

Efficient Regeneration of NADPH Using an Engineered Phosphite Dehydrogenase

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ABSTRACT: The in situ regeneration of reduced nicotinamide cofactors (NAD(P)H) is necessary for practical synthesis of many important chemicals. Here, we report the engineering of a highly stable and active mutant phosphite dehydrogenase (12x-A176R PTDH) from *Pseudomonas stutzeri* and evaluation of its potential as an effective NADPH regeneration system in an enzyme membrane reactor. Two practically important enzymatic reactions including xylose reductase-catalyzed xylitol synthesis and alcohol dehydrogenase-catalyzed (R)-phenylethanol synthesis were used as model systems, and the mutant PTDH was directly compared to the commercially available NADP⁺-specific *Pseudomonas sp.* 101 formate dehydrogenase (mut Pse-FDH) that is widely used for NADPH regeneration. In both model reactions, the two regeneration enzymes showed similar rates of enzyme activity loss; however, the mutant PTDH showed higher substrate conversion and higher total turnover numbers for NADP⁺ than mut Pse-FDH. The space-time yields of the product with the mutant PTDH were also up to fourfold higher than those with mut Pse-FDH. In particular, a space-time yield of 230 g L⁻¹ d⁻¹ xylitol was obtained with the mutant PTDH using a charged nanofiltration membrane, representing the highest productivity compared to other existing biological processes for xylitol synthesis based on yeast D-xylose converting strains or similar in vitro enzyme membrane reactor systems.

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Introduction

Enzymes are versatile catalysts with the capacity to synthesize milligram amounts of valuable compounds or produce bulk chemicals on the thousands-of-tons scale industrially. With recent advances in genomics and metagenomics, numerous new enzymes have been discovered which may have synthetic applications. However, many of these synthetically useful enzymes are underutilized because they require one or more costly cofactors (Woodyer et al., 2004; Zhao and van der Donk, 2003). In particular, oxidoreductases catalyze a number of industrially interesting reactions such as the production of unnatural amino acids, polyols, and chiral alcohols, but often require nicotinamide (NADH or NADPH) as cofactors (Hummel and Kula, 1989; Krix et al., 1997). These nicotinamide cofactors, especially in their reduced form, are too expensive to be used in a stoichiometric amount and thus require in situ regeneration for large-scale synthesis.

A number of chemical, electrochemical, photochemical, and enzymatic methods for regenerating reduced nicotinamide cofactors NADH and NADPH have been established and recently reviewed (van der Donk and Zhao, 2003; Wichmann and Vasic-Racki, 2005). Compared to enzymatic methods, chemical, photochemical, and electrochemical methods often lack the high selectivity required to achieve high total turnover numbers. For example, if reduction of NAD(P)⁺ to NAD(P)H is 95% selective for hydride donation to the four position of the nicotinamide ring, the residual activity of the cofactor after 100 turnovers would be $(0.95)^{100} \approx 0.6\%$. Therefore, most chemical, photochemical, and electrochemical methods are insufficiently selective to be practical. Currently, the most convenient and practical methods for regenerating the

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reduced nicotinamide cofactors are based on enzymatic regeneration systems where a second enzyme with high specificity for a sacrificial substrate is coupled with the production reaction (van der Donk and Zhao, 2003).

The currently available enzymatic systems for NAD(P)H regeneration include formate/formate dehydrogenase for NADH, isopropanol/alcohol dehydrogenase (*Pseudomonas sp.*) for NADH, isopropanol/alcohol dehydrogenase (*T. brockii*) for NADPH, H₂/hydrogenase (*M. thermoautotrophicum*) for both NADH and NADPH, glucose-6-phosphate/glucose-6-phosphate dehydrogenase (*L. mesenteroides*) for NADPH and glucose/glucose dehydrogenase for both NADH and NADPH (Hummel, 1999; Wichmann and Vasic-Racki, 2005). Of them, the best and most widely used system for enzymatic NAD(P)H regeneration is based upon the enzyme formate dehydrogenase (FDH). In fact, FDH from *Candida boidinii* is currently the only enzymatic system used industrially. Degussa uses FDH for the production of the amino acid derivative L-tert-leucine on the multi-ton scale (Tishkov and Popov, 2006). Unfortunately, wild-type FDH is not efficient enough for NADPH regeneration; however, rational design has been used to create a NADP⁺-specific FDH from the bacterium *Pseudomonas sp.* 101 (mut Pse-FDH) (Tishkov et al., 1999). This enzyme has been successfully used in combination with an alcohol dehydrogenase (Seelbach et al., 1996) and a monooxygenase (Rissom et al., 1997) to synthesize R-alcohols and chiral lactones, respectively. The more recently discovered enzyme phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri* (Costas et al., 2001) may have several practical advantages over its FDH counterpart in certain applications. Advantages of PTDH include a high thermodynamic equilibrium constant ($K_{eq} = 1 \times 10^{11}$), broad pH-rate maximum, inexpensive phosphite substrate, both the substrate phosphite and the product phosphate are innocuous and act as a buffer, and phosphate can be readily removed by calcium precipitation if needed (Relyea and van der Donk, 2005; Vrtis et al., 2002). Due to its very high equilibrium constant, PTDH catalyzes the nearly irreversible oxidation of phosphite to phosphate with concomitant reduction of NAD⁺ to NADH (Costas et al., 2001). Since the wild type PTDH accepts NADP⁺ as a substrate very poorly, rational design was used to create a double mutant PTDH (E175A and A176R) that can successfully reduce both NAD⁺ and NADP⁺ with high efficiency (Woodyer et al., 2003). In addition, directed evolution was used to significantly improve the thermostability (Johannes et al., 2005), expression level, and activity of PTDH (Woodyer et al., 2006). The engineered thermostable mutant PTDH (12x) showed a more than 7,000-fold longer half-life of thermal inactivation than that of the wild type PTDH at 45°C and contains one of the cofactor specificity mutations (E175A) and all but two of the mutations that helped improve the activity/expression of PTDH (T181S and A308T) (Johannes et al., 2005).

