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Performance Comparison of Commercial and Home-Made Lipases for Synthesis of Poly(δ-Valerolactone) Homopolymers

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Abstract: Novozyme 435, which is the commercially available immobilized form of *Candida antarctica* lipase B, has been successfully conducted ring opening polymerization of lactones in organic solvents. In this paper, it was aimed to introduce an alternative biocatalyst for Novozyme 435. *Candida antarctica* lipase B immobilized onto rice husk ashes via physical adsorption (with a specific activity of 4.4 U/mg) was prepared in previous studies and used as a biocatalyst for poly(δ-valerolactone) synthesis in the present work. Polymerization reactions were proceeded at various reaction temperatures and periods via both two immobilized enzyme preparations. The resulting products were characterized spectroscopically and thermally. The highest molecular weight (M_n = 9010 g/mol) was obtained via Novozyme 435 catalysis at 40℃ and 24 hours. The performance of home-made lipase, which resulted in a molecular weight of 8040 g/mol, was close to commercial one.

Keywords: Poly(δ-valerolactone); *Candida antarctica* lipase B; Novozyme 435; immobilization; enzymatic ring opening polymerization

1 Introduction

There is a great interest for aliphatic polyesters based on lactone monomers with different ring sizes, such as ε-caprolactone (7-membered) and δ-valerolactone (6-membered) [1,2]. Due to their excellent biocompatibility, biodegradability, and permeability, polymers and copolymers of these monomers are highly preferred for biomedical applications including drug delivery systems, surgical sutures, bone and tissue fixation devices [1-5]. Poly(δ-valerolactone) (PVL) has identical features with linear aliphatic polyester poly(ε-caprolactone) (PCL), such as hydrophobic nature, semi-crystalline structure, low melting point and low cytotoxicity. However, there are limited studies on synthesis and application of PVL in biomedical area in contrast to PCL [4,6]. There exist several attempts for ring opening polymerization (ROP) of δ-valerolactone (δ-VL) including organometallic catalysts or biocatalyts. Generally, organometallic catalysts (aluminum alkoxides, tin carboxylates, etc.) are used for the synthesis of PVL. However, for the synthesis of a polymer that will be involved in a biomedical application, metallic residues are not tolerated due to their toxicity. Therefore, there is an increasing attention for enzymatic ROP nowadays. Enzyme catalyzed synthesis provides production of PVL without any metallic residues via mild reaction conditions [4]. Due to the lower activity of δ-VL (relative to ε-caprolactone) in enzymatic ROP, there is an afford for the optimization of reaction conditions such as, enzyme, temperature, reaction medium and reaction period [4]. Kobayashi and co-workers applied various lipases (derived from *Pseudomonas fluorescens* and *Candida cylindracea*) for the ROP of δ-VL. They only reached a maximum molecular weight of 3200 g/mol at the end of a long reaction period (360 hours) by using lipase *Candida cylindracea* [7]. Moreover, Cao and colleagues have studied on recombinant technology to produce enzymes with increased thermal tolerance and they reached a maximum molecular weight of ~2225 g/mol in toluene at high temperatures (70-90℃) [4]. These previous studies have shown how hard enzymatic polymerization of δ-VL and the need of searching novel enzyme preparations.

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Lipases are the most preferred enzymes for biocatalysis, due to their specificity, capability of catalyzing wide range of reactions in different reaction media with high stability [8]. They have a α-helical oligopeptide chain which covers their active site (lid) and prevents the access of substrate. When there is no hydrophobic interface present, the active site of the enzyme is hidden from the reaction medium. This conformation of enzyme is called as "closed conformation". On the other hand, when there exists a hydrophobic interface, the enzyme goes on a conformational change to expose its catalytic triad to the hydrophobic phase. This conformational change of enzyme results in "open conformation". Thus, the activation process of lipase is named as "interfacial activation" [9]. By the immobilization of lipase onto a hydrophobic support material, it is possible to obtain fully exposed active sites [8]. This may result in increased specificity, stability, and activity [8,10].

