

Original Research Article

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Comparative Analysis of Different Biodegradable Wastes for Better Biogas Production

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Recycle and reusing waste takes little effort on our part, for a big difference to the world. Wastes samples like cow dung slurry, kitchen waste slurry and de-oiled seed cake slurry were collected from different places aseptically and microbiological analysis of the samples was undertaken. A total of 30 bacteria isolates were identified on the basis of cultural, morphological and bio chemical characteristics by using PIBwin software. The bacterial isolates belong to the genera *Bacillus*, *Paenibacillus*, *Virgibacillus*, *Streptococcus*, *Micrococcus* and *Enterobacter* sp. All the bacterial isolates were screened for different enzymatic tests. Fluorescence tests were investigated which revealed that 33.3% bacterial isolates were methanogenic. Biogas production was done by using anaerobic conventional digester. Kitchen waste slurry showed maximum production of bio gas than other samples. Final biogas production was done by using methanogenic isolates from cow dung slurry and the isolate A4 & A8 showed maximum bio gas production. Isolate A4 was identified as *Bacillus licheniformis*. Further molecular identification of isolate A8 was done. It was identified to be *Enterobacter cloacae* subsp. *dissolvens* strain A8 having GenBank accession number KM034810.

Introduction

Energy is one of the most important factors to global prosperity. In India alone, there are an estimated over 250 million cattle and if one third of the dung produced annually is available for production of biogas, more than 12 million biogas plants can be installed (Kashyap *et al.*, 2003).

Biogas is a readily available energy resource that significantly reduces greenhouse-gas emission compared to the emission of landfill gas to the atmosphere

(Murphy *et al.*, 2004). The gas is typically composed of 50-70% Methane, 30-40% Carbon dioxide, 1-10% Hydrogen, 1-3% Nitrogen, 0.1% Oxygen and Carbon monoxide and trace of Hydrogen sulphide (Hasimoto *et al.*, 1978).

Cow dung has high nitrogen content and due to pre-fermentation in the stomach of ruminant, and has been observed to be most suitable material for high yield of biogas through the study made over the years (Chonkor, 1983). Plant materials such as

crop residues are more difficult to digest than animal wastes (manures) because of the difficulty in achieving hydrolysis of cellulosic and lignin constituents with attendant acidity in the biogas systems leading to reduction and sometimes cessation of gas flammability / gas production etc (Ofoefule *et. al.*, 2010).

Currently, much of our biodegradable wastes such as kitchen wastes, agricultural wastes & animal wastes are used to produce biogas. The process consists of four steps, each of which carried out by different groups of bacteria- Hydrolysis, Acidogenesis, Acetogenesis, Methanogenesis.

India has a very huge potential of tree-born non-edible oil seeds. The non-traditional seedoils available in the country, which can be exploited for this purpose, are *Madhuca indica*, *Shorea robusta*, *Pongamia glabra*, *Mesua ferrera*, *Mallotus philippines*, *Garcinia indica*, *Jatropha curcas* and *Salvadora*. Out of these seeds, at the most 25% is converted into vegetable oil and the remaining 70% is rejected as toxic de-oiled seed cake and 5% losses in the oil extraction process using mechanical expeller. However, this could be a big source of bio-energy production from the generated waste (Ram Chandra *et al.*, 2006).

Microbiology of Biogas Production

Microbial diversity in biogas digesters is as great as that of rumen (Ramasamy *et al.*, 1991). A total of seventeen fermentative bacterial species have been reported to play important role for production of biogas (Wolin, 1979). Furthermore, it is the nature of the substrate that determines the type and extent of the fermentative bacteria present in the digester (Zinder, 1984). It was

observed that while cow dung-fed digesters supported higher amyloytic microorganisms, poultry waste-fed digesters showed higher proteolytic population (Preeti Rao *et. al.*, 1993). Among fermentative organisms, *Bacteroides succinogens*, *Butyrivibrio fibrisolvens*, *Clostridium cellobioparum*, *Ruminococcus albus* and *Clostridium* sp. were predominant in cattle dung-fed digesters (Godbole *et al.*, 1981).