In this study, we used site-directed mutagenesis to incorporate the other cofactor specificity mutation (A176R)

into the 12x mutant PTDH from *Pseudomonas stutzeri* to create a new stable and efficient NADPH regenerating enzyme. We then demonstrated the stability and effectiveness of the PTDH mutant in an enzyme membrane reactor for the production of the five-carbon sugar alcohol xylitol and the chiral alcohol (R)-phenylethanol. We also compared our system directly with the best available NADPH regenerating system based on a rationally engineered mut Pse-FDH.

Materials and Methods

Materials

Isopropyl-β-D-thiogalactospyranoside (IPTG), D-xylene, xylitol, lysozyme, sodium phosphite, sodium formate, dimethylsulfoxide (DMSO), and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO). Acetophenone and (R)-(+)-phenylethanol were purchased from Lancaster Synthesis, Inc. (Windham, NH). *Escherichia coli* BL21 (DE3) and plasmid pET15b were purchased from Novagen (Madison, WI). *PfuTurbo* DNA polymerase was obtained from Stratagene (La Jolla, CA). Restriction enzymes *Nde*I and *Bam*HI, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). PCR grade dNTPs were obtained from Roche Applied Sciences (Indianapolis, IN). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). The QIAprep spin plasmid mini-prep kit, QIAEX II gel purification kit, and QIAquick PCR purification kit were purchased from Qiagen (Valencia, CA). BD Talon™ metal affinity resin was purchased from BD Biosciences Clontech (Palo Alto, CA). Amicon® Ultra-15 centrifugal filter devices and YM3 ultrafiltration membranes (NMWL of 3 kDa) were purchased from Fisher Scientific (Pittsburgh, PA). NTR 7410-charged nanofiltration membranes were purchased from Somicon AG (Basel, Switzerland). NADP⁺, NADP⁺-specific formate dehydrogenase (mut Pse-FDH) from *Pseudomonas sp.* 101, alcohol dehydrogenase from *Lactobacillus brevis* (ADH-LB), and a 10-mL stainless-steel reactor were purchased from Jülich Chiral Solutions GmbH (Jülich, Germany).

Site-Directed Mutagenesis (A176R→12x)

A megaprimer method of site-directed mutagenesis was used to introduce mutation A176R into the 12x template (Sarkar and Sommer, 1990). Two oligonucleotide primers flanking the gene were used in combination with the mutagenic forward primer (underlined codon encodes desired amino acid substitution) 5'- CAG TAC CAC GCG CGG AAG GCT CTG GAT-3'. The site-directed mutant gene was cloned into pET15b using *Nde*I and *Bam*HI restriction sites. The resulting pET15b-12x-A176R construct is under the control of the T7 promoter and expresses

12x-A176R as an N-terminal His₆-tag fusion protein. The plasmid was then transformed into *E. coli* BL21(DE3) by heat shock and selected on Luria-Bertani (LB) solid media with ampicillin. The plasmid was sequenced using the BigDyeTM Terminator sequencing method and an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA).

Overexpression and Purification of 12x-A176R and ncXR

Protein purification of *Neurospora crassa* xylose reductase (ncXR) and the 12x-A176R mutant PTDH were carried out using a protocol described elsewhere with some modifications (Woodyer et al., 2005a). Both His₆-tagged enzymes were expressed and purified in a single step IMAC purification procedure using a BioLogic LP fast performance liquid chromatography system (Bio-Rad) and a column packed with 30 mL of IMAC Co²⁺ resin (Talon). Pooled active fractions were desalted and concentrated using a Millipore Amicon 8400 stirred ultrafiltration cell with a YM10 membrane at 4°C with 50 mM MOPS buffer (pH = 7.25). The enzyme was then stored as concentrated as possible (>10 mg/mL) in 10% glycerol at -80°C.

Enzyme Kinetics

The kinetic constants for the wild type and mutant PTDHs were determined as described elsewhere (Woodyer et al., 2003). The initial rates of reaction were determined by monitoring the increase in absorbance of NADPH ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) at 340 nm. Initial rates were carried out at 25°C using a Varian Cary 100 Bio UV-visible spectrophotometer. Reactions were initiated by addition of ~3 μg of PTDH. Enzyme concentration was determined by measuring A_{280} ($\epsilon = 30 \text{ mM}^{-1} \text{ cm}^{-1}$). The concentration of NADP⁺ stock solutions was determined by UV-visible spectroscopy ($\epsilon = 18 \text{ mM}^{-1} \text{ cm}^{-1}$ at 260 nm). Phosphite concentrations were determined enzymatically by measuring the amount of NADPH produced in the presence of saturating NADP⁺ and excess PTDH enzyme. At least five concentrations of each substrate were used varying from below the K_M value to at least five-times higher than the K_M value while the other substrate was kept at a constant saturating concentration. The data were used to calculate the kinetic constants by fitting of the Michaelis–Menten equation using Origin 5.0 (OriginLab Corporation, Northampton, MA). The kinetics parameters for mut Pse-FDH with NADP⁺ were determined using similar conditions as reported elsewhere, except at 25°C (Serov et al., 2002).

Stability in Organic Solvent

The stability of the wild-type (WT) PTDH, 12x-A176R mutant, or mut Pse-FDH in the presence of an organic solvent was determined after each enzyme was incubated for

10 h in concentrations varying from 0% to 60% DMSO. Enzyme activity remaining after solvent treatment was measured by monitoring the increase in the NADPH absorbance at 340 nm as previously described and expressed as percentages of the original activities.

