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Research Article

Efficient Immobilization of Milk Clotting Enzyme Produced by *Rhizomucor miehei* on Tricalcium Phosphate

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Abstract

Milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 under solid state fermentation was efficiently immobilized on Tricalcium phosphate (TCP) with immobilization degree of 90 % and activity retained of 93%. Optimum pH value for enzyme immobilization was pH 4. Stirring time of 30 min. was most suitable for the process at 30°C. 3 mg protein concentration was the best for ideal immobilization. Immobilized enzyme reacted optimally at pH 5 which represents the optimum value for the free enzyme preparation. Optimum temperature raised to 70°C for immobilized enzyme preparation in comparison to 60°C for free enzyme. Immobilized enzyme showed improved thermal stability.

Keywords: Milk Clotting Enzyme; *Rhizomucor miehei*; Immobilization; Tricalcium Phosphate

Introduction

Enzymes are biocatalysts that increasing the reaction rates of different chemical and biological processes [1]. Milk coagulation is the main step in cheese manufacturing. Calf rennet, is an aspartic protease enzyme (E.C. 3.4.23.4) and renin enzyme excreted by the calves fourth stomach. All cheese types are prepared by milk coagulated by renin like enzyme [2]. Specificity for K- casein cleavage is required for good coagulation process [3]. Obtaining natural milk coagulants is a challenge because of the huge amount of milk coagulants required for dairy industries requirements for milk coagulants worldwide [4]. Milk clotting enzyme (MCE) of animal origin are expensive, and their consumption has been restricted due to religious or dietary reasons. Therefore, searching for rennet substitutes and decreasing their cost is encouraged [5].

Among different rennet sources, Microbial rennet is considered a good source of calf rennin substitute. Many research articles had investigated fungal microbial rennet like enzymes. *Rhizomucor miehei* MCE production conditions and its properties [6] and recently, *Aspergillus oryza*e [7]. The protease produced by *Rhizomucor miehei* is an acid-asparatate protease with molecular weight of about 38.000. It consists of a poly peptide chain which is similar to calf rennet in 3-D-structure and its properties [4,8]. It is similar to the true calf rennet in its high specificity in splitting similar peptide bonds in kappa-casein, high milk coagulating activity, calcium requirement and high cheese quality [8].

Immobilized enzymes leads to easy and feed back of technology processes. Also they are more specific, are easier to separate the product than the soluble enzyme systems and can be reused or applied in continuous process [9,10]. These advantages of immobilized enzymes are important in the food industry. Furthermore, the immobilization may improve the enzyme characteristics such as the activity, thermal stability, selectivity and specificity to substrates and reduction of the inhibition and dissociation by reaction products and metal ions [1].

Nowadays, there is renewed interest in preparing immobilized enzymes for milk clotting [3]. Jacob., *et al*. [4] reported that using immobilized chymosin in cheese production is a promising approach to decrease the cost and shortage of calf rennet.

Many carriers were used for immobilization of enzymes via simple adsorption, ionic binding and covalent binding in addition to alginate entrapment. Solid supports with its low cost, large surface area and high thermal stability represents good carriers for immobilization process [11]. Simplest immobilization type is named adsorption method. It is characterized by preserving the enzyme from conformation [1]. Required solid supports must be available in moderate to cheap cost [1].

One of the most important salts of H_3PO_4 (phosphoric acid) is the tri calcium salt (TCP) with the chemical formula $Ca_3(PO_4)_2$. It is a low soluble white powder. TCP is mainly hydroxyapatite. It is one of the main burning products of bone. It is also commonly derive from mineral rocks. It is also naturally found in the skeletons, teeth and milk of vertebrate animals [12]. It is also used as a nutritional supplement [13].

Our team was successfully produced, *Rhizomucor miehei* NRRL 2034 milk clotting enzyme efficiently and economically under solid state fermentation techniques in trays using wheat bran as the carrier and substrate. The produced enzyme showed high MCE activity [6]. It was successfully applied in the production of UF-white soft cheese at lab scale [14].

This study aims to immobilize the MCE obtained from *Rhizomucor miehei* NRRL 2034 under solid state fermentation technique on TCP. Optimization of the process parameters, and characterization of immobilized enzyme were also studied.

Materials and Methods

Microorganism

Rhizomucor miehei NRRL 2034 was a gift from Northern Regional Research Lab., Peoria Illinois, USA.

