Research Article

Metabolic engineering of Clostridium acetobutylicum M5 for highly selective butanol production

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To improve butanol selectivity, Clostridium acetobutylicum M5(pIMP1E1AB) was constructed by adhE1-ctfAB complementation of C. acetobutylicum M5, a derivative strain of C. acetobutylicum ATCC 824, which does not produce solvents due to the lack of megaplasmid pSOL1. The gene products of adhE1-ctfAB catalyze the formation of acetooacetate and ethanol/butanol with acid re-assimilation in solventogenesis. Effects of the adhE1-ctfAB complementation of M5 were studied by batch fermentations under various pH and glucose concentrations, and by flux balance analysis using a genome-scale metabolic model for this organism. The metabolically engineered M5(pIMP1E1AB) strain was able to produce 154 mM butanol with 9.9 mM acetone at pH 5.5, resulting in a butanol selectivity (a molar ratio of butanol to total solvents) of 0.84, which is much higher than that (0.57 at pH 5.0 or 0.61 at pH 5.5) of the wild-type strain ATCC 824. Unlike for C. acetobutylicum ATCC 824, a higher level of acetate accumulation was observed during fermentation of the M5 strain complemented with adhE1 and/or ctfAB. A plausible reason for this phenomenon is that the cellular metabolism was shifted towards acetate production to compensate reduced ATP production during the largely growth-associated butanol formation by the M5(pIMP1E1AB) strain.

Keywords: Aldehyde/alcohol dehydrogenase · Butanol · Clostridium acetobutylicum · Coenzyme A transferase · Trade-off curve analysis

1 Introduction

Biobutanol, which can be produced by the genus Clostridium, has been widely used as an important industrial chemical. Recently, it has attracted more attention as a potential alternative fuel because it has several advantageous characteristics, including high energy density, phase stability on blending with other fuels, and non-corrosiveness [1–3]. However, producing biobutanol using the genus Clostridium needs processes for the separation of by-products including ethanol and acetone [4]. This is a critical issue for the economic viability of the industrial production of biobutanol [5]. To reduce separation costs, studies on developing strains with high butanol selectivity have been conducted with both top-down (based on the wild-type strain) [6–12] and bottom-up [13–16] approaches. The latter are based on a degenerate mutant. The degeneration refers to loss of solvent production, which in C. acetobutylicum ATCC 824 is the

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result of loss of the pSOL1 megaplasmid that carries the essential genes for butanol and acetone formation.

A ctfB antisense RNA, which down-regulates subunit B of the CoA-transferase in the acetone pathway, has been used to reduce acetone formation in the wild type of *C. acetobutylicum* [6]. Acetone concentration was successfully reduced by the ctfB antisense RNA in a previous study, but the butanol concentration was also reduced [6]. However, butanol concentration from the down-regulated strain by the ctfB antisense RNA was recovered up to the level of the wild type by adhE1 gene overexpression [7]. It has been known for many years that the butanol selectivity can be altered by modifying the electron flow and availability as demonstrated by the inhibition of hydrogen formation [8–11] or by employing substrates having different degrees of oxidation [12]. Specifically, by inhibiting the hydrogenase reaction [8, 9] or by using synthetic dyes affecting redox states [10], the excess amount of electrons available forces the organism to channel most carbon flow to butanol and ethanol production to maximize the electron use. The bottom-up approach based on a degenerate mutant, *C. acetobutylicum* M5, was also used to reduce acetone production. *C. acetobutylicum* M5 does not harbor the megaplasmid pSOL1, which contains several genes responsible for acetone-butanol-ethanol (ABE) production, including adhE1, ctfAB, and adc [13, 14]. According to a previous report, *C. acetobutylicum* M5 could be complemented with the adhE1 gene for butanol production without acetone [15]. The butanol concentration could be restored to its wild-type level by employing the ptb promoter controlling the adhE1 gene expression [16].