Batch Production of (R)-Phenylethanol

Small-scale batch regeneration reactions contained 50 mM sodium phosphite (100 mM sodium formate for mut Pse-FDH), 20 mM acetophenone, 0.2 mM NADP⁺, 1.4 U/mL of alcohol dehydrogenase from *L. brevis* (ADH-LB), and 0.67 mg/mL of the regeneration enzyme (wild-type PTDH, 12x-A176R, or mut Pse-FDH). All reactions were carried out at pH 7.0. Reactions involving mut Pse-FDH were carried out in 50 mM sodium phosphate buffer. The reactions were mixed gently and incubated at 25°C. Every 10 min, samples were removed from the reaction and immediately frozen at -20°C. Frozen samples were thawed immediately prior to analysis. The enzyme-catalyzed conversion of acetophenone into (R)-phenylethanol was monitored by HPLC. An Agilent 1100 series solvent selector, pump, column, and detector modules were utilized with a Zorbax 150 mm \times 3.0 mm SB C-18 (3.5 μm) column. The flow rate was 0.8 mL/min at a temperature of 25°C. Substrate and product were separated using an isocratic elution of acetonitrile/25 mM NaH₂PO₄ buffer pH 2.1 (40:60 v/v) and measured spectrophotometrically at 260 nm. Retention times were found to be 1.9 min for (R)-phenylethanol and 2.8 min for acetophenone. Each reaction was performed and analyzed at least twice and the reported values are the average of the two measurements with the associated standard deviation.

Continuous Production of (R)-Phenylethanol

The continuous production of (R)-phenylethanol was performed in a 10-mL stainless-steel enzyme membrane reactor (EMR) with an Amicon YM3 ultrafiltration membrane as described elsewhere (Kula and Wandrey, 1987). The reactor was continuously mixed by a magnetic stirrer. Bovine serum albumin (BSA) (10 mg) was initially pumped into the reactor to improve the efficiency of the membrane to retain the cofactor and to prevent the other enzymes from absorbing to the membrane. Enzymes were separately injected directly into the reactor through the sample port resulting in a final reactor concentration of 2.8 U/mL of ADH-LB and 1.6 mg/mL of the regeneration enzyme (either 12x-A176R or mut Pse-FDH). Sodium azide (0.025% (w/v)) was used as an antimicrobial agent in the substrate feed for the PTDH system. The azide ion is a strong inhibitor of all FDHs and thus no sodium azide was added during reactor runs with mut Pse-FDH (Serov et al., 2002). The reactor was continuously operated for more than 180 h. A substrate flow rate of 4.8 mL/h was used, resulting in a residence time of 2.1 h. The substrate (feed) solution was kept on ice and replaced daily. Reactor output samples were

analyzed using the same procedure described in the previous section. Each reactor run was performed and analyzed at least twice under identical conditions with no more than 5% variation between runs.

Continuous Production of Xylitol

The continuous production of xylitol was performed in a 10-mL stainless-steel EMR as described elsewhere (Kula and Wandrey, 1987) and setup under similar conditions as described in the previous section. The enzymes were injected directly into the reactor resulting in a final concentration of 3 mg/mL ncXR and 2 mg/mL of the regeneration enzyme (either 12x-A176R or mut Pse-FDH). A substrate flow rate of 2.4 mL/h was used, resulting in a residence time of 4.2 h. Two different types of membranes were used, an Amicon YM3 ultrafiltration membrane and a NTR 7410 charged nanofiltration membrane. Samples were taken periodically from the reactor outlet and the conversion was monitored by HPLC. D-Xylose and xylitol were separated using an Alltech Prevail 5 μ m Carbohydrate ES 250 \times 4.6 mm column on a Shimadzu-10A HPLC system. Substrate and product were separated using an isocratic elution of 49.96% water, 0.04% NH₄OH, and 50% acetonitrile at a flow rate of 0.8 mL/min. The samples were detected by an inline Shimadzu ELSD-LT detector using N₂ as the carrier gas and the peak area was used to calculate conversion based on a standard curve previously prepared from authentic xylitol. Reactor runs were performed and analyzed at least twice under identical conditions with no more than 5% variation between runs.

Results

Creation of a Stable NADP⁺-Specific Mutant PTDH

Previous work has demonstrated that two specific mutations (E175A and A176R) in PTDH resulted in significantly increased NADP⁺ specificity (Woodyer et al., 2003). During our work to improve various properties (activity, expression, and thermostability) of PTDH using directed evolution, we created a thermostable mutant (12x) that contains only one of the previously identified cofactor specificity mutations (E175A). Thus we used site-directed mutagenesis to incorporate the other cofactor specificity mutation (A176R) into the 12x template in the hope of creating a new stable NADP⁺-specific PTDH. In order to compare the catalytic and enzymatic properties of the PTDH enzymes and mut Pse-FDH, all enzymes were characterized with respect to their kinetic constants for the nicotinamide cofactor NADP⁺. Kinetic parameters of wild type PTDH (WT PTDH), double mutant (E175A, A176R) PTDH, 12x mutant, 12x-A176R mutant, and mut Pse-FDH towards NADP⁺ and phosphite or formate (mut Pse-FDH) were listed in Table I. The catalytic efficiency ($k_{cat}/K_{M,NADP}$) of

Table I. Comparison of the kinetic parameters of the various enzymes with NADP⁺ at 25°C.

Enzyme	k_{cat} (min ⁻¹)	K_M (μ M)	k_{cat}/K_M (mM ⁻¹ min ⁻¹)	K_M (μ M) (Pt-H or formate)
WT PTDH	85 \pm 0.5	2,510 \pm 410	0.034	1,880 \pm 325
E175A/A176R	114 \pm 33	3.5 \pm 0.5	32.5	21 \pm 3
12x	80 \pm 4	49 \pm 0.6	1.62	75 \pm 2
12x-A176R	82 \pm 4	5.5 \pm 0.7	14.9	36 \pm 14
mut Pse-FDH	37 \pm 2	168 \pm 3	0.232	28,000 \pm 7,000

the 12x-A176R mutant PTDH with NADP⁺ was about 440-fold higher than that of the WT PTDH enzyme. This is primarily due to the lowering of the K_M for NADP⁺ which is approximately 460-fold lower in the 12x-A176R mutant. Based on a homology structural model, by replacing Ala176 with a positively charged residue (Arg), the enzyme forms favorable ionic and H-bonding interactions with NADP⁺, thus significantly lowering the K_M for NADP⁺ (Woodyer et al., 2003). An additional 52-fold improvement in the K_M for phosphite in the presence of NADP⁺ was observed in the 12x-A176R mutant over the WT PTDH enzyme. The 12x-A176R mutant had a \sim 65-fold higher catalytic efficiency when compared to mut Pse-FDH and a $>$ 770-fold lower K_M for the second substrate (phosphite vs. formate).

