Isolation and characterization of thermophilic alkaline proteases resistant to sodium dodecyl sulfate and ethylene diamine tetraacetic acid from *Bacillus* sp. GUS1

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Abstract
Thermophilic *Bacillus* sp. GUS1, isolated from a soil sample obtained from citrus garden, produced at least three proteases as detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis. The enzymes were stable in the alkaline pH range (8.0-12.0), with the optimum temperature and pH range of the proteases being 70ºC and 6.0-12.0, respectively. All three proteases were also highly stable at 70ºC. After 60 min of incubation at 70ºC, the enzymes retained 100% of their original activities. Enzymes were mostly inhibited by phenylmethylsulfonyl fluoride (PMSF), however 80-90% enzyme activities were retained in presence of 2-mercaptoethanol and iodoacetate. Addition of SDS and ethylene diamine tetraacetic acid (EDTA) also marginally influenced protease activities, but addition of Ca^{2+} to the proteases did not bring about any change. The results suggeste that most of these proteases were not metalloproteases, but Ca^{2+}-independent serine alkaline proteases.
Keywords: *Bacillus* sp.; Alkaline serine proteases; Thermostability Resistance to EDTA and SDS

INTRODUCTION

Extracellular proteases are involved in the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz, 1988). At the same time, such enzymes have also been commercially exploited to assist protein degradation in various industrial processes (Gupta *et al*., 2002a; Kumar and Takagi, 1999). Hence, proteases are among the most important industrial enzymes accounting for nearly 60% of the total world-wide enzyme sales (Mala *et al*., 1998). Most commercially available proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus* (Schallmey *et al*., 2004). Alkaline proteases secreted by both neutrophilic and alkalophilic bacilli are of particular interest due to their wide applications in laundry detergents, leather processing, protein recovery or solubilization, organic synthesis, meat tenderization, production of certain oligopeptides, and recovery of silver from used X-ray films (Saeki Katsuhisa *et al*., 2007; Gupta *et al*., 2002a; Anwar and Saleemuddin, 1998; Mala *et al*., 1998). In recent investigations (Moradian *et al*., 2006; Sajedi *et al*., 2005; Sajedi *et al*., 2004) attempts were made to isolate certain industrial enzymes from newly isolated strains in Iran have been reported. In the present study, the efficiency of alkaline proteases recovered from *Bacillus* isolate was investigated.

Proteolytic enzymes produced by *Bacillus* sp. GUS1 were not only active at high temperatures but were also alkaline-stable and active in a wide range of pH. The enzymes were characterized to exploit their potential for use as effective additives in the detergent industry. The alkaline proteases produced by the GUS1 strain showed resistance to SDS and EDTA, and did not require Ca^{2+}; therefore, they have the potential of being used as detergent additives.

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MATERIALS AND METHODS

Isolation and identification of microorganisms: Soil was collected from a citrus garden in Fasa, Fars province of Iran. Ten grams of air-dried soil sample was added to 90 ml of sterile water in an Erlenmeyer flask. The flask was then heated in a water bath for 10 min at 80ºC while the contents were agitated. Subsequently, 1 ml of the suspension was added to 9 ml of sterile distilled water and a serial dilution (10^{-1}-10^{-8}) was prepared. One ml of each dilution was added and distributed onto skim milk agar plates containing peptone (0.1% w/v), NaCl (0.5% w/v), agar (2.0% w/v), and skim milk (10% w/v) and the pH was adjusted to 8.0. Plates were incubated at 50ºC for 24-72 h, different colony types were chosen and purified. The isolates were then screened by the skim milk protein hydrolysis test. Hydrolysis was recorded when clear zones appeared around the colonies. The promising strains were further examined for morphological, physiological and biochemical characteristics with reference to Bergey's Manual of Systematic Bacteriology (Sneath, 1986) and the Color Atlas of *Bacillus* species (Pany *et al*., 1983).

PCR amplification and 16S rDNA sequencing: Genomic DNA was extracted and purified according to Sambrook and Russell (2001) and its purity was spectrophotometrically assessed by the A260/A280 ratio. The universal 16S rDNA primers; forward (5´-AGTTTGATCCTGGCTCAG-3´) and reverse (5´-GGC/T TACCTTGTTACGACTT-3´) were used for amplification of the 16S rDNA gene (Desantis *et al*., 2003; Weisburg *et al*., 1991). Amplification of DNA was carried out with a DNA thermal cycler (Applied Biosystem, UK) under the following conditions: initial denaturation at 94ºC for 5 min; 30 cycles of denaturation at 94ºC for 45 sec, annealing at 48ºC for 45 sec, extension at 72ºC for 90 sec, and a final extension at 72ºC for 5 min. The amplified PCR product was analyzed on an agarose gel, purified and sequenced on both strands directly by SEQ-LAB (Germany) according to the super long run (>800 bases).

