Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 101 - 110

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

PRODUCTION, PURIFICATION AND FIBRINOLYTIC CHARACTERIZATION OF ALKALINE PROTEASE FROM EXTREMOPHILIC SOIL FUNGI

P. PALANIVEL[1], L. ASHOKKUMAR[2] AND R. BALAGURUNATHAN[2*]

[1]Department of Microbiology, Vivekananda College of Arts and Sciences for Women, Elayamlalayam,Thiruchengode, Namakkal- 637212 Tamilnadu, India.
[2]Department of Microbiology, PGP College of Arts and Science, Namakkal, Tamil Nadu, India.
[2]Department of Microbiology, Periyar University, Salem 11, Tamil Nadu, India*

ABSTRACT

The fungal populations in the alkaline soil samples collected from Eastern Ghats, Kolli hills region was studied by using potato dextrose agar medium. The samples possessed an average of $15X10^4$ colony forming unit per gram of soil. Among this population 23 strains of fungi belong to five genus such as *Trichoderma, Aspergillus, Penicillium, Rhizopus* and *Mucor* were isolated. Protease producing fungal species was screened by Casein and Skimmed milk agar. Among the isolates three strains of *Aspergillus* spp., one *Mucor* sp. and one strain of *Curvularia* sp. showed greater proteolytic activity in both of the medium. An isolate of *Aspergillus* strain KH 17 showed maximum zone of proteolysis in screening media and greater enzyme activity in submerged fermentation 215U/ml in complex media containing casein. The effect of physiochemical parameters studied includes different carbon, nitrogen sources, pH and temperature. Among the parameters tested 1 % starch, 3% soya bean meal, pH 9 and temperature of 25 to 30^0C showed greater induction in enzyme production. The crude enzyme of cell free extract was concentrated by ammonium sulphate and dialysis. The precipitate showed 4.1 fold greater activities and dialyzed precipitate showed 6.7 fold greater specific activity per milligram protein than the crude. The fibrinolytic activity of the partially purified enzyme was determined, it showed prompt fibrinolytic activity. The area of clearance by alkaline protease was observed to be 64.5 ± 2.4 mm^2. From this study it is found that the alkaline protease isolated from *Aspergillus* strain KH 17 has a great scope of medicinal, diagnostic and industrial applications.

KEYWORDS:Alkaline protease, *Aspergillus* spp., Partial purification, Characterization, Fibrinolytic action.

R. BALAGURUNATHAN
Department of Microbiology, Periyar University, Salem 11, Tamil Nadu, India

Corresponding author

INTRODUCTION

Proteases are a group of enzymes which break down a variety of proteins into simpler forms. Extracellular proteases have greater commercial application in medicine and various industrial sectors such as food, pharmaceutical, leather, detergent, diagnostics industries and waste management [1]. Alkaline Proteases find diagnostic and therapeutic applications. Among the total microbial proteases the most commercially important are the alkaline proteases, especially those from fungal source [2]. The use of immobilized alkaline protease from microbial source having therapeutic properties was studied for the development of soft gel-based therapeutic formulas, gauze, non-woven tissues and bandage materials [3]. Proteases from *Aspergillus oryzae* were orally used as a therapeutic and diagnostic aid to treat certain lytic enzyme deficiency syndromes [4]. The alkaline protease finds their largest use in household laundry with a worldwide annual production of detergents of approximately 13 billion tons.Several microbial strains including fungi (*Aspergillus flavus, Fusarium graminarum, Penicillium griseofulvin* etc.,) and bacteria (*Bacillus licheniformis, B. firmus, B. subtilis,* etc.,) are reported to produce protease[5].Among the various proteases fungal proteases are the most significant, compared with animal and bacterial protease [6]. Most fungi will grow over a broad pH range, however fungal proteases are active over a wide range of pH from 3-13 [7, 8]. Alkaline fibrinolytic proteases were reported to degrade fibrin suggesting its application in thrombolytic treatment and anticancer application [9, 10]. The main drawback with the production of bacterial protease is the requirement of cost intensive procedures for separation of enzymes from cells, on the other hand enzyme from fungal origin offer an advantage of the separation of mycelium by simple filtration. Besides, the fungus can be grown on inexpensive substrates [11]. For the production of enzymes in medicinal and industrial use, isolation and characterization of new promising strain is a continuous process. Proteases are generally produced by using submerged and solid state fermentation due to its apparent advantages in downstream in spite of the cost intensiveness for medium components [12].Considering these facts; we attempted to isolate new alkaline tolerant fungi from alkaline soil of the less explored ecosystem.

