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Immobilization of Rhizomucor miehei Lipase on High Density Polyethylene

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Abstract

Immobilization of Lipase produced from Rhizomucor miehei on HDPE fine powder was investigated. As compared to an aqueous system, immobilization in a non-aquous organic medium such as n-hexane was not successful and caused enzyme denaturation. Prewetting the support with ethanol increased the immobilized protein and enzyme activity as much as 31% and 34%, respectively. The maximum immobilized activity was obtained at the isoelectric pH of 4-5. The enzyme was suspected to have competition and/or interaction with other protein entities on the surface. Immobilization of the enzyme onto the support seems to be via shear sensitive weak physical adsorption. Proper duration of mixing was found to be around 6 minutes. Longer periods of shaking led to enzyme desorption, thereby reducing the immobilized activity. Neither efficiency nor stability was improved using glutaraldehyde as a cross-linking agent despite the fact that in some occasions, protein loading of the support was improved. This suggests the possible effect of glutaraldehyde on enzyme denaturation in these conditions. At optimum conditions, immobilized enzyme activity was enhanced almost 6-folds increasing from 8 units (per 0.5 ml of the enzyme liquor) to about 45.8 units (when 0.5 ml was immobilized on one gram of support).

Keywords: *Enzyme Immobilization; Rhizimucor miehei Lipase; HDPE Powder*

Introduction

Lipases (triacylglycerol ester hydrolase, E.C.3. 1.1.3) as a major group of industrial enzymes have been used as the catalytic agents for the hydrolysis/synthesis of triacylglycerols and esters. Lipases are very useful enzymes in organic chemistry. Successful commercial ap-plication of enzymes, and in particular, im-mobilized preparations, is due to taking an appropriate approach to solve the enzyme's instability problem, which may be considered as one of the inherent characteristics of these biocatalysts. Immobilization of enzymes is claimed to improve the enzyme stability in many cases [1,2,3]. Moreover, immobiliza-tion of enzymes facilitates its reuse, which in turn, provides a logical way to conduct continuous operations [4-7].

Different methods have been proposed for enzyme immobilization so far. Among these, adsorption has gained popularity because of its simplicity and low cost. However, weak forces in the adsorption and possible enzyme desorp-tion have imposed some constraints on their successful application. This phenomenon of course is not serious in organic media in which the proteins are not

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soluble. Enzymes used in these studies include those prepared from species such as Candida cylindraceae, Candida rugosa, Rhizopus oryzae. These lipases have been immobilized on carriers such as activated cellulose, polyethylene, polypropylene, silica gel, PVC and Celite [8,9]. It has often been claimed that enzyme activity and/or stability is enhanced through immobilization [10-14]. Among the numerous available supports, hydrophobic carriers appear to be very suitable for such applications as hydrolysis of fats and oils or transesterification reactions [3].

Hydrophobic microporous polyethylene powder has been used as an appropriate support in both organic and aqueous media, and received much attention due to their characteristics, including high surface area, proper pore size, cheapness and availability [3]. However, using the internal surface area of a support may introduce mass transfer limitations. One possible solution to lessen the magnitude of this problem is to grind the support down to a fine mesh size so that a large surface area is obtained externally without imposing mass transfer limitations.

In this work, fine powder of high-density polyethylene was used to immobilize a commercial microbial lipase preparation. Immobilization was carried out by physical and chemical means of adsorption. Effects of parameters such as support prewetting, pH, medium composition, mixing time and enzyme-to-support ratio in the reaction medium on the efficiency of immobiliza-tion were investigated.

Materials and Methods

Enzyme

The enzyme used in this study was a commercial liquid preparation (NOVO, lipozyme 100 ml, 1000 LU/mg the reported unit was based on μmol butyric acid liberated by tributyrin hydrolysis per minute at 30 ºC and pH=7, using gum arabic as emulsifier [24]). In this work, unit of activity was measured and reported based on the method by Yamada, *et al*. [17].

