Novel Butyric Acid Fermentation Strains and Their Performance in Fermenting Rice Straw by Pure and Mixed Culturing

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Keywords: Rice Straw, Bactrial Strain, Butyric Acid Fermentation, Screening, Co-Culture, Construction of Bacterial Consortium, Ratio Butyric Acid Production Rate.

Abstract. Synergistic metabolism among mixed culture can be utilized to hydrolyse and ferment cellulose and hemicellulose in rice straw to avoid high cost of saccharification and its complex procedure. Three buryric acid producing strains cellulose-decomposing bacteria named Clostridium cadaveris C4, cellulbiose fermenting bacteria named C. butyricum B20, xylan-degrading bacteria named C. butyricum X3 respectively were isolated from undefined mixed culture which was enriched and cultured from rice straw. The fermentation experiment of rice straw was carried out to increase the burytic acid conversion rate with three single strain and their mixed strains. Fermentation results showed that co-fermentation with two or three strains can significantly improve the degradation of rice straw and yield of butyric acid because of synergistic effect in or among different metabolic strains. Among the experiment, co-culture of three strains with inoculum of 1.1 g-DCW/L got the butyric acid production rate 0.18 g/(g-DCW⋅h) during logarithmic period which increased by 50%, 200% and 63.64% compared with C4, B20 and X3 respectively. The results of the study provide the methodological guidance and bacterial strain resources for the butyric acid biotransformation from lignocellulosic biomass.

Introduction

Butyric acid is an important resource and raw material which is widely used in food additives, chemicals and pharmaceutical products[¹-²]. At present, the industrial production of butyric acid mainly comes from n-butyraldehyde oxidation synthesis which is primarily based on non-renewable fossil resources such as oil, coal, natural gas and other fossil resources[¹]. However, these non-renewable fossil resources are not only limited and depletable but also causes environmental pollution. Consequently, a more environmentally friendly butyric acid production methodology is therefore required. Butyric acid production by fermentation of microorganisms with abundant straw as substrate is an alternative technology with great potential[³-⁴]. The main components that could be hydrolyzed and fermented in the straw biomass are cellulose and hemicellulose. However, it is difficult to effectively degrade and bio-transform by microorganism since these two components are intertwined with lignin and other substances and which subsequently forms complex and stable multimers[⁵-⁷]. Thus, pretreatment is usually required to disrupt the structure of the lignocellulose to expose the cellulose and hemicellulose prior to bio-fermentation[⁸]. Physical, chemical or biological treatment for saccharification is also needed to obtain the fermentable sugar, such as glucose, xylose and cellulbiose, etc after the pretreatment of the straw[⁹-¹⁰]. However, the high cost of saccharification offset the advantage of crop straw material[¹¹]. The research on hydrolysis and fermentation of cellulose and hemicellulose in straw by using the synergistic metabolism of mixed flora attracted the attention of many scholars in order to avoid high cost of saccharification and the complexity of the process[¹²-¹⁴]. Reportedly, fermentation by mixed cultures has the advantage of high utilization rate of substrate compared with fermentation by pure culture, but too many by-products are subsequently
produced\textsuperscript{[15,16]}. Construction of microbial flora with screened cellulose and hemicellulose degrading bacteria and butyric acid fermenting bacteriacan combines the simplicity of pure culture fermentation and high substrate conversion rate of mixed fermentation and thus improve the target product conversion rate and utilization rate of straw\textsuperscript{[17]}. Fermentation by microbial flora is not only a simple process but also additional saccharification treatment is not required. Thus fermentation by microbial flora could significantly reduce the cost of butyric acid production from rice straw by fermentation. Moreover, the system also has the advantages of stable operation, anti-bacterial contamination and easy management\textsuperscript{[18]}. However, it is quite rare to achieve transformation of butyric acid from rice straw by employing bacterial flora with certain known pure cultures\textsuperscript{[19]}.

In this study, three butyric acid producing strains with different functions were isolated and screened from undefined mixed culture which was enriched and cultured from rice straw. The study specifically explored the effects of microbial flora with three strains on the efficiency of butyric acid production from pretreated rice straw base on the performance of single strain and provided the methodological guidance and bacterial strain resources for the butyric acid biotransformation from lignocellulosic biomass.

**Materials and Methods**

**Pretreatment of Rice Straw**

The rice straw was collected from a local farm in Harbin, China, and cut into the length of 10~15 cm, 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%. The rice straw was soaked in 1% sodium hydroxide solution with a solid-to-liquid ratio of 1:15 (w/v) at 50 °C for 72 h in static status. The solid residue was separated by filtering and washed by tap water to neutral pH, dried and stored for further experiments\textsuperscript{[20]}.

