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# Immobilized microorganisms in the reduction of ethyl benzoylacetate

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The enantioselective reduction of ethyl benzoylacetate (EBA) into ethyl (*S*)-3-hydroxy-3-phenylpropanoate (*S*-HPPE) by nine yeast strains and three filamentous fungi strains is described. The conversion obtained was in the range 0–89% and the enantiomeric excess was 100% in many cases. Conversion levels were higher when the reduction was performed with microorganisms immobilized in calcium alginate and enantioselectivity remained excellent. Some reaction's conditions of bioreduction by immobilized cells of *Rhodotorula rubra* were studied using a  $2^{5-2}$  fractional factorial design.

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Optically pure  $\beta$ -hydroxyesters are important chiral building blocks in organic synthesis. Biocatalytic methods to obtain these compounds are attracting much attention, due to their high selectivity and environmentally friendly appeal. Biocatalytic approaches include asymmetric reduction and kinetic resolution.<sup>1</sup> Kinetic resolution has the limited yield of each enantiomer (maximum 50%) as disadvantage. On the other hand, asymmetric reduction can provide up to 100% of the desired isomer.<sup>2</sup> Isolated enzymes and whole cells are possible catalysts for B-ketoesters bioreductions leading to β-hydroxyesters. The use of microbial whole cells is advantageous because they contain the necessary co-factors (NADH and NADPH) and the metabolic pathways for their regeneration, as well as low cost.<sup>3</sup> Saccharomyces cerevisiae is the most used chiral catalyst for β-ketoesters bioreduction, but this microorganism does not always afford β-hydroxyesters with satisfactory enantioselectivity. So, other biocatalysts must be screened to be used as alternative.3-5

Ethyl (*S*)-3-hydroxy-3-phenylpropanoate (*S*-HPPE) is an important pharmaceutical intermediate for the synthesis of fluoxetine, a serotonin uptake inhibitor used against depression, anxiety, obesity, chronic pain, and bulimia.<sup>6</sup> This chiral synthon can be obtained by enantioselective bioreduction of ethyl benzoylacetate (EBA) (Fig. 1).

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In a previous work, we demonstrated that *Geotrichum* sp., *Dekera* sp., and *Kluyveromyces marxianus* could be also useful in EBA bioreduction.<sup>5</sup> Thus, in this work, we aimed to screen other possible microorganisms to be used as biocatalysts for this reaction.

Free cells of 12 microorganisms (nine yeast strains and three filamentous fungi strains) were tested for EBA reduction ability (Table 1).<sup>7</sup> There was no reduction in presence of *Hansenula* sp. and *Aspergillus niger* in the experimental conditions used. All other microorganisms were able to reduce the substrate with excess of the (*S*)-isomer, but only *Pichia* sp., *K. marxianus, Rhodotorula rubra, Rhodotorula minuta,* and *Mucor ramannianus* led to more than 50% of conversion. In the biotransformations catalyzed by *R. minuta, Pichia* sp., and *K. marxianus,* 100% of enantiomeric excess (ee) was obtained for *S*-HPPE.

These data were better than the ones reported by Milagre et al.<sup>8</sup> with various microorganisms, including another *R. minuta* strain, in the absence of inhibitor. Despite lower than 100% conversion, our



**Figure 1.** Microbial reduction of ethyl benzoylacetate (EBA) to ethyl-(*S*)-3-hydroxy-3-phenylpropanoate (*S*-HPPE).



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Table 1	
Microbiological reduction of EBA to S-HPPE with free co	ells

Microorganism	Conversion (%)	ee (%)
Saccharomyces cerevisiae 40	18	100
Saccharomyces cerevisiae 60	34	100
Saccharomyces cerevisiae 80	22	100
Pichia sp.	51	100
Hansenula sp.	0	-
Candida sp.	23	100
Kluyveromyces marxianus	59	100
Rhodotorula rubra	61	96
Rhodotorula minuta	57	100
Trichoderma harzianum	19	13
Mucor ramannianus	89	19
Aspergillus niger	0	_

Incubation: 30 °C, 150 rpm, 24 h.

results showed higher enantiomeric excess than obtained by Chênevert et al.<sup>9</sup> with *S. cerevisiae* (87–93%) and recently by He et al.<sup>10</sup> with *Bacillus pumilus* (95.7% ee). It was comparable with that obtained by Cha et al.<sup>4</sup> with *Kluyveromyces lactis*.

