Bioreduction of Some Common Carbonylic Compounds Mediated by Yeasts

Javier Silva[a], Julio Alarcón[b], Sergio A. Aguila[a], and Joel B. Alderete[a,*]

[a] Departamento de Organica, Facultad de Ciencias Químicas, Universidad de Concepción, Edmundo Larenas 129, Concepción, Chile. Fax: +56-41-245974. E-mail: jalderet@udec.cl
[b] Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad del Bio-Bio, A. Bello S/N, Chillán, Chile

* Author for correspondence and reprint requests

Bioreduction of several prochiral carbonylic compounds such as acetophenone (1), ethyl acetoacetate (2) and ethyl phenylpropionate (3) to the corresponding optically active *sec*-alcohols 1a–3a was performed using wild-type strains of *Pichia pastoris* UBB 1500, *Rhodotorula* sp., and *Saccharomyces cerevisiae*. The reductions showed moderate to excellent conversion and high enantiomeric excess, in an extremely mild and environmentally benign manner in aqueous medium, using glucose as cofactor regeneration system. The obtained alcohols follow Prelog's rule, but in the reduction of 1 with *P. pastoris* UBB 1500 the anti-Prelog enantiopreference was observed.

Key words: Yeast, Enantioselective Reduction

Introduction

The asymmetric synthesis is an area extensively explored by organic chemists. It is based on the production of synthetic derivatives with a specific stereochemistry. The synthesis of enantiomerically pure compounds has become an useful tool in the pharmaceutical and biologically active materials industries. Obtainment of chiral compounds is often quite difficult due to the number of steps required to reach the desired molecule, and often the use of expensive chiral reagents and environmentally dangerous heavy metals is necessary. For these reasons, the use of unconventional tools to solve this challenge should be evaluated (Adio, 2009; Crossley, 1992). Biocatalysis, alongside chemocatalysis, has now become a key component in the toolbox of chemical processes (Astudillo *et al.*, 2009; Hlavsova *et al.*, 2008; Pollard and Woodley, 2007). In comparison to most common methodologies, biocatalysis has the advantage of its chiral nature and mild reaction conditions. Biocatalysis is performed using either whole cells or isolated enzymes, whereas both applications have distinct different characteristic. Whole cells provide the enzyme with the perfect environment, and the main advantage is the *in vivo* recycling cofactors converting the whole cells into a readily cheap catalyst.

Optically active alcohols can be prepared by a number of routes including the reduction of carbonylic compounds. Reduction of prochiral carbonyl precursors to chiral *sec*-alcohols is special enzymatic reaction where cells have been also used (Goldberg *et al.*, 2007). Chiral alcohols are very useful materials especially in the chemical and pharmaceutical industries, where enantiopurity of drugs or building blocks synthesis is highly relevant (Chartrain *et al.*, 2001; Ishige *et al.*, 2005).

In general, bioreduction of prochiral carbonylic compounds with yeast generates the (*S*)-alcohol as predominant enantiomer. Thus, for example the production of (*R*)-1-phenylethanol, an important optically active compound which is widely used as fragrance in the cosmetic industry and also as ophthalmic preservative (Costa *et al.*, 2008), can not directly be obtained by yeast reduction of acetophenone.

This study reports the enantioselective bioreduction of acetophenone (1), ethyl acetoacetate (2) and ethyl phenylpropionate (3) mediated by *Pichia pastoris* UBB 1500, *Rhodotorula* sp., and *Saccharomyces cerevisiae*. The *sec*-alcohols obtained are useful intermediates in the asymmetric synthesis of biologically active compounds (Chenevert *et al.*, 1992; Zhu *et al.*, 2006), especially the reduction of 1 by *P. pastoris* UBB 1500 afforded the anti-Prelog (*R*)-alcohol.

