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Enhancements of enantio and diastereoselectivities in reduction of (*Z*)-3-halo-4-phenyl-3-buten-2-one mediated by microorganisms in ionic liquid/water biphasic system

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ABSTRACT

Reductions of (*Z*)-$C_6H_5CH{=}CXC(=O)CH_3$ (X = Cl, Br) mediated by *Saccharomyces cerevisiae*, *Candida albicans*, *Rhodotorula glutinis*, *Geotrichum candidum* and *Micrococcus luteus* gave the corresponding halohydrins through consecutive reduction reactions of C=C and C=O bonds. In general, the reactions performed in the biphasic system water/[(bmim)PF_6] gave better diastereoselectivity and enantioselectivity than in pure water.

1. Introduction

Enzymatic reactions using ionic liquids (ILs) with both whole cells and free enzymes were firstly described in the early 2000s and have recently been reviewed [1]. Ionic liquids, low-temperature molten salts, have been used as green reaction solvents due to their physical and chemical properties such as negligible vapor pressure, good chemical and thermal stability and relative low flammability and viscosity [2]. In general, ILs are used as solvent or co-solvent for biocatalytic reactions employing isolated enzymes, whereas whole cell reactions in aqueous medium usually employ hydrophobic IL as a second liquid phase [3]. In this biphasic system, the substrate and products are mostly in the IL and the cells are dispersed in the water phase. In case of hydrophobic substrates and products, the IL acts as a substrate reservoir, delivering the substrate to the aqueous phase and withdrawing the product from it. This implies a decrease of the concentrations of substrate and product in the aqueous phase, preventing cellular inhibition by them [3,4]. Moreover, the work up is very simple since the product is extracted from the IL using a volatile solvent and after that the IL may be recycled. Nowadays, a variety of ILs are commercially available in high purity [5].

There are very few examples of microorganism inhibition caused by IL [6], although studies show noninvasive effects of hydrophobic ILs on cellular membranes, especially those with hexafluorophosphate and bis(trifluoromethylsulfonyl)imide anions in biphasic IL/water systems [7]. 1-(*n*-Butyl)-3-methylimidazolium hexafluorophosphate [bmim(PF_6)] has been demonstrated to have a greater biocompatibility with *Saccharomyces cerevisiae* than *n*-hexane, and to be superior to the use of the water miscible 1-(*n*-butyl)-3-methylimidazolium tetrafluoroborate [bmim(BF_4)] as a co-solvent [8]. The use of hydrophobic IL in reduction of ketones mediated by whole cells is generally carried out with a second liquid phase, giving secondary alcohols in good yields and enantiomeric excesses [4,8,9].

The enones have been investigated as potential substrates for biocatalytic reductions that can lead to the introduction of one, two or three new chiral centers into an achiral structure [10], and the bioreduction of α- and β-haloenones was recently studied in deep by Fuganti et al. [11].

In this work, the results of (*Z*)-3-bromo-4-phenyl-3-buten-2-one **1a** and (*Z*)-3-chloro-4-phenyl-3-buten-2-one **1b** reduction mediated by five microorganisms in a water/[bmim(PF_6)] system are reported. The bioreduction of these substrates proceeds by two consecutive reactions (Scheme 1), beginning with a C=C bond reduction catalyzed by an enoate reductase, producing the transient species **2a** or **2b**, followed by C=O bond reduction catalyzed by an alcohol dehydrogenase to produce halohydrins **3a**

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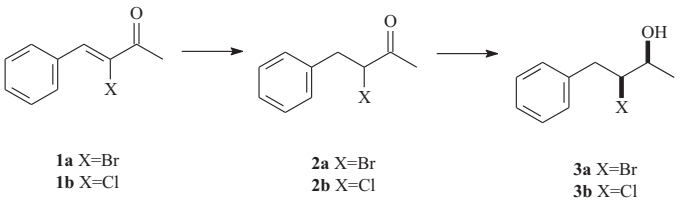

Scheme 1. Consecutive C=C and C=O bonds reduction of (*Z*)-3-halo-4-phenyl-3-buten-2-one mediated by microorganisms.

or **3b** [12]. Our interest is to study the effect of the biphasic system water/[(bmim)PF_6] in these consecutive reactions mediated by microorganisms.

2. Experimental

2.1. General

The substrates (*Z*)-3-bromo-4-phenyl-3-buten-2-one **1a** and (*Z*)-3-chloro-4-phenyl-3-buten-2-one **1b** were prepared following published methodologies [12]. The [bmim(PF_6)] was prepared following a literature procedure [13] using 1-butyl-3-methylimidazolium trifluoromethanesulfonate and potassium hexafluorophosphate as starting materials.

