Alkaline Protease from a Non-toxigenic *Vibrio* sp. (V26) and its Applications

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By

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Dedicated to the
Loving Memory of my
Grandfather

M. K Panikkar

Certificate

This is to certify that the thesis entitled ***"Alkaline Protease from a Non-toxigenic Vibrio sp. (V26) and its Applications"*** *is an authentic record of research work carried out by Ms. Manjusha K, under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and no part thereof has been presented before for the award of any degree, diploma, or associateship in any University.*

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Declaration

I hereby do declare that the thesis entitled **"Alkaline Protease from a Non-toxigenic *Vibrio* sp. (V26) and its Applications"**, is a genuine record of research work done by me under the supervision and guidance of Dr. A.V. Saramma, Associate Professor, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology and that no part of this work has been presented for the award of any degree, diploma or associateship in any University or Institution earlier.

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Abbreviations

A_{280}	Absorbance at 280 nm
°C	Degree Celsius
%	Percentage
ANOVA	Analysis of variance
APV26	Alkaline protease from *Vibrio* sp.(V26)
BATH	Bacterial adhesion to hydrocarbon test
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CaCo-2	Human colon carcinoma cells
CCD	Central Composite Design
Da	Dalton
DEAE	Diethyl amino ethyl
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOE	Design of Experiments
EDTA	Ethylene-diamine tetra acetic acid
FBS	Fetal bovine serum
FCCCD	Face Centered Central Composite Design
g	Gram
HA/protease	Hemagglutinin/protease
HeLa	Henritta Lacks, human cervical carcinoma cell lines
HEp-2	Human larynx epithelial cell lines
hrs	hours
IAA	Iodo acetic acid
kDa	kilo Dalton
LC_{50}	Lethal concentration, 50%
L	Litre

M	molar
MEM	Minimal essential media
mg	milligram
ml	Milli litre
mM	milliMolar
mol/L	Moles per litre
mOsm	milliosmole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide
nm	nanometer
O.D	Optical density
PBD	Plackett-Burman Design
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
pmol	Picomoles
PVP	Poly vinyl pyrrolidone
RBCs	Red Blood Cells
RNA	Ribonucleic acid
rpm	revolutions per minute
RSM	Response surface methodology
RTG-2	Rainbow trout gonadial cell lines
SAT	Salt aggregation test
SDS	Sodium dodecyl sulphate
sec	seconds
TCA	Trichloroacetic acid
U	Protease Unit
µ	microgram
µl	microlitre

Contents

Chapter 1

General Introduction

Conventional chemical processes with regard to their environment and cost issues are being subjected to considerable scrutiny these days. Biotechnology is rapidly gaining ground due to the various advantages it offers over the conventional technologies. Industrial enzymes represent the heart of these biotechnology processes. Enzymes are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries.

The global market for industrial enzymes was estimated at $3.3 billion in 2010. This market is expected to reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5-year forecast period (BCC research report, 2011). Of the industrial enzymes, 75% are hydrolytic. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes (Rao *et al.,* 1998).

Proteases

Proteases are a complex group of enzymes collectively known as peptidyl-peptide hydrolases and are responsible for the hydrolysis of peptide bonds in a protein molecule. They are a single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases constitute a group of enzymes which differs in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature for

activity and stability profiles. Proteases of commercial importance are produced from microbial, animal and plant sources, and have enormous applications in a range of processes which take advantage of the unique physical and catalytic properties of individual proteolytic enzyme types. Today, proteases account for more than half of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management, and silver recovery. This dominance of proteases in the industrial market is expected to increase further in the coming years.

Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as homoeostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions (Rao *et al.,* 1998). Extracellular proteases contribute to the nutritional well being of the producing organism hydrolyzing large polypeptides into smaller molecules that the cell can absorb. Proteases are also involved in the regulation of biological processes such as spore formation, spore germination, protein maturation in viral assembly, activation of certain viruses of importance for pathogenicity, various stages of the mammalian fertilization processes, blood coagulation, fibrinolysis, complement activation, phagocytosis and pressure control (Ward, 1983). Other complex physiological functions executed by proteases include cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumour growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas; intracellular proteases play a critical role in the regulation of metabolism (Rao *et al.,* 1998).

Classification of Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (IUB). However, proteases 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 the type of reaction catalyzed, chemical nature of the catalytic site, and evolutionary relationship with reference to structure (Barett, 1994).

Proteases are grossly subdivided into two major groups, i.e., 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 substrate.

Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases (E.C.3.4.21), cysteine proteases (E.C.3.4.22), aspartic proteases (E.C.3.4.23) and metalloproteases (E.C.3.4.24) (Hartley, 1960). There are a few miscellaneous proteases which do not precisely fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Menon and Goldberg, 1987). Based on their amino acid sequences proteases are classified into different families (Argos, 1987) and further, subdivided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Rawlings and Barrett, 1993). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo-, or unknown type, respectively.

Based on their pH optima proteases are classified into acidic, neutral and alkaline proteases.

Alkaline proteases

Of the various types of proteases alkaline proteases which are active from neutral to alkaline pH, find major (37%) commercial application (Gupta *et al.,* 2005a). It has been generally found that proteases that function under alkaline conditions are either serine protease or metalloprotease. While aspartic proteases are a group that commonly works under acidic conditions, and are referred to as acid proteases.

Alkaline serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases. Serine alkaline proteases are produced by several bacteria, molds, yeasts, and fungi. They are inhibited by inhibitors 3, 4 dichloroisocoumarin (3,4-DCI) diisopropyl fluorophosphate (DFP), phenylmethylsulphonyl fluoride (PMSF) and tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Barrett, 2001). The optimal pH of alkaline proteases is around pH 10, and their isoelectric point is around pH 9. 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* spp. (Boguslawski *et al.,* 1983), subtilisins produced by *Bacillus* spp. are the best known. Alkaline proteases are also produced by *Saccharomyces cerevisiae* (Mizuno and Matsuo, 1984) and filamentous fungi such as *Conidiobolus* spp. (Phadatare *et al.,* 1993) and *Aspergillus* and *Neurospora* spp. (Lindberg *et al.,* 1981). Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protease Nagase (BPN9), have been identified. Subtilisin Carlsberg is produced by *Bacillus licheniformis*. Subtilisin Novo or BPN9 is produced by *Bacillus amyloliquefaciens*. Subtilisin Carlsberg is widely used in detergents.

Alkaline metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Barett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria. About 30 families of metalloproteases have been recognized, of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo- and exopeptidases. Families of metalloproteases have been grouped into different clans based on the nature of the amino acid that completes the metal-binding site; e.g., clan MA has the sequence HEXXH-E and clan MB corresponds to the motif HEXXH-H (Rao *et al.,* 1998).

Matrix metalloproteases play a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing and may be useful in the treatment of diseases such as cancer and arthritis (Browner *et al.,* 1995).

Microbial Proteases

Though proteases are ubiquitous in occurrence and are produced by animals, plants and microbes alike, the commercial sources of these enzymes are today primarily microbial. This is mainly due to their multifold properties such as extracellular production that simplifies the downstream processing, unlimited supply, longer shelf life, less stringent storage requirements, broad biochemical diversity and their susceptibility to genetic manipulation.

Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to

8) and have relatively low thermotolerance. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases, which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, broad substrate specificity and high temperature. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao *et al.,* 1998).

A number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of serine type. Bacteria are the most dominant group of alkaline protease producers, with the genus *Bacillus* being the most prominent source (Kumar and Tagaki, 1999). The other important producers of this enzyme include *Pseudomonas, Vibrio, Flavobacterium, Serratia*, *Staphylococcus, Brevibacterium, Halobacterium, Alcaligenes, Arthrobacter* and *Clostridium* (Gupta *et al.,* 2005a). A rare, spiral shaped gram positive bacterium *Kurthia spiroforme* possessing distant relationship to the Genus *Bacillus* was also reported to produce this enzyme (Steele *et al.,* 1992). Among actinomycetes, strains of *Streptomyces, Nocardia* and *Nocardiopsis* are potential ones. Aspergilli are exploited the most among fungi as a protease producer. *Candida* and *Yarrowia* sp. are yeasts that have been identified as potent alkaline protease producers (Gupta *et al.,* 2005a).

With the recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Increasing demand of proteases with specific properties has led biotechnologists to explore newer sources of proteases (Sana *et al.,* 2006). Considerable efforts have been devoted to the selection of microorganisms via sophisticated screening techniques and process methodology for the production of enzymes with new physiological / physical properties and tolerance to extreme conditions used in the industrial processes (e.g. temperature, salts and pH). Marine microorganisms represent one such novel source of enzymes. The marine

environment has proven to be a rich source of both biological and chemical diversity (Pomponi, 1999).

Marine Microbial Enzymes

The potential of marine organisms for commercial development impinges on virtually every area of biotechnology (Cowan, 1997). Marine microorganisms have recently emerged as a rich source for the isolation of an array of industrial enzymes such as protease, chitinase, esterase, lipase, amylase, agarase and arylsulphatase. Due to their unique natural habitat these microorganisms show distinct physiological characteristics, metabolic patterns and nutrient utilization as compared to their terrestrial counterparts (Sana *et al.,* 2006). Apart from microorganisms like bacteria (including actinomycetes) and fungi, many other marine organisms such as fishes, prawns, crabs, snakes, plants and algae were also studied to tap the arsenal of the marine world.

A marine enzyme is a unique protein molecule with novel properties derived from an organism whose natural habitat comprises saline or brackish water (Sarkar *et al.,* 2010). Marine bacterial enzymes have several advantages for industrial utilization (Ventosa and Nieto, 1995). Properties like high salt tolerance, hyperthermostability, barophilicity, cold adaptability, and ease in large scale cultivation are exhibited by the marine enzymes. These properties may not be expected in terrestrial sources as marine organisms thrive in habitats such as hydrothermal vents, oceanic caves and in areas where high pressure and absence of light is obvious (Ghosh *et al.,* 2005). The optimum activity of marine bacterial enzymes usually occurs at high salinity, making these enzymes utilizable in many harsh industrial processes, where the concentrated salt solutions used would otherwise inhibit many enzymatic transformations. In addition, most marine bacterial enzymes are considerably thermotolerant, remaining stable at room temperature over long periods (Mohapatra *et al.,* 2003).

Vibrios a prominent genus in the aquatic environment have been found to be capable of producing a variety of enzymes including protease (Estrada-Badillo and Marquez-Rocha, 2003; Venugopal and Saramma, 2006; Vázquez *et al.,* 2006), agarase, (Sugano *et al.,* 1995), L-glutaminase (Prabhu and Chandrasekaran, 1995), alginate lyase (Xiaoting *et al.,* 2008) and amylase (Saramma and Babu, 1994; Najafi and Kembhavi, 2005).

Hyphomonas jannaschiana (Weiner *et al.,*1996), *Bacillus* strain TA39 (Narinx *et al.,* 1997), *Teredinobacter turnirae* (Beshay and Moreira 2003; Elibol and Moreira, 2003, 2005), *Vibrio harveyi* (Estrada-Badillo and Marquez-Rocha, 2003), *Bacillus clausii* (Kumar *et al.,* 2004), *Engyodontium album* BTMFS10 (Chellappan *et al.,* 2006) *Vibrio fluvialis* (Venugopal and Saramma, 2006) and *Bacillus cereu*s (Abou-Elela *et al*., 2011) are a few examples of marine microbes capable of producing the commercially important enzyme protease.

Purification and characterization

A number of alkaline proteases from different sources have been purified and characterized. There are no set rules for the purification of proteases. After separating the culture from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by various means such as ultrafiltration, salting out by ammonium sulphates or by solvent extraction methods. Further purification is done using a combination of one or more chromatographic techniques like ion exchange chromatography, gel filtration chromatography etc. Other methods like aqueous two phase extraction have also been employed (Gupta *et al.,* 2002b).

The properties of protease from different microbes have been studied with respect to the effect of pH and temperature on activity and stability, substrate specificity, effect of various additives as well as metal ions on activity. Kinetic

parameters of the enzyme have also been investigated. It is based on these properties that they are used in various industries (Gupta *et al.,* 2002b).

Protease production, optimization and scale up

Protease production is an inherent property of all microorganisms and these enzymes are generally constitutive; however, at times they are partially inducible (Gupta *et al.,* 2002b). The proteases are largely produced during stationary phase and thus they are generally regulated by carbon and nitrogen stress. Different methods in submerged fermentations have been used to regulate the protease synthesis by combinations of either strategies, such as fed-batch, continuous and chemostat cultures. Extracellular protease production in microorganisms is also strongly influenced by media components, e.g. variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose (Beg *et al.,* 2002a), and metal ions (Varela *et al.,* 1996). Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium. Besides these, several other 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).

In order to scale up protease production from microorganisms at the industrial level, biochemical and process engineers use several strategies to obtain high yields of protease in a fermenter. Controlled batch and fed-batch fermentations using simultaneous control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability (Mao *et al.,* 1992; Hubner *et al.*, 1993; Van Putten *et al.,* 1996; Hameed *et al.,* 1999) and chemostat cultures (Frankena *et al.,* 1985, 1986) have been successfully used for improving protease production using a number of microorganisms. The overall cost of enzyme production and downstream processing is the major obstacle against the successful application of any technology in the enzyme industry. Researchers and process engineers have used several methods to

increase the yields of alkaline proteases with respect to their industrial requirements. Recent approaches for increasing protease yield include screening for hyper-producing strains, cloning and over-expression, fed-batch, chemostat fermentations, and optimization of the fermentation medium through statistical approach, such as response surface methodology (Gupta *et al.*, 2002a).

Medium optimization by a conventional 'one-at-a-time-approach' does lead to a substantial increase in enzyme yields, however, this approach is not only cumbersome and time consuming, but also has the limitations of ignoring the importance of interaction of various physicochemical parameters (Beg *et al.*, 2003). The statistical approach using response surface methodology (RSM) for process optimization serves the purpose by finding out the optimal conditions of a set of independent variables over a specific region of interest in any given system, by establishing the relationship between more than one variable and a given response (Haaland, 1989). RSM is a collection of mathematical and statistical techniques that are useful for modelling and analysis; in applications where, a response of interest is influenced by several variables and the objective is to optimize this response. The first step in RSM is finding a suitable approximation for the true relationship between the response and independent variables (Myers and Montgomery, 2002).

Applications of alkaline protease

Alkaline proteases account for a major share of the enzyme market all over the world (Kalisz, 1988; Godfrey and West, 1996). Alkaline proteases from bacteria find numerous applications in various industrial sectors and different companies worldwide have successfully launched several products based on alkaline proteases

Food and feed industry

Proteolytic enzymes have been used to modify food proteins to improve their flavour, texture, functionality and nutritional quality. Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks (Ward, 1985; Neklyudov *et al.,* 2000). The immobilized enzyme has been used in cheese making (Ohmiya *et al.,* 1979). The most common application is in baking where it reduces the processing time and improves the dough handling properties. Keratinolytic activity of alkaline protease has also been exploited in the production of proteinaceous fodder from waste feathers or keratin-containing materials (Gupta *et al.,* 2005a).

Peptide synthesis

The proteases have frequently been used for peptide synthesis. Enzymatic peptide synthesis offers several advantages over chemical methods, e.g. reactions can be performed stereospecifically and reactants do not require side-chain protection, increased solubility of non-polar substrates, or shifting thermodynamic equilibria to favour synthesis over hydrolysis . There is less need for expensive protecting-groups, organic solvents, or hazardous chemicals, resulting in production costs competitive with those of chemical methods (Morihara, 1987).

Leather industry

Alkaline proteases with elastolytic and keratinolytic activity can be used in leather-processing industry. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic

proteases. However, recently, the use of microbial alkaline proteases has become popular (Varela *et al.*, 1997).

Management of industrial and household waste

Proteases solubilize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era in the use of proteases in waste management (Moreira-Gasparin *et al.*, 2009).

Medical usage

Alkaline proteases are also used for developing products of medical importance. The enzyme has been applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses (Kudrya and Simonenko,1994).

Textile industry

One of the least explored areas for the use of proteases is the silk industry and only a few patents have been filed describing the use of proteases for the degumming of silk. Sericin, which is about 25% of the total weight of raw silk, covers the periphery of the raw silk fibers, thus providing the rough texture of the silk fibers. This sericin is conventionally removed from the inner core of fibroin by conducting shrink-proofing and twist-setting for the silk yarns, using starch (Kanehisa, 2000). The process is generally expensive and therefore an alternative method suggested is the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing (Freddi *et al.*, 2003).

Detergent Industry

Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. Applications of detergent proteases have grown substantially and

the largest application is in household laundry detergent formulations. However, apart from their use in laundry detergents, they are also popular in the formulation of household dishwashing detergents and both industrial and institutional cleaning detergents (Godfrey and West 1996; Showell, 1999).

The success of detergent enzymes has led to the discovery of a series of detergent proteases with specific uses. Alkazym (Novodan, Copenhagen, Denmark) is an important enzyme for the cleaning of membrane systems. Other enzymes used for membrane cleaning are Terga- zyme (Alconox, New York, USA), Ultrasil (Henkel,Dusseldorf, Germany) and P3-pardigm (Henkel-Ecolab, Dusseldorf, Germany). Pronod 153L, a protease enzyme based cleaner is used to clean surgical instruments fouled by blood proteins (Gupta *et al.,* 2002a). Subtilopeptidase A is an enzyme based optical cleaner, presently marketed in India (Kumar *et al.,* 1998). Sakiyama *et al*. (1998) reported the use of a protease solution for cleaning the packed columns of stainless steel particles fouled with gelatin and β-lactoglobulin.

Photographic industry

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery.

Other uses of alkaline protease

In addition to these major applications, alkaline proteases are also used to a lesser extent for other applications, such as contact lens cleaning (Nakagawa, 1994), molecular biology for the isolation of nucleic acid (Kyon *et al.,* 1994), pest control (Kim *et al.,* 1999), and selective delignification of hemp (Dorado *et al.,* 2001), in biodiesel production (Anita and Rabeeth, 2010) which all may be technically interesting, but have not reached commercial success in terms of impressive sales figures.

Though enough data is available to prove the potential of marine derived enzymes, only very few of these have reached the market. Zonase X™, leading

product of Aqua Biotechnology (http://aquabiotechnology.com) is one such example. As a skin-care product, it gently removes the dead cells in the outer layer of the human skin, initiating and enhancing the renewal and healing process of the skin.

Although it has been realized that the marine realm is a rich and a largely untapped resource of products that are of potential interest to mankind, only a few of these marine natural products have reached the stage of commercial production. There exists a gap between discovery of marine enzymes and its commercialization which essentially needs to be bridged. There is still a lack of research into bioreactor engineering and bioprocess design in the area of cultivation of marine organisms to produce enzymes.

A previous investigation in our laboratory had revealed the potential of the mangrove sediment isolate *Vibrio* sp. (V26), as an excellent protease producer (Venugopal, 2004). Hence, this study was taken up with a view to explore the potential of this alkaline protease for commercial application.

Objectives of this study can be briefed as follows

- Biochemical, serological and molecular characterization of the producer strain *Vibrio* sp. (V26).
- Purification and characterization of the alkaline protease from *Vibrio* sp. (V26).
- A preliminary investigation of the gene coding for the alkaline protease
- Optimization of physical parameters and media components influencing alkaline protease production using the statistical approach of Response Surface Methodology followed by a scale-up of the operations under the optimized conditions.
- An investigation into the possible areas of commercial application of the enzyme.

Chapter 2

Phenotypic and Molecular Identification of the Producer Strain

Contents

Vibrios are abundant worldwide in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings (Urakawa *et al.,* 2000; Suantika *et al.,* 2001; Heidelberg *et al.,* 2002a, 2002b). They are also often found in association with aquatic animals like corals, fish, molluscs, sponges, shrimp, zooplankton and aquatic plants such as seagrass (Thompson *et al.,* 2004a).

Vibrios act as first decomposer of dead and decaying plants (Simidu *et al.,* 1974) and play a role in nutrient cycling in aquatic environments (Sherr and Sherr, 2000). Vibrios have been exploited in many ways. They have been used in environmental monitoring to assess effectiveness of remediation and in vaccine and probiotic production. They are also capable of carrying out

bioremediation of polyaromatic hydrocarbons (Thompson *et al.,* 2004a). Marine Vibrios have been recognized as producers of several of the commercially important enzymes such as agarase, L-asparaginase, L-glutaminase, protease, alpha-amylase and chitinase.

Vibrios are fairly easy to isolate from both clinical and environmental samples, although some species may require specific growth factors and/or vitamins. There are several commercial media which may be used for the isolation of vibrios, but tryptone soy agar supplemented with 1 to 2% NaCl and marine agar are commony used. Thiosulphate-citrate bile salt-sucrose agar (TCBS) is an ideal medium for the selective isolation and purification of vibrios. Vibrios grow well at temperatures between 15 and 30°C. Most vibrios (except *V. ezurae*, *V. gallicus*, *V. pectenicida*, *V. penaeicida*, *V. salmonicida* and *V. tapetis*) withstand the freeze drying process very well (Thompson *et al.,* 2004a).

As per *Bergey's Manual of Determinative Bacteriology* (Baumann *et al.,* 1984) and *Bergey's Manual of Systematic Bacteriology* (Farmer and Janda, 2005), vibrios (members of the family *Vibrionaceae*) belong to the group *Gammaproteobacteria*. They are gram negative straight / curved rods, non sporing usually motile with polar flagella. Vibrios are chemoorganotrophic, mesophilic and facultative anaerobes (both respiratory and fermentative metabolism), mostly oxidase positive and ferment D-glucose. Most species require Na^+ / seawater for growth.

Several species under this genus are well known pathogens. *Vibrio cholerae*, *Vibrio parahaemolyticus* and *V. vulnificus* cause serious infection in humans, while *Vibrio anguillarum*, *V. salmonicida*, *V. vulnificus, V. harveyi, V. campbellii* and *Vibrio splendidus*-related species are pathogens of various aquatic animals (Le Roux *et al.,* 2002; Thompson *et al.,* 2004a).

Virulence factors that could play a crucial role in the pathogenesis of an organism include, their ability to adhere to epithelial cells (Alam *et al.,* 1996), colonize intestine as well as produce toxin. The mechanism of pathogenicity induced by Vibrio infections is complex and related to several factors including cytotoxins, enterotoxins and lytic enzymes (Ottaviani *et al.,* 2001). The ability to adhere to epithelial cells is recognized in several *Vibrio* spp. (Alam *et al.,* 1996) as an auxiliary virulence associated factor (Baffone *et al.,* 2005) and is established as the first step of infection (Dawson *et al.,* 1981). Hydrophobicity of the microbial cell surface plays a vital role in adherence of the organism to the surface of various materials (Magnusson *et al.,* 1980).

The identification of genera and species of the family *Vibrionaceae* based purely on phenotypic characters has presented several difficulties; mainly due to the great variability of diagnostic phenotypic features (Thompson *et al.,* 2004a) and also due to the description of several new species that has led to a constantly changing taxonomy of *Vibrionaceae* (Alsina and Blanch, 1994). Moreover, as several members of this genus are pathogens; it is pertinent to differentiate between pathogenic and non-pathogenic strains. Therefore in this study, for the identification of the protease producer strain, a holistic approach has been adopted including phenotypic, molecular, serological and genotypic traits of the producer strain.

2.1 Review of Literature

2.1.1. Identification

2.1.1.1 Phenotypic Identification

Several of the numerical (phenetic) and / or polyphasic taxonomic studies have helped in laying the foundations of *Vibrio* taxonomy. Clustering of strains in these works were on the basis of their ability to utilize different compounds as sources of carbon and / or energy, hydrolytic activity, salt tolerance, luminescence, growth at different temperatures, antibiograms, DNA

base composition, morphological features, and other biochemical tests (Thompson *et al.,* 2004a). Fatty acids methyl ester (FAME) profiling was evaluated for the differentiation of *Vibrionaceae* species but due to the high degree of similarity of FAME profiles among the different species examined, this technique could be used only as an additional phenotypic feature (Lambert *et al.,* 1983; Bertone *et al.,* 1996). The use of Biolog as a means of phenotypic characterization has gained popularity in the recent years especially in the identification of Vibrios from aquatic environments (Miller and Rhoden, 1991; Klingler *et al.,* 1992; Vandenbergh *et al.,* 2003; Gomez-Gil *et al.,* 2004). The pattern of utilization of the 95 carbon sources forms the basis of identification in this technique. A great variability in the diagnostic phenotypic features like, arginine dihydrolase and lysine and ornithine decarboxylases, susceptibility to the vibriostatic agent 0/139, flagellation, indole production, growth at different salinities and temperatures, and carbon utilization poses serious difficulties in the identification of genera and species of *Vibrionaceae* (Thompson *et al.,* 2004a). Ample phenotypic variability within *Vibrionaceae* species points to the need for the use of classification and identification scheme based more on genomic data (Thompson *et al.,* 2004a). As a result of increase in data obtained with modern molecular biological techniques the taxonomy of *Vibrio* is in the process of revision with special emphasis on 16S rRNA sequence (Dorsch *et al.,* 1992; Gomez-Gil, 2004).

2.1.1.2 Phylogenetic identification and 16S rRNA

In the last two decades, bacterial phylogeny has been enriched with chronometers, like rRNAs (5S, 16S, and 23S). These chronometers not only find application in reconstruction of bacterial phylogenies but also, used as taxonomic markers for identification. In many cases, the phylogenies obtained by 16S rRNA sequencing pointed out the inadequacy of grouping bacteria by the classical criteria (Thompson *et al.,* 2004a).

The 16S rRNA molecule (about 1,500 bp in length) consists of highly conserved regions which may reveal deep-branching (e.g., classes, phyla) relationships but may also demonstrate variable regions which may discriminate species within the same genus. This feature has prompted researchers to use 16S rRNA both as a phylogenetic marker and as an identification tool (Wiik *et al.,* 1995). A huge number of 16S rRNA sequences are now available at The Ribosomal Database Project II which can be easily queried using software like BLAST and FASTA. Dorsch *et al.* (1992) determined the almost complete 16S rRNA sequences of 10 *Vibrio* species. Kita-Tsukamoto *et al.* (1993) presented a comprehensive phylogenetic study of the *Vibrionaceae* based on partial 16S rRNA sequences. The outcomes of a comprehensive phylogenetic study based on the 16S rRNA sequences of marine bacteria especially *Vibrionaceae* by Kita-Tsukamoto *et al.* (1993) were the circumscription of species (at least 99.3% 16S rRNA similarity), genus (95 to 96%), and family (90 to 91%) borders within the *Vibrionaceae* and the delineation of seven main groups of *Vibrionaceae* species that would correspond to different genera or families. Suggestions of reclassification were also proposed by Kita-Tsukamoto *et al*. (1993). Until now, the backbone of bacterial systematics has been derived from the 16S rDNA sequence-based phylogeny (Ludwig and Klenk, 2001). 16S rDNA is indeed the most useful chronometer to allocate strains to different branches of the family *Vibrionaceae*. Accurate identification of vibrios at the family and genus levels is obtained by 16S rRNA gene sequencing, whereas identification at the species and strain levels requires the application of genomic analyses (Thompson *et al.,* 2005).

Unfortunately, the 16S rRNA is unable to resolve closely related species (Nagpal *et al.,* 1998), such as the ones clustered in the Vibrio core group, namely *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, *V. natriegens* and the newly described *V. rotiferianus* (Gomez-Gil *et al.,* 2003).

Several other identification markers / genes such as *gyrB* (Venkateswaran *et al.,* 1998), 16S–23S intergenic spacer region (IGS) (Chun *et al.,* 1999), *recA* (Stine *et al.,* 2000; Thompson *et al.,* 2004b), 23S rRNA (Macian *et al.,* 2001), *hsp60* (Kwok *et al.,* 2002), *gapA* (Nishiguchi and Nair, 2003) and *ami B* (Hong *et al.,* 2007) have been employed for phylogenetic studies and the identification of *Vibrionaceae* species.

5S rRNA of superfamily I (*Vibrionaceae* plus *Enterobacteriaceae*) was analyzed by MacDonell and Colwell (1985). Studies in the later years indicated that the use of 5S rRNA in phylogenetic studies has its limitations (Thompson *et al.,* 2004 a).

2.1.1.3 Genotypic identification

Ribotyping and PCR-based techniques like amplified fragment length polymorphism (AFLP), fluorescence in situ hybridization (FISH), amplified ribosomal DNA restriction analysis (ARDRA) (Thompson *et al.,* 2004a), random amplified polymorphic DNA (RAPD), repetitive extragenic palindromes (rep), and restriction fragment length polymorphism (RFLP) have yielded valuable information about and new insights into the population structure of some species of the *Vibrionaceae* and have also provided a means of identifying these organisms at the species and strain level (Thompson *et al.,* 2004a, 2005). Unfortunately, their use is restricted to a few reference laboratories. Inter laboratory comparisons of fingerprint patterns are difficult. The sequencing of housekeeping genes is emerging as an alternative to overcome this problem (Thompson *et al.,* 2005).

Multilocus sequence typing (MLST) (Maiden *et al.,* 1998; Urwin and Maiden, 2003) was developed as an improved adaptation of Multilocus enzyme electrophoresis (MLEE) (Caugant, 2001; Vieira *et al.,* 2001). Both these techniques index the variation in housekeeping genes and have been advocated as the most reliable molecular tool for epidemiology (Thomoson *et al.,* 2004a). Nishiguchi and Nair (2003) in their attempt to complete a phylogenetic survey

of the *Vibrionaceae* and also to determine evolutionary patterns that are prevalent and influential for radiation used both molecular and biochemical approaches. In their study they have used DNA sequence data from three molecular loci - 16S rRNA, the intergenic region of the lux operon (luxRI) and glyceraldehyde phosphate dehydrogenase (gapA). Phylogeny and molecular identification of Vibrios on the basis of Multilocus Sequence Analysis of *rpoA*, *recA*, and *pyrH* gene sequences was carried out by Thompson *et al.* (2005). Sawabe *et al.* (2007) analyzed partial sequences of nine house keeping genes *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA*, and 16S rRNA, inorder to reconstruct the evolutionary history of vibrios. In a very recent study, on the basis of Multilocus sequence analysis (MLSA), Kirkup *et al*. (2010) have been able to conclude that two chromosomes of *Vibrio* share a common history.

2.1.2 Putative virulence traits

In *V. cholerae* O1 and O139 the pathogenesis has been found to depend on the synergistic effect of different properties, that include the ability to produce the enterotoxin - cholera toxin (CT, *ctx A* gene) and to adhere and colonize the small intestine (colonizing factor, toxin-coregulated pilus, TCP) (Herrington, 1988). Hemolysin (*hly A*) (Yamamoto *et al.,* 1984), heat stable enterotoxin (*stn/sto*) (Arita *et al.*, 1986; Ogawa *et al.,* 1990), hemagglutinins (Datta-Roy *et al.,* 1986), Tox R regulatory protein (Miller *et al.,* 1987) zonula occludens toxin (*zot*) (Fasano *et al.,* 1991), Shiga-like toxin (*stx*) (Kaper *et al.,*1994), and outer membrane protein (*ompU*) (Sperandio *et al.,* 1996) are the other factors associated with enteropathogenicity (Rivera *et al.,* 2001) of *Vibrio cholerae*.

Although the major features of the pathogenesis of *V. cholerae* are well established, there are still significant questions which are un-answered for several aspects of the disease process. Many workers in their study have attempted to investigate these factors not only of *V. cholerae* but also other species of Vibrios as well to get a clearer picture. Virulence related properties

of halophilic *Vibrio* spp have been studied by Baffone and group (2005) while Wong and Chang (2005) have investigated properties like hydrophobicity, cell adherence, cytotoxicity and enterotoxicity of starved *Vibrio parahaemolyticus*. Putative virulence-related genes in *Vibrio anguillarum* have been identified by Rodkhum *et al.* (2006) using random genome sequencing. Adhesive properties of environmental *Vibrio alginolyticus* strains to biotic and abiotic surfaces was evaluated in the work of Snoussi *et al.* (2008a) whereas the prevalence and virulence properties of non-O1 non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy was investigated by Ottaviani *et al.* (2009). The adhesion mechanisms of *Vibrio fluvialis* to skin mucus of *Epinephelus awoara* was studied by Qingpi and co-workers (2010).

2.1.2.1 Adherence and hydrophobicity

Kabir and Ali (1983) in their attempt to characterize the surface properties of *Vibrio cholerae* were able to conclude that the *V. cholerae* surface contains both specific (hemagglutinating) and nonspecific (hydrophobic and ionic) factors which may influence its eventual adherence to the host cell surface. The ability of *V. cholerae* to adhere to animal cells has long been studied (Datta-Roy *et al.,* 1989; Benitez *et al.,* 1997) and different ligands like virulence-associated toxin co-regulated pilus (TCP), outer membrane proteins and lipopolysaccharide (Chitnis *et al.,* 1982; Taylor *et al.,* 1987; Sperandio *et al.,* 1995; Tacket *et al.,* 1998) involved in intestinal colonization have also been investigated (Zampini *et al.,* 2005). Surface proteins of *V. cholerae* have been shown to mediate N-acetyl glucosamine (GlcNAc)-sensitive attachment to chitin particles in vitro (Tarsi and Pruzzo, 1999). The role of mannose-sensitive hemagglutinin (MSHA) pilus in biofilm formation on abiotic surfaces (borosilicate and cellulose) by *V. cholerae* O1 El Tor (Watnick *et al.,* 1999) and its involvement in *V. cholerae* O1 El Tor and O139 adhesion to the exoskeleton of the planktonic crustacean *Daphnia pulex* (Chiavelli *et al.,* 2001) have been investigated, while certain other ligands are hypothesized to be

utilized by *V. cholerae* O1 classical strains for zooplankton adhesion (Chiavelli *et al.,* 2001). Zampini *et al.* (2005) have hypothesized that sarkosyl-insoluble membrane proteins (siMPs) might have the function to mediate adherence to GlcNAc-containing substrates both in the aquatic environment and in human intestine.

The cell surface hydrophobicity of vibrios has been investigated by several workers (Amaro *et al.*, 1990; Wong and Chang, 2005; Zampini *et al.,* 2005). Lee and Yii (1996) have compared three different methods for assaying hydrophobicity of pathogenic vibrios to determine the most ideal method.

Toxin co-regulated pili (TCP)

For *V. cholerae* to establish an infection, it requires the bacteria to colonize the intestine and secrete cholera toxin. Bacterial colonization requires toxin-coregulated pili (TCP) which are related to type 4 pili of enterotoxigenic *E. coli* and *Citrobacter rodentium* (Giron *et al.,* 1991; Taniguchi *et al.,* 2001; Mundy *et al.,* 2003). The discovery of a pilus colonization factor (composed of long filaments, 7 nm in diameter laterally associated in bundles) in *V. cholerae* O1 was reported in 1987 by Taylor *et al.* The pilus was named TCP because expression of the pilus was correlated with expression of CT. TCP is the only colonization factor of *V. cholerae* whose importance in human disease has been proven. Volunteer studies carried out by Herrigton *et al*. (1988) showed that the intestinal colonizing factor was abolished when the gene *tcpA* was inactivated. Regulation of *tcp* gene expression is under the control of the ToxR system and is complex just like the synthesis of TCP which is incompletely understood (Taylor *et al.,* 1987; Miller and Mekalanos, 1988; Ogierman and Manning, 1992; Kaufman *et al.,* 1993; Ogierman *et al.,* 1993). TCP is found in both El Tor and classical strains, and 82 to 83% homology between the predicted protein sequences was observed among the biotypes (Iredell and Manning, 1994; Rhine and Taylor, 1994).

Assorted cellular functions, including motility, microcolony and biofilm formation, host-cell adhesion, cell signalling, transformation and phage attachment (Levine *et al.,* 1985; Herrington *et al.,* 1988; Bieber *et al.,* 1998; Tacket *et al.,* 1998) are mediated by TCP. In addition, TCP was also found to protect *V. cholerae* from complement-mediated cytolysis (Chiang *et al.,* 1995).

ompU (outer membrane protein)

Though it was suggested by Sperandio *et al.* (1995) that the adhesive properties of the outer membrane protein, OmpU may play a role in the pathogenesis of cholera, later investigations made by Nakasone and Iwanaga (1998) on OmpU revealed that it was not involved in adhesion of *V. cholerae* to the intestinal epithelium. The *ompU* gene product has more of a physiological role (Rivera *et al.,* 2001); they function primarily as channels for entry and exit of hydrophilic, low-molecular-weight molecules and these porins are also believed to contribute to *V. cholerae*'s resistance to bile, anionic detergents (Provenzano *et al.,* 2001) and antimicrobial peptides (Mathur and Waldor, 2004).

2.1.2.2 Toxins

ctxA (cholera toxin subunitA)

Cholera toxin CTX genetic element which comprises of the genome of a filamentous phage CTX Φ (Waldor and Mekalanos, 1996) is one of the major virulence-associated gene cluster regions in *V. cholerae* chromosome. This region carries the *ctx* AB gene that encodes for CT which is a typical AB5 toxin. CT consists of five identical B subunits (11.6 kDa) and a single A subunit (27.2 kDa). The mature A subunit is proteolytically cleaved to yield two polypeptide chains, A1 peptide (21.8 kDa) and A2 peptide (5.4 kDa), even after cleavage, the A1 and A2 peptides are still linked by a disulfide bond before internalization (Gill and Rappaport, 1979) The B subunits form a pentamer. A pore of 11 to 15 Aº is formed in the center of the pentamer; the C-

terminal end of the A2 peptide sits in this pore and binds to the B pentamer. The N-terminal half of the A2 is a long helix which extends outside the B pentamer and interacts with the A1 peptide. The interaction between CT and the receptor (ganglioside GM1) occurs via the B subunit (King and van Heyningen, 1973; Pierce, 1973). The intracellular target of CT is adenylate cyclase (Hepler and Gilman, 1992). There is a lag of 15 to 60 minutes before adenylate cyclase is activated (Gill and King, 1975). This is the time required for the A1 peptide to translocate through the membrane and to ADP ribosylate the G protein. This inturn activates adenylate cyclase that leads to increased intracellular levels of cAMP. cAMP activates a cAMP-dependent protein kinase, leading to protein phosphorylation, alteration of ion transport, and ultimately to diarrhea (Karper *et al.,* 1995).

zot (zonula occludens toxin)

The effect of this toxin unlike CT brings about a variation in transepithelial conductance that reflects in the modification of tissue permeability through the intercellular space (Diamond, 1977). Fasano *et al.* (1991) reported that *V. cholerae* produced a toxin that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens. Due to its prominent effect on zonula occludens it was named Zot, for zonula occludens toxin. By increasing intestinal permeability, Zot might cause diarrhea by leakage of water and electrolytes into the lumen under the force of hydrostatic pressure (Fasano *et al.,* 1991). The gene encoding Zot was cloned and found to be located immediately upstream of the *ctx* locus (Baudry *et al.,* 1992). The *zot* gene consists of a 1.3 kb open reading frame which could potentially encode a 44.8 kDa polypeptide. The predicted amino acid sequence of the Zot protein shows no homology to any other bacterial toxin (Koonin, 1992). *zot* gene sequences are present in both *V. cholerae* O1 and non-O1 strains, and strains that contain *ctx* sequences almost always contain *zot* sequences and vice versa (Johnson *et al.,* 1993; Karasawa *et al.,* 1993; Faruque *et al.,* 1994).

Cytolysin/hemolysin (VCC/HlyA)

Hemolysin from *V. cholerae* was initially purified by Honda and Finkelstein (1979) and shown to be cytolytic for a variety of erythrocytes and mammalian cells in culture and rapidly lethal for mice. Most *V. cholerae* isolates belonging to O1 biotype El Tor and to non-O1, non-O139 serogroups produce *V. cholerae* cytolysin (VCC) (Honda and Finkelstein, 1979; Goldberg and Murphy, 1984; Yamamoto *et al.,* 1984). Earlier studies showed that VCC is synthesized and secreted outside the bacterial cells as an 80 kDa pro-cytolysin which is proteolytically activated by different proteases including hemagglutin protease (HapA) of *V. cholerae* through removal of the 15 kDa polypeptide at the N-terminus (Alm *et al.,* 1988; Nagamune *et al.,* 1996, 1997). The *Vibrio cholerae* cytolysin, also called hemolysin A (HlyA) is one of the pore-forming toxins that appear to have a strict requirement for cholesterol (Ikigai *et al.,* 1996). This is encoded by a *hlyA* gene located on the *V. cholerae* chromosome II (Heidelberg *et al.,* 2000). Several contradictory data exist regarding the real role of VCC in the pathogenesis of CT-negative TCP-negative *V. cholerae* strains during infection (Ichinose *et al.,* 1987; Levine *et al.,* 1988; Zitzer *et al.,* 1993; Alm *et al.,* 1991; Figueroa-Arredondo *et al.,* 2001; Fullner *et al.,* 2002; Faruque *et al.,* 2004).

2.1.2.3 Regulation of virulence factors

Multiple systems are involved in the regulation of virulence in *V. cholerae*. The outer membrane protein controls expression of several critical virulence factors and has been the most extensively characterized. Regulation in response to iron concentration is a distinct regulatory system that controls additional putative virulence factors (Kaper *et al.,* 1995).

toxR

Expression of several virulence genes in *V. cholerae* O1 is coordinately regulated so that multiple genes respond in a similar fashion to environmental

conditions (Miller and Mekalanos, 1984, 1988). ToxR (32 kDa transmembrane protein) has been identified as the ‘‘master switch’’ for control of these factors in *V. cholerae* O1 (Miller *et al.,* 1987). ToxR binds to DNA sequence found upstream of the *ctxAB* structural genes and increases transcription of *ctxAB*, resulting in higher levels of CT expression (Miller and Mekalanos, 1984; Miller *et al.,* 1987). In addition to cholera enterotoxin, ToxR also controls expression of the TCP colonization factor (Taylor *et al.,* 1987), the accessory colonization factor (Peterson and Mekalanos, 1988), the outer membrane proteins OmpT and OmpU (Miller and Mekalanos, 1988), and three lipoproteins (Parsot *et al.,* 1991). The effect of ToxR on expression of most of these factors is to increase expression, but expression of OmpT is decreased in the presence of ToxR (Miller and Mekalanos, 1988). At least 17 distinct genes are found to be regulated by ToxR (Peterson and Mekalanos, 1988). These genes make up what is called ToxR regulon. The importance of ToxR in human disease has been demonstrated (Herrington *et al.,* 1988).

A very clear change in trend in the detection of virulence related properties can be seen over the recent years, with Polymerase chain reaction (PCR) becoming the method of choice of investigators over conventional techniques. PCR has gained popularity as it is highly sensitive, specific (Shangkuan *et al.,* 1995) and time saving; multiplex PCR a modification of basic PCR is now widely being used.

2.1.3 Antibiotic susceptibility

There are several reports on antibiotic resistance in Vibrios. Antibiotic resistance patterns of *Vibrio mimicus* isolated from human and environmental sources in Bangladesh was investigated by Chowdhury *et al.* (1986) while the interaction of *Vibrio cholerae* cells with β-lactam antibiotics and emergence of resistant cells at a high frequency was studied by Sengupta *et al.* (1992). The genes of El Tor strains corresponding to resistance of Furazolidone, Sulthanethzole, Trimethoprim, Chloramphenicol and Streptomycin genes were

found to be located in an integrating conjugative element that was closely related but not identical to the O139 SXT element (Waldor *et al.,* 1996; Hochhut and Waldor, 1999). Garg *et al*. (2001) reported of the emergence of fluoroquinolone- resistant strains of *V. cholerae* O1 biotype El Tor among hospitalized patients with cholera in Calcutta. The antibiotic susceptibility patterns of non-O1, non-O139 *V. cholerae* isolated in Cordoba, Argentina was investigated by Bidinost *et al*. (2004). Studies by Campos *et al*. (2004) on the genetic diversity and antibiotic resistance of clinical and environmental *V. cholerae* suggested that many serogroups are reservoirs of resistance. While Wang *et al*. (2006) investigated the antibiotic resistance and plasmid profile of environmental isolates of *Vibrio* species from Mai Po Nature Reserve, Hong Kong; Krishna *et al.* (2006) studied the Fluoroquinolone resistant *V. cholerae* isolated during a cholera outbreak in India. Multiple antibiotic resistances have been reported among *V. alginolyticus* strains (Snoussi *et al.,* 2008b). The Detection and molecular characterization of *V. cholerae* O1 Inaba biotype El Tor strain in Kerala was carried out by Thomas *et al.* (2008). A number of reports on the antimicrobial susceptibility patterns and molecular characterization of *V. cholerae* strains from North India (Chander *et al.,* 2009; Kingston *et al.,* 2009a) as well as from South India are available (Kingston *et al.,* 2009b; Goel *et al.,* 2010).

2.2 Materials and Methods

2.2.1 Experimental organism

The organism used in this study was isolated from mangrove sediments of Puduvyppu, Cochin and maintained in the Microbiology Laboratory of the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology (CUSAT). The strain *Vibrio* sp. (V26) was previously identified by Venugopal (2004) as a good producer of the enzyme alkaline protease.

2.2.2 Identification of the selected mangrove isolate *Vibrio* sp. (V26)

Identification of the alkaline protease producer was done on the basis of morphological, biochemical, serological and molecular characteristics of the strain. The phenotypic identification was carried out as per standard schemes based on Bergey's Manual of Systematic Bacteriology (2005) and as per the key of Alsina and Blanch (1994).

2.2.2.1 Morphology

Morphology of the culture was studied by Gram's staining and also by Scanning Electron Microscopy (SEM).

Scanning Electron Microscopy

An aliquot of 1.5 ml of bacterial culture was centrifuged at 8000 x g in a refrigerated centrifuge for 15 minutes. The pellet was washed with sterile physiological saline and fixed in 0.5 ml of 2.5% glutaraldehyde prepared in sterile saline at 4ºC overnight. The pellet was washed repeatedly with saline and dehydration was done through an acetone series of 70-100% and kept overnight in a dessicator. The particle was spread on SEM stubs, dried in critical point dried apparatus, platinum coated and observed under JEOL Analytical Scanning Electron Microscope, JSM 6390 LV, Tokyo, Japan (Sophisticated Test and Instrumentation Centre, CUSAT, Cochin-22).

2.2.2.2 Phenotypic characterization

Motility assay

a) Soft agar method

Motility was tested in soft agar medium. Culture was stab inoculated into the medium using a straight wire loop and incubated at 28 ± 0.5ºC for 24 to 48 hours. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility.

b) Hanging drop test

The organism was grown in nutrient broth. Petroleum jelly was placed at the four corners of the coverslip. A loop full of the 18 to 24-hour-old culture was placed in the centre of the cover slip. The cavity slide was placed with the concave surface facing down over the cover slip so that the depression covers the drop of culture. Slide was gently pressed to form a seal between the slide and the coverslip. This preparation was inverted quickly so that the drop of culture was seen hanging from the coverslip. The slide was placed under the objective lens and observed for actual movement of the cells that could very well be differentiated from Brownian movement.

Oxidase test (Cytochrome oxidase activity)

The organism was freshly grown on nutrient agar plate. A platinum loop / wooden applicator was used to pick a colony and make a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive reaction is indicated by the development of intense deep violet/ purple colour within ten seconds. Negative reaction is indicated by the absence of the characteristic colour within ten seconds.

Oxidation /Fermentation reaction

MOF medium (HiMedia Laboratories, Mumbai) was employed for this test. This reaction was determined by inoculating the organisms into basal media supplemented with 1% glucose in the culture tubes. The tubes were stabbed and streaked and incubated at 28 ± 0.4ºC.

The results were recorded as follows:

O- Oxidation (yellow colouration (acid production) in the slope)

F- Fermentation (yellow colouration in both butt and slant)

FG- Fermentation with gas production

Alk / N – alkaline reaction (pink or purple colouration in the slant and no reaction in the butt)

Growth on Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS)

Culture was streaked onto TCBS agar (Himedia, Bombay) which is both a selective and differential medium for the cultivation and isolation of Vibrios. Vibrios form yellow / green colonies on TCBS agar.

Hydrogen sulphide production

Triple sugar iron agar (TSI) was used for this test. The culture was inoculated by stabbing and streaking the TSI slant. The slant was incubated at 28 ± 0.5ºC for 18 to 24 hrs and then checked for H_2S production. Blackening of media is positive for H_2S production.

Utilization of amino acids

The aminoacid decarboxylase test demonstrates the bacterial decarboxylation of lysine, arginine, and ornithine, and these tests are of particular use in identifying members of *Enterobacteriaceae*. Decarboxylase basal broth (HiMedia Laboratories, Mumbai) incorporated with 1% of the L-aminoacid (L (+) Lysine dihydrochloride, or L (+) Ornithine monohydrochloride and Arginine dihydrolase broth (HiMedia Laboratories, Mumbai) was used for this test.

The test organism from a solid media was introduced into the specific broth with a straight inoculating wire through the paraffin overlay. Suitable controls were placed. Tubes were incubated and examined daily for 4 days. As a result of the bacterial fermentation of the glucose in the medium, the colour of indicator changes to yellow. The control tube without the amino acid remains yellow; but a subsequent change to violet or purple in the tests indicates that alkaline degradation products were produced in the course of decarboxylation of the particular amino acid. A colour change in medium from purple to yellow and back to purple is the positive result for this test.

