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Properties of Immobilized *Candida antarctica* Lipase B on Highly Macroporous Copolymer

(Sifat Pegun *Candida antarctica* Lipase B ke atas Kopolimer yang Sangat Mikroporos)

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ABSTRACT

In spite of their excellent catalytic properties, enzymes should be improved before their implementation both in industrial and laboratorium scales. Immobilization of enzyme is one of the ways to improve their properties. Candida antarctica *lipase B (Cal-B) has been reported in numerous publications to be a particularly useful enzyme catalizing in many type of reaction including regio- and enantio- synthesis. For this case, cross-linking of immobilized Cal-B with 1,2,7,8 diepoxy octane is one of methods that proved significantly more stable from denaturation by heat, organic solvents, and proteolysis than lyophilized powder or soluble enzymes. More over, the aim of this procedure is to improve the activity and reusability of lipase. Enzyme kinetics test was carried out by transesterification reaction between 4-nitrophenyl acetate (pNPA) and methanol by varying substrate concentrations, and the result is immobilized enzymes follows the Michaelis-Menten models and their activity is match with previous experiment. Based on the V_{max} values, the immobilized enzymes showed higher activity than the free enzyme. Cross-linking of immobilized lipase indicate that cross-linking by lower concentration of cross-linker,* FIC *(immobilized lipase that was incubated for 24 h) gave the highest activity and cross-linking by higher concentration of cross-linker,* PIC *(immobilized lipase that was incubated for 2 h) gives the highest activity. However, pore size and saturation level influenced their activity.*

Keywords: Candida antarctica *lipase B (Cal-B); cross-linking; enzyme immobilization*

ABSTRAK

Walaupun sifat katalitisis yang sangat baik, enzim perlu ditingkatkan keupayaannya sebelum diimplementasikan dalam skala industri maupun makmal. Imobilisasi enzim merupakan salah satu cara untuk memperbaiki sifat enzim. Candida antarctica *lipase B (Cal-B) telah dilaporkan dalam sejumlah penerbitan sebagai enzim yang dapat digunakan dalam mengkatalisis enzim dalam berbagai tindak balas termasuk sintesis yang bersifat regio- dan enantio-selektif. Dalam kajian ini didapati taut-silang dalam Cal-B terimobilisasi oleh 1,2,7,8 diepoksi oktana merupakan salah satu kaedah yang terbukti secara signifikan lebih stabil terhadap penyahaslian oleh pemanasan, pelarut organik, dan proteolisis dibandingkan dengan enzim dalam bentuk larutan maupun serbuk terliofilik. Selain itu, tujuan kajian ini adalah meningkatkan keaktifan dan kebolehulangan pemakaian lipase. Ujian kinetik enzim dilakukan melalui tindak balas transesterifikasi antara 4-nitrofenil asetat (pNPA) dan metanol dengan memvariasikan kepekatan substrat, dan hasilnya adalah bahwa enzim terimobilisasi mengikuti model Michaelis-Menten serta keaktifannya sesuai dengan hasil kajian sebelumnya. Berdasarkan nilai V_{max}, enzim yang terimobilisasi menunjukkan keaktifan yang lebih tinggi daripada enzim bebas. Taut-silang pada lipase yang terimobilisasi menunjukkan bahawa taut-silang oleh adanya rangkaian silang* FIC *(lipase terimobilisasi yang diinkubasi selama 24 jam) berkepekatan rendah menunjukkan keaktifan tertinggi, sedangkan taut-silang oleh adanya rangkaian silang* PIC *(lipase terimobilisasi yang diinkubasi selama 2 jam) berkepekatan lebih tinggi menunjukkan keaktifan tertinggi. Di samping itu, saiz liang dan aras ketepuan memberikan pengaruh terhadap keaktfian enzim tersebut.*

Kata kunci: Candida antarctica *lipase B (Cal-B); imobilisasi enzim; taut-silang*

INTRODUCTION

Enzymes are macromolecules that have many roles in accelerating chemical reactions in living cells. Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes are biocatalysts for supporting almost all of chemical reactions and play an indispensable role in many living systems. These biocatalysts have many specific features, such as highly controlled regioselectivity, stereolectivity, specificity of substrate, and their reaction generally carried out under very mild conditions (i,e., pH, temperature, pressure). Enzymes are found in all tissues and fluids of the body and carried out many reaction of catalysis (Kobayashi et al. 2006).

