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Reaction and strain engineering for improved stereo-selective whole-cell reduction of a bicyclic diketone

Ted Johanson · Magnus Carlquist · Cecilia Olsson · Andreas Rudolf · Torbjörn Frejd · Marie F. Gorwa-Grauslund

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Abstract Reduction of bicyclo[2.2.2]octane-2,6-dione to (1R, 4S, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one by whole cells of *Saccharomyces cerevisiae* was improved using an engineered recombinant strain and process design. The substrate inhibition followed a Han-Levenspiel model showing an effective concentration window between 12 and 22 g/l, in which the activity was kept above 95%. Yeast growth stage, substrate concentration and a stable pH were shown to be important parameters for effective conversion. The over-expression of the reductase gene YDR368w significantly improved diastereoselectivity compared to previously reported results. Using strain TMB4110 expressing YDR368w in batch reduction with pH control, complete conversion of 40 g/l (290 mM) substrate was achieved with 97% diastereomeric excess (de) and >99 enantiomeric excess (ee), allowing isolation of the optically pure ketoalcohol in 84% yield.

T. Johanson · M. Carlquist · M. F. Gorwa-Grauslund (✉)
Department of Applied Microbiology, Lund University,
P.O. Box 124, SE-22100 Lund, Sweden
e-mail: Marie-Francoise.Gorwa@tmb.lth.se

C. Olsson · T. Frejd
Department of Organic Chemistry, Lund University,
P.O. Box 124, SE-22100 Lund, Sweden

A. Rudolf
Department of Chemical Engineering, Lund University,
P.O. Box 124, SE-22100 Lund, Sweden

Present address:
A. Rudolf
Biosystems Department, Risø National Laboratory,
Technical University of Denmark—DTU,
P.O. Box 49, DK-4000 Roskilde, Denmark

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Introduction

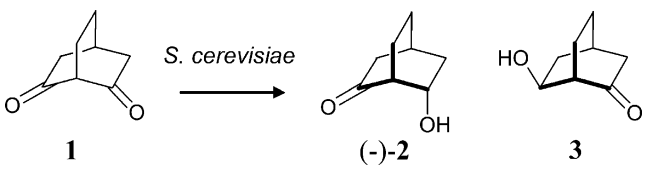
Pure chiral alcohols are important building blocks in the production of pharmaceuticals and fine chemicals (de Souza Pereira 1998; Schmid et al. 2001; Liese 2002; Patel 2006; Hilterhaus and Liese 2007). However, many chiral syntheses routes remain impractical, requiring many reaction steps or results in low yield or stereoselectivity—i.e. low enantiomeric excess [ee; (mol major enantiomer−mol minor enantiomer)/sum of enantiomers] or diastereomeric excess [de; (mol major diastereomer−mol minor diastereomer)/sum of diastereomers]. Under these circumstances, biocatalysis using either microorganisms or enzymes represents an attractive alternative (Chartrain et al. 2001; Patel 2006; Hilterhaus and Liese 2007).

So far, baker's yeast, *Saccharomyces cerevisiae*, has been the most commonly applied biocatalyst for the production of optically pure ketoalcohols (Servi 1990; Csuk and Glänzer 1991; D'Arrigo et al. 1997; Stewart 2000). Advantages include its availability, ease of use, low cost, GRAS (generally regarded as safe) status and its ability to catalyse a wide variety of carbonyl reductions. Still, the use of non-engineered strains under suboptimal reaction conditions has often resulted in low yields, reaction rates, product titres and/or poor stereoselectivity (Johanson et al. 2005).

