Enzymatic Synthesis of Polycaprolactone: Effect of Immobilization Mechanism of CALB on Polycaprolactone Synthesis

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ABSTRACT: Surface-modified rice husk ash was used as an inorganic support material for immobilization of Candida antarctica lipase B. (3-aminopropyl) trimethoxysilane was used for surface modification. Immobilization of CALB was performed via both physical adsorption and cross-linking. PCL synthesis was carried out by using these immobilized enzymes, free enzyme and Novozyme 435®. Molecular weight distribution of polymer samples was obtained by gel permeation chromatography (GPC) and chain structures of the polymer samples were observed by hydrogen nuclear magnetic resonance (^{1}H-NMR). The highest monomer conversion is generally obtained by using cross-linked enzyme, around 90%. PDI values for all polymer samples were approximately 1.5 which can be considered as acceptable. In general cross-linked enzymes were better than physically adsorbed enzymes in terms of average molecular weights. It can be concluded that PCL can be synthesized with these immobilized enzymes with high molecular weight and low PDI values.

KEYWORDS: Candida antarctica lipase B, cross-linking, physical adsorption, Polycaprolactone, rice husk ash

1 INTRODUCTION

Biomaterials are becoming attractive materials for various applications. Polycaprolactone (PCL) is a biopolymer which has attracted attention due to its mechanical properties and biodegradability [1]. PCL is synthesized via ring opening polymerization (ROP) which can be catalyzed with both chemical catalysts and enzymes. For enzymatic synthesis, Candida antarctica lipase B was successfully used in its immobilized forms [2, 3].

Candida antarctica lipase B (CALB) is a fungal lipase which is known for its ability to catalyze esterification and transesterification reactions. The commercially available CALB, Novozyme® 435, is physically adsorbed on a macroporous acrylic polymer resin [4] and is used for ring opening polymerization of several lactones. Even though it has many advantages in polymer synthesis, there are some major problems of utilization of Novozyme 435: High cost, leaching problem of the enzyme and diffusional limitations [5].
Rice husk ash (RHA) is a light and highly porous material with high silica content which has emerged as a cheap alternative to fumed silica [6]. It is obtained by burning of rice husk and the combustion temperature directly affects the structure of silica ash obtained. Rice husk is a by-product of rice production process which constitutes 20% of rice by weight. Its chemical composition is 50% cellulose, 25-30% lignin and 15-20% silica [7]. It was earlier reported that high content of amorphous silica (95%) can be obtained by burning at 700°C for 6 h [8-10]. Compared to the reaction conditions of fumed silica formation [11], the conditions to obtained RHA are much milder. Additionally, consumption of RHA is an advantageous application since disposal of this ash has harmful impacts on environment [12]. Rice husks find various application areas such as production of mesoporous sieves, insulating materials, fertilizers, silicon carbide, cement as additive and waste water treatment due to its high absorptivity [7, 8]. By the addition of functional groups to the surface through silanization process, RHA can become an active and suitable support material for enzyme immobilization. Silanization is a widely used technique for surface modification of materials. Organosilanes are generally used for surface modification of hydroxylated (silanol for silica-based materials) surfaces where hydroxyl groups on the surface react with active groups of the silanization agent, generally alkoxy groups. Trifunctional silanes have higher reactivity and monolayers of trifunctional silanes are believed to yield well-ordered and compact films [13, 14]. Most widely used trifunctional organosilanes are 3-aminopropyl triethoxysilane (3-APTES), 3-aminopropyl trimethoxysilane (3-APTMS) and 3-glycidyloxypropyl trimethoxysilane (3-GPTMS).

Physical adsorption is a simple and inexpensive immobilization method. In this method, enzyme attaches to the support material by weak forces such as Van der Waals, hydrophobic or ionic interactions [15, 16]. In case of lipase enzyme, generally hydrophobic interactions cause adsorption [17]. Adsorption is often preferred since it is a simple and low-cost procedure. There is no need for chemical

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addition to the process which eliminates the risk of undesired modification of the enzyme and high lipase activity recovery can be achieved [16, 18]. However, in case of adsorption the major disadvantage is that enzyme tends to leach out especially in aqueous media [15, 18]. On the other hand, immobilization by cross-linking is achieved by the formation of intermolecular cross-linkages. The procedure requires a multifunctional reagent such as glutaraldehyde. Such a chemical modification can provide multipoint covalent attachment between both enzyme and enzyme or enzyme and support material [19]. Moreover, the fact that glutaraldehyde may also take place as dimeric form (amino/glutaraldehyde/glutaraldehyde moieties) on the surface of carrier decreases the steric hindrance. Thus, the enzyme may react with more and relatively large substrates [20]. In the case of such a dimer formation, the modified surface may provides three kinds of interaction opportunity for enzyme attachment: Covalent, hydrophobic, anionic exchange according to reaction conditions. Due to this multifunctionality, the enzyme to be immobilized has three different and even simultaneous possibility to build an interaction with the surface of the modified support [21].

