Butyric Acid Fermentation of Sodium Hydroxide Pretreated Rice Straw with Undefined Mixed Culture

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Introduction

With the rising tightness of the world’s crude oil supply and the challenges of the environment and global warming, growing attention has been devoted to the conversion of carbon-neutral lignocellulosic biomass into biofuels and biobased products. There are three biorefinery platforms used to produce bioproducts from lignocellulose. The most well-known one is the sugar platform, in which cellulose and hemicellulose are converted by cellulolytic enzyme into six- and five-carbon sugars as intermediates that can be fermented to various chemicals by different microorganisms [8]. In the syngas platform, solid biomass is processed thermochemically under anaerobic conditions into gaseous components that can be converted into fuels or valuable chemicals [8, 12]. The third one is a carboxylate platform using an undefined mixed culture of microorganisms to produce a mixture of carboxylates as intermediate platform chemicals towards the generation of complex biofuels and biochemicals [1, 15]. The carboxylates are composed mainly of acetate and butyrate, with small amounts of propionate, lactate, and valerate. A patented process was developed to convert the carboxylates, mainly acetate, from organic wastes into mixed alcoholic fuels, which can be blended with gasoline [6, 14]. Physicochemical properties suggest that butyrate would be superior to acetate as intermediate products for further processing to biofuels, because butyrate has a longer hydrophobic carbon chain and a lower oxygen/carbon ratio, which increases the energy density. It was reported that supplementing butyric acid to an acetone–butanol–ethanol (ABE) fermentation process enhanced the butanol yield [2, 23, 31]. Furthermore, the mixed carboxylates rich in butyrate could also be used to produce polyhydroxybutyrates [3, 5], the dominant polyhydroxyalkanoates that are the raw materials for the production of biodegradable plastics. Butyrate is itself a valuable product when separated from the culture broth, and has been widely used in chemical, food, and pharmaceutical industries [39].

Pretreatment has been studied as a key technology for...
the efficient utilization of lignocellulosic biomass. An important effect of pretreatments is the delignification, because lignin is known to inhibit the hydrolysis of cellulose and hemicellulose [10]. Sodium hydroxide pretreatment is recognized as an effective method for delignification as well as swelling of biomass to increase digestibility [7, 18, 30, 36–38, 40]. This pretreatment can be carried out at a low temperature under atmospheric pressure but with a relatively long time. It was reported that alkaline pretreatment at lower temperature retained more cellulose and hemicellulose in solid [19, 20], which inferred less loss of fermentable sugars and no need of separate recovery of xylose from the pretreatment liquid. In this work, soaking in dilute NaOH solution as the pretreatment method of rice straw at relatively low temperature was attempted. Butyric acid fermentation with an undefined cellulose-degrading butyrate-producing microbial community was conducted to evaluate the pretreated rice straw’s potential for butyric acid production. Batch fermentation and repeated-batch fermentation were performed and compared. The main advantage of batch fermentation is the simplicity of setup and operation, but also with disadvantages of turnaround time for cleaning, medium introduction, inoculation, and cell lag phase [33]. A repeated-batch fermentation process, in which a part of the culture broth is used as inoculum for the subsequent batch, shortens or skips the nonproductive time [16]. It is more essential to keep cell productivity constant because repeated-batch operation often continues for a long time [29], especially concerning mixed culture fermentation. Owing to the use of undefined mixed cultures with high microbial diversity as inocula, more stringent operation conditions are needed to keep the processes stable and to generate narrow product spectrums [21]. Denaturing gradient gel electrophoresis (DGGE) analysis, followed by cloning and sequencing, was applied to monitor the changes of microbial community structure in the batch fermentations and repeated-batch fermentations.

Materials and Methods

Materials

The rice straw was collected from a local farm in Harbin, China, and cut into 10–15 cm lengths for storage. Before pretreatment, the stored rice straw was placed in an oven overnight at 50°C, after which the moisture content of the rice straw was less than 5%. Cellulase used in enzymatic saccharification was purchased from Imperial Jade Biotechnology Co., Ltd, China. The average activity of the enzyme was 10 filter paper units per milliliter (FPU/ml). One FPU is defined as the amount of cellulase that releases 1 µmol of glucose from filter paper per minute.

Sodium Hydroxide Pretreatment of Rice Straw

Size-reduced rice straw was soaked in 1% sodium hydroxide solution at 50°C or room temperature (18–28°C) for 24, 48, or 72 h in static status. A solid-to-liquid ratio of 1:15 (w/v) was applied. The solid residue was separated by filtering and thoroughly washed with tap water to near-neutral pH. The neutralized residue was squeezed and refrigerated at 4°C for further use. The moisture content of the pretreated rice straw was 81.8%.

