Metabolic Engineering of Escherichia coli: Increase of NADH Availability by Overexpressing an NAD$^+$-Dependent Formate Dehydrogenase

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Metabolic engineering studies have generally focused on manipulating enzyme levels through either the amplification, addition, or deletion of a particular pathway. However, with cofactor-dependent production systems, once the enzyme levels are no longer limiting, cofactor availability and the ratio of the reduced to oxidized form of the cofactor can become limiting. Under these situations, cofactor manipulation may become crucial in order to further increase system productivity. Although it is generally known that cofactors play a major role in the production of different fermentation products, their role has not been thoroughly and systematically studied. However, cofactor manipulations can potentially become a powerful tool for metabolic engineering. Nicotinamide adenine dinucleotide (NAD) functions as a cofactor in over 300 oxidation–reduction reactions and regulates various enzymes and genetic processes. The NADH/NAD$^+$ cofactor pair plays a major role in microbial catabolism, in which a carbon source, such as glucose, is oxidized using NAD$^+$ producing reducing equivalents in the form of NADH. It is crucially important for continued cell growth that NADH be oxidized to NAD$^+$ and a redox balance be achieved. Under aerobic growth, oxygen is used as the final electron acceptor. While under anaerobic growth, and in the absence of an alternate oxidizing agent, the regeneration of NAD$^+$ is achieved through fermentation by using NADH to reduce metabolic intermediates. Therefore, an increase in the availability of NADH is expected to have an effect on the metabolic distribution. This paper investigates a genetic means of manipulating the availability of intracellular NADH in vivo by regenerating NADH through the heterologous expression of an NAD$^+$-dependent formate dehydrogenase. More specifically, it explores the effect on the metabolic patterns in Escherichia coli under anaerobic and aerobic conditions of substituting the native cofactor-independent formate dehydrogenase (FDH) by an NAD$^+$-dependent FDH from Candida boidinii. The overexpression of the NAD$^+$-dependent FDH doubled the maximum yield of NADH from 2 to 4 mol NADH/mol glucose consumed, increased the final cell density, and provoked a significant change in the final metabolite concentration pattern both anaerobically and aerobically. Under anaerobic conditions, the production of more reduced metabolites was favored, as evidenced by a dramatic increase in the ethanol-to-acetate ratio. Even more interesting is the observation that during aerobic growth, the increased availability of NADH induced a shift to fermentation even in the presence of oxygen by stimulating pathways that are normally inactive under these conditions.

Key Words: cofactor; redox system; NADH regeneration; FDH; ethanol.

INTRODUCTION

The metabolic pathways leading to the production of most industrially important compounds involve reduction–oxidation (red–ox) reactions. Biosynthetic transformations involving red–ox reactions also offer a considerable potential for the production of fine chemicals over conventional chemical processes, especially those requiring stereospecificity.

Nicotinamide adenine dinucleotide (NAD) plays a central role in cellular metabolism by functioning as a cofactor in over 300 red–ox reactions (Foster et al., 1990). In addition, studies have shown that the NADH/NAD$^+$ cofactor pair has a regulatory effect on the expression of some genes and the activity of certain enzymes. Examples include, among others, the induction by NADH of the expression of the adhE gene that encodes the enzyme alcohol dehydrogenase (Leonardo et al., 1993, 1996), which catalyzes the production of ethanol during fermentation; the inhibition of high NADH/NAD$^+$ ratios on the pyruvate dehydrogenase complex (De Graef et al., 1999); and the regulation by the NADH/NAD$^+$ ratio on the shift between oxidation or reduction of L-lactaldehyde (Baldoma and Aguilar, 1988).

During catabolism, a cell oxidizes a carbon source, such as glucose, utilizing NAD$^+$ as a cofactor and producing...
reducing equivalents in the form of NADH. The cell regenerates NAD$^+$ from NADH to achieve a redox balance. Under aerobic growth, oxygen is used as the final electron acceptor. While under anaerobic growth and in the absence of an alternate oxidizing agent, the regeneration of NADH is achieved through fermentation, where NADH is used to reduce metabolic intermediates and to regenerate NAD$^+$ (Fig. 1). Therefore, in fermentation, alterations in the availability of NADH are expected to have a profound effect in the whole metabolic network.

The influence of cofactors in metabolic networks has been evidenced by studies in which the NADH/NAD$^+$ ratio has been altered by feeding carbon sources with different oxidation states (Alam and Clark, 1989; Leonar do et al., 1996); by supplementing anaerobic growth with different electron acceptors, such as fumarate and nitrate (De Graef et al., 1999); or by expressing an enzyme like NADH oxidase (Lopez de Felipe et al., 1998). Other previous efforts to manipulate NADH levels have included the addition of electron dye carriers (Park and Zeikus, 1999) and the variation of oxidoreduction potential conditions (Riondet et al., 2000).

