Fermentative metabolism of an anaerobic, thermophilic consortium on plant polymers and commercial paper samples

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ABSTRACT

The purpose of the study was to examine the feasibility and capacity of a thermophilic microbial consortium to produce fermentative metabolites from plant polymers. The consortium comprised of cellulolytic anaerobes that were originally enriched from a compost pile using cellulose as the substrate. Fermentative metabolism was examined with monosaccharides, disaccharides, hemicellulose, starch, pectin, chitin, and eight commercial paper samples without further enrichment of the culture to each specific substrate. In general, H₂, CH₄, CO₂, and organic acids were the main metabolites on all substrates but the metabolite profiles varied with the substrate. Similar H₂ yields of 2-3 mol mol⁻¹ substrate at 48 h were obtained with all monosaccharides and disaccharides. The CO₂ yields were higher with disaccharides than with monosaccharides, 4.5 vs 2 mol mol⁻¹ substrate. Metabolite yields were relatively low with glyceraldehyde, glycerol, and arabinose. Paper samples containing high amounts of chemical pulp produced the highest metabolite yields, and biodegradation accounted for ≤ 74% of total dry weight loss. The fermentative metabolism of the paper samples varied with the pulp composition and the amount of inorganic material. Bacterial community analysis using pyrosequencing analysis of 16S rRNA gene showed a predominance of members of the order Clostridiales, including members of genera *Clostridium* and *Lutispora*, which contain known cellulolytic organisms. Most differences among the samples were attributed to small taxonomic groups represented by ≤ 10% of total sequences.

Keywords: Anaerobic biodegradation, Biohydrogen, Cellulose biodegradation, Fermentation, Plant polymers

1. Introduction

Recent research into sustainable energy has focused on cellulose and cellulosic biomass as renewable resources. Agricultural and wood residues and other forms of plant biomass are complex mixtures of polymers that include cellulose, hemicelluloses, lignin, starch, and pectin. Cellulose microfibrils are buried within a multifaceted matrix that is covalently bound with other polymers [1-3]. The most readily biodegradable plant polysaccharide is starch, a polymer composed of glucose linked with α 1-4 and α 1-6 glycosidic bonds [4]. Hemicellulose is a heterogeneous substrate with a backbone of β 1-4 linked xylose with branches of pentoses and hexoses, including arabinose, mannose, and glucuronic acid. Some of the saccharides within the hemicellulose structure are modified through reactions such as esterification [5, 6]. Pectin, commonly present in fruit biomass, consists of an α 1-4 galacturonic acid backbone with side chains of fucose, xylose, and/or rhamnose with a high degree of methylation and acetylation [5, 7].

Residues containing plant polysaccharides have been shown to be good substrates for value-added products such as ethanol, methanol, methane, and biohydrogen [8-10]. Pulp and paper mills generate both liquid and solid residue streams that contain many biodegradable plant polysaccharides. Within US landfills, the paper and cardboard fraction has decreased from 38 to 28% of the total solid waste between 1999 and 2009 [11, 12]. Although recycling rates have increased, paper and other cellulose products still constitute a major fraction of municipal solid waste and could provide a large source of renewable feedstock for bioenergy purposes.

In paper and cellulose manufacturing, pulp is generated through mechanical, chemical, or combined processes. Mechanical pulping utilizes physical forces in the presence of water to strip fibers from wood and suspend the free fibers in water. This process recovers 90-95% of the

wood fiber but the pulp is of a low quality with hemicelluloses and recalcitrant lignin present [13, 14]. Chemical pulping utilizes either acidic, H_2SO_3 and HSO_3^- , or alkaline, NaOH and NaS_2 , chemicals along with heat and/or pressure to separate the fibers. While the pulp yield is 40-50%, much lower than that of mechanical pulping, the pulp is of higher quality as lignin and some hemicelluloses are dissolved during the chemical process [15]. After pulping, the slurry is diluted and mixed with the biologically inert mineral fillers necessary for mechanical strength and integrity before it is pressed into paper. Bleaching of the pulp prior to pressing is optional and likely affects the polymer characteristics. The paper can be surface coated, usually with conventional pigments (e.g., kaolin, CaCO_3 , TiO_2) or silica pigment and polyvinyl alcohol, to achieve the desired final paper quality, gloss, and void fraction of ink volume for printing. Different pulping and paper making processes can result in residue streams that vary in types and amounts of individual substrates, which can affect bacterial degradation and fermentation.

Paper products has been tested as a feedstock for simultaneous saccharification and fermentation to ethanol [16-18] or lactic acid [19-21]. Ntaikou and co-workers [22] tested paper biodegradation with *Ruminococcus albus*, a cellulolytic anaerobe, and reported a positive relationship between total carbohydrate content in the paper and high H_2 yields. To date, however, the composition of the pulp has not been considered in paper biodegradation studies.

