Biocatalysis and Agricultural Biotechnology 12 (2017) 275–285

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Fungi isolated from food samples for an efficient stereoselective production of phenylethanols

María F. Decarlini[a], Mario L. Aimar[b,*], Ana M. Vázquez[a], Silvana Vero[c], Laura I. Rossi[d], Pablo Yang[e]

[a] Laboratorio de Tecnología Química, Facultad de Ciencias Químicas, Universidad Católica de Córdoba, Córdoba, Argentina
[b] Cátedra de Química Aplicada, Departamento de Química, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Vélez Sarsfield 1611, Ciudad Universitaria (X5016GCA), Córdoba, Argentina
[c] Cátedra de Microbiología, Facultad de Química, Universidad de la República, Montevideo, Uruguay
[d] Departamento de Química Orgánica, Instituto de Investigaciones en Fisicoquímica de Córdoba (INFIQC), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina
[e] Unidad Asociada: Área de Cs. Agrarias, Ingeniería, Cs. Biológicas y de la Salud, Universidad Católica de Córdoba, Argentina

ARTICLE INFO

Keywords:
Galactomyces candidus
Biocatalysis
Stereoselective bioreduction
Acetophenones
Phenylethanols
Rivastigmine

ABSTRACT

Twenty strains of fungi isolated from food samples were screened for the asymmetric reduction of acetophenone. A dimorphic fungus denominated initially as GZ1 and isolated from a carrot sour rot showed very high reduction activities, and based on phenotypic and genetic characteristics was identified as *Galactomyces candidus*. The only product of the reduction was confirmed to be (*R*)-1-phenylethanol. The bioreduction conditions mediated by *Gal. candidus* GZ1 were investigated, and the optimal conditions in terms of acetophenone concentration, temperature, pH media, co-solvent, the charge of inoculum and shaking speed were established. Under the optimal conditions of 1.875 g/L of acetophenone dissolved in DMSO (1.25% v/v), 112.5 g/L of microorganism cells in 100 mM sterile phosphate buffer solution at pH 7.0, 25 °C and 150 rpm, the conversion and enantiomeric excess values after 48 h reaction were 99% and > 99.9%, respectively. A preparative scale reaction of (*R*)-1-phenylethanol was made and the isolated yield was 82% with > 99.9 e.e.%. Additionally, a study using several substituted acetophenones was carried out, and the advantages and scope of GZ1 in the production of chiral 1-phenylethanols were established. In this sense, good results were obtained with 3′-hydroxyacetophenone and a preparative scale reaction was performed which give (*R*)-1-(3′-hydroxyphenyl)ethanol (key intermediate of Rivastigmine) with 53% of isolated yield and > 99.9 e.e.%.

1. Introduction

Production processes that allow chemical transformations to be carried out which are compatible with the environment is a necessity of the first order (Sheldon et al., 2007). Additionally, because of safety, therapeutic and regulatory concerns, there has been an increasing interest in the development of processes capable of producing enantiomerically pure drugs (Rouhi, 2003; Hutt and Tan, 1996). Related to this, in recent decades, with the increasing demand for modern drugs, food ingredients and agrochemicals, more attention has been paid to the enantioselective synthesis of optically pure compounds using environmental friendly methodologies. In this sense, one of the most important reactions in the organic synthesis is the asymmetric reduction of ketones to produce chiral alcohols (Singh, 1992). Optically pure alcohols are useful as building blocks for the synthesis of bioactive compounds such as pharmaceuticals and agrochemicals. Moreover, chiral alcohols can be transformed into other functionalities without racemization (Nakamura et al., 2003).

Biochemical processes mediated by whole cells of microorganisms (fungus and yeasts (Bi-Hong et al., 2012), bacteria (Xie et al., 2010), algae (Utsukihara et al., 2006), endophytic microorganisms (fungi and bacteria) isolated from vegetable (Rodriguez et al., 2017)), isolated enzymes and co-factors from various sources (Goldberg et al., 2007), and more recently plants and their cell cultures (Cordell et al., 2007; Bennamame et al., 2014; Pavoković et al., 2017), have some comparative advantages over classical chemical procedures, including their low cost, high versatility and efficiency, in addition to highly desirable chemical aspects such as chemo-, regio-, and stereoselectivity (Nakamura and Matsuda, 2006). Moreover, there is the added advantage of using bioreagents for organic transformations, as they can be

* Correspondence author.
E-mail address: mlaimar@unc.edu.ar (M.L. Aimar).

http://dx.doi.org/10.1016/j.bcab.2017.10.014
Received 24 August 2017; Received in revised form 13 October 2017; Accepted 13 October 2017
Available online 16 October 2017

used on a sustainable basis, rather than depleting resources (Cordell et al., 2007). Another advantage is that these systems are more environmentally friendly than classical chemical processes, due to the reaction being carried out in water as the solvent and the catalyst being biodegradable or environmentally compatible. For these motives, these processes generate less waste than conventional chemical reagents (Kumaraswamy and Ramesh, 2003). However, sometimes the enzymatic and chemical approaches complement each other to obtain both enantiomers (Bennamame et al., 2015).

Over the past decade the application of biocatalytic processes in the commercial synthesis of chiral alcohols has undergone a revolution, with biocatalysts now often being the preferred tool for the synthesis of chiral alcohols via prochiral ketone reduction (Huisman et al., 2010), because chemical catalysis has disadvantages of undesired by-products, toxic effluents (as heavy metals and organic solvents are commonly used) and low substrate selectivity.

However, in order to meet the new demand for whole cell biocatalysts in pharmaceuticals, agrochemicals and natural products, it is still necessary to discover new strains which are able to reduce various substrates to produce excellent enantiomeric purity and high yield. With this in mind, recently a project was started with the aim of identifying green procedures in order to obtain chemical intermediates using whole microorganisms as biocatalysts, with the screening of the fungi isolated from different commercial food samples initiated to identify strains that could be used as biocatalysts in the reduction of prochiral ketones to obtain chiral phenylethanols as a sustainable and an inexpensive alternative to traditional chemical methods.

2. Materials and methods

2.1. General

Acetophenone, substituted acetophenones, 2-acetylpyridine, 2-acetylthiophene and $NaBH_4$ were purchased from Sigma-Aldrich S.A. (Argentina). (*S*)-1-phenylethanol was synthesized using a methology reported on the literature (Aimar et al., 2014). Sterile deionized water was used to prepare phosphate buffer, and ethyl acetate and hexane were purified by a fractional distillation prior to use. The crude reaction was extracted with ethyl acetate, the organic solutions evaporated, and the products were filtered on a short column with silica gel (70–230 mesh) using hexane – ethyl acetate 90:10 as the eluent. Gas chromatography (GC) analyses were carried out on a Buck Scientific Model 910/310 instrument with a flame ionization detector (FID), and GC – mass spectrometry (MS) analyses were carried out on a Hewlett Packard HP 5890 Series II gas chromatograph equipped with the Mass Detector HP 5970. The nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a Bruker Avance II 400 MHz, and optical rotations were measured in a JASCO P-1010 polarimeter. The products were identified by comparing its optical rotations, MS, and ^{1}H and ^{13}C NMR spectra with literature data.

2.2. Microorganisms and culture medium

The strains were isolated from samples of several fruits, vegetables and elaborated foods with swab samples being performed in 0.1% p/v peptone with 0.05% Tween 80, and in maltose broth in the case of beers. The yeasts were then insulated in 3 mediums: GPYA, PDA and SDA. Coloration with lactophenol for the colonies that presented a morphology corresponding to the yeasts meeting requirements was prepared, and then these were identified using the key of Pitt and Hocking (2009).

2.3. Screening for stereoselective reduction of acetophenone

Each strain to be tested was grown for 3 days at optimum temperature in GPY broth, and the yeast cells (≈ 3 g) were separated from the culture broth by filtration and put into a 250 mL sterile Erlenmeyer flask containing 80 mL of sterile 100 mM phosphate buffer pH 7.0. The substrate (50 μL), dissolved in 1 mL of dimethyl sulfoxide (DMSO) was added, and incubation was carried out on an orbital shaker at 100 rpm at 28 °C for 7 days. Samples (2 mL) were taken every 24 h, and were extracted with ethyl acetate (2 mL). Finally, anhydrous calcium chloride was added in order to remove the dissolved water, and the organic solution was filtered and analysed (1 μL) by chiral GC-FID and GC-MS. All assays were performed twice. The identity of the peaks of the enantiomers in the chromatogram was performed using (*S*)-1-phenylethanol as reference standard. In addition, the retention times of GC data were compared with those reported in the literature for the same column.

2.4. Identification of the selected strain by the PCR technique

The selected strain GZ1 was identified at the species level by sequence analysis of the ITS1-5.8S-ITS2 genomic region of the nuclear rRNA gene and the D1/D2 variable domains of the 26S rRNA gene at the 5′ end of the large subunit rRNA gene (Pereyra et al., 2014; Alper et al., 2011). DNA extraction was carried out as described by Schena et al. (1999) and PCR fragments were generated using the primers ITS1-ITS4 (White et al., 1990) and NL1-NL4 (Peterson and Horn, 2009). The thermal profile was 96 °C for 2 min, followed by 35 cycles of 96 °C 30 s, 51 °C 45 s, 72 °C 120 s, and a final extension step at 72 °C for 7 min. Nucleotide sequences of the PCR products were determined in both directions at Institute of Biotechnology (CICVyA, INTA, Hurlingham, Buenos Aires, Argentina), which were aligned with Chromas lite program 2.3 version, visually corrected and used as query sequences to search for similar ones from the NCBI/GenBank database by using the BLAST program (www.ncbi.nlm.nih.gov/blast/). Additionally, to confirm identification, the ability to grow at 35 °C was validated for the GZ1 strain, according to Kurtzman et al. (2011).

2.5. GC-FID and GC-MS analyses

To establish the chromatographic conditions, acetophenones were reduced with sodium borohydride in ethanol to obtain a racemic mixture of the 1-phenylethanols with very good yields. The GC separations were performed on a fused silica capillary column Supelco β-Dex 120 (phenyl polysiloxane with 20% of permethylated β-cyclodextrin, 30 m, 0.25 mm, 0.25 μm) with GC general conditions of split, 145 mL/min; injector, 220 °C; detector FID, 220 °C; carrier gas, N_2; and head pressure, 29 psi, and with the conversion percentages of the reactions being determined by using normalized peak areas without a correction factor. The GC-MS (70 eV) analyses were performed using the same conditions as those in the GC analysis, but utilizing a Hewlett Packard HP-5 (Crosslinked 5% PhMeSiloxane, 30 m, 0.32 mm, 0.25 μm film thickness) capillary column.

