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COMPARATIVE EVALUATION OF *Bacillus licheniformis* 5A5 AND *Aloe variegata* MILK-CLOTTING ENZYMES

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Abstract - The properties of a milk clotting enzyme (MCE) produced by bacteria *(Bacillus licheniformis* 5A5) were investigated and compared to those of rennet extracted from a plant (*Aloe variegata)*. Production of MCE by *B. licheniformis* 5A5 was better in static than in shaken cultures. Maximum activity (98.3 and 160.3 U/ml) of clotting was obtained at 75°C and 80°C with bacterial and plant rennet, respectively. In the absence of substrate, the clotting activity of Aloe MCE was found to be less sensitive to heat inactivation up to 80°C for 75 min, retaining 63.8% of its activity, while bacterial MCE was completely inhibited. $CaCl_2$ stimulated milk clotting activity (MCA) up to 2% and 1.5% for bacterial and plant enzymes. NaCl inhibited MCA for both enzymes, even at low concentration (1%). Plant MCE was more sensitive to NaCl at 3% concentration it retained 30.2% of its activity, whereas bacterial MCE retained 64.1%. Increasing skim milk concentration caused a significant increase in MCA up to 6% for both enzymes. Mn^{2+} stimulated the activity of bacterial and plant enzymes to 158.6 and 177.9%, respectively. EDTA and PMSF increased the activity of plant MCE by 34.4 and 41.1%, respectively, which is higher than those for the bacterial MCE (19.1 and 20.9%). Some natural materials activated MCE, the highest activation of bacterial MCE (128.1%) was obtained in the presence of Fenugreek (with acid extraction). However Lupine Giza 1 (with neutral extraction) gave the highest activation of plant MCE (137.9%). All extracts from Neem plant increased MCA at range from 105.6% to 136.4%. Plant MCE exhibited much better stability when stored at room temperature (25-30°C) for 30 days, retaining 51.2% of its activity. Bacterial MCE was highly stabile when stored under freezing (-18°C), retaining 100% of its activity after 30 days. Moreover, bacterial MCE was highly tolerant to repeated freezing and thawing without loss of activity for 8 months.
Keywords: Bacterial rennet; Plant rennet; Stability; MCE.

INTRODUCTION

Milk coagulation is the basic step in cheese manufacturing. Milk-clotting enzymes (MCE) are the primary active agents in cheese making. Chymosin (EC 3.4.23.4) is an aspartic protease that specifically hydrolyzes the peptide bond Phe_{105}-Met_{106} of *κ*-casein and is considered to be the most efficient protease for cheese industry (Ageitos *et al*., 2006). Milk-clotting process consists of three main phases: 1) enzymatic degradation of *κ*-casein; 2) micellar flocculation and 3) gel formation, the limiting step in milk-clotting being the degradation rate of *κ*-casein (Ageitos *et al*., 2006).

κ-casein → *ρ*-*κ*-casein (insoluble)↓ + macropeptide (soluble) Aggregation

Rennet is a term applied to any crude enzyme preparation of animal, plant or microbial origin

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which curdles milk (Sardinas, 1968). MCE produced from the abomasums of unweaned calves has traditionally been used since ancient times as a coagulant in most cheese manufacturing. However, the increasing consumption of cheese and the decreasing number of slaughtered calves have led to an increased price of calf rennet and a search for alternative milk coagulants. Those facts have been accompanied by the recent Bovine Spongiform Encephalomyelitis (BSE) disease in dairy cattle (Vioque *et al*., 2000). This situation encourages the search for other sources of rennet substitutes from microbial or plant sources to meet the needs of the expanding cheese industry. Coagulation of milk can be achieved by a number of proteolytic enzymes from various sources, such as animals (e.g., pig, bovine, and chicken pepsins), microorganisms (*Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica*), and plants (e.g., pineapple, papaya, and sodom apple (Llorente *et al*., 2004; Yu and Chou, 2005; Low *et al*., 2006 and El- Bendary *et al*., 2007). The present investigation aims at comparing the properties of microbial milk-clotting enzyme with those of plant origin for selecting the appropriate one as a substitute of calf rennet.

