Mesophilic Anaerobic Co-digestion of Cattle Manure with *Malus domestica* and *Dalbergia sissoo* during Biomethane Potential Assays

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The burning of cattle manure for domestic use, and plant biomass left out in fields, is a common practice in South Asia, specifically Pakistan. According to the 2014 government of Pakistan (GOP) survey, Pakistan had 171 million head of cattle that would produce 345 billion kg of manure, which could easily be converted into 150 billion m^3 of biogas. The focus of the present study was to evaluate the benefits from co-digestion of cattle manure (CM) with *Dalbergia sissoo* leaves (DSL) and *Malus domestica* leaves (MDL), with a focus on changes in the biodegradability, C/N ratio effect, and synergistic effect. The idea was to adjust the C/N ratio to increase biodegradability at mesophilic range to help the process to produce more methane than 100% manure-based digestion. First, the ideal pH and temperature conditions for mesophilic anaerobic digestion (AD) were optimized to carry out further co-digestion under the same conditions. The results of co-digestion revealed a 40% (251 NmL CH_4/g VS) increase in methane yield by replacing 20% of volatile solid in CM-based AD reactors with MDL. This combination also presented a biodegradability of 59% and a synergistic effect (θ) value of 1.40, which corresponded to highly positive synergism reflecting the optimum growth conditions. The DSL/CM co-digestion also followed the same pattern, and the maximum methane yield of 229 NmL CH_4/g VS was obtained using a 20/80 DSL/CM combination.

Keywords: Biomass; Lignocellulose; Methane Production; BMPs

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INTRODUCTION

The former most-used technologies for the application of manure were as burning material or fertilizer, and such uses have resulted in high environmental destruction due to the greenhouse effect and eutrophication. However, with the advancement of science and technology it is now feasible to reduce this waste and utilize it in an environmentally friendly way by digesting it anaerobically and producing biomethane (Awais *et al.* 2016). Currently, this anaerobic digestion (AD) and energy recovery from animal and plant waste is considered a smart solution socially, economically, and environmentally. Pakistan is an agricultural country supported with 171 million head of livestock producing 345 billion kg of manure that could easily be converted into 150 million m^3 of biogas annually (GOP 2014). While European countries such as Denmark already have well-established systems for biogas production, these countries are now focusing on lignocellulosic wastes to be added and co-digested with the manure (Biswas *et al.* 2012).

The 20th century was the era when the AD process attracted more focus and popularity as the best reported waste treatment and sustainable energy generating process. Moreover, in addition to the AD process, the co-digestion of manure with other lignocellulosic biomasses also created a focal point for researchers in the last couple of decades (Kretschmer *et al.* 2012; Rodriguez *et al.* 2017). Hence, this technique was later named as anaerobic co-digestion, which can be defined as the simultaneous digestion of two or more contrasting feedstocks in a single AD reactor. For example, this can be used to enhance methane augmentation of livestock manure, an otherwise low methane-yielding substrate (Macias-Corral *et al.* 2008). Augmentation is achieved through the addition of substrates with higher methane potentials, thus improving the economy of AD process (Plöchl and Heiermann 2006). Co-digestion benefits were first reported in 1981, when it was found that the buffering capacity of manure-based AD reactors was enhanced in addition to lesser ammonia inhibition, enriched balance of nutrients, and adjusted C/N ratios (Mshandete *et al.* 2004). Furthermore, other ideal materials for anaerobic co-digestion are agricultural and wild plant residues from non-arable lands, due to their low or no competency with the human food chain and a slightly higher biomethane augmentation potential compared to cattle manure (Holm-Nielsen *et al.* 2009). Furthermore, Somayaji and Khanna (1994) conducted a study with wheat straw (40%) and (60%) cattle manure (CM) on a total solid basis and reported a significant methane rise of 20 NmL CH_4/gTS after co-digestion at mesophilic conditions. Similarly, another study reported a significant volumetric methane rise of 16% compared to CM mono-digestion after the addition of 30% oat straw in an AD batch reactor (Johansson and Azar 2007).

Co-digestion has also been reported as an application for increasing the biodegradability of recalcitrant substrates, *e.g.*, lignocellulosic (LS) biomass (lignin presence and compact architecture), and as helpful in decreasing ammonia accumulation that negatively affects the AD process. Furthermore, it can decrease the net amount of nitrogen in the AD reactor that produces free and derived ammonical complexes, reduce the volatile fatty acid (VFA) accumulation, and stabilize the pH during anaerobic co-digestion (Somayaji and Khanna 1994).

