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An operative laboratory investigation of bioconversion route from waste coal to natural energy

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Abstract

Purpose: In the present research, the potential of reactivated consortium for the methane production consuming waste coal as a carbon source (1% w/v) in the modified media at mesophilic temperature (37 °C) was determined.

Methods: Media modification was conducted for the enhancement of methane production by selecting three different components from the two media, i.e., *Methanosprillium* sp. producing media (MSP) and methane-producing bacteria media (MPB). From MSP medium, $C_2H_2NaO_2$ (sodium acetate), KH_2PO_4 (potassium dihydrogen the phosphate), and $NaHCO_3$ (sodium bicarbonate) whereas from MPB medium; yeast extract, peptone, and NH_4Cl (ammonium chloride) were selected in the range of 0.5–2.5 (g/l). Analytical assay, i.e., Fourier transform infrared spectroscopy (FTIR), gas chromatography mass spectrophotometry (GCMS), scanning electron microscopy (SEM), and energy dispersive X-ray (EDX) were conducted. Further, compatibility study and pathogenicity was performed.

Results: In the present study, reactivated consortia was used therefore key components of the media were modified. In case of MPB medium, 2 g/l of yeast extract, 2 g/l peptone, and 1 g/l NH_4Cl showed the promising results; whereas for MSP medium, 1 g/l of KH_2PO_4, 0.5 g/l of $NaHCO_3$, and 1.5 g/l of $C_2H_2NaO_2$ were noted to be the suitable range for methane production. Analytical studies confirmed the presences of -OH and aliphatic groups which majorly belongs to alkane, alkene, and phenol derivative compounds whereas SEM and EDX studies delineated the active interaction of bacteria with coal particles and presences of carbon (C) as a major peak in untreated coal and absence of C peak in microbial treated coal. In addition, a compatibility study was performed and their successful results aid in the future approach of field implementation. Further, pathogenicity data indicated the non-virulent and non-toxic nature of the consortia.

Conclusions: The production of waste coal is one of the most problematic and common activities of the mining industry. They release toxic substances into the environment (water, air, and soil) and damage the local biodiversity. Therefore, the generation of biogenic methane from waste coal is an environmentally friendly approach to overcome this problem.

Keywords: Waste coal, Modified medium, Reactivated microbial consortia, Methane, Analytical methods

Introduction

Low calorific value coal-generated from the coal mining industries is identified as waste coal or low-grade coal. Sometimes these are considered as a discarded coal; they generally form piles near the industries and appear as dark hills or unproductive small mountains. Waste coal usually creates metal leaching problems such

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as iron, manganese, and aluminum in the water and further causes water pollution. It is also responsible for acid drainage. As these piles easily catch fire, they release toxic gases in the air and cause air pollution (www.energyjustice.net). Therefore, controlled production of methane from the waste coal is an economically valuable solution. Methane is one of the clean natural forms of energy which fulfills the need of many industries and households activity with less waste to the environment (Li et al., 2020). However, consistently expanding worldwide energy demands and limited fossil fuel sources has created enormous pressure for developing sustainable energy source for hydrocarbons (Gupta and Gupta 2014). Energy sources with low carbon emission, such as methane gas, are becoming important these days (Caposciutti et al., 2020).

Methane is usually trapped in the coal therefore its production becomes an alternative mode for the energy generation. Methane can be produced by thermogenic (abiogenic) and biological (biogenic) processes. Thermogenic, occurring in subsurface carbon deposits at early or late coalification stages by the thermal cracking whereas biological occurs usually at or near the earth's surface using microorganisms (Chena et al., 2017; Wang et al., 2019). Biogenic methane is the result of complex biochemical reactions by groups of bacteria and archaea during the decomposition of organic matter in the anoxic environment. Due to the complexity in process of biogenic methane production, the procedure was poorly understood, but still, they are pervasive in nature (Wolfe 1996). In the past few years, numerous researchers have investigated the biodiversity of microbes residing in the coal seams and coal beds. The reported bacteria mainly belong to the three functional different trophic groups: hydrolytic fermentative, syntrophic acetogenic, and methanogenic bacteria (Boone 1991; Ritter et al., 2015). Hydrolytic fermentative and syntrophic acetogens hydrolyze complex polymers (cellulose, polysaccharide, and protein) into monomers (fatty acids, sugars, amino acids, carbon dioxide, acetate, and hydrogen). These monomers are further utilized by methanogens to produce methane as depicted in Fig. 1 (Conrad et al., 1999; Davis and Gerlach 2018). Although, according to Enzmann et al. 2018, the universal mode of methane production is a hydrogen mediated reduction of carbon dioxide. Various, environmental (pH, salinity, temperature), and nutritional factors (inorganic and organic) can affect the process of methanogenesis (Boone 1991).

It has been reported that the rate of methane production depends on the maturity and functional microbial communities present in the coal. Configuration-wise, coal consists of condensed aromatic ring which makes it a complex and heterogeneous material. Lignin monolignols were considered as the main compound, whereas aromatic compounds considered as a derivative of coal which can further be substituted with hydroxyl, methoxy, and carboxyl groups. According to Mayumi et al. 2016, immature coals were commonly abundant in the methoxy groups. Since methanogenesis from coal tends to occur in immature coal rather than in mature coal, it was believed that coal-bed microorganisms may produce methane from methoxy groups (Rathi et al., 2015). Unlike coal mining, which required mechanical methods of extraction and processing, biogenic methane production is one of the conventional methods and found to be economically viable and environment-friendly.

In the present study, we proposed an approach for (1) the development and demonstration of the bioconversion process for the generation of methane from waste coal received from Tata Steel Jamshedpur, India. (2) To

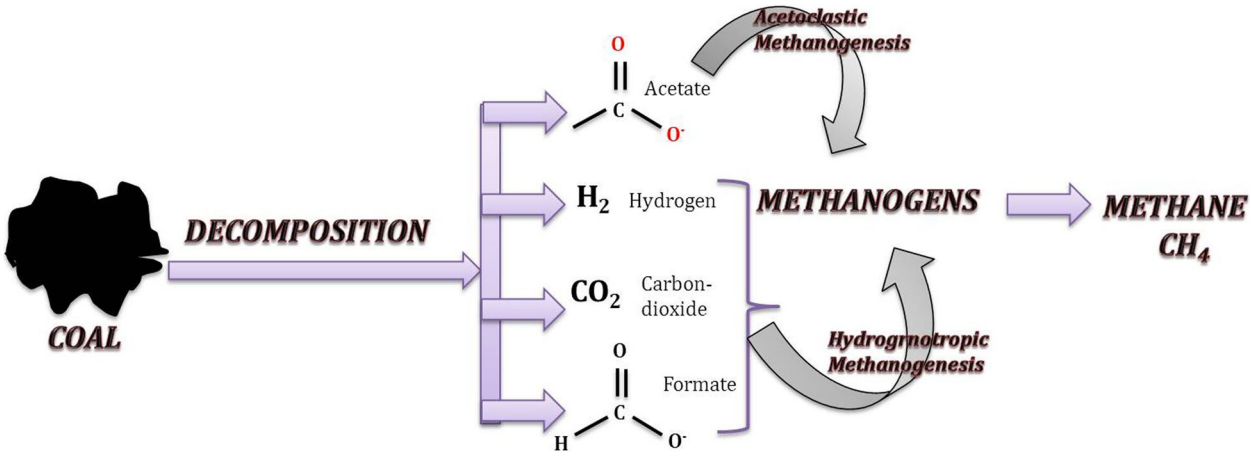

Fig. 1 Pictorial representation of microorganism interactions in the bio-conversion process of coal to methane

study the potential of developed consortia by modifying nutrient media (MSP and MPB) further, the analytical parameter of coal examination was conducted using FTIR and GCMS, followed by SEM and EDX techniques, and (3) pathogenicity assay and compatibility study were conducted. This study would help in proposing the suitable strategy for methane generation and possible future approach (Fig. 2).

