Appl Biochem Biotechnol (2010) 162:524–535
DOI 10.1007/s12010-009-8834-2

Evaluation of Methanogenic Activity of Biogas Plant Slurry for Monitoring Codigestion of Ossein Factory Wastes and Cyanobacterial Biomass

P. Chellapandi · D. Prabaharan · L. Uma

Received: 18 June 2009 / Accepted: 25 October 2009 /
Published online: 13 November 2009

Abstract Overall measurement of methanogenic activity of sludge and or slurry is thought as a key for understanding the basic physiology of anaerobic consortia involved in anaerobic digestion process of an alternative biomass. In this study, the methanogenic activity of biogas plant slurry was used to evaluate the anaerobic digestion of ossein factory wastes such as sinews and primary clarified bone waste (PCBW) and cyanobacterial biomass in standard assay conditions. A maximum methanogenic activity was reported here when ossein factory wastes mixed with cyanobacterial biomass in specific proportions in which sinews and PCBW alone also favored to a significant methane yield. Cyanobacterial biomass alone did not give a desirable methanogenic activity. Approximately 48% of total solids were destroyed from these wastes after 30 days. This study gives information on the use of these wastes with suitable proportions for taking an effort in a large-scale anaerobic digestion in an effective way of ossein factory.

Keywords Ossein factory waste · Cyanobacterial biomass · Methanogenic activity · Biogas · Methane · Anaerobic digestion

Introduction

Anaerobic digestion has been widely used for the treatment of organic sludge and wastewater, biomethanation and recycling, and degradation of organic pollutants [1–4]. Biochemistry and microbiology of anaerobic digestion are complex biogenic processes involving a number of microbial populations (hydrolytic, acetogenic, and methanogeneic bacteria), often linked by their individual substrate and product specificities [5]. Among those bacteria, maintenance of sufficient methanogenic populations in an anaerobic system

P. Chellapandi (✉)
Biogas Research Center, Gujarat Vidyapith, Sadra 382320 Gujarat, India
e-mail: pchellapandi@gmail.com

D. Prabaharan · L. Uma
National Facility for Marine Cyanobacteria, Bharathidasan University,
Tiruchirappalli 620024 Tamilnadu, India

is critical for stable performance [6]. Methanogenic species types and their relative population levels in reactor biomass depend on organic waste characteristics as well as operational/environmental conditions maintained [7]. The reactor performance is usually evaluated in terms of process efficiency and stability through the estimation of organic matter removal [5], volatile fatty acids levels [8], quantity, composition of biogas produced [9], and relative population levels of methanogenic species under varied operational/environmental conditions [6, 10]. Enumeration and counting of methanogens as well as nonmethanogens in reactor biomass have also been made by several investigators [7, 10, 11]. However, these techniques require a high level of skill, advanced equipment, and costly and specific growth media, which restrict its application at the plant site. Specific methanogenic activity (SMA) tests on anaerobic sludges (biomass) have been gaining importance. SMA tests have been mainly used to select an adapted sludge as inoculum [12], evaluate the behavior of sludge under the effect of potential inhibitory compounds and batch kinetic parameters [13, 14], estimate maximum applicable loading rate to a certain sludge [15], and establish the degree of degradability of various substances [10, 16]. Dolfing and Bloemen proposed methanogenic activity measurements as a tool to monitor the microbial composition of methanogenic environments using H_2, formate, acetate, and propionate as test substrates [17]. A number of methods have been suggested for the estimation of maximum methanogenic activity because of its quite simplicity, even though several automated monitoring processes are available [12, 18, 19]. Several anaerobic process variants having specific biomass retention mechanisms are available for field application [20, 21]. However, only a limited attempt has been made on evaluating anaerobic biodegradability of proteinaceous wastes by using such effort.

After collecting flesh and hides from cattle in slaughterhouse, the bones are brought to ossein factory wherein they are socked in water for at least 10 days. Hence, the same bones are clarified with alums in a clarification tank to separate out the minerals (calcium) from bones and to also neutralize it before gelatin extraction. During this process, a huge amount of bone waste having hairs, greases, and small bone pieces is discharged out wherein primary clarified bone waste (PCBW) and sinews are the major biological wastes causing odor due to deamination reactions in bulk storage. The organic matters of these wastes can be used as a substitute for value-added products such as methane by replacing conventional sources. Anaerobic digestion can reduce the volume of waste sludge and produce methane that can be utilized in supplying the thermal energy requirement in raw gelatin drying unit. Although a significant fraction of thermal input can be recovered using a heat exchanger, the thermal requirement is, of course, an important economic factor for operating the process in full scale [9].

