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Full Length Article

Butyric Acid Fermentation from Rice Straw with Undefined Mixed Culture: Enrichment and Selection of Cellulolytic Butyrate-Producing Microbial Community

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Abstract

The study was conducted with an objective to present an alternative strategy for bioconversion of low grade lignocellulosic biomass into butyric acid without supplementary cellulolytic enzyme. Eight inocula sampled from various natural sources (cattle manure, pig manure compost, biogas residue, soil from the corn field, sediment from ditch, soil from shrub, rotten wood and rotten leaf) were used to start the enrichment procedure, which continued for over 8 months. The microbial community from the combination of cattle manure, pig manure compost, soil from corn field and rotten wood was selected for its stable cellulose-degrading potentials and high selectivity of butyric acid production from NaOH pretreated rice straw. The PCR-DGGE and sequence analysis revealed that the microbial community included cellulolytic and xylanolytic bacteria, butyrate-producing bacteria and other acidogenic bacteria. © 2013 Friends Science Publishers

Keywords: Butyric acid production; Anaerobic microbial community; Cellulose degradation; Mixed culture fermentation

Introduction

Issues of energy, environment and sustainability emerged as a result of consumption of fossil resources. Plant biomass utilization for the production of fuels and chemicals could meet the energy needs and sustainable development. Lignocellulosic biomass is perceived as a renewable source with great potential for biofuels and bioproducts. However, the utilization of this low-cost bioresource in plentiful supply has been limited by the high cost of cellulolytic enzymes (Stephanopoulos, 2007). To overcome this impediment use of mixed culture fermentation technology to convert lignocellulosics into valuable products anaerobically is a promising strategy. In a mixed culture fermentation process, supplementation of cellulase is not necessary. A proven and widely applied example was anaerobic digestion aimed at the recovery of methane containing biogas (Kleerebezem and van Loosdrecht, 2007; Mussoline *et al.*, 2012). Mixed culture biotechnology has also been investigated for the production of hydrogen (Ren *et al.*, 2006), alcohols (Steinbusch *et al.*, 2008), short chain carboxylates (Fu and Holtzapple, 2010; Agler *et al.*, 2011), medium chain fatty acids (Steinbusch *et al.*, 2011) and poly hydroxyl butyrate (Salehizadeh and Van Loosdrecht, 2004). Using anaerobic mixed cultures as the fermenting organisms make it possible to convert cellulosic feed stocks into biobased products in a process termed as the consolidated bioprocess (CBP). In CBP, cellulolytic enzymes production, both cellulose and hemicellulose hydrolysis, and desired products fermentation are accomplished in a single process step (Lynd *et al.*, 2002). The process enjoys the benefit of higher activity of cell-associated cellulolytic enzyme and thus completely eliminating the cost of enzyme purification (Stephanopoulos, 2007). A mixed culture-based process, as the name implied, generates mixed products. Based on the ecological selection principles, mixed culture-based processes generated narrow product spectrums could be established by process operations (Kleerebezem and van Loosdrecht, 2007). Another approach to increase the target product accumulation is to select and enrich a special mixed culture with efficient substrate hydrolysis and product formation. Many undefined mixed cultures have been enriched and applied to the conversion of lignocellulosic materials to biogas (Wu and Conrad, 2001), hydrogen (García *et al.*, 2012), ethanol (Lin *et al.*, 2011), and electricity (Wang *et al.*, 2009).

Butyrate is one of the main liquid products in anaerobic acidogenic systems, generating from both primary and secondary fermentation pathways (Agler *et al.*, 2011). Butyrate is itself a valuable product when extracted from the culture broth that has been widely applied in chemical, food, and pharmaceutical industries (Zhang *et al.*, 2009). It was reported that butanol was produced continuously from butyric acid by *Clostridium saccharoperbutylacetonicum* with the co-feeding of glucose required as a source of ATP and electrons (Richter *et al.*, 2012). According to the physicochemical properties that butyrate has a longer hydrophobic carbon chain and a lower oxygen/carbon ratio,

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it can be deduced that butyrate is superior to acetate as intermediates for alcohol fuels or medium chain fatty acids with the previously proposed processes (Holtzapple *et al.*, 1999; Steinbusch *et al.*, 2011). Butyrate fermentation with undefined mixed cultures, however, has been largely ignored for a long time, caused by the low yields and product contamination with co-products, such as acetate, propionate and valerate (Zigová and Šturdík, 2000; Agler *et al.*, 2011). The objective of the present study was to obtain a stable undefined mixed community for butyrate production from rice straw. This cellulolytic butyrate-producing microbial community was required to possess the combination of both cellulose and hemicellulose utilization and butyric acid formation properties. The community was also expected to produce a high enough proportion of butyrate in product spectrum to compete with pure butyrate-producing bacteria species. Furthermore, the bacterial community structure and phylogenetic relationships were characterized using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis, combined with cloning and sequencing.

