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Effective pretreatment of lignocellulosic co-substrates using barley straw-adapted microbial consortia to enhanced biomethanation by anaerobic digestion

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Abstract

Microbial pretreatments have been identified as a compatible and sustainable process with anaerobic digestion compared to energy-intensive physicochemical pretreatments. In this study, barley straw and hay co-substrate was pretreated with a microaerobic barley straw-adapted microbial (BSAM) consortium prior to anaerobic digestion. The improved digestibility was investigated through 16S rRNA gene sequencing, microbial counts and C:N ratios. BSAM pretreatment resulted in 15.2 L kg^{-1} TS of methane yield after 35 days, almost 40 times more than the control. The methane content in total biogas produced were 58% (v/v) and 10% (v/v) in BSAM and control, respectively. This research demonstrated that BSAM-based pretreatment significantly increased the digestibility and surface area of the lignocellulosic material and considerably enhanced biomethanation. This study generates new potential bio-research opportunities in the emerging field of lignocellulosic anaerobic digestion-biorefineries.

Keywords: Barley straw, Natural hay, Microbial consortia pretreatment, Anaerobic digestion, 16s rRNA gene sequencing

1. Introduction

Recently, the detriment of fossil fuel use together with increasing global energy demand have spurred enhanced efforts for robust and affordable renewable energy alternatives. According to the International Energy Agency (EIA), this global energy demand will soon rise from 636 quadrillion Btu (British thermal units) (671 quadrillion kilojoule (kJ)) in 2020 to 911 quadrillion Btu (961 quadrillion kJ) by the year 2050 (EIA's International Energy Outlook, 2019). Lignocellulosic material plays a major role as a substantial constituent of global biomass and is therefore considered a low cost and very abundantly available feedstock. It offers great potential for the generation of renewable second-generation bioenergy products such as biomethane and liquid biofuels. Annually, about 181.5 billion tonnes of lignocellulosic biomass is produced worldwide (Kumar et al., 2008). The lignocellulose material comprises energy-rich polymers in the plant material, particularly cellulose (up to 55%) and hemicellulose (up to 35%) that intertwine with phenylpropane units of lignin (up to 40%). The structural properties and recalcitrant nature of these polymers limit the accessibility of microorganisms and enzymes, thereby limiting bioconversion of lignocellulosic polymers (Xu et al., 2019a). Although biomethane production through anaerobic digestion (AD) of lignocellulosic material has great potential, the efficiency of AD, and thus biogas production, is not always satisfactory due to the poor or incomplete digestion (hydrolysis) that often results in economic losses (Heyer et al., 2013) that make the process non-commercially viable. Process modeling identifies that improvements in accessibility and digestibility of lignocellulosic substrates rely on

effective pretreatment methods to assist in improved AD performance (Abraham et al., 2020), with a key approach to increase the surface area of feedstocks for microbial/enzymatic activity during the AD process.

Various pretreatment techniques have been investigated, including chemical, physical (mechanical) and biological pretreatment (microorganisms and enzymes), prior to the AD process. It has been shown in a number of reviews recently that these pre-treatments make a positive contribution towards improved biomethane production (Abraham et al., 2020; Wagner et al., 2018). However, the selection of a proper pretreatment method is crucial for commercially viable production of lignocellulosic-derived biomethane. For instance, most of pre-treatments are chemical and physically-based techniques that often require high cost and high energy input, thus may not be economically and environmentally friendly. Biological treatments, on the other hand, are relatively less explored, but are comparatively advantageous over the non-biological pre-treatments in many ways, such as it reduces the formation of inhibitory compounds, reduces the crystalline nature of cellulose and hemicellulose by increasing surface area, minimises energy input and potentially lowers the cost (Wagner et al., 2018). Thus, this approach is becoming an increasingly important topic of research as can be seen in Web of Science® where "anaerobic digestion", "biological pretreatment" and "lignocellulosic biomass" as topics, display an increase in the number of research articles during the year 2011-2020, from 35 to 140.

Biological pretreatment strategies utilize microorganisms or enzymes prior to the lignocellulosic feedstock being subjected to an AD process, where enzymatic

pretreatment has been identified as a cost-effective route in bioprocess development. Several microbial pretreatment approaches involving single/ multi-organisms (microbial consortia) have reported the efficacy on biodegradability of lignocellulose and subsequent improvement AD processes (Abraham et al., 2020). Many researchers consider that microbial consortia are an effective microbial pretreatment approach over single-organism-based pre-treatments to improve biomass degradation performance, since the degradation of lignocellulose in natural habitats requires the interaction of multiple microorganisms. Using adapted or constructed microbial consortia is seen to be the most likely successful approach, as it can improve bio-methane potential (BMP) much better than any other tested treatment approach (Shrestha et al., 2017). However, to our knowledge, to date, there have been no reports on the production of biomethane from agriculture waste pretreated with a microaerobic barley straw-adapted microbial (BSAM), as we demonstrate here.

The objective of this present study was to develop an effective BSAM consortia-based pretreatment of barley straw and hay (BSH) as a co-substrate under microaerobic conditions and to demonstrate the effectiveness of this novel microbial pretreatment in a subsequent anaerobic digestion process. The characteristic changes during the BSAM pre-treatment process were measured. Here, we also carried out 16S rRNA gene sequencing analysis of pretreated samples to characterize changes in the microbial community and identify its potential functions related to lignocellulose degradation during pretreatment. Finally, the effectiveness of BSAM-based pretreatment on AD performance was estimated *via* biogas & biomethane production rates.

2. Materials and Methods

2.1. Raw material selection for pre-composting

We chose a mixture of natural barley straw (*Hordeum vulgare*) and natural meadow hay straw (hereafter referred to as BSH co-substrate) as the feedstock subjected to a pretreatment and subsequent AD process, since these agricultural materials are easily available in the United Kingdom. Since hay contains a high nitrogen content, and other essential nutrients (total digestible nutrients, crude protein, calcium, potassium and phosphate etc.) versus mature barley straw, a proportion blended in a 2:1 ratio was used in this study. The co-substrate was natural (without chemical pretreatment) shredded to a particle size of 3-5 cm using scissors. The initial moisture content of the BSH co-substrate was 10%, determined by standard gravimetric method. The unsterilized BSH co-substrate corresponding to dry 100 g total solid (TS) was adjusted to a 80% moisture content taken in a 2 L capacity conical flask.

2.2. Enrichment of barley straw - adapted microbial consortia in a compost habitat

The compost containing BSAM consortia was collected from an optimally selected location in its natural habitat (barley straw composting located at a farm in Sheffield, U.K) was used as an inoculant to enriched BSAM consortia on BSH co-

substrate in the laboratory. The unsterilized BSH co-substrate corresponding to 100 g of dry BSH co-substrate was adjusted to 80% moisture content and mixed with 25 g of the wet compost containing BSAM consortia in the ratio of approx. 7:1. Urea (NH_2CONH_2) was also added to the concentration required to adjust the C:N ratio to 40 in the initial co-substrate. The mixture was incubated in a water bath at 25-27 °C for 35 days. To stimulate facultative microbial growth and to increase hydrolysis, microaerobic conditions were maintained using periodical aeration with an oxygen level between of 2-5 mg L^{-1}, measured using a portable dissolved oxygen meter (HQ30D HACH, Manchester, UK). This pre-digested material was subsequently used as an inoculum in pretreatment experiments as a BSAM consortia.

