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Practical applications of hydrogenase I from *Pyrococcus furiosus* for NADPH generation and regeneration

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Abstract

The soluble hydrogenase I (H_2:$NADP^+$ oxidoreductase, EC 1.18.99.1) from the marine hyperthermophilic strain of the archaeon *Pyrococcus furiosus* was partially purified by anion-exchange chromatography. This *P. furiosus* hydrogenase I preparation (PF H_2ase I) has been used as biocatalyst in the enzymatic production and regeneration of β-1,4-nicotinamide adenindinucleotide phosphate, reduced form (NADPH), utilizing cheap molecular hydrogen and forming protons as the only side-product. Any excess of dihydrogen can be removed easily. It could be demonstrated, that this hyperthermophilic hydrogenase exhibits a high stability under reaction conditions. Generation as well as regeneration of NADPH were performed in batch and repetitive batch experiments with recyclisation of the biocatalyst. In two repetitive batch-series 6.2 g l^{-1} NADPH could be produced with a total turnover number (ttn: mol produced NADPH/mol consumed enzyme) of 10,000. Utilizing the thermophilic NADPH-dependent alcohol dehydrogenase from *Thermoanaerobium spec*. (ADH M) coupled to the PF H_2ase I in situ NADPH-regenerating system, two prochiral model substrates, acetophenone and (2*S*)-hydroxy-1-phenyl-propanone (HPP), were quantitatively reduced to the corresponding (*S*)-alcohol and (1*R*,2*S*)-diol. An e.e. >99.5% and d.e. >98%, respectively, with total turnover numbers (ttn: mol product/mol consumed cofactor $NADP^+$) of 100 and 160 could be reached.

Keywords: *Pyrococcus furiosus* hydrogenase; NADPH; Cofactor regeneration; Dihydrogen; Enantioselective reduction

Abbreviations: ADH, alcohol dehydrogenase; CE, capillary electrophoresis; EC, enzyme class; DAD, diode array detector; (H)EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propane sulfonic acid; FDH, formate dehydrogenase; GC, gas chromatography; GDH, glucose dehydrogenase; HPP, (2*S*)-hydroxy-1-phenyl-propanone; IC-MS/MS, ion chromatography-tandem-mass spectrometry; KP_i, potassium phosphate buffer (KH_2PO_4/K_2HPO_4); NAD^+, β-nicotinamide adenindinucleotide, oxidized form; NADH, β-1,4-nicotinamide adenindinucleotide, reduced form; $NADP^+$, β-nicotinamide adenindinucleotide phosphate, oxidized form; NADPH, β-1,4-nicotinamide adenindinucleotide phosphate, reduced form; PF H_2ase I, *Pyrococcus furiosus* hydrogenase I; TRIS, 2-amino-2-hydroxymethyl-1,3-propane-diol (α,α,α-tris-(hydroxymethyl)-methylamine)

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Nomenclature

k_d	deactivation constant (h^{-1})
$t_{1/2}$	half life (h)
T	temperature (°C)
tof	turnover frequency (h^{-1}) (tof = ttn K_d)
ttn	total turnover number (mol mol^{-1})
U	unit (μmol min^{-1} mg^{-1})
P	pressure (bar)

Greek letter

τ	retention time (min)

1. Introduction

Enzymes as biocatalysts have captured an important place in organic synthesis [1–3]. In particular, oxidoreductases (EC 1.1.X.X) are effective catalysts and they can perform highly stereoselective reductions of a variety of functional groups. Thus, many hydroxy acids, amino acids and alcohols are synthesized to serve as chiral building blocks for the synthesis of pharmaceuticals, flavors, and agrochemicals [4,47,48]. The large majority of oxidoreductases that catalyze the enantioselective reduction of prochiral ketones require β-1,4-nicotinamide adenindinucleotide, reduced form (NADH) or β-1,4-nicotinamide adenindinucleotide phosphate, reduced form (NADPH) as cofactors, supplying the needed reduction equivalents [5,6]. Since these nicotinamide cofactors are too expensive to be used stoichiometrically, there has been significant interest in recent decades in developing efficient pyridine nucleotide cofactor generating and regenerating processes [7–9]. So far, the more valuable products are potentially obtained with NADPH-dependent enzymes.

Synthesis of NADPH can be carried out by means of chemical, electrochemical or enzymatic methods. However, not many processes for the direct generation of reduced β-nicotinamide adeninedinucleotide phosphate are published. The enzyme-free chemical and direct electrochemical methods suffer from low selectivity and undesirable side reactions [7,8,10]. Indirect electrochemical processes circumvent these problems by using mediators, but these agents often hamper downstream processing [11]. In 1983 Eguchi et al. [12] reported the utilization of resting methanogenic bacteria cells as biocatalysts in the reduction of β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) cofactor with formate or dihydrogen. With a maximum conversion of 60% they could achieve an analytical yield of approx. 6 g l^{-1} NADPH. However, the limiting factor was the cultivation of the strictly anaerobic cells. An enzymatic generation of NADPH from $NADP^+$ and L-malic acid was achieved with the soluble L-malic:$NAD(P)^+$ oxidoreductase from *Achromobacter parvulus* IFO-13182 (EC 1.1.1.39–40) [13]. Under optimized conditions quantitative conversion yielded about 8.9 g l^{-1} NADPH after 4 h. Another common enzymatic method is the D-glucose-6-phosphate dehydrogenase (G6P-DH; EC 1.1.1.49) catalyzed reduction of β-$NADP^+$ with glucose-6-phosphate [14]. Since the thermodynamics of this reversible reaction are in favor of the substrate, this process has to be carried out with surplus of expensive glucose-6-phosphate. More favorable thermodynamics and less interference of by-products are encountered in a method using a recombinant $NADP^+$-specific formate dehydrogenase (FDH) (EC 1.2.1.2), which has gained much attention, since it reduces $NADP^+$ utilizing formate as cheap substrate, generating only gaseous CO_2 and NADPH [15].

Among several in situ regeneration techniques enzymatic processes are presently applied with success [9,16]. Two different approaches are known, namely the substrate- and the enzyme-coupled system. In the substrate-coupled system the in situ enzymatic conversion of a cheap co-substrate, e.g. 2-propanol to acetone, regenerates the reduced cofactor, whereas in the case of the enzyme-coupled in situ regeneration a second enzyme, which regenerates the cofactor directly, is added to the reaction system. A well-known example for the enzyme-coupled in situ cofactor regeneration is the formate dehydrogenase that catalyzed NADPH-regeneration through conversion of formate to carbon dioxide [15,17]. Another approach is to use a hydrogenase, which produces the reduced cofactor at the expense of the cheapest reducing agent, dihydrogen [18–24]. To date, to our knowledge, no paper has described the direct regeneration of NADPH with a hydrogenase.