Enzymatic Digestibility of the Pretreated Rice Straw

The enzymatic digestibility tests of rice straw pretreated at different treatment intensities were carried out in 250 ml Erlenmeyer flasks containing 100 ml of 0.05 mol/l citrate buffer (pH 4.8) at 50°C in a rotary shaker (FLY-2102C; Shanghai Shenxian Thermostatic Equipment Factory, China) at 120 rpm. One gram of rice straw (dry weight) and 50 FPU of cellulase were loaded. All tests were run in triplicate. Liquid samples were taken periodically for reducing sugar analysis to calculate the enzymatic digestibility.

Inoculum Preparation

The butyric acid producer, an undefined cellulose-degrading butyrate-producing microbial community, was an enrichment culture derived from the combination of cattle manure, pig manure compost, soil from a corn field, and rotten wood. For inoculum preparation, the stored microbial community was transferred to fresh peptone cellulose solution (PCS) medium. The PCS medium comprised 10 g of pretreated rice straw (dry weight), 5 g of tryptone, 1 g of yeast extract, 5 g of NaCl, 2 g of CaCO3, and 0.5 g of d-cysteine hydrochloride per liter [13], and one filter paper strip (1.5 cm × 5 cm) was added as an indicator. The broth was purged with nitrogen gas for 10 min to maintain totally anoxic conditions, and then the 150 ml serum bottle containing 100 ml of broth was sealed and autoclaved at 115°C for 20 min. After inoculation with 5 ml of stored culture, the preculture was incubated at 35°C without agitation. When the filter paper strip was broken down, 5 ml of totally mixed preculture was transferred into another fresh PCS medium with a filter paper strip for preparing inoculum 2, and the rest was stored at 4°C as inoculum 1 (Fig. 1). Inoculum 3 was prepared from inoculum 2 by the same procedure.

Butyric Acid Fermentation of the Pretreated Rice Straw

Besides enzymatic digestibility tests, butyric acid fermentations were also conducted to evaluate the biodigestibility of the pretreated rice straw. The fermentation trials were performed in triplicate in 500 ml serum bottles with 200 ml of PCS medium with 20 g of rice straw (dry weight). The fermentation media were prepared in the same way as inoculum culture. Five milliliters of preculture was inoculated into each serum bottle followed by incubation at 35°C with agitation of 140 rpm for 6 days. During the 6 days of the culture, gas and liquid samples were taken every day for the analysis of biogas, pH, and volatile fatty acids (VFAs). At the end of the fermentation, the biomass and weight loss of the rice straw were determined.
Batch fermentation and repeated-batch fermentation were performed to estimate the two processes for butyric acid production. The operational procedure is outlined in Fig. 1. In batch operation, the inocula were the three consecutive generations of microbial community. In repeated-batch operation, the inocula were the culture broths of their last batches.

**Analytical Methods**

The cellulose, hemicellulose, lignin, and water contents of rice straw were measured using the method described by Van Soest et al. [35]. A scanning electron microscope (JEOL JSM-6380LV) was used to take images of treated and untreated rice straw at 10 kV acceleration voltage after gold coating. The 3,5-dinitrosalicylic acid (DNS) method was used to analyze the reducing sugar from saccharification of rice straw [11].

The total biogas volume was measured by releasing the gas pressure in the bottles using a 100 ml glass syringe to equilibrate with the room pressure. Gas analyses of hydrogen, methane, and carbon dioxide were analyzed by a gas chromatography system (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) [24]. Liquid samples were centrifuged at 8,000 × g for 5 min, and then 1 ml of supernatant was acidified with 0.1 ml of 25% phosphoric acid before VFA analysis. The VFA determination was performed using a gas chromatography system (SP-6800A; Shandong Lunan Instrument Factory, China) equipped with a flame ionization detector and a FFAP capillary column (30 m × 0.32 mm × 0.50 μm; Lanzhou ZhongKeKaiDi Chemical New-tech Co., Ltd, China). The temperatures of the injection port, column oven, and detector were set at 220°C, 180°C, and 220°C, respectively. Nitrogen was used as the carrier gas, with 0.05 MPa column head pressure and a flow constant rate of 50 ml/min. The split ratio was 50. The injection volume was 1 μl.

Microbial biomass was estimated by optical density at 260 nm as follows [26]. To 5 ml of the culture broth, 5 ml of 1 M/1 HClO₄ solution was added. If required, the samples were diluted to adjust the optical density within the readable range. The tubes were placed in boiling water for 20 min and then cooled to room temperature. The contents of the tubes were centrifuged and the OD of the supernatant was measured at 260 nm. The OD₂₆₀ of cultured broth was denoted by [A]. Uncultured broth was washed as a blank, and OD₂₆₀ was denoted by [B]. The microbial biomass was denoted as [A]–[B].

