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SHORT COMMUNICATION

Unique role of water content in enzymatic synthesis of ethyl lactate using ionic liquid as solvent[‡]

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Enzymatic synthesis of ethyl lactate was studied in organic solvents and in ionic liquids to determine optimal media for the reaction, and to investigate the effect of water content on the ester yield. Experiments proved that Cyphos 202 ionic liquid is the best solvent affording the highest ethyl lactate yields. Furthermore, 20 times less enzyme sufficed to carry out the reaction in this ionic liquid compared to organic solvents; ionic liquid could be applied as solvent. Using water removal, the ester yield decreased since a side reaction, dimerization of lactic acid, occurred as well. In contrast to these results, without water removal, the produced water was partly consumed by the decomposition of lactoyllactic acid to free lactic acid, increasing thus the substrate concentration of the mixture and enhancing the ester yield.

Keywords: ionic liquid, esterification, enzyme, water removal, lactic acid

Water content has a particular role in ester synthesis from organic acids (e.g. lactic acid (LA)) and short alkyl alcohols (e.g. ethanol) using non-conventional media. Esterification is an equilibrium reaction, hence the maximal ester yield can be influenced either by using an excess of one of the substrates (usually alcohol), or by removing the product, ester, or the side-product, water. In case of enzymatic synthesis, water content has also another role, since a monomolecular water layer is necessary for the enzyme to maintain its native conformation (Yang & Pan, 2005). Even so, water removal can increase the ester yield effectively.

In our earlier works, the effectiveness of enzymatic synthesis of ethyl acetate (Bélafi-Bakó et al. 2003; Gubicza et al., 2008) and isoamyl acetate (Fehér et al., 2009) was successfully enhanced by water removal using pervaporation or zeolite adsorption, respectively. However, using LA as substrate, a new role of water content emerges. In lower water content, LA undergoes self esterification producing its open chain dimer, lactoyllactic acid (LA_2), and other oligomers (Vu et al., 2005). Therefore, the commercially available 90 % LA solution contains a significant amount of LA_2 beside the monomeric form. Furthermore, dimerization/decomposition of LA (Eq. (1)) has to be considered as a side reaction in the reaction mixture beside ethyl lactate (EL) synthesis (Eq. (2))

$$\mathrm{LA} + \mathrm{LA} \leftrightarrow \mathrm{LA}_2 + \mathrm{H_2O} \tag{1}$$

$$\mathrm{LA} + \mathrm{EtOH} \leftrightarrow \mathrm{EL} + \mathrm{H_2O} \tag{2}$$

The reverse reaction (decomposition of LA_2 to LA) is very slow as highlighted by Eugin et al. (2003). Therefore, it is the rate limiting step in the ester synthesis, especially at higher conversions. In our recent publication (Major et al., 2009), we have pointed out that microwave irradiation enhances the decomposition of LA_2 to LA, increasing thus the rate of ester synthesis and achieving higher yield than that calculated from the monomeric acid content of the substrate.

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On the other hand, removal of the side-product, water, during the esterification reaction may influence the monomer LA content, and may also affect the ester yield. To study this effect, pervaporation (Delgado et al., 2008) and zeolite adsorption (Roenne et al., 2005) have been investigated and they seem to be suitable techniques for water removal.

Our aim was to compare the ester yield in two different types of media (organic solvents, ionic liquids (ILs)) and to determine the complex effect of water content on the ethyl lactate production using the best solvent.

