Evaluation of the Coal-Degrading Ability of Rhizobium and Chelatococcus Strains Isolated from the Formation Water of an Indian Coal Bed

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The rise in global energy demand has prompted researches on developing strategies for transforming coal into a cleaner fuel. This requires isolation of microbes with the capability to degrade complex coal into simpler substrates to support methanogenesis in the coal beds. In this study, aerobic bacteria were isolated from an Indian coal bed that can solubilize and utilize coal as the sole source of carbon. The six bacterial isolates capable of growing on coal agar medium were identified on the basis of their 16S rRNA gene sequences, which clustered into two groups; Group I isolates belonged to the genus Rhizobium, whereas Group II isolates were identified as Chelatococcus species. Out of the 4 methods of whole genome fingerprinting (ERIC-PCR, REP-PCR, BOX-PCR, and RAPD), REP-PCR showed maximum differentiation among strains within each group. Only Chelatococcus strains showed the ability to solubilize and utilize coal as the sole source of carbon. On the basis of 16S rRNA gene sequence and the ability to utilize different carbon sources, the Chelatococcus strains showed maximum similarity to C. daeguensis. This is the first report showing occurrence of Rhizobium and Chelatococcus strains in an Indian coal bed, and the ability of Chelatococcus isolates to solubilize and utilize coal as a sole source of carbon for their growth.

Keywords: Coal bed, coal solubilization, rep-PCR, 16S rRNA gene sequence, Chelatococcus

Coal is one of Earth’s most abundant fossil fuels, which contributes nearly 30% of global energy consumption [19]. The burning of coal creates a variety of problems to the environment including the release of atmosphere-polluting sulfur and nitrogen oxides, and leftover ash containing toxic metals, but it cannot be replaced or minimized by any other more efficient fuel in the near future. Although direct thermochemical liquefaction can convert coal into a cleaner liquid fuel, it is an energy intensive process requiring high temperatures and pressures, and, hence, is not cost effective [26]. In order to address the environmental concerns associated with the use of coal, alternative methods of coal-processing are being explored that would extend utilization of coal-derived biofuels as an alternative source of energy. It has been predicted that a microbial, enzymatic, or enzyme-mimetic technology would have greater advantage compared with the physical and chemical technologies for coal conversion.

Coal is a solid rock, which is often dominated by recalcitrant, partially aromatic, and largely lignin-derived macromolecules that tend to be relatively resistant to microbial degradation. It has a highly heterogeneous structure; lignite being more complex than the hard coal. Since lignin was the main parent material in the formation of lignite, typical structures of the original lignin are preserved in lignite, which is also designated as demethylated and dehydrated lignin [9, 17, 18]. The lignite consists of several distinct compound classes: mainly hydrophobic bitumen, the alkali-soluble humic and fulvic acids, and insoluble residue known as the matrix or humine. The aromatic structures of coal derived from cellulose and lignin are often interlinked by oxygen bridges and contain numerous oxygen-containing moieties (e.g., carboxyl, hydroxyl, or ketone functional groups). These oxygen-containing functional groups can be targeted by fermentative microbes, providing important intermediates of degradation, such as succinate, propionate, acetate, CO₂, and H₂, which can be utilized by methanogens to produce methane. The rate-limiting step of coal biodegradation is the initial fragmentation of a macromolecular, polycyclic, lignin-derived aromatic network of coal.

Lignin can be degraded by some fungi and bacteria that produce unspecific extracellular enzymes [8, 14]. Up to 40% of the weight of some coals can be dissolved using extracted microbial enzymes [32]. There are several reports on the ability of filamentous fungi to solubilize
solid particles of low-rank hard coal into black liquid droplets [5, 11, 14, 21], but there are very few reports on the ability of bacteria to degrade or utilize coal as the sole source of carbon and energy [7, 11–13, 20]. In recent years, bacterial communities present in the coalbeds, which can convert coal into the substrates that can be utilized by methanogens, have been described with the help of cultivation-independent methods [23, 28].

Coal beds contain water (known as formation water), which facilitates coal degradation by bacteria at the coal–water interface. Thus, the water extracted from the coal beds might harbour unique microbial communities possessing the ability to solubilize and degrade coal and other polyaromatic hydrocarbons. In the present study, bacteria were isolated from the formation water collected from a coal bed located in eastern India. The isolates were identified using molecular tools of taxonomy and characterized for their ability to solubilize and utilize coal as a sole source of carbon and energy. This study is the first attempt from India towards the isolation and characterization of bacteria from a coal bed.

