of pH while its pH optimum was 9.5 (Atalo and Gashe, 1993); *B.cereus* protease it was pH 6 to 9 (Ghorbel, 2003). Optimum pH was 9 for a thermostable alkaline protease reported by Manachini and Fortina (1998), and for a *Streptomyces* protease reported by Petinate *et al.*, (1999). Kwon *et al.* (1994) reported an alkaline protease from *V.metschnikovii* with broad range of pH with optimum pH at 10.5 and were exceptionally stable, retaining more than 80% of its original activity after incubation at pH 12 for 24 hr. pH optimum of 8.6 was observed for *Vibrio* protease (Wiersma *et al.*,1978 b). Kobayashi et al., (1985) reported an alkaline protease from *Pseudomonas maltophila* active over a broad pH range with the maximum observed around 10.5. *Aeromonas hydrophila* protease- I had optimum pH 8.5 and an aminopeptidase of optimum pH 8 (Pansare et al 1986). pH 7.8 to 8.2 was optimum for *Achromobacter lyticus* protease (Masaki *et al.*, 1986). pH optimum of 8.5 was observed for a serine protease from nemtophagous

fungus *Verticellium suchlasporium* (Lopez-Llorca, 1990) An extremely halophilic protease of *Halobacterium halobium* had broad pH optimum between pH 8 and 11,with maximum activity near 10 (Ryu *et al.*, 1994). The protease from a *Baillus subtilis* strain protease had a pH optimum of 8 and had pH stability between 7 and 9 (Yang *et al.*, 2000).

4.5.5 Effect of temperature on protease activity and stability

The three proteases studied showed considerable variation with respect to temperature optima for maximum activity. The optimum temperature ranged from mesophilic to thermophilic range. (The proteases form all the three strains, B15, V10, and V26 were stable at refrigeration temperature (4 °C) for more than six months, (the maximum period tested and not shown in graph). For B15 the optimum temperature of activity was 40°C, and it was active over a wide range of temperatures from 15°C to 75°C having 29% and 49% of the maximum activity, respectively. It had more than 50% activity at a range of 30°C to 70°C. This protease was stable up to 60°C as observed from the stability studies. It was absolutely stable up to 30°C, i.e. most stable within a range of 0°C and 30°C. Then it was slightly decreasing and reached a residual activity of 55% at 50°C and 26% at 55°C.

V10 protease was also active over a wide range of temperature, 25°C to 75°C. Optimum activity was at 55°C. At 60°C also it was highly active. V10 protease was absolutely stable up to a temperature of 50°C, and showed a decrease in stability when the temperature was increased further.

For V26 protease, the activity over an exceptional temperature range of 30°C to 80°C was observed, the optimum being at 75°C. At 70°C also about 99% of the maximum activity was obtained. The stability studies of this enzyme revealed that it was absolutely stable up to 50°C.

Among the three proteases, the *Bacillus* (B15) protease was promising with regard to its wide range of activity with its optimum activity at 40°C, for low temperature applications. Activity at low temperatures has become a

desirable feature in respect of their use in detergent industry. At the same time V10 and V26 proteases are exceptionally good for use at high temperatures being highly active up to 75°C, which is a very desirable character in many fields.

The results obtained in the present study are in accordance with several similar studies carried out elsewhere. The optimum temperature of alkaline proteases generally ranges from 50 to 70°C; *Aeromonas hydrophila* protease had an optimum of 48°C to 50°C (Pansare, 1986), 50°C for *Vibrio* sp. (Wiersma *et al.*, 1978 b) and *Bacillus subtilis* (Yang *et al.*, 2000), 60° C for *Bacillus alcalophilus* protease (Takii *et al.*, 1990) and 70°C for *B. licheniformis* (Manachini and Fortina, 1998). An aminopeptidase from *A. hydrophila* required a temperature of 70°C for its maximum activity (Pansare *et al.*, 1986). A protease from an alkalophilic *Bacillus* sp. B18' showed an exceptionally high optimum temperature of 85°C. Of the two thermophilic proteases from a strain of *Thermus aquaticus*, one had a temperature optimum between 70 to 80°C, and the other 95°C (Matsuzawa *et al.*, 1983). In contrast to these observations, a very low temperature optimum of 25°C was observed for *Streptomyces cyaneus* (Petinate *et al.*, 1999).

There are many reports on increase in thermal stability by the presence of Ca^{2+} (Sakata *et al.*, 1977; Chandrasekaran *et al.*, 1986; Lee *et al.*, 1996; Kumar, 2002), Mg^{2+} and Mn^{2+}(Paliwal *et al.*, 1994) Alkaline proteases from *Bacillus* sp., *Streptomyces* sp., and *Thermus* sp., are quite stable at high temperatures and the addition of Ca^{2+}, further enhanced enzyme thermo stability. The enhancement effect of calcium was reported for a protease of *Porphyromonas gingivalis* (Kontani *et al.*, 1996). Vap T and VapK, two alkaline proteases from *V.metshnikovii* (Kwon *et al.*, 1994) had a temperature optimum of 60°C and the addition of Ca^{2+} extended the half-life more than 3 fold and 10 fold respectively. A thermo stable protease of *Bacillus* sp. AH-101 had a temperature optimum of 80°C and 70°C in presence of and absence of Ca^{2+} respectively (Takami *et al.*, 1989). Temperature stability of

B.subtilis serine protease was in the rage 25°C to 50°C and optimum activity at 50°C (Yang *et al.*, 2000).

4.5.6 Effect of substrate concentrations on enzyme activity – Kinetic constants

Alkaline proteases have broad substrate specificities and are active against a number of synthetic substrates and natural proteins. However, the literature conclusively suggests that they are more active against casein than against azocasein, bovine serum albumin or hemoglobin (Gupta *et al.*, 2002a). Moreover there are specific types of alkaline proteases, viz. collagenase, elastase, keratinase (Friedrich *et al.*, 1999) which are active against specific protein substrates.

To develop an enzyme-based process, prior information about kinetic parameters of the enzyme in question is of utmost importance. To be precise, kinetic properties like Vmax and Km are important being not only enzyme specific, but also substrate and environment specific, and knowledge of these parameters is essential for designing enzyme reactors or quantifying the application of the enzyme under different conditions. Various complex substrates, viz., casein, azocasein etc., and synthetic substrates, viz., para nitroanilides esters; are used for determining kinetic parameters of alkaline proteases. The synthetic substrates are much more popular than complex substrates for this purpose as they are more convenient (Larcher *et al.*, 1996; Kumar 2002). For an alkaline protease from *B. mojavensis*, the Km for casein decreased with corresponding increase in Vmax, as the reaction temperature was raised from 45 to 60°C. (Beg *et al.*, 2002b). In contrast the Km and Vmax for an alkaline protease from *Rhizopus oryzae* increased with an increase in temperature from 37°C to 70°C. (Banerjee and Bhattacharya, 1993).

In the present study, casein was used as substrate for determining the kinetic properties. Among the three proteases B15 proteases showed maximum affinity towards the substrates followed by V10. The Km was only 0.101 mg/ml for B15 protease whereas V26 protease required higher concentration of substrates for maximum activity. However Vmax was

highest in the case of V26 protease (344.05U). For B15 protease Vmax was 203.9308 U. For V10 protease Km value was 0.168mg/ml and Vmax was 231.816 U. For V26 Km value was calculated as 0.656mg/ml.

From the Vmax of the three, it is quite evident that V26 protease is the one with the highest activity followed by V10.

For a Bacillus thermoruber protease, Km value was 2.5mM and Vmax was 2.4 umol, using p-nitro aniline as substrate (Manachini *et al.*, 1988). The Km value and Vmax of the alkaline protease of *Xenorhabus nematophila* were determined using synthetic substrates by Caldas *et al.*, (2002). *Fusarium* trypsin like protease had Km value 0.16mM and Vmax of 0.6umol/min/mg (Barata *et al.*, 2002). Km value of serine protease from a *Streptomyces cyaneus* was 1.86×10^{-15} mmol l^{-1} and Vmax 2×10^{-2} mmol l^{-1} for TAME (alpha-N-p-tosyl- L- arginine-methyl ester) as substrate. (Petinate *et al.*, 1999). These results, where synthetic substrates are used to determine the kinetic constants, could not be compared with that of the present study where casein was the substrate used.

4.5.7 Effect of metal ions on the activity and stability of proteases

The effect of metal ions on enzyme activity varied depending upon the type of metal and concentration used. In general mercury and copper were inhibitory where as other metal ions were showing different effects on different proteases. Hg^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} markedly inhibited the B15 protease, while V10 protease was inhibited completely by Hg^{2+} and Cu^{2+} and partially by Zn^{2+} (43-57%) and Co^{2+} was found to enhance the enzyme activity. For V26 protease Hg^{2+} and Cu^{2+} was completely inhibitory.

For B15 protease the enhancing effect of calcium was profound, giving 195% of retained activity at 5mM concentration followed by Mg^{2+} (132% at 5mM), K^{+} (118% at 5mM) and Fe^{3+} (114% at 5mM). Mn was showing an inhibitory effect.

For V10 Ca^{2+}, Co^{2+} and Fe^{3+} were enhancing the activity. There was no significant difference in the enhancing effect between these three metal ions.

All the ions tested except Hg^{2+} and Cu^{2+}, showed considerable enhancement on the activity of V26 protease, the maximum being shown by Co^{2+} (up to 152% activity after treatment at 5mM), followed by Mg^{2+} Fe was having neither enhancing nor inhibitory effect on V26 protease. So in this study only Hg^{2+} and Cu^{2+} were found to be inhibitory to all the three proteases and Co^{2+} caused complete inhibition of B15 while it was very good enhancers of the other two proteases. Zn^{2+} was inhibitory to B15 completely and to V10 partially, but enhanced V26 protease. Similar effects of metal ions on protease action were reported by several authors (Manachini *et al.*, 1988). Hg^{2+} inhibited catalytic activity of many proteases (Shimogaki *et al.*, 1991; Rahman *et al.*, 1994). The poisoning of enzymes by heavy metals has been well documented by Vallee and Ulmer (1972). The inhibitory effect of Cu^{2+} and Zn^{2+} was also reported (Ghorbel *et al.*2003). Besides Cu^{2+} and Zn^{2+} Fe^{2+} also inhibited the protease produced by *A.hydrophila*. (Pansare *et al.*, 1986). $ZnCl_2$ was found to be inhibitory, and Ca^{2+} and Mg^{2+} was stimulatory to the activity of trypsin like protease of *Fusarium oxysporum* var.lini (Barata *et al.*, 2002); *Alcaligenes faecalis* serine protease (Thangam and Rajkumar, 2002), *Achromobacter lyticus* protease (Masaki *et al.*, 1986).

There are several reports on the enhancing effect of different ions on protease activity. Calcium ions were found to be enhancing the activity of several proteases. (Kobayashi *et al.*, 1985; Takami *et al*, 1989; Secades and Guijarro 1999; Ghorbel *at al.*, 2002). Probably the Ca^{2+} and Mg^{2+} ions are involved in stabilizing the active structure but they are not needed for the catalytic function or for the protein chain folding process to form the active structure. Calcium ions are known to protect the protease against denaturation and proteolytic degradation. (Voodouw *et al.*, 1976; Frommel and Sander, 1989). Metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+}, and Zn^{2+} were used for stabilization of protease (Rattray *et al.*, 1995; Johnvesly and Naik 2001) For a *B. subtilis* protease Ca^{2+}, $Mn2^{+}$, Mg^{2+} Fe^{2+}, $Zn^{2+}$and

Co^{2+} were stimulatory while Hg^{2+} inhibited the activity (Yang *et al.*, 2000). Oberoi *et al.* (2001) reported an enhanced protease activity in presence of Mn^{2+}, in a *Bacillus* sp. These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at higher temperatures.

The effect of NaCl on a halophilic protease has been described. It needed NaCl concentrations near saturation. Salt effects the stability of the enzyme in a cooperative manner. (Ryu *et al.*, 1994). A *B. licheniformis* protease was strongly activated by 1 to 1.5 M NaCl with a three-fold increase in activity (Manachini and Fortina, 1998). However in the present study, sodium chloride was found not to have any influence on protease activity suggesting the non-halophilic nature of the organism.

It is evident from the earlier works and the present, that the different metal ions profoundly influence the protease activity of different strains of microorganisms and their effects vary from organism to organism.

