Ann Microbiol (2013) 63:1021–1027
DOI 10.1007/s13213-012-0557-x

ORIGINAL ARTICLE

Complementary microbial approaches for the preparation of optically pure aromatic molecules

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Received: 10 September 2012 / Accepted: 3 October 2012 / Published online: 14 October 2012

Abstract Different strategies for stereoselective microbial preparation of various chiral aromatic compounds are described. Optically pure 2-methyl-3-phenyl-1-propanol, ethyl 2-methyl-3-phenylpropanoate, 2-methyl-3-phenylpropanal, 2-methyl-3-phenylpropionic acid and 2-methyl-3-phenylpropyl acetate have been prepared using different microbial biotransformations starting from different prochiral and/or racemic substrates. (*S*)-2-Methyl-3-phenyl-1-propanol and (*S*)-2-methyl-3-phenylpropanal were prepared by biotransformation of 2-methyl cinnamaldehyde using the recombinant strain *Saccharomyces cerevisiae* BY4741ΔOye2Ks carrying a heterologous OYE gene from *Kazachstania spencerorum*. (*R*)-2-Methyl-3-phenylpropionic acid was obtained by oxidation of racemic 2-methyl-3-phenyl-1-propanol with acetic acid bacteria. Kinetic resolution of racemic 2-methyl-3-phenylpropionic acid was carried out by direct esterification with ethanol using dry mycelia of *Rhizopus oryzae* CBS 112.07 in organic solvent, giving (*R*)-ethyl 2-methyl-3-phenylpropanoate as major enantiomer. Finally, (*R*,*S*)-2-methyl-3-phenylpropyl acetate was enantioselectively hydrolysed employing different bacteria and yeasts having cell-bound carboxylesterases with prevalent formation of (*R*)- or (*S*)-2-methyl-3-phenyl-1-propanol, depending on the strain employed.

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Keywords Microbial biotransformation · Enoate reductase · Acetic acid bacteria · Oxidation · Esterase · Stereoselective

Introduction

Biocatalysts have gained importance in different fields of chemical transformations for their high enantio- and regioselectivity and as an alternative to chemical catalysts in more environmentally friendly processes, but the number of biocatalysts available for preparative transformations is still limited (Bommarius and Riebel-Bommarius 2004). Thus, the availability of new microbial methods for the preparation of optically pure molecules is very attractive for organic chemists. Reductive (Hollmann et al. 2011; Toogood et al. 2010), oxidative (Romano et al. 2012) and hydrolytic enzymes (Bornscheuer and Kazlauskas 2006) are particularly attractive for the production of stereochemically enriched alcohols, aldehydes and carboxylic acids.

The enantioselective preparation of 2-methyl-3-phenyl-1-propanol, 2-methyl-3-phenylpropanal and 2-methyl-3-phenylpropionic acid deserves importance since these molecules are valuable chiral building blocks and flavour components (Fuganti et al. 1975; Fardelone et al. 2004); previous attempts of preparing optically pure 2-methyl-3-phenyl-1-propanol and 2-methyl-3-phenylpropanal by biocatalytic methods exploited the use of whole cells of *Saccharomyces cerevisiae* for the reduction of 2-methyl cinnamaldehyde (D'Arrigo et al. 1994). The use of wild-type cells of *S. cerevisiae* gave a mixture of (S)-2-methyl-3-phenyl-1-propanol and 2-methyl cinnamyl alcohol in different ratios depending on the strain and conditions employed (Fronza et al. 1996). The enoate reductases (also known as Old Yellow Enzymes, OYE) of *S. cerevisiae* catalyse the formation of (*S*)-2-methyl-3-phenylpropanal, which is subsequently transformed into (*S*)-2-methyl-3-phenyl-1-propanol; alternatively, aldehyde dehydrogenase(s) of *S. cerevisiae*

reduce 2-methyl cinnamaldehyde directly to 2-methyl cinnamyl alcohol. The relative rates of OYEs and aldehyde dehydrogenase(s) determine the final ratio of the products.