Classical hydrogenases are bi-directional enzymes that catalyze the production and oxidation of molecular hydrogen. The enzymatic reaction is represented

by the equation:

$$H_2 \rightleftharpoons 2H^+ + 2e^-$$

Actually, the enzymatic cleavage of H_2 is heterolytic: $H_2 \Rightarrow H^+ + H^-$ [25]. Generally, hydrogenases are divided into two major groups on the basis of the metal content of their respective dinuclear catalytic centers: the "iron-only" [FeFe]-hydrogenases and the [NiFe]-hydrogenases [26–29]. [FeFe]-hydrogenases contain only [FeS]-clusters, of which one functions as the "H-cluster", binding the hydrogen [30]. It is evident, that with [NiFe]-hydrogenases the hydrogen activation takes place at the dinuclear catalytic center [31], although the exact catalytic mechanism remains to be developed.

The organism hosting the hydrogenase used in this study, *Pyrococcus furiosus*, is a strictly anaerobic hyperthermophilic archaeon and a heterotroph which grows optimally at temperatures above 90 °C [34]. It harbors two soluble [NiFe]-hydrogenases, namely H_2ase I and H_2ase II, in its cytoplasm [32,33].

The soluble PF H_2ase I, formerly known as PF sulfhydrogenase because of its S^0-reducing activity, is capable of generating the cofactor NADPH directly from the oxidized $NADP^+$ without producing any by-products other than protons. NADPH was found to be the physiological substrate [35–37]. Therefore, the high biotechnological potential of PF H_2ase I for the synthesis of NADPH, as well as for the enzyme-coupled in situ NADPH-regeneration is evident [38,39]. The reactions are shown in Fig. 1.

Here we present the practical application of the partially purified hydrogenase I from the hyperthermophilic strain of the Archaeon *P. furiosus* for the production and regeneration of NADPH, utilizing cheap molecular hydrogen. Two prochiral model components, acetophenone and (2*S*)-hydroxy-1-phenyl-propanone (HPP), are used in the *Thermoanaerobium spec.* alcohol dehydrogenase (ADH M) catalyzed enantioselective reduction.

2. Experimental

2.1. Materials

Thermoanaerobium spec. alcohol dehydrogenase (EC 1.1.1.2) and $NADP^+$ were obtained from Juelich

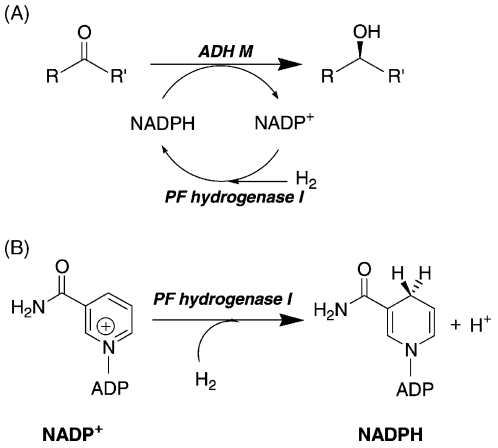

Fig. 1. (A) Regeneration and (B) generation of NADPH with hydrogenase from *Pyrococcus furiosus*; ADP: adenine dinucleotide phosphate; ADH M: *Thermoanaerobium spec*. alcohol dehydrogenase.

Fine Chemicals, Juelich, Germany. DNase and RNase were obtained from Roche Diagnostics GmbH, Mannheim, Germany. (2*S*)-Hydroxy-1-phenyl-propanone was synthesized according to the procedure described in [40,41]. Gases (helium, argon, hydrogen) were of 99.999% purity and obtained from Messer (Krefeld, Germany). All other materials were of highest grade and commercially available (Sigma, Steinheim, Germany and VWR International GmbH, Langenfeld, Germany).

2.2. Cultivation and protein purification

P. furiosus (DSM 3638) was cultivated in synthetic sea water supplemented with a vitamin and trace element mixture and potato starch as carbon source at 90 °C in a stirred 300 l fermenter (lp 300, Bioengineering, Wald, Switzerland) as previously described in [42,43]. Cells were collected by continuous centrifugation (Sharples, France) and stored at −80 °C until used. As hydrogenases are sensitive to oxygen during the purification process, anaerobic conditions were generally maintained by using argon to avoid damage to their structural integrity or inactivation [29,44]. All buffers were either degassed by alternately applying vacuum and argon or by helium, and handled under an argon atmosphere. Freshly thawed cells of *P. furiosus* were broken by osmotic shock. For this purpose, the

cell paste was diluted with 50 mM TRIS–HCl buffer (1:5 (v/v)), pH 8.0, containing 0.1 mg l^{-1} DNAse I, 0.1 mg l^{-1} RNAse and 5 mM $MgCl_2$. The suspension was stirred for 5 h under argon and was centrifuged at 25,000 × g for 1 h at 4 °C. The brownish supernatant was collected and passed through an anion-exchange Source 15Q™ column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM TRIS–HCl buffer, pH 8.0. Elution was carried out applying a linear gradient of 0–500 mM NaCl in standard buffer. PF H_2ase I eluted at 210–240 mM NaCl from the column. Fractions containing hydrogenase activity were pooled and concentrated by ultrafiltration using a YM10 membrane (Millipore, Eschborn, Germany) [37]. Fractions that eluted at higher salt concentration containing the less active PF H_2ase II were discarded [33]. The resulting PF hydrogenase I preparation was concentrated to final protein content of 51 mg ml^{-1} with a specific dihydrogen:$NADP^+$ oxidoreductase activity of 1.55 μmol min^{-1} mg^{-1}) and the yield of purification is about 38%. Activities were determined spectrophotometrically. One unit of PF H_2ase I activity equals the amount of protein needed to produce one 1 μmol NADPH per minute at 40 °C. Since no reproducible activity measurements were possible, because of the found activation effect described in chapter 3.2, PF H_2ase I concentrations are given in units of mg ml^{-1} instead of specific activities.

