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Optimization of (*R*,*S*)-1-phenylethanol kinetic resolution over *Candida antarctica* lipase B in ionic liquids

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ABSTRACT

The kinetic resolution of racemates constitutes one major route to manufacture optically pure compounds. The enzymatic kinetic resolution of (*R*,*S*)-1-phenylethanol over *Candida antarctica* lipase B (CALB) by using vinyl acetate as the acyl donor in the acylation reaction was chosen as model reaction. A systematic screening and optimization of the reaction parameters, such as enzyme, ionic liquid and substrates concentrations with respect to the final product concentration, were performed. The enantioselectivity of immobilized CALB commercial preparation, Novozym 435, was assayed in several ionic liquids as reaction media. In particular, three different ionic liquids: (i) 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6], (ii) 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4] and (iii) 1-ethyl-3-methylimidazolium triflimide [emim][NTf_2] were tested. At 6.6% (w/w) of Novozym 435, dispersed in 9.520 M of [bmim][PF_6] at 313.15 K, using an equimolar ratio of vinyl acetate/(*R*,*S*)-1-phenylethanol after 3 h of bioconversion, the highest possible conversion (50%) was reached with enantiomeric excess for substrate higher than 99%.

1. Introduction

Chiral intermediates and fine chemicals are in high demand both from pharmaceuticals and agrochemical industries for the preparation of bulk drug substances and agricultural products. Therefore, the production of single enantiomers of chiral intermediates has become increasingly important in the pharmaceutical industry [1], for which safety is of utmost importance. This led to a need for scientists to develop a process to produce the optically pure compound with the desired biological activity [2]. The demand of enantiopure compounds is expected to dramatically increase [1,3], the pharmaceutical industry being the main contributor and driving force of this tendency. The biocatalysis has been heralded as superior over chemical synthesis to obtain enantiopure substances due to the high regio- and enantioselectivity. Despite the impressive new progress in asymmetric synthesis, the dominant production method to obtain a single enantiomer in industrial synthesis consists of kinetic resolution of racemates [4]. The success of this method is dependent on the fact that the two enantiomers react at different rates with a chirality entity, the biocatalyst.

Room-temperature ionic liquids (ILs), ion containing liquids, which combine good and tuneable solubility properties with no measurable vapour pressure and excellent thermal stabilities, have rapidly found a place of choice as valuable environmentally benign media substitutes for many volatile solvents [5–9]. With respect to enzymatic reactions carried out in conventional solvents, reactions in ILs exhibited several thermodynamic and kinetic behaviours, which led to improved process performance [10–14]. Surprisingly, it is only recently that attention has been focused on the application of ILs as reaction media for enantioselective processes [15–17]. The variations possible for tailor-made solvents may have a similar impact as the pioneering work of the use of enzymes in pure organic solvents [18]. Moreover, ILs showed an over-stabilization effect on biocatalysts [19] on the basis of the double role played by these neoteric solvents. First, ILs act as a solvent, providing an adequate microenvironment for the catalytic action of the enzyme (mass transfer phenomena and active catalytic conformation); second, ILs may be regarded as liquid immobilization supports, since multipoint enzyme–IL interactions (hydrogen, Van der Waals, ionic, etc.) may occur, resulting in a flexible supramolecular net able to maintain active the protein conformation [20]. However, many enzymes were rapidly inactivated in ILs [21].

The disadvantages of using ILs as media for enzymatic reactions are their high costs. The question about how "green" are ILs, also appears, since their synthesis involves toxic reagents. The toxicity effect on humans/environment is still not yet clear, so they are rather considered as harmful.

Chiral phenylethanol derivatives are important chiral building blocks for pharmaceuticals, agrochemicals and natural products. Novozym 435, a commercially available *Candida antarctica* lipase B

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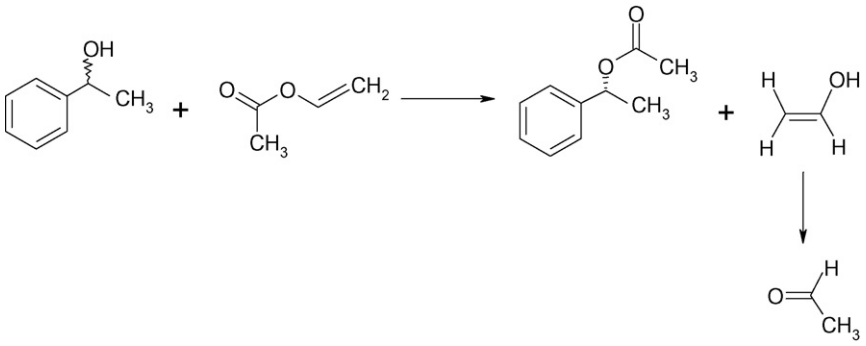

Fig. 1. Reaction scheme of kinetic resolution of racemic 1-phenylethanol.

