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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2020.726.734

Research Article
Biodegradation of Petroleum Hydrocarbons Using Indigenous Bacterial and Actinomycetes Cultures

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Abstract

Background and Objective: Hydrocarbon contamination is one of the important environmental problems resulting from activities related to the production and use of petrochemicals. The aim of current study was to isolate, characterize and evaluate the potential for treating petroleum hydrocarbons using actinomycetes and bacterial strains isolated from petroleum-contaminated soil. **Materials and Methods:** Two *Pseudomonas* isolates and three Actinomycetes isolates were isolated from contaminated soil and then identified using 16S rDNA gene sequencing. These isolates tested for high degradation capacity of petroleum oil. **Results:** Actinomycetes and bacterial isolates that showed biodegradation capacities were molecular identified as *Streptomyces sampsonii*, *Streptomyces orinoci*, *Streptomyces ferralities*, *Pseudomonas xanthomarina* and *Pseudomonas proteolytica*. These strains were subjected for further study to measure the quantitative degradation of saturated and polycyclic aromatic hydrocarbons (PAHs). *Streptomyces ferralities* showed the highest degradation percent of saturated hydrocarbons, reached 82.106 and 81.672 for normal-paraffin and iso-paraffin, respectively. Maximum biodegradation percent of polycyclic aromatic hydrocarbons (PAHs) was 54.798 by using *Streptomyces sampsonii*. **Conclusion:** The observed PAHs activities of these actinomycetes strains suggested their potential usage as an alternative sources of hydrocarbon chemical degradation in petroleum industry.

Key words: Saturated hydrocarbons, normal-paraffin, iso-paraffin, polycyclic aromatic hydrocarbons (PAHs), bioremediation, 16S rDNA gene, Actinomycetes, *Pseudomonas*

Citation: Hassan Alsberi, Asmaa A. Hamad and Mohamed M. Hassan, 2020. Biodegradation of petroleum hydrocarbons using indigenous bacterial and actinomycetes cultures. Pak. J. Biol. Sci., 23: 726-734.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Contamination of the environment by crude petroleum oil has occurred naturally since prehistoric times[1,2]. Biodegradation (bioremediation) is most often the primary mechanism for contaminant clean-up[3,4]. Growth stimulation of indigenous microorganisms, biostimulation, along with inoculation of foreign oil-degrading bacteria is a promising means of accelerating detoxifying and degrading activities at petroleum-polluted site with minimum impact on the ecological systems[5]. Crude oil may contain thousands of diverse chemical compounds including alkanes (normal, iso- and cyclo-), aromatics, polycyclic aromatics, heterocyclic and asphaltic compounds. Each of these compounds has a different biodegradation rate in the natural environment.

Microorganisms that use petroleum hydrocarbons (PHs) as a source of cell carbon and energy are widely distributed in nature. More than 100 strains of bacteria have been identified that degrade petroleum hydrocarbons[6,7]. Some of the more common crude oil-degrading bacterial species belong to the following genera: *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Brevibacterium*, *Corynebacteriun* and *Flavobacterium*. In an environment that is not under stress, bacteria are generally believed to be primarily responsible for the degradation of petroleum hydrocarbons. They generally are in greater numbers compared to yeasts and fungi in an environment contaminated with petroleum hydrocarbon compounds[8,9].

Bioremediation processes concerning to environmental protection are based on the ability of microorganisms to enzymatically oxidize petroleum hydrocarbons[10-12]. The degree of degradation of hydrocarbons correlates with an increase in the population and oxygenase activity of microorganisms[13,14]. The objectives of this study were therefore, to isolate and identity some of the indigenous actinomycetes and bacterial flora of oil contaminated soils and evaluate the biodegradation efficiencies of the potent oil-degrading strains.

