

UTILIZATION OF SALT WHEY FROM EGYPTIAN RAS (CEPHALOTYRE) CHEESE IN MICROBIAL MILK CLOTTING ENZYMES PRODUCTION

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ABSTRACT

Background. Microbial milk-clotting enzymes are valued as calf rennet substitutes in the cheese industry. The worldwide increase of cheese production coupled with a reduced supply of calf rennet has prompted a search for calf rennet substitutes, including microbial and plant rennets. However, most plant rennets have proved unsuitable because they impart a bitter taste to the cheese. Microbial rennet appears to be more promising because its production is cheaper, biochemical diversity is greater, and genetic modification is easier. Most cheese manufacturing facilities in Egypt perform land spreading of salt whey. However, this practice increases the chloride levels of soil, and elevates the risk of crop damage. One possible application for salt whey is to use it as a whole medium for growth and production of milk clotting enzyme from fungi.

Material and methods. *Mucor pusillus* QM 436 was identified to produce the highest milk-clotting activity during screening of 19 fungal strains. Salted whey results from Ras (Cephalotyre) cheese manufacture as a whole medium for growth of *Mucor pusillus* QM 436 and production of the enzyme.

Results. The milk-clotting enzyme from *Mucor pusillus* QM 436 was purified to 7.14-fold with 54.4% recovery by precipitation in ammonium sulfate, ethanol and fractionated by gel filtration on Sephadex G-100. The enzyme was active in the pH range 5.5-7.5 and was inactivated completely by heating 5 min at 70°C and 30 min at 65°C. The highest level of enzyme activity was obtained at 60°C, pH 5.5. A positive and proportional relationship occurred in the presence of CaCl₂ in milk, with inhibition which occurred in the presence of NaCl.

Conclusions. The high level of milk-clotting activity coupled with a low level of thermal stability suggested that the milk-clotting enzyme from *Mucor pusillus* QM 436 should be considered as a potential substitute for calf rennet.

Key words: milk-clotting enzymes, *Mucor pusillus* QM 436, Ras cheese, Salted whey, purification

INTRODUCTION

Calf rennet as a traditional milk-clotting enzyme is very important in the production of cheese. The enzyme clots milk and plays an important role in the process of cheese maturation [Kumar et al. 2005]. Calf

rennet has traditionally been used by the dairy industry for the manufacture of cheese with good flavour and texture. The worldwide increase of cheese production coupled with a reduced supply of calf rennet has

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prompted a search for calf rennet substitutes, including microbial and plant rennets [Cavalcanti et al. 2004]. However, most plant rennets have proved unsuitable because they impart a bitter taste to the cheese. Microbial rennet appears to be more promising because its production is cheaper, biochemical diversity is greater, and genetic modification is easier. Many species of microorganism are known to produce a milk-clotting enzyme that can potentially substitute for calf rennet, and *Rhizomucor pusillus*, *Rhizomucor miehei*, *Endothia parasitica* and *Irpex lactis* are used extensively as sources of milk-clotting enzymes in the manufacture of cheese O'Leary and Fox [1974] and Kurutahalli et al. [2010]. It is well accepted that discharging of whey from dairy industry as waste creates severe pollution due to its high biological oxygen demand (BOD) (35-40 g/l) Zayed and Winter [1995]. Annually, 110 million metric tons of whey is produced worldwide through cheese manufacture [Briczinski and Roberts 2002]. About 50 percent of Egypt's total milk production is utilized in commercial cheese. Cheese production in Egypt totaled 480,000 metric tons in 2001 and accounted for 2.9 percent of the world total cheese. In the 1990s, whey production in Egypt increased steadily by about six percent annually and the amount produced in 2000 was 1,452,500 41 metric tons Zhang et al. [2003]. Salt whey is produced from Domiati, Ras (Cephalotyre) and Cheddar cheese making. The salt level in whey from Domiati cheese is about 8-15%, whereas it is only about 2-5% in whey from Ras and Cheddar cheese making. Salt whey, unlike sweet whey, cannot be conveniently processed because of its high salinity level Sanderson et al. [1996]. Moreover, it has a high biological and chemical oxygen demand, which makes its disposal a problem Zayed and Winter [1995]. Most cheese manufacturing facilities in Egypt perform land spreading of salt whey. However, this practice increases the chloride levels of soil, and elevates the risk of crop damage. One possible application for salt whey is to use it as a whole medium for growth of milk clotting enzyme from fungi. The purpose of the present work has been carried out on 19 cultures of fungi with the object to select a specific strain capable to produce a milk clotting enzyme of suitable nature. Furthermore, the possibility of using salted whey resulting from Ras cheese manufacture as a whole medium for growth was investigated.

