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Effect of different levels of NADH availability on metabolite distribution in *Escherichia coli* fermentation in minimal and complex media

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Abstract A range of intracellular NADH availability was achieved by combining external and genetic strategies. The effect of these manipulations on the distribution of metabolites in *Escherichia coli* was assessed in minimal and complex medium under anoxic conditions. Our in vivo system to increase intracellular NADH availability expressed a heterologous NAD^+-dependent formate dehydrogenase (FDH) from *Candida boidinii* in *E. coli*. The heterologous FDH pathway converted 1 mol formate into 1 mol NADH and carbon dioxide, in contrast to the native FDH where cofactor involvement was not present. Previously, we found that this NADH regeneration system doubled the maximum yield of NADH from 2 mol to 4 mol NADH/mol glucose consumed. In the current study, we found that yields of greater than 4 mol NADH were achieved when carbon sources more reduced than glucose were combined with our in vivo NADH regeneration system. This paper demonstrates experimentally that different levels of NADH availability can be achieved by combining the strategies of feeding the cells with carbon sources which have different oxidation states and regenerating NADH through the heterologous FDH pathway. The general trend of the data is substantially similar for minimal and complex media. The NADH availability obtained positively correlates with the proportion of reduced by-products in the final culture. The maximum theoretical yield for ethanol is obtained from glucose and sorbitol in strains overexpressing the heterologous FDH pathway.

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Introduction

The NADH/NAD^+ cofactor pair plays a major role in microbial catabolism: a carbon source (CS), such as glucose, is oxidized using NAD^+ to produce reducing equivalents in the form of NADH. The cell regenerates NAD^+ from NADH to achieve a redox balance. Under oxic growth, NADH recycling occurs through the oxic respiratory chain, where oxygen acts as the oxidizing agent. Under anoxic growth and in the absence of an alternate oxidizing agent, the regeneration of NAD^+ is achieved through fermentation, using NADH to reduce metabolic intermediates (Fig. 1). Therefore, in fermentation, alterations in the availability of NADH should have a profound effect on the whole metabolic network.

Our previous studies established cofactor manipulations for the NADH/NAD^+ cofactor pair as an additional tool for metabolic engineering. We investigated different external and genetic means of increasing the availability of NADH and examined the effect of these manipulations on the distribution of metabolites in *E. coli*. The strategies included feeding CSs with different oxidation states (Alam and Clark 1989; Berríos-Rivera 2002a; San et al. 2002), overexpressing an enzyme that can regenerate NADH (Berríos-Rivera et al. 2002b, 2002c), overexpressing an enzyme in the NAD salvage pathway (NAPRTase, *pncB*; Berríos-Rivera et al. 2002d) and eliminating NADH-competing pathways (Berríos-Rivera et al. 2003).

In previous studies (Berríos-Rivera et al. 2002b, 2002c), we increased the availability of intracellular NADH in vivo by regenerating NADH through the heterologous expression of a NAD^+-dependent FDH from *C. boidinii* in *E. coli*. This NAD^+-dependent FDH pathway converted

