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Screening and reaction engineering for the bioreduction of ethyl benzoylacetate and its analogues

Cíntia D.F. Milagre, Humberto M.S. Milagre, Paulo J.S. Moran, J. Augusto R. Rodrigues*

State University of Campinas, Institute of Chemistry, CP 6154, 13084-971 Campinas, SP, Brazil

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ABSTRACT

Microbial reduction of benzoylacetates is already an established part of the synthetic toolbox to obtain chiral ethyl 3-hydroxy-3-phenylpropionate although bioreduction yields are low to moderate. A 30% increase in the enantioselectivity to 99% ee and a significant improvement in the yields to around 85% were achieved by combining simple screening procedures and a reaction engineering strategy. Three experimental parameters were selected for investigation: the influences of glucose, enzymatic inhibitor and biocatalyst immobilization. The screened yeasts *Pichia kluyveri*, *Pichia stipitis* and *Candida utilis* were found to give better yields and ee's for ethyl benzoylacetate **1a**, *p*-nitrobenzoylacetate **1b** and *p*-methoxybenzoylacetate **1c**, respectively, with addition of glucose, α-chloroacetophenone as inhibitor and immobilization of the yeasts in alginate beads. Our results demonstrate that the optimized process can be implemented on a preparative scale without any loss in yield and ee.

1. Introduction

Chiral alcohols are important synthons for the synthesis of numerous pharmaceuticals, agrochemicals, flavors and fragrances, and also play an important role in many biological reactions inside the human body [1–6]. The bioreductions of prochiral ketones offer significant advantages in the synthesis of optically pure alcohols such as environmentally benign reaction conditions, broad reaction scope, and high stereo- and regioselectivity [7–9]. Both isolated enzymes and whole cells have been used on a laboratory scale and industrially as biocatalysts, but in redox reactions the use of whole cells is particularly advantageous since cofactor regeneration is essential for sustained catalytic activity [3]. A recent successful application of whole cells processes for the commercial synthesis of chemicals has been described in the literature [10]. However, since a whole cell may contain many ketoreductases, not all ketone reductions provide a chiral alcohol product with high optical purity [11–15]. To circumvent these limitations, the main tools are (i) screening of the microorganisms, (ii) overexpression and directed evolution of the enzymes and (iii) the modifications of the reactions conditions [2]. A recent example is the enantioselective reduction of ethyl benzoylacetate to (*S*)-3-hydroxy-3-phenylpropionate mediated by a recombinant *Saccharomyces cerevisiae*, where the reaction conditions were optimized by combining simple screening procedures and a stochastic search strategy (genetic algorithm) to improve the yield and ee [16].

Herein, we describe the optimization of the reaction conditions for an efficient whole cell reduction of ethyl benzoylacetate and its analogues mediated by previously screened wild-type yeast strains. We demonstrate the applicability of the optimized process for a preparative scale bioreduction. The enantioselectivity of ethyl benzoylacetate and their *p*-nitro and *p*-metoxi substituted derivatives reduction products are of great importance since they afford key chiral building blocks for the synthesis of many compounds with pharmaceutical interest such as Fluoxetine [5,17–19], Chloramphenicol [20] and Diltiazem [21].

2. Materials and methods

2.1. Experimental

^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ solution on a Varian Gemini 300 spectrometer operating at 300 and 76 MHz, respectively. Chemical shifts are expressed in ppm using TMS as an internal standard. Chiral analysis was performed using Eu(hfc)$_3$ as lanthanide shift reagent. Infrared spectra were recorded on a Bomen MB-100 FT IR spectrophotometer from Hartmann & Braun. Gas chromatographic analyses and mass spectra were obtained

* Corresponding author. Tel.: +55 19 3521 3141; fax: +55 19 3521 3023.
E-mail address: jaugusto@iqm.unicamp.br (J.A.R. Rodrigues).

