African Journal of Biotechnology Vol. 10(7), pp. 1160-1164, 14 February, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.164
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Isolation of protease producing novel *Bacillus cereus* and detection of optimal conditions

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Accepted 29 July, 2010

Fifteen bacterium strains were isolated from soil samples and the one that had the highest proteolytic activity, from Susanoğlu village at Silifke-Mersin, was selected. The strain was identified and determined as *Bacillus cereus*. The bacterium, that had R type colony morphology, subterminal spores, motile and facultative anaerobe microorganism, was a gram positive with 10 μ length and 1 μ width. It could ferment glucose, fructose and maltose which formed acid but not gas. Catalase and urease were positive. The bacterium could grow on common medium easily and form β-haemolyze on blood agar. It was determined that the microorganism's growth continued up to the 12th hour and when it gets to the 18th hour, it was in a stationary phase. As a result, after the 18th hour, it was in a lytic phase. The optimum growth temperature range was 20 - 40°C and the optimum pH range was 6.0 – 10.0, respectively. Maximum growth was obtained at 30°C and at pH 7.0. The highest protease activity was determined at 30°C temperature and 6.4 pH conditions and after the 18th hour, it decreased evidently.

Key words: Protease, production, optimization, *Bacillus* sp.

INTRODUCTION

Enzymes have been produced in large industrial scale for several decades (Falch, 1991). Microbial enzyme production on an industrial scale was initiated by Takamine who, in 1890, settled in the U.S.A and started the production of Takadiastase enzyme preparation which was mainly an α-amylase preparation, but it contained a substantial amount of protease (Aunstrup, 1980). Since the initiation of microbial enzyme production, many protease preparations took part in the market. Although, variant microorganisms were used to produce protease, the genus *Bacillus* have being so far the most important group of enzymes produced commercially (Ferrero et al., 1996).

Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors (Gupta et al., 2002). The most important applications of protease are used in laundry detergents. However, proteases are also use in leather processing, brewing, food and pharmaceutical industries (Abdel-Naby et al., 1997).

This work was undertaken to obtain a new obligate microorganism which had a well known protease production. Fifteen bacterium strains were isolated from soil samples that were collected at different regions of Turkey. One, of which had the highest proteolytic activity, was selected and the identification of the new obligate bacterium was investigated. Temperature and pH conditions were studied to optimize protease production. The results were compared with other researches.

MATERIALS AND METHODS

Isolation and identification

Soil samples were collected from different regions of Turkey and 15 bacterium strains were isolated. Strains were assayed for proteolytic activity and a bacterium strain which had the most proteolytic activity was selected.

The selected strain was identified using biochemical and morphological tests. Bergey's manual of systematic bacteriology was used as reference (Sneath et al., 1986). Gram strain, endospore forming, motility test, catalase test, glucose fermentation and nitrat reduction characteristics were detected. The results showed that

