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Bioorganic & Medicinal Chemistry Letters 17 (2007) 921–924
Bioorganic & Medicinal Chemistry Letters

Kinetic resolution of (±)-1-phenylethanol in [Bmim][PF_6] using high activity preparations of lipases

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Received 20 September 2006; revised 16 November 2006; accepted 18 November 2006
Available online 22 November 2006

Abstract—Lipases from two different sources *Candida rugosa* (CRL) and *Burkholderia cepacia* (BCL) were formulated as enzyme precipitated and rinsed with organic solvents, organic solvent rinsed enzyme preparation, cross-linked enzyme aggregates (CLEAs) and protein coated micro-crystals (PCMCs). These various enzyme formulates were evaluated for the kinetic resolution of (±)-1-phenylethanol in ionic liquid [Bmim][PF_6] by transesterification with vinyl acetate. Of all the enzyme forms evaluated EPRP and PCMC in the case of CRL showed the best results with 26 % (*E* value = 153) and 53% (*E* value = 79) conversion, respectively, at 35 °C in 24 h. Carrying out this conversion with PCMC at lower temperature of 25 °C further improved the *E* value to 453 (with 44% conversion in 12 h). For BCL the acetone-rinsed enzyme preparation (AREP), CLEA and PCMC performed equally well with % conversion of 50 and 99 ee_p (%) (*E* value >1000) in just 2 h, whereas, the free lipase gave only 8% conversion.

The ionic liquids (ILs) are fast emerging as a useful reaction medium for enantioselective biocatalysis.[1] In many cases, especially with lipase-catalyzed transesterification reactions; higher reaction rates and greater enantioselectivity have been reported as compared to the corresponding performances in anhydrous organic media.[2] Most such studies have been carried out with pH-tuned lyophilized enzyme powders, either in free or immobilized forms.[2,3] In the last few years, it has become clear that structural changes during lyophilization are largely responsible for relatively low catalytic rates shown by enzymes in nearly anhydrous organic solvents.[4] Consequently, many enzyme formulations which 'dry' enzymes for use in such media have been described. Such enzyme forms include propanol-rinsed enzyme preparations (PREP),[5] cross-linked enzyme aggregates (CLEAs)[6] and protein coated micro-crystals (PCMCs).[7] PREP refers to propanol-rinsed immobilized enzymes, the corresponding free enzyme preparations rinsed with propanol (EPRP) also give higher reaction rates than pH-tuned lyophilized powders in low water-containing organic solvents.[8] Studies with such preparations in ILs have begin to be described.[1a] The present work shows that these enzyme preparations, which have proven efficiency in organic solvents, work well in a water-immiscible IL, 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]). The reaction chosen is the kinetic resolution of (±)-1-phenylethanol (1) by transesterification with vinyl acetate (2) (Scheme 1); this reaction has been often chosen for evaluating the reaction rates and enantioselectivity with various lipases.[2,3] The *Candida rugosa* lipase (CRL) is reported to give worst result in [Bmim][PF_6][3b] (for kinetic resolution of (±)-1-phenylethanol). The lipase from *Burkholderia cepacia* (BCL) has been found to give very poor reaction rates in [Bmim][PF_6] for transesterification of (±)-1-phenylethanol.[2b,c] Hence these two enzymes were converted into above-mentioned formulations and these enzyme forms were evaluated for their comparative performance in the ionic liquid.

The alcoholysis between (±)-1-phenylethanol (1 mmol) and vinyl acetate (1 mmol) was carried out in 1 ml ionic liquid (Acros Organics, USA; the purity of [Bmim][PF_6] as specified by the vendor was 99.6% (by HPLC) with water content of 0.05% v/v by Karl-Fischer) using different preparations of CRL/BCL (containing 10 mg of lipases) at a fixed temperature as mentioned in the legend with constant shaking at 250 rpm. CRL (lipase AYS 'Amano') and BCL (lipase PS 'Amano') were a kind gift from Amano, Nagoya, Japan. For analysis, 50 μl of the reaction mixture was extracted with 500 μl *n*-hexane/

