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Coexpression of *Lactobacillus brevis ADH* with *GDH* or *G6PDH* in *Arxula adeninivorans* for the synthesis of 1-(R)-phenylethanol

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Abstract The yeast *Arxula adeninivorans* was used for the overexpression of an *ADH* gene of *Lactobacillus brevis* coding for (R)-specific alcohol dehydrogenase (LbADH) to synthesise enantiomerically pure 1-(R)-phenylethanol. *Glucose dehydrogenase* gene from *Bacillus megaterium* (*BmGDH*) or *glucose 6-phosphate dehydrogenase* of *Bacillus pumilus* (*BpG6PDH*) were coexpressed in *Arxula* to regenerate the cofactor NADPH by oxidising glucose or glucose 6-phosphate. The yeast strain expressing LbADH and BpG6PDH produced 5200 U l^{-1} ADH and 370 U l^{-1} G6PDH activity, whereas the strain expressing LbADH and BmGDH produced 2700 U l^{-1} ADH and 170 U l^{-1} GDH activity. However, the crude extract of both strains reduced 40 mM acetophenone to pure 1-(R)-phenylethanol with an enantiomeric excess (ee) of >99 % in 60 min without detectable by-products. An increase in yield was achieved using immobilised crude extracts (IEs), Triton X-100 permeabilised cells (PCs) and permeabilised immobilised cells (PICs) with PICs being most stable with GDH regeneration over 52 cycles. Even though the activity and synthesis rate of 1-(R)-phenylethanol with the *BpG6PDH* and *LbADH* coexpressing strain was higher, the BmGDH–LbADH strain was more stable over successive reaction cycles. This, combined with its higher total turnover number (TTN) of 391 mol product per mole $NADP^+$, makes it the preferred strain for continuous reaction systems. The initial non-optimised semi-continuous reaction produced 9.74 g l^{-1} day^{-1} or 406 g kg^{-1} dry cell weight (dcw) day^{-1} isolated 1-(R)-phenylethanol with an ee of 100 % and a TTN of 206 mol product per mole $NADP^+$. In conclusion, *A. adeninivorans* is a promising host for LbADH and BpG6PDH or BmGDH production and offers a simple method for the production of enantiomerically pure alcohols.

Keywords 1-(R)-Phenylethanol · *Lactobacillus brevis* ADH · *Bacillus megaterium* GDH · *Bacillus pumilus* BpG6PDH · *Arxula adeninivorans* · Cell permeabilisation · Calcium alginate immobilisation

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Introduction

Although two enantiomers of a chiral compound have the same structure and physical and chemical properties, they have different biological activities depending on their stereochemistry, which makes the use of enantiomerically pure alcohols necessary for use in the fine chemical and pharmaceutical industries (Meyer 2010; Wohlgemuth 2011).

Enantiomerically pure alcohols, which cannot be produced by chemical synthesis, can be synthesized by biocatalysts such as alcohol dehydrogenases (ADH), which react stereospecifically (Hummel 1990; Leuchs and Greiner 2011; Wolberg et al. 2000; Daußmann et al. 2006; Rosen et al. 2004). The easiest and oldest method for producing ADHs is through the use of wild-type cells of an organism producing it innately. This avoids compliance with the regulations governing the use of genetically modified organisms but has the problem of low yields. (S)-configured 1-arylethanols can be synthesized by fermentative reductions by *Saccharomyces cerevisiae*, but the yields are only low to moderate with an enantiomeric excess

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(ee) of 82 to 96 % (Csuk and Glanzer 1991; Aragozzini et al. 1968; Meese 1986).

Higher yields and product purities have been obtained using genetically modified organisms overexpressing an *ADH* gene or enzymes isolated from those organisms. Bacterial ADHs are most often produced in *Escherichia coli* (Isotani et al. 2012; Zhou et al. 2013; Li et al. 2012) due to its capability to produce proteins in high concentrations, in a short time and at low cost. In addition, endogenous ADHs have low activities so that modified *E. coli* can be easily used as whole-cell catalysts with high yields and ee without the problem of by-products (Hildebrandt et al. 2002).

Rarely are yeasts used as a host system for bacterial proteins, because they need more complex media and grow more slowly. However, the non-conventional yeast *Arxula adeninivorans* is an excellent host for the production of a variety of proteins, which is attributed to its excellent growth characteristics and its ability to use a wide spectrum of carbon and nitrogen sources.

A. adeninivorans has already been used for the expression of the *ADH* gene of *Rhodococcus ruber* and a recombinant strain coexpressing RrADH and *Bacillus megaterium* GDH was used for the synthesis of enantiomerically pure 1-(S)-phenylethanol (Giersberg et al. 2012; Rauter et al. 2014a).

In the present work, the (R)-selective ADH of *Lactobacillus brevis* was expressed in *Arxula* for the synthesis of enantiomerically pure 1-(R)-phenylethanol. ADH from *L. brevis* is a versatile enzyme, which catalyzes the reduction of carbonyl compounds stereoselectively by oxidising the co-factor NADPH. It is currently used for the synthesis of methyl (R)-3-hydroxybutanoate, tert-butyl (S)-6-chloro-5-hydroxy-3-ketohexanoate and fluorinated hydrins (Wolberg et al. 2001, 2008; Borzęcka et al. 2013). It has a broad substrate spectrum which includes aromatic ketones and keto-esters and is the reason for the growing interest in the ADH of *L. brevis* as a catalyst for other redox reactions (Leuchs and Greiner 2011).

Different cofactor regeneration systems such as GDH of *B. megaterium* and G6PDH of *Bacillus pumilus*, which recycle NADPH using glucose or glucose 6-phosphate as substrates (Kizaki et al. 2001; Weckbecker and Hummel 2005a) were used by coexpressing these genes with LbADH in *A. adeninivorans*. The resulting crude extract (CE), immobilised crude extract (ICE), permeabilised cells (PCs) and permeabilised immobilised cell (PIC) of the yeast strains were analysed for stability to establish their suitability for industrial application and a semi-continuous reactor system was tested.