4.5.8 The mechanistic classes of proteases based on inhibitor studies

Proteases are classified in subgroup 4, in group 3 (hydrolases), of enzymes (International union of Biochemistry 1992). However proteases generally do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently proteases are classified on the basis of three major criteria. i) Type of reaction catalyzed, ii) Chemical nature of the catalytic site iii) and evolutionary relationship with respect to structure. (Barett 1994)

Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960). There

are a few miscellaneous proteases that do not fit into the standard classification. (Menon and Goldberg, 1987)

Diisopropylphosphofluoridate (DFP) is the most widely used inhibitor of serine protease. PMSF (Phenylmethylsulphonyl fluoride) is another well-known irreversible inhibitor of serine protease, which inhibits serine proteases by the sulphonylation of the serine residue at the active site. This is the least selective and therefore most useful as a general inhibitor of serine proteases. However, PMSF can also react reversibly with some cysteine proteases (Beynon, 1987). Some of the serine proteases are inhibited by thiol reagents such as p-chloromercuribenzoate due to the presence of cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH range with an optimum between pH 7 and 11. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases. Their molecular masses are in the range of 15 to 30 kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces* and *Flavobacterium* sp. (Boguslawski *et al.*, 1983) subtilisins produced by *Bacillus* sp. are the best known (Rao *et al.*, 1998).

Metalloproteases are the most diverse of the catalytic types of proteases (Barett 1995). The basis for the classification of the metalloproteinase is the presence of a metal ion (usually zinc), which participates in catalysis. This differentiates them from proteases such as calpains and some trypsin like serine proteases, whose activities are stabilized by, but not necessarily depend on, the presence of calcium. Both the zinc dependent metalloproteases and some calcium-stabilized proteases from other classes can be inactivated by chelating agents such as EDTA and EGTA (Salvensen and Nagase 1989), and also by dialysis (Rao *et al.* 1998). 1,10 Phenanthroline is preferred as an inactivator of metalloproteases as it has a much higher stability constant for zinc than for calcium. Thus 1mM 1,10 Phenanthroline will inactivate metalloprotease even in the presence of 10mM Ca^{2+}, whereas this concentration of 1,10 Phenanthroline will not remove Ca^{2+}

from calcium binding protein. Therefore this compound is usually diagnostic for a Zn^{2+} metalloprotease (Salvesen and Nagase 1989)

Common cysteine protease inhibitors are IAA (Iodoacetic acid), E64, cystatins and p-chloromercuribenzoate. The best-characterized aspartic proteases are from mammals (pepsin, chymosin, cathepsin D and rennin) and also some fungal acid proteases belong to this class, and are rarely represented by bacterial proteases.

In the present study, the three alkaline proteases from the three strains were treated with various inhibitors and the results obtained showed that they could not be assigned to a particular class of protease based solely on the effects of the inhibitors on their activity.

The B15 protease, which is a 20kDa protein, was almost completely inhibited by EDTA at all concentrations (up to 95% at 1mM) and PMSF (up to 88% at 1mM). 1mM phenanthroline did not inhibit this protease. It retained about 40 to 60 % activity with urea, and SDS and 55 to 65% with mercaptoethanol. Dithiothreitol was least inhibitory. At 1mM concentration both PMSF and EDTA were inhibiting this protease. These results suggest that it could belong to a group of calcium dependent serine proteases. Though serine proteases are not generally inhibited by metal chelating agents, there are examples for inhibition by EDTA (Kato *et al.*, 1974; Gnosspelius, 1978; Strongin *et al.*, 1979; Izotova *et al.*, 1983;). Manachini *et al.* (1988) reported a thermostable alkaline protease from *B. thermoruber,* which was inhibited by both EDTA and PMSF, and was not inhibited by o-phenanthroline, and suggested it to be a calcium dependent protease. *Pseudoperkinsus tapetis* protease also showed this kind of inhibition profile, being inactivated by both PMSF and EDTA but not by the classic metalloprotease inhibitor phenanthroline and it was suggested to be a Ca^{2+} or Mg^{2+} dependent serine protease (Ordas *et al.*, 2001). It was also true with the two proteases of *V. metschnikovii* (Kwon *et al.*, 1994). Protease-II from *Aeromonas hydrophila* was sensitive to both EDTA and PMSF and since it was more affected by PMSF it was suggested to be a serine protease. The

serine protease produced by marine *Vibrio* sp.was inhibited by EDTA (Merkel and Sipos, 1971) An alkaline protease from marine *Pseudomonas* sp. was not inhibited by 1mm EDTA, or DFP but was completely inhibited by 50mM EDTA and was reported to be a metal chelating agent sensitive alkaline protease (Makino *et al.*, 1983) according to the classification of proteases proposed by Morihara (1970). The alkaline protease of *P. maltophila* was inhibited both by PMSF and EDTA and claimed to be distinctly different from other microbial proteases so far found, and suggested to be a serine metalloprotease (Kobayashi *et al.*, 1985). Alkaline proteases, which are metal ion dependent in view of their sensitivity to metal chelating agents such as EDTA, have been reported by some other workers also (Kato *et al.*, 1974; Steele DB *et al.*, 1992; Dhandapani and Vijayaragavan1994; Shevchenko *et al.*, 1995).

B15 protease was inhibited by 1mM and 2.5mM concentrations of IAA, a thiol inhibitor. Similarly an alkaline metalloprotease from a fish pathogen *Yersinia ruckeri* was found to be inhibited by dithiothreitol, which is a thiol inhibitor (Secades and Guijarro, 1999). This suggests that disulphide bonds could be important in maintaining the molecular confirmation required for activity.

V10 protease was inhibited up to 58%, 84% and 97% by 1mM, 2.5 mM and 5mM EDTA respectively, and 63%, 65% and 73% respectively by 1mM, 2.5 mM and 5mM PMSF. Dithiothreitol and Mercaptoethanol were not inhibiting this protease though IAA at 5mM and 2.5mM concentrations were inhibitory. Urea and SDS were not inhibitory. In presence of 1,10 Phenanthroline which is an ideal inhibitor to diagnose zinc dependent metalloprotease, at 1mM concentration the protease retained 57% of activity, which showed that this does not belong to a group of metalloprotease, even though completely inhibited by 5mM EDTA. That is, at 1mM concentrations, only EDTA and PMSF were inhibiting this protease above 50%. So it could be suggested that this also belonged to a group of calcium dependent serine protease.

At 1mM concentration, the V26 protease was inhibited only by EDTA, though at the two higher concentrations it was affected by IAA and Phenanthroline. It retained more than 50% of activity in SDS, urea, mercaptoethanol and dithiothreitol. The complete insensitivity to PMSF excludes this from the serine protease group. It could be a metalloprotease. Generally metalloproteases lose the activity by dialysis (Rao *et al.,* 1998), while the V26 protease retained its complete activity even after dialysis as observed for a metalloprotease from *Aeromonas proteolytica* which was unaffected by dialysis (Schalk *et al.*, 1992).

Thus, an insight into some important physical and chemical properties of the alkaline proteases of interest could be obtained by these experiments, which would be useful in the further study regarding their structural aspects and designing their application in the suitable fields.

CHAPTER 5
APPLICATION OF THE ALKALINE PROTEASES AS DETERGENT ADDITIVES

Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives. Out of the vast pool of enzymes proteolytic enzymes from microorganisms are the most widely exploited enzymes in the detergent industries worldwide (Godfrey and West 1996; Showell, 1999). Over the past 30 years the importance of proteases in detergents has grown from being minor additives to being key ingredients. In 1994, the total market for industrial enzymes accounted for approximately $ 400 million, of which enzymes worth $ 112 million were used for detergent purposes (Hodgson, 1994). In Japan, alkaline protease sales were estimated to be 15000 million ¥ (equivalent to $ 116 million)(Horikoshi, 1996). An upward trend in the use of alkaline protease is expected so that by the turn of the decade the total value of industrial enzymes is likely to reach $ 700 million or more (Hodgson, 1994). Microbial alkaline proteases dominate with a significant share of the market captured by subtlisins and/ or alkaline proteases from *Bacillus* sp. for laundry detergent applications (Ward, 1985).

Alkaline proteases added to laundry detergent enable the release of proteinaceous materials from stains (Van Tilburg, 1983). The increased usage of these proteases as detergent 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 enzymes allows lower wash temperatures and shorter periods of agitation, often after a preliminary period of soaking. (Nielsen *et al.*, 1981).

Dirt comes in many forms and includes proteins, starches and lipids. Using detergents in water at high temperatures and with vigorous mixing, it is possible to remove most types of dirt but the cost of heating the water is high, and lengthy mixing or beating will shorten the life of clothing and other materials. Proteinaceous dirt will not always be removed in this process, and the protein remaining on the fabric will be able to retain other components. The reason is that proteins may coagulate in the fabric in such away that they are not dissolved at the pH value (9-10) of the washing process. This coagulation may be caused by the alkaline pH value, temperature, enzymic

processes such as coagulation of blood, and by the action of oxidising agents like the hydrogen peroxide that develop in perborate containing detergents above 50°C. This latter action is of particular importance since proteinaceous dirt is not removed from the fabric when the temperature is raised to 50°C. In general, enzyme detergents remove protein from clothes soiled with blood, milk, sweat, grass, etc. far more effectively than non-enzyme detergents. At present, only proteases and amylases are commonly used in detergents. Although a wide range of lipases is known, it is only very recently that, lipases suitable for use in detergent preparations have been described.

Detergent enzymes must be cost-effective and safe to use. Early attempts to use proteases foundered, as producers and users were developing hypersensitivity. This was combated by developing dust-free granulates (about 0.5 mm in diameter) in which the enzyme is incorporated into an inner core, containing inorganic salts (e.g. NaCl) and sugars as preservative, bound with reinforcing, fibres of carboxymethyl cellulose or similar protective colloid. This core is coated with inert waxy materials made from paraffin oil or polyethylene glycol plus various hydrophilic binders, which later disperse in the wash. This combination of materials prevents dust formation and protects the enzymes against damage by other detergent components during storage.

A major trend in the detergent industry is the development of non phosphate- based products that function at low washing temperatures (Van Tilburg, 1984), as to reduce the environmental problems caused by excess phosphate viz. eutrophication of water bodies. In addition, liquid laundry detergents do not contain effective water softening chemicals and they fail to significantly increase the alkalinity of wash water, which impedes detergent performance. To compensate for decreased detergency associated with detergents, enzymes like proteases are being incorporated in laundry products (VanTilburg, 1984).

Enzymes are used in surprisingly small amounts in most detergent preparations, only 0.4 - 0.8% crude enzyme by weight. Once released from

its granulated form the enzyme must withstand anionic and non-ionic detergents, soaps, oxidants such as sodium perborate which generate hydrogen peroxide, optical brighteners and various less-reactive materials (Table 5.1), all at pH values between 8.0 and 10.5. Although one effect of incorporating enzymes is that lower washing temperatures may be employed with consequent savings in energy consumption, the enzymes must retain activity up to 60°C.

In short, proteases and other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzyme used should be effective at low levels (0.4 to 0.8 %) and should also be compatible with various detergent components along with oxidizing and sequestering agents. They must also have a long shelf life (Ward, 1985).

Table 5.1 Compositions of an enzyme detergent in use

Constituent	Composition (%)
Sodium tripolyphosphate (water softener, loosens dirt)	38.0
Sodium alkane sulphonate (surfactant)	25.0
Sodium perborate tetrahydrate (oxidising agent)	25.0
Soap (sodium alkane carboxylates)	3.0
Sodium sulphate (filler, water softener)	2.5
Sodium carboxymethyl cellulose (dirt-suspending agent)	1.6
Sodium metasilicate (binder, loosens dirt)	1.0
Sodium metasilicate (binder, loosens dirt)	1.0
Fluorescent brighteners	0.3
Foam-controlling agents	Trace
Perfume	Trace
Water	to 100%

(Courtesy: http://www.lsbu.ac.uk/water/)

In addition to the granulated forms intended for use in detergent powders, liquid preparations in solution in water and slurries of the enzyme in a non-ionic surfactant are available for formulating in liquid concentrates, used for removing stubborn stains.

Though the use of enzymes in detergent formulations is now common in developed countries, with over half of all detergents presently available containing enzymes, they have not found widespread use in developing countries, which are often hot and dusty, making frequent washing of clothes necessary. There are opportunities to extend the use of enzymes in detergents both geographically and numerically. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents. Considering the vast diversity of microbial world, the chances of finding microorganisms producing novel enzymes with better properties suitable for commercial exploitation, is always great.

The alkaline proteases in the present study were shown to exhibit very good activity and stability over a wide range of pH and temperature. It is now well established that proteases exhibiting activity in the high alkaline and temperature range have potential in detergent and stain removing formulations (Smith and Turk, 1974; Samal *et al.*, 1990; Anwar and Saleemuddin, 1997) Their utility can be significant only if they also exhibit compatibility with various detergents (Samal *et al.*, 1990; Phadtare *et al.*, 1993). Taking these into consideration, in the present study, the effects of an oxidising agent (H_2O_2), a non-ionic (Triton-X-100) and an anionic (SDS) detergent, as well as commercial detergent formulations, on the selected proteases were investigated thereby an attempt has been made to evaluate the suitability and effectiveness of these three alkaline proteases as detergent additives.