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on a Shimadzu QP 5000 equipped with a J&W Scientific HP-5 (5% phenylmethylpolysiloxane, 30 m × 250 μm × 0.25 μm) capillary column. Optical rotations were recorded on a PerkinElmer 341 Polarimeter. High-resolution mass spectra were determined on a VG Auto Spec from Micromass. TLC was done on Kieselger 60F254 silica on glass plates. The mobile phase was hexane/ethyl acetate; the polarity of solvents was varied for the different compounds.

2.2. Chemicals

Ethyl benzoylacetate **1a** (97%) and ethyl *p*-nitrobenzoylacetate **1b** (98%) were purchased from Acros and *p*-methoxybenzoylacetate **1c** (97%) was purchased from Fluka. The culture media components: yeast extract, malt extract and bacto-peptone were purchased from Biobrás (Brazil). All other chemicals were at least analytical grade.

2.3. Yeast strains

Dry *S. cerevisiae* (baker's yeast) was purchased from Emulzint Ltd. (Belgium) and stored in a refrigerator. *P. stipitis* (CCT 2617), *P. kluyveri* (CCT 3365), *Pichia canadensis* (CCT 2636), *Rhodotorula minuta* (CCT 1751), *Rhodotorula glutinis* (CCT 2182) and *C. utilis* (CCT 3469) were supplied by the Tropical Culture Collection (CCT) of the André Tosello Foundation, Campinas, SP, Brazil (http://www.fat.org.br/).

2.4. Culture medium

The media used for culturing all the yeasts except for dry *S. cerevisiae* contained YMB (yeast malt broth): glucose (10 g L^{-1}), peptone (5 g L^{-1}), yeast extract (3 g L^{-1}) and malt extract (3 g L^{-1}). The yeast malt broth was sterilized in an autoclave at 121 °C and 1.0 kgf cm^{-2} pressure for 15 min.

2.5. Cultivation of yeasts

All strains were routinely maintained in agar slants (2.0% agar with the above mentioned culture media). The slants were preserved in a refrigerator at 4 °C.

2.6. Culture conditions

The pure culture of each yeast strain was inoculated with a loop into the YMB media. A working volume of 30 mL (YMB media, after inoculation) in 125 mL Erlenmeyer flasks was cultivated in an orbital shaker at 30 °C, 150 rpm. The cells were harvested by centrifuging the 48–72 h culture broth. The wet biomass was used for biotransformations. The volumes cited were altered depending on the experimental scale.

2.7. Screening experiments

Screening experiments with **1a–c** were performed in 200 mL shaking flasks containing 50 mL of distilled water (pH 6.5) and 5 g of wet yeast biomass. The flasks were incubated at 30 °C on an orbital shaker for 16–40 h.

2.8. Immobilization

The immobilization procedure used has been a previously reported [22].

2.9. Determination of the partition coefficients

An aqueous solution of sodium alginate (50 mL, 2%) was extruded using syringe nozzles with inner diameters of 0.8 mm into a solution of $CaCl_2$ (0.2 mol L^{-1}) to produce beads with 2 mm of diameter. After 20 min, the beads were filtered and washed with water to remove the excess of $CaCl_2$. The beads (without yeasts cells) were suspended in a 200 mL shaking flasks containing distilled water (50 mL, pH 6.5) with **1a** and **2a** (obtained by reduction of **1a** with $NaBH_4$) and stirred at 300 rpm at 30 °C. The initial concentration of **1a** and **2a** were the same in all experiments and was varied between 1 and 5 mmol. The aqueous phase was monitored by GC–MS in intervals of 1 h. The same procedure was repeated for **1b**–**2b** and **1c**–**2c**.

2.10. General procedure for reduction mediated by free yeasts

In a 100-mL bioreactor, 5.0 g (wet biomass) of yeast were suspended in distilled water (60 mL, pH 6.5) and stirred at 300 rpm at 30 °C. The substrate **1a** or **1b** (0.5 mmol in 2.0 mL of ethanol), or **1c** (0.5 mmol in 1.0 mL of ethanol–1.0 mL DMF) was added and the reaction was monitored by GC–MS. At the end of the reaction, the biomass was centrifuged, washed with ethyl acetate, and the reaction mixture was extracted with ethyl acetate, dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated. The crude material was purified by flash chromatography with different solvent polarities depending on the substrate.