Materials and methods

Chemicals

1-Phenylethanol, 1-(S)-phenylethanol and 1-(R)-phenylethanol were purchased from Acros (Germany). Acetophenone was from Sigma (Germany). $NADP^+$, NADPH, calcium alginate, calcium hydroxide and glucose 6-phosphate were purchased from Roth (Germany). Glucose and calcium chloride (water-free) was from Applichem (Germany). 1-Heptanol was purchased from Merck (Germany).

Strains and culture conditions

E. coli XL1 blue [*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [F'*proABlacI* q Z DM15 Tn10 (Tetr)], obtained from Invitrogen (USA), served as the host strain for bacterial transformation and plasmid isolation. The strain was grown on LB medium (Sigma, USA) supplemented with 75 mg l^{-1} ampicillin (Applichem, Germany) or 75 mg l^{-1} kanamycin (Roth, Germany) for selection.

The wild-type *Arxula* strain, LS3, was originally isolated from wood hydrolysate in Siberia and deposited as *A. adeninivorans* SBUG 724 into the strain collection of the Department of Biology of the University of Greifswald (Kunze and Kunze 1994). Auxotrophic mutants *A. adeninivorans* G1212 [*aleu2 ALEU2::atrp1*] (Steinborn et al. 2007), G1216 [*aleu2 ALEU2::aade2*] (Alvaro-Benito et al. 2012) and MS1006 [*aleu2 ALEU2::atrp1 ALEU2::aade2*] (Rauter et al. 2013) were used as host cells. All strains were grown in 100 ml of broth in 500-ml shake flasks at 30 °C, 180 rpm, either under non-selective conditions in a complex medium (YEPD) or selective conditions in yeast minimal medium supplemented with 20 g l^{-1} glucose as carbon source and 43 mM $NaNO_3$ (YMM–glucose–$NaNO_3$) as nitrogen source (Tanaka et al. 1967; Rose et al. 1990). Agar plates were prepared by adding 16 g l^{-1} agar to the liquid medium.

Transformation procedures, recovery of stable *A. adeninivorans* strains and isolation of nucleic acids

E. coli and *A. adeninivorans* cells were transformed according to Böer et al. (2009a). Stable yeast transformants were obtained after passaging on selective and non-selective media (Klabunde et al. 2003). MS1006/YRC104-BmGDH–YRC102-2LbADH (strain A) and MS1006/YRC104-BpG6PDH–YIC102-2LbADH (strain B) transformants were stabilised twice, once after transformation with the gene of the regenerating enzyme and then after integration of *LbADH* into the yeast genome. Isolation of plasmid DNA and DNA restriction were carried out as described previously (Wartmann et al. 2003).

Construction of *LbADH* and *BpG6PDH* expression plasmids

Plasmids were constructed to enable the expression of *LbADH* (accession no. LK055285) and *BpG6PDH* (accession no.

LK055287) genes in *A. adeninivorans* G1212 or G1216. The open reading frame (ORF) (using optimized codon usage for *A. adeninivorans*), fused with a His-tag encoding sequence at the 3′ end and *Eco*RI and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, was purchased from GeneArt. PCR was done with gene-specific primers on the GeneArt template to produce the ORF of *LbADH* gene and *BpG6PDH* gene without a His-tag (LbADH: primer 1, 5′-GCC**GAATTC**ATGTCT AACCGACTG-3′, nucleotide positions 1–15, *Eco*RI restriction site in bold type, and primer 2, 5′-GCC**GGATCC**CTAC TGAGCAGTG-3′, nucleotide positions 756–777, *Bam*HI restriction site in bold type; BpG6PDH: primer 1, 5′-GCC**GAATTC**ATGAACACCGAGACT-3′, nucleotide positions 1–15, *Eco*RI restriction site in bold type, and primer 2, 5′-GCC**GGATCC**CTAGATGTTCCACC-3′, nucleotide positions 1472–1494, *Bam*HI restriction site in bold type).

The *Eco*RI-*Bam*HI gene fragments, which correspond to the complete ORF, were inserted into the plasmid pBS-TEF1-PHO5-SS (flanked by *Spe*I-*Sac*II restriction sites) and in the case of LbADH also into pBS-TEF1-PHO5-SA (flanked by *Apa*I-*Sal*I restriction sites) between the *A. adeninivorans*-derived *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator (Böer et al. 2009b).

Construction of plasmids with the different expression modules, required the insertion of *TEF1* promoter–gene–*PHO5* terminator flanked by *Spe*I-*Sac*II and in LbADH, *Apa*I-*Sal*I restriction sites, into the Xplor2.2 or 2.4 plasmid to generate Xplor2.2-TEF1-(2)LbADH-PHO5 and Xplor2.4-TEF1-BpG6PDH(−6H)-PHO5. The 25S ribosomal DNA (rDNA) target sequences were interrupted by the selection marker modules (*ATRP1m* for Xplor2.2 and *AADE2* for Xplor2.4) and *Eco*47III, *Spe*I, *Sac*II, *Sal*I, *Apa*I multicloning restriction sites for insertion (Alvaro-Benito et al. 2012). Xplor2.2-2TEF1-LbADH-PHO5 was constructed by inserting the LbADH expression module flanked by *Apa*I-*Sal*I into Xplor2.2-TEF1-LbADH-PHO5.

Xplor2.2-TEF1-(2)LbADH-PHO5 and Xplor2.4-TEF1-BpG6PDH-PHO5 or Xplor2.4-TEF1-BmGDH-PHO5 (Rauter et al. 2014a) were used to coexpress *LbADH* and *BmGDH* (accession no. LK055286) or *BpG6PDH* gene in *A. adeninivorans* MS1006.

Screening of *LbADH*, *BpG6PDH* and *BmGDH* expressing transformants

After stabilization, transformants were cultivated for 48 h in 1.5 ml YMM–glucose–$NaNO_3$ at 30 °C and 200 rpm. One milliliter of culture was harvested (4600×*g*, 5 min), and the pellet was washed with water. The cells were disrupted in 100 mM sodium phosphate buffer pH 7.5 by silica beads using Mixer Mill MM400 (RETSCH) for 3 min, vibrational frequency of 30 s^{-1}. After elimination of cell debris (10 min, 16000xg), soluble intracellular proteins in the supernatant were tested for ADH and GDH or G6PDH activity as described in "Assay for determination of ADH, GDH and G6PDH activity".