In this perspective, the present work was focused on the application of a simple methanogenic activity assay to evaluate reactor biomass and its substitution in terms of relative production levels of methane by using two different test substrates such as ossein factory wastes (PCBW and sinews) and cyanobacterial biomass. This work was also aimed to utilize the biogas plant slurry to start biomethanation process wherein ossein factory wastes are used as substrates in batch mode. The results so obtained would be correlated with the performance of a laboratory-scale reactor.

Materials and Methods

Substrate Collection and Preparation

In wet (raw) form, the test biomasses sinews and PCBW were obtained from a discharging outlet of a bone clarification tank at Pioneer-Myagi Indo-Japan Company, Cuddalore, India,

and immediately stored in plastic container at 4 °C in a cold room until use. Cyanobacterial strain *Phormidium valderianum* BDU 20041 was procured from the National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, India, and maintained in ASN III liquid medium [22]. The cyanobacterial mass cultivation was carried out in 1-l conical flask containing 400-ml ASN III medium, and 25 ml of cyanobacterial biomass was used as inoculum. The inoculated flask was then incubated at 25 °C with 1,500 lx. After a 7-day incubation, cyanobacterial biomass was harvested, washed with distilled water, and then homogenized by vortex afterwards. This cyanobacterial biomass was preserved at 4 °C in a cold room until use.

Anaerobic Seed Collection and Preparation

Predigested slurry from a biogas plant (50 m^3) running in continuous mode at Pioneer-Myagi Indo-Japan Company, Cuddalore, India, was collected in a serum bottle. Sinews and PCBW were primary biomasses used in the biogas plant of that factory, which were partially substituted with fresh cattle dung. One volume aqueous portion of the slurry was diluted with four volumes of dilution medium containing $NaHCO_3$ (0.5%, *w*/*v*), $NaCO_3$ (1%, *w*/*v*), and resazurin (0.0001%, *w*/*v*) [22]. This diluted solid-free slurry (decanted) was preincubated at 38 °C (24 h) for activating anaerobic bacteria prior to inoculation. Slurry prepared herein was used as an inoculum throughout this study.

Specific Methanogenic Activity Assay

A serum vial with a volume of 134 ml was used as an anaerobic digester. About 70 ml of assay medium [22] was mixed with substrates as described in the text and 5 ml of preactivated seed (as inoculum) in a serum vial. The assay medium was composed of 1-ml macronutrient solution, 0.1-ml micronutrient solution, 1-ml phosphate solution (50 g KH_2PO_4/L), 0.57 g $NaHCO_3$, and 1 ml resazurin (0.0001%, *w*/*v*) in 100 ml distilled water. Macronutrient solution (g/l) has the following composition: 10 g NaCl, 50 g NH_4Cl, 10 g $MgCl_2{\cdot}6H_2O$, and 10 g $CaCl_2{\cdot}2H_2O$. Micronutrient solution has (g/l) 10 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.3 g H_3BO_4, 1.5 g $FeSO_4{\cdot}4H_2O$, 10 g $CoCl_2{\cdot}6H_2O$, 0.03 g $MnCl_2{\cdot}4H_2O$, 0.03 g $NiCl_2{\cdot}6H_2O$, and 0.1 g $Alk(SO_4)_2{\cdot}12\ H_2O$. This assay medium was boiled for 30 min at mild temperature and cooled to room temperature. The mouth of the serum vial was closed with butyl rubber and sealed with aluminum crimp. A mixture containing substrates and anaerobic seed in assay medium was subjected to anaerobic conditions by passing through nitrogen gas for 1 min. A serum vial containing the same assay medium and seed without the above substrates was served as a control in all experiments. All serum vials with different treatments were incubated at 38 °C in a temperature-controlled incubator (ORBITEK, India) for 30 days. These vials were frequently flushed with nitrogen gas for 1 min to maintain a suitable anaerobic condition in head gas phase, and the contents in each vial were mixed daily by swirling manually. Gas production was measured using an air-tight syringe. The activity tests were run in four similar setups (quartet) for every treatment. Total solids (Ts) reduction in substrate was determined by weighing constant dry weight (at 105 °C in an oven) of whole biomass which was retained in the vial after dismantling the setups.

