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

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ABSTRACT

Microbial milk-clotting enzymes are valued as calf rennet substitutes in the cheese industry. *Mucor pusillus* QM 436 was identified to produce the highest milk-clotting activity during screening of 19 fungal strains. Salted whey resulting from Ras (Cephalotyre) cheese manufacture as a whole medium for growth of *Mucor pusillus* QM 436 and production of the enzyme. 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 70ºC and 30 min at 65ºC. The highest level of enzyme activity was obtained at 60ºC, pH 5.5. A positive and proportional relationship occurred in presence of CaCL2 in milk, with inhibition occurred in presence of NaCl. 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.

Keywords: 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 has 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 flavor and texture. 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 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 Brizinski 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, the 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 oxygen demand and chemical oxygen demand, which make 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 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 mature. Furthermore, the possibility of using salted whey resulting from Ras cheese manufacture as a whole medium for growth was investigated. This by-product however, due to this high salt content, is not recycled in any significant industrial process so far in Egypt.

MATERIALS AND METHODS

Microorganisms ,Stock cultures

A total of Ninteen 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).

Table 1. Fungi cultures assayed for milk clotting activity

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

All the strains 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±4ºC for 3 days. The cultures were then, harvested by centrifugation at 3000 rpm for 15 min. in a cooling centrifuge (Janetzk, 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 Sama Milk Co., produced from Ras cheese. The composition of the sweet whey in table (2).

Table 2. The 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 USA was obtained from Ministry of agriculture, Giza.

Casein

Casein soluble in alkali was obtained from Adwic.

The milk clotting activity (M.C.A.)

The milk clotting activity (MCA) of the enzyme preparation was measured by the method described by Otani *et al.* (1991). A 5mL portion of the substrate (12% skim milk in 10 mM CaCl2 was incubated for 5 min at 35ºC 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: SU=2400×5×D/T×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 1mL of substrate within 40min at 35 ºC.

The proteolytic activity (P.A.)

The proteolytic activity (PA) of the purified enzyme was determined with light soluble casein as substrate, following essentially the technique by Otani *et. al,* [24]. 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 4ºC. 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 sub jected to Gel Sephadex G-100 column (30 cm×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 5ºC from 30-85ºC. 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 0.1M HCl or 0.1M 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 5ºC intervals from 40-70ºC, 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 0.1M 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 skim milk 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 first clot was noted down and compared with control.

Effect of some cations on milk-clotting activity

The effect of metal ions Mn2+, Zn2+, Mg2+, Cu2+, Fe3+ 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 Km 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 examined cultures to clot milk. Tables (3, 4 and 5) show the milk clotting enzymes produced 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 30oC was found better for Milk clotting activities than 6 and 9 days, respectively. However, 15% NaCl concentration stimulate the highest production of Milk clotting activities by *Mucor pusillus QM 346* culture. Similar observation were reported by Suganthi, *et al.* (2012) on producing milk-clotting enzymes by culture of *Aspergillus niger.*

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

Fungi cultures	concentration of NaCl (%)				
	0.0	5	7.5	10	**15**
	Milk clotting activity (MCA)				
A. oryzae	8	0	0	0	0
A. niger	0	0	0	0	0
A. awamori	0	0	0	0	0
A. flavus	3.5	0	3.8	0	0
A. terreus	4.6	3.4	0	0	0
A.ochraceus	0	0	0	0	0
A.vesicolor	22.2	3.4	0	0	0
A.tamarii	40	21	36.3	5.6	2.2
A. fumigates	0	0	7.4	0	0
M. pusillus	40	80	100	133	200
M.hiemalis	0	0	0	0	0
R.oryazae	0	0	0	0	0
R.chinensis	22.2	12.5	8.8	6.1	12.5
R.stolonifer	0	0	0	0	0
R. nigricans	0	0	0	0	0
P. citrinum	0	0	0	0	0
P. lilacinm	44.4	0	0	0	0
P.implicatum	6.1	40	4.7	23.5	2.2
P.verrucosum	0	0	0	0	0

Where:
M.C.A.= Milk Clotting Activity (Soxhlet Units /ml)