5.1 Review of literature

The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The preparation of the first enzymatic detergent "Burnus" dates back to 1913; it consisted of sodium carbonate and crude pancreatic extract. The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name BIO-40. The enzymes now in commercial use are all produced using species of *Bacillus*,

mainly by just two companies. Novo Industry A/S produces and supplies three proteases; Alcalase, from *Bacillus licheniformis* (first introduced in 1960, under the commercial name BIOTEX) Esperase, from an alkalophilic strain of *B. licheniformis* and Savinase, from an alkalophilic strain of *B. amyloliquefaciens*. Gist Brocades produces and supply Maxatase, from *B. licheniformis*. Alcalase and Maxatase (both mainly subtilisins) are recommended for use at 10-65°C and pH 7-10.5. Savinase and Esperase may be used at, up to pH 11 and 12, respectively.

Research efforts to develop novel proteases to be used in detergents are underway in various parts of the world. At present only very few published reports are available on the compatibility of the alkaline proteases with detergents.

Recognising the industrial need for stability at alkaline pH values typically associated with detergents, Anstrup *et al.* (1973) patented proteolytic enzymes from a variety of *Bacillus* sp. Durham (1987) studied the utility of subtilisin GX as a detergent additive. Thermo stability of high activity alkaline protease from the same species was studied by Bhosale *et al.* (1995) Kwon *et al.* (1994) reported two thermo stable and alkali stable proteases from *V.metschnikowii* strain, which were resistant to detergents, SDS and urea, and suggested the possibility to be used as detergent additives. The alkaline protease from a marine shipworm bacterium was reported to be stable with high concentrations of oxidants (Griffin *et al.*, 1994). The use of the same protease in industrial cleansing applications was reported by Greene *et al.* (1996). The future application of a bleach stable alkaline protease from *Bacillus* sp. was reported (Gupta *et al.*, 1999).

Oberoi *et al.* (2001) characterized and tested the wash performance of an SDS stable alkaline protease from a *Bacillus* sp. The application of protease as a laundry detergent additive from a *Nocardiopsis* sp. has been reported by Moriera *et al.* (2002). Purification and characterization of a thermo stable protease resistant to detergents were reported by Seong *et al.*

(2004). Protease autolysis identified as the primary mode of activity loss in heavy-duty liquid detergent formulations, was studied by Stoner *et al*, (2004).

There are a few reports on the use of fungal proteases in detergent industry. Samal *et al.* (1990) reported the possible use of protease from a fungus *Tritirachium album* in commercial laundry detergent formulations. Phadatare *et al.* (1993) investigated the stability of a protease from *Conidiobolus coronatus* with the commercial detergents. Compatibility of the alkaline protease of *Basidiobolus*, with commercial detergents has been reported by Ingale *et al.* (2002). The purification and characterization of a protease from *Aspergillus parasiticus* has been carried out and its stability towards different oxidizing and reducing agents and detergents were studied by Tunga *et al.* (2003).

Use of proteases of non-microbial origin, in detergent formulations was also reported. Anwar and Saleemuddin (2000) reported the application of a protease from the larvae of an insect *Spilosoma obliqua* alkaline protease in detergent industry.

5.2 Materials and methods

5.2.1 Effect of pH on the activity and stability of the proteases

The effect of pH on the activity and stability of the proteases was determined as described in section 4.2.5.2.1

5.2.2 Effect of temperature on the activity and stability of the proteases

Activity and stability of the proteases at various temperatures were determined as described in section 4.2.5.2.2

5.2.3 Activity of the proteases on different protein substrates

The ability of the proteases to hydrolyse different protein substrates like casein, gelatin and bovine serum albumin was ascertained. Casein hydrolysis was determined by the assay method described in section 2.2.5.4 and also by the hydrolysis of casein on casein agar medium (section 2.2.4.2). Gelatin

hydrolysis was determined by the hydrolysis of gelatin on gelatin agar medium (section 2.2.4.1). The hydrolysis of BSA was assayed by the same procedure as used for casein, where BSA was used as substrate, instead of casein.

5.2.4 Stability of the proteases in different commercial detergents

Measured quantity of enzymes was incubated with the solutions of the different commercial detergents at a detergent concentration of 7mg/ml (to simulate washing conditions) for one hour. The detergents tested were Ariel, Henko, Rin, Surf, Sunlight and Tide, which are widely used in India. Suitable aliquots were withdrawn at different time intervals for one hour, and the residual activity measured by standard assay procedure and compared with the control incubated under similar conditions, without any detergent and the relative activity was expressed in percentage taking the value given by control as 100%. Also the detergent solutions in the same concentration used for this study, but without incubating with the enzyme, were assayed for protease activity, to rule out the possibility of any protease (if at all present) as the ingredient of the detergents, contributing towards the residual activity tested (Several commercial detergents are claimed to contain protease enzyme, for e.g. Tide). The dialysate of the ammonium sulphate precipitated fraction was used for this study, as enzyme source.

5.2.5 Stability of the proteases in SDS, TritonX-100 and H_2O_2

The proteases were incubated with the different concentrations of SDS (0.05%, 0.1%, 0.2%, 0.4%), H_2O_2 (1%, 2%, 3%, 4%) and Triton X-100 (0.1%, 0.4%, 0.7%, 1%), for 30 minutes, and residual activity of the proteases were measured by the standard assay procedure.

5.3 Results

The results are presented in graphs and tables and values are given in Appendix 4.

5.3.1 Effect of pH on the activity and stability of proteases

B15

For B15 protease the optimum pH was 7, and was active over pH values up to 10. It had nearly 75% of the maximum activity at pH 8, 9 and 10. (Fig.4.1). It was stable between pH 7 and 11. Maximum stability was at pH 8 and even at pH 11, 52% of the maximum activity was retained. (Fig 4.4)

V10

For V10, the optimum pH was 8. It was active over a range of pH 7 to 11, (Fig.4.2). The protease showed a broader range of pH stability i.e. between pH 5 and 13. In this case also maximum stability was at pH 8. (Fig 4.5)

V26

For V26 protease the optimum pH was 9 the activity range being 7 to 11 (Fig 4.3). The pH stability range was between pH 6 and 13 (Fig 4.6). Maximum stability was obtained at pH values 9, 10 and 11, the difference in values being not significant.

5.3.2 Effect of temperature on the activity and stability of proteases

B15

For B15 protease the optimum temperature of activity was 40°C, and it was active over a range of 15°C to 75°C (Fig 4.4). The protease was stable up to 60°C (Fig 4.7).

V10

V10 protease was active over a wide range of temperatures, 25°C and 75°C; optimum activity was at 55°C. At 60°C also it was equally active having

no significant difference from that at 55°C (Fig 4.5). The protease was absolutely stable up to a temperature of 50°C (Fig 4.8).

V26

For V26 protease the maximum activity was obtained at 75°C, and then steeply decreased. It was active over a wide range of temperatures (30°C to 80°C) (Fig 4.6). The stability studies of this revealed that the enzyme was absolutely stable up to 50°C, and retained considerable activity up to 60°C (Fig 4.9).

5.3.3 Activity of the proteases on different protein substrates

The three proteases preferred mostly casein, as their substrate. A comparison was made between their affinity towards casein and BSA. The results are shown in table 5.2. Here we can see that although the proteases hydrolyse BSA, the affinity towards casein is much higher than that towards BSA. For B15 the BSA hydrolysis was only 14 % efficient as that for casein and for V10 it was 16% and for V26, 28%.

Table 5.2 Hydrolysis of casein and BSA by the three selected proteases.

Strain	Units of enzyme activity	
	Casein	BSA
B15	97.47441	13.89883
V10	207.5494	33.45509
V26	219.3662	63.28327

Gelatin was hydrolysed as can be seen from the zone of hydrolysis by protease on gelatin agar plates. (Plate 1). All the three strains were gelatinolytic.

5.3.4 Stability of the proteases in different commercial detergents

B15

B15 protease was considerably stable with all the commercial detergents tested. After one hour 72% activity was retained with Ariel and

Rin, 74%with Henko, 78% with Sunlight, 67% with Surf and 70% with Tide (Fig 5.1; Table 5.3).

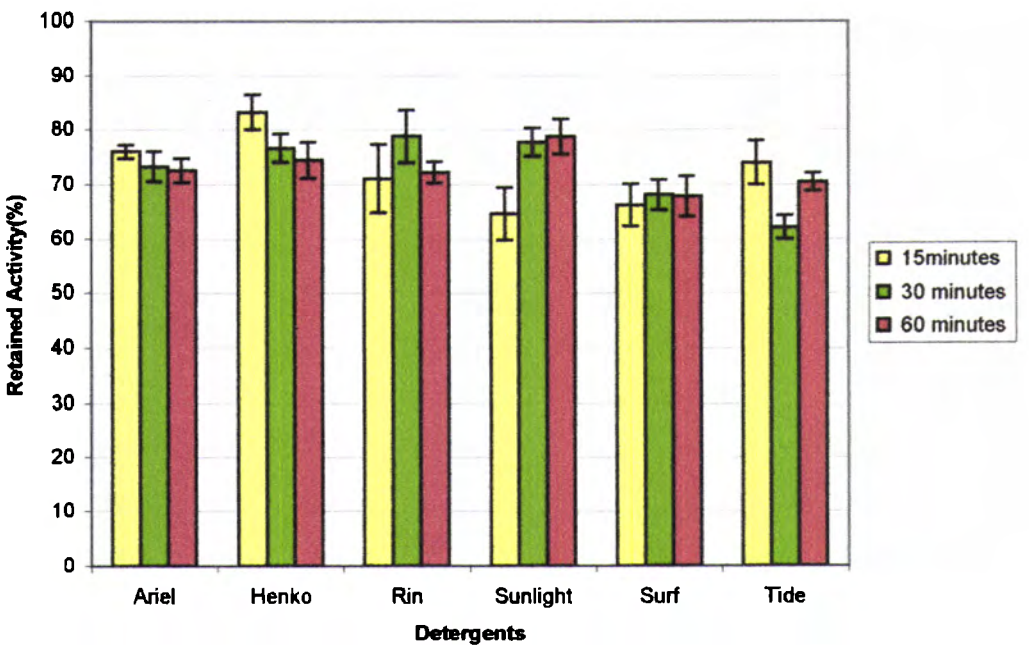


Fig.5.1 The stability of the *B. circulans* protease in the commercial detergents.

Table 5.3 ANOVA for the stability of the *B. circulans* protease in the commercial detergents.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Periods	29.00723	2	14.50362	0.209057	NS
Detergents	749.0603	5	149.8121	2.159411	NS
Error	693.7635	10	69.37635		
Total	1471.831	17			

ANOVA showed that the various detergents did not have any significant difference in their effect on the B15 protease, nor did the different periods of incubation i.e. in all the detergents after different period of incubation the B15 protease retained almost equal residual activity.

V10

The V10 protease retained 42% residual activity after one hour of incubation with Ariel and Henko, 51% with Rin, 47% with Sunlight, 61% with

Surf, and 47% with Tide. After 15 minutes they retained 53%, 63%, 57%, 54%, 60% and 64% respectively (Fig 5.2; Table 5.4)

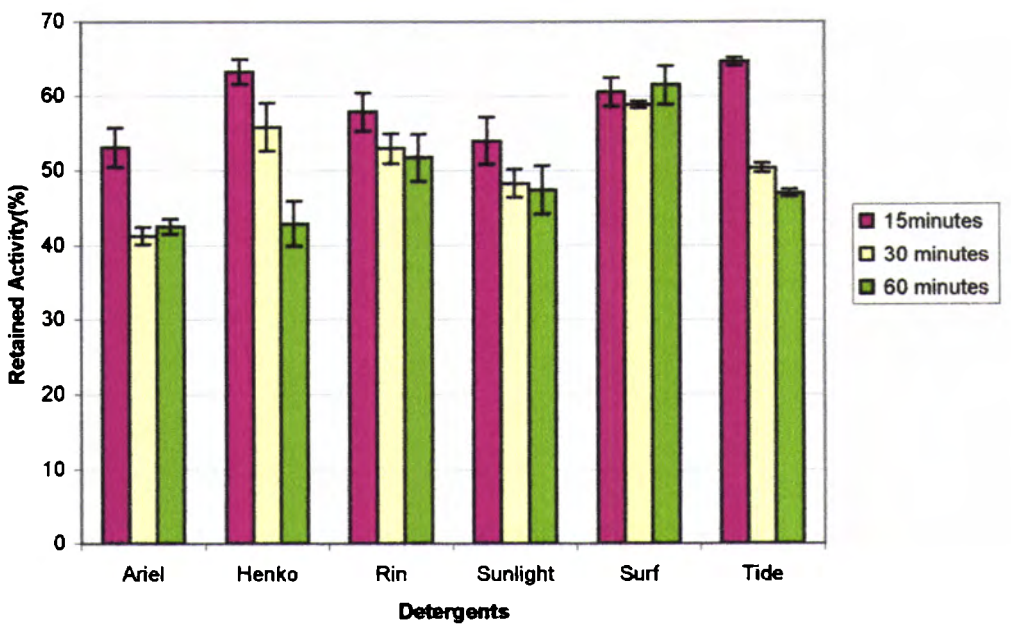


Fig.5.2 Stability of the *V. fluvialis* protease in the commercial detergents.