Gas Chromatograph Analysis

Gas samples were taken from the head phase of each anaerobic digester at time intervals of approximately 5 days during anaerobic digestion. Methane was determined by gas

chromatograph (Hewlett Packard 5890), equipped with a flame ionization detector and a data integrator [23]. One hundred microliters of diluted biogas sample was injected into a 2-m Poropak T steel column (80–100 mesh) using an air-tight syringe with an 18-gage needle. Nitrogen and hydrogen were used as a carrier gas and as a fuel at the flow rate of 30 ml/min^{-1}, respectively. The column, injection, and detector temperatures were adjusted to 75, 110, and 120 °C respectively. After calibrating with a standard methane gas, methane content was expressed as millimole of methane per milliliter head gas phase.

Results and Discussion

Physiochemical Properties of Substrates

An intensive and stable anaerobic digestion of partly hydrolyzed organic waste and protein-rich slaughterhouse waste was already achieved in the balance of inconsistent pH and buffering NH_4-N [24]. It suggested the possibility of using proteinaceous wastes sinews and PCBW as substitutes and predigested slurry obtained from industrial biogas plant, treating these wastes as a microbial source in this study. As listed in Table 1, physiochemical analyses of these wastes pointed out that protein was a major constituent in these organic materials and has a low content of lipids and oils. Protein content in sinews was much more than in PCBW whereas mineral content was lesser than in PCBW. It suggested that a substitution of carbon source is necessary to initiate biomethanation process due to more nitrogen percentage found in these wastes. Moreover, at low C/N ratio of organic materials, anaerobic microorganisms prefer to synthesize cellular energy from available protein constituents; not all organisms, only those whose metabolic regulations have to be adapted to such microenvironment, are being considered.

Nickel, iron, and cobalt form relatively strong organic complexes with proteinous substrate, particularly yeast extract in which the bioavailability of these essential metals in anaerobic batch reactors is dramatically increased [25]. An enhanced production of biogas has been reported earlier in the presence of cobalt, nickel, magnesium, calcium, iron, and manganese in the digesters [26–29]. Therefore, as a result of good metal ion composition found in PCBW, the additional metals are not optional for boosting the growth of anaerobic microorganisms due to the bioavailability of essential metals. This concern is thought to be

Table 1 Physicochemical characteristics of ossein factory wastes.

Constituents	Composition (%)	
	Sinews	PCBW
Total solid	42.5±1.0	4.88±1.0
Total volatile solid	78.4±0.5	71.5±0.5
Total minerals	21.6±0.5	28.5±0.5
Organic carbon	57±1.0	42±1.0
Total nitrogen	14.4±1.2	11.5±0.8
Total protein	90±3.0	72±2.0
Lipids and oils	7.0±0.5	2.6±1.0
C/N ratio[a]	4.0±0.3	3.64±0.3
pH[a]	6.5±0.2	7.15±0.5

Percent of TS in cattle dung was 27.6%

[a] Not expressed in percent

one of the advantageous of using ossein factory wastes as good substrates for biogas production. These organic wastes are usually treated with lime in ossein factory, which may contribute to the reduction of some toxicity and enduring buffering capacity in treatment plants [21, 23]. This provides an endeavor to use these wastes for running a stable reactor performance. The anaerobic biomass composition may be assessed using a simple methanogenic activity test procedure, selecting substrate on which the biomass is being maintained [10]. Therefore, this study was attempted to use a simple SMA test to evaluate sinews, PCBW, and cyanobacterial biomass with relative proportions to reveal their suitability in a stable reactor performance at ossein factory.