The reduction products **3a**–**b** were analyzed by ^{1}H NMR, ^{13}C NMR, IR spectra and are identical to those previously published [12]. ^{1}H NMR spectra were determined at 250 MHz (Varian Gemini 250) or 500 MHz (INOVA 500). ^{13}C NMR spectra were determined at 62.5 MHz (Varian Gemini 250) or 125.7 MHz (INOVA 500). IR spectra were recorded on a FT-IR Bomen MB-100 from Hartmann & Braun. GC–MS analyses were obtained on a QP 5000-Shimadzu instrument (70 eV) using a DB1 silica capillary column from J&W Scientific (30 m × 0.25 m ID × 0.25 μm film thickness) and helium as a carrier gas (0.8 mL/min). The split ratio was 1:30. The injector temperature was at 270 °C and the detector was at 280 °C. The column temperature was held at 80 °C for 3 min, increased to 290 °C at a rate of 29 °C/min and then kept constant for 8 min. One μL of a compound solution or extracted reaction solution (1 mg/mL) in ethyl acetate was injected and the retention times (min) for each compound are: **1a** (8.87), **2a** (8.27), *syn*-**3a** (8.43), *anti*-**3a** (8.59), **1b** (8.84), **2b** (8.93), *syn*-**3b** (8.61), *anti*-**3b** (8.45). Chiral GC-FID analyses were obtained on an Agilent 6850 Series GC System, using a Hydrodex chiral capillary column (30 m × 0.25 mm × 0.25 μm). Hydrogen was used as carrier gas (1 mL/min), the injector temperature was 200 °C and the detector temperature was 220 °C. The column temperature was held at 80 °C for 3 min, increased to 180 °C at a rate of 1 °C/min, and then kept constant for 5 min. One μL of a compound solution or extracted reaction solution (1 mg/mL) in ethyl acetate was injected and the retention times (min) for each compound are: (2*S*,3*S*)-**3a** (23.10), (2*R*,3*R*)-**3a** (23.28), (2*S*,3*S*)-**3b** (24.71), (2*R*,3*R*)-**3b** (24.85). Thin-layer chromatographic (TLC) analyses were performed with precoated aluminum sheets (silica gel 60 Merck), and flash column chromatography was carried out on silica (200–400 mesh, Merck). The ultraviolet spectra were obtained on an Agilent Spectrophotometer, model 8453, with diode array. The yeast strains *S. cerevisiae* CCT 3019, *Candida albicans* CCT 0776, *Geotrichum candidum* CCT 1205, *Rhodotorula glutinis* CCT 2182 and *Micrococcus luteus* CCT 2283 were obtained from the André Tosello Research Foundation (Campinas, SP, Brazil) [14].

2.2. Growth conditions of *C. albicans*, *R. glutinis*, *G. candidum* and *S. cerevisiae*

Microorganisms were cultivated in YM (yeast-malt extract) nutrient broth obtained from Merck (1000 mL) under aseptic conditions, incubation at 30 °C on an orbital shaker (180 rpm) for 1 day just before use. All materials and medium were sterilized in an autoclave at 121 °C before use and the yeast was manipulated in a laminar flow cabinet.

2.3. Growth conditions of *M. luteus*

M. luteus CCT 2283 was cultivated in NB (nutrient broth) obtained from Oxoid under aseptic conditions, incubation at 30 °C on an orbital shaker (180 rpm) for 1 day just before use. All materials and medium were sterilized in an autoclave at 121 °C before use and the yeast was manipulated in a laminar flow cabinet.

2.4. Test of inhibition activity

Aqueous dispersions of each microorganism (100 μL) were spreaded over a YMA (malt extract, yeast extract, peptone, glucose and agar) medium, and after that, sterile filter paper disks with [bmim(PF_6)] or **1a** or **1b** adsorbed (10 μL of a 1 mg/mL solution in ethyl acetate was used) were placed over them. The standards were fungicide (Cyclopirox amine®) and bactericide (Cloranfenicol®) for control. After 24 h at 30 °C, inhibition halos on the filter paper were observed for **1a** and **1b** and no halo of inhibition was observed for [bmim(PF_6)].