Materials and Methods

Samples Collection and Substrate Preparation

The samples used to start enrichment originated from various sources. Cattle manure, pig manure compost, biogas residue and soil from the corn field were collected from the suburb of Harbin, China. Sediment from ditch, soil from shrub, rotten wood and rotten leaf were collected from the campus of Northeast Forestry University, Harbin, China. All samples were taken at locations 10 to 20 cm below the surface.

The rice straw was collected from a local farm in Harbin, China, containing 39.7% cellulose, 24.8% hemicellulose and 15.3% lignin. The cellulose, hemicellulose and lignin were measured using the method described previously (Van Soest *et al.*, 1991). The rice straw cut into 10-15 cm lengths was soaked in 1% sodium hydroxide solution at 50°C for 72 h in static status. Solid-to-liquid ratio of 1:15 (w/v) was applied. The solid residue was separated by filtering and thoroughly washed with tap water to near-neutral pH. The neutralized residue was squeezed and the water content was measured. The treated rice straw was refrigerated at 4°C for further use as the substrate for butyric acid production. The substrate contained 53.0% cellulose, 27.4% hemicellulose and 8.0% lignin.

Enrichment and Selection Procedure

Between 1.5 and 5 g (on a dry weight basis of 1 g) of extracted samples from eight sources were inoculated into serum bottles containing 100 mL of peptone cellulose solution (PCS) medium, and one filter paper strip (1.5 cm × 5 cm) was added as an indicator of cellulose-degrading potential. The PCS medium was comprised of 10 g NaOH pretreated rice straw, 5 g tryptone, 1 g yeast extract, 5 g NaCl, 2 g $CaCO_3$ and 0.5 g D-cysteine hydrochloride per liter (Guo *et al.*, 2008). The broth was purged with nitrogen gas for 10 min to maintain totally anoxic conditions, and then the 150 mL serum bottle containing 100 mL of broth was sealed and autoclaved at 115°C for 20 min. After inoculation, the cultures were incubated at 35°C without agitation, and checked for degradation of the filter paper strip every day. When the filter paper strip was broken down, 5 ml of totally mixed broth was transferred into fresh PCS medium with filter paper strip, as described above. This procedure was repeated. Those cultures that took an obviously longer time to break the filter paper strip down would be eliminated, and those that took the shorter time to break the filter paper strip down would be mixed together as a new source for the future transferring.

Continuously transferring over 8 months, the enrichment cultures that were maintained to be stable and possess high cellulose-degrading potentials were selected to verify the capabilities of butyrate-producing. Butyrate fermentations were performed in triplicate in 500 mL serum bottles with 200 mL PCS medium with 20 g of pretreated rice straw. 10 mL of each selected enrichment was inoculated into serum bottles followed by incubation at 35°C with agitation of 140 rpm for 6 d. All fermentations were run in triplicate. During 6 d of culture, gas and liquid samples were taken every day for the analysis of biogas, pH and volatile fatty acids (VFAs). At the end of fermentation, biomass and degradation ratios of rice straw were determined.

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. Gas analyses of hydrogen, methane and carbon dioxide were analyzed by gas chromatography (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) (Li *et al.*, 2009). Liquid samples were centrifuged at 8000 *g* for 5 min and then 1 mL supernatant was acidified with 0.1 mL 25% phosphoric acid before VFAs analysis. The VFAs determination was performed using gas chromatography (SP-6800A, Shandong Lunan Instrument Factory, China) equipped with a flame ionization detector and a FFAP capillary column (30 m×0.32 mm×0.50 μm, Lanzhou ZhongKeKaiDi Chemical New-tech Co., Ltd, China). The temperatures of the injection port, column oven and detector were set at 220°C, 180°C and 220°C, respectively. Nitrogen was used as carrier gas, with 0.05 MPa column head pressure. The split ratio was 50. The injection volume was 1 μL.

