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Characterization of Hydrocarbon Degrading Bacteria Isolated from Hydrocarbon Contaminated Soil

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Polycyclic aromatic hydrocarbons (PAHs) are of particular concern due to its toxic and carcinogenic properties. The fate of these PAHs and other associated compounds present in the environment is directly related to abiotic and biotic processes; including oxidation, bioaccumulation and degradation by microbes. This study was conducted for the isolation of bacteria with potential biodegradation abilities. Nineteen different bacterial isolates were identified from soils contaminated with PAHs with biodegradation capabilities of different hydrocarbon compounds (1 %) (Xylene, Benzene, Naphylamine, Diphenylamine) added to nutritional media (L. agar and MM_2 Agar). These bacterial isolates were found able to grow in the presence of different metals stress along with hydrocarbon at different temperature (25 to 45) and pH (5 to 9) conditions. Majority of the isolates were found belonging to *Enterobacteriaceae* family, while some of them were from *Pseudomonas* and *Azotobacteriaceae*. The bacterial isolates were capable of degrading a verity of different hydrocarbons with its ability to grow in different metals stress environment. Such bacteria can be successfully used for the bioremediation of industrial wastes.

Key words: Bioremediation, Heavy metals, Industrial wastes, Environmental health, Biodegradation, Public health.

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants worldwide. Human activities are directly responsible for its accumulation[1]. These compounds are carcinogenic and carry the ability to stay in living organisms with long term resistibility to degrade[2]. Their bioavailability is limited by a low aqueous solubility, which causes specific adaptations in degrading bacteria[3]. Microorganisms are the scavengers in nature, responsible for recycling natural waste materials into harmless compounds[4, 5]. Microorganisms are highly adaptive and develop the ability to degrade such recalcitrant compounds through evolution of new genes, which encoded enzymes that can use these compounds as their primary substrates[6]. In most cases, this has been attributed to the production of extra cellular emulsifying agents during the hydrocarbon fermentation. A requirement for an extra cellular

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rhamnolipid in hydrocarbon utilization by strains of *Pseudomonas aeruginosa* has been reported[7,8].

Some microorganisms can use different complex hydrocarbons as their source of energy. The hydrocarbons (Benzene, Naphthalene, Phenanthrene, Xylene and Toluene) are readily degradable in the presence of oxygen. There is evidence, that under methanogenic conditions the activation of aromatic ring involves the addition of oxygen atom to the ring from water[9]. Reports showed that hydrocarbons degradation bacteria (Gram's positive and negative) are mainly associated with soil[10]. Mutants of *P. aeruginosa* which do not produce the rhamnolipid were found to grow poorly on the hydrocarbon substrate. Addition of rhamnolipid from the parental strain stimulated the growth of such mutants on hydrocarbons[11].

Presence and disposal of PAHs is highly concerned due to its toxicological effects in the environment, as the low molecular weight hydrocarbons are identifies as potentially toxic[12,13], whereas the higher-molecular weight hydrocarbons have genotoxicity and can accumulate in food chain[14]. Bioremediation of contaminated soil is an effective, environmentally and economically sustainable way to degrade the hydrocarbons. The microbes capable to biodegrad hydrocarbons can be isolated from hydrocarbon rich environment[1]. Researchers are investigating different tools and methods for an easy, economical and sustainable ways for treatment and bioremediation of naturally and anthropogenically contaminated environments[15].

Since microbes can be one important option to exploit for biodegradation and pollution eradication purpose. So it is highly needed to identify and investigate microbes with potential biodegradation abilities. The objective of the current study was to isolate bacteria from the hydrocarbon contaminated soil and to investigate its ability of degrading different hydrocarbons. The bacteria were also evolved for its growth in diverse stress conditions in the presence of different metals and pH.

MATERIALS AND METHODS

Isolation and identification of bacteria

Soil samples from the motor vehicles repairing workshop near Mozang Lahore Pakistan, were collected for isolation of hydrocarbons degrading bacteria. The soil was found enriched with different type of petroleum oils. All the samples were transported to laboratory in sterile plastic bags and further processed for chemical and microbiological analysis. Two different methods namely direct plating and dilution methods were used for bacterial isolation.