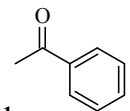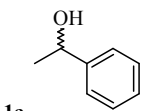## Results and Discussion
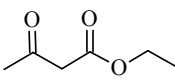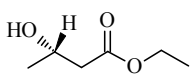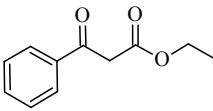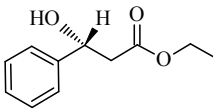
Reduction of acetophenone (1)

Acetophenone (**1**) is the most frequently used aromatic ketone in bio- or metal-mediated reduction studies. The enantioselective reduction of **1** using resting cells of *P. pastoris* UBB 1500 provided the respective (*R*)-(+)-1-phenylethanol (**1a**) with a high conversion rate (98%) and good enantiomeric excess (ee, 83%). The result showed the presence of different reductases in the cell (Fig. 1A). The (*R*)-enantiopreference according to the anti-Prelog rule is quite unusual. The studies on yeasts have shown that they transfer the pro-*R*-hydride to the *re*-face of the carbonyl group to give (*S*)-alcohols, a process described by Prelog's rule (Faber, 1997). To our knowledge, this is the first report on obtaining of (*R*)-1-phenylethanol by yeast reduction of **1** at higher conversion and enantiomeric excess, without the use of additive substances (Patel *et al.*, 2004; Zymanczyk-Duda *et al.*, 2005).

Rhodotorula sp. (UBB 2009, Laboratorio de Microbiologia, Universidad del Bio-Bio, Chillán, Chile) was the most enantioselective strain for bioreduction of **1**, which resulted in (*S*)-(-)-**1a** with a high conversion rate (99%) and excellent enantiomeric excess (> 99%) (Fig. 1B). This result shows high stereoselectivity of the *Rhodotorula* species. Thus, *Rhodotorula* sp. could be a good alternative towards acetophenone derivatives reduction. Bioreduction by *Rhodotorula* sp. obeyed Prelog's rule, showing the same enantiomeric preference already observed with baker's yeast (*S. cerevisiae*) (de Carvalho *et al.*, 1991).

Biocatalytic reactions carried out with *S. cerevisiae* yielded (*S*)-(-)-**1a** with low conversion (10%) and good enantiomeric excess (94%) (Fig. 1C). After 20 h the bioconversion reached just a transformation of 10%, and there was no significant change until the end of the reaction (48 h) (Table I). Under the established analytical methods any other compounds were detected in the reaction medium (Table II). In the case of baker's yeast (*S. cerevisiae*) it has been reported that reductions of acetophenone derivatives produce (*S*)-alcohols in low to moderate yield and higher enantiomeric excess (Csuk and Glaenzer, 1991). These results indicate that evaluation of the same *S. cerevisiae* species does not always show the same outcome, since the process depends on parameters like nature of nutrients, substrate concentration, temperature, cell age, pH or additives present in the medium (Fantin *et al.*, 1994; Nakamura *et al.*, 1984).

Table I. Reduction of prochiral carbonylic compounds by yeasts.

Substrate	Product	Micro-organism	Reaction time [h]	Confor-mation	Conversion (%)	ee (%)
1	**1a**	*P. pastoris* *Rhodotorula* *S. cerevisiae*	48	*R* *S* *S*	98 99 10	83 >99 94
2	**2a**	*P. pastoris* *Rhodotorula* *S. cerevisiae*	3	*S* *S* *S*	100 38 100	>99 >99 >99
3	**3a**	*P. pastoris* *Rhodotorula* *S. cerevisiae*	12	*S* – *S*	19 – 68	>99 – >99

