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Production of a thermostable alcohol dehydrogenase from *Rhodococcus ruber* in three different yeast species using the Xplor®2 transformation/expression platform

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Abstract The Xplor®2 transformation/expression platform was employed for comparative assessment of three different yeast species as hosts for synthesis of a thermostable nicotinamide adenine dinucleotide (NAD^+)-dependent medium-chain alcohol dehydrogenase from *Rhodococcus ruber* strain 219. Using yeast ribosomal DNA (rDNA) integrative expression cassettes (YRCs) and yeast integrative expression cassettes (YICs) equipped with a selection-marker module and one, two or four expression modules for transformation of auxotrophic *Arxula adeninivorans*, *Hansenula polymorpha*, and *Saccharomyces cerevisiae* strains, quantitative comparison of the yield of recombinant alcohol dehydrogenase RR-ADH6Hp in all three species was carried out. In all cases, the *RR-ADH6H* gene was expressed under the control of the strong constitutive *A. adeninivorans*-derived *TEF1* promoter, which functions in all yeast species analyzed. Recombinant RR-ADH6Hp accumulated intracellularly in all strains tested. The best yields of active enzyme were obtained from *A. adeninivorans*, with *S. cerevisiae* producing intermediate amounts. Although *H. polymorpha* was the least efficient producer overall, the product obtained was most similar to the enzyme synthesized by *R. ruber* 219 with respect to its thermostability.

Keywords Alcohol dehydrogenase · *Arxula adeninivorans* · *Hansenula polymorpha* · RR-ADH · *Rhodococcus ruber* · *Saccharomyces cerevisiae* · Yeast

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Introduction

Alcohol dehydrogenases (E.C. 1.1.1.1; ADHs) belong to the class of oxidoreductases, and represent an important group of biocatalysts due to their ability to stereospecifically reduce prochiral carbonyl compounds. Among other applications, they are used for synthesis of optically active alcohols, which are key building blocks for the fine chemical industry [1, 12, 21]. Alcohol dehydrogenases that use NAD as the cofactor are of particular importance. Coupling of the oxidation/reduction reaction with cofactor regeneration by formate dehydrogenase has proven to be economically feasible for production of fine chemicals [13]. An example for this kind of reaction, using a secondary ADH from *Rhodococcus erythropolis* (RE-ADH) and formate dehydrogenase, has been described [14]. However, for technical and economic reasons, a system based on a single dehydrogenase that simultaneously catalyzes the desired substrate conversion and regenerates the cofactor would be preferable [16].

Whole lyophilized cells of *Rhodococcus ruber* DSM44514, a member of the cosmopolitan bacterial genus *Rhodococcus*, have been shown to possess stereoselective ADH activity with exceptional tolerance towards organic solvents [27, 28]. The enzyme responsible for this activity (RR-ADH) has been purified from *R. ruber* and biochemically characterized. The isolated RR-ADH was confirmed

as an excellent candidate biocatalyst for oxidation of secondary alcohols, coupled to cofactor recycling at the expense of acetone as the hydrogen acceptor, on a preparative scale [16].

Despite these favorable properties of RR-ADH, the utility of the whole-cell catalysis system is limited by the fact that enzyme levels in whole cells are low, and efforts to enhance its expression in *R. ruber* by manipulating culture conditions have been unsuccessful. Gene technology offers an alternative approach to achieving low-cost production of the amounts of RR-ADH required for chemical applications. So far, recombinant expression of RR-ADH has been confined to *Escherichia coli*, and a yield of RR-ADH of 4.5 U (reduction of *p*-chloroacetophenone) per mg of cellular protein has been reported [2].

In the present work, we used the yeast-based Xplor®2 transformation/expression platform to identify a suitable host organism for efficient production of recombinant RR-ADH in active form. This platform has already been successfully applied for construction of transgenic *Arxula adeninivorans* strains that secrete high levels of recombinant *Klebsiella*-derived phytase, recombinant human interferon-α, and recombinant tannase [4–6]. In the present study, the system was used to explore the suitability of *A. adeninivorans*, *Hansenula polymorpha*, and *Saccharomyces cerevisiae* as hosts for production of RR-ADH. Xplor®2 allows the introduction of the same DNA cassette as a yeast rDNA integrative expression cassette (YRC) or a yeast integrative expression cassette (YIC) into the genomes of these three yeast species, so the most suitable host can be easily identified. The Xplor®2 transformation/expression platform can then be used for construction of transformants with improved expression by including host-specific, highly active promoters which accumulate the target recombinant protein to a biotechnologically acceptable level [32].

Materials and methods

Strains and culture conditions

Escherichia coli TOP 10 [F′ *mcra*Δ *(mmr-hsdRMS-mcrBC) F80*Δ*lacZDM15 DlacX74 deoR recA1 araD139* Δ(*ara leu*) *7697 galU galK Γ rspL andA1 nupG F*′], obtained from Invitrogen (Grand Island, NY, USA), served as the host strain for bacterial transformation and plasmid isolation. The strain was grown on lysogeny broth (LB) medium supplemented with ampicillin (50 μg mL^{-1}) for selection.

In this study, the wild-type *A. adeninivorans* strain LS3 [17] was used, together with the following auxotrophic mutants derived from it: *A. adeninivorans* G1211 [*aleu2*] [26], G1212 [*aleu2 atrp1::ALEU2*] [30], G1214 [*aleu2 aura3::ALEU2*], and MS1001 [*aleu2 atrp1::ALEU2 aura3::ALEU2*] (this work), *H. polymorpha* RB11 [*odc1* = *ura3*] [10], and *S. cerevisiae* SEY6210 [*ura3 trp1 leu2 his3 lys2 can suc2*-Δ*9*] [22]. All strains were grown at 30 °C under nonselective conditions in a complex medium [yeast extract peptone dextrose (YEPD)] or under selective conditions in yeast minimal medium (YMM) or synthetic dextrose minimal medium (SD medium), each supplemented with 2 % (w/v) glucose as carbon source [23, 31].

Agar plates were prepared by adding 1.6 % (w/v) agar to the liquid media.

Construction of new auxotrophic *A. adeninivorans* mutants

To construct the uracil auxotroph *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*], the *AURA3* gene was isolated from a plasmid-borne *Arxula* complementary DNA (cDNA) library (YEP112A1NEXs-cDNA; [33]). Plasmids (bearing Trp^+ as a selection marker) were transformed into *S. cerevisiae* SEY6210 [*ura3 trp1 leu2 his3 lys2 can suc2*-Δ*9*] according to Dohmen et al. [8], and grown on SD medium supplemented with 2 % glucose, all amino acids except tryptophan, and/or uracil. Out of 3.0×10^6 Trp^+ transformants recovered, 43 were able to grow in minimal medium without uracil. Plasmid DNAs isolated from these transformants were used to transform *E. coli* and analyzed by restriction analysis, and subsequently sequenced on an automatic laser fluorescence DNA sequencer (Pharmacia, Uppsala, Sweden). All 43 transformants contained the same 800-bp cDNA fragment, which was highly similar to the *S. cerevisiae URA3* gene. The nucleotide sequence has been submitted to the GenBank/EMBL data libraries (HE650146).

Subsequently, an *A. adeninivorans* LS3 genomic library (Charon 4A; [24]) was screened with a fragment of the cDNA. Seven of 95 positive plaques were found to contain the *AURA3* gene on a 20-kb DNA fragment. After sequence analysis, a 2,890-bp subfragment, the *AURA3* gene, was identified and cloned into the pBluescript II KS (−) vector (Stratagene, La Jolla, California, USA). One of the plasmids so derived was selected for this study.