MATERIALS AND METHODS

Microorganisms

Bacillus licheniformis 5A5, *B. licheniformis* 9A1 were obtained from the Department of Biochemistry, Ohio State University Columbus, OH, USA. *B. licheniformis* ATCC21415 was obtained from the American Type Culture Collection, USA. *Xanthomonas campestris* NRRL B-1459, *Kluyveromyces marxianus* NRRL Y-7571 and *Aspergillus amori* NRRL 3112 were provided by Northern Regional Research Laboratory, Peoria, Illinois, USA. *B. megaterium*, *B. macerans*, *B. coagulans*, *B. sterothermophilus*, *Streptococcus sp.*, *Streptococcus thermophilus*, *Echerichia coli*, *A. niger* were obtained from the culture collection of the National Research Centre, Egypt.

Media

The inoculum and production medium was defined as follows (g/L): Lactose 15.0; Yeast extract 1.0; Peptone 1.0; KH_2PO_4 1.0 and $MgSO_4.7H_2O$ 0.25. The pH was adjusted to 6.0 prior to sterilization. A transfer was made from stock culture to nutrient agar slants, which were then incubated at 30°C for 24 h. An inoculum culture was obtained by culturing the bacterial strain in 250-mL Erlenmeyer flasks each containing 25 mL of sterile medium at 37°C for 24 h using static culture technique in an incubator. The main cultivation was carried out by using 2.5 mL of the inoculum in the above medium incubated at 37°C for 48 h under static conditions. Bacterial cells were harvested by centrifugation in a refrigerated centrifuge at 5000 rev/min for 15 min at 4°C. The clear culture filtrates were assayed for enzyme activity.

Assay of Enzyme Activity

Milk-clotting activity was measured by the method of Berridge (1952). Unless otherwise specified, 2.5 mL of the enzyme sample were incubated with 10 mL of reconstituted skim milk (12 g dry milk/ 100 mL 0.01M $CaCl_2$) pH 6.5 at 40°C and the time necessary for the formation of curd fragments was measured. MCA is expressed in term of Soxhlet unit, which was 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 the curd fragment formation (sec). At least three measurements were made for each experiment and the data given are an average of these results.

Milk Powder

Spray dried skim milk powder (low-heat treated grade) made in USA was obtained from Ministry of Agriculture, Giza, Egypt.

Preparation of *Aloe variegata* Leaves Extract

Aloe variegata leaves, *Aloe vera* leaves, *Gasteria variegata* leaves, *Scindapsus aureus* leaves, *Phaseolus vulgaris* fruits, *Pisum sativum* peels. Enzyme solution was prepared according to Low *et al*., (2006), by washing 10 g of fresh plant leaves thoroughly with distilled water, then cutting them into small pieces and homogenization with 100 mL distilled water. Filtration is done through cheese cloth, and the filtrate was collected and used as the plant rennet solution.

Biochemical Properties of Milk-Clotting Enzyme

Temperature Profiles and Thermal Stability

Enzyme activity was determined at the indicated temperature (35- 85°C). The thermal stability of the

enzyme was ascertained by measuring the activity of the residual enzyme exposed at various temperatures (40- 80°C) for various times (15-120 min).

Effect of NaCl and $CaCl_2$ Concentrations

Effect of NaCl (1-12%) and $CaCl_2$ (0.5- 3.0%) concentrations on milk-clotting activity were tested. NaCl was added to reconstitute skim milk solution (12% in 0.01M $CaCl_2$) before renneting.

Effect of Substrate Concentrations

Various amounts of dry skim milk were dispersed in 0.01M CaCl2 to obtain different concentrations (1-14%), and the clotting time was recorded.

Effect of Enzyme Concentration

The experiment was performed at 40°C using 10 ml of 12% (w/v) skim milk and 2.5 ml of different enzyme concentrations made by dilution (0.5-2.5 ml).

Effect of Some Additives

Effect of some metal ions (Mn^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}) and some inhibitors, i.e., ethylene diamine tetra acetic acid (EDTA) and phenyl methyl sulfonyl fluoride (PMSF) on MCA was tested at a concentration of 0.1 M. The enzyme was incubated at 30°C for 1 h with metal ions and inhibitors separately. The residual activity was measured.

