Screening Some Local Egyptian Seeds Extract for Milk-Clotting Activity and Physicochemical Characterization of *Brassica Napus* Seed Extract.

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ABSTRACT

The aim of this study was to test the possible presence of potent milk-clotting enzymes with promising industrial application in local low cost Egyptian plant seeds. The aqueous extracts of 28 dry seeds representing eight families (*Cruciferae, Umbelliferae, Leguminosae, Rosaceae, Asteraceae, Gramineae, Tiliaceae*, and *Cucurbitaceae*) were screened in order to find the most promising source for potent milk-clotting activity. The screening for milk-clotting enzyme was applied by using the reconstituted milk in acetate buffer, pH 4.5 and in water (pH around 7) to exclude any possible ionic strength inhibition for milk-clotting enzyme. Results of screening experiments indicated that the milk-clotting enzymatic activity is family related. The highest value of milk-clotting-activity was offered through seeds *Brassica napus* (rape), *Raphanus sativus* (radish), *Eruca sativa* (arugula), *Brassica alba* (white mustard), where they are all members of the family *Cruciferae*. *Brassica napus* seeds were chosen as it shows the highest milk-clotting over proteolytic activity among the 28 screened seeds. The optimum conditions of the crude milk-clotting enzyme such as temperature, pH, and effect of different concentrations of substrate and enzyme of the crude extract of *Brassica napus* seeds were studied. The crude milk-clotting enzyme showed a narrow range of activity in only the acidic range with a total 95% loss of milk-clotting activity at pH 7 and an optimal temperature of 55 ºC. The crude rape seed milk-clotting enzyme had a linear relation between the enzyme concentration as expressed by seed weight in gram/reaction mixture which illustrates that the milk-clotting activity was performed without interference or combination of other proteolytic enzymes active at the acidic pH tested.

Keywords: Protease, Enzyme, Rennin-substitute, Plant, *Cruciferae,* Rape.

1. INTRODUCTION

Calf rennet, which contains chymosin (EC 3.4.23.4) as the main enzyme component, has been widely used as a milk-clotting enzyme [13]. The reduced supply of calf rennet, calf diseases like bovine spongiform encephalopathy (BSE) has led to an increase in the demand for alternatives sources of milk coagulants [4, 26]. Milk-clotting enzymes from different sources have been suggested. Fish and aquatic invertebrates as a source has been studied [31].Various microbial alternatives are used for chymosin production *Rhizomucor pusillus and Cryphonectria parasitica* [8], *Thermomucor indicae-seudaticae* N31 [19], *Nocardiopsis sp* [4], *Rhizomucor miehei* [33], *Penicillium oxalicum* [11]. Most of the companies produce recombinant rennet of cattle calf origin in different microbial hosts [22, 30]. Microbial rennet produced by genetically engineered bacteria has proven suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants [13]. The consumer constraints on the use of animal rennet for religious reasons (e.g., Judaism and Islam) as well as diet (vegetarianism), or consumer concern regarding genetically engineered foods (e.g., Germany, Netherlands and France forbid the use of recombinant calf rennet) have led to a growing interest in vegetable coagulants [8]. Plant coagulants includes enzymes from *Cynara cardunculus* [18, 34], seven papilionoideae species (*Eriosema shirense*, *E. ellipticum*, *E. pauciflorum*, *E. gossweilleri*, *E. psoraleoides*, *Adenolichos anchietae* and *Droogmansia megalantha* [17], *Solanum dobium fresen* [13] and *Centaurea calcitrapa* [24, 25], *Cynara scolymus* [32], *Calotropis procera* [29], *Helianthus annuus* [23], *Lactuca sativa* [15], fig (*Ficus carica*), paw paw (*Carica papaya*), pineapple (*Ananas sativa*) and castor oil seeds (*Ricinus communis*) coagulate milk [10]. The present study was conducted to investigate the possible presence of proteolytic enzymes with promising industrial application as regard milk-clotting activity in local low cost Egyptian plant seeds. Twenty eight plant seeds representing eight families were screened in order to find the most promising source for MCE. The plant families include *Cruciferae*, *Umbelliferae*, *Gramineae*, *Rosaceae*, *Asteraceae*, *Cucurbitaceae*, *Tiliaceae* and *Leguminosae*. The crude extract of *Brassica napus* seeds shows a potent milk-clotting activity (164 U/g dry seeds) with firm clotting and minimum proteolytic activity at pH 4.5. Determining the characteristics of the crude enzyme was regarded essential due to their potential industrial application since in industry enzyme purity is given less importance than cost [19, 5].

