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Effect of removal of inhibitors on microbial communities and biogas yield of *Jatropha curcas* seeds during continuous anaerobic digestion

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ABSTRACT

Jatropha curcas seeds, as an abundant lignocellulosic biomass, offer a highly promising and ideal alternative for producing energy in the form of methane. Use of *J. curcas* seeds has the potential to significantly bolster the biofuel sector, fostering a more sustainable circular economy. In the current study, different fractions of processed *J. curcas* seeds were investigated for biogas production. *J. curcas* seed pressed cake, a by-product of biodiesel production, was subjected to methanolic extraction. The remaining solids, referred to as methanolic residues, yielded more biogas in batch experiments than pressed cake and residues from aqueous and *n*-hexane extractions. The compounds extracted with methanol inhibited hydrolysis and reduced biogas production by 35.5% compared to the same setup without extracts. In continuous reactors fed with methanolic residues, the highest biogas yield occurred at an organic loading rate (OLR) of 1 g VS L^{-1} day^{-1} and a hydraulic retention time (HRT) of 20 days. The relative abundance of acetogenic bacteria was higher in reactors fed with methanolic residues than in those fed with seed pressed cake, seed oil, and whole seed. Jatropha seed oil and whole seed did not inhibit methanogens. A higher relative abundance of methanogenic communities was observed in all reactors at HRT of 20 days compared to those at HRTs at 15 and 10 days. These findings can be used to increase biogas production during anaerobic digestion of *J. curcas* seed components and suggests a zero-waste biorefinery production route for value added compounds derived from the removal of biogas-inhibiting components.

1. Introduction

Biogas production through anaerobic digestion of feedstocks is promising due to reduced environmental impacts (Nielsen and Ahring, 2006). Many feedstocks, both edible and non-edible, have been reported as substrates for biogas production. Edible feedstocks lead to food versus fuel competition; non-edible feedstocks do not have this burden and are considered ideal for biogas production (Scarlat et al., 2015). Ideal feedstocks are non-edible crops grown on land not suitable for food production (Kurade et al., 2019). *Jatropha curcas,* an oil-rich, pest- and drought-resistant shrub, is considered a strong candidate for biodiesel production (Haq et al., 2020). The pressed cake, which remains after oil extraction for biodiesel production, can be used for biogas production without the food-vs-fuel tradeoff because it cannot be used as animal fodder due to considerable toxicity (EFSA Panel on Contaminants in the Food Chain CONTAM, 2015). However, the unprocessed cake inhibits microbial activities due to the presence of compounds such as phorbol esters, curcin, and long chain fatty acids (Haq et al., 2019; Scarlat et al., 2015). Anaerobic digestion efficiency could be increased if the antimicrobial phytochemicals are removed from *J. curcas* pressed cake by extraction with organic solvents such as methanol. The resulting residues of pressed cake (called methanolic residues [MR]), contain fewer inhibitory compounds, preserve the richness of the digester microbial community, and ultimately increase biogas yield. Additionally, the extracted inhibitory compounds are potentially valuable pharmaceuticals (Haq et al., 2019). This extraction approach suggests a sustainable biorefinery production route for valuable products and biofuels.

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Abbreviations

HRT	Hydraulic retention time
OLR	Organic loading rate
MR	Methanolic residues
NR	n-hexane residues
AR	Aqueous residues
JPC	Jatropha pressed cake
JWS	Jatropha whole seed
JO	Jatropha oil
VFAs	Volatile fatty acids
VS	Volatile solids
TS	Total solids
FTIR	Fourier transform infrared
GC-MS	Gas chromatography coupled with mass spectrometry
QIIME	Quantitative insights into microbial ecology
OTUs	Operational taxonomic units
CCA	Canonical correspondence analysis
ANOVA	Analysis of variance
LCFAs	Long chain fatty acids

Even with an acceptable substrate, biogas yield is highly affected by operational parameters. For example, higher organic loading rates (OLRs) are associated with increased volatile fatty acids accumulation leading to microbial inhibition (Ferguson et al., 2016). Further, if the hydraulic retention time (HRT) is too short there may be a washout of important slow-growing species, leading to poor biogas yields. Furthermore, single-stage anaerobic digestion is challenged at higher OLRs and shorter HRTs. In a single-stage setup, the higher OLRs and shorter HRTs favor acid forming bacteria, resulting in the accumulation of volatile fatty acids (VFAs) and inhibition of biogas production (Alavi-Borazjani et al., 2020). In contrast, two-stage anaerobic digestion can be sustained for longer durations even under high OLRs and short HRTs (Aslanzadeh et al., 2014), and even for highly degradable substrates (Alavi-Borazjani et al., 2020**).** To date, several studies have been conducted on the biogas potential of *J. curcas* seed (Sinbuathong et al., 2010, 2012; Steinbrenner et al., 2020) but none have evaluated the effect of seed toxicity and solvent extraction on microbial communities and biogas production during anaerobic digestion.

The goals of this study were to (1) assess the inhibitory effects of *Jatropha curcas* methanolic extracts on different steps of anaerobic digestion, (2) associate changes in the microbial community with different operating parameters, processing fractions, and biogas yields, (3) evaluate the biogas potential of processed *Jatropha curcas* seeds, and (4) identify operational parameters associated with increased biogas yields.

2. Materials and methods

2.1. Inocula and substrate preparation and characterization

Inocula used in batch and continuous reactor experiments were collected from a bioreactor fed with fruit and vegetable wastes and cattle manure at the Sustainable Bioenergy and Biorefinery Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad and were incubated at 37 °C for 2–3 weeks to allow for degassing. Specific methanogenic activities of inocula were determined as described previously (Astals et al., 2020). Specific methanogenic activities of inocula showed that the microbial communities were active (Fig. S1). Inocula were collected for four different experiments: for all batch methane potentials, for studying the effects of methanolic extracts on anaerobic digestion steps, for continuous reactors treating methanolic residues, and for continuous reactors studying whole seed, oil, and pressed cakes (Table S1).

The *J. curcas* seeds were obtained from a local dealer in Lahore, identified at the National Herbarium of Pakistan, Quaid-i-Azam University, and processed according to the flow schematic shown in Fig. 1. Oil was extracted from *J. curcas* whole seeds (JWS) using a mechanical oil expeller. After oil extraction, the remaining de-oiled Jatropha pressed cake (JPC) was further ground to a powder form and preserved in sterile zip-lock bags at −20 °C until further use. Measurement of total solids (TS), volatile solids (VS) and pH of substrates and inocula were performed according to Standard Methods (Federation and Association, 2005). The aqueous, methanolic, and *n*-hexane extracts of de-oiled Jatropha pressed cake were prepared as described previously (Haq et al., 2019). Fine powdered *J. curcas* de-oiled pressed cake (100 g) was dissolved in 500 mL of the individual solvents and incubated at 30 °C for 48 h. The extracts were filtered and the solvents evaporated using a rotary evaporator (Rotary Evaporator RE300 Stuart®) at reduced pressure. The resulting crude extract was allowed to dry at room temperature to a constant weight. The residues obtained from aqueous (AR), *n*-hexane (NR), and methanolic (MR) extraction were stored at 4 °C

Preliminary qualitative tests of *J. curcas* seed oil (JO) and de-oiled pressed cake extracts detected the presence of balsams, flavonoids, saponins, glycosides, steroids, phenol, and tannins (Haq et al., 2019). The phytochemicals were removed from *Jatropha* pressed cake using aqueous, methanolic, and *n*-hexane extraction. Fourier transform infrared (FTIR) spectroscopic analyses of *J. curcas* seed oil and JPC extracts were performed using standard procedures (Haq et al., 2019). The chemical composition of *J. curcas* seed oil and JPC extracts was determined using gas chromatography coupled with mass spectrometry (GC-MS) technique (GC-MS – QP5050A, Shimadzu, Europe) according to previously described methods (Basri and Fan, 2005; Mu'azu et al., 2013), with the following modifications. A 2 μL aliquot of each sample was injected separately into the column using an automated injector split ratio 1/48 (for extracts) and 1/25 (for seed oil). The column (DB-5) had a length 30 m, internal diameter 0.25 mm, and thickness 0.25 μm with flow rates of 1 and 1.8 mL min^{-1} for extracts and seed oil, respectively. The analytes were detected using a thermal conductivity detector (TCD). The National Institute of Standards and Technology library (NIST 27 and NIST 147) was used for peak identification based on mass spectra.

