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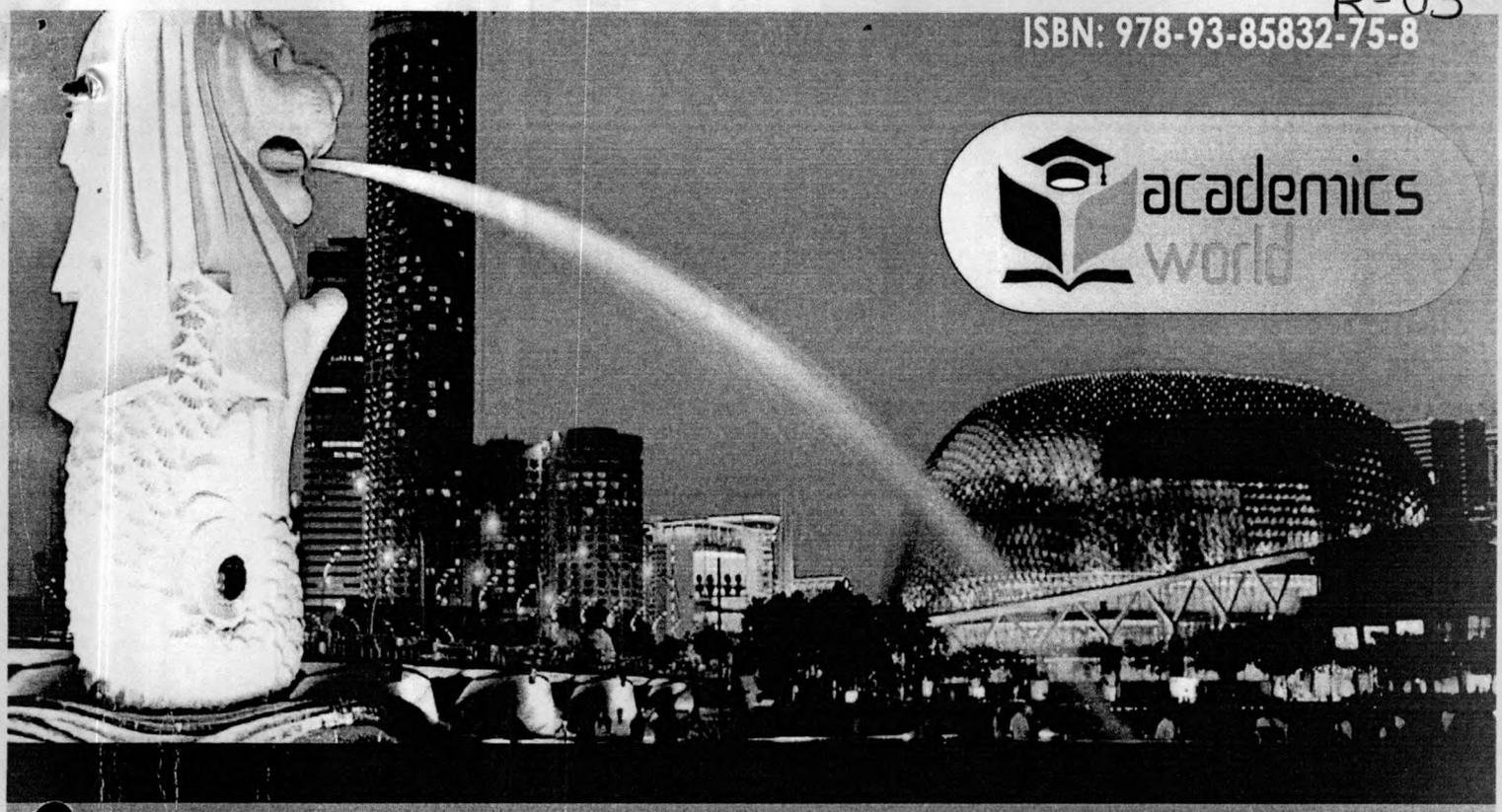
ISBN: 978-93-85832-75-8



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ROLE OF NATIVE BACTERIA AS A BIOREMEDIATING AGENT FOR NAPHTHALENE AND PHENANTHRENE