2.3. Pretreatment with the BSAM consortia

The purpose behind the pretreatment with BSAM was to achieve partial hydrolysis to gain access to cellulose and hemicellulose and to increase the digestibility of the lignocellulosic material for AD processes. Two pretreatment experiments were set-up in duplicates; BSH only (hereafter denoted as control) and BSH + BSAM. The unsterilized BSH co-substrate corresponding to 100 g of dry BSH co-substrate was adjusted to 80% moisture content and was used in both pretreatment experiments. The BSH + BSAM digesters were loaded at enriched BSAM (as mentioned in section 2.2) to the BSH co-substrate ratio of 1:7. In a control pretreatment, 25 g of wet BSH co-substrate was added instead of BSAM.

161 All pretreatment experiments were started at the same time and continued for 40 162 days at 25-27 °C. It is noted that during pretreatment, the oxygen level in the pre-163 digester was periodically maintained between 2-5 mg L^{-1} in every 2 days, as described 164 above. These selected microaerobic conditions in the compost have previously shown 165 an increase in abundance and diversity of facultative lignocellulolytic bacteria, with 166 accelerated solubilisation of lignocellulosic material (Shrestha et al., 2017). During 167 pretreatment, periodical sampling was carried out to assess microbial population, the 168 C:N ratio, and 16S rRNA gene sequencing to check the progress of pretreatment. The 169 significance between conditions was determined using two-tailed Student's t test at 95% 170 confidence.

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172 *2.3.1. Changes in viable bacterial population during pretreatment*

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174 Microbial population was estimated by counting colony forming units. Although 175 this is not the most accurate method of quantifying microbial abundance, it was deemed 176 appropriate here where we aimed to relatively compare the differences between the 177 treatment and control, and moreover, further microbial investigations were based on 178 molecular taxonomy. Briefly, plate counting was performed by a serial dilution 179 technique using R2A agar growth media as suggested elsewhere (Gibbs & Hayes, 180 1988). One gram (wet wt.) pretreated sample was dissolved in 4 mL sterilized saline 181 water (0.9%) (Gibbs & Hayes, 1988). This was mixed well and kept for 30 min with 182 constant shaking (70 rpm) at room temperature. The samples were diluted by serial 183 dilution (10^{-1} to 10^{-5} mL). A 100 µL of appropriate dilution were spread plated on R2A

agar plates. Colonies were counted and populations were expressed in terms of cfu g^{-1} of dry compost.

2.3.2. Changes in C:N ratio during pretreatment

A one gram of pretreated samples were taken at 0 days (Initial) and 40 days (Final) of pretreatment. The samples were dried in the oven at 60 °C for 3 days. Samples were ground into the powder and dried again for 1 day at 60 °C in the oven. 10 mg of each dried samples were analysed by a Vario MICRO cube CHN/S analyser *via* the combustion method to obtain the carbon-to-nitrogen (C:N) ratio as described elsewhere (James et al., 2019). Briefly, 2 mg of the dried samples were accurately weighed out on a Mettler-Toledo MX5 5 decimal point microbalance and sealed with the tin capsule. The tin capsule was introduced to the combustion tube of the CHNS analyser *via* a stream of helium. Within the oxygen-rich environment of the combustion tube at a temperature in excess of 1000 °C, the samples completely combust into gaseous NOx, CO_2, H_2O, SO_2. The resultant gases flow into the reduction tube packed with copper, where excess oxygen is removed and NOx is reduced to nitrogen. The gases are separated using a "Thermal Programmed Desorption" column and detected using a "Thermal Conductivity Detector", where the signal is integrated and the % of each element is calculated. Blanks & Daily Factors are used to ensure consistency between analysis runs and standards are run at regular intervals to confirm analyser function.

207 *2.3.3. Genomic DNA extraction, purification and quantification from*
208 *pretreated samples*

209

210 A total of 8 samples were selectively collected from control and BSH + BSAM
211 pretreatment conditions at day 10, 20, 30, and 40 days of pre-composting. One gram of
212 each pretreated sample was added to 10 mL of 10 mM sodium tripolyphosphate and
213 mixed for 10 min. The mixture was then centrifuged at 690 × g for 5 min in order to
214 remove the particles. The supernatant obtained from this was further centrifuged at
215 17,360 × g for 2 min to obtain the microorganisms as a pellet as described previously
216 (Yamamoto et al., 2009).

217

218 DNA extraction was carried out by the CTAB method as described previously
219 (Karunakaran et al., 2016). Briefly, the microbial cell pellets were suspended in 720 µL
220 of SET buffer (40 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris–HCl; pH
221 9 and 0.75 M sucrose). Subsequently, an aliquot of 81 mL of lysozyme (10mg/mL) was
222 added, vortexed for 10-15 sec and incubated at 37 °C for 30 min. After incubation, 90
223 µL of 10 % SDS plus 25 µL of freshly prepared proteinase K (20 mg mL^{-1}) were added
224 and vortex for 10-15 sec. Samples were incubated at 55 °C for 2 hours. The samples
225 were centrifuged at 5,000 g for 5 min, and the supernatant was collected into a new
226 Eppendorf tube. To the supernatant, 140 µL of 5 M NaCl solution and 115 µL
227 CTAB/NaCl solution (4.1 g NaCl and 10 g CTAB in 100 mL of distilled water) were
228 added, mixed well by inverting and incubated at 65 °C for 1 hour with gentle shaking.
229 After incubation, 838 µL of chloroform added to the lysate and mixed well. The
230 aqueous layer was obtained by centrifugation at 14,000 x g for 5 min and carefully

collected into another Eppendorf tube. Another 838 μL of chloroform again added to the aqueous layer, mixed and centrifuged at 14,000 x g for 5 min. An aqueous layer was carefully collected into another Eppendorf tube and at least three volumes of 100% isopropanol was added and incubated at -20 °C for overnight. The suspension was then centrifuged at the maximum speed of 21,000 x g for 20 minutes at 4 °C. The supernatant was decanted and DNA pellet was rinsed with 70% ethanol, centrifuged at maximum speed again and decanted. This step was repeated one more time. The pellet containing the DNA was air-dried and resuspended in 100 μL of TE buffer. DNA quantification was determined using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific).

2.3.4. Microbial diversity analysis by 16S gene sequencing

All 100 μL of 20 μg μL^{-1} of DNA samples were sent to the RTL Genomics laboratory (Lubbock, TX, USA) to perform PCR amplification, product pooling, purification and 16S gene sequencing using an Illumina MiSeq (Illumina, Inc. San Diego, CA, USA). Briefly, samples were first amplified for the sequencing. The forward primer was constructed (5'-3') with the forward Illumina overhang adapter (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) added to the CCTACGGGNGGCWGCAG primer (Klindworth et al., 2013). The reverse primer was constructed (5'-3') with the reverse Illumina overhang adapter (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) added to the GACTACHVGGGTATCTAATCC primer (Klindworth et al., 2013). Amplifications were performed in 25 μL reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1 μL of each 5μM primer, and 1μL of template. Reactions were

performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95 °C for 5 min, then 10 cycles of 94 °C for 30 s, 50 °C for 90 s (+0.5 °C per cycle), 72 °C for 1 min, followed by 25 cycles of 94 °C for 30 s, 54 °C for 90 s, 72 °C for 1 min, and finally, one cycle of 72 °C for 10 min and 4 °C hold. Products from the first-stage amplification were added to a second PCR based on qualitatively determined concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers as follows: Forward - AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and Reverse - CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The second stage amplification was run with the following thermal profile: 95 °C for 5 min, then 10 cycles of 94 °C for 30 s, 54 °C for 40 s, 72 °C for 1 min, followed by one cycle of 72 °C for 10 min and 4 °C hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled into equimolar units and each pool was size selected in two rounds using SPRIselect Reagent (BeckmanCoulter, Indianapolis, Indiana) in a 0.75 ratio for both rounds. Size selected pools were then quantified using the Qubit 4 Fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2 x 300 flow cell at 10 pM. A complete description of the applied bioinformatics filters is also available at http://www.rtlgenomics.com/docs/Data_Analysis_Methodology.pdf.

