Bioaugmentation Strategies to Enhance Methane Production from Lignocellulosic Substrates: Dynamics of the Prokaryotic Community Structure

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BIOAUGMENTATION STRATEGIES TO ENHANCE METHANE PRODUCTION FROM LIGNOCELLULOSIC SUBSTRATES: DYNAMICS OF THE PROKARYOTIC COMMUNITY STRUCTURE

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ABSTRACT: Optimization of anaerobic digestion (AD) of lignocellulosic substrates via bioaugmentation for enhanced biomethane production is imperative. Here, eight different screened bacterial isolates were used to augment different batch anaerobic digesters that comprised freshly chopped water hyacinth (WH) and cow dung (CD), at 2% total solids (w/v). Methane production was monitored at intervals and DNA metabarcoding of the 16S rRNA genes revealed the dynamics of the prokaryotic community structure of the anaerobic digesters. Obtained results suggest bioaugmentation improved the abundance and diversity of the prokaryotic community. The treatment that was inoculated with *Serratia marcescens* produced the highest cumulative methane of 0.68 L, 45.6% methane more than the consortium treatment. A shift from *Pseudomonas* to *Bacteroides* was observed in the bacterial community at the genus taxonomic level after AD while *Methanosarcina* dominated the archaeal genera. Furthermore, independent bioaugmentation of AD of different substrates such as water hyacinth, cow dung, and cellulose powder with identified model bioaugmentation agent, *Serratia marcescens* portrayed a positive effect with regards to methane production when compared with the control treatment. This study represents an economic and environmentally friendly approach of single isolate inoculation (bioaugmentation) in optimizing methane production from lignocellulosic substrates such as water hyacinth.
Keywords: Bioaugmentation, water hyacinth, *Serratia marcescens*, lignocellulosic, prokaryotic, methane.

1 INTRODUCTION

Biomass of lignocellulosic origin is widely available and provides the most abundant carbon-based feedstock, cellulose [1]. The complex physical and chemical structure of lignocellulosic biomass is due to the tightly connected polymeric components and the stabilized structure of cellulose (increased number of OH groups) as a result of the β-1,4-glycosidic linkages of cellobioses (glucose disaccharides) [2,3]. These pose a crucial challenge to the production of biogas during anaerobic digestion (AD) due to decreased bioavailability of the lignocellulosic components of the biomass [4, 5]. Previous studies have identified hydrolysis as the rate-limiting step of AD of lignocellulosic substrates; this could result in reduced methane production, generation of toxic metabolites during AD, or complete digester failure [6-8]. However, optimization techniques such as pretreatment, co-digestion, and bioaugmentation with microbial cultures could mitigate the aforementioned challenges [9-12]. Some pretreatment techniques are marred by challenges including cost intensiveness which relates to time consumption, labor cost, and high energy demands [13-15]. Possible loss of the cellulose fraction of the substrate [12], the toxicity of pretreatment chemicals on the indigenous microbial population and diversity [13,15], generation of toxic compounds [16] as well as subsequent processing of generated hydrolysates before AD [17] are all potential limitations of pretreatments methods. Incorporation of co-substrates (co-digestion) like animal manure during AD of lignocellulosic substrates is an economical strategy due to microbial abundance, nutrient variety, and improved synergistic effects [18-22]. Notwithstanding the inclusion of a co-substrate, the addition of active microbial cultures to enhance a specific stage or the overall AD process will be ideal for improved biogas production from lignocellulosic substrates.

The inclusion of active microbial strain/s to the indigenous microbial community of an anaerobic digester to improve the digestibility of substrates and enhance productivity is termed bioaugmentation [23]. Bioaugmentation enhances the bioavailability of the polymeric lignocellulosic fractions to hydrolytic enzymes through disruption of the glycosidic bonds that bind the lignocellulosic matrix. Additionally, bioaugmentation could potentially mitigate the challenges of inhibition posed by intermediary compounds and the problem of unfavorable microbial diversity shift during AD [24, 22]. The success of any bioaugmentation procedure is dependent on the metabolic efficiency, adaptability, survival, growth, and washout rate of the inoculated microbes [25]. The metabolism of lignocellulosic substrates is due to the action of cellulases secreted by microorganisms [26, 27]. Microorganisms such as fungi are potential cellulase producers [28], but their high production cost and slow or inhibited proliferation due to unfavorable environmental conditions pose a challenge to utilizing them as bioaugmentation agents [29]. Furthermore, the complex growth, energy, and respiratory needs of anaerobic fungi could result in the loss of carbohydrates during AD [30, 31]. Bacteria are ideal for anaerobic bioaugmentation processes due to their resilience and adaptability to different environments. Their high rate of proliferation and metabolic efficiency increase their suitability for such processes [29]. A previous study reported that bioaugmentation with cellulolytic bacteria caused a shift in the archaeal population and the out competition of the inoculated strain [32]. This is in contrast with the findings of Tsapekos et al. [33] which reported no effect on the microbial community structure after bioaugmentation with different cellulolytic bacteria. Understanding the metabolic features of microbial

community structure during bioaugmentation is required for an effective AD process. We hypothesize that bioaugmentation of anaerobic digesters with bacterial isolates improves methane production from water hyacinth. The study also investigates the effect of bioaugmentation on the diversity of the prokaryotic community structure of the digesters.

2 MATERIALS AND METHODS

2.1 Substrate sampling

The whole water hyacinth plant was harvested from the Hartbeespoort dam, Madibeng district of North West province, South Africa (25°44′ 51″S 27° 52′ 1″E) while the cow dung was obtained from the dairy parlor of Agricultural Research Council – Animal production (ARC – AP), Irene, Gauteng province South Africa (25º 53' 59.6" S 28º 12' 51.6" E). Samples were transported aseptically to the biogas laboratory of Agricultural Research Council – Soil Climate and Water for storage until usage. Some characteristics of substrates are as follows; Water hyacinth- Dry matter (5.97%); Volatile solids (4.46%); pH (8.11); carbon-nitrogen ratio (14.5). Cow dung- Dry matter (16.8%); Volatile solids (14.16%); pH (8.34); carbon-nitrogen ratio (23.7).