Effect of Onefold Ossein Factory Wastes and Fourfold Cyanobacterial Biomass Mixture

A maximum methanogenic activity (896.53±39.95 mmol methane per milliliter head gas phase) was obtained after 20 days when 1-ml PCBW was substituted with 4-g sinews (1+4-fold); after that, methanogenic activity declined. PCBW and cyanobacterial biomass mixture gave a methanogenic activity (673.65±89.80 mmol methane per milliliter head gas phase) which was significantly lower than in sinews and cyanobacterial biomass mixture (3,401.53±378.71 mmol methane per milliliter head gas phase). As shown in Fig. 1, a favorable methanogenic activity was observed after 20 days of digestion when a mixture containing 1-ml PCBW and 4-g sinews was used.

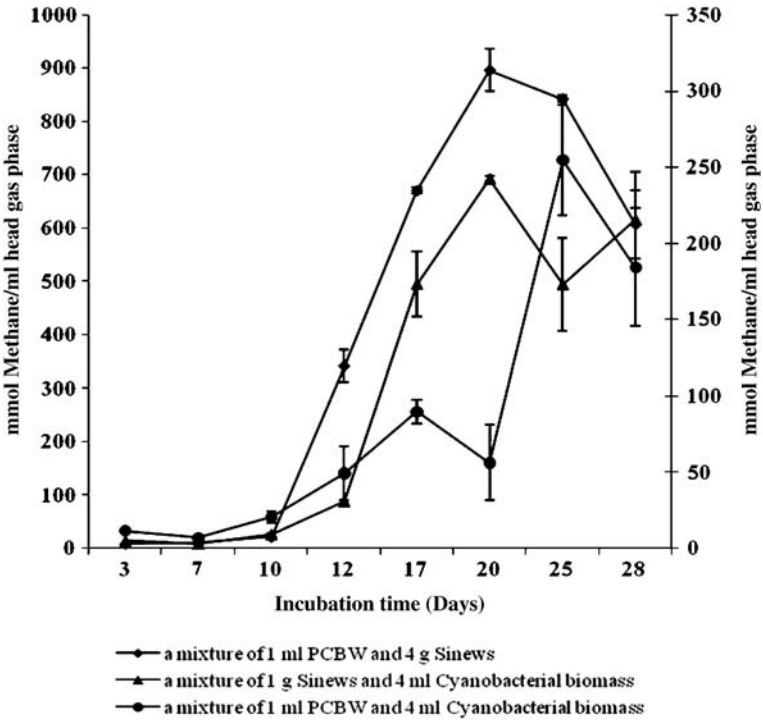

Fig. 1 Effect of a mixture containing 1 g of ossein factory waste (onefold) and 4 ml cyanobacterial biomass on specific methanogenic activity of biogas plant slurry at different incubation time (40 °C)

Effect of Twofold Ossein Factory Wastes and Threefold Cyanobacterial Biomass Mixture

As shown Fig. 2, the methanogenic activity of biogas plant slurry was gradually increased with increasing digestion time on using twofold proportion of each substrate in which a mixture of 2-ml PCBW and 3-g sinews resulted in a maximum activity (3,440.28± 392.68 mmol methane per milliliter head gas phase). It also revealed that the methanogenic activity on cyanobacterial biomass mixed with sinews (2,949.32±366.27 mmol methane per milliliter head gas phase) was better than PCBW when mixed with it (1,716.18± 227.62 mmol methane per milliliter head gas phase). At initial digestion period (up to 10 days), all the mixtures not supported to improve the methane productivity due to the specific growth rate should be attained for hydrolytic activity of anaerobic organisms. A huge amount of fibrous content and other solid matters in sinews [21] and delayed acclimatization of methanogens [9, 11, 30] on the substrate may be the reasons for the decline of the methanogenic activity at the initial digestion period.

Effect of Threefold Ossein Factory Wastes and Twofold Cyanobacterial Biomass Mixture

From the results obtained for the mixture of threefold ossein factory wastes and twofold cyanobacterial biomass from Fig. 3, we observed that methanogenic activity of biogas plant slurry was gradually increased to a maximum at 17 days, slowly declined until 25 days, and slightly raised again after its activity. An inadequate availability of methane precursors and delayed acclimatization process of anaerobic populations on organic wastes are concerned