**Isolation Source**

Butyric acid producing fermentative microbe was isolated from the microflora, which is preserved in the laboratory and could efficiently degrade the pretreated straw and produce the liquid end product dominated by butyric acid. The maximum production and selectivity of butyric acid was 6 g/L and 76% respectively\textsuperscript{[20]}. The undefined microflora included many cellulolytic and hemicellulolytic strains, mainly *Clostridium.sp, Bacteroides.sp* and *Oscillospira.sp*.

Under a continuous supply nitrogen for protection of microbes, 1 mL of the mixed culture liquor was taken out and diluted 1000 times to obtain the bacteria suspension with anoxic physiological saline.

**The preparation of Medium**

Basal medium (L^{-1}): yeast extract, 1.0 g; cysteine, 0.5 g; KH_{2}PO_{4} 1.5 g; K_{2}HPO_{4}·3H_{2}O 2.9 g; MgCl_{2}·6H_{2}O 0.2 g ; (NH_{4})_{2}SO_{4} 1.3 g; CaCl_{2} 0.056 g; FeSO_{4}·7H_{2}O, 0.00125 g; 1% resazurin solution 0.5 mL; vitamin solution, 1 mL.

Vitamin solution (L^{-1}): ZnCl_{2} 70 mg; MnCl_{2}·4H_{2}O 100 mg; H_{3}BO_{3} 6 mg; CoCl_{2}·6H_{2}O 190 mg; CuCl_{2}·2H_{2}O 2 mg; NiCl_{2}·6H_{2}O 24 mg; Na_{2}MoO_{4}·2H_{2}O 36 mg; FeCl_{2}·4H_{2}O 1.5 mg; 10 ml of 25% HCl.

The following six kinds of medium were prepared on the basis of the basal medium.(1)Cellulose-congo red solid medium(for isolation cellulose-degrading bacteria):α-cellulose, 10 g/L; congo red, 0.3 g/L; agar powder, 17 g/L. (2)Xylan solid medium(for isolation of xylan-degrading bacteria): xylan, 10 g/L; agar powder, 17 g/L. (3)Cellubiose solid medium(for isolation of cellubiose fermenting bacteria: cellubiose, 10 g/L; agar powder, 17 g/L. (4)Cellubiose liquid medium(for culture of cellubiose fermenting and cellulose-degrading bacteria): cellubiose, 10 g/L. (5)Xylan liquid medium(for culture of xylan degrading bacteria): xylan, 10 g/L. (6)Rice straw fermentation medium(for butyric acid fermentation by mono-culture or co-culture): pretreated rice straw, 10 g/L.
The solid medium was subpackaged in 25 ml Hungate-tube with working volume of 3 ml. Liquid medium was also subpackaged in Hungate-tube with working volume of 10 ml. The rice straw fermentation medium was subpackaged in 180 ml anaerobic bottle with working volume of 65 ml.

All Hungate-tubes and anaerobic bottles with subpacked medium were sterilized by autoclave at 115 °C for 20 min.

**Isolation, Purification and Selection of Strain**

The separation of objective strain was achieved by alternate cultivation method of solid-liquid medium. For isolation of cellulose degrading and butyric acid producing bacteria, operations were as follows: (1)0.3 ml bacteria suspension liquid was added in 45~50 °C sterile cellulose-congo red medium by syringe, rolled tubes, and cultivated at 37 °C until the formation of significant discernible colonies with degrading circles. (2) Selected and picked 30 colonies on the surface of solid medium and transfer them into the cellobiose liquid medium, cultivated at 37 °C 120 r/min for 2 d then detected the concentration of butyric acid in liquid. (3) Select the 10 cultures with higher butyric acid concentration among 30 colonies and dilute them 1000 times to get the bacteria suspension liquid. (4) Repeated the operation (2) and (3) 8 times. In the selecting process, the optical microscope (BX51, Olympus) and scanning electron microscope (S-3400N, Hitachi) were used for observing the purification of the bacteria.

For the separation and purification of xylan degrading bacteria, xylan solid and liquid medium was used, while for cellulbiose fermenting bacteria, cellulbiose solid and liquid medium was used.

The yield of butyric acid was selection criteria for xylan degrading and cellubiose fermenting bacteria and the enzyme activity of Carboxyl Methyl Cellulose (CMC) is another criteria besides the yield of butyric acid for the selection of cellulose degrading bacteria.

