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Enhancement of Crude Oil Biodegradation by *Pseudomonas reinekei* MT1

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ABSTRACT

One of the greatest challenges facing today is endangering living organisms as a result of environmental pollution caused by petroleum crude oil. Therefore it was necessary, to find out a way to remediate these pollutants biologically. An oil degrading bacterial isolate was isolated from oil polluted soils and optimized for oil biodegradation. *Psuedomonas reinekei* MT1 was able to produce biosurfactants which play a major role in the availability of oil for bacterial biodegradation. *Ps. reinekei* MT1 gives maximum oil degradation on 30ºC, pH 7, sodium nitrate as nitrogen source and addition of gasoline and triton X100. Oil degradation reaches 69.62% after 15 days of incubation. Salinity decreased oil degradation, although *Ps. reinekei* MT1 had the ability of growing and degrading oil in salinity reaches 2%, while there was neither bacterial growth nor oil degradation at 3%.

Keywords: Oil biodegradation, Biosurfactant, Salinity.

INTRODUCTION

Oil is used as the main source of energy, which is widely used in industry, transport and many of the daily activities of human life (Turner and Ranegar 2017). As a result of that, leakage of oil is causing harmful pollution in many environments. Oil considered as a major reason for pollution of many natural habitats like soils and aqueous environments causing harmful damage to the environment and human health (Mahjoubi *et al.,* 2018). The main causes of oil pollution in the oceans are the extraction of oil, transportation with ballast water release, pipeline leaks, storage tank rupture, and tanker accidents, and also war-related incidents (Margesin 2000 and Haapkyläl *et al.,* 2007).

Using microorganisms in oil degradation seems to be a promising method in curing oil pollution. There are a large number of current researches on the biochemistry and genetics involved in this activity (Michaud *et al.,* 2004). Fungi and Bacteria involved in the oil biodegradation (Rahman *et al.,* 2003). Crude oil biodegradation could be stimulated with three major categories: 1) addition of nutrients to stimulate indigenous microorganisms, 2) addition of special microorganisms that naturally could biodegrade crude oil and 3) using genetically engineered microorganisms with special properties of oil degradation (Westermeyer *et al.,* 1991). Many factors can affect microbial biodegradation of oil such as temperature, pH and nutrients (Al-Hawash *et al.*, 2018).

Temperature affects the activity of bacteria in biodegradation of crude oil. Also, temperature influences the physical and chemical properties of crude oil and the degradation ability of bacteria (Qin *et al*., 2012). High temperatures increase the hydrocarbon solubility, distribution, bioavailability, and diffusion rates that promote the ability of microbial biodegradation, as well increment of biodegradation rate. On the other hand, high temperature may cause decreases in soluble oxygen, leading to limitation in aerobic biodegradation activity (Mahjoubi *et al.,* 2018). In addition, low temperatures increase the viscosity of the oil and reducing the volatilization of the short-chain alkanes, decreases their solubility in water, which delays the biodegradation (Atlas and Baartha 1972). Besides the decreases of oil degradation in low temperatures may be due to the decreases of the microbial enzymatic activity (Margesin 2000).

Most heterotrophic bacteria favor neutral pH. Previous studies have shown that slightly alkaline conditions were optimum for most oil-degrading bacteria (Dibble & Batha 1979 and Foght & Westlake 1987). Extreme pH may have a passive influence on microbial population and so oil biodegradation (Leahy and Colwell 1990).Salinity affects the microbial activity of oil biodegradation. Qin *et al.,* (2012) suggested that there was a major impact of salinity on biodegradation process, affecting also on microbial growth and diversity. As well salinity has an adverse effect on some key enzymes activity which involved in hydrocarbon degradation. Kerr and Capone (1988) and Ebadi *et al.,* (2017) reported that a positive relationship between oil degradation and salinity in estuarine sediments, while Ward and Brock (1978) reported that a great reduction of oil degradation with increasing of salinity in range between 3.3-28.4%, as a result of the general decline in microbial metabolism.

Biosurfactants increase the contact between the polluted-oil and the microorganisms by reducing oil surface tension (Bento *et al.,* 2005 and Batista *et al.,* 2006). Rhamnolipids are the most useful biosurfactants which improve oil biodegradation (Salihu *et al*., 2009 and Aparna *et al*., 2012).