To assemble the *AURA3* replacement vector, the complete 2,890-bp genomic fragment, which includes promoter and terminator sequences, was first amplified by polymerase chain reaction (PCR) using the primers AURA3-1 (5′-GGATCCGTTGACACACAGCC-3′, 1,863–1,844) and AURA3-2 (5′-GGATCCTGGAGACCGGCTTT-3′, -1,027–1,008), and inserted into the *E. coli* vector pCR4 (Invitrogen, Grand Island, NY, USA). Subsequently, 5′- (900 bp) and 3′- (1,027 bp) segments were amplified on a single fragment by whole-vector PCR using a pair of outwardly directed primers: AURA3-3 (5′-**TCCGGA**CAT

CGGAGATAGGGGACGAT-3′, 9,64–9,83) and AURA3-4 (5′-**TCCGGA**TAGTACCTTTCTCTCGCAGT-3′, -1–20), with *Kpn*2I restriction sites (indicated in bold type and underlined) at their 5′-ends. After self-ligation of the resulting product, which lacks the sequence between nucleotide positions 33 and 1,449 corresponding to complete *AURA3* open reading frame (ORF), the *ALEU2* selection marker flanked by *Kpn*2I sites [30] was inserted into the *Kpn*2I site located between the 5′ and the 3′ segments of the *AURA3* gene fragment. Finally, the complete construct (900-bp 5′ *AURA3* fragment + *ALEU2* + 1,027-bp 3′ *AURA3* fragment) was amplified using the primers AURA3-1 and AURA3-2, and the 4,114-bp product was used to transform *A. adeninivorans* strain G1211 [33] to generate G1214.

Since *A. adeninivorans* is classified as imperfect yeast, parasexual processes were used to obtain the double auxotrophic *atrp1 aura3* mutant. First, the auxotrophic mutants *A. adeninivorans* G1214 [*aleu2 aura3::ALEU2*] and G1212 [*aleu2 atrp1::ALEU2*] were fused by the spheroplast technique [26]. The resulting heterozygous diploid *A. adeninivorans* fp1001 (*aleu2 aleu2 AURA3 aura3::ALEU2 ATRP1 atrp1::ALEU2*) was then induced to undergo mitotic segregation in the presence of benomyl, as described by Samsonova et al. [26]. Since the two auxotrophic markers are located on different chromosomes (*ATRP1* on chromosome 2, *AURA3* on chromosome 3), they segregate independently of each other. The haploid double mutant selected (*aleu2 atrp1::ALEU2 aura3::ALEU2*) was named *A. adeninivorans* MS1001 and used as host for the introduction of multiple RR-ADH6H expression modules (see "Construction of RR-ADH expression plasmids" section).

Construction of RR-ADH expression plasmids

Based on the published amino acid sequence of RR-ADH from *R. ruber* strain 219 (GenBank accession no. CAD36475), the ORF was assembled by de novo gene synthesis (GeneArt, Regensburg, Germany) with flanking *Eco*RI and *Bam*HI sites, and in the process streamlined for heterologous expression, as well as adapted to the *H. polymorpha* codon usage. A 6 × His-tag coding sequence was inserted between the last amino acid and the termination codon using PCR techniques. The whole gene construct, including the flanking restriction sites, was amplified by PCR from plasmid using primers RR-ADH6H-1 (5′-**GAATTC**ATGAAGGCCCTGCAGTACAC-3′, ORF positions 1–20) and RR-ADH6H-2 (5′-**GGATCC**TTATTAGTGATGGTGATGGTGGTGGCCTGGCACCACAACACCTC-3′, ORF positions 1,038–1,020; the His-tag coding region including the stop codon are underlined). The amplified *Eco*RI–*Bam*HI *RR-ADH6H* ORF was inserted into the plasmids pBS-TEF1P-PHO5-SS (flanked by *Spe*I-*Sac*II restriction sites; [4]) and pBS-TEF1P-PHO5-SA (flanked by *Sal*I-*Apa*I restriction sites; [33]) between the *A. adeninivorans*-derived *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator.

For construction of a plasmid with a single RR-ADH6H expression module, the *TEF1* promoter–*RR-ADH6H* gene–*PHO5* terminator fragment flanked by *Sal*I–*Apa*I restriction sites was inserted into the plasmid Xplor2.2 [5] to generate Xplor2.2-TEF1-RR-ADH6H, in which the selection-marker module (*ALEU2* promoter–*ATRP1m*, and *Eco47*III, *Spe*I, *Sac*II, *Sal*I, *Apa*I multicloning restriction sites for insertion) is flanked by 25S rDNA target sequences.

Xplor2.2-TEF1-2RR-ADH6H, which carries two RR-ADH6H expression modules in opposite orientations, was obtained by inserting the *TEF1* promoter–*RR-ADH6H* gene–*PHO5* terminator module flanked by *Spe*I–*Sac*II restriction sites into Xplor2.2-TEF1-RR-ADH6H.

The plasmid Xplor2.3 was obtained by replacing the *ALEU2* promoter–*ATRP1m* module in Xplor2.2 by the *Sal*I–*Afe*I *AURA3mmm* (*AURA3* gene with gene substitutions at positions 57 [T → A], 669 [C → T], and 848 [C → G] to remove the restriction sites for *Afe*I, *Sal*I, and *Spe*I, respectively) selection-marker module, and used to assemble Xplor2.3-TEF1-RR-ADH6H and Xplor2.3-TEF1-2RR-ADH6H, in which the selection-marker module (*AURA3mmm* and *Eco47*III, *Spe*I, *Sac*II, *Sal*I, *Apa*I multicloning restriction sites for insertion) is flanked by 25S rDNA target sequences.

To assemble the final constructs used for integration, these four plasmids (and the control plasmids Xplor2.2 and Xplor2.3 lacking a RR-ADH6H expression module) were cleaved with *Asc*I (YRC) or *Sbf*I (YIC) to remove the *E. coli* sequences including the kanamycin resistance marker. The resulting linear fragments depicted in Fig. 1, as well as YRC102, YIC102, YRC103, and YIC103 as controls (Fig. 1), were subsequently transformed into the different yeast species. YIC102/YIC103 and YRC102/YRC103 cassettes containing one or two RR-ADH6H expression modules, respectively, were transformed into *A. adeninivorans* G1212, G1214, *H. polymorpha* RB11, and *S. cerevisiae* SEY6210. *A. adeninivorans* MS1001 was cotransformed with YIC102-2ADH and YIC103-2ADH or YRC102-2ADH and YRC103-2ADH, resulting in transformants bearing four integrated copies of the RR-ADH6H expression module [4, 5, 29, 30].

Generation of stable recombinant strains and transcript analysis

Escherichia coli strains were transformed according to the procedure of Hanahan [11]. *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* transformants were generated according to Rösel and Kunze [24] and Dohmen et al. [8],