Effect of Some Natural Materials

Preparation of Neutral, Acidic and Alkaline Extract

According to Whistler and Saarina (1957) with some modifications, plant-parts (5 g) were extracted with 200 mL of extracting solvent (water, 0.5 N HCl, or 0.5 N NaOH) at 80°C for 3 h. After filtration, the extract was dialyzed against distilled water for 48 h, dried and weighed.

Preparation of Sulfated Extracts

Sulfation of the water-free extract was prepared according to Yang *et al*. (2005) with some modification. Water-free extract (0.1 g) was suspended in 0.5 mL dry formamide, and the mixture was stirred at room temperature for 24 h in order to disperse it into the solvent. A sulfating agent was prepared by 3 dropping of 1 mL of chlorosulfonic acid ($HClSO_3$) into 4 mL of formamide under cooling (in ice bath) and then added to the extract. The reaction was cooled in ice bath, neutralized with 30 % NaOH solution, and dialyzed against running water for 48 h and dried.

Plant natural extracts (0.01%) were incubated with enzyme solution at 30°C for 1 h and the residual activity was measured.

Storage Stability

Both bacterial and plant MCE were stored by freezing (-18°C), incubating at 4°C and at room temperature (25-30°C) for different periods and the residual activity was determined.

RESULTS AND DISCUSSION

The shortage of calf abomasums has led to a search for other coagulants such as aspartic acid proteinases of microbial and plant origins (Rogeli *et al*., 2001). Microbial rennet alternative to bovine chymosin are very desirable due to their lower cost, production via large scale fermentations, stability, availability and specificity (Repelius, 1998). On the other hand, plant enzymes are relatively safe, inexpensive, readily available and are generally acceptable for applications (Gandhi and Mukherjee, 2001). Microbiological and plant programs were instituted to search for an animal rennet substitute. Among 14 microorganism tested, *B.licheniformis* 5A5 yielded a suitable enzyme with highest productivity (21.9 U/ml) using static cultures after incubation at 37°C for 48 h. Variations in the abilities of these strains for the production of MCE were observed to be dependent on the strain as well as the incubation period (data not shown). Of several plants tested, the enzyme from *Aloe variegata* leaves was the most promising coagulant with highest enzyme production (43.7 U/ml) (data not shown). Yousif *et al*. (1996) used the MCE from berries (fruit and seeds) of *Solanum dobium* plant in cheese manufacture. Biochemical properties related to cheese making exhibited by the crude enzyme extracted from *B. licheniformis* 5A5 and *Aloe variegata* leaves were examined in comparison.

Effect of Incubation Temperature on MCE

Milk coagulation is strongly dependent on the temperature (Najera *et al*. 2003). It has been observed that the temperature of the milk affects

protein aggregation rate to a large extent and increased temperature increases the rate of gel firming (McMahon *et al*. 1984). The increase in temperature causes the protein matrix to shrink due to increased hydrophobic interaction, and also increases the rate of fermentation of lactose to lactic acid. The rate of enzyme action was markedly influenced by the variation in the temperature of the reaction (Figure 1). It is obvious from the data that the *B. licheniformis* 5A5 and *Aloe variegata* rennets were characterized by high thermal resistance. The maximum clotting activity for bacterial and plant MCE was at 75 and 85°C, respectively, which are higher than those reported for *Penicillium oxalicum* (60°C) by Hashem, (1999). El-Bendary *et al*. (2007) reported that MCE from *Bacillus sphaericus* was optimally active at 55°C. It is interesting to note that *Aloe variegata* rennet tends to be less heat-sensitive as compared to other plant rennets. El-Sayed (2002) suggested that the rennet extracted from fruits of *Phaseolus vulgaris* and *Pisum sativum* acted optimally at 45°C. On the other hand, in comparison, calf rennet showed lower thermal resistance, as reported by several investigators (Garnot and Molle, 1987; Wahba *et al*. 1995).

