

Pilot Scale Production and Isolation of Recombinant NAD⁺- and NADP⁺-Specific Formate Dehydrogenases

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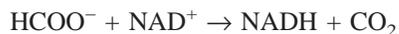
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Abstract: The expression of the recombinant wild-type NAD⁺- and mutant NADP⁺-dependent formate dehydrogenases (EC 1.2.1.2., FDH) from the methanol-utilizing bacterium *Pseudomonas* sp. 101 in *Escherichia coli* cells has been improved to produce active and soluble enzyme up to the level of 50% of total soluble proteins. The cultivation process for *E. coli*/pFDH8a and *E. coli*/pFDH8aNP cells was optimized and scaled up to a volume of 100 L. A downstream purification process has been developed to produce technical grade NAD⁺- and NADP⁺-specific formate dehydrogenases in pilot scale, utilizing extraction in aqueous two-phase systems. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* 64: 187–193, 1999.

Keywords: formate dehydrogenase; *Pseudomonas* sp. 101; expression in *E. coli*; optimization of expression; large-scale cultivation; aqueous two-phase extraction

INTRODUCTION

In methanol-utilizing microorganisms the NAD⁺-dependent formate dehydrogenase (EC 1.2.1.2, FDH) catalyses the last step in the dissimilatory pathway of methanol to carbon dioxide:



FDH consists of two identical subunits and does not contain metal ions or other prosthetic groups in the active center.

FDH is of great interest from scientific and practical considerations. The enzyme belongs to the superfamily of D-stereospecific 2-hydroxy acid dehydrogenases (Vinals et al., 1993) and can be regarded as a model dehydrogenase cata-

lyzing the reaction with the simplest substrate: the formate ion. Structures of the apo- and holo-FDH from *Pseudomonas* sp. 101 have been solved with high resolution (Lamzin et al., 1994), and site-directed mutagenesis experiments have been carried out to clarify the role of different amino acid residues in the catalytic mechanism of hydride transfer (Tishkov et al., 1993b, 1996).

From applied aspects FDH is the best catalyst for *in-situ* NADH regeneration in the enzymatic synthesis of amino acids, chiral hydroxy acids and esters, alcohols, and other fine chemicals using NAD⁺-dependent dehydrogenases (Hummel and Kula, 1989; Drauz and Waldmann, 1995; Peters, 1998). The reaction catalyzed by FDH is practically irreversible and 100% conversion of educts is possible in coupled reactions. Formate does not inhibit most other dehydrogenases and the co-product, carbon dioxide, can be easily removed from the reaction mixture. The enzyme has a wide pH optimum for activity and can be obtained from methanol-utilizing microorganisms in large amounts. A process for the production and purification of technical grade FDH from *C. boidinii* in large scale has been developed (Weuster-Botz et al., 1994) and the enzyme was used for the industrial production of L-*tert*-leucine (Bommarius et al., 1995).

NAD⁺/NADH are mainly involved in biodegradation processes, while NADP⁺/NADPH participate in biosynthetic pathways. Many interesting compounds can be obtained only using NADP⁺-specific dehydrogenases requiring for economic reasons an efficient and inexpensive NADPH regeneration system, which was previously not available (Drauz and Waldmann, 1995). Wild-type FDH cannot be used for NADPH regeneration. Enzymes produced by yeasts do not accept NADP⁺ at all and FDHs from bacteria have very low specific activity (about 5% compared to the activity with NAD⁺) and very high K_m values (0.10 versus 35 mM for NAD⁺ and NADP⁺, respectively). Using a rational design strategy we derived a NADP⁺-

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specific FDH based on the FDH gene of the bacterium *Pseudomonas* sp. 101 (Tishkov et al., 1993a). The new enzyme was successfully employed 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.

