Delignification and Enhanced Gas Release from Soil Containing Lignocellulose by Treatment with Bacterial Lignin Degraders

Goran M.M. Rashid[a], Maria Jesus Duran-Pena[a], Rahman Rahmanpour[a], Devin Sapsford[b], and Timothy D.H. Bugg[a]*

[a]Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K.

[b]School of Engineering, Cardiff University, Queen's Building, The Parade, Cardiff, CF24 3AA, U.K.

Corresponding author: Prof. T.D.H. Bugg, Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K. Email T.D.Bugg@warwick.ac.uk, Tel 44-2476-573018

Running title: Bacterial delignification in soil

Abstract

Aims: The aim of the study was to isolate bacterial lignin-degrading bacteria from municipal solid waste soil, and to investigate whether they could be used to delignify lignocellulose-containing soil, and enhance methane release.

Methods and Results: A set of 20 bacterial lignin degraders, including 11 new isolates from municipal solid waste soil, were tested for delignification and phenol release in soil containing 1% pine lignocellulose. A group of 7 strains were then tested for enhancement of gas release from soil containing 1% lignocellulose in small-scale column tests. Using an aerobic pre-treatment, aerobic strains such as *Pseudomonas putida* showed enhanced gas release from the treated sample, but four bacterial isolates showed 5-10 fold enhancement in gas release in an *in situ* experiment under microanaerobic conditions: *Agrobacterium* sp., *Lysinibacillus sphaericus*, *Comamonas testosteroni*, and *Enterobacter* sp..

Conclusions: The results show that facultative anaerobic bacterial lignin degraders found in landfill soil can be used for *in situ* delignification and enhanced gas release in soil containing lignocellulose.

Significance & impact of the study: The study demonstrates the feasibility of using an *in situ* bacterial treatment to enhance gas release and resource recovery from landfill soil containing lignocellulosic waste.

Keywords: Delignification; lignin degradation; bacterial treatment; methane gas release; municipal waste treatment.

Introduction

The commitment of land for municipal waste landfill, and the eventual recovery and reuse of that land for other purposes, are issues of social and commercial interest, especially in densely populated parts of the world. The potential recovery of valuable resources from such landfill sites, especially valuable metals, is also of growing interest, and represents a new technological challenge (Jones *et al*., 2013; Tonini *et al*., 2013). If the rate of biodegradation of the landfill contents could be enhanced using biotechnology (Mali *et al*., 2012; Ni *et al*., 2016), then the release of methane from landfill sites which provides gas for commercial or private energy generation would be accelerated, and the time needed for reuse of the land should be reduced, both of which would be valuable for commercial landfill operators, and for regional town planning purposes. Addition of bacterial culture to a compostable municipal solid waste (MSW) bioreactor has been shown to increase methane production by 25% (Mali Sandip *et al*., 2012), and aerobic pre-treatment of MSW has also been shown to improve digestibility and methane production (Ni *et al*., 2016).

Lignocellulosic waste present in landfill sites is broken down slowly, since the lignin fraction is recalcitrant to microbial breakdown (Sanchez, 2009). Moreover, the documented pathways for microbial breakdown are oxidative, aerobic pathways (Bugg *et al*., 2011), whereas degradation in landfill sites is largely anaerobic (Yazdani *et al*., 2010). The aromatic heteropolymer lignin is highly resistant to breakdown, since it is linked together via ether C-O and C-C bonds that are not susceptible to hydrolytic cleavage (Bugg *et al*., 2011a). Microbial degradation of lignin has been studied mainly in basidiomycete fungi (Sanchez, 2009; Bugg *et al*., 2011a), which grow only in aerobic environments, but in recent years there has been renewed interest in bacterial lignin degradation (Ahmad *et al*., 2010; Bugg *et al*., 2011b), which offer potential applications in biotechnology, due to the relative ease of protein expression for bacterial enzymes, and genetic tools available for genetic manipulation in bacteria (Bugg *et al*., 2011b). A number of lignin-oxidising bacteria have been isolated from environmental soil samples (DeAngelis *et al*., 2011a; Taylor *et al*., 2012), and bacterial DyP-type peroxidase enzymes have been discovered that can

oxidise lignin in *Rhodococcus jostii* RHA1 (Ahmad *et al*., 2011), *Amycolatopsis* sp. 75iv2 (Brown *et al*., 2012), and *Pseudomonas fluorescens* Pf-5 (Rahmanpour & Bugg, 2015), and extracellular manganese superoxide dismutase enzymes with activity for lignin oxidation have been discovered in *Sphingobacterium* sp. T2 (Rashid *et al*., 2015).

In this work we wished to examine the hypothesis that bacterial lignin-degrading strains or lignin-degrading enzymes could be used for delignification of lignocellulosic waste in soil, and hence could be used to enhance the rate of gas release from MSW-containing soil. Kumar and co-workers have previously shown that the application of fungal Mn peroxidase on a small scale could enhance the rate of gas release from lignin-rich waste materials (Jayasinghe *et al*., 2011), and addition of enzyme to anaerobic bioreactors also enhanced methane production (Hettiaratchi *et al*., 2014), and Feng *et al*. have shown that addition of fungal Mn peroxidase to lignocellulolytic waste composting enhanced carbon utilization (Feng *et al*., 2011), therefore it seemed feasible that an equivalent bacterial lignin-oxidising enzyme could be used in a similar fashion. The use of a bacterial strain for delignification *in situ* in soil is a more challenging application, since lignin degradation is currently believed to be an exclusively aerobic process (Bugg *et al*., 2011a), and while the topsoil layer of soil is aerobic, the micro-organisms responsible for gas production are strictly anaerobic (Yazdani *et al*., 2010). Here we report that selected bacterial isolates can be used for delignification and enhancement of gas production in small-scale and lab-scale experiments.

