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A study of *Raphanus sativus* and its endophytes as carbonyl group bioreducing agents

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Abstract

The reported anti-Prelog selectivity in *Raphanus sativus* hairy roots prompted us to search for similar activity in other *R. sativus* systems, such as *in vitro* germinated sprouts, edible roots, and its associated endophytic microorganisms. Two test substrates were used in these studies: acetophenone and *rac*-ethyl 2-ethyl-3-oxobutanoate. Endophytes were isolated under substrate selective pressure and individual strains were evaluated for their ability to reduce both substrates. Among the isolates, two bacteria and a fungus having the potential to be used as biocatalysts were found: *Bacillus megaterium* which reduces acetophenone with excellent anti-Prelog selectivity, and *Pseudomonas* sp. and *Penicillium chrysogenum* with enantioselectivity and stereoselectivity for the reduction of *rac*-ethyl 2-ethyl-3-oxobutanoate, respectively.

Keywords: Raphanus sativus, *sec-alcohols, diastereoselectivity, enantioselectivity, keto-reductase, endophytes*

Introduction

Efficient and sustainable access to optically enriched α-alkyl-β-keto esters and secondary alcohols is important for the synthesis of chiral drugs, complex natural products, and libraries of optically active molecules. Since the dawn of chiral technology, chemists and pharmacologists have looked at biocatalysis as a valid alternative to transfer chirality in a controlled fashion (Nestl et al. 2011; Turner 2011; Zheng & Xu 2011). Initially, only bacteria and fungi were utilized but in recent years, algae, cell cultures, and plant tissues have also been exploited in biocatalysis (Cordell et al. 2007; Chang et al. 2010; Suarez-Franco, Hernandez-Quiroz et al. 2010; Magallanes-Noguera et al. 2012; Majewska & Kozłowska 2013; Vandenberghe et al. 2013; Zampieri et al. 2013). Random searches of collection strains and plants or new strains isolated from selected environments have rendered a plethora of enzymes capable of catalyzing a wide range of reactions (Fischer & Pietruszka 2010; Garcia-Urdiales et al. 2011). However, the quest for novel stereoselective biocatalysts is a major goal, and genetic diversity is continuously being explored in the search for unusual or improved selectivity.

Most of the current research on endophytes aims to achieve a better understanding of the ecology of plant–microbe interactions, as well as the biosynthesis of their unique metabolites (Aly et al. 2011). Less frequently has the use of endophytic microorganisms

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been directed to biotechnological applications (Borges et al. 2009; Wang & Dai 2011). In the field of biocatalysis, some reports relate to the use of endophytic fungi for CH hydroxylation (Zikmundova et al. 2002; Agusta et al. 2005; Murgu et al. 2008; Fu, Yang et al. 2011; Fill et al. 2012; Molina et al. 2012). Besides our work concerning the application of endophytic bacteria and yeasts to achieve the stereoselective biotransformation of acetophenone (Rodriguez et al. 2007), α-alkyl-β-keto esters (Rodriguez et al. 2011), and β-substituted acetophenones (Coronel et al. 2014) only a few studies have addressed the potential of endophytes for the bioreduction of prochiral ketones (Pedrini et al. 2009; Pinedo-Rivilla et al. 2009; Prado et al. 2013; Sultan et al. 2014).

As with other types of whole-cell catalysts, only a minority of plants show anti-Prelog selectivity toward prochiral ketones (Andrade et al. 2006) or anti-Prelog reductions are limited to a few substrates such as some five-membered heteroaromatic acetophenone analogs (Aldabalde et al. 2007). Bioreductions with *Raphanus sativus* have been performed with edible roots, sprouts, and hairy roots. Matsuo et al. carried out the biotransformation of prochiral ketones with *R. sativus* sprouts (Matsuo et al. 2008). The production of the anti-Prelog alcohol with enantiomeric excess *(ee)* >99% was reported when using α,α,α-trifluoroacetophenone as substrate; in contrast, the reduction of *o*-chloroacetophenone with this catalyst yielded the *S*-alcohol with excellent optical purity. The particular electronic characteristic of the CF_3 group precludes making general inferences from this observation.

