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Bioaugmentation of Hydrocarbons using Bacterial species extracted from oil contaminated site

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Abstract: Bioaugmentation with hydrocarbon utilizing indigenous bacteria were employed as remedial options for 30 days in a crude oil-contaminated soil. The best ratio of crude oil (5 ml) was fixed and applied for final bioremediation to all six consortiums identified as *Coccus* and *Bacillus* species . Final results showed oil removal ranging from 80 to 97%. The present work deals with bioaugmentation aimed at the remediation of a soil contaminated with crude oil (5% v/w). Consequently, the bioaugmentation was performed by introducing microbial strains. They belong to a stable population isolated from a polluted site and are hydrocarbon degraders as microbial community.

Keywords: Bioaugmentation; *Bacillus* sp. *Coccus* sp. Hydrocarbons; Microbial consortium

1. INTRODUCTION

A sustainable approach to soil remediation requires technologies that restore soil while preserving its quality and thus its functions. Bioaugmentation is one of the possible ways to activate such natural potentials, provided that the biodiversity introduced to increase catabolically relevant capacity is identified also considering the ecological context. Bioaugmentation is influenced by several factors like strains selection, concentration and methods of inoculation and inoculum heterogeneity. A major problem associated with soil bioaugmentation is the inoculants survival, due to both the abiotic and biotic stresses.

There are several approaches that allow selection of microorganisms useful for bioaugmentation. Bacteria for this purpose may be isolated from given contaminated soils and after culturing under laboratory conditions pre-adapted pure bacterial strains return to the same soil. This approach is called reinoculation of soil with indigenous microorganisms. The second possibility is selection of appropriate microorganisms from sites with similar contaminants that are present in soils submitted to clean-up by these selected microorganisms. Many studies have indicated that the use of consortia of aromatic-degrading bacteria has been more effective in removing pollutants as compared with selected single strains (Ghazali *et al*. 2004; Goux *et al*. 2003). Most experiments dealing with bioaugmentation were carried out using gram-negative bacteria belonging to genus *Pseudomonas* (Heinaru *et al*. 2005), *Flavobacterium* (Crawford and Mohn 1985), *Sphingobium, Alcaligenes* (Halusˇka *et al*. 1995) and *Achromobacter* (Ronen *et al*. 2000). Increasing attention also needs to be directed to gram-positive bacteria belonging to the genera *Rhodococcus* (Briglia *et al*. 1990), *Mycobacterium* (Jacques *et al*. 2008) and *Bacillus* (Silva *et al*. 2009b). In turn, fungi potentially useful for bioaugmentation are represented by species from genus *Absidia* (Garon *et al*. 2004), *Achremonium* (Silva *et al*. 2009b), *Aspergillus* (dos Santos *et al*. 2008), *Verticillium* (Silva *et al*. 2009b), *Penicillium* (Mancera-Lo´pez *et al*. 2008) and *Mucor* (Szewczyk and Długon´ski 2009). There are no microorganisms or their groups universally applicable to bioaugmentation. Many microorganisms are metabolically versatile and are capable of degrading a wide spectrum of substrates. Bioaugmentation with single strains Studies on biodegradation of hydrocarbon-compound-contaminated soil under different conditions have been conducted using mainly gram-negative bacteria as well as fungi (Mrozik *et al*. 2010).

2. MATERIALS AND METHODS

Sampling site and sample collection

Crude oil contaminated soil was collected at different levels (on surface, 6 inches deep and 12 inches deep) from Tando Alam Complex of Oil and Gas Development Company Limited (OGDCL) Sindh Pakistan and preserved at 20 ^{0}C.



