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Screening of a newly marine bacteria producing alkaline protease from Qinhuangdao sea area and its characterization of alkaline protease

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ABSTRACT

A marine bacterium SD8 producing thermostable alkaline serine nonmetal protease was isolated from sea muds in Geziwo Qinhuangdao sea area, China. The crude extracellular protease produced by the isolate had optimal activity at about 60 ℃. The enzyme was stable at 20-50 ℃ and retained 58 and 43% of its initial activity after heating for 1 h at 60 and 70 ℃, respectively. The protease was highly active from pH 7.0-11.5 and stable from pH 7.0-11.0, with an optimum at pH 10.0. The activity was totally lost in the presence of PMSF(phenylmethylsulfonyl fluoride) and slightly increased in the presence of EDTA, suggesting that the preparation contains serine-protease(s) and nonmetal protease(s). Moreover, the crude protease was activated by the presence of 5mM Ca^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}. Furthermore, the enzyme showed excellent stability towards anionic surfactant SDS(5mM). In addition, the enzyme was also stable towards oxidizing agents, retaining 89% of its initial activity after 20 min incubation in the presence of 1% H_2O_2. The crude protease was found to be stable in the presence of several organic solvents too. The marine bacterium strain SD8 selected is thus promising thermostable alkaline protease producers for industrial use.

Keywords: Alkaline protease; marine bacteria; Thermostability; organic solvents; Qinhuangdao

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INTRODUCTION

Proteases, also known as peptidyl-peptide hydrolases, are the most important industrial enzymes accounting for approximately 60% of the total industrial enzyme market[1], with two-thirds of the proteases produced commercially being or microbial origin[2]. They have numerous applications in the industrial production of different items including detergents, foods, pharmaceuticals, leathers and diagnostic reagents. These enzymes have also been used for waste management and silver recovery[3, 4]. Forty percent of the enzymes used today are produced by microorganisms including bacteria, moulds and yeasts[5]. Microorganisms are an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods, and as they can be made to produce an abundant, regular supply of the desired product. Bacteria are the most dominant group of alkaline protease producers with the genus Bacillus being the most prominent source[3, 6, 7]. Microbial proteases, especially from Bacillus sp. are the most widely exploited industrial enzymes with major applications in detergent formulations[8]. Among them, alkaline proteases have been widely used in the detergent industry, because the pH of laundry detergents is generally in the range of 9.0-12.0, since their introduction in the 1914 as detergent additive[9].

The performance of proteases in detergents depends on number of factors including the pH of detergent, ionic strength, wash temperature, detergents composition, washing procedure, and wash-water hardness. However, the key challenge for the use of enzymes in detergents is their stability. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents to stimulate the search for new enzyme sources[10]. Although several studies have focused on the proteolytic enzymes of hot spring microorganisms, terrestrial Bacilli[11-13], relatively little work has been published on marine

microorganisms.

The oceans covering 71% of the planet represent an important bioresource for microorganisms. The existence of marine microorganisms was first reported in the late 19th century, and they were found to be metabolically and physiologically different from terrestrial microorganisms[14]. Isolation and screening of micro-organisms from marine environment are expected to provide new strains producing active and stable enzymes in highly alkaline conditions and resisting chemical denaturant agents present in detergents. During a screening programme on alkaline protease-producing strains, identified as Bacillus sp. SD8 producing a pH and temperature stability as well as organic solvent tolerance protease was isolated.

This study aimed at screening and isolation of marine bacteria with high protease activities and describing some biochemical characterization of the crude alkaline protease production by one of them.

EXPERIMENTAL SECTION

2.1 Isolation and Screening of alkaline proteases producing bacteria

For isolation of protease producing strains, samples were collected from sea muds in Geziwo Qinhuangdao sea area then diluted in sterile sea water. The diluted samples were then plated onto skimmed milk agar plates containing (g/L): peptone, 10; beef extract, 3; skimmed milk powder, 60; agar, 18; pH 8.5. plates were incubated at 37℃ for 24 h. Occurrence of clear proteinolytic zone around the colonies confirmed proteinase producing ability of the screened bacteria[15], which were selected for purification the subsequent investigation. One loops of the cells of the purified strains were transferred to 20ml of protease production medium consisting of (g/L): peptone, 10; beef extract, 3; NaCl, 5; pH 8.5 prepared with sea water in 100ml flask and aerobically cultivated by shaking at 150rpm and 37℃ for 24 h. The SD8 strain which produce maximum activity in liquid medium was selected for protease characterization. The organism was maintained on nutrient agar plates and stored at 4℃.