In our previous study, we investigated cellulose biodegradation by a thermophilic, anaerobic consortium TC52 [23]. A subculture of TC52, which was maintained on cellulose at 60 °C, was previously found to contain mostly *Clostridium*, *Acetivibrio*, and *Thermoanaerobacter* spp. [23]. Because of the phylogenetic diversity of the TC52 consortium, it was hypothesized that the culture can use a broad spectrum of plant-derived complex substrates. In view of the capacity of TC52 to produce value-added metabolites from cellulose, the present study was undertaken to

examine the fermentative metabolism of TC52 with multiple carbon sources including monosaccharides, disaccharides, plant polysaccharides, and eight types of commercial paper. Pyrosequencing of 16S rRNA genes of representative samples was used to characterize changes in the makeup of the consortium during the degradation of the polysaccharides.

2. Materials and Methods

2.1. Substrates

Simple sugar substrates were $\geq 98\%$ pure forms of glyceraldehyde, glycerol, ribose, arabinose, xylose, glucose, galactose, fructose, mannose, lactose, sucrose, cellobiose, and maltose. Polysaccharides analyzed were chitin from crab shells and amylose and pectin from apples (Sigma-Aldrich, St. Louis, MO). A heterogeneous hemicellulose of unknown composition was obtained from a paper mill company (UPM Corp., Helsinki, Finland). The hemicellulose was homogenized with a mortar and pestle before use. Commercial paper samples were obtained from two paper mill companies (UPM and M-Real Corp., Helsinki, Finland). Information provided by the companies on the standardized paper composition is listed in Table 1. Stock paper samples were manually cut to approximately 0.5 x 0.5 cm for use as a substrate and designated as paper types A through H.

Table 1 here

2.2. Culture Conditions

The microbial consortium, designated TC52, originated from the interior of a compost heap through enrichment on cellulose as the sole substrate at 55 °C. It has been maintained on cellulose for years and used in two previous studies [24, 25]. The TC52 consortium was grown anaerobically in medium containing (per L) 2 g trypticase, 1 g yeast extract, 4 g Na₂CO₃, 0.23 g

122 K_2HPO_4 , 0.18 g KH_2PO_4 , 0.36 g NH_4Cl , 0.04 g NaCl , 0.09 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$,
 123 5.66 g acetic acid, 1.62 g propionic acid, 0.68 g *n*-butyric acid, 0.23 g isobutyric acid, 0.20 g
 124 isovaleric acid, 0.20 g *n*-valeric acid, 0.20 g 2-methyl-butyric acid, 0.001 g rezasurin, 0.25 g
 125 cysteine-HCl, 0.25 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, and 4 g microcrystalline cellulose (Sigmacell Type 20, Sigma-
 126 Aldrich).

127 Monosaccharides and disaccharides were prepared anaerobically as sterile 0.05 mol L^{-1} stock
 128 solutions and added as single substrates to anaerobic media following autoclaving to a final
 129 concentration of $5 \cdot 10^{-3} \text{ mol L}^{-1}$. Polysaccharides were added in one of two ways: (i) the substrate
 130 was added prior to autoclaving of medium (AC), or (ii) after the anaerobic medium was
 131 autoclaved (NAC). Paper stock samples precut to approximately 5 x 5 mm squares were added to
 132 sterile medium. All polysaccharide and paper substrates were tested in duplicate cultures at 4 g
 133 dm^{-3} . Cultures were inoculated with 48 h old TC52 (5 ml for each 100 ml culture) and incubated
 134 in serum bottles at 52°C at 3 Hz. All experimental conditions were sampled daily for the
 135 headspace gas composition. Liquid samples of cultures were taken every 48 h and centrifuged at
 136 $16,000 \text{ g}$ for 10 min. The supernatant and pellet were separated and stored at -20°C until further
 137 analysis.

138 **2.3. Headspace Gas Analysis**

139 Immediately after removal of serum bottle cultures from the incubator, overpressured
 140 headspace was vented into a sterile syringe for gas volume measurement. Samples of headspace
 141 gas (0.2 cm^3) were analyzed for gas composition using a GC-2014 Shimadzu gas chromatograph
 142 equipped with a thermal conductivity detector and a Porapak N (2 m length x 2 mm ID) column
 143 (Sigma-Aldrich). The carrier gas, N_2 , was maintained at $2 \text{ cm}^3 \text{ min}^{-1}$. Temperatures for the
 144 injector, detector, and column oven were 110 , 110 , and 80°C , respectively. Cumulative gas

amounts were calculated based on temperature and overpressure as previously described [26]. The chromatographs were analyzed with GC Solution Analysis software (Shimadzu, Kyoto, Japan).