Materials & Methods

Growth media.

Bacteria were grown in either M9 minimal media (unbuffered) or Luria-Bertani media, at 30 ^{o}C, in some cases containing additives as described below.

Isolation of lignin-degrading bacteria from MSW-containing soil

Soil was collected from a municipal landfill site at Sandford Farm (Woodley/Reading, UK), and was stored in a sealed container at -20 ^{o}C prior to use. Method A: 25 mg of waste sample and 10 mg of wheat straw were mixed, to which 5 mL of M9 salts added. The mixture was incubated at 30°C for 21 days with shaking, then 200 µL of enriched sample was streaked on M9 plates containing 1.5% (w/v) Bacto-agar and 2% wheat straw). The plate was sprayed with nitrated pine lignin (nitrated lignin was prepared as described by Ahmad *et al*., 2010), colonies with different appearance (colour, size or shape) were picked and transferred into 10 mL Luria Bertani (LB) broth and grown overnight at 30°C with shaking at 180 rpm. Samples from selected colonies were taken for Gram staining and 16S rRNA gene was amplified by polymerase chain reaction, and submitted for DNA sequencing, and sequences analysed using the BLAST algorithm on the EBI server (www.ebi.ac.uk). Oligonucleotide primers for amplification of 16S rRNA: Forward 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse 5'-TACGGYTACCTTGTTACGACTT-3' .

Method B: Soil samples were incubated in M9 minimal media containing 0.5% alkali Kraft lignin (Sigma-Aldrich, ref 471003) for 3 days, and then plated on M9 agar plates containing 0.5% alkali Kraft lignin, and colonies picked, and re-streaked on the same media. This method resulted in 2 further isolates, whose 16S rRNA gene was amplified by polymerase chain reaction, and submitted for DNA sequencing.

Delignification of lignocellulose (pine) using lignin-degrading bacteria

Bacterial strains were grown overnight in Luria-Bertani broth at 30°C (10 mL), then cells were harvested by centrifugation (5000 rpm, 10 min), and the cell pellets were washed with M9 minimal media (2 mL) and re-pelleted by centrifugation, and then re-suspended in M9 minimal media (2 mL). 100 µL of suspended bacterial cells were inoculated in 10 mL of M9 salts containing 1g of pine powder and 0.1% glucose, then the cultures were grown at 30°C in a shaking incubator (180 rpm) for one week. The treated lignocellulose was filtered through Whitman no. 1 filter paper,

112 washed with distilled water, and dried prior to estimation of lignin content using the Klason method
113 (see below). The supernatant was filtered through 0.2 µm syringe filter and its phenolic content
114 estimated using Folin-Ciocalteau method (see below).

115 The Klason assay for lignin determination is based on a published method (Kirk & Obst, 1988).
116 Lignocellulose samples were dried in oven at 110°C ±2 for 2 hr, of which 0.25 g ± 0.001 placed in a
117 25 mL conical flask. The samples were digested with chilled concentrated H_2SO_4 (3.75 ± 0.02 mL)
118 for 2 hr at room temperature, with stirring. The hydrolysates were diluted with deionised water (140
119 mL), then refluxed for 4 hr in round bottom flasks. The residues were collected by filtration (using
120 Whatman® no.1 filter paper) and washed three times with deionised water. The residue (insoluble
121 lignin) was dried at 110°C for 1 hr and its weight was measured on a 4-figure balance, from which
122 the percentage lignin was calculated.

123 The Folin-Ciocalteau assay is adapted from a published method (Meda *et al*., 2005). The general
124 method involved the successive addition of 80 µL of deionised water and 50 µL Folin's reagent
125 (Sigma Aldrich) to 20 µL of supernatant from bacterial treatment, or standard (*p*-hydroxybenzoic
126 acid used as a standard with concentration of 50, 100, 200, 300 and 400 µg/mL). The mixture was
127 incubated for 3 min at room temperature, then 250 µL of 20% sodium carbonate was added, and
128 samples incubated in the dark for 30 min. Absorbance was then measured at 760 nm, and total
129 phenol content was expressed as g/L based on *p*-hydroxybenzoic acid as standard. For measurement
130 of phenol release at different times, bacterial strains were inoculated and grown on M9 minimal
131 media (10 mL) containing 1% pine lignocellulose as described above for 10 days. Samples (100
132 µL) for phenol assay were taken after 2, 4, 6, 8 and 10 days of incubation.

133

134 *Enhancement of gas production by addition of lignin-degrading strains (small scale)*

135 Syringe columns were made by adding 16 g of MSW soil mixed with 1% chopped pine to a 10 mL
136 plastic syringe, to which was added 6.25 g/L sodium acetate (see Supporting Information Figure S1

for illustration). The top of each syringe was sealed to be gas-tight using the rubber stopper from the syringe plunger, which was pierced with a plastic tube to supply fresh media, and a second plastic tube to collect gas. The second tube was connected to gas collection unit, comprising an upturned 5 mL syringe containing saturated NaCl solution, into which gas was bubbled. Bacterial culture (100 µL in M9 minimal media) was applied to all columns after 4 days of incubation except for a control column in which 100 µL in M9 salts was added. The volume of generated gas was measured by eye using the printed volume scale at 2-day intervals for 36 days.

Enhancement of methane production in two-step process (aerobic delignification followed by anaerobic digestion)

Bacterial strains were grown in M9 minimal media (10 mL) containing 150 mg pine powder (autoclaved), supplemented with 0.1% glucose, at 30°C with shaking at 180 rpm for 7 days. The grown bacterial cultures were used as starter culture for delignification experiments as described below.