2.2. Anaerobic digestion

The biogas potential of *J. curcas* seeds and the effect of methanolic extracts of JPC on several substrates representing different degradation steps in anaerobic digestion (i.e., hydrolysis, acidogenesis, and acetoclastic methanogenesis) were evaluated in batch mode. The effect of methanolic extraction from JPC on microbial communities during anaerobic digestion was evaluated in the continuous process.

2.2.1. Biogas potential and inhibitory effects of methanolic extracts of pressed cake on different stages of anaerobic digestion

Batch anaerobic digestion was carried out in triplicate in 500 mL reactors with working volumes of 400 mL at 37 °C. The pH of all reactors was adjusted to neutral using 1 M solutions of HCl and NaOH. Before incubation, the reactors were flushed with nitrogen gas and sealed with butyl rubber corks. The biogas was collected in airtight bags (UNO-GUARD, China) made of a flexible, gas-tight high-density polyethylene (HDPE) material that can withstand the pressure of the generated biogas. The bags were attached to each reactor and biogas production was measured daily using a gas-tight syringe (Fig. 2). The airtight seal of the bag prevented the escape of the gas. Fig. 2 shows the schematic diagram of the test reactors and biogas collection system. The biogas volumes were collected at 37 °C and later normalized to standard temperature and pressure (273.15 K and 101325 Pa). The background biogas production was estimated using negative controls containing only inocula and was subtracted from the biogas yield of each substrate.

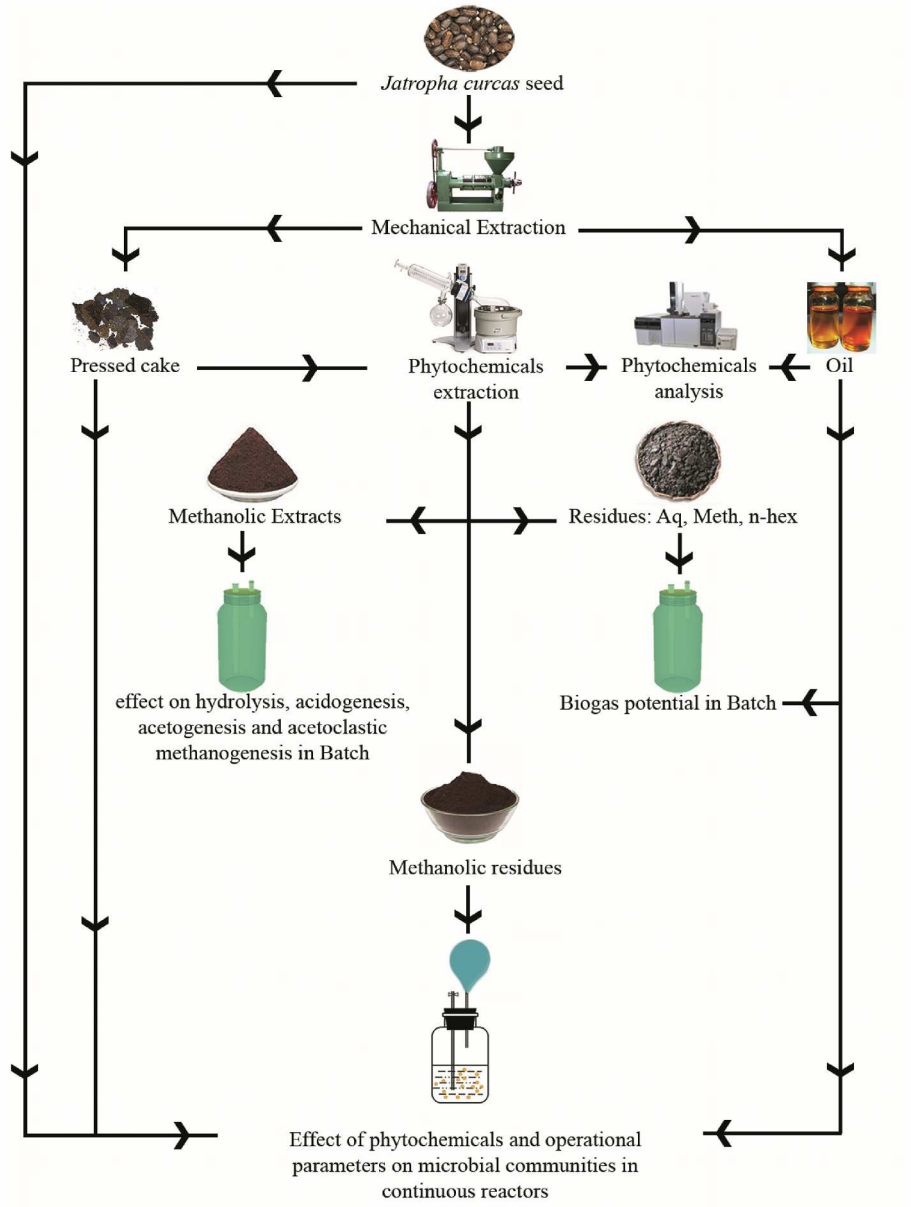

Fig. 1. Schematic representation of processing flow of *Jatropha curcas* seed.

The biomethane potentials of the substrates were evaluated by loading digesters at a ratio of 4 g VS of inocula to 1 g VS of substrate. The amounts of substrates added to their respective reactors were as follows; Jatropha whole seed (JWS, 2.22 g), Jatropha oil (JO, 1.89 g), Jatropha pressed cake (JPC, 2.5 g), methanolic residues (MR, 2.76 g), *n*-hexane residues (2.68 g), and aqueous residues (7.05 g). The positive controls, reactors with cooking oil (1.76 g) and cellulose (1.9 g) as substrates, were run in parallel with test reactors (Astals et al., 2014).

To evaluate the inhibitory effect of methanolic extracts on different steps in anaerobic digestion, cellulose (2.45 g, to investigate hydrolysis), glucose (2.9 g, to investigate acidogenesis), and sodium acetate (10.3 g, to investigate acetoclastic methanogenesis) were added to separate reactors with methanolic extract (2 mg mL^{-1}) and compared with the reactors without methanolic extracts. Methanolic extract with inoculum was used as negative control and the biogas produced was subtracted from the biogas produced by the test reactor. A reactor without methanolic extract was used as negative control in batch experiments.

2.2.2. Effect of operational parameters on biogas yield in continuous mode

In continuous mode, the anaerobic digestion of JWS, JO, JPC and MR was carried out at different organic loading rates (OLRs) and hydraulic retention times (HRTs) in 2.5 L reactors with working volumes of 2 L at 37 °C in an incubator. Substrate was fed every 24 h. The reactor treating MR was operated at OLRs of 1–7 g VS L^{-1} day^{-1}. The MR reactor was initially operated at OLRs of 1–3 g VS L^{-1} day^{-1} and a HRT of 20 days in single reactor with a an influent/effluent flow of 100 mL day^{-1}, but at OLR 3 g VS L^{-1} day^{-1}, a sudden decrease in the biogas production occurred due to rapid biodegradability of MR. Thereafter, the digestion of MR was further tested in two stage anaerobic digestion to address rapid biodegradability. In two stage anaerobic digestion, the MR treating reactors were operated at OLRs of 3, 4, 5, 6 and 7 g VS L^{-1} day^{-1}. To maintain equal volume as in one stage anaerobic digestion of MR, the reactors R_1 and R_2 were operated each at an HRT of 10 days with a flow rate of 200 mL day^{-1} for each reactor. In the two stage setup, both reactors in combination had the same total volume as in single stage digestion. The JPC treating reactor was operated at OLRs starting from 1 to 6 g VS L^{-1} day^{-1}; the reactor treating JWS was operated at 1, 1.5, 2,