For application in larger scale the enzyme has to be available in sufficient amounts. Presently the commercially available NAD⁺-dependent FDH is mainly produced from the yeast *Candida boidinii* (Weuster-Botz et al., 1994). Optimization of the cultivation medium resulted in a space/time yield up to 3600 U/L per day (Weuster-Botz and Wandrey, 1995). Production of target proteins using recombinant *E. coli* strains has some advantages compared to systems based on wild strains. During expression of recombinant proteins in *E. coli* concentrations of 8.5 g/L have been described (Joly et al., 1998). This approach is very flexible and the same expression system could be used for the production of different mutant FDHs with changed coenzyme specificity and increased thermal stability. FDH genes from the bacteria *Pseudomonas* sp. 101 (Tishkov et al., 1993b), *Mycobacterium vaccae* N10 (Galkin et al., 1995), *Moraxella* C-1 (EMBL accession number O08375) and the yeast *Hansenula polymorpha* (Hollenberg and Janowicz, 1989), *Candida methylica* (Allen and Holbrook, 1995), and *Candida boidinii* (Sakai et al., 1997, Slusarczyk et al., in press) have been cloned and expressed in *E. coli* cells. In shake flask experiments the highest level of FDH expression, 15% of total soluble *E. coli* proteins, was achieved for the enzyme from *C. methylica* under the control of the *tac* promoter (Allen and Holbrook, 1995). Large-scale production of FDH using recombinant *E. coli* cells has not been reported so far.

The aim of the present work was to develop up to the pilot scale a low cost process for the production of a biocatalyst for NAD(P)H regeneration based on the recombinant wild-type NAD⁺- and mutant NADP⁺-specific formate dehydrogenases from *Pseudomonas* sp. 101. During this research we wanted to achieve (1) optimization of the FDH expression system in *E. coli*; (2) optimization of the large-scale cultivation of the recombinant *E. coli*/pFDH strain to obtain high yield of biomass and high level of FDH expression; and (3) adaptation of a downstream process for FDH purification from *E. coli* homogenates.

MATERIALS AND METHODS

Enzymes and Biochemicals

Restriction endonucleases, T4 DNA kinase, T4 DNA polymerase, and T4 DNA ligase (400 U/ μ L) were obtained from New England Biolabs (Schwalbach, Taunus, Germany). All chemicals used for genetic engineering experiments and site-directed mutagenesis were molecular biology grade. ATP and dNTPs were purchased from Pharmacia Biotech (Freiburg, Germany), and NAD⁺ and NADP⁺ (\geq 98% purity both) were from Sigma (Deisenhofen, Germany).

Genetic Engineering Methods

All operations with DNA (digestion, analytical and preparative agarose gel electrophoresis, DNA extraction from gel, ligation, etc.) were carried according to Maniatis et al. (1982). The introduction of new restriction sites was accomplished by a site-directed mutagenesis using the method of Kunkel et al. (1991). All mutations were controlled by DNA sequencing of both strands of the entire FDH gene with an Applied Biosystems automated DNA sequencer 370A and PCR Taq dye-labeled terminators sequencing kit (Perkin Elmer–Applied Biosystems, Weiterstadt, Germany).

Expression

The level of the FDH expression in different *E. coli*/pFDH strains was studied in shake flasks experiments. A single colony was picked from a Petri dish and used to inoculate an overnight culture (37°C, 160 rpm) in 3 mL of 2YT medium (16 g of Bactotrypton, 10 g of yeast extract (Difco, Augsburg, Germany) and 5 g NaCl per liter, pH 7.0) containing 300 μ g/mL ampicillin. A 2-mL amount of the cell suspension was used to inoculate 200 mL of 2YT medium, 200 μ g/mL ampicillin in 1-L flasks. Cultivation was carried out at 37°C and 150 rpm. FDH biosynthesis was induced by supplementation of the medium with isopropyl- β -thiogalactoside (IPTG final concentration 0.25 mM) after 4 h when cell absorbance A_{600} reached values about 2.0. At certain time intervals probes were taken and prepared for analysis as described by Klyushnichenko et al. (1997). Enzyme activity was measured spectrophotometrically at 340 nm in 0.1 M K-phosphate buffer (pH 7.0), at 30°C following the production of the reduced coenzyme. The concentrations of NAD⁺ or NADP⁺ (for the measurement of corresponding activity of NAD⁺- and NADP⁺-specific enzymes) were 1.5 mM, and the concentration of sodium formate was 0.3 M in the assay mixture. The FDH concentration in probes was also estimated taking into account a specific activity 10 and 2.5 U/mg of protein of homogeneous NAD⁺- and NADP⁺-specific formate dehydrogenases, respectively (Tishkov et al., 1993c; Klyushynichenko et al., 1997). Total protein concentration in the probe was determined by the method of Bradford (1976). The level of FDH expression was expressed as a ratio of FDH and total protein concentrations in the probe. Some probes were subjected to SDS electrophoresis in polyacryl amide gel and the level of expression was estimated by scanning densitometry on the Shimadzu dual-wavelength Flying-Spot Scanner Model CS9000 (Shimadzu GmbH, Duesseldorf, Germany).