Microbial biomass was estimated by optical density at 260 nm as follows (Morikawa *et al.*, 1985): To 5 mL of culture broth 5 mL of 1 $molL^{-1}$ $HClO_4$ solution was added.

If required, the samples were diluted to adjust the optical density within the readable range. The tubes were placed in boiling water for 20 min and cooled to room temperature. The contents of the tubes were centrifuged and OD of the supernatant was measured at 260 nm. The OD260 of cultured broth was denoted by [A]. Uncultured broth was used as a blank, and OD260 denoted by [B]. The microbial biomass was denoted as [A]-[B].

DNA Extraction, PCR Amplification and DGGE Analysis

A total of 5 microbial communities with the high cellulose-degrading potentials were characterized. 1.5 mL culture broth was collected from the serum bottles showing snapping of filter paper strips. The culture broth was centrifuged at 13 000 *g* for 5 min, and the pellets were washed three times with 10 mM phosphate buffer solution (pH 7.4). Bacterial genomic DNA was extracted and purified using the Bacteria DNA Mini Kit (Watson Biotechnologies, Shanghai, China) according to the manufacturer's instructions.

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

Bacterial amplicons were subsequently separated by DGGE performed with a Bio-Rad DCode system (Bio-Rad Laboratories, USA) using a denaturant gradient of 30-60%. The denaturing gradient gel was prepared to contain 1 × TAE buffer, 8% acrylamide-bis-acrylamide and 40 to 60% denaturant (100% denaturant contains 7 M urea and 40% deionized formamide). Electrophoresis was performed at 120 V for 6 h in 0.5 × TAE buffer at 60°C. Gels were silver-stained.

Cloning, Sequencing and Phylogenetic Analysis

Prominent DGGE bands were cut from the gel and crushed in 50 μL of 1 × TAE buffer. After equilibration at 55°C for 3 h and centrifugation at 11000 *g* for 3 min, 1 μL of each supernatant was used as the template for PCR amplification as described above, but with the primer BSR 534 without a GC clamp. The PCR products were purified using a Gel Recovery Purification Kit (Shanghai Watson Biotechnologies, China) and cloned in E. coli competent cells using the pMD-T 18 plasmid vector system (Takara Dalian, China) according to the manufacturer's instructions.

One positive clone after the blue/white screening and colony PCR from each DGGE band was maintained in ager stab culture and sent to Shanghai Sangon, China for sequencing analysis. The analyzed 16S rRNA gene partial sequences were compared with sequences in the GenBank database using the NCBI BLAST search program. The closest cultured relatives were retrieved from the database with their degrees of similarity. A neighbor-joining phylogenetic tree was constructed with the Molecular Evolutionary Genetics Analysis package (MEGA 5). The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers JX271012 to JX271038.

Results

Preliminary Selection Based on Cellulose-degrading Potential

The cellulose-degrading communities were enriched from biogas residue, pig manure compost, rotten wood, rotten leaf, cattle manure, sediment from a ditch and soil samples from the corn field and a shrub by transferring repeatedly from one PCS medium containing pretreated rice straw and filter paper to another. The degree of degradation of filter paper strip observed from Fig.1c was set as the benchmark to transfer to fresh bottles for further enrichment. Shorter time required for breakdown of filter paper strip suggested better cellulose-degrading potential. The subculturing resulted in an increase in the time to break down the filter paper strips as shown in Table 1, and some lost their capabilities of cellulose degradation. After over 5 months of enrichment, enrichment cultures from cattle manure, pig manure compost, soil from the corn field and rotten wood were selected because of their relative higher cellulose-degrading potentials. The G 17 (17^{th} generation) of cattle manure, rotten wood and soil from the corn field and G 20 of pig manure compost were further subcultured. Combination of these served as a potential new source of consortia for cellulose degradation. All the five samples were subcultured by making transfers to fresh enrichment media periodically for three months. They were finally selected for butyric acid production.

