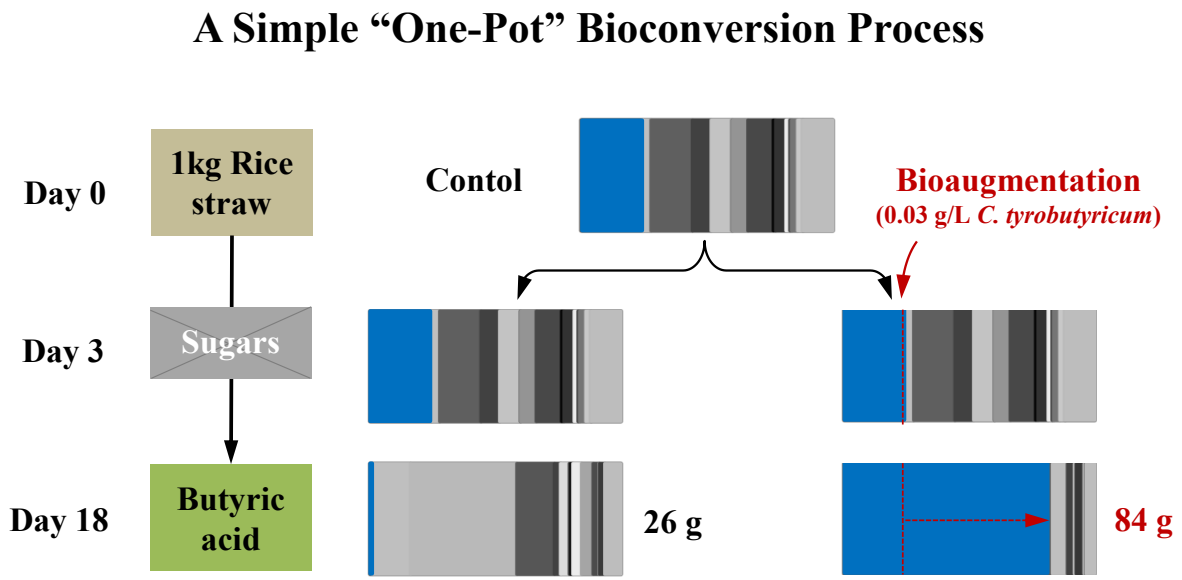

Figure Abstract

Highlights

- *Clostridium tyrobutyricum* plays a key role in rice straw – butyrate conversion.
- Bioaugmentation enhanced butyric acid concentration by 326%.
- High butyrate titer of 18.05 g/L was achieved with a selectivity of 60.7%.
- Significant shift of bacterial community was recorded after augmentation.

Bioaugmentation with *Clostridium tyroburicum* to improve butyric acid production through direct rice straw bioconversion

Xue Chi[a], Jianzheng Li[a,*], Xin Wang[b], Yafei Zhang[a], Shao-Yuan Leu[c,*], Ying Wang[c]

[a] State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, 73 Huanghe Road, Harbin 150090, China

[b] School of Resources and Environment, Northeast Agriculture University, 59 Mucai Road, Harbin 150001, China

[c] Department of Civil and Environmental Engineering, the Hong Kong Polytechnic University, Kowloon, Hong Kong, China

[*] Corresponding Authors. Phone: +86 (451)-86283761; Fax: +86 (451)-86283761; E-mails: syleu@polyu.edu.hk (S.-Y. Leu); ljz6677@163.com (J. Li);

Abstract

One-pot bioconversion is an economically attractive biorefinery strategy to reduce enzyme consumption. Direct conversion of lignocellulosic biomass for butyric acid production is still challenging because of competition among microorganisms. In a consolidated hydrolysis /fermentation bioprocess (CBP) the microbial structure may eventually prefer the production of caproic acid rather than butyric acid production. This paper presents a new bioaugmentation approach for high butyric acid production from rice straw. By dosing 0.03 g/L of *Clostridium tyrobutyricum* ATCC 25755 in the CBP, an increase of 226% higher butyric acid was yielded. The selectivity and concentration also increased to 60.7% and 18.1 g/L, respectively. DNA-sequencing confirmed the shift of bacterial community in the augmented CBP. Butyric acid producer was enriched in the bioaugmented bacterial community and the bacteria related to long chain acids production was degenerated. The findings may be useful in future research and process design to enhance productivity of desired bio-products.

Keywords: Bioaugmentation; Butyric acid; *Clostridium tyroburicum*; Rice straw; Microbial community

1. Introduction

Butyric acid is a valuable building-block precursor of many chemicals and materials (Baroi et al., 2015; Wei et al., 2013). This chemical has mainly been derived from unsustainable petroleum refinery. Producing cost-effective butyric acid from lignocellulosic feedstock has become a critical step towards a sustainable society. Lignocellulosic biomass is available in large quantities with widely distribution and low prices, although its complex cell wall structure can result in high recalcitrant to bioconversion (Tsapekos et al., 2017). In a typical biorefinery process exogenous enzymes are employed to hydrolyze lignocellulose in the separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) process. This practice could almost counteract the benefit of the low-cost feedstock (Jørgensen et al., 2007). Combining endogenous enzyme production, saccharification and fermentation in a single vessel, consolidated bioprocess (CBP) provides a potential approach to butyric acid production directly from lignocellulosic biomass (Wilson, 2009). Without involvement of exogenous enzymes, the lignocellulosic biorefinery process would be greatly simplified and get more cost-effective butyric acid production (Parisutham et al., 2014).

Microbes used in CBP can degrade lignocellulosic feedstock to bioproduct owing to the metabolic flexibility conferred by the many members of the community (Agler et al., 2011). However, conventional fermentation processes for butyric acid production usually suffer from the low final product concentration, yield and productivity, resulting in difficulties for large scale application. Bioaugmentation is a common practice conducted by adding specific microbes to a system to enhance desired activities. It has also been applied successfully to improve biodegradability and biogas production from lignocellulosic wastes with hydrolytic bacteria (Čater et al., 2015; Li et al., 2017; Tsapekos et al., 2017). Compared to the bioaugmentation with hydrolytic bacteria, adding butyric acid-producing bacteria to a

cellulolytic microbial consortium could be feasible to improve butyric acid production from lignocellulosic feedstock.