An alternative microbial mean for obtaining optically pure aromatic aldehydes and/or carboxylic acids is the enantioselective dehydrogenation of racemic mixtures of primary alcohols (Molinari 2006; Romano et al. 2012). Acetic acid bacteria can be advantageously used as enantioselective biocatalysts for the oxidation of primary alcohols (Romano et al. 2002); the transformation generally lead to carboxylic acids by a two-step dehydrogenation of primary alcohols. Achiral (Molinari et al. 1997a; Gandolfi et al. 2001a; Pini et al. 2009) or chiral (Molinari et al. 1999a; Borrometi et al. 2002) aliphatic and aromatic primary alcohols and *meso* diols (Molinari et al. 2003) are oxidized with high yields and stereoselectivity by acetic acid bacteria, depending on the strain and the conditions of growth and biotransformation. One-step oxidation leading to aldehyde can be obtained by using different strategies: mutant strains with low aldehyde dehydrogenase activity can be used (Manzoni et al. 1993; Molinari et al. 1995a; Villa et al. 2002; Wu et al. 2011), and/or further oxidation to acid can be avoided by reacting the intermediate aldehyde with condensing agents (Zambelli et al. 2012) or using two-liquid phase systems (Molinari et al. 1999b; Gandolfi et al. 2001a). The productivity of these biotransformations can be dramatically increased by using membrane bioreactors, where aldehyde is promptly and in situ removed, avoiding prolonged contact with the biocatalyst (Molinari et al. 1997b).

Another biocatalytic approach for obtaining optically pure chiral arylpropionic acid is the kinetic resolution of racemic substrates by enzymatic direct esterification. A number of extracellular lipases and carboxylesterases are available for enantioselective hydrolysis and synthesis of esters, but direct esterification is often hampered by low stability of the biocatalyst towards carboxylic acid and unfavourable equilibria (Bornscheuer and Kazlauskas 2006). Mycelium-bound carboxylesterases from different strains belonging to the species *Rhizopus oryzae* and *Aspergillus oryzae* had previously shown the ability of catalysing the direct esterification of different alcohols and acids (Molinari et al. 1995b, 1998a). The optimisation of the reaction conditions showed that mycelium-bound activity of *Rhizopus oryzae* and *Aspergillus oryzae* have good stability in hydrophobic organic solvents and at relatively high temperature (up to 80–90 °C), and that water partitions inside and outside the mycelia favourably alter the overall equilibrium of the biotransformation towards ester formation (Molinari et al. 2000; Converti et al. 2002a, b, c). These features were exploited for achieving the kinetic resolution of different chiral alcohols (Molinari et al. 1998b; Romano et al. 2006), sugars (Molinari et al. 1999c) and arylpropionic acids (Gandolfi et al. 2001b; Spizzo et al. 2007).

Finally, hydrolysis of racemic esters is a well-known means for obtaining optically pure alcohols, such as 2-methyl-3-phenyl-1-propanol. Many lipases show high enantioselectivity toward secondary alcohols, but only a few microbial ones (i.e. from *Burkholderia cepacia* and *Achromobacter* sp.) show high enantioselectivity toward primary alcohols (Bornscheuer and Kazlauskas 2006). Instead, carboxylesterases often show much higher stereoselectivity with racemic esters of primary alcohols (Bornscheuer 2002; Molinari et al. 1996) and can be advantageously employed for performing kinetic resolutions.

Scheme 1 summarises the different biocatalytic approaches investigated in this work for the obtaining of optically pure aromatic molecules.