Keywords: Cross-linked enzyme aggregates; Enantioselectivity; Enzyme precipitated and rinsed with propanol; Ionic liquids; Lipases; Propanol-rinsed enzyme preparation; Protein coated microcrystals.
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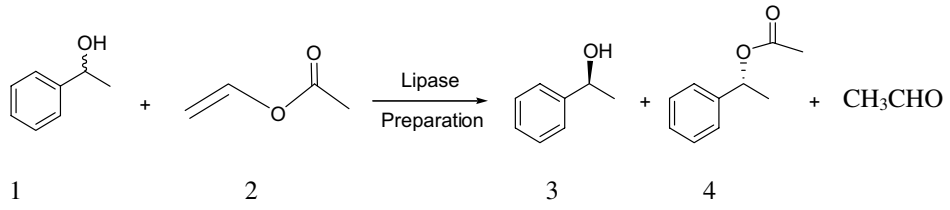
doi:10.1016/j.bmcl.2006.11.057



Scheme 1.

propan-2-ol (97.5:2.5) mixture. The extract was analyzed by HPLC using Chiracel OD-RH column (Diacel, Japan). The eluent consisted of 96.5% (v/v) *n*-hexane, 3% (v/v) propan-2-ol and 0.5% (v/v) ethanol with a flow rate of 1 ml min^{-1} and detection was carried out with a UV detector at 254 nm.[3b]

Figure 1 shows the steps involved in the various preparations of biocatalysts. All these preparations involve simple protocol and each involves either precipitation or washing with organic solvent for drying. All these preparations essentially avoid drying by lyophilization, which is now known to affect the secondary structure of proteins/enzymes.[4] Such changes are reversible when lyophilized enzymes are used in the conventional water-rich media, that is, aqueous buffers. However, in low water media such as nearly anhydrous media, these structural changes are not reversed and are an important factor for low enzyme activities.[4,5]

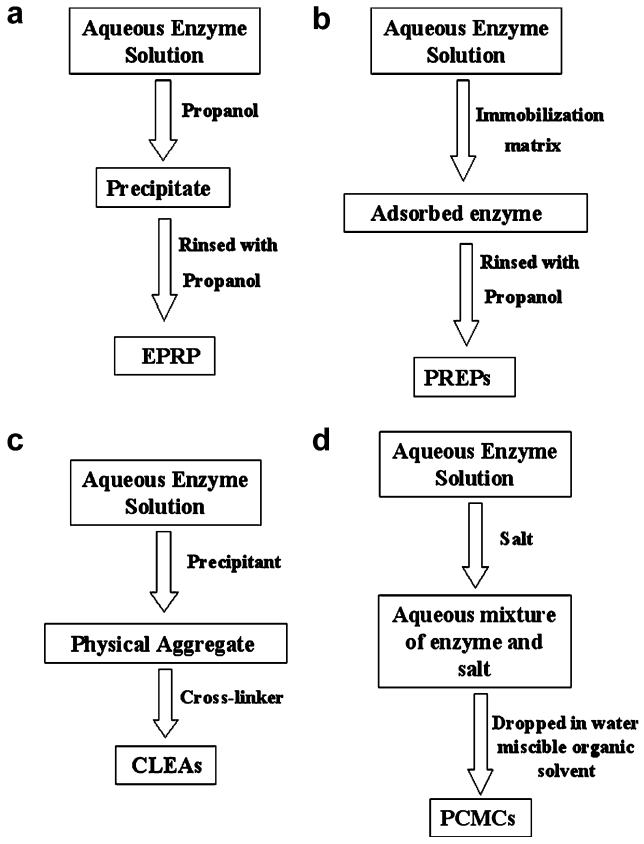


Figure 1. General protocol for the preparation of EPRP (a); PREPs (b); CLEAs (c); PCMCs (d).