A stable microbial consortium was obtained in our previous study to ferment alkali pretreated rice straw for butyric acid production (Ai et al., 2013). In the batch fermentation, the microbial consortium produced 6.01 g/L butyric acid from 10.67 g/L pretreated rice straw with by-products such as acetic, propionic, isobutyric, isovaleric, valeric and caproic acid. Although the butyric acid selectivity reached 75.4%, the fermentation was still suffered from low final product concentration. In order to further improve the butyric acid production, *Clostridium tyrobutyricum* was employed for bioaugmented fermentation (**Fig. 1**). Among all the butyric acid-producing bacteria, *C. tyrobutyricum* has received much attention over other species due to its simple medium requirement for cell growth, relatively high product purity and yield (Jiang et al., 2011; X. Liu & Yang, 2006). In line with our previous findings the main objectives of this work were to evaluate the potential of using the bioaugmentation with *C. tyrobutyricum* for enhanced butyric acid production from rice straw. To get a comprehensive insight into the biological mechanism for the effect of the bioaugmentation on the fermemtaetion processes, the microbial community structure was also investigated using Illumina Miseq platform.

2. Materials and methods

2.1 Preparation of pretreated rice straw

Rice straw was collected at a local farm which containing 39.7% cellulose, 24.8% hemicellulose and 15.3% lignin (Harbin, China) (Van & Wine, 1968). The air-dried rice straw was cut into 10-15 cm length and pretreated with 1% (*w/v*) NaOH solution with a solid: liquid ratio of 1:15 (*w/v*) (based on dry weight) (Ai et al., 2013). The pretreatment was conducted in a 21-L tank-reactor containing 20 L working volume and incubated at 50°C for 72 h. After

solid-liquid separation, the residual solid fraction was washed with distilled water to reach neutral pH. Composition per kilogram of the pretreated rice straw contained 530 g cellulose (53.0%), 274 g hemicellulose (27.4%), 80 g lignin (8.0%) and 15 g ash (1.5%), respectively.

2.2 Microorganism and media

2.2.1 Microbial consortium for lignocellulosic butyric acid production

The microbial consortium to produce butyric acid from pretreated rice straw was a stable mixed culture obtained previously (Ai et al., 2013) and was named as DCB. Consortium DCB was stored at 4°C as spores in fresh peptone cellulose solution (PCS) medium containing the following components (per 1 L distilled water): 5 g tryptone, 1 g yeast extract, 5 g NaCl, 2 g CaCO3, 0.5 g D-cysteine hydrochloride, and 10 g pretreated rice straw was added as substrate.

2.2.2 Strain for bioaugmentation

C. tyrobutyricum ATCC 25755 obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) was used for bioaugmentation. The strain was stored in the form of spores in fresh PCS medium at 4°C. The inoculum preparation was performed by transferring 0.5 mL of stored spore suspension to a 20-mL Hungate tube with 9.5 mL PCS medium containing 10 g/L glucose and incubating at 140 rpm and 37°C for 22 h.

2.3 Butyric acid fermentation

The control and bioaugmented fermentation were conducted in a 3-L stirred-tank reactor by feeding 1 L PCS medium containing 20 g/L pretreated rice straw under 37°C and 60 rpm with the constant pH of 6.5. Both fermentations were initiated by inoculation with 5% (*v/v*) consortium DCB. Then 2.5% (*v/v*) of bioaugmented strain *C. tyrobutyricum* was inoculated (2.5%, *v/v*) to one of the reactors, which was designed as bioaugmented consortium DCB

(BDCB) on the 3rd day. 20 g/L pretreated rice straw and 2 g/L peptone were fed every two days to the two reactors during the 3rd to 13th day. Both the biogas and aqueous samples were analyzed daily for composition of substrates and fermentation products. Weight losses of the fed pretreated rice straw were measured at the end of fermentation.

2.4 Analytical procedures

The biogas produced in each of the fermentations was measured by wet gas meter (LML-1, Changchun Automotive Filter Co., LTD., Changchun, China). 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 N_2. The temperature of the column oven was set at 50°C, meanwhile 80°C was set for both the injection port and the detector.

The concentrations of aqueous products in the culture supernatant were determined by another gas chromatograph (SP-6800, Shandong Lunan Instrument Factory, China) equipped with a flame ionization detector and a 30 m FFAP capillary column (i.d.0.32 mm, SHIMADZU, Japan), also with N_2 as the carrier gas. The oven temperature was 160°C, and both temperatures of the injector and the detector were set at 210°C.

The weight loss of rice straw during acidogenic fermentation was calculated as described by Ai et al. (2013).

2.5 Analysis of the microbial community

10 mL samples from each fermentation were collected at distinctly different experimental periods in order to determine the microbial diversity in response to the bioaugmentation strategies. Three group of samples were obtained: (1) before the bioaugmentation period at 3rd day (DCB-3), (2) after the end of the control fermentation at

134 18^{th} day (DCB-18), (3) after the end of the bioaugmented fermentation at 18^{th} day
135 (BDCB-18).
136 Genomic DNA from samples was isolated and purified using the DNA Isolation Kit
137 (MO BIO laboratories, Inc.) following the guidelines of the manufacturer's protocol. The
138 hypervariable V3-V4 regions of microbial 16S rRNA genes were amplified using primers
139 pairs 341f (5'-CCTACGGGAGGCAGCAG-3') and 805r (5'-GACTACHVGGGTATCT
140 AATCC-3') (Herlemann et al., 2011). Composition of the PCR products of V4 region was
141 determined by pyrosequencing using the Illumina Miseq sequencer platform (Shanghai
142 Sangon, China).
143
144 **3. Results and discussion**
145 3.1 Performance of fermentations
146 In our previous study, it was shown that the microbial consortium efficiently produce
147 butyric acid using alkali pretreated rice straw in a batch test, which indicated that the
148 consortium DCB was an excellent butyric acid producer from lignocellulose (Ai et al., 2013).
149 In an attempt to improve further butyric acid concentration, we initially described a
150 bioaugmentation strategy by supplementation with *C. tyrobutyricum* in a fed-batch mode of
151 CBP. The control and bioaugmented fermentations using consortium DCB and BDCB were
152 investigated for 18 days, respectively (Table 1, Fig. 2). It was noticed that the consortium
153 DCB degenerated remarkably during the fed-batch fermentation process, resulting in only
154 5.53 g/L butyric acid from total 140 g/L pretreated rice straw, which account for 20.8%
155 product selectivity (Table 1). As illustrated in Fig. 2a, the accumulation of butyric acid
156 reached maximum on the 3^{rd} day, and slightly decreased from that day onwards. With feeding
157 of the pretreated rice straw, the production of caproic acid continuously accumulated to a
158 high concentration of 8.49 g/L. It has been proved that butyric acid can convert to caproic

159 acid by some anaerobic bacteria, and the possible reactions are shown as the equations (1)
160 and (2) (Agler et al., 2011; Spirito et al., 2014; X. Zhu et al., 2017). Therefore, the produced
161 butyric acid was further converted to caproic acid by the microbes existed in consortium
162 DCB, resulting in the low butyric acid production in the fed-batch process.

