

## Optimization and Purification of Alkaline Proteases Produced by Marine *Bacillus* sp. MIG Newly Isolated from Eastern Harbour of Alexandria

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### Abstract

A marine *Bacillus* strain was isolated from the eastern harbour of Alexandria and identified as *Bacillus* sp. MIG. Maximum activity of studied proteases was obtained when the bacterium was grown in medium with 1% wheat bran and 0.5% yeast extract in addition to the mineral salts and incubated for 48 h at 30°C and 120 rpm. Two alkaline proteases (Pro 1 and Pro 2) were purified to homogeneity using cation exchange chromatography on CM-Sepharose CL-6B followed by Sephadex G-75 superfine. The optimum activities were at pH 11 or 12, and temperatures of 50 and 55°C for Pro 1 and Pro 2 respectively. These two enzymes were relatively stable over pH range from 7.0–11. Pro 2 was found to be more stable at 50°C in absence of Ca<sup>2+</sup> and retained about 47% of its activity after 3 h at this temperature, while Pro 1 lost its activity completely at the same conditions. The two enzymes were active against haemoglobin and casein; in addition, Pro 2 exhibited moderate activity against keratin. Both enzymes were partially inhibited by Ag<sup>+</sup> and Hg<sup>2+</sup>. PMSF completely inhibited the enzymes, while dithiothreitol and 2-mercaptoethanol stimulated their activities, suggesting to be thiol-dependent serine proteases. The enzymes were stable in the presence of the surfactants and bleaching agent (H<sub>2</sub>O<sub>2</sub>) and relatively stable in presence of some commercial detergents.

**Key words:** marine *Bacillus* sp. MIG; alkaline serine protease; thiol-dependent serine protease

### Introduction

Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives. This group of enzymes has other industrial applications such as in food, pharmaceutical, leather industry and recovery of silver from used X-ray films (Anisworth, 1994; Inhs *et al.*, 1999; Outtrup *et al.*, 1995). In 1994, the total market for industrial enzymes accounted for approximately 400 million \$, of which enzymes worth 112 million \$ used for detergent purposes (Hodgson, 1994). Alkaline protease added to laundry detergents plays a specific catalytic role in the hydrolysis of protein stains such as blood, milk, human sweat, *etc.* The increased usage of the protease as a detergent additive is mainly due to its cleaning capabilities in environmentally acceptable, nonphosphate detergents (Mei and Jiang, 2005).

Although microbes from terrestrial sources are employed for industrial production of enzymes, the potential for synthesis of several novel enzymes by marine microorganisms has been recognized (Chandrasekaran, 1997). Diverse techniques have been used for the screening of novel enzymes with new biocatalytic capabilities and great potential for several industrial processes and other applications (Manachini and Fortina, 1998). Large number of microorganisms produces proteases, but *Bacillus* strains are recognized as important sources of commercial alkaline proteases because of their ability to secrete large amounts of enzymes with high activity (Beg and Gupta, 2003; Joo *et al.* 2004).

Joo and Chang (2005) reported that around 30–40% of the production cost of the industrial enzymes accounted for the cost of the growth medium. Research efforts have been directed mainly towards evaluating the effect of various carbon and nitrogen nutrient cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation. In addition, no defined medium

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has been established for the optimum production of alkaline proteases from different microbial sources. Each organism has its own special conditions for maximum enzyme production (Kaur *et al.*, 2001; Kumar and Takagi, 1999; Razak *et al.*, 1994). In order to reduce medium costs, different low-cost substrates were screened and in course of this wheat bran, molasses, soybean meal and feather were used for cost-effective production of multiple forms of alkaline proteases by marine *Bacillus* sp. MIG. The present work also describes purification of two alkaline proteases from this strain to homogeneity. Some properties of the pure enzymes were also tested.

## Experimental

### Materials and Methods

**Microorganisms.** Thirty-five marine bacteria were isolated from the eastern harbour of Alexandria (Egypt). All isolates were cultured on skim milk agar plates. The isolates, which show clear zones on the plates, were cultivated in liquid production medium to test their ability to produce alkaline proteases.

