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ORIGINAL ARTICLE

Fermentation conditions of serine/alkaline milk-clotting enzyme production by newly isolated *Bacillus licheniformis* BL312

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Abstract

Purpose This study was conducted to find a microbial milk-clotting enzyme (MCE) with a high and stable milk-clotting activity (MCA) to proteolytic activity (PA) ratio suitable for the cheese industry.

Methods Microbial strains were isolated from soil suspensions cultured in solid casein medium. 16S rDNA of representative isolates were sequenced to identify the microbial species. Nutrition and fermentation conditions were systematically examined to optimize MCA of the selected MCE. Protease inhibitors were used to identify the type of MCE. The casein hydrolysis was analyzed through reversed-phase HPLC (RP-HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Results The *Bacillus licheniformis* BL312 was identified from 50 bacterial strains. BL312 MCE achieved a maximal MCA (460 ± 15 SU/mL) at 48 h that was 2.7-fold higher than the control, and the MCA/PA ratio (9.0) and pH (6.6) remained stable throughout the fermentation process. Medium containing 30 g/L wheat bran shorts, 5 g/L glucose, and 3 g/L corn steep liquor was sufficient for optimal BL312 MCE production. Fermentation conditions of an inoculum size of 7.0% (v/v), fermentation temperature of 37 °C, agitation speed of 210 rpm, and initial pH 6.6 were required to achieve maximal MCA. BL312 MCE was inhibited by phenylmethanesulfonyl fluoride (PMSF) and high concentrations of ethylenediaminetetraacetic acid (EDTA) (5–25 mM). The α_s-casein (α_s-CN) and β-casein (β-CN) hydrolysates generated by BL312 MCE and calf rennet were different.

Conclusions BL312 MCE is a serine/alkaline protease that exhibits high MCA and various hydrolysis for caseins in comparison with calf rennet.

Keywords Isolation · Fermentation · *Bacillus licheniformis* · Milk-clotting enzyme · Serine/alkaline protease · Casein hydrolysis

Introduction

Milk coagulation is an important step in cheese production, and MCEs play a major role in this process. MCEs facilitate milk clotting and may influence the flavor and texture of the cheese. Calf rennet, the most widely used milk coagulant, is obtained from the fourth stomach of suckling calves (Liburdi et al. 2018). However, the use of calf rennet substitutes is becoming necessary due to the increase in cheese consumption. The selection of a suitable calf rennet substitute is a critical factor, which influences the characteristics of the final product. The MCA/PA ratio is an inherent characteristic of MCE. The ideal MCE is characterized with a high MCA and a low non-specific PA (Meng et al. 2018). In fact, the high MCA/PA ratio leads to better textural and sensorial properties of cheese and a great reduction of bitterness. Consequently, the high MCA/PA ratio has shown to be crucial in evaluating the applicability of MCEs (Ben et al. 2017). Plant-derived coagulants can be produced from seeds, vacuoles, and extracellular spaces (Gagaoua et al. 2017; Luo et al. 2018), and can be used as calf rennet substitutes. However, most plant-derived coagulants, which typically exhibit low MCA/PA ratios, achieve poor cheese yield and form bitter substances during cheese ripening (Salehi et al. 2017). In contrast, recombinant chymosins exhibit high MCAs and good thermal stability. However, they are banned in many countries (Vallejo et al. 2012).

Recently, microbial MCEs have attracted great interest as promising calf rennet substitutes. Fungal MCEs, especially those from *Rhizomucor miehei*, *R. pusillus*, and *Endothia*

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parasitica, are already widely used in commercial cheese production (Celebi et al. 2016). Compared with fungal MCEs, bacterial MCEs are cheaper to produce, more biochemically diverse, and more easily modified genetically (Hang et al. 2016). Bacterial MCEs have been reported to exhibit high MCA/PA ratios (Luo et al. 2018; Li et al. 2019). Moreover, bacterial submerged fermentation is easier to control and shows a higher material utilization ratio compared with fungi. However, studies on bacterial MCEs have been less frequently conducted compared with those on fungal MCEs. Few commercial applications of bacterial MCEs as milk coagulants have been reported.

Shouguang (Shandong, China) is known as the hometown of Chinese vegetables, and its agricultural soil is rich in organic matter. Importantly, it houses a wide variety of microbial species (Zhou et al. 2011), many of which exhibit high protease activities. Microorganisms producing proteases (e.g., serine and threonine protein kinase) have previously been collected from the agricultural soil in Shouguang (Wang et al. 1998; Qiu et al. 2016). In this study, BL312 (a *Bacillus licheniformis* strain) was isolated and found to exhibit a high and stable MCA/PA ratio during fermentation. Various conditions and nutritional parameters were systematically examined to optimize the condition for MCE production and MCA. Large-scale fermentation (using a fermenter) was then used to investigate the economic feasibility of BL312 MCE production. The effects of different protease inhibitors on the MCA of BL312 MCE were examined. The hydrolysis behavior of BL312 MCE for caseins was analyzed.

Materials and methods

Chemicals and reagents

Skim milk powder was purchased from Fabrique par Fonterra Co., Ltd. (Auckland, New Zealand). Calf rennet was provided from Yuexiang Chemical Co., Ltd. (Shandong, China). PMSF, EDTA, pepstatin A, and DL-dithiothreitol (DTT) were obtained from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The other chemicals used in the study were analytical grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Isolation and identification of MCE-producing bacteria

Soil samples were collected from 10 to 15 cm-deep pits in greenhouses and different agricultural regions in Shouguang (36° 88′ N, 118° 73′ E). The samples were placed into sterile zipper polythene bags and stored at 4 °C until use. Soil samples taken to the laboratory were passed through a sieve (3–4 mm mesh) and then were stored in sterile centrifuge tubes (50 mL) at 4 °C. To prepare soil suspensions, 1 g of soil sample was suspended in 9 mL of normal saline. Tenfold serial dilutions of the initial suspension were prepared with normal saline until a final dilution of 10^{-4}-fold was achieved. A 120-μL aliquot of each diluted sample was cultured at 37 °C for 36 h in solid casein medium (pH 6.8) containing 2.5 g/L casein peptone, 10 g/L glucose, 1 g/L yeast extract powder, 25 g/L skim milk powder, and 20 g/L agar. Each strain was numbered, and the diameters of hydrolysis and precipitation rings were examined every 12 h.

The isolated strains with high ratios of (i) precipitation ring diameter to hydrolysis ring diameter and (ii) precipitation ring diameter to colony zone diameter were selected, and their 16S rDNA were sequenced. For species-level identification, sequences were compared with those in the GenBank database using the BLAST program (NCBI). For phylogenetic analysis, our data were aligned to a dataset containing 16S rDNA sequences using the BioEdit program. MEGA 7.0 software was used to construct the phylogenetic tree (He et al. 2012). The harmful bacteria (e.g., *Serratia marcescens*, *Acinetobacter*, and *Pneumococcus*) were excluded and others (e.g., *Bacillus licheniformis* and *Bacillus subtilis*) were maintained at − 80 °C in 20% (v/v) glycerin. MCA, PA, and fermentation pH were measured in subsequent fermentation experiments using the selected microorganism.

Measurement of MCA and PA

The fermentation broth was centrifuged at 5000×*g* for 10 min. The crude MCE was in the supernatant and used for MCA and PA determinations. MCA was determined according to the method of Arima et al. (1968). Skim milk 10% (w/v in deionized water) containing 10 mM $CaCl_2$ was used as the substrate. Curd formation was observed by manually rotating the test tube from time to time so as to form a thin film on its inner surface. The MCA is expressed in Soxhlet unit (SU), which is defined as the amount of MCE required to clot 1 mL of milk substrate at 35 °C. The MCA was calculated using the following formula: $SU = (2400 \times V_1 \times n) / (t \times V_2)$, where V_1 is the milk volume (mL), n is the dilution of MCE, t is the milk-clotting time (s), and V_2 is the MCE volume (mL).