Nucleotide sequence accession number: The nucleotide sequence data were submitted to GenBank at NCBI, (http://www.ncbi.nlm.nih.gov/) under the accession number EU183349.

Culture conditions and partial purification of protease: Two media were studied for alkaline protease production. The first containing glucose 0.5%, Peptone 0.75%, $MgSO_4,7H_2O$ 0.5%, KH_2PO_4 0.5% and $FeSO_4,7H_2O$ 0.01% (w/v) and the second containing citric acid 1%, $NaNO_3$ 1%, $MgSO_4.7H_2O$ 0.03%, K_2HPO_4 0.5%, $CaCl_2.2H_2O$ 0.02%, NaCl 0.5%, Na_2CO_3 1% (w/v). The pH of the two media was adjusted to 8. Incubation was carried out at 40ºC with agitation at 140 rpm and at different times ranging from 24-72 h. After 24-72 h, the media were centrifuged at 5000 ×*g* for 20 min at 4ºC. The cell-free supernatants were precipitated with 85% ammonium sulfate for 3 h at 4ºC and the pellets were dissolved in small quantities of 20 mM Tris-HCl buffer, pH 7.4 and then dialyzed for 6 h against the same buffer. The partially purified enzymes were used for further studies.

Protease assay: Protease activity was determined by the modified method of Kembhavi and Kulkami (1993). Supernatant (0.15 ml) was added to a tube containing 0.3 ml of 1% (w/v) casein (dissolved in 20 mM Tris-HCl buffer, pH 7.4) and incubated at 37ºC for 30 min. Subsequently, 0.45 ml of a 10% (w/v) trichloroacetic acid solution (final concentration of 5% (w/v)) was added to stop the proteolysis. The mixture was incubated at room temperature for 1 h. After incubation the reaction mixture was centrifuged at 12000 ×*g* for 5 min, and the absorbance of the supernatant was measured at 280 nm. One unit of protease is defined as the amount of enzyme that hydrolyses casein to produce equivalent absorbance to 1 µmol of tyrosine/min with tyrosine as standard.

Effect of pH on enzyme activity and stability: The optimum pH of the protease was determined with 1% (w/v) casein (as substrate) dissolved in a mixed buffer containing citrate, phosphate, Tris-HCl, and glycine (50 mM each). The pH stability of the protease was established by incubating the enzyme in above mixed buffers of different pH values in the range of 8.0-12.0 for 5, 15, 30, 60 min at room temperature. Aliquots were withdrawn and proteolytic activity was determined at pH 8.0 and room temperature.

Effect of temperature on enzyme activity and stability: The activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 20 to 90ºC. The irreversible ther-

moinactivation of each enzyme was examined by incubating the enzyme for different periods of time ranging from 0-60 min and then cooling on ice. Finally, the residual activity was determined under the abovementioned assay conditions.

Effect of inhibitors, sodium dodecyl sulfate, and Ca^{2+}: The effects of various protease inhibitors such as the serine inhibitor (Phenylmethylsulfonyl fluoride [PMSF], cysteine inhibitors (iodoacetate, 2-mercaptoethanol), Ca^{2+} ion and a chelator of divalent cations (ethylene diamine tetraacetic acid [EDTA]) were investigated. After 30 min of preincubation of inhibitor with enzymes at room temperature, residual protease activity was measured by the standard assay method.

Polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis: SDS-PAGE was carried out using a 12.5% (w/v) running gel by the method of Laemmli (1970). For zymography, 0.5% (w/v) gelatin was co-polymerized with the running gel and samples were then loaded onto the gel without heating followed by electrophoresis with 100 V at 4ºC. Following electrophoresis, the gel was incubated in 2.5% (v/v) Triton X-100 (renaturing solution) for 30 min at room temperature with gentle agitation, then was decanted and replaced with developing buffer (50 mM Tris, 0.2 mM NaCl, and 5 mM $CaCl_2$, pH 8.0). The gel was equilibrated for 30 min at room temperature with gentle agitation, then was decanted and replaced with developing buffer and incubated at 37ºC for at least 4 h. The gel was stained with Coomassie Brilliant Blue G-250 or R-250 (both at 0.5% (w/v)) for 30 min and finally destained.

