

# Isolation and Characterization of Protease Producing Marine Eubacteria

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**A** **STRACT** Twenty five bacterial isolates from soil samples collected from coast of Andhra Pradesh were screened for protease production, among which 8 strains showed proteolytic activity and one isolate (N2) was selected for further study. The proteolytic bacteria was identified as Bacterium VITKHRB based on morphological, biochemical tests and 16s rDNA sequencing. Enzyme was produced, purified up to 1.20 fold and its specific activity was found to be 1.83 IU/mg. Therelative molecular mass of enzyme was measured 36 kDa by SDS-PAGE. The best enzyme activity was observed at pH 8 and temperature 35°C, 6.5% NaCl concentration, xylose as carbon source and yeast extract as nitrogen source. This enzyme is expected to be a good industrial application as it was found to digest egg white and remove blood stain efficiently. This is the first report on protease production from marine eubacteria inhabiting coast of Bay of Bengal near Andhra Pradesh.

## Key Words

Protease, Eubacteria, Bacterium VITKHRB, SDS-PAGE.

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## 1 Introduction

Enzymes are the biocatalysts that are known for enhancing rate of metabolic reaction by lowering the activation energy in our body. A number of enzymes being important in vivo processes and have industrial importance too. Among these, protease is one of the industrially important enzymes. It is a naturally occurring enzyme present in all organisms constituting 1-5% of total protein content. It is present in all living organisms including human beings, plants, insects even micro organisms like bacteria, actinobacteria, viruses etc (Karthik *et al.*, 2011). Protease has been also reported in many pathogenic and infectious micro-organisms. It is responsible for proteolysis i.e., protein catabolism by hydrolysis of peptide bonds that link amino acids together in the polypeptide chain. They themselves being protein are cleaved by other and even same variety of protease molecules. They are capable of hydrolysing almost all protein as long as they are not the component of living cells. They are of great importance as they can lead to activation of a function or can be signal in a pathway. Extracellular proteases are important for the hydrolysis of external proteins and enable the cell to absorb and utilize the hydrolytic products. Simultaneously, they can be destructive as they can abolish a protein's function or digest it to its principal components. Moreover, they are simultaneously, they can be destructive as they can abolish a protein's function or digest it to its principal components. Moreover, they

are also considered as one type of exotoxins. Besides this they are of great value in food, detergent, leather, pulp and paper industry (Anonymous). They constitute about two-third of total enzymes used in industries for various purposes. It is also used in bioremediation processes. Microbial proteases are very important in wide variety of biotechnological applications and account for approximately 59 % of total enzymes used. Among bacteria, the *Bacillus* sp. is specific producers of extracellular proteases (Joshi, 2010). The lack of pathogenicity and the ability to grow in simple culture medium can also be accounted for their applications in industry (Daniel *et al.*, 1984).

Marine environment has been natural habitat for organisms known for bioactive compounds viz, enzymes, antibiotics, biosurfactants, etc. It has been found to be highly variable in respect of different parameters viz salinity, temperature, pressure, density, light and even sound because of which the bioactive substances synthesized by its inhabitants are also capable to sustain or retain their activity in such a flexible or extreme conditions (Karthik *et al.*, 2011). These organisms may be various plants, animals, fungi and even microorganisms. In fact now-a-days microorganisms are more focussed as compared to plants and animals for enzymes of industrial importance as they have been found to be more stable than enzymes derived from plants and animals. Unusual characteristics can be seen in these enzymes due to their habitat related properties such as salt tolerance (above 1.7M) (Marhuenda

