Screening of yeast species for the stereo-selective reduction of bicyclo[2.2.2]octane-2,6-dione

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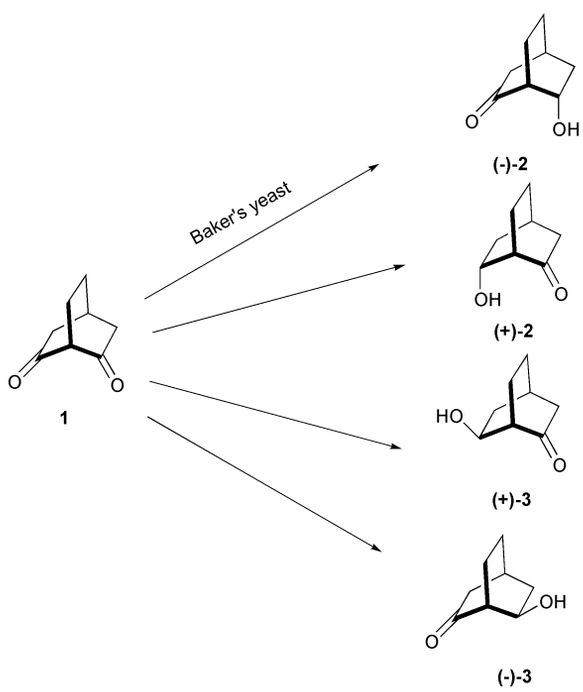
Yeast strains from more than 31 different genera were screened for the enantioselective reduction of bicyclo[2.2.2]-octane-2,6-dione (**1**). Reducing activity was found in 80% of the screened yeasts. Bicyclo[2.2.2]octane-2,6-dione was enantioselectively reduced (>98% ee) to (1*R*,4*S*,6*S*)-6-hydroxybicyclo[2.2.2]octane-2-one (−)-**2** by 69% of the strains. Enantioselective reduction of the diketone to (1*S*,4*R*,6*S*)-6-hydroxybicyclo[2.2.2]octane-2-one ((+)-**3**, >98% ee) as a major product is reported for the first time. *Candida tropicalis* UOFS Y-0534 and *Candida wickerhamii* UOFS Y-0652 displayed this unusual diastereoselectivity.

Introduction

In the synthesis of natural products there is a constant need for readily available optically active building blocks. Optically active bicyclo[2.2.2]octane derivatives have been used as rigid templates for regio- and stereo-controlled transformations towards natural products.[1,2] Several other applications for bicyclo[2.2.2]octane derivative in organic synthesis have been reported,[3] such as building blocks for mono- and sesquiterpenoids,[4,5] isopropyl hemiesters[6] and as synthons for taxanes.[7] Biotransformations are generally regarded as an efficient means to produce non-racemic building blocks. It is known that commercially available baker's yeast *Saccharomyces cerevisiae* asymmetrically reduces bicyclo[2.2.2]octane-2,6-dione (**1**) and predominantly forms the *endo*-product (−)-**2**[8,9] (Scheme 1). There are several advantages of working with baker's yeast, it is readily available, relatively inexpensive and non-pathogenic. However, it is uncertain as to which *S. cerevisiae* strain (or strains) actually are present in batches of commercially produced baker's yeast. Although reductions with baker's yeast are commonly used, it would be an advantage to select a strain to ascertain the reproducibility.

Even if the optically active keto-alcohol, (−)-**2**, is available *via* reduction of the diketone **1** by baker's yeast, the asymmetric formation of the other *endo*- enantiomer, (+)-**2**, and both the *exo*-enantiomers, (+)- and (−)-**3**, have not been reported as major products. Since it would be fruitful to gain access to all four isomers, particularly for the synthesis of natural products[10] and as starting materials for catalyst or ligands,[11–13] the Yeast Culture Collection of the University of the Free State (UFS) was screened for the asymmetric reduction of **1**. The aim of this study was (i) to find a strain for improved production of (−)-**2** and (ii) to find strains that could predominately produce any one of the other isomers (+)-**2**, (+)-**3** or (−)-**3**.



Scheme 1 The possible products from mono reduction of the diketone **1**.

In the current study, 327 yeast strains from more than 31 different genera were screened to establish the distribution of reducing activity towards **1** within the yeast domain, and to determine whether any strains could reduce **1** enantioselectively.

