Application of Enzymatic and Bacterial Biodelignification Systems for Enhanced Breakdown of Model Lignocellulosic Wastes

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ABSTRACT

This paper explores the extent to which enzymatic and bacterial biodelignification systems can breakdown lignocellulose in model wastes to potentially enhance biogas generation. Two representative lignocellulosic wastes (newspaper and softwood) commonly found largely undegraded in old landfills were used. A fungal peroxidase (lignin peroxidase) enzyme and a recently isolated lignin-degrading bacterial strain (*Agrobacterium sp.*) were used. Tests were conducted in stirred bioreactors with methanogens from sewage sludge added to produce biogas from breakdown products. Addition of lignin peroxidase resulted in ~20% enhancement in cumulative methane produced in newspaper reactors. It had a negative effect on wood. *Agrobacterium sp.* strain enhanced biodegradation of both wood (~20% higher release of soluble organic carbon and enhanced breakdown) and newspaper (~2-fold biogas yield). The findings of this paper have important implications for enhanced breakdown in old landfills that are rich in these wastes, and anaerobic operations utilising lignocellulosic wastes for higher degradation efficiencies and biogas production.

KEYWORDS: Lignocellulose, peroxidase enzyme, bacterial biodelignification

1 INTRODUCTION

The rate of landfill biogas production is initially high as the easily degradable fraction is broken down, the rate then decreases with a subsequent long 'tail' of emissions typically observed. This happens due to the accumulation of waste fractions that are difficult for landfill microbiota to break down, i.e. lignocellulose-containing wastes (Barlaz et al., 1989). Moreover, these slow rates of gas generation after the easily-degradable matter has depleted are insufficient for energy generation or flaring, and so the biogas typically escapes into the atmosphere. As landfill biogas mainly comprises of methane and carbon dioxide, which are two major contributors to climate change (O'Dwyer et al., 2017), this is not a sustainable situation.

The recalcitrance of lignocellulosic wastes results from the presence of lignin, which is a complex heteropolymer and the second most abundant biopolymer in nature. Due to its very complex structure, including ether and C-C double bonds, phenolic monomers etc, most microorganisms found in landfills find it extremely difficult to breakdown this polymer (Bugg et al., 2011a; Cragg et al., 2015). Lignin also forms a 'glue'-like structure around other easily degradable matter (e.g. cellulose, hemicellulose) in lignocellulosic wastes to, in effect, protect against microbial attack, hence further decreasing the bioavailability of the biomass (Brandt et al., 2013).

Accelerating breakdown of lignin and subsequent methanogenesis in the waste body would help to confine methane emissions to a shorter period of higher methane concentration in biogas, allowing more of the landfill gas to be used for energy generation/stopping escape into the atmosphere and reducing the long 'tail' of low emissions. This would also allow more rapid stabilization of sites and so reduce long-term

management costs for operators. Lignocellulosic wastes are also diverted to anaerobic digestion (AD) plants for production of biogas (Wyman et al., 2017). However, the same problem of recalcitrance of these wastes to biodegradation makes it difficult to utilise their full potential in AD operations (Hassan et al., 2018). Again, accelerating the breakdown of lignocellulose could also offer better biogas recovery resulting in improved biogas yields from lignocellulosic waste.

This acceleration may be achieved by biotechnological methods. For instance, some microorganisms have evolved specifically to degrade lignin, with white-rot fungi being one of the few well-studied organisms (Bugg et al., 2011a). White-rot fungi produce extracellular peroxidase enzymes to break down lignin. However, the process is extremely slow due to slow fungal growth over the biomass (Bugg et al., 2011a; Bugg et al., 2011b; Cragg et al., 2015) and is limited in typical waste environments such as landfill due, for example, to a lack of oxygen (Geoffrey, 2003; Leonowicz et al., 1999). Some bacteria are also able to metabolise lignocellulose (Mathews et al., 2015; Rahmanpour et al., 2016; Xu et al., 2018) and have been shown to be more flexible in terms of the conditions under which they can operate (Bugg et al., 2011a; Bugg et al., 2011b; Cragg et al., 2015; Rashid et al., 2017).

Acceleration of breakdown of lignin-rich materials will require more rapid enzymatic activity, which can be addressed in two ways: (i) extracellular enzymes similar to those produced by white-rot fungi, which are available commercially, can be added to the system thereby eliminating the need to use the organism itself in biodelignification (Hettiaratchi et al., 2014; Hettiaratchi et al., 2015; Jayasinghe et al., 2011; Jayasinghe et al., 2014; Jayasinghe et al., 2013). Researchers have applied this technique of enzymatic

71 biodelignification with the prospect of enhanced recovery of chemicals/biogas with varying
72 degrees of success (Schroyen et al., 2017; Schroyen et al., 2014; Schroyen et al., 2015)
73 although not to realistic, complex waste materials such as wood or newspaper. (ii) a second
74 approach is the application of bacteria for delignification, this has received only very
75 limited attention but is gaining popularity and shows promise in enhancing the breakdown
76 of lignocellulosic wastes for various biotechnological applications (e.g. biogas generation,
77 bioethanol production, renewable chemicals) (Bugg et al., 2011a; Bugg et al., 2011b;
78 Mathews et al., 2015; Rashid et al., 2017). However, the application of this technique to
79 complex waste materials likely to be found in significant quantities in landfills is yet to
80 attract significant attention.
81 Many of the aforementioned studies have been carried out under highly-controlled
82 conditions (e.g. highly buffered systems) and with idealised materials (e.g. model lignin
83 molecules, dissolved pure kraft lignin). The question of how well these enzymes and
84 bacteria can breakdown lignocellulose in realistic waste materials found typically
85 undegraded in landfills, with the goal of enhancing gas production and hence stabilising the
86 waste mass is considered in this study.
87 The objective of this paper is to test the hypothesis that certain enzymes and bacteria can
88 break down lignocellulose in complex and realistic waste materials, found to be largely
89 undegraded in landfills (Ximenes et al., 2015; Ximenes et al., 2017; Ximenes et al., 2008),
90 and so enhance rates of biogas production. Conversion of breakdown products to biogas
91 through methanogenic activity (provided through addition of methanogen-rich sewage
92 sludge) is recorded as volume of gas produced, alongside key parameters of the system
93 (chemical oxygen demand, organic carbon, pH etc.). As part of the bacterial

biodelignification experiments, the hypothesis that the solid:liquid ratio might also play an important role in the ability of the *Agrobacterium sp.* strain to enhance lignocellulosic breakdown and subsequent methanogenesis is also tested.

2 MATERIALS & METHODS

Two waste materials are considered: newspaper (52 g/m^2 standard recycled paper) and softwood (kiln-dried Nordic redwood pine timber, high heartwood proportion). For particle-size reduction, prior to starting an experiment, approx. 200g of waste were fed to a Fritsch 55743 rotary knife mill (approx. feed size: 2 cm long squares for newspaper and cubes for wood) with a 2mm screen. For experiments requiring powdered wood, the knife-milled material that passed through the 2mm screen was collected and passed through a 0.15 mm sieve. These wastes were chosen to represent the range of lignocellulosic wastes found in MSW landfills, with newspaper containing a smaller amount of lignin in comparison to softwood which is very lignin-rich.

Sewage sludge from an anaerobic digester at a wastewater facility in Cardiff, was used as a source of methanogens (5.52% ± 0.002 dry solids of which 59.46% ± 0.023 volatile solids) in order to model the presence of methanogens in the landfill environment. Sewage sludge was used due to its reported relatively more homogeneous behaviour as an inoculum across the literature for anaerobic biodegradation in comparsion to landfill leachate, which varies much more widely around the world (Pearse et al., 2018). The sludge was sampled from the digester in three 5-litre high-density polyethylene jerrycans, immediately transferred to the lab and used to start the bioreactor experiments. For experiments where the bacteria was acclimatisation at 30℃ for three days prior to starting an experiment, the jerrycans were immediately taken to the lab from the site, transferred to an incubator

maintained at 30℃ and shaken manually twice a day to ensure homogenisation. For every experimental run, fresh sludge was sampled from the same site and sampling point.

Commercially available lignin peroxidase (Merck product code: 42603-10MG-F) was used in the enzyme delignification experiments. *Agrobacterium sp.* (GenBank accession JX872342, bacterial phylum α-*Proteobacteria*), supplied by T. Bugg (Warwick University, UK), which is a facultative anaerobic lignin degrader isolated from landfill soil that grows optimally at 30 ºC (Rashid et al., 2017) was used in the bacterial biodelignification experiments.

2.1 Bacterial Culture

The *Agrobacterium sp.* cultures were maintained on Luria-Bertani (LB) agar. The bacterium was cultured in LB broth at 30 ºC and the cultures were harvested (centrifugation for 10 min at 3394 rcf – relative centrifugal force) in the exponential phase according to its growth curve. The cultures were then washed with M9 mineral media (6.78g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4CL, 0.5 g/l NaCL) to remove any carbon from the LB broth, followed by centrifugation again and resuspension in M9 before addition to the bioreactors.

2.2 Experimental Design

Preliminary Small-scale Bacterial Biodelignification: To identify optimal conditions for later bioreactor experiments on wood a series of preliminary experiments were undertaken. This is particularly critical for wood due to the typically lengthy degradation time-scales (experiments lasting years in some studies) (Wang et al., 2013) and the lignin content of wood and its various sizes found undegraded in landfills (small cm-scale chips to m-scale blocks) (De la Cruz et al., 2013; Wang & Barlaz, 2016; Ximenes et al., 2017; Ximenes et al., 2018). Since wood contains roughly 1.5 times more lignin than newspaper and is

140 unprocessed lignocellulose, where the structure is intact, it represents some of the most
141 difficult to degrade wastes in landfills and should prove to be a more challenging substrate
142 for biodegradation. To get an understanding of the impact of the physical state of the wood
143 on biodegradation by *Agrobacterium sp.*, small-scale tests in 50 ml sterile tubes were
144 conducted.

