Isolation and identification of actinomycetes strains from oil refinery contaminated soil, Basrah-Iraq

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Abstract: Six actinomycetes strains from genus Streptomyces isolated from hydrocarbon contaminated soil in Basra, Iraq were selected for their capacity to grow in the presence of crude oil. Their growth rates and biodegradation ability were investigated in mineral salt media supplemented with light crude oil. These strains consist of S. variabilis (two strains), S. cellulosae, S. parvus, S. bacillaris and S. flavoviridis.

Keywords: actinomycetes, biodegradation, contaminated soil

I. INTRODUCTION

Crude oil pollution and petroleum products are widespread in the soil and at present is a serious problem that represents a global concern for the potential consequences on ecosystem and human health [1]. Although the crude oil products are the major source of energy for industry and daily life, with a great interest in exploration and production it, reach to the environment by several ways, such as Leakages and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products [2]. Human activities that resulted from development of industrial are the main source of significant hydrocarbon release to the environment, increasing incidence of petroleum contamination [3].

Petroleum hydrocarbons composed of complicated mixtures of non-aqueous and hydrophobic components such as n-alkane, aromatic, resins and asphaltenes. Due to the adverse impact of these chemicals on human health and environment, they are classified as priority environmental pollutants by Environmental Protection Agency [4]. Soil contamination with toxic hydrocarbons causes extensive change of local system since accumulation of these in organisms tissue may cause death or mutations also have been known to belong to the family of carcinogens and neurotoxic organic pollutants also cause disruptions of natural equilibrium between the living species and their natural environment [5,6]. Many genus of actinomycetes can degrade different pollutants for example Streptomyces, Arthrobacter, Rhodococcus, Gordonia and Mycobacterium are known hydrocarbon degraders [7,8]. Mereena et al. [9] explain the major genus of actinomycetes showed hydrocarbon degrading activity was Streptomyces and Micromonospora.

The application of 16S rRNA gene sequencing to examine genetic relatedness of microbial species is well suitable due to sequence differences in the 16S rRNA gene [10]. The diversity analysis of actinomycetes by 16S rRNA phylogenetic marker showed that a group are dominant in environment [11].

II. MATERIAL AND METHODS

A. Sample collection

The oil samples were obtained from crude oil of South Refineries Company (SRC) in Basra-Iraq and stored in pre-sterilized glass bottles were used for ability of the isolated bacteria to degrade petroleum.

Oil contaminated soil samples in the present study were collected throughout the study period randomly from petroleum contaminated of (SRC). Samples were collected in sterile container at a depth of 10- 20 cm , samples were brought to the laboratory, where petroleum degrading bacteria were isolated form them and analysis , air dried and passed through 2mm sieve and mixed thoroughly and stored at 4ºC for further analysis [12].

B. Measurement of soil chemical and physical properties

Moisture content was measured by gravimetric dry weight soil, pH was measured in a 1:2 mixture of soil : water (0.01 M CaCl2 solution) using pH electrode [13], soil texture was determined by Particle size analyzer (Malvern, Germany), total petroleum hydrocarbons was extracted as described by Mishra et al., [14].

C. Selective isolation of actinomycetes

Soil samples were air dried at room temperature for 3 days and pretreated at 45-55°C in a hot air oven for 16 hrs, dilution plate was used to enumerate common actinomycetes in the soil, dilution spread onto actinomycete isolation agar (Maknur, Canada). This medium are supplied with antibiotics solution (75mg/l of Nalidixic acid and 80mg/l Cycloheximide), plates were incubated at 30°C for 7-14 day for the growth different forms of actinomycetes colonies at different periods and colonies of all separated plated were counted [15,16], colonies were recognized basis on the morphology under dissected and light microscope. Actinomycetes showed formed aerial and/or substrate vegetative mycelium. Isolates were maintained on International Streptomyces project media (ISP-5) at 4°C for 2 month. Isolation and enumeration of total heterotrophic bacteria count (THBC) in soil were performed by soil dilution plate technique [17]. Hydrocarbon degrading bacteria count (HDBC) were quantified by use spread plate technique on mineral salt medium (MSM) agar plates supplied with 0.5ml of an ethereal solution of the crude oil 10% w/v was transferred to the surface of the agar plate as a carbon source. Plates were incubated at 30°C for two weeks [18,12].