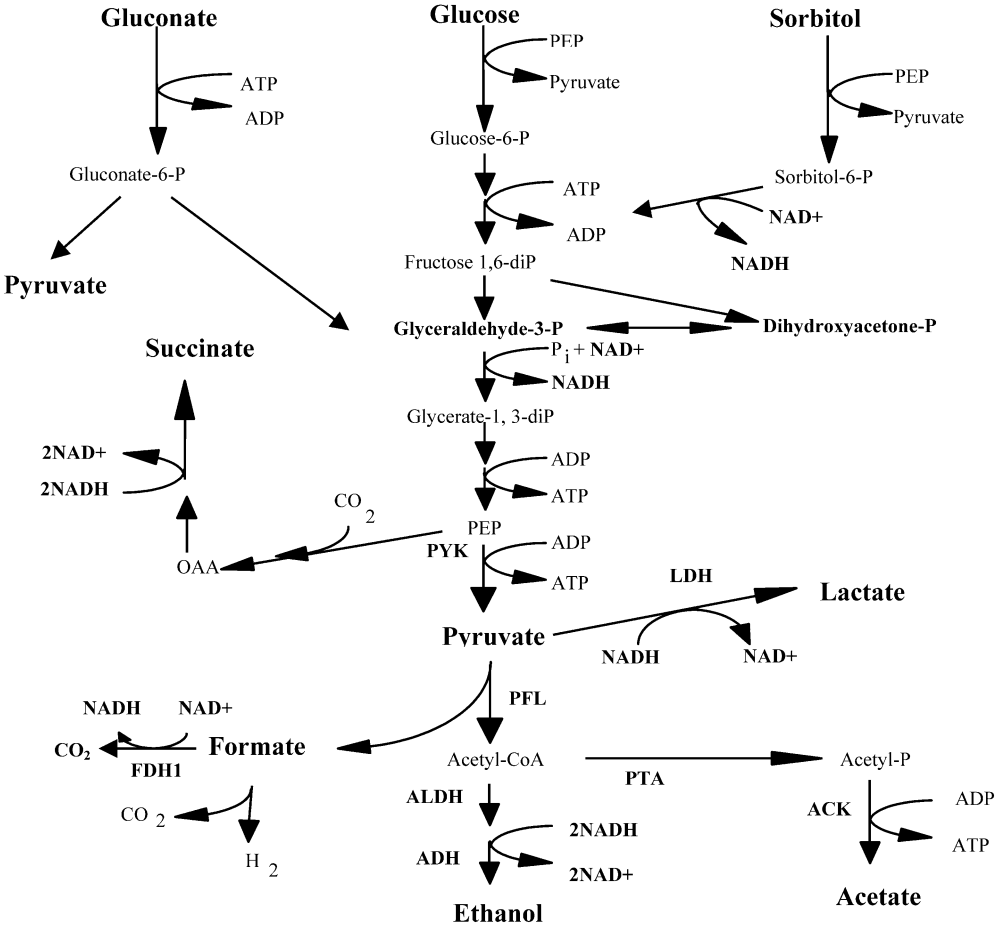

Fig. 1 Central anoxic metabolic pathway of *Escherichia coli* showing generation of NADH and regeneration of NAD^+, including the new NAD^+-dependent formate dehydrogenase (FDH) from *Candida boidinii* and the differences in the oxidation of glucose, sorbitol and gluconate as they enter glycolysis. Note the differences in NADH production

1 mol formate into 1 mol NADH and CO_2 (Fig. 1). In contrast, the native FDH converted formate to CO_2 and H_2 with no cofactor involvement. The new system allowed the cells to retain the reducing power that was otherwise lost by the release of formate or H_2 in the native pathway. When using glucose as a CS, this NADH regeneration system doubled the maximum yield of NADH from 2 mol to 4 mol NADH/mol substrate consumed. The higher NADH availability significantly changed the final metabolite concentration pattern under anoxic conditions. In this case, more reduced fermentation products were formed to consume additional reducing equivalents (in the form of NADH) in the same proportion as they were produced, to achieve a proper fermentation balance. To have a balanced fermentation, it is necessary to match the NADH produced with the NADH consumed by excretion of fermentation products (Clark 1989). The production of more reduced metabolites was favored, as evidenced by a dramatic increase in the ethanol-to-acetate ratio and a shift towards the production of ethanol as the major fermentation product.

The current study examines the effect of a range of NADH availability levels on the distribution of metabolites in *E. coli* using both minimal and complex medium. Different levels of NADH availability can be achieved by combining two strategies of cofactor manipulation, namely feeding CSs with different oxidation state and regenerating NADH by means of the NAD^+-dependent FDH pathway. Table 1 shows the maximum theoretical yield of NADH (moles of NADH produced per mole of substrate utilized) that can be achieved under anoxic conditions with the different combinations. This study analyses the effect of these different combinations on the NADH availability and distribution of metabolites in *E. coli*.

