Bioresource Technology 211 (2016) 319–326

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Bioaugmentation of *Hydrogenispora ethanolica* LX-B affects hydrogen production through altering indigenous bacterial community structure

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HIGHLIGHTS

- The added *Hydrogenispora ethanolica* LX-B caused a shift in metabolic pathway.
- The added *H. ethanolica* LX-B caused a shift in indigenous bacterial community.
- Enhanced hydrogen production was related to change in bacterial community structure.

ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form 15 March 2016
Accepted 17 March 2016
Available online 22 March 2016

Keywords:
Bioaugmentation
Hydrogen production
Bacterial community shift

ABSTRACT

Bioaugmentation can facilitate hydrogen production from complex organic substrates, but it still is unknown how indigenous microbial communities respond to the added bacteria. Here, using a *Hydrogenispora ethanolica* LX-B (named as LX-B) bioaugmentation experiments, the distribution of metabolites and the responses of indigenous bacterial communities were investigated via batch cultivation (BC) and repeated batch cultivation (RBC). In BC the LX-B/sludge ratio of 0.12 achieved substantial high hydrogen yield, which was over twice that of control. In RBC one-time bioaugmentation and repeated batch bioaugmentation of LX-B resulted in the hydrogen yield that was average 1.2-fold and 0.8-fold higher than that in control, respectively. This improved hydrogen production performance mainly benefited from a shift in composition of the indigenous bacterial community caused by LX-B bioaugmentation. The findings represented an important step in understanding the relationship between bioaugmentation, a shift in bacterial communities, and altered bioreactor performance.

1. Introduction

Hydrogen production from nonsterile organic wastes via an acidogenic fermentative pathway represents the potential bioenergy strategy using mixed cultures (Wong et al., 2014). The hydrogen production performance relies on properties of microbial communities and operation conditions (e.g. temperature, pH, hydraulic retention time), which control the bioconversion of organic waste to hydrogen (Wong et al., 2014). Among them, both the nature and structure of microbial community plays a crucial role in hydrogen production efficiency. With the available molecular techniques, numerous studies have sequenced clones of the most abundant hydrogen-producing bacteria in bioreactors, and the contributions of the key functional players for conversion of substrate to hydrogen are fully demonstrated (Wong et al., 2014). Thus inoculating excellent hydrogen production strains to the mixed cultures (defined as bioaugmentation), is expected to facilitate conversion of organic waste to hydrogen and improve the process efficiency (Goud et al., 2014).

Many studies have recently investigated how the added bacterial strains performed in terms of hydrogen production (Goud et al., 2014; Guo et al., 2010; Kotay and Das, 2010; Marone et al., 2012). The findings from these studies contributed to our understanding that bioaugmentation can enhance hydrogen yield, accelerate startup of a reactor, and counteract the adverse operation process. However, it is uncertain whether the added strains can compete with indigenous communities and establish the introduced property in a bioreactor system (Kuo et al., 2012). So far, the effect of bioaugmentation mostly based on analysis of metabolites (H_2 and/or volatile fatty acids) alone lacked information on bacterial community composition to demonstrate the role of the added bacteria (Guo et al., 2010; Kotay and Das, 2009; Marone et al., 2012). Using the available molecular techniques, some studies confirmed the survivability and persistence of the added

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http://dx.doi.org/10.1016/j.biortech.2016.03.097
0960-8524/

strains, implying the contribution of the added bacteria to the improved hydrogen production (Goud et al., 2014; Kotay and Das, 2010). However, the improved hydrogen production may not necessarily reflect that the added bacteria made a major contribution, as the developed dominant bacteria derived from the indigenous bacterial community might also make a substantial contribution to the improved hydrogen production (Goud et al., 2014). Thus, understanding the community structural properties may be particularly important in the context of bioaugmentation, but the knowledge available to date remains unclear.

To precisely evaluate the survival and activity of the added bacteria, the candidate bacteria should not share similar phylogenetic characteristic with the indigenous functional bacteria inhabited in bioreactors. Thus, the selection of candidate bacteria is crucial. *Hydrogenispora ethanolica* LX-B (named as LX-B) was a new genus and novel species with hydrogen yield of 2.52 mol H_2/mol glucose (Liu et al., 2014). LX-B were phylogenetically different from the well documented hydrogen-producers (e.g. *Enterobacter*, *Citrobacter*, *Bacillus*, *Clostridium*, *Enterococcus*, *Clostridium* and *Klebsiella*) that were frequently detected in bacterial consortia of hydrogen-producing reactors (Wong et al., 2014). Thus, LX-B with high hydrogen yield and unusual phylogenetic property was selected to investigate its contribution to the improved reactor performance and the response of indigenous microbial community after bioaugmentation. In this study, metabolite analysis and 16S rDNA sequencing approaches were applied to investigate the survival and fate of LX-B, bacterial community shift and their correlations with metabolite change through batch and repeated batch cultivations. DNA-sequencing approach showed that no related 16S rDNA sequences of LX-B were recovered in control without LX-B addition, indicating the novelty of LX-B in investigating bioaugmentation mechanisms.

