Isolation, screening and characterization of crude oil degrading bacteria isolated from Al- Dora refinery wastewater treatment plant

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Abstract:

Fifty two wastewater samples from wastewater treatment plant of Al-Dora refinery were collected at period from February 2012 to January 2013. 154 bacterial isolates were isolated from wastewater samples using liquid and solid BH medium with 1% crude oil as a carbon source. Primary screen were done using solid BH- medium with 1% crude oil, the bacterial isolates were inoculated and incubated at 30˚C for five days. Results shown that 45 isolates were appeared the highest growth ability. Secondary screen were done for bacterial isolates selected from primary screen using clear zone and redox indicator (DCPIP) techniques . Results shown that B.T.23, B.T.27 and A.T.90 isolates have the best ability for crude oil degradation .The three isolates were characterized based on morphological , cultural and biochemical tests , subsequently was identified and designated as *Pseudomonas aeruginosa* , *Bacillus cereus*and *Bacillus subtilis* respectively.

Key words: Biodegradation, crude oil, Al-Dora refinery, clear zone, 2,6- (DCPIP).

Introduction:

Waste water released from the refineries are characterized by the presence of large quantity of crude oil products, polycyclic and aromatic hydrocarbon, phenols, metal derivatives, surface active substances ,sulfides, naphthalene acids and other chemicals (1).

As a result of ineffectiveness of purification systems, waste water may become seriously dangerous, leading to the accumulation of toxic products in the receiving waster bodies with potentially serious consequences on the ecosystem(2, 3).

There are so many bacterial strains that can degrade or transform the components of crude oil products to the non-toxic, non- hazardous, biodegradable and environmentally friendly compounds such as CO_2,H_2O and biomass This action is known as a biodegradation.(4).

Rate of this action is critically depending on different factors include microbial composition, contaminant type, geology of polluted site and chemical conditions at the contaminated site(5).

The principle of enrichment culture is to provide growth conditions that are very favorable for the organisms of interest and as unfavorable as possible for competing organisms. Hence, the microbes of interest are selected and enriched (6).

In this study bacteria which degrade petroleum hydrocarbons (crude oil) were isolated from wastewater treatment plant and screened for their hydrocarbon degradation efficiency which was carried out using growth ability , formation of clear zone (7) and DCPIP method (10 , 8) ,they were further characterized by morphological, cultural and biochemical techniques.

Materials and Methods:

Samples collection:

Wastewater samples were taken from wastewater treatment plants(physical chemical treatment unit, biological treatment unit and final discharge unit) of AL-Dora refinery for biological studies, one sample per season with two replicates for each sample for a year from February 2012 to January 2013.

Wastewater samples were collected from surface water of each unit (30-40 cm depth) except the final discharge unit samples which were taken from site of subtraction of water using conical flasks. All the samples were then carefully transferred to laboratory and stored at 4˚Cbefore analysis.

Enrichment and isolation:

Selective enrichment technique was used for isolation of hydrocarbon degrading bacteria from wastewater samples. Bushnell-Hass broth was used in this technique.

About 25 ml of Bushnell- Hass medium which compose of (g/l):$MgSO_4$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1, $(NH_4)_3PO_4$ 1 , KNO_3 1 and Fe CL_3 0.05. Was dispensed in 100 ml Erlenmeyer flasks ,pH adjusted at 7.0 and supplemented with 1% crude oil as a substrate .Flasks were sterilized by autoclave at 121˚C ,15 psi for 15 min. After sterilization 1 ml from each wastewater samples was inoculated to 25 ml Erlenmeyer flask (triplicate for each waste water sample) with control (flasks were inoculated with 1ml of sterile distill water) (11,25) .

All flasks were incubated in shaker incubator at 30 ˚C for 7 days at 150 rpm. After a week of incubation 0.1 ml from each flasks (test and control) were subjected to appropriate dilution using 0.1% sterilized peptone broth (pH 7.0) from (10^{-2} - 10^{-5}) , 0.1 ml from each dilutions were spread on L-agar composed of (g / l): tryptone 15 , yeast extract 5.0 , NaCl 5 , glucose 1.0 and 2% (v/v) agar - agar, pH adjusted at 7.0**.**All plates were incubated with three replicate for each wastewater samples as well as control plates at 30 ˚C for 48-72 hour(9 ,12).

After incubation colonies with different morphologies (in shapes , size and color) were purified by streaking method on L- agar plates, this method was repeated until pure isolated colonies were obtained and stored on slants at 4 ˚C for further experiment(13, 14).