2.2 Characterisation of bacteria for bioaugmentation

Digestate was sampled from a 20 L semi-continuous stirred tank reactor after 80 days of AD. The reactor ran at a mesophilic temperature of 30°C with water hyacinth and cow dung as substrates. Bacteria were isolated from 1 ml of digestate samples according to the method reported in Mukhuba et al. [34]. Characterization of bacteria for hydrolytic abilities was conducted by spot plating 5 µl of nutrient broth overnight grown bacterial cultures on sterile carboxymethyl cellulose (CMC) agar as detailed in Obi et al. [35].

Bacterial isolates were identified based on their partial 16S rRNA gene sequences. Amplification of the partial 16S rRNA genes of the isolates was conducted with Colony Polymerase Chain Reaction (PCR) using universal primers 27F and 1492R, as described by Obi et al. [36]. Amplicons were verified by 1% agarose gel electrophoresis and the 16S rRNA genes were sequenced by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. The 16S rRNA gene sequence electropherograms were manually edited with BioEdit (v7.2.50) and CLUSTALW software. Taxonomy assignment was based on the alignment of the 16S rRNA gene sequence data with their closest relatives in the GenBank using the Basic Local Alignment Search Tool (BLAST) program of the National Centre for Biotechnology Information (NCBI). The partial 16S rRNA gene sequences are available in the GenBank under the accession numbers MK104459, MK104464, MK104469, MK104485, MK104517, MK104523, MK104525, and MK104526.

2.3 Batch culture analysis of methane production efficiency through different bioaugmentation approaches

Evaluation of the efficacy of different bacterial isolates to enhance methane production via different approaches was analysed with batch culture assays. A working volume of 250 ml comprising of freshly chopped water hyacinth (2cm x 2cm) and cow dung in the ratio of 2:1, at 2 % TS was set up in 500 ml Schott batch culture bottles fitted with screw caps containing septa (Silicone cream, Hardness 55°, shore A, Thickness 3.2mm) (Monitoring & Control Laboratories (PTY) LTD, Johannesburg, South Africa). Enumerated bacterial cells at OD_{600} = 1.5 (10^8 – 10^9 CFU/ml) [37, 38] were used to inoculate the batch bottles and treatments were set up in triplicate with appropriate controls as shown in Table I:

Table I: Bioaugmentation strategies with batch culture assay set up of water hyacinth and cow dung

Treatment name	Treatment composition
3H	water hyacinth + cow dung + 10^9 cfu/ml 3H
3M	water hyacinth + cow dung + 10^9 cfu/ml 3M
4F	water hyacinth + cow dung + 10^9 cfu/ml 4F
7B	water hyacinth + cow dung + 10^8 cfu/ml 7B
11H	water hyacinth + cow dung + 10^9 cfu/ml 11H
12H	water hyacinth + cow dung + 10^9 cfu/ml 12H
D31B	water hyacinth + cow dung + 10^8 cfu/ml D31B
D31D	water hyacinth + cow dung + 10^9 cfu/ml D31D
Consortium	water hyacinth + cow dung + 10^9 cfu/ml 3H + 10^9 cfu/ml 3M + 10^9 cfu/ml 4F + 10^8 cfu/ml 7B + 10^9 cfu/ml 11H + 10^9 cfu/ml 12H + 5% (v/v) 10^9 cfu/ml D31B+ 10^9 cfu/ml D31D
Control	water hyacinth + cow dung

The batch reactors were not flushed with nitrogen gas and the digestions were carried out anaerobically as batch cultures at 30°C pending reduced CH_4 and CO_2 production after 35 days at 120 rpm (revolutions per minute). Biomethane production was monitored through Gas chromatography (GC) (SR1 8610C, CHROMPEC, Canada). The gas chromatograph is equipped with a thermal conductivity detector (TCD) and HayeSep D packed column for the analysis. With reference flow of 20 ml/min and makeup flow of Helium carrier gas at 10 ml/min, the temperature of the TCD was set at 155°C. The initial oven temperature was set at 50°C and held for 4 min, initial ramp temperature of 20°C, and a final temperature of 220°C. Two milliliter aliquots of gas were sampled from the headspace of the batch culture bottles employing a gas-tight syringe with a Luer lock valve (SGE 10MDR-VLLMA-GT). Sampled aliquots were injected into the GC for analysis of biomethane production at three days intervals [24].

2.4 Anaerobic digestion of different substrates with bacterial isolate 11H

The bacterial isolate, 11H contributed to the highest cumulative methane generated from treatment 11H and was identified as *Serratia marcescens.* The isolate was further assessed for its metabolic abilities using different substrates. The substrates refer to water hyacinth, cow dung, and cellulose powder. For each treatment, 250 ml working volume of chopped water hyacinth (2 cm x 2 cm), cow dung, and cellulose powder all at 2% TS were respectively inoculated with 5% (v/v) of enumerated (10^9 CFU/ml) pure cultures of *Serratia marcescens* in 500 ml Schott Batch bottles. Appropriate controls without the bacterial isolate *Serratia marcescens* were set up as shown in Table II and the experiment was conducted in triplicates. Treatments were incubated for 35 days at 30°C in a

rotatory incubator. Methane production was monitored at intervals using the method outlined in section 2.3.

Table II: Anaerobic digestion of different substrates with *Serratia marcescens*

Treatments name	Treatment composition
WHSM	Water hyacinth + 10^9 cfu/ml of *Serratia marcescens*
CDSM	Cow dung + 10^9 cfu/ml of *Serratia marcescens*
CPSM	Cellulose powder + 10^9 cfu/ml of *Serratia marcescens*
WH	Water hyacinth
CD	Cow dung
CP	Cellulose powder

Treatment WHSM refers to mono-digestion of water hyacinth with *Serratia marcescens*; CDSM equates monodigestion of cow dung with *Serratia marcescens* and CPSM refers to the AD of cellulose powder with *Serratia marcescens*.

