Journal of Molecular Catalysis B: Enzymatic 59 (2009) 286–291

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Identification of a *Candida* sp. reductase behind bicyclic exo-alcohol production

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ARTICLE INFO

Article history:
Received 14 May 2008
Received in revised form 2 November 2008
Accepted 6 November 2008
Available online 14 November 2008

Keywords:
Bioreduction
Bicyclic diketone
Exo-alcohol
Candida albicans
Candida tropicalis
Membrane reductase
IPF4033
AYR1

ABSTRACT

Stereoselective baker's yeast-catalysed bioreduction of bicyclo [2.2.2]octane-2,6-dione generates (1R, 4S, 6S)-6-hydroxy-bicyclo [2.2.2]octane-2-one (endo-alcohol) with high enantiomeric and diastereomeric excess. In contrast, whole cells and crude membrane fractions of *Candida* sp. have been reported to produce the unusual (1R, 4S, 6S)-diastereomer (exo-alcohol) as a major product. Previous *in silico* screening has identified seven membrane or membrane-bound reductases in *C. albicans* as candidates for the exo-activity. In this work, purification of the corresponding exo-reductase(s) as well as the heterologous cloning of the seven candidate genes was attempted in *C. tropicalis*. The overexpression of IPF4033 (*AYR1*) gene generated an increased exo-to-endo ratio and exo-alcohol production in whole cells and membranes of *C. tropicalis*. In addition, a slight increased exo-to-endo ratio was observed when overexpressing IPF4033 in *S. cerevisiae*, although the reduction rate and exo-to-endo ratio were several fold lower compared to those obtained with *C. tropicalis*.

1. Introduction

Whole-cell microbial reduction of pro-chiral carbonyl compounds is a simple way to generate chiral building blocks for the production of drugs and fine chemicals [1]. One example is the yeast-catalysed stereoselective bioreduction of the bicyclic diketone bicyclo[2.2.2]octane-2,6-dione (BCO2,6D) to its corresponding ketoalcohols (1R, 4S, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one (endo-alcohol) and (1S, 4R, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one (exo-alcohol) (Figure 1) [2–4]. The produced ketoalcohols have applications as starting material for ligands of transition-metal based chiral chemical catalysts [5–7] and as synthons for taxane analogues [8].

Whereas the endo-alcohol has been obtained with high yield and enantiomeric excess (ee) using wild type and engineered baker's yeast *Saccharomyces cerevisiae* [9,10], only minor amounts of exo-alcohol have been detected [2,10,11]. A screening of yeast species has enabled the identification of several non-conventional yeasts, among others *Candida* sp. that formed the exo-alcohol as a major product [4]. In a follow-up study, the NADPH dependent exo- and endo-activities of *C. tropicalis* were shown to be separable after treatment with the mild detergent-based protein extracting reagent Y-PER resulting in a cytosolic supernatant fraction containing the endo-activity and a remaining crude cell debris fraction displaying exo-activity [12]. This led to the conclusion that the reductase responsible for the exo-activity in *C. tropicalis* likely was membrane-bound or membrane-associated [12].

An *in silico* screening was subsequently conducted to find candidate genes of the hypothetical exo-reductase in *Candida* sp. [12]. *Candida* sp. homologues of NADPH-dependent *S. cerevisiae* oxido-reductases were identified using the blast program and the resulting candidates screened for putative transmembrane helices as an indication of membrane origin. This narrowed down the putative NADPH-dependent membrane reductases to 8 in the partially sequenced *C. tropicalis* and 7 in the fully sequenced *C. albicans*, each containing 1–7 putative transmembrane regions [12].

The present study aimed at the identification of exo-reductase activity from *Candida* sp., by (i) purification of membrane or membrane-bound exo-reductase(s) from *C. tropicalis* and (ii) cloning and expression in *C. tropicalis* of candidate genes from *C. albicans,* and investigation of their putative exo-reductase activity.

