Biodegradation of BTEX by indigenous microorganisms isolated from UCG project area, South Sumatra

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Abstract. Recently Indonesia is conducting Underground Coal Gasification (UCG) project in South Sumatra for power generation. The potential of negative impacts from UCG on groundwater and the broader environment cannot be ignored since past similar projects were often confronted with pollution issues of BTEX and PAHs due to condensation of tar-loaded gas. This study focuses on finding indigenous microorganisms capable of BTEX degradation and evaluate their biodegradability. Several microorganisms were successfully isolated and screened. Pseudomonas putida and Bacillus cereus were chosen for this bioremediation study since the bacteria were predominant and highly viable on the screening test. The BTEX degradation has been studied in single component using single and mixed bacterial cultures in the concentration range of 250-500 ppm. The experimental results show that biodegradation of BTEX by P. putida ranged from 61.4-70.2% and by B. cereus ranging 63.9-74.7% at initial BTEX concentration of 500 ppm. Meanwhile, consortium of both isolates has the highest percentage of BTEX biodegradation (67.8-79.8%) during 14 days of retention time. The findings reveal that indigenous bacteria of P. putida dan B. cereus exhibit the potential to be used for decontamination of BTEX as an anticipated mitigation for potential pollution coming from the UCG project.

1. Introduction
The fast depletion of Indonesian resources of oil and natural gas, and the apparent immaturity of renewable energy sources, have brought "the old king coal" to the fore once again. However, the coal should be used with maximum energy efficiency - a much better efficiency than the traditional combination of conventional coal mining coupled with coal-fired power generation. Coal must be able to compete with any other energy sources in terms of cost, technical ability and also environmental performance.

Indonesia is currently conducting Underground Coal Gasification (UCG) project at pilot plant scale in South Sumatra as it has potential for long term energy supply in the future. UCG converts coal seams in the ground by extracting and making use of the vast unminable coal resources into combustible gas (syngas), eliminating the need to mine coal to use and it gives the highest energy efficiency compared to any other way of using coal. In chemical applications, a wide array of products can be economically synthesized from syngas; some of these are methanol, synthetic liquid
fuels, synthetic natural gas, ammonia and ammonia-derived products, dimethylether (DME), and monomers[1,2].

UCG is the promising technology having a lot of health, safety and environmental advantages over the conventional mining techniques. The major motivational aspects of UCG involves increased worker health and safety by using no man underground, larger coal reserves exploitation, no surface disposal of ash and coal tailings, low dust and noise pollution, low water consumption, and low volatile organic components, methane and green house gases emission to atmosphere. UCG is an inherently clean coal technology as it reduces sulfur and nitrogen oxide emissions to very low levels. Total solid waste from UCG is typically half the volume generated by conventional coal plants and water use is substantially lower as well [3]. Nevertheless, the potential for negative impacts from UCG on groundwater, public health and the broader environment is still exist and can not be ignored. The control of soluble metal elements and organic compounds in UCG by-product is of great importance for groundwater protection [4,5]. Several past UCG projects have been confronted with organic groundwater pollution issues in the form of enhanced benzene, toluene, ethylbenzene, and xylene (BTEX), polycyclic aromatic hydrocarbons (PAHs) and heterocyclic compound concentrations due the condensation of tar loaded gas-losses [6,7,8,9]. At the worst, BTEX release into the environment can causes very severe public health problems because these compounds have neurotoxic, mutagenic, and carcinogenic effects not only for humans but also for flora and fauna and other organisms[10,11]. This problem has led to the shutdown of the Australian UCG site Kingaroy, where enhanced benzene and toluene concentrations were detected in the local groundwater [12,13].