Further Selection Based on Butyrate-producing Ability

The results of butyric acid fermentation from NaOH pretreated rice straw with the five selected enrichments were summarized in Table 2. Although eight carboxylic acids were detected, acetic and butyric acid were absolutely the main products in all fermentation trials. The product

Table 1: Days that were taken to break the filter paper strips down by every generation of enrichment cultures from different sources during the continuously transferring over 8 months

	Cattle manure	Biogas residue	Pig manure compost	Sediment from a ditch	Soil from the corn field	Soil from a shrub	Rotten wood	Rotten leaf	Combination [b]
G 1 [a]	4	5	2	7	4	10	3	8	10
G 2	6	5	3	15	6	17	5	35	12
G 3	6	7	5	27	5	30	7	-	11
G 4	9	4	5	-	8	-	6	-	13
G 5	6	6	7	-	7	-	5	-	11
G 6	6	7	6	-	6	-	8	-	13
G 7	7	6	5	-	8	-	7	-	14
G 8	9	9	8	-	8	-	9	-	-
G 9	9	9	7	-	10	-	10	-	-
G 10	11	12	8	-	13	-	9	-	-
G 11	11	10	9	-	11	-	10	-	-
G 12	10	13	9	-	10	-	12	-	-
G 13	13	16	11	-	11	-	11	-	-
G 14	10	17	10	-	13	-	11	-	-
G 15	12	19	11	-	13	-	14	-	-
G 16	11	16	12	-	12	-	12	-	-
G 17	12	19	11	-	14	-	12	-	-
G 18	12	-	11	-	13	-	12	-	-
G 19	13	-	13	-	13	-	14	-	-
G 20	12	-	11	-	14	-	14	-	-
G 21	13	-	11	-	13	-	13	-	-
G 22	13	-	13	-	15	-	11	-	-
G 23	12	-	12	-	13	-	14	-	-
G 24	14	-	11	-	-	-	12	-	-
G 25	-	-	13	-	-	-	-	-	-
G 26	-	-	12	-	-	-	-	-	-
G 27	-	-	13	-	-	-	-	-	-

[a]The 1st generation. Likewise, G 27 stands for the 27th generation

[b]Combination of the G 17 of cattle manure, soil from the corn field and rotten wood and G 20 of pig manure compost as a new source for the next transferring

Table 2: Results of NaOH pretreated rice straw fermentation with five mixed cultures [a]

	Cattle manure	Pig manure compost	Soil from the corn field	Rotten wood	Combination
Initial pH	7.66	7.73	7.68	7.76	7.74
Final pH	5.06	5.20	5.14	5.07	5.04
Rice straw ($g\ l^{-1}$) [b]	-9.95	-10.97	-9.35	-10.05	-10.67
Acetic acid ($g\ l^{-1}$)	1.70	2.55	0.94	3.64	1.35
Propionic acid ($g\ l^{-1}$)	0.11	0.43	0.35	0.28	0.24
i-Butyric acid ($g\ l^{-1}$)	0.08	0.07	0.08	0.08	0.09
n-Butyric acid ($g\ l^{-1}$)	2.57	4.30	4.99	2.04	6.01
i-Valeric acid ($g\ l^{-1}$)	0.09	0.09	0.10	0.10	0.12
n-Valeric acid ($g\ l^{-1}$)	0.06	0.04	0.06	0.03	0.05
i-Caproic acid ($g\ l^{-1}$)	0.08	0.19	0.10	0.10	0.07
n-Caproic acid ($g\ l^{-1}$)	0.75	0.08	0.08	0.29	0.05
Total VFAs ($g\ l^{-1}$)	5.43	7.74	6.69	6.54	7.97
Product selectivity (%)[c]	47.36	55.58	74.55	31.23	75.40
Biomass (OD_{260} after $HClO_4$ hydrolysis)	4.13	4.62	4.41	4.61	4.53
Butyric acid yield ($g\ g^{-1}$ rice straw fed)	0.13	0.22	0.25	0.10	0.30
VFAs yield ($g\ g^{-1}$ rice straw fed)	0.27	0.39	0.33	0.33	0.40
Cumulative H_2 yield ($ml\ g^{-1}$ rice straw fed)	26.26	39.55	40.74	34.44	41.67
Cumulative CH_4 yield ($ml\ g^{-1}$ rice straw fed)	3.04	4.45	4.44	5.48	4.44

[a]All the numbers presented in the table was the measurements at the end of fermentation period

[b]Negative values indicated the consumption of rice straw

[c]Percent of butyric acid in total VFAs

distribution of fermentation for the five mixed cultures was distinctly different. The highest butyric acid concentration, which accounted for 75.4% of the total VFAs, was observed for the fermentation carried out by the consortia resulting from combination of G17 of cattle manure, rotten wood, soil from the corn field and G 20 of pig manure compost.

