POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

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Pol. J. Food Nutr. Sci.
2009, Vol. 59, No. 1, pp. 67-72

EFFICIENT IMMOBILIZATION OF MILK CLOTTING ENZYME PRODUCED BY *BACILLUS SPHAERICUS*

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Key words: *Bacillus sphaericus*, immobilization, milk clotting enzyme, silica gel

Milk clotting enzyme produced by *Bacillus sphaericus* NRC 24 was immobilized efficiently on silica gel with 73% activity retained. Optimum conditions for immobilization of milk clotting enzyme were as follows: pH 5, 4 h contact time and 0.5 mg/mL protein in bulk solution. Optimum pH of the immobilized enzyme was found to be 4 compared to 6 for free enzyme. Optimum temperature ranged from 60° to 70°C for both free and immobilized enzyme preparations. The activation energy (E_a) of the immobilized enzyme was lower than that of the free enzyme (E_a=12.5 and 16.5 Kcal/mol, respectively). Immobilized enzyme showed improved pH, thermal, storage and operational stabilities.

INTRODUCTION

Since the recovery yield and reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization which may offer advantages over soluble native or modified enzymes [Tischer & Kasche, 1999]. In fact, insolubility of immobilized enzymes allows for: (i) continuous operation of the enzymatic processes, (ii) controlled product formation, (iii) rapid termination of reactions, (iv) easy separation from reaction mixture, (v) improved enzyme stability, and (vi) greater variety of engineering designs [Cao *et al*., 2003].

The immobilization of the enzymes by adsorption onto an inorganic support has been proposed for simplicity, low cost and preservation of the enzyme/substrate specificity [Malcata *et al.,* 1990]. Among the existing supports, silica gel deserves a special attention due to the fact that this matrix presents a high thermal, chemical and mechanical stabilities as well as high surface area (around 800 m^2/g, [http://en.wikipedia.org/wiki/Silica gel]) [Kermasha *et al*., 2000; Nagata *et al.,* 1998]. Silica gel is an amorphous inorganic polymer having siloxane groups (Si-O-Si) on the inside and silanol groups (Si-OH) on its surface. These silanol groups react with distinct ionic species [Kondo *et al.,* 1993].

Use of immobilized enzymes in cheese production has been considered to be a promising approach [Shah *et al.,* 1995]. Several attempts have been made to carry out the milk clotting process in continuous systems using immobilized enzymes. Some of the first results showed that the milk clotting process of pepsin immobilized on protein-coated glass was actually due to a leaching of soluble enzyme as reported by Curcio *et al.* [2000]. Rennet was immobilized on sand and on Sepharose-4B without any appreciable leaching while a significant decrease in milk clotting activity (MCA) was noticed during the first hour of operation [Curcio *et al.,* 2000]. The same observation was reported by Mashaly *et al.* [1988] for the clotting activity of immobilized calf rennet, pepsin and milk clotting enzyme (MCE) of *Rhizomucor miehei*, on agarose beads. The immobilization of different milk clotting enzymes, such as papain, trypsin, chymotrypsin, pancreatin and chymosin (enzymes of animal origin) on aminopropyle-glass beads were stable both in a batch and continuous stirred tank reactor [Haque & Mozaffar, 1992]. There are numerous publications about immobilization of pepsin on different supports such as alumina, titania, glass, stainless steel, Teflon, ion oxide, poly methyl methacrylate, salicylic acid-resorcinol-formaldehyde resin, Sepharose-4B, agarose, chitin and chitosan [Ahmed, 2003; Altun & Cetinus, 2007; Frydlova *et al.,* 2004; Han & Shahidi, 1995; Kurimoto *et al.,* 2001; Li *et al*., 2004; Shah *et al.,* 1995; Ticu *et al*., 2005]. Literature data about immobilization of MCE of microbial origin are rather scarce. Recently, MCE produced by *Bacillus circulans* 25 and *Bacillus licheniformis* 5A1 has been successfully immobilized on chitosan and Amberlite IR-120, respectively [Ahmed, 2003; Esawy & Combet-Blanc, 2006].