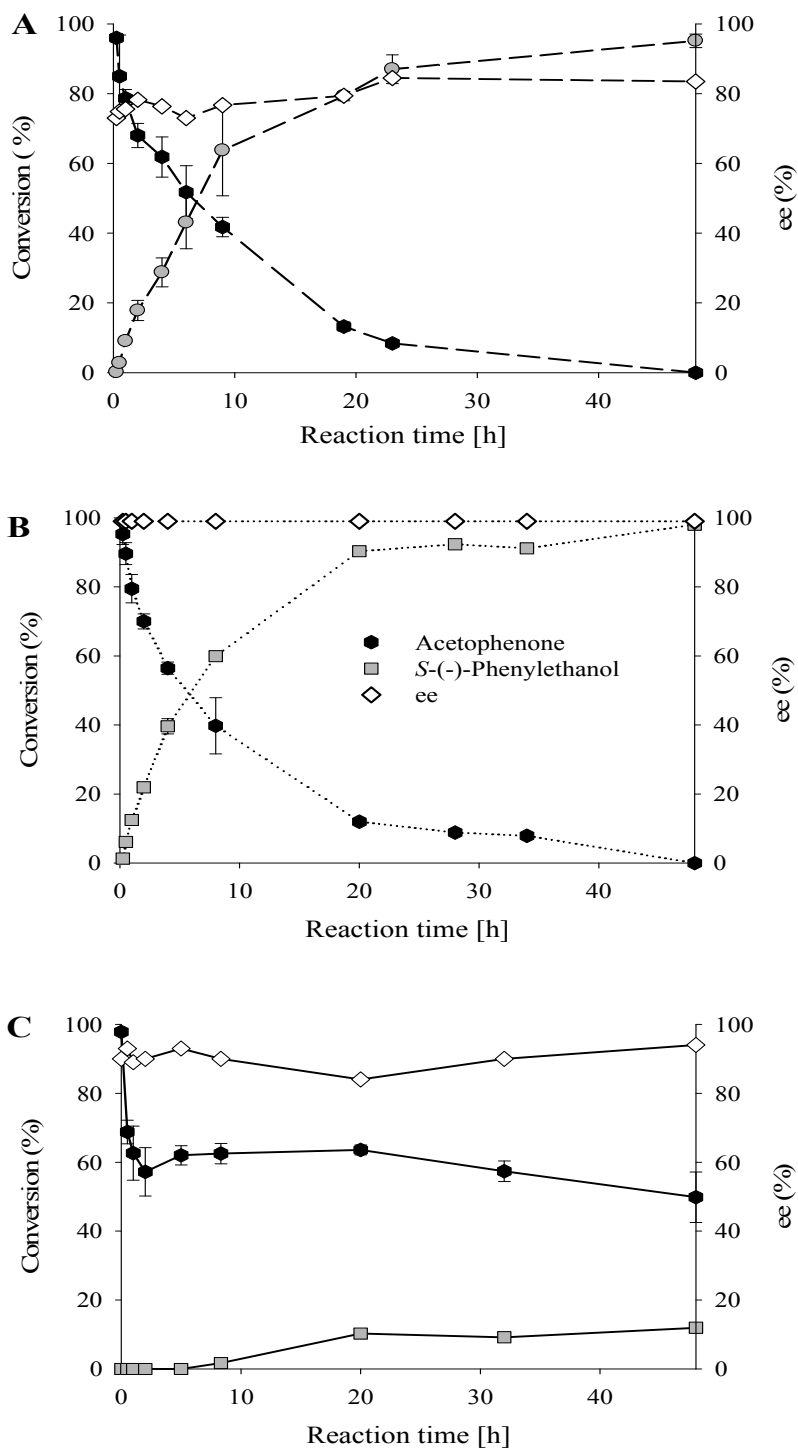The bioreductions were carried out with 200 mM glucose, at 30 °C and 120 rpm.

Fig. 1. Time course of conversion (200 mм glucose, at 30 °C and 120 rpm) and enantioselectivity (ee, enantiomeric excess) of acetophenone (**1**) reduction by resting cells of (A) *P. pastoris* UBB 1500, (B) *Rhodotorula* sp., and (C) *S. cerevisiae*.

Table II. Chromatographic analysis of substrates and products of biotransformations.

Substrate/ product	Analyzing method (HPLC/RP-18)/ conditions; retention time [min]	Determination method of (*R*) and (*S*)-alcohols/ GC/CP-cyclodextrin, inj.: 250 °C, det.: 280 °C/ conditions; retention time [min]
1/1a	H_2O/ACN (55:45), 0.9 ml/min, at λ = 220 nm; **1a**: 4.8, **1**: 7.1	column: 100 °C, hold 15 min, 20 °C/min to 120 °C; **1**: 10.8, (*R*)-**1a**: 16.9, (*S*)-**1a**: 17.8
2/2a	H_2O/ACN (70:30), 0.7 ml/min, at λ = 213 nm; **2a**: 5.2, **2**: 7.0	column: 70 °C; **2**: 33.6, (*S*)-**2a**: 43.6, (*R*)-**2a**: 45.9
3/3a	H_2O/ACN (55:45), 0.9 ml/min, at λ = 220 nm; **3a**: 6.2, **3**: 9.7	column: 150 °C; **3**: 12.3, Acyl-(*S*)-**3a**: 15.8, Acyl-(*R*)-**3a**: 17.7