MATERIALS AND METHODS

Screening and isolation of microorganisms with oil-degradation potential: The current study was carried out at Microbiology Department, Faculty of Science Lab, Taif University from May 2018-April 2019. Soil samples used for the isolation of the potential oil-degrading microorganisms were collected from areas contaminated with crude petroleum oil after cracking of petroleum pipelines. The soil samples were homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using 2 mm sieve. Serial dilution method was used for screening of potential oil-degrading microorganisms using selective media supplemented with crude oil as a sole carbon source[7]. Starch agar medium devoted of starch used to isolate the potential oil-degrading actinomycete populations. For bacteria, M9 Minimal Medium devoted of glucose was used. The solid media that have been poured in plates, 9 cm in diameter, were covered with layer of crude oil before inoculation with the three dilutions (10^{-1}, 10^{-2} and 10^{-3}) in triplicate. Plates were incubated at 28°C for 5 days for actinomycetes respectively and at 37°C for 3 days for bacteria. The grown cultures of bacteria and actinomycetes were carefully and aseptically sub-cultured onto fresh M9 minimal agar and starch agar plates, respectively to obtain pure cultures for identification and biodegradation assay of microorganisms with oil-degradation potential.

Identification of bacterial and actinomycetes isolates: Two cultures of the potential bacterial isolates and three cultures of the potential actinomycetes isolates were identified using molecular biology techniques. The DNA extraction was performed using protocol of Gene JET genomic DNA purification Kit (Thermo, USA). The purity of the obtained DNA was estimated using sub-marine gel electrophoresis.

The PCR were carried out according to Hassan and Ismail[8], using Maxima Hot Start PCR Master Mix (Thermo) in 50 µL reaction system containing, 25 µL of 2X Maxima Hot Start PCR Master Mix, 16S rRNA gene forward primer sequence was 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer sequence was 5'-GGT TAC CTT GTT ACG ACT T-3'. The PCR samples were performed using the thermal cycler conditions outlined[8]. The 5 µL of PCR mixture was loaded on 2% agarose gel against 100 bp plus ladder (Thermo) to examine the PCR product. For purification of PCR product Gene JET™ PCR Purification Kit (Thermo) was used.

Biodegradation assay of the selected cultures: To get fresh bacterial and actinomycetes cultures, 2 species of potential oil-degrading bacteria (*Pseudomonas proteolytica* and *Pseudomonas xanthomarina*) and 3 species of potential oil-degrading actinomycetes (*Streptomyces sampsonii, Streptomyces orinoci* and *Streptomyces ferralities*) were grown in liquid cultures by suspending a single colony from each individual organism in 50 mL liquid media. Liquid starch medium was used for actinomycetes cultures and M9 minimal medium was used for bacterial cultures. The different suspensions were incubated in shaking incubator at 150 rpm for 24 h at 37 and 28°C for bacterial and actinomycetes

cultures, respectively. Then 1 mL of each individual suspension was transferred to 250 mL flask contain 50 mL of liquid starch medium devoted of starch and supplemented with 0.25 g crude oil as a sole carbon source (for actinomycetes cultures) or M9 minimal medium devoted of glucose and supplemented with 0.25 g crude oil as a sole carbon source (for bacterial cultures). To obtain similar cell count of 1.0×10^4 CFU/50 mL, the bacterial and actinomycetes suspension were diluted with sterile distilled water. Inoculation of each organism was carried out in triplicate prior to incubation at 28 and 37°C for actinomycetes and bacteria, respectively for 30 days on shaking incubator at 150 rpm. Three replicate of control flasks without culture inoculation was prepared accordingly.

To extract the residual amounts of petroleum hydrocarbons from liquid media after incubation period, equivalent quantity of dichloromethane (1:1) were mixed with the content of each flask. Using a separating funnel, the PHs content of each flask was extracted by gentle shaking. The lower organic layer was collected into a clean dry conical flask. The content of each flask was extracted in 3 times. The extract was combined and completely evaporated at 65°C water bath in the hood and the amount of the extracted PHs was determined gravimetrically. The components of PHs were fractionated using column chromatography technique[9]. The extend of petrol biodegradation by potential species were performed using gas chromatography (GC) and high-performance liquid chromatography (HPLC) for saturated and aromatic fractions, respectively.

