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Investigation of Oil-Degrading Bacterial Growth in Salt Solution and Screening of Its Bio-Emulsifiers Production Ability

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Abstract
Background: Oil-degrading bacteria could be used for remediation of oil pollution as microbial degradation has a crucial role in oil removal, as well as evaporation and weathering procedure.
Objectives: This study aims to identify some oil-degrading bacterial strains and investigate their growth in salt solution with various concentrations and their ability to produce bio-emulsifiers.
Methodology:16 samples which were collected from sea water of different 4 locations in Egypt at, nearly, 1 meter depth. After bacterial growth and cultivation, the Bushnell Hass medium was made and deposited 10 mL in glass bottles 1 percent, 5 percent, 10 percent, 25 percent, and 50 percent, respectively of crude oil concentrations. 10 mL of basic medium was placed in glass bottles, along with various amounts of saline: 1.2 percent, 2.4 percent, 3.6 percent, and 5percent.For determining the ability of production bio-emulsifiers, bacterial filtration was added to 2 mL of various hydrocarbon compounds in sterile glass for each isolation type (toluene, hexane), and mixed for two minutes at room temperature and left to settle for 24 hours.The optimum emulsification effect was then determined using an equation. Identification of the selected isolates was done by 16S rRNA.
Results: Isolated bacteria had the ability for growing in a crude oil-containing medium with various concentrations up to [50%]. Excellent growth in all samples was detected in site 4. Excellent growth was observed in [34%] of the isolates at a concentration of [1%] and 5% crude oil. Excellent growth was possessed by 87.5% of the isolated bacteriaat the [1.2%] salt concentration, 75% at the [2.4%] concentration, and 75% of the isolated bacteria at the [3.6%] concentration. Isolated bacteria produced bio-emulsifiers on Toulene and Hexane. The identified bacterial isolates were*Acinetobactergrimontii, Aeribacilluspallidus, and Bacillus cereus*.
Conclusion: The concentration of crude oil concentration as well as salinity has a crucial role in the oil biodegradation.
Key words: Biodegradation bacteria, Crude oil, Salinity Concentrations, Bio-emulsifiers production.

1. Background

Petroleum oil is a vital strategic supply for which all nations struggle ferociously.Actually, the petrochemical sector develops as a result of human activity that dependent on oil to supply the energy demands. Nevertheless, the usage of oil has a negative impact on the ecosystem**(Xu et al., 2018)**.Oil pollution is one of the most common environmental dangers thatpossess harmful impacts

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on all aquatic living creatures**(Karlapudi et al., 2018)**.Blowouts during oilfield progress, leakage accidents of oil tankers, leakage from storage tanks or oil pipelines, and repairs of petrochemical manufacture equipment are altogether common reasonsfor spills and discharges of petroleum hydrocarbons through petroleum manufacture**(Wang et al., 2018)**.

Despite the difficulty of treating oil pollution, microorganisms that degrade petroleum hydrocarbons have arisen due tobeing living nearly to the petroleum hydrocarbons that present naturally within the environment. These microorganisms could be used forremediation of oil pollutionas microbial degradation has a crucial role in oil removal, as well as evaporation and weathering procedure**(Varjani, 2017)**,where the microorganisms (bacteria) convert pollutants (hydrocarbons) into energy, cell mass, and biological waste products. These bacteria involve*Staphylococcus*, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Rhodococcus*, *Serratia*and*Acinetobacter* in addition to some colonies similar to Actinomycetes**(Abdullaet al., 2019)**.

The marine environment, which includes oceans and oil reservoirs, has the characteristic great salinity that reaches over 150 g/l NaCl,and a relatively low temperature of 1 to 10 °C**(Raddadi et al., 2017)**. Microorganisms from marine habitats are a great source of new biosurfactants that are likely to work within marine environments with high temperature and limitedactivity of water **(Jackson et al., 2015)**.

As water and oil are incompatible, an emulsion is created when oil and water are mixed together. Oil droplets separate from water when an emulsion sits motionless for a long time. Emulsifiers are utilised to hinder this process. Emulsifiers, in fact, are needed to keep the emulsion from separating**(Johnson &Affam, 2019)**.

