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Novel milk-clotting enzyme from *Bacillus stearothermophilus* as a coagulant in UF-white soft cheese

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ABSTRACT

The Crude enzymatic extract produced by *Bacillus stearothermophilus* exhibited high milk-clotting activity (MCA). Optimized medium and conditions increased the enzyme production to 189.68%. Moreover, dissolving skim milk in acetate buffer and in acetate buffer containing $CaCl_2$ increased the activity to 353.01% and 394.01%, respectively. MCA was increased to 462.76% when assayed at higher temperature (60 °C). Mn^{+2} and Ca^{+2} ions stimulated MCA to 642.11% and 134.92%, respectively. In the presence of 0.1 M NaCl the activity was increased to 128.34%, however, at 0.8 M the activity was decreased to 38.40%. MCE showed excellent thermal and pH stability by retaining all of its activity after incubation at 40 °C for 60 min and at pH 4.0 for 90 min. Furthermore, milk-clotting enzyme (MCE) retained its complete activity when stored at −18 °C and 4 °C for 40 and 30 day, respectively. UF-white soft cheese chemical characteristics were similar of both *B. stearothermophilus* MCE and other commercial coagulants. Moreover, cohesiveness, springiness, gumminess and chewiness were similar of cheese made with all tested coagulants. The flavor of *B. stearothermophilus* cheese was similar with other commercial coagulants during all storage period. Also, the total score of organoleptic properties of cheeses made by *B. stearothermophilus* coagulant were lower than other commercial coagulants during all storage period. Thus this novel coagulant (26 ml/l milk) could be used to produce acceptable UF-white soft cheese.

1. Introduction

Rennet (MCE) is a term applied to any crude enzyme preparation of animals, plants or microbial origin which curdles milk and plays an important role in cheese maturation (El-Tanboly et al., 2013). Calf rennet, the conventional milk-clotting enzyme obtained from the fourth stomach of suckling calves, which consists of chymosin (EC 3.4.23.4), as the major component, and pepsin (EC 3.4.23.1), when rennet is extracted from adult animals this proportion is inverted, and there is predominance of pepsin (Sousa et al., 2001). Chymosin as an aspartic protease is used for milk clotting in cheese making by cleavage the bond at Phe_{105}–Met_{106} of bovine κ-casein in milk and causes destabilization of the casein micelles, resulting in milk coagulation to curdle (solids) and whey (liquid). The employed rennet in cheese production is derived from the abomasums of un weaned calves which varies seasonally and is becoming progressively more scantly as: 1) increasing cheese consumption and production, 2) decreasing slaughter of calves, 3) decreasing exports of calf rennets, 4) increasingly high prices of calf rennet, 5) religious factors, 6) diet (Dutt et al., 2008; Shieh et al., 2009; Shah et al., 2014). So, the need for a suitable rennet substitute is rapidly becoming acute from various sources. However, attention has been focused on MCE from microorganisms (microlant) considering its stability, availability, rapid growth, cheaper cost, greater biochemical diversity, easier genetic modification and offer a variety of properties permitting selection of those most suitable in cheese production (Patil et al., 2012; Wu et al., 2013; El-Tanboly et al., 2013). Besides, microlant has a better acceptance by people whose eating habits (vegetarian approved) and religious beliefs (kosher and Halal certification) against the use of animal coagulants which are expensive or derived from pigs (Merheb-Dini et al., 2010; Lemes et al., 2016). Recently, some non-pathogenic strains of *Bacillus species* have been successfully produced MCE (Ahmed and Helmy, 2012; Wu et al., 2013; Hang et al., 2016). On the other side, most plant rennet proved un suitable for cheese making lowers cheese yield and produce bitter flavors in the cheese and texture defects owing to their excess in proteolytic character (Shah et al., 2014; Imdakim et al., 2015). Therefore, this study reports the production of novel microbial coagulant and investigates its biochemical characteristics. Also, chemical,

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rheological and organoleptic properties of UF-white soft cheese made with *Bacillus stearothermophilus* MCE are compared to other commercial coagulants.

2. Materials and methods

2.1. Microorganism

B. sterothermophilus and *B. licheniformis* which were obtained from the Culture Collection of National Research Center, Egypt. *B. licheniformis* ATCC 21415 was obtained from American Type Culture Collection, USA and *Kluyveromyces marxianus* NRRL Y-7571 was obtained from Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA. The cultures were maintained on nutrient agar or potato dextrose agar media at 30 °C for 48 h and stored at 4 °C.

2.2. Materials

Milk retentate and permeate were obtained from Dairy industry unit, Animals Production Research Institute, Ministry of Agriculture, Giza, Egypt. Microbial rennet powder (RENIPLUS) from *Mucor miehei* was purchased from Gaglio Star, Spain. Liquid microbial rennet extra (CHY-MAX) was purchased from CHR-Hansen, Denmark. Liquid animal commercial rennet was purchased from local market, Egypt. Cheese starter culture used in the cheese manufacture is a mixture of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* were obtained from Egyptian Microbial Culture Collection (MIRCEN), Ain Shams University, Egypt.