2.4. Analysis of Short Chain Fatty Acids

Short chain fatty acids (SCFAs) were analyzed in sample supernatants following cleaning with solid phase extraction (C18-T) and filtration through a 0.2 μm PTFE filter (Pall, Port Washington, NY) as described previously [27].

2.5. Microbial Diversity Analysis

Bacterial cell biomass was recovered from 2 cm^3 culture at the end incubation by centrifuging at 16,000 g for 10 min. The cell pellets were then suspended in 0.3 cm^3 of 1X phosphate buffered saline. The RBB+C method was used as previously described [28] to extract DNA from the suspended pellets with minor modifications. The following changes were made to the extraction protocol in order to increase the extracted DNA concentration: the volume of elution buffer was reduced to 0.05 cm^3 and the elution step repeated by re-application of the initial elute to the column. The extracted DNA was checked on a 0.8% agarose gel and quantified in triplicate assays using the Quant-iT Broad Range dsDNA kit (Invitrogen, Carlsbad, CA).

Denaturing gradient gel electrophoresis (DGGE) was run using two sets of primers: EUB357f with a GC clamp and EUB519r for bacteria 16S rRNA genes and ARC344f with a GC clamp and EUB519r for archaeal 16S rRNA genes as described previously [28-30]. The PCR program was 94 $^{\circ}\text{C}$ for 4 min, 10 cycles of touchdown PCR (94 $^{\circ}\text{C}$ for 30 s, 61 $^{\circ}\text{C}$ with a decrease of 500 mK s^{-1} for 30 s, and 72 $^{\circ}\text{C}$ for 30 s), 25 cycles of PCR (94 $^{\circ}\text{C}$ for 30 s, 56 $^{\circ}\text{C}$ for 30 s, 72 $^{\circ}\text{C}$ for 30 s), with a final extension at 72 $^{\circ}\text{C}$ for 30 s to eliminate artificial double DGGE

bands [31]. PCR products were checked on a 1.5% agarose gel and quantified in triplicate with the Quant-IT kit (2-1000 ng, Invitrogen).

Prior to DGGE, bacterial PCR products were diluted to $2.5 \cdot 10^{-8}$ g μL^{-1} and approximately $3 \cdot 10^{-7}$ g was loaded per well. For archaeal PCR products, about $1.5 \cdot 10^{-7}$ g was loaded ng per well without dilution. Each sample was run twice to ensure reproducibility. The polyacrylamide gel (7.5%, 37.5:1 acrylamide:bis powder) was made with a denaturing gradient of 40-60% acrylamide (100% consisting of 40% (v v⁻¹) formamide and $7 \cdot 10^{-3}$ mol L⁻¹ urea) [28]. The gel was run at 60 °C with a program of 80 W A⁻¹ for 1 h and 160 W A-1 for 15 h in 0.5% TAE buffer in the INGENYphorU system (Ingeny, Netherlands). The gel was dyed with 2X SYBR Green I (Invitrogen) for 30 min with gentle rocking in the dark. Banding patterns were analyzed using Bionumerics software (Applied Maths, Austin, Texas) and similarity dendrograms determined with Dice and UPGMA algorithms.

For 454 pyrosequencing analysis, PCR amplicons encompassing the V1-V3 hypervariable regions of the 16S rRNA gene were generated using the genomic DNA extracts from duplicate TC52 cultures as described previously [32]. Sequencing was carried out at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign using the Roche 454 GS FLX Titanium sequencing kit. Returned sequences were processed and binned into operational taxonomic units (OTUs) at the 0.03 distance cut-off using QIIME v1.7.0. Chimeric sequences were eliminated using ChimeraSlayer in QIIME. Representative OTU sequences were compared to those of known species using RDP with a confidence cutoff of 0.80. Raw sequence information was deposited in the Sequence Read Archive (SRA) under accession ID SRX332554.

2.5. Assessment of Paper Degradation

After 17 days of growth of TC52 on the paper substrates, paper degradation was assessed using standard protocols for total suspended solids (TSS) and volatile suspended solids (VSS) [33].