2.2 Fermentation and crude enzyme preparation

The strain SD8 was cultivated in protease production medium consisting of (g/L): peptone, 10; beef extract, 3; NaCl, 5; pH 8.5. Media were autoclaved at 121℃ for 20 min. Cultivations were performed on a rotary shaker (150 rpm) for 24 h at 37℃, in 250 ml Erlenmeyer flasks with a working volume of 50 ml. The biomass produced was separated by centrifugation at 10000 rpm for 10 min at 4℃, and the supernatant subjected to ammonium sulfate precipitation using varying salt concentrations to attain different saturation levels (0-80%). Each precipitated fraction was dissolved in buffer(phosphate buffered solution, pH 7.4), dialyzed against the same buffer, and then examined for protein content and enzyme activity. Specific activity of the enzyme was expressed as activity per mg protein.

2.3 Determination of Enzyme Activity

Protease (caseinolytic) activity was assayed by a modification of the method of Kunitz[16]. The reaction mixture (0.6 ml) consisted of 150 μl of 1 % Hammerstein grade casein in 200 mM glycine-NaOH buffer (pH 10.0) 150 μl of enzyme solution or cultivated supernatant. The reaction was started by adding enzyme solution at 40℃. After incubation for 15min, the reaction was stopped by adding 300 μl of 0.4 M trichloroacetic acid. The sample was kept on ice for another 10 min, then centrifuged at 10000 rpm for 10 min at 4℃. The supernatant (0.3 ml), mixed 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, was incubated at 40℃ for 20 min. The concentration of digested casein in the supernatant was determined by monitoring an increase in absorbance at 680 nm. The calibration curve was made using L-tyrosine as a standard. One unit of protease activity was defined as the amount of enzyme that releases 1 μg/ml of tyrosine equivalent per min.

2.4 Determination of Protein

Protein concentration was determined according to the method described by Bradford[17] using bovine serum albumin as standard, and during the course of enzyme purification by measuring the absorbance at 280 nm.

2.5 Biochemical properties

2.5.1 Effect of pH on crude alkaline protease activity and stability

The optimum pH of the preliminary purified enzyme was studied over a pH range of 7.0-13.0 at 40℃ using casein 1% (w/v) as a substrate. The effect of pH on proteolytic stability was examined by incubating the enzyme in buffers of different pH values in the range of pH 7.0-12.0 for 1 h at 40℃. Aliquots were withdrawn at the desired time intervals to test the remaining caseinolytic activity at pH 10.0 and 40℃. The following buffer systems were used: 200 mM sodium phosphate buffer for pH 7.0-8.0, and 200 mM glycine-NaOH buffer for pH 9.0-13.0.

2.5.2 Effect of temperature on crude alkaline protease activity and stability

The effect of temperature on alkaline protease activity was studied from 20 to 70℃ using casein as a substrate for 20 min in 200 mM glycine-NaOH buffer, pH 10.0. Thermo-stability was examined by incubating the preliminary purified enzyme for 1h at different temperatures. Aliquots were withdrawn at desired time intervals to test the remaining activity at standard conditions. The non-heated enzyme was considered as control (100%)[18].

2.5.3 Effects of metal ions and inhibitors on crude enzyme activity

The effects of various metal ions (5 mM) on enzyme activity were investigated by adding the divalent(Ca^{2+}, Mn^{2+}, Zn^{2+} or Cu^{2+}) metal ions to the reaction mixture. The activity of the enzyme without any metallic ions was considered as 100%.

The effects of 5 mM EDTA or PMSF (phenylmethylsulfonyl fluoride) on protease activity were also studied. The preliminary purified enzyme was pre-incubated with EDTA or PMSF for 20 min at 40℃, and then the remaining enzyme activity was determined under standard conditions. The activity of the enzyme, determined without EDTA, was taken as control.