Methanogens possess very limited metabolic range, using only acetate or C₁ compounds (H₂ and CO₂, formate, methanol, methylamines or CO), with methane being the end product of the reaction. Of the methanogenic genera, *Methanosarcina* sp. and *Methanosaeta* sp. form methane by aceticlastic reaction (Preeti Rao *et al.*, 1993). Both carbon-dioxide-reducing and aceticlastic-methanogens play an important role in maintaining stability of the digester. The failure in a biogas digester can occur if carbon dioxide-reducing methanogens fail to keep pace with hydrogen production (Robinson *et al.*, 1984).

Materials and Methods

The present investigation was carried out in Department of Microbiology, O.U.A.T., Bhubaneswar. Different samples were collected from different places like Cow dung slurry and *Pongamia pinnata* (Karanja) de-oiled seed cake slurry from agricultural field and College of Agriculture & Engineering Technology, Orissa University of Agriculture and Technology respectively. Kitchen waste slurry was collected from College of Engineering Bhubaneswar (CEB), Patia, Bhubaneswar. Microbial analysis of the samples was carried out to isolate aerobic and facultative anaerobic bacteria. For this

purpose serial dilutions & spread plate method were carried out to obtain distinct colonies. For aerobic & facultative anaerobic bacteria, media like nutrient agar & thioglycollate agar were used respectively. Identification of bacterial isolates was done on the basis of their colony characteristics on the basal media & Gram staining.

Bio-chemical characterization of the isolates was done on the basis of the following bio-chemical tests including: - (1) Standard test (2) Enzymatic test (3) Sugar test. For all bio-chemical tests 24 hour fresh culture was used. One control was kept in the case of each test.

Fluorescence Test

Fluorescence is presumptive evidence for methanogenic bacteria, but definitive proof requires further characterization. To observe the presence of fluorescence, the isolated colony plates were directly placed in UV Trans-illuminator and observations were noted. Positive - Development of blue-green fluorescence, Negative – no fluorescence (Dhadse *et al.*, 2012).

Biogas Production and Setup

A bench scale study was conducted using cattle dung slurry and in combination of cow dung with kitchen waste & de-oiled seed cake to determine the volume of biogas generation. An anaerobic conventional digester had been taken for generation of biogas. The test was carried out by digesting 250ml of cattle dung and 500 ml of water (ratio 1:2) in a 1000ml conical flask. The flask was made air tight by using rubber cork. A plastic pipe of approx. 1 m long was attached to one glass tube (3" long) perforated into the cork of the conical flask (digester) and another

glass tube into the measuring cylinder in upside down position containing acidic solution of 20% sodium sulphate. The biogas formed in the sample collected in the measuring cylinder. When an acidic solution was used as the displacement liquid, CO₂ will not be absorbed and the displaced gas volume will be indicative of the total biogas generated (Dhadse *et al.*, 2012).

For this experiment different experimental sets were taken and biogas production was determined in a per day basis. The sets or consortia were performed on the basis of their characters like methanogenic, nonmethanogenic, aerobic and facultative anaerobic. The differentiation was on the basis of fluorescence test and growth on aerobic and anaerobic media. In each digester 2 ml of each culture was added to the cattle dung slurry to check the potential activity of the isolates on biogas generation. Sterilized cattle dung slurry was used as control

Molecular Identification

Molecular identification of the selected bacterial isolate was done by 16S r DNA sequencing. For DNA Isolation Bacterial DNA isolation Kit was used (Xcelgen).

Result and Discussion

Bacterial isolates from different slurries

A total of 16, 8 and 6 bacteria were isolated from cow dung slurry, kitchen waste slurry and de-oiled seed cake slurry respectively. The bacterial isolates were designated as A1-A12 and AN1-AN4 for aerobic and facultative anaerobic bacteria from cow dung slurry, KA1-KA4 and KAN1-KAN4 for aerobic and facultative anaerobic bacteria from kitchen waste slurry and

OA1-OA2 and OAN1-OAN4 for aerobic and facultative anaerobic bacteria from de-oiled seed cake slurry respectively.

Identification of Bacterial Isolates

Identification of bacterial isolates was done by cultural, morphological & biochemical characterization using Bergey's Manual of Systematic Bacteriology (Holt *et. al*, 1984) and PIBwin- software (Bryant, 2004). The bacterial isolates belong to the genera *Bacillus*, *Paenibacillus*, *Virgibacillus*, *Streptococcus*, *Micrococcus* and *Enterobacter* sp.