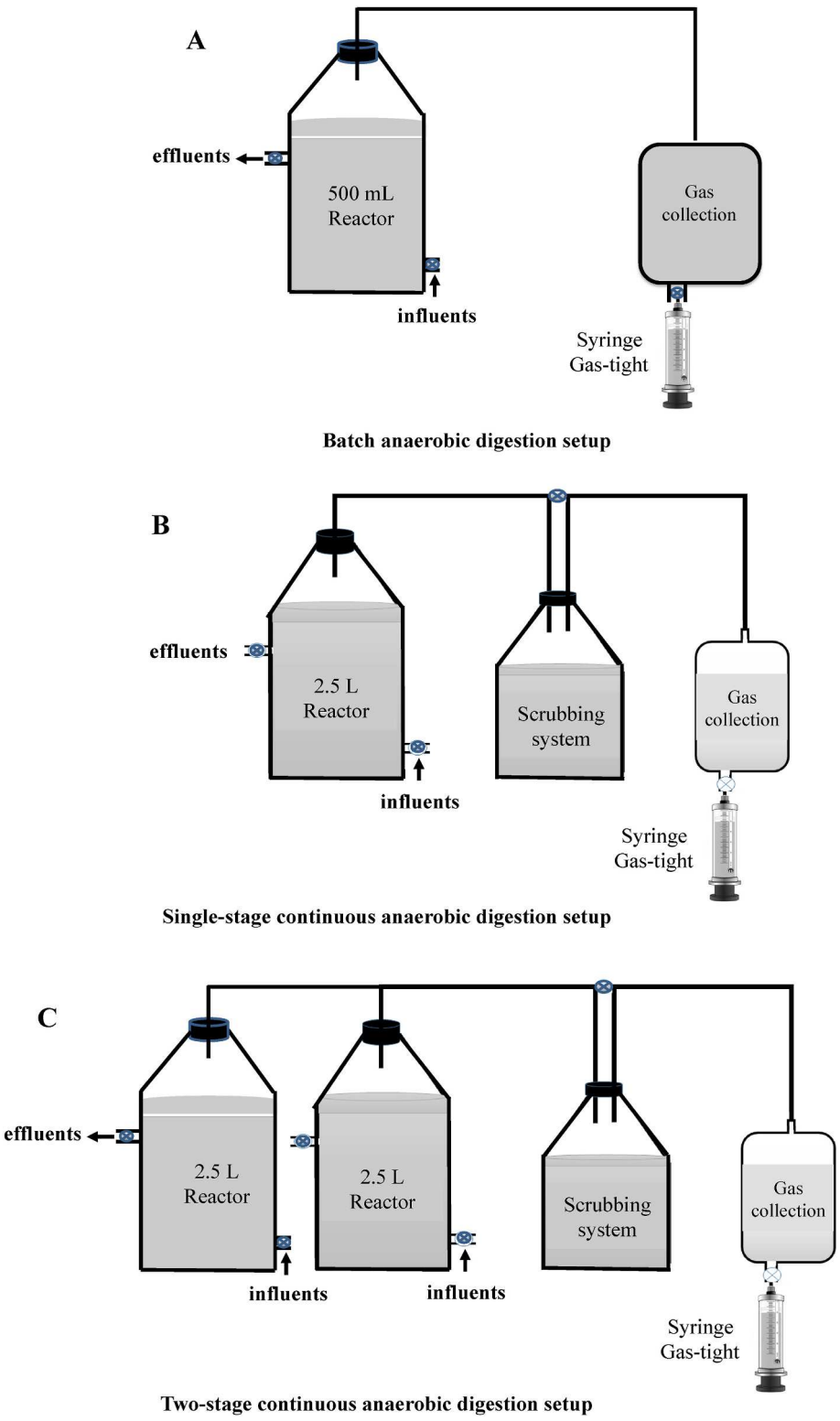

Fig. 2. Schematic representation of the test reactors and biogas collection system, **(A)** schematic representation of anaerobic digestion batch setup, **(B)** schematic representation of single-stage anaerobic digestion continuous setup (**C**) schematic representation of two-stage anaerobic digestion continuous setup. Scrubbing system contains a solution of 3M NaOH, and is connected to reactors through pipes to absorb the CO_2 leaving only methane to be collected in gas collection bag.

and 3 g VS L^{-1} day^{-1} and the reactor treating JO was operated at an OLR of 1 g VS L^{-1} day^{-1}, and HRT of 20 days with influent/effluent flow of 100 mL day^{-1}.

To determine the effect of HRT, the reactors were operated at HRTs of 10, 15 and 20 days with a daily flow rate of 200 mL, 130 mL and 100 mL, respectively, and the reactors were fed at the respective best performing OLRs determined in the previous experiment. During the startup phase, 1.5 L of temperature-acclimatized inocula was added to each reactor, flushed with nitrogen gas and plugged with butyl rubber cork attached to pipes for biogas collection, substrate feeding and

effluent removal. Each reactor was fed with their respective substrates at the specific HRT without taking any effluent until it reached its working volume (2 L). The biogas was collected in airtight bags (UNOGUARD, China) attached to reactors and was measured using a syringe. When the reactor reached a pseudo-steady state (defined as less than 5% variation in consecutive biogas volume production), the biomethane produced was measured at the end of each pseudo-steady state for 3–7 days by passing the biogas produced through a scrubbing solution (3M NaOH solution). The volume of biogas and biomethane were normalized at standard temperature and pressure (273.15 K and 101325 Pa). The effluent pH was measured on daily basis. The volatile fatty acids (VFAs) and alkalinity were measured at specific intervals using standard titrimetric procedures (Clesceri et al., 1998).

2.3. Microbial community analysis using high throughput sequencing

Samples (50 mL) for microbial DNA analyses were collected in sterile bottles at different intervals from each reactor operating at the best performing OLR and different HRTs (20, 15 and 10 days). The samples were frozen at −20 °C until further use.

2.3.1. DNA extraction, 16S rRNA gene amplification, sequencing, and data processing

Microbial DNA was extracted from pelleted biomass from the thawed samples using the DNeasy Powerlyzer Powersoil microbial DNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer instructions. The quantity and purity of extracted DNA were assessed using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA fragments of approximately 460 bp length flanking the V3 and V4 regions of the 16S ribosomal RNA (rRNA) gene of bacteria and archaea were amplified using forward and reverse primer pairs, modified 341F and modified 806R, respectively (Sundberg et al., 2013; Yu et al., 2005). Library preparation, quantification, normalization, and pooling were conducted following the Illumina 16S metagenomics protocol (Amplicon et al., 2013). Library quantity and quality were assessed using a D1000 ScreenTape® Tapestation (Agilent technologies, Santa Clara, CA). Prepared libraries were pooled and run on an Illumina MiSeq platform for 300 bp paired-end read sequencing at the Genomic Sciences Laboratory, North Carolina State University, NC. Raw sequences were deposited to the National Centre for Biotechnology Information Sequence Read Archive (BioProject ID PRJNA557512).

2.3.2. Sequence analyses

QIIME version 1.9.1 (Caporaso et al., 2010b) was used for qualitative screening of genomic sequences. Forward and reverse sequences were merged, de-barcoded and trimmed (Bolger et al., 2014). *Multiple_split_libraries_fastq.py* script was used for demultiplexing and quality filtering. Chimeric sequences were identified by *parallel_identify_chimeric_seqs.py* script using ChimeraSlayer and filtered using *filter_fasta.py* script (Haas et al., 2011). The screened sequences were then clustered into operational taxonomic units (OTUs) by *pick_open_reference_otus.py* script with UCLUST algorithm using 97% similarity index along with Greengenes database version (13_8) (Caporaso et al., 2010b). Taxonomic classification was done based on the latest Greengenes database version (13_8 and RDP classifier 2.2 with a confidence value of 0.8 using UCLUST (Edgar, 2010). Alignment was performed using PyNAST (Caporaso et al., 2010a) followed by generating phylogenetic trees using *make_phylogeny.py* script. The OTUs having less than 0.05% of the total sequence reads were filtered out for further analysis.