2.5.4 Effect of organic solvents and detergents on crude protease activity

The effect of various organic solvents on the activity of the preliminary purified enzyme was studied. The enzyme (in 100 mM glycine-NaOH pH 10.0 buffer) was incubated 20 min with 25 % (v/v) of various organic solvents (methanol, isopropanol and acetone) at 40℃ with shaking (150 rpm). Aliquots were withdrawn at desired time to test the remaining activity at standard conditions. The activity of the enzyme measured in absence of any organic solvents was taken as control.

The effect of 5 mM surfactant SDS and 1 % (v/v) oxidizing agent H_2O_2 on enzyme activity were studied by pre-incubating the preliminary purified enzyme for 20 min at 40℃. The residual activity was measured under the standard assay conditions. The activity of the enzyme, pre-incubated without any additive, was taken as 100 %.

RESULTS AND DISCUSSION

3.1 Isolation and Screening of alkaline proteases producing bacteria

In the current study, total 116 bacteria from sea muds were obtained but only 14 strains among them could form clear zone of hydrolysis of skimmed milk powder on agar plate (Fig. 1). This finding is analogous with VisÔtto et al.[19] where he found several bacterial strains including *B. subtillis* with proteolytic activity from the caterpillar gut on calcium caseinate agar. Using the ratio of the clear zone diameter (onto skimmed milk agar plates) and that of the colony, five isolates SDL5, SDL9, SD7, SD8 and SD10 exhibited the highest ration (>2). The results were showed in Table 1. And then, they were tested for protease production in liquid culture. Through Fig. 2, the results indicated that protease activity of strain SD8 was the highest 177.9 U/ml after 24 h incubation in a non-optimized medium. Therefore, strain SD8 was retained for all subsequent studies.

3.2 Alkaline protease partial purification

Table 2 illustrates the steps involved in the purification process. The cell-free supernatant obtained after growth of strains SD8 for 24 h had a total activiy of 1975.70 U and a specific activity of 185.86 U/mg protein and was considered as the control (100 %). The supernatant was then subjected to ammonium sulfate precipitation at 0-80 % saturation. Maximum activity of protease was obtained with 60 % ammonium sulfate saturation. This resulted in a total activity of 1343.48 U (68 % of yield) and the specific activity of 4199.55 U/mg protein and purification fold of 2.25,enhancing the specific activity to 418.53 U/mg protein with a yield of 68 %.

3.3 Effect of pH on activity and stability of crude alkaline protease

The effect of pH on the protease activity toward casein as substrate, was determined over the pH range of 7.0-13.0. The pH altered the protease activity at 40℃. The protease showed highly activity over a broad range of pH between 7.0 and 11.5 and the optimum pH for the enzyme activity was around 10.0 (Fig. 3a). The relative activity at pH 11.0 was 90 % and it decreased rapidly above pH 11.0. The enzyme showed 40 % and 6 % activity reductions at pH 12.0 and 13.0, respectively. These results suggested that the enzyme was alkaline protease[20] Since detergents-solution's pHs are generally between 9.0 and 10.5, this high activity at alkaline pHs is an important factor required in almost all detergent-enzymes such as those described by Hadj-Ali et al.[21], Li et al.[22] and Cheng et al.[23]. This demonstrated that the enzyme in the paper may be used in the detergents-solution. At the same time, the enzyme is almost more efficient at alkaline pH comparing to the main commercially detergent enzymes for example Alcalase™ (Novozymes A/S), produced by B. Licheniformis having a maximal activity at pH values from 8.0 to 9.0, Savinase™ (Novozymes A/S), produced by B. clausii, with a maximal activity at pH values from 8.0 to 10.0[24].

The pH stability was determined after 1 h incubation of the enzyme in different buffers pH ranging from 7.0 to 12.0 at 40℃, followed by the determination of residual activity at pH 10.0 and 40℃. As shown in Fig. 3b, the enzyme activity was very stable within a broad pH range from 7.0 to 11.0 (>80 %) and decreased rapidly at pH 12.0 (47 %). This confirmed its potential use in industrial purpose, which requires enzyme stability in wide pH (9.0-11.0) range[25]. This stability also very suitable for detergent application.