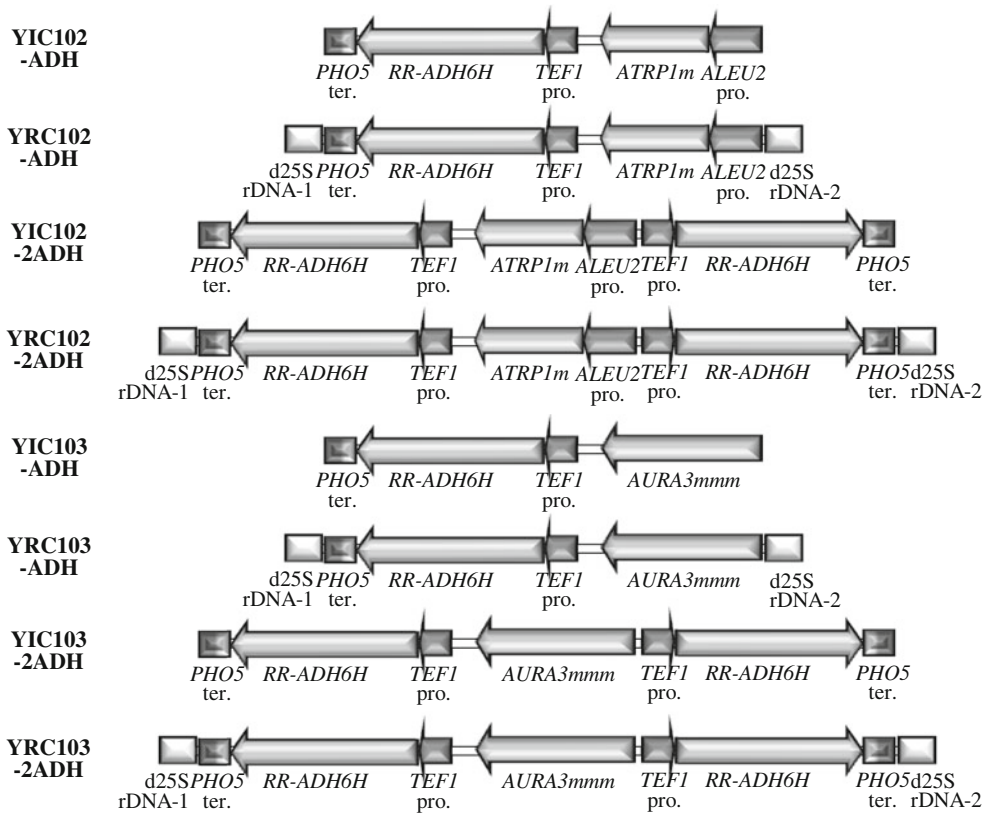

Fig. 1 Physical maps of the YRCs and YICs used for transformation of *A. adeninivorans* G1212, G1214, MS1001, *H. polymorpha* RB11, and *S. cerevisiae* SEY6210. Cassettes contain the selection marker *ATRP1m* fused to the *ALEU2* promoter (YRC102, YIC102, YRC102-ADH, YIC102-ADH, YRC102-2ADH, YIC102-2ADH) or the selection marker *AURA3mmm* (YRC103, YIC103, YRC103-ADH, YIC103-ADH, YRC103-2ADH, YIC103-2ADH) together with one or two copies of the expression module *TEF1* promoter–*RR-ADH6H*–*PHO5* terminator. In addition, YRCs (*Asc*I fragments) are flanked by 25S rDNA sequences for targeting, whereas YICs (*Sbf*I fragments) contain only the selection marker and expression modules

respectively. Stable yeast transformants were obtained after a sequence of passages on selective and nonselective media [15].

RNA isolation was carried out using a Nucleospin RNA Plant Kit (Macherey–Nagel, Düren, Germany) in accordance with the supplier's instructions. Agarose/formaldehyde gel electrophoresis and Northern transfer of RNA were performed according to Sambrook and Russell [25]. RNA was hybridized under high-stringency conditions at 60 °C, following the recommendations provided in the instruction manual for Hybond N+ nylon membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and hybridization and washing steps according to the protocol supplied by the manufacturer of the hybridization buffer (Roti Hybri Quick, Carl Roth GmbH, Karlsruhe, Germany).

The RR-ADH6H-specific probe and the AHSB4 internal control probe [34] [ADH-fwd (5′-ACACCTGGTTCCAATTGGTGACTTGGACCCAG-3′, 405–436), ADH-R (5′-TCTACCTCTGATCGAGCCCTCTCTCAATCTTCTG-3′, 1,020–987), AHSB4-fwd (5′-ACGCGGAAAAGGAGGAAAAGGACTTGGAAAGGGAG-3′, 609–643) and AHSB4-R (5′-ACCTCCAAAACCATAAAGAGTTCGTCCCTG-3′, 909–880)] were labeled with digoxygenin deoxyuridine triphosphates (dUTPs) using the High Prime Labeling Kit (Roche Diagnostics, Mannheim, Germany), hybridized to filter-bound RNA as described above, and visualized with the Digoxygenin Detection Kit in combination with the CSPD substrate for alkaline phosphatase-conjugated anti-Dig Fab fragments (Roche Diagnostics).

Biochemical analysis of recombinant RR-ADH6Hp

Crude yeast-cell extracts were prepared by disrupting the samples in an X-pressure cell (BIOX AB, Göteborg, Sweden). NADH-dependent RR-ADH6Hp activity was assayed by measuring the NAD produced from NADH by the action of the enzyme on *p*-chloroacetophenone, as described by Abokitse and Hummel [1] with a few modifications. The reaction (total volume 200 µl) was carried out in 100 mM potassium phosphate buffer (pH 6.0) in the presence of 3 mM *p*-chloroacetophenone, 0.25 mM NADH, and the enzyme (added as protein extract or purified enzyme) at 37 °C, and NAD^+ was measured at 340 nm. One unit of RR-ADH6Hp activity was defined as the amount of enzyme that converted 1 µmol NADH into NAD^+ per minute.

To analyze the thermostability of RR-ADH6Hp, the crude cell extract was incubated at different temperatures for various times, denatured proteins were removed by centrifugation for 5 min at 13,000 × *g*, and the enzyme activity in the supernatant was measured photometrically at 37 °C as described above.

For determination of the kinetic constants K_m, V_{max} and K_{cat} of RR-ADH6Hp synthesized in the three yeast species, the respective purified enzyme was incubated under optimal temperature conditions (*A. adeninivorans* G1212/YRC102-2ADH: 39 °C, pH 6.0; *H. polymorpha* RB11/YIC103-2ADH and *S. cerevisiae* SEY6210/YIC103-2ADH: 35 °C, pH 6.0) in the presence of different concentrations of *p*-chloroacetophenone and four additional substrates [1].

To analyze the stability of RR-ADH6Hp in the presence of organic solvents, whole cells (cell pellets drained of aqueous liquid) of *A. adeninivorans* G1212/YRC102-2ADH, *H. polymorpha* RB11/YIC103-2ADH, and *S. cerevisiae* SEY6210/YIC103-2ADH were exposed to pure *n*-hexane, acetone, and ethanol for up to 5 h. After removing the solvents, the yeast cells were permeabilized as described by Miozzari et al. [20] for detection of intracellular RR-ADH6Hp activity.

Partial purification of recombinant RR-ADH6Hp

Recombinant RR-ADH6Hp, which carries a His-tag, was purified by column chromatography on His-Bind resin, and gel filtration on a Superdex^{TM}200 column. For further analysis, the purified recombinant RR-ADH6Hp was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) under denaturing conditions [19].

Protein analytics

SDS-PAGE and Western analyses were performed as described by Kunze et al. [18]. Western blots were treated with an anti-His-tag specific primary antibody (Antibodies-online GmbH, Aachen, Germany) and a goat anti-rabbit IgG alkaline phosphatase conjugate, and subsequently stained by incubation with Nitroblue tetrazolium chloride (NBT)/bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) substrate (Roche Diagnostics, Mannheim, Germany).

The dye-binding method of Bradford [7] was used for protein quantification, with bovine serum albumin as standard.

Results

Generation of recombinant yeast strains

The Xplor®2 platform has been successfully established as a routine transformation/expression platform in *A. adeninivorans* [5], and allows construction of resistance marker-free transformants. The system is based on a bacterial vector backbone into which yeast modules such as selection and expression modules can be inserted. The cloning sites for insertion of these modules are located between two 25S rDNA segments that are arranged in the same orientation but in reverse order relative to the genomic rDNA. After insertion of the appropriate modules, the *E. coli* sequences in the plasmid construct can be removed by digestion with *Asc*I or *Sbf*I. Furthermore, the choice of restriction enzyme determines whether the linearized fragment is targeted to the rDNA (*Sbf*I) or not (*Asc*I), allowing one to obtain YRC or YIC integrants as desired.