Production of crude extracts

The production of LbADH, BpG6PDH and BmGDH was achieved by culturing strains A, B, C (G1212/YRC102-LbADH) and D (G1216/YRC104-BpG6PDH), in 100-ml YEPD shake flasks for 48 h at 30 °C, 180 rpm. Cells were harvested by centrifugation (5 min, 5000×*g*) and washed with water and were mechanically disrupted in 10 ml (1/10 of the culture volume) of 20 mM sodium phosphate buffer pH 6.5 as described in "Screening of *LbADH*, *BpG6PDH* and *BmGDH* expressing transformants".

Assay for determination of ADH, GDH and G6PDH activity

The assay for the determination of the ADH oxidation activity was performed in 50 mM sodium phosphate buffer pH 7.5 containing the substrate 1-phenylethanol (10 mM) and $NADP^+$ (1 mM). The activity of the best-performing transformants (see "Coexpression of *LbADH* with *BmGDH* or *BpG6PDH* in *A. adeninivorans*") over different incubation periods was determined for the reduction reaction with acetophenone (10 mM) and NADPH (0.4 mM) in sodium phosphate buffer pH 6.5. The reaction was monitored for 2.5 min at 340 nm, 30 °C. The change in absorbance was then converted to reduced $NADP^+$ or oxidised NADPH concentration using the Beer–Lambert equation (ε=6200 M^{-1} cm^{-1} for NADPH).

To establish GDH and G6PDH activity, 100 mM glucose or 100 mM glucose 6-phosphate and 1 mM $NADP^+$ in 50 mM sodium phosphate buffer pH 7.5 were used unless stated otherwise. Reactions were started and measured as for ADH activity. Coexpression activity was determined in 50 mM sodium phosphate buffer pH 6.5. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 µmol $NADP^+$ or oxidise NADPH per minute at 30 °C.

Enzymatic synthesis of 1-(R)-phenylethanol

The enzymatic synthesis of 1-(R)-phenylethanol was done at 30 °C in 500 µl of 50 mM Tris–HCl pH 8. Twenty millimolar acetophenone, 1 mM $NADP^+$, 1 mM $MgCl_2$, and 100 mM glucose or glucose 6-phosphate were added to permit regeneration with BmGDH or BpG6PDH. Reactions were started by the addition of enzymes, and at intervals, substrate and product were extracted from 400 µl reaction mixture with 1/5 volume ethyl acetate. The phases were separated by centrifugation for 5 min at 16,000×*g*, and the upper phase was

analysed by GC-FID (Gas chromatography- Flame ionisation detector, colum:Supelco β-DEX™ 110 L×I.D. 30 m× 0.25 mm, d_f 0.25 μm, Sigma, Germany). After 1 min at 110 °C, the temperature was ramped to 160 °C at 5 °C min^{-1}. Detection temperature was 220 °C. The % content of 1-(R)-phenylethanol was calculated from the ratio of acetophenone to 1-(R)-phenylethanol recovered. The R-configuration assessment of the enzymatically obtained 1-phenylethanol was made by comparison with 1-(S)-phenylethanol and 1-(R)-phenylethanol from Acros (Germany), which had retention times of 8.8 and 8.6 min, respectively.

Cell permeabilisation and immobilisation

Yeast cell permeabilisation by Triton X-100 was a modification of the method used by Miozzari et al. (1978). Cells were harvested (5 min, 5000×*g*), and the washed cell pellet was resuspended at an OD_{600} of 20 in 100 mM sodium phosphate buffer pH 7.5+0.05 % Triton X-100 concentrations. The suspension was frozen in liquid nitrogen and stored at −20 °C overnight. Before use, the cells were thawed at room temperature, centrifuged (5 min, 5000×*g*) and washed once with water.

Immobilisation of CE and PCs used a procedure that is a modification of the method described by Jobanputra et al. (2011), Talekar and Chavare (2012) and Anwar et al. (2009). CE or PCs were mixed 1:1 with 3 % (*w*/*v*) sodium alginate and dropped in 50-μl volumes into cold stirred 1 % $CaCl_2$. Curing time was 60 min for crude extract and 150 min for PCs. The drops were collected in a suction filter.

Time course analysis of 1-(R)-phenylethanol synthesis by CE, ICE, PCs and PICs

All synthesis of 1-(R)-phenylethanol was done in a 500 μl reaction volume. Crude extract was prepared by disrupting 256 mg dry cell weight (dcw) in 4 ml 20 mM sodium phosphate pH 6.5 as described in "Production of crude extracts", and 2 ml of the crude extract was used for immobilisation. A cell pellet of 256 mg dcw was permeabilised and resuspended in 4 ml water, and 2 ml of the suspension was immobilised as described in "Cell permeabilisation and immobilisation".

Two-hundred-microliter crude extract, 250 μl PCs or 10 beads of PICs and IE (which are all equivalent to 16 mg dcw cells) were used for synthesis reactions with 40 mM acetophenone, 200 mM glucose or 200 mM glucose 6-phosphate, 200 mM BIS-TRIS pH 7, 1 mM $MgCl_2$ and 1 mM $NADP^+$ as substrates. At various time intervals, soluble fractions of the reactions were prepared for 1-(R)-phenylethanol determination as described in "Enzymatic synthesis of 1-(R)-phenylethanol".

Determination of process stability

PCs, PICs and IEs were prepared as described in "Time course analysis of 1-(R)-phenylethanol synthesis" and used repeatedly over 1.5 h for the synthesis of 1-(R)-phenylethanol.

After each reaction cycle, immobilisates were collected in a suction filter and washed with distilled water. PCs were washed once with 1 ml water and centrifuged (5000×*g*, 5 min).

The synthetic activity of the enzyme preparations were determined by taking 200 μl of the 700 μl of the reaction solution after 15 min if regeneration was done by BpG6PDH or after 20 min with BmGDH regeneration and acetophenone and 1-(R)-phenylethanol content was measured by GC-FID analysis as described in "Enzymatic synthesis of 1-(R)-phenylethanol". The percentage of 1-(R)-phenylethanol produced in the time interval was compared with that of the first cycle and expressed as a % of the initial synthetic activity.