**Butyric acid Production Test from Fermentation of Straw**

One of each cellulose-degrading, xylan-degrading and cellubiose fermenting bacteria with best performance was selected after the isolation, purification and selection process. Three pure cultures were cultivated by corresponding liquid medium at 37 °C, 120 rpm for 24 h and stored for later experiments. And after fermentation, the dry cell weight (DCW) of each strain was around 1.1 g/L.

Fermentation experiment of straw by different inoculation programs with three single strain and their mixed cultures was carried out in 150 mL anaerobic bottles which contained 65 mL medium and 15 mL inoculum. Depending on the inoculation programme, the fermentation experiment was divided into three groups. (1) Fermentation by three single strain. (2) Fermentation by two mixed cultures. (3) Fermentation by three mixed cultures.

The above seven sets of fermentation experiments all had three parallel tests. The anerobic bottles were cultivated at 37 °C,130 rpm for 6 d, the fermentation liquid pH value, production of volatile fatty acids (VFAs) and biogas were measured each 24 h, and the average of three parallel test results were taken into account.

**Analyze Project and Method**

pH of liquid sample was determined by pH meter (FE28-Bio, Mettler Toledo); VFAs in fermentation liquid was measured by gas chromatography (SP6800A, Shandong Lunan Instrument Factory, China). The biogas production was tested by pressure balance method with the help of an air tight syringe while gas components was detected by another gas chromatography (SP6890, Shandong Lunan Instrument Factory, China). The CMCase was detected by 3,5-Dinitrosalicylic acid (DNS) colorimetric method, and optical density(OD) was measured by a spectrophotometer (UV-2401PC, Shimadzu). Both the dry cell weight and rice straw residue were determined by constant weight method.
Results and Discussion

Isolation and Identification of Strains

After continuous isolation and purification, 30 strains of cellulose-degrading, 10 strains of cellubiose fermenting and 6 strains of xylan-degrading bacteria were obtained from isolation source. The best performed strains were selected and marked as C4, B20, X3. Carbon source utilization tests showed that strain C4 could degrade cellulose, but couldn’t utilize xylan; strain B20 could utilize cellubiose, but couldn’t degrade cellulose and xylan; and strain X3 could degrade xylan but couldn’t degrade cellulose.

To elucidate the select strains, colonies of selected strains were investigated. The study revealed that, colony of strain X3 was round, yellow, smooth edge and had a approximate diameter ranged about 1.5~2 mm. The colony of strain B20 is grey, round, middle convex, edge smooth with diameter of about 1.5 mm. The colony formed on the solid medium of C4 strain is white and its diameter was about 1 mm. The edge of colony was zigzag, and there was a large degrading circle around it showing good degradability of cellulose[26]. As shown in Fig. 1, strains C4, B20 and X3 were all gram positive. In addition, scanning electron microscopy (SEM) was conducted to make further revlations on the morphology. SEM (Fig. 1) showed that the cell of strain X3 was rod-shaped, with a diameter of about 2 μm and length of 8~10 μm. The strain B20 was rod-shaped with a diameter of about 0.8 μm and a length of 4~7 μm. For strain C4, the cell was spindle shaped with length of 2~4 μm and maximum diameter of 0.4 μm. The 16S rRNA gene sequences of three strains were submitted to Genbank for homology comparison. The results showed that strain X3 and B20 belonged to C. butyricum, and strain C4 belonged to C. cadaveris.

![Figure 1. Gram Stain and Scanning Electron Microscopy Photographs of Three Strains.](image)

Butyric Acid Fermentation by Single Strains

The three strains of C4, B20 and X3 was inoculated and cultivated respectively to test the capability of butyric acid production by fermentation of rice straw. As shown in Fig 2, liquid end products of three strains were relatively simple. Only acetic acid and butyric acid were obtained in the experiment suggesting the neccessity to harness the butyric acid.

The production of VFAs accompanied with forming of H2 mainly occurred in the first day (logarithmic period) but did not experience an obvious change after 2 d (Fig. 2). After 4 day’s fermentation, pH of the system decreased to around 5.5 and straw degradation for strains C4, B20 and
X3 was 2.13, 1.30 and 2.04 g/L, respectively. Butyric acid yield was 0.70, 0.42 and 0.72 g/L for strains C4, B20 and X3, respectively, whereas proportion of butyric acid in total VFAs was 55.16%, 62.56% and 60.14% respectively. Although the butyric acid conversion rates from rice straw for three strains were all about 0.33 g/g, theirs maximum ratio butyric acid production rates had few difference and for strain C4, B20 and X3 it was 0.12, 0.06 and 0.11 g/(g-DCW-h) respectively. The above results indicated that strain B20 had a better selectivity of butyric acid production but inferior straw utilizing ability compared with strain C4 and X3. Due to the differences in straw utilization capacity, selectivity of butyric acid and conversion rate of butyric acid, combination of three strains could be a better choice to improve butyric acid production capabilities from a fermentation system.