HDPE and Other Reagents

The commercial HDPE (High Density Polyethylene) used in this study was a non-porous product purchased locally (Bandar Imam Petrochemical Complex, Iran), and ground to a mesh size of 80–1000. Olive oil and ethanol (98%) obtained from the local market were of commercial grade. Poly vinyl Alcohol (PVA), glutaraldehyde 25% , n-Hexane and all other reagents were of reagent grade (Merck, Germany).

Immobilization

In the physical immobilzation of aqueous enzyme solution on the hydrophobic HDPE support, an ethanol prewetting protocol was adopted [2,3,10,14,15]. To do this, 1 g of polyethylene powder was rinsed in 6 ml of ethanol for 15 minutes and then mixed with the enzyme and buffer solution after decantation. It was then filtered using the Wattman no. 2 filter paper and washed with 100 ml of distilled water. Various parameters influencing this procedure including media composition, enzyme- to- carrier ratio, mixing time and a range of pH (3-9) were studied and optimized in this research. Buffers used were all 20 mM and included citrate-phosphate [3-6], phosphate [6-8] and tris HCl [8,9]. Protein was measured using the Lowry method [16]. The amount of protein fixed onto the carrier was calculated by subtracting the filtrate protein from that of the mother liquor.

In the chemical method of adsorption, a suspension containing 1 g of prewetted polye-thylene powder and 0.5 ml of lipozyme in 4.5 ml of buffer (pH = 5) was shaken for 6 min. Then, glutaraldehyde was added to the mixture at various ratios of 0.05, 0.1, 0.5 and 1 percent of the medium (v/v). Shaking continued for 1 h at room temperature. Suspension was then filtered and the retentate was let to dry for 2 days in room temperature

after washing with 100 ml of distilled water.
To determine the reusability of the immobilized enzyme, a sequence of enzyme activity measure-ments was carried out in 3 steps. In the first two steps, a duplicate run was conducted such that one run was completed to the enzyme activity measure-ment protocol and the other run was terminated after completion of the hydrolysis without addition of acetone-ethanol mixture and the immobilized enzyme was filtered, washed with 100 ml of distilled water and dried in room temperature for two days to give the proper enzyme amount for the next step. This method ruled out the ambiguity of convolution of the adverse effect of aceton-ethanol mixture on the immobilized enzyme activity with that of the natural activity loss of the enzyme.

Measurment of Enzyme Activity

One volume of olive oil was blended just before use with 3 volumes of a 2% aqueous solution of PVA. 5 ml of this mixture was added to 1 ml of enzyme and 3 ml of buffer (phosphate, pH=7) and incubated at 37 °C in a water bath for 15 minutes. 20 ml of a 1:1 mixture of acetone and ethanol was then added to stop the reaction and break the emulsion. The mixture was titrated with 0.05 N aqueous sodium hydroxide using 1% phenolphethalein in ethanol as an indicator. One unit of activity was defined as the amount of enzyme which liberates one μmole of oleic acid from the above-mentioned mixture. Activity of the used commercial lipozyme measured by this method was 16 U/ml.
The immobilized enzyme activity was measured similar by except that instead of 1 ml of enzyme solution, 0.4 g of immobilized enzyme was used. Moreover, the mixture was mixed gently at 50 rpm during hydrolysis in the water bath. Efficiency of immobilization was defined as the ratio of the immobilized enzyme activity and the activity of the original soluble enzyme before immobiliza-tion.

Results and Discussion

In immobilization practice on a fine solid powder, the early concern is dispersion of the particles in a liquid media to facilitate the spread of the enzyme molecules onto its surface. Some possible choices in this regard could be distilled water, buffered solutions or alternatively, a hydrophobic hydrocarbon such as n-hexane. This non-polar solvent has been reported to have a positive effect on the stability of lipases [18]. To check the idea, 4.5 ml of the liquid media were mixed with 1 g of the polyethylene support and 0.5 ml of the lipase was added later on. Immobilization was allowed to proceed for 30 min at room temperature and a shaker speed of 70 rpm. In the case of n-hexane, the im-mobilized preparation was filtered with difficulty. After filtration, the retentate was washed with 100 ml of distilled water and dried at room temperature for about 2 days.