2. Experimental

2.1. Chemicals

BCO2,6D was synthesised as described previously [13]. Crude BCO2,6D was purified by silica gel chromatography (MATREX, 25–70 μm), re-crystallised and estimated to be of high purity

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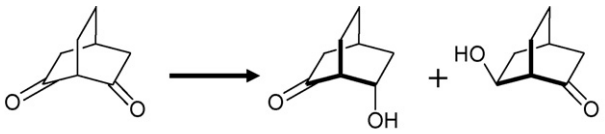
doi:10.1016/j.molcatb.2008.11.002



BCO2,6D endo alcohol exo alcohol

Figure 1. Bioreduction of bicyclo[2.2.2]octane-2,6-dione (BCO2,6D) to (1R, 4S, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one (endo-alcohol) and (1S, 4R, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one (exo-alcohol) by *S. cerevisiae* and *Candida sp.*

on a DRX400 NMR spectrophotometric system (Bruker, Fallanden, Switzerland) with solvent as internal reference [$CHCl_3$ (^{1}H, 7.27 ppm), $CDCl_3$ (^{13}C, 77.23 ppm)]. Exo-alcohol was produced using the crude membrane fraction of *C. tropicalis* CBS094, as described in "*in vitro* bioreduction". The product was extracted with ethyl acetate and purified with silica gel chromatography. 5-α-androstane-3,17-dione was purchased from Sigma–Aldrich (St Louis, Missouri) and the corresponding 3,17-diol was synthesised as described previously [12]. *D*-glucose was purchased from Sigma–Aldrich (St Louis, Missouri). Yeast protein extraction reagent Y-PER was purchased from Pierce (Rockford, Illinois).

2.2. Enzyme, co-factor and co-substrate

Glucose-6-phosphate dehydrogenase (G6PDH) (E.C. 1.1.1.49, Torula yeast, NADPH dependent), *D*-glucose-6-phosphate (G6P) and $NADP^+$ were purchased from Sigma–Aldrich (St Louis, Missouri).

2.3. Strains and plasmids

Escherichia coli DH5α was purchased from Life Technologies (Rockville, MD). *C. tropicalis* CBS094 (diploid strain) was a gift from Professor Martha S. van Dyk, University of the Free State, Bloemfontein, South Africa. *C. tropicalis* SU-2 [*ura3a*/*ura3b*] was purchased from the ATCC collection at LGC Promochem (ATCC number 20913) (Middlesex, UK). *S. cerevisiae* CEN.PK 113-7A [*MAT a, MAL-8c*; *SUC2*; *his3-Δ1*] was a gift from Dr P. Kötter, Institute of Microbiology, Frankfurt, Germany. *S. cerevisiae* strains TMB4091 overexpressing YMR226c, TMB4102 overexpressing YIL124w and the control TMB4103 with an empty plasmid were constructed previously [12,14]. Shuttle vector pRM2 was purchased from the BCCM/LMBP collection (BCCM/LMBP) and p423ADH [15] (ATCC number 87371) was obtained from LGC Promochem (Middlesex, UK).

2.4. Cell growth

Yeast strains were kept at −80 °C and streaked on plates containing 20 g/l glucose, 7 g/l yeast nitrogen base, 15 g/l agar and 100 mM phosphate buffer pH 6.0 except *C. tropicalis* CBS094, which was streaked on 20 g/l glucose, 20 g/l tryptone, 10 g/l yeast extract and 15 g/l agar. Colonies were taken from the agar plates with sterile cotton applicators and used to inoculate 100 ml of the same agar free medium. Yeast was grown in 1000 ml shake flasks at 30 °C and 200 rpm until an OD_{620} of 15–20 or stationary phase, corresponding to about 24 h. Cells were harvested by centrifugation at 4000 g for 5 min and washed twice with sterile Milli-Q water. Cell concentration was determined by dry weight according to [16] or estimated by optical density (OD_{620nm}) on a Hitachi U-1800 spectrophotometer.