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

Fungi cultures	concentration of NaCl (%)				
	0.0	**5**	**7.5**	**10**	**15**
	Milk clotting activity (MCA)				
A. oryzae	14	5	0	0	0
A. niger	0	0	0	0	0
A. awamori	0	0	0	0	0
A. flavus	5	7	33	27	0
A. terreus	6	4	4	0	0
A.ochraceus	0	13	0	0	0
A.vesicolor	5	4	0	0	5
A.tamarii	5.7	33	50	9	11
A. fumigates	5	0	7	0	0
M. pusillus	19	28	40	57	133
M.hiemalis	0	0	0	0	0
R.oryazae	0	0	0	0	0
R.chinensis	33	16	6	9	10
R.stolonifer	7	0	5	0	4
R. nigricans	9	27	19	25	13
P. citrinum	7	7	7	22	3
P. lilacinm	36	7	17	9	0
P.implicatum	9	80	36	40	20
P.verrucosum	0	9	25	7	20

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

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

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	(NH4)2SO4 (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**

Purification and Molecular Mass

Partial Purification of enzyme from culture supernatant fig. (1) shows flow sheet diagram of the purification steps of rennin like 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 the enzyme to 7.14-fold with 54.4% recovery (Table 6). The partially

purified enzyme was separated into two peaks but with 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), Shieha *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.

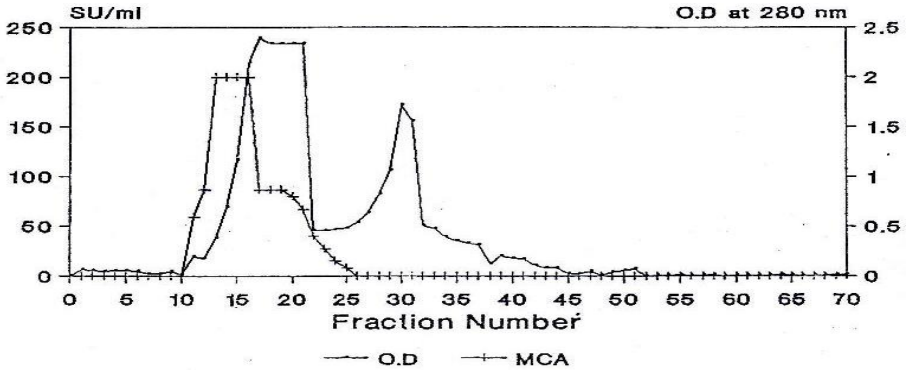


Fig.1: Purification of milk clotting enzyme from *M. pusillus QM 43*6 on Sephadex G-100

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 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).

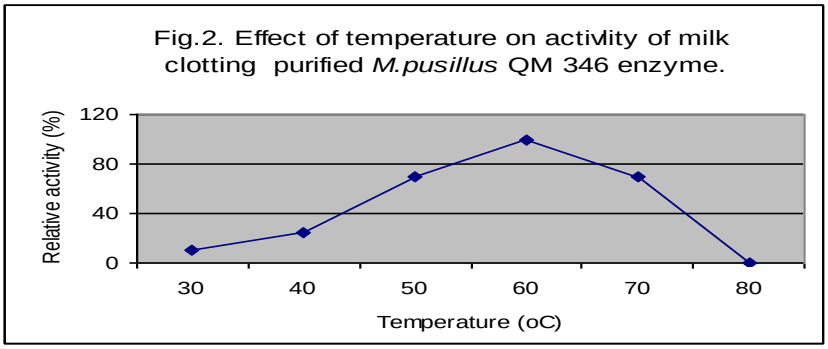


Fig.2. Effect of temperature on activity of milk clotting purified *M.pusillus* QM 346 enzyme.

