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Full Length Research Paper

Production of milk-clotting enzyme by *Bacillus subtilis* B1 from wheat bran

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Three strains, *Bacillus subtilis* B1, *B. subtilis* B18 and *Bacillus thuringiensis* B12, were screened from wheat bran to produce milk-clotting enzyme. Among them, *B. subtilis* B1 exhibited considerable milk-clotting activity with low proteolytic activity. After response surface methodology optimization, milk-clotting activity was **improved from 782 SU ml^{-1} to 1129.05 ± 74.55 SU mL^{-1} with a small inoculum size (0.130%, v/v). The** optimized **medium contained glucose (16.2 gL^{-1}), wheat bran (30 gL^{-1}), NaCl (5 gL^{-1}), $MgSO_4 \cdot 7H_2O$ (5 gL^{-1}), KH_2PO_4 (2 gL^{-1}) and $CaCO_3$ (3 gL^{-1}). The milk-clotting enzyme from *B. subtilis* B1 has the optimum pH 5.5 and $CaCl_2$ concentration 50 mmol L^{-1}. It is completely inactivated after 5 min at 70°C. The result showed that *B. subtilis* B1 was a promising strain for industrial milk-clotting enzyme production.**

Key words: Milk-clotting enzyme, *Bacillus subtilis*, submerged fermentation, wheat bran.

INTRODUCTION

Rennet (E.C. 3.4.24.4) from calf stomach is the largest single proteolytic enzyme in food processing. Rennet not only can clot the milk in the first step in cheese manufacture, but also plays an important role for the flavor and texture during cheese ripening (Souse et al., 2001)

Although, the calf rennet has been used widely in cheese manufacture industry for a long time, its preparation process is ethically arguable and lowly productive. Its possible alternatives have drawn more attentions due to an ever-increasing demand for cheeses. Various milk-clotting enzymes (MCEs) from animals, plants and micro-organisms have thus, been suggested as calf rennet substitutes (Ayhan et al., 2001; Libouga et al., 2008; Guiama et al., 2010). Microbial MCEs have some advantages over those obtained from plants or animals, such as cheaper cost, greater biochemical diversity and easier genetic modification. Nowadays, most research work about MCEs production has focused on solid state fermentation by fungus, such as *Mucor miehei*, *Rhizomucor pusillus* var and *Aspergillus oryzae* (Birkkjacer and Jonk, 1985; Crawford, 1985; Ayhan et al., 2001).

Although, only few research work on bacteria are reported, wild bacteria with a high milk-clotting activity (MCA) in submerged fermentation show a great potential, due to shorter fermentation period, larger capacity of extracellular secretion and higher material utilization ratio (Dutt et al., 2008). Some bacteria, such as *Bacillus subtilis*, *Bacillus licheniformis* and *Enterococcus faecalis*, have been suggested as potential rennet substitutes (Sato et al., 2004; Ageitos et al., 2007; Dutt et al., 2008). Further, Singh and McGugan indicated that, bacterial MCEs contributed to distinctive flavors in the formation of Cheddar cheese (McGugan, 1975; Singh et al., 2003).

Most of the microorganisms mentioned were screened from soil, natto, milk or glutinous rice wine samples (Rao and Mathur, 1979; D'souza and Pereira, 1982; Sato et al., 2004; Dutt et al., 2008). Microorganisms from different sources allow a variety of cheeses with different rheological and organoleptic properties (Silva and Malcata, 2005; Ageitos et al., 2006). However, there are no

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Abbreviations: MCA, Milk-clotting activity; **MCE,** milk-clotting enzyme; **PA,** proteolytic activity; **SU,** soxhlet unite.

reports about MCE-producing bacteria screened from wheat bran. In this paper, three strains (*B. subtilis* B1, *B. subtilis* B18 and *B. thuringiensis* B12) were isolated firstly from wheat bran. The fermentation conditions and enzyme characterizations of *B. subtilis* B1 were investigated.