The research focus on isolating capable and active bacterial strains to degrade or breakdown crude petroleum oils and optimal conditions to improve the efficiency of the isolate.

MATERIALS AND METHODS

Isolation of crude oil degrading bacterial strain:

Oil degrading bacterial isolate was isolated from oil polluted soil on Bushnel Hass (BH) medium supplemented with crude oil as a sole carbon source. Bacterial culture was subcultured and maintained on nutrient agar medium. Isolated strain was identified by Sigma Scientific Services Co., using 16s rRNA gene (Moore *et al*., 1996).

Inoculum preparation

Inoculum was prepared by growing the isolated strain on nutrient broth medium, consists of (g/l) 3.0 beef extract, 0.5 Peptone and 0.2 NaCl. The inoculum was incubated at 30°C for 48 h. with shaking at 150 rpm. Each one ml of the inoculum contains 10^8 bacterial cells.

Microbial ability to grow on crude oil and biosurfactant production

Hundred milliliter of Bushnell Hass (BH) broth medium in 250 ml Erlenmeyer flask was amended with

1% of crude oil. BH medium consists of (g/l) 0.2 $MgSO_4.7H_2O$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 0.05 $FeCl_3$, 1.0 $(NH_4)_2NO_3$, 0.02 $CaCl_2$, final pH adjusted to 7.0 ± 0.2 (Bushnell and Hass, 1941). fermentation medium was inoculated with One ml (1%) of inoculum culture and incubated at 30°C for 7 days to determine the ability of bacterial isolate to grow on crude oil as a sole carbon source. Residual crude oil was extracted (Saxena 1990) and bacterial culture was centrifuged at 10000 rpm for 20 min. to use bacterial culture supernatant to determine the ability of isolated strain for biosufractants produciton.

Drop collapse test

Onto parafilm, 25 µl of culture supernatant was pipetted as a droplet and left for seconds or minutes to flatten and spreading the droplet on the parafilm surface. Methylene blue was added on the droplet and allowed to dry. After dryness, diameter of the dried droplet was measured (Tugrul and Cansunar 2005).

Oil spill zone

A modification was carried out on oil spreading technique by Morikawa *et al.,* (2000). One ml of crude oil was added to 30 ml of distilled water poured in a Petri dish. Twenty µl of culture filtrate was added on the top of crude oil layer. A zone of displacement in the oil was observed and the diameter of oil displacement (cm) was measured.

Emulsification activity

Two ml of cell free supernatant were added to 2 ml of distilled water in a screw capped tube and 1 ml of soybean oil were added. The solutions were mixed in a vigorous vortex for 2 min. The tubes were kept standing for one hour and the aqueous phases were removed carefully and measured spectorphometrically at 540 nm (Satpute *et al.,* 2008).

Emulsification index (E_{24}%)

Two ml of cell free supernatant were added to 2 ml of diesel oil in a screw capped tube and mixed a vigorous vortex at high speed for 2 min. The tubes were kept standing at room temperature for 24 hr. The emulsification index (E_{24}%) was calculated as the ratio of emulsion zone height to the total height of the three phases (Yeh *et al.,* 2005).

Effect of time course on crude oil degradation

Bacterial strain was grown on 100 ml of BH medium supplemented with 1% of crude oil in 250 ml Erlenmeyer flask. Flasks were incubated at 30 ºC for 15, 20 and 25 days to determine oil degradation through time course.

Effect of Temperature on crude oil biodegradation

Bacterial strain was grown on BH medium supplemented with 1% of crude oil at 25, 30 and 35ºC for 5, 10 and 15 days to determine the effect of different temperatures on the crude oil degradation and bacterial growth.

Effect of initial pH on crude oil biodegradation

The effect of pH values on crude oil biodegradation was estimated by growing the oil degrading bacterial strain on BH medium with 1% crude oil. Initial pH was adjusted at 5, 6, 7, 8 and 9. Flasks were incubated at the optimum temperature for 10 and 15 days.

Effect of nitrogen sources on crude oil biodegradation

To study the effect of nitrogen source on oil degradation, ammonium nitrate was replaced with beef, peptone, sodium nitrate, ammonium phosphate and ammonium sulphate. Flasks were incubated at optimum temperature and optimum pH for 10 and 15 days.