Table 5.4 ANOVA for the stability of the *V. fluvialis* protease in the commercial detergents.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of squares	F-value	P-value
Periods	333.9364	2	166.9682	8.65646	<0.001
Detergents	361.8394	5	72.36787	3.75191	<0.05
Error	192.8827	10	19.28827		
Total	888.6585	17			

LSD for periods = 7.472

LSD for detergents = 10.567

ANOVA showed that there was significant difference in the effect of different detergents and the period of incubation on the V10 protease. The detergent Surf exerted a significant difference from the others i.e. V10 protease was more or less equally stable with surf at all the periods tested.

For all the others although they conferred almost equal stability at 15 minutes, at 30 and 60 minuets they showed reduction in stability.

V26

The residual activity of V26 protease after one hour in Ariel, Henko, Rin, Sunlight, Surf and Tide were about 37%, 58%, 64%, 63%, 60% and 45% respectively. After 45 minutes they were 45%, 71%, 68%, 67% and 71% respectively (Fig. 5.3; Table 5.5).

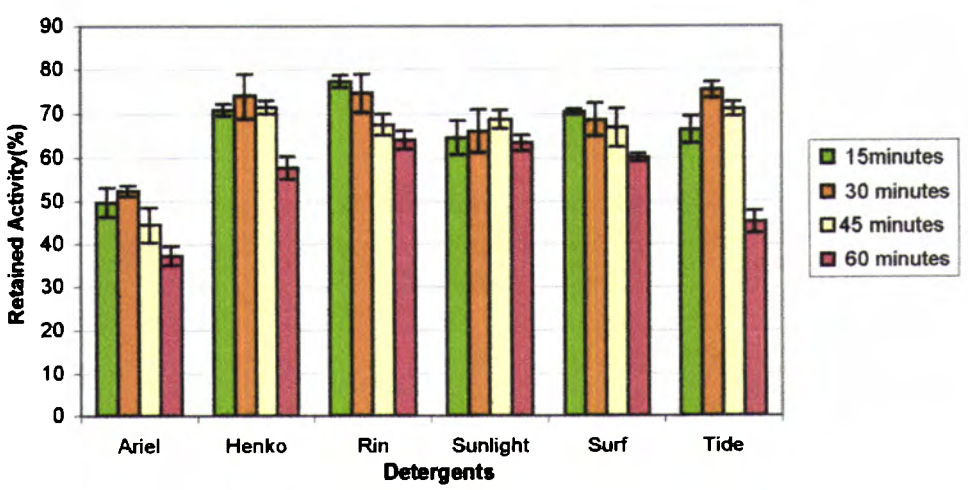


Fig.5.3 Stability of the *Vibrio* sp. protease in the commercial detergents.

Table 5.5 ANOVA for the stability of the *Vibrio* sp. protease in the commercial detergents.

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Periods	770.6377	3	256.8792	11.46313	<0.001
Detergents	1537.388	5	307.4776	13.72106	<0.001
Error	336.1375	15	22.40917		
Total	2644.163	23			

LSD for periods = 8.054

LSD for detergents = 8.26

ANOVA showed that there was significant effect of detergents as well as incubation periods on the activity of V26 protease. Ariel was found to

significantly lower the stability in comparison with the other detergents. Regarding incubation period, up to 45 minutes the residual activity of the protease was almost stable but at 60 minutes it significantly reduced.

5.3.5 The effect of H_2O_2 on the activity and stability of the proteases.

For B15 and V10 proteases, the activity was found to increase with in crease in H_2O_2 concentration. Activity was more or less similar for B15 and V10 proteases. For B15 it increased up to 116% and for V10 132% at the highest concentration of H_2O_2 tested. For V26 it was found to decrease with increase in concentration of H_2O_2, the residual activity being 74% at the highest concentration of H_2O_2 (Fig 5.4;Table 5.6-5.8).

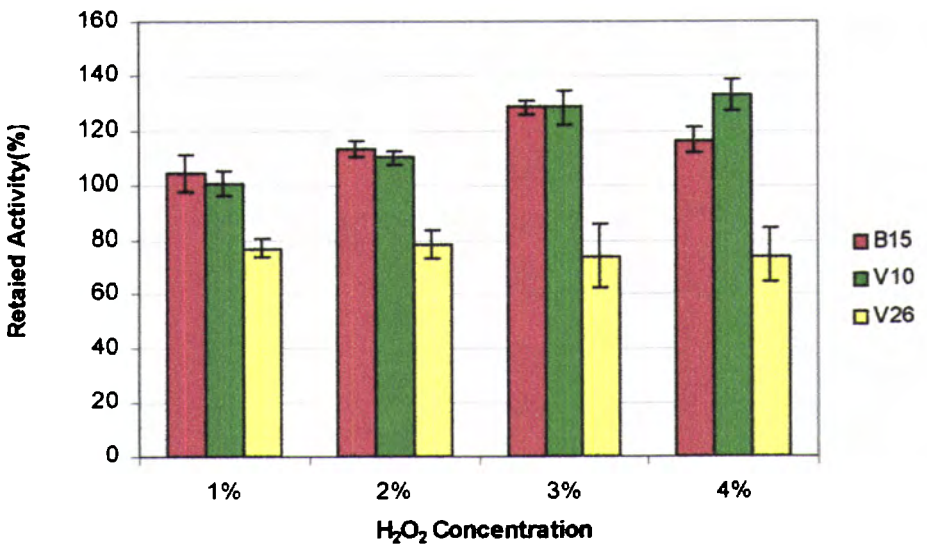


Fig.5.4 The effect of H_2O_2 on the proteases of *B. circulans*, *V. fluvialis and Vibrio* sp.

Table 5.6 ANOVA for the effect of H_2O_2 on the *B. circulans* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	1218.527	3	406.1757	19.71703	<0.001
Within Groups	247.203	12	20.60025		
Total	1465.73	15			

LSD=9.8046

ANOVA showed that for B15 protease there was significant difference between the residual activity at 2% and 3% concentrations and not between the others.

Table 5.7 ANOVA for the effect of H_2O_2 on the *V. fluvialis* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	2774.401	3	924.8004	36.108	<0.001
Within Groups	307.3421	12	25.61184		
Total	3081.743	15			

LSD=10.9324

ANOVA showed that for V10 protease there was significant difference between the residual activity at 2% and 3% and 4%concentrations.

Table 5.8 Results of ANOVA for the effect of H_2O_2 on the *Vibrio* sp. protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	57.82218	3	19.27406	0.27779	NS
Within Groups	832.602	12	69.3835		
Total	890.4241	15			

ANOVA showed that for V26 protease there was no significant effect for different concentrations on the residual activity.

5.3.6 The effect SDS on the activity and stability of the proteases

For B15 protease the retained activity after treatment with 0.05% SDS was 70% and with 0.4% SDS was 56%. For V10 protease, it was 65% and 62% respectively. And for V26 it was 57% and 63% respectively (Fig. 5.5; Tables 5.9-5.11)

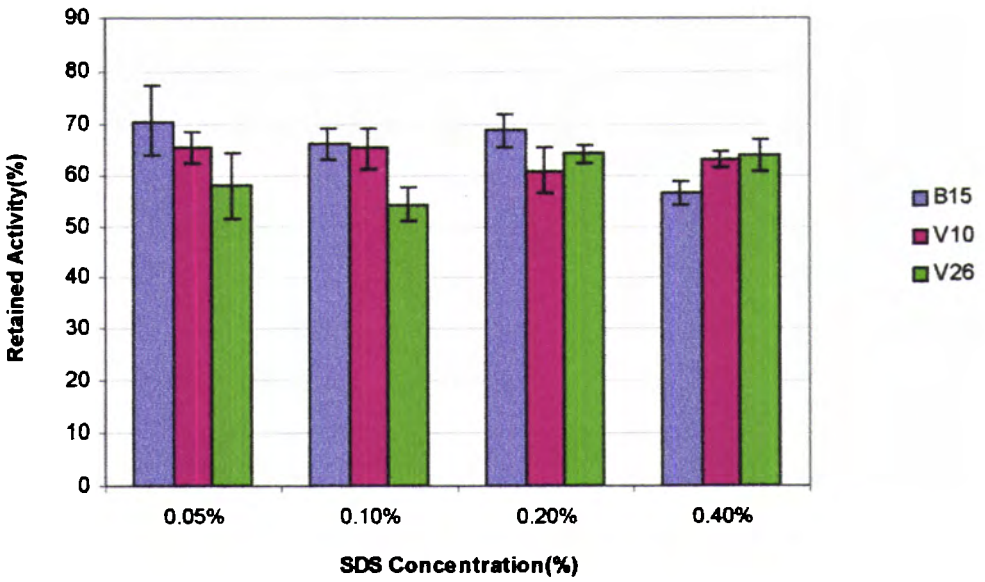


Fig.5.5 The effect of SDS on the proteases of *B. circulans*, *V. fluvialis and Vibrio* sp.

Table 5.9 ANOVA for the effect of SDS on the *B. circulans* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	470.5164	3	156.8388	8.80171	<0.001
Within Groups	213.8295	12	17.81913		
Total	684.3459	15			

LSD=9.11

ANOVA showed that for B15 protease there was significant reduction in residual activity at 0.4% SDS concentration with respect to the other concentrations tested. There was no significant difference between the residual activities at other concentrations.

Table 5.10 ANOVA for the effect of SDS on the *V. fluvialis* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	54.3656	3	18.12187	1.481641	NS
Within Groups	146.7713	12	12.23094		
Total	201.1369	15			

ANOVA showed that for V10 protease there was no significant effect for different concentrations of SDS on the residual activity.

Table 5.11 ANOVA for the effect of SDS on the *Vibrio* sp. protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	272.198	3	90.73266	5.682736	<0.01
Within Groups	191.5964	12	15.96637		
Total	463.7944	15			

LSD = 2.997

ANOVA showed that for V26 protease there was no significant difference in residual activity at 0.2% and 0.4% SDS concentrations. But there was significant difference in residual activities in 0.05%, 0.1% and 0.2% SDS.

5.3.7 Effect of Triton -X-100 on the activity and stability of the proteases

In the case of B15 protease the residual activity was found to increase with increasing concentrations of T X-100 up to 0.7% and then decreased on further increase in concentration. For V10 proteases, the residual activity was found to decrease with increasing concentrations of T-X-100. But for V26 there was a dramatic increase in residual activity, which ranged from 69% to 135% for 0.1% and 1% T-X-100 respectively (Fig.5.6; Tables 5.12 to 5.14)

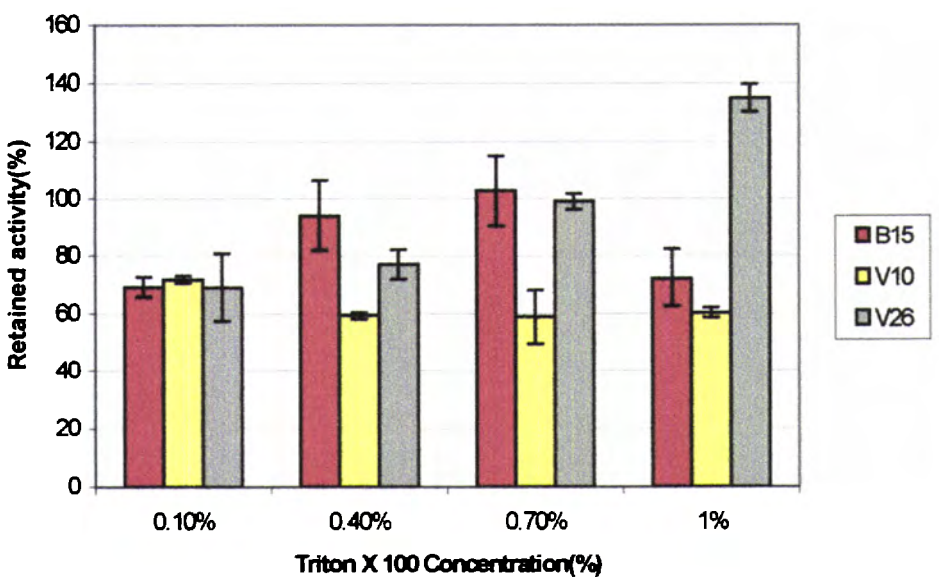


Fig.5.6 The effect of Triton -X-100 on the proteases of *B. circulans, V. fluvialis and Vibrio* sp.

Table 5.12 ANOVA for the effect of Triton -X-100 on the *B. circulans* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	3223.569	3	1074.523	14.8781	<0.001
Within Groups	866.6613	12	72.22178		
Total	4090.231	15			

LSD=18.3582

ANOVA showed that for B15 protease there was significant difference in residual activity at 0.1% and 0.4% and 0.7% T-X-100 concentrations. But there was no significant difference in residual activities at 0.1% and 1% concentrations.