EXPERIMENTAL

Strains isolation

The wheat bran samples were purchased in local market. The average percentages of starch, proteins, moisture, crud fibers, ash and others in sorghum are 19.71, 14.2, 8.5, 48.80, 5.57, and 8.22, respectively. An aliquot of 6 g samples was mixed with 100 ml sterile physiological saline and several glass beads. The mixture was incubated on a rotary shaker at 150 rpm for 20 min. About 10 ml of this mixture was transferred into the flasks containing 90 ml nutrient broth medium. The culture was placed on a rotary shaker in the dark at 35 ± 0.5°C for 12 h. By spreading over the diluted fermentation broth on skim milk plates (skim milk 30 g L^{-1}, agar 15 g L^{-1}), the enzyme-producing strains gave a brilliant clear circle of hydrolysis inside a precipitation circle. The isolated strains were further inoculated in nutrient broth medium to detect MCA. Then, they were grown and maintained at 35 ± 0.5 and 4°C, respectively, on agar slant (glucose, 20 g L^{-1}; yeast extract, 10 g L^{-1}; agar, 20 g L^{-1}).

Physiological and biochemical tests

The bacillus characters and bacterium configurations were observed by microscope. Phenotypic and biochemical characterizations of strains were performed as described in Bergey's manual of determinative bacteriology.

Phylogenetic analysis by using 16S rRNA gene sequence comparison

Cloning and sequencing of 16S rRNA genes were carried out as described by Weisburg et al. (1991). Oligonucleotides, 5′-AGAGTTTGATCCTGGCTCAG-3′ (primer F) and 5′-CGGTTA CCTTGTTAC GACTT-3′ (primer R), were used as the primers for the amplification of 16S rRNA gene fragments *in pMD18-T vector (TaKaRa). Then, the cloned genes were* sequenced with a dye terminator cycle-sequencing Fs ready reaction kit and a model ABI 3130 automatic DNA sequencer (Applied Biosystems). Primers, M13R (CAGGAAACAGCTATGACC) and M13F (TGTAAAACG ACGG CCAGT), were employed for sequencing. A homology search was performed with GenBank database. To further investigate the characterization of strains, a distance tree was built through neighbor-joining methods by Mega version 3.1, after preliminary multiple alignments of the sequences by Clustal W version 1.83 with *1000 rounds of bootstrapping (*Kumar et al., 1994; Thompson et al., 1994*).*

Medium and culture conditions

Each Erlenmeyer flask (500 ml), containing 40 ml fermentation medium was inoculated from the agar slant and incubated at 35 ± 0.5°C on a rotary shaker at 150 rpm. The fermentation medium contained wheat bran (30 g L^{-1}), NaCl (5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (5 g L^{-1}), KH_2PO_4 (2 g L^{-1}), $CaCO_3$ (3 g L^{-1}).

Optimization of the fermentation process

There are four main factors affecting MCA under submerged conditions, such as glucose concentration, inoculum size, fermentation time and medium volume. With full-factorial central composite design, a response surface methodology (RSM) was applied for improving MCA. Samples were withdrawn and centrifuged at 5900×g for 5 min with supernatant used for MCA assay. The statistical software package (Design-expert 7.0) was used to analyze the experimental design.

The fermentation broth was filtered by microstrainer and ultrafiltration, then lyophilisated. The crude enzyme powder was stored in 4°C.

Biochemical properties of crude enzyme

Since the skim milk coagulated at low pH even absence of the enzyme, investigation of pH values on MCA was done at pH 5.5 to 7.0 by appropriate buffer solutions (Ageitos et al., 2007; Vishwanatha et al., 2010).

To confirm optimum reaction temperature, the milk samples were previously equilibrated at temperatures from 35 to 75°C. The thermal stability was understood by the activity of residual enzyme exposed at various temperatures (50 to 70°C) for 5, 10, 15 and 20 min.

Calcium chloride was added to reconstitute skim milk at various concentrations (0, 1, 5, 10, 25, 50 and 100 mmol L^{-1}). The MCA was separately assayed in each sample at 35°C.

Assay for enzyme activity

Based on the appearance of the first clotting flakes, MCA was determined and expressed in terms of Soxhlet unite (SU) according to the method of Arima et al. (1970). One SU was defined as the amount of enzyme which clotted 1 ml of a solution containing 0.1 g skim milk powder in 40 min at 35°C (Shieh et al., 2009).

The proteolytic activity (PA) was determined at pH 6.0 by the casein digestion method (Shieh et al., 2009). One unit of proteolytic activity was defined as the amount of enzyme which yields the color equivalent to 1 µmol of tyrosine per minute. All the experiments were carried out in three times.

