3. Materials and Methods

3.1. Isolation, Screening and Characterization of Carbon dioxide Reducing Methanogens

3.1.1. Sample Collection

Sediments samples were collected from various locations of Tamil Nadu, Pondicherry, Karnataka and Andaman Island November 2013 to March 2014. Soil sediments were randomly collected using an open end soil borer (20 cm depth and 5 cm diameter) at depths 10-20 cm below the surface sediments. Samples were kept in sterile containers pre-flushed with nitrogen and stored at 4°C until processed (Ranadae and Gadre, 1988; Ahila et al., 2014).

3.1.2. Enrichment of Methanogens

The collected samples were immediately inoculated in 100ml vials containing pre reduced mah’s and bicarbonate yeast tryptone medium closed with butyl rubber stopper and sealed with an aluminium crimp (Balch and Wolfe, 1976; Ahila et al., 2014). The inoculated vials were flushed with H$_2$:CO$_2$/ N$_2$ gas and incubated at 37-40°C for 15 days to enrich the samples. Sterile gassing syringes and hypodermic needles were used for the addition and withdrawal of culture samples, which was used for minimize the oxygen contamination. Sampling of headspace gas was performed with the help of pressure lock syringes (Sowers, 1995; Sowers and Schreier, 1995).
3.1.3. Preparation of Pre-reduced Anaerobically Sterilized Medium (PRASM)

The Mah’s Media (Acetoclastic Methanogens) and BCYT medium (Carbon dioxide reducing methanogens) were used for isolation of methanogens. The distilled water was boiled and it was then cooled under passage of nitrogen to 40°C used for media reparation. The pH was adjusted to the desired value with any one of the three solutions viz., NaOH, HCl or NaHCO₃. Then the media evenly transferred to serum vials and then closed securely using butyl rubber stoppers with aluminium seals and autoclaved at 121°C for 20 minutes. The vials were cooled after autoclaving. The cysteine hydrochloride and sodium sulphide mixture was used as reducing agent, it was added at this stage using a sterile syringe. Within 5 minutes the medium would be devoid of oxygen, as indicated by decolourization of the indicator. The heat labile and volatile substrates were filter sterilized and added to the medium before inoculation. Gassing of the medium was done consecutively with suitable gas or gas mixture viz., N₂, N₂:CO₂ (80:20) or H₂:CO₂ (80:20) depending upon the final gas phase (Ramasamy et al., 1992).

3.1.4. Isolation of Methanogens

The liquid medium was prepared in the double strength, boiled, cooled to 50°C and the pH was adjusted as described above. To this double strength molten agar was added. The medium was then dispensed in roll tubes maintaining anaerobiosis as described and autoclaved. The containers were cooled and maintained at 50°C in a serological water bath and used for inoculation (Hucker, 1950; Hungate, 1966; Ramasamy et al., 1992).
The enriched samples were suspended in 100 ml of pre-reduced sterile distilled water and then incubated at 30°C for 30 min. Mixtures were subsequently diluted 10⁻⁵, then 1 ml portions of suspensions were transferred to roll tube under N₂:CO₂(1:1) atmosphere and the test tubes were rolled over the foam soaked in cold water till the medium uniformly solidifies on the sides of the test tube. After solidification, the test tubes were incubated at 37°C temperature (Hungate et al., 1966; Hungate, 1969; Wolfe, 2011). After incubation colonies were randomly selected and subcultured for further studies.

Colonies of the methanogens showed a greenish blue auto-fluorescence on roll tubes which was primary identification of methanogens (Adachi et al., 1996; Adachi, 1999). The well developed colonies were appeared on the surface of the roll tube. The isolated colony was then transferred suitable medium and incubated at an 37°C for 3-4days. The purity of the isolates was monitored at regular intervals by re-isolation in a roll tube. The growth of methanogens were monitored and confirmed periodically by observing turbidity, use of substrates, wet mount observation on epifluorescent microscopy and formation of methane gas (Leadbetter and Breznak, 1996; Randae and Gadre, 1988; Uchino and Ken, 2011; Katayama and Kamagata, 2015).