In this study, rice husk ash was used as a support material for CALB immobilization commercially known as Lipozyme®. Rice husk is high silica containing material which is obtained from rice production process as by-product. Additionally, it is a cheap and easily accessible material since it is obtained from a locally available product. 3-APTMS was used as the reagent for activation of RHA. The reason of this choice is the faster hydrolysis rate of methoxy groups of 3-APTMS, which may favor the formation of more siloxane bonds with the support material compared to similar agents with ethoxy group such as 3-APTES [22]. Immobilization of CALB onto surface-modified RHA was earlier performed via both physical adsorption and cross-linking. PCL synthesis was carried out by using these immobilized enzymes. Additionally, PCL synthesis was carried out by using free enzyme and Novozyme 435® for comparison. Molecular weight distribution of polymer samples was measured by gel permeation chromatography (GPC) and chain structures of the polymer samples were observed by hydrogen nuclear magnetic resonance (^{1}H-NMR). Polymers were also characterized with FT-IR, TGA, DSC and XRD.

2 MATERIALS AND METHODS

2.1 Materials

Free CALB was obtained from Novozymes (Denmark). Coomercial immobilized CALB (Novozyme 435®) was purchased from Sigma-Aldrich. Rice husk ash (RHA) was obtained by burning rice husks which were supplied by a rice production company in Edirne, Turkey. (3-Aminopropy) trimethoxysilane (3-APTMS) (95%, $C_6H_{17}O_3NSi$) were purchased from Aldrich and Acros, respectively. Phosphate buffer solutions were used during immobilization processes. These solutions were prepared with monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) which were purchased from Carlo Erba and Merck, respectively. Glutaraldehyde solution, used as the cross-linker in the immobilization method was purchased from Merck.

In PCL synthesis, ε-caprolactone (99%), dried over molecular sieves before employment, was obtained from Alfa Aesar. Molecular sieves were purchased from Sigma Aldrich. Toluene (99%), solvent in polymerization, and methanol (99%) were purchased from Merck. Chloroform (99%) was obtained from Sigma Aldrich. Tetrahydrofuran (THF-HPLC grade), used in GPC analysis, was purchased from Carlo Erba.

2.2 Preparation of Surface-Modified Rice Husk Ash for Immobilization Support Material

Rice husks were washed with distilled water and dried in an oven for 24 h. Then the dried husks were burned in a furnace at 600°C for 6 h in order to obtain RHA. The temperature of the furnace was increased stepwise until the burning temperature was reached. RHA was modified with 3-APTMS separately before it was used as support material for lipase immobilization. For this purpose, RHA was mixed with 15% (v/v) 3-APTMS in acetone. These mixtures were incubated in shaking water bath at 50°C and 160 rpm for 2 h. After incubation, surface modified RHA were filtered and washed with distilled for removal of unreacted compounds and dried at 60°C. The activation of RHA was accomplished by the reaction given in Figure 1.

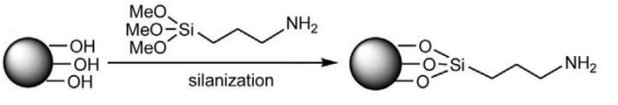

Figure 1 Surface activation of RHA with 3-APTMS [23].

2.3 Immobilization of CALB Onto Surface-Modified Rice Husk Ash

Free form of lipase enzyme was immobilized on surface-modified RHA by physical adsorption and cross-linking methods. In physical adsorption method, the activated RHA was mixed with free lipase enzyme (enzyme to support ratio of 2 μl/mg) and 0.015 M, pH 7.0 phosphate buffer solution on a magnetic stirrer at

room temperature for 5 h. At the end of mixing, immobilized enzyme samples were collected by filtrating the solution under vacuum. The immobilized enzyme samples were washed with 0.015 M, pH 7.0 phosphate buffer solution. They were dried at 30°C for 12 h. In order to obtain cross-linked lipase, activated RHA, subsequent to silanization, was treated with 2% (v/v) glutaraldehyde/phosphate buffer solution (pH 7, 0.015 M) at room temperature for 2 h on magnetic stirrer. The rest of the immobilization procedure was executed as the same in physical adsorption. This reaction, depicted in Figure 2, was run at neutral pH to enable the formation of Schiff bases towards the terminal amino groups of the enzyme.

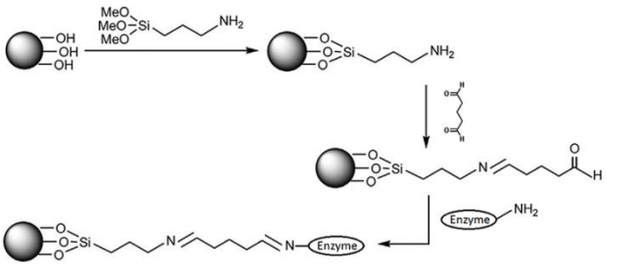

Figure 2 Two-step immobilization process by cross-linking [Adapted from 24].

2.4 Characterization of RHA and Activated RHA

Chemical structures and compositions of RHA were determined by FTIR analysis. Thermal gravimetric analysis was carried out for thermal characterization of surface modified RHA. Analysis was conducted with SEIKO TG/DTA 6300. 5-10 mg of samples was heated to 1000°C with a rate of 10°C/min and cumulative weight loss of samples was recorded. Nitrogen isotherms were measured at the temperature of liquid nitrogen (N_2) with a Micromeritics ASAP 2010 apparatus. Liquid N_2 used during analysis was at 77.30 K. The surface area of RHA was determined by the BET method. The total pore volume was calculated from the amount of vapor adsorbed at a relative pressure (P/P_0), where P and P_0 are the measured and equilibrium pressures, respectively.

