Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 798304, 8 pages
http://dx.doi.org/10.1155/2015/798304

Research Article

Production and Characterization of Alkaline Protease from a High Yielding and Moderately Halophilic Strain of SD11 Marine Bacteria

Hongxia Cui,[1,2] Liping Wang,[3] and Yang Yu[4]

[1]*College of Environmental and Chemical Engineering, Yanshan University, Qinhuangdao, Hebei 066004, China*
[2]*Hebei Province Key Laboratory of Applied Chemistry, Qinhuangdao, Hebei 066004, China*
[3]*Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia*
[4]*Flinders Centre for Nanoscale Science & Technology, School of Computer Science, Engineering and Mathematics, Flinders University, Adelaide, SA 5042, Australia*

Correspondence should be addressed to Hongxia Cui; hxcui@ysu.edu.cn

Received 16 December 2014; Revised 5 May 2015; Accepted 6 May 2015

Academic Editor: Murat Senturk

A marine bacterium SD11, which was isolated from sea muds (Geziwo Qinhuangdao Sea area, China), was used to produce thermostable alkaline serine nonmetal protease in the skim milk agar plate medium with 10% NaCl. The optimal temperature about the manufacture of the extracellular protease was ~60°C. The crude enzyme was stable at 20–50°C. The activity was retained to 60% and 45% after heating for 1 h at 60 and 70°C, respectively. The protease was highly active in a wide pH scope (8.0–10.0) and maximum protease activity exhibited at pH 10.0. The activity was restrained by phenylmethylsulfonyl fluoride (PMSF) but mildly increased (~107%) in the presence of ethylenediaminetetraacetic acid (EDTA), indicating that the production contains serine-protease(s) and nonmetal protease(s). Moreover, the crude alkaline protease was active with the 5 mM Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Na^{+}, and K^{+} that existed separately. In addition, the protease showed superduper stability when exposed to an anionic surfactant (5 mM SDS), an oxidizing agent (1% H_2O_2), and several organic solvents (methanol, isopropanol, and acetone). These results suggest that the marine bacterium SD11 is significant in the industry from the prospects of its ability to produce thermally stable alkaline protease.

1. Introduction

Protease and other carbohydrate-hydrolyzing enzymes are the most important among industrial enzymes and have been researched widely. Proteases constitute one of the third largest groups of enzymes in industry [1, 2], with two-thirds of the proteases produced by microorganisms commercially [3]. They are used in all kinds of industrial situations such as pharmaceuticals, drug manufacturing, detergents, surface cleaning formulations, waste treatment, silver recovery, digestive supplements, agrochemical additives, and diagnostic reagents [4–6]. It is said that 40% of the enzymes used today are produced by microorganisms including bacteria, molds, and yeasts [7]. The growth rate of microorganisms is very quick. A large number of microorganisms may be accumulated in a relatively short time through cultivation methods, while they can be used to generate a sufficient, regular supply of the expected product, such as proteases. Among them, bacteria are the most primary species of alkaline protease generators and *Bacillus* genus is the most dominant source [5, 8–10]. Proteases from microorganisms especially from *Bacillus* sp. are the most extensively developed industrial proteases that have been used mainly in detergent formulations [11, 12].

Recently, the enzymes produced by moderately halophilic microorganisms have gained significant attention [13]. Unlike other enzymes that can be inhibited by the concentrated salt solutions, they have optimal activities at high salinity conditions and, therefore, could be used in much severe

industrial procedures [14]. Certain species of halophilic microorganisms (such as *Pseudoalteromonas* sp. Strain CP76 [15], *Bacillus clausii* [16], and other halophilic isolates) have been commonly used for protease production [17].

The property of proteases used in abstergents relies on several indexes, such as ionic strength, pH, composition of the abstergent, washing temperature/procedure, and hardness of water. One of the most important features, for the enzymes used in detergents, is their stability, and different methods have been applied to better the washing performance and enzymatic stability against bleaching agents [18]. Nature is a good source for these enzymes, and current studies have been focusing on the proteolytic enzymes (such as hot spring microorganisms and terrestrial Bacilli [19–21]), and little attention was being given to marine microorganisms.

The oceans covering 71% of the planet possess an important bioresource for microorganisms. The metabolism and physiologic features of marine microorganisms were found to be different from that of terrestrial microorganisms [22]. Isolation and screening of strains from marine environment are hoped to supply new strains using the manufacture of stable and active proteases in highly alkaline environments with the presence of chemical reagents from detergents. In this paper, a high alkaline protease yielding, moderately halophilic bacterium SD11 was screened and isolated from marine bacteria. The effects of the activities and stabilities of the protease by pH, temperature, metal ions, organic solvents, and detergents were also studied.

In the paper, the screening of marine bacteria was reported. The moderately halophilic bacterium SD11 had the high ability to produce alkaline protease. The biochemical characterization of the crude enzyme was investigated too.