Effect of oil derivatives on crude oil biodegradation

Some oil derivatives; Benzene, Diesel, Petroleum Ether, and Acetone were mixed with crude oil to made oil-oil derivative mixture in concentration of 10%. One percent of oil-oil derivative mixture was added to BH medium. Crude oil without oil derivatives was used as control. Flasks were incubated at optimum temperature, optimum pH and best nitrogen source for 10 and 15 days.

Effect of surfactants on crude oil biodegradation

To determine the effect of surfactants on crude oil biodegradation, tween 80 and triton X100 were added in concentration of 5% to make mixtures with crude oil and oil-oil derivative mixture from the previous experiment. One percent of the surfactant-oil mixture oil or surfactant-oil-derivative was added to BH medium. Flasks were incubated at optimum temperature, optimum pH and best nitrogen source for 10 and 15 days.

Effect of salinity on crude oil biodegradation

Sodium chloride concentrations (1 and 2 and 3%) were added to determine the effect of salinity on the growth and oil degradation by the isolated bacterial strain. Flasks were incubated at optimum temperature, optimum pH, best nitrogen source, best oil derivative and best surfactant for 10 and 15 days.

Assay of oil degradation

Oil degradation was assayed gravimetrically by weighing the residual oil after degradation process. For estimation of oil degradation rates by gravimetric analysis, 10 ml of petroleum ether was added to residual oil in flasks and shook. The contents were transferred to a separating funnel and extracted. The separation was performed again using 10 ml of petroleum ether to ensure full oil recovery. The amount of residual oil was measured after extraction of oil from the medium and evaporating it to dryness in an oven and weighed (Saxena 1990). The percentage of oil degradation was calculated as follow:

$$\% \text{ oil degradation} = \frac{\text{amount of crude oil degraded}}{\text{amount of crude oil added in the medium}} \times 100$$

Bacterial growth determination

After oil extraction, the bacterial culture from separation process was obtained and centrifuged at 10000 rpm for 20 min. Bacterial cells were washed twice with distilled water and centrifuged again at 10000 for 20 min. Bacterial cells were collected and dried at 80ºC for 24h and weighted for constant weight.

RESULTS AND DISCUSSION

Identification of isolated bacterial strain

Nucleotide sequences data from Sigma Scientific Services Co. were entered to National Center for Biotechnology Information database (NCBI), website: www.ncbi.nlm.nih.gov/blast, to make close identification to the bacterial isolate. Data proved that the isolate is *Pseudomonas reinekei* MT1. Phylogenetic trees of *Pseudomonas reinekei* MT1 represented in fig. (1).

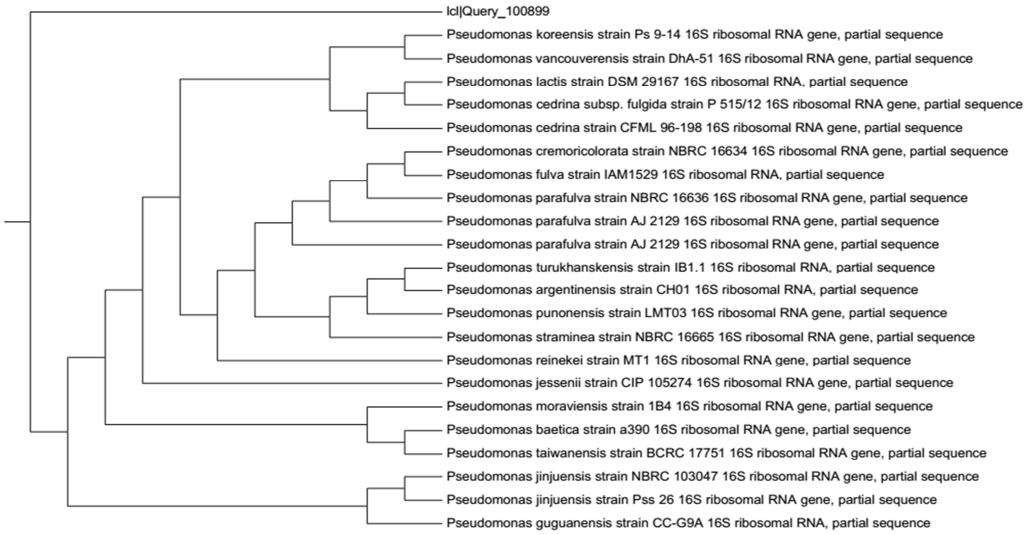


Fig. 1. Phylogenetic trees of isolate *Pseudomonas reinekei* MT1.

