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PCR-Based Investigation of Oxygenase Among Crude Oil Degrading Bacteria in Hilla City, Iraq

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Abstract : Forty five oil-contaminated soil samples were collected from fuel station in Hilla city-Iraq. All soil samples were cultivated on special medium (Bushnell and Hass mineral salts (BHMS) medium) to recover bacterial isolates with Biosurfactant activity.
According to cultural characteristics, morphological and biochemical tests 10 isolates with biosurfactant activity were recovered, 9 of them belong to *Pseudomonas aeruginosa* and one isolates belong to *Bacillus spp*. All isolates were subjected to conventional biosurfactant screening tests. Oil spreading test, emulsification Index (E_{24}), hemolysis activity and lipolytic activity. The detection of genes of the two important enzymes; phenol monooxygenase and xylene monooxygenase; were performed by PCR using specific primer pairs. All isolates positive for hemolysis activity(β -hemolysis) and positive for oil spreading assay except ps1, ps2 and ps7 isolates . All isolates produce lipase enzyme and have emulsification capcity (E_{24}) that ranged from 52.6 to 42.85. Polymerase chain reaction results revealed that all isolates were negative for toluene dioxygenase gene while 2 isolates of *Pseudomonas aeruginosa* (ps2 and ps9) were positive for phenol monooxygenase gene.
This study conclude the ability of isolated bacteria to degrade crude oil in enriched media and the ability of these isolates to produce biosurfactant.
Keywords: Oil-Contaminated soil, Biosurfactant, Pseudomonas aeruginosa, phenol monooxygenase.

Introduction

Biosurfactant is an amphiphilic molecules produced by microorganisms that act to reduce surface tensions or reduce the interfacial tension between two immiscible liquids like oil and water to facilitate hydrocarbon uptake and emulsification (emulsifier)[1,2]. Large amounts of hydrocarbon contaminants are released into the environments as a result of human activities. The industrial emissions, spillage from tankers, pipelines and storage tanks are largely accidental and occur frequently in present times leading to sever, immediate as well as long-term ecological and environmental hazard. The eco-hazard comes from fact that hydrocarbon components are toxic and persistent in terrestrial and aquatic environments. Most of hydrocarbons are insoluble in water. The degradation of hydrocarbons by microorganism have an important role in combating environmental pollution. Hydrocarbons degrading microorganism produce biosurfactant of different chemical nature and molecular size which can fulfill various physiological roles and provide different advantages to producing isolates. Biosurfactant have many advantages such as increasing the surface area of hydrophobic water-insoluble substrates by emulsification, increase the bioavailability of hydrophobic substrates, bind heavy metals by involved in pathogenesis , possess antimicrobial activity, regulate the attachment /detachment of microorganisms to and from surfaces[3].

Almost all surfactants currently produced are chemically derived from petroleum. These synthetic surfactants are toxic and hardly degraded by microorganisms .They are caused a potential source of pollution and damage to the environment. Biosurfactant derived from living microorganisms have advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity and lower CMC .These properties have led to several applications in the food , cosmetic and pharmaceutical industries[4]. In addition biosurfactant implicated in the field of environmental remediation processes such as bioremediation, soil washing, and soil flushing .

Biosurfactant produced by microorganisms are of many types such as glycolipids lipopolysaccharides, oligosaccharides and lipopeptides[5]. Biosurfactant impact these processes because of their efficacy as dispersion and remediation agents and their environmentally friendly characteristics such as low toxicity and high biodegradability. Although biosurfactant demonstrate such advantages, they have not yet been employed widely in industry because of high production costs[6,7]. Biosurfactant producing attributed to presence of many oxygenase (monooxygenase and dioxygenase) genes such as biphenyl dioxygenase, naphthalene dioxygenase, toluene dioxygenase, toluene/xylene monooxygenase, phenol monooxygenase, and ring-hydroxylating toluene monooxygenase genes[8]. In this study we focused on the two gene phenol monooxygenase and xylene monooxygenase.

The current study aims to isolate local bacterial isolates with high potential to degrade oil-contaminant in environment by producing biosurfactant and investigate the type of oxygenase gene at molecular level.