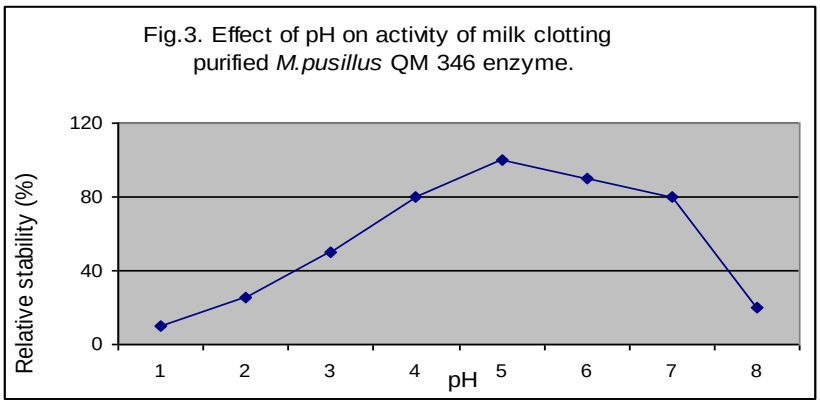
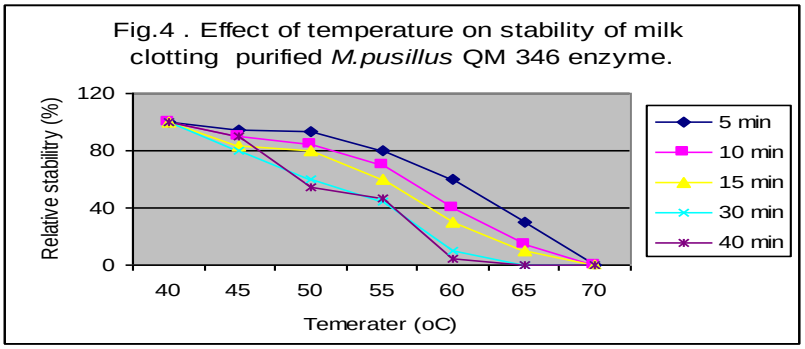


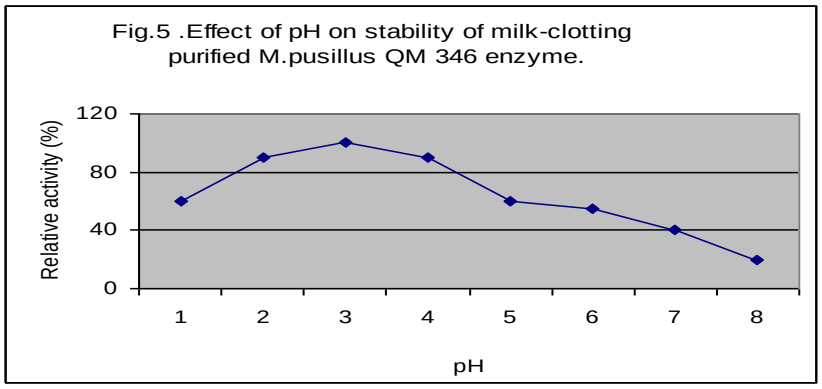
Fig.3. Effect of pH on activity of milk clotting purified *M.pusillus* QM 346 enzyme.

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.* (2009). In Fig. 4. is shown the thermal stability of purified milk-clotting enzyme produced by *Mucor pusillus QM 436*. The purified enzyme retained 96% of MCA after incubation for 40 min at 40◦C, and decreased to 47% after incubation for 40min 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 *Nocardiopsis 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 Fig. 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 *Nocardiopsis sp.* Cavalcanti, *et. al.,*[4] was 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 rang of pH stability has added advantage to its usefulness as cheese-making coagulant.

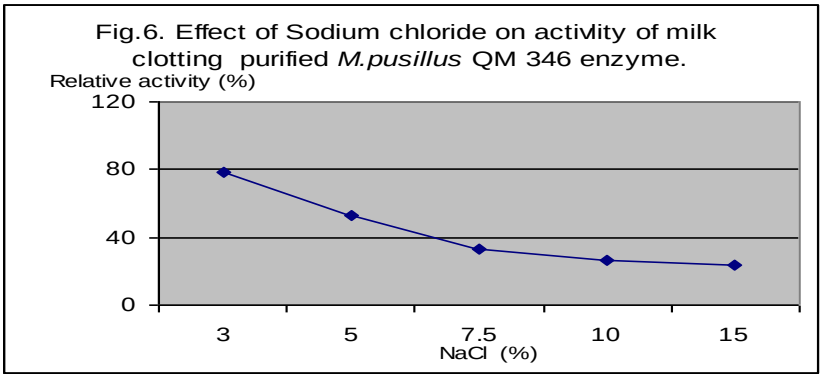
Michaelis-Menten Constant

The Km of purified enzyme was 19.5 mg/ml when casein used as a substrate suggesting a wide specificity of enzyme towards different substrates. Similarly Km 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

