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ASYMMETRIC LACTIC ACID ESTERIFICATION WITH BIOCATALYSTS IN IONIC LIQUID

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Biodegradability and environmentally friendly technologies recently came into prominence; this is the reason why we assayed to develop a new “green” technology for L-lactic-acid (LLA) production. Racemic lactic acid (rLA) mixture produced by chemical industry is difficult to handle. The product of esterification with low carbon chain alcohols has higher volatility than lactic acid (LA) itself, therefore it can be more effectively separated. Our reactions were carried out with biocatalysts (enzymes) — some of them prefer reactions with L-enantiomer — result in enantioselective esterification. After LLA ester production the hydrolysis leads to separated LLA, which is the starting material of a biodegradable plastic.

Our aim was to achieve enantioselectivity in phosphonium-type ionic liquid solvents by the optimization of several parameters, such as temperature, substrate molar ratio, amount of IL, water content. Reasonable results were achieved with three types (*Candida antarctica, Candida rugosa*, AMANO PS-IM) of lipases.

The use of enzymes and ionic liquids can make the technology “greener”, where an ingredient of a biodegradable plastic can be produced. The toxic heavy metals or hazardous acids can be replaced by biocatalyst (enzymes). These intermediates are re-usable, and they work at lower temperature, than conventional catalysts, thus the operational costs can be reduced. Ionic liquids — compared with conventional organic solvents — have insignificant vapour pressure, they are non-flammable and re-usable after a purification process, furthermore they can be tailor made for a certain application. It is not negligible that the structure affects the environmental features like biodegradability or toxicity. The high lactic acid dissolving capacity is the reason why phosphonium-type ionic liquids were used. There are research teams, apply them for lactic acid extraction from fermentation broth.

Keywords: lactic acid, enzyme, ionic liquid, enantioselectivity, esterification

Introduction

According to sustainable development environment needs to be protected beyond industrial production. This is the reason why non pollutant, biodegradable materials spread widely nowadays. Polylactides (PLA) and some of their derivatives are thermoplastic, biodegradable and biocompatible polymers with mechanical properties similar to the plastics which are commonly used, like polystyrene or polyethylene terephtalate. That is the reason why they were extensively studied in the last 20 years [1]. Economic studies show that PLA is an economically feasible material to use as a packaging polymer [2, 3]. Second generation PLA application could be seen mainly in the area of fresh products where PLA is being used as a food packaging polymer for short shelf life products, such as fruit and vegetables [4]. Currently, PLA is used in compostable yard bags to encourage recycling and composting efforts. In addition, new applications such as fibres [5-8], textiles, foamed articles and paper coatings [4] can be pursued.

Lactic acid (2-hydroxypropionic acid) is the simplest hydroxyl-carbonyl acid. It has an asymmetric carbon atom and exists in two optically active configurations; the L(-) and D(+) isomers. The chemical synthesis route can be used to produce large scale quantities of rLA [9]. The L(-) and D(+) isomers can be produced in bacterial systems. Mammalian systems produce only the L(-) isomer which is easily assimilated. The majority of lactic acid is made by bacterial fermentation of carbohydrates. The fermentation processes can be classified according to the type of bacteria used [3].

Poly(L-lactic acid) (PLLA) can be degraded by natural environments. The crystallinity of PLLA depends on the optical purity of the L-lactate units in the polymer, hence the higher the optical purity of the L-lactate units, the higher is its crystallinity. The production of highly crystalline PLLA requires the optical purity of L-lactic acid (LLA). Optical pure LLA can be produced by a particular microorganism in a selected medium [10]. Lee [11] observed that the biodegradable polymer produced from LLA does not have proper mechanical properties; it is hard and breakable. Polymerized DLA has the same disadvantages. Since there is no industrial technology for producing DLA, Németh and co-workers [12] started to develop a fermentation technology. *Lactobacillus coryniformis* bacterium was applied for the production of DLA. The experiments led to high yield at low glucose content, but the bacteria are need to be developed further.

Synthesis of PLLA with a wide range of molecular weight using toxic inorganic catalysts or inducers has been reported [13]. The use of enzyme biocatalysts is advantageous in catalysis because they proceed in mild reaction conditions without metal or toxic organic contaminations [14]. Matsumura et al. [15] observed first the bulk polymerization of LLA with low PLLA yields using lipase from *Burkholderia cepacia*. Recently, some authors have claimed that it is possible to synthesize PLLA in bulk [16] and in ionic liquids using the readily available immobilized lipase B from *Candida antarctica* (CALB) in its commercial form Novozym 435 [17, 18, 19].