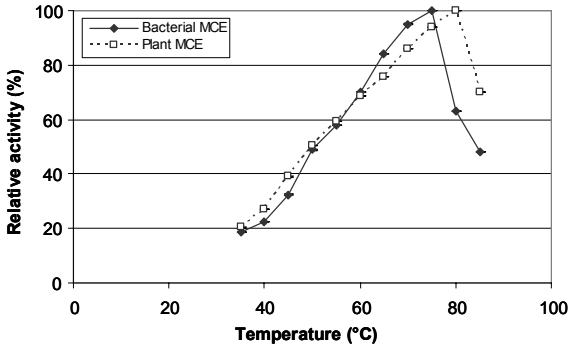


Figure 1: Effect of temperature on bacterial and plant MCE

Thermal Stability of MCE

Thermal stability of rennet enzyme was one of the most important criteria with respect to applications. In this study, MCE from *Aloe variegata* leaves showed a significant thermal stability compared to *B. licheniformis* 5A5 rennet (Figure 2). At 40°C for 1 h, the bacterial rennet retained 73.4% of its activity, whereas the plant rennet was still completely active. On the other hand, at 80°C for 75 min, all the activity of bacterial rennet was lost directly, whereas the plant rennet in the same conditions conserved 63.8%. These results are superior compared to other literature results. Sardinas (1968) reported that MCE from *Endothia parasitica* was destroyed completely by heating for 5 min at 60°C. Hashem (1999) noted that *Penicillium oxalicum* MCE still retained 58% of its original activity after heating at 40°C for 1 h, but 60°C caused a dramatic loss in activity. El-Sayed (2002) suggested that rennet enzymes from fresh fruits of *Phaseolus vulgaris* and *Pisum sativum* were considerably stable up to 40°C for 1h with only 33% loss of their activities. El-Bendary *et al*. (2007) reported that *B. sphaericus* MCE was quite stable at 40°C for more than 30 min, while it lost 35% of its activity after 10 min incubation at 60°C.

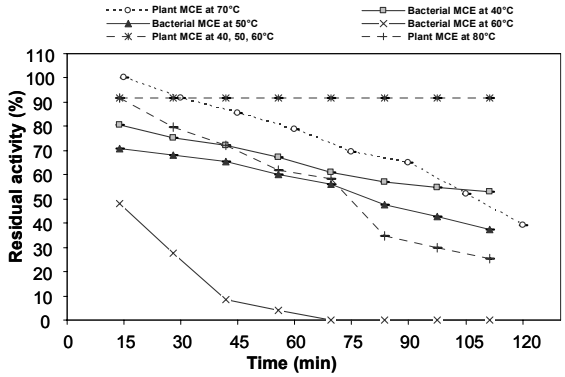


Figure 2: Thermal stability of bacterial and plant MCE

Effect of NaCl Concentration

Milk is sometimes salted with NaCl for protection against spoilage by various microorganisms. Sodium chloride is usually used during the process of cheese manufacture. The amount of salt required to reduce water activity to prevent microbial growth is 4-5%. The sensitivity of MCE from various sources to NaCl is not the same. Addition of NaCl to milk from 1-12% (Figure 3) resulted in a marked decrease in clotting activity with all rennets used. *B. licheniformis* 5A5 rennet appeared to be less sensitive to NaCl than *Aloe variegata*, particularly at high salt concentration. In the presence of NaCl (3%), bacterial rennet retained 64.1% of its activity; however, plant rennet retained 30.2%. These results agree with those reported by Shehata *et al*. (1996) and El-Sayed (2002). At higher concentrations of NaCl, no milk-clotting was observed with plant rennet, while at 12% NaCl bacterial rennet was still active, with retained activity of 40.4%. From these results, bacterial rennet is suitable for manufactured Domiati cheese.

