Journal of Agricultural Technology 2012 Vol. 8(5): 1633-1649
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Isolation and characterization of protease producing marine eubacteria

Singh, K., Bose, H., Richa, K., Karthik, L., Gaurav, K. and K.V. Bhaskara Rao*

Molecular and Microbiology Research Laboratory, Environmental Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamilnadu, India

Singh, K., Bose, H., Richa, K., Karthik, L., Gaurav, K. and K.V. Bhaskara Rao (2012) Isolation and characterization of protease producing marine eubacteria. Journal of Agricultural Technology 8(5): 1633-1649.

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.

Keywords: Protease, Eubacteria, *Bacterium VITKHRB,* SDS-PAGE.

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

*Corresponding author:K.V. BhaskaraRao; e-mail: kokatibhaskar@yahoo.com

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 areSimultaneously, 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 exo toxins. 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 (80-108ºC), barophilicity (60 MpA), cold adaptivity (Esterase can retain its 50% activity at freezing point of water) and pH. They also have novel chemical and sterochemical properties (Trincone, 2011). Enzymes derived from extremophilicarchaea 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 p^H, salt concentration and pressure and they have enzymeswhich 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 an 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 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.

Materials and methods

Chemicals

All the mediaused 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 (15º30’N, 80 º03’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 4ºC 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 37ºC.

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. Endosporestaining and Capsule stainingwere performed to observe the morphology of the cells.

Biochemical characterization

The bacterial isolates were characterized biochemically by Indole test, Methyl red test, VogesProskauer 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_2PO_4 2, $MgSO_4.7H_2O$ 2, Casein 10, at pH 8. Enzyme production was carried out by inoculating 10ml of bacterial inoculums 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 37ºC 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).

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.

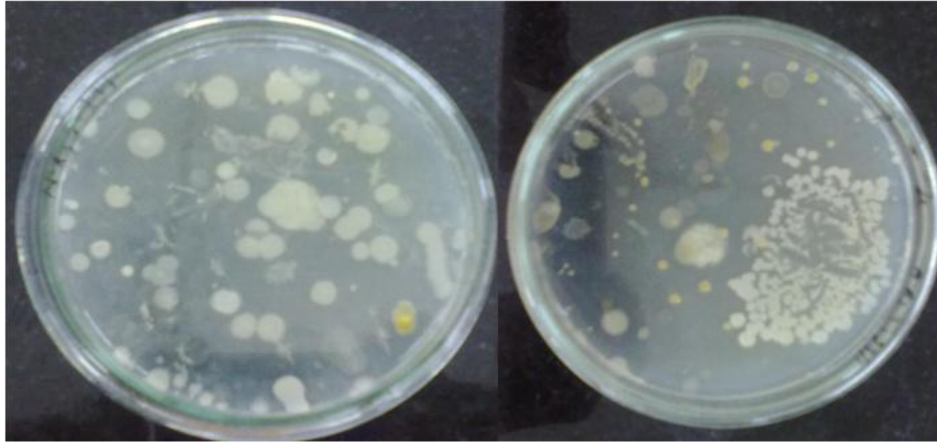

Fig. 1. Isolation of eubacteria

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).

