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Isolation and Enzyme Properties of a Newly Isolated *Bacillus licheniformis* HN1 Producing Milk-Clotting Enzyme Utilizing Wheat Bran

Zhang Weibing*, Luo Qiaoqiao, Ma Jiang,Yang Min, Wen Pencheng, Zhang Zhongmin, He Xiaoling
College of Food Science and Technology Engineering,
Gansu Agricultural University, Lanzhou 730070, China

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Abstract: Milk-clotting enzyme has an important role in the process of cheese procution and maturation. In this study, a bacterium producing extracellular milk-clotting enzyme was isolated from the soil in Tibetan Plateau and identified as *Bacillus licheniformis* using 16S rDNA. Then the milk-clotting enzyme from *B. licheniformis* HN1 was purified to 26.25-fold with 18.39 % recovery by precipitation in ammonium sulfate and ion-exchange chromatography. The molecular mass of the enzyme was 51.9 kDa as determined by SDS-PAGE, and the result of pepstatin A inhibition treatment showed that this enzyme was an aspartic proteas. The enzyme was active in the pH range 5.5-9.5 and was inactivated completely by heating at 60°C for 20 min. The highest level of enzyme activity was obtained at 55°C, pH 5.5, and in the presence of 20 mM $CaCl_2$. The high level of milk-clotting activity coupled with a low level of thermal stability suggested that the milk-clotting enzyme from *licheniformis* HN1 was a potential substitute for calf rennet.

Keywords: Milk-clotting enzyme; *Bacillus licheniformis*; Enzyme properties.

Introduction

Calf rennet, the traditional milk-clotting enzyme, has been widely used by the dairy industry for the manufacture of cheese with good flavour and texture [1-2]. The worldwide increase of cheese production coupled with a reduced supply of calf rennet has prompted a search for calf rennet substitutes, including milk-clotting enzyme from microbe and plant [3]. However, most plant rennets have proved unsuitable because they impart a bitter taste to the cheese [4]. Attention has been focused on the milk-clotting enzymes from microbial sources because of its extensive sources, lower cost, and easy extraction [1].

Beside various fungi, numerous bacteria belonging to *Bacillus* have been suggested as promising microbial rennet producers, such as *Bacillus subtilis* [5], *Bacillus amyloliquefaciens* [6], and *Bacillus sphaericus* [1]. Bacterial rennet appears to be more promising because its production is cheaper, its biochemical diversity is greater, and its genetic modification is easier [6]. However, to obtain the enzyme with high milk-clotting activity, many researchers focus on isolating new bacteria producing milk-clotting enzyme from special environment.

The Tibetan Plateau in China is home to a diversity of microbial species and plentiful microbial germplasm resources [7-8]. Qinghai pastoral area located in the northwest of the Tibetan Plateau in China. Local herdsmen produce a variety of dairy products such as yoghourt, cheese, Qula and butter from yak milk for thousands of years [9]. It is possible to find microorganisms producing milk-clotting enzyme in this region. The objectives of this study were to screen and isolate microorgan-

*Corresponding author (Zhang Weibing)
E-mail: < 453303011@qq.com >

isms producing milk-clotting enzyme using wheat bran as nutrient source in this region, and characterize the milk-clotting enzyme produced by B. *licheniformis* HN1.

Materials and Methods

Isolation and identification of bacterium producing milk-clotting enzyme

The soil samples were collected from the 5-10 cm layers below soil surface of the yak herding regions in Qinghai pastoral area of China. All soil samples were kept at 4°C and immediately taken back to the laboratory [11].

Soil samples were filtered through a sieve and one gram of soil was suspended with 9 mL of saline water and a series of ten-fold dilutions of the suspension was made by pipetting 1 mL aliquots into 9 mL saline water. 0.1 mL of each dilution of the series was cultivated at 37°C for 2 days in casein agar plates as the procedure described in Arima *et al.* [10].

Identification of the selected strain

The selected strain with highest milk-clotting activity was identified using 16S rDNA. Homology searches were performed against the sequences with the database using the BLAST program (NCBI). For phylogenetic analysis, a dataset containing GenBank 16S rRNA gene sequences were aligned using the CLUSTALX program [11]. To construct the phylogenetic tree, MEGA 4.0 software was used [12].

