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Poly (ε-caprolactone) synthesis by a novel enzymatic catalyst: *Candida antarctica* lipase B (CALB) immobilized on a modified silica-based material by physical adsorption

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ABSTRACT

In the present study, ring opening polymerization of ε-caprolactone was performed by a novel enzymatic catalyst, *Candida antarctica* lipase B (CALB) immobilized on a modified silica-based material by physical adsorption. Molecular weight distributions and chain structures were compared by using gel permeation chromatography (GPC) and hydrogen nuclear magnetic resonance (^{1}H NMR) analysis, respectively. In addition, for the determination of thermal properties, thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC) were performed. Scanning electron microscopy (SEM) was applied to observe the surface structure of the polymer. Effects of temperature, reaction time, and enzyme concentration on molecular weight of poly (ε-caprolactone) (PCL) were investigated and optimum conditions for the ring opening polymerization of ε-caprolactone via this new immobilized enzyme were obtained. Highest molecular weight was achieved as 14000 g/mol at the end of 48 hours at 60˚C. Moreover, considerably high molecular weights were successfully reached at lower temperatures by this novel enzyme, which makes this process low energy consuming besides being environmentally friendly. It is suggested that, CALB immobilized on a modified silica-based material by physical adsorption may be a great alternative for widely used commercial enzyme, Novozyme 435. This work also makes possible a new route for polymer synthesis. Copyright © 2016 VBRI Press.

Keywords: Enzymatic polymerization; poly (ε-caprolactone); lipase; enzyme immobilization.

Introduction

In recent years, aliphatic polyester synthesis via enzymatic ring opening polymerization (ROP) of lactones has become an attractive research area [**1**]. There is a considerable interest on utilization of enzymatically synthesized aliphatic polyesters for biomedical applications due to their biodegradability, biocompatibility, and outstanding mechanical properties [**2**]. In addition, catalysis with enzymes provides achievement of non-toxic materials which is essential for biomedical implementations such as drug delivery systems, tissue engineering, and medical devices [**2**].

Polycaprolactone (PCL) is aliphatic polyester that can be synthesized via ROP of ε-caprolactone (ε-CL) [**3**]. Among other aliphatic polyesters, PCL has a relatively low melting temperature (T_m), between 59-64˚C, and low glass transition temperature (T_g), between -60-10˚C. Moreover its decomposition temperature is about 350 ˚C which provides high thermal stability [**4**]. Besides good viscoelastic and rheological features, these thermal properties make PCL to be easily manufactured [**3**]. In addition, PCL is highly soluble in variety of solvents such as tetrahydrofuran (THF), toluene, chloroform, benzene and dichloromethane and it is compatible with other polymers which makes possible to obtain blends [**4, 5**]. Furthermore, PCL is a suitable biopolymer for biomedical purposes due to its biocompatibility and biodegradability. When compared with the other biodegradable polymers, PCL degradation process is slower *in vivo*. This long-term biodegradation behavior provides advantage for drug delivery application of PCL [**3, 6**]. As a result of these advantageous properties, PCL has become an important subject for polymer science and achievement of enzymatic PCL synthesis has received attention for biotechnological applications.

ROP of ε-CL can be catalyzed by both biocatalysts (enzymes) and organometallic initiators. Organometallic initiators such as Zn, Al, Sn, and Ge, cannot be removed completely from polymer matrix at the end of the polymerization which may lead toxic effects when used as a biomaterial. On the other hand by using biocatalysts, this problem can be overcome since they can be removed easily at the end of the reaction and they are non-toxic and eco-friendly catalysts. Moreover, biocatalysts can catalyze polymerization at mild reaction conditions (relatively low temperatures and pressures) [**7, 8, 9**]. However, biocatalysts may lose their activities after long reaction periods and may

have stability troubles [**10**]. Thus, enzyme immobilization is the most widely used strategy to overcome utilization problems of enzymes such as low stability and lack of ability to be reused. By the immobilization of enzymes, enzyme activity and stability can be improved, enzyme can be recovered at the end of the reaction and reused which may provide a potential to enzyme to be used in a continuous process [**11, 12**].

