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Identification of Phenanthrene and Pyrene degrading Bacteria from used Engine Oil contaminated Soil

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Abstract— The purpose of the study was to identify effective bacteria that degrade phenanthrene and pyrene from used engine oil contaminated soil. A total of 93 bacterial isolates were obtained and their degradations were determined. Based on culture-depended technique and tested for *in vitro* phenanthrene and pyrene degradations using spray plate technique and colorimetric assay with 2,6-dichloroindophenol (DCPIP). Fifty three isolates shows evidence of 5 g/l phenanthrene degradation within 72 hours in spray plate technique. Further 23 isolates were selected from 53 isolates after the colorimetric screening. Biodegradation study shows two isolates named MM045 and MM087 with 75.2% and 80.2% degradations for 500 mg/l phenanthrene and also 54.3% and 59.7% of 250 mg/l pyrene degradations within 24 hours respectively. These isolates were identified as *Cronobacter sakazakii* strain MM045 (Accession number: KT933253) and *Enterobacter* specie strain MM087 (Accession number: KT933254) using 16S rRNA gene sequencing and neighbour joining tree analyses. The identified isolates were proven to be fast effective in degrading high concentrations of phenanthrene and pyrene as their sole carbon and energy sources.

Index Terms— Biodegradation, contaminated soil, phenanthrene, pyrene.

1 Introduction

USED engine oil is a major occupational source of polycyclic aromatic hydrocarbons (PAHs) resulting from incomplete combustion process [20, 53]. PAHs are highly recalcitrant and persistent chemicals that are widely distributed within the environment due to their chemical stability and low water solubility [4, 9]. US Environmental Protection Agency classified 16 PAHs as the most toxic and hazardous [3]; phenanthrene, a low molecular weight (LMW) and pyrene, a high molecular weight (HMW) PAHs were included [23, 53]. Pyrene being HMW PAH is more toxic and difficult to degrade even with the use of microbial consortium [34] and it causes body weight changes, kidney lesions, neurological disorders, and mortality [12, 36]. Meanwhile, phenanthrene causes skin photosensitization with mild allergen [33]. The contamination level of phenanthrene and pyrene concentrations on soil environment was reported to be above 10 mg/kg and 20 mg/kg respectively [2].

Physical and chemical methods are used for the removal of PAHs on the environment [21, 55, 56] while bioremediation is more environmentally friendly and generally acceptable technology that completely detoxifies PAHs [29]. In bioremediation, phytoremediation plays significant contribution [7, 13] while bacteria and fungi degrade PAHs much effectively due to their possession of broad range of enzymes [32]. Many bacterial species such as *Kocuria, Brevibacterium, Sphingomonas, Rhodococcus,* and *Pseudomonas* were reported to degrade pyrene and phenanthrene [31, 46, 48].

Inadequate information about the toxicity of PAHs containing waste and its post-disposal behaviour significantly introduces severe effects on the environmental quality [45]. This causes extensive damage on the ecosystem which is one of the major environmental pollution problems [50, 54]. Moreover, studies on PAHs biodegradation have rarely focused on decreasing the PAHs degradation duration. An important factor that improves PAHs biodegradation is shortening slow degradation time caused by PAHs chemical stability [38, 52]. Additionally, the degrading bacteria need pre-exposed PAHs contaminated sites in order to induce the synthesis of PAHs degrading enzymes [17]. Hence, effective PAHs biodegradation required bacterial strains that rapidly recognise PAHs as sole carbon source [39].

A more significant way to improve the efficiency in PAHs degradation is to reduce the period of degradation. Recently, more than 48 hours degradation duration were reported to be applied even with lower PAHs concentrations [1, 24, 26, 46], due to the PAHs chemical stability and their resistance to biodegradation process [10, 15, 30, 42, 43]. Consequently, identification of bacteria with potentials to degrade PAHs within 24 hours may help to overcome such limitation and could be a more successful remediation strategy. In view of this, the present research aimed to isolate and identify bacteria with 24 hours degradation potentials for both LMW PAH phenanthrene and HMW PAH pyrene.