2.11. General procedure for reduction mediated by yeasts with addition of glucose

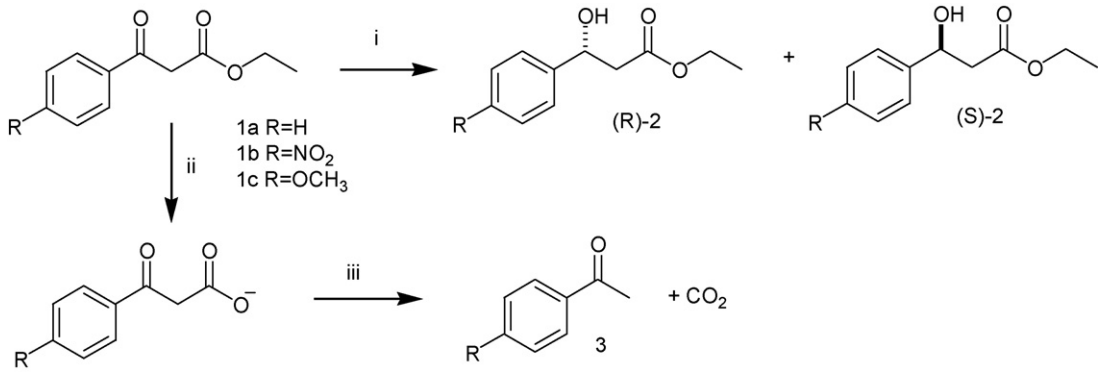
In a 100-mL bioreactor, 5.0 g (wet biomass) of yeast were suspended in distilled water (60 mL, pH 6.5) and stirred at 300 rpm at 30 °C, and then 1.5 g of glucose were added and the yeast was activated for 2 h. The substrate **1a** or **1b** (0.5 mmol in 2.0 mL of ethanol), or **1c** (0.5 mmol in 1.0 mL of ethanol–1.0 mL DMF) was added and the reaction was monitored by GC–MS. The procedures used for extraction and isolation of the product are the same as those described above.

2.12. General procedure for reduction mediated by yeasts with addition of inhibitor

In a 100-mL bioreactor, 5.0 g (wet biomass) of yeast were suspended in distilled water (60 mL, pH 6.5) and stirred at 300 rpm at 30 °C, and then 10 mg of chloroacetophenone was added and the yeasts were activated for 2 h. The substrate **1a** or **1b** (0.5 mmol in 2.0 mL of ethanol), or **1c** (0.5 mmol in 1.0 mL of ethanol–1.0 mL DMF) was added and the reaction was monitored by GC–MS. The procedures used for extraction and isolation of the product are the same as those described above.

2.13. Preparative scale biotransformation

A solution of sodium alginate (150 mL, 2%) was added to a suspension of *P. kluyveri* (23 g wet biomass) in distilled water (50 mL). This mixture was extruded using syringe nozzles with inner diameters of 0.8 mm into a solution of $CaCl_2$ (0.2 mol L^{-1}) to produce beads with 2 mm diameter. After 20 min the beads were filtered and washed with water to remove the excess of $CaCl_2$. The beads were suspended in distilled water (300 mL, pH 6.5) containing glucose (6 g) and α-chloroacetophenone (40 mg) in a 400-mL bioreactor and stirred at 300 rpm at 30 °C. After activation of the yeast for



Scheme 1. The main reactions during yeast-mediated reduction of ethyl benzoylacetate and analogues: (i) reduction; (ii) hydrolysis and (iii) decarboxylation.

2 h, the substrate **1a** (5.2 mmol in 3.0 mL of ethanol) was added. The reaction was monitored by GC–MS and after 24 h the beads were filtered and washed with ethyl acetate. The bead-free mixture was extracted with ethyl acetate, dried over anhydrous $MgSO_4$, filtered and then the solvent was evaporated. The crude material was purified by flash chromatography, eluting with 10% ethyl acetate in hexane (to recover **3a**) and 20% ethyl acetate in hexane (to recover **2a**). This procedure was repeated for substrates **1b** (dissolved in DMSO) and **1c** (dissolved in ethanol) using *P. stipitis* and *C. utilis*, respectively.