*et al.*, 2002), hyperthermostability (80-108°C), barophilicity (60 MPa), cold adaptivity (Esterase can retain its 50% activity at freezing point of water) and pH. They also have novel chemical and stereochemical properties (Trincone, 2011). Enzymes derived from extremophilic archaea have higher stability towards heat, pressure, detergents, solvents and they are often more resistant to cellulolytic attack (Egorova *et al.*, 2005). Marine enzymes have also been used in pollution monitoring (Van der Oost *et al.*, 2003). In a review by Trincone (2010) the entire marine enzyme has been highlighted. A number of extremophiles have been isolated from marine environments which can thrive on wide range of pH, salt concentration and pressure and they have enzymes which can act upon carbohydrates, proteins and lipids (Antranikian *et al.*, 2005; Demirjian *et al.*, 2001; Ferrer *et al.*, 2007). Several screening techniques and processes methodology are being adapted for isolation and detection of potent micro-organisms for production of enzymes with novel physiological properties (Karthik *et al.*, 2010). It has been found that marine bacteria has a unique light harvesting pigment called as Proteorhodopsin which mediates phototrophy that allows their survival even during starvation period. In present study we report the production of protease from marine eubacteria isolated from the soil samples collected from coast of Bay of Bengal near Andhra Pradesh. Earlier few groups have reported the effective production of marine eubacterial enzyme. But this is the first report of enzyme production from marine eubacteria belonging to coast of Bay of Bengal near Andhra Pradesh. The enzyme produced can be utilized effectively as an industrial application.

## 2 Materials and methods

### Chemicals

All the media used for this study and dialysis membrane was purchased from Hi Media chemicals, Mumbai, India and

chemicals were from Merck Specialities Private Limited, India and Sisco Research Private Limited, Mumbai, India respectively.

### Sample collection

Salt pan soil samples were collected from Ongole (15°30'N, 80°03'E) which is situated at coastal regions of Andhra Pradesh in sterile polybags at a depth of about 3-4 cm with the help of a sterile spatula. The bags were transferred to the labs in sterile conditions and were stored at 4°C till isolation.

### Isolation of Marine Eubacteria

Isolation was done by serial dilution method. Plating was done by spread plate method on Nutrient Agar medium supplemented with 50% distilled water and 50% marine water. The plates were incubated for 24-48 hrs at 37°C.

### Screening of potent Protease producing strains

The isolates were screened for proteolytic activity by growing them on Nutrient Agar Medium supplemented with 1% Casein and Skim milk. Casein is a type of phosphoprotein which is prepared by acetic acid precipitation. Skim milk powder is a milk made protein supplement which is made by dehydrating milk after the removal of its major fat components. These two proteins are generally used for screening of protease. After incubation period plates were observed for clear zone around the colonies.

### Identification of protease producing bacteria

#### Cultural characterization

The isolates were observed under the microscope, the colony morphology was noted down with respect to colour, shape, size, nature of colony and pigmentation.

#### Microscopic observation

The bacterial isolates were stained by Gram staining and observed under a high power magnifying lens in Light

microscope. Endospore staining and Capsule staining were performed to observe the morphology of the cells.

#### Biochemical characterization

The bacterial isolates were characterized biochemically by Indole test, Methyl red test, Voges-Proskauer test, Simmons Citrate test.

#### Molecular characterization

The strains were screened on the basis of above tests and the most efficient isolate was characterised based on 16S rDNA sequencing. Phylogenetic tree was constructed using the Tree view software.

#### Production of Enzymes

Fermentation medium for protease was prepared. Protease production media was prepared which contained, (g/L) Dextrose 10, Peptone 5, KH<sub>2</sub>PO<sub>4</sub> 2, MgSO<sub>4</sub>·7H<sub>2</sub>O 2, Casein 10, at pH 8. Enzyme production was carried out by inoculating 10ml of bacterial inoculum in 500 ml production medium and the flask was kept on rotary shaker incubator at room temperature for 24 hours.

After incubation, fermented broth was centrifuged at 10000 rpm for 10 minutes in a cooling centrifuge. Supernatant was collected and used for estimation of protease.

#### Optimization of temperature, pH, carbon source, nitrogen source and NaCl concentration on protease enzyme productivity and enzyme activity

Effect of temperature on enzyme production and enzyme activity was studied by adjusting the incubation temperature at 25, 30, 35, 40, and 45°C and production medium pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Similarly, effect of carbon source, nitrogen source and NaCl concentration was studied by adjusting different carbon sources (Sucrose, Fructose, Xylose, Starch and Lactose), nitrogen sources (Beef extract, Yeast extract, Nutrient Broth, Urea and Casein) and NaCl concentrations (2.5, 3.5, 4.5, 5.5, 6.5 %) in the production medium (Ashwini *et al.*, 2010).