RESULTS

Isolation and identification

Preliminary screening indicated that, 3 in 21 strains have MCA. In nutrient broth medium, the MCA of B1, B12 and B18 is 131.50, 64.17 and 114.09 SU mL^{-1}, respectively.

Several important physiological and biochemical charac-teristics were employed to identify the strains. The three strains, long rod-shaped Gram-positive bacteria, can form spores. On skim milk medium, the colonies of B1 and B18 were rough, diffusible and creamy in color, with a diameter of approximately 1.3 to 4.4 mm. There were droplets on the surface of B1 colonies, none on B18. The colonies of B12 appeared to be smooth, circular, lustrous and light yellow in color, with a diameter

Table 1. Biochemical characteristics of strain B1, B12 and B18.

Parameter	B1	B12	B18
MCA (SU/ml)[a]	131.50	64.17	114.09
Spore	+	+	+
Anaerobic growth	-	+	-
Gas from glucose	-	-	-
Hydrolysis of			
Casein	+	+	+
Gelatin	+	+	+
Starch	+	+	+
Utilization of			
Citrate	+	+	+
Phenylalanine deaminase	-	-	-
Growth temperature			
10℃	-	-	-
40℃	+	+	+
55℃	-	-	+
65℃	-	-	-
MCA per PA[a]	4.75	1.45	3.55
Parasporal crystal	-	-	-
Lecithinase	-	-	-
V-P	+	+	+
M.R			
D-Glucose	+	+	+
L-Arabinose	+	-	+
D-Xylose	+	-	+
D-Mannitol	+	-	+
Growth at pH			
6.8	+	+	+
5.7	+	+	+
GCN[b]			
2%	+	+	+
7%	+	+	+

[a]The medium was nutrient broth medium. [b]GCN means growth at concentration of NaCl.

of approximately 0.8 to 2.3 mm. Both B1 and B18 were aerobic. B12 was facultative aerobe. The results were listed in Table 1. B1 and B18 show typical characteristics of *B. subtilis,* while B12 was similar as *B. thuringiensis.*

Phylogenetic tree analysis

Almost the entire length of the 16S rRNA gene was amplified by polymerase chain reaction. A BLAST search against GenBank indicated that, the 16S rRNA gene nucleotide sequences of B1, B18 and B12 were similar to that of *B. subtilis* strain MZA75, *B. subtilis* strain ZHA9 and *B. thuringiensis* strain GS1, respectively. Phylogeny relationship with similar sequences in GenBank is shown in Figure 1.

Optimization of the fermentation process

The *B. subtilis* B1 had considerable MCA with low PA (Table 1). A new medium (part 2.4) was developed for enzyme production by *B. subtilis* B1. 782 SU mL^{-1} of MCA was obtained. The MCA was 5.57-fold as high as that in nutrient broth medium. After RSM optimization (data shown in Tables S1 and S2 and Figure S1), *B. subtilis* B1 yielded 1129.05 ± 74.55 SU ml^{-1} with 1.44-fold as high as 782 SU mL^{-1}. The ratio of MCA/PA was 6.5.