The course of the main liquid products and product selectivity (percentage of butyric acid in the total acid products) in the fermentations with the five mixed cultures is shown in Fig. 2. i-butyric acid, i-valeric acid, n-valeric acid, i-caproic acid and n-caproic acid were also produced but not shown in the figure due to their

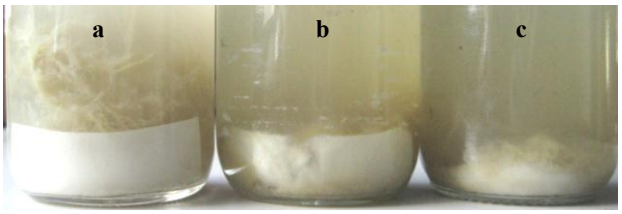

Fig. 1: Photographic images in PCS medium. (a) control, without inoculation of any enrichment culture, (b) filter paper strip that began to break down, (c) filter paper strip that was broken down

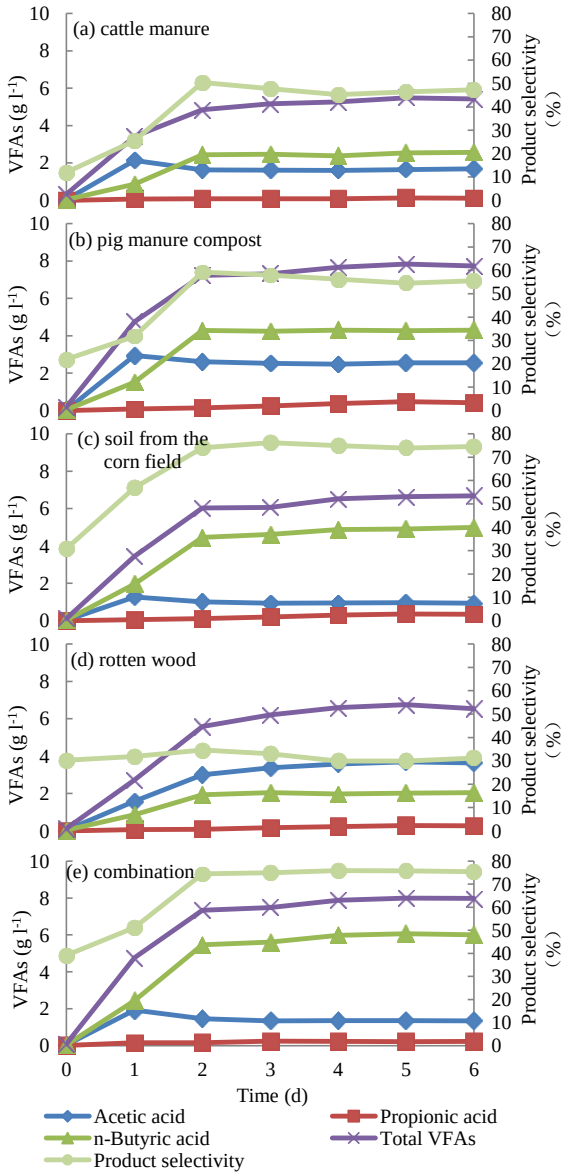

Fig. 2: The VFAs concentration and product selectivity (percent of butyric acid in the total acid products) profiles of pretreated rice straw fermentation with different mixed cultures

very low concentrations. As seen in the figure, production of butyric acid and total VFAs had already risen almost to the maximum levels in the first two days, which inferred that prolonging the fermentation period was helpless to the digestion of rice straw and the production of butyric acid. It was noticed that the acetic acid concentration, in the trials that butyric acid predominated and was of greatest concentration, followed the pattern of first rising in the first day and then falling to the basal levels.

DGGE Analysis of Microbial Community Structure and the Phylogenetic Characterization