Table 5.13 Results of ANOVA for the effect of Triton -X-100 on the *V. fluvialis* protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	470.904	3	156.968	6.718005	<0.001
Within Groups	280.3833	12	23.36527		
Total	751.2873	15			

LSD=10.4419

ANOVA showed that for V10 protease there was significant difference in residual activity between 0.1% and all other concentrations of T-X-100.

Table 5.14 Results of ANOVA for the effect of Triton -X-100 on the *Vibrio* sp. protease

Source of Variation	Sum of Squares	Degrees of freedom	Mean of Squares	F-value	P-value
Between Groups	10258.69	3	3419.564	38.06054	<0.001
Within Groups	1078.145	12	89.8454		
Total	11336.84	15			

LSD=20.4759

ANOVA showed that for V26 protease there was significant difference in residual activity at 0.1% and 0.7% and 1% TX-100 concentrations. But there was no significant difference in residual activities between 0.1% and 0.4% concentrations and between 0.4% and 0.7%.

5.4 Discussion

Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sale of enzymes (Rao *et al.*, 1998). The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains, due to blood, food and other body secretions. Activity and stability at high pH and temperature and compatibitlity with other chelating and oxidising agents added to the detergents are among the major prerequisites for the use of proteases in detergents. Alkaline proteases are of considerable interest in view of their activity and stability at alkaline pH. All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe- free enzyme.

5.4.1 Effect of pH on the activity and stability of proteases

pH is the key factor, which determines the effectiveness of an enzyme as a detergent additive. Generally all detergent compatible enzymes have their pH optimum at 9-12 (Gupta *et al.,* 1999). The optimum pH of the important detergent enzymes Subtilisin Carlsberg and Subtilisin Novo are 10.5.

In the present study also the protease showed considerable activity and stability in the pH range 7 to 11. The B15 protease was active over pH values up to 10 and the optimum pH was 7. Maximum stability was at pH 8. V10 protease was active over a pH range of 7 to 11 the optimum being pH 8. This is comparable to *Conidiobolus coronatus* protease, which was reported to be

used in detergents, with an optimum pH at 8.5 (Phadatare *et al.*, 1993); and the *Aspergillus* protease reported by Tunga *et al.* (2003) which had a pH optimum of 8 and the activity of which decreased with increasing pH. The V10 protease showed a broader range of pH stability i.e. between pH 5 and 13 as observed in a *Bacillus* protease described by Gupta *et al.* (1999), which was optimally active at pH 10 and stable between pH 5 to 12.

For V26 protease, the optimum pH was 9 the activity range being 7 to 11. The pH stability range for the enzyme was between pH 6 and 13. Maximum stability was obtained at pH values 9, 10 and 11, the difference in values being not significant. From these observations it can be inferred that among the three proteases, the maximum pH stability was shown by V10 protease (5-13), followed by V26 (6-13) and B15 (7-11).

All the three proteases became inactive at pH 12 similar to the alkaline protease from a marine shipworm bacterium, which was stable up to pH 11.9 and became inactive at pH 12 (Greene *et al.*, 1996). The pH optimum for a lepidopteran larvae protease, reported to be an effective stain remover was in the range 10-12. (Anwar and Saleemuddin, 2000). The protease of *Bacillus* sp. (Oberoi *et al.*, 2001) had a pH optimum of 11 and was stable up to pH 12. The *Basidiobolus* protease, which was tested for its efficacy as a detergent additive, was active and stable at pH 10 (Ingale *et al.*, 2002). The *Nocardiopsis* protease reported by Moreira (2002) showed an optimum pH of 10.5. With 45% of the maximum activity at pH 12, this enzyme was stable at alkaline pH, retaining more than 90% activity at pH 10.

5.4.2 Effect of temperature on the activity and stability of proteases

Most of the subtilisin type commercially available proteases are thermo stable and active over a range of 50-70°C. A recent trend in detergent industry is a requirement for alkaline proteases active at low washing temperatures; for example, Kanase- marketed by Novo enzymes- is active even at temperatures as low as 10-20°C (Gupta *et al.*, 2002a).

In the preset investigation the proteases showed very good activity and stability over a wide range of temperature. For B15 the optimum activity was at 40°C, and it was active over a range of 15°C to 75°C.

V10 protease was also active over a wide range of temperatures, between 25°C and 75°C. Optimum activity was at 55°C. V10 protease was absolutely stable up to a temperature of 50°C, and showed considerable stability up to 60°C.

For V26 protease, the maximum activity was obtained at 75°C, and then steeply decreased. It was active over a wide range of temperatures (30°C to 80°C). The stability studies of this revealed that it was absolutely stable up to 50°C and retained considerable stability up to 65°C.

The results obtained in the present study are in accordance with similar studies carried out elsewhere. The Subtilisin GX protease produced by an alkalophilic *Bacillus* sp. with good alkaline stability had high activity at moderate temperatures. Its temperature optimum was between 50°C and 60°C (Durham, 1987). The wash performance tests as measured by the reflectance measurement showed that the Subtilisin GX was effective at 20°C, 40°C and 50°C, however, the reflectance (a measure of effectiveness of washing) was much lower at 20°C than at 50°C. A shipworm bacterium protease, which was found to increase the efficacy of standard detergents, was active at 50°C but was ineffective at 70°C (Greene *et al.*, 1996). A fungal protease, which was suggested to be used in detergents, had heat stability up to 40°C (Phadatare *et al.*, 1993). The protease from a *Bacillus* sp. was optimally active at 60°C to 70°C and was stable up to 60°C. (Gupta *et al.* (1999). A broad temperature range of 20 to 80°C with an optimum at 60°C, was reported for *Bacillus* sp. by Oberoi *et al.* (2001). Moreira (2002) described a protease from *Nocardiopsis* sp. that could be used in detergents, and its optimum temperature of activity was 50°C and retained activity of 60% up to 80°C after 30minutes incubation at this temperature. The well-

established commercial detergent namely Subtilisin Carlsberg has a half-life of 2.5 minutes at 60°C. (Durham *et al.*, 1987)

In general, all currently used detergent compatible enzymes are alkaline and thermo stable in nature with a high pH and temperature optima. The present enzymes are promising detergent additives as besides being alkaline, they are also active over a broad temperature range, this would facilitate washing even at ambient temperatures which is a present day requisite to maintain fabric quality and lowering energy demands. Very few reports are known where proteases are active at room temperatures (28°C). Tamiya and Nakamura (1996) reported a protease from psychrophilic *Flavobacterium balustinum* P104 optimally active at 40°C and rapidly inactivated at 50°C. Kitayama (1992) reported a low temperature alkaline protease from *Bracteococcus* minor with optimum temperature at 37°C. The two proteases under study are very promising in this regard, the B15 protease has its temperature optimum at 40°C with very good activity in the range 30 to 60°C and moderately active up to 75°C and it was found to retain 66% of the maximum activity at 30°C. V10 protease is having its optimum at 55°C, and showed more than 64% activity at 30°C. The V26 protease is exceptional for its optimum at 75°C but have only 22% activity at 30°C. V26 protease is highly promising for high temperature application.

5.4.3 Activity of the proteases on different protein substrates.

Broad substrate specificity is a desirable feature of the proteases used in detergents, as they will have to remove a variety of protein stains from clothes soiled with milk, blood, sweat, cocoa, etc.

The three proteases cleaved casein, gelatin and BSA, and the preferred substrate was casein. A comparison made between their affinity towards casein and BSA showed that although the proteases hydrolysed BSA, the affinity towards casein was much higher than that towards BSA. For B15 the BSA hydrolysis was only 14 % efficient as that for casein and for V10 it was

16% and for V26, 28%. Similar studies have shown that casein was the most preferred substrate of the alkaline proteases and BSA was least preferred.

The insect protease was reported to have broad substrate specificity, the most preferred substrate being casein, followed by hemoglobin, BSA, ovalbumin and gelatin. It also cleaved synthetic substrates (Anwar and Saleemuddin, 2000). The *Conidiobolus* protease had broad substrate specificity, casein being the most preferred substrate (30U/ml), followed by haemoglobin (15U/ml), ovalbumin (3.6U/ml), and BSA (4.5U/ml) (Phadatare *et al.,* 1993). The *Bacillus* protease (Oberoi *et al.* (2001), was also found to be active on casein, hemoglobin and BSA. Hasan and Tamiya (1997) reported a cold adaptive protease CP70 active on casein, gelatin, albumin and haemoglobin. *Nocardiopsis* protease (Moreira, 2002) was also active against various substrates; the most preferred being casein, followed by azocasein, haemoglobin, and ovalbumin and least hydrolysed was BSA.

5.4.4 Stability of the proteases in different commercial detergents.

In order to assess the suitability of an alkaline protease to be used as detergent additive, it is very essential that the resistance of these proteases to the commercial detergents be tested, as this is a very effective method to know the overall stability and activity of the proteases under the normal washing conditions. The commercial detergents with all their ingredients will be exerting their effects on the proteases added, and those proteases, which could withstand these effects and still retain the activity, would be very much suitable to be used in detergent formulations owing to their stability.

In the present study the stability of the three proteases in presence of various detergents have been studied and the results were promising. B15 protease was considerably stable with all the commercial detergents tested. After an hour, 72% activity was retained with Ariel and Rin, 74% with Henko, 78% with Sunlight, 67% with Surf and 70% with Tide. This protease was almost equally stable with all the detergents tested. The V10 protease retained 42% residual activity after one hour of incubation with Ariel and

Henko, 51% with Rin, 47% with Sunlight, 61% with Surf, and 47% with Tide. After 15 minutes they retained 53%, 63%, 57%, 54%, 60% and 64% respectively. The residual activity of V26 protease after one hour in Ariel, Henko, Rin, Sunlight, Surf and Tide were about 37%, 58%, 64%, 63%, 60% and 45% respectively. After 45 minutes they were 45%, 71%, 68%, 67% and 71% respectively. Both V10 and V26 were least stable with Ariel. It was found to significantly lower the stability in comparison with the other detergents. For V26 protease, up to 45 minutes the residual activity of the protease was almost stable but at 60 minutes it significantly reduced.

Almost similar results were recorded by several workers. An insect protease reported by Anwar and Saleemuddin (2000) was found to retain 80% of its activity with Surf and 60% in Nirma, and least stable in Ariel retaining only 30% activity at pH 11 after 60 minutes, whereas at pH 9 it was more stable with Ariel, and more labile to surf. *Conidiobolus* protease retained 90% activity with the detergent Snow white, more than 80% in presence of detergents, Nirma and Revel and more than 56% in Wheel and Surf. In the detergent Det it lost almost 60% of activity (Phadateare *et al.*, 1993). The *Bacillus* protease reported by Gupta *et al.* (1999) retained 66% activity after 30 minutes and 35% after 60 minutes and other detergents like Fena, wheel and Robin liquid bleach were found to enhance the activity. The *Basidiobolus* protease reported by Ingale *et al.* (2002) was tested for its stability in the commercial detergent Surf (3mg/ml) and the retained activity was only below 40% after 40 minutes. The *Nocardiopsis* protease (Moreira, 2002) was found to be stable with various commercial detergents tested, the maximum retained activity being 64% after 1 hr. incubation.

Subtilisin GX, (Durham, 1987) was tested for its detergent efficacy by measuring the reflectance of the standard test fabric that was washed with the detergent plus enzyme combination and that washed with detergent alone, and found that the protease detergent combination was more effective as it showed higher reflectance than the fabric washed with detergent alone. The alkaline protease from a marine shipworm bacterium claimed to double

the cleaning power of a standard phosphate detergent, by this method (Greene *et al.*, 1996). Wash performance analysis was also done with a *Bacillus* protease by Oberoi *et al.* (2001), where the combination of the enzyme with the commercial detergents gave best results, and the protease retained more than 70% activity in presence of the commercial detergents like Surf, Ariel, Wheel, Fena and Nirma.

5.4.5 The effect of H_2O_2 on the stability of proteases

H_2O_2 is abundantly generated in the washing process. Hence the added proteases should be stable in the presence of this compound. Hence stability of the alkaline proteases in the presence of different concentrations of H_2O_2 was tested and very interesting results were obtained.

For B15 and V10 proteases, the activity was found to increase with increase in H_2O_2 concentration. For B15 it increased up to 116% and for V10 132% at the highest concentration (4%) of H_2O_2 tested. For V26 it was found to decrease with increase in concentration of H_2O_2, the residual activity being 74% at 4% H_2O_2 and this is more effective when compared to a protease reported by Manachini *et al.* (1998), which retained 70% of its activity in 3% H_2O_2 after 1 hour at 25°C and 45% after 30 minutes at 40°C.

