Petroleum and Coal

**Article** **Open Access**

# INVESTIGATION OF COAL-METHANOARCHAEAL ASSEMBLAGE IN DEEP SUBSURFACE UNDERGROUND USING ENRICHMENT CULTURE TECHNIQUE

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Received June 22, 2018; Accepted September 3, 2018

**Abstract**

Enrichment culture of coal samples from underground mines (Jitpur and Moonidih underground mines) located in Dhanbad region, the Indian state of Jharkhand was tested positive for the presence of living microbial lineage capable of producing methane from coal during energy metabolism under laboratory condition. Accordingly, two gram negative, mesophilic, penicillin G and streptomycin resistant strain (BC/CIMFR-ana-CH4-12 and 14) derived from preferred sites were produce methane from acetate. They assimilated complex proteinaceous substances (yeast extract, beef extract, tryptone, and peptone) and amino acids (alanine, cysteine, and methionine) for energy metabolism. The pH ranges for growth were 6.0-7.5 for strain BC/CIMFR-ana-CH4-12 and 6.5-8.5 for strain BC/CIMFR-ana-CH4-14, with the fastest growth at pH 7.5 for BC/CIMFR-ana-CH4-12 and pH 7.0 for strain BC/CIMFR-ana-CH4-14. During growth, doubling time was observed 13.702-14.005 hours under the optimal condition, and specific growth rate was recorded 0.021/hour for both strains. Based on the study strains were belongs to *Methanococcus spp.* and *Methanobacterium spp.*, associated with numerous aerobic and anaerobic microbial community in deep subsurface coal deposits as suitable habitat. The ecological implication of their habitat also discussed. The study suggests that there is intense biogenic methane generation activity in a selected environment which may me enhance by stimulating the activity of existing methanoarchaeal consortia.

***Keywords***: *Coal bed; Archaea; Methane; Habitat; Substrate; Growth.*

## 1. Introduction

Archaea constitute the third fundamental domain of life, it comprises methanogens, red extreme halophiles, and the thermoacidophiles, playing a great impact on global nutrient cycling as well as a geochemical cycle in the biosphere. These diverge from other bacteria at the very early stage of evolution and habitat under extreme condition [1-2].

Generally, coal is made up of decomposed plant materials that initially form peat. After peatification, it metamorphosed into different ranks of coal as a complex heterogeneous ultra structure according to its burial depth and temperature [3]. About 85 to 95% (wt/wt dry coal) organic materials in coal are known as macerals which composed of plant material, and the inorganic materials are present as aluminosilicates and pyrites. Therefore, coal could be considered a very attractive carbon source for microbial biodegradation [4-5]. Studies revealed that a portion of coal bed methane is biological origin occurred by microbial consortia habitat in coal deposits [6-7]. Accordingly, high diversity of bacteria, firmicutes, spirochetes, bacteroides and all subgroups of proteobacteria are reported in the coal bed. Contrasts with archaeal linage, methanogens are common habitat, and only a few are characterized [8-10]. For example, sub-surface coal deposits were found to be the habitat of diverse acetoclastic, methylotrophic and hydrogenotrophic methanoarchaeal assemblages [11]. The members of

*Methanosarcina spp*. can take up $H_2$/CO, acetate, methanol, and methylamines as substrate habitat in coal beds of Alberta, Canada [5]. The Methanosarcinales [8], Methanobacteriales and Methanococcales [12] were reported from Powder River Basin, Wyoming. Kai *et al.* [13] stated that rod shaped methanogens are generally affiliated to the order of Methanobacteriales, which belongs to three mesophilic genera as *Methanobacterium*, *Methanobrevibacter* and *Methanosphaera*, and two thermophilic or hyperthermophilic genera as *Methanothermobacter* and *Methanothermus*. Indeed, archaebacteria residences under extra eme environment are undertaken for extensively exploration [14].

The goals of the current study were to confirm the presence of methane generating archaea in coal bed (Jitpur and Moonodih underground coal mine) of Dhanbad coal fields using culture dependent method to explore their physiology, growth kinetics and to emphasize their habitat for ecological point of view. The study further expands our knowledge and the value of archaea kingdom.