Furthermore, the ctfAB genes encoding coenzyme A transferase (CoAT) in the sol operon (adhE1-ctfAB) catalyze the formation of acetoacetate by transferring CoA group of acetoacetyl-CoA to acetate and butyrate to form acetyl-CoA and butyryl-CoA, respectively [17–19]. In solventogenic phase, acetyl-CoA and butyryl-CoA are used as precursors for ethanol and butanol production, respectively, by aldehyde/alcohol dehydrogenase encoded by the adhE1 gene [20]. Acetoacetate is converted to acetone by the unique acetoacetate decarboxylase (AADC) encoded by the adc gene, also located on the pSOL1 plasmid divergently next to the sol operon. Although the CoAT of *C. acetobutylicum* has been cloned, purified, and characterized [21], it is not known what the impact of CoAT expression would be in the absence of the AADC enzyme, or what the fate of acetoacetate would be (which is a rather unstable metabolite naturally decomposing to acetone at high concentrations [22]).

To improve recovery performance and reduce associated costs, much effort has been put into developing separation processes that employ pervaporation, adsorption, liquid–liquid extraction, gas stripping, and reverse osmosis [1]. For the butanol recovery process, the ratio of butanol to total solvent is more important than the amount of organic acids including acetic and butyric acids for the efficient recovery of butanol. For this reason, several previous papers defined the butanol selectivity as the ratio of final butanol concentration to total solvent concentration; one can also define the butanol selectivity to a particular solvent, for example the butanol selectivity to acetone [16]. In this study, the butanol selectivity was defined as the ratio of final butanol concentration to the total solvent concentration.

We report the development of a recombinant *C. acetobutylicum* M5 strain capable of producing butanol with much reduced acetone production by adhE1 and ctfAB complementation. The effects of adhE1 and ctfAB co-expression in the M5 strain on the selectivity of butanol to total solvents were examined from batch fermentation profiles under various conditions including pH and initial glucose concentration. Finally, flux balance analysis (FBA) was conducted to further characterize the metabolism of the recombinant strain using a genome-scale metabolic model of *C. acetobutylicum* [23].

## 2 Materials and methods

### 2.1 Bacterial strains and culture conditions

The properties of the asporogenous *C. acetobutylicum* strain M5 and plasmid pCAAD (expressing the *C. acetobutylicum* adhE1 gene from its native promoter) have been reported previously [15]. Briefly, the M5 strain is a mutant strain that is not able to produce solvents due to the loss of the megaplasmid. The properties of the control plasmid pIMP1, and the methylating plasmid pAN1 (to overcome the restriction system in *C. acetobutylicum* 824) have also been reported [24]. Plasmid pAN1 contains a gene encoding the *Bacillus subtilis* phage Φ3T1 methyltransferase, which prevents the degradation of recombinant DNA by the restriction system present in *C. acetobutylicum* [24].

*E. coli* strains were grown aerobically in liquid Luria-Bertani (LB) medium at 200 rpm and 37°C and on solidified LB medium containing 1.5% agar. *C. acetobutylicum* strains were grown anaerobically in an anaerobic chamber (Forma Scientific, Marietta, OH, USA), which contained 96% nitrogen and 4% hydrogen at 37°C. *C. acetobutylicum* strains were
cultured in clostridial growth medium (CGM) containing 0.75 g/L K$_2$HPO$_4$, 0.75 g/L KH$_2$PO$_4$, 0.7 g/L MgSO$_4$·7H$_2$O, 0.017 g/L MnSO$_4$·5H$_2$O, 0.01 g/L FeSO$_4$·7H$_2$O, 2 g/L (NH$_4$)$_2$SO$_4$, 1 g/L NaCl, 2 g/L asparagine, 0.004 g/L p-aminoenzoic acid, 5 g/L yeast extract, 4.08 g/L CH$_3$COONa·3H$_2$O, and 80 g/L glucose. Plate cultures were performed on 2x YT medium (16 g/L Bacto tryptone, 10 g/L yeast extract, 4 g/L NaCl, and 5 g/L glucose) plates containing 1.5% agar. When necessary, ampicillin, chloramphenicol and erythromycin were added at concentrations of 100, 34 and 40 μg/mL, respectively. Frozen stocks were prepared as 15% glycerol stock of cells grown to an OD$_{600}$ of 0.8 to 1.0, and were stored at −80°C. All media components and antibiotics used in this study were purchased from Difco (Detroit, MD, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