There are only a few reports on H_2O_2 stable enzymes and there was no report available where the activity of enzyme was enhanced in presence of H_2O_2. Gupta *et al.* (1999) observed that the bacillus protease retained 85% activity after 1 hr incubation with 10% H_2O_2. The protease reported by Oberoi *et al.* (2001) was stable in presence 5% H_2O_2 retaining 60% of the maximum activity. For the alkaline protease of a marine shipworm bacterium, which was suggested to be used as a detergent additive, at 3% H_2O_2 a significant reduction in the stability of the proteases was observed (Greene *et al.*, 1996). The *Aspergillus* protease (Tunga *et al.*, 2003) retained 98% activity after incubation with 10mM H_2O_2. *Nocardiopsis* protease reported by Moriera *et al.*, (2002) retained 87% of the activity in presence of 10% H_2O_2 even after one hour.

In general, the majority of commercially available enzymes are not stable in the presence of bleaching or oxidising agents. Hence the latest trend in enzyme-based detergent is the use of recombinant DNA technology to produce bio-engineered enzymes with better stability (Abrige and Pitcher, 1989). Bleach stability has been introduced by protein engineering i.e. replacement of certain amino acids residues (Boguslawski and Shultz 1992; Wolff *et al.*, 1996) and site directed mutagenesis (Outtrup *et al.*, 1993;Tuschiya *et al.*, 1993; Outtrup *et al.*, 1995). The B15 and V10 proteases show not only inherent bleach stability but also an enhancement of activity in presence of H_2O_2 suggesting the possibility of these proteases to be included as detergent additives. The present study is highly significant in this regard.

5.4.6 The effect of SDS and Triton X –100 on the activity and stability of the proteases

SDS is an anionic detergent and T-X-100 is a non-ionic detergent. Hence it was appropriate to test the stability of the proteases towards these compounds to prove their effectiveness. The retained activity of the B15 protease after treatment with 0.05% SDS was 70% and with 0.4% SDS was 56%, for V10, it was 65% and 62% respectively and for V26 it was 57% and 63% respectively.

B15 protease showed an increase in residual activity with increase in concentrations of T X- 100 up to 0.7% and then decreaed on further increase. For V10 the residual activity was found to decrease with increasing concentrations of T-X-100. But for V26 there was a dramatic increase in residual activity, which ranged from 69% to 135% for 0.1% and 1% T-X-100 respectively.

The stability towards SDS is important because SDS stable enzymes are not generally available except for a few. A SDS resistant alkaline serine protease from *Vibrio alginolyticus* (Deane *et al.*, 1987) retained its full activity at 2.5% SDS. The *Bacillus* protease (Oberoi *et al.*, 2001) was stable in presence of high concentration of SDS i.e. retained 70% of its activity at

0.5% SDS, and in Triton –X-100 59% of the activity was retained after one hour. Tunga *et al.* (2003) reported an *Aspergillus* protease, which were very much stable in 2% SDS and Triton X 100 retaining more than 95% of the activity and this protease exhibited considerable stability with the heavy metals and metal chelators like EDTA. A *Streptomyces* protease was reported which was resistant to Triton-X- 100 (Seong *et al.*, 2004). Moriera *et al.* (2002) reported a *Nocadiopsis* protease whose activity was enhanced to 171%, in presence of 1% SDS after 30 minutes and decreased to 50% after 60 minutes. However, proteases from *Oerskovia xanthineolytica* TK-1 (Saeki *et al.*, 1994) and *Streptomyces* sp. YSA-130 (Yum *et al.*, 1994) are reported to be inhibited in presence of 0.1% SDS. In the present study all the proteases were found to be retaining around 60% activity in presence of 0.4% SDS. The enzymes were very active in presence of T- X- 100 also. The activity of proteases from V26 was even considerably enhanced in presence of the detergents suggesting their possible utilisation in the non-ionic detergents.

Most of the detergent proteases reported so far belong to the class of serine proteases. It is argued that thiol proteases (e.g. papain) would be oxidised by the bleaching agents, and metalloproteases (e.g. thermolysin) would lose their metal cofactors due to complexing with the water softening agents or hydroxyl ions. B15 and V10 proteases were found to be serine proteases and V26 protease a metalloprotease. But the fact that the proteases showed considerable stability in the commercial detergents, suggests that their susceptibility to EDTA was not a major factor affecting their suitability as detergent additives. However stability to metal chelating agents like EDTA is a desirable character for detergent enzymes. Even though the proteases studied here lacked this property, their properties like high pH and temperature stability, stability in commercial detergents, the stability and even an enhanced activity with H_2O_2, stability with ionic and non-ionic detergents and broad substrate specificity make them ideal candidates to be used in detergent formulations.

Considering the overall properties of different alkaline proteases of microbial origin and their evaluation, the reported alkaline proteases were found to be much promising with regard to pH and temperature stability, the detergent compatibility, stability to oxidising agents and substrate affinity, for their future application in detergent formulations. Moreover, their effectiveness at low wash temperatures makes them much valuable in view of the present energy crisis, particularly in developing countries like India where detergents are commonly used at room temperatures, as the cost of heating water is high.

CHAPTER 6
SUMMARY

This thesis presents a detailed account of the distribution of proteolytic bacteria in the sediments of Cochin estuary, their distribution, the optimum conditions of the selected bacterial strains to grow and produce alkaline protease, purification, and characterisation of the alkaline proteases and their application in detergent formulations.

The important findings of the study are

- Proteolytic bacteria are distributed widely in the sediments of Cochin estuary. The most abundant genus was *Bacillus* and the percentage of protease producing strains was also maximum in this genus, followed by *Vibrio* sp.
- The gelaltinolytic strains were 73% of the total isolates and caseinolytic strains were 35%.
- The most potent alkaline protease produers, selected for an in-depth study, were *Bacillus circulans* (B15), *Vibrio fluvialis* (V10) and a *Vibrio* sp. (V26).
- Mangrove station showed an abundance of proteolytic forms. The most potent strains selected were from the mangrove sediment at Puduvaippu.
- The culture conditions were optimized for the selected strains, for maximum growth and protease production.
- The optimum shaking speed was in the range of 50 rpm to 150 rpm for the three strains.
- The incubation period for maximum protease production for B15 was 60 hr. and for V10 and V26 were 36 hr and 72 hr. respectively.
- Optimum pH for protease production was 7 for all the three strains, and at pH 8 also remarkable production was observed in the case of V10 and V26

- Salinity did not significantly influence protease production. For B15 and V26 maximum protease production was at 0 salinity. For V10 maximum protease production was at 0.5% NaCl.
- The optimum temperature for enzyme production for all the strains was 25°C and very good enzyme production was obtained up to a temperature of 35°C. Though good growth was obtained at 20°C and 40°C there was only negligible enzyme production at these temperatures.
- The influence of metallic salts on the protease production was studied. Co^{2+} showed a complete inhibition of protease production by all the strains. Manganese also in high concentrations was found to be inhibitory to all the strains. For B15, Ca^{2+}, Mg^{2+} and K^{+} were stimulating protease production whereas for V10, all the salts (K^{+}, Ca^{2+}, Mg^{2+}, Fe^{3+} and the lower concentrations of Mn^{2+}) tested except Co^{2+} and Mn^{2+}, were enhancing protease production. The same was the case with V26.
- Growth and enzyme production were markedly influenced by the source of carbon and nitrogen. Easily metabolisable carbon sources, chiefly glucose, showed catabolite repression. Maltose was the best source with regard to its stimulatory effect on enzyme production. Molasses was giving comparatively good production.
- For all the strains organic nitrogen sources were better inducers of growth as well as protease production. For B15, casein was the best nitrogen source. For V10, casein and yeast extract were the best sources and for V26, beef extract induced maximum production.
- The alkaline proteases from the three strains were purified by ammonium sulphate precipitation. Ammonium sulphate up to 80% saturation was used.
- The molecular weights of the proteases as determined from SDS-PAGE analysis were, 20 kDa for B15 protease, 33.5 kDa for V10 protease and 22.4 kDa for V26 protease.

- All the enzymes were active as well as stable in the pH range 7 to 11. The optimum pH of B15 protease was 7, for V10 protease was 8 and for V26 protease it was 9.
- The optimum temperature of activity showed considerable variation from strain to strain. For B15 protease optimum temperature was 40°C, for V10 protease it was 55°C and for the protease of V26, optimum was 75°C. The proteases were absolutely stable up to a temperature of 50°C. V26 proteases exhibited more heat stability i.e. up to 60°C.
- Substrate concentrations significantly influenced protease action. Km value and Vmax were calculated for the three enzymes with casein as substrate, and by constructing Lineweaver - Burk plot. For B15 protease, Km value was 0.101mg/ml and Vmax was 203.9308 U. For V10 protease, Km value was 0.168mg/ml and Vmax was 231.816 U. For V26 protease, Km value was calculated as 0.656mg/ml and Vmax as 344.05U.
- For B15 protease Hg^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+}, completely and Mn^{2+} to some extent were inhibiting the enzyme activity; the best enhancer of protease activity was Ca^{2+} For V10 protease Hg^{2+} and Cu^{2+} were completely inhibitory, and Zn^{2+} also caused reduction in activity. Co^{2+} and Ca^{2+} were having an enhancing effect on the protease production. For V26 protease Hg^{2+} and Cu^{2+} were completely inhibitory, the enhancers of the activity were Co^{2+}, Mg^{2+}, Na^{+} and K^{+} in the descending order.
- Based on the inhibiton profile of these alkaline proteases, the class of protease they belonged to was determined. B15 and V10 could be placed in the class of calcium dependent serine proteases and V26 to the metalloprotease group.
- These proteases were tested to prove their efficacy as detergent additives, as they were found to have high activity and stability at a wide range of pH and temperature and several features suitable for detergent enzymes.

- They possess good hydrolytic ability over different substrates like casein, gelatin and bovine serum albumin.
- They exhibit good stability in different commercial detergents tested up to one hour. B15 protease was found better with respect to the stability in commercial detergents.
- For B15 and V10 proteases, H_2O_2 was found to be stimulatory. V26 retained up to 74% of activity with the highest concentration of H_2O_2 tested.
- The three proteases were moderately stable with SDS and Triton X-100. However, V26 was found to be stimulated by Triton X-100.
- The properties exhibited by the proteases, make them ideal agents to be used in detergent industry.

The present investigation suggests that proteolytic bacteria are distributed widely in aquatic environment, and they play an important role in biodegradation. The selected *Bacillus* and *Vibrio* species may be considered as potential commercial sources of alkaline proteases, and also as very promising candidates for use in detergent formulations. However, further studies on mass culturing of these strains using cheap raw materials, retention of activity while being incorporated along with the detergents in the powder form, ability to remove various types of stains from different types of fabrics etc. have to be undertaken in detail before recommending them as ideal agents to be used in detergent industry.

A century after the pioneering works of Louis Pasteur, the science of microbiology has reached its pinnacle. In a relatively short time, modern biotechnology has grown dramatically from a laboratory curiosity to a commercial activity. Advances in microbiology and biotechnology have created a favourable niche for the development of proteases and will continue to facilitate their applications to provide a sustainable environment for mankind to improve the quality of human life.

