Environmental Degradation of Cellulose Under Anaerobic Conditions

Eleanor G. Schut *

Cellulose is a primary structural component of plants and is one of the most abundant polymers on the Earth. Degradation of this recalcitrant component of plant biomass is an important process in the global carbon cycle and can potentially provide feedstock for biofuels. Fungi and bacteria are the primary organisms able to breakdown biomass-derived cellulose. Anaerobic bacteria, present in cellulose degrading ecosystems, such as compost piles, soils rich in organic matter, aquatic sediments, and digestive systems of herbivores, have developed efficient pathways to maximize metabolic energy from biomass degradation. In the absence of terminal electron acceptors, such as oxygen, hydrogen-producing pathways are common methods of electron carrier recycling. Electron bifurcating systems linked to hydrogen metabolism play an important role in anaerobic metabolism. In this study, samples from environmental cellulose-degrading microbial communities were collected, and the metabolic products produced during anaerobic cellulose degradation were examined. Samples from different environments produced different fermentation products from cellulose, suggesting flexibility in the fermentative degradation pathways. The most abundant products observed included hydrogen, acetate, propionate, butyrate, ethanol, and methane.

DOI: 10.15376/biores.17.4.6953-6969

Keywords: Cellulose; Carbon cycle; Anaerobic; Hydrogen; Ethanol; Acetate; Electron bifurcation

*Contact information: Department of Biology, University of North Georgia, P. O. Box 1358, Gainesville, GA 30503 USA; *Corresponding author: eleanor.schut@ung.edu*

INTRODUCTION

Cellulose is one of the most common sugar polymers on the Earth and plays a key role as a structural component of plant cell walls. The degradation of cellulosic biomass is important in sustaining the global carbon cycle and is primarily performed by aerobic fungi and bacteria (Stursova *et al.* 2012; Robak and Balcerek 2020; Sun *et al.* 2020). Although cellulose is difficult to metabolize, it can be broken down to fermentable glucose moieties by selected bacteria under aerobic and anaerobic conditions. During sugar metabolism under anaerobic conditions, two main electron carriers are used, NAD^+ and the redox protein ferredoxin (Fd). They are reduced to NADH and reduced ferredoxin (Fd_{red}), respectively. For glycolysis to continue, the NADH and Fd_{red} need to be reoxidized through the formation of fermentation products. From an energy yielding standpoint, a hydrogen producing pathway is a preferred method of electron carrier recycling. Thus, many anaerobic bacteria produce H_2, a potential biofuel candidate (Søndergaard *et al.* 2016). However, the reoxidation of NADH presents thermodynamic restrictions (Thauer *et al.* 1977; Thauer 2015). The redox potential of NADH (E_0' = -320 mV) is not low enough to

allow for H_2 formation (E_0' = -414 mV), while the redox potential of Fd (E_m = ~-500 mV, under physiological conditions) is low enough for H_2 formation to proceed (Buckel and Thauer 2013).

Metabolic yields of anaerobic organisms are much lower than their aerobic counterparts; however, they have developed both efficient pathways and interspecies relationships to maximize metabolic energy use. One way of maximizing cellular energy yields is through a process called electron bifurcation (Buckel and Thauer 2013, 2018; Peters *et al.* 2018a,b). Electron bifurcation couples exergonic and endergonic reactions to limit free energy loss. Bifurcating enzymes pair two electron donors with one receptor, or pair one electron donor with two receptors in simultaneous reactions involving four electrons per turnover. Many bifurcating enzymes utilize the low potential redox protein Fd (E_m = ~-500 mV), which is a key factor for driving the production of H_2 (E_0' = -414 mV) and other metabolic processes. Anaerobic H_2 production through a bifurcating hydrogenase maximizes the free energy in the anaerobe's metabolism (Fig. 1A). The bifurcating hydrogenase can pull the thermodynamically unfavorable oxidation of NADH (E_0' = -320 mV) to the production of H_2 (E_0' = -414 mV) when simultaneously paired with the favorable Fd_{red} (E_m = ~-500 mV) reaction allowing the production of H_2 as the major electron sink (Fig. 1A; Schut and Adams 2009). Anaerobic bacteria often contain multiple hydrogenases with various metabolic functions and can be linked to NAD(P), Fd, or for the bifurcating hydrogenase, NAD and Fd simultaneously (Søndergaard *et al.* 2016).

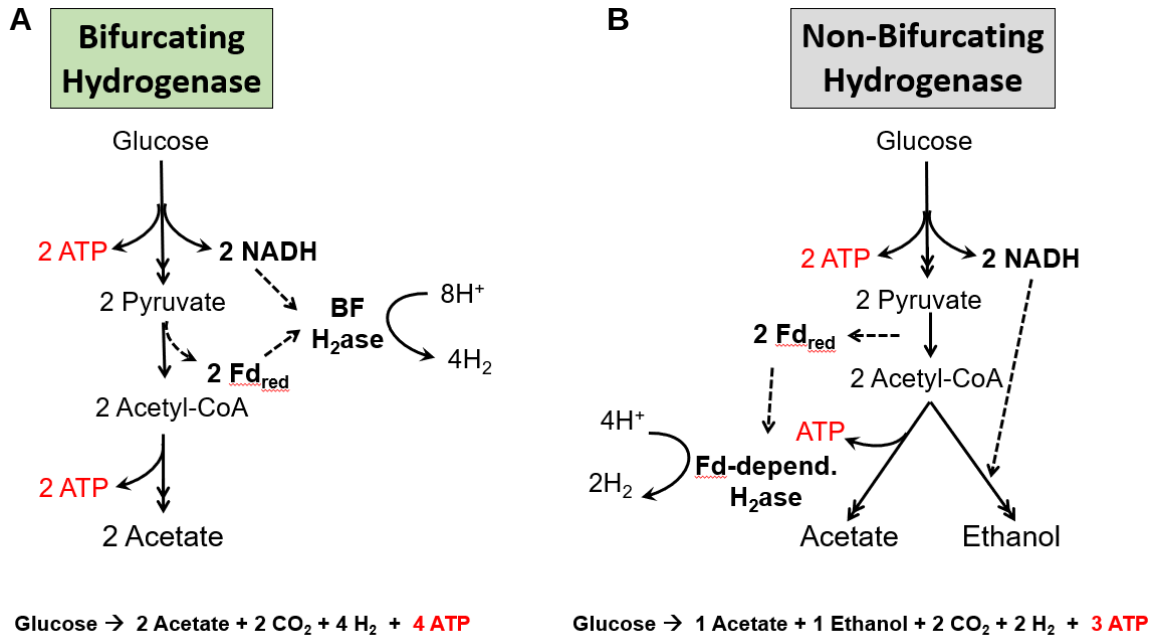

Fig. 1. Anaerobic glucose pathways in bacteria: **A.** Bifurcating hydrogenase pathway - Anaerobic glucose metabolism in which the electron carriers Fd and NADH are reoxidized using a bifurcating hydrogenase (BF H_2ase); and **B.** Ethanol pathway - Anaerobic glucose metabolism in which electron carriers are reoxidized using a Fd-dependent hydrogenase (Fd-depend H_2ase) and the production of the reduced carbon compound ethanol. Dual arrow heads indicate multiple reactions. Fd_{ox} and Fd_{red} indicates oxidized and reduced ferredoxin and for simplicity is presented as a 2-electron carrier (Buckel and Thauer 2013 and Zheng *et al.* 2014 were used as a guide to draw the pathways).

Carbohydrate degradation using a bifurcating hydrogenase is characterized by the conversion of 1.0 mole of glucose equivalents to 2 moles of CO_2, 2 moles of acetate and 4 moles of H_2 with a maximum yield of 4 ATP per glucose molecule (Fig. 1A; Thauer *et al.* 1977; Schut and Adams 2009; Thauer 2015). This can be observed in an experimental anaerobic system when acetate is the major carbon product detected in the liquid phase and H_2 (or methane) is formed in the headspace in a ratio of approximately 1.0 mole acetate to 2 moles H_2 (Fig. 1A). When conditions become less favorable, for example increased H_2 levels (Zheng *et al.* 2014), other fermentative pathways with a concomitant lower ATP yield take over, and reduced carbon products, such as ethanol and butyrate, are also produced (Figs. 1B and 2).