(CALB) preparation has been shown to be an excellent chiral biocatalyst for the stereo-selective acylation of racemic alcohols [22] due to its very high kinetic resolution (*R*)-enantiomer yields and selectivity [2,23–25]. Moreover, it is reported to withstand a great variation in experimental conditions [26].

In the present work, the enantioselectivity of commercially available immobilized CALB (Novozym 435) for the kinetic resolution of racemic *sec*-alcohols was assayed in different ILs. In kinetic resolution of (*R*,*S*)-1-phenylethanol via transesterification, the acyl donor of choice was an enol ester such as vinyl acetate. The vinyl alcohol formed as a by-product undergoes keto-enol tautomerization to yield the corresponding carbonyl compound, the acetaldehyde (Fig. 1). Thus this transesterification pathway is much faster compared to reaction using free carboxylic acids or simple esters such as ethyl acetate [27]. Previous studies on immobilized-lipase mediated kinetic resolution of racemic *sec*-alcohol in ionic liquids dealt chiefly with enzyme free form assay and the influence of the IL on the biocatalyst activity [15–17,28]. In this research, we set out to investigate the enzyme and solvent concentrations and the substrates molar ratio conditions on the enantiomerically pure compound (*R*)-1-phenylethyl acetate formation via kinetic resolution of racemic 1-phenylethanol in three different ILs, such as (i) 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6], (ii) 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4] and (iii) 1-ethyl-3-methylimidazolium triflimide [emim][NTf_2]. Chemical structures of the assayed ILs are depicted in Fig. 2.

2. Materials and methods

2.1. Materials

(*R*,*S*)-1-Phenylethanol (≥98%), vinyl acetate (≥99%), 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [emim]-[NTf_2] (≥98%), 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6] (≥96%) and 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4] (≥97%) were provided from Fluka (Buchs, Switzerland). *n*-Heptane (>99%) was purchased from Merck (Darmstadt, Germany). (*R*)-1-phenylethanol and (*S*)-1-phenylethanol were obtained from Sigma (Schnelldorf, Germany). Decane (99+%) was supplied by Aldrich Chemical Co. (Deisenhofen, Germany).

The commercially available enzyme preparation, CALB—Novozym 435, a lipase with 1,3-positional specificity (EC 3.1.1.3) immobilized on a macroporous anion exchange resin, was kindly donated from Novozymes AS (Bagsvaerd, Denmark). The crude enzyme consisted of spherical micronic granules with a mean particle size of about 500 μm. The biocatalyst was used without any pre-treatment.

2.2. Analytical methods

The enantiomers content during the reaction time course was monitored by an HP 5890 series A gas chromatograph, equipped with a FID detector, using helium as carrier gas and a capillary column with a 20% content of permethylated β-cyclodextrin (β-DEX 120, length × I.D. 30 m × 0.25 mm with 0.25 μm film thickness, Supelco, Schnelldorf, Germany), at following temperature program: 373.15 K hold for 5 min, rise up to 393.15 K at rate of 5 K/min, hold for 12 min and last rise up to 473.15 K hold for 10 min; detector and injector temperatures were set at 523.15 and 493.15 K, respectively.

