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Extraction of Milk-clotting Enzyme Produced by Solid State Fermentation of *Aspergillus oryzae*

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Abstract

Studies on the extraction of milk-clotting enzyme after solid-state fermentation (SSF) of wheat bran by a local strain of *Aspergillus oryzae* LS1 were done. The extraction of the enzyme was found to be depended on different parameters like nature of extractant, soaking time, temperature *etc*. From different inorganic and organic extractants, calcium chloride (0.05%) and glycerol (40%) were found to be the best solvents for leaching out milk-clotting enzyme. The optimum volume of calcium chloride was 5 ml/g biomass. An extraction time of 180 min. at 30°C and 100 rpm was sufficient to extract out nearly 28% of the enzyme (2666.7 U/g biomass). Most of the enzyme (about 98.71%) was recovered in four repeated extractions.

Key words: milk-clotting enzyme, extraction, solid-state fermentation, *Aspergillus oryzae*

Introduction

Solid-state fermentation is the one in which microorganisms secret the necessary enzyme for degradation of the available substrate molecules in order to meet their nutritional requirement. In this system the fermented mass consists of non-utilized solid substrate containing microbial cells, spores and product for which a number of co-metabolites are formed during the course of fermentation.

Solid-state fermentation is the fermentation in the absence of free liquid and recovery of fermentation products requires its extraction from the solid fermented medium with a suitable solvents or solutions (Tunga *et al.*, 1998). From the bulky solid mass, getting the product out of the system has many problems (Lonsane and Krishnaiah, 1992). Attempt has been made by researchers to isolate the desired product from the fermented mass by various techniques due to its implication on process economics (Bjurstrom, 1985 and Calton *et al.*, 1986). With the initial moisture levels used for fermentation, squeezing of the solid medium itself hardly yields any extract and even if there is any, the volume might not be sufficient for complete extraction. The medium must therefore, be soaked for some time in an adequate amount of a suitable extractant for total recovery of the product. A common extractant is distilled or deionized water (Silman, 1980; Wang *et al*., 1984; Yano *et al.*, 1991 and Ghidyal *et al.*, 1993). Bhumibhamon (1986) used distilled water for extraction of glucoamylase enzyme from solid-state on rice bran. Malathi and Chakraborty (1991) also used distilled water for extraction of alkaline protease from solid wheat bran fermentation. Tunga *et al.*, 1998 also reported that fermented mass was soaked with water for two hours at room temperature to extract proteolytic enzyme. Other extractants have also been used for extract other enzymes. Yang and Chiu (1987) used sodium chloride solution for protease extraction. Shata (1999) extracted glucoamylase by sodium chloride solution. Rivera-Munoz *et al.* (1991) used 0.002 M succinate buffer for lipase and protease while Castilho *et al*. (2000) extracted pectinases from wheat bran by acetate buffer at pH 4.4.

This paper presents, studies on milk-clotting enzyme extraction from wheat bran, a cheap agro-residue, fermented by a local strain of *Aspergillus oryzae* LS1.The study includes the effect of some factors which influence the efficiency of leaching out of the enzyme and its efficacy in the leaching technique.

Experimental

Materials and Methods

Microorganisms. *Aspergillus oryzae* LS1 a local strain obtained from the Microbial Resource Center at Cairo (MIRCEN), Ain Shams University was used through out this work.

Inoculum preparation. Fungal spores were prepared on Czapek's Dox agar medium in Petri dish incubated for 7 days at 30°C to assure good sporulation. The amount of inocula determined as colony-forming units (CFU) was prepared by scraping agar discs of 1.5 cm diameter aseptically from agar plate.

Fermentation. Wheat bran (10 gram) in 500 ml conical flask sterilized at 121°C for 20 min. were mixed with 15 ml of a sterile salt solution containing (g/l): magnesium chloride 15×10^{-3}, and fructose, 8 previously sterilized at 110°C for 10 min. Each flask was inoculated with 186×10^6 colony-forming units. Incubation was carried out at 30°C for three days.

Extraction. The extraction of the enzyme from the fermented biomass was carried out with distilled water, tap water and different salt solutions of, potassium chloride, magnesium chloride, calcium chloride and sodium chloride. The investigation was also, carried out to see the effect of different organic solvents like glycerol, methanol, ethanol and acetone in concentration of 5%.

