Modulation of the Acetone/Butanol Ratio during Fermentation of Corn Stover-Derived Hydrolysate by Clostridium beijerinckii Strain NCIMB 8052

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ABSTRACT Producing biobutanol from lignocellulosic biomass has shown promise to ultimately reduce greenhouse gases and alleviate the global energy crisis. However, because of the recalcitrance of a lignocellulosic biomass, a pretreatment of the substrate is needed which in many cases releases soluble lignin compounds (SLCs), which inhibit growth of butanol-producing clostridia. In this study, we found that SLCs changed the acetone/butanol ratio (A/B ratio) during butanol fermentation. The typical A/B molar ratio during Clostridium beijerinckii NCIMB 8052 batch fermentation with glucose as the carbon source is about 0.5. In the present study, the A/B molar ratio during batch fermentation with a lignocellulosic hydrolysate as the carbon source was 0.95 at the end of fermentation. Structural and redox potential changes of the SLCs were characterized before and after fermentation by using gas chromatography/mass spectrometry and electrochemical analyses, which indicated that some exogenous SLCs were involved in distributing electron flow to C. beijerinckii, leading to modulation of the redox balance. This was further demonstrated by the NADH/NAD+ ratio and trxB gene expression profile assays at the onset of solventogenic growth. As a result, the A/B ratio of end products changed significantly during C. beijerinckii fermentation using corn stover-derived hydrolysate as the carbon source compared to glucose as the carbon source. These results revealed that SLCs not only inhibited cell growth but also modulated the A/B ratio during C. beijerinckii butanol fermentation.

IMPORTANCE Bioconversion of lignocellulosic feedstocks to butanol involves pretreatment, during which hundreds of soluble lignin compounds (SLCs) form. Most of these SLCs inhibit growth of solvent-producing clostridia. However, the mechanism by which these compounds modulate electron flow in clostridia remains elusive. In this study, the results revealed that SLCs changed redox balance by producing oxidative stress and modulating electron flow as electron donors. Production of H2 and acetone was stimulated, while butanol production remained unchanged, which led to a high A/B ratio during C. beijerinckii fermentation using corn stover-derived hydrolysate as the carbon source. These observations provide insight into utilizing C. beijerinckii to produce butanol from a lignocellulosic biomass.

KEYWORDS corn stover, soluble lignin compounds, NADH/NAD+ ratio, A/B ratio, Clostridium

Acetone and butanol are both important feedstocks with many applications in the chemical industry. Solvent-producing strains of Clostridium spp. have been used to ferment acetone-butanol (AB) at a large industrial scale since the last century (1–4). This
traditional fermentation industry declined on the heels of rapid growth of the petrochemical industry. At present, acetone and butanol are mainly manufactured using petroleum feedstocks. There has been a resurgence of interest in microbial-based generation of butanol from lignocellulosic biomass, considering the impact of fossil oil use on climate change and environment (5).

Lignocellulosic biomass is primarily composed of cellulose, hemicellulose, and lignin, which account for >90% of its dry weight (6). Cellulose and hemicellulose are degraded by cellulase and hemicellulase, respectively, to hexose and pentose, which are subsequently converted to biofuels or other value-added chemicals by microbial fermentation. However, lignin is a noncarbohydrate aromatic heteropolymer that is difficult for microbes to degrade (7). Therefore, the lignin must be separated from cellulose through appropriate pretreatment methods, such as steam explosion, to increase the efficiency of enzymatic hydrolysis (8, 9). During this process, inhibitors such as furfural, hydroxymethylfurfural (HMF), soluble lignin compounds (SLCs), weak acids, and other organic acids are generated and are toxic to subsequent microbial growth and fermentation. Therefore, detoxifying of the hydrolysates prior to biological fermentation is essential (10–14).