Due to the lack of information on immobilization of MCE of microbial origin, and no study found on immobilization of this enzyme on silica gel, the present study was undertaken to investigate possible immobilization of new MCE produced by *Bacillus sphaericus* NRC 24 by adsorption on silica gel. The process parameters that affect the immobilized enzyme system were studied to evaluate its potential as a possible candidate for future industrial application.

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MATERIALS AND METHODS

Materials

Silica gel 60H (5-40 μm) was purchased from E-Merck Ltd. (Germany). Skim milk was purchased from local market. All chemicals used were of analytical grade and were purchased from Sigma (USA), BDH (UK) and Fluka (Switzerland).

Microorganism

An Egyptian isolate, *Bacillus sphaericus* NRC 24, was used in this study. It is a new and promising MCE producer. Characterization of the crude and purified enzyme has been presented elsewhere [El-Bendary, 2004; El-Bendary *et al.,* 2007].

Enzyme production

Inoculum was prepared by inoculating nutrient broth medium with a loopful of bacterial culture and incubated overnight under shaking conditions.

Fodder yeast medium (25 mL in 250 mL Erlenmyer flasks) which contained 4.0% fodder yeast, 0.001% $MnCl_2$, 0.01% $CaCl_2$ and 0.02% $MgCl_2$ was inoculated with 2.4×10^6 CFU of an overnight-culture and incubated for three days at 30°C on an orbital shaker at 150 rpm. The cells were harvested by centrifugation at 6000 $\times g$ for 20 min and the supernatant (crude enzyme source) was used for immobilization experiments.

Enzyme immobilization

Immobilization of MCE on silica gel by adsorption technique was employed according to Das & Prabbu [1990]. Two hundred milligrams of the support were stirred in 0.1 mol/L phosphate buffer (pH 6.0) with 5 mg of the enzyme in a total volume of 5 mL for 6 h at 4°C. The adsorbed enzyme was harvested by centrifugation at 4000 $\times g$ for 10 min, washed with a small amount of distilled water to remove free enzyme and finally with 0.1 mol/L phosphate buffer (pH 6.0).

The degree of immobilization (DI) and activity retained (AR) are important parameters to develop bioprocess using immobilized enzyme. They were calculated using the following equations [Chae *et al.,* 2000; Han & Shahidi, 1995]:

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

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

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.

Optimum pH for immobilization

Effect of pH value of the bulk solution on the immobilization process was studied by varying the pH of the immobilization mixture between 4 and 8.

Optimum contact time

The optimum contact time was determined by stirring the bulk solution for different intervals (1-16 h).

Optimum enzyme concentration

Effect of the enzyme concentration was studied by using different amounts of the enzyme (1.2 – 6.3 mg) in the immobilization mixture.

Protein estimation

The amount of immobilized protein was estimated by subtracting the amount of protein determined in the supernatant after immobilization from the amount of protein which was used for immobilization. The protein concentration was determined by the method of Ohnistti & Barr [1978].

Milk clotting activity

MCA was assayed as described by Greenberg [1957] with some modification. The enzyme source (0.1 mL of free enzyme or 25 mg of immobilized enzyme) was added to 2 mL of substrate solution (12% skim milk powder in 0.01 mol/L $CaCl_2$). The time necessary for the formation of curd fragment was measured. MCA was expressed in terms of Soxhlet unit.

Soxhlet units were calculated using the following equation: Soxhlet unit = $M\times35\times2400/ E\times t\times T$, where M is the volume of substrate (mL), E is the enzyme concentration (mg), t is the clotting time (sec) and T is the reaction temperature (°C).

Each experiment was repeated twice and assayed in triplicate.

Protease activity

Protease activity was measured using casein as a substrate. One mL of 1% casein in phosphate buffer (pH 7) and 50 mg of immobilized enzyme were incubated at 40°C for 30 min. The reaction was terminated by addition of 2 mL of 15% trichloroacetic acid solution and was centrifuged at 6000 $\times g$ for 10 min. To 1.0 mL of supernatant, 5.0 mL of 0.5 mol/L of sodium hydroxide and 0.5 mL of Folin reagent were added, followed by 30 min incubation at room temperature. Absorbance was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme, which released 1 μg of amino acid equivalent to tyrosine under the assay conditions.

pH and temperature profiles

Effect of pH value of the substrate solution on MCA of both free and immobilized forms was analysed at pH range of 3.5-8 by using appropriate buffer solutions.

