Journal of Applied Sciences Research 1(1): 1-8, 2005
© 2005, INSInet Publication

Optimization of Environmental and Nutritional Conditions for the Production of Alkaline Protease by a Newly Isolated Bacterium *Bacillus cereus* Strain 146

[1]Norazizah Shafee, [1]Sayangku Norariati Aris, [1]Raja Noor Zaliha Abd Rahman,
[2]Mahiran Basri and [3]Abu Bakar Salleh

[1]Department of Microbiology, [3]Department of Biochemistry,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia.
[2]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia,
43400 UPM Serdang, Malaysia.

Abstract: Optimization of the growth conditions for maximum growth rate and alkaline protease production was carried out using the newly isolated mesophilic bacterium *Bacillus cereus* strain 146. The bacterium produced protease at maximum rate after 48 h of incubation at 37°C with agitation speed of 170 rpm and 4% (v/v) starter culture. The best carbon and organic nitrogen sources for this bacterium were glucose and beef extract, respectively. While the most effective inorganic nitrogen sources were urea and lysine. Supplementation of the culture medium with Mn^{2+} improved the protease production substantially. Under these conditions *B. cereus* strain 146 was found to produce alkaline protease at a maximum rate of approximately 2.0 µg/mL/min.

Key words: Alkaline protease, *Bacillus cereus*, environmental factors, nutritional conditions

INTRODUCTION

Alkaline proteases, an important group of industrial enzymes are produced by a wide range of microorganisms including fungi, animal and bacteria. Bacteria which produce proteases include *Bacillus* sp., *Alcaligenes faecalis, Pseudomonas fluorescens* and *Aeromonas hydrophilia. Bacillus subtilis* is the main group that is used in international enzyme industry[1]. *Bacillus cereus* also was reported to produce proteolytic enzymes[2], however, few studies have been done on proteolytic enzymes from *Bacillus cereus*[3].

Studies on other strains of *Bacillus* showed that nutritional, chemical and physical factors can influence protease production. Nutritional factors include the sources of carbon, nitrogen and metal ions. In addition, availability of metal ions in growth media was also shown to affect protease production[4,5]. Fe^{3+} and Ca^{2+} were reported to increase enzymatic activity while Mg^{2+}, Na^{+}, Zn^{2+} and Cu^{2+} interfered with protease production by *Bacillus* sp. SSR1[6]. Besides nutritional factors, physical factors such as inoculum concentration, aeration[7], temperature[8], pH[9], and incubation time[10] also significantly affect protease production.

In the present study, various parameters of environmental and nutritional factors were tested. The resulting growth and protease activity in the newly isolated *Bacillus cereus* strain 146 were measured. The present investigation is aimed at optimization of growth conditions which have been predicted to play a significant role in enhancing the production of alkaline proteases.

MATERIALS AND METHODS

Microorganism: A new strain *Bacillus cereus* 146, isolated from contaminated soil of a wood factory in Selangor, Malaysia was inoculated onto 0.5% skim milk agar plate and incubated at 37°C for 24 h. Appearance of clearing zones formed by hydrolysis of skim milk was used as indication of an alkaline protease producer. The alkaline protease producer was then inoculated onto nutrient agar plates and incubation at 37°C for 24 h to obtain a pure culture. The resulting isolated colonies were subcultured onto nutrient agar slants, grown at 37°C for 24 h, maintained at 4°C and subcultured at four-week intervals.

Inoculum preparation: Inoculum was prepared by

Corresponding Author: Dr. Norazizah Shafee, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia.

inoculating one loopful of culture into 10 mL TSB medium and incubated at 37°C for 24 h on a rotary shaker at 170 rpm. The resulting culture was subjected to centrifugation at 13,000*g* for 10 min. The pellet was then resuspended in 0.85% w/v NaCl and absorbance was determined to obtain the value of 0.5 at 540 nm. The resulting supernatant was used for quantitative assay of proteases[11].

Analytical methods**:** Cell growth expressed as colony-forming unit (CFU) per mL was evaluated by plating ten-fold serial dilutions of the culture media onto nutrient agar plates and counting the colonies after 24, 48 and 72 h of incubation. Quantitative analysis of alkaline protease activity was performed according to Keay dan Wildi[11] with minor modifications. Briefly, the reaction mixture containing 0.25 mL enzyme and 0.75 mL distilled water was incubated at 37°C for 5 min. The reaction began when 1.0 mL casein 2.0% (w/v) pH 7.0 was added. The mixture was then incubated for 10 min and the reaction was terminated by addition of 2.0 mL 0.4 M Trichloroacetic acid (TCA). The mixture was then vortexed and incubated for 20 min followed by centrifugation at 14,000*g* for 10 min. The resulting supernatant (1 mL) was then mixed with 5.0 mL Na_2CO_3 and 1 mL of Folin-ciocalteau reagent: distilled water (1:3 v/v) to give bluish coloration. The colored suspension was then incubated for 20 min prior to absorbance measurement at 660 nm. The similar method was used to prepare a control, however, TCA was added at the surface and casein was added only after 10 min of incubation. One protease unit (PU) is defined as the amount of enzyme that releases 0.5 µg/mL/min tyrosine under the above conditions. The amount of tyrosine was obtained from tyrosine standard curve according to Keay dan Wildi[11].