### 2.2 DNA isolation, manipulation, and transformation

Plasmid DNA was isolated using a mini-prep kit (Solgent, Daejeon, Korea) or the QIAFilter plasmid maxi kit (Qiagen, Valencia, CA, USA). Total DNA including both the chromosomal DNA and the megaplasmid DNA of *C. acetobutylicum* ATCC 824 were isolated with the DNeasy blood and tissue kit (Qiagen) according to the manufacturer’s protocol. *E. coli* transformation was carried out with One Shot Top10 (Invitrogen, Carlsbad, CA, USA). Competent cells of *C. acetobutylicum* were prepared by harvesting cells grown anaerobically up to an OD$_{600}$ of 0.5 in CGM. Cell broth (60 mL) was stored on ice for 10 min before the centrifugation at 7000 × g for 10 min at 4°C (SUPRA22K, Hanil Science Industrial, Incheon, Korea). Cell pellets were washed three times with electroporation buffer (270 mM sucrose and 686 mM Na$_2$PO$_4$, pH 7.4, chilled to 0°C before use) and resuspended in 2 mL of the same buffer. Plasmid DNA (0.5–2.0 μg) prepared was added to competent cells for transformation. Electroporation was performed using Gene Pulser II (Bio-Rad, Hercules, CA, USA; 2.5 kV, 220 Ω, and 25 μF) and 4-mm electrode gap cuvettes (Bio-Rad). Immediately after the electroporation, 1 mL 2x YT medium was added to the mixture and incubated at 37°C for 4 h. Cells were then plated onto 2x YT agar plate containing 40 μg/mL erythromycin and incubated in an anaerobic chamber. All plasmids transformed into *C. acetobutylicum* were previously methylated in *E. coli* ER2275(pAN1) to protect them from the restriction system [24]. All restriction enzymes used in this study were purchased from New England Biolabs (Ipswich, MA, USA). T4 DNA ligase (Roche, Basel, Switzerland) was used for ligation and a proofreading Pfu DNA polymerase (Solgent) was used for PCR. PCR products and DNA fragments were purified with a PCR purification kit (Solgent).

### 2.3 Construction of plasmid pIMP1E1AB

The *C. acetobutylicum* adhE1 and ctfAB genes, including both the native promoter and the transcription terminator, were amplified by PCR with primers E1AB-F (5’-TACGCGTCGACGTTTCTTTTATGTATTATTCTACAAGA-3’) and E1AB-R (5’-TACGCCGTGCAGTTTCTTTTATGTATTATTCTACAAGA-GATTGTTTCTAGC-3’) using the total DNA of ATCC 824 as a template. The resulting adhE1-ctfAB fragment and pIMP1 were digested with Sall, and were ligated to construct pIMP1E1AB.

### 2.4 Fermentation

Batch fermentation of *C. acetobutylicum* was carried out in a LiFlus GX bioreactor (Biotron, Kyunggi-Do, Korea) containing 1.8 L CGM. When necessary, 40 μg/mL erythromycin and Antifoam 204 (Sigma-Aldrich) were added to the bioreactor. A single colony of *C. acetobutylicum* was inoculated into a test tube containing 10 mL CGM, grown to an OD$_{600}$ of 1.0, and was transferred to a 500-mL flask containing 200 mL CGM. When the cell concentration in the flask reached an OD$_{600}$ of 1.0, cells were inoculated into the bioreactor. The pH was controlled with ammonia solution above desired values (4.5, 5.0, 5.5 and 6.0) during the acidogenic phase, and was not controlled when it became higher than the set value during the solventogenic phase. During the fermentation, plasmid DNAs were isolated from the cell samples to ensure their presence and plasmid stability.