2 Materials and Methods

2.1 Chemicals preparation

Phenanthrene (98% purity) and pyrene (96% purity) used for the study were obtained from Sigma-Aldrich Co. and Merck chemicals USA respectively. All media and chemicals used were of analytical grade and obtained from standard manufactures. Stock solutions of filter sterilized phenanthrene and py-

rene (5 g/l each) in petroleum ether solvent were prepared and used as sole bacterial carbon source during the study. All media were dissolved in de-ionized water and sterilized at 121^{0}C for 15 minutes. Experiments were also conducted in triplicates and negative controls were prepared for each treatment.

2.2 Soil samples and bacteria isolation

Soil contaminated with used engine oil were collected from eight sampling locations within Puchong and Seri Kembangan, Selangor Malaysia (Figure 1) and preserved at 4^{0}C ± 0.5^{0}C within two hours of sampling.

Bacterial isolates were initially enriched and isolated from the soil samples [48]. The enrichment process was started with the addition of 5 g soil sample onto 50 ml mineral salts (MS) medium (KH_2PO_4, 1.5 g/L, NH_4Cl, 2.0 g/L, $MgSO_4 \cdot 7H_2O$, 0.2 g/L, Na_2HPO_4, 9.0 g/L, $MnSO_4 \cdot H_2O$, 3.0 g/L, Ferric citrate, 5.0 g/L, $ZnSO_4 \cdot 7H_2O$, 0.2 g/L, Titriplex III, 0.01 g/L, and $CoSO_4 \cdot 7H_2O$, 10µg) supplemented with 50 mg/l phenanthrene [22]. The enrichment culture was incubated for 24 hours at 37^{0}C ± 0.5^{0}C and 200 rpm. Then 1 ml from the culture was serially diluted in sterile de-ionized water before plated onto prepared Luria-Bertani (LB) agar and incubated for further 24 hours at 37^{0}C ± 0.5^{0}C. Colonies were purified on LB media using the same culture conditions and pure isolates were preserved on 20% glycerol at -80^{0}C for long term storage.

Bacterial resting cells were prepared [44] and used during the study. The selected bacterial isolates were initially grown on LB broth at 37^{0}C ± 0.5^{0}C and shaken at 200 rpm for 24 hours. The culture was then centrifuged at 4000 rpm for 15 minutes and the pellets was washed twice with phosphate buffer saline (NaCl, 8 g/L, KCl, 0.2 g/L, Na_2HPO_4, 1.44 g/L, KH_2PO_4, 0.24 g/L, pH adjusted to 7.4 using conc HCl) and resuspended on the same buffer before stored at 4^{0}C. Bacterial cells per ml were calculated using haemocytometer after diluting the resting cells with trypan blue dye.

2.3 Screening process

Screening process was initiated using spray plate technique [28], where 100µl of the 24 hours grown bacterial culture was spread on Bushnell Haas (BH) Agar (KH_2PO_4, 1.0 g/L, NH_4NO_3, 1.0 g/L, $MgSO_4 \cdot 7H_2O$, 0.2 g/L, K_2HPO_4, 1.0 g/L, $FeCl_3$, 0.05 g/L, $CaCl_2 \cdot 2H_2O$, 0.02 g/L, and agarose, 8.0 g/L). After 15 minutes of inoculation, 5 g/l phenanthrene solution was sprayed on the surface of the inoculated plates. The solvent was allowed to evaporate leaving behind thin layer of phenanthrene crystals on the BH Agar surface. Later, the inoculum was incubated at 37^{0}C ± 0.5^{0}C for 72 hours with close observation every 24 hours. Disappearance of phenanthrene crystals on the culture plates indicates the phenanthrene utilization.