To use this system for construction of transgenic RR-ADH6Hp strains, the *ATRP1m* or *AURA3mmm* selection-marker module, together with one or two expression modules for RR-ADH6Hp, were inserted into the Xplor®2 vector between the two 25S rDNA fragments. Since the vector system is designed for integration of YRCs and YICs lacking undesirable bacterial sequences, all plasmids were digested with *Asc*I or *Sbf*I to remove all *E. coli* sequences prior to transformation. In the resulting cassettes, the module combination was flanked (YRCs) or not (YICs) by the two 25S rDNA fragments. All YRCs and YICs were introduced in linear form (Fig. 1) into *A. adeninivorans* G1212, G1214, and MS1001, *H. polymorpha* RB11, and *S. cerevisiae* SEY6210. In addition, the strain *A. adeninivorans* MS1001 was cotransformed with YIC102 and YIC103 or YRC102 and YRC103, respectively, each of which carries two expression modules. Thus, recombinant yeast strains containing one or two copies (all three hosts) and one, two or four copies (*A. adeninivorans*) of the *RR-ADH* gene were generated.

All cassettes were successfully transformed into the three yeast species. Irrespective of the host used, between 50 and 200 transformants were obtained per µg of transforming cassette, although the 25S rDNA fragments had a weak positive influence on transformation efficiency in the case of the YRCs (data not shown).

After stabilization of the transgenic yeast strains by passaging (see "Materials and methods" section), no loss of YRCs or YICs was observed after cultivation on non-selective medium for a period of 30 days.

Expression of RR-ADH6H in the three yeast species

Transgenic yeast strains carrying between one and four RR-ADH6H expression modules were grown in YEPD at 30 °C for 48 h. Since RR-ADH6Hp does not contain an obvious secretion sequence, the recombinant protein should be localized intracellularly. Whole-cell extracts (50 transformants per construct) were therefore analyzed for the presence of recombinant RR-ADH6Hp activity in the initial screen. For a given construct, levels of intracellular RR-ADH6Hp activity in *A. adeninivorans* varied by up to sixfold, and showed no consistent correlation with the total

number of the RR-ADH6H expression modules (one to four) present. The range of variation observed in the other two species was lower, up to threefold in *H. polymorpha* and up to twofold in *S. cerevisiae* (data not shown). The maximum yields of RR-ADH6p obtained in the three yeast species are presented in Table 1.

The transgenic *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* strains with the highest recombinant RR-ADH6Hp activities were analyzed in time-course experiments. For this purpose, the strains listed in Table 1 were cultured in YEPD for 120 h at 30 °C, i.e., under conditions that allow high-level expression from the *TEF1* promoter and where cultures enter the stationary growth phase after approx. 24 h. Cell extracts as well as aliquots of culture medium were then analyzed for the presence of RR-ADH6Hp.

In contrast to the negative control strains (*A. adeninivorans*, *H. polymorpha*, *S. cerevisiae*) without RR-ADH6H expression modules, recombinant enzyme activity was detected in all transformants with RR-ADH6H expression modules. Irrespective of the host species considered, essentially all RR-ADH6Hp was localized intracellularly.

The recombinant enzyme activity in the *A. adeninivorans* transformants with RR-ADH6H expression modules reached its maximum level within approx. 48 h, before declining to a basal level at 120 h (Fig. 2). However, no correlation between the activity levels of YRC and YIC transformants containing the same number of expression modules or between the number of RR-ADH6H expression modules and the level of RR-ADH6Hp activity was detectable, except in the *A. adeninivorans* G1212 transformants. The product yields measured in G1212 strains with two RR-ADH gene copies (5.5 and 12.2 U mL^{-1} of culture) were four- and sevenfold higher, respectively, than in the G1212 strains containing only one RR-ADH gene copy (Table 1).

In contrast, maximum productivity varied considerably between the host strains and species. Among *Arxula* transformants, the auxotrophic double mutant strain MS1001 and the *ura3* mutant G1214, with 34–61 U g^{-1} dcw, exhibited relatively low maximum yield coefficients $Y(P/X)$ [product P in units of RR-ADH6Hp per biomass X in gram dcw]. However, the *A. adeninivorans* strains G1212/YRC102-2ADH (155 U g^{-1} dcw) and the corresponding YIC transformant G1212/YIC102-2ADH (351 U g^{-1} dcw) were notably more productive (Fig. 2; Table 1).

The maximum activity levels measured in *H. polymorpha* and *S. cerevisiae* transformants were much lower. Whereas all cultures entered stationary phase after 24 h at dcw levels similar to those of *A. adeninivorans* transformants,

Table 1 Maximum yields of RR-ADH6Hp activity obtained in recombinant strains of *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae*

Yeast strain[a]	Maximum yield of RR-ADH6Hp activity [U mL^{-1} culture]	Dry cell weight [g L^{-1}]	Yield coefficient (P/X) [U g^{-1} dcw]
A. adeninivorans			
G1212/YRC102-ADH	1.4	36.5	40.4
G1212/YIC102-ADH	1.5	35.7	41.6
G1212/YRC102-2ADH	5.5	35.2	155.4
G1212/YIC102-2ADH	12.2	39.9	350.7
G1214/YRC103-2ADH	1.9	37.8	49.9
G1214/YIC103-2ADH	2.1	35.7	61.3
MS1001/YRC102-2ADH–YRC103-2ADH	1.6	35.8	45.4
MS1001/YIC102-2ADH–YIC103-2ADH	1.3	36.5	34.1
H. polymorpha			
RB11/YRC103-ADH	0.4	37.6	10.2
RB11/YIC103-ADH	0.4	36.8	10.7
RB11/YRC103-2ADH	0.4	36.7	10.5
RB11/YIC103-2ADH	0.5	38.8	12.4
S. cerevisiae			
SEY6210/YRC103-ADH	1.0	36.4	27.3
SEY6210/YIC103-ADH	1.1	37.2	29.8
SEY6210/YRC103-2ADH	0.9	39.1	25.4
SEY6210/YIC103-2ADH	1.3	37.6	34.2

[a] Strains were grown in shake-flask cultures as described in "Materials and methods" section. For details of the expression cassettes present in the strains listed, see Fig. 1