Clostridium beijerinckii and Clostridium acetobutylicum are two well-known solvent-producing Clostridium species, and butanol, acetone, and ethanol are the main end products of the fermentation. The molar ratio of acetone to butanol in the end products is 0.5 after a typical C. beijerinckii batch fermentation with glucose as the carbon source. The biosynthetic pathway for butanol from acetyl-coenzyme A (acetyl-CoA) has been described in detail and published in some reviews (3, 15–17). This pathway contains a series of reduction reactions in which NADH plays an important role as a reduced coenzyme. In contrast, forming acetone from acetyl-CoA is a nonreducing, energy-consuming pathway. As a result, the acetone/butanol (A/B) ratio changes once extraneous elements interfere with the redox balance. C. acetobutylicum produces alcohol as the main product in the presence of artificial electron carriers, such as neutral red and methyl viologen (18, 19). The same phenomenon occurs when a culture is sparged with carbon monoxide (CO) or when glycerol is substituted for glucose in continuous culture (20–23). All of these techniques have been used to reduce the formation of molecular hydrogen, which affects the electron flow distributions in C. acetobutylicum and increases the availability of reducing equivalents (17). Therefore, the A/B ratio decreases in the end products compared with that of a typical batch fermentation. Interestingly, the A/B ratio can increase in the C. beijerinckii fermentation using a lignocellulosic biomass. C. beijerinckii P260 produces more solvents (acetone and butanol) using wheat straw, corn stover, and barley straw hydrolysates as the carbon source than when glucose is used as the carbon source. In particular, the acetone and butanol concentrations in the end products were both 12.5 g/liter when wheat straw hydrolysate was used as a carbon source in batch culture (24). C. beijerinckii NCIMB 8052 also produces comparable titer of acetone and butanol (both 7.5 g/liter) in batch culture using corn cob residue hydrolysate as the carbon source (25). A similar fermentation profile has been reported for C. beijerinckii BA101 fermentation using a corn fiber hydrolysate (12). It is unclear why the A/B ratio increases during butanol fermentation of a lignocellulosic hydrolysate.

In this study, we analyzed the A/B ratio of C. beijerinckii NCIMB 8052 by using corn stover hydrolysate as the substrate. The physiological responses of C. beijerinckii to inhibitors in the hydrolysate were also investigated. These results will provide insight into the studies of lignocellulosic biomass as a substrate for butanol and acetone fermentation by C. beijerinckii.

RESULTS

Compositional analysis of the steam-exploded corn stover (SECS) hydrolysate.

After steam explosion and enzymatic hydrolysis of the corn stover, the main sugars in the hydrolysate were glucose and xylose (see Table S2 in the supplemental material), and 3.3 g/liter cellobiose was detected, suggesting that β-glucosidase activity was
insufficient in this enzymatic system. Approximately 97.5 g/liter total reducing sugar content was detected in the hydrolysate, and the yield through the steam explosion and enzymatic process was 0.49 (grams of sugar/gram of corn stove biomass). Furfural, HMF, and SLCs also appeared in the hydrolysate with the soluble sugars, which were generated during pretreatment and derived from degradation of xylose, glucose, and lignin, respectively. SLC is a mixture of tens or hundreds of compounds which have not been characterized; however, they may have the following characteristics: (i) they were derived from degrading lignin in the pretreatment; (ii) they were soluble in the hydrolysate with sugars; and (iii) they potentially presented toxic effects on microbial fermentation (26). In this study, a modified overliming process was used to detoxify the lignocellulose-derived hydrolysates during sequential microbial fermentation. Overliming resulted in a 35% reduction in furfural, a 27% reduction in HMF, and a 0.04% decline in total reducing sugars in detoxified hydrolysate (DTH), compared to those in the nondetoxified hydrolysate (nDTH) (Table S2). In contrast, the concentration of SLC increased from 3.7 g/liter to 4.1 g/liter.

**Acetone and butanol fermentation of the SECS hydrolysate by *C. beijerinckii***.

Batch fermentation was carried out in P2 medium with 75 g/liter total reducing sugars (diluted with distilled water) to evaluate cell growth and the solvent production profiles of *C. beijerinckii* grown in nDTH, DTH, and glucose (as a control). As a result, *C. beijerinckii* produced 39.6 mM acetone and 49.9 mM butanol at the end of the fermentation when nDTH was used as the carbon source (see Table 2). In contrast, *C. beijerinckii* grew well in P2 medium with DTH and glucose as the carbon source (Fig. 1). Approximately 141 mM butanol was produced under both conditions, suggesting that overliming as a detoxification method mitigated the detrimental effects of the inhibitors in nDTH and facilitated growth and production of butanol by *C. beijerinckii*. Furthermore, *C. beijerinckii* consumed more carbon (302.2 mM) and produced more acetone (134 mM) in growth on DTH than in growth on glucose. Thus, the A/B ratio in the end products increased compared to that of the control (Fig. 2). H₂, CO₂, acetate, and butyrate were also identified in the end products under both conditions (Table 1). These results indicate that *C. beijerinckii* consumed more acetate and produced more H₂, CO₂, and butyrate in the end products in growth on DTH than in growth on glucose. Notably, the gas volume was determined independently without degassing to test the total volume in the entire fermentation run. Fermentation was partially inhibited due to the higher gas partial pressures of H₂ and CO₂. Moreover, considering that CO₂ could be resolved in the fermentation broth, the practical volume of gas may have been lower than the theoretical value.

