PROPERTIES OF THE IMMOBILIZED *Candida antarctica* LIPASE B ON HIGHLY MACROPOROUS COPOLYMER

NURRAHMI HANDAYANI[1], NEMANJA MILETIC[2], KATJA LOOS[2], SADIJAH ACHMAD[1], DEANA WAHYUNINGRUM[1]

[1]Chemistry Study Program, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Indonesia

[2]Zernike Institute for Advanced Materials, University of Groningen, Netherland

E-mail: ami_chemie2003@yahoo.com

ABSTRACT

In spite of their excellent catalytic properties, enzymes should be improved before their implementation both in industrial and laboratorium scale. Immobilization of enzyme is one of the way that purposed in their properties improvement. *Candida antarctica* lipase B (Cal-B) has been shown a great number 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 improvement of enzymes activity and reusability. Enzyme kinetics test of immobilized enzymes folows the Michaelis-Menten models, and their activity is match with previous experiment. According V_{max} values of immobilized enzymes and free enzyme indicated that immobilized enzymes show higher activity than free enzyme. The cross-linking of immobilized enzyme gives the result that for lower cross-linker concentration, FIC (Full immobilization Crystals) gives the highest activity and for higher cross-linker concentration, PIC (Part Immobilization Crystals) gives the highest activityy. However, pore size and saturation level give an influence of their activity.

Keywords: Enzyme immobilization, cross-linking, *Candida antarctica* lipase B (Cal-B), macroporous copolymer

INTRODUCTION

Enzymes are macromolecules that have many roles in acceleration the chemical reactions of living cells. Without enzymes, most biochemical reactions would be too slow to even carry out life

processes. Enzymes display great efficiently because of specificity and biodegradable behaviour. Many advantages from the enzymes, such as high or low specified characteristic of enzyme can be selected to desired function, just a little by-product formation is observed, and optimal activity occurs under very mild reaction condition (Kobayashi, 2001). Enzyme that has many roles in chemical catalysis, both in laboratories and in industries is *Candida antarctica* Lipase B (Cal-B). Cal-B is one of the most effective catalysts with high stereoselectivity and simplicity of processes. These properties make this enzyme become the most adequate for the resolution of primary amines bearing the amino group in the stereocenter (Gotor-Ferna´ndez, 2006). Beside of many advantages, this enzyme has limitation: (i) high cost of enzyme preparations, (ii) weak stability, and (iii) easy inhibitation (Govardhan, 1999). Concerning these reasons, the improvement of their activitiy, stability, and reusability is needed.

In the second half of the last century, numerous attempts were devoted to growth of 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, 2005). Miscellanous of experiments were reported that the immobilization method using cross-linking reaction between enzyme molecules are an attractive strategy because it affords stable catalysts with high retention of activity (Cao, 2000; Schoevaart, 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 (Sheldon, 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, 2004). However, CLEAs are mechanically fragile, and it is difficult to handle and fully recover the CLEA particles over repetitive uses (Mc-Kee-Mckee, 2004). Because of these reasons, optimation and various tests have been needed in acquiring the best performance of enzyme. The aim of this research is to investigate the Cal-B immobilization on poly (glycidyl methacrylate-*co*-ethylene glycol dimethacrylate) and check their reusability, kinetic properties, and activity after crosslinking.

MATERIALS AND METHODS

MATERIALS AND INSTRUMENTAL

Methanol 99.8% were purchased from Lab-Scan, 4-nitrophenyl acetate (*p*NPA) was purchased from Sigma Aldrich, $Na_2HPO_4.12H_2O$ 99% and $NaH_2PO4.H_2O$ were purchased from Merck. *Candida antarctica* Lipase type B was 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 diamater (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-d2[a]	300-150	560	13.2	1.125

[a]The particles do not have spherical shape.

ENZYME IMMOBILIZATION AND SEPARATION

The immobilization of Cal-B was done by following a protocol of Miletic, N., et al. (2008). Copolymer beads and Cal-B (4:1) were dissolved in 1.5 mL buffer tris-HCl pH 6.8 in a 4 mL screw-capped vial. The samples were incubated in a rotary shaker at 30^{o}C and 200 rpm for 24 hours until immobilized enzymes have been precipitated. Buffer solution for each samples was removed by filtering and placed into the vials. On the other hand, immobilized Cal-B was washed with buffer *tris*-HCl 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 could be estimated. The solid phases were dried with using liquid nitrogen and placed under vacuum condition for 24 hour at room temperature.

