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Enzymatic ring opening polymerization of ε-caprolactone by using a novel immobilized biocatalyst

Nurefsan Gokalp*, Cansu Ulker, Yuksel Avcibasi Guvenilir

Istanbul Technical University, Chemical Engineering Department, Istanbul, Turkey

*Corresponding author. Tel: (+90) 5556311298; E-mail: nurefsan.gokalp89@gmail.com

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ABSTRACT

In this study, an amorphous silica material was used as a carrier to immobilize *Candida antarctica* lipase B (CALB) by crosslinking method for ring opening polymerization of ε-caprolactone (ε-CL). The optimum temperature, enzyme concentration and time period were investigated for poly(ε-caprolactone) (PCL) synthesis via ring opening polymerization of ε-CL catalyzed by immobilized CALB (IMCALB). Molecular weights of PCLs were determined by using gel permeation chromatography (GPC) and hydrogen nuclear magnetic resonance (^{1}H-NMR) analysis. The surface morphologies of PCLs were analyzed by scanning electron microscopy (SEM). Besides, PCLs were successfully characterized by fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC) analysis. The results showed that the immobilized lipase by crosslinking method via glutaraldehyde possessed good activity and stability. By using this immobilized enzyme, high molecular weights and monomer conversions of PCLs were achieved about 9000 g/mol and 90 %, respectively. This work has showed that activity of CALB increased about 17 % dramatically after immobilization process, and PCL was synthesized via enzymatic polymerization catalyzed this novel enzyme, which provides an effective method for conducting "green polymer chemistry". Copyright © 2016 VBRI Press.

Keywords: *Candida antarctica* lipase B; ring opening polymerization; ε-CL; immobilization; crosslinking.

Introduction

Over the last two decades, "the green friendly method" enzymatic polymerization was greatly developed and became an important technique for biodegradable and biocompatible polymeric materials. Enzymatic polymerization has many benefits over chemical polymerization methods such as mild reaction conditions, high enantio-, chemo- and regioselectivity, high activity and few by-products. Furthermore, this polymerization method can help to overcome problems related to residues of metallic catalysts which have high toxicity and cannot completely remove from the resulted polymer.

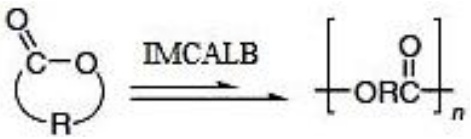

Fig. 1. Polymerization of ε-CL catalyzed by IMCALB **[4].**

Ring opening polymerization of lactones is one of the enzymatic polymerization techniques **[1].** In particular, several studies have showed that small, medium and large ring sized lactones could be successfully polymerized in enzyme catalyzed ring opening polymerization **(Fig. 1).** The medium sized monomer ε-caprolactone (ε-CL) is the most widely studied lactone in comparison to the other lactones because it has high activity in enzymatic polymerization reactions **[2].** Polycaprolactone (PCL) from Mn = 500 to 50,000 g/mol is produced with polymerization of ε-CL and it is an important polyester used in different biomedical applications because of its eco-friendly properties such as biodegradability, biocompatibility and permeability **[3].**

On the other hand, the use of lipases (E.C. 3.1.1.3) for the enzyme catalyzed synthesis of polymers has been increased rapidly **[5].** Lipases, especially *Candida antarctica* lipase B (CALB), are the most widely used group of enzymes that they can catalyze not only hydrolysis reactions of mono-, di- and triacylglycerols, but also esterification or transesterification reactions with very high efficiency. However, the free enzymes have very high costs and complicated downstream processing. Therefore, several immobilization techniques apply to make enzymes more useful in commercial applications by improving enzyme properties. They can be immobilized onto different support materials that can be divided in organic or inorganic and natural or synthetic. Specifically, organic particles such as polystyrene resins, octyl agarose, and inorganic particles such as silica, carbon materials and titania can be used to immobilize lipases **[6].**

In this paper, we synthesized poly (ε-caprolactone) by using home-made immobilized enzyme, which was immobilized onto an amorphous silica material by crosslinking method. Polymerization conditions such as temperature, time period and enzyme concentration were optimized, and samples possessed the highest number

average molecular weight were characterized by different analysis.