Materials and methods

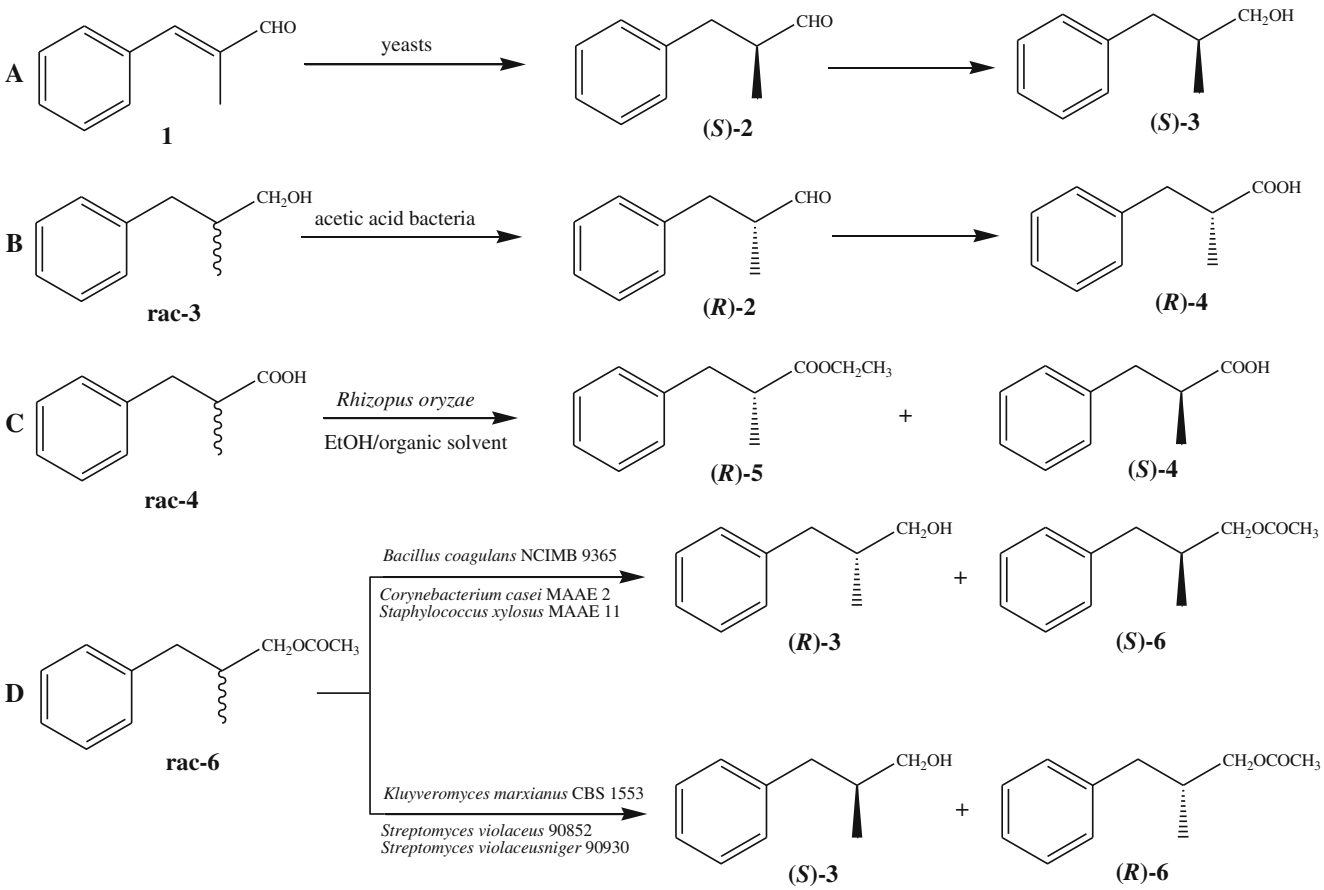
Chemicals were of reagent grade and purchased from Sigma Aldrich, Milano, Italy.

Biotransformation of 2-methyl cinnamaldehyde (**1**) with yeasts

Four strains of *Saccharomyces cerevisiae* were used for the biotransformation of **1**: wild-type *S. cerevisiae* BY4741, the mutant strains *S. cerevisiae* BY4741ΔOye2 (having the OYE2 gene deleted) and *S. cerevisiae* BY4741ΔOye3 (having the OYE3 gene deleted), and finally the recombinant *S. cerevisiae* BY4741ΔOye2Ks, which was derived from *S. cerevisiae* BY4741ΔOye2 after cloning of the OYE gene from *Kazachstania spencerorum* DBVPG6748 (Raimondi et al. 2011). The strains were maintained and cultured as previously described (Raimondi et al. 2011). Bioreductions of **1** were carried out in 100-ml Erlenmeyer flasks incubated in an orbital shaker at 180 rpm and 30 °C. Biotransformations were performed with growing cells of wild-type and recombinant strains: 10 ml of YPD medium were supplemented with 10 mg of **1** and inoculated with exponential phase precultures, to obtain an initial OD_{600} of 1.0. Samples (0.5 ml) were taken at intervals and extracted with an equal volume of EtOAc; the organic extract was dried over Na_2SO_4 and used for analysis. Conversions and stereochemical outcome of the biotransformations were analysed HPLC using a chiral column (Chiralcel OD, 4.6×250 mm, Daicel Chemical Industries, Tokio, Japan) mobile phase: *n*-hexane/2-propanol 90/10, flow 0.5 ml/min, temperature 28 °C, detection UV 254 nm.