2.5. General procedure for bioreduction of α-haloenones ***1a*** and ***1b*** mediated by microorganisms in aqueous media

Compounds **1a** or **1b** (100 mg) dissolved in 1 mL of ethanol was added to an Erlenmeyer flask containing 200 mL of the slurry medium of growing microorganism cells. The reaction mixture was incubated in an orbital shaker (200 rpm at 30 °C) for 120 h. Then, the product was extracted by a continuous liquid/liquid extractor with dichloromethane for 2 days. The solvent was dried over sodium sulfate, evaporated, and the products were purified by preparative TLC (hexane/ethyl acetate 9:1), analyzed by GC–MS and ^{1}H NMR, and the *ee* was determined by chiral GC-FID.

2.6. General procedure for bioreduction of α-haloenones ***1a*** and ***1b*** mediated by microorganisms in aqueous media with addition of ionic liquid

Compounds **1a** or **1b** (100 mg) dissolved in [bmim(PF_6)] (1 mL) was added to an Erlenmeyer flask containing 200 mL of the slurry medium of growing microorganism cells. The resulting mixture was stirred in an orbital shaker (400 rpm, 30 °C) for 24 h. Then, the products were extracted by a continuous extractor with dichloromethane for 2 days. The organic phase was dried over sodium sulfate, the solvent evaporated, and the products were purified by preparative TLC (hexane/ethyl acetate 9:1), analyzed by GC–MS and ^{1}H NMR, and the *ee* was determined by chiral GC/FID.

2.7. General procedure for detection of intermediaries during bioreduction of α-haloenones ***1a*** and ***1b*** mediated by *S. cerevisiae* in aqueous media

Compounds **1a** or **1b** (10 mg) dissolved in ethanol (0.5 mL) were added to each of seven Erlenmeyer flasks containing 20 mL of the slurry medium of growing *S. cerevisiae* cells. The reaction mixtures were incubated in an orbital shaker (200 rpm at 30 °C) for appropriate periods of time. After the flasks were withdrawn from the shaker, the mixtures were centrifuged and the supernatants free of cells were submitted to extractions with ethyl acetate (3 × 10 mL). The extracts were dried over sodium sulfate, the solvent evaporated, and the products were analyzed by GC–MS.

Table 1
Reagent and product percentages after 120 h of bioreduction of **1a** in 200 mL of slurries of growing microorganisms.

Microorganism	System	**1a** (%)[a]	**2a** (%)[a]	**3a** (%)[a]	
				syn (2*S*, 3*S ee*)[b]	*anti*
S. cerevisiae	H_2O[c]	–	26	74 (90)	–
	H_2O/IL[d]	28	10	62 (97)	–
C. albicans	H_2O[c]	21	18	61 (65)	–
	H_2O/IL[d]	32	13	55 (81)	–
R. glutinis	H_2O[c]	18	9	47 (70)	26
	H_2O/IL[d]	39	14	47 (92)	–
G. candidum	H_2O[c]	12	13	39 (65)	36
	H_2O/IL[d]	33	15	52 (91)	–
M. luteus	H_2O[c]	55	5	40 (38)	–
	H_2O/IL[d]	58	–	42 (42)	–

[a] Conversion determined by the relative areas of the chromatographic peaks using GC–MS.
[b] *ee* were determined by CG-FID using a chiral column.
[c] Reaction conditions: 10 mg **1a** in 200 mL of growing cell slurry, in an orbital shaker at 200 rpm and 30 °C.
[d] Reaction conditions: 100 mg of **1a** in 1 mL of [bmim(PF_6)] and 200 mL of growing cell slurry, in an orbital shaker at 200 rpm and 30 °C.

Table 2
Reagent and product percentages after 120 h of bioreduction of **1b** in 200 mL of slurries of growing microorganisms.

Microorganism	System	**1b** (%)[a]	**2b** (%)[a]	**3b** (%)[a]	
				syn (2*S*, 3*S ee*)[b]	*anti*
S. cerevisiae	H_2O[c]	13	20	67 (71)	–
	H_2O/IL[d]	18	21	61 (92)	–
C. albicans	H_2O[c]	22	8	47 (79)	23
	H_2O/IL[d]	49	10	41 (94)	–
R. glutinis	H_2O[c]	–	13	63 (72)	24
	H_2O/IL[d]	44	8	48 (95)	–
G. candidum	H_2O[c]	38	2	44 (67)	16
	H_2O/IL[d]	42	5	53 (95)	–
M. luteus	H_2O[c]	61	6	33 (32)	–
	H_2O/IL[d]	76	10	18 (39)	–

[a] Conversion determined by the relative areas of the chromatographic peaks using GC–MS.
[b] *ee* were determined by CG-FID using a chiral column.
[c] Reaction conditions: 10 mg **1b** in 200 mL of growing cell slurry, in an orbital shaker at 200 rpm and 30 °C.
[d] Reaction conditions: 100 mg of **1b** in 1 mL of [bmim(PF_6)] and 200 mL of growing cell slurry, in an orbital shaker at 200 rpm and 30 °C.

