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ENZYMATIC ESTERIFICATION OF LACTIC ACID UNDER MICROWAVE CONDITIONS IN IONIC LIQUIDS

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Ethyl lactate is a natural flavouring compound and can be used as an environmentally friendly solvent, as well. Lactic acid production requires costly downstream processes, which increases the price of the products. One of the latest purification methods is the extraction of the lactic acid from the fermentation broth by phosphonium type ionic liquids. This method gives the possibility to synthetise lactates in the extracting agent avoiding an expensive separation process. Microwave heating is widely used in organic chemistry because it usually shortens the reaction time and enhances the reaction rate, but its effect on enzymatic esterification reactions in ionic liquid media was hardly investigated. For comparison of the ethyl lactate synthesis in different media two organic solvents and 20 ionic liquids were tested. Eight suitable media were found: toluene and 7 ionic liquids. The reaction conditions of the enzymatic synthesis were optimised in toluene and in Cyphos 104. Using toluene the highest yield (80%) was achieved in a reaction mixture consisting of 1 mmol lactic acid, 5 mmol ethanol and 4.5 w/w% initial water content diluted by organic solvent to 5 cm^3. The enzyme amount needed was 250 mg. In Cyphos 104 medium 0.8 mmol ionic liquid, 2 mmol lactic acid, 7 times ethanol excess, 2 w% initial water content and 25 mg immobilised *Candida antarctica* lipase B was enough to carry out the reaction up to 95% yield in 24 hour on 40 °C. The obtained yields and reaction parameters were compared using the two previous media and enzyme reusability tests were done. This experiment gave the result that smaller enzyme amount is enough in ionic liquid than in toluene and the enzyme stability is also much better in it. The synthesis was studied under microwave conditions as well, and the following effects were observed: The optimal initial water content was shifted from 3.7 w/w% to 3 w/w% but the same yield was achieved. Microwave heating accelerated the hydrolysis of lactoyllactic acid providing the mixture with fresh lactic acid and enhancing the reaction rate.

Keywords: ethyl lactate, Cyphos type ionic liquid, *Candida antarctica* lipase B, microwave

Introduction

In recent years, there is increasing demand on using renewable materials instead of petroleum-based feedstocks because of the rising crude oil prices and the increasing necessity reducing dependence on petroleum. An important bio renewable building block is lactic acid (LA) (2-hydroxypropionic acid), an α-hydroxy acid containing both a hydroxyl and carboxylic acid functional group, which results its wide application field [1].

LA is mainly consumed by the food industry as an additive or preservative, but it is also used as a pharmaceutical intermediate and as the basic compound of poly-lactic acid, a biodegradable polymer. Its esters are alternative "green" solvents to glycol ether. Additionally, ethyl lactate is a natural flavouring compound, so a valuable food and perfumery additive [2, 3].

Although LA can easily be produced either via fermentation or via a chemical route and has several applications, this potential can only be realised if the cost of production is competitive. The main problem is that fermentation-derived LA requires extensive and costly purification processes because it is not volatile. Several downstream processes have been developed such as reactive distillation, reactive extraction, electrodialysis, adsorption and esterification [4] and one of the newest methods is the extraction by phosphonium type ionic liquids (ILs), because they form complexes with LA, so they are proper extracting agents for them [5].

Enzymes are normally used in water. However, one of their most interesting properties is their ability to possess excellent catalytic activity in non-aqueous media (e.g. organic solvents, ILs or supercritical fluids) if they contain trace amounts of water [6]. A major reason for applying enzymes (e.g.: lipases) under such conditions is to avoid hydrolysis when performing non-hydrolytic transformations, such as esterification.

Since ILs can be perfect media for enzymatic reactions because of their negligible vapour pressure, reusability and enzyme stabilization effect [7-9] our first aim was to test if there is a possibility to produce lactates in the extracting agent avoiding an expensive separation process.

Traditionally organic syntheses are carried out using external heat source, although it is not a really efficient way of energy transport because its velocity depends on

the heat conductivity of the vessel and the reaction mixture. In contrast to conventional heating microwave is independent of these factors. The result is a localized heating by dipole rotation or ionic conduction, which are the two fundamental mechanisms for transferring energy from microwaves to the reaction mass being heated. Microwaves transfer energy in 10^{-9} s with each cycle of electromagnetic energy. The kinetic molecular relaxation from this energy is approximately 10^{-5} s. This means that the energy transfers faster than the molecules can relax, which results non-equilibrium conditions and a greater number of energetic collisions. This leads to enhancement in reaction rates and product yields [10].