2.3.5. Statistical analysis

Richness was calculated as the alpha diversity measure to compare the diversity between the control and BSAM pretreatment samples. The observed diversity was based on unique OTU's found in each sample and reported as the alpha-diversity index with a statistical T-test to compare across all time points.

2.4. Anaerobic digestion of pretreated BSH substrates

After 40 days of pretreatment, whole partially digested BSH co-substrates obtained from control and BSAM pretreatment were subsequently subjected to the batch AD process each in duplicate. A 50 mL sample of freshly collected rumen fluid was added to pretreated BHS co-substrates as a methanogenic inoculum in the ratio of approx. 1:4 (w of wet co-substrate/v). The conical flasks were then sealed with a silicone rubber cork. The outlet of the flasks was connected to the water displacement unit using a silicon tube. The flasks were placed into a water bath and maintained at 33-36 °C. The AD process was carried out for 35 days. The rumen fluid is rich in diverse and dynamic methanogen communities (Patra et al., 2017). The rumen slurry was collected from a freshly slaughtered cow rumen at the local abattoir (Woolley Bros Ltd, Holbrook, Sheffield, UK). The slurry was filtered through 3 layers of cheesecloth into 250-mL glass bottles and flushed with carbon dioxide to produce the rumen fluid.

2.4.1. Biogas analysis

Biogas volume was monitored daily using the water displacement method, and the corresponding cumulative biogas volume was calculated. Using the ideal gas law,

the measured volume was then converted to a gas volume at standard temperature and pressure. Methane and carbon dioxide content of biogas was analysed as previously described by Raut et al. (Raut et al., 2016), with a few modifications. Briefly, gas samples from biological replicates were collected from the headspace of a gas collector using 10 mL gas-tight syringes at 5-day time intervals. The samples were injected into the gas chromatograph TRACE 1300 (Thermo Scientific, Paisley, UK) equipped with a thermal conductivity detector (TCD), of that, 250 µL of samples was automatically injected into the column. Separation of gas content was achieved using a precolumn Haysep Q (60-80) with 2 m x 1/16 SS packing connected with Molsieve 5A (60-80) column. Argon gas was used as a carrier gas at 36.25 psi pressure. Each run was performed for 13 min using a temperature gradient (with a hold at 50 °C for 2.5 min, followed by a ramp at a rate of 20 °C min^{-1} to 70 °C for 45 s, then hold at 70 °C for 8 min and finally ramp of 1 min up to 150 °C). The detector and oven temperature were 150 °C and 80 °C respectively. The GC was controlled and automated by the Chromeleon software (Dionex, Version 7). The instrument was calibrated using methane and carbon dioxide gas standards of 5, 20, 40, 60% (v/v) from BOC (Guildford, Surrey, UK). A total cumulative biogas, methane and carbon dioxide production was expressed as kg^{-1} TS co-substrate process in the AD. The significance between conditions was determined using two-tailed Student's t test at 95% confidence.

2.5. Kinetic study

In order to predict the performance of the AD process of BSAM pretreated material, the modified Gompertz and logistic models were used to compare

experimental methane yields as suggested by Yadav et al. (2019). Maximum biogas production rate (*Rm*) was obtained from the slope of the line during the exponential phase of gas production. Lag phase (Λ) is the x-axis intercept of this slope. The modified equations of Gompertz model (equation (1)) and logistic model (equation (2)) are as follows:

$$M = Pexp\left\{-exp\left[\frac{Rm.e}{P}(\Lambda - d) + 1\right]\right\} \quad (1)$$

$$M = \frac{P}{\left\{1 + exp\left[\frac{4Rm}{P}(\Lambda - d) + 2\right]\right\}} \quad (2)$$

M; Cumulative methane yield (L kg^{-1} TS)

d; Retention time (days)

P; Maximum specific methane production potential

Rm; Maximum methane production rate

Λ; Lag Phase

e; *Exp* (1) = 2.718

2.6. Energy and economic analysis

The assumptions for energy and GHG reduction potential are derived from the previous studies by Yadav et al. (2019) and Murphy et al. (2004). Briefly, up to 10000 kg of straw residues was considered for the analysis. The 1 m^3 of biogas was considered as equivalent to 2.04 kWh and 2.33 kWh of electricity and heat generation, respectively (Murphy et al., 2004). GHG reduction potential calculation were made based on the replacement of diesel generator with combined heat and power (CHP) units running on

biogas obtained from AD of pretreated straw residues. 1 kWh of energy generation was assumed as equivalent to 0.23 L of diesel. The present cost of 1 L of diesel in UK is 1.51 USD. One liter of diesel and 1 kg of residue burned were assumed to be contributed to 2.68 kg and 1.46 kg of CO_2 GHG emission respectively.

3. Results and discussion

In the present study, we obtained the compost containing BSAM consortia from its natural habitat specialized for barley straw composting located at a farm in Sheffield, U.K. and enriched on BSH co-substrate conditions by mimicking its natural oxygen and temperature conditions. This environment as a source of microbial consortia was chosen due to the continuous adaptation in conditions we deemed suitable for the pre-treatment. The microaerobic and temperature conditions of BSAM in its natural environment might be advantageous to achieve its superior performance in the *in-vitro* pretreatment (this refers here to the desire to achieve partial digestion of BSH co-substrate biomass). Therefore, the temperature and oxygen levels were maintained in the *in-vitro* pretreatment process at levels approximating those seen in their natural habitats: i.e. 22-27 °C and 2-5 mg L^{-1}, respectively. Microbial consortia enriched in microaerobic conditions are significantly diverse in microbial population compared to the microbial consortia enriched in anaerobic condition, (Lim et al., 2014). There are several studies that demonstrate that microaerobic conditions preferentially selects and stabilises a facultative microbial community during the pretreatment process and particularly promotes the growth of lignocellulolytic facultative anaerobes, which leads to a higher hydrolysis efficiency and digestibility of biomass (Fu et al., 2016; Lim et al., 2014).