3. Results and Discussion

The TC52 consortium was maintained on microcrystalline cellulose and a typical growth curve is shown in Figure 1. Growth on cellulose varied between subcultures with CH₄ present in some subcultures but not in others. An increase in the incubation temperature from 52 to 60 °C completely eliminated methanogenesis, and microbial cellulolytic activity was later found to be optimal at 60 °C [7]. Compared to other test substrates including polysaccharides, growth of the TC52 consortium with cellulose was much slower as the maximum CO₂ production was reached after 6 days. Unlike in the example shown in Figure 1, there were occurrences of more CH₄ production than H₂ after 5 days, with CO₂ accumulation occurring more slowly. In comparison, SCFA accumulation was similar as seen after 48 h of growth on all simple sugars. The final pH values were in the range of 6.4-6.8, similar to disaccharide conditions but somewhat lower than with other polysaccharides.

Figure 1 here

TC52 was not acclimated or subcultured on any of the substrates past the initial culture conditions presented here because optimizing the metabolite yields was outside the scope of this study. Overall, the most abundant fermentation products in the substrate experiments were H₂, CH₄, CO₂, and acetic and butyric acids although their relative proportions varied in some cases considerably.

3.1. Soluble Substrates: monosaccharides and disaccharides

TC52 readily adapted to each substrate, and their fermentative metabolism led to differences in gas and SCFA profiles even between duplicate samples. Thus the metabolite data, specifically with monosaccharides and disaccharides, varied greatly as apparent from standard errors (Figure 2). The cellulose-grown TC52 produced H_2 relatively fast, ≤ 24 h, and it accumulated until day 3 when TC52 switched to hydrogenotrophic methanogenesis. Most of the evolution of CO_2 took place in the first three days. The highest theoretical H_2 yields are between 2 and 4 mol mol⁻¹ substrate for butyric and acetic acid fermentation, respectively [8, 34, 35]. By day 5, the apparent yields for CH_4 and CO_2 were 0.5 and 4 mol mol⁻¹ substrate added, respectively. Similar gas yields occurred with all substrates except for glyceraldehyde, glycerol, and arabinose. Their gas yields were similar to background levels when the medium without substrate was tested (data not shown). At 48 h, the disaccharides yielded twice as much CO_2 but similar levels of H_2 as monosaccharides (Figure 2). This indicated that H_2 production was not directly dependent on saccharide concentration in the cultures.

Figure 2 here

SCFAs were analyzed on days 2, 4, and 6 and showed less variability than gas metabolites (Figure 3). Butyric acid was the predominant SCFA at 48 h, but by day 6 the concentration decreased by up to 50% in monosaccharide fed cultures. The disaccharide experiments yielded steady levels of butyric acid throughout the 7 day incubation, indicating a balance between acid production and further oxidation. Acetic acid concentrations increased over time with the highest levels on day 6, regardless of the substrate. This corresponded to the gradual increase in CO_2 throughout the incubation. The oxidation of butyric acid, indicated by decreasing concentrations, was often detected in TC52 cultures with low substrate concentrations (monosaccharides at 5-10⁻³ mol L⁻¹ concentration) or with poorly utilized substrates such as arabinose or glyceraldehyde.

The loss of butyric acid indicates either a regulatory mechanism of redox balance or a reaction to gas sparging which lead to low hydrogen partial pressure and consumption of butyric acid [36]. Acetate fermentation appeared to be the dominant metabolic pathway associated with H₂ production. Other SCFA concentrations remained similar across the time course. Comparable concentrations of metabolites were produced from common substrates such as glucose and the more recalcitrant pentoses such as ribose and xylose (Figures 2 and 3). TC52 grown on lactose showed the most growth according to high metabolite yields. Both lactose and maltose promoted high acetate and formate yields which correlated with high H₂ yields.

Figure 3 here

The pH and optical density at 660 nm were determined for each culture after 7 days of incubation. All monosaccharide cultures had final pH ≤ 7.0 , while disaccharide cultures reached lower pH values in the range of 6.3 and 6.8, with lactose being the lowest. Although the initial pH of the medium was 6.7, the fermentative metabolism in the disaccharide cultures yielded a higher acid concentration than in the monosaccharide-fed cultures. Microbial biomass in the disaccharide-fed cultures was twice as high as in the monosaccharide-fed cultures (OD₆₆₀ 0.228 - 0.311). These observations confirmed the gaseous metabolite results with more extensive growth in disaccharide-fed cultures.

3.2. Plant Polysaccharides

The metabolism of plant polysaccharides was assessed by monitoring changes in gas and SCFA production over time along with the pH of endpoint samples. The polymers analyzed in these experiments were chitin, pectin, starch, and heterogeneous hemicellulose in addition to cellulose. Chitin was included in this study because it is abundant biopolymer residue in prawn and crab industry with little beneficial application in biotechnology and sustainable energy area.

