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*Full Length Research Paper*

# Optimization of production of an extracellular alkaline protease by soil isolated *bacillus* species using submerged and solid- state fermentation with agricultural wastes

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**The present study focuses on the production of an extracellular alkaline protease enzyme from soil selected strains of various active solid substrates by *Bacillus* sp., screened from soil. Some fermentation conditions were studied aiming to improve enzyme production-substrate; pH 9.0 and incubation temperature of 37° C given the best results. For the enzyme activity stability, the following best conditions were found: substrate pH of 13.0 and temperature of 55° C. Enzyme production by solid-state fermentation were studied using different agricultural solid wastes like rice bran, wheat bran, coconut oil cake, groundnut oil cake and gingili oil cake. The maximum enzyme activity was achieved from groundnut oil cake with soybean meal as substrate.**

**Key words:** *Bacillus* sp., Solid state fermentation, alkaline protease.

## INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes. They are degradative enzymes, which catalyze the total hydrolysis of protein (Raju et al., 1994). These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by microorganisms like bacteria and fungi. Microbial proteases are predominantly extra-cellular and can be concentrated in the fermentation medium. Microbial proteases are the most important industrial enzymes (Chouyyok et al., 2005) that account for approximately 40% of the total worldwide enzymes sale (Godfrey and West, 1996).

Alkaline proteases of microbial origin possess consi-derable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Rao et al., 1998). The alkaline proteases find their largest use in house hold laundry with a worldwide annual production of detergents of approximately 13 million tons (Nehra et al., 2002). Alkaline proteases were in fact the first enzyme produced in bulk.

At present, the use of alkaline proteases has increased remarkably with large properties of commercially available alkaline proteases derived from *Bacillus* sp. About 30 to 40% of the production cost of industrial enzymes is estimated to account for the cost of the growth medium. Considering the medium for the production of alkaline proteases from an alkalophilic, *Bacillus* sp. is especially important.

Hence it becomes the need of the day to in this study isolate new strains of *Bacillus* sp., which are alkalophilic and evaluate their efficiency to use various substrates for

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the production of alkaline proteases. Having this in mind, the present study was undertaken aiming to produce the proteases by submerged fermentation as well as by solid state fermentation using various agricultural wastes (Paranthaman et al., 2009). Furthermore, the proteolytic activity of the crude enzyme obtained by both the type of fermentative procedure was determined separately and the obtained results suggest that solid state fermentation is a valuable method of alkaline proteases production to fulfill the future need of alkaline proteases. To fulfill the future need of alkaline proteases. Having this in mind, the present study was undertaken to produce the proteases by submerged fermentation as well as by solid state fermentation using various agricultural wastes. Furthermore, the proteolytic activity of the crude enzyme obtained by both the type of fermentative procedure is determined separately and suggesting good method of enzyme production to fulfill the future need of alkaline proteases.

## MATERIALS AND METHODS

### Isolation of proteolytic organism and screening for proteolytic activity

Soil samples were collected, serially diluted, and placed on Nutrient Agar and incubated at 37±1°C for 24 h. The colonies appeared on the agar were then sub-cultured on Skim milk agar medium (5 g casein, 1 g dextrose, 2.5 g yeast extract, 28 g skim milk powder, 15 g agar and 1000 ml distilled water). The inoculated plates were incubated at 37 ± 1°C for 24 h. After incubation of the plates, colony showing a zone of hydrolysis was selected for the present study.

### pH tolerance of the selected isolated culture

Skim milk agar plates were prepared with different pH values ranging from such as 7, 8, 9, 10, 11, 12, and 13. The medium were incubated at 37 ± 1°C for 24 h. The plates were then observed for zone of hydrolysis which indicated the tolerance towards a specific pH range.

### Identification of selected strain

The isolated bacterium was subjected to various tests including colony morphology, staining and biochemical tests. The organism used in the present study has been identified based on the test performed as species of *Bacillus* sp.

### Estimation of protein-protease enzyme assay

The proteolytic activity of extracted crude enzyme from both submerged fermentation and solid state fermentation was determined separately using casein as a substrate. Casein was dissolved in 0.1 M Tris buffer (pH 9) at a concentration of 1.5%. The assay mixture consisted of 450 µl of substrate and 50 µl of crude enzyme solution suitably diluted with 0.1 M Tris buffer (pH 9.0). The reaction mixture was incubated at 45°C for 20 min and the reaction was terminated by the addition of 500 µl of 10% TCA (trichloroacetic acid) and then centrifuged at 5000 rpm for 10 min to remove the consulting precipitate. Protease activity was determined as released tyrosine from the supernatant according to a modified Lowry et al. (1951) method. The same procedure was repeated for each crude extract. One unit of enzyme activity is defined as the amount of enzyme resulting in the release of µg of tyrosine per minute at 45°C under the reaction condition. One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 µg of tyrosine per min at optimum conditions.