145 Four wood sample types were tested in these experiments exploring the impact of waste
146 form/size and accessibility of biodegradable materials. Each consisted of one particle size
147 range (<2 mm or <0.15 mm) and was either tested as-is or autoclaved prior to the
148 experiment. In the latter case wood was autoclaved at 120℃ for 15 mins to test its effect on
149 deconstructing the lignocellulosic matrix for easier microbial access to key polymers, i.e.
150 lignin, cellulose and hemicellulose (Pecorini et al., 2016). Each type was then tested with
151 and without *Agrobacterium sp.* in duplicate specimens. Each specimen comprised 1 g of
152 wood added to 20 ml of M9 mineral medium in 50 ml tubes with either 1 ml of
153 *Agrobacterium sp.* starter culture or 1 ml sterile M9 medium (control) (Rashid et al., 2017).

154 Flasks were placed in a shaking incubator at 200 rpm and 30℃ for seven days. For these
155 small-scale tests, sludge was absent since the purpose was to test the ability of this strain to
156 breakdown lignocellulose, whilst the presence of other microbial communities from the
157 sludge could interfere with the monitoring of the activity of only this strain. Total carbon
158 released into the liquid phase during the test and the organic carbon content of the solid
159 residue after bacterial treatment were analysed. Biogas production, pH and chemical
160 oxygen demand were not monitored in these preliminary small-scale tests.

161 Bioreactor System: The experimental apparatus (supplied by Anaero Technology UK)
162 consisted of 15 one-litre reactors submerged in a water bath maintained at the required

163 temperature. Gas flow meters based on the water displacement method (Wickham et al.,
164 2016) combined with an Arduino (for data logging) were used to monitor biogas
165 production. Biogas was collected in 5 litre Tedlar® bags attached to the outlets of the gas
166 flow meter for each reactor, and all biogas data are reported at STP. The reactors were
167 continuously stirred at 45 rpm during the experimental work and the duration of the
168 experiments was between 30-35 days.

169 Bioreactor-scale Bacterial Biodelignification (Experiment 1): Mechanistic insights from
170 the preliminary small-scale tests were used to inform the design of larger-scale 1 L (total
171 volume) tests. The rationale behind larger-scale experiments was to be able to study
172 whether enhanced biodegradation could be carried out using the *Agrobacterium sp.* whilst
173 monitoring for key variables such as biogas production, release of organic carbon etc, all of
174 this done in the presence of methanogenic microbial communities from sewage sludge.
175 These bacterial experiments contained 4g of waste, and employed a mixture of sludge and
176 M9 minimal media (for the agrobacterium sp. (Rashid et al., 2017)).

177 Each bioreactor comprised 600 ml M9 medium (650 ml in controls), 50 ml suspension
178 of *Agrobacterium sp.* (0 ml in controls), 50 ml methanogen-rich sewage sludge
179 (acclimatised at 30 ºC for three days prior to introduction) and 4g of lignocellulosic
180 material (wood or newspaper), together with 300 ml of headspace. The headspace
181 comprised of air due to *Agrobacterium sp.* being a facultative anaerobe, i.e. able to work
182 under anaerobic conditions but preferring aerobic conditions (Rashid et al., 2017; Taylor et
183 al., 2012). This reflects typical conditions in bioreactor landfills, where the waste mass is
184 initially aerobic and the system slowly goes anaerobic with the passage of time (Benson et
185 al., 2007). Five cases were tested (Table 1), each in triplicate, with *Agrobacterium sp.*

186 applied to vessels containing wood (<0.15 mm, non-autoclaved), newspaper and no waste,
187 whilst control vessels with only methanogens (no *Agrobacterium*) were supplied with wood
188 or newspaper. All bioreactors were incubated in the water bath apparatus at 30 ºC for 35
189 days. Liquid samples (5 ml) were taken periodically for analysis (section 2.3) from the
190 sampling port using sterile pipettes and transferred to 15 ml sterile containers. Solid residue
191 was obtained from all bioreactors by collection on Whatman No. 42 ® filter paper (2.5 µm)
192 via vacuum filtration then drying at 105 ºC. Biogas collected during the experiment in 5
193 litre Tedlar gas bags was analysed for methane content.
194 Progressively diminishing rates of biogas and organic carbon release for newspaper
195 reactors prompted a 'restarting' of the experiment, where the contents of the bioreactors
196 were centrifuged at 3394 rcf for 10 min under aseptic conditions to remove the supernatant
197 and fresh autoclaved M9 was added in the same amounts as the beginning of the test (see
198 above). This allowed for the hypothesis that towards the end of the experiment (whilst there
199 is still carbon present in the liquid phase but gas production has almost stopped),
200 accumulation of recalcitrant or toxic substances in the liquid phase inhibits further
201 conversion/breakdown to biogas to be tested. 'Restarting'/removing the liquid and
202 introducing a fresh medium could be beneficial and allow for biogas production to continue
203 again from the left-over solid residue. 'Restarted' bioreactors were incubated under the
204 same conditions for 20 additional days, with liquid and gas sampling.
205 Impact of Solid:Liquid Ratio on Bacterial Biodelignification (Experiment 2): Previous
206 work has shown that microbial activity is affected by the waste to inoculum ratio (with
207 particularly low ratios shown to inhibit methanogenesis) (Moset et al., 2015). As mentioned
208 previously, to test the hypothesis that the solid:liquid ratio might also play an important role

209 in the ability of the *Agrobacterium sp.* to breakdown lignocellulose, the impact of the
210 Solid:Liquid (S:L) ratio on bacterial biodelignification of these wastes was also studied by
211 increasing the mass of waste added to 12 g. The experiments (Table 1) are otherwise the
212 same as the earlier bioreactor-scale tests, apart from there not being a 'waste-free' control
213 triplicate. A 4 g test (for wood and newspaper respectively) was also carried out as a repeat
214 of that in the previous experiment to allow comparability and to serve as controls to test the
215 above hypothesis.

216 Bioreactor-scale Enzymatic Biodelignification (Experiment 3):. 1 L (total volume) tests
217 to study whether enhanced biodegradation could be achieved using lignin peroxidase.
218 Treatments are summarised in Table 1. 4 g of waste ≤2 mm, i.e. newspaper or wood (non-
219 autoclaved), and 2.22 mg of lignin peroxidase (LiP) were added to 600 ml of sludge (source
220 of methanogens) for a volatile solids ratio of sludge to waste of 4:1 (Labatut et al., 2011).
221 The reason for a much larger sludge volume here pertains to the fact that inoculum to
222 substrate ratio is a major factor in the determination of the biomethane potential of a
223 substrate (Raposo et al., 2006), hence the enzyme experiments were conducted at the ideal
224 volatile solids ratio of the sludge to the waste based on previous studies (Moset et al., 2015;
225 Peña Contreras et al., 2018). The chosen waste:LiP mass ratio was based on previous work
226 (Hettiaratchi et al., 2014; Hettiaratchi et al., 2015; Jayasinghe et al., 2011; Jayasinghe et al.,
227 2014; Jayasinghe et al., 2013) where the volatile solids (VS):LiP ratio was optimised for
228 maximum biogas production. LiP was activated with H_2O_2 prior to addition at the optimal
229 LiP: H_2O_2 ratio (Hettiaratchi et al., 2014; Jayasinghe et al., 2011; Jayasinghe et al., 2013).
230 The controls comprised of exactly the same experimental setup but lacked the peroxidase
231 enzyme in 'waste-containing' controls. 'Waste-free' controls were also carried out with 0

232 and 2.22 mg of LiP. All bioreactors were incubated at 38 ºC, as this is the optimal
233 operational temperature employed by the anaerobic digester used as the source of
234 methanogens.

235 **Table 1.** Conditions tested in the three main experiments. Numbers refer to number of replicates for each treatment.

	Experiment 1		Experiment 2		Experiment 3	
	With *Agrobacterium*	**Without *Agrobacterium***	**With *Agrobacterium***	**Without *Agrobacterium***	**With enzyme***	**Without enzyme***
No waste	3				3	2
Newspaper (4 g)	3	3	3		3	2
Newspaper (12 g)			3			
Wood (4 g)	3	3	3		3	2
Wood (12 g)			3			

*Note: Wood sample size < 0.15 mm, apart from samples denoted with * which had size < 2mm. Other experimental conditions are as described in the text.*

236 **Table 2.** Waste Characterisation.

Sample	**VS (%)**	**OC (%)**	**Lignin (%)**
Wood	99.62 ± 0.00	50.60 ± 0.40	38.45 ± 0.13
Newspaper	84.26 ± 0.00	36.72 ± 8.09	25.73 ± 0.89

237 *Note: Data presented as percentage of dry mass.*

2.3 Analytical Methods

Liquids: All 5 ml liquid samples taken during bioreactor experiments were filtered through sterile 0.2 μm filters prior to analysis. The pH was measured on the entire sample post-filtration according to standard methods (APHA, 2012). The Folin-Ciocalteau method was used for measuring phenol release (Meda et al., 2005; Rashid et al., 2017). 0.8 ml of deionized water and 0.5 ml Folin-Ciocalteau's reagent (Merck F9252) were added to 0.2 ml filtered liquid sample. 2.5 ml of 20% Na_2CO_3 was then added and the samples incubated in the dark for 30 min. Then the absorbance was measured using a spectrophotometer at 760 nm and p-hydroxybenzoic acid was used to calibrate the absorbance as a standard. A 1 ml portion of the filtered sample was used for soluble organic carbon (sOC) analyses *via* a Shimadzu TOC-VCPH following the manufacturer's instructions whilst 0.5 ml was used for total and soluble chemical oxygen demand (COD and sCOD) analyses and 1ml was used for determining total/dry solids (TS and DS) for sludge characterisation all according to standard methods (APHA, 2012).