The effect of autoclaving of the plant polymer on the subsequent biodegradation by TC52 was also tested. Autoclaving has been shown to promote non-enzymatic Maillard reactions of polymers between sugars and amines, generating an irreversible ketoamine (Amadori) product [37]. In addition, autoclaving has been shown to release toxic degradation products from hemicellulose such as furfural and hydroxymethylfurfural [38, 39]. The effect of autoclaving was most prominent at 48 h (Figures 4 and 5). CH_4 and CO_2 yields from polymers were similar regardless of autoclaving; only the formation of H_2 was greatly affected by autoclaving. Without autoclaving (NAC) chitin, hemicelluloses, and starch yielded at least 50% more H_2 than the corresponding AC counterparts (Figure 4). While there clearly were differences in gaseous metabolites at 48 h, the SCFAs were similar between the AC and NAC cultures (Figure 5).

Figures 4 and 5 here

TC52 growth showed shorter lag times and yielded higher metabolite concentrations with polysaccharides (4 g dm^{-3}) in comparison to $5 \cdot 10^{-3} \text{ mol L}^{-1}$ simple sugars. Of specific interest were the CH_4 and CO_2 yields that were up to twice the amounts obtained with disaccharide substrates. The H_2 yields were $< 1 \cdot 10^{-3} \text{ mol g}^{-1}$ substrate added (Figures 2 and 4). All polysaccharides, except chitin, had lag times ≤ 1 day and higher metabolite yields as compared to cellulose. The chitin yields were comparable to those in the negative control with no added substrate (maximum CO_2 of $2.3 \cdot 10^{-4} \text{ mol}$ for no substrate, $2.2 \cdot 10^{-4} \pm 0.1 \cdot 10^{-4} \text{ mol}$ for chitin NAC, and $2.8 \cdot 10^{-4} \pm 0.2 \cdot 10^{-4} \text{ mol}$ for chitin AC). Lactate accumulated more with polymeric substrates than with simple sugars. In addition, starch fermentation resulted in approximately five times more formate than other substrates, about $5 \cdot 10^{-3} \text{ mol g}^{-1}$ substrate added. Otherwise, SCFA and gas products were similar with all polysaccharides.

The final pH was 7.6 for the chitin culture, confirming that it was a poor substrate for TC52. The final pH values were 5.2, 6.1, and 6.1 for starch, pectin, and hemicellulose cultures, respectively. The lower pH values indicate higher acid production in comparison to the cellulose-fed culture, pH 6.6. Thus, starch, pectin, and hemicellulose were good substrates for TC52, each showing different metabolic profiles without a preceding adaptation.

3.3. *Paper Substrates*

With all paper types, gas production was relatively rapid and the metabolite trends were comparable. As seen in Figure 6, the formation of H_2 peaked in many cases by 24 h at $3 \cdot 10^{-3}$ mol g^{-1} substrate while CH_4 production slowly peaked at 0.4 and $0.6 \cdot 10^{-3}$ mol g^{-1} substrate as H_2 was consumed. CO_2 levels peaked in the range of $1 \cdot 10^{-3}$ to $5 \cdot 10^{-3}$ mol g^{-1} substrate at around 120 h. Upon further incubation (≥ 17 days), some paper samples yielded up to $6 \cdot 10^{-3}$ mol CO_2 g^{-1} substrate. Paper H produced the highest H_2 and CO_2 yields at 72 h with $6 \cdot 10^{-3}$ and $9 \cdot 10^{-3}$ mol g^{-1} substrate, respectively. Moreover, a minimal amount of CH_4 was seen with papers G and H, allowing for high H_2 yields (Figure 6). The consumption of H_2 correlated with high levels of CH_4 in TC52 cultures that were fed other types of paper and substrates, indicating that a large fraction of methanogens were hydrogenotrophic.

Figure 6 here

As with other substrates, lactic, acetic, and butyric acids showed the most variation among the paper types (Figure 6). By 48 h, the concentrations of lactic acid either increased and usually correlated with high H_2 concentrations, or decreased as with substrates promoting hydrogenotrophic methanogenesis (Figure 6). By day 5, TC52 fed papers A, B, D, G, and H produced lactic acid but at lower concentrations than observed with microcrystalline cellulose. With extended incubation, the production of lactic acid leveled off. Butyric acid reached the

second highest SCFA concentration with yields between 2.5 and $3 \cdot 10^{-3}$ mol g⁻¹ substrate at 24 h. This was comparable to the yield of butyric acid of $2.5 \cdot 10^{-3}$ mol g⁻¹ cellulose. Regardless of the paper substrate, the concentration of butyric acid decreased with further incubation. Only the TC52 culture grown on paper H showed relatively steady butyrate levels at $2 \cdot 10^{-3}$ mol g⁻¹ substrate through 17 days of incubation. The most abundant SCFA, acetate, was present in similar amounts with all substrates, $2\text{-}3 \cdot 10^{-3}$ mol g⁻¹ substrate, as also seen with cellulose.