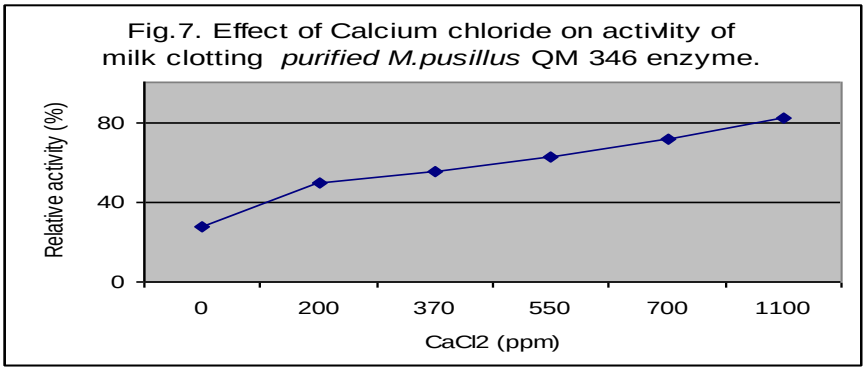
Results are shown in Fig. (6) the data indicated that the (MCA) of purified enzyme from *M. pusillus QM 436* decreased as the concentration of sodium chloride increased. The relative (MCA) declined sharply from 100% to 78.3% with the addition of 3% Nacl. However, the relative activity decreased smoothly with higher concentration of sodium chloride to reach 23.3% at 15% salt. Similar findings are reported in the literature Wang *et. al.* (1969), Kolaczkowska *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

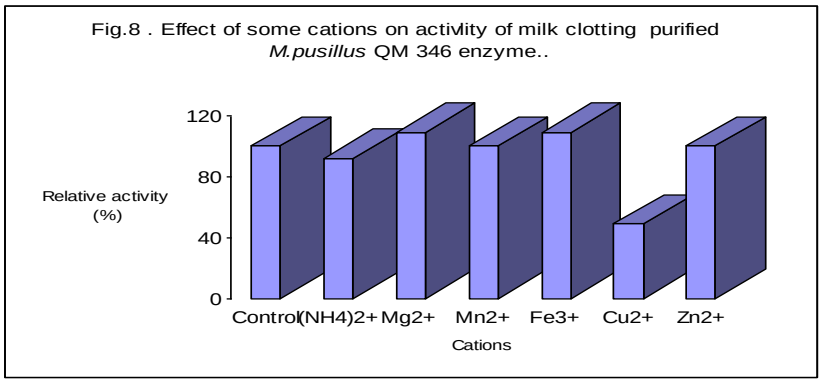
Calcium chloride is generally added to heated milk in the range from 0.01 to 0.02% overcome the difficulties sometimes encountered in the coagulation of such milks. This is regularly done in European countries where the milk is usually pasteurized at 75-80ºC for 12-20 seconds. Results are shown in the Fig. 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 CacL2 concentration within the range tested from 0 to 1600 p.p.m. In the other words, as the Ca++ ion concentration increased the clotting activity increased in a proportional manner.



Iwasaki *et al.* (1967), Kolaczkowska *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/100ml. milk was 41% with Mito rennet from *M. pusillus* lindt. Ismail *et. al.* (1978), El-Batawy (1991), Kumar *et al.* (2005) and El-Bendary *et al.* (2007) reported that the purified enzyme from *A. ochraceus* (MCA) was greatly enhanced by manganous ions and by increasing concentration of Cacl2. Addition of calcium also reduces pH of milk solution thereby hastening protein aggregation Mehaia and Cheryan (1983) Gastaldi *et al.* (1994). 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 Fig. (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 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.