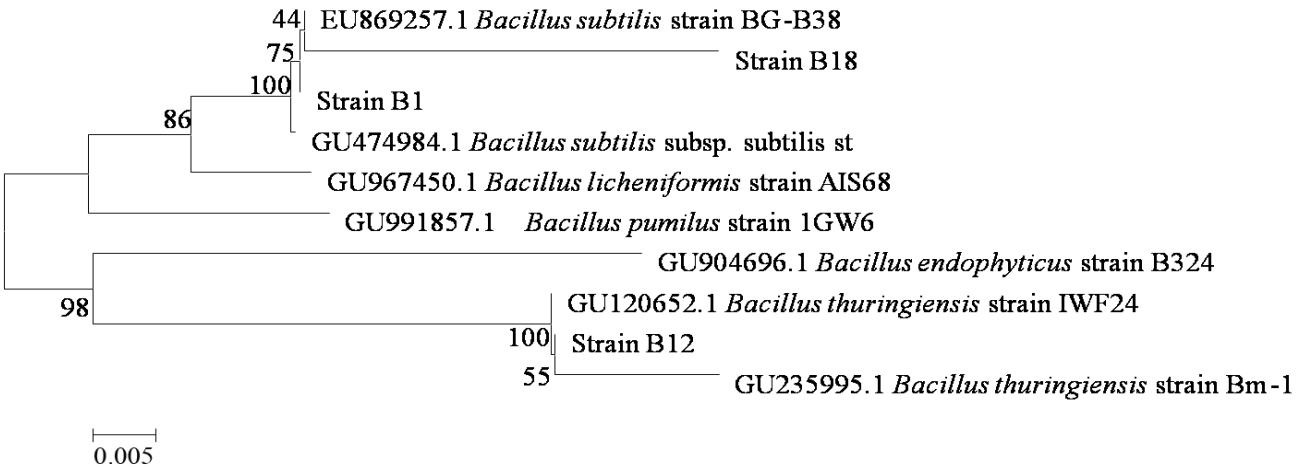

Figure 1. Phylogenetic relationship of MCE-producing strains *B. subtilis* B1, *B. thuringiensis* B12 and *B. subtilis* B18 to other related *Bacillus* sp. based on 16S rRNA gene sequences. (The branching pattern was generated by the neighbor-joining methods using Mega version 3.1 after preliminary multiple alignments of the sequences by Clustal W version 1.83 with *1000 rounds of bootstrapping. Bootstrap values of above 50% are shown at branch point. Bar, 0.005 substitutions per nucleotide position).*

Table S1. Experimental level and code of variables chosen for Full-factorial CCD.

Variable	Symbol		Coded level				
	Uncoded	Coded	-α	-1	0	1	α
Glucose concentration (g/l)	X_1	A	6.4	20	40	60	73.6
Medium volume (ml)	X_2	B	43.2	50	60	70	76.8
Inoculum size (%)	X_3	C	0.041	0.075	0.125	0.175	0.209
Fermentation time (h)	X_4	D	55.68	72	96	120	136.32

A=(X1-40)/2, B=(X2-60)/10, C=(X3-0.125)/0.05, D=(X4-96)/24°.

Table S2. CCD matrix of the four variables with the experimental and predicted MCE activity.

Run	Independent variable				MCA(SU/mL)	
	A	B	C	D	Experimental value	Predicated value
1	-1	-1	-1	-1	668.52	668.86
2	1	-1	-1	-1	441.99	443.65
3	-1	1	-1	-1	511.73	517.98
4	1	1	-1	-1	480.96	477.95
5	-1	-1	1	-1	691.64	694.14
6	1	-1	1	-1	458.02	456.78
7	-1	1	1	-1	604.53	601.28
8	1	1	1	-1	545.46	549.09
9	-1	-1	-1	1	1021.28	1022.13
10	1	-1	-1	1	682.27	683.98
11	-1	1	-1	1	882.54	882.24
12	1	1	-1	1	727.27	729.26
13	-1	-1	1	1	1033.12	1034.6
14	1	-1	1	1	686.07	684.30
15	-1	1	1	1	949.91	952.73
16	1	1	1	1	789.47	787.60
17	-α	0	0	0	941.18	936.24

Table S2. Continues

18	α	0	0	0	607.60	608.36
19	0	-α	0	0	848.06	846.19
20	0	α	0	0	808.53	806.22
21	0	0	-α	0	762.95	758.72
22	0	0	α	0	828.90	828.95
23	0	0	0	-α	464.22	461.54
24	0	0	0	α	960.13	958.64
25	0	0	0	0	956.18	958.83
26	0	0	0	0	958.52	958.83
27	0	0	0	0	956.72	958.83
28	0	0	0	0	956.18	958.83
29	0	0	0	0	960.39	958.83
30	0	0	0	0	960.07	958.83

The final empirical model to predict the MCA was $Y=958.83-97.58A-11.89B+20.90C+147.94D-66.09A^2-46.989B^2-58.4589C^2-88.1305D^2+46.29AB-3.04AC-28.23AD+14.51BC+2.75BD-3.20CD$.