It was seen that EPRP[9] (of CRL) did perform much better than pH-tuned lyophilized powder[10] (entries 1–4, Table 1). Longer time period of 24 h (entry 4, Table 1) resulted in only marginal increase in ee_p (%) but substantially improved *E* (*E* value was calculated by using Chen's equation).[11] The PREP[12] (of CRL) gave higher conversion than EPRP but poorer ee_p (%) and *E* value (entries 5–6, Table 1). CLEAs[13] prepared from free CRL did not show any dramatic improvement in % conversion (entries 7–8, Table 1). The % conversion was better than free enzyme but worse than both EPRP and PREP. Table 1 shows that ee_p (%) was higher than both pH-tuned lyophilized enzyme and PREP but lower than EPRP. PCMC[14] gave highest % conversion (entries 9–10, Table 1); ee_p (%) was quite good while ee_s (%) was highest of all enzyme preparations. The *E* value, however, was lower than that obtained with EPRP. Lowering of temperature reduces reaction rates but is often associated with improved enantioselectivity in organic solvents.[15] On the other hand, in the case of ILs, no significant difference in *E* with lowering of temperature has been reported.[16] Table 1 (entries 11–12) in the present instance shows that lowering of temperature to 25 °C further improved enantioselectivity of PCMC. The ideal time period was 12 h with 44% conversion, 99 ee_p (%) and *E* value of 453.

It is noteworthy that while PCMCs have shown enhanced catalytic activity in organic solvents, no change in enantioselectivity (*E*) was observed by others who compared the PCMC (prepared from 10 different lipases) with respective free enzymes for kinetic resolution of (±)-1-phenylethanol.[7]

In the case of BCL, the precipitation with propanol resulted in substantial loss of activity (up to 60%) as evaluated by hydrolysis of *p*-nitrophenylpalmitate.[17] However, substitution of propanol with acetone gave full recovery. Hence for BCL, enzyme precipitated and rinsed with acetone (EPRA) and acetone-rinsed enzyme preparation (AREP) were the correct formulations. For preparation of PCMC as well (of BCL), acetone was used as the solvent for precipitation.

The relative catalytic performances of EPRA, AREP, CLEA and PCMC of BCL were evaluated by a standard transesterification assay in [Bmim][PF_6].[1b] The initial rates obtained during the assay (Table 2) showed that AREP was the best catalyst followed by PCMC > CLEA > EPRA > pH tuned.

After this, these preparations were studied for kinetic resolution of (±)-1-phenylethanol. Again, the perfor-

Table 1. Kinetic resolution of 1-phenylethanol in [Bmim][PF_6] catalyzed by different preparations of *Candida rugosa* lipase

Entry	Lipase preparations	Time (h)	Conversion[a] (%)	ee_p[a] (%)	ee_s[a] (%)	E[b]
1	pH tuned	12	5	80	5	11
2	pH tuned	24	7	83	6	12
3	EPRP	12	22	97	28	123
4	EPRP	24	26	98	34	153
5	PREP	12	49	80	78	21
6	PREP	24	51	79	82	22
7	CLEA	12	10	91	10	30
8	CLEA	24	12	95	12	69
9	PCMC	12	51	87	93	53
10	PCMC	24	53	88	99	79
11	PCMC	6	34	99	51	283
12	PCMC	12	44	99	77	453

All reactions corresponding to entries 1–10 were carried, at 35 °C, whereas reactions corresponding to entries 11 and 12 were carried out at 25 °C.
[a] Conversion and ee's (%) were estimated by HPLC.
[b] $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$, where $c = ee_s/(ee_s + ee_p)$.

Table 2. Initial transesterification rates exhibited by different preparations of *Burkholderia cepacia* lipase in [Bmim][PF_6]

S. No.	Lipase preparations	Initial rates[a] (μmol min^{-1} mg^{-1})
1	pH tuned	8
2	EPRA	68
3	AREP	168
4	CLEA	100
5	PCMC	120

[a] Reaction conditions: ethylbutanoate (60 mM), 1-butanol (120 mM), different lipase preparations (prepared from 10 mg lipase) in [Bmim][PF_6] (0.5 ml) at 40 °C with constant shaking at 200 rpm. The substrate and product were extracted by hexane and hexane phase was analyzed by Agilent 6890 N GC with a flame ionization detector using Equity™ -5 fused silica capillary column (Supelco, USA).[6b]

Table 3. Kinetic resolution of 1-phenylethanol in [Bmim][PF_6] catalyzed by different preparations of *Burkholderia cepacia* lipase at 25 °C