2.14. Ethyl (−)-(S)-3-hydroxy-3-phenylpropionate, *2a*

Colorless oil, $[\alpha]_D^{20}$ − 20 (c 2.05, MeOH); IR (film) ν_{max} (cm^{-1}): 3420, 2982, 1727, 1493, 1452, 1370, 1196, 1158; ^1H NMR (300.0 MHz, $CDCl_3$) δ: 1.25 (t, 3H, J = 7.2 Hz), 2.70 (dd, 2H, J = 4.4 and 8.4 Hz), 4.16 (q, 2H, J = 7.2 Hz), 5.11 (t, 1H, J = 4.4 Hz), 7.31 (m, 5H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 14.1, 43.4, 60.7, 70.2, 125.5, 127.5, 128.2, 142.3, 172.1; EM m/z (%): 194 ($M^{\bullet+}$), 120 (14), 107 (100), 88 (24), 79 (66), 77 (59).

2.15. Ethyl (−)-(S)-3-hydroxy-3-p-nitrophenylpropionate, *2b*

Yellow oil, $[\alpha]_D^{20}$ − 12.0 (c 2.03, MeOH); IR (film) ν_{max} (cm^{-1}): 3474, 2987, 1730, 1601, 1521, 1348, 1192, 1105; ^1H NMR (300.0 MHz, $CDCl_3$) δ: 1.27 (t, 3H, J = 7.1 Hz), 2.75 (dd, 2H, J = 4.4 and 8.8 Hz), 4.19 (q, 2H, J = 7.1 Hz), 5.23 (t, 1H, J = 4.4 Hz), 7.56 (d, 2H, J = 8.4 Hz), 8.21 (d, 2H, J = 8.8 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 14.7, 43.5, 61.8, 69.9, 124.1, 126.9, 147.8, 150.0, 172.3, EM m/z (%): 239 ($M^{\bullet+}$), 222 (27), 210 (5), 165 (12), 150 (100), 107 (17), 105 (17); HRMS calc. 239.07937 found: 239.07934.

2.16. Ethyl (−)-(S)-3-hydroxy-3-p-metoxyphenylpropionate, *2c*

Pale yellow oil, $[\alpha]_D^{20}$ − 33.0 (c 2.04, MeOH); IR (film) ν_{max} (cm^{-1}): 3475, 2987, 2836, 1731, 1613, 1514, 1302, 1248, 1176, 1034; ^1H NMR (300.0 MHz, $CDCl_3$) δ: 1.26 (t, 3H, J = 7.0 Hz), 2.68 (dd, 2H, J = 4.0 and 9.1 Hz), 3.80 (s, 3H), 4.17 (q, 2H, J = 6.9 Hz), 5.07 (m, 1H), 6.88 (d, 2H, J = 8.8 Hz), 7.29 (d, 2H, J = 8.8 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 14.1, 43.3, 55.2, 60.2, 69.9, 113.9, 126.9, 134.7, 159.2, 172.4, EM m/z (%): 224 ($M^{\bullet+}$), 206 (48), 178 (10), 161 (100), 137 (76), 118 (14), 109 (19), 89 (33), 77 (60), HRMS calc. 224.10485 found: 224.10481.

