



Enhanced coproduction of hydrogen and butanol from rice straw by a novel two-stage fermentation process

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ABSTRACT

Lignocellulosic biomass has been increasingly used for biofuel production, such as biohydrogen and biobutanol. However, the enzymes used in hydrolysis result in a large increase in production cost. To reduce the enzyme involvement in lignocellulosic butanol, a novel two-stage fermentation process was constructed by combining a Carboxylate Platform and acetone-butanol-ethanol (ABE) fermentation. First, without enzyme involvement, lignocellulose was converted to volatile fatty acids (VFAs) using the Carboxylate Platform. Subsequently, the VFAs were used as feedstock to generate butanol by ABE fermentation of *Clostridium beijerinckii* NCIMB 8052. Fed with 20 g/L pretreated rice straw, the acidogenic fermentation produced 660 mL/L hydrogen and 6.87 g/L butyric acid, with a total VFAs of 9.52 g/L in the supernatant of fermentation culture (SFC). With 40% rice straw hydrolysate and 60% SFC as co-substrate, hydrogen and butanol productions of 5897 mL/L and 13.8 g/L, respectively, were obtained in the ABE fermentation. Mass balance and energy generation analysis showed that the specific butanol and energy yields from rice straw reached 230 g/kg and 9633.7 kJ/kg, respectively. Additionally, the carbon-flow distribution analysis indicated that the SFC supplement decreased the metabolic flux of acetone producing pathway and increased the metabolic flux of butanol producing pathway. The novel two-stage fermentation process was a more effective approach for energy generation from lignocellulose that avoided external addition of commercial butyric acid and reduced enzyme involvement for fermentation product.

1. Introduction

The rapid depletion of non-renewable fossil fuels has resulted in a global energy crisis, and the development of sustainable alternative fuels has attracted increasing interest all around the world. As the most abundant renewable source in nature, biomass is extensively developed for producing various alternative fuels, such as biohydrogen, bioethanol, biobutanol and biodiesel. Among the biofuels, biohydrogen is known as an ideal renewable energy because of its high energy content and lack of carbon emissions from combustion (Gonzales et al., 2016). Biobutanol is also a high-performance fuel and fits better than ethanol with the current fuel distribution infrastructure because of its low hygroscopicity (Chen et al., 2016).

Acetone-butanol-ethanol (ABE) fermentation of clostridia is well-known for butanol production from glucose and has been extensively investigated (Lertsriwong et al., 2017). In the fermentation process, produced butyric acid serves as the triggering substance to induce the transcription of solvent formation genes in the strains (Wang et al., 2013). In addition, external addition of butyric acid as the co-substrate

can prevent strains from degeneration and enhance sugar utilization and butanol yield (Regestein et al., 2015). However, most of the commercial butyric acid is produced from fossil fuels by chemical processes, which is not sustainable or economical (Zhang et al., 2009). Therefore, the fermentative butyric acid from lignocellulosic biomass is a promising alternative.

Generally, lignocellulosic biomass for biofuel production has to firstly undergo pretreatment (physical, chemical, or their combination) and enzymatic hydrolysis to produce fermentable sugars (Jin et al., 2015). However, the enzymes used for hydrolysis will inevitably result in a great increase in production cost (Green, 2011). Carboxylate Platform has been proposed to reduce or avoid enzymatic pretreatment by employing microbial consortia to convert biomass to short-chain carboxylic acids, such as acetic, propionic, butyric, and other acids (Chang et al., 2010; Zhao et al., 2015). The short-chain carboxylic acids can be used as feedstock to generate biofuels such as ethanol and butanol by bacterial fermentation or using electrochemical and thermochemical methods (Agler et al., 2011). Therefore, Carboxylate Platform was introduced to produce butyric acid, along with some other volatile

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fatty acids (VFAs) as by-products from rice straw by the fermentation of a microbial consortium in the present study. Then, the collected supernatant of fermentation culture (SFC) was used as a co-substrate to produce butanol by ABE fermentation of *Clostridium beijerinckii* NCIMB 8052 (Ye et al., 2012; Hartmanis et al., 1984; Harvey and Meylemans, 2011). The ABE-producing clostridia as an excellent hydrogen producer has been largely ignored. In fact, in addition to ABE fermentation, hydrogen can also be collected during acidogenic fermentation (Kuribayashi et al., 2017).

To reduce the enzyme involvement in lignocellulosic butanol and enhance energy generation, a novel two-stage fermentation process was constructed by combining a Carboxylate Platform and an ABE fermentation. The coproduction of hydrogen and butanol was evaluated, while the carbon-flow distribution, mass balance and energy generation were analyzed.

2. Materials and methods

2.1. Material preparation

The rice straw used for butyric acid fermentation and enzymatic hydrolysis was collected from a local farm in Harbin, China. The raw rice straw was cut into 10–15 cm lengths and pretreated with 1% NaOH solution as described previously (Ai et al., 2016). Mass fraction of cellulose, hemicellulose and lignin in the pretreated rice straw was 53.0%, 27.4% and 8.0%, respectively.

Enzymatic hydrolysis of the pretreated rice straw was carried out in 250 mL Erlenmeyer flasks located in an air-bath incubator (DLHR-Q200, Hadonglian, China) at 50 °C and 140 runs per minute (rpm). Each of the flasks was loaded with 80 g/L pretreated rice straw and 100 mL 0.05 mol/L acetate as buffer (pH 4.8). The cellulase (Sigma Chemicals) load in each flask was the same 30 FPU/g dry pretreated rice straw in terms of filter paper activity (FAP). To get a final substrate concentration of 120 g/L, the pretreated rice straw (20 g/L) was added twice at the 12th and 24th hour during hydrolysis with the cellulase (10 FPU/g dry pretreated rice straw). At the 72nd hour of hydrolysis, the flasks were autoclaved at 115 °C for 20 min, and then the rice straw hydrolysate was collected via centrifugation and stored at 4 °C (no longer than 4 h) for ABE fermentation tests. The reducing sugar concentration in the collected hydrolysate was approximately 94 g/L.