2.5 Enzymatic Ring Opening (e-ROP) Reaction of ε-Caprolactone

Ring opening polymerization (ROP) of ε-caprolacon (ε-CL) was performed in presence of lipase immobilized onto the support via the cross-linking and physical adsorption methods. Reaction was catalyzed by immobilized lipase; enzyme to monomer ratio was 20% (w/w). Toluene was used as organic medium for the ring opening polymerization, since it was reported to be more efficient for the ROP of ε-CL than other organic mediums due to its hydrophobicity and apolarity [25]. In order to determinate optimum reaction condition, reactions were conducted at various temperatures and reaction times. Reactions were terminated by the addition of chloroform. After that the enzyme was separated from the reaction medium by vacuum filtration; the supernatant, polycaprolactone solution, was put into the oven at 50°C and chloroform was evaporated in order to obtain pure polymer. The polymerization reactions were performed at different temperatures and reaction times. Additionally, the effect of enzyme concentration on the molecular weight and PDI of polymers was evaluated. The performance of immobilized enzyme was compared with commercial lipases, Novozyme 435® and Lipozyme® in terms of molecular weight and PDI value.

2.6 Characterization of Polycaprolactone

Perkin Elmer FT-IR Spectrum One B Spectrometer was used for the determination of chemical structure and composition of PCL samples. By using FT-IR, the synthesized polymer was verified to be PCL by comparing with characteristic functional groups and bonds of PCL. Each sample was analyzed by KBr pellet. The spectra were recorded by at least 32 scans with a resolution of 2 cm^{-1}. Thermal characterization of PCL samples were carried out by TGA. 5-10 mg of samples was heated from room temperature to 1000°C with a rate of 10°C/min under air flow. The apparatus used is SEIKO TG/DTA 6300. For the determination of Tm value of synthesized polymer samples SEIKO 7020 DSC was used. Thermal characterization was carried out between -50°C and 200°C at 10°C/min under inert nitrogen atmosphere. Crystallinity percentages (X_c) of polymer samples can also be obtained by DSC, using the following Equation [26].

$$X_c = \left(\frac{\Delta H_m}{\Delta H_m^\circ}\right) \times 100$$

In equation, ΔH°_m is the melting enthalpy of PCL and its value is 139.3 J/g [26]. ΔH_m is the enthalpy value at T_m temperature of the PCL sample. Gel permeation chromatography (GPC) was used for the determination of molecular weights and polydispersity indices of PCL samples. Measurements were carried out by Agilent 1100 model GPC apparatus. THF was used as an effluent with a flow rate of 1 ml/min. Analysis was carried out at 25°C. Samples were prepared by dissolving 3 mg of PCL in 1 ml THF. Before injection, all samples were filtered via 0.45 μm filter syringe. Hydrogen nuclear magnetic resonance spectroscopy (^{1}H-NMR) was used for the determination of molecular weight and molecular structure of PCL (Bruker

Ultrashield 300 MHz). Deuterated chloroform ($CDCl_3$) was used as a solvent. Molecular weight (M_n) value of PCL can also be calculated based on the areas of peaks obtained at characteristic chemical shift (δ) values of 4.07 ppm (CH_2O) and 3.65 ppm (CH_2OH, end group) by using the equation below [27].

$$M_{n,NMR} = \frac{5 \times I_{4.07}}{2 \times I_{3.65}} \times M_{\varepsilon-CL}$$

In this equation, $M_{\varepsilon\text{-CL}}$ is the molecular weight of ε-CL, $I_{4.07}$ and $I_{3.65}$ are the integrated peak areas [27]. Surface morphologies of immobilized enzymes and PCL samples were observed by JEOL JSM-6390LV SEM. Samples were coated with platinum and analysis was performed at 5 kV. For the investigation of crystal structures of PCL samples, X-Ray diffraction (XRD) analysis was performed by PANalytical XPER'T PRO XRD apparatus with CuKα radiation source (λ=1.5406 Å). Analyses were carried out between 15 and 30°C values with an increasing rate of 0.026°/s at 25°C.

3 RESULTS AND DISCUSSION

3.1 Characterization of RHA and Activated RHA

As shown in Table 1, the surface area (S_{BET}) of unmodified RHA is found to be 50.61 m^2/g. This value is significantly lower than that of fumed silica [28]. It is known that surface area of RHA decreases with increasing combustion temperature during preparation [29]. The surface area decreases to 31.88 m^2/g when the silica is modified with 3-APTMS. This may be due to deposition of small species formed by hydrolysis reactions [30]. Similar results were obtained with fumed silica. However, surface area of modified fumed silica is higher than RHA [28]. Cumulative pore volume is significantly increased after surface treatment.

Table 1 Surface areas and pore sizes of the RHA samples.

	RHA	3-APTMS-modified RHA
BET Surface Area (S_{BET}) (m^2/g)	50.61	31.88
BJH Adsorption Cumulative Pore Volume (pores 1.7-300 nm) (cm^3/g)	0.196	0.156
BJH Adsorption Average Pore Diameter (nm)	14.26	21.46

Structural characteristic peaks of silica-based materials were observed in FTIR spectrum of RHA (Figure 3). The first one was at about 800 cm^{-1} and it represents Si-O stretching peak [31]. The second one was found to be at around 1050 cm^{-1}. It is caused by asymmetric stretching vibrations of Si-O-Si bond structure [32]. Similar peaks were observed in FTIR spectrum of fumed silica [28], thus it was proved that RHA is a silica-based material.