## 2. Experimental

### 2.1. Study sites

Dhanbad is a city in the Indian state of Jharkhand and recognized as coal capital of India. The region is endowed with huge resources of bituminous coal and having an area of 453Sq. Km bounded by latitudes 23° 37' and 23° 50' N and longitudes 86° 07' and 86° 28' E [15]. It is well known for coal mining and having some of the largest mines in India. Accordingly, two different coal mines namely Jitpur underground mine and Moonidih underground mine located in Dhanbad region have been selected for the study (Figure 1).

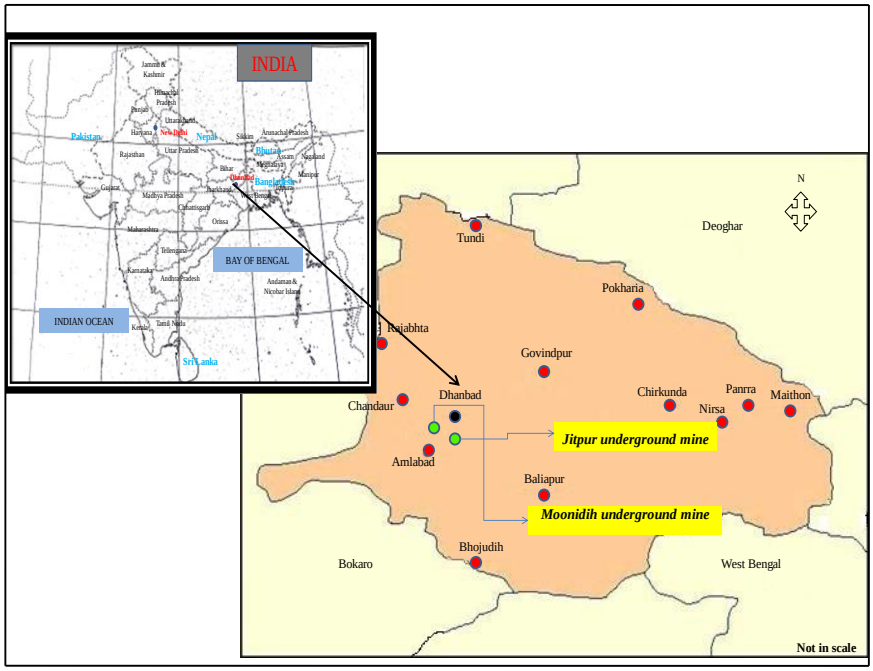


Figure 1. Map indicates the selected study sites

### 2.2. Microbial enrichment and isolation of a pure culture

Coal as inoculum source was collected (25-30cm deep) in polyethylene bag followed by flushing with 99.99% carbon dioxide ($CO_2$), sealed and kept at room temperature in dark condition until use. The basal medium [8-16] for enrichment was contained the following (g/L) ingredients was selected for enrichment and isolation: KCl (0.1), $MgCl_2$, $2H_2O$ (0.2), $NH_4Cl$ (1), $CaCl_2$, $2H_2O$ (0.04), $KH_2PO4$ (0.1), NaCl (0.8), and yeast extract (2.0). The final concentration of trace metal in the medium (mg/L) were: Nitrilotriacetic acid, 10; $MnSO_4$, $H_2O$ 5;

$Fe(NH_2)_4(SO4)_2.6H_2O$, 4; $CoCl_2.6H_2O$, 1; $ZnSO_4.7H_20$, 1; $CuCl_2.2H_2O$, 0.1; $NiCl_2.6H_2O$, 0.1; $Na_2MoO_4.2H_2O$, 0.1; $Na_2SeO_4$, 0.1 and $Na_2WO_4$, 0.1. Vitamin concentrations (mg/L) in the final medium were: Pyridoxine-HCl, 0.1; Thiamine-HCl, 0.05; Riboflavin, 0.05; Calcium pantothenate, 0.05; Thioctic acid, 0.05; p-aminobenzoic acid, 0.05; Nicotinic acid, 0.05; Vitamin B12, 0.05; Mercaptoethanesulfonic acid (coenzyme M), 0.05; Biotin, 0.02 and Folic acid, 0.02. The pH was adjusted 7.2 by 0.5N $H_2SO_4$ and NaOH. Finally, sodium bi-carbonate at a concentration of 2.0 g/L was added. A drop of resazurin (0.001g/L) was added as an oxygen indicator and autoclaved. After cooling 0.5mL reducing solution (1.25% cysteine-1.25% $Na_2S$) was added. Immediately 1g of powder sample was transferred into each flask and sealed by filling the headspace with pure $CO_2$. The flask was kept at 37°C in a dark place for one month. Pure culture was obtained by serial dilution technique on solid medium under anaerobic condition. Furthermore, screening was achieved through methane production potentiality by utilizing anaerobic basal liquid medium as described earlier supplemented with sodium acetate trihydrate at a concentration of 5g/L. The pure isolates were preserved at 4°C for characterization. Colony and cellular morphology were determined according to Balows *et al.* [17] using pure isolate. The cell aggregate formation of purely isolated methanogenic strains was determined in liquid basal medium supplemented with acetate at a concentration of 5gm/L. After commencing growth in liquid medium, the granule formation or cell aggregation was recorded [18].