Semi-continuous synthesis of 1-(R)-phenylethanol

Semi-continuous synthesis of 1-(R)-phenylethanol was done using PICs of strain A (see "Cell permeabilisation and immobilisation"). PCs-sodium-alginate mixture at an $OD_{600\ nm}$ of 100 was dropped through an aperture with 25 funnels, each of 1.2-mm diameter, into a stirred 1 % $CaCl_2$ solution. The beads were washed and mixed with the reaction solution containing 40 mM acetophenone, 200 mM glucose, 200 mM BIS-TRIS pH 7, 1 % $CaCl_2$, 1 mM $MgCl_2$ and 0.25 mM $NADP^+$. The beads and reaction solution was poured, avoiding air bubbles, into a column and incubated for 60 min at room temperature. Additional reaction mixture (700 ml) was then pumped through the column at a flow of 0.55 ml min^{-1} using a peristaltic pump. Exhausted reaction mixture exited the column through a tube at its lower end and entered the bottom of a second column filled with dichloromethane. This arrangement allowed the product and not reduced acetophenone to be extracted (Fig. 1). The exhausted reaction mixture was distilled with a rotary evaporator to eliminate dissolved dichloromethane and was recycled by the addition of acetophenone and glucose at the concentration of synthesized 1-(R)-phenylethanol and calcium hydroxide at half the concentration of formed product for pH stabilization, before using it for next reaction cycle.

After 300 h, dichloromethane from the second column was distilled and product was analyzed via GC-FID as described in "Enzymatic synthesis of 1-(R)-

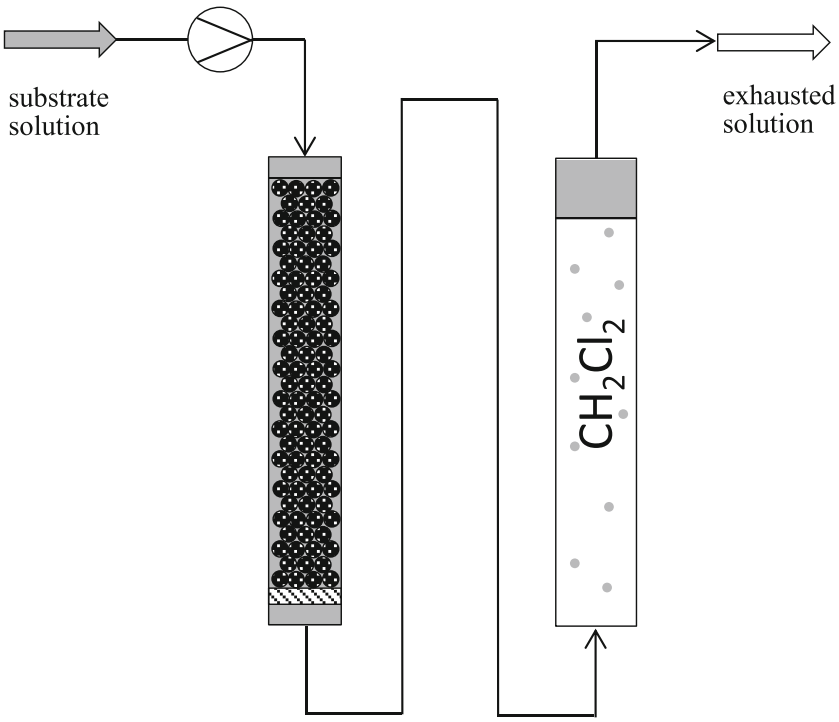

Fig. 1 Reactor design for semi-continous synthesis of 1-(R)-phenylethanol

phenylethanol". Quantification was performed with 1-heptanol as an internal standard.

Results

Expression of *LbADH* and *BpG6PDH* in *A. adeninivorans*

The Xplor®2 transformation/expression platform was used for the generation of yeast strains producing LbADH, BmGDH and BpG6PDH because it allows the construction of resistance marker-free transformants (Böer et al. 2009a).

BpG6PDH and LbADH producing yeast strains were produced as described by Rauter et al. (2014a). This process involves the ligation of the *LbADH* gene and ATRP1m or *BpG6PDH* gene and AADE2 selection marker module. Linearized DNA containing the expression and selection marker module without rDNA sequences (YIC102-(2)LbADH, YIC104-BpG6PDH(−6H)) or with rDNA sequences (YRC102-(2)LbADH, YRC104-BpG6PDH(−6H)) were integrated into the genome of the auxotrophic mutant strains *A. adeninivorans* G1212 (*atrp1*) or G1216 (*aade2*) (Fig. 2).

Both cassettes were successfully transformed into the parent yeast strains and stabilized by passaging (see "Transformation procedures, recovery of stable *A. adeninivorans* strains and isolation of nucleic acids" and "Construction of LbADH and BpG6PDH expression plasmids").

All yeast strains were grown in YMM–glucose–$NaNO_3$ at 30 °C for 48 h. Up to 1100 U l^{-1} culture (0.7 U mg^{-1} protein) ADH activity was detected in the soluble extract of strain C but not in that of the G1212/YRC102 cells. Activity of crude extract from BpG6PDH expressing strains was up to 1200 U l^{-1} culture (1.6 U mg^{-1} protein) irrespective of the presence of a His-tag on C-terminus. This was three times higher than the activity seen in the soluble extract from the control strain G1216/YRC104 (430 U l^{-1} culture).

Synthesis of 1-(R)-phenylethanol

Glucose dehydrogenase from *B. megaterium* and glucose 6-phosphate dehydrogenase from *B. pumilus* were tested as NADPH regeneration systems.

Coexpression of LbADH with BmGDH or BpG6PDH in A. adeninivorans

Cell-based production of 1-(R)-phenylethanol is less complex if both LbADH and a gene for a regenerating enzyme (BmGDH or BpG6PDH) are coexpressed in one cell so that only one growth and disruption step is required.

The double-mutant *A. adeninivorans* MS1006 [*atrp1*, *aade2*] was cotransformed with YRC104-BmGDH or YRC104-BpG6PDH and YIC/YRC102-2LbADH or YIC/YRC102-LbADH (Fig. 2, Rauter et al. 2013) to create recombinant yeast strains containing one or two copies of the LbADH and either a BmGDH or BpG6PDH expression module.

