Recent Research in Science and Technology 2009, 1(5): 250–254
ISSN: 2076-5061
www.recent-science.com

INDUSTRIAL BIOTECHNOLOGY

STUDIES ON THE PRODUCTION OF PROTEASE BY *BACILLUS* SPECIES

R. Nagalakshmi[1], S. Ramesh[2*]
[1]*Department of Biotechnology, PRIST University, Thanjavur - 613 403, Tamil Nadu, India*
[2]*Centre for Research and Development, PRIST University, Thanjavur - 613 403, Tamil Nadu, India*

Abstract

The different bacterial isolates such as *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus firmus* and mixed culture were screened for protease production. A basal medium containing peptone, beef extract and NaCl was used for this biomass cultivation. Among the different bacterial isolates screened, the maximum protease production was found in *B. coagulans* and minimum in *B. subtilis* in both quantitative and semi quantitative assay. The effect of various environmental conditions like pH, temperature, rpm and inoculum size on alkaline protease production was examined. The optimum condition for protease production upon inoculation of 1ml of overnight grown culture was found to be pH 9.2, 37º C, 175 rpm and 1% of inoculum for a time period of 24 h. One way analysis of variance showed significant differences ($P < 0.05$) in protease production between different species.

Key Words: *Bacillus* species; Basal medium; Optimum condition; Quantitative assay.

Introduction

Proteases are among the most valued commercial enzymes. The importance of thermostable proteolytic enzymes in a number of industrial processes is widely acknowledged. The thermostable proteolytic enzymes from the genus *Bacillus* are so far the most important group of enzymes produced commercially accounting for nearly 20% of the world enzymes market with the predominant application (35%) in detergents [1,2]. Other industrial uses of proteases are in food processing, pharmaceuticals, peptide synthesis, meat tenderization, medical diagnosis, baking, brewing, deproteinization of shrimp and crab shell waste and dehairing of hides and skins [3].

The proteases produced by different species of *Bacillus* vary not only in type but also in their pH and temperature optima [4,5] and also have the ability to function at high temperatures and pH values. According to their optimum pH, proteases are commonly classified as acidic protease, neutral protease and alkaline protease. One particular interest is the production of alkaline protease from *Bacillus* for application in detergent industry [6,7,8,9]. However there is no firm evidence to suggest that thermostable enzymes are particularly derived from thermophilic micro organisms, but there is a greater chance of exploring thermostable proteins from thermophilic bacteria.

Proteases are also a highly complex group of enzymes that vary enormously in their physico chemical and catalytic property. Several thermostable proteases have been reported from *Bacillus* species (7,10,11]. The present work was carried out to investigate the protease production by *B. subtilis*, *B. coagulans*, *B. firmus* & mixed culture (combination of three *Bacillus* species) and to study the effect of various environmental conditions on protease activity.

Materials and Methods

Micro organisms

The micro organisms i.e. *B. subtilis*, *B. coagulans* and *B. firmus* were obtained from the microbiology laboratory at PRIST University and maintained on nutrient agar slants at 4º C.

Protease production in liquid culture

Alkaline nutrient broth (pH 9.5) containing (g/l): peptone, 5; beef extract, 1.5; NaCl, 5 and yeast extract, 1.5 was used as a basal medium [12]. The sterilized production medium (100ml) in 4 different Erlenmeyer flask (250 ml) was inoculated with 1% (v/v) of overnight grown seed cultures and incubated at 36ºC under rotatory conditions for 24 h with 175 rpm. The medium

*Corresponding Author, Email: marineramesh2020@gmail.com

was prepared using marine water. Thereafter, the cell free supernatant was obtained by centrifugation at 12,000xg for 20 min and the protease yield was determined in the cell free supernatant.

Effect of various environmental conditions on protease activity

The effect of various environmental conditions like pH, temperature, aeration speed (rpm) and inoculum size on alkaline protease production by *B. coagulans* and *B. firmus* was studied. The basal medium was selected for analyzing enzyme activity due to its simplicity. The pH of the culture medium was regulated using NaOH and HCl with the pH ranged from 4 to 13. After injection with purified bacterium, the entire medium was incubated as before. The effect of temperature (10^0 C to 55^0 C), aeration speed (75 to 175rpm) and inoculum size (100 µl to 1000 µl) on alkaline protease production was measured.

Analytical Procedures

The protease was assayed at 90° C in 100 mM carbonate – bicarbonate buffer (pH 9.6). One ml of 1% casein solution was incubated with 1 ml of appropriately diluted enzyme for 20 min at 30° C. The reaction was stopped by addition of 4ml of 5% TCA. The tubes were centrifuged after 1 hour at 3000 X g for 10min and the degraded products were examined by Lowry's method [13]. One unit of protease was defined as amount of enzyme required to release 1µg/mol of tyrosine per ml per minute under assay conditions.

