Consolidated bioprocessing of microalgal biomass to carboxylates by a mixed culture of cow rumen bacteria using anaerobic sequencing batch reactor (ASBR)

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One supplementary table and three supplementary figures are available with the online version of this paper.

ABSTRACT

This study employed mixed-culture consolidated bioprocessing (CBP) to digest microalgal biomass in an anaerobic sequencing batch reactor (ASBR). The primary objectives are to evaluate the impact of hydraulic residence time (HRT) on the productivity of carboxylic acids and to characterize the bacterial community. HRT affects the production rate and patterns of carboxylic acids. For the 5-L laboratory-scale fermentation, a 12 -day HRT was selected because it offered the highest productivity of carboxylic acids and it synthesized longer chains. The variability of the bacterial community increased with longer HRT ($R^2=0.85$). In the 5-L laboratory-scale fermentor, the most common phyla were *Firmucutes* (58.3%), *Bacteroidetes* (27.4%), and *Proteobacteria* (11.9%). The dominant bacterial classes were *Clostridia* (29.8%), *Bacterioidia* (27.4%), *Tissierella* (26.2%), and *Betaproteobacteria* (8.9%).

Keywords:

Consolidated bioprocessing; algal biomass; carboxylates; cow rumen bacteria; ASBR reactor

1. Introduction

Microalgae can fix carbon dioxide and are a potential renewable source of liquid and gaseous biofuels. They can be converted using several different bioenergy productions options that produce various biofuels including trans-esterified biodiesel, photobiological biohydrogen, and biomethane (Sialve et al., 2009; Zhao et al., 2014). Among these options, anaerobic digestion of microalgae has reported biomethane yields from 0.09–0.45 L CH₄/g VS (volatile solids) (Mussnug et al., 2010; Zamalloa et al., 2012; Zhao et al., 2014). This wide range strongly depends on the microalgae species. However, one problem is the low C/N ratio in microalgal biomass, which results from its high protein content (39–71%). Consequently, the co-digestion of microalgal biomass supplemented with high-carbon content substrates should be considered. By adding waste paper (50% w/w) to algal sludge, the resulting C/N ratio was in the range of 20–25:1, and the methane production rate increased to 0.6–1.2 L/(L liquid·day) (Yen and Brune, 2007).

The MixAlco process is an example of the *carboxylate platform*, an advanced biorefining technology that uses undefined anaerobic acid-forming bacterial consortia (Holtzapple et al., 1999; Holtzapple and Granda, 2009). This technology converts complex biodegradable wastes into mixed carboxylate salts, an effective feedstock for downstream production of biofuels and specific valuable chemicals (Fu and Holtzapple, 2010a). During fermentation, the high concentrations of total ammonia nitrogen (TAN) from algal protein are easily acidified from the produced carboxylic acids (C₂–C₇) (Sialve et al., 2009). Furthermore, to neutralize the produced carboxylic acids, the ammonium from algal proteins is a superior buffer so less supplementation with bicarbonate salts is required (Fu and Holtzapple, 2010b). Cattle rumen bacteria that contain more than 1000 bacterial operational taxonomic units (OTUs) provide rapid fermentation rates from the complex feedstock with high productivity of short-chain volatile carboxylic acids, such as acetic, propionic and butyric acids. Therefore, an emerging and attractive concept is to use a mixed culture of cow rumen bacteria to convert microalgal biomass to carboxylate intermediates to generate biofuels and valuable chemicals.

To our knowledge, this is the first publication describing the production of biofuels from microalgal biomass using *carboxylate platform*. The primary objective is to establish a mixed culture of cow rumen bacteria that ferment microalgal biomass to carboxylates using an anaerobic sequencing

batch reactor (ASBR). Other objectives include the identification of efficient and stable operating regimes of this consolidated bioprocessing (CBP) technology. Furthermore, the bacterial population and the community structure of the *carboxylate platform* were investigated by Terminal Fragment Length Polymorphism (T-RFLP) technology and the clone library method, respectively. Understanding the microbial structure will help refine the design and operation of the bioreactor system, facilitate the development of inoculum for large-scale implementation of this process, and contribute to the body of knowledge regarding the conversion of microalgal biomass to biofuels.

