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Intracellular metabolic changes of *Clostridium acetobutylicum* and promotion to butanol tolerance during biobutanol fermentation

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ABSTRACT

During the fermentation process, *Clostridium acetobutylicum* cells are often inhibited by the accumulated butanol. However, the mechanism underlying response of *C. acetobutylicum* to butanol stress remains poorly understood. This study was performed to clarify such mechanism through investigating the butanol stress-associated intracellular biochemical changes at acidogenesis phase (i.e., middle exponential phase) and solventogenesis phase (i.e., early stationary phase) by a gas chromatography-mass spectrometry-based metabolomics strategy. With the aid of partial least-squares-discriminant analysis, a pairwise discrimination between control group and butanol-treated groups was revealed, and 27 metabolites with variable importance in the projection value greater than 1 were identified. Under butanol stress, the glycolysis might be inhibited while TCA cycle might be promoted. Moreover, changes of lipids and fatty acids compositions, amino acid metabolism and osmoregulator concentrations might be the key factors involved in *C. acetobutylicum* metabolic response to butanol stress. It was suggested that *C. acetobutylicum* cells might change the levels of long acyl chain saturated fatty acids and branched-chain amino acids to maintain the integrity of cell membrane through adjusting membrane fluidity under butanol stress. The increased level of glycerol was considered to be correlated with osmoregulation and regulating redox balance. In addition, increased levels of some amino acids (i.e., threonine, glycine, alanine, phenylalanine, tyrosine, tryptophan, aspartate and glutamate) might also confer butanol tolerance to *C. acetobutylicum*. These results highlighted our knowledge about the response or adaptation of *C. acetobutylicum* to butanol stress, and would contribute to the construction of feasible butanologenic strains with higher butanol tolerance.

1. Introduction

Energy crisis and environmental pollution caused by overutilization of traditional fossil fuel promote the demand for alternative novel fuel. As a renewable gasoline substitute, biobutanol is an attractive fuel due to its lower water absorption, higher energy content, and better blending ability; moreover, biobutanol can also be used in conventional combustion engines without any modifications (Dürre, 2007; Zheng et al., 2009).

Biobutanol can be produced by microbial fermentation (Gu et al., 2011), and *Clostridium acetobutylicum*, *C. saccaroperbutylacetonicum*, *C. beijerinckii*, and *C. saccharoacetobutylicum* embrace significant activity for production of biobutanol (Dürre, 2007; Kumar and Gayen, 2011; Green, 2011). Among these solventogenic clostridia, *C. acetobutylicum*, whose genome sequence has been published (Nölling et al., 2011), is the most used microbe for biobutanol production (Dürre, 2007; Lütke-Eversloh and Bahl, 2011). The biobutanol fermentation performed by *C. acetobutylicum* is characterized by acidogenesis phase and solventogenesis phase (Jang et al., 2014). Acidogenesis phase usually occurs during exponential growth, while solventogenesis phase usually occurs at stationary phase and the onset of sporulation as a response of acid accumulation (Amador-Noguez et al., 2011; Alsaker et al., 2010; Liao et al., 2015). During the fermentation process, *C. acetobutylicum* is often stressed by different

Abbreviations: BCAAs, branched-chain amino acids; DHAP, dihydroxyacetone phosphate; EMP, Embden-Meyerhof-Parnas pathway; GC–MS, gas chromatography–mass spectrometry; HCA, hierarchical cluster analysis; MSTFA, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide; PLS-DA, partial least-squares-discriminant analysis; RT-M/Z, retention time-mass to charge ratio; TCA, tricarboxylic acid; TIC, total ion chromatogram; VIP, variable importance in the projection.

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environmental factors, such as hyperoxia stress (Hillmann et al., 2008), high concentration of substrates and the accumulation of toxic products (acids and solvents) (Tomas et al., 2004; Alsaker et al., 2010). Among these stressors, the toxicity of accumulated butanol is an important obstacle for developing industrial feasible butanologenic strains (Ghiaci et al., 2013). Butanol can inhibit *C. acetobutylicum* cell growth mainly through disruption of membrane fluidity (Baer et al., 1987), inhibition of glucose uptake, nutrient transport and membrane-bound ATPase activity, partially or completely abolishing the approximately constant transmembrane pH gradient (Tomas et al., 2004), and lowering the intracellular ATP concentration (Tomas et al., 2004; Nicolaou et al., 2010).

To maintain the cell survival, microorganisms including *C. acetobutylicum* have evolved a series of stress responses to external stimuli (Ding et al., 2009). For example, increased levels of saturated fatty acid and longer acyl chain fatty acids at the expense of unsaturated chains and shorter acyl chains might confer butanol tolerance to *C. acetobutylicum* and counteract the increased membrane fluidity secondary to butanol challenge during solventogenesis (Baer et al., 1987; Zhao et al., 2003). Moreover, expressions of some stress response genes (*dnaKJ*, *groES*, *hsp18*, and *hsp90*), solvent formation genes (*adc*, *ctfA/B*, and *bdhA/B*), amino acid biosynthesis genes (*CAC0892-CAC0896*), fatty acid biosynthesis opern genes, glycolytic genes and sporulation genes also changed in response to the butanol challenge (Tomas et al., 2004; Alsaker et al., 2010); and overexpression of these genes would likely confer butanol tolerance to *C. acetobutylicum* (Alsaker et al., 2010). Based on these progresses, some efforts have been performed to improve butanol tolerance of *C. acetobutylicum* (Knoshaug and Zhang, 2009). For example, up-regulation of expression of *Spo0A* or *groESL* in *C. acetobutylicum* was performed to enhance butanol tolerance and subsequent solvent yield (Alsaker et al., 2004; Tomas et al., 2003). However, the mechanism underlying the butanol tolerance of *C. acetobutylicum* is very complicate and might involve many genes, mRNAs, proteins, and metabolites (Alsaker et al., 2010; Amador-Noguez et al., 2011). Therefore, though much progress has been made, the exact molecular mechanism still remains poorly understood, and a comprehensive understanding of the mechanism at the systematic level is needed (Amador-Noguez et al., 2011; Lee et al., 2014).