One example concerns the asymmetric reduction of the bicyclic diketone bicyclo[2.2.2]octane-2,6-dione (**1**) to (1R, 4S, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one ((-)-**2**) (Fig. 1), which is used as an intermediate in the synthesis

of transition metal based chiral chemical catalysts (Almqvist et al. 1997; Sarvary et al. 2001; Sarvary et al. 2002) and taxanes (Martin et al. 1982). This reduction was first described by Mori and Nagano who used 82 g/l commercial dry baker's yeast and 9 g/l diketone **1** to produce ketoalcohol (-)-**2** in 95% ee (Mori and Nagano 1990). However, the reduction turned out to have low diastereoselectivity and the obtained crude product was a mixture of ketoalcohol (-)-**2** and its diastereoisomer **3** (Fig. 1), giving a de of only 70%. After silica gel chromatography and crystallisation, the diastereomeric purity was increased to 84%, and the ketoalcohol mixture isolated in 52% yield. When repeating the experiment with compressed baker's yeast, ketoalcohol (-)-**2** was obtained in 92–97% ee and 92% de (Almqvist et al. 1993; Almqvist 1996). Due to the low reduction rate of commercial baker's yeast, a high yeast concentration, which leads to rapid accumulation of by-products, and a low substrate titre were used to attain acceptable conversion yields. The resulting work-up procedure was consequently difficult and time-consuming with water-phase extractions turning into thick emulsions of solvent mixed with water, yeast, metabolic by-products and surfactants, contributing to an overall poor yield (Almqvist 1996; our observation). In addition, the moderate dia- and enantioselectivity of the commercial baker's yeast required the use of silica gel chromatography and re-crystallisation to obtain pure ketoalcohol (-)-**2**, which was finally isolated in 59% yield in 98% de and 98% ee.

Katz et al. worked to improve the bioreduction by applying genetic engineering to develop more efficient yeast catalysts (Katz et al. 2002; Katz et al. 2003a). The best strain TMB4100, combined a low phosphoglucose isomerase (PGI) activity giving a low glucose consumption and by-product formation rate, with the over-expression of the reductase gene YMR226c. These changes led to a tenfold decrease in glucose utilisation rate and a more than tenfold increase in the specific reduction rate (Katz et al. 2003a). By employing TMB4100, bioreductions with lower amounts of yeast is possible, which could be important in terms of yield since high levels of yeast have been shown to result in severe product and substrate adsorption/absorption (Jayasinghe et al. 1994; Chin-Joe et al. 2000). Still, the substrate titres were limited to 5 g/l (36 mM) due to a previous study showing toxicity/inhibition by the substrate, which abolished yeast activity already at moderate concentrations (Katz et al. 2002). Moreover, no data on the production of the unwanted ketoalcohol by-product **3** was reported.



1 **(-)-2** **3**

Fig. 1 Reduction of bicyclo[2.2.2]octane-2,6-dione **1** to (1R, 4S, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-ol (-)-**2** and its exo-diastereomer **3** by *S. cerevisiae*

In the present study, we investigated the factors that influenced the bioreduction at higher substrate concentrations with the aim to find suitable biocatalysts and process conditions enabling the efficient production and isolation of enantio- and diastereomerically pure ketoalcohol (-)-**2** in high yield.

Materials and methods

Strains

S. cerevisiae strain RBY 7-1 (ENY.WA-1A, with 1% PGI activity, encoded by YBR196c) was a gift from Prof. E. Boles (Institute of Microbiology, Frankfurt, Germany). Strain TMB4100 (YMR226c, 1% PGI) was previously created from RBY 7-1 (Katz et al. 2003a). *S. cerevisiae* CEN.PK113-7A was kindly provided by Dr. P. Kötter (J. W. Goethe Univ. Frankfurt, Germany). *S. cerevisiae* strains over-expressing Open Reading Frames (ORFs; in brackets) TMB4091 (YMR226c, NCBI accession number NP 013953), TMB4093 (YDR368w, NP 010656), TMB4094 (empty plasmid) and TMB4096 (YOR120w, NP 014763) were previously constructed in a CEN.PK background (Katz et al. 2003a). *Escherichia coli* DH5α (Life Technologies, Rockville, MD) was used for all subcloning.

Nucleic acid manipulations

DNA extraction and purification were performed as described in (Katz et al. 2003a). Competent *E. coli* and *S. cerevisiae* were prepared and transformed as described in (Katz et al. 2003a).

