African Journal of Biotechnology Vol. 10(19), pp. 3894-3901, 9 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.774
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Increasing the alkaline protease activity of *Bacillus cereus* and *Bacillus polymyxa* simultaneously with the start of sporulation phase as a defense mechanism

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Accepted 1 April, 2011

In this research, the growth curve, curve of sporulation phase and alkaline protease activity of *Bacillus cereus* and *Bacillus polymyxa* were compared and examined simultaneously. These examinations showed that, the production of alkaline protease started with the beginning of the log phase in the growth curve simultaneously and after a small stop according to the stationary phase increased and then with the start of the death phase, increased rapidly again. The maximum alkaline protease activities of *B. polymyxa* and *B. cereus* were measured at the death phase of the growth curve and at the start of the sporulation phase of the two microorganisms. The mentioned experiments were made in glucose synthetic medium at 30°C with 100 rpm aeration speed for 144 h. The maximum alkaline protease activity of *B. cereus* and *B. polymyxa* occurred after 72 h simultaneously with the start of the sporulation phase of the two bacteria and were measured at 383 and 418 u/ml, respectively. The next experiments showed that, the enzyme was very stable in extreme conditions such as freezing, drying and lyophilization. Also, it protected 98 to 100% of its primary activity after 4 months in the mentioned conditions.

Key words: Sporulation phase, alkaline protease, *Bacillus cereus*, *Bacillus polymyxa*, extreme conditions.

INTRODUCTION

Bacterial spores are amongst the most resistant of all microbial forms to inactivation by chemical or physical agents. These resistant forms are created in extreme conditions such as famine, water deficiency and dryness, presence of harmful agents for instance chemicals, anti-biotics, radioactive and ultraviolet rays (Holt et al., 1994; Knaysi, 1951; Ram et al., 1994; Sneath et al., 1986). Among all the spore forming bacteria, the genus *Bacillus* is very outstanding and considerable due to its various physiological abilities and production of valuable biological products. The existence of resistant endospores in the *Bacillus* sp. has resulted in extensive distribution of these organisms through the nature and various ecosystems as well as isolation, passage, preservation and ultimately production of merit biological products such as hydrolyses (Beheshti Maal et al., 2009; Emtiazi et al., 2005; Holt et al., 1994; Sneath et al., 1986). *Bacillus* sp. are gram positive endospore forming straight rods that form individual endospore in each cell against extreme conditions. In these bacteria, the presence of oxygen could not prevent the sporulation phase. One of the significant biological substances that are produced by the genus *Bacillus* are alkaline proteases with several important applications in routine life affairs like detergent industries, alcohol and beer production industries, wastewater treatment, leathering, food industries, bio-transformation, debittering of hydrolyzed proteins, oil manufacturing, medical and pharmaceutical industries and cosmetic/sanitary industries (Beheshti Maal et al. 2009; Emtiazi et al., 2005; Aaslyng et al., 1990; Bierbaum

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Abbreviations: SIM, SH2-Indole-motility; **MR,** methyl red; **VP,** voges-proskauer; **TSB,** tripticas soy broth.

et al., 1994; Angelova et al., 1995; Atalo and Gashe, 1993; Geweley, 1996; Godfrey and West, 1996; Heitmann and Meyer, 1981). The industry of enzyme production in the world is improving; large amounts of these enzymes are produced in different countries and in the world and marketing of a primary sale have been accounted for it (Emtiazi et al., 2005; Aaslyng et al., 1990; Dosoretz et al, 1990; Heitmann and Meyer, 1981). It is known that for bacteria, the genera *Clostridium, Bacillus* and *Pseudomonas* and for fungi the genera *Aspergillus, Mucor* and *Rhizopus* are the best protease producers. In bacteria, the *Bacillus* sp. has shown various physiological capabilities which are one of the best alkaline protease producers (Beheshti Maal et al., 2009; Holt et al., 1994; Ram et al., 1994). The *Bacillus* sp. are detected and isolated using 80°C water bath that destroys all the vegetative bacteria and resistant forms except Bacilli and Clostridia endospores (Holt et al., 1994; Sneath et al., 1986; Vela, 1974). An alkaline protease has been produced by *Bacillus cereus* MCM B-326 using a medium containing soya flour, starch and wheat bran as the main com-ponents. The produced enzyme has been applied for the dehairing of buffalo hide (Zambareh et al., 2007). Several agro-industrial waste substances have been used to produce alkaline protease from an alkalophilic *Bacillus* sp. among which the green gram husk resulted in the highest production of the enzyme using solid-state fermentation (Prakasham et al., 2005). The increasing importance of these enzymes and their numerous appli-cations in different industries (Matoba et al., 1997; Moon and Parulekar, 1993) made us investigate and evaluate the alkaline protease production by *Bacillus* sp. and find any acceptable relation between sporulation phase in these bacteria and the production of alkaline protease. In this research, two native strains of *Bacillus cereus* and *Bacillus polymyxa* were isolated from the soils of Isfahan, Iran on the basis of their good production of alkaline protease. Then the growth curve, sporulation phase and the alkaline protease activity of the isolated strains were compared and examined simultaneously. Finally, the stability of alkaline protease from the mentioned bacteria against extreme conditions such as freezing, drying and lyophilization processes was studied.