*Reduction of ethyl acetoacetate (**2**)*

The *β*-keto esters is one of the most studied classes of compounds in enantioselective reactions (Mori, 1989; Nakamura *et al.*, 1995; North, 1996; Salvi and Chattopadhyay, 2004; Spiliotis *et al.*, 1990; Turcu *et al.*, 2007; Ushio *et al.*, 1991). Its products, *β*-hydroxy esters, are extremely useful as building blocks for the synthesis of a large number of bioactive compounds, intermediates and chiral auxiliaries (Ishihara *et al.*, 2003; Salvi and Chattopadhyay, 2006).

The reduction of ethyl acetoacetate (**2**) mediated by *P. pastoris* UBB 1500 produces ethyl (*S*)-(+)-3-hydroxybutanoate (**2a**) with a high conversion rate (100%) and excellent enantiomeric excess (>99%) (Fig. 2A). The high conversion and enantiopreference suggest that enzymes inside cells can easily differentiate between the small and large groups flanking the carbonyl function.

When **2** was reduced with *Rhodotorula* sp., only 38% of conversion to (*S*)-**2a** was reached (>99% ee) (Fig. 2B). This poor conversion should be the result of the action of other enzymes that can decarboxylate the substrate. The starting compound **2** is a substrate not only for keto reduction but also for esterases with far-reaching consequences. Hydrolysis of ester **2** results in ethanol and the corresponding 3-oxobutyric acid while the hydrolysis of (*S*)-**2a** produces 3-hydroxybutyric acid. These hydrolysis products can probably be metabolized further by the yeast cells into biomass or into products like acetic acid (via acetylCoA), acetone and carbon dioxide via chemical decarboxylation or acetoacetate decarboxylase (Chin-Joe *et al.*, 2000).

In the presence of *S. cerevisiae* compound **2** was biotransformed to (*S*)-**2a**. The *S*-enantiomer was obtained with a total conversion within 4 h with excellent enantiomeric excess (>99%) (Fig. 2C).

It proves that ethyl acetoacetate was a very good substrate for *S. cerevisiae*. Thus, *P. pastoris* and *S. cerevisiae* demonstrated to be excellent bioreduction systems for the enantiomeric synthesis of (*S*)-**2a** (Table I).

*Reduction of ethyl phenylpropionate (**3**)*

Ethyl phenylpropionate (**3**) is a very bad substrate or a non-substrate in most of bioreductions mediated by microorganisms (Havel and Weuster-Botz, 2006; Kroutil *et al.*, 2004). **3** possesses an ester-demanding group on the carbonyl moiety, that blocks the interaction with the active site of reductases. Our experiments showed that the concentration of **3** in the medium was instantaneously diminished (by around 70%) (Fig. 3). This behaviour could be explained as a fast absorption or adsorption of the ester by the yeasts due to its low solubility in water (Hussein *et al.*, 2007).

Bioreduction carried out with *P. pastoris* UBB 1500 produced ethyl (*S*)-(-)-3-hydroxy-3-phenyl-propionate (**3a**) with a low conversions (19%), but excellent enantiomeric excess (>99%) that remained constant during the entire course of the reaction (12 h) (Fig. 3A). Other polar compounds were observed during HPLC analysis, but they were not identified. It is possible that these compounds were products of other enzyme activity (lipases, estereases) (Ribeiro *et al.*, 2001).