Lipases (tryacylglycerol acyl hydrolases, EC 3.1.1.3) are the most interesting group of biocatalysts with many biotechnological applications. It is defined as a carboxylesterase, enzymes that catalyze hydrolysis

reaction and synthesis of long chain acylglycerols. There are many reasons that make lipases so attractive. Firstly, almost all of lipases display great regiosectivity, and stereoselectivity. Secondly, lipases could be produced in a high yield from microbial organisms (i.e. fungi and bacteria). Thirdly, the crystal molecules of many lipases have been solved, facilitating considerably the design of rational engineering strategies. Finally, lipases only require the catalyze side reactions and they do not require the cofactor like the other enzymes. Due to these properties lipases are the most widely used group of biocatalysts in organic chemistry (Jaeger & Eggert 2002).

Candida antarctica Lipase B (Cal-B) is one of lipases which has many applications both in the laboratory and the industry. This enzyme is one of the most effective catalysts for amines and amides production. Simplicity of use, low cost, commercial avaibility, and recycling possibility make this lipase as an ideal tool for many synthesis reaction. However, the use of enzyme as biocatalyst has many limitations, such as high production cost, weak stability, difficult to separate the enzyme from the reactants or products in its solution and easily inhibited (Govardhan 1999). Due to these reasons, the improvement of Cal-B performances in their activitiy, stability and reusability are needed.

In the second half of the last century, numerous attempts were devoted to develop various carrier-bound immobilized enzymes to facilitate their use in continuous processes and especially to overcome the cost constrains by reusing, recycling, facilitating efficient separation, and making easy control of the process. Moreover, the improvement of enzyme properties like their activity, stability, and reusability can often be achieved by enzyme immobilization (Sheldon et al. 2005). Several experiments reported that immobilization method using cross-linking reaction between enzyme molecules are an attractive strategy because it affords stable catalysts with high activity (Cao et al. 2000; Schoevaart et al. 2004).

The more recently developed cross-linked enzyme aggregates (CLEAs), on the other hand, are produced by simple precipitation of the enzyme from aqueous solution by the standard techniques to produce physical aggregates of protein molecules (Sheldonet al. 2005). CLEAs are very attractive biocatalysts, because of their facile, inexpensive, and effective production method. They can readily be reused and exhibit improved stability and performance (Wilson et al. 2004). However, CLEAs are mechanically fragile, and it is difficult to handle and fully recover the CLEA particles over repetitive uses (Mc-Kee, 2004). Because of these reasons, optimation and various tests is needed in acquiring the best performance of enzyme. The aims of this research were to investigate the Cal-B immobilization on poly (glycidyl methacrylate-co-ethylene glycol dimethacrylate) and to check their reusability, kinetic properties and activity after crosslinking.

Materials and Methods

Materials and Instrument

Methanol 99.8% were purchased from Lab-Scan, 4-nitrophenyl acetate (*p*NPA) were purchased from Sigma Aldrich, $Na_2HPO_4.12H_2O$ 99% and $NaH_2PO_4.H_2O$ were purchased from Merck. *Candida antarctica* Lipase type B were purchased from Codexis® (Codexis, CA, USA). BCA Protein assay kit was purchased from Pierce. Poly(GMA-*co*-EGDMA) resins were kindly provided by ICTM - Center for Chemistry, Belgrade, Serbia. There are eight different copolymers and all of the copolymers have the same chemical structure but different porosity parameters (pore size, specific volume and specific surface) and bead size. The copolymers were shown on the Table 1. UV-VIS data were recorded on SP8-200 UV/VIS spectrophotometer.

TABLE 1. Particle size and porosity parameters of poly(GMA-co-EGDMA): average pore diameter, specific surface area and specific volume

Resin	Sample name	Particle size (μm)	Average pore diameter (nm)	Specific surface area (m^2/g)	Specific volume (cm^3/g)
1	SGE-10/16-d1	630-300	30	82.0	0.923
2	SGE-10/16-d2	300-150	87	36.0	0.755
3	SGE-20/14-d1	630-300	92	36.0	1.111
4	SGE-20/14-d2	300-150	270	27.6	1.040
5	SGE-20/14-d3	150-100	59	46.7	1.088
6	SGE-20/14-d4	<100	48	55.2	1.100
7	SGE-20/16-d1	630-300	30	106.0	1.191
8	SGE-20/16-d2a	300-150	560	13.2	1.125

Note: aThe particles do not have spherical shape.