MATERIALS AND METHODS

Chemicals, microbiological culture media and instruments

The basic chemicals and culture mediums that were used included, NaOH, arabinose, xylose, manitol, glucose, urea agar, Simon citrate agar, nitrate broth, starch, gelatin, SIM, MR-VP, egg yolk agar, TSB, glucose synthetic medium [Glucose (6 g/l), $(NH_4)_2SO_4$ (10 g/l), Na_2HPO_4 (8 g/l), KH_2PO_4 (4 g/l), $MgSO_4$ $7H_2O$ (0.5 g/l) and $CaCl_2$ (0.02 g/l)], L-tyrosine, Caseine powder for enzyme assay (Merck 2241), trichloro acetic acid, Na_2CO_3, and Folin-ciocalteus phenol (all from Merck). The main instruments that were used were laminar air flow (Jahl 2100, Iran), high speed refrigerated centrifuge (Hitachi, 20PR, Japan), shaker refrigerator incubator (Jahl, JSH 20L, Iran), water bath (Dena 8000, Iran), autoclave (Iran Tolid, Iran), spectrophotometer (Milton Roy, Spectronic 1001, USA) and lyophilizer (HETO Birkkerod, HETOSICC, Denmark).

Screening and isolation of *Bacillus* sp.

For the isolation of *Bacillus* sp., several samples of soils from Isfahan, Iran were gathered in sterile containers, were dried and the fined. Serial dilution was made using 1 g of each soil sample and five tubes containing 9 ml of sterile water. The 5th dilution tubes were placed in an 80°C water bath for 10 min. Heat shock was then stopped using cool water. 1 ml of each sample was cultivated on nutrient agar media using surface culture method and incubated at 30°C for 24 h (Henriette et al., 1993; Shah et al., 1986). Gram reaction and endospore formation were confirmed using Gram and endospore staining, respectively.

Evaluation of alkaline protease production by bacteria

Protease producing *Bacilli* were isolated on casein agar with alkaline pH, 9, 10 and 11 and the pH of the culture media was optimized with NaOH (40 g/l). Screening of the best producers of alkaline protease was done by measuring of the largest transparent zone around the colonies. The evaluation of the alkaline protease activity was confirmed using alkaline skim milk agar culture media with pH 9, 10 and 11 and two colonies with the largest transparent zone around were purified on alkaline skim milk agar using streak plate method (Beheshti Maal et al., 2009; Emtiazi et al., 2005).

Identification and characterization of the best alkaline producers

Biochemical characterization and identification of the two isolated *Bacilli* with the largest transparent zone were carried out, following the procedures described by Bergey's manual of systematic bacteriology (Holt et al., 1994; Sneath et al., 1986). The selected biochemical tests included lecithinase, nitrate reduction, gelatin hydrolysis, starch hydrolysis, SIM, VP, citrate degradation, urea hydrolysis and fermentation of glucose, arabinose, xylose and manitol.