Unless otherwise stated, the extraction was conducted in 250 ml conical flask containing one gram of fermented biomass and 5 ml of solvent solution. Each type of solvent was added separately to the fermented biomass and kept for 180 min. on a rotary shaker at 100 rpm at 30°C. Then the extract was collected by filtration through Whatman No 1 filter paper and the clear solution containing milk-clotting enzyme was assayed.

To optimize the extraction process, different experiments were carried out by varying the volume and solvent concentration in addition to time, temperature and physical state (agitation or stationary) keeping all other conditions at optimum levels.

Milk-clotting enzyme assay. The assay of milk-clotting enzyme activity was carried out according to the standard procedure described by Carlson *et al*. (1985) with some modifications using 12% (w/v) dried skim milk in 0.01 M of calcium chloride solution as substrate. The reaction mixture contained 0.5 ml of enzyme and 1 ml of substrate and incubated at 70°C. The enzyme activity was calculated according to Otani *et al*. (1991) as follows: Milk-clotting activity units = 2400/TXS/E where T is the time necessary for the crude fragment formation, S is the volume of milk, and E is the volume of enzyme**.**

Protein estimation. Protein content was estimated by the method of Ohanistti and Bar (1978).

Results

Influence of solvent type. Effect of different solvents on milk-clotting enzyme extraction from solid-state fermented wheat bran is presented in Figs. 1 and 2. Inorganic and organic solvents beside tap and distilled water were used.

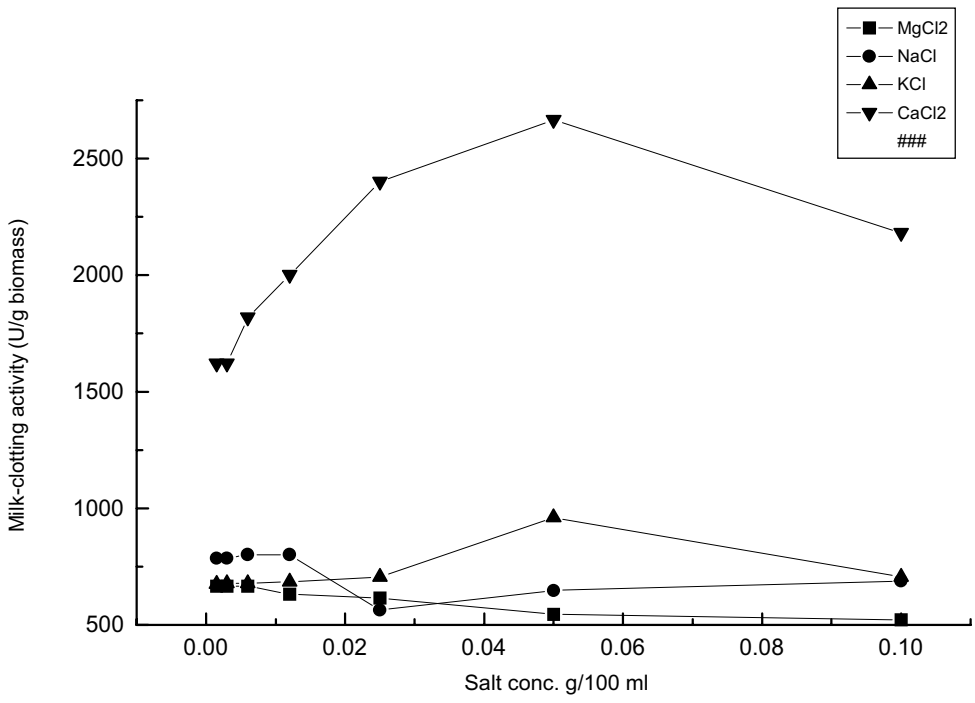

Fig. 1. Effect of different concentrations of magnesium chloride, sodium choride, potassium choride and calcium choride on extraction of milk-clotting enzyme from SSF