163 $$\text{Ethanol} + \text{Butyrate} \rightarrow \text{Caproate} + H_2O \quad (1)$$

164 $$\text{Butyrate} + 2CO_2 + 6H_2 \rightarrow \text{Caproate} + 4H_2O \quad (2)$$

165 On the other hand, when the bioaugmentation of *C. tyrobutyricum* ATCC 25755 was
166 evaluated by fed-batch mode of CBP, the production of acetic acid accumulated rapidly and
167 reached the maximum of 8.21 g/L on the 6^{th} day, during which no obvious butyric acid was
168 observed (Fig. 2b). With the end of acetic acid production phase, butyric acid accumulated
169 continually, and reached the maximum production rate of 3.32 g/L/d on the 7^{th} day. The
170 results indicated that the bioaugmentation with *C. tyrobutyricum* ATCC 25755 was a feasible
171 approach for enhanced butyric acid production from rice straw. Due to the accumulation of
172 rice straw residue and the deceleration of production rate of butyric acid, the supplement of
173 pretreated rice straw was ceased on the 13^{th} day, and subsequently the fermentation was
174 ceased two days later.. By the end of the fermentation, around 51% (equal to 71.33 g/L) of
175 the fed pretreated rice straw was consumed and the butyric acid concentration was
176 significantly increased to 18.05 g/L with a high selectivity of 60.7% (Table 1). Furthermore,
177 during the fermentation process no caproic acid was detected. The specific butyric acid yield
178 of consumed rice straw was increased from 0.09 g/g of control fermentation to 0.26 g/g of
179 bioaugmented fermentation, indicating that the designed consortium BDCB was more
180 efficient in converting rice straw to butyric acid compared with previous consortium DCB
181 study.

182 Considering the cost-efficiency of fermentative butyric acid production, renewable and

low-cost lignocellulosic substrates, including wheat straw, switchgrass (S. Liu et al., 2013), sugarcane bagasse (Wei et al., 2013) and corn fiber (Y. Zhu et al., 2002) have been applied in previous studies. However, due to the butyric acid-producing clostridia cannot utilize unhydrolyzed lignocellulose, SHF and SSF processes were conducted that involved cellulolytic enzymes to break down lignocellulosic biomass into fermentable monosaccharides. Although high butyric acid selectivity and less by-products were always investigated in the fermentation with lignocellulosic hydrolysate, the exogenous application of enzymes involved in hydrolysis almost counteracts the benefit from the cheap feedstocks (Jørgensen et al., 2007). Since the highly resistant to biological conversions, only few researches were investigated by CBP (Ai et al., 2013; Merklein et al., 2014). *Thermobifida fusca*, a cellulolytic butyric acid-producing actinobacterium once was introduced to butyric acid production form milled corn stover, whereas the low product concentration of 2.37 g/L was observed with three kinds of by-products (Merklein et al., 2014). Compared with the previous researches, the present fermentation strategy with consortium BDCB not only avoided the involvement of exogenous enzymes, but also obtained a high butyric acid production of 18.05 g/L.

3.2 Diversity of microbial community

It was reported that the addition of extra microbes into the bioreactor can lead to microbial community shifts which will further effect on the butyric acid production (Čater et al., 2015). We also further studied the reads and diversity indices of microbial community in the consortium DCB and BDCB of the control and bioaugmented fermentation, respectively. As shown in Table 2, the indices Chao1 and ACE of the DCB-3 were remarkably higher than that of the DCB-18, indicating that the species richness was seriously depressed by the degeneration which resulted in a poor butyric acid production (Table 1 and Fig. 2).

208 Meanwhile, Shannon index of the consortium DCB decreased from 2.98 on the 3rd day to
209 2.44 on the 18th day, suggesting that the accumulation of carboxylate product suppressed the
210 bacterial growth, which resulted in the lower diversity of microbial community and the
211 degeneration of consortium DCB.

212 For the culture of CBP with bioaugmentation, the indies Chao1 and ACE reached 352
213 and 373 in the BDCB-18, which similar to that of the DCB-3 (349 and 374, respectively).
214 The higher species richness in consortium BDCB laid a foundation for the high butyric acid
215 production. Whereas, Shannon diversity index of the BDCB-18 further decreased to 0.93,
216 was much lower than that of the DCB-3 (2.98) and DCB-18 (2.44). Although, the enrichment
217 of butyric acid-producing strain *C. tyrobutyricum* ATCC 25755 lowered the microbial
218 community diversity, resulting in the enhanced butyric acid production in the bioaugmented
219 fermentation (Table 1 and Fig. 2).

220

221 3.3 Microbial community structure

222 The results from all samples showed that the most predominant members of the
223 community belonged to bacterial phyla Firmicutes (72%-92%) and Proteobacteria (8%–27%).
224 The bacterial community profile obtained in the present study showing that the communities
225 were dominated by the phyla comprising species involved in cellulose degradation (Nyonyo
226 et al., 2014; Ozbayram et al., 2017). The enriched cellulose-degrading bacteria provided
227 fermentable substrates (pentose and hexose) for the acid-producing bacteria, which laid a
228 foundation for the high butyric acid production.

