Activated Carbon as Support for Lipase Immobilization

Mohd Basyaruddin Abdul Rahman*, Mahiran Basri, Mohd Zobir Hussein, Raja Nor Zaliha Raja Abdul Rahman, Yau Kim Yan and Abu Bakar Salleh.

Centre for Research in Enzyme and Microbial Technology, Faculty of Science and Environmental Studies, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

Abstract

Lipase from *Candida rugosa* was immobilized onto four different types of activated carbon; KI/2030, KI/3040, KI/5060 and KI/6070. The immobilized lipase was used in the esterification of oleic acid and 1-butanol in hexane. The effects of difference pore sizes, surface area, reaction temperature, thermostability of the immobilized lipases, storage stability in organic solvent and leaching studies were investigated. Among the four samples, KI/6070 gave the highest activities and stability in all the parameters investigated. Immobilized lipases generally exhibit activities higher than the native lipase for the parameters studied, with optimum temperature of 40°C. Immobilized lipases are more stable than native lipase in hexane at room temperature up to 12 days. Leaching study proved that the immobilization of lipase using physical adsorption is cheap and easy. This method was found to be suitable for the attachment of enzyme on the support.

Introduction

Activated carbon has stimulated studies in catalytic reaction due to its potential absorbency [1]. They are used as absorbent in wastewater treatment and are increasingly been applied as support for catalyst in advanced material and biotechnology [2]. Immobilizing a biocatalyst on support is a very effective way to stabilize the enzyme from being easily denatured at higher temperature or pressure and unstable in organic solvent environment [3]. Furthermore, immobilization leads to heterogeneous systems, which enable separation of products from the reaction medium and recycle for a continuous processing. Thus, the industrial and production costs can be reduced.

Effective use of support for immobilization of enzyme may involve chemical activation, covalent attachment, physical adsorption or a combination of these, while maintaining viability and metabolic activity. Physical adsorptions, either through ionic and electronic interactions have been used for enzyme and cell immobilization [4]. Activated carbon with large external surface area and high porosity can be used to adsorb enzyme from aqueous solution by simple physical adsorption.

Here, we reported our work on using activated carbons as support for immobilization of *Candida rugosa* lipase via physical adsorption. The characteristics and specific activity of the immobilized lipase prepared for the synthesis of butyl oleate were studied.

*corresponding author. E-mail: basya@fsas.upm.edu.my

Experimental

Materials

Lipase from *Candida rugosa* (E.C.3.1.1.3 Type VII) was purchased from Sigma Chemical Co. (St. Louis, MO). The activated carbons (KI/2030, KI/3040, KI/5060, KI/6070) were purchased from Kekwah Indah, Malaysia. All other chemicals were of analytical grade.

Characterization of the activated carbon

The activated carbons were put in an oven overnight to remove the moisture. Analysis of surface area and porosity of activated carbon were done using a Micromeritics, ASAP 2000. This study was carried out to investigate the best support among the four different types of activated carbon.

Immobilization of lipase

Crude lipase of *Candida rugosa* (1.50 g) was dis-

persed into distilled water (15 ml). The mixture was stirred for an hour, centrifuged at 5000 rpm for 15 minutes, and the supernatant was used as partially purified lipase. The immobilization of lipase was carried out by continuous shaking at 100 rpm of activated carbon (2.00 g) with purified free lipase solution (15 ml) for 1 h at room temperature. The immobilized lipase was then separated by filtration and washed with distilled water to remove the unadsorbed soluble enzyme. The amount of protein adsorbed onto the activated carbon was determined by Bradford method with bovine serum albumin as standard [5].