Assay of alkaline protease

The method which was used for the alkaline protease assay in all the experiments was based on a colorimetric technique for the assay of proteins and specifically for L-tyrosine and Lowry method (Atalo and Gashe, 1993) as follow: 5 to 10 ml of microbial broth medium were isolated and centrifuged for 20 min at 2500 rpm. Then, 1 ml of the supernatant including alkaline protease was mixed with 1 ml of alkaline casein substrate with pH 11 and was incubated at 40°C water bath for 10 min. The solution was mixed with 2 ml trichloro acetic acid (0.4 M), after blocking the enzyme activity and precipitating the intact casein and it was centrifuged in a high speed Hitachi refrigerated centrifuge at 12000 rpm for 10 min at 4°C. Then, 1 ml of supernatant including L-tyrosine was mixed with 5 ml Na_2CO_3 (0.4 M) and 1 ml of Folin-ciocalteus phenol (0.1 M) reagent and was incubated at 40°C water bath for 20 min in a dark condition. Ultimately, the OD of the mixed solution was measured at 660 nm wavelength using Milton Roy spectro-photometer. According to this method, one unit of alkaline protease activity is defined as the amounts of enzyme which can release 1 µg L-tyrosine from alkaline casein substrate (pH = 11) under the assay condition (40°C for 10´) and is expressed in u/ml. For all the experiments, we used a standard curve (L-tyrosine/OD) for converting the ODs to the enzyme activity (Atalo and Gashe, 1993; Lee and Chang, 1990; Mao et al., 1992).

Table 1. The characterizations of isolated *Bacillus* strains from Isfahan soil with high alkaline protease activity.

Main examination		*Bacillus cereus*	*Bacillus Polymyxa*
Macroscopic characterizations	Colony characteristics on nutrient agar	Mat, obscure, wavy surface, gray to light brown	Mucoid, shiny, irregular Margines, gray
	Gram reaction	+	+
Microscopic characterizations	Morphology	Long, thick rods	Mediate, thin rods
	Arrangement	Streptobacillus	Mono, diplobacillus
	Endospore type	Oval – central	Oval – terminal
Biochemical characterizations	LV test	+	-
	VP test	+	+
	Starch hydrolysis	+	+
	Nitrate reduction	+	+
	Gelatin hydrolysis	+	+
	Citrate degradation	+	-
	Urea hydrolysis	+	-
	Indole formation	-	-
	Acid from glucose	+	+
	Acid from Arabinose	-	+
	Acid from xylose	-	+
	Acid from manitol	-	+

Growth curve and sporulation phase measurements

In this experiment, the glucose medium was used as a basic medium and TSB was used as a preculture medium. For measuring the growth curve of these *B. cereus* and *B. polymyxa,* two 500 ml glucose medium were autoclaved and inoculated with 30 ml of 24 h *B. cereus* and *B. polymyxa* cultures in TSBs. Immediately after inoculation, the OD of the two glucose cultures were measured at 520 nm (T0). Then, the flasks were incubated in a shaker incubator at 30°C with 100 rpm aeration speed for 6 days. The ODs were measured at 520 nm every 1 to 24 h and then every 24 h to 6 days. Also, the enzyme activity was measured every 1 h using Lowry method in both culture media.

Stability of alkaline protease against cold and freezing conditions

For evaluating the stability of alkaline protease from *B. polymyxa* against cold and freezing conditions, the glucose medium was used as a basic medium and TSB was used as a preculture medium. The glucose media were inoculated with the bacteria and were incubated at 30°C with 100 rpm aeration speed for 72 h. 10 µl of the culture media were collected aseptically and centrifuged at 2500 rpm for 20 min. 6 µl of the cell free supernatant were distributed in six sterile tubes each. The six tubes were incubated in a refrigerator at 4°C for 1, 2, 4, 8, 12 and 16 weeks. After completion of each period, one tube was taken out from the refrigerator and enzyme activity was measured using Lowry method. The six tubes were incubated in freezer at -20°C for 1, 2, 4, 8, 12 and 16 weeks. After completion of each period, one tube was taken out from the freezer and enzyme activity was measured using Lowry method.

Stability of alkaline protease against drying and lyophilization conditions

For evaluating the stability of alkaline protease from *B. polymyxa* against drying and lyophilization conditions, the *B. polymyxa* was cultured and broth medium centrifuged as described earlier. 6 µl of cell free supernatant was distributed in 6 separate sterile tubes and was lyophilized for 72 h. After the complete dryness, the powder of alkaline protease was incubated at 50°C and dry atmosphere for 1, 2, 4, 8, 12 and 16 weeks. After completion of each period, 1 ml of sterile water was added to the dried enzyme and enzyme activity was measured in the provided suspension by Lowry method. All the aforementioned examinations in this research were repeated for 3 times and the averages were shown as the results.