Although, there are some studies about using enzymes for asymmetric reactions, for example enantioselective esterification of (±)-menthol [20], hydrolysis of (D,L)-phenylglycine methyl ester [21], esterification of racemic ibuprofen [22] and 2-substitued-propanoic acid [23], Ohara et al. was the only ones who [10, 24] investigated the optical resolution of lactic acid using enzymes. In the latest report [25] the optical resolution of butyl L- and D-lactate (BuLLA, BuDLA) using an immobilized lipase was investigated. BuLLA and BuDLA mixtures were used in the presence of Novozym 435 lipase. At 80 °C the oligomerization of BuDLA was induced enantioselectively, whereas BuLLA was not involved in the reaction.

Ionic liquids, which are liquids at ambient or far below ambient temperature, have been extensively used in the past decades as potential green alternatives for toxic, hazardous, flammable and highly volatile organic solvents. Indeed, their many attractive physicochemical properties, including negligible vapour pressure, excellent chemical and thermal stability and high ionic conductivity make ILs great candidates for replacing volatile organic compounds [26]. Biodegradability depends on the ions of the IL, the ligands and bonds in the cation and the selected anion [27]. All these interesting combinations of properties open the road to a wide range of applications, including organic and inorganic synthesis, catalysis, separation and enzymatic reactions.

Studies on enzymatic reactions in ILs over the last 10 years have revealed not only that ILs are environmentally friendly alternatives but that enzymes in these solvents exhibit excellent substrate, regio- and enantioselectivity [26].

Compared to polar organic solvents ILs surprisingly do not inactivate enzymes [28, 29]. This feature extends enzyme-catalyzed reactions to a solvent polarity range that was previously inaccessible. The ability to use solvents with greater polarity increases the solubility of polar substrates, such as glucose, maltose or ascorbic acid [28], leading to faster reactions and changes in selectivity. For example, in the CALB-catalyzed acylation of ascorbic acid with oleic acid in an ionic liquid the conversion was higher (83%) than the typical result in organic solvents (50%) [28]. The yield was higher in the case of some ionic liquids (80%) than in hexane (14%) during the esterification of LA with ethanol [30].

There are some reports about using phosphonium-type ILs. Extraction of LA has been studied by Marták et al. [31]. The phosphonium IL with the 2,4,4-trimethylpentylphosphinic anion (called Cyphos 104) is a new effective extractant of LA with a considerably higher value of the distribution coefficient compared to liquid extractants. Mainly the synergetic effect of the anion is responsible for the increased distribution coefficient of LA in Cyphos 104.

Separation of LA is quite difficult due to its low volatility; hence distillation, liquid extraction, esterification, salt processes, electrodialysis, thermal methods and ion exchange can be used for obtain lactic acid from fermentation broth [32]. Major et al. [30] studied esterification of LA and used phosphonium-type ionic liquids for the first time in the process. The two substrates to produce ethyl-lactate (EL) were lactic acid and ethanol and the applied biocatalyst was CALB. The reactions were carried out in a shaking incubator (150 1/min) at 40 °C for 24 h. The enzymatic EL synthesis was carried out in two different organic solvents (hexane and toluene) and in seven ILs. The best yields were observed in Cyphos 104 and Cyphos 202 without catalysing the reaction themselves. Five ILs (Cyphos 163, Cyphos 166, Cyphos 106, Cyphos 102, and Cyphos 110) showed high catalytic activity without enzyme loading. The substrates and products were completely miscible with the applied solvents, except for hexane and Cyphos 110 resulting in the lowest ester yields of 14% and 36%.

Water content has a particular role in ester synthesis from organic acids and short alkyl alcohols in non-conventional media. Esterification is an equilibrium reaction, hence the maximal yield can be influenced with the initial water content [30] or the control of the water content during the reaction by using zeolite [33] or pervaporation [34]. To reach high ester yield either one of the substrates (usually alcohol) should be used in excess or the product should be removed. Applying LA as substrate a new role of water content emerges, since in lower amount of water, LA undergoes self esterification producing its open chain dimmer, lactoyllactic acid and other oligomers [35]. Therefore the commercially available 90% LA solution contains a significant amount of dimmer LA beside the monomeric form. Furthermore, dimerization/decomposition of LA has to be considered as a side reaction in the reaction mixture beside ethyl lactate synthesis [30].