MATERIALS AND METHODS

Five different alkaline soil samples were collected from Kolli hills in Namakkal district, Tamil Nadu (Lat.$10^0 12'$ - $11^0 7'$N; Long.76^0 - $77^0 56'$E). The samples were serially diluted from 10^{-1} to 10^{-5}. The fungi were isolated by the spread plate method. The fungi were isolated, and tentatively identified by macroscopic and microscopic methods by lacto phenol cotton blue technique.

(i) Detection of Proteolytic activity [33]

The protease producing ability of the isolates was determined by Casein and Skimmed milk agar. The fungal isolates were inoculated in the centre of the plate then the plates were incubated at 28°C for 4-7 days. After incubation, the plates were observed for clear halo zone around the colonies, diameter of the zone shows extend of protease production by the isolate.

(ii) Submerged fermentation

Preparation of Protease Production Medium (Preinoculum): The inoculums were prepared by dispersing the spores from a week-old fungal slant culture in 0.1% Tween-80 solutions with a sterile inoculation loop. 1ml of the resulting cell suspension was transferred aseptically into 250 ml Erlen meyer flasks containing 49 ml of sterile inoculum medium (casein and minimal media) respectively [5]. The fungus was grown in Czapek dox medium containing (g/l) Sucrose, 30; KCl, 0.5; $FeSO_4$, 0.01; $MgSO_4$, 0.5; K_2HPO_4, 1.0; $NaNO_3$, 2.0 (pH 8.0). The medium without casein is used

as minimal media. Two different medium mediums were used in the study to find out the effect of inducing substrate casein; the flasks were kept in a shaker incubator at 220 rpm with 37°C for 7 days. The content of the flasks were centrifuged at 3000 rpm for 10 minutes and the culture filtrate (extracellular enzyme) was used as a crude enzyme source.

(iii) Effect of different physio chemical parameters on protease production by fungal isolate

Different physio chemical parameters used in this study on protease production includes different carbon sources such as starch, sucrose, xylose, glucose and maltose in the concentration (%) of 0.5, 1, 2, 3, and 4 and the nitrogen sources such as peptone, soy meal, yeast extract, casein and gelatin with different percentage such as 0.5, 1, 2, 3, 4 were used. Effect of different pH (7.5, 8.0, 8.5, 9, 9.5, 10, 10.5, and 11) and temperature from 20 to 45°C were also studied to determine optimal cultural conditions required to produce enzyme[34].

(iv) Protease assay

The alkaline protease activity was assayed by the method of Anson [13] and Folin & Ciocalteu [14] with some modifications. Reaction mixtures consist of Casein (0.65% w/v in 50mM potassium phosphate buffer, pH 7.5, Culture filtrate. The Reaction mixtures were incubated for 10 minutes at 37^{o}C. The reaction was terminated by adding 5 ml of 110mM TCA to both test and Blank preparation. The solution was filtered through Whatmann no.1 filter. For assay of tyrosine in the filtrate, 5 ml of 500mM sodium carbonate and 1ml of Folin & Ciocalteu's phenol reagent (1N) were added to 2 ml filtrate and incubated for 30 minutes at room temperature then the reaction mixture was read at 660 nm. Concentration of tyrosine in the filtrate was determined from a standard curve. One unit of alkaline protease activity was defined as the amount of enzyme that liberated one micromole of tyrosine per ml per minute under experimental condition.

