

Synthesis of Poly(L-) Lactide under Simultaneous Cooling and Microwave Heating by using Immobilised Candida Antarctica Lipase B

Attaullah Bukhari*, Madiha Atta, Ani Idris

Department of Bioprocess and Polymer Engineering, c/o Institute of Bioproduct Development (IBD), Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia
 bukhari_ppk@yahoo.com

Heavy metals based catalysts and time taking conduction heating mode is a challenge in poly(L-) lactide (PLLA) synthesis. In this study PLLA has been synthesised under simultaneous cooling and microwave heating using biocatalyst. Effect of immobilised lipase concentrations, polymerisation times and simultaneous cooling application was investigated in PLLA synthesis. Combination of techniques; intrinsic viscosity, proton nuclear magnetic resonance (HNMR), Fourier Transform Infrared Spectroscopy (FTIR) and Differential scanning calorimetry (DSC) was used to characterise PLLA. 8 % w/w biocatalyst relative to monomer was found an optimum concentration for ring opening L-lactide polymerisation. By the application of simultaneous cooling molar mass of PLLA was increased due to increased penetration of microwaves irradiations. PLLA of molar mass (M_n) 56,125 g/mol with 50.3 % crystallinity having specific rotation of $145 \text{ deg.dm}^{-1} \text{ g}^{-1} \text{ cm}^3$ was obtained in 8 h. Maximum 98 % monomer conversion was achieved in 8 h that is significantly short polymerisation time than several days of conventional heating. In toluene, 90 °C was found as optimum temperature for polymerisation at microwave power level of 200 W. Biocatalyst was recovered completely by centrifugation. Polymerisation reaction was faster due to rapid microwave energy transfer and increased stability of biocatalyst under simultaneous cooling.

1. Introduction

Among the polymers prepared from the lactic acid PLLA is more attractive and interesting due its excellent properties as biocompatibility, low toxicity, biodegradation, high melting temperature and semi crystalline behaviour. PLLA can be easily processed in the existing equipment. It is also used as degradable scaffold in tissue engineering in organ regenerating (Jardini and Lopes, 2014). Unlike the conventional plastics PLLA has the advantage of greater than 50 % reduction in CO₂ emissions. At present, industrially PLLA is synthesised via ROP of L-lactide using heavy metals catalysts. Metals induced racemisation and intensive energy is concerns of ROP. Temperatures in the range of 180 °C – 210 °C, tin catalyst concentrations of 100 – 1,000 ppm and reaction duration of 2 – 5 h are primary conditions required to reach 95 % conversion when synthesising PLLA by ROP (Mohanty et al., 2005). In bulk ROP up to 30 h reaction time is required when using tin (II) ethyl hexanoate as catalyst (Nikolic et al., 2010).

One of the main problems faced in the processing of PLLA is the difficulty to get rid of the toxic metal contaminants (heavy metals or heavy metals based catalysts e.g. Zn, Al, Sn or Ge). In order to solve the above mentioned problems and to make ROP sustainable, greener and eco-friendly; enzyme catalysed ring-opening polymerisation (eROP) is one of the achievable methods for high molar mass polyesters synthesis (Idris and Bukhari, 2012).

The use of biocatalysts as alternative to Ziegler catalyst in ROP is an advantage due to reduction of process temperatures from 200 – 250 °C to 60 – 110 °C, eventually reducing energy costs in ROP (Uyama et al., 1999). Currently lipase catalysed ROP is focused in biodegradable polymers and fine chemical synthesis. Enzymatic ROP (eROP) is an efficient technique to prepare polymers of substantial molecular weight in good yields and under mild reaction conditions (Manzini et al., 2010).

Candida Antarctica lipase B (CalB) is a best tool for resolution of racemates and in the synthesis of wide range of polyesters. A number of reports are presented the Novozym 435 (*Candida Antarctica* lipase B, CalB) catalysed ROP of L-lactide in volatile organic solvents under conventional heating (García-Arrazola et al., 2009). However, longer reaction time (5 – 10 d) required to complete eROP is highly undesirable in industry.