Methods

Sample collection and characterization

In the present study, waste coal was received from Tata Steel, Jamshedpur, India. Sampling was performed in sterilized bottles and stored at ambient temperature and further transported to the laboratory (The Energy and resources of Institute, New Delhi). The characterization of waste coal was conducted in terms of ash, moisture, volatile matter, and fixed carbon along with the specific carbon, hydrogen, nitrogen, sulfur, and oxygen (CHNSO). CHNSO analysis was determined using IS: 1350American Public Health Association guideline (Rathi et al., 2019).

Enrichment and modification of media

Initial optimization studies were based on one factor at a time analysis for enhanced methane production. In the modification studies, yeast & peptone (as a growth agent), KH_2PO_4 and $NaHCO_3$ (as a buffering agent) and NH_4Cl, CSL, and urea (as a nitrogen agent) were selected. And for the enhancement in methanation $C_2H_2NaO_2$ was added to the study.

To study the potential of developed consortia at mesophilic condition, four enrichment cycles were performed in two different media specific for different species of methanogens. MSP (*Methanosprillium* sp.) and MPB (methanogen specific). Components like KH_2PO_4, $C_2H_2NaO_2$, and $NaHCO_3$ belongs to MSP medium and components peptone, yeast, and NH_4Cl belongs to MPB medium. Detailed study based on urea and CSL was conducted (data presented in Supplementary). After

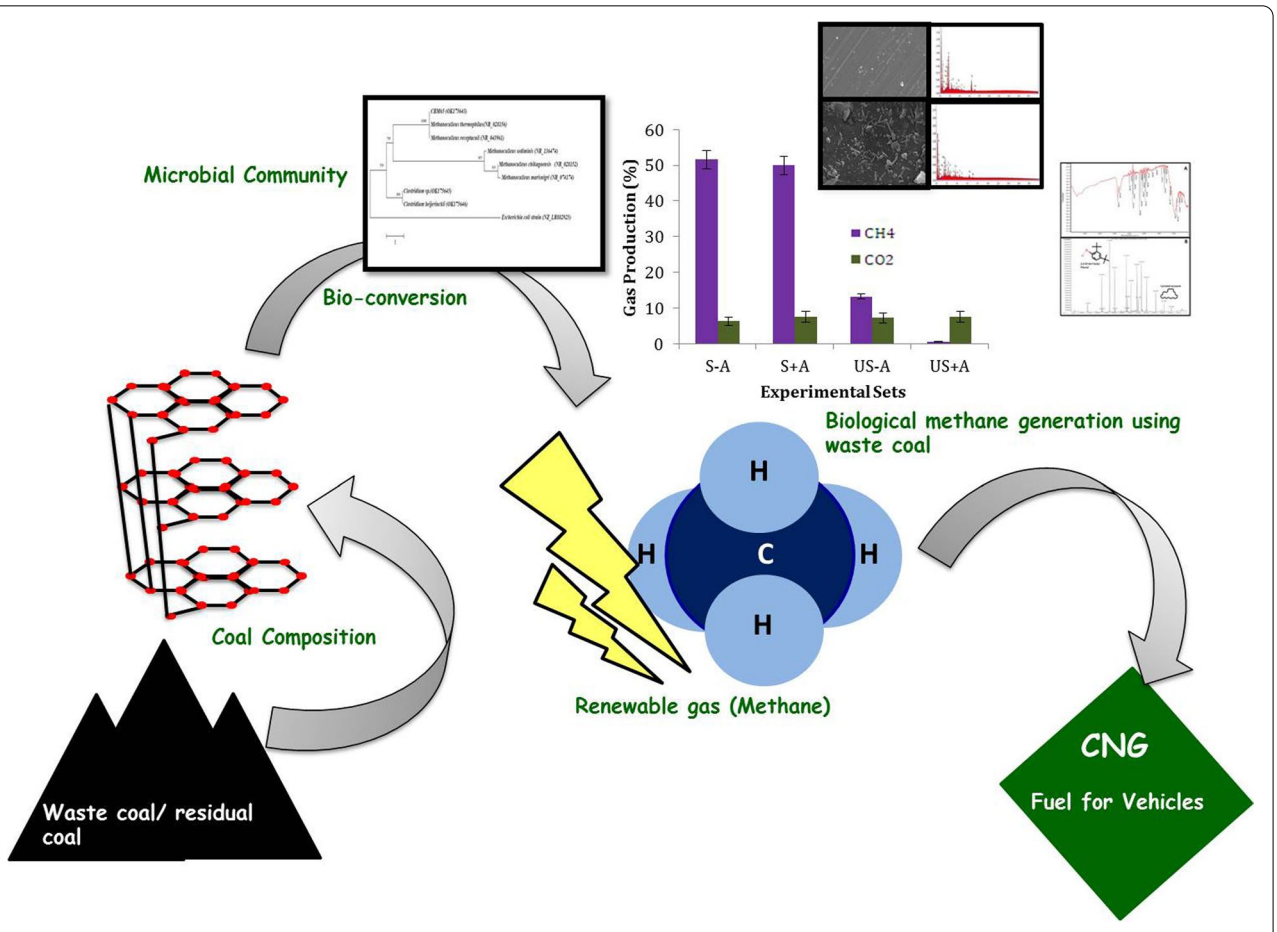

Fig. 2 Schematic demonstrations of bio-conversion process of microorganism interactions with coal and generation of methane

obtaining the best range for the selected components optimized media was used for the further studies.

The MSP medium contained (g/l in de-ionized water): KH_2PO_4, 0.5 g; $MgSO.7H_2O$, 0.4 g; NaCl, 0.4 g; $CaCl_2.2H_2O$, 0.05 g; $FeSO_4.7H_2O$, 0.002 g; yeast extract, 1 g; $C_2H_2NaO_2$, 1 g; sodium formate, 2 g; $NaHCO_3$, 4 g; resazurin, 0.001 g; and L-cysteine HCl, 0.5 g at pH 7.00 ± 0.2 (Lavania et al., 2014). The composition of MPB medium (g/l in de-ionized water) was K_2HPO_4, 0.3 g; KH_2PO_4, 0.3 g; NH_4Cl, 0.5 g; $MgSO_4.6H_2O$, 0.2 g; NaCl, 1.0 g; yeast extract, 1.0 g; casein peptone, 1.0 g; resazurin, 0.001 g; and L-cysteine HCl, 0.5 g at pH 7.00 ± 0.2. The pH was adjusted with 1 M NaOH/1 M HCL. The medium was then boiled under a stream of oxygen-free nitrogen gas to remove all the dissolved oxygen. After cooling under continuous nitrogen flow, the medium was dispensed into 100 ml serum bottles containing 1% w/v of waste coal (used as a carbon source). The bottles were sealed with butyl rubber stoppers and sterilized at 121 °C for 20 min. All the experiments were performed in triplicates, the inoculated serum (10% inoculum) bottles were incubated at 37 °C for 15–20 days.

For maximum production of methane, media modification studies were performed in which three components from two media (MSP and MPB) were selected in a range of 0.5–2.5 g/l. Ingredients from MPB medium were yeast extract, peptone, and NH_4Cl and from MSP medium; sodium acetate, KH_2PO_4, and $NaHCO_3$ were considered. Test range for ingredients was varied from 0.5, 1.0, 1.5, and 2.0 to 2.5 g/l. The medium was boiled under the inert environment (using nitrogen gas). Inoculated coal bottles were kept at 37 °C, and gas was monitored in 5th, 10th, 15th, and 20th day. Further, to study the effect of different nitrogen source on methane production CSL (corn steep liquor) and urea was also used. Modified medium contained (g/l in de-ionized water): KH_2PO_4, 1 g; NH_4Cl, 1 g; $MgSO_4.6H_2O$, 0.2 g; NaCl, 1.0 g; yeast extract, 2 g; Peptone, 2; $NaHCO_3$, 0.5 g; Sodium acetate, 1.5 g; resazurin, 0.001 g; and L-cysteine HCl, 0.5 g at pH 7.00 ± 0.2. Resazurin was added as an oxygen indicator (resazurin has a pink color at redox potentials of about150 mV). The pH was adjusted with 1 M NaOH/1 M HCL. The media was prepared anaerobically through nitrogen sparging. The medium was used for bacterial reactivation and scale-up analysis.