Experimental

Materials

The free form of *Candida antarctica* lipase B and acetone were supplied from Sigma Aldrich Company. 3-aminopropyltriethoxysilane (Merck) was used as organosilane agent and one of the most effective enzyme immobilizing agents glutaraldehyde (BDH Chemicals) used to immobilize CALB by crosslinking method. An amorphous silica material was supplied from a company in Istanbul. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) and sodium monohydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$) were used to prepare phosphate buffer solution and purchased from Carlo Erba Reagenti Company and Merck Company, respectively. Sodium hydroxide solution (Carlo Erba Reagenti) and ethanol (Merck) were used to determine lipase activity by titration. Ɛ-caprolactone (99 %) was used as monomer of the polymerization and supplied from Alfa Aesar Company. Toluene and methanol were purchased from Merck Company with more than 99 % purity. Chloroform was supplied from Spect Company, and was used without applying any pretreatment process. Tetrahydrofuran was used to dissolve the polymer in GPC Analysis and purchased from Labkim Company.

Modification of the amorphous silica material

3-aminopropyltriethoxysilane (3-APTES) is a most common used reactive silane for silanization because its amino groups are sensitive to the coupling reaction **[7]**. Therefore 3-APTES was used as an organosilane agent to modificate the silica support. Furthermore, acetone was used as a solvent for silanization step of immobilization. Firstly, silica material was activated by silanization. During silanization process, the silica support was activated by adjusting the ratio of 3-APTES to acetone 15 % (w/v) **[7]**. This suspension was stirred at 50 ºC for 2 hour in shaking water bath (Julabo SW22). The activated silica was washed with water and dried at 60 ºC for 2 hour in drying oven (BINDER). After that, the activated silica was stirred with 0.2 % (v/v) glutaraldehyde/phosphate buffer solution (pH 7, 0.015M). The mixture was kept under agitation for 2 hour at 25 ºC. Then, the modified silica was filtered under vacuum by washing with water.

Immobilization of CALB onto silica support

Candida antarctica lipase B and the modified silica (the ratio of enzyme to silica: 2 (w/w)) were suspended in 50 mL of phosphate buffer solution (pH 7, 0.015M) at 25 ºC for 5 hour. Immobilized enzymes were washed with phosphate buffer solution and dried at 30 ºC for 12-24 hour in drying oven.

Ring opening polymerization of ε-CL by using immobilized enzyme

Polymerization reactions were carried out by using immobilized enzymes at 40, 60 and 80 ºC temperatures. Toluene was used as organic media because of their excellent performance over other organic solvents used in polymerization reactions. The polymerization reactions were performed in 1000 mg toluene (toluene to ε-caprolactone, 2:1 (v/v)) and inert nitrogen was supplied in the reaction medium for 1 minute **[4]**. The reaction medium was stirred at 120 rpm with a magnetic stirrer during specified times which were 6, 24, 48, 72, 120, 150 and 170 h. After the specified time, the reaction was stopped by adding chloroform and enzyme was separated from the reaction medium by filtering with chloroform. Then, enzyme was washed also with phosphate buffer solution (pH 7, 0.015M) and dried at 50 ºC for 24 hour. On the other hand, the filtrate included the reaction medium in chloroform was dried to evaporate the chloroform at 50 ºC in drying oven. After evaporation of a large amount of chloroform, the solution was precipitated in cold methanol. Finally, polymer was filtered under vacuum and dried at 30 ºC for 12-24 hour. The dried polymers were stored to characterize in the desiccator. In addition, the ratios of enzyme to monomer were changed (enzyme to ε-caprolactone, % 2.5, % 5, % 10 and % 20 (w/w)) and it was obtained the optimum temperature, time period and enzyme concentration. The monomer conversions of polycaprolactones were gravimetrically calculated by using Equation 1, where Wp and Wm mean the weight of the resulted polymer and monomer, respectively.

