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

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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).

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 alternative 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,
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-benzoyloxy carbonyl 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) sulfo succinate (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 *Norcardiopsis* sp. 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 50-70°C (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 37°C (Patel et al., 2006) while an unusually high temperature optimum of 75°C 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°C (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 phenylmethylsulfonyl 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), *vme* 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, 1987a, 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 pathogenesis 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 *Vibrio* 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 121°C 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**

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<th>Ingredients</th>
<th>Concentration</th>
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<tr>
<td>Peptone</td>
<td>5 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Gelatin</td>
<td>10 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
</tr>
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3.2.1.3 Preparation of inoculum

The selected strain *Vibrio* sp. (V26) was inoculated onto nutrient agar slant and incubated at 28°C. 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 30°C at 130 rpm overnight (18 hrs).
### Composition of Nutrient agar

<table>
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<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
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</tbody>
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#### 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 30°C at 130 rpm for 48 hrs. The cell free supernatant was recovered by centrifugation (8000 x g, 4°C, 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 60°C for 30 minutes. The reaction was stopped by the addition of 500 µl of 20% trichloroacetic 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.

\[
\text{Relative activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Maximum enzyme activity (U)}}
\]

3.2.2.5 Residual activity

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

\[
\text{Residual activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Activity of control (U)}}
\]
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-equilibrated 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™ 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 4°C. 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 37°C, 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 30-80°C. The percentage relative activity was calculated (section 3.2.2.4) considering the activity at 60°C as 100%.

The temperature stability of the enzyme was determined by pre-incubating the enzyme at different temperatures (30-80°C) 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 (ZnCl₂, CaCl₂, MgCl₂, MnCl₂, PbCl₂, CoCl₂, HgCl₂, BaCl₂, and CuSO₄) at final concentration of 1mM and 5 mM at 60°C 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 60°C 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 (H₂O₂) and surfactants (SDS, Triton X-100 and Tween-80) on the enzyme stability, the purified protease was pre-incubated with different concentrations of H₂O₂ (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 erythrocyes 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 1x10⁵ 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.nlm.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 activity-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>
<thead>
<tr>
<th>Purification steps</th>
<th>Protease activity (U)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1115.16</td>
<td>0.91</td>
<td>1225.45</td>
<td>1</td>
</tr>
<tr>
<td>40-80% Ammonium sulphate fraction</td>
<td>2379.17</td>
<td>0.76</td>
<td>3130.48</td>
<td>2.6</td>
</tr>
<tr>
<td>DEAE Cellulose</td>
<td>1249.65</td>
<td>0.21</td>
<td>5950.73</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table 3.1

Summary of the purification of *Vibrio* sp. (V26) protease
Chapter 3  Purification and characterization of alkaline protease from *Vibrio* sp. (V26)

![Graph](image1.png)

**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.

![Image](image2.png)

**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).

![Figure 3.3: Effect of pH on the activity of protease from Vibrio sp. (V26)](image)

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)](image)

**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 (30-80°C) tested, with maximum activity recorded at 60°C, qualifying it to be designated as a moderately thermo-active protease. A sharp decline in activity at temperatures above 60°C was noted. Within the temperature range 40-60°C the protease retained more than 85% of its maximum activity. Even at temperatures 30 and 80°C 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).
### 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 30-50°C 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 70°C for 1 hour.

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

**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 (\(\rho >0.05\), 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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>IAA (Cysteine protease inhibitor)</td>
<td>1 mM</td>
<td>87.86 ± 2.09</td>
</tr>
<tr>
<td>1,10 Phenanthroline (Metallo-protease inhibitor)</td>
<td>5 mM</td>
<td>0</td>
</tr>
<tr>
<td>EDTA (Metalloprotease inhibitor)</td>
<td>5 mM</td>
<td>47.55 ± 3.65</td>
</tr>
<tr>
<td>PMSF (Serine protease inhibitor)</td>
<td>5 mM</td>
<td>94.48 ± 0.54</td>
</tr>
</tbody>
</table>