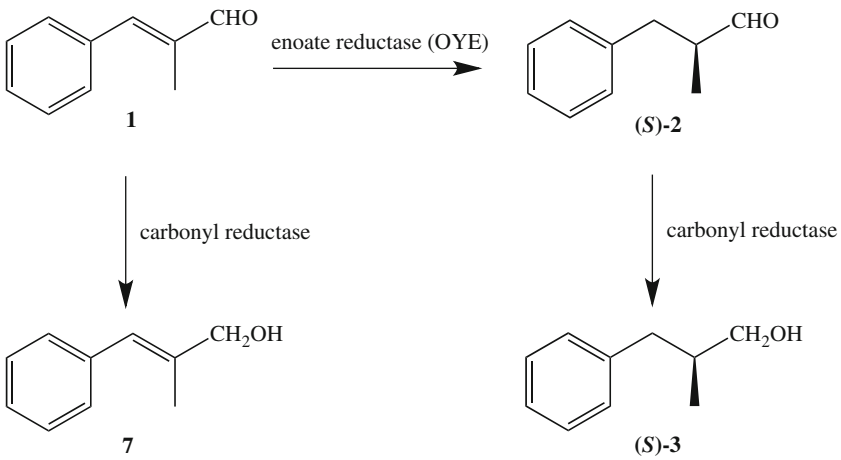
Biotransformations of racemic 2-methyl-3-pheny-1-propanol (**3**) with acetic acid bacteria

Acetobacter aceti MIM 2000/61 (Zambelli et al. 2012) and *Gluconobacter oxydans* DSM 2343 were routinely maintained on GYC slants (glucose 50 g/l, yeast extract 10 g/l, $CaCO_3$ 30 g/l, agar 15 g/l, pH 6.3) at 28 °C. The strains, grown on GYC slants for 24 h at 28 °C, were inoculated into 500-ml Erlenmeyer flasks containing 50 ml of the liquid



Scheme 1 Complementary biocatalytic approaches for the preparation of optically pure aromatic molecules. **A** Reduction of 2-methyl cinnamaldehyde (**1**) with wild-type and recombinant *S. cerevisiae* strains for preparing (*S*)-2-methyl-3-phenylpropanal (**2**) and (*S*)-2-methyl-3-phenyl-1-propanol (**3**); **B** oxidation of racemic-**3** with acetic acid bacteria for preparing (*R*)-**2** and (*R*)-2-methyl-3-phenylpropionic acid (**4**); **C** esterification of racemic-**4** and ethanol with *Rhizopus oryzae* dry cells for preparing of (*R*)-ethyl 2-methyl-3-phenylpropanoate (**5**) and (*S*)-**4**; **D** hydrolysis of racemic 2-methyl-3-phenylpropyl acetate (**6**) with cell-bound esterases of different bacteria and yeasts for preparing (*R*)-**3** and (*S*)-**6** or (*S*)-**3** and (*R*)-**6**

medium containing yeast extract (10 g/l) and glycerol (25 g/l) at pH 5 in distilled water, and incubated on a reciprocal shaker (100 spm). Biotransformations were directly carried out with growing cells. Neat racemic **3** (2.5 mg/ml) was directly added to the suspensions and flasks were shaken on a reciprocal shaker (100 spm) at 28 °C. Samples (0.5 ml) were taken at intervals, brought to pH 1 by addition of 0.5 M HCl, and extracted with an equal volume of EtOAc; the organic extract