Chemicals

The synthesis of diketone **1** was performed according to a method developed in our laboratory (Widegren et al. 2006). One batch (~25 g) was purified by column chromatography on a MATREX (25–70 μm) silica gel with heptane-ethyl acetate (1:3) as eluent, repeatedly re-crystallised from heptane-ethyl acetate and dried under vacuum. For the second larger batch (70 g), the crude product was dissolved in ethyl acetate and washed with saturated $NaHCO_3$ solution. The aqueous phase was extracted with ethyl acetate, and the combined organic phases were washed with brine, dried over Na_2SO_4 and the solvent removed at reduced pressure. Both batches were determined to be of

high purity according to ^{1}H-NMR analysis on a Bruker DRX 400 MHz spectrometer (Bruker, Fallanden, Switzerland) using the solvent as internal reference [$CHCl_3$ (^{1}H, 7.27 ppm), $CDCl_3$ (^{13}C, 77.23 ppm)]. The ketoalcohol (-)-**2** used as standard for high performance liquid chromatography (HPLC) analysis was produced via whole-cell yeast bioreduction of diketone **1** using TMB4100 (YMR226c, 1% PGI) and extracted and purified as described in Almqvist et al. (1993).

Construction of TMB4110 (YDR368w, 1% PGI)

The ORF YDR368w was amplified from chromosomal DNA of *S. cerevisiae* CEN.PK113-7A using the following primers, forward; 5′-GAAGATCTACCATGCCTG CTACG-3′ (*Bgl*II cleavage site underlined) and reverse; 5′-AACTGCAGCTCATTGGAAAATTGGGAAGG-3′ (*Pst*I cleavage site underlined). The amplicon was cut with *Bgl*II and *Pst*I endonucleases and ligated into the vector p423-GPD (ATCC 87355), previously cut with the same restriction endonucleases followed by transformation into *E. coli* DH5α. Recovered plasmids were transformed into *S. cerevisiae* RBY 7-1 and selected on plates containing 20 g/l fructose, 1 g/l glucose, 15 g/l agar and 7 g/l yeast nitrogen base without amino acids (Difco, Detroit, MI), supplemented with 50 mg/ml tryptophan. Successful expression in yeast transformants was determined by following the oxidation of NADPH at 340 nm in the presence of diketone **1** from crude cell extracts prepared according to (Katz et al. 2003b).

Cell growth

Yeast strains were grown in 100 ml selective medium with the appropriate amino acids in 1 l shake-flasks according to (Katz et al. 2003a). Cell mass for preparative scale and pH controlled reduction of diketone **1** was produced under sterile conditions at 30°C using a fed-batch approach. A 30-ml inoculum from a 48 h shake-flask culture was added into a 2.5 l bioreactor (BiostatA, B. Braun Biotech International, Melsungen, Germany) with constant stirring at 800 rpm, containing 0.7 l medium composing of 10 g/l fructose, 0.5 g/l glucose, 40 g/l YNB and 570 mg/l tryptophan dissolved in phosphate buffer (100 mM, pH 6.0). The aeration rate was started at 1.0 l/h and increased to 1.5 l/h when the feed was initiated. Dissolved oxygen tension (DOT) was monitored with a DOT electrode (Mettler Toledo, Grefensee, Switzerland) and the CO_2 content with a gas analyser (TanDem; Adaptive Biosystems, Luton, UK). The pH was maintained above 5.0 by the automatic addition of 3 M NaOH. The reactor was run in batch mode until all sugar and ethanol was depleted (about 20 h), which was indicated by a sharp drop in CO_2 production. The growth was subsequently switched to fed-batch mode by the continuous addition of 1.3 l media composing of 62 g/l fructose and 3.1 g/l glucose in phosphate buffer (100 mM, pH 6.0). The initial feed rate was 0.02 l/h and increased exponentially corresponding to a growth rate of 0.1 h^{-1}.

Dry weight (dw) was determined according to (Karhumaa et al. 2005).

Strain comparison for improved stereoselectivity

Comparison of strain stereoselectivity was performed in 10 ml plugged glass vials, equipped with syringes for outlet gas and sampling, containing 2 ml suspensions of 5 g/l dw yeast in citrate buffer (100 mM, pH 5.5) and a starting concentration of 5 or 20 g/l diketone **1** and 60 g/l glucose. Samples were withdrawn after 29 h, extracted with ethyl acetate and analysed with a gas chromatography (GC) system.