D. Biodegradation of crude oil by actinomycetes isolates

For examining the ability of actinomycetes degradation of crude oil, 84 Pure culture were confirmed by oil degradation in a liquid Mineral salt medium (50ml) that contain 1% (w/v) crude oil was used as the sole carbon source as described by Mirdamadian et al., [19]. All actinomycete strains were growing in yeast extract malt extract broth and incubated in rotary shaker at 120 rpm for 3 days at 30°C. 5% v/v of inoculums decant to MSM media, uninoculated media served as control. Flasks were incubated at 30°C in rotary shaker at 120 rpm for 10 days. Oil utilization in the enriched cultures was monitored by a decrease in the amount of crude oil concentration and an increase in bacterial biomass. After incubation, residual crude oil was extracted with chloroform in separating funnel and determined percent crude oil degradation gravimetrically. Finally, 6 isolates were appeared highest capacity for petroleum biodegradation.

E. Amplification of 16S-rRNA genes

The genomic DNA of actinomycetes isolates was extracted using Wizard Genomic Isolation DNA kit (Promega, USA) according to manufacturer instructions. The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using primer set 27f (5′-AGAGTTTGATCMTGGCTCAG 3′) [20] and 1525r (5′CGGCTACCTTGTTACGACTT-3′) [21] in 25 µl mixtures (Master mix, Bioneer, Korea) mixed with purified DNA 2 µl (approximately 50 ng/µl), 3 µl, forward and reverse primer (62.5 µmol/l) and d. H2O was added to a final volume of 50 µl . Thermal cycler (Bioneer, Korea) with the following thermal profile: A gene amplifier was used to incubate reaction through an initial denaturation at 94 ºC for 2 min. , followed by 35 cycles of amplification , denaturation at 94 ºC for 40 s, primer annealing at 55 ºC for 30 se., and primer extension at 72 ºC for 1 min., and finally at 72 ºC for 10 min, cooling to 4 ºC [22]. After the amplification the PCR products were examined by 1.5% w/v agarose gel electrophoresis with 100 bp DNA ladder at 65V and 120mA for 40 min. using TBE buffer 1x , the gels were stained with ethidium bromide solution. The nucleic acid on gel were visualized using UV transillumintor.

The PCR products and same primers were sequenced using Applied Biosystems 3730XL Genetic Sequencer (NICEM, USA). All the bacteria 16S rDNA sequences were analyzed and edited by using BioEdit software and sequences with noise were deleted, initially determined by comparing to 16S rDNA sequences available in the nucleotide databases of the GenBank using the basic local alignment search tool online service (http//www.ncbi.nlm.nih.gov/ BLAST) [23] at the National Center for Biotechnology Information (NCBI), to determined approximate phylogenetic position and percent homology scores were obtained to identify microorganisms.

III. RESULTS

A. Characteristic of Soil samples

The examined Soil contaminated with petroleum hydrocarbons derivatives originated from the oil refinery in Basra, where pollution is high and chronic. soil properties in five different area are presented in table 1. The

population of total heterotrophic bacteria count (THBC) was range from 1×106 to 3.3× 106 cfu.g-1 of indigenous soil microorganisms when plated on nutrient after two days, on the actinomycetes isolates agar the total actinomycetes bacteria count (TABC) about 1- 1.3×102 cfu.g-1 after incubation period and the population of hydrocarbon degrading bacteria count (THDBC) was 1.5x104 to 7x104 cfu.g-1 dry soil.

Table 1: Properties of contaminated soil samples

Property	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
TPH. mg.Kg^{-1}	652	4284	4272	2252	1364
pH	6.9	6.7	8.2	8.3	8.3
Water content%	7.5	1.5	2.3	1.8	1.8
Soil texture	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam
THBC cfu.g^{-1}	3.3×10^{6}	1×10^{6}	3×10^{6}	2.6×10^{6}	2.1×10^{6}
THDBC cfu.g^{-1}	6.1×10^{4}	1.5×10^{4}	7×10^{4}	3.5×10^{4}	5.2×10^{4}
TABC cfu.g^{-1}	1.3×10^{2}	1×10^{2}	1.2×10^{2}	1×10^{2}	1×10^{2}

B. Screening actinomycetes isolates for degradation of hydrocarbon

A total of 84 isolates belong to actinomycetes were obtained from soil contaminated samples using plate methods and selective actinomycetes media, resulted about 60% of Streptomyces strains which performed screening for capacity degradation rates of crude oil in liquid MSM contain 1% oil as sole carbon source, the results ten of these strain that code (A4, A5, A15, A19, A58, and A75) showed a highest capacity for biodegradation percentage was found to vary from 52% to 72.3% and growth range from 34.6 to 49 mg/50ml after 10 days incubation. These results are shown in the figure 1. Table 2 show each strain grew well in oil media and emulsified crude oil.

