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Combination of RNA sequencing and metabolite data to elucidate improved toxic compound tolerance and butanol fermentation of Clostridium acetobutylicum from wheat straw hydrolysate by supplying sodium sulfide

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HIGHLIGHTS

• Sodium sulfide (SS) can improve ABE fermentation from wheat straw hydrolysate.
• Supplementation with SS affected the central fermentative metabolic pathway.
• Supplementation with SS down-regulated metabolic flux toward acid formation branches.
• Supplementation with SS up-regulated metabolic flux toward ABE formation branches.
• Supplementation with SS demonstrated applied value for commercial ABE production.

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ABSTRACT

Sodium sulfide (SS) was added to the non-detoxified wheat straw hydrolysate for ABE fermentation by Clostridium acetobutylicum CICC8012. Biochemical measurements demonstrated that supplementation with SS promoted earlier and enhanced conversion of acid to ABE and led to a 27.48% improvement in sugar consumption, a 20.48% improvement in the sugar-based ABE yield, a 47.63% improvement in the butanol titer, and a 53.50% improvement in the ABE concentration. The response of C. acetobutylicum CICC8012 at the mRNA level was examined by a transcriptional analysis performed with RNA sequencing. The expression of genes involved in the membrane transport of carbohydrates, glycolysis, and ABE formation increased following SS-supplemented fermentation, whereas the expression of genes encoding enzymes involved in acid formation decreased, which indicates that supplemental SS affected the central fermentative pathway, down-regulated the metabolic flux toward the acid formation branches, and up-regulated the metabolic flux toward the ABE formation branches.

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1. Introduction

Biobutanol generated via acetone–butanol–ethanol (ABE) fermentation has been considered a potential biofuel because it has the potential to partially substitute for gasoline (Ezeji et al., 2003). However, the competitiveness of butanol as a renewable energy source is directly restricted by the cost of the sugar substrate needed for its generation. Therefore, lignocellulose, an abundantly available biomass derived from plants, such as wheat straw, corn stover, and rice straw, is considered a more economically favorable feedstock for use in butanol fermentation (Du et al., 2013).

Dilute-acid hydrolysis – an effective and probably the most commonly used pretreatment of lignocellulose – results in a highly digestible substrate that is amenable to further enzymatic hydrolysis of cellulose with a reasonably high sugar yield (Lee et al,
improved butanol production from non-detoxified corn fiber hydrolysate containing inhibitors directly. Guo et al. (Guo et al., 2013) of butanol production, recent research efforts have tended to utilize acid hydrolysate containing inhibitors directly. Guo et al. improved butanol production from non-detoxified corn fiber hydrolysate using an inhibitor-tolerant Clostridium beijerinckii mutant strain. With this strain, the final ABE concentration reached 10.6 g/L (Guo et al., 2013). Du obtained an improved butanol titer from non-detoxified corn fiber hydrolysate using a mutant strain and adding NH₄HCO₃ and CaCO₃ to the hydrolysate (Du et al., 2013). Leonard et al. enhanced ethanol titer by adding sodium sulfide to wood hydrolysate (Leonard and Hajny, 1945). In the previous study, a method in which sodium sulfide (SS) was supplemented during ABE fermentation was developed that significantly enhanced butanol production from non-detoxified wheat straw, rice straw and corn stover hydrolysate and exhibited great potential for use in the commercial fermentation process (Zhao et al., 2014). However, the mechanism underlying this method remained elusive.

The transcriptome is the comprehensive set of transcribed regions throughout the genome, and the information contained within the transcriptome is critical for revealing significantly differential gene expression. Transcriptome analysis by next-generation sequencing (RNA-seq) shows distinct advantages over previous methodologies and allows the investigation of the transcriptome at unsurpassed resolution. There are already several transcriptome analyses of solventogenic clostridia (Janssen et al., 2012; Wang et al., 2011). However, there is no published research on the transcriptome of the central metabolic pathway in ABE fermentation when clostridia have been grown in medium containing inhibitors. Moreover, previous studies have investigated the transcriptional state of solventogenic clostridia in synthetic medium, but clostridia grown in a natural medium, such as wheat straw hydrolysate, have not been analyzed.

To gain insight into the mechanisms underlying the SS-mediated improvement of ABE fermentation from non-detoxified wheat straw hydrolysate, ABE production by supplying fermentation cultures with SS was investigated. Next, gene expression patterns between control and SS-supplemented treatment cultures were compared using genome-wide RNA-seq.