The growth of *Pseudomonas reinekei* MT1 in BH broth medium supplemented with crude oil as a sole carbon source after 7 days of incubation as photographed in Fig. (2), which is insured that *Pseudomonas reinekei* MT1 was able to use crude oil as a sole carbon source.

Fig. 2. Growth of *Pseudomonas reinekei* MT1 in BH broth medium with 1% crude oil after 7 days of incubation at 30ºC and pH7.

Production of biosurfactants by *Pseudomonas reinekei* MT1

Data represented in Table (1) and photographs in Fig. (3) show the ability of *Pseudomonas reinekei* MT1 to produce biosurfactants after 7 days of incubation in BH broth medium with 1% of crude oil

Table 1. Drop collapse, Oil spill zone, Emulsification activity and Emulsification index E_{24}% test for *Pseudomonas reinekei* MT1 culture filtrate comparing with tween 80 and triton X100.

Tests Treatment	Drop collapse test (cm)	Oil spill zone (cm)	Emulsification activity	Emulsification index E_{24}%
Pseudomonas reinekei MT1	0.9	4.8	0.466	29.17
Tween 80	1.1	7.5	1.783	73.65
Triton X100	1.3	6.2	1.790	74.35

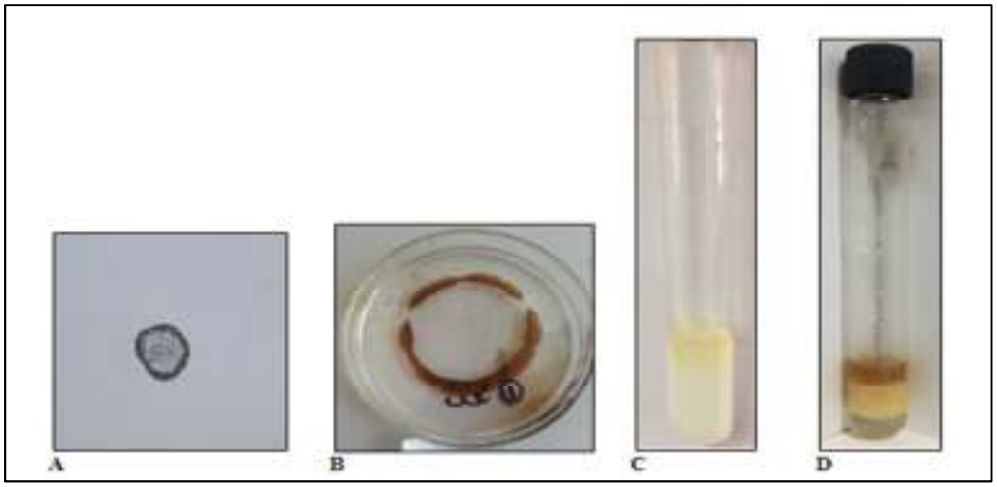


Fig. 3. Photographs showing Drop collapse test (A), Oil spill zone (B), Emulsification activity (C) and Emulsification index E_{24}% (D) by *Pseudomonas reinekei* MT1.

Crude oil degradation through time course

Data in Fig. (4) show that oil degradation by *Pseudomonas reinekei* MT1 grown on BH medium supplemented with crude oil reached 44.18, 48.17 and 48.7 % after 15, 20 and 25 days of incubation. Data show a little difference in oil degradation between 15 and 20 days, and there was almost no difference between 20 and 25 days of incubation.