The microbial community structures of mixed cultures originated from cattle manure, pig manure compost, soil from the corn field, rotten wood and the combination were analyzed by DGGE. The DGGE band patterns were presented in Fig. 3. Visual comparison of the band patterns showed that a same main frame existed in the microbial community structures of the five mixed cultures. As seen in the profile, the most prominent bands (band 1, 2, 12, 13, 14, 15 and 16) appeared in all the five lanes. Twenty-seven major DGGE bands were cloned and sequenced. Table 3 presented the closest relative of each band the first one listed in the BLAST hitsin which the uncultured and environmental sample sequences were excluded. The most abundant relatives of these clones were assigned to *C.*, *Bacteroidetes* and *Oscillospiraceae*. The other bacteria related to cellulose and hemicellulose degradation and carboxylic acid production, with our best knowledge, were selected from the BLAST hits. The cellulolytic and xylanolytic bacteria included *Bacteroides cellulosilyticus*, *C. cellulolyticum*, *B. graminisolvens*, *B. xylanisolvens*, *Cellulosilyticum ruminicola*, *Eubacterium xylanophilum*, *C. polysaccharolyticum*, *C. saccharolyticum*, *Thermincola carboxydiphila*, *C. xylanolyticum*, *C. algidixylanolyticum*, *Xylanibacter oryzae*. As expected, the well-known butyrate-producing species, *Butyrivibrio fibrisolvens*and *C. tyrobutyricum* were found in the hits. A phylogenetic tree was constructed to describe the relationship between the clones and other related species (Fig. 4).

The DGGE band patterns revealed that the community structures of different enrichments seemed to being becoming similar, which probably resulted by the long-term enrichment with the same simple substrates. The sequencing results showed that the community structure was dominated by cellulose-degrading bacteria and xylan-degrading bacteria, since NaOH pretreated rice straw was supplied as the main carbon source.

Discussion

Ligno cellulosic biomass, in nature, is degraded by the mixed culture of cellulolytic bacteria together with symbiotic non-cellulolytic bacteria (Haruta *et al.*, 2002). When the mixed cultures were transferred into new habitats that greatly differed from their natural ones, shifts of

Table 3: The closest relatives of bacterial 16S rRNA genes cloned from the DGGE bands

Band No. (Accession)	The closest relatives [a] (Accession)	Identity	Description
1 (JX271012)	*Bacteroide soleiciplenus* JCM 16102 (AB547644.1)	95	Major metabolic end products: acetic, succinic, formic and lactic acids (Watanabe *et al*., 2010)
2 (JX271013)	*Roseburia intestinalis* DSM 14610 (HM007565.1)	98	Acetate-converting, butyrate-producing bacterium (Falony *et al.*, 2006)
3 (JX271014)	*Oscillospiraceae bacterium* NML 061048 (EU149939.1)	99	-
4 (JX271015)	*Bacteroides graminisolvens* JCM 15093 (AB547643.1)	99	Xylanolytic anaerobe (Nishiyama et al., 2009)
5 (JX271016)	Cellulose degrading bacterium PS7 (AF087643.1)	93	-
6 (JX271017)	*Acetivibrio ethanolgignens* DSM 3005T (FR749897.1)	98	Ethanol as a major fermentation product (Robinson and Ritchie, 1981)
7 (JX271018)	*Bacteroides graminisolvens* JCM 15093 (AB547643.1)	100	Xylanolytic anaerobe (Nishiyama *et al.*, 2009)
8 (JX271019)	*Peptococcaceae bacterium* Ri50 (EU400652.1)	95	Isolated from microbial mat
9 (JX271020)	*Oscillibacter valericigenes*Sjm18-20 (AP012044.1)	98	Valerate-producing anaerobic bacterium (Iino *et al.*, 2007)
10 (JX271021)	*Methanospirillum hungatei* (AB517987.1)	98	Methane-producing archaeon
11 (JX271022)	*Bacteroides* sp. 22C (AY554420.1)	99	Cellulolytic anaerobe from a landfill leachate bioreactor
12 (JX271023)	*Clostridium bifermentans* GAL1_H (JQ271582.1)	97	Isolated from H_2 production enrichment culture
13 (JX271024)	*Oscillospiraceae* sp. GH1 (JF750939.1)	99	Isolated from rumen fluid
14 (JX271025)	*Anaerofilum agile* F (NR_029315.1)	95	Strictly anaerobic acidogenic bacterium (Zellner *et al.*, 1996)
15 (JX271026)	*Clostridium celerecrescens* N2 (AB601064.1)	100	Isolated from Italian ryegrass silage
16 (JX271027)	*Clostridium leptum* DSM 753T (AJ305238.1)	94	Fibrolytic and butyrate producing bacterium (Lay *et al.*, 2005)
17 (JX271028)	*Clostridium* sp. Z6 (AY949859.1)	99	Isolated from paper mill waste water
18 (JX271029)	*Clostridium* sp. BS-1 (FJ805840.2)	96	Isolated from an enrichment culture producing H_2, ethanol, butanol, acetic acid, butyric acid, and hexanoic acid (Jeon *et al.*, 2010)
19 (JX271030)	*Clostridium sporosphaeroides* DSM 1294 (NR_044835.1)	97	Converts glutamate to acetate and butyrate (Wilde *et al.*, 1997)
20 (JX271031)	Rumen bacterium R-7 (AB239481.1)	96	Rumen bacterium attached to cellulose powder
21 (JX271032)	*Clostridium* sp. AN-AS17 (FR872935.1)	82	Isolated from sediments
22 (JX271033)	*Clostridium ramosum* JCM 1298 (AB595128.1)	93	Human colonic commensal
23 (JX271034)	Acinetobacter junii NB5_3B (JN644576.1)	95	Isolated from midgut
24 (JX271035)	*Bacteroides cellulosilyticus*JCM 15632 (AB510698.1)	93	Cellulolytic bacterium
25 (JX271036)	*Xylanibacter oryzae* JCM 13649 (AB588019.1)	94	Xylanolytic bacterium
26 (JX271037)	*Prevotella* sp. RS2 (AY158021.1)	93	Isolated from pig colon mucosa
27 (JX271038)	*Eggerthella sinensis* HKU14 (NR_042840.1)	91	Human gut bacterium

