1 Potential enhancement of direct interspecies
2 electron transfer for syntrophic metabolism of
3 propionate and butyrate with biochar in
4 up-flow anaerobic sludge blanket reactors

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Abstract

Promoting direct interspecies electron transfer (DIET) to enhance syntrophic metabolism may be a strategy for accelerating the conversion of organic wastes to methane, but microorganisms capable of metabolizing propionate and butyrate via DIET under methanogenic conditions have yet to be identified. In an attempt to establish methanogenic communities metabolizing propionate or butyrate with DIET, enrichments were initiated with up-flow anaerobic sludge blanket (UASB), similar to those that were previously reported to support communities that metabolized ethanol with DIET that relied on direct biological electrical connections. In the absence of any amendments, microbial communities enriched were dominated by microorganisms closely related to pure cultures that are known to metabolize propionate or butyrate to acetate with production of H₂. When biochar was added to the reactors there was a substantial enrichment on the biochar surface of 16S rRNA gene sequences closely related to *Geobacter* and *Methanosaeta* species known to participate in DIET.

Keywords: Direct interspecies electron transfer (DIET); Syntrophic metabolism of propionate and butyrate; Up-flow anaerobic sludge blanket(UASB); Biochar

Strategies of the enrichment of DIET for enhancing syntrophic metabolism of propionate and butyrate in the UASB reactors

TOC

1. Introduction

An understanding of the potential mechanisms for the metabolism of propionate and butyrate in anaerobic digesters is important because of their central importance in carbon and electron flow in these systems (McInerney et al., 2009; Müller et al., 2010). Both substrates can be degraded in defined co-cultures in which one microorganism converts propionate or butyrate to acetate and H_2 and a methanogen consumes the H_2 with the reduction of carbon dioxide to methane (Stams and Plugge, 2009). This interspecies electron exchange with H_2 as the electron carrier is known as interspecies H_2 transfer (Stams and Plugge, 2009). A similar metabolism appears to take place in many anaerobic digesters as evidenced by the abundance of microorganisms closely related to *Syntrophobacter*, *Syntrophomonas*, and *Smithella* species that are known to metabolize propionate or butyrate with the production of H_2 (de Bok et al., 2004; Stams and Plugge, 2009; Stams et al., 2012) and the fact that high concentrations of H_2 , which inhibit this metabolism in defined co-cultures, also inhibit propionate and butyrate metabolism in anaerobic digesters (Gallert and Winter, 2008; Wang et al., 2006).

Some substrates can also be converted to methane via direct interspecies electron transfer (DIET) (Rotaru et al., 2014a; Rotaru et al., 2014b; Wang et al., 2016). DIET with ethanol as the electron donor has been documented in defined co-cultures of two *Geobacter* species (Shrestha et al., 2013; Summers et al., 2010) as well as in defined methanogenic co-cultures of *Geobacter metallireducens* with *Methanosaeta* (Rotaru et al., 2014a) or *Methanosarcina* species (Rotaru et al., 2014b). Studies of digesters

treating brewery wastes in which ethanol was the primary substrate suggested that DIET between *Geobacter* and *Methanosaeta* species was the primary mechanism for interspecies electron exchange (Rotaru et al., 2014a; Morita et al., 2011).

Subsequent studies with co-cultures with *Geobacter metallireducens* as the electron-donating partner and *Geobacter sulfurreducens* as the electron accepting partner demonstrated that propionate and butyrate, as well as acetate, propanol, and butanol, could be metabolized via DIET with fumarate as the electron acceptor (Wang et al., 2016). In similar studies with *Methanosaeta haurindaceae* or *Methanosarcina barkeri* as the potential electron-accepting partner, propanol and butanol were partially metabolized to propionate or butyrate, which could not be further metabolized (Wang et al., 2016). The biochemical pathways in *G. metallireducens* for propionate and butyrate metabolism with electron exchange via DIET are not sufficiently understood to provide an explanation for the lack of propionate and butyrate metabolism with a methanogen as the electron-accepting partner (Wang et al., 2016).

However, it has been suggested that propionate and butyrate can be metabolized via DIET under methanogenic conditions if magnetite is provided as an electrical conduit for interspecies electron exchange (Cruz Viggi et al., 2014; Li et al., 2015). In both studies, DIET was inferred from the faster metabolism of propionate or butyrate with magnetite amendments. In the study with butyrate, *Geobacter* species were enriched with magnetite (Li et al., 2015). However, the presence of *Geobacter* is not necessarily

evidence for DIET, because not all *Geobacter* species are capable of DIET (Rotaru et al., 2015). Furthermore, *Syntrophomonadaceae*, which have been described as metabolizing butyrate with the production of H₂ (Sousa et al., 2007; Zhang, 2004), were also highly abundant and appeared to be more metabolically active in the enrichments (Li et al., 2015).