2.4. Statistical analyses

Alpha and beta diversities of microbial communities in reactors treating JWS, JO, JPC and MR were analyzed using R (McMurdie and Holmes, 2013). For alpha diversity, the Chao1 index (richness only) and Shannon (richness and evenness) indices were calculated. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for multiple comparisons of Chao1 and Shannon indexes in reactors treating *J. curcas* seed's fractions at HRTs of 10, 15 and 20 days in R-Studio. The level of significance was $p < 0.05$, $p < 0.01$ and $p < 0.001$. Beta diversity was analyzed using Bray-Curtis distances and visualized using two dimensional ordination plots. Canonical correspondence analysis (CCA) was used to estimate correlations between microbial communities and the following parameters: VFA, Alkalinity, VFA-s/Alkalinity ratio, pH, and biogas yield. One-way ANOVA followed by Tukey's post-hoc test was used for multiple comparisons of biogas yield in batch and continuous setups using Prism Graphpad. The level of significance was $p < 0.05$. Relative abundances of microbial communities were analyzed using R.

3. Results and discussion

3.1. Phytochemical analyses of seed oil and extracts used in anaerobic digestion

Initially, *J. curcas* seeds were subjected to mechanical oil extraction and yielded 32.5% oil. The TS and VS contents of the substrates are shown in Table S1.

J. curcas seeds have a number of compounds such as phorbol esters, curcin, and long chain fatty acids (LCFAs) that have been reported to have antimicrobial activities (Haq et al., 2019; Mendonça et al., 2019). Preliminary phytochemical analyses of *J. curcas* seed oil and de-oiled JPC extracts (aqueous, methanolic, and *n*-hexane) detected flavonoids, steroids, tannins, and phenol. Saponins and glycosides were only detected in the aqueous and methanolic extracts and were absent in seed oil and *n*-hexane extract. No balsams were found in any extract or seed oil. A broad range of phytochemicals was detected during GC-MS (Supplemental Information, Tables S2–S5) and FTIR analyses of JO and JPC extracts (Supplemental Information, Figs. S3–S6). A number of LCFAs, known to inhibit microbial cells (Ma et al., 2015), were identified in JO and *n*-hexane extracts (Supplemental Information, Tables S4 and S5).

The methanolic extract is highly rich in medicinally important compounds such as beta-monolaurin, I-(+)-ascorbic acid 2,6-dihexadecanoate, 9-hexadecenal, bis (tridecyl) phthalate, 1-docosanol and diacetone alcohol (Table S3). Beta-monolaurin had been reported as having antimicrobial properties and may damage microbial cell membranes, targeting various proteins and nucleic acids and macromolecular synthesis processes resulting in cell damage (Skřivanová et al., 2006). Similarly, ascorbic acid 2,6-dihexadecanoate had been reported as having antioxidant activities, and 9-hexadecenal and 1-docosanol for antimicrobial activities (Bhardwaj, 2018). Methanolic solvent was a better extraction solvent than aqueous and *n*-hexane (Haq et al., 2019).

3.2. Biogas potential of J. curcas cake after solvent extraction and inhibitory effect of methanolic extract of pressed cake on different steps in anaerobic digestion

The biogas potential of *J. curcas* seed and the effect of methanolic extract of de-oiled JPC were evaluated on different steps of anaerobic digestion in batch reactors (Fig. 3A and B). The biogas produced by methanolic residues, Jatropha pressed cake, n-hexane residues and aqueous residues was 829 Nml, 560 NmL, 506 NmL and 314 NmL, respectively. Methanolic residues produced more biogas than Jatropha pressed cake (35.5%), *n*-hexane residues (39%), and aqueous residues (62%) (Fig. 3A). This suggests treating de-oiled JPC with a methanolic extraction may increase the seed cake anaerobic biodegradability by removing the compounds that inhibit anaerobic microbial communities, which is confirmed by the observed inhibition due to addition of methanol extracts to the reactor fed with cellulose (Fig. 3B). The higher biogas yield of methanolic residues (829 NmL) compared to residues

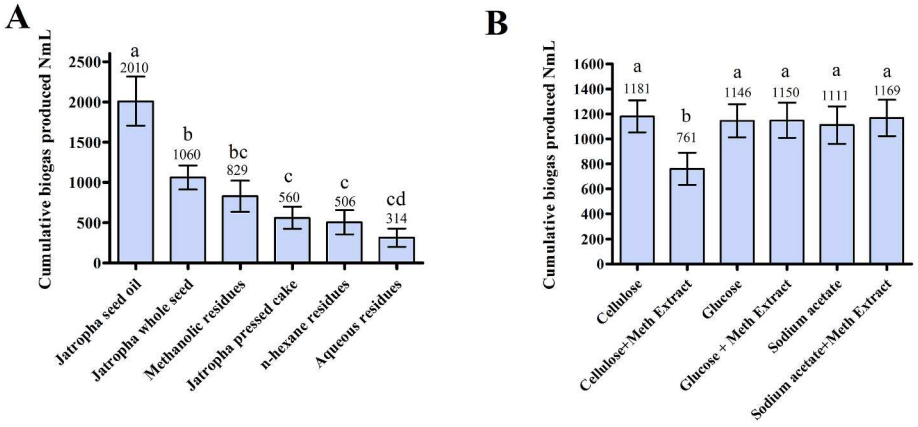

Fig. 3. Net cumulative biogas production of different treatments of *J. curcas* seed and the effect of methanolic extracts of de-oiled Jatropha pressed cake (JPC) on different steps of anaerobic digestion. (A) Biogas potential of different fractions of *J. curcas* seed. Inocula were used as negative control that was subtracted from biogas yield of substrates. (B) Inhibitory effects of methanolic extract of de-oiled JPC on net biogas production of cellulose, glucose and acetate representing different steps of anaerobic digestion. The background biogas produced by a negative control (inocula plus methanolic extract) was subtracted from biogas produced by each substrate. The experiments were carried out in triplicate and the data was presented as mean ± standard deviation. One-way ANOVA followed by Tukey's post-hoc test for multiple comparisons was used. The same lowercase letters on the adjacent bars indicate no significant differences; different letters indicate significant differences. Level of significance was $p < 0.05$.

from *n*-hexane (506 NmL) and aqueous residues (314 NmL) (Fig. 3 A, B) suggests more antimicrobials were removed by methanol extraction. This is likely because methanol, whose polarity index falls between the other solvents, has the capability to dissolve both polar and non-polar compounds (Haq et al., 2016).

The addition of methanolic extract to a cellulose-fed reactor, which necessitates hydrolysis, resulted in a substantial reduction in biogas production ($p < 0.05$). Specifically, the biogas yield decreased by 35.5%, with only 761 NmL of biogas being produced. However, the methanolic extract did not inhibit biogas production when added to substrates not requiring polymer hydrolysis (*i.e.,* sodium acetate and glucose) (Fig. 3B). This suggests that the microbial inhibitors in *J. curcas* seed selectively affect hydrolysis by either directly inhibiting the relevant bacterial communities or inhibiting their enzymes i.e., hydrolases. The effect of methanolic extract on microbial communities is explained in Section 3.4, where the *J. curcas* seed long chain fatty acids and phytochemicals inhibited the acetogenic bacterial groups.