BCA ASSAY

The working samples were prepared with added 1 volume of reagent B to 50 volumes of reagent A. In this research, 40 mL of reagent A was added with 0.8 mL reagent B until the color is changed. 2.0 mL of working samples were taken and putted into the flasks that contain 0.1 mL of each supernatant and washing fraction. The mixtures were incubated at 37^{o}C for 30 minutes and then determined the absorbance at 562 nm immediately by UV/VIS spectrophotometer. The loading of Cal-B on the copolymer was calculated from the amount of enzyme in the combined original solutions and washing

fractions. The loading is defined as the amount of Cal-B (mg) lost from the solution per unit of total weight of copolymer and the amount of enzyme.

CROSS-LINKING IMMOBILIZED ENZYMES

The immobilization onto 6 beads macroporous with chemically the same but different in porosity parameters was divided into two types. First type, immobilization was carried out for 2 hours which named part immobilized crystals (PIC) in order to make only physical connections between enzymes and macroporous copolymer. Second type, immobilization was carried out for 24 hours which named full immobilized crystals (FIC) in order to make not only physical connections but also chemical connections between enzymes and macroporous copolymers. Phosphat 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 those samples. Cross-linking was carried out during 3 hours, 30 ^{o}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 hours.

ACTIVITY AND REUSABILITY TEST

Activity and reusability test were carried out towards normal immobilized enzymes. These tests were followed the reaction between 4-nitrophenyl acetate (*p*NPA) and methanol in 1,4-dioxane. In the solution, containing *p*NPA (40mM) and methanol (80mM) in 1,4-dioxane, was added 0.5 mg of immobilized enzymes. The mixtures were incubated at 35 ^{o}C for 50 minutes (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 hours. The immobilized enzymes could be reused.

ENZYME KINETICS

Enzyme kinetics test was 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 is two times concentration of *p*NPA. These mixtures were added into 20 mL vials containing 0.5 mg immobilized enzymes (normal immobilization), then incubated at 35 ^{o}C for 50 minutes (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 at the λ_{max} (304 nm) of *p*NP.

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. Enzyme kinetic properties, reusability, and crosslinking the immobilized Cal-B were studied to know the influence of Cal-B immobilization to enzyme properties.

IMMOBILIZATION OF ENZYMES

Immobilization of Cal-B on eight of poly(GMA-*co*-EDGMA) was carried out during 24 hours to make chemical connections and physical connections 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 sum of Cal-B phisically adsorbed and amount of Cal-B chemically connected 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 perform the same tendency. Their increasing from 30 to 92 nm (resin 1 to 3, and resin 7 to 3) produce the decreasing of enzyme loading. Otherwise, for 630-300 μm beads with identical pore size of 30 nm, the increasing of the specific surface area from 82.0 (resin 1) to 106.0 m^2/g (resin 7) produced in decrease in enzyme loading from 204.69 (resin 1) to 179,96 μg/mg (resin 7). The differences of enzyme loading values are obtained from the differences of interaction between enzyme toward copolymer. Interaction of enzyme and water from the buffer during immobilization will cause the copolymer swelling and it can give the influence to the enzyme loading values (Smith, 1985). If the final loading of enzyme is still poor, it can caused by two reasons: (i) only the inner surface of mesopores is used for attachment of enzyme, (ii) 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 give the influence for 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 2).

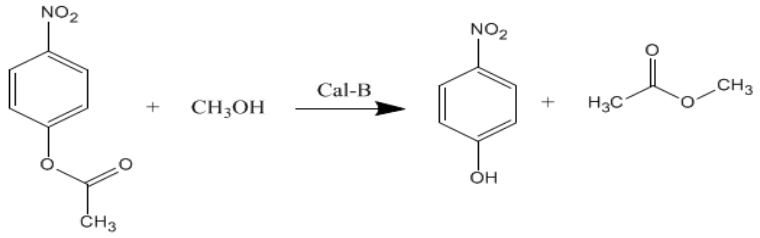

Figure 1 Transesterification between pNPA and methanol

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 (one sample is shown in the Figure 2 and 3).