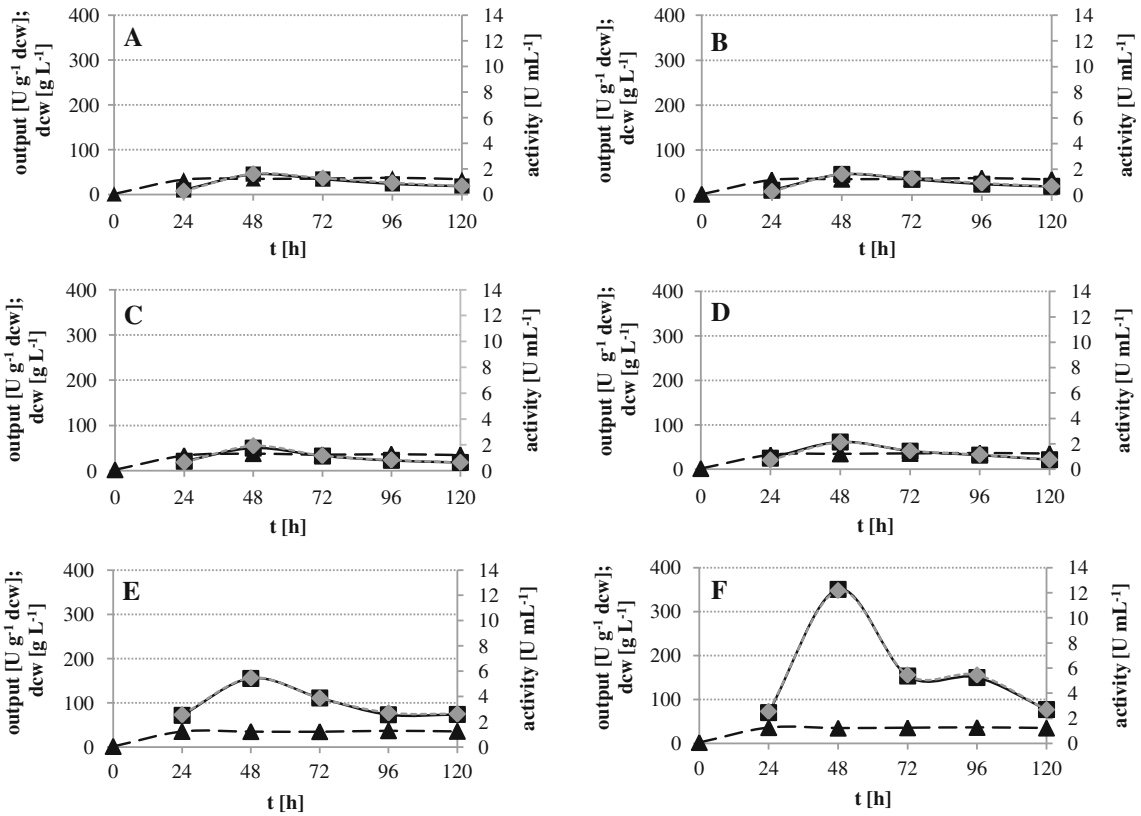

Fig. 2 Time courses of expression of His-tagged RR-ADH6Hp by the indicated transgenic strains of *A. adeninivorans*. Transformants **a** MS1001/YRC102-2ADH–YRC103-2ADH, **b** MS1001/YIC102-2ADH–YIC103-2ADH, **c** G1214/YRC103-2ADH, **d** G1214/YIC103-2ADH, **e** G1212/YRC102-2ADH, and **f** G1212/YIC102-2ADH were cultured in shake flasks for 120 h at 30 °C in the presence of YEPD. At the indicated times, 10-mL aliquots of the culture were used to determine biomass (dcw in g L^{-1}, *black triangles*) and to assay the intracellular RR-ADH6p activity (U mL^{-1} culture, *gray half block*) using *p*-chloroacetophenone as substrate, as well as to calculate the RR-ADH6p output *Y*(*P*/*X*) (U g^{-1} dcw, *black squares*)

with 10–12 U g^{-1} dcw (*H. polymorpha*) and 25–34 U g^{-1} dcw (*S. cerevisiae*), the maximum yield coefficients *Y*(*P*/*X*) were approx. 29- and 10-fold lower, respectively, than that of the best *A. adeninivorans* transformant (G1212/YIC102-2ADH). However, the level of RR-ADH6Hp remained nearly constant up to 120 h in all analyzed *H. polymorpha* transformants. *S. cerevisiae* transformants, however, achieved their maximum yield coefficients *Y*(*P*/*X*) at 96 h, and these decreased during further cultivation (Fig. 3).

To analyze the relationship between the recombinant ADH6Hp activities of the best *A. adeninivorans* YIC transformants (G1212/YIC102-ADH, G1212/YIC102-2A DH, G1214/YIC103-2ADH, and MS1001/YIC102-2ADH–YIC103-2ADH) with one, two or four RR-ADH6H modules and *ADH6H* transcription, the steady-state levels of *ADH6H* RNA were assayed by Northern hybridization. Total RNA was extracted from transformants grown in YEPD for 24 h at 30 °C, when the yeast cultures have entered the stationary growth phase. At this point, both *A. adeninivorans* transformants with two expression modules integrated in the genome had accumulated nearly the same amounts of *RR-ADH6H* transcripts as *A. adeninivorans* MS1001/YIC102-2ADH–YIC103-2ADH cells bearing four expression modules. Levels of the transcript in *A. adeninivorans* G1212/YIC102-ADH with one expression module, however, were some 50 % lower. In contrast, levels of RNA produced by the constitutively expressed single-copy *AHSB4* gene encoding histone H4 [34], which was used as control, were similar in all *A. adeninivorans* transformants analyzed (Fig. 4).

Characterization of recombinant RR-ADH6Hp

The RR-ADH6Hp synthesized by the *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* transformants with the highest activity (G1212/YIC102-2ADH, RB11/YIC103-2ADH, SEY6210/YIC103-2ADH) was purified by column chromatography on His-Bind resin, and analyzed in more detail by SDS-PAGE and Western blot using anti-His-tag antibodies. This experiment confirmed the accumulation of RR-ADH6Hp in *A. adeninivorans* G1212/YIC102-2ADH,

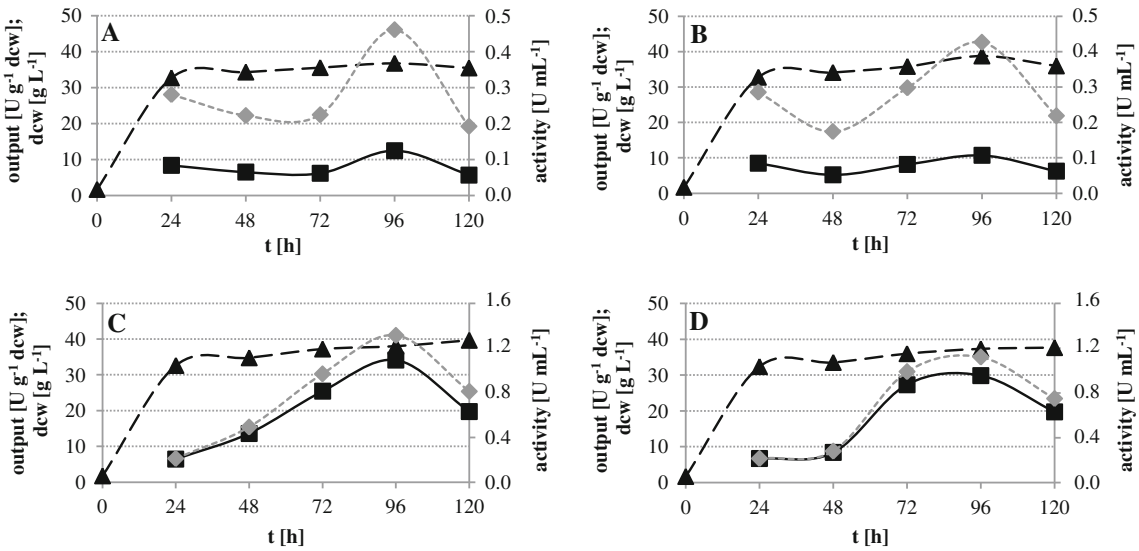

Fig. 3 Time courses of expression of His-tagged RR-ADH6Hp by the indicated transgenic *H. polymorpha* and *S. cerevisiae* strains. See legend to Fig. 2 for further details. Shake-flask cultivation of **a** *H. polymorpha* RB11/YIC103-ADH, **b** RB11/YIC103-2ADH, **c** *S. cerevisiae* SEY6210/YIC103-ADH, and **d** SEY6210/YIRC103-2ADH. All transformants were cultured in shake flasks for 120 h at 30 °C in the presence of YEPD. Aliquots (10 mL) of the yeast culture were used to determine biomass (dcw in g L^{-1}, *black triangles*) and to assay the intracellular RR-ADH6p activity (U mL^{-1} culture, *gray half block*) using *p*-chloroacetophenone as substrate, as well as to calculate the RR-ADH6p output *Y*(*P*/*X*) (U g^{-1} dcw, *black squares*)

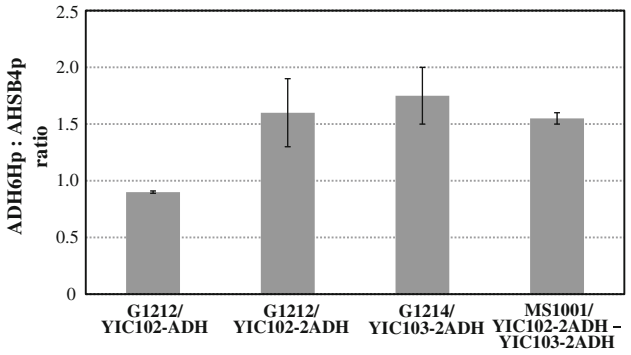

Fig. 4 Influence of RR-ADH6H expression module numbers on the level of *RR-ADH6H* transcripts. *A. adeninivorans* G1212/YIC102-ADH, G1212/YIC102-2ADH, G1214/YIC103-2ADH, and MS1001/YIC102-2ADH–YIC103-2ADH were cultured at 30 °C in YEPD for 24 h. After RNA isolation and Northern hybridization, the ratio of the *RR-ADH6H* signal to the *AHSB4* control was determined from the respective band intensities (Genesnap program from Syngen–Cambridge, UK). Standard deviations were calculated from three independent experiments