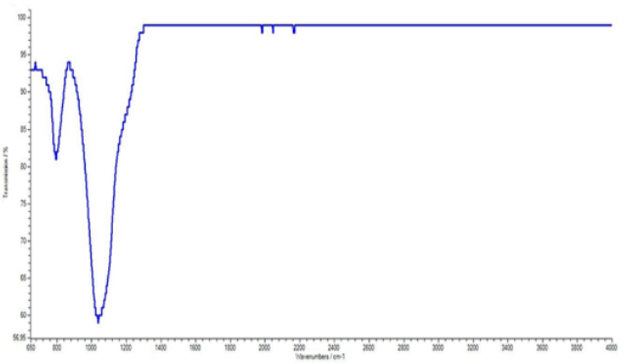

Figure 3 FTIR spectra of RHA.

Surfaced-modified RHA samples were thermally characterized by TGA in order to observe the presence of silane coupling agents. The cumulative weight loss of pure RHA up to this temperature was given as 1.8% in a previous study [33]. The thermogram given in Figure 4 showed a cumulative weight loss of 2.5% for 3-APTMS modified RHA as the temperature reaches 650°C. The difference of the two values gives the amount of evaporated 3-APTMS and it was calculated as 0.7%.

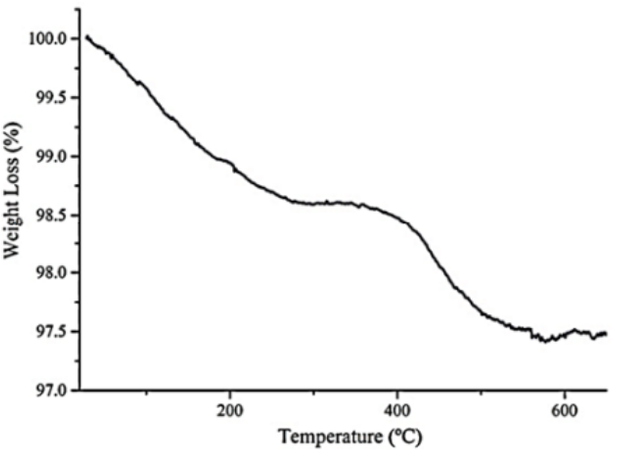

Figure 4 TGA curve of RHA.

3.2 Enzymatic Ring Opening (e-ROP) Reaction of ε-Caprolactone

Conditions of PCL polymerization reaction (time and temperarure) carried out with enzymes immobilized by physical adsorption and cross-linking were compared in terms of number average molecular weight (M_n), monomer conversion and polydispersity

indexes (PDI). Figure 5 depicts the comparison between different reaction conditions in terms of monomer conversion. It can be easily observed from the graphs that in general, monomer conversion was higher when the reaction was catalyzed by the cross-linked enzyme regardless of reaction conditions. It is also very clear that physically adsorbed enzymes were not very effective at low reaction times. Nevertheless, high monomer conversions (~90%) could be reached with both physically adsorbed and cross-linked enzymes. At reaction temperatures of 40°C and 60°C, there is a tendency of decrease in monomer conversion when reaction time reached to 120 h. It is known that CALB has also the ability of PCL degradation [34]. The decrease of monomer conversion at 120 h of reaction time may be attributed to product degradation by the enzyme.

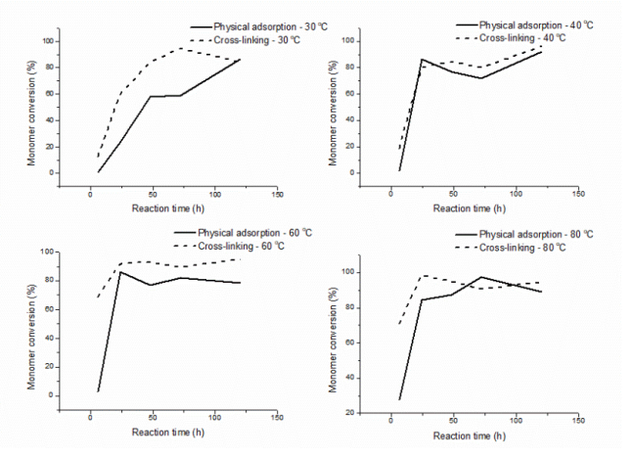

Figure 5 Monomer conversions at different reaction conditions (Monomer conversions were calculated gravimetrically).

Average molecular weight and polydispersity indices (PDI) of the polymer samples were determined by using gel permeation chromatography (GPC) (Table 1). As given in Table 2, PDI values were in a narrow range of 1.0-1.5. For natural polymers PDI value is equal to 1.0 which indicates a monodisperse structure [35]. PDI values that we obtained were close to 1.0, polymer samples can be considered as monodisperse.

For PCL synthesized with cross-linked enzyme, as reaction time increased from 6 h to 72 h keeping the temperature constant at 30°C, molecular weights increased. It is remarkable that even at a low temperature as 30°C, cross-linked enzyme successfully catalyzed the polymerization reaction with a high molecular weight end-product. When the reaction temperature was increased to 40°C, decrease in molecular weight between 48 h and 72 h of reaction times was observed in contrast to increase in monomer conversion in the same reaction time range. This may be caused by possible chain transfer from relatively long polymer chains to monomers [36]. At 60°C, for longer reaction times than 24 h, there was decrease in M_n value. This situation may be explained by the degradation activity of immobilized lipase above 24 h of reaction time at 60°C. In case of cross-linked enzyme, highest molecular weight was reached at 60°C and 24 h, and characterization of synthesized PCL was executed at this reaction conditions. For PCL synthesized with physically adsorbed enzyme, high molecular weights can only be achieved at higher reaction times such as 72 h and 120 h. Moreover, moderate temperatures as 40°C or 60°C yield higher molecular weights, reaching up to almost 15000 g/mol at 40°C and 72 h. Although the highest molecular weight was reached at this reaction conditions, shorter reaction times, such as 48 h, yield close molecular weight values. Thus 40°C and 48 h was decided to be more feasible choice as optimum reaction conditions.

