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Kinetic resolution of racemic 5,6-epoxy-bicyclo[2.2.1]heptane-2-one using genetically engineered *Saccharomyces cerevisiae*

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ABSTRACT

(+)-5,6-Epoxy-bicyclo[2.2.1]heptane-2-one, (+)-**1**, and *endo*-(−)-5,6-epoxy-bicyclo[2.2.1]heptane-2-ol, *endo*-(−)-**2**, were obtained by kinetic resolution of *rac*-**1** by asymmetric bioreduction catalyzed by whole cells of a genetically engineered *Saccharomyces cerevisiae* yeast strain. The strain, TMB4100, had 1% phosphoglucose isomerase (PGI) activity and overexpressed a specific short-chain dehydrogenase, encoded by the gene YMR226c. The whole cell biocatalyst was demonstrated to be significantly inactivated within 24 h, thus restricting the reaction to low concentration. Despite this, the resolution method could be used to produce optically pure (+)-**1** and *endo*-(−)-**2** from the racemic mixture at 5 g/L substrate. At optimal conditions, 1 g of *rac*-**1** was kinetically resolved to give (+)-**1** in 95% ee and 28% yield and *endo*-(−)-**2** in 74% ee, 80% de and 45% yield.

1. Introduction

The bicyclo[2.2.1]heptane motif is found in many structures with diverse fields of application [1]. For example, the structure occurs in natural products, such as camphor and fenchone, and in synthetic biologically active compounds [2]. Functionalized bicyclo[2.2.1]heptanes are used as starting materials for the synthesis of complex chiral compounds containing highly substituted cyclopentane moieties [1]. The conformationally restricted structure offers a rigidity that could be favourable in an enantioselective process and indeed many ligands containing the bicyclo[2.2.1]heptane subunit have been reported in the literature [3–8]. In the process of developing a new class of ligands based on bicyclo[2.2.1]heptane, an efficient way to obtain optically active bicyclic molecules, like **1** or **2** (Scheme 1), was required. Racemic alcohol **2** can be resolved by enzymatic transesterification using lipase from *Candida rugosa* [9] or Novozym 435 [10]. However, before resolution the pure endo-alcohol has to be isolated from a mixture of endo- and exo-alcohol, obtainable from commercially available 5-norbornen-2-yl acetate (80:20 mixture endo:exo), which results in a low yielding over all process [9].

Asymmetric carbonyl reduction of ketones by whole yeast cells is another way to produce enantiomerically pure substances from racemic mixtures [11]. Baker's yeast accepts a wide variety of xenobiotic carbonyl compounds as substrates and produces optically active alcohols at low temperature and neutral pH [12–14]. However, optical purity of the product may be limited by the presence of ketone reductases with overlapping substrate specificity resulting in products with mixed stereochemistries. In addition, bioreductions catalyzed by baker's yeast often demand large amounts of sugar as co-substrate to regenerate the NADPH required in the reaction [13]. This leads to the formation of inhibitory amounts of ethanol and a demand for more yeast biomass, sugar and time. Hence, baker's yeast is not a practical option for large-scale bioreduction to give optically active products.

As an alternative, genetically engineered yeast strains performing bioreductions with less biomass and co-substrate [15] and reach higher yield and purity of the desired enantiomer [16–19] are available. Most engineered strains overexpress one of the ca 50 different genes encoding existing NADPH-dependent yeast dehydrogenases that catalyze stereoselective carbonyl reductions [20]. To the best of our knowledge, only a few studies of yeast bioreduction in the presence of epoxide functionalities currently exist and

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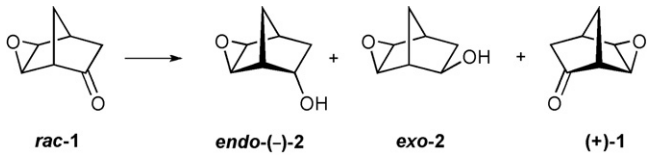

Scheme 1. Kinetic resolution of *rac*-**1** by baker's yeast catalyzed bioreduction.

none of them use engineered yeast strains [21–24]. Nevertheless, a few yeast enzymes that are encoded by the open reading frames (ORFs) YOR120w, YDR368w, YMR226c, YGL157w and YGL039w are known to accept bicyclic ketone derivatives with similar structure to **1** [20] and could potentially be used to kinetically resolve *rac*-**1**.