Table 1 NADH availability or theoretical yield (Y) of NADH for various strains and CSs under anoxic conditions. Yields were estimated based on the pathways shown on Fig. 1, assuming maximum flux through the pyruvate formate lyase (PFL) pathway

Strain	CS	$Y_{(NADH)\ T}$[a] (mol/mol)
GJT001 (pDHK29)	Gluconate	1
	Glucose	2
	Sorbitol	3
GJT001 (pSBF2)	Gluconate	3
	Glucose	4
	Sorbitol	5

[a]Moles of NADH produced from the oxidation of the CS and conversion of formate through the NAD^+-dependent FDH pathway per mole of CS consumed

Materials and methods

Bacterial strains and plasmids

Table 2 describes the strains and plasmids used in this study. Plasmid pSBF2 contains the *fdh1* gene from the yeast *C. boidinii* under the control of the *lac* promoter. The *fdh1* gene encodes an NAD^+-dependent FDH that converts formate to CO_2 with the regeneration of NADH from NAD^+. The heterologous pathway is referred in this paper as the NAD^+-dependent FDH, heterologous FDH or FDH.

Anoxic tube experiments

The anoxic tube experiments were performed using 40-ml glass vials with open-top caps and PTFE/silicone rubber septa. Each vial was filled with 35 ml of either minimal or LB medium supplemented with 20 g/l of CS (glucose, sorbitol or gluconate), 100 mg/l kanamycin and 1 g/l $NaHCO_3$ to reduce the initial lag time that occurs under anoxic conditions. The minimal medium consisted of 7 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl and 1 g/l NH_4Cl. This salt mixture was autoclaved and then supplemented with filter-sterilized solutions of thiamine, $MgSO_4$ and $CaCl_2$, which were added to a final concentration of 6 μg/l, 0.1 mM and 0.1 mM, respectively.

Complex medium anoxic tubes were inoculated in triplicate with 100 μl of a LB overnight culture (5 ml). Minimal medium anoxic tubes were inoculated in triplicate to an initial optical density at 600 nm (OD_{600}) of 0.2 to reduce the lag phase. After inoculation, 6 ml of air was removed with a syringe from the headspace to ensure anoxic 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 different time intervals (24, 48, 72, 144 h).

Analytical techniques

Cell density was measured with a Spectronic 1001 spectrophotometer (Baush & Lomb) at OD_{600} . Fermentation samples were centrifuged for 5 min in a microcentrifuge. The supernatant was filtered through a 0.2-μm syringe filter and kept at 4°C for HPLC analysis. The fermentation products and glucose were quantified using a 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 H_2SO_4 solution at a flow rate of 0.6 ml/min was used; and the column was operated at 55°C.

Results

Anoxic tube experiments—minimal and complex media

Anoxic tube experiments were performed with strains GJT001 (pDHK29) and GJT001 (pSBF2) utilizing gluconate, glucose, and sorbitol as CSs, to achieve different levels of NADH availability. Table 3 shows the maximum theoretical and experimental yields of NADH that were obtained for the different conditions studied.

As can be seen in Table 3, a range of NADH availability levels was achieved by combining the use of CSs with different oxidation states with or without overexpression of the NAD^+-dependent FDH. The maximum theoretical yield of NADH [$Y_{(NADH)T}$] ranged from 1 mol to 5 mol NADH/mol CS consumed, while the experimental yield of NADH, ($NADH_U$/CS), ranged from 1.23 mol to 4.11 mol NADH/mol CS for minimal medium and 1.75–4.56 mol NADH/mol CS for complex medium. The $Y_{(NADH)T}$ values were estimated based on the pathways presented on Fig. 1. In the calculation of the theoretical NADH yield for the FDH strain, it was assumed that all the carbon went through the PFL pathway and, therefore, 2 mol NADH/mol CS were formed by the heterologous FDH pathway.