Candida antarctica Lipase B (CALB) and its commercially available immobilized form, Novozyme 435 (Nov-435), have been widely used for ROP of lactones with different ring sizes [11,12]. In the present work, Nov-435, and CALB immobilized onto rice husk ashes (RHA) via physical adsorption (obtained in our previous work) were used as biocatalysts for ROP of δ-VL [13]. It was aimed to introduce the new home-made enzyme, that was shown to be successful for PCL (M_n = 14000 g/mol) and poly(ε-caprolactone-co-ω-pentadecalactone) (M_n = 20960 g/mol) syntheses previously, as an alternative for the commercial one [14,15]. In previous studies, performances of Im-CALB and Nov-435 had been compared for PCL and poly(ε-caprolactone-*co*-ω-pentadecalactone) syntheses. For PCL synthesis, highest molecular weight was obtained as 14000 g/mol at 60℃ at the end of 48 hours via Im-CALB catalysis. At same reaction conditions, Nov-435 catalysis resulted in 10630 g/mol molecular-weighted PCL [13]. Moreover, molecular weights of poly(ε-caprolactone-*co*-ω-pentadecalactone) (50% molar feed ratio) copolymers synthesized via Im-CALB and Nov-435 were measured as 20960 g/mol and 22000 g/mol, respectively (at 80℃ and 6 hours) [15].

In this study, synthesis of PVL with maximum molecular weight and suitable properties was purposed. Thus, polymerizations were carried out at varied temperatures (30, 40, 60, and 80℃) and periods (6, 24, 48, 72, and 120 hours) in order to determine optimum conditions.

2 Materials and Methods

2.1 Materials

The monomer δ-valerolactone (≥ 98%) was obtained from Sigma Aldrich and used as received. Toluene was purchased from Merck. Chloroform, methanol, and Novozyme 435 were acquired from Sigma Aldrich. The free form of *Candida antarctica* lipase B (CALB) was purchased from Sigma Aldrich and used as immobilized form (Im-CALB) [13].

2.2 Synthesis of Poly(δ-Valerolactone) Homopolymers

Ring opening polymerization of δ-VL was performed under dry nitrogen in 1 g of toluene (in order to favor esterification reaction and prevent hydrolytic activity of lipase) with a stirring rate of 120 rpm. Reactions were proceeded at various temperatures (30, 40, 60, and 80℃) and time periods (6, 24, 48, 72, and 120 hours). Calculated amount of lipase (Novozyme 435 or Im-CALB) and δ-VL monomer were introduced to the flask with 20% enzyme concentration (weight ratio of enzyme to monomer). Monomer to toluene ratio was arranged to be 1:2 (w:w). Reactions were terminated by the addition of excess chloroform and after filtration of enzyme from the polymerization medium, chloroform in the filtrate was evaporated in oven at 50℃. Then, the polymer was precipitated in cold methanol and filtrated for purification Finally, the product was dried in oven at 30℃ overnight.

2.3 Instrumental Methods

Molecular weights and polydispersity indexes (Đ) of polymer samples were measured by Gel Permeation Chromatography (GPC) using Agilent 1100 model apparatus equipped with a pump, refractive index detector, and Zorbax PSM colons (1000-S, 300-S, 60-S). The calibration curve was generated by polystyrene standards ranging from 580 g/mol to 504500 g/mol. Tetrahydrofuran (THF) was used as the

eluent and analyses were carried out at 25℃ with a flow rate of 1 ml/min. Before injection all samples were filtered via 0.45 μm filter syringe.

Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR) analysis was applied on Agilent VNMRS 500 MHz spectrometer at 25℃ for the determination of the chemical structure of samples based on characteristic chemical shifts. ^{1}H-NMR spectra were recorded in deuterated chloroform ($CDCl_3$) with respect to tetramethylsilane (TMS) standard.

Fourier Transform Infrared Spectroscopy (FTIR) analysis was applied on a Perkin Elmer spectrophotometer in order to define the chemical structure of the polymer samples. Each sample was analyzed by KBr pellet. The spectra were recorded by at least 32 scans with a resolution of 2 cm^{-1}.