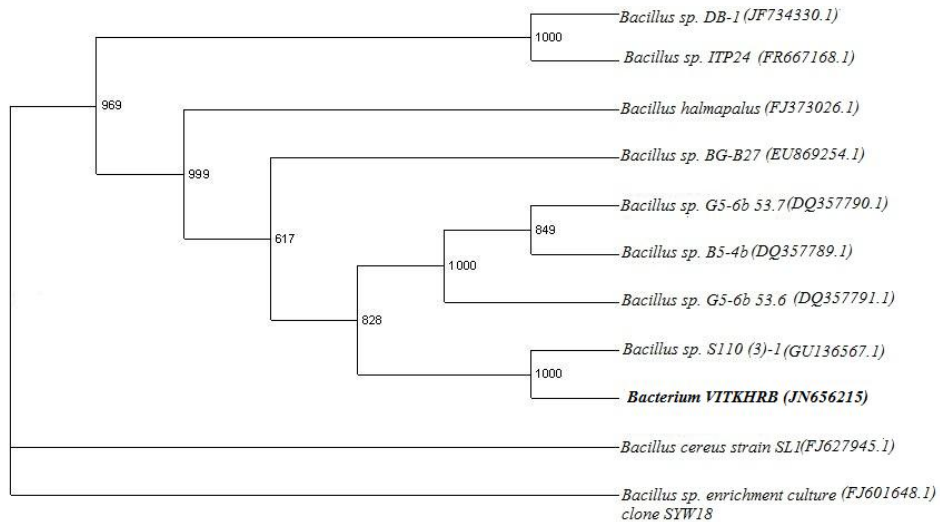

Fig. 2. Phylogenetic tree of *Bacterium VITKHRB*

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
Biochemical characters	Indole	Negative
	Methyl Red	Negative
	VogesProskauer	Positive
	Citrate Utilization	Positive
	Catalase test	Positive
	Oxidase test	Positive
	Starch Hydrolysis	Positive

Effect of temperature on enzyme activity and protein content

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

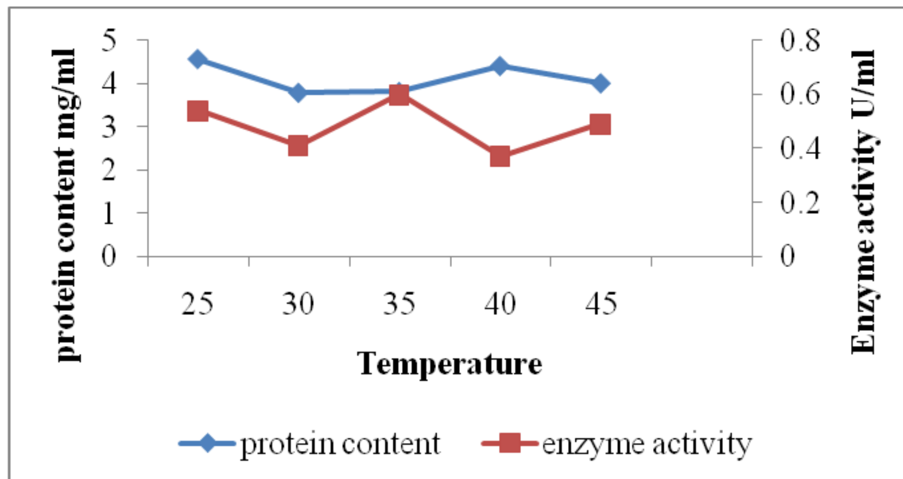

Fig. 3. Effect of temperature on enzyme activity and protein content

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.

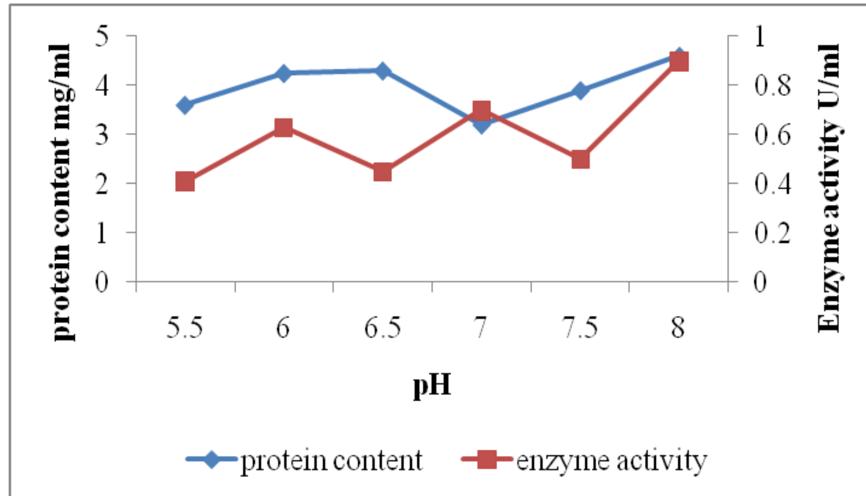

Fig. 4. Effect of pH on enzyme activity and protein content

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).