Microwave heating is rapidly developing potential alternatives of conventional. In 2012 Da Rós et al., reported that dielectric heating enhance speed of enzymatic reactions and play down the negative effects of operating conditions such as high temperature, stability, and specificity to its substrate and allow uniform heating (Da Rós et al., 2012).

Combine use of enzymes and microwave heating is more environmentally friendly and time and energy saving technique. Currently Mahapatro and Negron (2013) have reported Novozym 435 catalysed ROP of pentadecalactone under microwaves heating.

In PLLA synthesis, the simultaneous cooling, use of microwave heating and biocatalysts is highly unexplored up to now. The combination use of these technologies would be a development of total green synthesis process. Especially the eROP of cyclic ester would simplify the polymer synthesis processes and avoid the contamination, reduce the production cost in terms of energy and time.

2. Materials and methods

Enzymatic ring opening polymerisation of L-lactide was carried out in three necked round bottom (20 mL) flasks using continuous microwave irradiation workstation (MASS II). L-lactide (98 % pure, CAS No. 4511-42-6) and toluene was purchased from Sigma-Aldrich Malaysia. L-lactide was purified by recrystallisation in toluene and vacuum dried for 4 h. Analytical grade dimethyl sulphoxide (DMSO), methanol and anhydrous toluene were purchase from Merck Chemical and stored over the molecular sieve 3 Å in schott bottle for 24 h prior to use. Molecular sieve (3 Å) was purchased from Sigma-Aldrich.

The biocatalyst, covalently immobilised-CalB prepared in our laboratory reported elsewhere (Bukhari et al., 2014) was employed for eROP. For simultaneous cooling application in microwave assisted eROP jacketed flask was used. The jacket of flask was connected to chiller for circulating cold water to source cooling.

2.1 Lipase catalysed ROP of L-lactide

Reaction mixture was prepared in humidity free glove box under vacuum. ROP reactions were carried out at 90 °C (Chanfreau et al., 2010). All immobilised-CalB mediated ROP experiments were performed under dry nitrogen environment. For data reliability, each reaction was performed in triplicate.

Measured amount (1.44 g, 1 mol/L) of pure L-lactide and measured amount of immobilised-CalB were taken in 20 mL three neck flasks equipped with magnet bar stirrer. The whole contents of flask were vacuum dried at 00.0 mmHg for 4 h at room temperature. Then 10 mL of toluene was added. Various immobilised-CalB concentrations as 4 %, 8 % and 12 % w/w relative to L-Lactide were explored to find the optimum catalytic effect in terms of percentage conversion of L-lactide and molar mass of PLLA in ROP process. Control experiments were performed without immobilised-CalB under the identical conditions.

The effect of microwave heating mode was investigated on ROP for 4 h, 8 h, 12 h, and 16 h. All experiments were performed by using 200 W inputs of microwaves. After the predetermined time DMSO (20 mL) was added to decrease the viscosity and resultant mixture was centrifuged for 20 - 30 min at 3,000 rpm to separate the biocatalyst easily from solvent. PLLA was precipitated by adding cold methanol (4 °C) (10 mL). White creamy material obtained was vacuum dried at 40 - 50 °C. At optimum reaction time and enzyme concentration eROP was also performed in bulk (without solvent) to confirm the effect of solvent. The Effect of simultaneous cooling on eROP was investigated at optimum microwaves heating time and enzyme concentration.

3. Characterisation

3.1 Determination of viscosity average molecular weight (M_n)

The intrinsic viscosity of PLLA was determined by using the Ubbelohde viscometer by using the single point method as described by Al-Ahmad and Al-Deri (2012). The intrinsic viscosity $[\eta]$ was calculated using Eq(1). Where ' η_{rel} ' is relative viscosity and ' η_{sp} ' represents specific viscosity.

$$[\eta] = \sqrt{2(\eta_{SP} - \ln \eta_{rel})}/C \quad (1)$$

Where 'C' represents the concentration of PLLA (g/100 mL) in chloroform.

