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Production, purification and characterization of a milk clotting enzyme from *Bacillus methanolicus* LB-1

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Abstract *Bacillus methanolicus* LB-1 isolated from traditional rice wine was found to produce a milk clotting enzyme (MCE), and its fermentation conditions were optimized using response surface methodology. Then the MCE was produced by ethanol precipitation, and further chromatography separation resulted in a 10.46-fold purification with a 59.28% recovery. The MCA (milk clotting activity) of the purified MCE reached 597,310 ± 0.13 SU/g. The optimal temperature of the MCE was determined to be 50 °C and it was stable in the low temperature range of 40–45 °C. The MCE had an optimum pH of 6.5, and it was stable under neutral conditions. Calcium chloride at the concentration of 25 mM was found to be the most effective stimulus. The MCE was identified by LC–MS to be a putative protein (ID I3EB99) containing 759 amino acids with a molecular weight of 80.37 kDa and a pI of 9.23.

Keywords *Bacillus methanolicus* · Milk clotting enzyme · Production characterization identification

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Introduction

Milk coagulation is an important processing step in cheese manufacturing, and the milk clotting enzyme (MCE) plays the major role (Nasr et al., 2016). Although calf rennet has been conventionally employed as the milk clotting agent (Kobayashi et al., 1985), the choices of MCE are gradually migrating towards plant MCEs (Beka et al., 2014; Salehi et al., 2017) and microbial MCEs (Merheb-Dini et al., 2012). Owning to rapid growth of microbes and relatively inexpensive growth substrates, microbial MCEs have very attractive and promising application prospects (Fazouane-naimi et al., 2010; Hashem, 2000; Soltani et al., 2016). Many bacteria, especially the species belonging to *Bacillus* such as *B. subtilis* (Wu et al., 2013), *B. amyloliquefaciens* (An et al., 2014) and *Paenibacillus* spp. (Hang et al., 2016a; 2016b), have been reported to produce extra-cellular MCEs. Production of bacterial MCEs by submerged fermentation (SMF) was efficient with a high yield of MCE (Ding et al., 2012), and it was simple to operate compared to solid state fermentation (SSF) by fungus (Thakur et al., 1990).

The ideal MCE was characterized with a high milk clotting activity (MCA), equivalent to the commercial enzyme with a high ratio of MCA to proteolytic activity (PA) (Ding et al., 2012). Unfortunately, despite many reports on the production, purification and characterization of various bacterial MCEs, few of them met the requirement of an ideal MCE (Sato et al., 2004; Zhao et al., 2015). Production of bacterial MCEs was influenced by cultivation temperature, filled volume of growth medium, cultivation time and rotation speed in shaking-flask cultivation (Hang et al., 2016a; 2016b). Consequently, the ideal MCE could be produced by screening new bacterial producing strains and optimizing fermentation conditions. In this

study, a strain of *B. methanolicus* LB-1 isolated from traditional rice wine in our previous research (Li et al., 2017) was found to produce MCE for the first time. The fermentation conditions of the MCE from *B. methanolicus* LB-1 was optimized by response surface methodology (RSM), and it was further purified, characterized and identified to determine its potential as a milk clotting agent in cheese industry.

Materials and methods

Materials

The strain of *B. methanolicus* LB-1 was isolated and identified in our lab from rice wine and stored as freeze-dried powder at − 80 °C in the General Microorganism Center of Chinese Microbe Preservation Management Committee (Beijing, China), numbered CGMCC No. 14424. The constituents of the growth medium were 4.14 g/L of α-lactose, 7.47 g/L of casein, 8.31 g/L of yeast extract and 0.60 g/L of K_2HPO_4 (Teng and Yang, 2016). This medium was used for the activation of *B. methanolicus* LB-1 by transferring consecutively for three times, and then it was grown in 100 mL flask for shaking-flask experiment at 30 °C for 24 h.

Skim milk powder was purchased from Fonterra (Auckland, New Zealand). All chemical reagents used were of analytical grades.

Optimization of the fermentation conditions of *B. methanolicus* LB-1

Initial single factor experiments, controlling a single variable, were carried out to determine factors affecting the MCA of *B. methanolicus* LB-1 under the following condition: inoculation rate (1–7%), filled volume (10–60%), cultivation time (12–72 h), cultivation temperature (25–50 °C), culture rotating speed (80–180 r/min) and initial pH of medium (5 ~ 9). Then a two-level Plackett–Burman design was used to filter main factors, which had a significant effect on MCA, and the selected factors were chosen for the full-factorial face-centered central composite design (FCCCD) at five levels in RSM. The MCA (SU/mL) was taken as dependent variable or response (Y), and the relationship between independent variables and response function was deliberated by a second-order polynomial equation:

$$Y = B_0 + \Sigma B_i X_i + \Sigma B_{ii} X_i^2 + \Sigma B_{ij} X_i X_j \quad (1)$$

where Y, the predicted response; B_0, the constant; B_i, the linear effect; B_{ii}, the *square effect;* B_{ij}, the interaction effect; X_iX_j, the input variables which influence the response Y.