2.6. Preparative scale reaction of (+)-(R)-1-Phenylethanol

1.05 g acetophenone, 63 g mass of cells of *Gal. candidus* GZ1, 7 mL of DMSO (1,25% v/v) and 560 mL of sterile 100 nM phosphate buffer pH 7.0, were introduced into a 2 L sterile conical flask. Then, the flask was placed in a culture stove on an orbital shaker at a shaking speed of 150 rpm, the stove kept at 25 °C for 48 h. At the final time of reaction, the crude reaction system was extracted with ethyl acetate (5 × 100 mL) and the organic solution was dried with magnesium sulfate. Then, the filtrate organic solution was concentrated to dryness in a rotary evaporator to obtain 0.926 g of crude residue, which was filtered through a short silica gel column using hexane/ethyl acetate mixture at variable proportions as the eluent. Isolated yield: 0.875 g (82%). GC conditions: T_1 = 80 °C (1 min), ΔT = 2.5 °C/min,T_2 = 140 °C. GC Rt acetophenone: 14.3 min, Rt (*R*)-1-phenylethanol: 19.6 min, and Rt (*S*)-1-phenylethanol: 20.4 min (Orden et al., 2009; Aimar et al., 2014;

Supelco β-DEX 120), conversion: 99%, enantiomeric excess: > 99.9. $[\alpha]_D^{22}$ = +40.1, c = 0.51 MeOH ($[\alpha]_D^{25}$ = +39.1, *c* = 3.5 MeOH. (Yadav et al., 2002)). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.49 (d, 3H, CH_3), 2.03 (s, 1H, OH), 4.89 (q, 1H, CH), 7.28–7.39 (m, 5H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.16, 70.44, 125.38, 127.49, 128.52, 145.81. Electron ionization (EI) MS *m/z* (rel. int. %) = 123 (M^+ + 1, 4), 122 (M^+, 29), 121 (M^+ - 1, 6), 107 (M+- CH_3, 74), 105 (M^+ - OH, 13), 104 (M^+ - H_2O, 10), 79 (100), 78 (26), 77 (68), 57 (19), 51 (30).

2.7. Reduction of substituted acetophenones with GZ1

The same optimal proportions used in the scaling down of acetophenone were used to reduce substituted acetophenones but using 75 mg of substrate in a final volume of 80 mL. The final products were extracted with ethyl acetate and purified by filtered through a short silica gel column using hexane/ethyl acetate mixtures at variable proportions as the eluent.

2.7.1. (+)-(R)-1-(4′-Fluorophenyl)ethanol

GC conditions: T_1 = 100 °C, Δ*T* = 2 °C/min, T_2 = 200 °C. GC Rt 4′-fluoroacetophenone: 9.52 min, Rt (+)-(*R*)-1-(4′-fluorophenyl)ethanol: 13.95 min and Rt (-)-(*S*)-1-(4′-fluorophenyl) ethanol: 14.75 min (Orden et al., 2009; Aimar et al., 2014). $[\alpha]_D^{20}$ = +32, *c* = 1 MeOH, (Nakamura and Matsuda, 1998, $[\alpha]_D^{25}$ = −37.7, *c* = 0.931, MeOH for *S* enantiomer). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.47 (d, 3H, CH_3), 2.39 (b, 1H, OH), 4.87 (c, 1H, CH), 7.00–7.05 (m, 2H, ArH), 7.31–7.35 (m, 2H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.27, 69.79, 115.25 (d, J = 21.2 Hz), 127.04 (d, J = 8 Hz), 141.49 (d, J = 3.1 Hz), 163.34 (d, J = 243.8 Hz). EI MS *m/z* (rel. int. %) = 140 (M^+, 27), 125 (100), 123 (13), 97 (87), 96 (24), 95 (26), 77 (29), 75 (20), 51 (12).

2.7.2. (+)-(S)-2-Chloro-1-(phenyl)ethanol

GC conditions: T_1 = 110 °C, Δ*T* = 1 °C/min, T_2 = 145 °C (1 min). GC Rt 2-chloroacetophenone: 26.76 min, Rt (-)-(*R*)-2-Chloro-1-(phenyl) ethanol: 32.22 min and Rt (+)-(*S*)-2-Chloro-1-(phenyl)ethanol: 33.47 min $[\alpha]_D^{22}$ = +25.6, c = 0.1 $CHCl_3$ ($[\alpha]_D^{25}$ = −54.06, *c* = 1.0 $CHCl_3$ for (*S*)-enantiomer (Aguirre-Pranzoni et al., 2014)). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 2.36 (s, 1H, OH), 3.63–3.77 (m, 2H, CH_2), 4.89–4.92 (q, 1H, CH), 7.31–7.40 (m, 5H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 50.95, 74.09, 126.07, 128.49, 128.70, 139.90. EI MS *m/z* (rel. int. %) = 156 (M^+, 3), 107 (100), 79 (63), 77 (40), 70 (12), 61 (14), 51 (15).

2.7.3. (-)-(S)-1-(2′-Chlorophenyl)ethanol

GC conditions: T_1 = 110 °C, Δ*T* = 3 °C/min, T_2 = 210 °C. GC Rt 2′-chloroacetophenone: 10.88 min, Rt (+)-(*R*)-1-(2′-chlorophenyl) ethanol: 15.54 min and Rt (-)-(*S*)-1-(2′-chlorophenyl)ethanol: 16.88 min (Li et al., 2010; Aimar et al., 2014). $[\alpha]_D^{25}$ = −53, *c* = 1,23 $CHCl_3$ (Nakamura and Matsuda, 1998, $[\alpha]_D^{25}$ = −62.7, *c* = 0,894 $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.48 (d, 3H, CH_3), 1.89 (s,1H, OH), 4.87 (c, 1H, CH), 7.21–7.40 (m, 4H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 23.52, 67.00, 126.41, 127.22, 128.42, 129.42, 131.67, 143.04. EI MS *m/z* (rel. int. %) = 158 (M^+ + 2, 5), 156(M^+, 15), 143 (24), 141 (82), 113 (26), 77 (100), 51 (28).

2.7.4. (-)-(S)-1-(3′-Chlorophenyl)ethanol

GC conditions: T_1 = 110 °C, Δ*T* = 3 °C/min, T_2 = 210 °C. GC Rt 3′-chloroacetophenone: 12.94 min, Rt (+)-(*R*)-1-(3′-chlorophenyl) ethanol: 18.36 min and Rt (-)-(*S*)-1-(3′-chlorophenyl)ethanol: 18.85 min (Li et al., 2010; Aimar et al., 2014). $[\alpha]_D^{22}$ = −38.8, c = 1.27 $CHCl_3$ (Nakamura and Matsuda, 1998, $[\alpha]_D^{25}$ = −43.5, *c* = 1.08, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.47 (d, 3H, CH_3), 2.39 (s, 1H, OH), 4.85–4.90 (m, 1H, CH), 7.02–7.05 (m, 2H), 7.31–7.35 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.26, 69.83, 123.54, 125.64, 127.55, 129.80, 134.39, 147.86. EI MS *m/z* (rel. int. %) = 158 (M^+ + 2, 5), 156 (M^+, 16), 143 (16), 141 (54), 121 (11), 115 (11), 113 (38), 77 (100), 75(17), 51 (23).

2.7.5. (+)-(R)-1-(4′-Chlorophenyl)ethanol

GC conditions: T_1 = 110 °C, Δ*T* = 3 °C/min, T_2 = 210 °C (20 min). GC Rt 4′-chloroacetophenone: 13.26 min, Rt (+)-(*R*)-1-(4′-chlorophenyl)ethanol: 16.76 min and Rt (-)-(*S*)-1-(4′-chlorophenyl)ethanol: 17.36 min (Orden et al., 2009; Aimar et al., 2014). $[\alpha]_D^{22}$ = +41.6, *c* = 2.48 $CHCl_3$ (Nakamura and Matsuda, 1998, $[\alpha]_D^{25}$ = −49.0, *c* = 1.84, Ether, for *S* enantiomer). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.47 (d, 3H, CH_3), 1.83 (s, 1H, OH), 4.87 (q, 1H, CH), 7.26–7.31 (m, 4H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.29, 69.77, 126.80, 128.62, 133.09, 144.25. EI MS *m/z* (rel. int. %) = 158 (M^+ + 2, 4), 156 (M^+, 16), 143 (20), 141 (70), 121 (9), 113 (28), 77 (100), 75 (19), 51 (24).

2.7.6. (-)-(S)-1-(2′,4′-Dichlorophenyl)ethanol

GC conditions: T_1 = 140 °C, Δ*T* = 2 °C/min, T_2 = 180 °C (2 min). GC Rt 2′,4′-dichloroacetophenone: 9.81 min, Rt (+)-(*R*)-1-(2′,4′-dichlorophenyl)ethanol: 16.70 min and Rt (-)-(*S*)−1-(2′,4′-chlorophenyl) ethanol: 18.43 min $[\alpha]_D^{22}$ = −51.9, c = 0.1 $CHCl_3$ ($[\alpha]_D^{25}$ = −58.7, *c* = 1.03 $CHCl_3$ (Nakamura et al., 1999)). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.46 (d, 3H, CH_3), 1.95 (s, 1H, OH), 5.24 (q, 1H, CH), 7.26–7.55 (m, 3H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 23.61, 66.60, 127.46, 127.52, 129.11, 132.7, 133.39, 141.74. EI MS *m/z* (rel. int. %) = 192 (M^+ + 2, 10), 190 (M^+, 15), 177 (70), 175 (100), 149 (15), 147 (25), 113 (20), 11 (70), 87 (10), 75 (25).

2.7.7. (+)-(R)-1-(4′-Bromophenyl)ethanol

GC conditions: T_1 = 100 °C, Δ*T* = 2 °C/min, T_2 = 200 °C (20 min). GC Rt 4′-bromoacetophenone: 23.75 min, Rt (+)-(*R*)-1-(4′-bromophenyl)ethanol: 31.16 min and Rt (-)-(*S*)-1-(4′-bromophenyl)ethanol: 32.01 min (Orden et al., 2009; Aimar et al., 2014). $[\alpha]_D^{22}$ = +39.2, c = 2.32 $CHCl_3$ (Nakamura and Matsuda, 1998, $[\alpha]_D^{25}$ = −37.9, *c* = 1.13, $CHCl_3$ for *S* enantiomer). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.47 (d, 3H, CH_3), 1.92 (b, 1H, OH), 4.86 (c, 1H, CH), 7.23–7.26(m, 2H, ArH), 7.46–7.48 (m, 2H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.27, 69.80, 121.18, 127.16, 131.57, 144.78. EI MS *m/z* (rel. int. %) = 202 (M^+ + 1, 27), 200 (25), 187 (81) 185 (86), 183 (9), 159 (25), 157 (31), 156 (9), 121 (23), 103 (12), 102 (8), 78 (55), 77 (100), 76 (20), 75 (21), 51 (42).