2. Methods

2.1. Bacterial strains and inoculum development

Clostridium acetobutylicum CICC 8012 was obtained from the China Center of Industrial Culture Collection (CCICC, Beijing, China), which was originally separated by Shanghai solvent factory (Shanghai, China) in 1956. Stocks of C. acetobutylicum CICC 8012 spores were stored in sterile corn powder medium (CM) at 4 °C. To revive the spores, a 10-ml stock was transferred to 90-ml anoxic Wheat straw hydrolysate, 15 h after inoculation, the culture was subdivided into aliquots of 150 ml in six 250-ml screw-capped bottles. Three bottles in the treatment group were supplied with sodium sulfide (0.4 g/L), and the other three bottles were left unsupplied as a control. In all the experiments, the temperature was maintained at 37 °C for 72 h without agitation.

3 ml samples were taken during fermentation for sugar, acid and ABE analysis at 0 h, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, 24 h, 27 h, 30 h, 36 h, 42 h, 48 h, 54 h, 60 h, 66 h and 72 h post-inoculation. Sugars were measured using high-performance liquid chromatography (HPLC), Waters2795, Waters, USA) with an Evaporative Light-scattering Detector (All-Tech ESLD 2000, Altech, USA) and a column (Aminex HPX-87P, Bio-Rad, USA) (Huang et al., 2013). ABE and acids (acetic acid and butyric acid) were analyzed using gas chromatography (FUIJ 9790, FUIJ, China) on a Chromosorb 101 (80/100 mesh) column (Gao et al., 2012). Redox flux was measured using ORP combination electrodes (AURORA E-431Q, AURORA, China) connected to a mV meter (OAKTON Ph/mV/C meter, Singapore) to obtain readings in millivolts (Olsen and Ilenyem, 2009). Culture growth was measured by following optical density (OD) in the fermentation broth at A600

2.2. Wheat straw pretreatment and hydrolysis

Wheat straw, obtained from a farm product market in an urban area of Chengdu (Sichuan province, China), was ground to fine particles (filtered with a 0.5-mm sieve screen) using a mill. Every 100 g of wheat straw powder was suspended in 1 L of 0.5% (v/v) dilute sulfuric acid and heated in an autoclave at 121 °C for 1.5 h. Upon autoclaving, the mixture was cooled to room temperature, and the pH was adjusted to 4.8 with Ca(OH)₂. After this, 1 ml of cellulase and 1 ml of xylanase were added to 1 L of the mixture. Finally, the mixture was incubated at 50 °C for 48 h. After incubation, the mixture was centrifuged for 10 min at 3000 rpm to remove sediment. The clear liquid was stored at 4 °C for fermentation studies to be conducted later. The cellulase used was GC220 purchased from DuPont™ Genencor® Science and had a declared activity of 89 FPU/ml, 184 mg protein/ml. The Xylanase used was a Multifect Xylanase purchased from DuPont™ Genencor® Science and had a declared activity of 8000 GXU/ml, 47 mg protein/ml. The activity of Multifect Xylanase is expressed in GXU/ml. The GXU is based on the release of Remazol Brilliant Blue-dyed birch wood xylan at pH 4.5 and 30 °C in 10 min, using a xylanase reference standard.

2.3. Fermentation and fermentation product analysis

To evaluate the response of C. acetobutylicum CICC 8012 to SS, fermentation was conducted in a 2-L flask containing 1 L of anoxic medium in which wheat straw hydrolysate acted as the carbon source. One liter of fermentation medium contained 3 g of soybean meal, 1 g of CH₂COOH, 0.6 g of KH₂PO₄, 0.2 g of MgSO₄, 0.1 mg of biotin, 8 mg of vitamin B1, 8 mg of niacin, 8 mg of vitamin B6, and 20 mg of inositol. After adding the above mentioned substances to wheat straw hydrolysate acted, the fermentation medium was sterilized at 115 °C for 20 min. Next, the flask was purged with aseptic N₂ to remove dissolved O₂ when the medium was cooled to 37 °C. Next, 100 ml of highly motile C. acetobutylicum CICC8012 preculture was inoculated to the flask. At this time (0 h after inoculation), 21.05 g of glucose, 20.57 g of xylose, 3.21 g of cellobiose, 2.28 g of arabinose, 1.82 g of galactose, 1.94 g of mannose, 2.52 g/L of 5-hydroxyethyl furfural (HMF), 1.96 g/L of furfural, 0.13 g/L of syringaldehyde, 0.16 g/L of vanillin, 0.37 g/L of furfuryl acid, and 0.15 g/L of P-coumaric acid were detected in the fermentation medium made from wheat straw hydrolysate. 15 h after inoculation, the culture was subdivided into aliquots of 150 ml in six 250-ml screw-capped bottles. Three bottles in the treatment group were supplied with sodium sulfide (0.4 g/L), and the other three bottles were left unsupplied as a control. In all the experiments, the temperature was maintained at 37 °C for 72 h without agitation.
using a UV–Vis Spectrophotometer (754N, APL, China) (Wang et al., 2011). ABE yield was calculated as the total solvent produced divided by the total sugar utilized and was expressed in g/g. All the experiments were performed in triplicate, and all the data were reported as means.