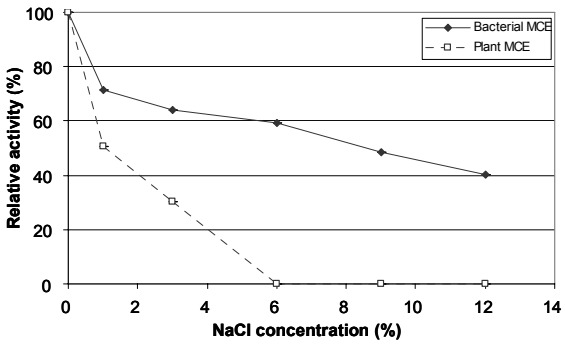


Figure 3: Effect of NaCl concentration on bacterial and plant MCE

Effect of $CaCl_2$ Concentration

Calcium chloride accelerated MCA at all the concentrations tested (Figure 4). It is known that Ca^{2+} combines with ρ-casein to form firm clot during the second phase of the clotting process. Addition of $CaCl_2$ to milk coagulation by rennet causes a reduction in rennet coagulation time and increases the rate of coagulation (Mohamed *et al*. 1988; Balcones *et al*. 1996; El-Bendary *et al*. 2007). Increasing $CaCl_2$ concentration resulted in an enhancement of the clotting activity of all coagulants. Ca^{2+} was found to be potent activator with 149.1% and 114.1% increase in bacterial and plant MCE, respectively, compared to the control at optimum $CaCl_2$ concentration.

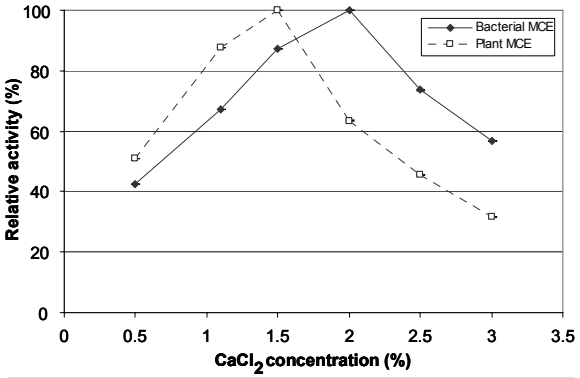


Figure 4: Effect of $CaCl_2$ concentration on bacterial and plant MCE

Effect of Substrate Concentration

The effect of skim milk concentration on the activity of bacterial and plant rennin was studied (Figure 5). Dilution of milk caused an increase in MCA, which was pronounced at low concentration of skim milk (6%), increasing the activity of bacterial and plant MCE to 138.7 and 136.9%, respectively. These results are in harmony with these found by Hashem (1999) for *Penicillium oxalicum* rennet. Wahba *et al*. (1995) reported that, over the range of 6% to 21% substrate concentration, the higher concentration increases the clotting time. In any case, such retardation of clotting of milk by the enzyme may be attributed to different factors. Thus, dilution of milk may result in scattering the casein particles and consequently the ability of the milk to clot becomes feeble. At the same time the insufficient amount of the substrate (casein particles) due to dilution actually restricts the enzyme to act at full capacity (Abdel-Fattah and Mabrouk, 1972). At higher concentrations of milk, the viscosity of solutions increases and the enzyme action decreases. This has been attributed by Dalgleish (1981) and Low *et al*., (2006) to a smaller amount of κ-casein that is hydrolysed as the coagulation commences.

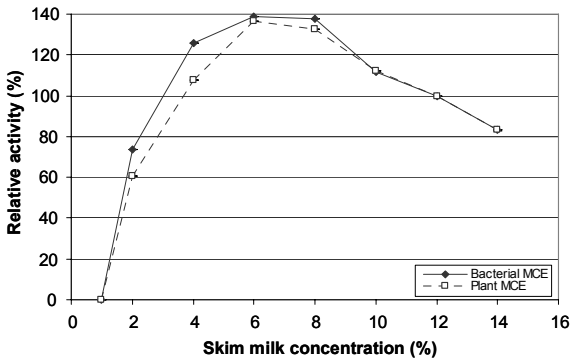


Figure 5: Effect of skim milk concentration on bacterial and plant MCE

Effect of Enzyme Concentration

The rennet coagulation of milk combines an initial enzymic hydrolysis reaction and a subsequent enzyme-independent protein aggregation reaction (Van Hooydonk & Watstra, 1987). From the results (Figure 6), it can be deduced that there was a parallel relationship existed between the enzyme concentration and MCA. There was a change in the coagulation parameters with the concentration of enzyme (Najera *et al*. 2003). The time of clotting decreased as the concentration of enzyme increased; these results agree with those reported by other authors (Hashem, 1999 and Najera *et al*. 2003). López *et al*. (1998) and Najera *et al*. (2003), attributed the decreases in clotting time when the enzyme concentration is increased to a higher level of proteolysis of κ -casein. On the other hand, at lower concentrations of enzyme, the activity decreases due to the insufficiency of enzyme to act on milk and form clot.