Stability of the Evolved PTDH Mutant in Organic Solvent

The 12x-A176R mutant was incubated in different concentrations of DMSO (v/v) to determine its tolerance to organic solvents. For comparison, wild-type PTDH and mut Pse-FDH were also tested for their stability in organic solvents (Fig. 1). The formate dehydrogenase from

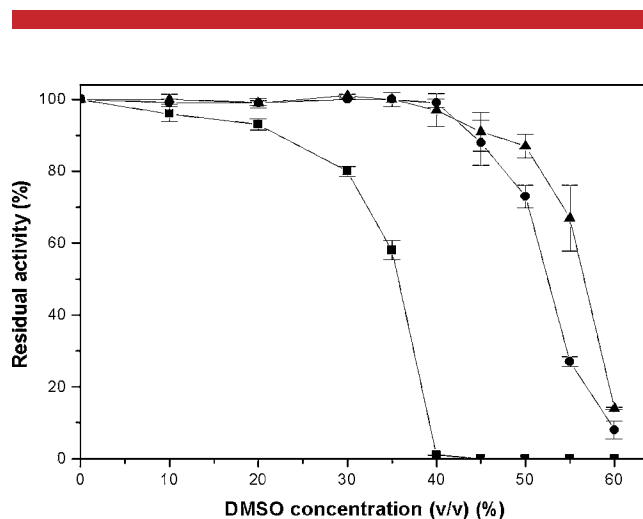


Figure 1. Stabilities of wild-type PTDH (■), 12x-A176R mutant PTDH (●), and mut Pse-FDH (▲) in DMSO. Enzymatic stabilities were determined after incubation at different concentrations (v/v) of DMSO for 10 h. The residual activities of the enzymes were measured and expressed as percentages of the original activities. All values are averages based on values from at least duplicate assays.

Pseudomonas sp. 101 has been shown to be very stable in the presence of organic solvents (Hofstetter et al., 2004). In 40% DMSO, wild-type PTDH was mostly deactivated after 10 h; whereas the 12x-A176R mutant was fully active. Furthermore, the 12x-A176R mutant displayed high solvent tolerance similar to mut Pse-FDH.

Batch Production of (R)-Phenylethanol

The effectiveness of the 12x-A176R mutant as a NADPH regeneration enzyme was studied by coupling it with an alcohol dehydrogenase from *L. brevis* (ADH-LB) for the production of (R)-phenylethanol. PTDH converts NADP⁺ into NADPH with the conversion of phosphite to phosphate and the phosphite/phosphate also functions as a buffer. The regenerated NADPH is then used by ADH-LB to convert acetophenone into (R)-phenylethanol. The coupled reaction system was shown in Figure 2A. Small-scale batch reactions containing 20 mM acetophenone were carried out using WT

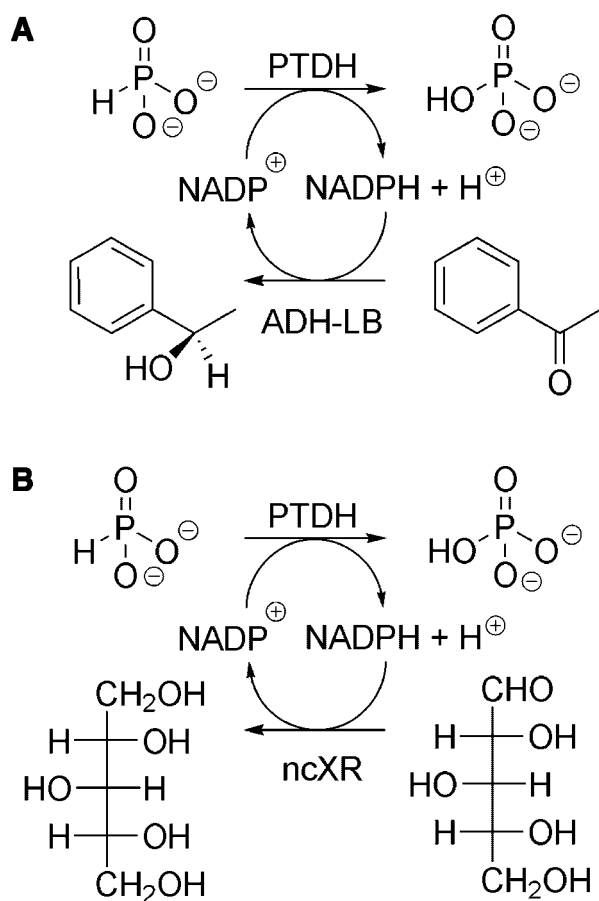


Figure 2. Reaction schemes for the enzymatic synthesis of (A) (R)-phenylethanol and (B) xylitol. PTDH, phosphite dehydrogenase; ADH-LB, alcohol dehydrogenase from *L. brevis*; ncXR, xylose reductase from *N. crassa*.

PTDH, the 12x-A176R mutant, and mut Pse-FDH. The reaction mixture (1 mL) contained 20 mM acetophenone, 0.2 mM NADP⁺, 1 mM MgCl₂, 1.4 U mL⁻¹ ADH-LB, and 0.67 mg/mL of the regeneration enzyme (wild-type PTDH, 12x-A176R, or mut Pse-FDH). Figure 3 showed the time course of production of (R)-phenylethanol with NADPH regeneration. The rate of reaction for the 12x-A176R mutant was about 1.8 and 2.2 times faster than WT PTDH and mut Pse-FDH, respectively. Furthermore, the 12x-A176R mutant reached 100% conversion after 40 min, whereas the WT PTDH and mut Pse-FDH had yet to achieve full conversion after 100 min. This resulted in a (R)-phenylethanol productivity of 88 g L⁻¹ d⁻¹ using the 12x-A176R mutant with a total turnover number (TTN) of 100 for NADPH.