Cultivation

Laboratory and pilot-scale fed-batch cultivations of *E. coli*/pFDH8a and *E. coli*/pFDH8aNP strains were performed in continuously stirred 20 and 300 L tank reactors (Chemap, Volketswil, Switzerland, working volumes 10 and 100 L,

respectively). The pH was maintained at 7.0 ± 0.1 by the controlled addition of ammonia. The dissolved oxygen level was maintained at 20–25% of air saturation by progressive increase in the agitation rate (40–550 rpm), the sparge volume (air, 0.1–4 L/min per liter of cultivation medium) and the reactor vessel pressure (0–1.3 bar). Foaming was suppressed by the controlled addition of the antifoam agent DEHYSAN Z2111 (Henkel, Düsseldorf, Germany). The cultivation medium (CM) contained (g/L): KH_2PO_4 , 3.0; Na_2HPO_4 , 6.0; NaCl , 0.5; HCOONa , 2.0; NH_4Cl , 0.25; MgSO_4 , 0.5, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.025. After heat sterilization and cooling to room temperature, a trace metal solution (g/L: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.58; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.30; H_3BO_3 , 0.12; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.10; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.10; KI , 0.17; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 6.0; EDTA, 0.5, and 4 mL of 0.25 M H_2SO_4 per liter) and thiamin solution (0.1 g/L) were added to the medium (1 mL/L of both solutions) by sterile filtration. Concentrated glucose solution (20% w/v) was added to the medium by sterile filtration just prior to inoculation to provide an initial concentration of 0.5 g/L. The feed medium added during the fed-batch process contained 650 g/L of glucose (heat sterilized), 12 mL/L of the trace element solution, and 12 g/L MgSO_4 . For induction, the feed medium was supplemented with 50 g/L lactose. Precultures were prepared by overnight cultivation at 37°C of the cells glycerol stock (50 μL of stock per liter of medium) in CM containing 1 g/L of glucose and 400 mg/L of ampicillin to a density of A_{600} 0.5–0.8. The inoculum volume was 5% and 1.3% of cultivation medium at laboratory and pilot scale, respectively. Cells were harvested by continuous flow centrifugation, and stored frozen at -20°C .

Extraction in Aqueous Two-Phase Systems

Aqueous two-phase systems were used for the large-scale purification of recombinant FDH. Frozen biomass was re-suspended in 0.1 M potassium phosphate buffer, pH 8.5, to get 25% wet cell mass concentration. The cell suspension was passed two times through a bead mill desintegrator (Netzsch, Selb, Germany). The first aqueous two-phase system had the following composition: 50% (w/w) cell homogenate, 18% (w/w) PEG 1550, 6% (w/w) K_2HPO_4 , 5% (w/w) sodium formate, and 21% additional water. After intensive mixing, the two-phase system was allowed to separate overnight. The upper phase was pumped to another vessel and the second two-phase system generated. It had a total composition of 50% top phase, 3% PEG 20,000, 7.5% KH_2PO_4 , 1.0% K_2HPO_4 , 2.5% sodium formate, and 36% additional water. The bottom phase obtained after mixing and phase separation was desalted and concentrated using an ultrafiltration module Sartocoon II (Sartorius AG, Göttingen, Germany) equipped with cellulose triacetate membranes (cut-off 20,000 Da), using 0.1 M sodium phosphate, 10 mM EDTA buffer, pH 7.0, for diafiltration.

