Short Communication

Isolation and identification of an alkaline protease producing *Bacillus* from soil

Fereshteh Eftekhar[1], Jamsheed Fouladi[2] and Mehrzad Faghihi[1]

[1]Biology Department, Faculty of Science, Shahid Beheshti University, Chamran Highway, Evin, Tehran, Iran.
[2]Biology Department, Alzahra University, Vanak Deh, Vanak Square, Tehran, Iran.

Received 5 May 2003; accepted 6 September 2003

Abstract
Soil samples were screened for alkaline protease producing bacteria on alkaline agar plates containing casein. Alkaline protease production was identified by clear zones of casein hydrolysis around colonies. Such colonies were grown in an alkaline broth for 48-72 h and the enzyme activity of the culture supernatants was determined by measuring the amount of tyrosine released from casein after 10 min at 35°C, at a pH of 10.5. A spore forming Gram positive aerobic Bacillus sp. which showed the best enzyme production was chosen (strain L2). The production medium was optimized for this strain. The best developed medium contained: 1% glucose, 0.5 % peptone, 1% yeast extract, 0.1% KH_2PO_4 and 0.02 % $MgSO_4$. $7H_2O$. Alkaline protease activity was highest at pH 11 and 45°C. Biochemical tests tentatively identified the organism as Bacillus licheniformis.
Keywords: *Alkaline protease, Bacillus, Soil.*

Alkaline proteases secreted by both neutrophilic and alkalophilic Bacilli are of interest because they represent the major source of commercially produced proteo-lytic enzymes (Horikoshi and Akiba, 1982; Markland and Smith 1971; Priest, 1977). The main industrial application for alkalophilic proteases are in the detergent industry (Ainsworth, 1994; Gupta *et al*., 1999; Kalisz, 1988), leather tanning process (Atalo and Gashe, 1993; Hameed *et al*., 1996; Malathi and Chakraborty, 1991), food industries (Kalisz, 1988, Outtrup and Boyce, 1990), and silver removal from X-ray films (Fujiwara *et al*., 1991). Most of the alkaline protease producers are *Bacillus* sp. and many of these enzymes have been isolated and characterized (Abdolrahman *et al*., 1994; Gupta *et al*., 2002; Yamagata *et al*., 1995). In this paper, we report isolation and characterization of an alkaline protease producing *Bacillus* sp. from soil which might have useful industrial applications.

Several samples of soil were plated on alkaline agar plates containing 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4$. $7H_2O$, 1% Na_2CO_3 and 1.5% agar, pH 10.5 (Horikoshi, 1990). Colonies were then transferred on to agar plates containing casein to screen for alkaline protease producing bacteria. The medium contained 0.5% tryptone, 0.25% yeast extract, 0.1% glucose, 1% sodium caseinate, 0.44% trisodium citrate. $2H_2O$, 20 mM $CaCl_2$. $6H_2O$ and 1.5% agar. The plates were incubated for up to 3 days at 35°C. The appearance of clear zones around the colonies as a result of casein hydrolysis was taken as an indication of alkaline protease production. Twenty six alkalophilic bacteria were isolated of which four were alkaline protease producers exhibiting large zones of casein hydrolysis after 48-72h. These organisms were then grown in an alkaline broth medium (same as the alkaline agar without the agar) at 35°C for 72 h with gentle shaking at 60 rpm (Taitec Bio-shaker, BR-300L). The cultures were centrifuged at 10000 ×g for 10 min at 4°C (Kokusan H2000BF). The Protease activity of the culture supernatants were then determined by adding 0.5 ml of the supernatant to 3 ml of the reaction mixture (6% casein in 10 mM borax buffer, pH 10.5) and incubating the mixture at 35°C for 10 min. The enzyme activity was stopped by adding 3.2 ml of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33

Correspondence to: **Fereshteh Eftekhar, Ph.D**
Tel: +98 21 29902721, Fax: +98 21 2403041
E-mail: f-eftekhar@cc.sbu.ac.ir

M acetic acid. The mixture was then incubated at 35ºC for 10 additional min and filtered. Alkaline protease activity was determined by measuring the amount of tyrosine released from casein at 275 nm (Milton Roy Spectronic 601). One enzyme unit was the amount of enzyme which produced 1 µg of tyrosine at 35ºC after 10 min of incubation (Shimogaki *et al.*, 1991). One of the isolates which produced the highest enzyme level (strain L2) was chosen for further studies.

Alkaline protease production was optimized for strain L2 by varying concentrations of glucose (0.5, 1, 2 and 3%), yeast extract (0.25, 0.5, 1, 1.5 and 2%), different temperatures (25, 30, 35, 40 and 45ºC), variable pH (7.0 to 11) and different aeration (shaker speed of 60, 120 and 180 rpm). Protease activity was measured under various experimental condition (enzyme units/ml supernatant) and the total cell mass was determined by measuring the optical density of the cultures at 660 nm.

