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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF METHANOGENIC BACTERIA FROM COWDUNG

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ABSTRACT

The focus of the present study is to isolate and identify the potential methanogenic bacteria from the cowdung procured from Namchi, South Sikkim, India. It was observed that, two bacterial isolates PRATIK-20 and VBC3 showed biogas (methane gas) production of 33.54% and 40.50% respectively using cow dung concentration 75% (w/v), inoculum 25% (w/v) at pH 7 and temperature 35°C in the fabricated anaerobic digester. Based on the 16S rDNA sequence, the bacterial isolates PRATIK-20 and VBC3 were identified as *Bacillus* sp. and *Proteus* sp. respectively.

INTRODUCTION

In today's energy demanding life style, need for exploring and exploiting new sources of energy which are renewable as well as eco-friendly is a must. In rural areas of developing countries various cellulosic biomass (cattle dung, agricultural residues, etc.) are available in plenty which have a very good potential to cater to the energy demand, especially in the domestic sector. 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 [1]. Biogas typically refers to a gas produced by the anaerobic digestion (AD) of biodegradable materials such as biomass, manure or sewage, municipal waste, green waste and energy crops [2]. It consists of varying percent of methane, carbon dioxide, nitrogen, hydrogen, ammonia and water vapor. Biogas is used for direct combustion in cooking or lighting applications, or to power combustion engines for motive power or electricity generation. Like natural gas, biogas can be directly used for heating. A cubic meter of biogas with 60% methane content can substitute approximately 0.6 cubic meters of natural gas or 0.6 L of fuel oil during electricity generation in a combined heat and power [3]. This type of energy generation is practically carbon dioxide-neutral as the greenhouse gases released during the combustion have been previously consumed by the plants. Considerable quantities of biogas have been generated by anaerobic digestion of poultry droppings, pig and cow dungs. Fermented substrate (digestate) from the biogas production can be used as high quantity fertilizer. There are a number of bacteria that are involved in the process of anaerobic digestion including acetic acid forming bacteria (acetogens) and methane forming archaea (methanogens). These organisms feed upon the initial feedstock, which undergoes a number of different processes converting it to intermediate molecules including sugars, hydrogen and acetic acid before finally being converted to biogas. Different species of bacteria are able to survive at different temperature ranges. The ones living optimally between 35 - 40°C are called mesophiles of mesophilic bacteria. Some of the bacteria can survive at the hotter and more hostile conditions of 55 - 60°C, these are called thermophiles or thermophilic bacteria [4]. Till today a number of reports are available on the biogas (methane) production from the cowdung, however a very little work has been carried out on monitoring the potential of methanogenic bacteria for biogas production from the cowdung. The present work is aimed at screening of methanogenic bacteria from cowdung for biogas production using cowdung as the substrate in the fed batch anaerobic digester. The potential methanogenic bacteria selected were further identified using phenotypic and phylogenetic analysis.

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MATERIALS AND METHODS

Sample collection: The cowdung sample of a native cow breed Siri was collected from Namchi, South Sikkim, India, in sterilized container and stored at 4°C until used for the experiment.

Fabrication of anaerobic digester: Anaerobic digester used for the biogas production from cowdung was a fixed batch prototype (Figure 1).

