IDENTIFICATION OF BACTERIAL STRAINS CAPABLE OF DEGRADING MALAYSIAN PETROLEUM SLUDGE

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ABSTRACT

In an organic sludge biodegradation process, identification of bacterial strains with effective biodegradation capabilities indigenous to the sludge is an important step as isolation and subsequent usage of such strains can enhance the rate of the biodegradation process. In this study, the identification of bacterial strains capable of degrading Malaysian petroleum sludge was carried out using a biolog system kit subsequent to enrichment, isolation and degradation processes. A total of 53 purified strains was obtained from the isolation process. Three strains, namely, *Clavibacter michiganesis ss insidiosus, Rhodococcus rhodochrus* and *Brevibacterium otitidis* showed favorable degradation results for nearly all hydrocarbons especially for polycyclic aromatic hydrocarbon (PAHs) (anthracene, phenathrane and dibenzothiophene),. *Brevibacterium otitidis* is especially interesting since it is the first time such a strain has been reported to have good PAHs degradation capabilities.

Keywords: Bacterial strains, biodegradation, petroleum sludge, enrichment, isolation, identification

1. INTRODUCTION

Petroleum refineries generate huge volumes of petroleum sludge during the refining process of crude oil. It is well-established that petroleum sludge contains toxic, mutagenic and carcinogenic compounds that constitute health hazard to human (Mishra *et al*., 2001). Recent advances in molecular biology have extended our understanding of the metabolic processes related to microbial degradation of petroleum hydrocarbons. Long recognized as substrates supporting microbial growth, hydrocarbons are both a target and a product of microbial metabolism (Jonathan *et al*., 2003). The biggest challenge in the bioremediation of oily sludge is to isolate, select and identify the best consortium of microorganisms that is capable of biodegrading the sludge. To date, there were several studies on biodegradation of oily sludge. Sabate *et al*. (2004) showed that bioremediation occurs through high metabolic activity of indigenous microbial populations in degrading total petroleum hydrocarbon (TPH). Marin *et al.* (2005) reported that the bioremediation of refinery sludge by landfarming can successfully degrade 80% of total hydrocarbons in 11 months. Ghazali *et al*. (2004) reported on usage of consortia of bacteria that include *Bacillus* and *Pseudomonas spp*. to degrade linear chain hydrocarbon.

While there are many similar studies on biodegradation of oily sludge, nonetheless, there is no identified literature that reports on biodegradation of polycyclic aromatic hydrocarbons (PAHs) using microbial populations indigenous to Malaysian petrochemical sludge. The main objective of the study is to identify microbial strains that are capable of degrading low-chain hydrocarbon and polycyclic aromatic hydrocarbons (PAHs) from a mixture of petroleum sludge subsequent to enrichment, isolation and degradation processes. Findings from this study will further assist in future quantitative degradation studies and design of petroleum sludge biodegradation systems.

2. METHODOLOGY

Petroleum sludge samples were obtained from *ExxonMobil* petroleum refinery treatment plant in Kerteh, Terengganu. Twenty kilograms of sample were collected, stored and transported to the laboratory in UiTM, Shah Alam. The storage and transportation of the sample were conducted in strict compliance to the requirements set by the Department of Environment (DOE), Malaysia.

The enrichment process was carried out to enhance degradation rates using standard culture enrichment technique recommended by Manee *et al*. (1998). Bacteria strains capable of degrading hydrocarbon were obtained from petroleum sludge via standard culture enrichment technique with individual hydrocarbon as the sole carbon and energy source. The isolation process involved the use of the streaking technique. After 21 days of enrichment, 0.1 ml of culture from each universal bottle was aseptically suspended in 1-ml centrifuge tube containing 0.9 ml sterile ultra pure water. The mixture was homogenized via a vortex mixer. Serial dilution was made ranging from 10^{-1} to 10^{-6}. Each dilution was streaked on agar plate supplemented with hydrocarbon. The plate was incubated for three weeks at 28 °C. Colonies showing a clear zone on the crystalline hydrocarbon layer were picked and cultured in new Bushnell Hass medium for seven days. The cultures were then streaked on new Bushnell Hass agar. A rapidly growing, visually distinct colony and a separate, morphologically unique isolate were selected for further analysis and purified by repeated plating.

Hydrocarbon degradation test in this study was done by using the microtiter plate technique. INT indicator was used in this study. Reduction of INT was used to detect positive wells where hydrocarbons where degraded. Five microtiter plates were set up with each well containing 180 µL of sterilized Bushnell Hass medium, 40 µL of cell and 7 µL of hydrocarbon. Each well was occupied by different strains and hydrocarbons. Seven (7) µL of INT indicator was added to each well after 21 day.