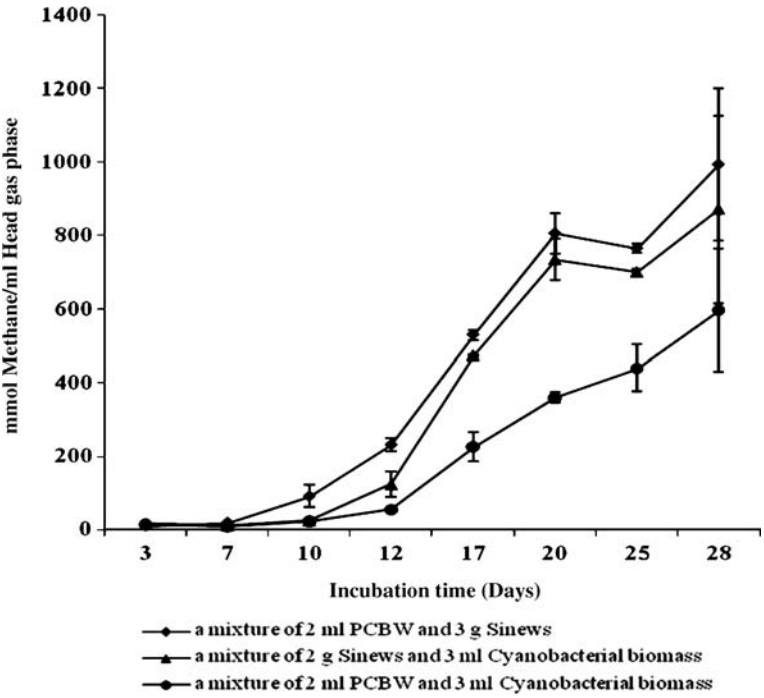

Fig. 2 Effect of a mixture containing 2 g of ossein factory waste (twofold) and 3 ml cyanobacterial biomass on specific methanogenic activity of biogas plant slurry at different incubation time (40 °C)

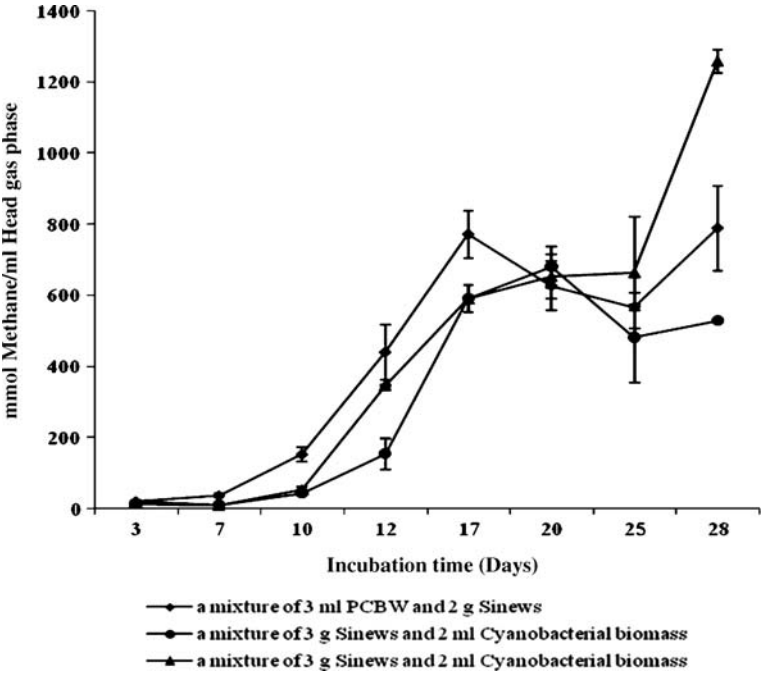

Fig. 3 Effect of a mixture containing 3 g of ossein factory waste (threefold) and 2 ml cyanobacterial biomass on specific methanogenic activity of biogas plant slurry at different incubation time (40 °C)

here to bring such shift in anaerobic batch digestion [18, 19, 30, 31]. Any imposed stress may also lead to a change in species types and their relative population levels which are ultimately reflected in the reactor performance [10, 12, 32]. It is suggested that due to overaccumulation of ammonia produced from deamination reactions at high methanogenic rate the growth of some potential methanogens could be inhibited by exerted ammonia toxicity. Cyanobacterial biomass mixed with PCBW and with sinews provided the methanogenic activity of 2,508.98±283.78 and 3,599.24±432.11 mmol methane per milliliter head gas phase, respectively. It is also reported that a maximum methanogenic activity was obtained when ossein factory wastes was mixed with cyanobacterial biomass. As a consequence of high soluble organic matter in ossein factory wastes as compared to cyanobacterial biomass, a considerable methanogenic activity was recorded as 3,409.83±316.41 mmol methane per milliliter head gas phase using a mixture of 3-ml PCBW and 2-g sinews.