When *K. marxianus* was used in the reduction of ethyl acetoacetate, the (*R*)-hydroxyester was the major product.<sup>11,12</sup> *A. niger* and *Hansenula* sp. were able to reduce ethyl acetoacetate with at least 85% of conversion.<sup>11,12</sup> So, conversion level and enantioselectivity depend on substrate's structure, as described previously by other researches.<sup>13-15</sup>

The entrapment of cells in calcium alginate, an immobilization technique frequently used in biocatalytic production of chemicals, can influence in product configuration, enantiomeric excess and conversion level.<sup>5,8,11,16</sup> To evaluate these effects, *S. cerevisiae* 40, *Pichia* sp., *Hansenula* sp., *Candida* sp. *K. marxianus*, and *R. rubra* were immobilized in calcium alginate spheres and tested again for EBA reduction ability (Table 2).<sup>17</sup>

Besides the excellent enantiomeric excess obtained (100% ee), product recovery was much easier after immobilization than when free cells were used. After immobilization, *S. cerevisiae* 40, *K. marxianus*, and *R. rubra* provided higher conversion levels in comparison with free cells used in the first step of this study. The most important difference was observed with *S. cerevisiae* 40: with free cells conversion was only 18%, while 78.5% of conversion was obtained with immobilized cells. Milagre et al.<sup>8</sup> also verified an increase on conversions of EBA after immobilization in calcium alginate spheres, probably due to the high affinity of the alginate matrix for this substrate and low affinity for the product.

The maximum conversion level was obtained with *R. rubra* immobilized cells that provided 84% conversion after 24 h-incubation-period. *K. marxianus* was also considered as a good biocatalyst and furnished 70% of conversion. These results were better than that obtained by our group in a previous study, when the best conversion was 76% obtained with free cells of *Geotrichum* sp.,<sup>5</sup> *Hansenula* sp., and *K. marxianus*, showed different conversion levels and enantioselectivity in comparison with the earlier work.<sup>5</sup> This difference could be attributed to pH: in the present study, the ini-

### Table 2

Microbiological reduction of EBA to S-HPPE with immobilized cells

Microorganism	Conversion (%)	ee (%)
S. cerevisiae 40	78.5	100
Pichia sp.	56	100
Hansenula sp.	0	-
Candida sp.	25	100
K. marxianus	70	100
R. rubra	84	100

Incubation: 30 °C, 150 rpm, 24 h.

tial pH of the reaction's medium was 5.5, while in the previous study it was adjusted to 6.5.

Due to good results obtained with immobilized cells of *R. rubra*, a  $2^{5-2}$  fractional factorial design<sup>18–21</sup> was used to study conversion levels and enantioselectivity of the reaction with this biocatalyst. Five variables were studied: biomass concentration (*Cell*), diameter of calcium alginate spheres (*diam*), substrate concentration (*EBA*), magnesium chloride concentration (*MgCl*<sub>2</sub>), and glucose concentration (*G*). Three central point replicates were accomplished. Experimental domain and results are shown in Table 3. Response variables were % conversion and % ee after 18 h-incubation-period. All experimental runs furnished excess of *S*-HPPE.

According to Table 3, excellent enantioselectivity (100% ee) was obtained in all cases. So, only conversion level should be maximized in the experimental domain studied. Conversions obtained after 18 h varied from 5.0% to 75.2%. The highest conversions ( $\geq$ 72.5%) were achieved in experimental entries 5 and 7, when maximum biomass concentration and minimum substrate concentration were used. In both conditions, conversion and enantioselectivity were better than obtained with free cells of *R. rubra* (61% conversion and 96% ee—Table 1).

Data were evaluated by means of analysis of variance (ANOVA), shown in Table 4. Confidence level was set at 95%. Only biomass concentration, diameter of calcium alginate spheres, EBA concentration and the interaction between concentrations of biomass and EBA were significant parameters in experimental domain studied and were considered in the model below (normalized variables).

$$\% \text{ conversion} = 33.1 + 17.2 \times Cell + 3.80 \times diam - 15.8 \times EBA - 8.25 \times Cell \times EBA$$
(1)

where *Cell*: biomass concentration; *diam*: diameter of calcium alginate spheres; *EBA*: substrate concentration.

The coefficient of determination  $(r^2)$  was 0.99, what means that the model explains 99% of variability in response. Adjusted  $r^2$  statistic was also high (0.98). Analysis showed that curvature was not important to the model (*p*-value = 0.378) and that the linear model obtained to describe % conversion was suitable for observed data.