Candida antarctica lipase B (CALB) is one of the most efficient and selective lipase that can be used for the synthesis of polyesters. Its immobilized form is widely used as a biocatalyst and commercially available as Novozyme 435®, which is known with its effectivity on ROP of ε-CL [**1**]. In this work, it was aimed to catalyze this polymerization reaction with an alternative immobilized CALB. For this purpose, PCL synthesis was catalyzed by a new lipase, which was immobilized on surface-modified silica-based material, rice husk ash (RHA), via physical adsorption method in previous studies. For the determination of optimum polymerization condition of immobilized lipase, polymerizations were performed for various reaction times (6, 24, 48, 72, and 120 hours) at different temperatures (30, 40, 60, and 80 ˚ C). After obtaining the optimum polymerization conditions of immobilized lipase, effect of different enzyme concentrations were investigated. In addition, polymerizations via Lipozyme® and Novozyme 435® were also carried out at best condition for the comparison of the performances of home-made and commercial enzymes. Molecular weight distributions and chain structures of the polymer samples were compared by gel permeation chromatography (GPC) and hydrogen nuclear magnetic resonance spectroscopy (^{1}H NMR), respectively. In addition, for the characterization of chain structures, FTIR was also used. Thermal properties of the polymer samples were obtained by TGA and differential scanning calorimetry (DSC) analysis. Moreover, for determination of surface structures, SEM was used.

This work is significant since PCL synthesis was successfully achieved with an alternative novel biocatalyst which was never used for such an application and obtained. Moreover, high molecular weights (for an enzymatic polymerization) were reached even at 30 ^{o}C.

Experimental

Material

Immobilized lipase: The free form of *Candida antarctica* lipase B (CALB), with its commercial name Lipozyme®, was immobilized on surface-modified rice husk ashes (RHA) via physical adsorption in laboratory before. Surface modification of RHA was achieved by using 3-aminopropyl triethoxysilane (3-APTES) for the addition of $–NH_2$ groups to the surface that will interact with the free lipase during immobilization process.

Materials for PCL synthesis: ε-caprolactone (99 %, $C_6H_{10}O_2$), monomer of the polymerization reaction, was provided from Alfa Aesar and stored over molecular sieves (3A˚) before polymerization reactions to avoid water. Molecular sieves were obtained from Sigma Aldrich. Toluene (99 %, $C_6H_5CH_3$) was purchased from Merck and used as a solvent in polymerization reactions. Chloroform (99 %, $CHCl_3$) was used for termination of the polymerization reaction and provided from Sigma Aldrich. For precipitation of the polymer at the end of the reaction, methanol (99 %, CH_3OH) was used and it was obtained from Merck. Tetrahydrofuran (THF, C_4H_8O) was used to solubilize polymer samples in GPC analysis and purchased from Carlo Erba with high purity (HPLC grade).

PCL synthesis via immobilized CALB

ROP of ε-CL was carried out by CALB immobilized onto surface-modified RHA. Reactions were performed in 1000 mg of toluene (ε-CL to toluene ratio 1: 2 (w: w)) under dry nitrogen. Enzyme concentration was 20 % (w: w) (enzyme to monomer ratio). Reaction medium was stirred at 120 rpm with a magnetic stirrer. Reactions were carried out at 30, 40, 60, and 80˚C for 6, 24, 48, 72, and 120 hours. At the end of the reaction time, reaction was terminated by the addition of excess chloroform to the mixture. Enzyme was separated from the reaction mixture by filtration. Then, chloroform was evaporated in oven. After that, by the use of cold methanol, polymer was precipitated. The polymer sample was washed with methanol and filtrated. Then, the sample was dried.

Characterization methods

Fourier transform infrared spectroscopy (FTIR): Perkin Elmer FTIR Spectrum One B Spectrometer was used for the determination of chemical structure and composition of polymer sample. It was verified to be PCL by comparing with characteristic functional groups and bonds of PCL.

Thermal gravimetric analysis (TGA): Thermal characterization of polymer sample was carried out by TGA. For this aim, 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.

Differential scanning calorimetry (DSC): For the further determination of thermal properties of polymer sample, SEIKO 7020 DSC was used. Under inert nitrogen atmosphere 10-15 mg samples were analyzed. The materials were exposed to thermal cycles (heat-cool-heat). Thermal characterization was carried out between -70 and 200˚ C at 10˚ C/min. By DSC analysis, crystallinity percentages of polymer samples can also be obtained besides T_g and T_m values. For the calculation of crystallinity percentage (X_c), Equation 3.1 was used [**13**].

$$X_c = \left(\frac{\Delta H_m}{\Delta H°_m}\right) x100 \qquad \textbf{(3.1)}$$

In Equation 3.1, $\Delta H°_m$ is the melting enthalpy of PCL where it has 100 % crystalline structure and its value is 139.3 J/g [**13**]. ΔH_m is the enthalpy value at T_m of the PCL sample.