$$Conversion = \frac{Wp}{Wm} \times 100 \quad (1)$$

Characterization techniques

The surface morphology of the polycaprolactones was characterized by Scanning Electron Microscopy (SEM). The assay samples were initially coated with a layer of platinum to provide conductivity between the samples and electrons. Analyses were performed on a JEOL JSM-6390LV SEM apparatus with accelerating voltage of 5 kV and images were recorded at different magnifications. Thermal characterization of polycaprolactones was carried out by thermogravimetric analysis (TGA) on 10-20 mg samples by heating from room temperature to 900 ºC at 10 ºC/min under nitrogen or air flow using a TG/DTA 6300 apparatus. The bond structures of polycaprolactones were characterized by using a Perkin Elmer Fourier Transform Infrared Spectroscopy (FTIR) apparatus. The analysis was performed by using the ATR kit of FTIR apparatus. Through FTIR analysis, the synthesized polymers possessed the highest Mn were analyzed whether the resulting polymer has the characteristic properties of PCL or not. Differential Scanning Calorimetry (DSC) was conducted on a Seiko 7020 DSC (Sensitivity, 0.2 µW) under inert atmosphere on 7-9 mg samples placed in aluminium pans. The materials were exposed to consecutive thermal cycles (heat-cool-heat) with the first heating scan allowing obliterating the thermal or thermomechanical history of the materials. Thermal characterization was performed between -80 and 100 ºC for PCL at 10 ºC/min. DSC was used to determine the thermal properties of polymers such as the fusion enthalpy (ΔH^0_f) and melting temperature (Tm). Crystallinity percentage was calculated by taking the ratio of fusion enthalpy of the

sample to the fusion enthalpy of purely crystalline polymer (ΔH^0_f) showed in the equation 2.

$$\chi\,(\%) = \frac{\Delta Hf sample\ (\frac{J}{g})}{\Delta H0f\ (\frac{J}{g})} \times 100 \quad (2)$$

where, ΔH^0_f PCL= 139 J/g (theoretical value for 100 % of crystallinity) and χ is crystallinity **[8]**.

An Agilent 1100 Series Gel Permeation Chromatography (GPC) was used to determine the number average molecular weight (Mn), the weight average molecular weight (Mw) and polydispersity (PDI) of the synthesized polycaprolactones. The samples were previously prepared by dissolving into tetrahydrofuran, and the dissolved samples were filtered then by using 0.45 µm pore size membrane. The analyses were performed during 20 or 45 minutes. On the other hand, the obtained results by using GPC are not completely clear, although GPC is one of the most common apparatus to determine molecular weight of polymers. The molecular weights can change depending on used solvents **[9]**. Therefore, the determined Mn values were compared to values obtained by using ^{1}H NMR. The ^{1}H NMR spectrums were recorded on an Agilent VNMRS 500 MHz ^{1}H NMR apparatus. The samples were prepared by adding sufficient amount of a deuterium solvent (deuterated chloroform, 0.6 – 0.75 mL) in 10 mg sample. The Mn values of polymers were calculated by using the equation 3. In this equation, the areas of two peaks on ^{1}H NMR spectrum were used for calculation **[10]**.

$$Mn, NMR = \frac{5 \times I4.07}{2 \times I3.65} \times M, \varepsilon - CL \quad (3)$$

The polymerization degree is calculated by the ratio of 4.07 ppm peak area to 3.65 ppm peak area. The reaction conversion rates are calculated also by using these polymerization degrees.

Table 1. The ring opening polymerization of ε-CL catalyzed by IMCALB.

Reaction Temperature (˚C)	Time (h)	6	24	48	72	120	150	170
40 ˚C	Conversion (%)	-	24	45	54	47	-	-
	Mn (g/mol)	-	3700	4100	4500	4000	-	-
	PDI (Mw / Mn)	-	1.4	1.5	1.6	1.6	-	-
60 ˚C	Conversion (%)	8	21	42	88	90	-	-
	Mn (g/mol)	2900	5400	3400	5000	5100	-	-
	PDI (Mw / Mn)	1.2	1.2	1.6	1.8	1.4	-	-
80 ˚C	Conversion (%)	7	45	23	58	63	67	69
	Mn (g/mol)	3500	4300	2900	2500	4700	9000	9400
	PDI (Mw / Mn)	1.2	1.5	1.3	1.4	1.6	1.4	1.5

Results and discussion

The effect of time and temperature on PCL polymerization catalyzed by IMCALB

The optimum time and temperature were determined to define best conditions for PCL polymerization. The results of PCL polymerization catalyzed by immobilized CALB by crosslinking method at 40 ºC can be seen in **Table 1**, where Mn and polydispersity values of polycaprolactones obtained from GPC analysis were given respectively. Monomer conversion rates, which also were noted in **Table 1**, were measured by using equation 1. From **Table 1**, the highest Mn and monomer conversion rate were obtained at the end of 72 h at 40 ºC, while any polymer could not be obtained for 6 h. It can be said that 6 h are not enough to polymerize ε-CL at 40 ºC. Furthermore, Mn values did not linearly increase or decrease with changing time period, in contrast a fluctuation was observed on Mn values at 40 ºC. Besides, changing between 1.4 and 1.6 PDI values were obtained at 40 ºC.

