IDENTIFICATION OF AN ALKALINE PROTEASE PRODUCING BACTERIUM ISOLATED FROM PANJIN RED BEACH, CHINA

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Abstract. Extremophilic bacteria surviving in extreme environmental conditions possess the special abilities to adapt adverse environment. There are very high salinization and alkalinization contents in Panjin Red Beach's soil. A productive alkline protease strain HHT597 was isolated from the soil samples and identified as *Bacillus altitudinis* based on the 16s rDNA sequence. The HHT597 strain was characterized as a gram positive, rod-shaped bacterial strain with flagellum. The optimum temperature for protease activity by the strain HHT597 was 60°C. There was a broad pH active range of protease from 7.0–11.0 with having optima pH 9.0. The media containing 2.0% glucose and 1% yeast extract as carbon and nitrogen sources could improve protease activity. The strain HHT597 could degrade casein, hemoglobin (HGB) and albumin (ALB) protein. The results indicated that the strain HHT597 might be potential for biotechnological applications.
Keywords: *extreme environment, isolation, 16S rRNA gene, identification, Bacillus*

Introduction

Proteases, which catalyze proteolysis by performing cleavage of peptide bonds, are widely used industrial enzymes, accounting for more than 65% of the total worldwide sale of the enzymes (Rao et al., 1998; Shankar et al., 2011; Annamalai et al., 2014). Proteases include alkaline, acid, thiol and metallo proteases. Among these categories of proteases, alkaline proteases are a class of extracellular enzyme that performs proteolysis and have wide applications as industrial catalysts in various of industries and research laboratories, which could be used in enzymatic peptide synthesis, biotransformation reactions, detergent, surfactants, food processing, diagnostic reagents, preparation of organic fertilizers, silver recovery from used X-ray film and wastes treatment (Kalisz, 1988; Anwar and Saleemuddin, 1998; Shah et al., 2010; Rathod and Pathak, 2016; Hakim et al., 2018). A wide range of sources was found to produce alkaline protease such as bacteria, molds, yeasts, certain insects or mammalian tissues (Mabrouk et al., 1999; Kumar et al., 2014). Bacteria producing

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alkaline protease are the most important approach relative to plants, animal, and fungus because of their extracellular nature, high yield of production, limited space, convenient cultivation and feasibility to genetic manipulation (Breithaupt, 2001; Selvamohan and Sherin, 2010).

With previous studies gaining a better understanding of the physical, chemical and biological properties of alkaline proteases, researchers nowadays focus more on searching for alkaline proteases with specificities such as high temperature and pH tolerance. Active ranges of temperatures of alkaline (serine) proteases were 35°C–80°C and pH was 7–12 (Rao et al., 1998). Morozkina et al. (2010) reported that the extremophiles could produce extremozymes. Through the long term of natural selection, extremophilic bacteria possess the special abilities associated tightly with their structures, physiological mechanisms, genetic characteristics and biochemical pathways. For example, they can produce extracellular proteases. At high pH and temperatures, there were remarkable activity and stability in alkaline protease produced by marine bacteria (Arastoo and Zahra, 2013). Therefore, extreme environments, in particular, marine environments are a valuable approach for bacteria producing microbial enzymes (Mirete et al., 2016).

Panjin Red Beach which locates in Liaoning, China, is a famous wetland landscape covering an area of 133.33 km^2 on a flat and broad terrain (Wang et al., 2011). Years of marine corrosion leads to the soil a sandy, humid and heavy texture with a high level of salinization and alkalinization. The inartificial edatope creates a unique ecosystem that provides native habitats for variety of halophilic and basophilic microorganisms. Therefore, it is likely to find functional strains that are adaptation to saline-alkali environment. Our research focused on isolating and identification bacteria producing alkaline protease with protease activity in the natural saline-alkaline soil of the Red Beach.

Materials and Methods

Isolation of Bacterial Strain Producing Alkaline Protease

Five representative plots were selected during September 2017 in Panjin Red Beach. Soil samples, five soil cores (5 cm diameter) were collected in triplicate from the topsoil (0-15 cm) in each sampling site and mixed evenly, then placed in aseptic polyvinyl chloride soil bag.