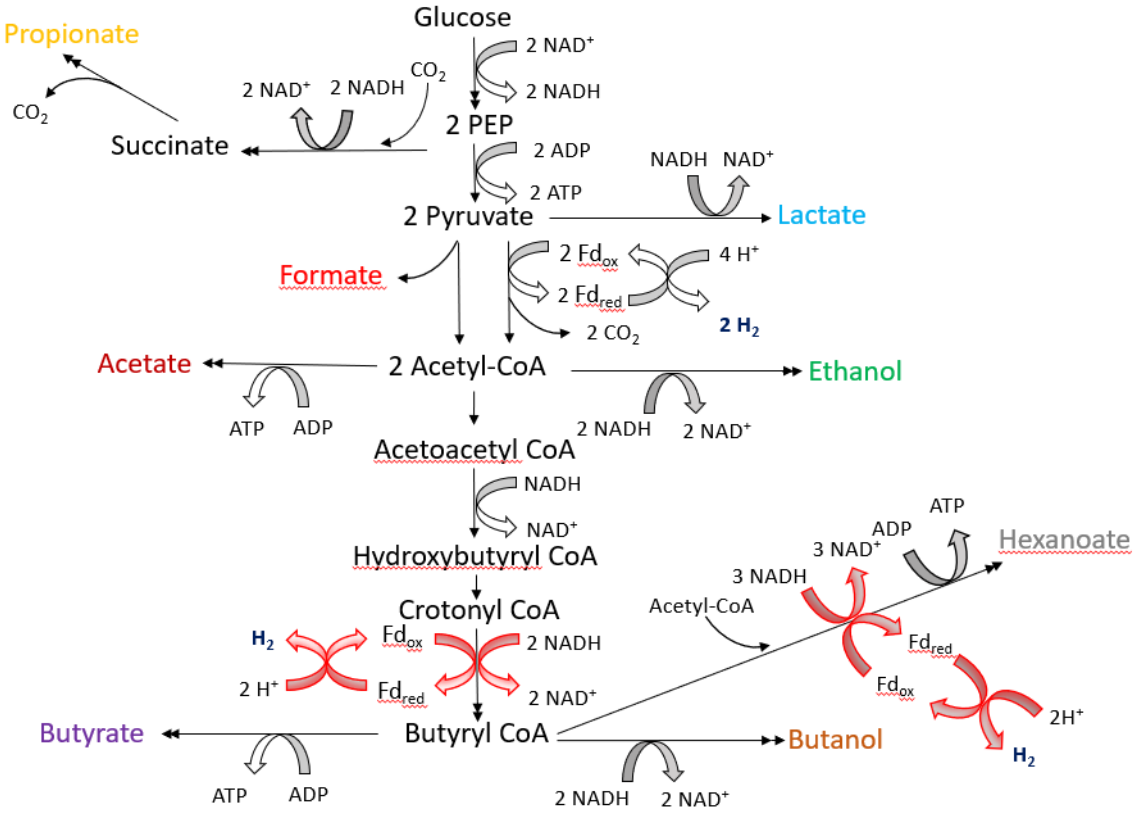

Fig. 2. Common anaerobic glucose fermentation pathways correlating with product observed in this study. The presented diagram is not balanced by carbon or electrons for clarity. Dual arrow heads indicate multiple reactions. Bifurcating reactions are indicated with red outlined curved arrows. Color of the metabolic product correlates with the products observed in Figs. 3 to 7. Fd_{ox} and Fd_{red} indicate oxidized and reduced ferredoxin, and for simplicity ferredoxin is presented as a 2-electron carrier (Buckel and Thauer 2013 and Gonzalez-Garcia *et al*. 2017 were used as a guide to draw the pathways).

In the ethanol pathway, ethanol is produced from glucose by anaerobic bacteria, which does not involve bifurcating enzymes (Fig. 1B). The NADH formed in glycolysis is recycled with the production of ethanol, while reduced Fd is recycled through the production of H_2 by a Fd-linked hydrogenase yielding a molar ratio of acetate:ethanol:H_2 of 1:1:2. Figure 2 gives an overview of the common products formed during anaerobic fermentation of glucose. The formation of butyrate, called the butyrate pathway, involves chain elongation in which acetyl-CoA units are merged, producing 4 carbon units and

reoxidizing 3 NADH molecules (Fig. 2; Spirito *et al.* 2014). The butyrate pathway also contains a bifurcating component, electron transferring flavoprotein butyryl-CoA dehydrogenase (EtfAB-Bcd), which catalyzes the favorable oxidation of crotonyl-CoA (E_0' = -10 mV) to butyryl-CoA. Oxidation of one NADH is coupled simultaneously to the endergonic reduction of Fd driven by a second NADH molecule linked to the exergonic reduction of crotonyl-CoA (Fig. 2; Li *et al.* 2008; Buckel and Thauer 2013). The reduction of Fd in this reaction can lead to the production of additional H_2, increasing the overall metabolic yield compared to butyrate formation without bifurcation (Li *et al.* 2008; Buckel and Thauer 2013). The enzymes of the butyrate pathway are promiscuous and can potentially act on longer carbon chains such as C5 and C6 intermediates (Fig. 2; Jeon *et al.* 2016). In butyrate metabolism the bi-functional alcohol dehydrogenase (AdhE), which is employed for the two-step production of ethanol from the acetyl-CoA, can also act on butyryl-CoA and form butanol which has potential applications for industry (Figs. 1B and 2; Nguyen *et al.* 2018).

Hydrogen is an abundant product in the anaerobic degradation of biomass. In nature the produced H_2 is typically used by anaerobic hydrogen oxidizing species, such as methanogens and acetogens (Blaut 1994; Robert *et al.* 2001; Gilmore *et al.* 2017), but it can also be used by aerobic bacteria at the air-soil boundary (Greening *et al.* 2022). A prime example of an anaerobic cellulose-degrading organism is the rumen bacterium *Ruminococcus albus*, which converts cellulose to acetate, ethanol, CO_2, and H_2 (Hungate 1957). When *R. albus* is grown with a H_2 utilizing methanogen (*Methanobrevibacter ruminantium*), it efficiently degrades cellulose without the production of ethanol. Instead, the major products are acetate, CO_2, and methane (Zheng *et al.* 2014). The *R. albus* is considered a keystone species in the ruminant's digestion of recalcitrant plant biomass-producing acetate that can be used by the ruminant host (Ze *et al.* 2013; Christopherson *et al.* 2014). The breakdown of cellulose by anaerobic microorganisms in the digestive tracts of herbivores provides host animals nutrients in the form of short chain organic acids (Ze *et al.* 2013). In anaerobes, the conversion of crystalline cellulose to smaller glucans is aided by highly efficient extracellular cellulose degrading structures, described as cellulosomes. They are covered with glycan binding units and degrading enzymes. Cellulosomes function by attaching to the cellulose fibers and hydrolyzing crystalline cellulose, thereby releasing metabolizable glucans. Their modular nature suggests that they could have biotechnological applications (Artzi *et al*. 2017; Alves *et al*. 2021; Joshi *et al*. 2022).

In this work, open cultures were analyzed containing cellulose-degrading consortiums from various environments, which are high in cellulosic biomass and tend to be limiting in aeration such as forest soils, pond sediments, and compost. The ability of such environmental samples to degrade cellulose under anaerobic conditions was compared, and the fermentation products formed were analyzed. The highly efficient cellulose degrader *R. albus* was used as a control.