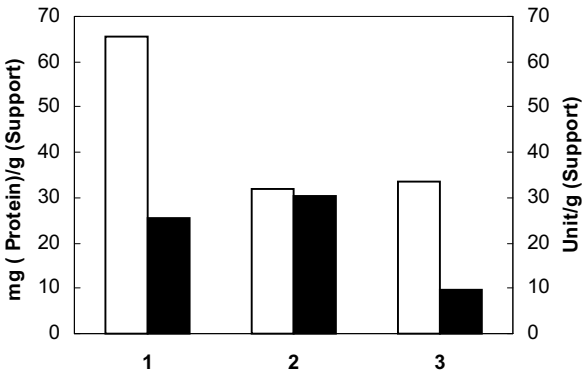

Figure 1. Protein and enzyme activity loading on HDPE fine powder using distilled water (treatment 1), citrate-phosphate buffer of pH=5 (treatment 2) and n-hexane (treatment 3), (□ is mg (Protein)/g (Support), ■ is Unit of activity/g (Support))

As illustrated in Figure 1, a lower activity was obtained with the preparation im-mobilized using n-hexane as compared to distilled- and buffered water.
Since the enzyme is insoluble in n-hexane, it may appear that the addition of enzyme may have caused its denaturation. In fact, it was observed that upon addition of the enzyme solution to n-hexane, the brown color of the Lipozyme vanished instantaneously and tiny

coagulated particles were seen to originate at the site of addition and stick to the glass flask bottom.These observations have also been reported by other researches in the literature [18].

It is believed that the structure of lipases is amphiphilic with a minor segment being hydrophobic [19,20]. Therefore, one may expect that proper treatment of the hydrophobic HDPE would increase the immobilization efficiency of the overwhelmingly hydrophilic enzyme. One possible pretreatment in this regard could be prewetting it with suitable polar organic solvents. Table 1 presents the data col-lected in studying immobilization of *C. rugosa* lipase on polypropylene, with the support being prewetted with pure organic solvents such as ethanol, acetone, acetonitrile, 2-propanol and methanol [2]. As shown in table 1, the yield of immobilization may increase several times upon solvent pretreatment.

Table 1. Effect of pretreatment of polypropylene support using various solvents[2]

Solvent used in pretreatment of supprt	Residual soluble activity and protein		Yield of Immobilized Activity (%)
	Activity(%) Protein(%)		
None	44.4	68	3.6
Methanol	0.05	22	12.6
Ethanol	0.16	13	15.2
2-Propanol	0.83	12	12.5
Acetone	0.16	16	16.3
Acetonitrile	0.21	25	13.0

In contrast, in another report, immobilization of *Candida cylindracea* lipase on polypropylene with the support being prewetted with polar solvents including ethanol, isopropanol, methanol, acetone and tetrahydrofuran has been shown to have only a minor positive effect on the adsorption efficiency [21]. Nevertheless, kinetics of enzyme adsorp-tion on the support has been claimed to improve in that report.

Figure 2 illustrates the effect of ethanol on the immobilization of *R. miehei* lipase on the prewetted HDPE support. Treatment of the support has improved immobilization, indeed. The improvement factor for the protein content and activity of the enzyme are approximately 31% and 34%, respectively.

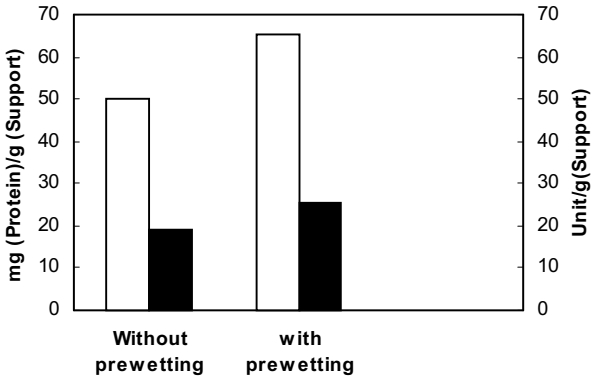

Figure 2. Protein and enzyme activity loading on HDPE fine powder with and without ethanol prewetting , (□ is mg (Protein)/g (Support), ■ is Unit of activity/g (Support))

Assuming that other protein entities are present in the enzyme liquor, this observation implies that improvement of adsorption on the support was not protein- selective. Based on this observation, ethanol prewetting was found to be beneficial and included in the immobilization procedure in this work hereafter.