Gel permeation chromatography (GPC): It was used for the determination of molecular weights and polydispersity indexes (PDI) of PCL samples. Measurements were carried

out by Agilent 1100 model GPC apparatus. THF was used as an eluent with a flow rate of 1 mL/min. Before injection, all samples were filtered via 0.45 µm filter syringe.

Hydrogen nuclear magnetic resonance spectroscopy (^{1}H NMR): It was used for the determination of molecular weight and molecular structure of polymer sample. The apparatus used for analysis was Bruker Ultra shield 300 MHz. Deuterated chloroform ($CDCl_3$) was used as a solvent during analysis. ^{1}H NMR spectra was obtained with respect to tetramethylsilane (TMS) standard. Molecular weight (M_n) value of PCL can 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). The formula used for $M_{n,NMR}$ calculation is given in Equation 3.2 [**14, 15**].

$$M_{n,NMR} = \frac{5xI_{4.07}}{2xI_{3.65}} xM_{\varepsilon-CL} \quad \textbf{(3.2)}$$

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 [**15**].

Scanning electron microscopy (SEM): Surface morphologies of polymer sample was observed by JEOL JSM-6390LV SEM. Sample was coated with platinum before observation. Analysis was performed at 5 kV with different magnifications.

Results and discussion

Monomer conversions, molecular weights (M_n), and polydispersity indexes (PDI) of PCL samples at different temperatures are given in **Table 1** depending on reaction time.

It can be clearly seen from **Table 1** that, at 30 ˚C M_n values of PCL samples increased with reaction time and reached 9000 g/mol at the end of 72 hours polymerization. However, after 72 hours M_n started to decrease. CALB has also the ability of catalysis of PCL degradation, therefore M_n decrement after a certain reaction time was an expected result [**16**]. In addition, at the end of 72 hours, 85.0 % monomer conversion was obtained and it began to decrease after this point. This result showed that monomer consumption could go further for longer reaction periods at these conditions, but after a while reaction shifts to degradation.

At 40 ˚C, M_n value reached to its highest value of 11160 g/mol after 48 hours reaction (**Table 1**). At longer reaction periods, M_n decreased only about 1000 g/mol. In addition, monomer conversion was 91.5 % at the end of 48 hours, but it started to decrease after this point. This may a result of degradation activity of enzyme [**16**].

When the polymerization reaction was carried out at 60 ˚C, M_n value increased with reaction time and reached 14000 g/mol at the end of 48 hours reaction (**Table 1**). However, after 48 hours M_n started to decrease which was a result of degradation activity of enzyme [**16**]. In addition, highest monomer conversion was 87.6 % which had been obtained from 48 hours reaction. After 48 hours, conversion started to decrease as a result of the beginning of polymer degradation.

Finally at 80 ˚C, highest M_n value was obtained at the end of 24 hours, which were 11820 g/mol (**Table 1**). After 24 hours, reaction shifted from polymerization to degradation and molecular weight started to decrease. On the other hand, monomer conversion reached to its maximum value of 85.5 % at the end of 24 hours and began to decrease after this point as a result of degradation activity of enzyme [**16**].

Table 1: Polymerization results obtained at different temperatures.

Reaction Temperature (˚C)	Time (h)	6	24	48	72	120
30 ˚C	Conversion (%)[a]	39.6	43.1	63.1	85.0	84.3
	M_n (g/mol) [b]	4000	6120	8670	9000	7300
	PDI [b]	1.1	1.5	1.5	1.4	1.4
40 ˚C	Conversion (%)[a]	32.7	65.8	91.5	81.7	77.0
	M_n (g/mol) [b]	4825	10050	11160	10010	10190
	PDI [b]	1.2	1.5	1.5	1.5	1.5
60 ˚C	Conversion (%)[a]	41.9	82.2	87.6	85.3	81.5
	M_n (g/mol) [b]	6750	9860	14000	9500	8790
	PDI [b]	1.3	1.5	1.5	1.5	1.5
80 ˚C	Conversion (%)[a]	50.0	85.5	83.9	79.3	77.3
	M_n (g/mol) [b]	6300	11820	10230	9710	9960
	PDI [b]	1.3	1.5	1.5	1.5	1.5

[a]Conversion was calculated gravimetrically
[b]M_n and PDI (M_w/M_n) were obtained by GPC

Moreover, in **Table 1** polydispersity indexes (PDI) can also be seen. As given in **Table 1**, PDI values were obtained in the range of 1.0-1.5. Since they are close to 1.0, polymer samples can be considered as monodisperse [**17**].