#### Protein Estimation

Protein estimation was done using Lowry's method (Lowry *et al.*, 1951).

#### Enzyme Assay

The original casein assay was first described by Kunitz (1947) and later

modified by **Detmar and Vogels (1971)**. 0.1 ml of enzyme was taken in test tube and 0.9 ml of 0.1 N TrisHCl (pH 8) was added to it. 1 ml of 1% Casein was added and it was incubated at 37°C for 30 minutes in a water bath. 2 ml of Trichloroacetic acid (TCA) was added to stop the reaction. The reaction mixture was centrifuged at 5000 rpm for 10 min. Supernatant was collected and absorbance was measured at 280 nm by spectrophotometer.

### Partial purification

#### Ammonium Sulphate precipitation

The 100 ml of cell free extract was saturated with ammonium sulphate. The desired saturation of 80% was achieved. The contents were centrifuged at 5000 rpm for 20 min and pellet was collected. The supernatant was saturated to 90% again. Again the contents were centrifuged at 5000rpm for 20 minutes. Now the supernatant was discarded and pellet was collected for further analysis.

#### Dialysis

The precipitate was desalted by dialysis. The enzyme solution was placed in a bag of selectively permeable membrane (Dialysis membrane-150) One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, dialysis bag was suspended in a beaker containing 0.5 M Tris-HCL buffers (pH 8) for 24 hours.

#### SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) with slight modification using a 10% cross linked polyacrylamide gel.

### Application of Protease

#### Digestion of natural protein

The crude enzyme (10 ml) was incubated with coagulated egg white at room temperature at different incubation time.

#### Removal of Blood Stain

Few drops of human blood were

taken on a clean piece of cloth and blood clot was allowed to dry. Then the cloth was incubated with crude protein at room temperature for 24 hours. After incubation cloth was rinsed with water for 2 minutes and then dried. The same procedure was done with control (detergents).

## 3 Results

In the present study a total of 4 marine sediments were collected and processed by serial dilution and spread plate method. A total 25 different bacterial strains (Figure 1) were isolated from the marine sediments of Bay of Bengal in the coastal regions of Andhra Pradesh. Out of these 25 isolates, 8 isolates showed proteolytic activity. Among them a strain N2 was selected based on its ability to produce largest zone of hydrolysis on Casein Agar plate. According to the results of primary

screening strain N2 was chosen for production.

### Identification of protease producing bacteria

The bacterial isolates were characterized on the basis of colony morphology, microscopic characteristics and biochemical tests. Taxonomical identification of the bacterial isolate was performed by 16S rDNA analysis. The 16S rDNA sequence of the bacteria was blasted using online tool blast of NCBI gene bank and the phylogenetic tree was constructed with other homologous sequences (Figure 2). According to the results obtained from morphological, biochemical (Table 1) and 16S rDNA sequence characteristics, the isolate had 97% similarity with *Bacillus* sp and named as *Bacterium VITKHRB* (Acc.no: JN656215).