Effect of Fourfold Ossein Factory Wastes and Onefold Cyanobacterial Biomass Mixture

After 10 days, a notable methanogenic activity was observed when a fourfold proportion of ossein factory wastes are mixed with onefold cyanobacterial biomass, and it slowly rose to reach maximum at the end of digestion period as represented in Fig. 4. The greatest methanogenic activity (3,665.95±327.08 mmol methane per milliliter head gas phase) was observed while 4-ml PCBW mixed with 1-g sinews and later on 4 g sinews with 1-ml cyanobacterial mixture (3,106.24±376.64 mmol methane per milliliter head gas phase). It was revealed that even a high proportion of ossein factory wastes is not affecting the methanogenic activity and so is probably suitable for methane production. Similarly, 4-ml

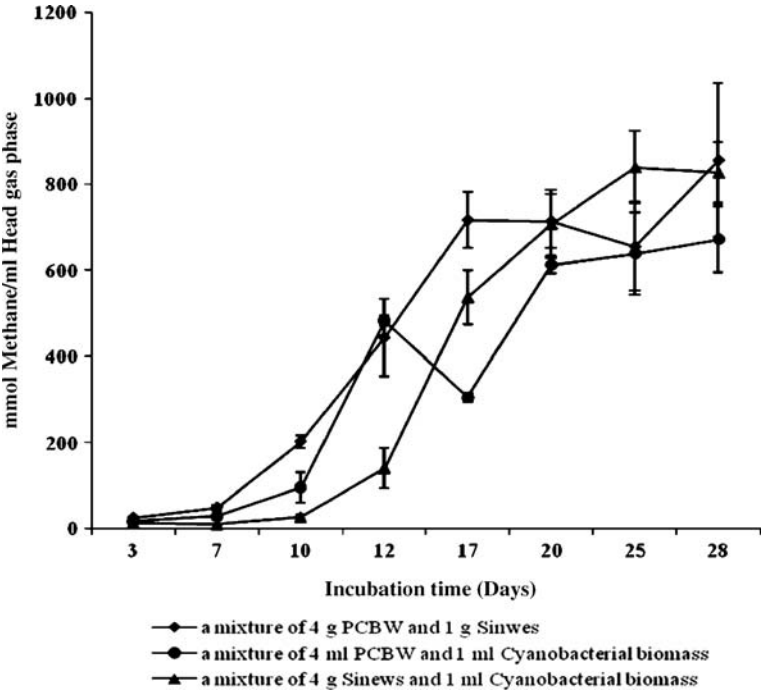

Fig. 4 Effect of a mixture containing 4 g of ossein factory waste (fourfold) and 1 ml cyanobacterial biomass on specific methanogenic activity of biogas plant slurry at different incubation time (40 °C)

PCBW and 1-ml cyanobacterial mixture also favored somehow to methanogenic activity, and it was 2,853.82±281.66 mmol methane per milliliter head gas phase. Methanogenic activity of granules has already been reported to increase steadily with increasing influent substrate concentration that may be attributed to an increased fraction of viable organisms in the more heavily loaded granules [19] and hydrogen-oxidizing methanogens [10]. Accordingly, a maximum methanogenic activity, even at an increased initial substrate load of ossein factory wastes, can be obtained by increasing numbers of viable and potential anaerobic consortium as existed in predigested biogas plant slurry of this study.