Figure 1: Screening of actinomycetes isolates for crude oil degradation and biomass

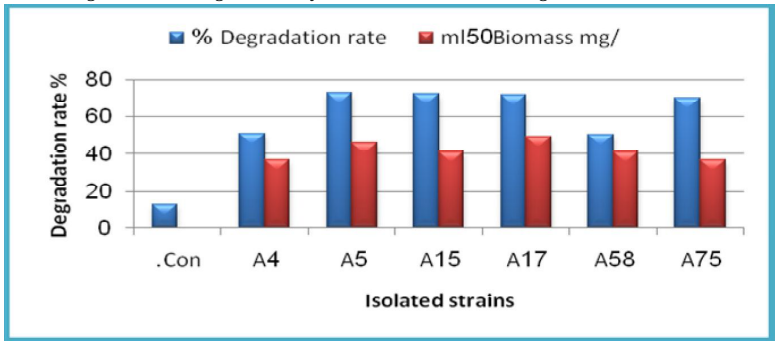
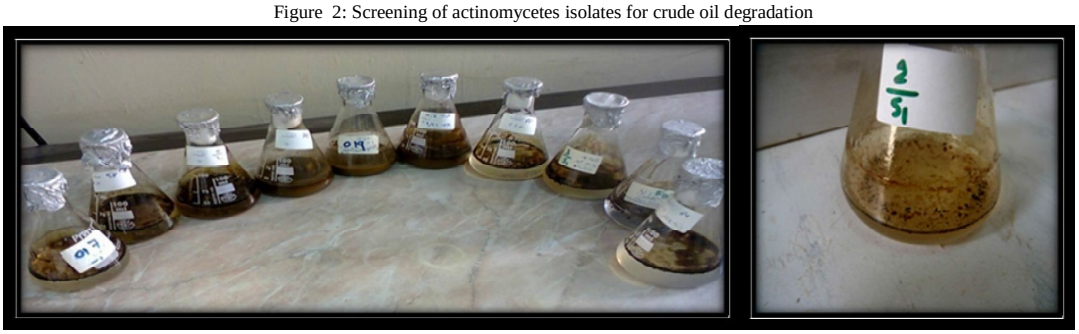


Table 2: Changes in crude oil by isolated strains in media after 10 days.

Strains	Changes in crude oil
A4	Forming dispersed flock and dense liquid
A5	Forming oil film and large flock
A15	Forming high small dispersed flock and dense liquid
A17	Complete emulsification and forming flock
A58	Complete emulsification and dense liquid
A75	Forming oil film and flock

Figure 2: Screening of actinomycetes isolates for crude oil degradation



C. Identification of crude oil degrader actinomycetes strains

Morphology of actinomycetes isolates (A4, A5, A15, A17, A58, and A75) was visually observed and their aerial and substrate mycelia are shown in Figure 3. Observation under microscope that the isolated was a Gram positive and filament shaped, the six strains represented members of genus Streptomyces, possessed spore chain morphology. All these isolates grew well ISP-5 medium with characteristic musty odor, non-motile colonies, produced disc shaped colonies with sporulation largely at the edges of the colony and produced snow white aerial mycelium and wheat color substrate mycelium figure 3. The results pigments were produced by isolates and growth characteristic showed in table 3. The Gram stained isolates were observed and pictures of the isolates are shown in figure 4, all isolates were gram positive filaments shaped.
These results also were consistent with results obtained for 16S rDNA gene sequences DNA, indicated that a actinomycetes were represented as Streptomyces species.

Figure 3: Streptomyces isolates grow on the ISP-4 medium.