Screening of bacterial isolates:

1-primary screening

Solid BH- medium supplemented with 1% of crude oil was used to express bacterial crude oil degrading ability .The medium were inoculated with loopfull from each bacterial

isolates in the middle of the agar plate, all plates were incubated at 30˚C for5 days. The growth ability of bacterial isolates and the diameter of bacterial colonies was graded as strong (+++), moderate (++) and weak (+), and the most active bacterial isolates were selected for further experiment by secondary screening(11).

2-Secodery screening

It was assessed by two methods:

1- Formation of clear zone

An ethereal solution of crude oil (10% v/v) was uniformly sprayed over the surface of BH-medium agar plates, the ether immediately vaporized and a thin layer of oil remained on the entire surface.

Pure bacterial isolates obtained were cultured by spreading technique over a 1 cm area on the middle of BHM agar plates (a triplicate for each bacterial isolate),then the plates were incubated at 30˚C for (24 - 144) hours. The bacterial isolates that formed clear zone around colonies were considered as crude oil degraders(15). The most active bacterial isolates were selected for further screening of biodegradation rates by colorimetric method.

2- Using 2,6-DCPIP indicator in liquid culture

8, 10 used DCPIP as redox indicator ,in this method consisted of addition to the medium an electron acceptor dye such as(2,6-DCPIP) to test the ability of bacterial isolates to utilize the substrate by observing the color change of the indicator from blue (oxidized) to colorless (reduced).

Inoculums' of bacterial isolates were prepared by transferring a loop full from stored L-agar slant medium to a test tube containing 10 ml of sterile BH-medium broth .Tubes were incubated at 30˚C for 24 hours at 150 rpm ,after incubation 0.5 ml from each bacterial cultures were inoculated into test tubes containing 10 ml of BH-medium ,all tubes were supplemented with 1% (v/v) of crude oil and (2mg/ ml) of 2,6-DCPIP indicator. (17 , 16).

BH-medium broth contains 1% of crude oil and (2mg/ml) of 2,6-DCPIP indicator without inoculum was used as control ,the tubes were incubated at 30˚C for (24-144) hours at 150 rpm.

Identification of selected isolates:

Each isolate was examined for its cell morphology, arrangement and Gram reaction as described in Bergey's Manual of Determinative Bacteriology (14). Biochemical properties tested include, production of catalase, indole, oxidase , methyl red test, VogesProskauer test , starch utilization test , gelatin utilization test, nitrate reduction test, citrate utilization , sugars fermentation tests ,pyoceyanin pigment production and motility test (11, 14).

Results and Discussion:

Isolation of crude oil degrading bacteria:

In this study wastewater samples from Al-Dora refineries considered important source for native bacterial capability of mineralize crude oil hydrocarbons in oil contaminated sites was confirmed before by many scientists (5, 18). 154 bacterial isolates isolated from 52 wastewater samples from (physical chemical treatment unit , biological treatment unit and final discharge unit) of AL-Dora refinery wastewater plant .Results in table (1) shown variation in isolates number obtained depending on the place and the period of sample collection, results shown that the highest number of isolates were from biological treatment tank, 89 isolates.

(19) isolated113 crude oil degrading isolates from different crude oil contaminated environment. While (20) isolated 20 bacterial isolates capable of degrading crude oil. According to the above result, there is a compatibility about the wastewater is a good source of bacterial isolates capable of utilized crude oil.

Table (1): Bacterial isolates isolated from wastewater samples obtained from AL-Dora refinery, using solid BH-medium with 1% of crude oil as a substrate. Plates were incubated for 3 days at 30˚C.

Sequence	Type of samples collected and sites	Samples number	Samples collecting date	Number of bacterial isolates that we obtained
1	Water - Physico-chemical treatment tank	Five samples	15 /4/2012	5
2	Water - Biological treatment tank	Three samples	15/4/2012	32
3	Water - Final discharge tank	Five samples	15/4/2012	18
4	Water - Physico-chemical treatment tank	Five samples	29/4/2012	3
5	Water- Biological treatment tank	Three samples	29/4/2012	18
6	Water - Final discharge tank	Five samples	29/4/2012	13
7	Water - Physico-chemical treatment tank	Five samples	5/6/2012	4
8	Water - Biological treatment tank	Three samples	5/6/2012	25
9	Water - Final discharge tank	Five samples	5/6/2012	10
10	Water - Physico-chemical treatment tank	Five samples	26/11/2012	3
11	Water - Biological treatment tank	Three samples	26/11/2012	14
12	Water - Final discharge tank	Five samples	26/11/2012	9

* Total sum of bacterial isolates that we obtained = 154 isolate.