2.3. Activity measurements, analytical procedures

2.3.1. Spectrophotometric dihydrogen:$NADP^+$ oxidoreductase assay

An anaerobic 3 ml quartz cuvette with septum and screw cap (Hellma, Muellheim, Germany) was filled with 50 mM EPPS buffer, pH 8.0 containing 0.4 mM $NADP^+$, degassed with argon/helium and saturated with dihydrogen. The cuvette was incubated at 40 °C and after 5 min 20 μM sodium dithionite were added to remove last traces of oxygen. Hydrogenase preparations were activated by pre-incubation of the samples at 80 °C for 5 min under dihydrogen atmosphere. The H_2 uptake reaction was initiated by addition of 10 μl of the activated hydrogenase preparation to a final volume of 1 ml. The increase in extinction was measured at 340 nm using a Gilford UV-Vis Spectrophotometer 240.

2.3.2. Capillary electrophoresis

Conversions of $NADP^+$ to NADPH were monitored by capillary electrophoresis (CE) according to [45] (P/ACE™ System MDQ with DAD, Beckman, Munich, Germany; CElect™ fused silica column (50 μm i.d., 363 μm o.d., SUPELCO Inc., Bellefonte, USA) with 40 mM KH_2PO_4/10 mM H_3BO_3, pH 8.5, as standard CE buffer. Samples were withdrawn from the reaction mixture, diluted with CE buffer, mixed with 1 mM uridine as internal standard to a total volume of 50 μl before analysis. Analyses were routinely run at 40 °C and 30 kV. Detection of the electrolytes $NADP^+$, NADPH and by-product was performed at 254 and 340 nm and with 360 nm as reference channel. Fig. 2 shows a typical electropherogramme of the analysis of NADPH at 254 nm.

2.3.3. Determination of protein content

The protein content was determined using the Microbiuret method with bovine serum albumine (BSA) fraction V as standard [46].

2.3.4. Gas chromatographic analysis

Samples of acetophenone and (1*S*)-phenyl ethanol were analyzed for conversion and enantiomeric excess without any work-up on a 50 mm × 0.32 mm FS-Cyclodex β-I/P column (CS Chromatographie Service GmbH, Langerwehe, Germany) using a gas chromatograph (Agilent 6890 series GC-System, Varian, Darmstadt, Germany) equipped with a flame ionization detector (250 °C) and dihydrogen as carrier gas; oven T = 120 °C, injection T = 250 °C. Typical retention times were: $t_{(\text{acetophenone})}$ = 6.7 min, $\tau_{((1S)\text{-phenyl ethanol})}$ = 10.6 min, (2*S*)-hydroxy-1-phenyl-propanone and the corresponding (1*R*,2*S*)-diol were also analyzed using the same GC set-up, but on a 25 m × 0.25 mm CP-Chirasil-Dex CB column (Varian, Darmstadt, Germany); oven T = 130 °C, injection T = 250 °C, $\tau_{(\text{RS-diol})}$ = 23.9 min.

2.4. NADPH synthesis

All buffers were either degassed by alternately applying vacuum and argon or by helium under argon atmosphere. For each enzymatic experiment the anaerobic reaction mixture is saturated with dihydrogen (p = 1.1 bar) and the PF H_2ase I preparation

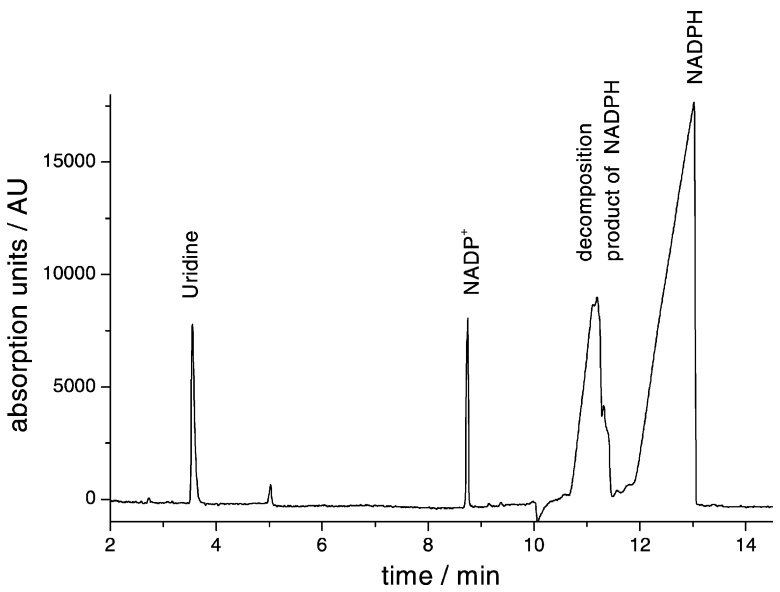

Fig. 2. Representative electropherogramme for the analysis of NADPH with capillary electrophoresis at 254 nm.

is incubated at 80 °C under dihydrogen atmosphere prior to use.

2.4.1. Batch experiments

The dihydrogen dependent reduction of $NADP^+$ to NADPH was performed in stirred 5 ml butyl rubber-stoppered glass vials at 40 °C. The anaerobic and dihydrogen saturated reaction mixtures contained in a final volume of 2 ml 12 mM $NADP^+$ in either 200 mM EPPS or 100 mM TRIS–HCl buffer, pH 8.0, under dihydrogen ($p = 1.1$ bar). The H_2 uptake reaction was initiated by the addition of 20 μl of the activated PF H_2ase I preparation (0.255 mg ml^{-1}).[3] Reactions were monitored by capillary electrophoresis and allowed to proceed until quantitative conversion in respect to the substrate were reached.

2.4.2. Repetitive batch experiments

In a modified ultrafiltration cell with a YM10 membrane (Amicon series, Millipore, Eschborn, Germany) 3 ml of 12 mM $NADP^+$ solution in 200 mM EPPS buffer, pH 8.0, were made routinely anaerobic and pre-purged with dihydrogen ($p = 1.1$ bar). After thermostating the solution to 40 °C the reaction was initiated by injection of activated PF H_2ase I preparation (1.02 mg/ml).[3] After >95% substrate conversion the product solution was filtered off. The permeate was analyzed using capillary electrophoresis. Results were corrected for the remainder of 0.2 ml reaction volume in the cell, which was necessary to keep the membrane wet. No protein could be determined in the permeate. For starting the next batch freshly prepared anaerobic 12 mM $NADP^+$ solution in 200 mM EPPS, pH 8.0, was added to the ultrafiltration cell. This procedure was repeated seven times without any further dosage of PF H_2ase I. Different dihydrogen pressures in the range of 2.5–7.5 bar were applied.