MCA was determined at different temperatures ranging between 25 and 80°C.

The activation energy (E_a) was determined from the slope of Arrhenius plot of log the enzyme activity (V_M) according to 1/T where, T is the temperature in degrees Kelvin. E_a was determined from the following relation: Slope = E_a / 2.303 R, where E_a is the activation energy, R is the gas constant (R = 1.987 cal/mol).

pH and thermal stabilities

pH stability of free and immobilized enzymes was ascertained by measuring the residual activity of the enzyme incubated at various pH values (4-8) for 48 h at 25°C.

The thermal stability of free and immobilized enzyme was determined by measuring MCA after heating the enzyme at various temperatures (25-80°C) for ten minutes.

Reusability and leaching of the enzyme

Immobilized enzyme (one gram) was put on a wound sheet hung with a thread in order to make a capsule shape. Then, one capsule was put into 10 mL of skim milk and kept at 15°C under stirring condition for 5 min. At the end of the reaction the capsule was removed and put into new 10 mL of fresh skim milk solution to start a new reaction. The clotting time was recorded for these enzymatic reactions at 60°C.

Soluble activity, clotting activity caused by desorbed enzyme, was determined by adding 2 volumes of the whey from the coagulated milk sample in each cycle to one volume of fresh milk solution. MCA in these mixtures was assayed as an index of leaching of the enzyme.

Storage stability

For testing the storage stability, both free and immobilized enzymes were stored at 4°C and room temperature (25°C) for 60 days. The activity was measured every week.

Biocatalysis of immobilized-MCE in a continuous system

Clotting of milk takes place in two stages. In the first enzymatic stage, proteolysis takes place, and this is followed by aggregation of micelles producing clotting in the secondary stage. The first stage of proteolysis was carried out at 15°C and the secondary stage of clotting was carried out at 60°C. The immobilized enzyme was used in 1x10 cm double jacketed glass column reactor. A low pressure drop was obtained and resistance to plugging problems was eliminated through the use of a fluidized-bed reactor. The fresh skim milk solution was pumped through the reactor by means of a peristaltic pump at a flow rate of 2 mL/min. The temperature of the reactor was maintained at 15°C. Fractions (10 mL each) of milk emerging from the reactor were collected in containers for two hours and assayed for enzyme activity.

RESULTS AND DISCUSSION

Immobilization of MCE on silica gel

Milk clotting enzyme produced by *Bacillus sphaericus* NRC 24 was efficiently immobilized on silica gel with 70% immobilization degree and 73% activity retained. The specific activity of the immobilized enzyme (328 U/mg protein) was 13% higher than that of the free enzyme (291 U/mg). Silica gel has been reported as a good carrier for enzyme immobilization since: (i) it is a cheap support, (ii) it has good mechanical properties, (iii) it has high thermal and pH stabilities, and (vi) it is resistant to microbial attack [Godjevargova *et al.,* 2006].

Mashaly *et al.* [1988] reported that MCA of immobilized calf rennet, pepsin and MCE of *Rhizomucor miehei* on agarose beads decreased as the reaction proceeded. Curcio *et al*. [2000] reported that the most active immobilized rennin resulted from using river-bed sand as a carrier without any appreciable leaching. MCE produced by *Bacillus circulans* 25 and *Bacillus licheniformis* 5A1 were efficiently immobilized on chitosan and Amberlite IR-120 with reserved 64.6%, and 100% of their original activities, respectively [Ahmed, 2003; Esawy & Combet-Blanc, 2006].

Optimum conditions for the immobilization process

Effect of pH value

The obtained results revealed that the optimum pH value for enzyme immobilization on silica gel was pH 5 (Table 1). At alkaline pH values, drastic decrease in immobilization rate was obtained. The lower immobilization rates at higher pH value could be associated with the deactivation of the reactive groups of the support under these conditions. Ahmed [2003] reported that the optimum pH for immobilization of MCE on chitosan was pH 6.

Effect of contact time

AS shown in Table 1, the optimum time required for the immobilization of MCE on silica gel was 4 h. After 16-h contact time the immobilization rate was decreased to 76.2%.