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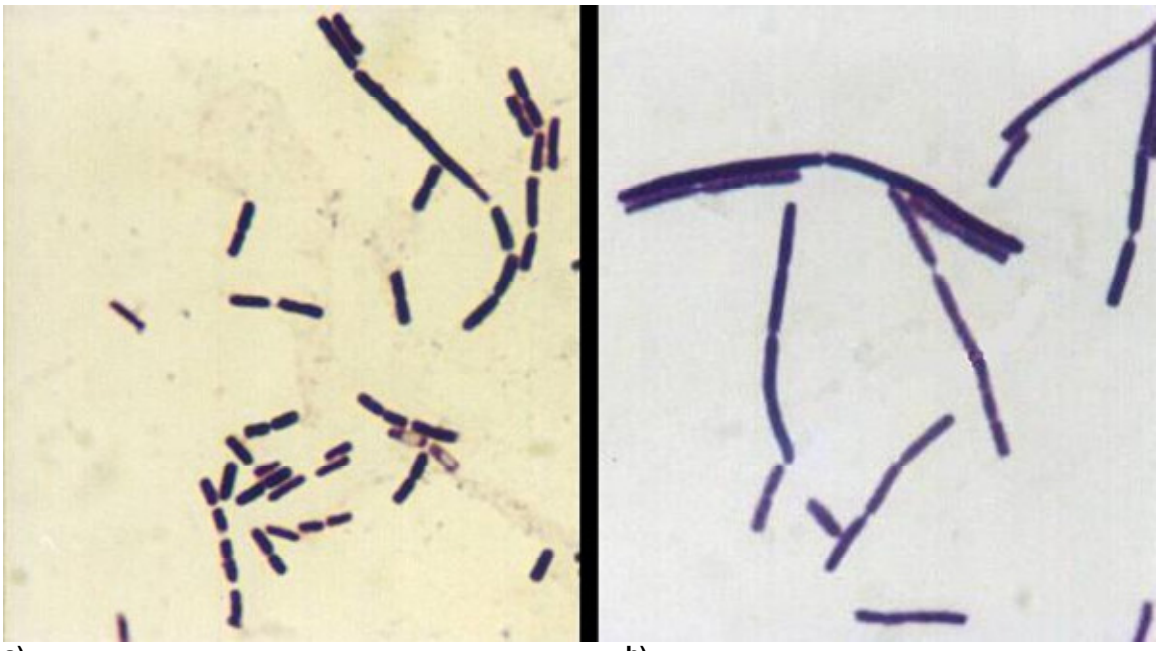

a) b)

Figure 1. Photographs showing typical cell morphologies of new *Bacillus* sp. isolate. a: After the 24th hour and b: 12th hour of incubation.

the bacterium was a *Bacillus* strain. The novel *Bacillus* strain was then identified as *Bacillus cereus* by 16S rDNA phylogenetic analysis.

Optimization

Nutrient broth (5 g peptone and 3 g meat extract, pH 7.0, Merck) was used as the common growth and stock media. Growth media was prepared at 250 ml erlenmeyer flasks containing 100 ml nutrient broth and stock media at 50 ml erlenmeyer flasks containing 25 ml nutrient broth. They were sterilized at 121°C for 15 minutes. 1 ml of stock medium was inoculated at the growth medium and incubated at 30°C with 150 rpm. Growth and proteolytic activity was measured at an interval of 2 h from the initiation of incubation. The growth and proteolytic activity curves were obtained.

Temperature and pH

Temperature and pH characteristics were determined respectively, while 10 to 50°C of incubation temperature and 3.0 to 11.0 pH ranges of culture media were measured. The maximum and optimum values were determined.

Protease assay

Proteolytic activity was carried out according to Casein-Pholine method (Boethling, 1975). Culture media was santrifugated at 7200 rpm for 10 min and supernatant was used as enzyme source. However, 1% casein (in 0.1 M phosphate buffer and pH 7.0) was used as substrate. 1 ml each of enzyme and substrate was incubated at 50°C for 60 min. The reaction was terminated by adding 3 ml of trichloroacetic acid (TCA). One unit of protease activity was defined as the increase of 0.1 unit optic density at 1 h incubation period. Total protein amount of supernatant was measured by Lowry et al. (1951) method.

Optimization of protease production

Enzyme localization was determined by sonication at 90 kc for 20 min. Temperature and pH effect of the culture medium on production of protease was detected. Optimum enzyme period, pH and temperature range was determined.

RESULTS AND DISCUSSION

Fifteen bacterium strains were isolated from collected soil samples. Their protease activity was assayed and one, of which had the most proteolytic activity, was selected. The isolate was motile, facultative anaerobic, subterminal sporulating, short chains forming, gram-positive and rod-shaped bacteria. The cells of isolates were 10 μ in length and 1 μ in width. It was determined as *Bacillus* sp. According to the biochemical and morphological analysis (Figure 1). 16S rDNA phylogenetic assay was performed to determine the species of the novel isolate and according to the assay result, it was determined as *B. cereus*. Fermentation tests with different carbon sources showed that the isolate could ferment glucose, fructose and maltose forming acid but not gas. The bacterium also had catalase and α-amilase activities, which could reduce nitrate through nitrite.