Since LA is a chiral molecule after esterification two molecules with the same consistence but different formation generates from industrial rLA mixture. The ratio of the enantiomers strongly depends on the activity of the applied enzyme. Enantioselectivity of enzymes is influenced by substrates and reaction conditions (water activity, temperature, pH, solvent, additives, etc.). In some experiments additional co-solvent increased activity, stability and enantioselectivity of the enzyme [36]. Enantioselectivity is calculated by the following equation of enantiomeric excess:

$$e.e. = \frac{|R - S|}{R + S} \times 100\%\,, \qquad (1)$$

where:

ee - enantiomeric excess

R and S - the ratios or values of the enantiomers

Our aim in this work was to achieve enantioselective lactic acid esterification in IL as a solvent and to use biocatalysts to achieve enantioselectivity. With asymmetric esterification of rLA it can be separated into the two enantiomers and after a cleaning procedure biodegradable PLA can be polymerized. Furthermore, the enzyme and the IL can be recycled. Lactic acid and ethanol are environmentally friendly materials, as well as enzymes and ILs. With the application of these materials a novel green technology can be developed.

Four reasonable parameters (temperature, alcohol excess, added IL, initial water content) were chosen to investigate their effects on enantioselectivity and yield. First the alcohol excess vs. temperature, then alcohol excess vs. IL, and finally alcohol excess vs. initial water content were investigated. In each case the two other parameters were constant.

Materials and methods

Enzymes and chemicals

Enzymes: Immobilized *Candida antarctica* lipase B (Novozym 435, triacilglycerol hidrolase, E. C. 3.1.1.3.) was a gift of Novo Nordisk (Basvaerd, Denmark). Lipase from *Candida rugosa* (liophylised, E. C. 3.1.1.3.) was from Sigma-Aldrich (Buchs, Switzerland) and Amano lipase PS-IM immobilised on diatomaceous earth from Sigma-Aldrich (St. Louis, USA).

Solvents: Trihexyl-tetradecyl-phosphonium-bis (2,4,4-trimethyl-pentyl)-phosphinate (Cyphos 104), Sigma-Aldrich, (Germany), tributyl-tetradecylphsphonium-dodecylbenzenesulphonate (Cyphos 201), IoLiTec GmbH, (Germany).

Reagents: (D,L)-Lactic acid (90%), Reanal (Hungary), absolute ethanol (>99%), Merck (Germany) were applied.

Reaction and analysis

The reactions were carried out in 10 ml vials with IKA KS 4000i shaking incubator at 150 min^{-1} in 24 hours. Every mixture contained rLA, ethanol and IL. After preparing the mixture the initial water content was checked and set with Karl-Fischer method. 0.5 μl samples were analysed by HP 5890 gas chromatograph, FID, LIPODEX-E column (Cyclodextrin) 30 m x 0.25 mm, head pressure 90 kPa at 90 °C constant temperature, injector 150 °C, detector 250 °C. Fiberglass and high surface adsorbent material were inserted to the injector inlet to protect the column from contamination by the IL.

Results and discussion

Selection of the reaction medium

The reaction medium was selected so that it neither reduces nor enhances catalytic activity of the enzymes. Therefore catalytic activities of two ILs were investigated. Previous report [30] shows that Cyphos 104 and Cyphos 202 were suitable solvents for lactic acid esterification with high ester yield. Cyphos 202 is no more available from the producer. That is why Cyphos 201 was applied, which is quite similar to Cyphos 202, but its catalytic activity had to be determined. Unfortunately, the catalytic activity of Cyphos 201 was too high and the conversion was close to 100%. In the case of Cyphos 104 the conversions without enzyme were low (<5%). These results were the start points for further investigations, hence the chosen solvent was Cyphos 104.

Investigation of the enantioselectivity of enzymes in Cyphos 104 ionic liquid

The aim of the further experiments was to determine the optimal parameter combination to achieve the highest enantioselectivity and yield in the presence of enzymes. Since alcohol:monomer LA molar ratio, initial water content, temperature and the amount of IL are the main parameters, which affect the activity of enzyme an experimental plan was composed with 13 measure points *(Table 1)*. First alcohol excess and IL amount was combined to 5 measure points, the other parameters were constant. Then the alcohol excess and temperature was combined, and in the end the alcohol excess and water content. The values of the parameters were: alcohol : monomer LA molar ratio = 3 : 1 (14 mmol), 7 : 1 (6 mmol) and 11 : 1 (4 mmol) (calculated for 2 ml / 46 mmol of ethanol); initial water content: 8 w/w%, 12 w/w% and 16 w/w%; temperature: 30 °C, 50 °C and 70 °C; amount of added IL: 0.5 g, 0.75 g and 1 g.