EXPERIMENTAL

Sample Collection and Growth

Samples were collected from environments in and surrounding Watkinsville, Georgia, USA. Samples were collected in sterile 1.5-mL centrifuge tubes with 1.0 mL of water. After mixing the samples, 100-mL serum bottles were inoculated with 0.5 mL of each sample's suspension. Cellulose-degrading consortia were transferred 3 times to

remove any substrate contained within the samples and to obtain stable cultures before experiments were executed. Anaerobic culture media included basic minerals, 200 μM of organic acid mix, 0.5 g/L yeast extract, 0.5 g/L cysteine as reductant, and a limiting amount (0.25 g/l) of the cellobiose was used to allow cellulose degrading organism to start growing and producing the complex enzymes systems needed to degrade the insoluble cellulose. For normal (1:1 liquid to gas phase) headspace cultures, 50 mL media was filter sterilized (0.2 μm PES syringe filters) in 100 mL serum bottles previously autoclaved with 0.5 g of blotting paper (GB004, Schleicher & Schuell/Whatman, Keene, NH, USA) acting as a defined and pure form of cellulose. Serum bottles were aseptically stoppered and closed with crimp seals, after which the headspace was flushed with N_2/CO_2 (80:20) gas mixture using sterile connections with 0.2-μm syringe filters. Four samples with obvious plant biomass degradation capability derived from: compost, pond sediment, soil under leaf litter, and soil under pine straw, were selected. Pure cultures of *R. albus* (DSM 20455) were used as cellulose-degrading control. Cultures containing 5 g/L of blotting paper were inoculated with 1.0 mL cellulose degrading cultures and were incubated at 35 °C for 4 weeks. For low headspace samples, 110 mL anaerobic cultures were incubated at 35 °C for four weeks, leaving ~3 mL of headspace. All cultures were stationary during incubation to promote stable interaction between cellulose and the organisms' cellulose degrading machinery (Christopherson *et al.* 2014; Iakiviak *et al.* 2016; Alves *et al.* 2021). Gas samples (0.5 mL) and liquid samples (0.5 mL) were collected at the indicated time points and analyzed for H_2, methane, organic acids, and alcohols using gas chromatography (GC) and high-performance liquid chromatography (HPLC). Photos were taken to allow visual estimation of cellulose degradation (Figs. S1 through S5).

Headspace and Liquid Sampling

A 1.0-mL gastight lock syringe (Vici, Baton Rouge, LA, USA) with a sterile 26-gauge needle was used to sample 0.5 mL of the headspace into a stoppered 8 mL serum vial filled with argon (Ar; ambient pressure). A disposable sterile 1.0-mL syringe with a 26-gauge needle was used to sample the liquid phase of the culture bottles (0.5 mL). The headspace of the low headspace cultures was only sampled at the end of growth to minimize the loss of gasses as pressures were much higher than the normal headspace cultures.

Determination of H_2, Organic Acids, and Alcohols by Gas Chromatography and High-Performance Liquid Chromatography

Gas sample vials (8 mL serum vials) were analyzed by injecting 100 μL into a Shimadzu GC-8A gas chromatograph equipped with a molecular sieve column (Restek 5A 80/100) at 80 °C with Ar as carrier gas and a thermal conductivity detector (TCD). Standards were prepared by injecting known quantities of pure H_2 or methane gas (at ambient pressure) in an uninoculated media bottle with either a normal headspace or a low headspace using a lock syringe (Vici, Baton Rouge, LA, USA) equipped with a 26-gauge needle. This was followed by sampling 0.5 mL gas phase into 8 mL serum vials in an identical process as used for the culture bottle experiments. The amount of measured gases was expressed as molar concentrations in the liquid phase to simplify the comparison with the soluble metabolites.

For GC analysis of liquid samples, 100 μL media were acidified with 0.2 M formic acid. The concentrations of metabolic products (organic acids and alcohols) were detected *via* GC (7890 A, Agilent Technologies, Santa Clara, CA, USA) fitted with a flame

ionization detector (FID) and equipped with a carbowax capillary column (Agilent stabilwax, 30 m, 0.32 mm, 0.25 µm). The 1.0 µL samples were injected in a 250 °C injection port and eluted with N_2 as the carrier gas and a temperature gradient from 40 to 200 °C over 6 min. Standards (10 mM) of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, hexanoate, octanoate, ethanol, propanol, butanol, and acetoin were obtained by weighing the appropriate amounts of pure compounds (obtained from Sigma-Aldrich, St. Louis, MO, USA).

Common fermentation products not detected with GC-FID analysis were analyzed by high-performance liquid chromatography (HPLC). Media samples (100 µL) were acidified with 0.2 M sulfuric acid, and the concentrations of metabolic products were detected by HPLC (7890 A, Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector (210 nm) using a Aminex HPX-87H column (100 × 7.8 mm, Biorad, Hercules, CA, USA). Samples (10 µL) were analyzed with 5 mM sulfuric acid as the mobile phase at room temperature over 25 min at 1 mL/min. Standards (10 mM) of formate, succinate, and lactate were obtained by weighing the appropriate amounts of pure compounds (obtained from Sigma-Aldrich, St. Louis, MO, USA).

RESULTS AND DISCUSSION

Ruminococcus albus Growth on Cellulose

Growth of *R. albus* on cellulose was much slower than its growth on glucose (Weimer 1996). While glucose batch cultures generally grow to full density overnight, the cellulose cultures took approximately 10 days to reach stationary phase (Fig. 3). The yellow *R. albus* cells adhered to the blotting paper substrate (Fig. S1).

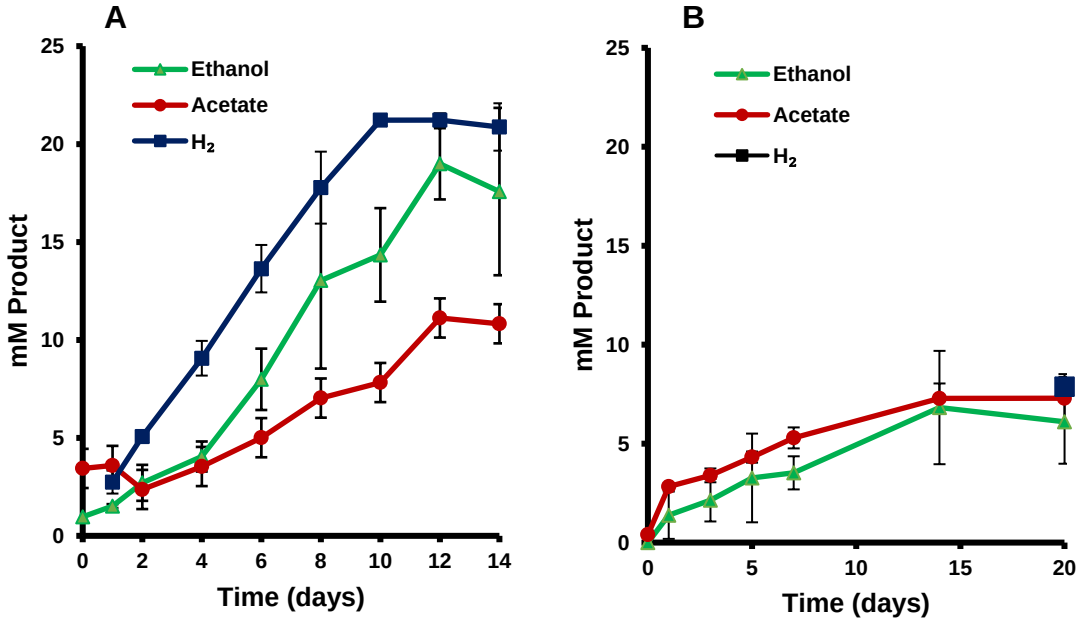

Fig. 3. Growth and product formation of *R. albus* with cellulose as carbon source: **A.** Culture conditions with 50% headspace; and **B**. Culture conditions with low headspace (~1% at inoculation). In the low headspace condition, H_2 was only sampled at the last time point.

The extracellular enzyme complexes converted this insoluble substrate to glucans that could then be absorbed by the cell (Christopherson *et al*. 2014). Glucose cultures, depending on the accumulation of H_2 in the headspace, typically produce more acetate than ethanol and in theory the ethanol to acetate ratio maximally reaches 1:1 (Zheng *et al.* 2014). Although cellulose is a glucan the *R. albus* cellulose cultures produced more ethanol than acetate (Fig. 3A), indicating that additional electron transfer pathways were employed. Overproduction of ethanol has also been observed in previous studies involving *R. albus* grown on polymeric substrates (Christopherson *et al.* 2014). In the rumen, *R. albus* produces limited ethanol because a methanogenic partner normally keeps the partial pressure of H_2 low. This allows *R. albus* to use the more energy efficient bifurcating hydrogenase system for cofactor recycling (Fig. 1; Miller and Wolin 1995; Zheng *et al.* 2014). When *R. albus* was grown with a small headspace, cellulose degradation and growth was impaired, indicating that the high partial pressure of H_2 hampered its ability to degrade cellulose (Figs. 3B and S1).

Diversity of Metabolic Pathways in Environmental Cultures

In the compost samples, interactions between cellulose degraders and methanogens were observed. Compost samples successfully degraded cellulose. However, H_2 levels did not rise above 100 μM. The presence of methane in the compost sample indicates that methanogens present in these samples were able to grow under the bottle conditions used in this study. Methanogens utilized the H_2 produced by the cellulose degraders, thus keeping the amount of H_2 minimal. Acetate levels by 28 days (~29 mM) were much higher than ethanol, propionate, and butyrate (all less than 3 mM), suggesting that the acetate/H_2 bifurcating hydrogenase pathway dominated (Figs. 1A and 4A).