(v) Estimation of protein

Total protein in the culture filtrate after the completion of fermentation in each step was determined according to the method of Lowry *et al*., with Bovine Serum Albumin (BSA) as standard [15].

(vi) Partial purification by ammonium sulphate precipitation

Different concentration of ammonium sulphate used in the preparation of enzyme concentrate. A concentration from 25% to approximately 75% saturation at 4^{o}C was used to precipitate protease [16, 17] in the crude extract. The centrifuged broth solution was taken in a beaker and added with ammonium sulphate solution then stirred by a magnetic drive at cold condition stirred for 60 minutes. Then the solution was transferred into a centrifuge tube and centrifuged at 10,000 rpm for 15 minutes at 4^{o}C. The supernatant was discarded. The precipitate was dissolved in 1 ml of phosphate buffered solution. Enzyme activity and protein content of the concentrate was determined.

(vii) Dialysis[35]

Membrane was boiled with 10 mM EDTA solutions to remove heavy metals and again boiled with 2% sodium bicarbonate for 10 minutes to remove sulphides. Then the membrane was placed in 1% sucrose solution for 30 minutes. The cell free crude extract was partially filled in the dialysis bag then the bag was placed in a vessel containing 50mM of phosphate buffer pH 7. Dialysis was carried out to remove the traces of ammonium sulphate. The procedure was performed by introducing the previous precipitate in solution after dissolving in phosophate buffer (pH 7.0) into a special plastic bag (10,000 Da) for a period of 12h, thus obtained enzyme preparation was concentrated against sucrose.

$$\text{Specific activity (Unit / mg, Protein Per ml)} = \frac{\text{Enzyme activity (IU/Ml}^{-1}\text{)}}{\text{Protein content (Mg/ ml}^{-1}\text{)}}$$

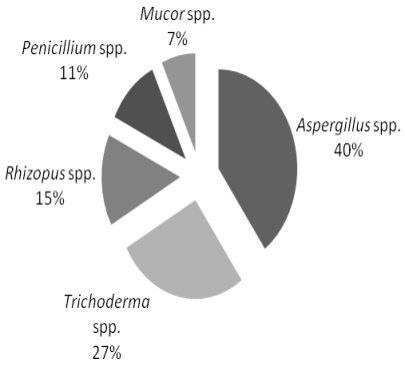
(viii) Fibrinolytic Assay[18]

The modified fibrin plate method was used to assay for fibrinolytic activity. Four ml of bovine fibrinogen solution (0.8% w/v bovine fibrinogen dissolved in 0.23% NaCl in 0.18 M boric acid buffer, pH 7.7) was gently mixed with 2 ml of thrombin solution (10 unit/ml in 0.1% CaCl2 in 0.18 M boric acid buffer, pH 7.7) in 1.2% agarose poured into a 9 cm diameter Petri dish and left undisturbed for 30 min at ambient temperature (28^{o}C) to form fibrin clots. For the enzyme assay, 30 µl of enzyme solution of varying concentrations (from 0 - 2 mg/ml) were carefully dropped onto the well (3.5 mm diameter) made in the gel of a fibrin plate. Plates were incubated at 35^{o}C for 18 h and the area of lysed zone was calculated. The mean value of the three fibrinolytic zones was used to represent the enzyme activity. A control was made for each experiment using buffer instead of the enzyme solution and plasmin as positive control for fibrinolysis. In this study, one unit of the enzyme activity was defined as the amount of enzyme in 30 microliters of enzyme solution that produced a clear zone of 1 mm^2 at pH 7.7 and 35^{o}C for 18 h [19].