ENZYME IMMOBILIZATION AND SEPARATION

The immobilization of Cal-B was done by following the protocol of Miletic et al. (2009). Copolymer beads and Cal-B (4:1) were dissolved in the 1.5 mL tris-HCl buffer pH 6.8 in a 4 mL screw-capped vial. The samples were incubated in a rotary shaker at 30°C and 200 rpm for 24 h until immobilized enzymes have been precipitated. Buffer solution for each samples was removed by filtering and placed into vials. On the other hand, immobilized Cal-B was washed with tris-HCl buffer pH 6.8 until no protein was detectable in the washing solution (more less 10 mL of buffer). Supernatant and washing solutions were collected and analyzed with BCA protein assay, the amount of immobilization enzyme then estimated. The solid phases were dried by using liquid nitrogen and placed under vacuum condition for 24 h at room temperature.

BCA ASSAY

The working solutions were prepared by adding reagent B to reagent A (1:50). In this research, 40 mL of reagent A was added by 0.8 mL of reagent B. After that, 2.0 mL of working solutions were taken and putted into the flasks that contain 0.1 mL of each supernatant and washing fraction. The mixtures were incubated at 37°C for 30 min then characterized by UV/VIS spectrophotometer at 562 nm. The loading of Cal-B (i.e., amount of Cal-B (mg) lost from the solution per unit of total weight of copolymer and the amount of enzyme) on the copolymer was calculated from the amount of enzyme in the combined original solutions and washing fractions.

CROSS-LINKING OF IMMOBILIZED ENZYMES

The immobilization of Cal-B onto six beads macroporous which were chemically the same but different in porosity was divided into two ways. First, immobilization was carried out for 2 h which was named Part Immobilized Crystals (PIC) in order to make only physical connections between enzymes and macroporous copolymer. Second, immobilization was carried out for 24 h which was named Full Immobilized Crystals (FIC) in order to make not only physical connections but also chemical connections between enzymes and macroporous copolymers. Phosphate buffer solution pH 8 was used during immobilization. After immobilization, 1.5 mL of crosslinker, in this case 1,2,7,8 diepoxy octane was added to the samples. Cross-linking was carried out for 3 h, 30°C, 200 rpm with different concentration of cross-linker (0.05%, 0.1%, 1%, and 10% (v/v)). After cross-linking, cross-linker solution was separated from immobilized enzyme. Cross-linking immobilized enzyme was washed with buffer PBS pH 8, dried, and keeped under vacuum pressure for 24 h.

ACTIVITY AND REUSABILITY TEST

Activity and reusability tests were carried out towards normal immobilized enzymes. The tests followed the reaction between 4-nitrophenyl acetate (*p*NPA) and methanol in 1,4-dioxane. In the solution, containing *p*NPA (40 mM) and methanol (80 mM) in 1,4-dioxane, was added 0.5 mg of immobilized enzymes. The mixtures were incubated at 35°C for 50 min (200 rpm) and the aliquotes (25 μL) were taken after certain periods of time. The concentration of the reaction product, *p*NP, was determined by UV-VIS at the λ_{max} (304 nm). After UV measurement, all of those immobilized enzymes were washed with 1,4 dioxane, then dried and keeped under vacuum pressure for 24 h. The immobilized enzymes could be reused.

ENZYME KINETICS

Enzyme kinetics test followed the reaction between 4-nitrophenyl acetate (*p*NPA) and methanol in 1,4-dioxane. This test was carried out for some concentration of *p*NPA solutions (20, 40, 60, 80, 150, 200 and 300 mM). The concentration of methanol was two times concentration of *p*NPA. The mixtures were added into 20 mL vials containing 0.5 mg immobilized enzymes (normal immobilization), then incubated at 35°C for 50 min (200 rpm) and the aliquotes (25 μL) were taken after certain periods of time. The concentration of the reaction product p-nitrophenol (*p*NP) was determined by UV-VIS spectroscopy at 304 nm.

RESULTS AND DISCUSSION

In this experiment, a series of poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) resins (see Table 1) with varied particle size which was synthesized in the shape of beads by suspension polymerization in various conditions were used as carrier for enzyme immobilization. In this research, enzyme kinetic properties, reusability, and crosslinking the immobilized Cal-B were studied.

IMMOBILIZATION OF ENZYMES

Immobilization of Cal-B on eight copolymers were carried out for 24 h to produce chemical bonds and physical interactions between enzymes and copolymers. The amount of enzyme that was immobilized onto copolymer was estimated with BCA protein assay. The results of BCA protein assay are presented in Table 2.