2. MATERIALS AND METHODS

2.1. Materials

Twenty eight dry seeds from eight families were used in the present study. They were brought from local markets. The dry skim milk powder was obtained from local market in Cairo. Soluble casein and L-Tyrosine were obtained from BDH, England. All the other chemicals were of analytical grade.

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2.2. Methods:

2.2.1. Preparation and extraction of the crude enzymes:

Twenty eight dry seeds in the rest states were separately crushed and mixed with dist. water at 9 °C with continuous shaking over a period of 12 hours. This extract was then centrifuged at 3000 r.p.m for 15 minutes, and the supernatant was collected, dialyzed against dist. water and then used as the crude enzyme preparation (milk-clotting enzyme, MCE and proteolytic enzyme, PE) (figure 1).

2.2.2. Enzyme assays:

2.2.2.1. Preparation of milk-clotting substrate:

Skim milk was used as a substrate for the assay of milk-clotting activity. It was prepared according to Kawai and Mukai (1970) with slight modification. Twelve gm of skimed milk powder were dissolved in 100 ml distilled water or in 0.1M acetate buffer, pH 4.5 containing 0.11 gm $CaCl_2$ (0.01M final concentration) and used as substrate for assaying milk-clotting activities.

2.2.2.2. Determination of milk-clotting activity (MCA):

The prepared enzyme solutions were assayed for their ability to produce extracellular milk-clotting activity using the standard assay procedure as described by Berridge (1955) with slight modification**.** The reaction mixture contained 0.5 ml of enzyme solution was added to 2.0 ml of milk-clotting substrate solution already incubated at 37 °C. The time necessary for the formation of curd fragment was measured. The activity of milk-clotting enzyme was expressed in term of Otani units.

Calculated as follows: MCA units = 2400/T x S/E

Where:

T = time (in sec) necessary for the curd fragment formation.

S = volume (in ml) of substrate (milk).

E = volume (in ml) of enzyme.

2.2.2.3. Determination of the proteolytic activity (PA):

The proteolytic activity of the prepared enzyme solutions was estimated according to Greenberg (1975) using soluble casein as a substrate. The assay used was as follows:

From 1% (w/v) soluble casein solution, 0.5 ml was pipette into a test tube followed by 1.0 ml of 0.1 M acetate buffer, pH 4.5 then 0.5 ml of the enzyme solution. The test tube was incubated in a water bath at 40 °C for 20 minutes. The reaction was stopped by adding one ml of 15% trichloroacetic acid and the tube was left 30 minutes, and then centrifuged. To 0.5 ml of the clear supernatant, blank and L-tyrosine solution (as standard), was added to 7.5 ml of 0.5 M NaOH followed by 0.5 ml of diluted Folin Ciocalteu's reagent with dist. water (1: 2) with continuous shaking. The absorbance of the samples and blank were read by spectrophotometer at 660 nm wave length against blank. One unit of protease enzyme was defined as the amount of enzyme that could liberate 1.0 μmole of amino acid per min under the conditions described above using L-tyrosine as standard.The activity of MCE and PE values of samples was average of three repeated measurements.

2.2.3. Estimation of protein:

The protein concentration was determined by Lowry *et al.* (1951) method using bovine serum albumin as a standard.

2.2.4. Physicochemical properties of the crude MCE:

2.2.4.1. Effect of pH value on the activity of the crude MCE:

Small aliquots of the purified enzymes were assayed with three buffering systems, namely acetate (0.1 M, pH 3.5-5.5), phosphate buffer (0.1 M, pH 4.5-8.0) and tris buffer (0.1 M, pH 8-9).

2.2.4.2. Effect of temperature on the activity of the crude MCE:

The maximum activity of the tested enzyme was determined at different temperatures ranged from 30 to 65 °C. Small aliquots of the purified enzyme were preheated at different temperatures (30-80 °C) for time intervals from 15 to 120 min. The remaining enzymatic activities were then assayed using the standard assay conditions.