The effective regeneration of used cofactors is critical in industrial cofactor-dependent production systems due to the high cost of cofactors such as NAD. In enzyme bioreactors, NAD$^+$-dependent formate dehydrogenase (FDH; EC 1.2.1.2) from methylotrophic yeast and bacteria is extensively used to regenerate NADH from NAD$^+$ in vitro. FDH catalyzes the practically irreversible oxidation of formate to CO$_2$ and the simultaneous reduction of NAD$^+$ to NADH. Cofactor regeneration has been successfully applied in vitro for the production of optically active amino acids (Kragl et al., 1996; Galkin et al., 1997), chiral hydroxy acids, esters, alcohols, and other fine chemicals synthesized by different dehydrogenases (Hum mel and Kula, 1989; Tishkov et al., 1999).

![Metabolic Pathway Diagram](https://example.com/fig1.png)

**FIG. 1.** Central anaerobic metabolic pathway of *Escherichia coli* showing generation of NADH and regeneration of NAD$^+$, including the new NAD$^+$-dependent formate dehydrogenase (FDH) from *Candida boidinii*. 

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In spite of these advances, biotransformation with whole cells is still the preferred method for the synthesis of most cofactor-dependent products industrially. In these systems, the cell naturally regenerates the cofactor. However, the enzyme of interest has to compete for the required cofactor with a large number of other enzymes within the cell. Therefore, in cofactor-dependent production systems, after the enzymes of interest have been overexpressed, the availability of the required form of the cofactor (reduced or oxidized) can become limiting, making cofactor manipulations crucial for optimal production.

Although it is generally known that cofactors play a major role in the production of different fermentation products, their role has not been thoroughly and systematically studied. Instead, metabolic engineering studies have focused on manipulating enzyme levels through the amplification, addition or deletion of a particular pathway. However, cofactor manipulations can potentially become a powerful tool for metabolic engineering.

This paper investigates a genetic means of manipulating the availability of intracellular NADH in vivo by regenerating NADH through the heterologous expression of an NAD$^+$-dependent formate dehydrogenase. We have developed a novel approach to increase the availability of intracellular NADH in vivo through metabolic and genetic engineering. In this approach, we overexpress a biologically active NAD$^+$-dependent formate dehydrogenase (FDH) from Candida boidinii in Escherichia coli. In the presence of this newly introduced formate dehydrogenase pathway, one mole of NADH will be formed when one mole of formate is converted to carbon dioxide (Fig. 2). In contrast, the native formate dehydrogenase converts formate to CO$_2$ and H$_2$ with no cofactor involvement. The new system allows the cells to retain the reducing power that otherwise will be lost by release of formate or hydrogen in the native pathway. The functionality of the new system for NADH regeneration was successfully demonstrated in anaerobic tubes and aerobic shake flask experiments.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**

Table 1 describes the strains and plasmids used in this study. The strain BS1 was constructed from the strain GJT001 by inactivating the native formate dehydrogenase. Plasmid pSBF2 contains the $fdh1$ gene from the yeast Candida boidinii under the control of the lac promoter. The $fdh1$ gene encodes an NAD$^+$-dependent formate dehydrogenase (FDH) that converts formate to CO$_2$ with the regeneration of NADH from NAD$. In contrast, the native formate dehydrogenase converts formate to CO$_2$ and H$_2$ with no cofactor involvement (refer to Fig. 2). The construction of the strains and plasmids is summarized below.
Strain BS1. Strain BS1 was constructed by replacing the wild-type fdhF gene with an fdhF-lacZ fusion by a P1 vir-mediated phage transduction with E. coli M9s as donor and E. coli GJT001 as recipient. The P1 phage transduction was performed following standard protocols (Maniatis et al., 1989). Ampicillin resistant transductants were selected for further analysis. The lack of formate dehydrogenase activity was confirmed by a previously described method with minor modifications (Mandrand-Berthelot et al., 1978). Briefly, wild-type and transduced GJT001 were grown on glucose minimal media plates for 2 days in an anaerobic chamber under an atmosphere of H2 and CO2. An overlay solution composed of 0.6% agar, 2 mg/ml benzyl viologen, 0.25 M sodium formate and 25 mM KH2PO4 (pH 7.0) was poured over the plates. The presence of formate dehydrogenase activity in the wild-type GJT001 was evidenced by a change in the color of the colonies, which turned purple. The colonies of the transductants remained white, thus indicating the lack of formate dehydrogenase activity. The presence of the mutation of fdhF in the transductants was also confirmed by PCR. Primers complementary to the ends of the fdhF gene (forward primer: 5'-GATTAACTGGAGCGAGACC-3'; reverse primer: 5'-TCCGAAAAGGGAGCCTG TAG-3') (Zinoni et al., 1986) were used to amplify this gene in both wild-type and transduced GJT001. The disruption of the fdhF gene in the transduced strain was confirmed by the absence of a PCR product as opposed to a 2.2-kb product corresponding to the complete gene in the wild-type strain.