RESULTS

Identification of bacteria and 16S rDNA Analysis: All bacterial strains were spore forming, Gram-positive and rod shaped. They were screened for alkaline protease activities at 50ºC. From a large collection, 4 strains were selected for further experimental studies. Finally, the isolate GUS1 was selected as the most potent producer of alkaline proteases. It was catalase-positive and capable of using sodium citrate and sodium propionate as the sole carbon source. It was also able to grow in nutrient media containing salt at various concentrations (up to 15% (w/v)). The strain was able to grow at temperatures as high as 50ºC. The

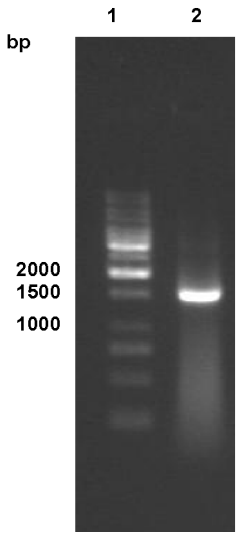

Figure 1. PCR product of 16S rDNA gene on 1% agarose gel. Lane 1, DNA size marker; lane 2, PCR product.

combination of morphological, physiological, and biochemical data suggested that this strain belonged to the *Bacillus* species.

The PCR amplification and sequencing of the 16S rDNA gene of strain GUS1 was also carried out. The product of PCR amplification was approximately 1500 bp (Fig. 1). 16S rDNA sequence was edited to a total length of 668 bp. Multiple sequence alignments were carried out using 16S rDNA sequences of 15 type strains. 16S rDNA sequences from the *Bacillus* species were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and the hypervariable multiple sequence alignments were carried out with ClustalW version 1.82 (Goto *et al*., 2000). A phylogenetic tree was constructed by the neighbor-joining method (data not shown). Multiple alignments and the phylogenetic tree showed that the GUS1 strain was closely related to *Bacillus licheniformis*.

The GUS1 strain grew at fast rate and exhibited maximum production of extracellular proteases after 48 h of growth. It could also grow at 35-50ºC, but the best temperature for protease production was 40ºC (Fig. 2). Protease activity measurements from culture supernatants showed enzyme production increase by approximately 6-fold when using the first production medium (203 IU/ml) as compared to the second one (32 IU/ml). Hence, the first production medium was employed for subsequent experiments.

The zymogram analysis of partial purified crude extract by 85% ammonium sulfate indicated the pres-

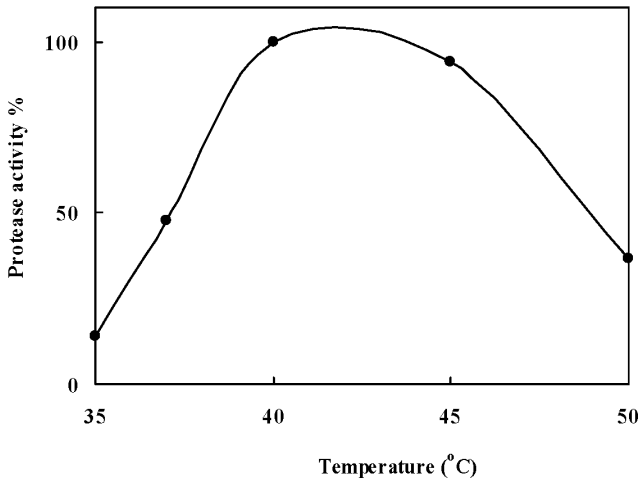

Figure 2. Effect of temperature on protease production. Incubation was carried out with agitation at 140 rpm and different temperatures for 48 h.

ence of at least three proteases. By staining the zymogram gel with Coomassei Brilliant Blue G-250, well resolved and clear marker bands appeared, hence the molecular weights of the proteases could be estimated by comparing the darkness of the bands (Fig. 3). The molecular weights of the proteases were approximately 30-47 kDa.

Effect of pH and temperature on protease activity and stability: The influence of temperature and pH on the activity of enzyme is shown in Fig. 4A. Two pH optima for alkaline proteases secreted by *Bacillus* sp. GUS1 were observed at 7 and 10. The result indicates that the crude enzyme contains several proteases. In addition, pH stability was determined by incubating the enzyme at different pH values in the range of 8.0-12.0 for different periods of time, at room temperature. The proteases were stable between pH 8.0-12.0 and retained more than 65% of their initial activities after 1h incubation even at pH 12.0 (Fig. 4B). Maximum stabilities were observed at a pH range of 8.0-10.