Solids: Lignin content and solid organic carbon analyses were carried out on the solid residue collected post-experiment. Lignin content analysis on the solid residue was adapted from Rashid et al., 2017. The samples were dried at 105℃ until constant weight, then 0.25 g of dry mass was added to 3.75 ml of 95% H_2SO_4 with stirring for 2 h at RTP. Then 140 ml of deionised water was added to the resulting solution followed by reflux for 4 h in round bottom flasks. The residual content was collected via Whatman No. 42 ® filter paper (2.5 μm) and washed with deionised water. Then the residue was dried at 105℃ until constant weight. The residue was then volatilised at 550℃ to correct for ash. The lignin content was determined by subtracting the mass of ash from that of the dry residue and then

calculating the ratio of this to the original sample dry mass. The solid total/organic carbon (TC/OC) were measured via a Shimadzu TOC-VCPH following the manufacturer's instructions. Volatile solids (VS) analysis was carried out according to standard methods.

Gases: The methane concentration of the biogas was determined via gas chromatography (GC) analysis on a Varian 450 GC equipped with a flame ionisation detector and a methaniser using a CP SiL5 CB column (50m, 0.33mm diameter, He carrier gas).

2.4 Statistical Analyses

Two-way ANOVA and Student's T-test were carried out. Statistical significance was attributed at $P<0.05$ (i.e. ANOVA F-value >F-critical). Correlations between data (specifically for the sCOD and sOC values) were identified via Pearson correlation initially and plotted against each other to further study the correlations.

3 RESULTS & DISCUSSION

3.1 Waste Characterisation

The composition of the model wastes is shown In Table 2. Volatile solids content of a substrate indicates the fraction that has the potential to be converted to biogas (Barlaz et al., 1989; Eleazer et al., 1997; Wang et al., 1994). The wood was almost entirely composed of volatile matter, whereas newspaper had some ash content. This is comparable to recent work studying the chemical composition of different wastes (Chickering et al., 2018; Krause et al., 2017; Krause et al., 2016). As the aforementioned studies also suggested, ash content likely comes from fillers and ink constituents within the newspaper. The wood was very rich in lignin and also has a higher organic carbon content compared to the newspaper. Bearing in mind the variability in waste composition that arises around the world, in

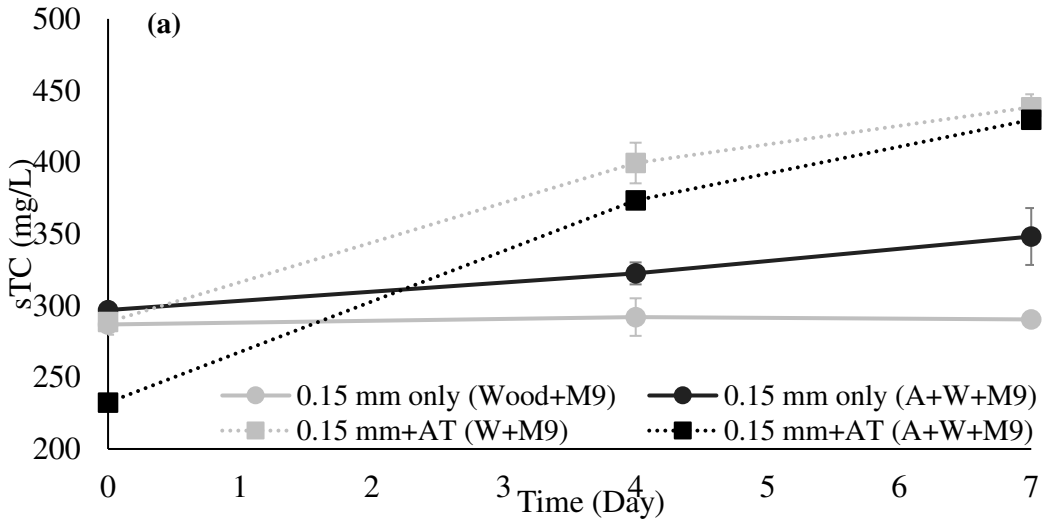
relation to recent work, these values fall within the typical ranges reported (De la Cruz et al., 2014; Wang & Barlaz, 2016).

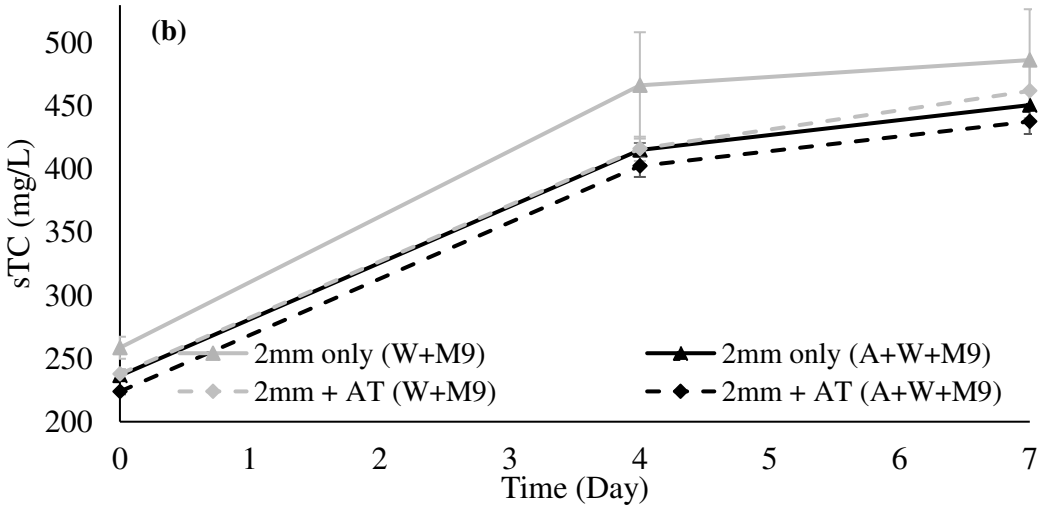
3.2 Bacterial Enhancement

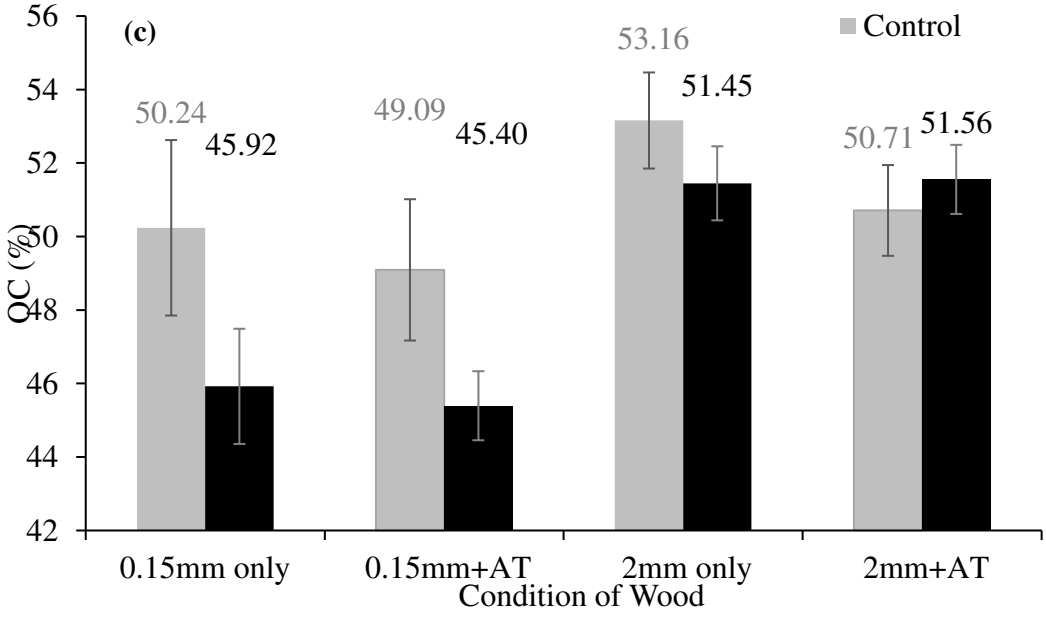
Small-scale: Total carbon release profiles from the preliminary small-scale experiments conducted on the non-autoclaved and autoclaved ≤0.15 mm and ≤2mm wood are shown in Figures 1a and 1b. In the presence of the *Agrobacterium sp.*, a steady and significant increase in the carbon present in the liquid phase occurred for the non-autoclaved ≤0.15 mm samples (black circle, Fig. 1a)) (~20% relative to the control, $P<0.05$, ANOVA F-values>F-critical) during the 7-day experiment. Since the only source of carbon in these experiments was lignocellulose from wood, and the soluble carbon content was determined post-filtration through 0.2 µm pore-size filters (even bacterial cells should not be passing through into the supernatant), it is highly likely that the release of carbon corresponds to biodegradation due to the bacterium. The profiles for the non-autoclaved <0.15 mm wood controls (grey circle, Fig. 1a)) remain steady and virtually unchanged, suggestive of no activity in the absence of the bacterium. No significant impact on the sTC release profiles due to the Agrobacterium *sp.* on 2 mm particles (Fig. 1b, autoclaved and non-autoclaved) was recorded ($P>0.05$, ANOVA F-values<F-critical). The sTC profile in the presence of the *Agrobacterium sp.* is consistently lower for 2 mm samples (non-autoclaved and autoclaved, dotted lines denote bacterium augmentation), which could be explained by the bacterium metabolising leached carbon from the wood.

