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Physiological response of *Clostridium ljungdahlii* DSM 13528 of ethanol production under different fermentation conditions

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HIGHLIGHTS

- A unique alternative acetate re-assimilation pathway triggered ethanol production.
- Putative aldehyde oxidoreductase *aor1* involved in this alternative pathway.
- Accumulation of external acids induced the shift to solventogenesis.
- A trace amount of $CaCO_3$ increased ethanol and acetate production.

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ABSTRACT

In this study, cell growth, gene expression and ethanol production were monitored under different fermentation conditions. Like its heterotrophical ABE-producing relatives, a switch from acidogenesis to solventogenesis of *Clostridium ljungdahlii* during the autotrophic fermentation with CO/CO_2 could be observed, which occurred surprisingly in the late-log phase rather than in the transition phase. The gene expression profiles indicated that *aor1*, one of the putative aldehyde oxidoreductases in its genome played a critical role in the formation of ethanol, and its transcription could be induced by external acids. Moreover, a low amount of $CaCO_3$ was proved to have positive influences on the cell density and substrate utilization, followed by an increase of over 40% ethanol and 30% acetate formation.

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1. Introduction

As an important renewable energy, ethanol production has been broadly studied in different organisms, such as yeast, *Clostridia*, *Caldicellulosiruptor*, etc. by using variant carbohydrates as carbon sources (Sanchez and Cardona, 2008; Sun and Cheng, 2002; Ying et al., 2013; Yuan et al., 2013; Zhang et al., 2014). Recently, fermentations with CO_2 containing gases are in the focus, which are important components in waste gases from steel mills, power plants, and refineries as well as syngas produced by gasification of biomass (Munasinghe and Khanal, 2010a). Acetogens are anaerobic bacteria occupying Wood-ljungdahl pathway for reduction of CO_2 to synthesis acetyl-CoA, for energy conservation and for fixation of CO_2 into cell carbon (Drake et al., 2008). *Clostridium ljungdahlii*, one of these strict anaerobes, is able to grow autotrophically by using H_2 and/or CO as electron donor and CO_2 as electron acceptor to produce bulk chemicals and biofuels such as acetate, ethanol and 2,3-butanediol (23BD), but not butyrate or butanol due to missing genes involved in butyrate or butanol synthesis such as *crt* (encoding crotonase) and *bcd* (encoding butyryl-CoA dehydrogenase) (Bengelsdorf et al., 2013; Kopke et al., 2011). In contrast, the heterotrophic *Clostridia* such as *Clostridiumacetobutylicum* and *Clostridiumbeijerinckii*, could produce acetone and butanol via the ABE (acetone–butanol–ethanol) fermentation. Using glucose or xylan as carbon source, these ABE-producers synthesize predominantly acetate and butyrate during acidogenesis (pH > 5.2), until the pH decreases sharply to about 4.5 and the fermentation switches from acidogenesis to solventogenesis. During the solventogenic phase, acetone and butanol are mainly conversed from acetate and butyrate and the pH increases lightly (Durre, 2008; Jang et al., 2014; Millat et al., 2013).

According to the genomic information from *C. ljungdahlii* DSM 13528, the metabolic pathways including the fixation of CO/CO_2 and production of ethanol could be illustrated (Kopke et al., 2010). Transcriptomic analysis of *C. ljungdahlii* DSM 13528 grown

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on fructose *versus* autotrophically on H_2/CO_2 or CO/CO_2 revealed that some hypothetical genes like *cooS1* may play a critical role in the carbon fixation process (Nagarajan et al., 2013; Tan et al., 2013). Different gas compositions were investigated for their influence on the yield of ethanol. During the fermentation under syngas containing 44% (v/v) CO, 32% (v/v) N_2, 22% (v/v) CO_2, and 2% (v/v) H_2, the major products were 1.8 g/l acetate, 0.9 g/l ethanol as well as a trace amount of 23BD and lactate (Kopke et al., 2010, 2011). Using 80% (v/v) H_2 and 20% (v/v) CO_2 fermentation could increase the production of acetate to about 7 g/l, but the ethanol production has no significant alternation (Ueki et al., 2014). The slow gas–liquid mass transfer rate is one of these barriers that hinders the utilization of carbon fixation process. To overcome this problem, several approaches on improving bioreactors, increasing CO concentration in the liquid phase by raising the partial pressure of CO or using nanoparticles to enlarge surfaces between gas and liquid phase were developed (Bredwell et al., 1999; Hurst and Lewis, 2010; Kim et al., 2014; Munasinghe and Khanal, 2010b; Ungerman and Heindel, 2007).

Despite these efforts, the productivity of chemical bulks such as ethanol from *C. ljungdahlii* is still very low in comparison with ABE-producers. Previous researches showed that fermentation broth additives such as acetate and calcium carbonate have successfully increased the yield and productivity during ABE fermentation (Han et al., 2013). Nevertheless, little is known about influences of variant compounds like acids or metal irons on the ethanol production and gene-expression profiles of key enzymes during the fermentation process of *C. ljungdahlii* DSM 13528.

In this study, *C. ljungdahlii* DSM 13528 was fermented by using a gas composition of CO/CO_2 in a 5 l bioreactor, meanwhile the gene expression profiles for key metabolic enzymes involved in the carbon fixation, biosynthesis of acetic acid and ethanol were analyzed at various time points. Combining with the effect of acids on the cell growth and ethanol productivity, it could be proposed that similar to the solventogenic *Clostridia*, the switch from acidogenic phase to solventogenic phase in the *C. ljungdahlii* DSM 13528 fermentation process did exist and could be shifted by external acids. Moreover, $CaCO_3$ played a crucial role in the cell density of *C. ljungdahlii* DSM 13528 and substrate utilization that led to increasing ethanol productivity.