RESULTS AND DISCUSSION

Optimization of FDH Expression in *E. coli* Cells

The native FDH promoter from *Pseudomonas* sp. 101 is not functional in *E. coli*, and it was necessary to select an expression system optimal for the host bacterial strain. Different expression systems can be used for the biosynthesis of recombinant proteins in *E. coli*, the most popular are based on *lac*, *tac*, and T7 RNA polymerase promoters (De Boer et al., 1983; Studier and Moffatt, 1986). To create an efficient expression system, high level of protein biosynthesis and high yield of biomass are required. In addition the level of the recombinant protein expression has to be considered, which depends on the initiation of transcription and translation. Furthermore it was desired to produce the recombinant enzyme as a soluble active protein. The soluble enzyme should be resistant to digestion by proteases in *E. coli* cells to avoid post-translational losses and the generation of fragments as contaminants.

Figure 1 and Table I present data improving the FDH expression in *E. coli*. The plasmid pFDH2 was obtained cloning a 2.3 kbp *HindIII*–*Bgl*II fragment carrying the FDH gene (Tishkov et al., 1993b) in plasmid pUC19 under the control of the *lac* promoter. This insert contained additional 460 and 670 bp in the 5'-upstream and 3'-downstream regions, respectively, of the FDH gene (Fig. 1). Deletion of a 150 bp *HindIII*–*Nru*I fragment in the 5'- region and insertion of the 81 bp *Bam*HI–*Hind*III block with the *tac* promoter from the plasmid pDR540 (final plasmid pFDH4, see Fig. 1 and Table I) resulted in enzyme expression comprising 5–7% of the total soluble *E. coli* proteins. An *Eco*RI restriction site was introduced by mutagenesis behind the TGA stop codon (plasmid pFDH5) to remove 660 bp in the 3'- region. Plasmid pFDH6 was generated cloning from pFDH5 the 1635 bp *Bam*HI–*Eco*RI fragment containing the *tac* promoter and the FDH gene, in the phagemid pUC119. Despite the use of the two strong promoters in tandem in pFDH6, the expression level of FDH was still rather low: 8–9% of total soluble protein. It could be explained by a low initiation of translation or by differences in the codon usage in *Pseudomonas* sp. 101 and *E. coli*. The (G+C) content in the FDH gene is 67% compared to 51% in the chromosome of *E. coli*. High level of FDH expression was achieved after substitution of the original ribosomal binding site (RBS) by a RBS with the enhancer of translation from the gene 10 of phage T7 (Olins and Rangwala, 1989) (plasmid pFDH6b). The high increase in the FDH expression level after optimization of the RBS in the plasmid pFDH6a shows that for plasmids pFDH2, pFDH4 and pFDH6 the initiation of translation was the limiting step in the FDH expression in *E. coli*. The best plasmid pFDH8a was obtained after optimization of the RBS and the exchange of some of the original codons in the starting region of the FDH gene for optimal codons from *E. coli*. This plasmid produced FDH corresponding to 45–50% of the soluble proteins in three different *E. coli* strains: TG1, JM109, and BL21/pLysS. A functional FDH

