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Thiol-Dependent Serine Alkaline Proteases From *Bacillus* sp. HR-08 and KR-8102

Isolation, Production, and Characterization

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Abstract

Two *Bacillus* sp. strains, HR-08 and KR-8102, isolated from soil of the west and north parts of Iran were screened on gelatin agar medium for their ability to produce alkaline protease. The enzymes were active in a wide pH range (6.0–11.0) and stable in the alkaline range (7.0–12.0). The optimum temperatures for the protease from HR-08 and KR-8102 were 65 and 50°C, respectively. The irreversible thermoinactivation of HR-08 and KR-8102 proteases showed that the stability of HR-08 enzyme was higher than that of KR-8102 and the half-lives of these enzymes were 95 and 32 min at 50°C, respectively. In the presence of 10 m*M* Ca^{2+}, HR-08 retained 100, 90, and 20% of its initial activity after heating for 30 min at 50, 60, and 70°C, respectively. Enzymes were inhibited by phenylmethylsulfonyl fluoride and iodoacetate. After inhibition by iodoacetate, both enzymes were reactivated by dithiothreitol. These data show that the enzymes seem to be thiol-dependent serine alkaline proteases. The enzymes especially from HR-08 were stable in the presence of H_2O_2, surfactants, and local detergents; their activities were enhanced in the presence of 5 m*M* Fe^{2+}; and the presence of 5 m*M* metal ions such as Mg^{2+}, Cu^{2+}, and Mn^{2+} produced almost no effect.

Index Entries: Proteolytic enzyme; thermostability; thiol-dependent protease; commercial detergent; inhibition.

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Introduction

Most commercially available proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus (1)*. Alkaline proteases secreted by both neutrophilic and alkalophilic bacilli are of particular interest owing to their wide applications in laundry detergents, leather processing, protein recovery or solubilization, organic synthesis, and meat tenderization *(2)*. Recently, the application of proteases to the production of certain oligopeptides *(3)* and recovery of silver from photographic plates *(4)* has also been explored. In recent studies *(5,6)*, we described our attempts to isolate some industrial enzymes from newly isolated strains in Iran.

In the present study, two alkalophilic *Bacillus* sp. were isolated from soil and optimized for maximum protease production. Proteolytic enzymes produced by both strains HR-08 and KR-8102 are not only active at high temperatures but are also alkaline stable and active in a wide range of pH values. The enzymes have been characterized to exploit their potential for use as effective additives for the detergent industry. Both alkaline proteases produced by HR-08 and KR-8102 are stable in bleach, EDTA, surfactant, and commercial detergents; therefore, they have the potential to be used as detergent additives.

Materials and Methods

Isolation and Screening

Soil samples were collected from the rhizospher and rhizoplane zone of potato cultivated in Hamadan, Iran. Twenty grams of air-dried soil samples were added to 100 mL of sterile water in Erlenmeyer flasks. The containers were heated in a water bath for 10 min at 80°C while the contents were agitated. Subsequently, 1-mL aliquots were added to 9 mL of sterile water and serial dilutions (10^{-1}–10^{-9}) were prepared. About 0.1 mL of each dilution was added and distributed on a sucrose-peptone agar medium. Plates were incubated at 37°C for 24–72 h, and different types of colonies were chosen and purified. These strains were screened by gelatin and/or casein hydrolysis tests. Gelatin hydrolysis was examined on agar medium containing 4 g/L of gelatin. After incubation at 37°C, plates were flooded with 12% (w/v) $HgCl_2$ in 20% (v/v) concentrated HCl, and hydrolysis was recorded when a clear zone appeared around the colonies. Casein hydrolysis was determined by supplementing nutrient agar with 10% skim milk, and the consequent production of a white precipitate around the colonies was confirmed by the appearance of a clear zone after flooding the plates with 10% (v/v) HCl. The promising strains were examined for morphologic, physiologic, and biochemical characteristics with reference to *Bergey's Manual of Systematic Bacteriology (7)* and *A Color Atlas of Bacillus Species (8)*.