**Carbon and redox balance stoichiometry.** To investigate the change in the A/B ratio during fermentation of *C. beijerinckii* grown on DTH and glucose, carbon flow
through the metabolic pathways was analyzed (Table 1) (17). *C. beijerinckii* grown on DTH as the carbon source consumed more acetate (15.2 mM) and produced more acetone and butyrate (62.6 mM and 20.4 mM, respectively) than when glucose was used as the substrate. However, less ethanol (8.9 mM) was produced when DTH was the

**TABLE 1** Stoichiometry of the carbon and redox balance during *Clostridium beijerinckii* batch fermentation

<table>
<thead>
<tr>
<th>Compound or process</th>
<th>Glucose uptake</th>
<th>Butanol</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Acetate consumption</th>
<th>Butyrate</th>
<th>H₂</th>
<th>CO₂</th>
<th>Glucose</th>
<th>Glucose uptake</th>
<th>Butanol</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Acetate consumption</th>
<th>Butyrate</th>
<th>H₂</th>
<th>CO₂</th>
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<tbody>
<tr>
<td>Glucose uptake</td>
<td>230.5 ± 9.3</td>
<td>140.7 ± 4.7</td>
<td>13.3 ± 2.3</td>
<td>71.7 ± 1.0</td>
<td>24.1 ± 0.8</td>
<td>41.1 ± 1.6</td>
<td>282.7 ± 9.8</td>
<td>402.1 ± 22.3</td>
<td>Glucose</td>
<td>230.5 ± 9.3</td>
<td>140.7 ± 4.7</td>
<td>13.3 ± 2.3</td>
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<td>41.1 ± 1.6</td>
<td>282.7 ± 9.8</td>
<td>402.1 ± 22.3</td>
</tr>
<tr>
<td>DTH</td>
<td>296.7 ± 7.7</td>
<td>140.7 ± 7.8</td>
<td>8.9 ± 0.4</td>
<td>134.3 ± 9.9</td>
<td>39.3 ± 3.8</td>
<td>61.5 ± 2.3</td>
<td>581.7 ± 13.5</td>
<td>590.5 ± 12.2</td>
<td>DTH</td>
<td>296.7 ± 7.7</td>
<td>140.7 ± 7.8</td>
<td>8.9 ± 0.4</td>
<td>134.3 ± 9.9</td>
<td>39.3 ± 3.8</td>
<td>61.5 ± 2.3</td>
<td>581.7 ± 13.5</td>
<td>590.5 ± 12.2</td>
</tr>
<tr>
<td>Observed difference</td>
<td>66.2 ± 17</td>
<td>0</td>
<td>−4.4 ± 2.7</td>
<td>62.6 ± 10.9</td>
<td>15.2 ± 4.6</td>
<td>20.4 ± 3.9</td>
<td>299 ± 23.3</td>
<td>188.4 ± 34.5</td>
<td>Observed difference</td>
<td>66.2 ± 17</td>
<td>0</td>
<td>−4.4 ± 2.7</td>
<td>62.6 ± 10.9</td>
<td>15.2 ± 4.6</td>
<td>20.4 ± 3.9</td>
<td>299 ± 23.3</td>
<td>188.4 ± 34.5</td>
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<tr>
<td>Predicted difference</td>
<td>72.2</td>
<td>209</td>
<td>120.4</td>
<td>260.4</td>
<td>260.4</td>
<td>260.4</td>
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<td>260.4</td>
<td>Predicted difference</td>
<td>72.2</td>
<td>209</td>
<td>120.4</td>
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<td>260.4</td>
<td>260.4</td>
<td>260.4</td>
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*Reaction 1, 1 glucose = 1 butanol + 2 CO₂; reaction 2, 1 glucose = 1 acetone + 3 CO₂ + 4 H₂; reaction 3, 1 glucose = 2 ethanol + 2 CO₂; reaction 4, 1 glucose = 2 acetate + 2 CO₂ + 4 H₂; reaction 5, 1 glucose = 1 butyrate + 2 CO₂ + 2 H₂.*

*Data represent the values obtained when the glucose values were subtracted from those for the detoxified hydrolysate (DTH).*

*Data represent the values obtained according to the reactions; 62.6 mM acetone, 20.4 mM butyrate, −4.4 mM acetate, and −15.2 mM ethanol should consume 72.2 mM glucose and produce 260.4 mM H₂ and 209 mM CO₂.*