2.8. Determination of the distribution coefficient of α-haloenones **1a–b** in water/[bmim(PF_6)]

The haloenone (100 mg) dissolved in IL (1 mL) was added to a 250 mL Erlenmeyer flask containing water (100 mL). The mixture was kept under orbital stirring at 400 rpm and 30 °C for 24 h. Then, an aliquot was removed from the aqueous phase. The concentration of haloenones in the water phase was determined by measuring the absorbance at 295 nm and with aid of calibrations curves ($R = 0.9999$) for **1a** and **1b** constructed from standard solutions. The obtained distribution coefficients values were 316 for enone **1a** and 257 for enone **1b**.

3. Results and discussion

Bioreductions of **1a** and **1b** were carried out in aqueous media and in aqueous/[bmim(PF_6)] biphasic systems mediated by the following microorganisms: *S. cerevisiae*, *R. glutinis*, *C. albicans*, *G. candidum* and *Micrococcus luteus*. For all experiments substrate concentrations of 100 mg in 200 mL of slurry medium of growing microorganism were used and the biotransformations were carried out for 120 h and then analyzed by GC–MS to determine the conversions and the diastereoselectivity, and by GC-FID using a chiral column to determine the enantiomeric excesses of **3a** and **3b**, as shown in Tables 1 and 2. Samples were withdrawn every 24 h (up to 120 h), and after 96 h no change was observed in the composition of the reaction mixture.

Even though the reactions in the presence of [bmim(PF_6)] resulted in slightly lower conversions of **1a** into **3a**, they gave overall better results, since in all biotransformations the corresponding halohydrins were obtained in higher enantiomeric excesses than in their monophasic system counterpart (see Table 1). When the reduction of **1a** was mediated by *S. cerevisiae* in aqueous media only the *syn* isomer (2*S*,3*S*)-**3a** is formed in 90% *ee*, in accordance with previous work [15]. This reaction in IL improved the enantiomeric excess up to 97% while the conversion was decreased from 74 to 62%. Experiments with *C. albicans* gave similar results while *M. luteus* gave unsatisfactory results. It is also noteworthy that, when *G. candidum* and *R. glutinis* were used as biocatalysts without addition of IL, both *syn* and *anti* diastereoisomers were formed, whereas in the presence of IL only the *syn* diastereoisomer (2*S*,3*S*)-**3a** was observed in higher *ee* than from the experiments without addition of [bmim(PF_6)].

The same protocol was applied for bioreduction of chloroenone **1b**, and the results are in Table 2. When the reduction is mediated by *S. cerevisiae* in aqueous media only the isomer (2*S*,3*S*)-**3b** was formed in 71% *ee* while in presence of IL the enantiomeric excess was improved up to 92%, while the conversion was decreased from 67 to 61%. Again, the bireduction of **1b** mediated by *R. glutinis*, *G. candidum* and also, in this case, by *C. albicans*, two diastereoisomers were observed when performed in a monophasic system, whereas the reaction in the presence of IL gave exclusively (2*S*,3*S*)-**3b** diastereoisomer in 94–95% *ee*.

The distribution coefficients of the substrates **1a** and **1b** in the two phases of the system water/[bmim(PF_6)] were determined as 316 for enone **1a** and 257 for enone **1b**, this implies in log *K* of 2.50 and 2.41, respectively. Therefore, in the reactions applying the biphasic system the substrates are mostly in the IL layer and thus the effective initial concentration of these compounds in the aqueous layer is much lower (2.3–2.5 times) than in the system that utilizes only water as solvent. This decrease in the concentration of substrates may be extended to the concentration of intermediates **2a** and **2b** since organochlorides with similar structures like chloroacetophenone, have the similar values of log *P* [16].

The above mentioned IL effects on the concentration of substrates and intermediates may be responsible for the diastereoselectivity and enantioselectivity of the reduction of **1a** and **1b**. Therefore, the IL is acting like absorbing resins in an *in situ* extractive biocatalysis, controlling the substrate concentration of hydrophobic substrates and improving enantio-chemoselectivity [17]. Considering the pull of oxidoredutases present in the microorganism cells, the enzyme with the lowest K_M is favored when the concentration of substrate is decreased. This effect may be occurring in the C=C bond reduction of **1a–b** and more effectively in the consecutive C=O bond reduction reaction of intermediates **2a–b**, increasing the enantioselectivity and diastereoselectivity when the bioreduction is performed in presence of IL (Fig. 1).