Production of Indole

Test organism was grown in Tryptone broth for 24 hrs. Two hundred microlitres of Kovac's reagent was added to 5 ml of culture medium. Formation of a cherry red ring is the positive result.

Methyl Red and Voges-Proskauer tests

These tests were carried out with cultures grown in glucose-phosphate peptone water.

Methyl Red test

A few drops of methyl red indicator were added to the culture broth and a resultant definite red colour was considered positive.

Voges-Proskauer test (acetoin production)

Barritt's reagent A and B were added in the ratio 3:1 to culture broth. A positive reaction was indicated by the development of a pink colour in 2-5 minutes, becoming crimson in 30 minutes. The tube was shaken at intervals to ensure maximum aeration.

Citrate utilization

This test demonstrates the ability of the microbes to utilize citrate as a sole source of carbon. Simmon's citrate agar slants were streaked with a loopful of culture and incubated. Colour of bromothymol blue indicator changes from green to blue on utilization of citrate.

Sodium chloride tolerance test

Growth at different concentrations of NaCl upto 10% (w/v) was tested by observing growth in 1% tryptone broth at pH 7.3 ± 0.3 containing varying amounts (0, 3, 6, 8 and 10%) of analytical grade NaCl. Growth was detected visually by observing turbidity.

Catalase

The test organisms were grown on a slope of nutrient agar. A thick smear of the organism was made from a 24 hr culture on a clean slide and a drop of hydrogen peroxide was placed on it. Immediate development of effervescence was the positive result.

Lactose utilization

Test culture was streaked onto Mac-Conkey agar plates and incubated at 28 ± 0.5ºC for 24 hrs. Culture that utilizes lactose in the medium turns pink.

Mannitol utilization

Mannitol motility agar was used for this test. Culture was stab inoculated onto mannitol motility agar tubes. Mannitol utilization was indicated by change in colour of the medium to yellow.

Utilization of various carbon sources using Biolog GN2 plates

The Biolog GN2 MicroPlate performs 95 discrete tests simultaneously. The culture was swabbed from the surface of the agar plate, and suspended to a specified density in GN / GP Inoculating Fluid (Biolog catalog # 72101). One hundred and fifty microlitre of bacterial suspension was pipetted into each well of the GN2 MicroPlate (Biolog catalog # 1101). The MicroPlate was incubated at 30ºC for 24 hours and results were read visually.

Production of Hydrolytic Enzymes

To determine the hydrolytic enzyme profile of *Vibrio* sp. (V26) the strain was checked for production of various enzymes viz. amylase, lipase, caseinase, chitinase, DNase and gelatinase. Nutrient agar supplemented with starch (1%), tributyrin (1%), colloidal chitin (5%) and DNA (0.2%) were prepared separately. Frazier's Gelatin Agar (modified method of Harrigane and McCane, 1972) and Skim Milk Agar (HiMedia Laboratories, Mumbai) were also prepared**.** Spot inoculation was performed on the various media plated.

After incubation at room temperature (28 ± 2ºC) for 2 days, observations were made. The gelatinase activity was observed as a clearing around the positive colonies when flooded with 15% $HgCl_2$ in 20% concentrated HCl. Starch agar plates were flooded with Gram's iodine solution and the appearance of a halo zone around the colonies was noted for a positive result. Tributyrin agar, Skim milk agar and Chitin agar plates were checked for a clear zone around the colonies, which indicated the production of the enzymes lipase, caseinase and chitinase respectively. The DNA agar plate was flooded with 1 M HCl and the appearance of clearance zone around the colony indicated a positive result.

Haemolytic assay

Haemolytic activity on Blood agar base (HiMedia Laboratories, Mumbai) containing 5% (v/v) human blood was examined by incubating the inoculated plate for 24 hrs at 37ºC. Based on the type of haemolytic activity of isolate it was categorized as alpha, beta or gamma haemolysis.

2.2.2.3 Molecular characterization

The 16S rRNA gene sequencing involves the following steps:

- Extraction of DNA
- PCR amplification
- Purification
- Sequencing

DNA extraction

Total genomic DNA extraction was carried out following the method of Lee *et al.,* (2003 a) with slight modification. Aliquots of 2 ml overnight culture of *Vibrio* sp. V26 grown in Nutrient broth at 30ºC were centrifuged at 8000 x g for 10 minutes and the resultant pellet was resuspended in 500 µl TNE buffer (Tris-HCl 10 mM, pH 8.0, EDTA 1 mM, NaCl 0.15 mM) and centrifuged again at 8000 rpm for 10 minutes at 4ºC. Subsequently, the pellet was

resuspended in 500 µl Lysis buffer (Tris-HCl 0.05 mM, pH 8.0, EDTA 0.05 mM, NaCl 0.1 mM, SDS 2%, PVP 0.2% and 0.1% mercaptoethanol) (Lee *et al.,* 2003a). Proteinase K (20 µg/ml) was added and incubated first at 37 ºC for 1 hr then at 55ºC for 2 hrs. After this step the DNA was further extracted by standard phenol-chloroform method of Sambrook and Russell (2001). The sample was deproteinated by adding an equal volume of phenol (tris-equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and aqueous layers were separated by centrifugation at 15000 rpm for 15 minutes at 4ºC. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once more. After this, an equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15000 rpm for 15 minutes at 4ºC to separate the aqueous phase which was transferred to a fresh tube. Then the DNA was precipitated by incubation at -20ºC overnight after adding equal volume of ice-cold absolute alcohol and 0.1% (v/v) 3 M sodium acetate. The precipitated DNA was collected by centrifugation at 15000 rpm for 15 minutes at 4ºC and the pellet was washed in ice-cold 70% ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes were left open until the pellet dried. DNA pellet obtained was resuspended in TE (Tris-HCl 0.5 mM, EDTA 0.5 mM, pH 8.0).

The isolated DNA was quantified spectrophotometrically (A_{260}) and the purity of DNA was assessed by calculating the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}), the value of which determined the amount of protein impurities in the sample. Electrophoresis was done using 0.8 % agarose gel. Concentration of DNA was calculated from the following formula:

Conc. of DNA (µg/ml) = OD at 260 nm x 50 x dilution factor.

16S rRNA gene amplification

Identity of the isolate *Vibrio* sp. (V26) was ascertained by partially sequencing a 1500 bp fragment of the 16S rRNA gene using the primers NP1F 5′-

GAGTTTGATCCTGGCTCA -3′and NP1R 5′- ACGGCTACCTTGTTACGACTT -3′, (complimentary to the conserved regions at the 5′- and 3′- ends of the 16S rRNA gene corresponding to positions 9-27 and 1477-1498 of the *Escherichia coli* 16S rRNA gene) (Reddy *et al.,* 2000). The amplification reaction was performed by using a DNA thermal cycler (Eppendorf).

Bacterial DNA (50 ng) was amplified by PCR in a total volume of 25 µl containing 2.5 µl of 10X Thermopol buffer (New England Biolabs), 0.5 U Taq DNA Polymerase (New England Biolabs), 10 pmol each of the two primers, and 200 µM each of dATP, dCTP, dGTP and dTTP. The amplification was carried out in a thermal cycler (Master Cycler, Eppendorf) which involved initial denaturation at 95ºC for 5 minutes followed by 35 cycles of (94ºC for 20 sec, 58ºC for 20 sec, 68 ºC for 90 sec) and final extension at 68 ºC for 10 minutes.

The PCR products were analyzed by electrophoresis on 1% agarose gel prepared in 1X TAE buffer and stained with ethidium bromide. PCR product was purified using Gen Elute PCR clean up kit (Sigma).

Sequencing

Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Microsynth AG, Switzerland.

16S rRNA gene sequence similarity and Phylogenetic analysis

DNA sequence data were compiled and analyzed. The Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.,* 1990) was used to search the GenBank database for homologous sequences (http:// www.ncbi.nlm.nih.gov/). The sequences were multiple aligned using the programme Clustal W (Thompson *et al.,* 1994). Then the aligned 16S-rDNA gene sequences were used to construct a phylogenetic tree using the neighbour-joining (NJ) method (Saitou and Nei, 1987), using the MEGA4 package (Tamura *et al.,* 2007). Bootstrap analysis was based on 1000 replicates.

2.2.2.4 Putative virulence traits

2.2.2.4.1 Serogrouping

Serogrouping was done using *Vibrio cholerae* O1 polyvalent antisera as per manufacturer's protocol (Murex Diagnostics Limited).

2.2.2.4.2 Virulence genes

Virulence-associated factors such as cholera toxin (*ctxA*), outer membrane protein (*ompU*), zonula occludens toxin (*zot*), toxin-coregulated pilus (*tcpA*), ToxR regulatory protein (*toxR*) and hemolysin (*hlyA*) were investigated in the isolate *Vibrio* sp. (26). The primers (Table 2.1) (Bioserve Biotechnologies, Hyderbad, India) employed for both multiplex PCR (*hlyA* (classical and E1Tor), *tcpA* (classical and E1Tor)) and simple PCR (*ctxA, ompU, zot, toxR)* were based on works of Fields *et al.* (1992) and Rivera *et al.* (2001).

DNA extraction

DNA extraction was carried out as described in the previous section ***2.2.2.3***

Amplification of Virulence Associated Genes

The PCR was carried out in 0.2 ml PCR tubes, using 25 µl reaction mixture consisting of 2.5 µl 10X Thermopol buffer (New England Biolabs/ Standard buffer), 2 µl (250 µM each of dATP, dCTP, dGTP and dTTP), 2 µl (10 µM) primer, 1.5 µl template (50 ng/µl) and 1 µl Taq DNA Polymerase (0.5 U, New England Biolabs) and Milli Q (to a final volume of 25 µl). The amplification was carried out in a thermal cycler (Master Cycler, Eppendorf) which involved initial denaturation at 95ºC for 5 minutes followed by 30 cycles of (95 ºC for 1 minute, 60ºC for 1 minute, 72ºC for 1 minute) and final extension at 72ºC for 10 minutes. The amplified products were separated on 1% agarose gel and stained with ethidium bromide. The DNA from *V. cholerae* strain MTCC 3906 was used as the positive control and the reaction mixture containing Milli Q and all other reagents except the template DNA was used as negative control.

Table 2.1

Primers used in amplifying toxin genes in *Vibrio* sp. (V26)

Gene(s), primers, and sequences (5´ to 3´)	Amplicon size (bp)	Reference
***ctxA* and *ompU* (CT subunit A and outer membrane protein)**		
94F, CGG GCA GAT TCT AGA CCT CCT G		
614R, CGA TGA TCT TGG AGC ATT CCC AC	**564 (*ctxA*)**	Fields *et al.,* 1992
80F, ACG CTG ACG GAA TCA ACC AAA G		
906R, GCG GAA GTT TGG CTT GAA GTA G	**869 (*ompU*)**	Rivera *et al.,* 2001
***zot* (zonula occludens toxin)**		
225F, TCG CTT AAC GAT GGC GCG TTT T		
1129R, AAC CCC GTT TCA CTT CTA CCC A	**947**	Rivera *et al.,* 2001
***toxR* (operon ToxR)**		
101F, CCT TCG ATC CCC TAA GCA ATA C		
837R, AGG GTT AGC AAC GAT GCG TAA G	**779**	Rivera *et al.,* 2001
***tcpA* (TCP A [Classical and El Tor])**		
72F, CAC GAT AAG AAA ACC GGT CAA GAG		
477R, CGA AAG CAC CTT CTT TCA CGT TG	**451(El Tor)**	Rivera *et al.,* 2001
647R, TTA CCA AAT GCA ACG CCG AAT G	**620 (Classical)**	
***hlyA* (hemolysin [Classical and El Tor])**		
489F, GGC AAA CAG CGA AAC AAA TAC C	**481(El Tor)**	Rivera *et al.,* 2001
744F, GAG CCG GCA TTC ATC TGA AT	**738/727**	
1184R, CTC AGC GGG CTA ATA CGG TTT A	**(ET/Clas)**	

2.2.2.4.3 Adherence Assay

The isolate was examined for its adherence to HEp-2 cells following the methods described previously (Snoussi *et al.,* 2008a) with certain modifications. The HEp-2 cells were grown in Eagle's MEM (Himedia) with 2 mM glutamine, 1.5 g/L sodium bicarbonate without fetal bovine serum to 50 to 70% confluence in a 24-well culture plate (Greiner Bio-One). 100 µl (10^7 CFU/ml) of bacterial culture was added to the wells and incubated with HEp-2 cell monolayers at 37ºC for 2 hrs. The monolayer were washed three times with PBS to remove nonadherent bacteria, fixed with 70% methanol, and stained with 10% Giemsa for 20 minutes. The adherence patterns were examined under an inverted phase contrast microscope (Leica DMIL, Germany). The adhesion index was assayed as: NA = non adhesive (0–10 bacteria/cells); W = weak adhesion (10–20 bacteria/cells); M = medium adhesion (20–50 bacteria/cells); S = strong adhesion (50–100 bacteria/cells).

2.2.2.4.4 Hydrophobicity

Cell surface hydrophobicity of bacteria was evaluated by their adherence to xylene (Rosenberg *et al.,* 1980) and salt aggregation test (SAT).

a. Salt aggregation test (SAT)

The test was performed as per the method described by Lindhal *et al*. (1981). To 30 µl of each ammonium sulphate concentration (0.05-4.0 mol/L) in 0.002 mol/L sodium phosphate buffer (pH 6.8) dispensed into test tubes, 30 µl bacterial cell suspension in 0.002 mol/L sodium phosphate buffer (A_{420} of 1) was added and mixed. The tubes were kept at room temperature for 3 hrs. The lowest concentration causing bacterial aggregation was recorded as SAT value.

b. Bacterial adhesion to hydrocarbons test (BATH)

Bacterial surface hydrophobicity was evaluated as described by Rosenberg *et al.* (1980) The overnight bacterial culture was harvested, washed twice with PBS (phosphate buffered saline) and resuspended in phosphate–urea–magnesium (PUM) buffer (K_2HPO_4. $3H_2O$ 22.2 g/L, KH_2PO_4 7.26 g /L, $MgSO_4$ 0.2 g /L, urea 1.8 g/L, pH 7.1) to $O.D_{560}$ = 0.5; 1.2 ml samples were then placed in test tubes and 0.5 ml p-xylene was added. Following 10 minutes preincubation at 30ºC, the tubes were vortexed for 1 minute and allowed to stand at room temperature for 20 minutes; then the lower aqueous phase was removed and the $O.D_{560}$ of the suspension measured. The results were expressed as the percentage decrease in absorbance (A_{560}) of the lower aqueous phase compared with A_{560} of the initial cell suspension.

2.2.2.5 Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed by the disc diffusion method (Bauer *et al.,* 1996) using commercially available discs (HiMedia) with 11 antimicrobial drugs on Mueller-Hinton agar (HiMedia). Isolates were considered susceptible, reduced susceptible, or resistant to a particular antimicrobial agent on the basis of the diameters of the inhibitory zones that matched the criteria of the manufacturer's interpretive table, which followed the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) (2002).

2.3 Results

2.3.1 Morphology

The cells were Gram negative slightly curved rods. Scanning electron microphotograph of the strain, *Vibrio* sp. (V26) is presented in Fig.2.1.

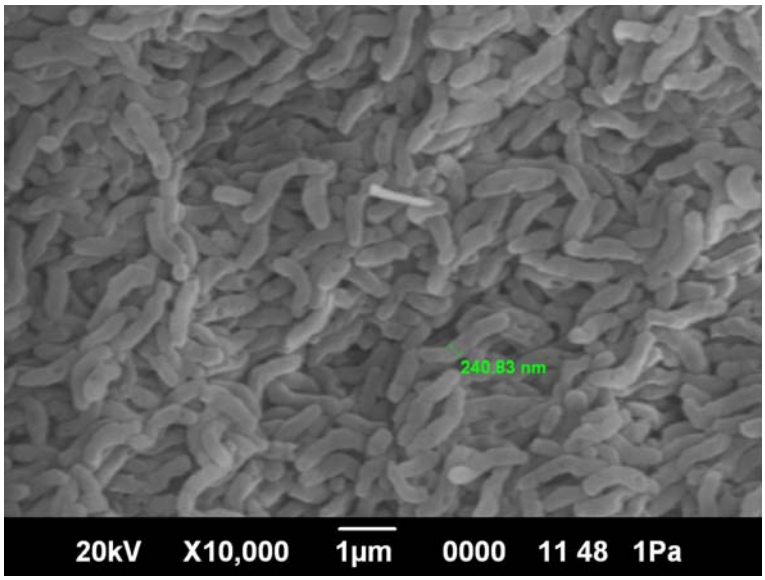


Fig. 2.1 Scanning Electron Micrograph of *Vibrio* sp. (V26)

2.3.2 Phenotypic Identification

The biochemical characteristics of *Vibrio* sp. (V26) are presented in Table 2.2. The isolate was motile, positive for oxidase, formed yellow colonies that turn green afterwards when cultured on TCBS and produced non-lactose fermenting colonies on MacConkey agar. The strain was capable of growing in the absence of NaCl. *Vibrio* sp. (V26) was also positive for the utilization of mannose as carbon source. The strain was capable of producing all the enzymes tested except amylase (Table 2.3). The strain *Vibrio* sp. (V26) was α-hemolytic. Based on the biochemical features it was difficult to identify the strain to the species level as it showed great similarity to *V. mimicus* and *V. cholerae.* Even when the software PIBWin bacterial identification program version: 2.0.0 was used it was difficult to identify the species.

Table 2.2 Phenoypic characteristics of *Vibrio* sp. (V26)

Test	Result	Test	Result	Test	Result
Gram Staining	-ve rod	m-Inositol	_	Propionic Acid	_
MOF	F	α- D-Lactose	_	Quinic Acid	_
Growth on TCBS	+	Lactulose	_	D-Saccharic Acid	_
Oxidase	+	Maltose	+	Sebacic Acid	_
Catalase	+	D-Mannitol	+	Succinic Acid	+
Growth in the presence		D-Mannose	+	Bromosuccinic Acid	+
0% NaCl	+	D-Melibiose	_	Succinamic Acid	_
3% NaCl	+	β-Methyl D-Glucoside	+	Glucuronamide	_
6% NaCl	_	D-Psicose	+	L-Alaninamide	_
8% NaCl	_	D-Raffinose	_	D-Alanine	_
10% NaCl	_	L-Rhamnose	_	L-Alanine	_
Citrate Utilization	+	D-Sorbitol	_	L-Alanyl gylcine	_
VP	+	Sucrose	+	L-Asparagine	+
Indole	+				
Mannitol	+	D-Trehalose	+	L-Aspartic Acid	+
TSI	**No H_2S**	Turanose	_	L-Glutamic Acid	+
Lactose Fermentation	_	Xylitol	_	Glycyl-L-Aspartic Acid	+
Luminescence	_	Pyruvic Acid Methyl Ester	_	Glycyl-L-Glutamic Acid	+
Argnine dihydrolase	_	Succinic Acid Mono –Methy-Ester	+	L-Histidine	+
Lysine decaroxylase	+	Acetic Acid	+	Hydroxy-L-Proline	_
Ornithine decarboxylase	+	Cis-Aconitic Acid	_	L-Leucine	_
α- Cyclodextrin	_	Citric Acid	+	L-Ornithine	_
Dextrin	+	Formic Acid	_	L-Phenylalanine	_
Glycogen	+	D-Galactonic Acid Lactone	_	L-Proline	_
Tween 40	+	D-Galacturonic acid	_	L-Pyroglutamic Acid	_
Tween 80	+	D-Gluconic Acid	+	D-Serine	_
N-Acetyl-D-Galactosamine	_	D-Glucosaminic Acid	_	L-Serine	+
N-Acetyl-D-Glucosamine	+	D-Glucoronic Acid	_	L-Threonine	_
Adonitol	_	α-Hydroxybutyric Acid	_	D,L-Carnitine	_
L-Arabinose	_	β-Hydroxybutyric Acid	_	γ-Amino Butyric Acid	_
D-Arabitol	_	γ-Hydroxybutyric Acid	_	Urocanic acid	_
D-Cellobiose	_	p-Hydroxy Phenylacetic Acid	_	Inosine	+
i-Erythritol	_	Itaconic acid	_	Uridine	+
D-Fructose	+	α-Keto Butyric Acid	_	Thymidine	_
L-Fucose	_	α-Keto Glutaric Acid	+	Phenylethylamine	_
D-Galactose	+	A-Keto Valeric Acid	_	Putrescine	_
Gentiobiose	_	D,L-Lactic Acid	+	2-Aminoethanol	_
α-D Glucose	+	Malonic Acid	_	2,3,-Butanediol	_
2,3,butanediol		Glycerol	+	D,L-α-Glycerol	
α- D-Glucose-1-Phosphate	_	D-Glucose-6-Phosphate	+	Phosphate	+

Table 2.3 Hydrolytic enzyme profile of *Vibrio* sp. (V26)

Exoenzyme activity	Observation
Amylase	-
Chitinase	+
Caseinase	+
Gelatinase	+
DNase	+

2.3.3 Molecular identification

The DNA of the strain was isolated (Fig.2.2A) and the 16S rRNA gene amplified using universal primers to obtain an amplicon with a product size of 1500 bp (Fig.2.2 B). This product was partially sequenced and the nucleotide sequence has been submitted to the GenBank data base and was assigned the Accession no: FJ665509 (Fig.2.2 C). When the sequence of this strain was compared with the GenBank database using the BLAST algorithm; 98% similarity (98% query coverage) was obtained to 16S rRNA gene of *Vibrio cholerae, V. mimicus, V. albensis* and certain Uncultured *Vibrio* clones (Fig.2.2 D).

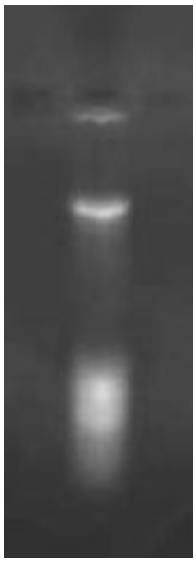


Fig.2.2 A. Agarose gel electrophoresis of the genomic DNA of *Vibrio* sp. (V26)

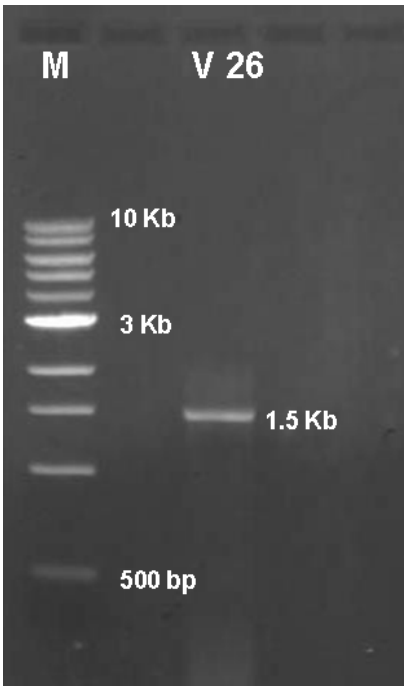


Fig.2.2 B. PCR product of 16S rRNA gene amplified from the DNA extracted from *Vibrio* sp. (V26). **M**-1 Kb DNA ladder, **V26**- 1.5 Kb PCR product of 16S rRNA gene gene of ***Vibrio* sp. (V26)**

1 actcttttga aatttgggtt aaatcccgca acgagcgcaa cccttatcct tgtttgccag
61 cacgtaatgg tgggaactcc agggagactg ccggtgataa accggaggaa ggtggggacg
121 acgtcaagtc atcatggccc ttacgagtag ggctacacac gtgctacaat ggcgtataca
181 gagggcagcg ataccgcgag gtggagcgaa tctcacaaag tacgtcgtag tccggattgg
241 agtctgcaac tcgactccat gaagtcggaa tcgctagtaa tcgcaaatca gaatgttgcg
301 gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca ccatgggagt gggctgcaaa
361 agaagcaggt gnttaac

Fig 2.2 C. Partial sequence of 16S rRNA gene (377 bp) of *Vibrio* sp. (V26) (Accession no: FJ665509)

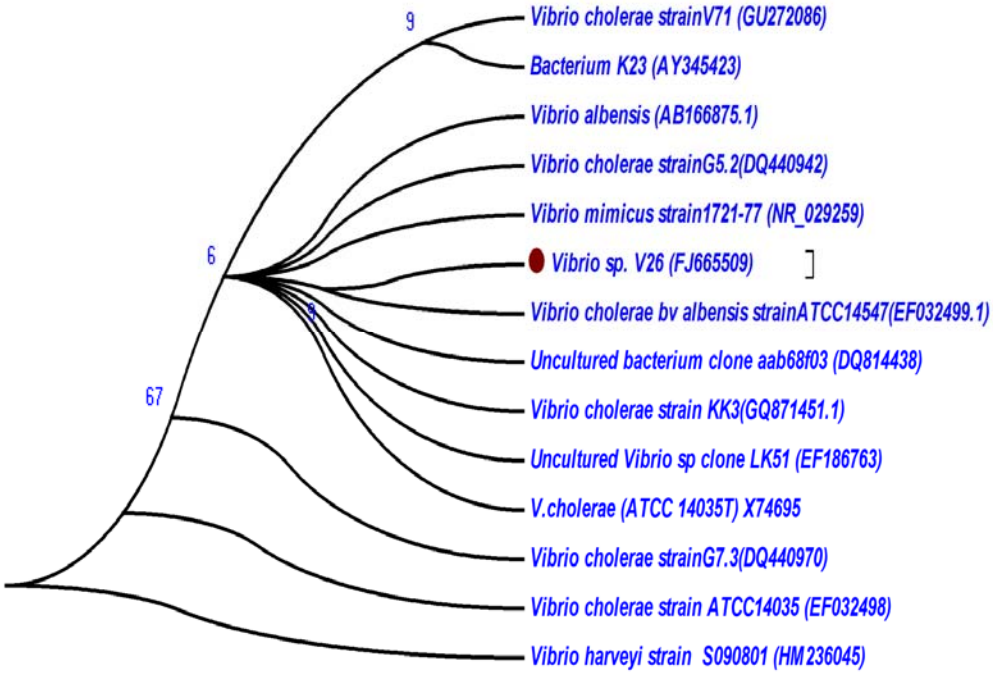


Fig.2.2 D. A bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the nucleotide sequences of the 16S rRNA gene of *Vibrio* sp. (V26) with relevant sequences of reference strains of *Vibrio* species downloaded from the National Center for Biotechnology Information Database (http:// www.ncbi.nlm.nih.gov/).The accession no: of the strains are given in brackets

2.3.4 Putative Virulence Traits

Pathogenicity is contributed by a combination of virulence associated factors. Therefore the traits such as the virulence associated gene profile, hydrophobicity, adherence pattern and antibiogram of *Vibrio* sp.(V26) were investigated.

2.3.4.1 Serogrouping

No agglutination was observed with *Vibrio cholerae* O1 polyvalent antisera. This revealed that the isolate *Vibrio* sp. (V26) did not belong to the O1 serogroup.

2.3.4.2 Virulence associated genes

The virulence genes *hlyA* and *tcpA* were sought out in the strains MTCC 3906 (positive control) and *Vibrio* sp. (V26) using multiplex PCR, while simple PCR was used for all the other genes. The PCR products were analyzed using agarose gel electrophoresis and results of this analysis is given in Fig.2.3.

The toxin gene cassette comprising of *cxtA* (564 bp) (lane 1) and *zot* (947 bp) (lane 5) were present only in the type strain MTCC 3906 whereas *Vibrio* sp. (V26) lacked both these genes. The primers used for targeting *tcpA* are designed to exploit the sequence difference between the *tcpA* of ElTor and Classical biotypes. Here in this study the gene *tcpA* was absent in *Vibrio* sp. (V26). While in the case of MTCC 3906, an amplicon of the same size (451 bp) (lane 7) as that obtained for *tcpA* ElTor gene was observed.

Both MTCC 3906 and V26 were found to possess the *toxR* regulatory sequence (amplicon of size 779 bp). The *hlyA* gene was also found both in positive control as well *Vibrio* sp. (V26). But in the case of MTCC culture only single band of 481 bp (lane 11) was observed whereas for *Vibrio* sp. (V26) dual amplification fragments of sizes 481 and 738 bp (lane 12) was seen. Apart from these two bands a faint band of ~ 1.3 Kb could also be seen.

The *ompU* fragment (869 bp) was amplified only from strain *Vibrio* sp. (V26) and not from the type strain. This was an unexpected observation.

The overall virulence profiles of the environmental isolate *Vibrio* sp. (V26) and the type strain MTCC 3906 are given below. The gene profile clearly revealed that the strain *Vibrio* sp. (V26) was non-toxigenic.

***Vibrio* sp. (V26)** $ctxA^{-}\ zot^{-}\ tcp\ A^{-}\ hly\ AET^{+}\ ompU^{+}\ tox\ R^{+}$

MTCC 3906 $ctx\ A^{+}\ zot^{+}\ tcp\ A^{+}\ hly\ AET^{+}\ ompU^{-}\ tox\ R^{+}$

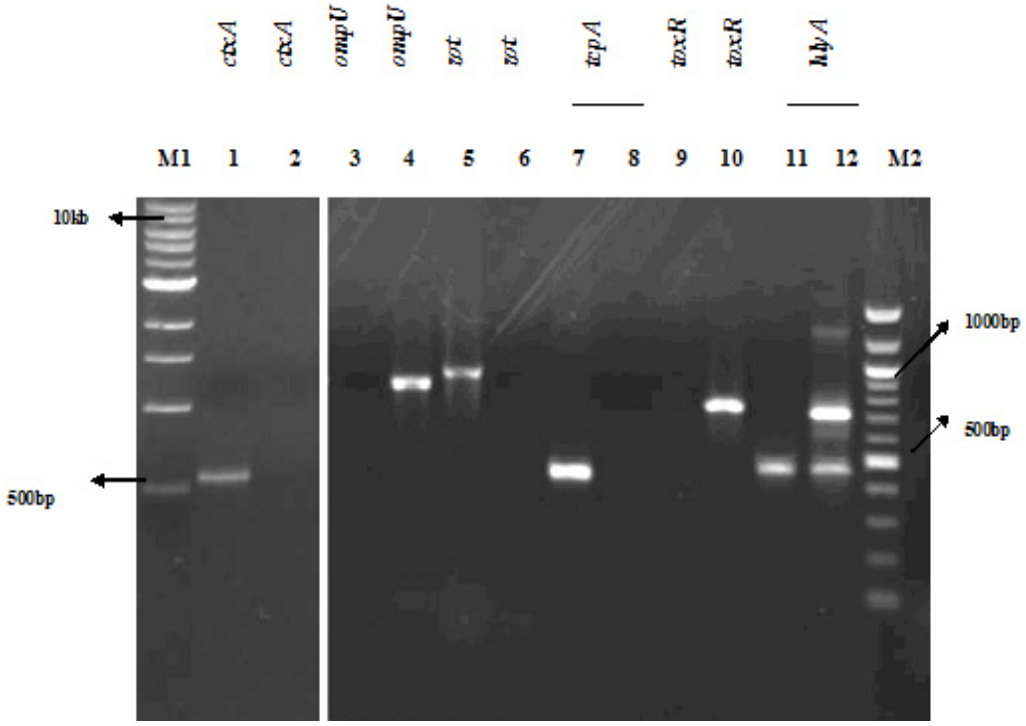


Fig.2.3 Analysis of PCR products of virulence genes.

Lane M1: 1 kb DNA ladder; lane M2: 100bp DNA ladder; lanes: 1,3,5,7 and 11 *V.cholerae* MTCC 3906; lanes: 2,4,6,8,10 and12 *Vibrio* sp. (V26) ; lanes: 1 to 6, 9-10 simple PCR for *ctxA*, *ompU*, *zot* and *toxR*; lane 9: *toxR* negative control; lanes: 7,8,11 and 12 amplicons obtained using multiplex PCR for *tcpA* and *hly*A.

2.3.4.3 Hydrophobicity and adherence

The strain was found to adhere to the HEp-2 cell lines, but the number of bacteria adhering to cell lines was only 10-20 per cell (Fig.2.4). This denoted that the *Vibrio* sp. (V26) possessed only a weak adherence capability. Moreover the pattern of adherence was diffuse (Fig.2.4).

Hydrophobicity was assessed by both SAT and BATH assay. During the SAT assay, no bacterial aggregation was observed in the 0.05 to 4.0 mol/L concentration of ammonium sulphate. BATH assay revealed a hydrophobicity value of 14.04 % for the strain, *Vibrio* sp. (V26). Both these tests concurrently pointed to the lack of surface hydrophobicity and that the strain was non-hydrophobic. This non-hydrophobic nature of *Vibrio* sp. (V26) could probably explain the weak adherence property exhibited by the strain.

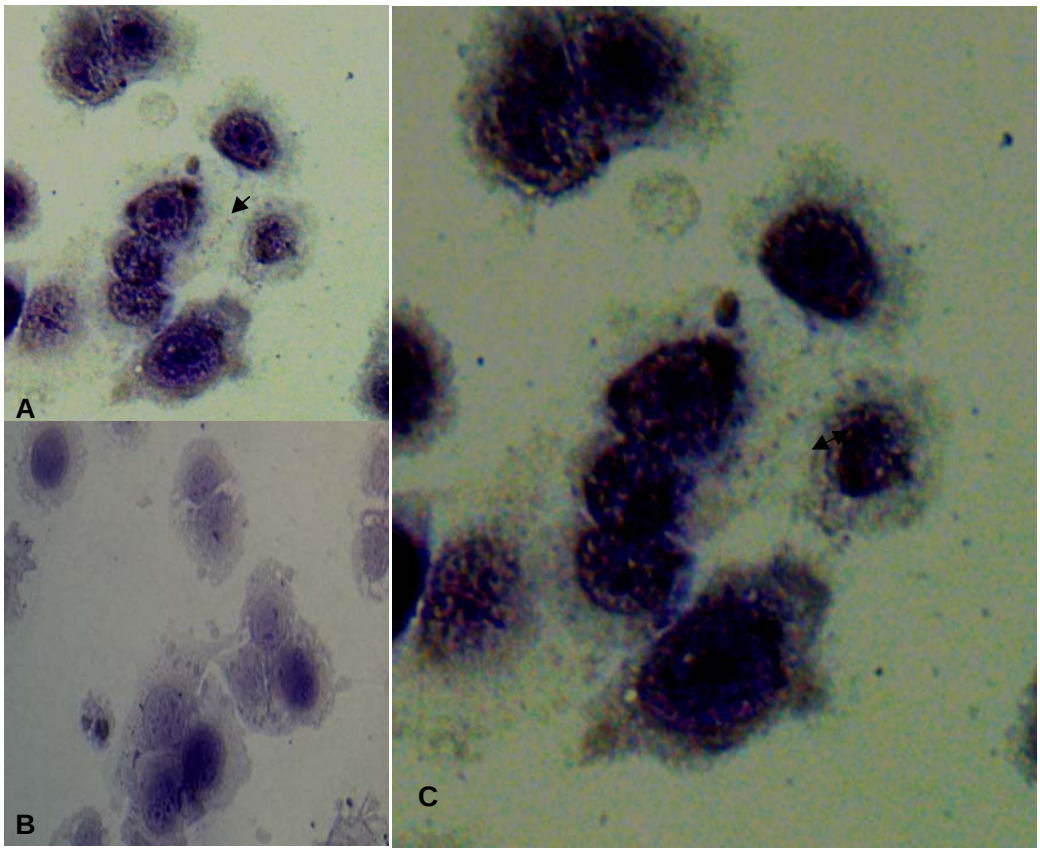


Fig. 2.4 Inverted phase contrast microscopy showing the weak adherence property of the strain *Vibrio* sp. (V26) on HEp-2 cells.
A) Diffuse adherence pattern was exhibited by the strain,B) control HEp-2 cells, C) Enlarged image of Fig 2.4A

2.4. Antibiogram

The antibiogram of the strain *Vibrio* sp. (V26) is presented in Table 2.4. The strain was resistant to Cefrodoxime (10 µg) and exhibited intermediate sensitivity to Ampicillin (10µg). *Vibrio* sp. (V26) was found to be sensitive to antibiotics such as norfloxacin, ciprofloxacin and tetracycline which are used in the treatment cholera (Fig 2.5)

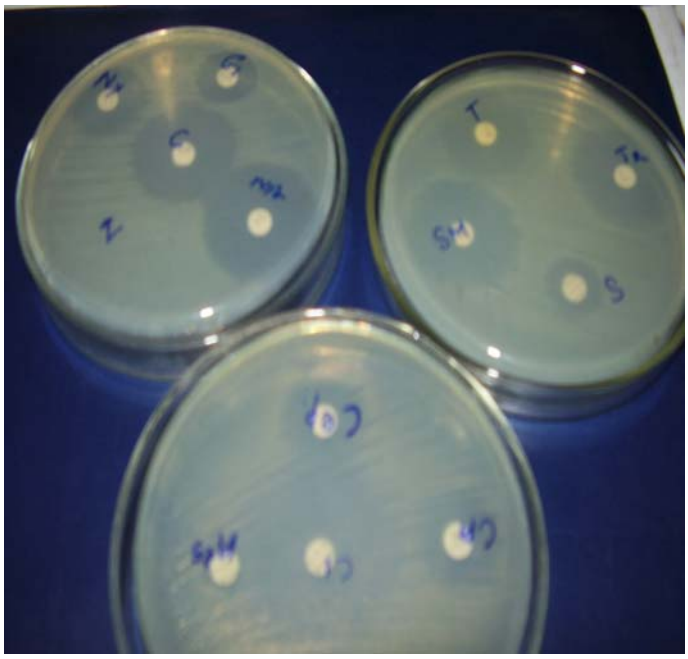


Fig. 2.5 Antibiotic susceptibility testing by the Disc Diffusion method
Clearance zones indicating sensitivity to antibiotics.

Table 2.4 Antibiogram of *Vibrio* sp. (V26)

Sl no:	Antibiotic	Diameter of zone (mm)	Result
1	Ampicillin (10 μg)	15	IMS
2	Ceftazidime (30 μg)	19	S
3	Ciprofloxacin (5 μg)	40	S
4	Cefrodoxime (10 μg)	16	R
5	Chloramphenicol (10 μg)	31	S
6	Gentamycin (10 μg)	20	S
7	Nalidixic acid (30 μg)	31	S
8	Norfloxacin (10 μg)	25	S
9	Tetracycline (30 μg)	25	S
10	Trimethoprim (32 μg)	27	S
11	Streptomycin (10 μg)	15	S
12	Sulfamethaxole (160 μg)	28	S

IMS – Intermediately Sensitive, R- Resistant, S- Sensitive

2.5 Discussion

In the present study, the biochemical and virulence traits of *Vibrio* sp. (V26) were extensively characterized with a view to identify the strain up to species level and to determine whether the strain is pathogenic or not.

The members of the genus *Vibrio* are Gram-negative curved motile rods, fermentative, facultative anaerobes, with the ability to grow on TCBS. These typical characteristic features were exhibited by *Vibrio* sp. (V26). The strain *Vibrio* sp.(V26) grew even in the absence of NaCl. Except for *V. cholerae* and *Vibrio mimicus*, Na^+ is an absolute requirement for the growth of all other Vibrios. This indicated that, *Vibrio* sp. (V26) belonged to either of these species. With regard to the utilization of mannose as carbon source it resembled non-01 /non-0139 serotypes O20, O64, O65 and certain untypeable *Vibrio cholerae* (Bag *et al.,* 2008).When the overall results of the biochemical tests were considered, a definite identification at the species level was not possible. Even when the software PIBWin bacterial identification program version: 2.0.0 was used, the required identification threshold could not be reached; it gave Id score of only 0.66, 0.32 and 0.008 to *V. cholerae, V. mimicus* and *V. alginolyticus* respectively. The Id score was not high enough for the assignment of species. This is in agreement with the findings of Thompson *et al.* (2004a) that the identification of species of the family *Vibrionaceae* based purely on phenotypic characters is quite difficult.

The hydrolytic profile of strain *Vibrio* sp. (V26) clearly revealed its potential to utilize a wide range of substrates which is in concurrence with the observation of Amaro *et al.* (1990). But with regard to amylase production the results of this study was in contrast with that of Amaro *et al.* (1990) where all *V. cholerae* non-O1 environmental strains were positive for amylase. Simpson *et al.* (1987), who studied O1 and non-O1 *V. cholerae* strains from environmental and clinical sources, found no differences between O1 and non-Ol strains since most of them displayed strong enzymatic and hemolytic

activities. Almost all isolates of non-O1 and non-O139 of *V. cholerae* are reported to produce hemolysins (Amaro *et al.,* 1990; Iyer *et al.,* 2000; Bag *et al.,* 2008). It was found that the hemolytic activities measured were also significantly correlated with lipolysis (Amaro *et al.*, 1990). Nearly 80% and 95% of *V. mimicus* strain isolated from the aquatic environments in Okayama, Japan were found to be positive for hemolytic activity and protease production (Alam *et al.,* 1996). Snoussi *et al.* (2008b) found no correlation between the production of virulence enzymes (lecithinase, amylase, DNase, protease and lipase), β-haemolysis and adherence to epithelial cells. So it is most likely that the ability to utilize a broad range of substrates by *Vibrio* sp. (V26) has more to do with its survival in the environment than its virulence.

As it was difficult to identify the strain *Vibrio* sp. (V26) purely on the basis of phenotypic characteristics, an attempt was made to further identify the strain by partial sequencing of the 16S rRNA gene. 16S rRNA sequencing is considered as the most reliable tool for the allocation of genera, species, and strains into the family *Vibrionaceae* (Thompson *et al.,* 2004a). The results of 16S rRNA partial gene sequence analysis confirmed the results of phenotypic identification that the strain under study belonged to the Genus *Vibrio.* At least 99.3% 16S rRNA similarity is required for a member of *Vibrionaceae* to be designated to a particular species (Kita-Tsukamoto *et al.,* 1993). As in this study, only 98% similarity was obtained, and that too with more than one species i.e., to *V. cholerae,* uncultured *Vibro* clones, *V. mimicus* and *V. albensis* the confirmation of the identity of the strain at species level was not possible. *V. albensis* is described as a biovar of *V. cholerae* whereas *Vibrio mimicus* is a biochemically atypical group of *Vibrio cholerae* strains (Davis *et al.,* 1981). Even on a full-length sequence comparison, *Vibrio mimicus* 16S rRNA gene (rDNA) was found to be nearly identical to those of *Vibrio cholerae*, differing only in 6 out of 1,456 nucleotides (Ruimy *et al.,* 1994), this could explain the similarity obtained in this study. Based on biochemical and molecular studies,

the strain may be grouped as a non toxigenic *V. cholerae,* as maximum strike rate was obtained to this group. However, a complete sequencing of 16S rRNA gene or use of the technique Multilocus sequence typing MLST is necessary for 100% confirmation.

For considering an organism for commercial application, it should not be pathogenic. To find out whether the protease producer strain is a pathogen or not, various putative virulence traits of the strain was studied. As pathogenicity is contributed by a combination of factors; the virulence association gene profile, hydrophobicity, adherence pattern and antibiogram of *Vibrio* sp. (V26) were investigated.

Serogrouping revealed that the strain belonged to a non-O1 serogroup suggesting that the strain is of environmental origin. Saravanan *et al.* (2007) have observed that majority of *V. cholerae* present in sea food and its environment in Manglore, India are of non-O1 serogroup. Non-O1 *V. cholerae* is an autochthonous micro flora of aquatic environment and their presence is not related to fecal contamination (Saravanan *et al.,* 2007). Over 200 'O' serogroups of *V. cholerae* are reported (Yamai *et al.,* 1997) of which only O1 and O139 serogroups are toxigenic and cause the devastating watery diarrhea, called cholera.

In this study the strain *Vibrio* sp. (V26) was found to lack the genes involved in toxin production, *ctxA* and *zot.* Similar observations were made by several workers among different environmental *Vibrio* strains. (Iyer *et al.,* 2000; Sechi *et al.* 2000; Karunasagar *et al.,* 2003; Bag *et al.,* 2008). While Rivera *et al.* (2001) observed that some environmental strains of non-O1, non-O139 *V. cholerae* possess the *ctx* gene, but not *zot* whereas the vice versa was reported from environmental *V. cholerae* by other workers (Karasawa *et al.,* 1993., Chowdhury *et al.,* 1994; Ghosh *et al.,* 1997). It is interesting to note that *ctxA* and/or *zot* have been reported even among environmental isolates of *Vibrio alginolyticus* strains (Snoussi *et al.,* 2008b). Cholera enterotoxin (CT)

is an important virulence marker (Finkelstein, 1988), while *zot is* another toxin gene, whose product (ZOT) increases the permeability of small intestinal mucosa by affecting the structure of intracellular tight junction (zonula occludens) (Fasano *et al.,* 1991). The absence of *ctxA* gene and *zot* (Fig.2.3) in *Vibrio* sp. (V26) indicated its non-pathogenic nature (Levine *et. al,* 1982).

The strain *Vibrio* sp. (V26) also lacked the *tcp*A gene of both the Classical and El Tor type. Contrary to this finding, there are some reports available on *tcp*A positive non-O1/non-O139 environmental isolates (Ghosh *et al.,* 1997; Rivera *et al.,* 2001). The absence of the *tcp*A gene in this strain gains significance in the light of the facts that TCP is the only colonizing factor of *V. cholerae* whose importance in human disease has been proven (Kaper *et al.,* 1995) and that it also acts as receptor for the phage CTXΦ (Levin and Tauxe, 1996; Waldor and Mekalanos, 1996). Volunteer studies carried out by Herrigton *et al.* (1988) showed that the intestinal colonizing factor was abolished when the gene *tcpA* was inactivated. This and the finding that the production of high levels HA/P by certain *V. cholerae* may function to protect *V. cholerae* from infection by CTXΦ (Kimsey and Waldor, 1998) points to the inability of *tcp*A negative *Vibrio* sp. (V26) to acquire CTXΦ by horizontal gene transfer and colonize intestine. Karunasagar *et al.* (2003) in their study too have inferred that strains of *V. cholerae* lacking *ctxA, zot and tcpA* are less likely to be toxigenic.

Vibrio sp. (V26) is positive for *hlyA* (both 481 bp and 738 bp amplified fragments). A dual amplification fragment pattern in the case of *hlyA* has been reported among E1 Tor biotypes and even among non-toxigenic *V. cholerae* O1 and *V. cholerae* O139 strains and occasionally, a larger fragment (1.4 kb) was observed in the El Tor biotype strains (Rivera *et al.,* 2001). The presence of the *hlyA* gene in non - O1 *Vibrio* sp. (V26) is consistent with earlier reports that *hlyA* gene is present in the majority of non-O1 / non-O139 *V. cholerae* isolates (Yamamoto *et al.,* 1986; Kaper *et al.,* 1995; Rivera *et al.,* 2001). Kaper

et al. (1995) found no correlation between with the presence of *hly* gene and *ctx* sequences. Levine *et al.* (1988) in their study showed that cytolysin/hemolysin is not the probable cause of diarrhea. The presence of *hlyA* gene alone cannot make the isolate *Vibrio* sp. (V26) pathogenic.

Investigations made by Nakasone and Iwanaga (1998) on the outer membrane protein OmpU suggested that it was not involved in adhesion of *V. cholerae* to the intestinal epithelium and that the *ompU* gene product has more of a physiological role (Rivera *et al.,* 2001). These porins are also believed to contribute to *V. cholerae*'s resistance to bile, anionic detergents (Provenzano *et al.,* 2001) and antimicrobial peptides (Mathur and Waldor, 2004). The strain under study was found to carry this gene.

Vibrio sp. (V26) was also positive for *toxR* that codes for ToxR. This is consistent with the previous studies (Ghosh *et al.,* 1997; Rivera *et al.,* 2001) where *toxR* was detected in all *V. cholerae* studied. The operon toxR was found even in nine out of 28 *V. alginolyticus* strains (Snoussi *et al.,* 2008b). ToxR a transmembrane protein is the "master switch" that controls expression of *ctxA*, TCP colonizing factor, *ompU* and at least 17 distinct genes (Miller *et al.,* 1987; Peterson *et al.,* 1988; DRita, 1992). In nonpathogenic strains, the ToxR protein controls only the biosynthesis of the outer membrane proteins OmpU and OmpT unlike in pathogenic strains where it is involved in the regulation of the virulence genes (Smirnova *et al.,* 2007). As *Vibrio* sp. (V26) is devoid of *ctxA* and *tcpA* the presence of *toxR* alone is unlikely to contribute to pathogenicity.

It is interesting to note that the virulence gene profile of *Vibrio* sp. (V26) (***ctxA*** $^{-}$ ***zot*** $^{-}$ ***tcp A***$^{-}$ ***hlyAET*** $^{+}$ ***ompU*** $^{+}$ ***tox R***$^{+}$ **)** matches with that of various non-O1 environmental isolates recorded previously from various parts of India (Karunasagar *et al.,* 2003; Bag *et al.,* 2008) and the world over (Iyer *et al.,* 2000; Sechi *et al.,* 2000). From the overall gene profile of *Vibrio* sp. (V26) it is evident that the strain is non-toxigenic as the majority of *V. cholerae*

strains in the environment are considered to be harmless estuarine microorganisms. (Rivera *et al.,* 2001)

The adherence properties of *Vibrio* spp. are a key factor of pathogenicity (Olafsen, 2001). This process is mediated by nonspecific (mainly hydrophobic) and specific (binding of the bacterial cells with its receptor on the epithelial cell) interactions with the host cells (Duguid and Old, 1980; Kabir and Ali, 1983).