### 2.5 Analysis of cell growth and fermentation products

Cell growth was monitored by measuring the OD$_{600}$ using an Ultrospec 3000 spectrophotometer (Pharmac Biotech, Uppsala, Sweden). Solvent concentrations were determined by gas chromatography system (Agilent 6890N, Agilent Technologies, CA, USA) equipped with a packed column (80/120 Carpack B AW glass column, Supelco, Bellefonte, PA, USA) and a flame ionization detector (FID). Helium was used as a carrier gas at a flow rate of 17 mL/min with the septum purge flow of 3 mL/min. Conditions for analysis were: inlet heater temperature at 220°C; oven temperature of 100°C for 30 s, ramping to 135°C at 10°C/min, ramping to 170°C at 30°C/min, and holding at 170°C for 9 min; and FID at 280°C. The retention time of n-butanol was 4.8 min.
2.6 FBA of the *C. acetobutylicum* genome-scale metabolic model

The genome-scale metabolic model of *C. acetobutylicum* ATCC 824 constructed previously [23] was employed for flux balance analysis. It was modified to simulate *C. acetobutylicum* M5(pIMP1E1AB), such that reactions encoded by genes residing on the megaplasmid pSOL1 were removed from the model, and CoA transferase (CtfAB), acetaldehyde/butyraldehyde dehydrogenase (Aldh), and ethanol/butanol dehydrogenase (Adh) reactions encoded in the plasmid were added to the model. The biomass composition of M5(pIMP1E1AB) was assumed to be the same as that of ATCC 824. To investigate the effect of butanol production in *C. acetobutylicum* M5 qualitatively, the glucose uptake rate was set at 10 mmol g dry cell weight (gDCW)$^{-1}$ h$^{-1}$. Then FBA were performed with maximizing cell growth and butanol production rate of 0, 1, 2 and 3 mmol gDCW$^{-1}$ h$^{-1}$. Trade-off analyses were carried out using the MetaFluxNet [23] and GAMS-CPLEX numerical system (GAMS Development Co., Washington). Experimentally measured cell growth rate was used as a constraint during the simulation. Trade-off analyses were performed by iterative FBA simulation with an objective function of maximizing butanol production and with varying acetate production rate. Trade-off analyses were carried out using the MetaFluxNet and GAMS with CPLEX solver. Experimentally measured cell growth rate was used as a constraint during the simulation. FBA was performed using linear programming with an objective function of maximizing butanol production.

3 Results and discussion

3.1 Effects of *adhE1* and *ctfAB* co-expression in *C. acetobutylicum* M5 on the selectivity of butanol to total solvent

To improve butanol selectivity with decreased acetone production, a degenerate mutant *C. acetobutylicum* M5 lacking in solventogenic ability was employed. Genes responsible for the formation of butanol in *C. acetobutylicum* ATCC 824 were selected from its genome sequence and metabolic pathways [25].

Batch fermentation of M5 containing pIMP1 in CGM resulted in no production of any solvent except for 2.8 mM ethanol (Fig. 1A). In the previous studies, the expression of the *adhE1* gene encoding aldehyde/alcohol dehydrogenase led to butanol and ethanol production without the formation of acetone in M5 [15, 16]. Batch cultivation of the *adhE1*-complemented *C. acetobutylicum* M5(pCAAD) strain resulted in the production of 69.2 mM butanol and 8.6 mM ethanol (Fig. 1B). To improve the butanol selectivity without acetone, genes responsible for the regeneration of butyryl-CoA (the precursor to butanol synthesis) were additionally co-expressed with the *adhE1* gene. CoAT is a key en-

![Figure 1](https://www.biotechnology-journal.com/)

**Figure 1.** Fermentation profiles of (A) *C. acetobutylicum* M5(pIMP1), (B) *C. acetobutylicum* M5(pCAAD) expressing the *adhE1* gene, and (C) *C. acetobutylicum* M5(pIMP1E1AB) expressing the *adhE1-ctfAB* genes at pH controlled above 5.5. ● acetone; ○ ethanol; ▼ butanol; ▲ acetate; ■ butyrate; ♦ OD$_{600}$; ◇ pH; □ glucose.
zyme for the uptake and conversion of butyrate and acetate to their CoA forms, which are the precursors of butanol or ethanol, respectively. While doing so, CoAT also catalyzes the conversion of acetoacetyl-CoA to acetoacetate, a precursor of acetone. However, acetone formation is prevented due to the lack of the adc gene encoding AADC in the final metabolic pathway.