Plasmid pSBF2. Plasmid pFDH1 was kindly provided by Dr. Y. Sakai (Sakai et al., 1997). It contains a 3 kb EcoRI insert containing the fdh1 gene from the yeast Candida boidinii in pBluescriptII SK+. The fdh1 gene in pFDH1 is under the control of its native promoter. Preliminary experiments with pFDH1 showed no FDH activity, suggesting that fdh1 from the yeast was not properly expressed in E. coli. For this reason, the open reading frame of the fdh gene from C. boidinii was amplified by PCR and placed under the control of the lac promoter for overexpression in E. coli.

XL-PCR was performed using the GeneAmp XL PCR kit from PE Applied Biosystems following the manufacturer’s protocol. This kit was chosen because of the proofreading ability of the enzyme Tth DNA polymerase, which not only promotes efficient DNA synthesis but also corrects nucleotide misincorporations. Plasmid pFDH1 was used as a template and the following were used as forward and reverse primers, respectively: 5'-GCGAATTCAGGAGGAATTTAAAATG-3'; 5'-CGC GGATCC TTAATTCTTTATCGTGTTTACCGTAAGC-3'. An EcoRI and a BamHI site were inserted in the forward and reverse primers, respectively, as represented by the underlined regions.

The program used for the PCR reaction consisted of an initial denaturation step at 94°C for 1 min and 30 s followed by 18 cycles of denaturation at 94°C for 30 s and combined annealing/extension at 55–66°C for 5 min. This was followed by 12 cycles in which the annealing/extension time was increased by 15 s in each cycle until it reached 8 min. A final step at 72°C for 10 min concluded the PCR.

The PCR product was verified by agarose gel electrophoresis. It was purified from the reaction mixture and

### TABLE 1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Significant genotype</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>GJT001</td>
<td>Spontaneous cadR mutant of MC4100, SmR</td>
<td>Tolentino et al. (1992)</td>
</tr>
<tr>
<td>DH10B</td>
<td>Cloning host</td>
<td></td>
</tr>
<tr>
<td>BS1</td>
<td>GJT001 fdhF-lacZ, ApR</td>
<td>This study</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Significant genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18</td>
<td>Cloning vector, ApR</td>
<td></td>
</tr>
<tr>
<td>pDHK29, pDHK30</td>
<td>Control, cloning vector, KmR</td>
<td>Phillips et al. (2000)</td>
</tr>
<tr>
<td>pFDH1</td>
<td>fdh1 in pBluescriptII SK+</td>
<td>Sakai et al. (1997)</td>
</tr>
<tr>
<td>pUCFDH</td>
<td>Intermediate plasmid, fdh1 in pUC18, ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pSBF2</td>
<td>fdh1 in pDHK30, KmR</td>
<td>This study</td>
</tr>
</tbody>
</table>
concentrated following the protocol of the StrataPrep™ PCR Purification Kit (Stratagene, La Jolla, CA). The purified fdh PCR product and the vector pUC18 were digested with EcoRI and BamHI. Both fragments were ligated to form pUCFDH. The ligation product was transformed into E. coli strain DH10B. White colonies from Ap/Xgal/IPTG plates were selected for further analysis and minipreps were performed. Insertion of the fdh gene was confirmed by agarose gel electrophoresis after digestion with EcoRI/SalI.

Plasmid pUCFDH served as an intermediate vector to facilitate the insertion of the fdh gene into pDHK30 in the right orientation. It was ultimately desired to have the fdh gene in the pDHK30 backbone because it is a high copy number plasmid with kanamycin resistance, which will not interfere with the ampicillin resistance of the BS1 strain. An additional advantage of the pDHK30 vector is that it can be co-transformed in a two-plasmid system together with the most common high copy number vectors bearing a ColE1 origin.

The intermediate plasmid containing fdh (pUCFDH), and pDHK30 were digested with EcoRI/XbaI and ligated to obtain plasmid pSBF2. The ligation product was transformed into DH10B and white colonies from Km/Xgal plates were analyzed. Minipreps were obtained and analyzed by agarose gel electrophoresis after digestion with EcoRI/XbaI. An appropriate plasmid was selected and transformed both into GJT001 and the fdh− strain BS1. Strain GJT001 was also transformed with pDHK29, and BS1 was transformed with pDHK30 to serve as negative controls.

FDH activity assay

The FDH activity of strains GJT001 (pSBF2) and BS1 (pSBF2) was determined as described below.