The selected bacterial isolates after 72 hours spray plate assay were further screened using colorimetric assay [19]. In this process, 9 ml MS medium containing 200 mg/L filter sterilized 2,6-dichloroindophenol sodium salt (DCPIP) in separate flasks were supplemented with 500 mg/L phenanthrene and 250 mg/L pyrene respectively. The DCPIP served as PAHs degradation indicator which changes the medium colour to deep blue and after degradation the medium colour becomes colourless [8, 14]. Then it was inoculated with 5% v/v of bacterial resting cells (1 x 10^6 cells/ml) and incubated at 37^{0}C ± 0.5^{0}C, 200 rpm, for 24 hours. Isolates with fast response based on colourless medium were selected for determination of the PAHs rate of degradations.

2.4 Biodegradation assessment

Assessment of phenanthrene and pyrene degradations started by supplementing the selected bacterial isolates with 500 mg/L phenanthrene and 250 mg/L pyrene separately using the same previous incubation conditions. Absorbance was measured at 600 nm within 24 hours of incubation using spectrophotometer against the treatment blank. The phenanthrene and pyrene degradations were measured based on the established linear standard curve and quantification was done using the formula:

$$D = \frac{C_1 - C_2}{C_1} \times 100\%$$

D : % degradation; C_1 : initial PAH concentration; C_2 : residual concentration

The linear standard curve for both phenanthrene and pyrene were constructed using colorimetric assay [18]. Different flasks containing 9 ml MS medium supplemented with varying concentrations of phenanthrene (200 mg/L to 2000 mg/L) and pyrene (100 mg/L to 1000 mg/L) separately. Then, 5% v/v of bacterial resting cells (1 x 10^6 cells/ml) were inoculated and incubated at 37^{0}C ± 0.5^{0}C, 200 rpm, for 24 hours. Absorbance readings were recorded at 600nm and standard calibration curve was then established and each of the linear equations was used in respective biodegradation assessments.

2.5 Identification of bacterial isolates

The identification of the selected isolates were done using 16S rRNA gene sequencing [47] with the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Genomic DNA was extracted and subjected to polymerase chain reaction (PCR) using thermal cycle conditions; Initial Denaturation: 95^{0}C for 5 minutes (1 cycle), Denaturation: 95^{0}C for 45 seconds, Annealing: 51^{0}C for 15 seconds (30 cycles), Extension: 72^{0}C for 2 minutes, Final extension: 72^{0}C for 10 minutes (1 cycle). The DNA size was determined using 1% TAE agarose against the positive and negative controls.

The PCR products were then purified by PCR cleanup and directly sequenced with primers 518F (5'-CCAGCAGCCGCGGTAATACG-3′) and 800R (5'-TACCAGGGTATCTAATCC-3′) using BigDye® Terminator

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v3.1 cycle sequencing kit (Perkin-Elmer Applied Biosystems, USA). The purified PCR products were sequenced by sanger dideoxy sequencing and the data was subjected to basic local alignment search tool (BLAST) using the databases from the National Centre for Biotechnology Information (NCBI) and multiple sequence alignment was done with ClustalW (1.6). Consensus neighbour joining tree was constructed with the software Molecular Evolutionary Genetics Analysis (MEGA) version 6 [16, 27, 47].

3 Results

A total of 93 bacterial isolates were obtained from the enrichment process with phenanthrene supplemented MS medium as the only source of carbon and energy. When these isolates were cultured on phenanthrene-coated mineral agar plates for 72 hours, 53 isolates showed degradation potentials based on phenanthrene crystals disappearance on the plates (Figure 2a & b). Further screening using colorimetric assay with DCPIP indicator reduced the number of the isolates to 23 after supplemented with 500 mg/L and 250 mg/L of phenanthrene and pyrene respectively (Figure 2c & d). However, rapid change in colouration to colourless was observed from two bacterial strains named MM045 and MM087 within 12 hours of incubation which indicate faster degradation response and were chosen for the biodegradation analyses and molecular identification.