Nowadays, biological methods such as microbial biodegradation were developed as the most influential, low-cost, and ecofriendly clean-up strategies for BTEX removal from contaminated sites[14]. Many research have focused on BTEX degradation in contaminated groundwater, where techniques such as natural attenuation are viable and sustainable options. In situ bioremediation of aromatic hydrocarbons contaminated groundwater by naturally occurring microorganisms is quite possible [15]. Currently, most efforts are directed towards finding bacterial communities which are able to mineralize contaminants into less harmful, non-hazardous compounds [16]. The main purpose of the present study was to find and isolate indigenous microorganisms capable of BTEX degradation from UCG project area in South Sumatra, and evaluate their biodegradability in the pure and mixed cultures. This work was taken up to develop a tolerant bacterial consortium that could degrade BTEX for field application in the near future.

2. Materials and Methods

2.1. Chemicals and media
BTEX were purchased from Merck. All other chemicals used in the present study were of all the highest purity available. The mineral salts medium (MSM) was composed of (g/L of distilled water) 1.0 (NH4)2SO4, 1.0 KH2PO4, 1.0K2HPO4, 0.2 MgSO4.7H2O, 0.02 CaCl2.2H2O and 0.05 FeCl3. The desired pH was adjusted using 0.1 mol/L H2SO4 or 0.1 mol/L KOH and measured by pH meter. After adjusting the pH to 6.8, the solution was autoclaved at 121°C for 15 minutes. BTEX individually were aseptically added to MSM as the sole carbon source using a microsyringe, directly from the stock solution to give desired final concentration.

2.2. Sample collection
Coal and soil samples were collected from several hydrocarbon rich spots at UCG project area, South Sumatra. Coal samples were taken from a bore hole at the depth of around 300 m. Soil samples were collected from the ground surface to a depth of 10-20 cm. Samples were collected in sterilized, labeled plastic bottles, kept on ice and were brought to the laboratory for analysis and bacterial isolation. The physical state, location, temperature and code number of these sites are given in Table 1.
2.3. Isolation and purification
Aerobic culturable bacteria were isolated by inoculating 1 g of the coal or soil in an amber serum bottle containing 100 ml of Mineral Salt Medium (MSM) broth amended with 250 mg/l of individual BTEX. The bottle was sealed with an aluminum crimp cap and placed on a shaking incubator (120 rpm) at room temperature of 25-30 °C for 48 h. After incubation, the MSM culture bacteria was serially diluted and 0.1 ml of the appropriate dilution was plated on nutrient agar using the spread plate technique. The plates were incubated at room temperature for 5-7 days and checked for bacterial growth. Morphologically distinct colonies were selected, purified and stored at 4 °C for further analysis.

2.4. Determination of BTEX tolerance
To determine the level of BTEX tolerance, 1% of the overnight grown enriched cultures were inoculated in 20 ml MSM broth supplemented separately with individual benzene, toluene, ethylbenzene and xylene in concentration of 250-500 ppm in 100 ml screw cap flask and incubated at room temperature under shaking (120 rpm) for 48 h. After incubation, 125 μl of broth culture was loaded into pre-prepared wells (8mm) at mineral salt medium agar. The plates were incubated at room temperature for 7 days. The organisms that formed clear zones around the wells were considered as BTEX degraders. All the experiments were carried out in triplicate.

2.5. Growth of bacteria in the presence of BTEX
To obtain a growth profile of potent strains, 1% of the overnight grown enriched cultures, that showed tolerance towards high concentration of BTEX, were inoculated in 100 ml MSM broth supplemented separately with benzene, toluene, ethylbenzene and xylene (500mg/L) in 250 ml screw cap flask and incubated at room temperature under shaking (120 rpm) for 5 days. Growth of the bacterial strains in the presence of BTEX was determined by estimating the number of colony forming units (CFU) per ml according to the method reported by Tehrani and Herfatmanesh [17] at a constant time interval of 8 h till 120h. All the experiments were carried out in triplicate.

2.6. Determination of BTEX degradation
The best two BTEX tolerant and adapted strains were further analyzed to determine their rates of BTEX degradation. A one ml log phase culture of each isolate was inoculated into separate, 100 ml serum bottles containing 59 ml of MSM. Benzene, toluene, ethylbenzene, and xylene were added individually for a final concentration of 500 mg/l each and the bottles sealed with aluminum crimp caps. Sets of 96 bottles were prepared for each bacterial isolate and incubated on a shaking incubator (120 rpm) at room temperature. Samples (40 mL) were removed at 1, 2, 4, 6, 9, 12, 14 days, and BTEX degradation were analyzed gravimetrically according to method performed by previous works.