Immobilized *Candida antarctica* lipase B (Novozym®435) was a generous gift from Novo Nordisk, Denmark. All the used ILs (95 % purity), trihexyl-tetradecyl-phosphonium-bis(2,4,4-trimethylpentyl)-phosphinate (Cyphos 104), trihexyl-tetradecyl-phosphonium-bromide (Cyphos 102), trihexyl-tetradecyl-phosphonium-dodecylbenzene-sulfonate (Cyphos-202), trihexyl-tetradecyl-phosphonium-hexafluorophosphate (Cyphos 110), tetrabutyl-phosphonium-bromide (Cyphos 163), tetraoktyl-phosphonium-bromide (Cyphos 166), and triisobutyl-methyl-phosphonium-tosylate (Cyphos-106), were bought from IoLiTec GmbH, Germany. LA (90 mass % water solution), ethanol (absolute), hexane, and toluene were purchased from Spektrum 3D, Hungary. All other chemicals used were of analytical grade. Composition of the LA solution was determined both by acid-base titration and by HPLC (53 mass % LA, 26 mass % LA_2, 7 mass % other acids, 14 mass % water). Esterification yields were calculated on the basis of the monomer acid content.

Reactions without water removal were carried out in a GFL 3031 type shaking incubator (150 min^{-1}) at 40 ℃ for 24 h. Reactions using microwave conditions (without water removal) were performed in a commercial microwave equipment (Discover series, BenchMate model, CEM Corporation, USA). The reaction mixture was stirred using a magnetic stirrer, and a fibre-optic sensor was used to monitor the temperature which was set by varying the microwave power. For the esterification reactions of LA, 10 W energy was used to keep the reaction temperature at 40 ℃. The volume and composition of the reaction mixture were the same as under conventional conditions.

To study the effect of water content, an integrated system was developed applying a pervaporation membrane (active area: 12 cm^2) or zeolite adsorption (40 g) for water removal. Reactions were carried out in a bioreactor (volume of the reaction mixture of 50 cm^3) equipped with a magnetic stirrer (500 min^{-1}), thermostat ($T = 40$ ℃), and cooling condenser. Reaction time was 13 h. Reaction mixture was circulated between the reactor and the membrane module/adsorber column by a peristaltic pump (flow rate of 30 cm^3 min^{-1}). In case of membrane separation, permeate was collected under vacuum (pressure of 1 kPa) in a cold trap using dry ice/acetone mixture as the cooling agent.

Table 1. Comparison of EL yields using different media

Medium	Yield (%)^a	
	With enzyme	Without enzyme
Hexane	14	0
Toluene	82	1
Cyphos 104	80	2
Cyphos 202	95	2
Cyphos 163	104^b	102^b
Cyphos 166	90	87
Cyphos 106	74	72
Cyphos 102	60	59
Cyphos 110	36	32

a) After 24 h; *b*) calculated on basis of the monomer acid content.

The applied STANDARD type flat sheet hydrophilic membrane was purchased from Beroplan GmbH, Germany, and the 4 Å molecular sieve from Zeolyst International, USA.

The reaction was monitored using a HP 5890 A gas chromatograph equipped with a HP-FFAP column (Macherey-Nagel, Germany) (head pressure of 80 kPa) and a FID detector. Water content was measured using a Mettler DL 35 Karl-Fisher titrator. To analyze the acid content of the reaction mixtures, HPLC was applied using a MERC type equipment with a Zorbax SB-Aq 76 column, and a L-7450 detector, at the monitoring wavelength of 215 nm.

Reaction conditions using organic solvents were as follows: 0.2 mmol cm^{-3} LA, 1 mmol cm^{-3} ethanol, 4.5 mass % water in organic solvent; total volume: 5 cm^3; enzyme: 250 mg per mmol LA. Using ILs, the reaction conditions were the following: 1.3 mmol cm^{-3} LA, 9.3 mmol cm^{-3} ethanol, 3 mass % water in IL; total volume: 1.5 cm^3; enzyme: 12.5 mg per mmol LA.

From the reaction mixture, 50 μL samples were taken. In case of IL media, EL was extracted by hexane (4 × 80 μL) before the gas chromatography analysis. In case of organic solvents, no preparation was used. Before the HPLC analysis, the samples were diluted with a phosphate buffer (5 mL, 6 % acetonitrile content, pH 2.3). All reactions were carried out in triplicate, and the average error was less than 6 %.