3.3. Effect of operational parameters and inhibitory compounds on biogas yield during anaerobic digestion

Operational parameters and inhibitors present in *J. curcas* seeds both affect biogas and biomethane yields. In continuous anaerobic digestion, all reactors were mainly sensitive to OLR, because it is directly correlated to loading of inhibitory compounds and, in the case of methanolic residues, over-acidification. HRT, although affecting yield and stability to a lesser extent than OLR, is also an important parameter. Previous studies have shown that higher OLR and shorter retention time favors over-acidification due to an increase in VFA accumulation, thus resulting in decreased biogas production (Dareioti and Kornaros, 2015; Nakasaki et al., 2015; Zhang et al., 2015; Razaviarani and Buchanan, 2014; Doğan and Demirer, 2009). Hence, low OLRs and extended HRTs have been considered ideal strategies to enhance the efficiency and stability of the reactors to avoid accumulation of VFAs. For instance, Lu et al. (2015) observed a sharp decline in pH (below 6.7) and biogas production from 3.95 to 0.5 $L/L_{reactor}/d$ by shortening HRT from 24 to 3 h and increasing the OLR from 1 to 8 g $COD/L_{reactor}/d$ in long term operation of a lab-scale reactor treating wastewater (Alavi-Borazjani et al., 2020). It appears that long-chain fatty acids (LCFAs), when present, produce rapidly failing reactors, likely by initially inhibiting microbial communities. Anaerobic digestion is a complex process that relies on the concerted efforts of various microbial communities, such as methanogens, acidogens, acetogens, and hydrolytic bacteria. These microbial groups are interdependent, and any slight variation in their composition can disrupt or modify the entire anaerobic digestion process (Pasalari et al., 2021). Previous studies have demonstrated inhibitory effects of LCFAs on methanogenesis and acetogenesis, while no inhibitory effects were reported for hydrolysis and acidogenesis (Rodríguez-Méndez et al., 2017). The methanogenesis was inhibited at concentrations of 0.11 g_{LCFA}/L but it was more prominent at the higher concentrations of 0.5 g_{LCFA}/L (Rodríguez-Méndez et al., 2017). Even when LCFAs concentrations are reduced after removing Jatropha oil (such as for biodiesel), remaining phytochemicals can inhibit the reactor. In the case where both LCFAs and phytochemical concentrations are reduced, the main cause of instability is organic overloading leading to acidification (Fig. 4 A and 4E). Previous findings are also in agreement with our statement that an increase in OLR leads to accumulation of VFAs, thus resulting over-acidification (Ferguson et al., 2016).

The maximum biogas (0.65 NL/g VS_{added}) yield for MR was obtained at an OLR of 1 g VS L^{-1} d^{-1} and a HRT of 20 days (depicted in Fig. 4A and B), which was significantly higher ($p < 0.05$) than at other OLRs (1.5, 2, 3, 4, 5, 6, and 7 g VS L^{-1} d^{-1} yielding biogas 0.18, 0.1, 0.4, 0.3, 0.24, 0.17, 0.1, 0.55, 0.22 NL/g VS_{added}, respectively) and HRTs (15 and 10 days with biogas yield of 0.55 and 0.22 NL/g VS_{added}, respectively). Further, the biomethane yield (0.536 NL/g VS_{added}) of MR treating reactor was significantly higher ($p < 0.05$) than the maximum yields from reactors treating jatropha pressed cake (0.38 NL/g VS_{added}), whole seed (0.47 NL/g VS_{added}), and oil (reactor failed). In reactors fed with methanolic residues, the biogas yield decreased at OLRs above 1 g VS L^{-1} day^{-1} likely due to its high biodegradability resulting in accumulating VFAs faster than they could be converted to methane. The VFAs/alkalinity ratio (Supplemental Information, Table S6) continued to increase with OLR; to address this issue the reactor was converted two stage anaerobic digestion for OLRs 4 g VS L^{-1} day^{-1} through 7 g VS L^{-1} day^{-1}, but the biogas yield did not fully recover. The methanolic residues treating reactor was stopped at OLR of 7 g VS L^{-1} day^{-1} as the small diameter pipes began to clog from the amount of methanolic residues used during feeding.

The reactor fed with Jatropha pressed cake produced maximum biogas (0.54 NL/g VS_{added}) and biomethane (0.38 NL/g VS_{added}) yields at an OLR of 1.5 g VS L^{-1} d^{-1} and HRT of 20 days (Fig. 4C and D). Although also inhibited with increased organic loading, Jatropha pressed cake degradation did not appear to produce VFAs as rapidly as

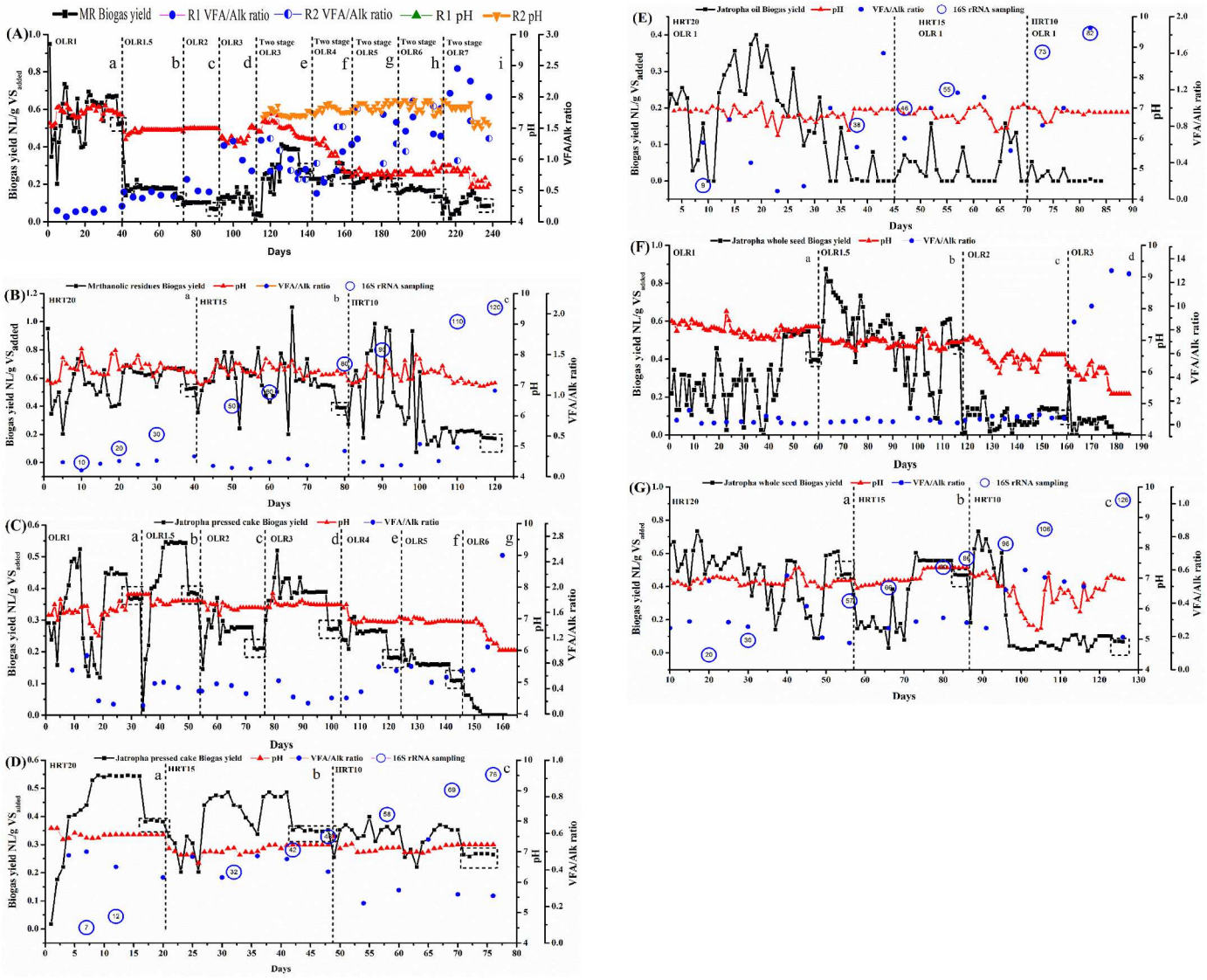

Fig. 4. Biogas yield of different treatments of *Jatropha curcas* seed in NL/g VS_{added}. **(A)** Biogas yield of methanolic residues (MR) at different organic loading rates, **(B)** Biogas yield of MR at different HRTs at an OLR of 1 g VS L^{-1} day^{-1}, **(C)** Biogas yield of jatropha pressed cake (JPC) at different OLRs, **(D)** Biogas yield of JPC at different HRTs at an OLR of 1.5 g VS L^{-1} Day^{-1}, **(E)** Biogas yield of jatropha seed oil (JO) at different HRTs at an OLR of 1 g VS L^{-1} Day^{-1}, **(F)** Biogas yield of Jatropha whole seed (JWS) at different HRTs at an OLR of 1.5 g VS L^{-1} Day^{-1}, **(G)** Biogas yield of JWS at different HRTs, The Biomethane yield is covered by dashed squares. The different letters show significant differences in biogas yields at steady state among different OLRs and HRTs. One-way ANOVA followed by Tukey's post-hoc test for multiple comparisons was used. The level of significance was ($p < 0.05$). Labelled blue circles show the days at which 16S rRNA samples, for microbial profile in the reactor, were taken from reactors and results are discussed in Section 3.4. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

methanolic residues degradation, leading to a less abrupt drop-off in yields. We believe this is caused by reduced biodegradation rates (and thus over-acidification) due to phytochemical inhibitors present in the Jatropha pressed cake that are not in the methanolic residues.