2.5. Nucleic acid manipulations

C. albicans genomic DNA (ATCC number 10231D) was obtained from LGC Promochem (Middlesex, UK). DNA polymerase *Pwo* was purchased from Roche (Basel, Switzerland). Plasmid DNA was isolated using the Biorad miniprep kit (Hercules, CA). DNA processing enzymes were obtained from Fermentas (St. Leon-Rot, Germany) or Life Technologies (Rockville, MD). DNA purification from PCR reactions and agarose gels was made using the QIAquick PCR cleanup and Gel extraction kits, respectively and recovery of oligonucleotides with the QIAquick Nucleotide removal kit (Qiagen, Hilder, Germany). Competent cells of *E. coli* were prepared using the $CaCl_2$ method [17] and transformed according to [18]. *E. coli* transformants were grown overnight and selected on Luria-Bertani (LB) medium plates [19] containing 100 μg/ml ampicillin (IBI Shelton Scientific Inc., Shelton, CT). *C. tropicalis* SU-2 was transformed using the lithium acetate procedure developed for *C. albicans* [20]. *S. cerevisiae* strains were transformed according to [21]. Yeast transformants were selected on plates containing 20 g/l glucose, 7 g/l yeast nitrogen base without amino acids (Difco, Detroit, MI) and 15 g/l agar.

2.6. Construction of plasmid pTJ1

Oligonucleotides For_MCS and Rev_MCS (Table 1) were dissolved in distilled water with a total DNA concentration of 0.44 μg/μl. The solution was boiled for 5 min, incubated at 72 °C for another 5 min and allowed to cool down to room temperature. The double-stranded DNA was restricted with *Hind*III and *Pst*I, purified and ligated with plasmid pRM2 that was previously cut with the same restriction enzymes. The ligation mixture was used to transform *E. coli* DH5α. Plasmids were recovered from the transformants and successful cloning confirmed by digestion analysis. The resulting plasmid was named pTJ1.

2.7. Construction of recombinant C. tropicalis strains

ORFs IPF1839, IPF4033, IPF6600, IPF11105 and IPF15523 were amplified from *C. albicans* genomic DNA using specific primers containing suitable restriction sites (Table 2). For IPF1839, IPF11105 and IPF15523, plasmid pRM2 was digested using corresponding restriction enzymes and ligated with the PCR products yielding pRM2-1839, pRM2-11105 and pRM2-15523 respectively. For cloning of the other ORFs, plasmid pTJ1 was digested using corresponding restriction enzymes and ligated with the PCR products yielding pTJ1-4033 and pTJ1-6600. The resulting plasmids and the control plasmid pRM2 were used to transform *C. tropicalis* SU-2, resulting in strains TMB8000 (empty pRM2 vector), TMB8001 (overexpressing IPF4033), TMB8002 (overexpressing IPF6600), TMB8003 (overexpressing IPF11105), TMB8004 (overexpressing IPF15523) and TMB8005 (overexpressing IPF1839).

Table 1
Oligonucleotides and restriction sites for the construction of the multiple cloning site of pTJ1.

Oligo-nucleotide	Sequence (restriction sites underlined)
For_MCS	5′-GGC**AAGCTTGGGCCC**T**AGATCT**C**ATCGATGGTACCGAGCTC**A**TCTAGA**G**CTCGAG**T**CTGCAG**GGC-3′
	*Hind*III *Apa*I *Bgl*II *Cla*I *Kpn*I *Sac*I *Xba*I *Xho*I *Pst*I
Rev_MCS	3′-CCG**TTCGAACCCGGG**A**TCTAGA**G**TAGCTACCATGGCTCGAG**T**AGATCT**C**GAGCTC**A**GACGTC**CCG-5′

Table 2
Primers and restriction sites for the construction of *C. tropicalis* strains overexpressing *C. albicans* reductase open reading frames (ORFs).