All strains were spore-forming, Gram-positive, and rod-shaped bacteria. They were screened for alkaline protease activity and 2 of 93 isolates, HR-08 and KR-8102, were selected for further experiments. Both strains were catalase and oxidase positive and capable of using sodium citrate and sodium propionate as the sole carbon source. They were also able to grow in salt-containing nutrient media at different concentrations up to 7% (w/v). Lecithinase activity test and indole production were negative. The strains were facultative anaerobic and broke down L-(+)-arginine in anaerobic conditions; were able to grow at temperatures as high as 55°C; and produced acid compounds from glucose, mannitol, xylose, and arabinose. These morphologic, physiological, and biochemical data combined suggest that these strains belong to *Bacillus* species.

Culture Conditions and Partial Purification of Proteases

Two isolates were grown in liquid preculture medium containing 8 g/L of nutrient broth, 10 g/L of starch, 10 g/L of yeast extract, and 5 g/L of NaCl. Incubations were carried out with agitation at 180 rpm for 20 h. The growth rate of the microorganisms was determined by measuring the absorbance at 600 nm. The liquid medium used for the production of protease was composed 5 g/L of sucrose, 5 g/L of citric acid, 10 g/L of yeast extract, 1 g/L of K_2HPO_4, 0.1 g/L of $MgSO_4 \cdot 7H_2O$, and 0.1 g/L of $CaCl_2 \cdot 2H_2O$. The pH of the medium was adjusted to 7.0 with 10% (w/v) Na_2CO_3 solution, and effect of pH on enzyme production was studied at 37°C in a pH range of 7.0–10.5. The medium was inoculated at 5% (v/v) with a 20-h-old culture and incubated at 37°C in a shaker (180 rpm) for 48 h. The culture medium was centrifuged at 8000*g* for 10 min at 4°C. The cell-free supernatant was precipitated with 80% ammonium sulfate for 1 h at 4°C. After centrifuging at 15,000*g* for 20 min at 4°C, the pellet was dissolved in a small amount of 5 m*M* Tris-HCl buffer, pH 7.0, and dialyzed overnight against the same buffer. This partially purified enzyme was used for further studies.

Protease Assay

Protease activity was determined by the modified method of Kembhavi and Kulkami *(9)*. Supernatant (0.25 mL) was added to a tube containing 0.5 mL of 1% casein (dissolved in 50 m*M* Tris-HCl buffer, pH 7.0) and 0.25 mL of 50 m*M* Tris-HCl, pH 7.0, and incubated at 37°C for 30 min. Subsequently, 1 mL of 10% trichloroacetic acid solution was added to stop the proteolysis. The mixture was incubated at room temperature for 1 h. After incubation, the reaction mixture was centrifuged at 12,000*g* for 10 min, and the absorbance of the supernatant was measured at 280 nm.

Effect of pH on Enzyme Activity and Stability

The optimum pH of the protease was determined with 1% casein as substrate dissolved in different buffers (citrate phosphate, Tris-HCl, and

glycine NaOH). The pH stability of the protease was established by incubating the enzyme in buffers of different pH values in the range of 6.0–13.0 for 48 h at room temperature. Aliquots were withdrawn and proteolytic activity was determined at pH 9.0 and 37°C.

Effect of Temperature on Enzyme Activity and Stability

The activity of the enzymes was determined by incubating the reaction mixture at different temperatures ranging from 20 to 80°C. The irreversible thermoinactivation of the enzymes was examined by incubating the enzymes at ambient temperatures in the presence or absence of 10 m*M* $CaCl_2$ for a series of time intervals and cooling on ice. Finally, the residual activity was determined under assay conditions.

Effect of Inhibitors and a Chelator

The effect of protease inhibitors such as serine inhibitor (phenylmethylsulfonyl fluoride [PMSF]), cysteine inhibitors (iodoacetate, 2-mercaptoethanol), and a chelator of divalent cations (EDTA) was investigated. After 30 min of preincubation of inhibitor with enzymes at room temperature, residual protease activity was measured by standard assay method.

Compatibility of Protease With Laundry Detergents

Local detergents such as Sepid 3 (Paksan, Iran), Shoma (Toolipers, Iran), Tage (Behdad, Iran), and Yekta (Pakname, Iran) were used. The detergents were diluted in distilled water (0.5% [w/v]) and incubated with protease in the presence of 10 m*M* Ca^{2+} for 1 h at 40°C, and the residual activity was calculated.

