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INTERNATIONAL CONFERENCE ON SUSTAINABLE INFRASTUCTURE AND BUILT ENVIROMENT IN DEVELOPING COUNTRIES
2-3 NOVEMBER 2009
BANDUNG, WEST JAVA, INDONESIA

*International Conference on Sustainable Infrastructure and Built Environment in Developing Countries*
*November, 2-3, 2009, Bandung, West Java, Indonesia*
*ISBN 978-979-98278-2-1*

# Isolation and Optimization of Napthalene Degradative Bacteria

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***Abstract***
*Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment and cause great environmental concern because of their persistent, toxicity, mutagenicity and carcinogenicity. Therefore they are listed by the United States Environmental Protection Agency (USEPA) as priority pollutants. The principle process for the successful removal and elimination of PAHs from contaminated environment is microbial degradation. Many studies from temperate countries had reported on biodegradation of PAHs but limited information could be found on tropical region. This study is carried out to isolate PAH degradative bacteria from municipal sludge and to optimize their degradation condition including pH, temperature and napthalene concentration required for bioremediation purposes. Several bacterial strains were isolated through enrichment and one strain, Micrococcus diversus that was tentatively identified by the Biolog system, demonstrated a high removal rate of naphthalene over other strains. Following one day lag phase more than 85% of naphthalene degraded after six days incubation. Maximum rate of naphthalene removal occurred in the culture containing 100 $mgL^{-1}$ of naphthalene. Media at a pH 7.0 was more favourable for the degradation of napthalene by Micrococcus diversus. Optimal temperature was determined as 30°C. The isolated Micrococcus diversus demonstrated to be a feasible strain for degradation of napthalene at a neutral pH, 30°C even up to a napthalene concentration of 100 $mgL^{-1}$.*

**Keywords:** Biodegradation, Bioremediation, Isolation, Napthalene, Optimization.

## 1. Introduction

PAHs constitute an enormously large and diverse class of organic compounds that are emitted into the atmosphere by a number of sources, anthropogenic and natural. They are thermodynamically and chemically stable, which distinguish them as persistent environmental pollutants (Juhasz and Naidu, 2000).

The fate of PAHs in nature is of great environmental concern due to their toxic, mutagenic and carcinogenic properties (Weissenfels *et al.*, 1990; Pahlmann and Pelkonen 1987). For example, phenanthrene is known to be a human skin photosensitizer and mild allergen. It has also been found to be an inducer of sistem chromatid exchanges and a potent inhibitor of gap junction intercellular communications. PAHs can sorb to organic rich sediments, accumulate in fish and other aquatic organisms, and may be transferred to humans through seafood consumption.

Possible fates for PAHs released into the environment include photolysis, chemical oxidation, photo-oxidation, bioaccumulation, adsorption on soil particle and volatilization. The major decomposition processes for their successful removal are currently believed to be microbial transformation and degradation (Cerniglia, 1984). PAH degradation by bacteria

occurs primarily under aerobic conditions involving oxygenase mediated ring oxidation and subsequent catabolic formation, ring fission and metabolism.

However, very few researches on biodegradation have been reported on studies in tropical countries like Malaysia (Ghazali *et al.*, 2004 and Dzulkifli, 2007). The present study aims to isolate bacteria from municipal sludge and to optimize the degradation condition.

**2. Experimental**

The biodegradation experiment was performed using 250 ml reactor flasks containing 50ml minimal media. Four parameters including the concentration of bacteria, concentration of PAH, pH and temperature were tested for investigating their effects on the degradation of PAHs of the isolated bacteria strains.

Amount of bacterial strains were varied from 1% to 12% (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%) from the total volume of minimal media broth. Concentrations of substrates were varied and value depended on characteristic study of sludge and previous researches (10ppm, 100ppm, 500ppm). The pH chosen were 5, 6, 7 and 8 for minimal media broth. The pH solution was adjusted to the desired pH glacial acetic acid and ammonium hydroxide. Temperatures chosen were 20°C, 25°C, 30°C, 35°C and 40°C.