RESULTS

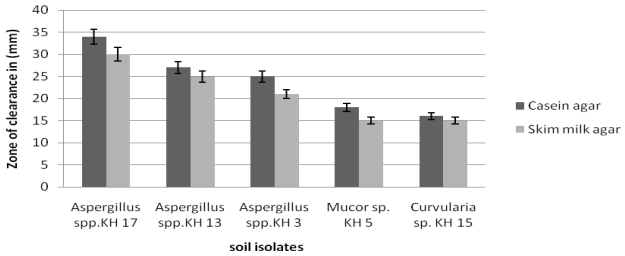
In the present investigation five different soil samples with the pH ranges from 8 to 8.5 were collected from Kolli hills in Namakkal district, Tamilnadu, the samples were processed for total fungal population and protease producing fungal population by using SDA medium. The samples contain an average of 15X10^4 colony forming unit per gram of soil. Among this population 23 strains of fungi belonging to five genuses such as *Trichoderma* spp. 27%, *Aspergillus* spp. 40%, *Penicillium* spp. 11%, *Rhizopus* spp. 15% and *Mucor* spp. 7% were isolated.

Graph 1
Percentage Occurrence of fungal population in soil samples



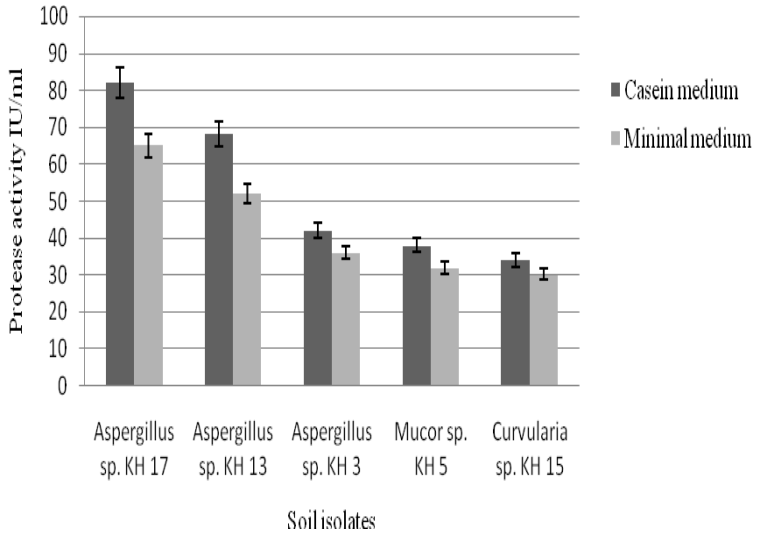
The Proteolytic activity was screened using Casein and skim milk agar and expressed as diameter of clear zone in mm. Among the total isolates strains of *Aspergillus* spp. showed higher proteolytic activity. Three strains of *Aspergillus* spp., one strain of *Mucor* spp.(KH 5) and one strain of *Curvularia* spp.(KH 15) showed proteolytic activity in both of the medium, the *Aspergillus* strain KH 17 showed maximum zone of proteolysis followed by *Aspergillus* spp. KH 13 and *Aspergillus* spp.KH 3.

Graph 2
Screening of Proteolytic activity on Casein and Skimmed milk Agar



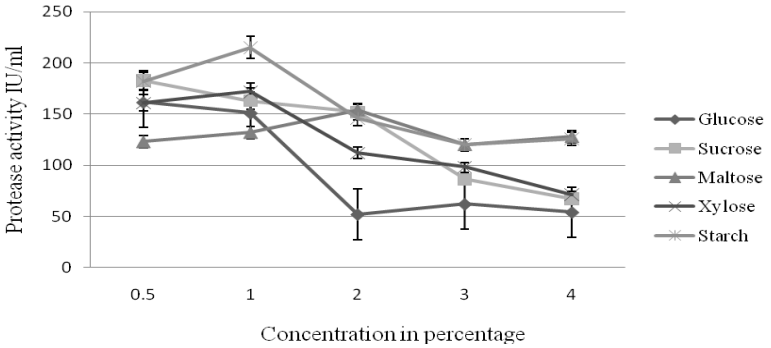
The strains those showed maximum zone of clearance in primary screening were tested for their ability in producing extracellular protease by submerged fermentation using Casein and Minimal medium at pH 8. *Aspergillus* strain KH 17 showed greater extracellular enzyme production than other isolates in both minimal and casein containing medium it showed that the use of casein enhances the production of enzyme and the strain KH17 was used in determining optimal condition required to over produce the enzyme in laboratory condition using optimal media with casein by submerged fermentation.