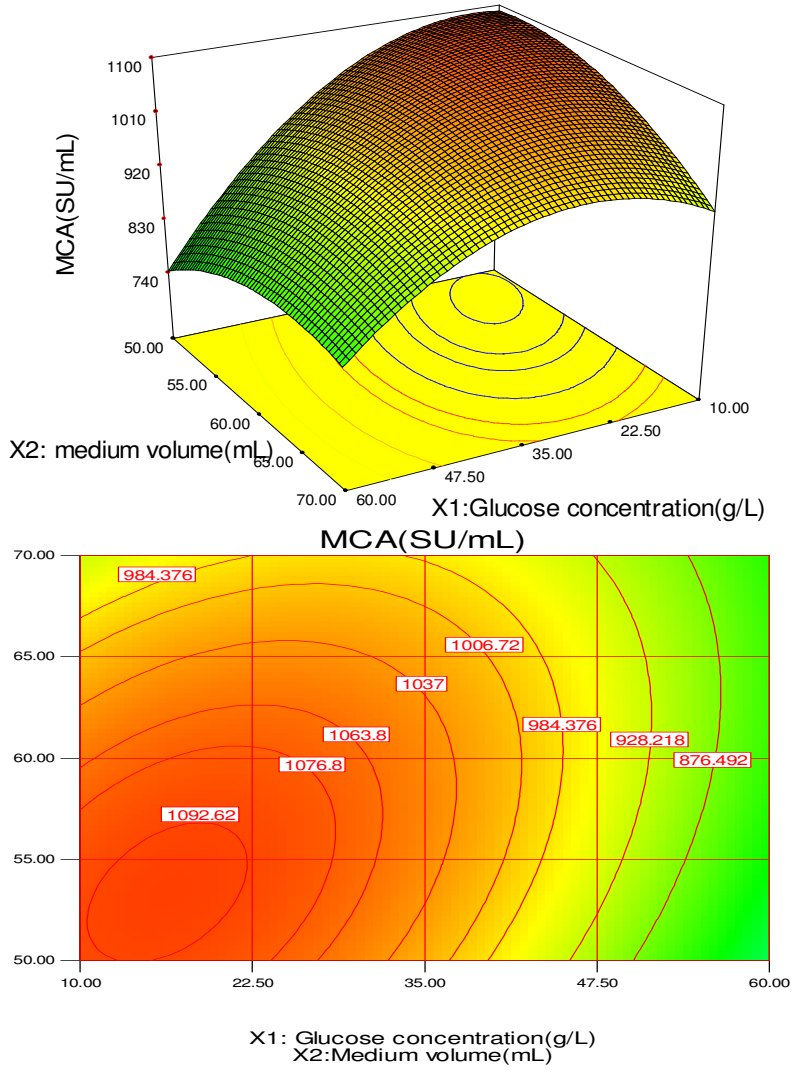

Figure S1. Response surface and their contour plots of MCE activity versus glucose concentration and medium volume.

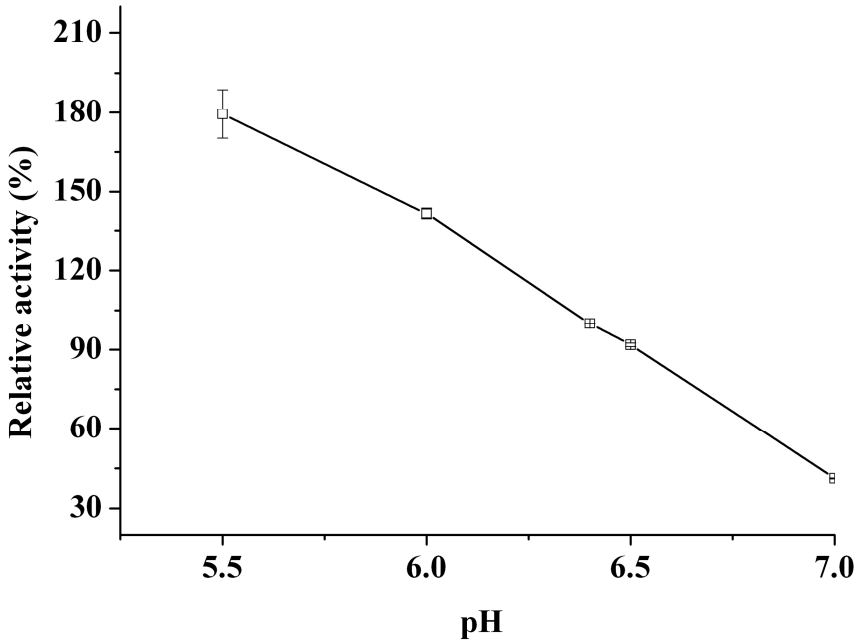

Figure 2. Effect of reaction pH on MCA (The MCA at original pH 6.40 is defined as 100%).

