Isolation of *Staphylococcus nepalensis* for Degradation of pyrene from Diesel Contaminated Site

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ABSTRACT
Pyrene is a relatively persistent 4-ring polyaromatic hydrocarbon (PAH) pollutant, and is often used as a model substrate in studies pertaining to high molecular weight PAH degradation. For degradation of pyrene, *Staphylococcus nepalensis* was isolated from diesel contaminated soil sample and it was tolerant to $50mgL^{-1}$ of pyrene. In 16S rRNA gene sequence *Staphylococcus* sp. showed 96% sequence similarity with *Staphylococcus nepalensis.* For efficient degradation of pyrene, various parameters such as pH, temperature and contact time were optimized. In order to enhance the degradation rates of pyrene, carbon sources such as glucose and sucrose at different concentration were also evaluated. *Staphylococcus nepalensis* showed maximum degradation of pyrene at pH 8 and temperature at 30°C within 5 days of incubation. The excellent bacterial growth and efficient pyrene removal was more when enriched with carbon co-substrates (glucose 4%, sucrose 2%).

General Terms
Polyaromatic Hydrocarbon, Pollutant, Enrichment technique.

Keywords
Pyrene, *Staphylococcus nepalensis*, Biodegradation, Co-substrate.

1. INTRODUCTION
Polyaromatic hydrocarbons are pervasive in air, water and soil. The fate of polycyclic aromatic hydrocarbons is of great environmental concern owing to their toxic, carcinogenic and mutagenic properties and their derivatives are widespread due to incomplete combustion of organic materials. They arise by natural combustion of forest fires, volcanic eruptions and also by human activities [1]. Due to the high hydrophobicity, pyrene is an important compound among the HMW PAHs (High molecular weight polyaromatic hydrocarbons), which are recalcitrant for microbial degradation and restricts its cellular uptake by microbes [2]. Pyrene, also referred to as benzo(def)phenanthrene is derived from coal tar. It is a tetracyclic aromatic hydrocarbon with a symmetrical structure. Pyrene is not a genotoxic compound by itself but it has four aromatic cores that are found in several PAH carcinogens, including benzo pyrene, indeno (1, 2, 3-cd) pyrene and 1-nitropyrene [3]. It is also a carcinogenic PAH as proven by Gong and Li [4] and causes crucial illness to human health and environment [5]. Using microorganism was an attractive methodology for restoration of the PAHs contaminated sites. Biodegradation by microorganisms is usually the dominant and most important way to degrade organic pollutants. Previous studies have shown that single isolates of either bacteria or fungi cannot allow rapid degradation of high-concentration PAHs such as pyrene and benzo[a]pyrene [6]. New microorganisms and their degradation features need further explorations to obtain more efficient approaches for pyrene biodegradation. Hence in this study the degradation of pyrene by single bacteria isolated from diesel contaminated soil was used for pyrene degradation. Also to enhance the bacterial growth and its pyrene degrading ability, enrichment technique were carried out using different carbon sources.

2. MATERIALS AND METHODS
2.1Chemical and Media
The Basal salt medium (BSM) medium used for experiments contained per liter 0.38g K_2HPO_4, 0.6g KH_2PO_4, 0.2g $MgSO_4.7H_2O$, 1g NH_4Cl and 0.05g $FeCl_3$. Solid plate was prepared with 2g agar in 100ml of distilled water. Luria Bertani (LB) medium (pH 7) containing 5g NaCl, 10g peptone, 5g yeast extract with 15g agar in 1000ml of LB plate was used.

All the chemicals used were of analytical grade and organic solvents were HPLC grade. Pyrene was purchased from SIGMA-ALDRICH. The stock solution was prepared in 45% of acetone (Eibes et al., 2006).

2.2 Isolation and Identification of Pyrene Degrading Bacteria
The bacterium was isolated from diesel contaminated soil sample on basal salt medium containing pyrene ($50mgL^{-1}$). The isolated bacterium was identified by biochemical characteristic using standard methods and 16Sr RNA gene sequence analysis by PCR amplification. The sequence was compared in GeneBank by using BLAST alignment tool. Multiple alignment of sequence and construction of phylogenetic trees were performed with the clustal X software. The stability of relationships was assessed by a

bootstrap analysis of 1000 trials. The culture was deposited in NCBI.