3. Results and discussion

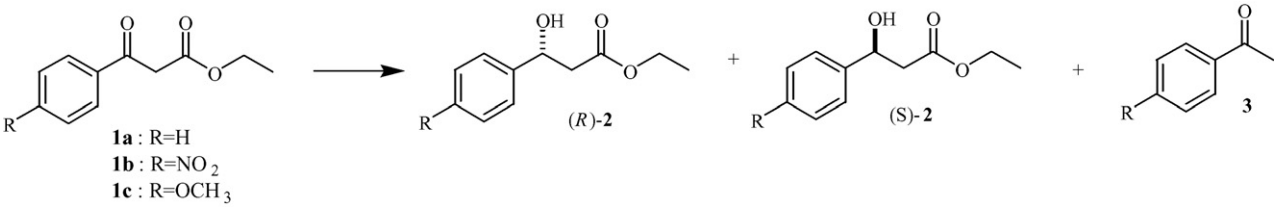
The asymmetric reduction of β-ketoesters mediated by microorganisms has become a standard method for the synthesis of chiral β-hydroxyesters [17,23]. In this study, we chose the β-ketoesters **1a–c** as substrates for bioreduction due to the adverse electronic effects of the aromatic substituent groups. β-Ketoesters bioreduction usually have low reaction rates and also low yields probably due to its toxicity to yeasts cells, low water solubility and substrate degradation [3,24,25]. The formation of undesired by-products **3a–c** (Scheme 1) can be rationalized by hydrolysis of **1a–c** followed by a decarboxylation reaction.

To evaluate the spontaneous hydrolysis of the starting materials **1a–c** under the same conditions used for the biotransformation of **1a** (citrate-phosphate buffer, pH 4.5), initial experiments were carried out without addition of the biocatalysts. The pH 4.5 was chosen based on Chênevert et al. previous studies [17]. After 24 h, the GC–MS analyses showed the formation of hydrolysis–decarboxylation products **3a–c** in 20, 32 and 100% yields, respectively. In view of these results, we looked for other conditions and found that the spontaneous hydrolysis of **1b–c** is diminished in distilled water, giving only 30% decomposition. Although spontaneous hydrolysis of **1a** increased from 20 to 30% in distilled water, we performed all biotransformation experiments (**1a–c**) in distilled water in order to maintain the same parameters for all three reactions. The reduction process mediated by the microorganisms must be as fast as possible to diminish the production of the undesired **3a–c** and to increase the production of **2a–c**.

In an attempt to find a suitable microorganism to circumvent the above difficulties, seven yeasts were screened for the reduction of **1a–c**. The results are shown in Table 1.

A relevant data were obtained from these screening experiments concerning enzymatic activity. *Rhodotorula* yeasts showed poor reductive activity for all three substrates while the yeasts *P. kluyveri* and *P. stipitis* had a similar behavior with **1c**. Also, *Rhodotorula* yeasts showed hydrolytic activity for the substrates **1a** and **1b** since the yields of **3a** and **3b** were higher than those afforded by spontaneous hydrolyses after 24 h of reaction. The screened yeasts *P. kluyveri*, *P. stipitis* and *C. utilis* were found to give best results as evaluated by yields and ee for the substrates **1a**, **1b** and **1c**, respectively. Electron withdrawing groups at the *para* position of the aromatic ring results in electron deficiencies at the carbonyl carbons which favors a nucleophilic attack by the cofactor hydride and decreases the time of reaction [26]. The opposite effect is observed with electron donating groups at the *para* position of the aromatic ring. However, the presence of these electron withdrawing (**1b**) or electron donating (**1c**) groups in the *para* position of the standard β-hydroxy ester (**1a**) does not seem to affect the optical purity of the product [24].

Table 1
Screening of biocatalysts to reduce **1a–c**



Substrate	Yeasts	2		3
		Conversion (%)	ee (%)	Conversion (%)
1a[x]	*S. cerevisiae*	60	51	40
	P. kluyveri (CCT 3365)	89	66	11
	P. stipitis (CCT 2617)	85	60	15
	P. canadensis (CCT 2636)	83	62	17
	R. minuta (CCT 1751)	13	60	87
	R. glutinis (CCT 2182)	10	60	90
	C. utilis (CCT 3469)	80	59	20
1b[y]	*S. cerevisiae*	70	72	30
	P. kluyveri (CCT 3365)	97	65	3
	P. stipitis (CCT 2617)	98	72	2
	P. canadensis (CCT 2636)	98	63	2
	R. minuta (CCT 1751)	60	65	30
	R. glutinis (CCT 2182)	1	65	99
	C. utilis (CCT 3469)	97	67	2
1c[z]	*S. cerevisiae*	63	69	37
	P. kluyveri (CCT 3365)	2	–	98
	P. stipitis (CCT 2617)	18	50	82
	P. canadensis (CCT 2636)	80	65	20
	R. minuta (CCT 1751)	5	–	95
	R. glutinis (CCT 2182)	10	40	90
	C. utilis (CCT 3469)	94	69	6