It is noted that the <0.15 mm autoclaved wood containing the *Agrobacterium sp.* (black dotted line, Fig. 1a) starts at a lower sTC on day 0 (black dotted line, Fig. 1a), however, the increase in total sTC released in solution is ~30% higher in comparison to the increase

306 found in the control, hence indicating lignocellulosic breakdown of the solid matrix due to
307 bacterial enhancement.
308 To confirm the liquid phase carbon release results, the organic carbon analysis of the
309 solid residue post-treatment with the *Agrobacterium sp.* for wood under different
310 conditions is shown in Figure 1c. The organic carbon in ≤0.15 mm samples is significantly
311 lower (two-way ANOVA, $P<0.05$, F-values>F-critical) in the treated samples for both
312 autoclaved and non-autoclaved wood samples (by 8.6 and 7.5% respectively), indicating
313 higher levels of breakdown of the lignocellulosic structure due to *Agrobacterium sp.*
314 treatment. With 2 mm wood there is no significant difference due to bacterial enhancement.
315 It is expected that the much higher surface area to volume ratio, and accessibility of the
316 <0.15 mm samples is the key cause of the much better solid OC breakdown due to
317 *Agrobacterium sp*. treatment in this set of samples in comparison to the 2mm size.
318 Overall, the preliminary small-scale data suggest that *Agrobacterium sp.* can break down
319 woody lignocellulose, and that the particle size is an important factor in the rate of reaction
320 when applying this *Agrobacterium sp.* strain for biodegradation. Treating wood by
321 autoclaving does not significantly impact the biodelignification ability of this bacterium.


(a)
sTC (mg/L)
Time (Day)
0.15 mm only (Wood+M9)
0.15 mm only (A+W+M9)
0.15 mm+AT (W+M9)
0.15 mm+AT (A+W+M9)


(b)
sTC (mg/L)
Time (Day)
2mm only (W+M9)
2mm only (A+W+M9)
2mm + AT (W+M9)
2mm + AT (A+W+M9)

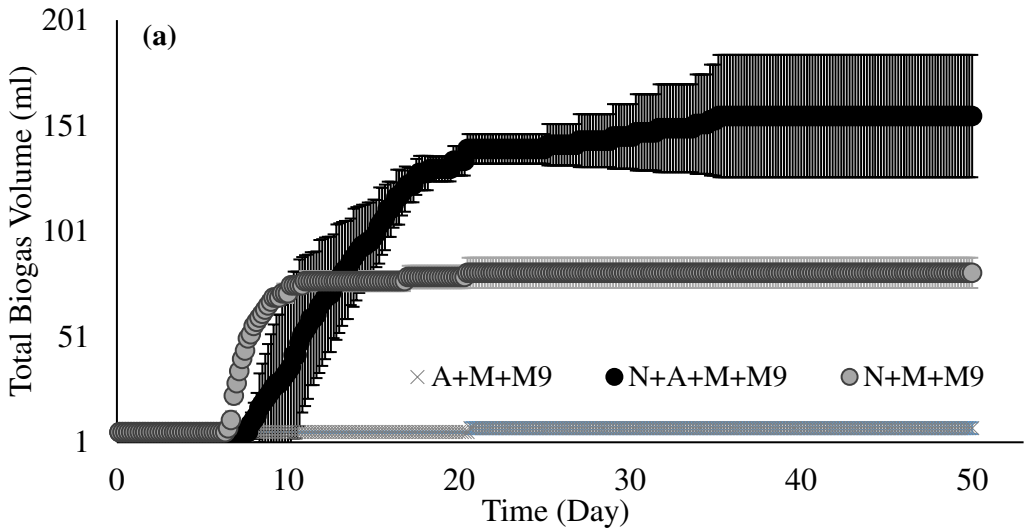

(c)
Control
QC (%)
50.24
45.92
49.09
45.40
53.16
51.45
50.71
51.56
0.15mm only
0.15mm+AT
2mm only
2mm+AT
Condition of Wood

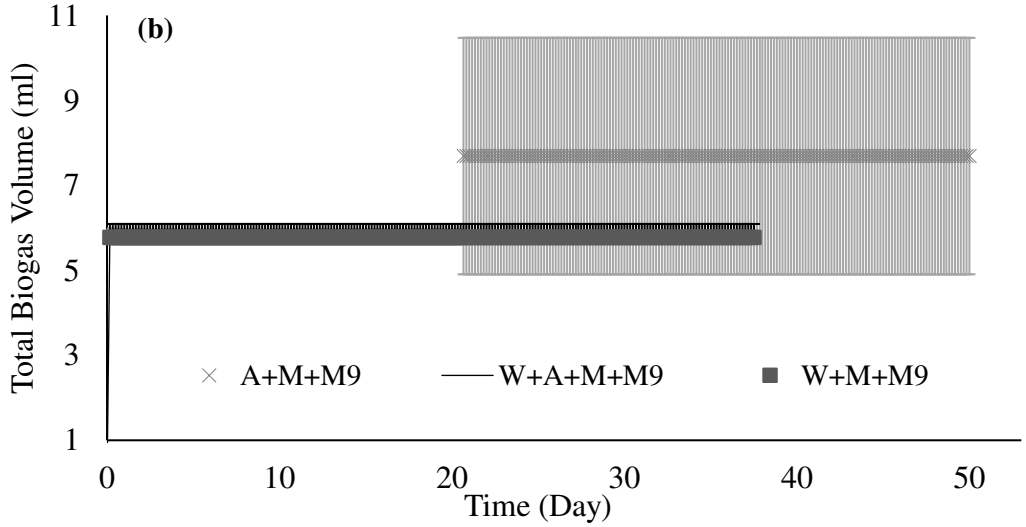
322

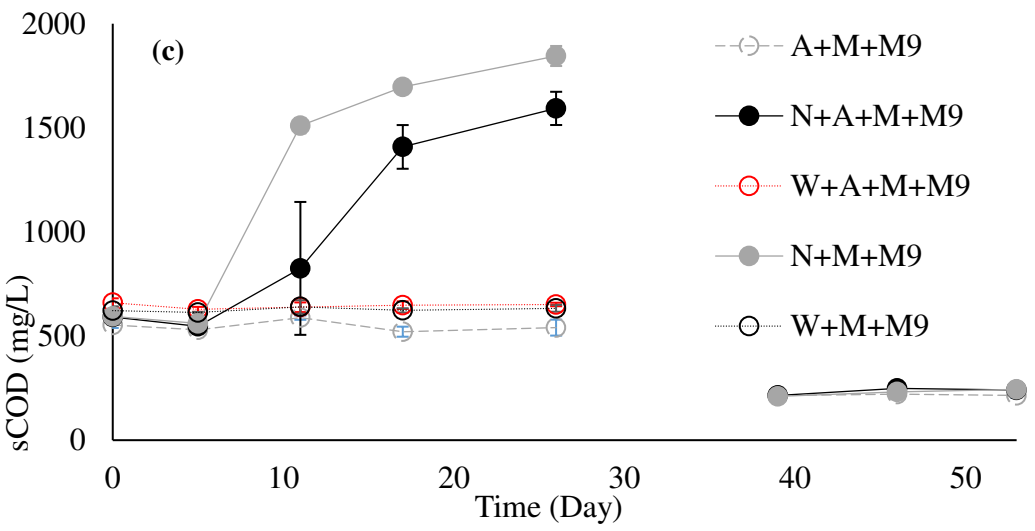
Figure 1. Assessing the impact of Agrobacterium sp. treatment on wood lignocellulose under different conditions. (a) Soluble liquid total carbon release results for the small-scale 0.15 mm tests. (b) Soluble liquid total carbon release results for the small-scale 2mm tests. (c) Solid phase organic carbon results for the small-scale experiments. All error bars represent +/- 1 standard deviation. Abbreviations: Agrobacterium, A; Autoclaved, AT; Wood, W; M9 solution, M9.