2. Materials and Methods

2.1 Feedstock and Inoculum Source

The feedstock used in the series of fermentations was *Scenedesmus dimorphus*, a freshwater unicellular microalga of class *Chlorophyceae*. The biomass of *S. dimorphus* was harvested, dewatered, and dried using technology from Algaeventure Systems (<http://www.algaevs.com/>). Subsequently, the biomass was desiccated in 105 °C to a constant weight. Before fermentation, the dried biomass was milled and passed through 1-mm screen using a direct-drive cyclone sample mill (Cole Parmer, Verner Hills, IL, USA) so as to reduce the level of compaction and reduce particle size.

The seed inoculum used in the ASBR system was obtained from a fistulated cow at Washington State University. The harvested rumen contents were predominantly mixed fiber and a slight amount of liquid. These samples for seeding were immediately transferred in sterile N₂-filled serum bottles, tightly sealed with butyl rubber stoppers, and then stored at 4 °C until use within one week.

2.2 Bioreactor Construction and Performance Monitoring

To optimize hydraulic residence time (HRT), 4 g dried microalgal biomass (*S. dimorphus*) and 10% seed inoculum (~0.4 g) were inoculated in four serum bottles, which served as the ASBR reactor. Each vessel had 100-mL working volume. No additional nutrients were supplied. Before fermentation, the anaerobic cultures in reactor were neutralized with aqueous ammonia to pH 7.0, flushed with O₂-free N₂ gas for 15 min to induce an anaerobic condition, and incubated at 39 ± 1 °C and 200 rpm. After 95 h of reactor operation, 95, 50, 33.3, and 25 mL of the cultured medium for 4.2, 8, 12, and 16 days of HRT was replaced by equal volumes of fresh medium containing 3.8, 2, 1.33, and 1 g algal biomass, respectively. From the second cycle, every operation was comprised of decanting period (10 min),

feeding period (20 min), neutralizing acids period (20 min), flushing period (10 min), and reacting period (varied by HRT). The initial volatile solids loading rate (VSLR) was decreased stepwise by increasing HRT from 4.2, 8, 12, and 16 days. Table 1 shows the experimental conditions for the different HRTs in the ASBR system. Constant substrate consumption and carboxylic acid production ($\pm 5\%$ variation) were considered as indicators for steady state.

The laboratory-scale ASBR bioreactor (Model Bioflo 110 Fermentor, New Brunswick Scientific Inc., NJ) was conducted with 5-L working volume supplying 3% seeds of 12-day HRT at 39 ± 1 °C and 200 rpm. The reactor was perfectly sealed and the head unit included functional ports such as feed inlet and outlet, temperature probe and pH probe. Prior to starting the reactor, it was sparged with O₂-free N₂ gas for 25 min. During 32 days fermentation, the substrates were added eight times to the 5-L experiment reactor with very 95 h and aqueous ammonia (300 g/L) was pumped automatically into the fermenter to maintain pH 7.0 ± 0.1 . Methanogens were inhibited by the concentration of 25 mg iodoform/L from the concentrated solution (25 g iodoform/L ethanol) during the fermentation (Fu and Holtzapple, 2010a). Iodoform was added through the butyl rubber stopper with a 1-mL syringe, and added into the reactor every two days to prevent methane production.

2.3 Analytical Methods

The elemental analysis for carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and sulfur (S) of *S. dimorpha*, the total metal concentration, the total lipid content, total ammonia nitrogen (TAN), the total solids (TS), and volatile solids (VS) were determined as described by Zhao et al., (2014).

Prior to measuring the total carboxylic acids, fermentation samples were first centrifuged at 13,000 rpm for 3 min. Subsequently, the supernatant liquids were mixed with an equal amount of internal standard solution (4-methyl-*n*-valeric acid, 1.162 g/L), acidified by formic acid (10 g/L) with the ratio of 1:4 (v/v) between sample and formic acid, and then filtered through Millipore 13-mm nonsterile membrane (0.22- μ m pore size; Fisher, PA, USA). The concentrations of carboxylic acids were analyzed using a Shimadzu gas chromatograph outfitted with an AOC-5000 auto-sampler and FID detector operated at 300 °C (GC-2014; Sunnyvale, CA, USA) using a HP-INNOWax column of 30-cm length, 0.25-mm ID, and 0.25- μ m film thickness. The start temperature was 70 °C, ramped at 15 °C per min to a hold temperature of 230 °C for 3 min. Split/split-less injector temperature was 250