APPENDIX

APPENDIX I

1.1 The generic composition of proteolytic bacteria isolated from Cochin estuary

Genus	Total no of isolates	% of total isolates	No. of Gelatinase +ve isolates	Gelatinolytic isolates(%)	No. of caseinase +ve isolates	caseinolytic isolates (%)
Bacillus	70	28	66	94	36	51
Vibrio	45	18	38	84	16	36
Enterobacteriaceae group	27	11	22	81	12	44
Arthrobacter	19	8	10	53	9	47
Aeromonas	16	6	14	88	5	31
Brevibacterium	16	6	6	38	2	13
Pseudomonas	10	4	7	70	3	30
Stapylococcus	7	3	4	57	2	29
Micrococcus	7	3	3	43	2	29
Flavobacterium	7	3	4	57	1	14
Acinetobacter	5	2	0	0	0	0
Chromobacterium	5	2	1	20	1	20
Alcaligenes	5	2	2	40	0	0
Bordetella	5	2	2	40	0	0
Achromobacter	3	1	2	67	0	0
Aerobacter	2	1	1	50	0	0
Aerococcus	2	1	0	0	0	0
TOTAL	251	100	182	73	89	35

APPENDIX 2

2.1 The effect of shaking speed on growth and protease production

RPM	B15		V10		V26	
	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)
0	3.59±0.52	1.45±0.1	2.47±0.08	1.59±0.07	4.50±0.01	1.52±0.05
50	23.09±1.19	2.22±0	29.07±0.94	2.18±0.01	50.01±0.21	1.75±0.56
100	24.31±0.04	2.21±0.	30.62±2.34	2.17±0	50. ±0.76	2.15±0
150	22.22±0.55	2.17±0	28.91±0.13	2.14±0.02	50.07±1.14	2.17±0
200	21.13±1.55	2.18±0	23.61±0.8	2.15±0.02	45.09±2.89	2.22±0

2.2 The effect of period of incubation on growth and protease production

Hours	B15		V10		V26	
	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)
2	2.41±0.21752	0.03±0.0007	5.38±1.11	0.27±0.01	0±0	0.02±0
4	3.03±1.1	0.13±0.006	5.88±1.64	0.55±0.02	0.57±0.21	0.14±0.01
6	10.823±0.951	0.48±0.009	11.56±1.52	0.79±0.02	1.37±0.33	0.39±0.01
8	11.15±1.90	0.73±0.03	17.15±2.09	0.98±0.03	8.21±2.74	0.65±0.02
10	10.04±1.77	0.93±0.04	23.69±1.55	1.18±0.03	13.38±3.81	0.97±0.00
12	13.08±1.66	1.09±0.02	27.33±1.42	1.3±0.02	18.99±2.41	1.19±0.05
14	14.33±1.97	1.26±0.02	26.99±3.84	1.45±0.01	20.82±2.77	1.31±0.06
24	26.31±2.65	1.74±0	36.38±2.71	1.86±0.03	19.41±2.66	1.49±0.03
36	35.57±3.61	1.95±0.01	41.97±4.148	1.86±0.01	28.67±1.85	1.81±0.04
48	39.36±2.87	1.90±0.06	30.98±0.64	1.69±0.02	29.44±0.38	1.85±0.10
60	53.9±1.44	1.98±0.03	38.30±2.57	1.67±0.05	37.76±2.79	1.39±0.01
72	27.21±1.25	1.8±0.05	40.40±3.21	1.73±0.02	38.97±2.59	1.39±0

2.3 The effect of pH on growth and protease production

pH	B15		V10		V26	
	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)
4	0.25±0.18	0.01±0	1.99±0.46	0.02±0	0.43±0.05	0.03±
5	0.38±0.13	0.02±0.01	30.86±1.90	1.77±0.02	0.65±0.43	0.02±
6	17.68±1.42	1.58±0.13	30.48±2.21	1.76±0.01	23.72±1.79	1.57±0.02
7	25.60±1.85	1.55±0.07	36.23±1.41	1.76±0	25.96±1.10	1.49±0.01
8	21.96±2.06	1.53±0.09	35.36±2.89	1.74±0.02	25.33±0.93	1.48±0.01
9	17.92±0.48	1.40±0.03	32.16±1.53	1.72±0	22.00±0.89	1.44±0.02
10	14.20±0.68	1.33±0.01	32.77±2.43	1.76±0.02	18.85±1.33	1.37±0.01

2.4 The effect of temperature on growth and protease production

	B15		V10		V26	
Temperature (°C)	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)	Enzyme Units	Growth (Abs.)
20	8.32±0.46	1.86±0.04	5.10±0.58	2.34±0.01	4.83±0.55	1.86±0.03
25	40.92±2.29	1.86±0.007	43.28±1.78	1.78±0.06	43.32±1.38	1.94±0.05
30	39.15±0.93	1.79±0.02	38.11±0.52	1.77±0.1	36.11±0.63	1.87±0.02
35	36.61±1.68	1.71±0.05	36.26±1	1.79±0.06	35.55±0.67	1.93±0.06
40	4.94±0.28	1.62±0.05	1.05±0.59	1.74±0.16	1.95±1.05	1.53±0.08

2.5 The effect of NaCl on growth and protease production

B15		
NaCl Concentrations (%)	**Enzyme Units**	**Growth (Abs.)**
0	33.29±3.51	2.05±0.05
0.5	20.15±1.65	1.82±0.19
1	16.14±3.38	1.76±0.02
1.5	18.50±3.71	1.77±0.02
2	20.11±2.78	1.82±0.06
2.5	19.05±1.93	1.77±0.01
3	17.10±3.07	1.78±0
3.5	11.24±1.73	1.64±0.03
4	13.26±3.63	1.63±0.02
4.5	16.91±2.45	1.56±0.02
5	15.41±4.03	1.62±0.05

V10		
NaCl Concentrations (%)	**Enzyme Units**	**Growth (Abs.)**
0	6.29±0.31	0.77±0.02
0.5	44.58±3.62	1.71±0.01
1	41.69±2.90	1.82±0.06
1.5	35.58±3.99	1.79±0.03
2	27.70±1.72	1.79±0.01
2.5	31.24±2.15	1.81±0.03
3	24.27±3.45	1.81±0.01
3.5	19.70±3.45	1.89±0.01
4	12.77±1.98	1.93±0.02
4.5	10.57±2.25	1.96±0.00
5	9.36±0.94	1.79±0.04

V26		
NaCl Concentrations (%)	**Enzyme Units**	**Growth (Abs.)**
0	49.08±3.09	1.54±0.04
0.5	39.77±2.01	1.67±0.15
1	34.95±2.23	1.66±0.02
1.5	34.07±3.79	1.81±0.03
2	32.75±2.07	1.81±0.06
2.5	33.72±1.69	1.79±0.06
3	31.81±2.44	1.40±0.18
3.5	21.13±5.88	0.72±0.14
4	10.72±2.08	0.39±0.02
4.5	2.71±1.69	0.19±0.06
5	1.86±1.19	0.08±0.01

2.6 The effect of metallic salts on growth and protease production

Effect on growth of B15					
	0.005mM	**0.01mM**	**0.02mM**	**0.05mM**	
Control					1.82±0.07
$CoCl_2$	0	0	0	0	
$CaCl_2$	1.78±0.05	1.80±0.07	1.95±	1.98±0.08	
$MnSO_4$	1.97±0.04	1.48±0.10	0	0	
$MgSO_4$	1.79±0.04	1.67±0.09	1.69±0.07	1.79±0.03	
KH2PO4	1.93±0.05	1.89±0.03	1.950.05±	1.93±0.13	
$Fe(PO_4)_3$	1.79±0.04	1.78±0.02	1.87±0.04	1.98±0.05	

Effect on protease production of B15					
	.005Mm	**.01mM**	**.02mM**	**.05mM**	
Control					35.07±3.32
$CoCl_2$	0	0	0	0	
$CaCl_2$	48.01±3.28	53.14±5.71	67.85±5.08	56.63±1.12	
$MnSO_4$	34.83±7.26	32.38±0.96	0	0	
$MgSO_4$	36.71±3.28	49±10.42	40.25±7.04	51.07±6.65	
KH2PO4	56.26±3.12	58.91±2.23	50.64±0.56	39.15±4.83	
$Fe(PO_4)_3$	29.18±1.24	22.05±3.75	24.29±3.13	17.09±2.13	

Effect on growth of V10					
	0.005mM	0.01mM	0.02mM	0.05mM	
Control					1.84±0
$CoCl_2$	0	0	0	0	
$CaCl_2$	1.86±0	1.88±0.03	2.03±0.19	1.90±0.02	
$MnSO_4$	1.87±0.03	1.87±0	1.96±0	0.07±0.02	
$MgSO_4$	1.84±0.03	1.84±0.02	1.57±0.04	1.88±0.03	
$KH2PO_4$	2.07±0.02	2.09±0.01	2.08±0.02	2.08±0.02	
$Fe(PO_4)_3$	1.85±0.02	1.85±0.01	1.90±0.01	1.68±0.02	

Effect on protease production of V10					
	.005Mm	.01mM	.02mM	.05mM	
Control					60.69±2.48
$CoCl_2$	0	0	0	0	
$CaCl_2$	67.84±1.88	79.77±12.32	71.83±6.51	68.26±4.25	
$MnSO_4$	70.00±3.15	71.52±2.08	96.98±6.03	0	
$MgSO_4$	73.32±4.26	73.24±5.48	74.93±3.84	87.31±3.29	
$KH2PO_4$	83.84±6.42	80.70±8.01	83.01±4.28	76.43±4.51	
$Fe(PO_4)_3$	88.39±0.80	69.32±1.32	75.40±2.39	70.95±4.83	

Effect on growth of V26					
	0.005mM	0.01mM	0.02mM	0.05mM	
Control					1.88±0.01
$CoCl_2$	0	0	0	0	
$CaCl_2$	1.86±0.02	1.93±0.01	1.95±0.04	2.00±0.01	
$MnSO_4$	1.91±0	1.89±0.03	0	0	
$MgSO_4$	1.87±0.03	1.85±0.03	1.88±0.01	1.88±0.01	
$KH2PO_4$	2.02±0.02	2.03±0	2.03±0.01	2.05±0.02	
$Fe(PO_4)_3$	1.85±0.06	1.86±0.01	1.86±0.01	1.95±0.12	

Effect on protease production of V26					
	.005Mm	.01mM	.02mM	.05mM	
Control					55.15±1.58
$CoCl_2$	0	0	0	0	
$CaCl_2$	56.36±5.54	51.04±1.21	62.29±8.58	68.55±5.37	
$MnSO_4$	63.98±3.73	58.96±2.55	0	0	
$MgSO_4$	61.09±1.11	67.57±1.03	73.63±3.32	63.96±2.17	
$KH2PO_4$	63.96±0.32	76.84±4.67	67.42±2.07	70.49±2.30	
$Fe(PO_4)_3$	69.68±1.32	70.57±2.66	74.98±4.69	66.58±3.82	

2.7 The effect of carbon sources on growth and protease production

Effect on growth of B15					
Concentration	**Glucose**	**Mannose**	**Maltose**	**Sucrose**	**Molasses**
0.05%					2.30±0.01
0.10%	2.28±0.01	2.23±0.01	2.19±0.02	2.15±0.08	2.34±0.06
0.20%	2.23±0.06	1.88±0.02	2.13±0.06	2.26±0.03	2.35±0.04
0.40%	2.22±0.04	1.61±0.01	2.16±0.05	2.29±0.04	2.40±0.00
0.60%	1.05±0.01	1.96±0.03	2.18±0.04	2.26±0.02	

Effect on protease production of B15					
Concentration	**Glucose**	**Mannose**	**Maltose**	**Sucrose**	**Molasses**
0.05%					11.86±1.97
0.10%	12.79±0.85	13.09±0.65	13.17±0.79	11.15±0.54	11.79±0.97
0.20%	12.79±0.70	13.03±1.23	14.28±0.27	17.63±0.17	8.91±0.20
0.40%	13.62±0.71	0.74±0.21	13.21±0.60	8.50±0.42	7.22±1.14
0.60%	0.47±0.11	1.44±0.91	12.08±0.77	8.68±0.35	

Effect on growth of V10					
Concentration	**Glucose**	**Mannose**	**Maltose**	**Sucrose**	**Molasses**
0.05%					2.33±0
0.10%	2.44±0.01	2.32±0.01	2.27±0.01	2.37±0	2.42±0.03
0.20%	2.42±0.02	2.41±0.04	2.34±0.01	2.44±0	2.54±0.01
0.40%	2.53±0.04	2.52±0.02	2.40±0.00	2.37±0.01	2.85±0
0.60%	2.00±0.00	2.13±0.03	2.51±0.00	2.01±0.01	

Effect on protease production of V10					
Concentration	**Glucose**	**Mannose**	**Maltose**	**Sucrose**	**Molasses**
0.05%					11.98±0.58
0.10%	12.36±0.94	11.73±0.47	12.99±0.52	13.42±0.30	11.95±0.58
0.20%	12.12±0.23	15.12±0.61	13.52±0.11	14.06±0.43	10.35±0.56
0.40%	15.06±0.20	12.96±0.62	13.14±0.19	9.79±1.02	11.96±1.21
0.60%	0.52±0.12	0.25±0.05	12.27±0.50	0.15±0.09	

Effect on growth of V26					
Concentration	**Glucose**	**Mannose**	**Maltose**	**Sucrose**	**Molasses**
0.05%					2.23±0
0.10%	2.27±0	2.05±0.01	2.05±0.01	2.13±0.02	2.22±0
0.20%	2.26±0.01	2.22±0.02	2.21±0.02	2.24±0.02	2.25±0
0.40%	2.32±0.01	2.35±0.03	2.35±0.02	2.45±0.01	2.31±0
0.60%	2.35±0.02	2.38±0.01	2.33±0.02	2.53±0.01	

Effect on protease production of V26					
Concentration	**Glucose**	**Mannose**	**Maltose**	**Sucrose**	**Molasses**
0.05%					43.18±2.80
0.10%	20.04±1.58	49.02±1.96	42.62±2.41	39.63±3.52	30.92±1.76
0.20%	18.31±0.95	46.34±1.61	41.73±0.85	32.95±0.34	29.53±1.47
0.40%	12.55±1.07	31.22±2.86	44.45±1.13	27.35±2.24	30.89±1.39
0.60%	0.41±0.13	24.95±1.54	47.55±1.76	26.52±1.07	

