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Short Communication

Whole cells in enantioselective reduction of *benzyl* acetoacetate

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Abstract

The β-ketoester benzyl acetoacetate was enantioselectively reduced to benzyl (*S*)-3-hydroxybutanoate by seven microorganism species. The best result using free cells was obtained with the yeast *Hansenula sp.*, which furnished 97% *ee* and 85% of conversion within 24 h. After immobilization in calcium alginate spheres, *K.marxianus* showed to be more stable after 2 cycles of reaction.

Key words: biotransformation, chiral reduction, β-ketoester, *Kluyveromyces marxianus*, immobilization.

Chiral β-hydroxy esters are important synthons for the synthesis of numerous pharmaceuticals (Amidjojo and Weuster-Botz, 2005; Banfi *et al.*, 1994; Chartrain *et al.*, 1996; Colle *et al.*, 1999) such as β-lactam antibiotics (Banfi *et al.*, 1994), reductase-inhibitors (Amidjojo and Weuster-Botz, 2005; Banfi *et al.*, 1994; Chartrain *et al.*, 1996; Colle *et al.*, 1999), fluoxetine (Corey and Reichard, 1989; Ribeiro *et al.*, 2005), HMGCoA dihydrokawain (a narcotic) (Spino *el al.*, 1996), L-carnitine (a nutraceutical) (Nakamura *et al.*, 2003) and enantiomerically pure sympatholytic non-selective beta blockers, as propranolol, alprenolol and 1-(isopropylamino)-3-*p*-methoxy-phenoxy-2-propanol (Wunsche *et al.*, 1996). Both enantiomers of ethyl 3-hydroxy butanoate and ethyl 3-hydroxy pentanoate are useful starting material for the synthesis of pheromones (Mori, 1989; Ramos *et al.*, 2009). This range of applications becomes very interesting to the asymmetric synthesis of β-hydroxy esters. Asymmetric reduction of prochiral ketoesters is an alternative route. Bioreductions are attractive methods, mainly due to high enantioselectivity, mild and safe reaction conditions, and lower environmental impact compared to conventional reactions in organic chemistry (Ramos *et al.*, 2011).

In recent years we have been investigating the microbial reduction of ketoesters (Ramos *et al.*, 2009a, 2009b, 2011; Ribeiro *et al.*, 2005, 2009). In the present work, seven wild-type microorganism species were employed in the asymmetric reduction of benzyl acetoacetate to benzyl (*S*)-3-hydroxybutanoate (Figure 1). Some yeasts were also tested after immobilization in calcium alginate spheres.

Microorganisms, medium, growth conditions, and biotransformation with free cells: *Saccharomyces cerevisiae, Hansenula* sp., *Geotrichum candidum*, *Kluyveromyces marxianus*, *Rhodotorula rubra*, *Aspergillus niger*, and *Trichoderma harzianum* belong to the collection of the `Departamento de Engenharia Bioquímica, Escola de Química, Universidade Federal do Rio de Janeiro (Cidade Universitária, CT Bloco E, Rio de Janeiro, Brazil)'. Cells were allowed to grow for 48 h, under 150 rpm at 30 °C in a medium containing 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% $(NH_4)_2SO_4$, and 0.1% $MgSO_4.7H_2O$. After this period, the cells were collected by centrifugation, re-suspended in water and used for the reaction without further purification. After centrifugation, the cells (4 g/L, dried weight) were added to the reaction medium containing: glucose (5%) in a final volume of 50 mL. After 30 min of addition of the microorganisms, the substrate (0.25 g diluted in 1 mL of ethanol 96%) was added to the medium. The reaction was carried out in 500 mL cotton-plugged Erlenmeyer flasks for 24 h at 30 °C and 150 rpm. After 24 h, the medium was centrifuged again to separate the cells and the liquid phase was extracted with

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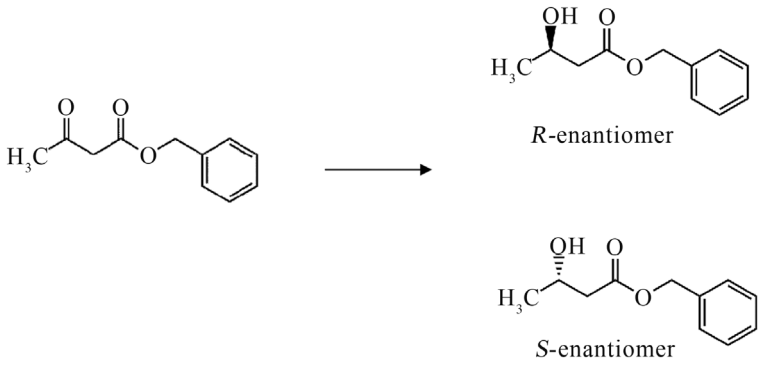
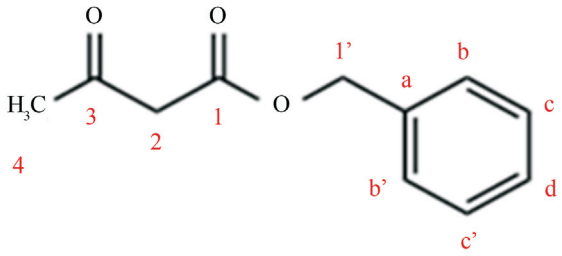