Production of milk-clotting enzyme

Prior to the cultivation of the isolate on the basal fermentation medium, it was cultivated on a seed culture medium (g/L) containing peptone 10, beef extract 3, NaCl 5, with pH 7.2. Incubation was carried out at 37°C, in a rotary shaker, with stirring at 170 rpm for 24 h. The seed cultivation medium was then inoculated aseptically at 2 % (v/v) of the basal fermentation medium, and incubated at 37°C, in a rotary shaker, with stirring at 170 rpm for 2 days. The basal fermentation medium contained glucose 5 g, Na_2HPO_4 0.2 g, with pH 6.2 (wD 100 mL wheat bran juice; wheat bran juice: 10 g wheat bran in 100 mL distilled water, boiled 10 min and filtered through gauze).

Enzyme purification

The crude enzyme was harvested by centrifugation at 8000*g* for 10 min and then precipitated with ammonium sulfate (30 % - 80 % saturation). The precipitate obtained after centrifugation at 12,000 g for 15 min and suspended in 50 mM sodium phosphate buffer (pH 7.0) and dialysed (7 kDa cutoff) overnight against several changes of distilled water to remove the salt.

Ten millilitres of the partially purified enzyme solution were subjected to ion-exchange chromatography on a DEAE-Sephadex A-25 column (30 cm × 2.6 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was eluted at a flow rate of 0.8 mL/min with an increasing linear gradient of NaCl from 0 to 0.5 M in 50 mM sodium phosphate buffer (pH 7.0) and 5 mL fractions were collected. The protein content of each fraction was determined by measuring the absorbance at 280 nm. The fractions with enzyme activity were pooled and dialysed overnight against distilled water and then lyophilized.

Effects of temperature on enzyme activity

The optimum temperature for the activity of the enzyme was determined by assaying the milk-clotting activity at intervals of 5! in the temperature ranging from 30°C to 75°C.

Effects of temperature on enzyme stability

To determine the thermal stability, the enzyme was incubated at 5°C intervals ranging from 35°C to 60°C, and the length of the incubation was varied from 0 to 60 min. After incubation, the residual milk-clotting activity was determined and the activity obtained with an incubation time of 0 min was taken to be 100 %.

Effects of pH on enzyme activity

The optimum pH for the activity of the enzyme was determined by assaying the milk-clotting activity in the pH range 5.5-8.5, by adjusting the pH of the substrate (skim milk) with 0.1 M HCl or 0.1 M NaOH as appropriate. The maximum milk-clotting activity obtained was taken to be 100 %.

Effects of pH on enzyme stability

To determine the pH stability, the enzyme was dispersed (1:1, v/v) in the following 0.1 M buffer solutions: glycine-HCl (pH 3.5-4.0), citrate/phosphate (pH 4.5-5.5), sodium phosphate (pH 6.0-8.5) and carbonate/bicarbonate (pH 9.0-11.0), and kept at room temperature for 24 h. The residual milk-clotting activity was determined and the maximum activity obtained was taken to be 100%.

Effect of $CaCl_2$ concentration

The effect of the concentration of $CaCl_2$ on the milk-clotting activity of the purified enzyme was determined by increasing the concentration of the calcium chloride solution at intervals of 5 mM from 0 to 35 mM. The maximum activity obtained was taken to be 100 %.

Effect of inhibitors

Different protease inhibitors, including a serine-protease inhibitor (phenylmethylsulfonyl fluoride (PMSF) at 10 mM), a metalloprotease inhibitor (ethylene-diaminetetraacetic acid (EDTA) at 10 mM), an aspartic protease inhibitor (pepstatin A at 20 µM) and a cysteine-protease inhibitor (iodoacetamide at 2 mM) were added separately to the purified enzyme. The mixture was incubated at room temperature for 30 min and the residual milk-clotting activity was tested. The milk-clotting activity obtained without the inhibitors was taken to be 100 %.

Milk-clotting activity determination

The milk-clotting activity was determined as described Arima *et al.* [13]. A 5 mL portion of the substrate (10 % skim milk in 10 mM $CaCl_2$) was incubated for 5 min at 35°C and then 0.5 mL of enzyme extract was added. The length of time starting from the addition of the enzyme extract to the formation of the first particles was recorded, and the milk-clotting activity was calculated. One Soxhlet Unit (SU) of milk-clotting activity was defined as the amount of enzyme required to clot 1 mL of substrate within 40 min at 35°C.

Proteolysis activity determination

The proteolytic activity of the enzyme was assayed after Arima *et al.* [13]. Enzyme extract (0.5 mL) was added to 2.5 mL of 1 % (w/v) alkali soluble casein in 20 mM potassium phosphate buffer (pH 6.5). The mixture was incubated at 35°C in a water bath for 10 min and the reaction was terminated by adding 2.5 mL of 0.44 M trichloroacetic acid, then the mixture was filtered through Whatman No.1 filter paper. One milliliter of 1 M Folin-phenol reagent and 2.5 mL of 0.55 M sodium carbonate solutions was added to 1 mL of the filtrate. This was further incubated for 20 min at 35°C for colour development; optical density (OD) at 660 nm was measured. One unit (U) of enzyme activity was taken as the amount of enzyme, which liberated 1 µg of tyrosine per mL per minute.