°C at a split ratio of 40/1 using helium carrier gas. Detector temperature was 300 °C. To express all carboxylic acids on a common basis, the carboxylic acid concentrations were converted to acetic acid equivalents (α) (Fu and Holtzapple, 2010b).

$$\alpha \text{ (mol/L)} = \text{acetic (mol/L)} + 1.75 \times \text{propionic (mol/L)} + 2.5 \times \text{butyric (mol/L)} + 3.25 \times \text{valeric (mol/L)} + 4.0 \times \text{caproic (mol/L)} + 4.75 \times \text{heptanoic (mol/L)}.$$

On mass basis, the acetic acid equivalent can be expressed as $A_e \text{ (g/L)} = 60.5 \text{ (g/mol)} \times \alpha \text{ (mol/L)}$.

The following equations were used to characterize fermentor performance:

$$\text{VSLR} = \frac{\text{VS fed (g)}}{\text{liquid in reactor (L)} \times \text{time (day)}} \quad (1)$$

$$\% \text{ Conversion} = \frac{\text{VS digested (g)}}{\text{VS fed (g)}} \times 100 \quad (2)$$

$$\% \text{ Yield} = \frac{\text{Total carboxylic acids produced (g)}}{\text{VS fed (g)}} \times 100 \quad (3)$$

$$\% \text{ Selectivity} = \frac{\text{Total carboxylic acids produced (g)}}{\text{VS digested (g)}} \times 100 \quad (4)$$

$$\text{Total acid productivity} = \frac{\text{Total carboxylic acids produced (g)}}{\text{liquid in reactor (L)} \times \text{time (day)}} \quad (5)$$

2.4 Molecular Analysis of Bacterial Communities

Using the Power Soil DNA Isolation Kit (MoBio Laboratories, CA), community genomic DNA was extracted from 2-mL slurry samples from the ASBR reactor. For T-RFLP analysis, bacterial 16S rRNA gene was amplified with the universal primer 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and primer 1492r (5'-GGTTACCTTGTTACGACTT-3'). The 5' end of the 8f primer was labeled with the fluorescent tag 6-FAM (6-carboxyfluorescein). Polymerase chain reaction (PCR) was performed as follows in a 50- μ L reaction volume using GoTaq Green Master Mix (Promega, USA): initial denaturation step at 95 °C for 5 min, followed by 20 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and primer extension at 72 °C for 1.5 min, with a final 5-min extension step at 72 °C. Three replicate PCRs were run per sample and pooled after amplification. The PCR products purified using the QIAGEN PCR purification kit (Valencia, CA, USA) were digested with *Hae* III (GG'AC, Takara Bio, USA) and *Rsa* I (GT'AC, Takara Bio, USA) for 8 h at 37 °C, respectively. In this study, *Hae* III was more suitable for further analysis because it generated the most

complex types. Separation of T-RFs was run on an ABI 3730xl DNA sequencer (GENEWIZ, Inc. USA) together with an internal size standard (GeneScan 500 LIZ; Applied Biosystems). T-RFs were analyzed using GeneMarker V 2.2 (SoftGenetics LLC., USA) including fragments with lengths ranging from 50 to 500 bp, an initial peak detection threshold of 50 relative fluorescent units, and a peak area with both >500 and its relative abundance >0.5%. Peaks of <0.5 bases apart from each other were merged. The T-RFs profiles were normalized by calculating the relative abundance (percentage) of each fragment (each T-RF peak area divided by the total peak area of each sample).