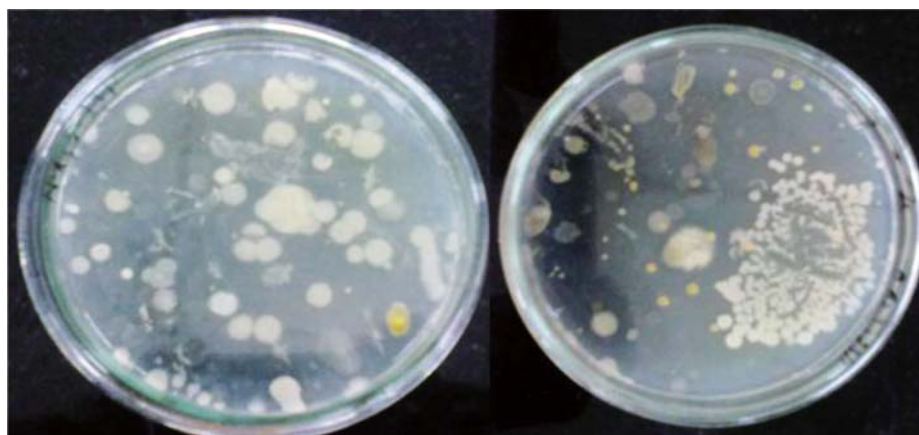


Fig.1 Isolation of eubacteria

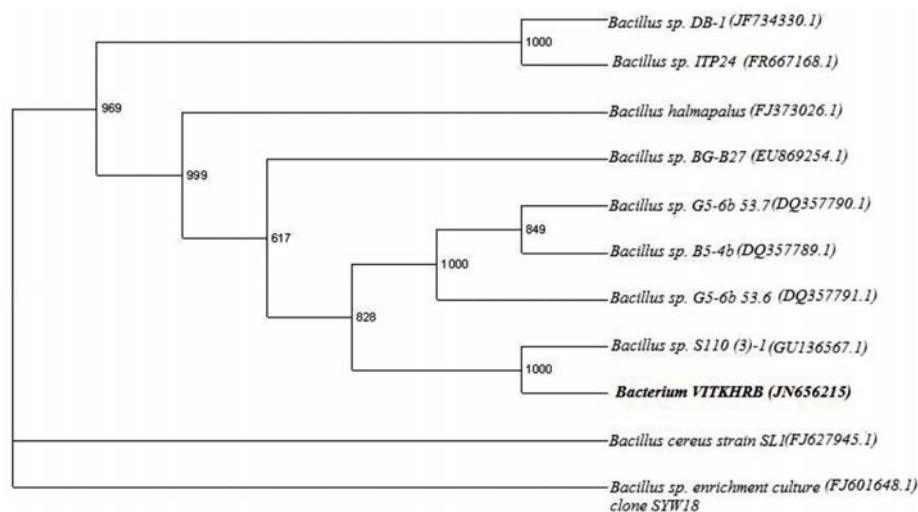


Fig.2 Phylogenetic tree of *Bacterium VITKHRB*



**Effect of temperature on enzyme activity and protein content**

Enzyme activity was maximum at 35°C. Enzyme production decreased as temperature was increased to 40°C and above (Figure 3).

**Effect of pH on enzyme activity and protein content**

Maximum enzyme activity was observed at pH 8 and as the pH was increased or decreased, there was gradual decrease in growth of the organism, protein content and enzyme activity (Figure 4). Organism did not grow at pH below 5 and above 10.

**Effect of carbon sources on enzyme activity and protein content**

The effect of Carbon source on protease production was characterized using five different sugars at 1 % (w/v) concentration. Maximum enzyme activity was observed in the presence of xylose as carbon source, whereas, the minimum enzyme activity and protein content was observed in the presence of fructose (Figure 5).

**Effect of nitrogen source on enzyme activity and protein content**

Production of protease has been studied in presence of five different organic and inorganic nitrogen sources. Among all, yeast extract showed maximum enzyme activity as compared to other organic nitrogen sources (Figure 6).

**Effect of NaCl concentration on enzyme activity and protein content**

At 6.5 % NaCl concentration, the enzyme activity was found maximum (Figure 7). As the NaCl concentration decreased, there was gradual decrease in enzyme production, enzyme activity and growth of organism.

**Partial purification of Protease**

Table 1. Morphological and biochemical characteristic of *Bacterium VITKHRB*

	Characterization of bacteria	Result
Culture characteristics	Colony Morphology on Nutrient Agar Medium	Medium, circular, off white mucoid, colonies
Microscopic characters	Gram staining	Gram positive, rods
	Endospore staining	Terminal Endospore forming
	Indole	Negative
Biochemical characters	Methyl Red	Negative
	VogesProskauer	Positive
	Citrate Utilization	Positive
	Catalase test	Positive
	Oxidase test	Positive
	Starch Hydrolysis	Positive