Activity assay

The assay system consisted of 1-butanol (4.0 mmole), oleic acid (2.0 mmole), immobilized lipase (0.30 g) and hexane (2 ml). The reaction mixture was incubated at 30°C for 5 h, with continuous shaking at 150 rpm in a horizontal shaker waterbath. The reaction was terminated by addition of acetone:ethanol (50:50 v/v, 3.5 ml). The remaining free fatty acid in the reaction was determined by titration with 0.15 M NaOH using autotitrator to an end point at pH 10.0. All experiments were done in triplicate. The control experiments were carried out with the native lipase. The specific activities are expressed as μmole of free fatty acid used/min/mg of immobilized lipase.

Characterization of the immobilized lipase

Effect of temperature on the esterification

The reaction mixtures were incubated at different temperatures (30°C, 40°C, 50°C, 60°C and 70°C) for 5 hours at 150 rpm. The relative yield was determined as:

$$\text{Relative Yield (\%)} = \frac{\text{\% Yield at different temperature}}{\text{Maximum \% Yield (40°C)}} \times 100$$

Leaching study of the immobilized lipase in hexane

The immobilized enzyme (0.30 g) was washed with hexane at 4, 8, 12, 16 and 20 ml by 4 ml at each washing. The relative yield was determined as:

$$\text{Relative Yield (\%)} = \frac{\text{\% Yield at different ml of hexane}}{\text{Maximum \% Yield (0 ml)}} \times 100$$

Thermostability of the immobilized lipase

The immobilized lipases were incubated at 30°C, 40°C, 50°C, 60°C and 70°C in sealed vials for an hour. The enzymes were left to cool before the esterification activity was determined. The relative yield was determined as:

$$\text{Relative Yield (\%)} = \frac{\text{\% Yield at different temperature}}{\text{Maximum \% Yield (40°C)}} \times 100$$

Stability of the immobilized lipase at room temperature

The immobilized lipases were incubated in hexane, without shaking for 1 to 12 days at room temperature. The relative yield was determined as:

$$\text{Relative Yield (\%)} = \frac{\text{\% Yield at number of days}}{\text{Maximum \% Yield (Day 1)}} \times 100$$

Results and discussion

Surface area and porosity of activated carbon

The BET surface area, micropore volume and BJH desorption pore size distribution of four types of activated carbon are summarized in Table 1. The BJH desorption pore size shows an average micropore size of activated carbon around 54-62 Å. This is very important for enzyme work, so that adsorption could occurr at the surface and the pore. In general, the plot of adsorption-desorption isotherms of nitrogen of each activated carbon is of typical Type 1 (Figure 1), indicating microporous nature of the activated carbon. The KI/6070 showed the highest BET surface area and have the largest pore size, which may be suitable for enzyme immobilization.

Immobilization of lipase

The highest amount of lipase adsorbed was on activated carbon (KI/6070) as shown in Figure 2. The percentages of protein adsorbed are more than 20% for all type of the supports, which suggests that all of the activated carbons are suitable matrix for lipase immobilization. The immobilization of lipase on the support was generally distributed within the support as the protein molecules of lipase were replacing the molecules of water. Highest amount of lipase absorbed was observed on KI/6070. The increased in the percentages of immobilization from KI/2030 to KI/6070 is related to the porosity and the surface area of the activated carbon.

Table 1
BET surface area, micropore volume and BJH Desorption Pore Size Distribution of Activated Carbon

Activated Carbon	BET Surface Area (m^2/g)	Micropore Volume (cc/g)	BJH Desorption (Å)
KI/2030	159	0.0592	34.5
KI/3040	811	0.3123	23.2
KI/5060	898	0.3018	28.2
KI/6070	1239	0.4473	29.2

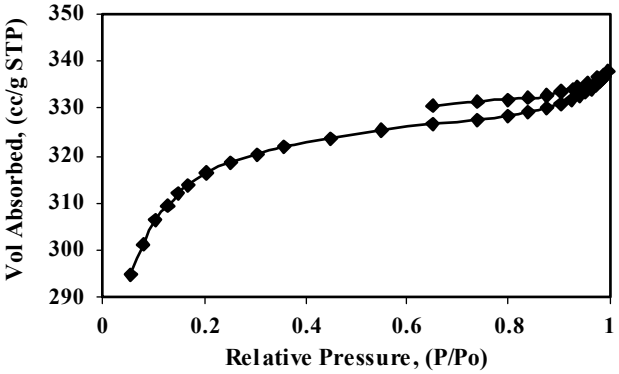

Fig. 1. Adsorption-desorption isoterms of nitrogen for activated carbon KI/6070.