1 375 This also helps the methanogenic microbial community present in the downstream AD
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3 376 process.
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6 377 The changes in a microbial population, the C:N ratio, and the microbial
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8 378 community analysis of the BSAM consortia during the pretreatment stage and the
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11 379 biomethane production in the downstream AD process are described in detail in the
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13 380 following sections.
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18 382 *3.1 Changes in viable bacterial population during the pretreatment stage*
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23 384 During the pretreatment stage, the changes in microbial population and C:N
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26 385 ratio were measured (**Fig. 1a, b**). The trend in microbial population growth in both BSH
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28 386 (hereafter referred to as the control) and BSH + BSAM consortia pretreatment process
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31 387 showed an initial increase in the total culturable microbial population up to days 8-10.
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33 388 This was typically followed by a brief decrease on day 10, followed by a steady and
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36 389 significant increase in microbial population number up to day 20, plateauing until ca. 40
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38 390 days of pretreatment. The microbial population of BSH + BSAM was always
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41 391 statistically significantly higher than the control pretreatment from day 2, which showed
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43 392 that the microbes added as an inoculum were able to thrive on the co-substrate. We
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46 393 presume that the growth of the microbial population seen in control pretreatment may
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48 394 attributed to the growth of majority of endophytes (non-phytopathogenic naturally
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50
51 395 associated organisms) that are already present on the BSH co-substrate. In the case of
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53 396 BSH + BSAM pretreatment, the bacterial population showed a steady and significant
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55 397 increase from day 10 onward and reached a maximum of log 10^{14} cfu g^{-1}dry BSH by
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58 398 day 20 as compared to control (log 10 ^{12} cfu g^{-1} dry BSH) pretreatment. The microbial
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population was always significantly higher in the BSH + BSAM pretreatment compared to control, which may indicate that the microbial population was dynamic and functional towards lignocellulose degradation during pretreatment. Our results suggest that at the early stage of the pretreatment process, the presence of readily available carbon substrates were used for microbial population growth, regardless of the material provided as a co-substrate. However, in the later stage, BSAM consortia selectively and functionally adapted to the BSH co-substrate over the time of pretreatment tested here. BSAM consortia may harbor a large number of lignocellulolytic microbes capable of degrading BSH co-substrates as seen in a previous study on lignocellulose composting, where the co-occurrence of vast majority of microbes are specialized in the degradation of lignocellulose (Wang et al., 2016) (refer to section 3.3 for more details).

3.2. Changes in C:N ratio during pretreatment

The C:N ratio is a direct indicator of biodegradation and also for the suitability of biomass for biogas production (Mishra et al., 2018; Raut et al., 2008). Previous studies have reported that the preferred C:N ratio that is effective for biomethanation is in the range of 20-to-30 (Wang et al., 2014; Yen & Brune, 2007), below which biodegradation reaches a maturation phase of compost that often ends with poor biomethanation (Gerardi, 2003; Raut et al., 2008).

The C:N ratio data obtained on initial (0 days; I) and final (40 days; F) of the BSH co-substrate pretreatment is given in **Fig. 1b**. There was a significant reduction in the C:N ratio achieved in the BSAM pretreated biomass compared to the control

423 pretreatment process. The initial C:N ratio of BSH co-substrate was 96 ± 29 and 81 ± 20
424 which had decreased to 60 ± 13 and 29 ± 7 by 40 days of pretreatment in the control and
425 BSH + BSAM pretreatment condition, respectively. It is found that the C:N ratio of
426 BSAM pretreated samples from the beginning was lower than the control. This indicates
427 that nitrogen present in the BSAM inoculum may be attributed to a lower C:N ratio in
428 BSAM pretreated samples. The significant decrease in the C:N ratio of the co-substrate
429 in the BSH + BSAM treatment indicates effective degradation of BSH residues by the
430 BSAM consortia, which resulted in increased in nitrogen content because of a loss of
431 total organic matter, as previously reported (Awasthi et al., 2018). Our results indicate
432 that the BSH + BSAM pretreatment achieved partial digestion of BSH co-substrate and
433 reduced the C:N to an optimal level for biomethanation, which is suitable for a
434 subsequent AD process. The decrease in the C:N ratio and increase in the relative
435 abundance of the microbial population in BSH + BSAM pretreatment means that both
436 parameters reflect the effectiveness of the BSAM consortia during the pretreatment
437 process, as seen in **Fig. 1a, b**. The functional microbial diversity for the BSH + BSAM
438 pretreatment process was inferred by a 16S rRNA gene sequencing taxonomy analyses.
439 The results are shown in the next section.

440

441 *3.3. Microbial diversity analysis during pretreatment*

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443 The pretreated samples from the control and BSH + BSAM pretreatment process
444 were collected every 10 days to investigate the diversity and composition of the
445 microbial communities related to lignocellulose degradation. A first overview of the
446 relative abundances of bacterial genera based on 16S rRNA gene sequencing of

1 447 extracted DNA is given in **Fig. 2**. Although there appears to be more observed alpha
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3 448 diversity in the treatment compared to the control, a *p-value* of 0.08 (T-test -2.313) was
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6 449 calculated over the 4 time points. A marked change in the profiling pattern of bacterial
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8 450 genera with the progression of pretreatment in both control and BSH + BSAM
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11 451 conditions were observed. The changes in structural composition of the microbial
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13 452 community were quite different in the BSAM treated samples, compared to the control
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16 453 pretreatment condition. Notably, in the control pretreatment samples, bacterial genera
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18 454 mostly belonged to the endophytes. Endophytes are non-phytopathogenic plant
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21 455 associated organisms that occur naturally. The endophyte genera include
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23 456 *Stenotrophomonas, Rhizobium, Cellvibrio, Microbacterium Sphingomonas,*
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26 457 *Ochrobacterium* and *Mesorhizobium,* all of which were predominantly found in the
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28 458 control pretreatment condition. The presence of these bacterial genera in the control
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31 459 condition may indicate that they are naturally associated with the barley straw that
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33 460 contributes to plant growth and development. Conversely, the genera of the bacterial
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36 461 community found in the BSAM treated samples were composed of different strains. The
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38 462 genera associated with the lignocellulose degradation such as *Fibrobacter, Variovorax,*
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41 463 *Cellvibrio, Sphingobacterium, Rheinheimera-, Flavobacterium, Brevundimonas,*
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43 464 *Hydrogenophaga, Caulobacter, Microbacterium, Sphingomonas, Mucilaginibac,*
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45
46 465 *Sphingobium* and *Variovorax* were predominantly found in the BSH + BSAM
47
48 466 pretreatment condition. The microorganisms of most of these genera are considered to
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51 467 be important in organic waste recycling and have often been found to associate with
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53 468 various environmental niches and organic waste treatment systems (Jordaan, 2015). As
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55
56 469 shown in **Fig. 2**, the BSH + BSAM pretreatment process showed remarkable changes in
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58 470 the pattern of bacterial genera over the time of pretreatment tested, reaching towards
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maturity and stability of the microbial community at the end of pretreatment process. The 16S gene sequencing results revealed that the genera belonging to the *Proteobacteria* were the most abundant in the BSH + BSAM pretreatment condition, followed by *Bacteroidetes, Actinobacteria, Firmicutes, Actinobacteria* and *Fibrobacteres.*

Notably, a large portion of the Operational Taxonomic Units (OTUs) belonged to unclassified and unknown genera. The presence of these unexplored prokaryotes may play key roles in geochemical cycle regulation during pretreatment and could provide insights into fundamental science that may have possible biotechnological applications. Therefore, it would be interesting to positively identify new potential functions of these unknown genera.

The correlation of the 25 top most abundant bacterial genera in the control and BHS + BSAM pretreated samples is given in **Fig. 3**. The correlation coefficient performed at the genus level demonstrated that the bacterial genera involved in lignocellulose degradation were found in both the control and the BSAM pretreatment condition. The bacterial species/strains of these genera (from both control and BSH + BSAM pretreatment) were constantly found to be associated with various environmental niches (e.g. compost, soil and wastewater) and organic waste treatment processes (e.g. sludge system). Notably, the dominant bacterial genera presented in the control conditions were significantly different from the BSAM pretreated samples. The bacterial genera predominantly presented in the control pretreatment condition were found to associated with various microbial systems involved in organics waste

degradation include *Sphingobium* (Xu et al., 2019b), *Sphingopyxis* (Silva et al., 2007), *Brevundimonas, Dyadobacter* (Photphisutthiphong & Vatanyoopaisarn, 2019), *Bosea* (Männistö & Puhakka, 2002), *Sphingomonas* (Wilhelm et al., 2019), *Phenylobacterium* (Wilhelm et al., 2018).