**FIG 2** Solvent production (top panel) and acid production (bottom panel) by *Clostridium beijerinckii* using the detoxified hydrolysate (DTH) and glucose as carbon sources. Solid symbols represent P2 medium supplemented with glucose as the carbon source; empty symbols represent P2 medium supplemented with DTH as the carbon source.
Batch fermentation was performed using P2 medium with 75 g/liter glucose as the carbon source than when glucose was the carbon source. Theoretical values for glucose consumption and gas formation were calculated according to the ethanol, acetone, and butyrate concentrations and the biochemical reactions (Table 1). The theoretical value was comparable to the practical value, suggesting there were no new products such as lactate or isopropanol (2, 3) generated during the fermentation. The increase in the A/B ratio seen when the bacteria were grown on DTH was probably due to the participation of some unknown compounds in the DTH.

**Response of** *C. beijerinckii* **to different additives.** Cellobiose, xylose, furfural, HMF, calcium ions, and SLCs were the main components added to DTH seen in comparing the compositions of normal glucose P2 medium and SECS hydrolysate P2 medium (Table S2). Batch fermentation was performed using P2 medium with 75 g/liter glucose as the carbon source and supplemented with selected compounds in the DTH to investigate which components affected AB production of *C. beijerinckii* (Table 2). *C. beijerinckii* challenged with 0.5 g/liter furfural or 0.5 g/liter HMF or a mixture of 0.5 g/liter furfural and 0.5 g/liter HMF did not show a decrease in glucose consumption or AB productivity. A sugar mixture of 60 g/liter glucose, 12 g/liter xylose, and 3 g/liter cellobiose was substituted for glucose in the P2 medium and was fermented by *C. beijerinckii* in batch culture. The sugar consumption and AB production results showed that the sugar mixture did not change the A/B ratio. *C. beijerinckii* produced more butanol and acetone and consumed more glucose when CaCO₃ was added to the P2 medium than when it was not added. The molar A/B ratios were 0.95 in the DTH P2 medium, 0.51 in the glucose P2 medium, and 0.58 in the glucose P2 medium with added CaCO₃, (Table 2). Based on these findings, furfural, HMF, mixed sugars, and calcium ions were unable to change the A/B ratio during *C. beijerinckii* fermentation.

SLC is a complex mixture formed during lignin degradation, but it is impossible to extract it from the hydrolysate to directly test its effects on the A/B ratio of the C. beijerinckii fermentation. Humic acid is a conglomeration of organic compounds formed when lignin is degraded. SLC and humic acid have similar original sources and chemical characteristics (27). Therefore, 1 mM humic acid was added to the P2 medium to study its effects on the A/B ratio. Only 108.3 mM glucose was consumed in the presence of humic acid, indicating that cell growth was inhibited. CaCO₃ was used to remove the toxic effect of humic acid on *C. beijerinckii* batch fermentation. The results showed that addition of humic acid increased the A/B ratio in the *C. beijerinckii* batch fermentation (Table 2).
Redox potential of the SECS hydrolysate and humic acid in P2 medium. Humic substances and high-molecular-weight organic materials are involved in anaerobic oxidation of organic compounds, and the proton is an electron acceptor in some microorganisms (28). Biologically reduced humic acid can provide reducing equivalents to generate hydrogen during *C. beijerinckii* glucose fermentation (29). SLCs in the DTH altered the A/B ratio of *C. beijerinckii* fermentation, indicating that humic acid and SLCs might act as electron carriers to influence fermentative physiology by the cycle of the reduced-oxidized states. To test this hypothesis, we measured the redox potential of P2 medium containing humic acid or the SECS hydrolysate. Moreover, the redox potential of the growth medium supernatant after 96 h with SECS hydrolysate as the carbon source was tested (Fig. 3). The $E_0'$ values for humic acid and SLC were $-298 \text{ mV}$ and $-399 \text{ mV}$ in P2 medium. Interestingly, the $E_0'$ value for growth medium supernatant was $-359 \text{ mV}$, which was different from the SLC value determined using P2 medium.