Solid state fermentation (SSF) for milk clotting enzyme production

Among all the industrial residues tested wheat bran gave the highest milk clotting activity [6]. Thus, it was dried at 60° C for one hour and used for enzyme production. Wheat bran medium (50% moisture content) was put in aluminum foil trays (20 × 25 × 5 cm^3) to 0.5 cm depths. Autoclave sterilization for 30 minutes were applied to the trays. The medium was inoculated with about 1.8×10^8 CFU/g of spore suspension and incubated at 40ºC.the Trays were inoculated. Incubated and the fermented culture was eluted with dist. Water to obtain the crude enzyme.

Milk clotting activity

MCA was determined according to Greenberg [15]. 2 ml of skim milk solution (12% in 0.01 M $CaCl_2$) was incubated at 35ºC. Then 0.2 ml of enzyme was added and the curd formation time was recorded. Three replica were examined and used in the calculation of enzyme activity. Enzyme activity was calculated as Soxhelt units. One milk clotting unit is defined as the amount of enzyme that clots 1 ml of skim milk in 40 min at 35°C.

Enzyme immobilization

Immobilization of MCE on Tricalcium phosphate (TCP) by simple adsorption technique was carried out according to [16]. 200 mg of the support were stirred in 0.1 M sodium acetate buffer (pH 5.2) with 5 mg of the enzyme protein in 5 mL total volume for 6 h at 4°C. After centrifugation at 6000 ×g for 10 min, enzyme was washed with distilled water and finally washed with 0.1 M sod. acetate buffer (pH 5.2).

The degree of immobilization (DI) and activity retained (AR) were calculated according to [17,18].

$$DI\ (\%) = A_a - (A_b - A_c)/A_a \times 100\%$$

$$AR\ (\%) = A_I/A_a \times 100\%$$

Where A_a is the milk clotting activity (MCA) of the enzyme before immobilization, A_b is the remaining MCA in the solution after immobilization, A_c is the MCA in washing solution and A_i is the MCA of immobilized enzyme.

Optimization of the physical conditions for immobilization

Effect of enzyme concentration, Effect of temperature, Effect of contact time.

Characterization of immobilized MCE

Optimum pH for immobilization

Effect of pH value on the immobilization process was studied in the range of pH 4 and 8.

Optimum contact time

The optimum adhering time was determined by stirring for different intervals (5- 200 min).

Optimum enzyme concentration

Different amounts of the enzyme (1-5 mg) were used in the immobilization mixture.

pH and temperature profiles

- Effect of pH value of the substrate solution on MCA of both free and immobilized enzymes were carried out at pH range of 3.5-8 by using appropriate buffer solutions.
- MCA was determined at different temperatures ranging between 30 and 70°C.

pH and thermal stabilities

- pH stability of free and immobilized enzymes was determined by measuring the residual activity of the enzyme incubated at various pH values (3-8) for 48 h at 25°C.
- The thermal stability of free and immobilized enzyme were evaluated by measuring MCA of heated enzyme at different temperatures (10-90°C) for ten minutes.

Results and Discussion

Enzyme immobilization

Milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 was efficiently immobilized on TCP with immobilization degree of 90% and activity retained of 93%.

Milk clotting enzyme produced by *Bacillus sphaericus* NRC 24 was immobilized on silica gel by simple adsorption with 70% immobilization degree and 73% activity retained [19].

Milk-clotting enzyme of *Bacillus licheniformis* 5A1 was immobilized on Amberlite IR-120 by ionic binding [20].

Optimization of immobilization conditions

The effect of MCE concentration on the immobilization process

To determine the best conditions of MCE immobilization, we studied the influence of the enzyme amount (in the range of 1-5 mg protein) to be adsorbed to 200 mg of TCP used as a support. As shown in figure 1, the activity of immobilized enzyme increased by increasing the enzyme loaded onto the support to reach a peak value at 3 mg protein/200mg TCP. At low enzyme loading, the enzyme maximize the contact with the surface, However, for a loading amount higher than 3 mg protein, multilayer adsorption might have occurred and effectively inhibited the active sites of enzyme.