Bioreactor-scale (Experiment 1): The cumulative biogas profiles released from newspaper in Experiment 1 are shown in Figure 2a. After a lag-phase of ~7-8 days, *Agrobacterium sp.* nearly doubled the biogas production in newspaper reactors compared to controls without *Agrobacterium* ~92% enhancement, $P<0.05$) whilst no response was observed without waste materials. At the same time, the sCOD and sOC profiles (Figure 2c, 2d) exhibit a consistently lower amount of organic carbon in solution with newspaper and *Agrobacterium sp.* than in newspaper controls. These data suggest that the *Agrobacterium sp.* enhance the release of carbon from the solid phase and increase the conversion of dissolved carbon to biogas. In controls with newspaper, the total amount of OC released into solution is ~0.32 g whilst the OC released in total as biogas is 0.04 g, giving a total of ~0.36 g. In specimens with *Agrobacterium sp.* and newspaper, ~0.31 g of OC was released into solution by the end of the experiment, but the amount of biogas was 0.08 g, giving a total of ~0.39 g. These data indicate greater release of solid organic carbon into solution and greater conversion of that soluble OC to biogas in the presence of *Agrobacterium sp.*, leaving less in solution. With 1.4 g of solid OC present initially, in the presence of the *Agrobacterium sp*. there is an ~8.2% increase in the total release of OC in relation to the

345 control. The observed effects most likely result from the *Agrobacterium sp.* breaking down
346 either solid phase or leached organic materials into a form utilisable by the methanogens.
347 For the newspaper reactors, the sOC and sCOD profiles increase gradually with
348 increasing biogas production and result in overall accumulation towards the end of the
349 experiment of organic carbon in the liquid phase. It is hypothesised that the cause was
350 accumulation of recalcitrant organics and other degradation products which are not
351 converted to the gas phase and which hinder the activity of the microorganisms. Therefore,
352 an attempt was made to 'restart' the experiments by replacing the liquid fraction with fresh
353 autoclaved M9 on Day 35. Following this, however, little to no additional activity was
354 observed (Figure 2). As such, the flat-lining of the biogas and sCOD/sOC curves is likely to
355 be due to a different limiting factor.
356 No significant effect was observed on biogas production due to the addition of the
357 *Agrobacterium sp.* strain to wood (Figure 2b). Similarly, no significant increase in sCOD or
358 sOC was observed in this experiment. A possible explanation for this, and an important
359 difference between the preliminary small-scale and bioreactor experiment 1 is the
360 concentration of wood in the overall volume. For the small-scale wood biodegradation
361 tests, the S:L ratio was 0.05 g/ml, and a significant impact of bacterial treatment on the
362 woody lignocellulose was observed, whereas here it was 0.006 g/ml (~8.3 times more
363 dilute). It is possible that the wood was not sufficiently concentrated in the bioreactors to
364 either observe a measurable response or to stimulate microbial activity.


(a)
Total Biogas Volume (ml)
201
151
101
51
1
0
10
20
30
40
50
Time (Day)
A+M+M9
N+A+M+M9
N+M+M9


(b)
Total Biogas Volume (ml)
11
9
7
5
3
1
0
10
20
30
40
50
Time (Day)
A+M+M9
W+A+M+M9
W+M+M9


(c)
sCOD (mg/L)
2000
1500
1000
500
0
0
10
20
30
40
50
Time (Day)
A+M+M9
N+A+M+M9
W+A+M+M9
N+M+M9
W+M+M9

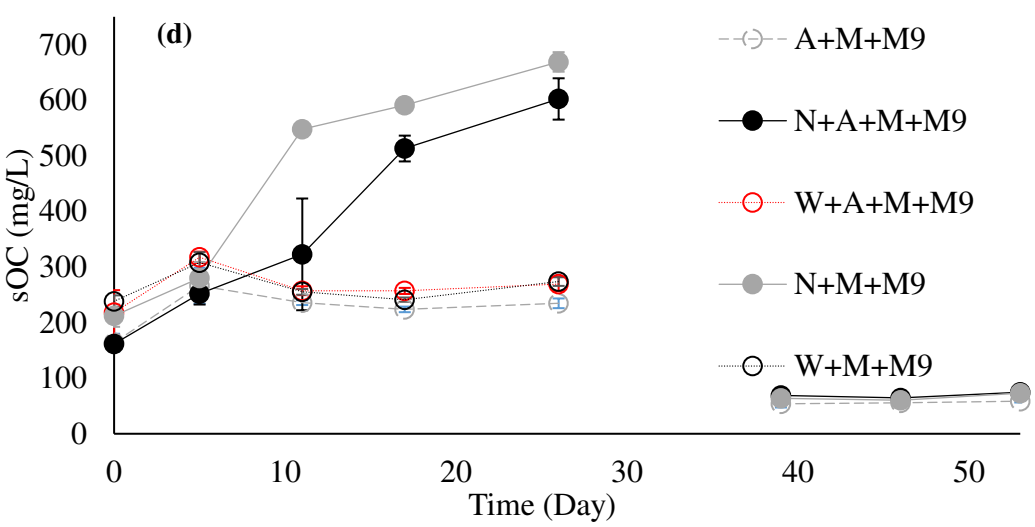
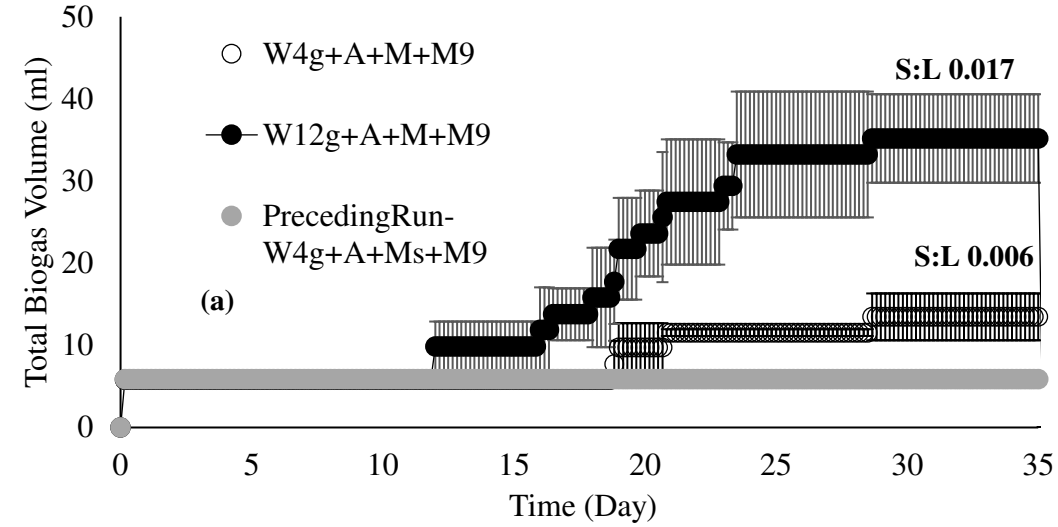

(d)
sOC (mg/L)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
Time (Day)
A+M+M9
N+A+M+M9
W+A+M+M9
N+M+M9
W+M+M9

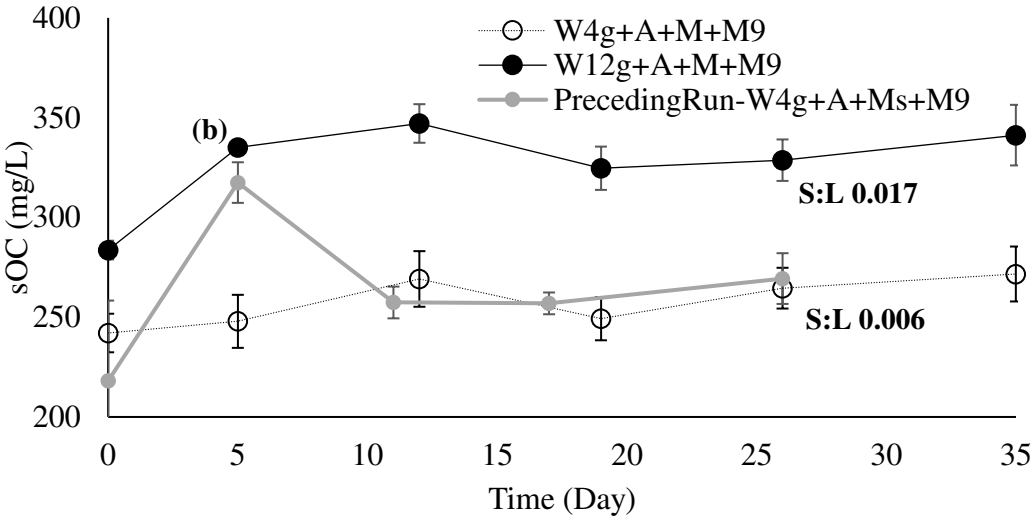
Figure 2. Assessing the impact of Agrobacterium sp. treatment on newspaper and wood lignocellulose in the presence of sludge. (a) Cumulative biogas volume results for newspaper. (b) Cumulative biogas volume results for wood. (c) Soluble chemical oxygen demand profiles. (d) Soluble organic carbon profiles. Note: system 'restarted' on Day 35, last liquid sampling point was at day 26 before the 'restart'. All error bars represent +/- 1 standard deviation. Abbreviations: Agrobacterium, A; Methanogens, M; Newspaper, N; Wood, W; M9 solution, M9.

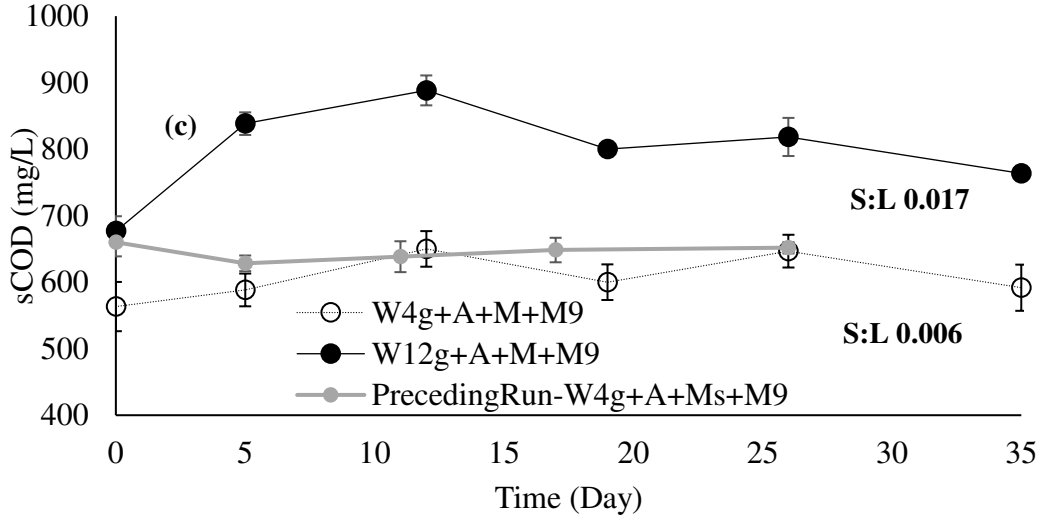
Impact of increasing solid:liquid (S:L) ratio (Experiment 2): The lack of a response from wood exposed to *Agrobacterium sp.* in the first bioreactor experiment was hypothesised to be due to a combination of a slow biodegradation rate and a low S:L ratio. Increasing the S:L ratio from 0.006 g/ml to 0.017 g/ml had a significant impact on the cumulative biogas volume for both lignocellulosic wastes (Figure 3a -wood, Figure 4a -newspaper).