Table 1: Experimental plan

Sample	**T (°C)**	**Alcohol molar excess**	**Initial water content (w/w%)**	**Added IL (g)**
1	50	3	8	0.5
2	50	3	8	1
3	50	7	8	0.75
4	50	11	8	0.5
5	50	11	8	1
6	70	3	8	1
7	70	11	8	1
8	30	3	8	1
9	30	11	8	1
10	50	7	8	1
11	50	3	16	1
12	50	11	16	1
13	50	7	12	1

Using Amano lipase PS-IM as biocatalyst no enantioselectivity was observed, therefore the data are not published. *Fig. 1* and *Fig. 2* represent the values of enantioselectivity in the presence of the two further enzymes after the first and 24 hours reaction time, respectively.

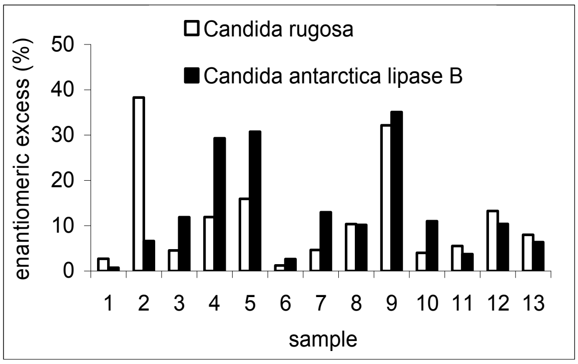

Figure 1: Values of enantioselectivity of the experimental design in the presence of *Candida rugosa* and *Candida antarctica* lipase B after 1 hour

Higher enantiomeric excess (e.e.) values were observed after 1 h reaction time than at the end of the reaction. There were parameter combinations where both enzymes showed reasonable selectivity almost as high as 40%. With time these high values began to reduce depending on the parameters. After 24 hours the selectivity values of *Candida rugosa* catalysed measure points reduced almost to 0%, but at three points: 4, 5 and 9 the CALB catalysed reactions kept their higher e.e. values of 14.74%, 18.95% and 22.09%, respectively.

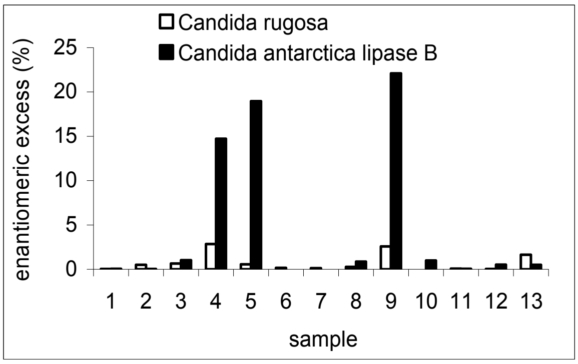

Figure 2: Values of enantioselectivity of the experimental design in the presence of *Candida rugosa* and *Candida antarctica* lipase B after the 24 hours

Determination of the optimal parameters for highest yield and enantioselectivity

Beyond selectivity a high ester yield is a requirement to obtain high amount of LLA, as well. Since the CALB was the suitable enzyme among the three investigated ones, *Fig. 3* shows conversion calculated for monomer LA and the e.e. values for the studied 13 points. Two of the earlier mentioned three best parameter combinations for enantioselectivity showed also high conversion rate. Conversion of 57% and 55% was measured for the points 5 and 9.

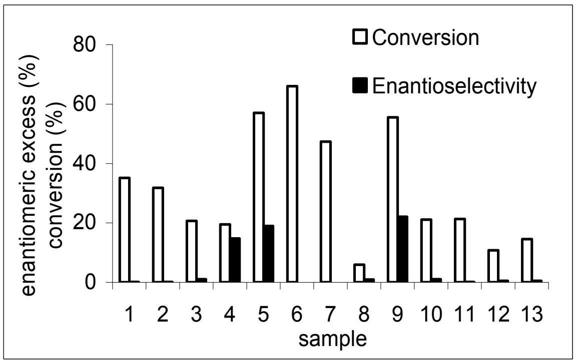

Figure 3: Enantioselectivity and conversion of CALB after 24 h

To obtain more information about the optimal parameters for the required results the experiments were widened with some more points. One of the parameters was changed and the values of other parameters remained constant. The fundamental parameters were 50 °C, 11 : 1 alcohol : LA molar ratio, 8 w/w% initial water content and 1 g IL. The effects of the variables, which generally correlate with the selectivity and conversion, are represented from *Fig. 4* to *Fig. 7.*