Continuous Production of (R)-Phenylethanol in an EMR

Based on the information extracted from the batch conversion data, a continuous synthesis of (R)-phenylethanol in an enzyme membrane reactor was investigated. Reactor conditions similar to those previously reported for the continuous production of (R)-phenylethanol using mut Pse-FDH were used (Seelbach et al., 1996). However, we chose to use the more stable and less active alcohol dehydrogenase from *L. brevis* instead of the less stable albeit more active alcohol dehydrogenase from *Lactobacillus kefir* (*L. kefir*) (Hummel, 1997; Hummel, 1999). Thus, the final enzyme concentrations in the reactor were 2.8 U mL⁻¹ of ADH-LB and 1.6 mg mL⁻¹ of the regeneration enzyme (either 12x-A176R or mut Pse-FDH). Table II showed the conditions and results for the continuous production of (R)-phenylethanol using either the 12x-A176R mutant or mut Pse-FDH. A mean conversion of ~98% was achieved during

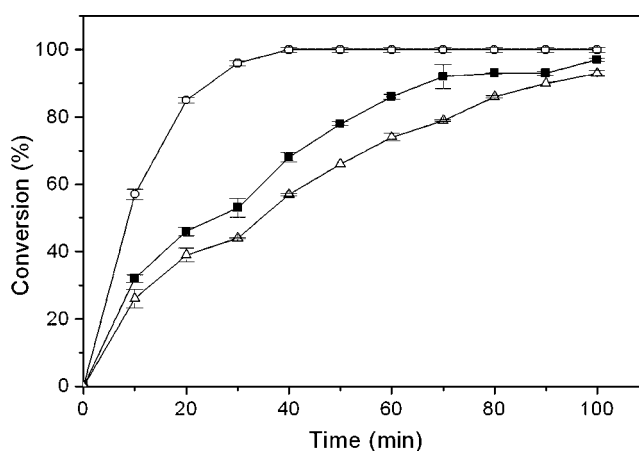


Figure 3. Batch production of (R)-phenylethanol from acetophenone with regeneration of NADPH using wild-type PTDH (■), 12x-A176R mutant PTDH (○), and mut Pse-FDH (△). The reaction mixture contained 20 mM acetophenone, 0.2 mM NADP⁺, 1 mM MgCl₂, 1.4 U mL⁻¹ ADH-LB, and equal molar amounts of either PTDH or mut Pse-FDH.

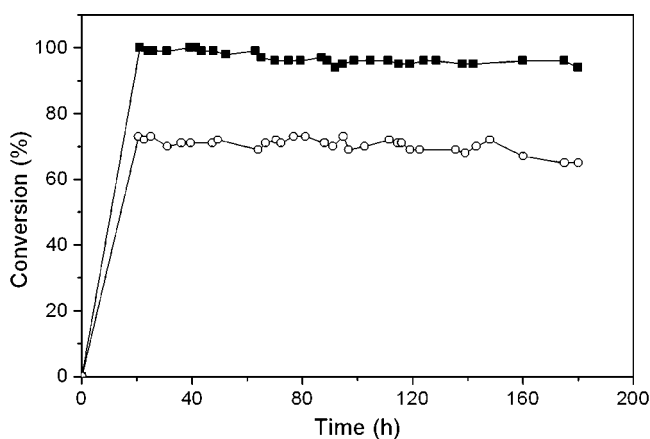
Table II. Conditions and results for the production of (R)-phenylethanol.

	12x-A176R	mut Pse-FDH
Feed concentrations		
(R)-phenylethanol (mM)	10	10
NADP ⁺ (mM)	0.10	0.10
Mean conversion (%)	98	70
Space-time yield (g L ⁻¹ d ⁻¹)	14	10
Total turnover number	100	70
Enzyme deactivation (% d ⁻¹)	0.8	1.1

the 180 h of continuous operation for the 12x-A176R mutant, whereas mut Pse-FDH reached only a mean conversion of around 70%. This resulted in a (R)-phenylethanol outlet concentration of 9.8 mM (1.2 g L⁻¹) for the 12x-A176R mutant and 7.0 mM (0.86 g L⁻¹) for mut Pse-FDH. The rate of loss of enzyme activity in both coupled systems appeared to be similar based on the data in Figure 4; however, a TTN of 100 was achieved using the 12x-A176R mutant whereas a TTN of only 70 was reached using mut Pse-FDH. The space-time yield of (R)-phenylethanol using the 12x-A176R mutant was also 40% higher than that using mut Pse-FDH.

Continuous Production of Xylitol in an EMR

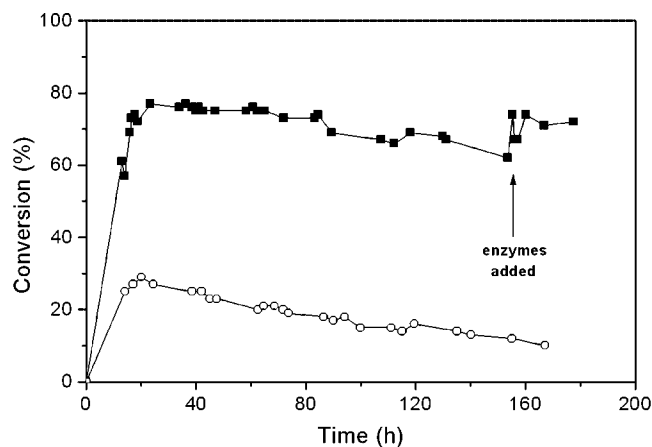
The stability and effectiveness of the 12x-A176R mutant were also demonstrated in a continuously operated enzyme membrane reactor for the production of xylitol from D-xylulose. The 12x-A176R mutant was coupled with a recently characterized xylose reductase from *N. crassa* (ncXR) (Woodyer et al., 2005a). This enzyme is one of the most

**Figure 4.** Continuous production of (R)-phenylethanol in an enzyme membrane reactor using an ultrafiltration membrane (YM3). Solid squares (■): 12x-A176R mutant PTDH; open circles (○): commercially available NADP⁺-specific FDH (mut Pse-FDH).**Table III.** Conditions and results for the production of xylitol.