2.3. Procedure

Enantiomerically pure compound (*R*)-1-phenylethyl acetate production by enzymatic transesterification reaction of (*R*,*S*)-1-phenylethanol with vinyl acetate as acyl donor in the acylation reaction was performed. Experiments were carried out in a batch stirred tank reactor (BSTR), consisting of 100 mL round bottom flask, magnetically stirred and thermostated in a water bath, by means

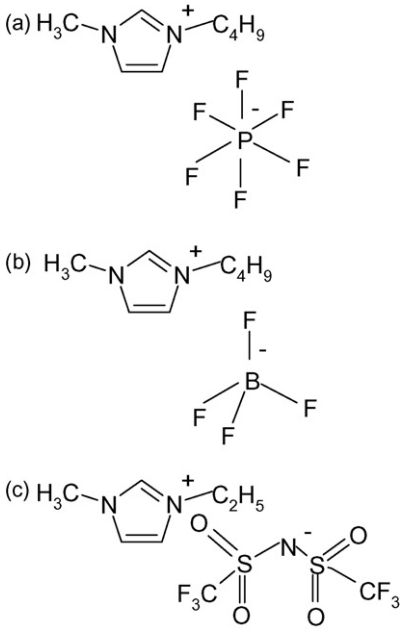

Fig. 2. Chemical structures of three tested ionic liquids: (a) 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6], (b) 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4] and (c) 1-ethyl-3-methylimidazolium triflimide [emim][NTf_2].

of a rotating heating mixing system (Rotamix 550 MMH, Tehtnica, Železniki, Slovenia). The reaction solution was continuously mixed to ensure a homogeneous dispersion of the enzyme particles in the reaction medium. The reaction started when the enzyme was added to the substrates and was carried out for at least 5 h. The experiments were carried out at temperature of 313.15 K and stirrer rate of 500 rpm, by modulating the operative variables. Aliquots of the sample were periodically withdrawn from the reaction vessel at fixed time intervals, suspended in decane (internal standard, IS) solution in *n*-heptane and analyzed by GC in order to establish the product formation profile. No reaction was detected in the absence of the enzyme. The enantiomers of the (*R*,*S*)-1-phenylethanol and of the product (*R*,*S*)-1-phenylethyl acetate were baseline separated in the GC-analysis. The conversion (*C*) was calculated by applying the equation, which is valid for irreversible reactions:

$$C = \frac{ee_R}{ee_R + ee_P} \times 100.$$

At least two experiments were run at each operative condition. The relative deviation was within ±1%.

3. Results and discussion

3.1. Effect of the enzyme concentration

The commercial immobilized form of CALB was assayed to catalyze the (*R*,*S*)-1-phenylethanol transesterification by using vinyl acetate as acyl donor. ILs based on the N,N′-dialkylimidazolium cation were chosen as model reaction media due to the wide spectrum of physico-chemical properties of this class [10–13]. Concerning the anions, bis(trifluoromethylsulfonyl)imide [NTf_2^-] has attracted particular interest, as it gives particularly thermally stable salts. In the research published to date, it has been regarded as one of the most suitable for biocatalysis. For this reason, firstly 1-ethyl-3-methylimidazolium-IL based on trifluoromethyl sulfonamide anion, [NTf_2^-], was assayed. CALB concentration was varied in the range 1.1–4.4% (w/w). At low CALB concentration a very low synthesis of (*R*)-1-phenylethyl acetate was observed. Above a Novozym 435 concentration of 4.4% (w/w), where after 6 h of reaction 43% conversion was observed, the reaction bulk appeared fully saturated by the immobilized enzyme preparation. For this reason, no further experiments were performed at higher CALB amounts. We expect higher resolution performances at higher biocatalyst contents. However, at higher enzyme concentrations the reaction could switch from being rate limited to mass transfer-limited [29].