For clone library construction for phylogenetic analysis, PCR products were obtained in triplicate as described above except that 8f primer was not labeled with 6-FAM. The amplified products were pooled, purified, cloned into pGEM-T Easy Vector (Promega, USA) and transformed into competent *E.coli* JM109. The colonies that contained an appropriately sized insert were sequenced bidirectionally using primers SP6 and T7. The obtained sequences per clone were assembled using the ContigExpress yielding almost full-length 16S rRNA gene. Subsequently, the sequences were trimmed using DNA Baser v2.9 (Heracle BioSoft, USA) and putative chimeric sequences were identified using DECIPHER tool. Sequences were assigned to OTUs (defined at 99%, 97%, and 95% sequence similarity) using the MOTHUR implementation (Schloss et al., 2009). Rarefaction curves were constructed at a distance cut-off of 0.01, 0.03, and 0.05 using rarefaction.single in MOTHUR. Representatives of each OTU at a threshold of 97% were selected using the MOTHUR get.oturep module and classified with the MOTHUR classify.seqs module using a Silva-compatible taxonomy database. The affiliation of each OTU representative was verified by comparing with sequences in Genbank database and in the ribosomal Database Project II (Cole et al., 2005). After merging the representative OTU sequences and the closest sequences of type strains, multiple sequences alignment were achieved using Clustal X program V 2.1. The distance matrix was calculated by the Jukes-Cantor model and the phylogenetic tree was constructed by neighbor joining (NJ) with MEGA V7.0 (Kumar et al., 2016).

2.5 Data Analysis

All data analyses were performed with SPSS Statistics V 19 for Windows (IBM, New York, USA). The statistical significances were examined using one-way analysis of variances (ANOVA) and the level of significance was defined at $P<0.05$.

3. Results and Discussion

3.1 Characteristics of *S. dimorphus* biomass

The biomass of *S. dimorphus* was harvested at ~0.5 g dry weight/L liquid, and subsequently the chemical composition was carefully determined. The dried biomass contained 0.291 g ash/g TS and abundant metal elements including sodium (1.90 mg/g), magnesium (5.71 mg/g), potassium (3.52 mg/g), calcium (20.13 mg/g), iron (3.02 mg/g), zinc (6.73 mg/g), copper (0.18 mg/g), and manganese (0.46 mg/g). In general, the concentration of metals derived from microalgal biomass was not high enough to inhibit microbial activities during anaerobic fermentation, whereas they met the required micro-metal nutrients for growth (Zhao et al., 2014). The significant non-mineral compositions were C (39.62 %), H (5.66 %), O (28.66 %), N (6.65 %), and S (1.25 %). Crude protein is generally regarded as 6.25× total nitrogen, indicating that approx. 41.6% of *S. dimorphus* biomass was protein, which is within the protein range (29–71%) given by Sialve et al., (2009). The total lipid content in *S. dimorphus* was only 7.22% because it was grown phototrophically without nutrient stress, which is significantly lower than the economic threshold of 40%. Therefore, anaerobic digestion of *S. dimorphus* whole biomass was suggested as a practical strategy for recovering sustainable bioenergy (Sialve et al., 2009).

Although there is still some debate, the recommended optimal C/N range for producing methane is 20:1 to 30:1 (Yen and Brune, 2007). Because of its low C/N ratio (5.96:1), *S. dimorphus* is not acceptable to generate methane. The reason is that the low C/N ratio might result in high concentrations of both TAN and carboxylic acids, which subsequently inhibit methanogen activity and therefore cause the anaerobic digestion to fail. Fortunately, the accumulated mixture of carboxylate salts produced by this fermentation can be utilized as intermediates for chemical and fuel production (Holtzapple et al., 1999). Thus, the anaerobic conversion of *S. dimorphus* biomass to carboxylates is an attractive option for producing renewable bioenergy and chemicals (Supplementary Figure S1).

3.2 Carboxylic Acids Productions and Profiles in ASBR with Different HRT

Continuous carboxylic acid production was operated simultaneously in ASBR with 100-mL working volume at different HRT. When HRT increased, VSLR decreased to 7.51, 3.96, 2.65, 1.98 g VS fed/(L liquid·day), respectively. Steady-state production of the total carboxylic acids was achieved

