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Bio-solubilization of the untreated low rank coal by alkali-producing bacteria isolated from soil

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Abstract−Coal is a hydrocarbon-rich fossil fuel considered as a possible replacement for petroleum as a feedstock for the production of fuel and valuable chemicals. In this study, bacteria capable of solubilizing untreated low rank coal were isolated from soil. A total of 19 microorganisms were isolated from soil enriched in MR medium with coal and were identified based on 16S rRNA sequencing. The identified soil isolates belonging to the genera *Citricoccus*, *Comamonas*, *Cupriavidus*, *Sphingomonas*, and *Sphingopyxis* were screened based on their growth in the chemically defined MR medium containing different concentrations of coal. Among the identified microbial strains, *Cupriavidus necator* S2A2, *Sphingopyxis ginsengisoli* S2B14 and *Sphingomonas* sp. S2B18 were further characterized for their ability to degrade low-rank coal. *Cupriavidus necator* S2A2, *Sphingopyxis ginsengisoli* S2B14 and *Sphingomonas* sp. S2B18 were found to solubilize untreated low-rank coal as indicated by the release of solubilized coal products detected at OD_{450} when they were grown in LB medium containing 1% coal. *Sphingomonas* sp. S2B18 showed the highest coal solubilization activity, based on the high absorbance of its culture supernatant (0.190). Although laccase-like activity was not detected in these strains when tested for RBBR dye degradation, increase in the pH of the culture medium up to 8.25-8.34 was observed. This may be attributed to the excretion of alkaline substances in the culture medium. Since biosolubilization of coal by microorganisms is a good alternative for the chemical conversion of coal, microorganisms screened in this study can be potentially used as biological catalysts for the conversion of coal into valuable chemicals.

Keywords: Untreated Low-rank Coal, Coal Biosolubilization, Coal Degradation, Alkaline Degrading Substances, Low Molecular Weight Coal Products

INTRODUCTION

Since the industrial revolution, petroleum has been the primary feedstock for the synthesis of most of the industrially important chemicals that we use today; however, its reserves are in the brink of depletion. Coal, one of the fossil fuels, has a larger amount of reserves than petroleum. It was estimated that the fossil fuel reserves for oil and coal would last for approximately 35 and 107 years, respectively [1]. Like petroleum, coal is also hydrocarbon rich, which makes it a possible substitute for petroleum as a feedstock for chemical production. However, due to the solid recalcitrant structure of coal, chemical processes involved in the conversion of coal into useful chemicals require extensive treatment. The current technologies for harnessing coal involve strategies such as carbonization, gasification and liquefaction [2]. All of these processes require high temperature and/or harsh chemical conditions that can also give off harmful gases such as sulfur oxides and nitrogen oxides, which contribute to air pollution. Thus, developing milder and cleaner processes to convert coal into valuable chemicals is needed. Biological degradation and utilization of coal by microorganisms is perceived to be the solution to address this issue [2].

Coal has a very complex and high recalcitrance structure, which is attributed to the natural chemical and physical processes that it has undergone for a long period of time beneath the soil. It is ranked based on its aromaticity or complexity, with low rank coal having the least recalcitrance followed by subbituminous, bituminous and anthracite [3,4]. Therefore, low rank coal is more preferable as a feedstock for microbial degradation and utilization since it is easier to degrade than its higher ranked forms. Aside from the recalcitrance of coal, its hydrophobicity, heterogeneity, acidity and toxicity are the major obstacles that have to be considered in developing a biodegradation process for the production of valuable chemicals [3].

Solubilization and degradation of coal by microorganisms are mediated by the released extracellular substances such as hydrolyzing and oxidative enzymes, chelators, surfactants and alkaline substances [5,6]. Several fungal species have been found to have the ability to solubilize coal. *Phanerochaete chrysosporium*, *Trichoderma* and *Trametes* species have been reported to produce ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase [6-10]. However, fewer studies have used bac-

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teria for coal degradation [11-14]. Bacteria have several advantages over fungi, such as ease and short period of cultivation and faster rate of reaction. Moreover, it is much easier to manipulate and engineer its metabolic system to make it favorable to its target application due to well-developed recombinant DNA technologies. Thus, it would be advantageous to search for bacteria that have good potential to degrade coal for the production of valuable and novel chemicals for the development of a biological process for coal.

We isolated several bacteria enriched in a chemically defined medium containing untreated low rank coal from soil and characterized it to evaluate its potential in the biosolubilization and degradation of untreated low rank coal.

