Linking microbial community structure and product spectrum of rice straw fermentation with undefined mixed culture

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Abstract. Undefined mixed culture-based fermentation is an alternative strategy for biofuels and bioproducts production from lignocellulosic biomass without supplementary cellulolytic enzymes. Mixed culture produces mixed carboxylates. To estimate the relationship between microbial community structure and product spectrum, carboxylate production was initiated by mixed cultures with different microbial community structure. All the inoculum cultures were derived from the same enrichment culture from the combination of cattle manure, pig manure compost, corn field soil and rotten wood. Due to the differences in the preparation method and culture time, the inoculum cultures for batch fermentation had high similarity in microbial community structure, while the community structure of each inoculum culture for repeated batch fermentation differed from that of another. The inoculum cultures with similar community structure led to a similar product spectrum. In batch fermentation, the selectivity of main product butyric acid stabilized around 76%. The inoculum cultures with different community structures resulted in different product spectra. In repeated batch fermentation, the butyric acid content gradually decreased to 27%, and the by-product acetic acid content steadily increased to 56%. The other by-products including propionic, valeric and caproic acids were also increased. It is deduced that keeping the microbial community structure stable makes the basic and key precondition for steady production of specific carboxylic acid with undefined mixed culture.

1. Introduction
Lignocellulosic biomass is a potentially huge renewable resource. The utilization of this renewable resource for biofuels and bioproducts is way to alleviate the crisis and challenges in the consumption of fossil resource [1]. However, the high cost of cellulolytic enzymes limits the commercial production of bioenergy from lignocellulosic feedstock [2, 3]. Undefined mixed culture-based fermentation technology is a newly raised strategy for biofuels and bioproducts production from lignocellulosic biomass, in which the cost of cellulolytic enzymes can be saved. Anaerobic digestion is a traditional mixed culture-based process for biogas. In this process, lignocellulosic feedstock can be hydrolyzed with no need of cellulase supplement and converted to short-chain carboxylic acids as intermediates, which will be finally transformed to methane [4, 5]. By stopping the methanogenesis, carboxylic acids
are accumulated. When extracted from the culture broth, the carboxylic acids themselves are high-
value chemicals, which can be further catalyzed to biofuels via thermochemical or biological process
[6, 7].

In the anaerobic digestion process, lignocellulosic feedstocks are converted to methane in the
synergy of among the celluloletic bacteria, acidogenic bacteria and methanogenic bacteria [8]. Compared
with pure culture-based fermentation process, this mixed culture-based process possesses
specific advantages including no requirement for sterilization, more resilient to environmental impacts,
ability to use mixed substrates owing to its microbial diversity, and possibility of a continuous
production [9]. The microbial diversity gives the mixed culture-based process stronger adaptive
capacity, but also broader product spectrum. The reported short-chain carboxylates generated in
anaerobic digestion systems include but are not limited to formate, methanol, acetate, ethanol,
propionate, i-butyrate, n-butyrate, i-valerate, n-valerate, i-caproate, n-caprate, and others [10, 11].
High selectivity of desired product is especially crucial for a mixed culture-based process, although
even for culture-based fermentation, it is impossible to obtain the desired product as the only product
in a single bioprocess. In our previous study in enriching and selecting a mixed culture for butyric acid
production from rice straw, with continuously transferring, some mixed cultures lost their ability of
-cellulose degradation partially or fully, and some obtained stable cellulose-degrading ability and
product spectrum [12]. The present study aims to investigate the relationship of microbial community
structure and product spectrum using a mixed culture with stable activity of both cellulose and
hemicellulose utilization and high selectivity of butyric acid production. Using different preparation
method and culture time, inoculum cultures with different community structure were prepared and
employed to initiate the carboxylate production. The product spectra were determined and compared
to find out whether the differences in product spectrum relate to the differences in microbial
community structure.

2. Materials and methods

2.1. Materials
The rice straw was collected from a local farm in Harbin, China, and cut into 10-15 cm lengths before
pretreatment. The carboxylate producer, a celluloletic acidogenic microbial community, is an
enrichment culture derived from the combination of cattle manure, pig manure compost, corn field soil
and rotten wood. The mixed culture included celluloletic and xylanolytic bacteria, butyrate-producing
bacteria and other acidogenic bacteria [12].

2.2. Sodium hydroxide pretreatment of rice straw
The rice straw was cut into 10- to 15-cm lengths and soaked in a 1% NaOH solution with a solid-to-
liquid ratio of 1:15 (w/v) at 50°C for 72 h in static state [13]. The solid residue was then separated by
filtering and thoroughly washed with tap water to near-neutral pH. The neutralized residue was
squeezed and stored at 4°C. The substrate contained 53.0% cellulose, 27.4% hemicellulose and 8.0%
lignin. The untreated rice straw contained 39.7% cellulose, 24.8% hemicellulose and 15.3% lignin.