between 60 to 80 days (data not shown). Production was impacted by HRT, leading to variation of total carboxylic acid concentration, productivity, yield, conversion, selectivity, and even metabolite patterns (Table 1). At 4.2- and 8-day HRT, low digestion occurred. The main reason is that the microalgal cell wall contain a diverse array of fibrillar, matrix and crystalline polymers that are not easily biodegraded by bacteria in a short operational period. In contrast, the digestion was significantly enhanced with 12- and 16-day HRT. The conversion increased about 1.4 to 2.9 fold, indicating that under the pressure of long HRT, specific microbial consortiums were selected and biodegraded *S. dimorphus* biomass during fermentation. The maximum conversion (0.598 g VS digested/g VS fed), yield (0.443 g total acids/g VS fed), and selectivity (0.742 g total acids/g VS digested) occurred at 16-day HRT (VSLR=1.98 g VS fed/(L liquid · day)) with a total carboxylic acids concentration of 14.06 ± 0.59 g/L liquid. The highest productivity of total carboxylic acid (1.029 g total acids/L liquid day) occurred at 12-day HRT (VSLR=2.65 g VS fed/L liquid day) with a concentration of 12.35 ± 0.53 g/L liquid. The reason is that, with more HRT in ASBR, microbial community has more time to digest the microalgal substrate while the productivity of total carboxylic acid simultaneously decreased. Because industry generally prefers higher productivity of total carboxylic acids, the 12-d HRT was selected in subsequent scale-up to the 5-L fermentor.

In the effluents, the profiles and proportions of individual acid were significantly different from those of sugarcane bagasse and sewage sludge (Fu and Holtzapfel, 2011). Table 1 shows that with increased HRT, the carboxylic acids in the fermentation effluent shifted from lower molecular weight acids to higher molecular weight acids. For instance, the percentages of the total carboxylic acids consisted of acetic acid (36.3–54.9 wt %), propionic acid (12.2–15.5 wt %), butyric acid (19.2–22.0 wt %), valeric acid (10.4–17.1 wt %), caproic acid (0–6.72 %), and heptanoic acid (0–6.58 %). Longer HRTs are also preferred because they generated long-chain carboxylic acids, although they possessed the relatively low proportion (<10%) in mixed carboxylic acid, which are energetically superior and facilitate downstream processing.

3.3 Carboxylic Acid Production in a Laboratory-Scale ASBR Bioreactor

To mimic industrial operating conditions, fermentations were conducted in a laboratory-scale ASBR with HRT of 12 days. After 32 days of incubation, the performance metrics were quite stable

and significantly greater at the 5-L scale than the 100-mL scale. At 20 days, the final total carboxylic acid concentration was 14.7 g/L liquid (Figure 1) and the yield was 0.462 g total acids/g VS fed. Total carboxylic acid productivity was 1.223 g total acids/(L liquid·day) with a biomass conversion of 0.632 g VS digested/g VS fed and selectivity of 0.731 g total acids/g VS digested. The reported parameter values of conversion, yield and selectivity in this study were similar to, or higher, than most of those data (i.e. 0.31–0.77 g VS digested/g VS fed, 0.20–0.55 g total acids/g VS fed, and 0.39–0.85 g total acids/g VS digested) in previous studies (Fu and Holtzappple, 2010b; Fu and Holtzappple, 2011; Liang and Wan, 2015; Rughoonundun et al., 2012). These data indicate that the performance of the laboratory-scale reactor using microalgal biomass as substrate and rumen microorganisms as inoculum at 12-day HRT is effective for producing carboxylic acids.

During anaerobic fermentation, proteins are degraded to ammonium, which is the main nitrogen form released into the liquid phase (Zamalloa et al., 2012). The average TAN in the liquid effluent was 1.165 g/L, which accounted for approx. 84.4% of the TAN in the total mixed solid and liquid effluent. This concentration is much lower than cyanobacteria *Spirulina maxima* (up to 7.0 g/L) (Samson and LeDuyt, 1986) and higher than marine microalgae *Phaeodactylum tricornutum* (0.546 g/L) (Zamalloa et al., 2012). The high concentration of ammonia from *S. dimorphus* fermentation also suggests the protein-rich microalgae might be unfeasible to yield biomethane because ammonia has toxic effects on methanogenesis. Ammonia can be recovered from the digestion effluent using sulfuric acid as an adsorbent by outgassing at elevated temperature of 50 °C (Supplementary Figure S1). In the aqueous effluent, soluble phosphates (PO₄-P) in the liquid phase averaged 0.267 g/L whereas total phosphate averaged 1.898 g/L, indicating that almost 86% of phosphates were in the solid effluent. Because N and P nutrients are expensive and cannot be sustainably discharged to the environment, the huge demand for these nutrients in microalgae cultivation can be satisfied by recycling N and P from waste streams exiting the *carboxylate platform*.