Therefore, the combination of feedstocks that have strikingly different chemical structures can stabilize and give an overall more efficient AD. Grass silage, for example, has been reported as a suitable co-substrate with manure to significantly increase the methane yield in both batch and continuous reactors (Lehtomäki *et al.* 2007). Nevertheless, the presence of lignin and the intricate architecture of lignocellulosic biomass negatively affects the AD and poses serious difficulties in reactor handling during the process, hence reducing the overall effectiveness of AD (Ashekuzzaman and Poulsen 2011). Moreover, another factor that can significantly regulate the co-digestion is the presence of different metal ions. Being an enzymatic process, the AD needs cofactors for the enzymes at different stages to work efficiently, and if these metal ions are provided in optimal concentration, they will significantly affect the overall AD. For example, some macronutrients like nitrogen (N), sulfur (S), magnesium (Mg), sodium (Na), calcium (Ca), and iron (Fe) are part of the basic needs for microbes to grow (Nizami *et al.* 2009). Furthermore, some of the other micronutrients or trace metals, *e.g.* chromium (Cr), cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), vanadium (V), and zinc (Zn), are usually the cofactors for various AD enzymes and hence bind to them, increase efficiency of the enzymes, and regulate the methane yield (Kim and

Holtzapple 2005). In previous studies with co-digestion, it was established that the microbial community in the reactors gets stabilized by the use of different substrates and when optimal nutrient ratios are also achieved (Angelidaki and Sanders 2004). The issue of sulfide and volatile fatty acid accumulation (VFA) accumulation that results in extension of the lag phase is also overcome by introducing co-digestion; hence the overall methane production rate is enhanced (Demirel and Scherer 2011).

The aim of the present study is to examine different co-digestion scenarios of *Dalbergia sissoo* (DS), commonly known as Sheesham in Pakistan, and *Malus domestica* (MDL), generally called apple, with cattle manure (CM). The study also focused on how, during mesophilic anaerobic digestion, a possible synergism and higher biodegradability would be achieved by varying volatile solid (VS) share of all the above-mentioned substrates during co-digestion. Moreover, the modified Gompertz equation (Nielfa *et al.* 2015) was used to model the kinetics of overall methane production from all co-digestion scenarios to specifically predict the methane augmentation and calculate the extent of synergistic effect.

EXPERIMENTAL

Materials

Collection of substrates

The DS and MDL leaves were collected from local plants on university premises and were identified by specialist botanists of the Department of Botany PMAS-Arid Agriculture University Rawalpindi (Rawalpindi, Pakistan), and were stored at 4 °C prior to use. The sieved mesophilic 37 °C inoculum was also obtained from the university biogas plant, and cattle manure (CM) was also obtained from the cattle farm situated within the university. The inoculum was degassed prior to further usage.

Methods

The total Kjeldahl (TKN) and ammonium nitrogen (NH_4-N), pH, VS, and total solids (TS) were measured according to the standard protocols described by the National Renewable Energy Laboratory (NREL). The pH of the CM and inoculum were only measured by a Fisher brand FE150 pH bench top meter (Fisher Scientific, New Hampshire, USA) by using the protocol specified by Kougias *et al.* (2014). A volatile fatty acids (VFA) analysis of the manure and inoculum was performed and accompanied with internal standard (4-methyl-valeric acid) and gas chromatograph (GC) analysis *via* an autosampler (Kugelman and McCarty 1965). A GC Clarus 500 Perkin-Elmer (Waltham, Massachusetts, USA) operational with a split/split-less injector and flame ionization detector (FID) was used for methane content determination of biomethane potentials assays (BMPs). The separation was done on a glass capillary column with dimensions of (3 m × 6 mm), with an inner diameter of 2.5 mm from Restek (Bellefonte, USA). For the detection of methane contents produced from BMPs, a FID detector was used. Furthermore, the injection and detection temperatures were 110 °C and 160 °C, respectively. The NREL standard protocol was used for the determination of Klason lignin and carbohydrates (Sluiter *et al.* 2004). Sugar contents of both the DS and MDL were measured with an Agilent 1200 Series HPLC system chromatograph (Hewlett-Packard, Waldbronn, Germany) with the Aminex HPX-78H column (dimensions of 300.0 mm × 7.8 mm) at pH 3.0 with 4 mM H_2SO_4 as an eluent, at a flow rate of 0.6 mL/min.