2.3 Optimization of Pyrene Degrading Bacteria

Pyrene uptake at different concentration (10 to 100 mgL^{-1}), the influence of hydrogen ion concentration (pH 2, 4, 6, 8 and 10), temperature variations (25 °C, 30 °C, 35 °C, 40 °C and 45 °C) and contact time (1-10 days) on bacterial growth and pyrene degradation were studied. The reaction mixtures were agitated in shaking incubator at 120rpm. The optical density value of each bacterium from above mentioned experiment was determined after 5 days of incubation. The broth culture was centrifuged at 6000 rpm for 20min. The bacterial biomass was measured spectrophotometrically at 595nm. The concentration of pyrene was determined by liquid-liquid extraction. From the collected supernatant, equal volume of hexane (v/v) was added and mixed thoroughly. The solvent in the upper layer was pooled out, this procedure was done trice. Extracted solvent was dried in the water bath and the residual was suspended with 5ml of methanol. It was measured spectrophotometrically at 254nm (Das and Mukerjee 2007).

2.4 Enrichment of Carbon Source in Pyrene Degradation

Biodegradation of pyrene by *Staphylococcus nepalensis* were performed by enriching the media with carbon (glucose and sucrose) sources from 1-5%. The culture was incubated at their optimum temperatures 30 °C and pH 8 with shaking condition at 120rpm for 5 days. The bacterial population and concentration of pyrene were determined by above mention procedure.

3. RESULTS AND DISCUSSION

The isolated strain showed effective growth on basal salt medium (BSM) by utilizing pyrene and it was identified on the basis of colony morphology, various staining reaction, biochemical activities (Table 1) and 16S rRNA gene analysis. The sequence and phylogenetic analysis of PCR amplified 16S r RNA gene fragment showed closest homologue to the *Staphylococcus nepalensis* (Figure 1). The 16S rRNA sequence were submitted in the Gen Bank under the accession number KF450166.

Characteristic	Strain
Color of colonies	White colony
Morphology	Cocci
Motility	Non motile
Gram's staining	+
Catalase activity	+
Oxidase test	-
Nitrate reduction	-
Indole production	-
Methyl Red	-
Voges proskauer	-
Citrate Utilization	+
Urease Activity	-
Anaerobic Growth	-
Glucose	-
Dextrose	+
TSI	K/A
H_2S Production	-
Gas Production	-
Starch hydrolysis	-
Casein hydrolysis	+
Gelatin liquefaction	-
Strain	***Staphylococcus* sp.**

Table 1. Biochemical characteristics of *Staphylococcus* sp.

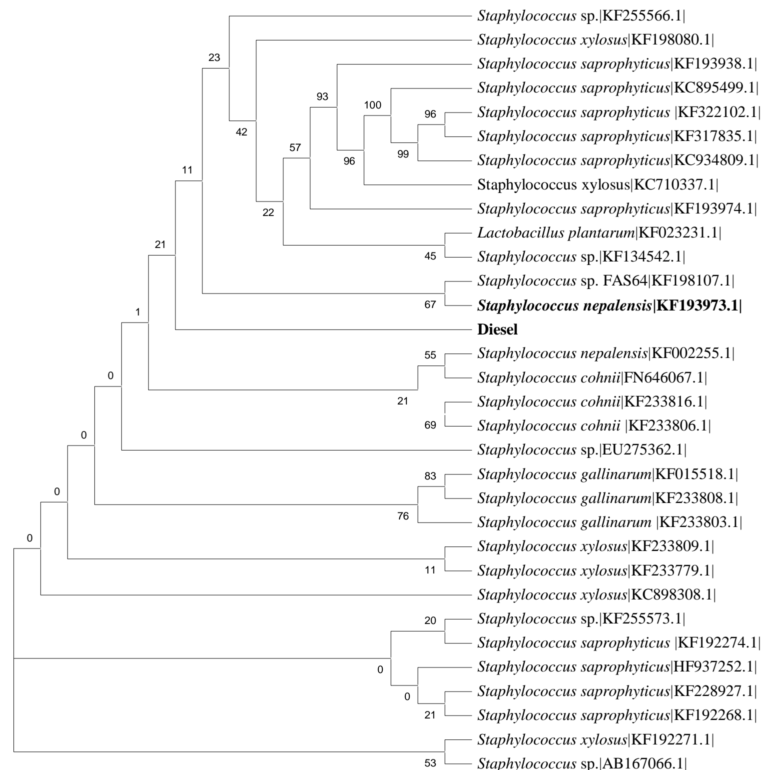

Fig 1: Neighbor-joining phylogenetic tree showing position of *Staphylococcus nepalensis* isolated from diesel contaminated soil based on 16S rRNA sequence