2.7.8. (+)-(R)-1-(4′-Cyanophenyl)ethanol

GC conditions: T_1 = 100 °C, Δ*T* = 2 °C/min, T_2 = 200 °C. GC Rt 4′-cyanoacetophenone: 29.10 min, Rt (+)-(*R*)-1-(4′-cyanophenyl)ethanol: 39.72 min and Rt (-)-(*S*)-1-(4′-cyanophenyl)ethanol: 40.83 min (Aimar et al., 2014). $[\alpha]_D^{22}$ = +40.2, c = 2.16 MeOH (Mathre et al., 1993, $[\alpha]_D^{25}$ = +41.7, *c* = 1.063, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.50 (d, 3H, CH_3), 2.07 (b, 1H, OH), 4.97 (c, 1H, CH), 7.48–7.50 (d, 2H, ArH), 7.64–7.66 (dd, 2H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.44, 69.69, 111.09, 118.89, 126.07, 132.38, 151.08. EI MS *m/z* (rel. int. %) = 147 (M^+, 7), 132 (64), 130 (10), 105 (12), 104 (100), 102 (22), 77 (21), 75 (13), 63 (6), 51 (12), 50 (10).

2.7.9. (+)-(S)-1-(2′-Nitrophenyl)ethanol

GC conditions: T_1 = 100 °C, Δ*T* = 2 °C/min, T_2 = 210 °C (20 min). GC Rt 2′-nitroroacetophenone: 29.20 min, Rt (-)-(*R*)-1-(2′ nitrophenyl) ethanol: 38.48 min and Rt (+)-(*S*)-1-(2′-nitrophenyl)ethanol: 40.45 min (Aimar et al., 2014). $[\alpha]_D^{22}$ = +28.1, c = 2.98 $CHCl_3$ (Comasseto et al., 2006, $[\alpha]_D^{25}$ = +30.6, *c* = 3.06 $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.56 (d, 3 H, CH_3), 2.41 (b, 1H, OH), 5.40 (c, CH), 7.38–7.42 (dt, 1H, ArH), 7.63–7.65 (dt, 1H, ArH), 7.81–7.87 (dd, 1H, ArH), 7.88 (dd, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 24.20, 65.59, 124.30, 127.58, 128.11, 133.59, 140.90, 147.89. EI MS *m/z* (rel. int. %) = 165 (M^+ − 2, 1), 150 (100), 123 (12), 104 (7), 91 (11), 77 (14), 76 (35), 74 (14), 63 (11), 51 (50).

2.7.10. (+)-(R)-1-(3′-Nitrophenyl)ethanol

GC conditions: T_1 = 100 °C, ΔT = 2 °C/min, T_2 = 210 °C (20 min). GC Rt 3′-nitroroacetophenone: 34.18 min, Rt (+)-(*R*)-1-(3′-nitrophenyl)ethanol: 41.61 min and Rt (-)-(*S*)-1-(3′-nitrophenyl)ethanol: 41.84 min (Aimar et al., 2014). $[\alpha]_D^{22}$ = +27.9, c = 3.28 $CHCl_3$ (Comasseto et al., 2006, $[\alpha]_D^{25}$ = −30.5, *c* = 2.99 $CHCl_3$, for *S* enantiomer). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.54 (d, 3H, CH_3), 1.91 (b, 1H, OH), 5.02 (c, 1H, CH), 7.50–7.54 (t, 1H, ArH), 7.71–7.73 (d, 1H, ArH), 8.11–8.14 (dm, 1H, ArH), 8.27 (t, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.51, 69.43, 120.46, 122.39, 129.46, 131.57, 147.91, 148.86. EI MS *m/z* (rel. int. %) = 167 (M^+, 1), 166 ($M^+ - 1$, 1), 152 (100), 121 (12), 107 (60), 106 (20), 105 (38), 78 (38), 77 (82), 76(19), 51 (32).

2.7.11. (+)-(R)-1-(4′-Nitrophenyl)ethanol

GC conditions: T_1 = 100 °C, ΔT = 2 °C/min, T_2 = 210 °C (20 min). GC Rt 4′-nitroroacetophenone: 36.38 min, Rt (+)-(*R*)-1-(4′-nitrophenyl)ethanol: 45.29 min and Rt (-)-(*S*)-1-(4′-nitrophenyl)ethanol: 45.89 min (Orden et al., 2009; Aimar et al., 2014). $[\alpha]_D^{23}$ = +29.0, c = 2.51 $CHCl_3$ (Yadav et al., 2002, $[\alpha]_D^{25}$ = −30.5, *c* = 4 $CHCl_3$ for *S* enantiomer). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.52 (d, 3H, CH_3), 2.09 (b, 1H, OH), 5.03 (c, 1H, CH), 7.54-7,56 (d, 2H, ArH), 8.20–8.22 (d, 2H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 25.54, 69.53, 123.78, 126.14, 142.21, 153.06. EI MS *m/z* (rel. int. %) = 167 (M^+, 3), 166 ($M^+ - 1$, 1), 152 (100), 122 (13), 107 (89), 106 (22), 105 (22), 94 (31), 78(35), 77 (74), 66 (13), 51 (33).

2.7.12. (+)-(R)-1-(3′-hydroxyphenyl)ethanol

GC conditions (Restek rt-βDEXsa column, split, 145 mL/min; injector, 220 °C; detector FID, 220 °C; carrier gas, N_2; and head pressure, 29 psi, T_1 = 100 °C, ΔT = 2 °C/min, T_2 = 200 °C, determined as monoacetate ester after derivatization). GC Rt 3′-hydroxyacetophenone: 48.44 min, Rt (+)-(*R*)-1-(3′-hydroxyphenyl)ethanol esterified: 41.06 min and Rt (-)-(*S*)-1-(3′-hydroxyphenyl)ethanol esterified: 41.33 min $[\alpha]_D^{22}$ = +10.8, *c* = 0.3 MeOH ($[\alpha]_D^{20}$ = +32, *c* = 1 MeOH, (Cazetta, 2014)). ^{1}H NMR (400 MHz, DMSO-D_6) δ (ppm) = 1.27 (d, 3H), 4.59–4.62 (q, 1H, CH), 5.04 (s, 1H, OH), 6.57–6.60 (m, 1H, ArH), 6.71–6.76 (m, 1H, ArH), 7.05–7.09 (s, 2H, ArH), 8.27 (s, 1H, ArOH), ^{13}C NMR (100 MHz, DMSO-D_6) δ (ppm) = 25.40, 68.47, 112.64, 113.81, 116.40, 129.32, 149.47, 157.61. EI MS *m/z* (rel. int. %) = 138 (M^+, 35), 123 (40), 120 (20), 95 (100), 91 (15), 77 (50), 65 (20).

2.7.13. (+)-(R)-1-(4′-methyphenyl)ethanol

GC conditions: T_1 = 100 °C, ΔT = 2 °C/min, T_2 = 200 °C. GC Rt 4′-methyacetophenone: 13.24 min, Rt (+)-(*R*)-1-(4′-methylphenyl) ethanol: 16.08 min and Rt (-)-(*S*)-1-(4′-methyphenyl)ethanol: 16.88 min (Orden et al., 2009; Aimar et al., 2014). $[\alpha]_D^{23}$ = +40.9, c = 2.05 MeOH (Nakamura and Matsuda, 1998, $[\alpha]_D^{25}$ = −43.5, *c* = 0.998, MeOH para el enantiómero *S*). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.47 (d, 3H, CH_3), 1.89 (b, 1H, OH), 2.33 (s, 3H, CH_3), 4.86 (c, 1H, CH), 7.15 (d, 2H, ArH), 7.26 (d, 2H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 21.10, 25.09, 70.27, 125.37, 129.18, 137.17, 142.90. EI MS *m/z* (rel. int. %) = 136 (M+, 39), 121 (98), 119 (9), 117 (5), 93 (100), 92 (21), 91 (77), 77 (45), 65 (27), 51 (15).

3. Results and discussion

3.1. Screening of microorganisms

A total of 20 strains of fungi isolated from commercial food products were assayed to determine their potential capabilities to transform stereoselectivity acetophenone (AP) to 1-phenylethanol (1-PE). The results are presented in Table 1, where it can be seen that all strains tested showed a reduction of AP but at different conversion and stereoselectivity grades. Many investigations have reported that most microorganisms biotransform prochiral ketones to (*S*)-alcohols in a way mainly following the Prelog rule (Quezada et al., 2009; Xie et al., 2010), as occurred in the present study. The reduction product of AP catalyzed by twelve strains revealed a predilection for the formation of (*S*)-1-PE (entries 1, 8–15, 17–19), while the other eight strains biotransformed AP with a preference for (*R*)-1-PE (entries 2–7, 16 and 20).

Saccharomyces cereviceae (Baker's yeast) is one of the most studied microorganisms to carry out bio-transformations (Rodrigues et al., 2004). For this reason, eleven strains of *S. cereviceae* isolated from several commercial samples were tested, and most of these presented a preference for the (*S*)-enantiomer (Table 1, entries 8–18), with the exception to this rule being *S. cereviceae* SCPA (Table 1, entry 16). Additionally, *S. cereviceae* SC6 and SC7 (both isolated from beers) were the only strains having a good stereoselectivity (Table 1, entries 8 and 18) but a poor efficiency of conversion (between 4% and 18%) in our reaction conditions.

Although few studies have been reported using strains of *Zygosaccharomyces*, one interesting example of the application of a microorganism of this genus has been described (Erdélyi et al., 2006). In our study, two species of *Zygosaccharomyces* were tested, which demonstrated contrasting stereoselectivities. In this sense, *Z. bailii* ZBC4 (isolated from Labrusca grape) had a strong preference for (*S*)-enantiomer (99 e.e.%) while *Z. rouxii* ZRC6 (isolated from Moscatel grape) revealed a preference for the (*R*)-enantiomer, but at low e.e.% (Table 1, entries 19–20). Nevertheless, the conversion percentages were low in our reaction media in both cases.

Of the all strains tested, only three showed a very good performance (Table 1, entries 2–4) with very high conversion percentages (86–96%) and an excellent enantiomeric excess (≥ 99%). Related to this, a strain initially named as GZ1 and later identified to be like *Galactomyces candidus* (isolated from a decaying carrot) produced the best performance (Table 1, entry 2) with an excellent yield and fully estereoselective reduction of AP to (*R*)-1-PE.

The four strains of *Geotrichum* genus were isolated from two different commercial samples and assayed. Two of these were from samples of Camembert cheese, one was from grapefruit and another was isolated from red glove grape. *Geotrichum* sp. GCQ1 and GCQ3 isolated from cheese did not produce a good performance (Table 1, entries 5–6) and showed a preference for (*R*)-enantiomer. These results are consistent with those reported by Nakamura and Matsuda (1998) in the production of (*R*)-1-PE, who observed that using resting cell of the strain of *Geotrichum candidum* IFO 4597 reduced AP to 52% of conversion and 28 e.e.% for (*R*)-enantiomer. Nevertheless, the other two strains of *Geotrichum* sp. had excellent behaviors in the reductive process (Table 1, entries 3–4), making both strains candidates for major studies. Similarly, excellent results were obtained by Nakamura et al. (2002) using *Geotrichum candidum* IFO 5767 in the production of chiral 1-PEs.