The proteolytic activity was measured following the method of Arima et al. (1968). In brief, 5 mL of 1.2% (w/v) casein solution in 0.05 M phosphate buffer (pH 6.5) was added 1 mL crude MCE. The mixture was then incubated at 35 °C for 10 min. After incubation, 2 mL (0.44 M) of trichloroacetic acid (TCA) was added to quench the reaction. The mixture was followed by centrifugation (6000×*g*) for 10 min at 4 °C. Two milliliters of the clear supernatant was added with 5 mL NaOH (0.28 M) solution and 1 mL Folin-Ciocalteu phenol reagent. After incubation at 35 °C for 15 min, the mixture was measured for optical density (OD) at 660 nm. One unit

(1 U) of MCE activity is defined as the amount of MCE that liberated 1 μg of tyrosine per 1 mL in 1 min.

Microbial growth and MCE production

The strain maintained in laboratory at −80 °C was grown on improved TYC medium, which contained 15 g/L casein peptone, 5 g/L yeast extract powder, 50 g/L glucose, 0.2 g/L L-cysteine, 1 g/L NaCl, 2 g/L $Na_2HPO_4{\cdot}12H_2O$, and 2 g/L $NaHCO_3$ and was sterilized at 115 °C for 20 min before use. The one single colony was inoculated on 50 mL of improved TYC medium in a flask of 250 mL and incubated at 37 °C for 16–18 h under shaking (150 rpm) as seed liquid.

MCE production was carried out by fermentation with a 5.0% (v/v) inoculum size in three flasks of 250 mL × 20 g/L of wheat bran shorts, which were autoclaved at 115 °C for 20 min. Wheat bran shorts contained 39.28% (w/w) carbon, 3.12% (w/w) nitrogen, and 6.51% (w/w) moisture. The fermentation conditions were as follows: temperature 37 °C, rotating speed 150 rpm, initial pH 6.6, and liquid volume 50 mL. Determinations on MCA, PA, and pH of fermentation broth were carried out in a time range of 36–108 h at a 12-h interval. At timed intervals, samples were collected and centrifuged at 5000×*g* for 10 min at 4 °C. The supernatant was used to measure MCA, PA, and pH of fermentation broth. Fermentation conditions were varied by one factor at a time.

The effects of wheat bran concentrations on BL312 MCE production

Different concentrations of wheat bran shorts including 20, 30, 40, 50, and 60 g/L were used to determine the optimal concentration for MCE production. MCA was measured in a time range of 36–96 h at a 12-h interval.

The effects of carbon and nitrogen sources on BL312 MCE production

To investigate the optimal carbon source for MCE production, different carbon sources (glucose, sucrose, soluble starch, maltodextrin, and lactose) at 5 g/L were individually added to the basal wheat bran shorts medium. The MCA in the basal wheat bran shorts medium (without other carbon sources) was the control and regarded as 100%. Different organic and inorganic nitrogen sources (corn steep liquor, casein peptone, urea, yeast extract powder, ammonium sulfate, and ammonium citrate) at 3 g/L were used to determine the optimal nitrogen source. The MCA of BL312 MCE in wheat bran shorts medium containing 5 g/L glucose (without nitrogen sources) was the control and taken as 100%. The MCA and MCA of following fermentation experiments were measured in a time range of 36–84 h at a 12-h interval, unless specified otherwise.

The effects of bioprocess parameters on BL312 MCE production

In order to determine bioprocess parameters on MCE production, inoculum size (1, 3, 5, 7, and 9%, v/v), fermentation medium volume (20, 30, 40, 50, and 60 mL), cultivation temperature (27, 32, 37, and 42 °C), and agitation speed (120, 150, 180, 210, and 230 rpm) were studied in 250 mL Erlenmeyer flasks. The initial pH of fermentation medium was adjusted to 4.6, 5.6, 6.6, 7.6, and 8.6 to determine the optimal initial pH for MCE production.

Time course of BL312 MCE production in a fermenter

The MCE production was carried out by fermentation with optimal parameters in a 7-L fermenter (LiFlus GM, Biotron, Korea) containing 4 L fermentation medium, which was autoclaved at 115 °C for 20 min. The initial concentration of dissolved oxygen (DO) was regarded as 100% of air saturation.

The effects of protease inhibitors on BL312 MCE

The supernatant of fermentation broth was brought to ammonium sulfate (60% saturation) at 4 °C for 2 h and harvested by centrifugation at 7000×*g* and 4 °C for 10 min. The precipitate was dissolved in Tris-HCl buffer (pH 7.0) and centrifuged at 7000×*g* and 4 °C for 10 min. The crude MCE in the clear supernatant was dialyzed in dialysis bags (8–14 kDa) and lyophilized to powder for further experiments. The effects of various protease inhibitors including pepstatin A (0.01 and 0.02 mM), EDTA (1, 5, 10, and 25 mM), PMSF (1 and

Table 1 The diameter of hydrolysis and precipitation rings on the solid casein medium

CN	CD	HD	PD	P/C	P/H
DB215	4.0 ± 1.0	7.0 ± 1.0	11.5 ± 1.5	2.9	1.6
DB218	2.0 ± 0.5	3.0 ± 0.5	10.5 ± 1.5	5.3	3.5
BL312	3.0 ± 0.5	3.5 ± 0.5	18.0 ± 2.0	6.0	5.1
BL211	2.0 ± 0.5	5.0 ± 1.0	11.0 ± 2.0	5.5	2.2
QD111	1.5 ± 0.5	5.5 ± 1.0	11.0 ± 1.5	7.3	2.0
QD212	3.0 ± 0.5	4.0 ± 1.0	16.5 ± 1.5	5.5	4.1
YN111	4.0 ± 1.0	5.0 ± 1.0	17.0 ± 2.0	4.3	3.4
YN213	4.0 ± 1.0	6.0 ± 1.0	14.0 ± 2.0	3.3	2.2
NX112	4.0 ± 0.5	8.0 ± 1.0	13.0 ± 1.0	3.3	1.6
GS113	2.0 ± 0.5	5.0 ± 1.0	10.0 ± 1.5	5.0	2.0
GL111	4.0 ± 1.0	5.0 ± 1.0	9.0 ± 1.0	2.3	1.8

The data are represented as mean ± SD ($n = 3$). CN, strain number; CD, colony zone diameter (mm); HD, hydrolysis ring diameter (mm); PD, precipitation ring diameter (mm); P/C, the ratio of PD to CD; P/H, the ratio of PD to HD. The culture time of all strains was 24 h

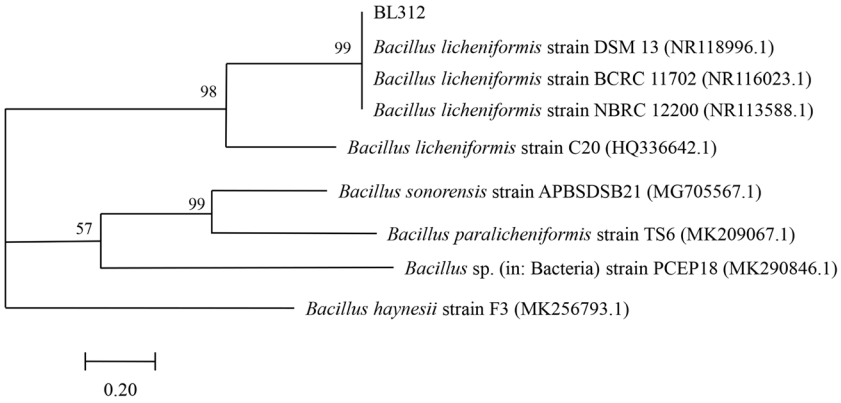

Fig. 1 Phylogenetic position of the isolated BL312 based on 16S rDNA gene sequence analysis. GenBank accession numbers are given in parentheses

2 mM), and DL-dithiothreitol (DTT) (1 mM) were examined on MCA of the crude MCE according to the method of Salehi et al. (2017) with some modifications. After addition of inhibitors, the mixtures were incubated at 25 °C for 30 min. The samples were evaluated for MCA as mentioned formerly. The control (without inhibitors) was regarded as 100%.