Thermal properties were determined by Differential Scanning Calorimetry (DSC) using a Perkin Elmer calorimeter. Under inert nitrogen atmosphere at a 20 ml/min flow rate 5-10 mg samples were analyzed. Sample scans were carried out between -70 and 200℃ at 10℃/min with heat-cool-heat thermal cycles and melting temperature (T_m) was measured at second heating. Crystallinity percentages (χ_c) were calculated from the ratio of fusion enthalpy (ΔH_f) of the sample to the fusion enthalpy of 100% crystalline polymer ($\Delta H°_f$) (Eq. (1)) [16].

$$\chi_c = \frac{\Delta H_f}{\Delta H°_f} \times 100 \quad (1)$$

where $\Delta H°_{f\ PVL}$ = 181.8 J/g [17].

Thermal Gravimetric Analysis (TGA) was applied on a Perkin Elmer apparatus for thermal characterization of the samples. The samples (5-10 mg) were heated from 25 to 550℃ at a heating rate of 10℃/min under nitrogen flow.

3 Results and Discussion

3.1 Synthesis of Poly(δ-Valerolactone) Homopolymers and Their Spectroscopic Characterızations

Previously, successful performance of Im-CALB for PCL synthesis have been shown and suggested as an alternative for Nov-435 [14]. In the current work, ROP of δ-VL was performed at varied reaction temperatures and periods via Nov-435 and Im-CALB. By comparing the performances of the commercial and home-made lipases, optimum reaction conditions were identified. Polymerization results are given in Fig. 1 and Fig. 2.

As seen from Fig. 1 and Fig. 2, polymerization could be achieved even at very low temperatures (e.g., 30℃) and short reaction periods via both two types of CALB. There exist studies with different types of enzymes, as mentioned before, which resulted in lower molecular weights even at higher reaction temperatures and longer reaction periods [4,7]. Highest molecular weight (M_n = 9010 g/mol) for Nov-435 catalyzed ROP was obtained as 40℃ and 24 hours (Fig. 1). However, for Im-CALB catalysis, higher temperature (80℃) and longer reaction period (120 hours) were needed in order to achieve highest molecular weight (M_n = 8040 g/mol) (Fig. 2). Thanks to thermal stability of Im-CALB which was enhanced via immobilization procedure described in previous work, it had proceeded polymerization of PVL at high temperatures [13]. Similarly, Im-CALB was found to be effective at high temperatures (e.g., 80℃) in a study of poly(ε-caprolactone-co-ω-pentadecalactone) synthesis [15]. An additional experiment was carried out via Im-CALB for 150 hours at 80℃, but the molecular weight tended to decrease (M_n = 7190 g/mol). Molecular weight decrease after a certain reaction period was seen also at different temperature series. This is because, both two lipases are capable of not only synthesis but also degradation of lactone based aliphatic polyesters, as well as PVL [18]. Fig. 1 and Fig. 2 also present conversion percentages. As seen from Fig. 1, over 65% conversion was achieved at all reaction conditions via Nov-435 catalysis. On the other hand, longer reaction periods (> 24 hours) were needed to achieve this conversion value via Im-CALB (Fig. 2). The decreases in monomer conversions may be resulted from inefficient enzyme-solvent interactions or water incorporated from atmosphere. It is known that, lipases catalyze esterification and transesterification

reactions in hydrophobic organic solvents, whereas they catalyze hydrolysis in aqueous media [19]. Additionally, polydispersity indexes were measured around 1.5 for both two immobilized CALB preparations by GPC analysis.