3.2. Screening of several ionic liquids

Then, a comparison of reactions performances obtained in [emim][NTf_2] with those obtained in ILs based on dialkylimidazolium cations associated with mononuclear anions, such as [BF_4^-] and [PF_6^-], was proposed. All the assayed ILs, [bmim][PF_6], [bmim][BF_4] and [emim][NTf_2], proved to be adequate media for Novozym 435-mediated transesterification of *rac*-1-phenylethanol. For all the three tested ILs, excellent enantioselectivity was observed. The straightforward interpretation of these successful results could be due to the exceptional solvent power, which results in an enhancement of mass transfer, exhibited by the three assayed ILs. Both the reactants and products exhibited to be soluble in the three assayed ILs, producing a single homogenous phase. Previous studies [30] reported that low hydrogen-bond basicity minimizes interference with the internal hydrogen bonds of the enzyme. However, due to their different properties [19] with regard to polarity, hydrophobicity and solvent miscibility behaviour through the combination of the different cation (i.e. 1-alkyl-3-methylimidazolium) and anion (i.e. [BF_4^-]; [PF_6^-]; [NTf_2^-]) ILs exhibited different performances as reaction media for (*R*,*S*)-1-phenylethanol kinetic resolution. The immobilized CALB showed good-to-excellent enantioselectivity with a decrease in polarity, whilst hydrophobicity and viscosity increase of the ILs, the best results being obtained with [bmim][PF_6]. Under the tested conditions, after 5 h of bioreaction in [bmim][PF_6] by using an enzyme concentration of 2.2% (w/w), a conversion of (*R*)-1-phenylethanol into its esterified form of 44% and an enantiomeric excess for reactants (ee_R) of 77% were registered. The (*R*)-1-phenylethanol conversion profile with time in [bmim][PF_6] was clearly higher than in [bmim][BF_4]. The kinetic resolution of *rac*-1-phenylethanol in [emim][NTf_2] was the less successful: conversions and enantioselectivity are lower than in [bmim][PF_6] and [bmim][BF_4], which might be due to a protein's conformational change, occurred in presence of [emim][NTf_2]: the inclusion of the biocatalyst in the IL matrix likely provide the protein with a conformation less suited to enantioselective action of CALB.

3.3. Effect of the IL concentration

IL concentration likely affects the reaction performance by changing the rate constant and the reactants solubility. To ascertain the influence of these enzymatic neoteric solvents media, the IL concentration effect was studied. The influence of IL concentration on the immobilized CALB enantioselectivity for (*R*,*S*)-1-phenylethanol kinetic resolution was ascertained to be a determining factor for the reaction performance. The (*R*)-1-phenylethyl acetate yield and the enantiomeric excess for reactants for three different [bmim][PF_6] concentrations, 9.520, 7.578 and 6.704 M, at 313.15 K, 500 rpm, dissolving an equimolar vinyl acetate/*rac*-1-phenylethanol solution, showed that by increasing the [bmim][PF_6] concentration, the conversion was enhanced. When the IL concentration of 9.520 M was used, the highest conversion of the enantiomeric (*R*)-1-phenylethanol into its esterified form increased up to 47%. The synthetic product (*R*)-1-phenylethyl acetate was obtained with an ee_R of 89, while (*S*)-ester product was never detected.

A series of experiments at the highest [bmim][PF_6] concentration (9.520 M) was performed by increasing the biocatalyst concentrations, assaying immobilized CALB concentration in the range 1.65–6.6% (w/w) (Fig. 3). The excellent stereo-selectivity of this enzyme was clearly demonstrated by the rapidly reached complete conversion of (*R*)-1-phenylethanol into the enantiopure (*R*)-1-phenylethyl acetate, whilst (*S*)-product was never observed. After only 3 h of bioconversion, approximately 50% yield in (*R*)-1-phenylethyl acetate was attained at the highest IL and enzyme concentrations. By halving the CALB concentration a lower reaction rate and yield in the desired enantiopure compound was observed: at CALB concentration of 1.65% (w/w) an enantiomeric excess for reactants (ee_R) of 91 was achieved after 5 h of bioconversion. Above a Novozym 435 concentration of 6.6% (w/w) the reaction bulk appeared fully saturated by the immobilized enzyme preparation. For this reason, no further experiments were performed at higher enzyme amounts.

3.4. Effect of the substrates molar ratio

Acyl donor/alcohol molar ratio is one of the most important parameters in enzymatic transesterification. In this reaction, a covalently linked acyl-enzyme intermediate is formed and the nucleophile attack by water results in ester hydrolysis, although the presence of another nucleophile, namely *rac*-1-phenylethanol, might involve the formation of the transesterification product. This latter synthetic pathway may be regarded as a kinetically con-