Considering the linear equations for the established standard curves, the two selected bacterial isolates (MM045 and MM087) were assessed using 500 mg/l phenanthrene and 250 mg/l pyrene for 24 hours incubation. Absorbance at 600nm was recorded in each case at one hour interval for 12 hours and then after 24 hours incubation respectively. The recorded absorbance was used for calculating the residual concentrations using standard linear equations. The linear equations for phenanthrene and pyrene degradations by isolate MM045 were: $y = 0.057x + 0.771$ ($R^2 = 0.991$) and $y = 0.125x + 0.542$ ($R^2 = 0.973$) respectively. The linear equations for phenanthrene degradation by isolate MM087 was $y = 0.056x + 0.687$ ($R^2 = 0.977$) and that of pyrene degradation by the same isolate was $y = 0.084x + 0.529$ ($R^2 = 0.982$).

During the initial five hours of incubation, isolate MM045 starts degrading phenanthrene while isolate MM087 started after four hours of the incubation as observed based on change in colouration. In the case of pyrene degradations, isolate MM045 started the degradation after three hours of incubation while MM087 started after 9 hours of incubation. This may be due to the initial biodegradation resistance of PAHs used. After 24 hours of incubation, Isolate MM045 was observed to have left 124 mg/l phenanthrene residual concentration from 500 mg/l which indicates 75.2% degradation. Isolate MM087 left 99 mg/l phenanthrene residual concentration from 500 mg/l, indicating 80.2% degradations (Figure 3a). Moreover, Isolate MM045 was observed to have 114.3 mg/l pyrene residual concentration from 250 mg/l, which represents 54.3% degradation. Isolate MM087 also left 100.7 mg/l pyrene residual concentration, representing 59.7% degradation (Figure 3b).

Based on the bacterial screening results, isolates MM045 and MM087 were selected and identified using morphology and 16S rRNA gene sequencing. Using the NCBI databases, isolate MM045 shows more close similarity with *Cronobacter sakazakii* of different strains (Figure 4a) while MM087 was observed to show 100% similarity with *Enterobacter* species as indicated in the neighbour joining tree analyses (Figure 4b). Morphologically, isolate MM045 was classified as gram negative straight rods with milk round colonies appearance on LB agar while MM087 appeared as gram negative rods with big flat colonies and rough edges.

4 Discussions

During the initial screening, 53 bacterial isolates were observed to degrade the supplemented phenanthrene by spray plate technique after 72 hours period. In a similar study conducted by Ahn *et al.*, [5], 83 out of 141 bacterial strains were observed to degrade phenanthrene using spray plate technique after 21 days of incubation. Moreover, colorimetric screening was performed for the purpose of selecting fast degrading isolates among the initially screened ones where DCPIP indicator was used to ascertain PAHs oxidation by the selected bacteria through redox reaction [8, 14].

In the colorimetric assay, two bacterial isolates MM045 and MM087 were observed to have greater degradation potentials in 500 mg/L phenanthrene and 250 mg/L pyrene within the initial 12 hours period and were further assessed for phenanthrene and pyrene degradations. Both isolates shows maximum degradations of more than 70% for phenanthrene and more than 50% for pyrene with MM087 recording the highest in both cases. This fast degradation response was never recorded in many previous literatures [1, 24, 26, 46] which could be a basis to ascertain the PAHs degradation potentials of more bacterial isolates in future research.

The two selected isolates were finally identified as *Cronobacter sakazakii* MM045 and *Enterobacter* sp. MM087 respectively. However, *Enterobacter* species were rarely reported to degrade PAHs [37] although very few reports showed bacterial consortium involving *Enterobacter* species to be capable of surviving in phenanthrene and pyrene as sole carbon and energy sources [6, 35, 40, 41, 49] while *C. sakazakii* was never reported to degrade any PAHs as it is opportunistic food borne pathogen that is widely distributed within the environment [25].