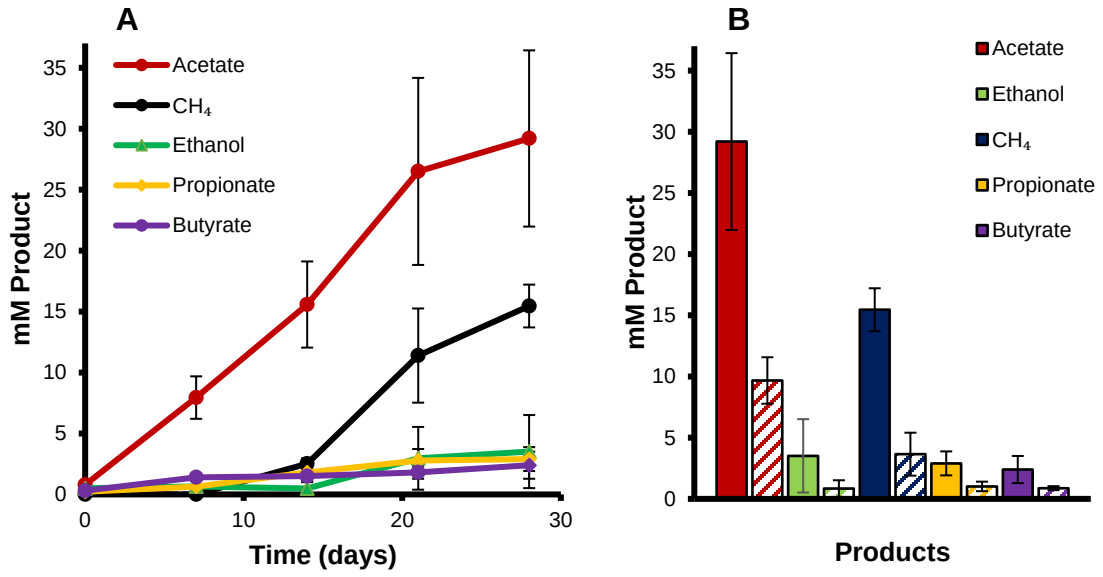

Fig. 4. Growth and product formation of mixed culture derived from compost with cellulose as carbon source: **A.** Growth and metabolite formation over time in culture conditions with 50% headspace; and **B.** Comparison of culture conditions with normal headspace (50%) indicated with solid bars *vs*. low headspace (~1%) presented with striped bars. Presented colors of the fermentation products correlate with Fig. 2.

Methanogens convert 4 moles of H_2 and 1.0 mole of CO_2 to 1.0 mole of methane (Blaut 1994; Deppenmeier 2002). In the compost samples, approximately 15 mM of methane were produced, which corresponds to ~ 60 mM of H_2 originally produced by the cellulose degraders. When matched with the 29 mM of acetate produced, the acetate:H_2 ratio was 1:2, as outlined in Fig. 1A. Compost cultures with a small headspace produced similar fermentation products but in lower amounts. About a third of the acetate and a fifth of the methane were produced, and the degradation of cellulose was clearly impaired (Figs. 4B and S2). In addition, the pH did not decrease as compared to the normal headspace cultures (5.6 *vs.* 7.0) corresponding to the lower acid formation. This indicates that in these cultures, increase of pressure by the formed methane and CO_2 in the headspace inhibited the degradation of cellulose overall. In both the normal and low headspace compost cultures the H_2 concentration was below 100 µM, indicating that the methanogenic organisms keep the H_2 levels low in both conditions.

Different cellulose degradation pathways were found in the pond sediment samples. The ratios of products suggest that the H_2 pathway was likely dominated by non-bifurcating hydrogenase and that the butyrate pathway was also involved in cellulose degradation. Pond environments often contain methanogens, but no methanogenic activity was observed in this study using pond sediment samples (Chaudhary *et al*. 2013). The pond sediment samples produced similar amounts of ethanol (16 mM) and acetate (18 mM) with a H_2 (28 mM) concentration close to twice that of acetate. This ethanol:acetate:H_2 ratio is close to 1:1:2 and is consistent with the ethanol pathway (Fig. 1B). Butyrate (6 mM) and butanol (4 mM) (Fig. 5A) were also produced. Production of both butyrate and butanol, suggests that the butyrate pathway was part of the cellulose degradation consortium and some of the butyryl-CoA was further reduced to butanol (Fig. 2). Cellulose was clearly well degraded anaerobically by this sample (Fig. S3). These results correlated with the pond sediment environment, which is in general rich in plant biomass and tends to lack oxygen.

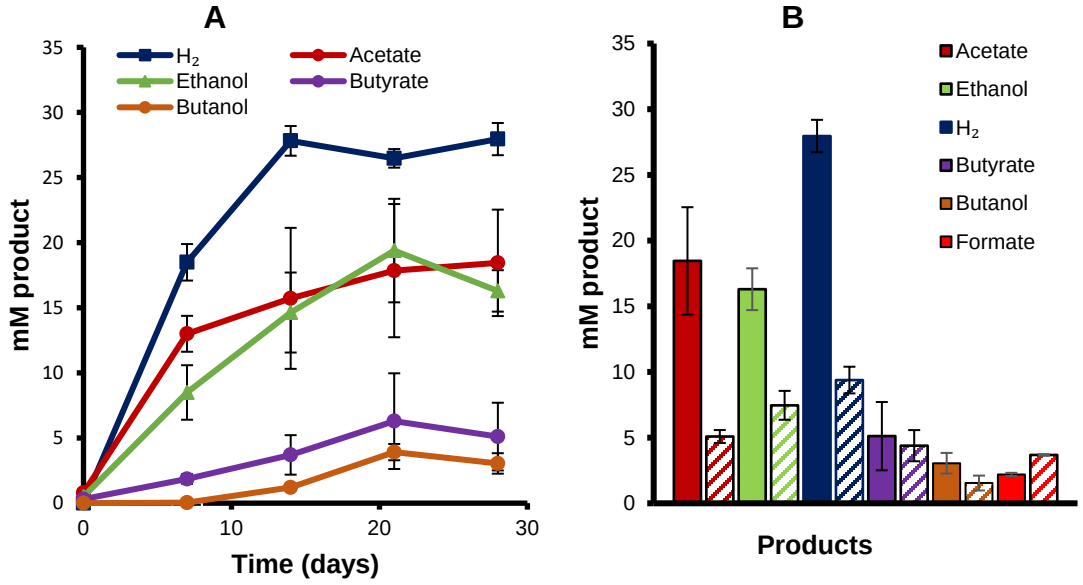

Fig. 5. Growth and product formation of mixed culture derived from pond sediment with cellulose as carbon source: **A.** Growth and metabolite formation over time in culture conditions with 50% headspace; and **B**. Comparison of culture conditions with normal headspace (50%) indicated with solid bars *vs.* low headspace (~1%) presented with striped bars. Presented colors of the fermentation products correlate with Fig. 2.

In the low headspace bottles, the ratios of fermentation products were similar, but production was reduced. Headspace pressure was high and cellulose degradation was decreased (Figs. 5B and S3). An overall decrease in the H_2 (3-fold), acetate (over 3-fold), and ethanol (2-fold) production was observed while the butyrate and butanol production were similar. Concomitantly, the pH had only a slight decrease in the low headspace cultures (5.6 *vs.* 6.9). A small increase in formate (2 to 4 mM) was observed, suggesting that there was only a slight shift in formate production at high H_2 pressure in the small headspace conditions. The production of butyrate includes a bifurcation step that adds to the production of H_2 (Fig. 2). Although lower overall amounts of H_2 (9 mM in 110 mL of medium) were measured, it led to higher pressure in the culture bottle at the end of growth because of the much smaller headspace. The pond sediment samples had the highest butyrate and H_2 production of the low headspace bottles, which might be analogous to conditions at the bottom of a pond where the water column and sediments can restrict the release of fermentation gasses.

The leaf litter soil samples were also dominated by the ethanol pathway. However, higher than expected production of acetate suggests that organisms may have also utilized the acetogenic pathway. The leaf litter soil samples produced high amounts of H_2 (21 mM), acetate (27 mM), and ethanol (12 mM) at a ratio approaching 2:2:1 and a small amount of formate (5 to 7 mM) (Fig. 6A).