2.2.4.3. Effect of the crude enzyme concentrations on the MCA activity:

The effect of enzyme concentrations was tested by incubating different enzyme concentrations (3.1-21.7 % w/v) with the substrate at the optimum temperature. The relation between enzyme concentrations and enzyme activity was plotted.

3. RESULTS

3.1. Screening for Milk-clotting enzyme (MCE) in different dry seeds:

The aqueous extracts of 28 dry seeds representing eight families (*Cruciferae, Umbelliferae, Leguminosae, Rosaceae, Asteraceae, Gramineae, Tiliaceae, and Cucurbitaceae*) were incubated separately with skimmed milk dissolved in water or 0.1 M acetic acid at pH 4.5 in order to assess the milk clotting activity (Table 1). Table (1) represent the results of screening experiments for the existence of milk clotting and proteolytic activities (MCA and PA, respectively) of the extract of 28 dry seeds at pH 4.5. These results illustrated the presence of MCA in appreciable amounts in the extract of seeds number 1, 2, 3, 4, 20 and 26 (ranging from 42.7 to 164 μ/g dry seed). Table (1) shows also that the extract of seeds number 2, 7, 8, 11, 13, 20 and 22 had higher PA at pH 4.5 (ranging from 1.73 to 6.00 μ/g dry seed).

3.2. Milk-clotting enzyme (MCE):

The above screening showed that *Brassica napus* (seed no 1) was the most promising seeds from which MCE could be isolated. It has high MCA (164 U/g dry seeds) with firm clotting and no proteolytic activity at pH 4.5.While *Raphanus sativus* (seeds no 2) has high MCA (160 U/g dry seeds) and has proteolytic activity (2.52 U/g dry seeds at the same pH (4.5). The aqueous extract of *Brassica napus* seeds was taken as a crude enzyme source.

3.2.1. Effect of pH on MCA:

Milk-clotting activity of the rape seed crude enzyme was determined at different pH range from 4.5 to 9.0 using the standard procedures. Three buffers namely acetate, phosphate and tris buffer were used. The obtained results are summarized in figure (2). The optimum pH value for milk clotting activity was at 4.5 whereas a notable decrease in MCA was progressively evident as the pH of buffer increased toward neutrality and above. At pH 6.5, no detectable milk-clotting activity could be detected.

3.2.2. Effect of incubation temperature on MCA:

Standard reaction mixture in thin-walled glass tubes were incubated in water-bath at different temperature sets in the range from 31 to 65 ºC. Three enzyme concentrations 3, 5 and 15% (w/v dry seeds / reaction mixture) were used. The MCA was determined from MCT as shown in figures (3, 4, 5). The MCA increased progressively with the increase in incubation temperature. At higher enzyme concentration (15% W/V), MCA reaching its maximum activity up to 55 ºC. At higher temperature, the enzyme lost its clotting activity gradually. It was also noticed that at higher enzyme concentration (15% w/v) high MCA was found.

3.2.3. Effect of enzyme concentration on MCA:

The crude rape seed enzyme was serially diluted with distilled water to make final concentration in the range from 3.1 to 21.7% (w/v) dry seeds / reaction mixture. The fractions obtained were then assayed for MCA using the standard procedures reported in the text. The results are summarized and graphically illustrated in figure (6). The enzyme activity was increased proportionally in a linear manner with the increase of the enzyme concentration within the range of concentrations tested.

4. DISCUSSION

The seeds were chosen as the target plant tissue to be studied as they contain high amounts of storage protein and hence a high percentage of proteolytic activity [21]. The screening for MCE was applied by using the reconstituted milk in acetate buffer, pH 4.5 and in water (pH around 7) to exclude any possible ionic strength inhibition for milk-clotting enzyme. The parameter texture was used as an enzyme specificity indicator. The more the milk proteolysis was targeted towards the κ –casein the more firmness the clot resulted. The highest value of milk clotting activity was offered through seeds *Brassica napus* (rape), *Raphanus sativus* (radish), *Eruca sativa* (arugula)*, Brassica alba* (white mustard), where they are all members of the family *Cruciferae*. With increasing reports on extraction of milk-clotting enzymes from plants, it was noted to be family related [14, 17]. The seed *Raphanus sativus* had the highest value of milk clotting activity but also had high proteolytic activity at pH 4.5. Hence, the rape seed was chosen as the best source tested among the twenty eight seed extract as the proteolytic activity was minimum. Generally, it was noticed a low milk-clotting activity in water than that at pH 4.5, which shows that the enzymes responsible are acidic proteases that make full activity at low pH. Thus, we can conclude that a number of low cost local seeds in Egypt could be considered a mine source for proteolytic enzymes to be used in industrial applications.