3.4 Effect of temperature on activity and stability of the crude protease

The effect of temperature on crude protease activity was examined at various temperatures. The alkaline protease was active between 30 and 70℃ with an optimum around 60℃ (Fig. 4a). The relative activities at 50 and 60℃ were about 98 and 100 %, respectively. Temperature optima at 60℃ have been reported for proteases from *B. pumilus*[26], *B. cereus* BG1[27] and *B. mojavensis*[24].

The thermal stability profiles of the crude protease showed that the enzyme is completely stable at temperatures below 50℃ after 1 h incubation. The enzyme at 40℃ remains fully active after 1 h of incubation. Even at 60 and 70℃, the residual activity of the enzyme were 58 and 43 %, respectively, indicating that this enzyme might be used under mild heating conditions (Fig. 4b). The thermal stability of crude protease is higher than the alkaline protease from *B. licheniformis* MP1[28]. In which, the enzyme remained fully active even after 2 h of incubation at 50℃, but retains only 41 and 5 % of its activity after 1 h preincubation at 60℃ and 20 min at 70℃, repectively. This stability might be an advantage for using this enzyme in industrial application such as laundry detergents formulations for example[3].

3.5 Effects of metal ions and inhibitors on crude enzyme activity

The effects of enzyme inhibitor and chelating agent on the enzyme activity were investigated and summarized in Table 3. The crude enzyme was completely inhibited by the serine protease inhibitor (PMSF, 5 mM), indicating that the crude enzyme contained serine-proteases. However, EDTA, a metalloprotease inhibitor, didn't inhibit the enzyme, indicating that didn't contained metalloprotease. In fact, the activity of enzyme was active to 111 % when preincubated with 5 mM EDTA. These findings are not in line with earlier reports which the enzymatic extract was slightly inhibited by the EDTA. This indicated that the crude enzyme was very useful for applications as detergent additive because chelating agents were useful components of most detergents. Chelating agents function as water softeners and also assist in the removal of stain[10].

The effects of several metal ions at a concentration of 5 mM, on the activity of the crude protease were also studied at pH 10.0 and 40℃ by the addition of the respective cations to the reaction mixture (Table 3). The addition of Ca^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} increased the enzyme activity by 150, 140, 110 and 115 % of the control, respectively.

3.6 Effect of organic solvents and surfactant on crude protease activity

In order to be effective during washing, a good detergent protease must be effective during washing, a good detergent protease must be compatible and stable with commonly used detergent compounds such as surfactants, oxidizing agents and other additives, which might be present in the formulation[29]. The crude protease was preincubated 20 min at 40℃ in the presence of SDS and H_2O_2 and the residual activity was assayed at pH 10.0 and 40℃ (Table 4). The enzyme was highly stable in the presence of the strong anionic surfactant (SDS) at 5 mM. This is not in accord with the report from Hadj-Ali et al[21], which inhibition with SDS was a common feaure of serine proteases. Oxidizing agents H_2O_2 at 1 % (v/v) caused a moderate inhibition 11 %. The present crude enzyme seems very stable in the presence of these detergent additives.

Table 1 The diameter of colony and hydrolyzed zone

Strains	Diameter of colony (D)	Diameter of hydrolyzed zone (d)	d/D
SDL5	1.21	2.54	2.10
SDL9	0.76	1.78	2.34
SD7	1.43	3.05	2.13
SD8	1.57	3.65	2.32
SD10	1.54	3.2	2.08

Enzymatic synthesis of peptides has attracted a great deal of attention because there are numerous advantages of ester and peptide synthesis[30]. Only proteases that are stable in the presence of organic solvents can be used as biocatalyst for ester synthesis[31]. Various water-miscible organic solvents at 25 % final concentration were tested for their effect on enzyme activity at pH 10.0 and 40℃. As seen in Table 4, crude protease activity was strongly enhanced by the addition of methanol and acetone in the reaction mixture. The relative activities were about 136 ant 148 % in the presence of methanol and acetone, respectively. However, the activity of crude enzyme was hardly affected by isopropanol From this study, it can be concluded that organic solvents stability of the enzyme depends of

the nature of organic solvents.