2.5 Preparation of 16S rRNA gene libraries and bioinformatics

Genomic DNA was extracted with a DNeasy PowerSoil kit (Whitehead Scientific (Pty) Ltd) according to the manufacturers' instructions from 2 ml aliquot of the feedstock and digestate samples from all the treatments in section 2.3 including controls. Extracted DNA from all samples was quantified with Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) and standardized to an equal concentration of 5 µg/ml with nuclease-free water [39]. Illumina barcodes were attached to the corresponding 5'-end of the primer sets 341F and 806R and the V3-V4 hypervariable region of the 16S rRNA was amplified with the barcoded primers [40, 41]. Preparation of 16S rRNA library was performed according to Van Wyk et al. [42] and the paired-end (2 x 300bp) 16S rRNA gene libraries were sequenced on the Illumina MiSeq Sequencer (Illumina, Inc., CA, USA) at the Agricultural Research Council – Biotechnology Platform, Pretoria, South Africa.

Generated sequences were imported into the Qiime2 pipeline (v. 2020.8, https://docs.qiime2.org/2020.8/) as paired-end sequences. Quality check of the generated reads with the 'qiime demux summarize' command of the Qiime2 pipeline revealed poor quality reverse reads, hence data analysis proceeded with just the forward reads as single-end sequences. Generation of amplicon sequence variants (ASV) were performed via denoising with the DADA2 plugin [43]. Annotation and organization of ASVs were conducted by training with q2-feature-classifier plugin in naïve Bayes classifier against the SILVA rRNA database (release 132) using the V3-V4 regions of the 16S rRNA genes [44]. Generation of taxonomic barplots was executed and normalization of data via subsampling without replacement (rarefaction) to even depth across samples was performed. Rarified data was applied in the diversity metrics analyses [45]. Prediction of metabolic abilities of the 16S rRNA community was performed with the software package, Piphillin (https://piphillin.secondgenome.com) [46, 47]. Generated SILVA-based sequence variants were converted into metabolic profiles via a pre-computed association matrix that relies on the prokaryotic Kyoto Encyclopedia of Genes and Genomes (KEGG) database (KEGG May 2020 version) (http://www.genome.jp/kegg/) at 99% identity cut-off. Functional attributes (enzyme-coding genes) with 16S rRNA genes were executed based on KEGG Orthology (KO). Generated sequences have been deposited in the Sequence Read Archive (SRA) NCBI within BioProject PRJNA703044 as an experiment, 'Prokaryotic community structure of anaerobic digesters' under the biosample accession numbers SAMN18008289 - SAMN18008308, SRA accession numbers SRR13754227 - SRR13754246.

2.6 Statistical analysis

One-way analysis of variance (ANOVA) was used to assess the mean differences between different treatment groups. Data were normalized for parametric tests with Log or square root transformation for evenness. Where normality could not be attained, Kruskal-Wallis non-parametric tests were used to evaluate statistics. Tukey Honest Significant Difference (HSD) was used for pair-wise comparisons at a 5% significance level ($P < 0.05$). Analyses of microbial community structure were based on 97% rRNA gene sequence similarity or otherwise stated. The microbial community structure was envisaged with non-metric dimensional scaling (NMDS) and metric dimensional scaling (MDS) in multivariate spaces. Differences between treatment groups in multivariate spaces were conducted based on the Bray-Curtis dissimilarity with permutational multivariate analysis of variance (PERMANOVA). All statistical analyses were performed in Excel 2013, R software (v.4.03) as well as Qiime2 (v. 2020.8, https://docs.qiime2.org/2020.8/).

3 RESULTS

3.1 Characterisation of bacteria for bioaugmentation

A total of eight hydrolytic bacteria obtained from the digestate was used for the bioaugmentation studies. Taxonomy assignment based on the 16S rRNA gene sequence data identified sequences to be homologous to the bacteria outlined in Table III below:

Table III: Identity and features of bacterial isolates used for bioaugmentation study

Bacterial Isolates' ID	Most likely taxonomic species	Sequence similarity (%)	Accession number	Cellulolytic index
3H	*Pseudomonas stutzeri*	99	MK104459	1.57
3M	*Exiguobacterium mexicanum*	99	MK104464	1.19
4F	*Bacillus cereus*	100	MK104469	1.84
7B	*Lysinibacillus fusiformis*	100	MK104485	1.17
11H	*Serratia marcescens*	99	MK104517	1.952
12H	*Brevundimonas vesicularis*	99	MK104523	0.46
D31B	*Acinetobacter iwoffi*	99	MK104525	0.5
D31D	*Planococcus maritimus*	100	MK104526	0.1

3.2 Batch culture analysis of biogas production efficacy

All treatments exhibited negligible methane production before day 13 of AD (Fig. 1). This could be due to acclimatization and unfavourable environmental conditions for methanogens. The highest methane production was observed in treatment 7B between days 13 and 25 while the highest cumulative methane of 0.68 L was

produced by treatment 11H. A 45.6% difference between the treatments that produced the highest methane and least methane was observed. Cumulative methane production remained below 0.63 L for other treatments. A significant difference was observed among treatments ($P < 0.05$). Further post hoc tests (Tukey HSD) showed a difference between 11H and other treatments.