Graph 3
Extracellular protease production by submerged process in two different media



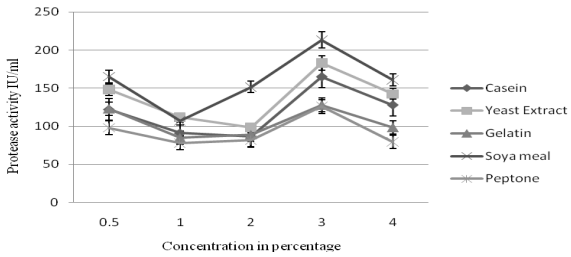
Aspergillus strain KH 17 produced maximum protease in casein medium with 1 % starch followed by the sucrose, xylose, glucose and maltose (Fig. 4). Inhibitory effect at higher concentrations of glucose might be due to catabolite repression [36].

Graph 4
Effect of different carbon sources on protease production



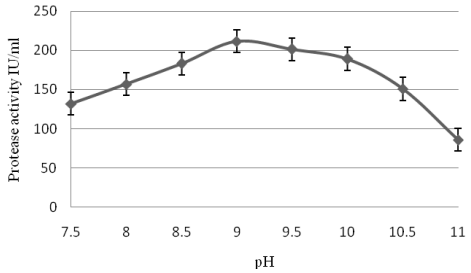
Maximum enzyme production was observed in casein medium with 3% soya bean followed by yeast extract, casein was found to be one of the good inducers of enzyme production (Fig. 5). The organic nitrogen sources such as peptone and gelatin were found to be poor inducers.

Graph 5
Effect of different Nitrogen sources on protease production



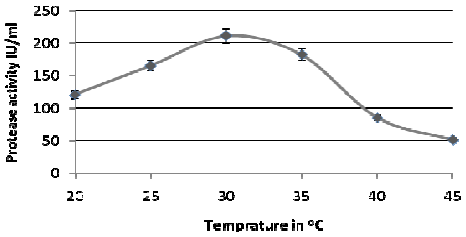
In case of minimal medium 1% yeast extract showed maximum enzyme production followed by soya bean meal, casein, gelatin and peptone. Significant enzyme production was observed at different pH range. A gradual increase in protease level was observed with increase in pH but reduced at pH 10. Enzyme production was higher in pH 9. Growth and enzyme production was observed from pH 7.5 to11. The results clearly showed that growth and protease production was significantly influenced by pH (Fig.6).

Graph 6
Effect of pH on protease production



Effect of temperature on enzyme production showed that the temperature range between 20°C to 35°C showed gradual increase in enzyme production and it was maximum at 30°C. Higher temperature of 40 to 45°C showed gradual decrease of protease alkaline production (Fig.7).

Graph 7
Effect of Temperature on protease production



The crude enzyme extract was analyzed by Lowry's method at all the experiments to determine total protein in the crude extract. Among all *Aspergillus* sp. KH17 strain showed more protein. i.e., 538.5 mg/ml in 1% starch, 3% soybean meal, pH 9.0 at 30°C temperature. The experiment result showed that if more protein present in culture filtrates it have more protease activity

Table 1
Purification of alkaline protease produced by Aspergillus sp. KH17 strain

Purification step	Protein Concentration (mg/ml)	Specific activity(U/mg of protein)	Purification (fold)
Culture supernatant	538.5	35.83	1
Ammonium sulfate precipitation	217.56	146.9	4.1
Dialysis	148.23	240.06	6.7

The alkaline protease from the cell free extract of *Aspergillus* sp. KH17 was precipitated by ammonium sulfate up to 75% saturation. The optimum ammonium sulphate fraction of 70% w/v showed 146.9 U/mg of protein i.e., it showed 4.1 fold increase in specific activity compared with crude supernatant. The present study showed the specific activity of 240.06U/mg protein. It was 6.7 fold greater activities than crude supernatant. The hydrolysis of fibrin by the isolated alkaline protease was analyzed by the fibrin plate method. The enzyme showed distinct clear zone that produced by an equal amount (mg) of plasmin used as a control in the assay, suggesting that fibrinolytic efficiency of partially purified enzyme in this investigation. The area cleared by the crude enzyme was relatively lesser than that of plasmin. The area of clearance by alkaline protease was observed to be 64.5 ± 2.4 mm^2, which is lesser than that produced by plasmin (73.4 ± 1.6 mm^2) after 18 h incubation at 35° C.

