Metabolic Engineering through Cofactor Manipulation and Its Effects on Metabolic Flux Redistribution in *Escherichia coli*

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Applications of genetic engineering or metabolic engineering have increased in both academic and industrial institutions. Most current metabolic engineering studies have focused on enzyme levels and on the effect of the amplification, addition, or deletion of a particular pathway. 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. It is conceivable that in cofactor-dependent production systems, cofactor availability and the proportion of cofactor in the active form may play an important role in dictating the overall process yield. Hence, the manipulation of these cofactor levels may be crucial in order to further increase production. We have demonstrated that manipulation of cofactors can be achieved by external and genetic means and these manipulations have the potential to be used as an additional tool to achieve desired metabolic goals. We have shown experimentally that the NADH/NAD+ ratio can be altered by using carbon sources with different oxidation states. We have shown further that the metabolite distribution can be influenced by a change in the NADH/NAD+ ratio as mediated by the oxidation state of the carbon source used. We have also demonstrated that the total NAD(H/+) levels can be increased by the overexpression of the *pncB* gene. The increase in the total NAD(H/+) levels can be achieved even in a complex medium, which is commonly used by most industrial processes. Finally, we have shown that manipulation of the CoA pool/flux can be used to increase the productivity of a model product, isomyl acetate.

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INTRODUCTION

The production of chemicals by biocatalysts has attained considerable attention. Technological advances for making genetically engineered strains, improvements in experimental measurement, and theoretical analysis of metabolic fluxes have contributed to the production of high value added products (for example, recombinant proteins for diagnostic and therapeutic uses) and the commercial introduction of new processes for the manufacture of certain novel chemicals (examples using engineered *Escherichia coli* are the production of indigo dye by Genencor and 1,3-propanediol by DuPont). The production of lactate, biopolymer PHB, and ethanol using metabolic engineering techniques is currently being pursued with commercial interest.

Metabolic engineering has the potential to considerably improve process productivity by manipulating the throughput of certain pathways. However, despite its powerful and precise nature, the current status of metabolic engineering is still hindered by the lack of our full understanding of cellular metabolism. The aspects of integrated dynamics and overall control structure are the common obstacles for the optimal design of pathways to achieve a desired goal.

Applications of genetic engineering or metabolic engineering have increased in both academic and industrial institutions and the area has been reviewed (reviews: Stephanopoulos and Vallino, 1990; Bailey, 1990; Carmeron and Chaplen, 1997; Stephanopoulos et al., 1998; Lee and Papousakis, 1999). Most current metabolic engineering studies have mainly focused on manipulating enzyme levels through the amplification, addition, or deletion of a particular pathway. However, cofactors play an essential role in a large number of biochemical reactions and their manipulation has the potential to be used as an additional tool to achieve desired metabolic engineering goals. Furthermore, it will also provide an additional means to study cellular metabolism, in particular the interplay between cofactor levels/fluxes and metabolic fluxes.

In this project, we focus on two common yet very important cofactors, nicotinamide adenine dinucleotide (NAD) and acetyl-CoA. NAD functions as a cofactor in over 300 oxidation–reduction reactions. The NADH/NAD+ cofactor pair plays a major role in microbial catabolism, in which a carbon source, such as glucose, is oxidized using NAD+ and producing reducing equivalents in the form of NADH. It is critically important for continued cell growth and product formation that this reduced NADH be oxidized to NAD+ and a redox balance be achieved. 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 (Fig. 1). Some previous efforts to manipulate NADH levels have included the use of the NADH oxidase system (Lopez de Felipe et al., 1998), the addition of an electron dye carrier (Park and Zeikus, 1999), or the variation of the redox-reduction potential conditions (Riondet et al., 2000).

Another important cofactor is coenzyme A, CoA, and its derivative acetyl-CoA. Acetyl-CoA is an essential intermediate in many energy-yielding metabolic pathways and is a substrate in enzymatic production of industrially useful compounds such as esters and lipid molecules.

The long-term goals of this project are threefold: (1) to implement these design concepts in a production strain; (2) to characterize their effects on host-cell physiology and metabolic patterns; and (3) to test their effects on process productivity in three different model systems.