Reactivation of developed consortia (reactivated consortia)

To study the efficiency of developed consortia, reactivation was conducted in the modified medium with 1% waste coal (w/v). To reactivate methanogens, an aliquot of developed consortia (5 ml) was added in 10 ml of the modified medium. Further, after obtaining 0.5 MacFarland standard turbidity of bacterial growth which was equivalent to 1.5×10^6 CFU/ml, subculturing was performed for inoculum preparation which was considered as reactivated consortium (Wayne 2003). All the inoculated serum bottles were incubated at 37 °C for 15–20 days.

Microbial community present in reactivated consortia

To identify the enriched/isolated microbial community from developed consortia, total genomic DNA was extracted and purified using a PowerSoil DNA Isolation Kit (MoBio) as instructed in the manufacturer's protocol. PCR amplification was done with universal bacterial primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-ACG GCT TAC CTT GTT ACG CTT-3′) as well as archaeal primers 109f (5′-ACK GCT CAG TAA CAC GT-3′) Met 915R (3′GTG CTC CCC CGC CAA TTC CT-5′) (Lavania et al., 2014). For gene sequencing, PCR product was outsourced (AgriGenome Labs Pvt. Ltd). Following quality check of the FASTQ files using FastQC (v0.11.9), the sequencing data files were analyzed using DADA2 package (version 1.14.0) which included quality filtering, trimming of barcode/adaptors, dereplication, learn error rates, and chimera removal, merging of paired reads. The SILVA version 138 16S rRNA gene reference database was used to assign bacterial taxonomic classification. The phylogenetic tree was constructed using the neighbor-joining method in MEGA (version 6.06) package. The tree topologies were estimated with 1000 bootstrap data sets. The similarity value used for the identification of microbial population was of 97%, from the assessed microbial consortia (Fuertez et al., 2018).

Analytical analysis of sample

Fourier transform infrared spectroscopy (FTIR)

FT-IR was carried out to identify the functional groups present in the bacterially degraded coal sample. Functional group was characterized by using Fourier transform infrared spectroscopy (Perkin Elmer). All spectra were recorded in an absorbance scale with a mid-measuring region of 400–4000 cm^{-1} (mid-infrared range). The resolution was set at 4 cm^{-1} with 64 scans per spectrum.

Gas chromatography (GC)

In the present analysis, concentration of gas produced in the headspace (methane and carbon-dioxide in %) of media bottles were analyzed with GC 7890A Agilent Ltd. USA equipped with a packed stainless steel column (2 m × 2 mm id NUCON, India) with a thermal conductivity detector (TCD), where argon acts as the carrier gas with flow rate of 1.0 ml/min. The operating temperatures of the injection port, oven, and the detector were 100, 50,

and 150 °C, respectively (Rathi et al. 2015). The incubated cultures were tested for CH_4 and CO_2 production after 15–20 days by taking 0.5 ml of headspace gas samples from the anaerobic serum bottles using gas-tight syringe.

Gas chromatography mass spectrophotometry (GCMS)

The sample was analyzed using GCMS (model GC-7890A, Agilent Ltd., United States) equipped with DB-WAX capillary column. Helium was used as the carrier gas. Temperature ranges between 230 and 325 °C. Initially, column temperature was set at 70 °C and further increased to 325 °C. Diluted sample (1/50 in methanol) of 0.1 µl was used. The components were identified on the basis of their mass spectra using NIST (National Institute for Standards and Technology) library data base.

Scanning electron microscopy (SEM)

Interactions between bacterial species and coal were studied by Scanning Electron Microscopy (Carl Zeiss) (Hayat 2000). Under aseptic conditions, sample was absorbed for 2 to 4 h in 2.5% glutaraldehyde solution. 0.1 M phosphate buffer was used for primary washing where pH maintained up to 7.2 further sample was dehydrated with ethanol solution in a series of 10–100% followed by acetone. Samples were air-dried overnight and coated with thin layer of metal (gold and palladium).

Energy dispersive X-ray (EDX)

The energy dispersive X-ray (EDX) is a known technique for detecting elemental present in the specimens. The X-ray revealed the true nature of the test sample. For the present study, the coal with or without treatment with bacteria was carried out in the Bruker X Flash 630 EDS detector using DX-700HS spectrometer (Shimadzu).

Pathogenicity test

The pathogenicity test of reactivated consortia was examined by acute oral toxicity under EPA 712-C-96-322 OPPTS 885.3550 guidelines at the National Toxicology Centre (APT Testing and Research Pvt. Ltd.), Pune. Twelve mice (6 males and 6 females) were designated to the dose groups: control and test ($1\,ml = 1.0*10^8$ CFU) were administrated by the gauge to six mice per sex. The mice were fasted overnight and 2 h after administration of the test material.

The mice were observed for 21 days after dosing. At the end of the inspection period, the surviving experimental animals were sacrificed for testing. Gross necropsy was performed and all animals were carefully examined for the presence of anaerobic bacteria. The body weight was recorded. All animals were observed for mortality throughout the observation period. RBC (red blood cell), WBC (white blood cell), hemoglobin, packed cell volume, glucose, BUN (blood urine nitrogen), total proteins, and albumin were studied on the 21st day of the experiment.

Compatibility studies

Before field implementation test, compatibility studies were conducted in the lab. In this analysis, obtained tube well water was used for media preparation (available near the washeries in Jharia). Experiment was conducted in four sets; in set 1: anaerobic condition was maintained without autoclaving (referred as S-A), in set 2: anaerobic condition was maintained with proper sterilization (referred as S + A), in set 3: aerobic condition without autoclaving (referred as US-A), and in set 4: aerobic condition with autoclaving (referred as US+A). While preparing the media, no precipitation was observed with commercial grade of chemicals in tube well water. In all sets, waste coal (1% w/v) was used. After inoculating with inoculum (10%), all sets were incubated at 37 °C for 15–20 days.

Statistical analysis

All the experiments were performed in triplicates. The data points are average of the triplicate ± standard deviation (less than5% of average) and calculated significance *p* values are ≤ 0.05.

Results

Coal characterization

The detailed analysis of collected waste coal samples in terms of ash, moisture, volatile matter, and fixed carbon along with the specific carbon, hydrogen, nitrogen, sulfur, and oxygen (CHNSO) was determined as per the guidelines of ASTM standard (Table S1) (Rathi et al., 2015). Proximate analysis data showed that waste coal contains 0.49% of moisture, 14.85% of volatile matter along with high 42.52% of ash, and 42.14% of fixed carbon. The calorific value of waste coal was 4092 kcal/kg. Obtained data of waste coal indicated the significant potential in the bioconversion process of methane. The ultimate analysis of the waste coal samples showed 44.71% of carbon, 2.55% of hydrogen, 0.04% of nitrogen, 0.28% of sulfur, and 9.41% of oxygen.

