ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 12 (1): 46-53, 2013
ISSN 1682-296X / DOI: 10.3923/biotech.2013.46.53

Biodesulfurization of Subbituminous Coal by Mixed Culture Bacteria Isolated from Coal Mine Soil of South Sumatera

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Abstract: Coal as fuel should be necessarily pre-treated by desulfurization in order to prevent excessive emissions of sulfur dioxide, a precursor of acid rain. Organic sulfur in coal can be eliminated by microbial action through the technology known as biodesulfurization. Source of microorganisms in the present study was coal mine soil in which microorganisms have been adapted to use the sulfur in coal. Coal mine in South Sumatra was choosen as source of microorganisms in this study, because it is an area in Indonesia with the largest of subbituminous coal reserves. The microorganisms were activated as mixed culture by culturing the soil sample in mineral salt medium containing subbituminous coal as the sole sulfur. Desulfurization activities were examined by using three variations of the initial coal concentration, i.e., 10, 15 and 20% weight per volume. Growth and activity of the mixed culture on the subbituminous coal were monitored by measuring of medium pH, cell concentration, sulfate and organic sulfur concentration. The result showed that desulfurization activity of the mixed culture on 15% of coal was able to reduce sulfur up to 82.36%. Isolation and identification of the mixed culture based on genotypic and phenotypic characterizations revealed that members of the mixed culture were identified as genera of *Enterobacter*, *Lelcersia* and *Bacillus*. Observation on growth curves showing that the culturable isolates grew in at least three overlapping stages when using coal as sulfur source suggested that the members of the consortium worked alternately on coal as substrate.

Key words: Biodesulfurization, mixed culture, subbituminous coal, organic sulfur

INTRODUCTION

Coal is an abundant source of energy with increasing demand. According to Statistical Review of World Energy 2012 (BP, 2012), total world proved coal reserves at the end of 2011 reached 456,176 million tons and the average consumption was continued to increase more than 5% per year in the range of year 2001 to 2011. Nevertheless direct combustion of coal may release emission of sulfur dioxide. It has been generally known that sulfur dioxide is a toxic compound that combines with water to form sulfuric acid, the cause of acid rain.

Sulfur compounds in coal consist of organic and inorganic forms. Unlike inorganic sulfur compounds which can be reduced through physico-chemical processes, organic sulfur compounds can only be reduced through biodesulfurization. Through a metabolic pathway known as the 4S, microorganisms cleaved carbon-sulfur bonds selectively and accessed these bonds, then released organic sulfur from coal matrix by using their enzymes, without much affected the carbon content and calorific value of the coal (Kilbane and Jackowski, 1992).

Many studies have been carried out in studying the desulfurization bacteria, either in use of cellular metabolism as well as their enzyme properties (Kirimura *et al*., 2001; Young *et al*., 2006; Li *et al*., 2007; Torktaz *et al*., 2012). The sulfur-oxidizing bacteria that have been used in biodesulfurization were derived from soil (Monticello, 2000; Prayuenyong, 2002; Mohebali and Ball, 2008). In previous study, bacteria isolated from coal-soil mixture of South Sumatra were shown to use organic sulfur dibenzothiophene (DBT) as the sole source of sulfur when growing as single cultures (Pikoli *et al*., 2012) but they has not been examined in mixed culture.

It has been known that microbial mixed cultures can increase activity of the microorganisms, because they can move in synergy in the use of complex materials, thereby

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increasing the yield of biotechnology, such as in food, energy and environmental biotechnologies (Grostern and Edwards, 2006; Sieuwerts *et al*., 2010). The use of mixed culture in biodesulfurization has also been conducted by some researchers (Kayser *et al*., 1993; Constanti *et al*., 1996; Jorjani *et al*., 2004). However, those studies typically used mixed cultures prepared from monocultures that were far different from composition resembling the original consortium in nature. Hence, in the present study, we tried to examine a mixed cultures obtained directly from soil sample, with no previous separation of its members, while they were grown on coal as the sole sulfur source. It was expected that the diverse bacteria in the soil were able to live and interact each other in the conditions in coal like their environmental origin. The mixed culture were obtained from coal mine soil in South Sumatra, an area which has the largest coal reserves in Indonesia with the largest of subbituminous coal (National Coal Study Team, 2006). Desulfurization activity derived from this research can be developed to improve the quality of subbituminous coal with reduced organic sulfur content.