In order to observe the effect of temperature on molecular weights of polymers and see the molecular weight distributions, **Fig. 1** is given.

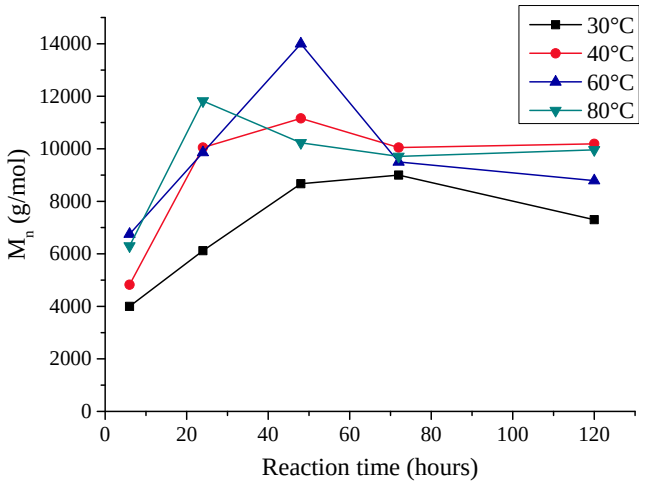

Fig. 1. Effect of temperature on M_n of PCL samples.

To summarize based on **Fig. 1**, highest molecular weight, which is 14000 g/mol, was obtained at 60 ˚C at the end of 48 hours. As also seen from this graph, molecular weights of polymer samples obtained from 30 ˚C reaction

were considerably high for a polymerization reaction. This makes the process low energy consuming besides being environmentally friendly.

Analyses were carried out for the polymer sample with highest molecular weight which was obtained at the end of 48 hours reaction at 60˚C. The FTIR spectrum of polymer sample is shown in **Fig. 2**.

The infrared bands of polymer sample synthesized by the new immobilized CALB was consistent with characteristic infrared bands of PCL, which were asymmetric CH_2 bonds at 2945 cm^{-1}, symmetric CH_2 bonds at 2866 cm^{-1}, carbonyl (C=O) bonds at 1720 cm^{-1}, C-O and C-C bonds seen in crystalline phase at 1293 cm^{-1}, asymmetric C-O-C bonds at 1238 cm^{-1}, and symmetric C-O-C bonds at 1167 cm^{-1} [**6**]. This situation proved that the synthesized polymer was PCL.

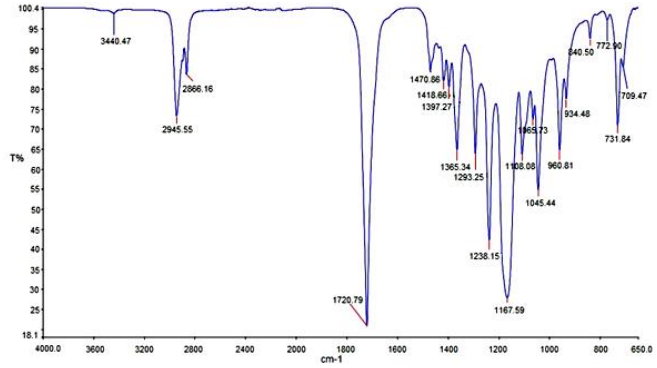

Fig. 2. FTIR spectrum of polymer sample.

Chemical structure of polymer sample was further characterized by ^1H NMR spectroscopy. The zoomed spectrum between 4.25 ppm and 3.5 ppm range, in which the characteristic peaks that are used for molecular weight calculation can be seen, is shown in **Fig. 3**. The chemical shifts (ppm) between 4.25 ppm and 3.5 ppm were as follow: 4.07 ppm (t, CH_2O) and 3.65 ppm (t, CH_2OH, end group) which were characteristic for PCL [**18**].

