Optimization of the production and characterization of milk-clotting enzyme from *Bacillus subtilis* isolated from marine sponge

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Background and objectives
Milk-clotting enzyme (MCE) has important applications in the dairy industry and in the cheese-manufacturing process. Because of the increase in cheese consumption and the low supply of calf rennets, there is a need for a suitable rennet substitute from an appropriate source. The present investigation aims at microbial production of MCE and medium optimization for maximal enzyme production by the most potent strain. Partial purification and the properties of the partially purified enzyme are also studied.

Materials and methods
In the present study several microorganisms were tested for production of the MCE. MCE/caseinase ratio was investigated and was used as the key parameter for selection of the most potent strain and for medium optimization. Medium optimization experiments were carried out in an attempt to increase the enzyme productivity by the most potent strain. The produced enzyme was partially purified using ammonium sulfate at 50% concentration and the properties of the partially purified enzyme were investigated.

Results and conclusion
Among all the tested bacteria the marine sponge *Pseudoceratina arabica* isolated bacterium (isolate I) showed the highest milk-clotting activity. This isolate was used for MCE production throughout this study and it was identified as *Bacillus subtilis* KU710517. Maximum enzyme productivity (581.8 U/ml) and maximum MCE/caseinase ratio were obtained using a medium containing 50 g/l wheat bran with xylose and yeast extract as carbon and nitrogen sources. For the partially purified enzyme, the optimal temperature and pH were 85°C and 5.0, respectively. The enzyme was thermally stable at 45°C and retained 100% activity after 90 min. At 50°C for the same period of time it retained about 82% activity. However, at 60°C, the enzyme lost about 70% of its original activity after 30 min. The activation energy (Ea) of the enzyme was calculated as 6.95 kcal/mol. K_m and V_{max} values were 4.6 mg/ml and 2933 U/mg protein, respectively.

Keywords:
Bacillus subtilis, medium optimization, milk-clotting enzyme

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Introduction

The word 'rennet' is applied to the crude enzyme that can coagulate milk either from animal, plant, or microbial source [1]. It is a protease enzyme that is used in cheese manufacture [2]. Milk coagulation occurs in two important phases: first, casein is hydrolyzed in an enzymatic phase and forms paracaseinate. It then forms a curd in the nonenzymatic phase at certain calcium ion concentrations [3]. Calf rennet, which contains chymosin (EC 3.4.23.4), is widely used as an important milk-clotting enzyme (MCE) [4]. Because of the reduced supply of calf rennet and calf diseases like bovine spongiform encephalopathy, there is an increase in the demand for new sources of MCEs to meet the needs of the cheese industry [5,6]. However, most plant rennets are unsuitable because they give a bitter taste to the cheese [7,8]. Microbial rennets are more promising enzymes because of its low production costs, greater biochemical diversity, and easier genetic modification [9]. Many fungal and bacterial sources are widely used for cheese production. About 60% of cheese in the USA is manufactured using fungal enzyme sources [10]. Bacillus species are known to produce many extracellular enzymes with a wide range of industrial applications [11].

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Wheat bran acts as a cheap source of energy in the fermentation process for enzyme production. This agroindustrial residue contains many ingredients such as cellulose, crude protein, starch, and trace elements that are used as carbon and nitrogen sources to stimulate the growth of microorganisms and enzyme productivity. Many researchers observed that wheat bran is a potent substrate for enzyme production [12,13].

Materials and methods

Microorganisms and media

All bacilli used in the screening, except *Paenibacillus macerans*, were obtained from the Culture Collection of the National Research Centre, Dokki, Cairo, Egypt. However, *P. macerans* was obtained from the Microbiological Resources Center (Mircen, Faculty of Agriculture, Ain Shams University). The bacterial isolates I, II, III, and IV were isolated from the marine sponge *Pseudoceratina arabica*. All microorganisms were grown on nutrient agar medium at 37°C. The basal medium used for MCE production contained the following (g/l): wheat bran, 50; yeast extract, 3.0; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 3.0; and glucose, 4.0. pH was 6.0 before sterilization.