According to the combination method of three strains described in section 1.5, microflora with two strains (X3+C4), (X3+B20), (C4+B20) and three strains [(X3+C4+B20)] were inoculated to test the production efficiency of butyric acid. The results depicted in Figure 3 indicated that all combined strains produced VFAs in first day as similarly noted with single strain fermentation. At the end of the fermentation, pH of (X3+C4), (X3+B20), (C4+B20) and (X3+C4+B20) was 5.28, 5.26, 5.28 and 5.22, respectively, as well their H₂ production was 1.32, 1.29, 1.23 and 1.30 L/L respectively.

Butyric Acid Fermentation by Mixed Strains

According to the combination method of three strains described in section 1.5, microflora with two strains (X3+C4), (X3+B20), (C4+B20) and three strains [(X3+C4+B20)] were inoculated to test the production efficiency of butyric acid. The results depicted in Figure 3 indicated that all combined strains produced VFAs in first day as similarly noted with single strain fermentation. At the end of the fermentation, pH of (X3+C4), (X3+B20), (C4+B20) and (X3+C4+B20) was 5.28, 5.26, 5.28 and 5.22, respectively, as well their H₂ production was 1.32, 1.29, 1.23 and 1.30 L/L respectively.
Regarding two strains of mixed fermentation systems (Fig.3), degradation of straw for (X3+C4) and (C4+B20) were almost same, thus 2.13 and 2.16 g/L respectively. Total VFAs was 1.34 and 1.29 g/L respectively. However, butyric acid yield of (C4+B20) was 0.79 g/L, slightly higher than that in (X3+C4) (0.74 g/L) indicating strain B20 had its positive effect of selectivity for butyric acid production in co-culture system. In contrast, co-fermentation of (X3+B20) had the best result of butyric acid production and degradation of straw. Total VFAs and yield of butyric acid was 2.83, 1.43 and 0.79 g/L, respectively. When it came to co-fermentation of (X3+C4+B20), the result of three items was 3.10, 1.53 and 0.93 g/L, respectively.

The fermentation results of straw by mono-culture and co-culture were listed in table 1. It could be deduced that, the efficiency of butyric acid production by fermentation of straw would increase by employing different type of strains under the same fermentation condition. Degradation of rice straw by mono-culture (C4), co-culture of two strains (X3+B20) and co-culture of three stains (X3+C4+B20) was 2.13 g/L, 2.83 g/L and 3.10 g/L, respectively. It was also observed that production of butyric acid increased with the adding of strain in fermentation system.

The maximum yield of butyric acid was 0.72 g/L(X3) for single strain fermentation, 0.79 g/L(C4+B20 or X3+B20) for two strains of mixed fermentation, and 0.94 g/L(X3+C4+B20) for three strains of mixed fermentation respectively. Furthermore, maximum ratio of butyric acid production rate in logarithmic phase of fermentation was 0.12, 0.15 and 0.18 g/(g-DCW-h) obtained from mono-culture of C4, mixture of two strains (C4+B20) and three strains (X3+C4+B20), respectively. The above results fully conclude that synergistic metabolism of different functional bacteria in constructed micoflora showed great advantage in fermentation of rice straw over mono-culture.
Table 1. Butyric Acid Fermentation Results by Three Single Strain and Their Mixed Cultures.