However, this by-product due to this high salt content, is not recycled in any significant industrial process so far in Egypt.

MATERIAL AND METHODS

Microorganisms, stock cultures

A total of Nineteen fungal strains were obtained from the stock culture collection laboratory of microbial chemistry, National Research Center, Cairo, Egypt. These organisms originally were obtained from various sources as shown in Table 1. All the strains

Table 1. Fungi cultures assayed for milk clotting activity

No.	Fungi cultures	Code number and source
1	<i>Aspergillus oryzae</i>	DSM 633
2	<i>Aspergillus niger</i>	DSM 823
3	<i>Aspergillus awamori</i>	DSM 734
4	<i>Aspergillus flavus</i>	DSM 818
5	<i>Aspergillus terreus</i>	NRRL 555
6	<i>Aspergillus ochraceus</i>	NRC 67
7	<i>Aspergillus vesicolor</i>	NCL
8	<i>Aspergillus tamarii</i>	NCL
9	<i>Aspergillus fumigates</i>	NCL
10	<i>Mucor pusillus</i>	QM 346
11	<i>Mucor hiemalis</i>	DSM 63297
12	<i>Rhizopus oryzae</i>	DSM 853
13	<i>Rhizopus chinensis</i>	DSM6 1834
14	<i>Rhizopus stolonifer</i>	DSM 6855
15	<i>Rhizopus nigricans</i>	DSM 907
16	<i>Penicillium citrinum</i>	DSM 62851
17	<i>Penicillium lilacinm</i>	MCL
18	<i>Penicillium implicatum</i>	MCL
19	<i>Penicillium verrucosum</i>	MCL

DSM – German Collection of Microorganisms Gottigen, West Germany.

NRRL – Culture Collection of Northern Regional Research Laboratory, Peoria, Illinois, USA.

NRC – Culture Collection of National Research Center, Egypt.

QM – University of Queensland, Brisbane, Australia.

MCL – Microbial Chemistry Laboratory, National Research Center, Egypt.

were grown for growth and enzyme production, suspensions from active slants of the organism, grown on Czapek-Dox agar medium, were used for incubation the 300 ml, conical flasks, containing 50 ml, of sterile medium of 5% salted whey. The flasks were incubated on rotary shaker at $30 \pm 4^\circ\text{C}$ for 3 days. The cultures were then, harvested by centrifugation at 3000 rpm for 15 minutes in a cooling centrifuge (Janetzki, k26 type); the mycelia discarded and the resulting supernatant was used as a source of enzyme.

Medium used for milk clotting enzyme production

Fresh sweet whey was obtained from Misr Milk and Food Co., produced from Ras cheese. The composition of the sweet whey presented in Table 2.

Table 2. General composition of sweet whey commonly used as a medium for growth of fungi

No.	Compound	Percent
1	water	92
2	lactose	5.2
3	lactic acid	0.05
4	fat	0.5
5	protein	1.1
6	ash	0.5
7	total solids	8.05
8	pH	6.4

Milk powder

Spray dried skim milk powder (high-high treaded grade) made in the USA was obtained from Ministry of Agriculture, Giza.

Casein

Casein soluble in alkali was obtained from Adwic.

The milk clotting activity (MCA)

The milk clotting activity (MCA) of the enzyme preparation was measured by the method described by Otani et al. [1991]. A 5 mL portion of the substrate

(12% skim milk in 10 mM CaCl_2 was incubated for 5 min at 35°C and then 0.5 mL of enzyme extract was added. The length of time starting from the addition of the enzyme extract to the formation of the first particles was recorded, and the milk-clotting activity was calculated by Kawai and Mukai [1970] as: $\text{SU} = 2400 \times 5 \times D/T \times 0.5$ (1), where T is milk-clotting time (s), and D is dilution of the enzyme. One Soxhlet unit (SU) of milk-clotting activity was defined as the amount of enzyme required to clot 1 mL of substrate within 40 min at 35°C .