2. Experiments

2.1. Isolation and Screening of Alkaline Proteases Producing Strains. In order to isolate the protease-producing bacteria, the samples were gathered from sea muds in the Geziwo Qinhuangdao Sea area and were diluted with sterile sea water. Then the diluents were spread onto skimmed milk agar plates containing beef extract (3 g/L), peptone (10 g/L), NaCl (10 g/L), skimmed milk powder (10 g/L), and 18 g/L agar, with pH adjusted to 8.5. Plates were incubated 24 h at 37°C. It was confirmed that the screened bacteria could produce proteinase when the clear proteolytic zone was found around the colonies [23], which were chosen for the purification the subsequent research. One loop of the cells of the purified strains was inoculated with 20 mL of protease production medium consisting of beef extract (3 g/L), peptone (10 g/L), and NaCl (7 g/L), with pH 8.5. The medium was prepared with sea water in 100 mL flask and aerobically cultivated by shaking at 150 rpm and 37°C for 24 h. Strain SD11 was found to generate maximum enzyme activity in the fluid medium. So it was used to investigate the characterization of protease. The organism was stored at 4°C.

2.2. Fermentations and Crude Enzyme Preparation. SD11 strain was fermented in protease production medium consisting of beef extract (3 g/L), peptone (10 g/L), and NaCl (7 g/L), with pH 8.5. The media were autoclaved for 20 min at 121°C. Fermentation processes were completed on a rotary shaker at 37°C for 24 h at 150 rpm, with 50 mL working volume in 250 mL triangular flasks.

The centrifugal machine was used to separate the biomass produced at 4°C for 10 min at 10000 rpm. The supernatant was added different concentrations ammonium sulfate to reach different saturation levels (0–80%) for precipitation. The fraction of precipitation was dissolved in phosphate buffer (PBS, pH 7.4). It was dialyzed against the same buffer. Then the contents of protein and activity of enzyme were determined. The specific activity of the protease was showed as enzyme activity per mg protein [24].

2.3. Denatured Polyacrylamide Gel Electrophoresis. The molecular weight of the protease was tested by SDS-PAGE with stacking gel (4%) and resolving gel (10%) as introduced by Laemmli [25] with slight modification. The enzymatic molecular weight was determined using a calibration molecular weight kit with markers containing of 14.4 kD, 18.4 kD, 25.0 kD, 35.0 kD, 45.0 kD, 66.2 kD, and 116 kD. Coomassie Brilliant Blue R-250 was used to stain and visualize the bands of the protein.

2.4. Determination of Enzyme Activity. The activity of protease (caseinolytic) was assayed by a modification method of Kunitz [26]. The reaction system (0.6 mL) consisted of 150 μL of 1% Hammerstein grade casein in 200 mM glycine-NaOH buffer (pH 10.0) and 150 μL of enzyme solution or cultivated supernatant. The reaction was beginning with adding an enzyme solution at 40°C. The reaction was terminated by adding 0.4 M trichloroacetic acid (TCA, 300 μL) after the reaction was sustained for 15 min. The reaction system was sustained on ice for another 10 min. Then it was centrifuged at 4°C for 10 min at 10000 rpm. 0.3 mL of supernatant, the hybrid with 1.5 mL of a 0.4 M Na_2CO_3 solution in 2.1 mL distilled water, and 0.3 mL of Folin-Ciocalteu reagent were incubated for 20 min at 40°C. The amount of L-tyrosine from digested casein was measured by determining the absorbance by using a UV-vis spectrophotometer at 680 nm in the supernatant. The standard curve was done using L-tyrosine as a standard substance. One unit of enzyme activity was defined as the quantity of protease that liberates 1 μg/mL of tyrosine per min [27].

2.5. Determination of Protein. The method described by Bradford was used to measure the protein concentration [28]. The bovine serum albumin was selected to as a standard. The absorbance was determined at 280 nm.

2.6. Biochemical Characteristics

2.6.1. Effect of Crude Alkaline Protease Activity and Stability by pH. The range of pH 7.0–13.0 was selected to observe the optimal pH of the preliminarily purified protease at 40°C. The casein 1% (w/v) was used as a substrate. The influence of pH

on protease stability was also determined. The protease was incubated in advance in different pH buffers of 7.0–12.0 for 1 h at 40°C. The incubations were taken at the suitable time to measure the residual activity at 40°C and pH 10.0. 200 mM sodium phosphate buffer was used for pH 7.0–8.0. 200 mM glycine-NaOH buffer was used for 9.0–13.0.

2.6.2. Effect of Crude Alkaline Protease Activity and Stability by Temperature. The influence of alkaline protease activity by temperature was investigated from 20 to 70°C in pH 10.0 for 20 min with casein substrate. Thermostability of the protease was also investigated. The preliminarily purified protease was incubated in advance for 1 h at different temperatures (20, 30, 40, 50, 60, and 70°C). The incubations were taken at suitable time to examine the residual enzyme activity under normal conditions. The unheated protease was regarded as 100% [29].