MATERIALS AND METHODS

Soil and coal samples: Soil and coal samples were sampled from coal mining area of Muara Tigo Besar Utara South Sumatera, authorized by Bukit Asam Company. The soil was categorized as silty-clay soil based on content of 46% silt and 54% clay. Result of proximate analysis (unpublished data) showed that the coal sample was categorized as brown coal lying between subbituminous and lignite, although the value of volatile matter and fixed carbon confirmed it as subbituminous coal (Speight, 2005). For obtaining bacterial mixed culture, natural particles of coal-mixed soil were filtered by 100-150 Mesh.

Medium, inoculum and culture condition: The medium used for isolating, maintaining and performing experiments with the mixed culture was a Mineral Salt Sulfur-free (MSSF) (Gunam *et al*., 2006) which sterilized by autoclaving at 121°C for 15 min after addition of 200 Mesh coal. The medium consisted of the following: 2.44 g of K_2HPO_4, 5.77 g of Na_2HPO_4, 2 g of NH_4Cl, 0,075 g of NaCl, 10 mL of mineral solution, dissolved in 1 L of demineralized water and added with 10 g of glucose. Source of mixed culture was prepared by culturing of 5 g of coal mine soil sample in 95 mL of MSSF containing 10% (w/v) of subbituminous coal. The soil culture was shake-incubated at 120 rpm in room temperature. After 24 h incubation, the culture was transferred to a fresh medium. The transfer was undertaken twice to the same kind of medium and incubated on the same condition. In the last transfer, the culture was incubated until its cells reached concentration of 10^6 cells mL^{-1}, by monitoring and counting the cells using a haemocytometer. This culture was ready to be inoculated in desulfurization examination. Beside MSFF medium, nutrient agar medium was also used to monitor cell growth during examination.

Growth and desulfurization examination: Three kinds of initial concentration of sub bituminous coal were used for examining growth and desulfurization activity, i.e., 10, 15 and 20% (w/v). Then each of the MSFF medium containing coal in accordance with the initial concentration was inoculated by 10% (v/v) of the soil culture inoculums. The mixed cultures were incubated at room temperature for 14 days whilst agitated at a speed of 120 rpm. Sampling was done every 3 h during the first 24 h and then continued every 24 h for the next day for 7 days, then once on the 14th day. Samples was measured their pH, cell concentration, sulfate and organic sulfur concentration.

Culture sample analysis: Each sample was filtered by membrane filter prior to analysis, to remove coal particles, except for cell growth analysis. Cell concentration (colony forming unit per mL) during growth on the coal medium was monitored by growing sample aseptically on nutrient agar by total plate count method. Each time interval of observation, different colonies were observed and counted. Sulfate concentration was measured by uv-vis spectrophotometer at wavelength of 420 nm. Concentration of organic sulfur used by the culture was known by measuring concentration dibenzothiophene (DBT). Liquid sample was acidified by HCl to pH 2 and extracted by ethyl acetate, in advance of measuring by uv-vis spectrophotometer at wavelength of 323.8 nm (Etemadifar *et al*., 2008).

Identification of culturable isolates: Identification was conducted by biochemical characteristics and molecular method. The mixed culture was grown on nutrient agar and purified in slant agar to have single cultures. Observation was performed on morphology of cell and colony and also biochemical activity and then matched to bacterial characteristics according to Bergey's Mannual of Determinative Bacteriology (Holt *et al*., 1994). To verify the identification results, each bacterial culture was subject to molecular identification as described below. A-48 h liquid culture was centrifugated at 7.5 K rpm for 5 min in 1.5 μL microtube to yield cells. The pellet was then suspended in 200 extraction buffer and freeze-thawed