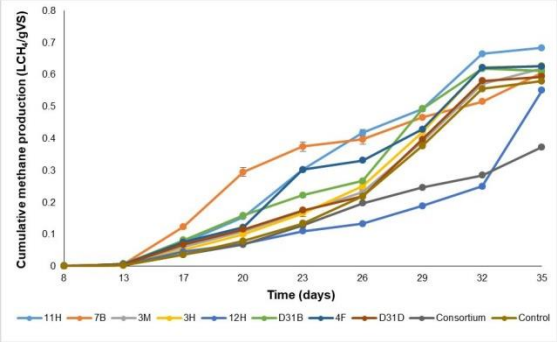

Figure 1: Cumulative methane yield from anaerobic co-digestion of water hyacinth and cow dung inoculated with pure bacterial isolates. Error bars represent standard deviation (n=3)

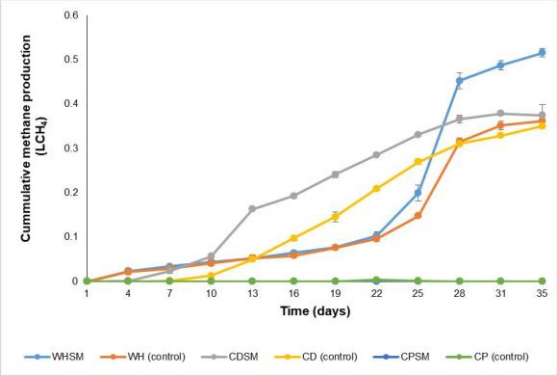

Figure 2: Cumulative methane yield from monodigestion of water hyacinth (WH), Cow dung (CD) and Cellulose powder (CP) respectively inoculated with *Serratia marcescens* 39_H1 (SM). Error bars denote standard deviation (n=3)

Fig. 2 displays the effect of inoculated bacterial strain, *Serratia marcescens* 39_H1, on methane production from respective monodigestion of water hyacinth (WHSM), cow dung (CDSM), and cellulose powder (CPSM). Enhanced metabolic effect of the inoculated model bacteria, *Serratia marcescens was* exhibited in treatments WHSM and CDSM, which produced the highest cumulative methane yield of 0.52 L and 0.37 L, respectively. Methane production was negligible in treatments CPSM and CP during the 35 day AD trial when measured with a GC as depicted in Fig. 2 but yielded some carbon dioxide (data not shown). Comparison of the treatments' means with regard to methane production indicated a significant difference among the treatments as $P = 0.00026$. Significant variation was observed between the following treatment pairs: WHSM and CPSM; WHSM and CP; CDSM and CPSM; CDSM and CP. However, on exclusion of treatments CPSM and CP from the variation test since they recorded no methane, no significant difference was observed amongst the treatments.

3.3 Preparation of 16S rRNA gene libraries and bioinformatics

Classification and rarefaction of generated partial 16S rRNA gene sequences (ASVs) to an even depth of 17440 revealed that greater diversity and abundance was observed in treatments after AD than treatments before AD (Fig. 3). Significant difference ($P < 0.05$) was observed in all the alpha diversity metrics, evenness index ($P = 0.038$), Faith's phylogenetic diversity ($P = 0.009$), Shannon diversity index ($P = 0.012$). Evaluation of the bacterial community structure via the beta diversity analyses showed distinct separation of species after AD (Fig. 4). As supported by the Bray Curtis dissimilarity amongst bacterial communities (NMDS and cluster dendrogram in Fig. 4A and Fig. 4B respectively), differences in bacterial community structure were exhibited. Significant distinction was also observed in the unweighted (PERMANOVA, $P = 0.001$; $R^2 = 0.284$) and weighted (PERMANOVA, $P = 0.001$; $R^2 = 0.724$) unifrac distance metrics (Fig. 5). This signifies the effect of AD on the bacterial community during biogas production.

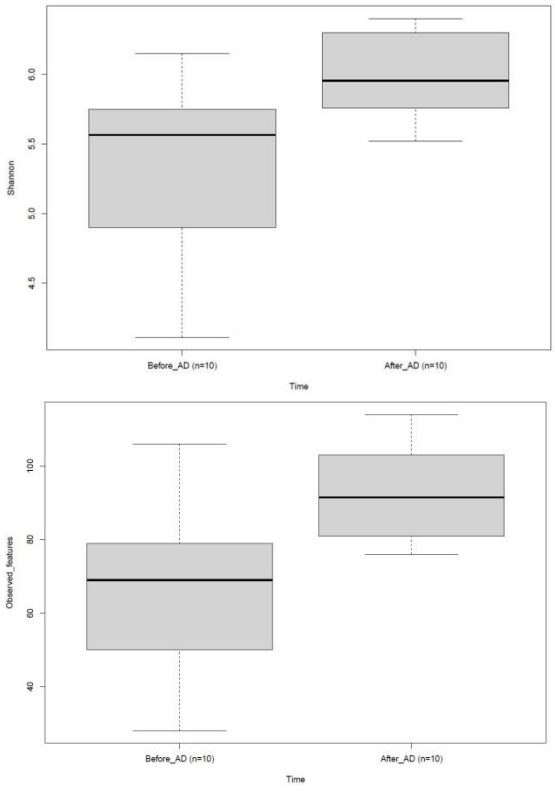

Figure 3: Alpha diversity analysis (quantitative measure of community richness) of bacterial community before and after AD. Diversity measure of (**A**) Shannon diversity index and (**B**) number of observed features (ASVs)

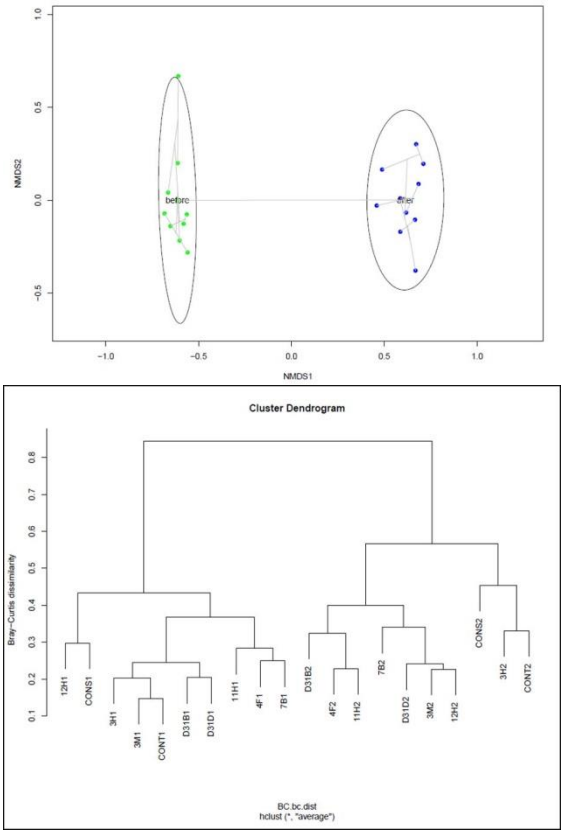

Figure 4: Bray curtis dissimilarity amongst bacterial communities (**A**) Non-metric dimensional scaling (NMDS) plot and (**B**) Agglomerative cluster dendrogram showing hierarchy. Stress plot is on Supplementary data, Fig. S1 with a stress of 0.055. Construction of NMDS and cluster dendrogram were respectively executed with vegan (v. 2.5.7) and dendextend (v. 1.14.0) packages of R software (https://cran.r-project.org/).