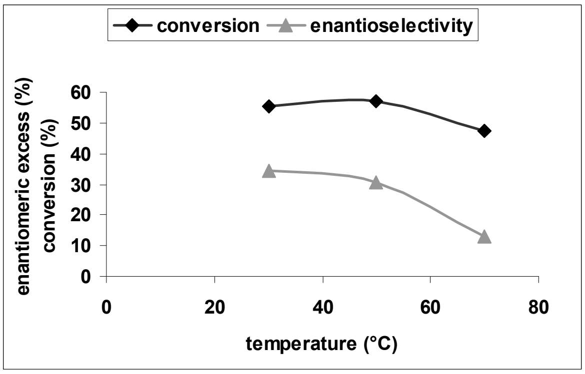

Figure 4: Effect of temperature on the enantioselectivity and conversion
(11 : 1, 8 w/w% initial water content, 1 g IL)

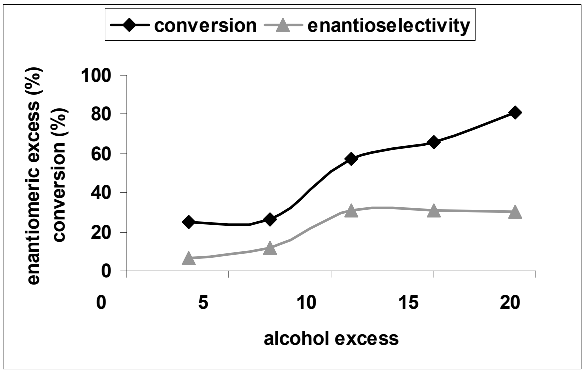

Figure 5: Effect of alcohol excess on the enantioselectivity and conversion
(50 °C, 8 w/w% initial water content, 1 g IL)

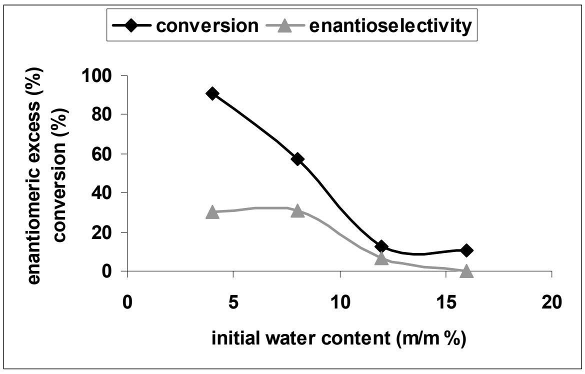

Figure 6: Effect of initial water content on the enantioselectivity and conversion (50 °C, 11 : 1, 1 g IL)

Lower temperature values and initial water content, higher alcohol excess and amount of IL were favourable. Taking these results into account the optimal reaction parameters could be determined: temperature of 30 °C, 11 : 1 alcohol : LA molar ratio, 8 w/w% initial water content and 1 g of IL.

The reason why enantiomeric excess decreases during the reaction is the CALB catalyses the esterification faster with LLA than with DLA and during the reaction the amount of DEL gets closer to LEL. To confirm our theory, pure LLA was applied for a parallel reaction with the above mentioned optimal parameters (30 °C, 11 : 1, 8 w/w% initial water content, 1 g IL). The initial reaction rates of the two reactions are $7.63\cdot10^{-2}$ mmol product/hour and $5.92\cdot10^{-2}$ mmol product/hour.

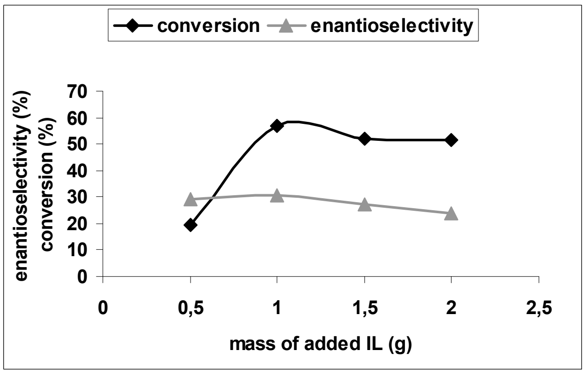

Figure 7: Effect of added IL on the enantioselectivity and conversion
(50 °C, 11 : 1, 8 w/w% initial water content)

