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Acetone–butanol–ethanol production by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) methods using acorns and wood chips of *Quercus acutissima* as a carbon source

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ABSTRACT

We investigated the production of acetone–butanol–ethanol (ABE) from acorns (starchy material) and wood chips (cellulosic material) of *Quercus acutissima* through separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) by *Clostridium acetobutylicum* NBRC13948. With acorns, 15.45 g/L of ABE was obtained in 96 h of fermentation time from the enzymatic hydrolyzate of acorns by SHF. From the SSF, it was found that ABE (16.70 g/L, 120 h) was produced without enzyme addition, given that *C. acetobutylicum* NBRC 13948 possesses an amylolytic enzyme. With wood chips, steam explosion was used for pretreatment. In the SHF, when the enzymatic hydrolyzate of residue of water and methanol extraction after pretreatment was used as the substrate, the maximum ABE concentration, 15.29 g/L, was obtained in 120 h. From the SSF, 13.41 g/L of ABE was obtained in 144 h, and it was determined that sufficient enzyme loading was 7.8 mg of protein to 1 g of dry substrate.

1. Introduction

Butanol is an important industrial fuel. Compared with ethanol, butanol has higher energy content and lower volatility and is less hygroscopic and less corrosive (Lee et al., 2008a,b). *Clostridium acetobutylicum* and *C. beijerinckii* are two bacterial strains often used for acetone–butanol–ethanol (ABE) fermentation. Recently, many researchers have studied ABE production by these two organisms via ABE fermentation of unutilized cellulosic and starchy biomass (Qureshi et al., 2010a,b; Sun and Liu, 2012; Liu et al., 2010; Survase et al., 2013).

Quercus acutissima, i.e., sawtooth oak, is a fast-growing tree. *Quercus* spp. are widespread across Asia, Europe, North America, and Africa. They have been used for different purposes such as bioenergy (Lee et al., 2008a,b), charcoal (Tang et al., 2009), timber (Tang et al., 2009), cultivation beds for mushrooms (Okamura et al., 2001), and acorn production (Canellas et al., 2007). In particular, *Q. acutissima*, native to eastern Asia, is an excellent hard broadleaved energy and timber tree species in China, Korea, and Japan. Trees of this species bear many acorns. Acorns are rich in starch and are unutilized, except as animal food. Their high starch content represents great potential as a fermentation substrate. L-Lactic acid has been produced from hydrolyzed acorn starch (Lu et al., 2010). However, there are no reports on the production of butanol from *Q. acutissima* as a cellulosic material and acorns as a starchy material. Advanced utilization of these materials as carbon resources holds promise for the bioproduction of butanol.

Production technology of bioliquid fuels such as ethanol and butanol from lignocellulosic biomass can be classified into two major process: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In SHF, enzymatic hydrolysis and fermentation are carried out in separate steps. This makes it possible to run each process under its optimum conditions, but end-products (cellobiose and glucose) of enzymatic hydrolysis inhibit cellulase activity, therefore, this factor results in a large reduction in reaction rate (Hong et al., 1981; Alfani et al., 1990). In SSF, enzymatic hydrolysis and fermentation are carried out in same reaction vessel where the glucose formed is rapidly converted into bioliquid fuel by the microorganisms. From these factors, the SSF has some advantages of lower equipment cost, lower contamination risk, lower process time, and higher production rates (Stenberg et al., 2000). Disadvantage of SSF is that the conditions for enzymatic hydrolysis and fermentation have to be the same, each process is carried out in suboptimal environment. For butanol production, there are few reports on the SSF method. In case of acorns (a starchy material), Jang et al. (2012) reported

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that *C. acetobutylicum* excreted α-amylase into the culture medium when starch was the carbohydrate source, therefore, SSF without a starch-hydrolytic enzyme may proceed. In case of wood (a cellulosic material), because cellulose is surrounded by a lignin network, it is necessary to decompose lignin using an environmentally friendly pretreatment method prior to the enzymatic conversion of cellulose into glucose. Recently, many pretreatment methods such as acidic treatment (García et al., 2014; Sasaki et al., 2014; Scordia et al., 2013), alkali treatment (Asgher et al., 2013), thermal treatment (Buruiana et al., 2014), and physical treatment (Khullar et al., 2013) were investigated to treat plant materials. Steam explosion is one of the hydrothermal pretreatment methods for lignocellulosic biomass that uses high-pressure and high-temperature steam without the addition of any chemicals to cause autohydrolysis, defibriation, and delignification (Sasaki et al., 2012; Ballesteros et al., 2011; Asada et al., 2011a). This process has several potential advantages over conventional chemical treatments; it eliminates the use of toxic substances such as strong acids or alkalis, chemical-tolerant equipment, waste processing systems, and high initial feedstock volume.

In the present study, to investigate efficient ABE production from unutilized plant materials, i.e., acorns and (steam exploded) wood of *Q. acutissima*, two types of fermentation modes, SHF and SSF, were applied using *C. acetobutylicum* NBRC 19438. ABE production performance from different resources and using different operation strategies was investigated and compared to identify preferable fermentation modes.