2.6.3. Effects of Crude Alkaline Protease Activity by Inhibitors and Metal Ions. The influences of protease activity by all kinds of metal ions (5 mM) were examined by adding the divalent (Zn^{2+}, Mn^{2+}, Ca^{2+}, or Cu^{2+}) metal ions and monovalent (Na^{+} and K^{+}) in the reaction system. The protease activity was regarded as 100% when there were no any metallic ions in the reaction system.

The effects of 5 mM EDTA or PMSF (phenylmethylsulfonyl fluoride) on the activity of protease were investigated too. The preliminarily purified protease was incubated in advance with EDTA or PMSF at 40°C for 20 min. The incubations were taken at the suitable time to determine the residual enzyme activity under normal conditions. The reaction mixture was regarded as control (100%) without EDTA or PMSF.

2.6.4. Effect of Crude Alkaline Protease Activity by Organic Solvents and Detergents. The influence of crude enzyme activity by various organic solvents was examined. The crude enzyme was incubated in advance in 100 mM glycine-NaOH buffer system (pH 10.0) with 25% (v/v) of several organic solvents (isopropanol, methanol, and acetone) and shaken at 150 rpm for 20 min at 40°C. The incubations were taken at the suitable time to determine the residual activity under normal conditions. The activity of the protease was regarded as controls without any organic solvents.

The effects of 5 mM surfactant SDS and 1% (v/v) oxidizing agent H_2O_2 on protease activity were investigated with incubating the crude protease in advance at 40°C for 20 min. The incubations were taken at the suitable time to determine the residual enzyme activity under normal conditions. The activity of the protease was regarded as 100% without SDS or H_2O_2.

3. Results and Discussions

3.1. Isolation and Screening of Alkaline Proteases Producing Bacteria. In this study, total 116 bacteria were obtained from sea muds obtained, but only 14 strains could form a clear zone of hydrolysis of skimmed milk powder on the agar plate. This finding is similar to the results reported by Visôtto et al. [30] where they found several bacterial strains including *B. subtilis* with proteolytic activity from the caterpillar gut on calcium caseinate agar. Among them, one strain named SD11 provided very clear hydrolysis zones around the bacterial colonies on the milk agar plates with 10% NaCl that is indicating it produces dominant amounts of protease (Figure 1). The ratio of the clear hydrolysis zone diameter and that of the bacterial colony on skimmed milk agar plate, six isolates SD7, SD8, SD9, SD10, SD11, and SDL9, exhibited the highest ratio (>1.5), which were shown in Table 1. And then, they were investigated for the production of protease in liquid medium. In Figure 2, the results indicated that protease activity of strain SD11 was the highest 171.9 U/mL in a nonoptimized medium after it was incubated for 24 h. Therefore, strain SD11 was selected for all subsequent researches.

Table 1: The diameter of colony and hydrolyzed zone.

Strains	Diameter of colony (D)	Diameter of hydrolyzed zone (d)	d/D
SD7	1.88	2.84	1.51
SD8	1.64	2.78	1.70
SD9	1.83	2.95	1.61
SD10	1.77	3.2	1.81
SD11	2.31	4.05	1.75
SDL9	2.15	3.4	1.58

Figure 1: Screening of strain SD11 for protease activity. Isolate was streaked on the agar plate containing skimmed milk powder and 10% NaCl, incubated for 24 h at 37°C. The clear hydrolysis zone indicated the hydrolysis of skimmed milk.

3.2. Alkaline Protease Partial Purification. The purification process of protease from strain SD11 was illustrated in Table 2. The supernatant contained a total activity of 1970.70 U after growth of strain SD11 for 24 h and a specific activity of 182.47 U/mg protein. It was regarded as the control (100%). And then, the supernatant was added with ammonium sulfate for precipitation under 0–80% saturation. The maximum activity of protease was obtained with 60% ammonium

Table 2: Purification of protease from strain SD11.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Culture supernatant	1970.70	10.80	182.47	100	1
Ammonium sulphate precipitation (60%)	1340.18	3.10	432.32	68	2.37

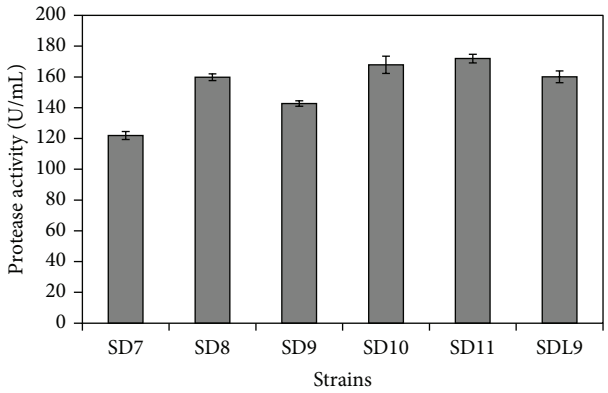

Figure 2: Protease activity of culture supernatant of different strains.