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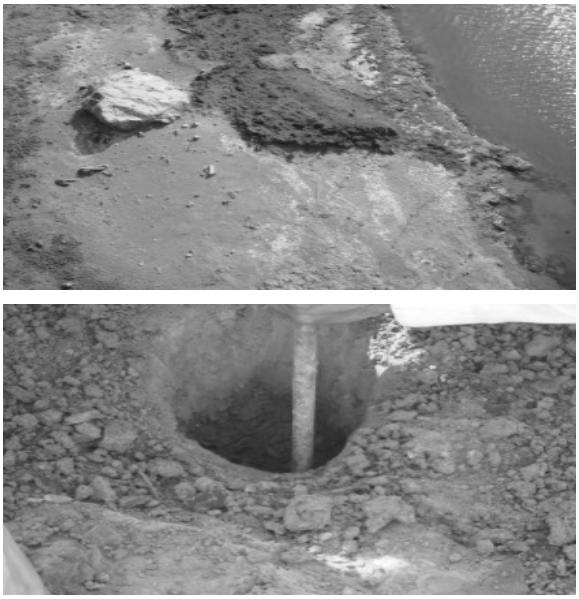


Fig. 1. Sample collection site pictures

Investigations of extracted crude oil and contaminated soil samples

Through Soxlet extraction, extracted oil detected through Gas chromatograph were found in higher concentrations (Aziza *et al* 2013). Soil characterized results showed calcareous texture and higher concentrations of soluble salts as well as organic matters as same discussed by Subhash Chandra *et al*., (2013). Soil moisture is very important factor for soil nutrients. All these parameter have been assessed where it has been observed that tensile strength of soil is directly proportional to organic matters and indirectly proportional to Electric Conductivity (EC) and Sodium Absorption Ratio (SAR).

Isolation and screening of bacteria from contaminated soil

Microbial culture were isolated and screened by serial dilution method, in which six dilutions were spread on sterilized nutrient agar plates incubated for 24 hrs at 37 ^{0}C as shown in **(Fig. 2)**. Later six isolates were selected for further studies (Aziza *et al* 2013).

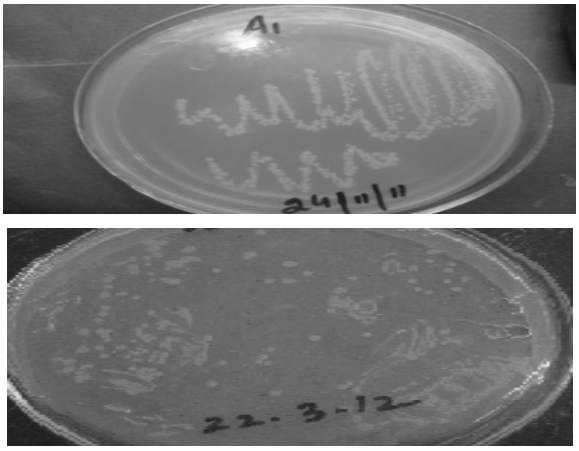


Fig 2 Isolation of pure culture on nutrient agar media (NAM) plates

Growth profile

Growth profile was followed by enumeration of inoculated media for 24 hrs on nutrient agar plates and also by reading the absorbance of inoculated broth media for 30 days in shaking incubator at 120 rpm and 37 ^{0}C.

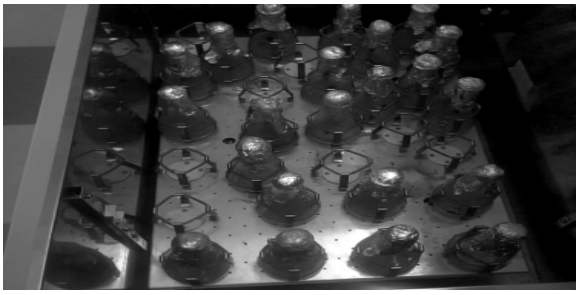
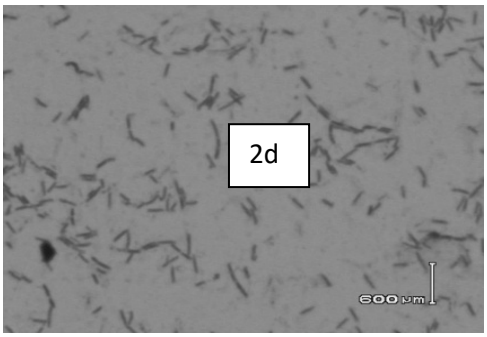
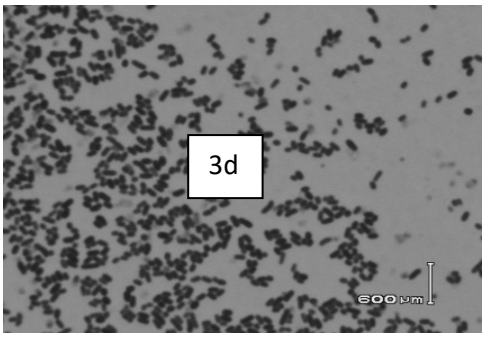