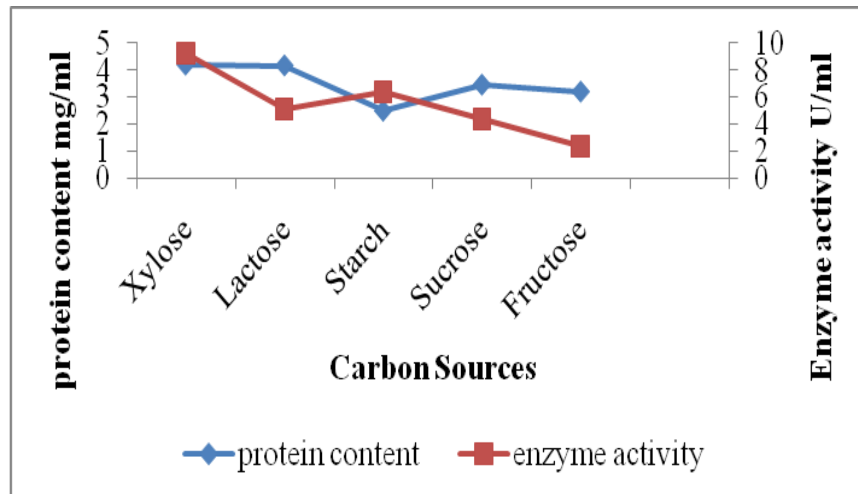

Fig. 5. Effect of carbon sources on enzyme activity and protein content

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).

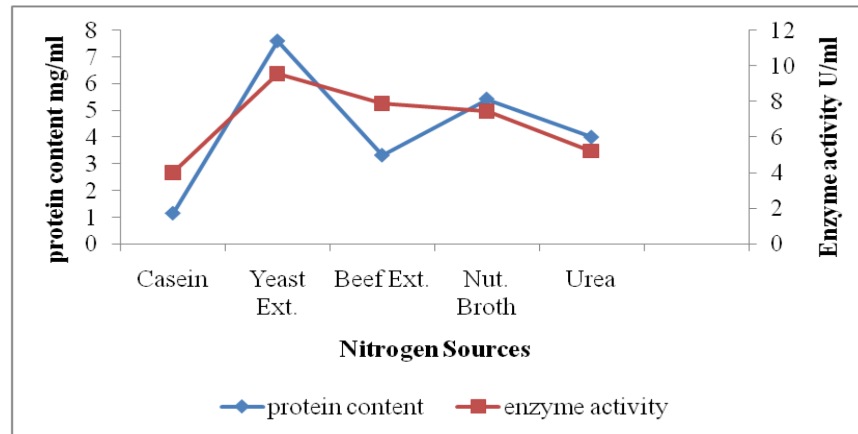

Fig. 6. Effect of nitrogen source on enzyme activity and protein content

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.

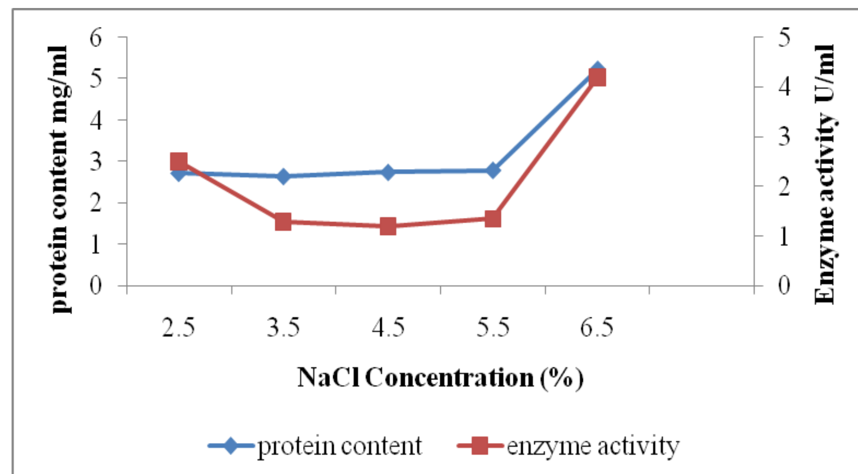

Fig. 7. Effect of NaCl concentration on enzyme activity and protein content

Partial purification of Protease

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).