In addition to magnetite (Kato et al., 2012; Liu et al., 2015), other conductive materials such as granular activated carbon (Liu et al., 2012), biochar (Chen et al., 2014a), and carbon cloth (Chen et al., 2014b) promote DIET in defined co-cultures and may aid in enrichment of microorganisms capable of DIET. In *Geobacter* co-cultures magnetite substituted for the cytochromes associated with electrically conductive pili (e-pili), but e-pili were still required for DIET (Liu et al., 2015). In contrast, in the presence of the conductive carbon materials e-pili are not required. Both electron-donating and electron-accepting partners attach to the carbon materials, which serve as a conduit for electron transfer between the cells.

Therefore, in order to further investigate whether propionate or butyrate might be metabolized via DIET under methanogenic conditions several enrichment strategies were investigated. One was to enrich in up-flow anaerobic sludge blanket (UASB) reactors similar to the UASB conditions previously found to enrich for *Geobacter* species metabolizing ethanol via DIET (Rotaru et al., 2014a; Morita et al., 2011; Shrestha et al., 2014). Another was to include biochar as a conductive carbon conduit to

potentially promote interspecies electron exchange in a manner different than that previously attempted with magnetite. Biochar is a granular activated carbon (GAC) precursor and chemical or physical activation of biochar produces GAC (Chen et al., 2014b). Although biochar is 1000 times less conductive than GAC and carbon cloth, biochar has a higher surface area and its highly porous structure should promote microbial attachment. A previous study (Zhao et al., 2015) found that biochar enhanced syntrophic metabolism under the high organic loading rates in the UASB reactors. The third strategy was to initially supplement the feed to the reactors with ethanol in addition to the organic acid because it is known that ethanol can be metabolized to methane via DIET.

2. Materials and method

2.1 Reactor Design.

Studies were conducted in four parallel continuous-flow UASB reactors (internal diameter of 70 mm and height of 300 mm) each of which had a working volume of 1000 mL. At the top of each reactor, a 2 L gas sampling bag was connected with the gas outlet of the three-phase separator described as our previous study (Zhao et al., 2015). All the experiments were operated at 37.0 °C. After a start-up period of 10 d, 5 g of biochar (Johnson; wesonline.com; 20-30 mm length × 5-10 mm width, 0.03-0.05g weight) was added into the two of the four reactors to construct the biochar-supplement reactors, respectively. The biochar was the same type used in a previous study on DIET (Chen et al., 2014b), with a Brunauer-Emmett-Teller (BET) surface area of 209 m²/g and an

electrical conductivity of 4.33 uS/cm per g. The other two reactors without biochar supplemented were used as the control reactors (see **Fig. 1**).

2.2 Sludge and wastewater.

Sludge obtained from the anaerobic digester of a municipal wastewater treatment facility in Pittsfield (Massachusetts) was used as the seed sludge. The total suspended sludge (TSS) of the seed sludge was 13100 ± 210 mg/L (mean $\pm$ standard deviation, $n = 3$) and the ratio of volatile suspended sludge (VSS) and TSS was 0.72. It was anaerobically stored at 4 °C prior to use. Each reactor received a 500 ml sludge inoculum at the start of experiments.

One of the two control reactors and one of the two biochar-supplemented reactors were fed with a wastewater in which ethanol and propionate were the primary substrates (see **Fig. 1**). The initial composition of this artificial wastewater (per liter) was as follows (Morita et al., 2011): ethanol, 2.5 mL; sodium propionate 0.88 g (ethanol: propionate = 4:1, as the ratio of chemical oxygen demand [COD]); KH_2PO_4 , 0.11 g; K_2HPO_4 , 0.17 g; Na_2SO_4 , 0.05 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g; trace element solution, 10 mL; vitamin solution, 10 mL. The composition of the trace element solution (in grams per liter) was as follows (Morita et al., 2011): $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.04; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.048; ZnCl_2 , 0.13; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01; $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.01; H_3BO_3 , 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.025. The composition of the vitamin solution (in grams per liter) was as follows: biotin, 0.002; pantothenic acid, 0.005; B-12, 0.0001; *p*-aminobenzoic acid, 0.005; thiocctic acid (alpha lipoic), 0.005;

168 nicotinic acid, 0.005; thiamine, 0.005; riboflavin, 0.005; pyridoxine HCl, 0.01; folic
169 acid, 0.002. After 30 days, the ratio of propionate and ethanol in the wastewater was
170 gradually adjusted from 4:1 to 3:2, 2:3, 1:4 and then to only propionate as carbon source
171 (see **Fig.1**). The COD of the feed was always maintained at 5137.5 mg/L throughout the
172 experiment.