### Table 1. The location and physical condition of collected samples

| No. | Sample type                  | Sample temperature | Sample pH | Sample code |
|-----|------------------------------|--------------------|-----------|-------------|
| 1   | Claystone outcrop            | 25 °C              | 6.8       | S1          |
| 2   | Coal outcrop                 | 25 °C              | 6.9       | S2          |
| 3   | Soil around outcrop          | 25 °C              | 6.8       | S3          |
| 4   | Coal seam D, 299 m           | 25 °C              | 6.8       | B1          |
| 5   | Coal seam D, 307 m           | 25 °C              | 6.8       | B2          |
| 6   | Claystone, 308 m             | 25 °C              | 6.8       | B3          |
| 7   | Coal seam D, 311 m           | 25 °C              | 6.8       | B4          |
| 8   | Coal seam D, 316 m           | 25 °C              | 6.8       | B5          |
| 9   | Coal tar as received         | 25 °C              | 6.9       | T           |

Sample pH was analyzed gravimetrically according to method performed by previous works.
Degradation was finally confirmed by Gas Chromatography analysis as previously described by Borah and Yadav [21] also Madhavi [22].

To observe the influence of single and mixed culture conditions on BTEX degradation, three set of batch experiments were conducted using single culture of *P. putida*, *B. cereus* and concorlatia at pH 6.8. Sterile 1 M NaOH and 1 M HCL were used to adjust MSM pH. During the incubation, concentrations of the BTEX compounds in the MSM were determined as previously described. These experiments were carried out in triplicate. Parallel experiments with serum bottles without inoculation were also prepared and served as controls to correct for BTEX removal due to abiotic losses. Liquid sample aliquots were periodically withdrawn to measure pH, and BTEX concentrations. The pH of the medium generally dropped slightly from the initial value of 6.8 to 6.2 at the end of each batch experiment. BTEX concentrations in the control bottles (without microorganisms) decreased by less than 1% at the end of each batch study.

3. Results and Discussion

3.1 Isolation
Potential hydrocarbon degrading microorganisms were isolated on the basis of their growth on MSM agar medium supplemented with BTEX. Almost 50 morphologically different colonies were successfully isolated from the coal and soil samples. Of these 50 bacterial strains, 9 microorganisms were screened on the basis of capability to tolerate high concentration of BTEX. Among those isolates, two of them exhibited maximum viability and tolerance towards BTEX compounds. Based on identification using API kit analysis and PCR, the two isolates were identified as *P. putida* and *B. cereus*. These species have also been reported as a potential hydrocarbon degrader in the previous works [18,21,23]. However, in the current study, *P. putida* and *B. cereus* should be new and novel strains of indigenous bacteria since they were isolated from unconventional source, i.e. UCG project site. For the very first time, the characterization of bacterial growth and BTEX degradation were then carried out in this work.

3.2 Bacterial growth
Growth of the bacteria is the primary requirement to carry out biodegradation. Figure 1 shows the growth of *P. putida* and *B. cereus* on individual BTEX in serum bottle at room temperature. The two strains exhibited almost the same growth pattern in the presence of individual BTEX. In the initial 24 hours they were in their lag phase as there was no gradual increase in the CFU number. *P. putida* attained their log phase between 24 to 84 hours of incubation and gradually entered the stationary phase from 84 hours. The CFU reaches the maximum value of 7.7×10^6/mL, and the steady state was achieved further. It is inferred that the growth of *P. putida* increased up to 84 hours and maintained till 96 hours and decreased thereafter.