This article describes the conceptual designs of various approaches that may lead to a change in the intracellular cofactor pools and/or fluxes. We also describe three different model systems that can be used in future studies to test the potential advantages of cofactor perturbation on process productivity. Furthermore, we present some early positive results based on these design strategies. We show experimentally that the intracellular NAD(H) pool, as well as its ratio within a host strain, can be perturbed; we further show that the host strain may respond to these perturbations by shifting its metabolic pattern. Finally, we demonstrate by using two host strains with different CoA levels that manipulation of the CoA pool/flux can be used
to increase the productivity of a model product, isoamyl acetate.

**MODEL SYSTEMS**

Three model systems were chosen to study the feasibility of improving process productivity through cofactor manipulations. These three systems are the biosynthesis of 1,2-propanediol, ester isoamyl acetate, and succinate (Figs. 2a–2c). All three systems require either NADH/NAD\(^+\) or acetyl-CoA in their synthesis pathways.

**Model System for NAD(H/\(^+\)) Study**

The production of 1,2-propanediol (1,2-PD) was used as a model system because its biological synthesis depends on NADH (Fig. 2a). The precursor dihydroxyacetone-P is a common glycolysis intermediate. It can be converted to methylglyoxal by the enzyme methylglyoxal synthase (MGS), from which it is converted to D-lactate through the glyoxalase system (Cooper, 1984). This pathway is normally not very active in *E. coli* due to the cytotoxicity of methylglyoxal (MG) (Ferguson et al., 1998). However, MG can be converted to 1,2-PD by a series of two reductions. In this study, the production of 1,2-PD was achieved by overexpressing MGS to increase the flux to MG and a dehydrogenase (glycerol dehydrogenase). Data on this system are not presented here, but the system is undergoing further study.

**Model System for CoA Study**

The biosynthesis of isoamyl acetate was selected as a model system because it uses acetyl-CoA as a substrate. Isoamyl acetate is not normally formed by wild-type *E. coli*. However, a genetically engineered *E. coli* strain bearing an alcohol acetyltransferase (ATF)-expressing plasmid grown in the presence of externally added isoamyl alcohol has been shown to be able to form isoamyl acetate (see Results and Discussion). Under anaerobic conditions, *E. coli* forms acetyl-CoA from pyruvate mainly via the pyruvate formate lyase pathway. The newly introduced ester-producing pathway competes directly with the existing ethanol- and acetate-forming pathways for acetyl-CoA (Fig. 2b). It is thus speculated that manipulation of cellular metabolism to increase CoA availability may enhance ester production. An example of this strategy is presented.

**Model System for NAD(H/\(^+\)) and CoA Study**

The biosynthesis of succinate was also chosen as a model system because of the involvement of both cofactors
NADH/NAD⁺ and acetyl-CoA. The biosynthesis of succinate from phosphoenolpyruvate (PEP) requires NADH. Two moles of NADH are consumed per mole of succinate formed (Fig. 2c). Note that the enzyme phosphoenolpyruvate carboxylase activity is activated by acetyl-CoA (Izui et al., 1981; Terada et al., 1991). It is therefore anticipated that both NADH/NAD⁺ and acetyl-CoA may play an important role in the synthesis rate of succinate. Experimental data on the effect of these manipulations on succinate production are not presented here but the system is currently under study.

**NADH/NAD⁺ Manipulation**

**NADH/NAD⁺ Manipulation Strategies**

Two distinct strategies can be used to alter the NAD(H⁺) pool and NADH/NAD⁺. The first approach is based on the use of carbon sources with different oxidation states. The other approach is based on genetic manipulations of the host cell. It should be noted that these strategies could be used in conjunction with the more traditional pathway manipulation approaches to achieve even more pronounced results.

