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## EVALUATION OF POTENTIAL FOR CONSIDERABLE DEGRADATION OF POLYAROMATIC HYDROCARBONS AND HETEROCYCLES UBIQUITOUS IN COAL TAR EFFLUENTS

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### ABSTRACT

Coal tar creosote is rich in various organic compounds which are reported to be potentially carcinogenic and harmful. Among the various compounds the predominant ones are assorted polycyclic aromatic hydrocarbons, phenols and heterocycles. Hence biodegradation of these recalcitrant constituents to reduce the toxicity of this multicomponent liquid is a major focus of research in this study. Coal tar effluent collected from an industrial site in West Bengal was subjected to microbial degradation by selected strains of mostly gram-negative bacteria viz. *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella abony* and a single gram-positive strain viz. *Staphylococcus aureus*. Reduction of the BOD<sub>5</sub> value from 250 to 30 and significant increase in colony forming unit count indicated *Staphylococcus aureus* as the most suitable resistant strain for degrading the effluent. Further evaluation of the degrading capacity of this strain and minimum inhibitory concentration (MIC) was done individually with 16 compounds comprising of 6 aromatic hydrocarbons (viz. benzene, toluene, *m*-xylene, naphthalene, phenanthrene, flourene), 3 phenols (viz. phenol, *o*-cresol, 2, 4-dimethyl phenol), 5 nitrogen heterocycles (viz. indole, quinoline, isoquinoline, carbazole, acridine), aniline and 1-naphthylamine. *Staphylococcus aureus* was found to degrade most of the compounds in variable extent except phenols (viz. phenol, *o*-cresol, 2, 4-dimethyl phenol), indole, isoquinoline, carbazole and aniline. Most effective degradation was found in case of aromatic hydrocarbons, especially in benzene followed by naphthalene and phenanthrene. Among heterocyclic compounds acridine followed by quinoline recorded a significant microbial growth.

### KEYWORDS

Aromatic hydrocarbon, Nitrogen heterocycles, Biological oxygen demand, Coal tar effluent and Biodegradation.

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### INTRODUCTON

Coal-tar creosote, a brownish liquid in appearance is actually a mixture of numerous organic compounds of which less than 20% of those compounds are present in amount more than 1% of the total<sup>1,2</sup>. Creosote contains six major classes of compounds<sup>3,4</sup>.

1. Aromatic hydrocarbons including polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs (which can constitute up to 90% of creosote)
2. Tar acids/phenolics including phenols, cresols, xylenols (about 2-17 weight %)<sup>5</sup>
3. Tar bases/nitrogen heterocycles including quinolines, benzoquinolines, acridines, indolines, and carbazoles (nitrogen-containing heterocycles, 4.4-8.2 weight %<sup>6</sup>
4. Aromatic amines including aniline, naphthylamine<sup>7</sup>
5. Sulfur heterocycles including thiophenes (1-3 weight %)
6. Oxygen heterocycles, including dibenzofurans (5-7.5 weight %).

Understanding the fate of potentially carcinogenic and mutagenic compounds within groundwater and subsurface soils is important in assessing the possible impact of these contaminants on an ecosystem<sup>8</sup>. Since these organic pollutants are toxic in nature their release into natural water bodies is dangerous to both the environment and human health<sup>9</sup>. Exclusion of these organic pollutants from water have been attempted through various strategies, following chemical (redox, complexation, and ion-exchange methods), biological (aerobic and anaerobic), and physical techniques (adsorption, precipitation, reverse osmosis, and membrane filtration)<sup>10,11</sup>. Degradation of coal tar-contaminated soils containing polycyclic aromatic compounds is highly challenging because of the low solubility and strong sorption properties of these aromatic compounds<sup>12</sup>. Microbial degradability of organic compounds to microbial destruction in nature may depend on its structure, concentration, solubility in water and other environmental factors<sup>13</sup>.