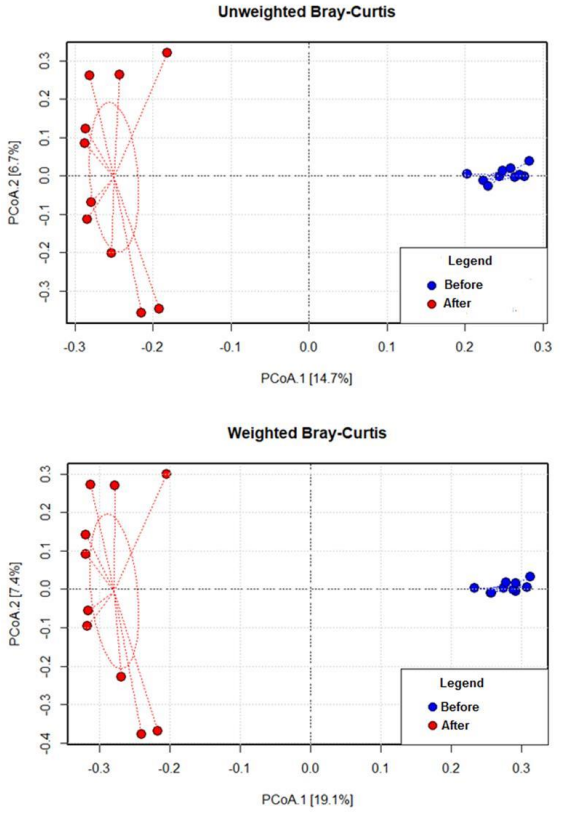

Figure 5: Principal coordinate analysis (PCoA) of Bray-Curtis dissimilarity between bacterial communities before and after AD (A) Unweighted unifrac distances and (B) Weighted unifrac distances. The ordispider in the plots display the connection of each treatment to the group centroids and the ellipses indicate a confidence interval of 95% around the centroids of the groups for the treatments 'before AD' and 'after AD'. PERMANOVA, P = 0.001.

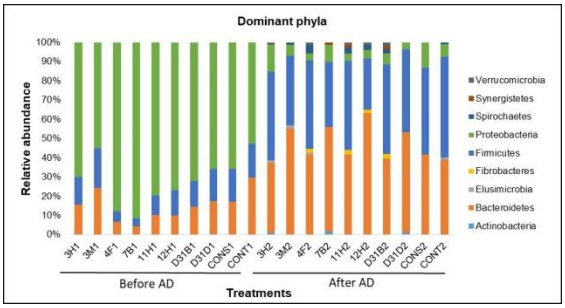

Figure 6: Relative abundance of dominant bacteria (≥1% average relative abundance) at the phylum taxonomic rank in different digestion treatments

Subsequent trained taxonomic classifier using the V3-V4 regions of the 16S rRNA genes from the SILVA database showed taxonomy barplots at the hierarchical taxonomic classifications. The phylum, Proteobacteria, dominated all treatments before AD (about 51% - 91%) (Fig. 6). Other relatively abundant phyla included *Bacteroidetes* and *Firmicute*s. However, significant dominance by *Bacteroidetes* and *Firmicutes* was observed after AD in line with a corresponding reduction of proteobacteria. Increased phylal classification was also observed after AD. In addition, phylal groups such as

Spirochaetes, *Verrucomicrobia*, *Synergistetes*, *Fibrobacteres*, *Elusmicrobia* as well as *Actinobacteria* were first observed in treatments after AD.

The genus taxonomic hierarchy of the bacterial community of the treatments as stated in Fig. 7 showed the dominance of *Pseudomonas* in most treatments before AD (≈ 21% - 77%). Treatments with a low concentration of *Pseudomonas* sp. (CONS2 and 12H) produced the least methane. However, *Bacteroides* became the dominant genus in treatments after AD. Increased diversity in genera after AD showed dominant genera (≥1% relative abundance) as *Advenella*, *Serratia*, *Ruminococcus*, *Enterobacter*, *Dysgonomonas*, etc. Comparable bacterial species richness is represented in the Supplementary data, Fig. S2. Similar microbiota was observed among the treatments before bioaugmentation. However, a shift in the microbiota was observed in some treatments after AD. Among the inoculated bacterial genera, *Serratia* was the only detected genera after bioaugmentation. Other inoculated bacterial genera were not identified.

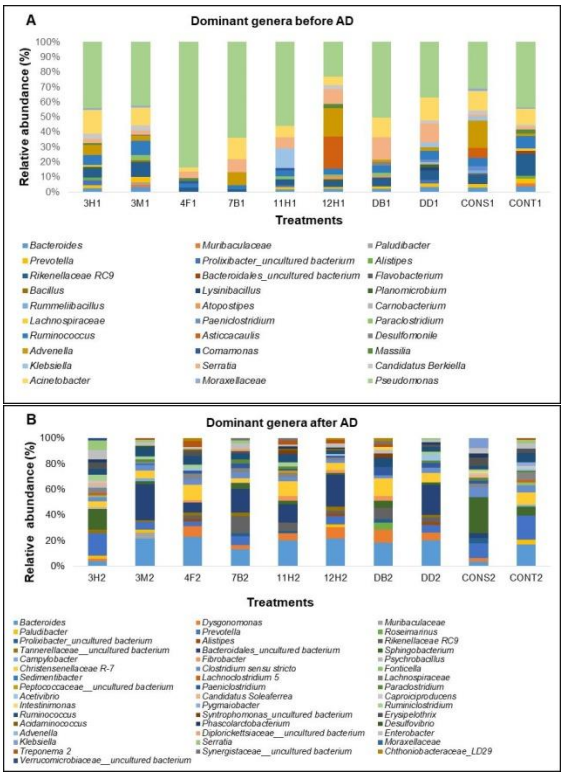

Figure 7: Relative abundance of dominant (≥1%) bacteria genera (A) Before AD (B) After AD. Genera with average relative abundance of below 1% were omitted.