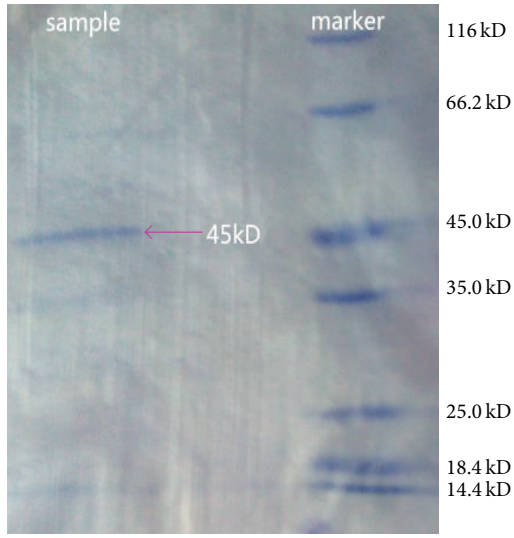

Figure 3: SDS-PAGE electrophoresis of the crude protease of strain SD11.

sulfate saturation. The total activity of protease was 1340.18 U, and purification fold was 2.37. The specific activity was increased to 432.32 U/mg protein with a yield of 68%. The production of ammonium sulfate precipitation was conducted by SDS-PAGE electrophoresis method (Figure 3). The results showed that one clear band existed, and the molecular weight of the band of the protease was about 45 kD. Then the crude protease of strain SD11 was used to study the characteristics.

3.3. Effect of Activity and Stability of the Crude Alkaline Protease by pH. The influence of protease activity by pH was examined with the pH from 7.0 to 13.0 at 40°C. The protease showed high activity in a broad pH range of 8.0–11.0 and the optimal pH was about 10.0 (Figure 4(a)). The relative activity of the enzyme was 90% at pH 11.0, and it was reduced rapidly when pH > 11.0. The relative activity of enzyme decreased to 45% and 10% at pH 12.0 and 13.0, respectively. These results showed that the enzyme was alkaline protease [31]. The range of pH is normal between 9.0 and 10.5 in detergents solution. The high enzyme activity in alkaline conditions is a significant element needed in nearly all detergent enzymes. These were also described by Li et al. [32], Hadj-Ali et al. [33], and Cheng et al. [34]. It proved that the enzyme produced by strain SD11 might be useful in the detergents solution. Meanwhile, the enzyme is more effective at alkaline environment compared to the detergent enzymes mainly used in the industry, such as Alcalase (Novozymes A/S) produced by *B. Licheniformis* which has a maximal enzyme activity at pH 8.0–9.0, and Savinase (Novozymes A/S) manufactured by *B. clausii* which has a largest activity at pH 8.0–10.0 [35].

The stability of pH was measured after the enzyme was incubated in advance for 1 h in different pH buffers between 7.0 and 12.0 at 40°C. Then the enzyme activity was examined at 40°C and pH 10.0. The results were shown in Figure 4(b). The activity of the enzyme was very stable when the pH was between 7.0 and 11.0 (>80%). However, the activity of enzyme reduced rapidly at pH 12.0 (45%). These results indicate the enzyme is suitable for potential industrial applications (e.g., protease in detergent), which requests broad pH (9.0–11.0) stability [36, 37].

3.4. Effect of Activity and Stability of the Crude Alkaline Protease by Temperature. The effect of the crude protease activity by temperature was investigated. The results showed that the crude alkaline protease from the strain SD11 was active from 30 to 70°C with an optimal temperature at ~ 60°C (Figure 5(a)). The relative activities of protease were about ~97% and ~100% at 50 and 60°C, respectively. The optimal temperature of the protease was 60°C which had been reported from *B. cereus* BG1 [38], *B. pumilus* [39], and *B. mojavensis* [35].

The thermostability investigations of the crude protease indicated that the protease was absolutely stable within temperatures 50°C after 1 h incubation. The enzyme remained fully active after 1 h incubation at 40°C. Even at 60 and 70°C, the residual protease activity was 60 and 45%, respectively,