2. Materials and methods

2.1. Feedstock, seed sludge and LX-B used for bioaugmentation

Fresh potatoes were purchased from the farmer's market (Qingdao, China) which were crushed and homogenized in a blender. The total solids (TS) content of potatoes was 13% (w/w), which contained 93.1% of the volatile solids (VS). Anaerobic granular sludge was collected from an upflow anaerobic sludge blanket (USAB) treating brewery waste at an brewery facility in Qingdao, China. VS of the sludge accounted for 90% (w/w) in TS. The sludge was first crushed and homogenized in a blender and then heated at 120 °C for 30 min prior to use.

LX-B was obtained from Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, China, which was stored at −80 °C. LX-B cells were grown with glucose-based fresh medium at 37 °C (Liu et al., 2014), which were harvested at logarithmic phase. The pure cultures were centrifuged at 5000 rpm for 8 min and resuspended in 10 mM phosphate buffer medium (pH 7.0) prior to use.

2.2. Hydrogen production by batch and repeated batch cultivations

Batch cultivation (BC) and repeated batch cultivations (RBC) were carried out at 37 °C in 120 mL of anaerobic bottles with a working volume of 50 mL. Each experiment was performed in triplicate. Both the heat-treated sludge and medium (pH 7.0) were added into the bottles, which were then purged with high purity of N_2 (99.99%) and sealed with butyl rubber stoppers. LX-B cells were then injected into the bottles using the sterile syringe. The medium contained 10 g-VS/L of potato slurry (final concentration), and other components were described previously (Yang et al., 2011).

BC was performed to evaluate the effects of LX-B/sludge ratio (w/w) on hydrogen production. RBC was conducted to determine the effects of bioaugmentation pattern of LXB on hydrogen production. Three experiments were conducted: (i) control without LX-B addition, (ii) one-time bioaugmentation of LX-B (named as NBA), and (iii) repeated batch bioaugmentation of LX-B (named as BA). The detailed experimental information was listed in Table 1. As for RBC, in the start-up stage, heat-treated sludge and/or LX-B were incubated in the medium. When hydrogen production reached midterm of logarithmic phase, RBC were commenced. Briefly, 50% (v/v) of the medium was replaced with the same amounts of the fresh medium when hydrogen production reaches a plateau at the end of each cycle. Gas stayed in the headspace of bottles was removed using a vacuum pump and aerated with N_2 (99.99%) before the start of the next cycle. Three cycles of harvesting and feeding were carried out.

2.3. Analytical methods

pH, TS and VS were measured as described previously (Yang et al., 2011). The gas volume was measured using water displacement method. The fraction of H_2 was periodically determined using gas chromatograph (SP6890, Shandong, China) according to the method developed by Yang et al. (2011). The maximum hydrogen production rate (Rm) and the lag-phase time (λ) were obtained using the modified Gompertz equation (Yang et al., 2011). In this work, VS was used to represent the organic content of fresh potato slurry. Hydrogen yield, Ps (unit of mL H_2/g-VS), was calculated by dividing cumulative hydrogen production (mL) with each gram of added feedstock, which was expressed as g-VS. The pH of fermentation broth was adjusted to pH 3 with 0.2 N HCl prior to analysis. Ethanol and volatile fatty acid (VFA) in the acidified fermentation broth were determined by a gas chromatograph (GC-2014, Shimadzu, Japan) equipped with a flame ionization detector (FID) and a 30 m × 0.32 mm × 0.5 μm WAX-DA column. Both the injector and detector of GC analyzed broth were 250 °C. The programmed column temperatures were as follows: 80 °C maintained for 2 min; 20 °C/min to 150 °C, maintained for 1 min;

Table 1
The conditions of bioaugmentation experiments for batch and repeated cultivations.

Bioaugmentation condition*	LX-B (g-VS L^{-1})	Sludge (g-VS L^{-1})
BC (LX-B/sludge ratio)		
0.06	0.06	1
0.12	0.12	1
0.24	0.24	1
Control	0	1
RBC (bioaugmentation pattern of LXB)		
Repeated batch bioaugmentation (BA)		
Start-up (LS)	0.12	1
Cycle 1 (BA1)	0.12	0
Cycle 2 (BA2)	0.12	0
Cycle 3 (BA3)	0.12	0
One-time bioaugmentation (NBA)		
Start-up (LS)	0.12	1
Cycle 1 (NBA1)	0	0
Cycle 2 (NBA2)	0	0
Cycle 3 (NBA3)	0	0
Control (C)		
Start-up (CS)	0	1
Cycle 1 (C1)	0	0
Cycle 2 (C2)	0	0
Cycle 3 (C3)	0	0

* LX-B and/or heat-treated sludge was added into the bottles at the beginning of each test.

10 °C/min to 180 °C, maintained for 1 min; 20 °C/min to 230 °C, maintained for 3 min.

2.4. Microbial community analysis

The total genomic DNA extraction of the experimental samples collected from fermentation process was performed using FastDNA®Spin Kit for Soil (CWBIO). DNA was then checked and quantified, and amplicon libraries were constructed for Illumina sequencing using primer pairs as described previously (Fu et al., 2015). Library construction and Illumina sequencing were performed at GENEWIZ, Inc., South Plainfield, NJ, USA. Sequence analysis was performed as described previously (Yang et al., 2015). An unweighted UniFrac distance matrix was constructed using QIIME and visualized via principal coordinates analysis (PCoA) as executed in R v3.2.3.