The $NADH_U$/CS values for GJT001 (pDHK29) with the three CSs were slightly higher than the theoretical values. Another observation is that the $NADH_U$/CS values for complex medium were significantly higher than the $NADH_U$/CS values for minimal medium for all the cases studied (Table 3). Most importantly, the NADH availability correlated positively with the ethanol yield (Table 3). The higher the $NADH_U$/CS, the higher the ethanol yield in almost all cases. Similarly, as the NADH availability increased, the ethanol levels and the ethanol to acetate (Et/Ac) ratio also increased, while the acetate levels decreased in both minimal and complex media (Figs. 2, 3). The only exception to this trend was the GJT001 (pSBF2) strain with sorbitol as CS in complex medium, which showed a lower level of ethanol and Et/Ac ratio than the same strain with glucose as CS (Fig. 3). However, the ethanol yield for complex medium was 2 mol ethanol/mol CS for both glucose and sorbitol (Table 3). This value represents the maximum theoretical yield for ethanol from these CSs.

In minimal medium, a yield of 1.94 mol ethanol/mol CS was achieved by the sorbitol–FDH combination, which is very close to the maximum theoretical yield of 2 mol/mol. This ethanol yield represents almost a five-fold (490%)

Table 2 List of strains and plasmids used in this study

	Significant genotype	Reference
Strain		
GJT001	Spontaneous *cadR* mutant of MC4100, Sm^R	Tolentino et al. (1992)
Plasmids		
pDHK29	Control, cloning vector, Km^R	Phillips et al. (2000)
pDHK30	Control, cloning vector, Km^R	Phillips et al. (2000)
pSBF2	*fdh1* in pDHK30, Km^R	Berríos-Rivera et al. (2002b)

Table 3 NADH availability and ethanol yield of various strains from anoxic tube experiments using minimal and complex media. Theoretical yields assumed maximum flux through the PFL pathway and were estimated based on the pathways shown in Fig. 1. Experimental yields were estimated from the concentrations of reduced metabolites by calculating the NADH used for their production according to the pathways shown in Fig. 1. Values shown are averages for triplicate cultures ± SD

Strain	CS	$Y_{(NADH)\ T}$ (mol/mol)[a]	Minimal medium		Complex medium	
			$NADH_U$/CS (mol/mol)[b]	$Y_{(ethanol)}$ (mol/mol)[c]	$NADH_U$/CS (mol/mol)[b]	$Y_{(ethanol)}$ (mol/mol)[c]
GJT001 (pDHK29)	Gluconate	1	1.23±0.01	0.22±0.01	1.75±0.04	0.19±0.01
	Glucose	2	2.06±0.06	0.33±0.03	2.33±0.01	0.41±0.01
	Sorbitol	3	3.42±0.04	1.42±0.02	3.77±0.20	1.47±0.07
GJT001 (pSBF2)	Gluconate	3	1.77±0.01	0.27±0.03	2.33±0.02	0.72±0.01
	Glucose	4	2.72±0.17	0.71±0.02	4.32±0.03	2.07±0.02
	Sorbitol	5	4.11±0.06	1.94±0.03	4.56±0.12	2.07±0.03

[a]Theoretical number of moles of NADH produced from the oxidation of CS and conversion of formate through the NAD^+-dependent FDH pathway per mole of CS consumed
[b]Experimental number of moles of NADH used for reduced product formation per mole of CS consumed, where $NADH_U$ is the total NADH used for product formation per unit volume at the end of fermentation (mmol/l)
[c]Ethanol yield (mol ethanol/mol substrate)