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Table 1 Distribution of reducing activity of **1** in the yeast domain

Genera	Number of species screened	Number of strains screened	Number with reducing activity[a]	Number enantioselective for (−)-**2**[b]	Number with **3** activity[c]
Arxiozyma	1	1	0	0	0
Brettanomyces	4	5	2	2	1
Bullera	1	2	1	1	1
Bulleromyces	1	1	1	1	0
Candida	28	47	46	40	17
Cryptococcus	1	1	1	1	0
Debaryomyces	1	26	20	20	1
Dekkera	1	2	0	0	0
Dipodascus	1	1	1	1	0
Endomyces	1	1	0	0	0
Endomycopsella	2	2	2	2	0
Filobasidium	1	1	1	0	0
Galactomyces	1	1	1	1	1
Geotrichum	6	8	2	2	1
Hasegawaea	1	1	0	0	0
Hormonema	1	1	1	1	1
Hyphopichia	1	1	1	1	1
Kluyveromyces	1	2	2	2	1
Lipomyces	3	6	5	5	1
Myxozyma	1	1	1	1	0
Pichia	5	17	17	15	5
Rhodosporidium	5	7	6	5	2
Rhodotorula	13	38	37	37	10
Saccharomyces	16	51	38	36	5
Saccharomycodes	1	1	1	1	0
Saccharomycopsis	3	8	5	4	2
Sporidiobolus	1	1	1	0	1
Trichosporon	7	33	21	17	10
Wickerhamiella	1	1	1	0	1
Wingea	1	1	1	1	0
Yarrowia	1	6	5	5	1
Unclassified		52	41	27	17
Total	**112**	**327**	**262**	**229**	**83**

[a] Strains which showed any reduction products by TLC analysis were rated as having reducing activity. [b] Strains which showed no traces of (+)-**2** by GC analysis were rated as enantioselective for (−)-**2**. [c] Strains which showed traces of **3** by GC analysis were rated as having **3** activity.

Table 2 Reduction of **1** (45 mM) by yeast strains

Strain	(−)-**2** formation		
	ee (%)	Yield (%)	Initial rate (nmol min^{-1} per g dry weight)
Baker's yeast	>98%	59	867
Saccharomyces cerevisiae CBS 3093	>98%	35	434
Cryptococcus albidus UOFS Y-2127	>98%	79	860
Rhodotorula sp. UOFS Y-0448	>98%	70	901
Unclassified UOFS Y-0515	>98%	67	653

Results and discussion

Screening for asymmetric reduction of bicyclo[2.2.2]octane-2,6-dione

The search for yeasts with the ability to reduce **1** enantioselectively, was initiated with a screening of 327 strains from more than 31 different genera. The results are summarised in Table 1. Of the 327 strains in the screening, 80% displayed reducing activity and 69% reduced **1** enantioselectively to (−)-**2**. For six genera, namely *Candida*, *Debaryomyces*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Trichosporon* more than 10 strains per genus were screened. Of these genera *Candida*, *Pichia* and *Rhodotorula* were remarkable because 97–100% of the tested strains had reducing activity. For the other three genera the percentage of strains with reducing activity varied between 64 and 76%. Strains having both **2** and **3** as products were identified from TLC and GC experiments (Table 1).

Time course reactions of bicyclo[2.2.2]octane-2,6-dione (1)

From the initial screening, the most promising yeast strains for the reduction of **1** to **2** were selected for time course reactions. From these experiments (Table 2) it was clear that most of the enantioselective strains preferentially reduced **1** to (−)-**2**, as baker's yeast is known to do. Excellent enantiomeric excesses (>98% ee) were found in all reactions rated as enantioselective. The results were compared to baker's yeast included in the study (Table 2). Baker's yeast proved to have higher initial reaction rate and yield than any *Saccharomyces cerevisiae* strain. However, yeasts from other genera were found that rival baker's yeast. *Rhodotorula* sp. (UOFS Y-0448) displayed the highest initial rate of (−)-**2** formation (901 nmol min^{-1} g dry weight yeast) and the highest yield, 79%, was produced by *Cryptococcus albidus* (UOFS Y-2127) (Table 2).