When the reaction was performed with *Rhodotorula* sp., **3a** was immediately observed together with acetophenone (**1**). The latter is a product of hydrolysis and subsequent decarboxylation of **3**. It is likely that the microorganisms, as well as keto reduction, also hydrolyzed the ester function of the substrate in a parallel reaction, the former being comparatively faster. The hydrolytic reaction supplied the corresponding *β*-keto acid, which on spontaneous decarboxylation produced **1** (Salvi
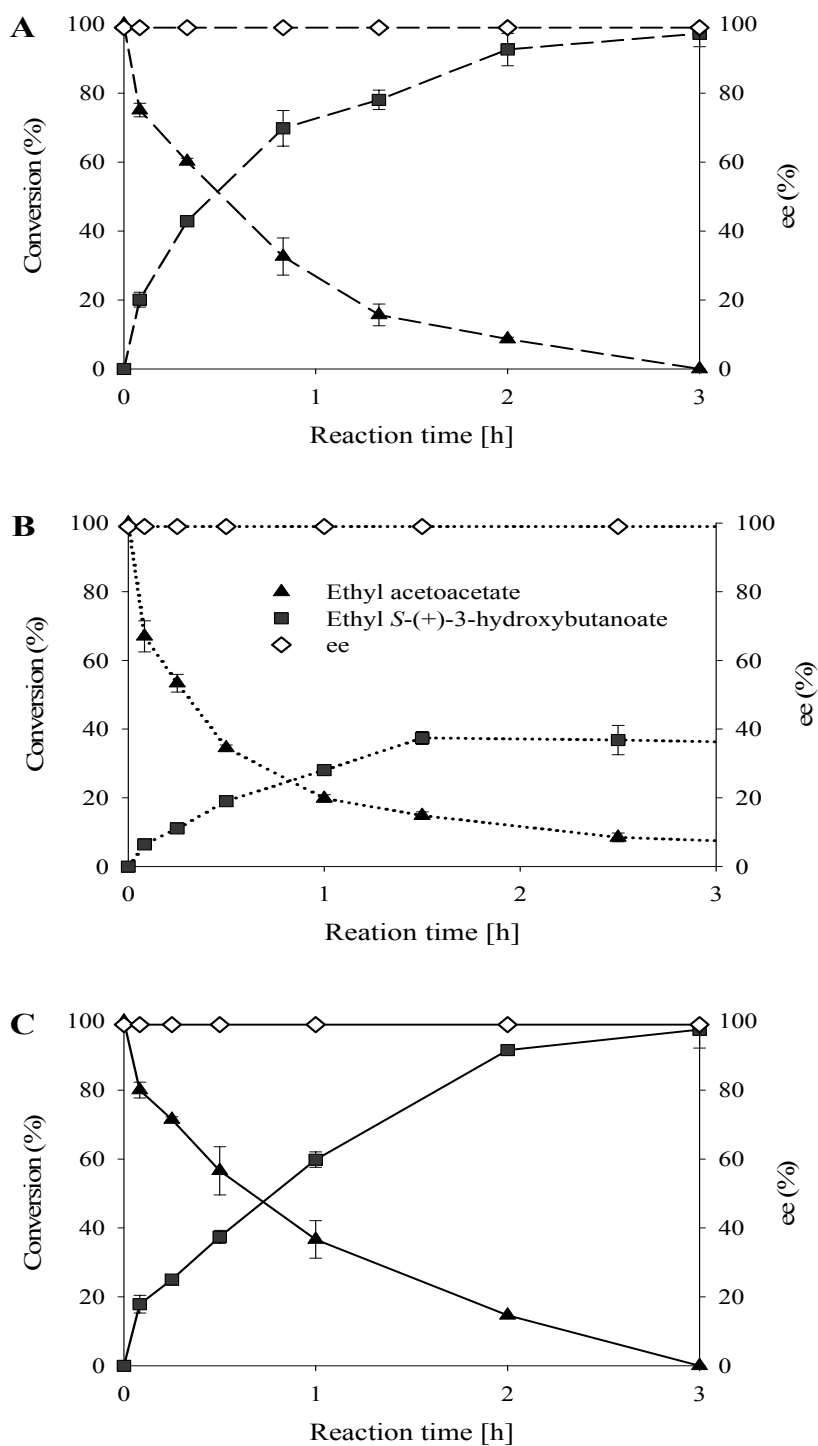