Notably, in the BSAM pretreated samples, the highly accumulated genera were specialized in lignocellulose degradation including: 1) cellulose/hemicellulose degradation; *Cytophaga*, *Fibrobacter*, *Cellulomonas* (Brethauer et al., 2020) and *Asticcacaulis* (Wilhelm et al., 2019), 2) aromatic ring/lignin degradation; *Hydrogenophaga* (Gan et al., 2011), *Demequina* (Pop Ristova et al., 2017) *Acholeplasma* (Braun et al., 2015), *Rheinheimera* (Sharma et al., 2017), *Oceanibaculum* (Sauret et al., 2014), and 3) nitrogen/phosphorous/sulphur regulation; *Hylemonella* (Spring et al., 2004), *Caldilinea* (Wang et al., 2016), *Hyphomicrobium, Aquicella, Dongia* (Antonelli et al., 2020). The genus belonging to the *Proteobacteria* phyla were the predominant microbial community (42%) in the BSH + BSAM treated samples, followed by phyla *Actinobacteria, Bacteroidetes, Firmicutes, Chloroflexi, and Fibrobacter*. In BSH + BSAM pretreatment, the microbial community was more specific to lignocellulose degradation than the consortium in the control pretreatment. This strongly suggests a synergistic action of multiple members of genera affiliated to various functions required to achieve more effective conversion of lignocellulosic biomass. For instance, the genera belonging to the *Cytophaga* and *Cellulomonas* are the best specialised cellulose/hemicellulose degraders in the microaerobic niche (Batool et al., 2018; Männistö & Puhakka, 2002). *Fibrobacter* is a dominant cellulose degrader of rumen ecosystem (Raut et al., 2019). A large group of bacterial genera associated with

aromatic compound/lignin degradation were also found in the BSAM treated samples. Notably, these bacterial genera involved lignin degradation belong to the *Proteobacteria and Actinobacteria* phyla that have been previously associated with lignin degradation (Brink et al., 2019). Generally, it is believed that the degradation of lignin is an oxidative or aerobic process, which is mostly dominated by fungi. However, the identified lignin/aromatic compound degrading bacterial genera under microaerobic condition in this study are facultative anaerobes, thus they may possibly have novel reductive or non-redox pathways to break down lignin as suggested previously (Rashid et al., 2017). Other genera identified are known to be involved in nitrogen/phosphorous/sulphur metabolism and these were also predominately found in the BSAM treatment, these are possibly forming an essential part of this consortium to function as microbial symbionts contributing to maintenance of optimal micronutrient conditions within the consortia. As a result, the synergetic effect of the bacterial population diversity on biomass pretreatment that focused on lignin, carbohydrate transformation and the regulation of nitrogen, phosphorous and sulphur is indeed an efficient way to fully promote biomass utilization. The microbial community in the BSAM pretreatment condition is suggested to be more diverse and dynamic in nature, comprising microbial genera involved in symbiotic biochemical cycling of macronutrients such as carbon, nitrogen and sulphur.

As expected, the majority of bacterial genera identified were facultative anaerobic types, since a microaerobic condition was maintained throughout the pre-treatment process. This suggests that the structure of the microbial community highly depends on the oxygen concentration in the pretreatment process. Based on these

observations, the expected mechanism of lignocellulosic biomass pretreatment can be hypothesised as follows: during pretreatment, lignin degrading facultative anaerobes partially removed/modified lignin, making cellulose/hemicellulose accessible to cellulose degrading microorganisms in the consortium that then converted cellulose/hemicellulose into nutrients for self-growth, with the balance being made available for downstream utilization during the downstream AD process. The other dominant bacterial genera found in the BSAM pretreatment were involved in regulating nitrogen, sulphur and phosphorous levels within the consortia. Our 16S rRNA gene sequencing data is very well aligned with the data on changes in microbial population and C:N ratio during the pretreatment processes.

3.4. Anaerobic digestion

The pretreated BSH co-substrate was subsequently used for the AD process. The BSAM consortia pretreated BSH co-substrate yielded more biogas than the control as seen in **Fig. 4 a**. The total biogas yield from BSAM consortia treated co-substrate reached 27 ± 2.9 L kg^{-1} TS of co-substrate after 35 days of AD. The value was significantly higher than the control that only produced 4.6 ± 2.0 L kg^{-1} TS of co-substrate. This result shows that BSAM consortia pretreatment is capable of significantly enhancing the methane production (15.2 ± 0.6 L in total biogas produced kg^{-1} TS) yield than control which only yielded 0.4 ± 0.15 L of methane in total biogas produced kg^{-1} TS (**Fig. 4b, c**). There were significantly higher production of methane and carbon dioxide in the BSAM treated condition compared to the control pretreatment condition. The total methane and carbon dioxide content of total biogas produced in

BSAM treatment were 58% and 11% respectively which is significantly higher than control treatment, which were 10% and 4% respectively (**fig. 4d, e**).

In recent years, the carbon dioxide content of biogas has become an important consideration for use as a feedstock to synthesize other biofuels or added-value byproducts and for biogas upgrading (Omar et al., 2019) *via* carbon dioxide sequestration from biogas and industrial effluents using waste gas fermenting microbes such as acetogens (Omar et al., 2019). This is a relatively new concept, the first investigation of biogas upgrading using *Actinobacillus succinogenes* 130Z was made by Gunnarsson et al. (Gunnarsson et al., 2014). The biogas upgrading by utilising the carbon dioxide by fermentation not only provides liquid biofuels and carbon dioxide content in the waste gas effluent/biogas but also potentially overcome the cost of CO_2 capture and storage and the cost of biogas upgrading thereby promoting a bio-based economy (Gunnarsson et al., 2014).

In order to achieve maximum theoretical methane yield, the effective pretreatment and digestion process needs to be developed that can produce greater methane density (biogas yield) with minimal energy and resource input. This will lead to the commercial expansion of biogas production from lignocellosic biomass. In previous studies, the improved methane yield in the AD process was recorded after physical, chemical, and biological pretreatment of lignocellulosic materials as reviewed recently by Abraham et al. (Abraham et al., 2020). It is concluded that pre-treatment of lignocellulosic biomass is an absolute requirement for lignocellulosic-AD performance. Microbial consortium pretreatment and improved biogas/methane production during

AD were positively correlated in the previous studies (Yuan et al., 2012; Kong et al., 2018; Li et al., 2020).

To date, Yuan et al. (2012), showed a 156% increase in methane yield in the AD process after a microbial consortium pretreatment when compared to an untreated (control) with newspaper as carbon substrate. In the present study, the cumulative methane yield after 35 days of AD of BSAM consortia-digested material represented a 3300% higher methane yield than the yield obtained from control pretreatment. Our results suggest that a significant increase in the methane yield is due to an increase in the digestibility of the BSH co-substrate during pre-treatment with BSAM consortia that subsequently resulted in the improved AD process. The significant abundance of a microbial population and significantly decreased in the C:N ratio during the pre-digestion stage evidently reflects the performance of the BSAM consortia. Correlating with that finding, is the 16S rRNA gene sequencing data analysis which revealed the dominant observed lignocellulolytic bacterial genera that synergistically enhanced the digestibility of BSH co-substrate during the BSAM pretreatment.