By substituting the value of intrinsic viscosity $[\eta]$ of PLLA solution in the Mark-Houwink Eq (2). The molar mass of PLLA was calculated. The values of constants 'K' and 'a' are reported in literature as 5.45×10^{-4} and 0.73 (Garlotta, 2001).

$$[\eta] = 5.45 \times 10^{-4} \times M^{0.73} \quad (2)$$

3.2 Measurement of Optical rotation power and monomer conversion calculation

Optical rotation power of synthesised PLLA was measured Polax-2L, Germany. The percentage conversion of L-lactide into polymer was determined by comparing the integral peak area of methine hydrogen of monomer.

3.3 Differential scanning calorimetry analysis

Melting temperature (T_m), heat of fusion (ΔH_m) and crystallinity (Crys.) of PLLA was determined with Perkin Elmer Differential Scanning Calorimeter 7 (DSC-7). DSC was performed under constant flow of nitrogen at the rate of 10 °C/ min. The chemical structure of PLLA was confirmed by FTIR spectrum.

4. Result and discussion

The reaction temperature of eROP was selected 90 °C. During the progress of eROP reaction it was observed that the viscosity of the mixture was increased gradually due the formation of polymer. Therefore, speed of magnetic stirrer decreased significantly at late eROP in reaction media. At the completion of eROP white creamy precipitates of PLLA were collected after the separation of biocatalyst and solvent.

The average viscosity molecular mass (M_η) of obtained PLLA was increased by increasing biocatalyst concentration. PLLA of maximum molar mass 44,651 g/mol with optical rotation power -148° ($\text{deg} \cdot \text{dm}^{-1} \cdot \text{gm}^{-1} \cdot \text{cm}^3$) and melting temperature (T_m) 157.37 °C was achieved at the expense of 8 % (w/w) biocatalyst relative to monomer.

Percentage conversion of monomer by using various concentrations of immobilised-CalB was calculated by the comparison of integral peak area of proton in methine group of PLLA and integral peak area of same proton in HNMR spectra of monomer. The distinguished quartet peak corresponding the monomer methine proton at $\delta = 5.0 - 5.1$ ($\delta =$ chemical shift) disappeared in HNMR spectra of PLLA. By comparing the integral of PLLA prepared under the conditions (12 h microwave heating at 90 °C), only 20 % conversion of L-lactide was achieved by using 4 % concentration of immobilised-CalB. The optimum monomer conversion 98 % was achieved by 8 % concentration of biocatalyst in 12 h of microwave heating (Figure 1).

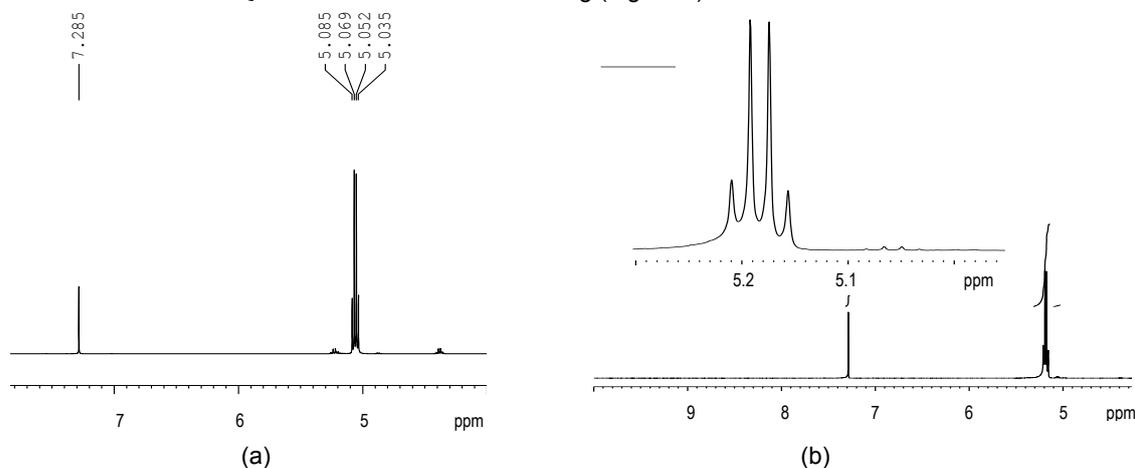


Figure 1: HNMR spectra (a) Pure L-lactide; (b) PLLA prepared by 8 % w/w biocatalyst in eROP