With the aim to develop an efficient synthesis of optically active **1** and **2** we have studied the kinetic resolution of racemic epoxy ketone **1** by asymmetric carbonyl reduction catalyzed by genetically engineered *Saccharomyces cerevisiae*. A screening of yeast strains overexpressing dehydrogenases, known to accept bicyclic diketones as substrate, was performed. The best strain was selected to study asymmetric carbonyl reduction of *rac*-**1**. Furthermore, the reaction kinetics was determined by following the progress of the bioreduction at various initial concentrations of substrate and product. Finally, preparative scale bioreduction of *rac*-**1** was performed under improved conditions and it yielded in a significant improvement of the reaction compared to commercial baker's yeast.

2. Results and discussion

2.1. Screening of S. cerevisiae strains overexpressing individual carbonyl reductases

Initial trials to kinetically resolve *rac*-**1** using commercial baker's yeast resulted in low conversion of *rac*-**1** (Table 1, entries 1 and 2). Therefore, a screening of *S. cerevisiae* strains overexpressing carbonyl reductases, known to accept bicyclic diketones with similar structure to **1** [20] was performed (Table 1, entries 3–7).

Strains TMB4092, TMB4093 and TMB4097, overexpressing reductases encoded by YBR149w, YDR368w and YOL151w, respectively, gave low conversion of *rac*-**1**. Strain TMB4096, overexpressing the reductase encoded by YOR120w, resulted in high conversion of *rac*-**1** (61%) and 99% ee of *endo*-(−)-**2**, but low diastereoselectivity (46% de). Since the diastereoisomers could not be separated by column chromatography, also this strain was discarded. TMB4100, overexpressing the reductase encoded by YMR226c, was shown to be the best strain for the kinetic resolution of *rac*-**1**. At approximately 70% conversion it gave unreacted (+)-**1** of >95% ee. This strain also gave higher diastereoselectivity of endo-(−)-**2** (88% de), compared to reduction with sodium borohydride, which gave only 50% de. TMB4100 (1% PGI, YMR226c) had been previously shown to reduce bicyclic diketones with high reaction rate, but with poorer stereoselectivity than for yeast strains overexpressing other reductases [16]. Nevertheless, the selectivity of the reductase encoded by YMR226c was in our case the most appropriate to resolve *rac*-**1**.

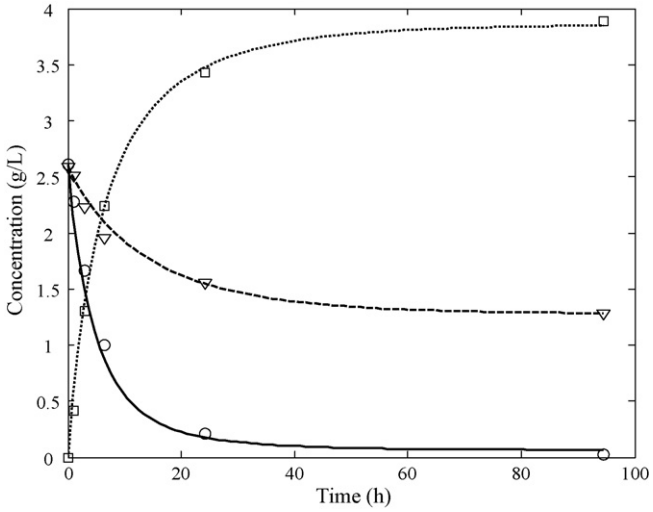

Fig. 1. Whole cell reduction of 5 g/L *rac*-**1** followed over time. The example illustrates inactivation of the reaction after approximately 24 h. Symbols represent experimentally determined concentrations of (−)-**1** (○), (+)-**1** (▽) and product **2** (□). Lines represent modelled concentrations (R^2 = 0.994). A detailed description of the model can be found in supplementary material.

Horak et al. studied baker's yeast catalyzed reduction of α,β-epoxy ketones and observed reduction of the epoxide in addition to the ketone [24]. However, we did not observe any diol formation with TLC or GC analysis. The possible production of triols, formed by a combination of ketone reduction and hydration of the epoxide [23], was not observed either. Due to the high polarity of a triol, its concentration in the organic phase may be below the detection limit. Therefore, we assumed that hydration was insignificant or did not occur at all.