The isolates were subcultured every two week interval in sodium bicarbonate slants. The isolated methanogens were purified using combination of antibiotics such as vancomycin and kanamycin. The pure cultures were maintained in serum vials for further investigations (Balch et al., 1979; Ranadae and Gadre, 1988; Adachi, 1999; Wolfe, 2011; Issazadeh et al., 2013). The cells were grown as pure culture in serum vials (50 ml or 100 ml or 500 ml) (Balch and Wolfe, 1976).
3.1.5. Detection of Methanogens by Epi-fluorescence Microscopic Analysis

The detection of methanogens was performed by production of unique co-enzymes including F$_{420}$, F$_{430}$ and methanoptrin by the organisms, which fluorescence under epi-fluorescent microscopy (Cheeseman et al., 1972). Methanogenic bacteria have F$_{420}$ which was one of the electron carriers. This cofactor F$_{420}$ was found to be in higher concentration in methanogens rather than other eubacteria (Cheeseman et al., 1972; Edmondson et al., 1972). The colonies exhibited fluorescence under epi-fluorescent microscope (NIKON, with Hg lamp at excitation at 420 and 430nm) were selected for further studies.

3.1.6. Identification and Characterization of CO$_2$ Reducing Methanogens

The carbon dioxide reducing methanogens were identified and characterized based upon its substrate preference. The type of substrates utilized by methanogenic isolates were checked in 50 ml serum vials containing 15 ml of appropriate liquid medium supplemented with acetate and H$_2$ + CO$_2$ (80:20 v/v) as carbon source respectively. Substrates were added to the final concentration of 100mM levels (duplicates were maintained). The formation of methane was checked for its identification.

3.1.7. Morphology and Physiological Tests

In many cases, motility, cell shape and size were observed in wet mounts. Cultures from different growth phases should be examined. Hanging drop preparations may be used, preferably prepared in an anaerobic laboratory and observed there by using a light microscope (Boone and Whitman, 1988). Gram character was done by the Hucker
method or the Burke method and the results compared with results for known gram-positive and gram-negative control organisms.

3.1.8. Growth in Aerobic and Anaerobic Liquid Media

Purity of the methanogenic bacteria was identified by inoculating the strains into aerobic nutrient broth. The isolated culture was obligate anaerobes, and then it did not grow in the aerobic media. So, this was considered as a supportive identification tool. Purity of the methanogenic isolates was confirmed by inoculation of strains into an anaerobic medium for heterotrophic bacteria, which contained glucose 5.0 g, polypeptone 1.0 g, and NaHCO₃ 0.8 g in 1000 ml the basal solution (head gas phase in test tubes: N₂). The isolate did not show growth after the 40 days of incubation were considered as pure cultures (Kepp and Leisinger, 1983; Adachi, 1999; Jablonski et al., 2005; Kendall and Boone, 2006).

The methanogenic isolates were inoculated in 20 ml bicarbonate broth in serum bottles. The growth of colonies in liquid bicarbonate culture was monitored and noted. The methanogens grown after 14 days of incubation were classified as fast growers. The isolates that formed visible colonies after 30 or more days of incubation were classified as slow growers. Those that grew intermediately were classified as medium growers (Adachi, 1999).

3.1.9. Resistance to Vancomycin

The methanogenic isolates were inoculated in 20 ml bicarbonate broth in serum bottles containing 10µgml of vancomycin. The growth of colonies in liquid bicarbonate culture was monitored and noted (Burlage et al., 1998).
3.1.10. Gas Detection Techniques

3.1.10.1. Moisture Syringe Method

After incubation period, the total gas content of the isolates was examined periodically (2, 4, 6 & 8 days) by moisture syringe method. 5 ml syringe was moistened with a drop of sterile water and a plunger was inserted on to the bottle. The excess methane gas in the culture bottle was allowed to flow in the syringe and it was detected (Ranade and Gadre, 1988).

3.1.10.2. Flame Test

In this process, methane gas was sucked through a sterile 2 ml syringe with needle. In the culture bottle, the head space contained the methane gas was plungered and injected before flame. The culture contained pure methanogens, based on their metabolism of pure methane gas was produced and flamed with bluish color (Ranade and Gadre, 1988).

3.2. Analysis of DNA Base Composition

The cells were harvested by centrifugation at 5000 rpm for 5 minutes. The pellet was suspended in 5 ml of saline-EDTA (0.8% NaCl; 0.1M EDTA) and centrifuged. The cell pellet was resuspended in 25 ml of saline-EDTA solution; 1.0 ml of lysozyme (10mg/ml) was added and incubated at 37°C for 45 minutes. Two ml of 25 % SDS solution was added to the mixture and heated at 60°C in water bath for 10 minutes. The mixture was then cooled to room temperature. About 9.0 ml of sodium perchlorate (5M) was added and mixed well. An equal volume of chloroform and isoamyl alcohol (24:1) was added to the above mixture and mixed in the separating funnel for 20 minutes. The mixture was
centrifuged at 12000 rpm for 5 minutes. The aqueous layer which contained nucleic acid was separated and precipitated with the addition of two volume of 95% ethanol. It was then spun at 12000 rpm for 10 minutes to obtain the DNA pellet. The DNA was resuspended in 10 ml of saline-EDTA buffer (Nagamani, 1996; Kumari, 2015).