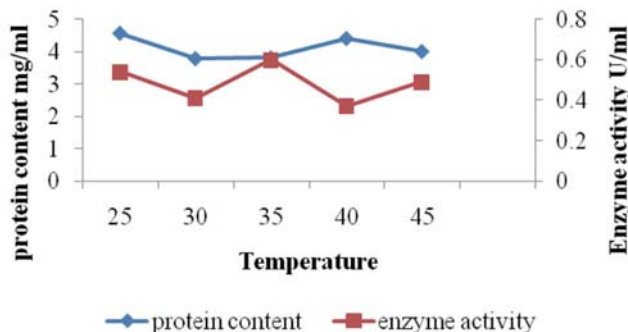


Fig.3 Effect of temperature on enzyme activity and protein content

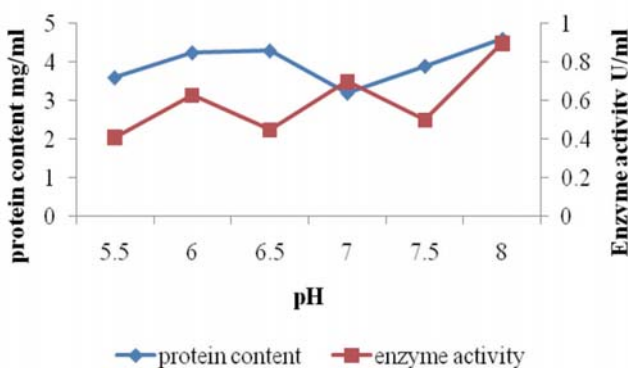


Fig.4 Effect of pH on enzyme activity and protein content

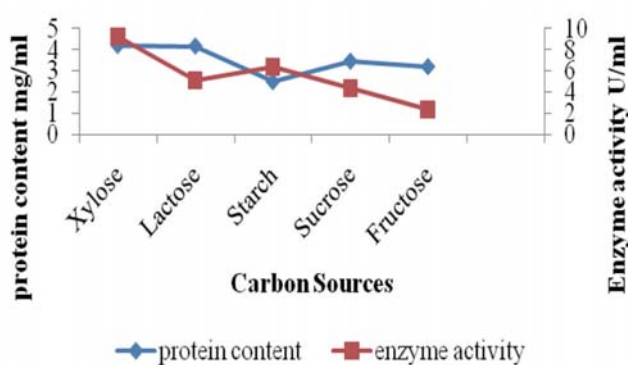


Fig.5 Effect of carbon sources on enzyme activity and protein content

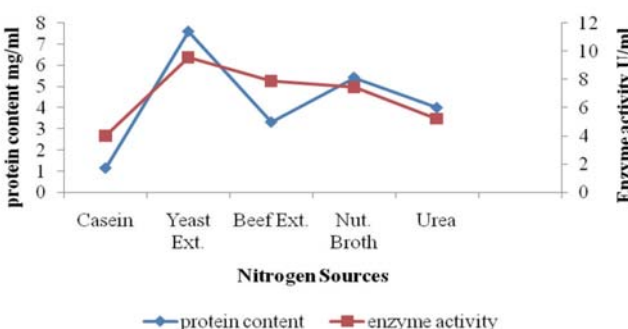


Fig.6 Effect of nitrogen source on enzyme activity and protein content

Table 2. Partial purification of protease from *Bacterium VITKHRB*

Purification steps	Total protein (mg/ml)	Enzyme Activity (mg)	Specific Activity (U/ml/mg)	Fold Purification	Yield (%)
Crude Extract	5.50	8.20	1.50	1.00	100
Ammonium precipitation	0.72	1.23	1.70	1.13	15
Dialysis	0.30	0.55	1.83	1.20	6.7