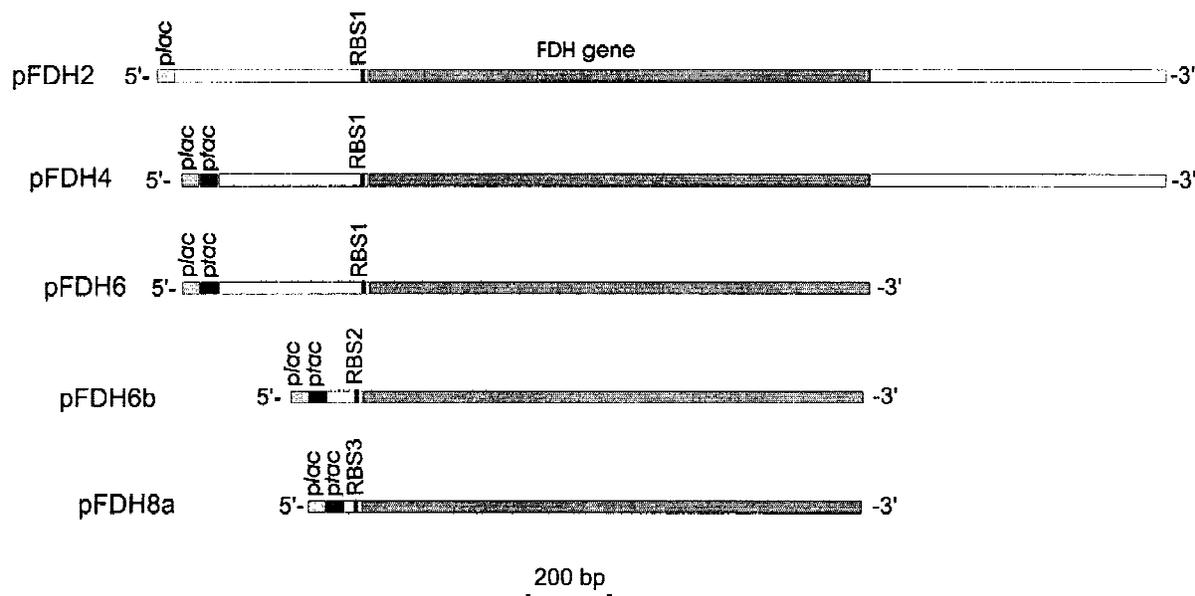


Figure 1. Optimization of the expression system of FDH in *E. coli* cells. RBS, ribosome binding site. Structures of RBS1, RBS2, and RBS3 are presented in Table I. Plasmids pFDH2 and pFDH4 are based on plasmid pUC19, and plasmids pFDH6, pFDH6b, and pFDH8a are derived from phagemid pUC119. The *lac* promoter is a part of the original plasmid pUC19 and phagemid pUC119. The *tac* promoter was inserted as 81 bp *Bam*HI–*Hind*III fragment from the plasmid pDR450. White boxes denote noncoding DNA sequences in 5'-upstream and 3'-downstream regions flanking the FDH gene in the *Pseudomonas* sp. 101 chromosome. At the 3'-end the inserted FDH gene is ligated through *Bg*III or *Eco*RI restriction sites in the polylinker of plasmid pUC19 and phagemid pUC119, respectively.

gene based on Tishkov et al. (1993b) but containing some site-directed mutations that altered the coenzyme specificity and thermostability of the FDH was cloned in plasmids with the expression systems described in Table I. Details of the construction of this gene and characterization of the different mutant proteins will be described elsewhere (Tishkov, Egorov, and Galkin, manuscript in preparation; Galkin, Rojkova, Fedorchuk, Serov, Kulakova, Matorin, Savitsky, Kula, and Tishkov, manuscript in preparation). There were no detectable differences in the expression of wild-type NAD⁺-dependent and mutant NADP⁺-specific FDHs. The enzyme was expressed solely as a soluble protein and efficiently extracted in solution. No evidence for inclusion bodies or aggregated FDH was found in the debris fraction after cell disruption and centrifugation as judged by SDS-electrophoresis in polyacryl amide gel (data not shown).

Pilot Scale Cultivation of FDH

Plasmids pFDH8a and pFDH8aNP containing the genes of NAD⁺- and NADP⁺-specific enzymes, respectively, were

used for pilot scale cultivation employing *E. coli* TG1, TG1/pLysS, or BL21/pLysS cell lines as the host strain. The strains TG1 as well as BL21 can be grown to high cell density (Korz et al., 1995). The use of the second plasmid pLysS expressing lysozyme from bacteriophage T7 facilitates cell disruption after cultivation (Studier, 1991). Ampicillin (400 mg/L) was used only to supplement the medium of the preculture but was omitted from the main culture. In the pilot cultivation in 100-L scale a sensitive glucose analyzer was not available. The addition of glucose was therefore based on the experimental data relating growth rate and biomass yield found in preliminary cultivations in 10-L scale (data not shown). The concentration of oxygen was maintained at 20–25% of saturation. Before induction cells were grown at 37°C. With the start of the induction the temperature was decreased to 30°C to prevent premature cell lysis. Lower growth temperature also facilitates the production of soluble recombinant proteins and prevents production of inclusion bodies (Schein and Noteborn, 1988). Instead of IPTG FDH biosynthesis was

Table I. Optimization of expression of the recombinant FDH from *Pseudomonas* sp. 101 in *E. coli* cells.