At 60 ºC, the highest Mn of PCL, 5400 g/mol was obtained at 24 h, while the highest monomer conversion rate 90 % was obtained at 120 h. For Mn values of PCLs were observed a fluctuation at 60 ºC similar to the obtained results at 40 ºC. At 60 ºC, from 5400 g/mol to 3400 g/mol reduced at 48 h, and 5100 g/mol was finally obtained at 120 h. Moreover, PDI values were obtained changing between 1.2 and 1.8 at 60 ºC. At the end of 6 and 24 h reaction times was obtained a dispersity of 1.2 which was the most approximate value to monodispersity.

ε-CL polymerization was performed for seven different times at 80 ºC, because Mn values of PCLs continued to increase after 120 h. The highest Mn and monomer conversion rate were obtained at 170 h, at which an achieved similar result to values was measured at 150 h. Therefore, it was accepted that Mn values were become stable, and the series of experiment was completed for 80 ºC. Additionally, PDI values were obtained changing between 1.2 and 1.6 at 80 ºC, a dispersity of almost 1 was achieved at 6 h.

The comparison of Mn values is shown in **Fig. 2(a)**, where the highest Mn value, 9400 g/mol was achieved at 80 ºC-170 h. At 40 ºC, Mn values of PCL increased until 72 h, decreased during 120 h, and became stable about 4000 g/mol. At 60 ºC, Mn value became stable between 72-120 h after fluctuation of values which is as similar as the results obtained at 40 ºC. ε-CL polymerization reaction is a reversible reaction during which degradation reaction can simultaneously take place **[4]**. Because of this the fluctuation may be happen in PCL polymerization at 40 and 60 ºC.

The comparison of monomer conversion rates is shown in **Fig. 2(b)**. The highest monomer conversions were observed at 60 ºC that 88 % and 90 % was obtained at 72 and 120 h, respectively. Although Mn values are high at 80 ºC, the highest monomer conversion is 69 % which is not enough compared the results obtained at 60 ºC. At 40 ºC, monomer conversion rates are generally lower than the rates measured at 60-80 ºC. The highest monomer conversion rate is 54 % at 40 ºC.

Mn of PCL is the most important parameter to optimize the reaction conditions for polymerization. Because it defines mechanical strength of PCLs, and also mechanical strength is an effective factor on usage of PCL in industrial application, especially biomedical applications. Polydispersity is another parameter which defines mechanical properties of biopolymers, and it means heterogeneity index which should be almost 1 for monodisperse systems, biopolymers **[9]**. During the series of experiments, low values of disperty were obtained generally at short time period. Moreover, time period is another significant factor to characterize polymerization reactions. As it can be seen in **Table 1**, the highest Mn values of PCL obtained at 150 and 170 h are too similar

which are 9000 and 9400 g/mol, respectively. However, polymerization at 150 h is more advantageous due to that short time period is more suitable for industrial applications. Conversely, monomer conversion rates are not high at 80 ºC like the reactions at 60 ºC, but the values are higher than the monomer conversion rates measured at 40 ºC. About 70 % monomer conversion rates, which are good enough for polymerization, were obtained at 80 ºC-150 h. All in all, 80 ºC and 150 h were determined as the optimum temperature and time period for PCL polymerization via immobilized CALB by crosslinking method.