Increasing the enantioselectivity of CALB

Ohara et al. [25] accomplished the lactic acid recycling after the selective polymerization. After the separation of not polymerized LLA it was reused and cleaned from PLA. Applying this method the produced ester was separated from other chemicals, hydrolysed and then the obtained lactic acid was recycled in our laboratory. The ratios of LLA and DLA in the further mixtures were set as they were observed after a 24 hours reaction. The reaction conditions were the same when the yield and the enantioselectivity were the highest: 50 °C, 11 : 1 alcohol : monomer LA molar ratio, 8 w/w% initial water content and 1 g of ionic liquid. The reactions were carried out with the same amount of initial LA (4 mmol) and with the e.e. values the previous reaction showed after 24 hours. The results can be seen in *Fig. 8* and *Fig. 9*. The first sample was prepared with rLA. After 24 hours enantiomeric excess of sample 1 reduced from 35% to 22%. The 2nd sample was prepared with the e.e. of 22% (3.3 mmol rLA and 0.9 mmol LLA). The initial e.e. of the 3rd sample was 51% (1.9 mmol rLA and 2.1 mmol LLA) and after one hour it was 73% and during the reaction it decreased to 65%. The last sample contained 1.4 mmol rLA and 2.7 mmol LLA, the selectivities were at the first hour and 24 hours 80% and 75%, respectively. In case of the yields the studied tendency could be observed, namely the more LLA the mixture contained the faster the reaction was.

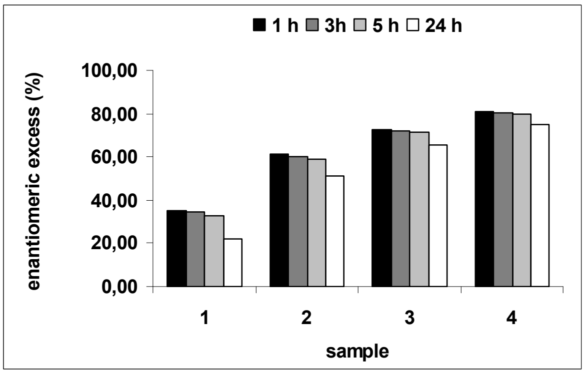

Figure 8: Enantiomeric excess values of each sample after recycling the LA

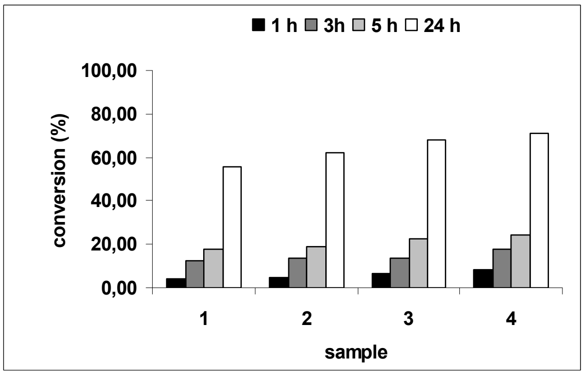

Figure 9: Conversion values of each sample after recycling the LA

Conclusion

Esterification of racemic lactic acid was investigated using three types of biocatalysts. First a suitable solvent, Cyphos 104 IL was selected so that it neither catalyses the reaction itself nor reduces the activity of the enzyme.

Then investigation of three types of enzymes was the next step, where CALB showed the highest enantioselectivity and yield by certain conditions.

Optimal reaction parameters were determined; which were temperature (30 °C), alcohol : monomer LA molar ratio (11 : 1), initial water content (8 w/w%) and the added ionic liquid to the mixture (1 g).

Finally, further investigations were carried out to increase selectivity by LA reusing. In these experiments LA was recycled to the start of the reaction three times. With this method e.e. value for the first hour increased from 35% to almost 80%.

NOMENCLATURE

PLA:	poly-lactic acid
PLLA:	poly-L-lactic acid
LA:	lactic acid
rLA:	racemic lactic acid
LLA:	(L)-lactic acid
DLA:	(D)-lactic acid
LEL:	(L)-ethyl-lactate
DEL:	(D)-ethyl-lactate
BuLLA:	butyl-(L)-lactate
BuDLA:	butyl-(D)-lactate
CALB:	*Candida antarctica* lipase B
IL:	ionic liquid
e.e.:	enantiomeric excess

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Nemzeti Fejlesztési Ügynökség

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