Moreover using microwave conditions enhances the reaction rate not only in chemical but in enzymatic reactions [11-13]. Although both microwave and ILs present several advantages only one article describes an enzymatic acylation reaction using the two special conditions simultaneously [14]. So our second aim was to test the influence of the microwave energy on the enzymatic synthesis of ethyl lactate in ILs.

Experimental

Chemicals

Enzyme: Novozym 435 (immobilised *Candida antarctica* lipase B) was received from Novozymes, Denmark as a gift.

Ionic liquids: All the utilized ILs, trihexyl-tetradecyl-phosphonium-bis(2,4,4-trimethylpentyl)-phosphinate (Cyphos 104), trihexyl-tetradecyl-phosphonium-bromide (Cyphos 102), trihexyl-tetradecyl-phosphonium-dodecyl-benzene-sulfonate (Cyphos-202), trihexyl-tetradecyl-phosphonium-hexafluorophosphate (Cyphos 110), tetrabutyl-phosphonium-bromide (Cyphos 163), tetraoktyl phosphonium-bromide (Cyphos 166), ,triisobutyl-methyl-phosphonium-tosylate (Cyphos-106) were bought from IoLiTec GmbH, Germany

Other chemicals: Ethanol (absolute) and lactic acid (90 w/w% solution) were purchased from Spektrum 3D, Hungary. Toluene, acetonitrile and hexane were received from Scharlau, Spain.

Methods and instrumentation

To avoid the inhibition effect of the water concentrated LA solution (90 w/w%) was used as a substrate which resulted the presence of LA dimers in the reaction mixture [1]. By acid-base titration the accurate monomer concentration of the acid solution was determined and the yields were correlated to this amount. Its composition was: 53 w/w% LA, 26 w/w% lactoyllactic acid, 7 w/w% lactide and 14 w/w% water.

A typical reaction mixture in organic solvents contained LA, ethanol in equimolar amounts or an excess of the ethanol, 0.5–5.5 w/w% water and organic solvent to get a total volume of 5 cm^3. To this mixture 100–500 mg enzyme was added.

In a typical reaction using IL media the reaction mixture contained 2 mmol LA, 4–16 mmol ethanol, 0,3–1,3 mmol IL, 1–4 w/w% water and 25–100 mg enzyme.

Sample preparation: Samples from organic media needed no extra preparation. Using IL 50 µl samples were taken and they were extracted with 4*80 µl hexane before GC analysis. As a preparation for HPLC analysis the samples were diluted in 5 ml phosphate buffer (pH: 2.3, 6% acetonitrile content).

Instrumentation: The reactions using conventional heating were carried out in a GFL 3031 shaking incubator at 150 rpm and on 40 °C.

Tests under microwave conditions were performed in a commercial microwave equipment *(Fig. 1)* (Discover series, BenchMate model, CEM Corporation, USA) with a capacity of 4 ml. It was provided with magnetic stirrer and a non-contact infrared temperature sensor to monitore the temperature, which was kept constant (±1°C) by altering the microwave power. For the esterification reactions of LA maximal energy was 10 W to maintain 40 °C.

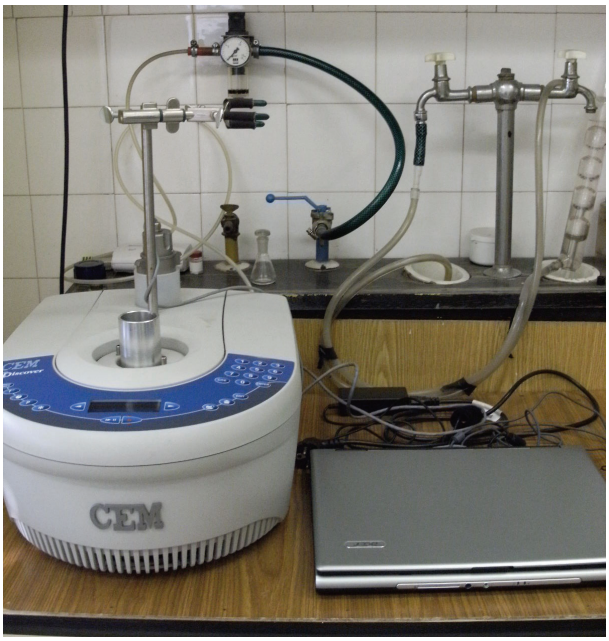

Figure 1: CEM Discover microwave equipment

Analytical methods: Water content of the substrates was measured by a Mettler Toledo DL31 type Karl Fisher titrator.