Enzymatic EL synthesis was carried out in two different organic solvents (hexane and toluene) and in seven ILs (Table 1). The self-catalytic activity of Cyphos 104, Cyphos 202, and of the organic solvents was negligible since the final EL yield after 24 h of reaction time was lower than 2 %, without enzyme loading. The other 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 therefore resulting in the lowest ester yields of 14 % and 36 %, respectively, in 24 h of the reaction time.

According to these preliminary investigations, toluene and Cyphos 202 IL were found to be the most suitable media for enzymatic esterification affording the highest yields of esters (82 % and 95 %, respectively). After comparing the optimal parameters of the reaction in these two solvents, the following conclusions were drawn: i) in an IL medium, 20 times less enzyme sufficed to complete the reaction and to achieve the same EL yield than in toluene, since 250 mg per mmol LA of the enzyme were necessary in toluene compared to only 12.5 mg per mmol LA in IL; ii) Cyphos 202 and Cyphos 104 can be applied as solvents reducing the amount of an expensive component since the reaction can be carried out in a more concentrated solution (LA concentration was 0.2 mmol cm^{-3} in case of toluene and 1.3 mmol cm^{-3} in case of ILs). According to these results, Cyphos 202 was proved to be the best medium for EL production; further experiments were carried out using this IL.

To study the effect of water content, water removal was performed using two different methods, which resulted in different water removal rates. Pervaporation represents the slower process removing 0.26 mol dm^{-3} of water in 13 h and zeolite adsorption the faster process removing 0.61 mol dm^{-3} of water in the same time (water concentration data are referred to the reaction mixture). Using zeolite, the amount of adsorbed water had to be calculated from a preliminary experiment since the water content of the reaction mixture was influenced by the water removal and other factors (e.g. esterification reaction, dimerization of LA). So, to estimate the adsorption capacity of the zeolite, a mixture of ethanol and water ($\varphi_r = 95 : 5$) was dehydrated under the same conditions as those of the reaction mixtures.

Reactions were started at optimized conditions, and the obtained EL yields were compared (Fig. 1).

As can be seen from Fig. 1, in this case, water removal decreased the ester yield unlike the water removal aided by the ethyl acetate (Gubicza et al., 2008) and the isoamyl acetate synthesis (Fehér et al., 2009) using on-line water removal processes.

Fig. 2 shows that water content of the reaction mixture was successfully decreased by the two processes. However, using zeolite adsorption, the water content was higher than in case of pervaporation. These unique water content results are in accordance with our hypothesis of water removal influencing the LA monomer content of the reaction mixture by shifting equilibrium (Eq. (1)) towards dimerization, where water is formed, too.

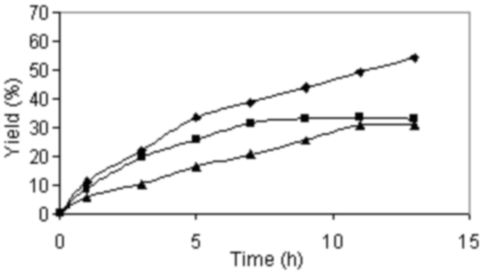

Fig. 1. Effect of water removal on ethyl lactate yield without water removal (♦), with pervaporation water removal (■), and with water removal by adsorption (zeolite) (▲) (solvent: Cyphos 202; $T = 40\,^{\circ}\text{C}$).

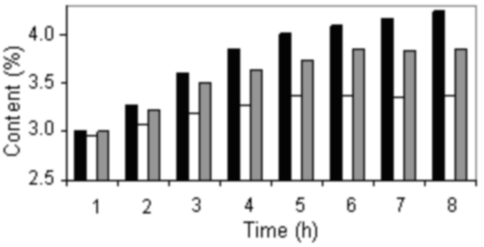

Fig. 2. Water content of the reaction mixtures without water removal (black columns), using pervaporation (white columns), and using zeolite adsorption (gray columns) (solvent: Cyphos 202; $T = 40\,^{\circ}\text{C}$).