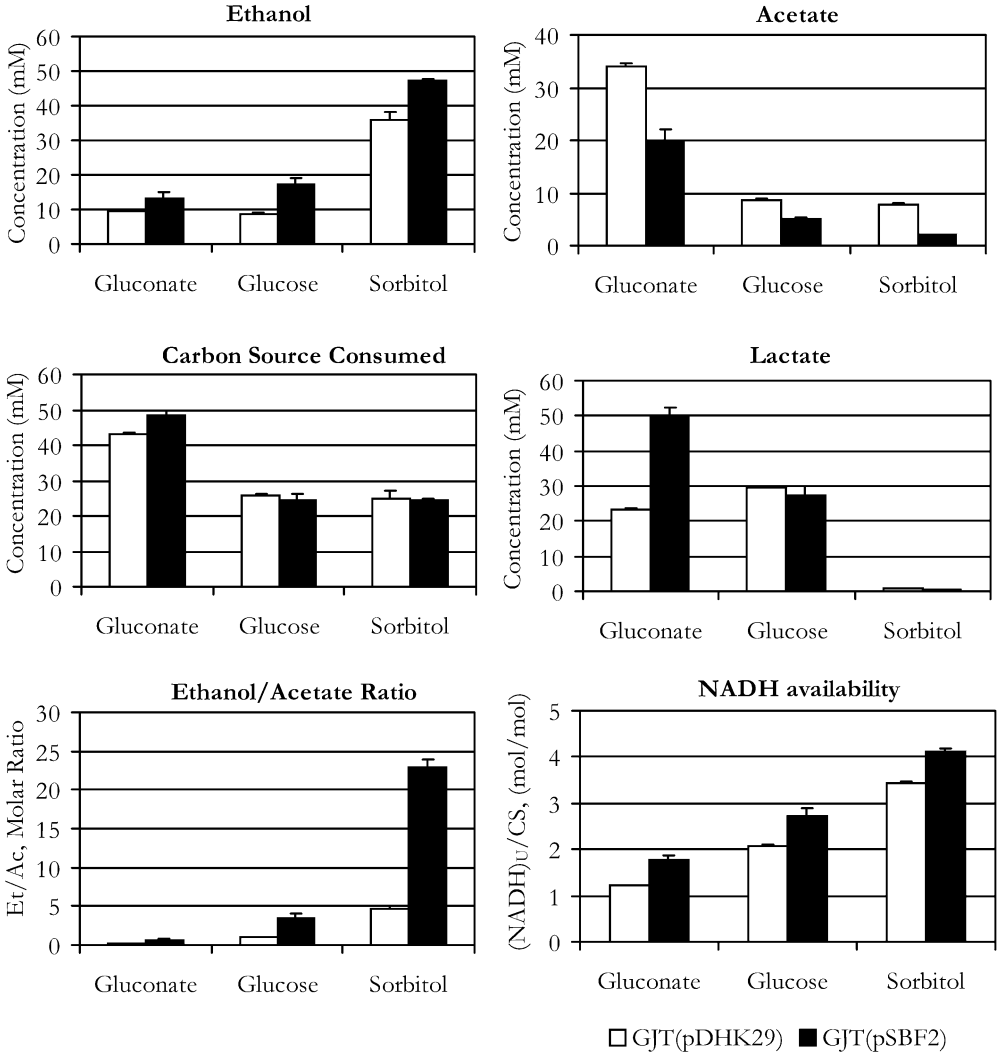

Fig. 2 Results of anoxic tube experiments with GJT001 (pDHK29) and GJT001 (pSBF2) with gluconate, glucose, or sorbitol as CS in minimal medium. The values shown are for triplicate analyses of the metabolites after 144 h of growth. The error bars correspond to the SD for the triplicates