Entry	Lipase preparations	Time (h)	Conversion (%)	ee_p (%)	ee_s (%)	E
1	pH tuned	1	5	99	5	114
2	pH tuned	2	8	99	8	187
3	EPRA	1	31	99	45	314
4	EPRA	2	49	99	96	736
5	AREP	1	44	99	78	415
6	AREP	2	50	99	99	>1000
7	CLEA	1	41	99	69	432
8	CLEA	2	50	99	99	>1000
9	PCMC	1	40	99	66	405
10	PCMC	2	50	99	99	>1000

mance of all the four preparations (EPRA, AREP, CLEA and PCMC) in case of BCL exceeded well beyond the pH-tuned lipase (Table 3). The EPRA gave 49% conversion, 99 ee_p (%) and the E value of 736 in 2 h. The AREP, CLEA and PCMC performed equally well with % conversion of 50 and 99 ee_p (%) (E value >1000) (entries 6, 8 and 10, Table 3) in just 2 h, whereas the free lipase gave only 8% conversion (entry 2, Table 3) in agreement with the earlier works which reported the slow conversion rates in [Bmim][PF_6] with BCL.[2b,c] Park and Kazalausks[2b] achieved ~30% conversion in 96 h in [Bmim][PF_6] with BCL.

The present work was limited to [Bmim][PF_6] which is a water-immiscible ionic liquid. This is because lipases are reported to give poor rates for this reaction in [Bmim][PF_6].[2b,c] The reaction when carried out in water-miscible (more hydrophilic) ionic liquid was adequately fast.[2b,c] It may be, however, added that in the case of subtilisin catalyzed transesterification, EPRP was found to give much higher rates in both [Bmim][PF_6] and [Bmim][BF_4] as compared to pH-tuned lyophilized enzyme.[18]

ILs are perceived as green solvents in view of their low vapor pressure. These are also called designer solvents, as different combinations of cations and anions can produce novel reaction media with desirable properties.[1,2] In order to fully exploit these advantages, it is necessary that the best ways of biocatalyst preparation are identified and tested with large number of systems. This work shows that with CRL (which did not show promising results when used as lyophilized powder), PCMC of the enzyme gives high enantioselectivity and conversion,

whereas all the four preparations were found to be highly active in the case of BCL.

Acknowledgments

The funds provided by Council of Scientific and Industrial Research (Extramural Division and Technology Mission on Oilseeds), Department of Science and Technology (DST), and Department of Biotechnology (DBT), all of which are Government of India Organizations, are gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.11.057.

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9. Preparation of EPRP; CRL (10 mg ml^{-1}, 50 mM sodium phosphate buffer, pH 7.0) was added to dry chilled 1-propanol at 4 °C. The precipitate was rinsed two times with 1 ml of ice-cold 1-propanol at 4 °C.[8] Before using, EPRP was washed two times with 1 ml of chilled vinyl acetate. A similar procedure was adopted for BCL where acetone was used in place of 1-propanol.
10. Preparation of pH-tuned lipase; CRL/BCL (10 mg) was dissolved in 1 ml of 0.02 M sodium phosphate buffer, pH 7.0, and was immediately frozen at −20 °C and lyophilized for 24 h.
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13. Preparation of CLEA: CRL/BCL (10 mg) along with bovine serum albumin (2.5 mg) was dissolved in 1 ml of 50 mM phosphate buffer, pH 7.0. This aqueous mixture was then added dropwise to a centrifuge tube containing 4 ml of acetone. After 30 min, glutaraldehyde (25% v/v in water) was added so that the final concentration of glutaraldehyde was 25 mM. The mixture was kept at 4 °C for 3 h with constant shaking at 300 rpm. The CLEAs formed were washed three times with 1 ml of acetone.[6b] Before using, CLEAs were washed two times with 1 ml of chilled vinyl acetate.
14. Preparation of PCMC: CRL powder (10 mg) was dissolved in 100 μl of phosphate buffer (50 mM, pH 7.0) and 300 μl of a saturated solution of K_2SO_4 was added. This combined solution was then added drop-wise to a vial containing 3.75 ml of 1-propanol with constant shaking at 150 rpm.[7] The PCMCs were washed two times with 1 ml of chilled vinyl acetate. Acetone was used in place of 1-propanol in case of BCL.
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