**Carbon source.** In the first strategy, we used three different carbon sources as a simple way to manipulate the cellular NADH/NAD⁺ ratio. It is noted that the oxidation state of glucose is 0 and those of sorbitol and gluconate are −1 and +1, respectively. Figure 3 highlights the differences in the oxidation of these carbon sources as they enter the glycolysis pathway. Sorbitol produces more reducing equivalents in the form of NADH than glucose while gluconate produces the least amount of NADH since half of every gluconate molecule goes directly to pyruvate, skipping the NADH-producing step in glycolysis (Fig. 3). It is thus expected that these three different carbon sources will have a significant effect on cellular NADH/NAD⁺ ratio and subsequently on the metabolic patterns.

**Genetic manipulation (enhancing the pyridine nucleotide salvage pathway).** The second strategy targets the genetic manipulation of the host cell. Two different approaches can be used; the first approach aims at increasing the total NAD(H⁺) pool while a second approach focuses on changing the NADH/NAD⁺ ratio. It is also conceivable that a combination of these two approaches may lead to both an increased NAD(H⁺) pool and an increased ratio.

*E. coli* maintains its total NAD(H⁺) intracellular pool by synthesizing NAD through the de novo pathway and the pyridine nucleotide salvage pathway. The salvage pathway recycles intracellular NAD breakdown products and preformed pyridine compounds from the environment, such as nicotinic acid (NA). The enzyme nicotinic acid phosphoribosyltransferase (NAPRTase; EC 2.4.2.11), encoded by the *pncB* gene, catalyzes the formation of nicotinate mononucleotide, a direct precursor of NAD, from NA (Fig. 4a).
This reaction is believed to be the rate-limiting step in the NAD salvage pathway. However, the product of the \(nadR\) gene regulates both biosynthetic pathways by negatively-controlling the transcription of the \(nadA\) and \(nadB\) genes, whose products catalyze the initial steps of the de novo biosynthetic pathway and the transcription of the \(pncB\) gene in the salvage pathway (Foster et al., 1990).

Based on these findings, we investigated the feasibility of increasing the total NAD(H) pool by substantially enhancing the salvaging pathway. This can be achieved by overexpressing NAPRTase. Direct cloning of the \(pncB\) gene on a multicopy plasmid may not be sufficient due to the transcriptional repression of \(NadR\) on \(pncB\) (Foster et al., 1990). Further manipulations have to be taken to ensure its overexpression: (1) by creating a mutation in the operator region where the repressor protein binds (Fig. 4b) or (2) by putting the transcription of the \(pncB\) gene under the control of another promoter, such as the \(lac\) promoter system.
Results and Discussion

Carbon Source

The feasibility of using carbon sources (glucose, sorbitol, and gluconate) with different oxidation states as a means to manipulate the intracellular NADH/NAD\(^+\) ratio was investigated by performing a series of chemostat experiments under anaerobic conditions. In addition, the subsequent effect on the redistribution of metabolic fluxes was examined. Chemostat mode of operation was chosen because it allows the determination of the concentrations of NADH and NAD\(^+\) and the metabolic fluxes under steady-state conditions, which generally provides a better data quality. Calculation of the specific metabolic fluxes at a given growth rate can be obtained by simply measuring the metabolite concentrations, cell density, and growth rate (dilution rate). Chemostat experiments have been successfully and routinely employed in our laboratories to study the effects of genetic and environmental perturbations on metabolic flux redistribution in E. coli (Aristidou et al., 1999; Yang et al., 1999; Berrios-Rivera et al., 2000). It should also be emphasized that the chemostat mode allows fixing the specific growth rate for each carbon source by fixing the dilution rate. As a result, it provides a better comparison than batch reactor runs because any growth rate effect due to the use of different carbon sources is eliminated.

Strain and medium. The strain used in this study is GJT001(pACYC184) (Tolentino et al., 1992), a derivative of MC4100. Luria-Bertani broth (LB) medium supplemented with 110 mM glucose, sorbitol, or gluconate was used for the chemostat runs. To reduce the initial lag time that occurs under anaerobic conditions, 1 g/L NaHCO\(_3\) was added to the LB medium. The medium was also supplemented with 25 mg/L tetracycline and 30 \(\mu\)L/L antifoam 289 (Sigma).