It is noteworthy that in our study, better results were observed using the strain of *Galactomyces candidus* GZ1 (Table 1, entry 2), which showed a nearly quantitative conversion and a complete preference for (*R*)-enantiomer (> 99.9 e.e.%). Finally, for all other strains tested, although some degree of conversion and stereoselectivity was observed, the efficiency of the reductions were poor (Table 1, entries 1 and 7). Thus, considering the results obtained during the screening, the strain of *Galactomyces candidus* GZ1 was selected for further studies.

3.2. Identification of the GZ1

The colonies of GZ1 were developed rapidly at 25 °C on PDA plates, which after 3 days were white, finely hairy and dense. Under optical microscope, GZ1 presented rectangular to cubic arthroconidia.

Molecular identification studies of GZ1 were carried out by using the corresponding analyses of the ITS1-5.8S-ITS2 and D1/D2 regions, and the sequence obtained from the ITS1-5.8S-ITS2 region of the strain GZ1 was about 275 bp. BLAST analysis of this sequence showed 100%

Table 1
Screening of fungi isolated from food samples.

Entry	Microorganism	Isolation source	Conversion %[a]	e.e.%[a,b]	Conf.[c]
1	*Debaryomyces hansenii DHC1*	Red glove grape	4	37	*S*
2	*Galactomyces candidus GZ1*	Carrot sour rot	96	> 99.9	*R*
3	*Geotrichum sp GP1*	Grapefruit	86	> 99	*R*
4	*Geotrichum sp GU1*	Red glove grape	89	> 99.9	*R*
5	*Geotrichum sp GCQ1*	Camembert cheese	22	75	*R*
6	*Geotrichum sp GCQ3*	Camembert cheese	18	69	*R*
7	*Penicillium roqueforti PR1*	Blue cheese	18	21	*R*
8	*Saccharomyces cereviseae SC6*	Yeast beer	5	99	*S*
9	*Saccharomyces cerevisiae SCLB*	Yeast beer	8	60	*S*
10	*Saccharomyces cerevisiae SCLW*	Yeast beer	12	62	*S*
11	*Saccharomyces cerevisiae SCF2*	Black beer	10	56	*S*
12	*Saccharomyces cerevisiae SCF3*	Light beer	7	65	*S*
13	*Saccharomyces cerevisiae SCF1*	Patagonic beer	8	75	*S*
14	*Saccharomyces cerevisiae SCC9*	Grape juice craft	15	85	*S*
15	*Saccharomyces cerevisiae* SCPF	Nutritional supplement[d]	18	64	*S*
16	*Saccharomyces cerevisiae* SCPA	Bakery yeast	15	20	*R*
17	*Saccharomyces cerevisiae SCU2*	Labrusca grape	5	62	*S*
18	*Saccharomyces cereviseae SC7*	Yeast beer	4	99	*S*
19	*Zygosaccharomyces bailii ZBC4*	Labrusca grape	23	99	*S*
20	*Zygosaccharomyces rouxii ZRC6*	Moscatel grape	20	39	*R*

[a] Conversion (%) and enantiomeric excess (e.e.%) were determined by chiral GC-FID and GC-MS analyses.
[b] Absolute configurations were assigned by comparing the retention times of chiral GC data with the reports in the literature and through the use of pure (*S*)-1-PE as a reference.
[c] Isolated from commercial beer's yeast tablets used as a nutritional supplement.

homology with the corresponding *Galactomyces candidus* CBS 557.83 (GenBank accession no. JN974288.1), and also with that of *Galactomyces geotrichum* ATCC 34614. Analyses of D1/D2 region of the strain GZ1 revealed a sequence of about 500 bp, with BLAST analysis of this sequence showing 100% homology with the corresponding *Galactomyces candidus* BS 606.85 (GenBank accession no. JN974267.1), and a similarity of 99% to the sequence of *Gal. geotrichum* ATCC 34614 (GenBank accession no. GQ458034.1). Similarities of 98% or less were obtained with sequences corresponding to other species, with that of *Geotrichum europaeum* CBS 866.68 being the nearest one.

Next, phylogenetic analyses of D1/D2 sequences of the selected isolates were conducted using MEGA version 6 and DNA sequences were aligned with sequences of homologous regions of closely related type strains retrieved from GenBank. The evolutionary distances were computed using the Jukes–Cantor method, and phylogenetic trees were obtained by neighbor-joining. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option), with stability of clades being assessed with 1000 bootstrap replications. All results can be observed in Fig. 1, where the phylogenetic tree showing placement of GZ1 among type strains of *Galactomyces* spp. and *Geotrichum* spp. *Dipodascus armillariae* NRRL Y-17580 was included as an out-group. The tree was constructed using the Neighbor-Joining method with bootstrap values (1000 tree interactions) indicated at the nodes. All but GZ1 are type strains. Moreover, phylogenetic analysis of the D1/D2 domain of rDNA sequences within a group representing the type strains of *Galactomyces* species accepted by Kurtzman et al. (2011) and related *Geotrichum* species, placed the strain GZ1 in the *Galactomyces candidus* clade to be closely related to *Galactomyces geotrichum* and *Geotrichum europaeum* (Fig. 1). On the other hand, it was demonstrated that the strain GZ1 could grow at 35 °C, which is a characteristic of *Galactomyces candidus* and constitutes a difference with *Galactomyces geotrichum* (Kurtzman et al., 2011). Therefore, according to these results, the strain GZ1 was identified as *Galactomyces candidus*.

It is worth mentioning that, on the basis of rDNA reassociation studies (Smith et al., 1995) and ITS sequence analyses (de Hoog and Smith, 2004) the species *Galalactomyces candidus* was introduced to accommodate the most common and widely distributed entity of the *Geotrichum* complex. This fungus is common in all kinds of moist substrates, particularly in fruits and tomatoes, and is the main cause of rot in ripe fruits, carrots and some other vegetables (Thornton et al., 2010).

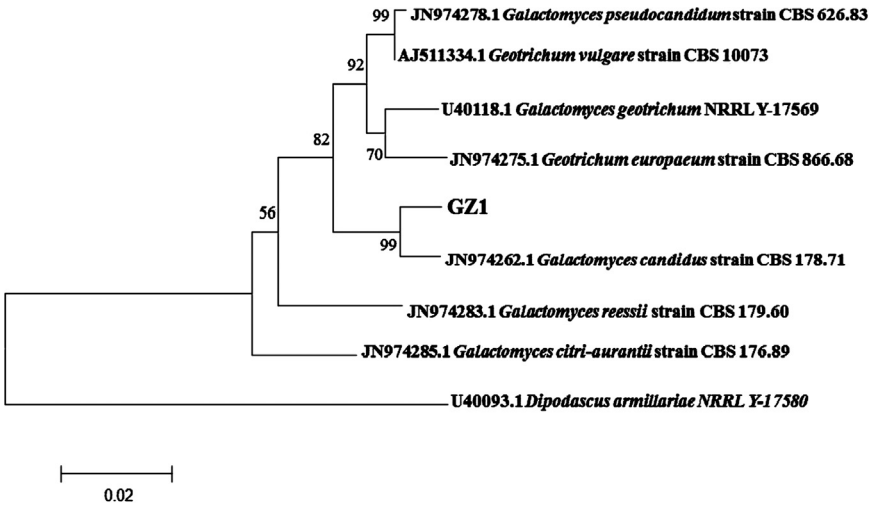


Fig. 1. Phylogenetic tree showing placement of GZ1.

3.3. Optimization of the methodology

Reduction of AP to chiral 1-PE is a biotransformation of high application potential because both enantiomers of this compound are important natural aromas. (*S*)-1-PE is characterized as a mild hyacinth, gardenia aroma with strawberry nuances (Farbood et al., 2003), while (*R*)-1-PE has a floral, earthy-green, honeysuckle odor (Homola et al., 2015, 2016). Moreover, (*R*)-1-PE is used as a chiral building block in fine chemical and in the pharmaceutical industry is used as an ophthalmic preservative (Suan and Sarmidi, 2004).

Additionally, the bioreduction of AP to the corresponding (*R*)- or (*S*)-1-PE is one of the most common reactions in organic chemistry occurring when a new biocatalyst is studied (Rodrigues et al., 2004). However, reduction of AP to (*S*)-1-PE, which is in agreement with the Prelog rule (Prelog, 1964), is more commonly observed and several works reported high yield and excellent optical purity of this enantiomer for different whole-cell biocatalysts (Hasegawa et al., 1998; Kurbanoglu et al., 2010; Cheng and Ma, 1996; Matsuda et al., 2008). As counterpart, *anti*-prelog reduction of AP to (*R*)-1-PE is less frequent. However there are a few microbial strains providing this bioconversion (Hummel, 1990; Schlieben et al., 2005; Inoue et al., 2005; Protsko et al., 2010).

On the other hand, parameters such as substrate concentration, reaction temperature, pH of the media, co-solvent used, inoculum charge, shaking speed and reaction time exert a critical effect on the performance of the biocatalytic processes, with a fine and delicate regulation of these parameters being necessary to identify the optimal working conditions of a biocatalytic system.

For all the reasons mentioned above, the effect of these parameters on the asymmetric reduction of AP to produce stereoselectively (*R*)-1-PE using *Galactomyces candidus* GZ1 was studied, and the results obtained are discussed below.

3.4. Effect of AP concentration

One of the major disadvantages of the biochemical methods is, in general, the lower charge substrate/biocatalyst relationship compared to that of classical chemical methodologies (Nakamura et al., 2003). Consequently, it is very important to determine the effect of substrate concentration on the product yield and stereoselectivity. To optimize the procedure, assays with 50, 75, 100, 125, 150, 175 and 200 µL AP in 80 mL of 100 mm phosphate buffer pH 7.0 (AP concentrations from 0.64 to 2.575 g/L with 37.5 g/L of cells), 3 g wet weight of cells of *Gal. candidus* GZ1, and 1.25% v/v DMSO, at 100 rpm, at 28 °C were used to determine their effects on the production of (*R*)-1-PE. The reaction was monitored for 96 hs, and samples were taken every 24 h and analysed by chiral GC-FID and GC-MS.

As shown in Fig. 2, increasing the concentration of AP in the reaction medium had a notable effect on the reductive process during the first 24 h, with the reaction only being efficient at lower charge (0.625 g/L). Similarly, the e.e.% was low too. On the other hand, and as can be seen in Fig. 2, the reductive process return was effective when a longer reaction time had elapsed. In effect, the higher load of AP increased the reaction time from 24 to 48 h in the range of 0.625–1.875 g/L. However, the higher load of 1.875 g/L of AP had an inhibitory effect on the reaction, probably due an increased toxicity of AP in the microorganism. Additionally, when the AP concentration was higher than 1.875 g/L, from 48 h the conversion notably decreased, but in all cases the e.e.% was very high. Considering both the conversion and e.e.%, the best AP concentration for the bio-reduction was 1.875 g/L and 48 h of reaction.