Profiles of pretreatment-derived compounds in the DTH hydrolysate. The redox potential in the fermentation with DTH as the carbon source changed during growth from $-399 \text{ mV}$ (0 h) to $-359 \text{ mV}$ (96 h), demonstrating that the electron carriers in DTH were transformed or modified during *C. beijerinckii* fermentation. In order to find the electron carriers, a gas chromatography/mass spectrometry (GC/MS) system was used to analyze the compounds in the DTH P2 medium prior to and after fermentation (see Fig. S1 in the supplemental material), and only those compounds whose structure changed after fermentation are listed in Table S2. The results show that 13 compounds occurred only in the hydrolysate prior to fermentation and that most were oxygen-containing heterocycle compounds. Among them, 2,3-dihydro-3,5-dehydroxy-6-methyl-4H-pyran-4-one, whose peak retention time was 15 min, possessed a carbonyl group in the heterocyclic ring (Fig. S1). This structure is similar to that of quinine.
moieties, which have been reported to be electron acceptors during reduction of humic acid (30). This result indicated that this compound possibly had redox potential and altered redox balance in C. beijerinckii fermentation as an electron carrier. In addition, 7 compounds which were present in the broth only after fermentation were detected (Table S3).

Determining the NADH/NAD$^+$ ratio in C. beijerinckii. Exogenous electron carriers can alter metabolism, the redox balance, and electron flow in clostridia (18, 19, 31). In the present study, the A/B molar ratio in the end products was approximately 0.5 in a C. beijerinckii batch fermentation with glucose as the carbon source and was 0.95 with DTH as the carbon source (Table 2). Considering that the butanol formation pathway involves a series of NADH-dependent reduction reactions, the differences in the A/B ratio should be related to NADH/NAD$^+$ ratio at the onset of solventogenesis. To investigate the differences in NADH/NAD$^+$ ratios in fermentations with glucose and DTH as the carbon source, samples were collected from batch cultures at the late exponential phase (24 h) (Fig. 1), and the NADH/NAD$^+$ ratios were determined (Table 3). The NADH/NAD$^+$ ratio in DTH P2 medium was 0.09, which was significantly lower than that of the control (0.23; Table 3). These results further indicated that SLCs in DTH decreased the NADH/NAD$^+$ ratio.

Gene expression analysis for C. beijerinckii batch fermentation. The expression profiles of seven genes were investigated in the acetone-inducing and butanol-inducing phenotypes of C. beijerinckii by quantitative PCR (qPCR). These genes encoded acetoacetate decarboxylase (adc, Cbei_3835), alcohol dehydrogenase (ald, Cbei_3832), thiolase (thl, Cbei_3630), butyryl-CoA dehydrogenase (bcd, Cbei_0325), nicotinic acid mononucleotide adenylyltransferase (nadD, Cbei_0513), thioredoxin reductase (trxB, Cbei_2681), and Ni/Fe hydrogenase (hyd, Cbei_3013). The adc, ald, thl, and bcd genes are located in the central butanol and acetone synthetic pathway (2, 32). The nadD gene is located in the NAD(H) biosynthesis pathway (33), and the hyd gene is involved in hydrogen formation in C. beijerinckii (34). Expression levels of adc, ald, and thl increased 2-, 1.9-, and 1.1-fold in DTH P2 medium compared to the levels of the control, and bcd, hyd, and nadD expression levels decreased slightly (Fig. 4). The mRNA fold change values of these six genes were all <-3. This result shows that the expression profiles of the genes involved in the biosynthesis of butanol, acetone, NAD(H), and hydrogen remained unchanged. In contrast, expression of the trxB gene was significantly (6.7-fold) upregulated in DTH P2 medium. TrxB is part of the Trx-dependent reduction system, which occurs widely in microbes, where it maintains a reduced environment in the cytosol (35). Upregulation of the trxB gene suggests that some compounds in DTH caused oxidative stress and that reactive oxygen species (ROS) accumulated, which were harmful to C. beijerinckii.

DISCUSSION

Butanol-producing clostridia are unable to directly utilize a lignocellulosic biomass; thus, it must be digested to soluble sugars by a cellulase cocktail prior to fermentation. Chemical or physical pretreatment is essential to enhance the susceptibility of cellulose to enzymatic hydrolysis and to ferment a lignocellulosic biomass to butanol. A side effect of pretreatment is formation of tens or hundreds of inhibitors of microbial fermentation. These inhibitors exhibit synergistic toxic effects on C. beijerinckii (36, 37).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (h)</th>
<th>NADH or NAD$^+$ (mM) ± SD</th>
<th>NADH/NAD$^+$ ratio$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>24</td>
<td>0.32 ± 0.08</td>
<td>1.38 ± 0.03</td>
</tr>
<tr>
<td>DTH$^b$</td>
<td>24</td>
<td>0.17 ± 0.05</td>
<td>1.95 ± 0.02</td>
</tr>
</tbody>
</table>

$^a$Data represent NADH concentration values divided by NAD$^+$ concentration values.