[a]The uncultured and environmental sample sequences were excluded

community compositions and functions happened to adapt to the different circumstances. Owning to the lopsided competition for nutrients and changes in micro scale spatial structure among the species in the bacterial communities, stable communities became unstable (Kim *et al*., 2008; Weibel, 2008). In the present study, some samples from various natural sources attained stability at the cost of decline in the cellulose-degrading potential. Compared with pure culture based fermentation, one of the specific advantages of mixed culture fermentation is the possibility of a continuous process with no risk of microbiological contamination, adaptive capacity to environmental impacts and to use mixed substrates owing to microbial diversity (Kleerebezem and van Loosdrecht, 2007). It was believed that the stability of inoculum was crucial for a continuous process.

Microbial diversity in a mixed culture-based process provided an adaptive capacity, but also resulted in a disadvantage that a wide product spectrum was generated. Eight short chain fatty acids had been detected in fermentation broth in this study. High selectivity of desired product production is more crucial for a mixed culture-based process, though it is impossible to obtain only one product in a single bioprocess even with a pure culture. In theory, *C. butyricum* and *C. tyrobutyricum* give selectivity of about 75% from glucose fermentation, with the byproducts such as acetic and lactic acids. Glucose fermentation by *C. butyricum* (Eq. 1) and *C. tyrobutyricum* (Eq. 2) follows the stoichiometric equations below (Zhang *et al.*, 2009):

Glucose→0.8 Butyrate+0.4 Acetate+2.4 H_2+2 CO_2 (Eq. 1).
Glucose→0.85 Butyrate+0.1 Acetate+0.2 Lactate+1.9 H_2+1.8 CO_2 (Eq. 2).

The highest product selectivity in this study was equivalent to the theoretical selectivity from glucose fermentation by those butyrate-producing strains. According to the metabolic pathway of the butyrate-producing *Clostridium* sp. (Zhang *et al.*, 2009; Zigová and Šturdík, 2000), four ATPs are produced in the acetate branch in which one glucose is converted into two acetic acids, and three ATPs for each butyrate are produced in the butyrate branch. In the growth phase, more acetic acid was produced for more ATP to meet the higher energy demand, by which was explained that acetic acid concentration reached its peak at the first day (Fig. 2a, b, c and e). 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 acetic acid concentration fell in the next day and butyric acid concentration climbed (Fig. 2a, b, c and e).

In this study the product concentration and yield were too low. It was reported that 29.9 g L^{-1} total carboxylic acid, mainly acetic acid, was produced from sugarcane trash by mixed culture fermentation (Nachiappan *et al.*, 2011). By