To see the effect of pH, 0.5 ml of the enzyme liquor was diluted to 5 ml in buffer solution and was immobilized on the support prewetted using ethanol. Data collected in this experiment is presented in figure 3. As expected, the maximum immobilized enzyme activity was obtained at pH 4-5. That coincides with the isoelectric point of this enzyme which happens at pH 4-5 [18]. Noticing the non-polar nature of the support, the largest amount of the enzyme immobilization may be expected at neutral status with higher hydrophobicity of the protein [22]. This finding was adopted to set the pH in immobilzation practice of this study using

citrate-phosphate buffer (pH=5).

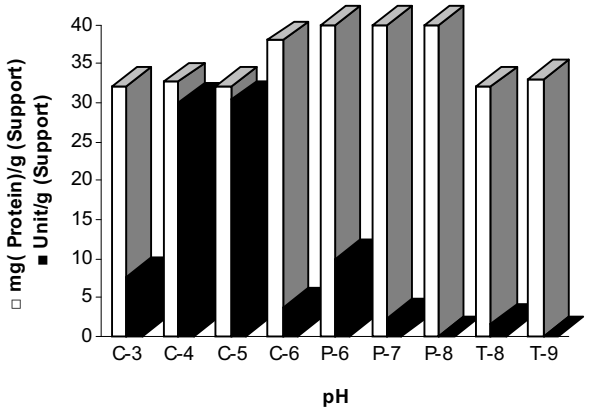

Figure 3. Protein and enzyme activity loading on HDPE fine powder at different values of pH (C is citrate-phosphate buffer, P is phosphate buffer, Tris-HCl buffer), (□ is mg (Protein)/g (Support), ■ is Unit of activity/g (Support))

The thermodynamic equilibrium of adsorption of the enzyme on the support was checked using 0.5 ml of the enzyme liquor diluted to 5, 10 and 15 ml buffer (pH=5).

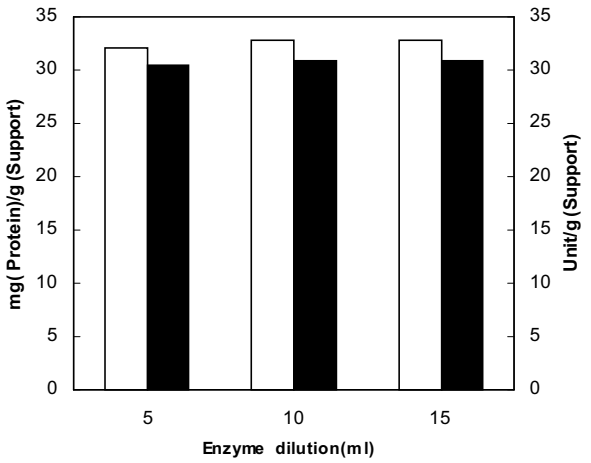

Figure 4. Protein and enzyme activity loading on HDPE fine powder at different enzyme dilutions, (□ is mg (Protein)/g (Support), ■ is Unit of activity/g (Support))

According to Figure 4, no significant change in the protein and activity loading was detected at various dilutions.

In a follow-up experiment, different amounts of lipozyme in the range of 0.1–2 ml were diluted to 5 ml in citrate phosphate buffer (pH=5) and were immobilized on 1 g of prewetted support. Figure 5 shows how the two parameters of enzyme and protein loading of the support respond to the changes implemented in the liquor.