Hydrolysis of caseins by BL312 MCE

The α_s-CN and β-CN hydrolysates generated by BL312 MCE and calf rennet were analyzed by RP-HPLC and SDS-PAGE with 12% (w/v) acrylamide according to the method of Merheb-Dini et al. (2010) and Wasko et al. (2012), respectively. The α_s-CN or β-CN (0.2%, w/v) was mixed with MCEs (100 SU/mL) in a proportion of α_s-CN or β-CN:MCEs = 15:1. For SDS-PAGE analysis, the proportions of α_s-CN or β-CN:BL312 MCE (10:1, 20:1, and 30:1) were also detected. The mixtures were incubated at 35 °C for 30 min for reaction. Reactions were quenched by heating the solutions in boiling water for 10 min.

Statistical analysis

Each experiment was performed in triplicate. The results were expressed as means ± standard deviations. Data obtained were analyzed by one-way ANOVA using the software SPSS 17.0. The level of statistical significance was set at $P < 0.05$.

Results and discussion

Separation and identification of *Bacillus licheniformis* BL312

Initially, 11 isolated strains were found to form large precipitation rings in the solid casein medium, which indicated high MCA (Table 1). Given that a high PA (as indicated by large hydrolysis rings in the solid casein medium) can lead to the formation of bitter substances in cheese (Merheb-Dini et al. 2010), strains with a high ratio of precipitation ring to hydrolysis ring and precipitation ring to colony zone were selected

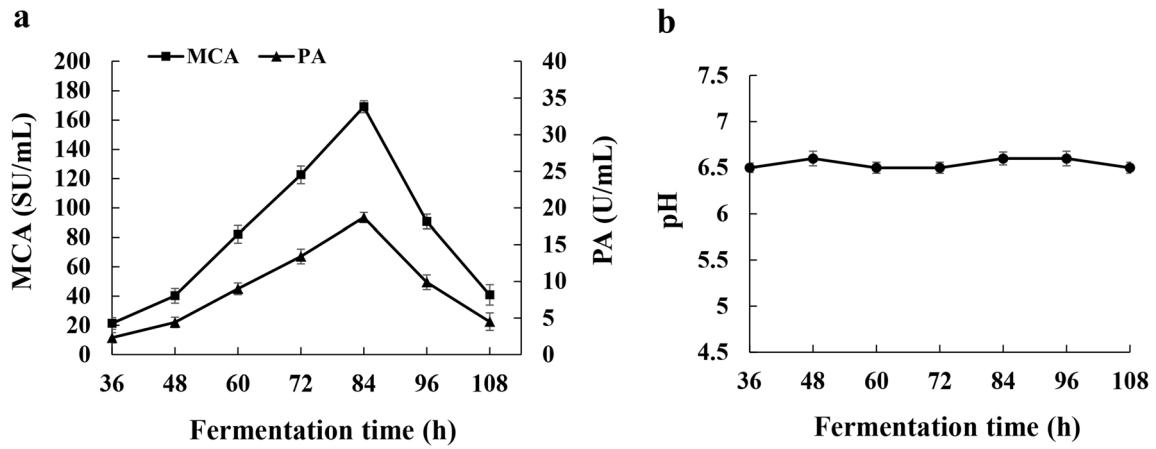

Fig. 2 The BL312 MCE production (**a**) and pH of fermentation broth (**b**) during different fermentation times (36–108 h). Data were presented as means of triplicate measurements; error bars were standard deviations

Table 2 Comparison of PA and MCA/PA ratio among different derived MCEs

Strain	Substrate	PA	MCA/PA ratio	References
B. licheniformis USC13	Casein	43 U/mL	6.7	Ageitos et al. (2007)
B. subtilis B1	Casein	174 U/mL	6.5	Ding et al. (2011)
B. amyloliquefaciens JNU002	Casein	1109 U/mL	5.9	Ding et al. (2012)
A. niger FFB1	Casein	3020 U/mg	0.6	Fazouane et al. (2010)
R. miehei (commercial MCE)	Casein	–	6.6	Thakur et al. (1990)
R. miehei (*Rennilase* L)	Casein	–	3.9	Thakur et al. (1990)
R. pusillus (Noury)	Casein	–	4.5	Thakur et al. (1990)
C. parasitica (Sure curd)	Casein	–	1.6	Thakur et al. (1990)
Calf rennet	Casein	–	12.8	Thakur et al. (1990)
M. mucedo DSM 809	Azocasein	7.9 U/mL	16.5	Yegin et al. (2012)
A. rouxii	Azocasein	0.7 U/mL	–	Marcial et al. (2011)
Nocardiopsis sp.	Azocasein	1.6 U/mL	2.9	Cavalcanti et al. (2005)
R. miehei	Azocasein	11.2 U/mL	23.9	Yegin et al. (2012)
R. miehei (Sigma)	Hemoglobin	–	2.0	Preetha et al. (1997)
A. oryzae MTCC 5341	Hemoglobin	172 U/mg	–	Vishwanatha et al. (2010)

and further examined using 16S rDNA sequencing and fermentation experiments. Accordingly, BL312 was selected from the 11 initial isolates for MCE production.

The 16S rDNA sequences of BL312 were compared to all sequences in the GenBank for species identification. Our obtained nucleotide sequence showed the best match (99% homology) to those of *Bacillus licheniformis* strain DSM 13 (NR118996.1), *Bacillus licheniformis* strain BCRC 11702 (NR116023.1), and *Bacillus licheniformis* strain NBRC 12200 (NR113588.1). A phylogenetic tree was constructed using the neighbor-joining method (Fig. 1), and BL312 was identified as *Bacillus licheniformis* using phylogenetic analysis. *Bacillus licheniformis* BL312 was deposited in the China General Microbiological Culture Collection Center (CGMCC No. 15009).

The MCA and PA of BL312 fermentation are shown in Fig. 2a. BL312 MCE achieved the maximal MCA (169 ± 6 SU/mL) and PA (19 ± 2 U/mL) at 84 h. The MCA/PA ratio was found to be 9.0 throughout the fermentation. As indicated in Fig. 2b, the pH of the fermentation broth was 6.6 and remained unchanged throughout. These results suggested that *Bacillus licheniformis* BL312 did not produce acid during fermentation, indicating that curd formation was caused by MCE. It is likely that BL312 is applicable for MCE production. Table 2 shows that the MCA/PA ratio of BL312 MCE was generally similar to that of commercial MCEs (e.g., calf rennet and *R. miehei* MCE). However, BL312 MCE exhibited a higher MCA/PA ratio in comparison with *B. amyloliquefaciens* JNU002, *A. niger* FFB1, and *C. parasitica* MCE. The MCA/PA ratio plays an important role in the selection of MCEs. The MCEs with high MCA/PA ratio achieve great cheese yield and decreased bitter substances in cheese production, indicating a good characteristic of BL312 MCE (Ben et al. 2017). In addition, for some organisms, the MCA/PA ratio fluctuates during fermentation (Hashem 1999). However, the MCA/PA ratio of BL312 MCE was found to be stable, indicating that BL312 MCE production and activity can be more easily controlled during fermentation compared with other MCEs.