**DGGE Analysis**

Microbial cells from 1.5 ml of totally mixed culture were collected by centrifugation at 13,000 rpm for 5 min, and the pellets were washed three times with 10 mM phosphate buffer solution (pH 7.4). Bacterial genomic DNA was extracted and purified using the Bacteria DNA Mini Kit (Watson Biotechnologies, Shanghai, China) according to the manufacturer’s instructions.

From extracted DNA, the bacterial 16S rRNA gene was amplified with primer pairs BSF 8 (5'-AGAGTTTGATCCTGGCTCAG-3', *E. coli* 16S rRNA position of 8–20) and BSR 534 (5'-ATTACCGCGGCTGCTGG-3', *E. coli* 16S rRNA position of 517–534) with a GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGGCA CGGGGGG-3') at the 5' end. Each 50 μl PCR mixture contained 5 μl of 10× Ex Taq buffer, 4 μl of 2 mM dNTP mixture, 1 μl of 20 μM each of forward and reverse primers, 0.5 U Ex Taq DNA polymerase (Takara, Dalian, China), 38 μl of sterile distilled water, and 0.5 μl of the DNA extract. The PCR cycling protocol included an initial denaturation step at 94°C (10 min), followed by 32 cycles of denaturation (94°C, 1 min), annealing (55°C, 45 sec), and extension (72°C, 45 sec), with a final extension at 72°C for 10 min. The amplicons were examined by electrophoresis on 0.8% (w/v) agarose gel.

Bacterial amplicons were subsequently separated by DGGE performed with a Bio-Rad DCode system (Bio-Rad Laboratories, USA). The denaturing gradient gel was prepared to contain 1× TAE buffer, 8% acrylamide-βis-acylamide, and 30% to 60% denaturant (100% denaturant contains 7 M urea and 40% deionized formamide). Electrophoresis was performed at 120 V for 6 h in 0.5× TAE buffer at 60°C. Gels were silver-stained according to the method described previously [4]. Prominent DGGE bands were cut from the gel and crushed in 50 μl of 1× TAE buffer. After equilibration at 55°C for 3 h and centrifugation at 11,000 × g for 3 min, 1 μl of each supernatant was used as the template for PCR amplification as described above, but with the primer BSR 534 without a GC clamp. The PCR products were purified using the Gel Recovery Purification Kit (Shanghai Watson Biotechnologies, China) and cloned in *E. coli* competent cells using the pMD-T 18 plasmid vector system (Takara Dalian, China) according to the manufacturer’s instructions.

One positive clone after the blue/white screening and colony PCR from each DGGE band was maintained in agar stab culture and sent to Shanghai Sangon, China for sequencing analysis. The
analyzed 16S rRNA gene partial sequences were compared with sequences in the GenBank database using the NCBI BLAST search program. The closest cultured relatives were retrieved from the database with their degrees of similarity. The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers KC905172 to KC905179.

Statistical Analysis
Statistical analysis was performed using Statistical Product and Service Solutions (SPSS, ver. 19.0).

Results
Improvement of Biodigestibility of Rice Straw by Sodium Hydroxide Pretreatment
Two different pretreatment temperatures (room temperature and 50°C) and three different pretreatment times (24, 48, and 72 h) were applied to investigate their effects on the composition changes and biodigestibility of rice straw. The results are summarized in Table 1. As shown in Table 1, an increase in alkali treatment intensity improved the delignification and the subsequent enzymatic and microbial digestibility, but compromised the fermentable sugars recovery in solid residue. Table 2 presents the results of the butyric acid fermentations with the rice straw untreated and pretreated at different pretreatment conditions. Acetic and butyric acids were the main products from the mixed culture fermentations. The two products contributed at least 85% to the total VFAs. As seen in the table, increasing pretreatment temperature and time enhanced the digestibility of rice straw significantly. In the fermentation of rice straw

![SEM pictures of raw rice straw (A), rice straw pretreated at 50°C for 72 h (B), raw rice straw after enzyme hydrolysis (C), rice straw pretreated at 50°C for 72 h after enzyme hydrolysis (D), raw rice straw after fermentation (E), and rice straw pretreated at 50°C for 72 h after fermentation (F).](image)
Table 1. Composition changes and biodigestibility of rice straw soaked in 1% NaOH solution at different pretreatment temperatures and times.