Bioreductions

Bioreductions were carried out at 30°C in 100 mM citric acid buffer pH 5.5 without aeration with constant agitation from magnetic stirrers in vessels equipped with syringes for outlet gas and sampling. Cells were harvested by centrifugation at 4,000×*g* for 5 min, washed twice with Milli-Q water and resuspended in citrate buffer. Batches were started by adding a suspension of yeast, corresponding to 15 g/l dw (in batch) of the final volume, to buffer solutions of 120 g/l glucose and either 40 or 50 g/l diketone **1**.

Small-scale bioreductions were performed in 5-ml scale in 10-ml plugged glass vials.

Substrate and product inhibition studies were performed in small scale using 5 g/l dw cell mass in 60-g/l glucose, 2.5–60 g/l diketone **1** and 0–80 g/l ketoalcohol **2**. Product inhibition was studied using a starting concentration of 18g/l diketone **1**. Preparative reductions were carried out in 600–625 ml scale in 1 or 2 l tempered reactors.

Reductions with pH control were performed in 50 ml buffer in 150 ml reactors. The pH was maintained at 5.5 by the addition of 5 M NaOH with a peristaltic pump controlled by a PHM 61 Laboratory pH meter (Radiometer, Copenhagen).

Samples (150–300 μl) were withdrawn at intervals with syringes, centrifuged at 13,000×*g* for 1 min and stored at −20°C. Non-sterile reaction vessels and substrates were used. However, the samples were routinely checked for contamination with a microscope, and no bacterial or fungal growth was ever observed.

After completed bioreduction, cells were removed by centrifugation at 5 000 g for 5 min and the resulting aqueous phases extracted five times with an equal volume of ethyl acetate. The organic phases were combined, washed with brine, dried over Na_2SO_4 and the solvent removed at reduced pressure. The crude product was dissolved in ethyl acetate

and filtered through a short plug of silica. Drying over night at 2 mmHg gave ketoalcohol (-)-**2** as white crystals.

Viability determination

Samples for viability determination were withdrawn every 24 h from the preparative bioreduction in 24-g (600 ml) scale, diluted in sterile distilled water and streaked on selective plates. A colony-forming-unit (CFU) count was made after 48 h of incubation at 30°C. Plates with counts between 30 and 300 CFUs were used to calculate the viability as a percentage of the count at the start of the bioreduction.

Analyses of substrates and products

Ethanol, diketone **1** and ketoalcohol **2** concentrations were measured with an HPLC system as described previously (Katz et al. 2003a). Diastereomeric purity of ketoalcohol **2** was analysed on a GC using the α-Dex column and set-up described in (Botes et al. 2002). The retention times of the peaks were as follows: diketone **1** 25.2 min, the two enantiomers of ketoalcohol **3** 39.4 and 39.9 min, respectively, ketoalcohol (+)-**2** 41.8 min and the major ketoalcohol product (-)-**2** 42.2 min. The enantiomeric excess was estimated with the same system. In some cases, ee was confirmed by Mosher ester synthesis followed by HPLC analysis (Botes et al. 2002), using a Hypersil HS silica column (Thermo Hypersil, Runcorn, UK). The retention times were as follows: ketoalcohol (+)-**2** 9.5 min, ketoalcohol (-)-**2** 11.4 min and the enantiomers of ketoalcohol **3** at 14.1 and 18.1 min, respectively.

Results

Reductase diastereoselectivity

Three *S. cerevisiae* reductases encoded by YMR226c, YDR368w and YOR120w were previously shown to catalyse the reduction of diketone **1** with high ee (Katz et al. 2003a). In the present study, the reductases were compared for conversion yield and diastereoselectivity in whole-cell reductions with 5g/l dw cells and 5- or 20-g/l diketone **1** (Table 1). High enantioselectivity was confirmed for all three strains with only one peak visible on GC. Whole-cell reductions with yeast over-expressing YDR368w and YOR120w also led to high diastereoselectivity (>95% de). In contrast, lower de was obtained with the strain over-expressing YMR226c. Moreover, the diastereoselectivity was negatively affected by increasing substrate concentrations. However, a higher conversion was achieved compared to the other strains at high substrate concentration. Due to its high diastereoselectivity and reasonably high conversion rate, YDR368w was selected for further evaluation and was over-expressed in a strain with a decreased PGI activity, generating strain TMB4110.