Because they are complex mixes of lipopolysaccharides, heteropolysaccharides, proteins, and lipoproteins, bioemulsifiers have a larger molecular weight than biosurfactants.Emulsifiers contain both lipophilic and hydrophilic characteristics. Emulsions, however, mayexist aswater-in-oil (W/O) or oil-in-water (O/W). Small droplets of oil form dispersed phase and are distinct in water in oil emulsions, whereas they are disseminated as small droplets of water within oil-in-water emulsions**(Alizadeh-Sani et al., 2018)**.

Bioemulsifiers, unlike synthetic surfactants, are environmentally friendly, less toxic, and biocompatible, have a better biodegradability, and are active at severe salinity, temperatures, and pH. Furthermore, bioemulsifiers can be made from inexpensive, renewable substrates**(Adetunji&Olaniran, 2019)**.Bioemulsifiers can be made by a broad range of microbes involving bacteria, fungi and yeasts, by utilizing various substrates, for example; hydrocarbons, carbohydrates, and glycerol for cellular growing**(Floris et al., 2018)**.

Bioemulsifiers are recognized to be the greatest used in bioremediation, cleaning up of oil-contaminated pipes, improved oil recovery, because of their various functional characters like emulsification, phase separation, cleansing, surface action and decrease in hydrocarbon viscosity**(Gudiña et al., 2015)**.

This study aims to identify some oil-degrading bacterial strains and investigate their growth in salt solution with various concentrations and their ability to produce bio-emulsifiers.

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2. Materials and methods

2.1. Sample collection:

In the present study, the crude oil from 4sites in Egypt; East of Suez, west of Alexandria, North of Port Said and North Damietta were collected. The study included 16 samples which were collected from sea water of different 4 locations in Egypt at, nearly; 1 meter depth within sterile dark bottles of 100 mL.Sample labeling was as the following:Site 1: (S1 A to S1 D), Site 2: (S2 A to S2 D), Site 3: (S3 A to S3 D), Site 4: (S4 A to S4 D).

2.2. Isolation and cultivation of bacteria:

Basic media was made, and 9 mL of it was put in glass bottles with a capacity of 25 mL. 0.1 % (v/v) crude oil was injected to the center. The bottles with the complex were then sterilisedfor 15 minutes at 121°C **(Kozai et al., 1988)**. Then, 1 mL of collected samples from sea water was added to the previously prepared glass bottles in the basic media and crude oil. For 7 days, crude oil was kept at 30-32 °C within incubator to limit bacterial development. After that, for the bacterial growth and cultivation, 0.1 mL was collected from the bottles and dispersed into Bushnell Hass media, to which this medium was added, in addition to[1% (v/v)] crude oil as the only supply of carbon and NaCl(3.5 %) percent. The plates were placed in the incubator for 18 - 72 hours at 30 - 32 °C **(Latha&Kalaivani, 2012)**.

The bacteria grown at the center were subsequently cultivated on nutrient agar. On nutritive agar medium, there was no growth. NaClwas added to the 3.5 % nutrition medium, and then incubated for 18-24 hours at 30 °C inside incubator to produce pure bacterial growth.Another subculture was performed for purifying the bacterial strains.

2.3. Isolated bacteria's capacity to grow in various crude oil concentrations:

The selected bacteria were initially cultivated on nutrient agar and put in the incubator for 18-24 hours at 32 °C. The Bushnell Hass medium was made and deposited 10 mL in glass bottles (1 %), (5 %), (10 %), (25 %), and (50 %), respectively. The prepared media were sterilised and incubated for seven days at 32 °C, with the growth checked by naked eye for turbidity of the media.

2.4. Selection of the best isolates have the ability to deconstruct various concentrations of crude oil:

The best 8 bacterial isolates were chosen and used for further identification.

2.5. Effect of different concentrations of salinity on selected bacterial isolates:

10 mL of basic medium was placed in glass bottles, along with various amounts of saline: [1.2 %], [2.4 %], [3.6 %], and [5%], and they were incubated for 18 to 24 hours at 30 °C. After that, the varied concentrations of the produced media were sterilized for 15 minutes at 121°C.