It was observed that as biocatalyst concentration was increased from 4 to 12 % the yield of polymer was also increased. At the optimum enzyme concentration (8 %) yield was increased to 40.8 %. The minimum yield of PLLA was recorded 5.6 % when the concentration of biocatalyst was 4%. Immobilised-CalB concentration beyond the 8 % does not have significant effect on the monomer conversion; It resulted in lower yield (36 %) and molar mass (41,099 g/mol) of PLLA. The reduced conversion of monomer and yield of polymer at higher concentration (12 %) of biocatalyst is expected due to hydrolysis behaviour of immobilised-CalB. Hydrolysis strongly depends on concentration of immobilised-CalB.

Unlike the 10 % concentration of Novozym 435 consumed for 98 - 99 % conversion of L-lactide reported by the authors (Yoshizawa-Fujita et al., 2008), in this study same percentage of conversion was achieved by using 8 % immobilised-CalB successfully. Control experiment was performed without immobilised-CalB in toluene under the same conditions. It was found PLLA was not formed without CalB catalyst which confirmed that PLLA

formation was due to CalB catalysed ROP only. In microwave oven, the predetermined temperature of toluene was achieved within 5 min and maintained at the expense of 100 W. The effect of varied (4 - 16 h) dielectric heating time on the conversion (%) of monomer, yield of PLLA and molar mass of PLLA in eROP reactions was explored at the optimum concentration (8 %) of biocatalyst. It was observed that as microwave heating time was increased from 4 h to 16 h monomer conversion and yield were also increased. After the 4 h of microwave heating time the percentage conversion of monomer reached to be 20 %. Monomer signals were not detected in the samples that subjected to 8, 12 and 16 h of microwave heating. That confirmed 99 - 100 % conversion of monomer. HNMR spectra of PLLA obtained in eROP at 8 h and 16 h of microwave heating times are given in Figure 2.

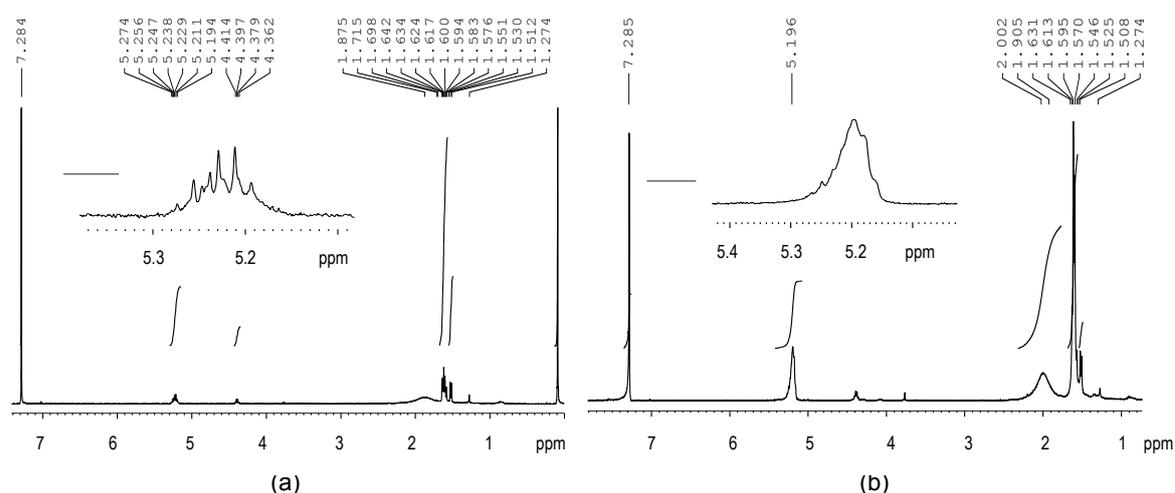


Figure 2: HNMR spectra of PLLA prepared in eROP at (a) 8 h and (b) 16 h of microwaves heating