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Weight loss (%)</th>
<th>Celluloseb (%)</th>
<th>Hemicelluloseb (%)</th>
<th>Ligninb (%)</th>
<th>Delignification (%)</th>
<th>Enzymatic digestibility</th>
<th>Microbial digestibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total reducing sugars (g/l)</td>
<td>Cellulose (%)</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.0 ± 0.0 A d</td>
<td>40.7 ± 1.3 A</td>
<td>24.8 ± 2.2 A</td>
<td>15.3 ± 0.5 A</td>
<td>0.0 ± 0.0 A</td>
<td>1.0 ± 0.1 A</td>
<td>16.6 ± 1.6 A</td>
</tr>
<tr>
<td>RT, 24 h</td>
<td>15.5 ± 1.2 B</td>
<td>39.5 ± 1.9 A</td>
<td>22.5 ± 1.8 AB</td>
<td>13.1 ± 0.4 B</td>
<td>14.4 ± 2.8 B</td>
<td>2.4 ± 0.5 B</td>
<td>34.2 ± 2.0 B</td>
</tr>
<tr>
<td>RT, 48 h</td>
<td>21.6 ± 1.9 C</td>
<td>37.5 ± 3.1 AB</td>
<td>21.7 ± 2.5 ABC</td>
<td>10.5 ± 0.4 C</td>
<td>31.4 ± 2.3 C</td>
<td>3.8 ± 0.1 C</td>
<td>54.5 ± 3.2 C</td>
</tr>
<tr>
<td>RT, 72 h</td>
<td>29.6 ± 2.8 D</td>
<td>35.4 ± 1.9 B</td>
<td>19.6 ± 1.9 BCD</td>
<td>7.8 ± 0.4 D</td>
<td>49.0 ± 2.6 D</td>
<td>5.4 ± 0.6 D</td>
<td>72.5 ± 4.8 D</td>
</tr>
<tr>
<td>50°C, 24 h</td>
<td>19.3 ± 1.6 C</td>
<td>37.4 ± 1.6 AB</td>
<td>23.9 ± 2.5 A</td>
<td>11.1 ± 0.6 C</td>
<td>27.5 ± 4.0 C</td>
<td>3.0 ± 0.2 E</td>
<td>43.2 ± 2.7 E</td>
</tr>
<tr>
<td>50°C, 48 h</td>
<td>30.4 ± 2.1 D</td>
<td>35.2 ± 1.0 B</td>
<td>18.5 ± 1.8 CD</td>
<td>6.7 ± 0.5 E</td>
<td>56.2 ± 3.0 E</td>
<td>6.2 ± 0.2 F</td>
<td>84.2 ± 2.7 F</td>
</tr>
<tr>
<td>50°C, 72 h</td>
<td>35.3 ± 1.6 E</td>
<td>34.3 ± 1.7 B</td>
<td>17.7 ± 1.0 D</td>
<td>5.2 ± 0.4 F</td>
<td>66.0 ± 2.6 F</td>
<td>6.8 ± 0.2 G</td>
<td>90.3 ± 1.6 G</td>
</tr>
</tbody>
</table>

aRoom temperature, 18–28°C.
bThe data in this column are based on the oven-dried untreated biomass. For example, the data 34.3 does not mean that the oven-dried solid residue contains 34.3% cellulose, but infers the residue (64.7% of untreated biomass) contains 53.0% cellulose.
cDigestibility (%) = ([grams of cellulose or hemicellulose in digested solid residue]/[grams of cellulose or hemicellulose in loaded solid residue]) × 100.
dMeans with standard deviation in the same column followed by the same letter do not differ significantly at P = 0.05, according to Duncan’s multiple range test.

Table 2. Fermentation results of rice straw pretreated with 1% NaOH solution at different pretreatment temperatures and times.