The proteolytic activity (PA)

The proteolytic activity (PA) of the purified enzyme was determined with light soluble casein as substrate, following essentially the technique by Otani et al. [1991]. To 2.5 mL of 1% casein solution prepared in 20 mM potassium phosphate buffer (pH 6.3), suitably diluted enzyme solution was added. The mixture was incubated at 35°C for 10 min, before adding 2.5 mL of 0.44 M trichloroacetic acid solution. The mixture was filtered using Whatman no. 1 filter paper. To 1 mL of this filtrate, 2.5 mL of 0.55 M sodium carbonate and 1 mL of three times diluted Folin reagent is added. This solution was kept at 30°C for 30 min and absorbance values are recorded at 660 nm. One unit is defined as unit increase in absorbance at 660 nm. Protein concentration was determined by Ohnishi and Barr [1978] using bovine serum albumin as standard.

Enzyme purification

All enzyme purification steps were done at 4°C . The crude enzyme was harvested by centrifugation at 8,000 g for 10 min and the supernatant was used for the study. The crude enzyme extract was precipitated with ammonium sulfate (40-80% saturation). The precipitate obtained after centrifugation was suspended in 50 mM sodium phosphate buffer (pH 6.0) and dialysed overnight against several changes of distilled water to remove the salt. Five milliliters of the partially purified enzyme solution were subjected to Gel Sephadex G-100 column (30×2.6 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was eluted at a flow rate of 0.3 mL/min and 5 mL fractions were collected. The protein content of each fraction was determined by measuring the absorbance

at 280 nm. The fractions with enzyme activity were pooled and dialysed overnight against distilled water and then used in all subsequent experiments.

Enzyme characteristics

Effects of temperature and pH on enzyme activity and stability. The optimum temperature for the activity of the purified enzyme was determined by assaying the milk-clotting activity at intervals of 5°C from 30-85°C. The optimum pH for the activity of the purified enzyme was determined by assaying the milk-clotting activity in the pH range 5.5-8.5, by adjusting the pH of the substrate (skim milk) with 0.1 M HCl or 0.1 M NaOH as appropriate. The maximum milk-clotting activity obtained was taken to be 100%. To determine the thermal stability, the purified enzyme was incubated at 5°C intervals from 40-70°C, and the length of the incubation was varied from 0 to 40 min. After incubation, the residual milk-clotting activity was determined and the activity obtained with an incubation time of 0 min was taken to be 100%. To determine the pH stability, the enzyme was dispersed (1:1, v/v) in the 0.1 M buffer solutions at different pH levels (pH 2-9). The MCE produced by *Mucor pusillus* QM 436, held at 30°C for 30 min and the residual milk-clotting activity was determined and the maximum activity obtained was taken to be 100%.

Effect of substrate concentration. The effect of substrate concentration on the milk-clotting activity of the purified enzyme was determined by increasing the concentration of the skim milk from 25 to 200 g/L. The maximum activity obtained was taken to be 100%.

Effect of NaCl concentration. In the manufacture of Domiati cheese, the most popular soft variety in Egypt, salt is added directly to milk before renneting in concentrations ranging from 9 to 15%. Accordingly, the effect of sodium chloride on the milk clotting activity of the enzyme from *M. pusillus* QM 436 was studied. Various concentrations of sodium chloride namely 0.0, 3.0, 5.0, 7.5, 10.0 and 15.0% were incorporated in the reconstituted skimmilk to be tested for enzyme clotting activity.

Effect of calcium on milk-clotting activity. To study the effect of calcium on clotting efficiency of the purified enzyme, assay was carried out with different concentrations of calcium (0.0, 200, 370, 550, 700, 1100 and 1600 p.p.m.) in the reaction mixture. Time taken

for appearance of the first clot was noted down and compared with control.

Effect of some cations on milk-clotting activity. The effect of metal ions Mn^{2+} , Zn^{2+} , Mg^{2+} , Cu^{2+} , Fe^{3+} and ammonium ions on the milk clotting activity was determined at metal ion concentrations of 1 mM. The milk-clotting enzyme was incubated at room temperature for 40 min with metal ions. The milk-clotting activity obtained without metal ions was taken to be 100%.

Michaelis-Menten constant. Solutions of casein at concentrations in the range 0.2-2% in 20 mM potassium phosphate buffer (pH 6.5) were used as the substrate and the proteolytic activity was determined as described Otani et al. [1991]. The Michaelis-Menten constant K_m was calculated from the double reciprocal Lineweaver-Burk plot [Lineweaver and Burk 1934].