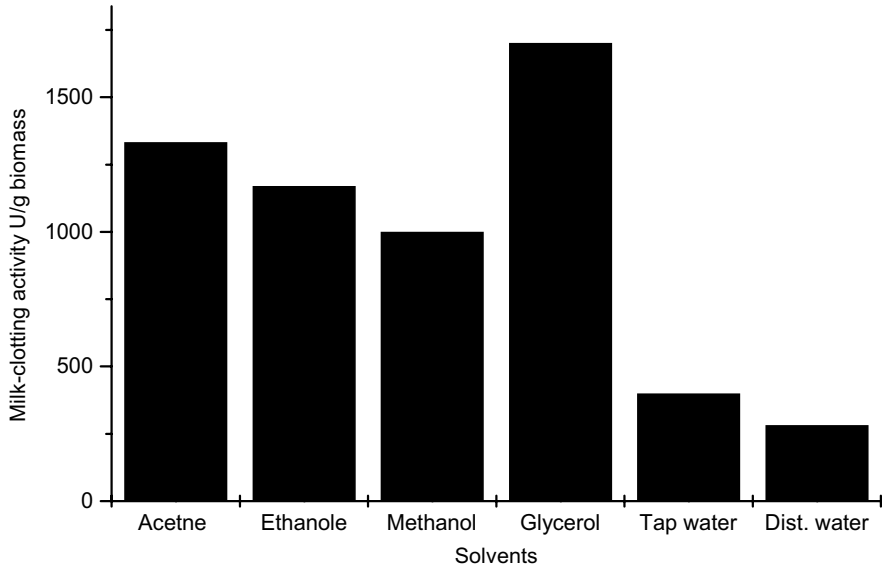

Fig. 2. Effect of different organic solvents and water extraction of milk-clotting enzyme from SSF

Among four inorganic salt solutions tested (sodium chloride, potassium chloride, calcium chloride and magnesium chloride), calcium chloride in concentration of 0.05% (w/v) gave the best extraction of milk-clotting enzyme from the fermented solids. As the concentration of calcium chloride increased, the amount of extracted enzyme increased reaching its maximum value at 0.05% (w/v). Addition of different concentrations of potassium chloride, sodium chloride or magnesium chloride, gave no significant results compared to calcium chloride solution.

Among four organic solvents (methanol, glycerol, ethanol, and acetone) investigated in concentration of 5% (v/v), glycerol gave the maximum leaching of milk-clotting enzyme. To optimize the concentration of glycerol in the solution used as solvent, experiment was carried out by varying the glycerol concentration from 5% to 60% keeping other parameters constant. As the concentration of glycerol increased milk-clotting enzyme recovery increased (Fig. 3). The increasing trend was observed up to 40%, beyond which there was a slight decrease. Therefore, calcium chloride of 0.05% (w/v) was used through the next experiments.

Optimization of solvent volume. Fig. 4 shows the effect of different volumes of 0.05% calcium chloride solution used as extractant on recovery of milk-clotting enzyme from the fermented wheat bran in SSF.

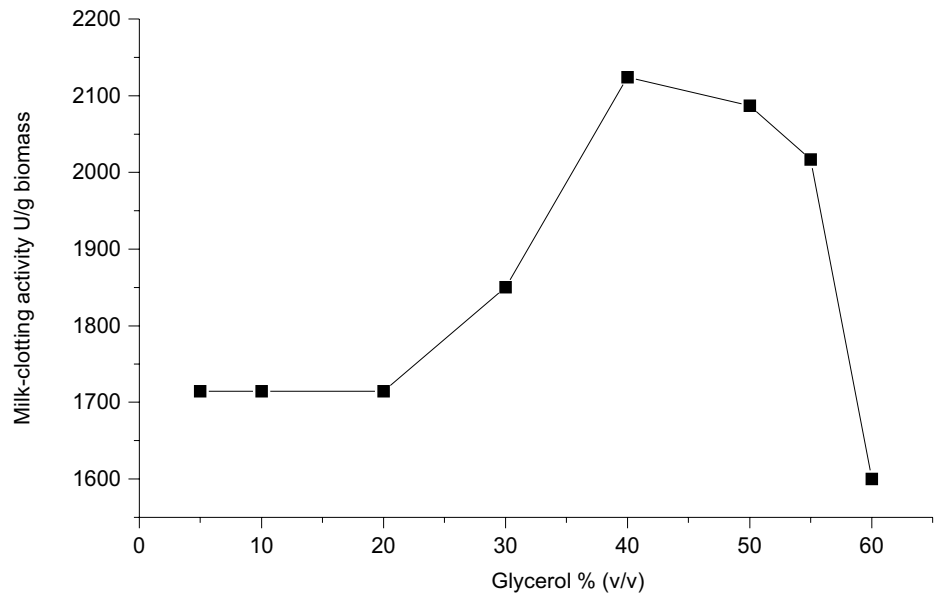

Fig. 3. Effect of different concentrations of glycerol on extraction of milk-cloting enzyme from SSF