A one day lag phase was also noted in the growth of *B. cereus* on individual BTEX. There was a sharp increase in growth from 24 hours and lasted until 96 hours. The maximum CFU of *B. cereus* at this time was 9.99×10^6 CFU/ml. It entered the stationary phase thereafter. The two isolates have shown very good growth on BTEX, suggesting that the strains could tolerate a higher toxic concentration of BTEX. Indigenous bacteria had been exposed to these toxic substrates (BTEX) for long period and thus must have been adapted well in the environment.
3.3 BTEX degradation

Figure 2 shows typical biodegradation kinetics for free cells of *P. putida* grown on individual BTEX as the sole carbon source in serum bottle. As seen in this figure, the culture used BTEX for cell growth; BTEX degradation occurred as cells grew in the serum bottle. During the second day until the last 14 days of experiments, an increase in the degradation was observed. The isolate was found to be degrading 66.7, 70.2, 68.2 and 61.3% of benzene, toluene, ethylbenzene and xylene respectively after 14 days. However, at the end of experiment after 14 days of retention time, cell growth still continued and BTEX degradation also still continued and was not complete yet. Therefore, biodegradation time tested was insufficient for the total elimination of BTEX, implying the need for periods exceeding of at least 5 days in order to achieve a complete degradation.

Similar degradation kinetics was also observed with *B. cereus* grown on individual BTEX as a sole carbon source in serum bottle culture (Figure 3). The isolate degraded 68.3, 74.7, 72.1 and 63.8% of benzene, toluene, ethyl benzene and xylene respectively after 14 days. At the end of the experiment, the degradation likely have not reached the maximum value and it needed some more several days to
be completed. In this study, the ability of *B. cereus* was slightly better than *P. putida* in degrading higher amount of BTEX, however, the difference was not significant.

![Figure 3. Typical BTEX biodegradation by B. cereus](image)

Table 2 shows the comparison of the BTEX degradation rates for single culture of *P. putida*, *B. cereus* and consortium achieved in this study. The difference between single and mixed cultures was especially evident for toluene biodegradation. Single culture of *P. putida* and *B. cereus* degrade 70.1 and 74.7% of toluene respectively at initial concentration of 500 mg/l, while consortia continued to degrade toluene at 79.7% mg/l, which was the highest degradation in this work.

**Table 2. Comparison of biodegradation rate of BTEX by *P. putida*, *B. cereus* and consortia**

| Bacteria   | Hydro-carbon | 0  | 2  | 4  | 6  | 8  | 10 | 12 | 14 |
|------------|--------------|----|----|----|----|----|----|----|----|
| **Control** | Benzene      | 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0|
|             | Toluene      | 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0|
|             | Ethylbenzene | 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0|
|             | Xylene       | 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0| 0.0|
| **P. putida** | Benzene      | 4.2| 10.8| 21.4| 40.3| 59.1| 64.6| 66.7| 66.7|
|             | Toluene      | 5.5| 11.7| 21.3| 44.6| 63.7| 67.0| 70.1| 70.1|
|             | Ethylbenzene | 4.0| 8.2| 22.7| 43.4| 64.0| 66.8| 68.2| 68.2|
|             | Xylene       | 2.1| 7.5| 15.9| 35.1| 46.8| 58.4| 61.3| 61.3|
| **B. cereus** | Benzene      | 5.7| 14.3| 31.3| 51.1| 59.9| 64.5| 68.3| 68.3|
|             | Toluene      | 7.7| 20.4| 44.1| 62.5| 66.6| 71.4| 74.7| 74.7|
|             | Ethylbenzene | 6.6| 18.3| 39.3| 54.5| 62.3| 66.8| 72.1| 72.1|
|             | Xylene       | 2.8| 13.1| 34.4| 51.8| 56.6| 60.9| 63.8| 63.8|
| **Consortia** | Benzene      | 9.9| 23.9| 43.6| 61.0| 64.8| 70.7| 72.5| 72.5|
|             | Toluene      | 13.8| 23.1| 43.7| 67.0| 73.0| 77.3| 79.7| 79.7|
|             | Ethyl-benzene| 11.6| 21.0| 40.0| 60.9| 66.5| 72.9| 75.6| 75.6|
|             | Xylene       | 4.2| 14.0| 36.9| 58.3| 62.1| 65.6| 67.7| 67.7|
Table 2 also shows the BTEX degradation as a function of time. It is observed that the degradation rate of BTEX increased along with the increase in incubation time. Degradation of BTEX was observed to be 2.1-13.8% in 2 day and 79.7% after 14 days, and seemingly still to be continued in longer time. The degradation rate grew between day 2 and 14. The degradation of BTEX follows the same trend as microbial growth. According to Bordenave et al. [24], the individual bacteria could metabolize only to a limited hydrocarbons and the complete biodegradation requires mixture of different bacterial groups. In contrast to this, the results of current study reveal that the individual culture of P. putida and B. cereus could degrade a wide range of hydrocarbons present in the solution. Both isolates probably have a diverse range of metabolic activities for degradation of various aromatic hydrocarbons. Therefore, the broad mineralization capacity exhibited by the mixed culture in this study is not surprising and may be attributed either to the presence of different microbial species with a number of metabolic pathways and/or to interspecies interactions. The mineralization of BTEX by the pure cultures is a unique result and merits further study. Finally, comparison of the mineralization potentials of the mixed and pure cultures revealed that the mixed culture is slightly more effective than the pure cultures in mineralizing BTEX. The two isolates in this study exhibited a biodegradation pattern that were a subset of that exhibited by the mixed culture. Mixed cultures therefore may be more effective than pure cultures in biotreatment systems because interspecies interactions may be necessary for the complete biodegradation of multicomponent hydrocarbon mixtures. Indeed, similar findings have been also reported in previous research by Sarkar, Rai and Ghosh [25], also Sarkar et al., [26].