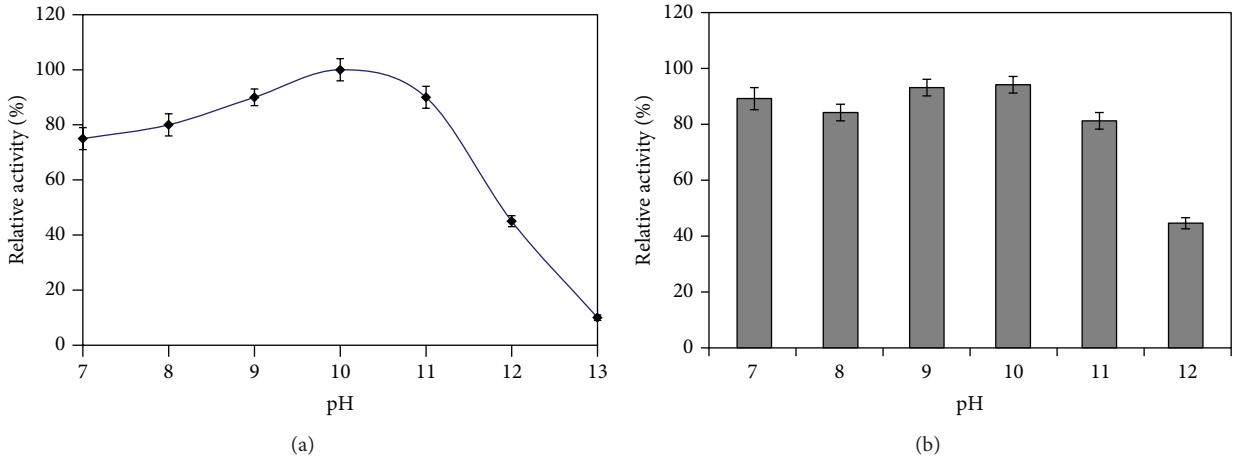

FIGURE 4: The effect of protease activity (a) and stability (b) by pH from strain SD11. (a) The enzyme activity was tested in different pH buffer solutions at 40°C. The enzyme activity was maximum at pH 10.0 regarded as 100%. (b) The protease stability against pH was examined after the enzyme was incubated in advance in different pH buffer solutions for 1 h at 40°C. The residual enzyme activity was tested at 40°C and pH 10.0. The enzyme activity without incubating was regarded as 100%. Each point indicates the average value of three times, and error bars show standard deviation.

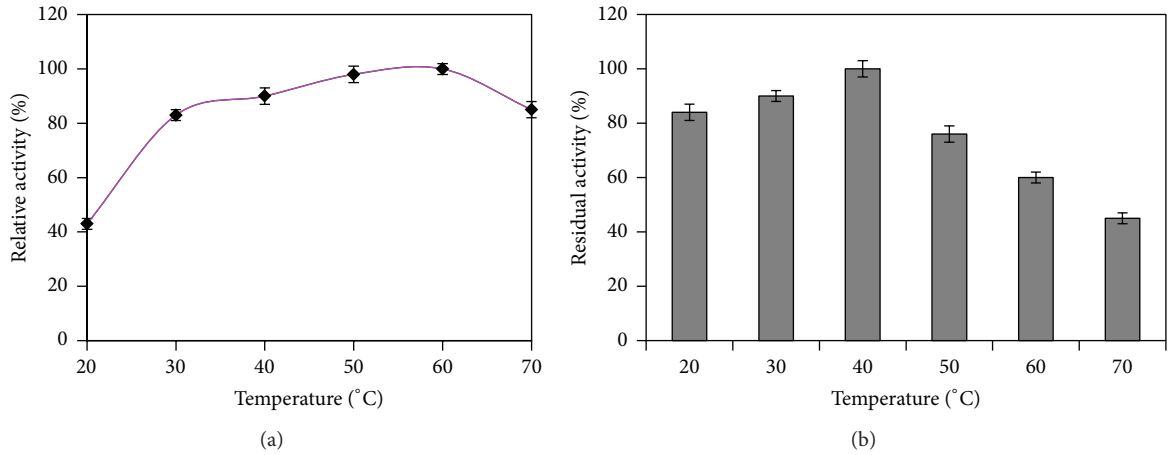

FIGURE 5: Effect of the activity (a) and stability (b) of the crude protease by temperature from strains SD11. (a) The activity of the enzyme was tested at various temperatures (20–70°C). The enzyme activity was maximum at 60°C regarded as 100%. (b) The protease stability against temperature was examined after the crude enzyme was incubated under temperatures between 20 and 70°C for 1 h. The residual activity was tested under the normal conditions. The enzyme activity without incubating was regarded as 100%. Each point indicates the average value of three times, and error bars show standard deviation.

showing that the enzyme would be used under moderate heating conditions (Figure 5(b)). The thermostability of crude protease was higher than that of alkaline protease from *B. licheniformis* MP1 [40], in which the enzyme activity was retained fully even incubated for 2 h at 50°C but remained only 41 and 5% after being incubated for 1 h at 60°C and 20 min at 70°C, respectively. This thermal stability would be a superiority of this enzyme application in the industry such as laundry detergent formulations [5].

3.5. Effects of Crude Alkaline Protease Activity by Inhibitors and Metal Ions. The influences of enzyme inhibitor and chelating agent on the enzyme activity were investigated. The results were summarized in Table 3. As we have seen, the crude enzyme was absolutely restrained by PMSF (phenylmethylsulfonyl fluoride, 5 mM), the serine protease inhibitor, demonstrating that the crude enzyme from strain SD11 contained serine-proteases. However, a metalloprotease inhibitor (EDTA) did not inhibit the enzyme activity, indicating the absence of metalloprotease in strain SD11. In fact, the activity of the enzyme from strain SD11 was active to 107% when preincubated with 5 mM EDTA. These findings are not in agreement with earlier publications in which the enzymatic activity was found slightly restrained by EDTA [40]. These results demonstrated that the crude protease would be useful as an abluent additive for there are always the chelating agents in most abluents. Chelating agents are used for the accelerant in the stain removal and water softeners [18].