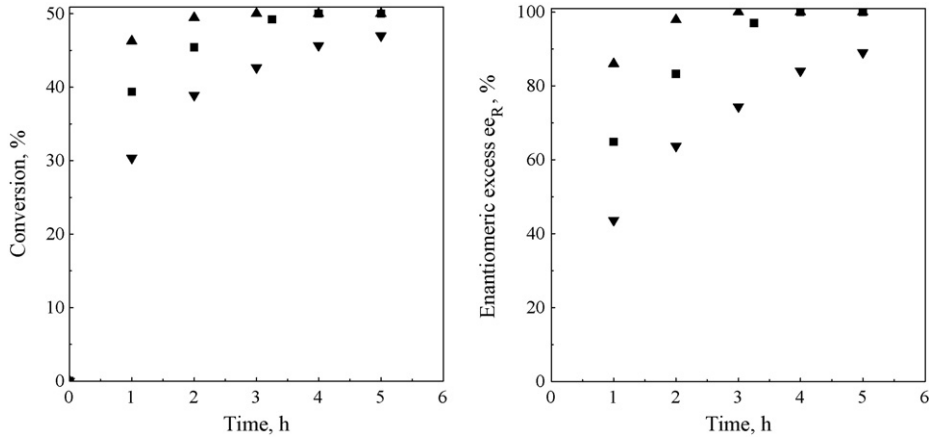

Fig. 3. Enzymatic kinetic (*R*,*S*)-1-phenylethanol resolution over immobilized CALB at (▲) 6.6% (w/w), (■) 3.3% (w/w), (▼) 1.65% (w/w), dispersed in 9.520 M of [bmim][PF_6] at 313.15 K, 500 rpm, using an equimolar ratio of vinyl acetate/(*R*,*S*)-1-phenylethanol. The volume of the reaction mixture was 1.7 mL.

trolled process, where the rapid accumulation of the acyl-enzyme intermediate and the preferential nucleophilic attack by the alcohol are essential. The first condition is enhanced by the use of activated acid acyl donors such as vinyl esters, since the vinyl alcohol released in the degradation of the vinyl ester tautomerizes to acetaldehyde, which cannot act as substrate for the enzyme [31]. Enol esters, such as vinyl and isopropenyl esters, are the most widely used irreversible donors [32]. The second condition may arise from using reaction media with very low water content (lower than 0.05%) with high nucleophile concentration. Since the reaction is reversible, an increase in the acyl donor concentration should result in higher product yields and shift the chemical equilibrium towards the synthesis. However, high substrates concentration may slow down the reaction rates due to inhibition phenomenon occurring. Therefore, in the present study the nucleophile concentration excess for the (*R*)-1-phenylethyl acetate formation was optimized. The effect of substrate vinyl acetate/(*R*,*S*)-1-phenylethanol molar ratio on the final conversion was studied at acyl donor/alcohol molar ratios of 1/2, 1/l and 2/l, under the following reaction conditions: 3.3% (w/w) of biocatalyst, 313.15 K and 500 rpm. Rising the acyl donor molar concentration with respect to the alcohol concentration a higher yield in the desired enantiopure compound was achieved. The highest activity in term of enantioselectivity by using an acyl donor/alcohol molar ratio of 2/1 was shown by the chiral catalyst. Under the tested conditions, after 3 h of bioconversion a complete conversion of (*R*)-1-phenylethanol into the enantiopure (*R*)-1-phenylethyl acetate was attained and an enantiomeric excess for reactants (ee_R) higher than 99 was registered.

4. Conclusions

Performing biocatalytic conversions in ILs can be beneficial with regard to activity, (enantio)selectivity and stability [33]. In the present work the kinetic resolution of racemic 1-phenylethanol over immobilized CALB as the chiral catalyst and vinyl acetate as the acyl donor was performed, screening several ILs as reaction media. ILs resulted excellent reaction media for enzyme-catalyzed resolution of racemic mixtures, exhibiting very widely tuneable properties with regard to polarity, hydrophobicity and solvent miscibility behaviour. This study has highlighted that by increasing the amount of the chiral catalyst, the suitable solvent and the acyl donor is a successful approach. The results presented here clearly demonstrated the potential of ILs for enzymatic biotransformations. Further tests are being conducted to investigate the enzymatic resolution of 1-phenylethanol racemates in ionic liquid/supercritical carbon dioxide biphasic systems in order to carry out an integral green biocatalytic processes.