TABLE 2. Enzyme loading values of each samples

No.	Sample	Enzyme loading (μg/mg)
1	SGE-20/14-d1	167.61
2	SGE-20/14-d2	199.88
3	SGE-20/14-d3	165.14
4	SGE-20/14-d4	182.01
5	SGE-20/16-d1	179.96
6	SGE-20/16-d2	176.38
7	SGE-10/16-d1	204.69
8	SGE-10/16-d2	217.48

Enzyme loading is the weight of Cal-B (μg) that is immobilized per unit of total weight of copolymer (mg). The amount of Cal-B adsorbed on copolymer is given as addition of the amount of Cal-B physically adsorbed and amount of Cal-B chemically binded to the copolymer in all samples (Rohandi 2007). Enzyme loading depends on the porosity parameters of carrier especially diameter size and specific surface area (see Table 1). Increasing the pore size of 300-150 μm beads from 87, 270, and 560 nm produce the decreasing of enzyme loading (217.5, 199.88, 176.38 μg/mg). Copolymer with the pore size of 630-300 μm beads also showed the same tendency. The increase from 30 to 92 nm (resin 1 to 3, and resin 7 to 3) produced the decrease of enzyme loading. Otherwise, for 630-300 μm beads with identical pore size of 30 nm, the increase of the specific surface area from 82.0 (resin 1) to 106.0 m^2/g (resin 7) produced a decrease in enzyme loading from 204.69 (resin 1) to 179.96 μg/mg (resin 7). The differences of enzyme loading values were obtained from the differences of interaction between enzyme toward copolymer. Interaction of enzyme and water from the buffer during immobilization makes the swelling of copolymer then give the influence to the enzyme loading values (Smith 1985). If the final loading of enzyme is still poor, it can be caused by two reasons: (1) only the inner surface of mesopores is used for attachment of enzyme, (2) received enzyme molecules can exert a steric hindrance against the other enzyme molecule penetrations into deeper mesoporous. From these results, it can be concluded that in the indentical pore size of beads, diameter size and specific surface area will influence the enzyme loading values.

ENZYME KINETIC TESTS

Enzyme kinetic tests were carried out to explore the effects of enzyme immobilization to kinetic properties of enzyme. In enzyme kinetic tests, the transesterification reaction between 4-nitrophenyl acetate (*p*NPA) and methanol in dioxane was followed (Figure 1).

This reaction was carried out using immobilized Candida antarctica lipase B (Cal-B) as biocatalyst at 35°C with taking the aliquots after certain time. The amount of enzyme was kept constant while concentration of substrate was varied. Saturation curve and Lineweaver-Burk plot were obtained using UV/VIS spectroscopy. Saturation curve and Lineweaver-Burk plot of immobilized lipase onto copolymer SGE/10-16-d2 as one example is shown in Figure 2.

Both saturation curve and Lineweaver-Burk plot of other samples have the same tendency as curves in Figure 2. According to our results, one can conclude that Cal-B mediated transesterification reaction between *p*NPA and methanol follows *Michaelis-Menten* kinetic model.