RESULTS

The results of the macroscopic, microscopic and biochemical examinations showed that, the two isolated strains from the soils of Isfahan, Iran that had made the greatest transparent zone around their colonies in alkaline skim milk agar with pH 9, 10 and 11 were *B. cereus* and *B. polymyxa*. The examinations that were made for identification of these species are shown in Table 1. The activities of the alkaline protease from *B. polymyxa* in glucose synthetic medium at 30°C and 100 rpm aeration speed after 24, 48, 72, 96, 120 and 144 h incubation were measured as 235.5, 285.5, 418, 350.5, 333 and 286

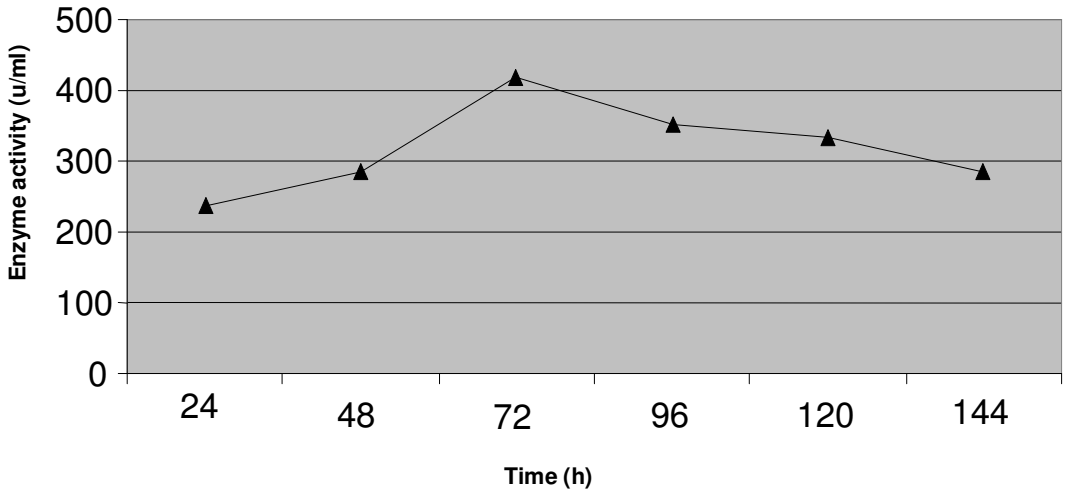

Figure 1. Alkaline protease activity of *B. polymyxa* at 30°C with 100 rpm aeration speed for 144 h in glucose synthetic medium.

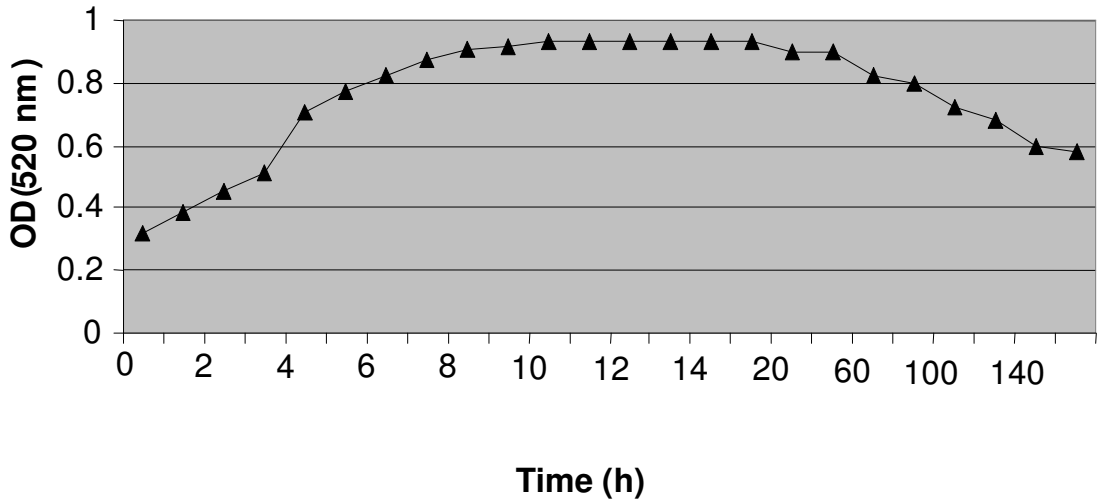

Figure 2. The growth curve of *B. polymyxa* using glucose synthetic medium at 30°C at 100 rpm aeration speed for 144 h.