Table 2 Average molecular weight and polydispersity index values of PCL synthesized at different reaction conditions.

Time (h)	Temperature (°C)	Physical Adsorption		Cross-linking	
		M_n	PDI	M_n	PDI
6	30	-	-	3700	1,1
	40	4300	1,1	4300	1,2
	60	1900	1,2	6600	1,4
	80	5000	1,2	7200	1,3
24	30	4600	1,2	11500	1,3
	40	9200	1.5	9600	1.4
	60	8500	1.5	13700	1.5
	80	9000	1,5	11300	1,4
48	30	6500	1,3	11500	1,4
	40	12000	1.4	9000	1.4
	60	8900	1.4	10300	1.4
	80	9500	1,5	9200	1,4
72	30	4500	1,3	9500	1,4
	40	14600	1,4	12500	1,3
	60	12900	1,6	10100	1,4
	80	10600	1,4	9200	1,4
120	30	12800	1,4	10800	1,5
	40	11400	1,4	12000	1,4
	60	12800	1,4	11100	1,5
	80	11000	1,4	12000	1,5

The effect of enzyme concentration on PCL polymerization efficiency in terms of average molecular weight, monomer conversion and PDI value was given in Table 3. Different enzyme concentrations were employed with the same amount of monomer, ε-caprolactone, at the best reaction conditions for each type of immobilized enzymes. Considering economic aspects, polymerization reactions catalyzed with higher enzyme concentration than 20% (w/w) were not performed. As the enzyme concentration was increased, an increase was observed on monomer conversions, molecular weight and PDI value. In experiments with 20% of enzyme concentration, molecular weight and monomer conversion reached to their highest values. Besides, PDI value was determinated as 1.5, indicating formation of almost monodisperse polymers. At low enzyme concentrations, physically adsorbed lipase performed a better monomer conversion and yielded high molecular weight PCL compared to cross-linked lipase. However, cross-linked lipase showed better performance at 20% enzyme concentration, reaching the highest molecular weight and monomer conversion. Depending on these results, it can be concluded that the catalytic activity cross-linked lipase is better than physically adsorbed lipase in terms of ROP of ε-caprolactone**.**

Table 3 Effect of enzyme concentration on polymerization reaction.

Enzyme concentration (%) (w/w)	Conversion[a] (%)		M_n[b] (g/mol)		PDI[b]	
	CR	ADS	CR	ADS	CR	ADS
2.5	10	22	860	5300	1.0	1.1
5	15	32	5300	6100	1.1	1.4
10	50	75	8600	9300	1.4	1.5
20	92	73	13700	12000	1.5	1.5

[a] Conversion was calculated gravimetrically.
[b] Mn and PDI were obtained by GPC.

3.3 Characterization of Polycaprolactone

Structural characterization of polymer chains obtained in polymerization at 60°C and 24 h catalyzed by cross-linked enzyme, were determined by Fourier transform infrared spectroscopy. As seen in the spectrum (Figure 6), the characteristic peaks of PCL were observed at around 1724 cm^{-1}, 2864 cm^{-1} and 2924 cm^{-1}, which are belong to crystalline carbonyl (C=O) stretching bonds, symmetric CH_2 bonds and asymmetric CH_2 bonds, respectively. Besides, backbone C-C and C-O stretching bonds in the crystalline phase was observed at 1293 cm^{-1} [3]. It can be concluded that PLC was synthesized successfully by immobilized CALB on RHA. In the spectrum of PCL which is synthesized in the presence of physically adsorbed enzyme at its optimum conditions, same characteristic peaks can be observed belonging to crystalline carbonyl (C=O) stretching bonds, symmetric CH_2 bonds and asymmetric CH_2 bonds, respectively. Thus, it is safely stated that both enzymes are effectively capable of catalyzing ring opening polymerization of ε-caprolactone.