229 To reveal the biological mechanism of the influence by bioaugmentation in butyric acid
230 production, further analysis of the microbial communities at the genus level was performed.
231 As illustrated in Fig. 3, significant changes of microbial community structures along with the
232 fermentation process were observed between DCB-3 and DCB-18. Average 12% in DCB-3

and 6% in DCB-18 of the total sequences were the unidentified bacteria at the genus level. As for the fed-batch fermentation with consortium DCB, the relative abundances of *Clostridium* IV, *Brevundimonas*, *Brucella*, *Soehngenia*, *Rummeliibacillus* and *Lysinibacillus* in DCB-18 were much lower than those in DCB-3, while the proportions of *Hungatella*, *Clostridium* XIVa, *Anaerotruncus*, *Clostridium* sensu stricto, *Oscillibacter* and *Clostridium* XIVb were higher than those in DCB-3. The genus *Clostridium*, including a large number of hemicellulose- and cellulose-degrading anaerobic bacteria, was a core group of microbes with the proportion of 29% and 23% in the DCB-3 and DCB-18, respectively. Their high abundance in the culture ensure that the rice straw could degrade to fermentable sugars efficiently (Dürre, 2005). Some bacteria in the core group, including genera *Clostridium* clusters IV and XIVa are the most active butyric acid producer in the microbial consortium (Abbeele et al., 2013; Antharam et al., 2013; Hold et al., 2003). The proportion of *Clostridium* IV and XIVa decreased from 27% in DCB-3 to 16% in DCB-18, resulting in the low butyric acid production in the fed-batch fermentation (Table 1 and Fig. 2a). In contract, the proportions of genus *Oscillibacter* and *Clostridium* sensu stricto in DCB-18 (8%) were higher than those in DCB-3 (2%). Members of *Oscillibacter* and *Clostridium* sensu stricto can produce medium and long chain fatty acids (Gupta & Gao, 2009; Iino et al., 2007). The high abundance of these microbes might contribute to the high concentration of caproic acid in the fed-batch fermentation with consortium DCB (Table 1 and Fig. 2a). Members of *Hungatella* and *Anaerotruncus* harbor the ability to ferment carbohydrate to acetic acids (Lawson et al., 2004; Kaur et al., 2014) that might be related to the increased acetic acid production (Fig. 2).

There are many factors limiting the application of bioaugmentation, such as the adaptation of the inoculums, competition from indigenous microbial populations, the insufficiency of substrate and grazing by protozoa (Bouchez et al., 2000; Tan. et al., 2016).

The survival of bioaugmented strain, and the structure and function breakdown of the indigenous microbial community are the primary factors influence on the performance of bioaugmentation. In the present study, the bioaugmentation of *C. tyrobutyricum* ATCC 25755 into consortium DCB resulting in an indigenous community shift, that corresponded to changes in the microbial distribution. The genus *Clostridium* IV increased to 83%, suggesting that the strain had acclimated and colonized the indigenous community successfully. The proportion of butyric acid-producing clostridia in BDCB-18 (*Clostridium* IV and XIVa) increased to 89%, resulting in a much higher butyric acid production when compared with that in the control fermentation (Table 1 and Fig. 2). Whereas, the genus *Oscillibacter* and *Clostridium* sensu stricto in BDCB-18 were both undetectable, which might be related to the negligible caproic acid production in the bioaugmented fermentation (Fig. 2b).

3.4 Functional genes

Accompanying changes of the taxonomic, shotgun metagenomic analysis revealed clear differences in the functional gene composition across communities between control and bioaugmented fermentations. Genes within each sample were further classified by aligning them against the COG (Cluster of orthologous groups) database (Tatusov et al., 2001; Gill et al., 2006). The cellular function and the percentage distribution of genes within each sample among the different COG categories are shown in Fig. 4. BLAST comparisons of all sequences yielded 4793 unique COGs. DCB-3, DCB-18 and BDCB-18 showed similar patterns of enrichment for each COG involved in metabolism. However, comparing with DCB-3 and DCB-18, majority exhibited genes in BDCB-18 mainly involved in cellular functions, such as energy production and conversion (C), amino acid transport and metabolism (E), carbohydrate transport and metabolism (G), general function prediction only (R), function unknown (S) and signal transduction mechanisms (T).

Fermentation of rice straw requires cooperation of groups of microbes in the community. Primary process was the hydrolysis of cellulose and hemicellulose into hexose and pentose (mainly glucose and xylose), The released monosaccharides were further fermented to short chain fatty acids. The consortium DCB was enriched for genes involved in cellulose, xylan, glucose, xylose and galactose metabolism, whereas these genes were further enriched in consortium BDCB. It suggested that the consortia can utilize wide-ranging substrates as the principal energy source (Gill et al., 2006). COG analyses demonstrated enrichment of key genes involved in generating acetate and butyrate in the consortium DCB and BDCB. The genes related to acetate and butyrate kinases in BDCB-18 were much higher than that in DCB-3 and DCB-18. It was known that acetic acid production pathway was more favorable to the synthesis of adenosine 5′ -triphosphate (ATP) for cell growth (Jo et al., 2008), therefore, the enrichment of gene related to acetate kinase ensured the ample energy for the fermentation process. Furthermore, the enrichment of gene related to butyrate kinase underscored the important commitment of the bioaugmented consortium (BDCB) to generate high concentration of butyric acid. The consortium DCB and BDCB were also enriched for a variety of COGs involved in amino acids metabolism, which might be contribute to the supplement of peptone during the fermentation.

4. Conclusion

This study serves as the first report on bioaugmentation with *C. tyrobutyricum* ATCC 25755 to improve butyric acid production in CBP process. A high butyric acid production of 18.05 g/L was obtained in the augmented process with a selectivity of 60.7% from rice straw. The high butyric acid production was attributed to the introduction of *C. tyrobutyricum* ATCC 25755, which shift the indigenous bacterial community and enriched the genes related to glycoside hydrolase and butyrate kinase. The process with consortium BDCB should be

more effective in butyric acid production for future applications.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (51478141), Open Project of State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology (HC201410), and Hong Kong General Research Fund (15212317).

Reference

[1] Abbeele, P.V.D., Belzer, C., Goossens, M., Kleerebezem, M., Vos, W.M.D., Thas, O., Weirdt, R.D., Kerckhof, F.M., Wiele, T.V.D. 2013. Butyrate-producing *Clostridium* cluster XIVa species specifically colonize mucins in an in vitro gut model. The ISME journal, **7**(5), 949-961.

[2] Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T. 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends Biotechnol., **29**(2), 70-78.

[3] Ai, B.L., Li, J.Z., Song J.L., Chi X., Meng J., Zhang L.G., Ban Q.Y. 2013. Butyric acid fermentation from rice straw with undefined mixed culture: enrichment and selection of cellulolytic butyrate-producing microbial community. Int. J. Agric. Biol., **15**(6), 1075-1082.

[4] Antharam, V.C., Li, E.C., Ishmael, A., Sharma, A., Mai, V., Rand, K.H., Wang, G.P. 2013. Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhea. J. Clin. Microbiol., **51**(9), 2884-2892.