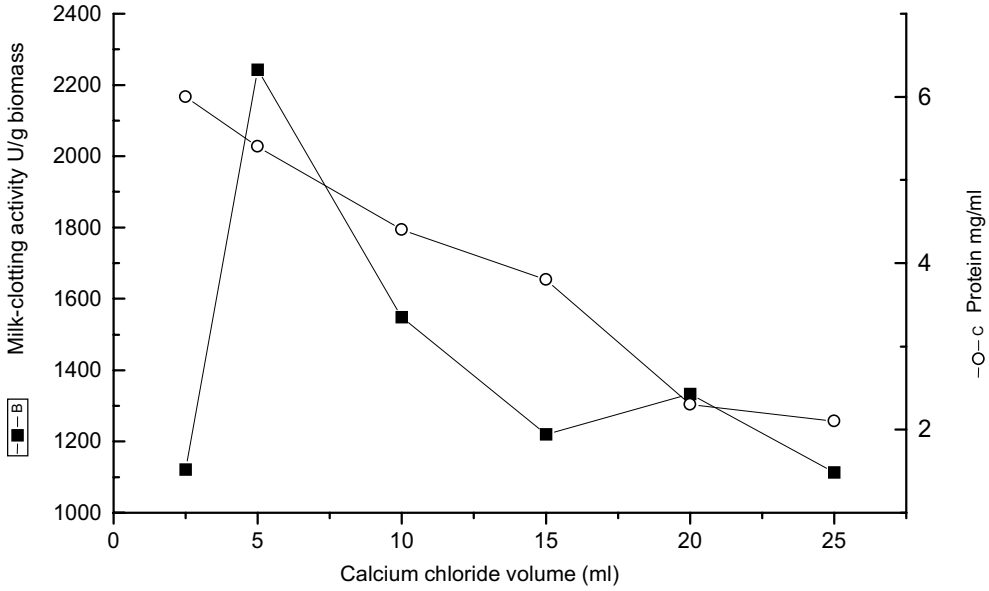

Fig. 4. Effect of calcium chloride volume on extraction of milk-cloting enzyme from SSF

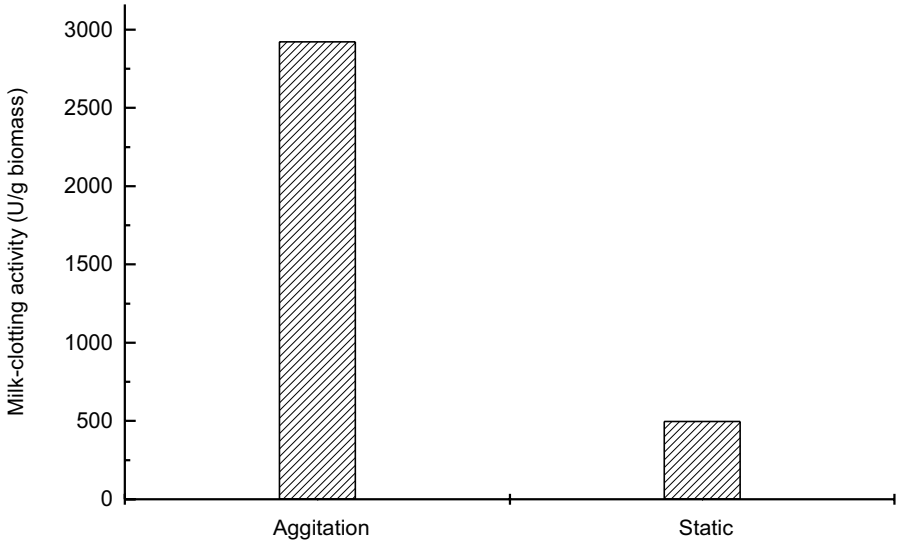

Fig. 5. Effect of phisical state on extraction of milk-cloting enzyme from SSF

The range investigated was 2.5–30 ml/g biomass with 3 h soaking time at 30°C and 100 rpm. The total activity of the extract increased up to 5 ml/g biomass, above which it remained more or less constant. However, the amount of extracted protein increased with the increase of volume of calcium chloride up to 20 ml.

Effect of physical state. Fig. 5 shows the effect of physical state on extraction of milk-clotting enzyme. It was found that agitation was quite satisfactory for maximum recovery of the enzyme. So it was selected as the best condition for enzyme recovery.