Sources of carbon, nitrogen and metal ions**:** Carbon sources chosen for the study were glucose, sucrose, starch, fructose, maltose and cellobiose. These carbon sources were used to replace the carbon source available in the media. Sources of nitrogen include organic nitrogen, inorganic nitrogen, and amino acid. The sources were soytone, casamino acid, beef extract, yeast extract, peptone, tryptone, ammonium nitrate, ammonium carbonate, urea, lysine, alanine, glutamic acid and glycine. Metal cations tested to replace metal ion source in the media were Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Li^{+}.

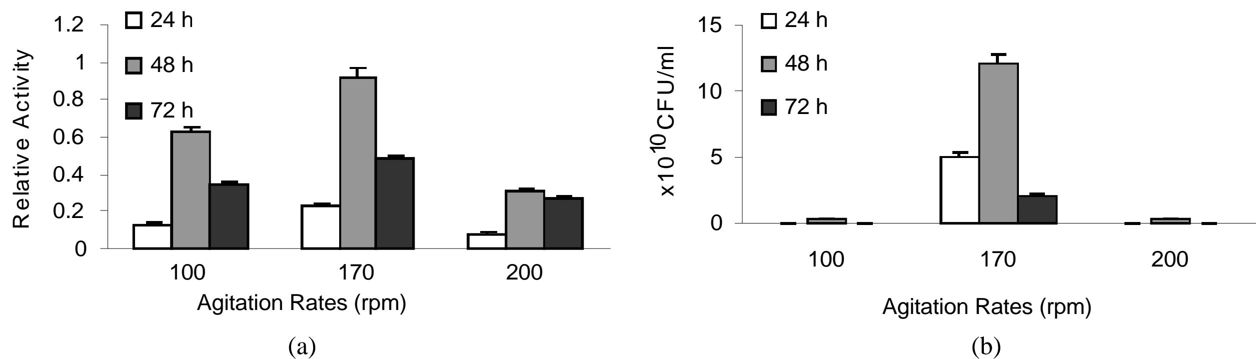
Culture conditions: Bacteria were grown in TSB media until optical density (OD) reading reached 0.5 at 540 nm absorbance. The cultures were then pipetted into growth media[12] with starting pH of 10 and at inoculum sizes of 2, 3, 4, 5 and 6% (v/v). The samples were then incubated at 37°C at the agitation speed of 170 rpm. The optimum inoculum size determined from this study was used to inoculate fresh media in order to test the effect of agitation speed. The agitation speeds tested were at 100, 170 and 200 rpm. For all the experiments, samples were harvested at 24, 48 and 72 h post-inoculation and processed as described previously.

Statistical analysis**:** For statistical analysis, standard deviation for each experimental results and student's t-test was calculated using Excel Spread-sheets available in Microsoft Excel. Results presented in this study are means of three independent determinations. Bars correspond to standard deviation.