Figure 1 - Reduction of Benzyl acetoacetate.

ethyl acetate. The organic phase was dried (anhydrous Na_2SO_4), filtered, and concentrated under vacuum. Conversions and enantiomeric excesses were determined by (chiral) gas chromatography (GC), on column Gama Dex 225 (30 m x 0.25 mm x 0.25 µm), at 110 °C (62 min). The elution order was: benzyl (*S*)-3-hydroxybutanoate (t_R = 49.9 min) followed by benzyll (*R*)-3-hydroxybutanoate (t_R = 51.0 min). Substrate was eluted at 57.3 min. The racemate obtained by $NaBH_4$ reduction was characterized by nuclear magnetic resonance (NMR).

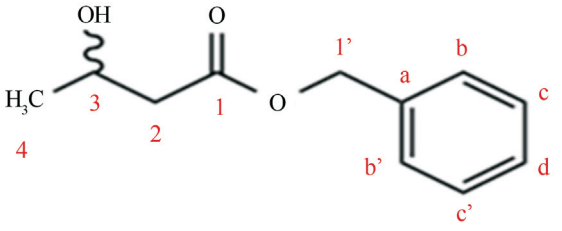
Immobilization of cells (*Kluyveromyces marxianus*, *Rhodotorula rubra* and *Hansenula* sp.) in calcium alginate and biotransformation. Cells grown during 48 h in the medium described before were centrifuged and 0.8 g (dry weight) was re-suspended in 3 mL of distilled water to obtain a cell-suspension. A 1.5% sodium alginate aqueous solution (20 mL) was added and this mixture (cell suspension sodium alginate aqueous solution) was dropped into a $CaCl_2$ aqueous solution (0.1 M) to form calcium alginate spheres. Spheres were filtered, washed with distilled water and added to the medium containing: glucose (5%) and substrate (0.25 g diluted in 1 mL of ethanol 96%) in a final volume of 50 mL. The reaction was carried out in 500 mL cotton-plugged Erlenmeyer flasks, at 30 °C, 150 rpm during 24 h. After this period, medium was filtered to separate the biocatalyst and the liquid phase was treated as described above. Immobilized cells were washed with distilled water and maintained at 4 °C in a $CaCl_2$ aqueous solution (0.1 M) for 7 days. Thus, the immobilized cells were incubated in growth medium under 150 rpm at 30 °C for 2 h, washed with distilled water again, and reused under the same reaction's conditions.

Optical rotations were measured from $CHCl_3$ solutions using a JASCO DIP-370 polarimeter at the sodium D line (589 nm) operating at room temperature and compared to literature (Medson *et al.*, 1997). $[\alpha]_D^{23}$ + 21.9 º (75% optical purity; *c* 1 g/100 mL; $CHCl_3$), literature: $[\alpha]_D^{23}$ + 29.0º ($CHCl_3$, c 1).



^{1}H NMR spectrum of benzyl 3-oxobutanoate (200 MHz, $CDCl_3$) δ (ppm): 2.25 (s,H, H4); 3.5 (s, 2H, H1'); 5.18 (s, 2H, H2); 7.36 (s, 5H, Hb, Hb', Hc, Hc', Hd).

^{13}C NMR spectrum of benzyl 3-oxobutanoate (50 MHz, $CDCl_3$) δ (ppm): 30.3 (C4); 50.1 (C1'); 67.3 (C2); 128.6 (Cc and Cc'); 128.7 (Cd); 128.8 (Cb and Cb'); 135.5 (Ca); 167.0 (C1); 200.5 (C3).



^{1}H NMR spectrum of benzyl 3-hydroxybutanoate (racemate obtained via $NaBH_4$ reduction). Characterization: (200 MHz, $CDCl_3$) δ (ppm): 1.23 (s, 3H, H4); 2.50 and 2.53 (m, 2H, H2); 2.79 (s, 1H, OH); 4.23 (m, 1H, H3); 5.15 (s, 2H, H1'); 7.36 (s, 5H, Hb, Hb', Hc, Hc', Hd).

^{13}C NMR spectrum of benzyl 3-hydroxybutanoate (racemate obtained via $NaBH_4$ reduction).Characterization: (50 MHz, $CDCl_3$) δ (ppm): 22.7 (C4); 43.1 (C1'); 64.5 (C3); 66.7 (C2); 128.4 (Cc and Cc'); 128.6 (Cd); 128.8 (Cb and Cb'); 135.8 (Ca); 172.8 (C1).