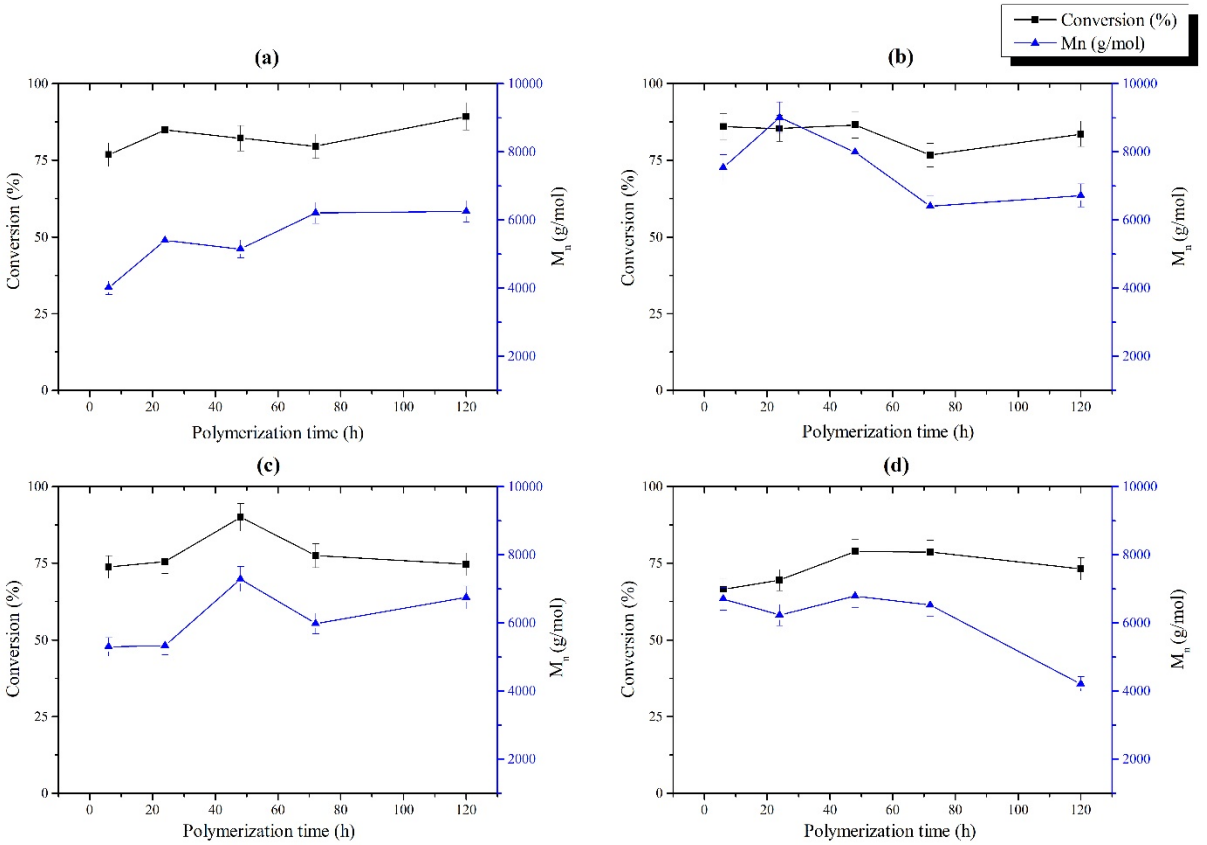

Figure 1: Results for ROP of δ-VL via Nov-435 catalysis: Polymerizations at (a) 30℃, (b) 40℃, (c) 60℃, (d) 80℃ (Monomer conversions were calculated gravimetrically. M_n were measured by GPC.)