Soil samples (10 g) collected were suspended in 90 mL of sterile distilled water. After a serial dilution (10^{-3} to 10^{-5}) of the soil suspension with sterile distilled water, 0.1 mL soil suspension was spread on selective agar plates and incubated at 37℃ for 2 d. The selective agent in the agar plates contained beef extract 0.3% (w/v), casein 1% (w/v), NaCl 1.5% (w/v), agar 2% (w/v). Ten plates were screened in soil samples and about 150 plates altogether were screened. The strain having the largest clear zone was selected by secondary screening for this study.

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Identification of Bacteria Strain

Bacteria strain was performed by Gram staining and biochemical tests such as methyl red test, V-P test, carbohydrate fermentation test and hydrogen sulfide production test following commonly used microbial identification manuals like Bergey's manual (Holt, 1994). Each treatment was conducted with three replicates.

The total genomic DNA of the bacteria strain was extracted and purified using Bacterial DNA Isolation Kit (Sangon Biotech Co., Ltd, China) according to maufacturer's instructions. The 16S rDNA of the isolate was amplified through PCR reaction using the universal primers 27f (5'-AGAGTTTGATCATCCTGGCTCAG-3') and 1492r (5'-TACGGTTACCTTGTTACGACTT-3') synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The PCR reaction process was as follows: 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. PCR product was sent to Sangon BiotechCo., Ltd (Shanghai, China) for sequencing. The similarities of the sequence were identified using online EzBioCloud database (https://www.ezbiocloud.net/). Construction of phylogenetic tree was carried out using MEGA 7 (Kumar et al., 2016).

Enzyme Assay Estimation

Alkaline protease activity was determined by using casein as a substrate by method of Yang and Wang (1999). One unit of protease activity was defined as the amount of the enzyme that produces 1μg/mL/min of tyrosine equivalent under the assay conditions.

Optimum Conditions for Protease Activity

Optimum Temperature and pH: Preheat 2.0% casein solution for 5 min by water bath at temperature ranging from 10°C to 80°C. Mix 1 mL of each casein solution with 1mL preheated crude protease solution and water bath at 40℃ for 10 min, then end the reaction with 5 mL TCA solution. The basal media was adjusted to seven different pH ranging 6.0-12.0. The seed culture (1.0% V/V) was inoculated to each 150 mL liquid medium and was carried out at 37°C, 160 rpm for 2 d. Enzyme assay determination was as stated above.

Carbon and Nitrogen Sources: 2.0% each of glucose, maltose, sucrose and lactose was added in the basal media to investigate the effects of different carbon sources. To observe the effects of various nitrogen sources on protease activity, 1.0% each of beef extract, peptone, yeast extract, sodium nitrate, ammonium nitrate and ammonium chloride was added to the basal media. Fermentation was carried out under optimum temperature and pH at 130 rpm for optimum period. Enzyme assay determination was as stated according to the method described above.

Substrate Specificity Assay: Prepare 2% (w/v) of each casein, gelatin, hemoglobin (HGB), albumin (ALB) solution as substrate. Mix 1 mL substrate solution with 1 mL preheated the crude protease solution and water bath at 40°C for 10 min, then end the reaction with 5 mL TCA solution. Enzyme assay determination was as already mentioned.

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Statistical analysis

All data were analyzed according to Duncan's multiple range tests using the SPSS 11.0 software package.

Results and Discussion

Isolation, Screening and Identification of Strain HHT597

Eight isolates from soil samples showed clear zone around bacterial colonies on the selective medium, indicating that these strains can produce extracellular alkaline protease. These eight strains were named for from HHT591 to 598. Finally, bacterium strain HHT597 was chosen for further study based on the intensity of clear zone.

The strain HHT597 was characterized as a gram positive, rod-shaped bacterial strain with flagellum (*Figure 1A*). Colony morphology was faint yellow, round, marginal tidy, dry surface (*Figure 1B, C*).