The medium contained glucose (16.2 g L^{-1}), wheat bran (30 g L^{-1}), NaCl (5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (5 g L^{-1}), KH_2PO_4 (2 g L^{-1}) and $CaCO_3$ (3 g L^{-1}). The inoculum size was small, 0.130% (v/v). The flask containing 53.3 ml medium was incubated at 150 rpm for 120 h.

Effect of pH on MCA

As the pH increased from 5.5 to 7.0, rapid decrease of MCA (Figure 2) was observed. The MCE exhibited the maximal activity at pH 5.5 with 179.45% retained.

Effect of reaction temperature and thermal stability

The MCA increased as temperature between 35 and 70°C. The optimal temperature was 70°C with more than 600% remained (Figure 3). Beyond 70°C, the MCA decreased rapidly as the temperature increased. It retained about 30% when the reaction temperature was 78°C.

The crude enzyme was stable below 50°C with more than 95% of MCA remained after incubation for 10 min at 50°C (Figure 4). The MCA decreased to 81.3% after incubation for 10 min at 55°C. It was deactivated completely after 5 min at 70°C.

Effect of $CaCl_2$ concentration on MCA

Ca^{2+} combined with para casein to form firm clot during second phase of clotting process. In our research, low concentrations (0 to 50 mmol L^{-1}) of $CaCl_2$ promoted the MCA, while high concentrations (>50 mmol L^{-1}) restrained the MCA (Figure 5). The maximum activity was obtained when 50 mmol L^{-1} was added to reconstituted skim milk.

DISCUSSION

Three *Bacillus* sp. were screened from wheat bran. Since wheat bran is the main part of koji in China, the enzyme-producing bacteria from glutinous rice wine also seems to come from wheat bran (Wang et al., 2009). The biochemical and phylogenetic tree analysis showed that, the three strains were *B. subtilis* B1, *B. subtilis* B18 and *B. thuringiensis* B12. As we known, there are few reports about *B. thuringiensis* to produce MCE. Furthermore, *B. subtilis* B1 was considered as a promising safe producer due to its high MCA with low PA and *B. subtilis* was generally regarded as safe by Food and Drug Administration (Schallmey et al., 2004). *Bacillus* sp. is one of the most important industrial enzyme producers owning to the capacity to secrete large amount (20 to 25 g L^{-1}) of extracellular enzymes (Rajagopalan and Krishnan, 2009). Schallmey estimated that, commercial enzymes from *Bacillus* sp. occupy about 50% of the market (Schallmey et al., 2004).

High MCA, 782 SU mL^{-1} in wheat bran medium, was obtained, only 131.50 SU mL^{-1} in nutrient broth medium. The high MCA may be attributed to wheat bran. The proteins content in wheat bran is 14.2%. In the medium,

