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Impact of zinc supplementation on the improved fructose/xylose utilization and butanol production during acetone–butanol–ethanol fermentation

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Lignocellulosic biomass and dedicated energy crops such as Jerusalem artichoke are promising alternatives for bio-butanol production by solventogenic clostridia. However, fermentable sugars such as fructose or xylose released from the hydrolysis of these feedstocks were subjected to the incomplete utilization by the strains, leading to relatively low butanol production and productivity. When 0.001 g/L $ZnSO_4 \cdot 7H_2O$ was supplemented into the medium containing fructose as sole carbon source, 12.8 g/L of butanol was achieved with butanol productivity of 0.089 g/L/h compared to only 4.5 g/L of butanol produced with butanol productivity of 0.028 g/L/h in the control without zinc supplementation. Micronutrient zinc also led to the improved butanol production up to 8.3 g/L derived from 45.2 g/L xylose as sole carbon source with increasing butanol productivity by 31.7%. Moreover, the decreased acids production was observed under the zinc supplementation condition, resulting in the increased butanol yields of 0.202 g/g-fructose and 0.184 g/g-xylose, respectively. Similar improvements were also observed with increasing butanol production by 130.2 % and 8.5 %, butanol productivity by 203.4% and 18.4%, respectively, in acetone–butanol–ethanol fermentations from sugar mixtures of fructose/glucose (4:1) and xylose/glucose (1:2) simulating the hydrolysates of Jerusalem artichoke tubers and corn stover. The results obtained from transcriptional analysis revealed that zinc may have regulatory mechanisms for the sugar transport and metabolism of *Clostridium acetobutylicum* L7. Therefore, micronutrient zinc supplementation could be an effective way for economic development of butanol production derived from these low-cost agricultural feedstocks.

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[**Key words:** Acetone–butanol–ethanol fermentation; *Clostridium acetobutylicum*; Zinc supplementation; Fructose; Xylose]

Butanol has been acknowledged as an advanced biofuel superior to fuel ethanol with respects to higher energy content, lower volatility and hygroscopy and good compatibility with existing facilities for fuels transportation and storage (1). As for microbial biosynthesis, butanol could be produced via clostridia-based fermentation from traditional sugar- and starch-based feedstocks (e.g., molasses, sugarcane juice, sago starch, cornstarch) (2). Clostridia can naturally utilize a broad spectrum of carbon sources such as mono- and disaccharides as well as polysaccharides via butanol-producing pathways (3), making them suitable for fermenting various feedstocks. Therefore, abundantly available and non-food related feedstocks, particularly lignocellulosic biomass and dedicated energy crops that can grow well without competing for arable land with grain plants, would be suitable for economic butanol production (4,5). Among these promising alternatives, Jerusalem artichoke is typically considered as an excellent feedstock due to its tolerance to environmental stresses such as drought and salinity (6,7). The Jerusalem artichoke tubers can be easily hydrolyzed into sugar mixture of fructose/glucose (4:1) by easy pretreatment with dilute acid. However, the corn stover is recalcitrant to degrade into complex mixture composed of xylose/glucose (1:2) as main sugars and inhibitory byproducts (e.g., formic acid and furfural) (8,9). Given the content of total sugars that is no more than 13%, the fresh Jerusalem artichoke tubers can be an industrially-feasible alternative for acetone–butanol–ethanol (ABE) production by solventogenic clostridia (10).

In the past decades, intensive studies have been carried out on the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), which plays an essential role in uptake and phosphorylation of sugars including hexoses and disaccharides (3), and 13 PTS have been identified in *Clostridium acetobutylicum* (11–13). However, *C. acetobutylicum* is incapable of co-utilizing multiple sugars derived from the hydrolysates of diverse feedstocks due to complex components and carbon catabolite repression (CCR) (14). Among these fermentable sugars, glucose is the most favorite by clostridia with a fast uptake rate (11,13,14), while fructose has a relatively low efficiency of utilization via PTS^{Fru} as the major fructose uptake system in *C. acetobutylicum* (15). In addition, xylose is the most abundant sugar released from the hydrolysis of hemicelluloses in lignocellulosic biomass. Although *C. acetobutylicum* is naturally capable of utilizing xylose via the pentose phosphate pathway (PPP), the fermentation efficiency is undesirable (16–18). Therefore, more efforts have been focused on engineering clostridia to improve sugar(s) utilization or co-utilization without CCR (19,20). Although genetic modifications on clostridia would be ultimate solutions for

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efficient utilization of these non-glucose sugars, the progress is slow and methodologies are specific with individual sugar, making them less practical from the industrial perspective.