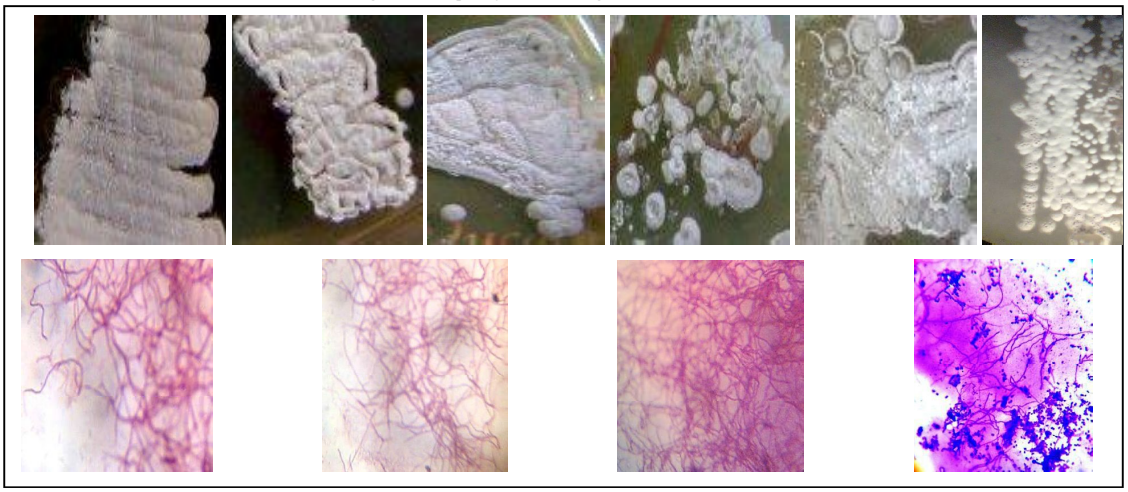


Table 3: Culture characteristic of Streptomyces isolates on ISP-5 medium

Isolates Code	Growth Degree	Pigment production	Color	Gram's reaction
A4	+++	Brown	Gray	G +ve
A5	+++	Red	Wheat-yellow	G +ve
A15	+++	Brown	Gray	G +ve
A17	+++	Pale-yellow	Golden-yellow	G +ve
A58	+++	Pink-yellow	Gray-yellow	G +ve
A75	+++	Brown	Gray	G +ve

D. 16S rDNA amplification

All actinomycetes were examined for the specific amplification of 16S rDNA gene sequences PCR amplification of 16S rDNA using a set of primers: 27f and 1525r yielded a single amplification of ~1500 bp for all the isolates. Show in figure 5. The sequences of the 16S rDNA were checked in BLAST program, the results of the studied isolates are shown in table 4. Isolates were identified as Streptomyces strains. After alignment with other 16S rDNA sequences in GenBank, it had a high degree of similarity (100%) to other members of genus Streptomyces. The 16S rDNA sequence was further aligned with the corresponding sequences from additional strains of species of Actinobacteria in Bergey's Manual of Determinative Bacteriology [24]. Phylogenetic analysis revealed that strains were first reported to be a pure strains capable of degrading crude oil.

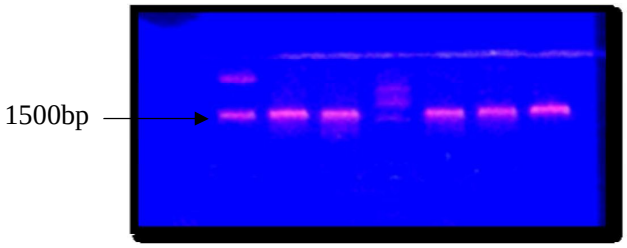


Figure 5: Agarose gel electrophoresis of 1500bp PCR fragment of 16S rRNA genes

Table 4: BLAST result of partial 16S rDNA gene sequence of Streptomyces

Isolates	Closed BLAST match	Strains	Accession no.	Identity
A4 (1073bp)	*Streptomyces variabilis*	7525	JN180216.1	100%
A5 (999bp)	*Streptomyces cellulosae*	XFB-T	KC429624.1	100%
A15 (1013bp)	*Streptomyces variabilis*	SW75	JN180216.1	100%
A17 (879bp)	*Streptomyces parvus*	S86	JN180216.1	100%
A58 (1048bp)	*Streptomyces bacillaris*	S4BW2	JN400100.1	100%
A75 (793bp)	*Streptomyces flavoviridis*	VITHM-1	KM588257.1	100%

IV. DISCUSSION

A. properties of Soil samples

Soil properties are presented in Table 1 pH of the collected soil is slightly alkaline, which is also typical for this area. Such pH values are not reported to be inhibitory for biodegradative abilities of microorganisms to hydrocarbons. [18]. Total heterotrophic bacterial counts in this soil are high enough, and above the recommended range for successful hydrocarbon degradation. Moreover, the soil contains high counts of bacteria 3.3x106 cfu.g_1 soil, this is probably a result of adaptation to the continuous addition of hydrocarbons contained in the treated oily wastewater applied to the soil. According to Mishra et al., [14], hydrocarbon-degrading bacteria that are indigenous to soil with populations exceeding 105 cfu.g_1 soil are usually enough to satisfactorily carry out the degradation process without the need for bioaugmentation. It has been reported that population numbers of hydrocarbon degrader and their population within the microbial community refers to be a clear index of environmental exposure to hydrocarbons [25].