Among the 4 strains selected for the production of both **2** and **3** and tested for time course reductions, two strains, *Candida tropicalis* (UOFS Y-0534) and *Candida wickerhamii* (UOFS Y-0652), were found to form the *exo*-isomer **3** as the major product (Fig. 1). These strains formed both (−)-**2** and the diastereomer (+)-**3** in the ratio of 1 : 2 (for absolute structure determination, see below). Both of the products were formed in excellent enantiomeric excess (>98% ee) for the four strains, *i.e. Candida rugosa* CSIR Y-0299, *C. tropicalis* UOFS Y-0534, *Rhodotorula philyta* UOFS Y-0134 and *C. wickerhamii*

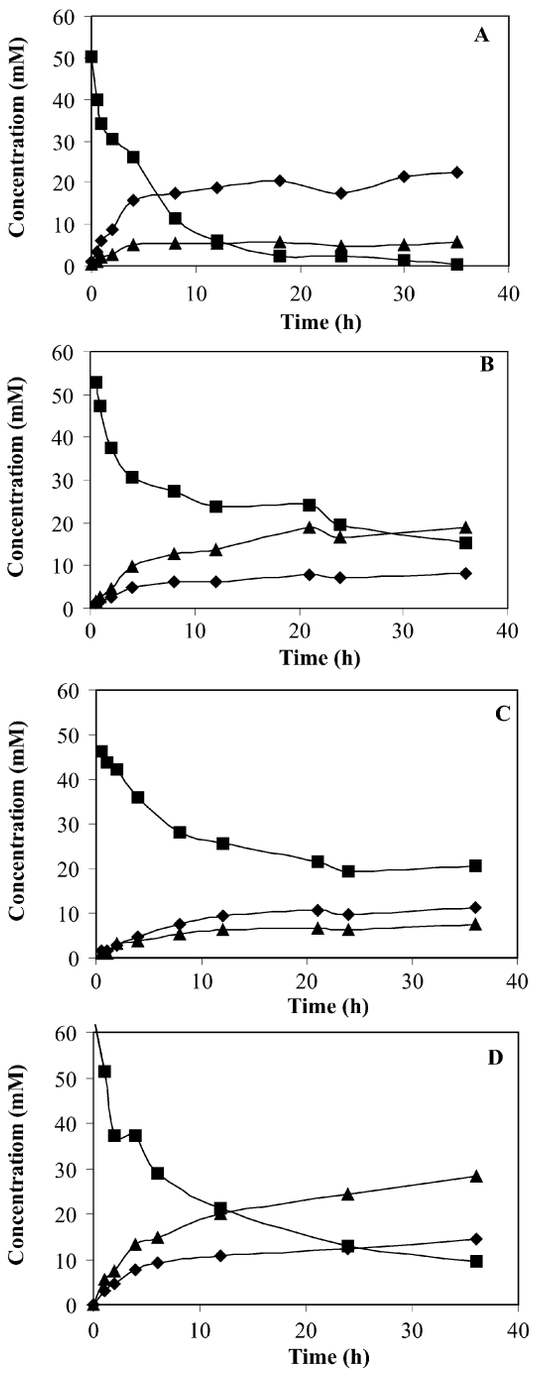

Fig. 1 Time course reductions of **1** (■) to **2** (◆) and **3** (▲) by different yeast strains. A: *Candida rugosa* CSIR Y-0299, B: *Candida tropicalis* UOFS Y-0534, C: *Rhodotorula philyta* UOFS Y-0134, D: *Candida wickerhamii* UOFS Y-0652.

UOFS Y-0652. (+)-**3** has never been reported as the major product from a bioreduction of **1**. Since (−)-**2** and (+)-**3** are diastereomers, they may be separated by conventional chromatography, thus allowing access to two of the four isomers in enantiopure form. This makes these strains very interesting for the synthesis of chiral building blocks.

Determination of the absolute stereochemistry of (+)-3

Candida tropicalis UOFS Y-0534 was selected for the preparative scale production of **3**. Mori and Nagano previously determined the absolute configuration of (+)-**3** to be 1*S*,4*R*,6*S* by a combination of chemical transformations and CD-spectroscopy.[9] Since it should be possible to use the octant rule[14] directly on **3** without chemical transformations, we took the opportunity to make an independent determination of the absolute configuration. A clear negative Cotton effect was noted for our sample of (+)-**3** and in orienting the 1*S*,4*R*,6*S*-enantiomer according to the octant rule placed the hydroxy group in the negative back lower left octant. The other enantiomer had the hydroxy group in the positive back lower right octant. Thus, our measurements confirmed that (+)-**3** has the 1*S*,4*R*,6*S*-configuration.