Scheme 2 Biotransformations of 2-methyl cinnamaldehyde (**1**) with wild-type *S. cerevisiae* BY4741 and mutant strains lacking OYE activities

Table 1 Biotransformation of 2-methyl cinnamaldehyde (**1**) into 2-methyl-3-phenyl-1-propanol (**3**) and 2-methyl cinnamyl alcohol (**7**); molar conversions and enantiomeric excess of **3** after 24 h

Strain	Molar conversion (%)		Enantiomeric excess (%)
	3	**7**	(*S*)-**3**
S. cerevisiae BY4741 (wild-type)	78	22	>98
S. cerevisiae BY4741ΔOye2	<5	>95	–
S. cerevisiae BY4741ΔOye3	79	21	75
S. cerevisiae BY4741ΔOye2Ks	97	–	>98

was dried over Na_2SO_4 and used for analysis. Analysis of the molar conversion was performed on HPLC Merck Hitachi 655A, with UV detector (254 nm) Merck Hitachi L-4000 using a Purospher® STAR RP-18e (5 μm) column. The solvent system consisted of a solution of water and acetonitrile (1/1) containing 0.1 % trifluoroacetic acid. The flow-rate was 0.8 ml/min; injection volume was 20 μl. Enantiomeric composition was routinely determined by gas chromatographic analysis using a chiral capillary column (column temperature of 120 °C, diameter 0.25 mm, length 25 m, thickness 0.25 μm, DMePeBeta-CDX-PS086; MEGA, Legnano, Italia).

Biotransformations of racemic 2-methyl-3-phenylpropionic acid (**4**) with *Rhizopus oryzae* CBS 112.07

Rhizopus oryzae CBS 112.07 was routinely maintained on malt extract (8 g/l, agar 15 g/l, pH 5.5) and cultured in 500-ml Erlenmeyer flasks containing 100 ml of medium and incubated for 48 h at 28 °C on a reciprocal shaker (100 spm). The liquid media contained a basal medium [Difco yeast extract 1 g/l, $(NH_4)_2SO_4$ 5 g/l, K_2HPO_4 1 g/l, $MgSO_4{\cdot}7H_2O$ 0.2 g/l, pH 5.8] added with Tween 80 (0.5 %). Suspension of spores (1.6×10^4/ml) were used as inoculum. Mycelium grown for 48 h in submerged cultures was harvested by filtration at 4 °C, washed with phosphate buffer (pH 7.0, 0.1 M) and lyophilised. Racemic **4** (30 mg) was dissolved in *n*-heptane (15.0 ml) and 125 mg lyophilised mycelium of *Rhizopus oryzae* CBS 112.07 were added; after 15 min under stirring, an equimolar amount of ethanol was added. The mixture was magnetically stirred at different temperatures (20, 30, 40, 50, 60 °C); samples (0.5 ml) were taken at intervals, paper-filtered and the organic phase evaporated. The residue was dissolved in EtOAc and used for analysis. Molar conversion and enantiomeric composition was determined by gas chromatographic analysis using a chiral capillary column (column temperature of 120 °C, diameter 0.25 mm, length 25 m, thickness 0.25 μm, DMePeBeta-CDX-PS086; MEGA).

Biotransformations of racemic 2-methyl-3-phenylpropyl acetate (**6**) with different microorganisms

Bacillus coagulans NCIMB 9365 (Molinari et al. 1996), *Streptomyces violaceus* 90852 and *Streptomyces violaceus-niger* 90930 (Gandolfi et al. 2000b; Molinari et al. 2005), *Corynebacterium casei* MAAE 2 and *Staphylococcus xylosus* MAAE 11 (Gandolfi et al. 2000a) and *Kluyveromyces marxianus* CBS 1553 (Monti et al. 2008) were maintained and cultured as previously described. Microbial cells (250 mg dry weight) were harvested by centrifugation and suspended in 20 ml of phosphate buffer (pH 6.8, 0.1 M); the reaction was started by addition of the substrate (2.0 mg). Samples (0.2 ml) were taken at intervals, centrifuged and extracted with an equal volume of CH_3COOEt containing an internal standard (2-pheny-1-propanol); molar conversion and enantiomeric composition was routinely determined by gas chromatographic analysis using a chiral capillary column (column temperature of 130 °C, diameter 0.25 mm, length 25 m, thickness 0.25 μm, DMePeBeta-CDX-PS086; MEGA).