**Enzyme production.** The production of alkaline protease from *Bacillus* sp. MIG was carried out in marine medium which have the following composition, (g l<sup>-1</sup>): peptone, 3; yeast extract, 1; casein, 10; ferric citrate, 0.1; NaCl, 19.45; MgCl<sub>2</sub>, 8.8; Na<sub>2</sub>SO<sub>3</sub>, 3.24; CaCl<sub>2</sub>, 1.8, the pH of the medium was adjusted to 7.5. The medium was inoculated with 1% of 24 h seed culture and incubated under shaken condition (120 rpm) and 30°C for different periods. Different carbon and nitrogen sources were used in the present work for optimization of nutritional factors. The physical parameters including (pH of the medium and temperature of incubation) were also tested. At the end of the incubation period, the cell-free enzyme supernatant was obtained by centrifugation at 10,000 g for 15 min at 6°C.

**Enzyme purification.** The cold cell-free supernatant obtained from the optimized medium after 48-h incubation was precipitated with two volumes of cold acetone and then collected by centrifugation. The precipitate was resuspended in a minimal volume of Tris-HCl buffer (0.02 M, pH 7.5) containing 5 mM CaCl<sub>2</sub>. After removing the insoluble materials by centrifugation for 15 min at 6°C and 10,000 g, the clear supernatant was applied to CM-Sepharose CL-6B ion exchange column (Pharmacia) per-equilibrated with the same buffer. The column was washed with the same buffer and eluted with stepwise gradient (0–0.5 M) of NaCl in the same buffer. Fractions of 5 ml were collected at a flow rate of 1 ml/min, and then the protein content and enzyme activity of each fraction were determined. The active fractions were collected and dialyzed against the same buffer without NaCl. The fractions, which are required further purification, were applied to Sephadex G-75 superfine gel filtration column. The column was equilibrated with the last mentioned buffer and eluted with the same buffer. Fractions of 2 ml were collected at a flow rate of 0.5 ml/min, and the protein content and enzyme activity were assayed. All active fractions were stored at –20°C for further study.

**Enzyme assay.** Protease activity was determined using casein as substrate. Casein was dissolved at 1% in glycine-NaOH buffer (0.1 M, pH 10). The assay mixture consisted of 650 µl buffer, 250 µl substrate and 100 µl of diluted enzyme and incubated at 37°C for 10 min. The reaction was terminated by the addition of 500 µl of trichloroacetic acid reagent (TCA), and then centrifuged in eppendorf centrifuge for 10 min to remove the undigested protein. Protease activity was determined as released tyrosine in the supernatant (Lowry *et al.*, 1951). One unit of the enzyme was defined as the amount of the enzyme resulting in the release of 1 µg of tyrosine ml<sup>-1</sup> min<sup>-1</sup> under the assay conditions. The protein content was also determined by Lowry *et al.* (1951) using bovine serum albumin as a standard.

**SDS-Polyacrylamide gel electrophoresis and detection of enzymatic activities in the gel.** The purity and relative molecular weights of the purified enzymes were estimated by SDS-PAGE using a 12% polyacrylamide gel according to the method of Laemmli (1970). The enzyme activities were tested in the gel following the method described by Heussen and Dowdle (1980).

**Substrate specificity.** Protease activities of purified enzymes against various protein substrates including BSA, gelatin, azocasein, azoalbumin, haemoglobin and keratin were assayed using 1% from each substrate dissolved in glycine-NaOH buffer, pH 11. The reaction was terminated with TCA and released products were measured at the suitable wavelength for each substrate. The activity toward casein was taken as control.

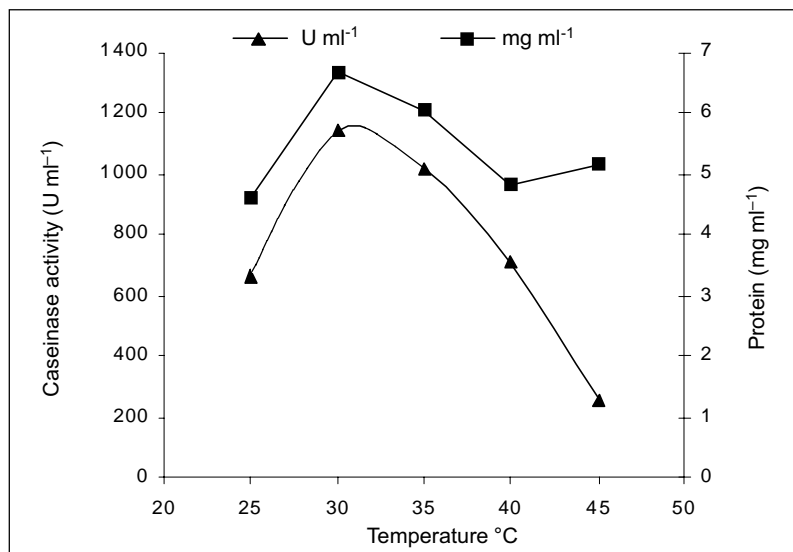
**Effect of metal ions, inhibitors, surfactants and detergents.** The effect of different metal ions, protease inhibitors, surfactants and commercial detergents on the activity of purified proteases was determined by incubating them with the enzymes for 30 min at 40°C and the residual activities were measured by the standard assay procedure.