Results and discussion

Isolation and identification of bacterium producing milk-clotting enzyme

Table 1 shows the milk-clotting activity and proteolysis activity of different strains isolated from the samples. A total of 21 isolates were obtained for milk-clotting enzyme production, milk-clotting activity of which range from 11.3 to 1215.6 SU/mL. Among them, an isolate HN1 have the highest milk-clotting activity (1215.6 SU/mL) and lowest proteolysis activity (14.6 U/mL), which is a rod-shaped and Gran-positive bacterial strain (Fig. 1a). As shown in Fig. 1b, fermentation broth of HN1 greatly coagulated the skim milk.

The 16S rDNA gene sequences of HN1 were compared to all sequences in GenBank and showed the closest match to that of *Bacillus licheniformis* HNL09, *Bacillus licheniformis* FR189 and *Bacillus licheniformis* MML2501 with a homology of 99 %. For phylogenetic analysis, the phylogenetic tree (Fig. 2) was constructed using neighbor-joining. Therefore, the isolate HN1 could be identified as *Bacillus licheniformis*.

Purification of the enzyme

Because the purity of an enzyme can impact the product quality, the milk-clotting enzyme of *B.licheniformis* HN1 was purified by ammonium sulphate precipitation and column chromatography with DEAE-Sephadex A-25. The results of the purification procedure are summarized in Table 2. Partial purification of the enzyme, 3.16-fold purification and 49.25 % recovery was achieved by precipitation in 30-80 % saturated ammonium

Table 1. Milk-clotting activity and proteolysis activity of different strains

Strain	Milk-clotting activity (SU/mL)	Proteolysis activity (U/mL)	Strain	Milk-clotting activity (SU/mL)	Proteolysis activity (U/mL)
HN1	1215.6±3.0	14.6±0.5	HB4	51.5±2.2	17.5±1.3
HN2	21.1±4.2	38.3±0.8	HX1	62.4±3.5	36.6±2.3
HN3	11.3±1.9	59.5±2.8	HX2	116.5±0.9	31.9±2.5
HN4	26.4±3.1	46.6±3.1	HX3	39.5±2.2	15.5±1.3
GL1	16.1±0.6	21.9±1.2	HX4	311.6±5.0	14.8±0.6
GL2	81.2±2.1	17.5±2.1	HM1	26.7±4.2	28.3±0.7
GL3	111.6±5.0	14.6±0.6	HM2	78.3±1.1	39.5±2.1
GL4	126.7±4.2	28.3±0.7	HM3	72.4±3.5	36.6±1.0
HB1	18.3±1.9	39.5±2.3	HM4	26.5±0.9	31.9±1.5
HB2	22.4±3.5	36.6±1.1	HM5	59.5±2.2	17.5±1.3
HB3	16.5±0.9	31.9±1.5			

Fig. 1a. Colony morphology of strain HN1

Table 2. Purification steps of milk-clotting enzyme from *B. licheniformis* HN1

Purification steps	Milk-clotting activity (SU)	Protein content (mg/ml)	Specific activity (SU/mg)	Purification fold	Recovery (%)
Crude enzyme	1215.6	0.315	3859.05	-	100.00
$(NH_4)_2SO_4$ Fractionation (30-80 %)	16021.3	0.821	12206.21	3.16	49.25
DEAE-SephadexA-25	721.5	0.0042	100357.14	26.25	18.39