The samples were analysed by HP 5890 A gas chromatograph, with HP-FFAP column, and FID detector. To test the enantioselectivity of the reaction an FP LIPODEX E column was necessary.

The HPLC analyses were done by a MERCK type equipment with Zorbax SB-Aq 76 column, and L-7450 detector. The monitoring wave-length was 215 nm.

Results and discussion

Experiments using organic solvents

For comparison of the results in ILs reactions were carried out in organic solvents. According to the literature data [15-16] toluene and hexane are the most appropriate solvents for the enzymatic esterification of LA. As mentioned by Parida et al. [16] straight-chain 2-hydroxy acids are highly reactive in esterification reactions with 1-butanol using 5000 mg *Candida rugosa* enzyme/mmol LA, while according to From et al. [15] esterification of one mmol LA in hexane needs 10 mg immobilised *Candida antarctica* lipase B. In our experiments ethyl lactate was produced with high yield in toluene, while in hexane the conversion remained under 15%. The needed enzyme amount was quite high *(Fig. 2),* at least 250 mg immobilised *Candida antarctica* lipase B was necessary for a measurable conversion of one mmol LA.

Increasing the initial water content from 2.5 w/w% to 4.5 w/w% the yield was increased up to 80% using 250 mg enzyme. The best result was achieved at 1:5 LA-ethanol molar ratio.

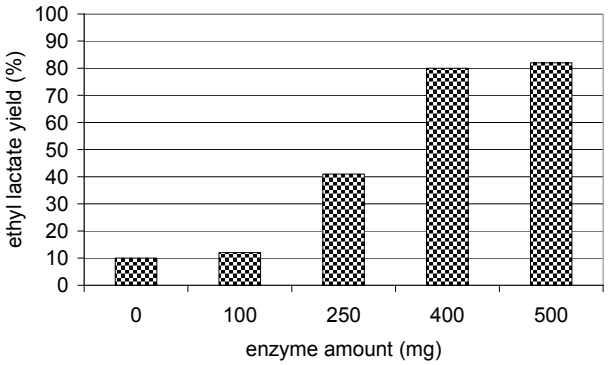

Figure 2: Ethyl lactate yield obtained after 24 h vs. enzyme amount used (LA 1 mmol, ethanol 5 mmol, initial water content 2.5 w/w% diluted with toluene to 5 cm^3)

Reusability of the enzyme was also tested in toluene where ethyl lactate yield was decreased completely after four cycles, which shows the fast deactivation of the enzyme.

Experiments using ILs under conventional heating

As a second step 20 different ILs were tested, but reaction could be carried out with considerable yield only in 7 media *(Table 1).*

These media could be divided into 3 groups. To the first one belonged Cyphos 104, where only the enzyme had catalytic effect on the reaction. There were some media (Cyphos 163, Cyphos 166, Cyphos 102, Cyphos 106 and Cyphos 110) where the reaction was catalysed by the IL as well, and similar ester yield was observed without enzyme. Finally, Cyphos 202 was situated between the two previous groups, because it slightly catalysed the reaction itself.

Table 1: Comparison of the ester yields in different ILs (40 °C, 25 mg enzyme, 3 w/w% initial water content)

Ionic liquid	Yield (%)	Catalyst
Cyphos 104	80	Enzyme
Cyphos 202	95	Enzyme + slightly IL
Cyphos 163	104	IL + slightly enzyme
Cyphos 166	90	IL + slightly enzyme
Cyphos 106	74	IL + slightly enzyme
Cyphos 102	60	IL + slightly enzyme
Cyphos 110	36	IL + slightly enzyme

All the listed ILs formed one phase system with the substrates and products, except Cyphos 110, which gave an emulsion. This two phase system was the reason for the obtained lowest product yield (36%). Increasing the reaction temperature the yield was growing, and this enhancement was the highest between 50 and 60 °C, where the reaction mixture became one phase.