Gas chromatography: The GC apparatus used was Perkin Elmer (Clarus 500), equipped with a hydrogen flame ionization detector and fused silica capillary column (60 m length×0.32 mm i.d), packed with poly (dimethyl siloxane) HP-1 (non-polar packing) of 0.5 µm film thickness[10]. In the chromatograph, the injector was heated at 350°C. The column temperature was programmed from 100 to 300°C at a fixed rate of 3°C/min and nitrogen (oxygen-free) was used as a carrier gas with a flow rate of 2 mL min^{-1}. The detector was heated at 350°C and operated with a hydrogen flow rate adjusted to optimize the detector sensitivity. The sample was melted and 0.1 µL of it was introduced into the injector. A mixture of pure n-paraffins was used as standard. The peak area of each resolved component (consisting of either n- and iso-paraffin) is determined individually. However, the unresolved complex mixtures (humps), composed of non n-paraffins presumably mainly cyclo-paraffins and aromatics with long side chains, were determined only as a total.

Polycyclic aromatic hydrocarbons analysis: Identification and quantification of polycyclic aromatic hydrocarbons (PAHs) were performed using high performance liquid chromatography (HPLC) for benzene fractions. The apparatus used was model Waters HPLC 600E, equipped with dual UV absorbance detector Waters 2487 and auto sampler Waters 717 plus attached to a computerized system with Millennium 3.2 software. PAHs standards were obtained from Supelco. The conditions of separation were according to Lal and Khanna[11], column: Supelcosil. LC-PAH, 5 µm particles, 15 cm length and 4.6 mm ID, Mobile phase: gradient acetonitrile: Water 60-100% Acetonitrile (v/v) over 45 min. Flow rate: 0-2min, 0.2 mL min^{-1}, 2-45 min, 1.0 mL min^{-1}. The detector was set at 254 nm.

Statistical analysis: The analysis of the variance of the antagonistic activity was done using one-way analysis of variance, using SPSS software var. 16. Duncan's multiple range tests, was used to detect variations among treatments at 5% probability. Means within a column, followed by the same letter, were not significantly different.

RESULTS

Isolation and identification of biodegradation strains: *Streptomyces* strains showed both the non-fragmented substrate mycelium and aerial mycelium with rectiflexibile (RF) arrangement. Under light microscope, the spores appear with a smooth surface and oval shape. Cultural morphology on ISP2 agar was white leathery brown and showed brown pigment. *Pseudomonas* strains were gram-negative, yellow-orange-pigmented rod-shaped bacteria motile by means of a single polar agellum.

Molecular identification of biodegradation strains: *Actinomycetes* and *Pseudomonas* isolates that showed biodegradation capacities (Fig. 1) were molecular identified using 16S rDNA gene sequencing as *Streptomyces sampsonii*, *Streptomyces orinoci*, *Streptomyces ferralities*, *Pseudomonas xanthomarina* and *Pseudomonas proteolytica*. These strains were subjected for further study to measure the quantitative degradation of saturated and polycyclic aromatic hydrocarbons (PAHs).

Biodegradation potential of saturated hydrocarbons: Two of the potential bacterial species (*Pseudomonas proteolytica* and *Pseudomonas xanthomarina*) and 3 of the potential actinomycetes species (*Streptomyces sampsonii*, *Streptomyces orinoci*, an Streptomyces *ferralities*) were used