Cultures were grown overnight in LB media supplemented with 20 g/L glucose and 100 mg/L kanamycin under anaerobic conditions. The cultures were inoculated with 100 μl of a 5 ml overnight LB culture and grown in a shaker at 37°C and 250 rpm. Cells were harvested by centrifugation of 20 ml of culture at 4000 g for 10 min. The pellet was suspended in 10 ml of 10-mM sodium phosphate buffer (refrigerated) at pH 7.5 with 0.1 M β-mercaptoethanol and centrifuged as described above. The cells were resuspended in 10 ml of 10-mM sodium phosphate buffer (refrigerated) at pH 7.5 with 0.1 M β-mercaptoethanol and sonicated for 6 min in an ice bath (Sonicator: Heat System Ultrasonics, Inc. Model W-255; Settings: 60% cycle, max. power = 8). The sonicated cells were centrifuged at 1500g and 4°C for 60 min to remove cell debris and reduce the NAD background. The formate dehydrogenase activity was assayed at 30°C by adding 100 μl of cell extract to 1 ml of a reaction mixture containing 1.67 mM NAD+, 167 mM sodium formate and 100 mM β-mercaptoethanol in phosphate buffer pH 7.5 and measuring the increase in absorbance of NADH at 340 nm (Schutte et al., 1976, modified). One unit was defined as the amount of enzyme that produced 1 μmol of NADH per minute at 30°C. Total protein concentration in cell extracts was measured by Lowry’s method (Sigma Kit) using bovine serum albumin as standard.

Growth experiment

Strains GJT001 (pDHK29) and BS1 (pSBF2) were grown in triplicate cultures aerobically in a rotary shaker at 37°C and 250 rpm. The cultures were grown in 250-ml shake flasks containing 50 ml of LB media supplemented with 10 g/L glucose, 100 mg/L kanamycin, and 0 or 100 mM formate. The OD at 600 nm was measured every 30 min during the exponential growth phase.

Anaerobic tube experiments

The anaerobic tube experiments were performed using 40- or 45-ml glass vials with open top caps and PTFE/silicone rubber septa. Each vial was filled with 35 ml (40-ml vials) or 40 ml (45-ml vials) of LB media supplemented with 20 g/L glucose, 100 mg/L kanamycin, and 0 or 50 mM formate, and 1 g/L NaHCO₃ to reduce the initial lag time that occurs under anaerobic conditions. The triplicate cultures were inoculated with 100 μl of a 5 ml LB overnight culture. After inoculation, air (6 ml) was removed with a syringe from the headspace to ensure anaerobic conditions. The cultures were grown in a rotary shaker at 37°C and 250 rpm. A sample of the initial media was saved for analysis and samples were withdrawn with a syringe at 24 h intervals (24, 48, and 72 h).

Aerobic shake flask experiments

Triplicate cultures were grown aerobically using either 125-ml shake flasks containing 25 ml of LB media or 250-ml shake flasks containing 50 ml of LB media. The LB media was supplemented with 10 g/L glucose, 100 mg/L kanamycin, and different amounts of formate. The cultures were inoculated with 50 or 100 μl of a 5 ml LB overnight culture and grown in a rotary shaker at 37°C and 250 rpm. A sample of the initial media was saved for HPLC analysis and samples were collected after 24 h of growth.

Analytical Techniques

Cell density (OD) was measured at 600 nm in a spectrophotometer. Fermentation samples were centrifuged for 5 min in a microcentrifuge. The supernatant was filtered through a 0.45 μm syringe filter and stored chilled for HPLC analysis. The fermentation products,
as well as glucose were quantified using an HPLC system (Thermo Separation Products) equipped with a cation-exchange column (HPX-87H, BioRad Labs) and a differential refractive index detector. A mobile phase of 2.5 mM H₂SO₄ solution at a 0.6 ml/min flow rate was used and the column was operated at 55°C.

RESULTS AND DISCUSSION

Characterization

The current study investigates the effect of increasing NADH availability intracellularly by genetic engineering on the metabolic patterns of E. coli under anaerobic and aerobic conditions. More specifically, this paper assesses the effect of regenerating NADH by substituting the native cofactor-independent formate dehydrogenase in E. coli by the NAD⁺-dependent FDH from Candida boidinii, as well as the effect of supplementing the culture media with formate.

Plasmid pSBF2, containing the fdh1 gene from Candida boidinii under the control of the lac promoter, was constructed and characterized by determining the activity of the new FDH. Table 2 shows the specific NAD⁺-dependent FDH activity of strains BS1 (pSBF2) and GJT001 (pSBF2) in U/mg of total protein. One unit is defined as the amount of enzyme that produced 1 μmol of NADH per minute at 30°C. The NAD⁺-dependent FDH activity of strain GJT001 (pSBF2) was 46% higher (0.416 U/mg) than the activity of BS1 (pSBF2) (0.284 U/mg). Strains GJT001 (pDHK29) and BS1 (pDHK30) showed no detectable NAD⁺-dependent FDH activity as expected.

To further characterize the effect of substituting the native FDH with the NAD⁺-dependent pathway, the specific growth rate (μ) of strains BS1 (pSBF2) and GJT001 (pDHK29) was determined in aerobic shake flask experiments. Table 3 presents the results of these experiments with and without 100 mM formate. The specific growth rate of strain BS1 (pSBF2) was 35% lower (0.986 ± 0.002) than that of GJT001 (pDHK29) (1.511 ± 0.016) without formate supplementation. However, by the end of the fermentation the cell density of BS1 (pSBF2) was comparable to or even higher than that of GJT001 (pDHK29).