The enantioselectivity of the reaction was tested as well, and Cyphos 104 was the only medium where a slight excess of ethyl L-lactate (e.e. 19%) was observed.

In the next step ethyl lactate production was optimized in Cyphos 104, because Marták et al. [5] it gave the best result as the extracting agent of LA. It was important to investigate the minimal amount of solvent necessary for the reaction. In the range from 200 mg (0.3 mmol) to 1000 mg (1.3 mmol) IL, the following effect was observed: Increasing of the amount of the IL to 600 mg the yield was increased extensively but its further addition had no influence on the ester yield. Based on these results for the further reactions 600 mg (0.8 mol) IL was used.

To investigate the effect of initial water content the LA was dehydrated using zeolite 3A, and different amounts of water were added to the reaction mixture. In the range from 1 to 2 w/w% water had positive effect on the enzyme activity providing the monomolecular water layer to the enzyme. More water shifted the thermodynamic equilibrium of the reaction towards hydrolysis.

The best LA : ethanol molar ratio was found at 1:7 unlike to toluene, where 1:5 was found optimal. The amount of immobilised enzyme was varied between 12.5–50 mg/mmol LA depending on the required reaction time, but using the smallest amount the reaction was completed in 24 hours.

The reusability of the enzyme was also tested and compared with the results in toluene *(Fig. 3).*

In this experiment reactions were carried out using the optimal parameters. After 24 hour reaction time and sample analysis the enzyme was filtrated, washed, dried and a new reaction was started with it. All the yields were correlated to the yield of the first cycle. It was found that in Cyphos 104 the ethyl lactate yield decreased only 20% after 6 cycles, while in toluene it dropped completely in four cycles.

From these experiments we can conclude: The reaction was carried out in an IL which can be used for the extraction of LA as well. In IL media smaller amount *Candida antarctica* lipase B was enough than in toluene, and the reusability of the enzyme was also much better.

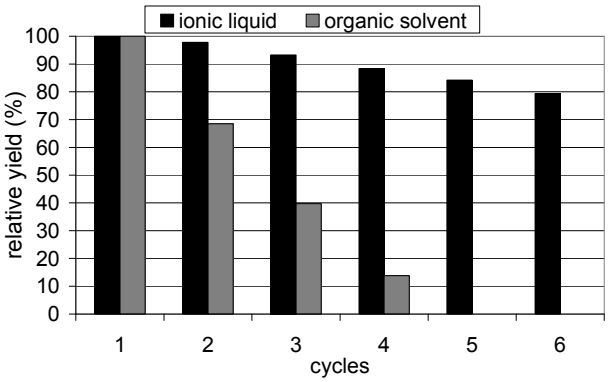

Figure 3: Reusability of the enzyme in toluene and Cyphos 104 IL

Experiments using ILs under microwave heating

Reactions were carried out under microwave heating in the 7 suitable IL media, but positive effect was observed only in four cases (Cyphos 202, 166, 163 and 102) where the reaction time decreased. In Cyphos 202 it was 7 h instead of 24h. In the other ILs the results did not change compared to conventional conditions, except Cyphos 104 where the yield decreased.

However, there are reports which describe that Novozym 435 weakly interacts with the microwave [12] and microwave can be used for example with imidasolium- and pyridinium-based ILs [14], control reactions were carried out to clarify our results in Cyphos 104. In our experiments different systems (IL, enzyme, IL with enzyme and IL with enzyme and ethanol) were irradiated by microwave energy for 2 hours. After this treatment, reactions were started with them in shaking incubator, and the obtained yields were compared *(Fig. 4)*.

The first column in Fig. 4 shows the control reaction carried out in shaking incubator without any previous incubation of the compounds. By the second column microwave irradiation had no effect on Cyphos 104, but after the incubation of the pure enzyme it reached only the 72% of the expected yield. Its reason was probably not the microwave, but the fact that enzymes are not really stable without a solvent, although they are immobilised. Using IL as a solvent for the enzyme the reaction was not successful, because the high viscosity hindered the mixing and local overheating caused denaturation of the enzyme. So this was the reason for the decreased yield under microwave conditions. After solving this problem by previously homogenised reaction mixture the reaction reached the same yield using microwave irradiation as in shaking incubator. To maintain the effect of microwave irradiation on *Candida antarctica* lipase B the viscosity of the IL was decreased by additional ethanol although it slightly damages the enzyme. The result was compared to the obtained yield in the same solution incubated in shaking incubator (control 2). By the fifth and sixth column of Fig. 4 they are equal, therefore microwave has no effect on immobilised *Candida antarctica* lipase B.