3. Results and discussion

3.1. Hydrogen production

The hydrogen production profiles in BC and RBC are shown in Figs. S1 and S2, and the total performances of all tests are outlined in Table 2. In all tests, no methane production was detected, because the pretreatment of seed sludge at high temperature can suppress the activity of methanogens (Wong et al., 2014; Yang et al., 2011). As shown in Fig. S1, compared with control, at the initial stage of fermentation from 0 h to 23 h, the initial lower hydrogen yield in groups amended with LX-B was obtained. However, after around 23 h of fermentation, the hydrogen yield increased rapidly in groups with LX-B/sludge ratio of 0.06 and 0.12. These findings, combined with the observation that longer lag-phase time was spent for LX-B/sludge ratio of 0.06 and 0.12 (Table 2), indicated that the added LX-B need long time to adapt new environment and to exert bioaugmentation effect.

Both Table 2 and Fig. S1 show that the LX-B bioaugmentation at a suitable ratio resulted in a significant increase in Rm and Ps. When the ratio was 0.06, Rm and Ps were, respectively, 75.4% and 31.7% higher than those in control without LX-B addition. When the ratio increased to 0.12, both Rm and Ps were over twice those of control. Similar to the well-studied hydrogen-producing strains, such as *Bacillus subtilis* (Goud et al., 2014) and *Ethanoligenens harbinense* B49 (Guo et al., 2010), the LX-B bioaugmentation also can promote hydrogen production from the complex substrates. However, when the ratio was increased to 0.24, both Rm and Ps were 15.8% and 74% lower than those in control, respectively. Previous reports also showed that excess addition of bacteria can result in a decrease in the hydrogen production rate (Guo et al., 2010). Thus, the findings clearly indicated that excess addition of LX-B had an adverse effect on hydrogen production. This was probably attributed to the changed microbial community structure, which was favored for hydrogen consumption rather than hydrogen production.

In the subsequent RBC, the LX-B/sludge ratio of 0.12 was used because the ratio showed the comparable Ps and Rm. Fig. S2 shows that the hydrogen yield in group BA and NBA was significantly higher than in control. Specifically, only average 31.2 mL/g-VS of hydrogen was produced over each cycle in control. In BA, average 54.3 mL H_2/g-VS was generated during each cycle, which was average 0.8-fold greater than in control. Average 66.6 mL H_2/g-VS in NBA was produced during each cycle, which was average 1.2-fold higher than in control. Table 2 shows that the LX-B bioaugmentation considerably improved Rm in RBC. Rm in group BA and NBA was at least average 1.4-fold and 1.2-fold higher than in control, respectively. These findings, combined with the hydrogen yield, suggested that the bioaugmentation of LX-B in the pattern of either BA or NBA was more favorable to hydrogen production.

Ps in group BA and NBA over each cycle during RBC was lower than that in group 0.12 from batch experiment, indicating that the part of the feedstock was not effectively converted to hydrogen. This may be due to the short retention time, as previous reports showed that decreasing retention times led to a decrease in the hydrogen yield and substrate removal (Kumar et al., 2014). Additionally, after three cycles of cultivation, the average Rm (7.4 mL H_2/h·g-VS) in BA was similar to that (6.9 mL H_2/h·g-VS) in NBA, while the average Ps in BA was slightly less than that in NBA. This suggested that one-time bioaugmentation of LX-B can obtain the similar bioaugmentation effect with the repeated batch bioaugmentation of LX-B in terms of improving hydrogen production during RBC.

It is noteworthy that the hydrogen yield for LX-B to degrade potato slurry was about 11 mL H_2/g-VS (Fig. S3), which was lower than the reported 313.6 mL H_2/g-glucose (Liu et al., 2014). This indicated that LX-B can poorly utilize organic matter in potato slurry to hydrogen. The improved hydrogen yield (Figs. S1 and S2) after bioaugmentation of LX-B further indicated that there might be a close cooperation between LX-B and indigenous bacterial consortia during the conversion of potato slurry to hydrogen. Additionally, the hydrogen yield ranged from 27 to 42 mL H_2/g-VS was obtained in group control, which was lower than the hydrogen yield of 200–218 mL H_2/g-VS for potatoes reported by Xie et al. (2008), and 72 mL H_2/g-VS for potato waste reported by Zhu et al. (2008). This might be attributed to several possible reasons. Firstly, the pretreatment temperature of seed sludge was too high. Previous report showed that the elevated pretreatment temperatures reduced the species diversity of seed sludge (Baghchehsaraee et al., 2008). Thus, most hydrogen-producing bacteria might be inhibited in this study. Furthermore, the substrate to inoculum ratio and fermentation conditions were not optimized. A previous report showed that hydrogen production from organic waste was affected by operation conditions (e.g. substrate to inoculum ratio, and pH) (Wong et al., 2014; Yang et al., 2011; Fan and Chen, 2004). The non-optimized operation conditions might therefore result in a low hydrogen yield in this work. Finally, hydrogen-consuming bacteria (e.g. homoacetogens) might co-exist with the hydrogen-producers, thus providing low hydrogen yield, as previous reports showed the possibility (Hussy et al., 2003).

Table 2
Hydrogen production rate and lag-phase time in BC and RBC.