In this work, a recombinant C. acetobutylicum M5 strain harboring pIMP1E1AB was constructed. This strain is able to co-express the adhE1 and the ctfAB genes under the control of their native promoters. As shown in Fig. 1C, M5(pIMP1E1AB) strain was able to produce butanol much more efficiently, and a final butanol concentration of 154.1 mM was obtained (Fig. 1C). Interestingly, concomitant production of 9.9 mM acetone was observed, even though the M5(pIMP1E1AB) strain lacks the adc gene. This suggests that acetoacetate produced by CoAT is labile at physiological pH, triggering the formation of acetone from acetoacetate [26]. Nevertheless, it is notable that the final butanol concentration of 154.1 mM obtained is similar to that of ATCC 824 strain, with significantly increased butanol selectivity (butanol/total solvent molar ratio) of 0.84, which is much higher than that (0.57 at pH 5.0 or 0.61 at pH 5.5) obtained with ATCC 824 (Table 1). Thus, the co-expression of the adhE1 and ctfAB genes in a megaplasmid-less M5 strain was an effective strategy for increasing the butanol selectivity.

### 3.2 Effects of pH and initial/residual glucose concentration on butanol fermentation

The medium pH is important in the biphasic clostridial fermentation. During the acidogenic phase, the formation of organic acids, including acetate and butyrate, causes a decrease in pH. Solventogenesis starts when the pH reaches a critical point, beyond which organic acids are re-assimilated and solvents are produced. Thus, low pH is a prerequisite for butanol production [1, 3].

To determine the effects of pH on the metabolism of C. acetobutylicum M5(pIMP1E1AB) strain, batch fermentations were performed at four controlled pH values, 4.5, 5.0, 5.5, and 6.0. The results shown in Table 2 clearly suggest that both cell growth and metabolites production were strongly pH dependent. Changes in pH values exerted strongest influences on the production of butanol and organic acids. Concentrations of butanol and organic acids were highest at pH 5.5 and 6.0, respectively (Table 2). The butanol selectivity was also increased with higher pH values up to 5.5, and there was a highly significant linear correlation between them (r=0.998). However, a relatively higher concentration of butanol (138.6 mM) was obtained with high concentration of acetate (250.6 mM) and butyrate (102.4 mM) when M5(pIMP1E1AB) strain was cultured at pH 6.0. The butanol selectivity of 0.76 was observed (Table 2). These results obtained with M5 were compared with those obtained with ATCC 824, a parent strain of M5 (Table 1). In general, an acidic pH (below 5.0) seems to be optimal for butanol and acetone production using C. acetobutylicum ATCC 824 [4, 6, 7]. Under this condition, the final butanol titer of 147.2 mM and the butanol selectivity of 0.57 were obtained. At the pH controlled above 5.5, the final butanol titer of 110.6 mM and the butanol selectivity of 0.61 were obtained (Table 1). It is also well known that only organic acids are generally produced at higher pH, for instance at 6.0 in ATCC 824 strain [4].

Most butanol fermentations have been performed with a sufficient amount of glucose at the beginning or during mid to late exponential growth phase to avoid premature termination of solvent production caused by glucose depletion. As a result of this excessive supply of glucose, residual glucose frequently remains over 100 mM, or even 200–300 mM in the fermenter [7, 16, 27, 28]. To deter-

<table>
<thead>
<tr>
<th>Strains</th>
<th>Butanol</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Butyrate</th>
<th>Acetate</th>
<th>Butanol selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 824 a)</td>
<td>147.2</td>
<td>93.4</td>
<td>17.7</td>
<td>47.1</td>
<td>98.1</td>
<td>0.57</td>
</tr>
<tr>
<td>ATCC 824 b)</td>
<td>110.6</td>
<td>50.2</td>
<td>19.5</td>
<td>13.9</td>
<td>201.2</td>
<td>0.61</td>
</tr>
<tr>
<td>M5(pIMP1)b)</td>
<td>0</td>
<td>0</td>
<td>2.8</td>
<td>248.4</td>
<td>122.9</td>
<td>0</td>
</tr>
<tr>
<td>M5(pCAAD)b)</td>
<td>69.2</td>
<td>0</td>
<td>8.6</td>
<td>84.6</td>
<td>167.9</td>
<td>0.89</td>
</tr>
<tr>
<td>M5(pIMP1E1AB)b)</td>
<td>154.1</td>
<td>9.9</td>
<td>19.6</td>
<td>54.4</td>
<td>226.8</td>
<td>0.84</td>
</tr>
</tbody>
</table>