The effects of different carbon and nitrogen sources on BL312 MCE production

Wheat bran shorts contains several sources of carbon and nitrogen. It is a low-cost raw material that supports the growth of many microorganisms. Importantly, wheat bran is an

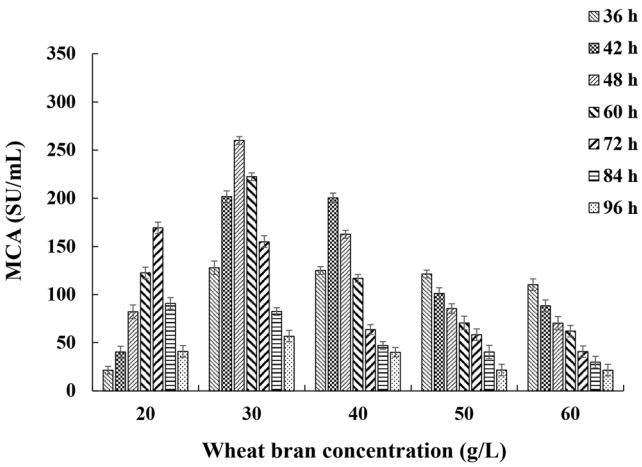

Fig. 3 The effects of different concentrations of wheat bran shorts on BL312 MCE production during fermentation (36–96 h). Data were presented as means of triplicate measurements; error bars were standard deviations

effective nutrient source for MCE production during fermentation (Chwen-Jen et al. 2009). Our results indicated that MCA of BL312 MCE increased with increasing concentrations of wheat bran shorts (up to 30 g/L), but decreased at higher concentrations (30–60 g/L) (Fig. 3). The maximal MCA (260 ± 9 SU/mL) was achieved after 48 h of fermentation using 30 g/L wheat bran shorts. Accordingly, 30 g/L of triturated wheat bran shorts was used as the basal fermentation medium in the following optimization experiments.

BL312 was cultured in conditions supplemented with different carbon sources, and MCE production was measured at different time points to select the most suitable source of carbon supplementation (Fig. 4a, b). The carbon sources included glucose, sucrose, soluble starch, maltodextrin, and lactose. Our results indicated that BL312 MCE achieved the highest MCA in the presence of glucose (275 ± 9 SU/mL, 6% higher than the control). While the MCA was not altered in the presence of lactose, it was reduced in the presence of soluble starch, maltodextrin, and sucrose (Fig. 4a, b). Given our results, 5 g/L glucose was used to supplement the basal fermentation medium in subsequent experiments. Next, the effects of four organic nitrogen sources (corn steep liquor, casein peptone, urea, and yeast extract powder) and two inorganic sources (ammonium sulfate and ammonium citrate) on MCE production were examined at different fermentation time points (Fig. 4c, d). Our data indicated that BL312 MCE achieved the maximal MCA in the presence of 3 g/L corn steep liquor (302 ± 10 SU/mL). Compared with the control, MCA increased by 10% and 5% in the presence of corn steep liquor and urea, respectively. However, MCA was reduced in the presence of casein peptone, yeast extract powder, ammonium sulfate, and ammonium citrate (Fig. 4c, d).

Taken together, these results indicated that glucose (5 g/L) and corn steep liquor (3 g/L) supplementation in wheat bran shorts fermentation medium produced the best culture condition for BL312 MCE production. MCE synthesis can be

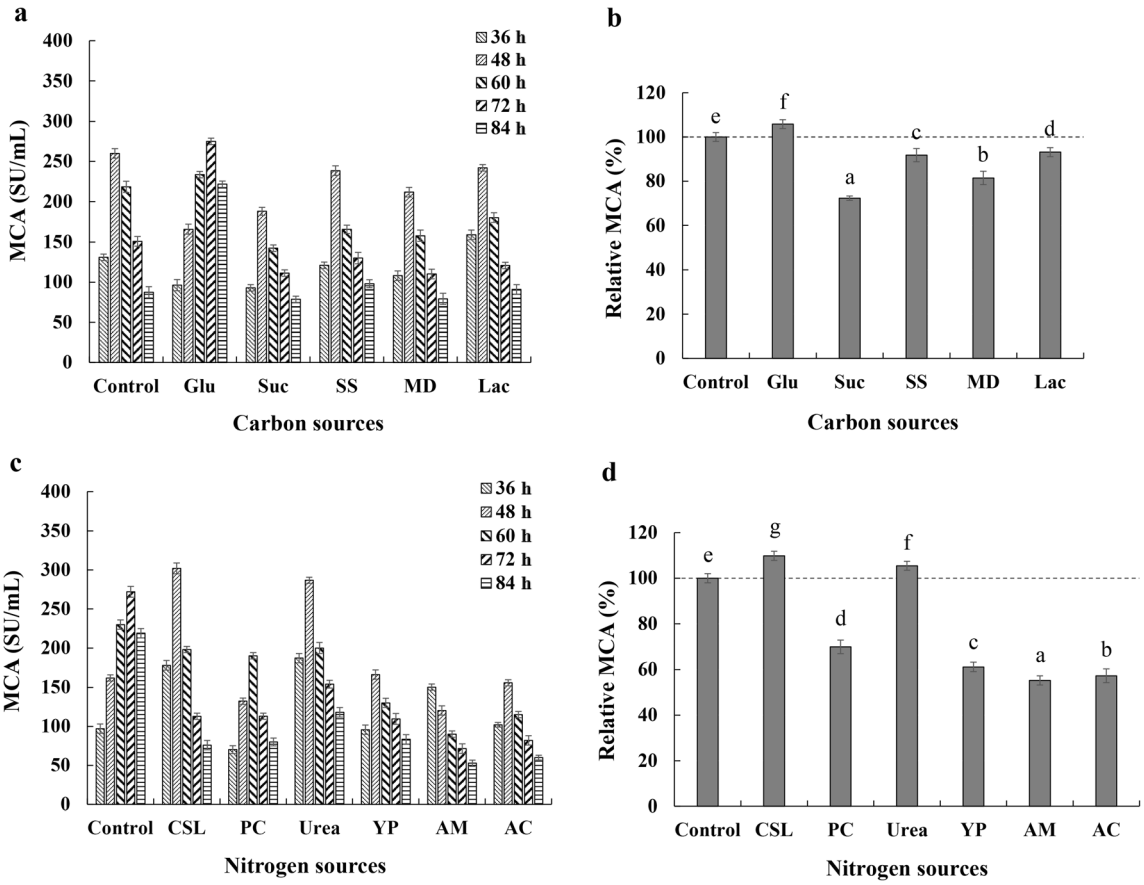

Fig. 4 **a**, **b** The effects of different carbon sources on BL312 MCE production during fermentation (36–84 h). Glu, glucose; Suc, sucrose; SS, soluble starch; MD, maltodextrin; and Lac, lactose. **c**, **d** The effects of different nitrogen sources on BL312 MCE production during fermentation (36–84 h). CSL, corn steep liquor; PC, casein peptone; YP, yeast extract powder; AM, ammonium sulfate; and AC, ammonium citrate. [a–g] Values in the same row with different superscripts are significantly different ($P < 0.05$). Data were presented as means of triplicate measurements; error bars were standard deviations

induced or suppressed by various materials and nutrient sources (Shata 2005). As BL312 growth and MCE production could be achieved using the low-cost wheat bran shorts with modest nutrient supplementation (Fig. 4), our results suggested that large-scale production of BL312 MCE would be economically feasible. Over-supplementation of carbon and nitrogen sources inhibits the growth of microorganisms and MCE production (Sen et al. 2009). According to our results, organic nitrogen sources (corn steep liquor and urea) produced better BL312 MCE yield than inorganic nitrogen sources (ammonium sulfate and ammonium citrate) (Fig. 4c, d). It could be possible that organic nitrogen contains kinds of amino acids, which can be absorbed directly by BL312. In contrast, BL312 first synthesized inorganic nitrogen into amino acids, reducing the growth of microorganisms. The MCE production is thus limited by the reduced biomass (Cai et al. 2004). These results were different from previous reports. Glucose and organic nitrogen sources are not considered good nutrient sources for MCE production in *Penicillium oxalicum* and *Nocardiopsis* sp. (Hashem 1999; Cavalcanti et al. 2005). However, glucose is considered a suitable carbon source for *Mucor miehei* (Sun et al. 2014).