Results

Biotransformation of 2-methyl cinnamaldehyde (**1**) with yeasts

The first approach investigated for the preparation of optically **2** and **3** was based on the use of OYE, which catalyse the

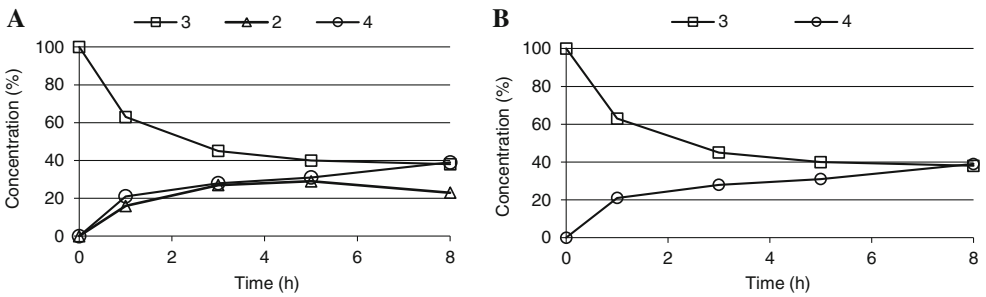


Fig. 1 Oxidation of racemic **3** (2.5 g/l) with *G. oxydans* DSM 2343 (**a**) and *A. aceti* MIM 2000/61 (**b**)

Table 2 Esterification of (*R*,*S*)-**4** with ethanol catalysed by dry mycelium of *Rhizopus oryzae* CBS 112.07 in *n*-heptane at different temperatures; molar conversion and enantiomeric excess of (*R*)-**5** after 4 days

Temperature (°C)	Molar conversion (%)	Enantiomeric excess (*R*)-**5** (%)
20	21	86
30	33	86
40	39	85
50	45	78
60	47	68

chemo- and stereoselective hydrogenation of the C-C double bonds of α-alkyl aldehydes, such as 2-methyl cinnamaldehyde (**1**). Wild-type strain *S. cerevisiae* BY4741 and *S. cerevisiae* BY4741ΔOye3 (having the OYE3 gene deleted) gave a mixture of **3** and unsaturated alcohol **7**, while *S. cerevisiae* BY4741ΔOye2 (having the OYE2 gene deleted) furnished **7** as the only detectable product (Scheme 2; Table 1); saturated aldehyde **2** was not observed during the reaction.

Saccharomyces cerevisiae BY4741ΔOye2 did not show enoate reductase activity towards **1**, therefore indicating that OYE2 is actually responsible for the activity observed with *S. cerevisiae* BY4741 and *S. cerevisiae* BY4741ΔOye3. The recombinant *S. cerevisiae* BY4741ΔOye2Ks (derived from *S. cerevisiae* BY4741ΔOye2 after cloning of the OYE gene from *K. spencerorum* DBVPG6748) was employed for the biotransformation of **1** furnishing (*S*)-**3** with high yield (97 % molar conversion) and high enantioselectivity (e.e. > 98 %). The reaction was completed within 8 h and saturated aldehyde **2** was observed only in traces (4–6 %) as a transient intermediate. This result indicates that the activity and enantioselectivity observed with *S. cerevisiae* BY4741ΔOye2Ks are mostly due to the action of the heterologous OYE.

The biotransformation was also carried out in the presence of a highly hydrophobic phase where **2** can preferentially partition, thus possibly delaying its oxidation, which takes place in the aqueous phase, and allowing its accumulation in the organic phase (Molinari et al. 1999b). Biotransformations were carried out in a two-liquid phase system composed of water and isooctane (phase ratio=1); it was previously proven that this two-liquid phase system is suited for biotransformations mediated by whole cells of different yeasts (Molinari et al. 1998c) . Under these conditions, maximum accumulation of enantiomerically pure *S*-**2** (35–37 %) was observed after 1 h under these conditions. The biotransformation in the two-liquid phase system was optimised taking into account different parameters (cell and substrate concentrations, phase ratio, pH, temperature): the biotransformation performed with 2 g/l of substrates using 20 g/l of cells (dry weight) at 28 °C, pH 6.5 and a phase ratio=4 (isooctane 4/water 1) gave the highest accumulation of (*S*)-**2** (65 % after 30 min).