In previous work, we reported the use of *R. sativus* hairy roots for the bioreduction of a series of prochiral alkyl aryl ketones to yield the corresponding anti-Prelog alcohols with an excellent degree of conversion and *R*-enantioselectivity (Orden et al. 2009). These results prompted us to search for this unusual activity in different *R. sativus* systems and its endophytes. Based on our previous results (Rodriguez et al. 2007; Rodriguez et al. 2011), we envisioned the possibility of isolating endophytes with anti-Prelog activity from *R. sativus*. We chose two model substrates: acetophenone (**1**), the most popular prochiral ketone used to search for keto-reductase activity, and the α-substituted-β-keto ester, *rac*-ethyl 2-ethyl-3-oxobutanoate (**3**), since the presence of an unresolved chiral center in the α-position provides an additional element to compare the stereochemical outcome of the reaction. Indeed, the reduction of ketoester **3** can potentially render up to four different stereoisomers (Figure 1).

Methods

Substrates and standards

Acetophenone (**1**) and *rac*-ethyl 2-ethyl-3-oxobutanoate (**3**) (>99%, gas chromatography [GC]) were purchased from Sigma-Aldrich. Standards of the corresponding racemic alcohols were synthesized by reduction of the substrates with $NaBH_4$ in methanol. Optically enriched mixtures of ethyl 2-ethyl-3-hydroxybutanoates with known diastereomeric composition were obtained by biotransformation of substrate **3** with *Escherichia coli* JM105 (pKTS3) and

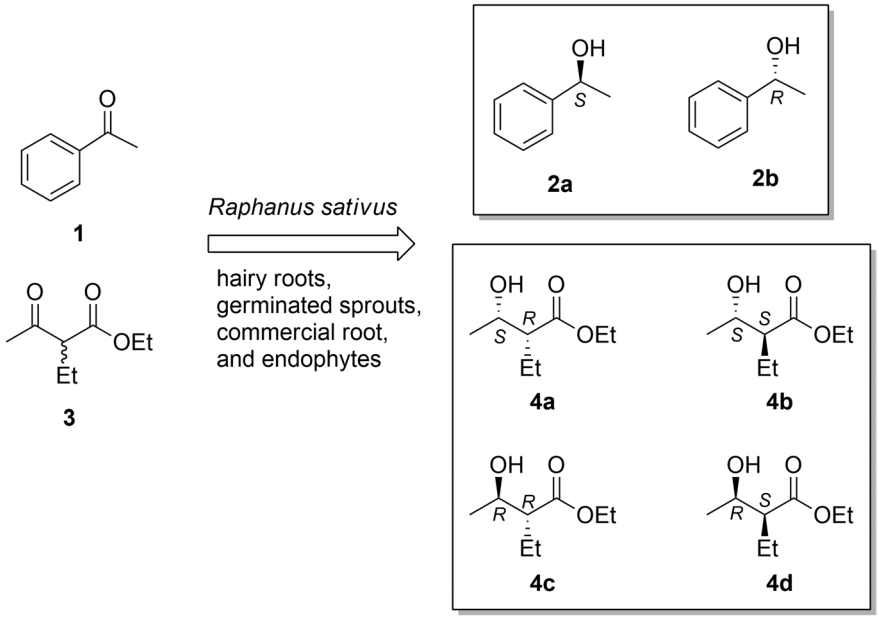

Figure 1. Tested substrates and all their possible reduction products.

E. coli BL21(DE3) Δ*yqhE* (pPP4) as previously described (Rodríguez et al. 2000; Panizza et al. 2007). *S*-1-Phenylethanol (**2a**) was obtained by bioreduction of ketone **1** with *Daucus carota* roots in water as described by the group of Yadav (Yadav et al. 2002).

Biotransformations with plant systems

General procedure for biotransformation with edible radish roots. Edible radish (*Raphanus sativus*) roots were purchased in a local market in Montevideo, Uruguay. The roots were thoroughly washed with running tap water. The external layer was removed with a knife. Then, the plant tissue was washed with distilled water, disinfected with 10% sodium hypochlorite for 3 min, and rinsed with sterile distilled water. Next, the roots were transferred to 70% aq. ethanol for 3 min and rinsed three times with sterile distilled water. Disinfected plant tissues were sliced with a sterile knife and 2 g of plant fragments were suspended in 10 mL of water in a 50-mL Erlenmeyer flask and the appropriate substrate was added to obtain a final concentration of 10 mM. The above-described procedures were performed under aseptic conditions, carried out in duplicate at 28°C and 150 rpm in an orbital shaker for 96 h. Blank assays without substrates and without plant material were also carried out for each experiment.