Enrichment studies

Figure 3 illustrated the gas production (methane and carbon dioxide) in all four successive enrichment cycles in both the specific media (MSP and MPB) at 37 °C. However, in the MSP medium, an increase in methane generation was observed from 9.99 to 27.4% in 1st and 4th enrichment cycle respectively, whereas carbon-dioxide was decreased from 5.2 to 4.9%. Further, $CH4{:}CO_2$ (methane to carbon-dioxide), the ratio was ranging from 1.8 to 5.5. Similarly, in the case of MPB medium, rises in

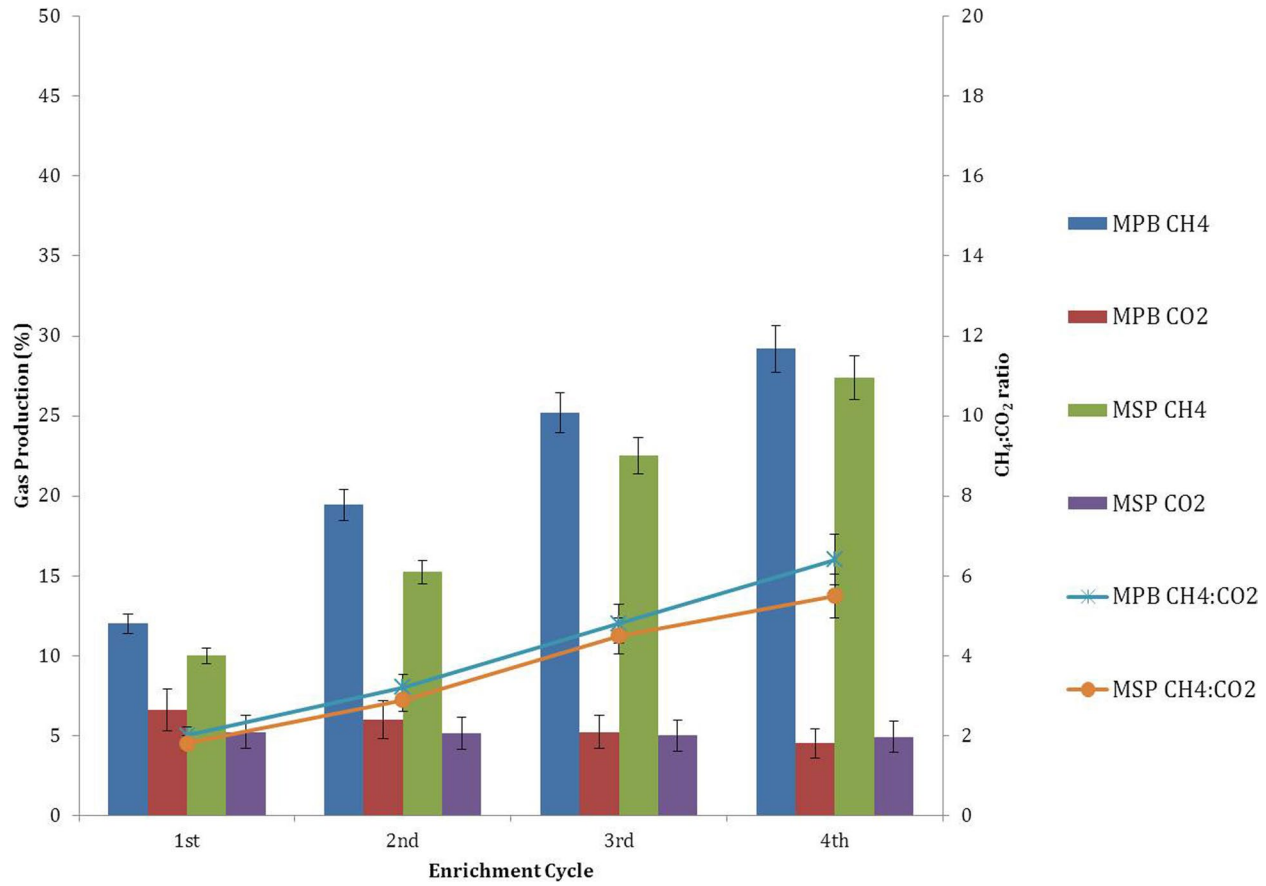

Fig. 3 Explained the percent of gas generation by developed consortia in MSP and MPB medium respectively where waste coal was used as carbon source in various enrichment cycles. Data recorded after 20 days of incubation

methane generation were noted from 12 to 29.2% and reduction in carbon-dioxide from 6.6 to 4.5% in the 1st and 4th cycles, respectively. Also, CH4:CO_2, the ratio was ranging from 2.0 to 6.4. In the developed consortium, both media showed almost the similar trends in methane production (29.2% in MPB and 27.4% in MSP).

Modification of nutrient media

Methane production by consortia was tested with different sets of MPB and MSP media. Gas was monitored at interval of 5 days during incubation period.

Figure 4 data demonstrated the methane production in selected range of components form MPB medium in with and without coal sets. Selected components were peptone (Fig. 4A), yeast extract (Fig. 4B), and NH_4Cl (Fig. 4C) in a range between 0.5, 1.0, 1.5, 2.0, and 2.5 g/l with waste coal (1% w/v). In this experiment, it was observed that methane was increased up to a concentration after that production was not supportive. In case of yeast extract and peptone, 2 g/l was obtained to be preeminent concentration for methane production (42.3% and 35.23%, respectively), whereas in NH_4Cl, 1 g/l was showed the respectable result with 38.1% of methane after the 20th day of incubation. Figure 4D depicted methane generation in set without coal (control). Methane was observed at the 20th day of incubation. By comparing the data of with and without coal, it was noted that methane generation was more in case of set containing coal.

Further in MSP medium (Fig. 5), 1 g/l of KH_2PO_4 (Fig. 5A), 0.5 g/l of $NaHCO_3$ (Fig. 5B), and 1.5 g/l of $C_2H_2NaO_2$ (Fig. 5C) indicated the suitable range for methane production with 23.34%, 23.45%, and 34.22%, respectively, after the 20th day of incubation. Figure 5D showed the methane production without coal (control) after the 20th day. As the range increased for selected components, the methane production was increased up to particular range beyond which it seems to be not favorable. Data obtained in with and without coal sets confirmed that a significant amount of coal converted into energy which was not in the case of set without coal. With coal, methane generation was observed to be more. Further, in modification study, impact of different

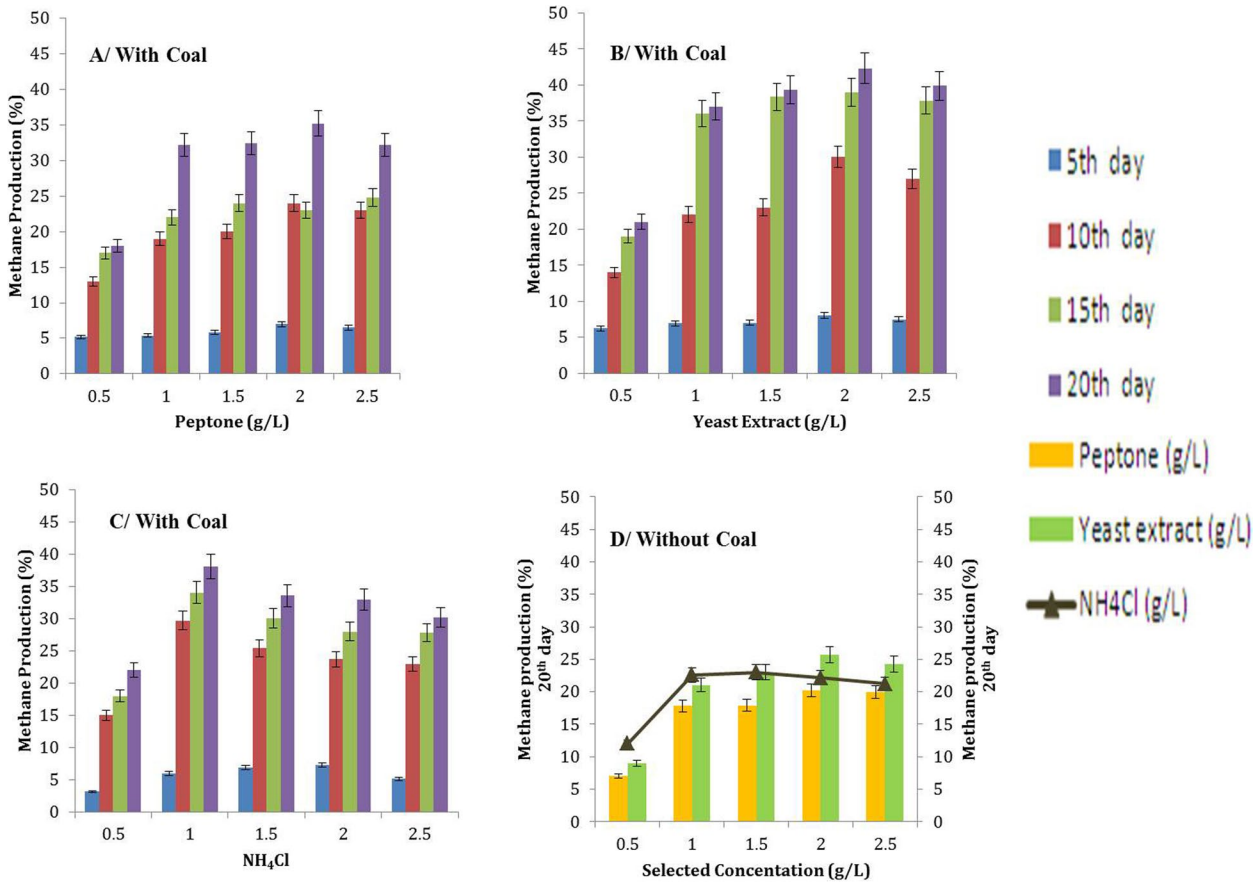

Fig. 4 **A**–**C** The methane gas production in peptone, yeast extract, and NH_4Cl selected components from MPB medium in range of 0.5–2.5 g/L with coal (1.0% w/v), whereas **D** depicted the methane production without coal