	Rm (mL h^{-1}·g-VS^{-1})	λ (h)	R^2
BC			
0.06	1	21.85	0.99
0.12	1.78	21.87	0.99
0.24	0.48	10.36	0.98
Control	0.57	13.42	0.98
RBC			
Control			
Cycle 1	3.58	1.06	0.96
Cycle 2	3.36	0.48	0.96
Cycle 3	2.81	0.05	0.98
NBA			
Cycle 1	5.64	1.08	0.99
Cycle 2	7.6	0.28	0.95
Cycle 3	7.47	0	0.97
BA			
Cycle 1	5.34	1.65	0.99
Cycle 2	6.73	0.52	0.96
Cycle 3	10.22	0.11	0.98

3.2. Intermediate metabolite accumulation

The production profiles and production rates of individual metabolites during BC are shown in Fig. 1 and Fig. S4 as well as Table S1. Although the LX-B bioaugmentation resulted in an ethanol production rate that was 21–93% higher than in control (Table S1), the ethanol concentrations did not improve substantially with the increase in the LX-B/sludge ratio up to 0.24 when compared with control (Fig. 1a). Additionally, the LX-B bioaugmentation resulted in the improved acetate yield and production rate, which was average 24.4% and 86% higher than in control, respectively (Fig. 1b and Table S1). However, the produced acetate was not proportional to hydrogen yield, which was only 7–47% of the theoretical value in all tests according to the equation ($C_6H_{12}O_6 \rightarrow 2CH_2COOH + 2CO_2 + 4H_2$). Homoacetogens can survive under the condition of heat treatment, which use hydrogen and CO_2 to produce acetate (Hussy et al., 2003; Wong et al., 2014). It was likely that homoacetogens in heat-treated sludge could survive and maintain metabolic activity, thus causing the decreased hydrogen yields in all tests. These findings indicated that ethanol and acetate used as indicators might interfere with the evaluation of the contribution of LX-B, though ethanol and acetate were the metabolites of LX-B during hydrogen production (Liu et al., 2014).

It is noteworthy that *n*-butyrate was produced in a considerable amount at all tests (Fig. 1d). Although increasing the LX-B/sludge ratio led to a stepwise decrease in the production rate of *n*-butyrate (Table S1), the *n*-butyrate yields in group 0.06 and 0.12 were substantially higher than those in control over the whole process of hydrogen production. The higher *n*-butyrate production is coupled with a higher hydrogen yield according to an equation ($C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$). Thus, these findings, in combination with hydrogen production data indicated that the bioaugmentation of LX-B might facilitate hydrogen production via shift of metabolic pathway to butyrate production.

As shown in Fig. 1c and Table S1, the propionate yield and production rate in control were higher than in groups with LX-B bioaugmentation. Increasing the LX-B/sludge ratio from 0.06 to 0.24 led to a stepwise decrease in both the propionate yields and production rates. These results indicated that the LX-B bioaugmentation can reduce the proportionate production, thereby establishing an environment that decrease the hydrogen consumption via propionate fermentation (Hawkes et al., 2002). Increased amounts of *n*-valerate and *n*-caproate were also detected in all tests after about 65 h of hydrogen production (Fig. S4). Fig. 1 shows that the ethanol, acetate, propionate and butyrate showed a declined levels at the end of fermentation. It was likely that parts of produced VFAs and ethanol was subsequently converted to *n*-valerate and *n*-caproate, as a report showed that high concentrations of VFAs and ethanol can induce production of *n*-valerate or *n*-caproate (Ding et al., 2010).

Figs. 2 and S5 illustrate that the major metabolites were ethanol, acetate, propionate, and butyrate with small amounts of *n*-valerate and *n*-caproate during RBC. Increasing the incubation cycles resulted in a gradual increase in yields of individual VFAs