Effect of Ossein Factory Wastes and Cyanobacterial Biomass Alone on SMA

A significant methanogenic activity (3.4777±0.2267 mmol methane per milliliter head gas phase) was obtained on 6.0-ml cyanobacterial biomass alone after 20 days, and overall methanogenic activity was 8.1443±1.558 mmol methane per milliliter head gas phase, but increasing proportion of cyanobacterial biomass did not improve the methanogenic activity (Table 2; Fig. 5). However, when different proportions of PCBW and sinews alone were tested, it showed the maximum cumulative methanogenic activity (2,842.41±359.60 mmol methane per milliliter head gas phase) on sinews, which was better than on using PCBW (2,399.57±261.76 mmol methane per milliliter head gas phase). As compared to ossein factory wastes in this study, cyanobacterial biomass alone did not serve as a good substrate for methanogenic activity that may be result due to low organic constituents (2.26% Ts) and minerals.

Table 2 Effect of different volume of cyanobacterial biomass on specific methanogenic activity (millimole methane per milliliter head gas phase) of biogas plant slurry at 40 °C.

Day	Cyanobacterial biomass volume									
	2 ml	SD	4 ml	SD	6 ml	SD	8 ml	SD	10 ml	SD
7	0.0106	0.0080	0.0185	0.0022	0.0321	0.0075	0.0262	0.0029	0.0167	0.0023
10	0.0234	0.0126	0.0198	0.0058	0.0190	0.0030	0.0247	0.0047	0.0360	0.0024
12	0.0240	0.0029	0.0296	0.0041	0.0305	0.0058	0.0356	0.0093	0.0327	0.0022
17	0.0228	0.0087	0.0215	0.0005	0.0236	0.0015	0.0261	0.0041	0.0136	0.0006
20	2.0342	0.5612	3.1416	0.0238	3.4777	0.2267	3.3891	0.2942	0.7505	0.0936
25	1.1361	0.0739	3.1334	0.0114	3.1599	0.4028	2.8021	0.0915	0.9150	0.3144
28	1.1427	0.0212	1.6501	0.0832	1.4015	0.3300	0.8989	0.2322	0.8643	0.2547

SD denotes standard deviation (±)

Effect of SMA on Total Solids Reduction in Substrates

Results of organic solids destroyed (milligram dry weight) in ossein factory wastes alone and their relative mixtures with cyanobacterial biomass is represented in Tables 3 and 4. It reported that the organisms in biogas plant slurry (seed) was readily consumed organic

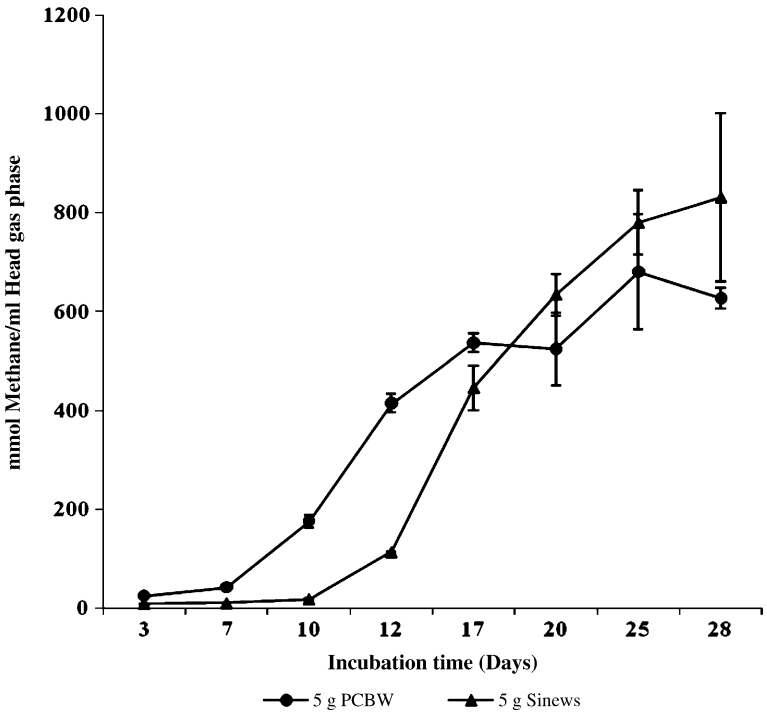

Fig. 5 Effect of ossein factory wastes alone on specific methanogenic activity of biogas plant slurry at different incubation time (40 °C)

Table 3 Effect of biogas plant slurry on total solids reduction (milligram dry weight) in different proportion of ossein factory wastes and cyanobacterial biomass after 30 days.