2.6. Evaluation of local isolates' potential to produce bio-emulsifiers

After bacterial filtration, 2 mL of bacterial filtration was put in to 2 mL of various hydrocarbon compounds in sterile glass for each isolation type (toluene, hexane). At room temperature, the contents were mixed for two minutes in a glass using a magnetic stirrer, and then the glass was left to settle for 24 hours**.**

A negative control containing hydrocarbon compounds was utilized without bacterial filtration, while a positive control comprising 4 m distilled water and Tween 80 was used. The optimum emulsification effect was then determined using the equation below to acquire the largest amount of bio-emulsifiers:

$$E24 = \frac{Height\ of\ the\ formed\ emulsion\ \times 100}{Total\ height\ of\ the\ solution}$$

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2.7. Identification of selected bacterial isolates

Identification of the selected isolates was done by 16S rRNA.

3. Results:

This study included 16 samples were collected from sea water of different 4 locations in Egypt; East of Suez, west of Alexandria, North of Port Said and North Damietta.

3.1. Isolated bacteria's capacity to grow in various crude oil concentrations

As shown in Table (1), the isolated bacteria had the ability for growing in a crude oil-containing medium. Excellent growth in all samples was detected in site 4. One excellent growth samplewas detected in site 2. Also, one excellent growth sample was detectedin site 3, while in site 1, good growth was observed in all samples.

Table (1):Bacterial growth of samples collected on basic media with a crude oil concentration of 0.1 percent (v/v).

Sample	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
S1A	-	+	+	+	+	++	++
S1B	+	+	+	+	+	++	++
S1C	-	+	+	+	+	++	++
S1D	+	+	+	+	+	++	++
S2A	+	+	+	+	++	+++	+++
S2B	+	+	+	+	+	++	++
S2C	+	+	+	+	+	+	+
S2D	+	+	+	+	+	++	++
S3A	+	+	+	+	++	+++	+++
S3B	+	+	+	+	+	++	++
S3C	-	+	+	+	+	+	+
S3D	+	+	+	+	+	++	++
S4A	+	+	+	+	+	+++	+++
S4B	+	+	+	+	+	++	+++
S4C	+	+	+	+	+	++	+++
S4D	+	+	+	+	+	+++	+++
Controlpositive	-	-	-	-	-	-	-
Control negative	-	-	-	-	-	-	-

3.2. Bacterial growth on Bushnell Hass media with [1 % (v/v) crude oil] + [NaCl 3.5 %]:

Table (2) revealed that excellent growth was possessed by 11 isolates; good growth was observed in 2 isolates and they were in site 3, and weak growth was observed in 3 isolates and they were in site 4. All bacterial isolates from all sites were grown on Bushnell Hass medium with 1% crude oil, and dense growth was seen in the majority of the sites.

Table (2): Bacterial growth on Bushnell Hass media with [1 percent (v/v) crude oil] + [NaCl 3.5 %]:

Sample	24 hr	48 hr	72 hr
S1A	+	++	+++
S1B	+	++	+++

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S1C	+	++	+++
S1D	+	++	+++
S2A	+	++	+++
S2B	+	++	+++
S2C	+	++	+++
S2D	+	++	+++
S3A	+	+	+++
S4B	+	+	+++
S3C	+	+	++
S3D	+	+	++
S4A	+	++	+++
S4B	-	+	+
S4C	-	+	+
S4D	-	+	+

3.3. Bacterial isolates growth on Bushnell Hass media after purification on nutrient agar:

After purification on the nutrition agar, intense growth of bacterial isolates was observed within all sites excepting two samples in site 2. Besides, 24 isolates were found.

3.4. Isolated bacteria's capacity to grow in various crude oil concentrations

The 24 isolates were investigated for their capacity to grow in various crude oil concentrations, [1 %], [5%], [10%], [25%], and [50%].

As revealed in Table (3), excellent growth was noticed in approximately [34%] of the isolatesat a concentration of [1%]as well as [5%] crude oil (S1Cb, S1Da, S2Aa, S2Ab, S2Ca, S3C, S3D, S4Aa).On the [10%] concentration, approximately [29%] of the isolates possessed excellent growth (S1Cb, S2Aa, S2Ab, S2Ca, S3C, S3D, S4Aa).On the[25%] concentration of crude oil,approximately [11%]of the isolates possessed excellent growth (S2Aa, S3C, S3D).On a concentration of [50%] crude oil, [11%] of the isolates possessed excellent growth (S2Aa, S3C, S3D), good growth was observed in nearly [7%] of the isolates(S3Ba), and weak growth was observed in roughly [25%] of the isolates at the same concentration (S1Aa,S1Bb, S1Ca, S2Ab, S2Ba, S2Bb, S2Cb, S2Da, S3Aa, S3Ab, S3Bb, S4Ab, S4Ba, S4Bb).