(u/ml), respectively. Figure 1 shows that, the maximum activity of alkaline protease from *B. polymyxa* occurred after 72 h with the activity of 418 u/ml. Figure 2 shows the growth curve of *B. polymyxa* using glucose synthetic medium at 30°C for 6 days. Also, the activities of the alkaline protease from *B. cereus* in glucose synthetic medium after 24, 48, 72, 96, 120 and 144 h incubation were measured as 235.5, 265.5, 383, 338, 330.5 and 263 u/ml, respectively. Figure 3 shows that, the maximum activity of alkaline protease from *B. cereus* occurred after 72 h with the activity of 383 u/ml. Figure 4 shows the growth curve of *B. cereus* using glucose synthetic medium for 144 h. Comparison of Figures 1 and 2 made clear that, the maximum alkaline protease activity in *B. polymyxa* occurred after 72 h, simultaneously, with the start of the stationary and death phases that were overlapped with the start of the sporulation phase of this bacterium. Similarly, comparison of Figures 3 and 4 clarified that, the maximum alkaline protease activity in *B. cereus* occurred after 72 h, simultaneously, with the start of the stationary phase and death phase that were overlapped with the start of the sporulation phase of *B. cereus*. The next experiments showed that, the alkaline protease from *B. polymyxa* was very stable in extreme conditions such as freezing, drying and lyophilization after about 3 to 4 months and protected 98 to 100% of its primary activity in the mentioned conditions. For example, the enzyme activities after 1, 2, 4, 8, 12 and 16 weeks of incubation at 4°C were measured as 358, 360.5, 355.5, 357.5, 355.5 and 355.5 u/ml. Figure 5 shows a

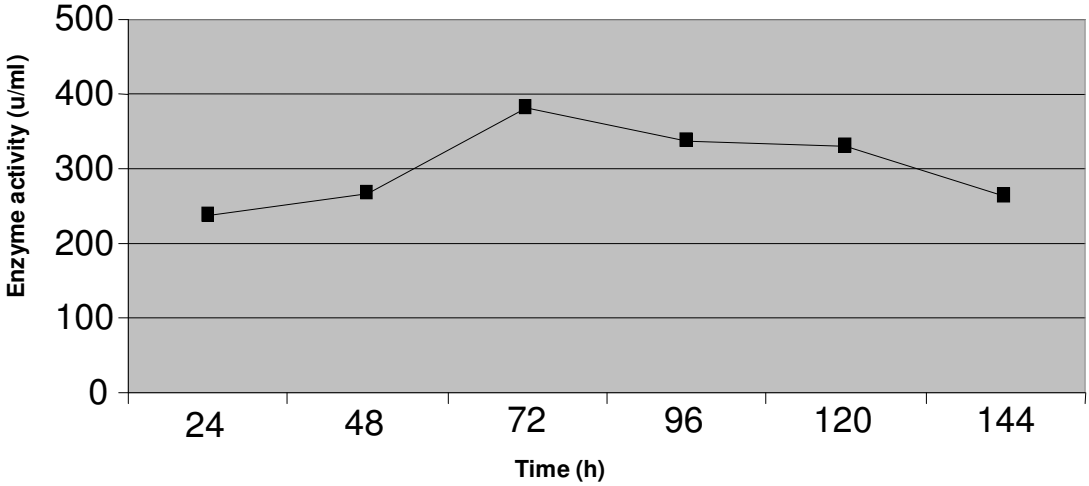

Figure 3. Alkaline protease activity in *B. cereus* at 30°C with 100 rpm aeration speed during 144 h.