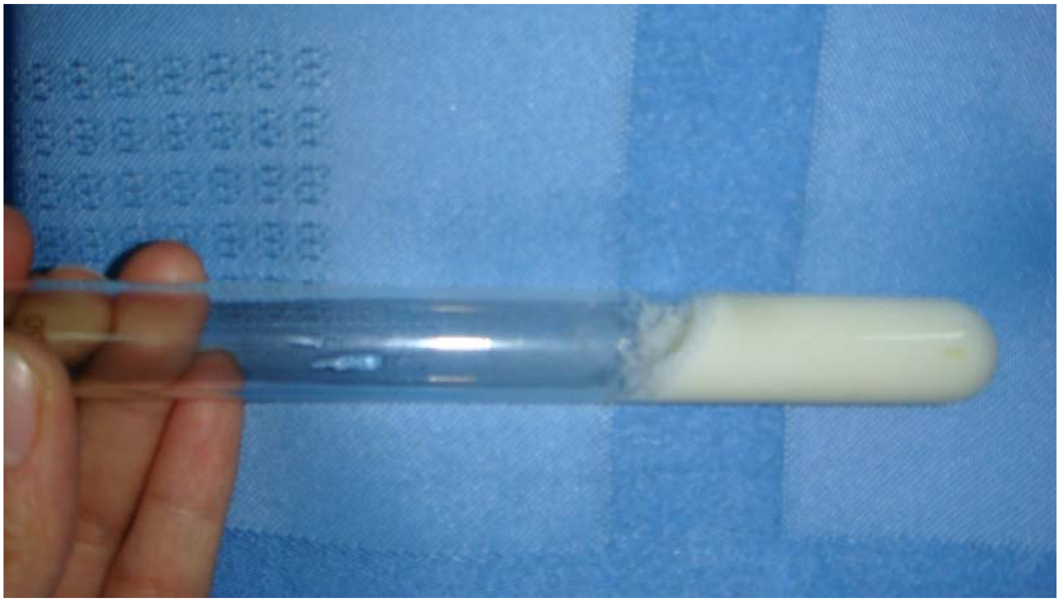

Fig. 1b. Coagulation of skimmed milk by crude enzyme from HN1 fermentation broth

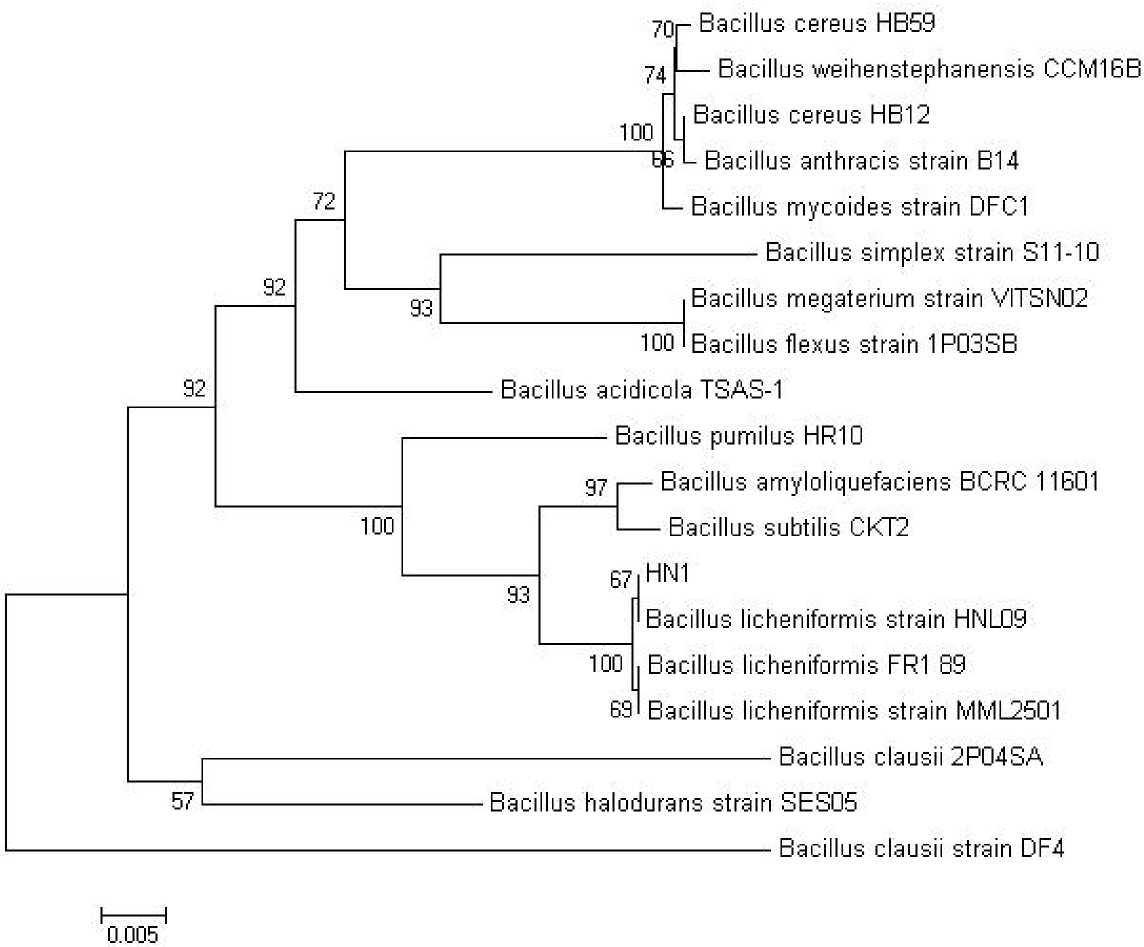

Fig. 2. Phylogenetic tree based on 16S rDNA sequence of homology

sulfate. Passage through a DEAE-Sephadex A-25 column eluted with a gradient of NaCl further purified the enzyme to 26.25-fold with 18.39 % recovery. The enzyme has a molecular mass of 51.9 kDa as determined by SDS-PAGE (Fig. 3a). The elution pattern of the milk-clotting enzyme after ion-exchange chromatography (DEAE-Sephadex A-25 column, elution with a linear gradient of 0-0.5 M NaCl) is shown in Fig. 3b.

