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Studies on methanogenic consortia associated with mangrove sediments of Ennore

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Abstract

In this study, methanogenic consortia were isolated and characterized from eight different sediment samples of mangrove ecosystem located in Ennore, Chennai. Out of eight samples, two consortia (SpR6 and SSS8) were consistent and good at methane production. The maximum methane (6.95%) was produced by the sample collected from the swampy sediments (SSS8) of mangrove environment on 5th day of incubation. The pure colonies of methanogenic consortia (SSS8) isolated in the roll tube produced maximum methane (5%) on 7th day. Further, all the isolated consortia produced methane when it was grown with and without gaseous (H_2 and CO_2) substrate and the maximum production of 15.1% (SSS8) on 12th day of incubation in the presence of gaseous substrate. Also, the methanogenic consortium isolated from the mangrove environment produced significant amount of methane in tyndallized cow dung (4.7%) than in raw cow dung. The fluorescence of isolated colony and individual cells of methanogenic consortia, methane production, and the amplification of archeal specific 16S rRNA genes confirmed the presence of methanogenic bacteria in the Ennore mangrove sediments.

Key words

Biogas, consortia, Methanogenic, Mangrove, Sediment

Introduction

Mangroves are complex coastal ecosystem under the influence of both marine and freshwater conditions, with numerous physiological adaptations. This has led to the assemblage of intricate ecosystem with highly interactive plants, animals and microbial communities, specially adapted to overcome the problems of anoxia, salinity and frequent tidal inundations (Logesh *et al.,* 2012). One of the most important constituents of this ecosystem are the sediments, rich in organic substrates, anaerobic and highly reduced. This optimal environment supports a complex anaerobic microbial community (Dar *et al.,* 2008), but little is known about their diversity, population and importance.

Methanogenic bacteria, one of the major components of anaerobic microbial population in the mangrove sediments (Shaoo and Dal, 2009), belong to archaebacteria. These diverse group of bacteria's are widespread in nature and highly abundant in extreme environments, tolerating variable factors *i.e.,* temperatures, salinity and pH. In anoxic mangrove sediments, where organic matter undergoes vigorous decomposition, methanogens (strict anaerobes) have the ability to reduce carbon dioxide with molecular hydrogen to produce methane and the process is called methanogenesis (Lekphet *et al.,* 2005). They utilize hydrogen as energy source and carbon dioxide as the only carbon source. In addition to this specific metabolism, they are able to use few organic substrates (acetate, formate, methanol and methylamines) to produce methane (Zinder, 1993; Barber and Ferry, 2001).

Along the intertidal margins of Ennore creek (backwater located in Ennore, Chennai along the Coromandel coast of Bay of Bengal), small patches of mangroves comprising *Avicennia marina, Excoecaria agallocha* and *Sonneratia optala* are available and *A. marina* is the dominant species. Studies

related to methanogenic consortia and their methane productions are inadequate in the mangrove sediments especially from the Ennore mangrove patches due to difficulty in isolation, long incubation period, strictly anaerobic cultivation and incomplete knowledge on nutritional requirements (Garcia *et al.,* 2005). Therefore, the present study was focused to isolate methanogenic consortia from the mangrove sediment and characterize their methane production.

Materials and Methods

Sample collection : Eight different sediment samples were collected from the Ennore mangrove environment, viz., rhizosphere samples and sub surface sediment samples near the mangrove plants such as *Avicennia marina* (AmR1&AmS2), *Suaeda monoica* (SmR3&SmS4), *Sesuvium portulacastrum* (SpR5&SpS6), pneumatophores of *Avicennia marina* (AmP7) and near shore region (SSS8). The collected samples were transferred into serum bottles, sealed immediately with butyl rubber stopper and aluminium crimps using sealing machine, transported to the laboratory in ice packs and stored at 4 °C for further study.

Culture enrichment : To enrich the methanogenic culture, 1 g of sediment samples were inoculated in 14 ml of anaerobic media (bicarbonate-buffered media - Boone *et al.,* 1989, filter-sterilized trace vitamin solution - Wolin *et al.,* (1963) with a headspace of gaseous substrate, H_2:CO_2 (80:20). The culture bottles were sealed tightly with butyl rubber stopper and aluminium crimps, and incubated at 37 °C (Mohanraju and Natarajan, 1992). Duplicates were also maintained. To create anoxic environment during inoculation and incubation, an anaerobic chamber (BACTRON IV) with gas phase as N_2, CO_2 and H_2 (90:5:5) was used.