Bacterial Isolates from Cow Dung Slurry

Out of 16 bacterial isolates, 11 and 5 isolates were found to be Gram positive rods and Gram negative rods respectively. Obtaining biochemical tests, all the bacterial isolates (100%) were found to be positive for citrate, nitrate, gas production and showed positive for VP, catalase, oxidase, mannitol by 56.25%, 68.75%, 68.75%, 50% isolates respectively while indole and methyl red negative for all the isolates. All the bacterial isolates showed sugar utilization positive.

Bacterial isolates from kitchen waste slurry

Out of 8 bacterial isolates, 4 isolates were found to be Gram positive rods, 4 isolates found to be Gram positive coccus. The bacterial isolates (50%) were found to be indole, methyl red, citrate, catalase, oxidase positive while 87.5% were mannitol & gas production positive. All the isolates were VP negative and nitrate positive. The isolates (75%) were positive to maltose, sucrose, raffinose, arabinose, cellobiose while 50% to xylose; 62.5% showed dextrose, lactose, inositol, rhamnose,

trehalose positive; 37.5% showed fructose positive; 87.5% showed glucose positive and 100% showed sorbitol and mellibiose positive.

Bacterial isolates from oil cake slurry

Out of 6 bacterial isolates, 2 and 4 bacterial isolates were found to be Gram positive rods, & Gram negative rods respectively. All the bacterial isolates (100%) were found to be VP, citrate, catalase, gas production, mannitol and nitrate positive while 66.6%, 33.3%, 83.3% isolates were found to be indole, methyl red, oxidase negative respectively. The isolates (83.3%) showed maltose, raffinose, rhamnose, inositol positive, 66.6% showed xylose, dextrose, sucrose, sorbitol, cellobiose positive while 16.6% showed lactose and fructose positive. All the bacterial isolates (100%) showed glucose, arabinose, trehalose positive and 50% showed mellibiose positive.

Enzymatic Characterization of Bacterial Isolates

All the bacterial isolates were screened for enzymatic activities by taking pseudo selective agar at 37°C for 72hours. The investigated enzymes were amylase, chitinase, caesinase, cellulase and gelatinase. (Table 1)

Fluorescence Test of Bacterial Isolates

All the bacterial isolates were investigated for methanogenic character by the fluorescence test. It was revealed that out of 30 bacterial isolates, 10 bacterial isolates (33.3%) were methanogenic. All the isolates obtained from de-oiled seed cake slurry were found to be non methanogenic. (Table 2) (Fig.1)

Biogas Production of Bacterial Isolates

All the bacterial isolates were investigated for biogas production by using an anaerobic conventional digester.

Table 3 depicts the biogas production form cow dung slurry bacterial isolates. It was observed that anaerobic methanogenic consortia showed a total of higher production of gas (402.876 cm^3) followed by cow dung slurry (no addition of bacterial isolates) of 298.623 cm^3 . Further methanogenic isolates were used for biogas production (Table 4). The combination of the methanogenic bacterial isolate A4 and

A8 showed maximum biogas production. Another experiment was done taking combinations of kitchen waste slurry as mentioned in Table 5. From this experiment it was observed that maximum bio gas production was found in set 7 (cow dung + facultative anaerobic non methanogenic isolates from kitchen waste slurry + kitchen waste) which was 568.974 cm^3 in three days followed by set 6 (cow dung + facultative anaerobic non methanogenic isolates from kitchen waste slurry) of 415.245 cm^3 and minimum was found in case of set 4 (cow dung + aerobic non methanogenic isolates from kitchen waste slurry) of 61.845 cm^3 in three days.