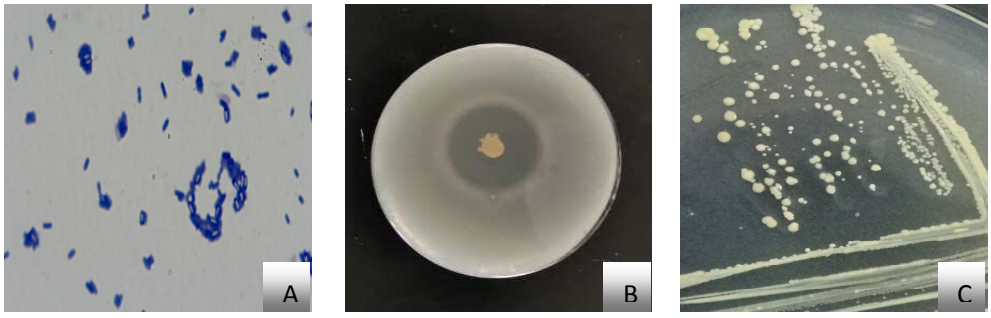

Figure 1. *The colony morphology (A, B) and Gram stain (C) of bacteria strain HHT597*

Biochemical characteristics were listed in *Table 1*. Based on the morphological and biochemical characteristics, isolate HHT597 was identified as *Bacillus* sp. Among all the alkaolphilic microorganisms, *Bacillus* spp. is the most predominant and a prolific source of alkaline proteases (Kumar and Takagi, 1999).

Table 1. *Biochemical characteristics of strain HHT597*

Glucose	Lactose	Acid production	Gas production	Voges Proskauer	Methyl red	H_2S production
+	-	-	+	+	+	+

To carry out the 16S rDNA gene sequencing analysis, the genome of strain HHT597 was used for PCR amplification of 16S rDNA. The molecular weight of PCR product of strain HHT597 was corresponding to 1500 bp of the DNA marker and the amplification was successful. The DNA sequence of 1456 bp product was uploaded to EzBioCloud database for phylogenetic identification and comparison. Phylogenetic tree was

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constructed based on neighbour-joining statistical algorithms. According to phylogenetic tree (*Figure 2*), the strain HHT597 were clustered with *Bacillus* altitudinis strain G65 with high (99%) 16S rDNA sequence similarity. The strain HHT597 was affiliated to the genus *Bacillus*.

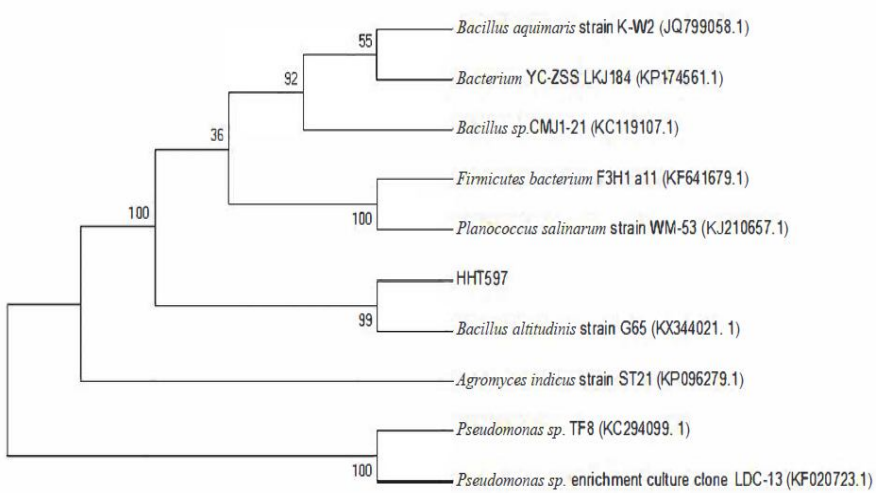

Figure 2. *Phylogenetic tree of HHT597 and B. altitudinis strain G65 with other B. species*

Effects of Temperature and pH on Protease Activity

Generally, the protease activity is easily decreased by high temperature. The temperature optima of protease depended on the bacterial species (Iqbal et al., 2018). In the present study, the alkaline protease activity first gradually increased then gradually decreased with increasing temperature (*Figure 3a*).