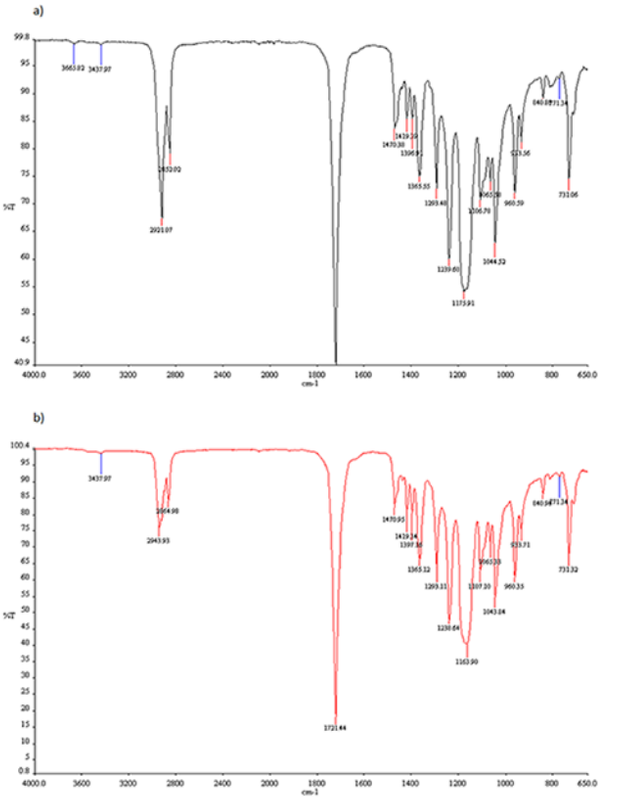

Figure 6 FTIR spectra of a) CR-PCL b) ADS-PCL.

The thermal degradation of PCL samples was analyzed by TGA. Ruseckaite and Jiménez has reported that, maximum degradation temperature of PCL is about 415°C under same thermal analysis conditions with the conditions applied in this study [37]. The TGA curves of PCL synthesized by cross-linked lipase were displayed in Figure 7. It seems that a sudden drop can be observed around 380°C on the TGA profile of PCL synthesized by cross-linked lipase, indicating initial degradation temperature (T_d) of PCL sample. Maximum weight loss temperature was observed as a single peak at 411°C. Initial degradation temperature of PCL synthesized by physically adsorbed lipase was found

to be 400°C as seen in Figure 7. Maximum weight loss temperature was 410°C. These results have shown that, PCL synthesized with these new immobilized lipases had a high thermal stability.

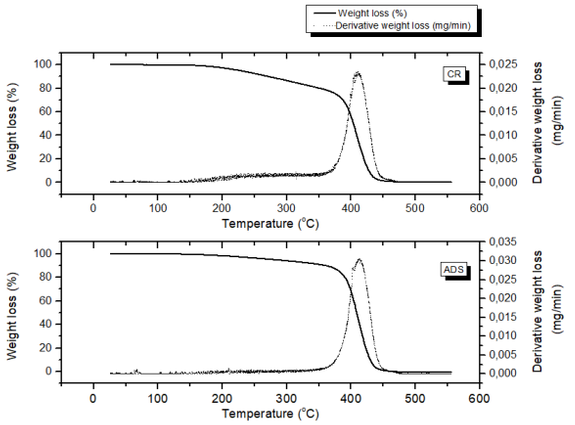

Figure 7 TGA and DTA curves of PCL synthesized with cross-linked lipase (CR-PCL) and physically adsorbed lipase (ADS-PCL).

The thermal transitions of PCL samples were evaluated by DSC and were depicted in Figure 8, on which melting points (T_m) were shown as a function of reaction temperature. The melting point of PCL samples were recorded as 61.21°C and 61.74°C for PCL synthesized by cross-linked and physically adsorbed lipase, respectively. These low melting temperatures provides easy manufacturing processes. The relative crystallinity (X_c) of the sample were calculated as 80% for PCL synthesized by cross-linked lipase. This result means that the PCL sample was mainly in crystalline structure. For PCL synthesized by physically adsorbed lipase, relative crystallinity was calculated as 62% which indicates to a semicrystalline structure.

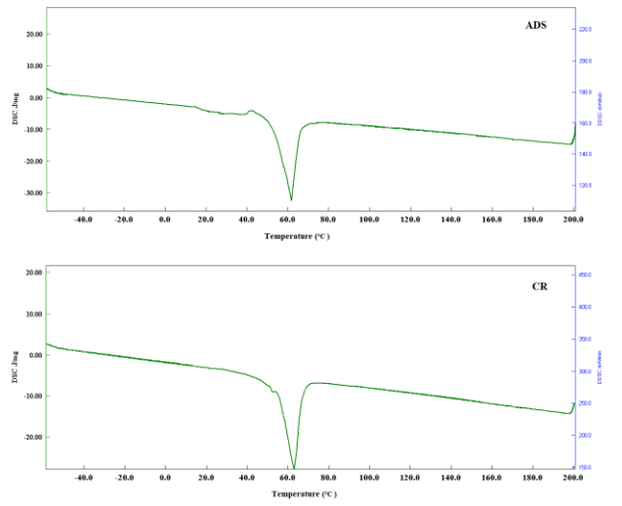

Figure 8 Melting points of ADS-PCL and CR-PCL.

Chemical structure of both polymer samples at their optimum reaction conditions was further characterized by ^{1}H-NMR spectroscopy. The spectrum was zoomed between 4.25 ppm and 3.5 ppm range, in which the characteristic peaks that were used for molecular weight calculation can be seen, was shown in Figure 9. The chemical shifts (ppm) observed from the given spectra were as follow: 4.07 ppm (CH_2O) and 3.65 ppm (CH_2OH, end group) which are characteristic for PCL [38]. Molecular weight of the PCL sample was also calculated from ^{1}H-NMR spectrum. $M_{n,\,NMR}$ value was obtained as 12700 g/mol and 11300 g/mol for PCL synthesized by cross-linked and physically adsorbed lipase, respectively. These values are consistent with the ones obtained from GPC, which were 13700 g/mol for PCL synthesized by cross-linked and 12000 g/mol for PCL synthesized by physically adsorbed lipase.