Table 1. Chemical Characterization of Inoculum, Cattle Manure, and Lignocellulosic Biomass

Parameters	Inoculum	CM	MD Leaves	DS Leaves
TS (%w/w)	23.6 ± 0.1	32.2 ± 0.1	91.6 ± 0.1	90.4 ± 0.2
VS (%w/w)	1.9 ± 0.0	27.6 ± 0.1	84.9 ± 0.4	87.8 ± 0.6
TKN (g/kg)	3.1 ± 0.1	2.4 ± 0.1	3.8 ± 0.1	16.3 ± 0.0
(NH_4-N) (g/kg)	3.9 ± 0.1	1.9 ± 0.0	0.9 ± 0.1	4.9 ± 0.2
VFAs (mg/L)	210.2 ± 13.2	7050.0 ± 17.0		
C/N Ratio		16	85	19
Cellulose (% TS)	-	-	37.0 ± 0.7	44.2 ± 8.2
Hemicellulose (% TS)	-	-	19.4 ± 0.5	29.8 ± 0.0
Klason lignin (% TS)	-	-	21.7 ± 2.7	29.7 ± 0.5

Strong acid hydrolysis and inductive coupled optical emission spectroscopy was used to determine macro- and micronutrients (trace metals) in the substrates (Awais *et al.* 2016). The determination of carbon, hydrogen, nitrogen, oxygen, and sulphur (CHNOS) elements and C/N ratio analysis was performed with elemental analyzer (Varion Macro Cube, Hanau, Germany). All determinations were performed in triplicate to reduce any experimental errors during the study.

Table 2. Representation of Predicted Methane Yield, Synergistic Effect, and Percentage Increase in Methane Production from Various Co-digestion Setups

CM (%VS Share)	DSL/MDL (%VS Share)	Sample Name	Theoretical CH_4 (P_{th})	BD (%)	C/N Ratio	Predicted CH_4 (P_{pred})	Exp. CH_4 (P_{exp})	Synergistic Effect (θ)	Increase (%)
100	0	CM	433	39	16	-	171 ± 13	-	-
80	20	MMD1	427	59	30	179 ± 11	251 ± 10	1.40	40*
60	40	MMD2	420	54	44	187 ± 10	228 ± 12	1.22	22*
40	60	MMD3	414	57	57	195 ± 12	236 ± 14	1.21	21*
20	80	MMD4	407	50	71	203 ± 13	202 ± 16	1.00	-**
0	100	MDL	401	53	85	-	211 ± 11	-	-
80	20	MDS1	429	53	16	177 ± 09	229 ± 11	1.29	29*
60	40	MDS2	425	50	17	182 ± 14	214 ± 13	1.17	17*
40	60	MDS3	422	52	17	187 ± 12	221 ± 15	1.18	18*
20	80	MDS4	418	45	18	192 ± 11	189 ± 17	0.98	-**
0	100	DSL	414	48	18	-	189 ± 06	-	-

*Indicates the significant ($p < 0.05$) results compared with CM, while ** are not significant ($p > 0.05$)

BMP assay

The protocol defined by Angelidaki *et al.* (2009) was used to set up mesophilic (33 °C to 39 °C) batch assays to determine the biomethane potentials (BMPs). All of the batch assays were performed using 542 mL ± 1 mL glass bottles with 200 mL of working volume with 40% of inoculum having 2 g VS/L organic loading (OLR). First, mono-digestion of CM, DS, and MDL was done separately to analyze their own potential for methane production during AD to be used as a control for co-digestion later on. For co-digestion, two setups were introduced, one using CM + MDL and the other using CM + DS, and in both co-digestion scenarios the 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0 ratios were examined to elucidate an expected synergism established during AD. To avoid the formation of dead zones in the bacteria community, the AD reactors were shaken twice a day, and every reactor's accuracy was validated by using Avicel® PH-101 cellulose (Sigma Aldrich, Darmstadt, Germany) as a positive control. A glass reactor with 80 mL of inoculum and 120 mL of water was used to determine the residual methane of all samples. Furthermore, the glass reactors were flushed with a nitrogen gas mixture and closed with rubber corks. Every BMP experiment was set up in triplicate to ensure reproducibility of the experiment.