RESULTS AND DISCUSSION

Screening for milk-clotting activity

A preliminary screening was done to detect the ability of the examined cultures to clot milk. Tables 3, 4 and 5 show the milk clotting enzymes produced

Table 3. Screening tests for the production of milk clotting enzymes in which media contain varying concentrations of sodium chloride after 3 days of incubation

Fungi cultures	Milk clotting activity (MCA)				
	concentration of NaCl, %				
	0.0	5	7.5	10	15
1	2	3	4	5	6
<i>A. oryzae</i>	8	0	0	0	0
<i>A. niger</i>	0	0	0	0	0
<i>A. awamori</i>	0	0	0	0	0
<i>A. flavus</i>	3.5	0	3.8	0	0
<i>A. terreus</i>	4.6	3.4	0	0	0
<i>A. ochraceus</i>	0	0	0	0	0
<i>A. vesicolor</i>	22.2	3.4	0	0	0
<i>A. tamarii</i>	40	21	36.3	5.6	2.2

Table 3 – cont.

1	2	3	4	5	6
<i>A. fumigates</i>	0	0	7.4	0	0
<i>M. pusillus</i>	40	80	100	133	200
<i>M. hiemalis</i>	0	0	0	0	0
<i>R. oryzae</i>	0	0	0	0	0
<i>R. chinensis</i>	22.2	12.5	8.8	6.1	12.5
<i>R. stolonifer</i>	0	0	0	0	0
<i>R. nigricans</i>	0	0	0	0	0
<i>P. citrinum</i>	0	0	0	0	0
<i>P. lilacinm</i>	44.4	0	0	0	0
<i>P. implicatum</i>	6.1	40	4.7	23.5	2.2
<i>P. verrucosum</i>	0	0	0	0	0

MCA – milk clotting activity, Soxhlet units/ml.

Table 4. Screening tests for the production of milk clotting enzymes in which media contain varying concentrations of sodium chloride after 6 days of incubation

Fungi cultures	Milk clotting activity (MCA)				
	concentration of NaCl, %				
	0.0	5	7.5	10	15
1	2	3	4	5	6
<i>A. oryzae</i>	14	5	0	0	0
<i>A. niger</i>	0	0	0	0	0
<i>A. awamori</i>	0	0	0	0	0
<i>A. flavus</i>	5	7	33	27	0
<i>A. terreus</i>	6	4	4	0	0
<i>A. ochraceus</i>	0	13	0	0	0
<i>A. vesicolor</i>	5	4	0	0	5
<i>A. tamarii</i>	5.7	33	50	9	11
<i>A. fumigates</i>	5	0	7	0	0
<i>M. pusillus</i>	19	28	40	57	133
<i>M. hiemalis</i>	0	0	0	0	0
<i>R. oryzae</i>	0	0	0	0	0
<i>R. chinensis</i>	33	16	6	9	10

Table 4 – cont.

1	2	3	4	5	6
<i>R. stolonifer</i>	7	0	5	0	4
<i>R. nigricans</i>	9	27	19	25	13
<i>P. citrinum</i>	7	7	7	22	3
<i>P. lilacinm</i>	36	7	17	9	0
<i>P. implicatum</i>	9	80	36	40	20
<i>P. verrucosum</i>	0	9	25	7	20

Table 5. Screening tests for the production of milk clotting enzymes in which media contain varying concentrations of sodium chloride after 9 days of incubation

Fungi cultures	Milk clotting activity (MCA)				
	concentration of NaCl, %				
	0.0	5	7.5	10	15
<i>A. oryzae</i>	20	21	4	0	0
<i>A. niger</i>	0	0	0	0	0
<i>A. awamori</i>	0	0	0	0	0
<i>A. flavus</i>	10	31	67	40	3
<i>A. terreus</i>	15	31	15	3	0
<i>A. ochraceus</i>	16	57	9	30	0
<i>A. vesicolor</i>	16	4	0	0	0
<i>A. tamarii</i>	67	133	200	22	40
<i>A. fumigates</i>	0	3	3	0	0
<i>M. pusillus</i>	13	13	19	21	40
<i>M. hiemalis</i>	0	0	0	0	0
<i>R. oryzae</i>	0	0	5	0	0
<i>R. chinensis</i>	25	27	7	9	15
<i>R. stolonifer</i>	13	3	4	0	0
<i>R. nigricans</i>	4	13	13	6	3
<i>P. citrinum</i>	3	6	0	12	0
<i>P. lilacinm</i>	57	13	16	10	0
<i>P. implicatum</i>	21	200	4	80	27
<i>P. verrucosum</i>	0	9	16	5	5