	12x-A176R		mut Pse-FDH
Feed concentrations			
Xylitol (mM)	300	300	300
NADP ⁺ (mM)	0.35	0.10	0.35
Type of membrane	YM3	NTR 7410	YM3
Mean conversion (%)	72	88	18
Space-time yield (g L ⁻¹ d ⁻¹)	190	230	48
Total turnover number	617	2,640	154
Enzyme deactivation (% d ⁻¹)	2.8	~3.7	2.7

highly active, stable xylose reductases characterized to date. The coupled reaction system was shown in Figure 2B. Several batch reactions were carried out to determine the optimal reaction conditions for xylitol production in the reactor (Woodyer et al., 2005b). Small-scale regeneration reactions carried out at an enzyme ratio of 3:2 (PTDH:XR), pH 6.9, NADP⁺ concentration of 0.35 mM, and a D-xylulose to sodium phosphite ratio of 0.8 yielded the highest production of xylitol per hour. Table III showed the feed concentrations and results for the continuous production of xylitol when the reactor was continuously operated for 180 h.

Figure 5 showed the results for the production of xylitol in the EMR using an Amicon YM3 ultrafiltration membrane. The loss of the enzyme activity under these reactor conditions was approximately 2.8% per day. A variety of enzymes used in enzyme membrane reactors have been found to have similar rates of enzyme activity loss of about 3% per day or less (Kragl et al., 1996). The conversion gradually decreased as time elapsed due to this loss of enzyme activity. For the PTDH system, after 160 h 25% of both enzymes were injected into the reactor to compensate for loss of enzyme activity and the conversion increased

**Figure 5.** Continuous production of xylitol in an enzyme membrane reactor using an ultrafiltration membrane (YM3). Solid squares (■): 12x-A176R mutant PTDH; open circles (○): commercially available NADP⁺-specific FDH (mut Pse-FDH).

from 60% to 73%, thus restoring the original conversion. An average space-time yield of $190 \text{ g L}^{-1} \text{ d}^{-1}$ was achieved during the 180 h of operation using the 12x-A176R mutant whereas only $48 \text{ L}^{-1} \text{ d}^{-1}$ was achieved using mut Pse-FDH. This resulted in a concentration of 216 mM (33 g L^{-1}) (R)-phenylethanol in the outlet for the 12x-A176R mutant, whereas only 54 mM (8 g L^{-1}) was obtained with mut Pse-FDH. Furthermore, the TTN reached 617 using the 12x-A176R mutant, whereas only a TTN of 154 was achieved using mut Pse-FDH.

The space-time yield and total turnover number could be increased for the PTDH system by using a different membrane. It was reported that selective retainment of the NADP(H) cofactor can be realized by using a negatively charged nanofiltration membrane that retains the cofactor based on electrostatic repulsion of negative charges between the membrane and the phosphate moiety of NADP(H) (Nidetzky et al., 1994). In particular, it was demonstrated that the NTR 7410 composite membrane is highly effective in retaining NADP(H) in the presence of BSA ($R = 0.96$, where R is the cofactor retainment ratio) (Ikemi et al., 1990). We experimentally determined the R -value of our system to be 0.89 for NADP⁺. Thus, the NTR 7410 membrane was chosen to substitute for the regular ultrafiltration membrane in the EMR. Consequently, the feed concentration of NADP⁺ was reduced from 0.35 mM (using the Amicon YM3 ultrafiltration membrane) to 0.1 mM (for the NTR 7410 membrane). Figure 6 showed the results for the production of xylitol in the EMR using the charged nanofiltration membrane, which increased the space-time yield to $230 \text{ g L}^{-1} \text{ d}^{-1}$ xylitol. The (R)-phenylethanol concentration in the reactor outlet also increased to 264 mM (40 g L^{-1}). The TTN using the charged membrane was 2,630, a fourfold increase compared to that using the ultrafiltration membrane. Additionally the percent yield was

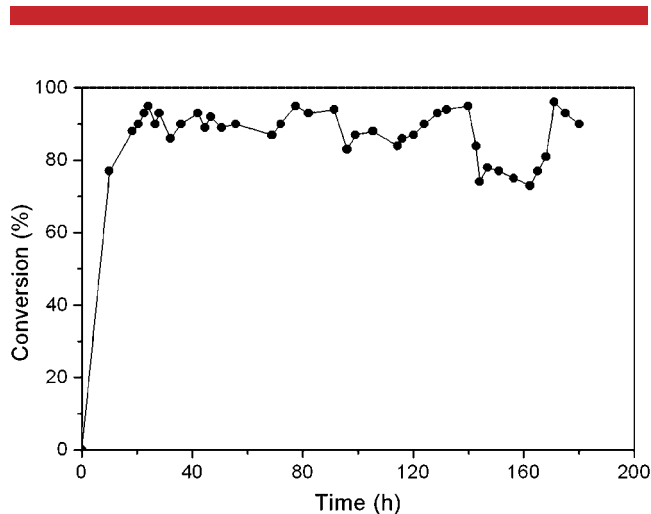


Figure 6. Continuous production of xylitol in an enzyme membrane reactor using a charged nanofiltration membrane (NTR 7410). The 12x-A176R mutant PTDH is used as the cofactor regenerating enzyme.

increased significantly, which was most likely the result of a higher in situ cofactor concentration as it was retained by the membrane.