Statistics

Statistical analyses were carried out with SPSS package (windows version 12.0). Data were subjected to one-way analysis of variance (ANOVA) and differences between means were assessed by Duncan's multiple range test (DMRT). All statistical tests were considered significant at 5% level ($P < 0.05$).

Results

The rate of protease production by three *Bacillus* species and its mixed culture studied are presented in Table 1. The concentration of protease produced in quantitative assay varied significantly ($P < 0.05$) in all the species studied.

The total protease yield was found to be high in *B. coagulans* (320 mg/l) followed by *B. firmus* (290 mg/l), mixed culture (270 mg/l) and *B. subtilis* (215 mg/l). In semi quantitative assay, extracellular protease was effectively produced by *B. coagulans* (5.25 cm^2) (Table 2).

The protease production was particularly sensitive to acidic pH (Fig.1). The maximum protease production in *B. coagulans* and *B. firmus* obtained at alkaline condition of pH 9 reaching to 300 mg/l and 270 mg/l respectively and the protease production was gradually decreased to 15 mg/l and 17 mg/l respectively at pH 12.

Table 1. The rate of protease production by *Bacillus* species and mixed culture in quantitative assay

Organism / Culture	Absorbance at 620 nm	Concentration (mg)
Bacillus coagulans	0.605	320 ± 33[a]
Bacillus firmus	0.565	290 ± 16[b]
Bacillus subtilis	0.408	215 ± 7[c]
Mixed culture	0.521	270 ± 23[b]

Mean values ± S.D. of determinations for duplicate samples
Means in the same column sharing different superscripts are significantly different ($P < 0.05$)

Table 2. The rate of protease production by *Bacillus* species and mixed culture in semi quantitative assay

Organism / Culture	Zone of inhibition (cm^2)
Bacillus coagulans	5.25[a]
Bacillus firmus	3.00[b]
Bacillus subtilis	0.69[c]
Mixed culture	2.00[d]

Mean values of determinations for duplicate samples
Means in the same column sharing different superscripts are significantly different ($P < 0.05$)

The results illustrated in fig.2 referred to a positive relationship between protease production and incubation temperature upto 55^0 C. About 90% of the original protease production was gained at 55^0 C in the present study. The maximum protease activity was observed in *B.coagulans* (516 mg/l) and *B.firmus* (497 mg/l) at 175 rpm after 24 hours of incubation (Fig.3).

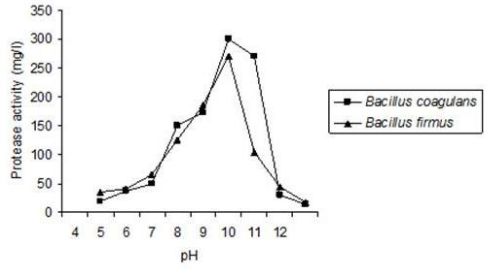

Fig.1. Effect of pH on protease activity by *Bacillus coagulans* and *Bacillus firmus*

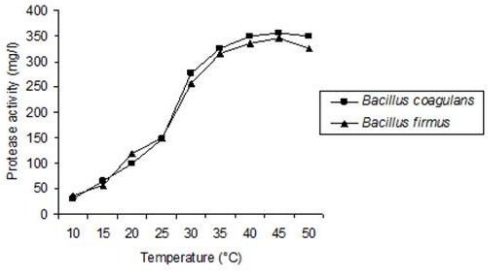

Fig.2. Effect of temperature on protease activity by Bacillus coagulans and Bacillus firmus

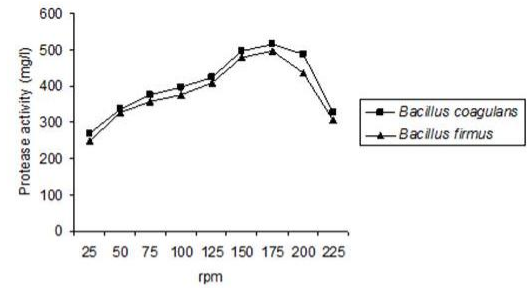

Fig.3. Effect of rpm on protease activity by *Bacillus coagulans* and *Bacillus firmus*

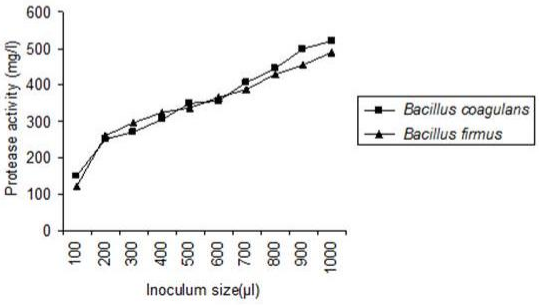

Fig.4. Effect of inoculum size on protease activity by *Bacillus coagulans* and *Bacillus firmus*

The results from present study showed that the optimum inoculum size for protease production in both the isolates were 1% (Fig.4). The protease activity was observed to be at 520 mg/l and 490 mg/l for *Bacillus coagulans* and *Bacillus firmus* respectively in this inoculum size.