In the direct plate method 50 mL of the sample was poured over L-agar (Tryptone 10.0 gm/L, Yeast Extract 5.0 gm/L, NaCl 5.0 gm/L, Agar 12.0 gm /L, pH 7) and MM_2- agars plates ($(NH_4)_2 S0_4$ 2.38 gm/L, $FeSO_4$. $7H_2O$ 0.28mg/L, $CaCl_2$. $2H_2O$ 14.70 mg/L, $MgSO_4$. $7H_2O$ 246.48 mg/L, NaCl 497.25 mg/L, Na_2HPO_4 3.58 gm/L, KH_2PO_4 1.36gm/L, Agar 15 %, pH 7) supplemented with (1 %) Xylene, Benzene, Diphenylamine and Naphylamine, and incubated at 37 °C. In dilution method, different dilutions were made in sterilized test tubes. One mL of the filtrate sample was added to sterile distilled water to make up the total volume of 10 mL. One ml from this dilution was added in sterile distilled water to attain the total volume of 10 mL. From each dilution 50m micro Liter was poured on L-agar plate and plates were incubated at 37 °C. The colonies were purified by continuous sub-culturing.

Identification and characterization of bacterial isolates

The bacteria with abilities of degrading hydrocarbons supplemented in the media were further identified with the help of shapes and colony morphology on L- agar along with biochemical tests. Gram's staining, spore staining, capsule staining and hanging drop techniques were performed for cell morphology and motility.

All the bacterial isolates were sub-cultured on MacConkey agar (Himdia, India), Brilliant green agar (Himedia), Eosine Methylene Blue agar (Himedia), King's agar (A) and (B) and Simmon citrate agar (Himedia) at 37 °C for 24-48 h, whereas anaerobic oxidation fermentation, urease, nitrate reduction, de-nitrification test, catalase, cytochrome oxidase, methyl red and H_2S production test were performed for bacterial identification.

Antibiotic, metals, pH and temperature sensitivity test

Antibiotic sensitivity and resistance was determined using disc diffusion test against common antibiotics (Oxytetracycline, Penicillin,

Chloramphenicol, Erythromycin, Gentamicin, Minocyclin). Inhibition zones were observed after 16-24 h and recorded.

Metals compound ($PbNO_3$, $ZnCl_2$, $COCl_2$, $CdCl_2$, KCl_2, Hg and NiCl2) were supplemented (50, 100, 150 mg/mL) to nutritional media, incubated at 37 °C for 24 48 h for determination of bacterial resistance to metals. Visible growth on solid media and optical density (OD) measurement at 600 nm for liquid media were observed.

The ability of the bacterial isolates to resist different pH were studied by introducing it to pH (ranged from 5.0 to 9.0) of L. Broth previously adjusted with 2M NaOH / HCl was incubated at 37 °C for 24-48 h. Bacterial growth was conformed with the help of measurement at 600 nm.

Fresh culture of the bacterial isolates were introduced to L-Broth and incubated at different temperature (ranged 25, 37°C, 45 °C) for 24-48 h. Bacterial growth was conformed with the help of measurement at 600 nm.

Hydrocarbons degradation ability determination

In this method bacteria from the cultures that were able to grow overnight on L-broth were transferred with sterile tooth pick onto an MM_2-agar plates. Solutions 10 % of the hydrocarbons i.e. Diphenylamine, Naphylamine, Xylene and Benzene, were prepared in petroleum ether and were used as follows.

Freshly prepared ether solution of Diphenylamine immediately sprayed uniformly over the surface of the agar plate. The ether immediately vaporized from the surface at room temperature and layer of Diphenylamine remained on the entire surface. The plates were then incubated at 37 °C for 4 to 5 days. Similar procedure was used for the growth of bacteria utilizing Xylene, Benzene and Naphylamine. Appearance of clear zones around bacterial growth indicated positive test i.e. the ability of bacteria to degrade this hydrocarbon.

RESULTS

The soil samples collected from hydrocarbons contaminated soil were analyzed chemically and microbiologically. Chemical analysis of the soil showed the pH level (8.0 ± 0.11), electric conductivity (4.6 ± 0.22 mS/Cm), amount of phosphorus (16.0 ± 0.09 ppm), organic matters (presence), available potassium (320 ± 0.31 ppm), and texture of soil (sanolly lome) and saturation percentage (26 %).