As shown in figure 1, protease production was highest at pH 11 (1a) and 35ºC (1b). Protease production was also maximum at concentrations of 2% yeast extract and 1% glucose (Fig. 2). Higher concentrations of glucose and yeast extract supported better growth and biomass but enzyme production was suppressed at glucose concentrations above 1%. This shows that protease synthesis is under catabolite repression as previously reported (Kalisz, 1988). Aeration had the reverse effect on protease production and maximum enzyme activity was observed when shaking speed was low (60 rpm). Protease production occurs in the stationary phase of growth and increases as GTP levels decrease (Gupta *et al.*, 2002, Bierbaum *et al.*, 1991). Hence, it has been suggested that optimization of nutritional contents of the medium (carbon and nitrogen), incubation period, pH, temperature and agitation are needed to determine the highest amount of enzyme production (Razak *et al.*, 1994).

Alkaline protease activity of the culture supernatants were tested against casein at varying pH values (7 to

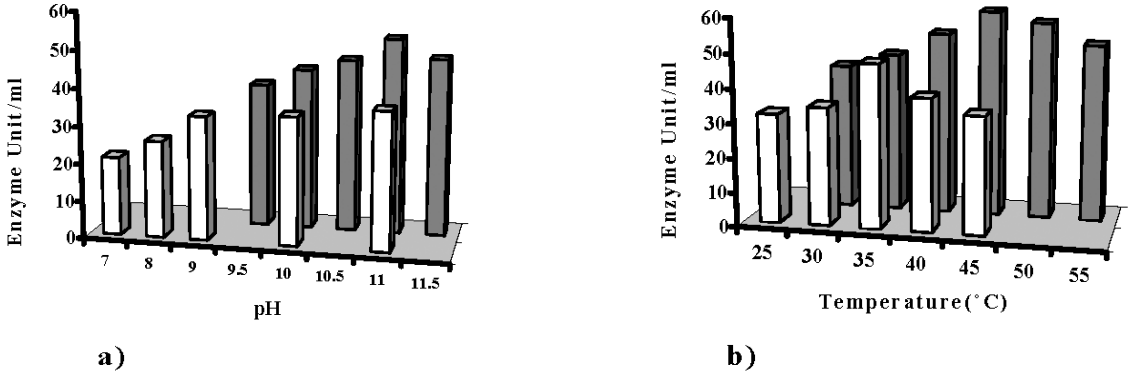

Figure1. The effect of pH (a) and temperature (b) on alkaline protease production (□) and alkaline protease activity (■) *in vitro*.

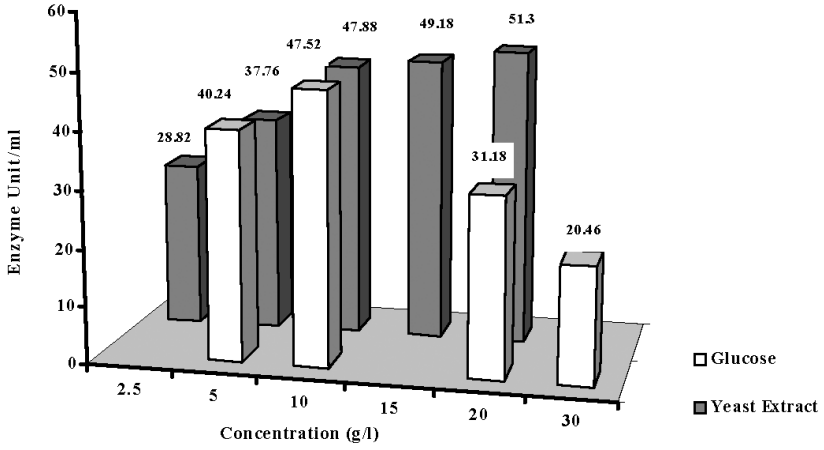

Figure 2. The effect of different concentrations of glucose and yeast extract on alkaline protease production.

12) and temperatures (30 to 55ºC). Figure 1 shows that the best activity was observed at pH 11 (1a) and 45ºC (1b). However, the enzyme was considerably active within the pH range of 9-12 and a temperature range of 35-55ºC. Other reports concerning alkaline protease production by different *Bacillus* species have shown a pH range of activity within 9-12 and temperatures between 40 to 70ºC (Johnvesly, Naik 2001; Kumar *et al*., 1999; Kumar, 2002). Our results were quite reproducible and showed a similar range of activity for the alkaline protease from strain L2. The wide range of the activity of this enzyme may be important for its potential use in the detergent industry.

Strain L2 was a Gram positive spore forming rod, catalase positive, Indole and VP negative. It produced acid from glucose but not from mannitol, arabinose, xylulose. The bacterium was able to hydrolyze casein, gelatin and starch. It grew in media containing citrate, 2-10% NaCl but not in the presence of lysozyme. The results from the biochemical tests tentatively identified strain L2 as *B. lichenoformis*. Isolation of thermostable alkaline proteases from other strains of *B. lichenoformis* has been previously reported and some have shown extremely good potentials for use in the detergent industry (Ferrero *et al*., 1996; Gupta *et al*., 1999). Further research is underway to determine the potential of the alkaline protease from strain L2 as a laundry detergent additive.