The high energy content of agriculture biomass offers a cheap and sustainable feedstock for bioenergy generation, but pretreatment is an absolute requirement to improve accessibility of polymers and increased surface area for microbial digestion during the AD process. The species diversity within the microbial consortia significantly depends on its environmental conditions that reaches functional stability over the period of time (Zegeye et al., 2019). Here we demonstrated that BSAM consortia enriched from its natural habitats on barley straw worked well *in-vitro*

pretreatment conditions, because of mimicking its microaerobic conditions allowing facultative anaerobes to work efficiently in the consortia, as shown in 16S rRNA gene sequencing analysis. Our study also suggests that the facultative nature of the BSAM consortia further helped in the AD process too. The significant increase in the methane yield indicated that the lignocellulosic material present in the feedstock had become more accessible and likely had a reduced crystallinity of polymers and increased surface area of substrate for microbial digestion during the AD process.

3.5. Kinetic study of methane production

The cumulative methane yield obtained from the batch AD of BSH pretreated residues was simulated by employing modified Gompertz and logistic models and the quality of fit for both models were analyzed. These are the most commonly used model for the determination of methane production kinetics. The lag phase (Λ) and maximum biomethane production rate (*Rm*) were 4 days and 0.739 L kg^{-1} TS d^{-1} respectively. The cumulative biogas yield calculated from experimental, modified Gompertz and logistic models were 15.2 L CH_4 kg^{-1} TS, 14.5 L CH_4 kg^{-1} TS and 14.9 L CH_4 kg^{-1} TS respectively. From visual inspection both the models were very well fits for the experimental cumulative methane production data (Fig. 5). The coefficient of determination (R^2) observed for both models were 0.999 depicting that the predicted value of cumulative biogas production yield obtained from both models fitted well with their experimental counterpart.

3.6. Energy and economic analysis

The BSAM pretreated BHS residues showed enhanced total energy (combined electricity and heat) generation potential (118 – 1180 kWh) as compared to control pretreatment (19.6 – 196 kWh) for 1000 – 10000 kg of straw residue (Table 1). The BSAM pretreatment also showed improve diesel savings (L) (27 - 271 L) compared to control pretreatment (4.5 – 45 L) along with saving of 320 USD for 1000 – 10000 kg of straw residue. Also, 15326 kg of CO_2 emission could be saved by BSAM pretreatment of 10000 kg of BSH residues (Table 2). The results showed that the BSAM pretreatment has positive impact on the biogas yield, energy generation and savings over the control pretreatment. Although the BSAM consortia-based pretreatment used in this study is promising, in the present scenario this approach is not suitable when considering the overall economics of the AD process. Therefore, further improvement by the optimization of pretreatment is needed. This would then also require a deep subsequent financial appraisal.

4. Conclusion

The microaerobic BSAM consortia significantly improved digestibility of co-substrate and biomethane production. Microbial population counts, C:N ratio and microbial diversity analysis of BSAM pretreatment clearly indicated that that BSAM consortia flourished well on co-substrate and the accumulated microbial genera were specialised in lignocellulose degradation over the period of pretreatment, thereby enhancing digestibility and suitability of co-substrate for biogas production. BSAM pretreatment enhanced biomethanation by 3300% over the control. However, we

identified that this pretreatment method needs further optimizations, and future research should be directed to improving production rates before the industrial application and therefore be vital in the AD research field.

E-supplementary data for this work can be found in e-version of this paper online.

CRediT authorship contribution statement

Mahendra P Raut: Conceptualization, Methodology, Investigation, Formal analysis, Software, Writing - original draft. **Jagroop Pandhal**: Resources, software, data analysis, drafting final manuscript. **Phillip C. Wright**: Resources, conceptualization, supervision and drafting final manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

Authors declare that they have no competing interests.

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

Supplementary Data 1.

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874 # Figure and Table captions

875

876 ## Figures:

877 **Fig. 1.** a) Changes in the bacterial population during pretreatment of BSH co-substrate.
878 b) C:N ratio during pretreatment. Results are expressed as the mean value and standard
879 deviation of two biological and two technical replicates. Results are expressed as the
880 mean value and standard deviation of duplicate samples (p-value *< 0.05; ** < 0.01).
881 NS indicates not significant.

882

883 **Fig. 2.** Relative abundances of bacterial genera based on 16s rRNA gene sequencing of
884 extracted DNA from control and BS + BSAM treatment condition at day 10, 20, 30 and
885 40.

886

887 **Fig. 3.** Top 25 bacterial genera were correlated with control and BS + BSAM treatment
888 condition. MiSeq reads of bacterial 16S rRNA genes were analyzed and assigned to
889 specific genera based on the sequence similarities to the NCBI 16S reference database.
890 The correlation coefficient, along with effect size measurements was applied to present
891 the enriched bacterial genera in BSH only (control) (blue) and BS + BSAM treatment
892 samples (red).

893

894 **Fig. 4.** Anaerobic digestion of pretreated BSH co-substrate with/without BSAM
895 consortia (orange circle indicates control and green square indicates BSH + BSAM
896 pretreatment). a) Cumulative biogas production b) cumulative methane yield c)

cumulative carbon dioxide yield d) % methane content of total biogas produced e) % carbon dioxide content of total biogas produced (Results are expressed as the mean value and standard deviation of duplicate samples. (p-value *< 0.05; ** < 0.01; ***<0.001).

Fig. 5. Cumulative methane production fitted with models.

Tables:

Table 1. Electricity and heat generation from control and BSAM pretreated BSH residues.

Table 2. Savings from biogas produced by utilizing control and BSAM pretreated BSH residues.

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921 # Figures:

922 **Fig. 1.**

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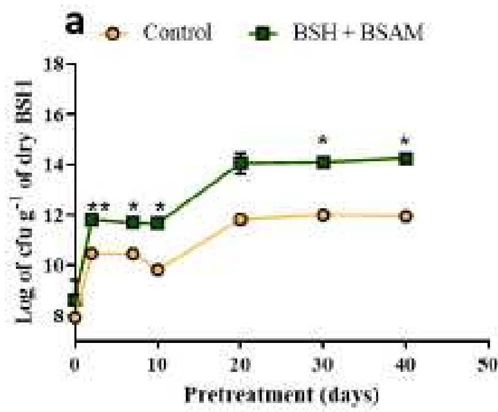