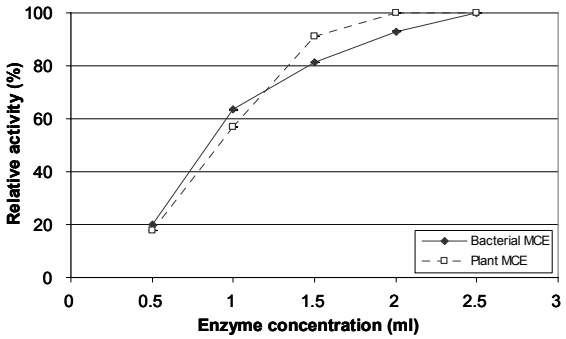
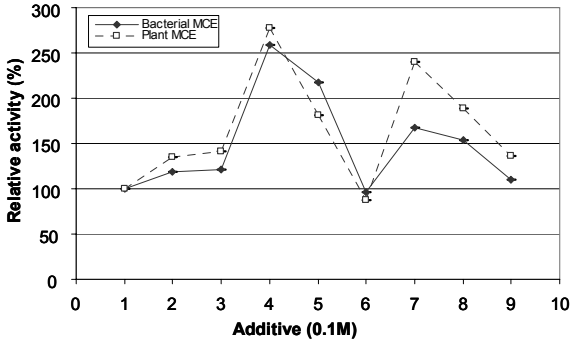


Figure 6: Effect of enzyme concentration on bacterial and plant MCE

Effect of Some Additive on the MCA

The effect of several metal ions, activators and inhibitors on MCA was studied (Figure 7). *B. licheniformis* 5A5 and *Aloe variegata* leaves MCE were activated by most of the metal ions tested. As shown by the results, Zn^{2+} ion activated bacterial and plant MCE by 67.7 and 139.7%. It is interesting to note that Mn^{2+} gave the highest activation for both bacterial and plant MCE (258.6 and 277.9%), respectively, for bacterial and plant MCE. Also, bacterial and plant MCE were activated by 19.2 and 34.4% in the presence of EDTA. Beside that, adding PMSF to bacterial and plant MCE increased the activity to 120.9 and 141.1%. Our results are higher than those obtained by Ageitos *et al*. (2006), who reported that *Bubalus arnee bubalis* MCE retained 100.5% and 105.22% of its activity in the presence of PMSF and EDTA. On the contrary, Ageitos *et al*. (2006) found that *B. Lichneformis* MCE was completely inhibited by PMSF and inhibited by 6.4% in the presence of EDTA. Fe^{2+} enhanced the activity by 54.1 and 89.0%, respectively, for bacterial and plant rennet. El-Bendary *et al*. (2007) suggested that Mg^{2+}, Cu^{2+}, Mn^{2+} and Fe^{2+} ions have no effect on the enzyme activity.

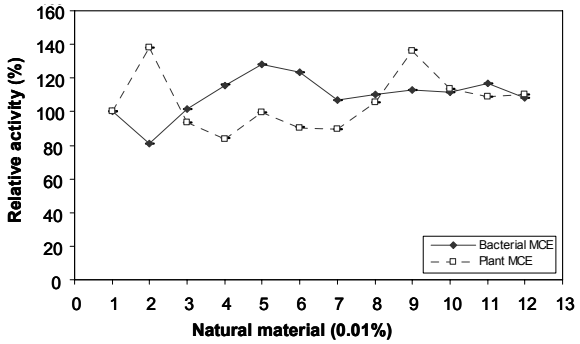


(1) None; (2) EDTA; (3) PMSE; (4) $MnCl_4.7H_2O$; (5) $CaCl_2$; (6) $MgSO_4.7H_2O$; (7) $ZnSO_4$; (8) $FeCl_3$, (9) $CuSO_4$