Theoretical biomethane potential, synergistic effect, and biodegradability calculation

Carbon, Hydrogen, Nitrogen, Oxygen, and Sulphur (CHNOS) analysis was also performed to calculate the theoretical methane potential (P_{th}) of substrates by using Buswell's formula (Symons and Buswell 1933), and the biodegradability of every sample was calculated by using Eq. 1,

$$BD\% = \left(\frac{Pex}{Pth}\right) \times 100 \tag{1}$$

where P_{ex} is experimental methane (NmL CH_4/g VS), P_{th} is theoretical biomethane (NmL CH_4/g VS), and BD% is percentage biodegradability (%).

Equation 2 was used to determine the value of the synergistic effect. Specifically, θ was calculated by dividing the methane yield from a BMP sample, and predicted the methane yield of that sample from a modified Gompertz model (Nielfa *et al.* 2015). The Gompertz equation was used to calculate the predicted methane yield and later this was used to calculate θ. The values of θ that were greater than one for a sample were considered for a positive synergism, and values lower than one were taken as antagonism.

$$\theta = \frac{Exp.Methane}{Pred.Methane} \tag{2}$$

Statistical analysis

A one-way analysis of variance (ANOVA) was used to establish difference among the means of each methane yield from a sample, and significant differences ($p < 0.05$) were identified using the Tukey *post hoc* analysis. Experimental data sets were analyzed with Graphpad Prism (Graphpad Software, Inc., version 5, San Diego, USA). The means and standard deviations were calculated using descriptive statistics.

RESULTS AND DISCUSSION

Proximate Analysis

Results from proximate analysis revealed VS contents of both the DSL and MDL were 87.8 ± 0.6 (% w/w) and 84.9 ± 0.4 (% w/w), respectively. The obtained values were also in accordance with the values 86.7 ± 4.0 (% w/w) and 89.6 ± 3.0 (% w/w) obtained for wheat straw and meadow grass, respectively, used as standard lignocellulosic materials during the experiments conducted by Awais *et al.* (2016). The apparent minor differences could have been because the substrates also had different lignin concentrations and other differences in composition, as shown in Table 1. The carbon nitrogen ratios of CM, DSL and MDL were found to be 16, 19, and 85 respectively. The strikingly different C/N values for lignocellulosic wastes were also reported in past as 23 for grass silage (Tsapekos *et al.* 2015) and 103 for wheat straw (Awais *et al.* 2016).

pH and Temperature Optimization for BMP Assays

Being the most crucial parameters for methanogenesis, the pH and temperature conditions were optimized to select the best pH and mesophilic temperature range for AD and carry out further co-digestion experiments on the same pH and temperature. For optimization during BMP assays, five different pH values ranging from 5 to 9, with equal intervals of one, and temperature ranges from 30 °C to 39 °C, were applied with equal intervals of 3 °C. So, at one pH four different BMP sets were assessed having different temperatures at single pH.

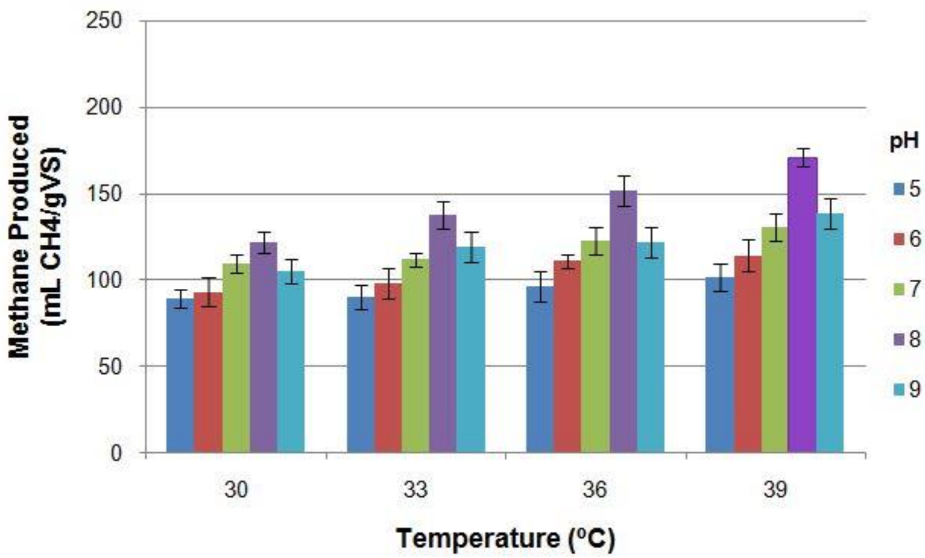

Fig. 1. Results of methane production from CM during pH and temperature optimization