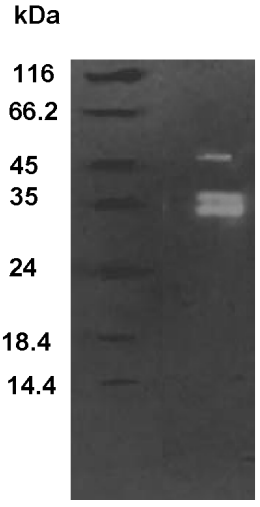

Figure 3. Zymogram of alkaline proteases from *Bacillus* sp. GUS1 stained with Coomassei Brilliant Blue G-250.

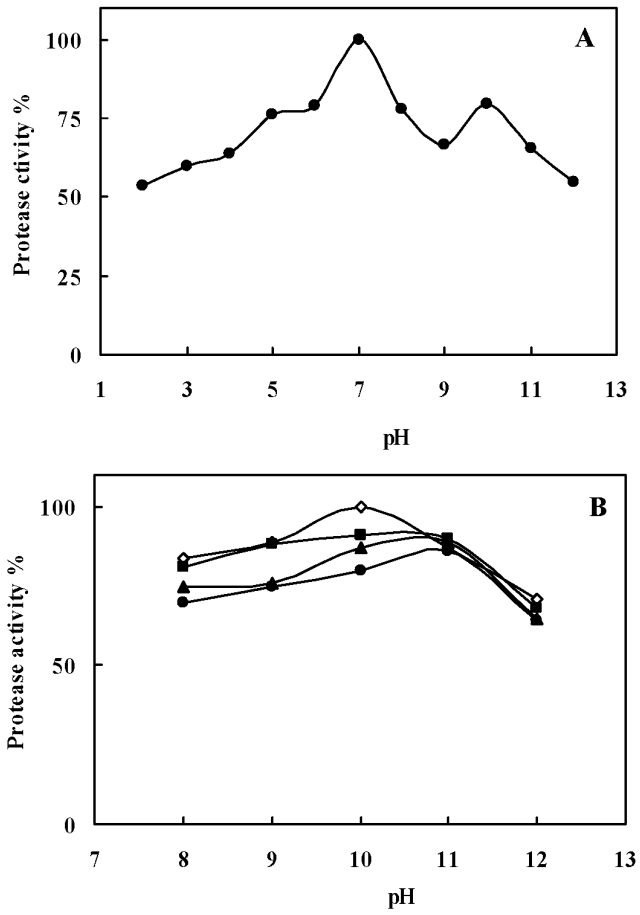

Figure 4. (A) Effect of pH on protease activity. Activities were determined in the mixed buffers adjusted to different values of pH at room temperature. The relative activities were based on the ratio of the activity obtained at a certain pH to the maximum activity obtained at that range and expressed as a percentage. (B) pH stability of *Bacillus* sp. GUS1 proteases at alkaline conditions for 5 (◊), 15 (■), 30 (▲), and 60 (●) min. The experiments were carried out by incubating the enzymes in the mixed buffers of different pH in the range of 8.0 to 12.0 for various times at room temperature. Aliquots were removed and proteolytic activities were determined at pH 8.0 and room temperature. The activity of the enzyme before incubation was taken as 100%.

Alkaline proteases from *Bacillus* sp. GUS1 were shown to be optimally active at 70ºC (Fig. 5A). Thermostability was examined by incubating the enzyme at 70ºC for different time periods. As