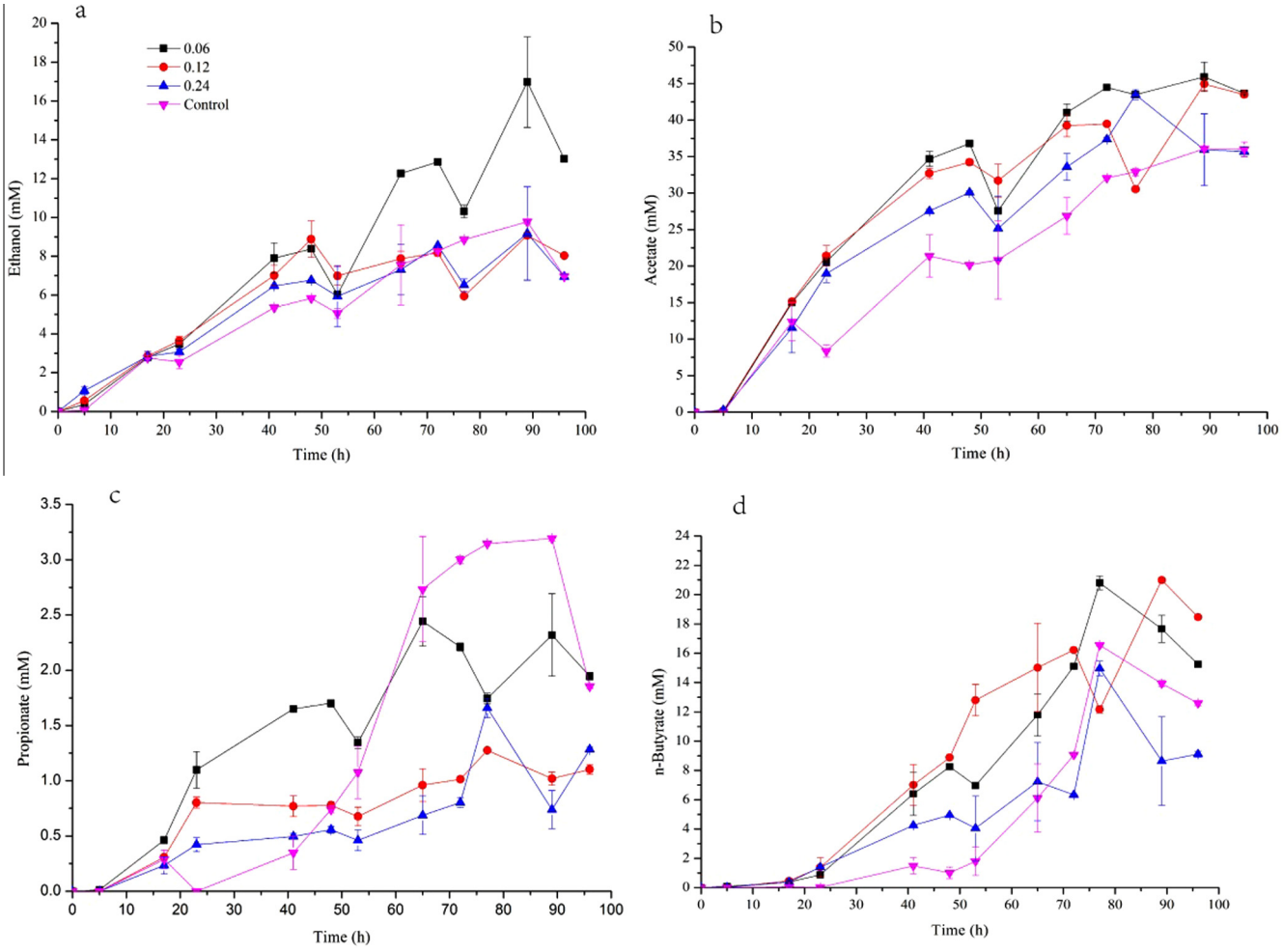

Fig. 1. Profiles of the intermediate metabolite production in BC. (a) Ethanol; (b) acetate; (c) propionate; (d) *n*-butyrate.