Some preliminary studies were carried out on the crude enzyme extract as the importance for industrial application is directed towards cost/effectiveness rather than purity. In agreement with previous findings, reducing the milk pH results in a significant decrease in clotting time [1]. An increase in the pH of the milk was accompanied by a loss of the milk clotting activity, an 84% loss at pH 6 and a total 95% loss at pH 7. Several authors have reported a similar decline in activity near neutrality [3, 5, 12]. A newly isolated milk-clotting enzyme from *Thermomucor indicae-seudaticae* [19] found the same steep decrease in activity upon raising the pH till a total loss at pH 7. Reports for milk clotting enzymes isolated from plants as *cyanara scolymus* L. flower showed a loss of activity of 87% at pH 7 [5]. Chymosin shows maximum activity at pH 5.5 and loss of activity at pH 7 [9, 20].

The increase in MCE activity was slower at temperature range 30-45 ºC than that from 45-60 ºC. This steep increase in activity with an optimal temperature of 55 ºC was followed by a slow decline as temperature increased. This is in agreement with other acidic proteinases extracted from *Centura calcitrapa* where maximum activity were detected at 52 ºC [28] and *Curcubita ficifolia* at 55 ºC [7] and *Maclura pomifera* at 58 ºC [27]. The difference in activity between different concentrations of milk in relation to temperature is attributed to the fact that the factor responsible for the decreased rennet clotting time of heated milk is the complex formed between κ–casein and β-lacoglobulin or α-lactalbumin [2]. An increase in concentration of milk proteins, increase the availability for complex formation and hence decrease clotting time upon enzyme use.This property of such an enzyme is of a significant importance in manufacture of cheese since it allows selecting the milk-clotting time as desired.

The milk clotting activity of an enzyme is dependent on different factors such as pH and temperature, as described above, as well as enzyme concentration. The milk clotting time decreases with increasing enzyme concentration [6]. The increase in enzyme concentration increases the rate of κ-casein proteolysis [16]. The higher the

κ-casein hydrolysis (more enzymatic specificity) the firmness the gel produced which is in agreement to the results of the present study. A linear relation between the enzyme concentration as expressed by seed weight in gram/reaction mixture which illustrates that the milk-clotting activity was performed without interference or combination of other proteolytic enzymes active at acidic pH. This linear proportionality is also of great importance to the industry as the use of low cost crude or partially purified seeds extract can yield an activity that is well determined and controlled.

This study indicated that many local plant seeds are rich in proteolytic activity (milk-clotting).The plant seeds were chosen to be a low cost source and of agricultural abundance. Also the use of seed part of the plant allows the continuous availability for industrial application.

Table 1: Milk-clotting activity (MCA) and proteolytic activity (PA) of twenty eight seed extract from eight families.