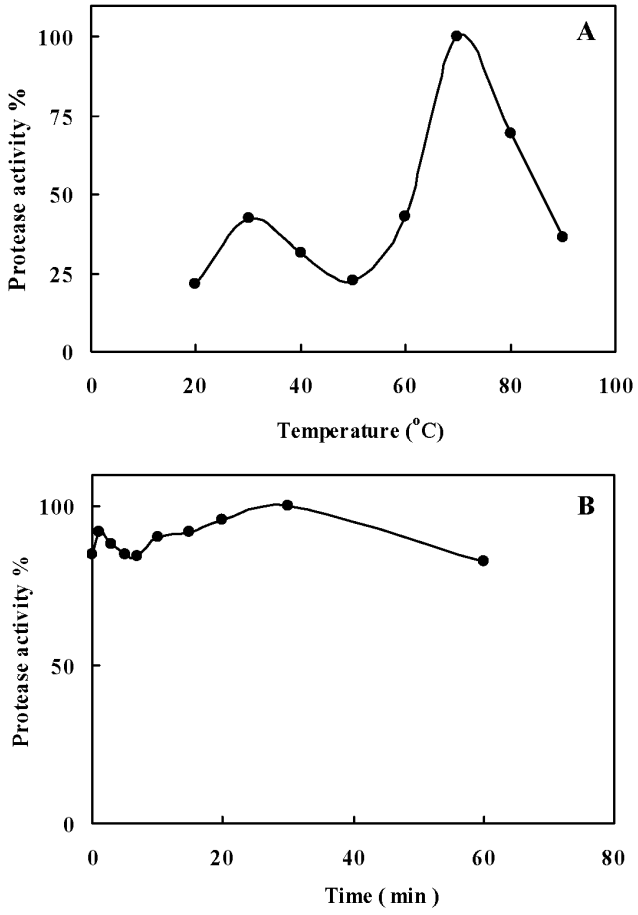

Figure 5. (A) Effect of temperature on protease activity. Activities were determined in 20 mM Tris-HCl buffer, pH 7.4 at different temperatures. The relative activities were based on the ratio of the activity obtained at a temperature to the maximum activity obtained at that range and expressed as a percentage. (B) Irreversible thermoinactivation of alkaline proteases from *Bacillus* sp. GUS1 at 70ºC. The enzyme samples were incubated at 70ºC, and at the different times; samples were removed and cooled on ice and activities determined in the usual manner. Activity of the same enzyme solution kept on ice was considered as a control (100%).

shown in Fig. 5B, after 60 min of incubation at 70ºC, the enzymes retained 100% of their original activities. However, some activation could be seen for alkaline proteases particularly after 30 min of incubation.

Effect of inhibitors, sodium dodecyl sulfate and Ca^{2+}: The effects of different reagents at different concentrations on the activities of the proteolytic enzymes were studied (Table 1). Zymogram analysis was also used to confirm the effects of these reagents (Fig. 6).

Table 1. Effects of various inhibitors and metal ions on activity of *Bacillus* sp. GUS1 proteases. Ca^{2+} was added as a chloride salt and EDTA and iodoacetate were used as sodium salts.

Chemical reagents (mM)	Relative activity
Ca^{2+}	
1	1.0
5	1.0
10	1.0
SDS	
1	0.90
5	0.90
10	0.76
EDTA	
1	0.93
5	0.83
10	0.87
PMSF	
1	1.0
5	0.20
10	0.20
Iodoacetate	
1	0.82
5	0.88
10	0.72
2-mercaptoethanol	
1	0.95
5	0.91
10	0.90

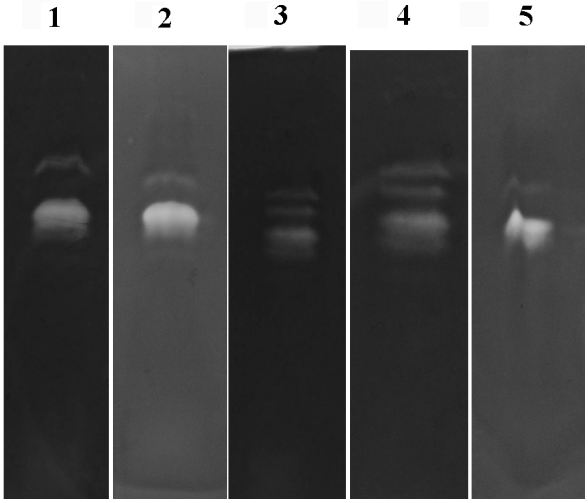

Figure 6. Zymogram of alkaline proteases from *Bacillus* sp. GUS1 stained with Coomassei Brilliant Blue R-250. lane 1, proteases in Tris buffer as a control; lane 2, in presence of Ca^{2+}; lane 3, in presence of PMSF; lane 4, in presence of EDTA and lane 5, in presence of 2-mercaptoethanol. For more details see Materials and Methods.

DISCUSSION

Protease production is an inherent property of all organisms and such enzymes are generally constitutive or partially inducible. Proteases are largely produced during the stationary phase and thus are generally regulated by carbon and nitrogen stress (Beg *et al*., 2002; Gupta *et al*., 2002b; Kalisz, 1988). Protease production is often related to the sporulation stage in many bacilli, such as *Bacillus subtilis* (O'Hara and Hageman, 1990) and *B. licheniformis* (Hanlon and Hodges, 1981).