General procedure for biotransformations with in vitro germinated sprouts. Radish seeds were purchased in a local market at San Luis, Argentina. The seed surfaces were rinsed three times with water, transferred to 70% aq. ethanol solution for 3 min, and then soaked for 15 min in a 20% sodium hypochlorite solution plus two drops of Tween 80. Next, the seeds were rinsed with sterile water and allowed to germinate in a moist filter paper at 20–23°C in the darkness for 1–2 d. The sprouts were transferred to flasks with 30 mL of LS medium (Linsmaier & Skoog 1965) and 3% sucrose and incubated for 10 days under a 16-h light/8-h dark cycle by fluorescent lamps at an irradiance of approximately 1.8 Wm^2. Finally, germinated sprouts were placed in flasks with 10 mL of LS medium (1 g per flask), 3% sucrose, and substrates (10% w/v in dimethyl sulfoxide [DMSO]) were added to achieve a final concentration of 2.5 mM. The experiments were carried out in duplicate and the reactions were allowed to progress under the same conditions for 96 h. Blank assays without substrates and without plant material were carried out for every experiment.

General procedure for biotransformations with hairy roots. R. sativus hairy roots, obtained as previously reported (Orden et al. 2009), were transferred to 30 mL of fresh MSRT medium consisting of MS medium (Murashige & Skoog 1962) plus RT vitamin complex (Flocco 1998) and incubated in an orbital shaker at 120 rpm and 22 ± 2°C in the dark. After 30 days, substrates (10% w/v in DMSO) were added to a final concentration of 2.5 mM and incubated in the dark at 22°C with orbital shaking at 120 rpm. The experiments were carried out in duplicate and the reactions were allowed to progress under the same conditions for 96 h. Blank assays without substrate and without hairy roots were carried out for every experiment.

Isolation of endophytic microorganisms

Microbial endophytic strains were recovered from the biotransformation media as previously described (Rodriguez 2011), in the presence of either substrate **1** or **3**. Serial dilutions of each culture were performed in physiological serum, and 100 μL of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions were spread onto agar plates (same composition as the broth but supplemented with 2% agar) and incubated at 28°C for 24–72 h. Isolated colonies were characterized as bacteria, yeasts, or filamentous fungi by macro- and microscopic observation. Fungal and bacterial strains were transferred to PDA (Potato Dextrose Agar, Sigma) and TSA (Tryptic Soy Agar, Sigma), respectively. Isolated bacterial strains were stored as frozen cultures in 15% glycerol at −20 and −70°C and fungal strains were grown in PDA slants (DIFCO, Detroit USA) at 28°C until sporulation, and then kept at 4°C.

Identification of isolated microorganisms

Only strains with intense or interesting biocatalytic potential were identified based on classical phenotypic analysis, and phylogenetic analysis of the 16 S rRNA gene. Extraction of genomic DNAs was carried out with PureLink™ Genomic DNA Mini Kit (Invitrogen) and amplification of almost full-length 16 S rRNA gene fragments was performed using primers 27F (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′) and 1492R (5′-ACGG(C/T)TACCTTGTTACGACTT-3′) as previously described (Weisburg et al. 1991). Extraction of fungal genomic DNAs was done by standard procedures (Wach et al. 1994). The ITS1–5.8 S–ITS2 region was amplified using primer pair ITS1–ITS4 (White et al. 1990). Amplification of D1/D2 domain of the LSU rRNA gene was performed with primers ITS1-F (TCCGTAGGTGAACCTGCGG) and NL -4 (5′-TCCTCCGCTTATTGATATGC-3′). The resulting PCR products

were sequenced with an Applied Biosystems automatic sequencer ABI 3730XL at Macrogen Corp., Seoul, Korea. Sequences were compared with other released sequences in the GenBank database using BLAST program (National Center for Biotechnology Information) (Altschul et al. 1990).