2.2. Bioreduction of rac-1

TMB4100 (1% PGI, YMR226c) was chosen to further optimize yield and ee of (+)-**1**. To establish reaction kinetics, bioreduction of *rac*-**1** was studied over time at varying initial substrate (2.5–50 g/L) and product concentrations (0–30 g/L), while keeping cell concentration constant (15 gdw/L). The experimental data indicated that the enzyme was subject to inhibition both by the product and excess of the substrate (see supplementary data). Furthermore, due to a severe inactivation of the cells within 24 h (Fig. 1), the reaction could only be driven to high enough conversion (and thus ee) at substrate concentrations below 5 g/L. The maximum yield and ee of (+)-**1** was achieved when the reaction was stopped at approximately 75–80%

Table 1
Screening of commercial yeast and yeast strains overexpressing specific reductases known to accept bicyclic diketones.

Entry	Strain	Conversion[a] *rac*-**1** (%)	ee (+)-**1** (%)	ee endo-(−)-**2** (%)	de endo-**2** (%)
1	Commercial Baker's yeast (red)[b]	6	8	95	66
2	Commercial Baker's yeast (blue)[b]	18	22	95	70
3	TMB4100 (1% PGI, YMR226c)[c]	73	96	73	88
4	TMB4092 (YBR149w)[c]	22	21	96	72
5	TMB4093 (YDR368w)[c]	28	22	98	84
6	TMB4096 (YOR120w)[c]	61	46	99	46
7	TMB4097 (YOL151w)[c]	13	9	93	92

[b] 24 h bioreduction of 15 g/L *rac*-**1** with 200 g/L yeast in water with 200 g/L glucose.
[c] 24 h whole cell bioreduction of 5 g/L *rac*-**1** with 5 g/L yeast in citrate buffer (100 mM, pH 5.5) with 100 g/L glucose.
[a] Analyzed by GC based on the assumption that endo- and exo-**2** were the only products. A calibration was performed to correct for the difference in response of the two compounds on the FID-detector.

conversion, corresponding to a biochemical stereoselectivity factor, *E*, of 6.5, as calculated with Eq. (1) [25].

$$E = \frac{Ln((1 - \text{conversion})(1 - \text{ee}))}{Ln((1 - \text{conversion})(1 + \text{ee}))} \quad (1)$$

2.3. Understanding the inactivation of the biocatalyst

To investigate whether the inactivation was caused by cell death and if so there was a correlation to the substrate concentration, cells were sampled from the reaction mixture after 24 h bioreduction of increasing concentrations of *rac*-**1**, and streaked on YPD-plates containing 2% fructose and 0.1% glucose. However, no significant difference in viability after 24 h at concentrations up to 30 g/L *rac*-**1** could be observed (see supplementary data). Only at 50 g/L of *rac*-**1** a decrease of approximately 2 units of log CFU/mL was observed compared to cells from a control experiment without epoxy ketone, which supported the fact that inactivation of the cells was not substrate dependent.

We speculated further that bioreduction terminated because of an arrest in glucose catabolism and thereby NADPH regeneration [26]. Therefore, glucose consumption was followed in three parallel bioreduction experiments: (i) no substrate added, (ii) 20 g/L of *rac*-**1** added and (iii) 20 g/L of *rac*-**1** added plus that the cells were replaced every 24 h (Fig. 2). New glucose was added to the initial concentration after depletion (followed continuously with glucose sticks during the reaction).

When cells were not replaced, glucose consumption stopped after approximately 50 h, which coincided with bioreduction arrest (Fig. 2a and b). When cells were replaced every 24 h, glucose consumption only terminated after 80 h, which also coincided with bioreduction arrest. Glucose consumption did not terminate because of cell death since the viable count was only slightly higher when epoxy ketone was present compared to the experiment without substrate (Fig. 2c).