The DNA preparation was diluted to an $A_{260}$ of 0.4. Water baths and oil bath were set at 35, 50, 70, 75, 80, 85°C and 90, 95, 100°C respectively. Aliquots of the DNA solutions were maintained at respective temperatures. One tube was maintained at room temperature and two tubes at 100°C. The tubes were incubated for 15 minutes in respective temperatures and were then quickly cooled to 25°C with ice water bath. One tube of 100°C was allowed to cool slowly to room temperature. The tubes were measured at $A_{260}$ (25°C) and values were recorded, calculated and a smooth curve was plotted. The mid-point at the absorbance increase was measured as Tm and the %GC was calculated by using the formula

$$\% \text{ GC} = 2.44 (Tm - 69.3)$$

Based on this identification and characterization studies, the methanogens were identified.

3.3. Optimization of Operational Parameters

3.3.1. Effect of Incubation Time and Temperature

The effect of incubation time and temperature was determined at 20- 65°C, using 5°C intervals at 2nd, 4th, 6th and 8th day. The isolates were inoculated in sodium bicarbonate medium and incubated at various temperatures. The vials were pressurized daily with H$_2$: CO$_2$ (80: 20). Gas analysis of these isolates showed whether methanogenesis was coupled
to growth. Triplicates were maintained and methane production was determined periodically using a gas chromatography. The incubation time and temperature, which gave the maximum methane production, was considered as the optimum incubation time and temperature of the isolate (Boone and Whitman, 1988).

### 3.3.2. Effect of pH

The experiments were performed in 35.50 ml serum vials with 15 ml of a bicarbonate free medium and 2 mM appropriate carbon source. The desired pH was controlled with 20mM phosphate-buffer and the various pH tested values ranging from 3.0-9.0±0.2. NaOH or HCl solution was added to give the desired pH values. Medium pH tends to increase during growth of carbon dioxide reducing methanogens. This can be avoided by repeatedly repressurizing vessels to the original pressure of hydrogen and carbon dioxide. All the isolates were incubated at optimized temperature. Triplicates were maintained and methane production was determined at 6th day using gas chromatography. The pH value, which gave the maximum methane production, was considered as the optimum pH of the isolate (Patel et al., 1990).

### 3.3.3. Effect of CO₂ Feeding Rate

All the carbon dioxide reducing methanogenic isolates were analysed the formation of methane with different CO₂ (H₂:CO₂ (80:20 v/v), H₂:CO₂ (60:40 v/v) and N₂:CO₂ (80:20 v/v)) feeding rate. It was determined once in three days. Vials with heat-treated inactive cells served as controls. Growth and activity were measured in terms of methane production respectively. Total gas production of the methanogenic cultures vials was measured and quantified by using gas
chromatography in head space of culture vials. The growth rates of methanogenic isolates were observed periodically (APHA, 1989).

Methane content of the system was confirmed and quantified by using “Varian” gas chromatography system, fitted with Thermal Conductivity Detector (TCD) and “Perkin-Elmer” gas chromatography having porapak “Q” column (6’ x 1/8” SS). The oven and injection/ detector temperatures were maintained at 70°C and 100°C respectively. Column condition was programmed as room temperature. Nitrogen gas was used as carrier gas at the rate of 40ml/ min. Air and Hydrogen were used for the flame (Ramasamy et al., 1992; Priebe et al., 2013; Ray et al., 2017). The biomethanation kinetic study was carried out for the entire isolates using one-way ANOVA.

3.4. Possible Enhancement of Sequestration of CO₂ in Fluidised Cell System and Trial Run of the Bioreactor for the Conversion of CO₂ to CH₄

With the optimized growth parameters, the methanogenic isolates which produced higher level methane was mass cultured using Applikon Bioreactor with Bio-flow control system in BCYT culture broth for carbon dioxide feeding rate study. The isolates were mass cultivated in 500 ml serum bottles sealed with serum caps on mineral salt medium with 10% carbon source. Cultures were routinely checked for purity by examination of stained cultures and wet mount using phase-contrast microscopy, and fluorescent microscopy for methanogens. The cultures were maintained in serum vials in the appropriate medium with the headspace containing hydrogen and carbon dioxide (80:20 v/v), and sealed with butyl-rubber stoppers and aluminum caps. The total biogas produced was observed at regular time interval.
About 10% (v/v) of the methanogenic isolates was added as inoculum in polymer matrix. The sequestration of carbon dioxide was studied under atmosphere such as H₂:CO₂ (80:20 v/v) which was the sole carbon source in the reactor. The gas analysis was done using gas chromatography (Ramasamy et al., 1992). The rate of methane production and carbon dioxide sequestration using methanogenic isolates at various calibrated gas mixtures were analysed. Magnetic stirrer with heating, Perkin Elmer Gas Chromatography fitted with TCD was used with H₂ as carrier gas using KSR-CVM method sequence equipments were used for this study. Gas washing bottle (250 ml) with BCYT medium (250 ml) was used for separate inoculum with standards gas area. The Incubation temperature was 35°C with in-flow and out-flow rate. Finally, total Carbon dioxide balance, total CO₂ consumed and methane produced rate was estimated. The rate of methane production and carbon dioxide sequestration of isolates also analyzed and estimated.