nitrogen source on methane generation was studied using CSL and Urea components. Obtained data was not favorable for methanogenesis as represented in Fig. S1.

Identification of microbial community present in reactivated consortia

The phylogenetic studies of reactivated CBM65 were studied, and it was observed that it shows the resemblance with published manuscript (Lavania et al. 2014). Both bacterial and archaeal domain were noted, and the tree was constructed (Fig. 6). The phylogenetic profile indicated that the consortium contained mixed phyla of anaerobic archaeal and firmicutes. These are available under the accession numbers OK175643, OK175645, and OK175646.

CBM65 shows maximum similarity with archaeal phylum containing *Methanoculleus thermophiles* and *Methanoculleus receptaculi* and minimum with *Methanoculleus sediminis*, *Methanoculleus chikugoensis*, and *Methanoculleus marisnigri*. Furthermore, bacterial domain was also noted in reactivated consortia which were comprised of fermicutes (*Clostridium* sp. and *Clostridium beijerinckii*). The obtained microbial community was able to generate methane at mesophilic condition.

Analytical analysis of sample (FT-IR and GC-MS)

In order to understand the functional groups present in the coal sample, FITR analysis was performed as shown in Fig. 7A. Spectra fragment lies between 1600 and 1800 cm^{-1} was attributed to aromatic group (C=C). Further the stretching of 3000–2800 cm^{-1} corresponded to -OH and -NH groups. The presence of aliphatic groups (methyl and methylene) illustrated by spectra range from 2400 to 2000 cm^{-1}. Smaller peaks beyond 1600 cm^{-1} were represented the presence of mineral compositions in the sample (Damin et al., 2010; Zhou et al., 2014; Singh and Zondlo, 2017).

Previous reports stated the presence of low molecular weight hydrocarbons in coal sample, where GCMS plays the important role in identifying those compounds (Fan et al., 2013). The organic moiety present in coal was observed to be cyclic and acyclic hydrocarbons mainly alkanes, alkenes, and

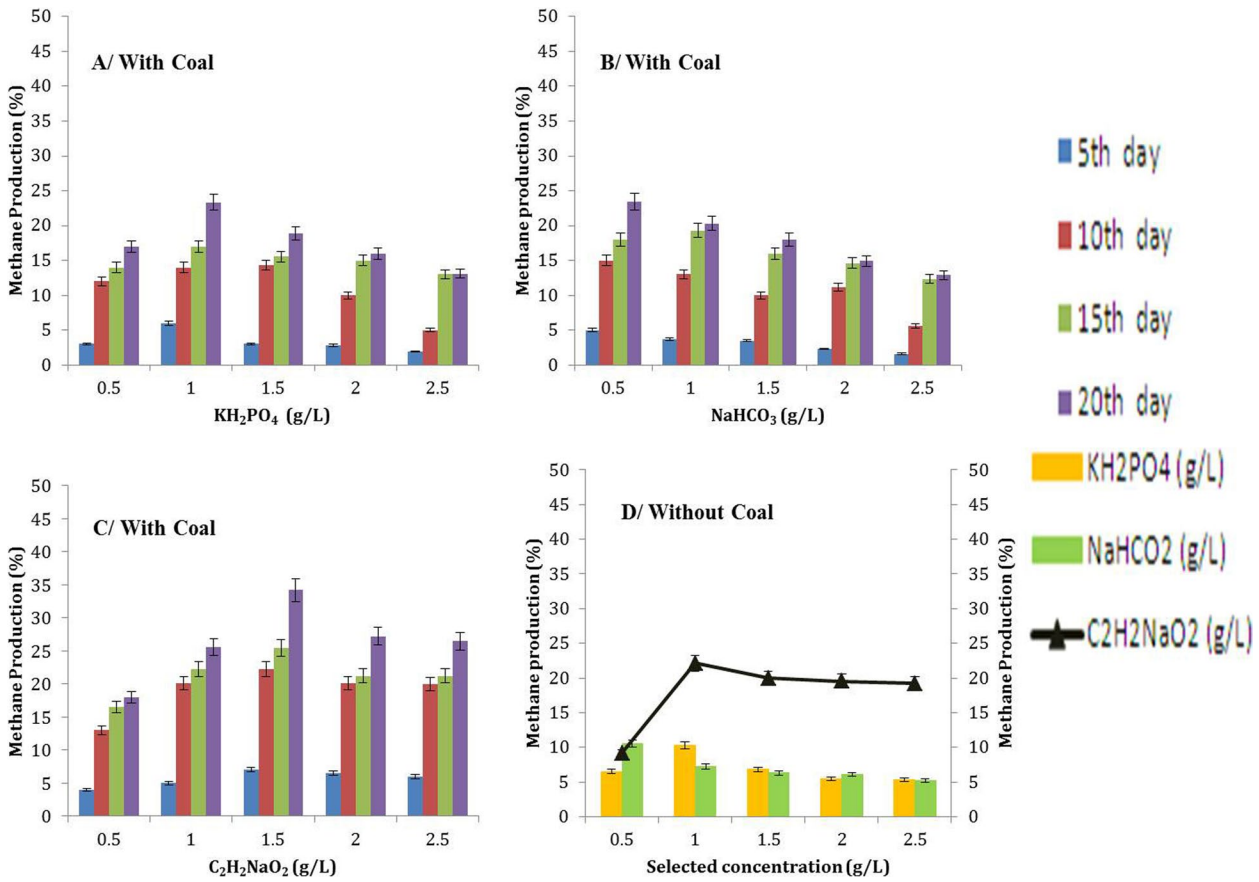

Fig. 5 **A**–**C** The methane gas production in KH_2PO_4, $NaHCO_3$, and $C_2H_2NaO_2$ selected components from MSP medium in range 0.5–2.5 g/L with coal (1.0% w/v), whereas **D** depicted the methane production without coal

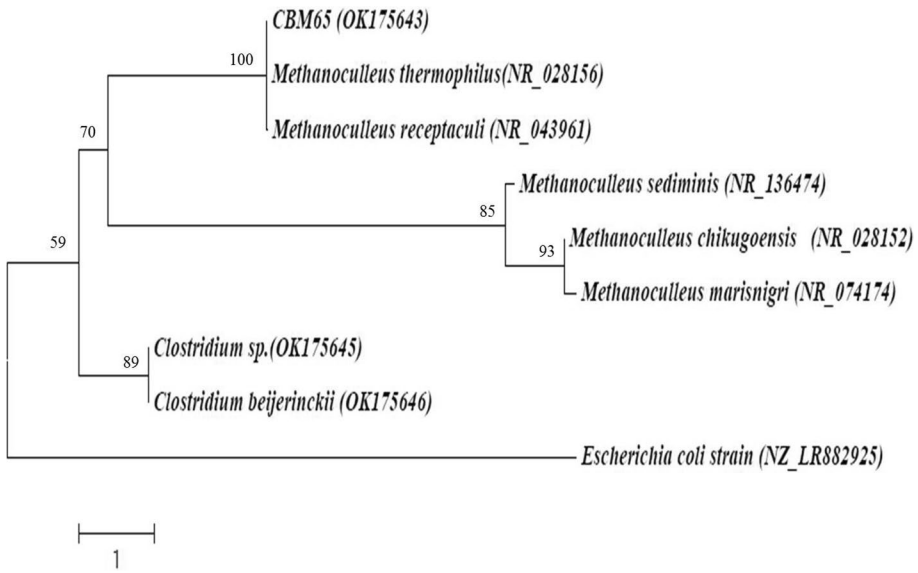

Fig. 6 The phylogenetic tree of the consortium was constructed using neighbor-joining. The tree topologies were estimated with 1000 bootstrap data sets. The tree shows the relationship within the archaeal and bacterial domain. GenBank accession numbers are provided in brackets. The scale bar indicates sequence divergence