Reaction conditions: distilled water, aerobic conditions, orbital stirring, 30 °C; [x]ca. 24 h of reaction; [y]ca. 16 h of reaction; [z]ca. 36 h of reaction. The enantiomeric excesses were determined by NMR using (+)-Eu(hfc)$_3$ (Fig. 1).

The three selected microorganisms were used in the following experiments in order to improve the preliminary results. Three variables were selected for investigation: (i) glucose addition, (ii) enzymatic inhibitor addition and (iii) biocatalyst immobilization. The results are shown in Table 2.

In all cases, the addition of glucose did not have a significant effect on yield but showed a significant improvement in the enantioselectivity. Various NAD(P)H-dependent oxyreductases may be involved in the reduction of ketoesters by yeasts [27]. Yeasts cells contain only catalytic amount of NAD(P)H and its regeneration must take place by means of metabolism of an electron donor [28,29]. To exhibit catalytic activities, these NAD(P)H-dependent dehydrogenases require a coenzyme such as NADH and/or NADPH, from which a hydride is transferred to the substrate carbonyl carbon, forming NAD^+ or $NADP^+$ and glucose is needed to recycle NAD^+ or $NADP^+$ [27,29,30].

α-Chloroacetophenone is a well-known enzymatic inhibitor in yeasts [31]. The addition of this enzymatic inhibitor considerably enhanced the ee while only a small increase in the yields was observed. Various compounds have been reported as enzyme

Table 2
Evaluation of the reaction conditions to improve the enantioselectivities and yields

Substrate	Yeast	Glucose	Inhibitor[a]	Imob.[b]	Yield (%) product 2	ee (%)[c]	Time (h)
1a	*P. kluyveri*	–	–	–	60	66	24
		+	–	–	65	80	24
		–	+	–	67	90	24
		–	–	+	80	70	24
		+	+	+	80	99	24
1b	*P. stipitis*	–	–	–	70	72	16
		+	–	–	70	90	16
		–	+	–	70	99	16
		–	–	+	85	70	16
		+	+	+	90	99	16
1c	*C. utilis*	–	–	–	63	69	36
		+	–	–	65	90	36
		–	+	–	65	99	36
		–	–	+	72	71	36
		+	+	+	81	99	36

[a] α-Chloroacetophenone.
[b] Calcium alginate beads.
[c] Absolute configuration (S).