RESULTS AND DISCUSSION

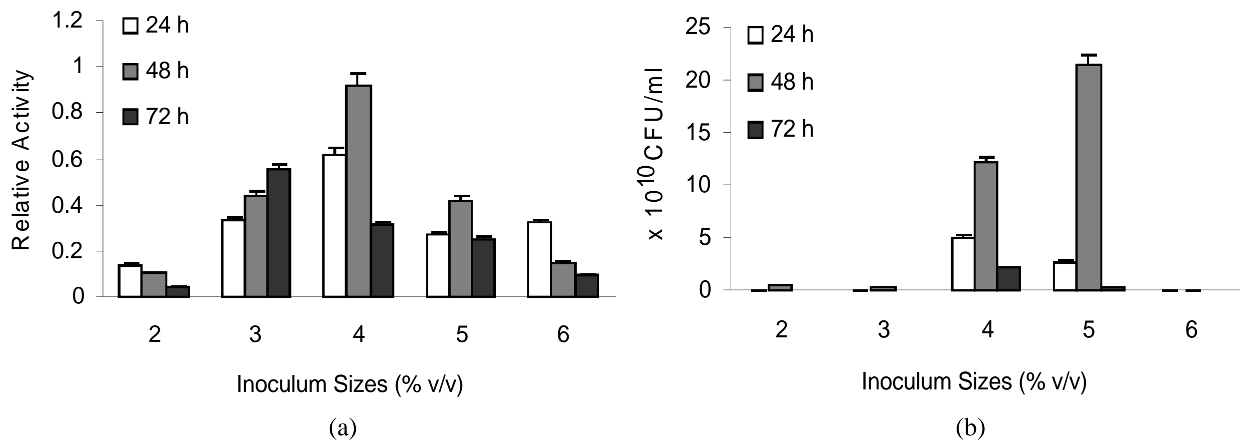
Effects of agitation rates on protease production**:** Environmental conditions could affect the production of extracellular proteolytic enzymes. Agitation rates have been shown to affect protease production in various strains of bacteria[13-16]. In the present investigation, *Bacillus cereus* strain 146 grown in culture media[12] with 4% inoculum size showed maximum protease activity at 170 rpm agitation speed after 48 h of incubation (Fig. 1a). At this speed, aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media[17]. Nutrient uptake by bacteria also will be increased[10] resulting in increased protease production. At 200 rpm, protease activity was found to be reduced. This was perhaps due to denaturation of enzymes caused by high agitation speed[18,19]. Agitation speed of 100 and 200 rpm affected the growth of the organism considerably (Fig. 1b). At 100 rpm, insufficient aeration and nutrient uptake perhaps caused the inability of bacteria to grow efficiently. At 200 rpm, however, excessive aeration and agitation could occur which led to cell lysis and increased cell permeability due to abrasion by shear forces[14]. Based on this finding, agitation speed of 170 rpm was used throughout the study.

Effects of inoculum sizes on protease production: The amount of inoculum used to culture the bacteria also affects protease production. Results from the present study showed that optimum inoculum size for protease production in *Bacillus cereus* strain 146 was 4.0%. At this size, protease activity was observed to be at 0.923 µg/mL/min after 48 h of incubation (Fig. 2a). The increase in protease production using small inoculum sizes was suggested to be due to the higher surface area to volume ratio resulting in increased protease



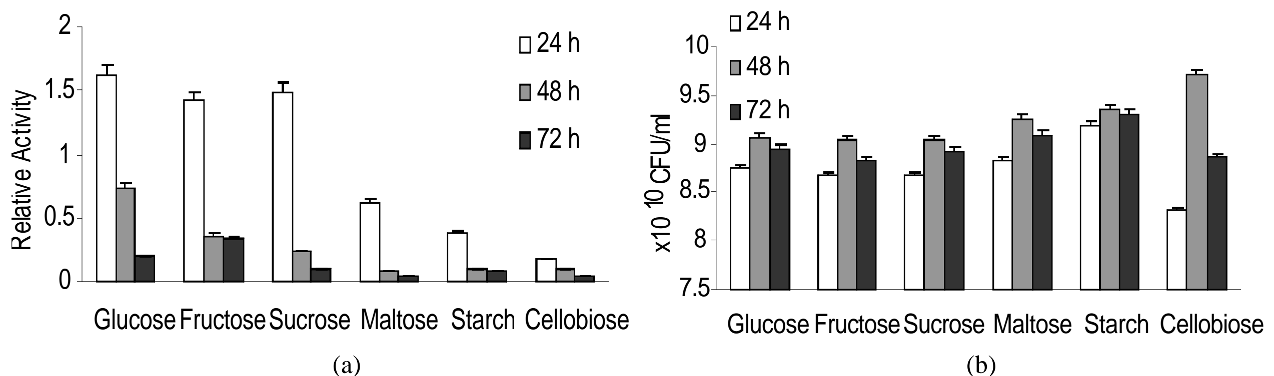
(a) (b)

Fig. 1: Effect of agitation rates on protease production and bacterial growth. *Bacillus cereus* strain 146 grown in culture media with 4% inoculum size and subjected for 24, 48 and 72 h of incubation. (a) Samples harvested after the different incubation period showed various levels of protease activity at the agitation rates tested. (b): Growth of the bacteria also was affected under these conditions. Results are means of three independent determinations. Bars correspond to standard deviation.



(a) (b)

Fig. 2: Effect of inoculum sizes on protease production and bacterial growth. Culture media were inoculated with 2.0, 3.0, 4.0, 5.0 and 6.0% starter inoculum sizes. (a): Protease activity of the harvested samples was assayed following 24, 48 and 72 h of incubation. (b): Growth of bacteria was quantitated as described in the Materials and Methods. Results are means of three independent determinations. Bars correspond to standard deviation.