Based on these analyses, the isolated strains were identified as *Bacillus megaterium* CM1 and *Penicillium brevicompactum* CM2 (isolated from *R. sativus* sprouts in the presence of acetophenone); and *Pseudomonas* sp. PR2, *Stenotrophomonas* sp. PR1, *Cladosporium cladosporioides* PR3, and *Penicillium chrysogenum* PR4 (isolated from *R. sativus* radish roots and sprouts in the presence of ethyl 2-ethyl-3-oxobutanoate).

General procedure for biotransformations with microorganisms

Biotransformations by fungi. Fresh plates of each fungal strain were cultured for 7 days in PDA at 28°C. Spores were diluted in physiological serum to a final concentration of 10^7 mL^{-1}. Spore solutions (5 mL) were used to inoculate 50 mL of PDB or Potato Dextrose Broth (Sigma); the culture was incubated at 28°C in an orbital shaker at 150 rpm. The appropriate substrate was added after 48 h of incubation at a concentration of 10 mM. The culture was incubated at 28°C and 150 rpm for 7–10 days. Samples were aseptically withdrawn from the reaction mixture at different times for GC analysis. Blank assays without substrates and without microorganisms were carried out. The results are the means of three replicate experiments.

Biotransformations by bacterial strains. Fresh plates of each strain were streaked from the frozen stock in TSA. A single colony was used to inoculate 5 mL of TSB or Tryptic Soy Broth (Sigma). Each culture was incubated at 28°C in a rotary shaker at 150 rpm overnight, and 500 µL of this culture was used to inoculate 5 mL of fresh TSB, containing the appropriate substrate at 10 mM and 1 mM (*B. megaterium* CM1 only), in 25-mL Erlenmeyer flasks. The fresh cultures were grown under the same conditions and sampled periodically for GC analysis. Blank assays without substrates and without microorganisms were carried out. The results are the means of three replicate experiments.

Analysis of the biotransformation products

Acetophenone (1) experiments. Analytical samples were prepared by mixing 200 µL of the biotransformation broth with 400 µL of ethyl acetate in a 1.5-mL tube. After mixing for 10 s, it was centrifuged for 2 min, the organic layer was separated and dried over anhydrous Na_2SO_4. The conversion of **1** was monitored by GC (FID) in a Perkin Elmer Clarus 500 instrument equipped with Quadrex 007 5% Phenyl/95% Methyl Silicone column (30 m, 0,25 mm, 0,25 µm), injector: 200°C, and FID: 300°C. The operating conditions were 85°C (2 min)/20°C/min/120°C (2 min)/10°C/min/150°C (1 min). Rt: **1** 5.66 min, **2** 5.53 min. For the measurement of the *ee* values, the alcohols were derivatized by the addition of 100 µL of acetic anhydride and a catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine (1 mg) to the organic phase. The reaction mixture was shaken for 90 min at 130 rpm and 22°C. Afterward, 400 µL of water was added. The solution was centrifuged (4 min) and the organic phase dried over anhydrous Na_2SO_4 and analyzed by chiral GC (FID) in a Perkin Elmer Clarus 500 instrument equipped with β-DEX-column (60 m, 0.25 mm ID and 0.25 µm df), injector: 200°C, and FID: 300°C. The operating conditions were 120°C (2 min)/1°C/min/126°C (1 min). Rt: **1** 3.94 min, **2a** (*S*-1-phenylethyl acetate) 5.33 min, **2b** (*R*-1-phenylethyl acetate) 5.92 min. Assignment of the absolute configurations was based on comparison of the retention times and co-injection with the above-mentioned standards.

rac-Ethyl 2-ethyl-3-oxobutanoate (3) experiments. Analytical samples were prepared as described for compound **1**. 1 µL was used for GC analysis on a GC2014 Shimadzu chromatograph equipped with a HP Carbowax (25 m, 0.25 mm) column and a FID detector. Temperature program: 60°C (5 min)/8°C/min/140°C/25°C/min/240°C (5 min). Injector 220°C, FID 250°C. *ee* and diastereomeric excess (*de*) values were determined by chiral GC on a Shimadzu 2010 chromatograph equipped with a Megadex DET-TBS (25 m, 0.25 mm) column (MEGA, Italy) and a FID detector. The operating conditions were 60°C (6 min)/15°C/min/90°C (20 min)/45°C/min/180°C (5 min). Rt: **3** 16.3 and 16.9 min, **4a** 25.0 min, **4b** 22.1 min, **4c** 26.0 min, and **4d** 29.2 min. Assignment of the absolute configuration was based on comparison of the retention times and co-injection with the standards mentioned above.