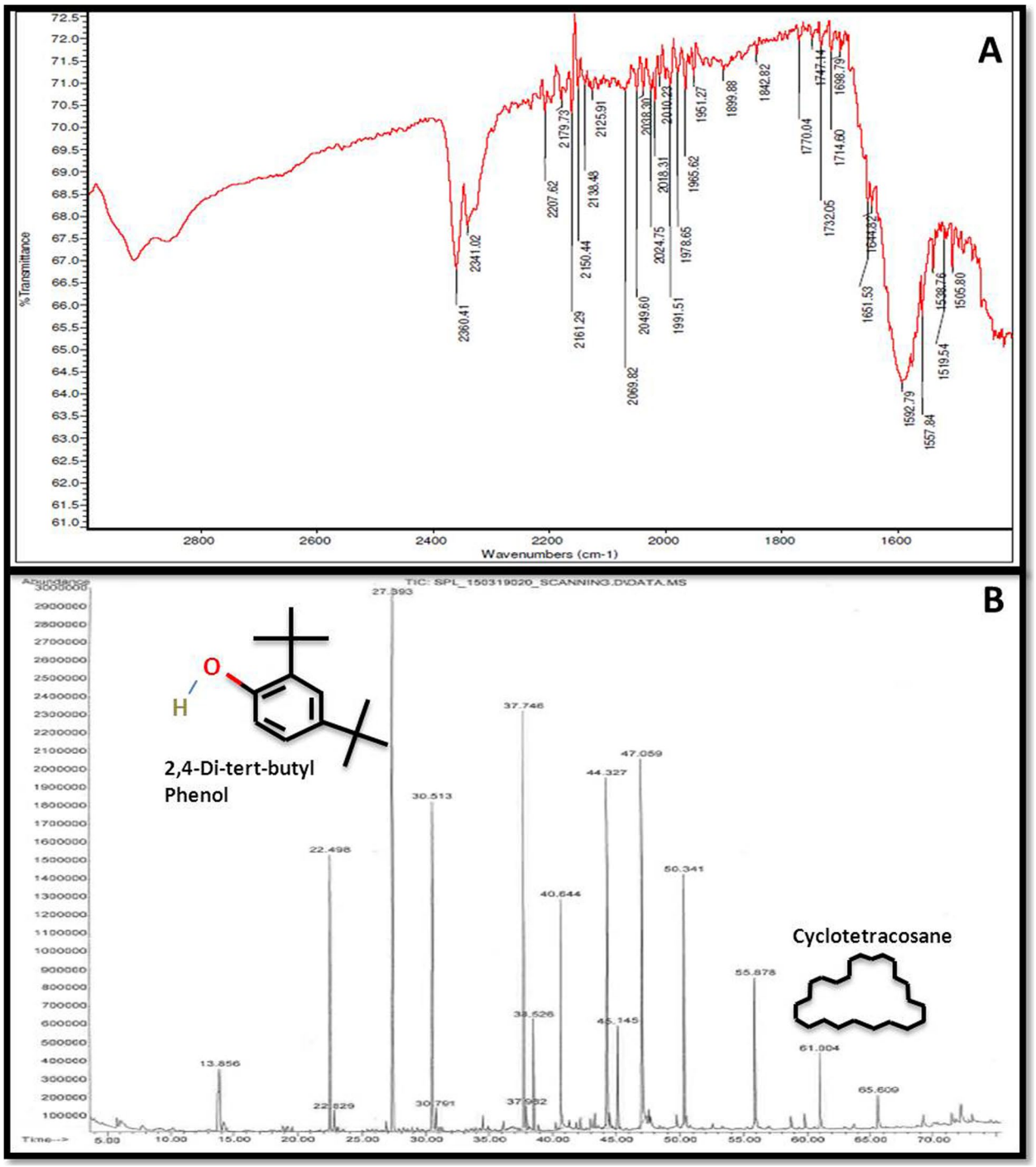

Fig. 7 **A** The FTIR spectra of coal sample. **B** The fragmentation pattern of coal sample

derivatives of phenols (Table S2). Figure 7B illustrated the fragmentation pattern of compounds present in the sample. Major peaks were recorded at RT 27.39, 37.74, 44.32, 47.05, and 61.00 which corresponds to 2,4-Di-tert-butyl phenol, 5-Octadecene, E-15 Heptadecenal, 1-Nonadecanol, and Cyclotetracosane, respectively (https://pubchem.ncbi.nlm.nih.gov/compound/2_4-Di-tert-butylphenol).

Scanning electron microscopy (SEM) and energy disruptive X-ray (EDX)

SEM micrographs depicted the coal particles were destructed and reduced in sized after the bacterial intervention (Fig. 8A, B). Figure 8A shows the intact coal piece without any bacterial treatment whereas the 8B has bacteria with coal particles. The reduced sized coal provided the large surface area which makes adherence easy for the bacteria. Morphology of bacteria and bacterial interaction with coal particles were clearly visible in Fig. 8b, where red arrows indicated the presence of bacteria, white arrows showed the coal particles and yellow circle illustrated the bacterial interaction with coal. Mixed natures of anaerobic bacteria were noted, where rod and coccus shaped were observed and bacteria in clusters were also recorded. By observing EDX graphs of coal and bacterial treated coal, major peaks were found to be missing. In coal samples, peaks for carbon (C), oxygen (O), silicon (Si), iron (Fe), titanium (Ti), magnesium (Mg), sodium (Na), sulfur (S), potassium (K), phosphorus (P), calcium (Ca), and aluminum (Al) were noted whereas in case of after bacterial treatment peaks of C and O were not observed (Fig. 8C, D).

Pathogenicity assessment

Reactivated consortia (1 ml of dose) did not show any case of mortality in the treated mice (both male and female). All the mice were appeared to be normal and showed no clinical signs of intoxication after dosing till the end of the study. No statistically significant difference in the hematological and blood chemistry parameters (red blood cells, white blood cells, hemoglobin, packed cell volume, BUN, albumin, total protein, and glucose) was observed in the test group. After evaluating the test groups with the control group, there were no numerically decreased in body weight was observed. The results from