Substrate and product inhibition

The sensitivity of TMB4110 (YDR368w, 1% PGI) towards the substrate diketone **1** was evaluated by expressing the initial reduction rate as a function of substrate concentration, displayed as percentage of the highest empirical rate (Fig. 2). Strain TMB4100 (YMR226c, 1% PGI) was included for comparison. Similar inhibition patterns were obtained for both strains, and the data could be adapted to a common Han-Levenspiel substrate inhibition model (Han and Levenspiel 1988):

$$R(S) = \frac{R_{\max} \times S}{K_S + S} \times \left(1 - \frac{S}{S_C}\right)^n \quad (1)$$

where $R(S)$ is the reaction rate expressed as a function of the substrate concentration S, $R_{\max}$ the maximum specific rate, K_s the half saturation constant, S_c the critical substrate concentration at which the reaction is completely inhibited and n the inhibition coefficient. By using the least square method, Eq. 1 was fitted to the experimental data (R^2=0.97) giving the following constants: K_S=9.81, $R_{\max}$=210, S_c=68.5 and n=1.11. The obtained parameters were used to plot the substrate inhibition curve (Fig. 2) and to calculate the substrate concentration at the theoretical maximum reduction rate, where the derivative of $R(S)$, $R'(S)$=0. The

Table 1 Comparison of recombinant *S. cerevisiae* strains with respect to diastereoselectivity for the reduction of diketone **1** using 5-g/l dw yeast with either 5- or 20-g/l diketone **1**

		5 g/l **1**		20 g/l **1**	
Strain	Overexpressed ORF	Conversion of diketone **1**	de %	Conversion of diketone **1**	de %
TMB4093	YDR368w	98%	99.3	66%	97.4
TMB4096	YOR120w	98%	98.2	47%	95.4
TMB4091	YMR226c	98%	90.1	80%	84.3

Reactions were stopped after 29 h.

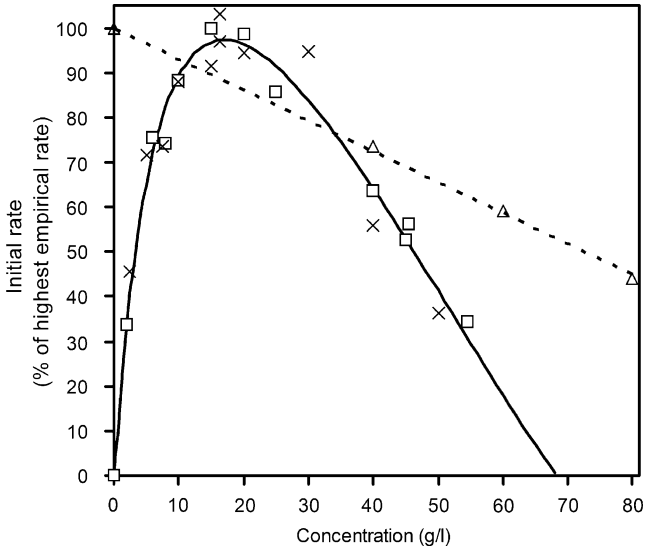


Fig. 2 Substrate and product inhibition models for the reduction of diketone **1** by *S. cerevisiae*. The Han-Levenspiel substrate inhibition model (*full line*) was adapted after empirical initial reaction rates of *S. cerevisiae* strains TMB4100 (YMR226c, 1% PGI; *open square*) and TMB4110 (YDR368w, 1% PGI; *cross mark*), plotted as a function of initial diketone **1** concentration. Product inhibition model (*dotted line*) is inferred from empirical initial reduction rates of *S. cerevisiae* strain TMB4100 (YMR226c, 1% PGI; *open triangle*) in reductions starting with 18 g/l diketone **1** and varying concentrations of ketoalcohol (-)-**2**

maximum rate was found at a substrate concentration of 17.0 g/l, and an effective range in which the reaction rate stayed above 95% could be inferred between 12 and 22 g/l of diketone **1**. The influence of purification method of diketone **1** was studied by comparing two different batches (see "Materials and methods"), for the reductions of 40 g/l diketone **1** using strain TMB4110 (YDR368w, 1% PGI). However, no significant difference was detected.