Plasmid	Promoter	Distance between promoter and RBS, bp	Ribosome binding site	Expression level, % of soluble protein
pFDH2	<i>lac</i>	460	AGGAG atgcccgATG	2–3
pFDH4	<i>lac + tac</i>	360	AGGAG atgcccgATG	5–7
pFDH6	<i>lac + tac</i>	360	AGGAG atgcccgATG	8–9
pFDH6b	<i>lac + tac</i>	90	TTAACTTAgAAGGAG atatacatATG	40–43
pFDH8a	<i>lac + tac</i>	45	AGGA tccatatATG	45–50

Table II. Dependence of total and specific yield of NADP⁺-dependent FDH in 10-L fermentations on the induction at different cell absorption.

Cell absorbance at induction, A ₆₀₀	Biomass yield (dry weight), g	Total yield, units	Specific yield, U/g of dry biomass
4.2	48	17300	360
5.1	60	21300	353
6.2	71	24850	350
7.0	71	25350	357
8.1	86	18100	210
10.0	92	4970	54

induced by the controlled addition of lactose. Usually, in shaking flasks and batch cultivation, induction is started at a cell absorbance at 600 nm (A₆₀₀) of less than 1. At higher A₆₀₀ values cells start to lose plasmids rather quickly. In fed-batch cultivation the limited growth rate provides better plasmid maintenance. In our case it was found that the optimal cell absorbance for induction is A₆₀₀ 6–7 (Table II). Induction at higher cell absorption resulted in a decrease of the total and cell-specific FDH yield. Induction at lower cell absorption did not influence the level of expression but decreased significantly the growth rate. Besides early cell lysis was observed.

After optimization of the cultivation conditions in 10-L scale, the level of FDH expression was about 50% of total soluble proteins (Fig. 2A). Data for the cultivation of NADP⁺-dependent FDH in 100 L are presented in Fig. 3. Taking into account that the total volume increased to 120 L by the feeding regime, the biomass yield was 8 g dry cells/L, the activity yield was 1650 U/L and the total yield about 200,000 U. The last value corresponds to 800,000 U for the NAD⁺-specific FDH. The enzyme content was more than 30% of total soluble protein (Fig. 2B, line 5). Due to

technical reasons the cultivation was stopped after 21 h when cells growth and FDH expression were still in the log phase (Fig. 3B). Therefore it should be possible to increase the biomass yield and total activity at least two times. As it was shown in our previous work (Klyushnichenko et al., 1997), both, NAD⁺- and NADP⁺-specific FDH are resistant to *E. coli* proteases. Using MALDI-TOF techniques (matrix-assisted laser desorption/ionisation time of flight mass spectrometry) it was demonstrated that FDH from *Pseudomonas* sp. 101 expressed in *E. coli* is a full size 400-aa protein and is not digested during the whole cultivation period (Klyushnichenko et al., 1997).

Aqueous Two-Phase Extraction System

Aqueous two phase extraction was successfully used for the large scale production (up to mega units range) of technical grade NAD⁺-dependent formate dehydrogenase from the yeast *C. boidinii* (Weuster-Botz et al., 1994) as well as numerous other enzymes. This method of purification is very flexible. It avoids difficult solid/liquid separation steps for the removal of cell debris and can be easily scaled up 10–10,000 times (Hustedt et al., 1985; Kula, 1990). Recombinant NAD⁺- and mutant NADP⁺-dependent FDHs from *Pseudomonas* sp. 101 expressed in *E. coli* were isolated by extraction in aqueous two phase systems based on polyethylene glycol (PEG)/salt (Table III). Identical conditions were used for the purification of NAD⁺- and NADP⁺-dependent enzymes and about equal results were obtained. In the first step FDH was extracted to the upper, PEG-rich phase (partition coefficient ~ 100) and separated from cell debris and DNA. The main difference of the first system compared to the isolation of FDH from *C. boidinii* is the use of PEG 1550 only instead of a mixture of PEG 400 and PEG