Figure 7: Effect of additives on bacterial and plant MCE

Effect of Some Natural Material on MCA

The results of rennet activation by extracts of Fenugreek (*Trigonella foenum*), Lupine (*Lupinus termis*) and Neem (*Azadirachtia indica*) were recorded (Figure 8). Many authors reported on the activation of rennet enzyme by metal ions, but no one has mentioned the activation using natural safe materials extracted from plants with low cost. As shown by the results, Fenugreek extracts (with different methods) activated bacterial rennet by 28.1, 23.1 and 6.6%, respectively, while plant rennet was not activated. All Neem extracts (from different parts, with different methods of extraction) activated both bacterial and plant MCE, which ranged from 105.6% to 136.4%. However, Lupine Giza 3 (neutral extract) increased the activity of bacterial MCE to 115.8%, but decreased the activity of plant rennet to 83.9%. On the other hand, Lupine Giza 1 (neutral extract) activated plant MCE by 37.9%, but inhibited bacterial MCE by 18.7%. The activation with natural materials is recommended for the enzyme to be used safety in industrial food applications. The activation of MCE in the presence of all extracts from Neem might be due to the calcium ions (4.5 mg/g) in the composition of aqueous Neem extract. Similar results were reported by Esawy *et al*. (2007) on the activation of *Geobacillus caldoxylosilyticus* IRDO alkaline protease in the presence of 0.05% extract of Henna, Ginger, Neem seeds, Curcuma and Cumin.

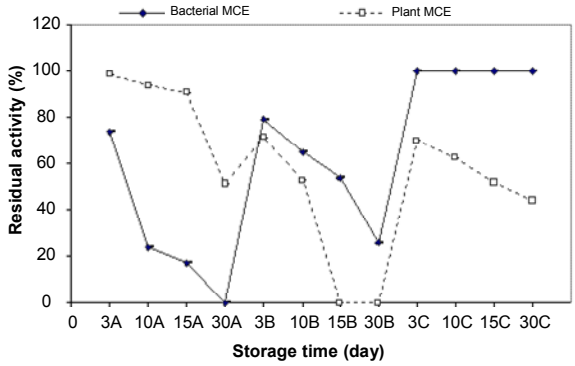


(1) None; (2) L.G1-2 (Lupine Giza 1, neutral extract); (3) L.G2-1(Lupine Giza 2,acid extract); (4) L.G3-2 (Lupine Giza 3, neutral extract); (5) F-1(Fenugreek, acid extract); (6) F-2(Fenugreek, neutral extract); (7) F-3(Fenugreek, alkaline extract); (8) SNL-3(Sulfated Leave Neem alkaline extract); (9) SNH-1(Sulfated Hull Neem acid extract); (10) SNH-2(Sulfated Hull Neem neutral extract); (11) SNH-3(Sulfated Hull Neem alkaline extract); (12) SNS-3(Sulfated Seeds Neem alkaline extract).

Figure 8: Effect of natural materials on bacterial and plant MCE

Storage Stability of MCE

After the enzyme preparations were stored at different temperatures, the enzyme activity was determined. As seen in Figure 9, about 83.1% loss of activity was observed for bacterial MCE after being kept 15 days at room temperature; in contrast, plant MCE still retained 90.8% of its activity under the same condition. On the other hand, bacterial MCE was highly tolerant to repeated freezing and thawing, with activity of 100% remaining for 8 months, while plant MCE retained only 44.0% of its activity after freezing for 30 days. Esawy and Combet-Blanc (2006) reported that *B. licheniformis* 5A5 MCE retained 100% of its activity after freezing for 6 months. At 4°C, bacterial and plant rennet retained 64.85 and 52.62% of their activities after 10 days.



Storage temperature: A (25-30°C), B (4°C), C (-18°C).

Figure 9: Storage stability of bacterial and plant MCE

CONCLUSIONS

In conclusion, it is important to remark that all the proteases used in this study produced milk clots. The coagulation properties of MCE from *Aloe variegata* and *B. licheniformis* 5A5 were compared to select the suitable one for chymosin substitution. Based on the data obtained, it was concluded that temperature, pH, $CaCl_2$, substrate concentration and enzyme concentration all affect the clotting process. Also MCE was actived by several substances extracted from natural sources (plants). Temperature sensitivity of bacterial and plant MCE appears to be one of its advantages in industrial applications. Aloe MCE exhibited much better stability when stored at room temperature compared with bacillus MCE, while bacillus MCE is highly tolerant to repeated freezing and thawing without loss in activity. Finally, *B. licheniformis* 5A5 and *Aloe variegata* rennet can be successfully used in cheese production.

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