Table 3: Influences of the activity of the crude protease by various metal ions and enzyme inhibitors.

Chemicals	None	PMSF	EDTA	Ca^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+}	Na^{+}	K^{+}
Concentration (mM)	—	5	5	5	5	5	5	5	5
Activity (%)	100	0	107 ± 3.3	145 ± 3.6	142 ± 3.1	115 ± 2.6	119 ± 2.3	136 ± 2.1	133 ± 2.9

The enzyme activity was tested under normal conditions after it was incubated in advance with various inhibitors for 20 min at 40°C. The enzyme activity was regarded as 100% without incubating. The influence of enzyme activity by metal ions was measured by testing the enzyme activity with metal ions (5 mM). The enzyme activity was regarded as 100% without metal ions. The enzyme activities indicate the mean of three experiments.

Table 4: Influence of crude alkaline protease activity by organic solvents and surfactant.

Additives	None	SDS	H_2O_2	Methanol	Isopropanol	Acetone
Concentration	—	5 mM	1% (v/v)	25% (v/v)	25% (v/v)	25% (v/v)
Relative activity (%)	100	95 ± 1.3	87 ± 1.1	138 ± 1.1	101 ± 1.6	141 ± 2.1

The enzyme activity was tested under normal conditions after crude enzyme was incubated in advance with various additives at 40°C and pH 10.0 for 20 min. The enzyme activity without incubating was regarded as 100%. The protease activities indicate the mean of three experiments.

The influences of crude protease activity were also investigated at 40°C and pH 10.0 by the addition of the metal ions (5 mM) to the reaction system (Table 3). The additive of Zn^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Na^{+}, and K^{+} increased the enzyme activity by 145, 142, 115, 119, 136, and 133% of the control, respectively.

3.6. Effect of Crude Alkaline Protease Activity by Organic Solvents and Surfactant. In order to enhance the washing performance, a good protease used in detergent must be effective during washing and be stable and compatible with common detergent components such as oxidizing agents, surfactants, and other additions [4]. The crude protease from strain SD11 was preincubated at 40°C for 20 min existing with SDS and H_2O_2, and the residual enzyme activity was determined at 40°C and pH 10.0 (Table 4). The enzyme was found to be very stable with the SDS at 5 mM, a strong anionic surfactant. This is not in agreement with the report from Hadj-Ali et al. [33], at which inhibition of enzyme activity with SDS was a common characteristic of serine-proteases. Oxidizing agents H_2O_2 at 1% (v/v) caused a moderate inhibition 13%. The present crude enzyme from strain SD11 seems very stable when these detergent additives were present.

Enzymatic synthesis of peptides has attracted great attention due to the numerous advantages of ester and peptide synthesis [41]. Proteases could be used for ester synthesis if they were stable with organic solvents [20, 42]. The influences of enzyme activity by different water-miscible organic solvents were measured at 25% final concentration at 40°C and pH 10.0. As shown in Table 4, the crude enzyme activity was enhanced significantly by methanol and acetone in the reaction system. The relative activities were ~138% and ~141% with methanol and acetone, respectively, while the activity of the crude enzyme was almost not affected by isopropanol. It can be concluded that the enzyme produced by strain SD11 is highly stable in different organic solvents.

4. Conclusions

In the paper, we reported the screening of strain SD11 producing alkaline protease. The characterizations of the crude alkaline protease from strain SD11 were also reported. The crude enzyme contained serine protease and had no metalloprotease. The crude protease had optimal activity at pH 10.0 and a broad range of pH stability (7.0–11.0). Furthermore, the protease was stable and active over a broad range of temperatures, had an optimum activity at 60°C, and was also stable until 60°C. The crude protease activity was improved by the addition of various 5 mM solutions of Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Na^{+}, and K^{+}. The enzyme also could tolerate and be stable against oxidizing agent H_2O_2, anionic surfactant SDS, and organic solvents. These characteristics suggested that this enzyme would be suitable for industrial applications such as the synthesis of peptides and detergent formulations. It also could be used in the processes performed at wide ranges of temperatures and in alkaline environments. In future work, we will further investigate the purification, 3D structure determination, and structure-function relationship through site-directed mutagenesis of the protease from strains SD11.

Conflict of Interests

The authors declare that they have no direct financial relation with the commercial identities mentioned in this paper that might lead to a conflict of interests for any of them.

Acknowledgments

The authors are very thankful for the Hebei Province natural Science Foundation of China (D2010001149, D2014203102) and Ministry of Education by the Specialized Research Fund for the Doctoral program of Higher Education (20131333120010). Liping Wang is supported by NHMRC Postgraduate Scholarship Grant (1094606).

References

[1] M. B. Rao, A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande, "Molecular and biotechnological aspects of microbial proteases," *Microbiology and Molecular Biology Reviews*, vol. 62, no. 3, pp. 597–635, 1998.