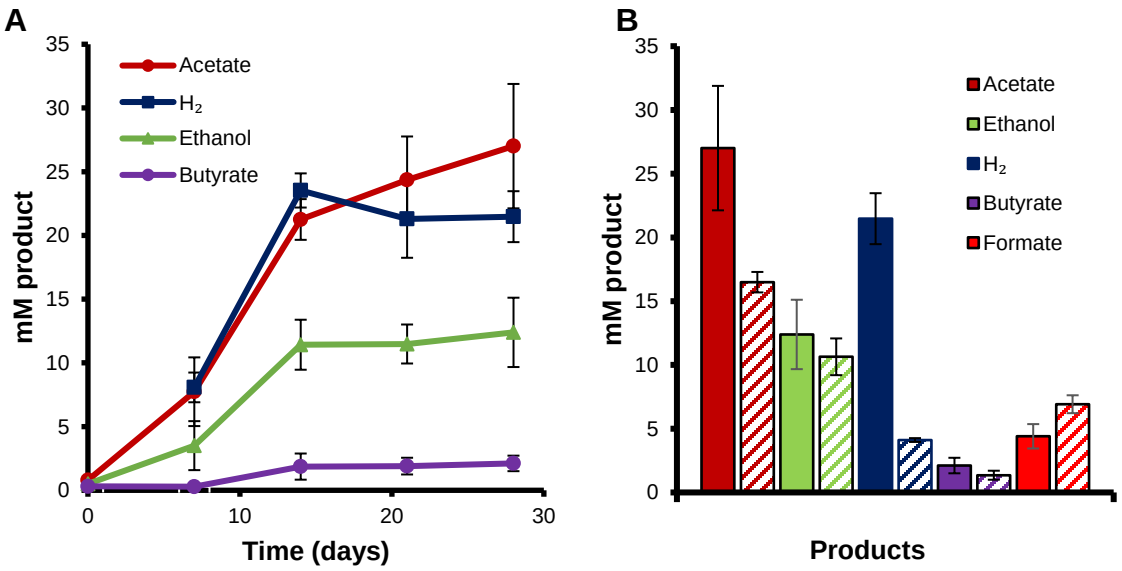

Fig. 6. Growth and product formation of mixed culture derived from leaf litter soil with cellulose as carbon source; **A.** Growth and metabolite formation over time in culture conditions with 50% headspace; and **B.** Comparison of culture conditions with normal headspace (50%) indicated with solid bars *vs* low headspace (~1%) presented with striped bars. Presented colors of the fermentation products correlate with Fig. 2

This was different from the expected 2:1:1 ratio of H_2:acetate:ethanol, as depicted in Fig. 1B. This suggests that additional products could be produced to satisfy the electron balance considering ethanol is used to recycle NADH in sugar metabolism (Fig. 1B). Indications for additional products were not observed in either the GC or HPLC analysis with standards of the normally observed fermentation products as described in Fig. 2, and no methane was observed with the GC analysis. Other possibilities include the presence of H_2 utilizing organisms, such as acetogens, or the cellulose degraders themselves could

possess the acetogenic pathway. Acetogens are bacteria that can obtain energy by converting H_2 and CO_2 to acetate (2 CO_2 and 4 H_2 results in one acetate) and often found with other anaerobic microorganisms that provide the H_2 and CO_2. The low pH observed (4.8) may have inhibited growth of acetogens and prevented complete conversion of H_2 to acetate, and this could explain the higher acetate to H_2 ratios (Miller and Wolin 1995; Schuchmann and Muller 2014; Regueira *et al*. 2018; Smith *et al*. 2019). In the low headspace samples, acetate (16 mM) was the major product and the ethanol:acetate ratio approached 1:1. Both H_2 (4 mM) and formate (7 mM) production were low, though more formate was produced than in the normal headspace samples (Fig. 6B). When visually compared to the normal headspace cultures, cellulose degradation decreased in the low headspace bottles (Fig. S4). Similar to the normal headspace bottles, acetogens may have played a role in utilizing H_2 and producing additional acetate. Low pH was also observed in the low headspace cultures (4.8) and may have inhibited both the cellulose-degrading bacteria as well as the acetogenic bacteria in the low headspace bottles

The pine straw soil samples appeared to be a community with more complex metabolic pathways. The major products were acetate (12 mM) and H_2 (22 mM) with smaller amounts of formate (5 mM), propionate (2.7 mM), butyrate (4 mM), ethanol (6 mM), and hexanoate (2.5 mM). A trace amount of valerate (0.5 mM, not shown) was also produced (Fig. 7A).

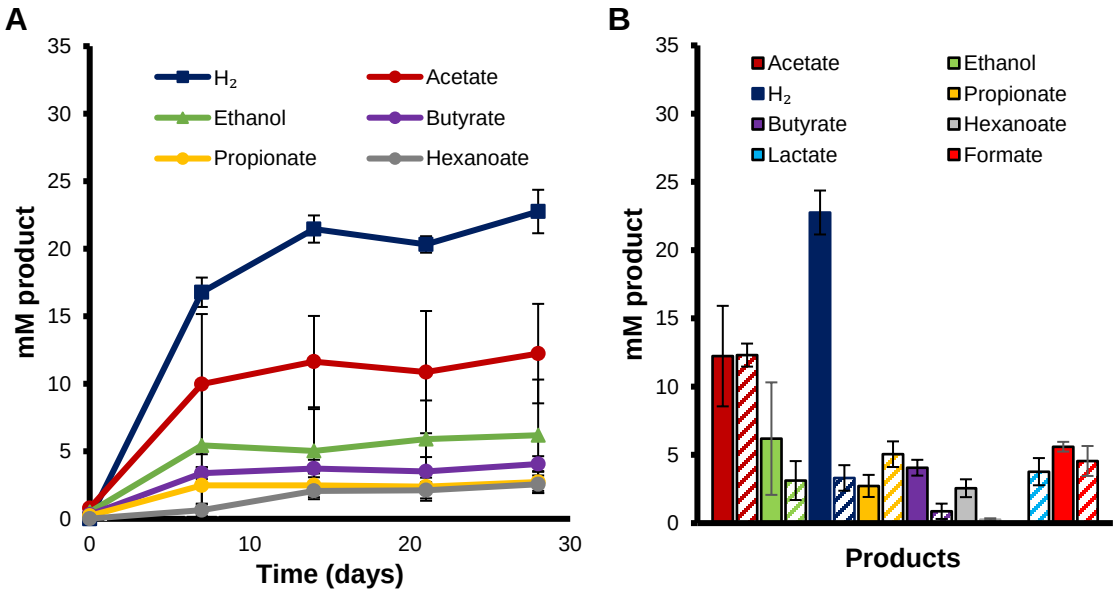

Fig. 7. Growth and product formation of mixed culture derived from soil under pine straw with cellulose as carbon source: **A.** Growth and metabolite formation over time in culture conditions with 50% headspace; and **B.** Comparison of culture conditions with normal headspace (50%) indicated with solid bars *vs* low headspace (~ 1%) presented with striped bars. Presented colors of the fermentation products correlate with Fig. 2.

The formation of hexanoate in addition to butyrate is rather unique. It is formed by the same pathway and enzymes as butyrate formation by adding an additional two carbon (acetyl-CoA) unit to the butyryl-CoA intermediate (Fig. 2). This so-called chain elongation process can be found in various environments and is often indicated by the pungent smell of organic acids (Spirito *et al*. 2014). The formation of propionate from glucose was likely achieved by the succinate/propionate pathway found in organisms such as

Propionibacterium spp. and *Bacteriodes* species (Fig. 2; Parizzi *et al*. 2012; Gonzalez-Garcia *et al*. 2017). The succinate/propionate pathway converts one mole of glucose to two moles of propionate without the release of CO_2, which means that no gas is produced in this process (Fig. 2; Reichardt *et al*. 2014; Ranaei *et al*. 2020). The observed small amount of valerate was likely produced by the incorporation of propionate into the chain elongation process, in which available organic acids in the medium can be incorporated in the chain elongation process as well (Spirito *et al*. 2014). The formation of medium chain organic acids (six and eight carbon length) from low grade biomass has some industrial value; however, the amounts produced here and in other studies are low (Steinbusch *et al*. 2011). At low headspace formate and acetate production had no noticeable change. Hydrogen (from 23 to 3 mM), butyrate (from 4 to 1 mM), and hexanoate (from 2.5 to < 1 mM) dramatically decreased, while propionate (from 3 to 5 mM) increased (Fig. 7B). In addition, lactate (4 mM) was produced. The pH of the low headspace decreased slightly compared to the normal headspace (4.7 *vs.* 5.0, respectively), reflecting the variety of organic acids that were produced. Cellulose degradation was decreased compared to normal headspace cultures (visual inspection Fig. S5).