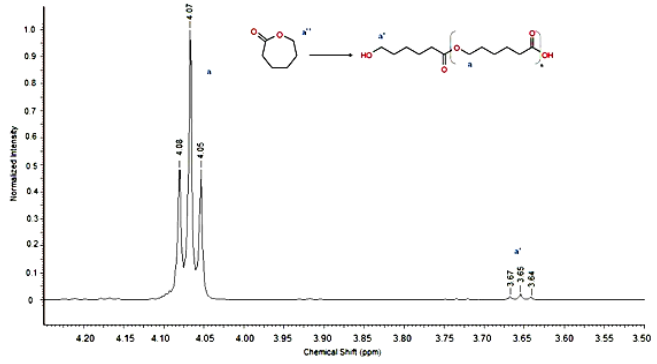

Fig. 3. ^1H NMR spectrum of polymer sample.

Moreover, molecular weight of the PCL sample was also calculated from ^1H NMR spectrum. $M_{n,\ NMR}$ value was obtained as 14880 g/mol by using equation 3.2.

For the thermal characterization of polymer sample, TGA and DSC analyses were applied. TGA and DSC curves are given in **Fig. 4**.

Ruseckaite and Jiménez has published that, maximum degradation temperature of PCL is about 415 ℃ under same thermal analysis conditions with the conditions applied in this study [**19**]. As seen from TGA curves given in **Fig. 4**, degradation temperature (T_d) of PCL sample was determined as 411.6˚C. This result showed that, PCL synthesized with this new immobilized lipase had a high thermal stability.

Moreover, DSC was also applied for the determination of T_m, ΔH_m, and X_c values of PCL sample (**Fig. 4**). As seen from DSC thermogram (endodown graphic), T_m value was obtained as 53.3˚C. This low T_m value made the polymer sample easily reshaped. From the area of melting peak, ΔH_m was computed as 83.69 J/g. By using Equation 3.1, crystallinity percentage (X_c) was calculated as 60 %, which showed that polymer sample had a semi-crystalline structure.

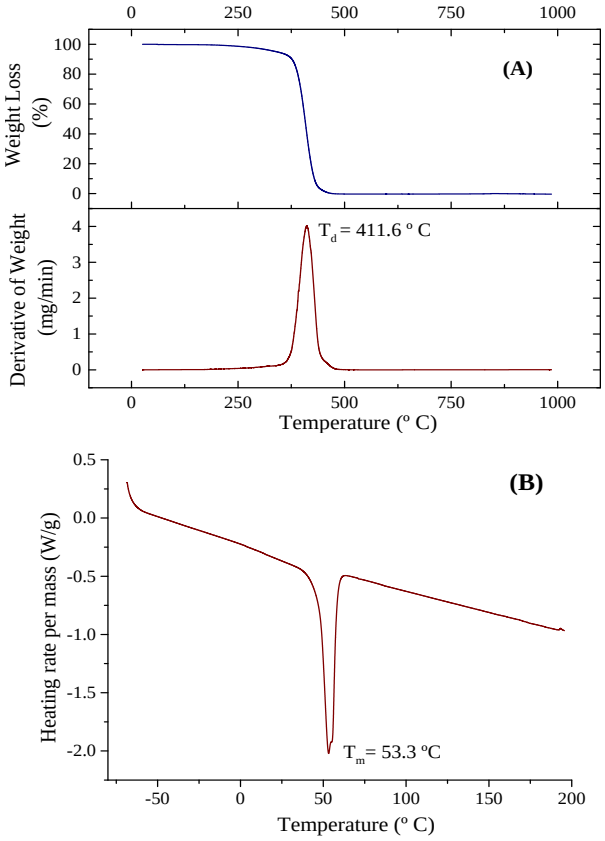

Fig. 4. TGA (A) and DSC (B) curves of polymer sample.

Surface morphology of polymer sample was observed by SEM analysis. The obtained SEM images are shown in **Fig. 5**.

As seen from **Fig. 5**, the polymer sample had a foam-like structure. It can be safely suggested that the PCL sample can be used as scaffold for tissue engineering successfully since its structure is suitable for cell attachment and growth [**3, 20**].

Effect of enzyme concentration on polymerization was investigated for the best synthesis conditions of the immobilized lipase. In addition to 20 % (w/w) enzyme concentration which was used in serial polymerization reactions, 2.5, 5, and 10 % (w/w) concentrations were also

tried. Since a higher enzyme concentration was not economic, polymerizations with such high concentrations were not performed.