Nineteen different strains were isolated on L-agar and MM_2 media supplemented with hydrocarbons (Xylene, Benzene, Diphenylamine, Naphylamine). These strains were labeled with identification code as XA, XB, X1OF, XF, XJ_1, XJ_2, XZ_1, XZ_{11}, DI, DG, DN, DH, ND, NK, NE, BO, BP, BC, and BM. The initial letter of the strains name representing the hydrocarbon name e.g. X is for Xylene, D for Diphenylamine, and N for Naphylamine and B for Benzene. These strains were characterized, classified and their hydrocarbons degrading ability were analyzed. The bacterial isolates were identified initially with the help of its morphology and cultural characteristics and finally confirmed to family level by using different biochemical testing media (Table 1). It was observed that majority of the isolates were Gram-negative rods, only two strains; XF and BO were coccus in shape. All the strains were found motile except XB, XJ1, DI, DN, and DH which were non-motile. All the strains were capsulated except BM. The bacterial isolates were able to survive in diverse conditions of different pH and temperature on MM_2 and L-broth with (1 %) (Xylene, Benzene, Diphenylamine, Naphylamine) supplementation and without hydrocarbons (Table 2).

The survivability of the isolates in the presence of metal supplemented in the nutritional media at concentrations 50, 100, 150 mg/mL revealed that majority of the isolates were growing in the presence of different metal stress. It was observed that all the bacteria sensitive to the metals has shown sensitivity even at lowest used concentration (50 mg/mL), whereas the resistant bacteria showed resistance to the highest concentration (150 mg/mL) used in the study. Detail of the isolates resistance and sensitivity to different metals are shown in Table 3. All the bacterial isolates were grown in the media in the presence of specific hydrocarbons substrate and metals. Antibiogram pattern of the isolates showed that all isolates were sensitive to Oxytetracyclin except DG, NK, NE and BO, whereas all were sensitive to Gentamicin and Minomycin except NK, NE, BP. All the isolates were found resistant to Penicillin and Erythromycin. The isolates XA, XB, XJ_1, XJ_2, XZi, XZii, ND, BM were sensitive to chloramphenicol

Table 1. Morphological and biochemical characteristics of the hydrocarbon degrading bacterial isolates

Isolates ID	Morphological characteristics							Biochemical characteristics										Isolates Family
	S	MT	GR	SS	CS	MKA	EMBA	CA	KAA	KBA	BGA	MR	VP	NT	UT	OT	DT	
XF	Co	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
XA	R	+	-	-	+	+	-	-	+	+	+	-	-	-	+	-	-	*Enterobacteriaceae*
XB	R	+	-	-	+	+	-	+	+	+	+	-	-	-	+	-	-	*Pseudomonas*
X1OF	R	-	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Pseudomonas*
XZi	R	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
XZii	R	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
XJ_1	R	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
XJ_2	R	-	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
NK	R	+	-	-	+	+	+	+	+	+	+	-	-	+	+	-	-	*Enterobacteriaceae*
NE	R	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	*Enterobacteriaceae*
DN	R	-	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
DH	R	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	*Enterobacteriaceae*
DG	R	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	*Enterobacteriaceae*
DN	R	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
DI	R	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Enterobacteriaceae*
BO	Co	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	+	*Azotobacteriaceae*
BP	Co	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	*Enterobacteriaceae*
BC	R	+	-	-	+	+	+	+	+	+	+	-	-	+	+	-	+	*Enterobacteriaceae*
BM	R	+	-	-	-	+	+	+	+	+	+	+	-	+	+	-	-	*Azotobacteriaceae*

Note: S= Shape, R= Rod, Co= Coccus, MT= Motility Test, GR= Gram Staining, SS= Spore Staining, CS= Capsule Staining, DT= Denitrification test, OT= Oxidation Fermentation Test, UT= Urease Test, NT= Nitrification test, MKA= MacConkey's Agar, EMBA= Eosin-Methylene Blue Agar, CA= Simmon Citrate Agar, KAA= King's A Agar, KBA= King's B Agar, BGA= Brilliant Green Agar, + = Positive, - = Negative