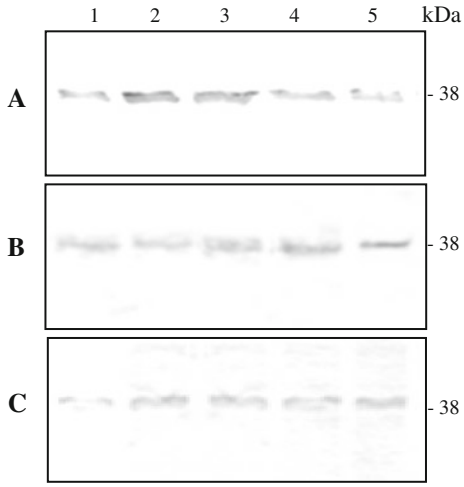

Fig. 5 Time course of RR-ADH6Hp accumulation during shake-flask cultivation of **a** *A. adeninivorans* G1212/YIC102-2ADH, **b** *H. polymorpha* RB11/YIC103-2ADH, and **c** *S. cerevisiae* SEY6210/YIC103-2ADH. The cultivation was performed as described in "Materials and methods" section. Aliquots of the cell suspension were harvested after 24 (1), 48 (2), 72 (3), 96 (4), and 120 h (5). Cells were harvested by centrifugation, and cell extracts were prepared. Aliquots (30 µg) of cell extract were separated on (15 %) SDS-PAGE gels, transferred to nitrocellulose filters, and probed with an anti-His-tag antibody (Antibodies-online GmbH, Germany)

H. polymorpha RB11/YIC103-2ADH, and *S. cerevisiae* SEY6210/YIC103-2ADH (Fig. 5).

The recombinant RR-ADH6Hp molecules synthesized in the different species all had native molecular mass of approx. 66–72 kDa by analytical gel filtration, while forming a distinct band at approx. 38 kDa on denaturing SDS-PAGE gels. These data indicate that the active enzyme is probably a homodimer. The pH optimum for enzyme activity was 6.0. The RR-ADH6Hp activity was observed to be stable in the temperature range of approx. 34.0–42.5 °C, although the *A. adeninivorans*-derived RR-ADH6Hp has a somewhat higher temperature optimum (39 °C) than the enzymes from *H. polymorpha* and *S. cerevisiae* transformants (Table 2).

Table 2 Properties of the recombinant RR-ADH6Hp synthesized in *A. adeninivorans* G1212/YRC102-2ADH, *H. polymorpha* RB11/YIC103-2ADH, and *S. cerevisiae* SEY6210/YIC103-2ADH

Property	*A. adeninivorans*	*H. polymorpha*	*S. cerevisiae*
pH optimum	6.0	6.0	6.0
pH stability[a]	5.8–6.6	5.5–6.3	5.2–6.3
Temperature optimum (°C)	39	35	35
Thermal stability (°C)[a]	38.5–42.5	34.0–38.0	32.0–41.0
M_r native (Da)	72,000	66,000	72,000
M_r denatured (Da)	38,000	38,000	38,000
Calculated M_r (Da)	37,000	37,000	37,000

[a] pH stability and thermostability were defined as the range over which at least 80 % of the initial activity was retained after 4-min incubation at given pH (at 30 °C) or temperature

The recombinant RR-ADH6Hp synthesized by transgenic *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* strains do however differ in thermostability. The *H. polymorpha*-derived enzyme exhibited the highest stability. It could be kept at 70 °C for up to 60 min without appreciable loss of activity. In contrast, exposure to the same conditions causes the recombinant RR-ADH6Hp activities from *A. adeninivorans* and *S. cerevisiae* to drop by approx. 30 and 60 %, respectively (Fig. 6).

To determine the effects of organic solvents on RR-ADH6Hp activity, whole yeast cells were exposed to pure *n*-hexane, ethanol or acetone for up to 5 h. Exposure to *n*-hexane, a nonpolar solvent, had no detectable effect on the recombinant enzyme. In contrast, treatment of the transgenic *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* strains with ethanol and acetone as polar solvents reduced the enzyme activity to 18, 25, and 28 % (ethanol) and 21, 38, and 31 % (acetone), respectively, of the values for untreated control cells.

The kinetic characteristics and binding affinities of the recombinant enzymes for a range of ketones and aldehydes are summarized in Table 3. For this purpose, the kinetics of reduction of various substrates by RR-ADH6Hp was measured photometrically, as described in "Materials and methods" section for *p*-chloroacetophenone. In all cases the highest activities were obtained with capronaldehyde as substrate. The enzyme is also able to act on acetophenone, phenylacetaldehyde, *p*-chloroacetophenone, and valeraldehyde, albeit at lower rates.

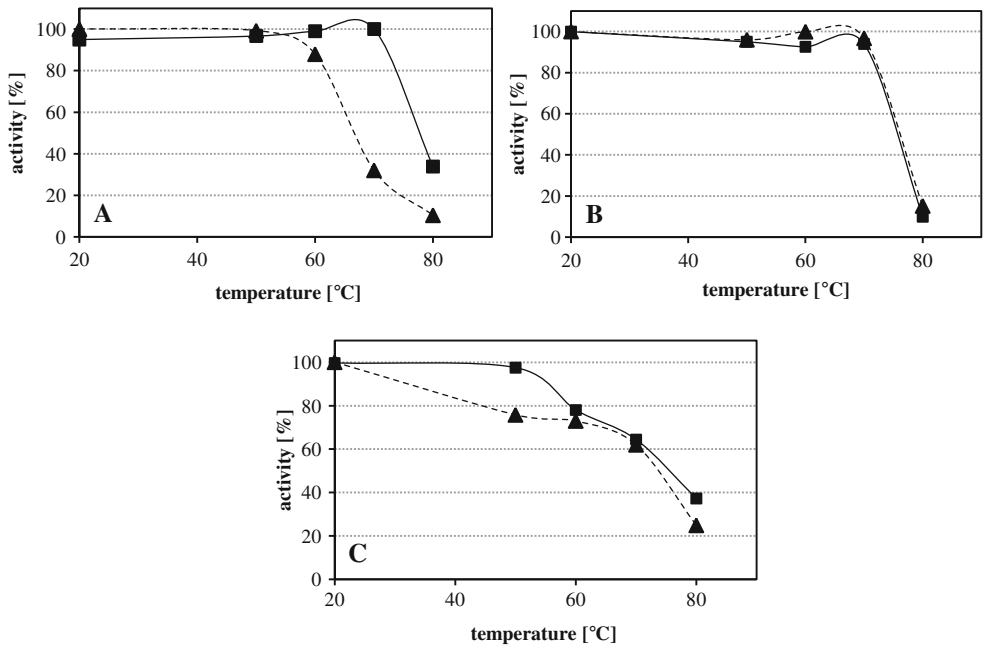

Fig. 6 Thermostability of purified recombinant RR-ADH6Hp synthesized by transgenic **a** *A. adeninivorans*, **b** *H. polymorpha*, and **c** *S. cerevisiae* strains. The recombinant purified proteins were incubated for (*squares*) 10 and (*triangles*) 60 min at the respective temperatures before the alcohol dehydrogenase activity was measured as described in "Materials and methods" section