Currently there was no biodegradation report where significant concentration of phenanthrene or pyrene was degraded within 24 hours of incubation. In this study, rapid degradation response within 12 hours of incubation was observed

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from each of MM045 and MM087 isolates. In both isolates, over 70% of 500 mg/l phenanthrene was degraded after 24 hours of incubation. They performed better than many bacterial species like *Pseudomonas* sp which took 7 days to degrade more than 70% of 500 mg/l phenanthrene [11]. The *Pseudomonas* sp performance was attributed to its adaptation to PAHs contaminated site which induces the synthesis of plasmid degrading PAHs. It was also shown that isolates MM045 and MM087 degraded over 50% of 250 mg/l pyrene within 24 hours. This exceeds the result of Sarma *et al.*, [37] where 61.5% of 200 mg/l pyrene was degraded by *Leclercia adecarboxylata* after 20 days of incubation.

Bacterial isolates were reported to degrade phenanthrene and pyrene, however, the time taken to degrade these PAHs underestimates the isolates performance [38, 52]. This is due to the isolates non pre-exposure to very high PAHs concentration in their natural environment [17]. Consequently, a very remarkable performance was observed in this study from isolates MM045 and MM087. This may be attributed to the isolates pre-exposure to highly contaminated soil with used engine oil which has 30% aromatic hydrocarbons [51]. The bacterial adaptation to aromatic hydrocarbons environment induces them to synthesize PAHs degrading enzymes and performed effectively well [17].

5 Conclusion

It is evident from the study that phenanthrene and pyrene degrading bacteria could be isolated from soil contaminated with used engine oil. The genus *Enterobacter* appeared to be hidden group of bacteria that efficiently remove PAHs from the environment individually without synergy with other strains of bacteria. The 24 hours degradation efficiency could also assist in shortening the long term microbial PAHs degradation. This suggests that further analyses of the isolates could improve their strength in degrading more aromatic compounds at very high concentrations.