Furthermore, HPLC analysis of the Mosher ester derivatives[15] showed that the minor diastereomeric product of the baker's yeast reduction corresponded to the major product from the reduction with *Candida tropicalis* UOFS Y-0534.

Conclusion

The ability to reduce **1** was found in a broad range of yeast genera, besides baker's yeast. Most of the yeasts formed (−)-**2** with excellent ees. Several yeasts were found that performed as well, or better, than baker's yeast.

Two strains of the genus *Candida*, formed (+)-**3** as the major product in excellent enantiomeric excess (>98% ee). This unusual diastereoselectivity has never been reported before. These results demonstrate that enantioselective reduction of **1** is not restricted to baker's yeast, *i.e.* the strains of *Saccharomyces cerevisiae*, and that other strains with different diastereoselectivities exist.

Experimental

General

Reactions were monitored by TLC (silica gel 60 F_{254} plates, Merck) in ethyl acetate–hexane (15 : 10) as mobile phase and visualised with anisaldehyde stain (anisaldehyde–ethanol–conc. H_2SO_4 1 : 9 : 1). Flash column chromatography was performed on Matrex (25–70 μm) silica gel. Melting points were taken on a Sanyo Gallenkamp melting point apparatus (MPD.350.BM3.5) and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 22 °C and are given in 10^{-1} deg cm^2 g^{-1}. CD-spectroscopic analysis was performed on a Jasco J-500A spectropolarimeter at 22 °C. The synthesis of the reference material from the baker's yeast reduction, (−)-**2**, was performed according to the literature.[16,17] The overall mixture of all possible stereoisomers of 6-hydroxybicyclo[2.2.2]octane-2-one was synthesised according to Mori *et al.*[9] *via* ring closure of the corresponding keto-aldehyde. ^{1}H NMR and ^{13}C NMR spectral data were in full agreement with those previously reported.[18] (*R*)-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride was purchased from Fluka and used as delivered. HPLC analysis was performed on a YMC-Pack SIL column (300 × 4.6 mm, 10 μm particle size) using hexane–*i*PrOH as the solvents with detection at 215 nm. All NMR spectra were recorded on a Bruker DRX 400 and the chemical shifts were measured using the solvent as reference (C_6H_6, 7.16 ppm, C_6D_6 ^{13}C, 128.39 ppm).

Optical purities were analysed by GLC (Hewlett-Packard 5890 equipped with FID) on fused silica cyclodextrin columns, using N_2 as carrier gas. Stereoisomeric analyses of the enantiomers of **2** and **3** were performed using both an α-DEX 120 and a β-DEX 120 column (Supelco, 30 m × 0.25 mm, 0.25μm film) at appropriate isotherm temperatures (see Table 3). Both columns had to be used since only the enantiomers of **2** were separated by α-DEX 120 and only those of **3** on β-DEX 120. Concentrations were derived from calibration curves.

Preparation of frozen yeast cells

Yeast strains were obtained from the Yeast Culture Collection of the University of the Free State, Bloemfontein, South Africa. Yeast cells were grown at 25 °C in 250 ml shake-flask cultures containing 50 ml YM-broth (10 g l^{-1} peptone, 20 g l^{-1} malt

Table 3 Chiral analysis of substrate and formed products

Compound	α-DEX 120 R_t(130 °C) /min	β-DEX 120 R_t(145 °C) /min
1	21.2	13.7
(−)-**2**	35.8	25.1
(+)-**2**	36.5	25.1
(−)-**3**	33.9	23.3
(+)-**3**	33.9	24.5

extract and 5 g l^{-1} yeast extract), supplemented with 15 g l^{-1} glucose. At exponential phase (24–48 h) the cells were harvested by centrifugation (5000 *g*, 10 min, 10 °C), washed and resuspended (1 : 4 w/v) in phosphate buffer (50 mM, pH 7.5) containing 20% glycerol. The cells were frozen at −78 °C in micro-centrifuge tubes (0.5 ml cell suspension per micro-centrifuge tube). The cells could be stored for several months without significant loss of activity.