2. Materials and methods

2.1. Acorns and wood chips of Q. acutissima

Acorns of *Q. acutissima* were collected from a park in Tokushima city and maintained at room temperature. Wood chips (1–2 cm) of *Q. acutissima* were kindly provided by Tokushima city and stored at room temperature. The moisture content were 51.0% for acorns and 8.8% of wood chips, respectively. Acorns were ground using a hammer mill (Fuji Pawdal Co., Ltd., Osaka, Japan) to a mesh size of 250 μm.

2.2. Steam explosion of wood chips

Wood chips were pretreated in a steam explosion apparatus (Japan Chemical Engineering and Machinery Co. Ltd., Osaka, Japan). The reactor was charged with 100 g (dry matter) of feedstock per batch. Saturated steam from the boiler was then allowed to enter the reactor to heat the bagasse at 35 atm (243 °C). Pressure was maintained for 5 min, following which the reactor was instantly depressurized. The exploded wood chips were recovered in a cyclone and cooled to room temperature.

2.3. Component analysis of acorns and wood chips

Component analysis of acorns was performed as follows:

The total lipid content was determined by continuous extraction in a Soxhlet apparatus for 12 h using ethyl ether as a solvent, and the ash content was determined using the AOCO methods (2000). Starch and crude protein contents were determined by a modified Southgate procedure (Nakaji et al., 1993). Nitrogen was determined using the Kjeldahl and AOAC methods, and a factor of 6.25 was used to convert nitrogen to crude protein. All the analytical determinations were performed in duplicate, and average results are shown.

Component analysis of untreated and steam exploded wood chips was performed using distilled water and methanol as extraction solvents based on previous reports (Sasaki et al., 2012). Water-soluble material, methanol-soluble material, acid insoluble material (contains Klason lignin, i.e., high molecular lignin), acid-soluble lignin, and sugars derived from cellulose and hemicellulose were determined as follows:

In total, 5 g of dry untreated or steam-exploded wood chips were added to 100 mL of distilled water and incubated for 24 h at room temperature. Solid and liquid materials were separated by filtration, and the filtrate was recovered from the liquid, concentrated, dried, and weighed (water-soluble material). This water-washed residue was treated at room temperature for 24 h in a Soxhlet extractor using 150 mL methanol to dissolve methanol-soluble material. After concentration and drying of the extract, the methanol-soluble material was weighed. Water- and methanol-extracted residues consisted of holocellulose and acid insoluble material, a high-molecular-weight lignin. Following this, 15 mL of 72% (w/w) H_2SO_4 was added to 1 g of this residue, and the solution was left at room temperature for 4 h. The residue was placed in a 100-mL conical flask, washed with 560 mL of distilled water, and autoclaved for 1 h. After the insoluble material was washed with distilled water, it was heat dried at 105 °C to constant weight, and its mass was recorded (acid insoluble material). Acid-soluble lignin in the hydrolyzed liquid was determined by UV spectrophotometry at 205 nm. The cellulose content was determined on the basis of the monomer content (glucose) measured after hydrolysis (72% (w/w) H_2SO_4 followed by dilution). Glucose in the hydrolyzate was evaluated by HPLC equipped with a refractive index detector and using a Bio-Rad HPX-87H column at 50 °C with 5.0 mM H_2SO_4 as the mobile phase at 0.6 mL/min. The hemicellulose content was determined by subtraction of the cellulose content from the holocellulose content. The holocellulose content was determined as the $NaClO_2$-delignified residue (Wise et al., 1946). All analytical determinations were performed in duplicate, and the means were calculated.

2.4. Enzymatic hydrolysis of acorns and wood chips

The milled acorns and steam-exploded wood chips were enzymatically hydrolyzed using glutase-AN (13,000 units/g, kindly provided by HBI Enzymes Inc.) and Cellic CTec 2 (150 FPU/mL, produced by *Trichoderma reesei*, kindly provided by Novozymes Japan Ltd.), respectively. Enzymatic hydrolysis was performed using 10 mL of 0.05 M sodium phosphoric acid buffer (pH 5.0) at 50 °C on a rotary shaker at 140 rpm for 48 h. The substrate concentration and enzyme loadings were 50 g/L and 1.0 mg/g substrate (glutase-AN) and 270 μl/g substrate (15.5 mg enzyme protein/g substrate, Cellic CTec 2), respectively. The supernatant was centrifuged to remove the solid wastes and analyzed for glucose. The protein content in enzymes was determined using the Bradford assay (Bradford, 1976). All enzymatic hydrolysis experiments were performed in duplicate, and the means were calculated.

The enzymatic saccharification rate was calculated as follows:

$$\frac{\text{Amount of glucose produced (g)}}{\text{Amount of starch or cellulose and hemicellulose contained in each substrate}/0.9\,\text{(g)}} \times 100.$$

2.5. Microorganisms and inoculum cultivation

C. acetobutylicum NBRC 13948 (obtained from the National Institute of Technology and Evaluation, Biological Resource Center, NBRC, Chiba, Japan) was used to produce ABE. The stock strain was subcultured every 4 weeks in Difco reinforced clostridial