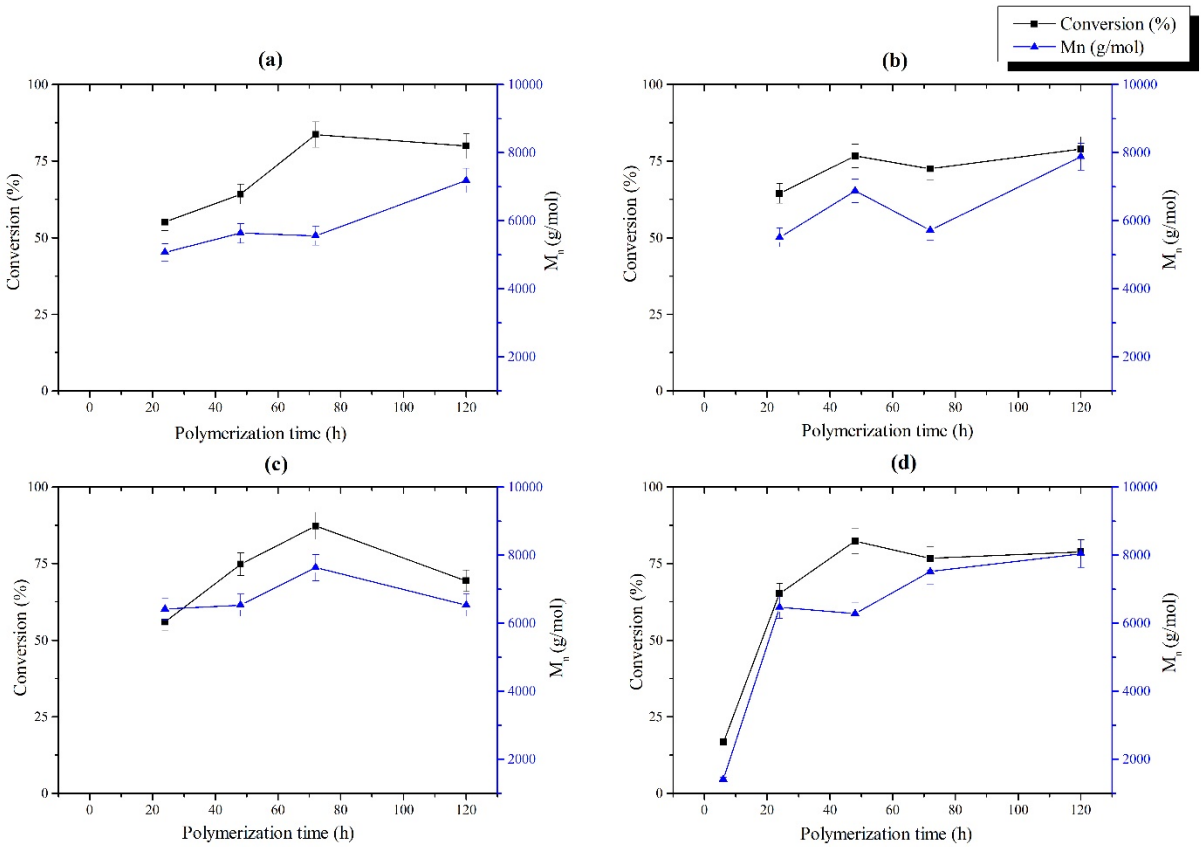

Figure 2: Results for ROP of δ-VL via Im-CALB catalysis: Polymerizations at (a) 30℃, (b) 40℃, (c) 60℃, (d) 80℃ (Monomer conversions were calculated gravimetrically. M_n were measured by GPC.)

^{1}H-NMR spectrum of PVL synthesized via Nov-435 at 40℃ and 24 hours is given in Fig. 3. The structure of PVL was confirmed by spectrum as follows (δ, ppm): 4.08 (t, -CH_2O-), 3.65 (t, -CH_2OH, end-group), 2.34 (t, -COCH2-), 1.68 (m, -COCH2CH2CH2CH2O-) [4,20,21] .

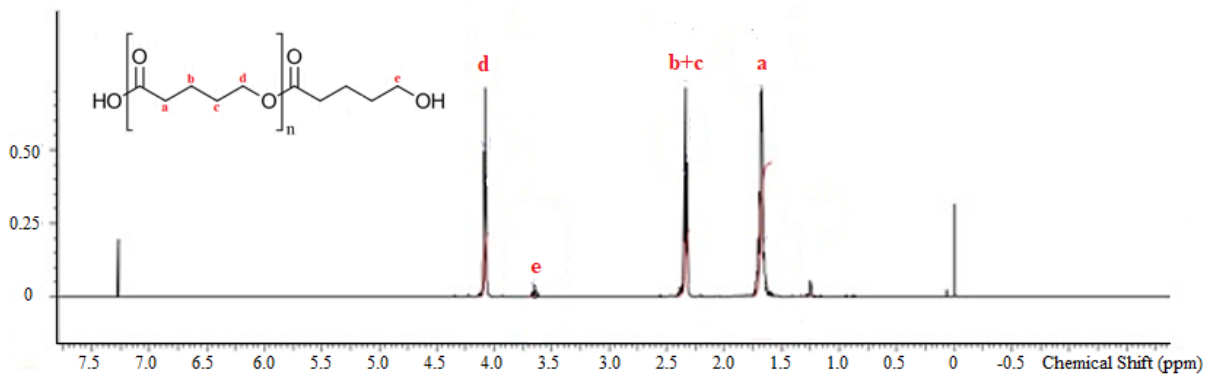

Figure 3: ^{1}H-NMR spectrum of PVL

In Fig. 4, FTIR spectra of PVL synthesized via both Nov-435 and Im-CALB at optimum reaction conditions are shown. Characteristic vibrational frequencies that validate the formation of PVL are as

follows: 2960 cm^{-1} for CH_2 stretching, 1720 cm^{-1} for C = O stretching, 1160 cm^{-1} for the C-O-C bond, and 1043 cm^{-1} for O-CH_2 stretching. These are compatible with literature [21].