Effect of enzyme concentration

The effect of initial protein concentration in the bulk solution on immobilization rate was determined by incubating the enzyme with support for 4 h at pH 5. Optimum enzyme concentration was 2.5 mg protein (0.5 mg/mL). Doubling the enzyme concentration resulted in a decrease in the immobilization rate up to 50%, as shown in Table 1.

Characterization of immobilized MCE

Proteolytic activity of immobilized enzyme (PA)

The ratio of milk clotting to proteolytic activities (MCA/PA) was assumed to be an important index for MCE selection and application in cheese industry. It was observed that

TABLE 1. Effect of pH values, contact time and enzyme concentration on immobilization of MCE.

Effect of	Relative activity (%)
pH values	
4.0	81.5
5.0	100
6.0	79.7
6.5	65.2
7.0	48.9
8.0	29.3
Contact time (h)	
0	0.0
1	54.5
2	81.4
4	100
6	96.0
16	76.2
Enzyme concentration (mg)	
1.2	91
2.5	100
3.7	79
5.0	50
6.3	50

the immobilized enzyme had fairly weak proteolytic activity. The proteolytic activity was 5.3 μg tyrosine/mg protein and MCA/PA ratio was 62. High MCA/PA ratio indicates that the immobilized MCE produced by *Bacillus sphaericus* could be used as a promising candidate in industrial application.

pH and temperature profiles

Optimum pH of the immobilized MCE was 4 as shown in Figure 1. Free enzyme showed bad, non-firmed clots at pH 3.5-5, while at pH 6-8, good curd firmness was evident and optimum pH was 6 (data not shown). Shifts in the optimum pH with immobilization have been reported for many enzymes [Altun & Cetinus, 2007]. This was explained usually by a conversion of the ionic microenvironment of the enzyme resulting in the adsorption of the enzyme and/or the chemical nature of the support [Chellapandian, 1998].

The results in Figure 2 showed that the immobilized and free enzyme exhibited the maximal relative activity at 60-70°C. The activation energy (E_a) of the immobilized MCE (12.5 kcal/mol) was lower than that of the free enzyme (16.5 kcal/mol). In a similar case, milk clotting activation energy decreased after immobilization on chitosan beads [Ahmed, 2003] and Amberlite IR-120 [Esawy & Combet--Blanc, 2006].

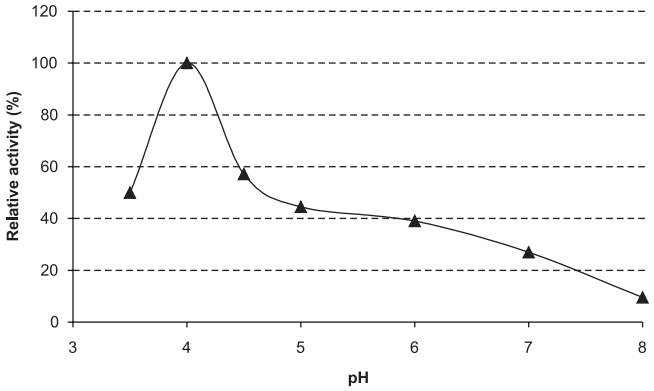

FIGURE 1. Effect of pH on MCA of the immobilized enzyme.

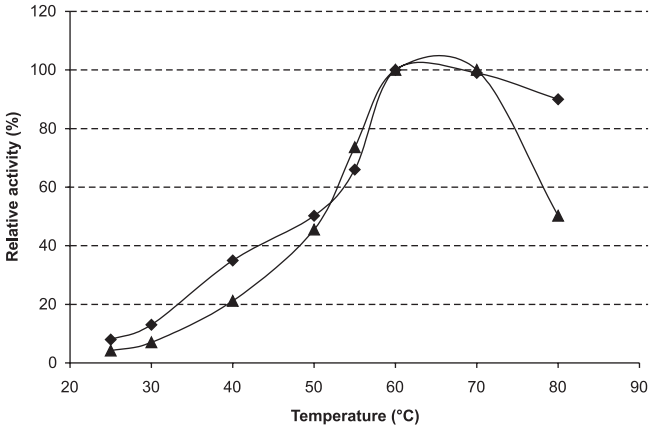

FIGURE 2. Effect of incubation temperature on MCA of free (■) and immobilized (▲) enzyme.