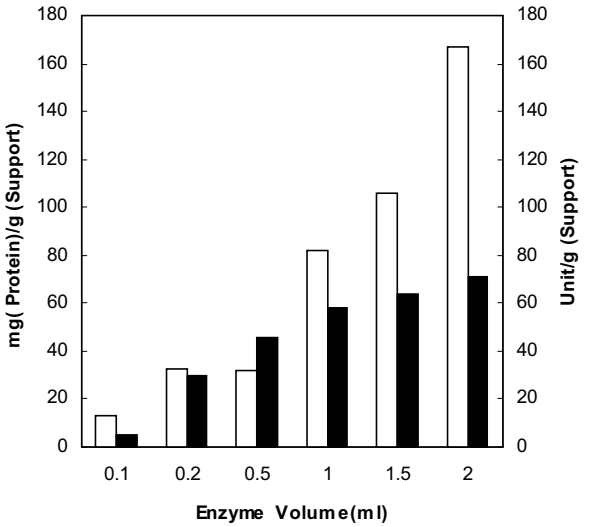

Figure 5. Protein and enzyme activity loading on HDPE fine powder at different enzyme amounts, (□ is mg (Protein)/g (Support), ■ is Unit of activity/g (Support))

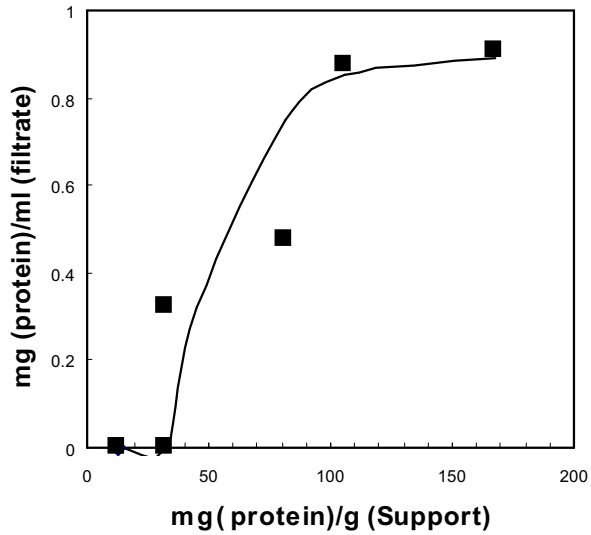

Figure 6. protein in filtrate versus protein loading on HDPE fine powder after immobilization of different enzyme amounts.

Assuming that adsorption of protein on the support is reversible, the amount of protein in

the medium would be in dynamic equilibrium with that adsorbed on to the support surface if all the surface has joined the interaction with the protein molecules forming a "Saturated" surface. If this hypothesis is valid, before the surface becomes saturated, more concentrated protein solutions are expected to lead to greater protein immobilization while after establishment of the surface saturation, increasing the protein concentration in the medium does not improve the loading of protein onto the particles.

Based on these grounds, one may expect a Michaelis-Menten type of the curve when investigating the effect of protein concentration in the liquor on the adsorbed protein on the support. Figure 6 illustrates data of Figures 4 and 5 when put on one single curve of soluble- versus adsorbed protein. As seen in this figure, coincidence of the curve with our expectation is fair except that in the vicinity of the origin, the curve lies on the X axis.

Figure 5 shows that up to 0.5 ml of lipozyme, both the adsorbed protein and immobilized enzyme activity increase upon an increase in the amount of enzyme. However, beyond this value no further increase is observed in the enzyme activity despite the fact that protein loading is increased monotonously. This leveling–off of activity at higher protein loading has been reported in the literature, as well [23].

This repeatedly checked observation may be described based on the enzyme-protein interac-tions on the support surface. Based on these grounds, one may speculate that the adsorbed enzyme on the support may have interactions with the other protein molecules on the surface such that beyond some specific concentrations, the negative effect of these interactions out-weighs the positive effect of the increase in the enzyme concentration on the surface.

Another explanation for the above-mentioned phenomenon may be developed based on the presence of a number of non-lipase proteins in the enzyme liquor. In this case, a competition may be expected between the lipase and other protein molecules in the system to settle on the Up to time of 6 minutes a mixing , the filtrate showed certain residual activity. In parallel, the amount of enzyme that was adsorbed onto the support showed an increase with time. After 6 min of mixing, no activity was found left in the filtrate indicating total adsorption of the enzyme onto the support. Beyond this time, there was a reduction in the immobilized activity. This may imply that a longer support surface. Therefore, at conditions prevailing above 0.5 ml preparations, more and more non-lipase molecules adsorb on the surface decreasing the availability of the support surface for the lipase.