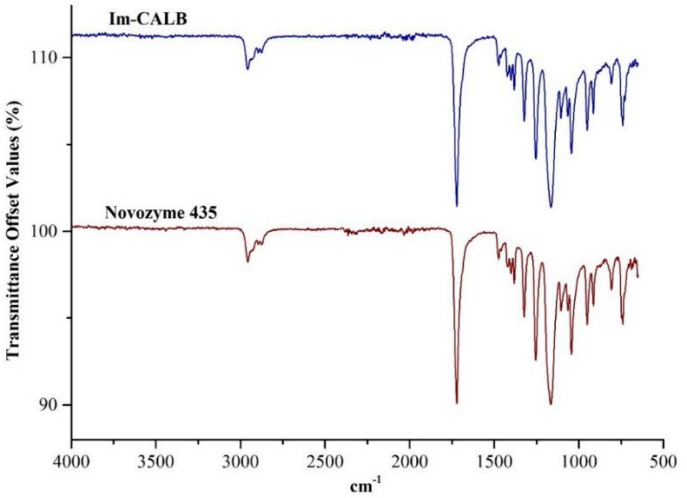

Figure 4: FTIR spectra of PVL synthesized via Im-CALB and Nov-435

3.2 Thermal Characterizations of Poly(δ-Valerolactone) Homopolymers

For the thermal characterization of PVL homopolymers, DSC and TGA were applied. Endo-down DSC curves, including both second heating and cooling, are given in Fig. 5. The thermal properties obtained from these curves are summarized in Tab. 1. Melting temperatures (T_m) of PVL samples obtained via both Nov-435 and Im-CALB catalysis were close to each other and around 52.5-53℃. Crystallization temperatures (T_c), that obtained from the exothermic peak formed during cooling, were 27.5℃ and 31.6℃ for PVL samples obtained via Nov-435 and Im-CALB catalysis, respectively. These results are compatible with previously published data [21]. Finally, crystallinity percentages (χ_c) of the samples were same and around 40%.

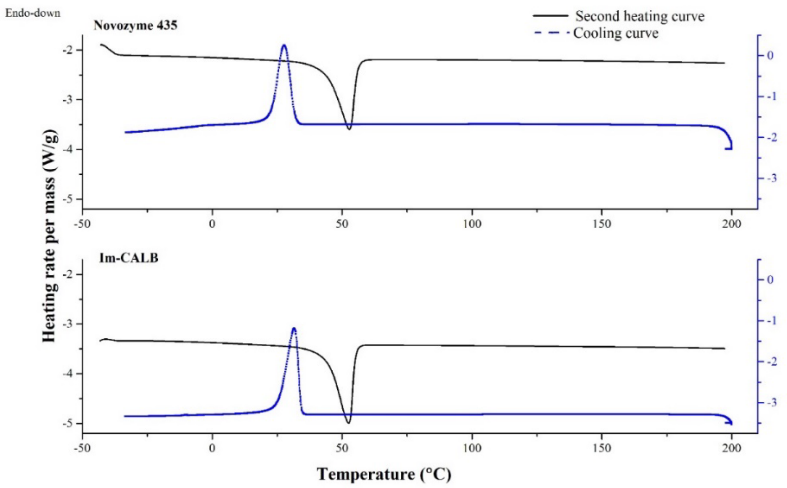

Figure 5: DSC thermograms of PVL synthesized via Nov-435 and Im-CALB

Table 1: Thermal properties of PVL samples obtained from DSC

Catalyst	T_m (℃)[a]	T_c (℃)[b]	ΔH_m (J/g)[c]	ΔH_c (J/g)[d]	χ_c (%)[e]
Nov-435	52.9	27.5	71.9	-70.7	39.5
Im-CALB	52.5	31.6	71.8	-70.3	39.5

[a] DSC melting peak at second heating cycle. [b] DSC crystallization peak. [c] Integral area under melting peak. [d] Integral area under crystallization peak. [e] Calculated from Eq. (1).