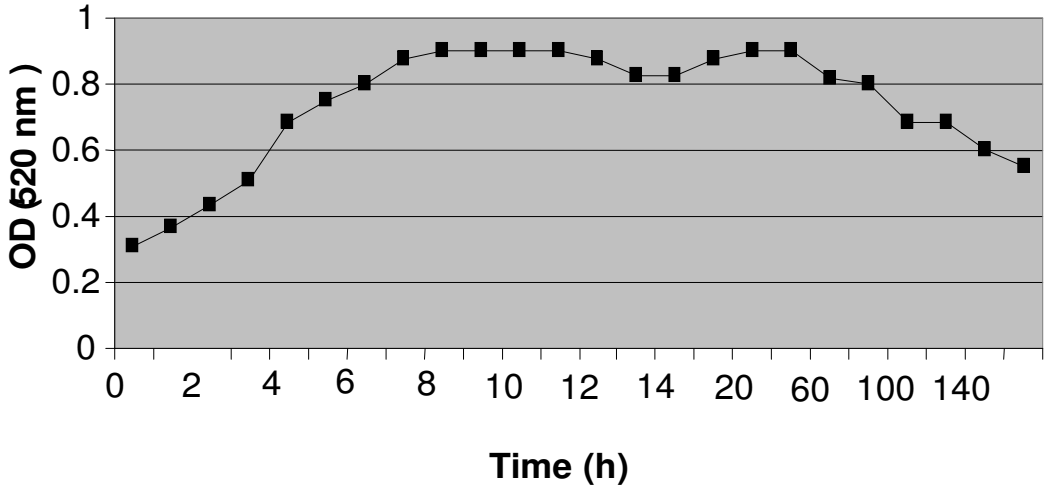

Figure 4. The growth curve of *B. cereus* using glucose synthetic medium at 30°C with 100 rpm aeration speed for 144 h.

comparison of the enzyme activities in the control sample and enzyme solutions after incubation at 4°C in the different periods. Also, the enzyme activities after 1, 2, 4, 8, 12 and 16 weeks of incubation at -20°C were measured as 356, 353, 354.5, 358, 355.5 and 357.5 u/ml. Figure 6 shows the stability of the enzyme in -20°C after one week to four months. Figure 7 shows that, the alkaline protease from *B. polymyxa* after 1, 2, 4, 8, 12 and 16 weeks of incubation at very dry conditions kept 98 to 100% of its primary activity. The enzyme activities were measured as 358, 357, 358 357.5, 357.5 and 355.5 u/ml, respectively.

DISCUSSION

Afify et al. (2009) studied the production of alkaline protease by an Egyptian *Bacillus sphaericus* isolate and reported that, *Bacillus sphaericus* produced a binary toxin as an inactive protoxin during its sporulation phase that was converted to active toxin by protease. Fedhila et al. (2003) and Chen et al. (2004) reported that, some proteases, and peptidohydrolyses group EC 3.4.4.4.1 to 3.4.4.20, from *Bacillus thuringiensis* and *B. sphaericus* were produced during the sporulation phase and had a considerable application in the physiology and regulation of sporulation processing in these two species. Rocha et al. (2007) reported that, the production of an extracellular protease by thermophilic *Bacillus* sp. strain SMIA-2 has reached a maximum level after 14 h using a liquid culture medium. Ram et al. (1994) suggested that, the best incubation temperature for alkaline protease production by *Bacillus coagulans* as a psychrotophic microorganism was 37°C. Beheshti Maal et al. (2009) and Emtiazi et al.