Another trivial question as to the efficiency of immobilization is the proper time of mixing of the enzyme solution with support. It seems that the already adopted time of 30 minutes is more than enough in this respect. To investigate this effect, 1 g of prewetted polyethylene powder was shaken with 5 ml of medium containing 0.5 ml of lipozyme in 4.5 ml of citrate-phosphate buffer (pH=5) for different periods and collected data were presented in Figure 7.

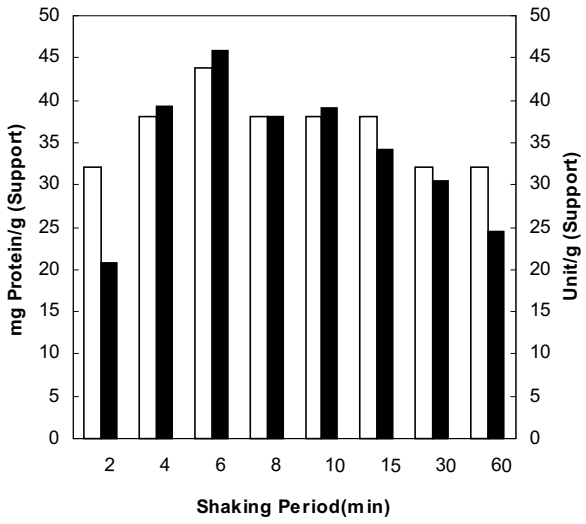

Figure 7. Protein and enzyme activity loading on HDPE fine powder after different shaking periods, (□ is mg (Protein)/g (Support), ■ is Unit of activity/g (Support))

period of shaking may lead to enzyme desorption due to prevailing shear stress [18]. Aside from the physical adsorption of enzymes onto supports, various chemical means could be adopted to increase the enzyme-support link. One possible choice is establishing a covalent bond using active agents such as glutaraldehyde. This should of course be carefully practiced to avoid any possible negative effect of the chemical agents on the enzymatic activity [2,12]. As previously reported, treatment with glutaraldehyde seems to stabilize the interactions of the enzyme with the support, although this probably decreases the conformational adaptability of the enzyme necessary for the conversion of the substrate [2,12,13].

In studying immobilization of *Candida rugosa* on ethanol-pretreated polypropylene, using 0.5%(v/v) glutaraldehyde decreased the original activity of the adsorbed enzyme by 80%, but the resulting cross - linked enzyme retained 88% of ito activity in the second hydrolysis assay [2]. In another study, immobilization of *Candida cylindraceae* on polyethylene and poly-propylene supports has been investigated. It has been claimed that enzyme has been Up to a glutaraldehyde concentration of 0.5%, im-mobilized protein decreases as much as 40%. A similar trend was observed in the immobilized enzyme activity. However, in a repeatedly observed and hence justified event, at 1% glutaraldehyde concentration a sudden rise in total immobilized protein and fall in immobilized enzyme activity was detected.

Immobilized very efficiently on polyethylene especially when glutaraldehyde was used to crosslink the lipase after physical adsorption. The activity retained after five reuses (86% of that of the original soluble enzyme) indicated very good attachment onto the support. Without glutaraldehyde, the enzyme had retained 66% of the original soluble enzyme after being reused five times [12].

To check the effect of chemical cross-linking in this work, physical immobilization procedure was added by a chemical step using various amounts of glutaraldehyde in a range of 0.05 to 1 percent of glutaraldehyde in the final immobilization media. Figure 8 illustrates the effect of this follow-up protocol on the immobilization efficiency.