2.4. RNA isolation, library construction and sequencing

C. acetobutylicum CICC 8012 samples for transcriptional analyses were collected from cultures at 0 h and 3 h after addition of SS (15 h and 8 h after inoculation). A parallel cultivation of C. acetobutylicum without SS was used as a control. In preparation for RNA isolation, 10-ml cultures were harvested at each time point. Aliquots of samples were placed on ice immediately after removal from the bottles and immediately centrifuged at 4000 g for 10 min at 4 °C (Wang et al., 2011; Zha and Ezeji, 2013). Total RNA was extracted from the cell pellet using Trizol reagent based on the manufacturer’s protocol (Invitrogen, USA) and was further purified using an RNeasy minikit (Qiagen, Germany). DNA was removed by DNase I (Fermentas, USA). The purity, concentration, and RNA integrity number (RIN) were measured by SMARD3000 and/or an Agilent 2100 Bioanalyzer. Bacterial 16S and 23S ribosomal RNAs were removed with Ribo-Zero™ rRNA Removal Kits from Epicentre (Madison, WI) following the manufacturer’s protocol. The enriched mRNAs were then submitted to the Beijing Genomics Institute (BGI)-Shenzhen, Shenzhen, China [http://www.genomics.cn/en/index] for mRNA-seq library construction and RNA sequencing.

2.5. RNA-seq data analysis

Clean reads of each sample were mapped to the reference genome of C. acetobutylicum ATCC 824 [GenBank assembly accession: GCA_000008765.1], with predicted protein-encoding genes, and rRNA and the plasmid were mapped separately using Bowtie2 software (V2.2.3) with default parameters; then, the mapping rates were calculated accordingly. TopHat V2.0.4 was used to map the inter-exon splice junctions. Cufflinks was used to assemble the aligned RNAseq reads into transcripts and estimate their abundance by quantifying the fragments per kilobase of transcript per million fragments mapped (FPKM value). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were retrieved from the KEGG web server [http://www.genome.jp/kegg/].

3. Results and discussion

3.1. Effect of supplemental SS on sugar consumption and ABE production

The effects of 0.2 g/L, 0.4 g/L, 0.6 g/L, 0.8 g/L and 1.0 g/L of SS on ABE fermentation was investigated. When the concentration of SS was lower than 0.4 g/L, butanol titer was lower than that under the treatment of 0.4 g/L of SS. When the concentration of SS increased to 0.6 g/L, the butanol titer did not increase, but decreased a little than that under the treatment of 0.4 g/L of SS. Therefore, to elucidate the effects of supplemental SS on ABE fermentation from non-detoxified wheat straw hydrolysate, 0.4 g/L of sodium sulfide was added to the wheat straw at 15 h after C. acetobutylicum CICC8012 was inoculated for ABE fermentation. Solventogenic clostridia are capable of utilizing a wide range of carbohydrate substrates. However, inhibitors existed in non-detoxified wheat straw hydrolysate medium may depress carbohydrate metabolism. One study reported that the ABE titer achieved by C. acetobutylicum CICC 8012 was 38.8% and 13.2% lower in the presence of 2 g/L furfural and HMF, respectively (Sun et al., 2010). In this study, 2.52 g/L 5-hydroxymethyl furfural (HMF), 1.96 g/L furfural, 0.13 g/L syringaldehyde, 0.16 g/L vanillin, 0.37 g/L ferulic acid, 0.15 g/L P-coumaric acid, and 3.90 g/L of acetic acid were detected at the beginning of fermentation in medium containing wheat straw hydrolysate as the carbon source. Other inhibitors may also exist in wheat straw hydrolysate that have not been detected. Therefore, the sugar-based yield of ABE in the unsupplemented control was only 0.29 g/g.