Bioreactor conditions. The fermentations were carried out under anaerobic chemostat conditions at a dilution rate of 0.1 h\(^{-1}\) in a 2.5-L bioreactor (New Brunswick Scientific; Bioflo III) initially with 1.3 L of medium during the anaerobic batch stage and maintained at 1.20-L working volume for the anaerobic chemostat stage. The pH, temperature, and agitation were maintained at 7.0, 32°C, and 250 rpm, respectively. A constant flow of nitrogen (10–12 ml/min) was maintained through the fermentor headspace to establish anaerobic conditions. The continuous culture reached steady state after four to six residence times. Samples were taken during the steady-state phase.

Analytical technique. The procedures for the determination of cell dry weight, metabolite concentrations (HPLC and GC), and the gases CO\(_2\) and H\(_2\) (GC) have been previously reported (Aristidou et al., 1999; Yang et al., 1999; Berrios-Rivera et al., 2000).

The NADH and NAD\(^+\) were extracted by collecting a sample from the reactor sample port and immediately pipetting 1 ml of the sample into two microcentrifuge tubes (one for NAD\(^+\) and one for NADH determination). The 1 ml samples were centrifuged in a microcentrifuge (Beckman Microfuge E) at 15,000 rpm for 1 min (12,535 g). The supernatant was removed and 300 \(\mu\)L of 0.2 M HCl (for NAD\(^+\) extraction) or 0.2 M NaOH (for NADH extraction) was added to the pellets to resuspend them. The samples were placed in a 50°C water bath for 10 min and then on ice to cool them to 0°C. The extracts were neutralized by adding 300 \(\mu\)L of 0.1 M NaOH (for NAD\(^+\) extraction) or 300 \(\mu\)L of 0.1 M HCl (for NADH extraction) dropwise while vortexing. The cellular debris was removed by centrifuging at 15,000 rpm for 5 min (12,535 g). Supernatants were transferred to new tubes and stored at −20°C for no more than 24 h.

The intracellular NADH and NAD\(^+\) concentrations were measured by a very sensitive cycling assay (Leonardo et al., 1996; Bernofsky and Swan, 1973). Figure 5 shows a schematic representation of the reactions involved in the assay. The cycling assay was performed using a reagent mixture consisting of equal volumes of 1.0 M Bicine buffer (pH 8.0), absolute ethanol, 40 mM EDTA (pH 8.0), 4.2 mM MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue), and twice the volume of 16.6 mM PES (phenazine ethosulfate), previously incubated at 30°C. The following volumes were added to 1-ml cuvettes: 50 \(\mu\)L neutralized extract, 0.3 ml water, and 0.6 ml reagent mixture. The reaction was started by adding 50 \(\mu\)L of yeast ADHII (500 or 100 U/ml in 0.1 M Bicine (pH 8.0) buffer). The absorbance at 570 nm was recorded for 10 min at 30°C. The assay was calibrated with 0.01–0.05 mM standard solutions of NADH and NAD\(^+\). The slope (AA/min) of the linear region of the absorbance versus time plot was correlated to the concentration of coenzyme (mM) by a linear fit equation. This equation was used to determine the concentration (mM) of NAD(H/\(^+\)) in the samples (extracts) from their slope (AA/min). The concentration in micromoles of NAD(H/\(^+\)) per gram of dry weight was obtained by multiplying the concentration of the extract by the corresponding dilution factor (0.6 ml extract/1 ml culture), dividing by the dry weight (g dry wt/L of culture), and converting the millimoles to micromoles.

Results

Table 1a shows the steady-state concentrations of various metabolites from the carbon source experiments as measured by the HPLC. Table 1b shows the calculated
metabolic fluxes in mmol/(g dry wt × h) and NAD(H/+) concentrations in μmol/g dry weight. In these experiments, the NADH/NAD+ ratio increased from 0.75 for glucose to 0.94 for sorbitol, which is more reduced and can therefore produce more reducing equivalents in the form of NADH. In a similar way, the NADH/NAD+ ratio decreased to 0.51 when gluconate, which is more oxidized than glucose, was used as the carbon source. These changes in the NADH/NAD+ ratio affected the distribution of metabolic fluxes in E. coli as is reflected in the ethanol to acetate ratio. This ratio increased from 1.00 with glucose to 3.62 with sorbitol and decreased to 0.29 with gluconate. It is evident from these results that the cell adjusts its partitioning at the acetyl-CoA node by changing the ethanol-/NADH (consumes 2 NADH) to-acetate (consumes no NADH) ratio to achieve a redox balance. Therefore, a change in the ethanol-to-acetate ratio can be used as an indirect indicator of a change in the NADH/NAD+ ratio.