(a) (b)

Fig. 3: Effect of carbon sources on protease production and growth of *Bacillus cereus* strain 146. Six different sources of carbon were tested in this experiment. Cultures containing 4% inoculum size were incubated at 37°C, 170 rpm for 24, 48 and 72 h. (a): Quantitative analysis of alkaline protease activity was performed on samples harvested after the incubation periods. (b): Bacterial growth in response to each carbon sources was also determined. Results are means of three independent determinations. Bars correspond to standard deviation.

production[8]. In addition, improved distribution of dissolve oxygen and more effective uptake of nutrient also contributed to higher protease production. If the inoculum sizes are too small (2.0 and 3.0%; Fig. 2b), insufficient number of bacteria would lead to reduced amount of secreted protease. At 5.0 and 6.0%, protease activity was also found to be decreased even though luxurious growth was observed at 5.0% after 48 h incubation. Previous study has suggested that higher inoculum sizes resulted in reduced dissolved oxygen, and increased competition towards nutrient[8]. Based on these results, inoculum size of 4.0% was used for the rest of the study.

***Effects of carbon sources on protease production*:** The present investigation was aimed at optimization of medium components which have been predicted to play a significant role in enhancing the production of alkaline proteases[20]. Various sources of carbon such as glucose, fructose, sucrose, maltose, starch and cellobiose were used to replace lactose which was the original carbon source in growth media. Results obtained showed that glucose instigated highest protease production compared to other carbon sources at 24 and 48 h of incubation (Fig. 3a). Fructose and sucrose also showed high protease expression at 24 h, but drastically reduced by 48 h of incubation. This observation agrees with previous report which suggested that sources of carbon affected production of enzymes by bacteria[21]. At 72 h incubation, low protease production was detected in all the samples. The prolonged incubation time perhaps led to autodigestion of proteases and proteolytic attack by other proteases[22,23]. Maltose, starch and cellobiose caused low protease production. This is in contrast to previous report which showed that starch caused high level of enzyme expression in *Bacillus* species[24]. Bacterial growth analysis showed that no statistically significant difference was observed between the different sources of carbon in the growth media ($P>0.001$, two-tailed student's t-test) (Fig. 3b). Even though cellobiose caused high bacterial growth, protease production was very limited. The similar result was also observed for maltose and starch. It has been reported that pure sugars affected protease production considerably[25]. Utilization of pure sugars as carbon and energy sources was also shown to result in good growth with low protease production. This observation is in agreement with previous studies which suggested that larger amount of enzyme was synthesized when carbon sources used were poorly utilized for growth purposes[26,27].

Effects of organic nitrogen sources on protease production: Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources[28,29]. In this study, three sources of nitrogen were used. They were organic nitrogen (soyton, casamino acid, beef extract, yeast extract, peptone and tryptone), inorganic nitrogen (ammonium nitrate, ammonium carbonate and urea) and amino acid (lysine, alanine, glutamic acid and glycine). In the present investigation, results obtained showed that beef extract resulted in the highest level of protease activity compared to other sources of organic nitrogen (Fig. 4a). The high level of protease production in the presence of beef extract was observed at all the incubation periods tested. This result is in agreement with Yang and Lee[30], which reported an increased production of protease by *Streptomyces rimosus* in the presence of beef extract. Previous studies also reported that protease production by *Bacillus stearothermophilus* F1 and *Bacillus mojavensis* was best in the presence of organic nitrogen sources[31,32]. Despite the luxurious bacterial growth (Fig. 4b), the presence of yeast extract, peptone and tryptone, resulted in low protease production. This observation contradicted Phadatare *et al.*[33] which reported that protease production in *Conidiobolus coronatus* was enhanced by organic nitrogen sources like yeast extract, peptone and tryptone. In general, the presence of organic nitrogen sources, except for beef extract, caused increased growth and repressed protease production of *Bacillus cereus* strain 146. This phenomenon was also observed in *Aeromonas hydrophila* and *Bacillus brevis*[34,35]. In some organisms, however, organic nitrogen sources were found to be better nitrogen sources both for growth and also protease production[36,33].

Effects of inorganic nitrogen sources on protease production: Inorganic nitrogen sources were also tested on the growth and protease production of *B. cereus* strain 146. Results obtained showed that amonium carbonate led to high protease activity at 24 h but reduced at 48 and 72 h (Fig. 5a). Growth was observed to be high in all the three sources of inorganic nitrogen and incubation periods tested except for ammonium carbonate and urea at 72 and 24 h, respectively (Fig. 5b). Urea did not enhance protease production at early stages of incubation but at later stages (48 and 72 h) protease production increased. Even though growth was stimulated, only moderate levels of enzyme activities were obtained when ammonium nitrate was used as a nitrogen source. This was perhaps due to the inability of bacteria to utilize ammonia in the media. The presence of ammonia was shown to interfere with