Discussion

Nutrient broth basal medium was employed in the preliminary studies to determine the growth and production of extracellular protease from 3 different species of *Bacillus* and its mixed culture after 24 hours of incubation in rotatory condition (175 rpm) at 36º C. Peptone was used as the nitrogen source which supported the protease production in the present study. Ferrero *et al* [7] concluded that the protease synthesis inhibited in the presence of urea and peptone.

The present work revealed that extracellular protease is efficiently produced by *B. coagulans* and *B. firmus* than *B. subtilis*. The total protease production was maximum in *B. coagulans* (320 mg) followed by *B. firmus* (290 mg), mixed culture (270 mg) and *B. subtilis* (215 mg). However, mixed culture of three species also showed significant efficiency in protease production. The productivity was confirmed from the result obtained from quantitative and semi quantitative assay analysis.

Choudhry *et al* [14] used *B. coagulans* for the synthesis of xylanase enzyme production. In contrast to the above, the higher protease production (5.25 cm^2) was noticed in *B. coagulans*. The high efficiency of protease production in *B. subtilis* was observed by Chengtoa Wang *et al* [15] and Kakoli Dutt *et al* [16]. The present study revealed that the *B. subtilis* is less efficient in producing protease compared with other three cultures studied. These results were confirmed with quantitative and semiquantitative assay in the present study. A similar kind of result was observed in the work performed by Moon and Parullekar [17].

The protease production was found to be high in *B. coagulans* and *B. firmus* obtained at alkaline condition of pH 9 reaching to 300 mg/l and 270 mg/l respectively and it was gradually decreased to 15 mg/l and 17 mg/l respectively at pH 12. These observations were in agreement with the result of El-Hawary and Ibrahim [18] and Joo *et al* [19] who concluded that the optimum pH must meet the requirements of the protease producing gene and the bacteria were more sensitive pH when used for the production of enzyme. On the other hand, Keivan *et al* [20] studied that pH 7 was found to be optimum for the production of alkaline protease from *B. polymixa* and *B. cereus*.

Temperature is the one of the most important factor affecting the enzyme production. In our present results, a positive relationship was observed between protease production and incubation temperature upto 55^0 C. About 90% of the original protease production was gained at 55^0 C in the present study. These results were in harmony with the findings of Atalo and Gashe [12] and Johnvesly *et al* [8].

Environmental conditions could affect the production of extracellular proteolytic enzymes. Aeration speed has been shown to affect the protease production in various strains of bacteria. In the present investigation, both the *Bacillus* isolates showed maximum protease activity of 516 mg/l for *B. coagulans* and 497 mg/l for *B. firmus* at 175 rpm after 24 hours of incubation. At this speed, aeration of the culture media was increased which could lead to sufficient supply of dissolved oxygen in the media. Nutrient uptake by bacteria also will be increased resulting in increased protease production. As the aeration speed is raised to 225 rpm the protease production is reduced in both the cases. This result correlates with those of Keivan *et al* [20] who found that the increase in aeration speed increased the enzyme production.

The amount of inoculum used to culture the bacteria also affects protease production. In the present investigation, the optimum inoculum size for protease production was found to be 1% in both the isolates. The protease activity was observed to be at 520 mg/ml and 490 mg/ml for *Bacillus coagulans* and *Bacillus firmus* respectively in the inoculum. The increase in protease production using small inoculum size was suggested to

be due to higher surface area to volume ratio resulting in increased protease production. In addition improved distribution of dissolved oxygen and more effective uptake of nutrients also contributed to higher protease production. This result is similar to those given by Norazizah *et al* [21] in which they said that 4% of inoculum provided a maximum enzyme production.

In summary, *B. coagulans* and *B. firmus* favoured high efficiency in producing protease enzyme. The optimum pH, temperature, aeration speed and required inoculum size were found to be 9, 45°C, 175 rpm and 1% respectively in the present study. Hence from the present results, these optimum environmental conditions could be used in the further studies.

Acknowledgements

The authors are grateful to the authorities of PRIST University for the facilities. They are thankful to Dr. S. Ravikumar, Dean, Faculty of Biotechnology, PRIST University for the encouragement and technical assistance all through the study.