It was observed that the 24 isolated bacteria were modified for growing at various crude oil concentrations and usedonly crude oil for carbon and energy. Then, eight different isolates were chosen for further studying.

Table (3): Isolated bacteria's capacity to grow in various crude oil concentrations; [1 %], [5%], [10%], [25%], and [50%]:

No	Sample	variouscrude oil concentrations				
		1%	5%	10%	25%	50%
1	S1Aa	+	+	+	-	-
2	S1Ab	+	+	+	+	+
3	S1Ba	-	+	+	+	+

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4	S1Bb	+	-	-	-	-
5	S1Ca	+	-	-	-	-
6	S1Cb	+++	+++	+++	++	+
7	S1Da	+++	+++	++	++	+
8	S2Aa	+++	+++	+++	+++	+++
9	S2Ab	+++	+++	+++	-	-
10	S2Ba	+	-	-	-	-
11	S2Bb	+	-	-	-	-
12	S2Ca	+++	+++	+++	++	+
13	S2Cb	+	-	-	-	-
14	S2Da	+	+	+	+	-
15	S3Aa	+	-	-	-	-
16	S3Ab	+	-	-	-	-
17	S3Ba	++	++	+	+	++
18	S3Bb	+	-	-	-	-
19	S3C	+++	+++	+++	+++	+++
20	S3D	+++	+++	+++	+++	+++
21	S4Aa	+++	+++	+++	+	+
22	S4Ab	+	-	-	-	-
23	S4Ba	+	-	-	-	-
24	S4Bb	+	-	-	-	-

3.5. Effect of different concentrations of salinity on selected bacterial isolates:

Table (4): Isolated bacteria's capacity to grow in various salinity concentrations (1.2%, 2.4%, 3.6%, 5%):

N	Sample	Different salt concentrations			
		1.2%	2.4%	3.6%	5%
1	S1Cb	+++	+++	+++	++
2	S1Da	+++	+++	+++	++
3	S2Aa	+++	++	+	+
4	S2Ab	++	++	+	+

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5	S2Ca	+++	+++	+++	++
6	S3Ba	+++	+++	+++	++
7	S3C	+++	+++	+++	++
8	S4Aa	+++	+++	+++	+
9	Control	-	-	-	-

Table (4) revealed that excellent growth was possessed by87.5% of the isolated bacteriaat the [1.2%] concentration of salt (S1cb, S1Da, S2Aa, S2Ca, S3Ba, S3C, S4Aa). Excellent growth was possessed by 75% of the isolated bacteria at the [2.4%] concentration of salt (S1cb, S1Da, S2Ca, S3Ba, S3C, and S4Aa), and about 25% possessed good growth (S2Aa, S2Ab). Excellent growth was possessed by 75% of the isolated bacteria at the [3.6%] concentration of salt (S1cb, S1Da, S2Ca, S3Ba, S3C, and S4Aa), where weak growth was observed by 25% (S2Aa, S2Ab). At the [5%] concentration of salt, around 62.5% of the isolates possessed a good growth, whereas the remaining isolates possessed weak growth.

3.6. The isolates' potential to produce bio-emulsifiers:

As shown in Figure (1); on Toluene, S1Cb possessed greater potential to produce very active bio-emulsifiers (72), while S1Da, S2Aa, S2Ab, S2Ca and S3Ba possessed good potential to produce bio-emulsifiers (55,50, 54,49, respectively). Mediumpotential for bio-emulsifiers' production was observed in S3C and S4Aa (44,43, respectively).

Also, on Hexane, S3C and S3Ba possessed greater potential to produce very active bio-emulsifiers (62, 56, respectively), while S2Ca, S4Aaand S1Cb, possessed good potential to produce bio-emulsifiers (56, 55, 54, respectively). Also, good potential for bio-emulsifiers' production was observed in S1Da, S2Aa and S2Ab (35, 30,43, respectively).