Material and Method

Screening and Isolation Bacteria :

Forty five oil-contaminated soil samples were collected from fuel station in Hilla city-Iraq. Bacterial isolates were isolated from oil contaminated soils by using the Bushnell and Hass mineral salts (BHMS)medium contain (Per liter of distilled water :1g of K_2HPo_4 ,0.02g of $CaCl_2$,2 drops of $FeCl_3$ 60% ,lg of KH_2 PO_4 0.2g of $MgSO_4$. $7H_2O$, lg of $(NH_4)_2SO_4$ .The pH was adjusted to 7.0.The bacteria was isolated by using an enrichment culture and a single colony isolation technique. The isolated culture was preserved in brain heart infusion broth with 10%glycerol and stored at 4cº for further use. For screening 1g of oil contaminated soils samples were separately suspended and vortexes in 10 ml of distilled water.1ml of this sample was used as an inoculum for isolation of oil degrading bacteria. Flask of (250 ml) was taken and 100 ml of BHMS broth medium[9] was transferred to each flask and Sterilized .5% crude oil 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, 1 ml samples 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 isolates was plated in duplicates into modified diesel agar medium The modified diesel medium using pour plate method composed of 1.4gm $KHPO_4$,0.2g of $(NH_4)_2So_4$,0.6 g of KH_2PO_4, 0.6g of $MgSo_4$. $7H_2O$ .4g of agar- agar and the mineral components of the medium were dissolved in 200 ml of distilled water .The medium was autoclaved at 121Cº for 15 min according to Okpokwasili and AnanChunkwu (1988)[10]. After solidified ,the plates were poured by 2ml of crude oil.The plates was incubated at 37Cº for 48 h.After incubation , the degraders isolates was defined as the diameter of the clearing zone on the oil surface in centimeters.

Bacterial Identification:

All bacterial isolates identified firstly using gram stain and some of the phenotypic and biochemical test and confirmed by Viteck2 compact system.

Screening for biosurfactant Production :

Pure isolates were cultured in mineral salts medium (MSM) with 5% crude oil as a sole source for carbon at 37cºand 120 rpm. For 2days. The broth cultures were centrifuged at 3000 rpm for 5min. the supernatant was subsequently subjected to preliminary screening methods .

Hemolytic activity :

Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37Cº for 48-72 hrs. Results were recorded based on the type of clear zone observed. i.e α -hemolysis when the colony was surrounded by greenish zone β -hemolysis when the colony was surrounded by a clear white zone and δ - hemolysis when there was no change in the medium surrounding the colony[11] .

Emulsification Test (E_{24})

All colonies of pure culture were suspended in test tubes containing 2 ml of mineral salts medium after 48h. of incubation , 2ml of hydrocarbon (crude oil) wa added to each tube .Then , the mixture was vortexed at high speed for 1 min and allowed to stand for 24h. The emulsion index (E24) is the height of the emulsion layer (cm) divided by total hight of liquid column (cm) multiplied by 100[12].

$$\text{Emulsification index } (E_{24}) = \frac{\text{Height of the emulsified layer}}{\text{Total Height of liquid column (mm)}} \times 100$$

Oil spreading assay :

Using a micropipette, 30 μL of crude oil was added to the surface of 40 ml of distilled water into a Petri-dish to form a thin oil layer.10 ml of culture supernatant was gently dropped on the center of the oil layer ,after one minute. If the biosurfactant was present in the supernatant ,the oil is displaced and a clearing zone was formed as described by Morikawa *et al* .(2000)[13] .

Lipolytic Activity assay:-

Preparation of agar plate the composition comprised of 2.5%agar- agar, Tween 20-80 (as a substrate)[14]. clear zone around the organism indicates the production of lipase which is the characteristic feature of biosurfactant producing bacteria according to Lakshmipathy, *et al* (2010)[15].

Polymerase Chain Reaction:

Specific primers were used to amplify oxygenase genes. PHE primer pair used for phenol monooxygenase while TOL primer pair used for xylene monooxygenase. The primer sequence and products size mentioned in the table (1) and the PCR condition mentioned in the table (2).

Table (1): Primer pair sequence and Amplicon size.