Polyacrylamide Gel Electrophoresis and Zymogram Analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with 10% running gel. For zymography, 0.1% (w/v) casein copolymerized with the running gel and samples was loaded into the gel without heating, and electrophoresis was performed at 4°C. Following electrophoresis, gel was incubated in 2.5% Triton X-100 (v/v) for 30 min at room temperature with gentle agitation, after which the zymogram denaturing buffer was decanted and replaced with developing buffer (50 m*M* Tris, 0.2 m*M* NaCl, 5 m*M* $CaCl_2$, and 0.02% Brij). The gel was equilibrated for 30 min at room temperature with gentle agitation, then replaced with fresh buffer and incubated at 37°C for at least 4 h. The gel was stained with Coomassie brilliant blue R-250 (0.5% [w/v]) for 30 min and finally destained *(10)*.

The results presented herein are the mean from at least three repeated experiments in a typical run. Experiments were repeated to confirm reproducibility.

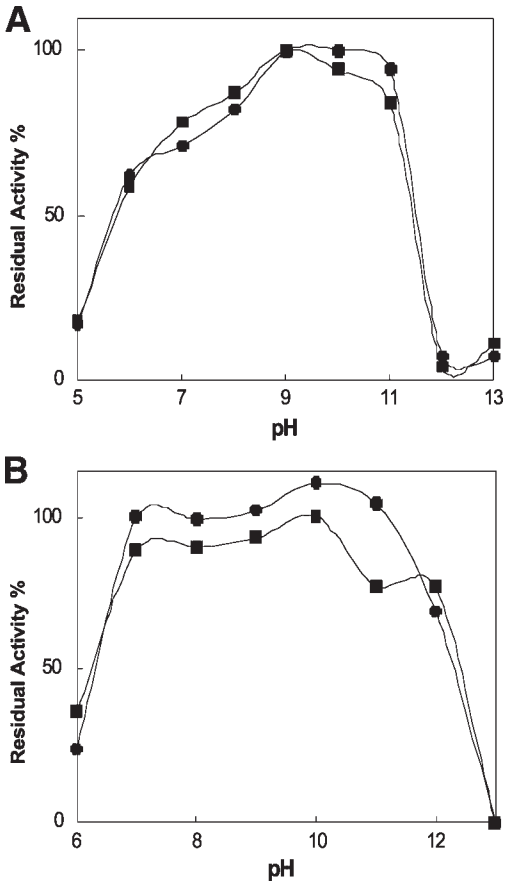

Fig. 1. Effect of pH on **(A)** activity and **(B)** stability of alkaline proteases from *Bacillus* sp. HR-08 (●) and KR-8102 (■).

Results and Discussion

Only 6 of 93 bacteria isolated from soil exhibited prominent clear zones around their colonies on gelatin agar medium. Two of them were identified as *Bacillus* sp. strains HR-08 and KR-8102. Both strains grew very fast and exhibited maximum production of extracellular proteases after 48 h of growth (data not shown).

Effect of pH and Temperature on Protease Activity

The results of pH studies indicate a broad pH activity range (7.0–11.0) for both enzymes. The optimum pH for alkaline proteases from *Bacillus* sp. HR-08 and KR-8102 was 10.0 and 9.0, respectively (Fig. 1A). Protease in strain KR-8102 at pH 10.0, 11.0, and 12.0 retained 90, 80, and 8% of its maximal activity, respectively. pH stability was determined by incubating the enzymes at different pH values in the range of 6.0–13.0 for 48 h at room