Fig 3 Shaking Incubator

Microbial characterization and identification

Selected species were characterized and identified as *bacilli* and *cocci* species before its final applications i-e bioremediation by studying its colonial, morphological and biochemical characters by using of microscopy as shown in **(Fig. 4)**., API20 KIT and Bergy's manual , all features were verified by Microbiological Department of Ojha, institute of chest diseases of Dow University of Health Sciences (DUHS) Karachi (Aziza *et al* 2014).





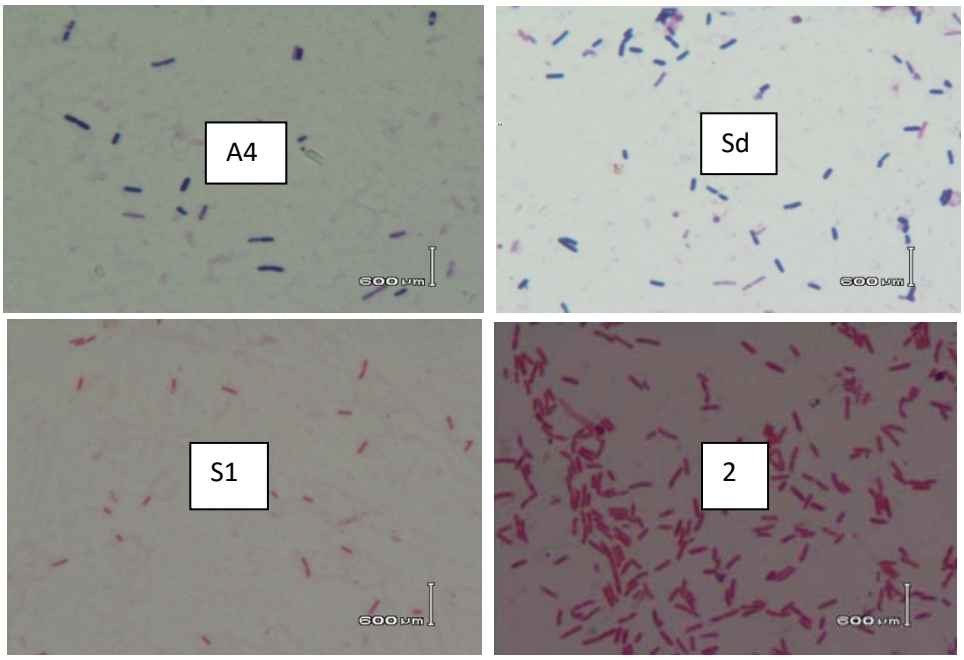


Fig 4 Microscopic view of all identified microorganism

Application of oil degrading identified Bacterial species to extracted oil

Final bioremediation six selected and identified species were activated and incubated of 100 ml makeup water, having 5 ml of extracted crude oil at different time intervals periods in broth medium for 30 days incubation period in shaken incubator, temperature 37 ^{0}C with rpm 120. Finally it was analyzed for estimation of oil degradation. All experiments were performed in triplicate.

Equipment Running Parameters

For the operation of laboratory equipments following parameters were fixed while performing experimental work in scientific laboratory of Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro. Soxlet extraction was performed by soxlet assembly, using hexane acetone (3:1) mixture as solvent for 24 hours at 80 °C. Gas chromatography systems having Flame Ionization Detector (FID) operated at 300°C, 30 meter capillary column with 0.53 mm diameter. Nitrogen gas was used as a carrier gas while 2% of hydrogen with air used as a fuel gas. Microscopy was performed through Microscope (Model Serial SA3000 50/60Hz, CE Made in China) to identify the shape, color and arrangement of the microorganisms. For proper biodegradation 30 days incubation was performed by Shaking incubator (Orbital Shaker Incubator, Horizontal Type Eyela England) at 120 rpm and 37°C. Optical Density (OD) was taken by double beam Spectrophotometer (UV-visible Double Beam Spectrophotometer, Shanghai Yiheng Technical Co. Ltd. 50 Hz) at wavelength 600 nm. Centrifugation (Hz 50/60, Kubota 8700, Japan) was performed at 4 °C, 10000 rpm for 10 minutes. Oil was recovered by distillation through Rotary vacuum evaporator (Laborota 4000 Heidolph ,Japan) at 40 °C, 100 rpm and 40 psi.

3. RESULTS AND DISSCUSSION

Growth profile at different time intervals of all screened species

A 30 days Incubated cultures in shaking incubator were monitored and checked by spectrophotometry at 600 nm. It was found that all species show maximum growth up to 15 days (log phase) and then up to 30 days stationary phase was observed except *Bacillus sphaericus* (2d) species which had continuous growth till 28 days and then declines. *Bacillus sphaericus* (S1) specie also shows different metabolic activities as shown in **(Fig 5** and **Table 1)**. The results are competent with Khan, *et al*., (2011).

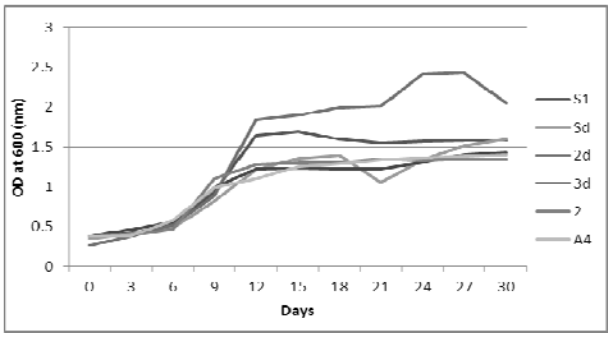


Fig. 5. Growth Profile of selected microorganisms in 30 days incubation period

Different microorganisms were grown on media with and without crude oil for 30 days given in Table 1 and the growth of these microorganisms were checked by taking absorbance at 600 nm. The best ratio of crude oil (5ml) was fixed and applied for final bioremediation to all consortiums. Our findings, according to results is maximum biodegradation ratio i-e 97%as shown in **(Fig.6)**, were achieved.

Table 1: Growth pattern of microorganism in oil and without oil medium

DAYs	Med + Oil	Medium	Med + Organism						Med + Organism + Oil					
			S1	Sd	2d	3d	2	A4	S1	Sd	2d	3d	2	A4
0	0.565	0.143	0.384	0.349	0.266	0.384	0.385	0.378	0.432	0.457	0.463	0.41	0.52	0.512
3	0.552	0.182	0.414	0.413	0.382	0.465	0.396	0.389	0.59	1.932	2.067	0.521	0.756	1.104
6	0.544	0.223	0.521	0.488	0.52	0.56	0.468	0.581	0.913	1.816	1.961	0.865	0.891	1.201
9	1.893	0.211	0.931	0.819	0.888	0.987	1.102	0.988	1.561	1.783	1.732	1.81	2.018	1.246
15	2.312	0.812	1.691	1.356	1.896	1.229	1.296	1.254	2.219	2.109	2.01	2.126	2.381	1.992
18	2.591	1.543	1.602	1.391	1.991	1.223	1.312	1.289	2.284	2.346	2.341	2.31	2.406	2.106
21	2.62	1.741	1.551	1.06	2.01	1.221	1.35	1.341	2.23	2.65	2.53	2.42	2.43	2.253
24	2.699	1.791	1.572	1.341	2.41	1.312	1.331	1.365	1.439	2.581	2.481	1.63	2.308	1.561
27	2.714	1.801	1.579	1.51	2.43	1.401	1.349	1.388	1.568	2.54	2.411	1.511	2.395	1.091
30	2.74	1.81	1.583	1.601	2.045	1.43	1.349	1.405	1.851	2.536	2.402	1.307	2.285	0.589