In the present study, alkaline proteases were extracted from a locally isolated strain, *Bacillus* sp. GUS1. The molecular weights of the proteases were estimated to be between 30-47 kDa, based on the zymogram gel stained by Coomassei Brilliant Blue G-250.

The results of pH studies indicate a broad pH range (5.0-12.0) for these enzymes at room temperature (Fig. 4A). These results are in accordance with several earlier reports showing pH optima being close to pH 10.0 (Patel *et al.,* 2006; Genckal and Tari, 2006; Beg and Gupta, 2003; Banerjee *et al*., 1999). This range of optimum catalysis, however, is relatively wide as compared to most previous reports (Patel *et al*., 2006; Ghorbel *et al.,* 2003; Banerjee *et al*., 1999). Generally, commercial proteases from microorganisms have maximum activities in the alkaline pH range of 8.0-12.0 (Ghorbel *et al*., 2003; Singh *et al*., 2001; Mehrotra *et al*., 1999). The proteases from *Bacillus* sp. GUS1 are very stable between pH 8.0-12.0. Maximum stabilities were observed at a pH range of 8.0-10. The alkaline protease from *Bacillus* sp. NCDC 180, *Bacillus* sp. JB-99 and *Bacillus* sp. SSR1 have also been reported to be stable at the above pH range (Johnvesly and Naik, 2001; Singh *et al*., 2001; Kumar *et al*., 1999). The results of this study differ from those of *Bacillus* sp. Ve1 and *Bacillus brevis* which exhibit a sharp decline in alkaline protease stability at pH 11.0 and 12.0, respectively (Patel *et al*., 2006; Banerjee *et al*., 1999).

Alkaline proteases from *Bacillus* sp. GUS1 were shown to be optimally active at 70ºC; similar to *Bacillus* thermophilic alkaline proteases reported earlier (Hawumba *et al*., 2002; Johnvesly and Naik, 2001; Hutadilok-Towatana *et al.,* 1999). The proteases from *Geobacillus* sp. PA-9, *B. mojavensis*, and *Bacillus* sp. SSR1 have been shown to exhibit a low optimum temperature of 65, 60, and 50ºC, respectively (Beg and Gupta, 2003; Hawumba *et al*., 2002; Singh *et al*., 2001). The higher optimum temperature of 70ºC has been reported for alkaline proteases belonging to *Bacillus stearothermophilus* F1 and *Bacillus* sp. JB-99 (Johnvesly and Naik, 2001; Rahman *et al*., 1994).

The alkaline proteases produced by the GUS1 strain are highly stable at 70ºC. After 60 min of incubation at 70ºC, the enzymes retained 100% of their original activities. Note that in the case of a thermophilic enzyme, activation is obtained, presumably owing to induction of flexibility in its relatively rigid structure (Khajeh *et al*., 2001; Sundaram *et al*., 1980). Such activation by incubating the enzyme at high temperatures has also been reported in a thermophilic alpha-amylase (Laderman *et al*., 1993). The present results show that proteases are highly themostable and suggest that they have good thermal stability as compared to previous findings (Patel *et al*., 2006; Ghorbel *et al*., 2003; Banerjee *et al*., 1999; Gupta *et al*., 1999). All currently used detergent enzymes are alkaline and thermostable in nature and possess high pH optima. The most commercially available subtilisin-type proteases are also active at pHs and temperature ranging between 8.0-12.0 and 50-60ºC, respectively (Saeki Katsuhisa *et al*., 2007; Ghorbel *et al*., 2003; Gupta *et al*., 2002a; Singh *et al*., 2001; Anwar and Saleemuddin, 1998).