Fig. 2. Time course of conversion (200 mм glucose, at 30 °C and 120 rpm) and enantioselectivity (ee) of ethyl aceto-acetate (**2**) reduction by resting cells of (A) *P. pastoris* UBB 1500, (B) *Rhodotorula* sp., and (C) *S. cerevisiae*.
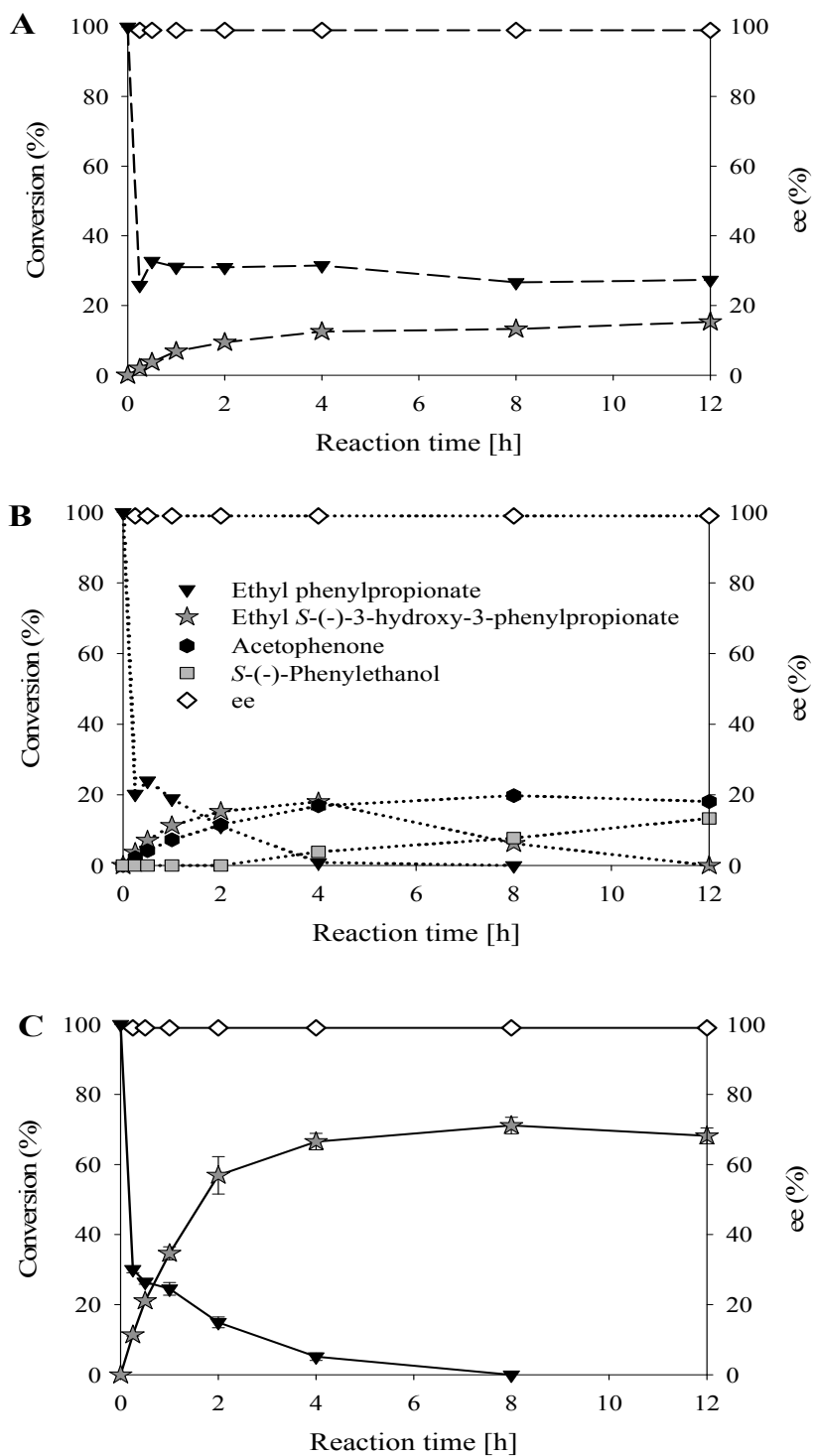
Fig. 3. Time course of conversion (200 mм glucose, at 30 °C and 120 rpm) and enantioselectivity (ee) of ethyl phenylpropionate (**3**) carried out by resting cells of (A) *P. pastoris* UBB 1500, (B) *Rhodotorula* sp., and (C) *S. cerevisiae*.