<table>
<thead>
<tr>
<th>Fermentation batches of pretreated rice straw</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>Consumption of rice straw (g/l)</th>
<th>Acetic acid (g/l)</th>
<th>Propionic acid (g/l)</th>
<th>Butyric acid (g/l)</th>
<th>Valeric acid (g/l)</th>
<th>Total VFAs (g/l)</th>
<th>Product selectivity (%)</th>
<th>Biomass (OD260 after HClO4 hydrolysis)</th>
<th>Butyric acid yield (g/g rice straw fed)</th>
<th>VFAs yield (g/g rice straw fed)</th>
<th>Cumulative hydrogen yield (ml/g rice straw fed)</th>
<th>Cumulative CH4 yield (ml/g rice straw fed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>7.03 ± 0.05 A d</td>
<td>7.74 ± 0.01 B</td>
<td>6.07 ± 0.04 A</td>
<td>3.5 ± 0.3 A</td>
<td>0.3 ± 0.0 A</td>
<td>0.6 ± 0.0 A</td>
<td>0.20 ± 0.02 A</td>
<td>3.3 ± 0.2 A</td>
<td>17.2 ± 1.7 A</td>
<td>2.0 ± 0.2 A</td>
<td>0.03 ± 0.00 A</td>
<td>0.17 ± 0.01 C</td>
<td>4.2 ± 0.6 A</td>
<td>3.1 ± 0.6 A</td>
</tr>
<tr>
<td>RT, 24 h</td>
<td>7.70 ± 0.08 B</td>
<td>7.62 ± 0.06 B</td>
<td>5.25 ± 0.04 CD</td>
<td>7.3 ± 0.3 C</td>
<td>0.4 ± 0.0 B</td>
<td>2.3 ± 0.1 B</td>
<td>0.02 ± 0.00 E</td>
<td>4.5 ± 0.0 C</td>
<td>58.3 ± 1.4 C</td>
<td>3.1 ± 0.1 B</td>
<td>0.11 ± 0.00 B</td>
<td>0.15 ± 0.01 C</td>
<td>4.3 ± 0.2 D</td>
<td>3.1 ± 0.4 A</td>
</tr>
<tr>
<td>RT, 48 h</td>
<td>7.65 ± 0.07 B</td>
<td>7.59 ± 0.15 B</td>
<td>5.15 ± 0.03 DE</td>
<td>8.4 ± 0.5 D</td>
<td>0.5 ± 0.0 B</td>
<td>2.9 ± 0.1 C</td>
<td>0.04 ± 0.02 DE</td>
<td>5.9 ± 0.1 D</td>
<td>58.1 ± 1.4 C</td>
<td>4.4 ± 0.0 D</td>
<td>0.19 ± 0.01 B</td>
<td>0.17 ± 0.00 D</td>
<td>4.3 ± 0.2 D</td>
<td>3.3 ± 0.4 A</td>
</tr>
<tr>
<td>RT, 72 h</td>
<td>7.65 ± 0.07 B</td>
<td>7.66 ± 0.09 B</td>
<td>5.27 ± 0.07 C</td>
<td>8.5 ± 0.4 D</td>
<td>0.3 ± 0.0 A</td>
<td>3.4 ± 0.1 D</td>
<td>0.04 ± 0.02 DE</td>
<td>4.9 ± 0.1 C</td>
<td>52.4 ± 2.2 B</td>
<td>4.4 ± 0.0 D</td>
<td>0.3 ± 0.00 A</td>
<td>0.13 ± 0.00 D</td>
<td>4.4 ± 0.2 D</td>
<td>3.3 ± 0.6 A</td>
</tr>
<tr>
<td>50°C, 24 h</td>
<td>7.59 ± 0.15 B</td>
<td>7.66 ± 0.09 B</td>
<td>5.08 ± 0.08 EF</td>
<td>10.0 ± 0.2 E</td>
<td>0.3 ± 0.0 A</td>
<td>2.6 ± 0.1 E</td>
<td>0.07 ± 0.01 CD</td>
<td>6.6 ± 0.0 E</td>
<td>69.0 ± 0.7 D</td>
<td>4.5 ± 0.1 D</td>
<td>0.11 ± 0.00 A</td>
<td>0.23 ± 0.00 C</td>
<td>4.5 ± 0.2 D</td>
<td>4.4 ± 0.2 B</td>
</tr>
<tr>
<td>50°C, 48 h</td>
<td>7.59 ± 0.15 B</td>
<td>7.66 ± 0.09 B</td>
<td>5.02 ± 0.06 F</td>
<td>11.6 ± 0.5 F</td>
<td>0.3 ± 0.0 A</td>
<td>4.5 ± 0.0 F</td>
<td>0.07 ± 0.01 CD</td>
<td>7.9 ± 0.1 F</td>
<td>75.9 ± 1.3 E</td>
<td>4.5 ± 0.1 D</td>
<td>0.10 ± 0.00 A</td>
<td>0.30 ± 0.00 G</td>
<td>4.7 ± 0.4 B</td>
<td>4.7 ± 0.4 B</td>
</tr>
<tr>
<td>50°C, 72 h</td>
<td>7.59 ± 0.15 B</td>
<td>7.66 ± 0.09 B</td>
<td>5.02 ± 0.06 F</td>
<td>11.6 ± 0.5 F</td>
<td>0.3 ± 0.0 A</td>
<td>4.5 ± 0.1 D</td>
<td>0.07 ± 0.01 CD</td>
<td>7.9 ± 0.1 F</td>
<td>75.9 ± 1.3 E</td>
<td>4.5 ± 0.1 D</td>
<td>0.10 ± 0.00 A</td>
<td>0.30 ± 0.00 G</td>
<td>4.7 ± 0.4 B</td>
<td>4.7 ± 0.4 B</td>
</tr>
</tbody>
</table>