Among the few inhibitors tested on the proteases from *Bacillus* sp. GUS1, PMSF was the most potent, while enzymes retained approximately 90% and 80% of their activities in presence of 2-mercaptoethanol and iodoacetate, respectively. PMSF inhibited total protease activity by up to 80%. In this regard, PMSF causes Sulphonation of the serine residues residing in the active site of the protease and has been reported to result in the complete loss of enzyme activity (Beynon and Bond, 2001). Results suggest that most of the proteases produced by *Bacillus* sp. GUS1 are serine alkaline proteases. Total protease activity is retained by up to 90% in the presence of 1mM EDTA and more than 80% in the presence of 5 and 10 mM of the chelator. On the other hand, addition of Ca^{2+} dose not influence the enzyme activity. These results suggest that the enzymes are not metalloproteases and are Ca^{2+}-independent. The resistance of the enzymes in the presence of EDTA and not requiring Ca^{2+} ions are advantages to be used as additives in the detergent industry. It is known that detergents containing high amounts of chelating agents function as water softeners and assist stain removal (Walsh, 2002). SDS also marginally influenced protease activity. The proteases from *Bacillus* sp. GUS1 retained 90% of their activities in 5

mM SDS. Therefore these alkaline proteases are stable against SDS. In the detergent industry, several chemical detergents are used to formulate industrial products, and hence detergent-stable enzymes are suitable for such an industry (Walsh, 2002). The stability toward SDS is important because SDS-stable alkaline proteases from wild-type microorganisms are not generally known except for few reports from *Bacillus mojavensis*, *Bacillus* sp. RGR-14, and *Bacillus* sp. KSM-KP 43 (Beg and Gupta, 2003; Saeki *et al*., 2002; Oberoi *et al*., 2001). The results obtained from the above mentioned experiments in the presence of different reagents are supported by zymogram analysis (Fig. 5). It is also worth mentioning that in most cases of protease application in industry, the enzyme can be used in a crude or partially purified form (Walsh, 2002).

In Conclusion, alkaline proteases isolated from *Bacillus* sp. GUS1 are thermostable serine proteases. They are stable at alkaline pH and at high temperature. These enzymes are stable against ionic surfactants such as SDS. One of the most important features of the proteases from *Bacillus* sp. GUS1 are their retained activities in presence of EDTA as well as no requirement for Ca^{2+} ions. It is also very important to obtain enzymes with high stabilities and activities under alkaline pH and high temperature. Further experiments purified proteases, including molecular genetic analysis are required to understand the mechanism(s) by which the enzymes resist SDS and EDTA effects.

Acknowledgments

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References

Anwar A, Saleemuddin M (1998). Alkaline proteases: A review. *Bioresour Technol*. 64: 175-183.

Banerjee UC, Sani RK, Azmi W, Soni R (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochem*. 35: 213-219.

Beg QK, Gupta R (2003). Purification and characterization of an oxidation-stable, thiol-dependent Serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb Technol*. 32: 294-304.

Beg QK, Saxena RK, Gupta R (2002). De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. *Process Biochem*. 37: 1103-1109.

Beynon R, Bond JB (2001). *Proteolytic Enzymes*, A Practical Approach, IRL Press, New York. PP: 113.

Desantis TZ, Dubosarkiy I, Murray SR, Andersen GL (2003). Comprehensive aligned sequence construction for automated design of effective probes using 16S rDNA. *Bioinformatics* 19: 1461-1468.

Genckal H, Tari C (2006). Alkaline protease production from alkalophilic *Bacillus* sp. Isolated from natural habitats. *Enzyme Microb Technol*. 39: 703-710.

Ghorbel B, Sellami-Kamoun A, Nasri M (2003). Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microb Technol*. 32: 513-518.

Goto K, Omura T, Hara Y, Sadaie Y (2000). Application of the partial 16S rDNA sequence as an index for the rapid identification of species in the genus *Bacillus*. *J Gen Appl Microbiol*. 46: 1-8.

Gupta R, Gupta K, Saxena RK, Khan S (1999). Bleach-stable, alkaline protease from *Bacillus* sp. *Biotechnol Lett*. 21: 135-138.

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

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

Hanlon GW, Hodges NA (1981). Bacitracin and protease production in relation to sporulation during exponential growth of *Bacillus licheniformis* on poorly utilized carbon and nitrogen sources. *J Bacteriol*. 147: 427-431.

Hawumba JF, Theron J, Brozel VS (2002). Thermophilic protease-producing *Geobacillus* from Buranga hot springs in Western Uganda. *Curr Microbiol*. 45: 144-150.

Hutadilok-Towatana N, Painupong A, Suntinanalert P (1999). Purification and Characterization of an Extracellular Protease from Alkaliphilic and Thermophilic *Bacillus* sp. PS719. *J Biosci Bioeng*. 87: 581-587.

Johnvesly B, Naik GR (2001). Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. *Process Biochem*. 37: 139-144.

Kalisz MH (1988). Microbial proteinases. *Adv Biochem Eng Biotechnol*. 36: 17-55.