### 2.3. Headspace gas analysis

Headspace gas was studied by using a gas analyzer (Madur, GA 21 Plus, Poland). For that, gas was sucked by 50mL injection syringe and directly injected to the sampling port of gas analyzer. Methane was detected through MadIR methane sensor and analyzed by Madcom software.

### 2.4. Phase-contrast and Scanning electron microscopy

Gram staining was performed according to Dubey, and Maheswari [19] and the cells were analyzed by phase-contrast microscope (Olympus BX53, Japan). The sample was prepared for scanning electron microscopy followed by user guideline of the instrument. For that, a thin bacterial smear was prepared on a glass slide, and it was dried completely. Then the smear was gold coated (15-20 nm) using a coater (Q1SOR). Finally, the slide was placed into vacuum created sample chamber of the instrument (Supra 55; Zeiss-Germany) and the image was taken at a suitable magnification.

### 2.5. Biochemical test, nutritional requirement, growth kinetics, and membrane study

Biochemical characterization as an enzymatic test, antibiotic sensitivity test, optimal growth condition, growth kinetics, and the nutritional requirement was performed as described by Dubey and Maheswari; Marteinsson *et al.;* Balows *et al.* [19-20-17]. Substrate utilization (as different compounds) was tested by using a basal medium with the addition of each tested compound and omitting one of the components in each test. All tests were performed in duplicate, and a control in which no substrate was added was used as a baseline. The pH of the medium was adjusted by 1N $H_2SO_4$ and NaOH. Penicillin G and streptomycin was added at a concentration of 0.5gm/L [9] into liquid basal medium. The turbidity of the medium was determined by UV/Vis spectrophotometer (CECIL C-7500 series, UK) at 610 nm. Doubling time ($t_d$), specific growth (μ) and generation time (g) were calculated according to Meher and Ranade, 1992; Tortara *et al.* [21-22]. The archaeal membrane lipid was extracted by a modification of Bligh and Dyer (chloroform-methanol) method [23-24] and analyzed by Fourier Transform Infrared Spectrophotometer (IRAffinity-1S, Shimadzu, Japan).

### 2.6. Habitat characterization

Characterization of archaeal habitat was performed by selecting different physicochemical and biological parameters. Accordingly, pH and electrical conductivity (EC) was measured by

pH meter (Thermo-Scientific, Orion Star-A214) and EC meter (PCS Tester35, Multi parameter). Total organic carbon was determined by rapid dichromate oxidation technique and total nitrogen content was analyzed by alkaline potassium permanganate method [25] using Kjeldahl instrument (Pelican, Kelplus, Distyl-EM). Humic acid (HA) was estimated according to Mesa Verde Resources humic acid methodology [26]. The optical density was measured at 450nm using spectrophotometer and quantified through standard curve. Biological parameter as dehydrogenase activity was determined by utilizing 2,3,5-triphenyltetrazolium chloride (TTC) as a substrate followed by standard curve [27]. Total aerobic and anaerobic microbes were counted on nutrient agar and anaerobic basal agar plate (Hi-Media) using serial dilution method [19]. Serial dilution for anaerobic organisms was made by using sterile anoxic water solution. For that, pure nitrogen gas was bobbling in boiling stage of pre-sterilized distilled water containing resazurin at a concentration of 0.001g/L in serum vial (20mL capacity, Borosil) under aseptic condition. When the solution turned pink to colorless the vial was capped using rubber stopper and crimped. After cooling, trace amount of reducing solution was added into it and dilution was achieved by using glass injection syringe. Active microbial biomass carbon (AMBC) was measured by glucose nutrient induced respiration method [28]. Media was composed of peptone, (10g); yeast extract, (5g); glucose, (5g); sodium chloride (5g); Potassium di-hydrogen ortho-phosphate (0.21g) in 1000ml de-ionized water. Basal soil respiration (BSR) was measured as the $CO_2$ evolved from moist soil (60% water holding capacity), over an incubation period of 10 days at 25°C, in the dark condition [28]. For element analysis, ICP-OES (Thermo Scientific, iCAP 6000 series spectrometer) was used and the sample was prepared as described in ASTM D-4638 method [29].