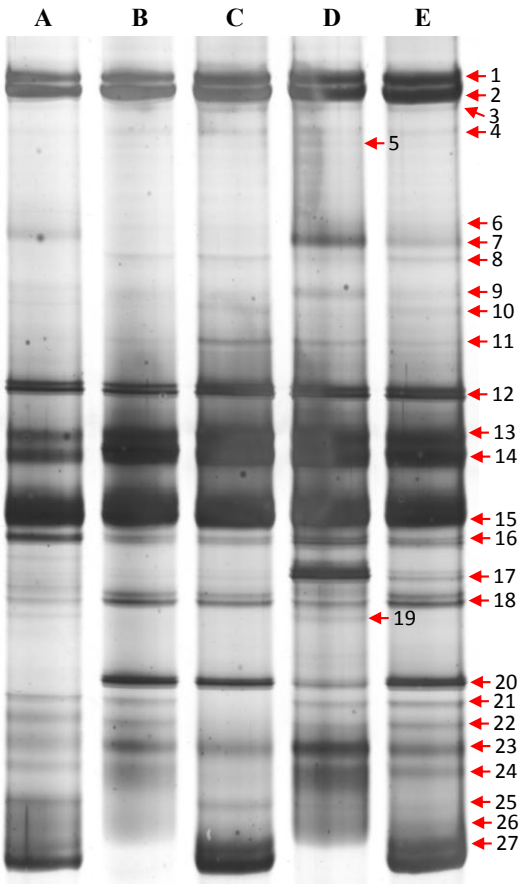

Fig. 3: DGGE profile of bacterial 16S rRNA gene in the microbial communities from cattle manure (A), pig manure compost (B), soil from the corn field (C), rotten wood (D) and the combination (E). The numbered bands were selected for cloning and sequencing

comparing and analyzing the fermentation parameters, the most probable cause to the low product yield in the present study was believed to be the pH down to nearly 5.0 in the fermentation systems. The neutral pH was favorable for the production of short chain fatty acids (Fu and Holtzapple, 2011; Nachiappan *et al.*, 2011). In order to increase the digestibility and butyric acid production, pH should be controlled at neutral range.

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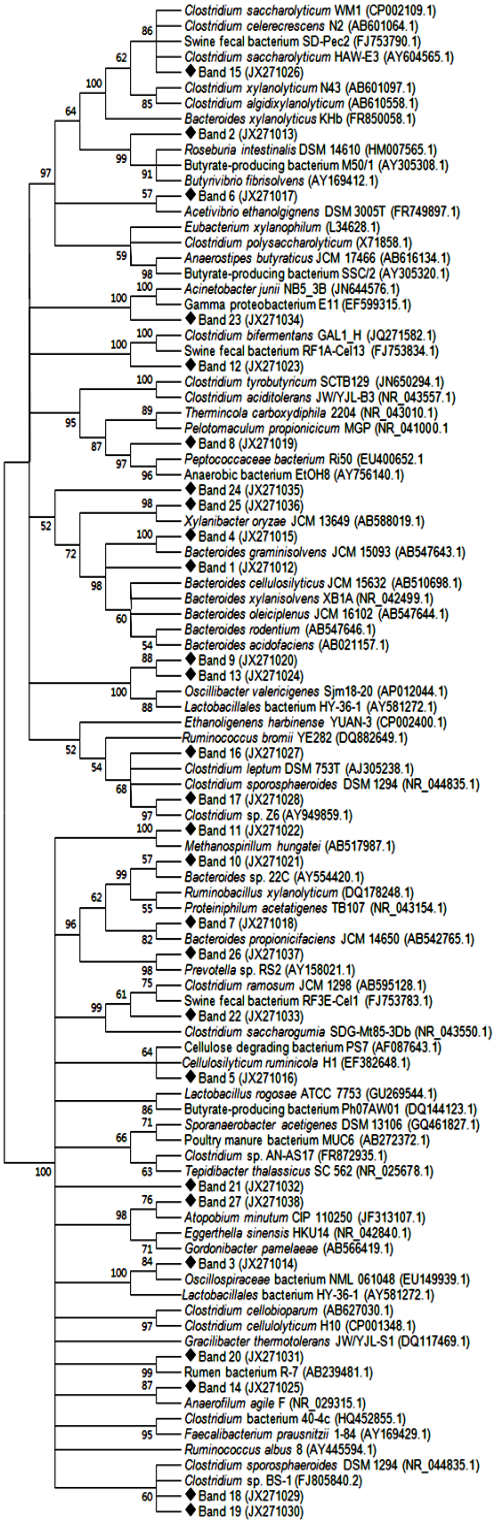

Fig. 4: Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences determined in this study and the related reference sequences. Sequences were aligned with ClustalW, and distances were computed using the Jukes-Cantor method. Bootstrap confidence levels greater than 50% are indicated above the nodes (replicate 1000 times)

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