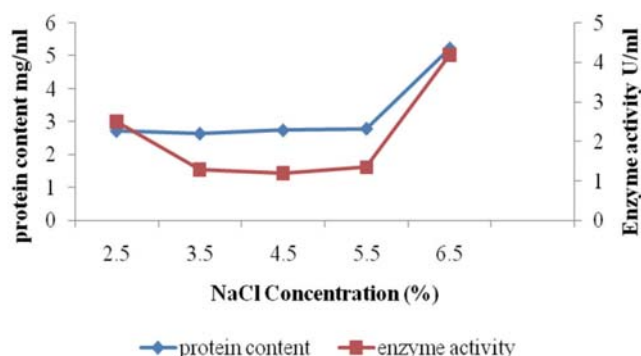


Fig.7 Effect of NaCl concentration on enzyme activity and protein content

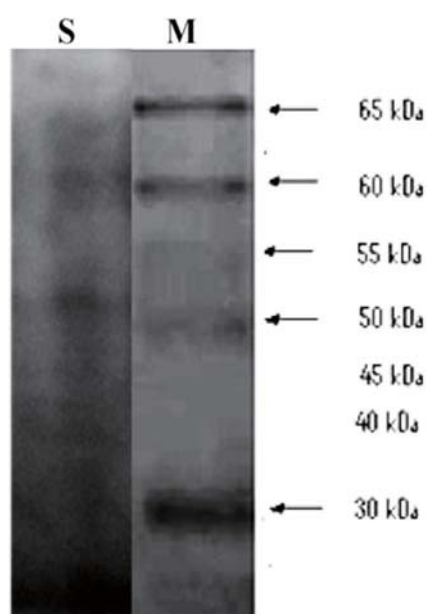


Fig.8 Polyacrylamide gel electrophoresis of partially purified sample (S – sample; M – Protein Marker).

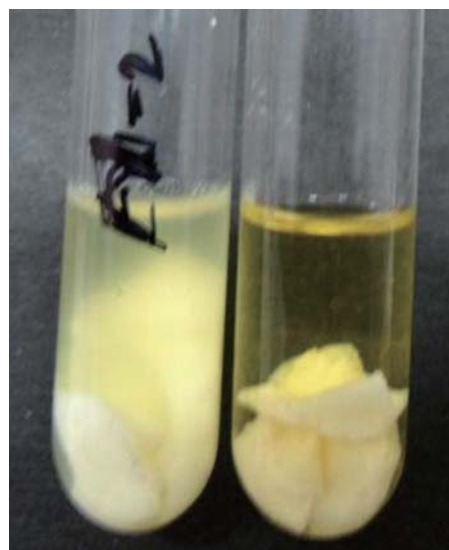


Fig.9 Degradation of egg white

Partial purification of protease enzyme was performed by Ammonium sulphate precipitation followed by dialysis. Increase in enzyme activity was observed after ammonium sulphate precipitation and dialysis. Partially purified protease exhibited specific activity of 1.83 U/ml/mg which corresponds to 1.20 purification fold and 6.7 % Yield (Table 2).

#### SDS-PAGE

SDS-PAGE was performed for the partially purified protease after dialysis (Figure 8). Multiple bands were observed in the gel at 36kd, 50kd and 60kd etc as the protein was not fully purified and only partially purified (Dialysis). The partially purified protein has to be subjected to further purification steps such as ion-exchange chromatography etc.

#### Digestion of Natural Protein

The egg white was completely digested after 24 hours of incubation at room temperature. Since the egg white contains coagulase so the protease may be of coagulase type (Figure 9).

#### Removal of Blood Stain

After 24 hours of incubation at room temperature partial removal of blood stain was observed on the cloth treated

with the crude enzyme. Stain removal rate was found to be high as compared to control.

## 4 Discussion

Proteases are considered among the most important enzymes to be produced commercially and are of great significance. They have their applications in food, detergent pharmaceuticals etc. Earlier several studies reported the biological production of protease from marine microorganisms. The present study is a preliminary screening report of soil samples obtained from coastal regions of Andhra Pradesh. Number of isolates was more as compared to earlier reports (Elela *et al.*, 2011). This suggests that the coastal regions of Andhra Pradesh are potent source of industrially important microorganisms. Out of all the isolates the isolate with the highest zone of inhibition was selected for protease production. It was identified as *Bacillus* spand named as *Bacterium VITKHRB*. Earlier reports also show efficient protease from *Bacillus* sp. (Das and Prasad, 2010; Joshi, 2010; Senthilraja, and Saravanakumar, 2011) isolated from different sources. Enzyme activity and production was checked in different conditions of temperature, pH, different carbon sources, different nitrogen sources and salt concentration. In case of temperature the results obtained are in accordance with that of results reported by El-Kastawy in 1998. Different optimum incubation temperatures were reported by other investigators such as 35°C (Gerze *et al.*, 2005), 50°C (Ammar *et al.*, 1991; Ali, 1991) 65°C, and 70°C (Sookkheo *et al.*, 2000). As far as pH is concerned the results are correlating with the other reports obtained for the optimum pH for enzymatic activity of other *Bacillus* species: pH 7.5 for *Bacillus subtilis* ITBCCB 148