Pollutants such as polycyclic aromatic hydrocarbons (PAHs) are recalcitrant in nature and are difficult to degrade. These hydrocarbons may be transferred to humans through fish and seafood consumption<sup>14</sup> as they can sorb to organic-rich soils and sediments and get accumulated in aquatic animals. Therefore, it is required to degrade or minimize some pollutants that are resistant to

biological degradation. Though a few species of bacteria and fungi are able to cause extensive degradation of relatively simple polycyclic aromatic molecules (biphenyl and naphthalene, for example) most microorganisms are unable to cause complete biodegradation of polycyclic aromatic compounds<sup>15,16</sup> due to the difficulty of isolating and removing PAHs from the environment, a better understanding of bioavailability, source apportionment, and degradation of these compounds will facilitate remediation efforts in contaminated areas<sup>17</sup>.

This research relates to estimation of potential for degradation of assorted polycyclic aromatic compounds found in coal tar effluent. In particular the objective of this investigation is to find a suitable microorganism capable of degrading the coal tar effluent as well as few predominant constituents occurring in coal tar creosote to provide an effective method of bioremediation. This study relates to a process for biodegrading coal tar and its assorted major constituents thereof. The process involves addition of different microbial strains with appropriate nutrients to coal tar and its polycyclic aromatic constituents separately under aerobic conditions and monitor the extent of biodegradation.

## MATERIAL AND METHODOLOGY

### Chemicals

All the solvents and reagents used were procured from Merck (India). The culture medium used was procured from Himedia Laboratories Ltd., India. All chemicals used were of analytical grade.

### Collection of Sample

Effluent samples were collected from the industrial site of Dankuni where large amounts of coal tar and creosote were discharged onto the site. The soils, surface, and groundwater are extensively contaminated with this material. The effluent water from the coal-manufacturing unit of the area was collected as the sample.

The other individual samples were 16 organic compounds comprising of 6 aromatic hydrocarbons (*viz.* benzene, toluene, *m*-xylene, naphthalene, phenanthrene, flourene), 3 phenols (*viz.* phenol, *o*-

cresol, 2, 4-dimethyl phenol), 5 nitrogen heterocycles (*viz.* Indole, quinoline, isoquinoline, carbazole, acridine), aniline and 1-naphthylamine. All these were procured from Merck (India).

#### PREPARATION OF TEST ORGANISMS

To determine biodegradability of the effluents and the organic compounds isolated strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella abony* were obtained from microbiology laboratory of Calcutta University, Kolkata.

The cultures of test organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella abony*) were maintained in agar slants at +4°C. Bacterial inoculums obtained from these reference stock cultures were inoculated in nutrient agar medium, which was incubated at 37°C for 18-24 hours. From the fresh grown cultures decimal dilutions were made in sterile lactose broth to the concentration of 10<sup>6</sup> CFU/mL and used for testing the effluent samples.

#### Determination of Biodegradability of Effluent

The individual strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella abony*) were tested separately for their degrading capability and their ability to use the components of the effluents by inoculation in the nutrient agar medium containing 1mL of 10% and 100% effluent solution after adjusting the pH to 7. Growth of bacterial colonies was recorded in the plates. Growth of the bacterial strains in the presence of effluent was determined by estimating the number of colony forming units (CFU) per mL. Sterile petri plates using nutrient agar medium were prepared and 1mL of effluent sample were poured in each plate before the agar had solidified. Then 10µL of inoculums prepared in lactose broth of each of four bacterial strains were added to the plate. They were kept at 37°C for 24 hours and the colony count (CFU/mL) was calculated.

Number of colonies

Number of cells per mL = ----- ..... (1)

Amount plated \* dilution

#### Determination of biological Oxygen Demand (Bods)

The strains, which were capable of utilizing the carbon from the effluents, were further subjected to harvesting by incubation on a rotary shaker at 200rpm under aerobic condition at 30°C. Approximately 10<sup>7</sup> cells of pure culture were inoculated in the nutrient medium containing the 10% effluent solution. After incubation in the dark for five days at 20°C, BOD<sub>5</sub> analysis was executed.