and Chattopadhyay, 2006), followed by its microbial reduction yielding **1a**. After 4 h, substrate **3** was totally consumed and **3a** reached its highest content (20%, >99% ee); 8 h later **3a** decreased until 0%. At the same time, **1** reached a content of 20% (8 h), and was later transformed into **1a** (18%, >99% ee) (Fig. 3B). It is noteworthy that the enzyme's hydrolytic activity present in the cell can hydrolyze either **3** or **3a** into high polar compounds (Csuk and Glaenzer, 1991).

S. cerevisiae reduced **3** to **3a** with the highest conversion rate (68%) and excellent enantiomeric excess (>99%) (Fig. 3C). This result showed that this microorganism is an appropriate system to reduce **3** (Table I).

Experimental

General

The carbonylic compounds **1**–**3** were obtained from Merck, enantiomerically pure standard (*S*)-(+)-**1a**, (*S*)-(+)-**2a**, and (*S*)-(-)-**3a** were purchased from Sigma-Aldrich. The cell concentration was adjusted with a Shimadzu UV-Vis 1603 spectrophotometer. Conversion of the biocatalytic reduction was monitored by RP-HPLC performed on a Merck-Hitachi L-4200 UV-Vis detector equipped with a reversed-phase C_{18} Merck-LiChrospher® column (4 mm x 250 mm, 5 μm), at 25–30° C with isocratic elution using a mobile phase comprised of water/acetonitrile. The enantiomeric excess (%) of the alcohols **1a**–**3a** was determined by GC-FID on a Shimadzu GC14A system equipped with a Supelco *β*-DEX™-225 column (0.25 mm x 30 m, 0.25 μm), using N_2 as carrier gas. The specific rotations were measured with an ATAGO POLAX-2 L semiautomatic polarimeter.

The ^{1}H NMR spectra were recorded on a Bruker AC250 NMR spectrophotometer; the compounds were dissolved in $CDCl_3$, and chemical shifts were reported in ppm. IR spectra were recorded on a Nicolect Nexus FT-IR spectrometer.

Microorganisms and source

The *P. pastoris* UBB 1500 strain was obtained from Laboratorio de Síntesis y Biotransformación de Productos Naturales, Universidad del Bio-Bio, Chillán, Chile. *Rhodoturula* sp., a pink microorganism isolated from the air, was identified by standard taxonomic methods based on physiological, morphological and nutritional properties (Barnett *et al.*, 2000). Briefly, the testing regime includes, in addition to morphological investigations, the following test: urea hydrolysis, D-glucose fermentation, growth at 30, 35 and 37 ºC, growth in the presence of cycloheximide (0.01 and 0.1%), growth in vitamin-free medium, and growth in media containing 50% glucose, and assimilation of carbohydrates (D-galactose, D-xylose, maltose, sucrose, trehalose, cellobiose, salicin, melibiose, raffinose, L-rhamnose, L-arabinose, lactose, melezitose, starch, erythritol, D-mannitol, 2-keto-D-gluconate, and citrate) and nitrogen (nitrate, nitrite, L-lysine and cadaverine). An assimilation test was performed by the pour-plate auxanographic method. The result was interpreted by the computer program ProleFood (Velázquez *et al.*, 2001). Additionally, the identification was confirmed by comparing the observed characteristics with the yeast identification key (Deak, 2007). All these studies of the yeast strain indicated that it belongs to the genus *Rhodotorula*. At the moment, the strain is a part of the collection of Laboratorio de Microbiología, Universidad del Bio-Bio, Chillán, Chile, with the accession number UBB 2009. *S. cerevisiae* was acquired from the local market, purified, and morphological and physiological identified.

Growth conditions

Cells were grown, shaken aerobically at 120 rpm and 30 °C for 60 h in 1-l Erlenmeyer flasks with 200 ml of MGYP medium (10 g/l malt extract, 20 g/l glucose, 10 g/l yeast extract, 3 g/l peptone). The MGYP medium was inoculated with a 48-h pre-culture.

Bioreductions

The bioreduction was performed with resting cells. Prior to the conversions, the cells were washed twice with water. After centrifugation at 4500 rpm for 10 min at 10 °C, the supernatant was removed. The cell pellet was resuspended in 200 mM glucose solution in order to obtain 50 g dry cell weight per liter [g_{DCW}/l]. The bioreduction was performed using open Erlenmeyer flasks (125 ml, x3) equipped with a cotton plug, on shaking flasks with 15 ml of cellular suspension at 30 °C, 120 rpm. The reaction was started by adding **1**–**3** at a concentration of 20 mM.