Biochemical measurements taken during fermentation demonstrated that C. acetobutylicum CICC 8012 in SS-supplied medium (+SS) exhibited more rapid sugar consumption than did cultures grown in unsupplemented control medium (−SS). At the end of fermentation (72 h), 39.26 g/L of sugars were consumed in +SS, whereas only 30.80 g/L of sugars were consumed in −SS. The biggest difference in sugar consumption existed in the consumption of cellobiose (Fig. 1C). After being supplied with SS (+SS), C. acetobutylicum CICC 8012 began assimilating cellobiose at a speed that was remarkably higher than in unsupplemented control medium (−SS), and most of the cellobiose in +SS was consumed by 48 h. At the end of fermentation, 3.00 g/L of cellobiose was consumed in +SS, corresponding to 93.71% of the initial content, whereas only 1.07 g/L of cellobiose was consumed in −SS, corresponding to 33.47% of the initial content. Glucose is a preferred sugar of C. acetobutylicum. There was no significant difference in glucose consumption between +SS and −SS before 36 h. However, the glucose utilization rate in −SS slowed after 36 h: 4.44 g/L of glucose remained at 72 h in −SS, whereas no glucose was detected in +SS after 66 h (Fig. 1A). Xylose is the most fermentable pentose found in lignocellulose hydrolysates (Aristidou and Penttila, 2000). Although clostridia can utilize xylose, they do so with a poor efficiency (Fond, 1986). The addition of SS also increased xylose consumption; the concentrations of residual xylose in +SS and −SS were 7.80 g/L and 9.69 g/L, respectively (Fig. 1B).

Little arabinose, galactose and mannose was consumed in both +SS and −SS, and the difference in the content of these sugars between +SS and −SS was not significant (data not shown).

Acetone, butanol and ethanol, acetic acid and butyric acid production levels in C. acetobutylicum CICC 8012 cultures with and without supplemental SS were examined (Fig. 2). When no SS was supplemented (−SS), 5.17 g/L of butanol, 3.31 g/L of acetone, 0.55 g/L of ethanol, 3.88 g/L of acetic acid and 0.82 g/L of butyric acid were produced by C. acetobutylicum CICC 8012, resulting in a total ABE concentration of 9.03 g/L and total acid concentration of 4.70 g/L. Therefore, the productivity and ABE yields were 0.13 g/L h and 0.29 g/g, respectively. When SS was supplied (+SS), 7.63 g/L of butanol, 4.74 g/L of acetone, 1.48 g/L of ethanol, 3.15 g/L of acetic acid and 0.61 g/L of butyric acid were produced by C. acetobutylicum CICC 8012, resulting in a total ABE concentration of 13.85 g/L and total acid concentration of 3.76 g/L. Therefore, the productivity and ABE yields were 0.19 g/L h and 0.35 g/g, respectively.

When the fermentation medium was supplemented with SS (+SS), a 180.37% improvement in cellobiose consumption, 26.73% improvement in glucose consumption, 17.38% improvement in xylose consumption, 47.63% improvement in butanol concentration and 53.50% improvement in final ABE titer were obtained. The supplemented SS provided a larger carbon feedstock pool for conversion to a higher final total solvent concentration. In other words, increased sugar consumption directly contributed to solvent formation. The calculation of carbon flux distribution showed that 54.95% of the consumed carbon was converted to ABE in +SS, whereas only 46.12% of the consumed carbon was converted to ABE in −SS. 10.10% of the consumed carbon was converted to acid in +SS, whereas 16.22% of the consumed carbon was converted to acid in −SS, indicating that the culture performed much better in +SS than it did in −SS.
Redox flux measurements taken during fermentation indicated that supplement of SS reduced oxidation-reduction potential (ORP) in the fermentation system. Before supplying SS (15 h), ORP of fermentation medium was −297 mV. After supplying SS (18 h), ORP was −307 mV in +SS, whereas it was −262 mV in −SS. Kim et al. indicated that lowering the redox potential increased electron flux in the direction of NADH/NAD(P)H in the cells, whose abundance then increased ABE production (Kim et al., 1988). As far as cell growth was concerned, OD600 of fermentation medium was 1.36 before SS was supplied (15 h). After supplying SS (18 h), OD600 was 1.79 in +SS, whereas it was 1.67 in −SS. The decrease of ORP may be one of the reasons that why SS improve cell growth and then central fermentation.

