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Genetically engineered *Saccharomyces cerevisiae* for kinetic resolution of racemic bicyclo[3.3.1]nonane-2,6-dione

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ABSTRACT

Whole cells of the genetically engineered *Saccharomyces cerevisiae* strain TMB4100 (1% PGI, YMR226c) were used as the biocatalyst for the kinetic resolution of racemic bicyclo[3.3.1]nonane-2,6-dione *rac*-**1**. The yeast's phosphoglucose isomerase activity was decreased, and the short-chain dehydrogenase/reductase encoded by YMR226c was overexpressed. This reduced the demand for the glucose to regenerate NADPH, while at the same time the reaction rate and selectivity towards (−)-**1** became higher. The demand for yeast biomass also decreased, facilitating down-stream processing, which is of considerable importance on a large scale. With 15 g dry weight/L of the genetically engineered yeast TMB4100 (1% PGI, YMR226c), 40 g/L *rac*-**1** was kinetically resolved within 24 h producing pure (+)-**1** with an enantiomeric excess (ee) of 100% after 75% conversion. This corresponds to a biochemical selectivity constant of $E = 10.3 \pm 2.2$. Thus, compared with conventional methods which use commercial baker's yeast as a biocatalyst, the reaction system was significantly improved, and would be superior in a large-scale process.

1. Introduction

The bicyclo[3.3.1]nonane (BCN) framework is a commonly occurring motif amongst natural products, displaying a wide scope of biological activities. For instance, the rather structurally complex polycyclic polyprenylated acyl phloroglucinol derivatives (PPAPs) have been shown to enhance in vitro choline acetyltransferase activity, cytotoxic properties and antibacterial activity.[1] The structural framework of BCN has also been exploited within the field of natural product mimetics, for example, in the synthesis of taxoid analogs.[2] Furthermore, derivatives of BCN have been used to introduce chirality, structural rigidity, and stereochemical properties within the field of supramolecular chemistry.[3–6] The characteristic rigidity of BCN and the possibility to strategically derivatize spatial defined positions have been employed in chiroptical investigations of numerous chromophores incorporated into the BCN framework.[7–11]

The broad versatility of the BCN skeleton has resulted in the development of several synthetic intermediates that can be functionalized. The most affordable and perhaps most popular intermediate is the chiral bicyclo[3.3.1]nonane-2,6-dione **1**. Racemic **1** is commercially available and if a large amount is needed, a large-scale synthesis is rather straightforward.[12,13] In addition to the easy accessibility of the racemate, enantiomeric enrichment has been accomplished in a number of ways: chromatographic separation,[14] fractional crystallization of diastereomeric derivatives,[15] desulfurization of 2-thia-adamantane-4,8-dione[16] and by different methodologies based on kinetic resolution utilizing enzymes and microorganisms.[10,17,18] These procedures add the additional steps of synthetic manipulation, and are only applicable on a semi-preparative scale or do not give useful enantiomeric excess (ee). The most convenient and often employed method is the approach of kinetic resolution using baker's yeast (Fig. 1), giving excellent ee's in acceptable yields (24–30%).[7,19]

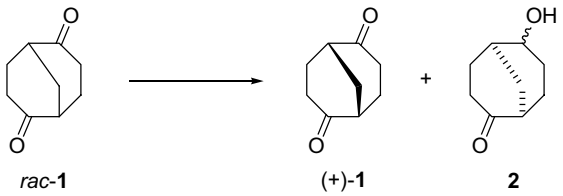

Figure 1. Kinetic resolution of racemic bicyclo[3.3.1]nonane-2,6-dione *rac*-**1** using baker's yeast.

However, the process includes a cumbersome work-up, and has to be performed twice over a period of two weeks to reach high ee.[7] A recently published method has circumvented this by performing the bioconversion in a highly diluted system with large

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amounts of baker's yeast.[20] However, low substrate concentration and work-up procedure prevent the application for the large-scale kinetic resolution of **1**.

Genetically engineered yeast strains have been proven to perform bioreductions better than commercial baker's yeast, by using less co-substrate[21] and reaching higher yield and ee of the desired enantiomer.[22–25] Baker's yeast contains ca. 50 different genes encoding NADPH-dependent dehydrogenases that could potentially catalyze stereoselective carbonyl reductions.[26,27] A number of the corresponding enzymes have been shown to accept bicyclic ketone derivatives with structures similar to **1**.[27] By overexpressing the mentioned genes, the specific reductase activity in whole cells was increased up to 40-fold, which improved the reaction rate and minimized the selectivity problems from competing enzymes.

With the aim of developing a more efficient synthesis of enantiomerically pure (+)-**1**, we have studied the kinetic resolution of racemic dione **1** by asymmetric carbonyl reduction catalyzed by genetically engineered baker's yeast *Saccharomyces cerevisiae*.