Effects of temperature on enzyme activity and stability

Milk-clotting enzymes of different origins have different optimum temperatures. As was showed in Fig. 4a, the milk-clotting activity increased with increased temperature in the temperature range 30-55°C and the optimum temperature for the purified enzyme was 55°C. The heat stability of the purified enzyme is shown in Fig. 4b. It was fully active after 10 min of incubation at 35°C and re-

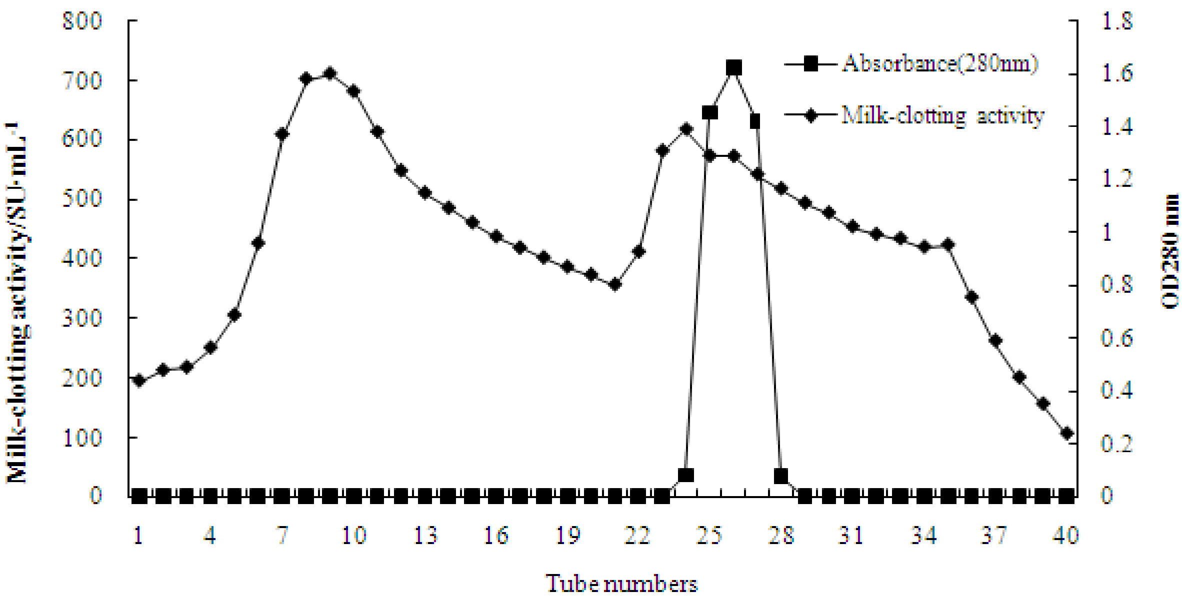

Fig. 3a. Ion-exchange chromatography through DEAE-Sephadex A-25 of MCE

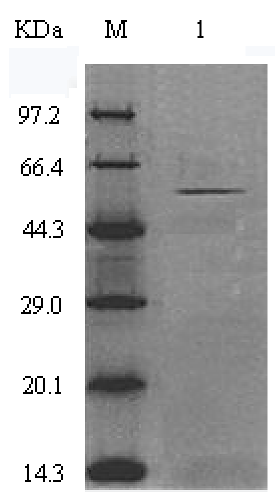

Fig. 3b. SDS-PAGE electrophoretogram of MCE after purification. Marker lane, standard molecular mass markers; lane 1, MCE purified by ion-exchange chromatography through DEAE-Sephadex A-25