Screening for asymmetric reduction of bicyclo[2.2.2]octane-2,6-dione

Frozen cells in micro-centrifuge tubes were thawed, washed with phosphate buffer (50 mM, pH 7.5) and resuspended (1 : 10 w/v) in 1 ml phosphate buffer (10 mM, pH 6.8). A solution of **1** [16,17] (5 µl of a 200 mg ml^{-1} in DMSO) was added to give a final concentration of 1 g l^{-1} (7.2 mM). The reaction mixtures were agitated on a shaking water bath at 30 °C and 200 µl samples were withdrawn after 1, 2 and 8 hours. The samples were saturated with NaCl and extracted with ethyl acetate (100 µl). After centrifugation (3000 *g*, 5 min) the organic phases were analysed by TLC analysis. Ethyl acetate extracts showing products were dried over Na_2SO_4 prior to chiral GC analysis at the appropriate isotherm temperature.

Time course reactions of bicyclo[2.2.2]octane-2,6-dione reduction

Frozen cells were thawed, washed with phosphate buffer (50 mM, pH 7.5) and resuspended (1 : 4 w/v) to a final volume of 5 ml in phosphate buffer (10 mM, pH 6.8) in 20 ml glass vials with screw caps fitted with a septa. A solution of **1** (150 µl of a 133 mg ml^{-1} in DMSO) was added to a final concentration of 45 mM. The reaction mixtures were agitated on a shaking water bath at 30 °C. The bioconversion was followed by withdrawing samples (200 µl) at appropriate time intervals. Samples were extracted as described above and the remaining **1** along with the formed ketoalcohols were quantified and the stereochemical compositions determined by chiral phase GLC analysis. Baker's yeast was used for comparison.

Preparative scale reduction of bicyclo[2.2.2]octane-2,6-dione with *Candida tropicalis* UOFS Y-0534

Previously optimised reduction conditions of **1** using *S. cerevisiae* [17] were used for preparative scale reductions of 5 g l^{-1} of **1** with *Candida tropicalis* UOFS Y-0534, with the following modifications: **1** (600 mg) was reduced in 120 ml of mineral medium [19] supplemented with 0.4 g l^{-1} Tween 80 and 10 g l^{-1}-ergosterol, and with a glucose concentration of 120 g l^{-1}. After 48 hours, **1** could not be detected and TLC analysis showed that the fermentation broth only contained the reduced products **2** and **3**. The fermentation broth was filtered through a pad of Celite, which was then rinsed with EtOAc (3 × 50 ml). The phases were separated and the water phase was saturated with NaCl and extracted with EtOAc (4 × 150 ml). The combined organic phases were thereafter dried (Na_2SO_4) and filtered. Removal of the solvent under reduced pressure gave a yellowish paste that was purified by chromatography (SiO_2, 80 : 20 EtOAc–heptane). Order of elution: (+)-**3** (358 mg) {R_f = 0.34} followed by a 1 : 1 mixture of (+)-**3** and (−)-**2** (198 mg) {R_f = 0.29, for **2**}. For (+)-**3**; {$[\alpha]_D^{22}$ +4.1 (*c* 1.0 in $CHCl_3$)} mp: 233–240 °C (sublimation followed by decomposition). Product confirmation was performed by NMR-spectroscopy. NMR analysis was carried out in the way described by De Santis *et al.* [18] The ^{1}H and ^{13}C NMR data for (−)-**2** and (+)-**3** were identical of those of the racemic compounds.

Mosher ester analysis of 2 and 3 (general method)

The alcohol (4.0 mg, 29 µmol) was added to dry pyridine (0.25 ml) followed by (*R*)-(−)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (19 µl, 0.10 mmol) The walls of the reaction vessel were rinsed with dry pyridine (0.25 ml). The reaction was stirred at rt for 22 h (until no unreacted ketoalcohol could be detected by TLC analysis) and then diethyl ether (30 ml) was added. The organic layer was washed with HCl (2 × 20 ml, 0.5 M), sat. $NaHCO_3$ (2 × 20 ml) and dried Na_2SO_4. Filtration through a small silica pad and evaporation of the solvent gave an oil that was analysed by HPLC; eluent: hexane–*i*PrOH 99 : 1 to 97 : 3 over 30 min. Retention times for the Mosher esters: (+)-**2**: 9.2 min; (−)-**2**: 11.0 min; (−)-**3**: 12.9 min and (+)-**3**: 15.3 min.

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