In the case of wood, the biogas for the higher S:L ratio was increased by ~205%. Changing the S:L ratio has been shown to have a significant impact on the biogas kinetics and cumulative volume of the organic fraction of MSW (Krause et al., 2017; Krause et al., 2016; Raposo et al., 2006). Although, to the best of our knowledge, this has not been investigated in the application of bacterial biodelignification systems on wood lignocellulose. The wood sCOD profile (Figure 3c) increases and reaches a peak around day 14 then decreases gradually. The sOC profile (Figure 3b) does not seem to strictly follow this exact trend of reaching a peak followed by gradual decrease. sCOD is indicative of the amount of oxygen required to oxidise the carbon present, and the more oxygen required, the more carbon is present in solution or roughly the same amount is present, but in a more complex structure which is more difficult to oxidise. The general trend in sCOD

390 is an increase then a gradual decrease. The sOC increases and then stays reasonably steady
391 with a higher value measured in the wood cases. A possible explanation of these
392 observations might have to do with the form of the carbon released. The fact that sCOD
393 lowers but the sOC does not perhaps indicates that the form of the soluble compounds
394 might have gone from more complex structures, to more simple structures that may be
395 relatively easier to convert to biogas, i.e. end-product of anaerobic digestion.
396 It is important to note that at the very high S:L of 0.05 g/ml in the small-scale tests, a
397 very flat line for the total carbon profile for the ≤0.15 mm wood controls was recorded.
398 Whereas here, the sCOD profile due to the addition of the bacterium is transient and
399 follows a logical pattern of lignocellulosic breakdown (i.e. ~31% increase in the first two
400 weeks) and methanogenesis to convert that carbon to the gas phase (i.e. evidence from
401 biogas production profile, and decrease of sCOD by ~16% relative to the maximum peak-
402 value reached in the first two weeks). As such, it is suggested that the liquid analyses
403 results discussed here are most likely due to the *Agrobacterium sp.* acting in synergy with
404 the microbes from the sludge for depolymerisation of wood.
405

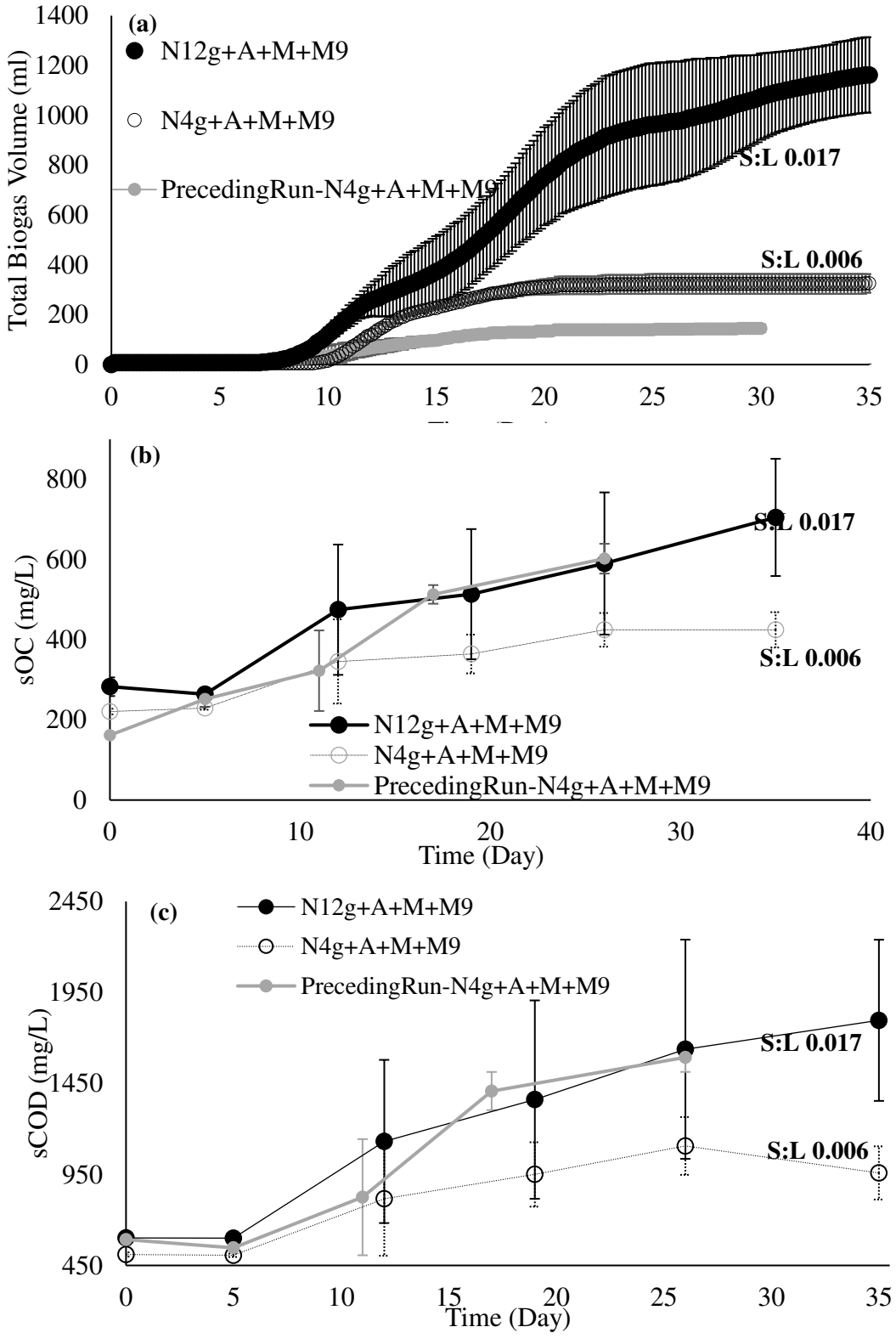

Total Biogas Volume (ml)
W4g+A+M+M9
W12g+A+M+M9
PrecedingRun-W4g+A+Ms+M9
S:L 0.017
S:L 0.006
(a)
Time (Day)


sOC (mg/L)
W4g+A+M+M9
W12g+A+M+M9
PrecedingRun-W4g+A+Ms+M9
(b)
S:L 0.017
S:L 0.006
Time (Day)


sCOD (mg/L)
(c)
S:L 0.017
S:L 0.006
W4g+A+M+M9
W12g+A+M+M9
PrecedingRun-W4g+A+Ms+M9
Time (Day)

406

Figure 3. Assessing the impact of increasing S:L ratio on Agrobacterium sp. treatment of wood lignocellulose with sludge. (a) Cumulative biogas volume for wood. (b) Soluble organic carbon profiles for wood. (c) Soluble chemical oxygen demand profiles for wood. All error bars represent +/- 1 standard deviation. Abbreviations: Agrobacterium, A; Methanogens, M; Wood, W; M9 solution, M9; 4 grams mass of waste, 4g; 12 grams mass of waste, 12g; PrecedingRun, data from the preceding run from Fig.2, identical experimental conditions to S:L 0.006 and included to account for inter-experimental variability, since sludge for the S:L experiments was sampled at a later date.


(a)
N12g+A+M+M9
N4g+A+M+M9
PrecedingRun-N4g+A+M+M9
S:L 0.017
S:L 0.006
Total Biogas Volume (ml)
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
(b)
S:L 0.017
S:L 0.006
N12g+A+M+M9
N4g+A+M+M9
PrecedingRun-N4g+A+M+M9
sOC (mg/L)
800
600
400
200
0
0
10
20
30
40
Time (Day)
(c)
N12g+A+M+M9
N4g+A+M+M9
PrecedingRun-N4g+A+M+M9
S:L 0.017
S:L 0.006
sCOD (mg/L)
2450
1950
1450
950
450
0
5
10
15
20
25
30
35
Time (Day)

415

Figure 4. Assessing the impact of increasing S:L ratio on Agrobacterium sp. treatment of newspaper lignocellulose with sludge. (a) Cumulative biogas volume for newspaper. (b) Soluble organic carbon profiles for newspaper. (c) Soluble chemical oxygen demand profiles for newspaper. All error bars represent +/- 1 standard deviation. Abbreviations: Agrobacterium, A; Methanogens, M; Newspaper, N; M9 solution, M9; 4 grams mass of waste, 4g; 12 grams mass of waste, 12g; PrecedingRun, data from the preceding run from Fig.2, identical experimental conditions to S:L 0.006 and included to account for inter-experimental variability, since sludge for the S:L experiments was sampled at a later date.

In Figure 4b and 4c the newspaper sOC and sCOD profiles are shown respectively. In the case of newspaper, the biogas volume was 280% greater than that at the lower S:L ratio. The cumulative overall volume for newspaper at the lower S:L ratio was significantly different ($P < 0.05$; approximately double) to that obtained previously with the same conditions (Figure 4a). This may have arisen due to variability in the newspaper composition (a different sub-sample was milled for this test) or variability in the microbial community from the sludge, since this was sampled 4 months after the previous test, albeit from the same site/digester/sampling point. However, it is clear that with all else equal the higher S:L ratio gives a significantly greater degree of biodegradation. For newspaper, the sOC and sCOD profiles follow a similar trend to the previously reported Figure 2b, 2c.

