Generation of high Calorific Fuel Gas by Photosynthetic Bacteria isolated from Cowdung

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Abstract

Cow dung is a valuable biomass and a natural source of different types of useful bacteria. Among these, photosynthetic bacteria are most important because it plays various important roles in the nature like, plant growth promotion, antimicrobial substrates secretion, extracellular carbohydrates production, etc. Some experiments have been carried out to study the methods of screening the presence of different types of photosynthetic bacteria like, cyanobacteria, green sulphur-bacteria, green non-sulphur bacteria, purple sulphur-bacteria, purple non-sulphur bacteria both from the fresh cow dung (FCD) and stored cow dung (SCD) using their reported selective culture media in aerobic and anaerobic conditions. Some biochemical tests have been conducted to find their characteristics and useful activities of these bacteria in the nature. Photosynthetic bacteria can produce high calorific fuel gas from cowdung in presence of light. Among all photosynthetic bacterial group green sulphur bacteria are main biomethane producer while purple non-sulphur and cyanobacterial groups of bacteria inhibit in biomethane production.

Keywords: Cowdung, photosynthetic bacteria, high calorific fuel gas, biomethanation, green bacteria.

Introduction

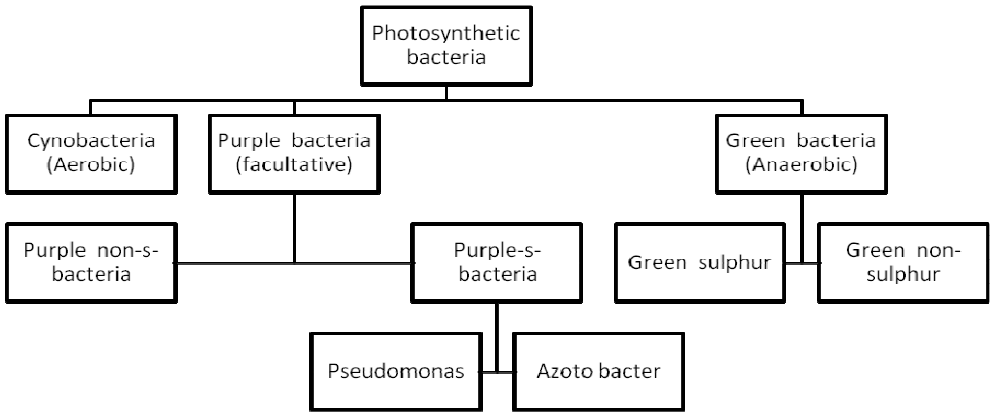
Cattle dung- such as cow dung, a waste material, is not only a cheap source of solid and gaseous fuels, but it is also widely used by the common people in solid and liquid forms for other various activities in social life. But in its fresh form and stored form, it contains some bacteria (Teo and Teoh, 2011; Swain et al., 2007) which have useful roles in nature such as – productions of biogas (Mandal et al., 1997; Yokoyama et al., 2007) increase in the fertility of the soil for plant growth (Punitha et al., 2010), germicide effect (Sreenivasa et al., 2009), etc. From the literature survey (Prescott, 2008; Stainer, 2007; Dubey and Maheshwari, 2007) it has been found that in the nature there exist various types of photosynthetic bacteria whose classification is shown in flow diagram- 1.

In this paper, an attempt has been made, to present the results of the experiments and tests conducted for the samples of FCD and SCD. For this, work has been divided into two stages. In first stage, some experiments and tests have conducted to isolate and screening of different photosynthetic bacteria using some standard methods. In second stage, some tests have been conducted to find the activities of the photosynthetic bacteria for their useful roles in nature like, gaseous fuel generation, plant growth promoting activities, etc.

Materials and Methods

- **Sample collection**

To conduct experiments both fresh cow dung (FCD) and stored cow dung (SCD) were collected from local dairy of Paschim Medinipur, West Bengal, India (Latitude- 22˚25'00'' to 22˚57'00'' north, Longitude- 87˚11' east, Altitude- 23 meters from mean sea level). Some biochemical tests have been conducted using above samples for isolation and identification of different types of photosynthetic bacteria.