Pine powder (4.5 g) was autoclaved with 150 mL deionised water, then 150 mL of M9 salts (2 x final concentration) was added, followed by 300 µL of the starter culture of the bacterial lignin-degrading strain. The resulting mixture was incubated with shaking at 180 rpm for another 7 days at 30°C. The solid pine residue was harvested by filtration on Whitman no. 1 filter paper and dried in oven at 110°C for 2 hr, to give a "treated pine lignocellulose" sample. As a control experiment, pine powder (5 g) was also de-lignified using thermochemical method by reflexing the lignocellulose with 95% ethanol containing 5% HCl (0.2 M) for 4 hr. The treated pine residue was isolated by filtration as described above, and dried at 110°C for 2 hr. The treated pine lignocellulose samples, thermochemically treated pine lignocellulose, and a sample of pine organosolv lignin were then used as substrates for methane generation via anaerobic digestion.

De-lignified (biologically or thermochemically) pine samples (2 g) or organosolv lignin (0.5 g) were mixed with 1 g of MSW soil, then deionised water (5 mL) was added to a plastic syringe as described above, sealed and connected to gas collection unit. Gas generation was monitored in 2 day intervals for 40 days. Samples (200 µL) for metabolite analysis by LC/MS analysis were collected at 7 day intervals, at which point 200 µL of deionised water was added to each column.

Large scale gas production

Commercially available softwood bark chips (J. Arthur Bowers, Wyevale Garden Centres, 50 g) were mixed with organic peat-free compost (New Horizon, Wyevale Garden Centres, 450 g) then placed into an apparatus constructed from a polypropylene 2 L plastic bottle (see Supporting Information Figure S2 for illustration) containing a gas-tight inlet at the top, connected to a gas measuring unit, and an outlet on the bottom to collect liquid samples for metabolite analysis. Deionised water was added (approximately 1 L) until all materials were submerged. A bacterial starter culture (5 mL overnight culture grown in Luria-Bertani media) was added to the column, then the column was sealed and connected to a gas collection unit and kept at room temperature. Gas production was monitored for 35 days. Samples for metabolite analysis were taken every 7 days up to 4 weeks. Control experiments were also prepared as follows: 1) experiment in which wood chips was replaced with compost; 2) experiment without bacterial inoculum; 3) experiment containing additional bacterial inoculum from anaerobic digester (5 mL).

Gas samples for GC analysis were taken after 7, 14, and 21 days after inoculation. The percentage of the generated methane in the gas samples was determined using an Agilent 7890B gas chromatograph equipped with FID detector. Samples from residue (compost and wood chips) were also taken after 6 weeks of inoculation for determination of its lignin content, using Klason assay (see above), which were compared to the corresponding controls (untreated compost and wood chips).

Metabolite analysis via LC-MS

Samples (0.2-5 mL) for LC/MS analysis were extracted with ethyl acetate (1-5 mL) after acidification to pH 1 with 1 M HCl, then dried (Na_2SO_4), and evaporated under reduced pressure. Samples were then re-dissolved in 300 µL of 1:1 MeOH: H_2O. Aliquots (50 µL) were injected onto a Phenomenex Luna C_{18} reverse phase column (5 µm, 100 Å, 50 x 4.6 mm) on an Agilent 1200 and Bruker HCT Ultra mass spectrometer, at a flow rate of 0.5 mL/min, monitoring at 310 and 270 nm. The solvents were water 0.1% formic acid as solvent A and MeOH 0.1% formic acid as solvent B. The gradient was 5-30% B from 0-30 min; 30-40% from 30-35 min, 40-70% from 35-40 min, 70-100% from 40-45 min, 100% solvent B continued from 45-57 min and followed by 100-5% solvent B for 3 min.

Incubation of ß-aryl ether lignin model compound with anaerobic consortium

Guaiacylglycerol-ß-guaiacyl ether (Tokyo Chemical Industry UK Ltd., 1 mM) was added to 30 mL M9 salts to which 1 mL of anaerobic culture from municipal solid waste was added, then the headspace gas replaced with nitrogen. Samples (200 µL) were taken at 0, 2, 8 and 16 days after incubation (at room temperature).

Results

Isolation of lignin-degrading bacteria from MSW-containing soil

We first examined whether lignin-degrading bacteria could be isolated from MSW-containing soil, and if so, whether they are similar classes of bacteria to those isolated previously from woodland soil (Taylor *et al*., 2012). Two screening methods were used: firstly, a method previously published by our group, involving the use of a nitrated lignin assay as a screen for lignin-degrading

bacteria on agar plates (Ahmad *et al*., 2010; Taylor *et al*., 2012); and secondly, growth of soil samples on minimal media containing Kraft lignin.

For the first method, samples of MSW-containing soil were incubated for 14 days in minimal M9 media containing 1% pine lignocellulose, in order to enhance the population of lignin-degrading bacteria. Samples were then plated out on M9 minimal media agar plates containing 1% pine lignocellulose for 3 days, then sprayed with a solution of nitrated lignin, and incubated overnight at 30 ^{o}C. Colonies showing yellow coloration were picked and re-streaked in order to obtain single bacterial isolates. This method resulted in 12 isolates, whose 16S rRNA gene was amplified by polymerase chain reaction, and submitted for DNA sequencing. For the second method, soil samples were incubated in M9 minimal media containing 0.5% Kraft lignin for 3 days, and then plated on M9 agar plates containing 0.5% Kraft lignin, and colonies picked. This method resulted in 2 further isolates, whose 16S rRNA gene was amplified by polymerase chain reaction, and submitted for DNA sequencing.