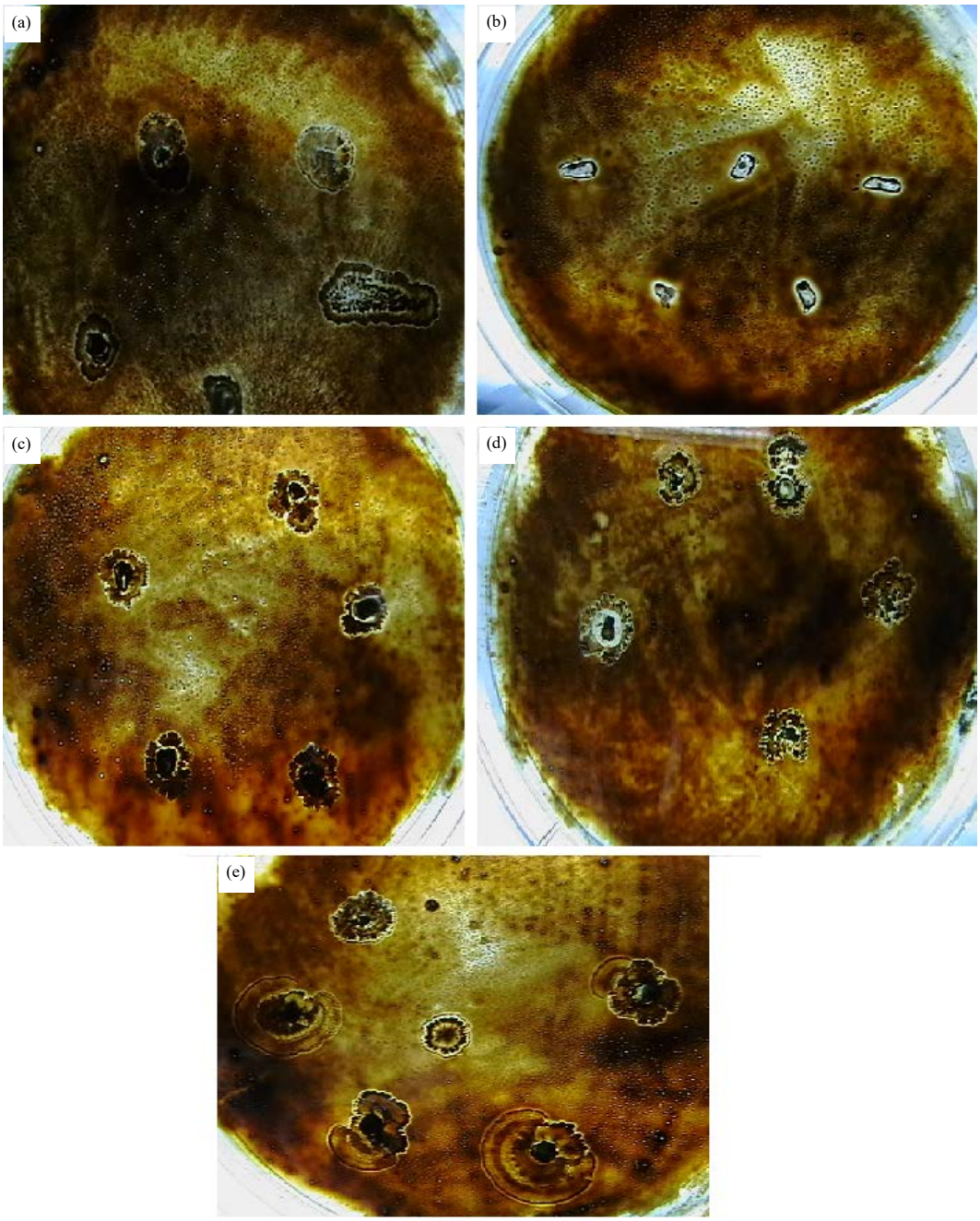

Fig. 1(a-e): Change in the appearance of crude oil that surrounding actinomycetes and bacterial colonies grown on media supplemented with crude oil as a sole carbon source, (a) *Streptomyces sampsonii*, (b) *Streptomyces orinoci*, (c) *Streptomyces ferralities*, (d) *Pseudomonas xanthomarina* and (e) *Pseudomonas proteolytica*

in this study. The ability of the 5 isolates to degrade saturated hydrocarbons and polycyclic aromatic hydrocarbons was confirmed using GC (Fig. 2, Table 1). The chromatogram appears as a number of peaks that represent the remaining paraffinic hydrocarbons on a hump which represent the unresolved compound mixture of high molecular weight hydrocarbons. Since the equal quantity of samples was injected per chromatogram, the response of each was proportional to the amount of soluble and insoluble hydrocarbons for each sample. Consequently, the decrease in peak areas from one sample to another reflects a decrease in GC detectable hydrocarbons.