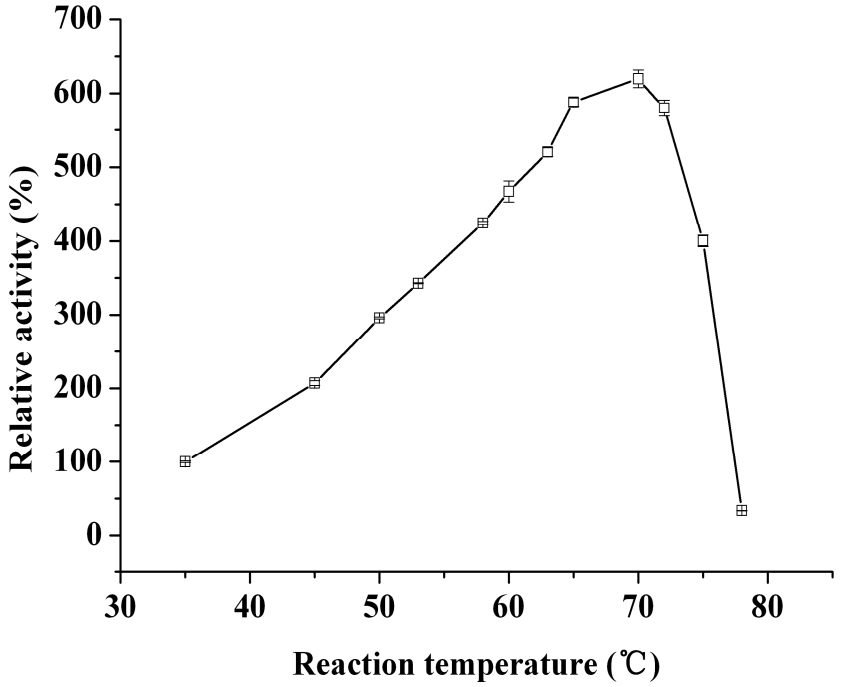

Figure 3. Effect of reaction temperature on MCA.

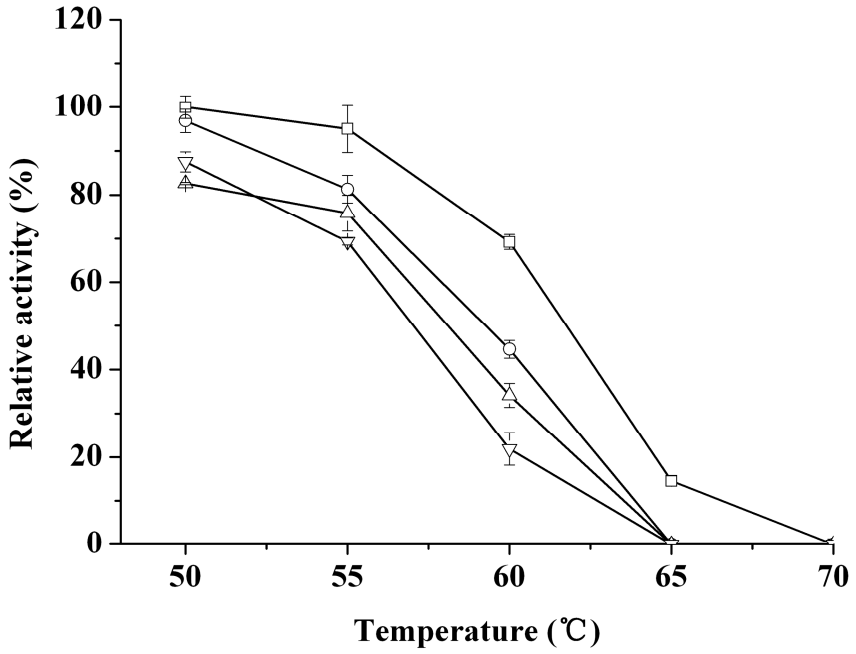

Figure 4. Thermal stability of MCE from *B. subtilis* B1. Symbols: –ϒ– 5 min; –⊄– 10 min; –≥– 15 min; –|- 20 min.

the easily-used nitrogen content is lower than 4.26 g L^{-1}. When the easily-used nitrogen is exhausted in batch culture, a slow release of nitrogen source from wheat bran is believed to support the bacterial growth (Kaur et al., 2001). The *B. subtilis* (natto) Takahashi also had high MCA under limited nitrogen supply (Shieh et al., 2009). Porto reported that, the excess of the nitrogen source or ammonia concentration could repress protease synthesis (Porto et al., 1996). Our researches firstly report that, wheat bran is the only nitrogen source in bacterial MCE