2. Results and discussion

The kinetic resolution of *rac*-**1** through carbonyl reduction (Fig. 1) with the aim to obtain enantiomerically pure (+)-**1** was studied using genetically engineered *S. cerevisiae*. The phosphoglucose isomerase (PGI) activity was reduced to 1% compared to the parental yeast, and a short-chain dehydrogenase/reductase encoded by YMR226c was overexpressed. Decreasing PGI activity reduces the glucolytic flux, and redirects carbon into the pentose-phosphate pathway, where NADPH is regenerated. Thus, the demand for glucose to regenerate NADPH is substantially lowered when compared with commercial baker's yeast. Decreased PGI activity has been combined with overexpression of YMR226c in strain TMB4100 (1% PGI, YMR226c),[24] which was successfully used as a biocatalyst. Improved asymmetric carbonyl reduction of bicyclic diones structurally related to **1**, including kinetic resolution of racemic bicyclo[2.2.2]octane-2,5-dione and bicyclo[2.2.2]oct-7-ene-2,5-dione, was achieved.[26,28]

To establish that the dehydrogenase encoded by YMR226c reduces **1** in *S. cerevisiae*, the biocatalytic activity of strain TMB4100 (1% PGI, YMR226c) and its control strain RBY7-1 (1% PGI) was compared. The control strain RBY7-1 (1% PGI) carries the same genetic information as TMB4100 (1% PGI, YMR226c), except that it lacks YMR226c overexpression. Therefore, RBY7-1 (1% PGI) should be a less active biocatalyst than TMB4100 (1% PGI, YMR226c) provided that the dehydrogenase accepts *rac*-**1** as a substrate.

Indeed, overexpression of YMR226c led to higher selectivity for (−)-**1**, and effectively increased the biocatalytic activity of the yeast cells (Table 1). Consequently, the *E*-value, as calculated from Eq. 1:

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

increased from 4 to 10 by overexpressing YMR226c, thus indicating a more selective system. The *E*-value is an intrinsic property of a biocatalyst that describes how efficiently it can differentiate between two stereoisomers in a kinetic resolution.[29] The higher *E*-value for TMB4100 (1% PGI, YMR226c) demonstrated that the overexpressed reductase accepted **1** as a substrate and that it had a greater selectivity than competing enzymes with similar activity.

Encouraged by the initial results, the biocatalytic activity of TMB4100 (1% PGI, YMR226c) towards the kinetic resolution of *rac*-**1** was further investigated. In an ideal kinetic resolution only one of the enantiomers would be converted, leading to a 50% yield of the enantiomerically pure product, in our case (+)-**1**. To maximize the obtainable yield of enantiomerically pure (+)-**1**, the time course of the reaction was followed to determine when it should be terminated. Furthermore, the maximum substrate load, which the modified yeast strain could tolerate with retained activity, was investigated. Hence, the highest possible substrate concentration was determined at constant yeast cell density (15 g dry weight/L) with increasing initial concentrations of *rac*-**1** (3–40 g/L).

An ee of 100% was reached at approximately 75% conversion with TMB4100 (1% PGI, YMR226c) as a biocatalyst (Fig. 2), which corresponds to an *E*-value of 10.3 ± 2.2, as determined with Eq. 1. The *E*-value was calculated as a mean of the *E*-values obtained with all experimental data points in Figure 2.

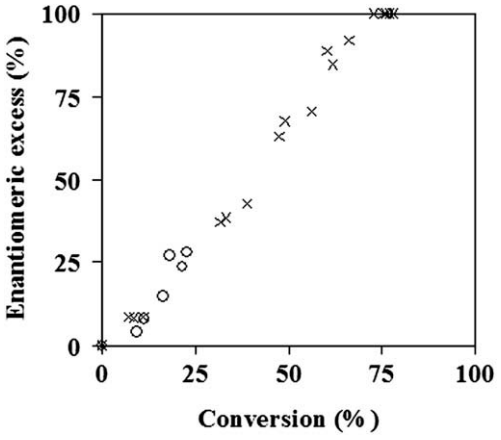

Figure 2. Enantiomeric excess of (+)-**1** (%) plotted against the degree of conversion of *rac*-**1** (%) with baker's yeast (open circles) or TMB4100 (1% PGI, YMR226c) (crosses) as a biocatalyst. Experimental values were determined by following the reaction over time at different concentrations of *rac*-**1** (3–40 g/L). 100% ee with highest possible yield was achieved at approximately 75% conversion.

There were no significant difference in the reaction rate from 3 to 20 g/L *rac*-**1** (Fig. 3), and even at 40 g/L *rac*-**1** 100% ee was

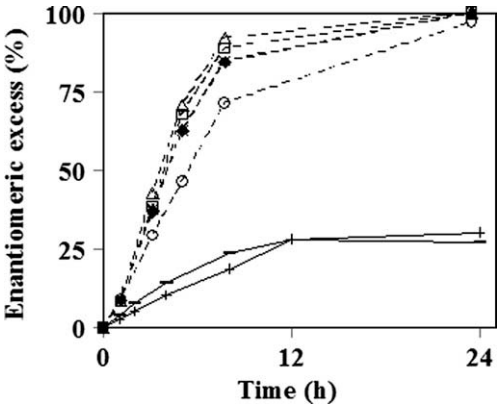

Figure 3. Enantiomeric excess of (+)-**1** followed over time during bioreduction of 3 g/L (□), 5 g/L (△), 10 g/L (∗), 20 g/L (●), and 40 g/L (○) g/L *rac*-**1** with TMB4100 (1% PGI, YMR226c) (dashed line) and of 5 g/L (−) and 50 g/L (+) *rac*-**1** with commercial baker's yeast (whole line).