Primer	Amplified ORF	Restriction site	Primer (restriction site underlined)
IPF1839-*Pst*I	IPF1839	*Pst*I	5′-ACGCTGCAGGCAATTGGGCTAAGATACGTAAG-3′
IPF1839-*Sal*I	IPF1839	*Sal*I	5′-CGTGTCGACGTGTTTTCTCAAGATATGGCACC-3′
IPF4033-BglII	IPF4033	*Bgl*II	5′-GACAGATCTGAAGTCGGAATAGAATGCACCG-3′
IPF4033-*Sal*I	IPF4033	*Sal*I	5′-CTGGTCGACCCATAATGAAGGATGAGCTGCC-3′
IPF6600-*Sal*I	IPF6600	*Sal*I	5′-GATCGTCGACAGGAAATGATACAGCAGGTTCG-3′
IPF6600-BglII	IPF6600	*Bgl*II	5′-CATCAGATCTAGTATCGACCTATTGTGCGTGAG-3′
IPF11105-*Pst*I	IPF11105	*Pst*I	5′-AGCCTGCAGTATGCCAAGTCTCAGTCATCAAC-3′
IPF11105-*Sal*I	IPF11105	*Sal*I	5′-GCTGTCGACATCTACCAGTAATTCACGAGCG-3′
IPF15523-*Pst*I	IPF15523	*Pst*I	5′-ACGCTGCAGCGATCCCTGATTTCTCCCCACT-3′
IPF15523-*Sal*I	IPF15523	*Sal*I	5′-CGTGTCGACATCAGCTCCTTCTTTACTTGTGAC-3′

2.8. Construction of recombinant S. cerevisiae TMB4111

C. albicans ORF IPF4033 was amplified from pTJ1-4033 and adapted for *S. cerevisiae* expression by changing the *Candida* serine codon CTG to the universal serine codon TCG in the C-terminal primer. For this purpose, the following primers (with restriction sites underlined and modified codon in bold) were used: Forward 5′-GAG AAT TCG CAT GTC AGA ACG TCA AAA GGT TGC ATT AG-3′ and Reverse 5′-CGA CGT GTC GAC CTA ATT AAC TTT CTC TTT **CGA** ATA CTT TTC ATG C-3′. The amplicon was cut and ligated into the *Eco*RI and *Sal*I sites of plasmid p423ADH, resulting in plasmid p423GPD-IPF4033. Further transformation of *S. cerevisiae* CEN.PK113-7A resulted in strain TMB4111.

2.9. In vivo bioreduction

Anaerobic whole-cell bioreductions were performed at 30 °C in 5 ml, magnetically stirred, plugged glass vials equipped with syringes for outlet gas and sampling. The reaction medium consisted of 50–100 g/l glucose and 5 g/l BCO2,6D (or 1 g/l androstanedione) in citrate buffer (100 mM, pH 5.5). An initial cell concentration of OD_{620} = 5 was used for *C. tropicalis* reductions, whereas *S. cerevisiae* reductions were carried out either at OD_{620} = 5 (i.e 0.8 g/l cell dry weight (cdw)) or OD_{620} = 30 (=5 g/l cdw). Screening for exo-alcohol generating mutants of *C. tropicalis* was performed in 2–5 replicates in 2 ml bioreductions.

2.10. In vitro bioreduction

Crude membrane and crude cytosolic fractions were obtained as described previously [12]. Bioreductions of BCO2,6D using cell extracts were performed in phosphate buffer (100 mM, pH 7.0) in 0.26–2 ml scale by adding 2 U/ml glucose-6-phosphate dehydrogenase, 12.5 mg/ml glucose-6-phosphate, 0.75 mg/ml $NADP^+$ and 5 mg/ml BCO2,6D (or 1 mg androstanedione) to the cell extracts. Incubation was performed at 30 °C and the vials were mixed on a rocking table (≤1 ml scale) or agitated with magnetic stirrers in small flasks (>1 ml scale). Samples for analysis were taken in 100 μl aliquots.

2.11. Analyses of substrates and products

BCO2,6D, endo-alcohol and exo-alcohol concentrations were measured with HPLC as described previously [9]. Stereoisomeric analyses and diastereomeric ratios of endo- and exo-alcohol were performed on GC with an α-Dex column as described in [4]. The retention times of the peaks were as follows: BCO2,6D 25.2 min, the two exo-alcohol enantiomers 39.4 min and 39.9 min, the two endo-alcohol enantiomers 41.8 min and 42.2 min. Bioreduction of androstanedione was evaluated using thin layer chromatography (TLC) on a silica plate (60 F254) using toluene/acetone 4:1 as mobile phase, developed with Seebach solution (25 g phosphomolybdic acid, 10 g cesium(IV) sulfate, 60 ml concentrated sulphuric acid and 940 ml water).