It is interesting to note that the lactate flux, which consumes 1 NADH, was affected in a different way by the oxidation state of the carbon source. In this case, the lactate flux was highest for gluconate and lowest for sorbitol. These results in combination with the ethanol results indicate that since lactate formation (1 NADH) is less NADH demanding than ethanol formation (2 NADH), it is preferred as a means to regenerate NAD+ under more oxidizing conditions. On the other hand, when there is an excess of reducing equivalents, ethanol formation is preferred since it provides a faster route to NAD+ regeneration.

These chemostat experiments show that feeding similar carbon sources with different oxidation states can be used to manipulate the NADH/NAD+ ratio, which is consistent with our earlier report (Berrios-Rivera et al., 1999). In addition, the oxidation state of the carbon source has a direct effect on the distribution of metabolic fluxes in E. coli, especially in those requiring NADH such as ethanol, lactate, and acetate (by its relationship with ethanol).

The method described here can also be used as a simple way of determining whether a particular pathway that requires NADH is limited by this cofactor. For example, if a product requires NADH for its synthesis, feeding a more reduced carbon source such as sorbitol will normally improve its yield if the system is cofactor limited. The opposite is also generally true. This information can help determine which strategy to use when trying to improve the production of cofactor-dependent chemicals.

**pncB Overexpression**

We have examined the effect of overexpressing the pncB gene from Salmonella typhimurium on the total levels of NAD and the NADH/NAD+ ratio in E. coli. Preliminary experiments were performed with E. coli strains DH10B and GJT001 transformed with the plasmids pPNCBIN and pPNCBIM, which contain the native or mutated pncB gene in the pBCSK+ backbone, or with pBCSK+ as a control. These six strains were grown anaerobically in glass vials sealed with an open-top cap and a septum. The medium consisted of 40 ml LB medium supplemented with 2% glucose, 1 g/L NaHCO3, and 34 mg/L chloramphenicol. After inoculation of 10 μl from an overnight culture, the
TABLE 2
Results of Anaerobic-Tube Experiments with Plasmids Overexpressing the pncB Gene

<table>
<thead>
<tr>
<th></th>
<th>DH10B (pBCSK)</th>
<th>DH10B (pPNCBIN)</th>
<th>DH10B (pPNCBIM)</th>
<th>GJT001 (pBCSK)</th>
<th>GJT001 (pPNCBIN)</th>
<th>GJT001 (pPNCBIM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD&lt;sub&gt;600mm&lt;/sub&gt;</td>
<td>1.200</td>
<td>1.305</td>
<td>0.702</td>
<td>0.57</td>
<td>0.486</td>
<td>0.561</td>
</tr>
<tr>
<td>NAD+ (μmol/g DW)</td>
<td>14.49</td>
<td>22.22</td>
<td>31.39</td>
<td>7.89</td>
<td>8.16</td>
<td>11.81</td>
</tr>
<tr>
<td>NADH (μmol/g DW)</td>
<td>15.57</td>
<td>18.26</td>
<td>37.85</td>
<td>12.27</td>
<td>18.82</td>
<td>11.51</td>
</tr>
<tr>
<td>NADH/NAD+ ratio</td>
<td>1.21</td>
<td>0.82</td>
<td>1.21</td>
<td>1.55</td>
<td>2.31</td>
<td>0.98</td>
</tr>
<tr>
<td>Total NAD(H+)</td>
<td>32.06</td>
<td>40.48</td>
<td>69.24</td>
<td>20.16</td>
<td>26.99</td>
<td>23.32</td>
</tr>
<tr>
<td>% change in total</td>
<td>—</td>
<td>26</td>
<td>116</td>
<td>—</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>NAD(H+)/+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Note. Percentage change in total NAD(H+)/+ is based on the concentration of the control experiments, DH10B(pBCSK) and GJT001(pBCSK), respectively. DW, dry weight.

tubes were incubated in a reciprocal shaker maintained at 250 rpm and 37°C. When the cultures reached an OD<sub>600</sub> of 0.5 to 1.0, samples were collected with a syringe through the septum and processed immediately for determination of NADH and NAD<sup>+</sup> levels.