3.5. Effect of temperature

Temperature is a critical factor when a biocatalytic process is used, because this parameter has a significant influence on the activity, selectivity and stability of a microorganism (Nakamura et al., 2003), with each microorganism having an optimal temperature at which all metabolic processes occur efficiently. To determine the temperature effect on the production of (*R*)-1-PE, assays were performed at 20, 25, 28 and 30 °C, with 150 µL of AP in 80 mL of 100 mm sterile phosphate buffer pH 7.0, 3 g wet weight of cells of *Gal. candidus* GZ1, and 1.25% v/v DMSO, at 100 rpm being used as the reaction condition (concentration of the substrate 1.875 g/L with 37.5 g/L of cells). Samples were taken over 48 hs and analysed by chiral GC-FID and GC-MS, and the results are shown in Fig. 3 were it can be seen that when the temperature was increased from 28 to 30 °C, a notable decrease in the conversion was observed with the yield falling from 96% to 51%. On the other hand, it is noteworthy that the e.e.% remained constant at all temperatures, and the reaction was fully stereoselective for the formation of (*R*)-1-PE. Also, in Fig. 3a stability zone between 20 and 28 °C can be seen. From these results, 25 °C turned out to be the most suitable temperature for carrying out the reductive process, which was within the stability zone for operation of GZ1.

3.6. Effect of pH

It has been well known that pH is a key factor in biocatalytic reactions as it can alter the ionic state of the substrate and / or the charging enzymes, and consequently affect the socket of the substrate at the enzyme's active site (Li et al., 2016). Moreover, when a reaction is catalyzed by several enzymes with different enantioselectivity at different pHs, alternation of pH will change the product yield and the e.e. % value. Here, to determine the pH effect, assays were carried out at 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0 upH using 80 mL of sterile 100 mM phosphate buffer, 150 µL of AP with 3 g wet weight of cells of *Gal. candidus* GZ1, and 1.25% v/v DMSO, 100 rpm, at 25 °C, over 48 h (concentration of the substrate was 1.875 g/L with 37.5 g/L of cells). Samples were taken and analysed by chiral GC-FID and GC-MS.

Fig. 4 shows the pH effect on the reaction, where it can be observed that the yield increased from 33% to 97% when the buffer pH increased from 5.0 to 6.5, with pH values between 6.5 and 7.5 providing optimal results. However, at pH 8.0 a notable decrease in the conversion was observed. It is noteworthy that the e.e.% remained significantly high and constant at all pH values tested, with this revealing that the stereoselectivity of the enzyme complex present in *Gal. candidus* GZ1 was not affected by the change of pH. Considering the strong pH effect on conversion, the optimal pH value for the synthesis of (*R*)-1-PE can be observed from Fig. 4 to be 7.0 (99% conversion).

3.7. Effect of co-solvent on bioreduction

Although biocatalytic systems have the environmental benefit that the solvent used as the reaction medium is water, this can also be an inconvenience when organic substrates whose solubility in water is low or zero are used. This situation can be resolved is saved by the use of a co-solvent to facilitate the solubilization of the substrate in the reaction media. However, the choice of co-solvent must be studied in order to minimize its possible toxic effect on the performance of the reaction. In fact, various co-solvents have been used as co-substrates to assist the recycling of enzymatic co-factors and thus make the interplay more efficient (Du et al., 2014). Considering both these ideas, we carried out a study using different organic co-solvents, with assays being conducted using eleven different co-solvents at 1.25% v/v of final concentration and a final volume of reaction of 80 mL of sterile 100 mM phosphate buffer pH 7,0. As control, a reaction without co-solvent was made. All these assays were performed using 150 µL of AP with 3 g wet weight of cells of *Gal. candidus* GZ1, 100 rpm, at 25 °C, for 48 hs (concentration of the substrate 1.875 g/L with 37.5 g/L of cells). Samples were taken at the end of the reaction and analysed by chiral GC-FID and GC-MS. Fig. 5 shows the co-solvent effect on the performance of the reaction.

As can be seen in Fig. 5, eleven co-solvents were tested, which had

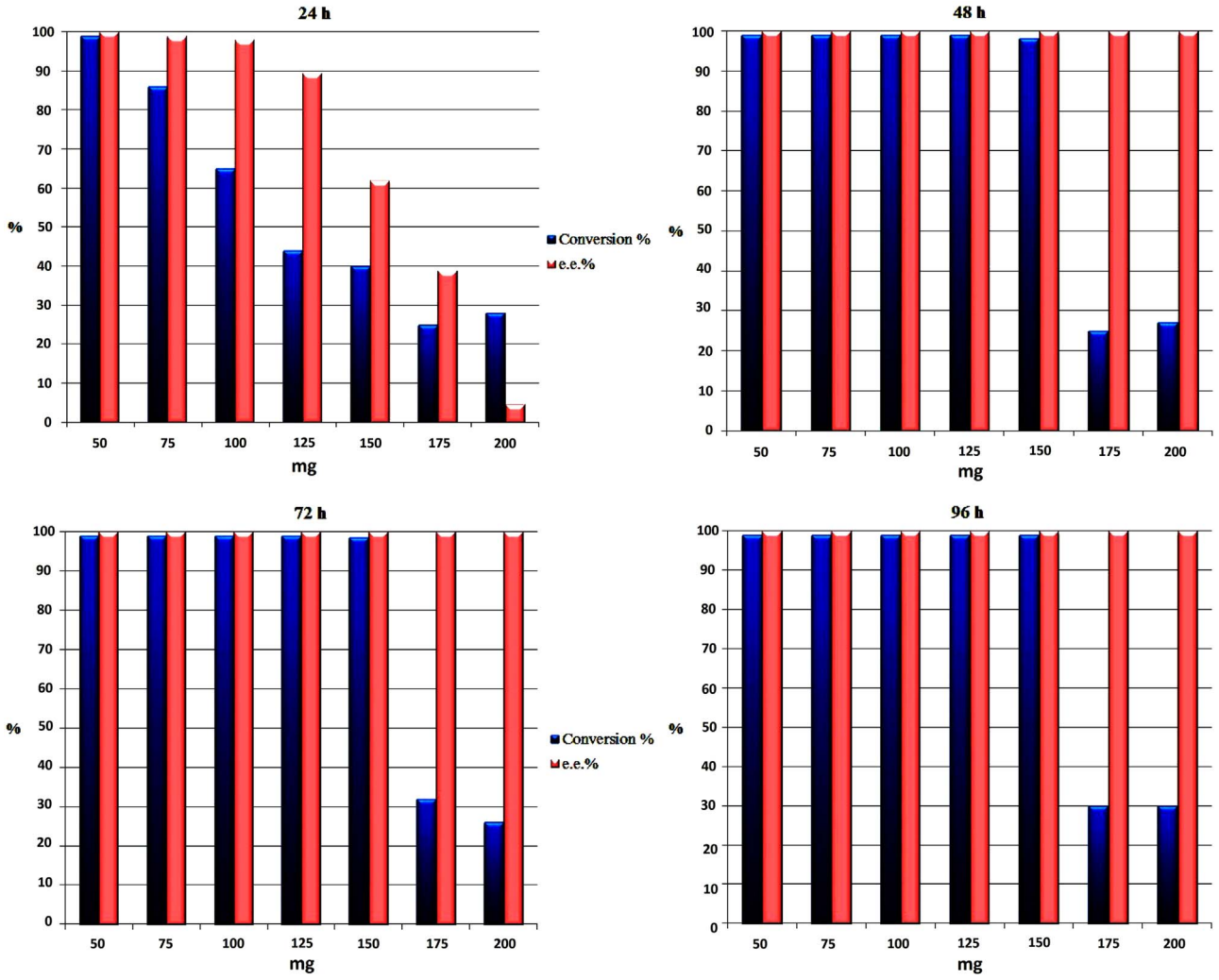


Fig. 2. Effects of acetophenone concentration on the production of (*R*)-1-phenylethanol mediated by *Gal. candidus* GZ1.

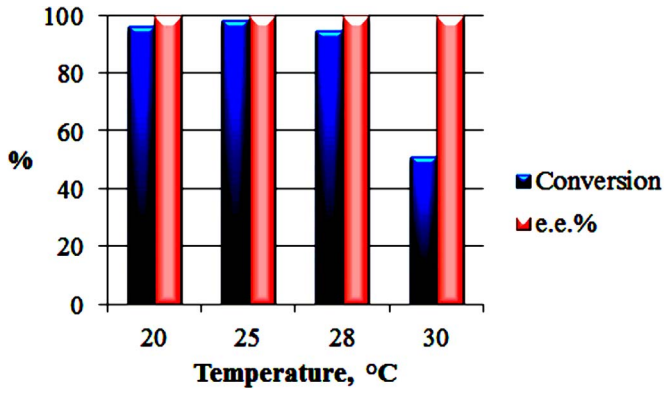


Fig. 3. Effects of temperature on the asymmetric reduction of acetophenone by cells of *Gal. candidus* GZ1.

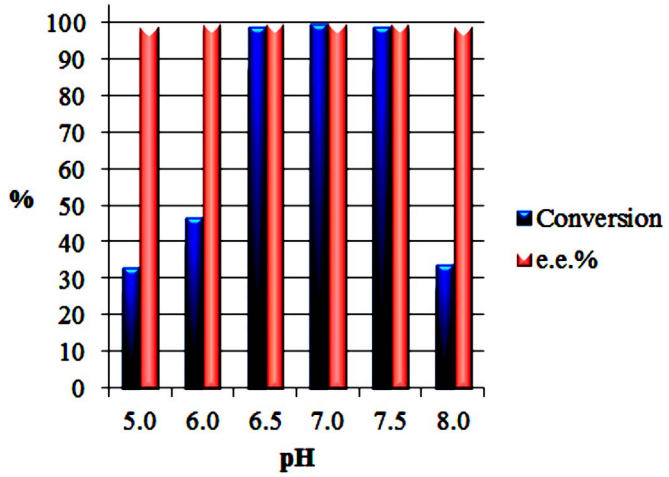


Fig. 4. Effects of pH on the asymmetric reduction of acetophenone by cells of *Gal. candidus* GZ1.

diverse effects not only on the percentage conversion but also on the e.e.%. Four co-solvents (1-butanol, cyclohexanol, benzyl alcohol and THF) had an inhibitory effect on the percentage conversion, and were detrimental to the formation of 1-PE, compared with the reaction without co-solvent. It is also noteworthy that benzyl alcohol had a fully inhibitory effect on the reaction at the concentration used, and additionally, these all had a negative effect on the stereoselectivity too. When 1-butanol and cyclohexanol were introduced in the reaction media, a change in the stereoselectivity was observed. In both cases, the (*S*)-enantiomer was preferred, with this situation being very notable when cyclohexanol was used as a co-solvent (91 e.e.% *S*). In this sense, it's probable that two or more enzymes with opposite enantioselectivities are present in *Gal. candidus* GZ1, and maybe also that cyclohexanol possesses an inhibitory effect on the enzyme that promotes the