The effects of bioprocess parameters on BL312 MCE production

As presented in Fig. 5, the bioprocess parameters were further optimized to achieve a maximal MCA (442 ± 12 SU/mL) in BL312 MCE. Our results indicated that the optimal inoculum size was 7% (v/v) and medium volume for BL312 MCE production was 40 mL (Fig. 5a, b). The maximal MCA was observed at a fermentation temperature of 37 °C and an agitation speed of 210 rpm (Fig. 5c, d). Deviations from the optimal inoculum size and fermentation temperature both decreased MCE production.

It is known that both the inoculum size and fermentation temperature profoundly affect the production of microbial MCEs. The biomass production was determined by inoculum size during fermentation (Sen et al. 2009). When the inoculum size was increased to 9% (v/v), MCA decreased significantly. It could be faster growth of BL312 and larger biomass, which caused the shortage in nutrients. To achieve the maximal MCE production, it is necessary to maintain the balance of biomass production and nutrient availability (Patel et al. 2005). The optimal temperature for MCE production in BL312 (37 °C)

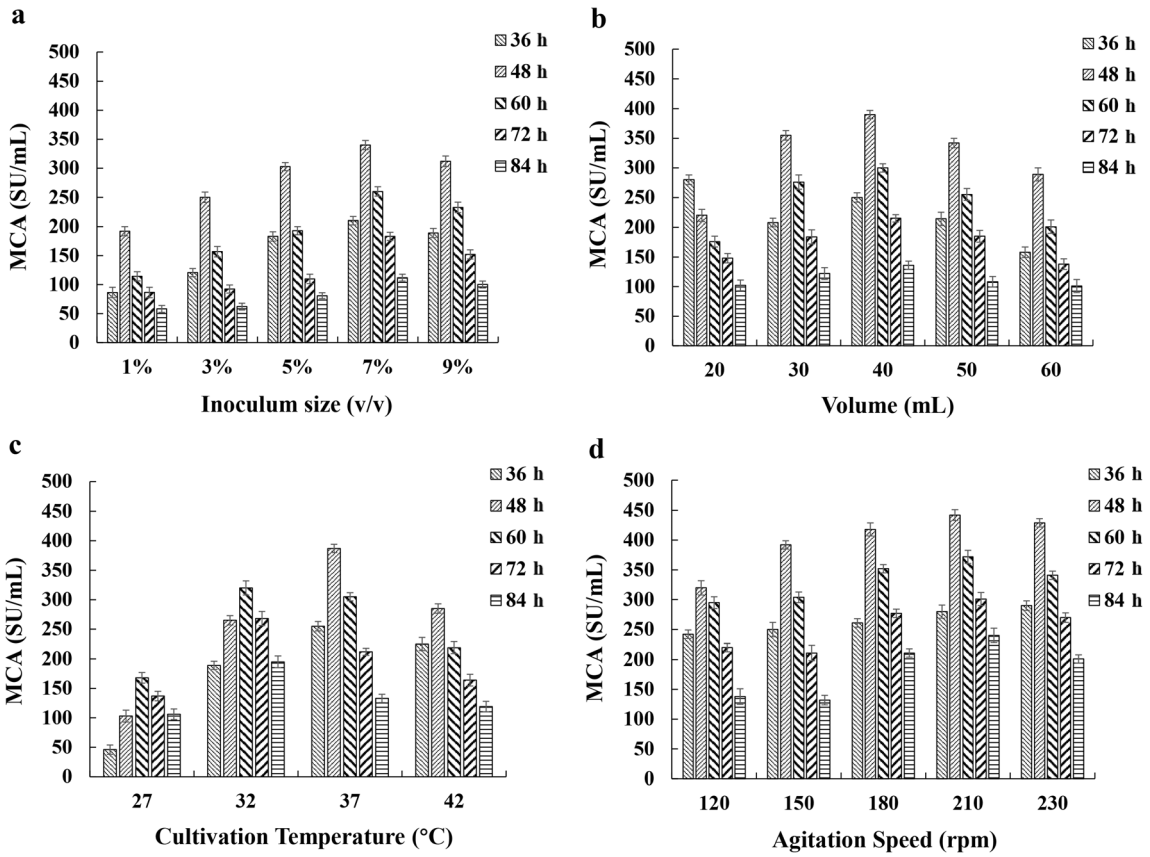

Fig. 5 The effects of inoculum size (**a**), volume (**b**), cultivation temperature (**c**), and agitation speed (**d**) on BL312 MCE production during different fermentation times (36–84 h). Dissolved oxygen (DO) and medium volume were the negative correlation. The medium composition was wheat bran shorts (30 g/L), glucose (5 g/L), and corn steep liquor (3 g/L). Data were presented as means of triplicate measurements; error bars were standard deviations

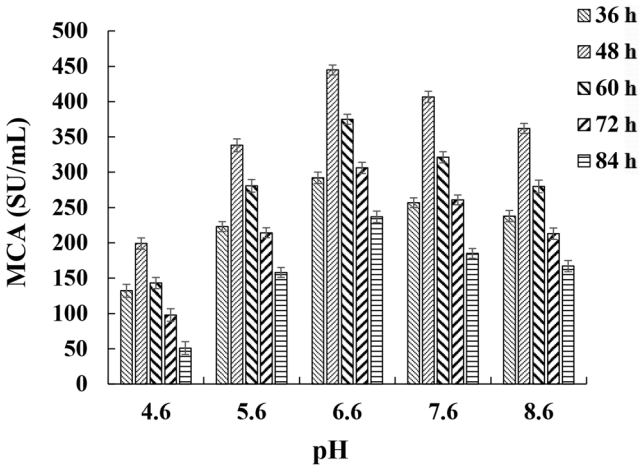

Fig. 6 The effects of initial pH of fermentation medium on BL312 MCE production during different fermentation times (36–84 h). The medium composition was wheat bran shorts (30 g/L), glucose (5 g/L), and corn steep liquor (3 g/L). Data were presented as means of triplicate measurements; error bars were standard deviations

was different from that for *Paenibacillus* sp. BD3526 (30 °C) (Hang et al. 2016) and *Bacillus methanolicus* LB-1 (30 °C) (Li et al. 2019). The agitation speed and liquid volume also played an important part in BL312 MCE production as they affected the amount of DO during fermentation.

The effects of initial pH on BL312 MCE production

BL312 MCE production reached the maximum when initial pH of the fermentation medium was adjusted to 6.6 (Fig. 6). Deviations from this initial pH decreased MCA. The initial pH is an important factor for many enzymatic and membrane transport processes (Moon and Parulekar 2010). The optimal initial pH for MCE production is dependent on the fermentation medium and the species of microorganism. The maximal MCA was achieved at the original medium pH (6.6). It was in favor of the production and industrialization of BL312 MCE. The optimal initial pH values for the maximal MCA were reported to be 4.5, 6.0, and 7.0 for *Chinese distiller's yeast* F34 (Wu et al. 2008), *Fusarium subglutinans* (Ghareib et al. 2001), and *Amylomyces rouxii* (Yu and Chou 2005), respectively.