The reactor treating Jatropha oil was inhibited during the early stages at OLR of 1 g VS L^{-1} day^{-1} at HRTs of 20, 15 and 10 days (Fig. 4E). This is likely due to high concentration of LCFAs in the Jatropha oil, which are known to decrease biogas yields and cause reactor failure (Xu et al., 2015). LCFAs, which is a product of hydrolysis of lipid is further degraded anaerobically through β-oxidation pathway to acetate and hydrogen. The acetate and hydrogen are subsequently converted to methane (Xu et al., 2015). The process of β-oxidation is initiated when the fatty acids are activated with coenzyme A, which subsequently oxidized to release acetyl-CoA and synthesis of a fatty acid chain. The fatty acids activation with coenzyme A starts the process of β-oxidation and further oxidation leads to the liberation of acetyl-CoA and the synthesis of a fatty acid chain (Madigan and Martinko, 2006). A notable concern arises from the potential detrimental impact of LCFAs on methanogenic bacteria when they are introduced at significant concentrations or loading rates. Researchers have posited that the adverse impact on microbial communities and biogas yield could be attributed to several factors, including sludge floatation and washout, transport limitation caused by a layer of LCFAs coating the bacteria, impeding their access to substrates and their ability to release biogas, as well as the potential toxicity effect of LCFAs on microbial communities (Nzila et al., 2019). Another operational concern is the digester foaming due to lipids during anaerobic digestion process (Long et al., 2012). Foaming can result in reduced gas recovery due to accumulation of high concentrations of solids at the top of a digester, thus resulting in blockage of gas mixers, fouling of gas pipes and so on (Ganidi et al., 2009).

Biogas yields from the reactor treating Jatropha whole seeds fluctuated, with maximum biomethane (0.47 NL/g VS_{added}) and biogas (0.6

NL/g VS_{added}) yields at an OLR of 1.5 g VS L^{-1} d^{-1} and a HRT of 20 days and the reactor failed at OLRs of 2 and 3 g VS L^{-1} day^{-1} (Fig. 4F). Because the failure occurred before the VFA/alkalinity ratio increased, we attributed the failure and variability primarily to inhibitory compounds rather than over-acidification. The increase in VFAs after methane generation ceased which suggests that methanogenesis was inhibited earlier than hydrolysis and acid production and this may have been caused in two ways. First, the methanogens themselves may have been directly and preferentially inhibited by the LCFAs (Nielsen and Ahring, 2006). Alternatively, the methanogens may have been starved for substrate either due to LCFA/phytochemical inhibited acetate-producers or additional upstream effects such as the potential hydrolysis inhibition suggested in Fig. 3B. The acetogenic activities are reduced as the LCFAs are accumulated, and subsequently lead to low methanogenesis (Pereira et al., 2003). Further, acetate starvation may also have occurred if the LCFA degrading acetogens were washed out at the lower HRTs. Regardless of the many potential explanations, the high toxicity of compounds in Jatropha whole seeds makes it an unsuitable direct substrate.

3.4. *Effects of methanolic extract of J. curcas pressed cake and hydraulic retention time on microbial diversity*

The total counts of raw reads were 7,786,706 sequences, with 97,334 mean raw reads per sample. Alpha diversities based on microbial richness (Chao1 index) and diversity (Shannon index) differed between reactors treating *J. curcas* seed fractions at HRTs of 10, 15 and 20 days (Fig. 5 A). The reactor treating methanolic residues generally had a significantly higher ($p < 0.001$) Shannon index (even diversity) than those fed with Jatropha oil and whole seed (*see* supplementary file Tables S7–S10), however, there were no significant ($p > 0.05$) variations in microbial richness based on the Chao1 index among them. As Chao1 index is not statistically different, the main effect seems to be on evenness of the microbial communities within the reactors. Those reactors, which treated Jatropha oil and whole seed, had generally the lowest Chao1 and Shannon diversity. We suggest both the evenness and richness were lowered by inhibitory compounds in the seeds, primarily the LCFAs present in the jatropha oil and, to a lesser extent, phytochemicals present before methanolic extraction. It is possible that the different inoculum batches contributed to different Shannon diversity values, however Jatropha pressed cake had a Shannon diversity closer to that an of the MR-associated inoculum than the one to which it was exposed. The lower diversity reactors were also the less stable ones, which correspond to established theory (Carballa et al., 2015). Higher richness and evenness are signs of functional stability of a reactor and the lower richness or evenness are usually considered as a warning indicator for reactor instability (Carballa et al., 2015). The statistical analysis of alpha diversity indices implies that proper and sustainable management of

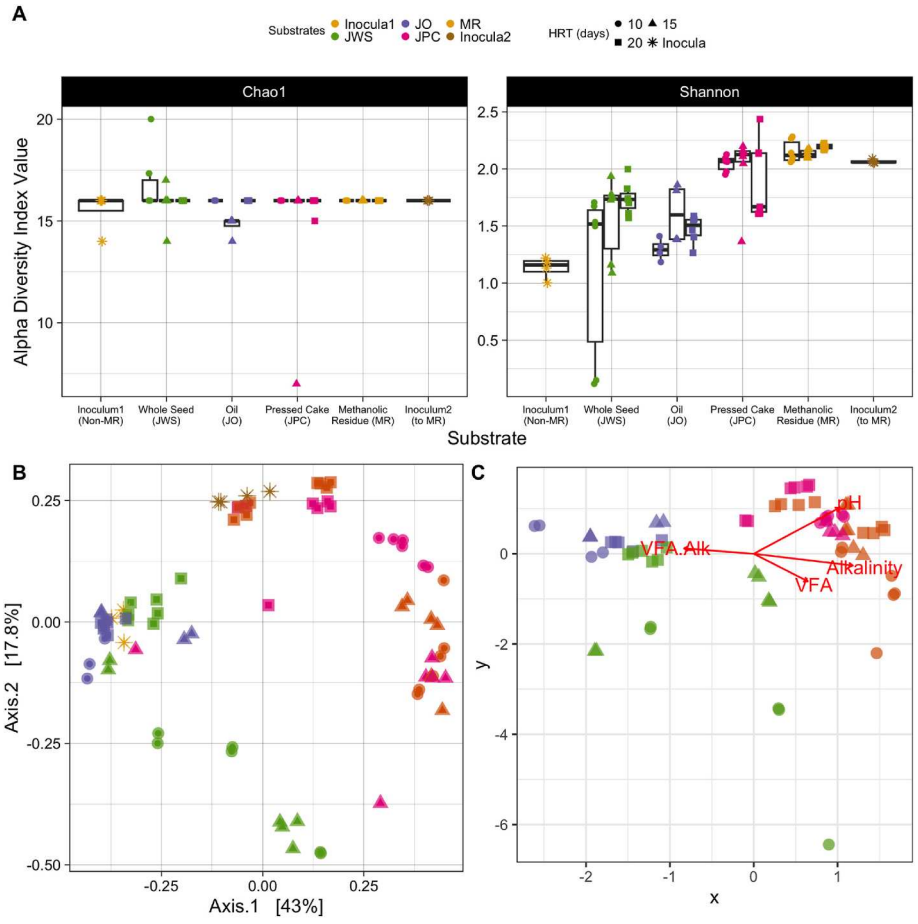

Fig. 5. 16S rRNA analysis of microbial communities during anaerobic digestion of *J. curcas* seeds. (**A**) Chao1 and Shannon indexes of alpha diversity (within sample diversity) of microbial communities within different anaerobic reactors treating jatropha oil (JO), whole seed (JWS), pressed cake (JPC) and methanolic residues (MR) substrates. (**B**) Principal coordinate analysis (PCoA) plot using Bray Curtis distances (dissimilarity) between *J. curcas* substrates (JO, JWS, JPC, and MR), inocula1 and inocula2 at different HRTs (20, 15 and 10 days). The shaded circle, triangle, square and plus signs in PCoA plot represent HRT 10, 15, 20 and inocula, respectively in continuous anaerobic digestion setup. The substrates are represented by different colors. (**C**) Canonical correspondence analysis to identify the relationship between operating parameters and microbial communities during anaerobic digestion of different setups of *J. curcas* seed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inhibitory compounds may greatly increase the microbial diversity inside the reactors, resulting in both more stable reactors and an enhanced biogas yield.