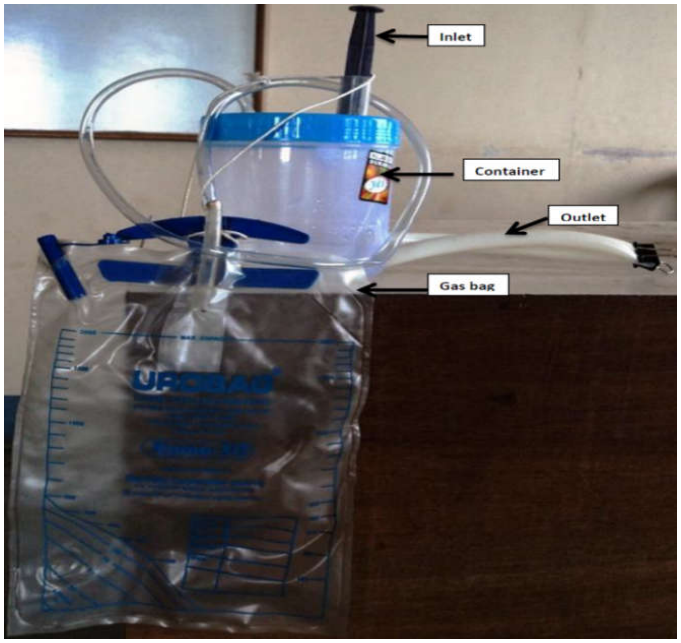


Figure 1: Depiction of fabricated fixed batch type anaerobic digester

Growth media: The isolation and routine sub-culturing of cow dung bacterial isolates were carried out on nutrient media (in g/L: Peptone, 10; Sodium chloride, 5; Yeast extract, 5 and Agar, 15) at pH 7.0 at 35ºC.

Isolation of bacteria from cowdung: For the isolation and screening of methanogenic bacteria, about 1 g of cowdung sample was weighed and poured into a test tube containing 9ml of autoclaved nutrient media and incubated under anaerobic condition for 72h at 35ºC to enhance the growth of the anaerobic methanogenic bacteria. Following incubation, the test tube was then manually agitated to form a uniform solution and allowed to stand for some seconds for the larger particles to settle down. The obtained dilution of 10^{-1} (w/v) was further serially diluted to 10^{-5} (w/v) and 100µl of the 10^{-5} dilution was then spread plated on the nutrient agar plates and further incubated at 35°C for 24h. Following incubation, distinct pure colonies obtained on the plates were isolated and purified using streak plate method and transferred to nutrient agar slants as stock. The bacterial isolates were then used as inoculum in the anaerobic digester containing cowdung as the substrate to check their individual potential for the biogas production.

Analysis of biogas (methane gas): The methane content in the biogas samples were quantitatively analyzed by gas chromatographic method [5]. The biogas samples were analyzed using gas chromatograph (GC 2014 series chromatograph Schimadzu) fitted with Forward tract ionization Detector (FTD) and SPB-1 (cross linked methyl silicon) column. The capillary column is used with a length of 30 m which is made of fused silica with a polyamide coating. The carrier gas contained Helium (0.8 Kg/cm^2), air (0.45 Kg /cm^2) and Hydrogen (0.6 Kg /cm^2). Column, injector and detector temperature were kept at 150^{o}C, 250^{o}C, 270^{o}C respectively. The area under the peak was measured by means of a microprocessor based integrator (Schimadzu GC 2014 series) attached to the chromatograph. The concentration of the methane produced was expressed in percentage (%).

Identification of potential methanogenic bacteria: The bacterial isolates showing maximum production of biogas were initially studied for phenotypic characteristic features on the nutrient agar followed by the classification based on the Gram's staining [6]. Further visualization of bacterial isolates under SEM was performed to determine their cell morphology. Following 24h incubation of bacterial cultures in nutrient broth at 35°C, cultures were centrifuged at 10,000 rpm for 10 min and the cell pellets obtained were fixed by the 2.5% (v/v) gluteraldehyde and dehydrated by successive immersion for 10 min in ethanol at the following concentrations: 30% (v/v), 50, 70, 80, 90 and 100%. This was then followed by critical point drying, gold palladium coating and then visualized under JEOL 840 SEM [7]. The potential isolates were outsourced to Bioserve, Hyderabad for 16S rDNA sequencing. The sequence obtained was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using nBLAST tool and corresponding sequences were downloaded (Altschul, 1990). The phylogenetic tree was constructed using MEGA4 package by the neighbor joining method [8].

RESULTS AND DISCUSSION

Isolation of bacteria from cowdung: Following serial dilution of the cowdung, seven morphological distinct bacterial colonies were picked up from the spread plate, named as PRATIK-20, PRATIK-30, PRATIK-40, PRATIK-50, VBC1, VBC2 and VBC3. Furthermore, the bacterial isolates were studied for the methane production.