## 3. Results and discussion

Generation of methane in empty space of enrichment culture flask was taken as evidence and screening tool for methanogenic archaea. Accordingly, two obligate anaerobic methane generating pure isolates were successfully received from selected habitat and referred as BC/CIMFR-ana-CH4-12 and 14 respectively.

### 3.1. Colony and cellular morphology

Isolated pure culture was considered for characterization in compare to colony and cellular morphology, summarized in Table 1.

Table 1. Characteristics of isolated methanoarchaeal strains from underground coal mines of Dhanbad

| Strains | Habitat | Colony characteristics | Methane generation | Gram reaction | Shape | Cell-cell aggregation | Endospore |
|---|---|---|---|---|---|---|---|
| BC/CIMFR-ana-CH4-12 | Coal surface of Jitpur underground mine | Colony was 0.5-1mm diameter, round and smooth. The color was off white with entire edge and texture was moist with flat elevation. | Positive | Gram Negative | Rod | Dispersed | Not found |
| BC/CIMFR-ana-CH4-14 | Coal surface of Moonidih underground mine | Round-smooth colony and 0.5-1mm elongated on solid medium with entire edge. It was off white in color and the elevation was flat with moist texture. | Positive | Gram Negative | Spherical or cocci | Dispersed | Not found |

Accordingly, the colony of both strains was found to be round shaped, off white color and exhibited 0.5-1.0mm in diameter with entire edge. The texture was noticed as moist with flat elevation on solid medium for the same. Methanogenic archaeal colony was observed grayish white color, opaque and round shaped with entire edges and the diameter was reached up to 0.5–1.0 mm [30]. The isolates grew as dispersed in liquid medium when supplemented with acetate and granule formation or cell aggregation was not observed during growth cycle. Cells

of both strain stained gram negative. Further, clear and details image for gross cell morphology was studied by scanning electron micrograph and revealed that strain BC/CIMFR-ana-CH4-12 was rod shaped and BC/CIMFR-ana-CH4-14 exhibited cocci in shape (Figure 2).

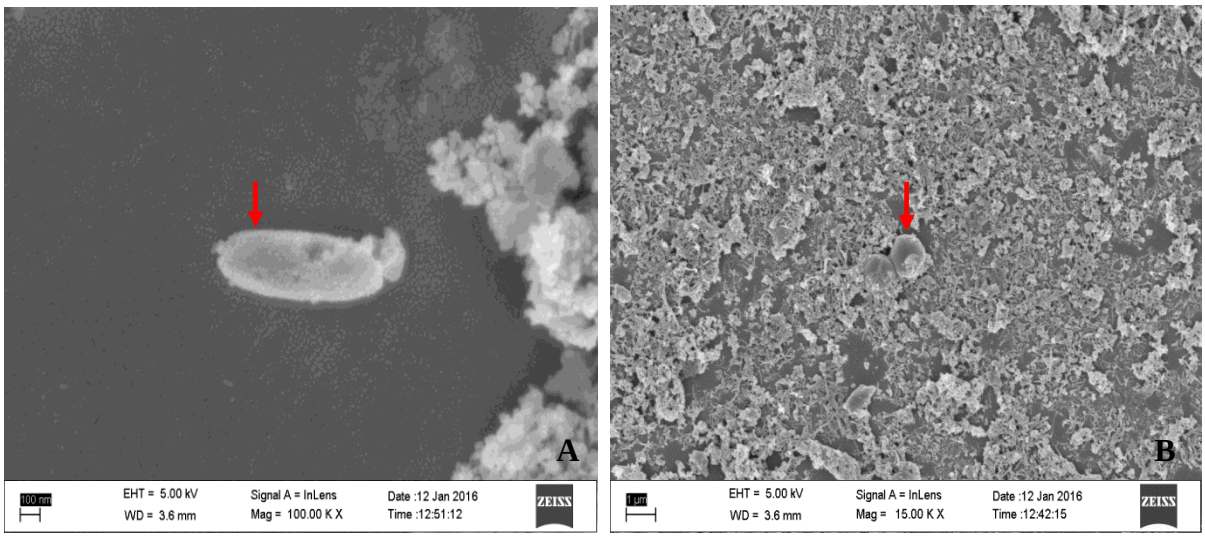


Figure 2. Scanning electron micrograph of isolated methanoarchaeal strains (A- BC/CIMFR-ana-CH4-12 and B- BC/CIMFR-ana-CH4-14)