173
174 The other two UASB reactors were fed with ethanol and butyrate (4:1, as the ratio of
175 COD) wastewater (see **Fig. 1**). The initial composition of this artificial wastewater (per
176 liter) was as follows: ethanol, 2.5 mL; sodium butyrate 0.71 g; KH_2PO_4 , 0.11 g;
177 K_2HPO_4 , 0.17 g; Na_2SO_4 , 0.05 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g; trace
178 element solution, 10 mL; vitamin solution, 10 mL. The composition of the trace
179 element solution and vitamin solution was the same as the ethanol-propionate feed. The
180 carbon source (butyrate and ethanol) of this wastewater was kept at a COD of 5137.5
181 mg/L. After 25 days experiments, the ratio of butyrate and ethanol in this wastewater
182 was gradually adjusted from 4:1 to 3:2, 2:3, 1:4 and then to only propionate as carbon
183 source.

184
185 All the feed was deoxygenated by flushing nitrogen gas for 1 h before use. The artificial
186 wastewater was pumped into the reactors with two two-channel peristaltic pumps
187 (ISMATEC, REGLO Digital MS-2/6, Switzerland). Before experiments the influent
188 flow between the two pumps was calibrated consistently. The hydraulic retention time
189 (HRT) of all the UASB reactors was always kept at 12 h throughout the experiments.

2.3 Analytical methods.

Analysis of total suspended solid (TSS) and volatile suspended solid (VSS) were conducted in accordance with Standard Methods for the Examination of Water and Wastewater. Volatile fatty acids (VFAs) were measured with a high-performance liquid chromatograph (Bio-Rad; Hercules; California) (Nevin et al., 2008). The ethanol concentration was measured with a gas chromatograph equipped with a headspace sampler and a flame ionization detector (Clarus 600; PerkinElmer; California) (Morita et al., 2011). The equivalent relationship between COD and substrates are as follows: 1.07 g-COD/g acetate, 1.51 g-COD/g propionate, 1.82 g-COD/g butyrate and 2.08 g-COD/g ethanol. The gas volume in the gas sampling bag was measured with a 100 mL glass syringe every eight hours (Zhao et al., 2015). The accuracy of this gas volume measurement was calibrated by measuring the given volume gas in the bag, showing that the errors were less than 2%. The composition of the biogas was analyzed with a gas chromatograph with a flame ionization detector (GC-8A; Shimadzu; Japan) (Morita et al., 2011).

2.4 Spatial fractionation of samples for microbial analysis.

To study the spatial distribution of bacteria and archaea, the sludge samples were divided into two parts: suspended sludge (bulk sludge) and biochar attached sludge. After 80 days experiments the suspended sludge of 10 mL was collected from the bottom of each of the four UASB reactors. Biochar taken from the UASB reactors were frozen with liquid nitrogen and then smashed into slags with a hammer. 2 mL of the biochar tightly attached sludge was collected by using phosphate-buffered saline (PBS;

0.13 M NaCl and 10 mM Na₂HPO₄ at pH 7.2) to wash the slags three times. All the sludge samples were rinsed again with phosphate-buffered saline (PBS; 0.13 M NaCl and 10 mM Na₂HPO₄ at pH 7.2) and then centrifugated (110 × 100 g for 10 min at 4 °C).

2.5 DNA extraction, PCR amplification and 454 pyrosequencing.

The total DNA in the bulk sludge and in the sludge tightly attached to the biochar was extracted with the MP-Bio FastDNA™ Spin Kit for Soil (MP-Bio; Laboratories Inc; California). The 16S rRNA gene V4 variable region PCR primers 515/806 were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed (Caporaso et al., 2011).

After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare DNA librarys by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary,

sequences were joined, depleted of barcodes, and then sequences < 150bp removed and sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTN against a curated database derived from GreenGenes, RDP II and NCBI (www.ncbi.nlm.nih.gov, <http://rdp.cme.msu.edu>) (DeSantis et al., 2006).