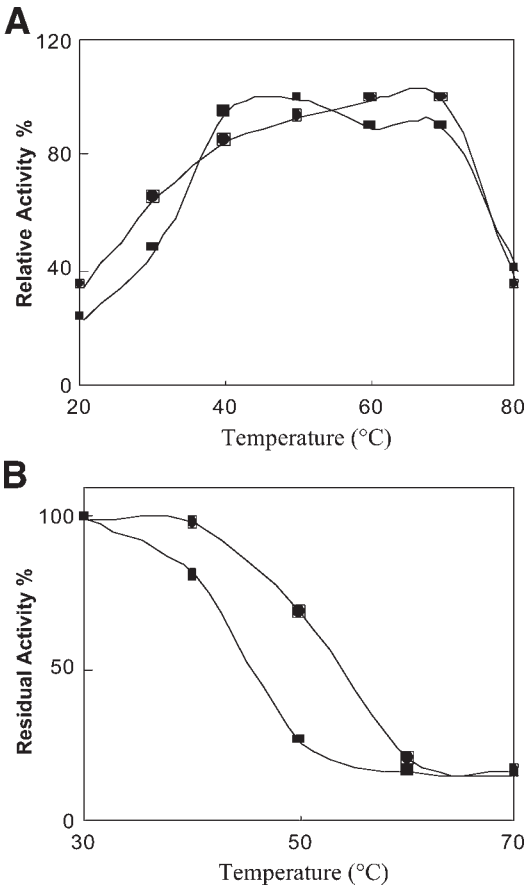

Fig. 2. **(A)** Effect of temperature on alkaline protease activity from *Bacillus* sp. HR-08 (●) and KR-8102 (■); **(B)** thermostability of alkaline proteases from HR-08 (●) and KR-8102 (■) at various temperatures. For more details, see Materials and Methods.

temperature (Fig. 1B). Both proteases were stable between pH 7.0 and 12.0 and retained more than 70% of their initial activity after 48 h.

Commercial proteases from microorganisms have maximum activity at pH 8.0–12.0 *(11)*. The pH required for both enzymes, HR-08 and KR-8102, is the same as for other alkaliphilic *Bacillus* sp. reported previously *(12)*. In other reports, a thiol-dependent serine alkaline protease from *Bacillus mojavensis (13)* and two alkaline proteases from *Bacillus* sp. strain GX 6638 *(14)* were shown to be optimally active at pH 11.0 and 10.3, respectively. The protease from *Bacillus* sp. KSM-K16 exhibited a much higher pH optimum of 12.3 at 55°C *(15)*. The alkaline proteases from *Pseudomonas aeruginosa* MN1 *(16)* and *Brevibacterium linens* ATCC 9174 *(17)* exhibited lower pH optima of 8.0 and 8.5 and were quite stable up to pH 10.0 and 12.0, respectively.

Alkaline proteases from strains HR-08 and KR-8102 were shown to be optimally active at temperatures of 65 and 50°C, respectively, with loss of

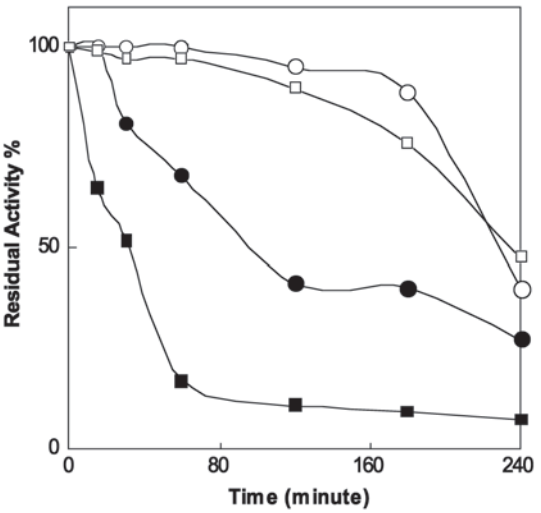

Fig. 3. Thermostability of proteases from *Bacillus* sp. HR-08 (circles) and KR-8102 (squares) in the absence (solid) and presence (open) of 10 m*M* $CaCl_2$ at 50°C.

activity above 70°C (Fig. 2A). The optimum temperature of proteases from *B. mojavensis (13)* and *P. aeruginosa* MN1 at pH 8.0 is 60°C, and temperatures up to 75°C have been reported for alkaline protease from *Bacillus stearothermophilus* F1 *(12)*.