Primer	sequence 5´-3´	Amplicon size (bp)	References
PHE-F PHE-R	GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC CGCCAGAACCA(C/T)TT(A/G)TC	206	[8]
TOL-F TOL-F	TGAGGCTGAAACTTTACGTAGA CTCACCTGGAGTTGCGTAC	475	

Table (2): PCR conditions

Step	Temperature (°C)	Time (minutes)	No. of Cycle
Initial denaturation	95	10	1
Denaturation	95	1	30
Annealing	49 (for PHE), 55 (for TOL)	1	
Extension	72	2	
Final extension	72	10	1
Hold	4	-	-

Results and Discussion:

Ten (22.22%) crude oil-degrading bacterial isolates were recovered from 45 oil-contaminated soil collected from the fuel stations (oil contaminated site). Many of the bacterial isolates that habit oil-contaminated soil have the capability to mineralize crude oil hydrocarbons in oil contaminated sites[16]. The figure (1) show the crude oil-degrading isolates grown on Bushnell and Hass mineral salts (BHMS) medium supplemented with 5% crude oil as a sole source for carbon. Confirmation of degradation achieved by cultivation of degrading isolates on modified crude oil agar[10]. Pure colonies were identified based on morphological cultural characteristics and biochemical tests and growing on *Pseudomonas* chromogenic agar to confirmed that a isolates related *Pseudomonas aeruginosa* one isolate identified as gram positive bacilli, spore forming related to genus *Bacillus* sp[17]. Finally identification confirmed by Viteck2 compact system using GP and GN card.

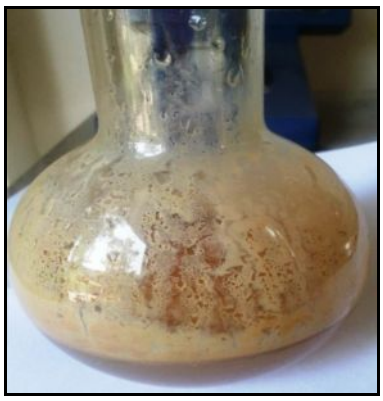

Figure (1): Bacterial growth on Bushnell and Hass mineral salts (BHMS) medium supplemented with 5% crude oil

Initially, all isolates were screened for its ability to produce biosurfactant using Emulsification Index E24, Hemolytic activity, Lipolytic activity and Oil displacement assays as shown in table (3). All isolates were showed a significant zone of clearance around the colony, confirming the production of surface active molecules as shown in figure (2). Lysis of blood agar has been recommended as a method to screen for biosurfactant activity. This method is useful in predicting the promising strains regarding biosurfactant production all *Pseudamonas aeraginosa* and *Bacillus* sp. showed a complete hydrolysis of blood so it is beta hemolytic organism this is based on surfactant interact strongly with cellular membranes and proteins, exotoxins called hemolysis cause lysis of the red blood cell[18] had recommended this method as a preliminary screening method, in addition, the hemolytic assay was a simple, fast and low- cost method for the screening of biosurfactant producers on solid medium. Many researchers have used this technique to screen for biosurfactant production[19].

Table (3): Primary screening assays for biosurfactant production.

Emulsification Index E24	Oil displacement assay	Lipolytic activity	Hemolytic activity	Isolate #
46.66	++++	+	B-hemolysis	B1
42.85	-	+	B-hemolysis	Ps.1
46.66	-	+	B-hemolysis	Ps.2
55	++	+	B-hemolysis	Ps.3
55	+	+	B-hemolysis	Ps.4
44.82	++	+	B-hemolysis	Ps.5
48.27	-	+	B-hemolysis	Ps.6
53.33	+++	+	B-hemolysis	Ps.7
51.42	+	+	B-hemolysis	Ps.8
55.26	+	+	B-hemolysis	Ps.9

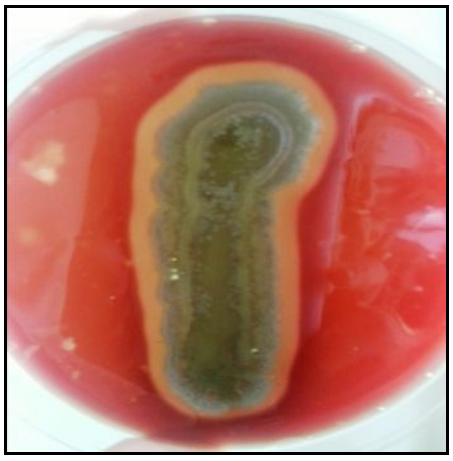

Figure (2): Bacterial grown on blood agar

Pseudomonas aeruginosa and *Bacillus* sp. Isolates was investigated its lipolytic activity data represented in figure (3). The results showed that all bacterial isolates produce lipase (table 3). In fact, the lipase producing microorganisms have the ability to degrade fat and oil pollutant sources this results were recommended by Sidkey *et al*. (2014)[18]