It was determined that the microorganism's growth continued up to the 12th hour and when it got to the 18th hour, it was in a stationary phase. Subsequently, after the 18th hour, the lytic phase begun (Figure 2). Also, subterminal endospore forming begun at the 12th hour and most of the bacteria were sporulated at the 24th

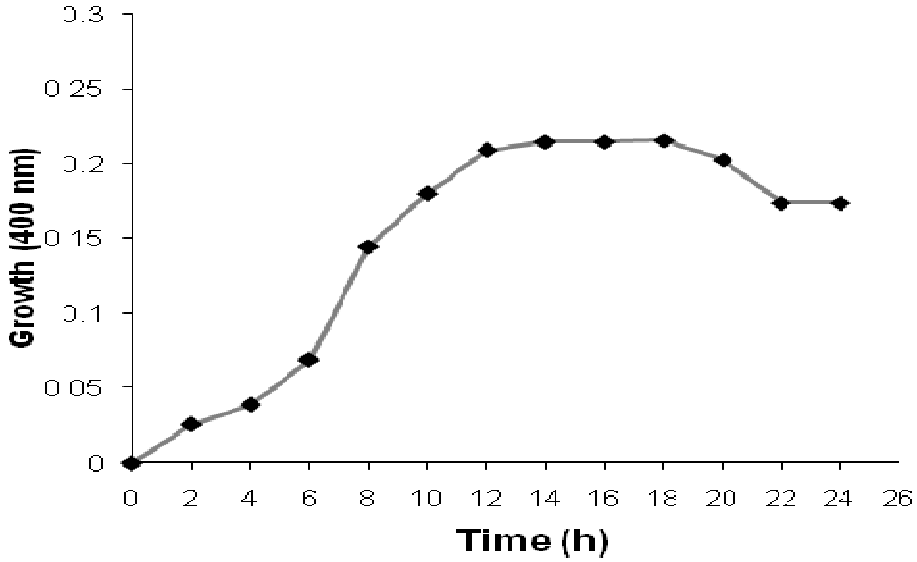

Figure 2. Growth curve of *Bacillus* sp.

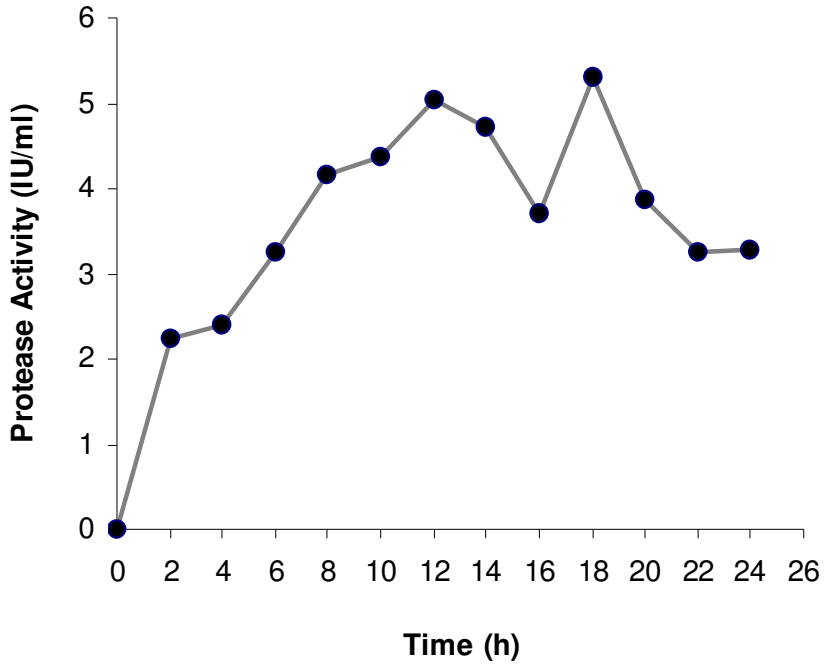

Figure 3. Protease activity of *Bacillus* sp. at 2 h intervals.