3. Results and discussion

3.1. Cloning of putative C. albicans exo-reductases in C. tropicalis

For the expression and evaluation of the seven previously identified exo-reductase candidates from *C. albicans* [12], the uracil auxotrophic *C. tropicalis* strain SU-2 was chosen as expression host to avoid the need for mutation of the non-conventional CUG codon used by *C. albicans* and *C. tropicalis*. First, uracil prototrophy was restored by transformation with the *C. albicans* plasmid pRM2. In addition, plasmid pTJ1 was constructed to facilitate cloning work by inserting a multiple cloning site (Table 1) between the *Hind*III and *Pst*I restriction sites in plasmid pRM2.

In order to overexpress each ORF in the presence of its original promoter and terminator regions, the *C. albicans* ORFs and flanking regions of 500–700 base pairs (bp) upstream and 200–500 bp downstream were amplified by PCR. The amplified segments were cut and ligated into either plasmid pRM2 (for ORFs IPF1839, IPF11105 and IPF15523) or pTJ1 (for ORFs IPF4033, IPF6600, IPF7602 and ARD8). The plasmids carrying ORFs IPF4033, IPF6600, IPF11105, IPF15523, IPF1839 were successfully recovered and used to transform *C. tropicalis* SU-2. The resulting strains were named TMB8000 (control), TMB8001 (IPF4033), TMB8002 (IPF6600), TMB8003 (IPF11105), TMB8004 (IPF15523) and TMB8005 (IPF1839). Despite repeated attempts using different primers and restriction sites, ORFs IPF7602 and ARD8 could not be obtained as non-truncated inserts in pTJ1.

3.2. Screening for exo-activity

Recombinant *C. tropicalis* strains TMB8001, TMB8002, TMB8003, TMB8004, TMB8005 and the control strain TMB8000 were screened for increased exo-activity by comparing the exo-to-endo product ratios in small scale bioreductions of BCO2,6D at 30 °C. After 20 h, strain TMB8001, carrying the ORF IPF4033, had a significantly higher exo-to-endo ratio (0.62) than the other strains, which all were within the 95% confidence limits of each other (Fig. 2). However lower conversion of BCO2,6D was observed for TMB8001 (40%) compared to the control strain (51%) (data not shown).

The exo-to-endo ratio in TMB8001 (IPF4033) was further evaluated by comparing *in vitro* reductions of crude membrane and cytosolic fractions with those of the control strain TMB8000. The reductions were carried out with 5 g/l BCO2,6D for 24 h using glucose-6-phosphate dehydrogenase, glucose-6-phosphate and $NADP^+$ for co-factor regeneration. A clear increase in exo-to-endo ratio was observed in the crude membrane fractions of the IFP4033 overexpressing strain, whereas only minor exo-alcohol

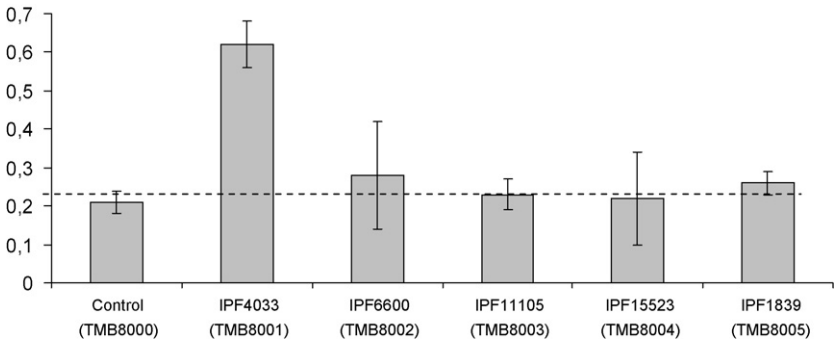


Figure 2. Exo-to-endo ratios from the screening of *C. tropicalis* strains over-expressing putative *C. albicans* exo-reductases. Reductions were performed at 30 °C with whole cells corresponding to an OD_{620} of 5 using 5 g/l BCO2,6D. Samples were taken after 20 h. Error bars: 95% confidence limits. Dotted line marks exo-to-endo ratio 0.23, which was within the 95% confidence limits of all strains, except TMB8001 (overexpressing IPF4033).