The samples were then agitated in an incubated shaker at 150rpm for two weeks. Samples were periodically collected from reactor flasks for day 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, and 15 for the` purpose of measuring residual concentration of PAHs.

The PAHs in the samples were extracted using solid phase microextraction (SPME) (Othman *et al.*, 2008a). 99% of n-hexane was used as solvent to dissolve napthalene. 20ml of the samples were aseptically transffered into 25ml glass bottle with septum cap. Then it was put in an ultrasonic water bath set at temperature 60°C.

The SPME fibre holder assembly equipped with a 7μm polydimethylsiloxane (PDMS) SPME fibre (purchased from Supelco, Sigma-Aldrich Chemie) was used to extract PAHs. The PDMS fibre was immersed in the sample for 60 minutes. The fibre was then retracted and transferred to the heated injection port of the gas chromatograph unit for analysis. In this analysis, each sample was tested in triplicate.

Extracts were analyzed by Perkin Elmer Clarus 500 gas chromatography. This unit, equipped with Elite column 5MS with 30m long x 0.25mm internal diameter x 0.25μm thickness, was used to separate the compounds. Flame ionization detector (FID) was adopted for the analysis. The injector was operated as follows; it was set at 250°C in the splitless mode with a 2 minute splitless period. Helium was used as the carrier gas with 1ml/min constant flow rate. The column temperature was initially set at 50°C for 1 minute, increased to 150°C at a rate of 15°C/min and held for 1 min, and finally ramped at 5°C/min to 300°C and held constant until 15 minutes of the total run time. Identification of analytes in the chromatograms was based on retention times.

**3. Results & Discusion**

Four parameters including the concentration of bacteria, pH, temperature, concentration of substrate were tested for investigating their effects on the degradation of PAHs using isolated bacteria strains. The degradation of PAHs varied according to isolates exposed.
There was about 98.9.% naphthalene degradation for cultures with the addition of 100 ppm pahs but only 95.1% naphthalene degradation at 500 ppm. The average removal rate of napthalene is 18.12 ppm/day at 100ppm phenanthrene, 99.48 ppm/day at 500ppm, 10.56 ppm/day at 50ppm, 1.8 ppm/day at 10ppm and 0.147 ppm/day at 1ppm.

There was no significance difference between 100 and 500 ppm naphthalene culture at removal rates of naphthalene. Nevertheless, 100ppm napthalene showed a higher efficiency to remove napthalene than 500ppm napthalene culture. This may be due to the fact that too high the concentration of naphthalene would saturate the bacteria cell with the substrate. Therefore, the optimum degradation on napthalene at 98.9% was obtained approximately at naphthalene concentration of 100ppm.

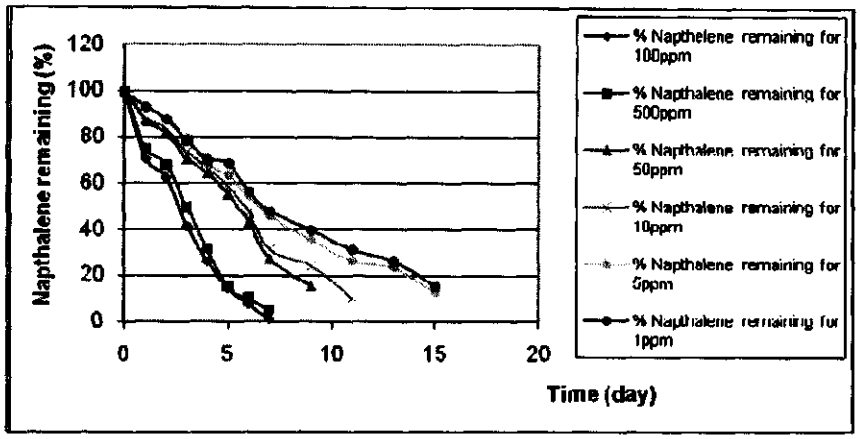


**Figure 1** Effects of substrate concentration on naphthalene degradation

Other researchers also reported that high concentration of PAHs were beneficial to the growth of isolated bacteria because PAHs could acts as a sole carbon source for bacterial growth. Therefore, too lower the concentration of PAHs would become a limiting factor for the bacterial culture to grow (Wong *et al.*, 2001).