In previous studies, these microorganisms that were used on this work were successfully used in the reduction of methyl acetoacetate (Ramos *et al.*, 2009a), ethyl acetoacetate (Ramos *et al.*, 2009a), ethyl 2-methylacetoacetate (Ramos *et al.*, 2009a), ethyl

benzoylacetate (Ramos *et al.*, 2009b; Ribeiro *et al.*, 2005) and ethyl 4-chloroacetoacetate (Ribeiro *et al.*, 2009). However, excess of the (*S*)-hydroxyester was obtained in almost all of the cases. Only *K. marxianus*, *T. harzianum* and *Aspergillus niger* led to the excess of the (*R*)-hydroxyester in the reduction of ethyl acetoacetate (67% *ee*, 51% *ee* and 19% *ee*, respectively) and *A. niger* maintained the (*R*)-enantioselectivity in the reduction of methyl acetoacetate (45% *ee*). So, enantioselectivity depends critically on the length of carbon chain, as also observed by other researchers (Ishihara *et al.*, 2003; Nakamura *et al.*, 2003).

In literature, there are few studies using benzyl acetoacetate. One of them was published by Medson *et al.* (1997), that related this reaction using an organic solvent and baker yeast (*Saccharomyces cerevisiae*) Using this system, they obtained 72% of isolated yield and 94% of *ee* of the (*S*)-enantiomer. In aqueous system, the substrate was not reduced. They also verified that the yield of isolated material varied considerably with the nature of the ester group: compounds with the larger ester groups react at a slower rate, possibly to increased steric interactions with the enzyme binding site. We also observed that, in general, ours results of conversion were better with smaller ester groups (Ramos *et al.*, 2009a), in decreasing order of conversion level with the following ester groups: ethyl > methyl > tert-butyl > benzyl. In other work, Chartrain *et al.* (1996) described the asymmetric reduction of benzyl acetoacetate using some yeasts species and they obtained preferentially the (*S*)-enantiomer, so we did. Chartrain *et al.* (1996) achieved conversions between less than 20% and up to 95%, while we achieved conversions between 27% and up to 99% using other yeasts strains. The *ee* obtained by them varied between 0.6 and 93% while our results varied between 51 and 97% *ee*.

In the first step of this work, free cells of five yeasts (*Saccharomyces cerevisiae* 40, *Hansenula* sp., *Geotrichum candidum*, *Kluyveromyces marxianus* and *Rhodorotula rubra*) and two filamentous fungi (*Trichoderma harzianum* and *Aspergillus niger*) were tested (Table 1). All strains were able to catalyze the reaction with excess of the (*S*)-hydroxyester (determined by measurement of optical rotation) (Medson et al., 1997) with conversion rate between 4 up to 99% within 24 h. The enantiomeric excess varied from 51% (with *A.niger*) to 97% (with *Hansenula* sp.).

In addition the immobilized biocatalyst is more expensive than the free cells. This new catalyst gives the same yield and *ee* (under experimental error) than resting cells. Finally the separation of whole cells from the reaction products is easy using organic solvents. Therefore, downstream processes are not improved. We used only the yeasts, *Hansenula* sp., *K. marxianus* and *R. rubra*, immobilized in calcium alginate spheres and tested for their benzyl (*S*)-3-hydroxybutyrate reduction ability in two cycles (Table 1). This entrapment technique makes the products recovery much easier and the biocatalyst can be readily reused. However, the immobilization can influence on enantiomeric excess and conversion level (Ramos *et al.*, 2009b;Ribeiro *et al.*, 2005; Ribeiro *et al.*, 2009).

As shown in Table 1, immobilized cells of *K. marxianus* exhibited the best results because they led to high enantioselectivity (62% *ee*) and conversion (78%) even after the second cycle, with storage of 7 days between cycles. The yeast *Hansenula* sp. gave the same conversion and enantioselective in the first cycle, but with decrease of conversion in the second cycle. In the first use of immobilized cells of *R. rubra* it was observed a little decrease in the enantioselectivity, but the conversion obtained was better and, in the second cycle, lower conversion was obtained. So, *K.marxianus* showed to be more stable, allowing the re-use of the microorganism even after 7 days of storage, *R.rubra* and *Hansenula* sp. showed low conversion after the 7 days of storage.

In conclusion, the yeast *G. candidum* led to high conversion of benzyl acetoacetate to benzyl (*S*)-3-hydroxybutyrate (> 99%) with high enantioselectivity (81% *ee*) and *Hansenula* sp. led to the high enantioselective, using this

Table 1 - Bioreduction of Benzyl acetoacetate using free and immobilized cells.

	Free cells		Immobilized cells			
			First cycle		Second cycle (after 7 days)	
Microorganisms	Conversion (%)	e.e. (%)	Conversion (%)	e.e. (%)	Conversion (%)	e.e. (%)
Hansenula sp.	85	97	85	91	8	86
K. marxianus	80	68	99	76	78	62
R. rubra	72	86	96	75	7	83
S. cerevisiae	27	83				
T. harzianum	61	56				
A. niger	4	51				
G. candidum	99	81				

microorganism was possible to obtain 97% of the *S*- isomer. Using immobilized cells, *K. marxianus* gave the best conversion. To our knowledge, this is the first Communication on the use of these microorganisms in the reduction of benzyl acetoacetate. The high conversion, high enantiomeric excess and the possibility of immobilization and reuse make *K. marxianus* a promising biocatalyst for industrial applications.

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