Results and discussion

Reduction of acetophenone (1)

In agreement with our previous report (Orden et al. 2009), the biotransformation of **1** with *R. sativus* hairy roots yielded **2b** with excellent conversion and optical purity (Table I). Attempts to isolate endophytic microorganisms from this system were unsuccessful, and the amplification of isolated DNA

Table I. Endophyte strains isolated from the experiments.

Entry	Isolated strain	Substrate	Remaining substrate
1	Rabix1	1	99
2	Rabix2	1	99
3	Rabix3	1	99
4	Rabix4	1	99
5	Rabix5	1	99
6	Rabix6	1	99
7	Rabix7	1	99
8	*B. megaterium* CM1	1	88
9	*P. brevicompactum* CM2	1	98
10	CM3	1	99
11	Eix1a TSA	2	92
12	Eix2b AR	2	93
13	Eix2a AR	2	81
14	Eix1c PDA	2	75
15	Eix2a PDA	2	93
16	*Stenotrophomonas* sp. PR1	2	90
17	Eix2b TSA	2	93
18	Eix2c TSA	2	91
19	Eix2c AR	2	80
20	Eix1b TSA	2	80
21	Eix1a AR	2	77
22	Eix2a TSA	2	79
23	Eix2b PDA	2	97
24	Eix2d TSA	2	100
25	Eix2d TSA	2	98
26	Eix2d TSA	2	94
27	Eiiix1a PDA	2	56
28	*Pseudomonas* sp. PR2	2	59
29	Eiiix2a TSA	2	70
30	Eiiix2b TSA	2	87
31	Eiiix2c TSA	2	100
32	*C. cladosporioides* PR3	2	65
33	*P. chrysogenum* PR4	2	14

Screening of reductase activity at 24- and 72-h reactions for bacteria and fungi, respectively.

with specific primers targeting the 16 S rRNA gene (Chelius & Triplett 2001) gave negative results. Therefore, we concluded that the *R. sativus* hairy root clone used for this experiment was free of endophytic microorganisms. As a consequence, the study of the biocatalytic potential of *R. sativus* endophytes was performed only with commercial edible roots and *in vitro* germinated sprouts. The biotransformation of **1** with edible roots resulted in a very low percentage conversion (6%). In this assay, seven bacterial strains were isolated and when they were screened for reductase activity, substrate **1** was recovered intact with a 99% recovery even after 24 h of reaction (Table I, entries 1–7). It was unexpected for us to observe such a low conversion of acetophenone by *R. sativus* commercial roots, since this substrate is easily reduced by many other plants as well as many bacteria and yeast strains (D'Arrigo et al. 2000). The fact that in this experiment the recovered endophytes closely followed the biocatalytic behavior of the original source plant is indicative of the wide diversity of ketoreductases and the usefulness of the plant screening to anticipate the activity present in the corresponding endophytic microorganisms.

Table II summarizes the results obtained with the different plant systems and the more promising endophytic strains. When *in vitro* germinated sprouts were tested for reduction of ketone **1**, a 30% conversion was observed but the metabolites were obtained with low enantioselectivity, with the *R*-alcohol **2b** being the major isomer (40% ee, entry 3). In this assay, it was possible to recover two bacterial strains (*B. megaterium* CM1 and CM3), and a filamentous fungus, (*P. brevicompactum* CM2). This fungus only delivered a low conversion reaction with substrate of **1** (20%), yielding **2a** with low optical purity (40% ee). On the other hand, *B. megaterium* CM1 exhibited anti-Prelog selectivity in this reaction. Reduction of **1** (10 mM) with this strain yielded 33% conversion after 48 h with preference toward **2b** (*R*, 82% ee). Further experiments were carried out with the substrate at a lower concentration (1 mM) and **2b** was obtained with excellent optical purity (>99% ee). However, working at this concentration only 15% conversion was observed (Table II, entries 4 and 5). Again, the isolation of a strain with strong anti-Prelog selectivity is remarkable and follows the indication of the experiment with the germinated sprouts. The anti-Prelog reductive selectivity of *B. megaterium* CM1 is an interesting result, considering that most reports on bioreductions of alkyl aryl prochiral ketones showed the opposite selectivity. This was exemplified by the screening of 120 soil isolated strains against substituted acetophenones,

Table II. Biotransformation of acetophenone (**1**) by *R. sativus* hairy roots, edible roots, *in vitro* germinated sprouts and their endophytic active strains.