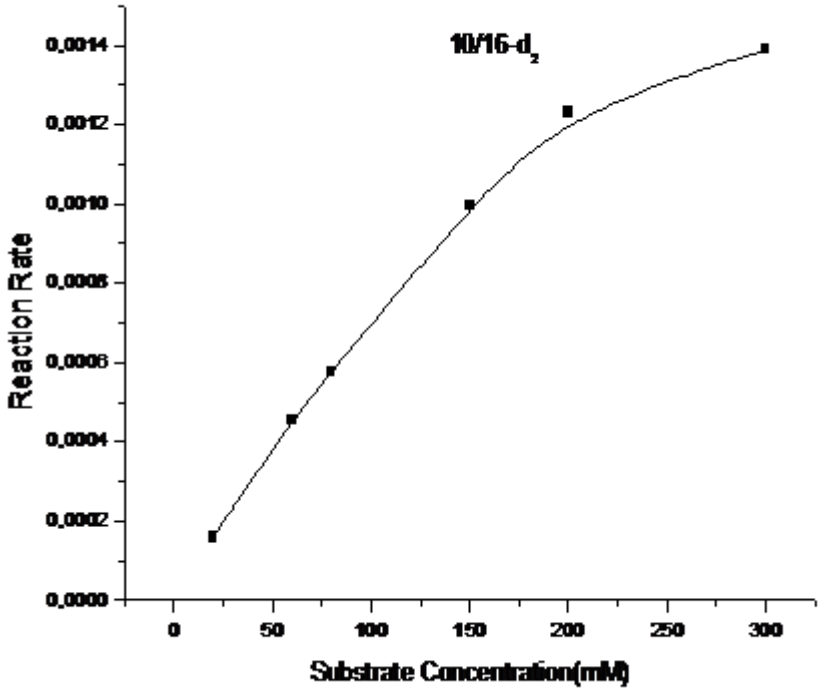

Figure 2 Saturation curve of SGE/20-16-d2

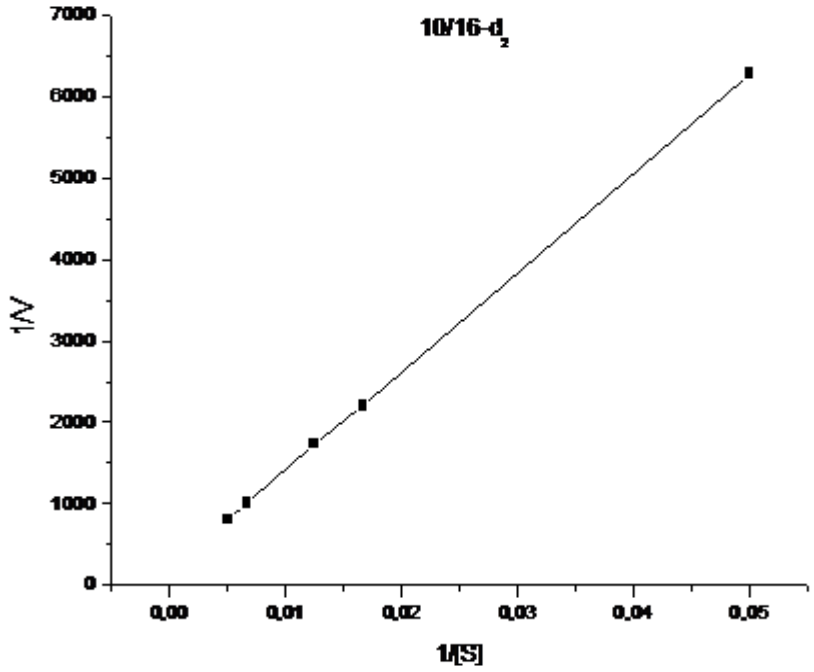

Figure 3 *Lineweaver-Burk* plot for SGE-10/16-d_2

Both saturation curve and *Lineweaver-Burk* plot of other samples are given the same tendency as curves in Figure 2 and 3. According to the obtained results, one can conclude that Cal-B mediated transesterification reaction between *p*NPA and methanol follows *Michaelis-Menten* kinetic model. *Michaelis-Menten* constant (K_M) describe 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 is changed 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 on 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 are higher than free enzyme. It means that amount of substrate that is required by immobilized enzyme to become saturated smaller than free enzyme. Moreover, the affinity of immobilized enzyme towards its substrate is smaller than free enzyme. On the other words, binding occured between immobilized enzyme and its substrate is more weak than free enzyme.

Table 3 K_M and V_{max} values of each immobilized enzyme

Immobilized Enzyme	K_M (mM)	Vmax (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}

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. On the other words, their efficiency is increase. 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 give the conclusion that enzyme kinetic properties were changed during the immobilization process.

REUSABILITY TEST

An important feature of immobilized enzymes is possiblity of reusability. Therefore, it is important to assay 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 IV.3 is shown the reusability test results.