Taxonomic classification of the 16S rRNA microbial community revealed the presence of the archaeal community in the treatments. The identified archaeal population represented ≤1% of the prokaryotic population in most of the treatments except treatments 3H2, 7B2, 11H2, and D31B2. Identified archaea belong to two archaeal phyla, *Crenarchaeota* and *Euryarchaeota*. The plot on Fig. 8 exhibited the absence of archaea in the treatments before AD except for treatment CONT1 and it consisted of just one archaeal
genus, *Methanocorpusculum. Methanosarcina* dominated the archaeal population in the treatments after AD.

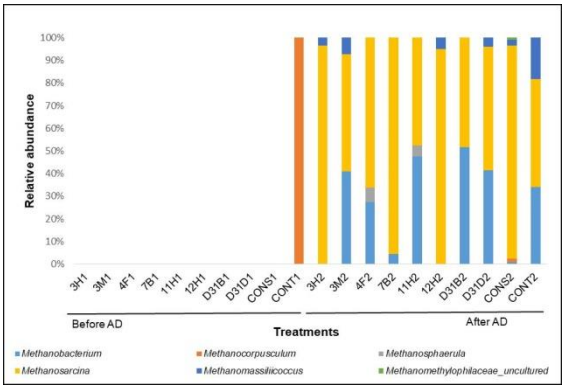

Figure 8: Average relative abundance of archaea at genus taxonomic rank in the different digestion treatments

3.4 Predicted functional diversity of 16S rRNA community

A total of 7690 KEGG Orthology (KO) terms was predicted from the entire 16S rRNA microbial community. Functional potential of the microbiome of the anaerobic digesters, as predicted by 16S marker genes, exhibited the presence of genes that encode the AD pathways. According to Fig. 9, identified genes of hydrolysis include endoglucanase (K01179), exoglucanase (K19668), and β-glucosidase (K05349) of which β-glucosidase (K05349) dominated across all treatments. Fig. 10 represents the genes of the three methanogenic pathways, acetoclastic, hydrogenotrophic, and methylotrophic methanogenic pathways. The range of abundance of the genes that encode the basic three AD methanogenic pathways is 35.9% - 90.38% acetoclastic, 2.5% – 46.19% hydrogenotrophic, 3.3% – 24.7% methylotrophic methanogenic genes. Acetoclastic methanogenic genes which include acetate kinase (K00925); phosphates acetyltransferase (K00625) and acetate-CoA synthetase were present but relatively not as abundant in the treatments as acetyl-CoA synthase and methyltransferase (K00194 and K14138).

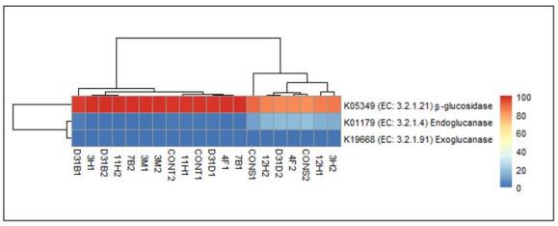

Figure 9: Relative abundance of KEGG Orthology (KO) features associated with hydrolysis

Genes related to hydrogenotrophic methanogenesis were identified to be more diverse but less abundant when compared with the genes related to acetoclastic methanogenesis. The treatment 11H which produced the highest methane was dominated by hydrogenotrophic methanogenic genes (46.2%) and has a relative abundance of 39.6% acetoclastic methanogenic genes and 14.1% methylotrophic methanogenic genes. In contrast, the least methane yield was obtained from treatment CONS which was dominated by acetoclastic methanogenic genes (90.3%) and a relative abundance of 0.36% and 9.25% of hydrogenotrophic and methylotrophic methanogenic

genes, respectively. Treatments 4F, 11H, and 12H had the least abundance of acetoclastic genes.

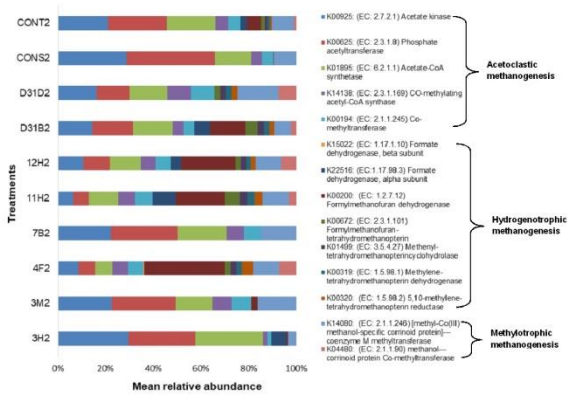

Figure 10: Relative abundance of KEGG Orthology (KO) features associated with predicted acetoclastic, hydrogenotrophic and methylotrophic methanogenic pathways

4 DISCUSSION

The metabolic features of the prokaryotic microbial community during bioaugmentation form the basis of an efficient AD process. The process of bioaugmentation with hydrolytic bacteria is a viable option to enhance methane production [48-50]. This study investigated the potential of different bioaugmentation strategies to improve productivity from co-digestion of water hyacinth and cow dung as well as improve the diversity of the prokaryotic microbial community after AD.