3. Results and Discussion

3.1 Effects of biochar on syntrophic metabolism of butyrate.

The performance of two reactors fed with butyrate and ethanol with equivalent ($P > 0.05$) methane production (**Fig. 2A**) and organic removal (**Fig. 2B**) diverged when biochar was added to one of them at day 11. The difference in performance was magnified as the proportion of ethanol in the feed was progressively reduced. From day 55 to 80, when butyrate was the sole electron donor, the methane production rate in the biochar supplemented reactor (143.3 ± 4.4 mL/h; mean $\pm$ standard deviation, $n = 10$), was about 25 % higher than that in the control reactor (115.2 ± 3.1 mL/h). The average effluent total VFAs in the biochar supplemented reactor was only 15.2 ± 6.5 mg/L COD, yielding a 99.7% COD removal efficiency. The average effluent total VFAs in the control reactor was 757.5 ± 44.6 , yielding a 85.3% COD removal efficiency. This differences in methane production and organic removal could primarily be attributed to more effective removal of acetate in the reactor with biochar (**Fig. 2D**). The acetate effluent in the biochar-amended reactor (15.2 ± 6.5 mg/L COD) was only 3% that in the

control reactor (702.6 ± 12.9 mg/L COD). Butyrate removal was comparable in the two reactors (**Fig. 2C**).

3.2 Effects of biochar on syntrophic metabolism of propionate.

Biochar also had an impact on propionate metabolism that was particularly notable once propionate was the only substrate in fed to the reactor (**Fig. 3**). From day 60 to 80, when propionate was the sole electron donor, the methane production rate in the biochar supplemented reactor (127.7 ± 2.7 mL/h), was about 16 % higher than that in the control reactor (110.2 ± 1.6 mL/h). The average COD removal efficiency in the biochar supplemented reactor (98.0%) was about 10% higher than that in the control reactor (87.8%). However, in contrast to the butyrate-fed reactors, acetate removal was highly effective in the absence as well as the presence of biochar (**Fig. 3D**). Instead, there was a significant difference in propionate removal rates. Once the reactors stabilized following the switch to propionate as the only substrate the propionate concentration in the reactor without biochar were more than 4-fold higher than those in the biochar-supplemented reactor (**Fig. 3C**). Propionate removal was less effective than removal of butyrate both in the presence and absence of biochar, which is consistent with the slower metabolism of propionate in most anaerobic digesters (Gallert and Winter, 2008; Wang et al., 2006). It should be noted that there was almost no ethanol detected in all the reactors throughout the studies with both butyrate and propionate.

3.3 Microbial community analysis.

After 80 days, archaeal and bacterial communities in the bulk sludge and attached to the biochar were analyzed with high-throughput 16S rRNA pyrosequencing. Sequences of

225 bacterial genera were detected, with the majority of sequences affiliated with 22 genera (**Fig. 4A**). The dominant bacterial genera (relative abundance > 5%) in the microbial communities and their reported metabolism are shown in **Table S1**.

In all of the reactors the dominant sequences (ca. 50 %) in the bulk sludge were most closely related to microorganisms known to metabolize organic acids to acetate with the production of H₂, such as *Smithella*, *Syntrophobacter*, *Syntrophomonas* and *Syntrophus* (**Fig. 4A and Table S1**). In the butyrate-fed reactors the dominant sequences were most closely related to known *Syntrophomonas* species, which are known to convert butyrate to acetate with the production of H₂ in co-culture with H₂-consuming methanogens (Sousa et al., 2007; Zhang, 2004) (**Table S1**). In the propionate-fed reactors the predominate sequences in the bulk sludge were most closely related to *Smithella* species, which are known for their ability to metabolize propionate to acetate and carbon dioxide with the production of H₂ (de Bok et al., 2001; Liu et al., 1999) (**Table S1**). Neither *Syntrophomonas* or *Smithella* species are known to be capable of extracellular electron transfer (Hatamoto et al., 2007; Sousa et al., 2007) and thus are unlikely participate in DIET. Furthermore, several other dominant bacterial genera (relative abundance > 5%), such as *Aminivibrio*, *Candidatus Cloacamonas* and *Thermanaerovibrio* species, were also detected in the microbial communities of bulk sludge (**Table S1**). Their possible role was to ferment the complex organic matters to organic acids with the production of H₂. Therefore, the UASB enrichment conditions did not appear to enrich for microorganisms capable of metabolizing butyrate or

propionate via DIET in the bulk sludge.

The microbial community attached to the biochar was different (**Fig. 4A**). *Syntrophomonas* and *Smithella* species remained abundant, but less so. Their decline was accompanied by the appearance of *Geobacter* species, which were not detected in the bulk sludge, but which accounted for ca. 20% of the community attached to the biochar in both the propionate- and butyrate-fed reactors (**Fig. 4A**). Four *Geobacter* species were detected with the most abundant being most closely related to *G. sulfurreducens* and *G. lovleyi* (**Fig. 5**). Although the attachment of *Geobacter* species to biochar is consistent with DIET, *Geobacter* species differ in their capacity to participate in DIET (Rotaru et al., 2015) and none of the sequences recovered were sufficiently closely related to known species that it can be conclusively determined whether the *Geobacter* species attached to the biochar were participating in DIET.