Materials and Methods

Collection of Sample

The sample was collected directly from the pipe extruding formation water from a depth of 700 m from a coal bed methane (CBM)-producing well located at Parbatpur in the Jharia coalfield of eastern India. Samples were collected in autoclaved bottles, which were brought to the laboratory under cooled condition. At the time of collection, the temperature of the formation water was 42°C and pH was 7.2; chloride and sulfate contents were 180 ppm and 22 ppm, respectively.

Isolation of Coal-Solubilizing Bacteria

For isolation of coal-solubilizing bacteria, formation water was serially diluted with sterile saline (0.89% NaCl) and spread onto coal agar medium [22] containing (per liter) KH2PO4, 1.2 g; Na2HPO4·12H2O, 10.8 g; MgSO4·7H2O, 0.04 g; FeSO4·7H2O, 0.02 g; MnCl2·4H2O, 0.02 g; NH4Cl, 3 g; lignite coal, 50 g; agar, 15 g; and cycloheximide, 0.5 g; pH 7.0. The lignite coal was oxidized with 10% hydrogen peroxide before using it in the coal agar medium [22]. The plates were incubated at 37°C for 3 days.

Nucleic Acid Extraction and 16S rRNA Gene Amplification

Total genomic DNA was extracted from the isolates using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The 16S rRNA gene was amplified using universal bacterial primers 8f (5'-AGA GTT TGA TYM TGG CTC AG- 3') and 1495r (5'-CTA CCT GCT TGT TAC G -3') [16]. A 50 µl reaction mixture included 0.5 g; MgSO4·7H2O, 0.04 g; FeSO4·7H2O, 0.02 g; MnCl2·4H2O, 0.02 g; NH4Cl, 3 g; lignite coal, 50 g; agar, 15 g; and cycloheximide, 0.5 g; pH 7.0. The lignite coal was oxidized with 10% hydrogen peroxide before using it in the coal agar medium [22]. The plates were incubated at 37°C for 3 days.

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Sequencing of 16S rRNA Genes

The amplified 16S rRNA gene was purified with a Wizard SV gel PCR purification kit (Promega, USA) and quantified by using a ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). Direct sequencing was performed with three primers, 8f (5'-AGA GTT TGA TYM TGG CTC AG- 3') and 1495r (5'-CTA CCT GCT TGT TAC G -3') [16], and 561f (5'-AATTACTGGGCAGGAAAG -3') [2] using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) in an ABI Prism 310 automated DNA Sequencer (Applied Biosystems, Rotkreuz, Switzerland).

Analysis of 16S rRNA Gene Sequence

The 16S rRNA gene sequence of the six isolates was edited using Bioedit software version 3.1 to make a complete sequence. The almost complete sequence (1,400 nt) was compared with the nucleotide sequences present in the NCBI database using the standard nucleotide BLAST search [1]. The programme ClustalW [35] was used to align the 16S rRNA gene of the isolates with the similar sequences retrieved from the NCBI database to construct a phylogenetic tree, as described by Chowdhary et al. [4].

Genomic Fingerprinting

rep-PCR. Primers corresponding to the conserved motifs in bacterial repetitive elements (REP, ERIC, and BOX) were used for PCR amplification to generate genomic fingerprints to differentiate the isolates further at the species, subspecies, or strain levels. All the six isolates were subjected to genomic fingerprinting using primer sets corresponding to REP, ERIC, and BOX elements. The PCR protocols with REP, ERIC, and BOX primers are collectively referred to as rep-PCR. The primer pairs REP1R-I (5'-IICGCIGICAICGCIG-3') and REP2-I (5'-ICGITITATCIGGCTAC-3') (where I is inosine); ERICIR (5'-ATGIAAGCTCCGATGCTAC-3') and ERIC2 (5'-AAATGAAAGCTGGTTGACCG-3'); and BOXAIR (5'-CTACG GCAAGGCAGCGTACG-3') [25] primer sequence corresponding to BOXA, a subunit of BOX element, were used to amplify REP-, ERIC-, and BOX-like elements from the isolates DNA. Approximately 50 ng of DNA was used as template, and PCR amplification reactions were performed with a Veriti 96-well Thermal cycler (Applied Biosystem, USA) using the following cycles: an initial denaturation at 95°C for 7 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 44, 57, or 60°C for 1 min with REP, ERIC, or BOX primer, respectively; and extension at 72°C for 1 min with single extension cycle at 72°C for 10 min before cooling at 4°C. A reaction containing all the components of PCR except genomic DNA was included as negative control in all the thermal cycles run. The 10 µl of PCR products was loaded on agarose [1.5% (w/v)] gel and amplification products were visualized under a UV light with Alpha imager.