Oil displacement assay also used to confirm the biosurfactant production. In this assay, the supernatant of the bacterial isolates was added to plates containing water and crude oil. Most of isolates displaced the oil showing a zone of displacement expect *Ps*.1 one *Ps*.2 and *Ps*. 7 and *Ps*. 5 as shown in figure (4). The isolates showed the highest displacement zone (table 3). In this study was similar to tambekar or and Gadakh (2013)[20]. The oil spreading method was reported as an indicative and wetting activities[21]. The advantage of oil spreading is very easy and less sample volume reach to 30 ML is required to check oil spreading method[22]. From earlier reports and results of this paper oil spreading assay is recommended as a reliable rapid simple and consistent analytical method for accurate measurement for biosurfactant production. According to Sriram *et al*., (2011)[23], the amount of biosurfactant is necessary to obtain a clear detection zone over an oil layer is called as the minimum dose (MAD) of the corresponding biosurfactant.

All bacterial isolates have the ability of emulsifying (E_{24}) crude oil (table 3) and showed that stabilization of oil and water emulsion was commonly used as a surface activity indicator as showed in figure (5). They isolated have an emulsification index ranged from 52-6 to42.85. This is in accordance to femi-Ola *et al*.(2015)[24]

Figure (3): Bacterial lipolytic activity assay.

Figure (4): Bacterial oil displacement assay.

Figure (5): Bacterial emulsification assay.

Many of researchers confirmed that *Pseudomonas aeruginosa* and *Bacillus* sp. As a potential producers of surface active agent and play role in the field of bioremediation and enhanced oil recovery. Femi-Ola *et al*. (2015)[24] Reported 22 bacterial biosurfactant producer isolates were recovered from 8 different sit from waste water oil-contaminated soil. The dominant species were *Bacillus* and *Pseudomonas* considered bacterial isolate (VSHUB005 as potential biosurfactant producer. This isolate was identified as *Pseudomonas aeruginosa*.

Affandi *et al*. (2014)[25] isolated 3 local bacterial isolates from wastewater of food processing, electrical and electronic and oil palm (POME) industries one of them *Bacillus cereus* in POME with the highest Biosurfactant value.

A tabatabaee et al. (2005)[26] isolated 45 bacterial isolates from oil wells one of the strains Bacillus sp. No.4 was showed the successful producer of biosurfactant production and is potential candidate for microbial enhanced oil recovery confirmed that Pseudomonas were the most of the strains that considered as the potential source for biosurfactant.

Polymerase Chain Reaction result revealed that all isolates were negative for xylene monooxygenase and only two isolates (ps2 and ps9) were positive for phenol monooxygenase figure (6). Phenol monooxygenase is very important for degradation of phenolic compounds. Many of phenolic compounds cause environmental pollution and hazard for human health[27].

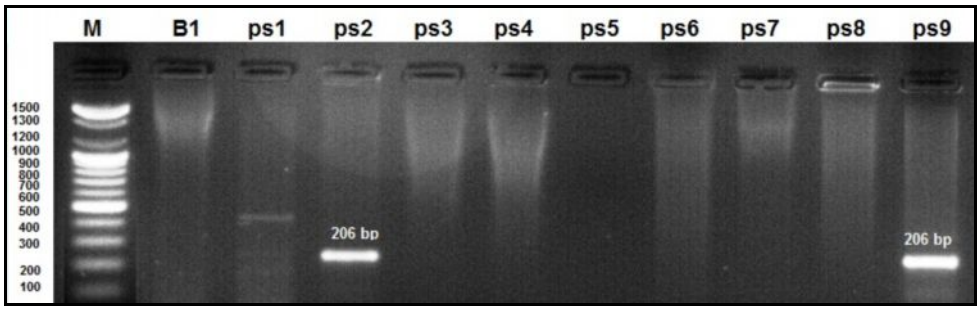

Figure (6):Agarose gel 2% for phenol monooxygenase gene amplicon. The product size 206 bp lane M (Ladder), Lane B1 represent *Bacillus* spp. while lane ps1-ps9 represent *Pseudomonas aeruginosa.*

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