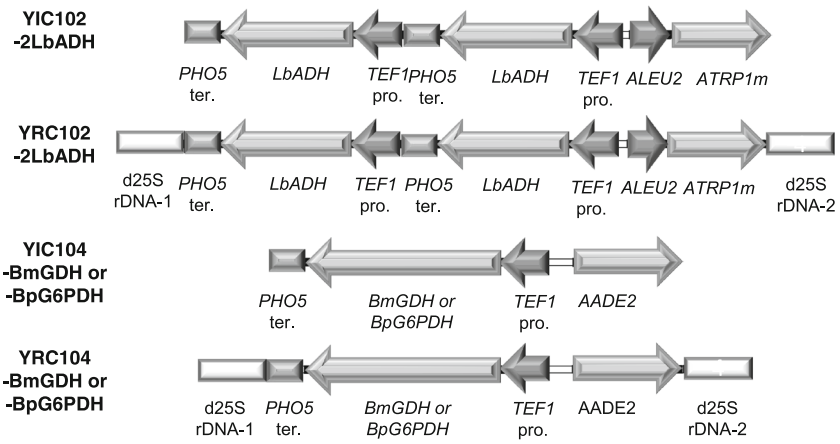

Fig. 2 Physical maps of the YRC and YIC used for transformation of *A. adeninivorans* MS1006. Cassettes contain the selection marker *ATRP1m* fused to the *ALEU2* promoter together with one or two copies of the expression module (YIC102-2LbADH, YRC102-2LbADH) *TEF1* promoter–*LbADH* gene–*PHO5* terminator or *AADE2* (YIC104-BmGDH/BpG6PDH, YRC104-BmGDH/BpG6PDH) with *TEF1* promoter–*BmGDH/BpG6PDH* gene–*PHO5*. 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

Crude extracts from stabilised transformants were screened for ADH and GDH or G6PDH activity. Transformants synthesising both enzymes were able to oxidise 1-phenylethanol and glucose or glucose 6-phosphate with $NADP^+$. The activity of the control cells, MS1006/YRC104 – YRC102, transformed with empty plasmids showed either no activity or in the case of G6PDH, two to three times lower activity.

Strains A and B which had 2.2 and 3.67 U mg^{-1} LbADH activity in their crude extracts were grown on YEPD at 30 °C for 96 h and the dcw, ADH, GDH or G6PDH activity and the yield Y(P/X) were determined each day (Fig. 3).

ADH and BpG6PDH activities for strain B were twice the level in strain A. The latter achieved a maximum 2700 U l^{-1} culture (330 U g^{-1} dcw) for ADH at 24 h of incubation, whereas the activity of strain B continued to increase to a maximum of 5200 U l^{-1} (450 U g^{-1} dcw) at 48 h and then remained constant until the end of the experiment (Fig. 3a, c). Dry cell weight increased slightly from the first to the second day and then remained constant (Fig. 3b, d). The activity of the regenerating enzymes was considerably lower than that of ADH with 170 U l^{-1} culture (16 U g^{-1} dcw) for GDH and 370 U l^{-1} (31 U g^{-1} dcw) for G6PDH activity. The GDH and G6PDH to ADH ratios were 1 to 16 and 1 to 14, respectively.

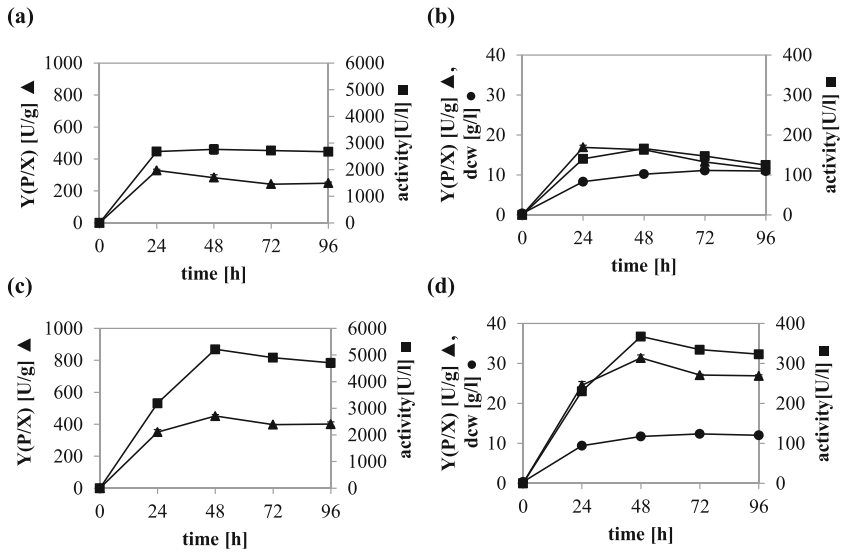

Fig. 3 Time course plots of ADH and GDH or G6PDH activities of transgenic *A. adeninivorans* strains. The transformants were cultured in shake flasks for 96 h in YEPD at 30 °C, 180 rpm. At the indicated times, 2 ml aliquots of the culture were used for determination of biomass (dcw in g l^{-1}), assay the intracellular soluble ADH and GDH or G6PDH activity (U l^{-1} culture) and to calculate ADH and GDH or G6PDH production, Y (P/X) (U g^{-1}dcw). ADH activity and Y (P/X) for strain A are shown in **a** and for strain B in **c**; dcw and GDH activity of strain A are shown in **b** and dcw and G6PDH activity of strain B **d**

1-(R)-Phenylethanol synthesis with different enzyme preparations

Acetophenone reduction is limited by its low solubility in water, so reusability of the enzymes is necessary to gain higher product yields in a one-phase water system. CE, IE, PCs and PICs of the two yeast strains A and B were tested for the synthesis of 1-(R)-phenylethanol. A dcw of 16 mg was used in 500 µl reactions, and the reaction was monitored over time (Fig. 4).

CE, PCs and PICs of strain A reduced acetophenone more rapidly than IE (4.4, 3.9 and 4.1 M product g^{-1} dcw h^{-1} and 3.2 M g^{-1} dcw h^{-1}, respectively), i.e. a 52–58 % and a 43 % conversion in a 20 min incubation (Fig. 4a).