MATERIALS AND METHODS

1. Materials and Chemicals

Soil sample was collected from a mountain slope in the vicinity of Korea Institute of Energy Research (KIER), Yuseong-gu, Daejeon, South Korea. The low rank coal used in this study, which was excavated from a coal mine in Indonesia, was also provided by KIER, Daejeon, South Korea. The coal sample was mechanically crushed and sieved using a steel sieve with a wire size of 50 μm and an aperture of 75 μm and dried to constant weight at 60 °C. The low rank coal used in all experiments was not subjected to any chemical treatment. The composition of the untreated low rank coal is summarized in Table 1.

2. Isolation of Coal Solubilizing Bacteria from Soil

For the isolation of coal solubilizing bacteria from soil, 0.5 g of soil sample was mixed with 10 mL saline solution (0.9% w/v). The soil in the solution was allowed to settle and 1 mL of the supernatant was serially diluted until 10^{-3} dilution was obtained. The dilution sample was added to 100 mL MR medium (1% v/v) containing 1% (w/v) coal at 28 °C for 10 days. MR medium (pH 7.0) contains (per liter) 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4{\cdot}7H_2O$, 0.8 g citric acid, and 5 ml trace metal solution. The trace metal solution contains (per liter of 0.5 M HCl) 10 g $FeSO_4{\cdot}7H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}4H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 0.02 g $Na_2B_4O_7{\cdot}10H_2O$. Coal and $MgSO_4{\cdot}7H_2O$ were autoclaved at 121 °C for 15 min, separately. After ten days, samples were taken and plated in MR agar medium with

Table 1. Composition of the untreated Indonesian low-rank coal

As received basis (wt%)				Dry ash-free basis (wt%)	
Moisture	Volatile	Ash	Fixed carbon	Volatile	Fixed carbon
17.36	43.19	6.57	32.88	56.78	43.22

Table 2. Growth characteristic of the isolates in MR medium supplemented with different concentrations of coal and in MR agar with RBBR dye (RBBR plate assay) and Simmons citrate agar (citrate utilization test)

Strain	Growth			RBBR plate assay	Citrate utilization test
	0.5% Coal	1% Coal	2% Coal		
Citricoccus sp. S2B15	+	+	+	x	✓
Comamonas jiangduensis S2A13	+	+	+	x	✓
Cupriavidus necator S2A1	++	+	+	x	✓
Cupriavidus necator S2A2	++	++	++	x	✓
Cupriavidus necator S2A3	++	++	++	x	✓
Cupriavidus necator S2A6	++	++	++	x	✓
Cupriavidus necator S2A9	++	++	++	x	✓
Cupriavidus necator S2A22	++	++	++	x	✓
Cupriavidus necator S2A23	++	++	++	x	✓
Cupriavidus necator S2A29	++	++	++	x	✓
Cupriavidus necator S2B6	++	++	++	x	✓
Cupriavidus sp. S2A4	++	++	+	x	✓
Cupriavidus sp. S2A8	+	+	++	x	✓
Cupriavidus sp. S2A19	+	++	+	x	✓
Cupriavidus sp. S2B8	++	++	+	x	✓
Cupriavidus sp. S2B16	++	++	+	x	✓
Sphingomonas sp. S2B18	++	++	+	x	✓
Sphingopyxis ginsengisoli S2B14	++	++	++	x	✓
Sphingopyxis taejonensis S2B26	+	+	+	x	✓

Note: Growth is designated as: (+) when OD_{600}=0.83-1.01 (0.5% coal), 0.83-1.01 (1% coal), 1.31-1.83 (2% coal); and (++) when OD_{600}=1.03-1.30 (0.5% coal), 1.37-1.98 (1% coal), 2.18-2.86 (2% coal). Strains showing positive results for the RBBR assay and citrate utilization test are designated as (✓), while strains showing negative results were designated as (**X**)

1% coal to obtain single colonies. Then stock cultures were made by maintaining the pure culture of the isolates at −80 °C in 15% glycerol

3. Identification of the Isolated Strains

The microorganisms isolated from soil were identified by 16S rRNA analysis. To identify the bacteria isolated from soil, bacterial cells were taken from the streak plates and dissolved in 100 µL sterile milli-Q water. Samples were boiled at 100 °C for 10 minutes and placed on ice for 20 minutes. Then, samples were centrifuged at 10,000 rpm for 10 minutes at 4 °C. The crude supernatant containing the bacterial DNA was used as a template in PCR for the 16S rRNA amplification reaction. For the amplification of 16S rRNA, the primers used were BAC-F (5′-AGAGTTTGATCMTGGCTC-3′) and BAC-R (5′-ACGGCTACCTTGTTACGACTT-3′). Amplified products were confirmed by gel electrophoresis, purified using Qiagen PCR purification kit and sequenced by Macrogen, Daejeon, South Korea. The sequences were then analyzed for the identification of the taxa of the isolated strains using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and EZTaxon (http://www.ezbiocloud.net/eztaxon).