In addition, the effect on the specific growth rate of formate supplementation at the level of 100 mM was examined. Formate addition to the media lengthened the duration of the lag phase for both strains, but more for BS1 (pSBF2). The difference in the specific growth rate between BS1 (pSBF2) and GJT001 (pDHK29) decreases with the addition of formate. Under these conditions, the specific growth rate of GJT001 (pDHK29) is only 10% higher. Addition of formate did not affect significantly the specific growth rate of BS1 (pSBF2); however, it decreased that of GJT001 (pDHK29) by 28%. As in the case without formate supplementation, the final cell density of BS1 (pSBF2) was comparable to that of GJT001 (pDHK29).

Anaerobic Tube Experiments: Effect of NAD⁺-Dependent FDH Overexpression

Anaerobic tube experiments were performed with strains GJT001 (pDHK29), GJT001 (pSBF2), BS1 (pSBF2), and BS1 (pDHK30) to investigate the effect on the metabolic patterns of the elimination of the native FDH and the addition or substitution of the new FDH. Figure 3 shows the results of these experiments, including the final cell density, glucose consumed (mM), and the concentration of different metabolites produced (mM) after 72 h of culture.

A comparison of the results for the control strains GJT001 (pDHK29) and BS1 (pDHK30) indicates the effect of eliminating the native FDH on the metabolic patterns of E. coli. As expected, an increase in residual formate was observed for the strain lacking FDH activity (data not shown). As can be seen from Fig. 3, glucose consumption for BS1 (pDHK30) decreased by 47% relative to GJT001 (pDHK29). A decrease in final cell density (29%), as well

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity (U/mg)</th>
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<tr>
<td>BS1 (pSBF2)</td>
<td>0.284 ± 0.002</td>
</tr>
<tr>
<td>GJT001 (pSBF2)</td>
<td>0.416 ± 0.004</td>
</tr>
<tr>
<td>GJT001 (pDHK29)</td>
<td>ND</td>
</tr>
<tr>
<td>BS1 (pDHK30)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note. One unit is defined as the amount of enzyme that produced 1 μmol of NADH per minute at 30°C. Values shown are average of triplicates from anaerobic tube cultures. ND: not detected (less than 0.001 U/mg).

### Table 3

<table>
<thead>
<tr>
<th>Strains</th>
<th>0 mM formate</th>
<th>100 mM formate</th>
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</thead>
<tbody>
<tr>
<td>BS1 (pSBF2)</td>
<td>0.986 ± 0.002</td>
<td>0.972 ± 0.014</td>
</tr>
<tr>
<td>GJT001 (pDHK29)</td>
<td>1.511 ± 0.016</td>
<td>1.086 ± 0.043</td>
</tr>
</tbody>
</table>

Note. Values shown are average of triplicates.
as in succinate (39%), lactate (66%), and ethanol (22%) production was also observed with the elimination of the native FDH activity. However, the level of acetate from BS1 (pDHK30) was very similar to that of GJT001 (pDHK29). As a result, the ethanol to acetate (Et/Ac) ratio decreased by 24%. The decrease in the Et/Ac ratio together with the decrease in other reduced metabolites (lactate and succinate) is indicative of a more oxidized environment for strain BS1 (pDHK30), which lacks formate dehydrogenase activity. These results suggest that under normal conditions GJT001 (pDHK29) can recapture some of the H₂ produced from the degradation of formate by the native FDH possibly by means of some hydrogenase, and this accounts for the slightly more reduced intracellular environment observed for the wild-type strain relative to BS1 (pDHK30).
An analysis of the results for BS1 (pSBF2) relative to BS1 (pDHK30) and for GJT001 (pSBF2) relative to GJT001 (pDHK29) provides an understanding of the effect of overexpressing the NAD\(^+\)-dependent FDH both alone or in conjunction with the native FDH, respectively. In both cases the trend is similar, but the effect is more pronounced for the BS1 strains due to the decrease in the metabolites observed for BS1 (pDHK30) relative to GJT001 (pDHK29). Both strains containing the new FDH present a significant increase in glucose consumption, cell density, ethanol, and succinate formation, accompanied by a decrease in lactate and acetate relative to the control strains. As a result, the ethanol to acetate (Et/Ac) ratio increased dramatically by 22-fold for GJT001 (pSBF2) and 35- to 36-fold for BS1 (pSBF2).