Based on our previous work about the effect of zinc supplementation on ABE fermentation using glucose as sole carbon source (21), the micronutrient zinc could lead to significant improvements with respect to cell growth, acids re-assimilation, butanol/ABE production and productivity. For the sake of providing an effective bioprocess engineering strategy to address the challenge with ABE fermentations from these fermentable sugars derived from the hydrolysates of Jerusalem artichoke tubers and corn stover, the objective of this work is to further evaluate the impact of micronutrient zinc supplementation on ABE fermentations by *C. acetobutylicum* L7 using fructose, xylose as well as sugar mixtures of fructose/glucose and xylose/glucose.

MATERIALS AND METHODS

Strain, pre-culture and media The strain used in this study was *C. acetobutylicum* L7 which was anaerobically pre-cultivated as previously described (21). The fermentation medium composed of (g/L): sugar(s), 70; yeast extract (Sangon Company, China), 2; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; CH_3COONH_4, 3.22; para-amino-benzoic acid, 0.01; biotin, 0.01. Each concentration of fructose, xylose or sugar mixtures of fructose/glucose (4:1) and xylose/glucose (1:2) added into the fermentation media were adjusted to about 70 g/L.

Based on previous study about the effect of micronutrient zinc supplementation on ABE fermentation by *C. acetobutylicum* using glucose as sole carbon source (21), all the fermentation media in this study were supplemented with 0.001 g/L $ZnSO_4 \cdot 7H_2O$ as zinc source to further evaluate its impact on the ABE fermentations from fructose, xylose as well as sugar mixtures of fructose/glucose and xylose/glucose compared to the control without zinc supplementation. All media were sterilized at 121°C for 15 min. After inoculation, the initial pH of the fermentation was adjusted to 5.5 with 1 N H_2SO_4.

Batch ABE fermentation Batch ABE fermentations by *C. acetobutylicum* were performed using a stirred tank (1.5BG-4-3000, Shanghai Baoxing Engineering, China) with a working volume of 1.1 L under anaerobic conditions established prior to inoculation as previously described (21). During ABE fermentations, 4 mL sample was collected for the analysis of biomass density, residual sugar(s), acids and ABE. All experiments were duplicated, and the averages were presented with standard error bars.

Analytical methods The cell density (OD_{620}) was measured with a spectrophotometer (Thermo Spectronic, USA). Glucose was analyzed by a glucose analyzer (Biosensor SBA-50, Institute of Biology, Shandong Academy of Sciences, Shandong, China). Fructose, xylose, acetate and butyrate were determined by HPLC (Waters 1525, Aminex HPX-87H, 300 × 7.8 mm at 50°C, UV detector at 210 nm and room temperature) equipped with an organic acids analysis column (Aminex HPX-87H, 300 mm × 7.8 mm) and 0.005 mol/L H_2SO_4 was used as the mobile phase with a flow rate of 0.5 mL/min. ABE were analyzed by the gas chromatography as previously described (21).

RNA isolation and transcriptional analysis Total cellular RNA was extracted from homogenized cells of *C. acetobutylicum* L7 grown on the medium containing sole glucose for 16 h with or without zinc supplementation using the RNeasy Mini Kit (Qiagen, Germany) according to the protocol of the manufacturer. The cells pellets for RNA isolation were collected by centrifuging at 4°C and 5000× *g* for 5 min, and immediately shock-frozen in liquid nitrogen. RNA quality and integrity were analyzed using a Nanochip 2130 bioanalyzer (Agilent Technologies). cDNA libraries construction and transcriptional analysis were both performed by Beijing Genomics Institute (BGI, Shenzhen, China). The final cDNA libraries were qualified and quantified using Agilent 2130 Bioanaylzer and ABI StepOnePlus Real-Time PCR System followed by sequencing via Illumina HiSeqTM 2000. To detect the differences of gene expressions among various samples, single-end technology was used to obtain about 40–50 bp reads in a single run. Furthermore, to screen the differentially expressed genes (DEGs) among these two samples, false discovery rate (FDR) ≤0.001 and the absolute value of log2 ratio ≥1 was used as the threshold to judge the significance of gene expression difference. Average linkage hierarchical clustering was performed using Cluster 3.0, and further visualized via Java TreeView.