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References

[1] R.N. Patel, Enzyme Microb. Technol. 31 (2002) 804–826.
[2] A. Overmeyer, S. Schrader-Lippelt, V. Kasche, G. Brunner, Biotechnol. Lett. 21 (1999) 65–69.
[3] B. Schulze, M.G. Wubbolts, Curr. Opin. Biotechnol. 10 (1999) 609–615.
[4] J. Carnell, J. Mol. Catal. B: Enzyme 19 (2002) 83–92.
[5] R. Madeira Lau, F. van Rantwijk, K.R. Seddon, R.A. Sheldon, Org. Lett. 2 (2000) 4189–4191.
[6] P. Wasserscheid, W. Keim, Angew. Chem. Int. Ed. 39 (2000) 3772–3789.
[7] T. Welton, Chem. Rev. 99 (1999) 2071–2083.
[8] L.A. Blanchard, D. Hancu, E.J. Beckman, J.F. Brennecke, Nature 399 (1999) 28–29.
[9] M.J. Earle, K.R. Seddon, Pure Appl. Chem. 72 (2000) 1391–1398.
[10] M. Eckstein, M. Sesing, U. Kragl, P. Adlercreutz, Biotechnol. Lett. 24 (2002) 867–872.
[11] M. Eckstein, P. Wasserscheid, U. Kragl, Biotechnol. Lett. 24 (2002) 763–767.
[12] P. Lozano, T. De Diego, D. Carrié, M. Vaultier, J.L. Iborra, Biotechnol. Prog. 19 (2003) 380–382.
[13] M. Persson, U.T. Bornscheuer, J. Mol. Catal. B: Enzyme 22 (2003) 21–27.
[14] C. Baudequin, J. Baudoux, J. Levillain, D. Cahard, A.C. Gaumont, J.C. Plaquevent, Tetrahedron: Asymm. 14 (2003) 3081–3093.
[15] S.H. Schöfer, N. Kaftzik, P. Wasserscheid, U. Kragl, Chem. Commun. (2001) 425–426.
[16] R. Sheldon, Chem. Commun. (2001) 2399–2407.
[17] U. Kragl, N. Kaftzik, S.H. Schöfer, M. Eckstein, P. Wasserscheid, C. Hilgers, Chim. Oggi-Chem. Today 19 (2001) 22–24.
[18] A.M. Klibanov, CHEMTECH 16 (1986) 354–359.
[19] P. Lozano, T. de Diego, J.-P. Guegan, M. Vaultier, J.L. Iborra, Biotechnol. Bioeng. 75 (2001) 563–569.
[20] T. de Diego, P. Lozano, S. Gmough, M. Vaultier, J.L. Iborra, Biomacromolecules 6 (2005) 1457–1464.
[21] S. Park, R.J. Kazlauskas, Curr. Opin. Biotechnol. 14 (2003) 432–437.
[22] J. Ottosson, K. Hult, J. Mol. Catal. B: Enzyme 11 (2001) 1025–1028.
[23] M.C. Almeida, R. Ruivo, C. Maia, L. Freire, T.C. de Sampaio, S. Barreiros, Enzyme Microb. Technol. 22 (1998) 494–499.
[24] G. Madras, R. Kumar, J. Modak, Ind. Eng. Chem. Res. 43 (2004) 7697–7701.
[25] T. Matsuda, K. Watanabe, T. Harada, K. Nakamura, Y. Arita, Y. Misumi, S. Ichikawa, T. Ikariya, Chem. Commun. (2004) 2286–2287.
[26] E.M. Anderson, M. Karin, O. Kirk, Biocatal. Biotransfor. 16 (1998) 181–204.
[27] A. Ghanem, H.Y. Aboul-Enein, Tetrahedron: Asymm. 15 (2004) 3331–3351.
[28] P. Lozano, T. de Diego, D. Carrié, M. Vaultier, J.L. Iborra, Chem. Commun. (2002) 692–693.
[29] M. Erbeldinger, A.J. Mesiano, A.J. Russell, Biotechnol. Prog. 16 (2000) 1129–1131.

[30] J.L. Kaar, A.M. Jesionowski, J.A. Berberich, R. Moulton, A.J. Russell, J. Am. Chem. Soc. 125 (2003) 4125–4131.
[31] P. Lozano, M. Daz, T. de Diego, J.L. Iborra, Biotechnol. Bioeng. 82 (2003) 352–358.
[32] M. Bakker, A.S. Spruijt, F. Van Rantwijk, R.A. Sheldon, Tetrahedron: Asymm. 11 (2000) 1801–1808.
[33] R.A. Sheldon, R.M. Lau, M.J. Sorgedrager, F. van Rantwijk, K.R. Seddon, Green Chem. 4 (2002) 147–151.