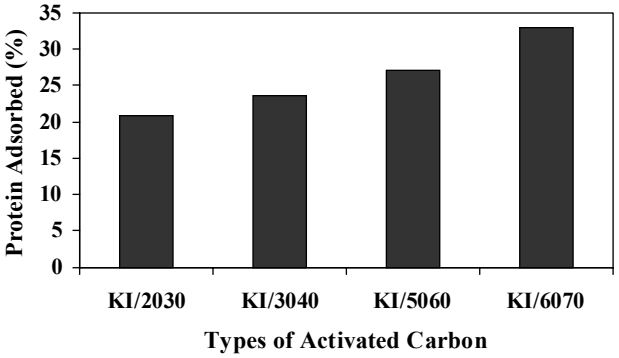

Fig. 2. Percentage of protein adsorbed on lipase immobilized on KI/2030, KI/3040, KI/5060 and KI/6070.

Characterization of the immobilized lipase

The increased of the BET surface area of the support from 159 m^2/g to 1239 m^2/g has increased the specific activities as shown in Figure 3. The highest specific activity at 9.45 μmol·min^{-1}·mg·protein^{-1} was observed when activated carbon (KI/6070) was used. Only slight difference in the specific activities between immobilized lipase on KI/5060 and KI/6070 was observed. The increased in activity of the immobilized lipase on KI/6070 may be due to the enzyme which was localized at the surfaces [6]. This will directly influence the increased in the interaction of the support with the enzyme. As expected, these may be due to the large surface area available from the support [7]. Smaller pore sizes restrict mass transfer and pore penetration of the protein, which limit the protein interaction with the total surface area of the activated carbon. In addition for a successful immobilization by adsorption, an electrostatic interaction is needed between the enzyme and support. The amount of lipase to be adsorbed on larger surface area is higher than the smaller ones due to their greater surface area [8]. Bosley [3] found that a support with diameter less than 25 nm and pores at least 35 nm were needed for the lipase to be able to act as best support for enzyme immobilization (approximately five times of the diameter of a protein molecule)

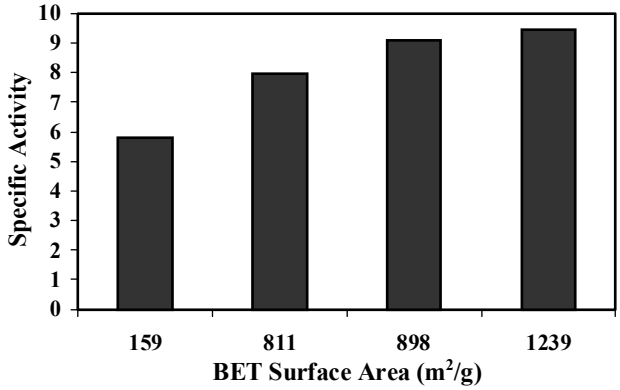

Fig. 3. Effect of BET surface area on the esterification activity of immobilized lipases. Specific activity was expressed as μmol·min^{-1}·mg·protein^{-1}.

The relative activity was found to be increased with the temperature from 30°C to 40°C as shown in Figure 4. At temperature below 30°C, decreased in activity was expected because of the diffusion limitation of the reactant due to increase in reactant viscosity [9]. The optimal reaction temperature was found at 40°C. At temperature above 50°C, the relative yield was slightly decreased, and further de-

creased was found at 70°C. This is due to denaturing of the lipase at high temperature. However, all immobilized lipases showed better relative activities for all the studied temperature compared to native lipase.