by heated at 100°C for 1 minute and freezed alternately for 3 times. After centrifugated at 14 K rpm for 10 min, the supernatant was transferred to a new microtube and was run onto 1% agarose gel electroforesis. The genomic DNA in the gel was then subject to purification by Geneaid® Gel/PCR DNA Fragments Extraction Kit. The purified DNA was PCR-amplified by using universal primer set of 27F and 1492R with the following conditions: initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 45°C for 30 sec, elongation at 72°C for 2 min and final elongation at 72°C for 10 min. PCR product was then sequenced comercially (Macrogen, Korea). The similarity of DNA sequences compared to genbank in NCBI (http://www.ncbi.nlm.nih.gov/) was analyzed by using Blast 2.2.28+ (Zhang *et al*., 2000). Phylogenetic tree of the bacteria was constructed by using Neighbor-Joining tree method with 1000 of bootstrap value, by software MEGA5 (Tamura *et al*., 2011).

RESULTS AND DISCUSSION

Growth of community members: The mixed culture grew on the three kinds of coal concentration, which are 10, 15 and 20% (w/v) and there were only 4 to 5 types of colony observed during the plate count procedure (Fig. 1). Isolate 1 to 4 were viable on medium containing all kind of coal concentration, whereas isolate 5 was only detected in medium with higher coal concentration (15 and 20%). Isolate 1 was of the most dominated member of the community because it grew fast since early incubation until the end. The most important thing of this isolate that it facilitated other members to grow in the next step. It was suggested that isolate 1 grew while using glucose as carbon source. But they need sulfur from coal as the only sulfur source, so they took readily available sulfur released by autoclaving. Although they might liberate sulfur from coal matrix for others, the organosulfur user did not metabolize it in excess of its requirement for growth (Kayser *et al*., 1993). It was also consistent with the knowledge that commonly different species in a consortia exhibit mutual metabolic dependencies that include the exchange of nutrients (Ruhl *et al*., 2011).

Isolates 2 seemed to be the next user of coal at the later stage. It could be the one who used product of isolate 1 or even the competitor against isolate 1. Isolate 3 and 4 were considered as members of minority in the mixed culture, as they appeared only in the middle to late of incubation time. They grew when pH was low and stable (Fig. 2).

Desulfurization activity: During mixed culture growth, desulfurization activity was monitored by measuring pH, sulfate concentration and DBT concentration. In all of the

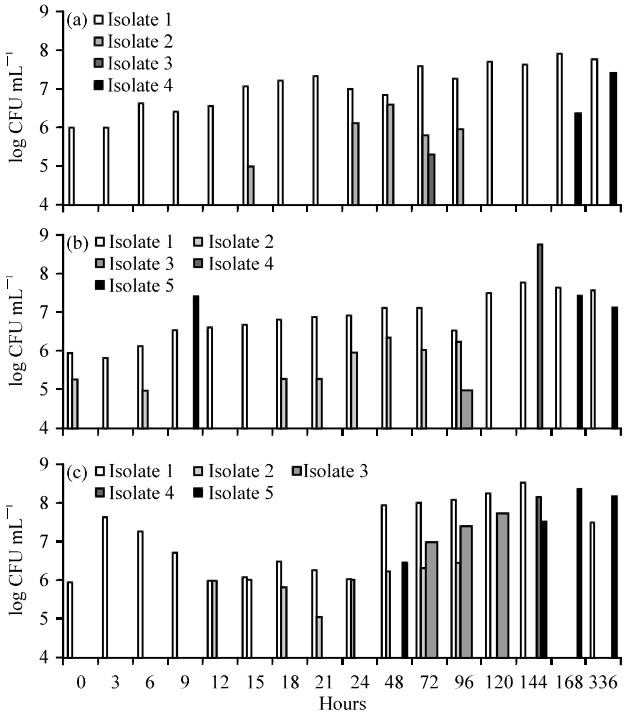

Fig. 1(a-c): Growth of mixed culture in MSFF containing subbituminus coal; (a) 10, (b) 15 and (c) 20%