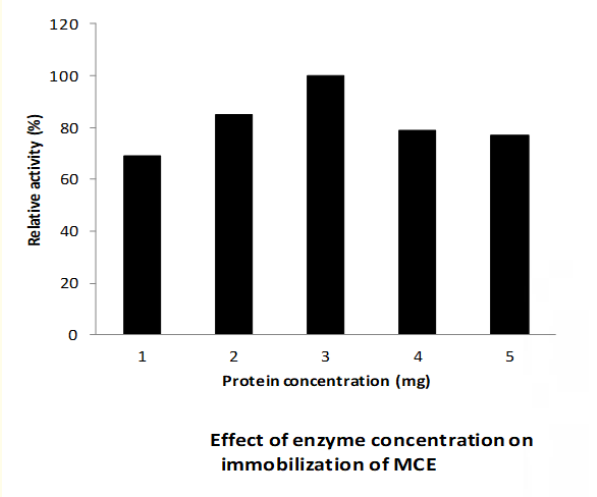

Figure 1

Das and Prabbu [16] found that Tricalcium phosphate gave best results for enzyme immobilization, may be due to its large number of hydroxy groups, high substrate affinity, complex nature, and effective surface area for adsorption.

The effect of the contact time

The activity of immobilized enzyme was measured at different stirring times of an enzymatic solution (3 mg protein) with 200 mg of support at 30°C. The immobilization was increased with increasing the contact time up to 30 min (Figure 2), followed by a stable state up to 60 min of incubation time. After that, it decreased with

increasing incubation for 90 min. more incubation time showed no effect on immobilization efficiency. As a result, 30 min incubation time of the enzymatic solution with the support was considered suitable to obtain the maximum adsorption onto TCP.

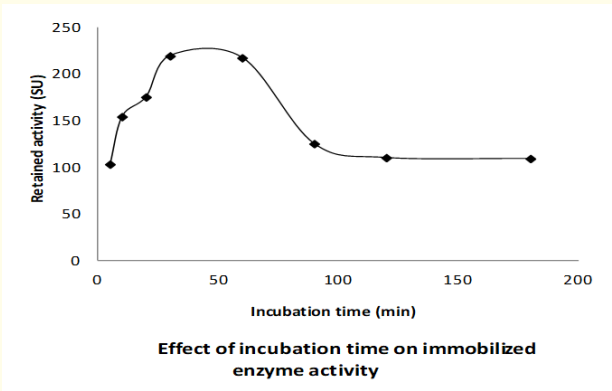

Figure 2

Influence of immobilization temperature

Effect of temperature (20-50^{0}C) on immobilization process was studied. Results (Figure 3) showed that 30°C was optimum for the highest immobilized enzyme activity. Thus all the next experiments were done at 30^{0}C. This is a promising result because this low temperature will decrease the cost of immobilization due to lower energy needed.

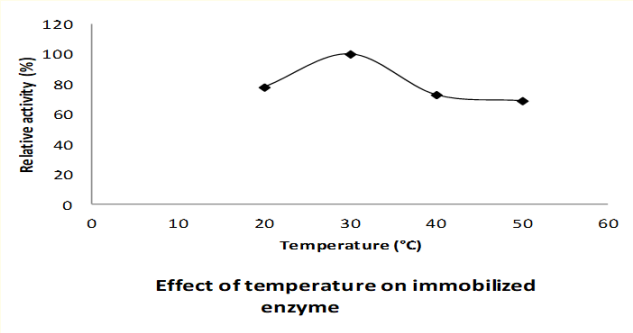

Figure 3

Characterization of free and immobilized

The immobilization process changes the original physicochemical properties and the kinetic parameters of the enzyme.

Thermal stability of the free and the immobilized MCE

To confirm the tolerance of the immobilized MCE towards high temperatures, its thermal stability was studied. Free and immobilized enzymes were incubated at different temperatures for 10 minutes and the residual activity was evaluated at 30°C. Immobilized MCE gave a higher thermal stability at all tested temperatures as shown in figure 4. It was observed that upon incubation for10 minutes at 60°C, the free enzyme retained 82% of its activity while the immobilized MCE displayed a residual activity of 100%. At 70°C after 10 minutes of enzyme heating, immobilized enzyme retained 80% of its activity while free form showed thermal inactivation to 47% of its activity. At 90°C, immobilized and free enzymes retained about 60 and 20 % of their activities, respectively indicating that the immobilized enzyme was more stable than the free one.