Where:

- Number of isolate from physico-chemical treatment tank = 15 isolate.

- Number of isolate from biological treatment tank = 89 isolate.

- Number of isolate from final discharge tank = 50 isolate.

Primary screening

45 isolates from154 hydrocarbon degraders were found to maintain its crude oil degrading ability, which was established by the isolates growing on BH-medium with 1% of crude oil. The efficiency to degrade was recorded depending on the diameter of bacterial colonies developing. (19). Results in table (9, 2) shown that most of those isolates shows strong growth ability with a diameter from 1.0 - 2.0 cm.

(21) mention that the ability of bacterial isolates to utilize crude oil was estimated by growth and that results ranging from strong to weak growth.

Table (2): Primary screening of crude oil degrading bacteria using solid BH-medium supplemented with 1% crude oil incubated at30 ˚C for 5 days.

Sequence	Number of bacterial isolates	Growth Ability of bacterial isolates		
		Strong	Moderate	Weak
1	45	+++		
2	77		++	
3	32			+

*the ability of bacterial isolates were measure depending on the diameter of bacterial colonies developing as the following:

- Strong = +++ were the diameter of developing colonies 1.0 - 2.0 cm .

- Moderate = ++ were the diameter of developing colonies 0.5 - 1.0 cm .

- Weak = + were the diameter of developing colonies 0.0 - 0.5cm .

*depending on diameter of developing bacterial colonies we select 45 bacterial isolates that characterized by their strong development on solid BH-medium.

Secondary screening:

1- Formation of clear zone

The efficiency to degrade which was recorded by the zone of clearance formed by the degraders on BH-medium plates (19). Results in table (3) shown that 15 from 45 bacterial isolates have the maximum degradation amount from(2.0 - 3.5 cm) as shown in figure(1) were estimated.

(22) mentioned that clearing of crude oil in the medium showed the bacterial growth and indicate the degradation which may be due to production of emulsifiers , surfactant.

Table(3) : Secondary screening for the most active 45 bacterial isolates from primary screening by formation of clear zone around the colonies on solid BH-medium sprayed with an ethereal solution of crude oil(10%) over the surface of the agar plate, then incubation at 30 ˚C for 6 days.

Degradation amount	Clear zone size	Number of bacterial isolates
Maximum	2.0 - 3.5 cm	15
Medium	1.5 - 2.0 cm	5
Minimum	1.0 - 1.5 cm	25

*clearing of crude oil in the medium showed the bacterial growth and it indicates the degradation according to clearing zone size.

*among the 45 isolates , 15 formed maximum clearing zone on BH-medium , those 15 isolates were selected for further screening of biodegradation rates by color metric method.

Figure (1): formation of clear zone on solid BH-medium with ethereal solution of crude oil (10% v/v) for 24 - 144 hours.

2- Using 2,6-DCPIP indicator in liquid culture

A rapid screening technique using a redox indicator 2.6- DCPIP used for further screening of bacterial isolates obtained from clear zone experiment (8).

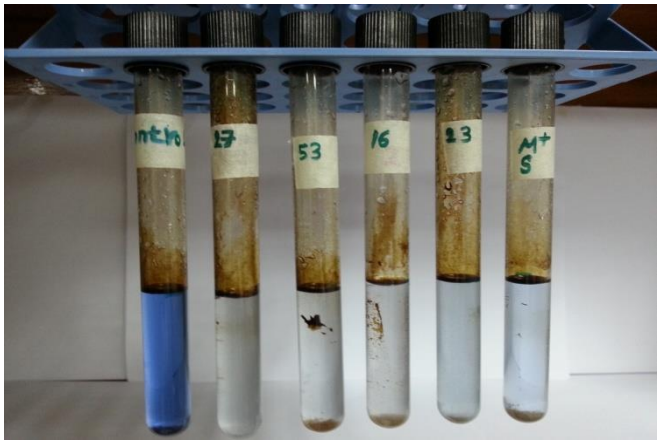
The 15 bacterial isolates were tested, and only three isolatesB.T.23, B.T.27 and A.T.90 were selected because they completely discolored the indicator within less than 24 h . as shown in table (4), while the other 12 isolates discolored the indicator after a period of 48 - 144 hours, which indicate slow response to biological oxidation, as shown in figure (2).