Isolation of marine sponge-associated bacteria

A single colony of *P. arabica* was collected from Red Sea, Sharm El Sheikh, south of Sinai, Egypt, by scuba diving at a depth of 15–20 m. Sponge specimens were transferred to a sterile plastic bag containing sea water, kept in ice, and transported to the laboratory. Afterwards, the specimens were rinsed several times with sterile sea water and cut into small pieces. The tissues were homogenized in sterile sea water. A volume of 100 μl of 10^{-1} dilution of the homogenate with sterile sea water was spread onto Zobell marine agar plates supplemented with 50% filtered sea water and cycloheximide (50 μg/ml) as antifungal agent. The plates were incubated at 28°C for 7 days. Colonies were selected on the basis of the morphological features and purified by making streak plates containing sterile Zobell marine agar medium.

Cultivation conditions and enzyme production

One slant was scratched with 10 ml distilled water, and 2 ml of cell suspension was used to inoculate a 250-ml Erlenmeyer flask containing 50 ml of sterile production medium. The flasks were incubated at 37°C on a rotary shaker at 150 rpm for 48 h. The cells were then centrifuged at 3000 rpm for 10 min and the supernatant was collected and assayed for milk-clotting activity (MCA).

Enzyme assays

Assay of milk-clotting activity

MCA was determined as follows: 0.5 ml of the enzyme solution was incubated with 2 ml of reconstituted skimmed milk (12 g dry skim milk/100 ml 0.01 mol/l calcium chloride) at 40°C. MCA was expressed in Soxhlet units, which were calculated using the following equation:

$$\text{Soxhlet units} = 2400/T \times S/E,$$

where S is the volume of milk (ml), E is the volume of enzyme (ml), and T is the time necessary for curd fragment formation (s) [14].

Assay of caseinase activity

One milliliter of enzyme was added to 1 ml of 1% casein solution in Tris-HCl buffer (0.2 mol/l, pH 8.5). The reaction mixture was incubated in a water bath at 40°C for 30 min. Then 2 ml of 15% trichloroacetic acid was added and the mixture was centrifuged at 4000 rpm for 10 min. The solubilized proteins in supernatant were measured using the Lowry method at 750 nm [15].

Survey of some bacterial cultures for milk-clotting enzyme production

In this experiment, 10 bacterial cultures were tested for MCE production by submerged fermentation using the basal medium under shaken culture conditions after a 48-h incubation period. The culture filtrates were assayed for both MCE and caseinase activities.

Optimization of some culture conditions for maximization of milk-clotting enzyme productivity by the potent strain

These experiments were undertaken to investigate the effect of different medium compositions on MCE productivity by the potent strain. Thereafter, an optimum culture medium was formulated for the production of MCE.

Effect of wheat bran concentrations

This experiment was undertaken to investigate the effect of wheat bran concentration on the production of MCE. The fermentation was performed for 48 h using different concentrations of wheat bran (10–70 g/l).

Effect of different carbon sources

For studying the effect of different carbon sources on the production of MCE, glucose from the basal medium was substituted by different carbon sources (i.e. fructose, sucrose, lactose, xylose, arabinose, starch, and cellobiose). The fermentation was performed for 48 h.

Effect of different nitrogen sources

On equivalent nitrogen basis, yeast extract from the basal medium was substituted with different nitrogen sources [i.e. peptone, casein soya bean, NH_4Cl, $(NH_4)_2S0_4$, and $NaNO_3$]. The fermentation was also performed for 48 h.

Effect of phosphate concentration

This experiment was undertaken to investigate the effect of phosphate level as K_2HPO_4 in the culture medium on MCE production. Different concentrations of K_2HPO_4 ranging from 2.0 to 3.5 g/l were used.