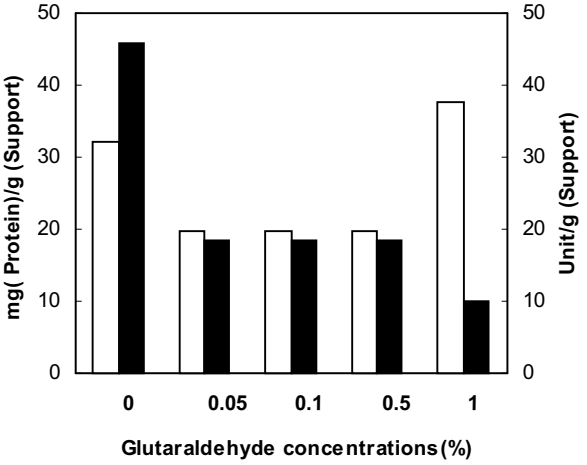

Figure 8. Protein and enzyme activity loading on HDPE fine powder at different glutaraldehyde concentrations, (□ is mg (Protein)/g (Support), ■ is Unit of activity/g (Support))

This may imply that at relatively higher levels of glutaraldehyde, the enzyme undergoes structural modifications which deteriorates its enzymatic activity while its immobilization tendency on the support is increased at the same time. One possible scenario in this case may be denaturation of the enzyme and loss of its solubility leading to percipitation on the support. Addition of glutaraldehyde had no positive effect on the immobilization efficiency in contrast to the results that has been reported elsewhere [2,12]. As presented in Table 2, glutaraldehyde had no positive effect on retaining the immobilized activity, either.

As observed in the table, the enzyme activity immobilized at the absence of glutaraldehyde falls to 68% of its initial value after the first recovery and 65% after the second one. The correaponding figures in the presence of glutaraldehyde are 60% and 50%,

respectively. Therefore, loss of immobilized activity is more serious when glutaraldehyde treatment was incorporated in the im-mobilization procedure. As described before and according to one hypothesis, glutaral-dehyde may have an effect on the partial denaturation of the enzyme such that part of the immobiliza-tion may be via percipita-tion of the insoluble enzyme on the support. If this is the case, removal of percipitated enzymatic activity upon recovery may be expected in subsequent application of the immobilized enzyme.

Table 2. Effect of glutaraldehyde treatment on the recovery of enzyme activity

Run	Immobilized activity [u/g Support]	Recovery percent	Glutaraldehyde treatment
1	33.45	-	-
2	22.74	68	
3	21.74	65	
1	18.22	-	+
2	10.93	60	
3	9.1	50	

Conclusion

In immobilization of Lipase on HDPE fine powder, dispersion of the support in the hydrophobic solvent n-hexane not only did not have any positive effect on im-mobilization, but also it may have introduced the negative effect of enzyme denaturation. Prewetting the support with hydrophilic ethanol increased protein loading and activity of the immobilized enzyme as much as 31% and 34%, respectively, implying a non-protein-selective effect of ethanol in improving the immobilization yield on the support. Maximum immobilized enzyme activity was obtained when immobilization was carried out at pH=4-5 that coincides with the neutral status of the enzyme at its isoelectric point. Using various enzyme dilutions in the imobilization protocol, it was shown that the soluble enzyme-support system is far from equilibrium at this condition. The adsorbed enzyme on the support is suspected to have interac-tions with the other protein molecules on the surface such that at relatively large enzyme loadings, the negative effect of these interactions outweighs the positive effect of the increase in the enzyme concentra-tion, leading to a fall in the overall immobilized activity. Aside from these interact-tions, a competition may be present between lipase and other protein molecules in the system to settle on the support surface. Non-lipase molecules being adsorbed on the surface limit the support surface available for lipase immobilization. In the immobilization protocol, proper time of mixing the enzyme solution with the powder was investigated and found to be around 6 minutes. Longer periods of shaking may lead to enzyme desorption reducing the immobilized activity. Feasibility of chemi-cal immobilization of lipase on HDPE fine powder using glutaraldehyde as a cross-linking agent was studied. Neither efficiency nor stability of the immobilization was improved in this practice. This was speculated to be due to the effect of glutaraldehyde on partial denature-tion of the enzyme and/or percipitation of the insoluble enzyme on the support. Based on the results obtained in this research, immobiliza-tion of lipase on polyethylene fine powder was shown to increase the activity of the enzyme around sixfolds. Therefore, the activity of 0.5 ml of the enzyme liquor when immobilized on 1 g of the powder increased from 8 to about 45.8 units.

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