<table>
<thead>
<tr>
<th>Single/mixed culturing</th>
<th>C4</th>
<th>B20</th>
<th>X3</th>
<th>(C4+X3)</th>
<th>(C4+B20)</th>
<th>(X3+B20)</th>
<th>(C4+B20+X3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum (g/L)</td>
<td>0.21±0.01</td>
<td>0.21±0.01</td>
<td>0.21±0.01</td>
<td>0.21±0.01</td>
<td>0.21±0.01</td>
<td>0.21±0.01</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>pH</td>
<td>6.67±0.02</td>
<td>6.53±0.02</td>
<td>6.55±0.02</td>
<td>6.54±0.02</td>
<td>6.61±0.02</td>
<td>6.63±0.02</td>
<td>6.57±0.02</td>
</tr>
<tr>
<td>Final</td>
<td>5.41±0.04</td>
<td>5.67±0.02</td>
<td>5.49±0.02</td>
<td>5.28±0.02</td>
<td>5.28±0.03</td>
<td>5.26±0.02</td>
<td>5.22±0.01</td>
</tr>
<tr>
<td>Degradation</td>
<td>2.13±0.11</td>
<td>1.30±0.10</td>
<td>2.04±0.05</td>
<td>2.52±0.04</td>
<td>2.16±0.16</td>
<td>2.83±0.07</td>
<td>3.10±0.10</td>
</tr>
<tr>
<td>Production of H2 (L/L)</td>
<td>0.94±0.06</td>
<td>0.35±0.04</td>
<td>0.99±0.04</td>
<td>1.32±0.06</td>
<td>1.23±0.09</td>
<td>1.29±0.06</td>
<td>1.30±0.09</td>
</tr>
<tr>
<td>Yield of total VFAs (g/L)</td>
<td>1.26±0.02</td>
<td>0.67±0.02</td>
<td>1.19±0.04</td>
<td>1.34±0.02</td>
<td>1.29±0.02</td>
<td>1.43±0.03</td>
<td>1.53±0.04</td>
</tr>
<tr>
<td>Yield of butyric (%)</td>
<td>0.70±0.03</td>
<td>0.42±0.03</td>
<td>0.72±0.02</td>
<td>0.74±0.02</td>
<td>0.79±0.01</td>
<td>0.79±0.01</td>
<td>0.94±0.01</td>
</tr>
<tr>
<td>Conversion rate (g/g)</td>
<td>0.33±0.02</td>
<td>0.32±0.03</td>
<td>0.35±0.01</td>
<td>0.30±0.00</td>
<td>0.37±0.03</td>
<td>0.29±0.01</td>
<td>0.30±0.01</td>
</tr>
<tr>
<td>Ratio rate [(g/(g⋅h)]</td>
<td>0.12±0.00</td>
<td>0.06±0.00</td>
<td>0.11±0.00</td>
<td>0.15±0.00</td>
<td>0.15±0.00</td>
<td>0.13±0.00</td>
<td>0.18±0.00</td>
</tr>
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</table>

1Proportion of butyric acid in total VFAs, 2Butyric acid conversion rate from straw

In the past ten years, the study of fermentation by constructed microflora to enhance the conversion of lignocellulose to butyric acid isn’t common. Bao et al. tested the fermentation property of microcrystalline cellulose by co-culturing of C. acetobutylicum X9 and Ethanoigenens harbinense B2 [27]. The results showed that the compound system with two strains got the yield of butyric acid 1.5 g/L, but the maximum ratio butyric acid production rate was 0.16 g/(g-DCW⋅h). Wang et al. used the co-culture system of Enterococcus gallinarum G1 and E. harbinense B49 to ferment cellulose and got the maximum ratio butyric acid production rate of about 0.16 g/(g-DCW⋅h)[28]. Liu et al. got the maximum ratio butyric acid production rate of 0.13 g/(g-DCW⋅h) by similar system with C. thermocellum JN4 and Thermoanaerobacterium thermosaccharolyticum GD17[29]. In contrast, the maximum ratio butyric acid production rate of 0.18 g/(g-DCW⋅h) obtained by co-culturing of (X3+C4+B20) has a significant advantage in the fermentation of lignocellulosic biomass in this study.

Conclusion

1) By screening and selection, three butyric acid producing strains were obtained, thus cellulose-degrading bacteria C. cadaveris C4, cellulose fermenting bacteria C. butyricum B20 and xylan-degrading bacteria C. butyricum X3, Which provides the strain resources for the biotransformation of butyric acid from lignocellulosic biomass.

2) The construction of microflora with C. cadaveris C4, C. butyricum B20 and C. butyricum X3 can fully utilize and strengthened synergistic effect between different metabolic strains, which can significantly improve the degradation of rice straw and increase the butyric acid yield.

3) The degradation rate of rice straw and yield of butyric acid were 3.10 g/L and 0.93 g/L respectively, and the maximum ratio butyric acid production rate was up to 0.18 g/(g-DCW⋅h) when fermenting with constructed microflora.

Acknowledgement

This research was financially supported by the National Natural Science Foundation of China (51478141). In view of this, the authors express their profound appreciation to all who have contributed in one way or the other in making this study a success.
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