RAPD. Amplification was performed in 25 µl with 20–50 ng of genomic DNA as template, 4 µM of 10 mer primer (5'-GGTTGCCCCCA-3') [36], 2.5 U of Taq DNA polymerase (Genei, Bangalore) with its 1× buffer, and 200 µM of each dNTPs. The reaction condition included an initial denaturation of 7 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C, with a final extension of 5 min at 72°C. The amplified product was analyzed in 0.8% agarose gel at 5 V/cm for 4 h and was visualized under UV light with an Alpha imager (Alpha Innotech Corporation, UK).
Carbon Source Utilization Ability of the Isolates

Bacterial inocula were prepared by growing the isolates on Biolog universal growth agar (BUG; Biolog) medium for 24 h. The cultures from the Biolog universal growth agar medium were collected using sterile swabs and resuspended in 0.85% saline. The inoculum density was adjusted with a Biolog turbidimeter within the limit of GN MicroPlate turbidity standards supplied by the manufacturer. The cell suspension was poured into a sterile disposable plastic reservoir, and Biolog-GN MicroPlates (Biolog Inc., Hayward, CA, USA) containing 95 different carbon sources were inoculated with 150 µl of bacterial suspension, and the microplates were incubated for 24 h at 37°C. Substrate utilization patterns were compared with Microlog database release 4.01 software.

Evaluation of the Ability of Isolates to Utilize and Solubilize Coal

In order to check coal utilization and solubilization capabilities, isolates were grown in mineral salt broth (MSB) supplemented with and without 5% coal. The constituent of MSB were g/l KH$_2$PO$_4$, 1.2 g; Na$_2$HPO$_4$·12H$_2$O, 10.8 g; MgSO$_4$·7H$_2$O, 0.04 g; FeSO$_4$·7H$_2$O, 0.02 g; MnCl·4H$_2$O, 0.02 g; and NH$_4$Cl, 3.0 g. Flasks were inoculated with equal number of overnight-grown log phase culture (same optical density) and the supernatant was used for spectrophotometric monitoring of coal solubilization at 450 nm [15, 37]. To determine the growth of isolates utilizing coal as sole carbon source, the protein concentration of cultures grown in MSB supplemented with and without 5% coal was measured. Cell pellets harvested at regular intervals of time were treated with 10% trichloroacetic acid for 30 min at 50°C. After incubation, tubes were centrifuged and pellets were incubated at 37°C for 4 h with 1 ml of 1 N NaOH. After centrifugation, 200 µl of supernatant was used for estimation of protein according to the Lowry method of protein estimation.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences of the six formation water isolates have been deposited in GenBank with accession numbers shown in brackets: Isolate #1 (JN182697), #2 (JN182699), #3 (JN182698), #4 (JN182700), #5 (JN182701), and #6 (JN182702).

RESULTS AND DISCUSSION

Isolation of Bacteria Capable of Growing on Coal Agar

In order to isolate bacteria capable of degrading coal and using coal components as the sole carbon source, we inoculated formation water collected from a 700 m depth of a coal bed onto coal agar plates containing minimal medium with oxidized coal [5% (w/v)] as the sole carbon source and cycloheximide (0.5 mg/ml) to suppress the growth of fungi. Since the pH of the formation water was 7.2, the pH of the agarized medium was also maintained at 7.0. Aerobic incubation of the plates at 37°C for 3 days produced bacterial colonies with different morphologies. Fourteen bacterial colonies were selected, which were thought to represent the morphological diversity on coal agar. Clonal cultures of 14 isolates (Isolate #1 – Isolate #14) were generated by repeated dilution streaking on coal agar. When the 14 isolates were
subcultured on coal agar, 6 of them showed relatively better and faster growth and were selected for further study. It might be possible that the remaining 8 colonies utilized the metabolic products produced as a result of coal solubilization by other microorganisms. Thus, when they were present in pure form in a medium with coal as the sole carbon source, they were not able to proliferate.