There was negligible production of methane at the initial stages of AD. The acclimatization of the inoculated microbes and no sparging of the digester with nitrogen gas before AD extended the time for methane production. Methane produced during AD resulted from the synthrophic activities of the complex digester microbiota. The prokaryotic community of the anaerobic digesters is of immense benefit to the AD process and this is attributed to their ability to survive in sensitive environments like anaerobic digesters and participate in various phases of AD. Treatment 11H produced the highest cumulative methane as opposed to the consortium treatment that generated the least methane, possibly due to the enhanced hydrolytic rate of the inoculated bacteria [35]. Further exploration of the bioaugmentation agent that enhanced the metabolism of the substrates in treatment 11H exhibited the potential of the bioaugmentation agent to enhance the metabolic process of AD of different substrates for subsequent methane production. The incorporation of this bacterium hydrolysed cellulose powder but the subsequent phases of AD (acetogenesis and methanogenesis) were inhibited due to absence of relevant microbes that promote such phases of AD.

Observed limited survival and metabolic characteristics of the inoculated consortial bacteria (CONS) were probably a result of the competition for nutrients among the inoculated bacteria due to the abundance of hydrolytic bacteria [32]. The significant distinctions in the microbial community structure of the treatments before and after AD as exhibited by the α-diversity indices described the increased abundance and diversity of the 16S rRNA gene sequence prokaryotic community. Improved prokaryotic community diversity correlates to improved microbial activities and subsequently increased methane production efficiency of the treatments. Further exploration of the bacterial community structure suggested a significant similarity at the phylum level among the 'before AD' treatments (Fig. 4 & Fig. 5). According to previous reports, the phyla, Proteobacteria, Firmicutes, and Bacteroidetes are renowned for their dominance in anaerobic digesters [51, 52], which is consistent with the result of this study where the 'before AD treatments' were dominated by Proteobacteria (>50%) and subsequently, Firmicutes and Bacteroidetes. The relative abundance of Proteobacteria before AD could be attributed to their natural occurrence in the mammalian gut (cow dung) and its inherent adaptability [53]. Proteobacteria are metabolically diverse and different groups of Proteobacteria have been associated with hydrolysis and acidogenesis during AD [54]. However, the reported decline in the relative abundance of Proteobacteria in the 'after AD' treatments and a corresponding increase in the abundance of Firmicutes and Bacteroidetes were suggestive of their adaptability and survival abilities during AD. The predominance of Bacteroidetes after AD is consistent with previous studies [55, 56]. The observation of a high abundance of Firmicutes in treatments that recorded high cumulative methane production suggests the fermentative abilities of Firmicutes [57, 58]. However, increased abundance of Firmicutes has been associated with increased ammonia concentration, which is a potent inhibitor of methanogenesis [22]. The increased relative abundance of Synergistetes in treatments with high methane is associated with organic and amino acid metabolism as well as synthrophic acetate oxidation by the aforementioned bacterial phylum [59].

The observed bacterial community shift at the genus taxonomic rank showed a significant reduction in the abundance of *Pseudomonas* across the 'after AD' treatments in comparison to their dominance 'before AD'. This contradicts the report of Makofane [60] where *Pseudomonas* was dominant at the end of the metabolic process of AD. *Pseudomonas* sp. is metabolically diverse and adaptable and has been linked with carbohydrate metabolism [61, 62]. *Serratia* amongst *Pseudomonas*, *Exiguobacterium*, *B acillus*, and *Lysinibacillus* are facultative anaerobes, however, the presence of *Serratia* sp. before and after bioaugmentation is a reflection of its survival abilities in such a complex and sensitive environment. The facultative anaerobes, though not resilient, potentially improved hydrolysis of substrates and subsequent increase in methane production in the majority of the treatments [35]. Inoculated microbes including *Serratia* did not thrive during the batch culture assay as they were not recorded as the predominant bacteria (>1%) after the bioaugmentation process. Suppression of the inoculated bacteria after bioaugmentation indicates their inability to acclimatize and thrive among the indigenous microbiome in the anaerobic digesters. Additionally, this could also be due to possible predilections experienced during sampling, high throughput sequencing techniques, and data analysis [33, 63]. The changes in the bacterial population are an indication of the impact of bioaugmentation and AD. Production of high cumulative methane from the treatment that was dominated by the genus, *Bacteroides* and production of the minimum quantity of cumulative methane from the treatment with the least abundance of

Bacteroides (2.6%) explains the bacteria's cellulolytic and fermentative characteristics of metabolizing cellobiose to acetic acids [64]. Based on hydrolysis and acidogenesis, identified genera *Ruminicoccus*, *Dysgonomonas*, *Ruminiclostidium* and *Enterobacter* have been associated with cellulose degradation for subsequent methane production [65-67]. The genera Ruminiclostridium was identified in most of the treatments, species of the genera contribute to the metabolism of cellulose and hemicellulose to different volatile fatty acids and sugars [68]. Some other identified bacterial genera have been previously observed in hydrolysis and they include *Bacillus*, *Butyrivibrio*, *Cellulomonas*, *Clostridium*, *Desulfovibrio*, *Enterobacterium*, *Micrococcus*, and *Streptococcus* [8, 26]. The dominance of Pretovella in the treatments relates to their adaptability and metabolic abilities [68]. The presence of *Christensenellaceae* R-7 in treatments with high methane production suggests their hydrolytic and acidogenic abilities [69]. *Sphingobacterium* sp. is known for the production of the CoA transferase enzyme of the citric acid cycle which catalyzes the acetogenic phase of AD [70].

The enzyme, β-glucosidases also known as cellobiases work interactively with endoglucanases and exoglucanases to release glucose from the non-reducing end during the breakdown of cellulolytic fractions of lignocellulolytic substrates [27]. β-glucosidases produced by microbes are known as the basic rate-limiting enzyme of cellulose hydrolysis and an essential group among the glycoside hydrolases that are classified under EC 3.2.1.21 [71, 72]. The abundance of β-glucosidases (cellobiases) across all treatments could be associated with the abundance of cellobiose in the AD system. As stated by Tao et al. [72], the intermediary product of cellulose degradation, cellobiose usually hinders the presence of endoglucanases and exoglucanases. This supports the limited abundance of endoglucanases and exoglucanases as shown in Fig. 9.