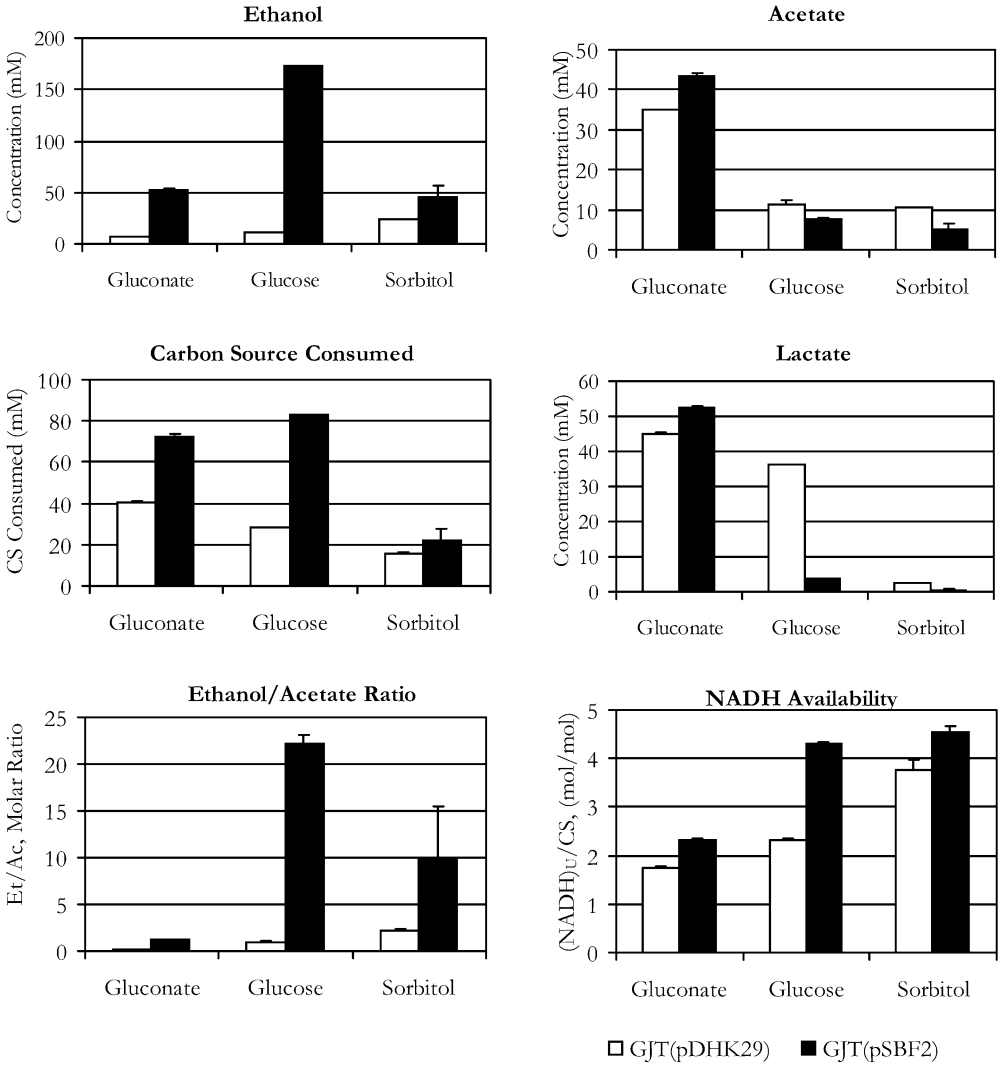

Fig. 3 Results of anoxic tube experiments with GJT001 (pDHK29) and GJT001 (pSBF2) with gluconate, glucose, or sorbitol as CS in complex medium. The values shown are for triplicate analyses of the metabolites analyzed after 72 h of growth. The error bars correspond to the SD for the triplicates

increase when compared with the glucose base case (0.33 mol/mol, Table 3). In complex medium, the effect of the NAD^+-dependent FDH incorporation, in terms of NADH availability, was more pronounced than that of NADH manipulation using different CSs. The NADH availability, $NADH_U$/CS, was increased from 2.33 mol/mol (control) to 4.32 mol/mol, approximately a 2 mol increase by the use of the NAD^+-dependent FDH. In minimal medium, the $NADH_U$/CS values of the NAD^+-dependent FDH with different CSs were further from the theoretical values than in complex medium. Thus, the effect of the NAD^+-dependent FDH was not as strong in minimal medium as in complex medium. Nevertheless, the general trends of the data were substantially similar for both growth conditions.