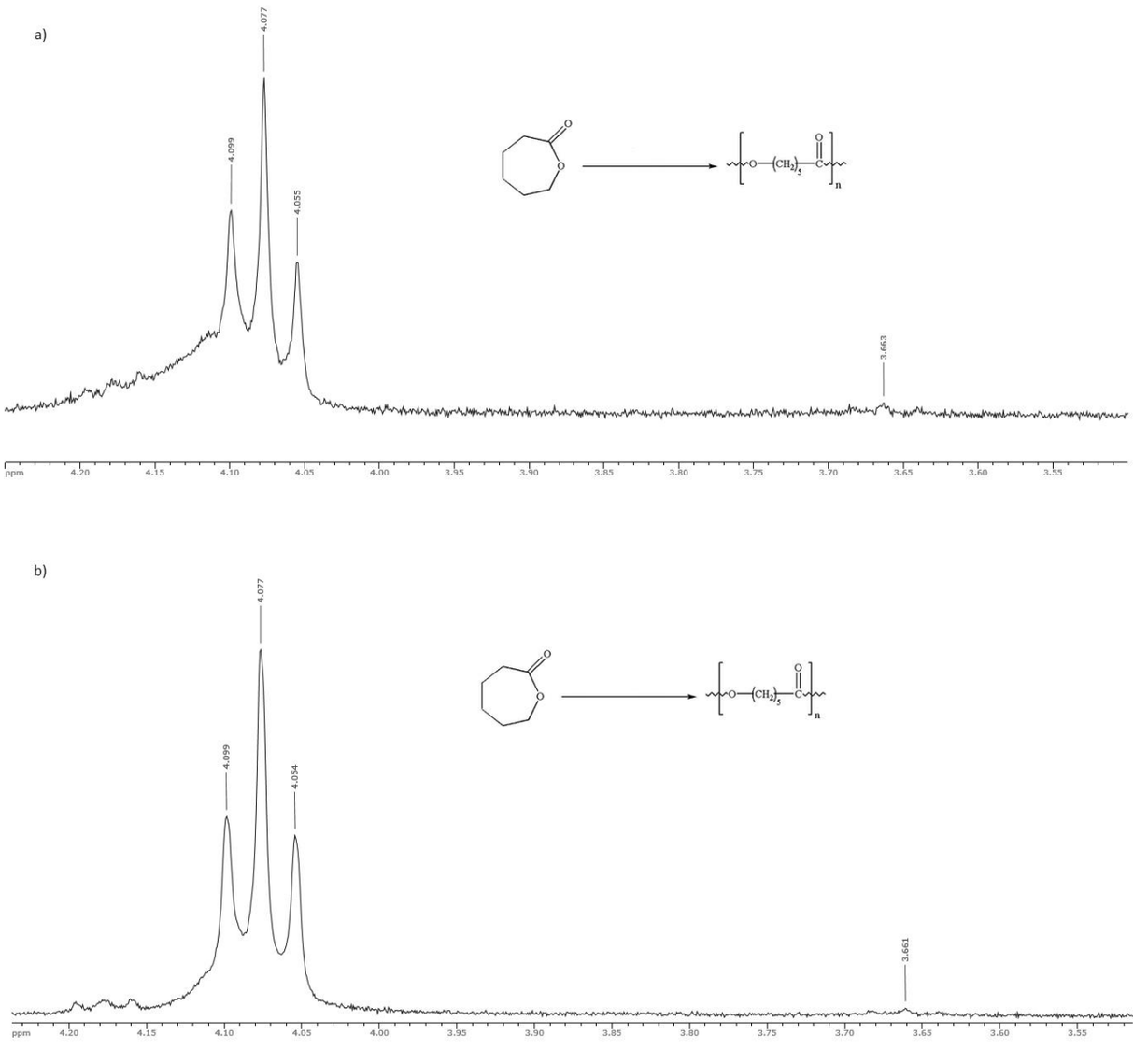

Figure 9 ^{1}H-NMR spectra of a) CR-PCL b) Pads-PCL.

Additional to the DSC analysis, XRD analyses were carried out in order to characterize the crystal structure of polymer samples. The XRD patterns were presented in Figure 10. The semi-crystalline structure of the samples was determined based on formation two diffraction peaks around 21.39 and 23.72, which are unique to PCL polymer.

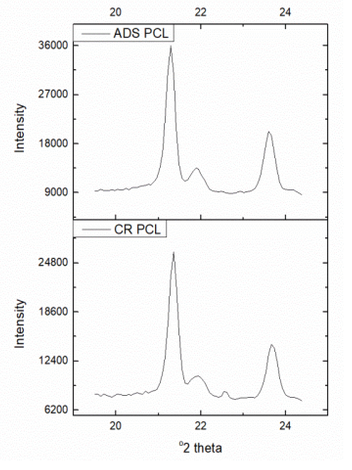

Figure 10 XRD patterns of CR-PCL and ADS PCL.

Finally, surface morphology of PCL samples was investigated by scanning electron microscopy (SEM) on Figure 11. Images of the samples were displayed at 3000x magnification. Poly(ε-caprolactone), synthesized in this study, has a lamellar structure within a range from approximate 1 μ to 5 μ diametered pores, which could provide surface area for cell adhesion.