hour. During the 24th hour growth, production of protease was assayed at 2 h intervals. Maximum growth was determined at stationary phase and at the 18th hour (Figure 3). The growth and protease rates of the isolate were determined within the temperature and pH range of 20- 50°C and 3.0 - 11.0, respectively. The highest growth and protease rate was detected at 30°C and pH 7.0 and of growth medium, respectively (Figures 4, 5 and 6). Enzyme localization was detected as extracellular. Before optimization, the new obligate *Bacillus* sp. isolate could produce 5.5 U/ml protease at 30°C and 7.0 pH, after it had produce 9.56 U/ml protease at 30°C and pH 6.4. The results showed that optimization was succesful at about 173.8%.

Bacillus sp. is a spore forming bacterium, thus during sporulation and also germination, it increases protease activity (Cihangir and Aksöz, 1988). Scientists acclaimed that during sporulation and germination, hydrolized

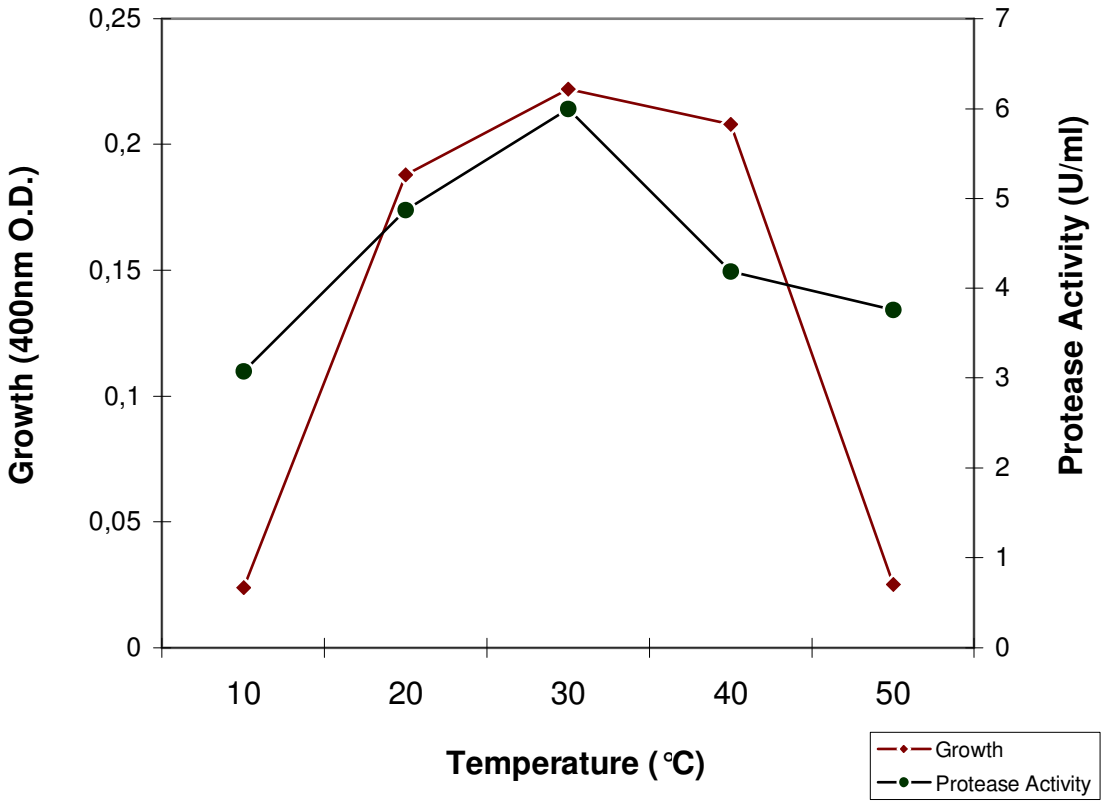

Figure 4. Effect of temperature on growth and protease production.