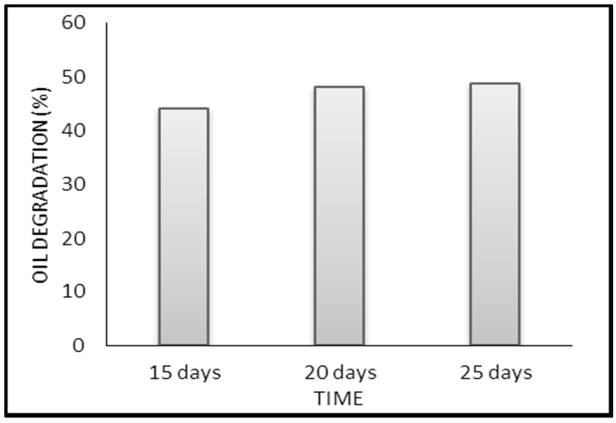


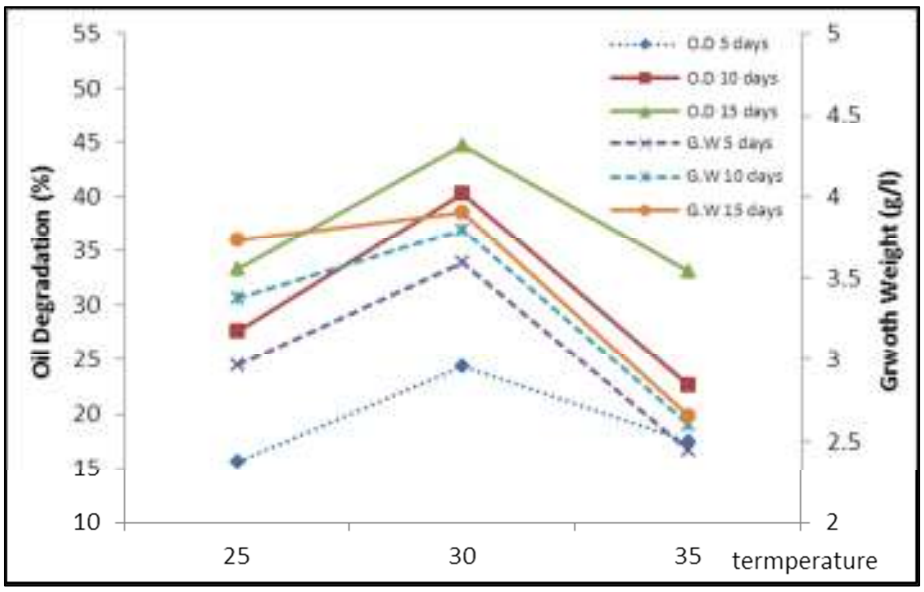
Fig. 4. Effect of incubation time on oil degradation (%) by *Pseudomonas reinekei* MT1 after 5, 10 and 15 days of incubation at pH7.

Effect of temperature on oil biodegradation

Temperature is a specific factor on crude oil biodegradation and limits bacterial activity in biodegradation of oil. High and moderate temperatures influence physical and chemical properties of crude oil, decreasing the bacterial ability for oil degradation. In addition, temperature influences microbial hydrocarbon metabolism and microbial community composition (Atlas 1981 and Qin *et al*., 2012).

Data graphically illustrated in Fig. (5) show the effect of temperature on oil degradation (%) and growth mass (g/l) after 5, 10 and 15 days of incubation by *Pseudomonas reinekei* MT1. Data reveal that *Pseudomonas reinekei* MT1 caused oil degradation at the three tested temperatures. The highest value of degradation (44.65%) obtained at 30ºC, while at 25 and 35, oil degradation decreased. The same trend was observed in microbial growth weight, where there was a relation between oil degradation and growth mass. The highest growth mass achieved at 30ºC, while growth mass decreased at 25 and 30ºC. Similar results were obtained by Sathishkumar *et al.,* (2008), they achieved 69% oil biodegradation by using *Pseudomonas* sp. BPS1-8 at 30ºC.

Also Al-Wasify and Hamed (2014) obtained 77.8% of crude oil degradation by using *Pseudomonas auroginosa* after 28 days at 30ºC. Chen *et al.*, (2017) found that optimum temperature for oil degradation with free and immobilized cells of *Pseudomonas auroginosa* ASW-2 was 30ºC.