2.3. Inoculum preparation

Inoculum medium (25 ml in 100 ml-Erlenmyer flask) contained (g/l): $CaCl_2$, 0.01; $FeSO_4$, 0.06; $MgSO_4 0.7H_2O$, 0.2; $(NH_4)_2HPO_4$, 2.0; KH_2PO_4, 2.1 and 20.0 g wheat bran. Inoculum was prepared by inoculating the inoculum medium with a loopful of bacterial culture (24 h at 30 °C) and incubated overnight at 30 °C and 150 rpm.

2.4. Liquid state fermentation

In the preliminary experiments, three media (A, B and C) were screened for MCE production. Medium A was composed of (g/l): $CaCl_2$, 0.01; $FeSO_4$, 0.06; $MgSO_4 0.7H_2O$, 0.2; $(NH_4)_2HPO_4$, 2.0; and KH_2PO_4, 2.1. Erlenmeyer flask (250 ml) contains 50 ml of the production media and 2.0 g wheat bran. Medium B contained the same composition of medium A and each flask contains 2.0 g wheat bran and 0.5 g skim milk. Medium C was composed of (g/l): lactose, 1.0; yeast extract, 1.0; KH_2PO_4, 1.0; $MgSO_4 0.7H_2O$, 0.25. The media were adjusted to pH 7.0 and sterilized in autoclave at 121 °C for 15 min. Each flask was inoculated with 2.5 ml of bacterial inoculum, followed by incubation at 35 °C on an orbital shaker at 200 rpm for 48 h. At the end of incubation period, the culture medium was centrifuged at 6000 × *g*, 4 °C for 20 min and the supernatant was used to assay for MCA.

2.5. The milk-clotting activity (MCA)

The milk-clotting activity (MCA) of the enzyme preparation was measured by the method described by (El-Tanboly et al., 2013). The crude enzyme preparation (0.5 ml) was added to the test tube containing 5.0 ml of pre-incubated substrate (skim milk 12% in 0.01 M $CaCl_2$) at 35 °C for 5 min. The time at which the first particles were formed was recorded.

and the MCA was calculated as:

$$SU = 2400 \times 5 \times D/T \times 0.5$$

where T is milk-clotting time (s) and D is dilution of the enzyme. One Soxhlet unit (SU) of MCA was defined as the amount of enzyme required to clot 1 ml of substrate within 40 min at 35 °C.

2.6. Different wheat bran concentration

The optimal wheat bran (wb) concentrations for express MCE were determined by adding varied wb from 1.0 to 5.0 g/flask. The maximum activity obtained was taken to be 100%.

2.7. Effect of supplementation with some natural additives

The effect of some natural additives (soy bean, leucena, lupine, fenugreek, radish seeds, watercress seeds, mustard seeds and turmeric) on the production of MCE was tested at concentrations of 1.0 g/flask.

2.8. Effect of rotation (rpm)

This experiment was undertaken to investigate the effect of rotation speed on the production of MCE. In this respect, different rpm were examined (0, 50, 100, 150 and 200).

2.9. Enzyme characteristics

2.9.1. Effect of solvent type on milk-clotting activity

MCA was determined using skim milk dissolved in different solvent 0.01 M and pH 4.5 ($CaCl_2$, acetate buffer, phosphate buffer, $CaCl_2$ in acetate buffer and $CaCl_2$ in phosphate buffer).

2.9.2. Effects of pH and temperature on milk-clotting activity

The optimum pH for the activity of MCE was determined by assaying the MCA in the pH range 4.0–7.0. The optimum temperature for the activity of MCE was determined by assaying the MCA at intervals of 10 °C from 30 to 70 °C (Ding et al., 2011).

2.9.3. Effects of enzyme substrate ratio on milk-clotting activity

To study the effect of enzyme/substrate (E/S ratio) on MCA, assay was carried out with different ratios of E/S (1.0:1.0, 1.0:2.0, 1.0:3.0, 1.0:4.0 and 1.0:5.0) in the reaction mixture.

2.9.4. Effect of some metal ions on milk-clotting activity

Different metal ions (0.2 M) were tested for their ability to activate or inhibit the activity of MCE. The milk-clotting enzyme (1.0 ml) was incubated at 30 °C for 30 min with 0.1 ml metal ions ($CaCl_2$, $MgSO_4$, $MnSO_4$, $CuSO_4$ and $FeSO_4$). The milk-clotting activity obtained without metal ions was taken to be 100%.

2.9.5. Effect of NaCl concentration on milk-clotting activity

To confirm the effect of NaCl concentration on MCA, assay was carried out after pre-incubating enzyme solution with equal volume of different NaCl concentrations (from 0.0% to 4.0%) at 30 °C for 60 min. Time taken for appearance of the first clot was noted down and compared with control (El-Tanboly et al., 2013).

2.9.6. Stability studies of MCE

2.9.6.1. Thermal stability. The thermal stability was determined by pre-incubating the enzyme in the temperature 40 and 50 °C. The incubation time of samples varied from 0 to 60 min. After incubation, the residual MCA was determined and the activity obtained at zero time was taken to be 100% (Merheb-Dini et al., 2010).