4. Screening of the Isolated Strains

The isolates were tested for their growth ability in 10 mL MR medium containing low rank coal at different concentrations (0.5%, 1%, and 2%). Isolates grown in MR containing 20 g/L of glucose were used in control experiments to examine the viability of the bacterial cells. The cultures were incubated for 24 hours in a shaking incubator at 30 °C, 180 rpm. The growth was noted as (+) or (++), depending on the turbidity of the culture medium (Table 2). Citrate utilization test were also performed in all the isolates by streaking in Simmons citrate agar (Difco), in which isolates positive for citrate utilization is indicated by the growth and changes in the color of the agar medium from green to blue [15].

To check if the isolates were capable of producing oxidative enzymes (e.g., LiP, MnP and laccase), overnight cultures were prepared for each isolate and streaked in RBBR plates. The RBBR plates were prepared by adding 0.04% (w/v) Remazol Brilliant Blue R dye (RBBR) in MR agar supplemented with glucose. The plates were incubated in a static incubator at 30 °C for 72 h. Coal isolates that are capable of producing oxidative enzymes can be observed by the formation of colorless zones around the colonies of the strains streaked in RBBR plates.

5. Shake Flask Cultivations

For further characterization of the coal degradation ability of the selected isolates, seed cultures of the selected isolates were prepared by inoculating a single colony in 10 mL LB medium and incubating for 14 h. Then 2 mL of the seed cultures, having an OD_{600} of 1, were inoculated to 500 mL flasks containing 200 mL LB medium with 1% (10 g/L) of coal and cultivated for 96 h. LB medium contains (per liter) 10 g tryptone, 5 g yeast extract and 5 g NaCl. Flask cultures were carried out in a rotary shaker at 180 rpm and 30 °C. Every 24 h, samples were collected, and filtered with 0.22 µm PES membrane syringe filter and analyzed for its pH and absorbance (450 nm). Culture medium without inoculum and cultures without coal were used as controls.

6. Analytical Procedures

The culture pH was measured using Orion Star™ A211 pH benchtop meter to detect if alkaline substances were present in the medium. While, the amount of solubilized coal products released in the medium was determined by measuring the absorbance of the filtered supernatant at OD_{450} using UV spectrophotometer (Shimadzu UV-1800).

RESULTS AND DISCUSSION

1. Isolation and Identification of Bacterial Isolates

Several bacteria were already found to degrade coal by producing coal solubilizing substances such as *Bacillus pumilus*, *B. subtilis*, and *Streptomyces griseus*, all of which are capable of producing alkaline solubilizing substances, laccase and MnP [6,16-18]. As mentioned, bacteria have several advantages over fungi when it comes to developing industrial microbial processes. Thus, in order to isolate bacteria that can degrade and solubilize low rank coal, soil samples were enriched in MR medium containing 1% coal for 10 days. The bacterial isolates from the enrichment culture were identified and found to belong in the genera: *Citricoccus*, *Comamonas*, *Cupriavidus*, *Sphingomonas* and *Sphingopyxis*. Based on Fig. 1, the *Cupriavidus* bacterial isolates dominated in the enrichment culture, accounting for 75% of all the identified isolates. Its dominance may be attributed to its predatory nature, especially *Cupriavidus necator*, which is a known non-obligate bacterial predator of bacteria in soil [19,20]. *C. necator* N-1 was found to be capable of attacking several gram positive and gram negative bacteria in soil such as *Escherichia coli*, *Staphylococcus aureus*, *B. subtilis* and *Bacillus thuringiensis* [19].

2. Screening of the Bacterial Soil Isolates

Based on the results summarized in Table 2, most of the isolates were able to have high growth (++) in different coal concentrations tested (0.5%, 1% and 2%). The ability of the isolates to utilize citrate, present in MR medium at low concentration (0.8 g/L), was determined by performing a Simmons citrate agar test. Most of the isolates showing good growth in the medium containing coal as carbon source were able to grow and change the color of the agar medium from green to blue, indicating the ability of the isolates to utilize citrate. This result suggests that citrate in MR medium could support the growth of the isolated microorganisms in the medium containing coal at concentrations up to 2% (w/v) [15].