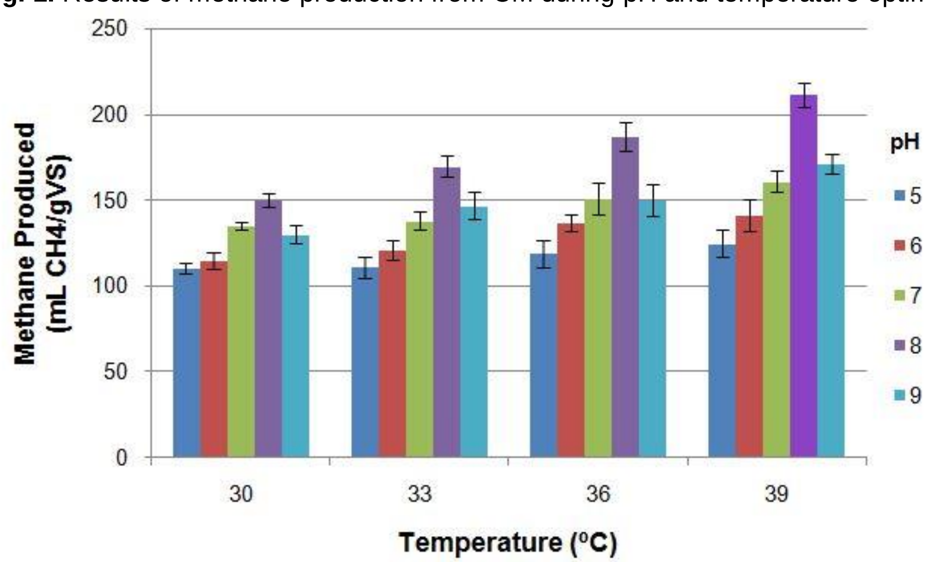

Fig. 2. Results of methane production from MDL during pH and temperature optimization

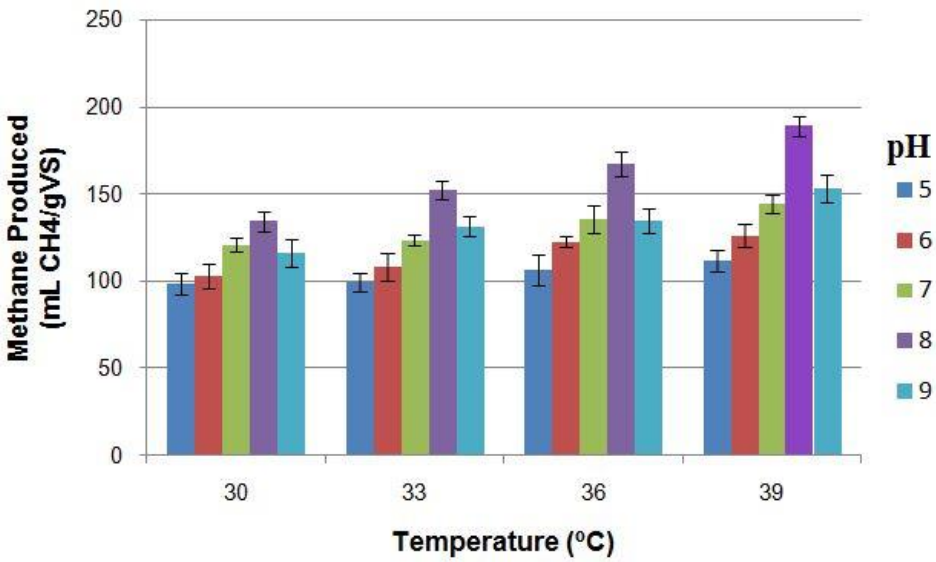

Fig. 3. Results of methane production from DS during pH and temperature optimization

First, the authors optimized the conditions for CM digestion, and the results revealed that the methane production was maximized at pH 8 and 39 °C with 171 ± 5 NmL CH_4/g VS, which was significant ($p < 0.05$) among all the other treatments conducted at this temperature. The methane yield at 5 pH and 30 °C was observed to be the lowest (121 ± 6 NmL CH_4/g VS) at 8 pH. As AD utilizes methanogen for both lignocellulosic biomass and sludge, in former experiments the results revealed that a pH around 7 to 8 was more suitable for AD of sludge. Furthermore, the authors' experimental trends followed the same pattern that increasing the pH until 8 increases the methane yield, but after this pH the increase in pH offers inhibition to methane production (Lay *et al.* 1997).