| No | Scientific name | English name | Family | MCA | | PA |
| | | | | In 0.1M acetate buffer, pH 4.5 | In water | In 0.1M acetate buffer, pH 4.5 |
				(U/g dry seeds)	(U/g dry seeds)	(U/g dry seeds)
1	*Brassica napus*	Rape	*Cruciferae*	164[F]	7[F]	0
2	*Raphanus sativus*	Radish	*Cruciferae*	160[F]	61[F]	2.52
3	*Eruca sativa*	Arugula	*Cruciferae*	68[W]	13.6[S]	1.07
4	*Brassica alba*	White mustard	*Cruciferae*	58.7[W]	13.53[ST]	0
5	*Brassica nigra*	Black mustard	*Cruciferae*	13.9[W]	5.2[ST]	0
6	*Coriandrum sativum*	Coriander	*Umbelliferae*	18.5[F]	6.9[SF]	0.72
7	*Anethum graveolens*	Dill	*Umbelliferae*	0	3.4[SF]	6.00
8	*Apium graveolens*	Celery	*Umbelliferae*	0[PD]	4.5[F]	1.74
9	*Petroselinum crispum*	Parsley	*Umbelliferae*	2.9[S]	2.9[W]	0
10	*Foeniculum vulgare*	Fennel	*Umbelliferae*	0[PD]	5.5[SF]	0
11	*Vicia faba*	Beans	*Leguminosae*	0	0	2.71
12	*Vigna unguiculata*	Cowpea	*Leguminosae*	10[SF]	0	0
13	*Trigonella foenum-graecum*	Fenugreek	*Leguminosae*	20.5[S]	0	2.71
14	*Phaseolus vulgaris*	White bean	*Leguminosae*	8.8[S]	0	0
15	*Lupines luteus*	Yellow lupin	*Leguminosae*	17.5	0	0
16	*Trifoluim L.*	Clover	*Leguminosae*	32[S]	0	0
17	*Glycin max*	Soya bean	*Leguminosae*	0	0	0
18	*Tamarindus indica*	Tamarind	*Leguminosae*	0	0	0
19	*Lens culinaris*	lentil	*Leguminosae*	0	0	0
20	*Prunus armeniaca*	Apricot	*Rosaceae*	44.4[F]	7.6[F]	1.73
21	*Prunus domestica*	Prune	*Rosaceae*	0	0	0
22	*Lactuca sativa*	Lettuce	*Asteraceae*	0[PD]	2.2[W]	4.17
23	*Triticum vulgare*	Wheat	*Gramineae*	0	0	0.26
24	*Echinochloa crusgalli*	Barnyard grass	*Gramineae*	0	0	0.54
25	*Panicum repens L.*	Torpedo grass	*Gramineae*	0	0	0
26	*Corchorus olitorius*	Corchorus	*Tiliaceae*	42.7[W]	12.8[F]	3
27	*Cucumis melo-flexuosus*	Snake cucumber	*Cucurbiaceae*	0	0	0
28	*Luffa aegyptiaca*	Egyptian Luffa	*Cucurbiaceae*	0	0	0

Texture parameter was indicated as F: firm; W: watery; PD: powerful digestion; S: soft; ST: semi-soft; SF: semi-firm.
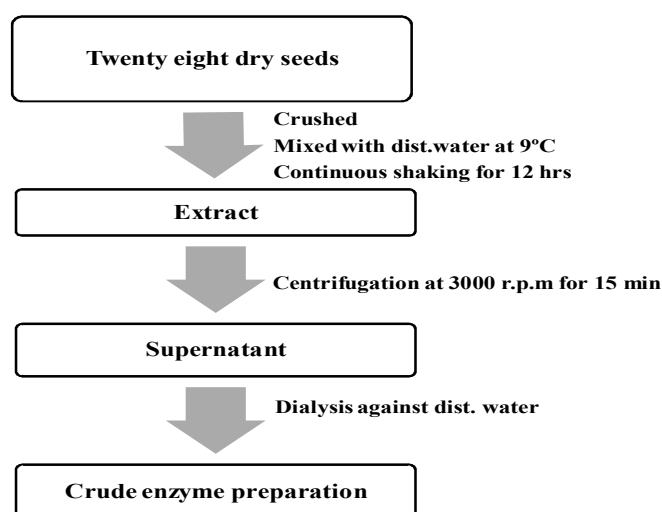