[2] O. Kirk, T. V. Borchert, and C. C. Fuglsang, "Industrial enzyme applications," *Current Opinion in Biotechnology*, vol. 13, no. 4, pp. 345–351, 2002.

[3] V. J. Mehta, J. T. Thumar, and S. P. Singh, "Production of alkaline protease from an alkaliphilic actinomycete," *Bioresource Technology*, vol. 97, no. 14, pp. 1650–1654, 2006.

[4] C. G. Kumar and H. Takagi, "Microbial alkaline proteases: from a bioindustrial viewpoint," *Biotechnology Advances*, vol. 17, no. 7, pp. 561–594, 1999.

[5] R. Gupta, Q. K. Beg, and P. Lorenz, "Bacterial alkaline proteases: molecular approaches and industrial applications," *Applied Microbiology and Biotechnology*, vol. 59, no. 1, pp. 15–32, 2002.

[6] P. Kamath, V. M. Subrahmanyam, J. Venkata-Rao, and P. Vasantha-Raj, "Optimization of cultural conditions for protease production by a fungal species," *Indian Journal of Pharmaceutical Sciences*, vol. 72, no. 2, pp. 161–166, 2010.

[7] C. Sandhya, A. Sumantha, G. Szakacs, and A. Pandey, "Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation," *Process Biochemistry*, vol. 40, no. 8, pp. 2689–2694, 2005.

[8] S. K. Singh, V. R. Tripathi, R. K. Jain, S. Vikram, and S. K. Garg, "An antibiotic, heavy metal resistant and halotolerant *Bacillus cereus* SIU1 and its thermoalkaline protease," *Microbial Cell Factories*, vol. 9, article 59, 7 pages, 2010.

[9] O.-H. Ban, S.-S. Han, and Y. N. Lee, "Identification of a potent protease-producing bacterial isolate, *Bacillus amyloliquefaciens* CMB01," *Annals of Microbiology*, vol. 53, no. 1, pp. 95–103, 2003.

[10] R. N. Z. R. A. Rahman, M. Basri, and A. B. Salleh, "Thermostable alkaline protease from *Bacillus stearothermophilus* F1; nutritional factors affecting protease production," *Annals of Microbiology*, vol. 53, no. 2, pp. 199–210, 2003.

[11] R. Gupta, K. Gupta, R. K. Saxena, and S. Khan, "Bleach-stable, alkaline protease from *Bacillus* sp.," *Biotechnology Letters*, vol. 21, no. 2, pp. 135–138, 1999.

[12] K. Jellouli, A. Bougatef, L. Manni, R. Agrebi, R. Siala, and I. Younes, "Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio metschnikovii* J1," *Journal of Industrial Microbiology & Biotechnology*, vol. 36, no. 7, pp. 939–948, 2009.

[13] A. Ventosa, *Halophilic Microorganisms*, Springer, Berlin, Germany, 2004.

[14] M. A. Amoozegar, A. Z. Fatemi, H. R. Karbalaei-Heidari, and M. R. Razavi, "Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004," *Microbiological Research*, vol. 162, no. 4, pp. 369–377, 2007.

[15] C. Sánchez-Porro, E. Mellado, C. Bertoldo, G. Antranikian, and A. Ventosa, "Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76," *Extremophiles*, vol. 7, no. 3, pp. 221–228, 2003.

[16] C. G. Kumar, H.-S. Joo, Y.-M. Koo, S. R. Paik, and C.-S. Chang, "Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate," *World Journal of Microbiology and Biotechnology*, vol. 20, no. 4, pp. 351–357, 2004.

[17] L. Lama, I. Romano, V. Calandrelli, B. Nicolaus, and A. Gambacorta, "Purification and characterization of a protease produced by an aerobic haloalkaliphilic species belonging to the *Salinivibrio* genus," *Research in Microbiology*, vol. 156, no. 4, pp. 478–484, 2005.

[18] A. Haddar, A. Sellami-Kamoun, N. Fakhfakh-Zouari, N. Hmidet, and M. Nasri, "Characterization of detergent stable and feather degrading serine proteases from *Bacillus mojavensis* A21," *Biochemical Engineering Journal*, vol. 51, no. 1-2, pp. 53–63, 2010.

[19] R. K. Patel, M. S. Dodia, R. H. Joshi, and S. P. Singh, "Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp," *Process Biochemistry*, vol. 41, no. 9, pp. 2002–2009, 2006.

[20] S. Ç. Aksoy, A. Uzel, and E. E. H. Kocabaş, "Extracellular serine proteases produced by *Thermoactinomyces* strains from hot springs and soils of West Anatolia," *Annals of Microbiology*, vol. 62, no. 2, pp. 483–492, 2012.

[21] R. K. Sarma, R. Debnath, R. Saikia, P. J. Handique, and T. C. Bora, "Phylogenetic analysis of alkaline proteinase producing fluorescent pseudomonads associated with green gram (*Vigna radiata* L.) rhizosphere," *Folia Microbiologica*, vol. 57, no. 2, pp. 129–137, 2012.