3.5. Isolation of Methane Oxidizing Methanotrophs from Different Ecological Niches of India

3.5.1. Sample Collection and Isolation

Samples were collected from different ecological niches of costal area of Tamil Nadu. The samples were enriched with 50% of methane and 50% of air in NMS medium. Nitrate mineral salt (NMS) medium was used for methylotrophs enrichment, a litre of which contained NaCl 5.0 g, Na₂HPO₄ 0.72 g, KH₂PO₄ 0.28 g, MgSO₄.7H₂O 0.2 g, CaCl₂.2H₂O 0.02 g, FeSO₄7H₂O 5.0mg, ZnSO₄.7H₂O 70 mg, MnCl₂. 4H₂O 30 mg, H₃BO₃ 30 mg, CoCl₂ 20mg, NiCl₂.6H₂O 20 mg, Na₂MoO₄.2H₂O 30 mg, CuCl₂.2H₂O 10 mg, Na₄-EDTA 1.0 mg, KNO₃ 1.0 g, methanol 0.1 % v/v or methane:air(1:1). Antifungal agent cycloheximide (40 mg/ml) was added at the rate of 500mg per liter, and pH was adjusted by inorganic (sodium;
NaHCO$_3$–Na$_2$CO$_3$) buffer as per pH of the sample at sampling. The isolation and characterization of methanotrophs were performed using conventional culture techniques using single carbon source (Methanol) on agar medium and Methane in a closed system as broth culture. Characteristic the methylotrophs colonies growing over the medium were identified (Corpe and Rheem, 1989). Further the methylotrophs were purified by the streak plate method and well isolated colonies on the plates were preserved in MMS agar slants, Isolates were maintained on NMS slants at 4°C in a refrigerator for further use.

3.5.2. Soluble Methane Monooxygenase Activity

For qualitative determination of soluble methane monooxygenase a slightly modified version of the naphthalene oxidation assay of was followed. Each culture was transferred in 1 ml aliquots to 10 ml screw-cap tubes and 1 ml of pre-filtered saturated naphthalene solution was added to each tube. The samples were prepared in triplicate keeping sterile medium control as blank. The reaction mixtures were incubated at 200 rpm on incubator shaker at 25°C for 1 to 3 hrs. After incubation, 100 μl of freshly prepared 4.21 mM tetrazotized-o-dianisidine solution was added to each tube and the intensity of coloured diazo-dye complex was immediately monitored by recording the A525 by spectrophotometry. The intensity of diazo-dye formation is proportional to the naphthol concentration (1-naphthol and 2-naphthol). The specific activity of sMMO was expressed as nanomoles of naphthol formed per milligram of cell protein per minute (Kalyuzhnaya et al., 2015).
3.5.3. Development of Process System for Bioconversion of Methane to Methanol

The methanotrophic isolates which produced higher level sMMO was mass cultured using Applikon Bioreactor with Bio-flow control system in MMS culture broth. Cultures were routinely checked for purity by examination of stained cultures and wet mount using phase-contrast microscopy. The cultures were maintained in serum vials in the appropriate medium with the headspace containing air and carbon dioxide (80:20 v/v), and sealed with butyl-rubber stoppers and aluminum caps.

About 10% (v/v) of the methanotrophic isolates was added as inoculum in polymer matrix and packed in columns. The reactor was flooded with MMS medium. Then the flow rate was adjusted and out flow of fluidized bed reactor (methanogenic phase) along with zero air used as gas phase. Methanol conversion rate was estimated. The rate of methanol production by isolate was analysed and estimated.

3.5.4. Methanol Recovery and Quantification

The methanol converted was detected in the medium as alcohol with positive (Methanol and Ethyl alcohol) and negative control (PRAS medium and uninoculated NMS medium). Methanol was extracted by distillation process at 65°C. The viability of the cells was determined by viable plate count method using appropriate culture medium. Presence of methanol was confirmed by GC.