Flow diagram1. Classification of photosynthetic bacteria

- **Isolation of Pseudomonas, Azotobacter and purple non-sulphur sp.s**

1gm of both FCD and SCD samples were dumped separately in 100ml King's B (KB), Ashby's mannitol (AM) and Acetate-Yeast extract (AYE) broth (Jayaprakashvel et al., 2010; Ramamoorthy et al., 2002; King et al., 1954; Torres-Rabio et al., 2004; Hoogewerf et al., 2003; Madigan et al., 1983) for screening the presence of *Pseudomonas, Azotobacter* and purple non-sulphur sp. respectively both in aerobic as well as anaerobic conditions and incubated at room temperature. After 3 days, 1ml of those screening liquid media were serially diluted up to 10^{-9} times using sterile distilled water. Each 100µl serially diluted fractions were pour-plated with respective-agar medium and all plates were incubated at 27^0C for 48hrs for *Pseudomonas* sp., 7 days for *Azotobacter* sp. and 2 weeks for purple non-sulphur sp. Distinct single colonies were picked up, sub-cultured for purify on same medium. The cultures were identified by following the tests given in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

- **Isolation and identification of Cyanobacteria**

1gm of both FCD and SCD samples were dumped separately

in 100ml Pringsheim's broth (PB) (Huang et al., 2010; Dubey and Maheshwari, 2007) and incubated at 27^0C. After two weeks, 1ml of those screened medium were serially diluted up to 10^{-9} times using sterile distilled water. Each 100µl serially diluted inoculums were pour-plated with PB-agar (1.5%) medium and incubated at 27^0C for 2 weeks. Distinct single colonies were picked up, sub-cultured for purify on same medium. The cultures were identified by following the tests given in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

- **Screening of the presence of Green bacteria and purple sulphur bacteria**

1gm of both FCD and SCD samples were dumped separately in 100ml Sulfanilate broth (SB) for green bacteria and Sodium-sulphide broth for purple sulphur bacteria (Anil Kumar et al., 2007; Biebel and Pfenning, 1978) and incubated both anaerobically using capped measuring cylinder and aerobically at 35^0C under a 60W tungsten-bulb. A similar separate set was also prepared and kept in dark.

- **Methane gas generation ability test**

This test was conducted for each of the photosynthetic bacteria during 1.5 month to find the biomethane production ability by burning the produced gas. A gas production unit was developed by taking seven different 500ml conical flasks inside the digester. Different conical flask (written as set) contain following bacteria:

Set1: 350ml FCD slurry (Cow dung: sterile distilled water = 1:1; w/v)

Set2: 300ml Autoclaved FCD slurry + 50ml aerobically screened KB

Set3: 300ml Autoclaved FCD slurry + 50ml aerobically screened AM

Set 4: 300ml Autoclaved FCD slurry + 50ml anaerobically screened SB

Set 5: 300ml Autoclaved FCD slurry + 50ml aerobically screened PB

Set 6: 300ml Autoclaved FCD slurry + 50ml aerobically screened AYE

Set 7: 300ml Autoclaved FCD slurry + 50ml anaerobically screened AYE

The volume of produced gas was measured by one week interval. The produced gases in different sets were burned to verify the presence of methane content (Mandal et al., 1999).

- **Gas composition analysis**

Hydrogen, methane and carbon dioxide gases percentage was determined by Gas chromatography (GC Agilent Technology 7890A U.S.A) equipped with a thermal conductivity detector (TCD) and a stainless steel packed with Porapak Q (80/100). The operational temperatures of the injection port, the oven and the detector were 80°C, 150°C and 200°C, respectively. Nitrogen was used as the carrier gas for biogas at a flow rate of 20 $ml.min^{-1}$.

Results and Discussions

The data obtained for the experiments and tests conducted, for the samples of FCD and SCD, as described in the previous section, can be used to study to confirm the presence of different types of photosynthetic bacteria and their phenotypic and biochemical characteristics. Table1 shows, the results of the experiments conducted for FCD and SCD in aerobic and anaerobic conditions with presence of light and also the absence of light i.e.; in dark condition. From this Table1, it can be seen that *Pseudomonas sp.* (see Table3) and *Azotobacter sp.* are present in both types of cow dungs. Under aerobic condition they are identified both in dark as well as in light. For SCD in anaerobic condition, the growth of purple sulphur bacteria (community identification was done by metagenome analysis) takes place in two weeks in presence of light but it takes place in one month in absence of light i.e.; in dark condition and in little amount. For FCD in aerobic condition, the growth of cynobacteria takes place in 2 months in absence of light, whereas it takes in 3 weeks in the presence of light (as shown