(Yandri *et al.*, 2008), *Bacillus* sp. HS08 (Huang *et al.*, 2006) and *Bacillus* sp. S17110 (Jung *et al.*, 2007), pH 8.0 for *Bacillus cereus* KCTC 3674 (Kim *et al.*, 2001), Thermophilic *Bacillus* SMIA2 (Nascimento and Martins, 2004) and *Bacillus cereus* BG1 (Ghorbel-Frikha *et al.*, 2005). Results observed in case of different carbon sources agree with one report which suggested that sources of carbon affected production of enzymes by bacteria (Juhasz *et al.*, 2003). Starch cause low protease production. This in accordance with one report which showed low protease production in presence of starch (Jadeja and Bhatiya, 2010). This is in contrast to one report which showed that starch caused high level of enzyme expression in *Bacillus* species (Mahmood *et al.*, 2000). It has been reported that pure sugars affected protease production considerably (Dahot, 1993). Production of protease in presence of different nitrogen sources are contrast to the result obtained by Yang and Lee (2001) which showed highest enzyme activity in presence of Beef Extract.

Related studies also reported that protease production by *Bacillus stearothermophilus* F1 and *Bacillus mojavensis* was best in the presence of organic nitrogen sources (Razzak *et al.*, 1995; Beg and Gupta, 2003) respectively. However, some organisms responded to organic nitrogen sources and found to be better nitrogen sources both for growth and protease production (Aleksieva *et al.*, 1981; Phadatare *et al.*, 1993). The organism shows a good growth and adaptability at 6.5% NaCl concentration. It also maintains high enzyme activity as compared to other concentrations, which is highly preferred for commercial purposes. Maximum enzyme activity at 1.5% NaCl concentration is in accordance with the earlier reports (Shaheen *et al.*, 2008). The enzyme was purified upto 1.20 fold by ammonium sulfate cut and dialysis and its specific activity was increased to 1.83 U/mg/ml which is very high thus this strain can be used for commercial production of protease. The increase in protease activity by using ammonium sulfate is same as reported earlier (McKevitt *et al.*,

1989; Sexton *et al.*, 1994). In literature, the alkaline proteases with molecular weight ranging from 16-36 KDa are reported from *Bacillus* sp. (Kaur *et al.*, 1998; Adinarayana *et al.*, 2004; Jaswal and Kocher, 2007; Almas *et al.*, 2009; Joshi, 2010). So it can be concluded that the band observed at 36kd is of protease as compared to earlier reports. Protease produced by this isolate can be efficiently used for industrial purposes for digestion or removal of proteins. It can also be used for detergent production since it is efficiently removing the blood stain. This is in accordance with the earlier reports which also show efficient digestive properties of protease (Malathi and Chakraborty, 1991). The protease produced is a coagulase protease since it can efficiently digest the egg white. So this enzyme can be utilized effectively as an industrial application.

## 5 Conclusion

Proteases are considered among the most important enzymes to be produced commercially and are of great significance. They have their applications in food, detergent pharmaceuticals etc. Earlier several studies reported the biological production of protease from marine microorganisms. The present study reports production of protease from *Bacterium VITKHRB*. The best enzyme activity was observed at pH 8 and temperature 35°C, 6.5% NaCl concentration, xylose as carbon source and yeast extract as nitrogen source. The protease enzyme produced is also capable of purified upto 1.20 fold and its specific activity is 1.83 U/mg/ml which is very high thus this enzyme can be utilized effectively as an industrial application.

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