Time course experiments indicated that the majority of metabolic activity occurred within the first 5 days. Metabolic activity was consistent with the previous observations of acetic acid fermentation and butyric acid oxidation. Both acetic and butyric acid fermentation are known to be coupled with hydrogenases [8, 34, 35]. Since the concentrations of butyric acid decreased, H₂ production is mainly attributed to acetic acid fermentation. Previously, lactic acid bacteria were shown to inhibit H₂-producing organisms via excreted bacteriocins [40]. However, the explanation does not apply in this the case because high levels of lactic acid occurred simultaneously with high H₂ yields, demonstrating a unique feature of microbial interplay in the enriched TC52 consortium. Dissecting the interactions in the consortium could yield interesting information on metabolic dynamics.

3.4. Paper Composition and Degradation

Most paper samples lost their shape and construction within a day or two of incubation with TC52; only samples A and B remained intact throughout the experiment. Thus, TC52 was capable of degrading cellulose and hemicellulose polymers in bulk paper in spite of the presence of more recalcitrant and inert components. The biodegradation was measured by analyses of TSS and VSS following 17 days of incubation. The amount of mass lost (on dry wt basis) was determined by subtracting TSS from the total amount of paper added (4 kg m^{-3}). VSS

encompassed any solids that were volatile between 105 and 550 °C. This fraction includes residual cellulose, hemicellulose, recalcitrant polymers, metabolites, and microbial biomass. Inert residue is the material remaining following combustion at 550 °C, such as pigments and fillers and other inorganic fractions.

The inert fraction was the largest in paper type G, with F, C, E, H, B, and D having lesser amounts (Table 2). This trend can be correlated with the pigment and filler amounts in each paper type (Table 1). The cultures grown on each paper sample, except for A, were analyzed for residual substrate using the TSS-VSS method. The culture with paper type A could not be sampled because the culture/paper slurry was not homogenous. The VSS fraction includes microbial biomass and recalcitrant carbon polymers present in mechanical and recycled pulp. Mechanical pulp is known to contain high amounts of hemicelluloses and lignin. Paper types E, C, B, F, D, H, and G had decreasing amounts of VSS. Papers E, C, and B had high levels of mechanical pulp, 60, 56, and 41%, respectively, while F and D had 46 and 92% recycled fiber content, respectively. G and H both had approximately 10% VSS, which likely consisted of microbial biomass and residual polysaccharides. Other than paper types G and H, a large amount of potentially biodegradable material remained in the cultures even after 17 days of incubation, especially portions of mechanical pulp and recycled fiber.

Table 2 here

TC52 produced the highest metabolite yields with paper samples containing only chemical pulp, i.e., paper types G and H, which lacked lignin and other recalcitrant polymers. Chemical pulp has been treated to specifically remove lignin and brown, undesirable fibers present in plant biomass leaving mostly cellulose behind [13]. In a preliminary test of an H-like paper with print present, TC52 was able to degrade the paper around the ink, leaving the ink behind. In

accordance with the data on VSS and inert fractions, the loss of mass varied between the paper types, with the highest loss seen with sample H followed by papers G, D, B, F, C, and E (Figure 8). High levels of chemical pulp in papers H and G, 68 and 44%, correlated closely with the loss of mass, 74 and 52% (dry wt), respectively. Although the sample D contained no chemical pulp, it ranked the third most degraded by TC52. This paper type contained 92% recycled fiber that was partially biodegradable as indicated by the 40% loss of dry weight. However, on the basis of 47% VSS, a relatively large residual of recycled fiber persisted in the TC52 culture.

The metabolic patterns of the three most biodegradable papers (D, G, and H), were similar to those of cellulose-fed TC52 (Figure 7). The H_2 yields were highest in these three samples at 48 h, $2.5\text{--}3.5 \cdot 10^{-3} \text{ mol g}^{-1}$ substrate added. When the cultures were fed paper, CO_2 levels were higher, $1\text{--}2 \cdot 10^{-3} \text{ mol g}^{-1}$ substrate, than those with cellulose, $< 0.5 \cdot 10^{-3} \text{ mol g}^{-1}$ substrate. According to CO_2 accumulation, TC52 metabolism was faster and the yields higher on paper D, G, and H than with microcrystalline cellulose. TC52 produced more gas and acid metabolites on the most recalcitrant paper (A) than on microcrystalline cellulose. Samples G and H had negligible CH_4 production during the first 5 days. In contrast, paper D with high recycled fiber content was a substantial source of CH_4 production (Table 2).