Table1. Screening of bacteria

Sample No.	Type* of cow dung	Situation Dark/Light	Condition Aerobic / Anaerobic	Retention time	Type of bacteria isolated/screen
1.	FCD	Dark	Aerobic	3 days	*Pseudomonas*, *Azotobacter*
				2 months	Cynobacteria
2.	FCD	Dark	Anaerobic	1 week	Green, Purple *Pseudomonas sp.* and Purple non-s-bacteria
3.	FCD	Light	Aerobic	1 day	*Pseudomonas*, *Azotobacter*
				3 weeks	Cynobacteria
4.	FCD	Light	Anaerobic	1 week	Green, Purple *Pseudomonas sp.* and Purple non-s-bacteria
5.	SCD	Dark	Aerobic	3 days	*Azotobacter and Pseudomonas sp.*
6.	SCD	Dark	Anaerobic	1 month	Purple non-s-bacteria
7.	SCD	Light	Aerobic	1 day	*Pseudomonas*, *Azotobacter*
				3 weeks	Cynobacteria
8.	SCD	Light	Anaerobic	2 weeks	Purple non-s-bacteria

*FCD = Fresh Cow Dung; SCD = Stored Cow Dung

Table2. Characteristics of different photosynthetic bacteria

Type of cow-dung	Type of bacteria	Colour	Population	Gas production rate	Active role in nature
FCD	*Azotobacter* sp.	Colorless/Brown	Lowest	Nil	Fixing atmospheric nitrogen
	Pseudomonas sp.	Yellowish/Brown	Lower	Medium	Increasing fertility of soil
	Purple non-s- bacteria	Brown	Medium	High	Increasing fertility of soil
	Cynobacteria	Green	Higher	Nil	Air-purification
	Green bacteria	Green	Highest	Highest	Fuel-gas production
SCD	*Azotobacter* sp.	Colorless/Brown	Higher	Highest	Fuel-gas Production
	Pseudomonas sp.	Yellowish	Highest	Medium	Increasing fertility of Soil
	Purple non-s-bacteria	Brown	Medium	Low	Increasing fertility of Soil
	Cynobacteria	Green	Low	Medium	Gas production, air purification

Table3. 16s rDNA characterization of Pseudomonas sp. isolated from both FCD and SCD

Isolates	**Isolates name**	**GenBank Accession Number**
E_{57}	*Pseudomonas sp* Strain GRTM	KC169988
B_{11}	*Pseudomonas sp* Strain GATS	KC169987
P_{64}	*Pseudomonas sp* Strain GNST	KC169994
P_{76}	*Pseudomonas sp* Strain TMGR	JX094352
P^{S}_{46}	*Pseudomonas sp* Strain GTRS	KC169990
P^{S}_{71}	*Pseudomonas sp* Strain TSSG	KC169991
P^{S}_{111}	*Pseudomonas sp* Strain GTNS	KC169989
Z^{S}_{16}	*Pseudomonas sp* Strain TSGR	KC169995

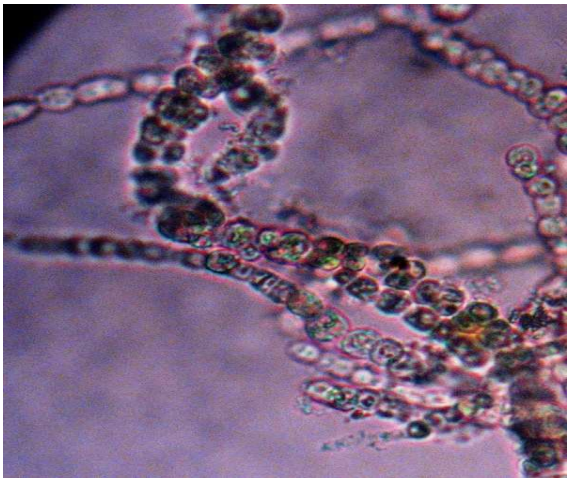


Fig.1. Cynobacteria isolates (*Nostoc sp.*) isolated from cow dung

Table4. Biomethane generation by different photosynthetic bacteria

Set	Set description	Volume of gas produced (in ml)	Gas started to burn after days	Duration of gas burned (in month)	Gas composition (in mL) [$H_2/CH_4/CO_2$]
1	Non autoclaved slurry	3060	15	1.25	143.21 / 1600.69 / 1268.37
2	Autoclaved slurry and *Pseudomonas* sp.	1680	20	0.75	0.00 / 1015.06 / 582.79
3	Autoclaved slurry and *Azotobacter* sp.	3020	10	1.00	146.13 / 1772.74 / 854.06
4	Autoclaved slurry and Green bacteria	3210	3	1.5	609.79 / 2119.98 / 248.53
5	Autoclaved slurry and Cyanobacteria	1100	-	-	-
6	Autoclaved slurry and Purple non-sulphur bacteria (aerobically isolated)	2800	-	-	-
7	Autoclaved slurry and Purple non-sulphur bacteria (anaerobically isolated)	2900	-	-	-

in Fig. 1). It can also be observed that there is no presence of green bacteria in SCD whether it is in dark or light and aerobic or anaerobic conditions. It indicates that green bacteria (community identification was done by metagenome analysis) are present only in FCD while it dies in SCD within a few days after growth due to the presence of oxygen. Table2 displays the results of some biochemical tests conducted with regards to find the colour, population and biofuel gas production rate and active roles in nature of different types of photosynthetic bacteria. Every time the produced gases were burned from the Set1 to Set4.

