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Isolation and Screening of Biosurfactant Producing Bacteria from Oil Contaminated Soils in Iraq

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Abstract: *Nine polycyclic aromatic hydrocarbons (PAH) degrading bacteria were isolated from oil field contaminated soil samples at Basarah city and automobile workshop sites at Babylon city. Isolation was occurred by enrichment procedure in crude oil as the sole source of carbon and energy. These isolates were screened for the biosurfactant production, three of them 1oil, 3H, and B23 which were found high active to utilize crude oil and biosurfactant production.*

Three isolates were characterized using morphological and biochemical tests to Bacillus spp. Three isolates showed the growths on mineral salts medium as the carbon source and energy as demonstrated by the increase in cells forming unit (CFUml) during the incubation period. Three isolates showed greater clearing zones (cm) on the modified oil agar medium. The three isolates were tested to producing biosurfactant by two assays (E24 assay and oil agar plate assay) these assays suggested that three isolates producing biosartactant. This study conclude the ability of Bacillus spp. isolated from oil contaminated soil to grow on the enriched media with the hydrocarbon as a sole source of energy with high potential capacity of oil degradation and Biosurfactant production.

Key word: *Bacillus, Petrol, Biodegradation, Biosurfactant.*

I. Introduction

Petroleum- based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products (Kvenvolden and Cooper, 2003). Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution (Hilliger, et al., 1997).Soil contamination with hydrocarbons causes extensive damage of local system since accumulation of pollutants in animals and plant tissue may cause death or mutations (Alvarez and Vogar, 1991).The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dispersion and washing However these technologies are expensive and can lead to incomplete decomposition of contaminants. Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Ulrici, 2000). Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Petroleum hydrocarbons can be divided into four classes: the saturates, the aromatics, the asphaltenes (phends, fatty acids, ketones, esters, and polyphyrins) and the resins (pyridines , quinolines, carbazoles, sulfoxides, and amides (Colweel, et al., 1977).

Different factors influencing hydrocarbon degradation. One of the important factors that limit biodegradation of oil pollutants in the environmcnt is their limited availability to microorganisms. Petroleum hydrocarbon compounds bind to soil component, and they are difficult to be removed or deqraded (Barathi and Vasudevan, 2001).Microbial degradation is the major and ultimate natural mechanism by which one can cleanup the petroleum pollutants from the environment (Atlas, 1992; Lal and Khanna,1996,and Amund and Nwokoye,1993).Bacteria are the most active agents in petroleum degradation , and they work as primary degraders of spilled oil in environment (Rahman et al., 2003; Brooijmans et al., 2009). Biosurfactants are heterogeneous group of surface active chemical compounds produced by a wide variety of microorganisms (Mahmound,et al.,2008,Ilori,et al.,2008,Kiran,et al.,andObayori,et al.,2009).

Hydrocarbon degrading microorganisms produced biosurfactant of different chemical nature and molecular size which are surface active compounds which increases the surface tension of the hydrophobic water- insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation (Pekdemir et al., 1999). Biosurfactant has received considerable attention in the field of environmental remediation processes such as bioremediation, soil washing and soil flushing Biosurfactant influence these processes because of their efficacy as dispersion and remediation agents and their environmentally friendly characteristics such as low toxicity and high biodegradability.

The current study aimed to isolate and screening biosurfactant producing microorganisms from oil contaminated soil samples.

II. Materials and Methods

Sampling:

About 10g soil samples were aseptically collected with a sterile spatula at the Babylon engine contaminated soil. Soil was sampled from 0-20 cm depth. Another soil was sampled at Basrah oil field contaminated soils from 2000-3000m depth underground. All samples were placed into sterile polythene bags and stored at 4Cº immediately they were brought to the laboratory.