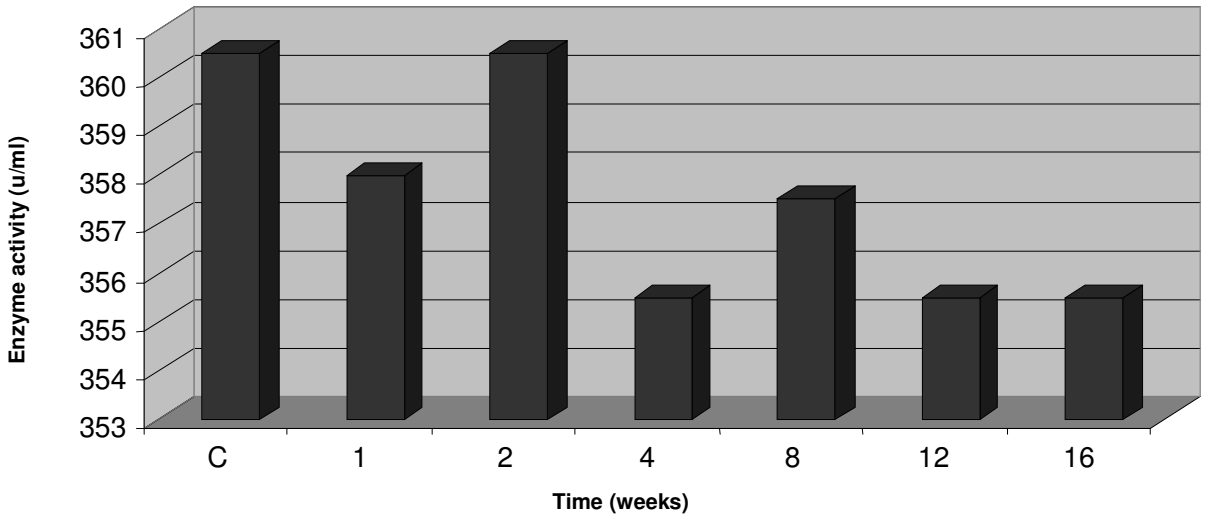

Figure 5. The stability of alkaline protease from *B. polymyxa* at 4°C in different durations.

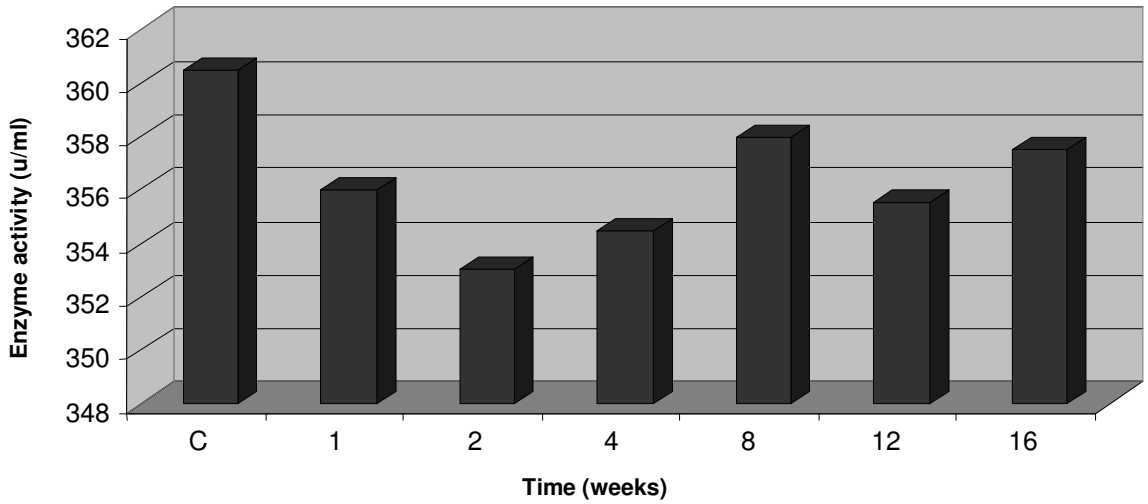

Figure 6. The stability of alkaline protease from *B. polymyxa* at -20°C in different durations.

(2005) reported that, the best temperature for alkaline protease production by *B. cereus* and *B. polymyxa* was 60°C. We showed that the maximum level for alkaline protease production and activity by *B. cereus* and *B. polymyxa* occurred after 72 h that was overlapped with the initiation of death phase and sporulation phase of the two microorganisms. While these two species were not theromophils, we suggested that the temperature of 60°C as an extreme condition for *B. cereus* and *B. polymyxa* resulted in faster start of the death phase in their growth curve and consequently, faster switch to sporulation phase initiation and ultimately, the more production of alkaline protease by them.

Production and release of alkaline protease by *B. coagulans* as a psychrophylic microorganism was related to the bacterial growth and the optimum temperature of the enzyme production was 20°C, but the ratio of alkaline protease amount to cell protein weight in 37°C was more than that for 20°C. As a matter of fact, the production of enzyme was increased with increasing bacterial growth and death of *B. coagulans* resulted in the reduction of the enzyme activity but the ratio of alkaline protease to cell total protein in the death phase was increased (Ram et al., 1994). Macfarlane and Macfarlane, (1992) and Atalo and Gashe (1993) reported that, the production of proteases by *Bacillus* sp. generally occurs during the end of logarithmic phase to stationary phase. We showed that, the production of alkaline proteases by *B. Polymyxa* and *B. cereus* started simultaneously with the logarithmic phase and reached the maximum pick in the death phase. Regarding the lack of protein in the glucose culture medium as a basic culture and the initiation of the