#### Determination of Biodegradability of Aromatic Compounds and Minimum Inhibitory Concentration (MIC)

Sterile petri plates using nutrient agar medium were prepared and 1mL of each of 15 aromatic compounds in concentrations 100mM and 1000mM were poured in each plate before the agar had solidified. Then 10µL of inoculums prepared in lactose broth of the most resistant strain *Staphylococcus aureus* was added to the plate. They were kept at 37°C for 24 hours and the colony count (CFU/mL) was calculated using equation (1). Minimum inhibitory concentration (MIC) of compounds was assessed using the broth micro-dilution method<sup>18</sup>. MICs were determined from the colony count after the incubation period and were taken as the sample concentration at and above which no growth microbial was recorded.

All the experiments were carried out in triplicate.

#### STATISTICAL ANALYSIS

The data was reported as mean (n=3) ± SD (n=3). Statistical analyses of the data were performed by split-plot design Analysis of Variance (ANOVA). Probability value of  $P \leq 0.05$  was considered to denote the statistically significant differences at 5% level of significance.

#### RESULTS AND DISCUSSION

##### Determination of Biodegradability of Effluents

In batch cultures the individual gram negative bacterial strains comprising of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella abony* were unable to substantially degrade the effluent along with nutrient as observed from the colony count represented in Figure No.1. Whereas

inoculation of the single gram-positive strain viz. *Staphylococcus aureus* in nutrient medium with the effluent was found to be highly effective since huge growth of bacteria was recorded from the colony forming unit count. Biodegradability of *Escherichia coli*, *Pseudomonas aeruginosa* as accounted from cell numbers was only 25% of *Staphylococcus aureus* whereas that of *Salmonella abony* was only 16% of the most resistant strain. Bacterial count varied inversely with change in effluent concentration. Maximum growth of all the bacterial strains were observed when the inoculation was done in 10% effluent solution compared to 100%. High concentrated growth exhibited by the resistant *Staphylococcus aureus* indicating capability of effectively degrading the effluent was also manifested from decline in dissolved oxygen (DO) concentration in the sample (Table No.1).

#### **Determination of biological Oxygen Demand (BOD<sub>5</sub>)**

The biological oxygen demand values indicate the decay of organic matters present in the effluent. It is the amount of dissolved oxygen consumed by the microbes while they decompose organic matter under aerobic conditions at a specific temperature. BOD<sub>5</sub> values of the waste before and after microbial treatment with all the four strains are depicted in Table No.1. Reduction in the BOD<sub>5</sub> values post remediation implies decrease in the amount of dissolved oxygen in the effluent and hence reinforces the degradation results shown in Figure No.1. On treatment with *Staphylococcus aureus* BOD<sub>5</sub> is reduced from about 250 to 30. Compared to the maximum reduction (88%) of the biological oxygen demand value recorded by *Staphylococcus aureus* the other strains viz. *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella abony* could only reduce by 51.8%, 44.5% and 26% respectively. Hence from the recorded BOD<sub>5</sub> values it can be inferred that *Staphylococcus aureus* could significantly deplete the organic wastes from the effluent to a satisfactory and acceptable value for discharge of the creosote to the soil<sup>19</sup>.