By comparison with database 16S rRNA sequences, the identity of the isolates was established, as shown in Table 1. Three of the isolates were found to be in the *Ochrobactrum* class of α-proteobacteria. Our group has previously reported the isolation of two *Ochrobactrum* isolates with activity for lignin oxidation from woodland soil (Taylor *et al*., 2012). A further *Agrobacterium* sp. isolate is also a member of the α-proteobacteria; there is a previous report of an *Agrobacterium* isolate able to degrade Kraft lignin (Deschamps *et al*., 1980). A *Comamonas testosteroni* isolate is a member of the β-proteobacteria; there is a recent report of a *Comamonas* isolate able to degrade Kraft lignin (Chen *et al.*, 2012). Two *Enterobacter* isolates are members of the γ-proteobacteria; there are reports of an *Enterobacter lignolyticus* strain isolated from tropical soils amended with Kraft lignin that is able to degrade lignin under anaerobic conditions (DeAngelis *et al*., 2011a; DeAngelis *et al.*, 2011b). One *Pseudomonas* isolate was found, which is also a member of the γ-proteobacteria; we and others have previously reported activity of *Pseudomonas putida* for lignin degradation (Ahmad *et al*., 2010; Salvachua *et al*., 2015). One *Microbacterium* isolate was found,

which is a member of the actinobacteria; our group has previously reported the isolation of three *Microbacterium* strains active for lignin breakdown from woodland soil (Taylor *et al*., 2012). Two isolates were found to members of the Firmicutes phylum. The *Lysinibacillus* isolate is from the bacilli class: although our group has found previously that *Bacillus subtilis* has no activity for lignin oxidation (Ahmad *et al*., 2010), there are reports of *Bacillus* and *Paenibacillus* isolates with activity for Kraft lignin oxidation (Chandra *et al*., 2008), and we have also identified here a *Paenibacillus* isolate able to grow on minimal media containing Kraft lignin.

Table 1. Bacterial lignin-degrading strains isolated from municipal landfill soil via either nitrated lignin spray assay (method A) or growth on minimal media containing Kraft lignin (method B).

Activity of bacterial strains for delignification

The collection of new bacterial isolates were tested for delignification activity alongside four further lignin-degrading bacterial strains (two further *Microbacterium* strains, a *Rhodococcus erythropolis* strain, and *Sphingobacterium* sp. T2) isolated from woodland soil (Taylor *et al*., 2012), and *Rhodococcus jostii* RHA1 and *Pseudomonas putida mt-2* known to have lignin degradation activity (Ahmad *et al*., 2010), and *Pseudomonas fluorescens* Pf-5 from which a lignin-oxidising Dyp1B peroxidase has been identified (Rahmanpour & Bugg, 2015). The assay involved incubation of a bacterial starter culture with 1 g of chopped pine lignocellulose for 7 days. The treated solid residue and untreated lignocellulose was then analysed for percentage lignin content using the Klason assay (Kirk & Obst, 1988). Samples of the treated aqueous supernatant after 4 days and 7 days were also analysed for total phenol content using the Folin-Ciocalteau colorimetric assay (Meda *et al*., 2005). The results are shown in Table 2.

The results obtained from Klason assay of the treated material showed that 4 strains gave 20-25% delignification after 7 days (*P. putida, P. fluorescens, E. cloacae, and L. sphaericus*), as

shown in Table 2, with another 4 strains giving 15-20% delignification (*O. pectoris, Agrobacterium* sp., *E. ludwigii, and Microbacterium* sp.).

Using the Folin-Ciocalteau assay, 10 strains gave >20% increase in phenol release after 4 or 7 days, with highest phenol release observed with *C. testosteroni* (74% increase) and *R. erythropolis* (63% increase). Significant differences between phenol release was observed between 4- and 7-day time-points, with some strains showing maximum phenol release after 7 days, whereas for other strains maximum phenol release was observed after 4 days, decreasing after 7 days. This is ascribed to the high phenol degradation activity of some strains, which would start to degrade low molecular weight phenols as their concentration builds up.

Table 2. Delignification and phenol release of bacterial lignin-degrading isolates, using milled pine lignocellulose as substrate.

Activity of recombinant bacterial lignin-oxidising enzymes for delignification

The activity of purified recombinant bacterial lignin-oxidising enzymes for delignification and phenol release from lignocellulose was also tested, comparing *P. fluorescens* Dyp1B (Rahmanpour & Bugg, 2015) with *Sphingobacterium* sp. T2 manganese superoxide dismutase (Rashid *et al*., 2015), and also comparing with commercially available lignin peroxidase from *Phanerochaete chrysosporium* (Sigma-Aldrich). Doses of 0.2-1.0 mg enzyme/g lignocellulose were incubated with 1.0 g pine lignocellulose for 1 hr, and the residual solid assayed for lignin content using the Klason method (Kirk & Obst, 1988), and the aqueous sample tested for total phenol content using the Folin-Ciocalteau colorimetric assay (Meda *et al*., 2005), as shown in Figure 1.

Using the Klason assay, dose-dependent delignification was observed for each enzyme, with highest activities at 1 mg/g dose. At this dose 26% delignification was observed for *P. fluorescens* Dyp1B, 31% for *Sphingobacterium* sp. T2 MnSOD, and 31% for *P. chrysosporium* lignin peroxidase. These levels of delignification are comparable to the 20-25% decreases in lignin

content for bacterial strain treatment after 7 days (see Table 2), but were achieved *in vitro* in a 1 hr treatment.