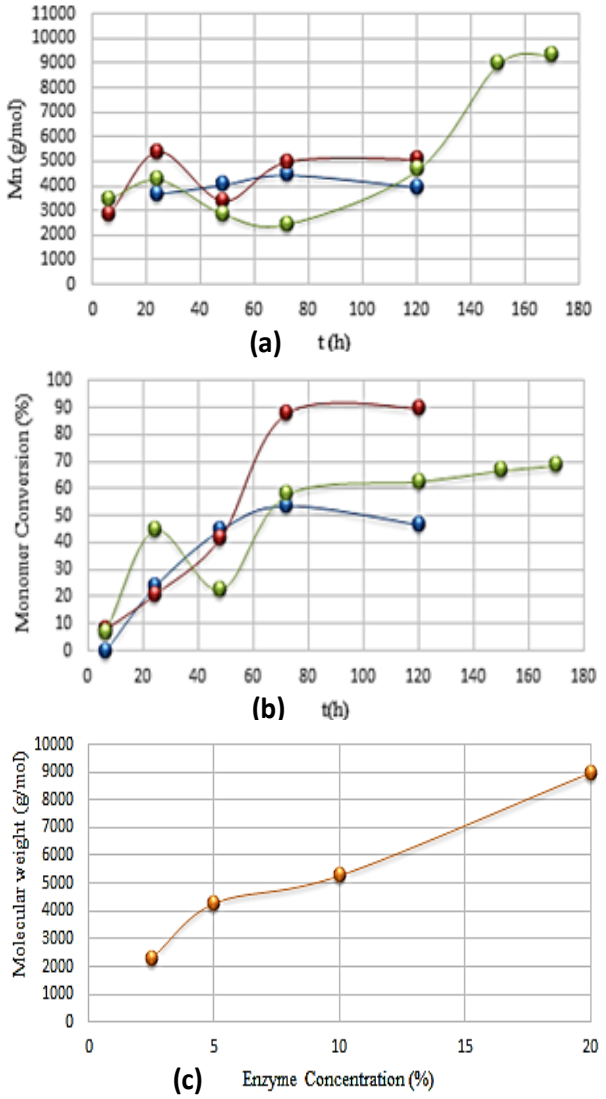

Fig. 2. (a) The comparison of Mn values for different temperatures, (b) the comparison of monomer conversion rates for different temperatures, (c) the comparison Mn values for different enzyme amount.

Table 2. The results at different enzyme loading.

Enzyme Concentration (%)	Mn (g/mol)	PDI (Mn/Mw)	Monomer Conversion (%)
2.5	2300	1.3	3.3
5	4300	1.6	27
10	5300	1.7	42
20	9000	1.4	67

The effect of enzyme concentration on PCL synthesis

The effect of enzyme concentration on PCL polymerization reactions was researched to determine the most effective enzyme amount for synthesis of PCL. The reactions were carried out under determined conditions (80 ºC-150 h). Mn, PDI and monomer conversion rates of PCLs were shown in **Table 2**. From **Table 2**, the monomer conversion rates of PCLs increased by increasing enzyme concentration. However, conversion rates at 2.5 % (w/w) were obtained 3,3 %, was very low. Furthermore, PDI values obtained also in the range of 1.3-1.7. Deng and Gross (1999) studied ring opening polymerization of ε-Cl catalyzed by Novozym®-435, and they reported similarly that monomer conversion rates increased with increasing enzyme concentration. In their study, a monomer conversion rate of 80 % was obtained in a 4 h time period for an enzyme concentration of 9.8 mg lipase per mmol monomer, while for an enzyme concentration of 1, 8 mg lipase per mmol monomer, 48 h was required to achieve 80 % **[11]**.

The comparison of number average molecular weights of PCLs can be seen more clearly in **Fig. 2(c)**. The molecular weights of PCLs went up with increasing enzyme concentration during reactions catalyzed by immobilized CALB. The best result was 9000 g/mol obtained in a reaction conducted at 20 % (w/w) enzyme concentration. According to these results, it can be said that immobilized enzymes content presented a positive effect on PCL polymerization regarding the molecular weights and also monomer conversions.

Scanning electron microscopy (SEM)

SEM was used to determine surface morphology of polycaprolactone which can be seen in **Fig. 3**, which shows SEM images of PCL catalyzed by IMCALB at 2000x and 3000x magnifications. Structure of polycaprolactone catalyzed by IMCALB looks like a foam, and has wide and long holes on the surface.