Assay for MCA

MCA was determined according to the method of Arima (Iwasaki et al., 1968), which was based on visual evaluation of the appearance of first clotting flakes, and expressed in terms of Soxhlet units (SU). Skim milk (10 g dry skim milk/100 mL, 0.01 M $CaCl_2$) was pre-incubated at 35 °C for 5 min, and 0.5 mL of tested material was added to a test-tube containing 5 mL of skim milk. The time between addition and appearance of clots was recorded, and the clotting activity was calculated using the following formula: SU = 2400 × 5 × D/T × 0.5; T = clotting time (s); D = dilution of test material.

Assay for PA

The PA was assayed as described by Arima (1970). Sample (0.5 mL) was added to 2.5 mL of 20 mM potassium phosphate buffer at pH 6.5, containing 1% (w/v) alkali-soluble casein. The mixture was incubated in a water bath at 35 °C for 10 min, and the reaction was terminated by adding 2.5 mL of 0.44 M trichloroacetic acid. One milliliter of 1 M Foline-phenol reagent and 2.5 mL of 0.55 M sodium carbonate solutions were added to 1 mL of the filtrate. This was further incubated for 20 min at 35 °C for color development and A_{660} was measured. One unit (1U) of enzyme activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per 1 mL in 1 min.

Production and purification of the MCE from *B. methanolicus* LB-1

The fermentation broth obtained from *B. methanolicus* LB-1 under optimal fermentation conditions was centrifuged (10,400×g, 30 min) at 4 °C. Pre-cooled ethanol was added into the supernatant to 20–80% with 10% intervals at 4 °C overnight. The precipitate was collected by centrifugal separation (10,400 × g, 30 min), and dissolved in 5 mM phosphate buffer (pH 7) to obtain the crude MCE solution. The volume fraction of ethanol with the highest MCA of the crude MCE was chosen for further purification, then the crude MCE was dialyzed in distilled water at 4 °C to remove small impurities and freeze–dried using a lyophilizer (OSTC, Beijing, China).

The dissolved and filtered (0.45 μm pore size) crude MCE was subjected to a DEAE Sepharose Fast Flow column previously equilibrated with 50 mM Tris–HCl buffer (pH 7). Elution was performed with a linear gradient of NaCl from 1 to 4 M at a flow rate of 2 mL/min in 50 mM Tris-HCl buffer (pH 10). The purity of the purified MCE

was confirmed by SDS-PAGE. The Kjeldahl method was used to determine the total protein content, then the recovery rate and other related parameters were calculated.

Enzymatic characterization of the MCE from *B. methanolicus* LB-1

Effect of temperature on MCA of the purified MCE from *B. methanolicus* LB-1 was studied at different temperatures from 25 to 70 °C to investigate the optimal temperature. The thermal stability of the purified MCE was measured by incubating the MCE solutions in a water bath at 40 °C, 45 °C, 50 °C, 55 °C, 60 °C and 65 °C for 10, 20, 30, 40, 50 and 60 min, respectively. Effect of pH on MCA of the purified MCE was determined in the pH range from 5 to 8. The pH stability was tested by adjusting the pH of the MCE solutions to 5.0–8.5 and incubating the solutions for 10, 20, 30, 40, 50 and 60 min, then the MCE solutions were adjusted to pH 6.5 and the residual MCA of each solution was determined. To study the effect of metal ions, skim milk with added metal ions (Ca^{2+}, Na^{+}, Al^{3+}, Fe^{3+}, K^{+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}) was studied for MCA of the purified MCE. The MCA of MCE at 50 °C, pH 6.5 and skim milk without ions (blank control) was set as 100%, and characterization of the MCE from *B. methanolicus* LB-1 was compared with a commercial enzyme (Chr. Hansen, Hess, Holm, Denmark) which had MCA at 3.5×10^5 SU/g.

The Michaelis–Menten constant was determined according to the method described by He et al. (2011) with slight modification. Casein solutions with concentrations in the range of 0–5 g/L with 0.5 intervals were measured for the PA. The K_m (Michaelis constant) and V_m (maximum velocity) were calculated from the double reciprocal plot of the Lineweaver–Burk equation.

Identification of the MCE from *B. methanolicus* LB-1

The purified MCE which displayed as a single band protein was identified by LC–MS (Thermo Fisher Scientific, Waltham, MA, USA). The peptide segments of enzymolysis after decoloration and dehydration were extracted, and through separation by HPLC, they were entered into the Q Exactive mass spectrometer for online detection directly (Mata et al., 2015). The raw MS files were analyzed and searched against protein database based on the species of the samples using Maxquant.

Statistical analysis

All experiments were done in triplicate and the results were expressed as mean ± standard deviation. The data were analyzed by Plackett–Burman design and RSM, using Mintab 15.0 and Design-Export V8.0.6.1. The level of statistical significance was set at $p < 0.05$.