Table 2 summarizes the results of these experiments. The levels of NAD<sup>+</sup> and NADH are expressed in μmol/g dry weight (DW) after converting the OD<sub>600 nm</sub> measurements to dry weight using the conversion factor 3 OD<sub>600</sub> = 1 g DW/L. For the DH10B strain, the total NAD(H)/+ levels increased with the overexpression of the native pncB gene compared to the control strain (about 26%) and they increased even further with the overexpression of the mutated gene (about 2.16 times), last row of Table 2. Similar results were obtained for the GJT001 strain; the total NAD(H)/+ levels increased by 34% with overexpression of the native pncB gene. However, overexpression of the mutated gene led to an increase of only 16%. The NADH/NAD<sup>+</sup> ratios do not exhibit a consistent trend among the different constructs/strains. The lack of an apparent trend may be due to the fact that these experiments were performed in anaerobic tubes, where the growth environment is not controlled. It is well known that the NADH/NAD<sup>+</sup> ratio is very sensitive to growth rate as well as other environmental factors. Note that the sampling optical densities for the GJT001 strains were lower than those of the DH10B. For this reason it is necessary to perform more batch and/or chemostat experiments. Nevertheless, the current results suggest that the total NAD(H)/+ levels can be increased by the overexpression of the S. typhimurium pncB gene. These results are consistent with an earlier study with E. coli pncB gene in a semisynthetic medium (Wubbolts et al., 1990). The current study also demonstrated that the increase in the total NAD(H)/+ levels can be achieved even in a complex medium, which is commonly used by most industrial processes.

In addition, experiments were performed under anaerobic chemostat and anaerobic tube conditions to investigate the effect of overexpressing the pncB gene under the control of the native, mutated, or lac promoter on the total NAD levels, the NADH/NAD<sup>+</sup> ratio, and the distribution of metabolites in E. coli. Overexpression of the pncB gene in chemostat experiments increased the total NAD levels, decreased the NADH/NAD<sup>+</sup> ratio, and did not significantly redistribute the metabolic fluxes. However, under anaerobic tube conditions, overexpression of the pncB gene led to a significant shift in the metabolic patterns as evidenced by a decrease in lactate production and an increase as high as twofold in the ethanol-to-acetate ratio. These results suggest that under chemostat conditions the total level of NAD is not limiting and the metabolic rates are fixed by the system at steady state. On the other hand, under transient conditions (such as those in batch cultivation) the increase in the total level of NAD can increase the rate of NADH-dependent pathways (ethanol) and therefore change the final distribution of metabolites (Berrios-Rivera et al., 2001).

COENZYME A PERTURBATION

CoA Perturbation Strategies

Figure 6a shows the involvement of pantothenate kinase (PanK) in the biosynthesis of CoA. Two important features should be noted: (1) pantothenate kinase is an upstream rate-limiting step in the acetyl-CoA biosynthesis pathway (Rock et al., 2000) and (2) recent results indicated that pantothenate kinase is repressed by CoA and acetyl-CoA (Rock et al., 2000). We illustrate the cofactor-manipulating strategy by using a strain with an increased CoA pool presumably from overexpression of pantotheinate kinase, an enzyme involved in the limiting step in the CoA biosynthesis pathway (Vallari and Rock, 1987). Further extension of this work would employ a plasmid-based system for overexpression of CoA biosynthetic enzymes and potentially a feedback-insensitive pantothenate kinase.