The beta diversity between samples, visualized as a PCoA ordination based on Bray-Curtis distances (Fig. 5B), also shows that both substrate composition and HRTs affected the microbial communities. Communities associated with the Jatropha whole seed and oil deviated from their inoculum due to the presence of phytochemicals. They indicated similar microbial community composition and tended to cluster together at long HRTs. On the other hand, MR and JPC clustered close to each other especially at HRT of 20 days, indicating similar microbial communities; interestingly, they clustered close to the inocula. This close adherence of MR and JPC to their inocula shows the strong adaptability and acclimatization of the microbial communities present in their respective inocula for breakdown of these substrates. Moreover, the microbial communities in all reactors at HRT of 20 days were dissimilar than those at HRT 10 and HRT 15 except for Jatropha oil, illustrating the effect of HRT on the microbial shifts between the reactors. The performance and stability of the reactors varies with HRTs. The microbial communities performed well and were more stable at longer HRTs compared to shorter ones as evident from biomethane yield.

Canonical correspondence analysis (CCA) shows that the microbial communities present in MR and JPC treating reactors exhibited positive correlation with increased operating pH (Fig. 5C). Microbial communities consuming methanolic residues and Jatropha pressed cake at HRT 20 days had a strong positive correlation with pH and negatively correlated with increased VFAs/Alkalinity ratios. HRT has strong effects on the pattern and composition of microbial communities during anaerobic digestion. Higher HRTs favor greater microbial diversity, while lower HRTs tend to lower the microbial diversity due to microbial washout (assuming no biomass retention mechanism which decouples mean cell residence time (MCRT) from HRT). Microbes that have longer doubling times than the HRTs are liable to washout at shorter retention time during anaerobic digestion (Xu et al., 2018). As previously mentioned, the Jatropha whole seeds in their natural form are enriched with several phytochemicals and LCFAs, which impedes the process of anaerobic digestion and affects the associated microbial communities. Similarly, Jatropha oil, enriched in LCFAs, has negative effects on the microbial communities involved in the process of anaerobic digestion as discussed earlier in section 3.3. In the other fractions, such as pressed cake and methanolic residues, the level of phytochemicals and LCFAs are reduced subsequently compared to the Jatropha whole seeds and oils. Particularly, the methanolic extraction has the tendency to extract both polar and non-polar phytochemicals from jatropha seeds, thus resulting in relatively lower phytochemicals or LCFAs. On the other hand, the microbial communities in Jatropha oil and whole seed reactors were positively correlated with increased VFAs/Alkalinity ratios. In addition, the methanolic residues and Jatropha pressed cake had positive correlation with VFA but this increase had no obvious negative effects on the biogas yield due to higher alkalinity (Eduok et al., 2017).

The methanolic residue reactors had higher relative abundance of acetogenic bacteria than the whole seed reactors (Fig. 6). The relative abundance of *Clostridium* was 0.7% in MR, while for JWS it was 0.4% (Table S11). *Clostridium* and *Syntrophomonas* were found in higher abundance in the methanolic residues compared to Jatropha whole seed treating reactors at HRT of 20 days; these organisms are known for their broader range of substrate utilization and higher abundance in well operating and stable anaerobic digesters (Papp et al., 2016). Both genera are syntrophic acetate oxidizers and are in general involved in proteolytic and saccharolytic conversions (Vanwonterghem et al., 2014). *Syntrophomonas* is a facultative bacterium that consumes acetate and converts it into H_2 and CO_2 via acetate oxidation and the resultant products are further utilized by hydrogenotrophic methanogens to produce CH_4 (Treu et al., 2019). *Syntrophomonas* is highly resilient, but it can be out-competed by acetoclastic methanogens, and is negatively correlated with higher VFAs (Treu et al., 2019). The methanogens were more abundant in Jatropha oil and whole seed compared to methanolic residues treating reactors. The percent relative abundance of *Methanosaeta* at HRT 20 was 44.4%, 37.9%, 17.7% and 12.5% in reactors treating Jatropha whole seed, oils, pressed cake and methanolic residues, respectively (Table S11). A similar trend was seen for other methanogenic microbial communities in reactors treating Jatropha whole seed, oils, pressed cake and methanolic residues at HRTs of 15 and 10 days (Table S11). The high abundance of the genus *Methanosaeta* in reactors treating high amount of LCFAs may be due to their acetoclastic activity and LCFA-resistant nature. Thus the genus *Methanosaeta* is the most dominant group in LCFAs-rich reactors (Usman et al., 2020).

Methanosaeta was relatively more abundant in Jatropha whole seed, oil and pressed cake reactors compared to the methanolic residues reactor community (Table S11). The lower relative abundance of

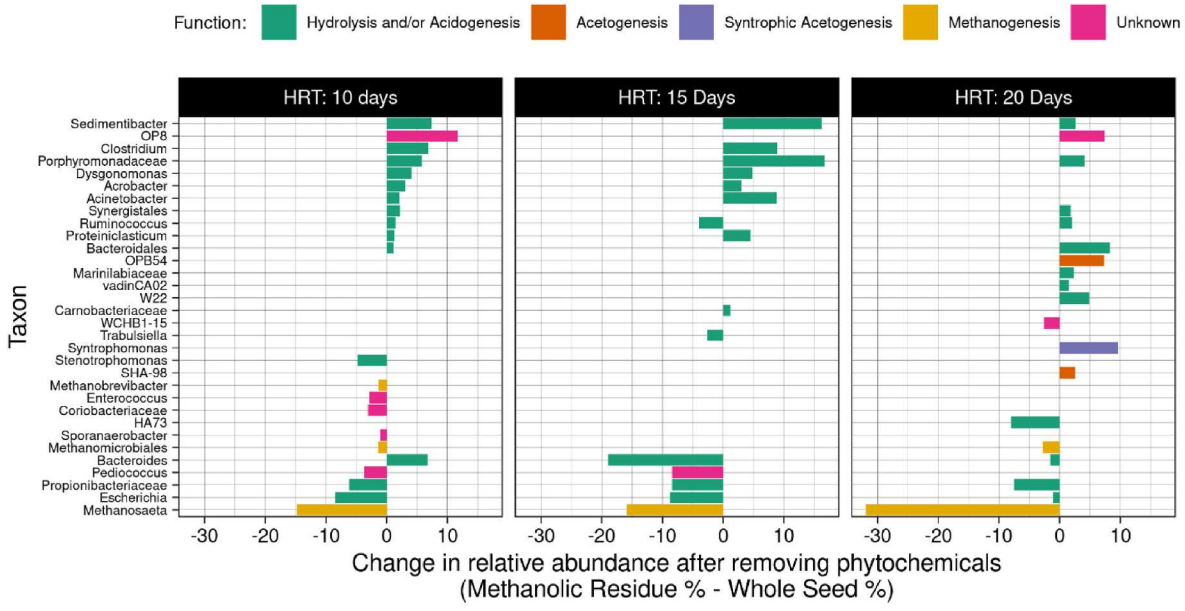

Fig. 6. Relative abundance of microbial communities in reactors treating jatropha whole seed (JWS) compared to those in methanolic residues (MR) at hydraulic retention time (HRT) of 20, 15 and 10 days.