Table 1: Quantitative degradation analysis, estimated by GC, of saturated hydrocarbons using 3 actinomycetes species and 2 bacterial species corresponding to un-treated control

Carbon number	Control	*S. sampsonii*	*S. orinoci*	*S. ferralities*	*P. xanthomarina*	*P. proteolytica*
C14	272.236	0.000	0.000	0.000	0.000	0.000
C15	104.644	0.000	0.000	0.000	0.000	0.000
C16	520.426	182.475	126.572	32.864	420.141	313.798
C17	551.560	508.498	336.282	147.015	574.580	562.954
C18	385.167	383.683	371.910	214.094	403.640	379.212
C19	232.124	25.608	36.054	23.270	117.080	101.642
C20	275.465	107.070	277.578	142.919	272.207	277.976
C21	270.583	0.000	0.000	1.504	0.000	0.000
C22	255.931	181.716	250.911	105.530	260.075	249.032
C23	223.251	0.000	20.359	0.000	0.000	0.000
C24	416.495	86.255	281.362	75.058	335.570	322.081
C25	192.239	0.000	0.000	0.000	0.000	0.000
C26	217.033	57.739	146.903	49.163	201.684	193.651
C27	150.592	0.000	0.000	0.000	0.000	0.000
C28	232.546	41.505	103.286	40.126	217.834	156.545
C29	121.139	0.000	0.000	4.521	0.000	25.518
C30	158.843	22.880	60.431	17.644	118.858	106.306
C31	114.574	29.270	29.923	9.813	89.259	0.000
C32	115.503	24.020	53.439	18.043	97.020	88.667
C33	68.620	8.613	18.108	5.413	36.842	0.000
C34	68.296	11.589	15.854	5.510	42.792	57.207
C35	52.560	0.000	35.112	0.000	0.000	0.000
C36	19.428	16.913	19.276	19.667	20.291	15.760
C37	30.769	0.000	0.000	0.000	0.000	0.000
C38	36.433	0.000	20.107	6.470	27.601	21.330
C39	20.665	0.000	0.000	0.000	0.000	0.000
C40	33.494	7.864	23.472	7.185	29.765	27.222
C41	11.886	0.000	0.000	0.000	0.000	11.325
C42	17.738	0.000	14.784	3.619	18.037	16.034
C43	8.002	0.000	0.000	0.000	8.464	0.000
C44	15.969	0.000	0.000	0.000	13.513	0.000
Total n-paraffin	5194.210	1695.699	2241.724	929.430	3305.253	2926.263
Degradation of n-paraffin (%)	67.354	56.842	82.106	36.367	43.663	
Total Iso-paraffin	1633.894	761.849	724.687	299.457	1632.336	1593.946
Degradation of iso-paraffin (%)	53.372	55.647	81.672	0.095	2.445	
Total saturated hydrocarbons	6828.104	2457.548	2966.410	1228.887	4937.589	4520.209
Degradation of total saturated hydrocarbons (%)	64.008	56.556	82.003	27.687	33.800	

Control sample have normal-Carbon14 (n-C14) as the initial carbon number. The initial carbon number for the other samples, treated with bacterial and actinomycetes cultures, was n-C16 indicating that n-C14 and n-C15 completely degraded by all the used cultures. Samples treated with the 3 *Streptomyces* spp., showed high percentage of both n-paraffin and iso-paraffin degradation. *Streptomyces ferralities* showed the highest degradation percent of saturated hydrocarbons among all tested species, reached 82.106 and 81.672 for normal-paraffin and iso-paraffin, respectively. Percent of saturated hydrocarbons degradation by using *Streptomyces sampsonii* and *Streptomyces orinoci* was 64.008 and 56.556, respectively. The degradation percent of n-paraffin exhibited by *Pseudomonas proteolytica* and *Pseudomonas xanthomarina* was 43.663 and 36.367 respectively, while the degradation percent of iso-paraffin was non-significant.