Biogas production analysis: The cowdung was autoclaved for 30 minutes at 120°C and diluted to 75% concentration using 24h old cowdung isolate culture in nutrient media, poured into the fabricated anaerobic digesters followed by incubation at 35°C for 30days. It was observed that among all the bacterial isolates inoculated in the anaerobic digesters, the isolate PRATIK-20 and VBC3 were the two potential methanogenic bacteria which produced significant concentration of 33.54% and 40.50% of biogas (methane) from the cow dung.

Methanogenic bacteria: The bacterial colonies of the isolate PRATIK-20 were, circular, smooth, opaque, 1 mm in diameter and while VBC3 was segregate, circular and whitish. The isolate PRATIK-20 and VBC3 were observed to be gram-positive and gram negative bacilli respectively. The scanning micrographs of the bacterial isolates PRATIK-20 and VBC3 were as shown in Figure 2(a,b).

Sequence analysis of 16S rDNA showed that the isolate PRATIK-20 had highest similarity of 99% with the strain *Bacillus* sp. SRC_DSF22 (GU797304) while strain VBC3 showed highest similarity of 100% with the uncultured *Proteus* sp. (JQ624316). Based on the phenotypic characteristics and phylogenetic analysis, strain PRATIK-20 and VBC3 were identified as *Bacillus* sp. and *Proteus* sp. as shown in Figure 3(a, b). The16S rDNA sequence of the isolate PRATIK-20 and VBC3 has been deposited in the Gene Bank with an accession numbers JQ726697 and JX089650 respectively.

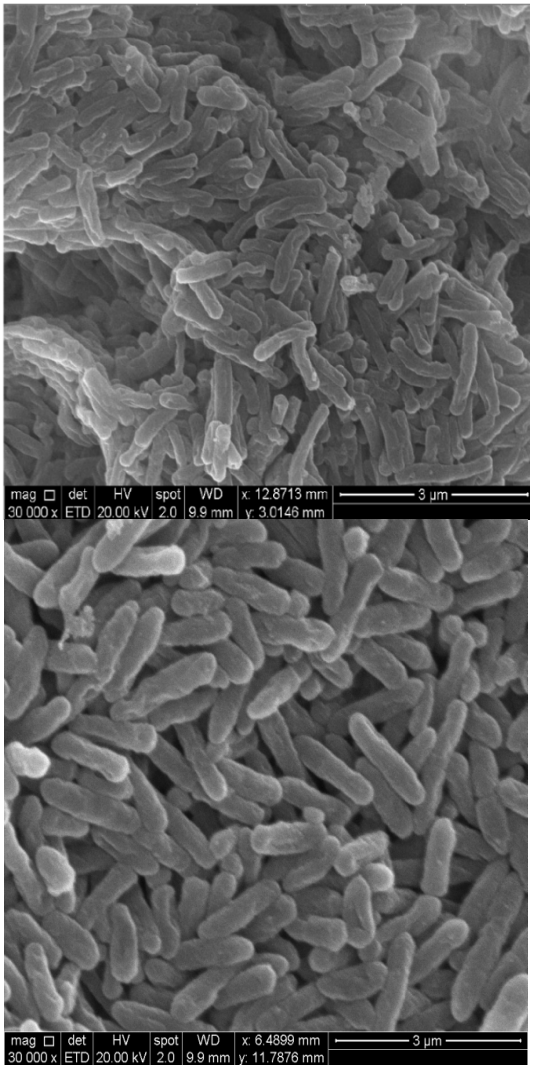
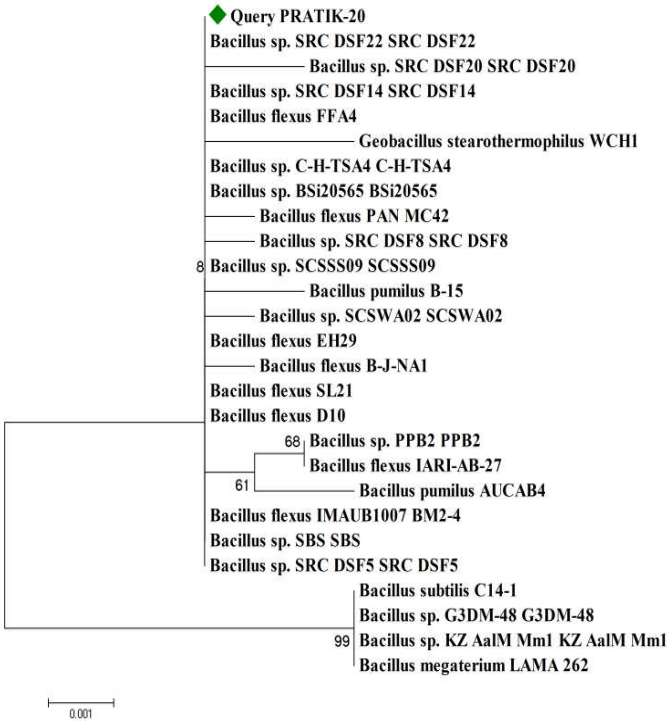