#### **Determination of Biodegradability of Aromatic Compounds**

Further investigations were carried out to identify the compounds actually undergoing biodegradation through the attempt of potential biodegradations of the individual compounds present in coal tar in predominant proportion. Different classes of compounds like aromatic hydrocarbon, tar acids/phenolics, tar bases/nitrogen containing heterocycles, aromatic amine etc. in their purest form was administered for the study of the microbial degradation individually by that particular strain of *Staphylococcus Aureus* which had already furnished excellent biodegradation of the coal tar effluent. Out of 16 aromatic compounds of different class only 9 compounds comprising of all the 6 aromatic hydrocarbons (viz. benzene, toluene, *m*-xylene, naphthalene, phenanthrene and fluorene), 2 nitrogen heterocycles (viz. quinoline, acridine) and the nitrogenous base named 1-naphthylamine underwent microbial degradation. Among all classes of aromatic compounds the hydrocarbons allowed maximum growth of microbes. Microbial degradation in the nitrogen heterocycles was much less than the hydrocarbons only with an exception for acridine which exhibited a substantial bacterial count. Higher microbial population was observed in the individual compounds at 100mM concentration compared to 1000mM and higher concentrations (Figure No.2). On decreasing the concentration of the organic compounds (benzene, toluene, *m*-xylene, naphthalene, phenanthrene, fluorene, quinoline, acridine and 1-naphthylamine) from 1000mM to 100mM percentage increase in the growth of bacteria recorded was 188.5%, 135.3%, 131.1%, 70.8%, 148.8%, 183.4%, 1058.8%, 477.8%, 778%, 1037.5% respectively. From this data it can be inferred that variation of concentration of individual compounds had lesser impact on the growth of microbes in case of aromatic hydrocarbons with an exception of fluorene and had higher impact in 1-naphthylamine and acridine.

### **Biodegradability of Aromatic Hydrocarbons and Minimum Inhibitory Concentration (MIC)**

Six aromatic hydrocarbons namely benzene, toluene, *m*-xylene, naphthalene, phenanthrene, fluorene which are generally present in the effluent in sizeable proportion was subjected to microbial degradation with *Staphylococcus Aureus* separately. The result of the biodegradability study along with the Minimum inhibitory concentration (MIC) data is represented in Table No.2. All the aromatic hydrocarbons had underwent biodegradation and to a substantial extent as expressed in Figure No.2. Both naphthalene and benzene showed a considerable microbial population at 1000Mm followed by phenanthrene, whereas *m*-xylene, toluene and fluorene were next in order and could allow less than 50% microbial growth compared to naphthalene, benzene and phenanthrene. MIC values recorded in Table No.2 indicate the concentration above which no microbial growth was observed. Naphthalene scored the highest MIC value of 290mg/mL whereas benzene recorded the least, 115mg/mL. Phenanthrene recorded 285mg/mL followed by fluorene and toluene with values 180mg/mL and 155mg/mL. Though both naphthalene and phenanthrene showed a substantial microbial growth and a similar high MIC value, benzene in spite of comparable microbial growth recorded the lowest MIC value. Hence it can be inferred that concentration of benzene higher than 115mg/mL is difficult to biodegrade while naphthalene and phenanthrene can be biodegraded upto a concentration of 285mg/mL.

### **Determination of Biodegradability of Tar Acids - Phenols**

Coal tar effluent comprises of tar acids which are also known as phenols. In this category 3 phenolic compounds namely phenol, *o*-cresol and 2, 4-dimethylphenol was evaluated for their biodegradability. *Staphylococcus aureus* which yielded good biodegradability results for aromatic hydrocarbons turned out to be ineffective in case of phenolic compounds upto a concentration of 100mM. None of the phenols could be biodegraded by this strain. Biodegradable potential at lower concentrations (<100mM) had not been studied.

### **Biodegradability of Tar Bases- Nitrogen Heterocycles and Amines**

Tar bases present in the creosote mainly comprises of two classes - nitrogen heterocycles and aromatic amines. Of these two categories 5 nitrogen heterocycles viz. indole, quinoline, isoquinoline, carbazole and acridine and 2 aromatic amines viz. Aniline and 1-naphthylamine were evaluated of their degradability separately with *Staphylococcus aureus* since they are present in slightly higher proportions in the effluent than the others. The minimum inhibitory concentration (MIC) data and result of the biodegradability study is represented in Table No.4.