The first and second subcultures were made on 20th and 40th day of incubation, respectively to refine the consortia from the mother inoculum. The third subculture was inoculated in pre-reduced basal carbonate yeast trypticase (BCYT) anoxic media with gas phase N_2 and CO_2 (Touzel and Albagnac, 1983) to reduce the sulfur reducing bacteria (SRB). Further isolation and routine maintenance of methanogenic consortia were carried out in BCYT.

Methane production and growth analysis : Methane production and growth of methanogens were monitored regularly on mother inoculum and respective subcultured samples. After incubation, 100 µl of gas from the headspace of inoculated sample bottles, using a gas-tight syringe, was injected into gas chromatography (Chemito 7610) (Boone, 1982) equipped with Flame-Ionization Detector (FID) and porapak Q column. The oven temperature was 45 ºC, for injector and detector 75º and 200 ºC, respectively with N_2 as a carrier gas, H_2 and air as flame igniters. The percentage of methane in the samples was calculated based on the peak retention time and response factor of standard methane gas (Kumar *et al.,* 2012). Growth of methanogenic consortia was monitored by measuring the optical density (∞ cell density) at 600 nm in UV-Vis spectrophotometer (Hausrath *et al.,* 2007). The optical density was considered as positive growth after methane estimation. Analysis of variance (two-way ANOVA) was performed for varying methane production on different days in different samples ($P<0.05$) using Microsoft excel -2007 and same with respect to growth analysis.

Evaluation of methane production in different substrates : To study the gaseous substrate preference of the refined methanogenic consortia, methane production was compared in the presence and absence of H_2 and CO_2 in anoxic media, incubated at 37 ºC. In addition, methanogenic consortia isolated from the mangrove sediments were analyzed for methane production in the natural substrate *i.e.* cow dung as raw and tyndallized.

Isolation of methanogens : The roll tube method (Hungate, 1969) was adopted to isolate the methanogenic bacteria from high methane producing consortia, the sample SSS8. The refined methane positive consortia were serially diluted, inoculated into Hungate tubes with double strength BCYT agar media and H_2:CO_2 (80:20), and then incubated at 37 °C. The colony morphology, cell morphology, Gram staining and fluorescence were recorded microscopically. The methanogenic colonies developed on the agar tubes were individually inoculated into the BCYT liquid media and further used for molecular studies.

Molecular characterization : The genomic DNA of isolated methanogens (SSS8) was extracted by using the methods given by Grosskoβf *et al.,* (1998) and Lepage *et al.,* (2004). The oligonucleotide primer system designed specifically to target archaeal 16S rRNA genes from positions 109 through 934 (Grosskoβf *et al.,* 1998; Lueders *et al.,* 2002). They were as follows: Forward primer: A109f (5'-ACTGCTCAGTAACACGT-3') 109–125, Reverse primer: Ar912rt (5'-GTGCTCCCCCGCCAATTCCTTTA-3') 915–935. Gradient PCR (Corbett GC1-96) was used to amplify the fragments. The PCR conditions were as follows. Denaturation – 94 °C for 60 s, Annealing – 45, 54, 56, to 60 ºC for 60 s, Extension – 72 °C for 90 s and final extension – 10 min at 72 °C. The reaction cocktail contained the following constituents (25 µl): Sterile glass distilled water-16.5 µl, PCR buffer 10X (including 1.5 mM $MgCl_2$)-2.5 µl, dNTP mix-2 µl, Sequence specific forward and reverse primers-1 µl, Taq DNA polymerase-1U, Template DNA-1 µl. The amplification was for 30 cycles and the amplified products were visualized in 1% agarose gel electrophoresis.

Results and Discussion

In mother inoculum, initially there was no methane production till 5th day, signifying longer incubation period.