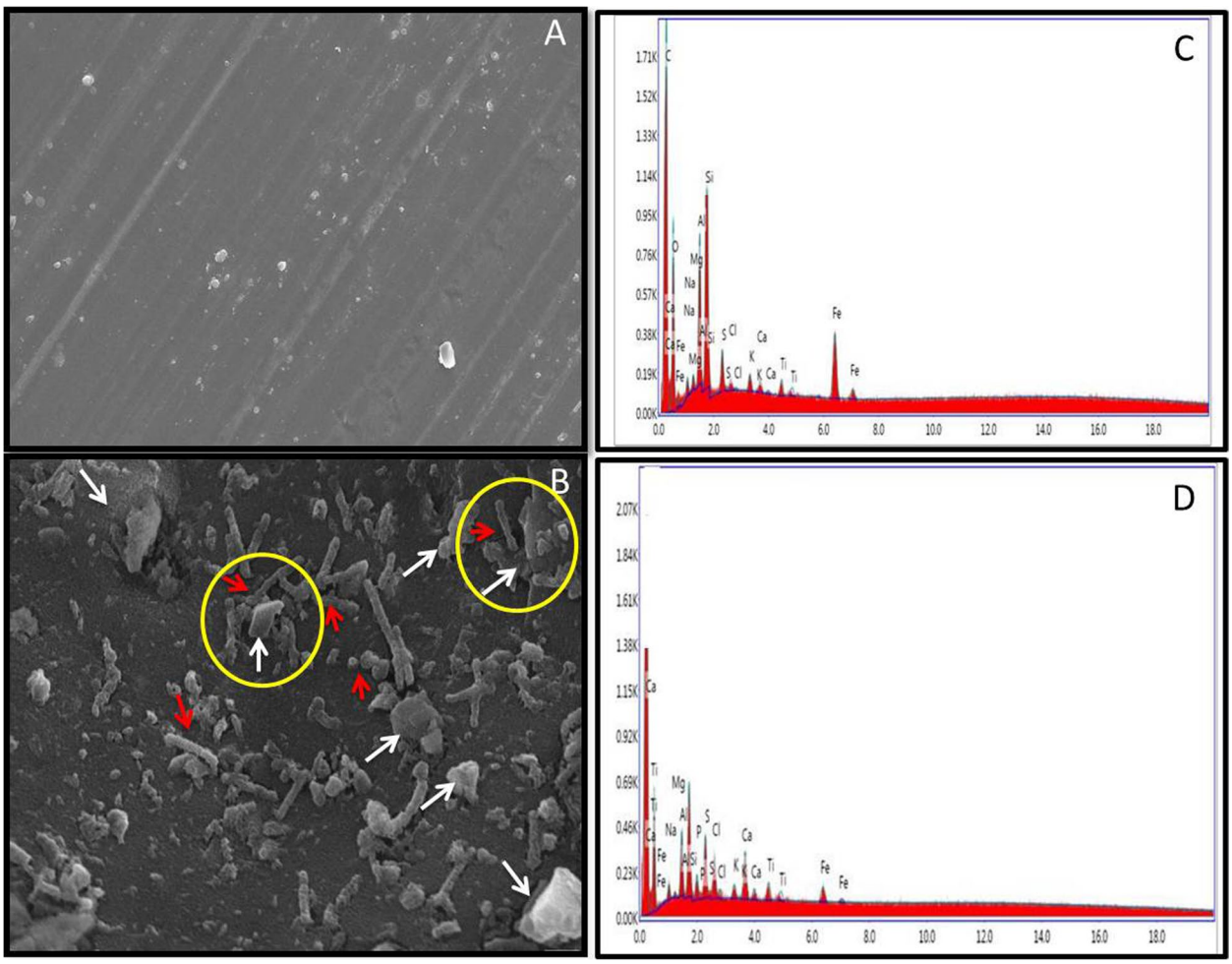

Fig. 8 **A** The untreated (before bacterial treatment) coal particles (scale bar 2 µm). **B** The treated (after bacterial treatment) coal particles (scale bar 2 µm)

the necropsy revealed no abnormalities in the test group when compared with the control group animals. The consortia did not induce any gross pathological alterations, in experimental models during their necropsy. The sacrificed mice's were thoroughly examined and were found to be completely free from any live anaerobic bacteria. After analyzing the data, reactivated consortia were considered to be non-toxic and non-virulent. Hence, it is safe for field implementation (Table S3–S6).

Compatibility studies

Compatibility study of waste coal in modified media with reactivated consortia was executed as represented in Fig. 9. Methane and carbon-dioxide were observed in all experimental sets with different percentage. In set 1 and set 2, significantly high amount of methane and low amount of carbon-dioxide was noticed with 51.6%, 49.91 methane, and 6.25%, 7.61% CO_2, respectively. In set 3, methane was found to be significantly reduced which was 13.2% with 7.21% of carbon dioxide, whereas in set 4, negligible amount of methane (0.54%) was observed with 7.56% of carbon dioxide.

Discussion

Bio-conversion of coal to methane can be considered as a healthy and feasible approach for the environment (Fig. 2), as studies reported by Ge et al. (2016), (Ribeiro et al. 2012), and Hao et al. (2016) suggested the toxic nature of waste coal piles generated near the industries during coal mining. Therefore, through this study recovery of significant methane was observed using waste coal.

In the present research, microbes from the developed consortia were reactivated and further used as a source for biogenic methane production. Figure 3 showed the production of methane gas (29.2% in MBP and 27.4% in MSP) along with the carbon-dioxide (5.2% in MBP and 6.6% in MSP). Therefore, to maintain the composition of gas (majorly methane) modification studies were conducted by selecting two specific media (MPB and MSP). In MPB medium concentration of yeast extract, peptone, and NH_4Cl and in MSP medium concentration of

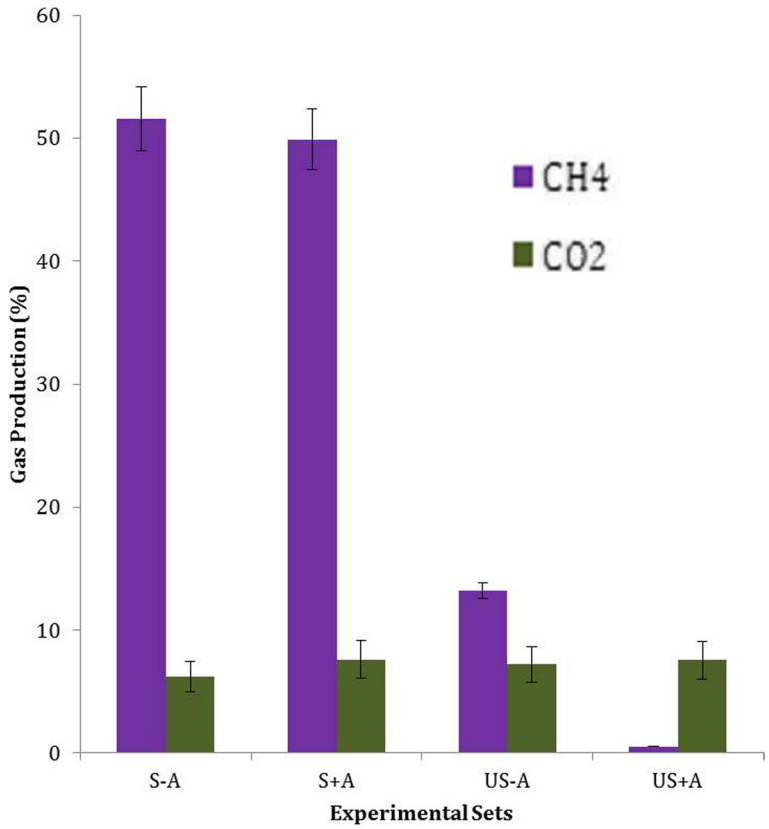

Fig. 9 The compatibility test in modified media with waste coal and tube well water (Set 1: S-A, where S signifies nitrogen sparge media (anaerobic) and -A represents without autoclave media; Set 2: S + A, where S signifies nitrogen sparge media and + A represents Autoclave media; Set 3: US-A, where US signifies without sparging media (aerobic) and -A represents without autoclave media; Set 3: US+A, where US signifies without sparging media (aerobic) and + A represents autoclave media

$C_2H_2NaO_2$, KH_2PO_4, and $NaHCO_3$ were altered. Modification provided promising results for methane generation in the scale-up analysis.