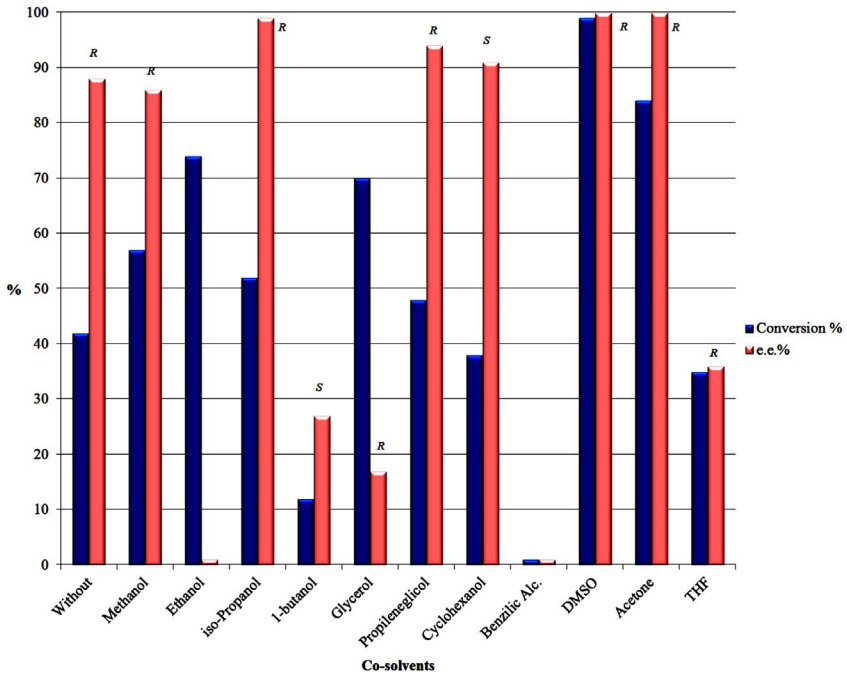


Fig. 5. Effect of co-solvents on the asymmetric reduction of acetophenone by cells of *Gal. candidus* GZ1.

formation of (*R*)-enantiomer. Nevertheless, this result opens up the possibility of inverting the stereoselectivity of this microorganism, and this way, obtaining the stereoselectively of the (*S*)-enantiomer, but more studies are necessary to examine this hypothesis. Related to this, Nakamura et al. (2002) using a strain of *Geotrichum candidum* IFO 5767 achieved the total inversion of the configuration using a nonpolar resin amberlite XAD-7 under a nitrogen atmosphere. Moreover, mechanistic interpretations were made by the same authors using kinetic studies on racemic samples of several chiral phenyethanols and the same strain IFO 5767 (Nakamura et al., 1995). Based on the results observed, two enzymes were postulated to co-exist with opposite stereoselectivities in this microorganism.

On the other hand, seven co-solvents (methanol, ethanol, *iso*-propanol, glycerol, propilenglicol, DMSO and acetone) had a positive effect on the conversion compared with the reaction without co-solvent. Furthermore, an excellent stereoselectivity with 1-propanol, DMSO and acetone were observed. In contrast, although the use of ethanol improved the conversion, the stereoselectivity of the process was totally lost. A similar situation with glycerol was also observed, but the loss of stereoselectivity was not as pronounced. Finally, for the pursuit of our studies, DMSO was selected as the best co-solvent.

3.8. Effect of cell concentration

In a whole cell catalytic system, the mass of the whole cells influences not only the initial reaction rates, but also the regeneration of the coenzymes. Additionally, large amounts of the mass of whole cells have an effect on the reaction media viscosity, and this situation can bring about a mass transfer limitation of the reaction system (Li et al., 2016). For these reasons, different cell concentrations from 37.5 to 125 g/L were chosen to study the effect of the microorganism concentration on the reduction of AP.

Fig. 6 shows the variation of the conversion and e.e.% with different cell concentrations. It can be seen that, when cell concentration increased from 75 to 125 g/L, the yield decreased and reached its minimum at 125 g/L of cell concentration. However, there was no variation of e.e.% of (*R*)-1-PE with the a change in cell concentration, and this remained independent of the amount of microorganism used. Despite these results, it was decided to perform experiments at different speeds of agitation using 112.5 g/L, and thus try to establish a greater useful load of the microorganism.

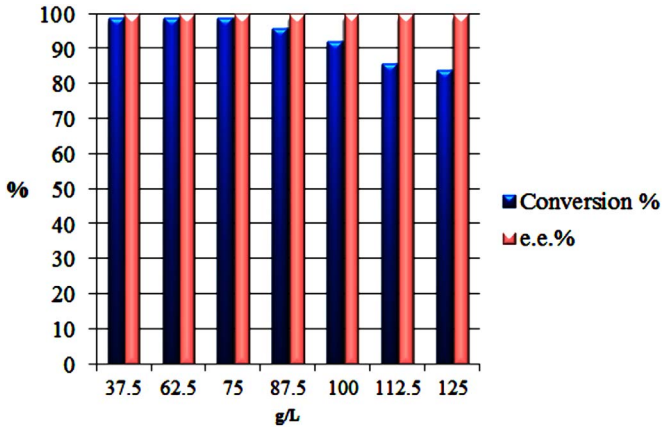


Fig. 6. Effects of cell concentration on the asymmetric reduction of acetophenone by cells of *Gal. candidus* GZ1.

3.9. The effect of shaking speed

The shaking speed can influence the dissolved oxygen content in the liquid and the diffusion of substrate and product in the reaction system. This can lead to changes in the reaction rate, the conversion and e.e.% of the product obtained (Li et al., 2016). The effect of three different shaking speeds (100, 125 and 150 rpm) on the reduction efficiency was investigated in order to select a suitable level. Samples were taken at 24 and 48 h. It can be seen in Fig. 7 that at 48 h the conversion increased to be quantitative when the shaking speed was 150 rpm. On the other hand, the e.e.% was constant and high for all levels of shaking. Therefore, a shaking speed of 150 rpm was selected for the our bioreduction system.

3.10. Preparative scale bioreduction of AP

A major scale reaction was performed using the same optimal conditions as in our previous studies. 1.05 g AP were used with 0.875 g of (*R*)-1-PE after purification being obtained (82% of yield, > 99.9 e.e.

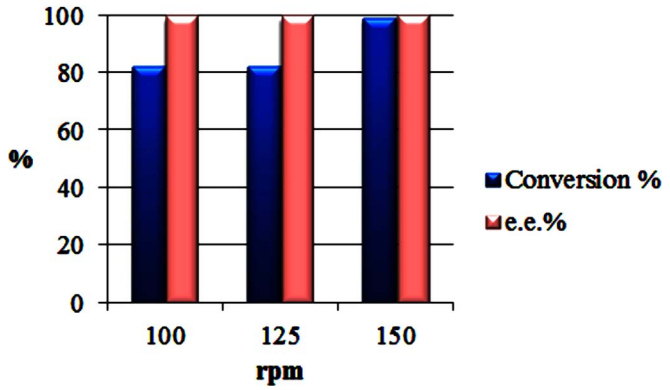


Fig. 7. Shacking effect on the asymmetric reduction of acetophenone by cells of *Gal. candidus* GZ1.

%). It should be noted that the extraction and purification procedures were not optimized.

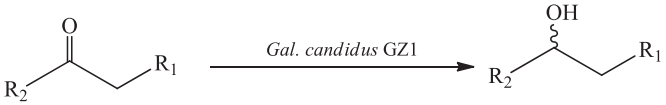
3.11. Advantages and scope of Galactomyces candidus GZ1 as a stereoselective bioreductor

After successfully optimizing the reaction conditions for obtaining (*R*)-1-PE, we turned our attention to the biocatalytic reduction of substituted APs. For this purpose, fifteen AP derivatives with different substituents (fluorine, chlorine, bromine, nitro, cyano, hydroxy, and methyl) on the benzene ring were selected to assess the advantage and scopes of *Gal. candidus* GZ1 as a biocatalyst. Moreover, 2-chloroacetophenone was used to determine the effect of a substituent located in the methyl group and 2-acetylthiophene and 2-acetylpyridine were also assayed as substrates. These results are shown in Table 2 where it can be seen that, two effects were observed depending on the nature and position of the substituent. On the one hand, there appeared to be a relationship between the reactivity and the electronic nature of the substituent groups. In fact, while GZ1 efficiently reduces both substituted APs with attractor or donor electron groups (Table 2, compare entry 1 with 6, 12 y 16), but substrates with electron donating groups were less reactive than those having attractors groups. This difference can be observed in the reaction times. In this sense, it seems that electron donor groups had a retarding effect on the reductive process (Table 2, entries 2–12 *vs* 14 and 16).

On the other hand, it is also possible to observe in Table 2 an effect exerted by the relative position of the substituents related to the carbonyl group and this situation could be observed in the reduction of chloroacetophenones (Table 2, entries 4–6). In fact, substituents in the 2′- and 4′-positions appeared to have no effect on the percentage reduction compared to AP. Conversely, the introduction of a substituent in the 3′-position had a slightly negative effect on the conversion. In the case of the 2′- and 4′-chloroacetophenone, the conversions were practically the same as those obtained when AP was used (Table 2, entry 1 compared with 4 and 6), whereas the conversion decreased with 3′-chloroacetophenone (Table 1, entry 5). Likewise, when nitro groups were used as the substituent (Table 2, entries 10 and 12 compared with entry 11) the behavior was similar to that observed with chloroacetophenones. In addition, it should be noted that the slightly lower conversion observed with 2′-Nitroacetophenone (Table 2, entry 10), compared to 4′-Nitroacetophenone (Table 2, entry 12), may be attributed to a steric effect due to the proximity of the nitro substituent to carbonyl group.

Another important observation relating to the relative position of the substituents with respect to the carbonyl group can be observed in Table 2. When the substituent was located in the 2′-position, the absolute configuration responded to the Prelog rule, which can be observed in the results obtained with 2′-Chloro-, 2′,4′-Dichloro-, 2′-Nitroacetophenone (Table 2, entries 4, 7 and 10). Similarly, 3′-Chloroacetophenone produced the formation of (*S*)-1-(*3*-chlorophenyl) ethanol (Table 2, entry 5). It is interesting to note that a similar

Table 2
Stereoselective reduction of substituted APs promoted by *Galomyces candidus* GZ1.