Metabolomics strategy assesses as many metabolites in a biological system as possible (Lee et al., 2014), and allows us to gain an insight into the biochemical changes followed by external stimulus or a pathological insult (Ku et al., 2010). For example, metabolomics strategy-based discovering of acidogenic-solventogenic transition-associated intracellular metabolite changes facilitated deeper understanding of remodeling of central metabolism in *C. acetobutylicum* during solventogenesis (Amador-Noguez et al., 2011). Many previous studies have proven that gas chromatography-mass spectrometry (GC–MS)-based metabolomics approach can effectively analyze metabolic changes. In our previous studies, GC–MS-based strategy was also used to evaluate biochemical changes in *Saccharomyces cerevisiae* (Cui et al., 2015; Li et al., 2012) or *Blakeslea trispora* (Hu et al., 2013). These previous results showed that GC–MS-based metabolomics strategy can provide us a powerful platform for effectively determining the butanol stress-associated biochemical changes in *C. acetobutylicum*.

In this study, to clarify the mechanism underlying response or adaptation of *C. acetobutylicum* to butanol stress, a GC–MS-based metabolomics approach combined with a multivariate analysis were performed to determine the butanol stress-associated *C. acetobutylicum* intracellular metabolic changes at two distinct fermentation phases (i.e., acidogenic and solventogenic phases).

2. Materials and methods

2.1. Strain, media and culture conditions

C. acetobutylicum ATCC 824 used in this study was purchased from China General Microbiological Culture Collection Center (Beijing, China). Inoculum preparation and batch fermentations were performed in an anaerobic chamber (YQX-II, CIMO, China) with a mixed atmosphere containing 90% nitrogen, 5% carbon dioxide and 5% hydrogen (Amador-Noguez et al., 2011).

C. acetobutylicum 824 was cultured anaerobically in PYG medium (5 g/L polypeptone, 5 g/L tryptone, 10 g/L glucose, 10 g/L yeast extract, 8 mg/L $CaCl_2$, 16 mg/L $MgSO_4{\cdot}7H_2O$, 40 mg/L K_2HPO_4, 40 mg/L KH_2PO_4, 0.4 g/L $NaHCO_3$, 0.08 g/L NaCl) at 37 °C for 72 h, and subsequently maintained as spores. Inoculant was prepared by heat-activating spores at 80 °C for 10 min followed by cooling on ice for 2 min and grown to saturation overnight (Baer et al., 1987).

2.2. Butanol challenge experiments

The precultured *C. acetobutylicum* cells were inoculated into fermentative medium and the initial optical absorbance of 600 nm was adjusted to 0.1. The fermentative culture was performed at 37 °C in 250 mL cotton-plugged flasks containing 100 mL of PYG broth either with or without butanol in an anaerobic chamber. For the butanol-treated cells, 0.5%, 1.0%, 1.5%, 2.0%, or 2.5% (v/v) butanol (final concentration) were respectively added into the PYG broth at the beginning of the primary culture.

2.3. Determination of the growth curve

Samples (700 μL) were respectively taken after 0, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 48, 60 and 72 h of incubation. *C. acetobutylicum* cell growth curve was determined by measuring the optical density at 600 nm with a 752 visible spectrophotometer (Shanghai Optical Instrument Factory, Shanghai, China). The experiment was conducted in triplicate.

2.4. Preparation of metabolome samples

It is shown that atmospheric metabolite sampling and processing resulted in quality and quantity of the metabolomics data similar to those obtained using anaerobic processing and pure methanol as the optimal extraction solvent for *C. acetobutylicum* (Lee et al., 2014). In this study, metabolome samples of *C. acetobutylicum* were prepared as previously described (Li et al., 2012) with some modifications. Six biological replicates were performed for each group. Two milliliters of cultures were quickly harvested from middle exponential phase (i.e., the acidogenic phase samples taken after 8 h of cultivation) and early stationary phase (i.e., the solventogenic phase samples taken after 24 h of cultivation) and immediately transferred to 15 mL-tubes containing 8 mL of −40 °C pre-chilled methanol-water (60:40, v/v) to quench the intracellular metabolism. Then the cells were collected by centrifugation (5000*g*, −4 °C, 5 min). The supernatant was discarded, and the pellet was washed by 1 mL of pre-chilled 60% methanol (−40 °C). Then the pellet spiked with 50 μL of internal standard (0.5 mg/mL of ribitol in water) was prepared for extraction of intracellular metabolites. The pellet was suspended in 0.75 mL of pre-chilled pure methanol (−40 °C) and the mixture was vortexed for 15 s. The suspension was frozen in liquid nitrogen for 5 min and then thawed in an ice bath for 3 min. The freeze-thaw process was repeated three times prior to centrifugation (8000*g*, −4 °C, 10 min). The supernatant was collected and an additional 0.75 mL of pre-chilled pure methanol was added to the pellet. The mixture was vortexed for 15 s before