Acetophenone reduction by CE and PCs from strain B had similar efficiencies with 6.4 and 5.9 M 1-(R)-phenylethanol g^{-1} dcw h^{-1} with over 90 % of the substrate being converted to 1-(R)-phenylethanol in 20 min. IE catalysed the reduction of 4.7 M acetophenone g^{-1} dcw h^{-1} (80 % conversion), whereas PICs were slowest with 2.4 M g^{-1} dcw h^{-1} (50 % conversion) in 20 min (Fig. 4b). After 60 min, 98 % of the substrate was converted to 1-(R)-phenylethanol (ee >99 %) by all preparations.

Strain B, using G6PDH-coupled regeneration, had on average 1.5 times higher activity compared to strain A coexpressing GDH. Maximum activities of 6.4 M g^{-1} dcw h^{-1} for strain B and 4.4 M g^{-1} dcw h^{-1} for strain A were obtained. The stability of the different enzyme combinations was assessed (Fig. 5).

All immobilisates were stabilized by the addition of 1 % $CaCl_2$. Highest stability was found for strain A as PICs which had an activity of over 80 % for 52 cycles, whereas strain B catalyzed reactions could only be repeated 14 times. PCs of strain A could be used 30 times, whereas activity ceased within 14 cycles for IE of the strain A (Fig. 5a). IE and PCs of strain B were 4.6 times less stable with an initial activity of more than 80 % for three reactions (Fig. 5b) but decreasing slowly over the next 8 or 16 cycles, respectively, until 1-(R)-phenylethanol was no longer synthesised. PICs showed an activity increase from 100 to 220 % after the first reaction,

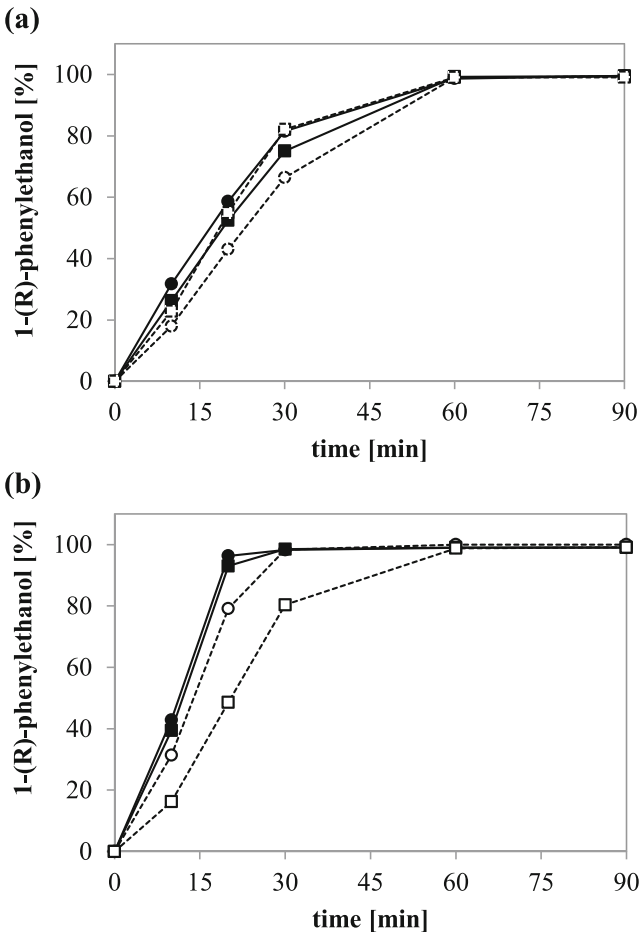

Fig. 4 Synthesis of 1-(R)-phenylethanol by reduction of 40 mM acetophenone. Enzyme preparations of **a** strain A and **b** strain B. CE (*black circles*, *solid line*), IEs (*white circles*, *dashed line*), PCs (*black squares*, *solid line*) and PICs (*white squares*, *dashed line*)

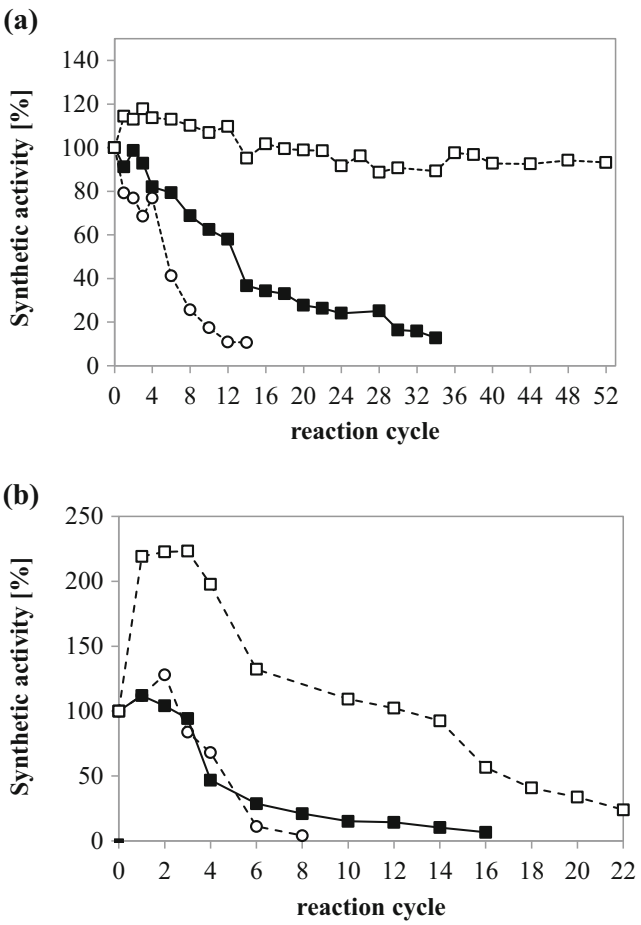

Fig. 5 Process stability of different enzyme preparations of strain A (**a**) and strain B (**b**). Synthetic activity after each cycle of synthesis was determined; IEs (*white circles*, *dashed line*), PCs (*black squares*, *solid line*) and PICs (*white squares*, *dashed line*). The synthetic activity before starting synthesis reactions was set to 100 %

which remained constant for 3 cycles and decreased within 12 cycles to 100 %. After 22 cycles, all immobilisates were practically inactive.