2.2. Inocula

2.2.1. Microbial consortium for lignocellulosic butyric acid production

The microbial consortium for fermentation of the pretreated rice straw to produce butyric acid was a stable mixed culture obtained previously (Binling et al., 2013) and was designed as consortium DCB17. Consortium DCB17 was dominated by cellulosic and xylanolytic bacteria, butyrate-producing bacteria and other acidogenic bacteria (Ai et al., 2016), which was maintained as spores in fresh peptone cellulose solution (PCS) medium at 4 °C. The PCS medium contained the following per 1 L distilled water: 10 g pretreated rice straw, 5.0 g tryptone, 1.0 g yeast extract, 5.0 g NaCl, 2.0 g CaCO₃, and 0.5 g D-cysteine hydrochloride.

2.2.2. Strain for ABE fermentation

C. beijerinckii NCIMB 8052 obtained from the China General Microbiological Culture Collection Center (CGMCC) was used for synchronous production of butanol and hydrogen by ABE fermentation. The strain was maintained in the form of spores in fresh peptone-yeast glucose (PYG) medium at 4 °C (Chen et al., 2013). For inoculum preparation, the stored spore suspension was transferred into fresh P2 medium containing 10.0 g/L glucose and then incubated at 140 rpm and 37 °C for 18–22 h. After incubation, the cells were harvested by centrifugation at 3000 rpm for 5 min. The harvested cells were suspended in 1 mL of P2 medium and the bacterial suspension was used as

inoculum. The P2 medium contained the following per 1 L distilled water: 30.0 g glucose, 3.0 g yeast extract, 0.5 g K₂HPO₄, 0.5 g KH₂PO₄, 0.001 g p-aminobenzoic acid, 0.001 g thiamine, 0.0001 g biotin, 0.2 g MgSO₄·7H₂O, 0.01 g MnSO₄·H₂O, 0.01 g FeSO₄·7H₂O, and 0.01 g NaCl.

2.3. Fermentation tests

2.3.1. Acidogenic fermentation

As the first stage in the proposed two-stage fermentation process, acidogenic fermentation of consortium DCB17 was performed in 500 mL serum bottles, each of which contained 200 mL PCS medium and 20 g/L pretreated rice straw in terms of dry weight. The broth was adjusted to a pH of 6.5 with 4 mol/L HCl solution and flushed with N₂ for 10 min. After autoclaving at 115 °C for 20 min and cooling to room temperature, 5% (v/v) of the stored consortium DCB17 was inoculated. All of the fermentation systems were incubated at 35 °C and 140 rpm for 6 days. Biogas in each of the fermentation systems was measured daily, and approximately 1.3 mL broths were sampled for pH and liquid product detection. Weight loss from the rice straw in each of the systems was measured at the end of fermentation.

2.3.2. ABE fermentation

ABE fermentation of *C. beijerinckii* NCIMB 8052 was performed in M-1, M-2 and M-3 mediums. In M-1, glucose was the only carbon source with a concentration of 30.0 g/L. In addition to the 30.0 g/L glucose, 6.25 g/L sodium butyrate (5.00 g/L in terms of butyric acid) was added in M-2 as co-substrate. M-3 was prepared with 40% (v/v) rice straw hydrolysate and 60% (v/v) SFC mixed with the nutrients in P2.

After acidogenic fermentation of the pretreated rice straw, SFC was collected via centrifugation to remove the insoluble and microbial residues. The SFC was used as co-substrate of the rice straw hydrolysate for hydrogen and butanol production by *C. beijerinckii* NCIMB 8052. 30 mL SFC and the nutrients in P2 medium were transferred into 160-mL screw capped bottles and flushed with N₂ for 10 min. The bottles were sealed and sterilized in an autoclave at 115 °C for 20 min. After naturally cooling down to room temperature, each of the bottles were inoculated with the bacterial suspension of *C. beijerinckii* NCIMB 8052 and 20 mL sterile rice straw hydrolysate in sequence (M-3). The inoculation amount of the strain was determined by the optical density at 600 nm (OD₆₀₀) of 1.0 in the 50-mL broth. M-1 and M-2 were inoculated in the same way, and 20 mL of 2.5-fold concentrated sterile glucose solution was added instead of the rice straw hydrolysate. The fermentation systems constructed with M-1 acted as the controls with a pH of approximately 6.8. An initial pH of approximately 5.0, adjusted with 4 mol/L HCl solution, was used in the M-2 and M-3 fermentations. Each test was performed in triplicate, and all of the cultures were incubated at 37 °C and 140 rpm.

During the 6-day fermentation process, biogas in each of the fermentation systems was measured daily with approximately 1.3 mL broths sampled with syringes. After the determination of pH, the samples were diluted and used to monitor the concentrations of sugar, VFAs and solvents. Carbon-flow distribution of ABE fermentations were analyzed, the detail calculation method were shown in the [Supporting file](#).

2.4. Analytical methods

The biogas produced in each of the fermentation systems was measured by releasing the gas pressure to equilibrate with room pressure (Binling et al., 2013). The composition of the biogas was analyzed with a gas chromatograph (SP-6800A, Shandong Lunan Instrument Factory, China) equipped with a thermal conductivity detector and a 2-m stainless column packed with Porapak Q (60/80 mesh, Lanzhou ZhongKeKaiDi Chemical Newtech Co., Ltd, China). The carrier gas was N₂. The temperature of the column oven was set at 50 °C, and the temperature was set at 80 °C for both the injection port and the

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