TGA curves (weight loss and first derivative of weight) of PVL synthesized via Nov-435 are given in Fig. 6. As seen, PVL sample started to degrade at about 180℃. The peak at derivative of weight graph indicated the degradation temperature (T_d) as 408℃. Moreover, there occurred a shoulder at 326℃ at which 30% of PVL was degraded.

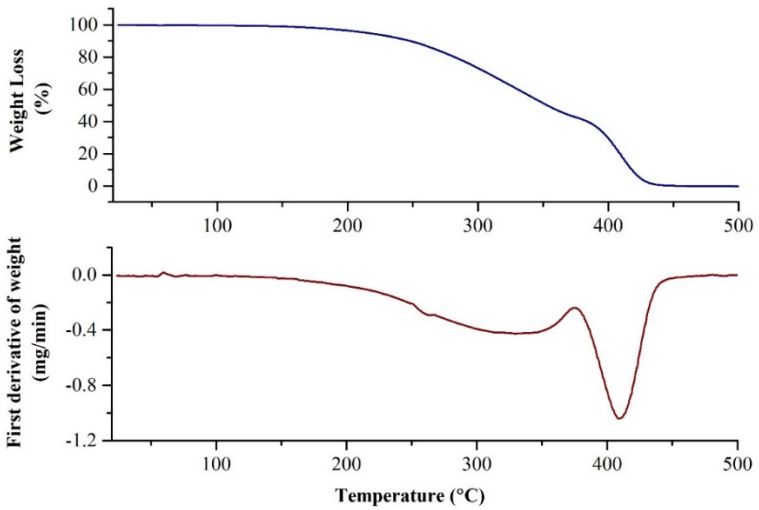

Figure 6: TGA curves of PVL synthesized via Nov-435

4 Conclusion

In this paper, ring opening polymerization of δ-valerolactone via Novozyme 435 and Im-CALB was successfully achieved. When compared with previous studies, higher molecular weights were obtained by the use of these *Candida antarctica* lipase B preparations. Moreover, the home-made immobilized CALB (Im-CALB) was found to be as successful as Novozyme 435. Further improvements in monomer conversions and molecular weights may be possible with enhancements in immobilized enzyme stability. This would be the subject of our next studies. Consequently, Im-CALB is suggested as an alternative biocatalyst for PVL synthesis.