[5] Baroi, G.N., Baumann, I., Westermann, P., Gavala, H.N. 2015. Butyric acid fermentation from pretreated and hydrolysed wheat straw by an adapted *Clostridium tyrobutyricum*

strain. Microb. Biotechnol., **8**(5), 874-882.

[6] Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Doré, J., Delgenès, P., Moletta, R., Wagner, M. 2000. Ecological study of a bioaugmentation failure. Environ. Microbiol., **2**(2), 179–190.

[7] Čater, M., Fanedl, L., Malovrh, Š., Logar, R.M. 2015. Biogas production from brewery spent grain enhanced by bioaugmentation with hydrolytic anaerobic bacteria. Bioresour. Technol., **186**, 261-269.

[8] Dürre, P. 2005. Handbook on clostridia. Taylor, Francis.

[9] Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraserliggett, C.M., Nelson, K.E. 2006. Metagenomic Analysis of the Human Distal Gut Microbiome. Science, **312**(5778), 1355-1359.

[10] Gupta, R.S., Gao, B. 2009. Phylogenomic analyses of clostridia and identification of novel protein signatures that are specific to the genus *Clostridium* sensu stricto (cluster I). Int. J. Syst. Evol. Microbiol., **59**(Pt 2), 285-294.

[11] Herlemann, D.P.R., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F. 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. The ISME journal, **5**(10), 1571-1579.

[12] Hold, G.L., Schwiertz, A., Aminov, R.I., Blaut, M., Flint, H.J. 2003. Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. Appl. Environ. Microbiol., **69**(7), 4320-4324.

[13] Iino, T., Mori, K., Tanaka, K., Suzuki, K., Harayama, S. 2007. *Oscillibacter valericigenes* gen. nov., sp. nov., a valerate-producing anaerobic bacterium isolated from the alimentary canal of a Japanese corbicula clam. Int. J. Syst. Evol. Microbiol., **57**(8), 1840-1845.

[14] Jørgensen, H., Kristensen, J.B., Felby, C. 2007. Enzymatic conversion of lignocellulose

into fermentable sugars: challenges and opportunities. Biofuels, Bioprod. Biorefin., **1**(2), 119-134.

[15] Jiang, L., Wang, J., Liang, S., Cai, J., Xu, Z., Cen, P., Yang, S., Li, S. 2011. Enhanced butyric acid tolerance and bioproduction by *Clostridium tyrobutyricum* immobilized in a fibrous bed bioreactor. Biotechnol. Bioeng., **108**(1), 31-40.

[16] Jo, J.H., Lee, D.S., Park, J.M. 2008. The effects of pH on carbon material and energy balances in hydrogen-producing *Clostridium tyrobutyricum* JM1. Bioresour. Technol., **99**(17), 8485-8491.

[17] Kaur, S., Yawar, M., Kumar, P.A., Suresh, K. 2014. *Hungatella effluvii* gen. nov., sp. nov., an obligately anaerobic bacterium isolated from an effluent treatment plant, and reclassification of Clostridium hathewayi as Hungatella hathewayi gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol., **64**(3), 710-718.

[18] Lawson, P.A., Song, Y., Liu, C., Molitoris, D.R., Vaisanen, M.L., Collins, M.D., Finegold, S.M. 2004. *Anaerotruncus colihominis* gen. nov., sp. nov., from human faeces. Int. J. Syst. Evol. Microbiol., **54**(2), 413-417.

[19] Li, Y., Zhang, Y., Sun, Y., Wu, S., Kong, X., Yuan, Z., Dong, R. 2017. The performance efficiency of bioaugmentation to prevent anaerobic digestion failure from ammonia and propionate inhibition. Bioresour. Technol., **231**, 94-100.

[20] Liu, S., Bischoff, K.M., Leathers, T.D., Qureshi, N., Rich, J.O., Hughes, S.R. 2013. Butyric acid from anaerobic fermentation of lignocellulosic biomass hydrolysates by *Clostridium tyrobutyricum* strain RPT-4213. Bioresour. Technol., **143**(6), 322-329.

[21] Liu, X., Yang, S.T. 2006. Kinetics of butyric acid fermentation of glucose and xylose by *Clostridium tyrobutyricum* wild type and mutant. Process Biochem., **41**(4), 801-808.

[22] Merklein, K., Fong, S.S., Deng, Y. 2014. Production of butyric acid by a cellulolytic actinobacterium *Thermobifida fusca* on cellulose. Biochem. Eng. J., **90**, 239-244.

[23] Nyonyo, T., Shinkai, T., Mitsumori, M. 2014. Improved culturability of cellulolytic rumen bacteria and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the bovine rumen. FEMS Microbiol. Ecol., **88**(3), 528-537.

[24] Ozbayram, E.G., Kleinsteuber, S., Nikolausz, M., Ince, B., Ince, O. 2017. Effect of bioaugmentation by cellulolytic bacteria enriched from sheep rumen on methane production from wheat straw. Anaerobe, **46**, 122-130.

[25] Parisutham, V., Kim, T.H., Lee, S.K., 2014. Feasibilities of consolidated bioprocessing microbes: from pretreatment to biofuel production. Bioresour. Technol. **161**(3), 431-440.

[26] Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J., Angenent, L.T. 2014. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Curr. Opin. Biotechnol., **27**(6), 115-122.

[27] Tan., W., Huang., C., Chen., C., Liang., B., Wang, A. 2016. Bioaugmentation of activated sludge with elemental sulfur producing strain *Thiopseudomonas denitrificans* X2 against nitrate shock load. Bioresour. Technol., **220**, 647-650.

[28] Tatusov, R.L., Natale, D.A., Garkavtsev, I.V., Tatusova, T.A., Shankavaram, U.T., Rao, B.S., Kiryutin, B., Galperin, M.Y., Fedorova, N.D., Koonin, E.V. 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic Acids Res., **29**(1), 22-28.

[29] Tsapekos, P., Kougias, P.G., Vasileiou, S.A., Treu, L., Campanaro, S., Lyberatos, G., Angelidaki, I. 2017. Bioaugmentation with hydrolytic microbes to improve the anaerobic biodegradability of lignocellulosic agricultural residues. Bioresour. Technol., **234**, 350-359.