Using the Folin-Ciocalteau assay, dose-dependent increases in phenol release were observed for *Sphingobacterium* sp. T2 MnSOD, up to 30% increase for the 1 mg/g dose. Only very small changes were observed upon treatment with *P. fluorescens* Dyp1B, with a 10% decrease in phenol content at low dose, and 8% increase at high dose, compared with the untreated lignocellulose control. This behaviour might be due to the tendency of lignin-oxidising peroxidases to catalyse repolymerisation as well as depolymerisation of lignin fragments (Rahmanpour *et al*., 2017). For fungal lignin peroxidase, a decrease in phenol content was also observed at low dose, perhaps for the same reason, but at higher dose a dose-dependent increase in phenol release was observed, with >2-fold phenol release at 1 mg/g dose.

Figure 1. Delignification and phenol release by *Sphingobacterium* sp. T2 MnSOD1, *P. fluorescens* Dyp1B and *P. chrysosporium* lignin peroxidase.

Enhancement of gas release from soil containing lignocellulose

We then examined whether addition of a bacterial lignin degrader to soil containing lignocellulose could enhance gas release. The experimental design (see Figure 2A) involved samples of soil collected from a municipal solid waste (MSW) site, mixed with 1% (w/w) chopped pine lignocellulose, packed in 2.5, 5, or 10 mL plastic syringes, to which sodium acetate (6.25 g/L) buffer, bacteria or enzyme was added. The syringe was then sealed, and released gas was collected via plastic tubing, and the volume of gas measured. Liquid run-off was collected from the bottom of the syringe for analysis, and additional buffer added via airtight needle at the top of the syringe.

Figure 2. Small-scale testing of gas release from endogenous microbial population in MSW soil. A. Schematic diagram of experimental set-up. B. Gas generation from MSW soil in the presence and absence of 6.25 g/L NaOAc, in 2.5, 5.0, and 10 mL syringes.

First, the generation of gas from the microbial population in the MSW soil was tested without addition of exogeneous bacteria. In order to stimulate methanogenic bacteria in MSW, 6.25 g/L sodium acetate was added to 2.5, 5, and 10 mL syringes containing MSW, and the experiment left at room temperature (20-25 ^{o}C) for up to 20 days. The data in Figure 2B show that, after a lag phase of 7 days, time-dependent gas production was observed in the samples supplemented with sodium acetate buffer, with optimum gas production observed in the 10 mL sample.

The addition of exogeneous bacteria, grown on Luria-Bertani media and then suspended in M9 salts (100 µL), was then tested using 10 mL MSW containing 1% (w/w) chopped pine lignocellulose, but without addition of sodium acetate, over 35 days. The 6 bacterial strains showing highest levels of delignification and/or phenol release (see Table 2) were tested in this experiment, namely *P. putida*, *Ochrobactrum* sp., *Agrobacterium* sp., *L. sphaericus*, *C. testosteroni*, and *Paenibacillus* sp.. As shown in Figure 3, after a lag phase of 20 days, enhanced gas production was observed initially with *Agrobacterium* sp. and *L. sphaericus*, giving 10-fold enhancement of gas production compared with a control lacking exogenous bacteria. After 25-30 days, enhancement of gas production was also observed to a lesser extent with *C. testosteroni* and *Paenibacillus* sp.., giving 4-fold enhancement of gas production compared to the control lacking exogenous bacteria. No significant enhancement of gas production under these conditions was observed using *P. putida* or *Ochrobactrum* sp.

Figure 3. Enhancement of gas production *in situ* from 10 mL MSW soil containing 1% chopped pine lignocellulose by addition of bacterial lignin degraders

An alternative two-stage treatment scenario was also tested, whereby samples of pine lignocellulose were pre-treated aerobically with six bacterial strains in minimal media for 7 days, and then the sample centrifuged, mixed with 10 mL MSW, and incubated as above without sodium acetate. In order to compare biological vs. thermochemical pretreatment, another sample was delignified thermochemically (ethanol organosolv method). Anaerobic digestion of pre-treated (biologically and thermochemically treated) pine samples resulted in generation of up to 3-fold more gas (see Figure 4) compared to untreated pine and ethanosolv lignin from pine, over a 40-day experiment. In this scenario, optimum enhancement was observed with *P. putida* and *Paenibacillus* (3-fold enhancement), followed by *C. testosteroni* and *L. sphaericus* (2.6-fold enhancement), then *Ochrobactrum* sp. and *Agrobacterium* sp. (1.4-1.7 fold enhancement), which were similar to the thermochemically treated pine (1.4-fold enhancement, compared with untreated pine). Of particular note was that *Pseudomonas putida*, which showed the highest activity in the 2-stage treatment, showed no activity in the *in situ* experiment above, whereas *Agrobacterium* sp. and *L. sphaericus* showed activity in both treatment scenarios

Figure 4. Enhancement of gas production from pine lignocellulose pre-treated aerobically with lignin-degrading strains, followed by anaerobic digestion with MSW soil.

The first *in situ* treatment method was then tested on a larger 0.5 kg scale, using commercially available organic compost in place of MSW soil, and supplementing with 10% (w/w) commercial softwood bark chips in place of chopped pine lignocellulose. Test experiments using *Agrobacterium* sp. confirmed that 3-fold enhanced gas generation was observed over a 30-day experiment containing organic compost supplemented with softwood bark chips, compared with experiments lacking softwood bark chips, or lacking additional bacteria, as shown in Figure 5A. Addition of a sample of anaerobic digester extract to the organic compost was found to make no

significant difference to the gas yield (see Figure 5A), indicating that there is an efficient microbial population for anaerobic gas production present in commercial organic compost.