DISCUSSION

Alkaline proteases produced by microorganism have the major industrial applications and the recent trend towards the use of alkaline proteases from these sources in different process like detergents, tanning, food, waste treatment and peptide synthesis has increased because of their high catalytic activity and high degree of substrate specificity [30]. Fibrinolytic proteases were used in the treatment of thromboembolic vascular disease However; these enzymes are often expensive, thermolabile and can produce undesirable side effects [20]. Intravenous administration did not

show any clear thrombolytic effect but oral administration enhanced fibrinolytic activity [21]. In a study, it is reported that when Nattokinase was given to human subject by oral administration, fibrinolytic activity and the amount of tPA and fibrin degradation product in plasma increased about two folds. On the basis of these reports, *B. subtilis* producing fibrinolytic protease enzyme were isolated from soil obtained from various regions of Kolkata. Among them *B. subtilis* showed strongest fibrinolytic, thermophilic and hydrophilic activity. Fibrinolytic enzyme from *Bacillus* sp. nov. SK006 degraded fibrin clot in plasminogen-rich plate by forming active plasmin from the plasminogen and plasmin-free plate by direct fibrinolysis; and the activity was slightly enhanced in the former case. A strong fibrinolytic activity of FP84 fungal isolate was observed in plasminogen-free fibrin plates, indicating that it was able to degrade fibrin clot by direct fibrinolysis. The fibrinolytic activity of FP84 expressed in terms of lytic area per mg protein was 1.38-fold higher than that of plasmin which is highly encouraging [22]. Alkaline proteases were studied in many organisms including snakes, earthworms, and bacteria such as *Streptococcus pyogenes, Aeromonas hydrophila, Serratia* E15, *B. natto*, *Bacillus amyloliquefacens*, Actinomycetes and fungi such as *Fusarium oxysporum*; *Mucor* sp, *Armillaria mellea* [23]. An investigation showed that paddy soil fungal isolate of *Aspergillus niger* from Manipal produces proteases [24]. Similar investigation showed that among thirty one fungal isolates collected from beef luncheon meat for their abilities to produce protease enzyme revealed that eleven isolates (35.48%) exhibited high protease production. *Aspergillus flavus, Aibberella fujikuroi and Penicillium chysogenum* were the most active producers of protease [25]. It is reported that sorbitol [31] was found to be the best source of carbon but previous studies [32] suggested that glucose is the best source of carbon because it can be easily metabolized by microorganisms. In the present study inhibitory effect at higher concentrations of glucose might be due to catabolite repression, one another study showed that carbon and nitrogen source of sucrose and peptone induced more enzyme production in submerged fermentation but in present study soya bean meal worked as good source of nitrogen. The protein fraction precipitated with 80% ammonium sulfate saturation had the highest fibrinolytic activity ($35.12x10^4$ units/mg proteins). Ammonium sulfate was found to activate the fibrinolytic activity after dialysis. Fibrinolytic enzyme was partially purified using anion exchange chromatography (DEAE-Sephacel). Purity was increased 86 fold and specific activity of 39.31 x104 units/mg protein was obtained [26]. *Bacillus sphaericus* produce 64 mg/l of the crude fibrinolytic protease enzyme and after purification it was 6.3 mg/l. The molecular weight of the compound was 18.6 kDa [27]. A strain of *Aspergillus tubingensis* NIICC-08155 isolated from soil and was subjected to natural protease production and its partial characterization. The maximum production of neutral protease i.e.68.50 u/ml was attained after 96h of incubation. The crude preparation of protease showed higher activity at 40^{o}C with the pH of 6.4 [6] and reported that *Aspergillus nidulans* was a highly potent fungus used in the production of alkaline protease [28, 29]. Industrial production and medicinal use of the enzyme needs large scale production by some alternative methods and high purity. So, isolation, production, purification, assay and characterization of fibrinolytic enzymes from microbial sources are very effective and useful. In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources.