Communities of microorganisms across different environments were able to degrade the recalcitrant cellulose substrate under strict anaerobic conditions. Acetate, ethanol, and H_2 were the core metabolic products produced during this process. Methane, and organic acids, such as butyrate, propionate, lactate, hexanoate, and formate were also observed. Methane was only found in the compost samples. Methanogens may not have been prominent members of the other sampled environments. Production of gaseous metabolites, especially H_2, during the degradation of biomass was important, and when the headspace was reduced, all the experimental sample cultures showed decreased cellulose degradation. This suggests that communities had limited alternative pathways to degrade cellulose without producing H_2. This was especially prominent with the *R. albus* control cultures, as well as the compost samples that produced methane due to the presence of a methanogen. Although the small headspace cultures produced much less H_2, there was no substantial increase in solvent production, such as ethanol and butanol, or longer chain fatty acids such as hexanoate. This simple adjustment of the culture condition was not sufficient to increase the production of valuable bioproducts.

CONCLUSIONS

1. Anaerobic bacterial cultures with the ability to degrade cellulose were easily obtained from soil and sediment samples.
2. Hydrogen, methane, acetate, and ethanol were found to be key metabolites in the anaerobic degradation of cellulose in soil and sediment samples. In addition, butyrate, propionate, formate, and butanol were also observed.
3. Restriction of the gas exchange, such as by limiting the headspace, in general decreases the cellulose breakdown.
4. Industrial relevant metabolites, such as hydrogen, methane, alcohols and medium chain organic acids, are produced by anaerobic cellulose-degrading communities.

5. In bacterial metabolism, electron bifurcation can improve the metabolic efficiency and can give organisms a competitive advantage in the environmental communities, especially involving recalcitrant substrates found in forest soils.

ACKNOWLEDGMENTS

This research was funded by a grant from the University of North Georgia College of Science and Mathematics Internal Faculty Development Funding. The author thanks Gerti Schut for technical help and critical reading of the manuscript and undergraduate research students, Rebecca L. Corley, Madison Prchal, and John M. Simmons. The author thanks the Adams Lab of the University of Georgia for use of GC, HPLC, and anaerobic gas manifold, and Alex La Reau and Garret Suen of the University of Wisconsin for providing a *Ruminococcus albus* culture.

REFERENCES CITED

Alves, V. D., Fontes, C. M. G. A., and Bule, P. (2021). "Cellulosomes: Highly efficient cellulolytic complexes," *Subcell. Biochem.* 96, 323-354. DOI: 10.1007/978-3-030-58971-4_9

Artzi, L., Bayer, E. A., and Morais, S. (2017). "Cellulosomes: Bacterial nanomachines for dismantling plant polysaccharides," *Nat. Rev. Microbiol.* 15, 83-95. DOI: 10.1038/nrmicro.2016.164

Blaut, M. (1994). "Metabolism of methanogens," *Antonie Van Leeuwenhoek* 66(1-3), 187-208. DOI: 10.1007/BF00871639

Buckel, W., and Thauer, R. K. (2013). "Energy conservation *via* electron bifurcating ferredoxin reduction and proton/Na^+ translocating ferredoxin oxidation," *Biochim. Biophys. Acta*. 1827(2), 94-113. DOI: 10.1016/j.bbabio.2012.07.002

Buckel, W., and Thauer, R. K. (2018). "Flavin-based electron bifurcation, a new mechanism of biological energy coupling," *Chem. Rev.* 118(7), 3862-3886. DOI: 10.1021/acs.chemrev.7b00707

Chaudhary, P. P., Brablcová, L., Buriánková, I., and Rulík, M. (2013). "Molecular diversity and tools for deciphering the methanogen community structure and diversity in freshwater sediments," *Appl. Microbiol. Biotechnol.* 97(17), 7553-7562. DOI: 10.1007/s00253-013-5102-8

Christopherson, M. R., Dawson, J. A., Stevenson, D. M., Cunningham, A. C., Bramhacharya, S., Weimer, P. J., Kendziorski, C., and Suen, G. (2014). "Unique aspects of fiber degradation by the ruminal ethanologen *Ruminococcus albus* 7 revealed by physiological and transcriptomic analysis," *BMC Genomics* 15(1), article no. 1066. DOI: 10.1186/1471-2164-15-1066

Deppenmeier, U. (2002). "The unique biochemistry of methanogenesis," *Prog. Nucleic Acid Res. Mol. Biol.* 71, 223-283. DOI: 10.1016/s0079-6603(02)71045-3

Gilmore, S. P., Henske, J. K., Sexton, J. A., Solomon, K. V., Seppala, S., Yoo, J. I., Huyett, L. M., Pressman, A., Cogan, J. Z., Kivenson, V., *et al.* (2017). "Genomic analysis of methanogenic Archaea reveals a shift towards energy conservation," *BMC Genomics* 18(1), article no. 639. DOI: 10.1186/s12864-017-4036-4

Gonzalez-Garcia, R. A., McCubbin, T., Navone, L., Stowers, C., Nielsen, L. K., and Marcellin, E. (2017). "Microbial propionic acid production," *Fermentation* 3(2), article no. 21.

Greening, C., Islam, Z. F., and Bay, S. K. (2022). "Hydrogen is a major lifeline for aerobic bacteria," *Trends Microbiol.* 30(4), 330-337. DOI: 10.1016/j.tim.2021.08.004

Hungate, R. E. (1957). "Microorganisms in the rumen of cattle fed a constant ration," *Can. J. Microbiol.* 3(2), 289-311. DOI: 10.1139/m57-034

Iakiviak, M., Devendran, S., Skorupski, A., Moon, Y. H., Mackie, R. I., and Cann, I. (2016). "Functional and modular analyses of diverse endoglucanases from *Ruminococcus albus* 8, a specialist plant cell wall degrading bacterium," *Sci. Rep.* 6, article no. 29979. DOI: 10.1038/srep29979

Jeon, B. S., Choi, O., Um, Y., and Sang, B. I. (2016). "Production of medium-chain carboxylic acids by *Megasphaera sp*. MH with supplemental electron acceptors," *Biotechnol. Biofuels* 9, article no. 129. DOI: 10.1186/s13068-016-0549-3

Joshi, A., Verma, K. K., Rajput, V. D., Minkina, T., and Arora, J. (2022). "Recent advances in metabolic engineering of microorganisms for advancing lignocellulose-derived biofuels." *Bioengineered* 13(4), 8135-8163. DOI: 10.1080/21655979.2022.2051856.

Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W., and Thauer, R. K. (2008). "Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*," *J. Bacteriol.* 190(3), 843-850. DOI: 10.1128/JB.01417-07

Miller, T. L., and Wolin, M. J. (1995). "Bioconversion of cellulose to acetate with pure cultures of *Ruminococcus albus* and a hydrogen-using acetogen," *Appl. Environ. Microbiol.* 61(11), 3832-3835. DOI: 10.1128/aem.61.11.3832-3835.1995

Nguyen, N. P., Raynaud, C., Meynial-Salles, I., and Soucaille, P. (2018). "Reviving the Weizmann process for commercial n-butanol production," *Nat. Commun.* 9(1), article no. 3682. DOI: 10.1038/s41467-018-05661-z

Parizzi, L. P., Grassi, M. C., Llerena, L. A., Carazzolle, M. F., Queiroz, V. L., Lunardi, I., Zeidler, A. F., Teixeira, P. J., Mieczkowski, P., Rincones, J., *et al.* (2012). "The genome sequence of *Propionibacterium acidipropionici* provides insights into its biotechnological and industrial potential," *BMC Genomics* 13, article no. 562. DOI: 10.1186/1471-2164-13-562

Peters, J. W., Beratan, D. N., Bothner, B., Dyer, R. B., Harwood, C. S., Heiden, Z. M., Hille, R., Jones, A. K., King, P. W., Lu, Y., *et al.* (2018). "A new era for electron bifurcation," *Curr. Opin. Chem. Biol.* 47, 32-38. DOI: 10.1016/j.cbpa.2018.07.026