Table 1
Ee, conversion, and *E*-value for the kinetic resolution of *rac*-**1** with TMB4100 (1% PGI, YMR226c) and its control strain RBY7-1 (1% PGI)

Strain	ee (+)-**1**[a] (%)	Conv. *rac*-**1**[a] (%)	*E*[a]
RBY7-1	18	24	4
TMB4100	35	32	10

[a] Determined after 24 h of bioreduction of 5 g/L *rac*-**1** with 5 g dry weight/L whole cells. The E-value was calculated from the ee of (+)-**1** and conversion of *rac*-**1** according to Eq. 1.[29]

reached after approximately 24 h, despite a somewhat slower reaction rate. The decrease in reaction rate could be caused by substrate inhibition of the enzyme or substrate toxicity to the cell. Furthermore, the solubility of the substrate in water may become a problem at higher concentrations.

For the sake of comparison, commercial baker's yeast was tested under the same experimental conditions. The selectivity did not notably differ between commercial baker's yeast and TMB4100 (1% PGI, YMR226c) (Fig. 2). In fact, the biochemical selectivity constant was determined to be $E = 8.2 \pm 6.8$, which is not significantly different from the *E*-value for TMB4100 (1% PGI, YMR226c), $E = 10.3 \pm 2.2$. However, while 100% ee was reached at 40 g/L within 24 h for TMB4100 (1% PGI, YMR226c), commercial baker's yeast did only reach an ee of about 30% after the same time (Fig. 3). Furthermore, commercial baker's yeast seemed to be inactivated after 12 h, despite the glucose still being in the reaction solution (tested with glucose sticks). The reason for this is unclear but commercial baker's yeast may be less viable than the freshly grown genetically engineered yeast, even though it had long lasting expiration date. Obviously, the lower efficacy of commercial baker's yeast could be compensated by using more yeast biomass during a longer period of time. However, the formation of organic solvent emulsions together with lysed cell content (e.g., proteins and lipids) complicates biomass separation from the reaction mixture, and consequently leads to a more cumbersome work-up of the product.

3. Conclusions

The kinetic resolution of *rac*-**1** with the genetically modified yeast TMB4100 (1% PGI, YMR226c) as a biocatalyst required less yeast biomass and co-substrate at higher substrate concentrations, which significantly improved bioreduction compared with commercial baker's yeast. The lower demand for yeast biomass and co-substrate (glucose) facilitates down-stream processing in terms of less organic solvents and less emulsion formation during extraction. At a substrate concentration of 40 g/L *rac*-**1**, enantiomerically pure (+)-**1** with an ee of 100% at 75% conversion was produced within 24 h. This corresponds to an *E*-value of 10.3 ± 2.2 for the biocatalyst. The performance of the genetically engineered yeast would thus compete favorably with the commercial baker's yeast system for the production of (+)-**1** on a large scale.

4. Experimental

4.1. 1 General

All chemicals were used as received from commercial suppliers. Bicyclo[3.3.1]nonane-2,6-dione was prepared in accordance with literature procedure.[13] Baker's yeast (Kronjäst, blue label)[30] was obtained from a local supermarket. *S. cerevisiae* strains RBY7-1 (1% PGI) and TMB4100 (1% PGI, YMR226c) were constructed as previously described.[24] Strains were stored in 15% glycerol stocks with liquid YNB-media (6.7 g/L YNB w/o amino acids, 20 g/L fructose, 1 g/L glucose, and 50 mg/L tryptophane) at −80 °C. Strains were plated on YNB-plates (YNB-media with 20 g/L agar) and incubated for 2 days at 30 °C. Cells were then grown in a 1-L Erlenmeyer flasks containing 100 mL liquid YNB-media, buffered with 100 mM potassium phosphate, pH 6.8, at 180 rpm and 30 °C. Cells were harvested in early stationary phase by centrifugation at 5000*g*, and washed once with distilled water. Cell dry weight was determined as described previously.[31] Conversions and ee:s were determined with a Perkin Elmer Autosystem XL Gas Chromatograph using an Alpha DEX™ 120 fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness).

4.2. Bioreduction of *rac*-1

Whole yeast cells (5 or 15 g dry weight/L), glucose (100 g/L), and *rac*-**1** (2.5–40 g/L) were mixed in citrate buffer (100 mM, pH 5.5) at a total volume of 2 mL. The mixture was agitated with a magnetic stirrer at 30 °C. Samples from the reaction mixture were centrifuged at 16,100*g* for 2 min. The supernatant was extracted with ethyl acetate, and the organic phase was dried with Na_2SO_4. Conversions and ee:s were determined by GC analysis.

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