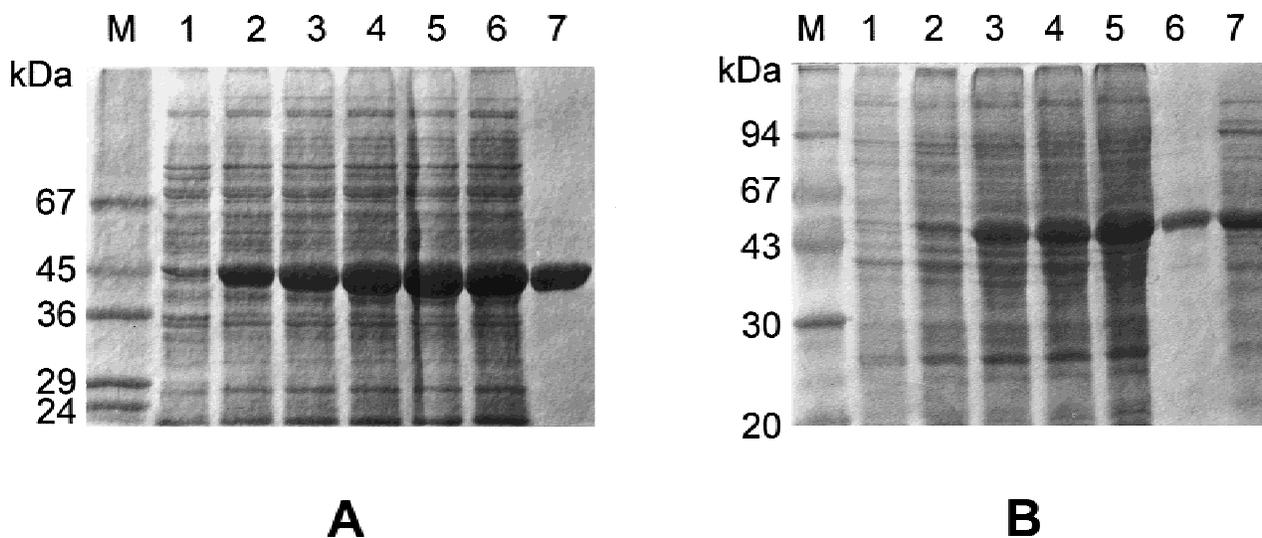


Figure 2. Expression of the mutant NADP⁺-specific FDH in *E. coli*/pFDH8aNP strain during fed-batch fermentations. (A) Cultivation in 10 L. M, molecular weight markers (Boehringer Mannheim); lines 1–6, 0, 3, 6, 9, 12, and 15 h after induction, respectively; line 7, purified FDH. (B) Cultivation in 100 L. M, molecular weight markers (Sigma); lines 1–5, 0, 3, 7, 11, and 15 h after induction, respectively; line 6, purified FDH; line 7, FDH preparation after extraction in aqueous two-phase systems.

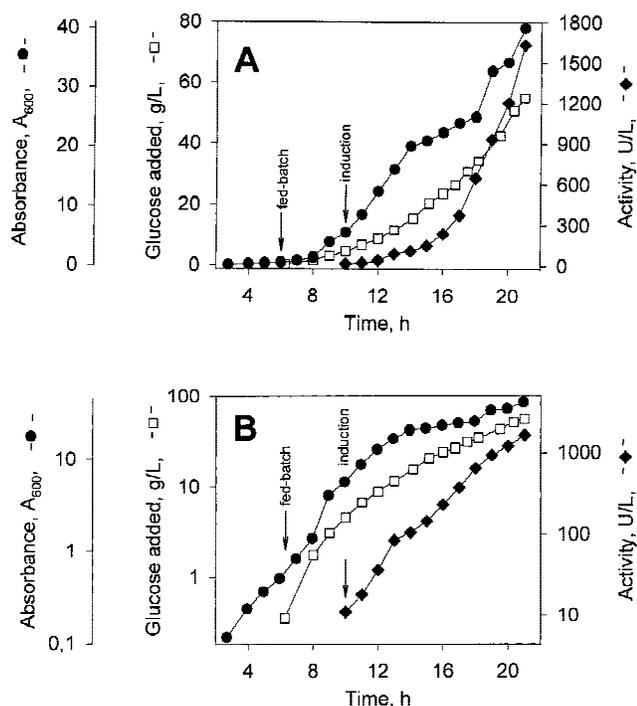


Figure 3. Fermentation of *E. coli*/pFDH8aNP by glucose fed-batch operation followed by induction with a glucose/lactose feed mixture. (A and B) normal and logarithmic ordinate scales, respectively.