Table 2 Purification of protease from strains SD8

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield[a] (%)	Purification (fold)
Culture supernatant	1975.70	10.63	185.86	100	1
Ammonium sulphate precipitation (60%)	1343.48	3.21	418.53	68	2.25

[a] *All values are expressed in terms of activity units in culture filtrate taken as 100 %*

Table 3 Effects of various enzyme inhibitors and some metal ions on the activity of the crude protease

Chemicals	None	PMSF	EDTA	Ca^{2+}	Mn^{2+}	Zn^{2+}	Cu^{2+}
Concentration(mM)	–	5	5	5	5	5	5
Activity (%)	100	0	111±3.3	150±3.6	140±3.1	110±2.6	115±2.3

Crude enzyme was pre-incubated with various inhibitors for 20 min at 40℃, and the residual activity was determined under standard conditions. Activity of the enzyme pre-incubated in the absence of any additive was taken as 100 %. The effect of metal ions on the activity of the crude protease was determined by measuring the proteolytic activity is expressed as a percentage level in the absence of metal ions (5 mM). The activity is expressed as a percentage level in the absence of metal ions. Protease activities represent the mean of three determinations, and bars indicate ± standard deviation.

Table 4 Effect of organic solvents and surfactant on crude protease activity

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	96±1.3	89±1.1	136±1.1	99±1.6	148±2.1

The enzyme was incubated at pH 10.0 and 40℃ in the presence and absence of various additives. The non-incubated enzyme was considered as control (100%). Protease activities represent the mean of three determinations, and bars indicate ± standard deviation.

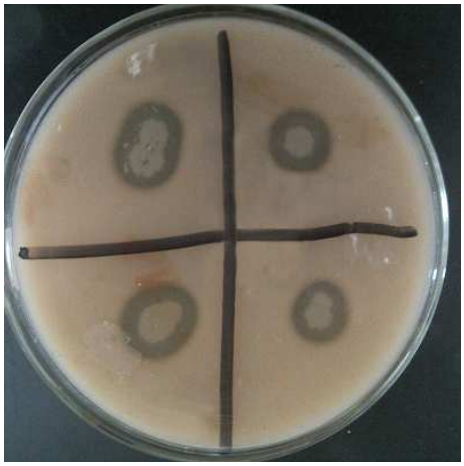


Fig. 1 Screening of strain SD8 for proteases activity. Isolate are streaked on agar plate containing skimmed milk powder, incubated for 24 h at 37℃. The clear zone indicated the hydrolysis of skimmed milk