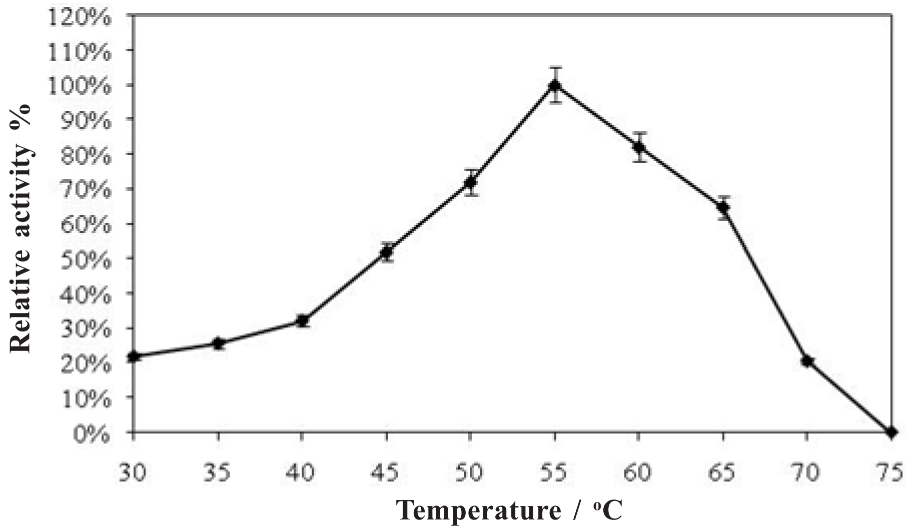

Fig. 4a. Effect of temperature on milk-clotting activity

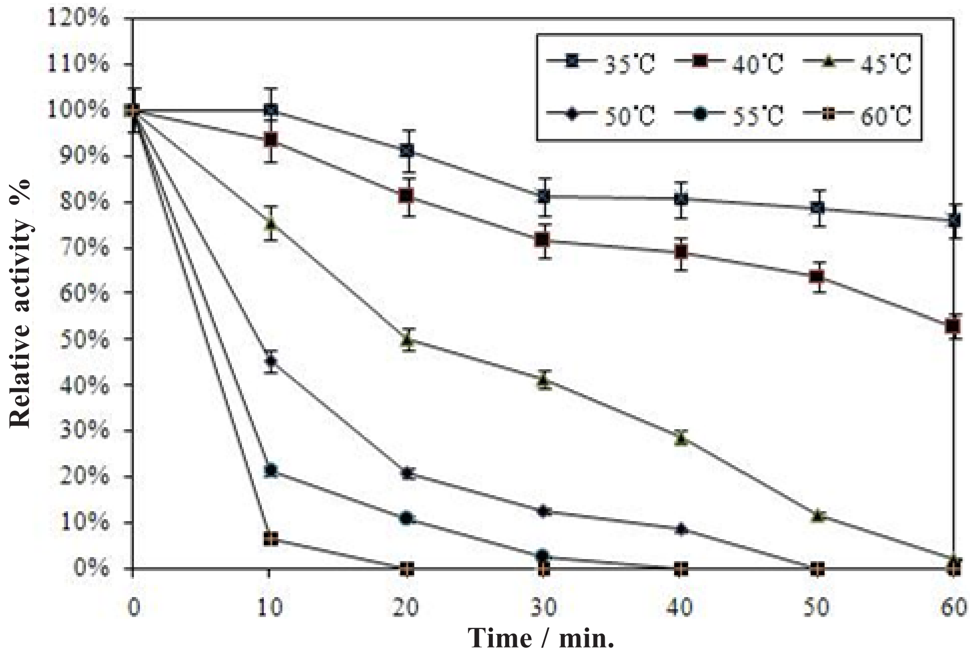

Fig. 4b. Thermo-stability of milk-clotting enzyme

tained 75.7 % activity after 60 min. At 40°C, the enzyme showed good stability and retained 81.1 % activity after 20 min. But above 50°C, it caused greatly loss in activity. As soon as the temperature was up to 60°C, the milk-clotting activity was completely lost.

Effects of pH on enzyme activity and stability

The maximum milk-clotting activity was at pH 5.5 for the purified enzyme, and the activity decreased with increasing pH (**Fig. 4c**). The stability of the purified enzyme at different pH values is shown in Fig. 4d. The enzyme was stable in a relatively wide range of pH 5.5-9.5, with maximum stability at pH 6.0. Outside either end of this range, the activity of the enzyme decreased drastically, which showed that the *B. licheniformis* HN1 enzyme was stable in the acidic to neutral range.