The inhibitory effect of product ketoalcohol (-)-**2** was also investigated. A linear decrease in activity was observed with increasing concentration of ketoalcohol (-)-**2**. Using the obtained data, the linear trend line (R^2=0.999) described in Eq. 2 was inferred:

$$R(P) = -0.6898 \times P + 100 \quad (2)$$

where $R(P)$ is the reduction rate as a function of the product concentration P.

Selecting parameters for bioreductions

The influence of initial cell biomass was investigated with small-scale bioreduction experiments (0.2 g diketone **1**) using 10g/l diketone **1** and 5-, 15- and 30-g/l dw yeast. The total amount of substrate plus product decreased at 30g/l dw (data not shown), therefore 15g/l dw was subsequently used for all bioreductions. In addition, the solubility of diketone **1** was found to be 67±4 g/l at room temperature (data not shown). Consequently, the feed concentration was limited to 60 g/l in all fed-batch experiments to (1) avoid local precipitation and (2) reach as high product concentration as possible.

Influence of scale, pH and yeast cultivation method on bioreductions

The performance of *S. cerevisiae* strain TMB4110 (YDR368w, 1% PGI) in the reduction of diketone **1** was compared in batch and fed-batch mode at small-scale, with shake flask grown cells. However limited advantages were achieved in terms of final product titre and co-substrate yield with the more advanced set-up of the fed-batch mode (data not shown). The possibility of using two-phase system for the bioreduction of diketone **1** was also evaluated. However, the hydrophilic nature of diketone **1** and ketoalcohol (-)-**2** favoured the equilibrium towards the aqueous phase in the two-phase systems (data not shown). Moreover, the best extractants had extremely poor biocompatibility. Thus, no solvent combining good extracting properties for both substrate and product and high biocompatibility was identified (data not shown). Consequently, a batch set-up was chosen for further evaluation.

Batch reduction of diketone **1** with TMB4110 (YDR368w, 1% PGI) was first evaluated at a substrate concentration of 50 g/l, but complete conversion was not achieved. Instead, full conversion (>99%) was obtained in 35 h by lowering the concentration to 40 g/l (Table 2). Furthermore, the influence of scale was investigated by comparing reduction of 40g/l diketone **1** in two large- (24 and 25 g of diketone **1**) and one small-scale (0.2 g) experiments.

Table 2 Summary of bioreductions of diketone **1** with *S. cerevisiae* strain TMB4110 (YDR368w, 1% PGI)

Scale (comment)	Substrate titre (g/l)	Conversion of **1** (%)	Glucose consumed (g/l)	Conversion time (h)	Stereoselectivity de (%)
Shake-flasks grown cells					
Small scale	50	64	47	65	94.5
Small scale	40	>99	104	35	96.9
Fed-batch grown cells					
Preparative scale	40	75	101–120	88–97	95.3
Small scale	40	76	95	97	95.1
Small scale (pH control)	40	>99	128	72	96.7

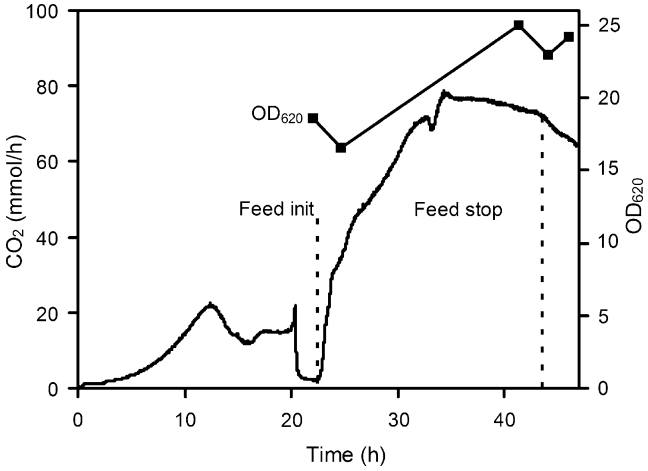


Fig. 3 CO_2 production profile during fed-batch growth of TMB4110 (YDR368w, 1% PGI) cells. Optical density (OD_{620}; *filled square*). First 20 h batch growth. Exponentially increasing feed initiated at 22.5 h and stopped at 43.5 h

For adequate biomass generation, large amount of yeast was prepared using a fed-batch cultivation mode. For comparison, shake-flask grown cells were prepared in parallel. The end dry weights of the fed-batch culture broths were all about 5 g/l, corresponding to 0.1 g biomass/g sugar. However, growth problems were observed, starting with a decrease in the CO_2 evolution rate after 34 h (Fig. 3).