### Effect of incubation time on enzyme production by submerged fermentation

About 100 ml of Tryptone soya broth (TSB) was prepared and pH was adjusted to 9.0. The medium was sterilized, cooled, inoculated with the selected strains and incubated for 72 h in an orbital shaker (Orbiteck, Chennai) at 37°C. Aliquots of the sample was removed every 12 h interval after inoculation and assayed for protease activity.

### Effect on temperature on enzyme production by submerged fermentation

Erlenmeyer flasks with 100 ml of Tryptone soya broth were inoculated with each colony. The flasks were incubated at the above mentioned temperature for 48 h. After inoculation, each flask was cultured in different temperature for incubation: 27, 37, 47 and 57°C.

The flasks were sterilized, cooled, and inoculated with isolated culture. Following inoculation, the flasks were incubated at the above mentioned temperature for 48 h. Following incubation time, the protease activity of each isolated bacteria was estimated for each aliquot. The temperature at which the maximum activity was found was considered the optimal best temperature for the protease production.

### Effect on pH on enzyme production by submerged fermentation

Eight Erlenmeyer flasks with 100 ml of Tryptone soy broth with different adjusted pH as 7, 8, 9,10,11,12 and 13 were inoculated with each isolated colony. The flasks were incubated at 37°C for 48 h. Following inoculation, the flasks were incubated at 37°C for 48 h. Following incubation, the protease activity was determined for each aliquot. The pH at which the maximum activity was found was the best optimal pH for the protease production (Joo et al., 2002).

### Determination of ideal optimum ph of bacterial enzyme activity

Nine clean beakers were taken and five sets of 100 ml of phosphate buffer were prepared in different pH as 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5,12 and 12.5 respectively. These nine buffers were then used for substrate preparation and then enzyme assay was carried out for all the five samples of each pH. The maximum enzyme activity was obtained and determined from the OD value among five sets and which was found to be indicating the best optimal pH for protease activity.

### Determination of optimum temperature of bacterial enzyme activity

Five clean beakers were taken. 100 ml of phosphate buffer with above the optimized pH was prepared and 100 ml of buffer was suspended to five each beaker. Buffers were then used for substrate preparation and then enzyme assay was carried out for

**Table 1.** The combination of solid state fermentation system with different supports.

| Support | Moistening solution |
|---|---|
| Rice bran | Distilled water, nutrient solution, Soya bean meal broth. |
| Wheat bran | Distilled water, nutrient solution, Soya bean meal broth. |
| Coconut oil cake | Distilled water, nutrient solution, Soya bean meal broth. |
| Ground nut oil cake | Distilled water, nutrient solution, Soya bean meal broth. |
| Gingili oil cake | Distilled water, nutrient solution, Soya bean meal broth. |

all the four sets. During each step, the solution of each set were incubated at different temperature: 40, 45, 50, 55, 60, 65 and 70°C respectively. Four samples of each determination were realized. The maximum enzyme activity was determined from the OD value of four sets. The maximal value obtained was related to be the optimal temperature for protease activity (Joo et al., 2002).

#### Enzyme production on solid state fermentation system

The solid supports such as rice bran, wheat bran, coconut oil cake, groundnut oil cake and gingili oil cake were selected. From each of the solid substrates (before sterilized), 10 g was taken in 250 ml conical flask. Then, the substrate was moistened with: (a) distilled water (b) nutrient solution and (c) soya bean meal solution unless otherwise mentioned which gave a 10:2 ratio of substrate to medium solution (Shyamala and Sreekanthiah, 1980) and mixed thoroughly. The flasks were then sterilized in an autoclave at 121° C for 15 min.

About 10 g of each agro waste used for the present study, were moistened with different supplement to attain 72-80% moisture content. The solid substrate was prepared in components given in Table 1.

The above mentioned combination of medium were prepared, sterilized and inoculated with a test organism. The solid substrate was mixed thoroughly with the inoculums and incubated at 37 ± 1°C for 72 h. After incubation, the crude enzyme was extracted and analyzed for enzyme activity.