Table 3 Kinetic constants of RR-ADH6Hp synthesized in *A. adeninivorans* G1212/YRC102-2ADH, *H. polymorpha* RB11/YIC103-2ADH, *S. cerevisiae* SEY6210/YIC103-2ADH, and *E. coli* BL21(DE3)/pRR-ADH1[a, b]

Substrate	K_m (mM)				K_{cat} (s^{-1})				K_{cat}/K_m (mM^{-1} s^{-1})			
	E. coli[b]	*A. adeninivorans*	*H. polymorpha*	*S. cerevisiae*	*E. coli*	*A. adeninivorans*	*H. polymorpha*	*S. cerevisiae*	*E. coli*	*A. adeninivorans*	*H. polymorpha*	*S. cerevisiae*
Acetophenone	0.76	0.40	0.20	0.66	303	288	211	259	399	720	1,055	392
Phenylacetaldehyde	0.17	0.91	0.33	0.19	158	303	264	202	929	333	800	1,063
p-Cl-Acetophenone	0.06	1.11	0.14	0.53	595	432	145	230	9,917	389	1,036	434
Valeraldehyde	n.d.	0.28	2.00	0.17	n.d.	432	528	418	n.d.	1,543	264	589
Capronaldehyde	n.d.	0.14	0.25	0.22	n.d.	317	410	576	n.d.	2,264	1,640	2,618

[a] The kinetic constants for RR-ADH6Hp were determined by enzyme assay as described in "Materials and methods" section in at least three independent experiments. Lineweaver–Burk plots were used to determine the K_m values, and the cosubstrate (NADH) was added in great excess. Substrate concentrations of up to 3.0 mM were tested

[b] Abokitse [2]

Discussion

So far, the Xplor®2 system has been used for construction of transgenic *A. adeninivorans* strains engineered for expression of recombinant proteins [5]. By using the same expression modules with *TEF1* promoter and *PHO5* terminator, auxotrophic selection markers, and appropriate auxotrophic mutants, the platform can be extended to allow transformation of the same YRC or YIC into different yeast species. In the present work, the platform was utilized for the first time to construct transgenic *H. polymorpha* and *S. cerevisiae* strains, which were compared with cognate *A. adeninivorans* strains, and the functionality of the expression modules described by Terentiev et al. [32] for the Xplor®1 platform was confirmed. In contrast to its predecessor, the Xplor®2 system is based on the transformation of linear cassettes (YRCs, YICs) that include no bacterial sequences and carry up to four expression modules. YRC fragments are terminated by 25S rDNA fragments arranged in the same orientation but inverted relative to chromosomal rDNA. After introduction into yeasts of different species, the free ends interact or are ligated by endogenous ligases, and can be integrated into the genomic rDNA (YRCs) or elsewhere in the genome (YICs) by homologous or nonhomologous recombination. In this model, YRC/YIC integration is achieved by single crossover events.

The transformation frequencies obtained in the three yeast species were similar, but the level of recombinant ADH6Hp expressed was generally higher in *A. adeninivorans* transformants than in the other two. Similar observations were made by Böer et al. [3] for the expression of a *MATα-IL6* ORF encoding human interleukin-6 in *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* using the Xplor®1 system. This suggests that the effect is due to the *TEF1* promoter, which exhibits optimal activity in the homologous system (*A. adeninivorans*) but operates less efficiently in *H. polymorpha* and *S. cerevisiae*.

To examine the effect of RR-ADH6H gene dosage, transgenic *A. adeninivorans* strains bearing one, two, and four expression modules were constructed. Analyses of *RR-ADH6H* transcripts in the best *A. adeninivorans* YIC transformants with one, two or four RR-ADH6H modules revealed limited correlation between transcript level and gene dosage. While cells carrying two RR-ADH6H expression modules expressed twice as much of the transcript as cells bearing a single module, increasing the number of expression modules to four had no further effect. This finding indicates that some mechanism acts at the transcriptional level to limit RR-ADH6H expression, perhaps because higher levels of the protein have toxic effects on the host cell.

The maximal RR-ADH6Hp activity obtained was 12.2 U mL^{-1} of culture (*A. adeninivorans* G1212/YIC102-

2ADH), an eightfold increase over the respective strain with a single RR-ADH6H expression module. However, this correlation was detected in *A. adeninivorans* G1212 transformants only, but not in the other *A. adeninivorans* strains tested. Likewise, a consistently positive correlation between gene dosage and product titer was not observed among *H. polymorpha* and *S. cerevisiae* transformants. One reason for this discrepancy probably lies in chromosomal position effects. YIC cassettes integrate randomly into the host cell DNA, and although YRC cassettes insert into the ribosomal DNA repeats, the expression level depends on the position of the interrupted rDNA repeat within the entire repetitive cluster [5]. Another frequently relevant reason for a nonlinear relationship between target gene dosage and expression level lies in the recombinant product itself. The target protein yield may be limited by structural requirements not sufficiently provided by the host cell (e.g., cofactors), by instability, or by harmful effects on host cell growth. One such example has indeed been observed with Xplor®2-mediated overexpression of endogenous tannase in *A. adeninivorans* G1212. In this case, a single-copy transformant yielded significantly more product than a transformant carrying two copies [6]. That an increase in the copy number is not necessarily accompanied by a corresponding increase in target protein yield has also been reported for other expression systems such as *H. polymorpha* [9]. In fact, there are indications that RR-ADH level may be restricted within any host cell: In *E. coli*, reduction of expression plasmid copy number resulted in a fivefold increase in product level of a related ADH from *R. erythropolis* (RE-ADH; [2]).

The molecular and biochemical properties of the recombinant, His-tagged RR-ADH6Hp produced in *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* differ from those of the natural enzyme synthesized by the *R. ruber* strains 219 or DSM 44541 in a number of respects. Notably, while the purified *R. ruber* ADHp displays a temperature optimum for maximal activity of 50–60 °C, the optima for the recombinant enzymes were 15–20 °C lower. The optimal pH of *R. ruber* ADHp determined for reduction was 6.5 [16], whereas the yeast-derived RR-ADH6Hp showed a slightly lower pH optimum of 6.0. Some or all of these differences could be due to the C-terminal His-tag, which also reduces RR-ADH activity by 20–30 % (A. Degelmann and M. Piontek, unpublished data).

The endogenous RR-ADHp is a remarkably thermostable enzyme, and its activity is not markedly affected by exposure to 60 °C for 60 min [28]. RR-ADH6Hp synthesized in *H. polymorpha* is equally stable, but the recombinant products from the other two species, especially *S. cerevisiae* RR-ADH6Hp, were less resistant to high temperatures. Host-specific variations in posttranslational modifications of the recombinant enzymes, which may influence the native structure of RR-ADH6p, could account for these differences.

In addition, like the endogenous RR-ADHp [27], the recombinant RR-ADH6Hp synthesized in *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae* was found to be relatively resistant to organic solvents. The effects of *n*-hexane, ethanol, and acetone on the RR-ADH6Hp synthesized by the three yeast species were similar to those previously determined for the RR-ADHp synthesized by the *R. ruber* strain [27].

Analysis of the substrate specificities of the recombinant enzymes revealed only minor interspecies differences. Independent of the yeast host considered, the highest catalytic efficiency was observed with capronaldehyde as substrate. *H. polymorpha*-derived RR-ADH6Hp yielded the lowest K_m value (with *p*-chloroacetophenone) among the yeast-produced enzymes and the five substrates tested, and thus is most similar to the *E. coli*-produced enzyme. On the other hand, *H. polymorpha*-derived RR-ADH6Hp also exhibited the highest K_m in this dataset (for valeraldehyde) of all the enzymes considered (Table 3). The differences in kinetic behavior and thermostability may reflect subtle differences in conformation owing to variations in folding of the polypeptide in the three species.