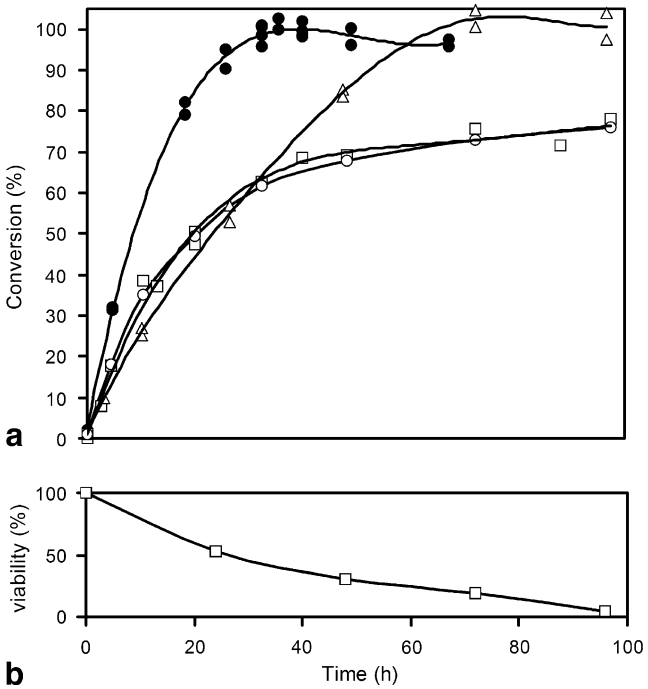


Fig. 4 Batch reductions of 40 g/l diketone **1** using *S. cerevisiae* TMB4110 (YDR368w, 1% PGI). *Full symbols*; shake-flask grown cells. Empty symbols; Fed-batch grown cells. **a** 0.2 g diketone **1**, buffered reductions (*filled circle*, *open circle*). 24 and 25 g diketone **1**, buffered reductions (*open square*). 0.2 g diketone **1**, pH maintained at 5.5 (*open triangle*). **b** Cell viability of 24 g diketone **1** reduction (*open square*)

An analysis of culture broth revealed accumulation of fructose (8–16 g/l) and ethanol (30–50 g/l), but no glucose and only little acetate (0.2–0.5 g/l) and glycerol (3 g/l).

In subsequent reductions, the influence of the cell cultivation mode was evident. Reductions with fed-batch grown yeast were considerably slower than the shake-flask grown counterparts, and full conversion was never reached (Fig. 4a). However, no significant difference could be detected between the large- and the small-scale experiments using fed-batch grown cells.

A decreased pH, ending at between 4 and 5, was observed at the end of these long reductions. An investigation of cell viability revealed a steady decline of viability throughout the reduction (Fig. 4b). A production of acetate by the yeast and the associated drop in pH was suspected to affect the activity. This was confirmed when the reduction activity was prolonged by maintaining the pH at 5.5. (Fig. 4a). Full conversion was reached in 72 h giving crude product in 97% de and >99% ee, confirmed by Mosher ester analysis. Normal workup (as described in "Materials and methods") gave ketoalcohol (-)-**2** in 84% yield without need for further purification.