### 3.2. Nutrient utilization

Methanogen can utilize various substrates for energy metabolism such as $CO_2$ reduction with hydrogen, formate as electron donors, methanol reduction, fermentation of acetate and dismutation of methylated compounds [31-32]. Substrate utilization by isolated strains as different carbon and nitrogen source was investigated during the course of growth and presented in Table 2. Accordingly, luxuriant growth was observed in presence of acetate to the growth medium for selected isolates. However, citrate was taken poorly by both strain and formate was moderately assimilated by strain BC/CIMFR-ana-CH4-12. Furthermore, the isolates were able to utilize selected complex proteinaceous substances (yeast extract, beef extract, tryptone and peptone) for energy metabolism. Yeast extract was luxuriantly assimilated by strain BC/CIMFR-ana-CH4-14, while tryptone was taken as good for both strains. The isolates further exploited selected amino acids (alanine, cysteine and methionine) as sole carbon and energy source. Although growth was generally weaker than the growth observed with complex substrates. Comparatively, strain BC/CIMFR-ana-CH4-12 respond luxuriantly in presence of L-methionine to the medium. Since, bacterial growth is depending upon the media composition as well as substrate composition. These differences may be attributed to differences in substrate composition [33].

Table 2. Utilization of different compounds as growth factor

| Strains | Formate | Acetate | Citrate | YE | BE | Tryptone | Peptone | L-alanine | L-cysteine | L-methionine |
|---|---|---|---|---|---|---|---|---|---|---|
| BC/CIMFR-ana-CH4-12 | + + | + + + + | + | + + + | + + + + | + + + | + + ++ | + + + | + + + | + + + + |
| BC/CIMFR-ana-CH4-14 | + | + + + + | + | + + + + | + + + | + + + | + + + + | + + + | + + + | + + + |

*YE- Yeast extract, BE- Beef extract; growth was expressed as ++++ (luxuriant growth), +++ (good), ++ (moderate), + (poor) and – (no growth). Concentration of each proteinaceous substrate was tested at 0.5% (wt/vol) and amino acid was at a concentration of 0.1 mM* [20]

### 3.3. Temperature and pH range

After temperature optimization in laboratory it was observed that isolated strains grew well under mesophilic condition in basal medium (Table 3). Culture of BC/CIMFR-ana-CH4-12 was found to sustain in a temperature range between 32-42°C while the optimum growth was recorded at 36°C. Strain BC/CIMFR-ana-CH4-14 was able to grow between 29-39°C and achieved fastest growth at 34°C. In addition, the pH is an important parameter for growth of microorganisms. Each bacterium is having a definite pH growth range and growth optimum [34]. Therefore, pH tolerance limit for isolates was found to be diverse. Strain BC/CIMFR-ana-CH4-12 thrived in a pH range 6.0-7.5 followed by optimal growth at pH 7.5. Further, the isolate BC/CIMFR-ana-CH4-14 tolerated pH range 6.5-8.5 and attained its fastest growth at pH 7.0.

Table 3. Optimal growth condition and biochemical characteristics of isolated strains

| Parameters | BC/CIMFR-ana-CH4-14 | BC/CIMFR-ana-CH4-12 |
|---|---|---|
| Temperature range (°C) | 29-39 | 32-42 |
| Optimum temperature (°C) | 34 | 36 |
| pH range | 6.5-8.5 | 6.0-7.5 |
| Optimum pH | 7.0 | 7.5 |
| Catalase test | - | - |
| Indole test | - | - |
| Lipase activity | - | - |
| Protease activity | - | - |
| Starch hydrolysis | - | - |
| Urea hydrolysis | - | - |
| Penicillin G | Resistant | Resistant |
| Streptomycin | Resistant | Resistant |
| Doubling time ($t_d$) | 14.005 | 13.702 |
| Specific growth rate (μ) | 0.021 | 0.021 |
| Generation time (g) | 32.255 | 31.556 |