Secondly, the same experimental design for pH and temperature was followed for optimization of MDL mono-digestion, and the results obtained from triplicate batches revealed that at the lowest pH value of 5, the methane production was the least at all temperatures. The highest methane yield 211 ± 7 NmL CH_4/g VS was revealed at 39 °C and 8 pH. Finally, the optimization was performed for DSL, and the findings of the BMP assays revealed the same trend as CM and MDL mono-digestion. The treatment at pH 8 and 39 °C produced the highest methane yield of 189 ± 6 NmL CH_4/g VS. From all the mono-digestion results, it can also be established that DSL was more recalcitrant than MDL leaves. Table 1 also shows that DSL leaves had more lignin compared to MDL, which influences the rate of methane production (Zeng *et al.* 2014). Therefore, being more resistant to digestion during AD, DSL produced the least methane compared to both MDL and CM. Lastly, the finding of these experiments were taken as the conditions for later co-digestion experiments.

Co-digestion

The co-digestion experiments with various combinations were performed according to the design mentioned in Table 2. The results of co-digestion experiments were compared with CM mono-digestion that produced 171 ± 13 NmL CH_4/g VS. This treatment was kept as the standard for comparison of all the co-digestion results, to evaluate the percentage increase or decrease in methane augmentation. The results of methane production from MMD1 (CM + MDL combination 1) revealed 251 ± 10 NmL CH_4/g VS, which was significantly higher than mono-digestion of CM. The combination MMD1 also had a C/N ratio of 30 that otherwise would have been 16 in CM based digester; introduction of 20% MDL resulted in increased C/N ratio. It was previously established that the C/N ratio around 45:1 is considered ideal for piggery waste based digester (Itodo and Awulu 1999). The increase in C/N ration also resulted in the marked increase of biodegradability from 39% to 59% that resulted in production of 40% more methane compared with CM only.