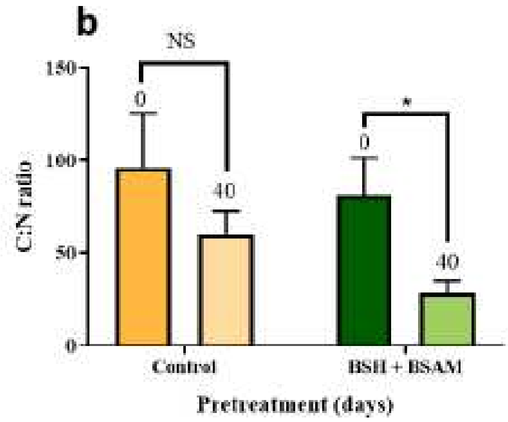

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939 **Fig. 2.**

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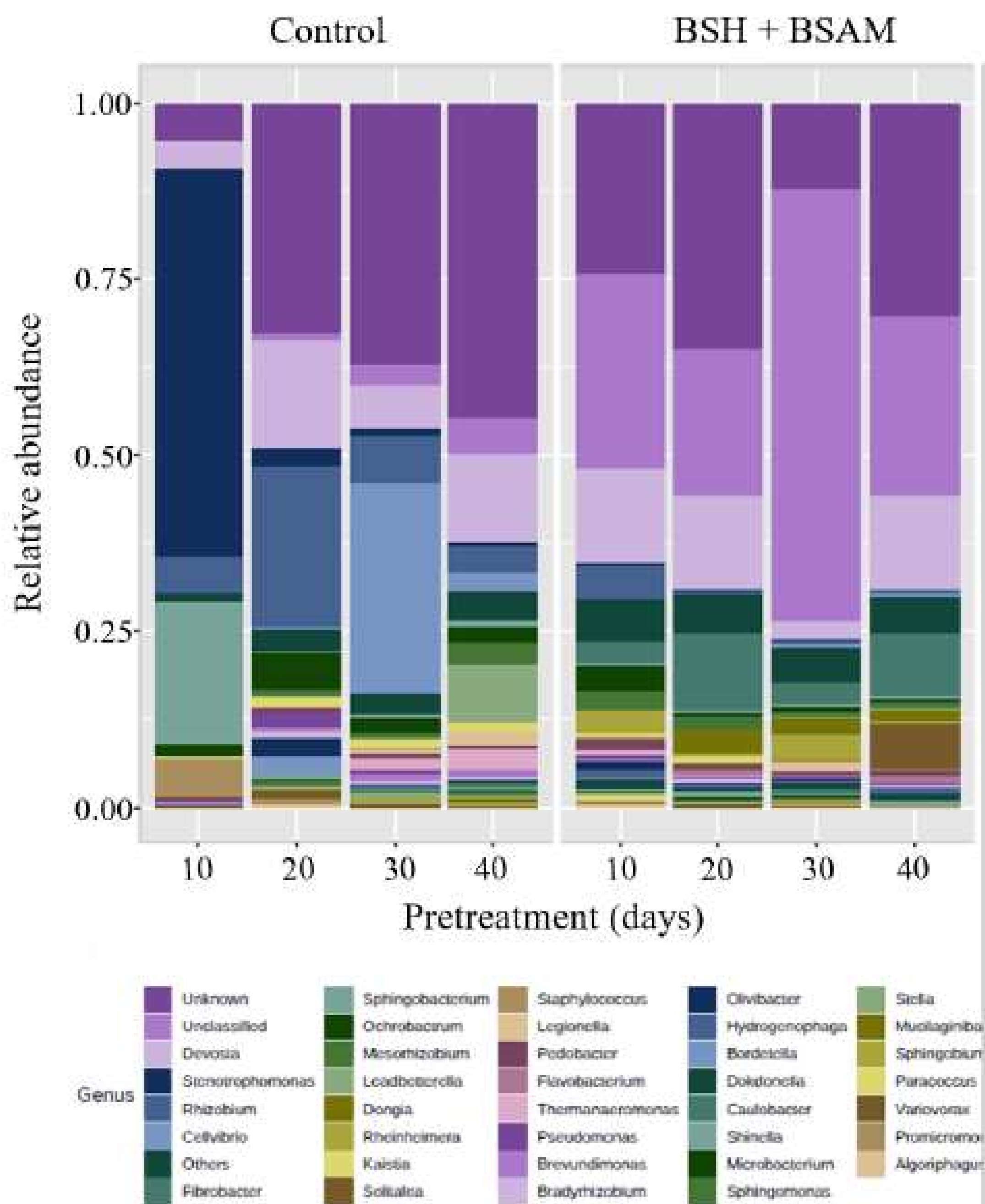

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946 **Fig. 3.**

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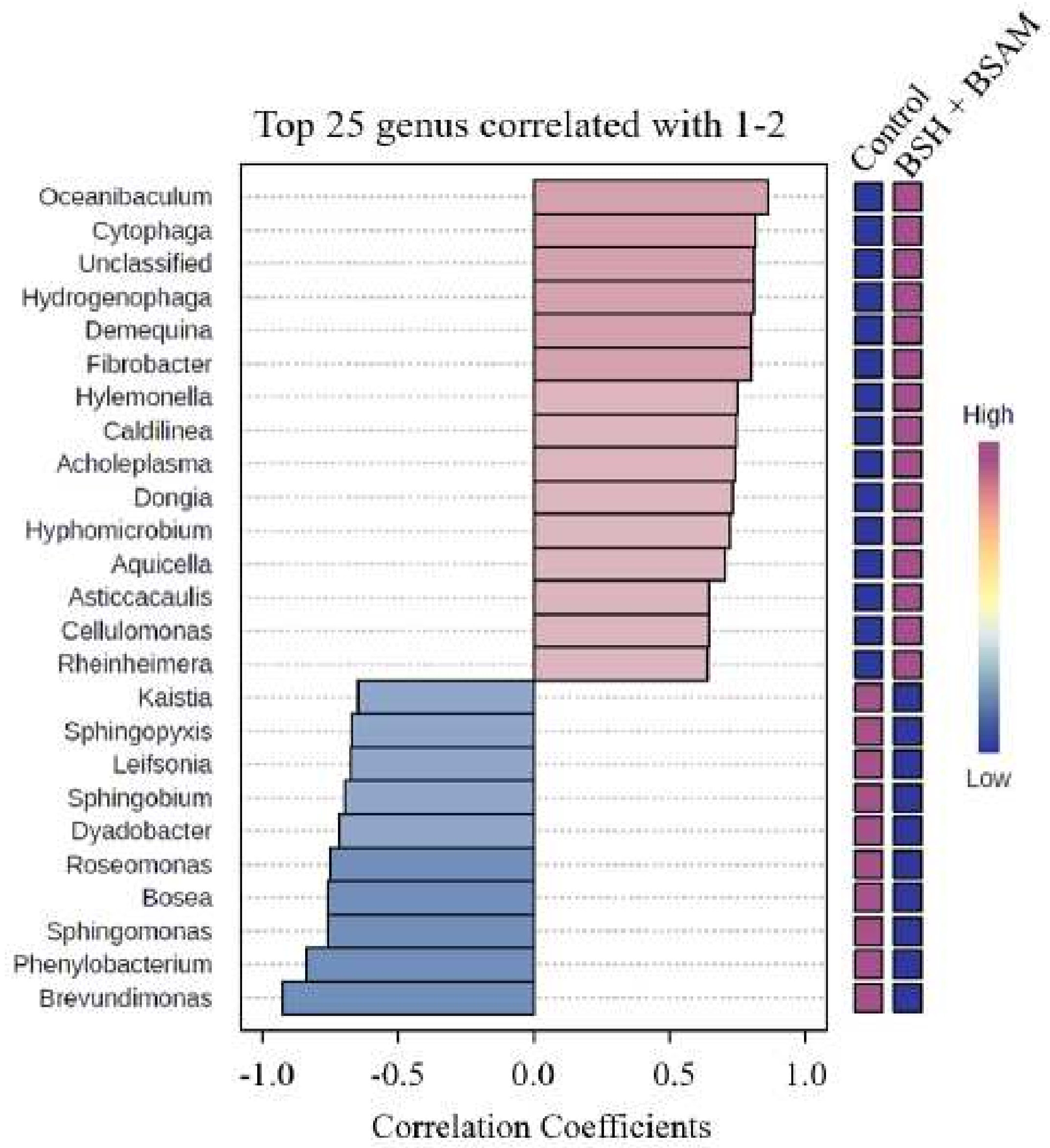