Thermostability of alkaline proteases was first examined by incubating the enzymes for 180 min at different temperatures before measuring residual activity (Fig. 2B). The enzymes were stable from 30 to 50°C, but the activity decreased at higher temperatures. However, in the presence of 10 m*M* Ca^{2+}, enzyme activity stabilized at certain temperatures. Irreversible thermoinactivation of the enzymes was examined at 50, 60, and 70°C in the presence of 10 m*M* Ca^{2+}. The data show that the alkaline proteases from HR-08 and KR-8102 were highly stable at 50°C (Fig. 3). In addition, the half-lives were 120 and 100 min at 60°C and 14 and 13 min at 70°C for alkaline proteases from strains HR-08 and KR-8102, respectively (data not shown). Other investigators have also reported half-lives of 30 min *(9)*, 7 h *(18)*, 12 h *(19)*, 25 min *(14)*, and in the case of subtilisin Carlsberg 2.5 min at 60°C *(14)*. The present results suggest that these enzymes have good thermal stability compared with previous findings. The increased rate of autolysis of proteases at elevated temperature is one of the factors responsible for the rapid thermoinactivation of enzymes. Several groups have reported that calcium ions play a major role in enzyme stabilization at high temperatures *(19,20)*. The improvement in protease thermostability against thermal inactivation in the presence of Ca^{2+} can be explained by the possible strengthening of interactions inside protein molecules and the binding of Ca^{2+} to autolysis sites, which could prevent autolysis.

Table 1
Effect of Various Metal Ions (5 m*M*) and Some Inhibitors on Activity of Proteases From *Bacillus* sp. HR-08 and KR-8102

Metal ion/inhibitor	Concentration (m*M*)	Residual activity (%)	
		HR-08	KR-8102
PMSF	10	4	1
Iodoacetate	2	35	12
EDTA	5	80	69
2-Mercaptoethanol	10	73	123
DTT	5	79	116
$CaCl_2$	5	97	100
$MgCl_2$	5	97	94
$ZnCl_2$	5	50	55
$CuSO_4$	5	77	73
$MnSO_4$	5	73	82
$FeCl_2$	5	136	155

Effect of Various Inhibitors and Metal Ions

One can use specific inhibitors to study the active site of enzyme that is involved in the reaction as well as its cofactor requirements. Table 1 presents the effects of different inhibitors and metal ions on enzymes. Among the few inhibitors tested on protease, 10 m*M* PMSF and 2 m*M* iodoacetate were the most potent (Table 1 and Fig. 4). The protease from strain HR-08 retained more than 70% of its activity in the presence of 2-mercaptoethanol and dithiothreitol (DTT). These agents enhanced the activity of protease from KR-8102 (Table 1). The protease inhibitor PMSF strongly inhibits the serine residue in the active site, causing complete loss of enzyme activity *(13)*. As shown in Fig. 4, both proteases were also inhibited by iodoacetate, which strongly inhibits the cysteine residue at the active site *(13),* but were reactivated by DTT.

Our results suggest that these enzymes are thiol-dependent serine proteases. We did not observe an inhibitory effect of EDTA, which suggests that the enzymes are not metalloproteases. The results of metal ion experiments showed that ions such as Ca^{2+}, Mg^{2+}, Cu^{2+}, and Mn^{2+} at a concentration of 5 m*M* retained enzyme activity more than 70%, Fe^{2+} stimulated the protease activity more than 40%, but Zn^{2+} almost inhibited the activity of both strains by 50% (Table 1). Singh et al. *(21)* reported similar effects for Fe^{2+} on the activity of thermostable alkaline protease.

Effect of NaCl on Enzymes

Figure 5 presents enzyme activities after preincubation at different concentrations of NaCl (0.5–4 *M*). In the presence of 0.5 *M* NaCl, protease from strain HR-08 showed no change in activity, whereas 50% activation of protease from strain KR-8102 was observed in this condition. On the other hand, in the presence of 3 *M* NaCl, both enzymes retained 45% of activity.