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
M. pusillus proteasea	33	147	0.22
FPCC*	170	25	7
Piglet chymosina	25	12	2.1
M. meihei proteasea	19	149	0.13
Bovine pepsina	124	2,731	0.05
Mucor pusillus rennet	20	11	1.81

MCA , PA (% of calf rennet), *FPCC fermentation produced camel chymosin , From reference (Kappeler *et al.* (2006).

CONCLUISSON

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

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الإستفادة من الشرش المملح الناتج من الجبن المصرية فى إنتاج الإنزيمات الميكروبية المجبنة للبن

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**** المركز القومى للبحوث- الدقى- القاهرة**

يعتبر تحويل اللبن من الحالة السائلة الى خثرة خطوة اساسية في صناعة جميع انواع الجبن ويتجبن اللبن بأحد الانزيمات المجبنة سواء كانت حيوانية (المنفحة) او ميكروبية او نباتية ثم تركيز بعض محتويات الحليب بأزالة كمية من الشرش وتمليحه ثم وضع الناتج تحت ظروف مناسبة للتسوية. إن صناعة الجبن شهدت تطورا كبيرا وأدخلت فيها تقنيات عالية لتحويل كميات هائلة من اللبن يومياً الى الجبن بأنواعه المختلفة وبالنظر لزيادة كمية الحليب المتوفرة لصناعة الجبن في العالم لذلك بدأ التوجه الى ايجاد بدائل للمنفحة الحيوانية وذلك لقلة العجول الرضيعه المذبوحة وعليه تم التوجه الى البدائل الاخرى مثل الأنزيمات الميكروبية والتي سوف نتطرق لها في بحثنا هذا لتخثير الحليب وتحويله الى جبن. استخدمت المنفحة البديلة للاسباب التالية -:انتاجها من مواد خام متوفرة بكميات كبيرة وباسعار مقبولة ومناسبة لانتاج انواع كثيرة من الجبن وسهولة انتاجها على نطاق تجاري واسع والامان من الناحية الصحية وليس لها تأثيرات جانبية .تم دراسة إمكانية تعظيم الإستفادة من الشرش المملح الناتج من الجبن المصرية فى إنتاج الإنزيمات الميكروبية المجبنة للبن وذلك حفاظا على البيئة من التلوث وبداية لمبدأ الإنتاج النظيف.لوحظ أن السلالة *Mucor pusillus* QM 436 إستطاعت أن تنتج الإنزيمات المجبنة للبن فى حالة إستخدام الشرش المملح كبيئة. تمت التنقيه الجزئيه للانزيمات المجبنه والمنتجه من الفطر السابق بواسطة الترسيب بكبريتات الأمونيوم المشبعه ثم التبادل الغشائى ثم الفصل الكروماتوجرافى على عمود SephadexG-100 وقد بلغت مقدار تنقية الأنزيم ٧,١٤ مره من المستخلص الأنزيمى الخام ونسبة استرداد النشاط الكلى ٥٤,٤% .وقد تم دراسة بعض خواص الأنزيم بعد التنقيه ويمكن تلخيص النتائج كما يلى:

كانت الدرجه المثلى للقوه التجبنيه للأنزيم عند pH ٥,٥ ودرجة حرارة ٦٠ °م بينما يفقد نشاطه تماما على٦٥°م لمدة ٣٠ دقيقة أو على٧٠°م لمدة ٥ دقائق. توجد علاقه عكسيه بين القوه التجبنيه للأنزيم المنقى وتركيز الملح فى اللبن. بينما وجدت علاقه ايجابيه مباشره بين القوه التجبنيه للأنزيم المنقى وتركيز كلوريد الكالسيوم.

يمكن أن نخلص إلى أنه يمكن استخدام شرش اللبن المملح الناتج من صناعة الجبن كبيئة لإنتاج الانزيمات المجبنة للبن من فطر *Mucor pusillus* QM 436 نظرا لكونه مصدرا رخيصا بالإضافة إلى الحفاظ على البيئة .

قام بتحكيم البحث

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