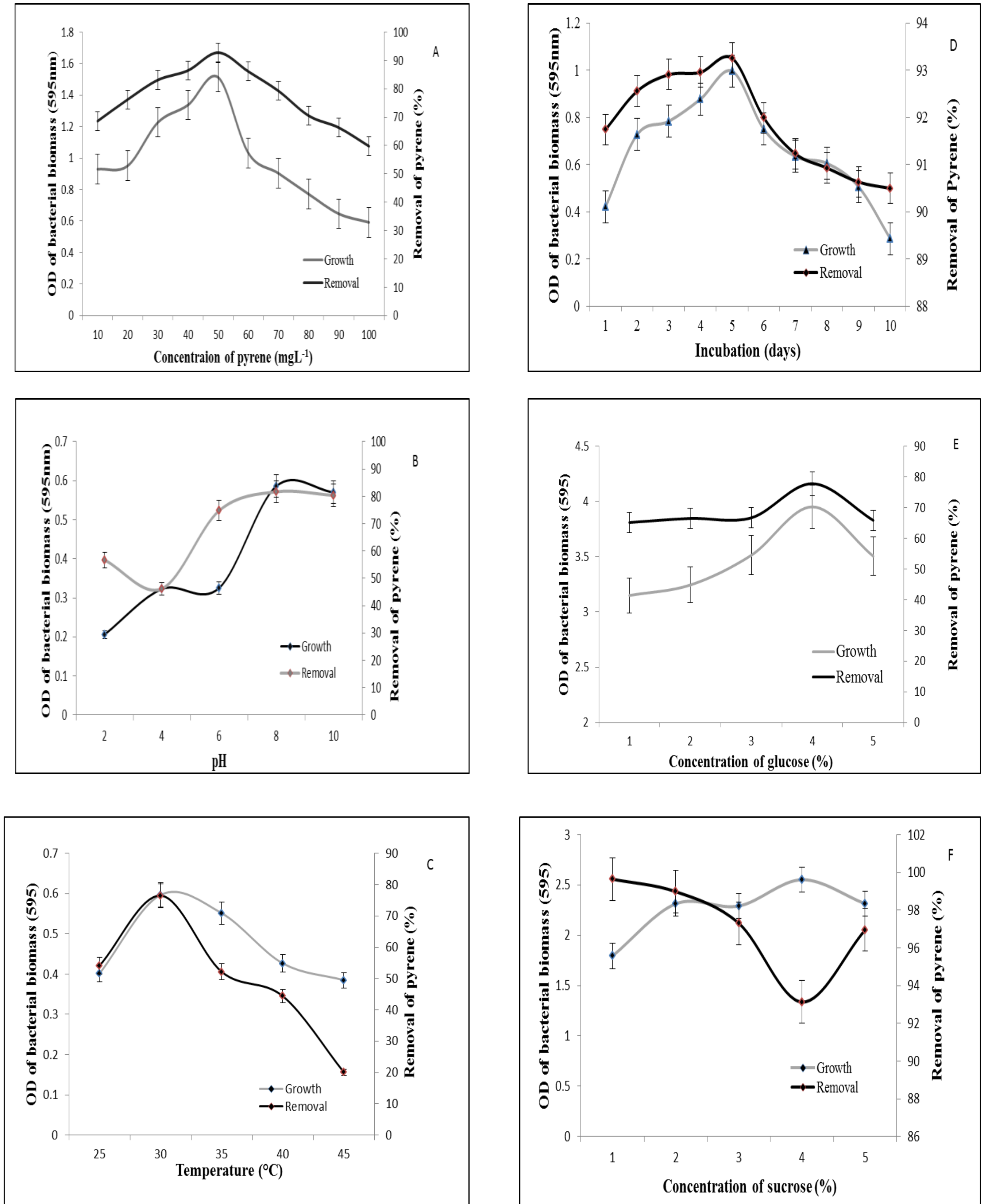

Fig 2: Effect of initial concentration (A), pH (B), temperature (C), incubaction time (D) and carbon sources (E, F) on bacterial growth and degradation of pyrene