HEp-2, CaCo-2, (human colon carcinoma cells) and Intestine 407 are few of the epithelial cell lines that are often used to investigate the property of adherence among *Vibrio* species. While in the case of fish pathogens like *V. fluvialis* adhesion to fish mucus has been looked into (Qingpi *et al.,* 2010). The strain *Vibrio* sp. (V26) exhibited weak adherence to HEp-2 cell lines and the pattern was diffuse. The weak adherence of this strain could be correlated with its non-hyrophobic nature and also the absence of *tcpA* gene. Barbieri *et al*. (1999) found that among the *Vibrio* strains recovered from the Foglia and Metauro estuaries along Italian Adriatic Coast, only 35.5% and 66.7% of the strains respectively were able to adhere to HEp-2 cells. Nearly 43% of *Vibrio mimicus* that was able to adhere to intestinal mucosa, was classified under the weak adherence group by Alam *et al.* (1996). Baffone *et al*. (2005) reported of both weak and moderate adhesion types among the strains of halophilic *Vibrio* spp isolated from seawater to CaCo-2 and HEp-2 cells. Only diffuse pattern was detected among them. While in another investigation all the adhesion types weak, moderate and strong were observed among the environmental isolates of *V. alginolyticus* (Snoussi *et al.,* 2008a). Baffone *et al.,* (2006) noted that all *V. parahaemolyticus* and *V. vulnificus* strains and 20/30 *Vibrio alginolyticus* isolates adhered to Caco-2 monolayers but with different efficiencies, depending on strains.

The ability to attach to different substrata is important for the survival of Vibrios in aquatic environments and it is not exclusively a virulence trait.

Studies have shown that adherence often of the weak type, is exhibited quite widely among environmental isolates of Vibrios (Datta-Roy *et al.,* 1989). In the present study also the strain exhibited weak adherence. This is could be a trait necessary for its existance in the aquatic environment. The study on the adherence property of *V. vulnificus* to HEp-2 cells demonstrated a correlation between attachment and the isolate source (Gandert and Larocco, 1989). However this is in contrast to observations of Datta-roy *et al*. (1989) and Carvalho *et al*. (1994) who found that adhesiveness could not distinguish the source of the strains.

The physicochemical properties (hydrophobic character) of the bacterial surface are important in host-parasite interactions. Hydrophobic interaction is likely to provide the driving force for host-parasite interaction through the displacement of water and formation of adhesive bonds (Lachica and Zink, 1984). As a single assay cannot be a reliable indication of the surface hydrophobicity of different bacterial isolates (Lee and Yii, 1996), two different methods of hydrophobicity assay were adopted in this study. Precipitation of cells by salts such as ammonium sulphate forms the basis of SAT assay (Lindahl *et al.,* 1981). SAT assay is one of the most useful assays to test the attachment ability of the potential pathogen (Amaro *et al.,* 1990). In this assay the order in which the cells are precipitated acts as measure of their surface hydrophobicity with the most hydrophobic being precipitated at the low salt concentrations (Lee and Yii, 1996). In the present study the strain *Vibrio* sp. (V26) was found to be non hydrophobic as no precipitation was observed upto 4 mol/L. Amaro *et al.* (1990) too have used SAT assay for assessing hydrophobicity of environmental non-O1 *Vibrio cholerae* strains isolated from Albufera Lake and they found 23.6% were non-hydrophobic. While the BATH assay using the hydrocarbon ρ-xylene revealed a value of 14.04 % for the strain *Vibrio* sp. (V26) which too indicated the strain was non-hydrophobic. Only an adherence value of ≥35 % to hydrocarbon is regarded as strong. A higher

percentage of hydrophobicity was observed for *V. cholerae* by Zampini *et al*. (2005). Other hydrocarbons like n-hexane or n-octane are also used in BATH assay (Lee and Yii, 1996). This lack of surface hydrophobicity could probably explain the weak adherence exhibited by the strain and also clearly indicating a correlation between these two traits. In this investigation, results obtained by SAT and BATH assays were concurrent, but some investigators have found a lack of correlation between different hydrophobicity assays employed in their study (Dillon *et al.,* 1986; Lee and Yii, 1996). A lack of direct relationship between cell surface properties and virulence of bacterial fish pathogens has been reported (Santos *et al.,* 1991) which is contradictory to the observation made in this study. The growth conditions of the cultures have profound influence on cell surface hydrophobicity (Kabir and Ali 1983; Amaro *et al.,* 1990). The hydrophobicity was accentuated when the strains were cultivated in a chemically defined synthetic medium, harvested at the exponential phase of growth, and cultured at a lower temperature. Rough strains were more hydrophobic than smooth strains (Kabir and Ali, 1983).

The drug resistance patterns of the clinical and environmental strains show remarkable differences, with the clinical strains being resistant to more drugs and exhibiting multidrug resistance unlike their environmental counterparts *(*Charkraborty *et al.,* 2001). *Vibrio* sp. (V26) an environmental isolate was found to be sensitive to most of the antibiotics tested, similar to the observation made among 15 non-O1 and non-O139 strains isolated in Cordoba, Argentina during the period 1991-96 (Bidinost *et al.,* 2004). In this study the strain *Vibrio* sp. (V26) exhibited intermediate sensitivity to ampicillin. A resistance to cefrodoxime, a third generation cephalosporin was noted, while at the same time the strain was sensitive to another third generation cephalosporin Ceftazidime. Resistance to ampicillin among environmental non-O1/non-O139 sergroups isolated from aquatic biotopes in Kerala has been observed earlier (Kumar *et al.,* 2008). Strong resistance to β-

lactam family of antibiotics has been reported among Vibrios (Wang *et al.,* 2006). Sengupta *et al.* (1992) observed that unlike other gram-negative enteric bacteria, *Vibrio cholerae* cells were equally susceptible to penicillin and ampicillin and in general more susceptible than *Escherichia coli* to most of the beta-lactam antibiotics. A fluctuation test indicated that this resistance might be due to adaptive mutation. Cells resistant to a beta-lactam antibiotic exhibited broad cross-resistance to other beta-lactam antibiotics. Though an increase in resistance towards to fluroquinones have been reported in India (Garg *et al.,* 2001; Krishna *et al.,* 2006; Chander *et al.,* 2009) several reports conflicting these observations are also available (Goel *et al.,* 2010; Kingston *et al.,* 2009a). The antibiogram of *V. cholerae* isolated in North India over the period of nine years (Chander *et al.,* 2009) showed that isolates were consistently sensitive to gentamicin and cefotaxime similar to the observation made in this study. Even among *V. mimicus* strains, a very high percentage was found to be sensitive to gentamicin (Chowdhury *et al.,* 1986, 1989). An O1 El Tor *V. cholerae* strain isolated from the stool sample of a patient in Piravam, Ernakulam district Kerala showed resistance to polymyxin B, trimethoprim, nalidixic acid, co-trimoxazole, ampicillin and streptomycin. Additionally it showed intermediate resistance to chloramphenicol, norfloxacin and cephataxime (Thomas *et al.,* 2008), whereas the strain used in this study was susceptible to all these antibiotics except ampicillin. An overview of the antibiotic sensitivity patterns indicates a great degree of resistance to the drugs such as furazolidone, co-trimoxazole, naldixic acid, polymyxin B, ampicillin, trimethoprim and sulphamethozole among *Vibrio cholerae* isolates (Bag *et al.,* 2008; Kingston *et al.,* 2009a, 2009b; Goel *et al.,* 2010). Antibiotics norfloxacin, ciprofloxacin, tetracycline and doxycycline are widely used in the treatment of watery diarrhea (Kingston *et al.,* 2009b) and multi-drug resistance among *V. cholerae* strains limits the therapeutic potential and is also of great public health concern. It is favorable that the strain *Vibrio* sp. (V26) is susceptible to all these drugs used in the treatment of cholera.

Though precise species level identification of the producer strain could not be done, the various studies conducted on *Vibrio* sp. (V26) such as presence of toxin genes, adherence and surface hydrophobicity, antibiotic susceptibility revealed that the strain is a non-toxigenic and a non-pathogenic environmental isolate.

Chapter 3

Purification and Characterization of Alkaline Protease from *Vibrio* sp. (V26)

Contents

Purification of protease is very important for developing a better understanding of the functioning of the enzyme. Strategies adopted for purification of enzymes are on similar lines as that of proteins. Despite the diversity in the origin of enzymes they are purified using a generalized overall approach, which involves initial recovery of protein, concentration / primary purification and finally high end resolution chromatographic purification (Walsh, 2004). At each stage of purification, an assay for the enzyme being

purified is performed on all fractions and the total protein is also determined (Palmer and Bonner, 2008). The exact purification scheme for any given protein depends on factors such as the source material chosen and location of the target protein (extra/intracellular), level of expression, physiochemical characteristics of protein and purpose of purification. However there are no set rules for the purification of proteases (Gupta *et al.,* 2002 b).

The first step of any purification procedure involves recovery of enzyme from its source- the complexity of this step depends on whether it is intra or extracellular. Microbial enzymes are mostly extracellular and are released into the fermentation media; in such cases the separation of whole cells from the media is generally carried out by centrifugation or in some cases by filtration. While in the case of intracellular microbial enzymes; steps such as appropriate cell harvesting and disruption techniques are adopted (Walsh, 2004).

As the enzyme of interest is usually present in the cell-free supernatant in very dilute concentrations, removal of water becomes necessary. The concentration of process liquids makes the volume manageable for subsequent purification steps (Walsh, 2004). Ultrafiltration is now being largely used for concentration as an alterative to evaporation. This pressure driven process is inexpensive, results in little loss of enzyme activity and offers both purification and concentration (Sullivan *et al.,* 1984). Diafiltration is also used for salt removal or for changing salt composition (Manachini *et al.,* 1988; Peek *et al.,* 1992). One drawback of this technique is its susceptibility to fouling or clogging of membrane (Walsh, 2004).

Concentration by precipitation is one of the oldest concentration methods known. Protein precipitation can be promoted by agents such as neutral salts, organic solvents, and high molecular mass polymers or by appropriate pH adjustments. Organic solvents and neutral salts (ammonium sulphate), which lowers the solubility of the desired proteins in an aqueous solution are the usual agents employed for precipitation (Kumar and Takagi,

1999; Walsh, 2004). Organic solvents frequently used to promote precipitation include, ethanol and acetone.

To further purify the enzyme a combination of one or more chromatographic techniques are applied viz. affinity chromatography (AC), Ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and gel filtration chromatography.

Once purified, most enzymes are subjected to a battery of characterization studies which include functional characteristics, evidence of purity, structural studies (molecular mass, amino acid composition, amino acid sequencing, analysis of secondary / tertiary / quaternary structure) etc., (Walsh, 2004). The properties of the enzyme identified during these studies help in determining the areas of its possible application.

Even though, extensive investigations on the enzymatic and physiochemical properties of alkaline protease from the genus *Bacillus* has been carried out, only very little data are available on the characterization and applications of proteases from the genus *Vibrio.*

As the recovery costs of enzymes are nearly 70% of the total manufacturing costs (Atkinson and Mavituna, 1991) it is necessary to identify the characteristics of an enzyme to determine whether it has the potential for being adopted as a commercial enzyme.

The protease from *Vibrio* sp. (V26) has been purified and its characteristics studied, with the aim of understanding the properties of the enzyme and assessing its worthiness as a commercial enzyme. The characterization of the gene coding for protease has also been carried out.

3.1 Review of Literature

Extensive studies have been carried out on the purification and characterization of alkaline proteases from microbes especially bacteria. Strategies adopted are seen to vary with the workers.

3.1.1 Concentration of the enzyme

Despite the availability of rapid and easy techniques such as ultrafiltration for concentration of enzymes; a review of the strategies adopted for the purification of alkaline protease over the last decade (2000-2010) clearly reveals that ammonium sulphate still remains a popular agent for the concentration of protease from microbes (Singh *et al.,* 2001; Lee *et al.,* 2002; Adinarayana *et al.,* 2003; Moreira *et al.,* 2003; Patel *et al.,* 2006; Chellappan *et al.,* 2006; Tremacoldi *et al.,* 2007; Kasana and Yaadav, 2007; Wang *et al.,* 2007; Tanskul *et al.,* 2009; Anita and Rabeeth, 2010, Cheng *et al.,* 2010; Joshi, 2010; Wan *et al.,* 2010). Popularity of ammonium sulphate is due to its high solubility, inexpensiveness, lack of denaturing property towards enzymes and its stabilizing effect on most enzymes. Various concentrations of the organic solvent, acetone such as 60% (Sana *et al.,* 2006), 70% (Arulmani *et al.,* 2007), 60-80% (Hajji *et al.,* 2007) too have been employed for the precipitation of protease from the cell free supernatant. While several other workers have used different volumes of acetone: 2 volumes (Tunga *et al.,* 2003) and 1.5 volumes (Mei and Jiang, 2005). Several workers have adopted ultrafiltration as a method of concentration of alkaline protease (Jellouli *et al.,* 2009; Manikandan *et al.,* 2009; Moreira-Gasparin, 2009).

3.1.2 Chromatography

DEAE-cellulose resin is quite widely employed in the purification of alkaline protease using Ion exchange chromatography (IEC) (Sana *et al.,* 2006; Patel *et al.,* 2006; Chellappan *et al.,* 2006; Arulmani *et al.,* 2007; Jellouli *et al.,* 2009; Tanskul *et al.,* 2009). Some of the improved (cellulose based,

sephadex based or agarose based) ion exchange resins that have been used by workers for purification include CM-Sepharose CL-6B (Kumar *et al.,* 1999; Hajj *et al.,* 2007), DEAE-Sephadex A-50 (Singh *et al.,* 2001), DEAE-Sepharose CL-6B (Wang *et al.,* 2007) and DEAE sephacryl (Almas *et al.,* 2009; Joshi, 2010).

The affinity adsorbents used for alkaline protease purification are casein agarose (Manachini *et al.,* 1988), feather keratin-covalently bound to controlled-pore glass (Grzywnowicz and Łobarzewski,1994), hydroxyapatite (Kobayashi *et al.,* 1996), N-benzoyloxycarbonyl phenylalanine agarose (Larcher *et al.,* 1996), aprotinin-agarose (Petinate *et al.,* 1999), benzamidine-sepharose (Joo *et al.,* 2001), or bacitracin–sepharose (Manikandan *et al.,* 2009). Though this technique is described as the most powerful highly selective method of protein purification available; the high cost of enzyme supports and labile nature of some affinity ligands are the major limitations that makes them un-recommendable for use at process scale (Kumar and Takagi, 1999; Walsh, 2004).

Phenyl sepharose (Lee *et al.,* 2002; Almas *et al.,* 2009; Joshi, 2010) is one of the most commonly used HIC matrixes in alkaline protease purification.

A review of purification strategies indicate that sephadex range (Sephadex G-75, G-100, G-200) of fractionation gels are used widely in the recovery of alkaline protease from microbes (Adinarayana *et al.,* 2003; Moreira *et al.,* 2003; Sana *et al.,* 2006; Patel *et al.,* 2006; Hajji *et al.,* 2007; Kasana and Yadav, 2007; Arulmani *et al.,* 2007; Tremacoldi *et al.,* 2007; Ma *et al.,* 2007; Manikandan *et al.,* 2009; Moreira-Gasparin, 2009; Anita and Rabeeth, 2010; Cheng *et al.,* 2010; Shrinivas and Naik, 2011). Sephacryl based gels such as Sephacryl S-200 (Kumar *et al.*, 1999; Wang *et al.,* 2007) and sepharose range of gels such as Sepharose 6B (Singh *et al.,* 2001) have also been employed by various workers.

In the recent years, Hydrophobic interaction chromatography matrix - Phenyl Sepharose 6 (Karan and Khare, 2010) and Ion exchange matrix - DEAE-Sepharose (Ma *et al.,* 2007; Wan *et al.*, 2010) have been used in fast flow chromatography. FPLC (Fast protein liquid Chromatography) using matrices Superose-12 (gel filtration) (Tunga *et al.,* 2003) and Mono Q (Tanskul *et al.,* 2009) too have been reported.

3.1.3 Other techniques used

Use of chromatographic techniques for purification is rather expensive; consequently, liquid–liquid extraction with a reversed micelle system or an aqueous two-phase system, have been investigated as a less expensive alternative. Extraction of an extracellular alkaline protease from *Nocardiopsis* sp. fermentation broth using reversed micelles of sodium di (2-ethylhexyl) sulfosuccinate (AOT) in isooctane was performed with equal phase volume ratio (Monteiro *et al.,* 2005). Alkaline protease produced by *Bacillus* sp. has been extracted from the fermentation broth using aqueous two-phase systems of polyethylene glycol 1000 (PEG) - potassium phosphate (Chouyyok *et al.,* 2005; Wongmongkol and Prichanont, 2006). A study on the recovery of an extracellular alkaline protease from fermentation broth produced by *Norcadiopsis s*p. was carried out using aqueous two-phase and reversed micelles systems by Porto and co-workers (2005). Aqueous two-phase system (ATPS) of PEG / citrate was used to remove proteases from a *Clostridium perfringens* fermentation broth (Porto *et al.,* 2008). Their results indicate that the aqueous two-phase extraction system was more attractive as a first step in the isolation and purification processes.

The use of activated charcoal for the recovery and purification of alkaline protease has been investigated by Kumar and Parrack (2003).

3.1.4 Characterization studies

The optimum pH range of alkaline proteases is generally found to be between pH 9 and 11 (Kumar and Takagi, 1999) with a few exceptions of higher (12) pH optima (Kumar *et al.,* 1999). The proteases of *Exiguobacterium* sp. SKPB5 a psychrotroph isolated from the soil of western Himalayas and a *Haloferax lucentensis* VKMM 007 a halophilic archaeon from solar saltern exhibited optimal activity at pH 8 (Kasana and Yadav, 2007; Manikandan *et al.,* 2009). The optimum temperature of alkaline protease from microbes generally ranges from 50-70ºC (Kumar *et al.,* 1999; Adinarayana *et al.,* 2003; Wang *et al.,* 2007; Almas *et al.,* 2009; Anita and Rabeeth, 2010; Joshi,2010; Shankar *et al.,* 2011). The alkaline protease from *Bacillus* sp. isolated from the soil of Veraval coast of the Gujarat (India) had a very low temperature optima of 37ºC (Patel *et al.,* 2006) while an unusually high temperature optimum of 75ºC was reported for the protease from *Bacillus laterosporus*-AK1 (Arulmani *et al.,* 2007). The protease of a γ-Proteobacterium isolated from sediments Lothian Island, Sundarbans had optimal activity at pH 9 and 40 ºC (Sana *et al.,* 2006).

Though the molecular mass of alkaline proteases are found in the range 15 to 30 kDa (Fogarty *et al.,* 1974) reports of exceptionally high molecular masses of 43 kDa (Lee *et al.,* 2002), 86.29 kDa (Arulmani *et al.,* 2007), 68 kDa (Anita and Rabeeth, 2010) and 52 kDa (Wan *et al.,* 2010) are available. Protease of an extremely low molecular weight of 8 kDa was reported from *Kurthia spiroforme* (Steele *et al.,* 1992).

Alkaline proteases often require a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations for maximum activity. These cations are believed to protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar and Takagi, 1999). Several workers have reported the role of Ca^{2+} in enzyme stabilization, by increasing the activity and thermal stability of alkaline

protease at higher temperatures (Lee *et al*., 1996; Kumar, 2002; Moradian *et al*., 2009). The inhibitory effect of heavy metals Cu^{2+} and Hg^{2+} on alkaline proteases are widely reported (Vallee and Ulmer, 1972; Johnvesly *et al.,* 2002; Mei and Jiang, 2005; Venugopal and Saramma, 2006). The alkaline protease of *Beauveria* sp. was inhibited by Cd^{2+}, Hg^{2+} and Mn^{2+} (Shankar *et al.,* 2011).

Inhibition studies give insight into the nature of the enzyme, its cofactor requirements and the nature of the active site (Sigma and Moser, 1975). Effect of inhibitors such as phenlymethylsulfonyl fluoride (PMSF), iodo acetic acid (IAA), ethylene-diamine tetraacetic acid (EDTA), 1, 10-phenanthroline and pepstatin on proteases have routinely been studied to determine the class of proteases. Most alkaline proteases from bacteria belong to the class serine proteases (Singh *et al.,* 2001; Lee *et al.,* 2002; Adinarayana *et al.,* 2003; Joo *et al.,* 2004; Sana *et al.,* 2006; Arulmani *et al.,* 2007; Tanskul *et al.,* 2009; Karan and Khare, 2010) while several others are reported to be of the class metalloproteases (Wang *et al.,* 2007; Wan *et al.,* 2010). Some rare reports of alkaline proteases belonging to the class cysteine proteases are also available (Liu *et al.,* 1997; Kasana and Yadav, 2007).

3.1.5 Microbial alkaline proteases

Microbial proteases, especially from *Bacillus* sp. are the most widely exploited industrial enzyme. This could be the reason for *Bacillus*-derived alkaline proteases for having been well documented and characterized. Purification and characterization studies from a wide variety of *Bacillus* species such as *Bacillus thermoruber* (Manachini *et al.,* 1988), *B. subtilis* (Yang *et al.,* 2000; Adinarayana *et al.,* 2003), *B. pumilus* (Kumar, 2002), *B. mojavensis* (Beg and Gupta, 2003), *B. clausii* (Joo and Chang, 2006), *B. laterosporus* (Arulmani *et al.,* 2007), *B. alcalophilus* (Cheng *et al.,* 2010) and *B. firmus* (Joshi, 2010) have been reported. There are also reports on purification of alkaline protease derived from *Bacillus* species isolated from unique environments like alkaline soil (Kumar *et al.,* 1999; Singh *et al.,* 2001),

Korean polychaete *Periserrula leucophryna* (Joo *et al.,* 2004), tannery waste (Almas *et al.,* 2009; Joshi, 2010), bat feces (Tanskul *et al.,* 2009) and slaughter house soil (Anita and Rabeeth, 2010). The alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 isolated from sugarcane molasses exhibiting dehairing activity was characterized by Shrinivas and Naik (2011).

Characterization studies of alkaline protease from Gram negative bacteria are much less compared to Gram positive bacteria. However quite a number of investigations are available on alkaline proteases from *Pseudomonas* species (Gupta *et al.,* 2005b; Najafi *et al.,* 2005). The alkaline protease of an actinomycete, *Nocardiopsis* sp. was characterized by Moreira *et al.* (2003) while the screening, characterization, and cloning of a solvent-tolerant protease from *Serratia marcescens* MH6 a Gram negative motile, catalase-positive bacterium was taken up by Wan *et al.* (2010).

The alkaline proteases from fungi such as *Aspergillus parasiticus, A. clavatus*, *Engyodontium album*, *Myrothecium verrucaria* and *Beauveria* have been purified and characterized (Tunga *et al.*, 2003; Chellappan *et al.,* 2006; Hajji *et al.,* 2007; Tremacoldi *et al.,* 2007; Moreira-Gasparin 2009; Shankar *et al.,* 2011). Ma *et al.* (2007) performed the purification and characterization of an alkaline protease from marine yeast *Aureobasidium pullulans* for bioactive peptide production from different sources.

There are reports on purification and characterization of the enzyme from extremophiles also. Kasana and Yadav (2007) studied the cysteine proteases from psychrotrophic *Exiguobacterium* sp. SKPB5 while the serine alkaline protease from moderately haloalkaliphilic bacterium *Geomicrobium* sp. EMB2 was investigated by Karan and Khare (2010). Manikandan *et al.* (2009) carried out the purification and biological characterization of a halophilic thermostable protease from *Haloferax lucentensis* VKMM 007 an archaeon from solar saltern.

3.1.6 Vibrio protease- Gene, Cloning and Characterization

Various extracellular proteases produced by a number of *Vibrio* species isolated from sea water, fish, and shellfish have been isolated and examined with regard to their enzymatic properties and / or virulence (Liu *et al.,* 1997). Investigations on the purification and / or characterization of *Vibrio* proteases as a putative virulence factor include those from species such as *V. alginolyticus* (Hare *et al.,* 1983*), V. harveyi* (Fukasawa *et al.,* 1988; Liu *et al.,* 1997), *V. mimicus* (Chowdhury *et al.,* 1990), *Vibrio anguillarum* (Farrell and Crosa, 1991), *V. cholerae* (Ichinose *et al.,* 1992), *V. pelagicus* (Farto *et al.,* 2002) and *V. parahaemolyticus* (Lee *et al.,* 2002; Ishihara *et al.,* 2002). However, only very few reports are available on the purification, characterization and evaluation of alkaline proteases from Vibrios for commercial application (Mei and Jiang, 2005; Venugopal and Saramma, 2006; Jellouli *et al.,* 2009).

As a part of the attempt to understand the role and significance of the enzyme protease in Vibrios; researchers have identified, isolated, cloned, sequenced and expressed the protease gene from various *Vibrio* species. The *proA* gene from *V. alginolyticus* (Deane *et al.,* 1989), metalloprotease gene from *V. proteolyticus* (David *et al.,* 1992), *vapT* and *vapK*, gene from *V. metschnikovii* strain RH530 (Kwon *et al.,* 1995; Chung *et al.,* 2001), *vvp* encoding a thermolabile protease from *V. vulnificus* (Cheng *et al.*,1996), *vmc* gene from *V. mimicus* ATCC 33653 (Lee *et al.,* 1998a), *empA* gene from *V. anguillarum* (Chen *et al.,* 2002), *vppC* from *V. parahaemolyticus* 04 (Kim *et al.,* 2002) and a *prtV*-like gene from *V. anguillarum* M3 strain (Mo *et al.,* 2010) have been cloned and sequenced. Hase and Finkelstein (1991) have cloned and sequenced the *Vibrio cholerae* hemagglutinin / protease (HA/ protease) gene and also constructed a HA/protease-negative strain. Chung *et al.* (2001) and Cai *et al.* (2007) have successfully cloned and expressed alkaline protease genes of *V. metschnikovii* strain RH530 and *V. alginolyticus* in *Escherichia coli*.

Many extracellular bacterial proteases play an important role in virulence of the organism (Lee *et al.,* 2002). Their role in virulence has been identified in the genus Vibrios as well. Therefore toxicity studies (in-vivo and/or in-vitro toxicity) have been included as an integral part of the characterization of proteases from several of the *Vibrio* species such as *V. alginolyticus* (Nottage and Birkbeck, 1987 a, 1987b; Lee, 1995; Cai *et al.,* 2007), *V. parahaemolyticus* (Ishihara *et al.,* 2002; Lee *et al.,* 2002), *Vibrio cholera* (Vaitkevicius *et al.,* 2008) and *V. anguillarum* (Mo *et al.,* 2010). Cytotoxic effects of certain recombinant enzymes like PrtV (recombinant collagenase) on mammalian cells and rVMC61 (*V. mimicus* metalloprotease) on fish cell lines CHSE-214 have also been investigated (Yu *et al.,* 2000; Lee *et al.,* 2003b). Vaitkevicius *et al.* (2006) have established *Caenorhabditis elegans* as a useful model system for identifying and assessing factors other than CT from *V. cholerae* that may be important for pathogenesis related studies.

The ability of the proteases from Vibrios to agglutinate a diverse range of erythrocytes has been reported by several workers (Finkelstein and Hanne, 1982; Honda *et al.,* 1989; Chowdhury *et al.,* 1990). In the case of *V. cholerae,* its protease has been described as a bifunctional molecule exhibiting the characteristics of both a proteolytic enzyme and a hemmagglutinin; therefore this property of hemagglutination of protease too has been investigated as a part of characterization studies of this enzyme (Finkelstein and Hanne, 1982; Young and Broadbent, 1982; Ichinose *et al.,* 1992). The contribution of HapA (hemmagglutinin protease) to pathogensis have been investigated using *hapA* mutants and based on these HapA is considered as an important virulence factor (Benitez *et al.,* 1999; Garcia *et al.,* 2005; Silva *et al.,* 2006).

3.2 Materials and Methods

3.2.1 Alkaline protease production

3.2.1.1 Organism Used

The selected strain *Vibro* sp. (V26) was used for this study.

3.2.1.2 Medium Used

Nutrient broth supplemented with 1% gelatin was used for alkaline protease production. Nutrient broth (50 ml) with gelatin was prepared in 250 ml flasks and sterilized at 121ºC for 15 minutes in an autoclave. The pH was adjusted to 8 as it was most ideal for the strain under study (Venugopal, 2004).

Composition of Nutrient Broth supplemented with gelatin

Ingredients	Concentration
Peptone	5 g
Beef extract	1.5 g
Yeast extract	1.5 g
Gelatin	10 g
NaCl	5 g
Distilled water	1000 ml
pH	8

3.2.1.3 Preparation of inoculum

The selected strain *Vibrio* sp. (V26) was inoculated onto nutrient agar slant and incubated at 28ºC. From the slant, a loop full of culture was inoculated into nutrient broth supplemented with 1% gelatin. This was treated as the pre-inoculum or mother culture. The inoculated, pre- inoculum culture flask was incubated in a rotary shaker at 30ºC at 130 rpm overnight (18 hrs).

Composition of Nurient agar

Ingredients	Concentration
Peptone	5 g
Beef extract	1.5 g
Yeast extract	1.5 g
NaCl	5 g
Agar	20 g
Distilled water	1000 ml
pH	8

3.2.1.4 Inoculation and Protease Production

The Optical density (O.D) of the pre-inoculum culture was read at 600 nm in a UV-VIS spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan). A volume adequate to obtain an absorbance of 0.02 at 600 nm for the total medium was added to 50 ml production broth (nutrient broth supplemented with gelatin) in a 250 ml conical flask (1O.D= 3.85 x 10^{10} cells / ml).

Inoculated production flasks were incubated on a rotary shaker at 30ºC at 130 rpm for 48 hrs. The cell free supernatant was recovered by centrifugation (8000 x g, 4ºC, 15 minutes) and was assayed for total and specific protease activity. This cell free supernatant was used as the crude enzyme. The pH of selected media, the inoculum size, the temperature and shaking speed were selected on the basis of the previous study (Venugopal, 2004).

3.2.2 Enzyme and Protein Assays

3.2.2.1 Assay of protease activity

Protease activity was measured by the modified method of Kembhavi *et al.* (1993) using casein as substrate. 500 µl of suitably diluted enzyme was added to 500 µl of 1% casein (Hammerstein casein, SRL) prepared in 100 mM Tris-Cl buffer (pH 9) and incubated at 60ºC for 30 minutes. The reaction was stopped by the addition of 500 µl of 20% tricholoroacetic acid (TCA). The

mixture was allowed to stand for 15 minutes at room temperature and then centrifuged at 8000 x g for 15 minutes. The absorbance of the supernatant was measured at 280 nm spectrophotometrically (UV-1601, Shimadzu Corporation, Tokyo, Japan). Control consisted of reaction mixture to which the enzyme was added after the reaction was stopped by addition of TCA. A standard curve was generated using tyrosine as standard (50-250 µg/ml). One unit (U) of protease activity is defined as the amount of enzyme required to liberate 1µg tyrosine per millilitre per minute under the standard assay conditions.

3.2.2.2 Assay for protein determination

Protein content was measured by the method of Hartree-Lowry (1972) with Bovine serum albumin (BSA) as the standard.

3.2.2.3 Specific activity

Specific activity of the sample was calculated by dividing the enzyme units (U) with the protein content.

$$\text{Specific activity} = \frac{\text{Total enzyme units (U)}}{\text{Total protein (mg/ml)}}$$

The activity of the sample is expressed in units (U) and wherever necessary as specific activity (U/mg).

3.2.2.4 Relative activity

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

$$\text{Relative activity} = \frac{\text{Activity of sample (U) x 100}}{\text{Maximum enzyme activity (U)}}$$

3.2.2.5 Residual activity

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

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

3.2.3 Purification of enzyme

The protease from *Vibrio* sp. (V26) was purified as per the standard protein purification procedures which involved various steps such as centrifugation, ammonium sulphate precipitation, diafiltration and ion exchange chromatography.

3.2.3.1 Ammonium sulphate precipitation

To the chilled crude enzyme, solid ammonium sulphate (40-80 % saturation) was added as per standard chart (Green and Hughes, 1955) to precipitate out the enzyme. Precipitation was done at 4ºC. The precipitate obtained was collected by centrifugation (8000 x g at 4ºC for 15 minutes) and dissolved in minimum quantity of Tris-Cl buffer (pH 8.5). This preparation was treated as partially purified enzyme.

3.2.3.2 Diafiltration

The partially purified enzyme was diafiltered using Amicon UF Stirred Cell (Model 8010) with 10 KDa cut off membrane against Tris-Cl buffer (pH 8.5). This was done to remove ammonium sulphate. The sample was concentrated to one third its original volume in the same stirred cell unit.

3.2.3.3 DEAE-cellulose Ion exchange chromatography

DEAE-cellulose was purchased from Sigma and activated as per manufacturer's instructions. The resin was packed into C 10/20 column **(**AKTA prime, Amersham). Care was taken to avoid trapping of air bubbles. All the buffers used were filtered and degassed before each run. The column was pre-equilibrated with the 20 mM Tris-Cl buffer, pH 8.5.

One ml of the sample was loaded onto the pre-equlibrated column. The column was then washed with the same buffer (20 mM Tris-Cl buffer, pH 8.5) to remove the unbound proteins (indicated by zero absorbance at 280 nm). The bound protein was eluted by applying a linear gradient of 0-0.8 M NaCl in the same buffer at a flow rate of 0.5 ml/minute and monitored at 280 nm. 1 ml

fractions were collected and the peak protein fractions were analyzed for protease activity. The active fractions were pooled, assayed for protease activity (section ***3.2.2.1***) and protein content (section ***3.2.2.2***) and used for further characterization studies.

3.2.4 Determination of molecular weight of the enzyme

3.2.4.1 Sample preparation

Enzyme sample collected at each stage of purification (crude, ammonium sulphate and ion exchange chromatography) was subjected to SDS PAGE. The enzyme samples (crude, ammonium sulphate fraction and purified enzyme) and the broad range molecular weight markers (Genei, India) were treated with sample buffer (0.125 M Tris-Cl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue, pH-6.8). The sample and marker tubes were then placed in boiling water bath for 1½ minutes. They were cooled to room temperature.

3.2.4.2 Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS PAGE)

One dimensional SDS–PAGE was carried out for determination of molecular mass of the protease in a 4 % stacking gel (pH-6.8) and 10 % resolving gel (pH- 8.8) according to the method of Laemmli (1970). Electrophoresis was carried out at a constant current of 12 mA.

The treated sample (containing 50-100 µg of the protein) were loaded into the wells and electrophoresis carried out in a Hoefer^{TM} miniVE vertical electrophoresis system (Amersham BioSciences, Sweden) until the dye front reached the bottom of the gel.

After electrophoresis, gel was carefully removed from between the glass plates and stained with 0.025% Coomassie Brilliant Blue R-250 (methanol 40%, acetic acid 7%, Coomassie Brilliant Blue, 0.025%) and then destained initially with destain solution I (40% methanol, 7% acetic acid) for 30 minutes

followed by destain solution II (5% methanol, 7% acetic acid) until the band became clear.

Documentation and analysis of the gels were done by using Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad) and molecular weight of the protease was determined.

3.2.5 Zymogram/ Activity staining

Casein zymography was carried out according to the methods previously described by Kim *et al.* (1998) with slight modifications. Casein (0.12% w/v) was dissolved in 20 mM Tris-Cl buffer (pH 8.5) and co-polymerized with 10 % resolving gel. Samples were prepared by diluting the enzyme in zymogram buffer (0.125 M Tris-Cl, 2% SDS, 10% glycerol, 0.02% bromophenol blue, pH-6.8). The samples were then loaded into wells and electrophoresed at a constant current of 12 mA at 4ºC. After electrophoresis, the gel was incubated for 30 minutes at room temperature in reactivation buffer (100 mM Tris-Cl buffer, pH 9) containing 2.5% (v/v) Triton X-100. The gel was then washed with distilled water to remove Triton X-100, incubated in reaction buffer (Tris-Cl, pH 9) for 30 minutes at 37ºC, stained with Coomassie Brilliant Blue for 30 minutes and destained as described in Section ***3.2.4.2***. The protease activity was detected as clear colourless zone against dark blue background.

3.2.6 Characterization of enzyme

3.2.6.1 Effect of pH on enzyme activity and stability

The effect of pH on protease activity was evaluated over a pH range 7-12, using different buffers such as Sodium phosphate 0.1 M (pH 7), 0.1 M Tris-Cl (pH 8-9) and 0.1 M Glycine NaOH (pH 11-12) in the reaction mixture. The activity of the sample was expressed in terms of relative activity calculated as per section ***3.2.2.4***.

Stability of the enzyme at various pH was studied by pre-incubating the enzyme in buffers of different pH (7-12) for 1 hr and the residual enzyme activity (%) was measured. The percentage residual activity was calculated (section ***3.2.2.5***) by comparing the activity of treated enzyme with that of the untreated enzyme (control), which is taken as 100%.

3.2.6.2 Effect of temperature on the enzyme activity and stability

The effect of temperature on the enzyme activity was assessed by carrying out the assay at different temperatures from 30-80ºC. The percentage relative activity was calculated (section ***3.2.2.4***) considering the activity at 60ºC as 100%.

The temperature stability of the enzyme was determined by pre-incubating the enzyme at different temperatures (30-80ºC) for one hour and then assaying the residual activity (%) under the standard assay conditions. Residual activity was calculated as per section ***3.2.2.5*** .The activity of untreated enzyme (control) is taken as 100%.

3.2.6.3 Effect of metal ions and inhibitors on enzyme activity

The influence of various metal ions on the purified enzyme was studied by incubating the enzyme in the presence of various metal ions ($ZnCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $PbCl_2$, $CoCl_2$, $HgCl_2$, $BaCl_2$, and $CuSO_4$) at final concentration of 1mM and 5 mM at 60ºC for 30 minutes. The percentage relative activity was calculated (section ***3.2.2.4***) by considering the activity of enzyme (in absence of metal ions) at 60ºC and pH9 as 100% activity.

To study the effect of different protease inhibitors on the purified enzyme, aliquots of enzymes were pre-incubated with the different enzyme inhibitors such as phenylmethylsulphonyl fluoride (PMSF) (5 mM), iodo acetic acid (IAA) (1mM), ethylene-diamine tetraacetic acid (EDTA) (5 mM) and 1, 10 phenanthroline (5 mM) for 30 minutes at room temperature. Residual

activities (%) were measured and calculated (section ***3.2.2.5***). Suitable control was placed (without inhibitors).

3.2.6.4 Effect of surfactants and oxidizing agent

To investigate the effect of oxidizing agent (H_2O_2) and surfactants (SDS, Triton X-100 and Tween-80) on the enzyme stability, the purified protease was pre-incubated with different concentrations of H_2O_2 (0.1 and 0.5 %), SDS (0.1 and 0.5%), and Tween-80 (0.5 and 1 %) for 30 minutes and then their residual activities were measured by standard procedure (section ***3.2.2.5***).

3.2.6.5 Hemagglutination assay

The hemagglutinating activity was assayed using human (O type) and chick erythrocytes. The cells (RBCs) were washed twice in Alsever's solution (2.05 % dextrose, 0.8 % sodium citrate, 0.42 % sodium chloride and 0.05 % citric acid in distilled water) and resuspended in fresh Alsever's solution to prepare a 5 % (v/v) suspension of erythrocytes. Two fold serial dilutions of the purified alkaline protease and ammonium sulphate fraction in Alsever's solution were made in round bottomed microtitre plates, and aliquots of equal volume (25 µl) of 5 % (v/v) suspension of erythrocytes were added and mixed. After 45 minutes of incubation at room temperature (28 ± 2ºC) the extent of agglutination was examined and reported in terms of hemagglutination titre, the highest dilution at which agglutination was visible.

3.2.6.6 Cytotoxicity of *Vibrio* sp. (V26) protease

HEp-2 cells were seeded into 96 well plate (Greiner Bio-One) at a density of approximately $1x10^5$ cells/ml and cultured for 24 hours at 37ºC in Eagle's MEM (Minimal Essential Media, Himedia) with 2 mM glutamine, 1.5 g/l sodium bicarbonate and 10% fetal bovine serum (FBS). Different concentrations of the purified protease 5, 10, 50, 100, 250, 500, 1000 U were added to the wells. Triplicates were kept for each concentration. After 14 hours incubation MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium

bromide) assay was carried out. Percentage of cells inhibited at each concentration of protease was calculated.

For MTT assay, after replacing the media in the wells, 50 µl MTT (Sigma) solution (5 mg/ml in PBS (720 mOsm)) was added to each well and incubated for 5 hours in dark. MTT was added to the control wells with the medium alone. After 5 hours of incubation the medium was removed and MTT-formazan crystals were dissolved in 200 µl dimethylsulfoxide. Absorbance was recorded immediately at 570 nm in microplate reader (TECAN Infinite Tm, Austria). Probit analysis was done with SPSS software package.

3.2.7 Comparison of *Vibrio* sp. (V26) alkaline protease with a commercial alkaline protease Savinase ® (P3111)

The specific activity of the purified protease from *Vibrio* sp. (V26) was compared with the alkaline protease SAVINASE ® (P3111, Sigma Co; USA) from *Bacillus* sp. under the standard assay conditions (pH 9 and temperature 60ºC).

3.2.8 Protease gene Identification

Primers designed previously (Milton *et al.*, 1992) were used to amplify the zinc binding conserved region of the metalloprotease. Primer of the following sequence was used:

F-5'-CTCGAGCTCTAGACATGAGGTCAGCCACGGTTTTACTGAGCAG-3'

R-5'-CTCGATATCGATCGCGCGGTTAAACACGCCACTCGAATGGTGAAC-3'

The amplification was carried out in a thermal cycler (Master Cycler, Eppendorf) which involved initial denaturation at 95ºC for 5 minutes followed by 30 cycles of (94ºC for 20 sec, 55ºC for 20 sec, 72ºC for 1 minute) and final extension at 72ºC for 5 minutes. The amplified products were separated on 1% agarose gel and stained with ethidium bromide. The amplified product was purified and sequenced at Xcleris (India). The sequence homology and

deduced amino acid sequence comparisons were carried out using BLAST algorithm at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nim.nih.gov/blast). Gene translation and prediction of protein were performed with ExPASy (http://www.au.expasy.org/). The multiple sequence alignments were performed on amino acid sequences of known metallo proteases from bacteria using CLUSTALW computer program (Thompson *et al*., 1994). Amino acid sequences of bacterial metallo proteases were retrieved from the NCBI GenBAnk and phylogenetic tree was constructed by Neighbor –Joining (NJ) method based on these amino acid sequences (Saitou and Nei, 1987). Phylogenetic tree was constructed using MEGA version 4.0 (Tamura *et al.,* 2007).

3.2.9 Statistical Analysis

Data generated from the above experiments were analyzed using one-way Analysis of Variance (ANOVA) with post-hoc multiple comparison analysis performed using Tukey's HSD. Mean of the results was compared using SPSS 13.0 package for windows at a significance level of $p< 0.05$. Data are presented as mean ±standard deviation (SD).

3.3 Results

3.3.1 Purification of the enzyme

The cell free supernatant of the culture broth of *Vibrio* sp. (V26) was used as the source of the crude enzyme. The protease was purified to homogeneity by ammonium sulphate precipitation, diafiltration followed by DEAE-cellulose ion exchange chromatography. The results of the purification procedure are summarized in Table 3.1. 40-80% ammonium sulphate saturation fraction that exhibited highest activity was diafiltered and concentrated (specific acitivity-3130.48 U/mg). At this point 2.6 fold purification of the protease was attained. The concentrated enzyme was successively subjected to ion exchange chromatography on DEAE-cellulose

column. The elution profile (Fig.3.1) revealed one minor (fractions 2-4) and one major (22-39 fractions) protein peak. Maximum protease activity was detected in the fractions 26-31. The active fractions were pooled and used for further study. The ion exchange chromatography with DEAE cellulose enhanced the specific activity of the enzyme to 5950.73 U/mg (Table 3.1). At the end of the purification procedure a 4.9 fold purification of the protease was attained.

Protein purification was successfully achieved to homogeneity, as evident by a single band corresponding to 32 kDa on SDS-PAGE (Fig.3.2 A). In lanes marked 1 and 2 where the crude enzyme and ammonium sulphate fractions respectively were electrophoresed, multiple bands were observed. The proteolytic activity of the purified enzyme was confirmed with the help of zymogramphy/ activity staining. The analysis of zymogram revealed two very closely placed clearance bands of higher molecular mass (~96 kDa) than that observed during SDS-PAGE, which denoted the existence of the enzyme as an oligomer in its native state.

Table 3.1

Summary of the purification of *Vibrio* sp. (V26) protease

Purification steps	Protease activity (U)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold
Crude extract	1115.16	0.91	1225.45	**1**
40-80% Ammonium sulphate fraction	2379.17	0.76	3130.48	**2.6**
DEAE Cellulose	1249.65	0.21	5950.73	**4.9**

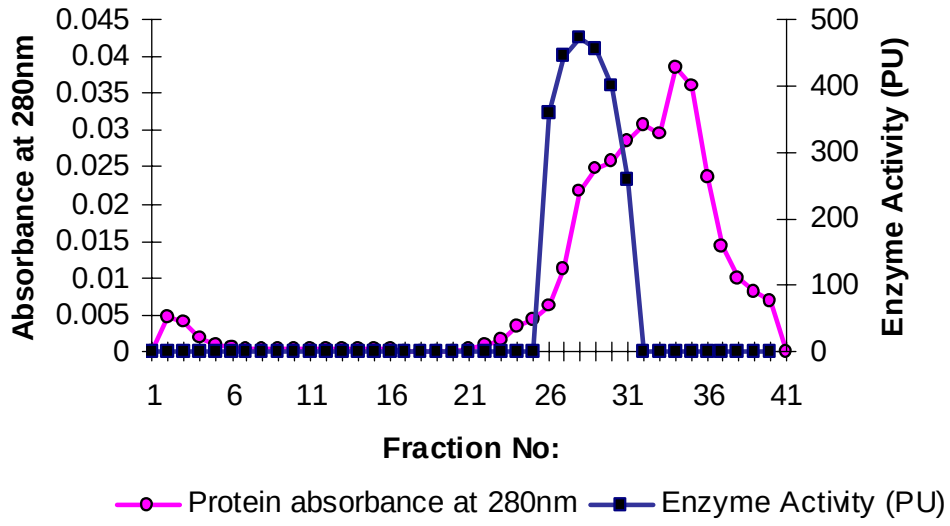


Fig.3.1. Elution profile of *Vibrio* sp. (V26) protease from DEAE-Cellulose column. The enzyme was eluted with a linear gradient of NaCl (0-0.8 M) in 20 mM Trs-Cl buffer (pH 8.5) at a flow rate 0.5 ml/minute.

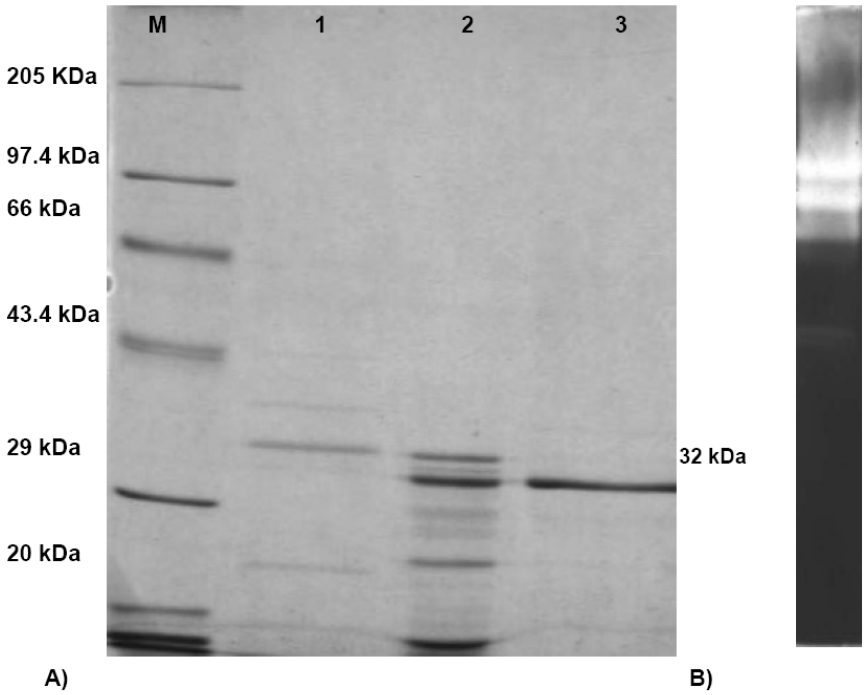


Fig.3.2. A) SDS-PAGE of the *Vibrio* sp. (V26) protease B) Zymogram of purified protease. M- molecular mass markers; lane 1 crude enzyme; lane 2 40-80% ammonium sulphate saturation fraction; lane 3 purified protease.