The yields and molar masses of PLLA obtained at 4, 8, 12, 16 h of microwave heating were found as 15,958, 50,482, 44,654, 38,637 g/mol and 16.5, 50.5, 39.8, 28.4 %. Similarly, the other properties as melting temperature and crystallinity of obtained PLLA were transformed (Table 1). Thus, optimum monomer conversion 99 % in term of energy and time saving was significant after 8 h of microwave heating. The effect of microwave heating on physical properties of PLLA is mentioned in Table 1. Further the chemical structure of PLLA prepared under microwave heating was confirmed by FTIR analysis. FTIR spectra of PLLA prepared under 8 h microwave heating is presented in Figure 3. The FTIR spectra shows the strong characteristic peak at 1,749.39 cm^{-1} that corresponds to carbonyl group (C=O) bond stretching mode. The strong IR band in the region of 2,999 cm^{-1} , 2,947 cm^{-1} corresponds the stretching of C-H in CH_3 which confirmed the existence of CH_3 group in PLLA. The peak in the regions of 1,383.94 cm^{-1} is originated from the bending vibration C-H from CH_3 . The other band at 1,458.08 cm^{-1} confirmed the existence of CH group in PLLA. FTIR results confirm the structure and purity of PLLA prepared in eROP at different microwave heating intervals.

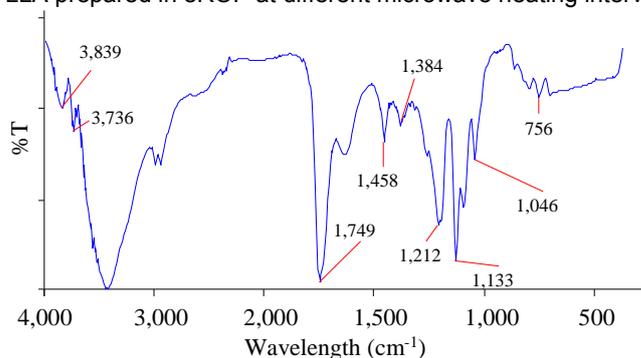


Figure 3: FTIR spectra of PLLA prepared under microwaves heating time

Table 1: Effect of microwave heating time on Properties of PLLA prepared by CalB catalysed ROP

eROP time (h)	Molar mass (g/mol)	Opt. Rot. (deg.dm ⁻¹ .cm ³)	DSC		
			T _m	ΔH_m (J/g)	Cryst. %
4	15,958	-155°	112.33	22.20	23.87
8	50,482	-155°	172.37	46.78	50.30
12	44,654	-148°	157.37	19.83	21
16	38,687	-142°	137.33	1.01	1.0

Microwave heating is known for the decrease in reaction time from several hours to few hours due to dielectric interaction with subject matrix. Reaction rate of enzyme catalysed reactions are lower than inorganic catalytic

reaction. Under conventional heating 5 - 9 d is required to reach 99 - 100 % conversion of L-lactide at the expense of 10 % Novozym 435. The findings of this work clearly confirmed up to 99 conversion of L-lactide in eROP was achieved in 8 h that was contributed by the exceptional advantage of microwave heating and nanobiocatalytic system. Unlike the convectional heating the direct and rapid heating by microwave irradiations is originated by the interaction of dipole with rapidly alternating electric field of microwaves. As a result, the number of activation collisions is enhanced in short time leading to increased rate of ROP. It was expected that microwaves induced the flexibility in CalB peptides chains that stabilised the CalB and enhanced its activity with transformation rate of product and substrate. Very little information is available on the enzymatic reactions assisted by microwave heating and it mainly focused on transformation of small organic molecules (Osuna and Rivero, 2012).

Unlike the several days required to reach 95 - 100 % conversion of L-lactide in conventional eROP as reported in related work (Mahapatro and Negrón, 2013), this study proved microwave assisted synthesis of PLLA using CalB is time efficient technique. Possibly higher capacity of L-lactide to absorb the microwaves and increased flexibility of biocatalyst induced by dielectric interaction enabled its conformation changes to allow the substrate to approach the active site more easily against conventional heating.