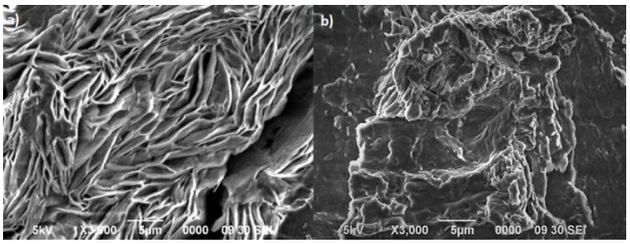

Figure 11 SEM images of PCL synthesized by a) cross-linked b) physically adsorbed lipase.

3.3 Comparison with Commercial Lipases

The best reaction conditions determined for both types of immobilized lipases were applied for commercial lipases; Lipozyme® and Novozyme 435® following the same PCL synthesis process explained earlier. Figure 12 compares M_n (g/mol) values of each type of enzyme. It is very obvious that the both type of immobilization procedure improved the catalytic activity and stability of free lipase so that the synthesized PCL reached higher average molecular weight. At its best reaction conditions (40°C and 48 h), average molecular weight of PCL synthesized by physically adsorbed lipase was close to that of PCL synthesized by Novozyme 435®. However, Novozyme 435® seems to be more thermally stable than our physically adsorbed lipase. When the reaction temperature was increased to 60°C, average molecular weight of PCL was increased in case of Novozyme 435® catalysis, whereas it was decrease in case of our ADS lipase catalysis. Additionally, monomer conversion of Novozyme 435® and Lipozyme® (95% and 98% respectively) was significantly higher than ADS lipase (77%). The average molecular weight of PCL synthesized with cross-linked lipase was lower than the average molecular weight of PCL synthesized with Novozyme 435® which reaches 17000 g/mol. Monomer conversion was very close to that of Novozyme 435®, at optimum reaction condition for CR lipase (60°C, 24 h). Immobilized CALB onto RHA by both physical adsorption and cross-linking can be considered as an alternative to commercially available CALB (Novozyme 435®) for enzymatic ROP of ε-caprolactone.

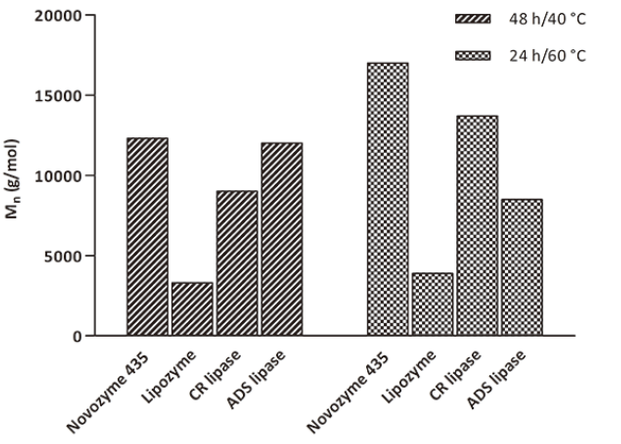

Figure 12 Comparison of CR lipase and ADS lipase to commercial lipases.

4 CONCLUSION

In this work, CALB was successfully immobilized onto rice husk ash via two different mechanisms: physical adsorption and cross-linking. RHA used in this study was characterized in order to understand its structure and observe the effect of surface modification. FTIR spectrum of RHA showed similarities with the spectrum of fumed silica. These similarities were considered as a proof that RHA contains silica. Surface modified RHA was characterized by TGA in order to determine whether the modification was successful or not. The result showed that 3-APTMS modification was successful. The immobilized enzymes were then used for enzymatic synthesis of PCL. The highest monomer conversion is generally obtained by using cross-linked enzyme, around 90%. Additionally, PDI values for all polymer samples were approximately 1.5 which can be considered as acceptable. In general cross-linked enzymes were better than physically adsorbed enzymes in terms of number average molecular weights. The maximum average molecular weight was reached with cross-linked CALB catalysis. Also, monomer conversion of cross-linked lipase was significantly higher than monomer conversion of physically adsorbed CALB, at their optimum reaction conditions (60°C, 24 h and 40°C, 48 h for cross-linked CALB and physically adsorbed CAL, respectively). These results suggest that cross-linked CALB has higher catalytic activity for polymerization of ε-caprolactone. The effect of enzyme concentration on polymerization reaction in terms of monomer conversion, average molecular weight and PDI values of PCL. It was observed that average molecular weight and monomer conversion were increasing with increasing enzyme concentration. The polymerization reactions were also performed with two commercial

CALB, Lipozyme® (free CALB) and Novozyme 435® (commercial immobilized CALB). The results showed that both immobilized enzymes improved stability and catalytic active of free form of CALB. Additionally, physically adsorbed CALB reached average molecular weight close to Novozyme 435®. The synthesized PCL samples were characterized with several analysis methods. ^{1}H-NMR analysis demonstrated characteristic peak of PCL chains. Moreover, molecular weights of samples calculated from ^{1}H-NMR were consistent with the ones obtained from GPC. The polymer samples were also thermally characterized. TGA and DSC analysis showed thermal characteristics of PCL. Low melting temperatures for both PCL samples were observed on DSC thermograms which may provide easy shaping of polymer material thus lower production cost. SEM images revealed suitable surface structures for tissue engineering applications.

This study offers an environmental friendly process for PCL synthesis by utilizing immobilized enzymes. Additionally, immobilizing CALB onto a cheap and renewable material provides an economic process. These novel immobilized forms of CALB showed significantly better performance than free CALB. Finally, characterization results suggest possible application methods and ease of processing of synthesized PCL.

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