Results and discussion

Factors affecting the MCA of *B. methanolicus* LB-1

The effect of different fermentation parameters on MCA was shown in Fig. 1. The inoculation rate of *B. methanolicus* LB-1 was determined to be an optimal level of 3%, corresponding to the maximal MCA [Fig. 1(A)]. Higher inoculation rates decreased the MCA due to limitation of nutritional supply in the growth medium (He et al., 2012). Figure 1B showed that the MCA of *B. methanolicus* LB-1 was the highest when the rate of filled liquid medium in the flask was at 30%. The amount of fermentation broth affected the dissolved oxygen, thus influencing the MCA. The MCA tended to increase linearly with the increasing of fermentation time, and reached the maximum value (623.38 SU/mL) at 60 h (Fig. 1C). Extension of fermentation time accompanied with gradual depletion of nutrients in the medium, preventing normal metabolism of microorganisms and MCA (Narwal et al., 2017). The MCA reached the maximum at 35 °C (Fig. 1D), as the fermentation temperature affected the reaction rate of various enzymes and protein properties, and temperatures lower or higher than the optimal one could significantly inhibit their activity, thus the MCE production (Chwenjen et al., 2009). With the increasing rotation speed, the MCA of *B. methanolicus* LB-1 reached the maximum at 140 r/min (Fig. 1E), and it decreased at higher speeds due to increased mechanical shear force that was not conducive to cell growth (Hashem, 2000; Narwal et al., 2017). Proper agitation of culture would facilitate MCA by letting in more air, therefore oxygen, but some other studies presented otherwise different results, that still culture would yield a higher MCA (Hashem, 2000; Narwal et al., 2017; Sun et al., 2014), which was possible due to the differences in the nature of strains. He et al. (2012) indicated that the initial pH of growth medium strongly affected enzyme reaction and transport of various components across the cell membrane, thus the MCA of *B. methanolicus* LB-1. It was evident from Fig. 1F that the maximum MCA was observed at the initial pH 7.

Optimization of the MCA of *B. methanolicus* LB-1 by RSM

Based on the appropriate values of fermentation conditions obtained from the above six single-factor experiments, a total of 12 fermentation experiments were carried out using

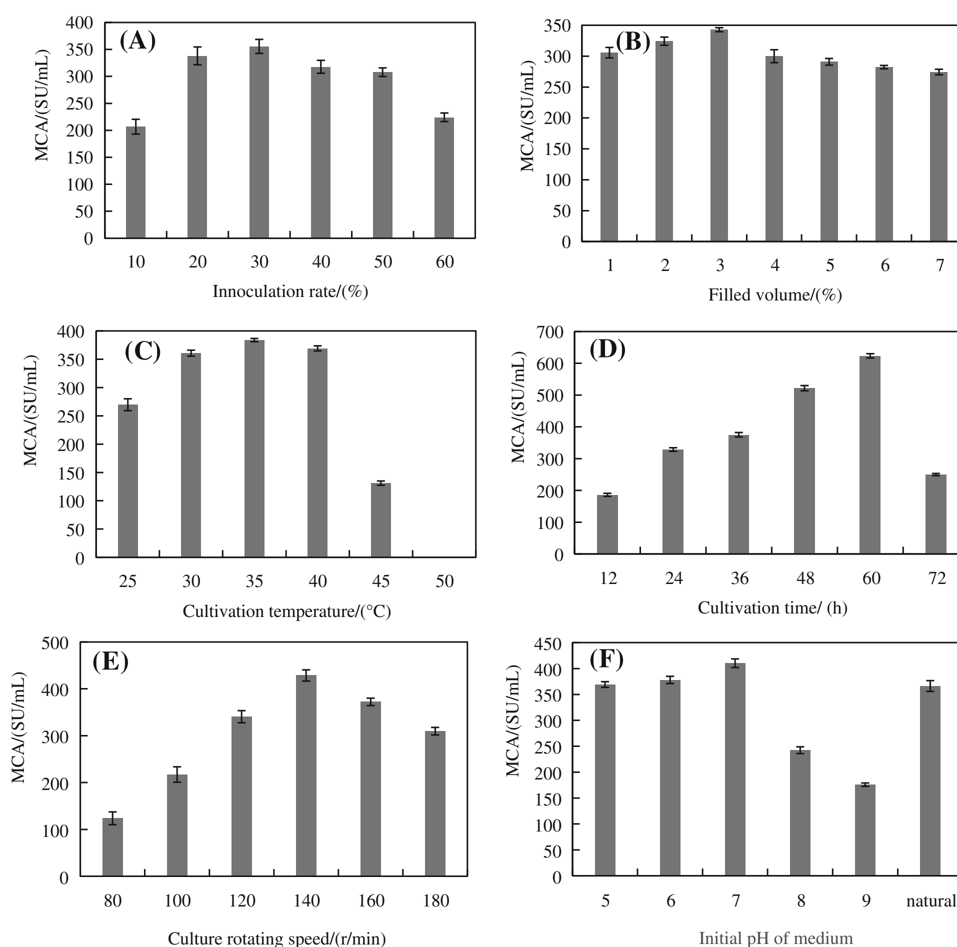


Fig. 1 Effect of different fermentation parameters on MCE produced by *B. methanolicus* LB-1. (**A**) Inoculation rate; (**B**) filled volume of growth medium; (**C**) cultivation time; (**D**) cultivation temperature; (**E**) culture rotating speed; (**F**) initial pH of growth medium. Values presented are mean ± standard deviation of samples analyzed in triplicate. Vertical lines represent standard deviations

the Plackett–Burman test design. Further analysis about the *p* value of main effect in Table 1 was 0.000, indicating that the factors in the Plackett–Burman design had significant effect on the MCA of *B. methanolicus* LB-1 in the selected range. The R^2 was 99.58%, pointing out that the regression was effective and the experiment design was reliable. The *p* values of cultivation temperature, cultivation time and initial pH were 0.000, 0.001 and 0.046, respectively, which were all < 0.05, indicating that these three factors had significant effect on the MCA and could be used in the screening of the main factors. The RSM with the three factors selected above was carried out to determine the optimal parameters. A FCCCD was performed on the selected non-compositional factors, and the data were fitted with a second-order polynomial function to get the following regression equation which was used to investigate data of FCCCD for forecasting MCA, as given in Eq. (2).