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Abstract- Polycyclic Aromatic Hydrocarbons (PAH) are major contaminants of the environment, associated with common anthropogenic activities such as oil refineries and incomplete combustion of fossil fuel.Naphthalene and phenanthrene are types of simplest PAH. The existence of these pollutants in aquatic environments is toxic and dangerous for human and other organisms. The microbial populations in contaminated sites are able to degrade the stable compounds like PAH. The goal of this study is to evaluate the degradation potential of naphthalene and phenanthreneby native bacteria isolated from oil contaminated coastal water and sediments in Sri Lanka. The isolated strains were identified as *Bacillus cereus, Enterobacter*sp. *and Enterobacterludwigii*by 16s RNA.Each bacterial strain was inoculated into LB broth incorporated with PAH (1% v/v) and redox indicator (2% v/v) and incubated at room temperature (28°C) with constant shaking at 180rev/min for 14 days with control without bacterial inoculation. The degradation efficiency of strains was assessed spectrophotometrically by measuring absorbance at 609nm for the residual hydrocarbon. The results showed that the highest degradation achieved by the bacterium*Bacillus cereus* (73%) whilst:*Enterobacter*sp. and *E. ludwigii*showed descending order of the naphthalene degradation at 63% and 61% respectively after 14 days of incubation. The highest degradation percentage of phenanthrene (87%) was record for *E.ludwigii*where*B.cereus*and *Enterobacter*sp.showed 86% and 79% degradation respectively. These PAH degraders identified in the present study could potential remediates for bioremediation of polluted marine environment and in oil contaminated fields in future.

Keywords- Naphthalene,Phenanthrene, Bioremediation, *Bacillus cereus, Enterobacter*sp., *Enterobacterludwigii.*

I. INTRODUCTION

Environment pollution is a worldwide problem and its potential to influence the health of environment is great [1]. Pollution reaches its most serious proportions in the densely settled urban-industrial centers of more developing countries [2]. From different type of pollutants, organic compounds are highly persistence and causes severe damage to the environment.Organic compounds are generally divided into three groups: Aliphatic, Salicylic, and Aromatic.

Polycyclic AromaticHydrocarbons (PAHs) are the most important environmental pollutants. PAHs are a group of organic compounds consisting of two or more fused aromatic rings. The main structural unit in aromatic compounds is benzene [3]. Phenanthrene and naphthalene are PAHs which are widely distributed environmental contaminant and cause detrimental ecological effect. PAHs originate mainly from anthropogenic processes, particularly from incomplete combustion of organic fuels. Natural processes such as volcanic eruptions and forest fires, contribute to an ambient existence of PAHsas well [4].

Due to widespread sources and persistent characteristics, PAHs disperse through atmospheric transport and exist almost everywhere.Moreover, due to their hydrophobic characteristics, PAHs are less soluble in water therefore these compounds settle in the sediments of rivers, lakes, and oceans. However, they are soluble in non-polar organic solvents. Thus, the existence of these pollutants in aquatic environments is very toxic and dangerous for humans and other creatures[5].

Human beings are exposed to PAH mixtures in gaseous or particulate phases in ambient air. Long-term exposure to high concentrations of PAHs is associated with adverse health problems. Since some PAHs are considered carcinogens, inhalation of PAHs in particulates is a potentially serious health risk linked to an excess risk of lung cancer. They are harmful for useful microorganisms of plants in contaminated soils as well [6].

The oil compounds are very resistant to evaporation, due to having aromatics derivatives intheir structures and remained in the environment for a long time. However, fortunately microbial population in contaminated sites results in removal of those stable compounds by microbial degradation.Marine bacteria capable of degrading PAHs have been recorded in genera: *Cycloclasticus, Neptumonas, Pseudoalteromonas, Marinomonas, Halomonas, Sphingomonas, Enterobacter, Bacillus* and *Burkholderia*[7, 8]. Moreover, number of studies showed that native microorganisms in oil contaminated areas are more effective than other organisms for biodegradation of oil pollutants[6].

Nduka et al., (2010) reported that biodegradation rates incontaminated areas depend on factors including microbial populations, pollutant compoundstype, contamination values and type, and chemical and geological conditions of the contaminatedarea [9].

Thus, the goal of the present study is to evaluate the biodegradation potential and rate of naphthalene and

phenanthrene by nativebacteria isolated from coastal environment in Sri Lanka.

II. MATERIALS AND METHODS

2.1 Enrichment study for bacterial cultures

Preserved bacteria cultures were obtained for the present study which were documented as potent crude oil degraders.The bacteria isolates were identified as *Bacillus cereus*, *Enterobacterludwigii* and*Enterobacter* sp. respectively[10]. To identify the viability of bacterial strains . LB broth cultures were prepared and incubated 24h at25°C ± 1 °C while constant shaking at 100rpm.Bacterial cultures were subjected to centrifugation and subsequent washing with PBS to remove carbon. Turbidity of the bacteria cell suspensions were equalized (A_{540}=0.35) using spectrophotometer (Model: Labomed. Inc. USA) [11].