Efficiency of cofactor regeneration by GDH and G6PDH

Synthesis of 1-(R)-phenylethanol was done with different activities of ADH and GDH or G6PDH in crude extracts from strain C and G1216/YRC104-BmGDH or strain D (Fig. 6). Regeneration by BpG6PDH is more efficient with up to 90 % 1-(R)-phenylethanol formed in 10 min. BmGDH regeneration yield was 66 % in the same time.

Acetophenone reduction increased with the increase of ADH or BmGDH/BpG6PDH activity. This increase is more rapid with BpG6PDH than BmGDH, although product formation is nearly the same in the saturation phase.

PICs of strain A and B were used for synthesis of 1-(R)-phenylethanol with different cofactor concentrations (Tables 1 and 2). The $NADP^+$ cofactor concentration was 1 mM; however, this could decrease to 0.2 or 0.1 mM with activity lowered to 71 and 47 % or 48 and 29 % in strain A and B, respectively. Simultaneously TTN increases from 39 to 391 or 388 in strain A and B, respectively, because same product concentration is formed after 24 h with less cofactor. An even lower $NADP^+$ concentration of 0.01 mM increases TTN further with up to 871 and 2066 for strain A and B, but the activity is lower with 10 or 5 %, respectively.

In conclusion, product formation is faster if BpG6PDH is used as regeneration system. However, strain A reduces $NADP^+$ more efficiently at low concentrations (0.1 mM) whereas strain B needs double cofactor concentrations to achieve the same synthetic activity. The use of 0.1 mM $NADP^+$ increases the TTN of around 40 to 400.

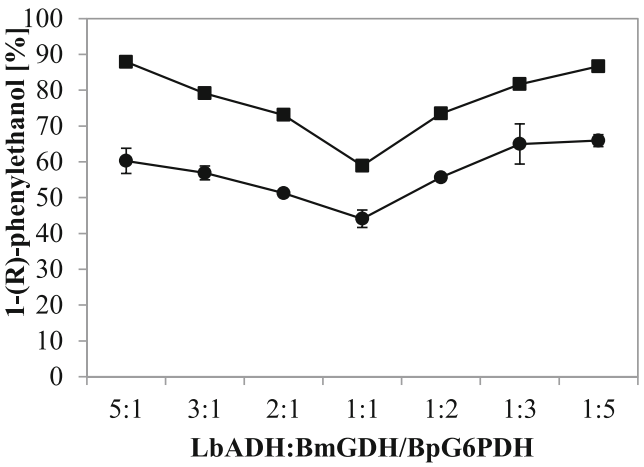

Fig. 6 Efficiency of BmGDH and BpG6PDH regeneration. Synthesis of 1-(R)-phenylethanol by different ADH and BmGDH (*black circles*) or BpG6PDH (*black squares*) concentrations of crude extract in 10 min. Activities of 0.5 U BmGDH or BpG6PDH and 0.5 U LbADH were used as 1:1 and were increased in 0.5 U steps

Table 1 Activity, conversion and TTN of acetophenone reduction by PICs of strain A

$NADP^+$ (mM)	Activity (U g^{-1} dcw)	Activity (%)	1-(R)-Phenylethanol (mM)	TTN
1	49.6	100.0	39.3±0.2	39
0.2	35.4	71.3	39.1±0.2	196
0.1	23.7	47.7	39.1±0.1	391
0.05	16.0	32.3	16.5±0.3	329
0.01	4.8	9.7	8.7±0.1	871

Semi-continuous synthesis of 1-(R)-phenylethanol by PICs of strain A

Synthesis of 1-(R)-phenylethanol was performed semi-continuously by PICs of strain A as described in "Materials and methods". Cofactor concentration was reduced from 1 to 0.25 mM.

Conversion of acetophenone was 98 % after 60 min pre-incubation of the immobilisates with substrate solution. Continuous flow was then initiated, and samples were taken daily from the dichloromethane column to track product enrichment. After 300 h, 38.5 g product was isolated from dichloromethane of which 12.78 g was 1-(R)-phenylethanol (33.2 %). The space-time yield (STY) was 9.74 g l^{-1} day^{-1} or 406 g kg^{-1} dcw day^{-1} and a TTN of 206 mol product per mole $NADP^+$ was achieved. The ee value was 100 %.

Discussion

The potential of *L. brevis* ADH for the synthesis of secondary alcohols was discovered by Riebel (1996) and has been used for a variety of industrial applications including biotransformations (Hummel and Riebel 1997; Hildebrand and Lütz 2006; Wolberg et al. 2000; Hummel 1997) reviewed by Leuchs and Greiner (2011). However, the capacity of an enzyme to synthesise is only one factor for its application, with productivity and low costs being the determining factors.

Table 2 Activity, conversion and TTN of acetophenone reduction by PICs of strain B

$NADP^+$ (mM)	Activity (U g^{-1} dcw)	Activity (%)	1-(R)-Phenylethanol (mM)	TTN
1	87.2	100.0	38.8±0.2	39
0.2	41.1	47.1	38.9±0.1	195
0.1	25.0	28.7	38.8±0.1	388
0.05	17.0	19.5	29.2±5.5	585
0.01	4.4	5.1	20.7±0.3	2066

In this work *LbADH* was expressed in the yeast *A. adeninivorans* and used for the synthesis of enantiomerically pure 1-(R)-phenylethanol.

Highest activity in cells that coexpress BmGDH or BpG6PDH for NADPH regeneration at pH 6.5 were 5200 U l^{-1} (450 U g^{-1} dcw) for ADH and 370 U l^{-1} (31 U g^{-1} dcw) for G6PDH activity. In contrast, ADH and GDH activities of strain A were only 2700 U l^{-1} (330 U g^{-1} dcw) and 170 U l^{-1} (16 U g^{-1} dcw), respectively. ADH activity was around 15 times higher than that of GDH or G6PDH. Weckbecker (2005) obtained the best yields of ADH from *Lactobacillus kefir*, which also had between 3 and 10 times higher activity than GDH from *Bacillus subtilis*.