RESULTS AND DISCUSSION

ABE fermentations from fructose and xylose Compared to the performance of batch ABE fermentation using 70 g/L glucose as sole carbon source (21), as illustrated in Fig. 1, both fructose and xylose were slowly utilized by *C. acetobutylicum* L7. During the ABE fermentation using 70 g/L fructose as sole carbon source, 21.2 g/L of fructose was utilized relatively fast within the first 48 h of fermentation while only 3.3 g/L of fructose was consumed from 48 h to 100 h (Fig. 1A), indicating that a lag phase of fructose utilization occurred. Therefore, as low as 0.463 g g-DCW^{-1} h^{-1} of specific fructose consumption rate was obtained as well as much lower specific growth rate. The reason for this phenomenon was speculated to be the deleterious inhibition resulting from drastic acids accumulation and the much lower pH levels ranging from 4.49 to 4.68 as shown in Fig. 1B. More interestingly, the fructose uptake was restored till the end of fermentation owing to possible adaption of *C. acetobutylicum* L7 to the inhibitory environment. It should be significantly noted that an apparent diauxic growth was observed, along with the ORP fluctuations ranging from −449 to −498 mV at the time frame of 24 h–100 h (Fig. 1B), suggesting the slightly restored cellular metabolic activities of *C. acetobutylicum* L7. As a result, only 4.5 g/L of butanol and 7.6 g/L of ABE were produced from a total utilization of 43.6 g/L fructose within 160 h with a sugar utilization rate of 62.3%. Correspondingly, as shown in Fig. 1C and D, when 70 g/L xylose was used as sole carbon source, 6.3 g/L of butanol and 11.2 g/L of ABE were slowly produced from 38.1 g/L xylose within 84 h with a sugar utilization rate of 54.3%. Similarly, both specific xylose consumption rate of 1.010 g g-DCW^{-1} h^{-1} and specific growth rate of 0.076 h^{-1} were still much lower than those of 1.816 g g-DCW^{-1} h^{-1} and 0.123 h^{-1} obtained from glucose. With regard to the acids produced by *C. acetobutylicum* L7, more acids were accumulated from xylose (1.7 g/L acetate and 3.0 g/L butyrate) and fructose (2.7 g/L acetate and 2.4 g/L butyrate) compared to those deriving from glucose (0.9 g/L acetate and 0.8 g/L butyrate) when the maximum butanol concentrations were achieved (21).

In recent years, more efforts have been focused on the xylose metabolism of *C. acetobutylicum* while little is known about the regulatory mechanism of fructose uptake that is different from that of glucose or xylose (16,21–23). As a matter of fact, three PTS have been confirmed to be responsible for the fructose uptake, one of the fructose/mannitol family and two other of the mannose/fructose families, which were independent from those for glucose and consequently led to different translocation (12,14). Among them, a polycistronic operon (*cac0231*–*cac0234*), namely the *fru* operon, encoded the fructose transport system PTS^{Fru} containing a putative DeoR-type transcriptional regulator (*cac0231*), a 1-phosphofructokinase (*cac0232*), an enzyme EIIA (*cac0233*) and an enzyme EIIBC (*cac0234*). In a recent study, 46.3 g/L fructose was consumed by wild-type strain *C. acetobutylicum* ATCC 824 within 160 h, whereas only 4.8 g/L fructose was slowly consumed by the deletion strain Δ*fru* (Δ*cac0231*–*cac0234*) constructed using homologous recombination, indicating the *fru* operon was significantly involved in the regulation of fructose uptake in *C. acetobutylicum* (15). On the other hand, ABE fermentation from the xylose-based feedstocks is usually unfavorable with respect to low butanol production, productivity and yield, which were ascribed to not only insufficient xylose utilization but also drastic butanol inhibition in xylose transport and metabolism of *C. acetobutylicum*, and thus resulted in poor competitiveness compared to the petroleum-based butanol (16,18,19).

Surprisingly, when 0.001 g/L $ZnSO_4 \cdot 7H_2O$ was supplemented into the media, ABE fermentation from fructose was significantly improved. As illustrated in Fig. 2A, the lag phaseof fructose utilization was also observed but the diauxic growth gave the cell density to the maximum with OD_{620} of 1.9, along with the ORP fluctuations ranging from −427 to −498 mV at the time frame of 20 h–96 h (Fig. 2B), indicating micronutrient zinc could stimulate cell growth and metabolic activities of *C. acetobutylicum* L7. As a consequence, dramatic improvements on specific fructose

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