Table 1 Effect and coefficient estimation for MCA

Sum	Effect	Coefficient	Standard error of coefficient	T	P
Constant		216.3	4.310	50.19	0.000
Installed volume	− 7.4	− 3.7	4.310	− 0.86	0.441
Inoculation amount	5.6	2.8	4.310	0.65	0.553
Cultivation temperature	− 251.5	− 125.8	4.310	− 29.18	0.000
Cultivation time	− 74.6	− 37.3	4.310	− 8.66	0.001
Initial pH	− 24.6	− 12.3	4.310	− 2.85	0.046
Agitation speed	− 10.3	− 5.2	4.310	− 1.20	0.297
R^2 = 99.58%		Adjusted R^2 = 96.18%	Predicted R^2 = 98.83%		

$$Y = -6703.07265 + 25.71094 \times A + 225.43016 \times B + 839.55370 \times C + 0.27398 \times A \times B + 0.80073 \times A \times C - 1.23675 \times B \times C - 0.34950 \times A^2 - 3.58779 \times B^2 - 62.43036 \times C^2 \quad (2)$$

where Y, MCA (SU/mL); A, fermentation time; B, fermentation temperature; C, initial pH of medium.

Analysis of variance (ANOVA) was employed for evaluating the suitability of the model. The p value of model was lower than 0.0001, indicating that this model was valid, and the lack of fit was not significant, showing that the model could reflect the actual situation of the experiment. This method of analysis was based on previously reported studies (Hang et al., 2017). Factor interaction between the response surface 3D graphs was shown in Fig. 2. The contour map close to the ellipse and three-dimensional response surface plot was the steepest in Fig. 2A, B, indicating that the interaction between fermentation temperature and fermentation time was the most significant (Teng and Yang, 2016).

Validation of the statistical model and regression equation was performed by taking fermentation temperature (32.43 °C), fermentation time (57.23 h) and initial pH (6.77) in the experiment. The predicted response of 530.68 SU/mL was close to the actual experimental response of 578.40 ± 0.017 SU/mL, thus proving validity. Under this condition, the PA value was 4.82 ± 0.07 U/mL.

Purification of the MCE from *B. methanolicus* LB-1

The MCE produced under the optimal fermentation conditions was extracted with different volume fractions of ethanol first (Fig. 3A). When precipitating with 70% volume of ethanol, the MCA was the highest, while about 64.83% of the total MCA was obtained, and the MCE was purified about 2.12-fold (Fig. 3D). The elution profile of the MCE on DEAE Sepharose Fast Flow chromatography column was shown in Fig. 3C. Three major protein peaks were separated and one of them had MCA. The active fractions were eluted in 2 M NaCl, which was similar to the purification of MCE from *Paenibacillus* spp. BD3526 using a DEAE Sepharose Fast Flow chromatography column (Hang et al., 2016a; 2016b). The MCE from this step gave a single protein band on SDS-PAGE (Fig. 3B line3), indicating that the MCE sample was purified after the DEAE purification. The MCA of the purified MCE was 597,310 SU/g, which was higher than the activity of the commercial enzyme (3.5×10^5 SU/g) used in this study. Comparing with other MCEs reported earlier (El-Bendary et al., 2007; Sun et al., 2014), the C/P value (59.82) of the purified MCE (Fig. 3D) was relatively high, indicating the suitability of the MCE for potential application in cheese.

Characterization and identification of the MCE from *B. methanolicus* LB-1

Effect of temperature, pH and metal ions on MCA of the MCE

Effect of temperature. The MCA of the MCE from *B. methanolicus* LB-1 increased and then decreased when the temperature increased from 25 to 75 °C with the maximal activity at 50 °C, which was similar to that of the commercial enzyme (Fig. 4A). Both enzymes exhibited higher MCA at a relativity low temperature, and they were different from the MCEs produced by *Paenibacillus* spp. BD3526 (Hang et al., 2016a; 2016b) and *Rhizopus microsporus* (Sun et al., 2014), which had the maximal MCA at relative higher temperatures (65 °C and 60 °C). The thermal stability of the MCE from *B. methanolicus* LB-1 and the commercial enzyme was shown in Fig. 4B and C, respectively. The MCA of the MCE from *B. methanolicus* LB-1 was relatively stable at lower temperatures (40 °C, 45 °C), and the MCA of the commercial enzyme remained stable at less than 50 °C, but they almost completely lost their activity at higher temperatures (60 °C, 65 °C). The MCE inactivation at high temperature could facilitate removal of residue proteolytic activity to avoid formation of bitterness in cheese during ripening.