Effect of magnesium sulfate concentration

The present experiment deals with the effect of magnesium sulfate concentration on the production of MCE. In this experiment, magnesium sulfate was added to the culture medium at different concentrations (0.1–0.3 g/l).

Partial purification of the enzyme

This was achieved by precipitation of the concentrated culture filtrate by salting out with ammonium sulfate at 35, 50, 70, and 90% concentrations. The obtained fractions were dried and assayed for enzyme activity.

Properties of the partially purified enzyme

Some properties of the partially purified MCE are studied. These include the effect of reaction temperature, pH, thermal stability, and substrate concentration.

Identification and phylogenetic analysis of sponge-associated strain based on the 16srRNA gene sequence

The gene coding for 16srRNA was amplified by PCR from the isolated genomic DNA using forward (5′-TCACGGAGAGTTTGATCCTG-3′) and reverse (5′-GCGGCTGCTG GCACGTAG TT-3′) primers. Considering the PCR conditions, genomic DNA was initially denatured at 95°C for 3 min, followed by 30 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 48°C for 30 s, and elongation at 68°C for 90 s. Final extension of the amplified products was performed at 68°C for 5 min. The reaction mixture was kept at 4°C [16].

Results and discussion

Survey of some bacterial cultures for milk-clotting enzyme production

After the 48-h incubation period, maximum MCA (564.7 U/ml) and MCA/caseinase ratio (3422.4) were obtained from the bacterial isolate I (Table 1). Therefore, this bacterial strain has been used for MCE production in this work.

Optimization of some cultural conditions for maximization of milk-clotting enzyme productivity

Effect of wheat bran concentrations

Wheat bran has been reported as an ideal medium for microbial protease production [17,18]. The highest MCE production yield (640 U/ml) was reached at wheat bran concentration of 70 g/l. At higher concentrations, the medium is thicker, which may limit cell growth and affect the enzyme productivity. However, the highest MCA/caseinase ratio (3422.4) was obtained with a wheat bran concentration of 50 g/l (Fig. 1). Thus, we conclude that this wheat bran concentration is favorable for MCE production. Many authors also used wheat bran as a potent substrate for MCE production by microorganisms [19–22]. However, some authors reported different optimum concentrations of wheat bran for MCE production from other microorganisms (*Paenibacillus* spp. BD3526, 30 g/l and *Bacillus amyloliquefaciens* D4, 180 g/l) [20,22].

Effect of different carbon sources

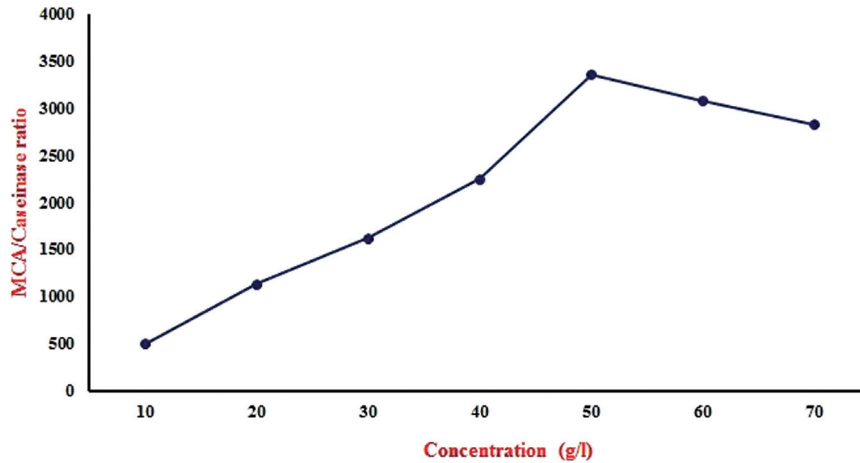
The maximal MCA (581.8 U/ml) was obtained with xylose, arabinose, and cellobiose. However, maximum MCE/caseinase ratio (4216) was obtained with xylose as indicated in Fig. 2. Thus, xylose proved to be the most favorable carbon source for the production of MCE. This activity is higher than that reported by other authors, who reported other sugars as the best carbon source for MCE production (lactose 58.5 U/ml, glucose 21.5 U/ml) [23–25].