by a total of 19 fungi for different period namely 3, 6 and 9 days, respectively. Also the same tables show the effect of varied concentrations of NaCl on the milk clotting enzymes production by the same collection of fungi. The enzyme was produced by *Aspergillus oryzae* DSM 633, *Aspergillus flavus* DSM 818, *Aspergillus terreus* NRRL 555, *Aspergillus vesicolor* NCL, *Aspergillus tamari* NCL, *Mucor pusillus* QM 346, *Rhizopus chinensis* DSM6 1834, *Rhizopus stolonifer* DSM 6855, *Rhizopus nigricans* DSM 907, *Penicillium citrinum* DSM 62851, *Penicillium lilacinm* MCL, *Penicillium implicatum* MCL and *Penicillium verrucosum* MCL. The incubation period for 3 days at 30°C was found better for Milk clotting activities than 6 and 9 days, respectively. However, 15% NaCl concentration stimulates the highest production of Milk clotting activities by *Mucor pusillus* QM 346 culture. Similar observations were reported by Suganthi et al. [2012] on producing milk-clotting enzymes by culture of *Aspergillus niger*.

Purification and molecular mass

Partial purification of enzyme from culture supernatant Figure 1 shows flow sheet diagram of the purification steps of rennin such as enzyme from *M. pusillus* QM 436. It was ratio purified 1.68-fold and 88% recovery with respect to the crude culture supernatant by precipitation in 40-80% saturated ammonium sulfate. Passage through a Gel-Sephadex G-100 column eluted purified enzyme to 7.14-fold with 54.4% recovery (Table 6). The partially purified enzyme was separated

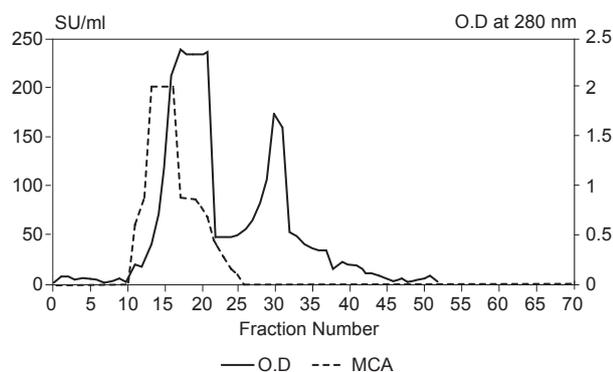


Fig. 1. Purification of milk clotting enzyme from *M. pusillus* QM 436 on Sephadex G-100

Table 6. Purification step of milk clotting enzyme from *M. pusillus* QM 436 using ammonium sulfate and gel filtration (Sephadex G-100)

Purification step	Crude enzyme	(NH ₄) ₂ SO ₄ (40-80%)	Sephadex G-100
Volume, ml	25	11	40
MCT, min	8	4	23
MCA, SU/ml	50	100	17
PC, mg/ml	6.3	7.5	0.3
SP.MCA	7.93	13.33	56.66
Total activity units	1250	1100	680
Recovery, %	100	88	54.4
Ratio purification	1.0	1.68	7.14

MCT – milk clotting time, min; SU – Soxhlet units; PC – protein content, mg/ml; SP.MCA – specific activity = MCA/PC.

into two peaks but with the only one having milk clotting activity. Earlier studies reported that the milk-clotting enzymes from different sources have different chromatographic activity results. However, this result was different from the milk-clotting enzyme from *Mucor bacilliformis* Areces et al. [1992], where the ion-exchange chromatography elution pattern showed two peaks of proteins but both fractions had milk-clotting activity. Similar results obtained from by Otani et al. [1991], Salem et al. [1998], Abbas et al. [1998], Cavalcanti et al. [2004], Shieh et al. [2009] and Xiaoling et al. [2011]. Needless to add, that the observed differences recorded in some cases between the present results and those of other investigator could be attributed to variations in both strains and growth the media.