Table 2. Growth of the isolates at different temperature and pH in L-broth

Isolates	Temperature (°C)	pH						
	25 °C	37	45	5.0	6.0	7.0	8.0	9.0
XA	+	+	+	+	+	+	+	+
XB	+	+	+	+	+	+	+	+
XF	+	+	+	+	+	+	+	+
X10F	+	+	+	+	+	+	+	+
XZi	+	+	+	+	+	+	+	+
XZii	+	+	+	+	+	+	+	+
XJ_1	+	+	+	+	+	+	+	+
XJ_2	+	+	+	+	+	+	+	+
NK	+	+	+	+	+	+	+	+
NE	+	+	+	+	+	+	+	+
ND	+	+	+	+	+	+	+	+
DI	+	+	+	+	+	+	+	+
DG	+	+	+	+	+	+	+	+
DN	+	+	+	+	+	+	+	+
DH	+	+	+	+	+	+	+	+
BO	+	+	+	+	+	+	+	+
BP	+	+	+	+	+	+	+	+
BC	+	+	+	+	+	+	+	+
BM	+	+	+	+	+	+	+	+

+ = Positive, - = Negative

Table 3. Effects of different metals on hydrocarbon degrading bacterial isolates survival

Isolates ID	$PbNo_3$ (150mg/ml)	Hg (150mg/ml)	$Cocl_2$ (150mg/ml)	$CdCl_2$ (150mg/ml)	$K_2Cr_2O_7$ (150mg/ml)	$ZnCl_2$ (150mg/ml)	$NiCl_2$ (150mg/ml)
On Xylene supplemented media							
XA	Rt	Sen	Rt	Rt	Rt	Rt	Rt
XB	Rt	Rt	Rt	Rt	Rt	Rt	Rt
XF	Rt	Sen	Rt	Rt	Rt	Rt	Rt
X10F	Rt	Sen	Rt	Rt	Rt	Rt	Rt
XZi	Rt	Sen	Rt	Rt	Rt	Rt	Rt
XZii	Rt	Sen	Rt	Rt	Rt	Rt	Rt
XJ_1	Rt	Sen	Rt	Rt	Rt	Rt	Rt
XJ_2	Rt	Rt	Rt	Rt	Rt	Rt	Rt
On Diphenylamine supplemented media							
DI	Rt	Rt	Rt	Rt	Rt	Rt	Rt
DG	Rt	Rt	Rt	Rt	Rt	Rt	Rt
DN	Rt	Rt	Rt	Rt	Rt	Rt	Rt
DH	Rt	Rt	Rt	Rt	Rt	Rt	Rt
On Naphylamine supplemented media							
NK	Rt	Rt	Rt	Rt	Rt	Rt	Rt
NE	Rt	Rt	Rt	Rt	Rt	Rt	Rt
ND	Rt	Rt	Rt	Rt	Rt	Rt	Rt
On Benzene supplemented media							
BO	Rt	Sen	Rt	Rt	Rt	Rt	Rt
BP	Rt	Rt	Rt	Rt	Rt	Rt	Rt
BC	Rt	Sen	Rt	Rt	Rt	Rt	Rt
BM	Rt	Rt	Rt	Rt	Rt	Rt	Rt

Rt = Resistant,Sen = Sensitive

Table 4. Antibiotic sensitivity pattern of the bacterial isolates

Isolates ID	Oxytetracycline	Penicillin	Chloramphenicol	Erythromycin	Gentamicin	Minocyclin
XA	Sen	Rt	Sen	Rt	Sen	Sen
XB	Sen	Rt	Sen	Rt	Sen	Sen
X10F	Sen	Rt	Rt	Rt	Sen	Sen
XF	Sen	Rt	Rt	Rt	Sen	Sen
XJ_1	Sen	Rt	Sen	Rt	Sen	Sen
XJ_2	Sen	Rt	Sen	Rt	Sen	Sen
XZi	Sen	Rt	Sen	Rt	Sen	Sen
XZii	Sen	Rt	Sen	Rt	Sen	Sen
DG	Rt	Rt	Rt	Rt	Sen	Sen
DN	Sen	Rt	Rt	Rt	Sen	Sen
DH	Sen	Rt	Rt	Rt	Sen	Sen
DJ	Sen	Rt	Rt	Rt	Sen	Sen
NK	Rt	Rt	Rt	Rt	Rt	Rt
ND	Sen	Rt	Sen	Rt	Sen	Sen
NE	Rt	Rt	Rt	Rt	Rt	Rt
BO	Rt	Rt	Rt	Rt	Sen	Sen
BP	Sen	Rt	Rt	Rt	Rt	Rt
BC	Sen	Rt	Rt	Rt	Sen	Sen
BM	Sen	Rt	Sen	Rt	Sen	Sen

Rt = Resistant, Sen = Sensitive

(Table 4). The hydrocarbon degradability of the isolates were reconfirmed by observing clear zone around the colonies of isolates after the inoculation of MM_2-Agar supplemented with 10 % Xylene, Benzene, Diphenylamine and the Naphylamine.