Biodegradation potential of PAHs: Qualitative and quantitative identification of the individual PAHs for the extracted aromatic fractions of un-treated control and samples treated with bacterial and actinomycetes cultures was shown in Table 2. The brief identification of PAHs for control un-treated sample revealed the predominant of high molecular weight PAHs which exists in very high percentage, 56.967, 29.450 and 13.110 for hexa-, penta- and tetra-aromatic rings PAHs respectively, while low molecular weight PAHs existed in traces.

Maximum biodegradation percent of PAHs was 54.798 and 17.429 by using *Streptomyces sampsonii* and *Pseudomonas proteolytica* respectively. The percent of high molecular weight PAHs in sample treated with *Streptomyces sampsonii* greatly decreased from 56.967 and 29.450 to 31.317 and 28.317, respectively corresponding to un-treated control, while the percent of the lower molecular PAHs

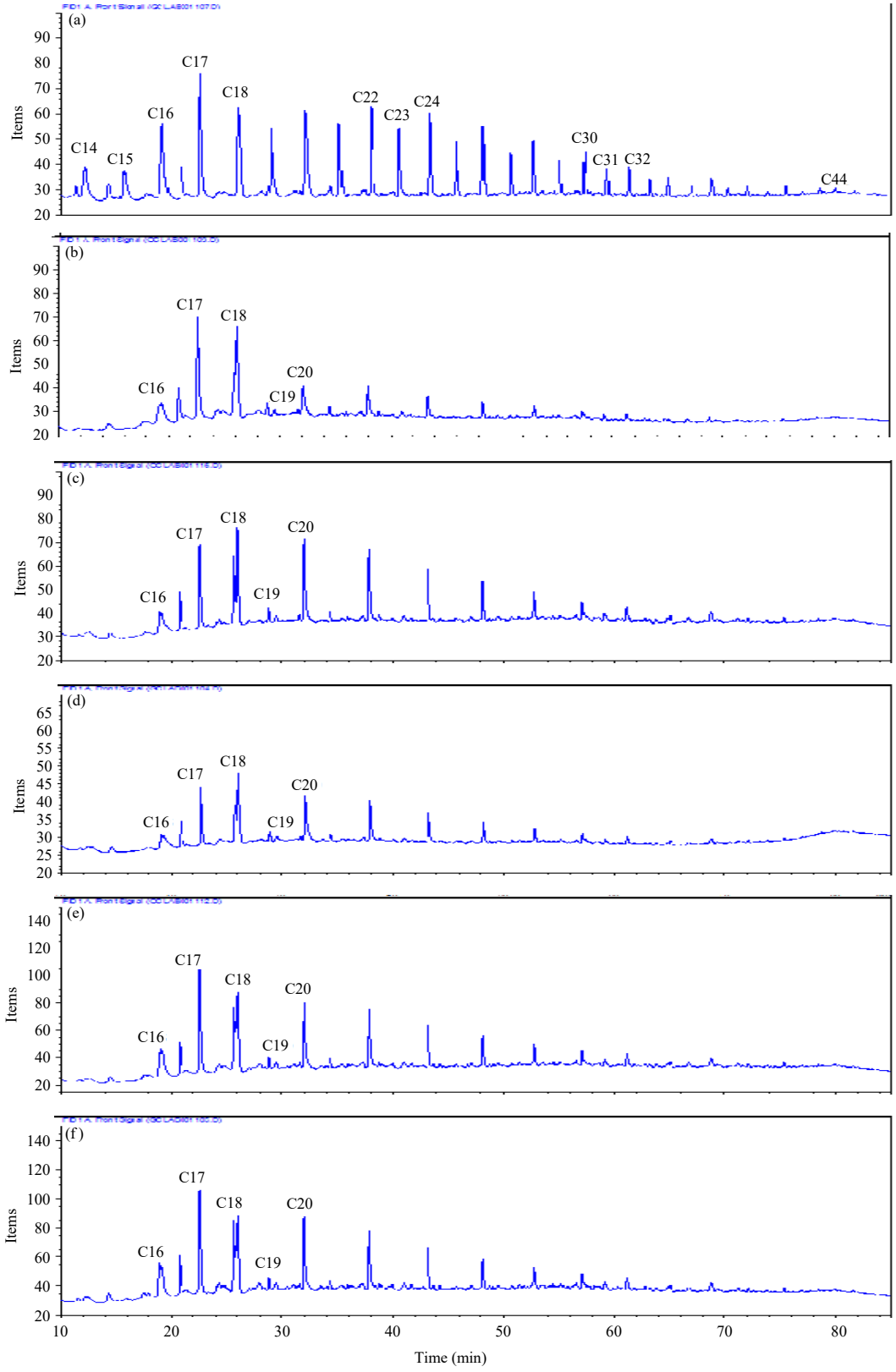

Fig. 2(a-f): Gas chromatograms of the saturated fractions extracted from samples treated with 3 actinomycetes species and 2 bacterial species and un-treated control, (a) Un-treated control, (b) *Streptomyces sampsonii*, (c) *Streptomyces orinoci*, (d) *Streptomyces ferralities*, (e) *Pseudomonas xanthomarina* and (f) *Pseudomonas proteolytica*

Table 2: Quantitative degradation analysis, estimated by HPLC, of PAHs using 3 actinomycetes species and 2 bacterial species corresponding to un-treated control

Number of rings	PAHs	Control	*S. sampsonii*	*S. orinoci*	*S. ferralities*	*P. xanthomarina*	*P. proteolytica*