Effect of pH. Both the MCE from *B. methanolicus* LB-1 and the commercial enzyme exhibited the maximal activity at pH 6.5 (Fig. 4D), and acidic or alkaline condition could reduce the MCA, which was inconsistent with the properties of metallic-protease (Hang et al., 2016a; 2016b). The pH stability of the MCE from *B. methanolicus* LB-1 (Fig. 4E) was similar to that of the commercial enzyme (Fig. 4F), displaying a good stability under neutral pH conditions (6.5–7.5). However, under acidic (pH 5.0) or alkaline (pH 8.5) condition, the commercial enzyme completely lost MCA, but the MCE from *B. methanolicus* LB-1 could retain lower MCA, which was a superiority for application in cheese making due to its less sensitivity to acidic pH.

Effect of metal ions. Among several common metal ions tested (Fig. 4G), the metal ions of Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+} could increase MCA of the MCE from *B. methanolicus* LB-1. As to the commercial enzyme, Cu^{2+} and Mn^{2+} strongly inhibited the MCA, but Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+} activated the MCA. Among all the metal ions, calcium ion showed the strongest stimulating effect on both the MCE from *B. methanolicus* LB-1 and the commercial enzyme at the concentrations of 25 mM and 20 mM (Fig. 4H).

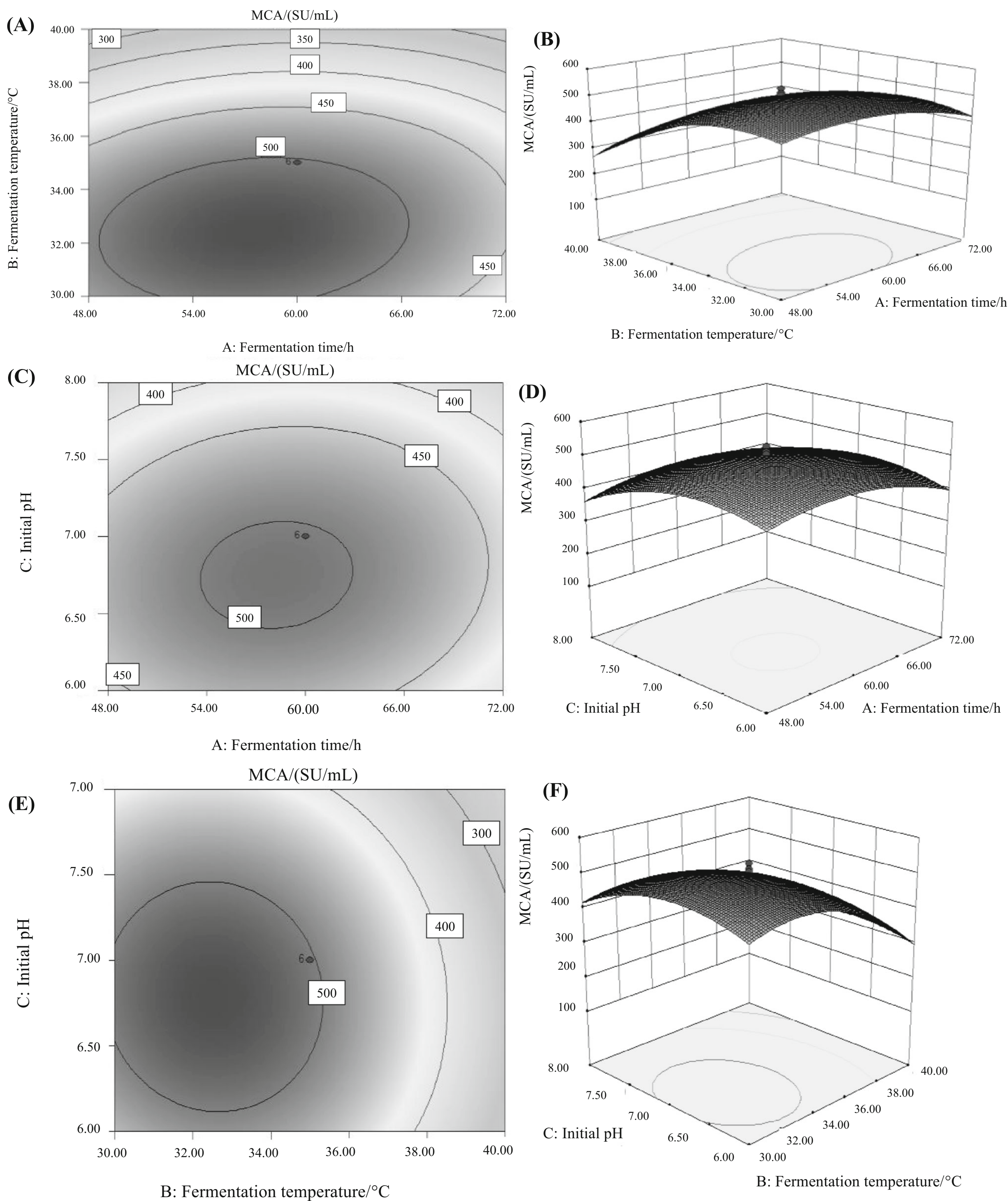


Fig. 2 Response surface plots showing the interactions between non-compositional factors. (**A**) Contour map of time and temperature interaction; (**B**) response surface of time and temperature interaction; (**C**) contour map of temperature and pH interaction; (**D**) response surface of temperature and pH interaction; (**E**) contour map of time and pH interaction; (**F**) response surface of time and pH interaction