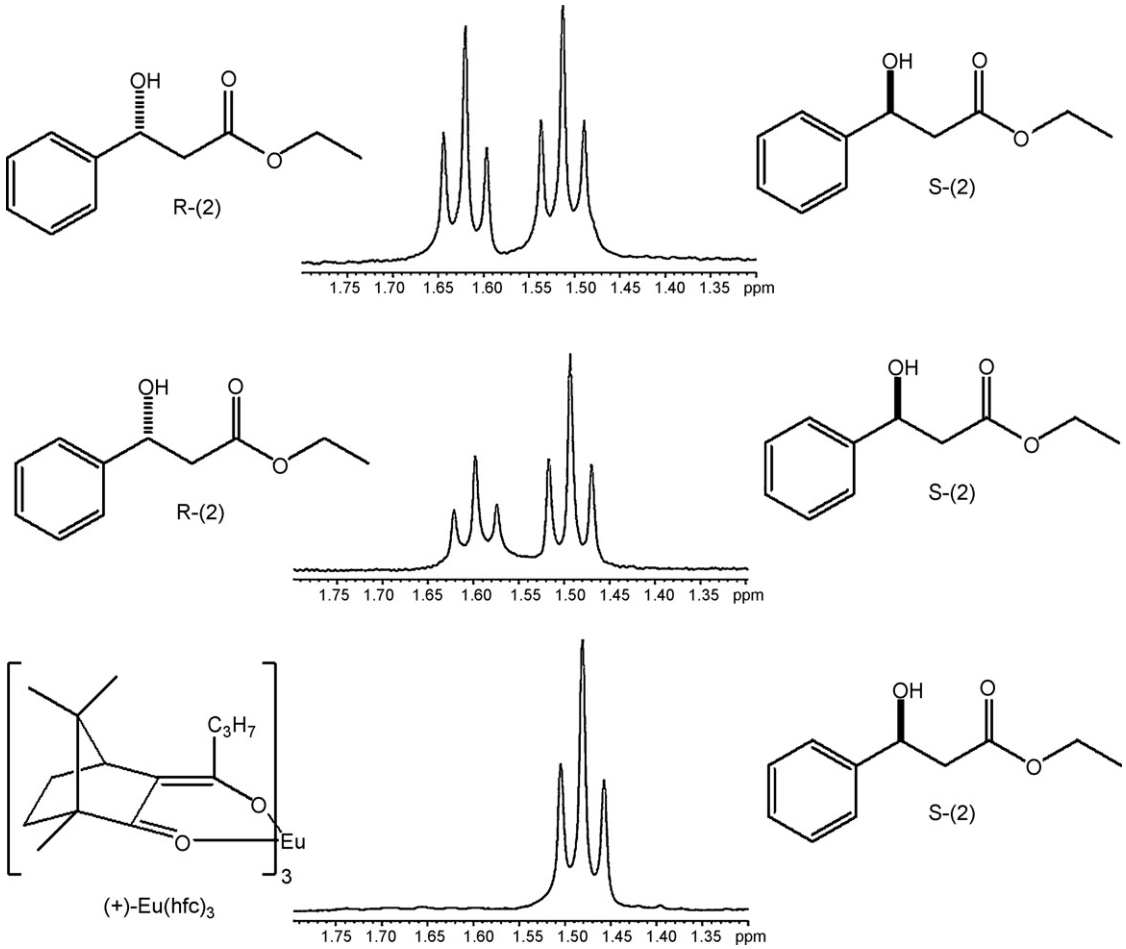


Fig. 1. Enantiomeric excess determination by NMR using the tris[3-(heptafluoropropylhydroxymethylene)-(+)-canforate of europium III, ($Eu(hfc)_3$), as a shift reagent. (±)-**2a** (upper); (*S*)-**2a** with 70% ee (middle) and (*S*)-**2a** with 99% ee (bottom).

inhibitors for baker's yeast and these inhibition mechanisms are reported to be non-competitive [32–36]. Although we observed the reduction product of α-chloroacetophenone in GC–MS analysis, the detailed inhibition mechanism was not evaluated in this study.

The substrate/yeast cell concentration ratio is another parameter that affects the enantioselectivity and yield of a bioreduction. In an attempt to decrease the toxicity effect of the substrate, the cells were immobilized in alginate beads, creating a barrier at the border of the cellular membrane [37–40]. The alginate matrix is inexpensive, readily available and has a high affinity for water and also has the ability to form gels under mild conditions that are suitable for these cells. Experiments were performed to measure the partition coefficient between substrate/alginate/product. Fig. 2 shows the high alginate affinities for substrates **1a**–**c** and low affinities for the products **2a**–**c**, indicating that biocatalyst immobilization in the alginate matrix is indicated for these reactions.

Biocatalyst immobilization in alginate beads caused an improvement in yields for all reactions (60–85%) and essentially did not influence the ee, which is already high. The yield improvement could be explained by the fast substrates **1a**–**c** absorption into the alginate beads (in about 1 h in Fig. 2) diminishing the extension of substrate decomposition by spontaneous hydrolyses. Also, the reduction products (*S*)-**2a**–**c** were easily separated from the biocatalyst due to their low affinity towards alginate beads as shown in Fig. 2.

The above optimized conditions were used for a scaled up biotransformation of **1a**–**c** (1.0 g of substrate per batch) in a 400 mL stirred-tank reactor (Fig. 3) with no loss in yield (79, 87 and 79% for **1a**, **1b** and **1c**, respectively, and ee, 99% for all three products) demonstrating the applicability of this optimized process for larger scale bioreduction.