pH and thermal stabilities

In this study, MCE immobilized on silica gel showed a significant increase in pH stability compared to the free enzyme (Table 2). Immobilized enzyme retained 100% of its activity at pH 6 after 48 h. While, free enzyme lost about 53% of its activity at the same pH value. At higher pH values (7 and 8), the immobilized enzyme lost about 20% of its activity, whereas free enzyme lost about 60-70% of its activity. At lower pH values (4 and 5), the free enzyme was completely inactive; however, the immobilized enzyme retained about 40--50% of its activity.

Thermal stability of the immobilized enzyme was one of the most important criteria with respect to application. In this study, immobilized and free MCE showed high stability after heating at 25-50°C for 10 min (Table 2). At 60°C, almost all the activity of the free enzyme was lost after 20 min, whereas the immobilized enzyme conserved 63% of its original activity under the same conditions.

The immobilization can enhance the stability of enzymes by rigidification of their three-dimensional structures, which results in a higher resistance to conformational changes induced by pH, heat and organic solvents [Altun & Cetinus, 2007; Godjevargova *et al.,* 2006; Gupta, 1991; Jiang & Zhang, 1993; Kermasha *et al*., 2000; Shah *et al*., 1995].

Reusability and leaching of the enzyme

Because of the importance of repeated applications in a batch and continuous reactor, the reusability of immo-

TABLE 2. pH and thermal stabilities of free and immobilized enzymes.

Exposure to	Residual activity (%)	
	Free enzyme	Immobilized enzyme
pH for 48h		
4.0	0.0	40
5.0	0.0	50
6.0	47	100
7.0	40	86
8.0	30	80
Temperature (°C) for 10 min		
25	100	100
30	100	100
40	100	100
50	100	100
60	50	81
70	0.0	18
80	0.0	0.0
Temperature at 60°C for (min)		
0.0	100	100
10	50	83
20	13	63
30	5.0	33
45	0.0	28
60	0.0	20

bilized enzymes is important for economical use. Immobilized MCE was active for three reuses and retained 50% of its activity after four cycles (Figure 3). It was found that there was no leaching of the enzyme after 6 cycles. Bickerstaff [1980] reported that the immobilization restricts the movement of the backbone and side chains of the enzyme molecule thereby preventing intermolecular interaction and unfolding of the polypeptide chain. These advantages increase the stability of the enzyme and retain its activity after repeated use. Ahmed [2003] and Esawy & Combet-Blanc [2006] reported that the immobilized MCE could be used for 5 cycles without the loss of its activity.

Storage stability

One of the most important characteristics of the immobilized enzyme is the storage stability. The results in Figure 4 indicated that the immobilized enzyme was quite stable when stored as a moist cake at 4°C and room temperature

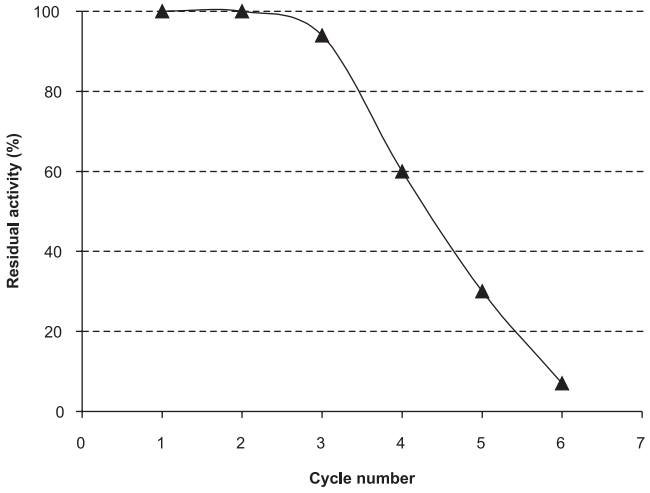

FIGURE 3. Reusability of immobilized MCE.