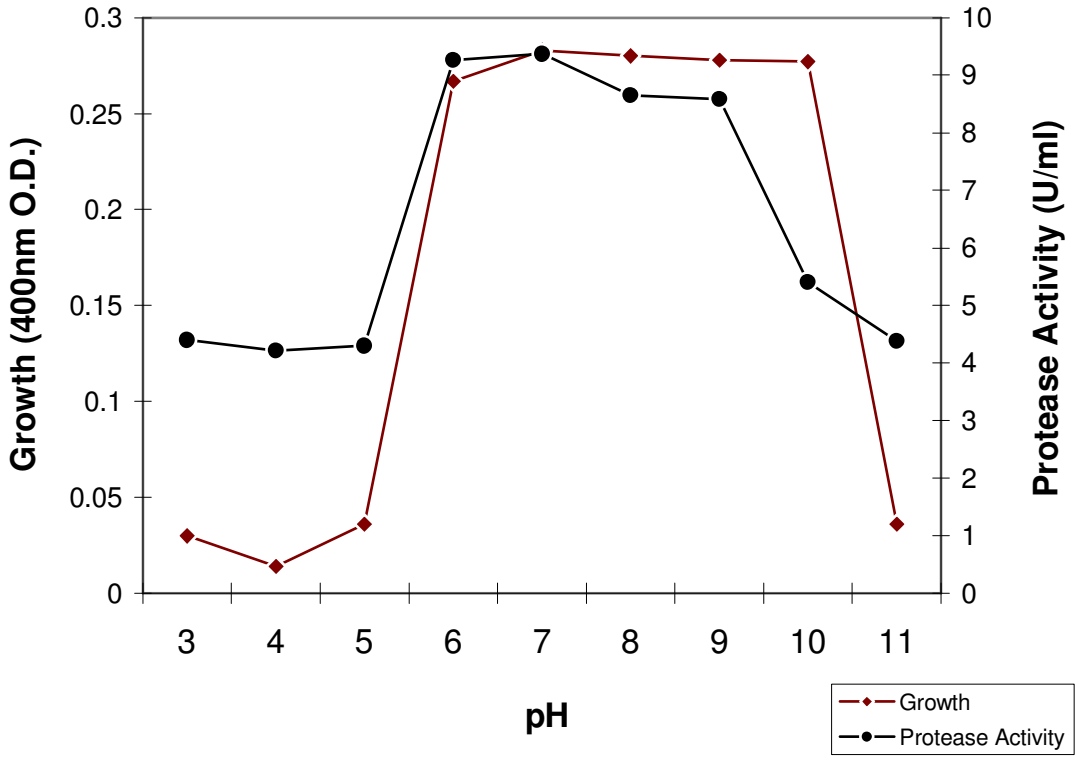

Figure 5. Effect of pH (3.0 - 11.0) on growth and protease production.