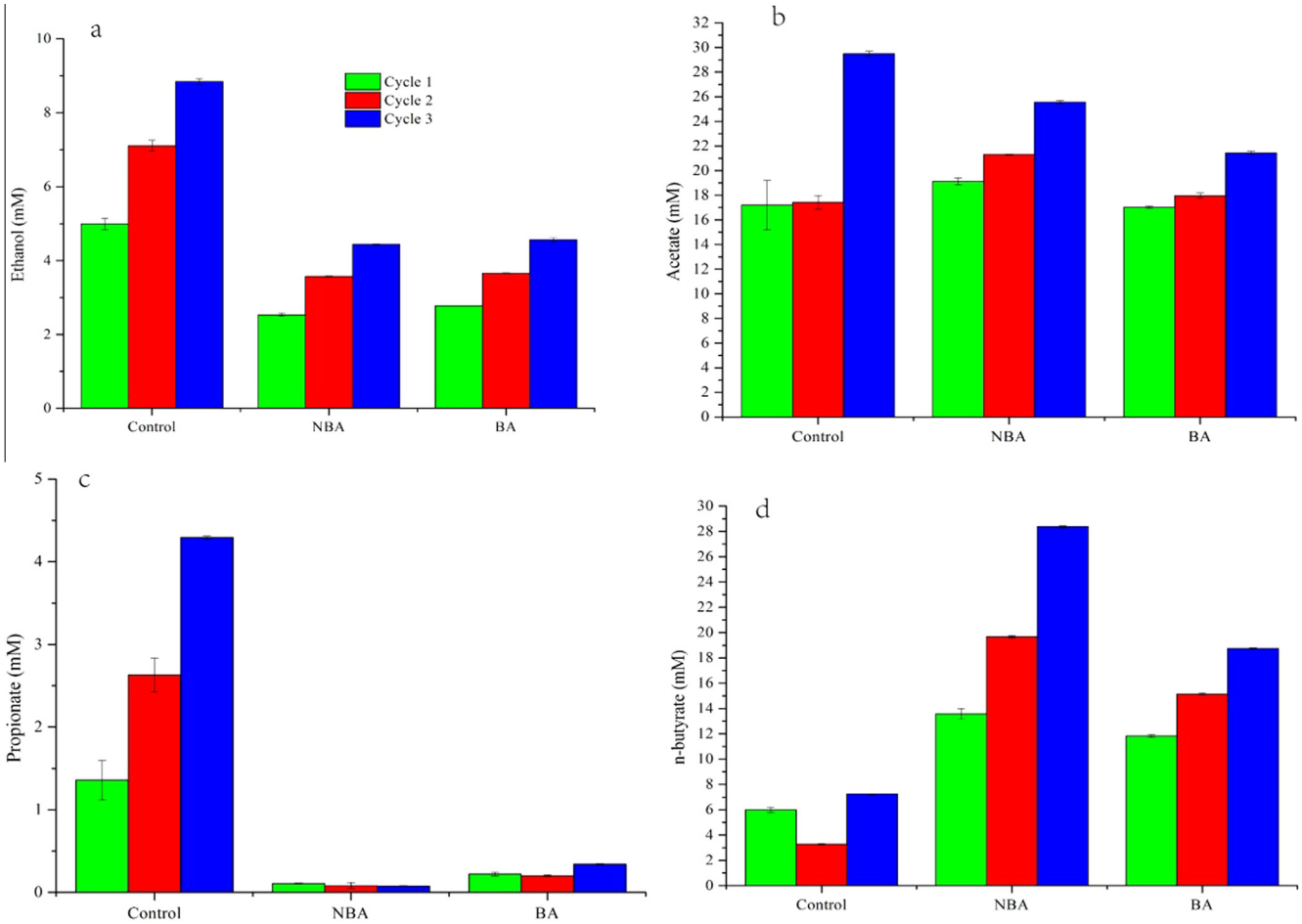

Fig. 2. The distribution of intermediate metabolites in RBC. (a) Ethanol; (b) acetate; (c) propionate; (d) *n*-butyrate.

and ethanol at each test. However, after three cycles of incubation, the ethanol, acetate and propionate levels in control were at least 0.93-fold, 0.15-fold, and 11-fold higher than in both NBA and BA, but the *n*-butyrate levels and hydrogen yields in NBA and BA were at least 1.6-fold and 0.79-fold greater than in control, respectively. This suggested a positive correlation between hydrogen yields and butyrate production. The high hydrogen yield in group NBA and BA further indicated that the LX-B bioaugmentation led to a metabolic shift toward butyrate production dependent on bioaugmentation pattern. Previous report also showed that *B. subtilis* augmented system showed a change in the metabolic pathway toward acidogenesis, providing high hydrogen yield (Goud et al., 2014).

3.3. Analysis of microbial community

To look at whether the metabolic shift was attributed to a change in the dominant microbial population, the microbial community structures were next determined. Fig. S6 and Table S2 show that the LX-B bioaugmentation had a slight influence on the diversity and richness of microbial communities. The taxonomic classification of bacterial operational taxonomic units (OTUs) showed that average 7.2% (BC) and 8.9% (RBC) of the total bacterial sequences were unclassified bacteria at the genus level (Fig. 3).

Fig. 3a shows that the relative abundance of LX-B ranged from 6.4% to 18.5% after LX-B bioaugmentation, indicating the acclimation and survivability of LX-B in the mixed cultures. However, the LX-B abundance did not increase when the LX-B/sludge ratio increased from 0.12 to 0.24. It is noteworthy that there was a stepwise increase in *Clostridia* from 41.6% to 60.4% with the increasing of the ratios, suggesting that increasing the inoculation amounts of LX-B were favorable for the growth and proliferation of *Clostridium* species. It was likely that LX-B cannot probably outcompete with the dominant *Clostridium* species with regard to competition of substrate at the ratio of 0.24, providing no increase in the relative abundances of LX-B compared with the ratio of 0.12. Additionally, Figs. 1 and S1 show that the levels of acetate and *n*-butyrate continuously increased, while hydrogen yield reached a plateau after about 40 h of fermentation in the ratio of 0.24. *n*-Butyrate production is positively related to hydrogen yield (Hawkes et al., 2002). The findings indicated that H_2, which was produced by LX-B, *Enterobacter* (Kumar and Das, 2000) and hydrogen-producing *Clostridium* (e.g. *Clostridium butyricum*) with *n*-butyrate fermentation (Wong et al., 2014), was likely further converted to acetate. Some *Clostridium* species (e.g. *Clostridium aceticum*) were identified as homoacetogens, which can utilize H_2 and CO_2 to produce acetate (Ohwaki and Hungate, 1977). Additionally, Fig. 1d shows that the *n*-butyrate levels in group 0.24 were lower than in other groups, probably indicating the existence of the low percentage of *Clostridium* producing hydrogen via *n*-butyrate pathway. Thus, the high acetate yield concomitant with low hydrogen production might attribute to co-existence of the high percentage of *Clostridium*-related homoacetogens with hydrogen-producers in group 0.24. The findings further indicated that the high inoculation ratio of LX-B/sludge over 0.12 might establish an environment which