a) Lower pH limit of 5.0.
b) Lower pH limit of 5.5.
c) Butanol selectivity is defined as the molar ratio of butanol to total solvents.
mine the effects of initial and residual glucose concentrations on butanol production by M5(pIMP1E1AB) strain, fed-batch fermentations were conducted in the medium containing three different glucose concentrations (130, 230, and 360 mM). A concentrated feeding solution containing glucose was intermittently supplied when the glucose concentration in the fermenter decreased to ~56 mM. In all cases, approximately 442 ± 12 mM glucose was consumed and 138 ± 2 mM butanol was produced (Fig. 2). These results suggest that the initial and residual glucose concentrations in the fermentation by M5(pIMP1E1AB) strain did not much affect the final butanol concentration and yield.

3.3 Characterization of C. acetobutylicum M5(pIMP1E1AB) metabolism by FBA

Even though the C. acetobutylicum M5(pIMP1E1AB) strain was able to produce butanol with high selectivity as desired, further investigation is required due to the production of high concentration of acetate (Table 1 and Fig. 1C); acetate is not usually accumulated to this high level in fermentations of wild-type C. acetobutylicum ATCC 824 [4, 6, 7, 29]. However, acetate accumulation has been observed in genetically engineered strains of C. acetobutylicum, e.g., in previous studies when the adhE1 gene was expressed under either native- or ptb-promoter in C. acetobutylicum M5 [15, 16]. In addition, the ratio of acetate to butyrate was significantly increased when adhE1 was overexpressed in C. acetobutylicum ATCC 824 even though acetate was not accumulated [20].

Although FBA might not accurately describe the fermentation performance, it is still possible to predict its overall tendency in a qualitative manner [30]. Using the previously constructed genome-scale model [23], the relationship between acetate accumulation and butanol production was examined (Fig. 3A). Acetate production rate increased significantly, and butyrate production rate decreased as butanol production rate was increased. Interestingly, the in silico cell growth rate did not show a significant difference. This simulation qualitatively explains the acetate accumulation phe-

<table>
<thead>
<tr>
<th>pH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentrations of fermentation products (mM)</th>
<th>Glucose consumption (mM)</th>
<th>Maximum cell growth (OD&lt;sub&gt;600&lt;/sub&gt;)</th>
<th>Butanol selectivity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Butanol</td>
<td>Acetone</td>
<td>Ethanol</td>
<td>Butyrate</td>
</tr>
<tr>
<td>4.5</td>
<td>27.1</td>
<td>3.8</td>
<td>20.4</td>
<td>45.7</td>
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<tr>
<td>5.0</td>
<td>76.3</td>
<td>10.2</td>
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<td>5.5</td>
<td>154.1</td>
<td>9.9</td>
<td>19.6</td>
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<tr>
<td>6.0</td>
<td>138.6</td>
<td>7.3</td>
<td>35.8</td>
<td>102.4</td>
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</table>

<sup>a</sup> Lower pH limit.

<sup>b</sup> Butanol selectivity is defined as the molar ratio of butanol to total solvents.
The effects of introducing both \( \text{adhE1} \) and \( \text{ctfAB} \) genes could not be studied in silico because there were multiple solutions when acetate and butyrate uptake reactions by CtfAB were added. This problem can be resolved by using a priori fermentation data and adding further constraints [31].