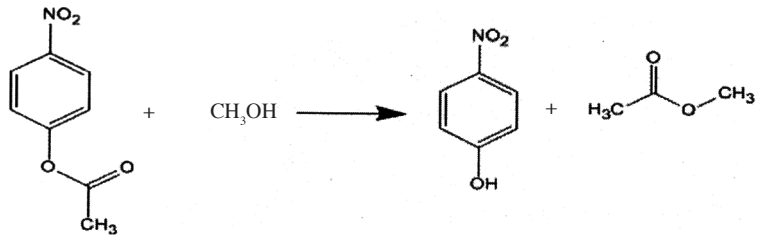

FIGURE 1. Transesterification between *p*NPA and methanol

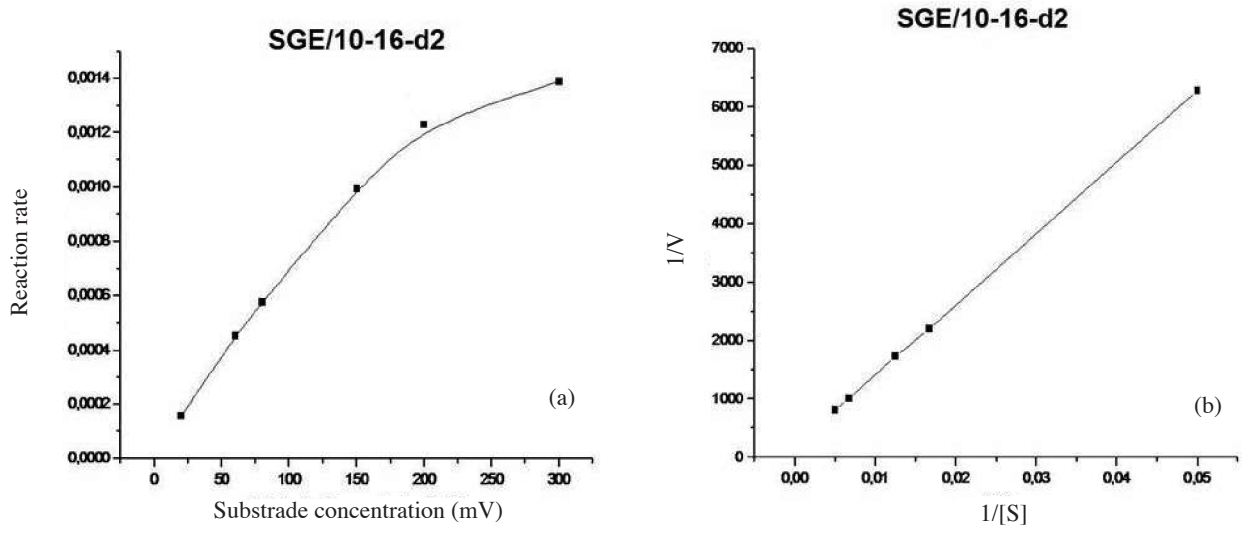

FIGURE 2. Saturation curve of (a) SGE/10-16-d2 and (b) Lineweaver-Burk plot for SGE-10/16-d2

Michaelis-Menten constant (K_M) describes the affinity between enzyme towards its substrate, is almost equal for above mentioned reaction catalyzed by immobilized form of Cal-B. On the other hand, *Michaelis-Menten* parameter was changed for the same reaction catalyzed by free enzyme powder. Change of *Michaelis-Menten* constant (K_M) and maximum velocity (V_{max}) are listed in the Table 3.

From the results in Table 3, all of K_M values are higher than concentration of substrates. It means the initial velocity is independent of [S]. K_M is related with the affinity of the enzyme for its substrate and also informs about the rate of reaction. The binding constant is approximated by K_M; small K_M means tight binding, while high K_M means weak binding. In all of the cases in this experiment, K_M values of immobilized enzymes were higher than free enzyme. It means that the amount of substrate that is required by immobilized enzyme to become saturated is smaller than free enzyme. Moreover, the affinity of immobilized enzyme towards its substrate is smaller than free enzyme.

On the other hand, V_{max} values of immobilized enzymes are different from free enzyme as can be seen in Table 3. V_{max} is the theoretical maximum rate of the reaction. However, this condition cannot be achieved, since V_{max} would require that all enzyme molecules have tightly bound substrate. V_{max} relates to efficiency. Higher V_{max} value means greater rates of reaction. In other words, their efficiency increased. From results in Table 3, V_{max} values of immobilized enzymes are higher than free enzyme. It means the tranesterification reaction between *p*NPA and methanol catalyzed by immobilized enzyme is more efficient than free enzyme. From the results of kinetic parameters, it can be concluded that enzyme kinetic properties changed during the immobilization process.

REUSABILITY TEST

An important feature of immobilized enzymes is the possiblity of reusability. Therefore, it is important to investigate their reusability performances after immobilization on poly (GMA-*co*-EDGMA). Reusability tests were performed by transesterification reaction between *p*NPA and methanol. After enzyme activity test (conversion of 4-nitrophenyl acetate into *p*-nitro phenol), immobilized enzymes were washed several times with dioxane and placed in the vacuum overnight. The day after, the same kind of activity test was carried out. The procedure was repeated for 7 days.

Figure 3 shows the decrease of enzyme activity after certain days. From this curve, the largest pore size of carrier showed the highest reusability. On the other hand, the smallest pore size of carrier showed the lowest reusability. Copolymer with the large pore size was better endured in the filter layer than the copolymer with the small pore size. That is the reason why the largest pore size carrier gave the highest reusability. However, immobilized Cal-B onto poly(GMA-*co*-EGDMA) showed higher reusability than free enzyme. According to the literature, after two or three days, free enzyme shows zero activity.

CROSS-LINKING OF IMMOBILIZED CAL-B

Cross-linking is based on the formation of covalent bonds between enzyme molecules, by means of bi- or multifunctional reagent, leading to three dimensional cross-linked aggregates. It is best used mostly as a means to stabilize adsorbed enzymes and also for preventing leakage (Kim et al. 2006). Cross-linked enzyme aggregates (CLEAs) is one of cross-linking procedures that is produced by simple precipitation of the enzyme from aqueous solution by the standard techniques to produce physical aggregates of protein molecules (Sheldon et al. 2005). CLEAs are very attractive biocatalysts, because of their facile, inexpensive, and effective production method. Because of this reason, cross-linking immobilized Cal-B was carried out by CLEAs method.