The performance of each isolate on degradation of individual BTEX was evaluated. The specific degradation rate for each substrate by single culture of P. Putida, B. cereus, as well as mixed culture increased in the order of X < B < E < T. This differed slightly from Shim et al. [27], who showed BTEX degradation by P. putida increased in the order of E < X < B < T. Differences between the degradation order can be attributed to differences in the strains which originated from different environmental conditions, which can have different BTEX degradation pathways [27]. The reduced degradation rate of xylene may be due to toxicity to the isolate. A little bit prolonged lag and less growth of the isolate P. putida and B. cereus in media containing xylene further confirmed the toxicity of this compound. The degradation of BTEX can be expected to vary among bacterial species. Lee and Cho [28] reported BTEX degradation by Rhodococcus sp. in the order of E < X < T. Shim et al.,[27] reported BTEX degradation by P. putida in the order of E < X < B < T.

Biodegradation time tested was insufficient for the total elimination of BTEX, implying the need for periods exceeding 5 days in order to achieve a complete degradation. The results demonstrated that the selected bacterial isolates could be effective in biodegradation BTEX individually and showed better biodegradation abilities when they are used together in mixed consortium. More effective bioremediation of highly recalcitrant compounds like BTEX, is most likely to rely on a consortia of microorganism rather than on the action of a single microorganism.

The study of microbial communities in a specific area in South Sumatra, in relation to their natural ecology with special condition of coal, soil, weather and water has initially conducted for future field application of UCG project. Microorganisms in an environment that never having been exposed to such factors would not have the ability to resist. In specific environments, the phenomena of horizontal/vertical transfers, mutations and adaptation to environmental stressors increase the metabolic capabilities of bacteria, including expanding their range of substrates, and allow their adaptation to new substrates. All bacterial cell components (physical structure and the functions of the organelles and metabolic activities) are regulated and controlled by the microenvironment. This approach will help answer the question on the different microbial communities that are available in UCG’s coal and soil and how they are unique in some biological activities. Here, it is showed that the isolation and screening strategy affected a lot, the selection of the hydrocarbon-degrading bacteria, by combining the bacterial tolerance to hydrocarbon toxicity to the hydrocarbons degradative activities. Strains’ response to altered growth conditions would be varied substantially, although they were from the same taxonomical group.
The wide varieties of aromatic compounds released into the environment through different human activities can be metabolized by coal and soil bacteria that have specialized metabolic capacities. This is of great importance in environmental cleanup technologies. It is evident from this study that hydrocarbon degrading organisms are ubiquitous in the environment and they can be isolated from hydrocarbon rich sites.