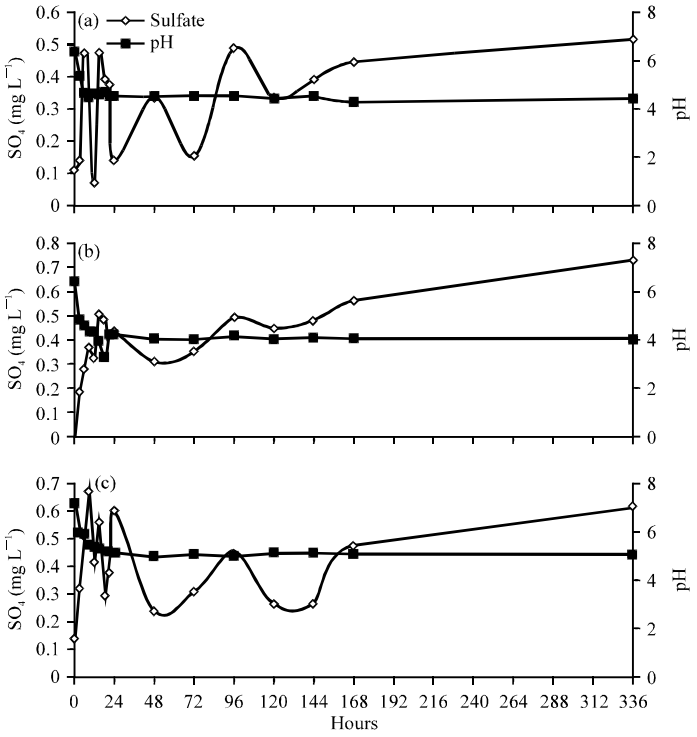

Fig. 2(a-c): Sulfate concentration and pH during growth of the mixed culture in MSFF containing subbituminus coal; (a) 10, (b) 15 and (c) 20%

three kind concentrations of coal, decrease in pH showed a similar pattern, i.e., decreased sharply in the first day, then relatively constant in the range of pH 3-4. Similarly, sulfate concentrations in the three medium showed a similar pattern, which showed fluctuations at similar points (Fig. 2). These were connected to DBT utilization by the first users when the DBT concentrations also decreased in those times (Fig. 1, 3) that could be related to the activity of microorganisms. Fluctuations in sulfate production were attributed to microorganisms metabolizing alternately in using coal as a substrate. Coal is a complex material, as well as bacteria in the soil sample; a complex set of members of the consortium, each member has a certain nutrition and therefore a specific role. This was in accordance with that proposed by Jones *et al.* (2010) that coal mineralization to simpler compounds happened through a series of changes in coal by different microorganisms.

Fluctuations in sulfate concentration associated with organic sulfur desulfurization and depyritization. When those mechanisms happened concomitantly, the oxidation of sulfur would lead to high concentrations of sulfate. But when one mechanism occurred more than other mechanisms, it would result in lower sulfate concentration. In metabolic pathway known 4S pathway, organosulfur users secreted degrading enzymes which broke carbon bonds, releasing organic sulfur from coal bound and produced sulfate as final byproduct (Del Rio *et al.*, 1994). Pyrite-using microorganisms are different from organosulfur-using microorganisms. Since the mixed culture was soil-source, the pyrite users were very possibly contained in it. In the process that produced sulfate, microorganisms first oxidized pyrite (FeS_2) into ions Fe^{3+} which then reacted with the pyrite (FeS_2) and produced ions Fe^{2+} (Gunam *et al.*, 2006). It would also happen that $2Fe^{2+}+2H^{+}+0.5O_2 \rightarrow 2Fe^{3+}+H_2O$, yielding $Fe(OH)_3$ which was alkaline (Prayuenyong, 2002). Decrease in pH value might also occur due to content of high humic substances in coal. Pyrolysis and oxidative degradation on low rank coal released various acid compounds, so when the desulfurization process took place, the coal would be separated into groups in medium and caused decrease in pH value of the medium. Meanwhile, the decline in the measured concentration of sulfate ions can result from the ability of the bacteria were able to reuse the SO_4^{2-} as a source of sulfur for growth, so that the amount of SO_4^{2-} in the medium was not much (Omori *et al.*, 1992).