Peters, J. W., Beratan, D. N., Schut, G. J., and Adams, M. W. W. (2018). "On the nature of organic and inorganic centers that bifurcate electrons, coupling exergonic and endergonic oxidation-reduction reactions," *Chem. Commun. (Camb)* 54(33), 4091-4099. DOI: 10.1039/c8cc01530a

Ranaei, V., Pilevar, Z., Khaneghah, A., M., and Hosseini, H. (2020). "Propionic acid: Method of production, current state and perspectives," *Food Technol. Biotechnol*. 58(2), 115-127. DOI: 10.17113/ftb.58.02.20.6356

Regueira, A., González-Cabaleiro, R., Ofiţeru, I. D., Rodríguez, J., and Lema, J. M. (2018). "Electron bifurcation mechanism and homoacetogenesis explain products yields in mixed culture anaerobic fermentations," *Water Res*. 141, 349-356. DOI: 10.1016/j.watres.2018.05.013

Reichardt, N., Duncan, S. H., Young, P., Belenguer, A., Leitch, C. M., Scott, K. P., Flint, H. J., and Louis, P. (2014). "Phylogenetic distribution of three pathways for propionate production within the human gut microbiota," *ISME J.* 8(6), 1323-1335. DOI: 0.1038/ismej.2014.14

Robak, K., and Balcerek, M. (2020). Current state-of-the-art in ethanol production from lignocellulosic feedstocks," *Microbiol. Res.* 240, article ID 126534. DOI: 10.1016/j.micres.2020.126534

Robert, C., Del'Homme, C., and Bernalier-Donadille, A. (2001). "Interspecies H_2 transfer in cellulose degradation between fibrolytic bacteria and H_2-utilizing microorganisms from the human colon," *FEMS Microbiol. Lett.* 205(2), 209-214. DOI: 10.1111/j.1574-6968.2001.tb10949.x

Schuchmann, K., and Muller, V. (2014). "Autotrophy at the thermodynamic limit of life: A model for energy conservation in acetogenic bacteria," *Nat. Rev. Microbiol.* 12(12), 809-821. DOI: 10.1038/nrmicro3365

Schut, G. J., and Adams, M. W. W. (2009). "The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: A new perspective on anaerobic hydrogen production," *J. Bacteriol.* 191(13), 4451-4457. DOI: 10.1128/JB.01582-08

Smith, N. W., Shorten, P. R., Altermann, E. H., Roy, N. C., and McNabb, W. C. (2019). "Hydrogen cross-feeders of the human gastrointestinal tract," *Gut Microbes* 10(3), 270-288. DOI: 10.1080/19490976.2018.1546522

Søndergaard, D., Pedersen, C. N., and Greening, C. (2016). "HydDB: A web tool for hydrogenase classification and analysis," *Sci. Rep.* 6, article no. 34212. DOI: 10.1038/srep34212

Spirito, C. M., Richter, H., Rabaey, K., Stams, A. J. M., and Angenent, L. T. (2014). "Chain elongation in anaerobic reactor microbiomes to recover resources from waste," *Curr. Opin. Biotechnol.* 27, 115-122. DOI: 10.1016/j.copbio.2014.01.003

Steinbusch, K. J. J., Hamelers, H. V. M., Plugge, C. M., and Buisman, C. J. N. (2011). "Biological formation of caproate and caprylate from acetate: Fuel and chemical production from low grade biomass," *Energy Environ. Sci.* 4, 216-224.

Stursová, M., Zifčáková, L., Leigh, M. B., Burgess, R., and Baldrian, P. (2012). "Cellulose utilization in forest litter and soil: Identification of bacterial and fungal decomposers," *FEMS Microbiol. Ecol.* 80(3), 735-746. DOI: 10.1111/j.1574-6941.2012.01343.x

Sun, S., Weng, Y., Di, X., Liu, Z., and Yang, G. (2020). "Screening of cellulose-degrading fungi in forest litter and fungal effects on litter decomposition," *BioResources* 15(2), 2937-2946. DOI: 10.15376/biores.15.2.2937-2946

Thauer, R. K. (2015). "My lifelong passion for biochemistry and anaerobic microorganisms," *Annu. Rev. Microbiol.* 69, 1-30. DOI: 10.1146/annurev-micro-091014-104344

Thauer, R. K., Jungermann, K., and Decker, K. (1977). "Energy conservation in chemotrophic anaerobic bacteria," *Bacteriol. Rev.* 41(1), 100-180. DOI: 10.1128/br.41.1.100-180.1977

Weimer, P. J. (1996). "Why don't ruminal bacteria digest cellulose faster?," *J. Dairy Sci.* 79(8), 1496-1502. DOI: 10.3168/jds.S0022-0302(96)76509-8

Ze, X., Le Mougen, F., Duncan, S. H., Louis, P., and Flint, H. J. (2013). "Some are more equal than others: The role of "keystone" species in the degradation of recalcitrant substrates," *Gut Microbes* 4(3), 236-240. DOI: 10.4161/gmic.23998

Zheng, Y., Kahnt, J., Kwon, I. H., Mackie, R. I., and Thauer, R. K. (2014). "Hydrogen formation and its regulation in *Ruminococcus albus*: Involvement of an electron-bifurcating [FeFe]-hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative hydrogen-sensing [FeFe]-hydrogenase," *J. Bacteriol.* 196(22), 3840-3852. DOI: 10.1128/JB.02070-14

Article submitted: June 28, 2022; Peer review completed: August 21, 2022; Revised version received: September 8, 2022; Accepted: October 11, 2022; Published: October 25, 2022.
DOI: 10.15376/biores.17.4.6953-6969

APPENDIX

Supplementary Material

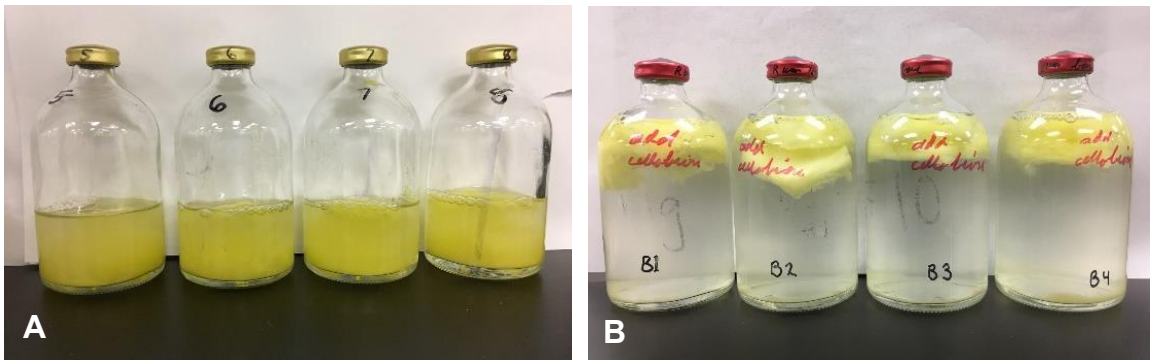

Fig. S1. Anaerobic cellulose degradation in *R. albus* cultures. Samples were incubated at 35 °C. *R. albus* produces a characteristic yellow pigment: **A.** Samples with 1:1 (normal) headspace at end of experiment; and **B**. Samples with low headspace at end of experiment.