Entry	Substrate		Time (days)	Conversion %[a]	e.e.%[a,b]	Yield %[c]
	R_1	R_2				
1	H	phenyl	2	99	> 99,9 (*R*)	82
2	H	4′-fluorophenyl	1	70	95 (*R*)	61
3	Cl	phenyl	0.67	> 99,9	66 (*S*)	42
4	H	2′-chlorophenyl	0.67	98	95 (*S*)	72
5	H	3′-chlorophenyl	0.67	67	85 (*S*)	57
6	H	4′-chlorophenyl	2	98	92 (*R*)	98
7	H	2′,4′-dichlorophenyl	0.67	99	97 (*S*)	49
8	H	4′-bromophenyl	2	87	99 (*R*)	50
9	H	4′-cyanophenyl	2	97	> 99,9 (*R*)	62
10	H	2′-nitrophenyl	2	84	99 (*S*)	73
11	H	3′-nitrophenyl	2	70	> 99.9 (*R*)	70
12	H	4′-nitrophenyl	1.7	99	99 (*R*)	92
13	H	2′-hydroxyphenyl	4	nr[d]	–	–
14	H	3′-hydroxyphenyl	3	74	> 99.9(*R*)	51
15	H	4′-hydroxyphenyl	4	nr	–	–
16	H	4′-methylphenyl	13.3	90	99 (*R*)	42
17	H	*2*-pyridyl	4	nr	–	–
18	H	2-thyenyl	4	nr	–	–

[a] Conversion (%) and enantiomeric excess (e.e.%) were determined by chiral GC-FID and GC-MS analyses.
[b] Absolute configurations are shown between parenthesis and were assigned by comparing the retention time of chiral GC data with the reports in the literature and by comparison of the sign of optical rotations with the literature values.
[c] Isolated yield by chromatographic column.
[d] Not reaction.

situation was observed by Kurbanoglu et al. (2007) and Comasetto et al. (2006) using a strains of *Aspergillus niger* EBK-9 and a strain of *Aspegillus terreus* CCT 3320, respectively. In this sense, Comasetto et al. (2006) carried out desracemization studies of (*R*,*S*)-1-(nitrophenyl)ethanols and using a strain of *Aspegillus terreus* CCT 3320, postulated the presence of two enzymes with opposite stereoselectivities. If both enzymes are operating simultaneously and competitively, it would be possible to imagine a difference in affinity depending on the spatial conformation in which the carbonyl group is located respect to the substituent. An inversion in the configuration of the final product would therefore be observed if a particular spatial conformation of the substrate had a higher affinity for the enzyme pro-*S*. For these reasons, it is possible that several enzymes are present in *Gal. candidus* GZ1, and this might explain the different preference for enantiomers formed for 2′- and 4′-substituted APs. Evidently, these results clearly demonstrate the need for complementary studies using 2′-substituted APs with groups of different sizes as well as kinetic studies to establish the reaction mechanism involved in order to justify the results presented here.

On the other side, very good results were obtained using 4′-Fluor, 4′-Bromo, 4′-CH_3, 4-NO_2 and 4′-Cyanoacetophenone (Table 2, entry 2, 8 and 9) with a very good conversion (70 - > 99.9%) and an excellent enantioselectivity (95 - > 99.9%) being obtained. In the five cases, the absolute configuration of the products was *R*. As counterpart, the reaction failed completely when 2-acetylthiophene and 2-acetylpyridine (Table 2, entries 17 and 18) were used as the substrate.

It is interesting to note the results achieved when hydroxyacetophenones were subjected to reduction with GZ1. The reaction failed completely when 2′- and 4′-hydroxyacetophenone were used in our reaction conditions (Table 2, entries 13 and 15 respectively). In contrast, 3′-hydroxyacetophenone was transformed into the corresponding (*R*)-alcohol with good conversion and excellent enantioselectivity (Table 2, entry 14). This marked difference in the behavior of hydroxyacetophenones might be explained by the formation of a quinone intermediate (Bell, 1969) in the reaction media by deprotonation of the phenolic hydroxyl. In this way, removal of the phenolic hydrogen would make a phenoxide ion. Thus, the charge is stabilized by resonance into the aromatic ring. The negative charge is spread over the 2′- and 4′-positions to the hydroxyl group; in addition, both positions also could interact with the carbonyl group giving additional stabilization and this situation could alter its reactivity. This situation is not possible with 3′-isomer and perhaps for this reason 2′- and 4′-hydroxyacetophenone do not react when *Gal. candidus* GZ1 when was used as the catalyst. However, more studies are needed to support this hypothesis.

Although biocatalytic reduction is recognized as being a highly efficient approach towards enantiomerically pure alcohols, studies on bioreduction of non-protected hydroxyacetophenones, which represent several substructures in a range of pharmaceutical drugs, are so far surprisingly rare (Neupert et al., 2010). For this reason, it is necessary to highlight the importance of the results obtained in the reduction of 3′-hydroxyacetophenone because (*R*)-1-(3-hydroxyphenyl)ethanol is the key intermediate in the synthesis of Rivastigmine (anti-Alzheimer drug, Yan et al., 2013).

Although 2-chloroacetophenone produced an excellent conversion when *Gal. candidus* GZ1 was used as the bioreductor, the stereoselectivity was lower than that observed in its reaction with AP (Table 2, entry 1 and 3). This result shows that the introduction of a substituent (chlorine) on the methyl group had a steric effect on the stereoselective transfer of a hydride ion at the active site of the enzymatic complex.

Finally, it should be clarified that the procedure carried out for the isolation of the products was not optimized, which resulted in the observed differences between the percentages of conversion and the isolates yields.

3.12. Preparative scale bioreduction of 3-hydroxyacetophenone

Due to the importance of the results obtained when 3-hydroxyacetophenone was reduced with GZ1, a preparative scale reaction was performed. To carry this out, 0.563 g of 3-hydroxyacetophenone was reacted with 66.7 g GZ1, 8 mL of DMSO in a final volume of 560 mL of phosphate buffer pH 7,0. The reduction was performed in three days and (*R*)-1-(3-hydroxyphenyl)ethanol was obtained with 66% of conversion and > 99.9 of e.e.%. The extraction of the product was performed with ethyl acetate using a liquid-liquid extractor. The dried residue was purified by column chromatography using hexane / ethyl acetate mixtures in variable portions as the eluent and the isolated yield of the product was 53% (0303 g). It should be noted that the extraction and purification procedures were not optimized.

4. Conclusions

Galactomyces candidus GZ1 isolated from a decaying carrot proved to be a very efficient biocatalyst for the asymmetric bioreduction of acetophenones, and this strain can be used for developing protocols to achieve “grams” scale production of (*R*)-1-PE in a reasonable time period. In effect, (*R*)-1-PE was obtained in a ≈ 2 g/L scale. Moreover, the result achieved using 3′-hydroxyacetophenone is very encouraging because the product formed is the key intermediary in the synthesis of the commercial drug Rivastigmine. Additionally, because of the appreciable bioreduction potential of this indigenous strain, this makes it a potential candidate for major studies to expand its use as an environmentally friendly tool for the synthesis of other desired chiral synthons. In fact, some other studies are currently being realized by our laboratory with respect to this issue.

Acknowledgments

M.F. Decarlini and A.M. Vazquez gratefully acknowledges financial support from the Catholic University of Cordoba. M. L. Aimar gratefully acknowledges financial support from Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (Grant number: 30720150100338CB) (SECyT-UNC). M.F. Decarlini and M.L. Aimar gratefully acknowledge Dr Nestor Soria for their technical assistance with the PCR analyses. Also, we thank Dr Paul Hobson, native speaker, for the revision of this manuscript. This work is dedicated to the memory and the teaching career of Dr. Adriana B. Pierini.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bcab.2017.10.014.

References

Aguirre-Pranzoni, C., Bisogno, F., Orden, A.A., Kurina-Sanz, M., 2014. Lyophilized *Rhodotorula* yeast as all-in-one redox biocatalyst: Access to enantiopure building blocks by simple chemoenzymatic one-pot procedures. J. Mol. Catal. B: Enzymatic 114, 19–24.

Aimar, M.L., Bordón, D.L., Formica, S.M.F.R., Cantero, J.J., Vazquez, A.M., Velasco, M.I., Rossi, L.I., 2014. Fruits of the glossy privet (*Ligustrum lucidum* – Oleaceae) as biocatalysts for producing chiral aromatic alcohols. Biocat. Biotrans. 32, 348–357.

Alper, I., Frenette, M., Labrie, S., 2011. Ribosomal DNA polymorphisms in the yeast *Geotrichum candidum*. Fungal Biol. 115 (12), 1259–1269.

Bell, K.H., 1969. Reductive removal by sodium borohydride of side-chain oxygen from phenolic ketones. Aust. J. Chem. 22, 601–605.

Bennamame, M., Zeror, S., Aribi-Zouioueche, L., 2014. Asymmetric reduction of ketones by biocatalysis using medlar (Mespilus germanica L) fruit grown in Algeria. Biocat. Biotrans. 32 (5-6), 327–332.

Bennamame, M., Zeror, S., Aribi-Zouioueche, L., 2015. Asymmetric reduction of ketones by biocatalysis using clementine mandarin (citrus reticulata) fruit grown in annaba or by rutheniumcatalysis for access to both enantiomers. Chirality 27, 205–210.

Bi-Hong, J., Ke, L., Ya-Ge, X., Ming-Yuan, L., Li, M., Zhen-Ming, C., Wen-Liang, X., 2012. *Rhodotorula mucilaginosa* as a new biocatalyst for asymmetric reduction of acetophenone. Afr. J. Microbiol. Res. 6 (46), 7324–7331 and references cited here.

Cazetta, T., 2014. Desracemização de álcoois secundários por estereoinversão e redução de metilenocetonas por micro-organismos. Tese de Doutorado apresentada ao Instituto de Química da UniCamp para obtenção do título de Doutora em Ciências, pages 28, 66 and 91. Universidad Estadual de Campinas. <http://repositorio.unicamp.br/jspui/handle/REPOSIP/250620>. (Accessed 08.08.17).

Cheng, C., Ma, J.-H., 1996. Enantioselective synthesis of S-(-)−1-phenylethanol in *Candida utilis* semi-fed-batch cultures. Process Biochem. 31, 119–124.

Comasseto, J.V., Assis, L.F., Andrade, L.H., Schoenlein-Crusius, I.H., Porto, A.L.M., 2006. **Biotransformations of *ortho*-, *meta*- and *para*-aromatic nitrocompounds by strains of *Aspergillus terreus*: Reduction of ketones and deracemization of alcohols**. J. Mol. Cat. B: Enzymatic 39, 24–30.

Cordell, G.A., Lemos, T.L.G., Monte, F.J.K., de Mattos, M.C., 2007. Vegetables as chemical reagents. J. Nat. Prod. 70, 478–492.

Du, P.-X., Wei, P., Lou, W.-Y., Zong, M.-H., 2014. Biocatalytic anti-Prelog reduction of prochiral ketones with whole cells of *Acetobacter pasteurianus* GIM1.158. Microb. Cell Fact. 13 (84), 1–9.

Erdélyi, B., Szabó, A., Seres, G., Birincsik, L., Ivanics, J., Szatzkerd, G., Poppe, L., 2006. Stereoselective production of (S)-1-aralkyl- and 1-arylethanols by freshly harvested and lyophilized yeast cells. Tetrahedron: Asymmetry 17, 268–274.

Farbood, M.I., Blocker, R.W., Arvizzigno, J., Muralidhara, R., 2003. Mixtures of optical isomers of styralyl alcohol or styralyl acetate, processes for preparing same and organoleptic uses thereof. US Patent 6511686 B1. <www.google.com/patents/US6511686>. (Accesed 18 August 2017).