BL312 MCE production using a 7-L fermenter

A large-scale production of BL312 MCE was next conducted in a 7-L fermenter using the optimized condition (Fig. 7). The BL312 MCE production was initiated using 4 L of the optimal fermentation medium (wheat bran shorts with glucose and corn steep liquor supplementation). The fermentation condition was as follows: inoculum size 7.0% (v/v), temperature 37 °C, agitation speed 210 rpm, and ventilation 1.7 vvm. Compared with using shake flasks, BL312 MCE achieved a higher maximal MCA (460 ± 15 SU/mL) in the fermenter. Throughout the fermentation process, the MCA/PA ratio and pH remained at 9.0 and 6.6, respectively. During the growth phase of BL312, DO concentration drastically decreased by 78% compared to the initial concentration, but increased after 36 h of fermentation. The maximal MCA of BL312 MCE observed here was higher than that reported for *B. licheniformis* USC13 (290 SU/mL) (Ageitos et al. 2007), *B. subtilis* YB-3 (200 SU/mL) (Li et al. 2012), and *M. mucedo* DSM 809 (130 SU/mL) (Yegin et al. 2012). The BL312 MCE exhibited lower PA compared with *B. subtilis* B1 and *B. amyloliquefaciens* JNU002 MCE (Table 2). Besides, the stable MCA/PA ratio and pH suggested that the submerged fermentation of BL312 was easy to control, implying that it is valuable in potential application.

The effects of protease inhibitors on BL312 MCE

The MCA values of crude BL312 MCE treated with different protease inhibitors are shown in Table 3. Our results indicated that BL312 MCE was not affected by pepstatin A (0.01–0.02 mM), DTT (1 mM), and low concentrations of EDTA (1 mM). However, it was significantly inhibited by PMSF (1 and 2 mM) and high concentrations of EDTA (5–25 mM)

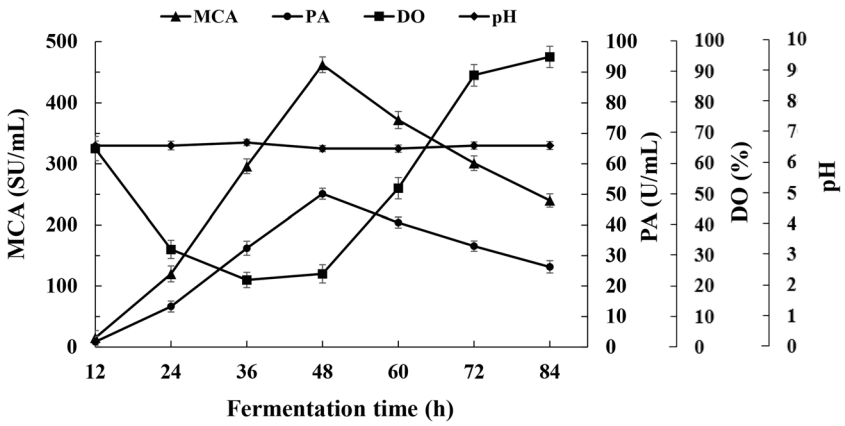

Fig. 7 Changes in the MCA, PA, DO, and pH in a 7-L fermenter with respect to fermentation times. Data were presented as means of triplicate measurements; error bars were standard deviations

Table 3 The effects of different protease inhibitors on BL312 MCE

Inhibitor	Concentration (mM)	Relative MCA (%)	Inhibition rate (%)
None	–	100	0
Pepstatin A	0.01	95.7 ± 1.5	4.3
	0.02	96.9 ± 1.0	3.1
EDTA	1	97.1 ± 2.1	2.9
	5	83.0 ± 2.3	17.0
	10	70.6 ± 3.2	29.4
	25	59.6 ± 2.1	40.4
PMSF	1	9.7 ± 1.7	90.3
	2	7.1 ± 2.2	92.9
DTT	1	98.6 ± 1.4	1.4

The data are represented as mean ± SD ($n = 3$)

(Table 3). Protease inhibitors can be used to reveal the type and active site of enzymes. It is known that PMSF, pepstatin A, and DTT were serine, aspartate, and cysteine protease inhibitor, respectively (Uda et al. 2017). The alkaline proteases were inhibited by high concentrations of EDTA (Tang et al. 2004). Our results showed that BL312 MCE exhibited the properties of serine and alkaline protease, indicating that it was a serine/alkaline protease. Unlike BL312 MCE, the MCA of *Bacillus licheniformis* 5A5 MCE is enhanced in the presence of PMSF and EDTA (Ahmed and Helmy 2012). In addition, the BL312 MCE identified in this study is different from the MCEs of *Enterococcus faecalis* TUA2495L (Sato et al. 2007), *B. subtilis* YB-3 (Li et al. 2012), and *Paenibacillus* sp. BD3526 (Hang et al. 2016).

Hydrolysis behavior of BL312 MCE

The RP-HPLC chromatograms of peptides were shown in Fig. 8. The BL312 MCE was more hydrolytic on α_s-CN and β-CN than calf rennet. As shown in Fig. 8c–f, the α_s-CN and

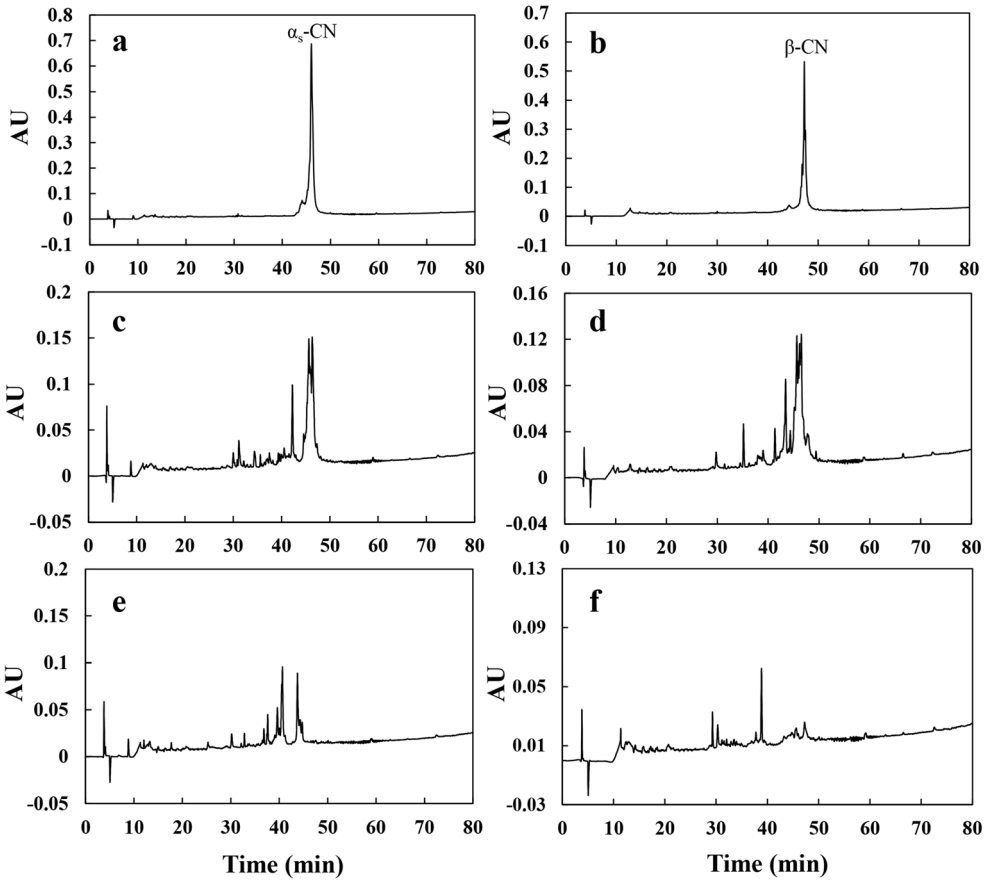

Fig. 8 RP-HPLC chromatograms of α_s-CN (**a**) and β-CN (**b**); RP-HPLC chromatograms of α_s-CN (**c**) and β-CN (**d**) hydrolysates generated by calf rennet; RP-HPLC chromatograms of α_s-CN (**e**) and β-CN (**f**) hydrolysates generated by BL312 MCE