#### Extraction of crude enzyme from fermented solid substrate

A solution of Tween 80 (0.1%) was added to 100 ml of distilled water. 10 ml of the water was added to the 2 g of fermented substrate and the substrate was homogenized on a rotary shaker at 180 rpm for 1 h. The solids were removed by centrifugation of the homogenate at 8000 rpm for 15 min and the resultant clear supernatant was used for analytical studies.

## RESULTS AND DISCUSSION

Proteases constitute one of the most important groups of industrial enzymes. The use of alkaline proteases in a variety of industrial processes involving detergents, food, leather and silk has increased remarkably in recent decades. Though several fungal sources are being increasingly employed, large properties of the commercially available alkaline proteases are derived from *Bacillus* sp. (Yang et al., 2000).

**Table 2.** Biochemical Results for *Bacillus* species.

| Biochemical Test | Results |
|---|---|
| Methyl Red test | Negative |
| Voges Proskar test | Negative |
| Citrate utilization test | Positive |
| Urease test | Positive |
| Triple Sugar Iron agar test | K/A |
| Oxidase test | Positive |
| Catalase test | Negative |
| **Carbohydrate test** | |
| Glucose | AG |
| Lactose | A |
| Sucrose | Negative |
| Manitol | Negative |
| Motility test | Motile |

K/A, Alkaline slant acid butt; AG, Acid Gas; A, Acid.

Many of the bacteria species of *Bacillus* sp., in particular are capable of growing in alkaline pH and hence they are capable of secreting enzymes which are active at alkaline pH values. Of the many isolates selected from the soil sample, one of them was founded to at pH values beyond 11. Then, that strain was used in the present study for alkaline protease production.

The selected strain of *Bacillus* sp. was grown on skim milk agar medium ranging in pH values from 7-13. In almost all the pH values, the organism was survived and produced the protease enzyme. Disease confirmative confirms the fact that the selected isolate strains is an alkaline tolerant organism and hence is capable of producing alkaline protease. The organism used in the study was identified as species of *Bacillus* sp. based on the staining and biochemical reactions (Table 2).

Alkaline proteases account for approximately 25% of world wide enzyme consumption (Gessesse, 1997). For this purpose, soybean meal was recognized as a potentially useful and cost effective medium ingredient, because it is largely produced as a byproduct during oil extraction (Gattinger et al., 1990). So soya bean meal is an inexpensive and radially available substrate. For this reason, an extracellular protease production soya bean meal has been used as culture medium ingredient.

When the time taken for maximum enzyme production was studied, maximum yield production was found to be at 48 h of incubation (Figure 1). The optimum best temperature for enzyme production with the selected isolate of *Bacillus sp*. was found to be 37°C (Figure 2) and the ideal optimum pH for enzyme production was found to be 9.0 (Figure 3).