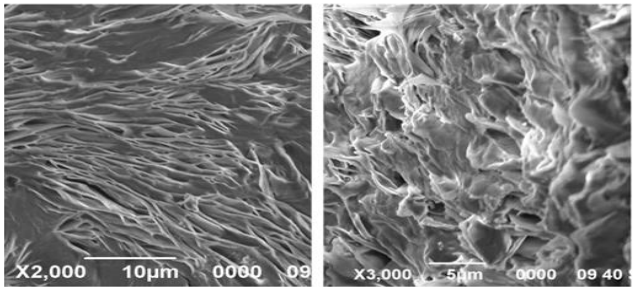

Fig. 3. SEM images of PCL catalyzed by IMCALB.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of PCL catalyzed by IML are demonstrated in **Fig. 4**, which belonged to PCL polymerized at 80 ºC 150 h with a molecular weight of 9000 g/mol. The characteristic peaks of PCL are asymmetric CH_2 bonds, symmetric CH_2 bonds and carbonyl bonds (C=O), which can be seen in **Fig. 4,** the IR band at 2943.98, 2865.31 and 1721.12 cm^{-1}, respectively. Furthermore, the C-O and C-C bands can be seen the IR band at 1292.96 cm^{-1}, asymmetric symmetric C-O-C bands can be also seen the IR band at 1238.34 and 1168.22 cm^{-1}, respectively **[9]**. Additionally, the IR band at

1470.89 and 1365.08 cm^{-1} are related to stretching of CH_2 and OH group, separately **[13]**.

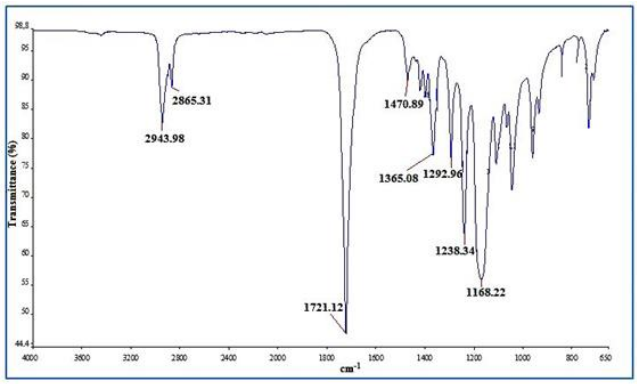

Fig. 4. FTIR spectra of polycaprolactone polymerized by IMCALB.

Ranjha *et al.* (2011) studied synthesis and characterization of polycaprolactone/acrylic acid hydrogel for controlled drug delivery, and they characterized the result polymers by using FTIR. The FTIR spectra in their study showed the peak at 1752 cm^{-1}, which belong to carbonyl bonds. Moreover, they obtained that the peaks at 1460-1375 cm^{-1} are because of stretching of CH_2 and OH group, respectively **[13]**. Wu (2005) studied thermal properties and biodegradability of polycaprolactones/chitosan and acrylic acid grafted polycaprolactone/chitosan, and characterized these polymers by using FTIR. In this study is obtained that the characteristic peaks of PCL in the wave length at 1710-1720 cm^{-1}, which belong to carbonyl stretching **[14]**. Based on these studies, it can be said that the FTIR spectra of PCL catalyzed by IMCALB have all characteristic bands of PCL, and can be concluded that PCL was successfully polymerized in this study.

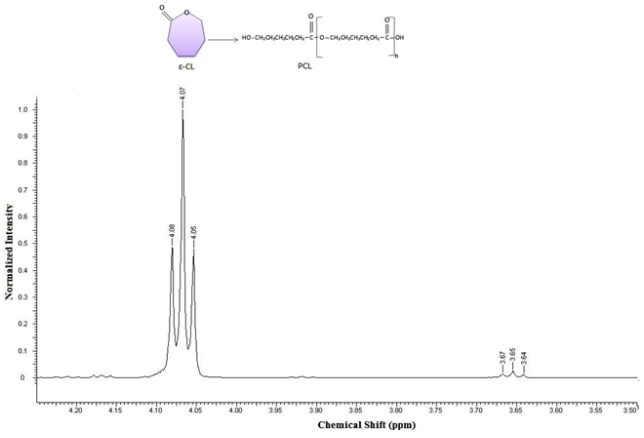

Fig. 5. ^1H NMR spectra of PCL catalyzed by IMCALB.

Proton nuclear magnetic resonance (^1H NMR)

^1H NMR analysis was applied for PCLs possessed the highest molecular weight, which was 9000 g/mol. **Fig. 5** shows that ^1H NMR spectra of PCL catalyzed by IMCALB. It was noted that the structural groups of PCL on **Fig. 5**: (δ, ppm): 4.07 (t, CH_2O, main chain) and 3.65 (t, CH_2OH, chain-end). The other groups are 2.32 (t, CH_2CO), 1.6-1.7 (m, CH_2) and 1.37 (m, CH_2) **[9]**. Additionally, the number average molecular weights of PCL were calculated 7500 g/mol by using equation 3. This result is lower than the result obtained by GPC. However, it is possible obtaining different values by using GPC and ^1H NMR because of measuring based on different standards.