## Results and Discussion

**Selection of the best alkaline protease-producing organism.** From the tested isolates, four have the ability to produce alkaline proteases. The best alkaline protease producer was identified using 16S rRNA methodology. Part of 16S rRNA gene was amplified, sequenced and deposited in the GeneBank database with accession number DQ076248 as *Bacillus* sp. MIG. Comparing the obtained sequence with the sequences available in the NCBI revealed 97% similarity with *Bacillus pumilus* strain: M1-9-1. This organism was selected for production and purification of alkaline proteases.

**Correlation between growth and protease production.** The protease production by the test organism started in the exponential growth phase and enzyme activity showed linearity with growth. The maximum activity (1070.86 Uml<sup>-1</sup>) was obtained in the stationary phase after 48 h and remains more or less stable

Fig. 1. Effect of incubation temperature on the production of proteolytic enzymes by *Bacillus* sp. MIG



until 72 h, and then decreased with increasing the incubation time (data not shown). A similar trend was reported for extracellular protease production by *Bacillus* sp. (Oberoi *et al.*, 2001), *Bacillus subtilis* PE-11 (Adinarayana *et al.*, 2003) and by *Bacillus clausii* (Kumar *et al.*, 2004). The cell density increased with time and reached its maximum after 24 h.

**Effect of temperature and pH on the enzyme production.** The effect of incubation temperature and pH of the production medium are critical factors and need to be optimized. The optimum temperature for enzyme production was found to be 30°C, increasing the temperature led to decrease the enzyme production (Fig. 1). Higher temperatures at 40 and 45°C caused more than 37 and 77% loss in the enzyme compared to 30°C. The protein content increased with the increase in enzyme activity except at 45°C, while the enzyme activity decreased, the protein content was increased. Ray *et al.* (1992) reported that temperature could regulate the synthesis and secretion of extracellular proteases by microorganisms. The effect of the initial pH of the culture medium on the enzyme production was studied in pH range of 4–10, the initial pH was adjusted with NaOH or HCl. No growth was found at pH 4. The enzyme was produced over pH range from 6–10 with a maximum value at pH 7, in which no pH adjustment was made. In order to prove that the enzyme was produced in neutral to weak alkaline pH, the initial pH was adjusted using sodium carbonate. In this experiment, maximum enzyme activity was obtained in pH range from 7–8. Drastic decrease in bacterial growth and enzyme production was observed, when the initial pH was in the alkaline range (data not shown). These results indicate that the *Bacillus* sp. MIG is a neutrophilic organism and produces alkaline proteases.

**Effect of carbon sources on enzyme production.** The effect of carbon sources was investigated in medium containing (3 g l<sup>-1</sup> peptone and 1 g l<sup>-1</sup> yeast extract). The tested carbon sources were supplemented to the medium at 1%. The results presented in Figure 2 showed that the addition of wheat bran, molasses,

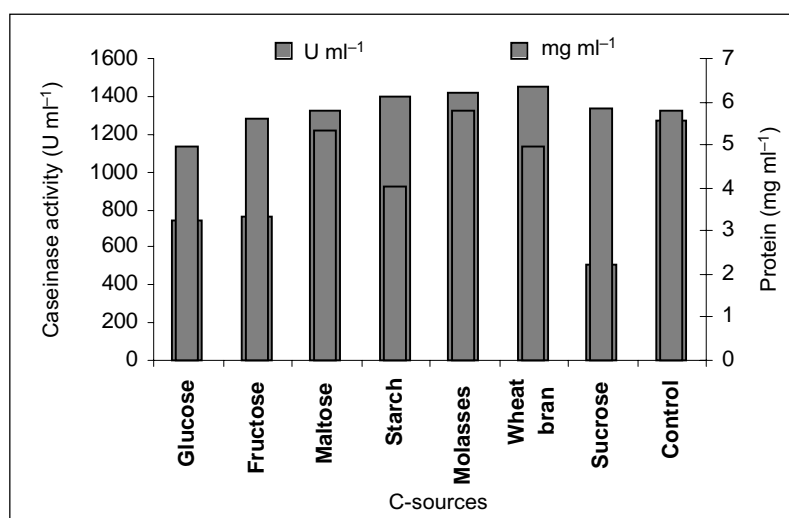


Fig. 2. Effect of supplementation of different C-sources to the control on the proteolytic activity of *Bacillus* sp. MIG, the C-sources were added at 1%; control: basal medium without casein.