When the optimum temperature of enzyme activity was

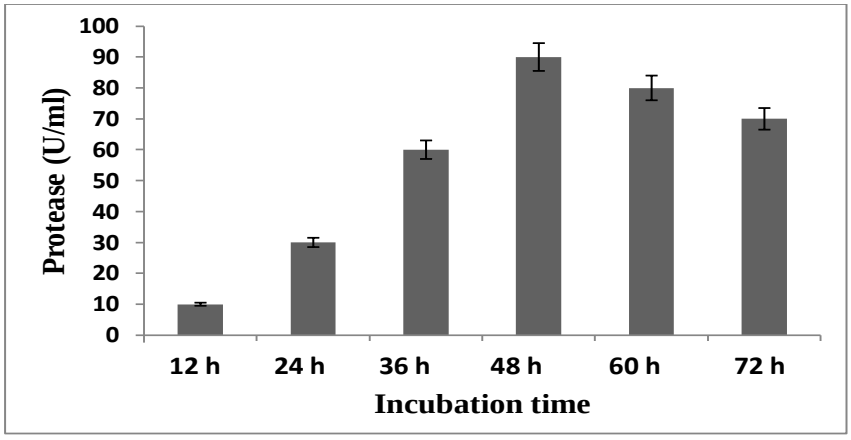


**Figure 1.** Effect of Incubation time on Bacillus sp protease production by submerged fermentation.

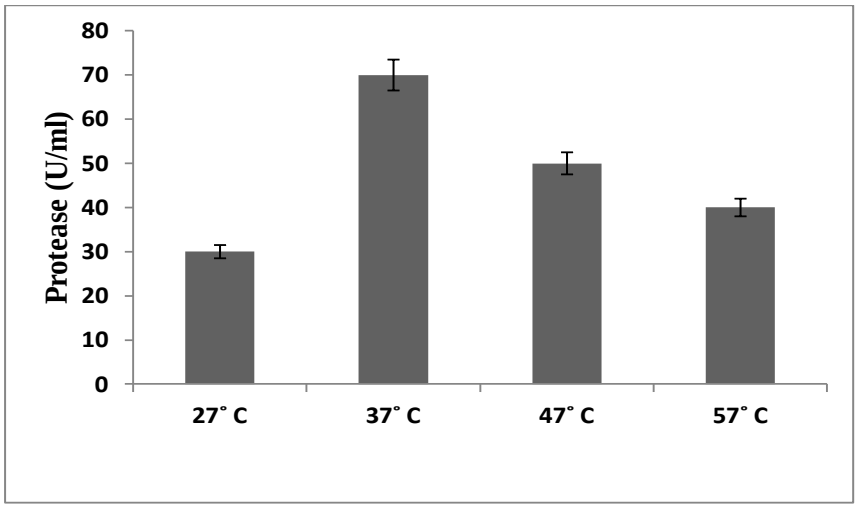


**Figure 2.** Effect of Temperature on Bacillus sp protease production by submerged fermentation.

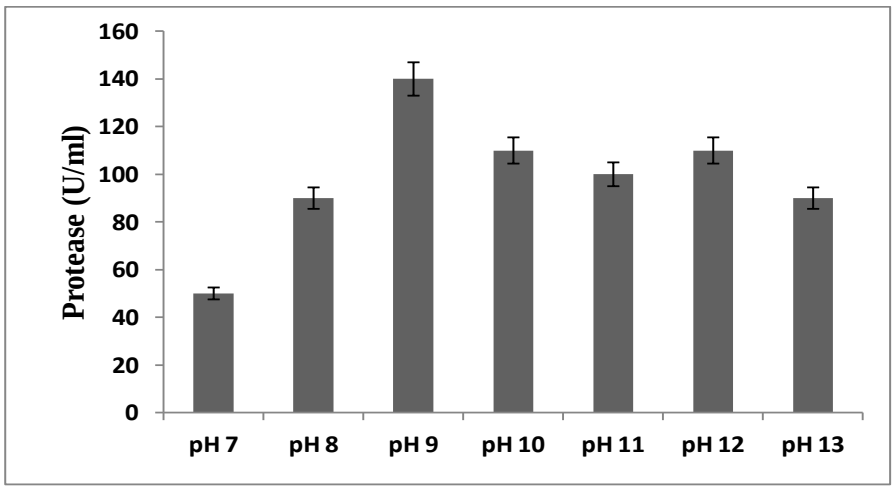


**Figure 3.** Effect of pH on *Bacillus* sp. protease production by submerged fermentation.

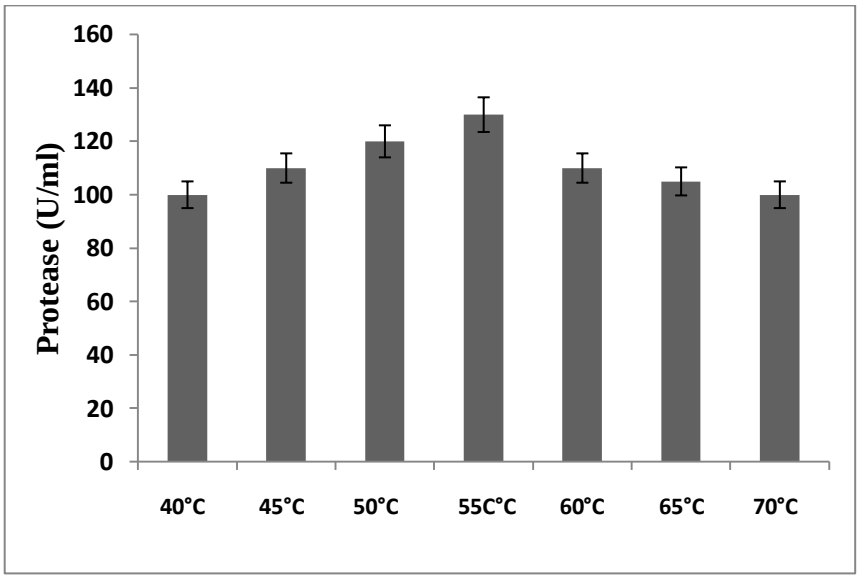


**Figure 4.** Influence of substrate temperature on Bacillus sp protease activity.

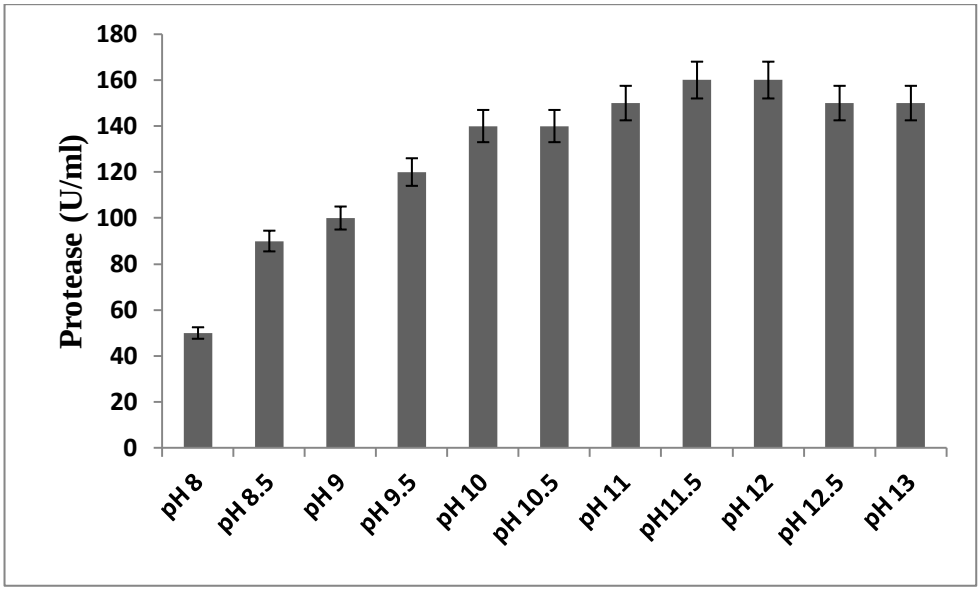


**Figure 5**. Influence of substrate pH on Bacillus sp protease activity.

studied, maximum activity was obtained at 55°C (Figure 4). From the experiment of the present study, the optimum pH of enzyme activity was found to be 11.5 and 12.0. However, the activity was mentioned high until up to pH value of 13.0 (Figure 5). Similar result has been reported by Joo et al. (2002). The high optimum pH is a feature of alkaline protease (Gessesse, 1997).

From these results, the protease secreted from *Bacillus* sp. isolated strains was highly alkaline protease with moderate activity at higher temperature (less heat stability). More than 30% of production cost of industrial enzymes is estimated to be accounted for by the cost of the growth medium. Hence, it becomes necessary to use cost effective substrate for enzyme production. Bearing this in mind in the present study, aiming the use of different solid, supports have been tried such as substrates for enzyme production on solid state fermentation. We studied the use of solid substrate such as rice bran, wheat bran, ground nut oil cake, gingili oil cake and coconut oil cake. The solid substrates were