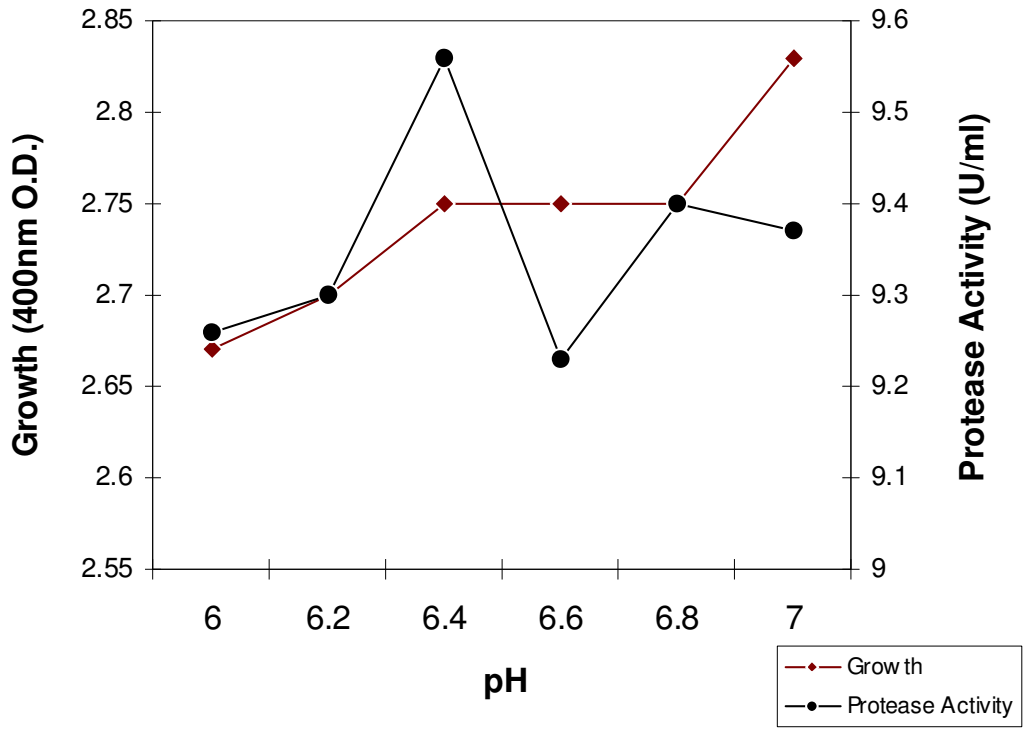

Figure 6. Effect of pH (6.0 - 7.0) on growth and protease production.

proteins were used to compose proteins for endospores or vegetative cells (Prestidge et al., 1971; James and Mandelstam, 1985). This process requires an increase of protease production. In this study, maximum protease activity was determined at the 18th hour, which occured in the late stationary phase, when most of the bacteria sporulated. Scientists prefered studying new isolates because they could be alternative for commercial use (Mehrotra et al., 1999; Dube et al., 2001; Hawumba et al., 2002). Particularly, thermostable alkaline proteases were used in laundry detergents and from a new obligate str-ain, they should be used commercially at detergents (Ferrero et al., 1996). Consequently, this study showed that *Bacillus* sp. protease could be used commercially because growth conditions are simple. As a result, it could protect its significance up to 60°C and have a well known activity at neutral and alkaline pH.

REFERENCES

Abdel-Naby MA, Ismail A-MS, Ahmed SA, Abdel FAF (1998). Production and immobilization of alkaline protease from *Bacillus mycoides*, Bioresour. Technol. 64: 205-210.

Aunstrup K (1980). Proteinases, Microbial Enzymes and Bioconversions, Edited by: Rose AH, Academic Press, pp. 49-114.

Boethling RS (1975). Regulation of protease secretion in *P. maltophila*, J. Bacteriol. 123: 954-961.

Cihangir N, Ve Aksöz N (1988). *Bacillus sp*. proteazının sentezi ve etkili bazı kültürel parametrelerinin saptanması, Kükem Dergisi, 11: 27-34.

Dube S, Singh L, Alam SI (2001). Proteolytic anaerobic bacteria from lake sediments of Antarctica, Enzyme Microb. Technol. 28: 114-121.

Falch EA (1991). Industrial enzymes-developments in production and application, Biotechnol. Adv. 9: 643-658.

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

Gupta R, Beg QK, Lorenz P (2002). Bacterial alkaline proteases: molecular approaches and industrial applications, Appl. Microbiol. Biotechnol. 59: 15-32.

Hawumba JF, Theron J, Brözel VS (2002). Thermophilic protease-producing *Geobacillus* from Buranga Hot Springs in West Uganda, Curr. Microbiol. 45: 144-150.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent, J. Biol. Chem. 193: 265-275.

Mehrotra S, Pandey PK, Gaur R, Darmwal NS (1999). The production of alkaline protease by a *Bacillus* species isolate, Bioresour. Technol. 67: 201-203

Prestidge L, Gage V, Spizizen J (1971). Protease activities during the cource of sporulation in *Bacillus subtilis*, J. Bact. 107: 185-213

Sneath PHA, Mair MS, Sharpe ME, Holt JG (1986). Bergey's Manual of Systematic Bacteriology, Williams & Wilkins, 2: 1104-1139.