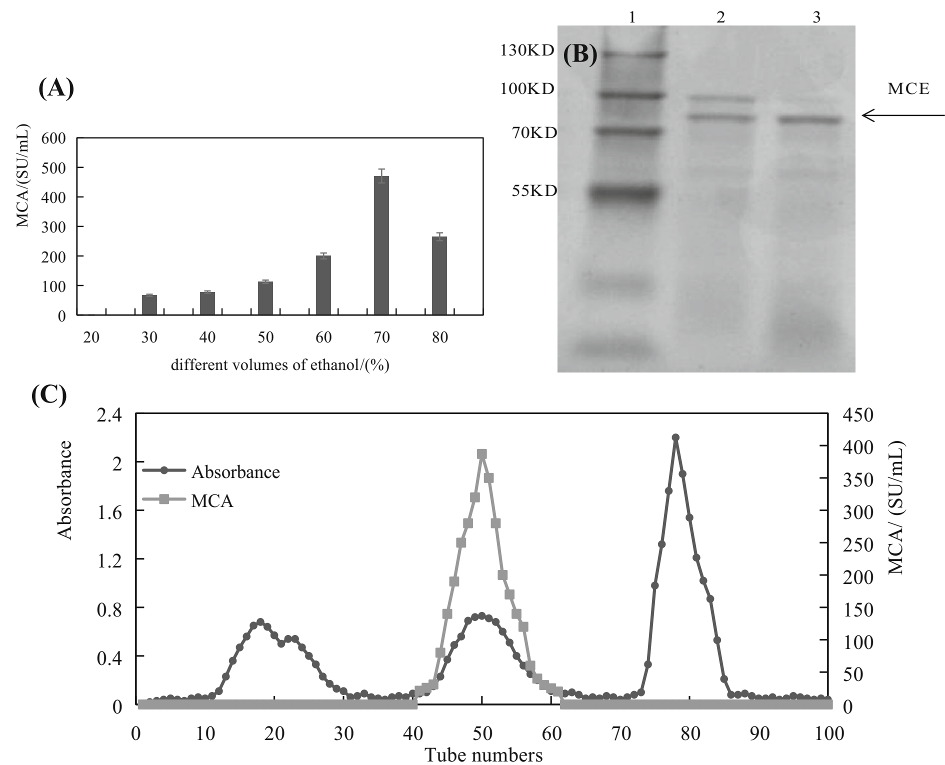


Fig. 3 Purification of the MCE produced by *B. methanolicus* LB-1. (**A**) Precipitation by ethanol; (**B**) elution profiles by DEAE Sepharose Fast Flow chromatography; (**C**) SDS-PAGE of the samples from each purification step; line1, markers; line2, protein precipitate with 70% ethanol; line3, purified MCE sample from DEAE Sepharose Fast Flow chromatography; (**D**) purification and recovery of MCE. Values presented are mean ± standard deviation of samples analyzed in triplicate. Vertical lines represent standard deviations

(D)

purification steps	Total protein (mg)	Total activity (SU)	Specific activity (SU/mg)	C/P	Recovery (%)	fold
Crude enzyme	101.29	5784.32	57.11	30.32	100	1.0
Ethanol precipitation	30.99	3750	121.01	57.50	64.83	2.12
DEAE Sepharose FF	5.74	3428.57	597.31	59.82	59.28	10.46

Kinetic characteristics of the MCE

Double reciprocal plots of velocity versus the amount of substrate according to Lineweaver–Burk resulted in linear regression curves (Fig. 4I) with intercepts at the x axis ($-1/K_m$) and y axis ($1/V_{max}$), from which the K_m and V_m could be calculated. The values of K_m and V_{max} for the MCE from *B. methanolicus* LB-1 were 0.93 g/L and 20.49 mL/U, respectively. By comparison, the MCEs reported by Hang et al. (2016a; 2016b) and Zhao et al. (2015) had the K_m values of 1.36 g/L and 22.90 g/L, respectively. A high K_m value generally corresponds to a low affinity, and vice versa. The lower value of K_m of the MCE from *B. methanolicus* LB-1 indicated that this MCE had a good affinity and reaction rate for the casein substrate under suitable condition.

Identification of the MCE

Identification of the MCE from *B. methanolicus* LB-1 was performed by LC–MS, and a secondary mass spectrum corresponding to a characteristic peptide segment of the MCE was demonstrated (Fig. 5A). By comparing the actual mass spectrum with the theoretical mass spectrum of protein library data of the species, a sequence of 8 amino acid residues of this peptide segment was determined (Fig. 5B). By using Maxquant software, the MCE from *B. methanolicus* LB-1 was therefore identified to be a putative protein (I3EB99_BACMT) matching the sequence from the start position of 202 to the end position of 209 with the matching score of 63.98 (more than 60), which was considered to be at a significant matching level (Hang et al., 2016a; 2016b). The identified complete amino acid