However, enriched cultures such as AmR1, AmS2, SmS4 and SpR6 showed methane production on 10th day. On 15th day, two more samples (AmP7, SSS8) also showed methane production. From the results, it is evident that the samples (SmR3, SpR5) collected near the rhizosphere surface were not positive for methane production (Taketani *et al.,* 2010). In 1st subculture, among the two samples (SmR3, SpR5) which did not produce methane initially, the sample SpR5 produced methane after 9 days of incubation. Two samples viz., SpR6 and SSS8 were high and consistent in methane production. Maximum methane production (6.95%) was reported by SSS8 on 5th day (Fig. 1). Methane production was statistically more significant between the samples (F = 5.32261; P<0.0001) on different days of incubation (F = 8.038476; P<0.0001).

In 2nd subculture, almost all the samples produced methane but the percentage was less AS compared to first. This variability could be attributed due to the presence of sulfur reducing bacteria (SRB). After 3 days of first incubation, black sediments associated with rotten egg smell from the gas phase (hydrogen sulphide production) was observed in the culture bottles, indicating the presence of SRB (Lymio *et al.,* 2002a). These sulfate reducers have higher affinity for acetate, formate and hydrogen in the anoxic marine sediments. Thus, outcompete methanogens for these substrates and methanogenesis is limited in these sulfate rich sediments (Holmer and Kristensen, 1994). To reduce SRBs from the consortium, composition of the media and gas phase were changed to BCYT.

In 3rd subculture, SmR3, SmS4, SpR6 and SSS8 showed significant methane production. Maximum (4.5%) methane production was recorded in SSS8 consortia on 13th day (Fig. 2) of incubation. Methane production between samples (F = 2.699472; P<0.05) on different incubation period (F = 17.14091; P<0.0001) was statistically significant. During all subcultures, the methanogenic consortia in this sample comparably produced maximum methane. This consistency may be because this sample might be rich in organic matter, as it was collected near shore swampy region. However, series of subcultures in BCYT media which does not contain either acetate or formate and the presence of gas phase (N_2:CO_2) resulted in increased methane production and subsequent decrease of SRBs and rotten egg smell in the gas phase. Thus, by continuous subcultures, the pure methanogenic consortia were refined.

The consortial growth during 1st subculture is shown in Fig. 3 and 3rd subculture in Fig. 4. Comparing methane production with consortial growth, it was observed that methane production in 1st subculture was high on 5th day of incubation when the absorbance was less. It might be due to methanogens use their energy source to grow maximally and produce high methane when other bacterial groups get depleted (Weijma *et al.,* 2002). The consortial growth was not statistically significant among the samples but more significant on different incubation period (F =

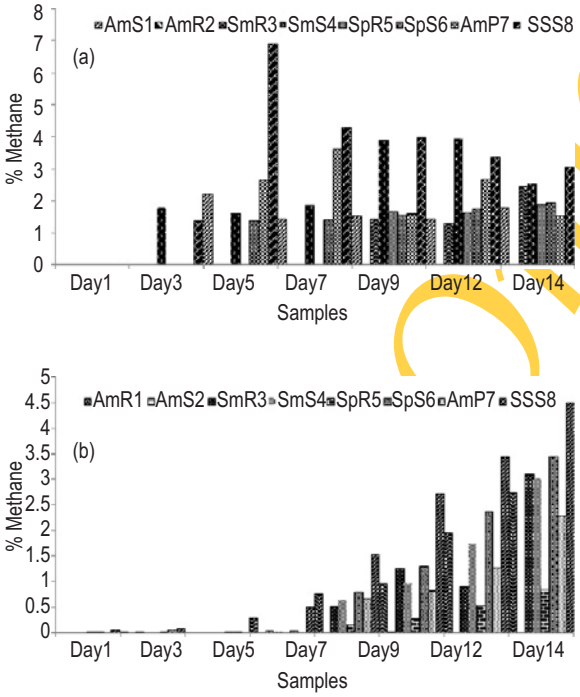

Fig. 1 : Methane Production by methanogenic consortia (a) in the first subculture (b) in the third subculture