Instrumental analyses

The reactions were routinely monitored by periodic sampling of aliquots (1.0 ml), which were centrifuged at 4500 rpm, 5 min, and filtered (0.2-μm disposable filter). 20 μl of the aqueous phase were analyzed by HPLC. At the same time, for enantiomeric excess determinations, 500 μl of the aqueous phase were mixed and extracted with 500 μl of ethyl acetate, the organic phase was separated and dried, and an aliquot (1 μl) was injected into a gas chromatograph, (Table II). In the last step, the cells were centrifuged the supernatant was collected and extracted with ethyl acetate (x3). The extracted solvent was dried and concentrated under vacuum. The crude reaction mixture was purified by silica gel chromatography and the products were confirmed by GC-MS, FT-IR, and ^{1}H NMR spectroscopy. The absolute configurations of all the optically active alcohols, **1a–3a**, were determined by comparing the retention time on a chiral chromatographic column with that of the authentic standard, and the sign of their specific rotations with that of the reported specific rotations.

The alcohol **3a** required acetylation prior to GC analysis for resolution of the optical isomers, which was carried out with excess Ac$_2$O, catalyzed by pyridine, overnight at room temperature. The racemate standards were obtained via NaBH$_4$ reduction of the carbonylic compounds **1–3**.

S-(-)-1-Phenylethanol (**1a**): Colourless oil. – $[\alpha]_D^{22}$ −42° (*c* 5, CH$_3$OH) (>99% ee). – IR: ν = 3340.5, 3028.0, 759.9 cm^{-1}. – ^{1}H NMR (CDCl$_3$): δ = 1.49 (d, 3H, CH$_3$, *J* = 6.4 Hz), 2.07 (s, 1H, OH), 4.88 (q, 1H, CH, *J* = 6.4 Hz), 7.26 – 7.41 (m, 5H, Ph). –

GC/EI-MS: *m/z* = 107, 91, 79, 63, 51, 43 ([M$^+$] 122 for C$_8$H$_{10}$O).

Ethyl S-(+)-3-hydroxybutanoate (**2a**): Colourless oil. – $[\alpha]_D^{22}$ +39.3° (*c* 2.3, CHCl$_3$) (>99% ee). – IR: ν = 3433.1, 2974.0, 1730.5 cm^{-1}. – ^{1}H NMR (CDCl$_3$): δ = 1.21 (d, 3H, CH$_3$, *J* = 6.3 Hz), 1.26 (t, 3H, CH$_3$, *J* = 7.1 Hz), 2.39 [dd, 1H, CH$_2$, *J*(d) = 7.5 Hz, *J*(d) = 17.5 Hz], 2.48 [dd, 1H, CH$_2$, *J*(d) = 2.5 Hz, *J*(d) = 17.5 Hz], 3.02 (d, 1H, OH, *J* = 3.2 Hz), 4.16 (q, 2H, CH$_2$, *J* = 7.1 Hz), 4.05 – 4.25 (m, 1H, CH). – GC/EI-MS: *m/z* = 117, 87, 71, 60, 43 (131 [M$^+$−H] for C$_6$H$_{12}$O$_3$).

Ethyl (S)-(-)-3-hydroxy-3-phenylpropionate (**3a**): Colourless oil. – $[\alpha]_D^{22}$ −47.5° (*c* 2, CHCl$_3$) (>99% ee). – IR: ν = 3434.6, 3032.3, 2961.0, 1726.1 cm^{-1}. – ^{1}H NMR (CDCl$_3$): δ = 1.26 (t, 3H, CH$_3$, *J* = 7.1 Hz), 2.69 [dd, 1H, CH$_2$, *J*(d) = 4.6 Hz, *J*(d) = 16.3 Hz], 2.77 [dd, 1H, CH$_2$, *J*(d) = 8.2 Hz, *J*(d) = 16.3 Hz], 3.32 (s, 1H, OH), 4.18 (q, 2H, CH$_2$, *J* = 7.1 Hz), 5.13 [dd, 1H, CH, *J*(d) = 4.6 Hz, *J*(d) = 8.2 Hz], 7.28 – 7.37 (m, 5H, Ph). – GC/EI-MS: *m/z* = 176, 131, 107, 77, 51 (193 [M$^+$−H] for C$_{11}$H$_{14}$O$_3$).

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