2	Naphthalene	0.341	5.753	0.701	1.166	0.765	0.507
2 rings (%)/total PAHs		0.258	9.626	0.535	0.989	0.700	0.380
3	Acenaphthylene	0.000	0.000	0.581	2.169	2.422	0.495
	Acenaphthene	0.128	0.000	0.000	0.129	0.007	0.000
	Fluorene	0.000	1.004	1.598	1.095	1.717	0.914
	Phenanthrene	0.000	5.429	1.032	1.739	1.461	0.816
	Anthracene	0.497	0.000	0.869	2.818	0.000	0.515
3 rings (%)/total PAHs		0.472	10.764	3.117	6.737	5.135	2.055
4	Fluoranthene	2.252	4.157	3.573	2.140	2.154	3.456
	Pyrene	0.000	5.605	5.502	4.662	0.724	0.632
	Benzo (a) anthracene	9.576	7.619	11.258	9.317	8.738	9.738
	Chrysene	5.508	0.558	5.811	4.541	5.135	4.622
4 rings (%)/total PAHs		13.110	30.015	19.972	17.510	15.342	13.835
5	Benzo (b) fluoranthene	11.469	7.395	13.500	10.282	10.237	15.047
	Benzo (k) fluoranthene	13.319	1.243	7.048	10.091	11.897	12.700
	Benzo (a) pyrene	0.000	3.240	5.693	0.273	0.163	0.453
	Dibenzo (a,h) anthracene	14.154	5.048	8.408	12.540	7.688	9.786
5 rings (%)/total PAHs		29.450	28.317	26.469	28.126	27.463	28.487
6	Benzo (g,h,i) perylene	50.065	11.707	38.208	30.018	26.064	50.660
	indeno	25.262	7.012	27.122	25.009	20.012	23.004
6 rings (%)/total PAHs		56.967	31.317	49.907	46.638	42.201	55.243
	Total PAHs	132.229	59.770	130.904	133.347	117.989	109.182
Degradation (%) of total PAHs			54.798	1.002	-0.845	10.769	17.429

significantly increased. On the other hand, HPLC analysis for aromatic fractions showed low percent of PAHs degradation exhibited by *Pseudomonas xanthomarina*, *Streptomyces orinoci* and *Streptomyces ferralities*.