Figure 5. Enhancement of gas release from 0.5 kg organic compost supplemented with 10% (w/v) softwood bark chips, to which was added bacterial culture.

Using these optimised conditions, the four bacterial isolates that showed activity in small-scale trials (*Agrobacterium* sp., *Lysinibacillus sphaericus, Comamonas testosteroni, Paenibacillus sp*.) were tested on a 0.5 kg scale experiment over 30 days. Enhanced gas production was observed with all four isolates, as shown in Figure 5B, showing 4-5 fold enhancement of gas production, compared with a control experiment lacking bacteria. Greatest enhancement of gas release was observed with *Lysinibacillus sphaericus* at 10-30 days. In this experiment, samples of gas at 7 day intervals were analysed by gas chromatography, revealing that the methane content was 15% after 7 days, 14% after 14 days, and 42% after 21 days.

Incubation of ß-aryl ether lignin model compound with anaerobic consortium.

Our observation that lignin degradation occurs under microanaerobic conditions is surprising, since the known lignin degradation pathways are oxidative and aerobic (Bugg *et al*., 2011a). Since the strains that are most effective under these conditions are facultative anaerobes, it is possible that these organisms might use reductive or non-redox pathways to break down lignin. In order to probe the molecular basis of the biotransformation of lignin under anaerobic conditions, a sample of anaerobic consortium collected from the 0.5 kg scale experiment described above (supplemented with *Agrobacterium* sp.) was incubated with ß-aryl ether lignin model compound guaiacylglycerol-ß-guaiacyl ether in M9 minimal media under anaerobic conditions for 16 days. Analysis of supernatant samples by LC/MS showed that a peak at 27.5 min corresponding to the

lignin model compound (MNa^+ 343) was reduced in size after 2 days, and consumed after 8 days (see Supporting Information Figure S3).

Extracted ion chromatographic analysis of the LC-MS data showed the formation of new compounds of reduced molecular mass: a new species at *m/z* 284.9 was observed after 2 days (Supporting Information Figure S4); while several new species were observed after 8 days at *m/z* 329, 270.9, 258.9, 274.9, 244.9 and 315 (Supporting Information Figure S5). The products at *m/z* 329 and 315 indicate two successive demethylation reactions occurring on compound **1**, consistent with the formation of compounds **2** and **3**, shown in Figure 6. The new product observed at *m/z* 275 is consistent with loss of formaldehyde via C-C fragmentation of the β,γ-bond to form compound **4**, which could be rationalised by oxidation of the α-hydroxyl group to a ketone, followed by retro-aldol cleavage. Demethylation of **4** followed by reduction of the α-keto group to the alcohol would give compound **5**, consistent with the observed peak at *m/z* 285. The observed species at *m/z* 245 is consistent with dehydroxylation of compound **4** in the *para* position, and demethylation, to form compound **6**. Although aromatic dehydroxylation is unusual, it is precedented in the bacterial anaerobic degradation of phenol via dehydroxylation of 4-hydroxybenzoyl-CoA (Glöckler *et al*, 1989; Boll *et al*., 2014). Hence the observed metabolites are consistent with demethylation, β,γ-fragmentation, and *para*-dehydroxylation reactions, as shown in Figure 6.

Figure 6. Proposed degradation route of ß-aryl ether lignin model compound by anaerobic consortium from MSW.

Discussion

We have identified 11 new bacterial lignin-degrading isolates, 7 using a nitrated lignin spray assay method previously published (Taylor *et al*., 2012), and 4 that are able to grow on Kraft lignin as a sole carbon source. Of these strains, three *Ochrobactrum* isolates and one *Microbacterium* isolate are of similar type to those isolated from woodland soil, while *Pseudomonas* strains are

414 known to have activity for lignin degradation (Ahmad *et al*., 2010). The majority of bacterial lignin-
415 degrading strains isolated previously are in the actinobacteria or α- or γ-proteobacteria phyla (Bugg
416 *et al*., 2011b; Tian *et al*., 2014; Wang *et al*, 2016). We have also isolated several new facultative
417 anaerobic lignin-degrading strains in this study: an *Agrobacterium* sp. isolate from the α-
418 proteobacteria phylum, consistent with two previous reports of lignin-degrading *Agrobacterium*
419 strains (Deschamps *et al*., 1980, Si *et al*., 2015); two *Enterobacter* isolates from the γ-
420 proteobacteria phylum, consistent with reports of a lignin-degrading *Enterobacter lignolyticus*
421 strain (DeAngelis *et al*., 2011a; DeAngelis *et al*., 2011b); a *Comamonas testosteroni* strain from the
422 β-proteobacteria, consistent with a lignin-degrading *Comamonas* isolate (Chen *et al*., 2012); and
423 *Paenibacillus* and *Lysinibacillus sphaericus* strains in the Firmicutes phylum, consistent with
424 Paenibacillus strains reported to degrade industrial lignins (Chandra *et al*., 2008; Mathews *et al*.,
425 2016). We note that the bacteria that we isolated previously from woodland soil were all aerobic
426 bacteria (Taylor *et al*., 2012), whereas some of the bacteria isolated here are facultative anaerobes,
427 which might reflect the sampling of topsoil in the earlier study vs. subsoil in this study.