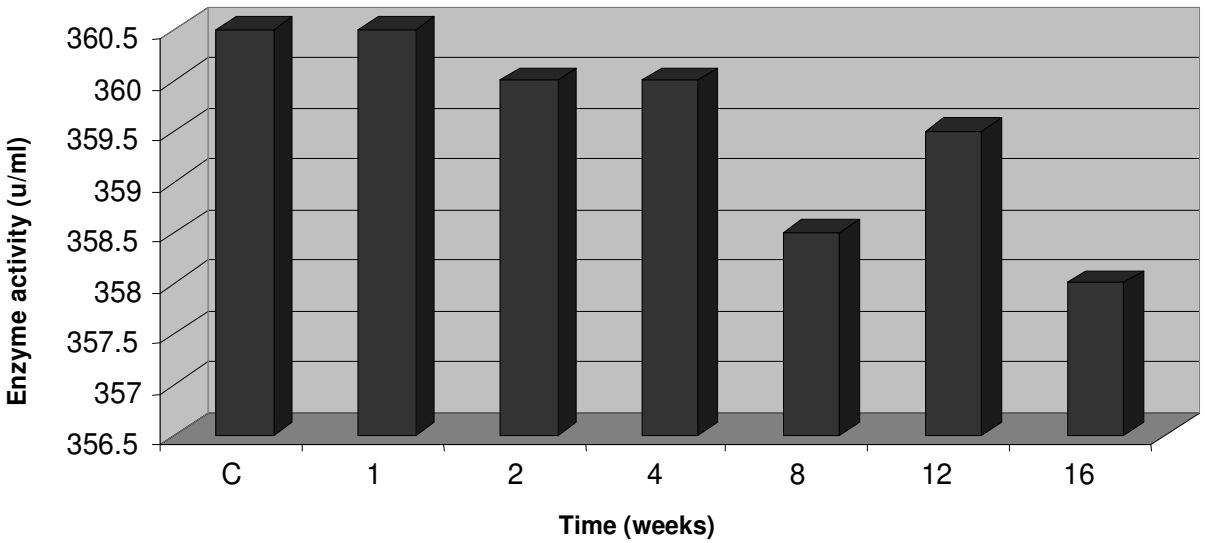

Figure 7. The stability of alkaline protease from *B. polymyxa* at dry condition in different durations.

enzyme leakage simultaneously, at the beginning of the log phase, we proposed that the production of this enzyme in the bacteria are not inducible and probably the operon of alkaline protease will turn on, once the bacterial growth occurs. On the other hand, the maximum release of the alkaline protease at the death phase and with the start of the sporulation phase probably suggested that, the elevated amounts of this enzyme in that time was considered as a defense mechanism in the creation of endospore for destroying the obtrusive and harmful proteins that probably affect the sporulation procedures. While the sporulation process in *Bacillus* sp. was so complex and more than 200 genes from several loci throughout the chromosome as well as many biochemical and physiological procedures were involved (Errington, 1993; Predich et al., 1992), we suggested that, the increment of the alkaline protease activity contemporarily with the sporulation phase could be best described in the protease, as necessary to provide building blocks of specific spore proteins, peptides and amino acids, from degrading protein residues of the mother cell. Kohlmann et al. (1991) studied the stability of the protease produced by the two psychrotrophic bacteria, *Pseudomonas fluorescence* and *Pseudomonas fraggi*, in milk and reported that, the enzyme was stable in 7°C. We showed that, the storage of the enzyme solution at 4 and -20°C had no effects on the alkaline protease activity from *B. polymyxa* and this enzyme preserved its activity for a long period of time (4 months). Also, it was shown that the alkaline protease from *B. polymyxa* preserved 98 to 100% of its primary activity in drought condition for a long time (4 months). It can be suggested that one of the proper approaches to decrease the volume of the enzyme solution without affecting the enzyme activity is lyophilization and formal drying.

In conclusion, this is the first report of the possible relationships between the production of alkaline protease by two native strains; *B. polymyxa* and *B. cereus*, isolated from the soils of Isfahan, Iran and its probable roles in the sporulation phase in the two endospore forming species. The resistance potentials of alkaline protease produced from *B. polymyxa* against high temperatures (50°C) and for a long time (4 months) could be an advantage for using this enzyme in the detergent industry and microbial biotechnology.

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