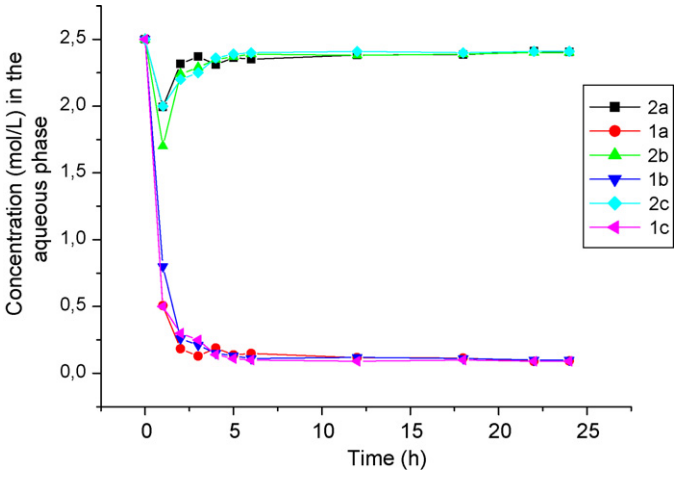


Fig. 2. Interaction of starting materials (**1a**–**c**, 2.5 mmol L^{-1}) and products (**2a**–**c**) with alginate beads in aqueous phase after 25 h.

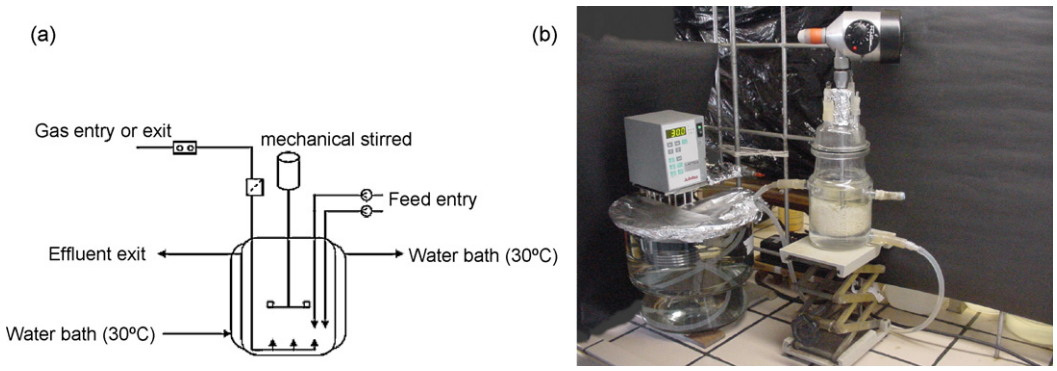


Fig. 3. (a) Schematic representation of the bioreactor and (b) photo of 400 mL home built stirred-tank bioreactor used in scale up bioreductions.

4. Conclusion

Screening experiments were carried out in order to identify the most active yeast for enantioselective reduction of each substrate **1a–c** with low hydrolytic activity and to determine the most favorable reaction conditions. The screened yeasts *P. kluyveri*, *P. stipitis* and *C. utilis* gave the best results as evaluated by yields and ee for ethyl benzoylacetate **1a**, *p*-nitrobenzoylacetate **1b** and *p*-methoxybenzoylacetate **1c**, respectively. The presence of electron donating and electron withdrawing groups at *para* position does not affect the enantioselectivity while the reaction rates were in agreement with the electronic nature of the substituent. Among the three variables that were selected for investigation, both glucose and α-chloroacetophenone improved the enantioselectivity giving products with 99% ee and showing no significant effect on reactions yields and a significant improvement in the yields was achieved by immobilizing the biocatalysts. Alginate is a good matrix for the biocatalyst immobilization due to the favorable substrate/product/alginate partitions. The scale up reaction with **1a–c** was carried out successfully in a 400 mL stirred-tank reactor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2008.04.009.

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