Figure 2: Scanning micrograph of *Bacillus* sp. PRATIK- 20 (a) and *Proteus* sp.VBC3 (b) isolated as methanogenic bacteria from the cowdung.



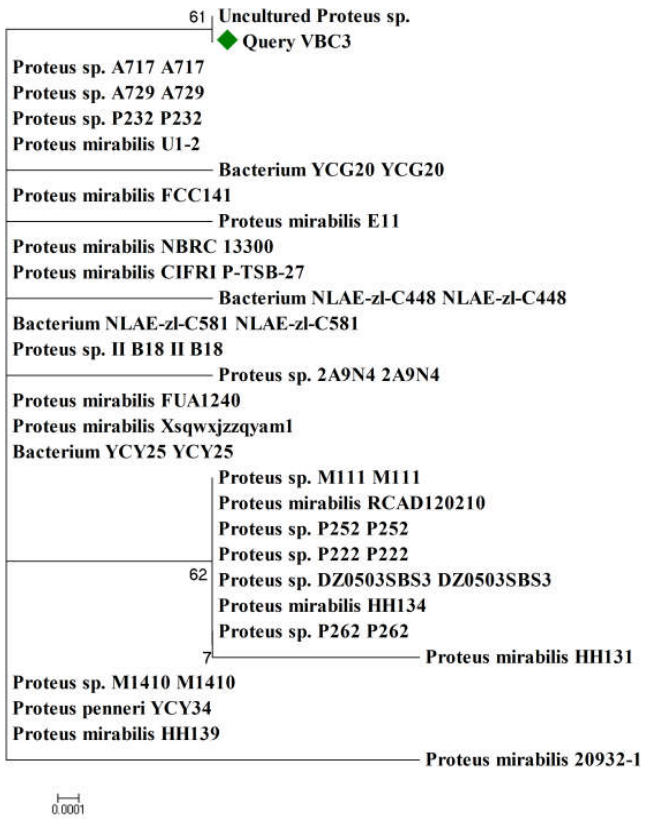


Figure.11: Phylogenetic tree of the isolate PRATIK-20 (a) and VBC3 (b) in relation to *Bacillus* and *Proteus* species, respectively. The tree was constructed using the Neighbor-joining algorithm. Numbers on the tree refer to bootstrap values on 1000 replicates. Nucleotide accession numbers are given in parentheses. The bar indicates a 0.1% estimated difference in nucleotide sequences.

It was reported that the *Bacillus* sp. *and Proteus* sp. were isolated from the anaerobic digestion of cassava peel mixed with pig waste for biogas and bio fertilizer generation [9], which provides an evidence for methanogenic nature of bacterial isolates *Bacillus sp.* PRATIK-20 and *Proteus sp*.VBC3.

Conclusion

A critical analysis of study concluded that the isolates PRATIK-20 and VBC3 are the potential methanogenic bacteria present in the cowdung, hence the study recommends the utilization of isolated strains for biogas production. The present study also suggests further optimization of the physiochemical parameters and the use of isolated strains along with the acidogenic and fermentative bacteria for effective, volumetric and faster production of biogas from the anaerobic digesters for commercial scale.

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