We also tested if the bacterial isolates could produce enzymes

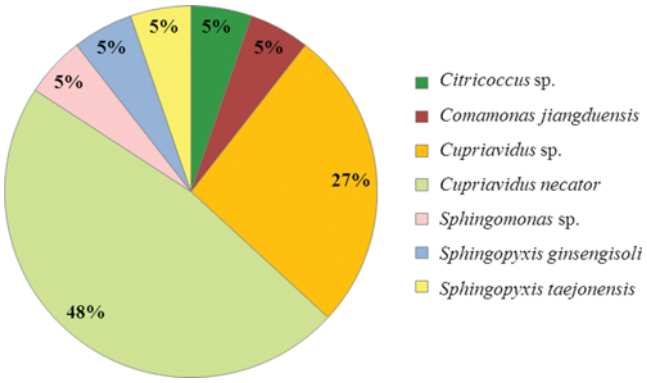

Fig. 1. Population of the identified microbial strains isolated from soil sample after enrichment with 1% coal for ten days.

that may aid in degrading coal, thereby allowing them to grow in coal despite its toxicity. RBBR dye plate assay is usually used for detecting the presence of oxidative ligninolytic enzymes [21,22]. Several studies reported that fungal and bacterial organisms capable of producing ligninolytic enzymes can oxidize coal, resulting in the formation of soluble low-molecular weight products, which are more available for bacterial utilization and/or conversion [3,6]. Thus, RBBR plate assay was performed to check if the isolates could produce these enzymes. However, decolorization of RBBR dye in the agar plates was not observed in all the isolates. Thus, the isolated strains cannot excrete oxidative enzymes like laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP), which have been reported to be produced by *Bacillus* sp., *Trametes hirsuta* and *Phanerochaete chrysosporium* to decolorize RBBR in liquid or solid agar media containing RBBR dye [21-23].

3. Shake Flask Cultivation

Even though citrate seemed to support the growth of all isolates in the medium containing coal, not all isolates have good growth in the presence of higher concentration of coal in the medium (Table 2). For the shake flask cultivations, *C. necator* S2A2, *Sphingopyxis ginsengisoli* S2B14, and *Sphingomonas* sp. S2B18 belonging to different genera, were selected for further characterization. The strains were selected for their high tolerance for coal, based on their excellent growth in MR medium containing 0.5%, 1% and/or 2% coal (Table 1). Since many isolates were identified as *C. necator* and S2A2, S2A3, S2A6, S2A9, S2A22, S2A23, S2A29, S2B6, all share the common characteristic of having an excellent growth in culture containing 0.5%, 1% and 2% coal; S2A2 was selected as a representative for the genus *Cupriavidus*.

The three selected isolates were cultivated in 200 mL LB medium containing 1% (w/v) coal for 96 h for further characterization. As shown in Fig. 2, although increase in absorbance at 450 nm can be observed in the cultures of the selected isolates grown in LB medium alone, the isolates grown in LB with 1% coal have considerably higher absorbance values. This indicates that the isolates were able to solubilize the untreated low rank coal.

Among the isolates, the supernatant obtained from *Sphingomonas* sp. S2B18 has the highest absorbance of 0.190 after 96 h, although it had the lowest absorbance in the first 48 h. This trend differs from the isolate *Sphingopyxis ginsengisoli* S2B14, which also belongs to the family, Sphingomonadaceae. On the other hand, the culture supernatants of *C. necator* S2A2 and *S. ginsengisoli* S2B14 have a close range of absorbance values throughout the cultivation period (0.171-0.182). If the equation for determining the coal solubilization ratio is applied (y=12.97x+13.19), it can be assumed that *Sphingomonas* sp. S2B18, *C. necator* S2A2, and *Sphingopyxis ginsengisoli* S2B14 solubilized the low rank coal by 1.30-1.48% [24].

Even though the isolates cannot produce lignin-oxidizing enzymes, based on the result of the RBBR assay, the degradation of coal seems to be due to the alkaline substances released by the bacteria. This is evident in the increase in pH of the culture medium during shake flask cultivation (Fig. 3). In the presence of coal, the highest pH was observed in the culture of *C. necator* S2A2 (pH 8.34) followed by, *S. ginsengisoli* S2B14 (pH 8.31) and *Sphingomonas* sp. S2B18. Fig. 3 also shows that the release of alkaline substances in the medium is not induced by the presence of coal, which was observed in the culture supernatants of the isolates grown in LB without low rank coal (control set-up) having a higher pH compared to the isolates grown in the presence of coal. The lower pH in the cultures of the isolates grown in the presence of coal can be attributed to the release of the solubilized coal products, which are acidic in nature, by the alkaline substances released in the culture medium.