Kembhavi AA, Kulkami A (1993). Salt tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No. 64. *Appl Biochem Biotechnol*. 38: 83-92.

Khajeh K, Khezre-Barati S, Nemat-Gorgani M (2001). Proteolysis of mesophilic and thermophilic α-amylase. *Appl Biochem Biotechnol*. 94: 97-109.

Kumar CG, Takagi H (1999). Microbial alkaline proteases: from a

bioindustrial viewpoint, *Biotechnol Adv*. 17: 561-594.

Kumar CG, Tiwari MP, Jany KD (1999). Novel alkaline serine proteases from alkalophilic *Bacillus* spp.: purification and some properties. *Process Biochem*. 34: 441-449.

Laderman KA, Davis BR, Krutzsch HC, Lewis MS, Griko YV, Privalov PL, Anfinsen CB (1993). The purification and characterization of an extremely thermostable alpha-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. *J Biol Chem*. 268: 24394-24401.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Mala B, Rao AM, Deshpande VV (1998). Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiol Mol Biol Rev*. 62: 597-635.

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

Moradian F, Khajeh K, Naderi-Manesh H, Ahmadvand R, Sajedi RH, Sadeghizadeh M (2006). Thiol-dependent serine alkaline proteases from *Bacillus* sp. HR-08 and KR-8102: isolation, production, and characterization. *Appl Biochem Biotechnol*. 134: 77-87.

Oberoi R, Beg QK, Puri S, Saxena RK, Gupta R (2001). Characterization and wash performance analysis of an SDS-resistant alkaline protease from a *Bacillus* sp. *World J Microbiol Biotechnol*. 17: 493497.

O'Hara MB, Hageman JH (1990). Energy and calcium ion dependence of proteolysis during sporulation of *Bacillus subtilis* cells. *J Bacteriol*. 172: 4161-4170.

Pany JM, Tumbull PCB, Gibson JR (1983). *A Color Atlas of Bacillus Species*. London, Wolfe medical.

Patel RK , Dodia MS, Joshi RH, Singh SP (2006). Purification and characterization of alkaline protease from a newly Isolated haloalkaliphilic *Bacillus* sp. *Process Biochem*. 41: 2002-2009.

Rahman RNZA, Razak CN, Ampon K, Basri M, Yunus WMZW, Salleh AB (1994). Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1. *Appl Microbiol Biotechnol*. 40: 822-827.

Saeki Katsuhisa S, Hitomi J, Okuda M, Hatada Y, Kageyama Y, Takaiwa M (2002). A novel species of alkaliphilic *Bacillus* that produces an oxidatively stable alkaline serine protease. *Extremophiles* 6: 65-72.

Saeki Katsuhisa S, Katsuya O, Tohru K, Susumu I (2007). Detergent Alkaline Proteases: Enzymatic Properties, Genes, and Crystal Structures. *J Biosci Bioeng*. 103: 501-508.

Schallmey M, Singh A, Ward OP (2004). Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol*. 50: 1-17.

Sajedi RH, Naderi-Manesh H, Khajeh K, Ahmadvand R, Ranjbar B, Asoodeh A, Moradian F (2005). A Ca-independent α-amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104. *Enzyme Microb Technol*. 36: 666-671.

Sajedi RH, Naderi-Manesh H, Khajeh K, Ranjbar B, Ghaemi N, Naderi-Manesh M (2004). Purification, characterization, and structural investigation of a new moderately thermophilic and partially calcium-independent extracellular alpha-amylase from *Bacillus* sp. TM1. *Appl Biochem Biotechnol*. 119: 41-50.

Sambrook J, Russell D (2001). *Molecular Cloning*: a laboratory manual. Cold Spring Harbor, NewYork.

Singh J, Batra N, Sobti RC (2001). Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. *Process Biochem*. 36: 781-785.

Sneath PHA (1986). *Bergeys Manual of Systematic Bacteriology*. The Williams, Wilkins, Baltimore, USA.

Sundaram TK, Wright IP, Wilkinson AE (1980). Malate dehydrogenase from thermophilic and mesophilic bacteria. Molecular size, subunit structure, amino acid composition, immunochemical homology, and catalytic activity. *Biochemistry* 19: 2017-2022.

Walsh G (2002) *Proteins; Biochemistry and Biotechnology*. J. Wiley and sons, Chichester UK. PP: 421-436.

Weisburg WG, Barns Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacterial*. 173: 697-703.