Discussion

The bioreduction of diketone **1** to ketoalcohol (-)-**2** reported herein represents, to the best of our knowledge, the best result described in literature with respect to stereoselectivity, product titre and isolated yield. By the use of a recombinant strain and a pure substrate, a product titre of 40 g/l was reached, which is an improvement with over 300% compared to reductions reported with baker's yeast (Mori and Nagano 1990; Almqvist et al. 1993). Moreover, the over-expression of the reductase gene YDR368w increased the diastereomeric excess of the crude product from 70–92 to 97% and the enantiomeric excess from 92–96 to >99% compared to previous studies (Mori and Nagano 1990; Almqvist et al. 1993; Almqvist 1996; Katz et al. 2003a). The high stereoselectivity, conversion and product titre taken together with the relatively low amounts of yeast used, contributed to a significantly facilitated workup and the isolated yield of ketoalcohol (-)-**2** could consequently be improved from the previously obtained 48–59% (Mori and Nagano 1990; Almqvist et al. 1993; Almqvist 1996) to 84%.

Three enantioselective enzymes had previously been identified as being responsible for the stereoselective conversion of diketone **1** to ketoalcohol (-)-**2** in *S. cerevisiae* (Katz et al. 2003a). However, data on diastereoselectivity had not been reported. Our study indicated that the YDR368w over-expressing strain displayed the highest diastereoselectivity and also only slightly lower conversion than the previously identified strain over-

expressing YMR226c (Katz et al. 2003a). Consequently, the YDR368w gene product was chosen as the most suitable reductase for exploring the whole-cell bioreduction of diketone **1**. The generation of ketoalcohol **3** by cells over-expressing YMR226c, had not previously been reported (Katz et al. 2003a). The reason could be the low substrate concentration used in previous studies in combination with the use of an HPLC/refractive index detector system, which is significantly less sensitive than the GC-FID system used in the present work. We also observed that the substrate concentration significantly affected diastereoselectivity in the strain over-expressing YMR226c.

Clear benefits with regard to glucose requirements are achieved when reducing PGI activity in whole-cell yeast bioreductions (Katz et al. 2002, 2003a). PGI works as a shunt between the pentose phosphate pathway (PPP) and the glycolysis; therefore, strains with reduced PGI activity display a restricted *in vivo* glucose consumption rate, which significantly lower co-substrate requirement (Katz et al. 2002). However, strains with altered PGI level grow poorly if not provided with a well-balanced mixture of fructose and glucose to supply metabolites for both pathways (Aguilera 1986). Our fed-batch yeast cultivation indeed indicated that further optimisation would be required for large-scale cultivation of strains with reduced PGI activity.

Substrate inhibition has previously limited the bioreduction of diketone **1** by recombinant yeast strains. Both reduction rate and metabolism were reported to gradually decrease at concentrations above 5 g/l, ending with an almost complete stop at 16 g/l (Katz et al. 2002). On the contrary, we observed very high yeast reduction rates above 30 g/l for strains with similar background. One difference between the two studies concerned the substrate preparation. In the previous study, diketone **1** was produced by the method of Almqvist et al. (1993), and reported to be of only >90% purity, whereas an improved method (Widegren et al. 2006) and an additional purification step were used in the present study. It is conceivable that inhibitory hydrophilic side products generated in the synthesis of diketone **1** may have been present in earlier work. We therefore recommend that acidic residues from the reagents in the synthesis of diketone **1** are removed by washing with sodium hydrogen carbonate. The comparison of the inhibition patterns of the two strains expressing YDR368w and YMR226c revealed a similar response to elevated substrate concentrations, indicating a general inhibition of yeast rather than specific enzymatic inhibition. The reduced glucose consumption observed at higher substrate concentrations also pointed in the same direction.

In addition to batch experiments, two-phase and fed-batch systems were evaluated as means to improve yield and product titre by decreasing the substrate/product inhibition. However, no solvent combining good extracting properties for both substrate and product and high biocompatibility was identified for the two-phase systems. In addition, the fed-batch approach showed little difference compared to the batch mode whilst requiring careful fine-tuning of the feed rate as well as numerous experiments for optimisation. Therefore, the batch mode was found to be the more suitable set-up for bioreduction of diketone **1**. In batch mode, a difference of two orders of magnitude in scale did not influence the reaction rate, whereas pH was shown to be very important for the yeast activity. Using pH control, it was possible to reach full conversion with high stereoselectivity, even when using fed-batch grown cells. In conclusion, the excellent stereoselectivity, high product titre and convenient workup described herein make this process well suited for laboratory scale production of pure ketoalcohol (-)-**2**.

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