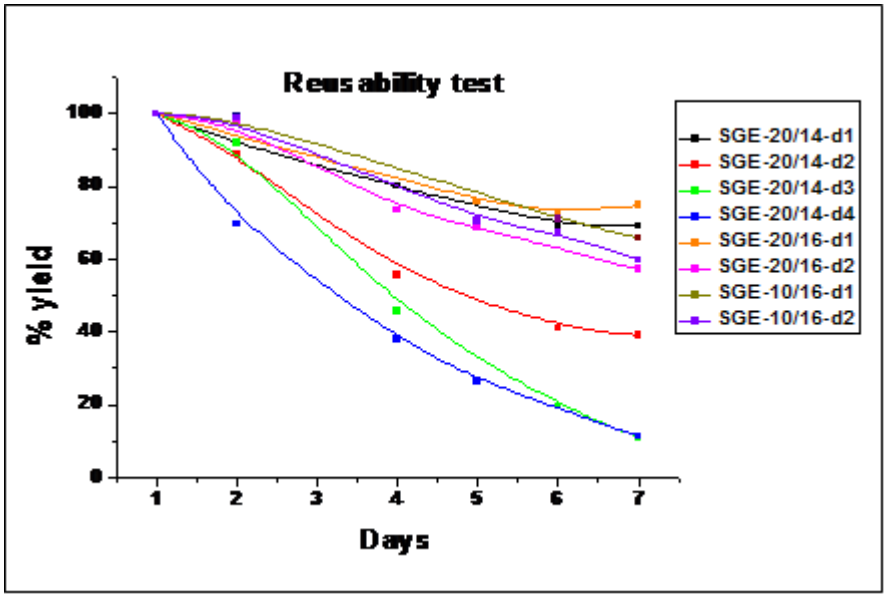

Figure 4 Result of reusability test

Figure 4 shows the decrease of enzyme activity after certain days. From this curve, the largest pore size of carrier performes the highest reusability. On the other hand, the smallest pore size of carrier performes 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 of carrier gives the highest reusability. However, immobilized Cal-B onto poly(GMA-*co*-EGDMA) perform the higher reusability than free enzyme. According to the literature, after two or three days, free enzyme shows the zero activity.

CROSS-LINKING 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 of stabilizing adsorbed enzymes and also for preventing leakage (Kim, 2006). Cross-linked enzyme aggregates (CLEAs) is one of cross-linking procedure that is produced by simple precipitation of the enzyme from aqueous solution by the standard techniques to produce physical aggregates of protein molecules (Sheldon, 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 hours incubation that is called PIC (Part Immobilization Crystals) and 24 hours 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.

The cross-linker that used in this procedure is 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. The results are presented in Figure 5.

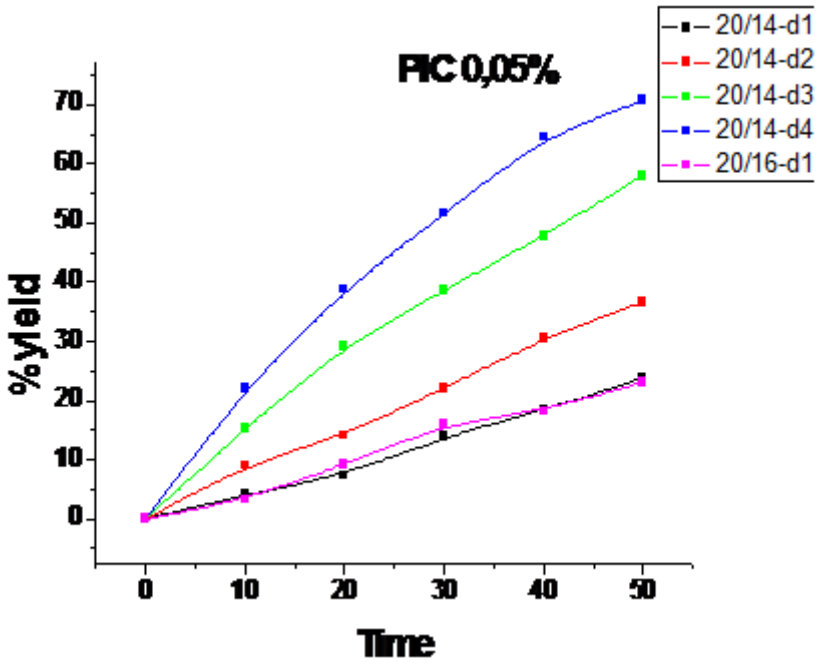

Figure 5 The representation of carrier influence towards enzyme activity for part immobilization with 0.05% CL

PIC with 0.05% cross-linker give the same performance which is the SGE-20/14-d4 shows the highest activity. The same tendency 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 will be increase. Immobilized enzyme onto SGE-20/14-d_3 and SGE-20/14-d_4 are significantly increase 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 6.