Entry	Biocatalytic system	Conv.(%)	2a (*S*) (%)	2b (*R*) (%)	Isolated strains	Active strains
1	Hairy roots	98	–	100	–	–
2	Edible roots	6	33	67	7	–
3	*In vitro* germinated sprouts	30	30	70	2	*B. megaterium* CM1 *P. brevicompactum* CM2
4	*B. megaterium* CM1 (10 mM)	33	9	91	N/A	N/A
5	*B. megaterium* CM1 (1 mM)	15	0	100	N/A	N/A
6	*P. brevicompactum* CM2	20	70	30	N/A	N/A

Table III. Biotransformation of ethyl 2 ethyl-3-oxobutanoate (3) by *R. sativus* hairy roots, edible roots, in vitro germinated sprouts and their endophytic active strains.

Entry	Biocatalytic system	Conv.(%)	4a (2*R*,3*S*) (%)	4b (2*S*,3*S*) (%)	4c(2*R*,3*R*) (%)	4d (2*S*,3*R*) (%)	Isolated strains	Active strains
1	Hairy roots	66	36	53	3	8	–	–
2	Edible roots	32	75	25	–	–	20	*Stenotrophomonas* PR1 *Pseudomonas* PR2
3	*In vitro* germinated sprouts	>99	57	38	2	3	2	*C.cladosporioides* PR3 *P.chrysogenum* PR4
4	*Pseudomonas* PR2	>99	60	–	40	–	N/A	N/A
5	*Stenotrophomonas* PR1	>99	50	28	10	12	N/A	N/A
6	*C.cladosporioides* PR3	>99	40	57	3	0	N/A	N/A
7	*P. chrysogenum* PR4	>99	21	79	–	–	N/A	N/A

which resulted in only one with *R*-selectivity (Zilbeyaz et al. 2010).

Reduction of rac-ethyl 2-ethyl-3-oxobutanoate (3)

Biotransformation of ketoester **3** with the axenic clone of *R. sativus* hairy roots gave a mixture of alcohols **4a** (2*R*,3 *S*) and **4b** (2 *S*,3 *S*) with modest conversion (66%) even after 48 h of reaction, and low diastereoselectivity, favoring the *anti*-product **4b**. The biocatalyst exhibited Prelog selectivity, although minor percentages of both anti-Prelog alcohols **4c** and **4d** were also detected (Table III, entry 1). In the experiment with edible roots, ketoreductase activity on **3** showed an even stricter Prelog selectivity, affording only **4a** and **4b** with moderate yield, both obtained with excellent optical purities (*ee* > 99%) since the corresponding enantiomers were not observed. Isolation of endophytic microorganisms was also performed in this experiment and a total of 21 bacterial strains were recovered and later screened for ketoreductase activity (Table I, entries 11–31). Although most of them displayed poor reductase activity (conversions lower than 30% in 24 h), two of them (*Stenotrophomonas* sp. PR1 and *Pseudomonas* sp. PR2) yielded the anti-Prelog alcohols as minor products and were selected for further study.

Pseudomonas sp. PR2 showed 61% conversion of ketoester **3**, yielding a diastereomeric mixture of **4a** and **4c** (60:40). We performed a time course study of this biotransformation in order to understand the biocatalytic process. Figure 2 shows the relative concentration of the substrate and the four products. After 24 h of reaction 40% conversion was achieved and the *anti*-isomer (2 *S*, 3 *S*) accounted for more than 90% of the products. However, this metabolite