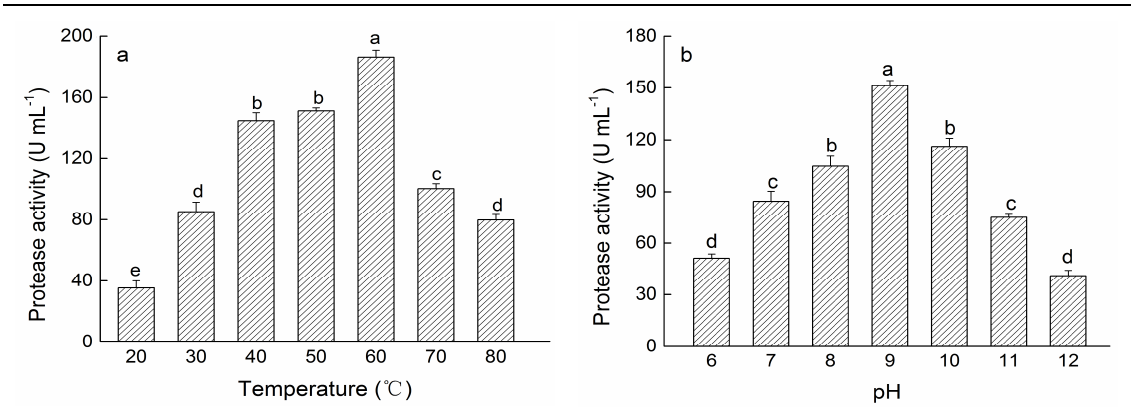

Figure 3. *Effects of temperature (a) and pH (b) sources on alkaline protease activity*

The optimum temperature of protease activity produced by strain HHT597 was 60°C and protease activity reached 186.35 U/mL. When temperature was up to 70°C, the protease activity began gradually to decrease. The results indicated that the protease

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produced by the strain HHT597 could work at high temperatures. Nevertheless, almost similar protease activity was found at 40–50°C (Hakim et al., 2018).

Many enzymatic processes are strongly influenced by the pH (Ellaiah et al., 2002; Hakim et al., 2018). In the present study, the pH active range of protease was from 7.0 to 11.0. The optimum pH for alkline protease activity was 9.0, and the protease activity was 151.21 U/mL (*Figure 3b*). Rao et al (1998) reported that pH optima of alkaline protease in commerce were from 8.0 to 12.0.

Effects of Carbon and Nitrogen Sources on Protease Activity

The carbon and nitrogen sources used in the media highly influenced production of alkaline protease (Hakim et al., 2018). The media containing glucose at 2% was the most favorable source supported the highest alkaline protease production compared to other carbon sources (*Table 2*). The results were similar with other reports (Ellaiah et al., 2002; Hakim et al., 2018). Among the different nitrogen sources studies, 1% yeast extract was the most benefit for alkaline protease production (*Table 2*), which is consistent with previous results of *B*. subtilis AKAL7 (Hakim et al., 2018) and *B*. sp MA6 (Azad and Hoq, 2000). However, the strain HHT597 could not use inorganic nitrogen compounds as nitrogen sources.

Table 2. *Effects of carbon source, nitrogen source and substrate on alkaline protease activity*

Treatment		Protease activity（U/mL）
Carbon source	Glucose	89.26±1.73a
	Maltose	79.37±2.38b
	Sucrose	35.43±1.2c
	Lactose	78.34±1.34b
Nitrogen source	Beef extract	117.26±4.16b
	Yeast extract	186.24±8.56a
	Peptone	109.32±6.52b
	Ammonium nitrate	—
	Sodium nitrate	—
	Ammonium chloride	—
Substrate	Casein	136.63±7.65a
	Gelatin	—
	Hemoglobin	112.06±4.31b
	Albumin	108.63±2.62b

The different letters indicate significant differences at $P < 0.05$

Optimum Substrate on Protease Activity

Casein, HGB, AHL and gelatin was used as substrate to detect the protease degradation ability, respectively. Among four substrates, casein was the most appropriate substrate and the maximum protease activity was 136.63 U/mL (*Table 2*). HGB and AHL could also be the substrate of alkline protease extracted from HHT597. However, gelatin was not used as the protease substrate.

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Conclusion

A productive alkline protease, gram positive bacterial strain of the genus *Bacillus* was isolated from the soil samples of Panjin Red in China and identified as *B. altitudinis* strain HHT597. The optimum temperature for maximum protease activity produced by the strain HHT597 was 60°C. There was a broad pH active range of protease from 7.0-11.0. The optimum carbon and nitrogen sources for protease activity produced by the strain HHT597 was 2% glucose and 1% yeast extract, respectively. The strain HHT597 could degrade casein, HGB and AHL protein. The research results showed that the strain HHT597 might be potential for industrial applications.

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