Enrichment of phenanthrene and naphthalene was done by inoculating 10 ml of equalized bacteria suspension into cotton-plugged Erlenmeyer flasks which containing LB broth medium with napththalene and phenanthrene at final concentration of 0.4g/l [12]. Triplicate samples were prepared for the each bacterium and incubate at 25°C ± 1 °C while shaking at 100rpm. After 14 days of incubation the streak plate method was done to incubate cultures for identify viability of bacteria isolates in naphthalene and phenanthrene mediums.

2.2Degradation kinetics of the isolated bacteria

Isolated bacterial strains were grown in liquid LB medium as described and starved overnight using Phosphate Buffer Solution (PBS). Following equalizing the turbidities of the bacterial strains at A_{540}=0.35: 5 ml of the bacterial suspension was inoculated into filter sterile (0.2um nucleopore) sea water containing naphthalene and phenanthreneat a final concentration of 0.4 g/l respectively.

Samples were incubated at room temperature (25°C ± 1°C) with constant shaking at 180rev/min, for 14 days with a control which was prepared without bacterial inoculation. Five milliliter of aliquots were removed from each flask and centrifuged at 6000rpm for five minutes. The recovered supernatants were analyzedspectrophotometrically measuring absorbance at 609nm for the residual hydrocarbon [13]. Replicates wereperformed for each bacterial strain for naphthalene and phenanthrene to determine the degradation percentage. The following equation was used for calculation[13].

$$\text{Percentage of degradation} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.3 Growth assessment of isolated bacteria

The optimal density of the each sample, which prepared by inoculating isolates was determined usingspectrophotometric method and measurements were taken at 590 nmat 2 days intervals for a period of 14 days[14].

2.4 Determination of the Emulsification index

Emulsion capacity measures the maximum amount of fat emulsified by a given isolate under standard conditions [15]. Emulsification index (E_{24})was used to evaluate the emulsifying capacity of each bacterial strain.

To determine the emulsion index. 2ml of the bacteria isolate was centrifuged at 3000rpm for 15min and the supernatant was removed. 2 ml of naphthalene and phenanthrenewere added to each fraction to determine the emulsification index according to the Abbasi and Amiri (2008)[16]. The tubes were properly vortexed at 2000 rpmfor 2min and allowed to stand for 24h. The emulsification index was calculated at 2 days interval for 14 days of incubation. The emulsification index was calculated using the following equation [16].

E_{24} = height of emulsion Height of supernatant X 100

III. RESULTS AND DISCUSSION

Naphthalene and phenanthrene are types of simplest PAHs. Phenanthrene, a tricyclic aromatic hydrocarbon, has a structure that is shared with several carcinogenic PAHs [17] and it has also been found to be toxic to aquatic organisms [18].

In the present study each isolate(*B. cereus*, *E. ludwigii* and*Enterobacter* sp.) was able to grow on naphthalene and phenanthreneas the sole carbon source when screened for hydrocarbon utilization. Interestingly, these same groups of organisms have been implicated in PAHs degradation, particularly *Bacillus* and *Enterobacter* by several workers [19, 20].More than 78% and 60% degradation of the residual phenanthrene and naphthalene in the media was detected after 14 days of incubation respectively (figure1 and figure 2).

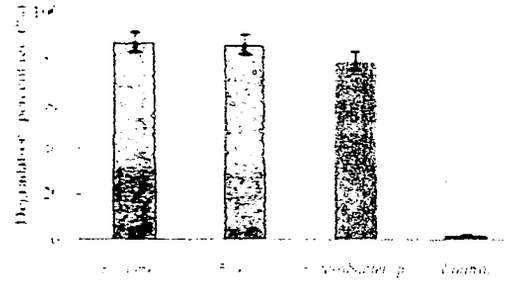

Fig 1: Degradation percentage of phenanthrene in bacterial cultures at 14 days of incubation

The results shows that the phenanthrene degradation was greater than 78% for all bacterial species and the highest percentage was achieved by the bacterium *E.ludwigii* (87%). The other strains *B. cereus*and

*Enterobacter*sp. showed 86% and 79% degradation respectively. Control showed 2% reduction of phenanthrene after 14 days of incubation.