aPercentage of butyric acid in total VFAs.
bMeans with standard deviation in the same row followed by the same letter do not differ significantly at P = 0.05, according to Duncan’s multiple range test.
pretreated at 50°C for 72 h, 6.0 g/l of butyric acid was produced, which accounted for 75.9% of the total VFAs. Moreover, it was noticed that the pretreatment intensity influenced the distribution of VFAs. The percentage of butyric acid was higher at higher intensity.

Rice straw samples were observed using SEM in order to determine what occurred on the physical structure of rice straw after pretreatment, enzymatic hydrolysis, and microbial digestion (Fig. 2). Fig. 2B clearly presents that an unorganized and loose structure of the rice straw fiber bundles was generated by this pretreatment compared with the untreated one shown in Fig. 2A. The figures of residual solids after enzymatic hydrolysis and microbial digestion suggested that NaOH pretreatment enhanced digestibility significantly. It could be observed in Figs. 2C and 2E that the surface of untreated rice straw became rough and lusterless, but those changes were limited. Figs. 2D and 2F show that the irregular fragments were digested into muddy sediment and small fibers. It was speculated that the improvements in enzyme hydrolysis in the pretreated biomass were due to the separation and exposure of the rigid and ordered fibers.

**Batch and Repeated-Batch Fermentation for Butyric Acid Production**

The results of butyric acid production in batch and repeated-batch fermentations are summarized in Table 3, and the time courses of the concentrations of the main liquid products are presented in Fig. 3. The results showed that modes of fermentation operation greatly affected the selectivity of butyric acid production. The concentration of total VFAs hardly changed between batch and repeated-batch operations. However, in the repeated-batch fermentation trials, butyric acid production had dropped considerably.

### Table 3. Batch and repeated-batch fermentation results of rice straw pretreated at 50°C for 72 h.

<table>
<thead>
<tr>
<th>Mode</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>Consumption of rice straw (g/l)</th>
<th>Acetic acid (g/l)</th>
<th>Propionic acid (g/l)</th>
<th>Butyric acid (g/l)</th>
<th>Valeric acid (g/l)</th>
<th>Total VFAs (g/l)</th>
<th>Product selectivity</th>
<th>Biomass (OD260 after HClO4 hydrolysis)</th>
<th>Butyric acid yield (g/g rice straw fed)</th>
<th>VFAs yield (g/g rice straw fed)</th>
<th>Cumulative H2 yield (ml/g rice straw fed)</th>
<th>Cumulative CH4 yield (ml/g rice straw fed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>7.59 ± 0.10 A</td>
<td>7.74 ± 0.11 A</td>
<td>7.74 ± 0.12 A</td>
<td>7.74 ± 0.13 A</td>
<td>7.74 ± 0.14 A</td>
<td>7.74 ± 0.15 A</td>
<td>5.08 ± 0.04 A</td>
<td>5.03 ± 0.04 A</td>
<td>5.10 ± 0.17 A</td>
<td>5.03 ± 0.06 A</td>
<td>5.07 ± 0.04 A</td>
<td>4.99 ± 0.05 A</td>
<td>10.1 ± 0.6 A</td>
<td>10.3 ± 0.5 A</td>
</tr>
<tr>
<td>B2</td>
<td>7.74 ± 0.11 A</td>
<td>7.74 ± 0.12 A</td>
<td>7.74 ± 0.13 A</td>
<td>7.74 ± 0.14 A</td>
<td>7.74 ± 0.15 A</td>
<td>7.74 ± 0.15 A</td>
<td>5.03 ± 0.04 A</td>
<td>5.03 ± 0.04 A</td>
<td>5.10 ± 0.17 A</td>
<td>5.03 ± 0.06 A</td>
<td>5.07 ± 0.04 A</td>
<td>4.99 ± 0.05 A</td>
<td>10.3 ± 0.5 A</td>
<td>9.9 ± 0.3 A</td>
</tr>
<tr>
<td>B3</td>
<td>7.74 ± 0.12 A</td>
<td>7.74 ± 0.13 A</td>
<td>7.74 ± 0.14 A</td>
<td>7.74 ± 0.15 A</td>
<td>7.74 ± 0.15 A</td>
<td>7.74 ± 0.15 A</td>
<td>5.03 ± 0.04 A</td>
<td>5.03 ± 0.04 A</td>
<td>5.10 ± 0.17 A</td>
<td>5.03 ± 0.06 A</td>
<td>5.07 ± 0.04 A</td>
<td>4.99 ± 0.05 A</td>
<td>10.4 ± 1.6 A</td>
<td>9.8 ± 1.1 A</td>
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<tr>
<td>R1</td>
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<td>5.07 ± 0.04 A</td>
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<tr>
<td>R2</td>
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<tr>
<td>R3</td>
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<td>4.99 ± 0.05 A</td>
<td>10.4 ± 1.6 A</td>
<td>9.8 ± 1.1 A</td>
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</table>