Each selected component plays a vital role in the methanation process as depicted in Figs. 4 and 5. Selected components from the MPB medium were yeast extract, peptone, and NH_4Cl which behaves like a common complex and defined growth and nitrogen source for the microorganism. Previous studies have been examined for their potential to enhance coal-to-methane conversion (Verstraete et al., 1984; Wagner et al., 2012; Davis et al., 2018). The preceding researches also investigated urea and CLS (corn steep liquor) compounds as a respectable nitrogen source (Yang et al., 2014; Tan et al., 2016). But in this investigation, yeast extract, peptone, and NH_4Cl showed promising results (Fig. 4) whereas urea and CLS were not found to be that effective (Fig. S1). In MSP medium, $C_2H_2NaO_2$, KH_2PO_4, and $NaHCO_3$ were elected. According to Ulrich & Bower 2008 study, $C_2H_2NaO_2$ was considered as an essential ingredient for methanogenesis. Furthermore, pH also plays an important role in the methanation process. And with the proper buffering system, optimized pH can be achieved (Gupta and Gupta 2014; Yang et al., 2018). KH_2PO_4 and $NaHCO_3$ were considered as the chief components in maintaining the pH of the medium (Eduok et al., 2018). As the selected components of MPB and MSP media had a significant role in the methane generation process, they were varied in a certain range (0.5–2 g/l) for the modifying study. The reactivated consortium showed the highest methane production at 37 °C in the modified medium when waste coal was used as a carbon source. By comparing Figs. 3, 4, and 5, noteworthy differences in methane generation were noted. In the case of MBP and MSP media, the methane production was observed to be 29.2% and 27.4%, respectively (Fig. 3), whereas in modified medium methane generation was in the range of 40–50%. These results prove that the nutrient amendment was a successful strategy for methane production.

Figures 4 and 5 data also illustrate the importance of coal in the medium. By observing with coal (Figs. 4A–C and 5A–C) and without coal (Figs. 4D and 5D) data sets, maximum production of methane after the 10th day of incubation was noticed in sets having coal. This study emphasized the importance of the methane production in a low incubation period in comparison to the previous literature on waste coal showed more than a month of the incubation period (Opara et al., 2012; Gupta and Gupta 2014).

The microbial community present in the reactivated culture showed a CBM65 has maximum similarity with those species which was obtained in the developed consortia (Fig. 6). Both bacterial and archaeal domain were observed. The bacterial domain was comprised of firmicutes (*Clostridium beijerinckii* and *Clostridium* sp.). Similar species was reported by many scientists for methane generation (Bi et al., 2017). Further, methanation by similar species at 23 °C was also observed (Fuertez et al., 2018). The archaeal domain includes majorly *Methanoculleus* sp. which are responsible for methane production was also noted. According to Zellner et al. (1998) and Zhu et al. (2011), research on methanation similar archeal species was reported. The genera *Methanoculleus* were related to the family Methanomicrobiaceae, this family contains methanogens of highly irregular coccoid shape with optimal growth temperature 25–60 °C (Spring et al., 2005). By looking into the mechanism of methane production by the microbial community, it was reported by previous researchers that acetogenic microorganisms oxidize organic compounds partially into acetate which was further consumed by methanogens for methane production (Kushkevych et al., 2017) (Fig. 1). *Clostridium* sp. is a well-known acetogenic species; it utilized the organic component from the environment and produces acetate (Schmidt and Cooney, 1985). Further, the byproduct of *Clostridium* sp. (acetate) is consumed by methanogens for methane production.

In analytical studies, FTIR provided the details of functional groups present in the coal sample (Fig. 7A). As reported by Reddy and Vinu (2016) and Sonibare et al. (2012), the organic part of coal contains aromatic, aliphatic, and oxygen groups. The spectrum obtained from FTIR of coal sample attributed the presence of –OH and C=C groups. The presence of aromatic C=C stretch demonstrated that the carbon content was more in the sample. The CHNS data also proved the same, the possible reason for high carbon content could be the reduction of oxygen due to the conversion of C=O to CH_2 or decarboxylation (Manoj et al., 2009). FTIR spectra reported by Li et al. (2018) and Zhang et al. (2018) showed similar trends. Further, extending the analysis in identifying the chemical groups of coal sample GC-MS was considered as a powerful tool (Fig. 7B). Aliphatic compounds present in the sample contained various range of hydrocarbons, alkene, and cyclic or acyclic compounds (Table S2). By observing the fragmentation pattern, the peak at RT 24.4 corresponds to 2,4-Di-tert-butyl which has a role in bacterial metabolites (National Center for Biotechnology Information NIH). Damin et al. (2010) and Shi et al. (2013) demonstrated the presence of alkenes, cyclic, and acyclic organic species in the coal sample. FTIR and GCMS data revealed that bacteria can utilize the components from coal for the production of methane. Further, an SEM micrograph depicted the interaction between coal and bacteria (Fig. 8). Stephen et al. (2014) study explained the

interactions between bacteria (rod-shaped, anaerobic) and coal. According to Wang et al. (2017), SEM images illustrated the growth of microflora on the surface of coal and their effects on the coal surface in terms of morphological change.

By analyzing the data set of EDX graph, it was confirmed that untreated coal contain majorly C, O, Si, F, Ti, Mg, Na, S, K, P, Ca, and Al (Fig. 8C). Long C peak is clearly visible in the graph with other element. Similar data was reported by Sellaro et al. (2015), according to their studies, the EDX of coal dust from mining area contains 85% carbon and 15% oxygen due to the coal particles in the mine dust. The coal dust was obtained after pulverizing the sample; further, it was collected in the polycarbonate (PC) filter and EDX was conducted the results showed the presence of C, O, Na, Mg, Al, Si, K, Ca, Ti, Fe, and Cu peaks. In another research conducted by Essex et al. (2017) in coal mine dust, their studies illustrated the presence of C because of coal particles. A study in India coal using SEM/EDX was conducted by Manoj (2016); in his investigation, he revealed that a coal contains C, N, O, and H in significant amount and Al and Si show their presence.

Figure 8D depicted the case of a treated coal. The peak that corresponds to C and O was missing; the possible reason for this could be the behavior of microorganism towards the coal particle as it can be said that microbes were actively participated in utilizing coal and generating methane as a byproduct. The presence of other peaks corresponds to the residual of media and coal.

Further, pathogenicity data of consortia revealed that consortium was safe for the large scale analysis or the field trial (Table S3-S6). In the experiment of compatibility (Fig. 9), the potential of waste coal for methane production was noted significantly. The nitrogen sparged sets; set 1 and set 2 demonstrated the maximum production of methane (51.6% and 49.91%, respectively); this study also proved that an anaerobic environment is a vital factor for methanogenesis, whereas sets without sparged (set 3 and set 4) showed considerably low and negligible methanation (13.2% and 0.54%, respectively). This research provides an idea for establishing a feasible way for creating a pollution-free environment from waste coal to clean and natural energy.

Conclusions

Our study emphasizes the production of renewable energy (methane) from the waste piles of coal present near the coal mining area. This study proves the enhancement of methane generation in the presence of coal containing medium. The data of FTIR and GCMS illustrated the complex nature of the coal sample. Moreover, active interactions of bacteria with coal particles were detected in the SEM micrograph and the compositional analysis was studied by EDX. Further, pathogenicity assay explained the non–pathogenic nature of the consortium. The results highlighted the potential of the bioconversion process from waste to renewable energy generation. This study can be seen as a promising alternative method for energy generation through coal waste piles.

Supplementary Information

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ESM 1 Table S1: Proximate and ultimate analysis of collected waste coal sample. Fig. S1 A and B, illustrated the percent of gas generation by reactivated consortia with variable nitrogen sources (Urea and CLS). Data recoded after 15-20th days of incubation. **Table S2**: Chemical moiety present in coal containing cultured bottles, identified through GCMS. **Table S3:** Group mean clinical signs data of male and female mice. **Table S4:** Group mean hematology data of male (M) and female (F). **Table S5:** Group mean body weight data of male (M) and female (F). **Table S6:** Group mean mortality data.

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Authors' contributions

PB and ML designed the experiments. PB conducted the experiments as per design and data generation and further wrote the manuscript. ML critically reviewed the manuscript. OS and SKS involved in the technical guidance in waste coal washeries and their selection and characterization of waste coal. BL provided the resources for performing the experiments. The authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Completing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The pathogenicity was studied by acute oral toxicity under EPA 712-C-96-322 OPPTS 885.3550 guidelines. The study was performed at the National Toxicology Centre (APT Testing and Research Pvt. Ltd.), Pune.

Consent for publication

Not applicable.

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