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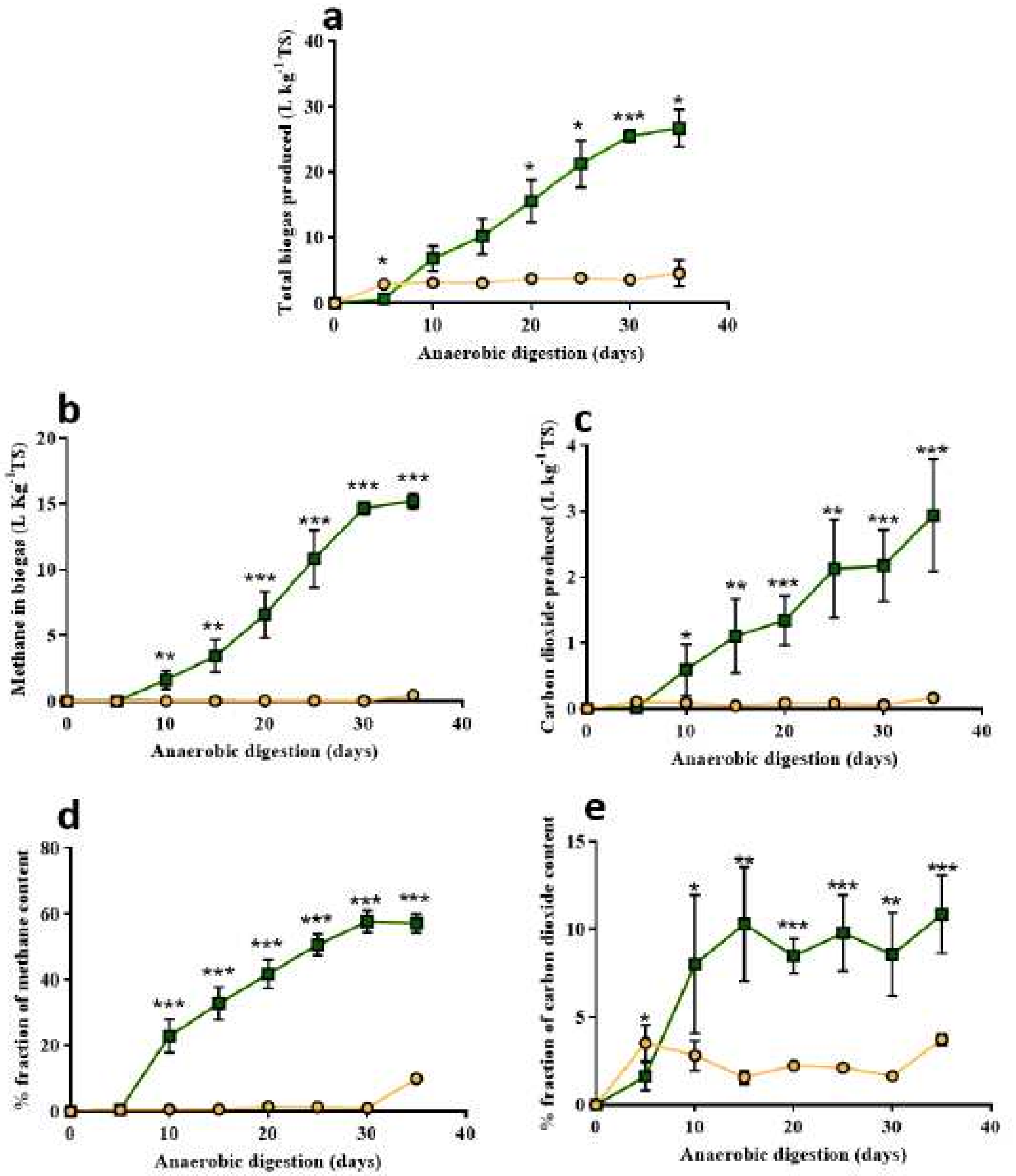
1 953 **Fig. 4.**



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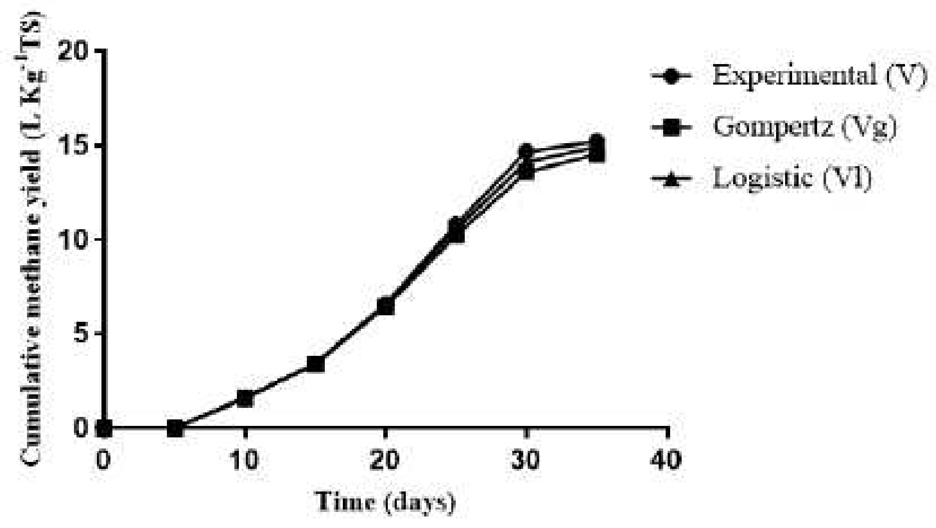

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Table 1. Electricity and heat generation from control and BSAM pretreated BSH residues.

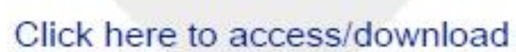
BSH residues (kg)	Biogas (m^3)		Electricity (kWh)		Heat (kWh)		Total energy (kWh)	
	Control	Pretreatment	Control	Pretreatment	Control	Pretreatment	Control	Pretreatment
1000	4.6	27	9.4	55	10.2	62.9	19.6	118
5000	23	135	47	275	51	314.5	98	590
10000	46	270	94	550	102	629	196	1180

Table 2. Savings from biogas produced by utilizing control and BSAM pretreated BSH residues.

BSH residues (kg)	Savings									
	Diesel (L) (0.23 L kW h^{-1})		USD (1.51 USD L^{-1})		GHG[a] (kg of CO_2)		GHG[b] (kg of CO_2)		Total GHG (kg of CO_2)	
	Control	Pretreatment	Control	Pretreatment	Control	Pretreatment	Control	Pretreatment	Control	Pretreatment
1000	4.5	27.1	6.8	41	12.06	72.62	1460		1472	1533
5000	22.54	136	34	205	60.40	364.48	7300		7360	7664
10000	45	271.1	68	409	120.6	726.2	14600		14721	15326

[a] Kg of CO_2 saved from diesel burned (2.68 kg CO_2 per L of diesel burned).
[b] Kg of CO_2 saved from burning of residues (1.46 kg CO_2 per kg of residues burned).

Electronic Annex



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Electronic Annex
Supplementary materials- BITE-D-20-07007R1.docx

Declaration of Interest Statement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: