Research Article

Indigenous Oil Degrading Bacteria: Isolation, Screening and Characterization

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Abstract: Background: Oil contamination is increasing at an alarming rate and to overcome to this problem the most efficient method used is biodegradation. Biodegradation is a natural mechanism that can be used effectively for degradation of recalcitrant hydrocarbon pollutants prevailed in the environment by employing environmentally friendly microbes.

Methods: Strain CMG 457 was isolated from petrol pump site from Firdous Colony, Karachi and was identified as *Enterobacter* sp. after morphological and biochemical characterization. Strain resistance towards different organic hydrocarbons (paraffin, aromatics and pesticides) and utilization of various organic compounds as sole carbon source were analyzed on Nutrient agar. MIC of antibiotics and heavy metals was checked on Bushnell Haas (BH) agar at 37°C for 24 hrs.

Results: Research investigated the role of *Enterobacter* sp. in biodegradation of petroleum hydrocarbons. The results showed that the bacterium is a gram-negative aerobic bacillus. *Enterobacter* sp. designated as CMG 457 was screened for oil degradation using BH agar. Resistance of strain against different organic hydrocarbons was tested and CMG 457 showed high resistance of upto 50% against paraffin. The Minimum Inhibitory Concentration (MIC) of heavy metals and antibiotic sensitivity were investigated for bacterial strains. Screening for antibiotic resistance revealed that CMG 457 was resistant to ampicillin and erythromycin and was able to tolerate heavy metals like $CuSO_4$, $CdCl_2$ and $CrCl_2$ upto 3mM.

Conclusions: From the study, it is concluded that oil contaminated areas are the best source for isolation of hydrocarbon degrading bacteria. *Enterobacter* sp. has a potential for oil degradation as it showed better hydrocarbon degrading ability.

Keywords: Biodegradation, Biotransformation, Naphthenes, Oil pollution, Paraffins, Polyaromatic hydrocarbons.
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INTRODUCTION

Petroleum hydrocarbon pollution has become a global problem due to vast urbanization and industrialization especially in developing countries causing water and soil contamination [1]. Due to immense utilization of petroleum based products, increased anthropogenic and industrial activities, there are irreversible and devastating effects on biodiversity as well as on human health [2]. Oil pollution is a form of pollution that leads to the release of poly aromatic hydrocarbons (PAHs) into the natural environment especially in water and soil disturbing ecosystem [3].

Petroleum is a complex mixture of thousands of compounds mainly consisting of carbon and hydrogen [4]. Crude oil composition varies considerably in different oils consisting of saturated, aromatic and polar compounds in an average proportion of 58.2%, 28.6% and 14.2% respectively as quoted after analysis of 527 crude oil samples [5]. But, on average, there exists a rough similarity in composition between paraffins, aromatics and naphthenes. Paraffins are characterized by linear or branched hydrocarbons chains, aromatics are saturated cyclic molecules with attached aromatic rings and naphthene molecules are saturated ring or cyclic hydrocarbons also called as cycloparaffins [6].

Generally, several physico-chemical methods have been exploited in order to remove hydrocarbon contamination but these methods are known to have limited effectiveness and negative consequences as compared to biological methods [7]. Biological means involve the use of microorganisms with hydrocarbon degradation ability. Some microorganisms cause biotransformation of hydrocarbons into simpler metabolites while others mineralize hydrocarbons either aerobically or anaerobically. Hydrocarbon contamination of water and soil stimulates indigenous microbial population to degrade hydrocarbon contaminants utilizing hydrocarbons substrate as a soul source of carbon and energy [8].

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Hydrocarbon degradation susceptibility to microorganisms may be ranked on the basis of hydrocarbon structural complexity. Polyaromatic hydrocarbons with high molecular weight and polycyclic structures are less susceptible to microbial degradation, while linear and branched alkanes are easily degraded by microorganisms [9]. Biodegradation capability is known to be found mostly in bacterial species. As reported by Das and Chandaran [10] bacterial genera isolated from oil contaminated soil namely, *Brevibacterium*, *Gordonia*, *Aeromicrobium*, *Mycobacterium* and *Burkholderia* were potentially capable of degrading hydrocarbons. Whereas, Godgift and Fagade [11] reported three species of bacteria namely *Pseudomonas aeruginosa* SA3, *Citrobacter* sp. SB9 and *Bacillus subtilis* SA7, alongwith two species of microalgae *Chlorella minutissimma* and *Aphanocaps*. Among fungal genera, *Amorphoteca*, *Neosartorya*, *Talaromyces* and *Graphium*, *Candida*, *Yarrowia* and *Pichia* are crude oil hydrocarbon degraders [10]. Thus, microorganisms have proved to be preferably best substituted for degradation of petroleum compounds. The overarching goal of this study is isolation, screening and identification of bacterial specie capable of efficiently degrading hydrocarbons from petroleum oil contaminated soils.

MATERIAL AND METHODS

Sampling

Soil sample was aseptically collected in a sterilized polyethylene bag from heavily contaminated petrol pump of Firdous colony, Karachi and was screened for hydrocarbon degrading bacteria.

Isolation and Maintenance of Oil Degrading Bacteria

For the isolation of oil degrading bacteria, 1 gm of sample was dissolved in 100 ml sterilized distilled water, shaken thoroughly and was allowed to settle down. Supernatant (1 ml) was then inoculated in 100 ml nutrient broth and incubated at 37°C for 24 hrs at 80 rpm in a shaking incubator. Morphologically distinct colonies were cultured on MacConkey agar and Bushnell Hass (BH) mineral salt medium (g/l): $MgSO_4$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (10), K_2HPO_4 (1.0), NH_3NO_2 (1.0), $FeCl_3$ (0.05) and pH was adjusted to 7.0 ± 0.2. Cultures were purified after repeated streaking on Nutrient agar (NA) plates. One strain was selected and characterized further on the basis of its high oil degrading ability. The culture was maintained at 4°C and was repeatedly streaked after one month interval.

Identification of Selected Strain

The selected isolate was characterized and identified by cultural (shape, size, colour, margin, opacity *etc*.) and cellular morphology *i.e.* gram staining.

Biochemical Characterization of Selected Strain

Different biochemical tests such as Urease production, nitrate reduction, oxidase, H_2S production, indole, TSI, motility and carbohydrate fermentation (lactose, glucose and sucrose) tests were performed to identify the oil degrading bacteria.

Determination of Strain Resistance Against Different Hydrocarbons

In order to check strain resistance against different hydrocarbons such as paraffin, aromatics (toluene and xylene) and pesticides (ripcord and thiodon) overnight grown culture was inoculated in NB containing varying concentrations of respective hydrocarbon source, while in case of pesticide a 10% stock solution of both pesticides were prepared and overnight grown culture was streaked on NA plate with various concentration of pesticides.

Screening for Heavy Metal Resistance and MIC Determination

Heavy metal salts like $CdCl_2$, $NiSO_4$, $CuSO_4$ and $CrCl_2$ were used to study the resistance of isolate to heavy metals. Culture was streaked on metal impregnated BH plates and incubated at 37°C for 24-48 hrs.

To find out the MIC of heavy metals, overnight grown culture was streaked on BH plates containing varying concentrations of metal salts (1mM, 2mM, 3mM, 4mM) and plates were incubated at 37°C for 24-48 hrs.

Screening for Antibiotic Resistance and Determination of MIC

Antibiotic resistance was investigated by plate incorporation method on NA plates containing streptomycin, kanamycin, chloramphenicol, ampicillin, erythromycin, rifampicin and tetracycline in varying concentrations (10 µg/ml, 25 µg/ml, 75 µg/ml and 100 µg/ml). Overnight grown culture was aseptically streaked on NA plate containing the desired antibiotic concentration and incubated at 37°C for 24-48 hrs. Varying concentrations of ampicillin and erythromycin (100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 600 µg/ml) were used to determine minimum inhibitory concentration (MIC) of antibiotics. NA plates containing antibiotics were streaked with overnight grown culture and plates were incubated at 37°C for 24-48 hrs.

Utilization of Organic Compounds as Sole Carbon Source

To study the effect of organic compounds on the growth of positive isolate, paraffin, pesticides and sugars were used. For paraffin utilization capability, culture was inoculated in BH media having various concentrations of paraffin. Two controls were prepared one without any carbon source while another containing 5% glucose. Culture was inoculated in all the flasks and incubated in a shaking water bath at 37°C for 48-72 hrs. To study pesticide utilization as an alternate carbon source, culture was streaked on BH plates containing pesticides like ripcord and thiodon in varying concentrations

(25 µg/ml, 100 µg/ml, 200 µg/ml). Plates were incubated at 37°C for 24-48 hrs. Similarly, glucose, lactose and sucrose were also used as a carbon source.

RESULTS

Morphological Characteristics of Bacterial Strain

For the identification of any microbe, morphological (cultural and cellular) characteristics are of importance because it is the first step in characterization. Therefore, the cultural or colonial morphology (shape size, margin elevation and chromogenicity) and cellular morphology (gram reaction, arrangement and shape) of the selected bacterial strain was studied (Table **1**). Microscopic studies showed scattered, gram negative rods (Fig. **1**).

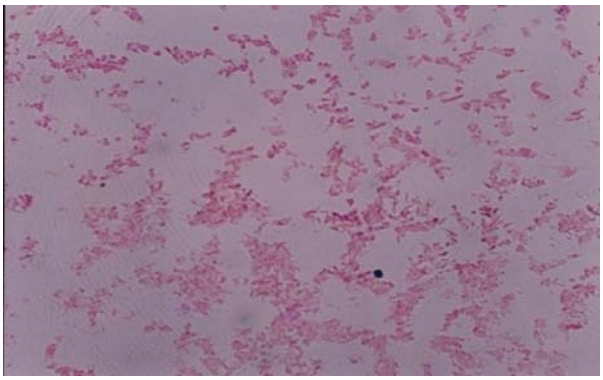

Fig. (1). Cellular morphology of isolated strain.

On NA plate, the isolated colony was off white, small, dark centered with entire margin having convex elevation. On BH plate colonial morphology was white, very small, round with no dark centered. On MacConkey's agar, yellowish centered pink colonies were observed (Fig. **2**).

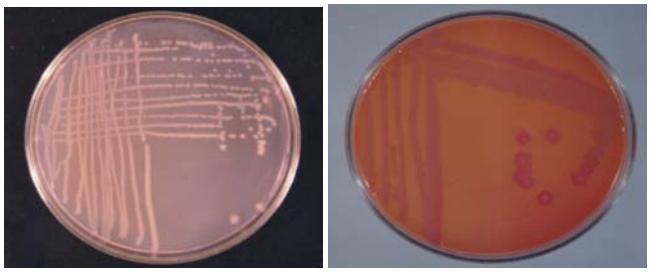

Fig. (2). Colonial morphology of the isolate on Nutrient agar (left) and MacConkey's agar (right).

On the basis of cellular and colonial morphology, isolate was identified upto genus level as belonging to *Enterobacter* sp. using Bergey's Manual of Determinative Bacteriology. This identification was further confirmed by biochemical testing. Results for biochemical testing are tabulated in (Table **2**). The identified isolate *Enterobacter* was designated as CMG 457.

Table **2**. Biochemical characteristics of *Enterobacter* sp. CMG 457.

Tests	Strain CMG 457
Urea	+ve
Nitrate	+ve
TSI	Acid
H_2S	-ve
Indole	-ve
Motility	+ve
Oxidase	-ve

Resistance Against Different Hydrocarbons

The results of strain resistance against different carbon sources showed that CMG 457 was resistant to paraffin, aromatic hydrocarbon and pesticides (Fig. **3**). Paraffin was tolerable upto 50%. Resistance against aromatic hydrocarbon i.e. toluene and xylene was upto 20% while tolerance to ripcord and thiodon was upto 1% at 200μL/ml. CMG 457 was unable to degrade toluene and xylene. (Table **3**)

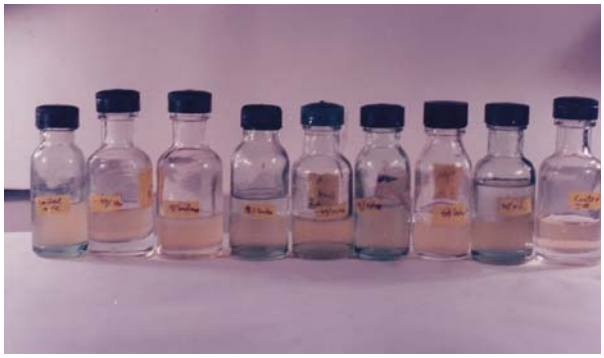

Fig. (3). Resistance of isolate to various components of petroleum.

Table **1**. Colonial and cellular morphology of *Enterobacter* sp. CMG 457.

Organism	Strain Code	Colonial Morphology			Cellular Morphology
		Nutrient Agar	Bushnell and Haas Agar	MacConkey's Agar	
Enterobacter sp.	CMG 457	Small, round, dark centered, translucent, convex, smooth	Very small, smooth, round and white	Small, round, yellowish centered pink colonies	Rod-shaped cells having scattered arrangement

Table **3**. Resistance and/or degradation of various compounds.

Nature of Compound	Compound Used	Resistance % (NB)						Degradation % (BH)			
		0.1	1	5	10	20	50	0.1	1	5	10
Petroleum based hydrocarbons	Paraffin (Aliphatic)	++	++	++	++	++	++	++	++	++	++
	Toluene (Aromatic)	++	++	++	++	++		_	_	_	_
	Xylene (Aromatic)	++	++	++	++	++		_	_	_	_
Pesticides	Ripcord	++	++					++	++	++	++
	Thiodon	++	++					++	++	++	++

+ = Degradation, − = No degradation

Table **4**. Metal tolerance of *Enterobacter* sp. CMG 457.

Metal salts	Concentration (mM)				
	0.5	1	2	3	4
$CuSO_4$	+	+	+	+	−
$CdCl_2$	+	+	+	+	−
$CrCl_2$	+	+	+	+	−
$NiSO_4$	−	−	−	−	−

+ = Resistance, − = Sensitive

Table **5**. Antibiotic resistance/susceptibility profile of *Enterobacter* sp. CMG 457.

Antibiotic	Abbreviations	Concentrations of antibiotics *μg/ml*							
		10	**50**	**100**	**200**	**250**	**300**	**350**	**400**
Ampicillin	Ap	+	+	+	+	+	+	+	−
Erythromycin	Em	+	+	+	−	−	−	−	−
Kanamycin	Km	−	−	−	−	−	−	−	−
Rifampicin	Rif	−	−	−	−	−	−	−	−
Tetracycline	Tc	−	−	−	−	−	−	−	−
Streptomycin	Sm	−	−	−	−	−	−	−	−
chloramphenicol	Cm	−	−	−	−	−	−	−	−

+ = Resistance
− = Sensitive

Effect of Heavy Metals

MIC's revealed that CMG 457 showed heavy metal resistance against $CuSO_4$, $CdCl_2$ and $CrCl_2$ upto 3mM but was sensitive to Ni at 0.5 mM concentration. MIC value was taken at lowest heavy metal concentration at which microbial growth was inhibited. (Table **4**).

Effect of Antibiotics

MIC for each antibiotic was studied at concentrations ranging from 10 µg/ml to 400 µg/ml .This assay was performed with most frequently used laboratory antibiotics. MIC's revealed that CMG 457 was resistant to ampicillin (350 µg/ml) and erythromycin (100 µg/ml) while was fairly sensitive to all of the other antibiotics tested. (Table **5**).