3.3.2 Characterization of enzyme

3.3.2.1 Effect of pH on activity

The pH activity profile of the purified protease of *Vibrio* sp. (V26) was determined using different buffers of varying pH values. The purified enzyme was active in the pH range of 6.0–11.0, with an optimum at pH 9 as indicated by the peak in Fig.3.3. The activity of the enzyme was found to increase proportionally with the increase in pH from 6 to 9 with a drop in activity beyond pH 9. The protease exhibited 70.22 %, 90.72 %, 60.38 % of the maximal activity at pH 7, 8 and 10 respectively. The relative activity of the enzyme at pH 6 (25.44%) and pH 12 (6.25 %) were minimal. These results clearly indicate that the enzyme is an alkaline protease. The statistical analysis revealed that pH had significant ($p < 0.01$) influence on the activity of the protease (Appendix 2).

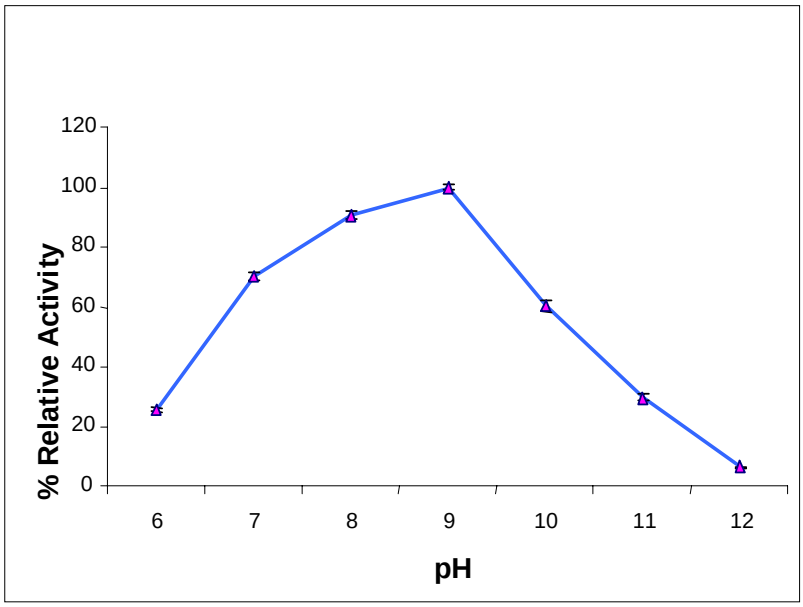


Fig.3.3. Effect of pH on the activity of protease from *Vibrio* sp. (V26)

3.3.2.2 Effect of pH on stability of the enzyme

It can be deduced from the data obtained on pH stability (Fig.3.4) that, the protease exhibited a great deal of stability (> 60% residual activity) in the

pH range 7-10 with the highest residual activity observed in the sample incubated at pH 9. In the pH range 7-9 more than 78 % of activity was retained by the enzyme with no significant ($p > 0.05$)difference in the residual activity at pH 7 and 8 (Appendix 2).

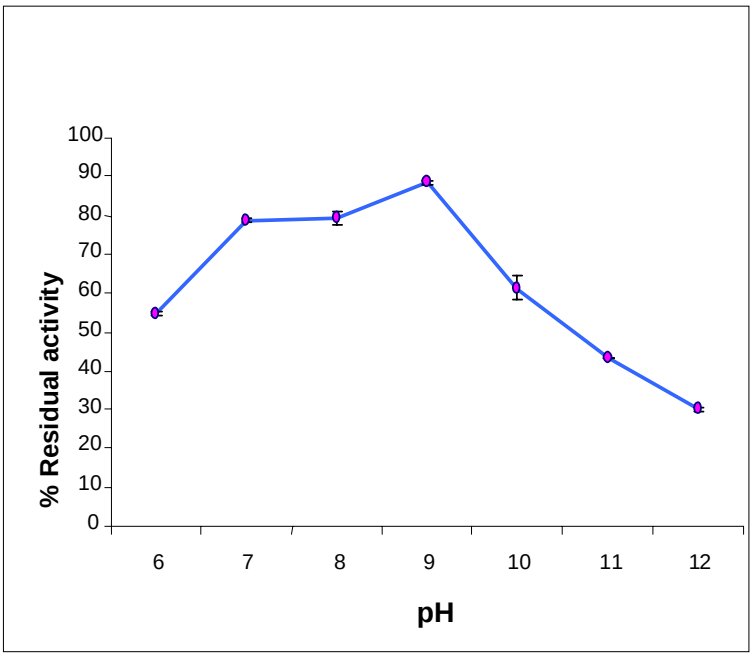


Fig.3.4. Effect of pH on the stability of protease from *Vibrio* sp. (V26)

3.3.2.3 Effect of temperature on the activity of the enzyme

From the data presented in Fig.3.5 it is clear that the alkaline protease of *Vibrio* sp. (V26) was active at all the temperatures (30-80ºC) tested, with maximum activity recorded at 60ºC, qualifying it to be designated as a moderately thermo-active protease. A sharp decline in activity at temperatures above 60ºC was noted. Within the temperature range 40-60ºC the protease retained more than 85% of its maximum activity. Even at temperatures 30 and 80ºC it exhibited 38.33 and 36.68% relative activity respectively. The statistical analysis revealed that temperature had significant ($p < 0.01$) influence on the activity of the protease (Appendix 2).

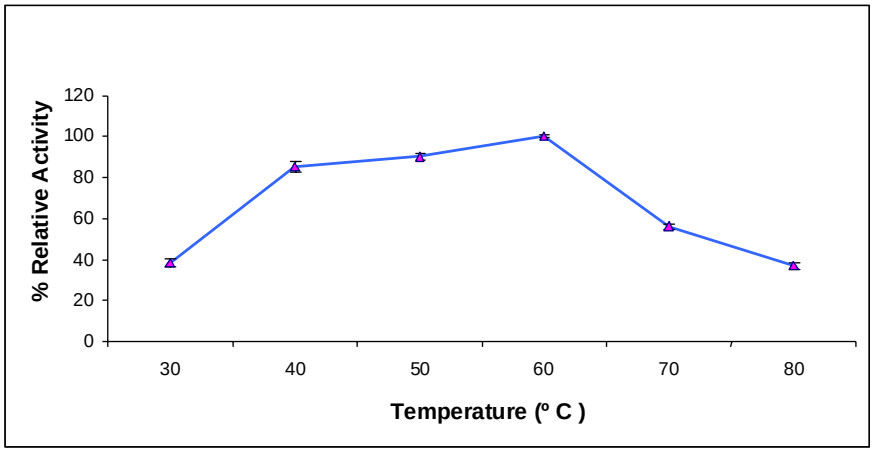


Fig.3.5. Effect of temperature on the activity of protease from *Vibrio* sp. (V26).

3.3.2.4 Effect of temperature on stability of the enzyme

The enzyme's temperature stability profile (Fig.3.6) revealed a great deal of stability in the temperature range 30-50ºC and the stability exhibited in this range did not vary significantly ($p > 0.05$, Appendix 2). However the protease was found to be unstable at its optimal temperature for action. Moreover the alkaline protease was almost completely inactivated when incubated at 70ºC for 1 hour.

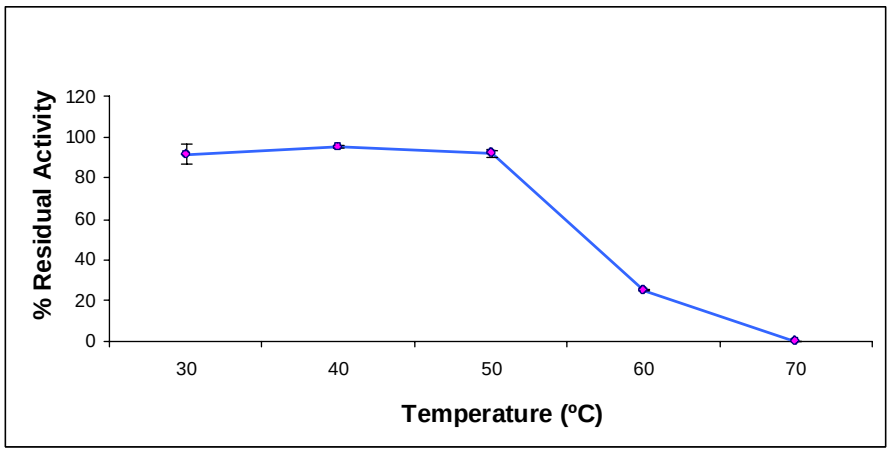


Fig.3.6. Effect of temperature on the stability of protease from *Vibrio* sp. (V26)

3.3.2.5 Effect of metal ions on enzyme activity

The results of the effect of metal ions on the activity of the protease are presented in Fig.3.7. In presence of Ca^{2+} (1 mM) and Ba^{2+} (1 mM) the activity of the protease was not significantly different from the untreated control (p >0.05, Appendix 2). Even in the presence of Mn^{2+} (86.2%), Pb^{2+} (71.5%), Mg^{2+} (88.9%) and Co^{2+} (86.5%) ions the enzyme showed a great deal of activity. At both the concentrations (1 mM and 5 mM), Hg^{2+} and Cu^{2+} were found to be the inhibitory, while Zn^{2+} had a negative effect at 5mM concentration. All the metal ions at 5 mM concentration were found to have a negative influence on the activity of the alkaline protease from *Vibrio* sp. (V26).

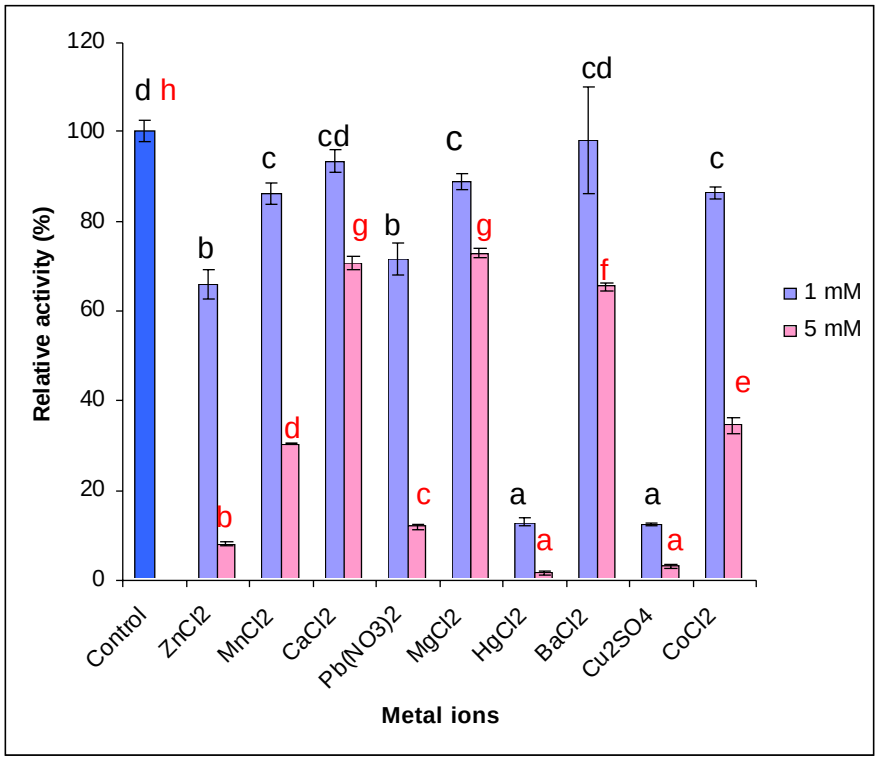


Fig. 3.7. Effect of various metal ions (1mM and 5 mM) on the activity of alkaline protease from *Vibrio* sp. (V26).

Values with the same superscripts donot vary significantly. Superscripts in black are the comparison of effect of 1mM concentration of metal ion on enzyme with the control. Superscripts in red are the comparison of effect of 5 mM concentration of metal ion on enzyme with the control

3.3.2.6 Effect of inhibitors on enzyme activity

Studies on the effect of inhibitors on the enzyme help in determining the nature or the class of the proteases. *Vibrio* sp. (V26) protease was inhibited up to 53% by EDTA (5 mM), a metal chelator and completely by 1, 10 phenanthroline (5 mM), a zinc specific chelator (Table 3.2) while PMSF and IAA did not drastically affect the activity of the enzyme. This clearly indicated that the protease is a zinc- metallo protease.

Table 3.2

Effect of inhibitors on the activity of protease from *Vibrio* sp. (V26)

Inhibitors	Concentration (mM)	Residual Activity (%)
None		100
IAA (Cysteine protease inhibitor)	1 mM	87.86 ± 2.09
1,10 Phenanthroline (Metallo-protease inhibitor)	5 mM	0
EDTA (Metalloproteaseinhibitor)	5 mM	47.55 ± 3.65
PMSF (Serine protease inhibitor)	5 mM	94.48 ± 0.54

3.3.2.7. Effect of oxidizing agent and surfactants on protease activity

The enzyme was found to be stable at low concentrations of oxidizing agent and surfactants. At a concentration of 0.1% of H_2O_2, a strong oxidizing agent; only less than 10% inhibition in activity was observed. Even at 0.5% of H_2O_2, the enzyme retained 57% of its maximum activity (Table 3.3). The nature of surfactant seemed to influence its effect on the protease. SDS (anionic detergent) had negative effect on the protease while Tween 80 (non-ionic detergent) had a slight enhancing effect on the enzyme. The enzyme retained nearly 70.7% and 32 % activity in presence of the 0.1% and 0.5% of

SDS respectively. With the increase in concentration of SDS the inhibitory effect was also found to increase. Tween 80 at both 0.5% and 1% concentration enhanced the activity of protease. Moreover no significant difference in activity was noted with the increase in concentration of Tween 80.

Table 3.3

Effect of oxidizing agent and surfactants on activity of protease from *Vibrio* sp. (V26)

Additive	Concentration (%)	Residual Activity (%)
SDS	0.1	70.72 ± 0.47
	0.5	32.25 ± 1.25
H_2O_2	0.1	90.27 ± 3.28
	0.5	57.41 ± 3.27
Tween 80	0.5	111.41 ± 0.99
	1	110.48 ± 3.68

3.3.2.8 Hemagglutination assay

The ability of the protease from *Vibrio* sp. (V26) to agglutinate human (O blood group) and chick RBC's were assessed using hemagglutination assay. Neither the ammonium sulphate fraction nor the purified enzyme was able to agglutinate the RBCs (human and chick). This was indicated by the clear button formation at the bottom of the wells of the microtitre plate (Fig.3.8).

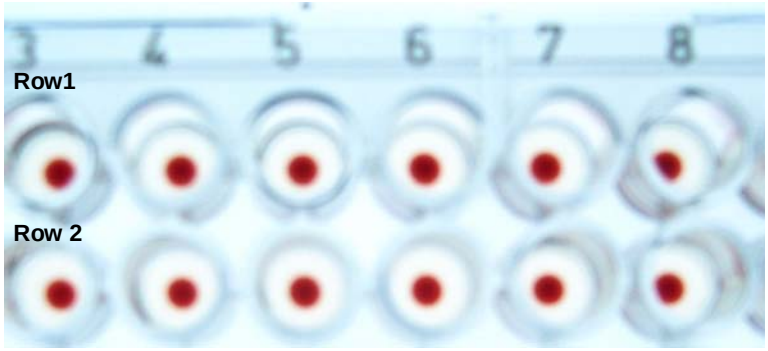


Fig.3.8. Hemagglutination assay.

Row 1 ammonium sulphate fraction, Row 2 purified enzyme.

3.3.2.9 Cytotoxicity of Vibrio protease

Cyotoxicity of *Vibrio* sp. (V26) protease was found to be dose dependent. As the concentration of the enzyme increased the toxicity was also found to increase (Fig. 3.9 A). When HEp-2 cell lines were incubated for 14 hours with high concentrations (250-1000 U) of the enzyme nearly 100% cell inhibition or death was observed. The LC_{50} of the purified *Vibrio* sp. (V26) proteases was determined to be 50 U. Cell rounding was observed as a cytotoxic effect (Fig.3.9 B, c). An interesting observation that was made during this investigation was that after the first few hours (4 hrs) of incubation with the enzyme, the HEp-2 monolayer was found to first detach initially from the edges of the wells and then subsequently as a whole sheet (Fig 3.9 B a&b).

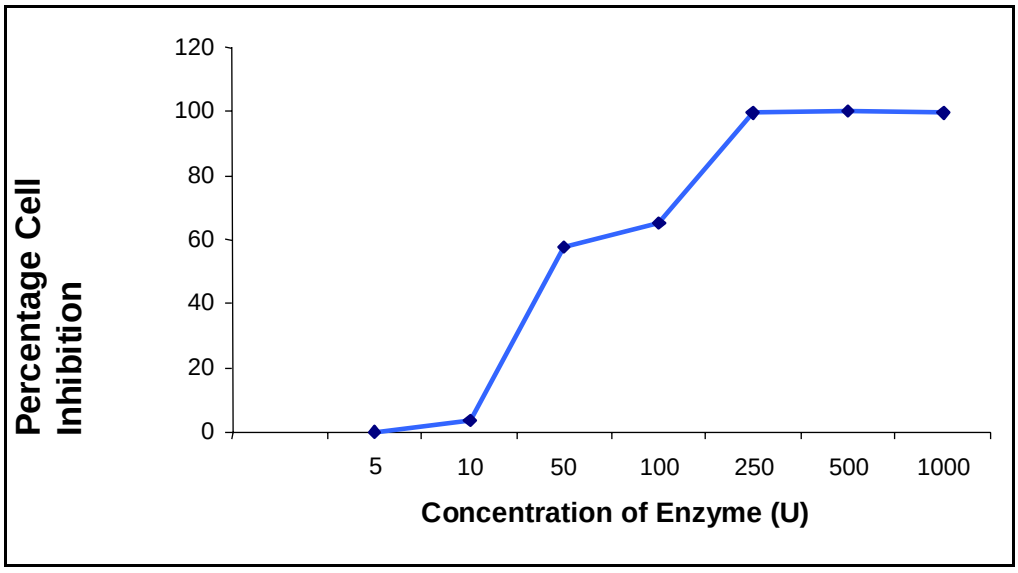


Fig.3.9A Sigmoid curve of the cytotoxicity assay

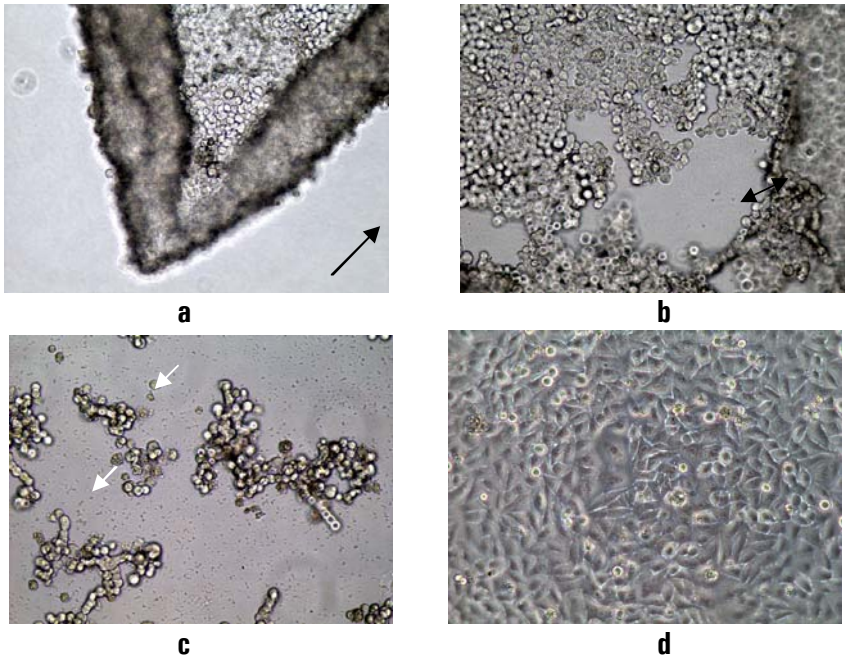


Fig.3.9 B Morphological changes of HEp2 cells

a & b). Shows the 'detachase' activity of *Vibrio* sp. (V26) protease on cells after 4 hrs of incubation, c) Cell rounding, d) Control-HEp2 monolayer.

3.3.3 Comparison of *Vibrio* alkaline protease with a commercial alkaline protease Savinase ® (P3111)

Purified *Vibrio* sp. (V26) protease was 31-fold more active than the commercial Savinase® under the standard assay conditions (Table 3.4). Savinase ® is a commercial enzyme that is claimed to be active over a wide range of pH.

Table 3.4

Comparison of *Vibrio* sp. (V26) alkaline protease with the commercial alkaline protease SAVINASE® (P3111)

Enzyme	Specific Activity (U/mg)
Alkaline protease from *Vibrio* sp.(V26)	5950.73
Savinase from *Bacillus* sp.	190.83

3.3.4. Protease gene

Primers used in this study were designed to amplify the zinc binding conserved region of the protease gene. A PCR product of amplicon size 304 bp was obtained (Fig.3.10).

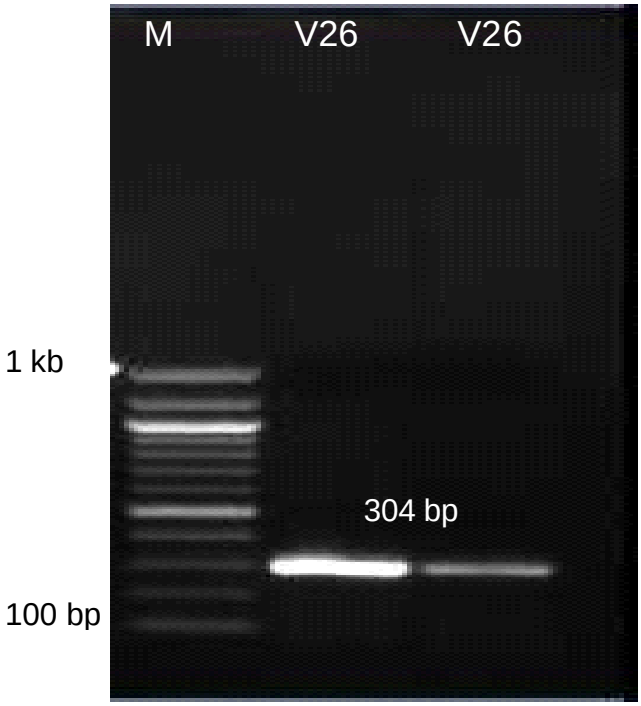


Fig.3.10. PCR product of the protease gene amplification M- 100 bp DNA ladder, V26 –PCR product of protease gene of *Vibrio* sp. (V26)

The PCR product obtained was sequenced. The nucleotide sequence as well the deduced amino acid sequence is given in Fig 3.11**.** The nucleotide sequence has been submitted to the GenBank data base and was assigned the Accession no: JN091086 (Appendix1). BLAST analysis of the nucleotide sequence revealed the similarity of *Vibrio* sp. (V26) metallo-protease gene with that of HA/protease gene of *V. cholerae* and *Helicobacter pylori* (Table 3.5)

The deduced amino acid sequence of *Vibrio* sp. (V26) protease was also compared with metalloproteases from other bacteria and it revealed maximum identity to that of the neutral precursor *V. cholerae bv albensis* (ZP04416044.1)

as well as HA/protease precursor of *V. cholerae*. Multiple alignment and the bootstrap distance tree calculated for the metallo-protease sequences and BLAST analysis confirmed that it was highly similar to HA/protease of *V. cholerae* or its precursor protein (ZP 06048800.1, ZP 04411813.1, ZP 01955135) , the neutral precursor *V. cholerae bv albensis* (ZP04416044) as well vibriolysin (ACX48920.1). From Fig.3.12 it is clear that protease of *Vibrio* sp. (V26) shared a great deal of similarity to the metalloproteases from several other *Vibrio* species including *V. mimicus* (BAG 30958.1), *V. fluvialis*-(BAB86344), *V. furnissii*-(ZP 05878240.1) and HA/protease *V. mimicus*-(ZP 05717625.1). The protease of this study was most distantly related to the elastase of *Pseudomonas aeruginosa* and zinc metalloprotease of *V. caribbenthicus* (Fig.3.12).

The zinc binding motif (HEXXH consensus motif) His-Glu-Tyr-Thr-His (HEVSH) was identified in the sequence. Putative zinc-binding residue, the active site residues, identical amino acid sequence it shares with other metallo-protease have been indicated in Fig. 3.11.

```
cat gag gtc agc cac ggt ttt act gag cag aat tca ggc ctc gtt tac cga gat atg tcc ggt ggc att aac gaa
H  E  V  S  H  G  F  T  E  Q  N  S  G  L  V  Y  R  D  M  S  G  G  I  N  E
*  $     *                          $                          *

gca ttc tcg gat atc gca ggg gaa gcg gca gag tac ttt atg cgt ggc aat gtc gac tgg att gtc ggc gcg
A  F  S  D  I  A  G  E  A  A  E  Y  F  M  R  G  N  V  D  W  I  V  G  A

gat att ttt aaa tcc tcc ggt ggt cta cgt tat ttc gat cag ccg tca cgt gat ggc cgc tcg ata gat cat
D  I  F  K  S  S  G  G  L  R  Y  F  D  Q  P  S  R  D  G  R  S  I  D  H

gct tca cag tat tac agt ggt att gat gtt cac cat tcg agt ggc gtg ttt aac cgc gcg
A  S  Q  Y  Y  S  G  I  D  V  H  H  S  S  G  V  F  N  R  A
```

Fig 3.11 Nucleotide and amino acid sequence of the protease gene of *Vibrio* sp. (V26) (Ac no: JN091086) Putative zinc-binding residue, active site residues, identical amino acids and HEXXH motif are indicated by asterisks, dollar symbol, bold black letters and bold red letters, respectively

Table 3.5

Result of Nucleotide BLAST analysis of *Vibrio* sp. (V26) protease

Accession	Description	Query coverage	E value	Max ident
CP002556.1	*Vibrio cholerae* LMA3894-4 chromosome II, complete sequence	100%	7e-140	99%
GQ912701.1	*Vibrio cholerae* O1 strain Ogawa vibriolysin (hap) gene, partial cds	100%	7e-140	99%
CP001486.1	*Vibrio cholerae* MJ-1236 chromosome 2, complete sequence	100%	7e-140	99%
CP001234.1	*Vibrio cholerae* M66-2 chromosome II, complete sequence	100%	7e-140	99%
AE003853.1	*Vibrio cholerae* O1 biovar eltor str. N16961 chromosome II, complete sequence	100%	7e-140	99%
DQ776042.1	Synthetic construct *Vibrio cholerae* clone FLH200370.01F hap gene, complete sequence	100%	7e-140	99%
M59466.1	*V.cholerae* HA/protease gene, complete cds	100%	7e-140	99%
CP001236.1	*Vibrio cholerae* O395 chromosome II, complete sequence	100%	7e-135	98%
CP000626.1	*Vibrio cholerae* O395 chromosome 1, complete genome	100%	7e-135	98%
Z27239.1	*H. pylori* HAP gene for haemagglutinin/protease	89%	1e-117	98%

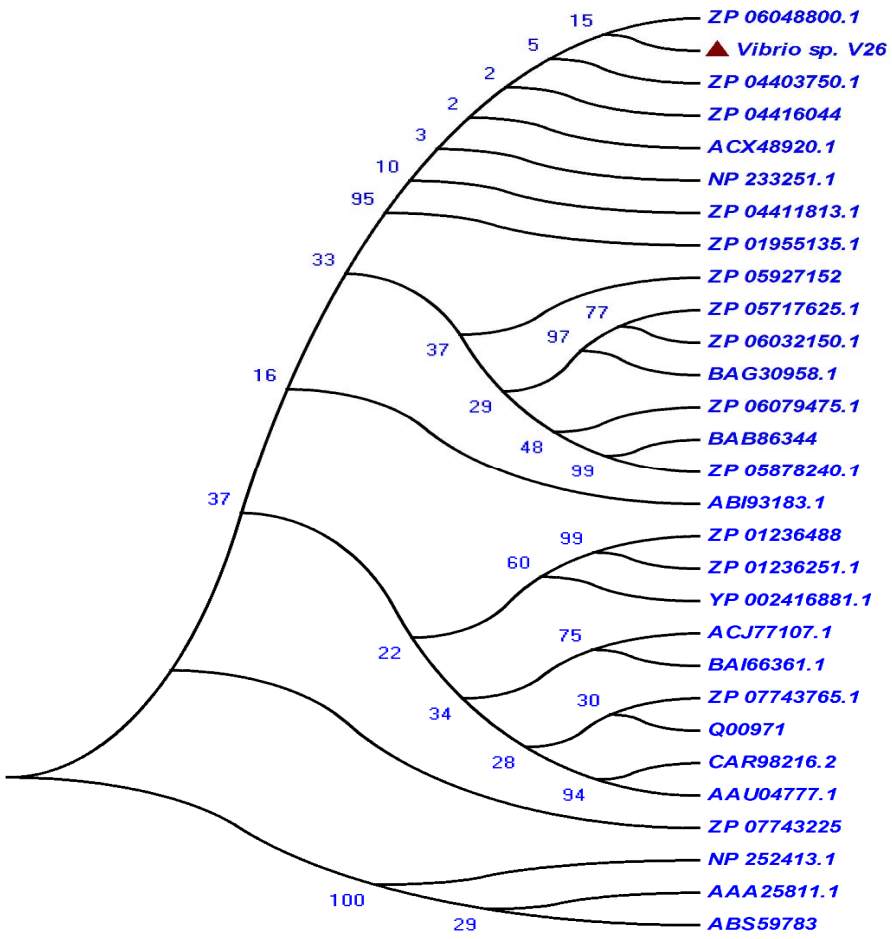


Fig 3.12 A bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequence of the *Vibrio* sp. (V26) with other bacterial metallo-proteases (HA/proteinase precursor *V. cholerae* T536993- **ZP 06048800.1**; neutral protease precursor- **ZP 04403750.1**; *V. cholerae bv albensis*-**ZP04416044**; vibriolysin-**ACX48920.1**; HA/protease *V. cholerae* O1 biovar-**NP 233251.1;** neutral protease precursor-ZP 04411813.1; HA/protease-ZP 01955135.1; Zn metalloprotease *Vibrio* sp. RC341-**ZP05927152**; HA/protease *V.mimicus*-**ZP 05717625.1**; Zn metalloprotease-**ZP06032150.1**; metalloprotease of *Vibrio mimicus* **-BAG 30958.1**; Zn metalloprotease elastase *Vibrio* sp. RC586-**ZP06079475.1**; metalloprotease *Vibrio fluvialis*-**BAB86344**; metalloprotease *V. furnissii*-**ZP 05878240.1**; Zn metalloprotease precursor *Salinivibrio proteolyticus*-**AB191383.1**; zn metalloprotease *V. angustum*-**ZP 01236488** & **ZP 01236251**; extracellular Zn metalloprotease *V. splendidus*-**YP 002416881.1**; vtp A *V. tubiashii*-**ACJ771071**; metalloprotease *V. vulnificus*-**BAI 66361.1**; extracellular Zn metalloprotease *V. caribbenthicus*-**ZP 07743765.1** & **ZP 07743225**; neutral protease *V. proteolyticus*- **Q 00971**; metalloprotease *Listonella anguillarum*;- **CAR 98216.2**; metalloprotease precursor *V. aestuarianus*- **AAU04777.1**; elastase Las B *Pseudomonas aeruginosa*-**NP 252413.1**; elastase precursor- **AAA 25811.1**; organic solvent tolerant elastase *P. aeruginosa*- **ABS59783**). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

3.4. Discussion

The alkaline protease from *Vibrio* sp. (V26) was purified by a two-step procedure with a nearly 5 fold increase in specific activity. The molecular mass of the protease was found to be 32 kDa which is in close agreement with the observation of previous workers on proteases from *V. cholerae* (Finkelstein and Hanne, 1982; Ichinose *et al*., 1992, Vaikkevicius, 2007), *V. mimicus* (Chowdhury *et al.,* 1990) as well as other Vibrios (Lee *et al.,* 1997; Venugopal and Saramma, 2006; Jellouli *et al.,* 2009). An alkaline serine protease of the same molecular weight has been isolated from the Gram positive bacteria *Bacillus cereus* VITSN04 (Sundararajan *et al.,* 2011) and the fungus *Aspergillus clavatus* ES1 (Hajji *et al.,* 2007). Though generally alkaline protease of microbes fall within the size range 15-30 kDa there are reports available on proteases from *Vibrio* species such as *V. parahaemolyticus* (Ishihara *et al.,* 2002; Lee *et al.,* 2002), *V. harveyi* (Liu *et al.,* 1997) and *V. pelagicus* (Farto *et al.,* 2002) having a molecular weight greater than 33 kDa. The protease (32-33 kDa) of *V. cholerae* as well as that of *V. mimicus* is believed to be a bifunctional molecule having hemagglutinating and proteolytic activities referred to as HA/protease (Finkelstein and Hanne, 1982; Honda *et al.,* 1989; Chowdhury *et al.,* 1990; Benitez *et al.,* 2001).

When the molecular mass of *Vibrio* sp. (V26) protease was analyzed using zymogram (~96 kDa) and SDS-PAGE (32 kDa) a considerable variation was observed. It is most likely that the protease exists as an oligomer of larger mass (~ 96 kDa) that gets dissociated into 32 kDa subunit when it is subjected to denaturation during SDS-PAGE. In a similar study, Finkelstein and Hanne (1982) reported that the HA/protease of *Vibrio cholerae* is a large molecular weight oligomer that dissociates into identical subunits of 32 kDa on treating it at 100ºC for 2 minutes. The protease of *V. harveyi* strain FLA-11 has also been found to exist as oligomer of 84,000 Da, comprising a tetramer of 21,000 molecular weight subunits (Fukasawa *et al.,* 1988).

The analysis of the zymogram revealed two very closely placed clearance or activity bands. This may be due to the existence of isoforms of the protease with slightly different electrophoretic mobility, as reported in several members of the genus *Vibrio* like *V. mimicus* (Lee *et al.,* 1998a), *V. cholerae* (Wu *et al.,* 2000; Halpern *et al.,* 2003), *V. anguillarum* (Staroscik *et al.,* 2005) and *V. fluvialis* (Miyoshi *et al.,* 2002), or due to the occurrence of zymogen or proenzyme (Milton *et al.,* 1992) along with the fully active protease, that becomes activated during the reactivation step or it may also be because of autoproteolysis of the enzyme. Norqvist *et al.* (1990), too have observed two active forms when the purified metalloprotease from the *V. anguillarum* wild type strain NB10 was electrophoresed on SDS-denaturing gels without prior denaturation of the sample by heating.

The protease from *Vibrio* sp. (V26) recorded maximum activity as well as maximum stability at pH 9 which entitles it to be classified under the category alkaline protease. Meanwhile proteases from most species of *Vibrio* were reported to have their optimum pH at 8.0 (Lee *et al.,* 2002; Lee *et al.,* 2003b; Venugopal and Saramma, 2006). However *V. fluvialis* TKU005 (Wang *et al.,* 2007) and *V. cholerae* (Ichinose *et al.,* 1992) were found to produce proteases with a similar pH optimum of 9. An extremely high pH optimum of 12 has been recorded for the protease from *V. metschnikovii* (Mei and Jiang, 2005). The high activity of *Vibrio* sp. (V26) protease in the alkaline pH is a very important characteristic for its eventual use as a laundry detergent additive. The pH stability profile of the protease of *Vibrio* sp. (V26) also meets the basic criteria for its possible application as detergent ingredient, in leather processing and other industrial processes that are carried out in the alkaline pH range. A highly pH stable serine protease was also reported from *V. metschnikovii* J1 (Jellouli *et al.,* 2009).

The purified protease from *Vibrio* sp. (V26) showed optimal activity at 60ºC. A similar optimum temperature for action of protease has been reported

from *V.fluvialis* TKU005 (Wang *et al.,* 2007), *V. metschnikovii* DL 33-51 (Mei and Jiang, 2005) as well as from different members of the genus *Bacillus* (Adinarayana *et al.,* 2003; Almas *et al.,* 2009; Tanskul *et al.,* 2009; Anita and Rabeeth 2010; Deng *et al.,* 2010). However, proteases from most Vibrios have their optima well below 60ºC (Ishihara *et al.,* 2002; Lee *et al.,* 2002; Lee *et al.,* 2003b; Venugopal and Saramma, 2006). Exceptionally high temperature optima of 75ºC had been observed for an alkaline protease isolated from *Bacillus laterosporus*-AK1 (Arulmani *et al.,* 2007). The protease from *Vibrio* sp (V26) was active over a wide range of temperature. A high degree of activity (> 85%) was exhibited by the protease in the temperature range 40-60ºC and even at 30ºC it was quite active. This property could be of great advantage in the detergent industry, which is now looking for alkaline proteases that work well under low temperature or room temperature conditions (Maurer, 2004), as this would facilitate washing under ambient temperatures, a pre-requisite to maintain fabric quality and also for reducing the energy demand (Venugopal and Saramma, 2006).

An investigation of the temperature stability profile of the protease from *Vibrio* sp. (V26) revealed that it was highly stable upto 50ºC (Fig. 3.6) and the stability exhibited did not vary significantly in the temperature range of 30-50ºC. This high degree of stability of the enzyme can come in handy in areas where the proteases is exposed to these temperature conditions for long durations; such as in the recovery of silver from X-ray films and as an ingredient of detergents etc. However it was found that the alkaline protease from *Vibrio* sp. (V26) V26 was quite unstable when pre-incubated at its optimum temperature (60ºC) for an hour. The proteases from *Salinivibrio* sp., *V. fluvialis,* and *Bacillus* strain SAL1 too have been found to be unstable at their optimum temperatures for action (Karbalaei-Heidari *et al.,* 2007; Wang *et al.,* 2007; Almas *et al.*, 2009). A drop in activity of the proteases on prolonged exposure to temperatures above 50ºC has been reported among Vibrios (Lee *et*

al., 2002; Lee *et al.,* 2003b). Even alkaline protease used in commercial detergents tends to get inactivated on extended exposures to temperature of 60ºC or more. The denaturation, followed by inactivation of the enzyme due to the prolonged exposure at high temperatures is responsible for this drop in activity.

At 1mM concentration, the effect of ions such as Ba^{2+} and Ca^{2+} were not significantly different from the control indicating practically no effect of these ions on the protease. Similar observation was made by Kumar *et al.* (1999). In general all metal ions were found to be inhibitory at the higher concentrations (5 mM) tested, however Cu^{2+} and Hg^{2+} exerted a high degree of inhibitory activity on the alkaline protease from *Vibrio* sp. (V26) at both the concentrations. The inhibitory effect of heavy metals especially that of Cu^{2+} and Hg^{2+}, on alkaline protease are well documented (Vallee and Ulmer, 1972; Johnvesly *et al.,* 2002; Mei and Jiang, 2005; Venugopal and Saramma, 2006). The ions of mercury, cadmium and lead react with the protein thiol groups (converting them to mercaptides) and with histidine and tryptophan residues. Moreover, by the action of silver and mercury, the disulphide bonds are hydrolytically degraded (Torchinsky, 1981). All these effects lead to the inactivation of enzyme. The inhibitory potential of Zn^{2+} was more prominent at higher concentration which indicated that it is most likely a zinc metallo protease. High concentrations of Zn^{2+} inhibits metalloprotease (Teo *et al.,* 2003) by the formation of zinc monohydroxide that bridges the catalytical zinc ion to the side chain of the active site of the enzyme (Larsen and Auld, 1991).

Enzyme inhibition studies primarily give an insight in to the nature of the enzyme, its cofactor requirements and the nature of the active centre (Sigma and Mooser, 1975). In the present study**,** *Vibrio* sp. (V26) protease was completely inhibited by 1, 10 phenanthroline (5 mM), the zinc specific chelator and up to 53% by EDTA (5 mM) which showed that the alkaline protease of *Vibrio* sp. (V26) is a metalloprotease. There are several reports available on

metalloproteases from vibrios including *V. cholerae* (Ichinose *et al.,* 1992), *V. mimicus* (Lee *et al.,* 2003b) and *V. fluvialis* (Miyoshi *et al.,* 2002; Wang *et al.,* 2007). While most other alkaline proteases reported from *Vibrios,* were found to belong to the class serine proteases (Ishihara *et al*., 2002; Lee *et al*., 2002; Venugopal and Saramma, 2006; Jellouli *et al.,* 2009), there is also a rare record of a cysteine protease from *Vibrio harveyi* (Liu *et al.,* 1997).

The major application of alkaline protease is in detergent industry and it is always desirable for the enzyme to be stable in the presence of various detergent ingredients such as surfactants and bleaches. *Vibrio* sp. (V26) alkaline protease was found to be highly stable in the presence of Tween 80; actually a slight enhancement in activity was noted. A similar observation was made by Kumar *et al.* (1999). This increase in enzyme activity is most likely due of the effect of the surfactants on the unfolding of the substrate moeity (Vita *et al.,* 1985; Chaphalkar and Dey, 1998). In certain other characterization studies, nonionic surfactants were found to have very little effect on protease activity (Joo *et al.,* 2001, 2004). In presence of the strong ionic surfactant such as SDS (0.1%) the enzyme retained nearly 71% activity. Stability of alkaline protease from *Vibrio* sp. (V26) towards the surfactant SDS gains importance in the light of reports that SDS has in general a strong inhibitory effect on proteases (Tremacoldi *et al.,* 2007). Combined effects of factors such as reduction in the hydrophobic interactions and the direct interactions with the protein molecule are believed to be the cause for the inhibition by SDS (Creighton, 1989). The alkaline metallo-protease from *Vibrio* sp. (V26) exhibited quite a reasonable degree of stability towards H_2O_2 an oxidizing agent. At concentrations of 0.1% and 0.5% of H_2O_2 the enzyme retained 90% and 57% of its maximum activity.

As the proteases from *V. cholerae* and *V. mimicus* of molecular mass 32-33 kDa were reported to exhibit hemagglutination property (Benitez *et al.,* 2001), the ability of *Vibrio* sp. (V26) protease to agglutinate human and chick

RBCs were assessed. However in this study, neither the ammonium sulphate fraction nor the purified alkaline protease from *Vibrio* sp. (V26) displayed the hemagglutination property. Quite a different observation was made by Ichinose *et al.* (1992); they noted that bacterial culture supernatant exhibited hemagglutination activity but not the purified protease from *V. cholerae* O1. While Fukuda *et al.* (1998) observed that the metalloprotease from *Vibrio* sp. NUF-BPP1 failed to agglutinate human erythrocytes but it was able to weakly agglutinate the chick erythrocytes. The degradation of the hemagglutination site of the purified enzyme with other proteases in the culture supernatant or the lowering of the hemagglutination property as an effect of storage (4ºC) (Ichinose *et al.,* 1992) could well be responsible for the loss of hemagglutination property of *Vibrio* sp. (V26) protease. The chemical and structural differences in the cell surfaces of erythrocytes from diverse origin have also been suggested as a reason for the dissimilarity noted in the hemagglutinating property of the proteases (Fukuda *et al.,* 1998).

In this study, the cytotoxicity was found to increase with the increase in the concentration of protease indicating that the degree of toxicity was found to be dose dependent. A similar observation was made in the case of Prt V, a metalloprotease produced by *V. cholerae* that is deficient in HA/protease on human intestinal cell lines HCT8 (Vaitkevicius *et al.,* 2008). Cytotoxic effects such as cell rounding and cell death at higher enzyme concentration could be clearly observed in this investigation. Tissue damage caused by the proteases was probably due to direct degradation of substrate proteins in host tissues thereby inducing cell rounding (Vaitkevicius *et al.,* 2008). The LC_{50} value of the purified *Vibrio* sp. (V26) protease on the HEp 2 cell lines was found to be 50 U. Young and Broadbent (1982) too noted that the protease of *V. cholerae* had cytotoxic effect at concentrations greater than 50 U/ml. Morphological changes as well as cell lysis were also observed by Lee *et al.* (2003 b) during the cytotoxic assay of the recombinant *V. mimicus* metalloprotease (rVMC61)

on CHSE-12 (Chinook salmon) fish cells. Lee *et al.* (2002) studied the invitro effects of the purified protease on mammalian cell lines (CHO, HeLa, Vero and CaCo-2) and found the cytotoxicity dose to vary with cell lines tested, however cell rounding was observed in all the cell lines. The recombinant zinc metalloprotease rEmpA too exhibited cytopathic effects like morphological damage to flounder gill cells (Yang *et al.,* 2007). A preliminary study on serine protease VPP1 from *V. parahaemolyticus* suggested that the enzyme caused exfoliation of cultured CaCo-2 cells and digested various proteins which compose the mammalian cells or tissues (Ishihara *et al.,* 2002). From this study and the previous reports it is clear that certain amount of cytotoxicity is exhibited by all proteases regardless of the species that produces it.

Protease from *Vibrio* sp. (V26) was found to act on HEp-2 cell lines, causing it to detach initially from the edges of the wells and then subsequently as a whole sheet (Fig 8, B). A similar effect of *V. cholerae* protease on epithelial cells MDCK-1 was noticed by Wu *et al.* (1996). The protease of *V. cholerae* has the ability to act on substances like mucin, fibronectin and lactoferrin. It is considered to be a type of 'detachase' which degrades protein structures required for attachment of *V. cholerae* to the intestinal epithelium and thus its detachment, for further transmission (Finkelstein *et al.,* 1992). Detachment of HEp-2 cell lines observed in this study could be due to a similar mechanism action of the *Vibrio* sp. (V26) protease ('detachase'). This study indicated that this 'detachase' property of the protease could find application in the field of animal cell culture.

The nucleotide sequence of *Vibrio* sp. (V26) metalloprotease gene was compared with other known protease sequences. This sequence comparison showed that it shared 99% similarity to *V. cholerae* HA / protease gene and 98% similarity to *H. pylori* HAP gene for haemagglutinin / protease. Sequence homology study of the deduced amino acid revealed that the protease from *Vibrio* sp. (V26) showed homology to neutral protease precursor of *V. cholerae*

bv albensis, neutral protease precursor of *Vibrio cholerae* and hemagglutinin/protease of *V. cholerae*. It also exhibited similarity to zinc metalloprotease of *V. mimicus.* The studies further confirmed that the protease from *Vibrio* sp. (V26) is a metalloprotease. The structural gene for the extracellular HA/protease of *V. cholerae* was cloned and sequenced by Hase and Finkelstein (1991). The deduced amino acid sequence of the mature HA / protease showed 61.5% identity to *Pseudomonas aeruginosa* elastase. Mo *et al.* (2010) cloned and sequenced a protease gene from *Vibrio anguillarum* M3 strain, which was found to encode for a putative protein of 918 amino acids that was highly homologous to the *V. cholerae prtV*gene.

The deduced amino acid sequence of *Vibrio* sp. (V26) protease includes a zinc metalloprotease HEXXH consensus motif, which is HEVSH (His-Glu-Tyr-Thr-His). The critical role of the amino acid structure HEXXH with bound zinc in catalysis was previously described in the mammalian and bacterial zinc metalloprotease family (Vallee and Auld, 1990). This motif HEXXH is important to transfer electron with zinc, for the hydrolysis of peptide bonds (Lee *et al.,* 1998a). In the general classification of zinc proteases, five groups are present. Three groups out of these five have the HEXXH motif (Lipscomb and Strater, 1996).

Based on the amino acid sequence similarities the *Vibrio* metalloproteases have been classified in two classes by Lee *et al.,* (1998 a). The zinc binding domain of class I contain HEXXH amino acids and an extra glutamic acid that is located near this motif (HEXXH+E type) whereas the class II showed only the HEXXH motif in the zinc binding domain (HEXXH). Class I metalloproteases were found from *V. cholerae*, *V. anguillarum, V. vulnificus* and *V. proteolyticus* and the class II included the metalloproteases from *V. mimicus*, *V. parahaemolyticus* and *V. alginolyticus.* The multiple alignment for the class I enzymes from several *Vibrio* sp. showed 68–72%

sequence similarity while the sequence similarity of the class II enzymes was 30–78% (Lee *et al.,* 1998a).

Kim *et al.* (2002) have proposed another classification, where *Vibrio* metalloproteases are grouped into three distinct classes instead of two. They actually divided the class II enzymes (as per Lee *et al.,* 1998a) further into two distinct groups based on amino acid sequence differences in the HEXXH region. VMC (*V. mimicus* metalloprotease), PrtV (*V.parahaemolyticus* strain 93 metalloprotease), and VCC (*V. cholerae* 569B Exoprotease) consists of one group (class II) which shares 70–78% sequence similarity. Whereas, VppC and *V. alginolyticus* collagenase belonged to another group (class III) which has 88% similarity and include an extended N- and C-terminal region. The comparison of the molecular mass and the substrate specificity of the metalloproteases also supported this classification. The molecular mass of the class I (HEVSH+E) enzymes is approximately 36–38 kDa while that of the class II (HEYTH) enzymes is about 62– 71 kDa while that of the class III (HEYVH) enzymes is close to 89 kDa, the exact size of these enzymes has not been determined yet. The smaller molecular sizes of the purified class I and II enzymes, compared with those predicted from the nucleotide sequence is due to the autocatalytic cleavage of these proteins during secretion. Many extracellular bacterial proteases are synthesized as inactive precursors with an additional polypeptide segment to keep the protease inactive inside the cell, and undergo several stages of processing, including cleavage of signal peptide to form a mature protein (Peterkofsky, 1982; Wandersman, 1989).