*Batch fermentation.
Repeated batch fermentation.
Percentage of butyric acid in total VFAs.
Means with standard deviation in the same row followed by the same letter do not differ significantly at P = 0.05, according to Duncan’s multiple range test.

### Table 4. The results from batch fermentation of butyric acid with step-wise addition of CaCO3.

| Initial pH | Final pH | Buffer added (g) | Consumption of rice straw (g/l) | Acetic acid (g/l) | Propionic acid (g/l) | Butyric acid (g/l) | Valeric acid (g/l) | Total VFAs (g/l) | Product selectivity (%) | Butyric acid yield (g/g rice straw fed) | VFAs yield (g/g rice straw fed) |
|------------|----------|-----------------|--------------------------------|-----------------|---------------------|-------------------|------------------|-----------------|-------------------|---------------------------------|--------------------------------|--------------------------------|
| 6.64 ± 0.03 | 5.77 ± 0.13 | 16 ± 0.4 | 14.2 ± 1.5 | 2.1 ± 0.2 | 0.3 ± 0.1 | 7.6 ± 0.8 | 0.3 ± 0.03 | 10.8 ± 1.1 | 70.2 ± 6.0 | 0.38 ± 0.04 | 0.54 ± 0.06 |

*In these trials, buffers followed step-wise addition of CaCO3, added at every 12 or 24 h when the bottles were opened.
Percentage of butyric acid in total VFAs.
The production of acetic, valeric, and caproic acids, and methane on the other hand increased. Findings from this study suggest that batch operation is more suitable for butyric acid production by undefined mixed culture than the unoptimized repeated-batch operation. Furthermore, it was noticed that more than one quarter of fermentable carbohydrates remained after the 6 days fermentation period, which was thought to be closely related to the pH being down to nearly 5.0. Neutral pH has been reported to be favorable for the production of short-chain fatty acids [9, 28]. It can be deduced that more pH buffer should be added during the fermentation to enhance the digestibility and carboxylates production. This deduction was confirmed by the following study. The results showed that neutral pH improved rice straw hydrolysis (up to 70%) and hence carboxylic acid production (up to 10.8 g/l), and a higher butyric acid production (~7.6 g/l) was achieved (Table 4). In view of the fact that methanogenesis had occurred, methane inhibitors should be employed for the accumulation of carboxylic acid.

**DGGE Analysis of Microbial Community Structure**

The microbial community structure was assessed by DGGE analysis. The DGGE band patterns are presented in Fig. 4. In the transferring of inocula, the microbial community was assumed to be stable (Lanes I 1, I 2, and I 3), but the microbial community structure during the repeated-batch fermentation clearly underwent a shift (Lanes R 1, R 2, and R 3). The bands with significant changes represented by the band intensity were selected for cloning and sequencing. The phylogenetic relationship between the sequenced clones and other related species is depicted in Fig. 5. In the repeated-batch trials, bands 1 and 2,

**Fig. 3.** Time courses of the concentrations of the main liquid products in the butyric acid production in batch and repeated-batch fermentations.

**Fig. 4.** DGGE profile of bacterial 16S rRNA gene in the microbial communities in the batch fermentations and repeated-batch fermentations.

The numbered bands were selected for cloning and sequencing.
respectively, closely relating to \textit{Oscillibacter valericigenes} and \textit{Clostridium hathewayi}, increased, which coincided with the increase of valeric and acetic acids production. \textit{Oscillibacter valericigenes} is a valerate-producing anaerobic bacterium [17], and \textit{Clostridium hathewayi} is an acetate-producing bacterium [34]. A dominant band, band 3, was found in the inocula of batch fermentation (Lanes I 1, I 2, and I 3), while it became extremely weak as fermentation progressed. Band 3 was closely related to \textit{Clostridium leptum} (100% similarity), a fibrolytic and butyrate-producing bacterium [22]. In contrast to band 2 becoming much stronger, during the fermentation some xylanolytic (Band 4-2, \textit{Ruminobacillus xylanolyticum}) and cellulolytic (Band 5, \textit{Clostridium cellulosi}) bacteria became weak or disappeared. It was believed that the shift in microbial population, especially the decline in butyrate-producing species (Band 3, \textit{Clostridium leptum}) caused, at least in large part, the decrease of butyric acid production in the repeated-batch operation.