Degradation and Utilization of Organic Compounds as Sole Source of Carbon

Enterobacter sp. CMG 457 was capable of degrading and utilizing paraffin and pesticides as sole carbon source while unable to grow in minimal media without any carbon source. CMG 457 was also able to utilize sugars (glucose, lactose and sucrose) as sole source of carbon. (Table **6**).

DISCUSSION

Oil degrading microorganisms are wide spread in nature. . They are well known for their ability to degrade variety of hydrocarbons present in the crude oil and harbor catabolic enzymatic activity to utilize organic contaminants as sole carbon and energy source converting them into less harmful substances [12]. In present study, bacterial strain CMG 457 was identified as gram negative *Enterobacter* after morphological and biochemical characterization. The potential of gram negative bacteria as efficient petroleum oil degraders as compared to gram positive bacteria is reported [13]. *Enterobacter* sp. as a bacterial hydrocarbon degrader is reported in the literature on the basis of oil hydrocarbon utilizing capability [14, 15].

Table **6**. Utilization of different carbon sources.

Paraffin (%)				Sugars (5%)			Pesticide (µg/ml)					
0.45	0.9	16.4	50	Lactose	Glucose	Sucrose	Thiodon			Ripcord		
							50	100	200	50	100	200
+	+	+	+	+	+	+	+	+	+	+	+	+

The bacterial strain CMG 457 was able to tolerate 50% paraffin due to the adaptation of indigenous bacteria and this adaptation might have genetic or extracellular basis. CMG 457 was resistant to aromatics like xylene and toluene upto 20% but could not degrade them. The high resistance to aromatics might be due to the fact that xylene and toluene are the constituents of petroleum like paraffin, however, the isolate could not degrade xylene and toluene because it is possible that CMG 457 might be lacking some unknown factors essentially required for the expression of degradative genes. Our finding is contrary to *Rajei et al.* [16] who found that *Enterobacter* is capable of degrading aromatic hydrocarbons as they possess aromatic catabolic pathway.

The strain was found to be sensitive to all the antibiotics tested except ampicillin and erythromycin. The highest level of resistance towards ampicillin indicated its previous exposures to antibiotics and also indicated the well spread resistance of microorganisms towards ampicillin and related drugs. Resistance of *Enterobacter cloacae* towards ampicillin is also reported [17].

Enterobacter sp. showed resistance to various heavy metals. This resistance to metals might be attributed to metal pollution in local environment. Several other bacterial species like *Gemella* sp. and *Micrococcus* sp. showed resistance to heavy metals like lead (Pb), chromium (Cr) and cadmium (Cd) [18]. CMG 457 was highly resistant to the pesticides ripcord and thiodon. This resistance might be attributed to the sharp utilization of pesticides during last few decades.

Paraffin is utilized by the strain as sole source of carbon which is due to the adaptation of indigenous bacteria to oil polluted environment. The result is in accordance with the results of Rahman *et al.* [19] who reported utilization of aliphatic hydrocarbons such as paraffin by bacteria. The results are in agreement with the findings of Singh *et al.* and Latifi *et al.* [20] and [21] who reported utilization of chlorpyrifos by *Enterobacter* as sole source of carbon and phosphorus for their growth and energy. Therefore, use of microorganisms for biodegradation as a clean-up strategy of hydrocarbon polluted environment is gaining much importance and is believed to be an excellent approach for future remediation.

CONCLUSION

In the present world, cleaning up of the environment polluted with petroleum hydrocarbons is the matter of major concern. Use of indigenous microorganisms aids in the removal of organic contaminants due to the specific enzyme systems synthesized by microorganisms. From the present study it can be concluded that *Enterobacter* sp. CMG 457 will serve as an effective degrader against oil hydrocarbons predominately PAHs. Further study is needed to characterize the strain on molecular and genetic level.

CONFLICT OF INTEREST

Declared none.

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