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Results and Discussion

The effect of altered CoA levels/fluxes on metabolic flux distribution and ester formation was investigated by using two strains, DV62 and DV79 (Vallari and Rock, 1987), that have different pantothenate kinase activities. DV79 is a spontaneous revertant of the temperature-sensitive DV62 strain. DV79 has a higher CoA pool than DV62 (Vallari and Rock, 1987).

The CoA level of two strains, DV62 and DV79, was examined under both aerobic and anaerobic conditions. These two strains were grown aerobically in 250-ml flasks and anaerobically in 40-ml amber-colored glass vials sealed with an open-top cap and a septum. The medium consisted of 35-ml LB medium supplemented with 2% glucose and an appropriate amount of ampicillin. After inoculation with 10 μl overnight culture, the flasks or the tubes were incubated in a reciprocal shaker maintained at 250 rpm and 30°C. Samples were collected and processed immediately when the cultures reached an OD₆₀₀ of about 0.5 to 1.0. The results of these experiments are shown in Table 3. The CoA concentration of DV79 is 149% higher than that of the DV62 strain.

The effect of CoA perturbation on isoamyl acetate production was also investigated. For this purpose a plasmid encoding ATF2 (Yoshimoto et al., 1999) was constructed, pTAAT, and was transformed into these two strains (Fig. 6b). The final metabolite concentrations for both strains bearing the pTAAT plasmid grown in LB medium without and with 10 mM isoamyl alcohol supplementation in 35-ml anaerobic tubes are shown in Tables 4a and 4b, respectively.
TABLE 3
Concentration of CoA of DV62 and DV79 in Aerobic Shake Flask and Anaerobic Tube Cultures

<table>
<thead>
<tr>
<th></th>
<th>DV62 CoA concentration (nmol/mg)</th>
<th>DV79 CoA concentration (nmol/mg)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>0.49 ± 0.11</td>
<td>1.22 ± 0.14</td>
<td>149</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0.51 ± 0.06</td>
<td>1.15 ± 0.22</td>
<td>124</td>
</tr>
</tbody>
</table>

It should be noted that under normal conditions the newly introduced pathway would not function since E. coli does not produce isoamyl alcohol, i.e., isoamyl acetate would be formed only when isoamyl alcohol is added externally.

The final biomass concentrations for the two strains bearing the pTAAT plasmid without isoamyl alcohol supplementation were very similar. Both the final acetate and the final ethanol concentrations for the DV79 strain were slightly higher than those of the DV62 strain. However, the overall metabolite distributions remained relatively unperturbed; most of the changes are within a 5% level (Table 4a). These results suggest that the degree of change in the CoA concentration/flux, as mediated by these two strains, has very little effect on the normal host cell metabolism.

The final biomass concentrations for the two strains bearing the pTAAT plasmid are still very similar, even when isoamyl alcohol was added. Measured metabolite concentrations, such as glucose, lactate, and succinate, remain relatively unchanged. However, a significant increase, about 33%, in the final isoamyl acetate concentration was observed in the DV79 strain. These results suggest that the productivity of isoamyl acetate may be improved by increasing the CoA pool/flux.

SUMMARY

We have outlined several strategies that can be used to manipulate the pools/fluxes of two common yet important cofactors, NAD(H) and acetyl-CoA. We have also discussed three potential model systems that can be adapted to study the effects of these cofactor manipulations.

In this article, we have shown experimentally that the NADH/NAD+ ratio can be altered by using carbon sources with different oxidation states. We have further shown that the metabolite distribution can be influenced by a change in the NADH/NAD+ ratio as mediated by the oxidation state of the carbon source used. We have also demonstrated that the total NAD(H) levels can be increased by the overexpression of the pmcB gene. The increase in the total NAD(H/) levels can be achieved even in a complex medium, which is commonly used by most industrial processes. Finally, we have shown that manipulation of the CoA pool/flux can be used to increase the productivity of isoamyl acetate.

In summary, we have shown in this article that manipulation of cofactors can be achieved by genetic means and these manipulations have the potential to be used as an additional tool to achieve desired metabolic engineering goals.

ACKNOWLEDGMENTS

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