Activity test of cross-linking immobilized Cal-B was done by various concentration of cross-linker. Immobilization of enzyme was carried out during 2 h incubation that is called PIC (Part Immobilization Crystals) and 24 h incubation that is called FIC (Full Immobilization Crystals). PIC was done in order to make only physical connection between enzymes toward copolymer. On the other hand, FIC was done in order to make not only physical connection between enzymes toward copolymer but also their chemical connection.

TABLE 3. KM and Vmax values of each immobilized enzyme

Immobilized Enzyme	KM (mM)	V_{max} (moles/seconds)
SGE-20/14-d1	170.55	1.71×10^{-3}
SGE-20/14-d2	149.90	2.45×10^{-3}
SGE-20/14-d3	143.00	4.17×10^{-3}
SGE-20/14-d4	164.54	4.71×10^{-3}
SGE-20/16-d1	168.19	1.70×10^{-3}
SGE-20/16-d2	144.49	2.87×10^{-3}
SGE-10/16-d1	138.76	1.39×10^{-3}
SGE-10/16-d2	155.45	1.55×10^{-3}

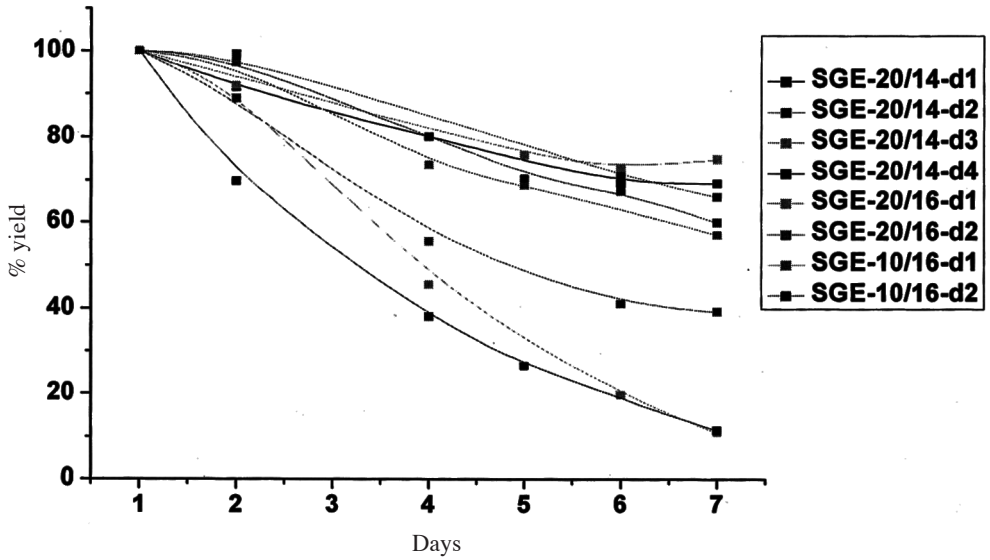

FIGURE 3. Result of reusability test

The cross-linker used in this procedure was 1,2,7,8-diepoxyoctane. The amine groups in Cal-B will react with the epoxy groups in cross-linker and make the bridge conformation. For this research, cross-linking of immobilized enzyme was done by 0.05%, 0.1%, 1%, and 10% (v/v) of cross-linker (CL) in the phosphate buffer solution.

The activity tests of cross-linked products were carried out by the transesterification reaction between *p*NPA and methanol. After incubation of the mixtures at 35°C for certain times, the amount of aliquots were taken out and measured by UV spectroscopy with 304 nm as maximum wavelengths (Figure 4).

PIC with 0.05% cross-linker give the same performance whereas the SGE-20/14-d4 shows the highest activity. The same tendency was also shown in the other concentration of cross-linker (0.1%, 1%, and 10% v/v) for PIC and FIC. In all of cases, the activity of immobilized enzyme after cross-linking would be increased. Immobilized enzyme onto SGE-20/14-d3 and SGE-20/14-d4 are significantly increased in their activity. It can be concluded that immobilization of enzyme onto carrier with small pore sizes perform the greater activity. The comparison between PIC, FIC, and normal immobilization with various concentration of cross-linker is shown in Figure 5.