To optimised the technique, simultaneous cooling was applied in eROP of L-lactides at optimum enzyme concentration (8 wt%) and dielectric heating time (8 h). In microwave assisted synthesis processes the simultaneous cooling presented a significant impact on product yield, stability of product and rate of reaction. For eROP reaction, initially 200 W of microwave input power was employed that decreased to 100 W after achieving the target temperature (90 °C). It was observed by applying the simultaneous cooling the microwave power again increased to 140 W to maintain the predetermined temperature. The temperature difference of cold circulating water was recorded as 5 - 10 °C decrease, which confirmed the cooling effect on reaction contents, while the increase of input power to 140 W indicated the more penetration of microwaves irradiations.

Monomer signals could not be traced in the HNMR spectra of product obtained in eROP under the effect of simultaneous cooling. It confirmed that 99 - 100 % monomer conversion was maintained in toluene and bulk even applying the cooling to reaction contents. The yield of PLLA was surprisingly increased from 50.5 % to 55.3 % under microwave heating by the application of simultaneous cooling. In bulk experiment maximum 32.4 % yield of PLLA (34,269 g/mol) was achieved. Under the effect of simultaneous cooling molar mass of PLLA was increased to 56,125 g/mol at 90 °C. Higher microwaves penetration power accelerates the reaction rate at reduced hot spot and carbonisation due to stirring process.

Due to increased microwave absorbed power by lactides under simultaneous cooling the enzymatic ring opening polymerisation reaction was accelerated many folds. Under simultaneous cooling the degradation of product was minimised thus resulting in the increased yield and molar mass of PLLA. Although circulating cold water absorbed a fraction of input power the effect of cooling reflected the increased penetration of microwaves was more significant on the yield and molar mass of PLLA compare to microwave heating only. From the results, it is concluded that simultaneous cooling significantly improved the molar mass and yield of PLLA under microwave heating.

Compared to the PLLA prepared under microwave heating the quartet peaks of methine proton of PLLA prepared under simultaneous cooling were found superimposed and broader. PLLA prepared under 8 h of microwave heating is more optically pure than those obtained by 12 and 16 h of microwave heating. It was inferred that higher absorption of microwaves irradiation under simultaneous cooling might induced racemisation. Specific rotation power of PLLA was decreased from -155° to -145° by the application of simultaneous cooling for 8 h. More than 8 h of microwave heating resulted many superimposed quartet peaks which indicate PDLLA formation. It concludes that 8 h of microwave heating is optimum time for the synthesis of optically pure PLLA through eROP in toluene. The effect of microwave irradiation on optical purity in bulk polymerisation was more prominent and in HNMR spectrum the quartet peak was fully superimposed and the optical purity was decreased to -142°.

It was observed that increasing melting temperature was consistent with molecular weight, since the highest melting temperature (173.83 °C) was shown by the PLLA of 56,125 g/mol obtained. The lowest melting temperature of PLLA was recorded 112.3 °C with the heat of fusion of 22.2047 J/g. Unlike the single melting temperature of PLLA under simultaneous cooling double melting temperature (173.83 °C and 156.83 °C) of PLLA was observed.

When the PLLA was annealed for 1 h at 80 °C it gave only one sharp melting temperature 173.93 °C. It confirmed that more than one melting temperature are due to recrystallisation/ phase transition. Such behaviour of PLLA is also recorded by other authors (Xiang et al., 2016). The highest percentage of crystallinity was found 50.30 %. It is concluded that PLLA synthesised in this work is semicrystalline nature polymer. Rapid and homogenous microwave heating and simultaneous cooling accelerated the eROP. The non-thermal effects of dielectric heating and promiscuous behaviour of biocatalyst under microwaves irradiations cannot be excluded.

5. Conclusion

PLLA of molar mass 56,125 g/mol at 90 °C was synthesised by applying the simultaneous cooling with microwave heating in eROP. PLLA was successfully synthesised from L-lactide by microwave assisted eROP using immobilised-CalB. Reaction time of eROP was significantly reduced to 8 h under microwave irradiation comparing to several days required in conventional eROP. More than 98 % monomer conversion was confirmed by HNMR analysis. FTIR analysis confirmed the chemical structure of PLLA. Crystallinity of synthesised PLLA was found 50.30 % by DSC analysis. The PLLA synthesised in eROP under microwave heating is optically pure and semicrystalline. Microwave assisted eROP can be applied for poly(ϵ -caprolactones) as well poly(macrolactones). Synergistic behaviour of biocatalyst and dielectric heating observed in ROP is novel approach for eco-friendly and metals free synthesis. As a result, PLLA of enhanced molecular weight was prepared without racemisation in short time by employing clean mode of heating compared to conventional heating system.