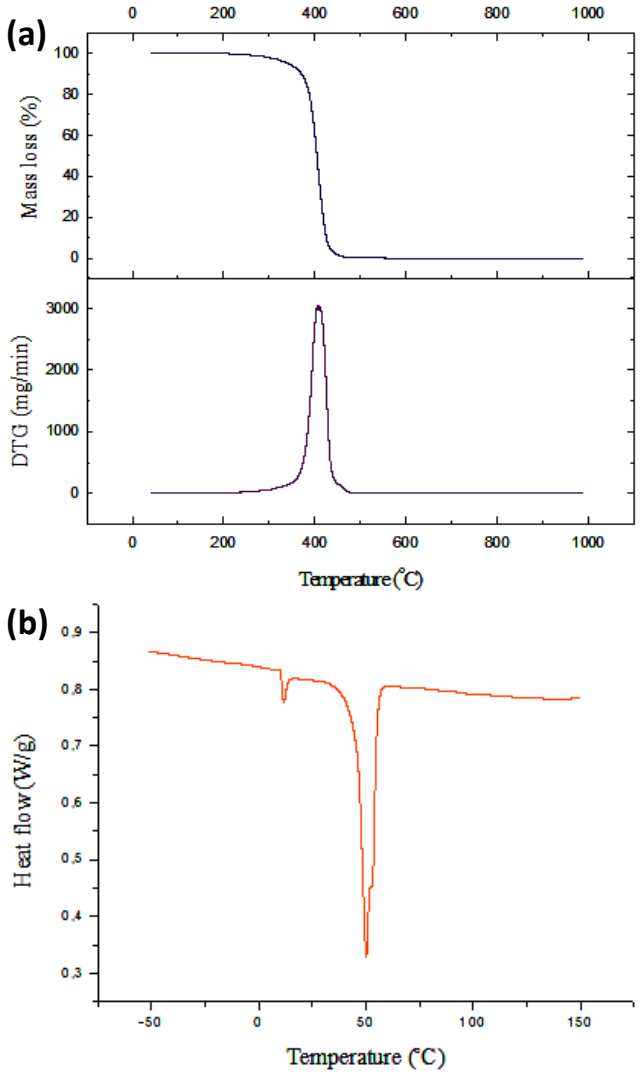

Fig. 6. TGA curves for PCL catalyzed by IMCALB (a), DSC scans of PCL catalyzed by IMCALB.

Thermal gravimetric analysis (TGA)

PCLs synthesized by IMCALB were characterized by TGA analysis. Its degradation behavior occurs one main degradation process with an inflection point that can be seen in **Fig. 6(a)**. PCL degradation was recorded at 408.9 ºC, and Tmax was calculated 435.7 ºC from TGA curves. Persenaire *et al.* (2001) studied thermal degradation of polycaprolactone, and DTG curves of PCL polymerized in their study are very similar to the curves stayed in **Fig. 6(a) [15]**. Parallelly, Düşkünkorur *et al.* (2014) studied lipase catalyzed synthesis of polycaprolactone and clay-based nanohybrids. They obtained similarly one main degradation for PCL, and degradation process occured at 400-420 ºC **[16]**.

Differential scanning calorimetry (DSC)

DSC scans of PCL catalyzed by IMCALB can be seen in **Fig. 6(b)**. From DSC thermogram of PCL, Tm and ΔH_f values were calculated, which are 57.08 ºC and 23.40 J/g respectively. Crystallinity percentage of polycaprolactone was measured by using equation 2, which were 17 %. It can be seen in **Fig. 6(b)** there are two peaks on DSC thermogram of PCL catalyzed by IMCALB. It is possible that PCL could be including a little amount of monomer. Thus, DSC analysis of PCL can be repeated to obtain more clear datas.

Kuo *et al*. (2001) has studied hydrogen-bonding strength in polycaprolactone blends by DSC and FTIR. They obtained similar DSC thermogram of PCL included between 60-70 ºC of Tm values **[17]**.