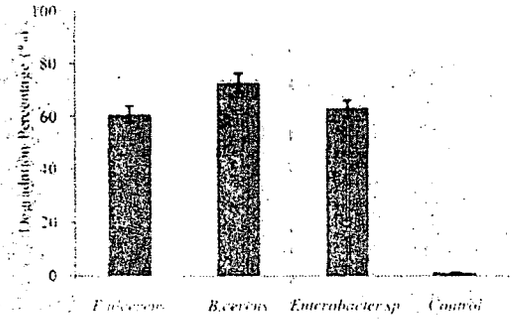

Fig2: Degradation percentage of naphthalene in bacterial cultures at 14 days of incubation

Naphthalene degradation by the same bacteria species were less than the results obtained for phenanthrene. Three bacterial species namely *B.cereus*, *Enterobacter* sp. and *E. ludwigii* showed greater than 60% degradation of naphthalene after 14 days of incubation at 25 ^{0}C ± 1^{0}C. The highest degradation was recorded by *B. cereus* (73%) where, other two bacteria strains;*Enterobacter*sp.(63%)and *E. ludwigii*.(61%) showed descending degradation trend respectively.1.2% reduction of naphthalene was detected in control experiment after 14 days of incubation as well.

However there is little information available for phenanthrene degrading bacteria. *Rhodococcus*, *Pseudomonas aeruginosa*,*Sphingomonas* sp.[4] and *Mycobacterium vanbaalenii* [21] were recorded as phenanthrene degrading bacteria so far and common naphthalene-degrading bacteria include *Pseudomonas* spp., *Vibrio* spp., *Mycobacterium*spp., *Marinobacter*spp., and *Sphingomonas*spp. *Micrococcus* spp.were documented [22]. Pawar et al., (2013) isolate genus *Enterobacter*spp. and *Bacillus* spp. as naphthalene degraders[23] and other several studies were also recorded *Bacillus* as naphthalene degrading genus[24, 25]. *Staphylococcus* (11.5%),*Corynebacterium*(5%), *Bacillus* (10%), *Proteus*(21.6%), *Pseudomonas*(8.3%), *Escherichia*(33.3%),*Klebsiella*(10 %) were resulted as naphthalene degrading bacteria (Sharma et al. (2014). Farshad et al. (2011) studied naphthalene-degrading bacteria in oil-contaminated soils and isolated 60 species of bacteria, which were belong to the genera *Enterobacter*, *Arthrobacter*and *Nocardia*. In many of previous studies *Bacillus* sp. was identified as the best naphthalene degrader among where at the present study *B.cereus*was capable of degrading naphthalene (73%) after 14 days' incubation.

Bio surfactant activities indirectly represent quantity of bio surfactant produced and directly involved in the process of hydrocarbon removal from the environment through increased bioavailability and subsequent biodegradation of the hydrocarbons by direct cell contacts [26, 27]. Thus the emulsification activity and emulsification index were used to determine bio surfactant producing activity of bacteria [28].

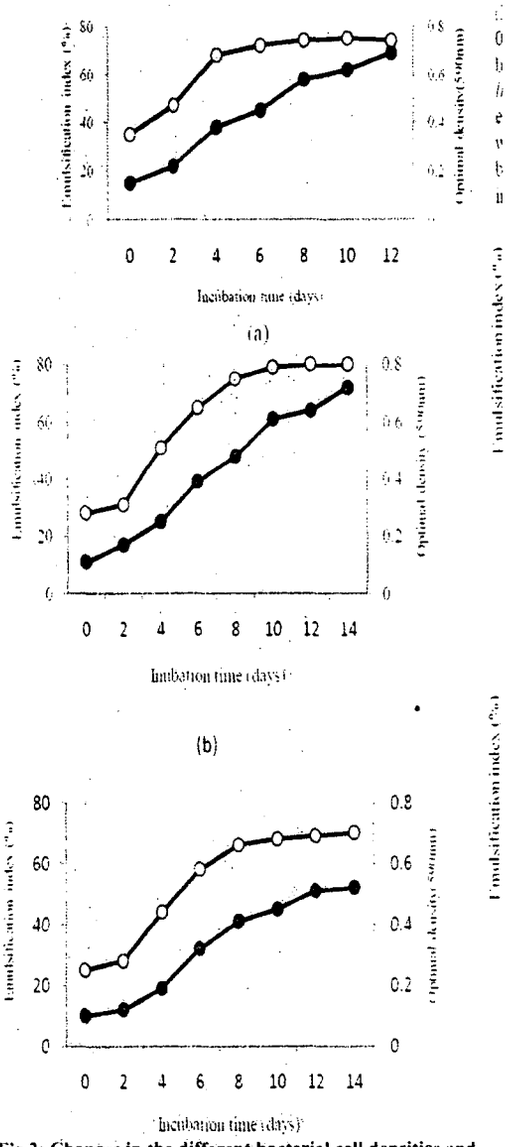

Fig 3: Changes in the different bacterial cell densities and emulsification index for phenanthrene. (a)*B.cereus* (b) *E. ludwigii*(c) *Enterobacter*sp. Optimal density (open circle),Emulsification index(close circle). When error bars are not shown, standard deviation was less than the width of symbol.