### 3.4. Biochemical tests

Microorganisms play extremely versatile role to their lifecycle and their range of metabolic capacities are diverse [35]. The metabolic diversity of isolates was demonstrated by utilizing exceptional biochemical tests (Table 3). Accordingly, nitrogen metabolism was determined by means of iodole test and both strains were negative for the test, representing that they cannot act upon amino acid tryptophan due to the deficient in tryptophanase in their cell. Further, catalase and urease activity were also accounting negative for the isolates due to lack of that particular enzymes [19]. Additionally, the enzyme activity such as lipase, protease and starch hydrolysis were also considered for the study and none of the strain was found to be positive for those tests. Moreover, both strains were observed to be penicillin G and streptomycin resistant at a concentration of 0.5g/L, may be due to the diverse structure of their cell wall [36].

### 3.5. Growth kinetics

Microbial growth refers to increasing the cell number and it was performed in anaerobic basal medium supplemented with acetate under optimum growth condition. Consequence to this, growth kinetics was demonstrated as doubling time ($t_d$), specific growth rate (μ) and generation time (g), listed in Table 3. The isolates grew with a doubling time between 13.702-14.005 hours under optimal condition. The doubling time was 14 hours [37] and 11hours was reported for formate grown *Methanobacterium formicicum* [38]. Further, specific growth rate (μ) was recorded 0.021/hour for both strains. However, generation time (g) was noticed longer for isolated methanoarchaeal strains (31.556-32.255/hour). The specific growth rate of methanogenic archaea was reported as 0.049, 0.030, 0.023 and 0.021/hour which were variable according to the substrate composition [30]. Additionally, archaebacteria especially

methanogens are very slow growing organism [39]. Therefore, doubling time and generation time was found to be longer during the course of growth.

### 3.6. Membrane study

The membrane of strain BC/CIMFR-ana-CH4-12 was analyzed by FT-IR and the data were collected over the frequency range of 3600-400 $cm^{-1}$ in order to display the details of spectral changes (Figure 3). During study, the bands such as alkyl (1377, 1463, 2856, 2926 and 2952$cm^{-1}$), primary carbinol (1051$cm^{-1}$), ether (1115$cm^{-1}$) and hydroxyl functional groups (3450$cm^{-1}$) are very much identical in membrane spectrum [24]. Archaea especially methanogens characteristically possess isoprenoid branched ether linkethe d membrane lipids which were also identical [40].

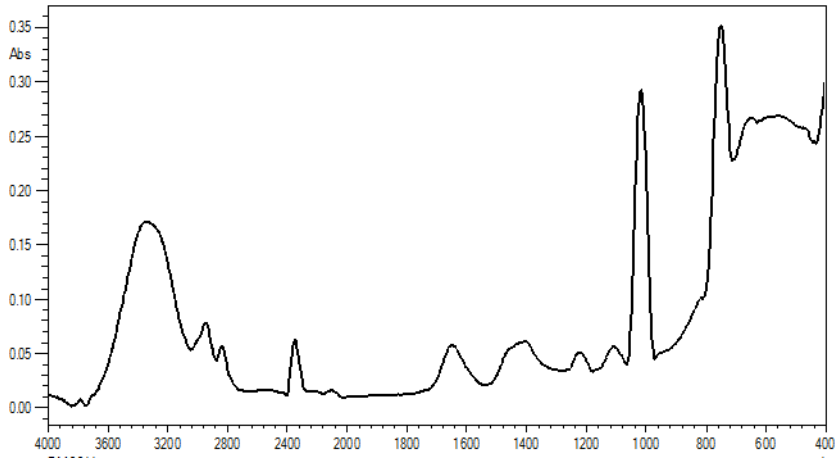


Figure 3. FT-IR spectra of isolated methanoarchaeal (BC/CIMFR-ana-CH4-12) membrane