Acetic acid and butyric acid were produced mainly during the early phase, and then acids were re-assimilated to produce acetone, butanol and ethanol. Acid production in +SS and −SS displayed different tendencies (Fig. 2D and E). In +SS, acetic acid and butyric acid increased significantly before 15 h and decreased after 18 h. In −SS, acetic acid and butyric acid increased significantly before 24 h and decreased after 27 h. This indicates that SS promoted the transition from acidogenesis to solventogenesis, indicating a dramatic shift in the biochemical pathway toward ABE production. Therefore, transcriptome analysis was conducted in order to obtain more comprehensive explanation about how SS influence ABE fermentation besides decreasing ORP.

3.2. RNA-seq data analysis

To investigate the transcriptomic response to SS addition in C. acetobutylicum CICC 8012, samples collected at 15 h, 18 h (+SS) and 18 h (−SS) were used for RNA-seq analysis (Table 1). 6,304,025, 6,325,577, and 6,271,268 paired-end (PE) 90-bp reads from the 15 h, 18 h (+SS) and 18 h (−SS) samples were obtained, respectively, corresponding to 1.7 Gbp of sequence data in total. Because there is still no published genome sequence of C. acetobutylicum CICC 8012, the reads obtained from sequencing were mapped onto the reference genome of C. acetobutylicum ATCC824, which belongs to the same species as C. acetobutylicum CICC 8012. For each of the three samples, >95% of all the reads were mapped to either the genome or to plasmid DNA. Based on the sequence data, 3569, 3595, and 3554 out of 3911 protein-coding genes had detectable expression in 15 h, 18 h (+SS) and 18 h (−SS) samples, respectively (Additional file 1).

3.3. Expression of membrane transporter genes

Fermentation of materials composed of carbohydrate mixtures will inevitably depend on the regulatory response of the organism to the carbohydrates present. Membrane transporter genes play an active role in the transportation and metabolism of sugars. The presence of SS in the ABE fermentation medium altered the

Fig. 1. Sugars consumption of C. acetobutylicum CICC 8012 with 0.4 g/L SS addition in +SS and no SS addition in −SS. (■••) +SS: 0.4 g/L SS was added to the fermentation medium. (–○–) −SS: no SS was added to the fermentation medium. (A) Glucose, (B) xylose, (C) cellobiose, (D) total sugars.
expression of genes encoding the membrane transport system, including the phosphotransferase system (PTS) and ATP-binding cassette transporters (ABC-transporter), in *C. acetobutylicum* CICC 8012.

PTSs are the most prevalent uptake route for sugars in solventogenic clostridia (Mitchell, 1998; Nolling et al., 2001). The most prominent changes among the membrane transporter genes are the PTS system cellobiose family of transporters involved in cellobiose metabolism (Fig. 3). Cellobiose was imported to the cell by two putative PTSs (Fig. 3) (Gu et al., 2014). Genes for one PTS are located in an operon encoding a β-glucosidase-specific PTS IIBC (CAC1407). Genes for a second potential cellobiose PTS are located in an operon that encodes a PTS IIA (CAC0383), a PTS IIB (CAC0384), and a PTS IIC (CAC0386) (Servinsky et al., 2010).
addition resulted in significantly increased expression of one PTS, including CAC0383, CAC0384, and CAC0386. Expression of these genes in +SS was up-regulated by up to 53.42 times over expression in –SS (Fig. 3).

Glucose was also imported to the cell by two putative PTSs (Fig. 3). PTS IICBA (CAC0570) is one of the important glucose PTS families (Gu et al., 2014). It was induced by the addition of SS, and in the presence of SS, its expression level was 8.26 times greater than in the unsupplemented control. A second putative glucose PTS is encoded by two divergently transcribed genes, CAC1353 and CAC1354, which encode PTS IICB and PTS IIA components, respectively (Tangney and Mitchell, 2007). Nevertheless, neither of these genes displayed significantly altered expression in the presence of SS (Fig. 3).

Xylose was transported via non-PTS in C. acetobutylicum. The ABC-type xylose transporter may be missing in C. acetobutylicum, and only several sugar–proton symporter genes that might be involved in xylose uptake are annotated in the genome (Grimmler et al., 2010; Gu et al., 2014). These symporter genes (CAC1339, CAC1345, CAC1530, CAC3422 and CAC3451) were all significantly up-regulated when SS was added to the medium, and the expression levels increased between 2.27 and 23 times in +SS as compared with –SS (Fig. 3).