Unlike most of the coal degrading fungal organisms, which produce oxidizing and/or ligninolytic enzymes, bacteria found to degrade coal commonly produces alkaline solubilizing substances, resulting in an increase in pH of the culture medium as previously reported in the strains, *B. cereus*, *B. pumilus*, *Bacillus* sp. Y7, *B. subtilis*, *Pseudomonas putida*, and *Streptomyces setonii* 75Vi2 [6,25-28]. The increase in pH of the culture medium by alkaline substances

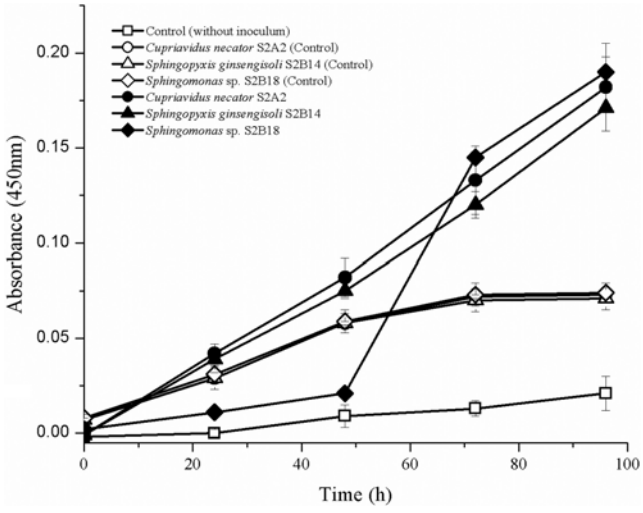

Fig. 2. Absorbance of the culture supernatants from the cultures indicating the degree of biosolubilization of low rank coal by *C. necator* S2A2, *S. ginsengisoli* S2B14 and *Sphingomonas* sp. S2B18 grown in LB medium with 1% (w/v) coal.

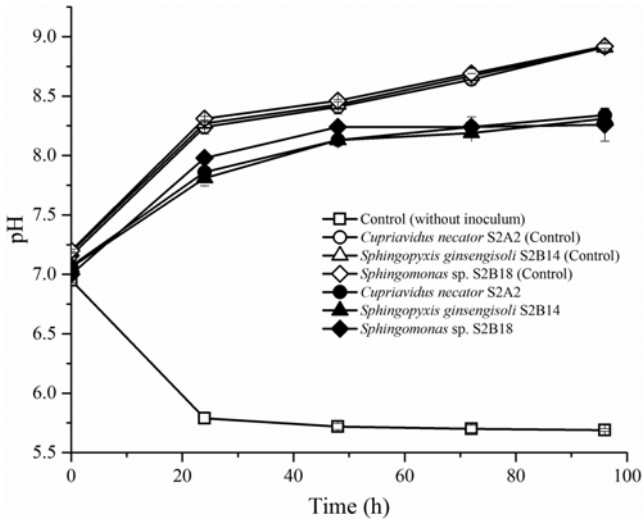

Fig. 3. pH of the culture supernatants of *C. necator* S2A2, *S. ginsengisoli* S2B14 and *Sphingomonas* sp. S2B18 during the cultivation period (96 h) in LB medium with 1% (w/v) coal.

may increase the oxidative degradation of low rank coal, thereby releasing the carboxylic acids and humic substances, which are highly present in low-rank coal [26,29,30]. Aside from alkaline substances, other coal solubilizing substances such as detergents, and esterases, which are known to aid in coal solubilization and/or degradation, may be produced by the isolates [6].

Cupriavidus, *Sphingomonas*, and *Sphingopyxis* are known aromatic degraders. Bacteria belonging to these genera are reported to have the capability to degrade and convert phenols, catechols, phenanthrene and other polyaromatic hydrocarbons (PAHs), which can be derived from coal [31-35]. Thus, it is not surprising that these soil bacteria have the capability to solubilize coal, resulting in the release of low molecular weight products in the medium. More study is required to understand the mechanism of the degradation of low-rank coal by the selected isolates, which can be useful for the development of metabolic engineering strategies of these strains to produce industrially important products from coal, as these strategies have successfully been employed for the biomass-based production of polymers, fuels, and chemicals [36].

CONCLUSION

C. necator S2A2, *S. ginsengisoli* S2B14 and *Sphingomonas* sp. S2B18 isolated from soil enriched with coal were found to solubilize untreated low-rank coal, as indicated by the release of solubilized coal products detected at OD_{450} when they were grown in LB medium containing 1% (w/v) coal. The release of alkaline substances, as indicated by the pH of the medium, aided in the solubilization of low rank coal. Thus, these bacteria have the potential to solubilize coal and produce low-molecular products that can be used as feedstock for the production of valuable chemicals.

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