This growth profile of microorganisms in broth medium with crude oil shows instant growth in 2nd and 3rd day period like *Bacillus megaterium* (Sd) and *Bacillus sphaericus* (2d) and both come in their stationary phase till 30th days as shown in Table1. However, rests of four (04) species gradually were grown till 12th days period and then start stationary phase till 21st day period. Then *Bacillus sphaericus sp. (2*) only remains in stationary phase while *Bacillus megaterium* sp. (A4), 3d and *Bacillus sphaericus* (S1) start decline. This *Bacillus megaterium* (A4) is capable to degrade the oil continuously increase of growth period up to 18 days and it is around 60 %. Then it increase rapidly from 18 days till 30 days with marginal growth around up to 84 %.

Final analysis of recovered oil after bioremediation

The experiment was conducted to study the biodegradation of crude oil consortium with 06 bacterial isolates. Culture prepared in nutrient broth for growth of cell biomass. The cultures were incubated for 3 days as shown in Fig.6. showed a less degree of degradation and then cultures were incubated for 30 days keeping a three days gap between the sample as shown in **(Table 2)**.

Table 2. Oil recovery of all species

Specie Name	Oil Recovery after 30 days
S1	0.747 ml
Sd	0.487 ml
2d	0.278 ml
3d	0.612 ml
2	0.106 ml
A4	0.747 ml

These fractions were also removed without inoculum, indicating abiotic removal by physical factors. When degradation was assayed and measured as removal by biotic fractions by subtracting the percentage removal in the test (with inoculum) from the control (without inoculum) different rates of removal were observed. The similar findings are also reported by Malik and Ahmed (2012).

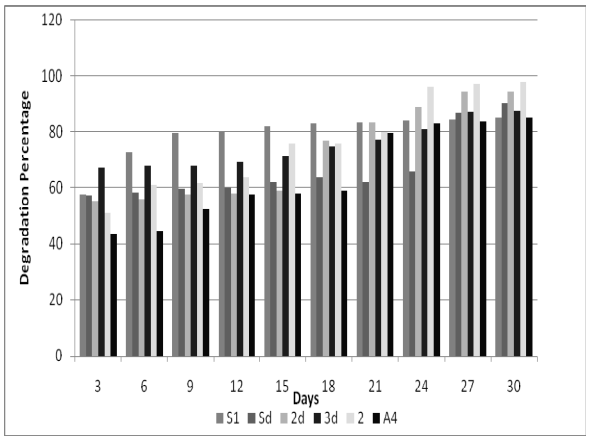


Fig.6. Biodegradation % using 5 ml oil by all microbial species

Taxonomic characteristics of these isolates identified them as *Micrococcus sp and Bacillus sp.* There are other reports of such organisms also reported by Sathishkumar *et al*., 2008; Lal and Khanna, 1996; Rahman *et al*., 2002. Micrococcus sp, was also described to degrade diesel oil (Pepi *et al*., 2003). The pattern of degradation exhibited that the microorganisms first attacked the lower and higher hydrocarbon chains and those of middle length were attacked later in the course of incubation (Bello, 2007).

4. CONCLUSION

Six bacteria species, tentatively named as (A4), (Sd), (3d), (2d), (S1) and (2) were identified as *Bacillus megaterium, Micrococcus luteus and Bacillus sphaericus,* isolated from the contaminated soil collected from Tando Alam oil fields of Oil and Gas Development Company Limited (OGDCL) Sindh Pakistan.

Consortium was incubated by shake flask transformation in nutrient broth medium by shaking incubator at 37 °C for 30 days with 5% optimized ratio of crude oil extracted by Soxhlet assembly. Total removal of aliphatic and aromatic hydrocarbons was observed through absorbance by spectrophotometer as 97% and 95% respectively, which indicate that these entire indigenous microorganisms are active degraders of crude oil i.e. able to feed on hydrocarbon fractions.

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