So based on HEXXH region in the sequence of the protease gene of *Vibrio* sp. (V26) it is clear that this protease belongs to the Class I *Vibrio* metalloprotease based on both Lee *et al.,* (1998a) and Kim *et al.,* (2002) classification.

The BLAST analysis of *Vibrio* sp. (V26) protease also indicated that the enzyme belonged to GluZincin peptidase super family and M4 peptidase

family (metalloprotease), also known as thermolysin family (http:// www. merops.sanger.ac.uk / famcards/M4.html). Thermolysin-like proteases (TLPs) are members of the peptidase family M4 (Barret *et al.,* 1998) of which thermolysin (TLN; EC 3.4.24.27) is the prototype. Pseudolysin, aeurolysin and bacillolysin are also included in this family. The family contains secreted eubacterial endopeptidases from both Gram-positive and Gram-negative sources. All members of this comprehensive family are produced as pre-pro-proteins. The mature enzymes are all of moderate size, around 35 kDa (316 amino acids for thermolysin). These proteases contain the typical HEXXH amino acid motif, require Zn^{2+} ions for their activity, and contain multiple Ca^{2+} ions (up to four) for stability. All enzymes are optimally active at neutral pH. The findings of this study seem to agree with all characteristics of M4 family expect with regard to pH. Here in this study enzyme was identified as an alkaline protease and not a neutral one.

As it is clear that the protease from *Vibrio* sp. (V26) belongs to M4 family, it is most likely that it is produced as a pre-pro-protein. The presence of more than one activity band in the zymogram can now be more substantially explained. The occurrence of zymogen form of the enzyme along with mature protease that also gets activated during the reactivation step in zymography, results in two clearance bands of slightly different molecular masses.

Savinase® a product of Novozyme Corp., is an endo-protease of the serine type. It is a protease derived from *Bacillus* sp. and has very broad substrate specificity. It is active throughout the pH range of interest for most detergent applications, namely pH 8-12. It functions between 30-60°C, above which the activity falls rapidly. The activity of the protease from *Vibrio* sp. (V26) was compared to this commercially used enzyme. The comparative study with this commercial enzyme (Savinase®) has clearly indicated that the *Vibrio* sp. (V26) protease was 31 fold more active. This investigation clearly

reveals how potent the alkaline protease from *Vibrio* sp. (V26) is and also indicates its worthiness as a commercial enzyme.

Some of the important features of *Vibrio* sp. (V26) alkaline protease are its high activity and stability at high pH and temperature, as well as in the presence of surfactants, oxidizing agents and metal ions. Apart from these features the enzyme exhibited 'detachase property' and an activity more than Savinase®**.** All these properties of the alkaline protease from *Vibrio* sp. (V26) indicates that, it is sure to find application in areas such as in detergent industry, in the recovery of silver from X-ray films, in animal cell culture etc. .

Chapter 4

Optimization of Physical Parameters and Media Components for Alkaline Protease Production: A Statistical Approach

Contents

Protease production is an inherent capacity of all microorganisms, however only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance (Beg *et al.,* 2003). Microbial protease production is influenced by both chemical constituents of the medium and the physical factors such as temperature, pH, agitation etc. Physical factors are important in promoting, stimulating, enhancing and optimizing the production of proteases (Rahman *et al.,* 2005). Media components for maximum enzyme production have been found to be different for each microorganism. Therefore, the required chemical constituents and their concentrations have to be optimized accordingly (Oskouie *et al.,* 2008). In general, no defined medium has been established for the best production of alkaline protease from different

microbial sources (Kumar and Takagi, 1999; Gupta *et al.,* 2002a, 2002b). Each organism or strain has its own requirement of special conditions for maximum enzyme production (Kumar and Takagi, 1999; Beg *et al.,* 2003).

Approximately 90% of the industrial enzymes are produced using submerged fermentation (SmF) (Joo and Chang, 2006) which involves the cultivation of microbes on substrates dissolved or submerged in an aqueous phase. This is also true in the case of alkaline protease where SmF is more widely employed, while solid state fermentation has been exploited to a lesser extent (Malathi and Chakraborthy, 1991; Chakraborty and Srinivasan, 1993; George *et al.,* 1995) despite its advantages such as lower production cost and less energy requirement. When SmF is used, the scale-up from laboratory level to industry is much more simplified, with parameters more easily monitored and controlled. Moreover SmF is intrinsically less problematic (Paul, 2005).

30 to 40% of the production cost of industrial enzymes is estimated to be accounted for the cost of the growth medium (Joo *et al.,* 2002). Therefore, it is important to improve the performance of the systems and to increase the yield of the processes without increasing the cost through optimization (Bas and Boyacı, 2007). When designing an industrial cultivation medium, a large number of probable cultivation substrates are available for selection. Though the media cost can be brought down by the use of cheap substrates such as agro residues, it tends to increase impurities in the production media resulting in an increase in the purification costs which in turn result in overall cost of enzyme production. An ideal balance has to be struck between these two aspects to keep the production costs under check. Moreover each component of the medium could potentially have a beneficial or detrimental effect on the growth of the microorganism or on the medium cost as well as on enzyme productivity. The only way to assess this is by medium optimization studies.

Several strategies have been adopted for the optimization of both physical factors as well as media components. The most commonly used

method being the 'one-variable-at-a-time' technique where there is a parameter change in the general practice of determining the optimal operating conditions, while keeping the others at a constant level. The major disadvantage of this technique is that it does not include interactive effects among the variables and, eventually, it does not depict the complete effects of the parameters on the process. It is also very time consuming. The other methods of optimization include factorial experiments, partial factorial experiments and Response Surface Methodology (RSM). Regardless of which optimization strategy is chosen, a large number of experiments are needed. Since a large number of flasks can fit on a rotary or orbital shaker, these experiments are performed in shake flask cultures (Sarkar *et al.,* 2010).

Industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better controlled process (Singh *et al.,* 2004). Statistical approaches offer ideal ways for process optimization studies in biotechnology and industrial processes (Haaland, 1989; Gupta *et al.,* 2002b; Beg *et al.,* 2003). Statistical experimental design, also known as design of experiments (DOE) is the methodology of how to conduct and plan experiments in order to extract the maximum amount of information with lowest number of analyses (Decaestecker *et al.,* 2004). Typical characteristics of an experimental design are planned testing, data analysis, simultaneous factor variability and scientific approach. As DOE is based on sound and logical statistical principles, it is possible to handle experimental error, determine important variables that need to be controlled from among the unimportant ones that need not be controlled, measure interactions, allows extrapolation of data and search for the best possible product within the test variable ranges and allows plotting graphs to depict how variables are related and what level variables give the optimum product (Vanaja and Rani, 2007). Response surface methodology (RSM) is one of the most popularly used statistical optimization procedures.

RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response. RSM has important application in the design, development and formulation of new products, as well as in the improvement of existing product design. It defines the effect of the independent variables, alone or in combination, on the processes. In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model which describes the chemical or biochemical processes (Myers and Montgomery, 1995; Anjum *et al.,* 1997).

The relationship between the response and the input is given in Eq.

$\eta = f(x_1, x_2 \ldots . x_n) + \varepsilon$

Where η is the response, f is the unknown function of response, $x_1, x_2 \ldots . x_n$ denote the independent variables, also called natural variables, n is the number of the independent variables and finally ε is the statistical error that represents other sources of variability not accounted for by f. These sources include the effects such as the measurement error. It is generally assumed that ε has a normal distribution with mean zero and variance.

It is possible to separate an optimization study using RSM into three stages. The first stage involves preliminary work, in which the determination of the independent parameters and their levels are carried out. The second stage is the selection of the experimental design, prediction and verification of the model equation. The last one is obtaining the response surface plot and contour plot of the response as a function of the independent parameters and determination of optimum points (Bas and Boyaci, 2007).

In the first stage of RSM, the parameters that have major effects on the biochemical process are identified from among several others affecting it. Screening experiments are useful to identify these independent parameters.

Factorial designs may be used for this purpose (Bas and Boyaci, 2007). Plackett- Burman design (PBD), is a screening design which yields unbiased estimates of all main effects in the smallest design possible. Various number or 'n' factors can be screened in an 'n+1' run PB design. A characteristic feature is that the sample size is a multiple of four (4k observations with k =1, 2…n). PB designs are proposed for more than seven factors. Such designs are known as saturated designs. The main advantage of saturated designs is that only a minimum number of observations are needed to calculate an effect for a certain factor.

After the identification of the important parameters, the direction in which improvements lie is determined and the levels of the parameters are identified. Determination of these levels is important because the success of optimization process directly relates to these levels. The units of the independent variables differ from one another and even if the units are the same, not all of these parameters will be tested over the same range. Since parameters have different units and/or ranges in the experimental domain, the regression analysis should not be performed. Instead, one must first normalize the parameters before performing a regression analysis. Each of the coded variables is forced to range from -1 to +1, so that they all affect the response more evenly, and so the units of the parameters are irrelevant. Commonly used equation for coding is seen below:

$$X = \frac{x - [x_{max} + x_{min}]/2}{[x_{max} - x_{min}]/2}$$

where x is the natural variable, X is the coded variable and x_{max} and x_{min} are the maximum and minimum values of the natural variable (Bas and Boyaci, 2007).

The second stage involves the selection of the experimental design, and prediction and verification of model equation. Some computer packages offer optimal designs based on the special criteria and input from the user. These designs differ from one another with respect to their selection of experimental

points, number of runs and blocks. After selection of the design, the model equation is defined and coefficients of the model equation are predicted. The model used in RSM is generally a full quadratic equation or the diminished form of this equation.

The second order model can be written as follows:

$$Y= \beta_0 + \Sigma \beta_i Xi + \Sigma \beta_{ii} X_i^2 + \Sigma \Sigma \beta_{ij} X_i X_j$$

Where Y is the predicted response where β_0, β_i, β_{ii} and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction coefficients respectively and X_i and X_j are coded independent variables. The system of equations given above is solved using the method of least squares (MLS). Method of least squares is a multiple regression technique (Bas and Boyaci, 2007). Once the regression coefficients are obtained, the estimated response could be easily calculated using model equation.

The central composite design (CCD) is the most frequently and extensively used RSM design. CCD is a well established, widely used statistical technique for determining the key factors from a large number of medium components by a small number of experiments (Soni *et al.,* 2007). The CCD has three groups of design points:

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

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

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

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

The advantages that RSM offers are that, a large amount of information can be obtained from a small number of experiments. Indeed, classical methods are time consuming and a large number of experiments are needed to explain the behaviour of a system. In RSM it is also possible to observe the interaction effect of the independent parameters on the response. Especially in biochemical processes, the interaction effect of the parameters would be more critical such as synergism, antagonism, and addition.

A huge amount of data is available on the production and scale-up operation of enzymes especially proteases from terrestrial microbes. But, their marine counterparts still remains a largely untapped resource of products that are of potential interest to mankind. Though a few enzymes from marine organisms have reached the market, there is still a lack of research into bioreactor engineering and bioprocess design in the area of cultivation of marine organisms to produce enzymes. There are only a few reports of

quantitative evaluation of protease production by marine bacteria especially Vibrios in a bioreactor. Therefore an attempt has been made to develop a bioprocess design for optimizing the physical parameters as well as the media components that are necessary for maximum production of alkaline protease from the marine bacterium *Vibrio* sp. (V26) using the statistical approach of RSM . An effort has also made to scale up the operation from a shake flask to fermenter level.

4.1 Review of literature

4.1.1 Production of proteases

Protease production is an inherent property of all organisms and these enzymes are generally constitutive, however at times they are partially inducible (Gupta *et al.,* 2002a). Under most culture conditions, microorganisms produce protease during post-exponential and stationary phases.

Members of the genus *Bacillus* from many different environments have been explored and exploited for alkaline protease production. Some of the most potent protease producing bacilli strains are *Bacillus licheniformis, B. subtilis, B. amyloliquefaciens* and *B. mojavensis* (Gupta *et al*., 2005a). Another major bacterial source is *Pseudomonas* sp. (Bayoudh *et al.,* 2000).

Among fungi strains of *Aspergillus, Neurospora, Pencillium, Cephalosporium, Myxococcus, Rhizopus* and *Basidiobolus* have been identified as sources of alkaline protease (Gupta *et al.,* 2005a). A highly active alkaline protease from *Conidiobolus coronatus* has been studied by Phadatare *et al.* (1993). Damare *et al.* (2006) have been successful in isolating deep sea fungi *A. ustus*, capable of producing alkaline protease. Chi *et al.,* (2007) have been able to optimize media and conditions for the production of alkaline protease from marine yeast *Aureobasidium pullulans*.

Different methods of submerged fermentations such as batch (Moon and Parulekar, 1991; Chu *et al.,* 1992; Beshay and Moreira, 2003; Reddy *et al.*,

2008), fed batch (Moon and Parulekar, 1991; Beg *et al.,* 2002b; Beshay and Moreira, 2005), continuous (Christiansen and Neilsen, 2000a, 2000b) and chemostat cultures have been adopted for the production of alkaline proteases.

Batch submerged processes have been widely adopted for the production of different types of proteases like alkaline protease, metalloproteases and subtilisins (serine protease) from different marine microbial sources (Narinx *et al.,* 1997; Estrada-Badillo and Marquez-Rocha, 2003; Kumar *et al.,* 2004; Joo and Chang, 2006; Venugopal and Saramma, 2006; Chi *et al.*, 2007; Reddy *et al.,* 2008).

Continuous culture provides constant reactor conditions for growth and product formation and supplies uniform-quality products. The application of continuous culture techniques has not been studied extensively and data about growth parameters correlated with product formation are very scarce. The efficiency of growth and production of exocellular protease in *Bacillus licheniformis* using different limitations in the chemostat, at a variety of growth rates have been investigated by Frakena *et al.* (1986).

In fed-batch culture, nutrients are continuously or semi-continuously fed, while effluent is removed discontinuously. This type of operation is intermediate between batch and continuous processes, increasing the duration of batch cultivation and the overall reactor productivity (Blanch and Clark, 1996; Shluer and Kargi, 2003). The use of fed-batch operations for the production of protease from *B. mojavensis* and *B. sphaericus* has been reported (Beg *et al.,* 2002b; Singh *et al.,* 2004). Beshay and Moreira (2005) reported enhanced production of a novel alkaline protease from a newly isolated strain of *Teredinobacter turnirae* via fed-batch cultivation using a constant feeding strategy of C and /or N sources. The maximum protease activity was achieved after about 25 hours and the fed-batch culture was found to be superior to batch culture of *T. turnirae* in producing alkaline protease with a production nearly 2.6-fold greater than those observed in batch operations.

Apart from free cells, immobilized cells have also been utilized for production of proteases from microbes. In immobilization, the producer cells are restricted in a fixed space. Immobilized whole cells of *Teredinobacter turnirae* in calcium alginate and on different inorganic matrices beads were used to produce alkaline protease (Elibol and Moreira, 2003; Beshay and Moreira, 2003). The alkaline protease activity produced by immobilized whole cells was higher than that produced by freely suspended cells under the same cultivation conditions. Adinarayana *et al*. (2005) investigated the production of alkaline proteases with immobilized cells of *B. subtilis* PE-11 in various matrices by entrapment whereas, Potumarthi *et al.* (2008) evaluated the various parameters of calcium-alginate immobilization method for enhanced alkaline protease production by *Bacillus licheniformis* NCIM-2042 using statistical methods.

The term solid-state fermentation (SSF) denotes cultivation of microorganisms on solid, moist substrates in the absence of a free aqueous phase (Sarkar *et al.*, 2010). SSF technology has been more often employed for the production of enzymes from fungi than bacteria. SSF technology has been known for centuries; from approximately 2,600 BC, it was used by the Egyptians for making bread, and information on the "koji process" dates back to 1,000 BC. The koji process is still used in the soy-sauce industry and can be considered as the prototype of SSF. Application of SSF for production of marine enzymes is a recent development. The nature of the solid support used may either be organic and utilizable by the producing microorganism or inert and not assimilated by the cultivated organism (Ooijkaas *et al.*, 2000; Hölker and Lenz, 2005). The production of extracellular alkaline protease by a marine shipworm bacterium *Teredinobacter turnirae* under solid-state process was optimized by Elibol and Moreira (2005). Various process parameters influencing protease production by *Engyodontium album* an alkalophilic and salt tolerant fungus isolated from marine sediment were evaluated for maximal

enzyme production using wheat bran as solid substrate and aged seawater incorporated with various nutrients as moistening medium (Chellappan *et al.,* 2006). Whereas, air pressure pulsation solid state fermentation (APPSSF) was applied to produce alkaline protease by *Bacillus pumilus* AS 1.1625 and the enzyme yield obtained by this method was more than twice that produced by static SSF (Aijun *et al.,* 2005).

Research efforts in the field of alkaline protease production have been mainly directed towards optimization of environmental and fermentation parameters, evaluation of effects of various carbon and nitrogenous nutrients on enzyme yields and requirement of metal ions in the fermentation medium.

4.1.2 Physical parameters

4.1.2.1 Aeration and Agitation

The growth of microorganisms and enzyme production is significantly affected by agitation and aeration (Garciade Fernando *et al.,* 1991). For aerobic fermentation, oxygen transfer is a key variable and is a function of aeration and agitation. Therefore, it is essential to establish optimum combination of airflow and agitation for maximum yield (Potumarthi *et al.,* 2007). The aeration rate indirectly influences the dissolved oxygen levels in the fermentation broth. Different dissolved oxygen profiles can be obtained by varying the aeration rate or variations in agitation speeds or use of the oxygen rich or oxygen-deficient gas phase (Kumar and Takagi, 1999).

The variation in the agitation speed influences the extent of mixing in the shake flasks or bioreactors and will also affect the nutrient availability (Kumar and Takagi, 1999). The agitation rate required for maximum alkaline protease production varies with the organism used. Agitation rates that have been found optimal for protease production from bacteria such as *Vibrio fluvialis* VM 10, *Pseudomonas aeruginosa* Pse A, *Bacillus cereus* and *Bacillus*

sp.103 were 100, 140, 200 and 250 rpm respectively (Banik and Prakash, 2004; Joo *et al.*, 2004; Gupta *et al.,* 2005b; Venugopal and Saramma, 2006).

Mixing is important in the microbial synthesis of protease enzyme in free cell bioreactor and can be imparted by means of aeration and agitation (Potumarthi *et al.,* 2007). Maximum protease production from *V. harveyi* was noted at an agitation rate of 700 rpm and at an aeration rate of 0.5 vvm, whereas, from *B. clausii* I-52 it was at 700 rpm and 1.5 vvm (Estrada-Badillo *et al.,* 2003; Joo and Chang, 2006). The best combination of airflow and agitation rate for the production of alkaline protease by submerged fermentation in a batch STR using *Bacillus licheniformis* NCIM-2042 was at an airflow rate of 3 vvm and agitation speed of 200 rpm (Potumarthi *et al.,* 2007).

4.1.2.2 pH

Culture pH strongly affects many enzymatic processes and transportation of various components across the cell membrane. In view of a close relationship between protease synthesis and the utilization of nitrogenous compounds (Moon and Parulekar, 1991), pH variations during fermentation might indicate kinetic information about protease production, such as the start and end of the protease production period (Kumar and Takagi, 1999). Alkaline protease production is often reported in the neutral to alkaline range (Johnvesly and Naik, 2001; Johnvesly *et al.,* 2002; Gupta *et al.,* 2005b; Asokan and Jayanthi, 2010). pH as high as 10.5 has been found ideal for protease production by *Vibrio metschnikovii* DL 33-51 (Mei and Jiang, 2005) while pH 7 was found optimal for *Vibrio fluvialis* VM 10 (Venugopal and Saramma, 2006).

4.1.2.3 Temperature

Temperature is a crucial parameter that has to be controlled and the optimum temperature for enzyme production varies from organism to

organism. Temperature strongly affects the synthesis of proteases either non-specifically, influencing the rates of biochemical reactions, or specifically, inducing or repressing their production. Ray *et al.* (1992) had shown that temperature can regulate the synthesis of extracellular proteases by microorganisms. Studies by Frankena *et al.* (1986) showed that a link existed between enzyme synthesis and energy metabolism in bacteria, which was controlled by temperature and oxygen uptake. Temperature was also proven to regulate enzyme synthesis at mRNA transcription and probably translation levels (Votruba *et al.,* 1991). It thus regulates the synthesis of several enzymes, including intracellular and extracellular enzymes. For extracellular enzymes, temperature influences their secretion, possibly by changing the physical properties of the cell membrane.

Optimum temperature values reported for maximum protease production is often in the mesophilic range (Peek *et al.,* 1992; Engel *et al.,* 1998; Secades and Guijarro, 1999; Joo *et al.,* 2004; Mei and Jiang, 2005; Venugopal and Saramma, 2006; Asokan and Jayanthi, 2010). A temperature optimum as high as 55ºC for enzyme secretion has also been reported (Johnvesly and Naik, 2001).

4.1.3 Media components

In commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation (Kumar and Takagi, 1999).

4.1.3.1 Complex and defined media

As the complex pathways within the cell are regulated through single or multiple intermediary metabolites or / and end products, the composition of the bioprocess medium that directs the intracellular reaction rates is critical (Çalık and Özdamar, 2002). Alkaline protease production has been reported in both

complex (Hubner *et al.,* 1993; van Putten *et al.,* 1996; Joo *et al.,* 2002; Çalık *et al.,* 2003) and chemically defined /synthetic medium (Mao *et al.,* 1992; Çalık *et al.,* 1999; Johnvesly and Naik, 2001), though investigations in complex media are clearly much more. On an industrial scale, enzymes such as alkaline protease are produced in complex media (Shikha *et al.,* 2007).

4.1.3.2 Nitrogen source

In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. The alkaline protease comprises 15.6% nitrogen and its production is dependent on the availability of both carbon and nitrogen sources 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.

Low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Chandrasekaran and Dhar, 1983; Sen and Satyanarayana, 1993; Yum *et al.,* 1994). Production of alkaline protease was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids (Litchfield and Prescott, 1970; Chu *et al.,* 1992). At the same time there are reports that the use of ammonium sulphate, potassium nitrate (Sinha and Satyanaryana, 1991), and sodium nitrate (Banerjee and Bhattacharyya, 1992) had resulted in an increase or stimulation in protease production. Contradictory to this are the observations of Fujiwara and Yamamoto (1987a), Chauhan and Gupta (2004) and Rao *et al.* (2006) where organic nitrogen sources were found to better than inorganic nitrogen sources for protease production. Corn steep liquor (Fujiwara and Yamamoto 1987a; Sen and Satyarayana, 1993), soybean meal (Sen and Satyarayana, 1993; Joo and Chang, 2006; Tari *et al.,* 2006), defatted-soybean (Çalık *et al.*, 2003), Soyabean (Elibol and Moreira, 2005) and soy powder (Rao *et al.,* 2006) have been used as cheap source of nitrogen for protease production. Some workers

have even reported the use of more than one nitrogen source for alkaline protease production. Hubner *et al*. (1993) have reported the use of soybean and ammonium phosphate as the nitrogen sources for production of alkaline serine protease subtilisin Carlsberg by *Bacillus licheniformis* while Rao *et al.* (2006) used a complex mixture of nitrogen sources.

4.1.3.3 Carbon source

Reduction in protease production due to catabolite repression by glucose has been reported (Frankena *et al.,* 1985, 1986; Hanlon *et al.,* 1982; Kole *et al.,* 1988). Zamost *et al.* (1990) correlated the low yields of protease with lowering of pH brought about by the rapid growth of the organism. Glucose and lactose were reported as effective carbon sources for protease production in some strains of *Bacillus* (Gessesse, 1997; Mabrouk *et al.,* 1999). There are a few reports where glucose was found to enhance protease production (Adinarayan and Ellaiah, 2002). As in commercial practice high carbohydrate concentrations repressed enzyme production (Kumar and Takagi, 1999), carbohydrates are either added continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited and thereby reduce power requirements (Aunstrup, 1980).

Though sugars like maltose, sucrose and lactose have been found to increase protease production, repression in enzyme synthesis was observed with these ingredients at high concentrations (Kumar and Takagi, 1999). Organic acids like acetic acid (Ikeda *et al.,* 1974) and citric acid (Johnvesly and Naik, 2001) have been found to enhance protease production. Whey has been used as a carbon source for alkaline protease production (Donaghy and Mc Kay, 1993).

4.1.3.4 Metal Ions

Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese and molybdenum are required in fermentation medium for optimum production of alkaline protease. A high concentration of

potassium phosphate (> 2g/L) was found to inhibit cell growth and repress protease production (Ikemura *et al.,* 1987). Potassium phosphate has been used as a source of phosphate in most studies and has shown to be involved in buffering the medium (Kumar and Takagi, 1999). Quite a number of workers have investigated the influence of metal ions on protease production (Liao and Mc Callus, 1998; Chauhan and Gupta, 2004; Abdel-Fattah *et al.,* 2009; Akolkar *et al.,* 2009).

4.1.4 RSM and Optimization

A popular class of screening designs is the Plackett-Burman design (PBD), developed by Plackett and Burman in 1946. It was designed to improve the quality control process that could be used to study the effects of design parameters on the system state so that intelligent decisions can be made.

PBD has been widely used for screening of media components as well as physical factors essential for extracellular protease production by different organisms. Starch concentration, casamino acid concentration, phosphate ion concentration, inoculum percentage and period of incubation were identified as the five key determinants necessary for maximizing extracellular protease production by *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004) while they were corn starch, yeast extract, corn steep liquor and inoculum size for *Bacillus* sp. RKY3 (Reddy *et al.,* 2008). Soybean flour and $FeCl_3$ were identified as significant factors influencing the protease production by *Halobacterium* sp. SP1(1) as screened by Plackett–Burman method (Akolkar *et al.,* 2009). To screen the bioprocess parameters significantly influencing the alkaline protease activity, a 2-level PBD was applied by Abdel-Fattah *et al.* (2009) Among 15 variables tested, the pH, peptone, and incubation time were selected based on their high positive significant effect on the protease activity.

RSM was developed by Box and his colleagues at Imperial Chemical Industries (1951). The graphical perspective of the mathematical model has led to the term Response Surface Methodology (Bas and Boyaci, 2007). RSM has

become a very popular technique for optimization studies in recent years. Many scientists have reported satisfactory optimization of protease production from microbial sources using statistical approach. Beg *et al*. (2003) reported the use of RSM to study the effects of various physicochemical factors on alkaline protease production from *B. mojavensis*. Further, the predicted model of RSM for protease production was validated in a 14 L bioreactor. The response surface approach involving a face-centered central composite design (FCCCD) was adopted for improving protease production by the *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004). Ramnani *et al.* (2005) too used the FCCCD to improve the production of protease and biosurfactant by *B. licheniformis* RG1. The effects of soyabean meal, maltose 50, Tween 80 and initial pH on protease production were evaluated using RSM by Tari *et al.* (2006). The central composite design (CCD) was used in the case of *Bacillus* sp. RKY3 (Reddy *et al.,* 2008) for improving protease production. The three variables, sucrose, yeast extract and KNO_3, which had played a significant role in enhancing the production of alkaline protease, was optimized with response surface methodology (Oskouie *et al.,* 2008). CCD of RSM was applied for the optimization studies of media parameters for extracellular protease production by *Halobacterium* sp. SP1(1) (Akolkar *et al.,* 2009). Zhou *et al.* (2009) in their study optimized the mycelial biomass and protease production by *Laccocephalum mylittae* in submerged fermentation using RSM and orthogonal matrix method. Rai and Mukherjee (2010) too have used response surface methodology to attain an optimum protease production of 518 U by *Bacillus subtilis* DM-04 in submerged fermentation. Manikandan *et al.* (2011) carried out the optimization of growth medium for protease production by *Haloferax lucentensis* VKMM 007 with help of RSM.

Apart from proteases, the other marine enzymes whose production has been optimized using the statistical design of experiments with the future prospect of large scale processing include chitinase (Gohel *et al.*, 2006; Patel *et*

al., 2007; Han *et al.,* 2008), phytase (Li *et al.,* 2008), esterase (Ping *et al.,* 2008), laccase (Bonugli-Santos *et al.,* 2010) etc.

RSM has also been used for the optimization of biochemical processes such as : cholesterol oxidase production by *Rhodococcusequi* no. 23 (Lee *et al.,* 1998b), butylgalactoside synthesis by galactosidase from *Aspergillus oryzae* (Ismail *et al.,* 1999), hydrolysis of pectic substrates (Panda and Naidu, 1999), enzymatic synthesis of fatty esters (Aracil *et al.,* 1999), lipase-catalyzed incorporation of docosahexanoic acid (DHA) into borage oil (Senanayake and Shahidi, 2002), phytase production by *Pichia anomala* (Vohra and Satyanarayana, 2002), determination of reaction parameters for damaged starch assay (Boyaci *et al.,* 2004), biotransformation of 2-phenylethanol to phenylacetaldehyde in a two-phase fed-batch system (Celik *et al.,* 2004), lipase catalyzed esterification reactions (Manohar and Divakar, 2004), pectinase usage in pretreatment of mosambi juice for clarification (Rai *et al.,* 2004), laccase catalyzed degradation of reactive textile dyes (Cristo´va˜o *et al.,* 2010) and enhanced decolourization of Direct Red-80 dye by the white rot fungus *Phanerochaete chrysosporium* (Singh *et al.,* 2010).

4.2 Materials and Methods

4.2.1 Experimental Organism

The strain *Vibrio* sp. (V26) was used.

4.2.2 Medium Used

Nutrient broth supplemented with 1% gelatin (composition, section *3.2.1.2* was used as culture medium (whereever there is any change in composition it has been mentioned).

4.2.3 Protease Production

4.2.3.1 Preparation of inoculum

The inoculum was prepared as described in section ***3.2.1.3***

4.2.3.2 Inoculation and Incubation

The Optical density (O.D) of the pre inoculum culture suspension was read at 600 nm in a UV-VIS spectrophotometer (UV-1601, Shimadzu Corporation, Tokyo, Japan). A volume adequate to obtain an absorbance of 0.02 at 600 nm for the total medium was added to 50 ml production broth in a 250 ml conical flask (1 O.D corresponds to 3.85 x 10^{10} cells / ml). Inoculated flasks were incubated in a rotary shaker.

4.2.3.3 Estimation of growth

Growth was determined by measuring the optical density of the culture at 600 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601).

4.2.3.4 Estimation of Protease production

The culture broth was centrifuged at 8000 x g for 15 min at 4ºC. The cell free supernatant obtained was assayed for alkaline protease activity as mentioned in section ***3.2.2.1***

4.2.4 Part A: Optimization of physical parameters

Optimization of process parameters was carried out using Nutrient broth supplemented with gelatin as the production medium.

4.2.4.1 Experimental Design

The process parameters pH, temperature, salinity and agitation were optimized statistically using the full factorial Face Centered Central Composite Design (FCCCD) of the RSM. The software Design expert (Version 6.0.9, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis and the quadratic model building. The optimal levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using the same software.

4.2.4.2 Optimization by RSM

The effect of the process parameters pH, temperature, agitation and salinity on alkaline protease production by *Vibrio* sp. (V26) had been previously studied (Venugopal, 2004) using 'one variable at a time' approach. These parameters had been identified as important factors influencing protease production under batch fermentation. The minimum and the maximum limits of these key process variables were fixed on the basis of this study. Face-centered central composite design (FCCCD) of RSM was utilized to find out the alkaline protease production by *Vibrio* sp. (V26) at different combinations of these process parameters and to determine ideal values of these parameters that would maximize the production of the enzyme. A 2^4-full factorial FCCCD, with eight axial points and six replications at the centre points (n_0=6) leading to a total number of 30 experiments was carried out for the optimization of the four chosen independent process variables. The lower and higher limits of these parameters were taken as -1 and +1 respectively in Face Centered Central Composite Design of RSM. Each of the four process parameters were varied over three different levels (+1, 0,-1). All the variables were taken at a central coded value which is considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form are listed in Tables 4.1 and 4.2.

Media were prepared in all the combinations of pH and salinity mentioned in Table 4.2. After inoculation the flasks were incubated in the respective combinations of temperature and shaking speeds. The growth measurements (section ***4.2.3.3***) and estimation of alkaline protease production (section ***4.2.3.4***) was done after 24 hrs (based on standardization) of incubation.

Upon completion of experiments, the average maximum protease production was taken as the dependent variable or response (Y). A second order polynomial equation was then fitted to the data by multiple regression procedure. This resulted in an empirical model that related the response measured in the independent variables to the experiment. For a four factor system, the model equation was:

$$Y= \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

Where, Y, predicted response;, β_0 intercept; β_1, β_2, β_3, β_4,, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, squared coefficients; β_{12}, β_{13}, β_{14}, , β_{23}, β_{24}, β_{34}, interaction coefficients.

The statistical significance of the model equation and the model terms were evaluated via Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination, R^2, and the adjusted R^2. The fitted polynomial equation was then expressed as three-dimensional surface plots to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study

Table 4.1

Levels of the variables tested in FCCCD

Variables	Symbol	Coded Levels		
		-1(Low)	0(Mid)	+1 (High)
Agitation (rpm)	(A)	50	100	150
Initial pH (H+ ions)	(B)	6	7.5	9
Temperature (°C)	(C)	25	30	35
Salinity (%)	(D)	0	1.5	3

Table 4.2

Face-Centered Composite Design Matrix of the four physical parameters

Run order	Coded Levels			
	Agitation (A) (rpm)	Initial pH (B) (H+ions)	Temperature (C) (°C)	Salinity (D) (%)
1	0 (100)	1 (9)	0 (30)	0 (1.5)
2	-1 (50)	-1 (6)	-1 (25)	-1(0)
3	-1 (50)	0 (7.5)	0 (30)	0(1.5)
4	0 (100)	0 (7.5)	0(30)	0(1.5)
5	-1 (50)	1(9)	-1(25)	1(3)
6	0 (100)	0 (7.5)	-1(25)	0(1.5)
7	1 (150)	-1(6)	1(35)	-1(0)
8	-1 (50)	-1(6)	-1(25)	1(3)
9	0 (100)	0 (7.5)	0(30)	0(1.5)
10	0 (100)	0 (7.5)	0(30)	-1(0)
11	0 (100)	-1(6)	0(30)	0(1.5)
12	0 (100)	0 (7.5)	1(35)	0(1.5)
13	-1 (50)	-1(6)	1(35)	1(3)
14	1 (50)	0 (7.5)	0(30)	0(1.5)
15	0 (100)	0 (7.5)	0(30)	0(1.5)
16	0 (100)	0 (7.5)	0(30)	1(3)
17	1(150)	-1(6)	1(35)	1(3)
18	-1 (50)	-1(6)	1(35)	-1(0)
19	1(150)	1(9)	1(35)	1(3)
20	1(150)	-1(6)	-1(25)	-1(0)
21	1(150)	1(9)	1(35)	-1(0)
22	0 (100)	0 (7.5)	0(30)	0(1.5)
23	-1 (50)	1(9)	-1(25)	-1(0)
24	0 (100)	0 (7.5)	0(30)	0(1.5)
25	1(150)	-1(6)	-1(25)	1(3)
26	-1 (50)	1(9)	1(35)	-1(0)
27	1(150)	1(9)	-1(25)	1(3)
28	0 (100)	0 (7.5)	0(30)	0(1.5)
29	1(150)	1(9)	-1(25)	-1(0)
30	-1 (50)	1(9)	1(35)	1(3)

The actual values of factors are given in brackets

4.2.4.3 Validation of model

The validation of statistical model was done by carrying out the experiment at optimum values of the process parameters (agitation, pH, temperature and salinity) as determined from the model. For this media was prepared with 1.37% NaCl and pH 7.66. After inoculation the production flasks were incubated in an incubator shaker at 29.7ºC (± 0.5) with a shaking speed of 147 rpm. After 24 hrs the growth (section ***4.2.3.3***) and alkaline protease production (section ***4.2.3.4***) were determined. The experimental values obtained were compared to the values predicted by the model.

4.2.4.4 Inoculum size

To investigate the effect of inoculum size on protease production; the protease production was carried out using various inoculum sizes i.e., 0.02, 0.04, 0.08 and 0.1 O.D. A volume adequate to obtain desired absorbance (0.02, 0.04, 0.08 and 0.1 O.D) at 600 nm for the total medium was added to 50 ml production broth in a 250 ml conical flask. The inoculated flasks were incubated at the optimized conditions. The results were analyzed statistically using one-way ANOVA. Mean of the results was compared at a significance level of $p < 0.05$ (Appendix 3).

4.2.5 Part B: Optimization of media components

The range of each of the media components used in the optimization studies (PBD) were selected based on the experimental results of the first step optimization done using the traditional "one variable-at-a-time" approach (Appendix 3).

4.2.5.1 Shake flask studies

4.2.5.1.1 Identification of the significant variables using Plackett–Burman designs

Plackett–Burman design (PBD) is an efficient way to screen out the main physico-chemical parameters from among a large number of process

variables, affecting protease production. Nutrient broth with 1% gelatin was supplemented with metal ions $CaCl_2$, $MgSO_4$ and KH_2PO_4 for this study. The ranges of peptone, beef extract, yeast extract and gelatin were fixed on the basis of first step optimization (Appendix 3) and that of metal ions on the basis of previous study on this strain (Venugopal, 2004).

The Plackett–Burman design allows the evaluation of N (11) variables in N+1(12) experiments; each variable was examined at two levels: –1 for a low level and +1 for a high level. The variables chosen for the present study were peptone, beef extract, yeast extract, gelatin, $CaCl_2$, $MgSO_4$ and KH_2PO_4. Total of seven components (Table 4.3) were selected for the study with each variable being represented at two levels, high (+) and low (-), and four dummy variables in 12 trials (Table 4.3). Each row represents (Table 4.4) a trial and each column represents an independent (assigned) or dummy variable (factor whose level does not change/ not chosen as a variable).

Table 4.3 illustrates the factors under investigation as well as the levels of each factor used in the experimental design. Table 4.4 represents the design matrix. "Design Expert® 6.0.9" Stat-Ease, Inc., Minneapolis, USA, was used to analyze the experimental Plackett–Burman design. Media was prepared in all the combinations of media components mentioned in Table 4.4.

After inoculation (as per section ***4.2.3.2***) the production flasks were incubated in an incubator shaker at 29.7^{o}C (± 0.5) with a shaking speed of 147 rpm. The growth measurements (section ***4.2.3.3***) and alkaline protease production assays (section ***4.2.3.4***) was done after 24 hrs of incubation.

Table 4.3

Levels of the variables tested in Plackett–Burman Design

Variables	Symbol	Coded Levels	
		-1(Low)	+1(High)
Peptone (g/100 ml)	(A)	0	1
Beef Extract (g/100 ml)	(B)	0	0.3
Yeast Extract (g/100 ml)	(C)	0	0.3
Gelatin (g/100 ml)	(D)	0.2	1
KH_2PO_4 (mM)	(E)	0.01	1
$CaCl_2$ (mM)	(F)	0.01	1
$MgSO_4$ (mM)	(G)	0.05	1

Factors H, J, K and L are dummy variables. The letter I is absent by default in the software

Table 4.4

Plackett–Burman Design Matrix for media components

Run order	A	B	C	D	E	F	G	H	J	K	L
1	-1	1	1	-1	1	-1	-1	-1	1	1	1
	(0)	(0.3)	(0.3)	(0.2)	(1)	(0.01)	(0.05)				
2	-1	1	1	1	-1	1	1	-1	1	-1	-1
	(0)	(0.3)	(0.3)	(1)	(0.01)	(1)	(1)				
3	-1	-1	1	1	1	-1	1	1	-1	1	-1
	(0)	(0)	(0.3)	(1)	(1)	(0.01)	(1)				
4	-1	-1	-1	1	1	1	-1	1	1	-1	1
	(0)	(0)	(0)	(1)	(1)	(1)	(0.05)				
5	1	1	-1	1	-1	-1	-1	1	1	1	-1
	(1)	(0.3)	(0)	(1)	(0.01)	(0.01)	(0.05)				
6	1	-1	1	1	-1	1	-1	-1	-1	1	1
	(1)	(0)	(0.3)	(1)	(0.01)	(1)	(0.05)				
7	1	-1	1	-1	-1	-1	1	1	1	-1	1
	(1)	(0)	(0.3)	(0.2)	(0.01)	(0.01)	(1)				
8	1	1	1	-1	1	1	-1	1	-1	-1	-1
	(1)	(0.3)	(0.3)	(0.2)	(1)	(1)	(0.05)				
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
	(0)	(0)	(0)	(0.2)	(0.01)	(0.01)	(0.05)				
10	1	1	-1	1	1	-1	1	-1	-1	-1	1
	(1)	(0.3)	(0)	(1)	(1)	(0.01)	(1)				
11	1	-1	-1	-1	1	1	1	-1	1	1	-1
	(1)	(0)	(0)	(0.2)	(1)	(1)	(1)				
12	-1	1	-1	-1	-1	1	1	1	-1	1	1
	(0)	(0.3)	(0)	(0.2)	(0.01)	(1)	(1)				

The actual values of factors are given in brackets

4.2.5.1.2 Optimization of media by Face-Centered Central Composite Design

The next step in the optimization of the medium was to determine the optimal levels of the significant variables in alkaline protease production. The FCCCD of RSM was adopted for the augmentation of total protease production. The most significant variables as determined from PBD were as follows: Peptone, yeast extract and $MgSO_4$, each of which was assessed at three coded levels (–1, 0, +1), as shown in Table 4.5. A total of 20 trials were employed with six replicates at the centre point. All variables were taken at a central coded value that was defined as zero. The coded and actual values of the variables at various levels are given in Table 4.5 and the full experimental plan with respect to their values in actual and coded form is listed in Table 4.6.

Media were prepared in all the combinations of the three components peptone, yeast extract and $MgSO_4$ as mentioned in Table 4.6 (the other media components that were not further optimized were added in the following concentrations: beef extract: 0.15 g/ 100 ml, gelatin: 0.2 g/ 100 and KH_2PO_4: 0.01 mM).

After inoculation (as per section ***4.2.3.2***) the production flasks were incubated in an incubator shaker at 29.7 ^oC (± 0.5) with a shaking speed of 147 rpm. The growth measurements (section ***4.2.3.3***) and alkaline protease production assays (section ***4.2.3.4***) was done after 24 hrs of incubation.

The data obtained from the RSM on protease production were subjected to analysis of variance (ANOVA). After running the experiments and measuring the activity levels, the experimental results of RSM were fitted with the response surface regression procedure using the following second-order polynomial equation:

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

Where, Y is the response; β_0 intercept; β_1, β_2, β_3 linear coefficients; β_{11}, β_{22} and β_{33} squared coefficients; β_{12}, β_{13} and β_{23} interaction coefficients.

The statistical significance of the model equation and the model terms were evaluated via Fisher's test. The quality of fit of the second-order polynomial model

equation was expressed via the coefficient of determination, R^2, and the adjusted R^2. The fitted polynomial equation was then expressed as three-dimensional surface plots to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

Table 4.5

Levels of the variables tested in FCCCD for media components

Variables	Symbol	Coded Levels		
		-1(Low)	0 (Mid)	+1(High)
Peptone (g/100 ml)	(A)	0.1	1.3	2.5
Yeast Extract (g/100 ml)	(B)	0.15	0.57	1
$MgSO_4$ (mM)	(C)	0.01	0.04	0.07

Table 4.6

Face-Centered Composite Design Matrix of three media variables

Run order	Coded Levels		
	Peptone (A)	Yeast extract (B)	$MgSO_4$ (C)
1	0 (1.3)	0 (0.57)	0 (0.04)
2	-1 (0.1)	-1 (0.15)	1 (0.07)
3	1 (2.5)	-1 (0.15)	1(0.07)
4	0 (1.3)	0 (0.57)	0 (0.04)
5	1 (2.5)	-1 (0.15)	-1 (0.01)
6	1 (2.5)	0 (0.57)	0 (0.04)
7	0 (1.3)	0 (0.57)	-1 (0.01)
8	0 (1.3)	0 (0.57)	0 (0.04)
9	0 (1.3)	0 (0.57)	1(0.07)
10	0 (1.3)	0 (0.57)	0 (0.04)
11	1 (2.5)	1 (1)	1(0.07)
12	0 (1.3)	-1 (0.15)	0 (0.04)
13	-1 (0.1)	0 (0.57)	0 (0.04)
14	0 (1.3)	1 (1)	0 (0.04)
15	1 (2.5)	1 (1)	-1 (0.01)
16	0 (1.3)	0 (0.57)	0 (0.04)
17	-1 (0.1)	1 (1)	1(0.07)
18	-1 (0.1)	-1 (0.15)	-1 (0.01)
19	0 (1.3)	0 (0.57)	0 (0.04)
20	-1 (0.1)	1 (1)	-1 (0.01)

Values in brackets are the actual values of variable

4.2.5.1.3 Validation of model

The validation of statistical model was done by carrying out the experiment at optimum values of the media parameters (peptone, yeast extract and $MgSO_4$) as determined from the model. The final composition of media is given in Table 4.7. 50 ml of media was prepared in 250 ml flasks and sterilized. Pre-inoculum was prepared and inoculation done as mentioned in section ***4.2.3.1*** and ***4.2.3.2.*** After inoculation the production flasks were incubated in an incubator shaker at 29.7°C (± 0.5) with a shaking speed of 147 rpm. After 24 hrs, the growth (section ***4.2.3.3***) and alkaline protease production were assayed (section ***4.2.3.4***). The experimental value was compared to the value predicted by the model.

4.2.5.2 Bioreactor studies

The conditions obtained as optimum through RSM were finally examined and verified in a 5 L Bench top bioreactor with a working volume of 2 L (media composition Table 4.7).

Fermenter

A continuous stirred tank bioreactor with a total volume of 5 L (B-Lite, Laboratory fermenter system, Sartorius, Germany) was used.

Medium Preparation

Modified nutrient broth

Two Litre (2 L) medium was prepared. All the ingredients in required amounts were dissolved in 2 L of distilled water (Table 4.7) and transferred into the fermenter. The vessel was assembled as directed in the operation manual. The vessel and the reagent/ sampling bottles already connected by silicone tubing were sterilized at 121ºC for 60 minutes.

Inoculation

The inoculum was prepared as mentioned in previous section (Section ***4.2.3.2***). A volume adequate to obtain an absorbance of 0.02 at 600 nm for the total medium was added to 2 L of medium in the bioreactor. The inoculum (~20 ml) was aseptically transferred into the fermenter through the inoculum port.

Fermentation conditions

Fermentation was performed under the following conditions: temperature- 29.7^{o}C, pH- 7.66 (as derived from RSM), aeration rate- 0.5v/v/m and agitation speed 300 rpm.

pH was under automated control. For the control of pH 500 ml of sterile 1N NaOH and HCl were provided. pH was maintained at 7.66 ± 0.1 by the automatic addition of the acid or base.

Sterile 10 % silicone (Himedia Laboratories Limited, Mumbai) constituted the antifoam.

Air was provided from an oil-free pump (Model OF01080, (Elgi Equipments Limited, Coimbatore) with a working pressure of 8 kgf/cm^{3}). Rotameter controlled the air flow rate into the fermenter vessel. A sterile filter (0.22 μm) was used as a bridge between the tubing from the rotameter and that connected to the air sparger of the fermenter. A second filter on the exit gas cooler stopped the microbes from releasing into the laboratory air as the gas left the fermenter under a slight positive pressure.

Table 4.7

Composition of optimized media

(Modified Nutrient broth)	
Peptone	12.8 g/L
Yeast Extract	5.6 g/L
Beef Extract	1.5 g/L
Gelatin	2 g/L
NaCl	1.37%
$MgSO_4$	0.07 mM
KH_2PO_4	0.01 mM
pH	7.66

4.3 Results

4.3.1 Part A: Optimization of physical parameters

4.3.1.1 Face Centered Central Composite Design of RSM for optimization of physical factors

The statistical design approach using response surface methodology was used to study the individual and interactive effects of various process parameters on alkaline protease production by *Vibrio* sp. (V26). The ranges of the independent process variables were selected on the basis of a previous study (Venugopal, 2004) on this strain using the 'one variable at a time strategy'.

The study showed that the conditions that promoted protease production were found to be different from those that enhanced the growth of the cell. Fig.4.1.

Table 4.8 summarizes the protease production (response in terms of protease activity) of the experiment (FCCCD) for each individual run along with the predicted response. The experiments were performed in triplicates and average values were taken. The results obtained after FCCCD were then

analyzed by standard analysis of variance (ANOVA), which gave the following regression equation (in terms of coded factors) of the levels of protease produced (Y) as a function of Agitation (A), pH (B), temperature (C) and salinity (D) .