Taken together, the results confirm the suitability of the Xplor®2 transformation/expression system as a platform for comparative analysis of recombinant protein production in different yeast species. The productivity level achieved with *H. polymorpha* was the lowest, but the recombinant enzyme exhibited the highest similarity to the natural enzyme synthesized by *R. ruber*. *A. adeninivorans* expressed higher recombinant protein levels, but the enzyme was less thermostable than the *H. polymorpha* product. The host *S. cerevisiae* synthesized ADH6Hp at intermediate levels, but the enzyme was also more thermolabile than in either of the other host species. Based on these results, *H. polymorpha* or *A. adeninivorans* would be suitable hosts for production of recombinant ADH6Hp. In *H. polymorpha*, the heterologous *TEF1* promoter could be replaced by a strong homologous promoter for construction of ADH6Hp producer strains.

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References

1. Abokitse K, Hummel W (2003) Cloning, sequence analysis, and heterologous expression of the gene encoding a (S)-specific

alcohol dehydrogenase from *Rhodococcus erythropolis* DSM 43297. Appl Microbiol Biotechnol 62:380–386
2. Abokitse K (2004) Biochemische und molekularbiologische Charakterisierung von Alkoholdehydrogenase und einer Oxygenase aus *Rhodococcus* Spezies. Dissertation Heinrich Heine Univ Düsseldorf, p 158
3. Böer E, Steinborn G, Matros A, Mock HP, Gellissen G, Kunze G (2007) Production of interleukin-6 in *Arxula adeninivorans, Hansenula polymorpha* and *Saccharomyces cerevisiae* by applying the wide-range yeast vector (CoMed™) system to simultaneously comparative assessment. FEMS Yeast Res 7:1181–1187
4. Böer E, Bode R, Mock HP, Piontek M, Kunze G (2009) Atan1p-an extracellular tannase from the dimorphic yeast *Arxula adeninivorans*: molecular cloning of the *ATAN1* gene and characterization of the recombinant enzyme. Yeast 26:323–337
5. Böer E, Piontek M, Kunze G (2009) Xplor®2-a new transformation/expression system for recombinant protein production in the yeast *Arxula adeninivorans*. Appl Microbiol Biotechnol 84:583–594
6. Böer E, Breuer FS, Weniger M, Denter S, Piontek M, Kunze G (2011) Large-scale production of tannase using the yeast *Arxula adeninivorans*. Appl Microbiol Biotechnol 92:105–114
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
8. Dohmen RJ, Strasser AM, Höner CB, Hollenberg CP (1991) An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. Yeast 7:691–692
9. Gellissen G, Janowicz ZA, Merckelbach A, Piontek M, Keup P, Weydemann U, Hollenberg CP (1991) Heterologous gene expression in *Hansenula polymorpha:* efficient secretion of glucoamylase. Biotechnology 9:291–295
10. Gellissen G, Piontek M, Dahlems U, Jenzelewski V, Gavagan JE, DiCosimo R, Anton DL, Janowicz ZA (1996) Recombinant *Hansenula polymorpha* as a biocatalyst: coexpression of the spinach glycolate oxidase (*GO*) and the *S. cerevisiae* catalase T (*CTT1*) gene. Appl Microbiol Biotechnol 46:46–54
11. Hanahan D (1983) Studies on transformation of *E. coli* with plasmids. J Mol Biol 166:557–580
12. Hilborn JW, Lu ZH, Jurgens AR, Fang QK, Byers P, Wald SA, Senanayake CH (2001) A practical asymmetric synthesis of (R)-fluoxetine and its major metabolite (R)-norfluoxetine. Tetrahedron Lett 42:8919–8921
13. Hummel W (1997) New alcohol dehydrogenases for the synthesis of chiral compounds. Adv Biochem Eng Biotechnol 58:145–184
14. Hummel W, Abokitse K, Drauz K, Rollmann C, Gröger H (2003) Towards a large-scale asymmetric reduction process with isolated enzymes: expression of an (S)-alcohol dehydrogenase in *E. coli* and studies on the synthetic potential of this biocatalyst. Adv Synth Catal 345:153–159
15. Klabunde J, Kunze G, Gellissen G, Hollenberg CP (2003) Integration of heterologous genes in several yeast species using vectors containing a *Hansenula polymorpha*-derived rDNA-targeting element. FEMS Yeast Res 4:185–193
16. Kosjek B, Stampfer W, Pogorevc M, Goessler W, Faber K, Kroutil W (2004) Purification and characterization of a chemotolerant alcohol dehydrogenase applicable to coupled redox reactions. Biotechnol Bioeng 86:55–62
17. Kunze G, Kunze I (1994) Characterization of *Arxula adeninivorans* strains from different habitats. Antonie Van Leeuwenhoek 65:607–614
18. Kunze I, Nilsson C, Adler K, Manteuffel R, Horstmann C, Bröker M, Kunze G (1998) Correct targeting of a vacuolar tobacco chitinase in *Saccharomyces cerevisiae*—post-translational modifications are dependent on the host strain. Biochim Biophys Acta 1395:329–344
19. Laemmli UK (1970) Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature (London) 227:680–685
20. Miozzari G, Niederberger P, Hütter R (1978) Permeabilization of microorganisms by Triton X-100. Anal Biochem 90:220–233
21. Patel RN (1997) Sterioselective biotransformations in synthesis of some pharmaceutical intermediates. Adv Appl Microbiol 43:91–140
22. Robinson JS, Klionsky DJ, Banta LM, Emr SD (1988) Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol Cell Biol 8:4936–4948
23. Rose MD, Winston F, Hieter P (1990) Methods in yeast genetics. A laboratory manual. Cold Spring Harbor Laboratory: Cold Spring Harbor, New York
24. Rösel H, Kunze G (1998) Integrative transformation of the dimorphic yeast *Arxula adeninivorans* LS3 based on Hygromycin B resistance. Curr Genet 33:157–163
25. Sambrook J, Russell DW (eds) (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
26. Samsonova IA, Kunze G, Bode R, Böttcher F (1996) A set of genetic markers for the chromosomes of the imperfect yeast *Arxula adeninivorans*. Yeast 12:1209–1217
27. Stampfer W, Kosjek B, Moitzi C, Kroutil W, Faber K (2002) Biocatalytic asymmetric hydrogen transfer. Angew Chem Int Ed Engl 41:1014–1017
28. Stampfer W, Kosjek B, Kroutil W, Faber K (2003) On the organic solvent and thermostability of the biocatalytic redox system of *Rhodococcus ruber* DSM 44541. Biotechnol Bioeng 81:865–869
29. Steinborn G, Wartmann T, Gellissen G, Kunze G (2007) Construction of an *Arxula adeninivorans* host-vector system based on *trp1* complementation. J Biotechnol 127:392–401
30. Steinborn G, Gellissen G, Kunze G (2007) A novel vector element providing multicopy vector integration in *Arxula adeninivorans*. FEMS Yeast Res 7:1197–1205
31. Tanaka A, Ohnishi N, Fukui S (1967) Studies on the formation of vitamins and their function in hydrocarbon fermentation. Production of vitamin B6 by *Candida albicans* in hydrocarbon medium. J Ferment Technol 45:617–623
32. Terentiev Y, Pico AH, Böer E, Wartmann T, Klabunde J, Breuer U, Babel W, Suckow M, Gellissen G, Kunze G (2004) A wide-range integrative yeast expression vector system based on *Arxula adeninivorans*-derived elements. J Ind Microbiol Biotechnol 31:223–228
33. Wartmann T, Stoltenburg R, Böer E, Sieber H, Bartelsen O, Gellissen G, Kunze G (2003) The *ALEU2* gene—a new component for an *Arxula adeninivorans*-based expression platform. FEMS Yeast Res 3:223–232
34. Wartmann T, Bellebna C, Böer E, Gellissen G, Kunze G (2003) The constitutive *AHSB4* promoter—a novel component of the *Arxula adeninivorans*-based expression platform. Appl Microbiol Biotechnol 62:528–535