The ADH activities attained by transgenic *A. adeninivorans* strains were 2 to 4 times higher than wild-type *L. kefir* (1200 U l^{-1} ADH) (Hummel 1997) and 15 to 28 times higher than RrADH producing *A. adeninivorans* strain (183 U l^{-1}) (Rauter et al. 2014a). Regenerating enzyme activity was 2.7 to 1.3 times lower with 170 or 370 U l^{-1}, respectively (Rauter et al. 2014a). Functionality of the different enzymes was not only determined by observing NADH oxidation assays, but also by product analysis with GC-FID. Acetophenone was reduced completely and enantiomerically pure 1-(R)-phenylethanol was detected.

There are no cross-reactions of the substrate acetophenone with *A. adeninivorans* wild-type ADHs. In addition $NADP^+$ reduction with G6PDH is supported by the yeast native enzymes, increasing G6PDH activity and regenerative capacity.

1-(R)-Phenylethanol synthesis by G6PDH-coupled regeneration was twice as fast as GDH-coupled regeneration because ADH and G6PDH activities in strain B were twice that of crude extract from strain A, which agrees with Hummel's findings (Hummel 1990), who obtained three times more 1-phenylethanol if $NADP^+$ was reduced by glucose 6-phosphate dehydrogenase than by glucose dehydrogenase.

In addition to the processes involved in the synthesis of 1-phenylethanol, the economics of the process must be considered. Enzymes and the cofactor $NADP^+$ used for LbADH conversions are expensive. A constraining factor in efficiency of production is the limitation imposed by the relative insolubility of 1-phenylethanol in aqueous solution, which is around 40 mM. Reactions in a water/organic solvent two-phase system could be an alternative to the water system, but often stability of cells is limited as observed by Weckbecker and Hummel (2005b).

So, CE, IE, PCs and IPCs were reused by centrifugation or filtration from the water system after one reaction cycle. The recycled catalysts were active and could be used for the reduction of 40 mM acetophenone with a yield of 98 % and an ee of >99 %. PICs were most stable with over 52 cycles for strain A and 14 cycles for strain B, which is in agreement with Rauter et al. (2014b), who used BmGDH and *R. ruber* ADH coexpressing yeast strain for up to 53 reaction cycles. The significantly lower stability of PCs and IEs compared to PICs seems to be due to the elution of enzymes with repeated use. PICs in contrast contain cells embedded in calcium alginate, so enzymes have to overcome two elution barriers, elution from cells and from calcium alginate.

The lower stability of the G6PDH-coupled regeneration was perhaps due to swelling of the immobilisates in G6PDH reaction mixture, which made them fragile and may have led to leaching of enzyme and instability of the regenerating enzyme. However, this was not observed in the GDH-coupled regeneration.

Both cofactor regeneration systems work well for NADPH recycling. BpG6PDH-coupled regeneration was more efficient than that of BmGDH. External $NADP^+$ is necessary for efficient synthesis, if PICs are used, which was also observed by Zhang et al. (2006). The lowering of the cofactor concentration from 1 to 0.2 for strain B and to 0.1 for strain A decreased activity to 47 and 48 %, respectively, with simultaneous increase of TTN from 39 to 195 and 391 and almost full conversion of acetophenone, respectively. Cell internal consumption of the cofactor could be decreased by knocking out other $NADP^+$-dependent enzymes meaning that lower $NADP^+$ concentrations could be used for synthesis. *E. coli* glucose 6-phosphate isomerase, glutamate dehydrogenase and phosphoenolpyruvate carboxylase, which require $NADP^+$ as a cofactor, were knocked out resulting in an increase in productivity by twofold to fourfold (Chemler et al. 2010).

Synthesis of 1-(R)-phenylethanol is faster with BpG6PDH as regeneration system, but twice the concentration of $NADP^+$ is needed for efficient regeneration. Due to the high cost of the cofactor, use of the cosubstrate glucose 6-phosphate and lower stability of PICs, strain A is favoured for industrial applications. Zhang et al. (2006) increased TTN from 59 to 402 by decreasing cofactor concentration with BpG6PDH regeneration, which is in the same range as our results. A further increase of TTN to more than 4000 was demonstrated by further decreasing the cofactor concentration and simultaneously increasing the cell density, but this led to a loss of activity of 90 %. In this work, a maximum TTN of 871 for strain A and 2066 for strain B was reached with activities of 10 and 5 % compared to higher cofactor concentrations. Zhang et al. (2009) obtained TTNs of up to 4200 with an initial addition of 0.005 mM $NADP^+$; however, the influence of cofactor concentration on synthetic activity was not discussed. Both authors used up to 60 mM substrate; however, acetophenone is not soluble at this concentration and the addition of organic solvents or the use of two-phase systems would be necessary to increase substrate concentration.

Alternative regeneration systems for NADH recycling are possible, however. Examples include changing the cofactor from the more expensive $NADP^+$ to NAD^+ is achievable through protein engineering (Machielsen et al. 2009) and the coexpression of pyridine nucleotide transhydrogenase, which reduces $NADP^+$ to NADH (Weckbecker and Hummel 2004).

The initial semi-continuous reaction process used 0.25 mM $NADP^+$ at the beginning to attain full ADH activity and achieved a TTN of 206. TTNs of 10^3 to 10^5 would be sufficient for a production process (Zhao and van der Donk 2003), which is five times more than for the process reported here. Additionally a STY of 9.74 g l^{-1} day^{-1} or 406 g kg^{-1} dcw day^{-1} was reached in this non-optimized semi-continuous process. Rissom et al. (1999) reported that *Rhodococcus erythropolis* ADH coupled with FDH yielded 88 g l^{-1} day^{-1} 1-(S)-phenylethanol, which is nine times higher than our productivity, and Hildebrand and Lütz (2006) obtained an even higher STY of 144 g l^{-1} day^{-1} 1-(R)-phenylethanol with *L. brevis* ADH.

However, improvement of the process is necessary for application in industrial production. Automated reverse flow extraction of the product and addition of new substrate to the reactor, with automated pH control is necessary to optimize the fermentation process. Expression levels in the yeast strain could be improved by the integration of more expression cassettes.

However, *A. adeninivorans* as presented here is an excellent host for the production of ADHs and 1-(R)-phenylethanol synthesis. An ee of 100 % was found for the isolated product, and no by-products were formed.

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