Staphylococcus nepalensis showed efficient removal of pyrene and good bacterial growth for 50mgL^{-1} concentration (Figure 2A). At 10mgL^{-1} of pyrene concentration the organism showed 68.61% of pyrene removal and the degradation was gradually increased to 92.78% at 50mgL^{-1} concentration of pyrene and growth of the *Staphylococcus nepalensis* was also showed high. Further increasing in the concentration of pyrene from 60 to 100mgL^{-1} the growth of the bacteria was significantly inhibited and removal of the pyrene was also reduced to 59.76%. The optimized degradation conditions were obtained for *Staphylococcus nepalensis* by varying pH and temperatures. The maximum removal of pyrene was 81% at pH 8 (Figure 2B) and 76.5% at 30 °C temperature (Figure 2C) when compared with other pH and temperatures. At pH 2 the removal of pyrene was 56% and it reaches highest at pH8 with 81%. The pH of culture medium can affect microbial diversity and activity through altering the enzymatic activity, transporting processes and the nutrient solubility [7], hence the pH plays a major role in degradation of pyrene. At 25 °C optical density value of bacterial growth was 54 % and it reaches to 76.5% at 30 °C. It showed 52.14, 44.3 and 20% removal at 35, 40 and 45 °C. With increase in temperature the bioavailability of PAHs molecule will increase. In addition, the oxygen solubility decreases with increase in temperature, which will reduce the metabolic activity of aerobic microorganisms [8]. The bacterial growth and pyrene removal was very low on the first day of incubation. The removal of pyrene was gradually increased to 93.25% on fifth day of incubation (Figure 2D). After fifth day, the removal of pyrene remained constant and growth of bacteria was rapidly decreased. Thus the optimum incubation time for maximum microbial growth and removal of pyrene was obtained at fifth day. In another study by Ping and *al* [9] 50% pyrene degradation by strain *Pseudomonas putida PL2* occured after 6 days of incubation and also 71.5% of pyrene was degraded in 7 days by Proteus *vulgaris* [10]. Nnamchi and *al* [11] reported that bacteria are able to degrade aromatic compounds in their surroundings because they possess all the necessary enzymes which are needed to degrade PAHs. Furthermore, co-metabolites (glucose and sucrose) were added to the experimental system for enriching the degradation rate by *Staphylococcus nepalensis* and every single co-metabolite showed different effects on pyrene biodegradation. Biodegradation of sole organics may be enhanced or restrained when other organic carbon sources exist, because of the co-metabolism or competition among these matters [6]. Glucose showed 77% of pyrene removal at 4% of concentration (Figure 2E) and 2% of sucrose showed 98.97% (Figure 2F) of pyrene degradation. In this experiment 2% of sucrose showed the maximum pyrene removal when compared with glucose. Carbon sources functions as co-substrate and it is an easy nutrient for bacteria. It may produce some metabolites upon metabolizing the carbon sources that may enhance the bioavailability of PAH or help bacteria to degrade [12]. Hence enrichment method yielded more bacterial population for degrading the pyrene at optimum conditions. In this study *Staphylococcus nepalensis* showed the maximum degradation rate of pyrene (50mgL^{-1}) with favorable conditions and on the addition of co-substrates namely 2% sucrose.

4. CONCLUSION

Staphylococcus nepalensis from diesel contaminated soil was capable of degrading pyrene to 93.25% (50mgL^{-1}) at an optimum condition of pH 8 and temperature 30°C. With additional nutrient of carbon source and at optimal conditions the bacteria's capacity to degrade pyrene increased to 98.97%. By enriching the basal salt media, bacterial growth was good and removal was also high when compared with normal media. Therefore, it was concluded that *Staphylococcus nepalensis* strain is an efficient degrader of pyrene which can be employed for the removal of pyrene in diesel contaminated sites. Also the application of this microbe in the contaminated ecosystem help to reduce the health risks associated with exposure of other PAHS also.

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