In general, overexpression of the NAD^+-dependent FDH increased the ethanol yield and Et/Ac ratio, while it decreased acetate in both minimal and complex media. This trend is similar to those previously described (Berríos-Rivera et al. 2002b, 2002c). In complex medium, CS consumption and ethanol production were increased dramatically with FDH overexpression, particularly when glucose was the CS. In complex medium with glucose as CS, the final ethanol concentration was one order of magnitude higher for the strain overexpressing the NAD^+-dependent FDH, compared with that of the control strain (Fig. 3).

Lactate levels also decreased with the overexpression of the NAD^+-dependent FDH, particularly in complex medium. Using gluconate as CS, the levels of acetate and lactate increased slightly with overexpression of the NAD^+-dependent FDH, but the yields of these two products actually decreased. This was due to the increase in gluconate consumption caused by overexpression of the NAD^+-dependent FDH (Fig. 3). In minimal medium, the gluconate consumption increased but not as dramatically as in complex medium and the lactate level and yield increased with the overexpression of the NAD^+-dependent FDH.

Table 4 Metabolite concentrations (mM), NADH availability and ethanol yield, showing results from anoxic tube experiments using gluconate as CS on complex medium with and without 25 mM formate supplementation. Concentrations shown are from samples collected after 72 h culture (average of triplicate cultures ± SD)

Initial formate	GJT001 (pSBF2)	
	0 mM	25 mM
Gluconate consumed	72.41±1.11	88.62±0.00
Succinate	7.23±0.23	10.45±0.05
Lactate	52.32±0.40	19.93±0.83
Residual formate	0.00±0.00	0.89±1.25
Acetate	43.34±0.74	42.08±0.58
Ethanol	52.20±1.10	117.77±3.87
Et/Ac ratio	1.20	2.80
OD_{600}	1.22±0.03	1.76±0.16
$NADH_U$/CS	2.36	3.12
$Y_{ethanol}$ (mol/mol)	0.72	1.33

Anoxic tube experiments—complex medium with exogenous addition of formate

For the gluconate–FDH case, the pathway distribution could go either through the PFL or the LDH pathway in order to achieve its redox state but, since the conversion of gluconate to two molecules of pyruvate was effectively redox balanced, the cells did not need to go through the PFL pathway to recycle their NADH. It seems that lactate is the preferred escape route to recycle available NADH and achieve proper fermentation balance, even in the presence of the NAD^+-dependent FDH. As a result, a reduced carbon flux through the PFL pathway results in less formate, the substrate for the NAD^+-dependent FDH. A lower level of formate flux decreases the amount of NADH that can be regenerated by the NAD^+-dependent FDH pathway. For this reason, an anoxic tube experiment was performed with strain GJT001 (pSBF2) using gluconate as CS and supplementing the media with 25 mM formate. The $NADH_U$/CS of gluconate increased from 2.36 mol to 3.12 mol NADH/mol CS, a more than 30% increase, upon the addition of 25 mM formate (Table 4). This higher NADH availability reduced the lactate formation and doubled the ethanol concentration, the molar yield and the Et/Ac ratio (Table 4).