Identification of the Isolates on the Basis of 16S rRNA Gene Sequence
The 6 selected isolates were identified using 16S rRNA gene sequencing. The identification based on comparison of the 16S rRNA gene sequence of the isolates with that of other bacterial sequences existing in the GenBank database showed that isolates #1, 3, and 5 showed 99% sequence identity, whereas isolate #4 showed 98% identity to *Rhizobium* sp. 525W (Accession No. AB262326). However, isolates #2 and 6 showed 98% sequence identity to *Chelatococcus daeguensis* (Accession No. EF584507). A phylogenetic tree based on the 16S rRNA gene sequences of our isolates and other related sequences clustered isolates into two groups; Group I isolates showing close similarity to the genus *Rhizobium* (isolates #1, 3, 4, and 5), and Group II isolates showing maximum similarity to the genus *Chelatococcus* (isolates #2 and 6) (Fig. 1).

Genomic Fingerprinting of the Bacterial Isolates
Since the 16S rRNA gene is a very small fraction of the whole genome, the techniques of whole genome fingerprinting are considered more sensitive for differentiating the isolates to the species, subspecies, and strain levels [34]. Hence, we used whole genome fingerprinting methods, namely, rep-PCR (REP-, ERIC-, and BOX-PCR) and RAPD, to differentiate the isolates of the two groups from each other at the strain level. The genomic fingerprints of the isolates revealed distinct patterns with 5–15 fragments in the range of 100–3,000 bp size and revealed differences among the isolates. Out of the 4 genomic fingerprinting methods used in this study, REP-PCR showed maximum differentiation among the isolates, followed by ERIC-PCR and RAPD, which were also able to show some differentiation within each of the two groups, whereas BOX-PCR failed to show any notable differentiation (Fig. 2).

Keeping in view the fact that rhizobia are normally known to form nodules in legumes, occurrence of the members of the genus *Rhizobium* in Indian coal bed was surprising. However, the presence of *Rhizobium* and *Bradyrhizobium* strains in the coal beds has already been shown with the help of cultivation-independent methods [23, 28]. *Agrobacterium* sp. was also reported from the formation water of an oil field in China [24]. These organisms are known to be associated with plant roots, and it seems that during the process of coalification, the plant-associated bacteria were also buried along with the remnants of plants under the Earth’s surface and were trapped in the coal seams. They might have survived the slow process of wood coalification to lignite, adapting themselves to the extreme conditions of coal seams [29].

Comparison of the *Chelatococcus* Strains with Other Species
The two strains of *Chelatococcus* isolated in this study from the formation water of the coal bed showed almost identical carbon source utilization abilities (Table 1). Like *Chelatococcus asaccharovorans*, both of them were urease-positive, but *C. daeguensis* was urease-negative [38]. Similar to *C. asaccharovorans* and *C. sambhunathii*, but unlike *C. daeguensis*, both the isolates also utilized nitrilotriacetate.
As a carbon source. Unlike \textit{C. asaccharovorans} and \textit{C. sambhunathii}, both our isolates were similar to \textit{C. daeguensis} in utilizing galactose, rhamnose, sorbitol, inositol, sucrose, adonitol, glycerol, arabinose, xylitol, and mannitol. However, unlike \textit{C. daeguensis}, they could not utilize cellobiose and mannose [27, 38]. In view of the above characteristics and the phylogenetic tree based on the 16S rRNA gene sequences, \textit{C. daeguensis} appears to be the most closely related, validly described species.

\textit{Chelatococcus asaccharovorans} is known to utilize the metal-chelating NTA as a sole source of carbon, energy, and nitrogen. It was described as a chelating coccus, a bacterium that forms claw-like complexes with divalent cations [10]. It is likely that inside the coal bed, the bacterial consortia involved in coal degradation produce metal ion chelators [14, 20], which are utilized by \textit{Chelatococcus} as a source of carbon, energy, and nitrogen, or else \textit{Chelatococcus} might be directly involved in the utilization and solubilization of coal by producing metal ion chelators, which lead to the weakening of coal matrix [3] and release of substance in the supernatant, rendering it the yellowish brown colour. \textit{C. daeguensis} has been shown to grow under anaerobic condition on trypticase soy agar plate supplemented with 0.01% potassium nitrate [38].