As shown in Figure 2, the results reveal that napthalene degradation reaches the optimum level at 10% of bacteria concentration. This is as predicted because the increase in bacteria amount could synthesize more enzymes. Each enzyme has a certain area on its surface that is active sites. In this case, the active site is the region at which the enzyme forms a loose association with PAHs as its substrate. Therefore, the more enzymes secreted more active site provided for PAHs. Enzymes also act as substance that remains unchanged while they speed up degradation of PAHs (Black, 2002).

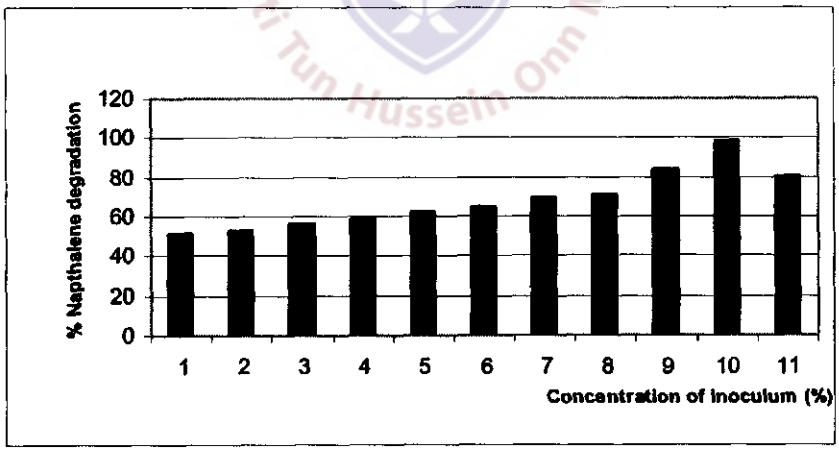


**Figure 2** Concentration of inoculum at 7 days degradation

The percentage of degradation was increased as the concentration of bacteria increased and finally reached a steady state known as chemical equilibrium. At this point the enzyme's active site or binding site is working at full capacity.

At equilibrium, biodegradation of PAHs on *Micrococcus diversus* as a function of pH is shown in Figure 3,which indicated that at pH 5 and pH 6, poor biodegradation was observed. While pH value was higher than 7 the biodegradation process decreased. The result shows

that the optimum degradation was accomplished at pH 7. The equilibrium PAHs degradation was 99.32% at this pH value