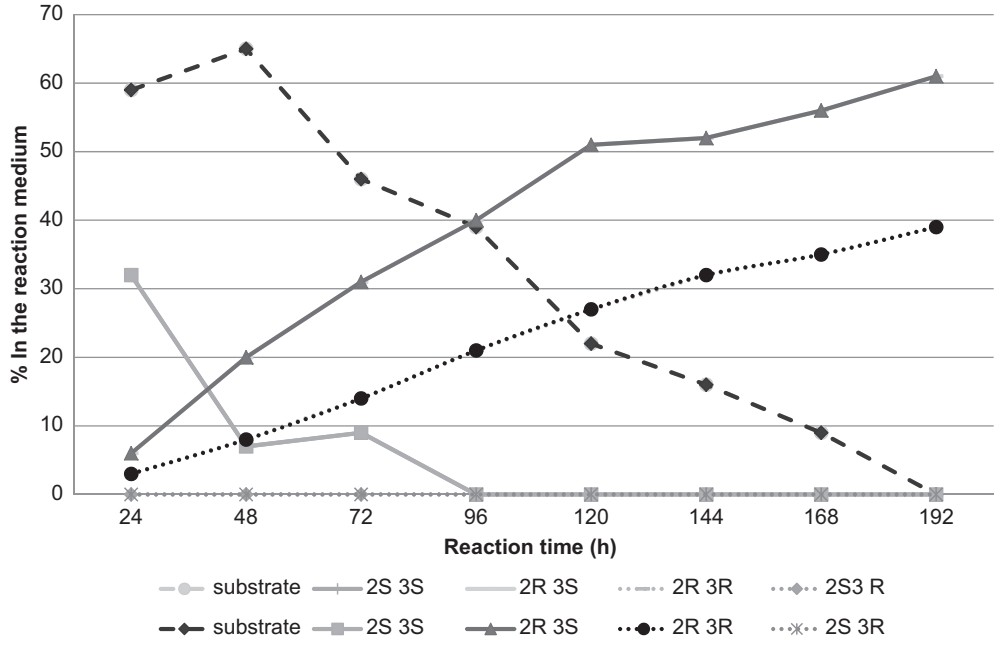

Figure 2. Time course of the biotransformation of rac-ethyl 2-ethyl-3-oxobutanoate (3) by Pseudomonas sp. PR2. Dotted lines: anti-Prelog activity. Solid lines: Prelog activity.

decreased rapidly and disappeared after 96 h of reaction while the *2R* isomers (*syn* 2*R*, 3 *S* and *anti* 2*R*, 3*R*) increased their concentration increased in a nearly linear fashion. At the end of the studied period (192 h) they were the only products, and total conversion of the starting material was achieved. The observed behavior could be explained by the presence of various reductases that are expressed at different stages of the culture. The disappearance of metabolite **4b** could be explained by oxidation of the hydroxyl group by a specific ketoreductase followed by reduction of the substrate by a different enzyme with opposite stereoselectivity. This concurs with the modest increase in the concentration of the substrate observed at 48 h of reaction.

The *Stenotrophomonas* strain gave very high conversion of the ketoester but yielded a mixture of the four possible products with **4a** (2*R*, 3 *S*) being the major isomer (50%). Interestingly, a combined yield of 22% of the rare 3*R* isomers (**4c** and **4d**) was observed in this experiment, indicating the possibility of obtaining an anti-Prelog selective catalyst from this strain (Table III, entry 5). This result is noteworthy not only because of the technological possibilities, but also because the commercial *R. sativus* root was strictly Prelog selective toward the substituted ketoester under study (entry 2). Evidently, the combined effect of the plant and the endophyte community in the root overwhelmed the action of the *Stenotrophomonas* strain. We also performed a time course study for this strain and observed that there was no evidence of reversion or reoxidation in this case. The Prelog metabolites 3 *S* increased gradually as the starting material was metabolized. From this study it was evident that the process was complete after 72 h, since the substrate was fully consumed and the concentration of products was essentially unchanged after this point (Figure 3).

C. cladosporioides PR3 and *P. chrysogenum* PR4 isolated from *in vitro* germinated sprouts exhibited a strong Prelog selectivity (Table III, entries 6 and 7) following the indication obtained from the experiment with the sprouts (entry 3). In the case of *P. chrysogenum* PR4, the anti-Prelog *R* isomers were completely undetectable. Regarding diastereoselectivity, the strains behaved in a different fashion with *P. chrysogenum* PR4 being strongly selective for the *anti*-isomer **4b** that was formed in an approximately 4:1 ratio (entry 7). *C. cladosporioides* PR3 followed the trend, but the selectivity toward **4b** was low and predominated in only 3:2 ratio.