Figure 7 here

Efficient biodegradation of paper as a substrate may be hindered by the presence of lignin, which can physically block cellulase from accessing the cellulose surface. Lignin, an aromatic polymer, is known for its recalcitrance to biodegradation [41, 42] and therefore chemical pulping, which eliminates lignin, produced a paper that was more readily biodegradable. Results of this study demonstrate that enrichment and selection pressure through substrate-temperature combination yielded a microbial consortium capable of metabolizing a wide range of potential

bioenergy feedstocks. While a pilot scale study was outside the scope of this study, a next step could be the optimization of bioprocess parameters for paper and other cellulose products and fibers in paper mill effluents. Optimization of chemical and physical pretreatments for bioprocessing of feedstocks of wood residues could also provide a possible avenue for TC52 to generate bioenergy.

3.5. Microbial Diversity

Bacterial DGGE banding patterns of 16S rRNA genes from TC52 cultures grown with monosaccharides and disaccharide indicated that a diverse consortium adapted to each test substrate (Figures S1-S3). The similarity between the patterns was 68% or higher. Duplicate cultures were not always similar to one another, which might reflect the stochastic nature of mixed microbial community. The most unusual banding pattern observed with arabinose as the substrate was only 59% similar to those observed with the sucrose and glucose sample. For archaea, the DGGE gels showed comparable banding pattern (100% similarity) regardless of substrate, indicating a consistent group of methanogens that were present even if CH₄ yields were absent.

16S rRNA genes from TC52 grown with polysaccharide was also analyzed through bacterial and archaeal DGGE. The results showed extensive change in the diversity of the TC52 consortium even though the similarity was $\geq 65\%$. Similar DGGE patterns were observed between autoclave and non-autoclave substrates and between the two duplicates on each substrate except for two exceptions, i.e., hemicellulose AC and starch NAC. Again, the archaeal 16S rRNA gene DGGE banding patterns were the same regardless of substrate or CH₄ production (data not shown).

The bacterial and archaeal communities in TC52 fed different paper types were also examined using DGGE. For bacteria, the DGGE banding patterns were similar for all but two (G and H) paper samples. Papers G and H resulted in a slightly different bacterial community that was 88% similarity to that of the other samples. These results showed that a base consortium was able to degrade all commercial paper samples. DGGE of archaeal PCR products revealed identical banding patterns. The archaeal methanogenic community was relatively simple and similar in the cultures regardless of CH₄ production.

To examine the effect of different substrates on the predominant bacterial populations in TC52, the V1-V3 region (about 500 bp long) of 16S rRNA genes was sequenced and compared among the cultures fed hemicelluloses, starch, and pectin, both AC and NAC. DNA extracted from the culture fed autoclaved starch did not produce the expected PCR amplicons and thus sequencing was not completed on these samples. The results of pyrosequencing and RDP classification of the sequences revealed a minimal level of diversity (Figure 9). From an initial 10,616 raw sequences, 6,649 passed initial quality tests and ChimeraSlayer identified 61 chimeric sequences, which were removed (Table S1). Pectin samples 1 and 2 had around 1000 sequences each while sample hemicellulose A had only 196 sequences. Due to unequal numbers of sequence among the samples, two rarefaction levels were tested, using 196 or 256 sequences (Table S1). For nearly all the samples, rarefaction using 196 sequences did not allow to capture the OTUs represented by the sequences so rarefaction using 256 sequences was used to compare the diversity. Samples Hemicellulose_1 and Paper G_2 had less than 256 sequence so they did not have rarefaction results. Hemicellulose_2 showed higher phylogenetic diversity (PD) than either pectin sample. Hemicellulose and pectin grown cultures contained 51-80% sequences classified to the order Clostridiales. Only one major difference between the two communities is

apparent; pectin samples had more sequences classified in the family Clostridiaceae than hemicellulose, 8-13% compared to 5%. All samples had a small subset of sequences, 1%, which could not be classified to any bacteria phyla. A strong subset of organisms belonging to at least the order Clostridiales, many also in family Clostridiaceae, contained the functional diversity to degrade hemicellulose and/or pectin [10]. Organisms in this order and family are well-known anaerobic cellulose degraders with the ability to degrade a variety of polysaccharides [10].

Figure 8 here

16S rRNA genes were sequenced for the cultures that were grown with the three most readily degraded papers (D, G, and H). While the 16S rRNA gene sequences for most paper samples were similar in phylogenetic diversity and observed species, one culture replicate (paper H_B sample) had low similarity of 0.849. Whether this was an outlier due to DNA extraction or sequence preparation stages cannot be determined. RDP classification showed several distinct differences between polysaccharide and paper fed cultures. Fewer of the sequences belonged to order Clostridiales, 39-48%. Up to 50% of 16S rRNA gene sequences from cultures grown with paper samples was assigned to family Lachnospiraceae, with many versatile polysaccharide degraders [10]. More sequences from paper grown cultures (2.7-7.5%) were only binned to kingdom bacteria as compared to TC52 grown with hemicellulose and pectin. Bacteria of the order Clostridiales were present, but unique organisms in the family Bacillaceae were only present in papers G and H and not D.