[30] Van S.P.J, Wine R.H. 1968. Determination of lignin and cellulose in acid-detergent fiber with permanganate. Journal of the Association of Official Analytical Chemists., **51**, 780-785.

[31] Wei, D., Liu, X., Yang, S.T. 2013. Butyric acid production from sugarcane bagasse hydrolysate by *Clostridium tyrobutyricum* immobilized in a fibrous-bed bioreactor. Bioresour. Technol., **129**(2), 553-560.

[32] Wilson, D.B., 2009. Cellulases and biofuels. Curr. Opin. Biotechnol. **20**(3), 295-299.

[33] Zhu, X., Zhou, Y., Wang, Y., Wu, T., Li, X., Li, D., Tao, Y. 2017. Production of high-concentration n-caproic acid from lactate through fermentation using a newly isolated *Ruminococcaceae* bacterium CPB6. Biotechnol. Biofuels, **10**(1), 102.

[34] Zhu, Y., Wu, Z., Yang, S.T. 2002. Butyric acid production from acid hydrolysate of corn fibre by *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. Process Biochem., **38**(5), 657-666.

Figure Captions

Fig. 1 Concept diagram of experiment design

Fig. 2 Performances of the control and bioaugmented fermentation by the consortium DCB and BDCB. (a) Fermentation by consortium DCB; (b) Fermentation by consortium BDCB.

Fig. 3 Bacterial community structure at genus level of consortium DCB and BDCB

Fig. 4 Functional distribution of differentially expressed genes

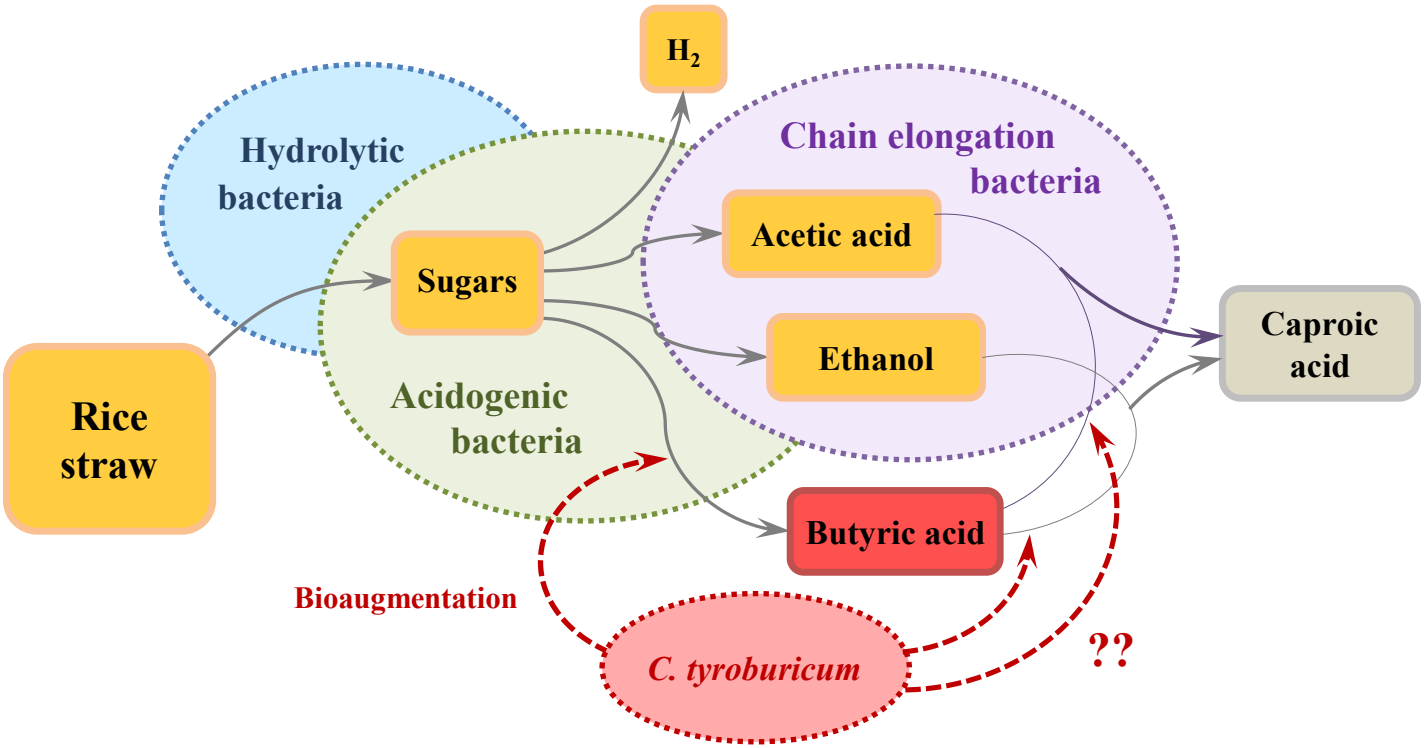
H2
Hydrolytic bacteria
Chain elongation bacteria
Acetic acid
Sugars
Ethanol
Caproic acid
Rice straw
Acidogenic bacteria
Butyric acid
Bioaugmentation
C. tyroburicum
??