2.3. Preparation of inoculum
For the inoculum preparation, the stored mixed culture was transferred to seed medium which was
composed of 10 g pretreated rice straw, 5 g tryptone, 1 g yeast extract, 5 g NaCl, 2 g CaCO3 and 0.5 g
D-cysteine hydrochloride per liter, and one filter paper strip (1.5 cm × 5 cm) as an indicator [14]. The
broth was purged with nitrogen gas for 10 min to maintain anaerobic conditions, after which the 500-
ml serum bottle containing 300 mL of broth was sealed and autoclaved at 115°C for 20 min.
Following inoculation with 10 mL of the stored culture, the bottle was incubated at 35°C without
agitation until the filter paper strip was broken down.

2.4. Carboxylates fermentation from pretreated rice straw
The carboxylates fermentation was performed in triplicate in the 500-mL serum bottles with 200 mL PCS medium containing 20 g of rice straw. The fermentation media were prepared in the same way as inoculum culture. 5 mL of preculture was inoculated into each serum bottle followed by incubation at 35°C with agitation of 140 rpm for 6 d. At the end of fermentation, biogas content, carboxylic acids, pH, biomass and weight loss of rice straw were determined.

2.5. Bacterial community structure analysis
Bacterial community samples were collected by centrifugation. Bacterial genomic DNA was extracted and purified using the Bacteria DNA Mini Kit (Watson Biotechnologies, Shanghai, China). Bacterial 16S rRNA genes was amplified with primer pairs BSF 8 and BSR 534 with a GC clamp at the 5’ end. The Bacterial amplicons were subsequently separated by DGGE performed with a Bio-Rad DCode system (Bio-Rad Laboratories, USA). Gels were silver-stained. Individual bands were excised, and the DNA was recovered for sequencing. The detailed operation was described previously [13].

2.6. Analytical methods
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. Carboxylic acids in the fermentation broth were measured using a gas chromatography (SP-6800A, Lunan Instrument Factory) equipped with a flame ionization detector and a FFAP capillary column (30 m × 0.32 mm × 0.50 μm, ZhongKeKaiDi Chemical New-tech Co., Ltd.). Microbial biomass was estimated by optical density at 260 nm of fermentation broth after HClO4 hydrolysis. The detailed operating conditions for determination of biogas composition, carboxylic acids and microbial biomass were as described previously [13]. Statistical analysis was performed using Statistical Product and Service Solutions (IBM SPSS Statistics for Windows, Version 19.0, IBM Corp., New York, US).

3. Results and discussion
To estimate the relationship between microbial community structure and product spectrum including the kinds of carboxylates and their contents, batch fermentation and repeated-batch fermentation were performed. The carboxylate production was initiated by mixed cultures with different microbial community structure. The operational procedure is outlined in Figure 1. In batch operation, the inocula were the three consecutive generations of the enrichment culture derived from the combination of cattle manure, pig manure compost, corn field soil and rotten wood. In repeated batch operation, the inocula were the culture broths of their last batches. The fermentation results are presented in Table 1.

![Figure 1. Flowchart of the batch fermentation and repeated batch fermentation (I, inoculum; B, batch fermentation; R, repeated batch fermentation).](image-url)
Table 1. Carboxylate fermentation results from pretreated rice straw.

|                        | Batch fermentation | Repeated batch fermentation |
|------------------------|-------------------|-----------------------------|
| Initial pH             | 7.69±0.09 A       | 7.74±0.10 A                 |
| Final pH               | 5.07±0.04 A       | 5.03±0.04 A                 |
| Consumption of rice straw (g/L) | 10.43±0.76 A     | 10.1±0.30 A                 |
| Acetic acid (g/L)      | 1.31±0.11 A       | 4.13±0.57 B                 |
| Propionic acid (g/L)   | 0.27±0.06 A       | 0.37±0.12 B                 |
| i-butyric acid (g/L)   | 5.83±0.17 A       | 2.63±0.91 B                 |
| n-butyric acid (g/L)   | 0.10±0.03 A       | 0.25±0.08 B                 |
| i-valeric acid (g/L)   | 0.15±0.01 A       | 0.22±0.09 B                 |
| n-valeric acid (g/L)   | 0.05±0.01 A       | 0.15±0.07 B                 |
| i-caproic acid (g/L)   | 0.05±0.01 A       | 0.11±0.03 B                 |
| n-caproic acid (g/L)   | 0.02±0.01 A       | 0.06±0.03 B                 |
| Total VFAs (g/L)       | 7.77±0.15 A       | 7.91±0.26 A                 |
| Biomass (OD260 after HClO4 hydrolysis) | 4.65±0.05 A     | 4.71±0.10 A                 |
| Carboxylate yield (g/g rice straw fed) | 0.39±0.01 A     | 0.39±0.02 A                 |
| Cumulative H2 yield (mL/g rice straw fed) | 37.67±8.8 A    | 31.47±2.84 B                 |
| Cumulative CH4 yield (mL/g rice straw fed) | 4.20±0.17 A    | 6.03±0.81 B                 |

*a* 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.