Only two nitrogen heterocycles - quinoline and acridine could be degraded, whereas only one aromatic base- 1-naphthylamine allowed microbial growth. The other compounds tested did not report any growth upto 100mM concentrations. Tolerance of microbes at lesser concentration was not tested. From Figure No.2 it is also found that between two nitrogen heterocycles microbes utilized acridine more as their source of carbon and nitrogen than quinoline. Microbial population recorded at 100mM in quinoline is 30% than in acridine whereas at a higher concentration of 1000 Mm, bacterial population in quinoline is 15% of acridine. Microbial growth in 1-naphthylamine was still lesser recording only 17.5% and 9% of microbial population in acridine at 100mM and 1000mM concentration.

MIC values recorded in Table No.4 indicate the concentration above which the microbial growth was inhibited. It is thus observed from the table that acridine did not allow any microbial growth above 213mg/mL whereas for quinoline and 1-naphthylamine the MIC values are 140mg/mL and 155mg/mL respectively. Hence it is inferred that acridine can be degraded even at higher concentrations than 1-naphthylamine and quinoline.

### **STATISTICAL ANALYSIS**




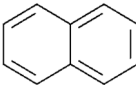
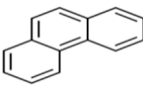
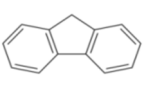
The overall view of the results of the ANOVA indicated that the variation in the chemicals had a control on the degradation of the effluent. The

bacterial strains responded to the variation in concentration as well as the chemicals.


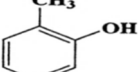
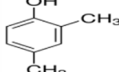
**Table No.1: Determination of bods**

S.No	Name of the strain	Initial BOD <sub>5</sub>	Final BOD <sub>5</sub>	% reduction
1	<i>Escherichiacolli</i>	250	120	51.8
2	<i>Pseudomonas arsenigenous</i>	250	138	44.5
3	<i>Staphylococcus aureus</i>	250	30	88
4	<i>Salmonella abony</i>	250	185	26


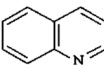
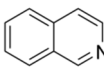
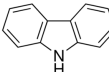
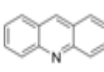

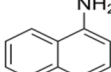
**Table No.2: Biodegradability of aromatic hydrocarbons**

S.No	Name of aromatic hydrocarbons	Benzene	Toluene	<i>m</i> -Xylene	Naphthalene	Phenanthrene	Fluorene
1	Structure of compounds						
2	Microbial degradation	+	+	+	+	+	+
3	Minimum Inhibition concentration in mg/mL	115	155	180	290	285	245

**Table No.3: Biodegradability of tar acids - phenols**

S.No	Name of phenols	Phenol	<i>o</i> -cresol	2,4-dimethylphenol
1	Structure of compounds			
2	Microbial degradation	-	-	-
3	Minimum inhibition concentration in mg/mL	-	-	-

**Table No.4: Biodegradability of tar bases- nitrogen heterocycles and amines**

S.No	Name of nitrogenous heterocycles and bases	Indole	Quinoline	Isoquinoline	Carbazole	Acridine	Aniline	1-naphthylamine
1	Structure of compounds							
2	Microbial degradation	-	+	-	-	+	-	+
3	Minimum inhibition concentration in mg/mL	-	140	-	-	213	-	155