References

1. Kalisz HM (1988). Microbial Proteinases. Adv. Biochem. Eng. Biotechnol. 36:1- 65.

2. Huang G., Dai Dehni, Hu Welian and Jilag Jiaxin (2008). Optimization of medium composition for thermostable protease production by *Bacillus* sp. HSO8 with a statistical method. African Journal of Biotechnol. 7:1115 – 1122.

3. Varela H., Ferrari MP, Bielobradjic L, Weyrauch R, Loperena ML (1996). Effect of medium composition on the production by a new *Bacillus subtilis* 100 late of protease with promising enhancing activity. World J microbial. Biotechnol. 12:643-645.

4. Dhandapani R. Vijyaragavan R (1994). Production of thermophilic extracellular alkaline protease by *Baciilus stearo thermophilus*. World J. Mocrobiol. Biotechnol. 10:33-35.

5. Udandi, B, Rajendran Rajkumar, Palanivel, K.V. Sivakumaar and M.M. Joe (2009) Optimization of protease enzyme production using *Bacillus* sp. isolated from different wastes. Botony Research International 2:83 – 87.

6. Manachini PL, Fortina MG, Parimi C (1989). Proteinase production by halophilic isolates from marine sediments Appl. Microbiol. Biotechnol., 28:409.

7. Ferrero MA, Castro GR, Abate CM, Baigori MD, Sineriz F (1996). Thermostable alkaline protease of *Bacillus lichniformis* MIR – 29 Isolation, production and characterization. Appl. Microbial. Biotech. 45:327 -332.

8. Johnvesly B, Manjunath BR, Naik GR (2002) Pigeon pea waste as a novel, in expensive, substrate for production of a thermostable alkaline protease from thermoalkalophilic *Bacillus* sp. JB – 99. Bio Resour. Technol. 82:61-64.

9. Fu.Z, Hanid.BA, Razak CN, Basru M, Sallch AB, Rahman RN (2003), Secretory expression in E.Coil and single step purification of heat – stable alkaline protease.Protein exp.purification.28:63-68.

10. Gupta, R., Q. Beg, S. Khan and B. Chauhan, (2002). An overview on fermentation, downstream processing and properties of microbial alkaline protease. Appl.Microb. Biotech., 60: 381-395.

11. Sandeep, K, R.M. Vohra, Mukesh Kapoor, Qusim Khalil Beg G.S. Hoondal (2000). Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus* sp. P – 2. World J. Microbiol. & Biotechnol. 17:125 – 129.

12. Atalo K and Gashe B A (1993). Protease production by a thermophilic *Bacillus* sp. (P-001A) which degrades various kinds of fibrous proteins. Biotechnology.lett.11:1151-1156.

13. Lowry OH, Rose brought NJ, Farr Al, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biol.Chem. 193 : 265 – 275.

14. Choudhry B, Sunita Chanran, S.N. Singh and P. Ghosh (2005). Production and xylanase of *Bacillus coagulans* and its bleaching potential. World J. Microbiol. Biotechnol. 22:283-288.

15. Chengtoa Wang, Bao Ping ji, Bo Li Rob Nout, Ping Lan Li, Hong ji, Long Fei Chen (2005). Purification of Characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional Douchi. J. Ind. Microbiol Biotech., 33:750 -758.

16. Kakoli Dutt, Pritesh Gupta, Saurabh Saran, Swati Misra and Rajendra Kumar Saxena (2009). Production of Milk-Clotting Protease from *Bacillus*

subtilis. Applied Biochem. and Biotechnol. [DOI 10.1007/s12010-008-8504-9].

17. Moon, S. and H. Parulekar (1993). A. Parametric study of protease production in batch and fed batch cultures of *Bacillus firmus*. Biotech. Bioeng. 37: 467 – 483.

18. El-Hawary, F.I. and I.I. Ibrahim (1992). A comparative study on protease of three thermophilic Bacilli. Zagazig Journal. Agric. Res. 19: 777-787.

19. Joo, H., C. Kumar, G. Park, K. Kim, S. Paik and C. Chang (2002). Optimization of the production of extracellular alkaline protease from *Bacillus horikoshii*. Process Biochem., 38: 155-159.

20. Keivan B maal,Giti Emtiazi and Iraj nahvi (2009). Production of alkaline protease by *Bacillus cereus* and *Bacillus polymixa* in a new industrial culture medium and its immobilization.African journal of mic.bio. research vol.3(9) pp.491-497.

21. Norazizah S, Sayangku N Aris, Raja noor zahila Abd rahman and Abu baker (2005).Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* (strain 146).Journal of app. science research 1(1):1-8.