References

Abdolrahman RNZ, Razak CN, Ampon K, Basri M, Zin WM, Yunus W, Salleh AB. (1994) Purification and characterization of a heat stable alkaline protease from *Bacillus stearothermophilus* F1. *Appl. Microbiol. Biotechnol*. 40: 822-7.

Ainsworth SJ. (1994) Soap and detergents. *Chem. Eng. News*.72: 34-59.

Atalo K and Gashe BA. (1993) Protease production by a thermophilic *Bacillus* sp.(P-001A) which degrades various kinds of fibrous proteins. *Biotechnology Letter*. 15:1151-6.

Bierbaum G, Giesecke UE, Wandrey C. (1991) Analysis of nucleotide pools during protease production with *Bacillus licheniformis*. *Appl. Microbiol. Biotechnol.* 35: 725-30.

Ferrero MA, Castro GR, Abate CM, Biagori MD, Sineriz F. (1996) Thermostable alkaline proteases of *Bacillus lichenoformis* MIR 29: isolation, production and characterization. *Appl. Microbiol. Biotechnol*. 45(3): 327-332.

N, Yamamoto K, Masui A. (1991) Utilization of a thermostable alkaline protease from an alkalophilic thermophile for recovery of silver from used X-ray film. *J. Ferment. Bioeng*. 72: 306-8.

Gupta R, Gupta K, Saxena RK, Khan S. (1999) Bleach stable, alkaline protease from *Bacillus* sp. *Biotechnology Letter*. 21: 135-8.

Gupta R, Beg Q, Khan S, Chauhan B. (2002) An overview on fermentation, downstream processing of microbial alkaline proteases. *Appl. Microbiol. Biotechnol*. 60(4): 381-395.

Hameed A, Natt MA. Evans CS. (1996) Production of alkaline protease by a new *Bacillus subtilis* isolate for use as a bating enzyme in leather treatment. *World J. Microbiol. Biotechnol*. 12: 289-91.

Horikoshi K. Akiba T. (1982) Enzymes of alkalophilic bacteria. In: *Alkalophilic Microorganisms*. Japan Scientific Scientific Societies Press, Tokyo, pp.93-101.

Horikoshi K. (1990) Enzymes of alkalophiles. In: Fogarty, WM. And Kelly, CT. (eds). *Microbial enzymes and biotechnology*, pp. 275-94.

Johnvesly B, Naik GR. (2001) Studies on production of thermostable alkaline proteases from thermophilic and alkalophylic *Bacillus* sp. JB-99 in a chemically defined medium. *Process Biochem*. 371: 139-144.

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

Kumar CG, Tiwari MP, Jang KD. (1999) Novel alkaline serine proteases from alkalophylic *Bacillus* sp.: purification and some properties. *Process Biochem*. 34: 441-9.

Kumar CG. (2002) Purification and characterization of a thermostable alkaline protease from alkalophylic *Bacillus pumilis*. *Lett. Appl. Microbiol*. 34: 13-17.

Malathi S, Chakraborty R. (1991) Production of alkaline protease by a new *Aspergillus flavus* isolate under solid-substrate fermentation conditions for use as a depilation agent. *Appl. Environ. Microbiol*. 57: 712-16.

Markland FS, Smith EL. (1971) Subtilisins: primary structure, chemical and physical properties. In Boyer, PD. (ed), *The enzymes*, 3rd Edn, vol 3, Academic Press, New York, pp. 586-92.

Outtrup H. Boyce COL. (1990) Microbial proteinases and biotechnology. In: Fogarty, WM. And Kelly, CT. (eds), *Microbial enzymes and biotechnology*. pp. 227-54.

Priest FG. (1977) Extracellular enzyme synthesis in the genus, *Bacillus*. *Bacteriol. Rev*. 41: 711-53.

Razak NA, Samad MYA, Basri M, Yunus WMZW, Ampon K, Salleh AB. (1994) Thermostable extracellular protease of *Bacillus stearothermophilus*: factors affecting its production. *World J. Microbiol. Biotechnol*. 10: 260-3.

Shimogaki H, Takeuchi K, Nishino T, Ohdera M, Kudo T, Ohba K, Iwama M. Irie M. (1991) Purification and properties of a novel surface-active agent and alkaline-resistant protease from *Bacillus* sp. Y. *Agric. Biol. Chem.* 55(9): 2251-8.

Yamagata Y, Isshiki K, Ichishima E. (1995) Subtilisin from alkalophilic Bacllus sp.:Molecular and enzymatic properties of the enzyme and molecular cloning and characterization of the gene, aprs. *Enzyme Microb. Technol.* 17: 653-63.