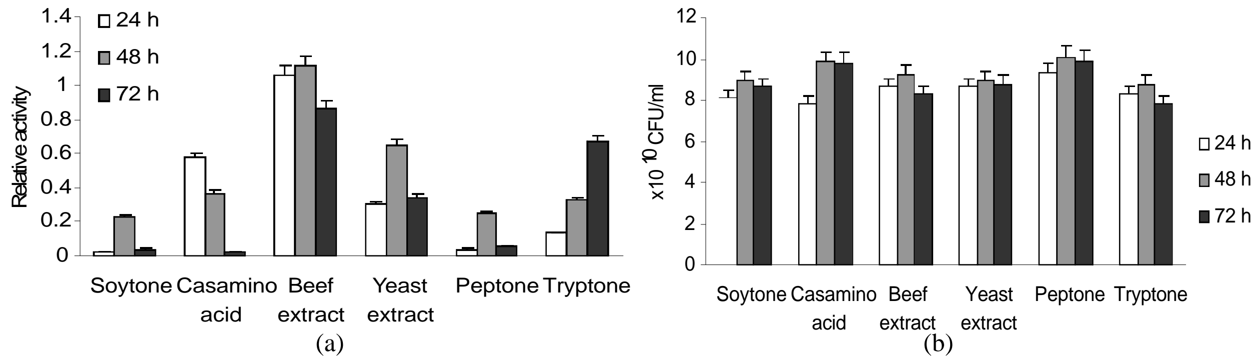


Fig. 4: Effect of organic nitrogen sources on protease production and bacterial growth. Selected organic nitrogen sources were added to culture media and incubated at 37°C, 170 rpm with 4.0% starter inoculum size. (a): Following the selected incubation periods, protease activity was assayed as described in the Materials and Methods. (b): Under these growth conditions, bacterial growth expressed as colony-forming unit (CFU) per mL was also evaluated.

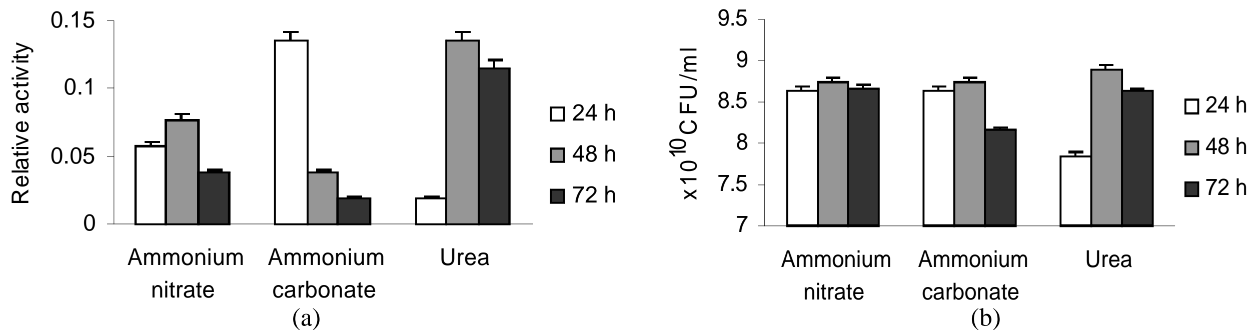


Fig. 5: Effect of inorganic nitrogen sources on protease production and growth of *Bacillus cereus* strain 146. Three different inorganic nitrogen sources were added into culture media and incubated at 37°C with 4% inoculum size and subjected to 170 rpm agitation speed. After 24, 48 and 72 h of incubation the samples were harvested. (a): Various levels of protease activity were detected in the samples. (b): Under these growth conditions, bacterial growth expressed as colony-forming unit (CFU) per mL was also evaluated. Results are means of three independent determinations. Bars correspond to standard deviation.

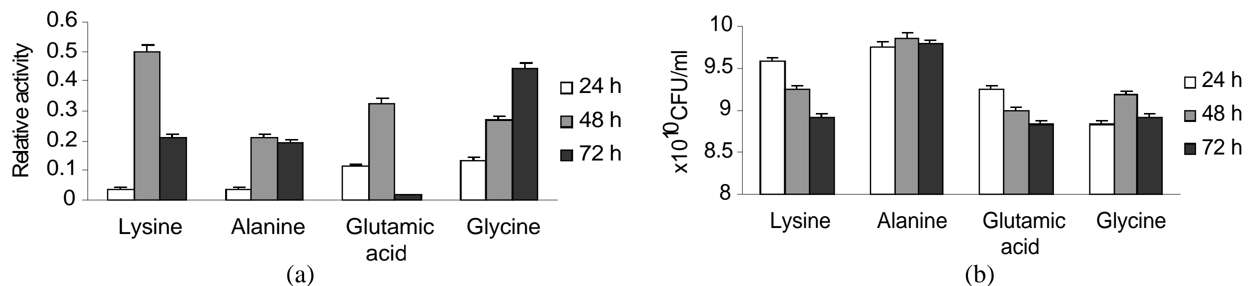


Fig. 6: Effect of amino acids on protease production and bacterial growth. Lysine, alanine, glutamic acid and glycine were tested as sources of inorganic nitrogen for protease production in *B. cereus* strain 146. (a): Various levels of protease activity were detected in the samples harvested after 24, 48 and 72 h incubation. (b): Cell growth expressed as colony-forming unit (CFU) per mL was evaluated by plating ten-fold serial dilutions of the culture media onto nutrient agar plates and counting the colonies. Results are means of three independent determinations. Bars correspond to standard deviation.

protease production[37] which led to significant decrease in protease production. This effect of different inorganic nitrogen sources was also observed for *A. hydrophila*[38], *A. salmonicida*[39], and *Vibrio* strain SA1[40]. These reports strongly suggest that ammonium-specific repression was likely to be the factor involved.