From this Table2, it can be seen that for FCD, the *Azotobacter sp.* has low population with respect to SCD. Population of purple non-sulphur bacteria (as shown in Fig.2) is more than *Pseudomonas sp.* but both can help in increasing the fertility of the soil. Cynobacteria in FCD has higher population than other bacteria and it can help in purification of air with their oxygenic photosynthesis process. Green bacteria, in FCD, have highest population and it can help in generation of biomethane gas. For SCD, the *Azotobacter* has higher population than cynobacteria. In SCD, purple non-sulphur bacteria and *Pseudomonas sp.* can help in increasing the fertility of the soil with their antimicrobial substances (HCN, siderophore), indole-3-acetic acid, various extracellular enzymes, exo-polysaccherides production and phosphate solubilization. Aerobic and anaerobic study reveals that *Pseudomonas*, *Azotobacter* and Cyanobacteria are aerobic, purple non-sulphur are facultative while green bacteria are strictly anaerobic in nature.

Biomethane production ability study in different sets revealed that *Pseudomonas* (Set2), *Azotobacter* (Set3) and green (Set4) bacteria can produce more methane enriched fuel gas than cyanobacteria (Set5) and purple non-sulphur (Set6 and 7) bacteria.

Quantitative analysis of gas product samples (See Table4), was obtained from Set1 to Set4, confirmed the presence of

biomethane along with biohydrogen. The amount of carbon dioxide in the produced gas sample from Set4 is remarkable less than the product gas obtained from normal cowdung slurry (Set1). The product gas from Set4 (green bacterial set) burns with a high flame temperature than other gas samples.

Fig.2. (a) Purple non-sulphur bacteria isolation, screening media turned into deep purple colourand, (b) Green bacteria screening media turned into green colour.

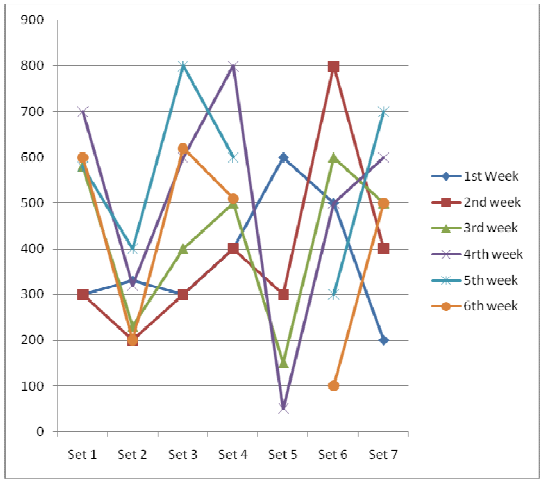


Fig.3. Week wise gas production record of different sets.

Conclusion

Above study concluded that, cow dung is a huge source of different types of eco-friendly photosynthetic bacteria. Their growth rates are different in different situations i.e.; aerobic/ anaerobic and light/dark conditions with different retention time. These bacteria have useful roles in nature like in generation of biomethane gas, increasing the fertility of soil and purification of atmosphere, etc.

Green bacteria are both autotrophy and methanogens in nature. It can alone produce methane gas with higher methane percentage than other methanogens present in cow dung.

Each green bacterium behaves like a powerful solar cell. It traps photon under diffused light where as solar cell cannot do so. Using these bacteria we can convert waste polluted carbonaceous materials into valuable fuel gas to control the environmental pollution. So, in future green bacteria can be used as a potential biocatalyst in upgradation technology of biogas production.

References

[1] Teo, K.C. and Teoh, S.M. (2011). Preliminary biological screening of microbes isolated from cow dung in Kamper. *Afr. J. Biotechnol.*, 10, 1640-1645.

[2] Swain, M.R., Naskar, S.K. and Ray, R.C. (2007). Indole-3-acetic acid production and effect on sprouting of Yam (*Dioscorea rotundata L.*) minisetts by Bacillus subtilis isolated from culturable cow-dung micro-flora. *Pol. J. Microbiol.*, 56, 103-110.

[3] Mandal, T., Mandal, N.K. and Rao, V. (1997). Comparative study of biogas production from different waste material. *Energy Convers. Manage.*, 38, 679-683.