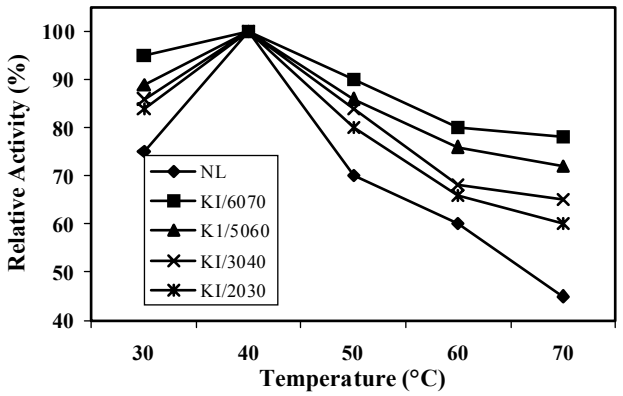

Fig. 4. Effect of temperature on the relative esterification activity of immobilized lipases on KI/2030, KI/3040, KI/5060 and KI/6070 (NL=Native Lipase).

The immobilized lipases have retained most of their catalytic activity even after 4 cycles (20 ml of hexane) as shown in Figure 5. Only a slight leaching of less than 1% were detected for all the immobilized lipase after fifth cycles which may be due to some loss of enzyme during washing and be an inherent consequence of its immobilization on each support. This finding proved that activated carbons are suitable support for immobilization of enzyme as it prevents enzyme from being washed away even after washing with 20 ml of hexane. This also proved that the physical adsorption method adopted in this study is an easy and suitable method of immobilization.

The immobilized lipases were significantly more

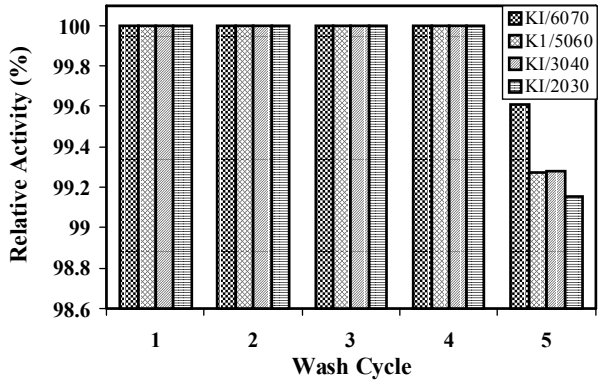

Fig. 5. Leaching study of immobilized lipases on KI/2030, KI/3040, KI/5060 and KI/6070.

thermostable than the native lipase after 1 h of incubation at temperature ranging from 40°C to 70°C as shown in Figure 6. The native lipase can undergo aggregation at higher temperature, which lead to a loss of enzymatic activity at temperature higher than 40°C. In addition, immobilized lipase (KI/6070) showed the highest thermostability at 40°C but decreased at 50°C. This may be due to a concurrent denaturation of the protein molecule due to a large and positive heat capacity change associated with the protein unfolding at high temperature. The decreased in the activity of the immobilized lipases, may also be due to the decrease in electrostatic interactions between lipase and activated carbon at high temperature. Immobilization had increased the thermostability, as all of the immobilized lipases showed better relative activities at all the incubated temperatures compared to native lipase. Although heat considerably reduce conformational flexibility of native and immobilized lipase, but immobilized lipase is still capable of performing its vibrational and more complex movement required for efficient enzymatic activity.