Enzyme activity = -26456.86749 + 0.66418 * A +3078.29470 * B + 1043.23165 * C + 738.06035 * D + 0.023395 * A^2 - 199.49257 * B^2 - 17.42493 * C^2 - 216.08812 * D^2 - 0.23292 * A * B + 0.15243 * A * C-0.33976 * A * D - 1.63508 * B * C +3.68417 * B * D - 4.13675 * C* D

ANOVA for the model was performed and is summarized in Table 4.9. The model *F*-value was found to be 76.55. High Model *F*-value with a very low probability value [(Prob >*F*) less than 0.0001] implied that model was highly significant. The linear terms A and D and the quadratic terms B^2, C^2, D^2 of the model were found to be significant (Prob > F less than 0.0500) while interactions were found to be 'not significant'. The value of multiple correlation coefficient R^2 was found to be 0.987, which indicated that the model can explain 98.7% variation in the response. Adjusted R^2 and Predicted R^2 values were 0.974 and 0.930 respectively. Also, the model had an "adequate precision value" of 25.608, this suggested the model can be used to navigate the design space. The model showed coefficient of variation (CV), standard deviation, mean and predicted residual sum of squares (PRESS) values of 10.02% 99.96, 997.15 and 7.586E+005 respectively. For this model the 'lack of fit' was found to be 'not significant'.

The software Design-Expert suggested solutions based on the data generated. The optimum values of the physical parameters and predicted response of the selected solution are given in table 4.10.

The regression equation is represented in 3-D response surface plots and 2-D contour plots. The yield for different values of the variables can also be predicated from the respective response surface plots (Fig. 4.2 A-F). Three-dimensional (3D) response surface curves were plotted to determine the optimum concentration of each factor for maximum protease production. Fig.4.2 shows the relative effects of six different sets of factors—agitation and pH (Fig. 4.2. A), agitation and temperature (Fig. 4.2. B), agitation and salinity (Fig. 4.2. C), pH and temperature (Fig.4.2. D), pH and salinity (Fig.4.2. E) and temperature and salinity (Fig.4.2.F)—when all the other factors were kept at their optimum levels. The results clearly showed a fairly strong degree of curvature of 3D surface, from where the optimum was determined.

4.3.1.2 Validation

The validation of the solution (optimized condition) suggested by the model was done experimentally. Experimental value 2064.375 U (±23.74) was very close to the predicted value of 2073.04 U validating the model.

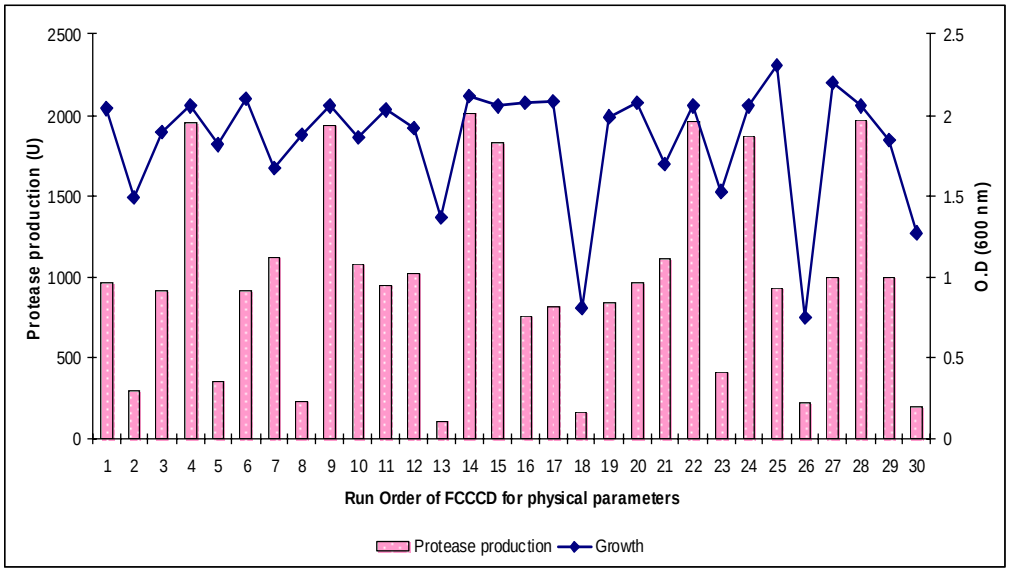


Fig. 4.1 Relationship between growth and protease production

Table 4.8

Face-centered central composite design of the four independent physical parameters along with the observed and predicated values of alkaline protease production

Run order	Coded Levels				Protease Activity (U)	
	Agitation (A)	pH (B)	Temperature (C)	Salinity (D)	Observed	Predicted
1	0	1	0	0	967.97	984.67
2	-1	-1	-1	-1	299.48	247.27
3	-1	0	0	0	908.85	1080.40
4	0	0	0	0	1957.29	1922.50
5	-1	1	-1	1	362.39	355.06
6	0	0	-1	0	916.49	996.72
7	1	-1	1	-1	1121.46	1130.81
8	-1	-1	-1	1	229.82	221.76
9	0	0	0	0	1940.00	1922.50
10	0	0	0	-1	1075.39	979.67
11	0	-1	0	0	944.10	927.40
12	0	0	1	0	1022.05	941.82
13	-1	-1	1	1	109.36	53.12
14	1	0	0	0	2017.91	1846.36
15	0	0	0	0	1828.13	1922.50
16	0	0	0	1	762.00	857.72
17	1	-1	1	1	823.00	879.27
18	-1	-1	1	-1	163.67	202.74
19	1	1	1	1	839.41	893.64
20	1	-1	-1	-1	962.93	1022.91
21	1	1	1	-1	1106.00	1112.03
22	0	0	0	0	1966.67	1922.50
23	-1	1	-1	-1	405.72	347.42
24	0	0	0	0	1871.00	1922.50
25	1	-1	-1	1	926.95	895.47
26	-1	1	1	-1	220.33	253.84
27	1	1	-1	1	1000.00	958.90
28	0	0	0	0	1971.88	1922.50
29	1	1	-1	-1	994.92	1053.18
30	-1	1	1	1	199.39	137.38

Table 4.9

Analysis of variance (ANOVA) for the fitted quadratic polynomial model of alkaline protease production

Source	Sum of Squares	DF	Mean Square	F value	Prob > F
Model	1.071E+007	14	7.648E+005	76.55	< 0.0001 *
A	2.640E+006	1	2.640E+006	264.23	< 0.0001 *
B	14755.33	1	14755.33	1.48	0.2444
C	13559.20	1	13559.20	1.36	0.2635
D	66926.77	1	66926.77	6.70	0.0215 *
A^2	8786.27	1	8786.27	0.88	0.3643
B^2	5.175E+005	1	5.175E+005	51.79	< 0.0001 *
C^2	4.874E+005	1	4.874E+005	48.78	< 0.0001 *
D^2	6.072E+005	1	6.072E+005	60.77	< 0.0001 *
AB	4882.87	1	4882.87	0.49	0.4960
AC	23235.67	1	23235.67	2.33	0.1495
AD	10389.22	1	10389.22	1.04	0.3252
BC	2406.15	1	2406.15	0.24	0.6312
BD	1099.42	1	1099.42	0.11	0.7450
CD	15401.43	1	15401.43	1.54	0.2348
Residual	1.399E+005	14	9991.72		
Lack of Fit	1.224E+005	9	13602.24	3.89	0.0743
Pure Error	17463.91	5	3492.78		
Cor Total	1.092E+007	29			

DF – Degrees of freedom, R^2 = 0.987, Adjusted R^2 =0.974, CV=10.02%.
* indicates significant terms of the model

Table 4.10

Solution for the Model

Solution	Agitation	pH	Temperature	Salinity	Predicted Response
1	147 rpm	7.66	29.7 ° C	1.37%	2073.04

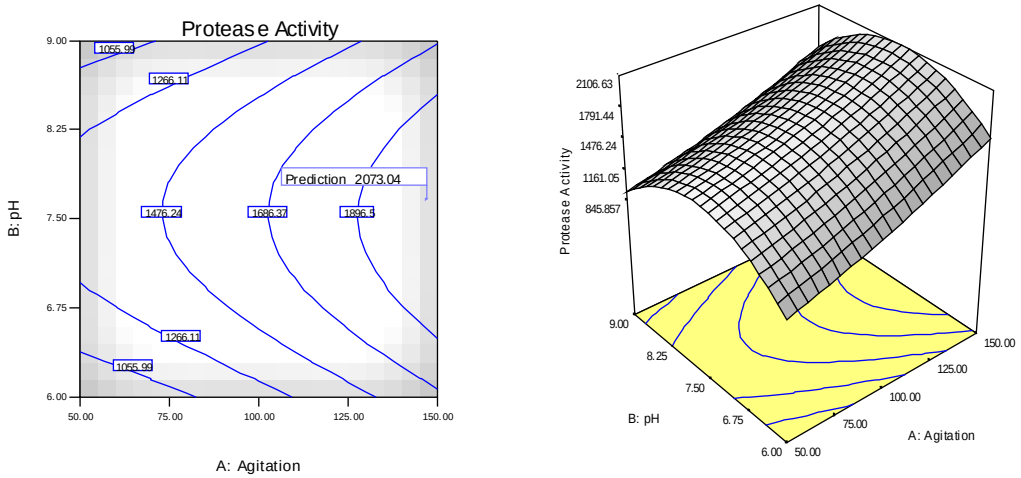


Fig.4.2 A) Contour and Response Surface Plots showing the relative effects of agitation and pH on the production of alkaline protease. The other two variables temperature and salinity are held at their optimal levels.

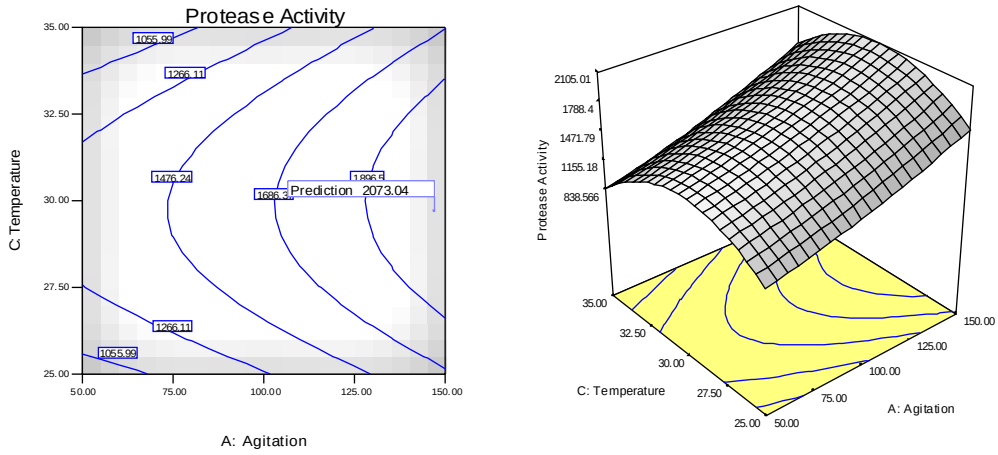


Fig.4.2 B) Contour and response surface plots showing the relative effects of agitation and temperature on the production of alkaline protease. The other two variables pH and salinity are held at their optimal levels.

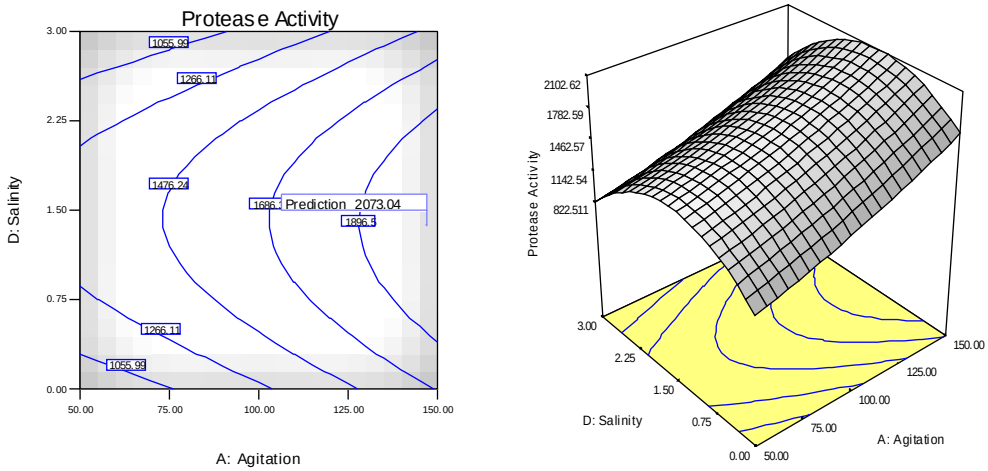


Fig.4.2 C) Contour and response surface plots showing the relative effects of agitation and salinity on the production of alkaline protease. The other two variables pH and temperature are held at their optimal levels.

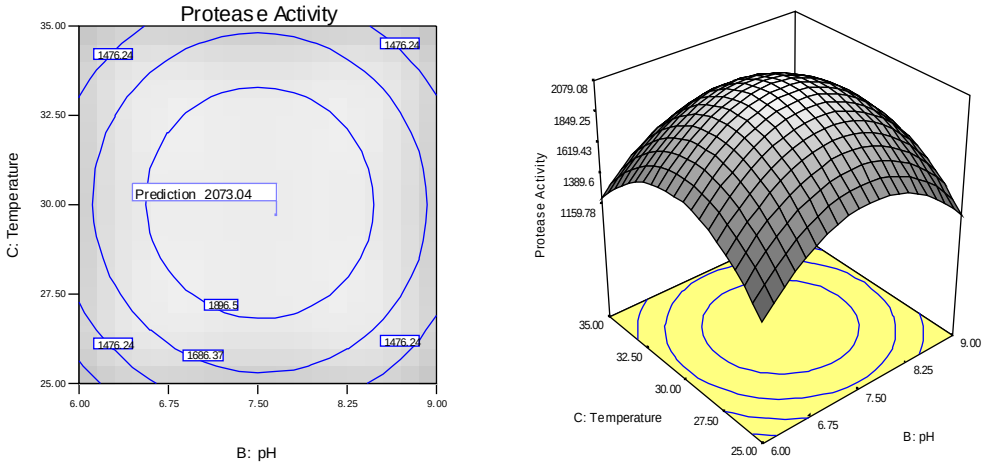


Fig.4.2 D) Contour and response surface plots showing the relative effects of temperature and pH on the production of alkaline protease. The other two variables salinity and agitation are held at optimal levels

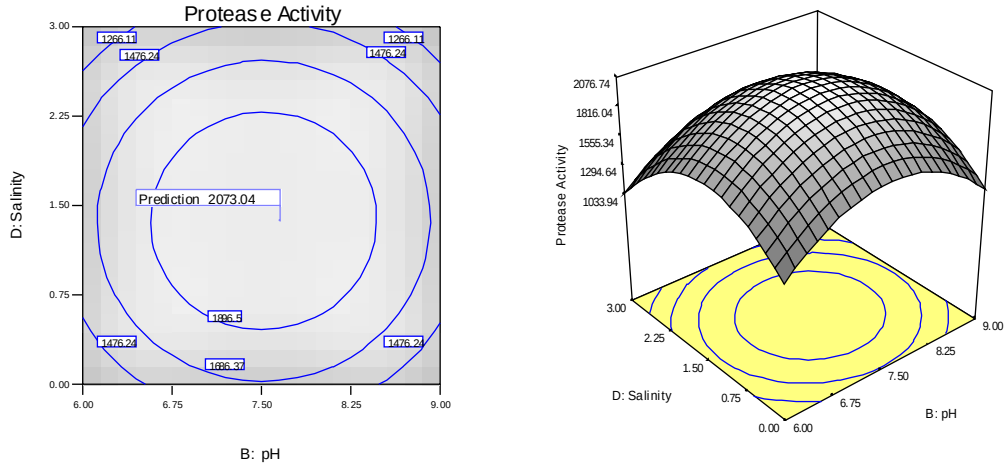


Fig.4.2 E) Contour and response surface plots showing the relative effects of salinity and pH on the production of alkaline protease. The other two variables temperature and agitation are held at optimal levels.

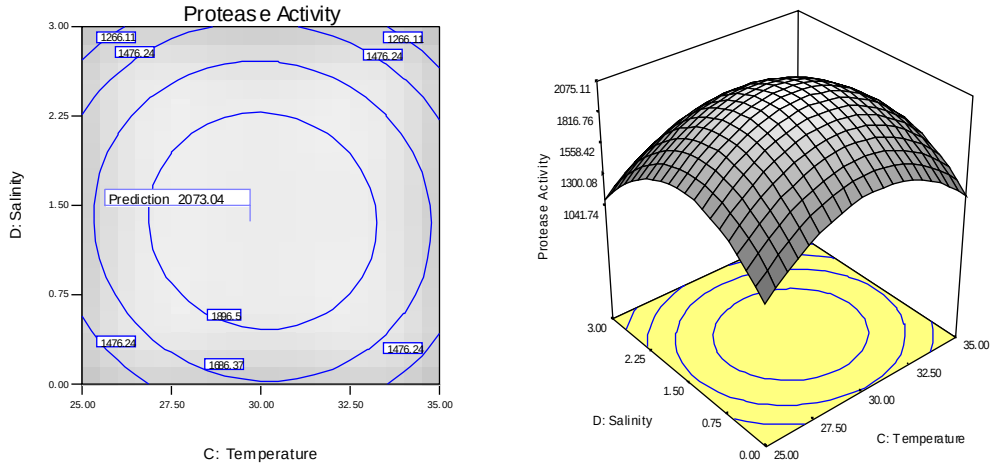


Fig.4.2 F) Contour and response surface plots showing the relative effects of salinity and temperature on the production of alkaline protease. The other two variables pH and agitation are held at optimal levels.

Fig.4.2. (A to F) The two-dimensional contour plots and three-dimensional response surface plots showing the effect of process parameters and their interaction on alkaline protease production by *Vibrio* sp. (V 26)

4.3.1.3 Inoculum size

The inoculum size was found to affect protease production (Fig.4.3). Higher inoculum size (0.08 and 0.1 O.D) decreased the protease production. 0.02 O.D was chosen as the optimal inoculum size as it protease production under these condition was not significantly different from 0.04 O.D were maximum enzyme production was noted

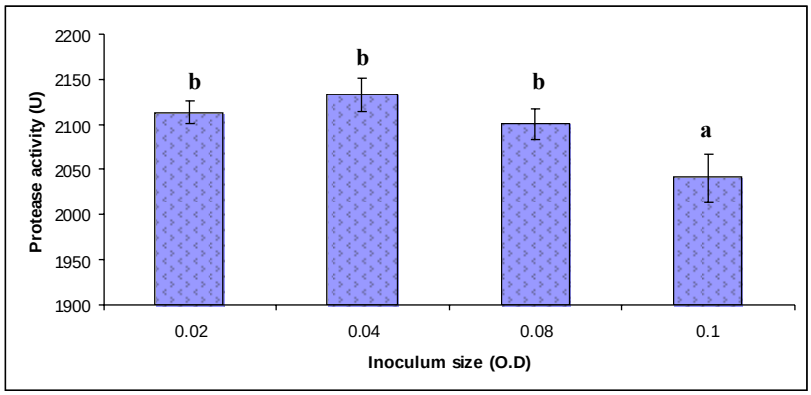


Fig.4.3 Effect of inoculum size on alkaline protease production
Values with same superscripts did not vary significantly

4.3.2 Part B: Optimization of Media components

4.3.2.1 Shake flask studies

4.3.2.1.1 Screening of media components using PB Design

A total of seven variables (media components) were analyzed with regard to their effects on protease production using a Plackett–Burman design (Table 4.11). The adequacy of the model was determined. The "Model *F*-value" of 19.14 and low probability value of 0.0063 [(Prob >*F*)] implied that the model was significant. The variables evidencing statistically significant effects were screened. Factors evidencing values of "Prob > F" less than 0.05 were considered to have significant effects on the response and they were A (Peptone, *p*-value =0.0014), B (Beef extract, *p*-value= 0.0285), C (Yeast extract, *p*-value=0.0059), D (Gelatin, *p*-value =0.0436) and G ($MgSO_4$, *p*-value= 0.0184). On analysis of the values of the regression coefficients of all the factors, it was

found that all of them had a positive effect on protease production. But on the basis of percentage contribution it was found that the contribution of $CaCl_2$ was negligble so this ingredient was avoided from the media in the next round of optimiization. Among the significant media components Peptone, Yeast Extract and $MgSO_4$ having confidence level (%) > 98 % were further optimized using FCCCD.

Table 4.11

Plackett–Burman Design for media components with the corresponding observed and predicted values of alkaline protease production

Run order	A	B	C	D	E	F	G	H	J	K	L	Protease Activity (U)	
												Actual	Predicted
1	-1	1	1	-1	1	-1	-1	-1	1	1	1	1645.49	1623.58
2	-1	1	1	1	-1	1	1	-1	1	-1	-1	2012.15	2128.36
3	-1	-1	1	1	1	-1	1	1	-1	1	-1	1854.43	1739.27
4	-1	-1	-1	1	1	1	-1	1	1	-1	1	977.083	997.95
5	1	1	-1	1	-1	-1	-1	1	1	1	-1	2094.27	1979.11
6	1	-1	1	1	-1	1	-1	-1	-1	1	1	2015.63	1943.20
7	1	-1	1	-1	-1	-1	1	1	1	-1	1	2224.17	2295.55
8	1	1	1	-1	1	1	-1	1	-1	-1	-1	2193.75	2215.65
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	40.36	207.09
10	1	1	-1	1	1	-1	1	-1	-1	-1	1	2201.56	2367.25
11	1	-1	-1	-1	1	1	1	-1	1	1	-1	2013.75	1942.37
12	-1	1	-1	-1	-1	1	1	1	-1	1	1	1151.04	984.32

4.3.2.1.2 Face Centered Central Composite Design for optimization of media components

FCCCD of RSM was used to optimize further the three most significant factors from the previous screening experiment using PB design i.e., Peptone (A), Yeast Extract (B) and $MgSO_4$ (C).

Table 4.12 summarizes the response (protease production in terms of protease activity) for each individual run along with the predicted response of the FCCCD. The results obtained after FCCCD were then analyzed using

ANOVA. The RSM gave the following regression equation for the Protease production Y as a function of Peptone (A), Yeast Extract (B) and $MgSO_4$ (C).

Final equation in terms of coded factors:

Enzyme activity Y = +840.23586 +1180.09055 * A + 1901.29214 * B - 4050.08299 *C - 237.05022 * A^2 - 843.54001 * B^2 + 1.04096E+005 * C^2 - 705.83384 * A * B -2405.82873 * A * C - 790.33906 * B * C

The results of ANOVA for the model are summarized in Table 4.13. The values of *F*- statistics was found to be 59.02. High Model *F*-value with a very low probability value [(Prob >*F*) less than 0.0001] implied that the model was highly significant. The linear terms A and C , the quadratic terms A^2 and B^2 and interaction terms AB and AC of the model were found to be significant ((Prob > F) less than 0.0500). In this model 'A' stands for Peptone, 'B' for Yeast Extract and 'C' for $MgSO_4$. The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient R^2 = 0.983, i.e. the model can explain 98.3% variation in the response. Adjusted R^2 and Predicted R^2 values were 0.967 and 0.821 respectively. Also, the model has an "adequate precision value" of 22.11 which suggested that the model can be used to navigate the design space. The model showed coefficient of variation (CV), standard deviation, mean and predicted residual sum of squares (PRESS) values of 3.01%, 59.68, 1985.66 and 3.438E+005 respectively. For this model the 'lack of fit' was found to be 'not significant' ((Prob > *F*) = 0.1127).

The software suggested solutions based on the data generated. The optimum values of the media parameters and predicted response of the selected solution is given in Table 4.14

The regression equation is represented in 3-D response surface plots and 2D contour plots (Fig.4.4).The contour plots were elliptical indicating that the interactions between Peptone and Yeast extract (Fig.4.4A) and Peptone and $MgSO_4$ (Fig. 4.4.B) were significant. (Only the significant interactions have

been plotted). Three-dimensional (3D) response surface curves were plotted to determine the optimum concentration of each factor for maximum protease production. Fig.4.4 A shows the response for the interactive factors peptone and yeast extract when $MgSO_4$ (0.07 mM) was kept at its optimal value. At low concentration of peptone when the concentration of yeast extract was increased, there was proportional increase in protease production. While at a low concentration of yeast extract when the concentration of peptone was increased upto 1.3 g/100ml there was a corresponding increase in the production of the enzyme. However a further increase in peptone concentration did not result in any proportional increase in production and at about 2.5 g/100 ml the production was seen to drop. A simultaneous increase or decrease in the concentration of both peptone and yeast extract resulted in a decrease in production. Maximum protease production predicted (2274.25 U) was observed near the mid-point (0) values (concentrations) of both peptone (1.3 g/100 ml) and yeast extract (0.57 g /100 ml).

Figure 4.4.B shows response for the interactive factors peptone and $MgSO_4$, when yeast extract was kept at its optimal value (0.56 g/100 ml). Up to 1.3% concentration of peptone, when the levels of $MgSO_4$ was increased the protease production was seen to increase. However at higher concentrations of peptone, even low levels of $MgSO_4$ had a negative effect on enzyme production.

4.3.2.1.3 Validation

The validation of the solution (optimized condition) suggested by the model was done experimentally. Experimental value 2262.15 U (± 36.11)) was very close to the predicted value of 2274.25 validating the model.

4.3.2.2 Enzyme Production in a Bioreactor

Further scale up studies was done in 5 L fermenter (Fig. 4.5)**.** The experimental value 2257.8 U (±64) was also very close to the predicted value

of 2274.25 U validating the model. The production was found to peak at 21-22 hrs and then a drop was noted.

Table 4.12

Face-centered central composite design matrix of the three independent media components along with actual and predicted values of alkaline protease production

Run order	Coded Levels			Protease Activity (U)	
	Peptone (A)	Yeast extract (B)	$MgSO_4$ (C)	Actual Value	Predicted Value
1	0	0	0	2196.88	2243.52
2	-1	-1	1	1309.38	1350.65
3	1	-1	1	2242.19	2247.92
4	0	0	0	2206.25	2243.52
5	1	-1	-1	2078.13	2065.40
6	1	0	0	1912.50	1961.74
7	0	0	-1	2037.50	2053.72
8	0	0	0	2260.42	2243.52
9	0	0	1	2216.67	2157.91
10	0	0	0	2181.25	2243.52
11	1	1	1	1500.78	1489.17
12	0	-1	0	2157.00	2100.48
13	-1	0	0	1934.38	1842.60
14	0	1	0	1836.46	1850.44
15	1	1	-1	1609.00	1578.36
16	0	0	0	2291.67	2243.52
17	-1	1	1	2239.84	2263.20
18	-1	-1	-1	1262.24	1284.48
19	0	0	0	2239.58	2243.52
20	-1	1	-1	2001.04	2005.95

Table 4.13

Analysis of variance (ANOVA) for the fitted quadratic polynomial model of alkaline protease production

Source	Sum of Squares	DF	Mean Square	F value	Prob > F
Model	1.892E+006	9	2.102E+005	59.02	< 0.0001*
A	35488.08	1	35488.08	9.96	0.0116*
B	828.72	1	828.72	0.23	0.6410
C	27138.67	1	27138.67	7.62	0.0221*
A^2	2.517E+005	1	2.517E+005	70.68	< 0.0001*
B^2	63667.25	1	63667.25	17.88	0.0022*
C^2	17327.13	1	17327.13	4.87	0.0548
AB	1.037E+006	1	1.037E+006	291.08	< 0.0001*
AC	48388.43	1	48388.43	13.59	0.0050*
BC	812.34	1	812.34	0.23	0.6443
Residual	32052.26	9	3561.36		
Lack of Fit	23196.77	4	5799.19	3.27	0.1127
Pure Error	8855.49	5	1771.10		
Cor Total	1.978E+006	19			

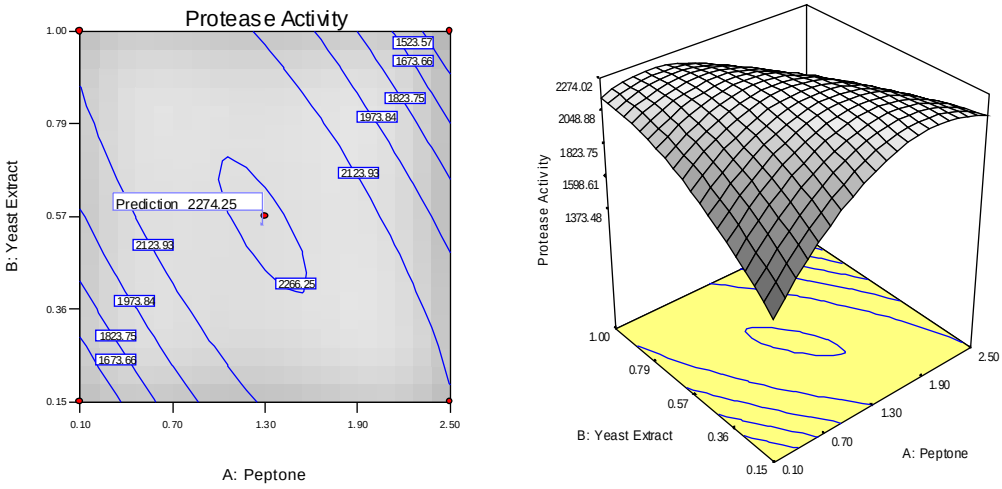
DF – Degrees of freedom, R^2 = 0.983, Adjusted R^2 =0.967, CV=3 %

* Indicate significant model terms

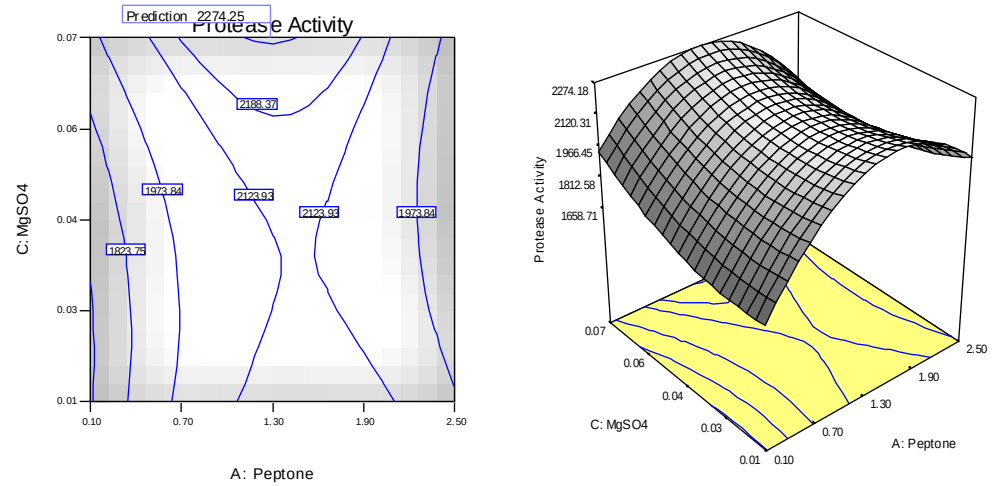
Table 4.14

Solution for the model

Solution	Peptone	Yeast Extract	$MgSO_4$	Predicted Response
1	1.28 g/ 100 ml	0.56 g/ 100 ml	0.07 mM	**2274.25 U**



A) Contour and response surface plots showing interactions of Peptone and Yeast extract on the production of alkaline protease. The third variable $MgSO_4$ is held at its optimal level.



B) Contour and response surface plots showing the interaction of Peptone and $MgSO_4$ on the production of alkaline protease. The third variable Yeast extract is held at its optimal value.

Fig.4.4 A-B The two-dimensional contour plots and three-dimensional response surface plots showing the effect of media components and their mutual interactions on alkaline protease production by *Vibrio* sp. (V 26)

Fig.4.5 Alkaline protease production by *Vibrio* sp. (V26) in 5 L stirred-tank fermenter

4.4 Discussion

There has been a tremendous increase in the use and acceptance of statistical experimental designs in biotechnology. Statistical experimental planning, factorial design and design of experiments investigate defined input factors to a converting system from which mostly common and well-defined output factors or responses such as product yield and productivity are generated.

In the present investigation, based on RSM study, various physical parameters and media components have been found to have profound effects on protease production. An overall study of physical parameters indicates that production of alkaline protease by *Vibrio* sp. (V26) is non-growth dependent (Fig.4.1) which is well in agreement with the observation of others (Moon and Parulekhar, 1991; Genckal and Tari, 2006).

The effects of process parameters on alkaline protease production by *Vibrio* sp. (V26) were analyzed using ANOVA. The analysis revealed that the quadratic regression model was highly significant as it had a high Fisher's F-value (76.55) and a very low probability value ((Prob> F) less than 0.0001). The goodness of fit of the model was determined from the value of R^2 (multiple correlation coefficient/determination coefficient), which gives measure of how much variability in the observed response could be explained by the environmental/physical factors and their interactions. This value was very high for this model (0.987) which indicates that 98.7 % variation can be explained, while only 1.3% cannot be explained by the model. R^2 values more than 0.9 indicates a high correlation and a model value with R^2 value between 0.7 and 0.9 is considered having a good correlation (Guilford and Frucher, 1973; Haaland, 1989). This meant, in the present case, that an R^2 value of 0.987 reflected a good fit between the observed and predicted responses, and it was reasonable to use the regression model to analyze the trends in the responses.

The 'adequate precision value' is an index of the signal (controllable factors) to noise (uncontrollable) and a value greater that 4 is an essential prerequisite for a model to be a good fit. In this model the adequate precision value was found to be 25.608, indicating adequate signal. A relatively lower value of the coefficient of variation (CV=10.02%) indicated a good precision and reliability of the experiment. The 'Lack of fit' is a measure of how well the model fits the data. If the model has a significant lack of fit, then it is not a good predictor of the response and should not be used. In this model the 'lack of fit' (*p* value = 0.0743) was found to be 'not significant'.

Enviromental factors have been found to affect the production of extracellular proteolytic enzymes. In this study, it was found that the linear terms of the regression equation agitation (A) and salinity (D) were found to significantly influence the production of alkaline protease by *Vibrio* sp. (V26). Whereas the other two process parameters pH and temperature (B and C) were found to be 'not significant' within the experimental range (design space). The quadratic terms B^2, C^2, D^2 too were found to be significant. If factor is significant at the quadratic level, it can act as a limiting factor (Elibol, 2004)

The 3D response surface plots and the 2D contour plots generated by the software are given in Fig 4.2 (A-F). These plots help to understand the interaction of the factors in the model and optimum values of each factor required for maximum protease production. In this, the response surface plots are plotted holding two variables constant while varying the other two within their experimental range. The response curves (especially 4.2D, 4.2E,4.21F) were convex in nature suggesting a well defined optimum for protease production (Rao *et al*., 2006). From the nature of the response surface contours whether elliptical, circular or saddle point, interaction between the variables may be predicted (Singh *et al*., 2010). In this study, the circular plots (Fig.4.2D, 4.2E, 4.2F) indicated that the interactions between physical parameters are 'not significant'.

Salinity was found to be significant at both linear and quadratic levels, which highlights its influence on protease production by *Vibrio* sp. (V26). Vibrios are primarily aquatic microorganisms and most species require Na^+ / seawater for growth. In the present study (Fig. 4.2C, 4.2E and 4.2 F) maximum protease production was noted at 1.37% salinity which is close to the mid-point value (1.5%) of salinity and production was found to drop towards both its low level (0%) and high level value (3%). Kim (2004) too observed that the production of extracellular proteases in *Vibrio* sp. strain 60 was affected by the concentration of NaCl; its production in the presence of 3% NaCl was severely inhibited when compared to that in the presence of 1% NaCl. In this study also 3% NaCl concentration was found to be more inhibitory than 0% NaCl. Protease production was not affected by Na+ electrochemical potential but was instead affected by the H+ electrochemical potential (Kim, 2004). The maximum production of protease by *Bacillus subtilis* BS1 was found to occur in presence of 1.5% NaCl (Shaheen *et al*., 2008) a concentration very similar to that observed in this study.

Agitation rate has been shown to affect protease production (Hare *et al*., 1981; Fernando *et al*., 1991). In the present study, the production of alkaline protease was seen to increase sharply from 50 to 150 rpm and the response surface curve (Fig4.2A, 4.2B and 4.2C) almost becomes almost flat as it reaches 150 rpm, indicating that maximum enzyme yield was observed near maximum level (+1) of agitation. The optimum agitation speed was identified as 147 rpm. An increase in aeration of the media at the optimal agitation speed resulted in increased nutrient uptake, which turn could be responsible for the hike in protease production (Kumar and Takagi, 1999; Beg *et al*., 2003). Agitation was found to be the most significant ($p < 0.0001$) linear term in the regression equation clearly revealing its influence. The optimum agitation rate for protease production was found to be 150 rpm for *Bacillus* sp. (No.1) (Razak *et al*., 1997) while it was 170 rpm for *B. cereus* (Shafee *et al*., 2005). In the

present study, production of the enzyme was low at 50 rpm and it was found to fall drastically at stationary conditions (Appendix 3). This could be due to lower availability of dissolved oxygen with low mixing rates (Potumarthi *et al*., 2007). This is consistent with the observations of Hare *et al*. (1981) for *Vibrio alginolyticus,* Fernando *et al.* (1991) for *Enterococcus faecalis* subsp. *liquefaciens* and Beg *et al.* (2002b) for *B. mojavensis* where shaking was required to stimulate protease production. In contrast, Dainty *et al.* (1978) and Rahman *et al*. (2005) reported that *Chromobacterium lividum* and *Pseudomonas aeruginosa* respectively produced protease under static conditions. Even higher agitation rate (200 rpm) was found to negatively affect the response in this study (Appendix 3). A possible explanation for this observation is the denaturation of enzymes at high agitation rates (Burkert, *et al.,* 2005). Similar observations were made by Shafee *et al*. (2005).

pH 7.66 was found to be most optimal for the enzyme production. However, in this study it was found that pH within the range 6-9 did not significantly affect alkaline protease production by *Vibrio* sp. (V26). The production of protease over a wide range of pH (5-8) has been reported (Razak *et al*., 1997; Rahman *et al*., 2005). At the same time there are reports of high pH requirements for enzyme production by microbes (Kumar *et al*., 2004; Ellaiah *et al*., 2005; Genckal and Tari 2006; Jaswal *et al*., 2008). The influence of pH could be due to its effect on the stability or conformation of the plasma membrane, which might indirectly affect the membrane-bound ribosomes involved in the protease synthesis (Razak *et al*., 1997).

Temperature is one of most important bioprocess parameters. Incubation temperature 29.7ºC i.e., 30^{o} C was found to be optimal in this study indicating the mesophilic nature of the strain. Many mesophilic protease producers such as *P. aeruginosa* (Engel *et al*., 1998), *Bacillus brevis* (Banerjee *et al*., 1999), *Yersinia ruckeri* (Secades and Guijarro, 1999) *Bacillus clausii* I-52 (Joo *et al*., 2003), *Bacillus* sp. (Genckal and Tari, 2006), *Halobacterium*

sp. SP1(1) (Akolkar *et al.,* 2009) have been reported. In this study, the production of protease was found to drop at temperatures above its optimum. Chaloupka *et al.* (1987) suggested that at high temperatures the inhibition might be due to repression at the level of mRNA transcription or translation. However among bacteria especially *Bacillus,* very high temperature optima such as 50^{o}C and 60^{o}C have been reported (Johnvesly and Naik, 2001, Beg *et al*., 2002b, Rai and Mukherjee, 2010).

Though protease production was maximum at an inoculum size of 0.04 O.D, it was not found to be significantly different from that at 0.02. Therefore lower inoculum size (0.02 O.D) was fixed as the optimal inoculum density. Higher inoculum sizes have been found to negatively affect protease production in the study. This is in agreement with previous observation that higher inoculum sizes might not necessarily give higher protease yield or cell growth. Higher inoculum sizes could result in the lack of oxygen and nutrient depletion in the culture media (Rahman *et al*., 2005).

As no single defined media has been established for optimal protease production from different microbial sources (Rao *et al*., 2006); nutrient broth supplemented with gelatin, a complex media was used in this study. Joshi and Ball (1993) reported protease activity by different species of alkaliphilic *Bacillus* in nutrient broth containing 4% gelatin. Complex media have been found suitable for protease production by several workers (Takami *et al*., 1989; Banerjee *et al*., 1999).

The biochemical and nutritional environment in a bioreactor is strongly influenced by growth conditions and medium composition, namely carbon and nitrogen sources, mineral salts, trace elements, peptides, amino acids, vitamins and/or other factors. Therefore, the optimization of culture medium is essential for the effective production in a bioreactor (Swift *et al*., 2000, Haq *et al*., 2003). The production of biomolecules, such as enzymes for food industry, often requires the use of complex and cheap industrial media based on yeast

extract and bactopeptone as nitrogen sources. Complex organic nitrogen sources are less expensive than pure amino acids and nitrogen bases and, additionally, can supply a variety of vitamins, minerals and other growth co-factors. Furthermore, cell growth is generally more rapid and efficient when these nutrients are used, since they reduce the number of components that cells would otherwise have to synthesize de novo (Calado *et al*., 2002). Therefore, nutrient broth supplemented with gelatin and metal ions was selected as the media to be used in this study.

When more than five independent variables are to be screened, the Plackett–Burman design is used to find out the most significant variables influencing the response (Stanbury *et al*., 1997). Based on this design (PBD) A (peptone), B (beef extract), C (yeast extract), D (gelatin) and G ($MgSO_4$) were found to be significant factors influencing protease production. The values of the coefficient of each of terms indicated that all factors used except $CaCl_2$ had a positive effect on alkaline protease production by the strain *Vibrio* sp. (V26). The overall percentage contribution of the factor $CaCl_2$ was found to be negligible and also the confidence level of this factor was found to be low, so this component was removed from the media in the next round of optimization experiments. The three most significant media components peptone, yeast extract and $MgSO_4$ were further optimized using FCCCD of RSM.

The effects of media components on protease production were analyzed using ANOVA. A high Fisher's F-value with low probability value [(Prob > *F*) < 0.0001] demonstrated that the quadratic regression model for the media components was highly significant. A R^2 (determination coefficient) value of 0.983 indicated a high 'goodness of fit' of the model and that only 1.7 % of the total variations are not explained by the model. The value of adjusted R^2 (Adjusted R^2 =0.967) was also very high. High R^2 value pointed to high correlation. The 'adequate precision value' of 22.114 denoted adequate signal; a value greater than 4 is essential. A relatively low value of the coefficient of

variation (CV=3%) indicated improved precision and reliability of the experiment. In this model the 'lack of fit' (*p*-value= 0.1127) was found to be 'not significant'. All the above considerations indicated an excellent adequacy of the regression model.

ANOVA revealed that the media components namely peptone (A) $MgSO_4$ (C) and interactions between peptone and yeast extract (AB) as well as between peptone and $MgSO_4$ (AC) have significant (p <0.05) influence on protease production. The quadratic terms of A^2 (peptone) and B^2 (yeast extract) were also found to be significant and the values of coefficients of both the quadratic terms were found to be negative; this indicated that the high concentrations of both peptone and yeast extract may inhibit enzyme synthesis. Repression of protease production by high concentrations of nitrogen sources have been reported (Frankena *et al*., 1986; Johnvesly and Naik, 2001; Adinarayana and Ellaiah, 2002). Peptone being significant at both linear and quadratic levels clearly revealed the influence of this ingredient on the production of alkaline protease by *Vibrio* sp. (V26). Adinarayana and Ellaiah (2002) too have found the factors Peptone and salt solutions as significant factors influencing alkaline protease production. Yeast extract is an inexpensive organic source of amino acids, proteins and vitamins for cell growth and synthesis of enzymes such as amylase and protease (Mehta *et al.,* 2006). In this study 0.56 % yeast extract was found to be optimum, while at high concentration it repressed enzyme secretion. A similar observation was made by Mehta *et al.* (2006).

In this investigation, carbon sources such as glucose and other saccharides were avoided due to widespread report in literature of repressive effect of readily metabolizable sugars on the mechanism of protease production (Johnvesly *et al.,* 2002; Joo *et al*., 2002; Elibol and Moreira, 2005). Peptone and yeast extract which are generally regarded as sources of nitrogen also seems to act as sources of carbon as well. Organic nitrogen sources such as

casein, soyabean meal and yeast extract are well known inducers of protease production (Beg *et al*., 2002b; Chauhan and Gupta, 2004; Reddy *et al*., 2008). In this study, gelatin was incorporated in the medium as an inducer for protease production. Kanekar *et al*. (2002) also reported that gelatin was a suitable carbon and nitrogen source for protease production by *B. alcalophilus.* In contrast are the reports of Ferrero *et al*. (1996) and Gusek *et al*. (1988) where the production of the enzyme was inhibited by organic nitrogen sources. In the present investigation (based on PBD) mixture of complex nitrogen sources seemed to work better than single source; similar observations were made by Rao *et al*. (2006). While Hubner *et al*. (1993) used a combination of inorganic and organic nitrogen source for enzyme production. The amino acid composition of the protein source is important in the fermentation process for enzyme (Çalık *et al*., 2003)

Inorganic compounds can contribute to the structures of metabolites or/and act as the cofactor of enzymes. Among the three metal ions tested only Mg^{2+} was found to be significant, and increase in its concentration resulted in an increase in the enzyme production. This could be due to the fact that Mg^{2+} is a trace element that regulates the reactions of the glycolysis pathway, i.e., glucose-6-phosphate synthesis from glucose; glucose-1,6-diphosphate synthesis from glucose-6-phosphate; and 3-phospho glycerate synthesis from 1,3-diphospho glycerate (Çalık *et al*., 2003). Uyar *et al*. (2011) too, observed that the addition of Mg^{2+} ions in the medium enhanced protease production. However Mg^{2+} ion supply to the production medium decreased serine alkaline production by recombinant *Bacillus subtilis* (Çalık *et al*., 2003). The different concentrations of Ca^{2+} did not have any effect on the enzyme production in this study. Sufficient supply of Ca^{2+} from other media ingredients could be a plausible explanation for this. Çalık *et al*. (2003) also noted that higher activity was obtained in the medium without $CaCl_2$ as Ca^{2+} supplied by soybean was sufficient for the bioprocess. This is quite different from the observations of

Vidyasagar *et al*. (2006) in which they found that Ca^{2+} at 200 mmol l^{-1} concentration enhanced protease production.

The peak production of alkaline protease by *Vibrio* sp. (V26) was noted at 21-22 hrs in the fermenter. The majority of the alkaline protease was secreted within the first 24 hrs, and this result indicates that alkaline protease production was associated to the late-exponential growth phase of the bacteria. Similar results have been reported in previous investigations (Kaur *et al*., 2001; Uyar and Baysal, 2004; Reddy *et al*., 2008)

Decline in protease production was noted both in the shake flask cultures and in the bioreactor (24 hrs) after maximum enzyme production. Autoproteolysis (Jang *et al*., 2001) and protease degradation by some proteolytic activity on the cell surface of nitrogen-starved cells in the cultivation broth (Chu *et al*., 1992) might be the reason for this observation. Similar observations were made by several workers (Chu *et al*., 1992; Gupta *et al*., 2002b; Rao *et al*., 2006).

An overall 2 fold (200%) increase in alkaline protease production was achieved under conditions optimized by RSM as compared with the conditions optimized by 'one-factor at a time' method. With regard to just the physical factors only 0.54 fold (54%) increase in production was noted. In a similar study an overall 2.3-fold increase in protease production was achieved in the optimized medium when compared to the un-optimized basal medium by Reddy *et al*. (2008).

The solution suggested by the model was validated both under shake flask and 5 L stirred tank bioreactor. Though the peak in enzyme production occurred slightly earlier in the fermenter; the enzyme production values obtained in reactor was slightly less. However the difference between these two production conditions was not found to be statistically significant (p value =0.282). Rao *et al*. (2006) in their study made note of similar observation that, though the extracellular protease production in 5 L stirred-tank peaked earlier

(i.e., in 6 days) when compared to that in shake flask (on the 7 th day) there was slight reduction in enzyme production (shake flask 281.14 U/ml; STR 238.77 U/ml). The activity however obtained in 30 L airlift bioreactor was close to that attained in shake flask with a cultivation time shorter by 4 days. They have suggested the use of airlift bioreactors as a possible alternative to stirred-tank bioreactors. Contradictory to this, Rai and Mukherjee (2010), observed that maximum protease yield of 518 U by *B. subtilis* DM-04 obtained by batch culture in 5 L fermenter post 60 hrs of incubation was slightly higher than the observed highest experimental value in shake-flask study as well as the predicted value of the protease yield by response surface method. Furthermore, the optimal time requirement for maximum protease production in a process-controlled fermenter was less than that observed under shake-flask study (60 hrs vs 72 hrs).

Only a few reports are available on bioreactor level studies as compared to shake flask experiments and the data is even more limited with regard to protease production by marine microbes (Narinx *et al*., 1997; Kumar *et al*., 2004). The marine bacterium *Vibrio harveyi* has been used to produce extracellular proteases (Estrada-Badillo and Marquez-Rocha, 2003). The bioreactor (1.5 L reactor volume) was operated in the batch culture mode and the effect of agitation rate on biomass and protease production was investigated. In their study peptone 5 g/L, yeast extract 1 g/L supplemented with skim milk served as carbon source. Process parameters were as follows: pH 7.2, temperature- 30 ºC, aeration rate (L/L/min) - 0.5 and agitation rate 700 rpm. The pH and temperature conditions used in their study were very similar to those obtained in this investigation.