Effect of extraction time. Soaking of the fermented solid substrate with calcium chloride solution at the optimum ratio 5 ml/g biomass was done at 30°C and at 100 rpm for different periods varying from 15 min to 48 hours. As shown from Fig. 6 maximum amount of milk-clotting enzyme was extracted after 180 min. Longer extraction time did not result in significant gain of recovery.

Effect of temperature on extraction process. To evaluate the effect of temperature on leaching process, a series of experiments were carried out at 10, 30, 40, 50, 60 and 70°C, keeping the other experimental conditions at optimum. The results presented in Table I show that 30°C was the optimum temperature for extraction of the enzyme. At higher temperature above 30°C the activity decreased.

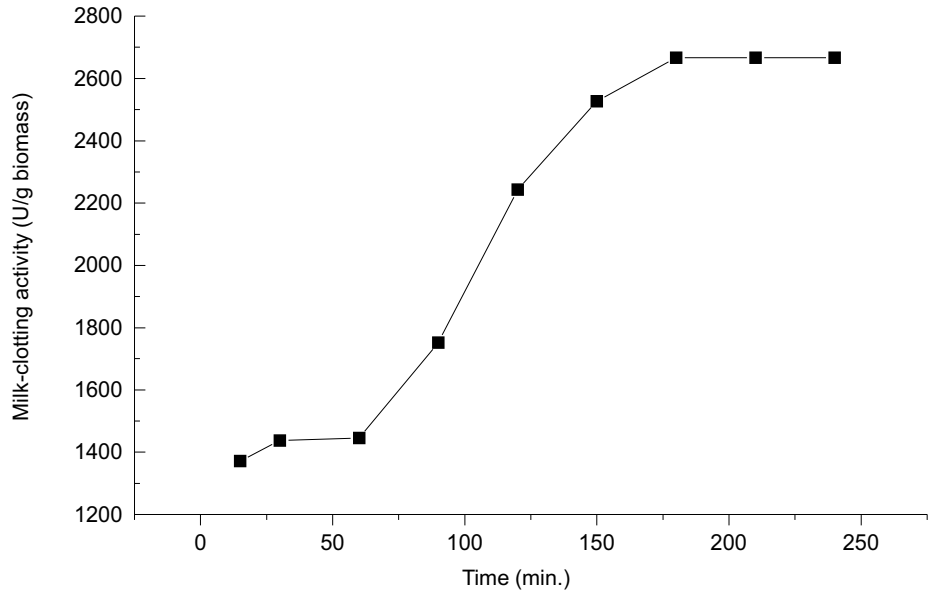

Fig. 6. Effect of soaking time on extraction of milk-cloting enzyme produced in SSF

Repeated extraction. Repeated extractions were carried out for recovery of the most milk-clotting enzyme from the fermented solid mass. From Table II, it was observed that most of milk-clotting enzyme was recovered during four repeated extractions. About 28% of the total activity was found in each of the first three extractions.

Table I
Effect of temperature on extraction of milk-clotting enzyme

Temperature °C	MCA		Remaining activity %	Loss of activity %
	U/ml	U/g		
10	16	80	3	97
20	26	133	5	95
30	505	2526	100	0
45	231	1154	46	54
50	53	267	11	89
60	20	100	4	69
70	0	0	0	0

MCA = Milk-clotting activity

Table II
Milk-clotting activity recovery in five repeated extractions with fresh calcium chloride solution

Number of recovery stage	MCA		Cumulative MCA U/ml	MCA U/ml %	Cumulative MCA U/ml %
	U/ml	U/g			
Recovery in the 1st washing	533	2667	533.3	28	28
Recovery in the 2nd washing	527	2637	1060.8	29	57
Recovery in the 3rd washing	527	2637	1588.3	28	86
Recovery in the 4th washing	264	1319	1852.0	13	99
Recovery in the 5th washing	5	27	1857.3	1	100