In C. acetobutylicum, both cellobiose and glucose were imported to the cell by two putative PTSs. Interestingly, only one PTS was strongly induced by SS and the expression of another PTS barely changed, no matter for cellobiose or glucose. This phenomenon demonstrates that one cellobiose (or glucose) transporter is highly inducible by addition of SS and another cellobiose (or glucose) transporter may be constitutively expressed by C. acetobutylicum. In addition, all five sugar–proton symporter genes potentially involved in xylose uptake are induced by SS supplementation. Therefore, the increased expression level of genes encoding membrane transporters involved in cellobiose, glucose and xylose uptake may be an important mechanism underlying the improved sugar consumption that was observed in the presence of SS.

### 3.4. Expression of sugar metabolism genes

Addition of SS increased carbohydrate consumption (Fig. 1). Expression analysis revealed that the expression of most of genes involved in glycolysis increased in +SS compared to –SS (Fig. 4),

**Table 1**

| Items                  | 15 h | Coverage (%) | 18 h (+SS) | Coverage (%) | 18 h (–SS) | Coverage (%) |
|------------------------|------|--------------|------------|--------------|------------|--------------|
| Map to genome          |      |              |            |              |            |              |
| Total reads            | 6,304,025 | 100.00      | 6,325,577  | 100.00       | 6271268 | 100.00       |
| Unique match           | 5,989,454 | 95.01       | 5,940,982  | 93.92        | 4,916,674 | 78.40        |
| Unique match           | 5,673,530 | 90.00       | 5,625,313  | 88.93        | 4,649,310 | 74.14        |
| Multi-position match   | 315,924  | 5.01        | 315,669    | 4.99         | 267,364  | 4.26         |
| Total unmapped reads   | 314,571  | 4.99        | 314,571    | 4.99         | 267,364  | 4.26         |
| Map to genes           |      |              |            |              |            |              |
| Total mapped reads     | 4,780,342 | 75.83       | 4,492,425  | 71.02        | 3,668,065 | 58.49        |
| Unique match           | 3,848,939 | 61.70       | 3,757,088  | 59.39        | 3,106,341 | 49.53        |
| Multi-position match   | 890,903  | 14.13       | 735,417    | 11.63        | 561,751  | 8.96         |
| Total unmapped reads   | 1,523,683 | 24.17       | 1,833,152  | 28.98        | 2,603,203 | 41.51        |
| Map to rRNA            |      |              |            |              |            |              |
| Total mapped reads     | 43,498   | 0.69        | 89,823     | 1.42         | 72,120   | 1.15         |
| Map to plasmid         |      |              |            |              |            |              |
| Total mapped reads     | 143,101  | 2.27        | 243,534    | 3.85         | 1,102,489 | 17.58        |
and these transcriptional results corroborate the greatly increased carbohydrate consumption observed in SS-supplemented cultures of *C. acetobutylicum* CICC 8012.

Cellobiose and glucose are metabolized in a similar manner because cellobiose is composed of glucose and varies only in its chain length. The primary differences in the metabolism of these two sugars lie in their transport mechanisms and the hydrolysis of the glycosidic bonds in cellobiose. After import into the cell, cellobiose was firstly converted to glucose, and then was ultimately assimilated to pyruvate through glycolysis (Servinsky et al., 2010). β-glucosidase plays an important role in the entrance of cellobiose to glycolysis by hydrolyzing cellobiose to β-glucose. β-glucose then enters into glycolysis directly or enters into glycolysis after being converted to α-glucose by aldose 1-epimerase. Impressively, the expression level of genes encoding β-glucosidases and aldose 1-epimerase were found to have increased in +SS as compared to −SS (Fig. 4). Three β-glucosidase genes (CAC0385, CAC0743, and CAC1408) were up-regulated when SS was supplied in the medium. Their respective expression levels increased by 9.99, 1.42 and 1.47 times in +SS over those in −SS. The gene encoding the β-glucosidase-like protein (CAC1075) was also strongly induced by SS, with a 16.5-fold increase in expression. Aldose 1-epimerase (mutarotase) is the enzyme responsible for the anomer interconversion of α-glucose and other aldoses between their alpha- and beta-forms. The gene encoding aldose 1-epimerase (CAC1349) had significantly higher expression levels in +SS than in −SS (13.39 times greater in +SS than in −SS) (see Fig. 4).