3.4 T-RFLP Analysis of Bacterial Community at Different HRT

T-RFLP profiles of bacterial fragments digested with *Hae* III (Supplementary Figure S2) indicate that the size and relative abundance of each T-RF varied significantly in each fermentor, suggesting the dynamic shifts in the bacterial community were affected by operating conditions. In this investigation,

the number of T-RFs positively correlate with HRT (number of T-RFs = $1.35 \times \text{HRT} - 2$; $R^2 = 0.85$), indicating that high microbial population diversity occurs with long HRTs. The primary reason for this result was the higher loading rate above 3.96 VSLR caused a relatively short HRT leading to more long-chain fatty acids (LCFA) that form adsorptive layers around microbial surfaces (Zhao et al., 2014). The reason is that the microorganisms from the rumen inoculum can easily transform lignocellulose, but might not degrade lipids at short HRT. As a consequence, some microbial species were inhibited and disappeared at shorter HRT, which decreased the microbial diversity.

Three T-RFs (261, 274, and 340 bp) were common to all four samples, but all were less important at HRT of 12 and 16 days based on the low relative abundances (<5 %) (Supplementary Figure S2). Two major T-RFs (175 and 233 bp) dominated (relative abundance of peak area >15 %) at HRT 8, 12, and 16 days, but were not detected at HRT 4 days. At HRT 4 days, three T-RF peaks of 64, 223, 272 bp (>10 %) were observed, but were absent at HRT 8, 12, and 16 days. Furthermore, the peak of T-RF at 339 bp was present in all samples and predominated at HRT 8 days (~33 %).

3.5 16S rRNA Gene Library and Phylogenetic Analyses

From 168 non-chimeric sequences in the library constructed from 5-L digester, 40 unique sequences were obtained at an OTU definition of 99%, 33 unique sequences at 97%, and 32 unique sequences at 95%. The rarefaction curves at all three thresholds showed that the plateau was reached (data not presented). Furthermore, the coverage was 0.95, indicating that the library dataset had thoroughly sampled diversity at three levels.

The bacterial sequences were assigned to different taxa from genus to phylum. *Firmicutes* (58.3% of the library), *Bacteroidetes* (27.4%), and *Proteobacteria* (11.9%) dominated and accounted for 97.6% of the bacterial community (Figure 2 and Supplementary Figure S3). The overrepresented phylogenetic groups are the classes *Clostridia* (29.8% of the library), *Bacterioidia* (27.4%), *Tissierella* (26.2%) and *Betaproteobacteria* (8.9%), and orders *Clostridiales* (28.8%), *Bacteroidales* (27.4%), *Tissierellales* (26.2%), *Rhodocyclales* (6.5%) and *Burkholderiales* (2.4%) (Supplementary Table S1). *Clostridia* had the highest biodiversity and comprised 16 bacterial OTUs at the 97% similarity threshold (Supplementary Table S1). Previous studies demonstrated that members of *Clostridia* generally were the most abundant bacterial order in anaerobic digestion (Smith et al., 2014). They are

known to play an important role in digesting polysaccharides and oligosaccharides (e.g., cellulose) to produce butyrate as major fermentation products (Ren et al., 2007), whereas *Bacterioidia* utilize xylans and xylose to generate propionate (Hollister et al., 2011). The genus *Tissierella* has already been detected in other anaerobic digesters (Jaenicke et al., 2011), but their specific function is not clear yet. As described in a previous study (Ariesyady et al., 2007), *Betaproteobacteria* affiliated with the *Variovorax* group could utilize propionate, butyrate, and acetate. Recently, *Betaproteobacteria* were also found to be strongly positively correlated with total VFA concentration in anaerobic digestion (De Vrieze et al., 2015), whose role in the platform from microalgal biomass to carboxylic salts will be addressed in a future study.