2.5. Synthesis of (1S)-phenyl ethanol

2.5.1. 2-Propanol coupled NADPH regeneration system

The stirred solutions of 10 mM acetophenone in 50 mM potassium phosphate buffer (KP_i), pH 8.0, with 300 mM 2-propanol and 0.5 mM (0.1 mM) $NADP^+$ was thermostated at 40 °C. The reaction was started by addition of 1 U *Thermoanaerobium spec.* alcohol dehydrogenase (0.33 U ml^{-1}) to the reaction mixture. One unit corresponds to the reduction of

[3] All given PF H_2ase I concentrations (mg/ml) refer to the protein content per ml reaction volume.

1 μmol (2*S*)-hydroxy-1-phenyl-propanone per minute at 40 °C and pH 8. Samples were analyzed for yield and conversion by means of gas chromatography.

2.5.2. *Pyrococcus furiosus hydrogenase I coupled NADPH regeneration system*

The stirred solutions of 10 mM acetophenone in 50 mM potassium phosphate buffer (KP_i), pH 8.0, 0.5 mM (0.1 mM) $NADP^+$ and 1 U *Thermoanaerobium spec*. alcohol dehydrogenase (0.33 U ml^{-1}) was degassed using helium under an inert atmosphere of argon and thermostated at 40 °C. Argon was exchanged against dihydrogen ($p = 1.1$ bar) and reaction was started through injection of activated PF H_2ase I (1.0 mg ml^{-1}).[3] Samples were analyzed by gas chromatography.

2.5.3. *Synthesis of (1S)-phenyl ethanol in a repetitive batch mode*

The same procedure as in the case of the NADPH synthesis was applied with freshly prepared anaerobic 10 mM acetophenone in 50 mM KP_i buffer, pH 8.0, with 0.5 mM $NADP^+$, 1 U ADH M and 1 mg ml^{-1} activated PF H_2ase I preparation. This procedure was repeated five times without any further dosage of PF H_2ase I, but additional ADH M (0.33 U ml^{-1}) injection prior to the third run. The conversion was followed analyzing the permeate by gas chromatography. Additionally, no protein was found in the permeate.

2.6. *Synthesis of (1R,2S)-phenyl-propane-1,2-diol (repetitive batch operation)*

Three milliliters of anaerobic and dihydrogen saturated 10 mM (2*S*)-hydroxy-1-phenyl-propanone in 50 mM KP_i buffer, pH 8.0, with 0.5 mM $NADP^+$ and 1 U ADH M (1 U; 0.33 U ml^{-1}; 1 U of ADH M reduces 1 μmol of (2*S*)-hydroxy-1-phenyl-propanone to diol per minute at 40 °C, pH 8) were thermostated to 40 °C in the previously described modified ultrafiltration cell. The reaction was initiated by injection of 1 mg ml^{-1} of activated PF H_2ase I preparation and kept under a dihydrogen pressure of 1.1 bar. The permeate was analyzed for conversion and enantiomeric excess by means of GC. As soon as quantitative conversion of the substrate was reached, freshly prepared anaerobic 30 mM (2*S*)-hydroxy-1-phenyl-propanone in 50 mM KP_i buffer, pH 8.0, with 0.5 mM $NADP^+$ were added. For the third run a solution containing 80 mM (2*S*)-hydroxy-1-phenyl-propanone was used. Results were corrected for the remainder of approx. 0.2 ml. This procedure could be performed without any further dosage of PF H_2ase I and ADH M.

3. Results and discussion

3.1. *NADPH regeneration*

Oxidoreductase catalyzed enantioselective reductions of prochiral ketons often suffer from low operational stability and their NADPH cofactor dependency. Therefore, practical methods for the regeneration of expensive NADPH are significant in biosynthesis [49,50]. Although nicotinamide cofactors are preferentially regenerated in situ through enzymatic processes, not many systems are known, involving a hydrogenase as regenerating enzyme [18,19,21–24,51]. This restriction stems from the availability of $NADP^+$-specific hydrogenases. To date there are only the *Alcaligenes eutrophus* H16 hydrogenase, the *Desulfovibrio fructosovorans* hydrogenase, a hydrogenase from *Anacystis nidulans* and a membrane-bound hydrogenase from *Klebsiella pneumoniae* besides the PF H_2ase I and PF H_2ase II known to be capable to utilize NADPH as a redox equivalent [52–56]. For this reason, we were interested in applying the *P. furiosus* hydrogenase I in a NADPH generating and regenerating enzymatic system.

In our study we have investigated the in situ NADPH regeneration using the thermophilic NADPH-dependent alcohol dehydrogenase from *Thermaoanaerobium spec*. to enantioselectively reduce acetophenone at 40 °C to (1*S*)-phenyl ethanol in a substrate-coupled and an enzyme-coupled in situ NADPH-regenerating system with PF H_2ase I under comparable conditions (see Fig. 3).

Fig. 3A shows the progression curves of the 2-propanol-coupled NADPH regeneration using two different initial cofactor concentrations. Regardless of the initial $NADP^+$ concentration applied, conversions of 93% with an enantioselectivity >99.5% were reached after 2 and 2.5 h, respectively. In contrast, quantitative conversions >99% (>99.5% e.e.) were reached after 3 h with the PF H_2ase I catalyzed NADPH-regenerating system (see Fig. 3B). These