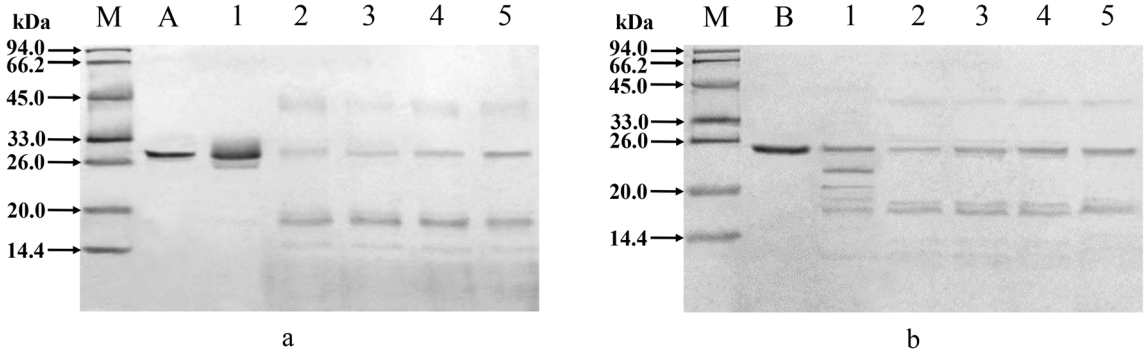

Fig. 9 SDS-PAGE patterns of α_s-CN (**a**) and β-CN (**b**) hydrolysates generated by BL312 MCE and calf rennet. Lane M: molecular weight marker proteins; lane A: α_s-CN; lane B: β-CN; lane 1: calf rennet; lanes 2–5: BL312 MCE. The α_s-CN:MCEs or β-CN:MCEs ratios were 15:1 in lanes 1 and 3, 10:1 in lane 2, 20:1 in lane 4, and 30:1 in lane 5

β-CN hydrolysates generated by BL312 MCE and calf rennet were significantly different at retention times of 10–50 min. However, the hydrolysates were generally similar at retention times of 50–80 min, where few peaks could be observed in chromatograms. The β-CN hydrolysates at retention times of 60–80 min represent the bitter peptides, which are important for the flavor during cheese ripening (Lee and Warthesen 2010). Although lack of the sensory analysis, the likely peptide compositions were indicated through the comparison of two chromatograms of hydrolysates generated by BL312 MCE and calf rennet (Fig. 8d, f). It is concluded that the β-CN hydrolysates generated by BL312 MCE were few bitter peptides.

For SDS-PAGE analysis, Fig. 9 shows that the hydrolytic abilities of BL312 MCE were getting weaker with the increase of α_s-CN:MCE or β-CN:MCE ratios. The α_s-CN was mainly hydrolyzed into apparent fragments (12–18 kDa) by BL312 MCE, which were much smaller than those by calf rennet (Fig. 9a). As presented in Fig. 9b, the β-CN hydrolysates generated by BL312 MCE were about molecular masses of 12–14 kDa and 17–18 kDa. Different casein hydrolysates generated by BL312 MCE and calf rennet indicated different cleavage sites in caseins and might lead to various cheese flavors (Majumder et al. 2015). In addition, the BL312 MCE hydrolyzed α_s-CN to a greater level than β-CN, which was in line with RP-HPLC analysis result. It is known that the hydrophobic peptides generated by β-CN hydrolysis were responsible for the bitterness in cheeses (An et al. 2014). Weak hydrolysis level of β-CN for BL312 MCE led to a decreased amount of bitter peptides in cheese production. Besides, various MCEs exhibited different preferences for α_s-CN and β-CN hydrolysis. As reported with researchers, the hydrolysis of β-CN was stronger than that of α_s-CN for *Paenibacillus* spp. BD3526 MCE (Hang et al. 2016). Importantly, the meltability and hardness of cheese were closely related to the α_s-CN and β-CN hydrolysis. The cheese meltability increased with α_s-CN degradation. In contrast, the cheese was hard at a high β-CN hydrolysis level (Hayaloglu et al. 2014). BL312 MCE preferred to hydrolyze α_s-CN, indicating it was potential to be used in making soft or semi-hard cheese.

Conclusions

In this study, a *Bacillus licheniformis* BL312 strain was identified and exhibited high MCA. A remarkable MCA (460 ± 15 SU/mL) was achieved after 48 h of fermentation in culture medium containing low-cost wheat bran shorts (30 g/L), glucose (5 g/L), and corn steep liquor (3 g/L). An initial pH of 6.6, fermentation temperature of 37 °C, agitation speed of 210 rpm, and ventilation of 1.7 vvm were required for the optimal MCA. The BL312 MCE exhibited a stable MCA/PA ratio of 9.0 and pH of 6.6 throughout the fermentation process, suggesting that it is suitable for prolonged fermentation. The MCA of BL312 MCE was significantly inhibited by PMSF and high concentrations of EDTA, implying that it was a serine/alkaline protease. Importantly, our results revealed that BL312 MCE was characterized with various hydrolysis for caseins. Further characterization and application of BL312 MCE should be conducted in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent This manuscript is approved by all authors for publication.

References

Ageitos JM, Vallejo JA, Sestelo ABF, Poza M, Villa TG (2007) Purification and characterization of a milk-clotting protease from *Bacillus licheniformis* strain USC13. J Appl Microbiol 103(6): 2205–2213

Ahmed SA, Helmy WA (2012) Comparative evaluation of *Bacillus licheniformis* 5A5 and *Aloe variegata* milk-clotting enzymes. Braz J Chem Eng 29(1):69–76

An ZG, He XL, Gao WD, Zhao W, Zhang WB (2014) Characteristics of miniature cheddar-type cheese made by microbial rennet from *Bacillus amyloliquefaciens*: a comparison with commercial calf rennet. J Food Sci 79:M214–M221

Arima K, Yu J, Iwasaki S, Tamura G (1968) Milk-clotting enzyme from microorganisms: V. purification and crystallization of *Mucor* rennin from *Mucor pusillus* var. Lindt Appl Microbiol 16(11):1727–1733

Ben AA, Makhlouf I, Flaviu PR, Francis F, Bauwens J, Attia H, Besbes S, Blecker C (2017) Effect of extraction pH on techno-functional properties of crude extracts from wild cardoon (*Cynara cardunculus L.*) flowers. Food Chem 225:258–266

Cai QH, Yue XY, Niu TG, Ji C (2004) The screening of culture condition and properties of xylanase by white-rot fungus *Pleurotus ostreatus*. Process Biochem 39(11):1561–1566

Cavalcanti MTH, Martinez CR, Furtado VC, Neto BB, Teixeira MF, Lima Filho JL, Porto ALF (2005) Milk-clotting protease production by *Nocardiopsis* sp. in an inexpensive medium. World J Microbiol Biotechnol 21(2):151–154

Celebi M, Topuzogullari M, Kuzu H (2016) Thermal destabilization of *Rhizomucor miehei* rennet with aldehyde dextran sulfate: purification, bioconjugation and milk-clotting activities. Appl Biochem Biotechnol 180(2):261–273

Chwen-Jen S, Lan APT, Ing LS (2009) Milk-clotting enzymes produced by culture of *Bacillus subtilis natto*. Biochem Eng 43(1):85–91

Ding ZY, Liu SP, Gu ZH, Zhang L, Zhang KC, Shi GY (2011) Production of milk-clotting enzyme by *Bacillus subtilis* B1 from wheat bran. Afr J Biotechnol 10(46):9370–9378

Ding ZY, Ai LZ, Ouyang A, Ding ML, Wang WF, Wang BD, Liu SP, Gu ZH, Zhang L, Shi GY (2012) A two-stage oxygen supply control strategy for enhancing milk-clotting enzyme production by *Bacillus amyloliquefaciens*. Eur Food Res Technol 34(6):1043–1048

Fazouane F, Mechakra A, Abdellaoui R, Nouani A (2010) Characterization and cheese-making properties of rennet-like enzyme produced by a local Algerian isolate of *Aspergillus niger*. Food Biotechnol 24(3):258–269