Figure 1: Schematic presentation of crude enzyme extraction from plant dry seeds.
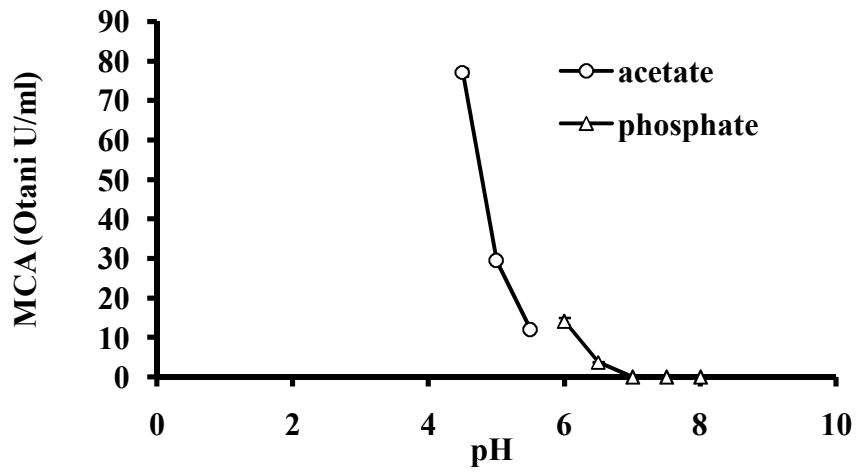
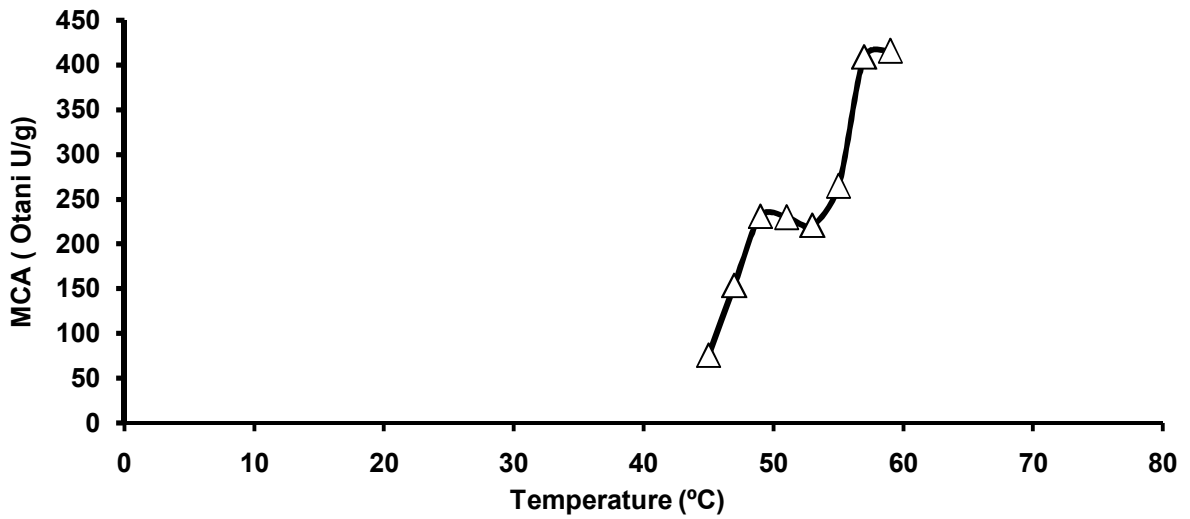Figure 2: Effect of different pH values on the activity of the crude MCE

Figure 3: Effect of incubation temperature on MCA at 3% (w/v) milk concentration.
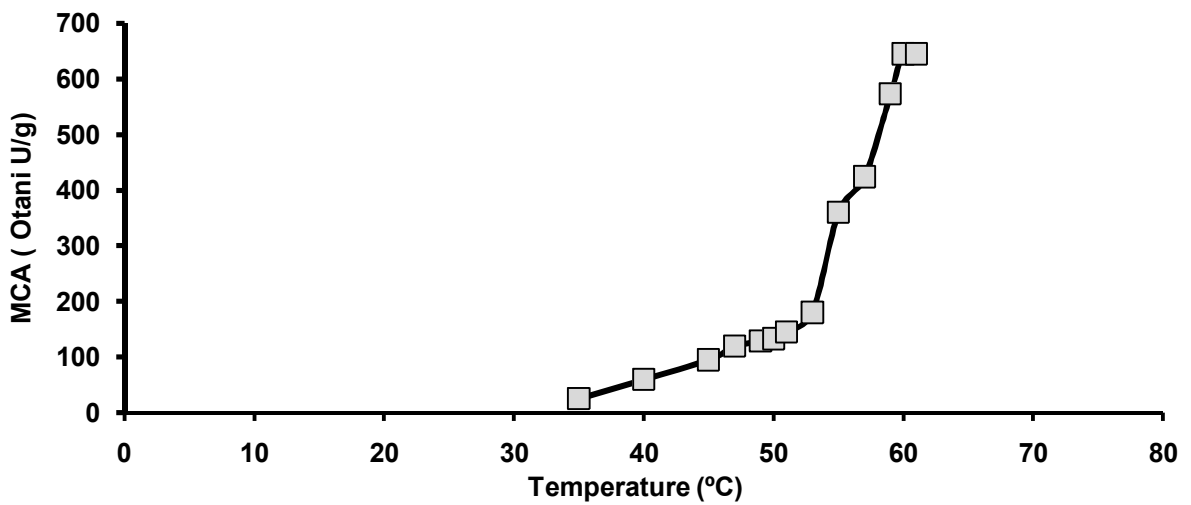