(9) mentioned that color change of DCPIP was observed visually till 144 h. and they found that six isolates decolorize the dye in about 120 h. While (16) mentioned that time taken for decolonization differ according to the biodegradability of bacterial isolates.

Table(4): Secondary screening of the most active 15 bacterial isolates for biodegradability of crude oil using redox indicator2,6- Dichlorophenol Indophenol in liquid BH-medium incubation at 30 ˚C for (24 - 144) hours at 150 rpm .

Seq.	Isolate name	Sampling site	isolates biodegradability of crude oil by using redox indicator (2,6-DCPIP) .					
			Decolourisation time (in hrs)in liquid BHM .					
			24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.
1	B.T.3	Biological treat.tank			+			
2	B.T.4	Biological treat.tank		+				
3	B.T.6	Biological treat.tank						+
4	B.T.16	Biological treat. tank					+	
5	B.T.21	Biological treat.tank		+				
6	B.T.23	Biological treat.tank	+					
7	B.T.27	Biological treat. tank	+					
8	B.T.31	Biological treat.tank						+
9	B.T.32	Biological treat.tank		+				
10	B.T.33	Biological treat.tank		+				
11	B.T.43	Biological treat. tank			+			
12	B.T.45	Biological treat.tank			+			
13	A.T.90	Final discharge tank	+					
14	P.C.145	Physico-chemical treat. tank				+		
15	P.C.147	Physico-chemical treat. tank					+	

* According to the secondary screening of the most active 15 bacterial isolates biodegradability of crude oil by using redox indicator (2,6-DCPIP) in liquid BHM , we select the most active 3 bacterial isolates which have the ability of decolonization of blue DCPIP indicator to colorless in liquid BH-medium .



Figure(2): The ability of decolonization of blue DCPIP indicator to discolor in liquid BH-medium.

Characterization of selected isolates:

Morphological and microscopic observations appeared that the three bacterial isolates were rod shape, one of them was Gram negative, motile and non spore former while others were Gram positive, motile and spore former. Biochemical tests shown in table (5), results suggested these isolates as *Pseudomonas aeruginosa* ,*Bacillus cereus* and *Bacillus subtilis* according to Bergey's Manual of Determinative Bacteriology (14).

Both *Pseudomonas aeruginosa* and *Bacillus subtilis* bacterial isolate were isolated from crude oil contaminated sites and identified as biodegraded bacteria (22). Also *Bacillus cereus* was isolated from petroleum hydrocarbon contaminated soil and water around oil refinery and show the best oil degrader compared to other isolates belonging to *Micrococcus varians* ,*P. aeruginosa* , *Vibrio sp.*and *Alcaligenes sp.*(24). *Bacillus* species are more tolerant to high levels of hydrocarbons due to their resistant end spores (23) .

Table (5): Morphological and biochemical characteristics of bacterial isolates.

Biochemical test name	Code number of bacterial isolates		
	B.T.23	**B.T.27**	**A.T.90**
Shape and arrangement of the cells	Rods in chains	Rods in chains	Swarming
Gram reaction	+	+	-
Spore staining and location	Sub terminal	Central	-
Motility test	+	+	+
Gelatin hydrolysis	+	+	+
Starch hydrolysis	+	+	-
Catalase test	+	+	-
Oxidase test	-	+	+
Indol test	-	-	-
Nitrate reduction	+	+	+
Citrate utilization	+	+	+
Voges – Proskauer test	+	+	+
Methyl red	-	+	-
Glucose fermentation	+	+	+
Lactose	-	-	-
Maltose	+	-	+
Manitol	-	+	-
Arabinose	-	+	+
Pyoceyan pigment production	-	-	+

+ positive reactions , - negative reactions

Conclusions:

According to results from this research, the following conclusions have been reached:

1- One hundred and forty five bacterial isolates were isolated from fifty two Al-Dora refinery wastewater samples, which collected at different period. All bacterial isolates showed ability to degrade crude oil.

2-This study has demonstrated a very good biodegradation capability of crude oil hydrocarbons as a source of carbon when use B.T.23, B.T.27 and A.T.90 isolates, which subsequently was identified and designated as *Pseudomonas aeruginosa* ,*Bacillus cereus* and *Bacillus subtilis* respectively.

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