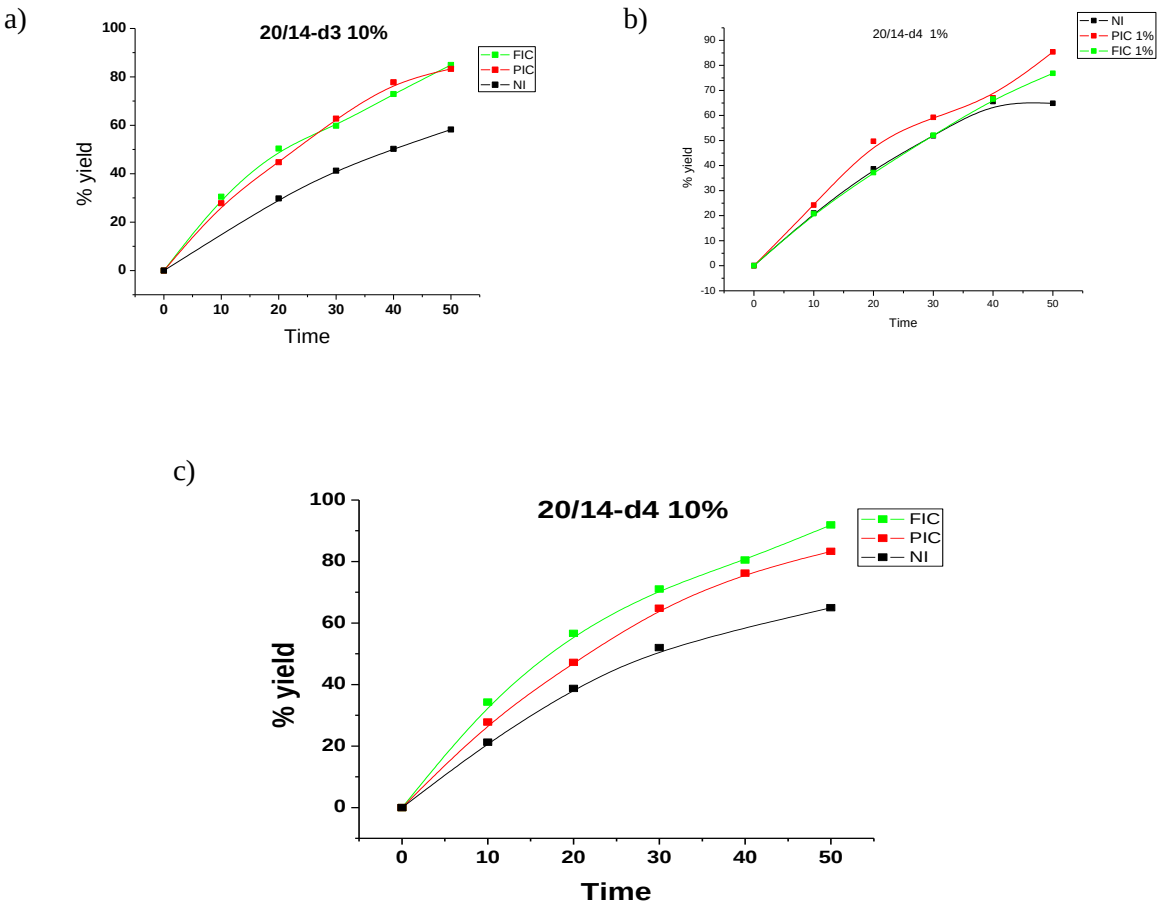

Figure 6 Comparison between PIC, FIC, and normal immobilization for : a) 20/14-d4 with 0.05% CL, b) 20/14-d4 with 1% CL, c) 20/14-d4 with 10% CL

Figure 6 gives the conclusion that cross-linking of full immobilized Cal-B with lower cross-linker concentration gives the highest activity and the cross-linking of part immobilized Cal-B with higher cross-linker concentration gives the highest activity. This regularity can be happened because the different characteristic of FIC and PIC. FIC is the rigid system which have the tight connection between enzymes toward substrate. On the other hand, PIC is the mobile system which almost of interaction between enzymes toward substrate is physical connection. For the PIC system, the enzymes are easily move and attack the cross-linker to make a stable conformation. 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 is still gives the highest activity. This phenomena can be found for the cases cross-linking 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 give an influence in enzyme activity. Moreover, the excess of cross-linker will denaturate the enzyme easily. This phenomena is found in the case of cross-linking of immobilized enzyme by 10% cross-linker.