To study the impact of S:L ratio on the activity of the *Agrobacterium sp.* and overall biogas generation as a continuum, this ratio could be further increased or decreased under similar experimental conditions. This would perhaps allow for optimisation of the microbial processes studied. However, since the aim of this paper has been to gain mechanistic insights, optimisation has been beyond the scope of this study.

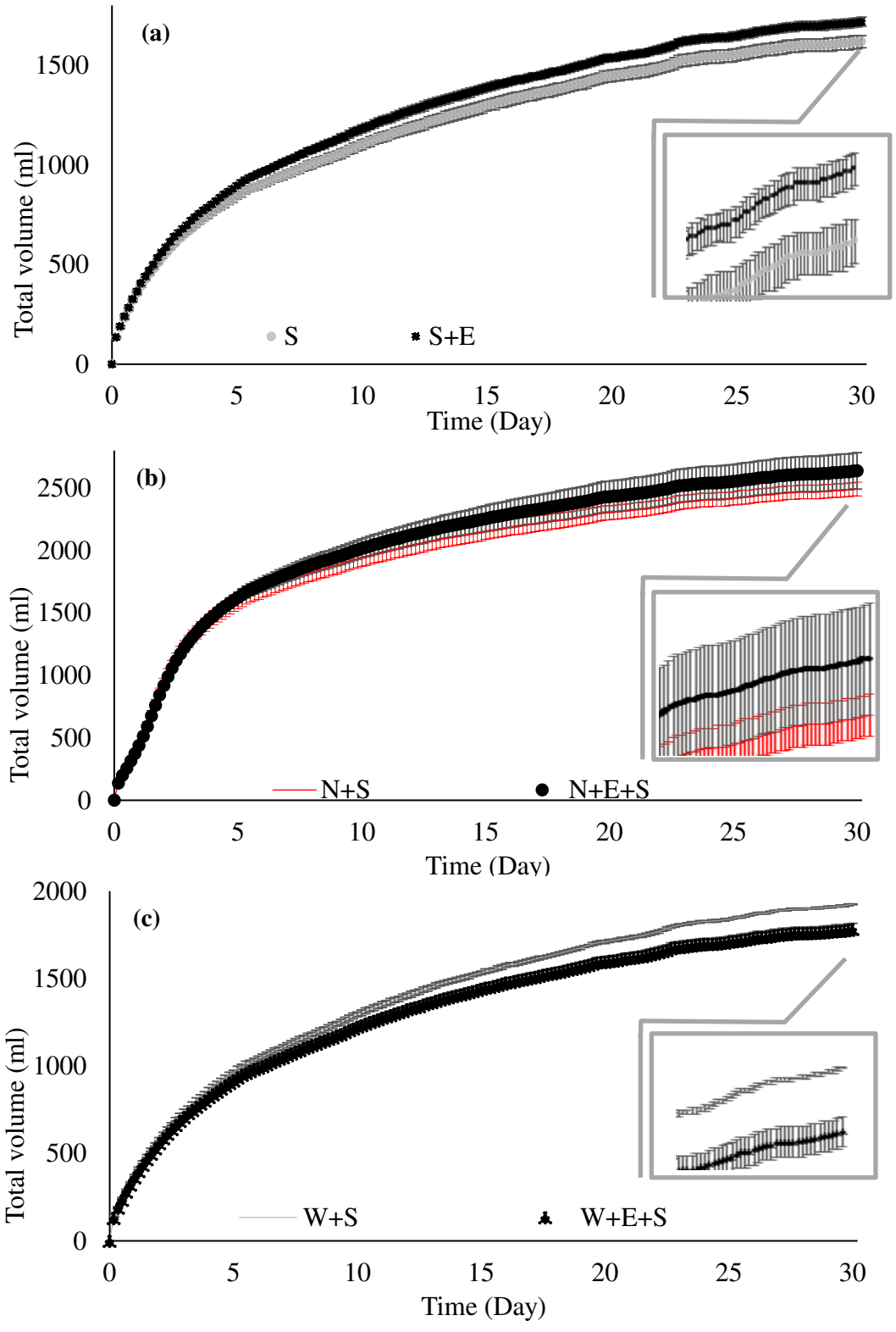
From the above bioreactor experiments, it is obvious that under all conditions, newspaper clearly produces much higher biogas yields than wood and shows a greater degree of enhancement (Fig. 2, 3, 4). This is likely due to a combination of two main factors. Firstly, due to the much higher lignin content of softwood (~1.5 times more than newspaper) (Table 2), which results in more recalcitrant biomass in comparison to wood. Secondly, newspaper, is a mechanical pulp which in relation to softwood is more processed, this likely also results in better accessibility to key polymers for degradation (Baldwin et al., 1998; Barlaz, 2006; Eleazer et al., 1997; Stinson & Ham, 1995; Wang et al., 1994).

3.3 Enzymatic Enhancement

In Figures 5 and 6 biogas profiles/values and net biomethane yields per gram of VS due to the action of LiP are shown. A small positive effect on biogas/biomethane yields due to the enzyme for just the bioreactors containing sludge and no waste took place ($P<0.05$) (Figures 5 and 6). This is likely a result of the enzyme breaking down suspended and/or dissolved recalcitrant organics present in the sludge which would otherwise not be broken down by the microbial communities present, which can then be utilised for metabolism and eventually be converted to biogas.

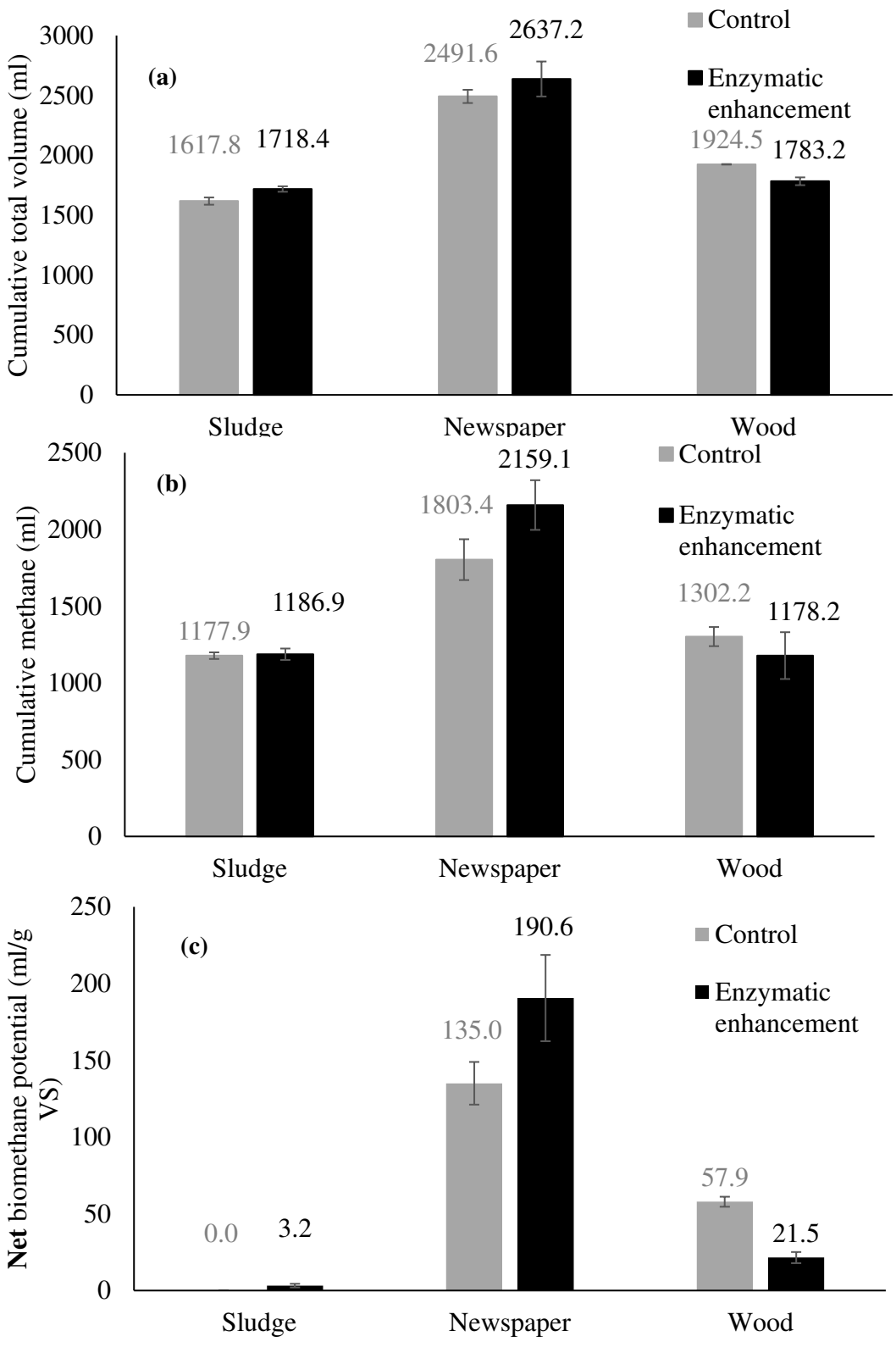
A significant ($P<0.05$) enhancement in biomethane potential from newspaper of ~41% was achieved with LiP present. LiP is thought to form reactive free radicals that attack the non-phenolic parts of the lignocellulosic structure (Bugg et al., 2011a; Bugg et al., 2011b; Cragg et al., 2015). The enhancement is possibly a result of the enzyme attacking the lignocellulosic structure of newspaper, thereby providing additional substrate to the microorganisms from the sludge which can then be converted to biogas.