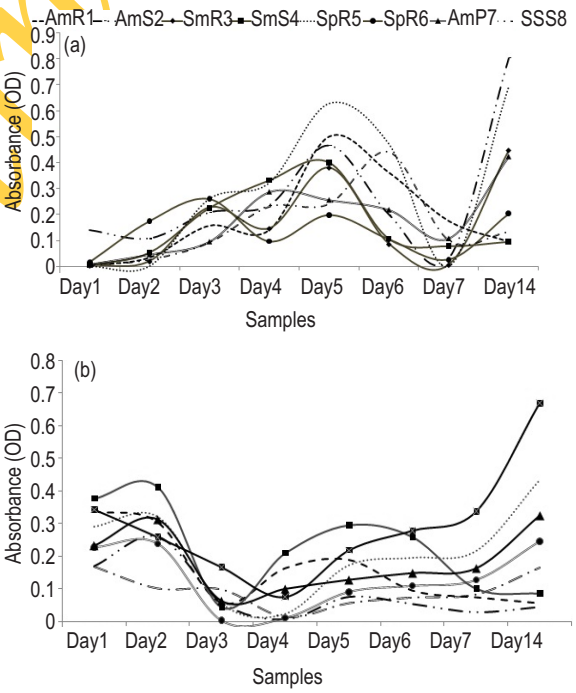

Fig. 2 : Growth of methanogenic consortia during the (a) first subculture (b) third subculture

12.90316; P<0.0001). In 2nd subculture, there was no considerable change in methane production and growth of methanogenic consortia. Conversely, in 3rd subculture, BCYT media showed maximum absorbance on 14th day of incubation, when methane production was also high. This might be due to series of subcultures had refined the methanogenic consortia. So, when methanogens had developed in the inocula, methane production also started and increased, consecutively (Lymio *et al.,* 2002b). Growth was statistically more significant among the samples (F = 5.87039; P<0.001) on different incubation period (F = 17.81245; P<0.0001) Though, the samples differed in initiation and quantity of methane production, stability in growth and methane production was observed after three successive subcultures of consortia to fresh pre-reduced media.

Consortia from SSS8 sample recorded high methane production (15.1%) in the presence of H_2:CO_2 (80:20) with acetate and formate in anoxic culture media on 12th day of incubation (Fig. 5) than in the absence of H_2:CO_2. This might be due to the action of syntrophs and hydrogenotrophs together in the consortia. This result is in accordance with the suggestion of Sirohi *et al.* (2010) that methane production depends on the gaseous substrate H_2 and CO_2, and its optimum ratio 8:2 (at pH 6.8) may enhance the production rate. In general, all the samples produced methane in the presence and absence of H_2 and CO_2. This indicates the existence of hydrogenotrophic methanogens in H_2:CO_2 samples and higher activity of syntrophs in the other samples (Chauhan *et al.,* 2004).

In general, all the methanogenic consortia isolated from different mangrove sediment samples showed better methane production in tyndallized cow dung rather than in raw cow dung. The methanogenic consortia from SSS8 sample produced maximum (4.7%) methane in the tyndallized cow dung, which was comparably higher than the methane produced in raw cow dung (3.2%). This could probably be due to native microflora in the raw cow dung that inhibited the growth of methaogenica bacteria. Whereas in the tyndallized cow dung, native flora was killed and the substrate favored inoculated mangrove methanogens to grow better and produce methane. The present study proved that the methanogenic consortia isolated from mangrove sediments can be used as a source of biogas production by using inexpensive substrate like tyndallized cow dung.

The presence of pure methanogenic colonies in SSS8 sample were evidenced by various criteria. In the roll tubes, two types of isolated methanogenic bacterial colonies were observed