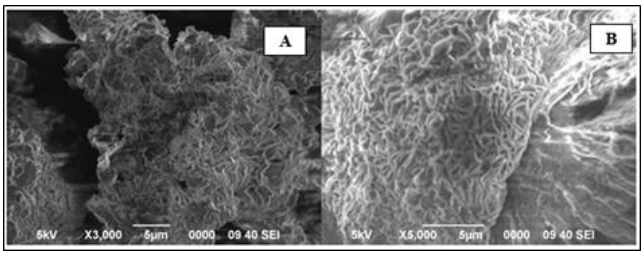

Fig. 5. SEM images of polymer samples at 3000x (A) and 5000x (B) magnifications.

Polymerization results obtained with different enzyme concentrations at 60˚C for 48 hours are given in **Table 2**.

As it is given in **Table 2**, highest molecular weight and monomer conversion were obtained when immobilized CALB was used with a concentration of 20 % (w/w). Monomer conversion and molecular weight decreased with decreasing the enzyme concentration.

Table. 2. Effect of enzyme concentration on polymerization.

Enzyme concentration (%) (w/w)	Conversion (%)[a]	M_n (g/mol)[b]	PDI[b]
2.5	23.1	6340	1.2
5.0	39.9	11020	1.6
10.0	78.1	11330	1.5
20.0	87.6	14000	1.5

[a]Conversion was calculated gravimetrically
[b]M_n and PDI (M_w/M_n) were obtained by GPC

At best polymerization conditions, PCL synthesis was also carried out by using commercial lipases; Lipozyme® and Novozyme 435®. Molecular weights of polymer samples obtained via commercial lipases and new immobilized CALB catalyzed reactions at 60℃ for 48 hours are given in **Fig. 6**.

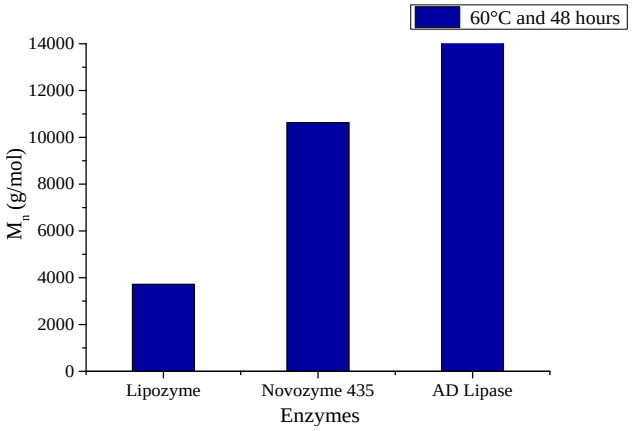

Fig. 6. Molecular weights of polymer samples obtained via commercial lipases and new immobilized CALB.

As seen from **Fig. 6**, M_n values obtained from the reaction carried out at 60˚C for 48 hours with Lipozyme® and Novozyme 435® were 3720 g/mol and 10630 g/mol, respectively. Lipozyme®, which is the free form of CALB, resulted in lower molecular weight and monomer conversion when compared with Novozyme 435®, which is the commercial immobilized form of CALB. This may a result of enhanced enzyme activity and stability by immobilization [**11, 12**]. Furthermore, at these conditions new immobilized CALB catalyzed reaction resulted in a polymer sample with the highest molecular weight, which was even higher than Novozyme 435®. This result indicated that, the new immobilized lipase showed higher activity for the ROP of ε-CL than Novozyme 435 ® which is known with its high catalytic activity for this reaction [**21**].

Conclusion

The objective of this study was achievement of PCL synthesis by the catalysis of CALB immobilized on surface-modified RHA via physical adsorption, which was a home-made enzyme. By using this enzyme highest molecular weighted PCL was successfully obtained at the end of 48 hours reaction at 60˚C with a value of 14000 g/mol. This molecular weight was even higher than the molecular weight of PCL synthesized via Novozyme 435® at same conditions. The highest molecular weighted sample was verified to be PCL by FTIR and ^{1}H NMR analyses. By TGA and DSC analysis, its superior thermal properties were characterized and the PCL sample was shown to be highly thermal stable and easily to be manufactured. Moreover, SEM pictures showed that, synthesized PCL had a suitable structure to be used for tissue engineering applications. Consequently, this work makes possible a new route for PCL synthesis in addition to providing a low energy consuming and green process.

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