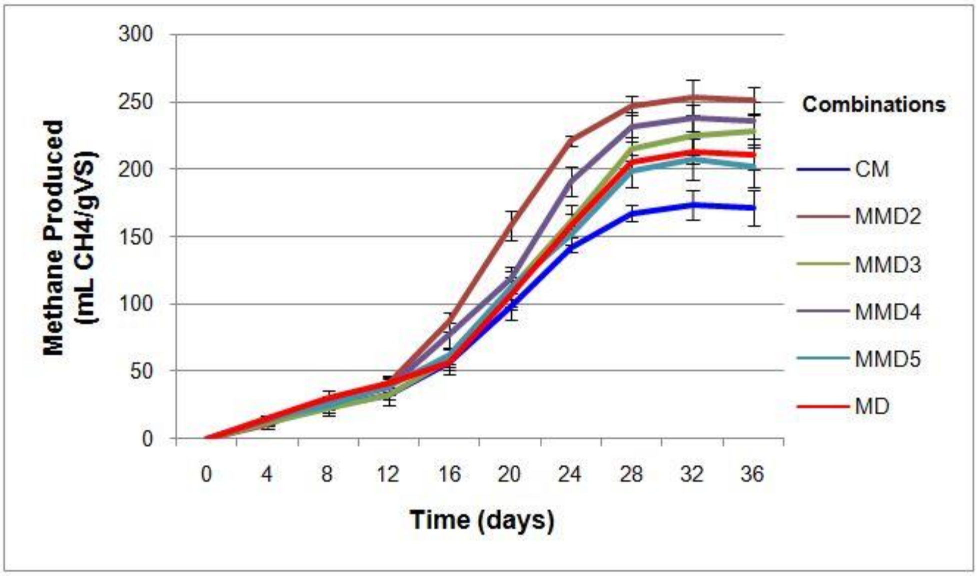

Fig. 4. Methane production from co-digestion of MDL and CM

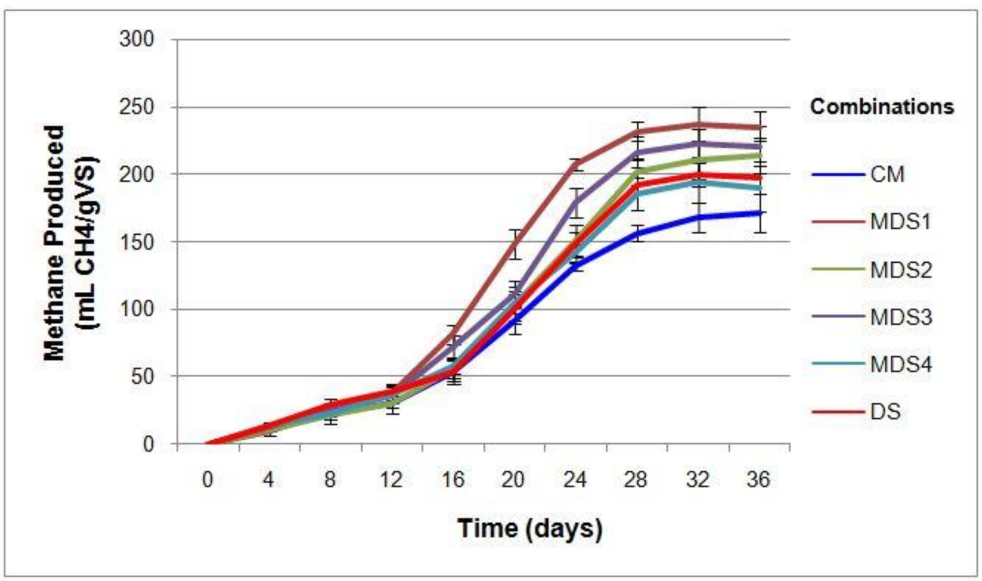

Fig. 5. Methane production from co-digestion of DSL and CM

The MMD3 and MMD2 were also found to have a methane yield of 236 ± 14 NmL CH_4/g VS and 228 ± 12 NmL CH_4/g VS and biodegradability of 54% and 57%, respectively. Meanwhile, MMD4 did not show any positive synergism, as it contained 80% VS of more recalcitrant lignocellulosic biomass MDL and 20% VS of CM. These results indicated that increasing lignocellulosic biomass resulted in decreased methane production.

The increase in lignocellulosic feedstock increases the carbon source and also the C/N ratio and the higher C/N ratios provoke VFA, which is unfavorable for methanogenesis (Bah *et al.* 2014). In previous studies, it was reported that adding 20% VS from lignocellulosic biomass for CM co-digestion increases the nitrogen contents and lowers the C/N ratio that otherwise leads to fatty acid accumulation (Angelidaki and Ellegaard 2002). It was also reported previously that similar combinations like MMD1 can lead to positive synergism (Awais *et al.* 2016), as the MMD1 combination revealed the θ value of 1.40 (Table 2), which showed not only an additive but synergistic effect.

The results of CM/DSL co-digestion also revealed a maximum methane yield of 229 ± 11 NmL CH_4/g VS from MDS1 that contained 80% VS share from CM and 20% from DSL. Subsequent increases in DSL percentage and decrease in CM (80% to 20% on VS basis) decreased the methane augmentation compared with MDS1. For example, MDS2, MDS3, and MDS4 revealed 214 ± 13 NmL CH_4/g VS, 221 ± 15 NmL CH_4/g VS, and 189 ± 17 NmL CH_4/g VS, respectively, but only the results of MDS2 and MDS3 were found to be significant ($p < 0.05$) compared with CM mono-digestion. The results of MDS1, and the trends followed by MDS2, MDS3, and MDS4, can be explained by the fact that the presence of CM provides buffering of metal ions in the AD reactor that plays an important role in nutrient balance for methanogenesis (Awais *et al.* 2016). Therefore, in light of this fact, MDS1 exhibited the optimum nutrient balance, ideal C/N ration, pH, and temperature parameters that resulted in 29% more methane augmentation as compared with CM, and showed a positive synergism θ value of 1.29. The overall C/N ratios of CM/DSL co-digestion did not change much markedly as compared to CM/MDL co-digestion because their combinations could only have C/N from 16 to 18 that are significantly lower than the optimum. This also explains the lower carbon and increased nitrogen contents that negatively affect the biodegradability of the system (Angelidaki and Ellegaard 2002).

Purification of Biogas

The purification of biogas was also done by taking the best co-digestion combination in five different glass reactors to increase the percentage of methane content.