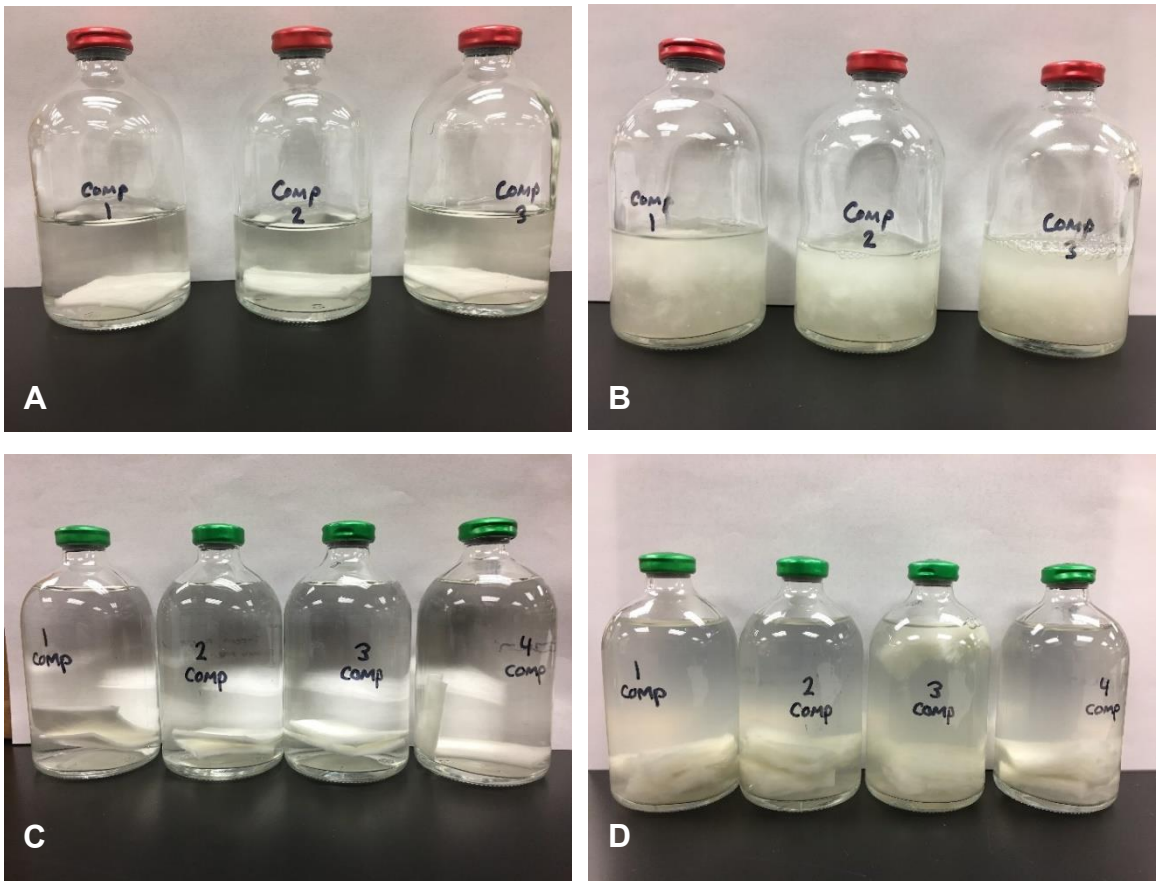

Fig. S2. Anaerobic cellulose degradation in samples derived from compost: Samples were incubated at 35 °C; **A.** samples with 1:1 headspace at beginning of experiment; **B.** samples with 1:1 headspace at end of experiment; **C.** samples with low headspace at beginning of experiment; and **D.** samples with low headspace at end of experiment.

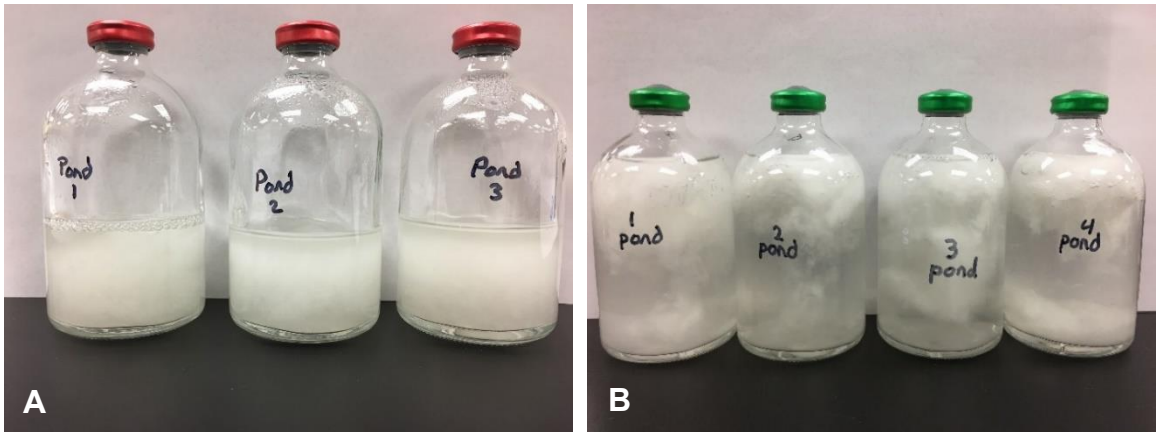

Fig. S3. Anaerobic cellulose degradation in samples derived from pond sediment: Samples were incubated at 35 °C; **A.** samples with 1:1 headspace at end of experiment; and **B.** samples with low headspace at end of experiment.

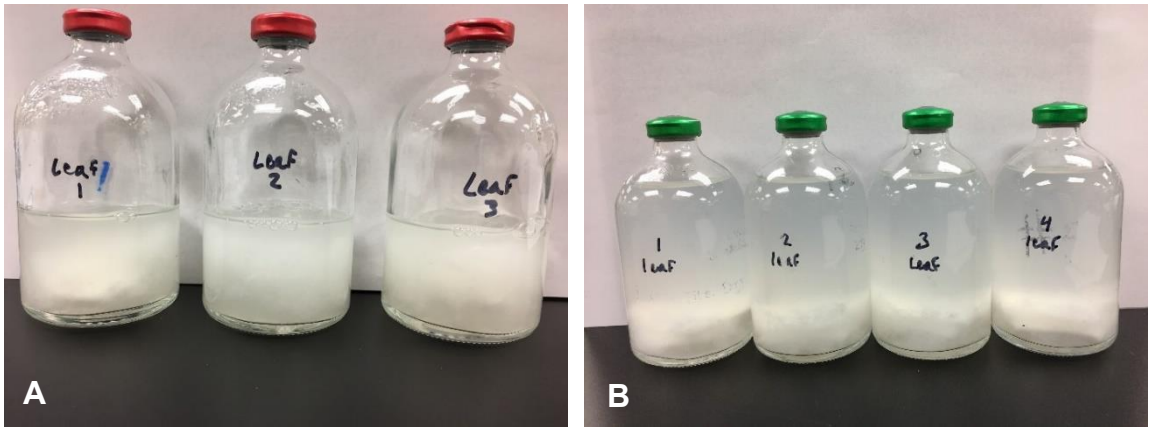

Fig. S4. Anaerobic cellulose degradation in samples derived from soil under leaf litter: Samples were incubated at 35 °C; **A.** samples with 1:1 headspace at end of experiment; and **B** samples with low headspace at end of experiment.

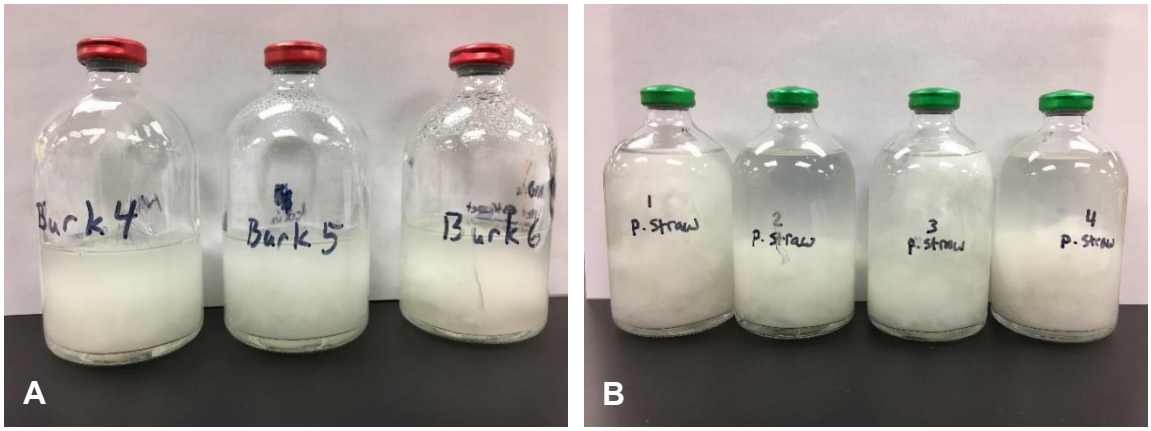

Fig. S5. Anaerobic cellulose degradation in samples derived from soil under pine straw: Samples were incubated at 35 °C; **A.** samples with 1:1 headspace at end of experiment; and **B.** samples with low headspace at end of experiment.