Discussion

By combining beneficial mutations discovered during the rational design and directed evolution of PTDH, we hoped to create a stable effective NADP⁺-specific PTDH for regenerating the costly NADPH cofactor in situ. Catalytic and enzymatic properties of the new PTDH mutant and mut Pse-FDH were determined for the NADP⁺ cofactor and directly compared. As shown in Table I, the 12x-A176R mutant PTDH appeared to have better kinetic parameters than mut Pse-FDH. The catalytic efficiency was ~ 70 -fold higher and the K_M for the second substrate (phosphite vs. formate) was >770 -fold lower. We then investigated the potential of the 12x-A176R mutant PTDH for its ability to regenerate the reduced NADPH cofactor for the continuous production of (R)-phenylethanol and xylitol in an enzyme membrane reactor. Again, we directly compared the new PTDH mutant to a rationally engineered FDH from *Pseudomonas sp.* 101 (mut Pse-FDH) that is considered to be the best NADPH regeneration system. For the continuous production of (R)-phenylethanol in an enzyme membrane reactor, the mean conversion of the 12x-A176R mutant was 98% compared to 70% for the mut Pse-FDH and the space-time yield was 1.4-fold higher (Table II). Previous studies have shown that conversions of 90–95% were possible using mut Pse-FDH; however, these results were obtained using a more active albeit less stable alcohol dehydrogenase from *L. kefir* (Seelbach et al., 1996). This ADH had to be repeatedly injected into the reactor to maintain high conversion and this eventually led to a dramatic drop in conversion after 90 h (Seelbach et al., 1996). Previous results have also shown that using a similar enzyme membrane reactor setup as described in this work but with a glucose-6-phosphate/glucose-6-phosphate dehydrogenase regeneration system, only a 90% conversion into phenylethanol was obtained (Hummel, 1990). The limited solubility of both acetophenone and (R)-phenylethanol diminishes space-time yields and TTNs for all of these enzymatic regeneration schemes and makes a traditional reactor setup unrealistic. However, by using a unique reactor setup, it has been possible to produce (S)-1-phenyl-2-propanol at high yield, a sparingly soluble chiral alcohol similar in structure to (R)-phenylethanol, in an enzyme membrane reactor with integrated extraction of the hydrophobic product (Kragl et al., 1996). Without recirculation of the water phase, the TTN was only 54 for this process. By recirculating the water phase containing the cofactor and through continuous extraction of the product, the TTN was improved 25-fold up to 1,350 and a space-time yield of $63 \text{ g L}^{-1} \text{ d}^{-1}$ was achieved.

We also investigated the potential of the 12x-A176R mutant PTDH to regenerate NADPH for the continuous

production of xylitol in an enzyme membrane reactor. Xylitol, a five-carbon sugar alcohol, has received much attention recently as an alternative natural sweetener in food products. It also has been shown to have anticarcinogenic properties and can be an insulin-independent sugar substitute for diabetics (Emodi, 1978). Xylitol is currently industrially produced by catalytic hydrogenation of D-xylose in hemicellulose hydrolysates (Roca et al., 1996). This process uses a heterogeneous Raney-nickel catalyst (Ni/Al₂O₃) under high pressure and high temperature and requires several purification steps using ion exchange chromatography to obtain xylitol in high purity (Melaja and Hamalainen, 1977). As a result of the byproducts formed and necessary downstream processing, typical yields of xylitol based on xylan are only ~50–60%. The limitations and difficulties of the traditional process have led to increasing interest in exploring cleaner, more efficient biochemical routes of synthesis. These biological routes of synthesis primarily involve either fermentation processes or in vitro reactions using an enzyme membrane reactor. Natural D-xylose fermenting yeasts have been used for the microbial synthesis of xylitol (Gong et al., 1981; Mahler and Guebel, 1994; Vongsuvanlert and Tani, 1989). Productivities of approximately 25 to 35 g L⁻¹ d⁻¹ are common for D-xylose converting yeasts (da Silva and Afschar, 1994; Meinander et al., 1994). The current best reported productivity for yeast D-xylose converting strains, 122 g L⁻¹ d⁻¹, was obtained by integrating an NADH-preferring XR into an industrially used strain of *Candida tropicalis* (Lee et al., 2003). Xylitol has also been produced in a charged membrane reactor by coupling the conversion of D-xylose with a glucose dehydrogenase/glucose regeneration system (Nidetzky et al., 1996), and a productivity of approximately 12 g L⁻¹ d⁻¹ was obtained by using a substrate feed of 300 mM D-xylose and glucose. Thus the productivity of the 12x-A176R mutant for the production of xylitol in an enzyme membrane reactor (230 g L⁻¹ d⁻¹) is twofold higher than the best yeast D-xylose converting strain and 20-fold higher than the most efficient continuous enzyme membrane reactor system found in the literature.

In conclusion, the effectiveness and stability of the new NADP⁺-specific mutant PTDH as a NADPH regeneration enzyme was demonstrated in an enzyme membrane reactor for the production of both (R)-phenylethanol and xylitol. These results indicate that the 12x-A176R mutant PTDH appears to be a very promising NADPH regeneration system when compared to established enzymatic NADPH regeneration systems. Based on productivity, the NADP⁺-specific PTDH mutant also appears to be an attractive alternative for the production of xylitol when compared to the best engineered yeast strains and most efficient enzyme membrane reactor systems.