The samples used for isolation contain high concentrations of hydrocarbon above 500mg.kg-1 there are significant differences between them in the type of matrix. According to the mineralogical composition, the all sample belongs to sandy loam soils. Nevertheless that soil properties in the study area affected remain of crude oil from the refinery, but the current study showed isolate crude oil degradable bacteria some of those belonging to the actinomycetes species and this corresponded to the Gogic-Cvijovic et al., [26] study to isolate the bacteria utilize of petroleum from sandy soil polluted an oil refinery consisting of strains of actinomycetes.

B. Degradation of hydrocarbons

The capacity of hydrocarbon degradation by actinomycetes isolates in MSM media supplemented with crude oil are illustrated in Fig. 2 All isolates showed the ability to utilize crude oil as sole carbon source however, the efficiency of utilization varied among strains after 10 days. In this study 6 strains showed a remarkable high ability above 50% to degrade crude oil in liquid medium.

Many studies performed about hydrocarbons degrading actinomycetes show ability of these bacteria for utilize compounds of crude oil isolated from contaminated sites [16,27,28]. Latha and Kalaivani [12] isolated microbial degradation of petroleum hydrocarbons from crude oil contaminated sites, some of these bacteria belong to actinomycetes. Ramadha et al., [29] were isolated two Streptomyces isolates from different soil contaminated with diesel in Iraq that showed the ability to utilize crude oil were cultivated in mineral salt medium with both complex hydrocarbons as the sole source of carbon and produced biosurfactant in medium and its effect was detected using surface tension as an indicator for this process. In other study by Radwan et al., [30] were isolated of Streptomyces strain capable of hydrocarbon uptake and utilization from the polluted desert of Kuwait, shown by transmission electron-micrographs of the cellular membrane was different from that of the non-utilizer control strain after incubation in the presence of n-hexadecane, metabolized into the corresponding fatty acid and transported these compounds more efficiently across their membranes and accumulated them as inclusions in the cytoplasm.

The results set forth in a table 2 indicate a clear change in the nature of crude oil and this explain to produce enhancer dispersion, emulsification and reduce surface tension of oil and make it available for microbial attachment, which is called biosurfactants. Several studies showed susceptibility to produce biosurfactants [31,32].

C. Identification of Actinomycetes isolates

In this study has been the adoption of the second edition of the Bergey's Manual of Systematic Bacteriology, volume 5 edited by Goodfellow et al., [24], arrange of manual in phylogenetic groups depend on the analyses of the 16S rRNA that presented in Road map of the phylum Actinobacteria. Occurred substantial modification to this phylum since the publication of volume 1 in 2001, which indicates the presence of many experimental data and a different method of analysis. Therefore volume 5 includes only the phylum Actinobacteria, taxa are arranged by class, order, family, genus and species.

Identification through morphology of colony and cells addition to pigment production, indicated that all the strains were in the Actinobacteria, genus of Streptomyces. This was confirmed using 16S rDNA gene sequencing analysis. The 16S rRNA sequence analysis of all the strains as representatives of the high crude oil degradable isolates and partially sequenced is summarized in the Table 3 a BLAST analysis carried out through Blastn search through GenBank revealed that six strains (A4, A5, A15, A17, A58 and A75) are the members of the genus Streptomyces.

Molecular tools have a great potential to assist in isolating bacteria with known rRNA sequences, one of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rDNA [33]. In this present study, 16S rRNA amplified products were sequenced, which revealed that six strains belong to the members of the genus Streptomyces, which was the dominant actinobacterial genus. Zhang and Zhang [21]

isolated actinomycetes from soil and found that the common genus was Streptomyces . Similar predominance of Streptomyces was also reported from marine sediments and alkaline environments [34]. Many reports that used of the 16S rRNA gene sequencing to examine genetic relatedness of actinomycetes species [35,36,37].

V. CONCLUSION

In this study, actinomycetes were isolated from contaminated soil in Basra have capacity for degradation of crude oil, identification of these isolates by 16S rRNA gene were assigned to six strains of Streptomyces.

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