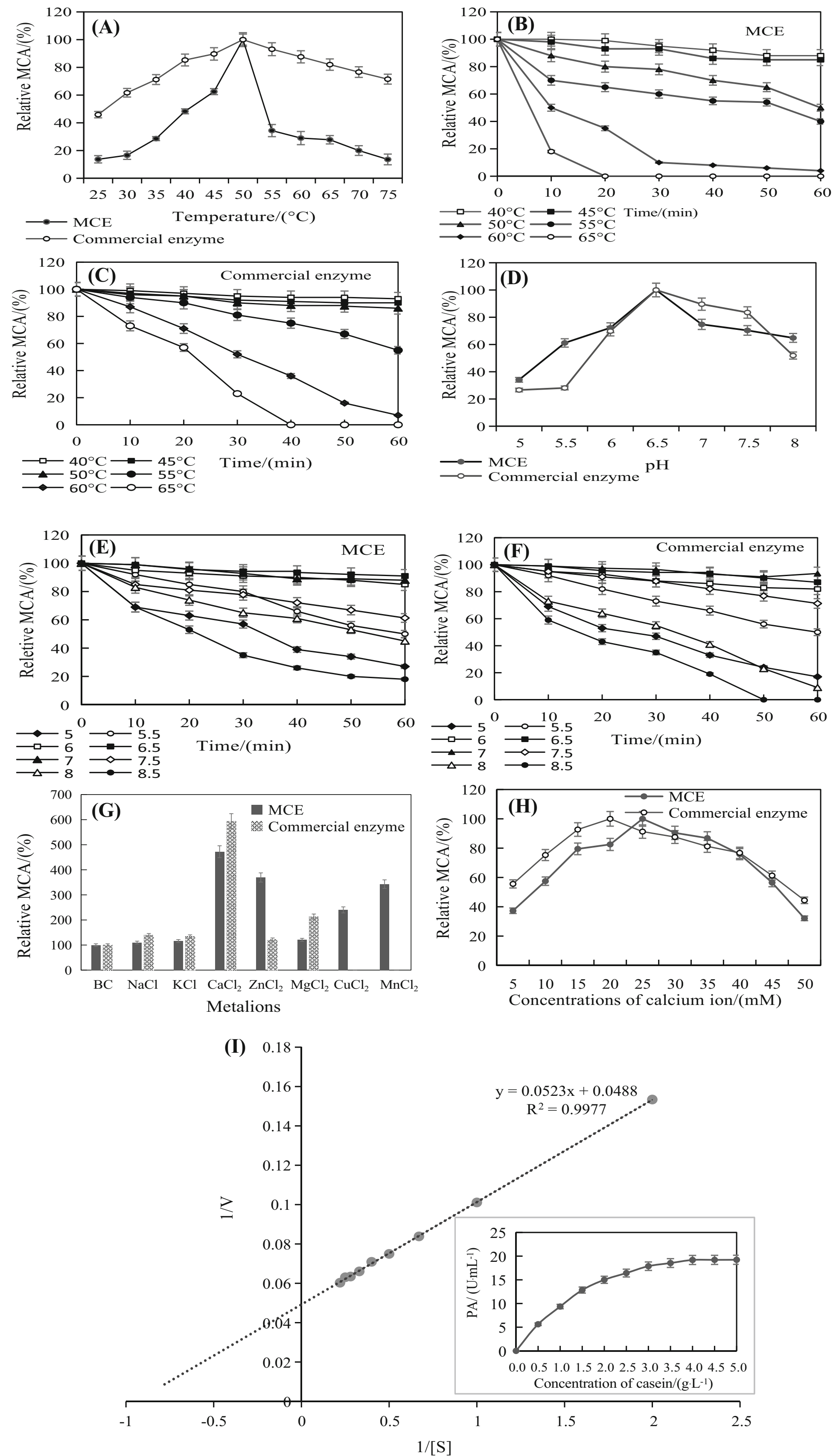
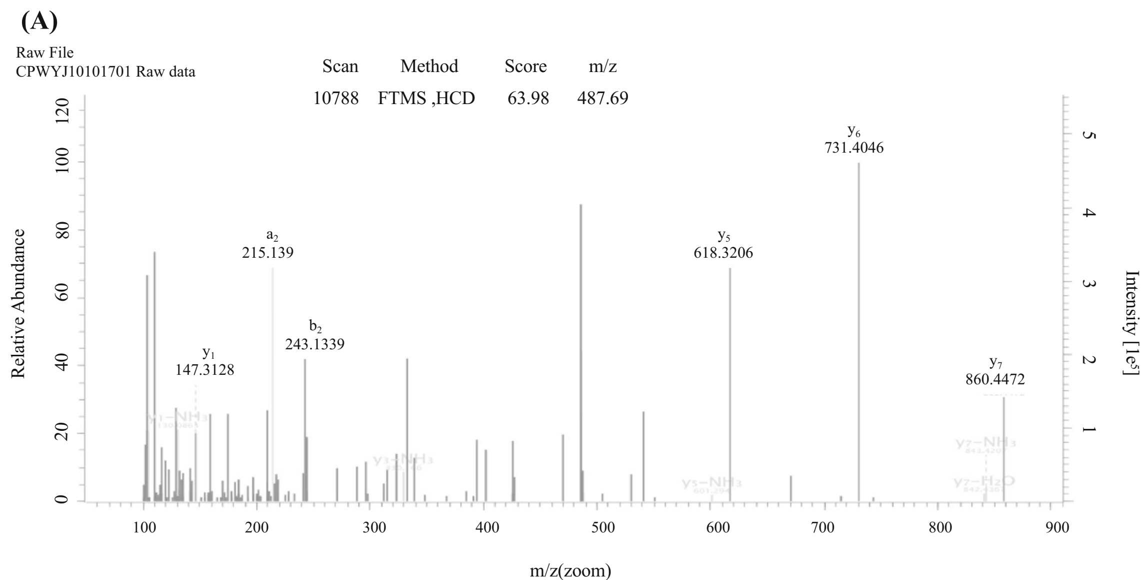