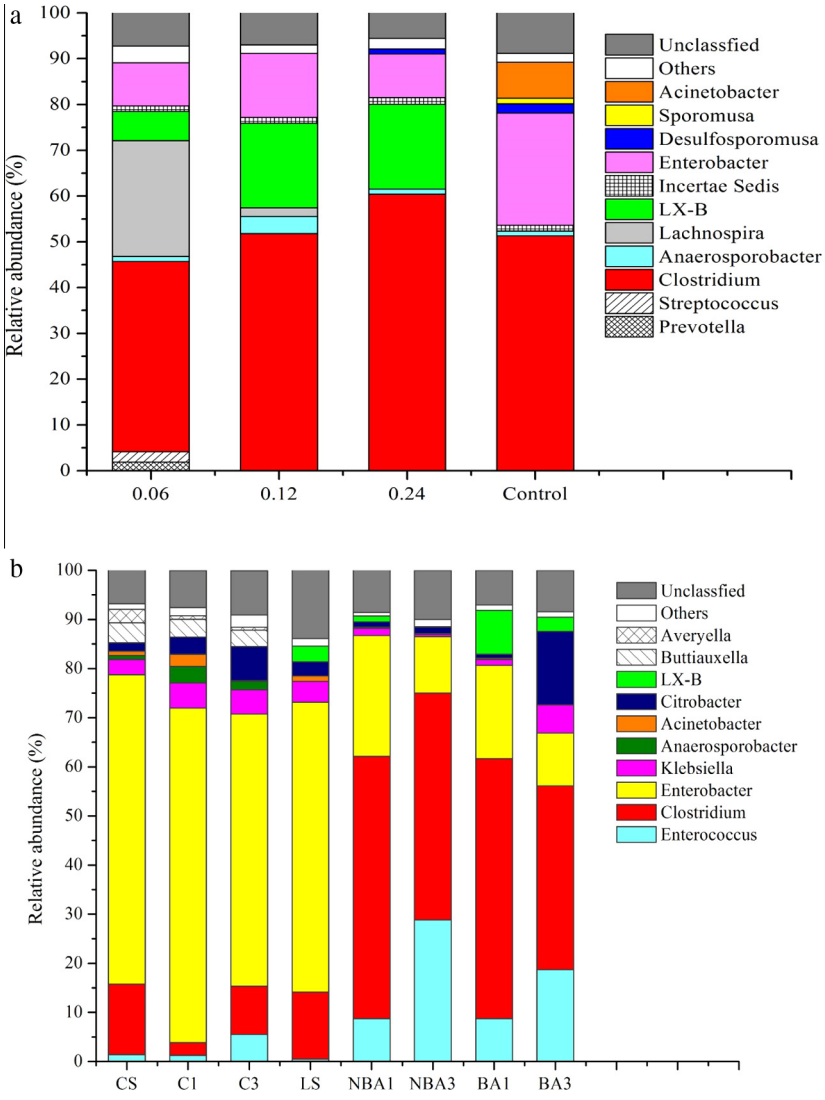

Fig. 3. The taxonomic classification of bacterial communities at the genus level. Genus accounting for less than 1% of total composition was identified as "others". (a) BC; (b) RBC. CS and LS represents start-up stage for control and LX-B addition, respectively.

was more favorable for hydrogen consumption by homoacetogenesis rather than hydrogen production.

The observation that the percentages of *Enterobacter* in the LX-B/sludge ratio of 0.06, 0.12, and 0.24 were 61.5%, 42.6%, and 60.7% lower than that in control, combined with the changed abundance of *Clostridia*, indicated that the LX-B bioaugmentation led to a change in microbial community structure. Although *Anaerosporobacter* and *Lachnospira* are known to convert carbohydrates to H_2 (Dušková and Marounek, 2001; Jeong et al., 2007), low percentage of *Anaerosporobacter* and low hydrogen yield of *Lachnospira* indicated that their less contribution to hydrogen production in this work. A lower percentage of *Desulfosporomusa*, *Sporomusa*, *Prevotella*, *Streptococcus Incertae Sedis* and *Acinetobacter* was observed in all tests. *Desulfosporomusa* and *Sporomusa* can convert H_2 and CO_2 to acetate (Breznak et al., 1988; Sass et al., 2004), but the low relative abundance indicated their less contribution to hydrogen consumption. *Prevotella* can produce VFAs (e.g. acetate) from polysaccharide (Venkata Mohan et al., 2010). *Streptococcus* probably acted as seeds for aggregate formation (Hung et al., 2007). *Incertae Sedis* and *Acinetobacter* were reported to have cellulolytic activity (Yuan et al., 2015) and produce cellulolytic enzymes (Lo et al., 2010), respectively. These two species may be related to degradation of cellulose of potato tuber (Abe and Yano, 2009).

As with bacterial communities during BC, bacterial community composition differed strongly among groups, irrespective of LX-B bioaugmentation pattern during RBC (Fig. 3b). These differences may be partially likely due to increasing cycles of transfer, as has been previously observed (Sivagurunathan et al., 2016). The abundant genera, including *Enterococcus*, *Clostridium*, and *Enterobacter*, all varied in their relative percentages among the groups with the increasing cycles of transfer. However, cycles of transfer caused marginal changes in bacterial community structure compared with that of LX-B bioaugmentation pattern (NBA and BA). For example, the relative abundances of *Clostridia* in BA increased by 2.9-fold as the feeding cycle increased from the start-up stage (LS) to cycle 1 (BA1) and decreased by 30% when the feeding cycle further increased to cycle 3 (BA3), while the relative abundances of *Clostridia* in BA were always greater (19.3-fold greater in BA1 and 2.8-fold greater in BA3) than in the corresponding control.