In the present study, the use of RSM, helped in carrying out the optimization of both physical parameters and media components in a limited number of runs. Under conditions optimized by RSM, *Vibrio* sp. (V26) was able to produce 2257.8 U of protease in a 5-L bioreactor and 2262.15 U in

shake flasks. The level of enzyme production achieved is approximately two fold higher than that attained using 'one factor at a time' approach. The FCCCD of RSM helped in understanding the influence of physical parameters, especially agitation and salinity on the production of protease. Unlike the classical approach, FCCCD helped in recognizing the effects of media components and their interactions on the production of the enzyme by *Vibrio* sp. (V26). Hence RSM can be considered as a very reliable methodology for the optimization of culture parameters for alkaline protease production by *Vibrio* sp. (V26).

Chapter 5

Applications of Alkaline Protease from *Vibrio* sp. (V26)

Contents

Proteases are the most important class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. They account for more than 65% of the total industrial enzyme market (Banik and Prakash, 2004). Alkaline proteases are robust enzymes which have large variety of industrial applications, mainly in the detergent, food, pharmaceutical, silk and leather industries (Rao *et al.*, 1998). Three entirely different areas of application of the alkaline protease from *Vibrio* sp. (V26) which include; the use in animal cell culture, in bioprocessing of used X-ray films and as an ingredient in detergents have been studied. The properties of the enzyme required for its application in peptide synthesis and food processing too have been investigated.

5.1 Application of alkaline protease from *Vibrio* sp. (V26) in animal cell culture

One of the fields where proteases find application is in animal cell culture. The use of trypsin to detach growing cells from plasma clots was first introduced by Rous and Jones in 1916, a method that preceded the use of proteolytic enzymes for preparing separated cells from tissue fragments for primary culture. Trypsin has since remained a popular agent for primary dissociation of tissues for detaching cells from monolayers for subsequent passaging. The use of enzymes such as pancreatin, elastase (Rinaldini, 1958) and accutase (Bajpai *et al*., 2008) in tissue culture has also been investigated. As the above are of animal origin, their sources are restricted and they turn out to be expensive as well. Trypsin's use is often limited by its narrow range of pH for action. Meanwhile 'Pronase' (Foley and Aftonomos, 1970), Collagenase (Hilfer, 1973), and Dispase (Kitano and Okada, 1983) are the microbial proteases that have found application in cell culture. Each of these enzymes has their own limitations as collagenase acts only on tissues containing collagen, while pronase with regard to its completeness of dispersion of certain continuous epithelial cell lines is inferior to trypsin (Foley and Aftonomos, 1970).

In the past 20 years or so, there have been a very few investigations on the application of proteases from microbes in animal cell culture (Chiplonkar *et al.*, 1985; Nestler *et al.*, 2004; Shankar *et al.*, 2011) and they have all been from fungal origin.

So far, there have been no reports on the use of proteases from *Vibrio* species in this field. Moreover, as the alkaline protease from *Vibrio* sp. (V26) exhibited 'detachase' property during characterization studies; its application in the dissociation of cells from monolayer cell cultures and tissues of primary cell culture were investigated. The cell dissociating properties of protease from

Vibrio sp. (V26) was also compared to trypsin, an enzyme widely employed in animal cell culture.

5.1.1 Materials and Methods

5.1.1.1 Assay of protease

As the application is in animal cell culture; the activity assay was performed at pH 7.2 and 37^{o}C as they were the conditions optimal for the cell lines. For the assay of the enzyme 500 µl suitably diluted enzyme was added to 500 µl casein (1%) prepared in PBS (phosphate buffered saline) (pH 7.2 ± 0.2) and incubated at 37^{o}C for 30 minutes. The rest of the procedure is as mentioned in section ***3.2.2.1***.

5.1.1.2 Production of enzyme

Alkaline protease was produced by growing *Vibrio* sp. (V26) in modified Nutrient broth (Table 4.7) under optimized conditions.

5.1.1.3 Cell Lines and tissues used

HEp-2 (Human larynx epithelial cells), HeLa (Henritta Lacks, human cervical carcinoma) and RTG-2 (gonadial cell line derived from *Oncorhynchus mykiss*) cell lines were used in the investigation. Cell lines were maintained in Eagle's MEM medium along with 2 mM glutamine, 1.5 g/L sodium bicarbonate and 10% FBS. HEp-2 and HeLa cells were maintained at 37^{o}C while RTG-2 cells were grown at 25^{o}C. Nine day old chick embryo was used for the development of chick embryo fibroblastic primary cell culture. The methods followed for the application of the enzyme in cell cultures and their maintenance was as per the standard protocols described by Freshney (2000).

5.1.1.4 Enzymes for cell culture study

Alkaline protease from *Vibrio* sp. (V26) (APV26): The cell free supernatant was precipitated with ammonium sulphate (40-80%) and the precipitate dissolved in minimum amounts of Tris-Cl buffer (pH 8.5). This was

then diafiltered and concentrated using Amicon UF stirred cell (Model 8010) with 10 kDa cut off membrane against Tris-Cl buffer (pH 8.5). The concentrated enzyme was lyophilized and dissolved in phosphate buffered saline (pH 7.2). This was treated as alkaline protease stock. Stock was suitably diluted with PBS to get the desired enzyme concentrations (400, 300, 200, 100 and 50 U at 37^{o}C).

Trypsin from Sigma Aldritch Inc.was used as the control for comparison. Aliquot of 0.025% trypsin was found to have an activity of ~ 400 U. From this stock further dilution were made to obtain 300, 200, 100 and 50 U working trypsin concentrations.

5.1.1.5 Dissociation of monolayer

The cells were seeded into 24 well plates at a density 1 x10 ^{5}cells/ml. The plates were incubated till confluent monolayers were established. Different concentrations of APV26 were tested on the cell lines in-order to determine an ideal concentration of the enzyme for cell dissociation. Cells were treated with each of these concentrations (400, 300, 200, 100 and 50 U) until they were dissociated / detached from the wells. Time required for detachment was noted at each concentration. For a comparative study, the same procedure was repeated using trypsin. Cells were then washed with PBS to stop the action of the enzyme and the viable count noted at each concentration of APV26 and trypsin. All the observations were made under an inverted phase contrast microscope (Leica DMIL fitted with camera- Leica DFC420C, Germany). Photographs were analyzed using the software Leica ApplicationSuite (LAS) version 3.0.0.

5.1.1.6 Primary cell culture

A nine day old chick embryo was carefully dissected to remove head, appendages and viscera retaining the body alone (Fig.5.1). The body was cut into two parts using a sterile scalpel. Each half was further minced into smaller

pieces. One half (~100 mg) of the cut pieces were transferred into a tube containing 1 ml (200 U) of APV26 and the other half to the same quantity of trypsin (0.025 %). Tubes were incubated at 37ºC until the tissue pieces were fully dissociated. The suspension was allowed to stand for 1 minute at 4ºC for the sedimentation of undissociated smaller tissue pieces. The supernatant of cell suspension was carefully transferred to another test tube and centrifuged for 10 minutes at 200 x g at 4ºC. The supernatant was discarded and deposited cells were resuspended in fresh medium. Viable counts were made by trypan blue exclusion method and the number of cells were adjusted and seeded into cell culture bottles, incubated at 37ºC in 5% CO_2 atmosphere.

5.1.1.7 Viable count-Trypan blue dye exclusion method

Cell suspensions obtained after enzyme treatment (APV26 and trypsin) were centrifuged and re-suspended in fresh medium. This was then mixed with an equal volume of trypan blue (0.4%) prepared in PBS having the same osmolarity of the medium. Sample was loaded into a counting chamber (Improved Neubauer) and viable and dead cells were counted. The dead cells take up the stain and appear blue, while the live ones remain unstained.

5.1.1.8 Statistical Analysis

Data generated from the above experiments were analyzed using Two-way Analysis of Variance (ANOVA). Mean of the results was compared at a significance level of $p < 0.05$. Data are presented as mean ±standard deviation (SD).

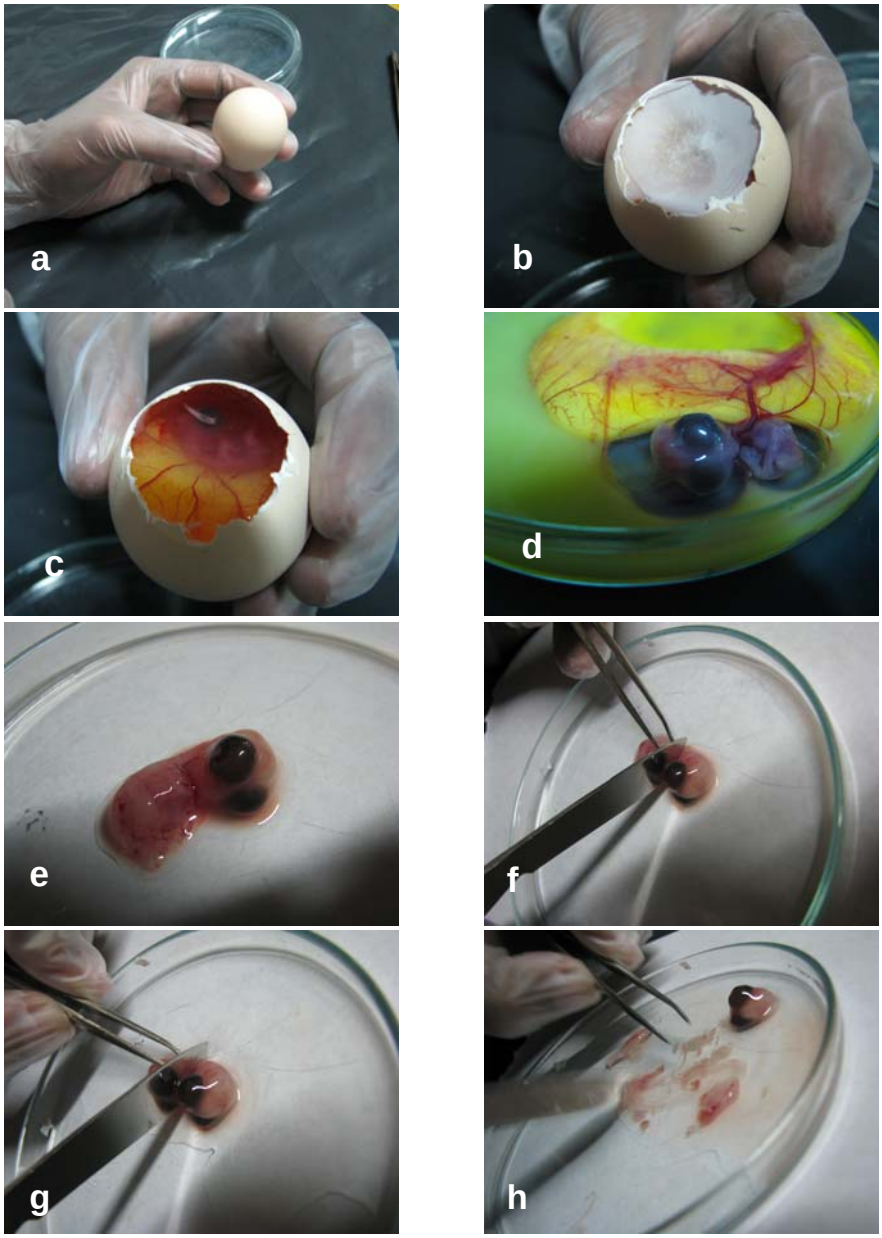


Fig.5.1 Steps in primary cell culture

a) Surface sterilized 9-day old embryonated egg
b) Egg shell removed to reveal the underlying Chorio-allantoic membrane (CAM)
c) CAM removed to reveal embryo along with the yolk sac
d) Embryo (along with yolk sac) transferred to a sterile plate
e) Embryo after the removal of yolk sac
f) Dissection of embryo to remove head
g) Dissection of embryo to remove head
h) Dissection of embryo to remove appendages

5.1.2 Results

APV26 was highly effective in dissociating the monolayer of all the cell lines tested (HeLa, HEp-2 and RTG-2) and the cell detaching property is clearly evident in Fig.5.2 (A, B and C). APV26 was also found to disperse the tissue of the chick embryo (fibroblast cells) effectively at 37ºC. The dispersed cells where then seeded into fresh culture bottles. The freshly seeded cells started attaching in about 2 hrs. After 24 hrs, the cells were seen to grow and migrate from the clone of attached cells at the centre (Fig. 5.2D).

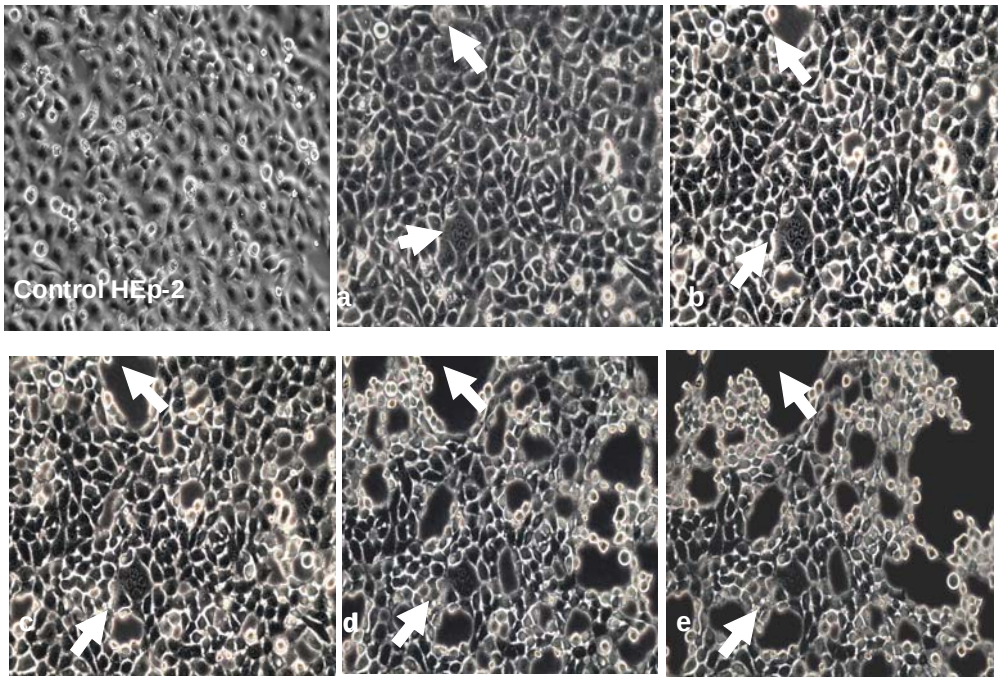


Fig.5.2 A) Time lapse image showing the cell dissociating and detaching property of APV26 on HEp-2 cell monolayers

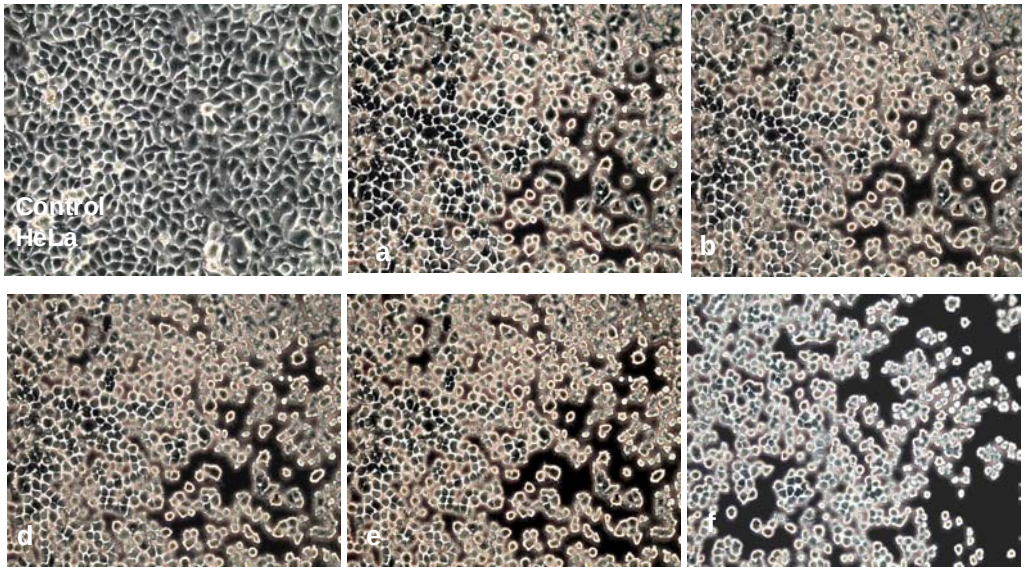


Fig.5.2 B**) Time lapse image showing the cell dissociating and detaching property of APV26 on HeLa cells monolayers**

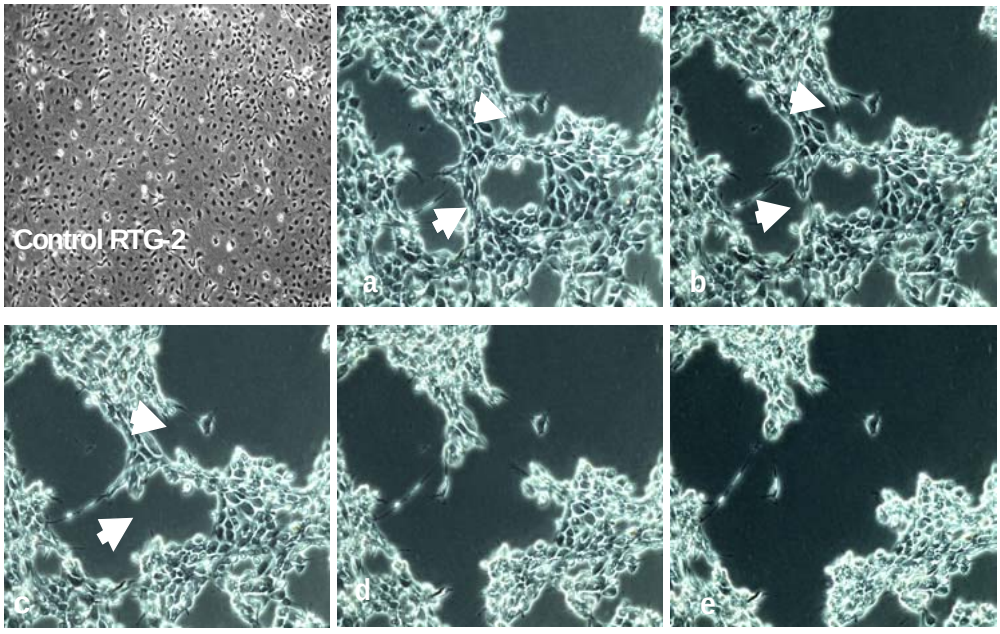


Fig.5.2 C) Time lapse image showing the cell dissociating and detaching property of APV26 on RTG-2 cell monolayers

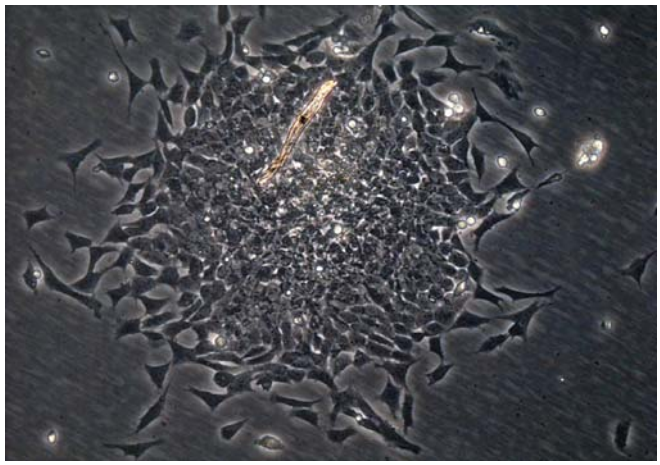


Fig.5.2 D) Primary culture of chick embryo fibroblasts after 24 hrs.

With the increase in concentration of APV26 the time required for dissociation of monolayer decreased (Table 5.1). HeLa cell lines took the longest time to dissociate both in the case of both APV26 and Trypsin. Two-way ANOVA indicated that the time required by APV26 for the detachment of all the cell lines except RTG-2 was significantly ($p < 0.01$) lower than that of trypsin (Appendix 4), while the effect of concentration of enzyme used was found to be not significant.

Viable cell yields of APV26 and trypsin for all the three cell lines were compared (Fig.5.3). The viable cell counts (%) of APV26 (different concentration) ranged between 91.6- 95.5%, 90.9-100% and 100% for the cell lines HeLa, HEp-2 and RTG-2 respectively. Two-way ANOVA indicated (Appendix 4) that there was no significant ($p>0.05$) effect of concentration of enzyme as well as the type of enzyme trearment used (APV26 or trypsin) on the viable cell yields of all the cell lines except RTG-2, where type of enzyme used was significant factor. From this it can be concluded that viable yields obtained using APV26 was comparable to trypsin, a standard enzyme used in animal cell culture.

Table 5.1

Comparison of time required by APV26 and trypsin for monolayer dissociation

Cell Lines	Enzyme concentration (U)	Time required for monolayer dissociation (minutes)	
		APV26	Trypsin
HeLa	50	7	12
	100	4	12
	200	1	10.4
	300	0.5	10.4
	400	0.4	10.4
HEp-2	50	1.3	7.4
	100	1.3	6
	200	1.3	6
	300	1	6
	400	0.56	6
RTG-2	50	4.3	1.22
	100	1.3	1.22
	200	0.56	0.51
	300	0.36	0.35
	400	0.31	0.26

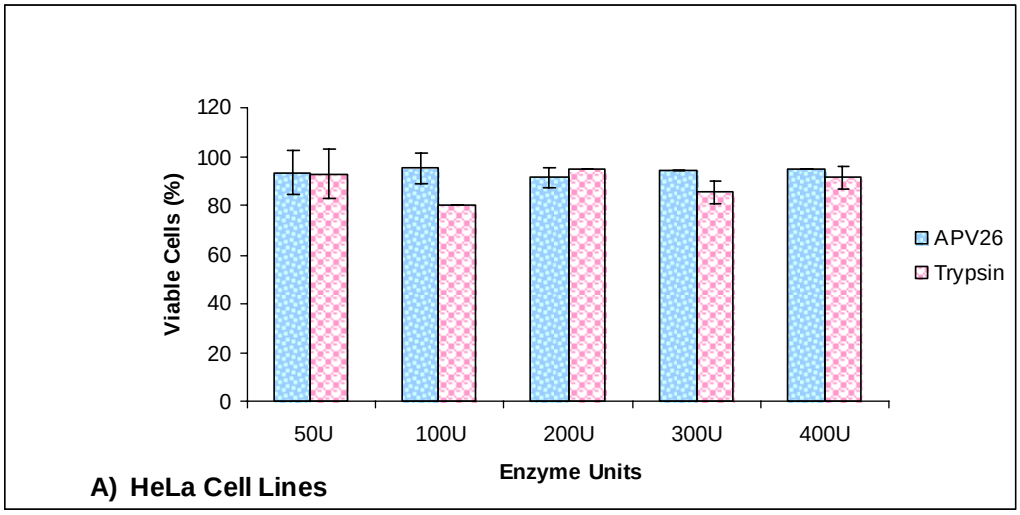


Fig.5.3 A Comparison of Viable cell yield of APV26 and Trypsin

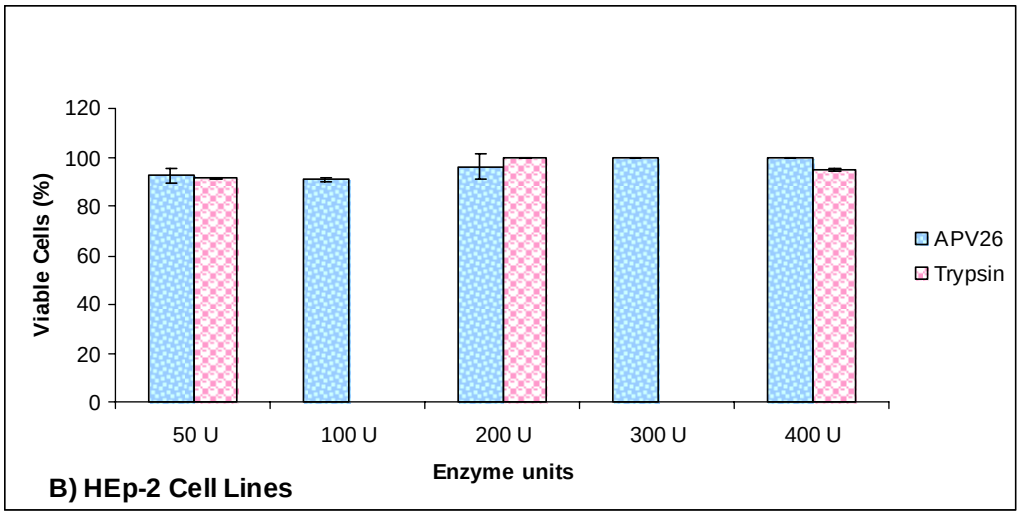


Fig.5.3 B Comparison of Viable cell yield of APV26 and Trypsin

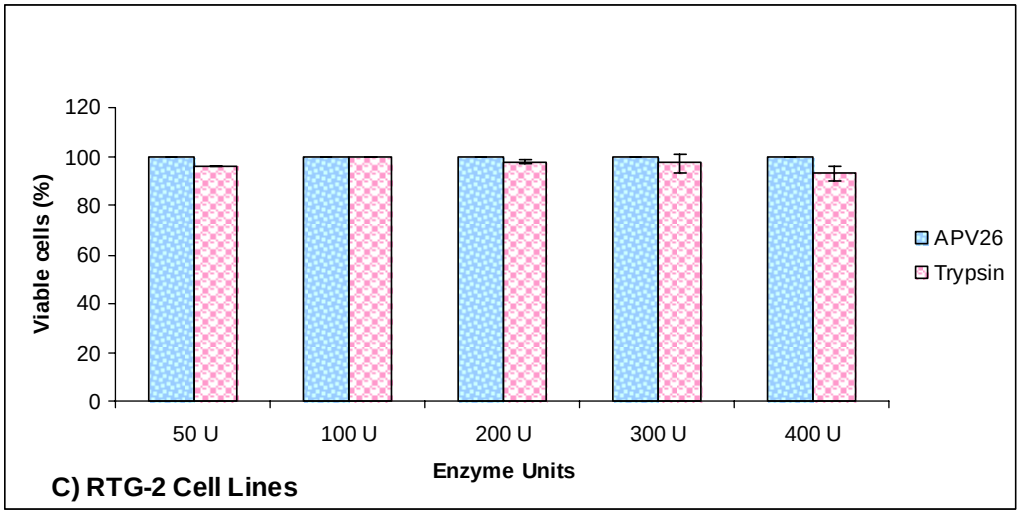


Fig.5.3 C Comparison of Viable cell yield of APV26 and Trypsin

5.1.3 Discussion

APV26 was found to be very effective in dissociating all the cell lines tested. The mechanism of action of APV26 which is a metalloprotease is likely to be different from that of trypsin which is a serine protease. The mechanism of action of APV26 could be the same as that of haemagglutinin/protease of *V. cholerae*, which degrades occludin in tight junctions (TJ) of epithelial cells (Wu *et al*., 1996, 2000; Mel *et al*., 2000). Though this action on TJ is believed

to be important in pathogenesis, this very same action of the protease can be put to beneficial use i.e., dissociation of cells in animal cell culture.

It is evident that the time needed to remove cells from culture vessel surface is dependent on the cell type, with cancer cell lines being more difficult to dislodge than the other cell line. Duration of exposure to proteolytic enzyme is a very significant factor in cell culture procedures. It is recommended that the exposure to proteolytic enzymes be kept to a minimum, as longer exposures can lead to cellular damage. As APV26 requires less time to dislodge cells than trypsin it is of great advantage in animal cell culture procedures. In the case of RTG-2, though APV26 took a longer duration to detach the cells than trypsin it was found to be less toxic. In fact the viable cell yield (%) observed for RTG-2 using APV26 was found to be 100% at all concentrations tested. The average viable cell yield of APV26 was found to be 94, 96 and 100 for HeLa, HEp-2 and RTG-2 cell lines respectively with an overall average greater than 96.7%. These results are indeed very promising for its application in animal cell culture. TrypLE™ Express a high purity recombinant fungal enzyme with cell dissociating property was found to have cell viability greater than 95% (Nestler *et al.*, 2004). Though not significant the overall viable cell yield of APV26 (96.7%) was slightly higher than trypsin (93.7%) (Fig.5.3) which indicate that APV26 was less toxic. Edwards and Fogh (1959) demonstrated that severe exo and endo-membrane damage and nuclear distortion resulted from treatment of human amnion cells with 0.25% trypsin. There are several such reports available on the toxicity of trypsin (Allen and Snow, 1970; Pumper *et al*., 1971) to cells, cell cultures and cell lines.

The time taken by APV26 for dissociation of chick fibroblast (primary culture) was the same as trypsin. However the yield of viable cells was 25% greater than the standard trypsin treatment. The dispersed chick fibroblast cells when seeded in to fresh culture bottles, were found to be capable of attaching and then multiplying.

Several reports on similar grounds are available. The ability of alkaline protease from *Conidiobolus* (Chiplonkar *et al*., 1985) and Pronase (mixture of proteinases) from *Streptomyces griseus* (Foley and Aftonomos, 1970) to dissociate cell lines have been compared to trypsin and found as possible substitutes for trypsin in animal cell culture. Dispase from *Bacillus polymyxa* (Kitano and Okada, 1983) and Collagenase from *Clostridium histolyticum* are some of the other microbial proteases that have been put to various cell culture applications.

An interesting observation made in this study was that APV26 exhibited very little toxicity (practically nil) towards RTG-2 (fibroblast-like cells) and chick-fibroblast. This indicates that APV26 holds potential for use in dissociation of animal cell lines, specifically, those which are fibroblastic in nature. So far, no reports are available on the application of alkaline metalloprotease from *Vibrio* species in the field of animal cell culture.

Metal ions (calcium and magnesium) and serum are essential components of cell culture media. Trypsin which is inactivated by both metal ions and serum cannot therefore be used along with ordinary cell culture media. As the protease from *Vibrio* sp. (V26) is neither inhibited by metals ions (Ca^{2+} and Mg^{2+}) nor serum, it has no special media requirements for action. Moreover trypsin acts only over narrow pH range (7.2-7.4) while APV26 has broader pH range. Therefore it is possible to use APV26 in cell culture media with higher pH. Unlike trypsin, the action of APV26 is stopped by washing and does not require the help of inhibitors. Therefore it can be concluded that alkaline metalloprotease from *Vibrio* sp. (V26) could be brought out as a product with application in animal cell culture.

5.2 Application in the recovery of silver from used X-ray films

Silver a precious and noble metal, finds application in the photographic industry in large quantities. It has been reported that 25% of the world's silver needs are met by recycling, out of which 75% is obtained from photographic waste. Photographic film in general consists of a support layer, such as glass, plastic sheet or paper coated with emulsion layer, usually a suspension of silver halide crystals in gelatin (Moore *et al.*, 1996). The amount of silver in the X-ray film varies between 1.5 and 2.0% (w/w) (Nakiboglu *et al.*, 2001).

Methods adopted for silver recovery from waste films include burning the films directly (Ewell and Piper, 1970), oxidation of the metallic silver following electrolysis (Ajiwe and Anyadiegwu, 2000), enzymatic hydrolysis of gelatin (Nakiboglu *et al.*, 2001) and stripping the gelatin-silver layer using different chemical solutions (Syed *et al.*, 2002). Recovery of silver by burning the films directly, a conventional method is a primitive one that generates foul smell and environmental pollution (Nakiboglu *et al.*, 2001). Moreover, the polyester film on which emulsion of silver and gelatin is coated cannot be recovered. Stripping the gelatin-silver layer by chemical methods or organic compounds cause environmental hazards and are either time consuming or very expensive while the use of NaOH at high temperatures poses a serious industrial safety problem (Sankar *et al*., 2010). As the emulsion layer containing silver also contains the protein gelatin, it is possible to break down the gelatin layer using proteases and release the silver (Nakiboglu *et al*., 2001). The enzymatic hydrolysis of the gelatin layers on the X-ray film enables not only the recovery of the silver, but also the polyester base which can be recycled. Basically enzymatic processes are more specific and remove gelatin layer from X-ray film in a few minutes; without damaging the polyester film base (Sankar *et al*., 2010). Protease enzyme based methods serve as an eco-friendly alternate option for the recovery of silver from X-ray/photographic waste.

Gelatin molecules are cross linked with hardeners and it is difficult for the usual proteases to degrade it in a short time. Fujiwara and Yamamoto (1987a) reported that an alkaline protease (AprN) from an alkaliphilic *Bacillus* sp. B21-2 could more rapidly break down gelatin layers on X-ray film than alkaline proteases or neutral proteases from neutrophiles (Fujiwara and Yamamoto, 1987b). Most of the proteases used so far for silver recovery are from bacteria such as *B. sphaericus* (Singh *et al*., 1999), *Bacillus subtilis* (Nakiboglu *et al*., 2001; Ramakrishnan *et al*., 2010), and fungi such as genetically - engineered *Aspergillus oryzae* (Samarntan and Tanticharoen, 1999) and *Conidiobolus coronatus* (Sankar *et al*., 2010). Fujiwara *et al*. (1989) have constructed a continuous and automatic process using the alkaline protease to recover both silver and polyethylene terephthalate film base independently from used X-ray film. Fundamental studies on the kinetics and mechanism of the enzymatic hydrolysis of gelatin layers on film and release of silver particles were carried out by Ishikawa *et al*. (1993). So far no reports are available on the use of proteases from Vibrios in this regard. In the present study the use of the alkaline protease from *Vibrio* sp. (V26) in the bioprocessing of used X-ray films for the recovery of silver has been investigated.

5.2.1 Materials and Methods

5.2.1.1 Decomposition of gelatin and release of silver

Used X-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in an oven at 40^{o}C for 30 minutes (Nakiboglu *et al*., 2001). One gram of X-ray film (cut into 2 x 2 cm pieces) was then incubated with suitably diluted partially purified alkaline protease in buffer (Tris–Cl, pH 9) (1000 U) with continuous shaking at 50^{o}C. Turbidity of the reaction mixture increased with time (as the hydrolysis progressed). Hence, the progress of hydrolysis was monitored by measuring the absorbance at 660 nm (Shankar *et al*., 2010). Samples were removed at regular

intervals and time required for complete removal of gelatin layer was noted.Visual examination of films after complete hydrolysis was also done.

5.2.1.2 Effect of Temperature

Optimum temperature for action was determined by carrying out the hydrolysis at pH 9 and at temperatures ranging from 30-60ºC.

5.2.1.3 Effect of enzyme dilution medium

The enzyme was diluted in Tris-Cl buffer (pH 9), tap water and distilled water. The gelatin hydrolysis was then carried out at 50ºC as previously described (section ***5.2.1.1***) in these three different dilution medium.

5.2.1.4 Repeated utilization of enzyme for gelatin hydrolysis from waste X-ray film

Repeated utilization of enzyme was studied by changing the X-ray films every 30 minutes. Each 30 minutes change of X-ray sheets was recorded as one run and in total four runs were carried out. The weight of X-ray films before and after each run was noted. The extent and time of decomposition was also noted.

5.2.2 Results

Visual examination of the films clearly revealed the ability of alkaline protease from *Vibrio* sp. (V26) to hydrolyse the gelatin in X-ray films leaving a clean polyester sheet while releasing silver into the hydrolysate (Fig.5.4).

5.2.2.1 Effect of temperature and enzyme dilution medium on hydrolysis of gelatin from X-ray films

Alkaline protease from *Vibrio* sp. (V26) was able to hydrolyse gelatin in X-ray films better at higher temperature (50 and 60ºC) which is reflected in a shorter time for action at these temperatures (Fig.5.5). Tris-Cl (pH 9) buffer was found to be most ideal for enzyme action while tap water was found to be the least effective among the three dilution medium (Fig.5.6). Maximum

gelatin hydrolysis was observed in the initial 15 minutes and duration of 25 minutes was required for the complete stripping of gelatin from the X-ray films.

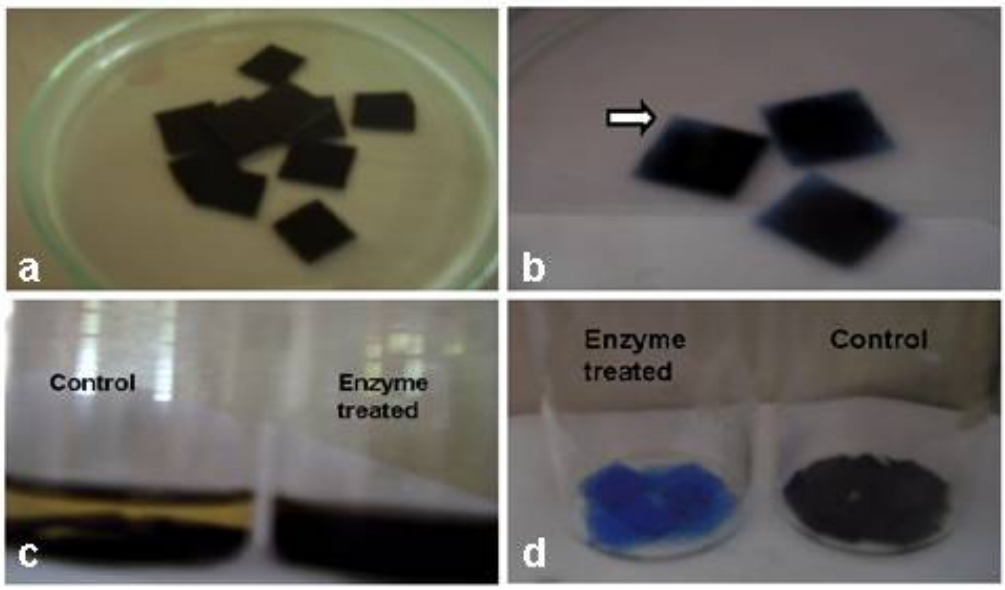


Fig.5.4 Decomposition of gelatin and release of silver

a) Cut X-ray film **b)** Edges of film change colour after the initial few minutes of exposure **c)** Control solution remains clear while enzyme solution turns turbid due to release of black metallic silver **d)** Clean bluish polyester sheets obtained after enzyme treatment of X-ray sheets while control films remained unchanged.

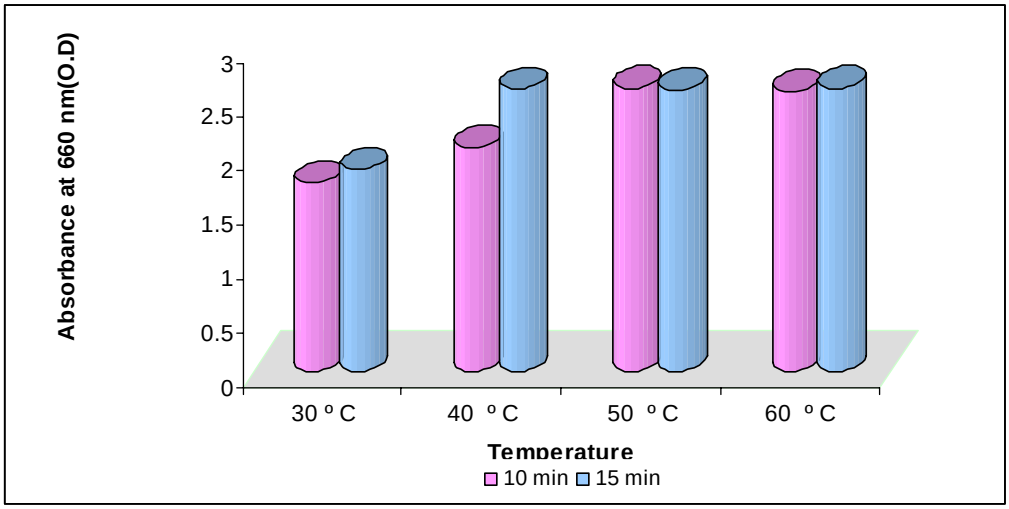


Fig. 5.5 Effect of temperature on the hydrolysis of gelatin over a time course

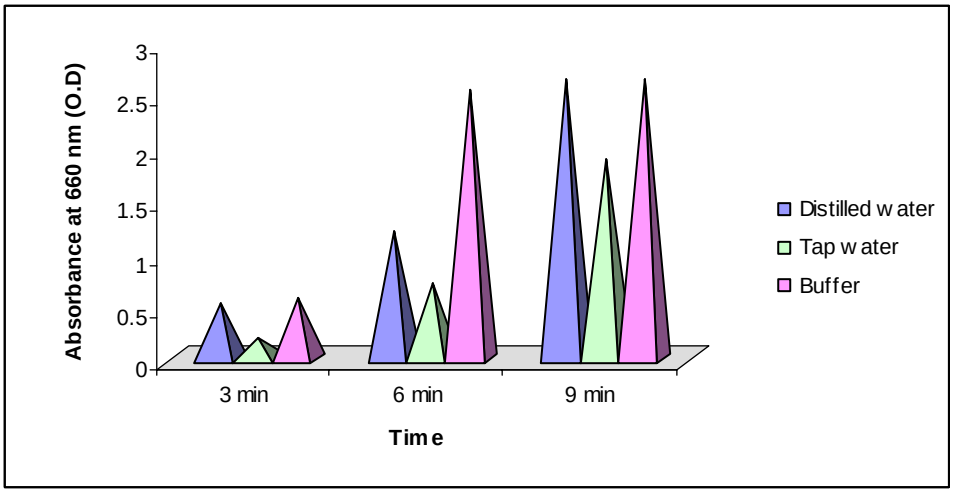


Fig.5.6 Effect of dilution media on the hydrolysis of gelatin over a time course

5.2.2.2 Reuse of enzyme for gelatin hydrolysis from waste X-ray film

Reuse of alkaline protease of *Vibrio* sp. (V26) was assessed at 50^{o}C and pH 9. The protease could be used upto 4 runs (Table 5.2). More than 30 minutes of exposure time was required to achieve completely clean sheets after the 3rd run.

Table 5.2

Reuse of enzyme for gelatin hydrolysis from waste X-ray film

Run	Loss in weight after the 30 minute treatment based on initial weight of the film.(w/w)	Observation
1	4%	Clean polyester sheets obtained
2	3%	Clean polyester sheets obtained
3	2%	Almost all sheets were clean except a few
4	3%*	*Clean sheets were obtained when duration of exposure was prolonged (total of 40 min)

5.2.3 Discussion

Alkaline protease produced by *Vibrio* sp. (V26) was found to be effective in the recovery of silver from X-ray films. With the increase in the incubation temperature from 30ºC to 60 ºC, a corresponding increase in the rate of hydrolysis of gelatin was noted. In a similar study, Sankar *et al*. (2010) too have reported of an increase in gelatin hydrolysis with increase in temperature. There was however no major difference in action at 50 and 60ºC in this study. Therefore 50ºC was chosen as the optimal temperature for hydrolysis due to lesser heating requirements. Nakiboglu *et al*. (2001) too selected 50ºC as the stripping temperature for the enzyme from *B. subtilis* ATCC 6633. In the present study, maximum gelatin hydrolysis was observed in the initial 15 minutes. The protease from *Vibrio* sp. (V26) took 25 minutes for the complete removal of gelatin, whereas the protease from a fungal isolate *Conidiobolus coronatus* ATCC PTA-4132 (Sankar *et al.*, 2010) took only 6 minutes. However the enzymes of the latter study, showed hydrolytic action only at 40ºC unlike the protease of this study which functioned over broad temperature range (40-60ºC). Masui *et al*. (1999) reported that the alkaline protease from the *Bacillus* sp. B21-2 mutant took 30 minutes for the complete hydrolysis of gelatin layer at 50ºC.

Hydrolysis of gelatin occurred best when Tris-Cl buffer of pH 9 was used; indicating that it was the most ideal pH for hydrolysis of gelatin. The optimum pH for stripping of the waste film by the enzymatic extract of *B. subtilis* ATCC 6633 was found to be 8 (Nakiboglu *et al*., 2001). Most other workers (Masui *et al.*, 1999; Sankar *et al.*, 2010) have found pH 10 or above to be ideal for gelatin hydrolysis. The ability of the protease used in this study, to work in different pH environments (tap water, distilled water and buffer) is highly advantageous from the industrial point of view. The conditions pH9 and 50ºC were found to be most ideal for the alkaline protease from *Vibrio* sp.

(V26) for carrying out the hydrolysis of gelatin and the release of silver from X-ray films.

The ability of an enzyme to retain its activity for repeated use makes it suitable for commercial application. It was found that the protease from *Vibrio* sp. (V26) could be reused for 4 runs. The efficiency was found to drop after the 3rd run resulting in longer treatment time for achieving complete hydrolysis in the 4th run. Sankar *et al*. (2010) too noted that the protease from *C. coronatus* could be reused 4 times and that, lowering the enzyme concentration resulted in longer reaction time for complete gelatin removal and also reduced the number of recycles. Masui *et al*. (1999) observed that the treatment time increased after every reuse of enzyme and the first decomposition was complete in 60 minutes while 2nd use required more than 2 hrs. When compared to the protease reported by Masui *et al*. (1999) the alkaline protease used in this study seems to be more efficient as it was capable of withstanding reuse to a greater extent.

In this era where natural mineral resources are getting depleted rapidly all over the world; reuse or recycle remains the most feasible option to slow down this exhaustion. This study clearly indicated that the alkaline protease of *Vibrio* sp. (V26) has the potential of being applied for recycling of silver from X-ray films and that too in an eco-friendly manner. As single dose of enzyme could be repeatedly used for recovery of silver from X-ray films, it clearly exhibited an important trait that is essential for commercial application.

5.3 Application as detergent additive

Proteolytic enzymes from microorganisms are the most widely exploited enzymes in the detergent industry world wide (Godfrey and West, 1996; Showell, 1999; Maurer, 2004). Microbial alkaline protease dominates with a significant share of the market captured by subtilisins and /or alkaline protease from *Bacillus* sp. for laundry detergent applications (Ward, 1985).

Over the years the importance of protease in detergents has grown from being minor additive to a key ingredient (Hodgson, 1994).

Alkaline proteases added to detergent formulations enable the release of proteinaceous dirt (blood, sweat, egg) that appear as stains on fabric (Masse and van Tilburg, 1983; Saxena *et al*., 1999). It is possible to remove these stains using detergents in water at higher temperatures and vigorous shaking but the cost of heating water, lengthy mixing and beating that shorten the life of the cloth are the main disadvantages.

'Green Chemicals' is a term applied to refer to enzyme based detergents (Kumar *et al.,* 1998) to highlight their eco-friendly nature. They are preferred over the conventional synthetic detergents. The increase in the usage of proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes in environmentally acceptable, non-phosphate detergents. Apart from improved washing efficiency the other benefits include lower wash temperatures and shorter periods of agitation often after a preliminary period of soaking (Nielson *et al*., 1981).

It was way back in 1913 that enzymes, specifically proteases were first used in laundry detergents. It was the German Chemist Otto Rohm who patented the application of pancreatic enzyme in presoaking detergent composition, it was brand named "Burnus" (Barfoed, 1981). It consisted of sodium carbonate and crude pancreatic extract. In 1957 the first detergent with bacterial proteases became available under the trade name BIO-40 (Rao *et al*., 1998). Only with availability of proteases of bacterial origin did its use become more efficient (Maurer, 2004) and practical. Mid 1960's saw the emergence of several new enzyme based detergents. Novo Industries A/S introduced Alcalase, an alkaline protease produced by *Bacillus licheniformis* under the commercial name BIOTEX (Rao *et al*., 1998; Saxena *et al*., 1999). It was stable and active at alkaline pH 8-10. Maxatase was another such product brought by Gist brocades (Gerhartz, 1990). Esperase and Savinase T (Novo

Industry) produced by alkalophilic *Bacillu*s spp., are two commercial preparations that withstand higher pH ranges.

In early 1970's the rapidly growing enzyme detergent industry suffered a serious set back when industrial workers as well as users developed allergies. This problem was overcome by developing dust free protease formulations. The dusting properties of the powdered enzyme in detergents were reduced by enzyme encapsulation or granulation (Barfoed, 1981). In mid 1980's the subtilisins (most studied bacterial serine proteases) were shown to be a good model for testing the genetic engineering approaches and by the end of the decade they were amongst the first technical enzymes to be manufactured using recombinant strains. Protein engineering approaches such as site directed mutagenesis based on rational design or various methods of random mutagenesis have been adopted for engineering of subtilisins. Since 1997 several gene shuffling approaches have been performed with subtilisins. These protein engineered enzymes entered the market in early 1990's (Maurer, 2004). They have carved a niche for themselves and are here to stay.

An ideal detergent protease should possess broad substrate specificity to facilitate the removal of a variety of stains (food, blood and other body secretions) (Rao *et al*., 1998). Incorporating enzymes into detergent formulations poses several practical problems (Crutzen and Douglass, 1999) with regard to its stability and compatibility. Enzymes are used in surprisingly small amounts in most detergent preparations, 0.4-0.8% crude enzyme by weight (Ward, 1985). Proteases are susceptible to autolytic degradation, oxidation and denaturation- processes that are often enhanced by surfactants, bleaches and water-softening builders that must be included in laundry detergent product (Crutzen and Douglass, 1999). Protease and other enzymes in detergent formulations should be alkaline in nature with a high pH optimum because the pH of laundry detergents is commonly in the range of 9.0-11.0 (Banerjee *et al*., 1999). In detergents, enzymes with lower temperature optima

may be employed with subsequent savings in energy consumption. However, protease and other enzymes used in detergents should have high activity and stability over broad range of pH and temperature. The enzyme should also be effective at low concentrations and be compatible with various detergent ingredients (Ward, 1985).