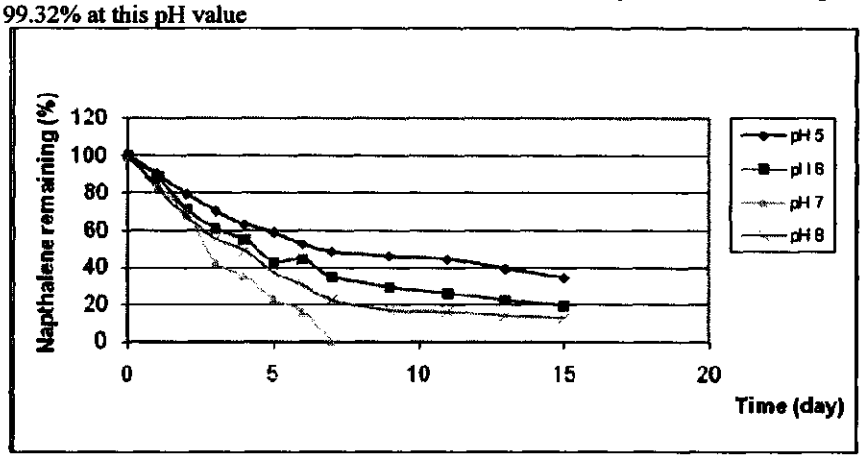


**Figure 3** Effects of pH on naphthalene degradation

Changes in pH can alter the electrical charge on various chemicals groups in enzymes molecules. In this manner it probably altering the enzyme's ability to bind its substrate and catalyze a reaction. Imbalance of the electrical charges in very acidic and alkali condition can disrupt hydrogen bonds and other weak forces that maintain enzyme structure. Such disruption of enzyme structure is called denaturation. Thus, this phenomenon resulted in poor biodegradation process. This explanation also shared with previous researchers Yuan *et al.*, (2000) and Wong *et al.*, (2001). They also had pointed out that microbial enzymes have an optimum pH at which they function most effectively, thus it is related to an organism normal environment.

Figure 4 shows biodegradation of PAHs by *Micrococcus diversus* at equilibrium condition as a function of temperature. It is clearly shown that at low temperature such as 20°C low degradation process was observed and at 25°C the biodegradation process started increased. The results show that the optimum temperature was attained at 30°C. The equilibrium PAHs biodegradation was 98.81 % at this temperature value.

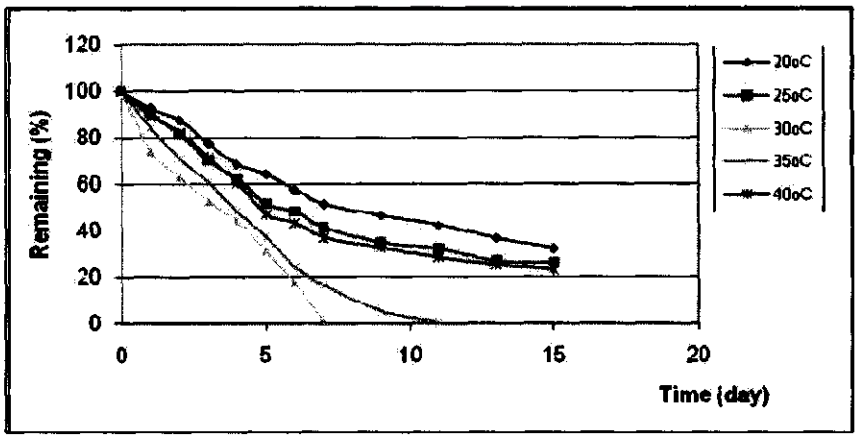


**Figure 4** Effects of temperature on naphthalene degradation

Low degradation process at low temperature suggests that this temperature value is not sufficient for enzyme in order to speed up the reaction rate. Therefore, increased the temperature will result to faster reaction rate in degrading PAHs. In general, enzymes as

catalysts have certain temperature that it withstands and normally optimum temperature will catalyze reaction most rapidly. Microbial enzymes likewise function best at optimum temperature which related to an organism's normal environment. Thus, reaction such as degradation process normally optimum at mild temperature in microbial cell. Above $40^0$C, however enzyme is rapidly denatured, and its activity decreased accordingly (Black, 2002).

**4. Conclusion**

This study established that *Micrococcus diversus* has the capability to degrade napthalene in a range varying depend on environmental conditions provided. Maximum rate of naphthalene removal occurred in the culture containing 100 $mgL^{-1}$ of naphthalene. Media at a pH 7.0 was more favourable for the degradation of napthalene by *Micrococcus diversus*. Optimal temperature was determined as 30°C. The isolated Micrococcus diversus demonstrated to be a feasible strain for degradation of napthalene at a neutral pH, 30°C even up to a napthalene concentration of 100 $mgL^{-1}$.

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