PIC with their weak connection between enzyme and copolymer will give the decreasing of activity, but it is not happened for FIC. The reason is PIC 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 7. This comparison have a function to show the optimum values of cross-linker that is added in the immobilized enzyme.

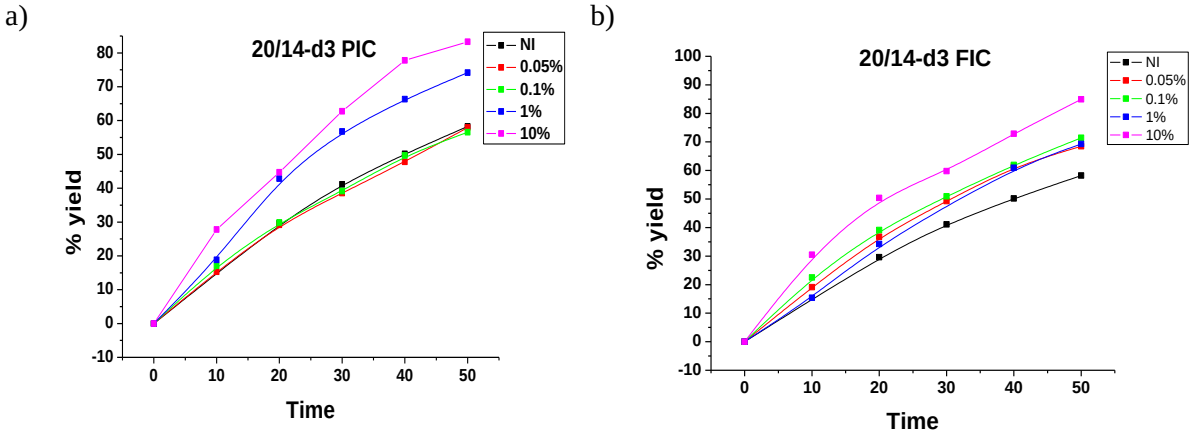

Figure 7. Comparison between normal immobilization and cross-linking activity with various concentration; a) Part Immobilization Crystals, b) Full Immobilization Crystals

Figure 7 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 increase the amount of cross-linker concentration, enzyme activity will increase. However, the saturation level and porosity parameters of immobilized Cal-B onto poly(GMA-*co*-EDGMA) give an influence in enzyme activity.

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

ACKNOWLEDGEMENTS

Dr. Sadijah Achmad, Dr. Deana Wahyuningrum, Dr. Katja Loos, Nemanja Miletic, DIKTI, and all of the staffs in Department of Chemistry ITB.

REFERENCES

Cao, L., van Rantwijk, F., and Sheldon, R.A.(2000), *Organic Letter*. **2**(10), 1361-1364.

Gotor-Ferna´ndez, V., Busto, E., Gotor, V. (2006), *Adv. Synth. Catal*. **348**, 797-812.

Govardhan, C.P. (1999), *Current Opinion in Biotechnology*, **10**, 331-335.

Kim, M.I., Kim, J., Lee, J., Jia, H., Na, H.B., Youn, J.K., Kwak, J.H., Dohnalkova, A., Grate, J.W., Wang, P., Hyeon, T., Park, H.G., and Chang, H.N. (2006), *Biotechnology and Bioengineering*, **96**(2), 210-218.

Kobayashi, S., Uyama, H., and Kimura, S. (2001), *Chem. Rev*. **101**, 3793-3818.

Mc Kee-Mc Kee. (2004), *The molecular basis of life*, 3th edition, McGraw Hill Comp., 125-126.

Schoevaart, R., Wolbers, M.W., Golubovic, M., Ottens, M., Kieboom, A.P.G., van Rantwijk, F., van der Wielen, L.A.M., and Sheldon, R. (2004), *Biotechnology and Bioengineering*. **87**(6), 755-762.

Sheldon, R. A., Schoevaart, R., and Van Langen, L.M. (2005), *Biocatalysis and Biotransformation*. **23** (3/4), 141-147.

Wilson, L., Illanes, A., Abia, O., Pessela, B.C.C., Ferna'ndez-Lafuente, R., and Guisa, J.M. (2004), *Biomacromolecules*. **5**, 852-857.