The results for GJT001 (pSBF2) and BS1 (pSBF2) show the effect of having both the native and new FDH active in the same strain or just the new FDH, respectively. A comparison of these results shows that these strains behave very similarly. The largest difference between these two strains is a 16% decrease in acetate, and consequently a 21% increase in Et/Ac ratio for BS1 (pSBF2) relative to GJT001 (pSBF2). The similarity in the metabolic patterns of strains GJT001 (pSBF2) and BS1 (pSBF2) implies that the NAD\(^+\)-dependent FDH is competing effectively with the native FDH for the available formate. This observation is possibly due to the gene dosage effect and/or the lower Km value for formate of the NAD\(^+\)-dependent FDH (13 mM) relative to that of the native FDH (26 mM) (Schutte et al., 1976; Axley and Grahame, 1991). Although these results suggest that the fdh mutation is not necessary to observe the effect of overexpressing the NAD\(^+\)-dependent FDH, the decrease in acetate levels observed for BS1 (pSBF2) suggests that eliminating the native FDH activity may be slightly beneficial in some cases.

Analyzing the results of BS1 (pSBF2) relative to GJT001 (pDHK29) can better elucidate the effect of substituting the cofactor-independent native formate-degradation pathway in *E. coli* by the NAD\(^+\)-dependent pathway. Substitution of the native FDH by the new FDH increased glucose consumption (three-fold), final cell density (59%), as well as the production of ethanol (15-fold) and succinate (55%), while it decreased lactate (91%) and acetate (43%) production. The higher NADH availability dramatically increased the ethanol to acetate (Et/Ac) ratio by 27-fold.

These results suggest that overexpression of the NAD\(^+\)-dependent FDH increases intracellular NADH availability, and this in turn leads to a drastic shift in the metabolic patterns of *E. coli*. Intracellular NADH availability almost doubled for the strains containing the NAD\(^+\)-dependent pathway from approximately 2 to 4 mol of NADH available for reduced product formation per mole of glucose consumed (Table 4). The increase in NADH availability favored the production of more reduced metabolites, particularly, those requiring 2 NADH molecules per molecule of product formed, like ethanol and succinate. The preferred product was ethanol, with a final concentration reaching as high as 175 mM for BS1 (pSBF2), as compared to 11.5 mM for the wild-type control, GJT001 (pDHK29). Consequently, ethanol becomes the major fermentation product for BS1 (pSBF2) anaerobic cultures, accounting for 91% of the metabolites produced based on mM concentrations, as opposed to 18% for GJT001 (pDHK29). Simultaneously, lactate was converted from a major product, representing 57% of the produced metabolites in the wild-type strain to only a minor product, accounting for less than 2% of the metabolites. The shift towards the production of ethanol as a major product is comparable to that obtained with overexpression of the ethanologenic enzymes from *Zymomonas mobilis* in the pet operon in *E. coli* (Ingram and Conway, 1988). What makes the current results so remarkable is the fact that no enzymes for ethanol production have been overexpressed in the present study. These results demonstrate how powerful cofactor manipulations can be as a tool for metabolic engineering.

The dramatic increase in ethanol production combined with a decrease in acetate levels led to the drastic increase in the Et/Ac ratio observed, which reached as high as 27 for BS1 (pSBF2), as compared to 1.0 for GJT001 (pDHK29). It is evident from these results that the cell adjusts its partitioning at the acetyl-CoA node by changing the ethanol (consumes 2 NADH) to acetate (consumes no

### TABLE 4

<table>
<thead>
<tr>
<th>Strain</th>
<th>(NADH)(_U)/Gl (mol/mol)</th>
</tr>
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<tbody>
<tr>
<td>GJT001 (pDHK29)</td>
<td>2.40</td>
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<tr>
<td>GJT001 (pSBF2)</td>
<td>4.34</td>
</tr>
<tr>
<td>BS1 (pSBF2)</td>
<td>4.35</td>
</tr>
<tr>
<td>BS1 (pDHK30)</td>
<td>2.38</td>
</tr>
<tr>
<td>GJT001 (pDHK29) + F</td>
<td>2.33</td>
</tr>
<tr>
<td>BS1 (pSBF2) + F</td>
<td>4.39</td>
</tr>
</tbody>
</table>

Note. (NADH)\(_U\)/Gl=mol of NADH available for reduced product formation per mole of glucose consumed, where (NADH)\(_U\)=total NADH used for product formation per unit volume at the end of fermentation (mmol/L). It was estimated from the concentrations of reduced metabolites by calculating the NADH used for their production according to the pathways shown in Fig. 1. +F=50 mM initial formate supplementation. Values shown are from average of triplicate cultures.
NADH) ratio to achieve a redox balance, as was previously observed in experiments utilizing carbon sources with different oxidation states (San et al., 2002). These findings also support the idea that NADH induces expression of alcohol dehydrogenase (adhE) (Leonardo et al., 1996).

The significant decrease in lactate levels obtained with overexpression of the NAD⁺-dependent FDH can be explained by noting that although lactate formation also requires NADH, it only consumes 1 NADH, while ethanol formation consumes 2 NADH. These results suggest that when there is an excess of reducing equivalents, ethanol formation (2 NADH) is preferred over lactate formation (1 NADH) since it provides a faster route to NAD⁺ regeneration. These observations support previous findings in experiments utilizing carbon sources with different oxidation states (San et al., 2002).