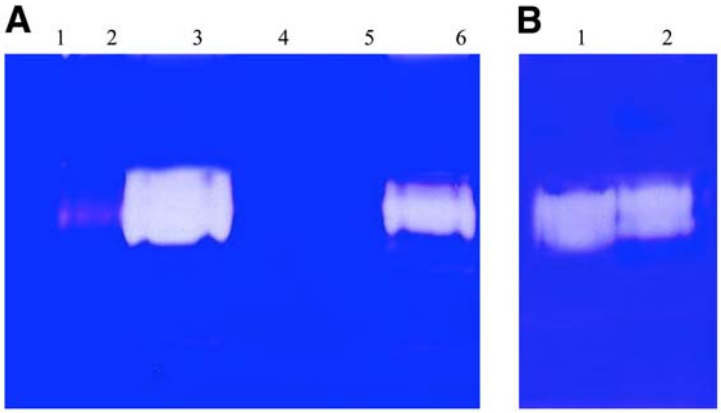

Fig. 4. Zymogram of alkaline proteases from *Bacillus* sp. HR-08 and KR-8102. **(A)** Lanes 1 and 4, KR-8102 and HR-08 proteases in presence of iodoacetate, respectively; lanes 2 and 5, KR-8102 and HR-08 proteases in presence of PMSF; lanes 3 and 6, KR-8102 and HR-08 proteases in Tris buffer. **(B)** Zymogram of alkaline proteases from KR-8102 (lane 1) and HR-08 (lane 2) after inhibition by iodoacetate (2 m*M*) and incubation in reactive buffer including 10 m*M* DTT. For more details, see Materials and Methods.

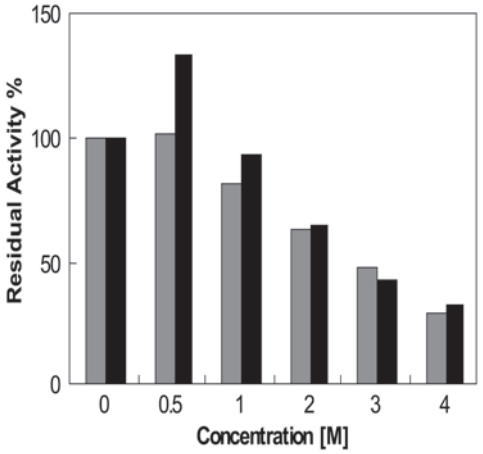

Fig. 5. Stability of alkaline proteases HR-08 (dotted bars) and KR-8102 (black bars) in NaCl. The enzymes were preincubated in different concentrations of NaCl (0.5–4 *M*) for 1 h at room temperature. The residual activity was measured by standard methods as described in Materials and Methods.

Similar reports on salt tolerance capacities of alkaline proteases are also available in the literature *(9,22,23)*.

Effect of Surfactants, Bleaching Agent, and Detergents

After preincubation with 0.5% SDS and 5% Triton X-100 for 30 min at room temperature, protease activity was determined and residual activity

Table 2
Effects of Laboratory Surfactants, Bleach Agent, and Local Detergents on Proteases[a]

Detergent	Concentration (%)	Residual activity (%)	
		HR-08	KR-8102
SDS	0.5	63	8
H_2O_2	5	82	90
Triton X-100	5	80	73
Sepid 3	0.5	85	78
Shoma	0.5	81	87
Tage	0.5	86	62
Yekta	0.5	95	88

[a]The enzymes were preincubated with 0.5% (w/v) SDS, 5% (v/v) H_2O_2, and 5% (v/v) Triton X-100 for 60 min at room temperature. Relative protease activities were measured under standard conditions.

was calculated. In the presence of SDS, the measured activity was 63% for HR-08 and 8% for KR-8102, whereas the activity was more than 80% in the presence of Triton X-100 for both enzymes (Table 2). In the presence of 5% H_2O_2, the activity of both enzymes remained more than 90%, showing that these alkaline proteases are also bleach stable. In the detergent industry, several oxidizing agents that may release H_2O_2, such as sodium perborate, are used as detergent compositions, and, hence, bleach-stable enzymes are suitable for the detergent industry *(24)*. Proteases from HR-08 and KR-8102 show high stability and compatibility with commercial detergents at 40°C for 1 h. The enzymes retained more than 80% of their initial activity in HR-08 and 60% in KR-8102 (Table 2). Based on these characteristics, *Bacillus* sp. alkaline proteases, especially strain HR-08, show promise for use in the detergent industry.

Conclusion

One of the most important features of the proteases that we studied from *Bacillus* sp. HR-08 and KR-8102 is their optimal activity at pH 9.0–10.0 and stability in an alkaline pH range of 7.0–12.0. These enzymes are stable at ionic/nonionic surfactants and in this respect show good compatibility with commercial detergents. Furthermore, the alkaline proteases, especially from strain HR-08, are stable at high temperature. All currently used detergent enzymes are alkaline and thermostable in nature with a high pH optimum. The most commercially available subtilisin-type proteases are also active in pH and temperature ranges between 8.0 and 12.0 and 50 and 60°C, respectively *(20,21,25–28)*. Further experiments on these proteases toward their purification and molecular genetic analyses are currently under way.

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