In the enzyme-containing bioreactor cases discussed above, in addition to the overall biogas volume being enhanced, the proportion of methane also seems to be higher in the presence of the enzyme. This observation suggests that enzymatic enhancement in these reactors not only increased the overall biogas yield, but also the specific methane production, thereby increasing its concentration in the biogas mixture.


(a)
Total volume (ml)
1500
1000
500
0
S
S+E
0
5
10
15
20
25
30
Time (Day)
(b)
Total volume (ml)
2500
2000
1500
1000
500
0
N+S
N+E+S
0
5
10
15
20
25
30
Time (Day)
(c)
Total volume (ml)
2000
1500
1000
500
0
W+S
W+E+S
0
5
10
15
20
25
30
Time (Day)

466

Figure 5. Cumulative biogas generation profile results. Assessing the impact of lignin peroxidase application on newspaper and wood. (a) Sludge only. (b) Newspaper. (c) wood. All error bars represent +/- 1 standard deviation. Abbreviations: Sludge, S; lignin peroxidase enzyme, E; Newspaper, N; Wood, W. Inset figures included for closer examination of the gas generation profiles towards the end of the test.


(a)
Cumulative total volume (ml)
3000
2500
2000
1500
1000
500
0
Control
Enzymatic enhancement
1617.8
1718.4
2491.6
2637.2
1924.5
1783.2
Sludge
Newspaper
Wood
(b)
Cumulative methane (ml)
2500
2000
1500
1000
500
0
Control
Enzymatic enhancement
1177.9
1186.9
1803.4
2159.1
1302.2
1178.2
Sludge
Newspaper
Wood
(c)
Net biomethane potential (ml/g VS)
250
200
150
100
50
0
Control
Enzymatic enhancement
0.0
3.2
135.0
190.6
57.9
21.5
Sludge
Newspaper
Wood

472

Figure 6. Biogas and methane yield results. Assessing the impact of lignin peroxidase application on newspaper and wood. (a) Cumulative biogas volume comparison for bioreactors. (b) Cumulative methane generated in bioreactors over the 30-day experimental run. (c) Net methane yield per gram of VS. All error bars represent +/- 1 standard deviation.

In bioreactors containing wood, there was a significantly lower amount of biomethane production ($P<0.05$, Figures 5,6) in the enzyme-augmented reactors compared to the control. This effect was also found by Schroyen et al. (Schroyen et al., 2017; Schroyen et al., 2014; Schroyen et al., 2015), where not all lignocellulosic substances responded positively to pre-treatment through peroxidase enzymes prior to anaerobic digestion, in some instances the treatment negatively impacted methane production (e.g. with corn stover, wheat straw, maize). No significant changes in phenolics (which at high concentrations can inhibit methanogenesis (Hernandez & Edyvean, 2004)), TCOD or any transient changes in pH were observed within the first two weeks in comparison to the controls (data not shown). At low concentrations of LiP and similar fungal peroxidases in relation to the amount of lignin, the peroxidase enzyme has been said to catalyse repolymerisation of lignin alongside depolymerisation (Cragg et al., 2015; Rahmanpour et al., 2017; Rahmanpour et al., 2016; Rashid et al., 2017). Repolymerisation of lignin means that lignin would precipitate back onto the surface and recent work has shown that this has a negative impact on the accessibility of the lignocellulosic structure to biodegradation (Li et al., 2007; MacAskill et al., 2018; Oliva-Taravilla et al., 2016; Wiman et al., 2012). Due to the very high lignin content of softwood (nearly double that of newspaper), it is possible that the concentration of LiP was low for these reactors, and that depolymerisation and simultaneous repolymerisation led to the lower methane yield in the enzyme-amended

reactors. The lignin content of the wood residue is slightly higher (38.17±0.42) in the enzyme reactors in relation to the controls (37.22±0.02), one possible reason for which could be re-polymerisation, although this difference is not statistically significant (two-way ANOVA P>0.05, F-values<F-critical). It might be that lignin droplets (post-depolymerisation) coalesced onto the surface of the wood (re-polymerisation) and resulted in slightly higher lignin contents in relation to the controls and lesser access for the microbes to the cellulose fibres resulting in lower methane yields as shown in Figures 5 and 6.

3.4 General Discussion of Bacterial and Enzyme Experiments

Agrobacterium sp., isolated from MSW soil, was tested(Rashid et al., 2017). for its ability to break down softwood bark chips in compost under aerobic conditions, showing an approximately 2.3-3.5-fold enhancement in biogas production The enhancement of a ~2-fold enhancement reported here is comparable to this, despite the methanogenic microbial communities from organic compost used by (Rashid et al., 2017) and the anaerobic sewage sludge (this study) being very different. In addition, compost certainly contains aerobic strains which could result in much better initial hydrolysis of the lignocellulose making it easier for the various strains tested to then attack the structure. Much of the work carried out on bacterial breakdown of lignocellulosic wastes in the literature (Chandra et al., 2007; Mathews et al., 2016; Mathews et al., 2015; Mathews et al., 2014; Mnich et al., 2017; Tsapekos et al., 2017) has not specifically paired this process with simultaneous methanogenesis as has been done in the current present study and that of (Rashid et al., 2017) as such this study has helped to shed more light into this gap in understanding.

518 Schroyen et al. applied peroxidase enzymes to lignocellulosic materials (Schroyen et al.,
519 2017; Schroyen et al., 2014; Schroyen et al., 2015). Interestingly, the case where they were
520 able to obtain maximum enhancement of biogas from corn stover (4.5% lignin) was with a
521 laccase treatment, not peroxidase or their mixture, and it resulted in a 17% increase in
522 methane yield. In comparison in this study a ~41% enhancement was obtained in
523 biomethane potential (25.7% lignin in this newspaper, Table 2). In Schroyen et al.'s
524 mixture of laccase and peroxidase pre-treatments, they studied 7 substrates and obtained
525 biogas enhancement only in 4 (hemp: 4.8%, miscanthus: 9.5%, flax: 14%, willow: 40%),
526 whilst the pre-treatment had a negative impact on the biogas production of the rest of the
527 substrates (in ensilaged maize, the pre-treatment instead of enhancing biogas production,
528 nearly halved it). Their work suggested that enzymatic biodelignification is a treatment that
529 depends highly on the nature of the substrate, it may actually have a negative impact in
530 some lignocellulosic wastes, the reasons for which are not clear as of yet. Interestingly, the
531 substrates studied in their study and ours suggest that it is not entirely clear as to why the
532 peroxidase enzyme treatment works with some wastes, and not with others. Looking at the
533 lignin contents, there does not seem to be a relationship between high lignin contents and
534 proportionally negative impact on peroxidase enzyme action. However, lignin is
535 predominantly made up of guaiacyl and syringyl monomers, where the former is less cross-
536 linked than the latter and in comparison, is an easier lignin-monomer to breakdown.
537 Syringyl-rich lignin has also been shown to negatively impact the growth of some lignin-
538 degrading fungi (Hooker et al., 2018). It might be the case that the monomeric composition
539 of lignin plays an important role in the success of peroxidase treatment, whereby wastes

with high syringyl monomeric units (e.g. softwood) may be more difficult to attack than say wastes made up predominantly of guaiacyl units (e.g. hardwood).

Jayasinghe et al. applied LiP and similar fungal peroxidases to partly degraded 30-year old excavated MSW (Hettiaratchi et al., 2014; Hettiaratchi et al., 2015; Jayasinghe et al., 2011; Jayasinghe et al., 2014; Jayasinghe et al., 2013). They also observed a positive impact of LiP on biomethane generation. However the increase in biogas yields recorded by them was nearly an order of magnitude larger than the control compared to the 41% increase in biomethane potential in the case of newspaper in this study. This may be due to the age of their waste, since for very old landfilled waste, significant degradation of the biomass likely occurred making the structure of the waste more accessible to enzymatic attack. On the other hand, this study used virgin materials and since newspaper is made from a mechanical pulp, much of the lignocellulosic structure is still intact (Eleazer et al., 1997; Wang et al., 2015; Wang et al., 1994).

From the experimental work carried out in this paper, it has been obvious that both the lignocellulosic wastes, wood and newspaper, responded to treatment by the *Agrobacterium sp*., whilst only newspaper responded positively to the peroxidase enzyme treatment. Possible reasons for these results and biogas yields obtained have been discussed. It has been highlighted that one of the major factors resulting in the different response exhibited by wood and newspaper has been due to the lignocellulosic structure and particularly, lignin content of the individual wastes. Finally, in comparison with enzymatic biodelignification, delignifying bacteria are present in landfills (the strain used here was isolated from landfill soil) and so there may be the potential to enhance their activity to encourage enhanced biogas recovery.

4 CONCLUSIONS

Agrobacterium sp. enhances the biodegradation of lignin-containing wastes, specifically newspaper and softwood, under idealised small-scale conditions, containing numerous other microbial communities. The solid:liquid ratio is a potentially important variable for the application of *Agrobacterium sp.* and should be considered for pilot-/field-scale trials (e.g. by adjusting leachate table). Lignin peroxidase enhances the biodegradation of newspaper (not wood), in conjunction with methanogenic bacteria. These results suggest that enhanced breakdown of real wastes in MSW landfills and processes such as anaerobic digestion is feasible using either technique but that the waste form is an important factor in the rate and extent of breakdown.

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