Effective production of methane during AD is reliant on the activities of the archaeal community as the correlation of methane production and the concentration of archaea revealed that the top methane-producing treatments had the highest concentration of archaea (≥ 1%). The fact that no archaeal community was identified in the treatments before AD supports the notion that the metabolic process of AD enhances the growth and diversity of the archaeal community due to their potential utilization of hydrolyzed products as well as providing an environment that is conducive to archaeal proliferation [73]. Suppressed archaeal community before AD is not indicative of their complete absence but, as a result of their characteristics as strict anaerobes, and sensitivity to environmental conditions such as pH changes which could result from VFA or ammonia accumulation [74]. The archaeal community of the digesters was generally dominated by the phyla Crenarchaeota and Euryarchaeota. Archaeal community distribution implied that the AD process of methanogenesis was catalyzed by acetoclastic, methylotrophic, and hydrogenotrophic pathways. The genus, *Methanosarcina* was predominant in most of the treatments and its relative abundance was as high as 95% in some treatments. This is due to the metabolic diversity of the genus as they have been known to catalyze the generation of methane via all three basic methanogenic pathways [75]. The genus, *Methanosarcina* is highly adaptive and tolerant to high level of VFAs and ammonium nitrogen hence their abundance, however, these compounds are inhibitory to methanogenesis [76]. This could relate to the low production of methane by certain treatments with high *Methanosarcina* concentration (about 94%) [6, 77]. This also suggests a low abundance of acetoclastic methanogenic genes in treatments that portrayed optimal methane. The presence of *Methanosarcina* has also been associated with the stability and efficiency of the AD process owing to their potential to reduce acidification of anaerobic digesters via utilization of accumulated VFAs [78, 79]. The treatment with the highest cumulative methane yield showed co-occurrence of equal concentrations of *Methanosarcina* and *Methanobacterium* (47.7%) and 4.6% of *Methanosphaerula* which is known as a typical abundant methanogen in anaerobic digesters [70]. *Methanobacterium* and *Methanomassiliicoccus* iden tified in this study have been associated with enhancing the degradation of hemicellulose [80, 81] and *Methanobacterium* secretes enzymes that are encoded by genes of the hydrogenotrophic and methylotrophic methanogenic pathways [82]. The fact that the hydrogenotrophic methanogenic genes dominated the treatment with the highest cumulative methane yield ascribes to the efficiency of the dominant genes considering that the treatment with the least cumulative methane was dominated by acetoclastic methanogenic genes. Dominance by hydrogenotrophic methanogens indicated their potential to tolerate the toxicity of possible high ammonia content [7, 19]. The dominance of *Methanosarcina*, an acetoclastic methanogen in the treatment that produced the least methane (Fig. 8) correlates to its functional attributes (Fig. 10) as 90.36% of acetoclastic genes were identified in the same treatment. Overall, the bacterial and archaeal community was enhanced by bioaugmentation due to the observed shift in microbial community dynamics. Previous findings have shown the diversity of 16S rRNA prokaryotic communities in anaerobic digesters and the effect of bioaugmentation on such communities. This study displayed the occurrence of similar core microbiota in different digesters irrespective of the bacterial species used for bioaugmentation. In addition, functional prediction of genes associated with hydrolysis and methanogenesis was based on the prokaryotic community phylotypes. In view of the optimization of the metabolic process of AD of water hyacinth from the Hartbeespoort dam and its effect on the prokaryotic community, this study is remarkable.

Bioaugmentation of batch reactors with *Serratia marcescens* improved cumulative methane production by 45.6% as opposed to the consortium treatment that produced the least cumulative methane. The study thus infers the potential strategy of optimizing biogas production from lignocellulosic substrates via bioaugmentation with *Serratia marcescens*. Analysis of the prokaryotic community revealed a favorable microbial shift which suggests the beneficial effect of bioaugmentation on the diversity and metabolic abilities of the prokaryotic community. However, the inoculated bacteria portrayed a negligible effect on the prokaryotic community structure. Anaerobic digesters are prospective sources for bioprospecting of potential beneficial species due to the abundance and diversity of the prokaryotic community. Evaluation of the thriving or out-competition of the inoculated bacterial isolates during the continuous

anaerobic digestion process could provide more insight into their metabolic efficiencies.

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6 ACKNOWLEDGEMENTS

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7 SUPPLEMENTARY DATA

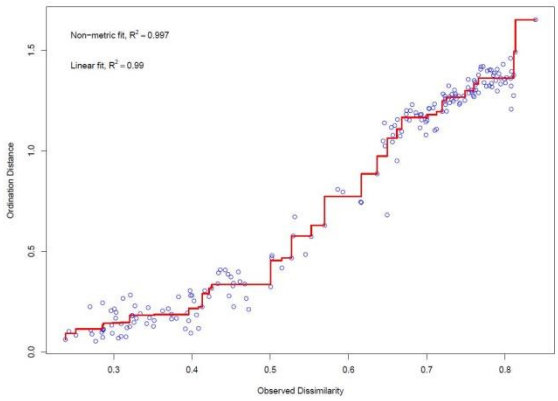

Figure S1: Stessplot for the NMDS plot on Fig. 4. Plot was generated to ascertain the integrity of the ordination fit. The 'stressplot' function of the vegan package of R software was used to create the stress plot.

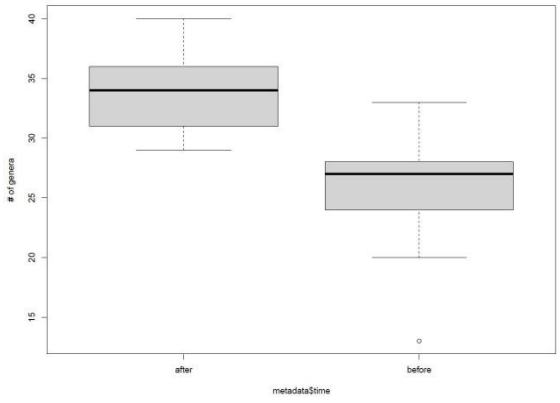

Figure S2: Comparison of bacterial species richness before and after AD