Screening and Isolation Bacteria:

Microbes were isolated from oil contaminated soils by using the Bushnell Hass Microbial salts (BHMS) medium. BHMS contain (per liter of distilled water) 0.2g of MgSO4.7H2O, 0.02g of CaCl2, 1g of KH_2PO_4, 1g of $NH_4(SO_4)_2$, 2 drops of $FeCl_3$ 60%. The pH was adjusted to 7.0-7.8. The bacteria were isolated by using an enrichment culture and a single colony isolation technique. The isolated cultures were preserved in nutrient agar slants and stored at 4Cº for further use. For screening 1 gm oil contaminated soils samples were separately suspended and vortexes in 10 ml of sterile distilled water, 1ml of this sample was used as an inoculum for isolation of oil degrading bacteria. Flask of (250ml) was taken and 100ml of BHMS broth medium (Bushnell and Hass, 1941; Atlas and Bartha,1991) was transferred to each flask and sterilized. 5% crude oil was used as the carbon source and incubated in shaker orbital incubator at 37Cº at 120 rpm for one week. All these screening experiments have done in triplicate. After that, 1ml sample was taken from each culture and transferred into fresh BHMS medium, followed by incubation as described above for one week. The enrichment procedure was repeated for the third time. Each bacterial isolate was plated in duplicates into modified oil agar medium composed of 1.4gm K_2HPO_4, (NH4)2SO4. 0.6gm KH_2PO_4, 0.6gm $MgSO_4$. $7H_2O$, 4gm agar- agar and. The mineral components of the medium were dissolved in 200ml of distilled water and mixed with 4gm agar-agar powder. The medium was autoclaved at121Cºfor 15min according to Okpokwasilli and Ananchukwu (1988). After solidified, The plates were poured by 2ml of crud oil. The plates were incubated at 37Cº for 48h. After incubation, the degraders isolates was defined as the diameter of the clearing zone on the oil surface in centimeters.

Biosurfactant Production Tests:

A-**Emulsification test (E24)**: Several colonies of pure culture were suspended in test tubes containing 2ml of mineral salt medium after 24hr of incubation, 2ml of hydrocarbon (oil) was added to each tube, then the mixture was vortexes at high speed for 1min and allowed to stand for 24h. The emulsion index (E24) is the height of the emulsion layer (cm) divided by height (cm), multiplied by 100 (Bodour et al., 2004) .

$$\text{Emulsification index (E24) \%} = \frac{height\ of\ the\ emulsion}{Total\ high} \times 100$$

B-Oil agar plate tests

Mineral salt agar medium coved by crude oil was used to detection of biosurfactant production. 10 μ of cell free supernatant was added into the each well prepared into mineral salt agar medium using cook borer (4mm). The plate was then incubated at 37Cº for 48hr. A clear zone around culture was considered positive result for biosurfactant production.

Determination of Crude Oil Degrader Bacteria Growth:

One ml of inoculum (Bacterial number was adjusted to give initial cell number 1×10^8 CFU ml^{-1}) for each degrader isolates studies was inoculated into 250ml flask containing 100ml of Bushnell and Hass medium with 5% crude oil as a source of carbon and energy. The flasks then incubated at 37Cº at 120 rpm for 15 days, 1ml of each sample were withdrawn periodically was diluted to 10^{-6} using phosphological saline, 1ml of inoculated by pour plate method in nutrient agar and incubated at 37Cº for 2 days and determination of growth by increasing in colony forming unite (CFU/ml) during the period of incubation .Colony forming unit (cfu): (number of colonies $\times$ dilution factor)/ volume of inoculums used.

Identification of Isolates:

The isolates were identified according to Gram staining, morphological characteristics and biochemical reactions according to MacFaddin (2000).

III. Result and Discussion

Nine crude oil degrader bacteria were isolated from oil contaminated soils using enrichment process on crude oil and they streaked on nutrient agar plate to obtain pure cultures and then taken for further study. Nine bacterial isolates were tested to determine the active bacterial isolates by the measuring of clear zone diameter (cm) growing on modified mineral salt agar medium plates as showed in figure (1). To identify of isolates ,

morphological characterization of 1 oil , 3H, and B23 isolates were done by Gram's staining and biochemical characterization by different biochemical tests, from results it was found that 1 oil , 3H, and B23 isolates are positive Gram stain with different bacilli shaped, spore forming and identified as Bacillus spp. according to table (1).