Table 1 Survey of some microorganisms for milk-clotting activity

Microorganisms	Final pH of CF	Protein content (mg/ml)	MCA (U/ml)	Caseinase activity (U/ml)	MCA/caseinase ratio
Bacillus licheniformis	6.22	1.95	42.7	0.021	2033.4
Bacillus subtilis	6.25	2.29	27.4	0.0185	1481
Bacillus macerans	6.34	2.89	39.3	0.022	1786.3
Bacillus megaterium	6.52	1.908	Negative	Negative	–
Bacillus circulans	6.36	1.468	Negative	0.066	–
Paenibacillus macerans	6.39	1.126	Negative	Negative	–
Isolate I	6.32	2.56	564.7	0.165	3422.4
Isolate II	6.46	2.229	355.6	0.183	1943.1
Isolate III	6.10	2.220	408.5	0.130	3142.3
Isolate IV	6.39	2.183	426.7	0.164	2601.8

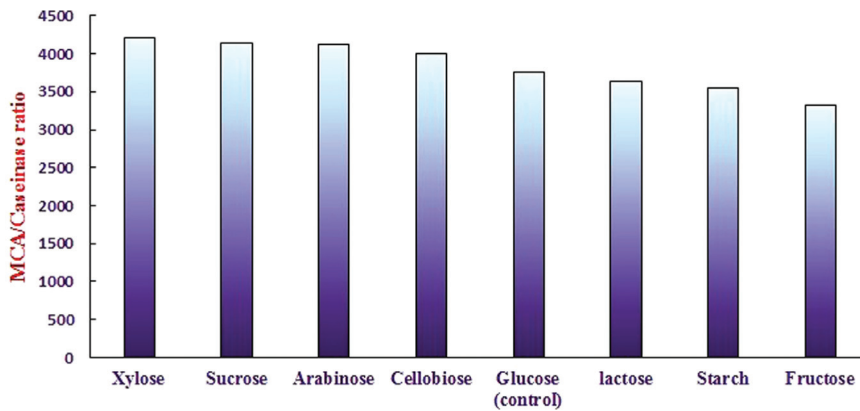
CF, culture filtrate; MCA, milk-clotting activity.

Figure 1



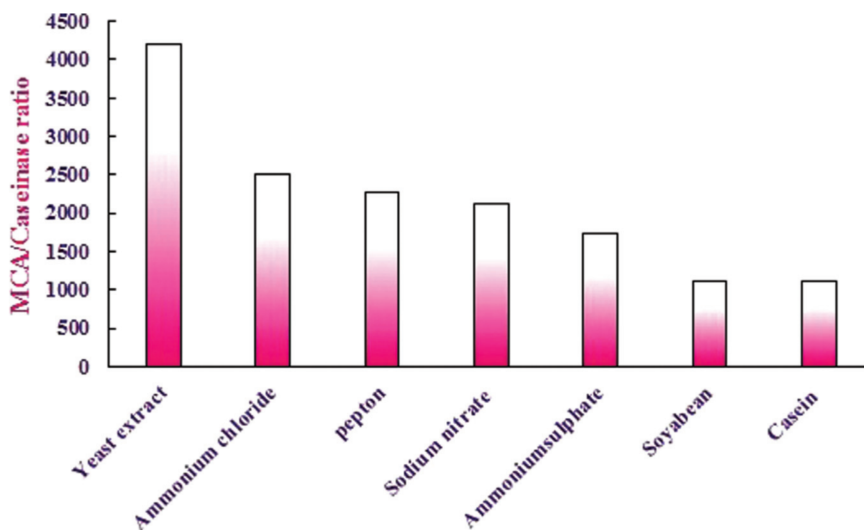
Effect of wheat bran concentrations on milk-clotting activity (MCA)/caseinase ratio.