MCA = Milk-clotting activity

Discussion

Solid-state fermentation is fermentation in the absence of free liquid, and recovery of the fermentation product requires its extraction from the solid fermented medium. Among the salt solutions tested, calcium chloride (0.05% w/v) gave the best extraction of milk-clotting enzyme from the fermented solids. However, it's interesting to notice that the extraction of milk-clotting enzyme increased with all salt solutions, as compared with tap or distilled water. The above observation was recorded for the protease enzyme produced by *Rhizopus oryzae* in solid-state fermentation of wheat bran, (Aikat and Bhattacharyya, 2000). They mentioned some weak ionic binding of the enzyme with either cell or substrate. On the other hand Wang (1967) reported from the studies on *Mucor hiemalis* that most of the proteolytic activity is cell surface bound and could be released by elution with sodium chloride suggesting that the enzyme was probably bound to the cell by weak ionic bonds. Castilho *et al*. (1999 and 2000) reported that acetate buffer gave better results for extraction of pectinases and from the fermented solids by *Asp. niger* than glycerol or distilled water. The poor performance of glycerol can be explained by its higher viscosity, which has a negative effect on mass transfer. Corpe and Winters (1972) found most of the protease activity to be cell bound, largely being associated with the cell envelop. They mentioned also that about 50% of the specific protease activity of the cell envelope could be removed with three washes with sea water, indicating a fairly weak binding.

The effect of calcium chloride concentration on enzyme extraction was a remarkable observation. Up to 0.05% (w/v), extraction increased probably due to the salting-in effect of electrostatic effect of the salt (Aikat and Bhattacharyya, 2000). Beyond the above mentioned concentration a slight salting-out effect due to hydrophobic effect began to show up resulting in a slight decrease in enzyme activity.

In the present investigation the presence of organic solvent in water was more effective in milk clotting enzyme extraction than tap or distilled water. Tunga *et al.* (1999) reported that in SSF protease was present in solid fermented mass due to some binding force. Water has the highest dielectric constant compared with other organic solvents. They mentioned that as the dielectric constant decrease, the force of interaction between the enzyme and solvent may increase. Therefore, leaching out of the enzyme by inorganic solvent was more effective.

However, it was noticed that with different concentrations of glycerol, a small increase in enzyme activity was recorded at higher concentration as shown in Fig. 3. This was due to the protective effect of glycerol on enzyme activity. Scopes (1982) mentioned that glycerol forms strong hydrogen bonds with water, reducing the water activity. On the other hand, Tunga *et al*. (1999) reported that protease extraction from SSF was maximally achieved using ethanol-glycerol mixture. They suggested that the hydroxyl group of ethanol and glycerol may form hydrogen bonding with protein molecules and this gives the stability of the enzyme molecule. As the literature report (Stryer, 1975, and Bailey and Ollis 1986) the stability of enzymes can be improved by using sorbitol as solvent.

A contact time of 180 min. at 30°C provided the best enzyme activities for most of the conditions tested. Periods of 15, 30 or 60 min. seem not to be enough for total solubilization of milk-clotting enzyme present in wheat bran medium. Increasing the time of extraction up to 24 hours has no promising value on extraction of the enzyme. This loss could have been due to the prolonged mechanical or to a greater extraction of denaturant agents (Ghildyal *et al.*, 1991). Castilho *et al.* (2000) investigated the effect of incubation time on protease extraction in SSF and they found that 30 min. provided the best time for enzyme extraction. Also, Ikasari and Mitchell (1996) studied the effect of incubation time on extraction of protease in SSF. They concluded that maximum enzyme recovery was achieved at 22°C with 60 min. contact time.

According to Aikat and Bhattacharyya (2000) the amount of solute increases with the increase of solvent volume. Our results showed that the level of milk-clotting activity reaches the maximum at 5 ml/g biomass and then decreased. These results can be explained from the calculated specific activity since more solvent cause the release of non specific protein. Excessively large volume of extractant used for greater extraction would also yield enzyme solutions to be too dilute to be profitably utilized.

Agitation of the fermented biomass with the extractant at 30°C and 100 rpm gave appreciable amount of milk-clotting enzyme compared with stationary condition. Agitation helps to reduce enzyme adhesion to cell biomass and also disperses the fermented mass uniformly in the continuous phase of the solvent (Tunga *et al.*, 1999).

Maximum yield of enzyme recovery was obtained at 30°C but at higher temperature it was less. Higher temperatures may have inhibitory effect on the enzyme activity and make it less stable. This fact could be explained by the two opposite effects on enzyme extraction. On one hand, greater temperatures increase

the solute solubility and diffusivity, therefore, a higher activity is attained in the extract. On the other hand, enzyme is susceptible to deactivation, which will increase as temperature and contact time increase.

In order to maximize enzyme recovery from solid-state culture, repeated extractions were investigated. It was verified that about 98.7% of milk-clotting activity were recovered in four repeated extractions using calcium chloride solution. As the extract obtained from the fifth washing contained about 1.3%, the recovery of the enzyme was very small. Therefore, four repeated extractions are sufficient, although dilute extracts were obtained.

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