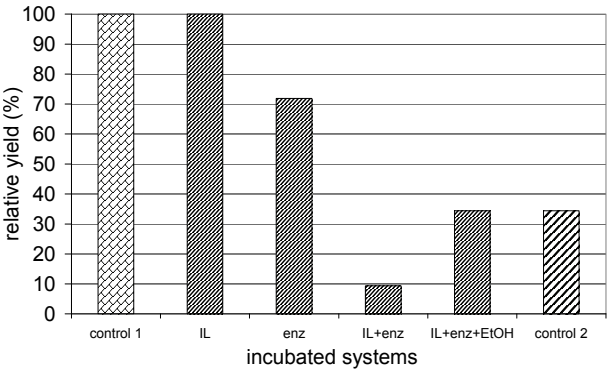

Figure 4: Enzyme and IL stability under microwave conditions

Further experiments were carried out in Cyphos 202, because it was the only media where the reaction was catalysed by enzyme and microwave had positive effect on it.

Since the influence of the initial water content is very important in esterification reactions [17] and the polar water molecules can influence the energy conduction under microwave conditions [13], the most significant parameter was the optimal initial water content. In our experiments with both methods (conventional and microwave heating) small (2 w/w%) initial water content decreased the yield dramatically (38% and 45% respectively). Under conventional conditions the highest ethyl lactate content was achieved at 3.7 w/w% while under microwave conditions at 3 w/w% initial water content with identical yield (105%).

As Table 1 and the experiments using microwave conditions show, in some cases ester yield exceeded the monomer LA content of the reaction mixture, which can be only possible if the dimers are able to decompose to monomers and form ethyl lactate. Engin et al. describes [18] neither temperature change, nor catalyst addition alters the dynamic equilibrium between LA, lactoyllactic acid and water, but in an esterification reaction the formation of water causes the hydrolysis of the dimer. They have found that lactoyllactic acid hydrolysis is a very slow reaction and may be a rate-limiting step in ethyl lactate formation. By our experiments an advantage of the microwave heating is that it accelerates the hydrolysis of the dimer *(Fig 5)*.

By the results of HPLC analysis presented in Fig. 5 not only the amount of LA but the amount of lactoyllactic acid decreased in the esterification reaction, while their ratio did not changed (about 47% LA, 43% lactoyllactic acid and 10% lactide). So the dimer can decompose fast enough, and the rate of hydrolysis is not a limiting step any longer. This effect results in faster reaction using microwave irradiation than under conventional conditions.

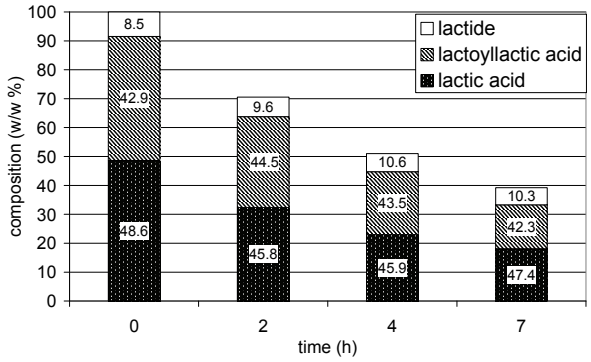

Figure 5: Composition of the LA solution in the reaction mixture vs. reaction time and the ratio of the compounds after certain reaction times (0.7 mmol Cyphos 202 IL, 2 mmol LA, 14 mmol ethanol, 3 w/w% initial water content)

Conclusion

For the esterification of LA different media were tested. The reaction was successful in toluene (yield 80%) and in 7 ILs. After the optimisation of the parameters and the comparison of the two media ILs were found better solvents because of the needed smaller enzyme amount (12.5 mg enzyme/mmol LA instead of 250 mg) and its enhanced reusability. In toluene the enzyme could be recycled only 3 times, while in Cyphos 104 the yield remained 80% after 6 cycles. It was determined that microwave heating harms neither *Candida antarctica* lipase B, nor Cyphos type ILs and it promotes the ethyl lactate production accelerating the hydrolysis of lactoyllactic acid. As a result the reaction time was shortened from 24 h to 7 h.

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