Figure 2



Effect of carbon sources on milk-clotting activity (MCA)/caseinase ratio.

Figure 3



Effect of nitrogen sources on milk-clotting activity (MCA)/caseinase ratio.

Effect of different nitrogen sources

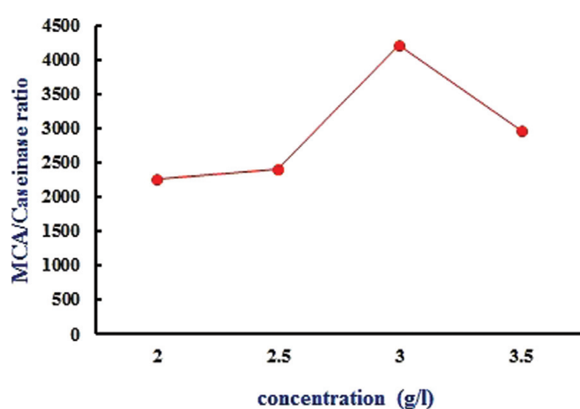
As shown in Fig. 3, maximum MCE production yield (581.8 U/ml) with maximum MCA/caseinase ratio of 4216 was reached with yeast extract. Therefore, the yeast extract that was used in the basal medium as a nitrogen source is used throughout the work for MCE

production. This result differs from those of other authors who observed maximum MCE production with other nitrogen sources (beef extract, whey powder, casein) [25–27]. Other authors observed that the presence of nitrogen sources is not critical for the production of MCE [28]. In general, the effect of nitrogen source on MCE production differs from organism to organism [25].

Effect of phosphate concentration

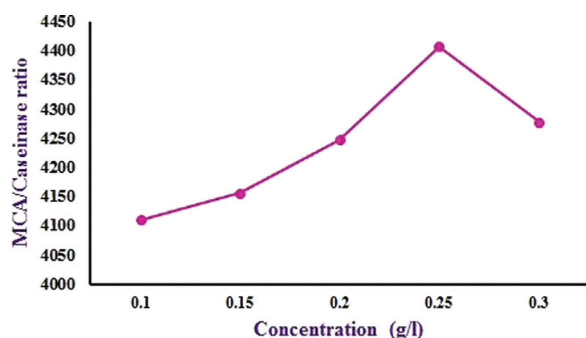
It is well known that phosphate is utilized to form various cellular components [29]. As illustrated in Fig. 4, the highest MCE yield (581.8 U/ml) with an MCA/caseinase activity ratio of 4216 was reached at a phosphate concentration of 3 g/l, which was previously used in the basal medium. This concentration is higher than that investigated by other authors for maximum MCE production (1.3 g/l) [24]. The requirement of phosphorus for protease production was very evident as reported by many researchers [30,31].

Figure 4



Effect of phosphate concentrations on milk-clotting activity (MCA)/caseinase ratio.

Figure 5



Effect of $MgSO_4$ concentrations on milk-clotting activity (MCA)/caseinase ratio.

Effect of magnesium sulfate concentration

Magnesium is essential for bacterial growth and cell division [32]. Thus, it was used in many media. The highest MCE yield (600 U/ml) was obtained at low magnesium sulfate concentration (0.1 g/l). Further increase in concentration caused a slight decrease in MCE yield. However, the highest MCA/caseinase ratio (4407.5) was obtained with a magnesium sulfate concentration of 0.25 g/l (Fig. 5) and hence this concentration was used in this work. From the previous results, the optimum culture medium for MCE production contained the following (g/l): wheat bran, 50; yeast extract, 3.0; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 3.0; and xylose, 4.0. The pH was 6.0 before sterilization.