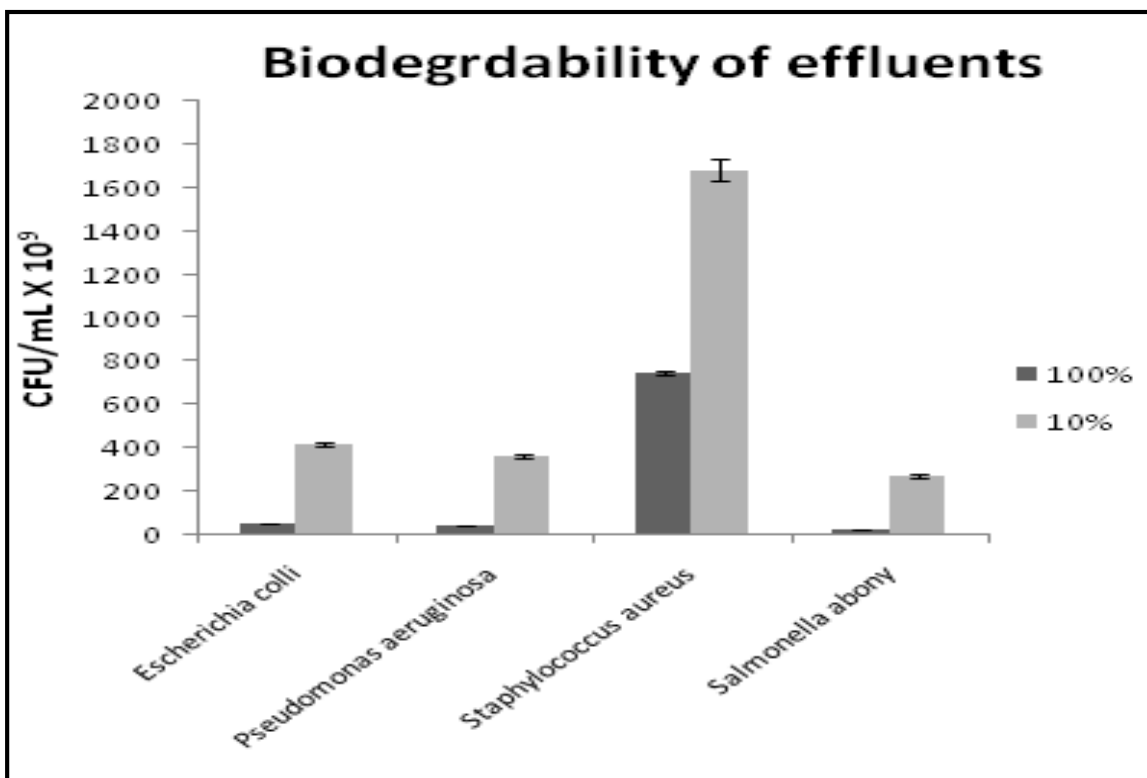


Figure No.1: Determination of biodegradability of coal tar effluent by individual bacterial strains