1550 for the yeast enzyme (Weuster-Botz et al., 1994). In the second extraction step FDH was partitioned to the bottom, salt-rich phase. To achieve a low partition coefficient ($k < 0.06$) we had to decrease the pH value to 6.0–6.2 instead of pH 8.0 used for *C. bovidinii* FDH (Weuster-Botz et al., 1994). After phase separation the enzyme solution

was subjected to diafiltration to concentrate the protein and remove salts as well as residual PEG. The purity of the FDH preparations after these steps is about 45–55% and depends on the level of FDH expression in *E. coli*. Line 7 in Fig. 2B shows the NADP⁺-specific FDH purified from biomass obtained by cultivation in 100-L scale. The final enzyme preparations had a specific activity of 0.9 and 3.6 U/mg for NADP⁻- and NADP⁺-specific FDHs, respectively (Table III). High yield at each step ($\geq 90\%$) resulted in 75% overall recovery of activity. The enzyme can be used for *in situ* coenzyme regeneration processes. After addition of 20% glycerol to prevent bacterial contamination, technical grade FDH may be stored at +4°C at least 10–12 months without loss of activity. If desired, the specific activity of the final FDH preparation can be increased by heat treatment of the cell suspension before the first extraction step. In preliminary small scale experiments it was found that 5 min heat treatment at 60°C in the presence of 10% w/w sodium formate removed 17–20% of *E. coli* proteins by heat coagulation without any loss of FDH activity.

CONCLUSION

The mutant FDH from *Pseudomonas* sp. 101 is one of the best catalyst for NAD(P)H regeneration. This enzyme has higher specific activity, better affinity to formate and the highest stability compared to FDH from other sources. The system developed for the pilot scale production of recombinant NADP⁻- as well as NADP⁺-dependent FDH from *Pseudomonas* sp. 101 gives higher space time yield (U/L per day), activity yield (U per g of dry biomass), and higher enzyme recovery after purification compared to the continuous production of wild type FDH with the yeast *C. bovidinii*

Table III. Purification of NAD⁻- and NADP⁺-specific formate dehydrogenases using aqueous two-phase systems.

Purification step	System components ^a	Step yield, %	Total yield, %
Cell homogenate after bead-mill desintegration	25% wet weight cell suspension in 0.1 M potassium phosphate buffer, pH 8.5	100	100
Phase system 1	Cell homogenate, 50% PEG 1550, 18% K ₂ HPO ₄ , 6% HCOONa, 5% Additional water, 21% pH 8.0–8.5 Partition coefficient, ^b ~100	92	92
Phase system 2	Upper phase from system 1, 50% PEG 20000, 3% KH ₂ HPO ₄ , 7.5% K ₂ HPO ₄ , 1.0% HCOONa, 2.5% Additional water, 36% pH 6.0–6.2 Partition coefficient ^b <0.06	90	83
Diafiltration	0.1 M potassium phosphate buffer, 10 mM EDTA, pH 7.0	90	75

^aAll percentage values are shown as a w/w %.

^bRatio of FDH concentration in top and bottom phases.

as described by Weuster-Botz et al. (1994). Large-scale cultivations and two-phase extraction isolation of our recombinant *Pseudomonas* FDH and the formate dehydrogenase from *C. boidinii* (Weuster-Botz et al., 1994) were done in the same equipment and our calculations show that the production cost of the recombinant bacterial NAD⁺- and NADP⁺-dependent FDHs should be similar or less than the same wild type enzyme from *C. boidinii* (Weuster-Botz et al., 1994).

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