Substrate proportion	Total solids reduction (mg dry weight)		
	Sinews[a]	PCBW[b]	Cyanobacterial biomass[b]
Onefold	394	60	374
Twofold	679	90	393
Threefold	812	155	416
Fourfold	1,088	157	443
Fivefold	1,164	209	452

[a] The proportion (fold) of sinews was used in gram (1, 2, 3, 4, and 5 g)

[b] The proportion (fold) of PCBW and cyanobacterial biomass was used in milliliter (1, 2, 3, 4, and 5 ml)

constituents of PCBW and cyanobacterial biomass that show the significant results in solids reduction (48.6%) even at their high proportion. The best reduction in solids (50.8%) was found on sing twofold proportion of PCBW alone and overall average was 42–44%. This revealed that the anaerobic digestion rate is increased with decrease in organic loading rate (concentration) of sinews, which in turn increases in proportion to PCBW and its substitution with cyanobacterial biomass. It may be the nature of substrate to determine the type and extent of the fermentative bacteria present in the digesters. The presence of proteolytic organisms in cow-dung-fed digesters and other-animal-waste-fed digesters contributed to the use of proteinous substrates as reported earlier [33]. This suggested that occurrence of some proteolytic organisms could be have a role on degrading protein contents in PCBW and converting it into amino acids. Generally, Stickland pathway is a

Table 4 Effect of biogas plant slurry on total solids reduction (milligram dry weight) in different proportion of ossein factory wastes and cyanobacterial biomass mixtures after 30 days.

Proportion	Total solids reduction (mg dry weight)
A mixture of PCBW and sinews	
A mixture of 1-ml PCBW and 4-g sinews	446
A mixture of 2-ml PCBW and 3-g sinews	760
A mixture of 3-ml PCBW and 2-g sinews	541
A mixture of 4-ml PCBW and 1-g sinews	510
5-g PCBW	460
A mixture of PCBW and cyanobacterial biomass	
A mixture of 1-ml PCBW and 4-g cyanobacterial biomass	416
A mixture of 2-ml PCBW and 3-g cyanobacterial biomass	373
A mixture of 3-ml PCBW and 2-g cyanobacterial biomass	397
A mixture of 4-ml PCBW and 1-g cyanobacterial biomass	409
A mixture of PCBW and cyanobacterial biomass	
A mixture of 1-g sinews and 4-ml cyanobacterial biomass	620
A mixture of 2-g sinews and 3-ml cyanobacterial biomass	688
A mixture of 3-g sinews and 2-ml cyanobacterial biomass	787
A mixture of 4-g sinews and 1-ml cyanobacterial biomass	936
1-g sinews	1,247

predominant metabolic route to convert 40% amino acids, as coupled with other amino acid reactions, into methane from protein waste, and the rest of the amino acids are utilized, as uncoupled reactions with molecular hydrogen by hydrogen-consuming methanogens [33]. In Stickland reactions, the electron donor amino acid (glycine, alanine, etc.) is oxidized to a volatile carboxylic acid (acetate) one carbon atom shorter than the original amino acid, which can further be converted into methane. According to that metabolic reaction, methane can be produced by microbial consortium existing in predigested biogas plant slurry on ossein factory wastes after hydrolyzing protein contents.

Conclusion

Despite of exploiting cyanobacterial biomass alone, substituting with ossein factory wastes will prove more noteworthy to methanogenesis. This present effort will also provide a new vision to control odor in ossein factory. Moreover, the activities so obtained correlate well with the reactor performance and clearly demonstrate the specific proportion of these substrates needed for higher methanogenic activity with maximum applicable loading rate in operational conditions. Herein, SMA tests can be used to monitor the biomass substitution, organic loading rate, and advantages of preactivated seed along with usual reactor performance evaluation parameters for giving a better insight into reactor stability and performance. Overall, this study concluded that ossein factory wastes and cyanobacterial biomass will be alternative feedstocks not only to methane production in a large-scale reactor but also for solid waste management in industrial sectors.

Acknowledgements The corresponding author thanks Vice-Chancellor and Registrar of Gujarat Vidyapith, Ahmedabad, India for providing the study leave to carry out this investigation. Department of Biotechnology, India is duly acknowledged.

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