$^b$DTH, detoxified hydrolysate of the steam-exploded corn stover.

TABLE 3 NADH/NAD$^+$ ratios at the late exponential phase under various conditions during Clostridium beijerinckii fermentation
In the present study, *C. beijerinckii* grew poorly in toxic hydrolysates and consumed less glucose than the strains in the control group (Table 2). Overliming is a cost-effective technology that removes the inhibitory effects of lignocellulose-derived compounds on cell growth and is extensively used for *C. beijerinckii* fermentation when using a lignocellulosic biomass as the carbon source (12, 13, 24, 25, 38). The results of the present study revealed that the inhibitory effects on cell growth were removed completely by overliming. Calcium ions were brought into the medium as well, as they can stimulate solvent fermentation and alleviate the toxic effects of lignocellulose-derived inhibitory compounds during *C. beijerinckii* fermentation (39, 40).

SLCs can decrease NADH/NAD\(^+\) ratios in *C. beijerinckii* fermentation (Table 3). The redox balance during fermentation of clostridia is maintained through various central metabolic reactions. Reducing equivalents (NADH) are generated by the glycolytic pathway and reoxidized (NAD\(^+\) generation) through alcohol and H\(_2\) production, as well as other NADH-consuming reactions (18, 22, 41). Therefore, considering that the butanol synthetic pathway is involved in a series of NADH-dependent reduction reactions, a lower NADH/NAD\(^+\) ratio "pushed" the carbon flow toward acetone, rather than butanol, leading to the high A/B ratio in the end products (Table 1). The issue is that of how SLCs consume NADH in *C. beijerinckii* fermentation. It has been reported that lignocellulose-derived inhibitors can generate oxidative stress, including in *C. beijerinckii*, such as was seen after adding furfural to the medium, leading to increased expression of genes related to perturbations in redox balance (37, 42, 43). Moreover, furan aldehydes inhibit yeast fermentation by causing oxidative cellular damage (44). Lignocellulose hydrolysates contain many heterocyclic aromatic compounds, such as furfural and HMF, and some of these participated in metabolic reactions, according to the GC/MS results (see Table S3 in the supplemental material). These compounds could cause reactive oxygen species (ROS), which are known to damage DNA, lipids, and proteins, to accumulate (35). The Trx-dependent reduction system reduces ROS and consumes NAD(P)H during the reaction (42). Although it was unclear which types of ROS were produced by lignocellulose-derived inhibitors, the Trx-dependent reduction system was upregulated in response to the challenge raised by the compounds in the hydrolysate in the present work (Fig. 4), which explains why the NADH/NAD\(^+\) ratio was lower in the fermentation with DTH as the carbon source than in the control (Table 3).

Some lignocellulose-derived compounds in the DTH could function as electron donors with redox potential in *C. beijerinckii* (Fig. 3). On the basis of our knowledge, our work represents the first time that the redox potential of corn stover-derived hydrolysate for acetone-butanol-ethanol (ABE) fermentation was tested in *C. beijerinckii*. In the

![FIG 4 Comparison of genes expressed by Clostridium beijerinckii at the late exponential phase and grown in the detoxified hydrolysate (DTH) and glucose P2 media.](image-url)
previous studies in ABE fermentation, methyl viologen and neutral red, the artificial electron carriers, were able to substitute for ferredoxin, the native electron donor for hydrogenase in *C. acetobutylicum* (18, 19). Thus, hydrogen formation is suppressed and butanol formation is stimulated when neutral red is added to the medium. However, in the present study, humic acid and SLC in the hydrolysate exhibited opposite effects on hydrogen and butanol formation in *C. beijerinckii* (Table 1). One explanation is that the redox potentials of humic acid and SLC were more positive than that of ferredoxin (−450 mV). However, the redox potential of neutral red was more negative than that of ferredoxin (Fig. 3). Therefore, humic acid and SLC could not replace ferredoxin to suppress the formation of hydrogen. It has been reported that adding reduced humic substances increases hydrogen production during *C. beijerinckii* fermentation (29). Here, hydrogen formation was also stimulated by the SLCs. These results revealed that extracellular electron transfer molecules influenced the fermentative metabolism of butanol-producing clostridia on different scales by stimulating or interfering with hydrogen formation. On the other hand, *C. acetobutylicum* and *C. beijerinckii* have similar butanol synthetic pathways and regulatory mechanisms (2, 3, 17). However, *C. acetobutylicum* grew poorly and produced less solvent than *C. beijerinckii* using DTH as the carbon source (data not shown). Thus, *C. beijerinckii* may have evolved differently in response to the lignocellulose-derived inhibitors. Interestingly, a trimeric hydrogenase has been reported in *Thermatoga maritima*, which utilizes ferredoxin and NADH simultaneously during hydrogen formation via electron bifurcation (34). *C. beijerinckii* is the only solventogenic strain that potentially encodes a homologous bifurcation hydrogenase (17), indicating that the mechanisms of hydrogen formation in *C. acetobutylicum* and *C. beijerinckii* might be different.