Conclusions

The results obtained with the different *R. sativus* systems showed high variation in yields and optical purities. While hairy roots possess excellent activity and stereoselectivity in the reduction of acetophenone, *in vitro* germinated sprouts and edible roots

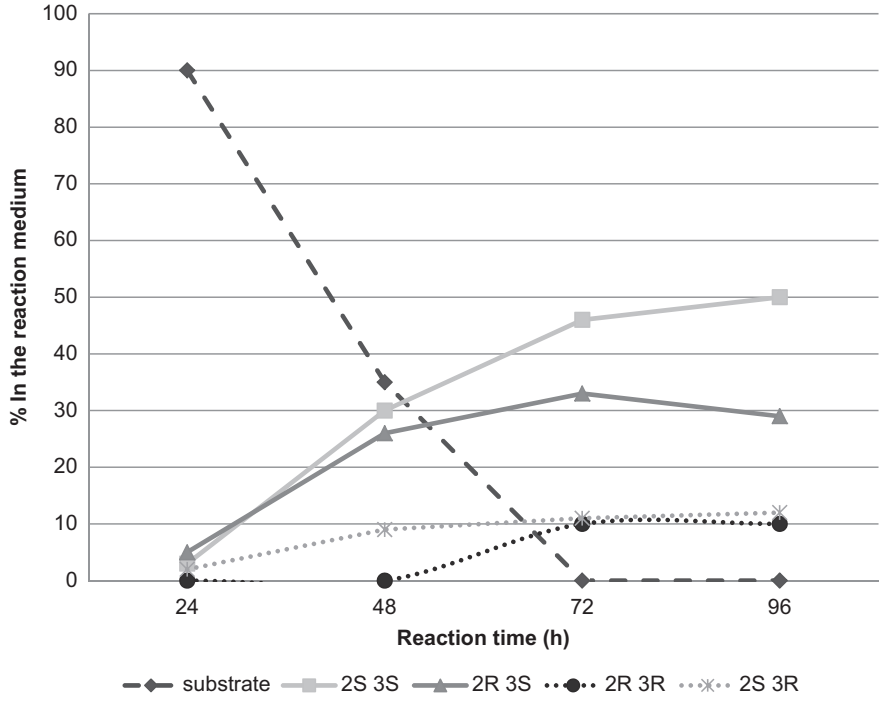

Figure 3. Time course of the biotransformation of *rac*-ethyl 2-ethyl-3-oxobutanoate (**3**) by *Stenotrophomonas* PR3. Dotted lines: anti-Prelog activity. Solid lines: Prelog activity.

presented moderate to low conversions and poor optical purities, which is agrees with previous literature reports on the use of similar systems. The different behavior of the three analyzed biocatalytic systems could be associated with differential expression and/or activity levels of the involved reductases in the diverse plant tissues and/or the concomitant activity of the endophytic consortium, whenever present.

By using hairy roots, 66% conversion of ketoester **3** was achieved. However, contrary to what was expected, the system yielded the Prelog alcohols **4a** and **4b** as major products, demonstrating that the stereoselectivity was largely dependent on the nature of the substrate. When in vitro germinated sprouts were used, the conversion was complete, unlike edible roots that gave only 32% conversion also following Prelog's rule.

When working with non-axenic plant systems, the potential influence of endophytic microorganisms on the outcome of the biotransformation must be taken into account. The isolation of endophytic microorganisms and the study of their biocatalytic activity can shed light on interpreting the results obtained with these complex systems. Furthermore, this approach can lead to interesting biocatalysts from an underexplored niche.

Since radish hairy roots are axenic, they constituted a proper system to exploit the predominant anti-Prelog stereoselectivity of their plant enzymes toward alkyl aryl ketones with whole cells. On the other hand, non-axenic plants offer the opportunity to isolate novel selective biocatalysts. In this work, we isolated three new microbial strains with potential biotechnological applications: *B. megaterium* CM1 possessing anti-Prelog reducing activity toward a model alkyl aryl prochiral ketone, and *P. chrysogenum* PR4 and *Pseudomonas* sp. PR2 showing excellent Prelog stereoselectivity in the reduction of the model *rac*-α-alkyl-β-ketoester.

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