production was observed in the cytosolic fractions (Table 3). These results clearly indicated that IPF4033 encodes a membrane or membrane bound protein involved in the reduction of BCO2,6D to the exo-alcohol product.

The conversion rate was again lower in the strain expressing IPF4033 for both the cytosolic and the membrane fractions (Table 3), which suggested that the overexpression of IFP4033 gene affected the expression levels or activity of other BCO2,6D reductases in *C. tropicalis*. No information on the effect of IFP4033 overexpression on cell metabolism is currently available. However it has been shown that a 5–10 times overexpression of *S. cerevisiae* YIL124w, which is the closest homolog to IFP4033 (54% identity and 68% similarity at the protein level), caused growth arrest in *S. cerevisiae*, indicating a severe stressing effect on the cell [22].

3.3. Exo-to-endo ratios vs. conversion in C. tropicalis

The above experiments with the *C. tropicalis* strains overexpressing IPF4033 and an empty plasmid showed that the exo-to-endo ratio in BCO26D reductions increased with time (data not shown). When plotting the exo-to-endo ratio against the fraction of converted BCO2,6D, the exo-to-endo ratio for both strains was shown to be almost linearly dependent on the substrate conversion, increasing with the conversion (Fig. 3). The increased ratio in the IPF4033 expressing strain compared to the control, which was observed throughout the conversion (Fig. 3), originated from both increased exo-alcohol and reduced endo-alcohol production (data not shown).

Time or conversion dependent enantioselectivity has already been observed in whole-cell bioreduction of racemic bicyclo[2.2.2]-oct-7-ene-2,5-dione with recombinant *S. cerevisiae* [23]. In that case, the decreasing ee of one of the formed ketoalcohol diastereomers was caused by the further reduction of its dominating enantiomer to a diol. Conversion dependence can occur whenever two or more independent asymmetric catalysts, with differences in kinetic profiles, compete for the same substrate [24]. In our case, this can correspond to two enzymes with different affinity constants and reaction rates, where the endo-reductase(s) have lower affinity for BCO2,6D than the exo-reductase(s). This would favour exo-alcohol production with decreasing BCO2,6D level. The conversion dependent selectivity may also arise from the late induction of exo-reductase gene expression or the repression (or inactivation) of endo-reductase(s) gene expression over time. A possible induction of IPF4033 during whole-cell bioreduction may for instance result from glucose depletion over time. In fact, the *S. cerevisiae* homologue YIL124w has been shown to be involved in the biosynthesis of phosphatidic and phospholipid acids [22] that are key intermediates in glycerolipid biosynthesis [25] and glycerolipids have been reported to accumulate during the stationary growth phase [26]. The time or conversion dependent stereoselectivity highlights the need to perform time-course experiments in order to identify when optimal stereoselectivity is obtained.

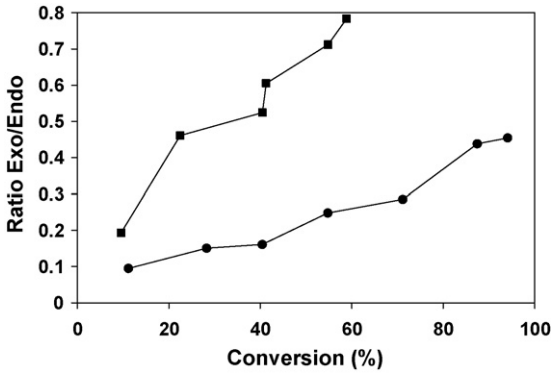


Figure 3. Exo-to-endo ratios plotted against conversion of 5 g/l BCO2,6D for *C. tropicalis* SU-2 carrying *C. albicans* IPF4033 (■) and the control plasmid pRM2 (●). Cell concentrations corresponding to OD_{620} = 5 were used.