From Figure (1), there were 4 isolates that were bio-emulsifier producer on Toluene (S1Cb, S1Da, S2Aa, S2Ca), whereas there were 4 isolates that were bio-emulsifier producer on Hexane (S1Da, S2Aa S2Ca, S4Aa).

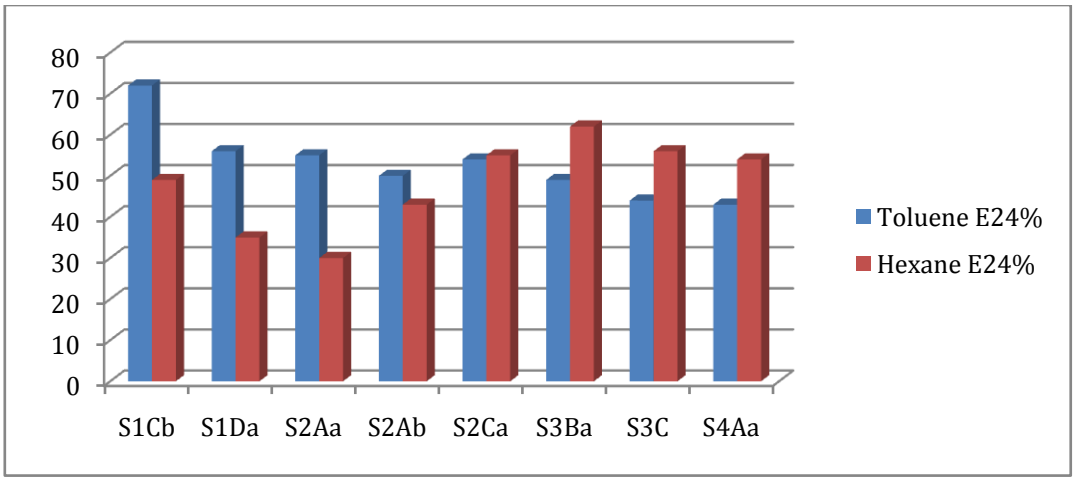

Figure (1): The potential of isolates to produce bio-emulsifiers in Toluene and Hexane.

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3.7. Identification of selected bacterial isolates

The 16R rRNA technique was used to identify some of the bacterial isolates, and the results of the bacterial identification were shown in table (7), Figure (1).

Table (7) Identification of bacterial isolates by 16S rRNA:

No	Bacterial isolates	Identified bacteria
1	**S2Aa**	*Acinetobactergrimontii*
2	**S3C**	*Aeribacilluspallidus*
3	**S3D**	*Bacillus cereus*

4. Discussion

Bacteria have become a promising technology among the recent years to be used in remedyingthe contamination of petroleum hydrocarbon as it has alittle cost and environmentally friendly **(Dvořák et al., 2017)**.A broad range of microorganisms involving bacteria have the ability to produce bioemulsifiers which are known to be the greatest used in bioremediationof petroleum hydrocarbon pollution **(Floris et al., 2018)**.The goal of the current study was to identify some oil-degrading bacterial strains and investigate their growth in salt solution with various concentrations besides their ability to produce bio-emulsifiers.16 samples were collected from sea water of different 4 locations within Egypt; East of Suez, west of Alexandria, North of Port Said and North Damietta.

This study revealed that the isolated bacteria had the ability for growing in a 1% crude oil-containing medium. Excellent bacterial growth was shown inthe majority of the sites in which the samples were collected. This indicates that most of the isolates in this study were able to degrade crude oil in addition to utilize the petroleum composites instead of just tolerate it. Likewise, **Pawliket al. (2017)** reported that forty percent ofthe bacterial isolates possessed the ability for utilizing for hydrocarbon biodegradation. This also was in agreement withseveral studies;**Abdullaet al.(2019), Tremblay et al. (2017),** and**Xue et al. (2015)** where it was revealed that there are several types of bacteria that have the abilityfor degrading petroleum hydrocarbons. The ability of the bacteria for degradation of crude oil could refer to some factors such as formation of emulsifiers and surfactants**(Alizadeh-Sani et al., 2018)**.