Effect of the concentration of $CaCl_2$

Calcium had a positive effect on the activity of the milk-clotting enzyme. Calcium has been de-

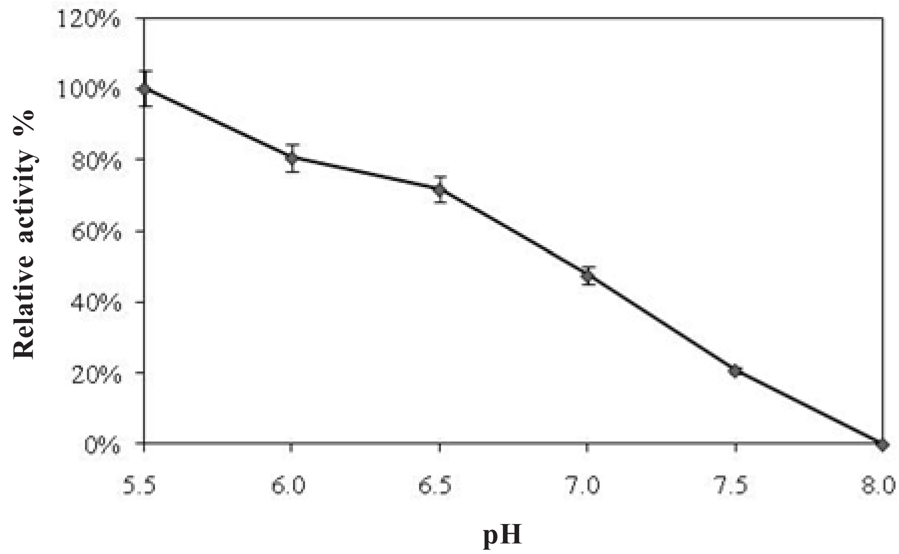

Fig. 4c. Influence of different pH on enzyme activity

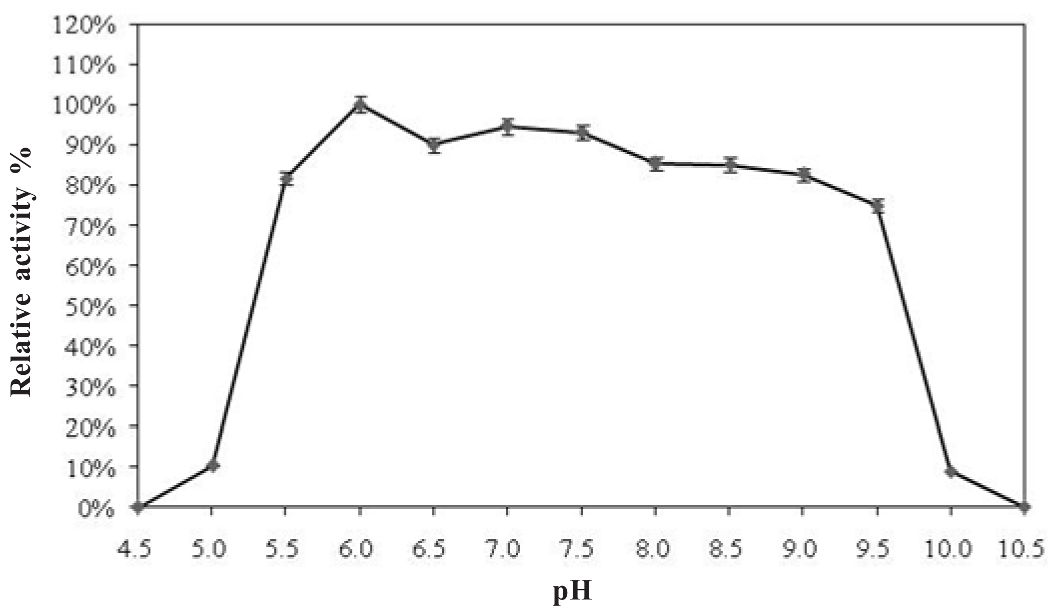

Fig. 4d. pH stability of milk-clotting enzyme

scribed as important in milk clot formation, when its concentration is high enough [14]. Fig. 4e shows that the milk-clotting activity was highest at 20 mM $CaCl_2$. In the range 0-20 mM $CaCl_2$, the coagulation rate increased with increasing concentration of Ca^{2+}. Milk-clotting activity decreased at concentrations of $CaCl_2$ higher than 20 mM.