Of the top 12 OTUs, accounting for approx. 78.7% of the library (Supplementary Table S1), the classes are *Bacterodia*, *Clostridia*, *Tissierella*, and *Betaproteobacteria*, suggesting these populations play a large role in acid production. The two most abundant OTUs (OTU001, 13.7% and OTU002, 12.5%) were affiliated with the family *Porphyromonadaceae*. Figure 2 shows that the closest cultured representatives to these two OTUs are *Proteiniphilum acetatigenes* DSM 18083^T and *Petrimonas sulfuriphila* BN3^T at 97.3% and 99.7% similarity, respectively (Chen et al., 2005; Grabowski et al., 2005). The third most abundant OTUs (OTU003, 11.3%) belong to the family *Sporanaerobacter*, which has highly similar (98.7%) to *Sporanaerobacter acetigenes* DSM 13106^T (Hernandez-Eugenio et al., 2002), a strictly anaerobic, moderately thermophilic bacteria isolated from an anaerobic sludge and could produce acetate, isovalerate, and isobutyrate from peptone fermentation.

4. Conclusions

This study employed the *carboxylate platform* to convert algal biomass to carboxylate salts. Using rumen bacteria as inoculum, the production rates and profiles of carboxylic acids were affected by different HRTs. The highest productivity was demonstrated with 12-day HRT. Compared with shorter HRTs, this study showed the proportions of longer chain carboxylic acids (C5–C6) were enhanced with longer HRTs. In the ASBR, the bacterial community structure was highly affected by HRT, with greater diversity at longer HRT. In the 5-L laboratory-scale fermentor, the dominant bacterial classes of *Clostridia*, *Bacterioidia*, *Tissierella* and *Betaproteobacteria* play an important role in acid production.

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Table 1 Operating parameters of ASBR system and reactor performance metrics at different HRT following 80 days incubation. n.d.–Non detected. All data are means $\pm$ standard deviation (SD) of triplicate determination.

ASBR fermentation	A	B	C	D
Hydraulic residence time (days)	4.2	8	12	16
VSLR (g VS fed/(L liquid·day))	7.51	3.96	2.65	1.98
Total carboxylic acid concentration (g/L liquid)	3.62 $\pm$ 0.63	7.50 $\pm$ 0.81	12.35 $\pm$ 0.53	14.06 $\pm$ 0.59
Product distribution				
Acetic acid (wt %)	54.85 $\pm$ 7.96	49.16 $\pm$ 9.88	44.90 $\pm$ 3.33	36.28 $\pm$ 3.50
Propionic acid (wt %)	15.48 $\pm$ 2.76	15.23 $\pm$ 1.83	14.56 $\pm$ 1.52	12.21 $\pm$ 1.69
Butyric acid (wt %)	19.23 $\pm$ 1.41	22.04 $\pm$ 1.94	21.48 $\pm$ 0.75	21.48 $\pm$ 2.17
Valeric acid (wt %)	10.43 $\pm$ 1.35	13.57 $\pm$ 0.82	13.88 $\pm$ 1.49	17.12 $\pm$ 3.29
Caproic acid (wt %)	n.d.	n.d.	4.18 $\pm$ 2.91	6.72 $\pm$ 1.39
Heptanoic acid (wt %)	n.d.	n.d.	n.d.	6.58 $\pm$ 0.68
Conversion (g VS digested/g VS fed)	0.208	0.381	0.526	0.598
Yield (g total acids/g VS fed)	0.115	0.237	0.388	0.443
Selectivity (g total acids/g VS digested)	0.552	0.621	0.738	0.742
Total carboxylic acid productivity (g total acids/(L liquid·day)	0.862	0.938	1.029	0.879
Acetic acid yield (g acetic acid/g VS fed)	0.063	0.116	0.174	0.161
Methane productivity (g CH ₄ /(L liquid·day)	0.0002	0.0009	0.0006	0.0010

373 **Figure 1.** The total acid concentration in the 5-L laboratory-scale ASBR bioreactor with 12-day HRT

375 **Figure 2.** Phylogenetic distribution of clones observed in 5-L fermentor using neighbor-joining (NJ)
376 method based on 16S rRNA gene sequences showing the relationship between OTU and the closest
377 related strains. Sequence data of reference strains were obtained from the GenBank/EMBL and/or
378 RDP databases. Accession numbers are indicated in parentheses. Bootstrap values (%) based on 1000
379 replicates are indicated at the branch nodes and are shown for branches with more than 70% bootstrap
380 support. The scale bar indicates 5.0 nt substitutions per 100 nt.