Fig. 1

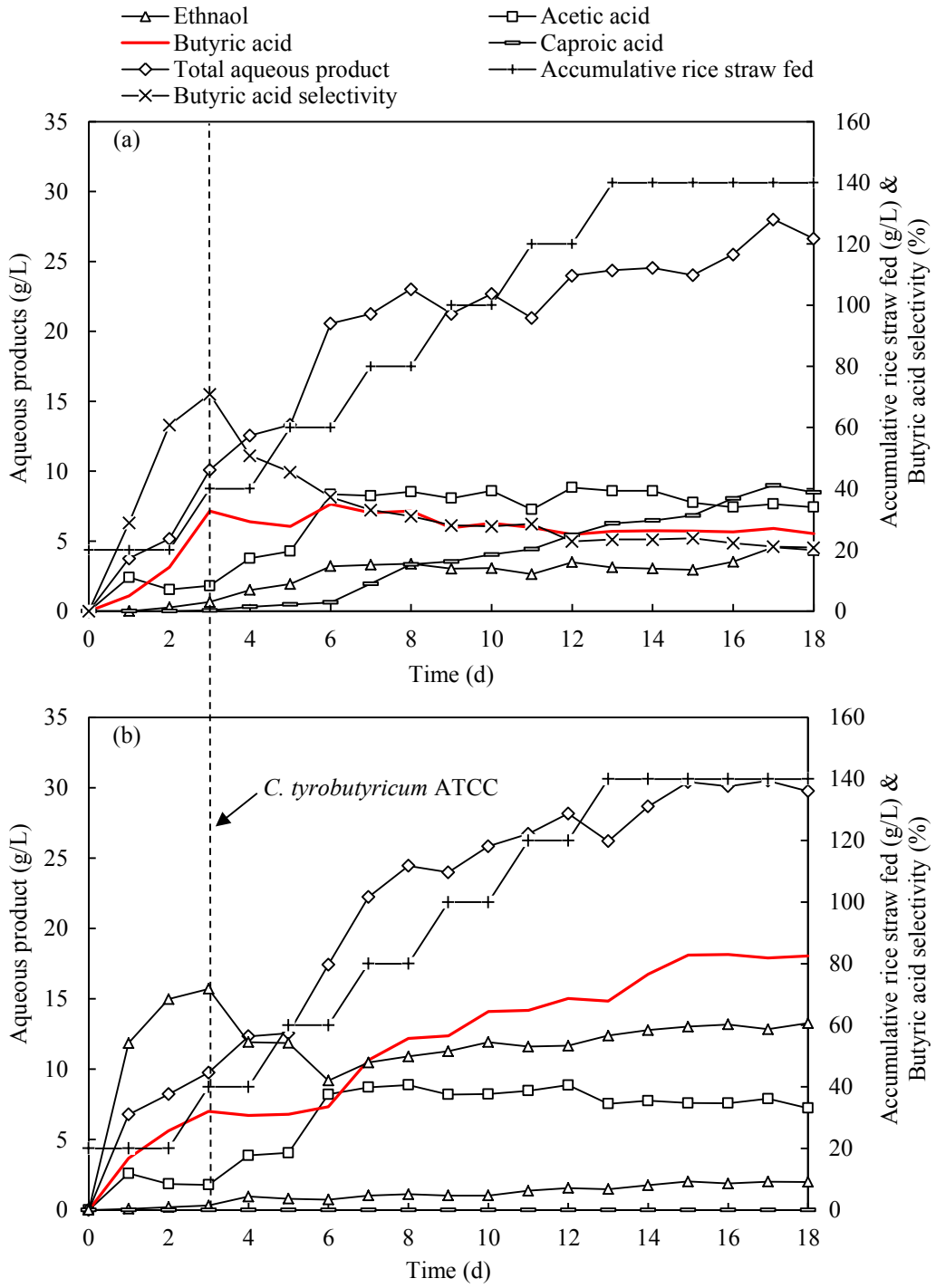
Ethnaol
Acetic acid
Butyric acid
Caproic acid
Total aqueous product
Accumulative rice straw fed
Butyric acid selectivity
(a)
Aqueous products (g/L)
Accumulative rice straw fed (g/L) & Butyric acid selectivity (%)
Time (d)
(b)
C. tyrobutyricum ATCC
Aqueous product (g/L)
Accumulative rice straw fed (g/L) & Butyric acid selectivity (%)
Time (d)

Fig. 2

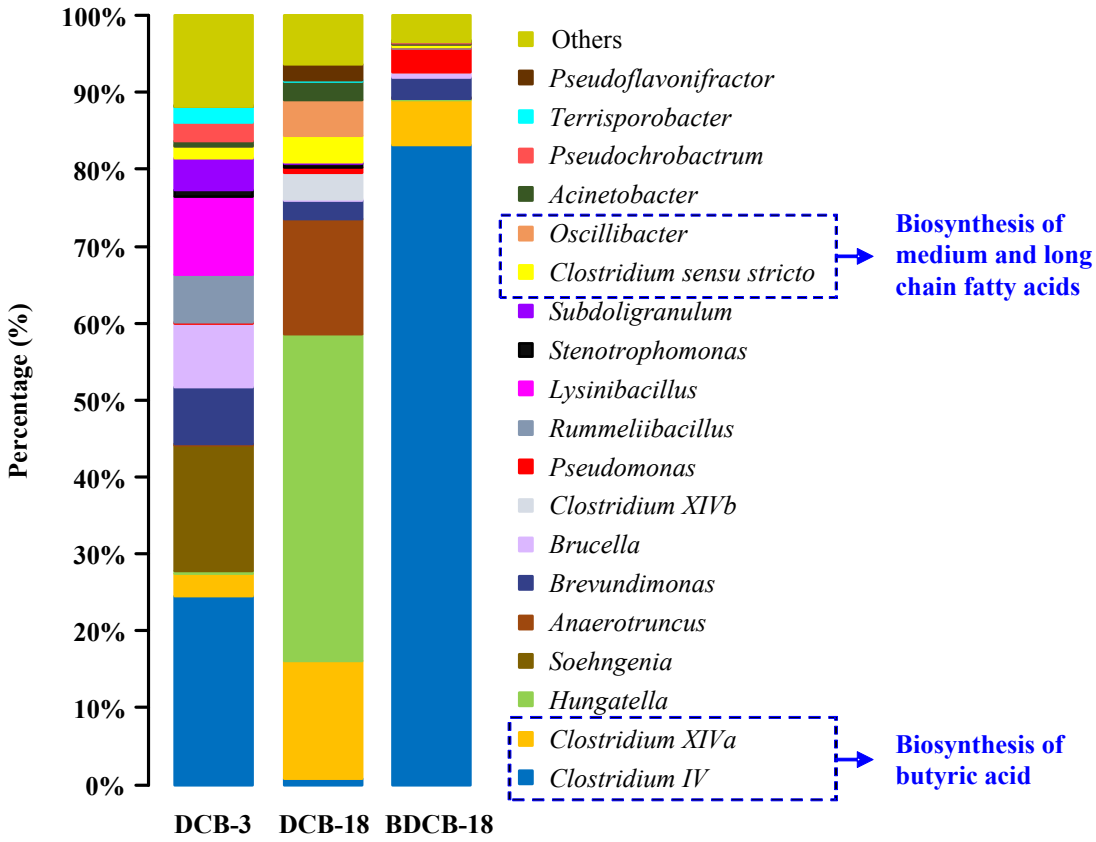
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage (%)
DCB-3
DCB-18
BDCB-18
Others
Pseudoflavonifractor
Terrisporobacter
Pseudochrobactrum
Acinetobacter
Oscillibacter
Clostridium sensu stricto
Subdoligranulum
Stenotrophomonas
Lysinibacillus
Rummeliibacillus
Pseudomonas
Clostridium XIVb
Brucella
Brevundimonas
Anaerotruncus
Soehngenia
Hungatella
Clostridium XIVa
Clostridium IV
Biosynthesis of medium and long chain fatty acids
Biosynthesis of butyric acid