CONCLUSION

From this study it is found that the alkaline protease isolated from *Aspergillus* strain KH 17 has a great scope of medicinal, diagnostic and industrial applications. Purification and further investigation on strain improvement studies will provide better yield of enzyme and a promising strain for the industrial production of alkaline protease.

REFERENCES

1. Nehra KS, Dhillon S, Chaudhary K, Singh R, Production of alkaline protease by *Aspergillus* species under submerged and solid state fermentation. Ind. J. Microbio*,* 42: 43-47, (2002).
2. Mohan FN, Dileep D and Deepthi D, Potential application of proteases isolated fron Psedomonas auriginosa PD 100. Biotechnol. ind*,* 8: 197-203, (2005).
3. Davidenko T, Immobilization of alkaline protease on polysaccharides of microbial origin. Pharm Chem J, 33 (9):487-489,(1999).
4. Rao MB, Tanksale AM, Ghatge MS and Deshpande VV, Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev, 62(3): 597- 635, (1998).
5. Elliah P, Srinivasulu B, Adinarayana K, A review on microbial alkaline proteases. J Sci Ind Res, 61: 690-704, (2002).
6. Morya V and Yadav D, Production and partial characterization of neutral protease by an indigenously isolated strain of *Aspergillus tubingensis* NIICC-08155. The internet J Microbial, 8(1): 1-5, (2010).
7. Sutar I, Vartak HG and Siva Raman H, Production of alkaline protease by immobilized mycelium of *Condiobolus.* Enzyme Microbial Technol, 8: 632-634, (1986).
8. Tunga RB, Influence of temperature on enzyme production. Tech M Thesis Indian journal of microbiology, 5: 345-349, (1995).
9. Mukherjee AK and Rai SK, A statistical approach for the enhanced production of alkaline protease showing fibrinolytic activity from a newly isolated Gram-negative *Bacillus* sp. strain AS-S20-I. New Biotechnol, 28(2):182-189 (2011).
10. Simkhada JR, Mander P, Cho SS and Yoo JC, A novel fibrinolytic protease from *Streptomyces* sp. CS684. Process Biochem, 45(1):88-93, (2010b).
11. Layman PL, Industrial enzymes: battling to remain specialties. Chem Eng News, 64:11–14 (1986).
12. Jayasree D, Kavi kishor B, Vijayalakshmi M and Lakshmi narasu M, Optimization of production protocol of alkaline protease by *Streptomyces pulvereceus.* Journal of microbiology, 13: 2123-2126 (2009).
13. Anson ML, The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J Gen physiol, 22: 79-89, (1938).
14. Folin O and Ciocatteu V, On tyrosine and tryptophan determination in proteins. J Biol chem., 73: 627(1929).
15. Palanivelu P, Analytical biochemistry and separation techniques. Indian chemical Engr, 48: 3, (2004).
16. Fujiwara N, masui A and, Imanaka T, Purification and properties of the highly thermostable alkaline protease from an alkalophillic and thermophillic *Bacillus* sp. J Biotechnol, 30: 245-256(1993).
17. Sumantha A, Sandhya C, Szakacs G, Carlos R Soccol and Ashok P, Production and partial purifi cation of a neutral metalloprotease by fungal mixed substrate fermentation. Food Technol Biotechnol, 43(4):313–319, (2005).
18. Astrup, T and Mullertz S, The fibrin plate method for estimating fibrinolytic activity. Archives of Biochemistry and Biophysics, 40: 346-351, (1952).
19. Giron ME, Salazar AM, Aguilar I, Perez JC, Sanchez EE and Arocha-Pinango CL, et al., Hemorrhagic, coagulant and fibrino (geno) lytic activities of crude venom and fractions from mapanare (Bothrops colombiensis) snakes. Comp Biochem Physiol C Toxicol Pharmacol, 147:113–21, (2008).
20. Chitte RR and Dey S, Potent fibrinolytic enzyme from a thermophilic *Streptomyces megasporus* strain SD 5. Lett. Appl. Microbiol, 31: 405- 410, (2000).
21. Sumi H, Hamada H, Nakanishi K and Hiratani H, Enhancement of the fibrinolytic activity in plasma by oral administration of Nattokinase. Acta Haematol, 84: 139-143, (1990).