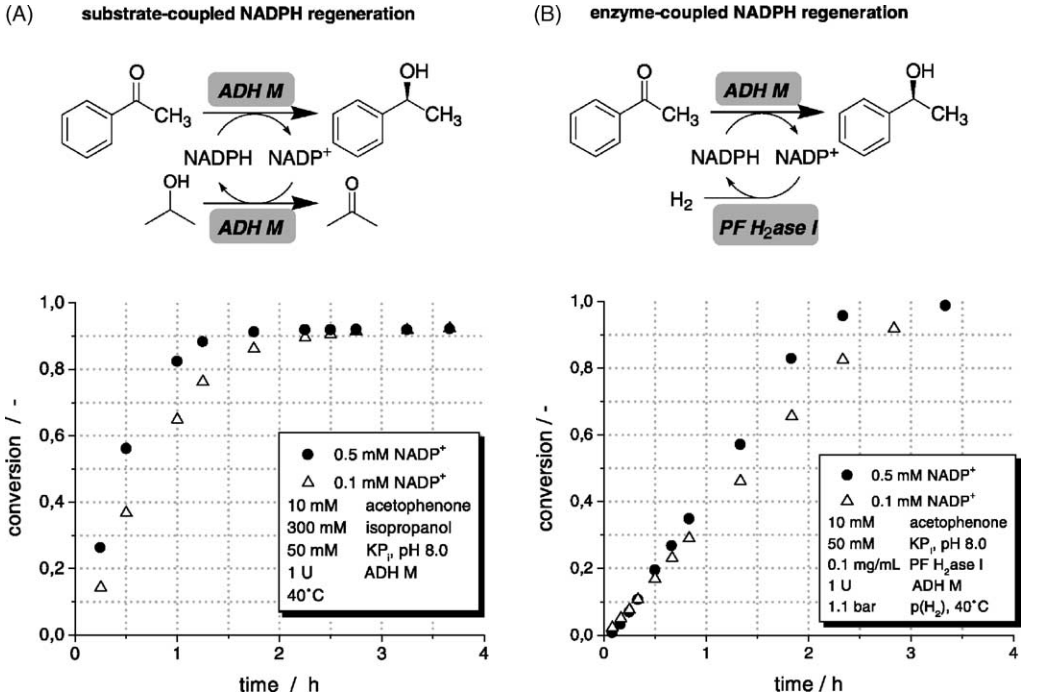

Fig. 3. Substrate-coupled NADPH regeneration (A) and enzyme-coupled NADPH regeneration using PF H_2ase I (B) batch experiments.

results lead to the conclusion, that the substrate-coupled NADPH regeneration reaction is thermodynamically limited, whereas the enzyme-coupled system overcomes this limitation.

The total turnover number (ttn) amounts to 93 and 19, concerning the oxidized nicotinamide cofactor ($NADP^+$) in both experiments. The product alcohol could be produced with an enantioselectivity of over 99.5% throughout all the experiments. By these means, it could be proved, that the stereoselectivity of the alcohol dehydrogenase catalyzed reduction of acetophenone to (1*S*)-phenyl ethanol is not influenced by the type of regenerating system used.

With these results in hand, we set out to investigate the PF H_2ase I coupled enzymatic reduction of acetophenone in a repetitive batch mode while recycling the enzymes in a modified ultrafiltration cell (see Fig. 4). Reactions were stopped as soon as quantitative conversion of acetophenone was reached.

Fig. 4 shows the conversion-time plots of the repetitive batch production of (1*S*)-phenyl ethanol, which could be performed in six consecutive experiments over 57 h without any further dosage of PF H_2ase I. Due to the obvious decrease in reaction rate during the third experiment, additional 1 U ADH M was added to the system after the third cycle. Since the initial reaction rates could be increased again, we could verify that the PF H_2ase I was still active in the given reaction system. Additionally, we could demonstrate, that the crude PF H_2ase I preparation is suitable for the side-product free reductive regeneration of NADPH for the thermophilic alcohol dehydrogenase. A maximum ttn of 100 referring to $NADP^+$ and a maximum space–time yield of $10\,g\,l^{-1}\,day^{-1}$ (1*S*)-phenyl ethanol could be reached. It is noteworthy, that throughout the repetitive batch experiment the regeneration reaction is not the rate limiting reaction, in contrast to the previously shown batch experiment.

To increase the total turnover number of the cofactor a substrate of higher solubility than acetophenone[4] was applied. The enantioselective reduction of

[4] The maximum solubility of acetophenone in 50 mM KP_i buffer, pH 8.0, $T = 40\,^{\circ}C$, totals 20 mM [57].

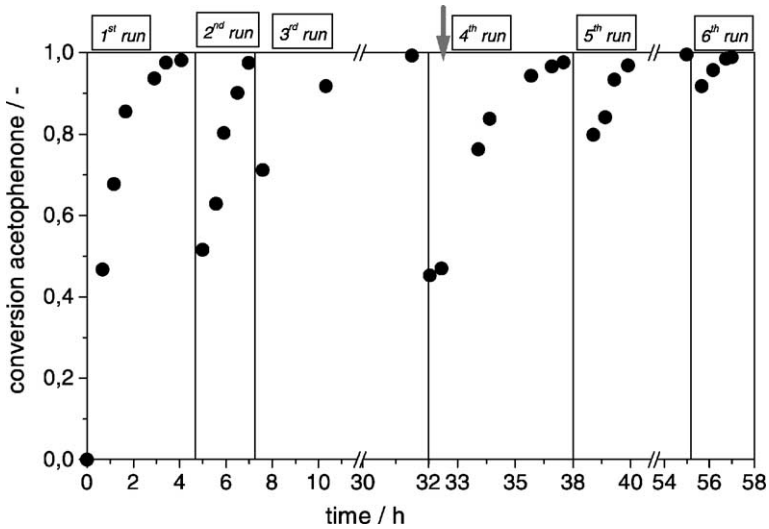

Fig. 4. Repetitive batch production of (1*S*)-phenyl ethanol coupled with PF H_2ase I catalyzed NADPH regeneration. The vertical black lines represent the beginning of a new cycle, i.e. dosing of acetophenone. The arrow indicates the additional dosage of 1 U ADH M prior the fourth cycle. (Initial conditions: 10 mM acetophenone, 0.5 mM $NADP^+$ in 3 ml 50 mM KP_i buffer (pH 8.0), 1 U ADH M, 1.0 mg/ml PF H_2ase I, $p(H_2) = 5$ bar, $T = 40°$, YM10 membrane.)

(2*S*)-hydroxy-1-phenyl-propanone[5] to the corresponding (1*R*,2*S*)-diol was catalyzed by ADH M with a d.e. >98% (see Fig. 5). NADPH was recycled with the PF H_2ase I-coupled cofactor regeneration system. The reaction was carried out as a repetitive batch regarding the enzymes.

Fig. 5 shows the progression curves of the repetitive batch experiment. Each of the three runs yielded quantitative conversion. The initial concentration of the (2*S*)-hydroxy-1-phenyl-propanone (HPP) was subsequently increased from 10 to 80 mM and a maximum ttn of 160 for NADPH could be reached.

In summary, the regeneration of the costly cofactor NADPH has been achieved enzymatically by PF H_2ase I. The combined use of a thermophilic ADH and PF H_2ase I should make these enzymatic reactions suitable for preparative scale transformations of prochiral carbonyl compounds. The use of the coupled enzymatic reaction showed to improve transformation yields since the reaction can be assumed to be irreversible and therefore the overall equilibrium constant value should be in favor of the product.