Acknowledgments

Authors are much grateful for research grant support by the Ministry of Higher Education (MOHE) Malaysia, Ministry of Agricultural (MOA) and Universiti Teknologi Malaysia.

Reference

- Bukhari A., Idris A., Atta M., Loong T. C., 2014, Covalent immobilization of *Candida antarctica* lipase B on nanopolystyrene and its application to microwave-assisted esterification, *Chinese Journal of Catalysis* 35, 1555-1564.
- Chanfreau S., Mena M., Porras-Domínguez J. R., Ramírez-Gilly M., Gimeno M., Roquero P., Bázquez E., 2010, Enzymatic synthesis of poly-L-lactide and poly-L-lactide-co-glycolide in an ionic liquid, *Bioprocess and Biosystems Engineering* 33, 629-638.
- Da Rós P. C., de Castro H. F., Carvalho A. K., Soares C. M., de Moraes F. F., Zanin G. M., 2012, Microwave-assisted enzymatic synthesis of beef tallow biodiesel. *Journal of Industrial Microbiology & Biotechnology* 39, 529-536.
- García-Arrazola R., López-Guerrero D. A., Gimeno M., Bázquez E., 2009, Lipase-catalyzed synthesis of poly-L-lactide using supercritical carbon dioxide, *The Journal of Supercritical Fluids* 5, 197-201.
- Garlotta D., 2001, A literature review of poly (lactic acid), *Journal of Polymers and the Environment* 9, 63-84.
- Idris A., Bukhari A., 2012, Immobilized *Candida antarctica* lipase B: Hydration, stripping off and application in ring opening polyester synthesis. *Biotechnology advances* 30, 550-563.
- Jardini A., Lopes M., 2014, Synthesis and characterizations of poly (lactic Acid) by ring-opening polymerization for biomedical applications, *Chemical Engineering Transactions* 38, 331-336.
- Mahapatro A., Negrón T. D. M., 2013, Biodegradable poly-pentadecalactone (PDL) synthesis via synergistic lipase and microwave catalysis, *American Journal of Biomedical Engineering* 3, 9-13.
- Manzini B., Hodge P., Ben-Haida A., 2010, Entropically-driven ring-opening polymerization of macrocyclic esters with up to 84-membered rings catalysed by polymer-supported *Candida antarctica* lipase B, *Polymer Chemistry* 1, 339-346.
- Mohanty A. K., Misra M., Drzal L. T., 2005, *Natural fibers, biopolymers, and biocomposites*: CRC Press, Taylor & Francis Group, Boca Raton, FL.
- Nikolic L., Ristic I., Adnadjevic B., Nikolic V., Jovanovic J., Stankovic M., 2010, Novel microwave-assisted synthesis of poly (D, L-lactide): the influence of monomer/initiator molar ratio on the product properties, *Sensors* 10, 5063-5073.
- Osuna V., Rivero I. A., 2012, Study of the influence of microwave and conventional heating on the lipase-catalyzed esterification of lauric acid with different alcohols, *Journal of the Mexican Chemical Society* 56, 176-182.
- Uyama H., Yaguchi S., Kobayashi S., 1999, Enzymatic synthesis of aromatic polyesters by lipase-catalyzed polymerization of dicarboxylic acid divinyl esters and glycols, *Polymer Journal* 31, 380-383.
- Xiang S., Jun S., Feng L.-d., Chen X.-s., Liu F.-q., Huang S.-y., 2016, Effects of molecular weight on the crystallization and melting behaviors of poly (L-lactide), *Chinese Journal of Polymer Science* 34, 69-76.
- Yoshizawa-Fujita M., Saito C., Takeoka Y., Rikukawa M., 2008, Lipase-catalyzed polymerization of L-lactide in ionic liquids, *Polymers for Advanced Technologies* 19, 1396-1400.