Glucose consumption stopped after approximately 70 h also in the control experiment without epoxy ketone present. The yeast strain used, TMB4100 (1% PGI, YMR226c), has only 1% phosphoglucose isomerase (PGI) activity, which results in either accumulation of glucose-6-phosphate or in enhanced carbon flux through the pentose-phosphate pathway and intracellular accumulation of NADPH. It has previously been shown that regeneration of NADPH, e.g. through ketone reduction, is necessary for a PGI deleted yeast cell to consume glucose for cell growth [27]. However, the need for an electron acceptor is fulfilled through NADPH-dependent reduction of the epoxy ketone, so other mechanisms than an imbalance of NADPH/NADP+ may also be involved in the inactivation. The arrest in glucose consumption could be speculated to be caused by inactivation of yeast enzymes involved in glucose metabolism or transport inside the cell. Since inactivation was related to the whole cell system an enzyme system with artificial recycling of NADPH might be a better choice for the kinetic resolution of *rac*-**1**.

In addition to glucose consumption, the inactivation of the overexpressed carbonyl reductase encoded by YMR226c could also stop the reaction. However, the carbonyl reductase (CR) remained active even after 96 h (Fig. 2a) in accordance with experiments performed on the purified enzyme, where the epoxy ketone had no detrimental effect on the enzymes activity (data not shown).

2.4. Preparative scale bioreduction of rac-1

The bioreduction was performed in a preparative scale, using 1 g of *rac*-**1**, at a concentration of 5 g/L, and with 15 gdw/L cells. After 48 h the reaction was stopped and the ketoepoxide (+)-**1** was isolated in 28% yield and 95% ee. The epoxy alcohol *endo*-(−)-**2** was isolated in 45% yield, 74% ee and 80% de. Even though a laborious and unpractical procedure had to be followed, the reaction could be successful at even higher substrate concentration if yeast cells were replaced. In fact, replace of the cells three times increased the ee of (+)-**1** from 47% to 83% (Fig. 2b). If the cells had been replaced more times, conversion and thus ee of (+)-**1** would probably have been higher.