[5] The maximum solubility of (2*S*)-hydroxy-1-phenyl-propanone in 50 mM KP_i buffer pH 7.0, $T = 20\,°C$, totals 120 mM [58].

Surprisingly, the alcohol dehydrogenase ADH M turned out to be not sufficiently stable under the applied reaction conditions. Further investigations on this behalf are in progress.

3.2. NADPH generation

As mentioned above, in syntheses of NADPH most often side-products are formed, which might hamper downstream processing. To circumvent this a cheap reducing agent is needed, which does not leave any interfering products in the reaction mixture. Molecular hydrogen is such an agent and hydrogenases are capable to utilize dihydrogen to selectively reduce double bonds by transferring hydride-ions (H^-). Therefore, we were interested to investigate the direct synthesis of NADPH from $NADP^+$ with PF H_2ase I. Following this strategy, NADPH can be produced very efficiently from $NADP^+$ with molecular hydrogen as reduction equivalents. Additionally, any excess of the gaseous reduction reagent H_2 can easily be separated.

Fig. 6A shows two typical conversion plots of a 0.26 mg ml^{-1} PF H_2ase I preparation at 40 °C in two different buffer systems. Ttn's of 4600 (EPPS buffer) and 7060 (TRIS–HCl buffer) in terms of NADPH with

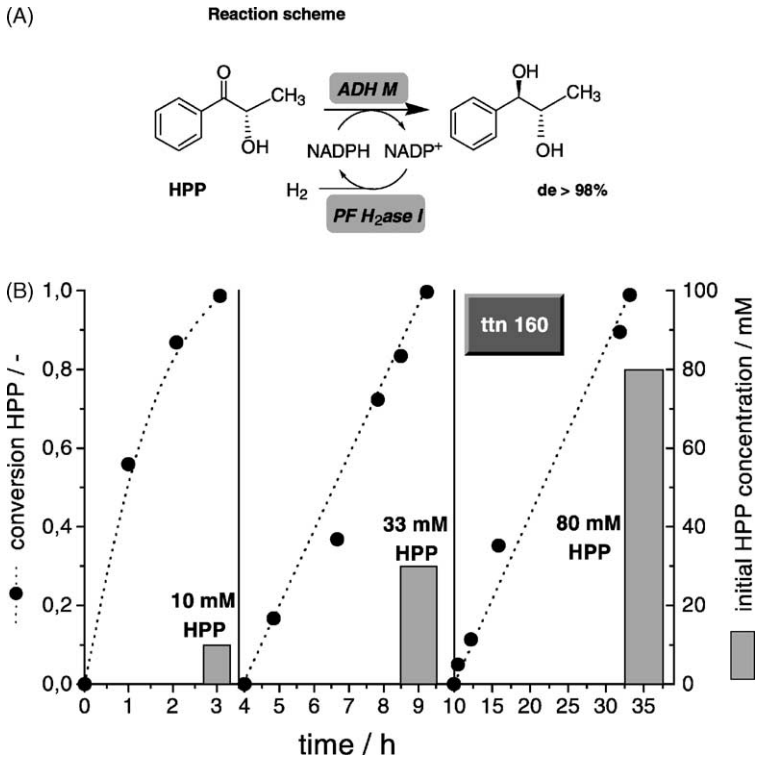

Fig. 5. Enantioselective reduction of (2*S*)-hydroxy-1-phenyl-propanone (HPP) to (1*R*,2*S*)-phenyl-propane-1,2-diol with PF H_2ase I catalyzed NADPH regeneration in a repetitive batch mode consisting of three cycles with different initial substrate concentrations. (Initial reaction conditions: 10 mM HPP, 0.5 mM $NADP^+$ in 3 ml 50 mM KP_i buffer, pH 8.0, 1 U ADH M, 1.0 mg ml^{-1} PF H_2ase I, 1.1 bar $p(H_2)$, YM10 membrane.)

turnover frequencies (tof) of 28.7 h^{-1} and 44.0 h^{-1}, respectively, could be reached. The turnover frequency is defined as mol substrate converted per mol catalyst (assuming one active site per molecule catalyst) and unit time.

Although the activity of the hyperthermophilic enzyme increases exponentially with temperature and has its maximum above 80 °C [35,37], we performed reactions at lower temperature (40 °C) due to the thermal instability of NADPH [19,59]. Nevertheless, the limiting factor still seems to be the low stability of NADPH at elevated temperatures. The decay of the product NADPH is accompanied by an obvious decline in the NADPH concentration, which can be deduced from the course of the yields in the shown batch experiments (see Fig. 6B). The yields pass through a maximum after 3 h (TRIS–HCl buffer) and 3.5 h (EPPS buffer) reaction time, respectively. This decrease is initiated by the chemical decomposition of the cofactor under these reaction conditions, which are necessary in order to achieve a sufficient enzyme activity. The amount of decomposition product formed in the EPPS-buffered process is higher than in the TRIS–buffered reaction. The mechanism and decomposition product still have to be elucidated. It might be, that NADPH decomposes following a reaction cascade, as published by Chenault and Whitesides [7], resulting in an inactive cyclic product. First analytical measurements using capillary electrophoresis and IC-MS/MS techniques showed the unknown substance to have the same molecular mass as NADPH, but with an absorption maximum at 266 nm instead of 340 nm (data not shown here). No decay products of $NADP^+$ could be detected. Whether the decomposition product inhibits the enzyme has not been investigated, yet.