In summary, a corn stover hydrolysate contained fermentable sugars, furfural, HMF, and SLC after steam explosion pretreatment and enzymatic hydrolysis. The SLC not only inhibited cell growth and sugar consumption but also changed the redox balance and electron distribution in *C. beijerinckii*, which led to a high A/B ratio in the end products. Although overliming removed the growth inhibitory effect of the corn stover hydrolysate and stimulated *C. beijerinckii* to consume more sugar, it was unable to eliminate the effects of SLC on redox balance in *C. beijerinckii*. Through analysis of redox balance and *trxB* gene expression levels, we concluded that SLC was oxidized to provide more electrons for H₂ production. More sugars were consumed to produce acetone, and more reducing equivalent NAD(P)H⁺ was utilized for removing ROS generated by SLC. This study provided evidence of and insight into understanding how the SLC modulates butanol fermentation in *C. beijerinckii*.

**MATERIALS AND METHODS**

**Chemicals and microorganisms.** All analytical grade chemicals were purchased from Sangon Co., Ltd. (Shanghai, China). Chipped corn stover was obtained from Jilin, China, and soaked in diluted sulfuric acid (2% [vol/vol]) before the steam explosion pretreatment, which was carried out in a screw extrusion device with continuous steam explosion at 0.7 Mpa and 150°C for 15 min. Approximately 75 g steam-exploded corn stover (SECS), with moisture content of 60%, was soaked in 150 ml sodium citrate buffer (50 mM) to obtain 20% (wt/vol) solid loading in a 500-ml flask and was adjusted to pH 5 with 5 M NaOH. The flasks were autoclaved at 121°C for 20 min. After cooling to room temperature, the flasks were heat shocked at 75°C for 10 min. The P2 medium was assembled with the appropriate concentration of Ca(OH)₂ to adjust the pH to 7 with 5 M NaOH. The mixture was incubated in a shaker at 110 rpm and 50°C for 72 h, after which the hydrolysates were centrifuged. The supernatant was named the detoxified hydrolysate (DTH).

A modified overliming procedure was used to remove the inhibitors (12, 45, 46). Briefly, the pH of SECS enzymatic hydrolysate was adjusted to 10 with Ca(OH)₂. The mixture was incubated in a shaker at 110 rpm and 50°C for 1 h followed by adjustment of the pH to 7 with 6 M H₂SO₄. Then, the mixture was centrifuged, and the supernatant was named the detoxified hydrolysate (DTH).

*C. beijerinckii* NCIMB 8052 (ATCC 51743) was purchased from the American Type Culture Collection (Manassas, VA, USA). *C. beijerinckii* stocks were routinely maintained as spore suspensions in screw-cap bottles at 4°C. Before inoculation, the spore suspension was transferred to anoxic P2 medium and was heat shocked at 75°C for 10 min. The P2 medium was assembled with the appropriate concentration of the carbon source, 1 g/liter yeast extract, and three kinds of P2 stock solutions as described previously (12, 47, 48).
**C. beijerinckii** batch fermentations under various conditions. Batch fermentation was performed in a 250-ml screw-cap bottle with a 50-ml working volume of F2 medium in triplicate. To investigate the factors modulating the A/B ratio, CaCO₃ (4 g/liter), furfural (0.5 g/liter), HMF (0.5 g/liter), and humic acid (1 mM) (calculated molecular weight, 2,000) were added into the bottles.

**Analytical methods.** The glucose, xylose, cellobiose, ethanol, acetic acid, and butyric acid concentrations were determined using an Agilent 1100 high-performance liquid chromatography (HPLC) system and an Agilent Hi-Plex H column (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector operated at 35°C. Column temperature was maintained at 65°C. Slightly acidified (5 mM H₂SO₄) water was used as the mobile phase at a flow rate of 0.6 ml/min. The HMF and furfural concentrations were determined by HPLC (Waters, Beijing, China) using a UV detector working at 284 nm and a SB-C₁₈ column (Waters), which was maintained at 35°C. The mobile phase consisted of methanol and water (1:4) at a flow rate of 1 ml/min. Total SLCs were identified by UV spectra. A Biophotometer Plus instrument (Eppendorf, Hamburg, Germany) was used to analyze the absorbance of samples at 280 nm, and a formula was used to calculate SLC concentrations based on the HMF and furfural concentrations (26, 49). Total reducing sugar levels were determined according to the 3,5-dinitrosalicylic acid method (50).