Table.1 Enzymatic characterization of the bacterial isolates

Bacterial Isolates	Enzymatic Tests				
	Amylase	Chitinase	Caesinase	Cellulase	Gelatinase
A1	-	+	-	+	-
A2	-	+	-	+	-
A3	-	+	-	+	-
A4	-	+	-	+	-
A5	-	+	-	+	-
A6	-	+	-	+	-
A7	-	+	-	+	-
A8	-	+	-	+	-
A9	-	+	-	+	-
A10	-	+	-	+	-
A11	-	+	-	+	-
A12	-	+	-	+	-
AN1	-	+	-	+	-
AN2	-	+	-	+	-
AN3	-	+	-	+	-
AN4	-	+	-	+	-
KA1	-	+	+	-	+
KA2	-	-	+	-	-
KA3	-	+	+	-	+
KA4	-	+	-	-	-
KAN1	-	+	+	-	+
KAN2	-	-	+	-	+
KAN3	-	-	-	-	+
KAN4	-	-	+	-	+
OA1	-	+	+	-	=
OA2	+	+	+	-	-
OAN1	-	+	+	-	-
OAN2	+	-	+	-	-
OAN3	-	+	+	-	-
OAN4	+	+	+	-	+

Table.2 Fluorescence test for the bacterial isolates

Bacterial Isolates	Methanogenicity	Bacterial Isolates	Methanogenicity
A1	-	AN4	-
A2	+	KA1	-
A3	+	KA2	+
A4	+	KA3	-
A5	-	KA4	+
A6	-	KAN1	-
A7	-	KAN2	-
A8	+	KAN3	-
A9	+	KAN4	-
A10	-	OA1	-
A11	+	OA2	-
A12	-	OAN1	-
AN1	+	OAN2	-
AN2	+	OAN3	-
AN3	-	OAN4	-

Table.3 Combinations of bacterial isolates for biogas production from cowdung slurry

Days	Biogas production (cm ³)					
	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6
1	72.447	0	26.505	61.845	100.719	0
2	98.952	56.544	35.340	93.651	141.36	0
3	127.224	29.971	47.709	130.758	160.797	0
Total	298.623	79.515	109.554	286.254	402.876	0

Set 1: Slurry (only cow dung); **Set 2:** cow dung + aerobic non methanogenic isolates from cow dung slurry; **Set 3:** cow dung + aerobic methanogenic isolates from cow dung slurry; **Set 4:** cow dung + facultative anaerobic non methanogenic isolates from cow dung slurry; **Set 5:** cow dung + facultative anaerobic methanogenic isolates from cow dung slurry, **Set 6:** control (sterilized cow dung).

Table.4 Different sets for biogas production from methanogenic isolates from cow dung slurry

DAYS	Biogas production (cm ³)			
	A 2 & A 3	A 4 & A 8	A9 & A 11	AN1 & AN2
1	22.971	73.330	40.641	56.544
2	23.854	84.816	33.573	83.932
3	25.621	100.719	44.175	116.622
Total	72.446	258.865	118.389	257.098

Table.5 Different sets of bacterial isolates for biogas production from kitchen waste slurry

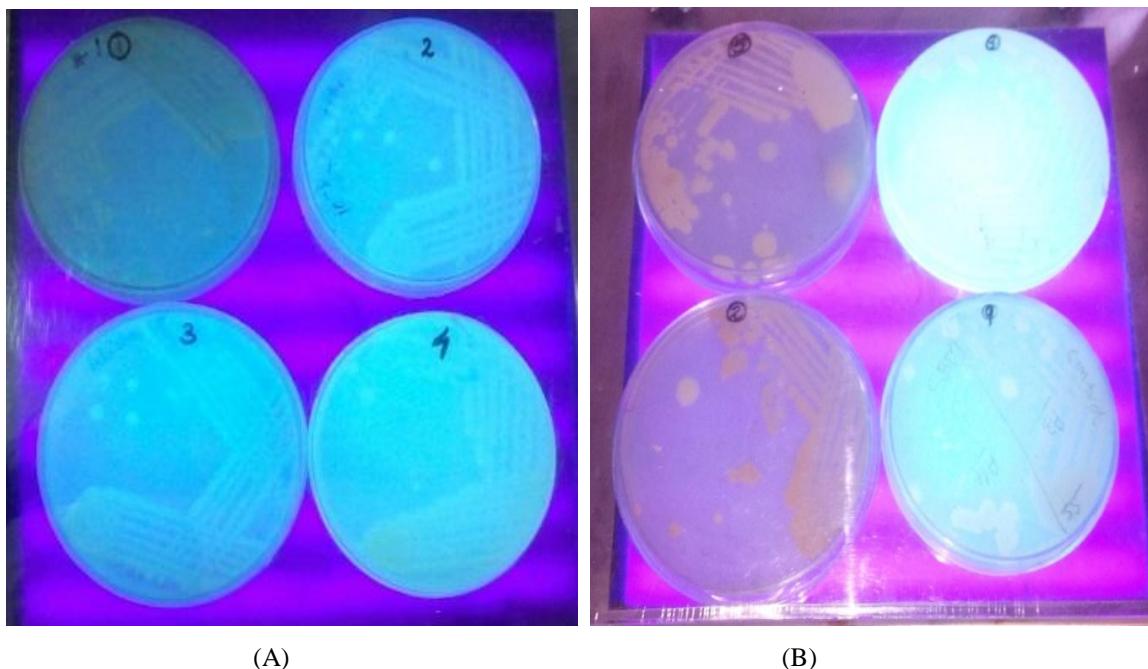
DAYS	Biogas production (cm ³)							
	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Set 7	Set 8
1	53.010	26.505	77.748	0	30.039	79.515	91.884	0
2	63.612	68.913	91.884	30.039	37.107	121.923	171.399	0
3	74.214	80.399	111.321	37.107	61.845	213.807	305.691	0
Total	190.836	175.816	280.953	61.845	128.985	415.245	568.974	0