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$_2$O$_2$, a strong oxidizing agent; only less than 10% inhibition in activity was observed. Even at 0.5% of H$_2$O$_2$, 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)

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration (%)</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>0.1</td>
<td>70.72 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>32.25 ± 1.25</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0.1</td>
<td>90.27 ± 3.28</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>57.41 ± 3.27</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.5</td>
<td>111.41 ± 0.99</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>110.48 ± 3.68</td>
</tr>
</tbody>
</table>

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.](image)

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](image-url)
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>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline protease from <em>Vibrio</em> sp. (V26)</td>
<td>5950.73</td>
</tr>
<tr>
<td>Savinase from <em>Bacillus</em> sp.</td>
<td>190.83</td>
</tr>
</tbody>
</table>
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).

![PCR product of the protease gene amplification](image)

**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 (Appendix 1). 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 metalloprotease have been indicated in Fig. 3.11.

**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

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Query coverage</th>
<th>E value</th>
<th>Max ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP002556.1</td>
<td><em>Vibrio cholerae</em> LMA3894-4 chromosome II, complete sequence</td>
<td>100%</td>
<td>7e-140</td>
<td>99%</td>
</tr>
<tr>
<td>GQ912701.1</td>
<td><em>Vibrio cholerae</em> O1 strain Ogawa vibriolysin (hap) gene, partial cds</td>
<td>100%</td>
<td>7e-140</td>
<td>99%</td>
</tr>
<tr>
<td>CP001486.1</td>
<td><em>Vibrio cholerae</em> MJ-1236 chromosome 2, complete sequence</td>
<td>100%</td>
<td>7e-140</td>
<td>99%</td>
</tr>
<tr>
<td>CP001234.1</td>
<td><em>Vibrio cholerae</em> M66-2 chromosome II, complete sequence</td>
<td>100%</td>
<td>7e-140</td>
<td>99%</td>
</tr>
<tr>
<td>AE003853.1</td>
<td><em>Vibrio cholerae</em> O1 biovar eltor str. N16961 chromosome II, complete sequence</td>
<td>100%</td>
<td>7e-140</td>
<td>99%</td>
</tr>
<tr>
<td>DQ776042.1</td>
<td>Synthetic construct <em>Vibrio cholerae</em> clone FLH200370.01F hap gene, complete sequence</td>
<td>100%</td>
<td>7e-140</td>
<td>99%</td>
</tr>
<tr>
<td>M59466.1</td>
<td><em>V.cholerae</em> HA/protease gene, complete cds</td>
<td>100%</td>
<td>7e-140</td>
<td>99%</td>
</tr>
<tr>
<td>CP001236.1</td>
<td><em>Vibrio cholerae</em> O395 chromosome II, complete sequence</td>
<td>100%</td>
<td>7e-135</td>
<td>98%</td>
</tr>
<tr>
<td>CP000626.1</td>
<td><em>Vibrio cholerae</em> O395 chromosome 1, complete genome</td>
<td>100%</td>
<td>7e-135</td>
<td>98%</td>
</tr>
<tr>
<td>Z27239.1</td>
<td><em>H. pylori</em> HAP gene for haemagglutinin/protease</td>
<td>89%</td>
<td>1e-117</td>
<td>98%</td>
</tr>
</tbody>
</table>
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. RC386-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. caribbenticus-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 100°C 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 60°C. 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 60°C (Ishihara *et al.*, 2002; Lee *et al.*, 2002; Lee *et al.*, 2003b; Venugopal and Saramma, 2006). Exceptionally high temperature optima of 75°C 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 40-60°C and even at 30°C 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 50°C (Fig. 3.6) and the stability exhibited did not vary significantly in the temperature range of 30-50°C. 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 (60°C) 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 50°C 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 60°C 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 moiety (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$_2$O$_2$ an oxidizing agent. At concentrations of 0.1% and 0.5% of H$_2$O$_2$ 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 (4°C) (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 LC₅₀ 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
by 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 except 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. .