However, additional evidence that the biochar was promoting DIET was the finding that *Methanosaeta* species comprised a much higher percentage of the methanogen community on biochar than in the bulk sludge with either butyrate or propionate as the substrate (**Fig. 4B**). In the bulk sludge *Methanosaeta* species would, in the absence of direct biological electrical connections, be restricted to acetate as a growth substrate and, as observed (**Fig. 4B**), H₂-utilizing methanogens would be expected to be most abundant. However, with an electrical connection through biochar to *Geobacter* species, or potentially other microorganisms, also attached to the biochar and metabolizing

propionate or butyrate, *Methanosaeta* species could also consume electrons derived from the oxidation of propionate or butyrate to acetate. This would be expected to increase the abundance of *Methanosaeta* species with a corresponding decrease in H₂-utilizing methanogens, as was observed.

3.4 Implications.

The results demonstrate that, under the conditions employed, a UASB reactor alone was not sufficient to enrich for microbial communities that can convert propionate or butyrate to methane via DIET. However, the enrichment on the biochar of both *Geobacter* and *Methanosaeta* species, which are known to participate in DIET with other substrates (Rotaru et al., 2014a; Wang et al., 2016), suggest that propionate and butyrate might be converted to methane with DIET if an electrically conductive material is available to facilitate electron transfer. The abundance of *Syntrophomonas* and *Smithella* species, even on the surface of biochar, demonstrates that the conditions chosen to enrich propionate and butyrate metabolism via DIET did not exclude metabolism via interspecies H₂ transfer. Further efforts are underway to identify better enrichment strategies that are more selective for DIET in order to obtain more defined cultures to study propionate and butyrate-based DIET.

The results suggest that biochar-mediated DIET promoted propionate metabolism. Propionate accumulation is often associated with digester failure (Gallert and Winter, 2008; Wang et al., 2006), thus a better understanding of propionate metabolism with DIET might suggest alternative strategies for anaerobic digestion. In the butyrate-fed

culture the enhanced methane production in the presence of biochar was associated with improved removal of acetate. One possible explanation for this is that *Methanosaeta* species might metabolize acetate better when they can also obtain additional energy from DIET-derived electrons. Alternatively, *Geobacter* species are expected to have higher affinity acetate transporters than methanogens because of their ability to maintain lower acetate levels than methanogens when Fe(III) is available (Lovley and Phillips, 1987). *G. metallireducens* can oxidize acetate via DIET with *G. sulfurreducens* reducing fumarate as the electron-accepting partner (Wang et al., 2016). Acetate is oxidized syntrophically in some methanogenic environments with H₂ as the electron carrier (Kimura and Okabe, 2013; Nusslein et al., 2001; Schnurer et al., 1999). Therefore, the possibility that *Geobacter*, or other organisms, may oxidize acetate with DIET to a methanogen, as previously suggested (Kato et al., 2012), warrants further investigation.

5. Conclusions

Results in this study suggested that DIET could be enriched with initially feeding the enrichments with ethanol in addition to organic acids and biochar for the effective improvement of syntrophic metabolism of propionate and butyrate in UASB reactors. The finding that, under the enrichment conditions employed, DIET was only effective with biochar as a conduit, suggest that establishing DIET that relies on direct biological electrical connections may be more difficult with propionate or butyrate as the electron donor than has previously been observed for DIET with ethanol.

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Figure.1. Schematic diagram of the experimental system and change of the feeding composition during the experiments.

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Figure.2. Methane production rate (A), effluent total VFAs (B), effluent butyrate (C) and effluent acetate (D) in the biochar-supplemented and control reactors during syntrophic metabolism of butyrate. Error bars in methane production rate represent standard deviations of triplicate measurements.

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Figure.3. Methane production rate (A), effluent total VFAs (B), effluent propionate (C) and effluent acetate (D) in the biochar-supplemented and control reactors during syntrophic metabolism of propionate. Error bars in methane production rate represent standard deviations of triplicate measurements.

Figure.4. Bacterial community structure (A) and archaeal community structure (B) at genus level associated with six different conditions. Genus level with relative abundance lower than 2.00% were classified into group ‘others’.

Figure.5. Proportion of various *Geobacter* species in all the sludge samples.