Set 1: Slurry i.e. cow dung + kitchen waste; **Set 2 :** cow dung + aerobic methanogenic isolates from kitchen waste slurry; **Set 3:** cow dung + aerobic methanogenic isolates from kitchen waste slurry + kitchen waste; **Set 4:** cow dung + aerobic nonmethanogenic isolates from kitchen waste slurry; **Set 5:** cow dung + aerobic nonmethanogenic isolates from kitchen waste slurry + kitchen waste; **Set 6:** cow dung + facultative anaerobic non methanogenic isolates from kitchen waste slurry; **Set 7:** cow dung + facultative anaerobic non methanogenic isolates from kitchen waste slurry + kitchen waste; **Set 8:** control (sterilized cow dung).

Table.6 Different sets of bacterial isolates for biogas production from de-oiled seed cake slurry

DAYS	Biogas production (cm ³)					
	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6
1	0	14.136	100.719	65.379	125.457	0
2	22.971	45.942	125.457	118.389	157.263	0
3	61.845	88.350	144.894	144.894	219.108	0
Total	84.816	148.428	371.070	328.662	501.828	0

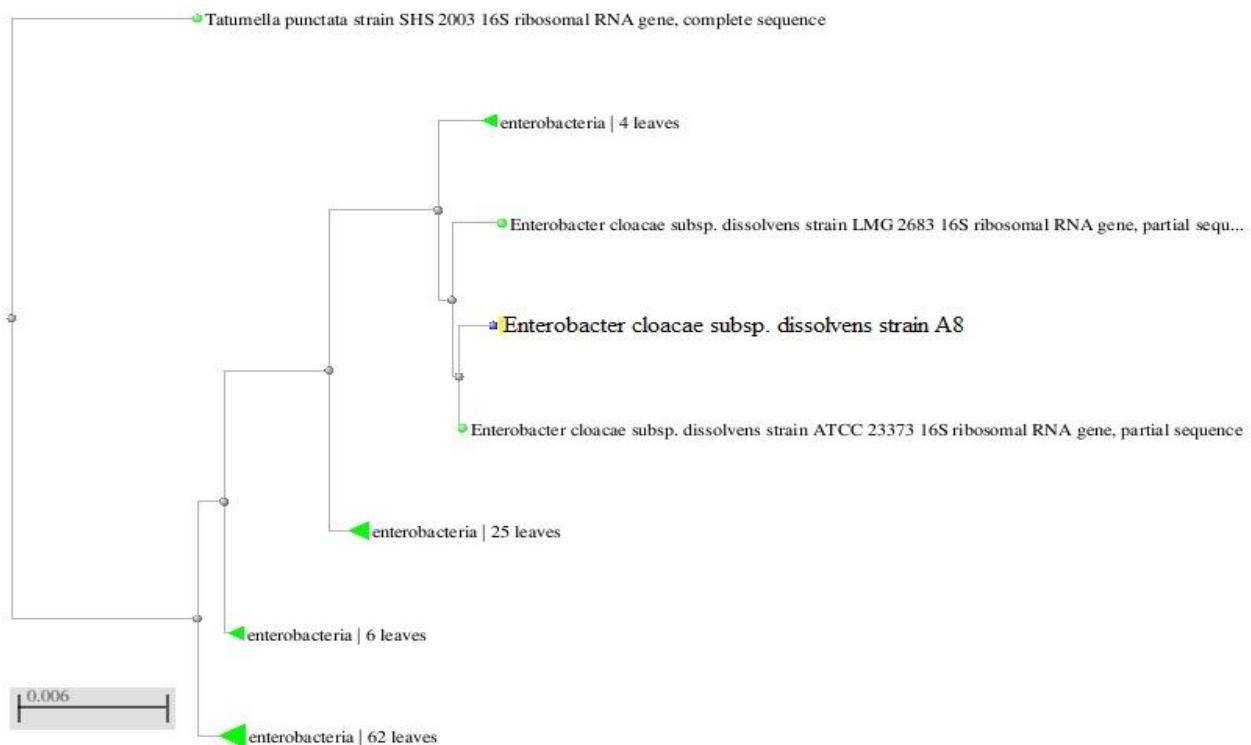
Fig.1 Fluorescence test for the bacterial isolates.