Gagaoua M, Ziane F, Nait RS, Boucherba N, Ait KEEA, Bouanane-Darenfed A, Hafid K (2017) Three phase partitioning, a scalable method for the purification and recovery of cucumisin, a milk-clotting enzyme, from the juice of *Cucumis melo var.* reticulatus. Int J Biol Macromol 102:515–525

Ghareib MH, Hamdy HS, Khalil AA (2001) Production of intracellular milk-clotting enzyme in submerged cultures of *Fusarium subglutinans*. Acta Microbiol Pol 50(2):139–147

Hang F, Liu PY, Wang QB, Han J, Wu ZJ, Gao CX, Liu ZM, Zhang H, Chen W (2016) High milk-clotting activity expressed by the newly isolated *Paenibacillus* spp. strain BD3526. Molecules 21(1):73

Hashem AM (1999) Optimization of milk-clotting enzyme productivity by *Penicillium oxalicum*. Bioresour Technol 70(2):203–207

Hayaloglu AA, Karatekin B, Gurkan H (2014) Thermal stability of chymosin or microbial coagulant in the manufacture of Malatya, a halloumi type cheese: proteolysis, microstructure and functional properties. Int Dairy J 38(2):136–144

He XL, Zhang WB, Ren FZ, Gan BZ, Guo HY (2012) Screening fermentation parameters of the milk-clotting enzyme produced by newly isolated *Bacillus amyloliquefaciens* D4 from the Tibetan Plateau in China. Ann Microbiol 62(1):357–365

Lee KD, Warthesen JJ (2010) Mobile phases in reverse-phase HPLC for the determination of bitter peptides in cheese. J Food Sci 61:291–294

Li Y, Liang S, Zhi DJ, Chen P, Su F, Li HY (2012) Purification and characterization of *Bacillus subtilis* milk-clotting enzyme from Tibet Plateau and its potential use in yak dairy industry. Eur Food Res Technol 234(4):733–741

Li L, Zheng Z, Zhao X, Wu FY, Zhang J, Yang ZN (2019) Production, purification and characterization of a milk clotting enzyme from *Bacillus methanolicus* LB-1. Food Sci Biotechnol. https://doi.org/10.1007/s10068-018-0539-2

Liburdi K, Emiliani SS, Benucci I, Lombardelli C, Esti M (2018) A preliminary study of continuous milk coagulation using *Cynara cardunculus* flower extract and calf rennet immobilized on magnetic particles. Food Chem 239:157–164

Luo J, Xiao C, Zhang H, Ren FZ, Lei XG, Yang ZB, Yu ZQ (2018) Characterization and milk coagulating properties of *Cynanchum otophyllum* Schneid. proteases. J Dairy Sci 101(4):2842–2850

Majumder R, Banik SP, Khowala S (2015) Purification and characterisation of κ-casein specific milk-clotting metalloprotease from *Termitomyces clypeatus* MTCC 5091. Food Chem 173:441–448

Marcial J, Pérez De Los Santos AI, Fernández FJ, Díaz-Godínez G, Montiel-González AM, Tomasini A (2011) Characterization of an aspartic protease produced by *Amylomyces rouxii*. Rev Mex Cienc Geol 10(1):9–16

Meng FQ, Chen R, Zhu XY, Lu YJ, Nie T, Lu FX, Lu ZX (2018) A newly effective milk-clotting enzyme from *Bacillus subtilis* and its application in cheese-making. J Agric Food Chem 66(24):6162–6169

Merheb-Dini C, Gomes E, Boscolo M, Silva RD (2010) Production and characterization of a milk-clotting protease in the crude enzymatic extract from the newly isolated *Thermomucor indicae-seudaticae* N31 (milk-clotting protease from the newly isolated *Thermomucor indicae-seudaticae* N31). Food Chem 120(1):87–93

Moon SH, Parulekar SJ (2010) A parametric study ot protease production in batch and fed-batch cultures of *Bacillus firmus*. Biotechnol Bioeng 37(5):467–483

Patel R, Dodia M, Singh SP (2005) Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: production and optimization. Process Biochem 40(11):3569–3575

Preetha S, Boopathy R (1997) Purification and characterization of a milk clotting protease from *Rhizomucor miehei*. World J Microbiol Biotechnol 13(5):573–578

Qiu WW, Curtin D, Johnstone P, Beare M (2016) Small-scale spatial variability of plant nutrients and soil organic matter: an arable cropping case study. Commun Soil Sci Plan 47(19):2189–2199

Salehi M, Aghamaali MR, Sajedi RH, Asghari SM, Jorjani E (2017) Purification and characterization of a milk-clotting aspartic protease from *Withania coagulans* fruit. Int J Biol Macromol 98:847–854

Sato S, Tokuda H, Koizumi T, Nakanishi K (2007) Purification and characterization of fan extracellular proteinase having milk-clotting activity from *Enterococcus faecalis* TUA2495L. Food Sci Technol Res 10(1):44–50

Sen S, Veeranki VD, Mandal B (2009) Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1. Ann Microbiol 59(3):531–538

Shata HMA (2005) Extraction of milk-clotting enzyme produced by solid state fermentation of *Aspergillus oryzae*. Pol J Microbiol 54(3):241–247

Sun Q, Wang XP, Yan QJ, Chen W, Jiang ZQ (2014) Purification and characterization of a chymosin from *Rhizopus microsporus* var. *rhizopodiformis*. Appl Biochem Biotechnol 174(1):174–185

Tang XM, Shen W, Lakay FM, Shao WL, Wang ZX, Prior BA, Zhuge J (2004) Cloning and over-expression of an alkaline protease from *Bacillus licheniformis*. Biotechnol Lett 26(12):975–979

Thakur M, Karanth NG, Nand K (1990) Production of fungal rennet by *Mucor miehei* using solid state fermentation. Appl Microbiol Biotechnol 32(4):409–413

Uda K, Abe K, Dehara Y, Mizobata K, Edashige Y, Nishimura R, Radkov AD, Moe LA (2017) Triple serine loop region regulates the aspartate racemase activity of the serine/aspartate racemase family. Amino Acids 49(10):1743–1754

Vallejo JA, Ageitos JM, Poza M, Villa TG (2012) Short communication: a comparative analysis of recombinant chymosins. J Dairy Sci 95(2):609–613

Vishwanatha K, Rao AA, Singh SA (2010) Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters. J Ind Microbiol Biotechnol 37(2): 129–138

Wang J, Li C, Yang H, Mushegian A, Jin S (1998) A novel serine/threonine protein kinase homologue of *Pseudomonas aeruginosa* is specifically inducible within the host infection site and is required for full virulence in neutropenic mice. J Bacteriol 180(24):6764–6768

Wasko A, Kieliszek M, Targonski Z (2012) Purification and characterization of a proteinase from the probiotic *Lactobacillus rhamnosus* OXY. Prep Biochem Biotechnol 42:476–488

Wu JJ, Chen HB, Chen WP (2008) Fermentation parameter and partial biochemical characterisation of milk clotting enzyme from *Chinese distiller's yeast*. Ann Microbiol 58(4):717–722

Yegin S, Goksungur Y, Fernandez-Lahore M (2012) Purification, structural characterization, and technological properties of an aspartyl proteinase from submerged cultures of *Mucor mucedo* DSM 809. Food Chem 133(4):1312–1319

Yu PJ, Chou CC (2005) Factors affecting the growth and production of milk-clotting enzymes by *Amylomyces rouxii* in rice liquid medium. Food Technol Biotechnol 43(3):283–288

Zhou J, Wang RQ, Guo WH, Zhou GJ, Wang Q, Wang W, Han XM, Pang XG, Zhan JC, Dai JR (2011) Soil microbial community diversity and its relationships with geochemical elements under different farmlands in Shouguang, China. Commun Soil Sci Plan 42(9): 1008–1026