2. Methods

2.1. Bacteria strain and growth conditions

C. ljungdahlii DSM 13528 was purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and conserved by freezing mid-exponential phase cultures at −80 °C with 20% glycerol for long-term storage. *C. ljungdahlii* DSM 13528 was generally cultivated at 37 °C and 200 rpm in a modified DSMZ 879 medium, supplied with 0.5 g/l L-cysteine and 5 g/l fructose (unless otherwise stated). The original recipe includes both cysteine and sodium sulfide as reductant, but no significant difference was found in growth without sodium sulfide. The basal DSMZ 879 medium with the following composition (per liter): 1.0 g NH_4Cl, 0.1 g KCl, 0.2 g $MgSO_4 \times 7H_2O$, 0.8 g NaCl, 0.02 g $CaCl_2 \times 2H_2O$, 0.1 g KH_2PO_4, 0.2 mg $Na_2WO_4 \times 2H_2O$, 1 g $NaHCO_3$, 0.3 g cysteine-HCl $\times H_2O$, 1 g yeast extract, 0.5 mg resazurin, 10 ml trace element solution and 10 ml vitamin solution. Trace element solution contains 1.5 g nitrilotriacetic acid, 3 g $MgSO_4 \times 7H_2O$, 0.5 g $MnSO_4 \times H_2O$, 1 g NaCl, 0.1 g $FeSO_4 \times 7H_2O$, 0.18 g $CoSO_4 \times 7H_2O$, 0.18 g $ZnSO_4 \times 7H_2O$, 0.01 g $CuSO_4 \times 5H_2O$, 0.02 g $KAl(SO_4)_2 \times 12H_2O$, 0.1 g $CaCl_2 \times 2H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \times 2H_2O$, 0.03 g $NiCl_2 \times 6H_2O$ and 0.3 mg $Na_2SeO_3 \times 5H_2O$ in 1 l distilled water. Vitamin solution involves 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine–HCl, 5 mg thiamine-HCl $\times$ 2 H_2O, 5 mg riboflavin, 5 mg Nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B_{12}, 5 mg p-Aminobenzoic acid and 5 mg lipoic acid in 1 l distilled water. Cells from frozen stocks were recovered in 50 ml modified DSMZ 879 medium for 72 h, and then re-transferred into a fresh medium until OD_{600nm} of 0.4 as pre-culture for the further work.

2.5 ml pre-culture was inoculated into 50 ml modified DSMZ 879 medium. As cells grew to an OD_{600nm} of 0.4–0.5, a final concentration of 20 mM acetic acid, 4 mM HCl or 20 mM sodium acetate buffer (pH 5.4) was applied in cultures for monitoring gene-expressions of *C. ljungdahlii* under extra acids. Cell density and pH value were monitored after 30 min and 10 ml of cells were harvested and stored at −80 °C for RNA preparation.

To examine effects of $CaCO_3$ on the growth of *C. ljungdahlii*, 5 ml pre-culture was inoculated into 100 ml modified DSMZ 879 medium containing 0.01 or 0.02 g/l $CaCO_3$. As negative control, no supplement of $CaCO_3$ was used. Cell density and pH value were measured immediately after sampling. For fructose, ethanol and acetic acid analysis, 2 ml samples were centrifuged at 4 °C and 10,000×g for 5 min and supernatants were collected and stored at −80 °C.

2.2. Fed batch fermentation with CO/CO_2

The gas fermentation was carried out anaerobically in a FUS-XL bioreactor (5 l; Guo Qiang, Shanghai, China) containing 3 l of modified DSM 879 base medium (without fructose and yeast extract). The continuously supplied gas was composed of 20% (v/v) CO_2 and 80% (v/v) CO with a constant pressure of 0.16 MPa. The temperature and stirring rate were controlled at 37 °C and 60 rpm. Before inoculating, 150 ml pre-culture of *C. ljungdahlii* was centrifuged at 5000×g and room temperature for 5 min, washed twice with DSMZ 879 base medium to remove the yeast extract and fructose, and then resuspended in the same medium to retain the original starting volume. Cell density and pH value were monitored every 12 h before entering late-log phase and then every 24 h till the end of the fermentation. 15 ml samples were collected for products analysis and RNA preparation.

2.3. Monitoring culture pH, cell growth and metabolic products

The pH was measured with a Sartorius PB-10 standard pH meter (Sartorius, Göttingen, Germany). The cell density was measured by using a 2600 spectrophotometer (Unico instrument, Shanghai, China) at OD_{600nm}. Ethanol, acetic acid and fructose were routinely detected by using an Agilent 1200 Infinity series HPLC system (Agilent Technologies, Santa Clara, USA) equipped with a refractive index detector (RID) (Agilent Technologies, Santa Clara, USA) operated at 35 °C. All samples, prepared by centrifugation at 12,000×g and 4 °C for 5 min and filtration with 0.22 µm filters, were separated by a Hi-Plex H column with the dimension 300 × 7.7 mm and particle size 8 µm (Agilent Technologies, Santa Clara, USA). The column was kept at 60 °C. Slightly acidified water (0.005 M H_2SO_4) was used as the mobile phase, with a flow rate of 0.6 ml/min. Fructose, ethanol and acetate peaks were identified by their standard products. The concentrations were calculated according to peak areas by using their standard curves.

2.4. Gene expression by quantitative real-time PCR

The cell pellets were harvested by centrifugation at 6000×g and 4 °C for 5 min, snap-frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted with the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration and quality were determined with a Biophotometer

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