Figure 5 shows that cross-linking of full immobilized Cal-B (FIC) by lower cross-linker concentration gives the highest activity and the cross-linking of part immobilized Cal-B (PIC) by higher cross-linker concentration gives the highest activity. This regularity happened because of the characteristic of FIC and PIC. FIC is a rigid system which have a tight connection between enzymes and substrate to form chemical connection by covalent bond. On the other hand, PIC is a mobile system and almost all of interaction between enzymes toward the substrate is physical interaction such as hydrophylic or hydrophobic interaction. For the PIC system, the enzymes were easily moved and attack the cross-linker to make a stable conformation. However, small amount of cross-linker is not enough to make a stable conformation between enzyme and cross-linker. That is the reason why for lower concentration, FIC gives the highest activity. This phenomenon can be found also in the cross-linking of immobilized enzyme by 0.05% and 0.1% cross-linker. From these results, 1% of cross-linker is enough to make a stable conformation between enzyme and cross-linker. Because of that, for higher cross-linker concentration, PIC performs the highest activity. However, the saturation level and porosity parameters of immobilized Cal-B influenced enzyme activity. Moreover, the excess cross-linker will denaturate the enzyme easily. This phenomenon was found in the case of cross-linking of immobilized enzyme by 10% cross-linker. PIC with their weak connection between enzyme and copolymer will decrease the activity, but not for FIC. The reason is PIC is easy to denaturate by excess cross-linker concentration.

Comparison between normal immobilization and cross-linking activity with various cross-linker concentration are presented in Figure 6. This comparison showed the optimum values of cross-linker that was added in the immobilized enzyme.

Figure 6 shows the activity comparison between normal immobilization and cross-linking with various cross-linker concentration. From both of curves, cross-linking with 10% cross-linker concentration gives the highest activity. This comparison give a conclusion that the amount of cross-linker during cross-linking process have an important role to increase their activity. By increasing the amount of cross-linker concentration, the enzyme activity will increase. However, the saturation level and porosity parameters of immobilized Cal-B onto poly(GMA-*co*-EDGMA) influenced the enzyme activity.

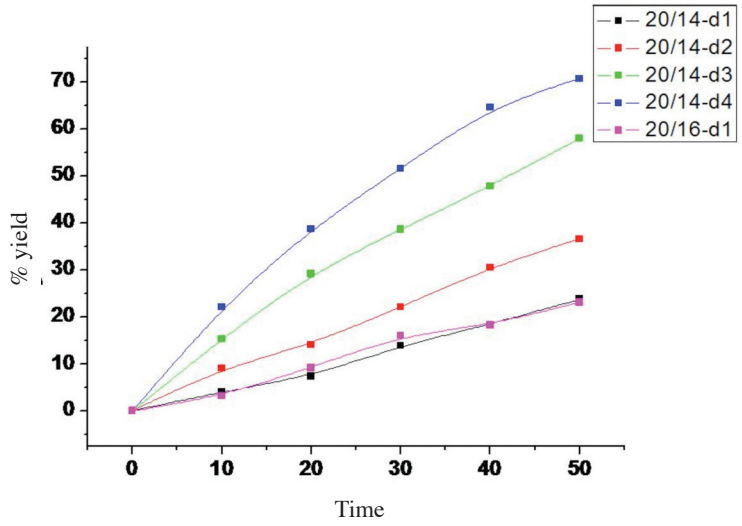

FIGURE 4. The representation of carrier influence towards enzyme activity for part immobilization with 0.05% CL