4. Conclusions

This study explores the phylogenetic diversity and biochemical capacity of the thermophilic consortium TC52, demonstrating its anaerobic metabolism of plant polymers, paper, mono- and disaccharides. Plant polymers are prospective for sustainable bioenergy production through

anaerobic bioconversions. The thermophilic consortium in this study has a core of anaerobic bacteria and archaea, with dominant members changing with the substrate. The molecular diagnostic analysis based on 16S rRNA gene sequencing suggested that methanogens persisted in the consortium even when CH₄ production was not detected. Anaerobes in the order Clostridiales dominated in the consortium, many members being known for cellulolytic capacities. Anaerobes in the families Lachnospiraceae and Bacillaceae were dominant in the consortium when cultured with the paper samples. Substrate induced selection was a key feature in changes in phylogenetic diversity when different polymers and carbohydrates were tested. The main metabolites measured in this study were H₂, CH₄, CO₂, and acetic and butyric acids, but the relative amounts and proportions varied with the substrate. CO₂ formation was taken to as a surrogate for measure of growth because the biomass in the slurries could be directly determined. This study illustrates the potential of anaerobic, thermophilic consortia for the degradation of a variety of polysaccharides and commercial paper samples for value-added products.

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Captions to Figures 1-8

Fig. 1. Growth curve of TC52 showing gas production over 7 days.

Fig. 2. Gas metabolite yields after 48 h of growth on various monosaccharides and disaccharides.

Fig. 3. SCFA metabolite yields after 48 h of growth on various monosaccharides and disaccharides.

Fig. 4. Gas metabolite yields after 48 h of growth on various AC and NAC polysaccharides.

Fig. 5. SCFA metabolite yields after 48 h of growth on various AC and NAC polysaccharides.

Fig. 6. Gas production over time by TC52 fed eight different paper samples.

Fig. 7. SCFA production over time by TC52 fed eight different paper samples.

Fig. 8. Relative abundance of select polysaccharide- and paper-fed TC52 samples and the taxonomic definitions at RDP confidence level of 0.8. A and C: hemicellulose- and pectin-fed TC52 diversity; B and D: paper-fed TC52 diversity; A and B: relative abundance of all phyla identified; C and D: relative abundance of phyla consisting of less than 10% of sequences.

TABLES

Table 1. Relative mass fractions (%) of the dry paper samples tested in this study. The number of digitals varies depending on the manufacturers' product information.

Paper Type	Mechanical Pulp	Chemical Pulp	Pulp from Recovered Fiber	Pigments and Fillers	Moisture	Binders
A	48.6	32.4	0	7.1	8.5	3.4
B	41.1	36	0	10.7	8.5	3.6
C	60	3	0	28	6	3
D	0	0	92	0	8	0
E	56	12	0	25	6	1
F	18	2	46	28	5	1
G	0	44	0	46	4	6
H	0	68	0	22	5	5

Table 2. Fractionation by weight of paper substrates after 17 days contact with TC52 cultures.

Each culture received paper at 4 g l⁻¹. Samples that were not homogenous on day 17 were eliminated from statistical analysis.

Paper type	Biodegraded		VSS		Inert Material	
	Average ± SD (g l ⁻¹)	Wt-% of Sample Added	Average ± SD (g l ⁻¹)	Wt-% of Sample Added	Average ± SD (g l ⁻¹)	Wt-% of Sample Added
A	0.17	4.3	3.43	85.8	0.39	9.8
B	0.82 ± 0.86	20.6	2.61 ± 0.62	65.2	0.57 ± 0.24	14.2
C	0.20	5.0	2.56	64.0	1.24	31.0
D	1.60 ± 0.24	40.0	1.89 ± 0.19	47.3	0.51 ± 0.05	12.7
E	0.15 ± 0.28	3.8	2.87 ± 0.19	71.7	0.98 ± 0.09	24.6
F	0.48 ± 0.00	12.1	2.11 ± 0.04	52.7	1.41 ± 0.04	35.2
G	2.06 ± 0.17	51.5	0.41 ± 0.06	10.3	1.53 ± 0.11	38.3
H	2.94 ± 0.15	73.6	0.43 ± 0.24	10.8	0.63 ± 0.09	15.6

FIGURES

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8