\textbf{Growth of the Coal Bed Isolates on Coal as Sole Carbon Source}

For characterizing the coal utilization ability of the isolates, their growth was monitored in minimal medium supplemented with or without coal as the sole carbon source. Since coal components create problems in measuring growth on the basis of turbidity or absorbance at 560 or 600 nm, we estimated the protein content of the culture at different stages of growth. A comparison of the growth of the isolates revealed that the \textit{Rhizobium} isolates showed very little growth in minimal medium supplemented with or without coal, whereas \textit{Chelatococcus} isolates showed much higher growth in the medium supplemented with 5% coal compared with the medium without coal (Fig. 3). From the growth pattern of the isolates, it became clear that \textit{Rhizobium} isolates were inefficient users of coal as a carbon source. The fact that \textit{Chelatococcus} isolates grew much better in coal-supplemented medium than that without coal indicated that \textit{Chelatococcus} isolates were able to utilize some components of coal as a carbon source for their growth.

\textbf{Coal Solubilization Ability of the Coal Bed Isolates}

In some cases, the utilization of brown coal is accompanied by its solubilization. Evolution of $^{14}$CO$_2$ from radioactively labeled coal ($^{14}$C-methoxylated german lignite) by a coal-solubilizing fungi was also used as an indicator of their coal utilization ability [20]. The browning of the supernatant of the cultures grown on hard coal was used for the first time to show coal solubilization [11, 12]. We found that \textit{Chelatococcus} isolates demonstrated coal solubilization, as shown by an increase in absorbance at 450 nm and browning of the culture supernatant (Fig. 4). However, \textit{Rhizobium} isolates did not show such coal solubilization ability. Thus, it can be concluded that \textit{Chelatococcus} strains isolated in this study are capable of the solubilization and utilization of coal. Biosolubilization of coal involves various mechanisms such as production of alkaline substances, biocatalysts, metal ions chelators, detergents, and esterases [14, 20]. The microorganisms growing on rich media were shown to produce alkaline metabolic products, which lead

<table>
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<tr>
<th>Carbon source</th>
<th>Isolate</th>
<th>\textit{Chelatococcus} sambhunathii DSM 1867$^T$</th>
<th>\textit{Chelatococcus} asaccharovorans DSM 6462$^T$</th>
<th>\textit{Chelatococcus} daeguensis CCUG 54519$^T$</th>
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<tr>
<td>Nitrilotriacetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>d-Galactose</td>
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<td>Rhamnose</td>
<td>+</td>
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<td>Sorbitol</td>
<td>+</td>
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<td>Inositol</td>
<td>+</td>
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<td>Sucrose</td>
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<td>Adonitol</td>
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<td>Glycerol</td>
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<td>Arabinose</td>
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\((+), \text{Positive}; (-), \text{Negative.}\)
to the ionization of acidic groups in low-rank coal to render coal humates water-soluble [30]. Removal of metal ions from the coal leads to the cleavage of ionic linkages between structural elements and an increase in the number of free acidic groups, enhancing the hydrophilicity of the coal [3].

In a preliminary characterization, we found that *Rhizobium* isolates produced some kind of biosurfactants, which are known to increase the surface area of hydrophobic water-insoluble substrates to increase their bioavailability. It is likely that *Rhizobium* isolates participate in the process of degradation of hydrocarbons by producing bioemulsifiers and thereby making the components of coal more water-soluble. In a consortium, the biosurfactant-producing bacteria provide emulsifier (surfactant) to other bacteria that carry out degradation of hydrocarbons. This is why the cultures of *Acinetobacter radioresistens*, which produce bioemulsifier but are unable to use hydrocarbon as a carbon source, were included in the mixture of oil-degrading bacteria to enhance oil bioremediation [31]. Therefore, further study is required to elucidate the role of *Rhizobium* isolates in coal degradation.

Advances in the understanding of coal biosolubilization processes and the discovery of enzymatic degradation of coal macromolecules offer new possibilities for utilizing the carbon from low-rank coals. Several Gram-positive and Gram-negative bacteria have been shown to produce non-oxidative hydrolytic enzymes, which might be involved in the depolymerization of humic acids obtained from
weathered lignite [6]. The bacteria progressively convert the coal polymers into solubilized coal, which can serve as substrate for anaerobic bacteria to produce clean methane as fuel. This study is the first of its kind to describe the occurrence of bacteria belonging to the genera *Rhizobium* and *Chelatococcus* in Indian coal beds, which might be useful in the biotransformation of coal.

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