22. Hua Y, Jiang B, Mine Y and Mu W, Purification and characterization of a novel fibrinolytic enzyme from Bacillus sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste. J Agric Food Chem, 56:1451–7, (2008).
23. Jian Sha CL, Galindo V, Pancholi VL, Popov Y, Zhao CWH and Chopra AK, Differential expression of the enolase gene under *in vivo* versus *in vitro* growth conditions of *Aeromonas hydrophila.* Microbial pathogenesis, 34: 195-204, (2003).
24. Subrahmanyam VM, Kamath P, Rao J and Raj PV, Optimization of cultural conditions for protease production by a fungal species. Indian J Pharm sci, 72(2): 161-166, (2010).
25. Saleem A and El-Said AHM, Proteolytic activity of beef Luncheon fungi as affected by incorporation of some food preservatives. J Acta Microbiologica et Immunologica Hungarica, 56(4): 417-426, (2009).
26. Patcharaporn Pandee, Aran H-Kittikul1, Ohsugi, Masahiro and Yaowaluk Dissara, Production and properties of a fibrinolytic enzyme by *Schizophyllum commune* BL23 Songklanakarin. J. Sci. Technol, 30(4): 447-453, (2008).
27. Balaraman K and Prabakaran G, Production & purification of a fibrinolytic enzyme (thrombinase) from *Bacillus sphaericus.* Indian J Med Res, 459-464, (2007).
28. Hui Yuan Zhu , Yong Tian , Yun-Hua Hou and Tian hong Wang, Purification and characterization of the cold-active alkaline protease from marine cold-adaptive *Penicillium chrysogenum* FS010. Mol Biol Rep, 36:2169–2174, (2009).
29. Charles P, Devanathan V, Periasamy A, Ponnuswamy MN, Kalaichelvan PT and ByungKihur R, Purification, Characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10. J Basic Microbiol, 48: 347-352, (2008).
30. Kumar CG and Takagi H, Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv, 17:561–594, (1999).
31. Santosh Kumar Yadav, Deepali Bisht, Shikha and Nandan Singh Darmwal, Oxidant and solvent stable alkaline protease from *Aspergillus flavus* and its characterization. African Journal of Biotechnology, 10(43): 8630-8640, (2011).
32. Srinubabu G, Lokeswari N and Jayaraju K, Screening of Nutritional Parameters for the Production of Protease from *Aspergillus Oryzae.* E-Journal. Chem. 4(2): 208-215, (2007).
33. Saha N, Bhattacharya B C, Strain improvement mutagenesis and random screening procedure for Rhizopus oryzae IIT KG-1, Proceeding of International symposium on industrial biotechnology, Department of Microbiology, Osmania Univ., Hyderabad (1990).
34. Shivasharana CT, Naik GR, Production of alkaline protease from a thermoalkalophilic *Bacillus* sp.jb-99 under solid state fermentation. Int j pharm bio sci, 3(4): (b) 571 – 587, (2012).
35. Usharani B, Muthuraj M, Production and characterization of protease enzyme from *Bacillus laterosporous.* Afr. J. Microbial. Res*,* 4(11): 1057-1063,(2010).
36. Elibol M, Moreira A.R, Optimizing some factors affecting alkaline protease production by a marine bacterium Teredinobacter turnirae under solid substrate fermentation. *Process Biochem*., 40: 1951-1956, (2005).