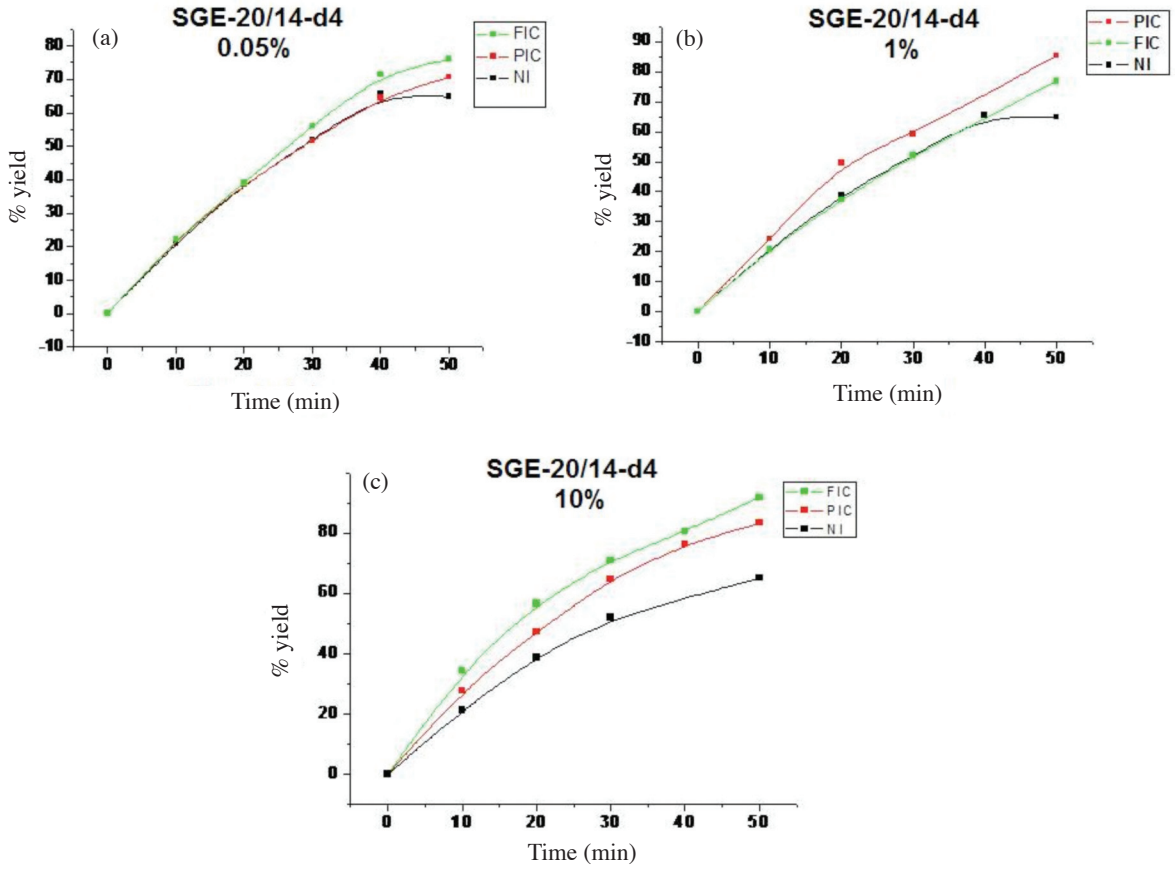

FIGURE 5. Comparison between PIC, FIC, and normal immobilization for SGE-20/14-d4 with (a) 0.05% CL, SGE-20/14-d4 (b) 1% CL, and (c) SGE-20/14-d4 with 10% CL

Immobilization of *Candida antarctica* lipase B onto poly(GMA-*co*-EGDMA) displayed greater activity than free Cal-B. Immobilized Cal-B can be recovered with great reusability. Enzyme kinetic tests indicate changes in the enzyme properties during immobilization process. After cross-linking with 1,2,7,8- diepoxyoctane, the activity of Cal-B is increase.

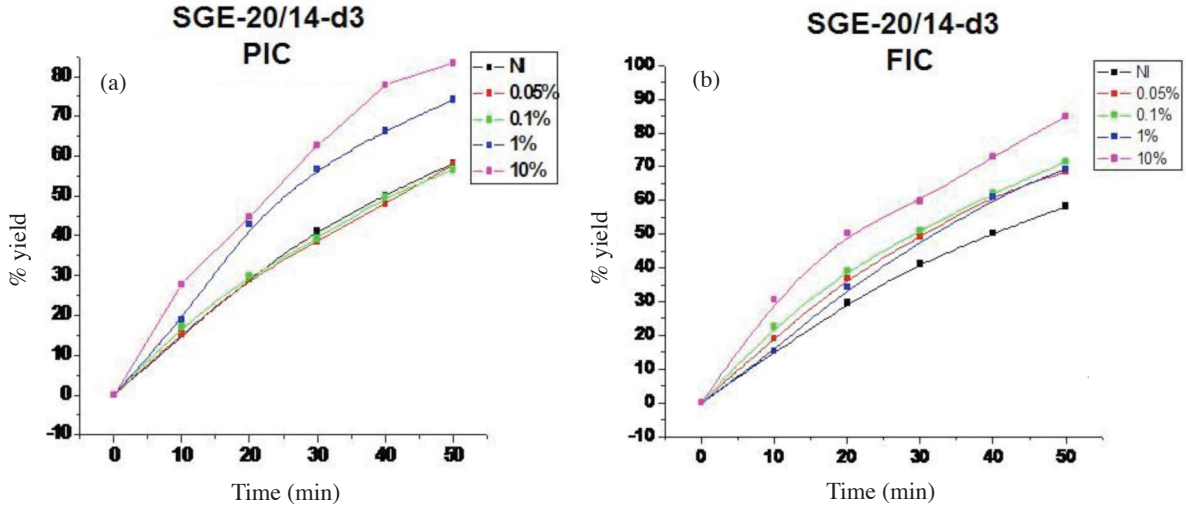

FIGURE 6. Comparison between normal immobilization and cross-linking activity with various concentration (a) for Part Immobilization crystals and (b) full immobilization crystals

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