After purification of the isolated bacteria on the nutrition agar, 24 isolates were obtained. The study by **Abdullaet al. (2019)** also isolated 27 bacterial isolatesin Baghdad that were able to degrade crude oil. **Adetunji, &Olaniran (2019)** also isolated 20 different bacterial isolates in South Africa.

With regard to the bacterial ability for growing in different concentrations of crude oil, this study revealed that the majority of the isolates [34%] showed excellent growth at a concentration of [1%] as well as [5%] crude oil, whereas; the growth rate diminished with rising the concentration of crude oil to reach to be [11%] in the concentration of [25%] as well as [50%]. These findings agreed with**Jensen et al. (2017)** as it was found that there is a direct association between crude oil concentration and total biomass production as, in terms of biomass production, a lower crude oil concentration was found to be less hazardous. The findings also were in line with**Chen et al. (2017)** where it was foundthat an inverse association was found between crude oil concentration and oil degradation. This could be attributed to the uncomplimentary environmental changes like nutrition or oxygen scarcity that could be occurred due to the increase in concentration of crude oil, or by the

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damaging effects of the volatile hydrocarbons which could be lethal for the bacterial and alters their growth**(Chen et al., 2017)**.

Salinity is considered a criticalissue that has a significant on the biological activity with the marine nature and it canaffect the oil biodegradation via changing the bacterial survival and metabolism **(Pugazhendiet al., 2017)**. The present study revealed that excellent bacterial growth was shown by a wide range of salinity (from 1.2% to 3.6%), and the majority of the bacterial isolates (87.5%) showed anexcellent growth in the salt concentration of [1.2%], while it was lower (around 62.5%) at [5%]salt concentration. Thiswas in agreement with **Adetunji al. (2019)** who reported that the stability of bio-emulsifiers was at a broad range of salinity (1 to 6%). The present results are also supported by **Chen et al. (2017**) who reported that revealed that the firstly, there was high efficacy of oil degradation by free bacterial consortium and next, by elevating salinity, there was a decrease in the degradation.**Zavareh et al. (2016)**,likewise, stated thatthe greatest biodegradation efficacy was proposed to happen at 2% salinity concentration.

On the other hand, the findings were in disagreement with **Xia et al. (2019)** wherethe maximum efficacy of degradation of crude oil was found at 32% salinity concentrations. **Chen et al. (2017**) also reported that degradation efficacieswere at salinity concentrations of 15 percent to 35 percent. This difference could be attributed to the difference in the country at which this studywasperformed; where **Chen**'s study was performed in China, and hence, the salinity concentrations in the marine environment could be different.

With regard to the isolates' ability for producing bio-emulsifiers, the present study revealed that the majority of the bacterial isolates possessed greater potential to produce very active bio-emulsifiers in the presence of Toluene and Hexane to be the only supplement for carbon and energy. This agreed to**Adetunjiet al. (2019)** where isolates were found to be good bio-emulsifiers. The findings also were in line with **Gupte&Sonawdekar (2015)**who reported an oilemulsification index from 3 to 54 percent.

Bacteria that have the ability to produce bioemulsifier have two physiological characteristics: first; their capability of enhancing the complexation of non-polar substrates and make them more soluble, therefore increasing substrate bioavailability. Second;their potential to promote the oil-water interface layer to be deformedby enhancing the attraction between oil-water interfaces and cell surfaces via metabolism**(Xu et al., 2018)**.

From the 24 isolates of this study, eight isolates were investigated for the most effective bacteria for growing in crude oil. Three bacterial isolates (S2Aa, S3C, S3D) were found to have the greatest efficacy for growing in crude oil up to [50%] concentration and have the potential for biodegradation. These isolates were *Acinetobactergrimontii, Aeribacilluspallidus, and Bacillus cereus.***Adetunji et al. (2019)** identified *Acinetobacter spp.* **Abdulla et al. (2019)**identified *Bacillus cereus***,** and**Chen et al. (2017**) identified *Bacillusspp.*

5. Conclusion:

From the findings of the present study, it is concluded that bacterial degradation might be regarded a major component of a petroleum hydrocarbon remediation cleanup technique. The concentration of crude oil concentration as well as salinity has a crucial role in the oil biodegradation.

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