Table 3
Exo-to-endo ratio after 24 h reductions with crude membrane and cytosolic fractions of *C. tropicalis* strain TMB8000 (control) and TMB8001 (overexpressing IPF4033).

	Conversion	Exo-to-endo ratio
Crude membrane fractions		
Control (TMB8000)	92%	0.30
IPF4033 (TMB8001)	46%	0.49
Crude cytosolic fractions		
Control (TMB8000)	28%	0.064
IPF4033 (TMB8001)	13%	0.075

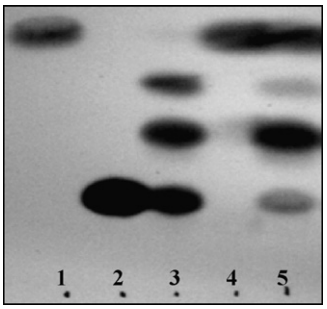


Figure 4. Reduction of androstanedione with whole-cell recombinant *S. cerevisiae* expressing *AYR1* gene from *S. cerevisiae* (YIL124w) or *C. albicans* (IPF4033). Lane 1 and lane 2: reference compounds androstanedione and androstanediol, respectively. Lane 3: YIL124w, lane 4: empty plasmid, lane 5: IPF4033.

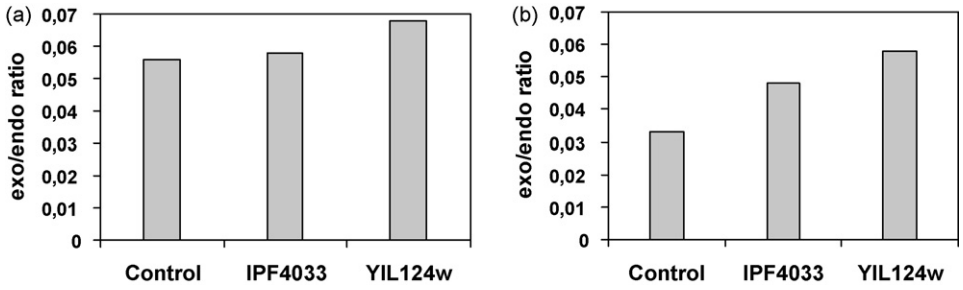


Figure 5. Exo-to-endo ratios from *S. cerevisiae* strains overexpressing *AYR1* gene from *C. albicans* (ORF IPF4033) and *S. cerevisiae* (ORF YIL124w). Reductions were performed at OD_{620} = 5 (a) and OD_{620} = 30 (b) with 5 g/l BCO2,6D. Samples were taken after 24 h.

In this case the generation of large amount of endo-alcohol during the whole conversion prohibited the use of *C. tropicalis* expressing IPF4033 as an efficient strain for the production of pure exo-alcohol. To improve the exo-to-endo ratio one possibility would be to increase the conversion using reactor engineering. However, this was not deemed worthwhile as the endo activity would have to be nearly completely removed in order to obtain an exo-alcohol product of sufficient purity for easy work-up and crystallization. This prerequisite was not considered realizable using whole-cells of TMB8001 (IPF4033). Only with *C. tropicalis* cell debris separated from the cytosol, has exo-alcohol been produced without the co-generation of significant amounts of endo-alcohol [12]. Another option would be to identify and delete the endo-alcohol producing enzymes. However, this is not a trivial task. *C. tropicalis* harbours many potential endo-reductases [27], is diploid and lacks many of the genetic tools available in *S. cerevisiae*, which makes multiple gene knockouts difficult.

3.4. Expression of IPF4033 in S. cerevisiae and assessment of exo-alcohol production

S. cerevisiae has the advantage that the reductases responsible for endo-alcohol generation are known [9] and that convenient tools for the deletion of genes are readily available [29]. In addition, *S. cerevisiae* shows higher tolerance to BCO2,6D compared to *C. tropicalis* (data not shown) and recombinant strains with efficient NADPH regeneration and low glucose consumption are available [9,30]. Therefore, expression of IPF4033 was attempted in *S. cerevisiae* to assess whether exo-alcohol production could be achieved in this host.