Acetone, butanol, and ethanol levels were determined using a gas chromatography (GC) system (Agilent 7890B) equipped with a flame ionization detector and a HP-INNOWax column (30-m length, 0.32-mm inner diameter). Samples were extracted with a 3-fold volume of ethyl acetate containing isomyl alcohol as the internal standard (51). A GC/MS system (Agilent 7890AGC, with a 5975 C mass selective detector) was used to analyze the compounds in the SECS hydrolysate. A column (DB-5ms; Agilent Technologies) was used to separate the compounds, and helium was used as the carrier gas. The sample was analyzed with a mass spectrometer (MS) (InertXL MSD; Agilent Technologies). The compounds were identified by comparing their mass spectra with those in the Wiley and the National Institute of Standards and Technology (NIST08) electronic libraries. Molecular hydrogen and carbon dioxide were detected using another GC system (SP-6890; Rainbow Chemical Instruments Co., Ltd., Tengzhou, China) equipped with a Porapak Q column (2-m length, 4-mm inner diameter). The gas mixture was detected with a thermal conductivity detector, and high-purity nitrogen was used as the carrier gas.

The NADH/NAD⁺ ratio was measured by using an Amplite colorimetric assay kit (AAT Bioquest, Inc., Sunnyvale, CA, USA). The cell pellets were washed with 0.1 M ice-cold phosphate-buffered saline (PBS) and then suspended in lysate buffer at room temperature for 15 min prior to the analysis. The reaction was carried out in 96-well plates in the dark at room temperature for 10 h. NADH and NAD⁺ concentrations were monitored at 460 nm using a microplate reader (BioTek, Winooski, VT, USA).

**Quantitative reverse transcription-PCR (qRT-PCR) gene expression profiles.** Triplicate samples were collected from the batch cultures at the late exponential phase of growth. The bacterial pellets were harvested after centrifugation (6,000 × g, 10 min, 4°C), snap-frozen in liquid nitrogen, and stored in a freezer at −80°C until RNA extraction. Total RNA was extracted with an RNeasy minikit (Tiangen, Beijing, China). RNA concentrations were determined with a Biophotometer Plus instrument and checked by gel electrophoresis. After DNase I treatment (TransGen Biotech, Beijing, China), cDNAs were generated using a Transcript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech) and random primers. The gene-specific primers were mixed with LightCycler 480 SYBR green I master mix (Roche Diagnostics GmbH, Mannheim, Germany), and all qPCR products were designed with lengths of 100 to 150 bp. All qPCR analyses were performed in triplicate using a LightCycler 480 real-time PCR system (Roche). The reaction conditions were 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 59°C for 10 s, and 72°C for 15 s. The quantities of transcripts from these genes were normalized with that of 16S rRNA as the internal standard (39, 42). Relative transcript levels of the studied genes were calculated using with the threshold cycle (2⁻ΔΔCT) method (52). The primers used in this study are listed in Table S1 in the supplemental material.

**Electrochemical analysis of the mediator.** Electrochemical analysis was performed in a three-electrode cell format (CH Instruments, Austin, TX, USA), consisting of a 3.0-mm-diameter glassy carbon working disk, a saturated Ag/AgCl reference, and platinum wire counter electrodes. The glassy carbon electrodes were polished successively with 1.0- and 0.05-μm-grain-size alumina (CH Instruments) and sonicated for 5 min in deionized water after each step, 3 μl of 2 mg/ml polycrystalline (PC) solution was deposited on the freshly polished electrodes, and the electrodes were allowed to air dry (27). Then, all instruments were brought into an anaerobic chamber. The electrodes were placed in an electrochemical cell filled with growth media with or without the mediator as described above and tested. All data were collected on a CH Instruments electrochemical workstation (Model 760b) and plotted using the International Union of Pure and Applied Chemistry format. The redox potential (E_r) value was transformed to the standard redox potential (E_r') value by adding 0.197.

**Accession number(s).** The GenBank accession numbers for the genome of *C. beijerinckii* NCIMB 8052 are NC_009617 and NZ_AALO01000000 to NZ_AALO01000089.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03386-16.

**SUPPLEMENTAL FILE 1,** PDF file, 0.1 MB.
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