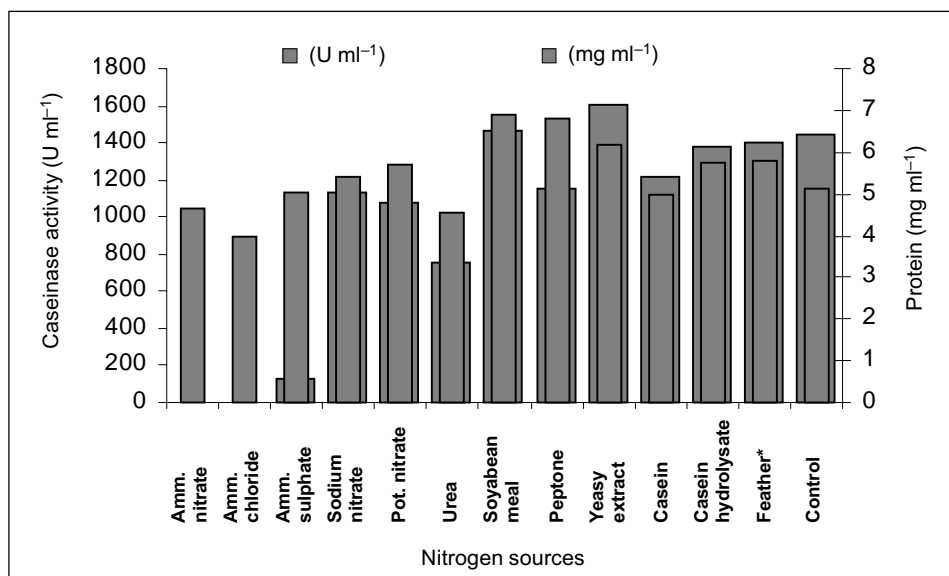


Fig. 3. Effect of different  $N_2$ -sources on the production of proteolytic activity by *Bacillus* sp. MIG, the  $N_2$ -sources were added on the basis of nitrogen equivalent to  $3 \text{ g l}^{-1}$  peptone and  $1 \text{ g l}^{-1}$  yeast extract. \* 4-days incubation.

starch or maltose enhanced the enzyme production with the maximum value in the presence of wheat bran ( $1449.15 \text{ U ml}^{-1}$ ). The use of wheat bran in the production medium is very important, because it is one of the cheap and readily available carbon sources. The estimated cost of wheat bran was found to be  $0.002 \text{ \$}$  for one liter production medium. More recently, alkaline protease production from *Bacillus* sp. was investigated in solid-state fermentation using wheat bran and lentil husk, where wheat bran was found to be a better source (Uyar and Baysal, 2004). Studies on alkaline proteases reported that the addition of starch to the culture medium induced enzymes synthesis (Chauhan and Gupta, 2004; Fang *et al.*, 2001). On the other hand, addition of other carbon sources like sucrose, glucose or fructose reduced the activity by about 60, 42 and 40% (respectively) compared to the control. Removal of casein from the medium with peptone and yeast extract had no effect on the enzyme production, indicating the constitutive nature of *Bacillus* sp. MIG enzyme. Production of microbial proteases has been found to vary from being constitutive to partially inducible in nature (Gupta *et al.*, 2002; Puri *et al.*, 2002). Increasing the concentration of wheat bran up to 2.5% had no effect on enzyme activity.