428 We have tested 20 lignin-degrading bacteria for delignification of pine lignocellulose, and
429 have observed 15-24% delignification via Klason assay after a 7-day treatment with 8 of the strains
430 (see Table 2). Phenol release was also observed with >10 strains, indicative of lignin breakdown,
431 but there is not a clear correlation between the data for phenol release and delignification in Table
432 2. We suggest that this is because some of these strains are also very efficient aromatic degraders
433 (Taylor *et al*., 2012), hence they would rapidly consume phenolic compounds that are produced
434 from lignin breakdown, therefore a lack of phenol release is not necessarily indicative of a lack of
435 lignin breakdown. Treatment with white-rot fungi such as *Phanerochaete chrysosporium* is known
436 to improve biomass digestibility (Akin *et al*., 1993), but bacteria such as *Streptomyces griseorubens*
437 have also been shown to improve enzymatic saccharification yields (Saritha *et al*., 2013), and there
438 is interest in the use of microbial treatment for delignification in the context of cellulosic bioethanol
439 production (Moreno *et al*., 2015).

We have also tested recombinant lignin-oxidising enzymes for delignification activity, and we have observed a dose-dependent reduction in Klason lignin content using *P. fluorescens* Dyp1B (Rahmanpour & Bugg, 2015) and *Sphingobacterium* sp. T2 MnSOD1 (Rashid *et al*., 2015), as shown in Figure 1. Similar levels of delignification were observed using fungal *P. chrysosporium* lignin peroxidase (31.1% delignification at 1 mg/g lignocellulose), although the fungal enzyme showed higher levels of phenol release (Figure 1). The loadings of enzyme required to see this level of delignification (1.0 mg/g lignocellulose) are fairly high, but are comparable with doses of 0.1-0.15 mg/g used in previous treatments with fungal MnP and LiP enzymes (Jayasinghe *et al*., 2011; Hettiaratchi *et al*., 2014).

We have then tested whether using a lignin-degrading bacterium as an additive, enhanced gas generation can be achieved during anaerobic lignocellulose breakdown. We have examined two different scenarios for carrying out a bacterial treatment. Carrying out microbial treatment under aerobic conditions, and then using the treated lignocellulose for anaerobic digestion, aerobic degraders such as *Pseudomonas putida* show highest activity (see Figure 4), but for an *in situ* bacterial treatment under microanaerobic conditions, facultative anaerobes such as *Agrobacterium* sp. and *Lysinibacillus sphaericus* show highest activity (see Figures 3 and 6). For a treatment of landfill soil in the environment, an *in situ* treatment would have the advantage of not needed to disturb the landfill soil, hence the ability of the bacteria used in Figure 6 to operate under microanaerobic conditions could be very useful.

It is surprising that lignin degradation occurs under microanaerobic conditions, since the known lignin degradation pathways are oxidative and aerobic (Bugg et al., 2011a), hence we have studied the degradation of a β-aryl ether lignin model compound by an anaerobic consortium. The observed metabolites are consistent with demethylation, β,γ-fragmentation, and *para*-dehydroxylation reactions taking place (see Figure 6). Anoxic oxidation of alkyl sidechains is precedented in anaerobic aromatic-degrading bacteria, via either flavocytochrome c hydroxylases or molybdenum-dependent hydroxylases (Boll *et al*, 2014), hence such enzymes might be responsible

for the demethylation reactions observed here, or a non-redox demethylation reaction could be taking place, similar to the tetrahydrofolate-dependent demethylase LigM from *Sphingobium* SYK-6 (Rosini *et al*., 2016). It seems plausible that the 1-carbon unit released via anaerobic demethylation may ultimately be converted to methane gas by the anaerobic consortium. Interestingly, the majority of bacteria known to carry out anaerobic aromatic degradation are in the α-proteobacteria and β-proteobacteria (Boll *et al*., 2014), in which phyla the *Agrobacterium* and *Comamonas testosteroni* strains identified here respectively belong. A strain of *Dysgonomonas* sp. from the Bacteroides phylum has recently been reported to carry out degradation of Kraft lignin under anaerobic conditions (Duan *et al.,* 2016), though the biochemical pathways used by this organism are not known. The ability to delignify lignocellulose *in situ* offers possible applications for treatment of landfill waste, and for delignification of biomass or lignocellulosic waste generated from agriculture, pulp/paper manufacture, or industries utilising lignocellulosic feedstocks.

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593
594

595 Table 1. Bacterial lignin-degrading strains isolated from municipal landfill soil via either nitrated
596 lignin spray assay (method A) or growth on minimal media containing Kraft lignin (method B).
597 Strain identification by sequence alignment of 16S rRNA gene sequence to Genbank sequence
598 database.

Isolation method	Growth temp (ºC)	Highest identity sequence match from 16S rRNA sequence	GenBank accession	Sequence identity	Bacterial phylum
A	30	*Pseudomonas* sp.	HM 219617	98%	γ–Proteobacteria
A	30	*Microbacterium oxydans*	JF 730219	99%	Actinobacteria
A	30	*Ochrobactrum pituitosum*	NR 115043	99%	α-Proteobacteria
A	30	*Comamonas testosteroni*	KJ 806363	96%	β-Proteobacteria
A	30	*Enterobacter ludwigii*	GQ 284566	99%	γ-Proteobacteria
A	30	*Enterobacter cloacae*	KF 017288	98%	γ-Proteobacteria
B	30	*Ochrobactrum* sp.	KF 737375	99%	α-Proteobacteria
B	30	*Ochrobactrum pecoris*	NR 117053	99%	α-Proteobacteria
B	30	*Agrobacterium* sp.	JX 872342	99%	α-Proteobacteria
B	30	*Paenibacillus sp.*	FR849917.1	99%	Firmicutes
A	45	*Lysinibacillus sphaericus*	HQ 259956	99%	Firmicutes

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600

601 Table 2. Delignification and phenol release of bacterial lignin-degrading isolates, using milled pine lignocellulose as substrate. Lignin content
602 measured by Klason assay (Kirk & Obst, 1988), and phenol release measured by Folin-Ciocalteau assay (Meda et al., 2005), as described in Materials
603 and Methods section. NT, Not tested.