Fig. 3

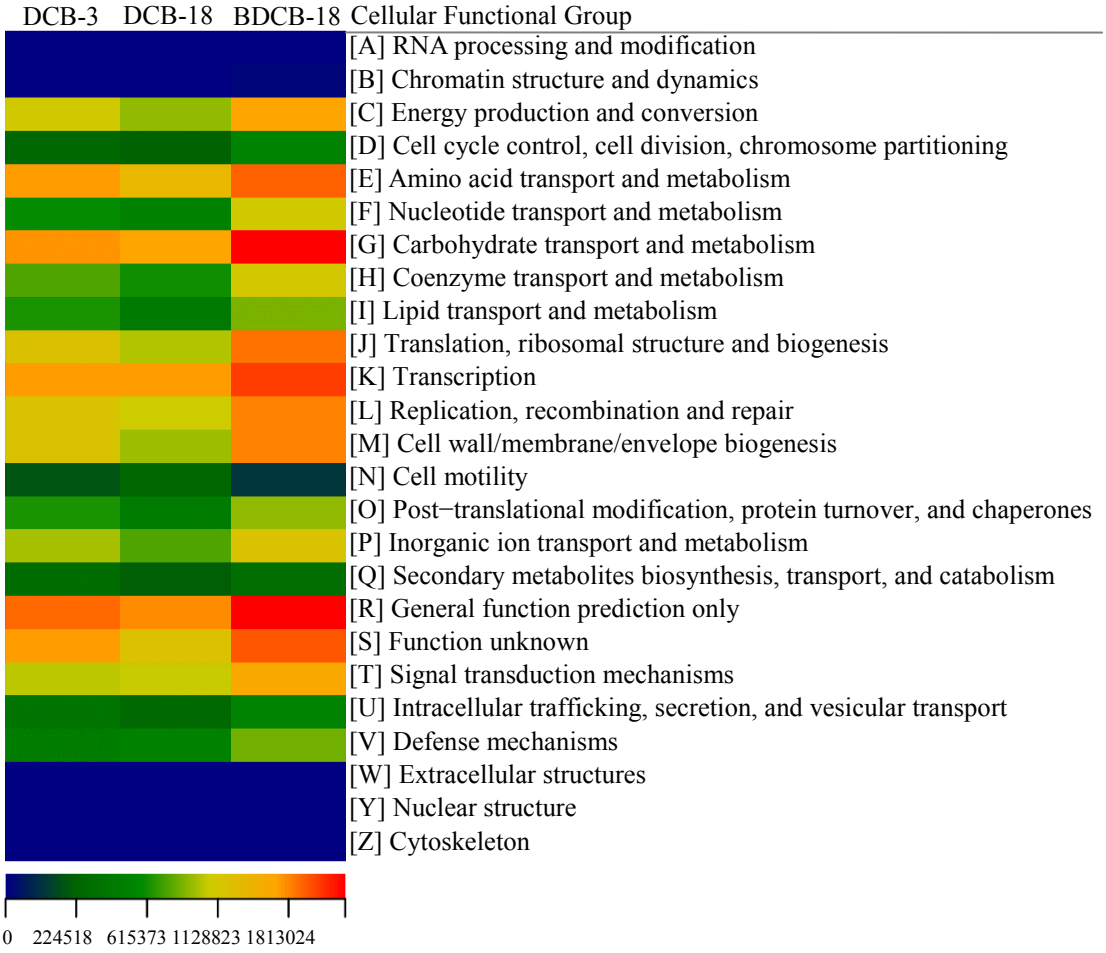
DCB-3
DCB-18
BDCB-18
Cellular Functional Group
[A] RNA processing and modification
[B] Chromatin structure and dynamics
[C] Energy production and conversion
[D] Cell cycle control, cell division, chromosome partitioning
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[G] Carbohydrate transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[J] Translation, ribosomal structure and biogenesis
[K] Transcription
[L] Replication, recombination and repair
[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[O] Post−translational modification, protein turnover, and chaperones
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport, and catabolism
[R] General function prediction only
[S] Function unknown
[T] Signal transduction mechanisms
[U] Intracellular trafficking, secretion, and vesicular transport
[V] Defense mechanisms
[W] Extracellular structures
[Y] Nuclear structure
[Z] Cytoskeleton
0
224518
615373
1128823
1813024

Fig. 4

Table 1. Characteristics of consortium DCB and BDCB

	DCB		BDCB
	Batch mode [a]	Fed-batch mode	Fed-batch mode
Rice straw fed (g/L)	20	140	140
Rice straw consumption (g/L)	10.67	60.21	71.33
Ethanol (g/L)	0.00	4.36	1.99
Acetic acid (g/L)	1.35	7.43	7.23
Propionic acid (g/L)	0.24	0.19	1.32
Isobutyric acid (g/L)	0.09	0.11	0.37
Butyric acid (g/L)	6.01	5.53	18.05
Isovaleric acid (g/L)	0.12	0.17	0.46
Valeric acid (g/L)	0.05	0.35	0.32
Caproic acid (g/L)	0.05	8.49	0.00
Hydrogen (L/L)	0.56	3.73	1.90
Total aqueous products (g/L)	7.97	26.63	30.47
Product selectivity [b] (%)	75.4	20.8	60.7
Butyric acid yield (g/g consumed rice straw)	0.56	0.09	0.26
Total products yield (g/g consumed rice straw)	0.75	0.35	0.43
Accumulative hydrogen (L/L)	0.57	3.73	1.90

[a] Results of previous research

[b] Percent of butyric acid in total aqueous products

Table 2. Diversity of microbial community in consortium DCB and BDCB

Sample	Sequences	OTUs	ACE	Chao1 index	Shannon index	Simpson index	Good's coverage (%)
DCB-3	60065	307	374	349	2.98	0.09	97
DCB-18	54494	176	222	209	2.44	0.21	98
BDCB-18	74143	290	373	352	0.93	0.66	97

Electronic Annex

Click here to download Electronic Annex: 03 Sup Chi BA.DOCX