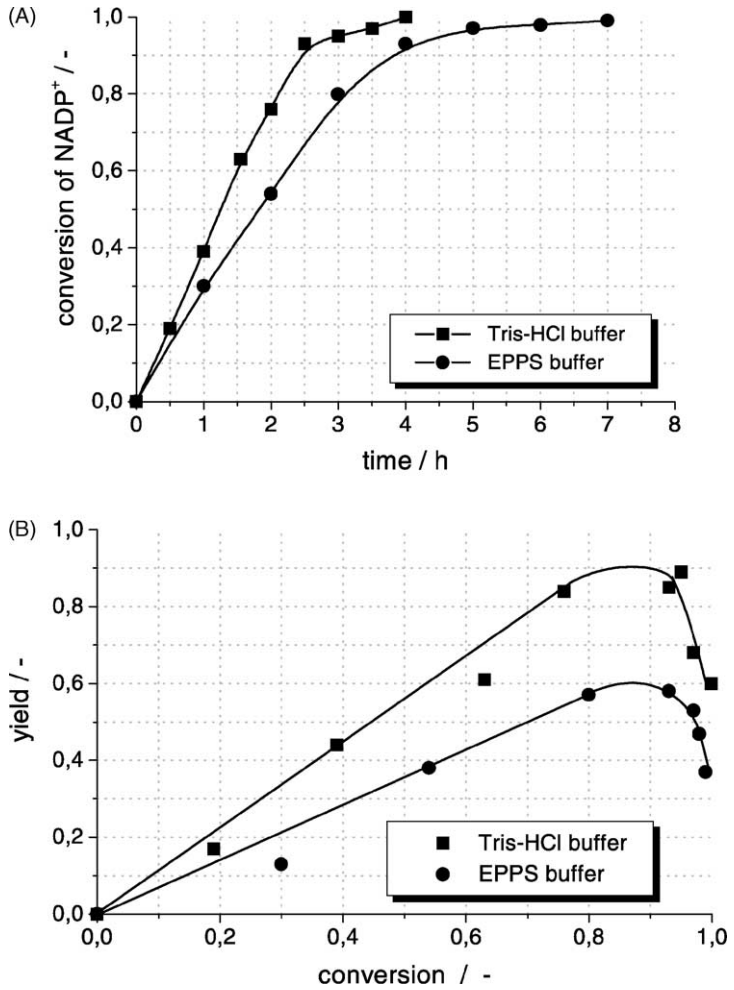

Fig. 6. NADPH production in two different buffer systems (batch experiments). (Reaction conditions: 12 mM $NADP^+$ in 2 ml 200 mM EPPS-buffer (●) or 100 mM TRIS–HCl buffer (■), pH 8.0, 0.26 mg ml^{-1} PF H_2ase I, 1.1 bar H_2, 40 °C.)

Furthermore, the PF H_2ase I catalyzed hydrogenation of $NADP^+$ was carried out as a repetitive batch process in a membrane reactor, equipped with an ultrafiltration membrane to retain the enzyme. Two separate experiments of eight runs each could be performed. The progression curves are shown in Fig. 7A and B.

After reaching >95% conversion,[6] the reactor content was concentrated to approx. 0.2 ml volume and refilled with freshly prepared substrate solution. Within the first repetitive batch-series (see Fig. 7A) a dihydrogen pressure of 5.0 bar was applied for the first six runs. Since the initial reaction velocity began to decrease, we applied a higher dihydrogen pressure of 7.5 bar to see, if there was any impact. This seemed not to be the case. During the second repetitive batch-series (see Fig. 7B) the dihydrogen pressure has been varied as well and the first repetitive batch experiment could be reproduced in general, including the rise in PF H_2ase I activity during the first three runs.

The PF H_2ase I was relatively stable ($t_{1/2} = 76$ h and $t_{1/2} = 208$ h)[7] and no further dosage during the

[6] Conversion $= ([NADP^+]_0 - [NADP^+])/[NADP^+]_0$.

[7] Half lives ($t_{1/2}$) were calculated by fitting the slope of initial reaction rates in Fig. 8 and using the first-order rate law: $t_{1/2} = ln\,(2/k_d)$.