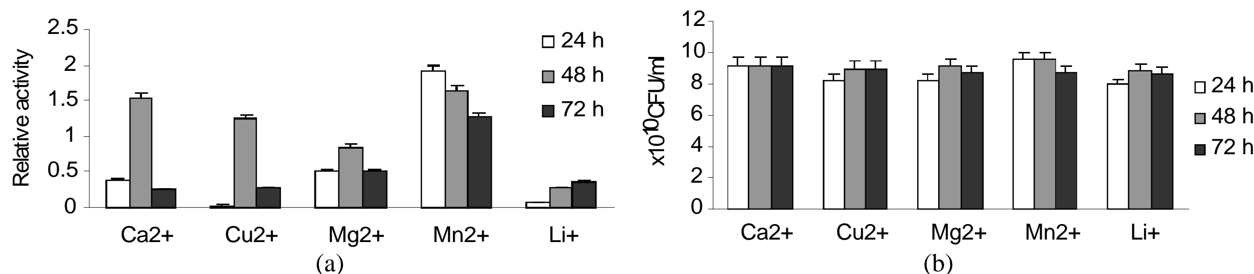


Fig. 7: Effect of metal ions on protease production and bacterial growth. Several metal ions were added to culture media which was inoculated with 4.0% starter inoculum size and incubated for at 37°C at the agitation speed of 170 rpm. (a): Following selected incubation periods, protease activity was assayed as described in the Materials and Methods. (b): Under these growth conditions, bacterial growth expressed as colony-forming unit (CFU) per mL was also evaluated. Results are means of three independent determinations. Bars correspond to standard deviation.

Effects of amino acid on protease production: Lysine, alanine, glutamic acid and glycine were also tested as sources of inorganic nitrogen for protease production in *B. cereus* strain 146. Results obtained showed various levels of protease were produced in these different inorganic nitrogen sources. In the presence of lysine and glycine, protease production was observed to be high (Fig. 6a). This observation is in agreement with previous report which showed that protease production in *Pseudomonas sp*. B45 was enhanced considerably in the presence of lysine and glycine[41]. Alanine, on the other hand, did not increase the protease production at 24 h, however increased slightly by 48 and 72 h of incubation. Even though protease production in the presence of alanine was reduced, bacterial growth remained high (Fig. 6b). This phenomenon was also observed in alkaline protease production by recombinant *Bacillus licheniformis*[42]. The study reported that alanine supplement resulted in highest cell growth without considerable effect on protease production.

Effects of metal ions on protease production: Supplementation of culture medium with metal cations improved substantially the protease production of *B. cereus* strain 146 (Fig. 7a). This observation strongly suggested the requirement of some metal ions for protease production by this organism. These results are in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions[4,5]. It was suggested that these metal ions increased stability of proteases[43,44]. The highest level of protease activity was observed at aproximately 2.0 µg/mL/min in the presence of Mn^{2+} at 24 h incubation. The activity, however, reduced slightly as incubation time increased. Addition of Ca^{2+}, Cu^{2+} and Mg^{2+} resulted in high protease production only at 48 h incubation. Among the heavy metal ions tested, Cu^{2+} and Li^{+} caused inhibition at 24 h of incubation. This observation is corroborated by previous studies which suggested inhibitory effect of Cu^{2+} and Li^{+} on proteases[45,4,46]. Even though effects of the different metal cations on protease production vary, their presence in the culture medium improved the growth of *B. cereus* strain 146 (Fig. 7b). These implied that there was no definite relationship between protease production and growth. Proteases formed at different growth phases were shown to differ in their chemical nature[47,25].

In summary, *Bacillus sp.*, particularly *Bacillus subtilis*, are known for their ability to produce proteolytic enzymes with potential use in industries. However, few studies have been done on proteolytic enzymes from *Bacillus cereus*. In addition to the limited number of reports, protease production by this microorganism also was shown to be affected by various environmental and nutritional conditions. In the present investigation we have determined the optimum parameters for maximum production of alkaline protease by the newly isolated mesophilic bacterium *Bacillus cereus* strain 146. This information has enabled the ideal formulation of media composition for maximum protease production by this organism. Further characterization of the protease produced is currently being carried out.