### 3.7. Habitat characterization

Physico-chemical characterization of the environmental sample can influence the growth, behavior, interactions, and existence of the organisms where they residence that environment. During energy metabolism, they obtain nutrients from that particular habitat for the biosynthesis of cellular macromolecules [41-34]. Therefore, an attempt has been to understand the ecological significance of preferred archaeal habitat by exploration of different physico-chemical and microbiological parameters, presented in Table 4. Accordingly, the pH was found to be slightly basic in nature for both habitats. Further, EC was recorded 137 µS for Jitpur underground mines and 343.5 µS Moonidih underground mines. Carbon, nitrogen, hydrogen, phosphorus, and sulfur are the essential ingredients for biosynthesis of protein, nucleic acid, coenzymes and phospho-lipids [41]. Total organic carbon was measured at 3.13% and 9.55% for Jitpur and Moonidih underground mines. Comparatively, nitrogen content was found to be higher in Moonidih underground coal deposit. In addition, P and S were found to be rich in selected habitat for a cellular biosynthetic pathway in the archaeal cell. Further, Humic acid content was recorded nearly same for both chosen habitats which serve as a catalyst for microbial activity [42]. The element such as Fe, Ni, Co, Mo, Cu, Zn, W, Se, and B is vital for bacterial growth and metabolism [43-44]. Among them, Fe, Ni, Co, Zn, Cu, Mo, and W are very significant for enzymatic activity in the methanogenic pathway [44-46]. After analysis, it was experimental that Fe, Ni, Cu, Zn, B, and Se are sufficient to support methanoarchaeal growth in the selected environment. Microbiological data revealed that methanogenic isolates were found to associate with numerous aerobic and aerobic microbial linages in deep subsurface coal deposits. Further, actively microbial biomass carbon and basal soil respiration also reflect the availability of carbon for microbial maintenance in selected coal deposits [47].

Table 4. Physicochemical and microbiological characteristics of isolated methanoarchaeal habitat located in Dhanbad coal field

| Parameters | Habitat | |
|---|---|---|
| | Jitpur underground mines | Moonidih underground mines |
| pH | 8.22 | 8.15 |
| Electrical conductivity (µS) | 137 | 343.5 |
| Total Organic Carbon (%) | 3.13 | 9.55 |
| Total nitrogen (%) | 0.228 | 0.342 |
| Humic acid (%) | 0.0075 | 0.008 |
| Sodium (Na) | 30.612 | 22.286 |
| Phosphorous (P) | 75.608 | 91.052 |
| Potassium (K) | 7.756 | 6.350 |
| Calcium (Ca) | 31.053 | 36.651 |
| Magnesium (Mg) | 16.608 | 26.288 |
| Iron (Fe) | 72.009 | 61.026 |
| Zinc (Zn) | 0.124 | 0.153 |
| Copper (Cu) | 0.105 | 0.103 |
| Manganese (Mn) | 1.001 | 1.005 |
| Nickel (Ni) | 0.100 | 0.011 |
| Boron (B) | 0.030 | 0.031 |
| Selenium (Se) | 0.593 | 0.824 |
| Sulfur (S) | 48.356 | 41.299 |
| *Microbiological parameters* | | |
| Aerobic microbial count | $1.2x10^8$ | $9.5x10^7$ |
| Anaerobic microbial count | $2.0x10^7$ | $2.8x10^7$ |
| Dehydrogenase activity (µg TPF/g/h) | 10.7 | 10.7 |
| *Soil respiration:* | | |
| AMBC (mg/kg) | 31.13 | 62.26 |
| Total BSR (mg/kg) | 1.0 | 1.4 |
| BSR (mg/kg) | 484 | 396 |

*Data are taken as mean value of triplicate. Elements were expressed as ppm. Microbial count was expressed as CFU (Colony Forming Unit); AMBC- Actively Microbial Biomass Carbon; BSR- Basal Soil Respiration; TPF- Tri-phenyl fomazon*

## 4. Conclusion

The study confirms the presence of active methane generating archaeal lineage in underground coal deposits located in Dhanbad which have great ecological importance. Additionally, the archaeal assemblage was found to be associated with numerous aerobic and anaerobic microbial communities in coal bed as significant habitat. Based on the study isolated strains belonged to the genera of *Methanococcus spp.* and *Methanobacterium spp.* The research is also suggesting that there is intense methane generation activity through the microbial origin in a particular environment. Further, it is recommended for chemical measurements to stimulating the activity of existing methanoarchaeal consortia which can improve the understanding of these organisms in the environmental sample.

## Acknowledgments

The authors would like to thank the CSIR, Government of India for its financial support and Dr. P. K. Singh, Director of CSIR-CIMFR for permitting this paper to be published.

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