DISCUSSION

Microbial xenobiotic removal has received much attention in the last years due to the potential use of microorganisms for cleaning anthropogenically polluted environment[16]. In some instances effective expression of the genes involved in biodegradation of toxic hydrocarbons leads to better utilization of these substances by bacteria[17]. This maximized or optimized genes expression involves in operon regulation, and gene transfer through plasmid[18]. Nineteen hydrocarbon degrading bacterial strains were isolated in this study. These bacterial isolates were found capable of degrading the pollution causing hydrocarbons. Bacteria with similar properties have also been reported by other researchers[19,20]. Dyksterhous *et al.*,[21] reported gram-negative motile rods having the ability to degrade naphthalene, phenanthrene and anthracene. Colonies capable of clearing the opaque PAH layer by solubilization of the substrates were readily detected. The most prominent genus of bacteria, frequently reported for the degradation of environmental hydrocarbons is *Pseudomonas*[7].

Hydrocarbons used in this study were Diphenylamine, Xylene, Benzene and Naphylamine. These PAH are regularly encountered in environment, and have carcinogenic chemical structure[2]. The hydrocarbons degrading bacteria isolated from environment XA, XB, XF, X10F, XZi, XZii, XJ_1, XJ_2, NK, NE, ND, DN, DI, DH, DG, BO, BP, BC, and BM were able to degrade diphenylamine after 4 days of incubation at 37 C. Leahy and Colwel[22] studied the conditions for the hydrocarbon degradation. They found that rates of biodegradation depend greatly on the composition, state, temperature, oxygen, moisture, salinity and pH. They reported pH 7 as optimum pH for all strain. The strains isolated in this study were mesophilic in nature and grown well on 37 °C. In a study, Yanase *et al.*,[23] reported that thermophilic (50-60 °C) bacteria could degrade benzoate and phenol. The present study proved that mesophilic bacteria can degrade hydrocarbons as well.

It has been established by many studies

that bioremediation, i.e., the exploitation of microorganisms for detoxifications of heavy metal ions, aromatic hydrocarbons, petroleum products, pesticides and other toxic organic molecules is the method of choice owing to fewer secondary hazards and generally low cost[24]. Studies showed that hydrocarbon degrading bacteria are ubiquitously distributed in soil and aquatic environments. However, their populations constitute less than 1 % of total microbial communities. Many of the microorganisms proposed for biodegradation and bioremediation have been isolated from contaminated soils and waters[25, 26]. Rapid adaptation of microbes has been observed in the presence of xenobiotic pressure when the pollutant load is raised. Increased number of hydrocarbon degrading microorganisms has been reported at sites within the path of the oil slick as compared with reference sites after the famous Exxon Valdez oil spill[27]. In a relevant study, benzene degrading bacteria were readily isolated from benzene contaminated work place[28].

The microbial profile of certain polluted areas is very informative concerning the type of pollution present is such areas. As these microorganisms are directly affected by the selection pressure exerted by the particular toxic substances, a related resistance, tolerance or metabolic capability develops in such microorganisms. This degradative ability gives good information concerning the type of environmental pollution prevalent in that area. In the present study the isolated bacterial strains are collectively resistant to Xenobiotic pollutants including heavy metals, antibiotics and hydrocarbons (Benzene, Xylene, Naphylamine and Diphenylamine) and are adaptable to local conditions of environment (temperature and pH). So they can be used for the bioremediation of contaminated soil such as aromatic hydrocarbons and for proper treatment of industrial effluents[29].

CONCLUSION

In this study a group of bacteria were isolated and identified from hydrocarbon contaminated soil of automobile workshop in Mazang area Lahore, and its ability for bioremediation of hydrocarbon (Benzene, Xylene, Naphylamine and Diphenylamine) were characterized for the treatment of hydrocarbon contaminated soils. The bacteria were found with significant ability of bioremediation of different hydrocarbon compound supplemented in agar. The bacteria were also able to survive in the presence of different metals and antibiotics. The growth of isolates in the presence of metals on hydrocarbon supplemented media can be helpful for its use in soil and industrial waste bioremediations. The ability of such bacteria can be exploited efficiently for sustainable environment management.

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