Growth study of freshly inoculated strains was studied under 37Cº and at 120rpm for 15 days the readings were plotted against time and was taken on interval 2 days.fiq2,3,4. Three of them isolates 1 oil, 3H, B23 were selected to further test figure (5) . 1 oil , 3H and B23 were showed the highest biosurfactant production on crude oil by biosurfactant detection methods: By oil agar plate method supernatant 1oil , 3H, and B23 isolates were add to the wells in mineral salt medium agar olate covered with 5ml of crude oil, 1oil, 3H, and 2/3000 showing a clear zone around the wells in mineral salts agar medium as showed in figure (5). Emulsification assay another method was used to detection biosurfactant production. 1oil , 3H, and B23 isolates showed positive result were tested for the abilities emulsify crude oil (table: 2) .

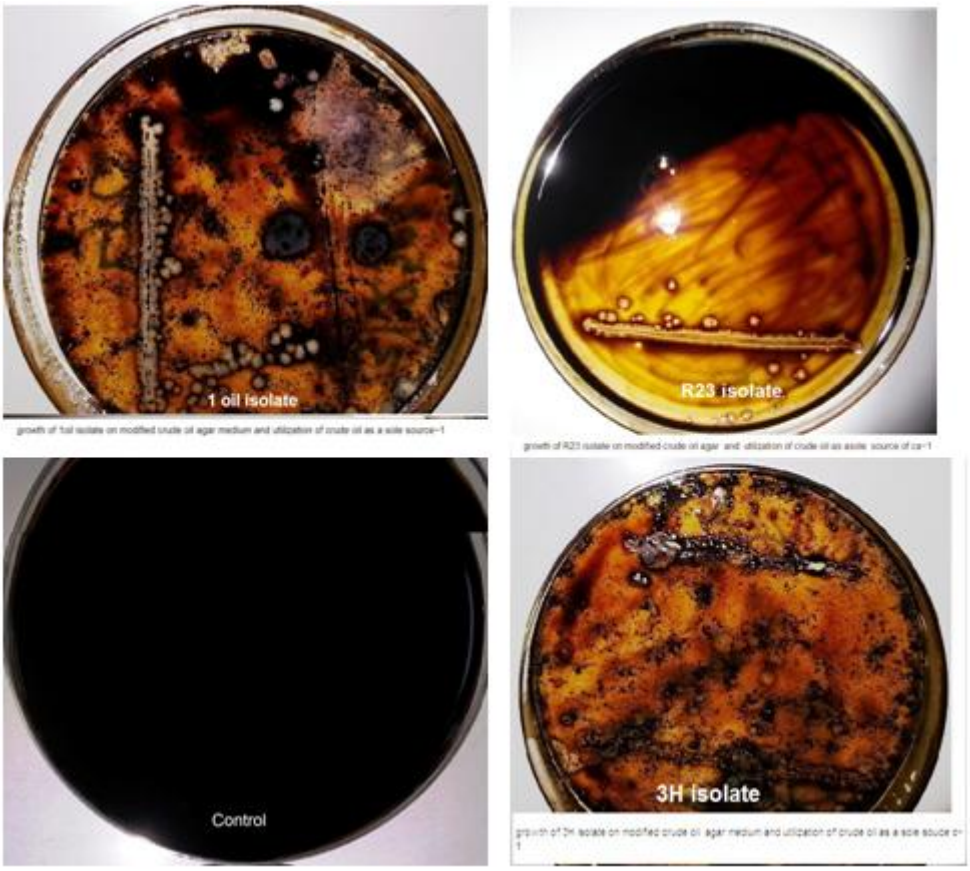
This was in accordance with the result of Sheshtawy ,etal work in (2013) that isolated Bacillus lichneformis and showed the biodegradation capacit in mineral salt medium containing crude oil as a sole source of carbon and energy . Anandarj and Thivakaran (2010) isolated five microorganisms and screened for the purpose of biosurfactant by oil spreading and blood haemolysis test. Ghayyomi et al (2012) isolated a strain of Bacillus sp. from petroleum contaminated soils of SirriIsland producing biosurfactant. Vijaya,et at (2013)isolated 42 isolates and selected for biosurfactant production.six of isolates showed highest CFU ml and biosurfactant production.