Effect of inhibitors

In order to determine the type of milk-clotting protease produced by HN1, inhibition experiments with different protease inhibitors were conducted. Table 3 showed the sensitivity of the purified enzyme to a serine protease inhibitor (PMSF), a cysteine protease inhibitor (iodoacetamide), a metalloprotease inhibitor (EDTA) and an aspartic protease inhibitor (pepstatin A). The fact that PMSF, EDTA and iodoacetamide did not inhibit the enzyme activity showed that the enzyme was not a serine protease, metalloprotease or cysteine protease. The strong inhibition, 97.83 % at 5 mM pepstatin A, showed that the enzyme belongs to

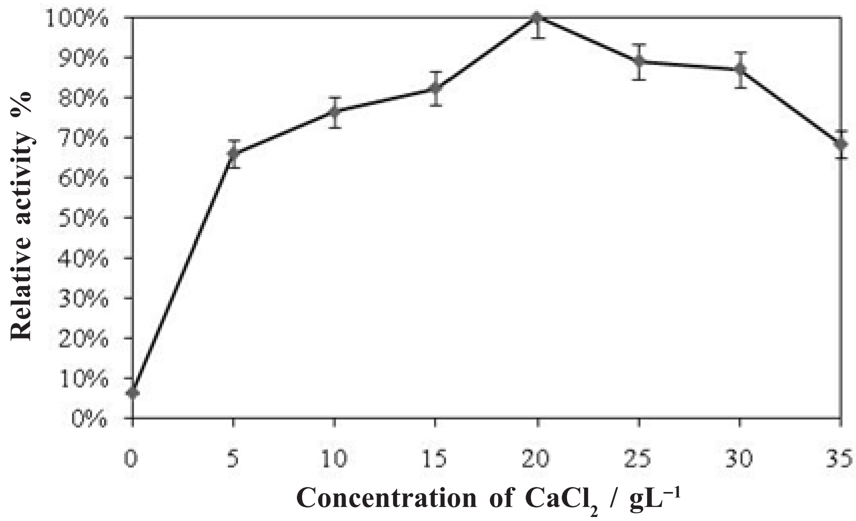

Fig. 4e Effect of $CaCl_2$ concentration on the milk-clotting activity

Table 3. Effect of inhibitors on the milk-clotting activity of the purified enzyme from *B. licheniformis* HN1

Inhibitor	Concentration (mM)	Residual activity (%)
Control	0	100±0.23
EDTA	10	94.38±0.93
Iodoacetamide	2	95.76±0.93
PMSF	10	96.99±1.0
Pepstatin A	0.02	1.95±0.15

the aspartic protease group.

Discussions

Many bacteria especially several species belonging to *Bacillus* are known to produce variety of extracellular enzymes, such as amylases, protease, and xylanase [15, 16-17]. Numerous bacteria such as *B. amyloliquefaciens*, *B. subtilis* and *B. subtilis natto* have been suggested as promising microbial rennet producers [18-20, 21]. In the present study, several bacteria producing milk-clotting enzyme isolated from soil samples collecting from Qinghai pastoral area in Tibetan Plateau of China. Among these bacteria, *B. licheniformis* HN1 possessed high rennet-producing capacity. To our knowledge, this is the first report on *B. licheniformis* producing milk-clotting enzymes that was isolated from Qinghai pastoral area in Tibetan Plateau of China.

Milk-clotting enzymes from different sources have different enzyme properties. The enzyme from *B. licheniformis* HN1 has a molecular mass of 51.9 kDa, which is lower than milk-clotting enzymes from *B. amyloliquefaciens* D4 [21] and higher than others (34-49 kDa) [22-26]. The optimum temperature of the enzyme from *B. licheniformis* HN1 was different from calf rennet, which has an optimum temperature in the range 40-42°C. Compared with the milk-clotting enzymes from other microorganism such as *B. sphaericus* [1, 27], *Mucor pusillus* [25] and *Rhizomucor miehei* [23], the B. *licheniformis* HN1 enzyme has a lower level of thermostability. Similar to calf rennet, the purified enzyme from B. *licheniformis* HN1 had a higher level of milk-clotting activity in the acidic range. The low pH-sensitivity and thermostability of the enzyme is useful for cheese making.

Wheat bran, an agro-industrial residue, contains cellulose material, starch, crude protein, trace elements and other certain ingredients. Wheat bran is abundant and cheap, usually used as feedstuff. There are several reports describing wheat bran as potent substrate for enzyme production [21]. In the present study, *B. licheniformis* HN1 possessed high rennet-producing capacity. Moreover, the medium used for enzyme production is wheat bran. These results suggest that *B. licheniformis* HN1 is a possible commercial source of milk-clotting enzyme for cheese making.

Conclusion

In this study, a total of 21 isolates were obtained for milk-clotting enzyme production and an isolate with good milk-clotting activity identified as *Bacillus licheniformis* and the properties of the milk-clotting enzyme were investigated. The purified enzyme from *B. licheniformis* HN1 belongs to the aspartic protease group with a molecular mass of 56.1 kDa, and has low thermostability and low sensitivity to pH. The result suggest that *B. licheniformis* HN1 is a possible commercial source of milk-clotting enzyme for cheese-making. An evaluation of its potential for use in cheese-making will be the subject of future work.

Acknowledgements

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