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References

- Costas AM, White AK, Metcalf WW. 2001. Purification and characterization of a novel phosphorus-oxidizing enzyme from *Pseudomonas stutzeri* WM88. *J Biol Chem* 276:17429–17436.
- da Silva SS, Afschar AS. 1994. Microbial production of xylitol from D-xylose using *Candida tropicalis*. *Process Biochem* 11:129–134.
- Emodi A. 1978. Xylitol: Its properties and food application. *Food Technol* 32:20–32.
- Gong CS, Chen LF, Tsao GT. 1981. Quantitative production of xylitol from D-xylose by a high xylitol producing yeast mutant *Candida Tropicalis* HXP2. *Biotechnol Lett* 3:125–130.
- Hofstetter K, Lutz J, Lang I, Witholt B, Schmid A. 2004. Coupling of biocatalytic asymmetric epoxidation with NADH regeneration in organic-aqueous emulsions. *Angew Chem Int Ed* 43(16):2163–2166.
- Hummel W. 1990. Enzyme-catalyzed synthesis of optically pure R(+)-phenylethanol. *Biotechnol Lett* 12:403–408.
- Hummel W. 1997. New alcohol dehydrogenases for the synthesis of chiral compounds. *Adv Biochem Eng/Biotechnol* 58:145–184.
- Hummel W. 1999. Large-scale applications of NAD(P)-dependent oxidoreductases: Recent developments. *Trends Biotechnol* 17:487–492.
- Hummel W, Kula MR. 1989. Dehydrogenases for the synthesis of chiral compounds. *Eur J Biochem* 184(1):1–13.
- Ikemi M, Koizumi N, Ishimatsu Y. 1990. Sorbitol production in charged membrane bioreactor with coenzyme regeneration system: I. selective retention of NADP(H) in a continuous reaction. *Biotechnol Bioeng* 36:149–154.
- Johannes TW, Woodyer RD, Zhao HM. 2005. Directed evolution of a thermostable phosphite dehydrogenase for NAD(P)H regeneration. *Appl Environ Microbiol* 71:5728–5734.
- Kragl U, Kruse W, Hummel W, Wandrey C. 1996. Enzyme engineering aspects of biocatalysis: Cofactor regeneration as example. *Biotechnol Bioeng* 52:309–319.
- Krix G, Bommarius AS, Drauz K, Kottenhahn M, Schwarm M, Kula MR. 1997. Enzymatic reduction of alpha-keto acids leading to L-amino acids, D- or L-hydroxy acids. *J Biotechnol* 53:29–39.
- Kula MR, Wandrey C. 1987. Continuous enzymatic transformation in an enzyme-membrane reactor with simultaneous NADH regeneration. *Methods Enzymol* 136:9–21.
- Lee JK, Koo BS, Kim SY. 2003. Cloning and characterization of the xyl1 gene, encoding an NADH-preferring xylose reductase from *Candida parapsilosis*, and its functional expression in *Candida tropicalis*. *Appl Environ Microbiol* 69:6179–6188.
- Mahler GF, Guebel DV. 1994. Influence of magnesium concentration on growth, ethanol and xylitol production by *Pichia Stipitis* Nrrl Y-7124. *Biotechnol Lett* 16:407–412.
- Meinander N, Hahnagerdal B, Linko M, Linko P, Ojamo H. 1994. Fed-batch xylitol production with recombinant xyl-1-expressing *Saccharomyces cerevisiae* using ethanol as a cosubstrate. *Appl Microbiol Biotechnol* 42:334–339.
- Melaja AJ, Hamalainen L. 1977. Process for making xylitol. US Patent 4,008,285.
- Nidetzky B, Schmidt K, Neuhauser W, Haltrich D, Kulbe KD. 1994. Application of charged ultrafiltration membranes in continuous, enzyme-catalyzed processes with coenzyme regeneration. In: Pyle DL, editor. *Separations for biotechnology III*. Cambridge, UK: Royal Society of Chemistry. p 351–357.
- Nidetzky B, Neuhauser W, Haltrich D, Kulbe KD. 1996. Continuous enzymatic production of xylitol with simultaneous coenzyme regeneration in a charged membrane reactor. *Biotechnol Bioeng* 52:387–396.
- Relyea HA, van der Donk WA. 2005. Mechanism and applications of phosphite dehydrogenase. *Bioorg Chem* 33:171–189.
- Rissom S, Schwarz-Linek U, Vogel M, Tishkov VI, Kragl U. 1997. Synthesis of chiral E-lactones in a two-enzyme system of cyclohexane monooxygenase and formate dehydrogenase with integrated bubble-free aeration. *Tetrahedron: Asymmetry* 8:2523–2526.

- Roca E, Meinander N, HahnHagerdal B. 1996. Xylitol production by immobilized recombinant *Saccharomyces cerevisiae* in a continuous packed-bed bioreactor. *Biotechnol Bioeng* 51:317–326.
- Sarkar G, Sommer SS. 1990. The megaprimer method of site-directed mutagenesis. *Biotechniques* 8:404–407.
- Seelbach K, Riebel B, Hummel W, Kula M-R, Tishkov VI, Egorov AM, Wandrey C, Kragl U. 1996. A novel, efficient regenerating method of NADPH using a new formate dehydrogenase. *Tetrahedron Lett* 37:1377–1380.
- Serov AE, Popova AS, Fedorchuk VV, Tishkov VI. 2002. Engineering of coenzyme specificity of formate dehydrogenase from *Saccharomyces cerevisiae*. *Biochem J* 367:841–847.
- Tishkov VI, Popov VO. 2006. Protein engineering of formate dehydrogenase. *Biomol Eng* 23:89–110.
- Tishkov VI, Galkin AG, Fedorchuk VV, Savitsky PA, Rojkova AM, Gieren H, Kula MR. 1999. Pilot scale production and isolation of recombinant NAD(+)- and NADP(+)-specific formate dehydrogenases. *Biotechnol Bioeng* 64:187–193.
- van der Donk WA, Zhao HM. 2003. Recent developments in pyridine nucleotide regeneration. *Curr Opin Biotechnol* 14:421–426.
- Vongsuvanlert V, Tani Y. 1989. Xylitol production by a methanol yeast, *Candida Boidinii* (Kloeckera Sp) No-2201. *J Ferment Bioeng* 67: 35–39.
- Vrtis JM, White A, Metcalf WW, van der Donk WA. 2002. Phosphite dehydrogenase, a new versatile cofactor regeneration enzyme. *Angew Chem Int Ed* 41:3257–3259.
- Wichmann R, Vasic-Racki D. 2005. Cofactor regeneration at the lab scale. *Adv Biochem Eng Biotechnol* 92:225–260.
- Woodyer R, van der Donk WA, Zhao HM. 2003. Relaxing the nicotinamide cofactor specificity of phosphite dehydrogenase by rational design. *Biochemistry* 42:11604–11614.
- Woodyer R, Johannes T, Zhao HM. 2004. Cofactor regeneration for biocatalytic applications. In: Pandey A, Webb C, Soccol C, Larroche C, editors. *Enzyme Technology*. New Delhi, India: Asiatech Publishers, Inc. p 83–101.
- Woodyer R, Simurdiak M, van der Donk WA, Zhao HM. 2005a. Heterologous expression, purification, and characterization of a highly active xylose reductase from *Neurospora crassa*. *Appl Environ Microbiol* 71:1642–1647.
- Woodyer R, Zhao H, van der Donk WA. 2005b. Mechanistic investigation of a highly active phosphite dehydrogenase mutant and its application for NADPH regeneration. *FEBS J* 272:3816–3827.
- Woodyer R, van der Donk WA, Zhao HM. 2006. Optimizing a biocatalyst for improved NAD(P)H regeneration: Directed evolution of phosphite dehydrogenase. *Comb Chem High Throughput Screening* 9:237–245.
- Zhao HM, van der Donk WA. 2003. Regeneration of cofactors for use in biocatalysis. *Curr Opin Biotechnol* 14:1–7.