The *C. albicans* IPF4033 gene was first mutated for expression in *S. cerevisiae* by changing the *Candida* serine codon CTG for the universal TCG codon. The mutated IPF4033 fragment was then cloned in *S. cerevisiae* shuttle vector p423ADH [15] and transformed into *S. cerevisiae* CEN.PK 113-7A, generating strain TMB4111. Functional expression was evaluated by following its whole-cell bioreduction of androstanedione compared to the control strain TMB4103 that carried an empty vector and TMB4102 expressing YIL124w, the closest *S. cerevisiae* homologue and a reductase known to reduce androstanedione [12]. The strain overexpressing IPF4033 produced, after 24 h reduction, three new spots on TLC compared to the strain with an empty plasmid (Fig. 4). Furthermore, crude membrane and crude cytosolic extracts were prepared to compare *in vitro* reductions of androstanedione. Three new spots were observed in the membrane fractions of *S. cerevisiae* TMB4111 and TMB4102 overexpressing IPF4033 and YIL124w, but not in the control strain nor in any of the cytosolic fractions (data not shown). This result indicated functional expression of the IPF4033 ORF in *S. cerevisiae*.

S. cerevisiae TMB4111 (IPF4033) and TMB4102 (YIL124w) were subsequently compared with the control strain TMB4103 in whole-cell bioreductions of BCO2,6D, using approximately the same amount of cells as in the *Candida* experiments (i.e OD_{620} = 5). After 24 h, approx. 11% of the added BCO2,6D was converted in all strains, which was significantly lower compared to conversions with *C. tropicalis*. Strains overexpressing IPF4033 and YIL124w gave slightly higher exo-to-endo ratios than the control strain (Fig. 5a), although the ratios were about tenfold lower than those obtained with *C. tropicalis* strain overexpressing IPF4033 (Fig. 3). The cell concentration was increased to 5 g/l cdw (OD_{620} = 30), in order to achieve a comparable conversion to the *C. tropicalis* reductions. The BCO2,6D conversion now reached 50% with exo-to-endo ratios clearly higher for the overexpressing strains (Fig. 5b).

Physiological differences that could explain the much higher increase in exo-to-endo ratio obtained when overexpressing IPF4033 in *C. tropicalis* might stem from its extensive ability to utilise and grow on unusual carbon sources such as alkanes and fatty acids [31,32] and its potentially larger numbers of reductases [33], which may include efficient BCO2,6D reductases. Also, the ability of *C. tropicalis* to efficiently grow on fatty acids hints at the presence of higher levels of liposomes and membrane reductases to process these metabolites. Considering the *S. cerevisiae* homologue YIL124w has been localised to lipid particles [22], additional liposomes in *Candida* may provide space for a considerably higher induction and expression of IPF4033. This could allow for a significant increase in exo-to-endo ratio, even with a high background of endo-activity. A better transport of xenobiotic compounds in *C. tropicalis*, or a slower transport out, is another possibility that could explain the higher reduction rate as well as the observed higher sensitivity toward BCO2,6D with *Candida* sp.

4. Conclusions

The *in vivo* screening of putative exo-reductase genes from *C. albicans* enabled the identification of ORF IPF4033 (*AYR1* gene), which generated significantly higher exo-to-endo ratio than any other candidate, when expressed in *C. tropicalis*. The effect, which was a combination of increased exo production and decreased endo production, was observed in both whole cells and crude membrane fractions. The exo-to-endo ratio was shown to be time or conversion dependent, increasing with increasing conversion. In addition, a slight increased exo-to-endo ratio was observed when overexpressing IPF4033 in *S. cerevisiae*, although the reduction rate and exo-to-endo ratio were several fold lower compared to those obtained using *C. tropicalis*.

Acknowledgements

We thank the Swedish Research Council (VR) and the Swedish foundation for international cooperation in research and higher education (STINT) for financial support.

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