Esterification reaction was also carried out using microwave irradiation instead of conventional heating. The mass balance of the reaction was calculated from the water content and yield data (Table 2). Data in Table 2 confirm two facts: i) without water control, the amount of produced ester exceeded the amount of produced water although the stoichiometry of the esterification reaction (Eq. (2)) predicts equal product formation. This is in accordance with literature data (Eugin et al., 2003) and confirms that the hydrolysis of LA$_2$ (Eq. (1)) proceeds at a low rate under the reaction conditions considered. Using microwave irradiation, this reaction is enhanced as it was described earlier (Major et al., 2009); ii) in contrast to these data, in both cases when water removal was used, the amount of produced water was higher than the amount of produced ester although the stoichiometry of the reaction (Eq. (2)) predicts equal production.

The explanation of this phenomenon is as follows: using water removal, the higher total water content proved that, beside the EL synthesis, dimerization of LA (Eq. (1)) occurred and water was formed resulting in higher water than ester production. The higher water removal rate was applied (by adsorption) the more LA was dimerized and formed, respectively. Thus, higher water production was observed in case of higher water removal rates (zeolite adsorption). Furthermore, since LA was consumed by this side-reaction, a lower EL yield was observed than without water removal.

To verify our hypothesis, acid concentration of the reaction mixture was analyzed by HPLC and the results for water adsorption by zeolite and ester production were compared (Fig. 3). Fig. 3 shows that the con-

Table 2. Amount of total produced water and EL after 13 h of reaction time

Reaction type	Concentration (mol dm^{-3})			
	Water			Ester
	In the reaction mixture	Removed	Total	Total
Microwave irradiation	1.00	0	1.00	1.20
Shaking flask reaction	0.65	0	0.65	0.70
Pervaporation	0.24	0.26	0.5	0.39
Zeolite adsorption	0.46	0.61^{a}	1.07	0.36

a) Estimated from the water control of the ethanol/water solution.

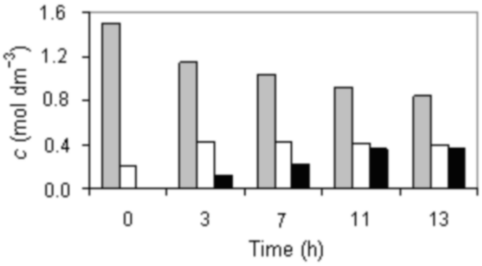

Fig. 3. Concentrations of lactic acids and ethyl lactate in the reaction mixture using water adsorption by zeolite: LA content (gray columns), LA_2 content (white columns), and ester content (black columns); solvent: Cyphos 202; $T = 40\,^{\circ}C$.

centration of LA decreases during the reaction, while the LA_2 content increases. After 13 h, 0.66 mol dm^{-3} of LA was reacted but only 0.36 mol dm^{-3} of ester was produced. The remaining part of LA was consumed by the dimerization reaction increasing the LA_2 content of the reaction mixture as it was predicted by the water content data (Table 2).

In conclusion, based on the experiments it was found that the water content has a characteristic role in the esterification of lactic acid with ethanol in an ionic liquid solvent. While the removal of water, formed in similar esterification reactions, e.g. synthesis of ethyl acetate (Gubicza et al., 2008) or isoamyl acetate (Fehér et al., 2009), enhanced the ester yield significantly; in case of the ethyl lactate synthesis, the effect was just the opposite. The presence of open chain dimer lactoyllactic acid (beside the lactic acid monomer) in the initial lactic acid and of water formed during the esterification facilitated the hydrolysis of dimers. It was proven that the removal of water decreased the ester yield in this reaction; therefore, water should not be removed in this particular case.

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