Bacterial strain	Phylum	Strain reference	% lignin decrease in 7 days	% phenol increase in 4 days	% phenol increase in 7 days
Ochrobactrum sp.	α-Proteobacteria	This study	12.9	**26.8**	6.7
Ochrobactrum pectoris	α-Proteobacteria	This study	**17.4**	NT	18.0
Ochrobactrum pituitosum	α-Proteobacteria	This study	5.6	NT	18.5
Agrobacterium sp.	α-Proteobacteria	This study	**16.1**	**22.8**	**22.7**
Comamonas testosteroni	β-Proteobacteria	This study	9.8	17.6	**74.6**
Pseudomonas putida mt-2	γ-Proteobacteria	Ahmad et al, 2010	**21.2**	18.8	3.0
Pseudomonas fluorescens Pf-5	γ-Proteobacteria	Rahmanpour & Bugg, 2015	**20.6**	NT	0.6
Enterobacter ludwigii	γ-Proteobacteria	This study	**17.3**	NT	**20.0**
Enterobacter cloacae	γ-Proteobacteria	This study	**22.7**	9.7	14.1
Sphingobacterium sp. T2	Bacteroides	Taylor et al, 2012	8.5	13.2	**43.0**
Microbacterium phyllosphaerae	Actinobacteria	Taylor et al, 2012	7.8	16.3	**31.3**
Microbacterium sp.	Actinobacteria	Taylor et al, 2012	**17.6**	NT	**31.3**
Microbacterium oxydans	Actinobacteria	This study	4.8	NT	1.6
Rhodococcus jostii RHA1	Actinobacteria	Ahmad et al, 2010	9.5	NT	**38.7**
Rhodococcus erythropolis	Actinobacteria	Taylor et al, 2012	7.3	**39.1**	**63.7**
Paenibacillus sp.	Firmicutes	This study	1.0	**21.0**	1.8
Lysinibacillus sphaericus	Firmicutes	This study	**24.0**	19.1	10.0

604

Figure Legends.

Figure 1. Delignification (panels A-C) and phenol release (panels D-F) by *Sphingobacterium* sp. T2 MnSOD1 (panels A,D), *P. fluorescens* Dyp1B (panels B,E) and *P. chrysosporium* lignin peroxidase (panels C,F), at ratios of 0.2-1.0 mg enzyme/g lignocellulose, using milled pine lignocellulose as substrate. Lignin content measured by Klason assay, and phenol release measured by Folin-Ciocalteau assay, as described in Materials and Methods section.

Figure 2. Small-scale testing of gas release from endogenous microbial population in MSW soil. A. Schematic diagram of experimental set-up. B. Gas generation from MSW soil in the presence of 6.25 g/L NaOAc, in 2.5 mL (purple crosses), 5.0 mL (blue diamonds), and 10 mL (red squares) syringes; control experiment in absence of NaOAc (green triangles).

Figure 3. Enhancement of gas production *in situ* from 10 mL MSW soil containing 1% chopped pine lignocellulose by addition of bacterial lignin degraders. A (green squares), *Ochrobactrum sp.*; B (orange diamonds), *Lysinibacillus sphaericus*; C (dark blue circles), *Comamonas testosteroni*; D (red crosses), *Paenibacillus sp.*; E (purple diamonds), *Pseudomonas putida*; F (yellow/blue circles), *Ochrobactrum sp.*; G (blue circles), control (no bacteria added).

Figure 4. Enhancement of gas production from pine lignocellulose pre-treated aerobically with lignin-degrading strains, followed by anaerobic digestion with MSW soil. A (purple crosses), pine treated with *Agrobacterium sp.*; B (orange circles), pine treated with *Paenibacillus sp.*; C (red dots), pine treated with *Comamonas testosteroni*; D (pale blue crosses), pine treated with *Lysinibacillus sphaericus*; E (light green triangles), pine treated with *Agrobacterium sp.*; F (green lines), pine treated with *Ochrobactrum sp.*; G (dark green triangles), pine delignified by thermochemical treatment; H (blue diamonds), untreated pine incubated with MSW soil; J (red squares), organosolv lignin incubated with MSW soil.

631

632 Figure 5. Enhancement of gas release from 0.5 kg organic compost supplemented with 10% (w/v)
633 softwood bark chips, supplemented with bacterial culture. A. Testing of gas production with
634 *Agrobacterium sp*. (1, green triangles), with added anaerobic digest extract (2, purple crosses),
635 versus control incubations lacking wood chips (3, red squares) and compost only (4, blue
636 diamonds). B. Testing of *Lysinibacillus sphaericus* (5, green triangles), *Comamonas testosteroni* (6,
637 puple crosses), *Paenibacillus sp.* (7, red squares), or *Agrobacterium sp*. (8, blue circles) over 30
638 days, versus compost only control (9, blue crosses).

639

640 Figure 6. Proposed degradation route of ß-aryl ether lignin model compound by anaerobic
641 consortium from MSW.

642 **Supporting Information**.

643 Figure S1-S2. Apparatus for small-scale (S1) and 500 mL scale (S2) methane generation
644 experiments

645 Figure S3-S5. LC-MS data for analysis of incubation of ß-aryl ether lignin model compound with
646 anaerobic consortium. S3, Disappearance of *m/z* 343 species; S2, selected ion chromatograms after
647 2 days incubation; S3, selected ion chromatograms after 8 days incubation.

648