Partial purification of milk-clotting enzyme

MCE was precipitated with ammonium sulfate and the most active fraction was obtained at 50% concentration. The partially purified enzyme retained a specific activity of 512.8 U/mg protein with 5.7 purification fold (Table 2).

Properties of the partially purified enzyme

Optimum temperature

The effect of the temperature of the reaction MCE was investigated at pH 4.5. The temperature profile of MCE showed that MCA was increased with increase in the reaction temperature up to 85°C. Further increase in reaction temperature resulted in loss in MCA. This may be due to the denaturation of the enzyme above this temperature. This value is higher than those reported by other authors (55 and 60°C) [33,34]. Other authors also have reported high optimum temperatures for MCE (75°C from *Bacillus licheniformis* and 85°C from *Aloe variegate*)

Table 2 Fractional precipitation of milk-clotting enzyme with ammonium sulfate

Ammonium sulfate concentration (%)	Protein content mg/fraction	Activity of fraction (U)	Specific activity U/mg protein	Recovered protein (%)	Recovered activity (%)	MCA/ caseinase	Fold purification
CF	1920	1 71 430	89.28	100	100	1251.3	1.00
35	70.38	4320	61.38	3.66	2.52	258	0.69
50	158.4	81 230.6	512.82	8.25	47.38	2725	5.74
70	140.52	41 143.2	292.79	7.32	24	1565.5	3.28
90	151.06	6399.4	42.36	7.86	3.73	346.3	0.47
Total	520.4	1 33 093.2		27.1	77.6		

CF, culture filtrate; MCA, milk-clotting activity.

[35]. The Arrhenius plot (Fig. 6) for MCE was linear. The activation energy was calculated as 6.95 kcal/mol from the slope using the following equation:

$$\text{Slope} = \frac{\text{Activation energy}}{2.303\,R},$$

where R is the gas constant (R=1.976 cal/mol).

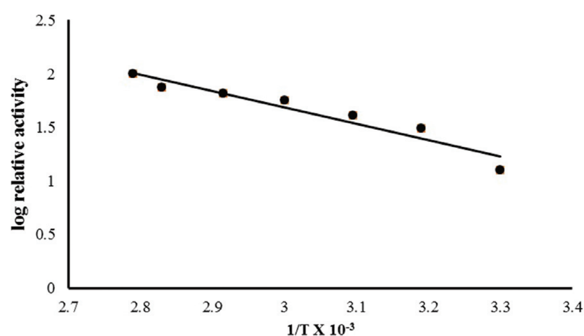
Optimum pH

The effect of the pH of the reaction on the activity of MCE was investigated at pH ranges of 4.0–7.5. MCE was optimally active at pH 5.0 (Fig. 7). Above this pH the activity decreased gradually, which was due to denaturation of the enzyme. This optimum pH is similar to that reported by other authors [26]. This result is also in agreement with previous findings, in which a reduction in the milk pH resulted in a decrease in the milk-clotting time [36,37]. Several authors have also reported a decline in MCA at pH near neutrality [38,39]. The higher activity of the enzyme in acidic pH is highly advantageous, and it can be easily controlled in industrial applications [40].

Thermal stability of milk-clotting enzyme

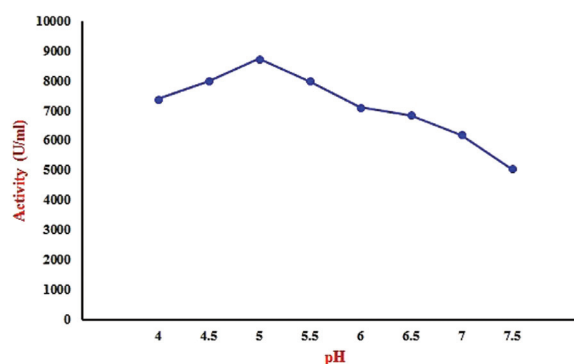
The enzyme solution was heated at different time intervals (15–90 min) at different temperatures (45–60°C) in the absence of substrate. As indicated (Fig. 8), the enzyme was stable after heat treatment for 90 min at 45°C and retained 100% activity. The enzyme retained about 73 and 47% of its original activity after heating for 15 min at 55 and 60°C, respectively. It was completely deactivated after 90 min of heating at 60°C. These results are better than those reported by other authors, as MCE produced from *B. licheniformis* retained 73% of its activity when heated for 1 h at 40°C [35]. Also the enzyme produced from *Penicillium oxalicum* retained only 58% of its activity at the same conditions [34]. However, MCE from *B. amyloliquefaciens* was completely deactivated when heated at 55°C for 20 min [21].