Enzyme characteristics

Effects of temperature and pH on enzyme activity and stability. The milk-clotting activity increased with increased temperature in the temperature range 30-60°C and the optimum temperature for the purified enzyme was 60°C (Fig. 2). Different enzymes have different optimum temperatures, mainly depending on the enzymes' structure. This result was in accord with the milk-clotting enzyme from *Bacillus subtilis* (natto) [Chwen-Jen et al. 2009] and *Bacillus*

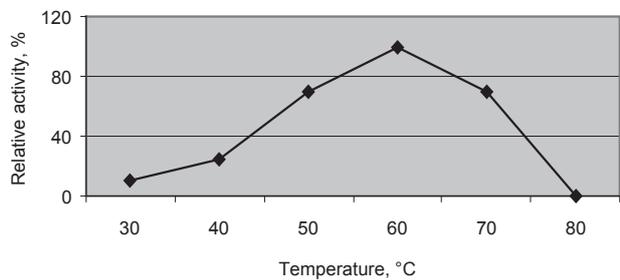


Fig. 2. Effect of temperature on activity of milk clotting purified *M. pusillus* QM 346 enzyme

amyloliquefaciens D4 [Xiaoling et al. 2011], but different from calf rennet, which has an optimum temperature in the range 40-42°C. This substantial difference in optimum temperature between the microbial milk-clotting enzyme and calf rennet suggests strongly that they are suitable for use under different conditions. The maximum milk-clotting activity was at pH 5.5 for the purified enzyme, and the activity decreased with increasing pH (Fig. 3).

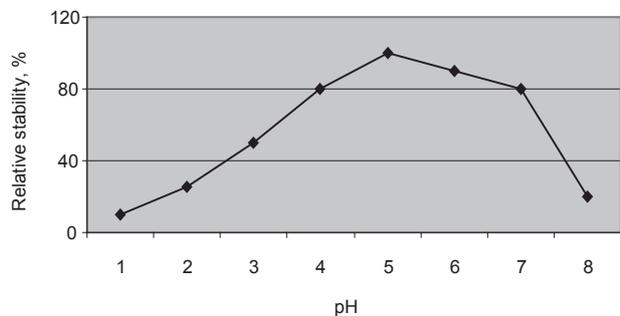


Fig. 3. Effect of pH on activity of milk clotting purified *M. pusillus* QM 346 enzyme

This result was similar to what is reported for the milk-clotting enzymes from *Mucor miehei*, *Rhizopus oryzae* [Kumar et al. 2005] and glutinous rice wine mash liquor [Wang et al. 2009]. Like calf rennet, the purified enzyme from *B. amyloliquefaciens* D4 had a higher level of milk-clotting activity in the acidic range Xiaoling et al. [2011]. In Figure 4 is shown the thermal stability of purified milk-clotting enzyme produced by *Mucor pusillus* QM 436. The purified

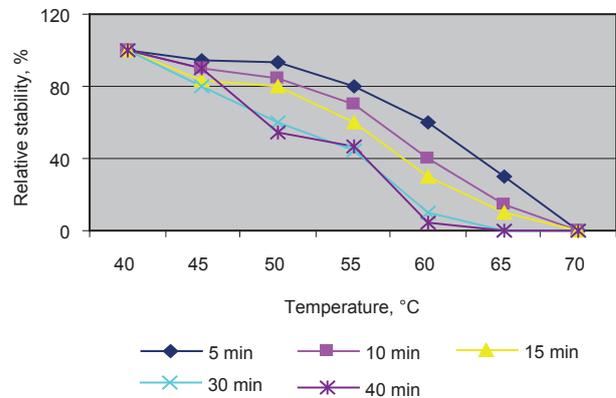


Fig. 4. Effect of temperature on stability of milk clotting purified *M. pusillus* QM 346 enzyme

enzyme retained 96% of MCA after incubation for 40 min at 40°C, and decreased to 47% after incubation for 40 min at 55°C. It was stable up to 45°C with more than 90% of the activity remained after 40 min of incubation, and it was deactivated completely after 5 min at 70°C and 30 min at 65°C. The results shown here are comparable to those reported previously for the thermo-stabilities of milk-clotting enzymes MCE (s) produced by various microorganisms.

The MCE by *P. oxalicum* displayed a dramatic loss in activity after 20 min of incubation at 55°C [Hashem 2000], and that by *M. pusillus* [O'Leary and Fox 1974] and *Mucor J20* [Hosoi and Kiuchi 2004] was completely inactivated after heating at 60°C for 20 min and 10 min, respectively. In addition, the crude MCE by *Nocardiosis* sp. Cavalcanti et al. [2004] lost its activity almost entirely when it was incubated at 65°C after 30 min. The pH effects on the activity of MCE produced by *Mucor pusillus* QM 436, are shown in Figure 5. The results indicated that the purified enzyme retained more than 85% of its activity between pH 4 and pH 7 and more than 60% of its activity between pH 7 and pH 9 for more than 30 min at 30°C, but the activity decreased dramatically below pH 4. In addition, the optimum pH for the purified enzyme is at pH 6, which is different from those of milk-clotting enzymes produced by various microorganisms.