Syntrophomonas in Jatropha whole seed and oil compared to methanolic residues and pressed cake treating reactors may have caused a decrease in acetate production (Regueiro et al., 2015). *Syntrophomonas* is specialized in converting butyrate and propionate to acetate, therefore, a lower relative abundance of *Syntrophomonas* indirectly indicates lower acetate concentration (Regueiro et al., 2015). *Methanosaeta* has higher affinity for acetate but can be sustained only at a lower concentration of acetate (Liu and Whitman, 2008). The *Methanosaeta* having a high affinity for acetate are only capable to produce methane by consuming it at concentrations not exceeding 100–150 mg/L (Kim et al., 2014; Lim et al., 2013). The higher relative abundance of *Methanosaeta* and lower relative abundance of *Syntrophomonas* is an indication of perturbation and instability of anaerobic digestion process. The *Actinobacteria* with unidentified genera were dominant in Jatropha oil and whole seed compared to pressed cake and methanolic residues treating reactors (Table S11) and have been reported to consume propionate; they can sustain in higher VFAs/Alkalinity ratios during anaerobic digestion (Cabezas et al., 2015). *Sedimentibacter* is a non-carbohydrate dependent bacterium, supported by pyruvate or amino acids fermentation (Gulhane et al., 2017). They were found in higher abundance in methanolic residues and pressed cake compared to Jatropha oil and whole seed treating reactors. The Cloacimonetes (WWE1) genus W22 is thought to play a role in protein and cellulose fermentation and oxidizes VFAs during anaerobic digestion (Chojnacka et al., 2015). They were found in higher abundance in methanolic residues followed by pressed cake, whole seed and Jatropha oil (Table S11). Their relative abundance indicated that the *J. curcas* seed long chain fatty acids and phytochemicals inhibited acetogenic bacterial groups (Fig. 7); however, no obvious decrease in methanogenic abundance was observed, contrary to previous literature (McMurdie and Holmes, 2013).

At lower HRTs, the methanogens were at low levels, resulting in reactor instability and lower biogas yields (Fig. 7). Conversely, the relative abundances of *Methanosaeta* (methanogens) and *Bacteroidetes* (hydrolyzers) were adequate to keep the anaerobic digestion process stable at HRT 20, in contrast to the instability at HRTs of 15 and 10 days. The relative abundances of genera *Pediococcus*, *Proteiniclasticum*, *Clostridium*, *Sedimentibacter* and *Sporanaerobacter* within *Firmicutes* were higher at shorter HRTs compared to longer HRTs (Table S11). The genus *Firmicutes* possess thick cell wall and bear harsh condition by forming endospore surrounding their cells, thus allowing them to thrive under different operational and environmental conditions (De Sa et al., 2011). Therefore, they have the capability to sustain in higher relative abundances at shorter HRTs. *Firmicutes* play a major role in hydrolysis and hydrogenogenic acidogenesis of different feedstocks during anaerobic digestion (Fitamo et al., 2017), as they are involved in a variety of roles such as degradation of cellulosic materials and secretion of ligninolytic enzymes (Laccases) (Dassa et al., 2014; Ze et al., 2015; Lage and Bondoso, 2014). They highly influence the composition of methanogens and the process of hydrogen (H_2) removal to maintain a low H_2 partial pressure (Garcia-Peña et al., 2011; Palatsi et al., 2011). *Sporanaerobacter* is a sulphur reducing acetogen and is considered fast grower compared to methanogens (Hernandez-Eugenio et al., 2002). The *J. curcas* seed being rich in oil has a low level of sulphur (Islam et al., 2015), which can be used by the sulphur reducing acetogen during anaerobic digestion. The perturbation in the Jatropha whole seed and oil reactors at shorter HRTs (15 and 10 days) may be due potentially to an increase in relative abundance of sulphur reducing *Sporanaerobacter,* which outcompeted the methanogens. On the other hand, some of the *Firmicutes* genera including *Syntrophomonas* and *Ruminococcus* were found in higher abundance at longer HRTs compared to shorter HRTs (Table S11). Synergistetes with genera HA73 and vadinCA02 had higher relative abundance at longer HRT compared to shorter HRT. The HA73 and vadinCA02 can convert protein into acetic acids during anaerobic digestion (Yamashita et al., 2016).

4. Conclusions

For *Jatropha curcas* seeds to be a suitable feedstock for biogas production, phytochemicals in the seeds that affect methane production, specifically by inhibiting hydrolytic and acetogenic bacterial communities, need to be removed. The phytochemicals extracted with methanolic solvent inhibited hydrolysis, reducing biogas production by 35.5% compared to the same treatment without extracts. Residues from which the phytochemicals were removed using methanolic extraction (methanolic residues) appear to not inhibit microbial communities and produced higher methane yields than other seed preparations. In the continuous setup, the reactor utilizing methanolic residues (MR) consistently demonstrated superior stability in biogas production compared to reactors treating Jatropha whole seeds, oils, or pressed cake. This heightened stability can be attributed to the notably higher abundance of syntrophic acetogens in the MR-treated reactor, with a relative abundance of 12.1%, compared to 9.6% in reactors fed with Jatropha pressed cake, 4.0% with seed oil, and only 2.5% with whole seeds. Enhanced biogas yield of 0.65 NL/g VS_{added}, 0.60 NL/g VS_{added} and 0.54 NL/g VS_{added} was achieved at longer HRTs (20 days), which we attribute to reduced washout of key slow-growing communities, and lower OLRs. The extracted phytochemicals and oil are themselves potentially valuable to the pharmaceutical industry and biodiesel production, respectively. This approach will increase the economic value of *J. curcas* for the biofuel and biorefinery sectors, by providing a

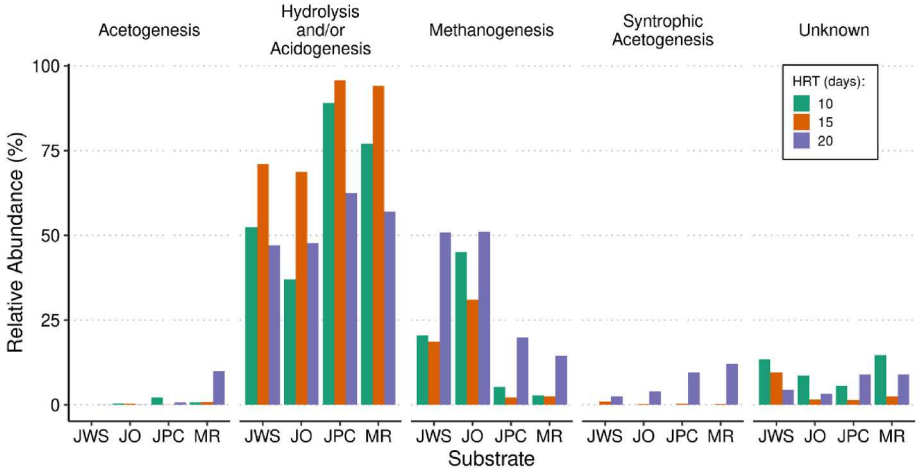

Fig. 7. Relative abundance of major microbial groups in different anaerobic reactors treating jatropha oil (JO), whole seed (JWS), pressed cake (JPC) and methanolic residues (MR) substrates at hydraulic retention time (HRT) of 20, 15 and 10 days.

potentially sustainable cleaner solution to mitigate waste and enhance the value of the feedstock for production of energy carriers and value-added compounds.

CRediT authorship contribution statement

Abdul Haq: Conceptualization, Methodology, Software, Formal analysis, Investigation, Visualization, Writing – original draft. Ayesha Malik: Methodology, Formal analysis. Alam Khan: Conceptualization. Joseph E. Weaver: Experimental Work, Software, Formal analysis. Ling Wang: Experimental Work, Software, Formal analysis. Haji Khan: Results interpretation. Samiullah Khan: Supervision. Aamer Ali Shah: participated substantially in discussion and modifications. Safia Ahmed: Supervision. Asif Jamal: Results interpretation. Francis L. de los Reyes III: Supervision, Writing – review & editing, Formal analysis, Resources. Malik Badshah: Conceptualization, Supervision, Writing – review & editing, Formal analysis, Resources, Funding acquisition, Project administration.

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Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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