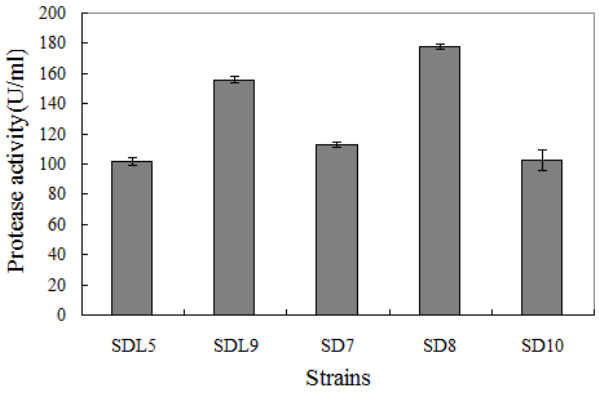
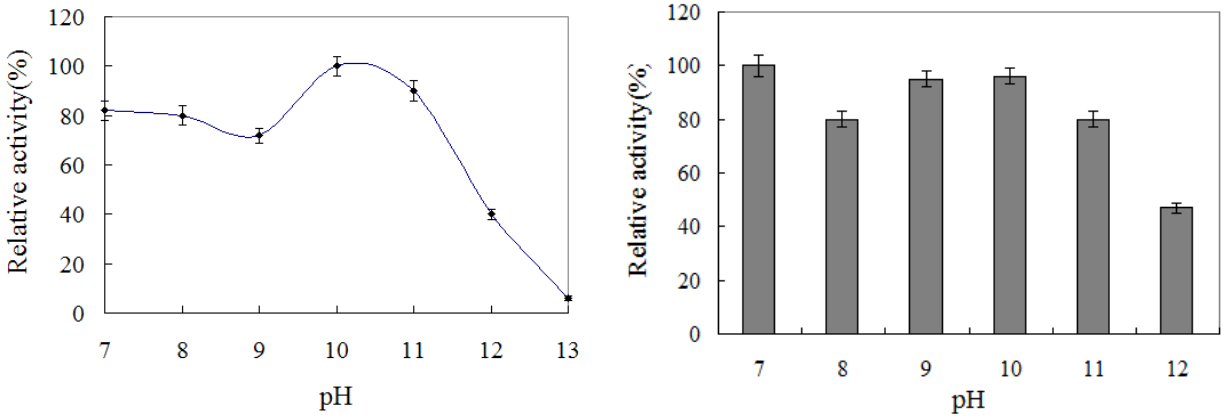
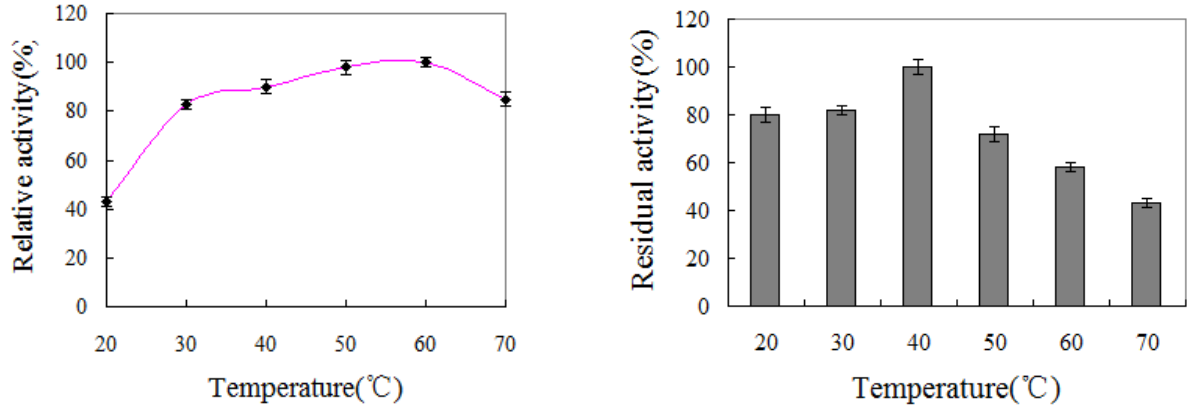


Fig. 2 Protease activity of culture supernatant of different strains



a **b**

Fig. 3 The effect of pH on protease activity (a) and stability (b) from strain SD8. (a) The pH profile was determined in different buffers by varying pH values at 40℃. The maximum activity at pH 10.0 was considered as 100 % activity. (b) The pH stability of the protease was determined by incubating the enzyme in different buffers for 1 h at 40℃ and the residual activity was measured at pH 10.0 and 40℃. The activity of the enzyme before incubation was taken as 100 %. Each point represent the means of three experiments, and bars indicate±standard deviation. Absence of bars indicates that errors were smaller than symbols



a **b**

Fig. 4 Effect of temperature on activity (a) and stability (b) of the crude protease from strains SD8. (a) The temperature profile was determined by assaying protease activity at temperatures between 20 and 70℃. The activity of the enzyme at 60℃ was taken as 100 %. (b) The temperature stability was determined by incubating the crude enzyme at temperatures from 20 to 70℃ for 1 h. The residual enzyme activity was measured under the standard conditions assay. The original activity before preincubation was taken as 100 %. Each point represent the means of three experiments, and bars indicate±standard deviation

CONCLUSION

In this study, we report the screening of producing strain SD8 of alkaline protease and characterization of a new crude alkaline protease from strain SD8. The crude enzyme contained serine protease and non metalloprotease. The crude protease was active and stable over a wide range of temperatures, demonstrated optimal activity at 60℃, and was stable until 60℃. Furthermore, the protease had optimal activity at a pH of 10.0 and a wide rang of pH stable (7.0-11.0). The activity of crude protease was enhanced by the addition of various 5 mM Ca^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}. The enzyme also could tolerant and stabile with anionic surfactant SDS, oxidating agent H_2O_2, and organic solvents. These properties suggest that this enzyme would be suitable for use in industrial application such as detergent formulations and synthesis of peptides. It also would have applications in processes performed at wide ranges of temperature and in alkaline environments. This would require further investigation of the purification, structure-function relationship through site-directed mutagenesis and 3-D structure determination.

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