Most of the proteases that have been reported to be capable of stain removal and / or compatible with commercial detergents or surfactants have been from the different species of *Bacillus* (Singh *et al*., 2001; Adinarayana *et al*., 2003; Banik and Prakash, 2004; Venugopal and Saramma, 2007; Sellami-Kamoun *et al*., 2008; Haddar *et al*., 2009; Mala and Srividya, 2010; Ramakrishna *et al*., 2010; Abou-Elela *et al*., 2011) while there are only a few from *Vibrio* species (Mei and Jiang, 2005; Venugopal and Saramma, 2006; Wang *et al*., 2007). Proteases from other bacteria like *Pseudomonas* (Najafi *et al*., 2005), *Virgibacillus pantothenticus* (MTCC 6729) (Gupta *et al*., 2008) have also been examined as a potential detergent additive. Chen and Wang (2008) have investigated the application of cellulase and protease from rumen bacteria *Fibrobacter succinogenes* S85, and *Prevotella ruminicola* 23, respectively as detergent additives. They have also assessed the combined use of cellulase and protease in detergents. An extremely halophilic archaeon *Haloferax lucentensis* VKMM 007 was found to produce a protease that worked well in the presence of anionic and cationic detergents and was stable in the presence of reducing agents and oxidizing agents (Manikandan *et al*., 2009).

A highly bleach stable and halo-tolerant alkaline protease has been reported from *B. pumilus* JB05 (Johnvesly and Naik, 2001). A novel species of alkaliphilic *Bacillus* that produces oxidatively stable serine proteases was isolated by Saeki *et al*. (2000, 2002); they have also worked on sequencing the genes coding for such a stable enzyme. Works on the stability of the enzyme in the presence of bleaching agents (such as H_2O_2, sodium hypochlorite) have been done by Gupta *et al*. (1999), Beg and Gupta (2003), Joo *et al*. (2004) and

Mei and Jiang (2005). Joo and Chang (2006) have studied the feasibility of an oxidant and SDS stable alkaline protease from *B. clausii* I-52 as a laundry detergent additive. Whereas Sana *et al*. (2006) have studied salt, detergent and bleach tolerant proteases from gamma-Proteobacterium.

Proteases from several fungi and actinomycetes have also been investigated as biocleaning agents. The application of protease as a laundry detergent additive from a *Nocardiopsis* sp., newly isolated from a soil sample collected in Northeast Brazil was reported by Moreira *et al*. (2002). Vishalakshi *et al*. (2009) have used the alkaline protease from *Streptomyces gulbargensis* in the removal of blood from cotton fabric and surgical instruments.

Efforts have also been made to isolate alkaline proteases which are thermostable or thermotolerant (Durham *et al*., 1987; Tsuchiya *et al*., 1991a, 1991b; Banerjee *et al*., 1999; Kaur *et al*., 2001; Kumar, 2002; Johnvesly *et al*., 2001, 2002; Damare *et al*., 2006). Though generally detergents have been used at elevated temperatures, at present there is considerable interest in identification of alkaline protease which are effective over a wide range of temperature (Gupta *et al*., 2005a).

Despite the fact that it has been over 50 years since the use of microbial proteases as a detergent ingredient, the detergent industry is in constant search for better performing enzymes as well as good producer strains isolated from novel environments. This forms the very reason for investigating the potential of alkaline protease from *Vibrio* sp. (V26) to be used as detergent additive.

5.3.1 Materials and Methods

5.3.1.1 Preparation of stained fabric

Wash performance analysis of protease was done on square (dimension-4x4 cm) pieces of cotton fabric. Samples of human blood and egg were used to stain the fabric. Fabric was air dried followed by oven drying (Kanekar *et al*.,

2002). Wash performance was done using partially purified alkaline protease (40-80% ammonium sulphate fraction prepared as per section ***3.2.3.1***).

5.3.1.2 Wash performance with protease preparation

Application of protease as detergent additive was studied on stained (blood and egg) cloth pieces. The stained cloth pieces were taken in separate flasks. The following sets were prepared:

A) 100 ml heat inactivated detergent (7 mg/ml) + stained cloth piece

B) 100 ml heat inactivated detergent (7 mg/ml) + 500 µl enzyme solution (final dilution-250 U) + stained cloth piece

C) 100 ml tap water + 500 µl enzyme solution + stained cloth piece

D) 100 ml tap water + stained cloth piece

Two such sets of flasks (each set in triplicates) were prepared. One set was incubated at 50ºC for 30 minutes while the other at room temperature. Later the cotton fabric was taken out rinsed in tap water, dried and examined.

5.3.1.3 Compatibility with commercial detergents

The commercial detergents Surf excel and Surf excel matic (Hindustan Lever), Ariel and Tide (Procter and Gamble), Ujala (Jyothi Laboratories Ltd.) and Sunlight (Hindustan Unilever) were dissolved in tap water to give a final concentration of 7 mg/ml (Banerjee *et al*., 1999) to simulate washing conditions. The endogenous proteases contained in these detergents were inactivated by incubating the detergent solutions at 65ºC prior to the addition of exogenous purified protease from *Vibrio* sp. (V26) (Banik and Prakash, 2004; Sellami-Kamoun *et al*., 2008). After the addition of exogenous protease in detergent solution (in a ratio 1:4) (Banik and Prakash, 2004), the mixture was incubated for 1 hour at 40ºC and the residual activity determined under standard assay conditions (Hadj-Ali *et al*., 2007). The enzyme activity of control sample (without detergent) was taken as 100%.

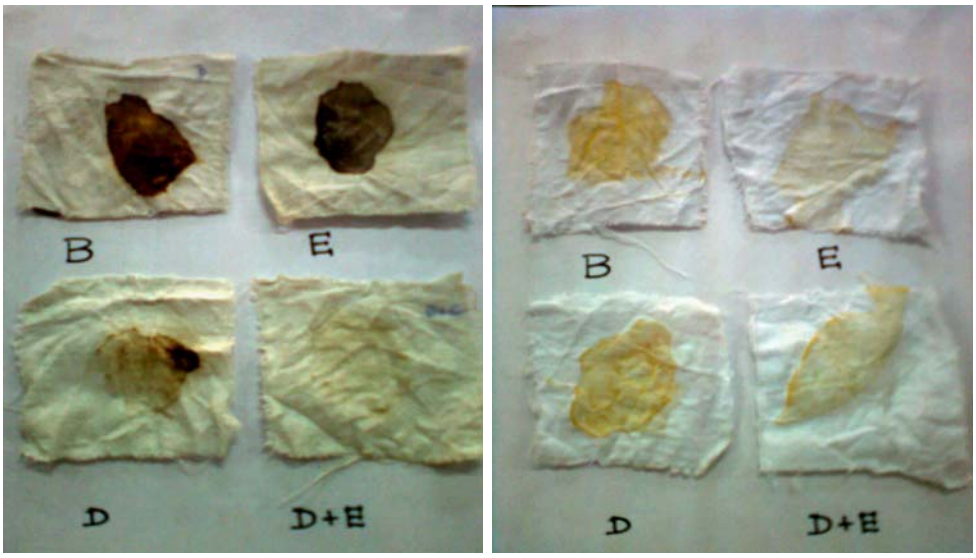
5.3.1.4 Statistical Analysis

Data generated from the above experiments were analyzed using one-way ANOVA with post-hoc multiple comparison analysis performed using Tukey's HSD. Mean of the results were compared using SPSS 13.0 package for windows at a significance level of $p<0.05$. Data are presented as mean ± standard deviation.

5.3.2 Results

5.3.2.1 Stain removal

Wash performance revealed that the partially purified alkaline protease of *Vibrio* sp. (V26) was very effective in removing blood and egg stains from cotton fabric when used in combination with detergent (Fig.5.7). In the case of egg stain removal the enzyme even in the absence of detergent was found to be quite effective. The protease was capable of removing stains both at 50ºC and room temperature.



A B

Fig.5.7 Wash Performance A) Blood stain removal B) Egg stain removal

B - Blank (without detergent or enzyme), E- Enzyme protease alone, D - Detergent alone, D+E - Detergent and enzyme in combination.

5.3.2.2 Compatibility with commercial detergents

The alkaline protease from *Vibrio* sp. (V26) was compatible with most of the commercial laundry detergents at 40ºC temperature (Fig.5.8). The compatibility with detergents in the following order Surf excel (78.83%) >Ariel (77.45%) > Sunlight (63.15%) >Ujala (50.43%) > Tide (48.92%) > Surf excel matic (42.42%) was noted after 60 minutes of incubation. ANOVA showed that the detergents had a significant effect on the compatibility of the protease. However, the compatibility exhibited by the protease did not vary significantly in the presence of Surf excel and Ariel (Appendix 4).

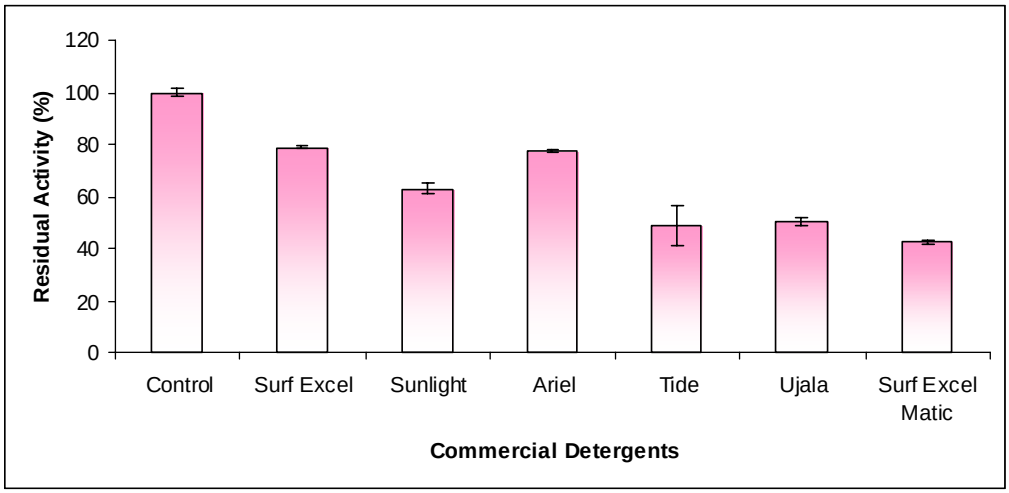


Fig.5.8 Compatibility with commercial laundry detergents

5.3.3 Discussion

Wash performance of partially purified alkaline protease and / or detergent solution were evaluated by subjecting it to blood and egg stain removal test from cotton fabric at 50ºC. The alkaline protease from *Vibrio* sp. (V26) was highly capable of removing both blood and egg stains. The combination of detergent and protease solution was most effective in the removal of blood stain indicating that the enzyme aided the detergent in its cleansing action or improving the wash performance of the detergent. The protease removed the egg stain quite effectively even in the absence of

detergent. Moreover, the alkaline protease of this study was capable of removing stains that had been made really stubborn by oven drying. With drying and ageing proteinaceous stains strongly resist action of most low cost and high volume detergents (Saxena *et al*., 1999). These stubborn stains can be easily removed only by detergents incorporated with proteases that attack hydrolytically, the peptide bonds in the proteins. The ability of the protease to act on different types of stains satisfies the primary criteria of broad substrate specificity for its application as laundry detergent ingredient.

The alkaline protease from *Vibrio* sp. (V26) exhibited its stain removing capabilities even at room temperature. This of advantage, as the detergent industry is now looking for enzymes that work under lower wash temperatures. However, the washing efficiency of the *Vibrio* sp. (V26) protease was found to increase with the increase in the temperature of incubation. The use of alkaline protease for the removal of proteinaeous stains from cotton fabric have been reported by Anwar and Saleemuddin (1997), Banerjee *et al*. (1999), Najafi *et al*. (2005), Abidi *et al*., (2008) and Abou-Elela *et al*. (2011).

From the characterization study (Chapter 3) it is clear that the alkaline protease from *Vibrio* sp. (V26) is a promising detergent additive as it is active at alkaline conditions and also over a broad temperature range which would facilitate washing even at room temperature. Apart from these properties, a good detergent protease is expected to be stable in the presence of commercial detergents as well. The compatibility of the alkaline protease varied with the different detergent brands, however more than 42% residual activity was observed with all the commercial detergents after 60 minutes of incubation at 40ºC. A similar observation (43%) was made by Rai and Mukherjee *et al*. (2009). When compared to protease of *V. fluvialis* (Venugopal and Saramma, 2006), the protease from *Vibrio* sp. (V26) exhibited greater compatibility with the laundry detergents Ariel (77.45%), Sunlight (63.15%) and Surf excel (78.83%). The enzyme produced by *B.cereus* retained more than 80% of its

activity in the presence of detergents Surf excel, Ariel and Henko (Banik and Prakash, 2004). When weighed against the protease from *Conidiobolus coronatus* (NCL 86.8.20) (Bhosale *et al*., 1995), *Bacillus* sp. SSR1 (Singh *et al*., 2001), and *Basidiobolus* (Ingale *et al*., 2002) the protease from *Vibrio* sp. (V26) was found to be more compatible with commercial detergents.

In the present study, the properties of the enzyme such as the alkaline pH optimum (pH 9), stability in the presence of surfactants and oxidizing agents, stain removing capabilities along with its detergent compatibility indicated the suitability of alkaline protease from *Vibrio* sp. (V26) for its application in laundry detergent formulations.

5.4 Other possible areas of application

The great economic value of protease still gives an impetus to search for new proteases with novel properties such as solvent and NaCl tolerance; apart from usual pH and temperature stability aspects.

Salt tolerant proteases are significant from an industrial perspective (Joo and Chang, 2005). They find application in the food industry where they are used in the processing of dry cured meat.

The application of enzymes to reactions performed under non-aqueous conditions has become a common practice in biocatalytic processes (Rolland and Lazaro, 2001; Rai and Mukherjee, 2009). Organic solutions are ideal for synthesis reactions such as that for peptides, since the solubility of polar substrates increases in such solutions. Apart from this, reversal of the thermodynamic equilibrium favors synthesis over hydrolysis, suppresses water-dependent side reactions, and decreases microbial contamination. Non-aqueous reactions are also characterized by high selectivity, specificity, and catalytic rates (Kato *et al*., 1996; Iyer and Ananthanarayan, 2008). To suit the conditions that prevail in industrial production, enzymes must be stable in the presence of organic solvents. These organic solvent stable proteases have been applied to

the synthesis of several peptides such as aspartame precursor (Sareen, *et al*., 2004) and kyotorphin precursor (Tsuchiyama, *et al*., 2007).

The new era in enzymology is marked by the search for proteases which are naturally stable in the presence of organic solvents (Geok *et al*., 2003; Abd Rahman *et al*., 2005; Gupta *et al*., 2005b; Gupta and Khare, 2006; Li *et al*., 2009). Salt and water miscible organic solvent tolerant protease has been reported from a marine bacterium *Halobacterium halobium* (Kim and Dordick, 1997). Some marine bacteria belonging to the genera *Vibrio*, *Pseudomonas* and *Bacillus* have been reported to produce protease with novel attributes like salt tolerance, thermostability and solvent stability (Makino *et al*., 1981; Qua *et al*., 1981; Marcello *et al*., 1996; Manachini and Fortina, 1998; Ogino *et al*., 1999). The screening, characterization, and cloning of a solvent-tolerant protease from *Serratia marcescens* MH6 has been reported by Wan *et al*. (2010). Singh *et al*. (2011) have reported of a solvent tolerant psycho-thermo alkaline protease from a psychotroph *Pseudomonas putida*.

So as to investigate the other possible areas of application of the alkaline protease from *Vibrio* sp. (V26), the effect of organic solvents and NaCl on the stability of the enzyme was assessed.

5.4.1 Materials and Methods

5.4.1.1Enzyme used

The cell free supernatant was precipitated with ammonium sulphate (40-80%) and the precipitate dissolved in minimum amounts of Tris-Cl buffer (pH 8.5). This was then diafiltered and concentrated using Amicon UF stirred cell (Model 8010) with 10 kDa cut off membrane against Tris-Cl buffer (pH 8.5).

5.4.1.2 Effect of NaCl on enzyme stability

To study the effect of NaCl on the enzyme stability, the partially purified enzyme was dissolved in 100 mM Tris-Cl buffer (pH 9) containing NaCl of different concentrations (0.5 M to 2.5 M NaCl) and incubated for 30

minutes. The residual activity was calculated as per section ***3.2.2.5***. The activity of the control (without NaCl) was taken as 100%.

5.4.1.3 Effect of organic solvents on protease stability

One ml of organic solvent (dimethyl sulphoxide (DMSO), isopropanol, ethanol, acetonitrile, ethyl ether and acetone) was added to 3 ml of protease solution and the reaction mixtures were incubated at 30ºC in closely stoppered vials (Wan *et al.*, 2010). Aliquots were withdrawn after 1 hr, 24 and 72 hrs to determine the residual activity. The stability was expressed as the residual proteolytic activity relative to the non solvent- containing control (section ***3.2.2.5***).

5.4.2 Results

5.4.2.1 Effect of NaCl on enzyme stability

Though the alkaline protease showed greatest stability in the absence of NaCl, it exhibited quite a high level of stability in the presence of 0.5 M (91.2%) and 1 M (88.4%)NaCl. The enzyme retained 77.08% and 72.63% of its original activity at 2 and 2.5 M NaCl respectively (Fig.5.9).

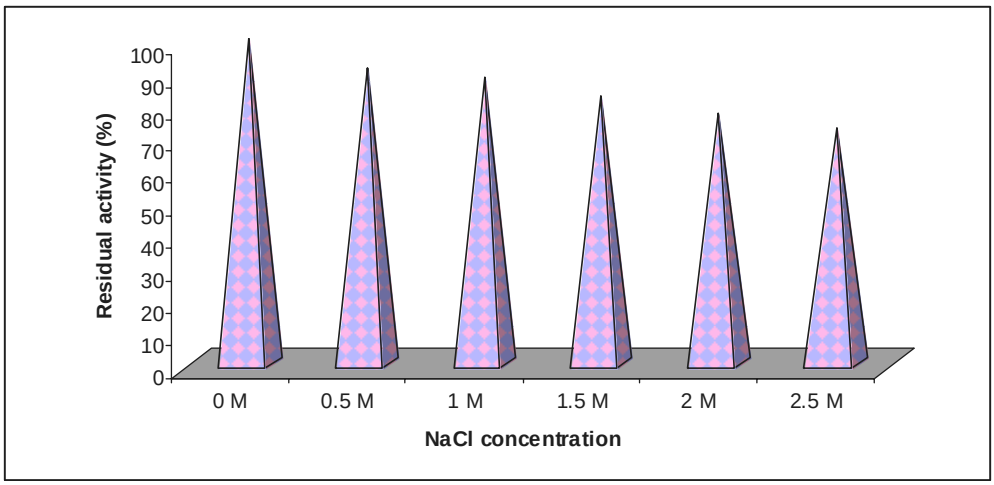


Fig.5.9 The effect of NaCl on the stability of alkaline protease from *Vibrio* sp. (V26).

5.4.2.2 Effect of organic solvents on enzyme stability

The alkaline protease from *Vibrio* sp. (V26) exhibited very good stability towards almost all the organic solvents tested in the study. Actually a slight enhancement in enzyme activity was observed with an increase in the incubation period from 1 to 72 hrs. Even though the enzyme was least stable in the presence of acetone, the alkaline protease retained more than 81 % of its activity in the presence of this solvent (Fig.5.10).

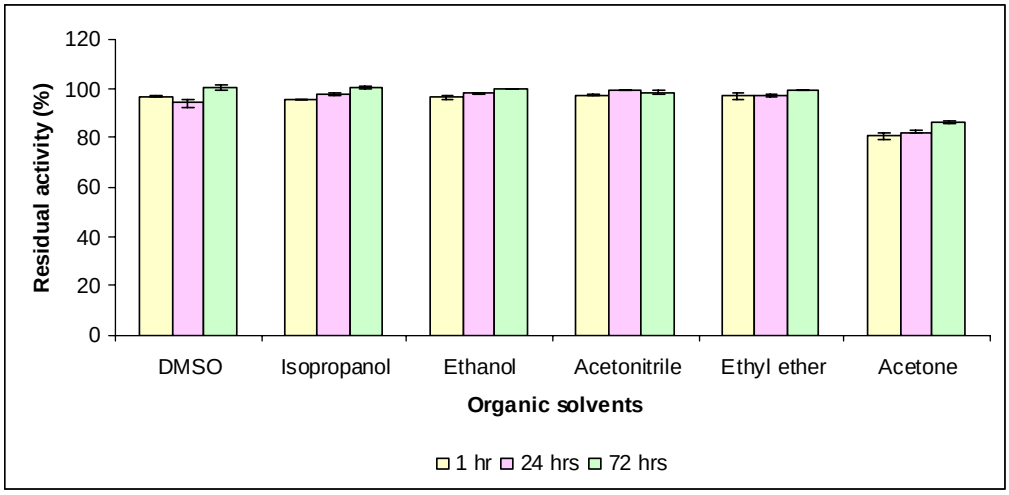


Fig. 5.10 Effect of different organic solvents on the stability of alkaline protease from *Vibrio* sp. (V26)

5.4.3 Discussion

The alkaline protease of *Vibrio* sp. (V26) was found to retain 91.2, 88.4, 82.7, 77.1 and 72.6% of its maximum activity in the presence of 0.5, 1, 1.5, 2 and 2.5 M NaCl respectively. This property of the enzyme can be exploited in the food industry for the processing of dry cured meat products. These meat products usually contain 1-2 M NaCl which act as a powerful inhibitor of endogenous proteolytic enzymes (Ordonez *et al*., 1999), which therefore requires the addition of NaCl tolerant exogenous proteases for its processing. In a similar study, Kim and Dordick (1997) reported of a metalloprotease from a marine *Vibrio* which retained 84% activity in the presence of 0.5 M NaCl. A

new gamma-Proteobacterium, DGII isolated from the marine environment of the Sundarbans, retained more than 70% of its maximum activity after 18 hrs pre-incubation with 35% NaCl (Sana *et al*., 2006). Though most proteases reported from halophiles are stable under high salt concentration, they are quite inactive in the absence of NaCl (Qua *et al*., 1981; Manikandan *et al*., 2009). Unlike this group of proteases, the enzyme reported in this work was found to have highest activity in the absence of NaCl and still retained more than 72 % of its maximum activity at 2.5 M concentration of NaCl. Patel *et al*. (2006) have studied the effect of NaCl on crude and the purified protease and found that while the enzyme activity was marginally enhanced at 0.2% (w/v) NaCl, the further increase in salt concentrations led to a significant reduction in the activity. However, the extent of adverse effect of salt on the enzyme activity was more pronounced in purified preparation as compared to crude.

Apart from the salt tolerance, the protease of *Vibrio* sp. (V26) exhibited remarkable stability in the presence of organic solvents. The enzyme showed high degree of stability (almost 100%) in presence of ethanol. Oda *et al*. (1996) too have reported of a metalloprotease from a marine *Vibrio* which was quite stable in presence of ethanol; however this enzyme was not salt tolerant. In contrast to this observation, the activity of chymotrypsin (a serine protease) was reported to decrease in presence of ethanol (Simon *et al*., 2004). Actually, in this study the organic solvents were seen to have a stabilizing effect on the enzyme. Longer the incubation with the organic solvents, greater was the residual activity. Stabilizing effect of organic solvent has also been reported by Sana *et al*. (2006). This stabilizing effect on the protease could be due to the replacement of some water molecules in an enzyme with organic molecules, which results in stabilizing the structure of the enzyme (Frikha *et al*., 2005).

Peptide synthesis was found to be enhanced by the addition of organic solvents in the reaction mixture (Geok *et al*., 2003; Ogino and Ishikawa 2001; Abd Rahman *et al*., 2005). However the reduced activity of enzymes under

anhydrous conditions is a major shortcoming of this approach and this can be overcome only by the use of organic solvent tolerant protease. The high degree of organic solvent stability of alkaline protease from *Vibrio* sp. (V26) indicated its potential for application in peptide synthesis.

Chapter 6

Summary

The thesis presents a detailed account of the alkaline protease produced by *Vibrio* sp. (V26) a mangrove isolate, and the application of this enzyme in different fields. The protease producer strain was identified on the basis of biochemical characteristics, putative virulence traits and 16S rRNA gene sequencing. The purification and characterization of the protease has been carried out. Along with this, an attempt has been made to identify the protease gene. The physical parameters as well as the media components influencing protease production were optimized using Response Surface Methodology (RSM). The scale up of the enzyme production was done in a Bench top fermenter. The scope of application of the protease from *Vibrio* sp. (V26) in the dissociation of cells in animal cell culture, in the recovery of silver from used X-ray films as well as an ingredient in commercial detergents were investigated.

The important findings of the study are summarized as follows:

- Identification of the species of the protease producer strain purely on the basis of phenotypic characteristic was difficult. It showed great similarity to both *Vibrio mimicus* and *V. cholerae*.
- The sequence of 16S rRNA gene was compared with the GenBank database using the BLAST algorithm and 98% similarity was obtained to 16S rRNA gene of *Vibrio cholerae, V. mimicus, V. albensis* and

certain Uncultured *Vibrio* clones. The nucleotide sequence of the 16S rRNA gene has been deposited at the GenBank data base and assigned the Accession no: FJ665509. Multilocus sequence typing (MLST) mighty be necessary for the species level identification of the strain.

- Serogrouping study revealed that the strain belonged to the non-O1 serogroup. The virulence associated gene profile showed that the strain lacked the major toxin genes *ctxA, zot* and also the gene for toxin-coregulated pilus *tcpA.* This demonstrated the non-toxigenic nature of the strain.
- The cell surface hydrophobicity and adherence assays clearly indicated that the strain was non-hydrophobic and exhibited weak adherence properties. The strain was also susceptible to most antibiotics tested. The study on all these putative virulence traits clearly illustrated the non-pathogenic nature of the strain *Vibrio* sp. (V26).
- The alkaline protease was purified by ammonium sulphate precipitation, diafiltration followed by DEAE-cellulose ion exchange chromatography. At the end of purification procedure a 4.9 fold purification of the protease was attained.
- The molecular mass of the protease as determined from SDS-PAGE was 32 kDa. The existence of the enzyme as an oligomer was indicated during the analysis of the zymogram.
- The protease was active as well as stable in the pH range 7-10 and optimum pH for the action of the enzyme was identified as 9.
- 60ºC was found as the optimum temperature for action of the alkaline protease. The enzyme was highly stable in the range 30-50ºC.
- Hg^{2+} and Cu^{2+} were found to strongly inhibit the alkaline protease from *Vibrio* sp. (V26) while in presence of Ca^{2+} (1 mM) and Ba^{2+} (1 mM) the activity of the protease was not significantly different from the untreated control. Inhibitory effect of Zn^{2+} was more pronounced at 5 mM than at 1 mM concentration.

- The inhibitors 1, 10-phenanthroline and EDTA strongly inhibited the activity of the alkaline protease indicating that the enzyme belonged to the class metalloprotease.
- The surfactant Tween 80 (non-ionic detergent) had a slight enhancing effect on the enzyme while SDS (anonic detergent) had negative effect on the protease. The protease was quite stable in the presence of the oxidizing agent H_2O_2.
- The purified protease as well as ammonium sulphate fractions failed to exhibit hemagglutination property.
- Cytotoxicity assay of the enzyme on HEp-2 cell lines revealed a LC_{50} value of 50 U. Morphological changes such as rounding cell and cell detachment were noticed.
- When compared to the commercial alkaline protease Savinase ® (P3111) the alkaline protease from *Vibrio* sp. (V26) was several folds more active.
- Protease gene was amplified using primers designed to amplify the conserved zinc binding region and the PCR product was sequenced. The sequence has been deposited at the GenBank data base and assigned the Accession no: JN091086. The BLAST analysis of the nucleotide sequence revealed the similarity of *Vibrio* sp. (V26) metallo-protease gene to the HA/protease gene of *Vibrio cholerae* and *Helicobacter pylori*.
- The deduced amino acid sequence of *Vibrio* sp. (V26) protease was also compared with metalloproteases from other bacteria. The study revealed maximum identity to that of the neutral precursor of *V. cholerae bv albensis* (ZP04416044) and also *V. cholerae* HA/protease and its precursor protein (ZP 06048800.1, ZP 04411813.1, ZP 01955135).
- The deduced amino acid sequence includes a zinc metalloprotease HEXXH consensus motif which is HEVSH (His-Glu-Tyr-Thr-His).

The BLAST analysis of *Vibrio* sp. (V26) protease gene indicated that the enzyme belongs to the peptidase family M4.

- The physical parameters agitation, pH, temperature and salinity were optimized statistically by Response Surface Methodology using the software Design-Expert (version 6.0.9, Stat-Ease, Minneapolis, MN, USA).
- Face centered central composite design (FCCCD) of RSM was employed. A total of 30 different experiments (2^4full factorial points, 8 axial points and 6 center points) were suggested by the software for optimization of culture conditions.
- Statistical analysis (ANOVA) showed that the model was significant ($p < 0.0001$) with an F value 76.55. The multiple correlation coefficient of alkaline protease production $R^2 = 0.987$ which indicated that the model can explain 98.7% variation in the response.
- The adequate precision value was found to be 25.608, indicating adequate signal. A relatively lower value of the coefficient of variation (CV=10.02%) denoted a good precision and reliability of the experiment. In this model the 'lack of fit' ($p = 0.0743$) was found 'not significant'. All this indicated adequacy of the model.
- The linear terms of the regression equation agitation (A) and salinity (D) and the quadratic terms pH (B^2), tempearture (C^2) and salinity (D^2) were found to be significant while interactions among these factor were found to be 'not significant'.
- The optimum conditions identified for alkaline protease production by *Vibrio* sp. (V26) were: agitation -147 rpm, pH 7.66, temperature 29.7^{o}C and salinity- 1.37%. Validation of the model was also carried out. The validation of the solution (optimized condition) suggested by the model was done experimentally. Experimental value 2064.375 U (±23.74) was very close to the predicted value of 2073.04 validating the model.

- The amount of inoculum was found to affect protease production. Higher inoculum size (0.08 and 0.1 O.D) decreased the protease production
- The media components were screened using Plackett-Burman Design (PBD). 12 different runs were suggested by the model. Peptone, yeast extract and $MgSO_4$ were identified as the most significant variables.
- Peptone, yeast extract and $MgSO_4$ were further optimized using FCCCD of RSM, a total of 20 runs (2^3full factorial points, 6 axial points and 6 center points) were carried out for the optimization of media components as suggested by the model.
- Statistical analysis (ANOVA) showed that the model for media components was significant ($p < 0.0001$) with an F-value 59.02. The multiple correlation coefficient of alkaline protease production R^2 = 0.983, which indicated that the model can explain 98.3% variation in the response.
- The adequate precision value was found to be 22.11 which indicated that the model can be used to navigate the design space. A relatively lower value of the coefficient of variation (CV=3.01%) denoted a good precision and reliability of the experiment. In this model the 'lack of fit' (p=0.1127) was found 'not significant'. All this indicated adequacy of the model.
- The linear terms of the regression equation peptone (A) and $MgSO_4$ (C), the quadratic terms A^2 (peptone) and B^2 (yeast extract) and interactions between peptone and yeast extract (AB) as well as peptone and $MgSO_4$ (AC) were found to be significant.
- The optimum values of the media components peptone, yeast extarct and $MgSO_4$ were 1.28 g/100 ml, 0.56 g/100 ml and 0.07 mM respectively. The validation of the solution (optimized condition) suggested by the model was done experimentally. Experimental value

2262.15 (± 36.11)) was very close to the predicted value of 2274.25, validating the model.

- Scale up studies was done in 5 L fermenter**.** The solution (optimized condition) suggested by the model was also further validated in the 5 L stirred tank reactor. The production was found to peak at 21-22 hrs of incubation. A two-fold (200%) increase in alkaline protease production was achieved under conditions optimized by RSM as compared to the conditions optimized by 'one-factor at a time' method.
- The application of protease of *Vibrio* sp. (V26) in animal cell culture was investigated and it was also compared to trypsin. The study revealed that it was capable of dissociating the cell lines HEp-2, HeLa, RTG-2 as wells as primary cell culture of chick fibroblasts.
- With the increase in concentration of APV26 the time required for dissociation of monolayer decreased. APV26 was found to take less time than trypsin, to detach cells of HEp-2 and HeLa. The overall average viable cell yield was 96 %.
- The application of alkaline protease in the recovery of silver from used X-ray films was also studied. The enzyme was capable of hydrolysing the gelatin in X-ray films and releasing silver into the hydrolysate. High incubation temperature (50-60 ^{o}C) and Tris-Cl buffer (pH 9) were found to be ideal conditions to carry out the gelatin hydrolysis. The alkaline protease could be reused effectively upto 4 runs.
- The alkaline protease was capable of removing both blood and egg stains from fabric. The purified enzyme was compatible with commercial detergents Surf excel and Surf excel matic (Hindutan Lever), Ariel and Tide (Procter and Gamble), Ujala (Jyothi Laboratories Ltd.) and Sunlight. The alkaline protease of *Vibrio* sp. (V26) was identified as a promising candidate for application in detergent industry.

- The protease was found to be quite salt-tolerant while it exhibited a remarkable stability towards organic solvents.

The present investigation indicates that the alkaline protease of *Vibrio* sp. (V26) is a robust enzyme with considerable industrial potential in detergents, silver recovery, animal cell culture, peptide synthesis as well as in food processing. The application in animal cell culture is definitely an area that is promising and requires further exploration. In this study, though the large scale enzyme production of the enzyme has been optimized, there is a definite need to standardize the downstream processing of this protease in an economically feasible manner.

By far, *Bacillus* sp. is the most popular source of commercial alkaline protease. However, there is an ever growing demand for novel proteases that function under highly arduous industrial conditions. This need can be met only by exploring the vast microbial diversity of our planet, which is least investigated. The need of the hour is not only to discover newer sources of proteases, but also to improve as well as develop a proper bioprocess design for its large scale production. Inorder, to keep pace with the competitive enzyme market, the use of recombinant technology, enzyme engineering and statistical methodology such as RSM are imperative.

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* not referred in original

APPENDIX

Appendix 1

Genbank Submissions from this work

Vibrio sp. V26 16S ribosomal RNA gene, partial sequence

GenBank: FJ665509.1

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BCT 14-MAR-2009
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SOURCE      Vibrio sp. V26
  ORGANISM  Vibrio sp. V26
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Vibrionales;
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REFERENCE   1  (bases 1 to 377)
AUTHORS   Manjusha,K., Divya,J., Seena,J., Priyaja,P.,
Sreelakshmi,B., Saramma,A.V. and Bright Singh,I.S.
TITLE     Purification and characterization of alkaline protease
from a non-toxigenic environmental isolate Vibrio sp. V26
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 377)
  AUTHORS   Manjusha,K., Priyaja,P., Meera,V., Divya,J.,
Seena,J., Sreelakshmi,B., Saramma,A.V. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-JAN-2009) National Centre for Aquatic
Animal Health, Cochin University of Science and Technology,
Fine Arts Avenue, Cochin, Kerala 682 016, India
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Vibrio sp. V26 zinc metalloprotease gene, partial cds

GenBank: JN091086.1

FASTA Graphics Item in clipboard

Go to:

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BCT 18-JUN-2011
DEFINITION  Vibrio sp. V26 zinc metalloprotease gene, partial
cds.
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VERSION     JN091086.1  GI:335929333
KEYWORDS    .
SOURCE      Vibrio sp. V26
  ORGANISM  Vibrio sp. V26
            Bacteria; Proteobacteria; Gammaproteobacteria;
Vibrionales;
            Vibrionaceae; Vibrio.
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Appendix 2

Table 2.1 (a)

Effect of pH on the activity of the protease from *Vibrio* sp. (V26)

pH	% Relative Activity
6	25.44 ±0.70[b]
7	70.22 ±1.35[e]
8	90.72 ±1.24[f]
9	100 ±1.10[g]
10	60.38 ±2.01[d]
11	29.71 ±0.99[c]
12	6.25 ±0.30[a]

Values with same superscripts did not vary significantly.

Table 2.1 (b)

ANOVA for the effect of pH on enzyme activity

	Sum of Squares	df	Mean Square	*F*	Sig.
Between Groups	22351.924	6	3725.321	2556.553	.000
Within Groups	20.400	14	1.457		
Total	22372.324	20			

Table 2.1 (c)

Homogenous subsets for effect of pH on enzyme activity

	pH	N	Subset for alpha = .05						
			1	2	3	4	5	6	7
Tukey HSDa	12.00	3	6.2480						
	6.00	3		25.4372					
	11.00	3			29.7138				
	10.00	3				60.3816			
	7.00	3					70.2226		
	8.00	3						90.7154	
	9.00								100.0000
	Sig		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subset are displayed

a. Uses Harmonic Mean sample size = 3.000

Table 2.2(a)

Effect of pH on the stability of the protease from *Vibrio* sp. (V26)

pH	% Residual Activity
6	54.58 ±0.60[c]
7	78.61 ± 0.47[e]
8	79.21 ± 1.58[e]
9	88.53 ± 0.54[f]
10	61.40± 3.16[d]
11	43.34± 0.09[b]
12	30.23± 0.45[a]

Values with same superscripts did not vary significantly

Table 2.2 (b)

ANOVA for the effect of pH on enzyme stability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8065.410	6	1344.235	694.492	.000
Within Groups	27.098	14	1.936		
Total	8092.507	20			

Table 2.2 (c)

Homogenous subsets for effect of pH on enzyme stability

	pH	N	Subset for alpha = .05					
			1	2	3	4	5	6
Tukey HSD a	12.00	3	30.2282					
	11.00	3		43.3371				
	6.00	3			54.5754			
	10.00	3				61.399		
	7.00	3					78.6083	
	8.00	3					79.2069	
	9.00	3						88.5297
	Sig		1.000	1.000	1.000	1.000	.998	1.000

Means for groups in homogeneous subset are displayed

a. Uses Harmonic Mean sample size = 3.000

Table 2.3 (a)

Effect of temperature on the activity of the protease from *Vibrio* sp. (V26)

Temperature (°C)	% Relative Activity
30	38.33 ± 2.01 [a]
40	85.43 ± 2.85 [c]
50	90.27 ± 1.61 [c]
60	100 ± 0.87 [d]
70	56.29 ± 1.53 [b]
80	36.68 ± 1.97 [a]

Values with same superscripts did not vary significantly

Table 2.3 (b)

ANOVA for the effect of temperature on enzyme activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11464.608	5	2292.922	632.431	.000
Within Groups	43.507	12	3.626		
Total	11508.115	17			

Table 2.3 (c)

Homogenous subsets for effect of temperature on enzyme activity

	Temp	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD[a]	80.00	3	36.6847			
	30.00	3	38.3257			
	70.00	3		56.2926		
	40.00	3			85.4262	
	50.00	3			90.2656	
	60.00	3				100.0000
	Sig		.890	1.000	.075	1.000

Means for groups in homogeneous subset are displayed

a. Uses Harmonic Mean sample size = 3.000

Table 2.4 (a)

Effect of temperature on the stability of the protease from *Vibrio* sp. (V26)

Temperature (°C)	% Relative Activity
30	91.46 ± 5.02 [c]
40	95.24 ± 0.51 [c]
50	92.02 ± 1.65 [c]
60	24.99 ± 0.43 [b]
70	0.167 ± 0 [a]

Values with same superscripts did not vary significantly

Table 2.4 (b)

ANOVA for the effect of temperature on enzyme stability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24179.503	4	6044.876	1064.556	.000
Within Groups	56.783	10	5.678		
Total	24236.286	14			

Table 2.4 (c)

Homogenous subsets for effect of temperature on enzyme stability

	Temp	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	70.00	3	.1669		
	60.00	3		24.9896	
	30.00	3			91.4615
	50.00	3			92.0178
	40.00	3			95.2441
	Sig		1.000	1.000	.356

Means for groups in homogeneous subset are displayed

a. Uses Harmonic Mean sample size = 3.000

Table 2.5 (a)

Effect of metal ions on the activity of the enzyme

Metal ions	Concentration	
	1 mM	5 mM
$ZnCl_2$	66.04 ± 3.25	7.94 ± 0.24
$MnCl_2$	86.23 ± 2.24	30.35 ± 0.34
$CaCl_2$	93.51 ± 2.47	70.85 ± 1.54
$Pb(NO_3)_2$	71.50 ± 3.62	11.89 ± 0.58
$MgCl_2$	88.92 ± 1.80	72.88 ± 1.05
$HgCl_2$	12.85 ± 0.92	1.43 ± 0.39
$BaCl_2$	98.09 ± 11.80	65.38 ± 1.15
Cu_2SO_4	12.24 ± 0.41	3.03 ± 0.63
$CoCl_2$	86.51 ± 1.41	34.40 ± 1.85

Table 2.5 (b)

ANOVA for the effect of metal ions on the activity of the enzyme

		Sum of Squares	df	Mean Square	F	Sig.
1mM	Between Groups	28249.748	9	3138.861	148.411	.000
	Within Groups	422.995	20	21.150		
	Total	28672.743	29			
5mM	Between Groups	33222.592	9	3691.399	2505.926	.000
	Within Groups	29.461	20	1.473		
	Total	33252.053	29			

Table 2.5 (c)

Homogenous subsets for 1 mM concentration of metal ions

			Subset for alpha = .05			
	Metalion	N	1	2	3	4
Tukey HSD[a]	cu	3	12.2434			
	Hg	3	12.8549			
	Zn	3		66.0358		
	Pb	3		71.4951		
	Mn	3			86.2280	
	Co	3			86.5046	
	Mg	3			88.9212	88.9212
	Ba	3			92.4443	92.4443
	Ca	3			92.5462	92.5462
	Control	3				100.0000
	Sig		1.000	.895	.792	.155

Means for groups in homogeneous subset are displayed

a. Uses Harmonic Mean sample size = 3.000

Table 2.5 (c)

Homogenous subsets for 5 mM concentration of metal ions

metalions		N	Subset for alpha = .05							
			1	2	3	4	5	6	7	8
Tukey HSD(a)	Hg	3	1.4267							
	cu	3	3.0281							
	Zn	3		7.9342						
	Pb	3			11.8940					
	Mn	3				30.3538				
	Co	3					34.4009			
	Ba	3						65.3807		
	Ca	3							70.8546	
	Mg	3							72.8781	
	control	3								100.0000
	Sig.		.826	1.000	1.000	1.000	1.000	1.000	.583	1.000

Appendix 3

Table 3.1

First Step Optimization

Effect of the concentration of various media components on protease production

Ingredient / Peptone concentration (g/100 ml)	Protease activity (U)
0	677.43 ± 70.50
0.5	975.52 ± 56.50
1	844.79 ± 11.91
Yeast extract concentration(g/100 ml)	**Protease activity (U)**
0	946.35 ± 42.04
0.15	975.52 ± 56.49
0.3	890.36 ± 172.15
Beef extract concentration (g/100ml)	**Protease activity (U)**
0	1129.16 ± 151.26
0.15	975.52 ± 56.49
0.3	916.40 ± 42.81
0.9	792.97 ± 22.25
Gelatin concentration (g/100 ml)	**Protease activity (U)**
0	1645.83 ± 7.37
0.2	1691.67 ± 0
0.4	1853.13 ± 8.10
0.6	1955.99 ± 23.94
0.8	2044.53 ± 60.03
1	2097.92 ± 1.47
1.5	1877.08 ± 22.83

Table 3.2

Protease production under extreme agitation conditions

Agitation (rpm)	Initial pH (H+ions)	Temperature (°C)	Salinity (%)	Protease production (U)
200	7.5	30	1.5	1065.97±17
0	7.5	30	1.5	72.48 ± 8.27

Table 3.3 a

Effect of Inoculum size

OD	Protease activity (U)
0.02	2113.19 ± 12.86
0.04	2133.33 ± 19.29
0.08	2100.69 ± 16.61
0.1	2040.63 ± 26.70

Table 3.3 b

ANOVA for the effect of inoculum size on protease production

	Sum of square	DF	Mean	F	Sig.
Between groups	14322.465	3	4774.155		
With in groups	3052.662	8	381.583	12.511	.002
Total	17375.127	11			

Table 3.3 c

Homogenous subsets for inoculum size

Tukey HSD[2]

		Subset for alpha = .05	
Inoculumsize	N	1	2
.10	3	2040.6250	
.08	3		2100.6944
.02	3		2113.0944
.04	3		2133.3333
		1.000	.249

Means for groups in homogenous subsets are displayed

Appendix 4

Table 4.1 a

Comparison of viable cell yields of APV26 and Trypsin for HeLa cell lines

Enzyme concentration	Percentage viable cell yields	
	APV26	Trypsin
50 U	93.75 ± 8.84	92.86 ± 10.10
100U	95.45 ± 6.43	80 ± 0
200U	91.67 ± 3.93	95.24 ± 0
300U	94.44 ± 0	85.35 ± 5
400U	95 ± 0	91.62 ± 4.78

Table 4.1 b

Comparison of viable cell yields of APV26 and Trypsin for HEp-2 cell lines

Enzyme concentration	Percentage viable cell yields	
	APV26	Trypsin
50 U	92.61 ± 3.01	91.29 ± 0.54
100 U	90.90 ± 0.59	
200 U	96.15 ± 5.44	100 ± 0
300 U	100 ± 0	
400 U	100 ± 0	94.84 ± 0.56

Table 4.1 c

Comparison of viable cell yields of APV26 and Trypsin for RTG-2 cell lines

Enzyme concentration	Percentage viable cell yields	
	APV26	Trypsin
50 U	100	96.08 ± 0.11
100 U	100	100 ± 0
200 U	100	97.87 ± 1.02
300 U	100	97.32 ± 3.79
400 U	100	93.27 ± 2.78

Table 4.1 d

Two-way ANOVA table for the effect of enzyme concentration and the type of enzyme used on time required for detachment of various cell lines

HeLa Cell lines					
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***
Enzyme concentration	27.444	4	6.861	3.134308	0.147213
Enzyme used	178.929	1	178.929	81.74006	0.000829
Error	8.756	4	2.189		
Total	215.129	9			
HEp-2 cell lines					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Enzyme concentration	1.28584	4	0.32146	1.827931	0.286697
Enzyme used	67.28836	1	67.28836	382.6246	4.03E-05
Error	0.70344	4	0.17586		
Total	69.27764	9			
RTG-2 cell lines					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Enzyme concentration	8.60214	4	2.150535	2.337754	0.2155
Enzyme used	1.06929	1	1.06929	1.162379	0.341651
Error	3.67966	4	0.919915		
Total	13.35109	9			

Appendix 5

Table 5.1 e

Two-way for the effect of enzyme concentration and the type of enzyme used on viable yields of cell lines

HeLa cell lines					
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***
Enzyme concentration	54.25182	4	13.56295	0.495506	0.743408
Enzyme used	63.75239	1	63.75239	2.329116	0.201675
Error	109.4877	4	27.37193		
Total	227.4919	9			
HEp-2 cell lines					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Enzyme concentration	5616.954	4	1404.238	1.038823	0.48572
Enzyme used	3745.056	1	3745.056	2.770506	0.171347
Error	5407.036	4	1351.759		
Total	14769.05	9			
RTG-2 cell lines					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Enzyme concentration	12.28415	4	3.071038	1	0.5
Enzyme used	23.91113	1	23.91113	7.786009	0.049295
Error	12.28415	4	3.071038		
Total	48.47943	9			

Table 5.2 Compatibility with commercial detergents

Detergent	Residual activity (%)
Control	100 ± 1.67 [d]
Surf Excel	78.83 ± 0.23 [c]
Sunlight	63.15 ± 1.95 [b]
Ariel	77.45 ± 0.34 [c]
Tide	48.92 ± 7.47 [a]
Ujala	50.43 ± 1.44 [a]
Surf Excel Matic	42.42 ± 0.69 [a]

Table 5.2 b

ANOVA for compatibility with commercial detergents

	Sum of squares	Df	Mean square	F	Sig.
Between groups	5099.600	6	849.933	91.372	.000
Within groups	65.113	7	9.302		
Total	5164	13			

Table 5.3

Effect of NaCl on enzyme activity

NaCl concentration	Residual activity (%)
0 M	100 ± 0
0.5 M	91.18 ± 1.07
1 M	88.36 ± 0.11
1.5 M	82.67 ± 1.83
2 M	77.09 ± 0.85
2.5 M	72.64 ± 1.05

Table 5.4

Effect of organic solvents on enzyme activity

	Residual activity (%) at after different periods of exposure		
Organic solvent	1hr	24 hrs	72 hrs
DMSO	96.62 ± 0.43	94.06 ± 1.61	100.51 ± 1.49
Isopropanol	96.02 ± 0	97.56 ± 0.67	100.46 ± 0.60
Ethanol	96.48 ± 0.50	98.40 ± 0.33	99.83 ± 0
Acetonitrile	97.29 ± 0.25	98.97 ± 0d	98.19 ± 0.72
Ethyl ether	97.07 ± 1.30	97.15 ± 0.94	98.95 ± 0
Acetone	81.01 ± 1.47	82.50 ± 0.36	86.21 ± 0.78
Control	100	100	100