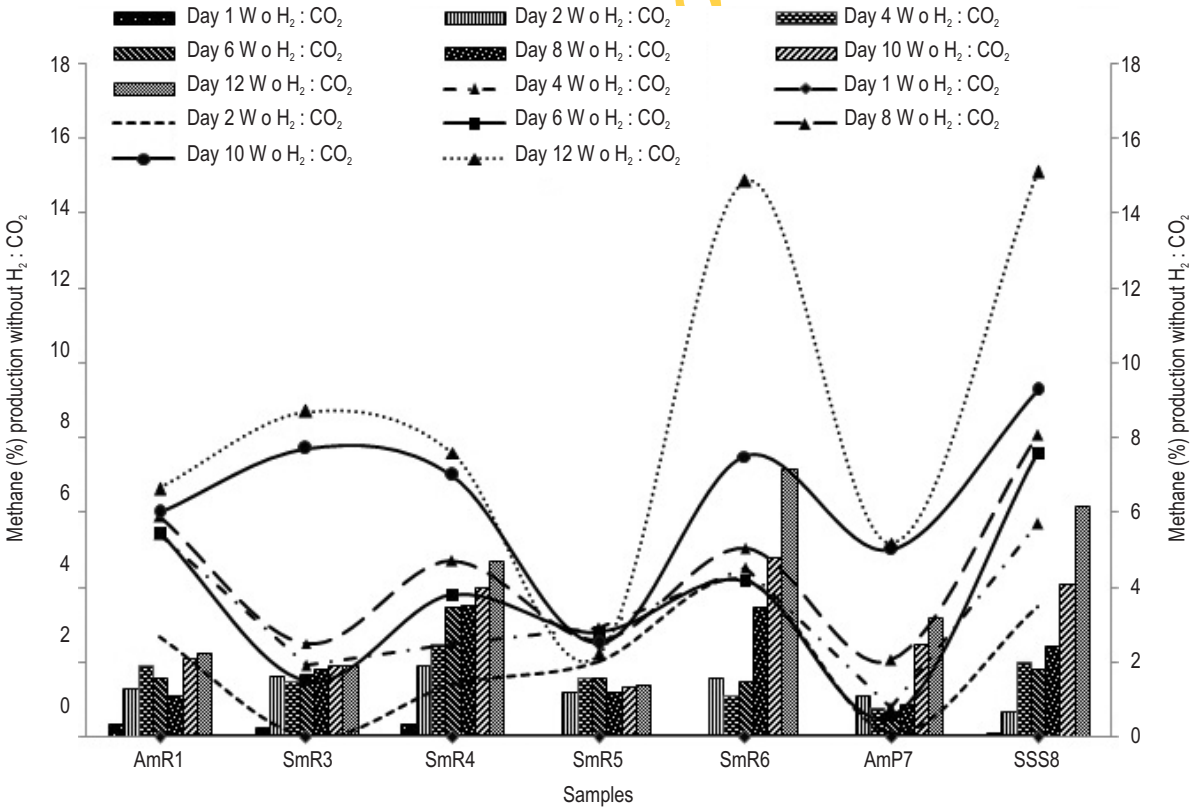

Fig. 3 : Effect of gaseous substrate (in the presence and absence of H_2 and CO_2) on methane production

on the sub surface of agar medium at 10^{-5} and 10^{-7} dilution after 3rd day of incubation. The shape and margin of the colonies were filamentous, circular, entire, transparent and pale yellow in colour (Dhadse *et al*., 2012). The elevation was flat for filamentous and raised for circular colony. The colonies of 0.5 mm diameter fluoresced when UV light was passed through the tube. Similar kind of colony morphology was observed by Mikucki *et al.* (2003). Different shapes of bacteria were observed *viz.,* cocci, cocci in clusters, rods of different sizes (slender long, stout, small). All the cells were Gram positive. The cocci were non-motile and the rods were motile. All the cells emitted blue green fluorescence when viewed under fluorescence microscope (Cheng *et al*., 2011) due to the presence of cofactors F_{420} which are unique to the methanogens. Also, the isolated colonies produced a maximum of 5% methane on 7th day of incubation in roll tube.

The molecular weight of amplified gene was assessed as 750 - 800 base pairs (Grosskoβf *et al.,* 1998; Lueders *et al.,* 2002) which confirms the amplification of archeal specific genes in methanogens isolated from the SSS8 sample. The methanogens isolated from the mangrove sediment samples produced methane by utilizing different chemical and natural substrates. Maximum methane was produced by the consortia developed from swampy soil (SSS8) of mangrove environment and also recorded maximum absorbance than other samples collected from the same environment. Hence, the methanogenic consortia isolated from the mangrove sediments can be further used as a biological source for biogas production from the least expensive raw materials.

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