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	100
Ammonium precipitation	0.72	1.23	1.70	1.13	15
Dialysis	0.3	0.55	1.83	1.20	6.7

SDS-PAGE

SDS-PAGE was performed for the partially purified protease after dialysis (Fig. 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.

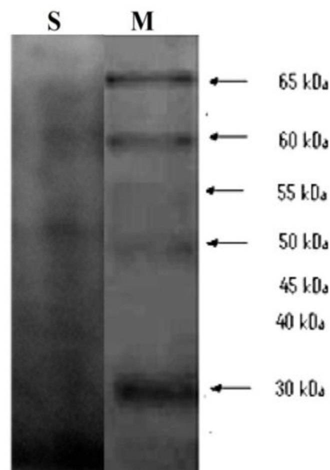

Fig. 8. Polyacrylamide gel electrophoresis of partially purified sample (S – sample; M – Protein Marker).

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 (Fig. 9).

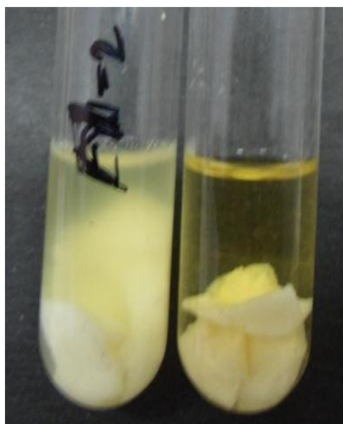

Fig. 9. Degradation of Egg White

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.

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 35ºC (Gerze *et al.,* 2005), 50ºC (Ammar *et al*., 1991; Ali, 1991) 65ºC, and 70ºC (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.

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.

Acknowledgement

Authors wish to thank management of VIT University, Vellore, TN, India, for providing necessary facilities and support for the completion of this work.

References

Adinarayana, K., Bapi-Raju, K.V.V.S.N., Ellaiah, P. (2004). Investigation on alkaline protease production with *B. subtilis*PE-11 immobilized in calcium alginate gel beads. Process Biochemistry 39(11): 1331-1339.

Aleksieva, P., Djerova, A., Tchorbanov, B. and Girarov, J. (1981). Submerged cultivation of a strain of *Humicolalutea* 72 producing acid protease. European Journal of Applied Microbiology and Biotechnology 13: 165.

Ali, O.A. (1991). Extracellular thermostable protease produced by thermophilic*Bacillus* sp. Az.J. Microbiol. (11): 78-95.

Almas, S., Hameed, A., Shelly, D. and Mohan, P. (2009). Purification and characterization of a novel protease from *Bacillus* strain SAL1. African Journal of Biotechnology 8(15): 3603-3609.

Ammar, M.S., El-Louboudy, S.S. and Abdulraouf, U.M. (1991). Protease (s) from *Bacillus anthracis* S-44 and *B. cereus* var. mycoids, S-98 isolated from a temple and slaughter house in Aswan city. Az. J. Microbiol. (13): 12-29.

Anonymous Protease: Production and Process, Technology and Applications.Primary Information Services. Retrieved March 25, (2011) www.primaryinfo.com/protease.html.

Antranikian, G., Vorgias, C.E. and Bertoldo, C. (2005). Extreme environments as a resource for microorganisms and novel biocatalysts.Advances in Biochemical Engineering/ Biotechnology 96: 219–262.

Ashwini K., Gaurav K., Karthik L. and BhaskaraRao K.V. (2011). Optimization, production and partial purification of extracellular α- amylase from *Bacillus* sp. *Marini.*Archives of Applied Science Research 3(1): 33-42.

Beg, Q.K. and Gupta, R. (2003). Purification and characterization of an oxidation-stable, thioldependent serine alkaline protease from *Bacillus mojavensis*.Enzyme and Microbial Technology 32: 294-309.