ACKNOWLEDGEMENTS

This research project was supported by IRPA Grant No. 09-02-04-0258-EA001

REFERENCES

1. Gupta R., Q.K. Beg, and P. Lorenz, 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biot. 2002, 59, 15-32.

2. Hayano K., M. Takeuchi and E. Ichishima, 1987. Characterization of a metalloproteinase component extracted from soil. Biol. Fertil. Soils., 4, 179-183.
3. Ghorbel, B., A. Sellami-Kamoun and M. Nasri, 2003. Stability studies of protease from *Bacillus cereus* BG1. Enzyme Microb. Tech. 32 513-518.
4. Adinarayana, K., P. Ellaiah, D.S. Prasad, 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. AAPS PharmSciTech. 4, 1-9.
5. Thangam, E.B. and G.S. Rajkumar, 2002. Purification and characterization of alkaline protease from Alcaligenes faecalis. Biotechnol. Appl. Bioc. 35, 149–154.
6. Singh, J., N. Batra and R.C. Sobti, 2001. Serine alkaline protease from a newly isolated *Bacillus sp.* SSR1. Process Biochem. 36, 781-785.
7. Votruba, J., J. Pazlarova, M. Dvorakova, L. Vachora, M. Strnadova, H. Kucerova, V. Vinter, R. Zourabian and J. Chaloupka, 1991. External factors involved in the regulation of synthesis of an extracellular proteinase in *Bacillus megaterium*: effect of temperature. Appl. Microbiol. Biot. 35, 352–357.
8. Rahman, R.N.Z.A., L.P. Geok, M. Basri, A.B. Salleh, 2005. Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. Bioresource Technol. 96, 429-436.
9. Chen. L., R.M. Daniel and T. Coolbear, 2002. Detection and impact of protease and lipase activities in milk and milk powders. Int. Dairy J. 13, 255-275.
10. Beg, Q.K., V. Sahai and R. Gupta, 2003. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in bioreactor. Process Biochem. 39, 203-209.
11. Keay, L. and B.S. Wildi, 1970. Proteases of genus *Bacillus*. I: neutral proteases. Biotechnol. Bioeng. 12, 179–212.
12. Singh, J., N. Batra and R.C. Sobti, 2000. Serine alkaline protease from a newly isolated *Bacillus sp*. SSR 1. Process Biochem. 36, 781-785.
13. Pourrat, H., C. Barthomeut, O. Texier and A. Pourrut , 1988. Production of semi-alkaline protease by *Aspergillus niger*. J. Ferment. Technol. 66, 383-388.
14. Darah, I. and C.O. Ibrahim, 1996. Effect of agitation on production of lignin-degrading enzymes by *Phanerochaete chrysosporium* grown in shake-flask cultures. As. pac. J. Mol. Biol. Biotechnol. 4, 174-182.
15. Mehrotra, S., P.K. Pandey, R. Gaur and N.S. Darmwal, 1999. The production of alkaline protease by a *Bacillus* species isolate. Bioresource Technol. 67, 201-203.
16. Mabrouk, S.S., A.M. Hashem, N.M.A. El-Shayeb, A.M.S. Ismail and A.F. Abdel-Fattah, 1999. Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415. Bioresource Technol. 69, 155-159.
17. Kumar, C.G. and H. Takagi, 1999. Research review paper: microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv., 17, 561-94.
18. Lee, P.G., C.N.A. Razak, R.N.Z.A. Rahman, M. Basri, and A.B. Salleh, 2002. Isolation and screening of an extracellular organic solvent-tolerant protease producer. Biochem. Eng. J., 13, 73-77.
19. Burkert, J.F., R.R. Maldonado, F.M. Filho and M.I. Rodrigues. Comparison of lipase production by *Geotrichum candidum* in stirring and airlift fermenters. J. Chem. Technol. Biotechnol., 80, 61-67.
20. Gupta, R., W. Beg, S. Khan and B. Chauhan, 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl. Microbiol. Biot. 60, 381 - 395.
21. Juhasz, T., K. Kozma, Z. Szengyel and K. Réczey, 2003. Production of beta- Glucosidase in mixed culture of *Aspergillus niger* BKMF 1305 and *Trichoderma reesei* RUT C30. Food Technol. Biotechnol., 41, 49-53.
22. Priest, F.G., 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. , 41, 711–753.
23. Chu, I.M., Lee, C. and T.S. Li, 1992. Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. Enzyme Microb. Tech. 1992, 14, 755–761.
24. Mahmood, A.U., J. Greenman and A.H. Scragg, 2000. Effects of macromolecular growth substrates on production of extracellular enzymes by Bacillus species in continuous culture. Microbios. , 103, 85-96.
25. Dahot, M.U., 1993. Cultivation of penicillium expansum on rice husk powder for protease production. J. Islamic Acad. Sc., 6.
26. Walker, W.E. and L.L. Campbell, 1983. Induction of a-amylase of *Bacillus sterothermophilus* by maltodextrin. J. Bacteriol., 86, 687-691.
27. Prasad, R., P.K. Malik and D.K. Mathur, 1984. Optimization of nutritional and environmental factors for the production of caseinolytic enzyme of *Micrococcus Sp* isolated from cheddar cheese. Asian J. Dairy Res., 3, 25-36.
28. Haulon, G.W., N.A. Hodges and A.D. Russell, 1982. The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. J. Gen. Microbiol., 128, 845-851.