(A) – Plate 1 showed no fluorescence while plate 2, 3 & 4 showed fluorescence.

(B) – Plate 1 & 2 showed no fluorescence while plate 3 & 4 showed fluorescence.

Fig.2 Phylogenetic tree of *Enterobacter cloacae* subsp. *dissolvens* strain A8.



From the experiment performed using combinations of bacterial isolates from de-oiled seed cake slurry observed that maximum bio gas production was found in set 5 (cow dung + facultative anaerobic non methanogenic isolates from seed cake slurry + seed cake) of 501.828 cm³ in three days followed by set 3 (cow dung + aerobic non methanogenic bacterial isolates from oil cake slurry + seed cake) of 371.070 cm³ and minimum was found in case of set 1 (cow dung + seed cake) of 84.816 cm³ in three days (Table 6).

Molecular Identification of the Potent Bacterial Isolate

The 16S r DNA based molecular technique revealed that the isolate A8 was *Enterobacter cloacae* subsp. *dissolvens* strain A8 having GenBank accession number KM034810. Phylogenetic tree reveals that *Enterobacter cloacae* subsp.

dissolvens strain ATCC 23373 is nearer to the bacterial isolate A8 (Fig 2).

It was reported that methanogenic bacteria isolated from cow dung slurry contributed more biogas production than non methanogenic bacteria (Dhadse *et al.*, 2012). The 16S r DNA based molecular technique revealed that the isolate A8 was *Enterobacter cloacae* subsp. *dissolvens* strain A8. There is another report of using *Enterobacter cloacae* for improved bio gas production by Kovacs *et al.* (2013). Facultative anaerobic isolates have the potential to metabolize methane and takes part in methane production. Kitchen waste with cow dung produced more gas. This result corroborates with the report of Ranade *et al.* (1987) who used market waste and reported a 200 litres capacity biogas plant produced 30.2 litres kg per day at 20 days HRT (hydraulic retention time). Biogas production is established using de-

oiled seed cake plus cow dung which corroborates with the result of Kumar *et. al.* (2013) that the maximum biogas production was observed for the treatment S1 (Cow dung 0.680 kg, Volume of water 0.820 lit) followed by S5 (pongamia de-oiled seed cake 0.160 kg, Volume of water 1.340 lit).

In conclusion, biogas is a promising renewable energy carrier as the technology of its production combines the elimination of biodegradable waste such as animal wastes, kitchen wastes and agricultural waste. Anaerobic digestion is a treatment that composts these wastes in the absence of oxygen producing biogas which can generate heat and energy. Although the combination of the bacterial isolate A4 and A8 showed maximum biogas production, the combined effect of cow dung and kitchen waste proved to be a better substrate for biogas production.

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