Fig. 4 Characterization of the MCE produced by *B. methanolicus* LB-1 comparing with a commercial enzyme. (**A**) Effect of temperature; (**B**) thermal stability of the MCE; (**C**) thermal stability of the commercial enzyme; (**D**) effect of pH; (**E**) pH stability of the MCE; (**F**) pH stability of the commercial enzyme; (**G**) effect of metal ions; (**H**) effect of different concentration of calcium ion; (**I**) Lineweaver–Burk plot for reaction velocity versus substrate concentration. Values presented are mean ± standard deviation of samples analyzed in triplicate. Vertical lines represent standard deviations

(A)



(B)

Sequence	Length	Mass	Proteins	Start position	End position
IEIDRAEK	8	972.52401	I3EB99	202	209

(C)

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        10         20         30         40         50
MKKKKYIVGK TVLAATVATS AFAVANTADA ASVSEAEKAV AKAEKLAGAL
        60         70         80         90        100
KWEVSIEYRK TKYPNNLVGY PNMKLFNDTK KALAEAKKSV SALKGKEKEV
       110        120        130        140        150
LQARLDANVA TYVNRAAAYI DAVSSGLKIQ KKYSELKSRF DKNIVDDQTD
       160        170        180        190        200
KLYHELSKEI KKNAFMLDRV YGATTRNEFR NYYKHSAENL MKQLLYPVSI
       210        220        230        240        250
KIEIDRAEKA LKEKNTDLAA THLSNVDFLV REAAKKGQKE TSAIMKSAMS
       260        270        280        290        300
KLENTQKEFN KFGVLYIAKS TNSASPSTFG PVSGSETINK TVYIVAGKNQ
       310        320        330        340        350
HVKLRNVTIN GNLVVKGSLD GAGTVYLENV KVNKVNGTSG SIIVEDVADH
       360        370        380        390        400
SLYLKGVEAD SVVVNDANGS NLVAQEGVKV KSLVVSEKAG AKGSVALESK
       410        420        430        440        450
AAGSFETITI AAKGSANSQG VVLKGNLSAT SVNVTGENAK IAISEGATVK
       460        470        480        490        500
QIKLNAVASV AVAKGAVVEE IKKDPSVTGK VEVDNKGTIK KADEGIEVKG
       510        520        530        540        550
NKPEVVTPPA PNTPPSVPGG GGGGPSVVIP SLTSVNAIIG GVSVPVQNNT
       560        570        580        590        600
LNLAGKSDND VLTEIQIGTN PSTNVKVKVT SLTARGYTNW ISSPIELPGT
       610        620        630        640        650
KITTKDLFGP LDTGAPGISL GNLRTAFLTD PIIIKGVLVG SGGGQSSEIT
       660        670        680        690        700
ITINLGASKE AITTFTNEFG TITKKGDNTI EVVINKNKLD TTIGDIEQET
       710        720        730        740        750
KVDFSQILIS FAESASPEVA KLLNQLINNQ LGKALEDIKL GELAGRTFQA

NGFTVEFKK
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Fig. 5 Identification of the MCE produced by *B. methanolicus* LB-1. (**A**) Mass spectrum of the peptide segment corresponding to the MCE from *B. methanolicus* LB-1; (**B**) Sequence information of the peptide segment corresponding to the MCE produced from *B. methanolicus* LB-1; (**C**) The complete amino acid sequence of the MCE from *B. methanolicus* LB-1

sequence of the MCE based on this putative protein was presented in Fig. 5C, showing 759 amino acid residues with the formula of $C_{3559}H_{5855}N_{967}O_{1125}S_6$, of which the molecular weight was 80.37 kDa and the pI was 9.23. Regarding subsections of the MCE from *B. methanolicus* LB-1, the signal peptide of the MCE was composed of 30 amino acids, which were boxed in Fig. 5C. The extended chain of the MCE was a peptide with 729 amino acids

which were not boxed in Fig. 5C. This result was different from the MCEs produced by *M. pusillus* (49 kDa) and *B. amyloliquefaciens* JNU002 (28 kDa) (Ding et al., 2012; Nouani et al., 2009), indicating that the MCE from *B. methanolicus* LB-1 could be a new protease. Based on cross-reference to National Center for Biotechnology Information (NCBI), it shared 50% similarities with a protein secreted by *B. firmus*, and the one by *Paenibacillus* sp. FSL R5-0490, but these proteins were still not characterized.

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