It was crucial to prove that the intermediates **2a–b** have permeability through cells membrane of microorganism during the reduction of **1a–b**. Thus, an experiment was designed to detect the intermediates **2a–b** in the aqueous media, just after the cells been withdrawn by centrifugation, during the reduction of **1a–b** mediated by *S. cerevisiae* using only water as solvent. Fig. 2 clearly shows the presence of **2a** as transient in the aqueous media.

The toxicities of these α-haloenones to the microorganisms were determined by inhibition assays in Petri dishes measuring the inhibition halo. Table 3 shows the inhibitory effects of

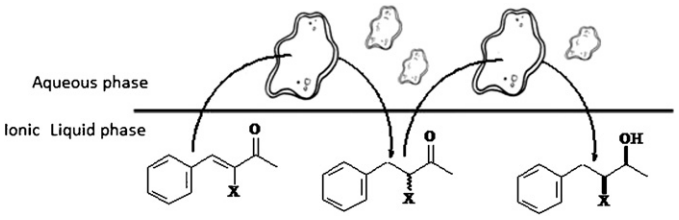

Fig. 1. The consecutive bioreduction reactions of **1a**–**b** performed in a water/ionic liquid biphasic system.

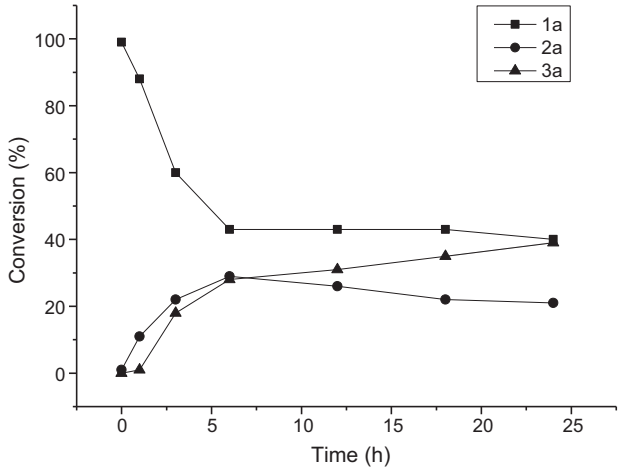

Fig. 2. Percentage of reagent, intermediate and product in the aqueous media during the reduction of (*Z*)-3-bromo-4-phenyl-3-buten-2-one **1a** (■) mediated by *Saccharomyces cerevisiae* using water as solvent giving 3-bromo-4-phenyl-2-butanone **2a** (●) as transient and 3-bromo-4-phenyl-2-butanol (▲) as final product.

Table 3
Microorganism inhibition halos[a] for **1a**, **1b** and [bmim(PF_6)].

Compound	*S. cerevisiae*	*G. candidum*	*C. albicans*	*R. glutinis*	*M. luteus*
1a[b]	2.5	4.0	3.0	1.9	5.0
1b[b]	2.4	2.3	3.1	2.7	5.0
[bmim(PF_6)][c]	0.0	0.0	0.0	0.0	0.0
Control	2.0[d]	3.0[d]	2.0[d]	2.7[d]	2.0[e]

[a] Values of the halo diameter in cm.
[b] Concentration 1 mg/mL.
[c] Pure.
[d] Cyclopirox amine (fungicidal).
[e] Cloranfenicol (bactericidal).

compounds **1a** and **1b**, fungicide (Cyclopirox amine®) and bactericide (Cloranfenicol®) on the six studied microorganisms. No formation of inhibition halos was observed for [bmim(PF_6)]. On the other hand, enones **1a** and **1b** have toxicity at the same level as the fungicide and bactericide used for comparison. Since the use of the biphasic system decreases the concentrations of enones **1a** and **1b** and as a consequence the toxicity, this is another advantage of the bioreduction of these substrates being performed in water/IL.

4. Conclusion

The reductions of **1a** and **1b** mediated by *C. albicans*, *G. candidum*, *M. luteus*, *R. glutinis* and *S. cerevisiae* in the two-phase system water/[bmim(PF_6)] gave the corresponding halohydrins with better enantiomeric excesses and diastereoselectivities than the reductions accomplished in pure water. This result may be due to the dramatic reduction of the substrate concentrations in the aqueous phase due to their better solubility in IL. The inhibitory effect of substrates on the biocatalysts was decreased in the presence of IL due to the reduction of substrate concentration in the aqueous phase. The extractive methodology employing the IL biphasic system can be of general use in biocatalysis for toxic substrates, as exemplified by the compounds described in this paper.

Acknowledgements

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