Glucose is converted to pyruvate via the glycolytic pathway, and then, pyruvate is converted to butanol via ABE fermentation. Three key enzymes of glycolysis are glucokinase (CAC2613), phosphofructokinase (CAC0517), and pyruvate kinase (CAC0518, CAC1036). After adding SS, the expression of CAC2613 and CAC0517 increased 1.91 and 1.51 times as compared to...
unsupplemented control. Unexpectedly, the expression levels of the other key enzyme, pyruvate kinase (CAC0518, CAC1036), was slightly decreased in +SS as compared to /C0 SS, and the reason need to be further researched.

For xylose metabolism, it has been supposed that xylose is converted to D-xylulose 5-P via the pentose-phosphate pathway before being metabolized by glycolysis. Xylose isomerase (xylA) and xylulokinase (xylB) play an important role in the entry of xylose to glycolysis by converting xylose to D-xylulose 5-P. C. beijerinckii harbors more sets of xylose metabolic pathway genes, including xylA and xylB, than does C. acetobutylicum (Nolling et al., 2001). xylA-II (CAC2610) was identified in C. acetobutylicum (Gu et al., 2010). However, both xylA-II and xylB may be essential for xylose metabolism in C. acetobutylicum because inactivation of either gene resulted in the loss of xylose consumption (Gu et al., 2010; Xiao et al., 2011; Zhang et al., 2012).

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Therefore, transcriptional analysis of the expression patterns of key enzyme-encoding transcript involved in carbohydrate import and glycolysis clearly showed that SS addition enhances solvent production by increasing the uptake and assimilation of sugars.

3.5. Expression of acidogenesis and solventogenesis genes

Supplying SS to cultures of C. acetobutylicum led to an earlier and enhanced solventogenic metabolism. There was only 20.48% improvement in the sugar-based ABE yield, but a 53.50% improvement in the final ABE concentration were found when SS was supplemented. This indicates that SS addition increased solvent yield rather than acid formation from pyruvate, which led to the further investigation of the transcriptional patterns with respect to central metabolism.

It has been well accepted that C. acetobutylicum produces ABE through its characteristic biphasic fermentation. Two butanol-forming pathways, the direct formation route (hot channel) and the acid reassimilation route (cold channel), contribute to solvent...
production in \textit{C. acetobutylicum}. In the hot channel, butanol can be formed directly from acetyl-coenzyme A (CoA) through butyryl-CoA. In the cold channel, acetic acid and butyric acid first formed during the acidogenic phase are subsequently reassimilated to form acetone–butanol–ethanol. \cite{Cornillot et al., 1997; Jang et al., 2012a; Jones and Woods, 1986; Wiesenborn et al., 1989}.

The direct butanol-forming hot channel plays a more important role in enhanced butanol production than the acid-reassimilating cold channel \cite{Jang et al., 2012a}. The sol operon of \textit{C. acetobutylicum} is the essential transcriptional unit for the formation of solvents by hot channel \cite{Thormann et al., 2002}. The sol operon is formed by structural genes encoding the acetoacetyl coenzyme A: acetic acid/butyric acid:coenzyme A transferase (ctfA and ctfB, CAP0163 and CAP0164) and an alcohol/aldehyde dehydrogenase (adhE1, CAP0162), together with a small open reading frame (ORF) of unknown function \cite{Ralf et al., 1993}. Most strikingly, the addition of SS led to increased transcription of the tricistronic sol operon of \textit{C. acetobutylicum} in this study. When supplementing with SS, the expression level of CAP0162-0164 was increased up to 10.76-fold over that in –SS (Fig. 5). It has been reported that overexpression of the adhE1 gene (CAP0162) increased butanol formation and the ratio of hot- to cold-channel butanol \cite{Jang et al., 2012a; Sillers et al., 2009}. Therefore, SS addition may reinforce the hot channel by inducing the adhE1 gene. In addition to the sol operon, several other genes are also important for solvent formation. Acetoacetic acid decarboxylase (adc, CAP0165) is the key enzyme in the pathway involved in acetone formation from acetoacetyl-CoA, and its expression level increased 3.66 times more in +SS than in –SS. A second NADH-dependent aldehyde/alcohol dehydrogenase (adhE2, CAP0035) is involved in ethanol and butanol formation, and the addition of SS led to a 12.08-fold increase in this gene. Two chromosomal butanol dehydrogenases bdHA and bdHB (CAC3299 and CAC3298) are also involved in ethanol and butanol formation. As compared to unsupplemented medium, CAC3298 exhibited a significantly higher expression level in SS-supplemented medium, whereas the expression of CAC3299 did not change. Therefore, in fermentations supplemented with SS (+SS), the genes encoding three out of four key enzymes in ABE formation pathways – including \textit{adc} (CAP0165) for acetone formation and \textit{adhE1} (CAP0162), \textit{adhE2} (CAP0035), \textit{bdHA} (CAC3299) and \textit{bdHB} (CAC3298) for butanol and ethanol formation – were significantly up-regulated, which reinforced a direct forming route (hot channel) for solvents (Fig. 5).