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6 Figures and Tables

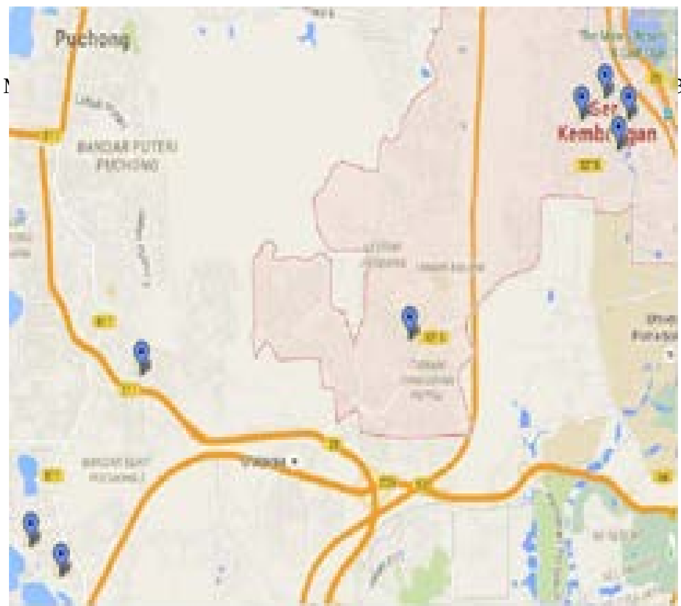

Figure 1: Sampling locations within Seri Kembangan and Puchong, Malaysia

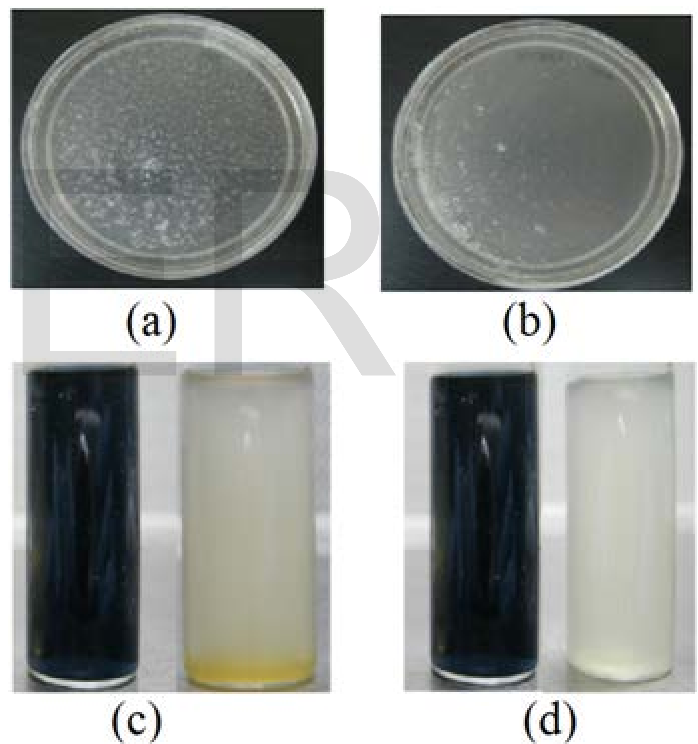

Figure 2: Reduction of phenanthrene crystals in spray plate assay (a) before and (b) after the degradation: Colorimetric assay screening showing negative control for (c) phenanthrene and (d) pyrene degradations

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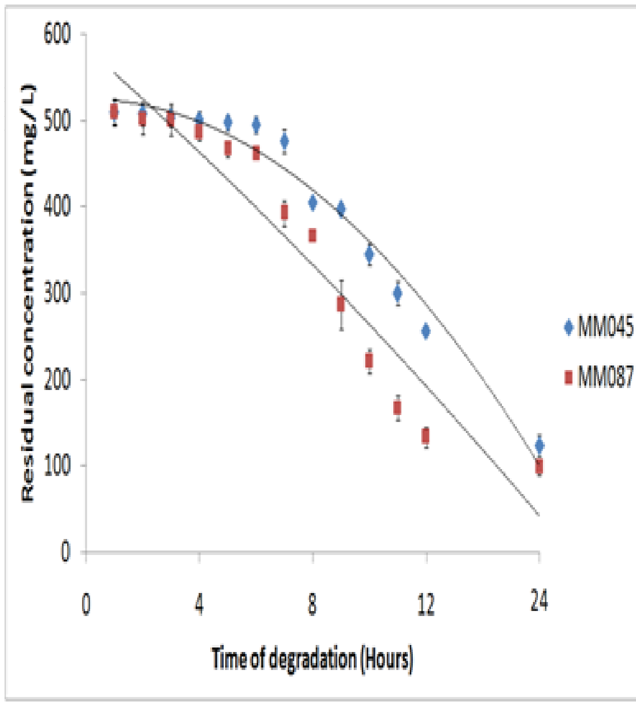

Figure 3a: Residual phenanthrene degradations within 24 hours by isolates MM045 and MM087