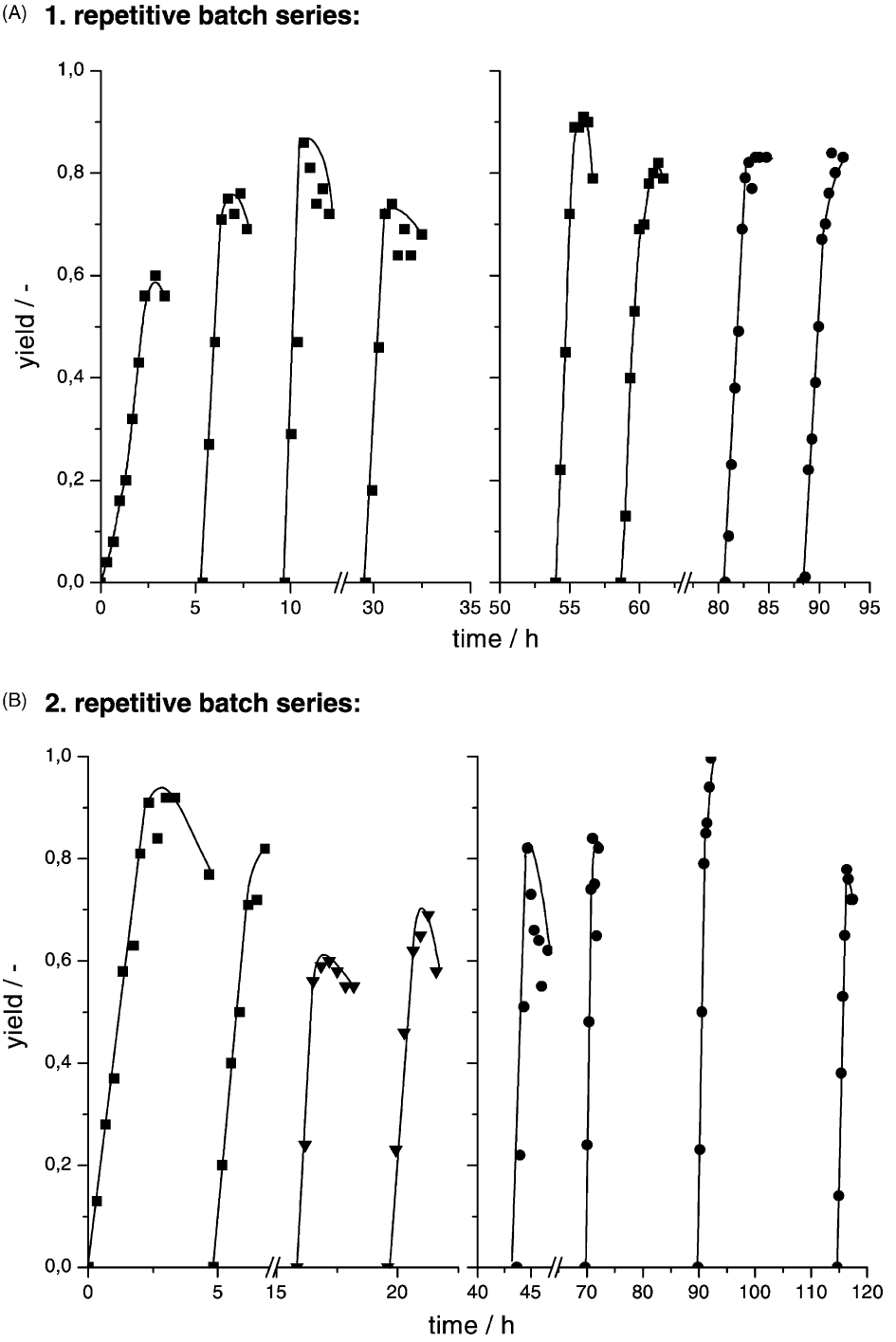

Fig. 7. NADPH production in two repetitive batch-series. Each experiment (A and B) consisting of eight runs at different hydrogen pressures as indicated: (▼) 2.5 bar H_2, (■) 5.0 bar H_2, (●) 7.5 bar H_2. (Initial reaction conditions: 12 mM $NADP^+$ in 3 ml 200 mM EPPS buffer, pH 8.0, 1.0 mg ml^{-1} PF H_2ase I, $T = 40\,^{\circ}C$, YM10 membrane.)

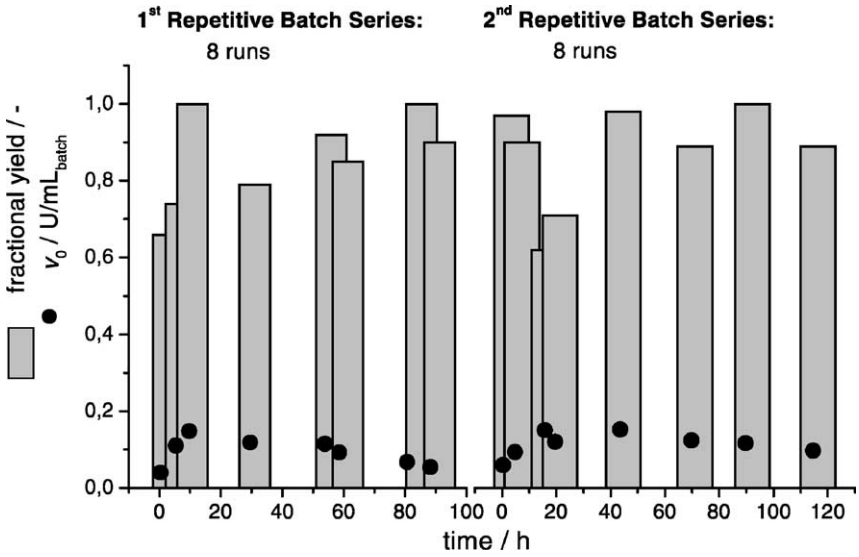

Fig. 8. Initial reaction rates and fractional yields of PF H_2ase I catalyzed NADPH synthesis in repetitive batch mode. Deactivation constants and half lives: first repetitive batch-series $k_d = 9.13 \times 10^{-3}\,h^{-1}$, $t_{1/2} = 76\,h$; second repetitive batch-series $k_d = 3.33 \times 10^{-3}\,h^{-1}$, $t_{1/2} = 208\,h$. Conditions as indicated in Fig. 7.

proceeding of the reactions was necessary. In the two repetitive batch-series 196.2 μmol (0.146 g) and 200.1 μmol (0.149 g) NADPH, respectively, could be produced. Over 8 runs, with the maximum analytical NADPH yield taken as a basis and the partially purified PF H_2ase I preparation treated as if it was pure hydrogenase, total turnover numbers[8] of 9800 and 10,000, respectively, were achieved. Thus, the biocatalyst performed 90 and 33 catalytic cycles per hour (tof) with a catalyst to substrate ratio of 1:1800 (mol/mol).

Since it is known, that [NiFe]-hydrogenases in general and the PF H_2ase I in particular need to be reductively activated prior to use, we pre-incubated the crude PF H_2ase I preparation at 80 °C for 5 min under dihydrogen atmosphere before transferring it anoxically into the reaction mixture [28,60]. Nevertheless we observed a reproducible increase in PF H_2ase I activity throughout the course of the first three runs in the repetitive batch experiments (see Fig. 8).

Fig. 8 shows the initial reaction rates as well as the fractional yield of both repetitive batch experiments. It visualizes the approx. three-fold increase in reaction rate during the conversion of $NADP^+$ at 40 °C in 200 mM EPPS buffer (pH 8.0) within the first 10–15 h reaction time, before a decrease, probably caused by deactivation of the PF H_2ase I, could be encountered.

It is unclear why this activation effect occurs. Presumably, the applied [NiFe]-hydrogenase was not fully converted to the catalytically active state by temperature-induced activation. However, these findings are interesting, since the PF H_2ase I preparation was purified under anaerobic and reducing conditions. Therefore, it was assumed, that the catalytic sites of the [NiFe]-hydrogenase would predominantly be in their EPR-silent "ready" redox state (Ni–B), which can be quickly converted into the "active" state through thermal incubation under H_2 or in the presence of trace amounts of low-potential reductants [60,61]. Only the "unready" or Ni-A redox state of oxidized [NiFe]-hydrogenase is known to have a long lag-phase, taking hours to be catalytically active at physiological temperatures [60,61]. There are still many questions to be answered concerning the mechanism of the enzyme. Although a consensus is slowly being reached, the exact roles of the different active

[8] With total turnover number = mol produced NADPH/mol consumed enzyme.

site components have not yet been fully established [62].

It is also conceivable, that the observation of promoted enzyme activity is related to the presence of NADPH besides $NADP^+$ and dihydrogen throughout the course of the experiments, since NADPH is an electron donor to the PF H_2ase I [35]. We conclude from our results, that a change in the heat activation protocol is necessary and the applied conditions need to be investigated further.

4. Conclusions

Within the aim of achieving an alternative method to generate and regenerate the cofactor NADPH, we have developed a procedure to apply the crude PF H_2ase I preparation efficiently for the production of NADPH as well as for the enzyme-coupled in situ regeneration with alcohol dehydrogenase ADH M, catalyzing enantioselective reductions of prochiral ketones.

These results show the potential of the hydrogenase I from *P. furiosus*. A by-product free and benign enzymatic NADPH generation and regeneration process utilizing cheap molecular hydrogen is now available. Because of its thermophilic character, the PF H_2ase I can be used over a broad temperature range between 20 and 80 °C. However, at higher temperatures the instability of NADPH becomes limiting. As consequence short residence times and special downstream processing is required for the production of NADPH.

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