Conclusion

As a result, *Candida antarctica* lipase B was immobilized onto an amorphous silica-based material in the first step of this study. Secondly, polycaprolactone was successfully synthesized via enzymatic ring opening polymerization catalyzed by the novel immobilized biocatalyst. The optimum temperature, time and enzyme content for polymerization reactions were determined 80 ºC, 150 h and 20 % (w/w), respectively. The product was characterized by using GPC, SEM, FTIR, ^{1}H NMR, TGA and DSC analyses. The analyses showed that the result polymer has all characteristic properties of polycaprolactone. All in all, this study has demonstrated that polycaprolactone can be synthesized via green friendly methods with high molecular weights and monomer conversion rates.

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Reference

1. Zhang, J.; Shi, H.; Wu, D.; Xing, Z.; Zhang, A.; Yang, Y.; Li, Q. *Process Biochem.* **2014**, *49*, 797. **DOI:** 10.1016/j.procbio.2014.02.006
2. Yang, Y.; Yu, Y.; Zhang, Y.; Liu, C.; Shi, W.; Li, Q. *Process Biochem.* **2011**, 46, 1900. **DOI:** 10.1016/j.procbio.2011.07.016
3. Li, Q.; Li, G.; Yu, S.; Zhang, Z.; Ma, F.; Feng, Y. *Process Biochem.* **2011**, *46*, 253. **DOI:** 10.1016/j.procbio.2010.08.019
4. Uyama, H. Enzymatic polymerization, Future Directions in Biocatalysis; Tomoko Matsuda (Ed.); Osaka University; Suita; Japan, **2007**, pp. 205. **DOI:** 10.1016/B978-044453059-2/50010-8
5. Santos, R. D.; Comim, S. R. R.; Oliveira, D.; Treichel, H.; Luccio, M. D.; Ferreira, S. R. S.; Oliveira, J. V. *Biocatal. Agric. Biotechnol.* **2012**, *1*, 280. **DOI:** 10.1016/j.bcab.2012.04.002
6. Mihailović, M.; Stojanović, M.; Banjanac, K.; Carević, M.; Prlainović, N.; Milosavić N.; Bezbradica, D. *Process Biochem.* **2014**, *49*, 637. **DOI:** 10.1016/j.procbio.2014.01.013
7. Park, S.; W., Kim, Y. I.; Chung, K. H.; Hong, S. I.; Kim, S. W. *React. Funct. Polym***.** 2002, *51*, 79. **DOI:** 10.1016/S1381-5148(02)00028-7
8. Öztürk-Düşkünkorur, H. *PhD Thesis*, ITU, Istanbul, Turkey, **2012**.
9. Özsağıroğlu, E. *M.Sc. Thesis*, ITU, Institute of Science and Technology, Istanbul, **2011**.
10. Sha, K.; Qin, L.; Li, D.; Liu, X.; Wang, J. *Polym. Bull.* **2005**, *54*, 1. **DOI:** 10.1007/s00289-005-0341-1
11. Deng, F.; Gross, R. A. *Int. J. Biol. Macromol.* **1999**, *25*, 153. **DOI:** 10.1016/S0141-8130(99)00029-X
12. Woodruff, M. A.; Hutmacher, D. W. *Prog. Polym. Sci.* **2010**, *35*, 1217. **DOI:** 10.1016/j.progpolymsci.2010.04.002
13. Ranjha, N. M.; Mudassır, J.; Majeed, S. *B. Mater. Sci.* **2011**, *34*, 1537. **DOI:** 10.1007/s12034-011-0356-1
14. Wu, C. S. *Polymer*, **2005**, *46*, 147. **DOI:** 10.1016/j.polymer.2004.11.013
15. Persenaire, O.; Alexandre, M.; Dege´, P.; Dubois, P. *Biomacromolecules*, **2001**, *2*, 288. **DOI:** 10.1021/bm0056310
16. Öztürk-Düskünkorur, H.; Pollet, E.; Phalip, V.; Güvenilir, Y.; Avérous, L. *Polymer*, **2014**, *55*, 1648. **DOI:** 10.1016/j.polymer.2014.02.016
17. Kuo, S. W.; Huang, C. F.; Chang, F. C. *J. Polym. Sci. Pol. Phys.* **2001**, *39*, 1348. **DOI:** 10.1002/polb.1107

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