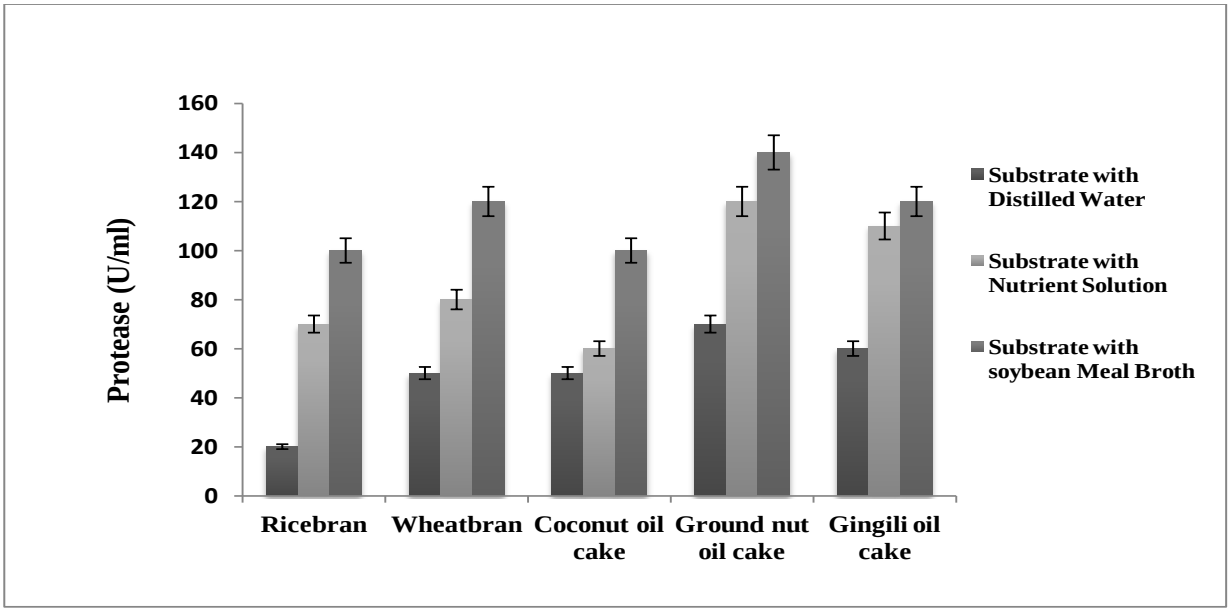


**Figure 6.** Protease production by *Bacillus* sp. cultivated on on solid state fermentation system with different supports and substrates.

moistened with distilled water, and distilled water with supplement (as mentioned in materials and methods). From the result, it has been observed that, groundnut oil cake as substrate supplemented with soybean meal broth had a higher yield of enzyme (Figure 6).

## Conclusion

In conclusion, the selected isolates of *Bacillus* species is a good candidate for alkaline protease production and groundnut oil cake supplemented with soy bean meal could serve as an effective substrate than the other food supports. The organism is capable of producing a highly alkaline stable enzyme which is a demand of many industries. It was more applicable for many industry especially dairy and food beverage industries, etc. The obtained results will be exploited for further synthesizes of more amount of enzyme through solid-state fermentation as which is inexpensive.

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## REFERENCES

Chouyyok W, Wongmongkol N, Siwarungson, Prichnont S (2005). Etraction of alkaline proteases using an aqueous two phase system from cell free *Bacillus subtilis* TISTR 25 fermentation broth. Process Biochem. 40:3514-3518.

Gattinger LD, Duvniak A, Khan AW (1990). The use of canola meal as a substrate for xylase production by *Trichoderma reesei.* Appl. Microbial. Biotechnol. 33:21-25.

Gessesse A (1997). The use of nug meal as low cost substrate for the production of alkaline protease by the alkalophilic *Bacillus* species. AR-009 and some properties of enzymes. Bioresour. Technol. 62:56-61.

Godfrey T, West S (1996). Industrail Enzymology, Second Edition. Macmillian Publisher. Inc. New York.

Joo HS, Ganeshkumar C, Park GC, Kim KT, Seung R, Paik Chang CS (2002). Optimization of the production extracellular alakaline protease from *Bacillus horikoshii*. Process Biochem. 38:155-159.

Lowry OH, Rousenbough HI, Fair AL, Randall RJ (1951). Protein measurement with the folin phonal reagent. J. Biol. Chem. 193:265-275.

Nehra KS, Dhilon S, Chaudhary K, Singh R (2002). Production of alkaline protease by Aspergillus species under submerged and solid state fermentation. Ind. J. Microbial. 42:43-47.

Paranthaman R, Alagusundharam K, Indhumathi J (2009). Production of protease from rice mill waste by *Aspergillus niger* in soid state fermentation. World J. Agric. Sci. 5(3):308-312.

Raju K, Jaya R, Ayyanna C (1994). Hydrolysis of Casein by Bajara Protease importance. Biotechnol. Coming Decadea 181:55-70.

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998). Molecular and biotechnological aspects of microbial proteases, Microbiol. Mol. Biol. Rev. 62:597-635.

Shyamala TR, Sreekarthiah KR (1986). Production of Cellulase and Protease by Selected Fungal isolates. Enzyme Microb. Technol. 8:178-182.

Yang JK, Shih IL, Tzeng YM, Wang SL (2000). Production and purification of protease from a bacillus subtilis that can deprotenize crustaceans wastes. Enzyme Microb. Technol. 26:406-413.