Figure 6



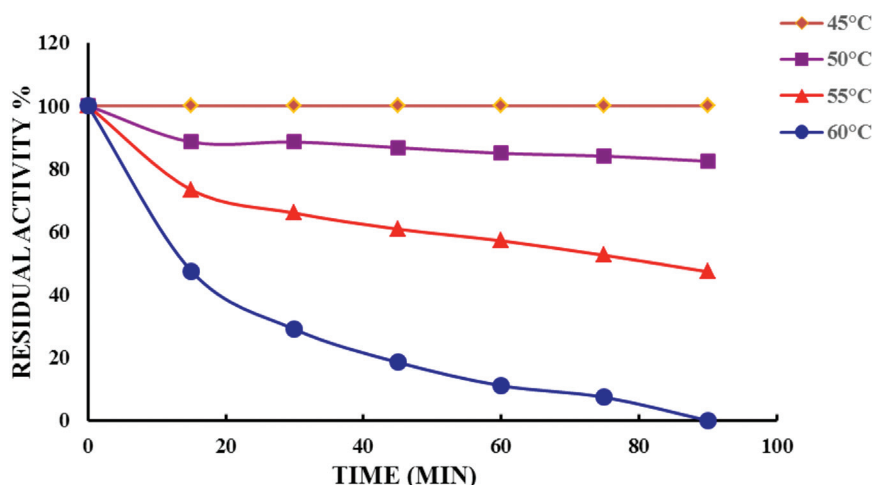
Arrhenius plot for milk-clotting enzyme (MCE).

Figure 7



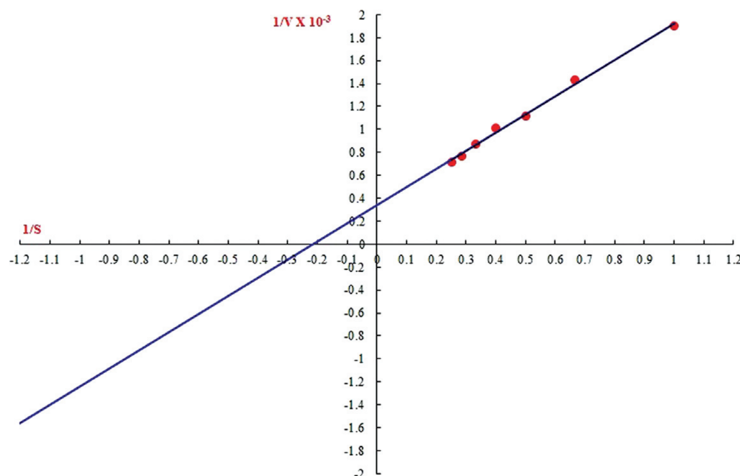
Effect of pH on milk-clotting enzyme (MCE).

Figure 8



Thermal stability of milk-clotting enzyme (MCE).

Figure 9



Lineweaver–Burk plot of milk-clotting enzyme (MCE).