[22] C. Imada, "Enzyme inhibitors of marine microbial origin with pharmaceutical importance," *Marine Biotechnology*, vol. 6, no. 3, pp. 193–198, 2004.

[23] R. Salwan, A. Gulati, and R. C. Kasana, "Phylogenetic diversity of alkaline protease-producing psychrotrophic bacteria from glacier and cold environments of Lahaul and Spiti, India," *Journal of Basic Microbiology*, vol. 50, no. 2, pp. 150–159, 2010.

[24] B. K. Bajaj and P. Sharma, "An alkali-thermotolerant extracellular protease from a newly isolated *Streptomyces* sp. DP2," *New Biotechnology*, vol. 28, no. 6, pp. 725–732, 2011.

[25] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[26] M. Kunitz, "Crystalline soybean trypsin inhibitor. II. General properties," *The Journal of General Physiology*, vol. 30, no. 4, pp. 291–310, 1947.

[27] M. Cheng, S. Takenaka, S. Aoki, S. Murakami, and K. Aoki, "Purification and characterization of an eggshell membrane decomposing protease from *Pseudomonas aeruginosa* strain ME-4," *Journal of Bioscience and Bioengineering*, vol. 107, no. 4, pp. 373–378, 2009.

[28] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[29] W. Zhu, D. Cha, G. Cheng, Q. Peng, and P. Shen, "Purification and characterization of a thermostable protease from a newly isolated *Geobacillus* sp. YMTC 1049," *Enzyme and Microbial Technology*, vol. 40, no. 6, pp. 1592–1597, 2007.

[30] L. E. Visôtto, M. G. A. Oliveira, A. O. B. Ribon, T. R. Mares-Guia, and R. N. C. Guedes, "Characterization and identification of proteolytic bacteria from the gut of the *Velvetbean* caterpillar (Lepidoptera: Noctuidae)," *Environmental Entomology*, vol. 38, no. 4, pp. 1078–1085, 2009.

[31] A. Anwar and M. Saleemuddin, "Alkaline proteases: a review," *Bioresource Technology*, vol. 64, no. 3, pp. 175–183, 1998.

[32] S. Li, B. F. He, Z. Z. Bai, and P. K. Ouyang, "A novel organic solvent-stable alkaline protease from organic solvent-tolerant

Bacillus licheniformis YP1A," *Journal of Molecular Catalysis B: Enzymatic*, vol. 56, no. 2-3, pp. 85–88, 2009.

[33] N. E. Hadj-Ali, R. Agrebi, B. Ghorbel-Frikha, A. Sellami-Kamoun, S. Kanoun, and M. Nasri, "Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1," *Enzyme and Microbial Technology*, vol. 40, no. 4, pp. 515–523, 2007.

[34] K. Cheng, F.-P. Lu, M. Li, L.-L. Liu, and X.-M. Liang, "Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations," *African Journal of Biotechnology*, vol. 9, no. 31, pp. 4942–4953, 2010.

[35] Q. K. Beg and R. Gupta, "Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*," *Enzyme and Microbial Technology*, vol. 32, no. 2, pp. 294–304, 2003.

[36] Y. C. Tsai, R. Y. Juang, S. F. Lin, S. W. Chen, M. Yamasaki, and G. Tamura, "Production and further characterization of an alkaline elastase produced by alkalophilic *Bacillus* strain Ya-B," *Applied and Environmental Microbiology*, vol. 54, pp. 3156–3161, 1988.

[37] B. Jaouadi, S. Ellouz-Chaabouni, M. Rhimi, and S. Bejar, "Biochemical and molecular characterization of a detergent-stable serine alkaline protease from *Bacillus pumilus* CBS with high catalytic efficiency," *Biochimie*, vol. 90, pp. 1291–1305, 2008.

[38] B. Ghorbel-Frikha, A. Sellami-Kamoun, N. Fakhfakh, A. Haddar, L. Manni, and M. Nasri, "Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1," *Journal of Industrial Microbiology and Biotechnology*, vol. 32, no. 5, pp. 186–194, 2005.

[39] C. G. Kumar, "Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*," *Letters in Applied Microbiology*, vol. 34, no. 1, pp. 13–17, 2002.

[40] M. N. Gupta and I. Roy, "Enzymes in organic media: forms, functions and applications," *European Journal of Biochemistry*, vol. 271, no. 13, pp. 2575–2583, 2004.

[41] K. Jellouli, O. Ghorbel-Bellaaj, H. B. Ayed, L. Manni, R. Agrebi, and M. Nasri, "Alkaline-protease from *Bacillus licheniformis* MP1: purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization," *Process Biochemistry*, vol. 46, no. 6, pp. 1248–1256, 2011.

[42] D. Kumar and T. C. Bhalla, "Microbial proteases in peptide synthesis: approaches and applications," *Applied Microbiology and Biotechnology*, vol. 68, no. 6, pp. 726–736, 2005.