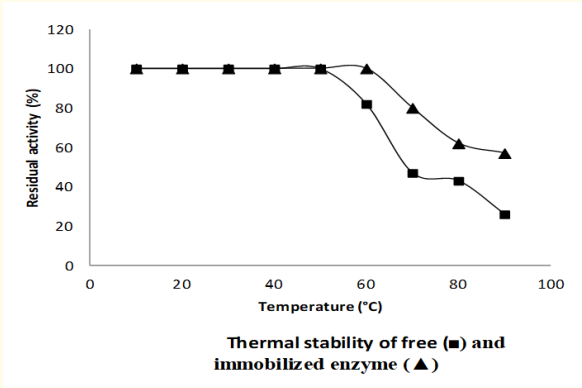

Figure 4

Thermal stability of the immobilized enzyme is the most important criteria with respect to industrial applications. Esawy and Combet-Blanc [20] reported that milk-clotting enzyme immobilized on Amberlite IR-120 showed a higher thermal stability compared to the free enzyme.

Effect of temperature on MCE activity

The influence of coagulation temperature on the free and the immobilized MCE was studied. It was found that increasing the temperature from 40 to 60°C increased the activity of both free and immobilized forms (Figure 5). At 60°C, this activity reached its peak value for free enzyme. With the immobilized enzyme it reached 70^{0}C (which corresponds to 100%). Thus, the optimum temperature for immobilized MCE is higher by 10°C. The influ-

ence of temperature depends on the type of rennet. The optimum temperature for the coagulation of milk by calf rennet at pH 6.6 is 45–48°C; presumably, the optimum for the hydrolysis of κ-casein is about this value [21].

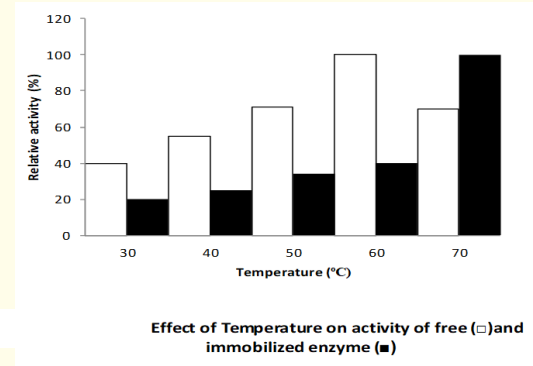

Figure 5

In comparable with our results, Milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341 had an optimum temperature of 55°C for activity, however, the entrapped enzyme was active at a higher temperature (60°C) [22].

Effect of pH value on MCE activity

The effect of pH on the activity of both free and the immobilized milk clotting enzymes was checked in the pH range of 3-8 at 30°C. As shown in figure 6, the maximum activity was found to be at pH 5 for both free and immobilized preparations. These results may due to the nature of the support material, (TCP) which occur naturally in cow milk.

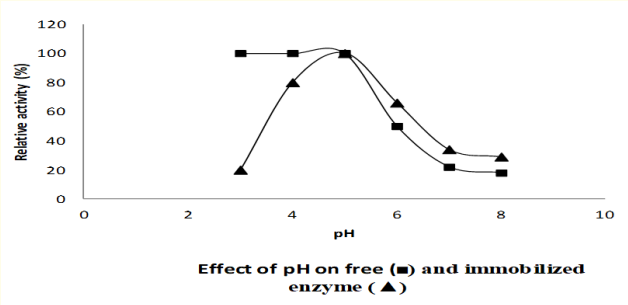

Figure 6

Both free and immobilized milk-clotting enzyme, showed neutral pH for their activity while, under slightly acidic condition (pH 6.5), the activity of the free enzyme decreased obviously compared to that of the immobilized enzyme. This was explained usually by a change of the ionic microenvironment of the enzyme leading to binding of enzyme and/or the chemical nature of the support [23].

Optimum pH for activity of milk-clotting enzyme from *Aspergillus oryzae* was found to be 6.3. Optimum pH for activity of the entrapped enzyme reached pH 6.9 while it was stable in the pH range 5-8 [22].

Ahmed., *et al.* [1] stated that the optimum pH of the immobilized protease showed acidic value (pH 5.5) than the optimum for free enzyme (pH 7.5). In contrast [24] found that immobilization of protease turned its optimum pH to more alkaline value.

At lower pH values than the optimum, the free enzyme was more stable than the immobilized one. However at higher pH values both free and immobilized enzymes showed decrement of activity. This decrease in activity at higher pH values was more in free enzyme than the immobilized enzyme.

Conclusion

In this study, milk clotting enzyme produced by *Rhizomucor miehei* NRRL 2034 under solid state fermentation was efficiently immobilized on tricalcium phosphate (TCP). We recommended this immobilized MCE as a promising candidate for cheese manufacture In addition, a significant improvement in pH, thermal, and storage stabilities was experimentally achieved compared to the free enzyme.

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