Discussion

Table 3 shows that the experimental NADH yield, $NADH_U$/CS, for GJT001 (pDHK29) from the three CSs was slightly higher than the theoretical value. This finding was presumably due to a small contribution from the PDH pathway that converts pyruvate to acetyl-CoA and NADH. It has been suggested that the PDH pathway is slightly active under anoxic conditions (Snoep et al. 1990; Kaiser and Sawers 1994). It was also seen that the $NADH_U$/CS values for complex medium were significantly higher than the $NADH_U$/CS values for minimal medium for all cases studied (Table 3). This may have been in part due to the extra reducing power that could be derived from the yeast extract and tryptone in the complex medium. Another probable explanation for this behavior is that, in minimal medium, a small fraction of the available NADH provided by the CS might be diverted towards the endogenous biosynthetic pathway (formation of building blocks, biomass; Tao et al. 1999). NADH can be interconverted to the biosynthetic precursor NADPH through native transhydrogenases present in *E. coli* (Lundquist and Olivera 1971; Boonstra et al. 1999; Canonaco et al. 2001). Cells in minimal medium face the need to synthesize their building blocks from the available CS and this burden is reflected in the growth rate of the bacterial cell (Tao et al. 1999). In complex medium, most of the NADH derived from the CS is available for product formation. This could be evidenced by slightly higher carbon recoveries from product formation obtained in complex medium experiments relative to those in minimal medium (data not shown; all estimated carbon recoveries were above 90%). However, the metabolite redistribution trend in both minimal and complex media was essentially the same.

As mentioned in the Results, NADH availability correlates positively with ethanol yield (Table 3), with the exception of the sorbitol–FDH combination. Since the cells have already attained the maximum theoretical yield of ethanol in complex medium with NAD^+-dependent FDH and glucose as a CS, increasing the NADH availability further by using sorbitol as a CS cannot increase the ethanol yield. The use of a combination of strategies (e.g. combining the use of the more reduced CS, sorbitol, with the overexpression of the NAD^+-dependent FDH) might be useful in the production of a product that is more reduced than ethanol. In the absence of a more reduced product alternative, the cells cannot handle the excess reducing power for the sorbitol–FDH combination and they decrease their consumption of CS, thus lowering the final ethanol level (Fig. 3).

In minimal medium, the highest level of ethanol was obtained using the sorbitol–FDH combination, indicating that extra available NADH is still manageable by the cells under these growth conditions. These results demonstrate that increasing NADH availability by using our combined system of CS and genetic manipulation can increase the production of more reduced metabolites.

In addition, overexpression of the heterologous FDH in complex medium, resulted in a higher CS consumption and the formation of more reduced by-products relative to minimal medium. This is because, in complex media, most of the NADH derived from the CS is available for product formation.

The effect of NADH availability observed in glucose-complex medium by means of the NAD^+-dependent FDH overexpression was more striking than that of NADH manipulation using different CSs. This effect was expected since NAD^+-dependent FDH can theoretically provide an extra 2 mole NADH/mol CS, while each different CS

provides only an extra 1 mol NADH when compared with its preceding oxidized sugar derivative.

In conclusion, the anoxic tube experiments demonstrated that it is possible to achieve different levels of NADH availability by combining the use of CSs which have different oxidation state with the overexpression of a NAD$^+$-dependent FDH in both minimal and complex media. The distribution of metabolites follows a similar trend in both types of medium. The availability of NADH correlates positively with the ethanol yield. However, the FDH–glucose system in complex medium readily achieves the maximum theoretical yield of ethanol. In minimal medium, using sorbitol as the CS in combination with the FDH system achieves this same maximum yield. It is possible that the FDH–sorbitol combination could be more beneficial for the production of compounds more reduced than ethanol. Moreover, it is also possible to increase the NADH availability by adding formate exogenously, as in the gluconate case, or in cases where formate is not produced by the system, as in oxic conditions (Berríos-Rivera et al. 2002b).

The present system could potentially be used to improve the production and/or productivity of NADH-dependent products, such as succinate. In addition, our current system can be extended to the production of biosynthetic products that require NADPH. The reducing equivalents in the form of NADH can be used to convert NADP$^+$ into NADPH to improve the production and yield of NADPH-dependent products, such as some biodegradable polymers, amino acids and other biosynthetic products.

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