Table (1): Summary of morphological, cultural characteristics and biochemical tests for identification Bacillus sp. According to MacFaddin (2000):

Test	1 oil	3H	B23
Gram stain	+	+	+
Spore former	+	+	+
Oxidase test	-	-	-
Catalase test	+	+	+
Gelatin hydrolysis	-	-	-
Citrate utilization	+	-	-
Nitrat reduction	-	-	-
Urea hydrolysis	+	-	+
Vp test	+	-	+
Indole production			
Xylose	-	-	-
Arabinose	+	+	+
Mannitol	+	-	-
Glucose	+	-	-
Lecithinase test	+	+	+
Growth in 5% NaCl	+	+	+
Growth in 7% NaCl	+	+	+
Starch hydrolysis	+	+	+
Mannose oxidation	+	-	-
Lactose oxidation	-	-	-
Raffinose oxidation	-	-	-
Sucrose	+	-	-
Maltose fructose	-	-	-
Arginine dihydrolyse	+	-	-
Lysine	-	-	+
Ornithine decarboxylase	+	+	+

Table (2): Detection of biosurfactants producing isolates by preliminary and complementry screening methods screening methods

Isolate	Oil agar plate method diameter of clear zone	E24 index E24 in presence of erude oil
3H	1.3 cm	25%
1 oil	1.5 cm	30%
2/3000	1.9 cm	45%



Figure(1): Showed Positive Result of 1oil, 3H and R23 Isolates on Modified Oil Agar Test for Biosurfactant producing

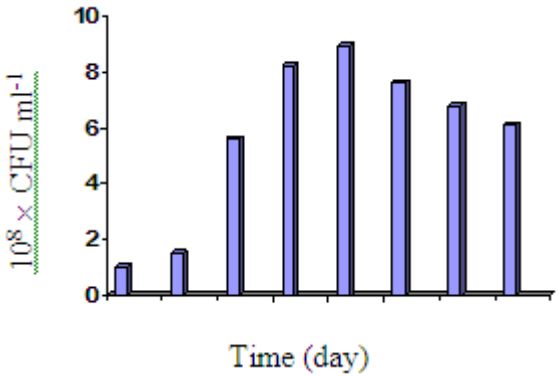


Figure (2): growth of 1oil isolate on BHMs medium contain 5/ crude oil during incubation period

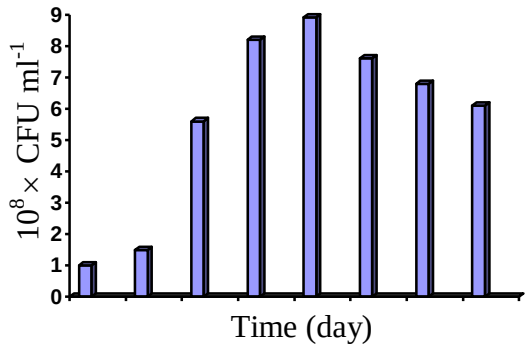


Figure (3): growth of e3H isolate

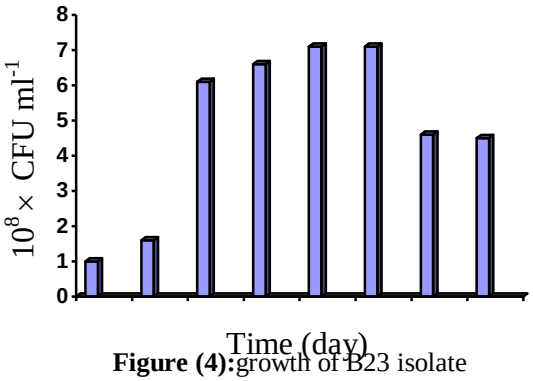


Figure (4):growth of B23 isolate

Figure(5): Showed Positive Result of 1oil, 3H and R23 Isolates on Oil Agar Test for Biosurfactant producing

IV. Conclusion

This study can conclude the ability of Bacillus spp. isolated from oil contaminated soil to grow on the enriched media with the hydrocarbon as a sole source of energy with high potential capacity of oil degradation and biosurfactant production.

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