Trade-off curve analysis using FBA has also been used for physiological studies in gene knockout experiments or adaptive evolution [32–34]. The effects of production rates of acetate on the butanol production rate were analyzed using the trade-off analysis. For this, butanol production rate was maximized at varied cell growth rates to obtain the trade-off curve of production rates of acetate vs. butanol; in this study, four different growth rates (0.08, 0.16, 0.32, and 0.48/h) were used (Fig. 3B). The glucose uptake rate was taken from the exponential phase of the fermentation data, which was found to be 18.3 mmol gDCW\(^{-1}\) h\(^{-1}\). It was not feasible to maintain both growth and butanol production rates at higher acetate production rates. This phenomenon is partly due to the NAD\(^+\)/NADH balance. NADH produced through glycolysis can be consumed via butyrate production or alcohol production. Our model uses the irreversible ferredoxin:NAD reductase reaction, which donates electrons of reduced ferredoxin to NAD\(^+\) [23], and NADH cannot be consumed through hydrogen production. As shown in Fig. 3B, the trade-off curve revealed that the butanol production rate decreased at the cell growth rate of 0.48/h and lower acetate production rate due to the competition between butyrate and butanol production. As acetate production rate decreased, butyrate production rate must be increased to compensate for the lower ATP production required for cell growth and maintenance. This result is consistent with the fermentation results of an engineered M5 strain, in which the \( \text{adhE1} \) gene was expressed and the acetate kinase gene was deleted [16].

Although there are many transcriptional regulators, sigma factors and various transporters encoded in the megaplasmid pSOL1, the altered metabolic phenomena observed might not be solely due to the absence of the megaplasmid in \( C. \) acetobutylicum M5. It was reported that not much acetate was produced in \( C. \) acetobutylicum M5 strain when the acetate kinase gene was knocked out. Interestingly, however, a significant amount of acetate was produced in acetate kinase-deleted \( C. \) acetobutylicum M5 strain when the \( \text{adhE1} \) gene was overexpressed [16]. Thus, there seems to be an alternative acetate-producing pathway operational in the butanol-producing acetate kinase mutant \( C. \) acetobutylicum M5. Also, CtfAB, which is overexpressed in this study, might affect the fluxes through the reductive TCA cycle [25] and might facilitate biosynthesis of several amino acids. This might in turn affect a better growth rate, cofactor utilization and regeneration affecting the fermentation profiles.

Figure 3. Flux balance analysis. (A) Flux distributions in \( C. \) acetobutylicum M5 at the butanol production rate of 0, 1, 2, and 3 mmol gDCW\(^{-1}\) h\(^{-1}\) (from top to bottom). Glucose uptake was set at 10 mmol gDCW\(^{-1}\) h\(^{-1}\). FBA were performed with maximizing the cell growth rate. Relative fluxes and cell growth rates were shown in black boxes. All fluxes except cell growth rate were normalized with respect to the glucose uptake rate of 100. Cell growth rates in each case were normalized with respect to that with no butanol production. (B) Trade-off curve of acetate production rate vs. butanol production rate obtained by the genome-scale metabolic simulation of \( C. \) acetobutylicum M5(pIMP1E1AB) strain. Results obtained at the growth rates of 0.08/h (solid line), 0.16/h (dot dot dash), 0.32 h (dashed line), and 0.48 h (dotted line) are shown. The glucose uptake rate was set to be 18.29 mmol gDCW\(^{-1}\) h\(^{-1}\) based on the fermentation result.
4 Concluding remarks

In this work, a recombinant C. acetobutylicum M5(pIMP1E1AB) strain was constructed by the complementation of the adhE1 and ctfAB genes in a megaplasmid-less C. acetobutylicum M5 strain. This resulted in the increase of both butanol concentration and butanol selectivity. The concentrations of butanol and acetone obtained by batch culture of M5(pIMP1E1AB) were 154.1 mM and 9.9 mM, respectively, while those obtained with C. acetobutylicum ATCC 824 were 147.2 mM and 93.4 mM, respectively. The butanol selectivities of ATCC 824 and M5(pIMP1E1AB) obtained were 0.57 and 0.84, respectively. In contrast to the wild-type ATCC 824 strain, C. acetobutylicum M5(pCAAD) and M5(pIMP1E1AB) strains produced acetate to a high concentration. This was found to be due to the cell’s effort to compensate ATP production as found from experimental correlations as well as FBA of the genome-scale metabolic model. Genome-wide studies are underway to elucidate an alternative acetate synthesis pathway, or a regulatory mechanism that causes acetate accumulation, and thereby reduce acetate formation, while developing a new way of increasing ATP formation by systems metabolic engineering [35].

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5 References