The optimum pH for the MCE of *P. oxalicum* Hashem [2000], *M. baciliformi* Venera et al. [1997] and *Nocardiosis* sp. Cavalcanti et al. [2004] was

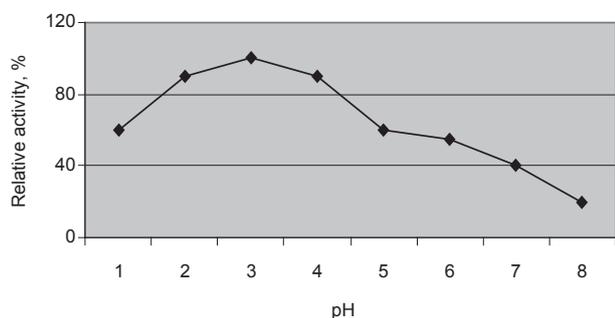


Fig. 5. Effect of pH on stability of milk clotting purified *M. pusillus* QM 346 enzyme

at pH 4, pH 5.5 and pH 11, respectively. It is expectant that coagulants should not be sensitive to variations in milk composition and pH in that the use of highly pH-sensitive rennet can lead to reduced yields and defective cheese due to soft coagulum at cutting Cavalcanti et al. [2004]. The fact that MCE produced in this study showed a wide range of pH stability has added advantage to its usefulness as cheese-making coagulant.

Michaelis-Menten constant. The K_m of the purified enzyme was 19.5 mg/ml when casein was used as a substrate suggesting a wide specificity of enzyme towards different substrates. Similarly K_m values for milk clotting enzymes from *Streptomyces remoseus* NRRL 12907 were reported to be 20 mg/ml El-Tanboly and Selim [1995].

Effect of NaCl concentration. Results are shown in Figure 6 the data indicated that the (MCA) of purified enzyme from *M. pusillus* QM 436 decreased

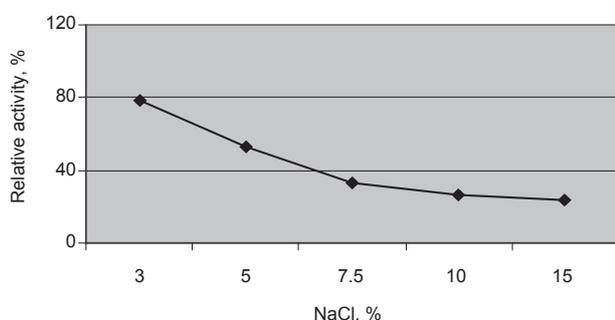


Fig. 6. Effect of sodium chloride on activity of milk clotting purified *M. pusillus* QM 346 enzyme

as the concentration of sodium chloride increased. The relative (MCA) declined sharply from 100% to 78.3% with the addition of 3% NaCl. However, the relative activity decreased smoothly with higher concentration of sodium chloride to reach 23.3% at 15% salt. Similar findings are reported in the literature Wang et al. [1969], Kołaczowska et al. [1985], El-Batawy [1991], El-Tanboly and Selim [1995] and El-Bendary et al. [2007] quoted that the (MCA) of both rennin and *Rhizopus* NRRL 3271 enzyme decreased as the concentration of sodium chloride increased. They also added that rennin appeared to be more sensitive to NaCl than microbial enzyme. In conclusion, the presence of NaCl in milk, even at low concentrations, would cause appreciable loss in the clotting activity of the studied purified microbial protease. This may be taken into consideration when manufacturing Domiati cheese using the previous enzyme.

Effect of calcium on milk-clotting activity. Calcium chloride is generally added to heated milk in the range from 0.01 to 0.02% overcome the difficulties sometimes encountered in the coagulation of such milks. This is regularly done in European countries where milk is usually pasteurized at 75-80°C for 12-20 seconds. Results are shown in the Figure 7. It could be observed that a positive and proportional relationship occurred between the clotting activities of the purified enzyme from *M. pusillus* QM 436 and the $CaCl_2$ concentration within the range tested from 0 to 1600 p.p.m. In other words, as the Ca^{++} ion concentration increased the clotting activity increased in a proportional manner.