**Effect of nitrogenous compounds.** Effect of different nitrogen containing compounds was evaluated in medium with wheat bran as the sole carbon source (Fig. 3). The nitrogen source in the basal medium ( $3 \text{ g l}^{-1}$  peptone and  $1 \text{ g l}^{-1}$  yeast extract) was replaced on their nitrogen equivalent by different organic and inorganic nitrogen sources. It was found that yeast extract was the best organic nitrogen source and gave the highest activity ( $1601.34 \text{ U ml}^{-1}$ ), while sodium and potassium nitrate were the best inorganic nitrogen source. Ammonium nitrate and chloride totally repressed the enzyme production and ammonium sulphate gave only 8.6% compared to the control medium. Johnvesly and Naik, (2001) reported that protease production by *Bacillus* sp. JB-99 was supported by nitrate nitrogen viz. sodium and potassium nitrate, while ammonium nitrogen completely inhibited the production. Soybean meal, casein hydrolysate and casein also supported high enzyme activity. Peptone and urea reduced the activity by about 20 and 47% (respectively) compared to the control. The addition of 1% feather to the medium with 1% wheat bran supported the enzyme production and gave high activity, but the incubation time was 4-days. *Bacillus* sp. P-001A was found to degrade feather after at least 5-days (Atalo and Gashe, 1993). It was also found that the use of medium with 1% feather as the sole carbon and nitrogen source gave about 60% of the activity obtained in presence of wheat bran and feather and the addition of 1% glucose to the feather reduced the activity by about 30% (data not shown). On the other hand, the protein content in the culture filtrate was not greatly affected by the nitrogen sources.

**Enzyme purification.** The purification scheme of the extracellular proteases produced by *Bacillus* sp. MIG is summarized in Table I. Two proteases were purified to homogeneity using cation exchange chromatography on CM-Sepharose CL-6B, followed by gel filtration on Sephadex G-75 superfine. The steps were very effective and combined to give overall purification of 19.3 and 16.1-fold for the protease 1 (Pro 1) and protease 2 (Pro 2) respectively. Figure 4 shows that two fractions were obtained after the cation exchange. The first one was eluted using  $0.1 \text{ M NaCl}$  and contained three main protein bands, in addition to minor proteins and the second was eluted with  $0.5 \text{ M NaCl}$  and contained one major band and some minor bands.

Table I  
Summary of purification steps

Steps	Total activity (U)	Total protein (mg)	Specific activity (Umg <sup>-1</sup> )	Yield (%)	Fold purification
Crude extract	400250	1542	259.6	100	1.0
Acetone precipitation	300650	589	510.4	75.1	1.9
CM-Sepharose CL-6B					
Eluted with 0.1M NaCl	121300	90	1347.78	30.3	5.2
Eluted with 0.5M NaCl	30213	15	2014.2	7.5	7.8
Sephadex G-75 superfine					
Pro 1	50231	10	5023.1	12.5	19.3
Pro 2	54412	13	4185.5	13.6	16.1

The rechromatography of the first fraction from the cation exchange on Sephadex G-75 gave two pure bands. The specific activities for both proteases are 5023.1 and 4185.5 Umg<sup>-1</sup> respectively. The purified enzymes were also confirmed to be homogeneous by SDS-PAGE with molecular weights of about 36 and 23 kDa respectively (Fig. 4). The molecular masses of microbial alkaline proteases ranged between 15 and 36 kDa, with few exceptions of high molecular mass, such as 42 kDa from *Bacillus* sp. PS719 (Towatana *et al.*, 1999) and very high (90 kDa) from *Bacillus subtilis* (Kato *et al.*, 1992). Zymogram activity staining revealed three major clear zones of proteolytic activities that are active over broad range of pH and fourth one that only active in the alkaline range (Fig. 5).

**Effect of pH on activity and stability of both enzymes.** The effect of pH on the activity of both purified proteases was determined at 40°C in the pH range from 6.0–12.0. The two proteases were more active in the alkaline range with maximum activity at pH 11 and 12 for Pro 1 and Pro 2 respectively (Fig. 6a). The two enzymes were relatively stable over a pH range from 7–10 for 20 h at 30°C and retained about 88 and 92% at pH 10 after the same time (Fig. 6b). Two alkaline proteases AP-1 and AP-2 from alkalophilic *Bacillus* spp. were also optimally active at pH 11 and 12, respectively, and were stable for 4 h in pH range of 6–12 for AP-1 and 6–9 for AP-2 (Kumar *et al.*, 1999). The optimum pH obtained for enzymes reported in this study, are higher than the values pH 9 (Rahman *et al.*, 1994) and pH 10.5 (Beg and Gupta, 2003) for other alkaline proteases.