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References

1. Yue, Z., Yan, Z., Meidong, L. (2011). Synthesis and characterization of poly(ε-caprolactone-co-δ-valerolactone) with pendant carboxylic functional groups. *Chinese Journal of Chemistry 29*, 343-350.
2. He, Y., Li, J. J., Luo, Y. K., Song, F., Wang, X. L. et al. (2015). Coating Novozyme435 with an ionic liquid: more than just a coating for the efficient ring-opening polymerization of δ-valerolactone. *RSC Advances, 5(84),* 68276-68282.
3. Kan, Z., Jin, S., He, Y., Chen, X., Wu, J. et al. (2013). Imidodiphosphoric acid as a bifunctional catalyst for the controlled ring-opening polymerization of δ-valerolactone and ε-caprolactone. *Polymer Chemistry, 4(21),* 5432-5439.
4. Cao, Q., Han, H., Li, H., Yang, G., Zhang, J. et al. (2012). Biocatalytic synthesis of poly(δ-valerolactone) using a thermophilic esterase from archaeoglobus fulgidus as catalyst. *International Journal of Molecular Sciences, 13(10),* 12232–12241.
5. Kakuchi, T., Tsuji, R., Chiba, Y., Fuchise, K., Sakai, K. et al. (2010). Controlled/living ring-opening polymerization of δ-valerolactone using triflylimide as an efficient cationic organocatalyst. *Macromolecules, 43(17),* 7090-7094.
6. Ren, Y., Wei, Y., Wu, Z., Bian, T., Leng, Y. et al. (2016). Synthesis of highly branched poly(δ-valerolactone)s: a comparative study between comb and linear analogues. *RSC Advances, 6(51),* 45791-45801.
7. Kobayashi, S., Takeya, K., Suda, S., Uyama, H. (1998). Lipase-catalyzed ring-opening polymerization of medium-size lactones to polyesters. *Macromolecular Chemistry and Physics, 199(8),* 1729-1736.
8. Manoel, E. A., dos Santos, J. C., Freire, D. M., Rueda, N., Fernandez-Lafuente, R. (2015). Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. *Enzyme and Microbial Technology, 71,* 53-57.
9. Mendes, A. A., Freitas, L., de Carvalho, A. K. F., de Oliveira, P. C., de Castro, H. F. (2011). Immobilization of a commercial lipase from *penicillium camembertii* (Lipase G) by different strategies. *Enzyme Research, 2011,* 1-8.
10. Melo, R. R. D., Alnoch, R. C., Vilela, A. F. L., Souza, E. M. D., Krieger, N. et al. (2017). New heterofunctional supports based on glutaraldehyde-activation: A tool for enzyme immobilization at neutral pH. *Molecules, 22(7),* 1-18.
11. Kumar, A., Kalra, B., Dekhterman, A., Gross, R. A. (2000). Efficient ring-opening polymerization and copolymerization of ε-caprolactone and ω-pentadecalactone catalyzed by Candida antartica lipase B. *Macromolcules, 33,* 6303-6309.
12. Bankova, M., Kumar, A., Impallomeni, G., Ballistreri, A., Gross, R. A. (2002). Mass-selective lipase-catalyzed poly(ε-caprolactone) transesterification reactions. *Macromolecules, 35(18),* 6858-6866.
13. Ulker, C., Gokalp, N., Guvenilir, Y. (2016). Enzymatic synthesis and characterization of polycaprolactone by using immobilized lipase onto a surface-modified renewable carrier. *Polish Journal of Chemical Technologies, 18(3),* 134-140.
14. Ulker, C., Gokalp, N., Guvenilir, Y. (2016). Immobilization of Candida antarctica lipase B (CALB) on surface-modified rice husk ashes (RHA) via physical adsorption and cross-linking methods. *Biocatalysis and Biotransformation, 34(4),* 172-180.
15. Wilberth, H. K., Manuel, C. U. J., Tania, L. C., Manuel, A. V. (2015). Effect of reaction temperature on the physicochemical properties of poly(pentadecanolide) obtained by enzyme-catalyzed ring-opening polymerization. *Polymer Bulletin, 72(3),* 441–452.
16. Loriot, M., Linossier, I., Vallée‐Réhel, K., Faÿ, F. (2016). Syntheses, characterization, and hydrolytic degradation of P(ε-caprolactone-co-δ-valerolactone) copolymers: influence of molecular weight. *Journal of Applied Polymer Science, 133(7),* 1-9.
17. Ulker, C., Guvenilir, Y. (2018). Enzymatic synthesis and characterization of biodegradable poly(ω-pentadecalactone-co-ε-caprolactone) copolymers. *Journal of Reneawable Materials*. (Article in press)
18. Ozsagiroglu, E., Iyisan, B., Avcibasi Guvenilir, Y. (2013). Comparing the in-vitro biodegradation kinetics of commercial and synthesized polycaprolactone films in different enzyme solutions. *Ekoloji, 22(86),* 90-96.

19. Yenenler, A. (2018). Investigating the structural properties of the active conformation BTL2 of a lipase from Geobacillus thermocatenulatus in toluene using molecular dynamic simulations and engineering BTL2 via in-silico mutation. *Journal of Molecular Modeling, 24(229),* 1-13.
20. Khalil, S. S., Al-Shamary, M. I., Al-deyab, D. O. H. (2015). Synthesis of poly (δ-valerolactone) by activated monomer polymerization, its characterization and potential medical application. *Asian Journal of Biochemical and Pharmaceutical Research, 5(4),*137-147.
21. Kasyapi, K., Bhowmick, N. (2014). Nanolamellar triblock of poly-D, L-lactide-d-valerolactone-D, L-lactide with tuneable glass transition temperature and crystallinity for use as a drug-delivery vesicle. *RSC Advances, 4,* 27439-27451.