Biotransformations of racemic 2-methyl-3-pheny-1-propanol (**3**) with acetic acid bacteria

The production of **2** was also investigated by oxidation of racemic **3** using two acetic acid bacteria (*Acetobacter aceti* MIM 2000/61 and *Gluconobacter oxydans* DSM 2343) previously selected for their ability to efficiently perform the oxidation of primary alcohols (Gandolfi et al. 2001a; Zambelli et al. 2012) (Scheme 1B).

Acetobacter aceti MIM 2000/61 furnished (*R*)-2-methyl-3-phenyl propionic acid (**4**) as the only product of biotransformation with 97 % enantiomeric excess, while *Gluconobacter oxydans* DSM 2343, which has low expression of aldehyde dehydrogenase, also gave **2** as a transient product (Fig. 1).

Biotransformations of racemic 2-methyl-3-phenylpropionic acid (**4**) with *Rhizopus oryzae* CBS 112.07

Following the approach **C** described in Scheme 1, lyophilised mycelium of *Rhizopus oryzae* CBS 112.07 was employed for obtaining the kinetic resolution of (*R*,*S*)-**4** by direct esterification with ethanol in *n*-heptane for the preparation of ethyl 2-methyl-3-phenylpropanoate (**5**). The biotransformation was performed at different temperatures (Table 2), since it is known that temperature dramatically influences the stereochemical outcome of enzymatically-catalysed esterification (Bornscheuer and Kazlauskas 2006).

The highest enantioselectivity was observed at lower temperatures; the reaction carried out at 40 °C gave the best compromise between conversion and enantioselectivity, furnishing (*R*)-**5** with 85 % enantiomeric excess.

Table 3 Hydrolysis of (R,S)-**6** with using different microorganisms; molar conversion and e.e. of (*R*)-**3** at different times

Microorganism	Molar conversion (%)	e.e. of **3** (%)	Time (h)
Bacillus coagulans NCIMB 9365	22	80 *R*	24
Streptomyces violaceus 90852	34	85 *S*	3
Streptomyces violaceusniger 90930	39	75 S	5
Corynebacterium casei MAAE 2	30	25 *R*	5
Staphylococcus xylosus MAAE 11	41	83 *R*	6
Kluyveromyces marxianus CBS 1553	20	79 *S*	2

Biotransformations of racemic 2-methyl-3-phenylpropyl acetate (**6**)

Finally, hydrolysis of racemic 2-methyl-3-phenylpropyl acetate (**6**) was studied using different microorganisms with cell-bound esterases (Scheme 1D), previously used for the kinetic resolution of different esters of racemic primary alcohols (Molinari et al. 1996; Gandolfi et al. 2000a, b; Monti et al. 2008).

Both the enantiomers of **3** could be obtained, depending on the strain employed. Yields and enantiomeric excesses were not excellent, but, being a kinetic resolution, optically pure compounds can be obtained depending on the conversion of the biotransformation (Table 3).

Conclusions

Different biocatalytic approaches for the efficient production of enantio-enriched aromatic molecules (2-methyl-3-phenyl-1-propanol, ethyl 2-methyl-3-phenylpropanoate, 2-methyl-3-phenylpropanal, 2-methyl-3-phenylpropionic acid and 2-methyl-3-phenylpropyl acetate) have been investigated. Overall, the reported strategies ensure the possibility of preparing both the enantiomers of these molecules with good yields. The availability of a preparative platform using microorganisms for the synthesis of chiral *R*- or *S*- aromatic molecules, which are relevant chiral synthetic building blocks or flavour/fragrance components, is of general interest for industrial microbiologists and organic chemists. The microbial processes reported here meet the requirement for the need for new selective, green and environmentally friendly reactions.

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