Biomass and diameter of calcium alginate spheres constituted positive effects on conversions within the experimental domain. Quite the contrary, substrate concentration showed a negative effect. The addition of the salt MgCl<sub>2</sub> did not affect conversions, as also observed by Houng et al.<sup>22</sup> in the ethyl 4-chloroacetoacete reduction employing baker's yeast as biocatalyst. Variation of

Table 3

Experimental design to improve reaction conditions of EBA reduction to S-HPPE by immobilized cells of *R. rubra* 

Entries	Factors				Responses		
	Cell (gdw/L)	diam (mm)	EBA (mM)	MgCl <sub>2</sub> (g/L)	G (g/L)	Conversion (%)	ee (%)
1	2.0	2.4	26	0	10	15.8	100
2	2.0	2.4	52	2	50	5.0	100
3	2.0	3.8	26	2	50	30.1	100
4	2.0	3.8	52	0	10	10.7	100
5	6.0	3.8	26	0	50	75.2	100
6	6.0	3.8	52	2	10	29.6	100
7	6.0	2.4	26	2	10	72.5	100
8	6.0	2.4	52	0	50	21.9	100
9	4.0	3.1	39	1	30	33.9	100
10	4.0	3.1	39	1	30	36.8	100
11	4.0	3.1	39	1	30	32.3	100

Incubation: 30 °C, 150 rpm, 18 h.

\* *Cell* (biomass concentration—g dry weight/L); *diam* (diameter of calcium alginate spheres); *G* (glucose).

# Table 4 ANOVA for EBA bioreduction by R. rubra immobilized cells

Factor	Sum of squares	df	Mean squares	F-ratio	p-value
Cell	2366.72	1	2366.72	454.85	0.002
diam	115.52	1	115.52	22.20	0.042
EBA	1997.12	1	1997.12	383.82	0.003
$MgCl_2$	23.12	1	23.12	4.44	0.170
G	1.62	1	1.62	0.31	0.633
$Cell \times diam$	11.52	1	11.52	2.21	0.275
$Cell \times EBA$	544.50	1	544.50	104.64	0.009
Lack of fit	6.56	1	6.56	1.26	0.378
Pure error	10.41	2	5.20		
Total (corr.)	5077.08	10			

Response: % conversion ( $r^2 = 0.99$ ,  $r^2$  adjusted for df = 0.98).

\* *Cell* (biomass concentration—g dry weight/L); *diam* (diameter of calcium alginate spheres); *G* (glucose).



**Figure 2.** Chiral GC analysis on column BGB-176B ( $25 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ µm}$ ), at 140 °C: (A) EBA; (B) *S*-HPPE (racemate obtained via NaBH<sub>4</sub> reduction); (C) immobilized *R. rubra* reduction (reaction conditions selected according fractionary factorial design).

glucose concentration had no significative effect on response and it was used at minimum level.

Based on these results, the following conditions were selected to EBA reduction by immobilized cells of *R. rubra*: biomass, 6.0 g dry weight/L; diameter, 3.8 mm; EBA, 26 mM; glucose, 10 g/L.

Experiments, performed in triplicate, were conducted to verify conversion level and enantiomeric excess in the reaction conditions described above. After 18 h, it was obtained 81% conversion (SD = 1.3%) and 100% ee. This result matched the model prediction. The high stereoselectivity achieved in this reaction is shown in Figure 2. Optimization studies are in progress.

In conclusion, a screening study indicated some wild yeast strains with excellent enantioselectivity in EBA reduction. Beyond providing good and easy recovery of product, immobilized cells in calcium alginate spheres led to higher conversion levels in comparison with free cells used. After a fractionary factorial design to study some reaction's conditions, calcium alginate immobilized cells of *R. rubra* furnished 81% conversion with 100% ee. There are few studies with immobilized yeasts in EBA reduction and, to our knowledge, this is the first Letter on the use of *R. rubra* for enantioselective reduction of EBA.

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- 21. Experimental design: A 2<sup>5-2</sup> fractionary factorial design<sup>18</sup> was used to study five variables in eight runs with three replicates of the central point. Variables and domain were: biomass concentration (*Cell*): 2.0–6.0 g dry weight/L; diameter of calcium alginate spheres (*diam*): 2.4–3.8 mm; EBA: 26–52 mM; MgCl<sub>2</sub>: 0–2 g/L; glucose: 10–50 g/L. Response variables were % conversion and % ee. Cells of *R. rubra*, obtained as described above,<sup>7</sup> was re-suspended 1.5% sodium alginate solution (50 mL). Immobilization was performed as already described.<sup>17</sup> After 30 min of immobilization, the substrate in aqueous-ethanol was added to the medium. Reaction's medium composition was according experimental design. The reaction was carried out in 500 mL cotton plugged Erlenmeyer flasks containing 100 mL of medium for 18 h at 30 °C and 150 rpm. After that period, medium was filtered to separate the biocatalyst and the liquid phase was treated as described above.<sup>7</sup> Statistical analyses were performed using Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA).
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