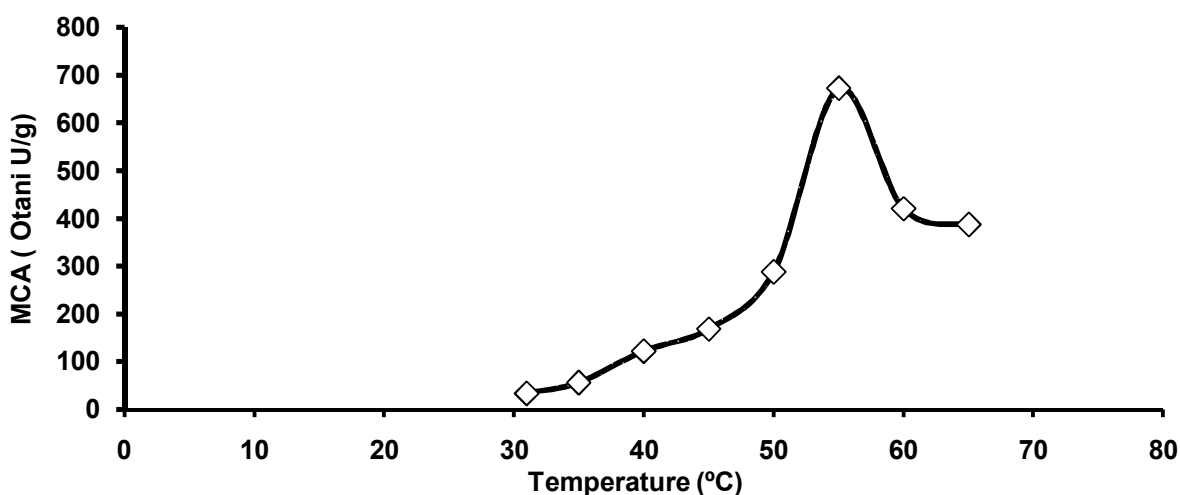Figure 4: Effect of incubation temperature on MCA at 5% (w/v) milk concentration.

Figure 5: Effect of incubation temperature on MCA at 15% (w/v) milk concentration.
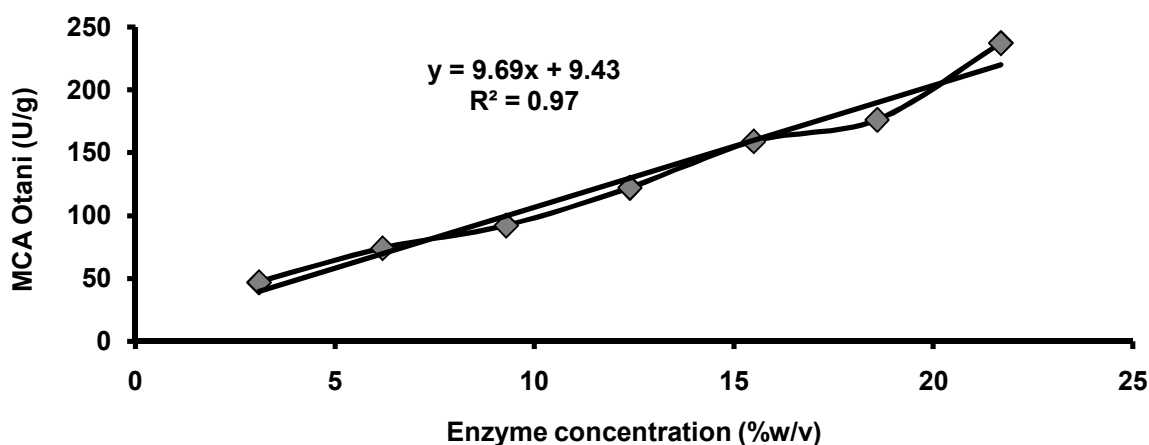

$$y = 9.69x + 9.43$$
$$R^2 = 0.97$$

Figure 6: Effect of enzyme concentration on the activity of the crude MCE.

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