**Effect of temperature on activity and stability of both enzymes.** The optimum temperatures for Pro 1 and Pro 2 were found to be 50 and 55°C respectively (Fig. 7a). It was also found that Pro 2 was relatively

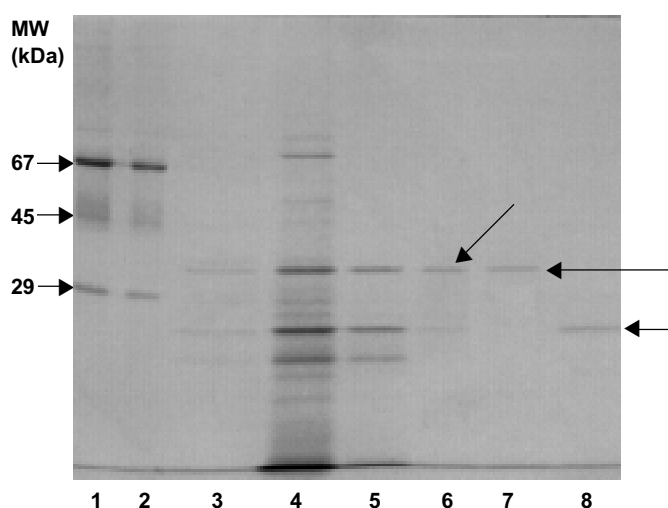


Fig. 4. SDS-PAGE of purified proteases produced by *Bacillus* sp. MIG. Lane 1 and 2: molecular mass marker proteins; lane 3: crude extract; lane 4: acetone fraction; lane 5: CM-Sepharose fractions (0.1M NaCl); lane 6: CM-Sepharose fractions (0.5M NaCl); lane 7 (pro 1) and 8 (pro 2) obtained after gel filtration using G-75 superfine.

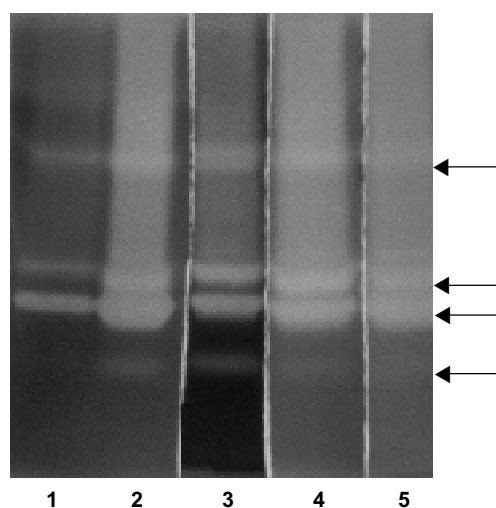


Fig. 5. Activity pattern of *Bacillus* sp. MIG proteases in SDS-PAGE containing 0.05% casein. After electrophoresis the gels were washed in Triton X-100 to remove the SDS, then incubated at different pH's. Lane 1: crude extract (pH 11); lane 2, 3, 4 and 5: acetone fraction (pH 11, 9, 7.5 and 6 respectively).

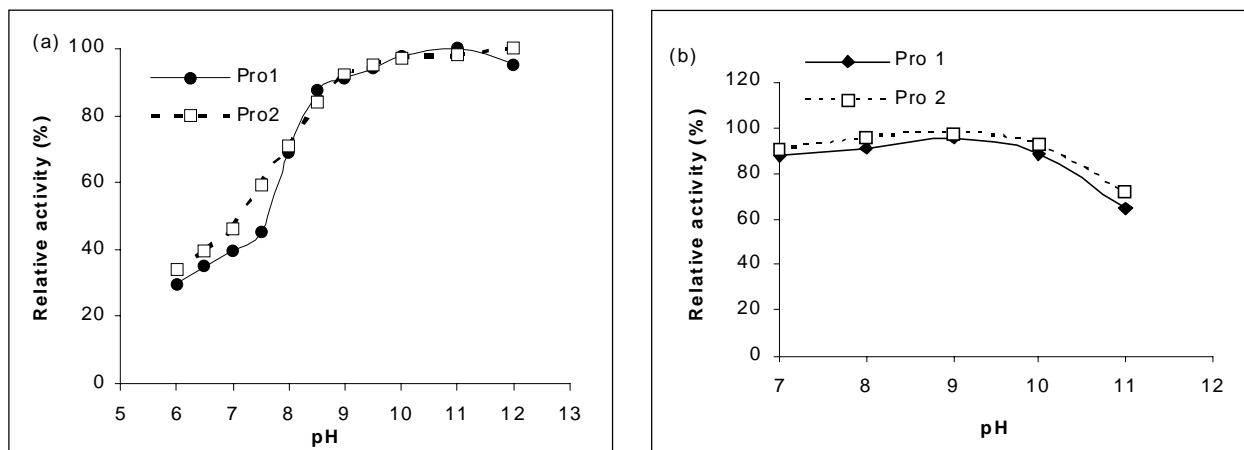


Fig. 6. Effect of pH on the activity (a) and stability (b) of Pro 1 and Pro 2 produced by *Bacillus* sp MIG. Activity was assayed at 40°C. Buffers used were phosphate, pH 6.0–7.5; Tris-HCl, 8.0–9.0 and glycine-NaOH, 9.5–12, each at 100 mM. In case of stability, the enzymes were preincubated in different pH values for 20 h at 30°C.