The LX-B bioaugmentation caused a change in the bacterial community structures regardless of bioaugmentation pattern. For example, compared with the corresponding control, the relative abundances of hydrogen-producing *Enterococcus* (Wang et al.,

2009) and *Clostridium* increased respectively by 5.7-fold and 19.4-fold on average in cycle 1 and by 3.32-fold and 3.27-fold on average in cycle 3, whereas the abundances of *Enterobacter* decreased respectively by an average of 68% and 80% in cycle 1 and cycle 3. Furthermore, when compared with the corresponding control, the abundances of hydrogen-producing bacteria *Citrobacter* (Kotay and Das, 2010) and *Klebsiella* (Wong et al., 2014) in NBA decreased respectively by 81% and 90%, while those in BA increased by 120% and 16%, respectively, after three cycles of transfer. Finally, the hydrogen-producing bacteria *Anaerosporobacter* (Jeong et al., 2007) and *Buttiauxella* (Marone et al., 2012) were nearly undetectable in BA and NBA compared with control.

It is important to note that, when compared with NBA, the relative abundances of *Enterococcus* and *Clostridium* decreased respectively by 35% and 19%, whereas the abundances of both *Citrobacter* and *Klebsiella* increased by 10-fold in BA after three cycles of transfer. Additionally, Fig. 4 shows that CS and LS were clustered together, which were close to C1 and C3 and far away from NBA1, BA1, NBA3 and BA3, but there was a distinction between CS and LS. BA1 and NBA1 were clustered closely, which were well separated from NBA3 and BA3, but there was a clear difference between NBA3 and BA3. These results suggested that the LX-B bioaugmentation pattern also led to a shift in the microbial community structure.

After three cycles of transfer, the relative abundances of LX-B in BA3 were 35.3-fold higher than in NBA3, indicating the importance of BA pattern in maintaining the survivability and persistence of LX-B. However, the relative abundances of LX-B in BA and NBA during RBC were substantially lower than those in the LX-B/sludge ratio of 0.12 during BC at the end of hydrogen fermentation. This indicated that the operation conditions might not favor the growth of LX-B, probably due to the fact that the short retention times during RBC resulted in a substantial wash-out of LX-B from anaerobic bottles. To prevent a wash-out of LX-B, strategies such as immobilization of the inoculum within supporting materials should be further considered in the future.

DNA-sequencing approaches showed that the LX-B bioaugmentation resulted in a detectable shift in the composition of indigenous bacterial consortia, which corresponded to the change in the relative abundances of dominant bacteria (e.g. *Clostridium* and/or *Enterococcus*) during BC and RBC. This finding, combined with the observation that the relative abundance of LX-B was still lower than the dominant hydrogen producers during BC and RBC, suggested that the high relative abundances *Clostridium* and/or *Enterococcus* made a major contribution to the improved hydrogen production. The shifted composition of the indigenous bacterial community subsequently led to a metabolic shift to acetate and *n*-butyrate production, as confirmed by the increased levels of acetate and *n*-butyrate as well as the declined propionate levels after LX-B bioaugmentation (Figs. 1 and 2). However, the low hydrogen yield concomitant with high levels of acetate showed that the hydrogen-consuming *Clostridium*-related homoacetogens might co-exist with the hydrogen-producers. The findings from BC and RBC indicated that controlling the LX-B/sludge ratio and curtailing retention times were crucial for successful LX-B bioaugmentation based hydrogen-producing system, providing low hydrogen consumption. Thus, the comparative analysis clearly showed that the LX-B bioaugmentation might affect hydrogen production mainly through altering the composition of indigenous bacterial consortia, resulting in highly variable hydrogen production depending on the inoculation ratio and bioaugmentation pattern of LX-B.

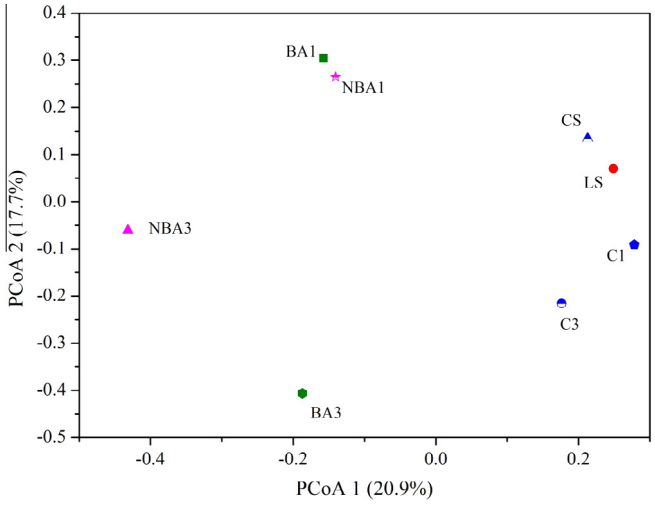

Fig. 4. Phylogenetic distances between samples assessed via principal coordinates analysis (PCoA).

4. Conclusions

The results demonstrated that both the LX-B bioaugmentation at a suitable LX-B/sludge ratio and bioaugmentation pattern (BA and NBA) substantially accelerated hydrogen production from fresh potato slurry, concomitant with detectable changes in metabolite distribution and bacterial community structure. The accelerated hydrogen production was positively related to the increased butyrate levels rather than the increased acetate levels. Together, the findings demonstrated that the LX-B bioaugmentation might affect hydrogen production mainly through altering the composition of the indigenous bacterial community, which subsequently led to a shift in acidogenic metabolic pathway.

Acknowledgements

This work was funded by 863 Project (2011AA060905), the National Natural Science Foundation of China (No. 21307143), Key Research & Development Project of Shandong (No. 2015GSF117016).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.03.097.

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