Anaerobic Tube Experiments: Effect of Formate Addition

Figure 3 also shows the results of anaerobic tube experiments performed with strains GJT001 (pDHK29) and BS1 (pSBF2) in which the media was supplemented with 50 mM formate. Addition of formate to both strains increased lactate levels and decreased the total acetate and ethanol production. The change in the metabolic pattern might be due to a bottleneck at the pyruvate node caused by the extra formate present. The increased pyruvate pool activates the LDH pathway and redirects some glucose consumed to lactate and away from the PFL pathway. The results for BS1 (pSBF2) and GJT001 (pDHK29) with 50 mM formate supplementation indicate a six-fold increase in ethanol accompanied by a 69% decrease in acetate levels, which leads to a 21-fold increase in the Et/Ac ratio with the substitution of the native FDH for the NAD⁺-dependent FDH.

In spite of the slight differences relative to the cultures with no formate addition, the moles of NADH utilized for reduced product formation per mole of glucose consumed (Table 4) remained unchanged with the addition of formate. Strain BS1 (pSBF2) was still operating at the new maximum theoretical yield of approximately 4 mol NADH/mol glucose as compared to 2 mol NADH/mol glucose for GJT001 (pDHK29). Therefore, it is not necessary to supplement the culture with formate anaerobically.

In addition, Fig. 3 presents the amounts of formate converted to CO₂ for the different strains with and without formate addition under anaerobic conditions. These values were calculated by subtracting the measured residual formate concentration from the concentration of formate produced plus the initial formate concentration in the media for the experiments with formate supplementation. The amount of formate produced was obtained based on the assumption that 1 mol of formate is produced per mol of acetyl-CoA formed through the PFL pathway (Fig. 2). Therefore, the amount of formate produced was calculated by adding the concentrations of ethanol and acetate formed from acetyl-CoA.

As can be seen from Fig. 3, overexpression of the NAD⁺-dependent FDH drastically increases the conversion of formate almost equally for both strains BS1 (pSBF2) and GJT001 (pSBF2) indicating that this new enzyme competes very effectively with the native FDH for the available formate. These two strains as well as GJT001 (pDHK29) converted all the formate produced during fermentation when there was no external formate added to the media, while strain BS1 (pDHK30) converted only minimal amounts of formate as expected.

It is also interesting to note that external addition of formate to the media had opposite effects on the native and new FDH. Formate supplementation of GJT001 (pDHK29) cultures significantly increased (2- to 3-fold) the amount of formate converted by the native enzyme, although only 78% of the available formate was converted. These results suggest that addition of extra formate has a stimulatory effect on the native FDH pathway or that initially the pathway was limited by the amount of formate, while after formate supplementation it became limited by the enzyme activity instead.

In contrast, addition of formate to BS1 (pSBF2) anaerobic cultures decreased the amount of formate converted, with only 69% of the available formate being degraded, suggesting possible inhibition of the new FDH at these levels of formate. The decrease in formate conversion can also be the indirect consequence of a lower glucose consumption and optical density. Although the total levels of formate produced by BS1 (pSBF2) without external formate addition were higher than with the 50 mM supplementation, the cells do not experience high levels of formate at a given time because it is being degraded as it is produced. In contrast, in the supplementation experiment the cell experiences a higher initial formate concentration. It is also possible that the cells are experiencing an excess of NADH produced from the externally added formate; and they cannot get rid of it through the formation of more reduced metabolites because they are already operating at the maximum theoretical yield for the available carbon.

Aerobic Shake Flask Experiments

Shake flask experiments were performed with strains GJT001 (pDHK29) and BS1 (pSBF2) to investigate the effect of increasing intracellular NADH availability by
substituting the native FDH in *E. coli* by the NAD$^+$-dependent enzyme on the metabolic patterns under aerobic conditions. These experiments were performed with and without 100 mM formate supplementation. Addition of formate as a substrate for the new FDH during aerobic growth was necessary because under these conditions the cells normally do not produce formate due to lack of activity of the pyruvate formate lyase (PFL) enzyme. Figure 4 presents the results of these experiments, including the final cell density, glucose consumed (mM), and the concentration of different metabolites produced (mM) after 24 h of culture. For both strains only minimal amounts of residual formate (less than 6 mM) were detected.

As can be seen from Fig. 4, addition of formate to BS1 (pSBF2) aerobic cultures induced the production of ethanol, lactate, and succinate, metabolites that are normally produced only under anaerobic conditions. The data indicate a 36-fold increase in ethanol, seven-fold increase in succinate, and the appearance of lactate. Glucose consumption increased by 50% and acetate levels by 11%. The Et/Ac ratio increased by 32-fold.