DISCUSSION

The bacteria and actinomycetes used in the present study were isolated from oil-polluted soil samples and tested qualitatively and quantitatively for their ability of petroleum hydrocarbons degradation. A diverse group of bacteria and fungi have been shown to have ability for PHs degradation[12]. Some researchers found that more than 100 species representing 30 microbial genera has been shown to be capable of utilizing hydrocarbons[13] and other author observed greater rates of uptake and mineralization for microorganisms collected from oil-polluted sites than for microorganisms from relatively un-polluted regions[14].

According to the results obtained, *Streptomyces orinoci* and *Streptomyces ferralities* were the most potent isolated strains in saturated hydrocarbons degradation. For PAHs degradation, *Streptomyces sampsonii* are the most potent strains. The bacterial genus; *Pseudomonas* as an important oil-degrading taxon. A large number of *Pseudomonas* spp.[15,16,17]. which are capable of utilizing petroleum hydrocarbons have been isolated[18,19]. The genetic information for hydrocarbon degradation in these organisms generally has been found to occur on plasmids[20]. *Pseudomonas* spp. has been used for genetic engineering to be involved in utilization of petroleum hydrocarbons[21]. In other study on oil-utilizing microorganisms, actinomycetes identified as a potent group of oil-degrading microorganisms[22]. It recorded also, the actinomycetes; *Streptomyces rimosus* as a potent oil-utilizing species[15].

The data obtained in this study revealed the high degradation percent of saturated hydrocarbons, especially n-alkanes, exhibited by all tested species, especially *S. sampsonii* and *S. ferralities*. Hydrocarbons within the saturate fraction including n-alkanes, branched alkanes and cyclo-alkanes (naphthenes). The n-alkanes are generally considered the most readily degraded components in a petroleum mixture[13]. Biodegradation of n-alkanes with molecular weights up to n-C44 have been previously demonstrated[23,24,25].

The results of the present study indicated the lower percent of isoprenoid (iso-paraffin) degradation, in correspondence to n-paraffin, by most of the tested species. However, the percent of n-paraffin and iso-paraffin degradation exhibited by *S. ferralities* and *S. orinoci* almost the same, indicating that the 3 species attack both n-paraffin and iso-paraffin in the same rate. Highly branched isoprenoid alkanes, such as pristane, have been found to undergo omega oxidation, with formation of di-carboxylic acids as the major degradative pathway[26]. The microbial metabolism of cyclic hydrocarbons and related compounds has been reviewed by Perry[27]. Up to 6 membered condensed ring structures have been reported to be subjected to microbial degradation[28].

The degradation of aromatic hydrocarbons has been reviewed by Gibson and Chapman[29] and Hopper[30]. The bacterial degradation of aromatic compounds normally involves the formation of a diol followed by cleavage and formation of a di-acid such as cis, cis-muconic acid. Light aromatic hydrocarbons are subject to evaporation and to microbial degradation in a dissolved state[31]. Several investigators have examined the potential activities of hydrocarbon-degrading bacteria by using ^{14}C-radiolabeled hydrocarbons[20]. The rates of mineralization were greater for hexadecane than for naphthalene, which were greater than those for toluene, which were greater than those for cyclohexane[14,32]. The observed PAHs activities of these Actinomycetes and *Pseudomonas* strains suggested their potential usage as alternative sources of hydrocarbon chemical degradation in petroleum industry.

CONCLUSION

The actinomycete; *Streptomyces ferralities* and *Streptomyces sampsonii* showed the highest degradation percent of saturated hydrocarbons. Additionally, *Streptomyces sampsonii* showed the highest PAHs degradation efficiency. The selected organisms can be used, after large scale production, in field application for remediation of petroleum hydrocarbons polluted soil.

SIGNIFICANCE STATEMENT

The current study discovered the effect of different actinomycetes strains and their potential in hydrocarbon degradation. This study will help the researcher to uncover the critical area of potential biodegradation of PAHs as alternative sources of hydrocarbon chemical degradation of PAHs. Thus, a new Actinomycetes strain will help improving the petroleum industry.

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