Colony that appeared was used for identification test by using Biolog System Kit. Bushnell Hass Agar plates were prepared and spread with 2 % of a hydrocarbon source. One loop of each active strain was streaked on the agar plate. The plates were incubated at room temperature (about 28° C) for one week. The appeared colony was subsequently transferred to inoculating fluid using sterile swab and the optical density was adjusted to 66 %. The inoculating fluid (150 µL) was then transferred to each Microlog plate's well and incubated for four hours. Results were obtained from MicroStation Reader.

3. RESULTS AND DISCUSSION

3.1 Enrichment process

The growth of microbes was monitored by comparing the medium's turbidity in the control universal bottle against universal bottles with samples. Medium in the control universal bottle shows clear solution while medium in universal bottles with sample turns turbid. Turbidity of medium indicates that the enrichment process was successful.

3.2 Isolation of bacterial strains

On hydrocarbon-coated agar plates, clear yellowish, brownish or cream-colored zones appear indicating formation of bacterial colonies and hydrocarbon degradation. Serial dilution in the range of 10^{-1} to 10^{-6} is made for each colony to decrease the intensity of microbes for each streaking. By decreasing the intensity of microbes in streaking technique, better purification process is obtained. A total of 53 purified strains is obtained and labeled. Figure 1 illustrates an example of an obtained colony.

Figure 1: Example of an obtained colony.

3.3 Hydrocarbon degradation test

In the hydrocarbon degradation test, the hydrocarbon sources used are n-decane, tetradecane, n-pentadecane, do-decane, anthracene, phenanthrene, dibenzothiophene and motor oil. Reduction of INT is used to detect positive wells in which there are degraded hydrocarbons. The degradation results show that single species of microbes are capable of degrading several types of hydrocarbon. Eight strains gave positive degradation result on n-decane, 15 strains on tetradecane, 15 strains on heptadecane, 13 strains on do-decane, 12 strains on anthracene, 12 strains on phenathrane and 17 strains on dibenzothiophene. Strains capable of degrading hydrocarbon compounds in this study are scored as positive result. Table 1 shows total positive scores for each hydrocarbon. Based on number of positive scores, the hydrocarbon most amenable to biodegradation is heptadecane, followed by dibenzothiophene, phenanthrane, do-decane, anthracene, tetradecane and n-decane.

Table 1: Positive scores for each hydrocarbon.

Hydrocarbon	Positive scores
Heptadecane	61
Dibenzothiophene	59
Phenanthrane	57
Do-decane	51
Anthracene	49
Tetradecane	44
n-decane	31

3.4 Identification

Only 3 strains are selected to undergo the identification process using Biolog system as these 3 strains consistently show favorable degradation results for nearly all hydrocarbons especially PAHs (anthracene, phenathrane and dibenzothiophene). PAHs are a type of hydrocarbon with mutagenic and carcinogenic properties which can be found in high concentration in petrochemical oil sludge. The Biolog System Kit is a standardized identification system and characterizing microorganisms. Its databases include over 1900 species of anaerobic bacteria, aerobic bacteria, fungi and yeast. Biolog's patented microbial identification technology with 95 carbon source utilization tests in a microtiter plate. The three identified species are *Clavibacter michiganesis ss insidiosus, Brevibacterium otitidis* and *Rhodococcus rhodochrus.*

This result verifies the findings of Sabate *et al*., (2004) with regards *to Clavibacter michiganesis ss insidiosus*, which is reported to degrade biodiesel compound. In relation to *Rhodococcus rhodochrus*, this study verifies the findings of Margesin and Schinner (2001) in establishing the capability of the bacteria to degrade alkane compounds. A significant finding is made through the present study in relation to *Brevibacterium otitidis*, as there is previous report on this bacterial strain used in hydrocarbon degradation.

5. CONCLUSIONS

Classification of bacteria strains was successfully achieved in this study by using enrichment, isolation and biolog identification processes. Microbes in petroleum sludge sample can be enriched using standard culture enrichment technique with Bushnell Hass medium as the selected medium. The enrichment process takes 21 days, which correspond to one of the fastest duration for enrichment process. Three strains show favorable degradation results for nearly all hydrocarbons especially polycyclic aromatic hydrocarbon (PAHs) (anthracene, phenathrane and dibenzothiophene), namely, *Clavibacter michiganesis ss insidiosus, Rhodococcus rhodochrus* and *Brevibacterium otitidis*. The last bacterial strain is especially interesting since it is the first time such strain is reported to have PAHs degradation capabilities.

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