2.8 The effect of nitrogen sources on growth and protease production

B15		
Nitrogen Source	**Enzyme Units**	**Growth (Abs.)**
Beef extract	94.30±5.04	2.19±0.06
Casein	159.96±4.00	2.32±0.04
Gelatin	117.44±3.87	2.08±0.01
Peptone	136.01±10.44	2.42±0.09
Tryptone	106.75±5.11	2.18±0.03
Yeast extract	81.77±2.72	2.40±0.01
$NaNO_3$	28.76±1.40	1.35±0.04
NH_4Cl	27.96±0.82	1.44±0.32
NH_4NO_3	16.46±0.08	1.58±0.19
$(NH4)_2SO_4$	18.18±1.40	1.60±0.04
Urea	15.07±1.61	1.32±0.04

V10		
Nitrogen Source	**Enzyme Units**	**Growth (Abs.)**
Beef extract	127.08±8.99	2.36±0
Casein	159.88±4.91	2.54±0.05
Gelatin	105.04±4.03	2.09±0.05
Peptone	111.30±1.89	2.49±0.01
Tryptone	79.62±8.33	2.46±0.05
Yeast extract	158.66±2.44	2.50±0.00
$NaNO_3$	3.99±0.98	1.24±0.01
NH_4Cl	12.22±6.65	1.38±0.02
NH_4NO_3	9.19±0.42	1.39±0.07
$(NH4)_2SO_4$	10.98±2.88	1.32±0.00
Urea	8.59±0.93	1.14±0.00

V26		
Nitrogen Source	**Enzyme Units**	**Growth (Abs.)**
Beef extract	43.48±6.38	2.26±0.07
Casein	38.47±4.98	2.26±0
Gelatin	13.53±0.92	1.88±0.10
Peptone	24.97±3.16	2.28±0.07
Tryptone	36.33±0.99	2.14±0.02
Yeast extract	34.10±4.39	2.43±0.02
$NaNO_3$	5.55±2.64	1.45±0.03
NH_4Cl	2.29±0.71	1.60±0.07
NH_4NO_3	5.30±1.99	1.23±0
$(NH4)_2SO_4$	2.47±0.47	1.36±0.01
Urea	3.11±0.73	1.25±0.01

APPENDIX 3

3.1 Effect of pH on the activity of proteases

	Enzyme Units		
pH	B15	V10	V26
7	36.45±2.88	46.40±1.70	131.85±7.79
8	27.33±1.50	50.06±2.53	144.20±3.49
9	26.71±1.32	49.52±1.17	172.93±6.69
10	27.33±1.50	23.05±1.44	131.75±4.90
11	5.56±0.71	15.98±1.67	113.36±3.09
12	3.61±0.00	4.50±0.87	5.05±0.51
13	2.13±0.68	2.13±1.26	1.41±0.53

3.2 Effect of pH on the stability of proteases

	Retained activity (%)		
pH	B15	V10	V26
4	2.54±0.60	8.16±1.21	3.68±1.14
5	0.93±0.87	39.43±3.40	8.70±2.12
6	13.17±1.80	67.37±4.55	48.29±1.04
7	42.83±6.81	66.78±2.47	85.48±2.84
8	100±0.86	100±3.58	88.15±4.89
9	52.66±1.58	92.27±5.46	100.00±3.62
10	44.93±5.81	86.54±3.60	99.13±2.41
11	52.15±3.46	80.93±1.23	98.98±4.01
12	22.77±1.96	76.46±1.42	62.37±3.13
13	17.58±2.64	68.66±3.46	46.25±3.21

3.3 Effect of temperature on the activity of proteases

	Enzyme Units		
Temperature (°C)	B15	V10	V26
15	27.05±1.17	8.49±0.34	10.236±1.12
20	32.75±1.17	21.56±0.41	11.67±1.81
25	42.44±2.56	41.71±1.39	21.18±9.95
30	61.29±0.86	72.78±1.58	87.19±11.81
35	68.99±4.29	74.96±1.57	105.57±4.51
40	92.93±5.40	80.63±6.05	123.56±1.84
45	76.93±1.51	91.90±3.93	151.35±3.47
50	74.58±0.22	105.35±3.66	215.42±5.69
55	72.65±4.01	113.55±3.93	257.89±2.94
60	67.67±2.14	111.99±2.93	303.20±13.13
65	54.31±1.63	101.57±3.21	315.26±4.95
70	50.79±3.31	101.93±3.70	367.49±20.72
75	43.85±2.36	96.70±2.07	381.03±18.59
80	0.13±0.00	13.98±2.43	185.51±3.87

3.4 Effect of temperature on the stability of proteases

Retained activity (%)			
Temperature (°C)	B15	V10	V26
15	99.58±1.24	98.39±2.50	100±2.36
20	99.79±0.63	96.34±0.39	97±3.25
25	100±1.34	95.03±2.29	98.91±2.45
30	97.57±2.27	98.95±2.02	97.74±3.65
35	83.70±4.10	99.37±2.13	94.67±2.35
40	72.82±3.02	100±0.80	93.07±4.56
45	60.97±3.32	97.89±0.53	87.76±2.89
50	55.11±4.95	98.82±1.45	96.98±2.92
55	26.51±2.45	42.67±1.94	71.19±6.32
60	10.52±2.29	29.23±1.49	42.97±3.56
65	5.53±2.83	0.35±0.15	17.62±2.35
70	2.23±1.49	1.93±0.84	1.97±1.36074

3.5 Line Weaver Burk Plot

1/v			
1/s	B15	V10	V26
1	0.049	0.030	0.0065
0.50	0.023	0.018	0.0056
0.33	0.016	0.012	0.0045
0.25	0.014	0.011	0.0041
0.20	0.012	0.009	0.0036
0.17	0.011	0.009	0.0033
0.14	0.010	0.008	0.0032
0.13	0.008	0.007	0.0032
0.11	0.008	0.007	0.0033
0.10	0.020	0.007	0.0034

3.6 Effect of various metallic salts on the activity of proteases

V10			
	1mM	2.5mM	5mM
$HgCl_2$	1.28±0.91	0.32±0.20	1.37±0.95
$ZnCl_2$	67.12±1.36	49.50±0.67	43.78±0.63
$CoCl_2$	109.81±1.12	113.99±1.93	124.88±1.93
$CuSO_4$	5.27±0.52	0	0
CaCl2	120.25±0.31	114.30±10.68	122.66±0.876
$MnSO_4$	97.64±2.21	88.78±2.99	80.75±3.00
$MgSO_4$	83.56±10.47	95.28±0.87	94.23±0.89
NaCl	90.24±3.07	103.54±1.93	97.73±1.96
KCl	97.18±0.34	88.33±1.81	96.73±3.07
$Fe(PO_4)_3$	112.29±3.40	102.66±2.58	104.45±1.89

B15			
	1mM	2.5mM	5mM
$HgCl_2$	3.98±1.58	1.17±1.46	4.56±0.92
$ZnCl_2$	8.29±1.41	4.56±3.85	4.45±1.07
$CoCl_2$	13.65±1.40	9.81±4.12	6.43±1.41
$CuSO_4$	8.17±1.41	0.83±0.20	6.19±0.20
CaCl2	171.08±13.12	193.22±2.33	195.90±1.79
$MnSO_4$	77.51±1.99	38.42±1.58	16.56±4.50
$MgSO_4$	104.64±4.88	115.96±1.01	132.98±3.32
NaCl	76.23±0.70	93.94±3.59	100.23±3.36
KCl	125.87±7.22	127.85±2.22	118.18±2.80
$Fe(PO_4)_3$	112.02±3.85	110.32±2.00	114.72±10.49

V26			
	1mM	2.5mM	5mM
$HgCl_2$	2.10±0.49	1.75±0.30	0.28±0.07
$ZnCl_2$	119.43±2.17	98.81±3.98	108.15±1.56
$CoCl_2$	130.90±0.27	146.46±9.62	152.79±2.68
$CuSO_4$	9.86±0.66	5.21±0.48	0.49±0.31
CaCl2	101.58±1.61	104.12±1.32	112.94±6.17
$MnSO_4$	111.55±6	92.44±2.16	102.69±1.13
$MgSO_4$	125.64±0.38	129.32±0.81	130.31±0.61
NaCl	121.61±1.53	124.81±4.28	123.78±2.10
KCl	112.27±10.17	109.97±0.60	109.73±0.60
$Fe(PO_4)_3$	97.38±1.06	98.80±3.36	100±1.43

3.6 Effect of various chemicals inhibitors on the activity of proteases

B15			
	1mM	2.5mM	5mM
EDTA	5.67±1.55	3.51±1.55	3.27±1.49
PMSF	11.83±3.38	11.83±4.45	16.76±2.47
IAA	64.14±4.15	3.30±1.55	2.07±0.64
Urea	37.73±0.64	36.02±1.67	57.97±3.74
SDS	40.09±4.54	47.59±5.27	54.37±3.83
Dithiothreitol	114.90±9.38	91.88±9.91	96.80±1.71
Mercaptoethanol	65.50±2.17	60.33±3.09	56.94±1.17
Phenanthroline	92.60±4.51	30.73±5.38	0.46±0.19

V10			
	1mM	2.5mM	5mM
EDTA	41.96±1.40	16.03±0.44	3.98±0.87
PMSF	37.43±2.62	35.20±3.01	24.59±1.84
IAA	65.99±2.11	3.21±1.66	3.71±1.37
Urea	87.05±9.41	75.16±7.68	74.14±1.25
SDS	76.03±4.53	77.03±4.33	67.71±4.08
Dithiothreitol	89.96±5.05	81.86±3.47	71.13±5.26
Mercaptoethanol	89.04±0.55	89.79±4.10	88.40±4.33
Phenanthroline	57.68±1.72	59.14±0.67	32.87±1.37

V26			
	1mM	2.5mM	5mM
EDTA	11.01±0.59	6.92±0.53	5.81±1.36
PMSF	62.72±3.86	56.55±2.45	65.75±3.18
IAA	32.60±2.70	0.50±0.00	0.25±0.00
Urea	57.53±0.95	60.95±1.19	56.65±6.35
SDS	72.95±2.32	57.00±3.80	57.25±5.12
Dithiothreitol	56.19±3.05	38.42±2.20	42.66±5.79
Mercaptoethanol	52.00±1.31	54.64±2.38	57.99±7.44
Phenanthroline	49.03±3.28	0.76±0.00	0.25±0.01

APPENDIX 4

4.1 The stability of the proteases in commercial detergents

B15			
	15minutes	30 minutes	60 minutes
ARIEL	76.00±1.20	73.31±2.72	72.54±2.23
HENKO	83.23±3.2	76.64±2.61	74.43±3.25
RIN	71.05±6.22	78.81±4.82	72.22±1.92
SUNLIGHT	64.58±4.78	77.74±2.56	78.75±3.2
SURF	66.2±3.84	68.09±2.8	67.82±3.75
TIDE	73.97±4.00	62.15±2.14	70.45±1.72

C10			
	15minutes	30 minutes	60 minutes
ARIEL	53.15±2.63	41.31±1.18	42.53±0.99
HENKO	63.38±1.68	55.85±3.23	42.92±2.97
RIN	57.91±2.55	52.96±2.02	51.77±3.17
SUNLIGHT	54.01±3.2	48.3±1.89	47.41±3.23
SURF	60.6±1.92	58.84±0.42	61.53±2.63
TIDE	64.75±0.52	50.37±0.62	47.03±0.5

C26				
	15minutes	30 minutes	45 minutes	60 minutes
ARIEL	49.80±3.40	52.53±1.24	44.55±4.06	37.33±2.16
HENKO	70.84±1.29	73.95±5.053	71.45±1.47	57.78±2.62
RIN	77.25±1.43	74.62±4.29	67.57±2.35	64.06±2.10
SUNLIGHT	64.61±3.83	66.08±4.88	68.70±2.13	63.42±1.80
SURF	70.48±0.64	68.66±3.75	66.86±4.37	60.11±0.86
TIDE	66.39±3.05	75.35±1.79	71.12±1.62	45.07±2.6

4.2 The stability of the proteases in H_2O_2

H_2O_2 (%)	B15	V10	V26
1	103.92±6.61	100.62±4.50	77.39±3.25
2	113.00±3.04	109.9±2.39	78.54±4.99
3	128.3±2.49	128.11±6.31	74.12±11.85
4	116.29±4.82	132.96±6.06	74.39±10.08

4.3 The stability of the proteases in SDS

SDS (%)	B15	V10	V26
0.05	70.4±6.81	65.31±3.14	57.78±6.36
0.1	66.21±3.07	65.17±4.12	54.25±3.13
0.2	68.57±3.20	60.81±4.41	63.96±1.89
0.4	56.35±2.28	62.96±1.64	63.78±3.18

4. 4The stability of the proteases in Triton-X-100

T-X-100 (%)	B15	V10	V26
0.1	69.35±1.76	71.96±1.20	69.17±6.36
0.4	94.18±10.48	59.29±1.08	77.12±3.13
0.7	102.81±10.47	58.90±9.37	99.06±1.89
1	72.36±8.14	60.25±1.74	134.77±3.18

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