Concentration of salts, organic and inorganic, due to evaporation in the cavity is unlikely to have any significant impact, as these will be mostly extracted in cleanup or rapidly diluted to near background levels when the cavity refills. Meanwhile, the potential for groundwater contamination by BTEX from UCG project is a complex consideration, as it is strongly dependent on the quality of the cleanup process. Contamination of the groundwater due to dispersal of soluble organics, such as BTEX, from residual non-aqueous phase organics in the affected area is more complex to analyse. There is insufficient data to accurately model the decay in the release rates of these compounds and the rates of adsorption and reaction that may remove them from the water, so the modelling assumed they would continue to be released at a constant rate and flow. Therefore, the contaminants are predicted to slowly spread into the coal seam and overlying aquifers. The rate of spread is extremely slow, so additional cleanup operations could be performed a considerable time after the operations without widespread contamination, but it will require careful placement of monitoring wells to detect the spread early. The dilution of contaminants with flow is likely to mean that concentrations will not be significant unless a significant source of drinking quality groundwater is close to the coal seam. It is expected that a well monitoring system would be required for a number of years after operation of a UCG project site.

As a general view so far, the UCG project has not found any critical issues that are likely to prevent implementation of UCG in South Sumatra. However, there are some areas of design and operation of UCG processes and social engagement that require care. The impact of UCG in terms of groundwater use and subsidence appear acceptable, but the operating techniques used, especially cleanup, require careful planning and monitoring to minimise the risks of groundwater contamination. No strongly hindering social or legislative issues were found, but further engagement with the public and regulatory authorities is advised.

The current study showed successful BTEX degradation in liquid culture by indigenous bacteria *P. putida* and *B. cereus* strains isolated from an unconventional source, i.e., the UCG project site. For the very first time, characterization of BTEX degradation was carried out, showing its significant potential which may attract its future filed application toward UCG project in South Sumatra. These findings prove the isolated strains as potential tool for BTEX bioremediation purpose.

### 4. Conclusions

The present study was carried out to obtain indigenous BTEX tolerant microorganisms from UCG project site that could degrade BTEX compound, and evaluate their degradation efficiency in single and mixed culture condition. It has been successfully isolated almost 50 different types of bacterial strains from different spots of UCG project area in South Sumatra. Of these 50 bacterial strains, 9 microorganisms were screened on the basis of capability to tolerate high concentration of BTEX. Two isolated bacteria were then selected for testing BTEX biodegradation from liquid culture media. Each of these bacteria was able to degrade BTEX but with different efficiencies. Maximum biodegradation efficiency for *P. putida* was 70.2%, *B. cereus* was 74.7% and 79.8% by mixed culture. With the knowledge of degradation of BTEX using bacteria in the laboratory scale experiments, it would be possible to develop approaches for using bacteria for the removal of BTEX from polluted water. All of the results demonstrated that the bacteria selected in this study is favorable for the bioremediation of sites contaminated with BTEX. This investigation had achieved its primary objective of finding indigenous microorganism that could be employed in the bioremediation of water polluted by BTEX as anticipated mitigation for UCG project in South Sumatra. Further studies are needed to explore more their efficiency, increase degradation rate and reduce time span of biodegradation to develop a robust bioremediation process.
5. References

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**Acknowledgements**

The authors are thankful to the UCG Project of R&D Centre for Mineral and Coal Technology, Ministry of Energy and Mineral Resources for financial support to carry out this research. The authors acknowledge Biology Department of Padjadjaran University for providing some laboratory facilities to conduct the experiments, also acknowledge LIPI Serpong for successfully analyzing the DNA sequences for the identification of the isolates mentioned in the manuscript.