Goldberg, K., Schroer, K., Lütz, S., Liese, A., 2007. Biocatalytic ketone reduction—a powerful tool for the production of chiral alcohols—part I: processes with isolated enzymes. Appl. Microbiol. Biotechnol. 76, 237–248.

Hasegawa, Y., Adachi, S., Matsuno, R., 1998. Asymmetric reduction of acetophenone by immobilized *Hansenula capsulata* cells. J. Ferment. Bioeng. 85, 322–327.

Homola, P., Kurák, T., Illeová, V., Polakovič, M., 2015. Cultivation of *Pichia capsulata* as a whole-cell biocatalyst with NADH-dependent alcohol dehydrogenase activity for R−1-phenylethanol production. Food Bioprod Process 96, 126–132.

Homola, P., Kurák, T., Illeová, V., Polakovic, M., 2016. Kinetics of acetophenone reduction to (*R*)-1-phenylethanol by a whole-cell *Pichia capsulata* biocatalyst. Biocatal Biotrans. 0 (0), 1–10.

de Hoog, G.S., Smith, M. Th, 2004. Ribosomal gene phylogeny and species delimitation in *Geotrichum* and its teleomorphs. Stud. Mycol. 50, 489–515.

Huisman, G.W., Liang, J., Krebber, A., 2010. Practical chiral alcohol manufacture using ketoreductases. Curr. Opin. Chem. Biol. 14, 122–129.

Hummel, W., 1990. Enzyme-catalyzed synthesis of optically pure *R*-(+)-phenylethanol. Biotechnol Lett. 12, 403–408.

Hutt, A.J., Tan, S.C., 1996. Drug chirality and its clinical significance. Drugs 52, 1–12.

Inoue, K., Makino, Y., Itoh, N., 2005. Purification and characterization of a novel alcohol dehydrogenase from *Leifsonia sp.* strain S749: a promising biocatalyst for an asymmetric hydrogen transfer bioreduction. Appl. Environ. Microbiol. 71, 3633–3641.

Kumaraswamy, G., Ramesh, S., 2003. Soaked *Phaseolus aureus* L: an efficient bio- catalyst for asymmetric reduction of prochiral aromatic ketones. Green Chem. 5, 306–308.

Kurbanoglu, E.B., Zilbeyaz, K., Kurbanoglu, N.I., Kilic, H., 2007. Enantioselective reduction of substituted acetophenones by *Aspergillus niger*. Tetrahedron: Asymmetry 18, 1159–1162.

Kurbanoglu, E.B., Zilbeyaz, K., Ozdal, M., Taskin, M., Kurbanoglu, N.I., 2010. Asymmetric reduction of substituted acetophenones using once immobilized *Rhodotorula glutinis* cells. Bioresour. Technol. 101, 3825–3829.

Kurtzman, C.P., Fell, J.W., Boekhout, T., 2011. The Yeasts, A Taxonomic Study, 5th ed. Elsevier Science B.V., Amsterdam, pp. 2080.

Li, H., Li, Z., Ruan, G., Yu, Y., Liu, X., 2016. Asymmetric reduction of acetophenone into R-(+)-1-phenylethanol by endophytic fungus *Neofusicoccum parvum* BYEF07 isolated from Illicium verum. Biochem. Biophys. Res. Comm 473, 874–878.

Li, W., Hou, G., Wang, Ch, Jiang, Y., Zhang, X., 2010. Asymmetric hydrogenation of ketone catalyzed by ruthenium(II)–indan-ambox complex. Chem. Comm. 46, 3979–3981.

Mathre, D.J., Thompson, A.S., Douglas, A.W., Hoogsteen, K., Carroll, J.D., Corley, E.G., Grabowski, E.J.J., 1993. A practical process for the preparation of Tetrahydro-l-methyl-3,3-diphenyll *H*, 3*H* -pyrrolo1[,2-c]-[1,3,2]oxazaborole-Borane. A highly enantioselective stoichiometric and catalytic reducing agent. J. Org. Chem. 58, 2880–2888.

Matsuda, T., Marukado, R., Mukouyama, M., Harada, T., Nakamura, K., 2008. Asymmetric reduction of ketones by *Geotrichum candidum*: immobilization and application to reactions using supercritical carbon dioxide. Tetrahedron: Asymmetry. 19, 2272–2275.

Nakamura, K., Matsuda, T., 1998. **Asymmetric reduction of ketones by the acetone powder of *Geotrichum candidum***. J. Org. Chem. 63, 8957–8964.

Nakamura, K., Matsuda, T., 2006. Biocatalytic reduction of carbonyl groups. Curr. Org. Chem. 10, 1217–1246.

Nakamura, K., Inoue, Y., Matsuda, T., Misawa, I., 1999. Stereoselective oxidation and reduction by immobilized *Geotrichum candidum* in an organic solvent. J. Chem. Soc. Perkin Trans. 1 (16), 2397–2402.

Nakamura, K., Takenaka, K., Fujii, M., Ida, Y., 2002. Asymmetric synthesis of both enantiomers of secondary alcohols by reduction with a single microbe. Tetrahedron Lett. 43, 3629–3631.

Nakamura, K., Yamanaka, R., Matsuda, T., Harada, T., 2003. Recent developments in asymmetric reduction of ketones with biocatalysts. Tetrahedron: Asymmetry 14, 2659–2681.

Nakamura, M., Inoue, Y., Matsuda, T., Ohno, A., 1995. Microbial deracemization of 1-Arylethanol. Tetrahedron Lett 36 (35), 6263–6266.

Neupert, A., Ress, T., Wittmann, J., Hummel, W., Grögera, H., 2010. Enantioselective biocatalytic reduction of non-protected hydroxyacetophenones. Z. Naturforsch. 65b, 337–340.

Orden, A.A., Magallanes-Noguera, C., Agostini, E., Kurina-Sanz, M., 2009. Anti-prelog reduction of ketones by hairy root cultures. J. Mol. Catal. B: Enzym. 61, 216–220.

Pavoković, D., Buda, R., Andrašec, F., Roje, M., Bubalo, M.C., Redovniković, I.R., 2017. Plant-mediated asymmetric reduction of 1-(3, 4-dimethylphenyl) ethanone. Tetrahedron: Asymmetry 28 (5), 730–733.

Pereyra, V., Martinez, A., Rufo, C., Vero, S., 2014. Oleaginous Yeasts form Uruguay and Antarctica as Renewable Raw Material for Biodiesel Production. Am. J. Biol. Sci. 2 (6), 251–257.

Peterson, S.W., Horn, B.W., 2009. *Penicillin pabulum* and *Penicillium Georgians*, Sep. nov., isolated from the conidial heads of *Aspergillus* species. Mycologia 101, 71–83.

Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage, 3rd ed. Springer Science + Business Media, LLC, 233 Spring Street, New York, NY 10013, USA.

Prelog, V., 1964. Specification of the stereospecificity or some oxido-reductases by diamond lattice sections. Pure Appl. Chem. 9, 119–130.

Protsko, C., Vieille, C., Laivenieks, M., Prasad, L., Sanders, D.R., Delbaere, L.T.J., 2010. Crystallization and preliminary X-ray diffraction analysis of the *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase I86A mutant. Acta Crystallograph. Sect. F, Struct. Biol. Cryst. Commun. 66, 831–833.

Quezada, M., Carballeira, J., Sinisterra, J., 2009. *Monascus kaoliang* CBS 302.78 immobilized in polyurethane foam using *iso*-propanol as co-substrate: Optimized immobilization conditions of a fungus as biocatalyst for the reduction of ketones. Bioresour. Technol. 100, 2018–2025.

Rodrigues, J.A.R., Moran, P.J.S., Conceição, G.J.A., Fardelone, L.C., 2004. Recent advances in the biocatalytic asymmetric reduction of acetophenones and α, β-unsaturated carbonyl compounds. Food Technol. Biotechnol. 42 (4), 295–303 and references cited here.

Rodriguez, P., Gonzalez, D., Rodriguez Giordano, S., 2017. Endophytic microorganisms: a source of potentially useful biocatalysts. J. Mol. Catal. B: Enzym. http://dx.doi.org/10.1016/j.molcatb.2017.02.013. (In press).

Rouhi, A.M., 2003. Chiral business. Chem. Eng. News 81, 45–56.

Schena, L., Ippolito, A., Zahavi, T., Cohen, L., Nigro, F., Droby, S., 1999. Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots. Postharvest Biol. Technol. 17, 189–199.

Schlieben, N.H., Niefind, K., Muller, J., Riebel, B., Hummel, W., Schomburg, D., 2005. Atomic resolution structures of R-specific alcohol dehydrogenase from *Lactobacillus brevis* provide the structural bases of its substrate and cosubstrate specificity. J. Mol. Biol. 349, 801–813.

Sheldon, R.A., Arends, I., Hanefeld, U., 2007. Green Chemistry and Catalysis. Wiley-VCH Verlag GmbH & Co., KGaA, Weinheim, Germany ISBN: 978-3-527-30715-9.

Singh, V.K., 1992. Practical and useful methods for the enantioselective reduction of unsymmetrical ketones. Synthesis 7, 605–617.

Smith, M.Th, de Cock, A.W.A.M., Poot, G.A., Steensma, H.Y., 1995. Genome comparisons in the yeastlike fungal genus *Galactomyces* Redhead et Malloch. Int. J. Syst. Bacteriol. 45 (4), 826–831.

Suan, C.L., Sarmidi, M.R., 2004. Immobilized lipase-catalyzed resolution of (*R*, *S*) −1-phenylethanol in the recirculated packed bed reactor. J. Mol. Catal. B: Enz. 28, 111–119 and references cited here.

Thornton, C.R., Slaughter, C.D., Davis, R.M., 2010. Detection of the sour-rot pathogen Geotrichum candidum in tomatoes fruit and juice by using a highly specific monoclonal antibody-based ELISA". Int. J. Food Microbiol. 143, 166–172.

Utsukihara, T., Misumi, O., Kato, N., Kuroiwa, T., Horiuchi, C.A., 2006. Reduction of various ketones by red algae. Tetrahedron: Asymmetry 17, 1179–1185.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.

Xie, Y., Xu, J.-H., Xu, Y., 2010. Isolation of a Bacillus strain producing ketone reductase with high substrate tolerance. Biores. Technol. 101, 1054–1059 and references cited here.

Yadav, J.S., Nanda, S., Reddy, P.T., Rao, A.B., 2002. Efficient enantioselective reduction of ketones with *Daucus carota* root. J. Org. Chem. 67, 3900–3903.

Yan, P.-Ch, Zhu, G.-L., Xie, J.-H., Zhang, X.-D., Zhou, Q.-L., Li, Y.-Q., Shen, W.-H., Che, D.-Q., 2013. Industrial scale-up of enantioselective hydrogenation for the asymmetric synthesis of rivastigmine. Org. Process Res. Dev. 17, 307–312.