\textbf{Discussion}

In the most of the plant biomass, the cellulose, with a content in the range of approximately 35\% to 50\% of dry weight, is embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20\% to 35\% and 5\% to 30\% of dry weight [25]. The structure of lignocellulosic biomass determines the difficulties in the widespread utilization of this important resource. The goal of any pretreatment technology is to liberate cellulose and hemicellulose from their complex with lignin to improve the enzymatic hydrolysis and increase yields of fermentable sugars in the downstream processing steps [27]. It is also of great concern to maximize the recovery of cellulose and hemicellulose from the pretreatment processes themselves without extra mechanical, thermal, and chemical operations. In this study, a higher recovery of cellulose and hemicellulose was achieved, compared with those of NaOH pretreatments with higher alkaline concentrations at the elevated temperatures [18, 36, 38]. The pretreatment preformed at $-15^\circ$C, as expected, achieved less weight loss of cellulose and hemicellulose [40]. It is true that the lower temperature of alkaline pretreatment came with less loss of fermentable components, but a longer treatment time is necessary.

Undefined mixed culture fermentation, as the name implies, generates mixed products. High selectivity of the desired product is especially crucial for a mixed culture-based process. In this study, the product selectivity in batch fermentation was about 76\%, and the theoretical product selectivity from glucose fermentation by pure butyrate-producing bacteria species was about 75\%. Glucose fermentation by \textit{Clostridium butyricum} (Eq. (1)) and \textit{Clostridium tyrobutyricum} (Eq. (2)) follows the stoichiometric equations below [39]:

\begin{equation}
\text{Glucose} \rightarrow 0.8 \text{Butyrate} + 0.4 \text{Acetate} + 2.4 \text{H}_2 + 2 \text{CO}_2 \tag{1}
\end{equation}
Glucose $\rightarrow$ 0.85 Butyrate + 0.1 Acetate + 0.2 Lactate + 1.9 H$_2$ + 1.8 CO$_2$ (2)

In laboratory research, a higher product selectivity (nearly 90%) from glucose fermentation by Clostridium tyrobutyricum was achieved [32]. Whatever its producer, mixed cultures or pure species, it is impossible to obtain butyrate as the only product in a single bioprocess. In the acetate branch of metabolic pathways of the butyrate-producing clostridia, four ATPs are produced during the conversion of glucose to two acetic acids (Eq. (3)). In the butyrate branch, three ATPs for each butyrate are produced (Eq. (4)).

Glucose $\rightarrow$ 2 Acetate + 4 H$_2$ + 2 CO$_2$ + 4 ATP (3)

Glucose $\rightarrow$ Butyrate + 2 H$_2$ + 2 CO$_2$ + 3 ATP (4)

In the growth phase, cells have a higher energy demand, and so acetate is the main product for more ATPs. At the end of the exponential growth, excreted acetate is taken up and converted into butyrate. The metabolism shift, from acetate formation with more energy production to butyrate formation with less carboxyl group production, may be related to detoxifying the medium by reducing the total hydrogen ion concentration [39, 41]. From the metabolic pathways, an explanation of why a higher percentage of butyric acid was obtained from the rice straw with higher pretreatment intensity (Table 2) could be found. In the fermentation of rice straw with low pretreatment intensity, less fermentable carbohydrate was available. Thus, more acetic acid was produced to equate the ATP demand.

In conclusions, sodium hydroxide pretreatment was shown to be an effective method for the bioconversion of rice straw. By soaking in 1% NaOH solution at 50°C for 72 h, about 66% of the lignin was removed, which greatly increased the rice straw digestibility. With this pretreated rice straw, about 6 g/l butyric acid dominating about 76% of the total VFAs was produced, which indicated that it was feasible to convert pretreated rice straw into butyric acid using an appropriate undefined mixed culture. In the mixed culture-based processes, keeping the microbial community structure stable is the key to achieving high selectivity of the desired product. In this study, owing to the instability of the microbial population in repeated-batch fermentation, batch operation is more suitable for butyric acid production. This study corroborates a promising strategy for the bioconversion of low-grade cellulosic feedstocks into biofuels and bioproducts without supplementary cellulolytic enzyme by using undefined mixed cultures.

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References