active at high temperatures than Pro 1. The thermal stability of the two enzymes was examined by preincubation of the enzymes at pH 11 for 3 h at temperatures 40 and 50°C in presence and absence of 5 mM  $\text{CaCl}_2$ , then the residual activities were determined under the standard assay conditions. Both enzymes are stable in presence of  $\text{CaCl}_2$  at 40°C and relatively stable in its absence at the same temperature (Fig. 7b). At 50°C, in the absence of calcium chloride, Pro 1 lost about 46 and 100% of its activity after 90 and 180 min, while Pro 2 lost only 24 and 53% after the same time. On the other hand, the two enzymes retained more than 80% after 2 h at 50°C in presence of 5 mM  $\text{CaCl}_2$ . These results revealed that calcium chloride increased the thermal stability of both enzymes. Similar optimum temperatures were reported for AP-1 and AP-2 from *Bacillus* spp. (Kumar *et al.*, 1999). Alkaline protease from *B. mojavensis* was optimally active at temperature of 60°C, with rapid loss of activity above 65°C (Beg and Gupta, 2003), while lower optimum temperature (45°C) was reported for other protease (Rattary *et al.*, 1994). Alkaline proteases from *Nocardiosis* sp. retained only 60% after 2 h at 50°C (Moreira *et al.*, 2003). Generally, most of the commercial available Subtilisin-type proteases are active in the pH and temperature range between 9.0–12.0 and 50–60°C, respectively (Beg and Gupta, 2003).

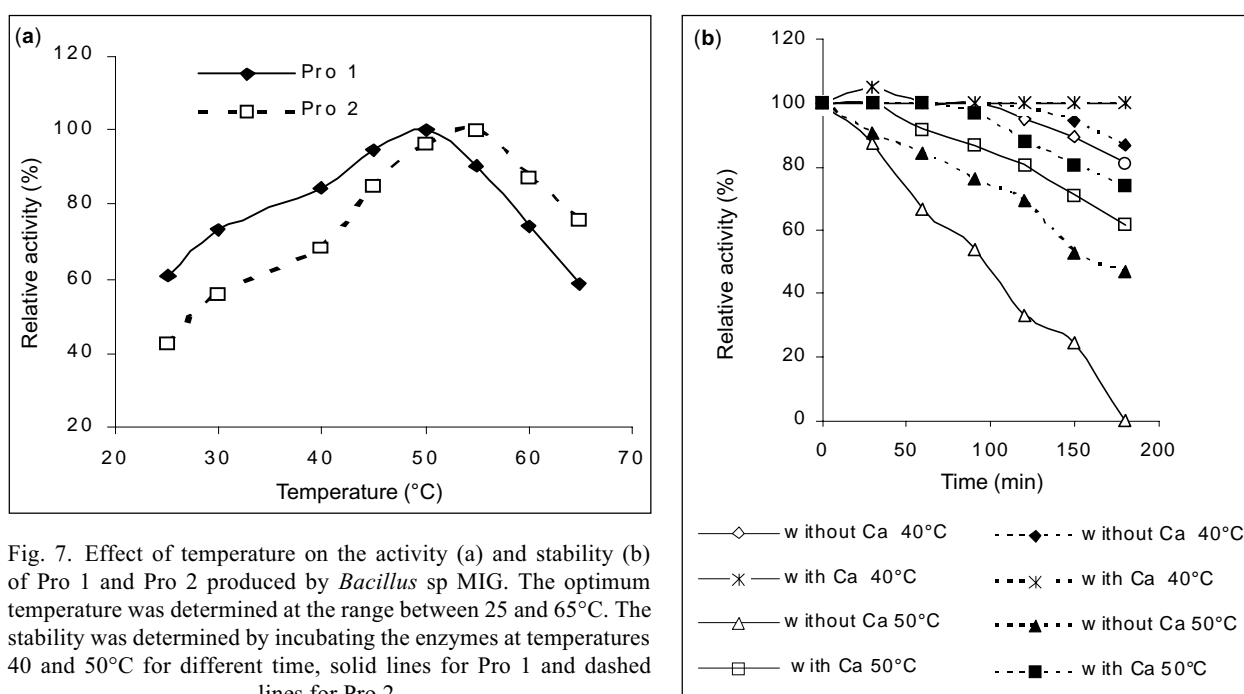


Fig. 7. Effect of temperature on the activity (a) and stability (b) of Pro 1 and Pro 2 produced by *Bacillus* sp MIG. The optimum temperature was determined at the range between 25 and 65°C. The stability was determined by incubating the enzymes at temperatures 40 and 50°C for different time, solid lines for Pro 1 and dashed lines for Pro 2.