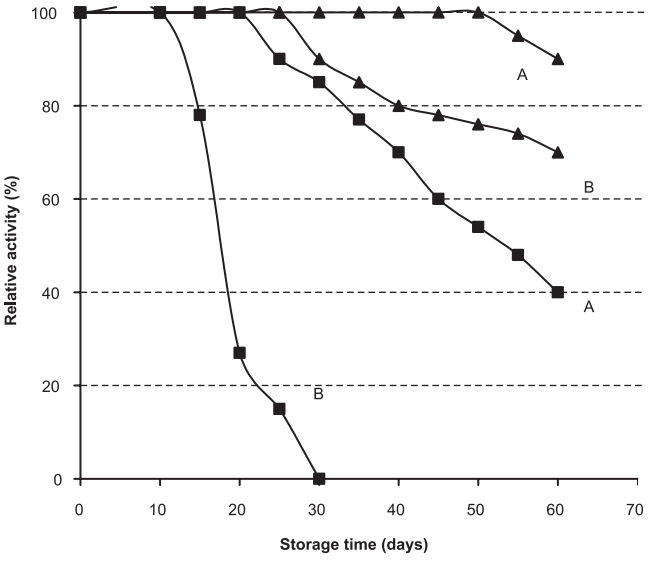

FIGURE 4. Storage stability of free (■) and immobilized (▲) enzyme at 4°C (A) and at room temperature (B).

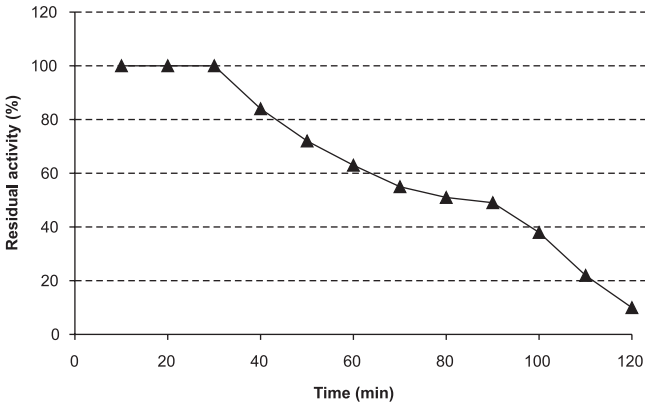

FIGURE 5. Biocatalysis of immobilized MCE in a continuous system.

(25°C). A 60% loss of activity was observed for the free enzyme after storage for 60 days at 4°C. This might be due to protein-protein interaction (autolysis) [Kumar & Gupta, 1998; Sharma *et al.,* 2003; Zhang *et al.,* 2001]. On the other hand, MCE immobilized on silica gel lost only 10% of its activity under the same conditions. This enhanced stability is probably a result of the prevention of autolysis by immobilization.

At room temperature (25°C), the free enzyme completely lost its activity in 30 days while the immobilized enzyme still retained about 70% of its activity after storage for 60 days. Goradia *et al*. [2005] reported that trypsin immobilized onto mesoporous silicates was able to retain 80% and 60% of its original activity after 30 days at 4°C and 25°C, respectively. Pepsin immobilized on resinous material showed 50% activity retention even after three months of moist storage [Shah *et al.*, 1995].

Biocatalysis in a continuous system

Figure 5 shows the relative activity of the immobilized enzyme in continuous system for 120 min. The relative activity was maximal during the first 30 min and continued to decrease gradually with time obtaining a minimum at 120 min. Kermasha *et al*. [2000] reported that the specific activity of the immobilized chlorophyllase in a continuous system was maximal during the first 5 min of reaction, after that it continued to decrease obtaining a minimum at 35 min.

The results of the continuous reactor showed that the immobilization of MCE on silica gel could be used as a prelude for further investigation of potential industrial applications.

CONCLUSIONS

Milk clotting enzyme produced by *Bacillus sphaericus* NRC 24 was successfully immobilized on amorphous inorganic polymer, silica gel by simple adsorption with 70% immobilization degree and 73% activity retained. With its high MCA/PA ratio, we recommended this immobilized MCE as a promising candidate for industrial application. In addition, a significant improvement in pH, thermal, and storage stabilities was experimentally achieved compared to the free enzyme.

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Received February 2008. Revision received July and accepted November 2008.