As shown in Table 1, the total carboxylate yield and the consumption of rice straw had no significant difference between the two operations. But the product spectrum, including eight kinds of carboxylic acids and two kinds of biogas components, has greatly changed. The percentages of different carboxylic acids from the three duplicates operated in batch fermentation are presented in Figure 2. The main product was butyric acid and its selectivity was more than 76%. The product spectra of the three duplicates were highly stable.

![Figure 2](image-url)

**Figure 2.** The Percentages of carboxylic acids produced by the continuous three generations of microbial community. One solid square represent one percent.

The microbial community structures of the inocula initiating the batch fermentation were characterized using DGGE analysis (Figure 3). The structure of inoculum culture was relatively stable.
It was thought the stable product spectrum was the result of the stable microbial community structure of inoculum culture. This thought was confirmed by the results from repeated batch fermentation.

Figure 3. DGGE profile of bacterial 16S rRNA gene of the inoculum cultures for batch fermentation.

The repeated batch fermentation was initiated with the culture broth of its last batch and the carboxylate production results are presented in Figure 4. In repeated batch operation, the yield of butyric acid the main product in batch operation decreased gradually, and the acetic acid production increased steadily. The butyric acid content in total carboxylic acids decreased from 76% to 27%, and the acetic acid content increased from 16% to 56%. The other by-products including propionic, valeric and caproic acids were also increased. As shown in the DGGE profile of the inoculum culture (Figure 5), the microbial community structure underwent a marked shift during the repeated batch fermentation, corresponding to the changes in product spectrum (Figure 4).
| Acetic acid | Propionic acid | Butyric acid | Valeric acid | Caproic acid |
|------------|---------------|--------------|--------------|--------------|
| Repeated batch fermentation 1 |                  |              |              |              |
| Repeated batch fermentation 2 |                  |              |              |              |
| Repeated batch fermentation 3 |                  |              |              |              |

**Figure 4.** The Percentages of carboxylic acids in the fermentations inoculated with the broth from different fermentation batches. One solid square represent one percent.

**Figure 5.** DGGE profile of bacterial 16S rRNA gene of the inoculum cultures for repeated batch fermentation.

In a natural environment, lignocellulosic biomass is degraded by the mixed culture of cellulolytic bacteria together with symbiotic noncellulolytic bacteria [8]. When a microbial community is transferred to a new habitat, due to the imbalance in competition for nutrients and the changes in the spatial structure of different microorganisms, the original stable microbial community become unstable, and the structure and function of the microbial community have to adjust to adopt the new environment [15, 16]. Under certain condition, the microbes with specific function multiply rapidly, and some that cannot adapt to the new condition are suppressed. For example, when an anaerobic mixed culture is exposed to oxygen, the growth and physiological activity of strict anaerobic
methanogens and anaerobic acidogenic bacteria will be blocked, and then the acidogenesis and methanogenesis stop. On the other hand, aerobic microbes will be activated. Besides the microbial community structure, the physiological function shifts, which also causes product spectrum changes. According to the metabolic pathway of Clostridium sp.[17, 18], four ATPs are produced in the acetate branch in which one glucose is converted into two acetic acids (Eq. 1), and three ATPs for each butyrate are produced in the butyrate branch (Eq. 2).

\[
\text{Glucose} \rightarrow 2 \text{Acetate} + 4 \text{H}_2 + 2 \text{CO}_2 + 4 \text{ATP} \quad \text{(Eq. 1)}
\]

\[
\text{Glucose} \rightarrow \text{Butyrate} + 2 \text{H}_2 + 2 \text{CO}_2 + 3 \text{ATP} \quad \text{(Eq. 2)}
\]

Under the nutrient-limited conditions, more acetic acid was produced for more ATP to meet the energy demand, by which was explained that higher selectivity of butyric acid production was obtained from the pretreated rice straw with more fermentable carbohydrate available [13]. The acetic acid producing causes pH decrease. As a result of the marked drop in pH, the excreted acetic acid was taken up and converted into butyric acid, and the metabolism shifted to butyric acid production for less acidic end groups producing, by which was explained that lower pH led to higher butyric acid selectivity [19]. Based on the ecological selection principles, mixed culture-based processes generated narrow product spectrums could be established by process operations [9, 20]. But first, to increase the target product accumulation is to select and enrich a structurally stable mixed culture with efficient substrate hydrolysis and high selectivity of product generation.

4. Conclusion

All the inoculum cultures for both batch fermentation and repeated batch fermentation were derived from the same enrichment culture. Due to the differences in the preparation method and culture time, the inoculum cultures for batch fermentation had high similarity in microbial community structure, while the community structure of each inoculum culture for repeated batch fermentation differed from that of another. The inoculum cultures with similar community structure led to a similar product spectrum, and the inoculum cultures with different community structures resulted in different product spectra. It is deduced that the stability of microbial community structure makes the basic and key precondition for stable product spectrum of carboxylate production with mixed culture.

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