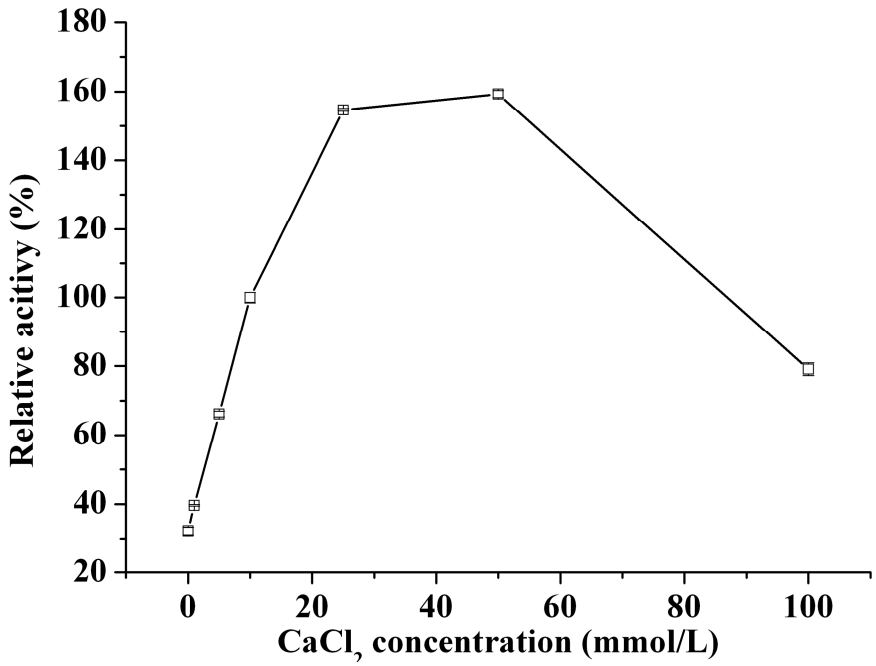

Figure 5. Effect of $CaCl_2$ concentration on MCA.

production with a high MCA.

Based on RSM optimization, 1129.05 ± 74.55 SU mL^{-1} was obtained as 1.44 times the MCA before optimization. The inoculum size was small, 0.130% (v/v). To our knowledge, such a small inoculum was firstly employed in MCE production by bacterial. It could simplify the preparation progress of inoculum in industrial applications. Further research is needed to understand why small inoculum size goes with high MCA.

The enzyme characterizations of *B. subtilis* B1 were investigated. The MCE from *B. subtilis* B1 was proved as an acid protease with optimum pH 5.5, although, commercial proteases from the genus *Bacillus* were mainly neutral and alkaline (Dutt et al., 2008). Its optimum pH was consistent with that of *M. miehei*, *E. faecalis* TUA2495L and *B. subtilis* K-26 (Rao and Mathur, 1979; D'souza and Pereira, 1982; Sato et al., 2004). Gels made in low pH appeared to have a denser and more interconnected structure (Esteves et al., 2003). The optimum reaction temperature was 70°C, the same as *E. faecalis* TUA2495L and *B. cereus* (Melachouris and Tuchey, 1987; Sato et al., 2004). The thermal stability of MCE from *B. subtilis* B1 was similar to that previously reported. The MCEs of *B. subtilis* (natto) Takahashi and *B. cereus* displayed a dramatic loss in activity after 40 min at 55°C (Melachouris and Tuchey, 1987; Shieh et al., 2009). The MCE of *B. licheniformis* was completely inactivated after 3 min at 70°C (D'souza and Pereira, 1979). This result showed that, the MCE from *B. subtilis* B1 can be inactive by pasteurization (70°C for 5 min). Low concentrations (0 to 50 mmol L^{-1}) of $CaCl_2$ promoted the MCA when high concentrations (>50 mmol L^{-1}) of $CaCl_2$ restrained the MCA. When 50 mmol L^{-1} $CaCl_2$ was added to skim milk solutions, the maximum activity was obtained. Similar phenomenon was observed in *B. subtilis* K-26, *E. faecalis* TUA2495L and *B. licheniformis* (Rao and Mathur, 1979; D'souza and Pereira, 1982; Sato et al., 2004). So, it can be confirmed that the enzyme from *B. subtilis* B1 is a typical microbial MCE with fine quality.

In conclusion, we represent the first report that bacteria (*B. subtilis* B1, *B. subtilis* B18 and *B. thuringiensis* B12) from wheat bran could produce MCEs. *B. subtilis* B1 is a promising strain for industrial MCE production. The MCE from *B. subtilis* B1 is an ideal enzyme for cheese-making industry with; (1) high MCA (1129.05 ± 74.55 SU mL^{-1}) in submerged fermentation with a small inoculum size (0.130%, v/v), using wheat bran as the only nitrogen source; (2) low optimum pH, making gels denser; (3) appropriate thermal stability for pasteurization.

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