Dahot, M.U. (1993). Cultivation of penicilliumexpansum on rice husk powder for protease production.Journal of Islamic Academy and Sciences, pp 6.

Daniel, C.S., Kopec, L.K., Yasbein R.E. and Young, E.F. (1984). Characterization of *Bacillus substilis*DSM 704 and its production of 1-Deoxynojiirimycin.Applied and Environmental Microbiology 281: 280-284.

Das, G. and Prasad, M.P. (2010). Isolation, purification & mass production of protease enzyme from *Bacillus subtilis.* InternationalResearch Journals of Microbiology 1(2): 026-031.

Demirjian, D.C., Morís-Varas, F. and Cassidy, C.S. (2001). Enzymes from extremophiles.Current opinion in Chemical Biology 5: 144–151.

Detmar, D. and Vogels, A.D. (1971). Automated determination of protease for laundry compounds. Journal of American Oil Chemists' Society 48: 7–79.

Egorova, K. and Antranikian, G. (2005). Industrial relevance of thermophilicArchaea. Current Opinion in Microbiology 8: 649–655.

Elela, G.M.A., Ibrahim, H. A. H., Hassan, S.W., HananAbd-Elnaby, H.A. and El-Toukhy, N.M.K., (2011). Alkaline protease production by alkaliphilic marine bacteria isolated from Marsa-Matrouh (Egypt) with special emphasis on *Bacillus cereus* purified protease. African Journal of Biotechnology 10(22): 4631-4642.

El- Kastawy, S.F.M. (1998). Application of enzyme inhibitor technique in regulating the activities of purified constitutive protease produced by *Staphylococcus aureus* 1 -10 isolated from a clinical source. Egyptian Journal of Biomedical Sciences 2: 53-66.

Ferrer, M., Golyshina, O., Beloqui, A. and Golyshin, P.N. (2007). Mining enzymes from extreme environments.Current Opinion in Microbiology 10: 207–214.

Gerze, A., Omay, D. and Guvenilir, Y. (2005). Partial purification and characterization of protease enzyme from *Bacillus subtilis* and *Bacillus megatherium.*Applied Biochemistry and Biotechnology 121-124:335-345.

Ghorbel-Frikha, B., Sellami-Kamoun, A., Fakhfakh, N., Haddar, A., Manni, L. and Nasri, M. (2005). Production and purification of a calcium protease from *Bacillus cereus* BG1.Journal of Industrial Microbiology and Biotechnology 32: 186-194.

Huang, G., Ying, T., Huo, P. and Jiang, Y.Z. (2006). Purification and characterization of a protease from thermophilic*Bacillus* strain HS08. African Journal of Biotechnology 5: 2433-2438.

Jadeja, G.R. and Bhatiya, R. (2010). Optimization of environmental and nutritional factors for alkaline protease production.Electronic Journal of Environmental, Agricultural and Food Chemistry 9(3): 594-599.

Jaswal, R.K. and Kocher, G.S. (2007). Partial characterization of a crude alkaline protease from *Bacillus* circulans and its detergent compatibility.*The* International Journal of Microbiology 4: 1.

Joshi, B.H. (2010). Purification and Characterization of a Novel Protease from *Bacillus Firmus*Tap5 Isolated from Tannery Waste.Journal of Applied Sciences Research 6(8): 1068-1076.

Juhasz, T., Kozma, K., Szengyel, Z. and Réczey, K. (2003). Production of beta- Glucosidase in mixedculture of *Aspergillusniger*BKMF 1305 and *TrichodermareeseiRUT C30*. Food Technology and Biotechnology 41: 49-53.

Jung, S. C, Paik, H., Kim, M.S., Baik, K. S., Lee, W., Seong, C.N. and Choi, S.K. (2007). InhA-like protease secreted by *Bacillus* sp. S17110 inhabited in turban shell. Journal of Microbiology 45: 402-408.

Karthik, L., Gaurav, K., Siva Kumar, K., Thangaradjou, T. and BhaskaraRao, K.V. (2011).Isolation and characterization of protease producing actinobacteria from marine crab. Elixir Bio Tech. 39: 5098-5102.