**Substrate specificity.** The ability of the Pro 1 and Pro 2 to hydrolyze different proteins was tested. It was found that Pro 1 exhibited the highest activity towards casein and bovine serum albumin and good activity with haemoglobin, while low level of activity was detected with modified substrates ( azocasein and azoalbumin). On the other hand, Pro 2 showed the highest activity toward casein but no activity with bovine serum albumin and azoalbumin. Pro 2 also showed a moderate activity against keratin, while Pro 1 had no activity on the same substrate. Both enzymes hardly hydrolyze gelatin. Many of the previous studies also revealed that alkaline proteases showed highest activity towards casein relative to other proteins including BSA, haemoglobin, keratin and azocasein (Freeman *et al.*, 1993; Rahman *et al.*, 1994). The two alkaline proteases isolated from some *Bacillus* spp. exhibited very low keratinolytic activity (Kumar *et al.*, 1999). The ability of the Pro 1 and Pro 2 produced by the test organism to hydrolyze a wide range of proteins substrate may be advantageous for its use in detergents against a wide variety of stains.

**Effect of metal ions.** The effect of different metal ions on the activity of both purified enzymes was also tested. Of all the tested ions  $Hg^{2+}$  and  $Ag^{+}$  inhibited the activity of both enzymes by about 50–70%. Many alkaline proteases were reported to be inhibited by mercury and silver (Banerjee *et al.*, 1999; Beg and Gupta, 2003; Kumar *et al.*, 1999). However, metal ions such as  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $Co^{2+}$  and  $Ca^{2+}$  increased or stabilized the activity of the two enzymes, confirming that these cations take part in the stabilization of the protease structure and are required for protection against thermal denaturation (Paliwal *et al.*, 1994). Another important feature of the enzymes used in leather industry was the salt tolerant capacities. In the presence of 1 M NaCl, the two enzymes retained 100% of their activities after 60 min preincubation at 40°C (data not shown). It was reported that alkaline protease produced from haloalkaliphilic *Bacillus* sp. lost about 20% when perincubated with 0.17 M NaCl for 30 min at 37°C (Gupta *et al.*, 2005), while *Bacillus* sp. JB-99 protease lost only 16% in the presence of 1 M NaCl after 2 h at 45°C (Johnvesly *et al.*, 2002).

**Effect of inhibitors, surfactants and detergents.** The results obtained in this study revealed that both enzymes were completely inhibited by 2 mM PMSF, a serine protease inhibitor, while 2-mercaptoethanol and dithiothreitol stimulate the activity suggesting that both are thiol-dependent serine proteases. Similar results were observed for *B. mojavensis* protease (Beg and Gupta, 2003). The two proteases were found to be very stable against non-ionic surfactants (such as Tween 80 and Triton X-100) and stable in the presence of anionic surfactants, SDS. Matta and Punj (1998) reported that protease from *B. polymyxa* B-17 lost approximately 10% of its activity on treatment with 1 mM SDS. The enzymes also exhibited strong stability against bleaching agent, hydrogen peroxide and relatively stable in presence of commercial detergents. Singh *et al.* (2001) reported that SSR1 protease retained 40–90% of its activity in the presence of local detergents. The stability of both enzymes in the presence of EDTA suggesting that metal cofactors are not required for enzyme activity. This property of the enzyme was very useful for application as detergent additive since chelating agents, which function as water softeners and are involved in the removal of stains are components of the detergent and then specifically bind divalent cations (Beg and Gupta, 2003).

From these results, it is envisage that *Bacillus* sp. MIG can be a potential source of alkaline proteases for use in different industrial application, because it produced the enzymes at a prominent level using cost-effective medium. The broad substrate specificity of the proteases from *Bacillus* sp. MIG may be also advantageous for its use in detergents against wide variety of stains and the stabilities in the presence of high sodium chloride concentration permit their application in leather industry as an unhairing agent, removing the need to use toxic reagents.

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