29. Levisohn, S. and A.I. Aronson, 1967. Regulation of extracellular protease production in *Bacillus cereus.* J. Bacteriol., 93, 1023-1030.
30. Yang, S.S. and C.M. Lee, 2001. Effect of culture media on protease and oxytetracycline production with mycelium and protoplasts of *Streptomyces rimosus.* World J. Microbiol Biotech., 17, 403 - 410.
31. Razak, C., R. Rahman, A. Salleh, W. Yunus, K. Ampon and M. Basri, 1995. Production of a thermostable protease from a new high pH isolate of *Bacillus stearothermophilus*. J. Biosci., 6, 94–100.
32. Beg, Q.K. and R. Gupta, 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enzyme Microb. Tech., 32, 294-304.
33. Phadatare, S.U., V.V. Deshpande and M.C. Srinivasan, 1993. High activity alkaline protease from *Conidiobolus coronatus* (*NCL* 86.8.20): Enzyme production and compatability with commercial detergents. Enz. Microbiol. Technol., 15, 72-76.
34. Ponsare, A.C., V. Venugopal and N.F. Lewis, 1985. A note on nutritional influence on extracellular protease synthesis in *Aeromonas hydrophila*. J. Appl. Bacteriol., 58, 101-104.
35. Banerjee, U.C., R.K. Sani, W. Azmi and R. Soni, 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Proc. Biochem. 35, 213-219.
36. Aleksieva, P., A. Djerova, B. Tchorbanov and J. Girarov, 1981. Submerged cultivation of a strain of *Humicola lutea* 72 producing acid protease. Eur. J. App. Microbiol. Biotech., 13, 165-169.
37. Frankena, J., H.W. Van-Verseveld and A.H. Stouthamer, 1985. A continuous culture study of the bioenergetic aspects of growth and production ofexocellular protease in *Bacillus licheniformis*. Appl. Microbiol. Biot., 22, 169–176.
38. O'Reilly, T. and F. Day, 1983. Effects of culture conditions on protease production by *Aeromonas hydrophila*. Appl. Environ. Microbiol. , 45, 1132-1135.
39. Liu, P.V. and M.C. Hsieh, 1969. Inhibition of protease production of various bacteria by ammonium salts: its effect on toxin production and virulence. J. Bacteriol. 99, 406-413.
40. Wiersma, M., T.A. Hansen and W. Harder, 1978. Effect of environmental conditions on the production of two extracellular proteolytic enzymes by *Vibrio* SA1. J. Microbiol. Serol., 44, 129-140.
41. Chakraborty, R. and M. Srinivasan, 1992. Production and regulation of a thermostable protease by *Pseudomonas* sp. B45. Acta Microbiol. Hung. 39, 181-191.
42. Calk, P., A. Bayram and A.H. Ozdamar, 2003. Regulatory effects of alanine-group amino acids on serine alkaline protease production by recombinant *Bacillus licheniformis.* Biotechnol. Appl. Biochem. 37, 165–171.
43. Rahman, R.N.Z.A., C.N. Razak, K. Ampon, M. Basri , W.M.Z.W. Yunus and A.B. Salleh, 1994. Purification and characterization of a heat stable alkaline protease from *Bacillus stearothermophilus* F1. Appl Microbiol Biotechnol. 1994, 40, 822-827.
44. Paliwal, N., S.P. Singh and S.K. Garg, 1994. Cation-induced thermal stability of an alkaline protease from a Bacillus species. Bioresource Technol., 50, 209-211.
45. Palmieri, G., C. Bianco, G. Cennamo, P. Giardina, G. Marino, M. Monti and G. Sannia, 2001. Purification, characterization, and functionalr role of a novel extracellular protease from *Pleurotus ostreatus.* Appl. Environ. Microbiol. 67, 2754-2759.
46. Rice, A.M. and A.C. Sartorelli, 2001. Inhibition of 20 S and 26 S proteasome activity by lithium chloride. Impact on the differentiation of leukemia cells by all-trans-retinoic acid. J. Biol. Chem. 276, 42722-42727.
47. Dahot, M.U. and A.R. Memon, 1989. Lipase activity of *Penicillium expansum*. J. Pure Appl. Sci. 8, 7-13.