Karthik, L., Gaurav, K. and BhaskaraRao, K.V. (2010). Mutational effects on the protease producing marine actinomycetes isolated from Scylla serretia. Pharmacologyonline 1: 221-227.

Kaur, M., Dhillon, S., Chaudhary, K. and Singh, R. (1998). Production purification and characterization of thermostable alkaline protease from *Bacillus polymyxa.*Indian Journal of Microbiology 38: 63-67.

Kim, S.S., Kim, Y.J. and Rhee, I. (2001). Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC 3674.Archives of Microbiology 175: 458-461.

Kunitz, M. (1947). Crystalline soybean trypsin inhibitor. The Journal of General Phsiology 30: 291–310.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lowery, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement by Folin Phenol reagent.The Journal of Biological Chemistry 193: 265-275.

Mahmood, A.U., Greenman, J. and Scragg, A.H. (2000). Effects of macromolecular growth substrates on production of extracellular enzymes by Bacillus speciesin continuous culture.International Microbiology 103: 85-96.

Marhuenda-Egea, F.C. and Bonete, M.J. (2002).Extreme halophilic enzymes in organic solvents.Current Opinion in Biotechnology 13: 385–389.

Malathi, S. and Chakraborty, R. (1991). Production of Alkaline Protease by a new *Aspergillusflavus* isolate under Solid Substrate fermentation condition for use as depletion agent. Applied Environmental Microbiology 57: 712-716.

McKevitt, A., Bajaksouzian, S., Klinger, J.D. and Woods, D. (1989). Purification and Characterization of an Extracellular Protease from *Pseudomonas cepacia.*Infection and Immunity 57(3): 771-778.

Nascimento, W.C. and Martins, M. L. (2004). Production and properties of an extracellular protease from thermophilic*Bacillus* sp. SMIA2. Brazilian Journal of Microbiology 35: 91-96.

Phadatare, S.U., Deshpande, V.V. and Srinivasan, M.C. (1993). High activity alkaline protease from *Conidioboluscoronatus* (*NCL* 86.8.20): Enzyme production and compatability with commercial detergents.Enzyme and Microbial Technology 15: 72-76.

Razak, C., Rahman, R., Salleh, A., Yunus, W., Ampon, K. and Basri, M. (1995). Production of athermostable protease from a new high pH isolate of Bacillus stearothermophilus.Journal of Biosciences 6: 94-100.

Senthilraja, P. and Saravanakumar, K. (2011). Purification and characterization of protease from mangroves derived strain of *Bacillus cereus.*International Multidisciplinary Research Journal 1(1): 13-18.

Sexton, M.M., Jones, A.L., Chaowagul, W. and Woods, W. (1994). Purification and characterization of a protease from *Pseudomonas pseudomallei.*Canadian Journal of Microbiology 40(11): 903-910.

Shaheen, M., Shah, A.A. and Hasan, F. (2008). Influence of culture conditions on production and activity of proteases from *Bacillus sublitisBS 1.* Pakistan Journal of Botany 40(5): 2161-2169.

Sookkheo, B., Sinchaikul, S., Phutrakul, S. and Chen, S.T. (2000). Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33.Protein Expression and Purification 20: 142-151.

Trincone, A. (2010). Potential biocatalysts originating from sea environments.Journal of Molecular Catalysis B: Enzymatic 66: 241–256.

Trincone, A. (2011). Marine Biocatalysts: Enzymatic Features and Applications. Marine Drugs 9: 478-499.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacolology 13: 57–149.

Yandri, T.S., Dian, H., and Sutopo, H. (2008). The chemical modification of protease enzyme isolated from local bacteria isolate, *Bacillus subtilis*ITBCCB148 with cyanuric chloride polyethylenglycol. European Journal of Scientific Research 23: 177-186.

Yang, S.S. and Lee, C.M. (2001). Effect of culture media on protease and oxytetracycline production with mycelium and protoplasts of *Streptomyces rimosus*.World Journal of Microbiology and Biotechnology 17: 403-411.

(Published in September 2012)