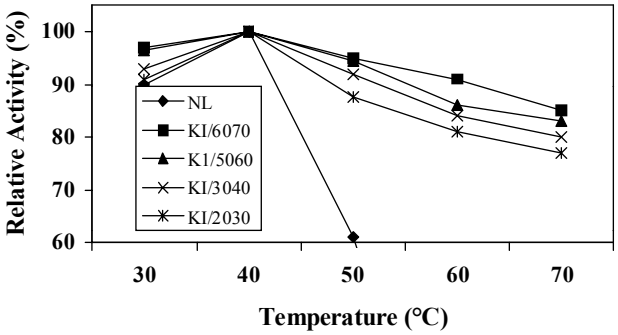

Fig. 6. Thermostability of the immobilized lipases incubated for 1 hour (immobilized lipases on KI/2030, KI/3040, KI/5060 and KI/6070, NL = Native Lipase). Relative activity of native lipase dropped to 0 at 60 and 70°C.

The immobilized enzymes were found to be stable in hexane at room temperature for 12 days as compared to native lipase (Figure 7). Immobilization seemed to protect the enzyme from denaturation caused by the presence of organic solvent. Although the polar solvents tend to strip water from enzyme molecule, the supports may trap and prevent the disruption of the enzyme-bound water essential to maintain the three-dimensional structure of the enzyme for catalysis [10]. A large amount of water may result in undesirable side reactions such as hydrolysis of acid anhydrides and halogenates. Therefore, the

immobilized enzymes are more stable in organic solvents than in water as thermodynamic equilibrium of most processes is unfavorable in water. So, we can conclude that the activated carbon has protected a monolayer of water for the enzyme molecule to be active.

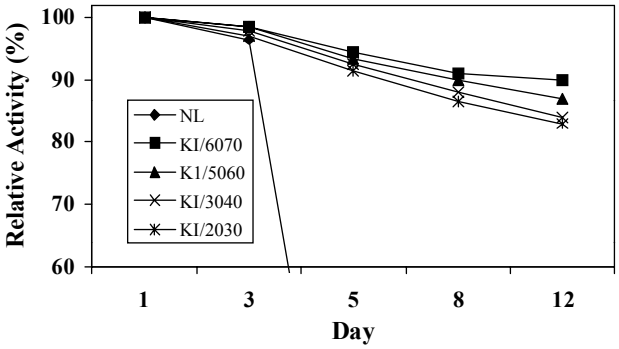

Fig. 7. Stability of the immobilized lipases at room temperature in hexane (immobilized lipases on KI/2030, KI/3040, KI/5060 and KI/6070, NL = Native Lipase). Relative activity of native lipase dropped to 0 after 5 days.

Conclusions

Lipase from *Candida rugosa* was successfully immobilized on activated carbon using physical adsorption method as shown by the enhancement of the total activity and increase in thermostability and storage stability of the lipase after immobilization. The cheap, simplicity and effectiveness of the technique may open up the possibility of immobilization of other protein on various type of activated carbons.

Acknowledgments

This project was financed by the Ministry of Science, Technology and Environment, Malaysia.

References

1. Gergova, K., J Chem Tech Biotechnol 56:77 (1993).
2. Bandosz, T.J., Jagiello, J. and Contescu, C., Carbon 31(7):1193 (1993).
3. Bosley, J., Biochem Soc Transactions 25:174 (1997).
4. Taylor, R.F., Industrial applications of immobilized proteins. Protein Immobilization. Marcel Dekker, New York , 1991, 339.
5. Bradford, M.M., Anal. Biochem. 72: 248 (1976).
6. Ampon, K., J Chem Biotechnol Bioeng 55:185 (1992).
7. Ruckenstein, E. and Wang, X., Biotechnol Bioeng 42:821 (1992).
8. Basri, M., Ampon, K., Razak, C.N.A., Salleh, A.B., Yoong, W.S. and Yunus, W.M.Z., J. Chem Tech Biotechnol 66:169(1996).
9. Fagain, C.O., Manipulating protein stability in Stabilizing Proteins Functions, Heidelberg, Berlin, Springer, New York, 1997, 67.
10. Gorman, L.A. and Dordick, J.S., Bioeng 39:392 (1992).

Received 12 December 2002.