It was detected that both optimal density and emulsification index were increasing with incubation time (Figure 3). Initial optimal density of bacteria was around 0.27 and rapid increase of optimal densities of the bacterium *B. cereus*(0.74), *Enterobacter*sp.(0.70) and *E. ludwigii*(0.8) were recorded at14 days of incubation. The emulsification index of each bacteria inoculated sample was increased from 10% to 69% by *B. cereus*, 52% by*Enterobacter*sp. and 72% by *E. ludwigii* at 14 days of incubation respectively.

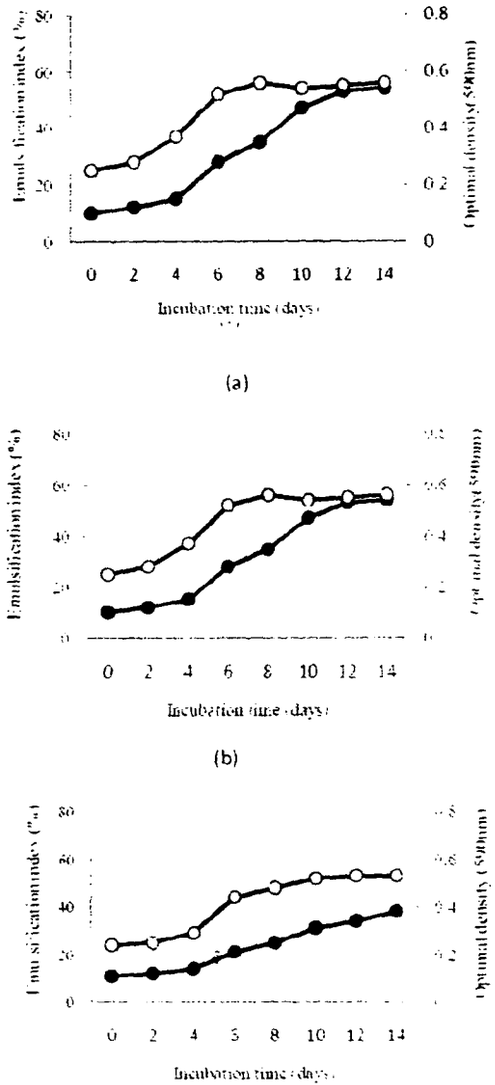

Fig 4: Changes in the different bacterial cell densities and emulsification index for naphthalene (a) *B. cereus* (b) *E. ludwigii* (c) *Enterobacter*sp. Optimal density (open circle) .Emulsification index(close circle). When error bars are not shown, standard deviation was less than the width of symbol.

Increment of emulsification index (E.I) and optimal density (O.D) of bacteria was detected when bacteria treatedwith naphthalene. The highest emulsification index and optimal density was recorded for*B. cereus*(E.I: 59%, O.D: 0.61), where *E.ludwigii*(E.I: 54%, O.D: 0.56) and *Enterobacter*sp. (E.I: 38%, O.D: 0.53), followed descending trend of emulsification index at 14 days of incubation.

Kostka et al.,(2011) reported that 96% of hydrocarbon-utilizing bacteria isolated from freshwater lakes wereable to emulsify PAHs, and it has been observed thatmixed cultures of marine (108) and soil (77) bacteria whicheffectively degrade naphthalene and phenanthrene also exhibit strong emulsifyingactivity [19]. Omatayo*et al.*, (2012) recorded the emulsification index values of *Corynebacterium* sp., *Bacillus* sp. and *Micrococcus* sp. were 48.80%, 48.25% and 48.84% after 30 days of incubation[29].

Peterz et al. (2010) and Rajesh et al. (2013) have reported that the degradability of naphthalene and phenanthrene by native bacteria [30, 31]. In the present study showed high emulsification index (Figure 3 and 4) which ensured the ability of the isolates to clean up oil polluted environmental effectively. Among three isolates, the most dominant naphthalene and phenanthrene degrading bacteria were identified as *B.cereus*and *E.ludwigii*respectively. Therefore, the results of this study showed that the bacteria are potential to remove the aromatic compoundssuch as naphthalene and phenanthrene. Furthermore, each isolate exhibited higher phenanthrene degradation compared with phenenthrene degradation.

Further researches studies related to PAHs degradation can result in more efficient and less time consuming microbial technologies which are important for developing countries like Sri Lanka.

ACKNOWLEDGEMENTS

The study was financially supported by the University of Sri Jayewardenepura, Sri Lanka.

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