[4] Mandal, T., Kiran, B.A. and Mandal, N.K. (1999). Determination of the quality of biogas by flame temperature measurement. *Energy Convers. Manage.*, 40(11), 1225-1228.

[5] Yokoyama, H., Waki, M., Ogino, A., Ohmori, H. and Tanaka, Y. (2007). Hydrogen fermentation properties of undiluted cow dung. *J. Biosci. Bioeng.*, 104, 82-85.

[6] Punitha, S., Balamurugan, I., Kuberan, T. and Kumar, R.S. (2010). Isolation and characterization of agriculturally important microbes from Panchakavya and their enzymatic activity. *J. Biosci. Res.*, 1(3), 194-201.

[7] Sreenivasa, M.N., Naik, N. and Bhat, S.N. (2009). Beejamrutha: A source for beneficial bacteria. Karnataka J. Agric. Sci., 22(5), 1038-1040.

[8] Prescott, H. and Klein's. (2008). "Microbiology". 7th edition published by Mc Graw- Hill International, a business unit of the Mc Graw–Hill companies, Inc.

1221 Avenue of the Americas, New York, NY 10020.

[9] Stainer, R.Y., Ingraham, J.L., Wheelis, M.L. and Painter, P.R. (2007). "General Microbiology", 5th edition published by MACMILLAN PRESS LTD., Houndmills, Basing stoke, Hampshire RG21 6XS & London, 344–382.

[10] Dubey, R.C. and Maheshwari. (2007). "A Textbook of Microbiology" First Multicolour Illustrative Revised Edition published by Chand, S., & Company Ltd. 7361, Ram Nagar, New Delhi-110 055, 335–349.

[11] Jayaprakashvel, M., Muthezhilan, R., Srinivasan, R., Hussain, A.J., Gobalakrishnan, S., Bhagat, J., Kaarthikeyan, C. and Muthulakshmi, R. (2010). Hydrogen cyanide mediated biocontrol potential of *Pseudomonas sp.* AMET1050 Isolated from the rhiosphere of coastal sand dune vegetation. *Advanced Biotech*., 9(10), 39-42.

[12] Ramamoorthy, V., Raguchander, T. and Samiyappan, R. (2002). Induction of defence related proteins in tomato roots treated with *Pseudomonas fluorescens* pf1 and *Fusavium. oxyporum* f. Sp. *Lycopersici V. Plant and soil*,239, 55-68.

[13] King, E.O., Ward, M.K. and Raney, D.E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Microbiol*., 44, 301-307.

[14] Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). "Bergey's Mannual of Determinative Bacteriology", 9th edition published by Williams and Wilkins, A. Wavely company, Baltimore.

[15] Torres-Rabio, M.G., Valencia-Plata, S.A., Bernal-Castillo, J. And Martinez-Nieto, P. (2004). Isolation of *Enterobacteria, Azotobacter* sp. and *Pseudomonas* sp., Producers of Indole-3-Acetic Acid and Siderophores, from Colombian Rice Rhizosphere. *Rev. Latinoam. Microbiol*., 42, 171-176.

[16] Madigan, M., Cox, S.S. and Stegeman, R.A. (1984). Nitrogen Fixation and Nitrogenase Activities in Members of the family

Rhodospirillaceae. *J. Bacteriol.*, 157(1), 73-78.

[17] Hoogewerf, G.J., Jung, D.O. and Madigan, M.T. (2003). Evidence for limited species diversity of bacteriochlorophyll b-Containing purple nonsulfur anoxygenic phototrophs in freshwater habits. *FEMS Microbiol. Lett.*, 218, 359-364.

[18] Huang, Y., Zhao, L., Dong, T. and Tan, X. (2010). Optimization of enzyme - producing conditions of *Micrococcus* sp. S-II for L-Cysteine production. *Afr. J. Biotechnol.*, 10(4), 615 - 623.

[19] Dubey, R.C. and Maheshwari. (2007). "Practical Microbiology". 2nd edition published by Chand, S., & Company Ltd. 9361, Ram Nagar, New Delhi- 110 055, 82–83 & 184–185.

[20] Biebel, H. and Pfenning, N. (1978). Growth yields of green sulphur bacteria in mixed cultures with sulphur and Sulfate reducing bacteria. *Arc. Microbiol.*, 117, 9-16.

[21] Anil Kumar, P., Srinivas, T.N.R., Sasikala, C., Ramanna, C.V., Süling, J. and Imhoff, J.F. (2007). Selective enrichment of green sulphur bacteria in the presence of 4–aminobenzene sulfonate (sulfanilate). *W. J. Microbiol. Biotechnol.*, 23, 393-399.