The genes involved in acid formation pathways, including phosphate acetyltransferase (CAC1742) and acetic acid kinase (CAC1743) for acetic acid formation and phosphate butyryltransferase (CAC3076) and butyric acid kinase (CAC3075 and CAC1660) for butyric acid formation, were up-regulated very slightly in +SS compared to –SS (Fig. 5). However, acetic acid and butyric acid formed during the acidogenic phase were subsequently reassimilated to form acetone–butanol–ethanol through cold channel. The key enzyme for the cold channel flux, CoA transferase (encoded by the ctfA gene CAP0163 and CAP0164), is responsible for the uptake of acids produced during the acidogenic phase \cite{Jang et al., 2012b; Jones and Woods, 1986; Lehmann et al., 2012}. Anaerobic cultivation of the \textit{C. acetobutylicum} mutant strains CKW, CEKW, and CBK, which lack the cold channel fluxes, exhibited butanol production only via the hot channel, but the butanol yields obtained were rather low. Moreover, these strains produced more acetic acid and butyric acid than the wild-type strain \cite{Jang et al., 2012a}. By contrast, SS addition promoted cold channel fluxes in this study by inducing CoA transferase, which reinforced the acid re-assimilation for solvents and then directed acetyl-CoA and butyryl-CoA to the ABE biosynthesis branch. All these led to a 18.81% decrease in acetic acid, a 25.94% decrease in butyric acid and a correspondingly enhanced ABE titer.

Therefore, transcriptional analysis of the expression patterns of key enzyme-encoding transcript involved in ABE formation clearly showed that SS addition enhances solvent production by reinforcing the direct formation route (hot channel) and the acid reassimilation route (cold channel), which reduced acetic acid and butyric acid metabolic flux and increased butanol, ethanol and acetone metabolic flux in response to SS addition.

In this study, the transcriptomics data and ABE titer variation were integrated to elucidate the mechanisms underlying the dramatic shift in biochemical pathway toward butanol production from wheat straw hydrolysate by \textit{C. acetobutylicum} CICC 8012 under SS addition. These two levels of data were analyzed and compared. Investigation of ABE fermentation parameters showed that the performance of \textit{C. acetobutylicum} CICC 8012 in non-detoxified wheat straw hydrolysate was greatly improved by the addition of SS. Further genome-wide transcriptomic analysis of the expression patterns of key enzyme-encoding transcripts involved in ABE formation supported the results described above. SS addition increased pyruvate generation via enhancing the carbohydrate metabolic status and redirected metabolic flux toward the biosynthesis of ABE. These results demonstrate the significant potential application value of SS supplementation for commercial butanol production. Comparison of gene expression patterns in relation to ABE production is expected to facilitate strain development, optimize fermentation conditions, and provide insights that will lead to better utilization of lignocellulosic biomass hydrolysates.

This is the first transcriptomic study of the central metabolic pathway in ABE fermentation from a natural medium (non-detoxified wheat straw hydrolysate) containing inhibitors, whereas all the previous transcriptomic studies have investigated solventogenic clostridia in synthetic medium. More research about the mechanisms underlying the SS-mediated improvement of ABE fermentation from lignocellulose, such as transcription factor, will also be carried out in the future.

4. Conclusion

This study elucidates the effects of SS on ABE fermentation from non-detoxified wheat straw hydrolysate by \textit{C. acetobutylicum} CICC8012. SS addition led to significant improvements in sugar consumption, final ABE titer, sugar-based yield and productivity. Transcriptional analysis indicated that supplemented SS affected the central fermentative pathway, down-regulated the metabolic flux toward the acids formation branches, and up-regulated the metabolic flux toward the ABE formation branches. Comparison of gene expression patterns in relation to ABE production is expected to provide insights that will lead to better utilization of lignocellulosic biomass hydrolysates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.08.139.
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