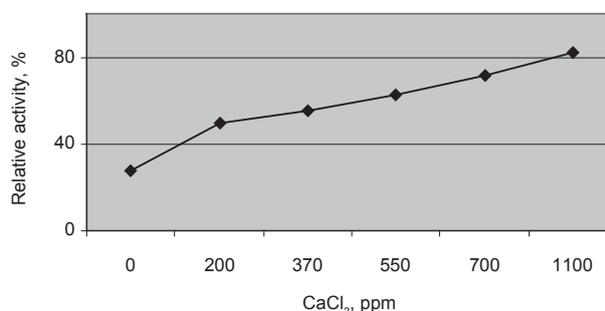


Fig. 7. Effect of calcium chloride on activity of milk clotting purified *M. pusillus* QM 346 enzyme

Iwasaki et al. [1967], Kołaczowska et al. [1985] and Najera et al. [2003] arrived to the same conclusion with *M. pusillus* protease which increased in its clotting activity the increase in Ca⁺⁺ ion. Park et al. [1987] and Selim et al. [1991] noted that the clotting time decreased progressively with increasing CaCl₂ concentrations, the percentage of reduction with 25 mg calcium/100 ml. Milk was 41% with Mito rennet from *M. pusillus* lindt. Ismail et al. [1978], El-Batrawy [1991], Kumar et al. [2005] and El-Bendary et al. [2007] reported that the purified enzyme from *A. ochraceus* (MCA) was greatly enhanced by manganese ions and by increasing concentration of CaCl₂. Addition of calcium also reduces pH of milk solution thereby hastening protein aggregation Gastaldi et al. [1994], Mehaia and Cheryan [1983]. Curd firmness, gel strength, aggregation rate, and adhesiveness are improved with the addition of calcium Patel and Reuter [1986], Solorza and Bell [1998] and Cavalcanti et al. [2004].

Effect of some cations on milk-clotting activity. From Figure 8 it is evident that most cations gave no effect on the enzyme activity at the concentration tested except Cu²⁺ which proved to be a potent inhibitor. On the other hand, Mg²⁺ and Fe³⁺ showed a pronounced effect on relative activity reached about 109%. These results were similar to those reported by Kurutahalli et al. [2010].

A comparison of activities of some of the rennets. A comparison of activities of some of the rennets is listed in Table 7. Purified milk-clotting enzyme from *Mucor pusillus* QM 436 has an MC/P ratio of

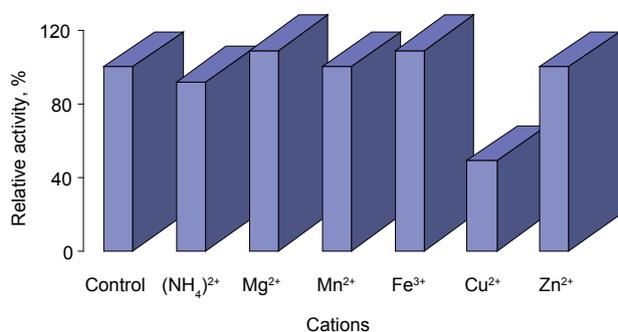


Fig. 8. Effect of some cations on activity of milk clotting purified *M. pusillus* QM 346 enzyme

Table 7. Comparison of the MCA produced by *Mucor pusillus* QM 436 with commercial rennet

Rennet source	MCA	PA	MC/PA
Calf rennet (sigma)	100	100	1
Mucor rennet (sigma)	11.3	126	0.089
<i>M. pusillus</i> protease	33	147	0.22
FPCC	170	25	7
Piglet chymosin	25	12	2.1
<i>M. meihe</i> protease	19	149	0.13
Bovine pepsin	124	2,731	0.05
<i>Mucor pusillus</i> rennet	20	11	1.81

MCA, PA, % of calf rennet, FPCC – fermentation produced camel chymosin [Kappeler et al. 2006].

1.81 in comparison to 7 in case of recombinant camel chymosin Kappeler et al. [2006]. Other proteases had an MC/P ratio in the range of 0.05 to 2.1. Thus, milk-clotting enzyme form *Mucor pusillus* QM 436 showed medium activity and high MC/P ratio, when compared to other commercial rennets.

CONCLUSION

It could be concluded that dairy companies wastes (salted whey resulting from Ras cheese manufacture) could be used as a cheap source of the milk clotting enzyme as a whole medium for growth *Mucor pusillus* QM 436, in addition to preserving the environment. These enzymes possessed high milk-clotting activity and low proteolytic action could be applied in manufacture of different kinds of cheese as calf rennet substitutes.

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