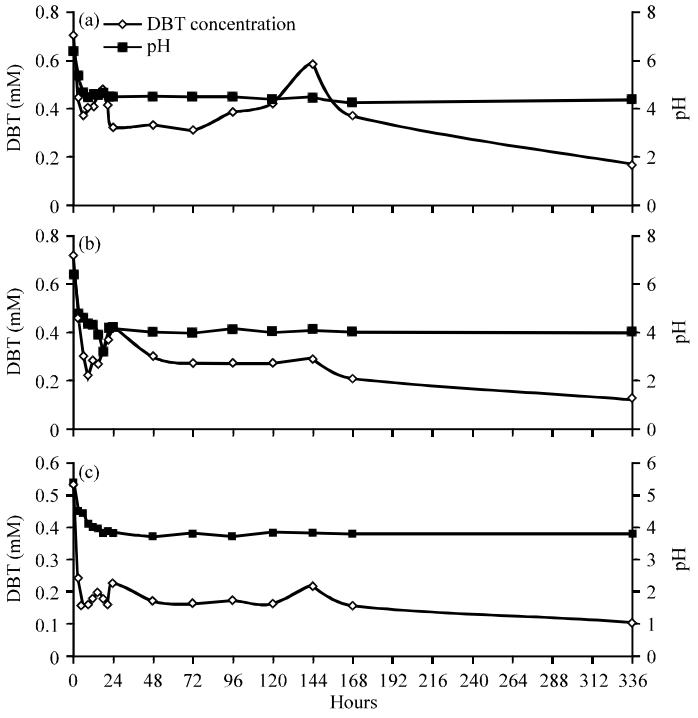

Fig. 3(a-c): DBT concentration and pH during growth of the mixed culture in MSFF containing subbituminus coal; (a) 10, (b) 15 and (c) 20%

Desulfurization activity by the mixed culture could also be shown by reduction in DBT concentration. DBT is an organic sulfur compound that makes up to 40-60% of organic sulfur in coal (Constanti *et al*., 1996). DBT concentration curve shown in Fig. 3 showed similar pattern among the three media with different concentrations of coal. At the start of incubation to maximum of the first day, DBT concentrations were decreased, which could be due to the massive use of DBT by dominant members of consortia in the mixed culture. DBT would dissolved in the medium due to autoclaving treatment. And then, the concentration of DBT experienced some increase points, followed by constant decline lines. This occurred due to release of DBT from coal caused by enzymatic activities produced by members of the mixed culture, aided by the agitation. This suggested that the mixed culture of coal mine soil that was used as a source of inoculum had ability in desulfurizing coal as one important substrate for growth of bacterial cells. The total decrease since the beginning of incubation until the fourth day i.e., up to 75.89, 82.36 and 80%, in medium containing of 10, 15 and 20%, respectively of coal showed no significant difference among them.

Morphological and phenotypic characteristics of isolates: Characterization of bacterial isolates phenotypically done in an effort to determine the reference strain for phylogenetic identification. Phenotypic characterization performed in this study include: colony morphology, cell morphology, biochemical activity assay. The results were then identified with the character of the genus or species of bacteria in the book Bergey's Mannual of Determinative Bacteriology (Holt *et al*., 1994). Description of the five isolates is shown in Table 1.

Morphological and biochemical characterizations of culturable members of the mixed culture showed that isolates 1, 2, 3 and 4 belonged to family Enterobacteriaceae, while isolate 5 to family Bacillaceae. Sequencing and analysis of genes supported those results. 16S rRNA sequences of the five isolates were compared with the corresponding sequences of strains obtained from the GenBank database. Phylogenetic tree showed that the five culturable bacteria in mixed culture isolated from coal mine soil were divided into two clusters (Fig. 4). The results showed that isolate 1 had 99% similarity to *Enterobacter hormaechei* strain WW2, isolate 3 to *Leclersia* sp. OTU28, isolate 4 to