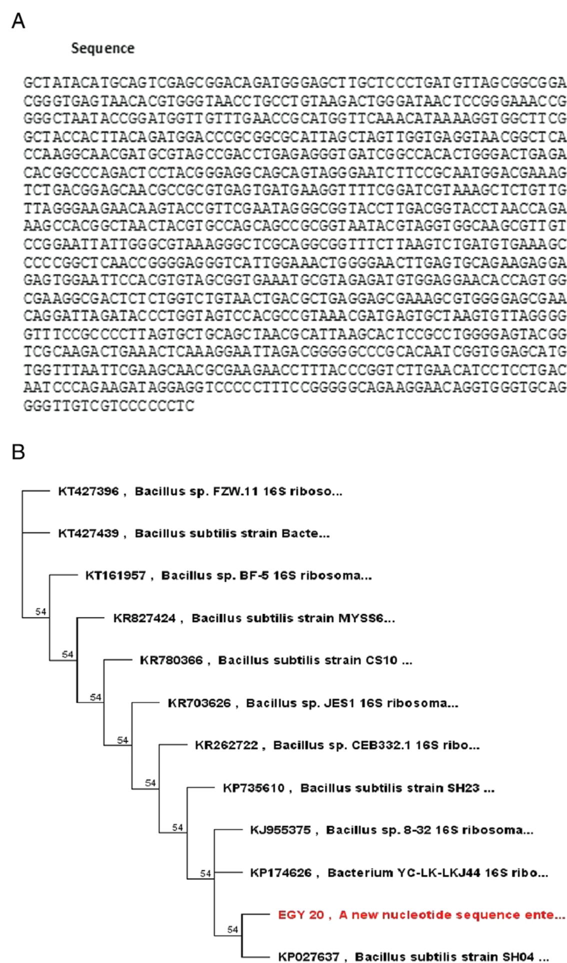
Effect of substrate concentration

MCE activity was assayed at different substrate concentrations (1–5 mg/ml) at optimum assay conditions. The gradual increase in the substrate concentration caused an increase in the enzyme activity. Maximal enzyme activity was attained at substrate concentration of 4 mg/ml. Further increase in substrate concentration did not cause any increase in the enzyme activity. This may be due to saturation of the enzyme active site by the substrate. This result is in agreement with those found by other investigators [34,41]. From the Lineweaver–Burk plot (Fig. 9) the K_m and V_{max} values of MCE were calculated as 4.6 mg/ml and 2933 U/mg protein, respectively.

Identification and phylogenetic analysis of sponge-associated strain

A specific band of 1256 bp was observed when resolved on agarose gel. The PCR products were purified with a GeneJET Genomic DNA Purification Kit (Thermo Scientific Company, USA) according to the instruction manual. Partial gene sequences were obtained using the forward primer. The nucleotide sequences were compared with those in the NCBI Gene Bank database (http://www.ncbi.nlm.nkh.gov/) with the basic local alignment search tool (BLAST) algorithm to identify known closely related sequences (Fig. 10a). The sequence was analyzed with FinchTV software, and thereby a phylogenetic tree was generated by the neighbor-joining algorithm implemented in Geneious R8 software (Fig. 10b). The results revealed that the aligned sequence had ~98% identity to *Bacillus subtilis* strain. The corresponding sequence was submitted in the NCBI Gene Bank under accession number KU710517. Many authors used the genus Bacillus for MCE production [42–44]. Other authors also used the strain *B. subtilis* for production of MCE [12,28,45–48].

Figure 10



(a) Nucleotide sequence. (b) Phylogenetic tree for marine sponge bacillus strain.

Conclusion

From the present study, it can be concluded that MCE produced by the marine sponge isolate *B. subtilis* KU710517 can be considered an alternative to calf rennet in the cheese industry as it has a high MCA/caseinase ratio, which is an important factor in milk clotting. Medium optimization resulted in a high increase in MCE productivity, and MCA/caseinase ratio was increased from 3422 to 4407 after optimization. The partially purified enzyme has high specific activity (512.8 U/mg protein) and high thermal stability at 45 and 50°C, which makes it a promising enzyme in industrial applications. Additional studies must be undertaken in the future to evaluate the enzyme's safety and benefits in the food industry.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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