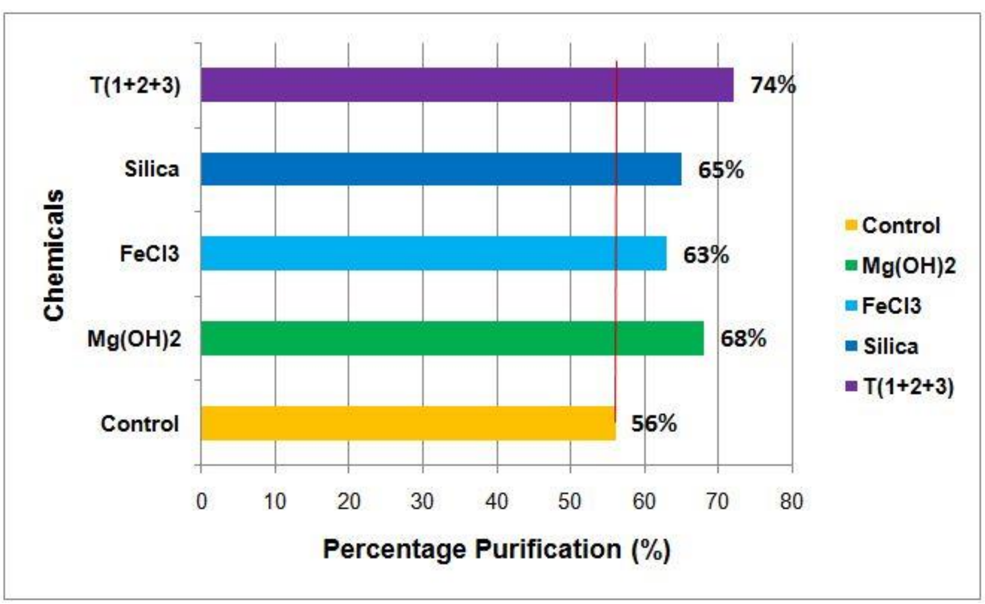

Fig. 6. Chemical treatments for biogas purification

One reactor was used as the control, and the biogas from other reactors was allowed to pass from magnesium hydroxide ($Mg(OH)_2$) solution (T_1), iron trichloride ($FeCl_3$) solution (T_2), silica (SiO_2) beads (T_3), and a combination of all three treatments ($T_1 + T_2 + T_3$). The results of percentage methane contents were compared with the reactor taken as the negative control in which no upgradation process was done.

Methane purification results from the reactor taken as the control revealed a maximum of 56% ± 0.84% methane content. Significantly ($p < 0.05$) higher results were observed when biogas was allowed to pass from the $FeCl_3$ solution, which enhanced the methane contents up to 63% ± 0.77%. Both T_1 and T_3 also enhanced the methane contents up to 68% ± 0.69% and 65% ± 0.38%, respectively. These results corresponded to the previously mentioned findings where 3 M sodium hydroxide could enhance the methane contents up to 62% due to CO_2 absorption in sodium hydroxide solution (Maile *et al.* 2017). Finally, the T_4 glass reactor that yielded 72% ± 0.21% content was the most effective biogas purification method.

CONCLUSIONS

1. Replacing the contents of manure-based batch reactors with 20% lignocellulosic biomass yielded up to 40% increased methane yield. The introduction of lignocellulosic materials increases the carbon contents and raises the C/N ratio, offering more biodegradability to produce more methane.
2. *Dalbergia sissoo* (DSL) had more lignin and nitrogen as compared with *Malus domestica* (MDL); hence it was estimated that (DSL) was more resistant to anaerobic digestion (AD) than MDL. Moreover, the co-digestion of MDL with cattle manure (CM) in 20/80 ratio is more feasible than CM/DSL 20/80 combination. Hence, all the lignocellulosic biomasses cannot be mixed in 20/80 combination with CM to get highest methane production results due to their different compositions.
3. The results from methane production revealed that CM/DSL co-digestion is not favorable as compared with CM/MDL co-digestion. CM/DSL co-digestion did not offer any marked adjustment in C/N ratio and biodegradability. Initial proximate analysis revealed low C/N ratios of both CM and DSL. Therefore, it is recommended to use MDL leaves for co-digestion with CM in batch reactors.
4. Furthermore, co-digestion created a synergism at mesophilic temperatures. In South Asia, where temperatures range from 33 °C to 40 °C or more, mesophilic co-digestion of substrates with highly different C/N ratios is able to replace all the digesters that currently use only manure. Such digestion systems produce less methane due to ammonia inhibition and low biodegradability.
5. Moreover, during the purification experiments it was also established that allowing the biogas to pass from $FeCl_3$, $Mg(OH)_2$, and silica beads columns together increased the methane content up to 74%.

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