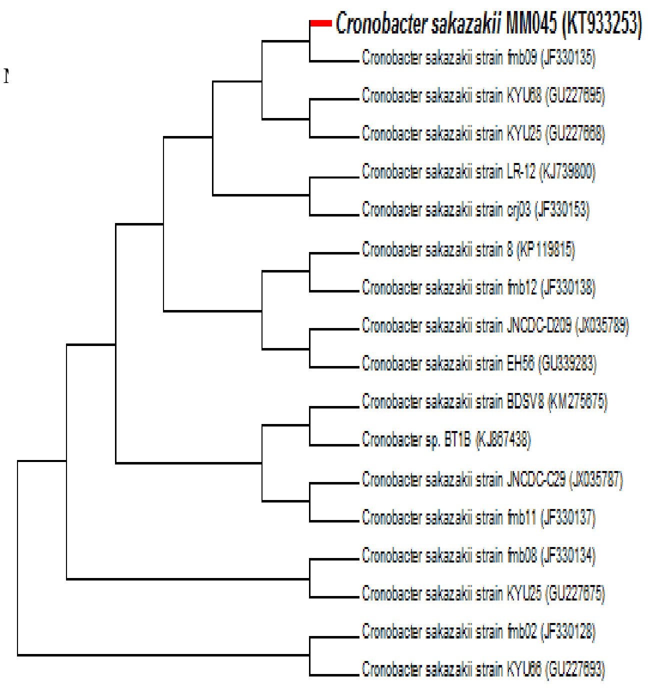

Figure 4a: Neighbour joining tree obtained from NCBI for *Cronobacter sakazakii* strain MM087 (Accession Number: KT933253)

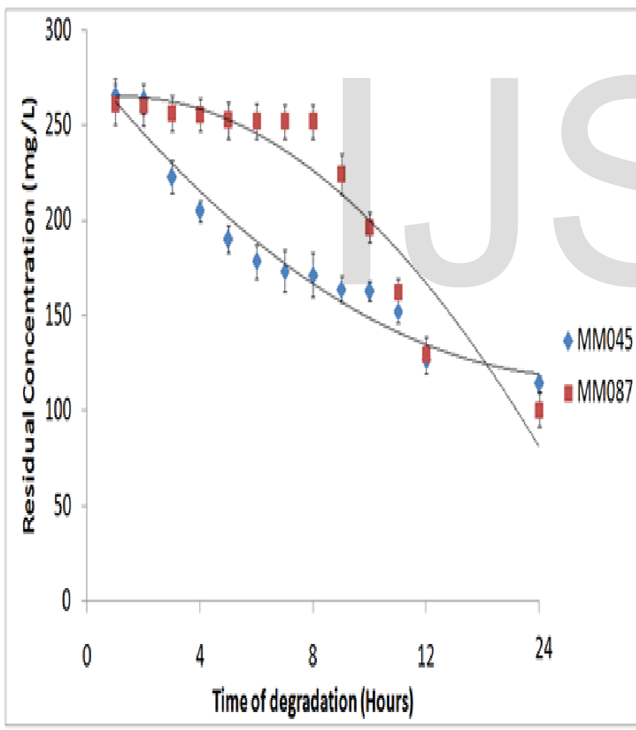

Figure 3b: Residual pyrene degradations within 24 hours by MM045 and MM087

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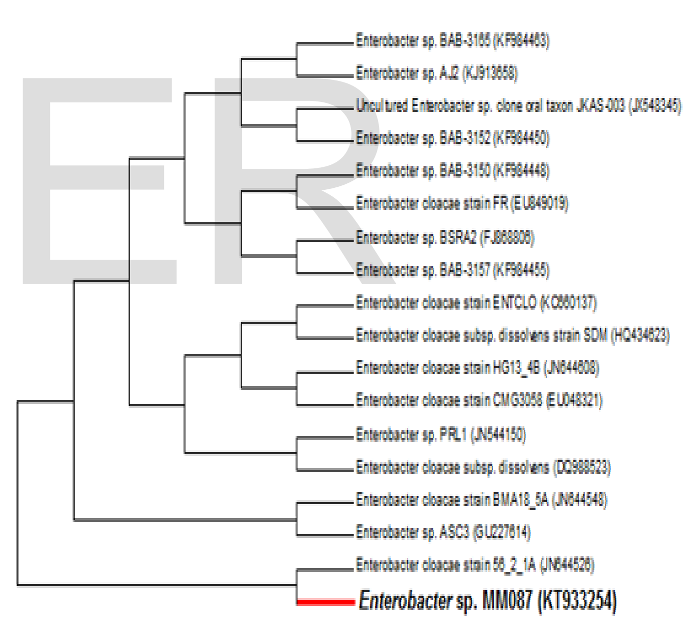

Figure 4b: Neighbour joining tree obtained from NCBI for *Enterobacter* sp. strain MM087 (Accession Number: KT933254)

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