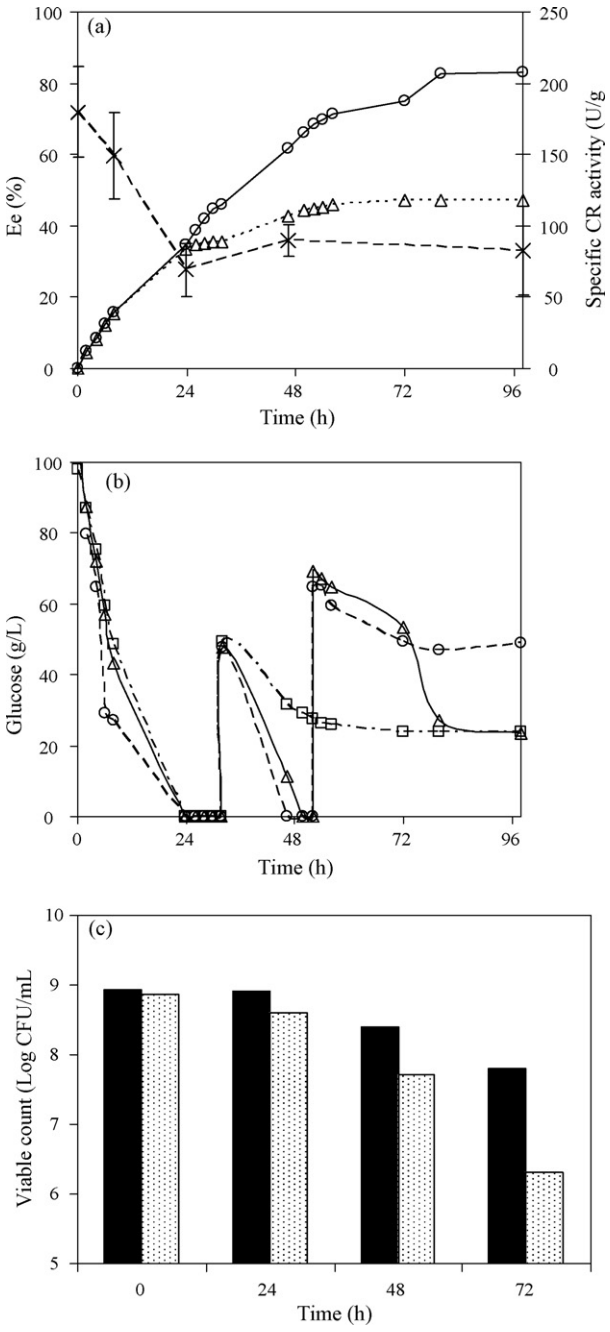

Fig. 2. Bioreduction of 0 g/L or 20 g/L *rac*-**1** with 15 gdw/L TMB4100 (1% PGI, YMR226c) that was unchanged or replaced every 24 h. (a) ee for (+)-**1** during bioreduction; with (○) and w/o (△) replaced cells. Specific carbonyl reductase (CR) activity (×) in cell extract of cells w/o replaced cells. (b) Glucose consumption during bioreduction of 0 g/L *rac*-**1** w/o changed cells (○), 20 g/L *rac*-**1** w/o changed cells (□) and 20 g/L *rac*-**1** with changed cells (△). (c) Viable count of unchanged cells followed over time during bioreduction of 0 g/L *rac*-**1** (black bars) and 20 g/L *rac*-**1** (grey bars).

3. Experimental

3.1. General

Commercial compressed baker's yeast for sweet (Red) and normal (Blue) dough (Kronjäst, Jästbolaget AB, Sollentuna, Sweden) were purchased from a local grocery store. *S. cerevisiae* TMB-strains (Table 1) had been constructed previously [11,19]. Strains were stored in 15% glycerol stocks with liquid YNB-media (6.7 g/L YNB w/o amino acids, 20 g/L glucose or 20 g/L fructose and 1 g/L glucose, and amino acids) in a −80 °C freezer. Yeast strains were plated on YNB-plates (YNB-media with 20 g/L agar) and incubated for 2 days at 30 °C before use. Yeast cells were grown in 1 L Erlenmeyer flasks containing 100 mL liquid YNB-media, buffered with 100 mM potassium phosphate pH 6.8 by continuous shaking at 180 rpm and 30 °C. Cells were harvested at early stationary phase by centrifugation at 5000 × *g*, and then washed once with distilled water. Cell dry weight and concentrations of glucose, glycerol, acetate and ethanol were determined as described previously [28]. Viability was determined as described earlier by streaking diluted cell suspensions on YPD-plates (20 g/L yeast extract, 10 g/L peptone, 20 g/L fructose, 1 g/L glucose and 20 g/L agar). For the determination of specific enzyme activity cells were sampled and harvested by centrifugation at 5000 × *g* for 5 min. Cells were then washed once with cold distilled water and centrifuged at 5000 × *g* for 5 min. Wet cells were dispersed (0.5 mg/mL) in Y-per yeast extraction protein reagent (Pierce, Rockford, IL, USA) in a 2-mL Eppendorf tube and incubated on a turning table at room temperature for 50 min. Cell debris was separated by centrifugation for 5 min at 16,100 × *g* (Hermle Labortechnik Z160M table centrifuge, Wehingen, Germany). The supernatant was collected and kept on ice. Protein concentration was determined with Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA). Bovine serum albumin (BSA) was used for the standard curve. Enzyme activity measurements were performed by following the oxidation of NADPH at 340 nm using an Ultrospec 2100pro spectrophotometer (Amersham Biosciences, Sweden). Data were collected using the software program SWIFTII (Amersham Biosciences, Sweden). Samples were diluted such that the decrease of absorbance was linear during 5 min. One unit of activity corresponds to 1 μmol NADPH consumed per minute at 30 °C. The assay contained diluted cell extract in potassium phosphate buffer (100 mM, pH 6.8), NADPH (200 μM) and the substrate *rac*-**1** (6 g/L). GLC analysis for determining enantiomeric excess was performed on a Perkin-Elmer Autosystem XL equipped with a Supelco alfaDEX 120 fused silica capillary column (30 m × 0.25 mm × 0.25 μm). Samples were analyzed at 100 °C (iso, 25 min) then 5 °C/min to 200 °C. Retention times at 1 mL/min He-flow: (−)-**1** 26.3 min. (+)-**1** 26.6 min, exo-2 35.2 min and 35.5 min, (−)-*endo*-**2** 36.0 min and (+)-*endo*-**2** 36.2 min. NMR spectra were recorded on a Bruker Ultrashield plus 400 MHz spectrometer using $CDCl_3$ (1H 7.16 ppm) as solvent. Column chromatography was carried out on Matrex (25–70 μm) silica gel. TLC was carried out on silica gel (60 F254, Merck), the plates were impregnated with a solution of p-methoxybenzaldehyde (10 mL), conc. H_2SO_4 (50 mL) and ethanol (95%, 950 mL) and visualized upon heating. Optical rotation was measured on a Perkin-Elmer (Model 341) polarimeter at 20 °C and is given in 10^{-1} deg cm^2/g.