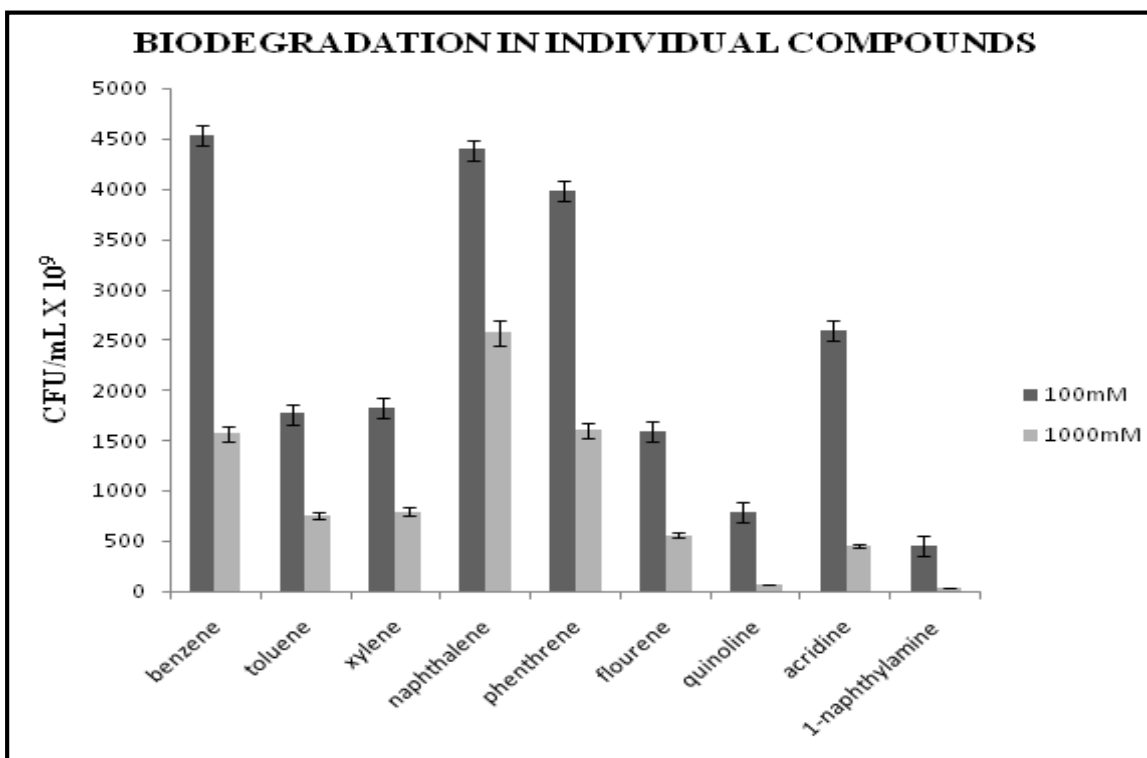


Figure No.2: Determination of biodegradability of individual organic compounds by *staphylococcus aureus*

## CONCLUSION

From the results of this study we find that the coal tar effluent collected as sample from industrial area of Dankuni, West Bengal could be substantially degraded by gram-positive strain viz. *Staphylococcus aureus* while the of the three gram-negative bacterial strains comprising of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella abony* were unable utilize the organic compounds as the sole source of carbon and nitrogen under aerobic condition. Growth of bacteria in 10% sample solution as assessed from the colony forming units in case of these gram-negative bacteria was just 16% of *Staphylococcus aureus* in case of *Salmonella abony* and 25% in the other two. Similar result was confirmed from the recorded BOD<sub>5</sub> values which indicated a maximum value of 88% reduction in the amount of dissolved oxygen in the creosote by the resistant strain *Staphylococcus aureus*. BOD<sub>5</sub> value noted after the degradation was within the acceptable range.

Further investigations to determine the resistance of *Staphylococcus aureus* to 16 predominant specific organic compounds present in this effluent revealed significant depletion of all the assorted aromatic hydrocarbons leading with naphthalene, followed by benzene, phenanthrene, *m*-xylene, toluene and fluorene respectively. Among the 5 nitrogen heterocycles, acridine was most utilized by the resistant strain compared to quinoline, while isoquinoline, carbazole and indole exhibited no growth upto 100mM concentration. Of the aromatic bases evaluated only 1-naphthylamine underwent microbial degradation whereas aniline did not allow microbial growth till 100mM concentration. Phenols which are identified as predominant tar acids reported no degradation upto 100Mm concentration in compounds phenol, *o*-cresol, 2, 4-dimethyl phenol. From the series of MIC values recorded for the degradable tolerant compounds it can be concluded that naphthalene and phenanthrene can be degraded even at higher concentrations compared to other aromatic compounds evaluated. Statistical results established the control of variation of chemicals, concentration

and bacterial strains on extent of degradability of the effluent.

Finally it can be concluded that this study has been able to provide an acceptable remediation of coal tar effluent with *Staphylococcus aureus* to decrease the toxicity by degrading most of its recalcitrant aromatic hydrocarbons along with few other nitrogen heterocycles and aromatic bases. Moreover it suggests the scope of further study of improving the resistance of the strains and hence degradability of these organic compounds by application of different biotechnological measures.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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