The effect of formate supplementation on the native FDH was also investigated (Fig. 4). Addition of formate to

![Graphs of Optical Density, Glucose Consumed, Ethanol Concentration, Acetate Concentration, Succinate Concentration, Lactate Concentration](image)

**FIG. 4.** Results of aerobic shake flask experiments. The cultures were analyzed 24 h after inoculation. Values shown are averages of triplicate cultures.
GJT001 (pDHK29) aerobic cultures caused an increase of 50% in glucose consumption, the same percentage of increase observed for BS1 (pSBF2). However, the increase in acetate levels was much higher (47%) with formate supplementation, as well as the increase in final cell density (48%). On the other hand, the production of ethanol was much lower, only 5.15 mM after 24 h, and succinate levels increased only by 72% compared to a seven-fold increase for BS1 (pSBF2).

The results obtained for both strains with formate supplementation shows a 27-fold increase in lactate, four-fold increase in ethanol, three-fold increase in succinate, accompanied by a 30% decrease in acetate (five-fold increase in Et/Ac) for the NAD\(^+\)-dependent FDH relative to the native FDH. The glucose consumption was similar for both strains, while the final cell density was slightly higher for BS1 (pSBF2) (refer to Fig. 4).

These results demonstrate that it is possible to increase the availability of intracellular NADH through the substitution of the native FDH in *E. coli* by an NAD\(^+\)-dependent FDH. The higher intracellular NADH levels provide a more reduced environment even under aerobic conditions. As a result, the cells utilize the extra NADH to reduce metabolic intermediates leading to the formation of fermentation products in order to achieve a redox balance. Conversely, under normal aerobic conditions, the environment is so oxidized that reduced fermentation products are not formed. Under aerobic conditions, only acetate, a more oxidized metabolite that does not require NADH, is normally produced. The results presented in Fig. 4 also suggest that although the cells can recapture some of the reducing power from the formate added by means of the native FDH, the new FDH pathway is a lot more effective because it recaptures the extra reducing power directly as NADH.

In addition, the effect of supplementing the media with different levels of formate (0, 50, 100, 150, and 200 mM) was investigated in aerobic cultures of BS1 (pSBF2). It is interesting to note that lactate was absent at 0 and 50 mM initial formate, but it was produced at 100, 150, and 200 mM initial formate (Fig. 5). The concentration of lactate increased with an increase in the initial formate levels. The same trend was evident in succinate production with the difference that similar levels were produced at 0 and 50 mM initial formate (Fig. 5). On the other hand, the cells produced ethanol only after formate supplementation, but the levels did not significantly increase with an increase in formate levels (data not shown). Acetate production and final cell density did not follow any notable trend with increasing levels of formate supplementation (data not shown). Glucose consumption increased with the addition of formate, but since all the glucose was consumed by 24 h in all the formate supplemented cultures, the effect of different formate levels cannot be ascertained (data not shown).

It was also observed that the concentration of residual formate reached 63.5 mM for the 200 mM initial formate experiment, a 10-fold increase from the residual levels in the 150 mM experiment. The levels of residual formate were lower than 12 mM for all other initial formate levels. These observations possibly indicate that the culture is past saturation at 200 mM initial formate level. In addition, based on the formate conversion levels observed, more NADH is being generated by the NAD\(^+\)-dependent pathway than that used to produce reduced metabolites. The cells are possibly using the extra NADH formed for ATP generation through the electron transport system since they are growing aerobically.

The results of the formate supplementation experiment show that different formate levels can be used to provide different levels of reducing power. Higher levels of reducing power aerobically mainly increased lactate production. In contrast, in anaerobic cultures with no formate supplementation, where the environment was a lot more reduced, ethanol production was highly increased, while lactate
levels decreased. However, formate supplementation in anaerobic cultures provoked an increase in lactate levels, which is consistent with the aerobic case.

CONCLUSIONS

This study demonstrates that it is possible to increase the availability of intracellular NADH through metabolic engineering and therefore provide a more reduced environment both under anaerobic and aerobic conditions.

The substitution of the native cofactor-independent FDH pathway by the NAD\(^+\)-dependent FDH provoked a significant metabolic redistribution both anaerobically and aerobically. Under anaerobic conditions, the NADH availability increased from 2 to 4 mol NADH/mol glucose consumed, and this increase favored the production of more reduced metabolites, as evidenced by a dramatic increase in the ethanol to acetate ratio for BS1 (pSBF2) as compared to the GJT001 (pDHK29) control (Fig. 3). The higher NADH availability led to a shift towards the production of ethanol as the major fermentation product.

Even more interesting is the finding that during aerobic growth, the increased availability of NADH induced a shift to fermentation even in the presence of oxygen by stimulating pathways that are normally inactive under these conditions. To increase NADH availability aerobically, formate was added to the media since it is not a normal product under aerobic conditions. As can be seen from Fig. 4, addition of formate to BS1 (pSBF2) aerobic cultures induced the production of ethanol, lactate, and succinate, metabolites that are normally produced only under anaerobic conditions.

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