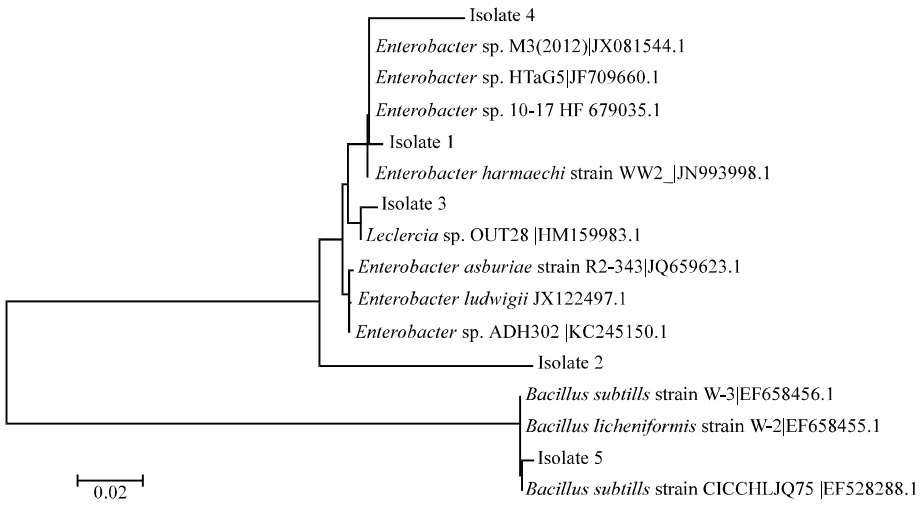

Fig. 4: Phylogenetic tree of five isolates related to two families, Enterobacteriaceae and Bacillaceae

Table 1: Characteristics of isolates

Characteristics	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Colony					
Form	Circular	Circular	Circular	Circular	Irregular
Color	White	White	Brownish white	Yellowish white	White
Elevation	Raised	Raised	Raised	Raised	Raised
Edge	Entire	Entire	Entire	Entire	Undulate
Cell					
Form	Rod	Rod	Rod	Rod	Rod
Endospore	No	No	No	No	Yes
Gram reaction	Negative	Negative	Negative	Negative	Positive
Biochemical					
Starch hydrolysis	-	+	-	-	+
Fat hydrolysis	+	+	+	+	-
Casein hydrolysis	-	-	-	-	+
Glucose fermentation	+	+	-	+	+
Sucrose fermentation	+	+	-	+	+
Lactose fermentation	+	-	+	+	-
Methyl Red	+	-	+	+	-
Voges Proskauer	+	+	-	+	+
Nitrate	+	+	+	+	+
Urea hydrolysis	+	+	+	+	-
Tryptone	-	-	-	-	-
Citrate	+	-	-	+	-
Catalase	+	+	+	+	+
H_2S production	-	-	-	-	-
Triple sugar iron	Yellow, gas	Yellow	Pink surface, yellow bottom	Yellow	Yellow
Motility	+	+	-	-	+
Gelatin hydrolysis	+	+	+	+	+
Litmus milk reaction	Litmus reduction	Alkaline reaction	Alkaline reaction	Alkaline reaction	Litmus reduction

Enterobacter M3 (2012), as well as isolate 2 which belonged to Enterobacteriaceae. Isolate 5 had 99% similarity to *Bacillus subtilis* CICCHLJ Q75.

CONCLUSION

The mixed culture derived from coal mine soil showed biodesulfurization activity based on ability to grow in free sulfur medium but containing subbituminous coal, decrease in pH, fluctuations in sulfate concentration and decrease in DBT concentration. Monitoring on the growth of culturable bacteria showed that sulfur in complex medium such as coal can be reduced when it was used by mixture of microorganisms which grew alternately in overlapping stages, so it can achieve a high percentage of sulfur reduction.

ACKNOWLEDGMENTS

Technical and administrative assistance in sampling of the coal and coal mine soil were greatly appreciated to Ir. Eko Pujiantoro, Drs. Supaimin and their staff from PT. Bukit Asam Tbk.

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