3.2. 5,6-Epoxy-bicyclo[2.2.1]heptane-2-one (rac-**2**)[29]

Norborn-5-ene-2-ol (a mixture of endo/exo) (6.60 g, 59.9 mmol) dissolved in CH_2Cl_2 (10 mL) and MCPBA (15.5 g, 90.0 mmol) dissolved in CH_2Cl_2 (150 mL) were mixed at 0 °C. The cooling-bath was removed and the reaction was stirred at rt for 30 min, subsequently the hydrochloric salt of tetramethylpiperidine (TMP·HCl) (3.0 mL, ≈0.2 M) was added followed by MCPBA (15.8 g, 64.0 mmol) in CH_2Cl_2 (150 mL). The reaction was stirred at rt for 3 h then more TMP·HCl (3.0 mL, ≈0.2 M) was added and the reaction was stirred for 30 min. The organic phase was washed with 2 M NaOH, water and brine, and then dried over Na_2SO_4. The solvent was removed at reduced pressure and the crude product (7.0 g) was purified by sublimation giving the product as white crystals (5.45 g, 73%). The ^{1}H NMR analysis was in accordance to the literature [30].

3.3. Bioreduction of rac-**1**

Whole yeast cells (5–20 gdw/L), glucose (100 g/L) and *rac*-**1** (2.5–50 g/L) were mixed in citrate buffer (100 mM, pH 5.5). The mixture was agitated with a magnetic stirrer at 30 °C. Samples were collected from the reaction mixture and centrifuged at 16,100 × *g* for 2 min with a table centrifuge. The supernatant was extracted with ethyl acetate and the organic phase was dried with Na_2SO_4. Conversion and ee were determined by GC analysis.

3.4. Preparative scale synthesis of (+)-(**1**) and endo-(−)-**2** by yeast reduction

Whole yeast cells of TMB4100 (YMR226c, 1% PGI) (15 gdw/L), glucose (100 g/L) and *rac*-**1** (1.04 g, 8.4 mmol) were mixed in 200 mL citrate buffer (100 mM, pH 5.5). The mixture was agitated with a magnetic stirrer at 30 °C. Sugar concentration was followed with glucose sticks and after 24 h more glucose (100 g/L) was added. After 48 h cells were separated at 5000 × *g* for 5 min and the supernatant was collected. The pellet was washed once with distilled water. The combined aqueous phases were saturated with NaCl and extracted with ethyl acetate (3 mL × 200 mL). The combined organic phases were washed with brine, dried over Na_2SO_4 and the solvent was removed at reduced pressure. (+)-**1** (0.29 g, 28%, 95% ee, $[\alpha]_D^{20}$ +339 (c 1.2, $CHCl_3$)) and (−)-**2** (0.48 g, 45%, 74% ee, 80% de) were isolated by column chromatography (SiO_2, heptane/ethyl acetate 1:1).

4. Conclusions

The genetically modified yeast TMB4100, having 1% PGI activity and overexpressing YMR226c gene, was found among a series of yeast strains to be the best strain to reduce racemic epoxy ketone *rac*-**1** and produce (+)-**1** and *endo*-(−)-**2** with high purity. The limitations of the reaction consisted of a combination of product and substrate inhibition of the enzyme plus a time-dependent inactivation of the cells. Furthermore, the inactivation of the reaction was shown to be correlated to an arrest in glucose consumption and hence NADPH regeneration. The arrest of glucose consumption was not connected to cell death but instead to another more complicated mechanism, which remains to be elucidated. Finally, TMB4100 was used to reduce *rac*-**1** in preparative scale and ketoepoxide (+)-**1** was isolated in 28% yield and 95% ee. The epoxy alcohol (−)-**2** was isolated in 45% yield, 74% ee and 80% de. Thus, the bioreduction system is well suited for the production of enantiomerically pure (+)-**1** and (−)-**2** in gram scale. This is to the best of our knowledge the first detailed description of kinetic resolution of a racemic bicyclic ketone, also carrying an epoxide moiety, through asymmetric carbonyl reduction catalyzed by genetically engineered *S. cerevisiae*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2008.11.014.

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