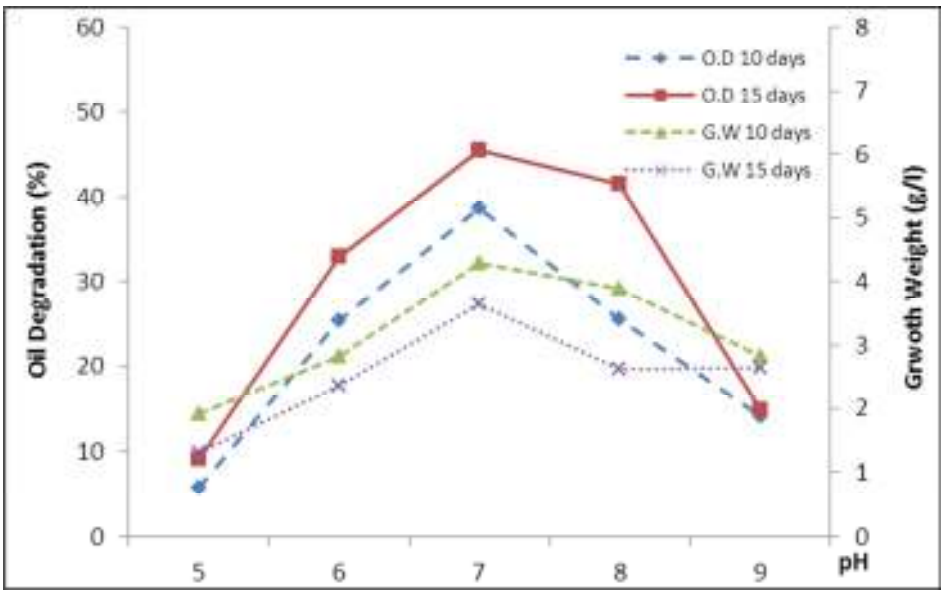
O.D: oil degradation, G.W: growth weight

Fig. 5. Effect of temperature on oil degradation (%) and bacterial growth mass (g/l) by *Pseudomonas reinekei* MT1 after 5, 10 and 15 days of incubation at pH7.

Effect of initial pH on oil biodegradation

Most heterotrophic bacteria favor neutral pH. Certain studies have shown that slightly alkaline conditions were optimum for most degrading oil bacteria (Dibble & Batha 1979 and Foght &Westlake 1987). Extreme pH may have passive influence on microbial population and subsequently oil degradation (Leahy and Colwell 1990).

Data represented in Fig. (6) illustrate that initial pH affects oil degradation and mass growth of *Pseudomonas reinekei* MT1, where *Pseudomonas reinekei* MT1 gave good oil degradation at pH values 6, 7 and 8. The highest oil degradation achieved at pH 7, it reached 38.67 and 44.55% after 10 and 15 days of incubation, respectively. Also data show that there were large decreases in oil degradation at pH values of 5 and 9. Also bacterial mass growth gave highest weights at pH 7, which reached 3.644 and 4.282 g/l after 10 and 15 days, respectively. Similar results were obtained by Sathishkumar *et al.,* (2008), they found that pH 7 gave maximum oil biodegradation by strains; *Pseudomonas* sp. BPS1-8 and *Pseudomonas* sp. HPS2-5. Also, Verstraete *et al.,* (1976) reported that adjusting pH to 7.4 doubled biodegradation rate of gasoline in acidic soil pH 4.5, however biodegradation dropped significantly with raising pH to 8.5. On the other hand and in contrasts with our results, whereas some reports mentioned that *Pseudomonas* prefer alkaline pH in oil biodegradation (Mahjoubi *et al.,* 2018).



O.D: oil degradation, G.W: growth weight

Fig. 6. Effect of initial pH on oil degradation (%) and bacterial growth mass (g/l) by *Pseudomonas reinekei* MT1 after 10 and 15 days of incubation at 30ºC.

Effect of nitrogen source on oil biodegradation

The release of hydrocarbons such as oil pollutants to poorly organic matter aquatic environments causes excessively high carbon/nutrients ratios, such as nitrogen or phosphorus, which is unfavorable to microbial population and decrease the microbial growth Atlas (1980) and Cooney (1980). Addition of different nitrogen sources to oil medium affects the activity of *Pseudomonas reinekei* MT1 for oil degradation. Data represented in Fig. (7) appear that *Pseudomonas reinekei* MT1 gave highest oil degradation when BH oil broth medium was supported with Na_2NO_3 instead of NH_4NO_3, where oil degradation percent reached 50.53 and 54.20 % after 10 and 15 days, respectively. Also sodium nitrate as a nitrogen source gave good growth that reached 5.7 and 6.331 g/l after 10 and 15 days, respectively. Moreover, obtained data show that addition of beef extract and peptone did not achieve high oil degradation, even though they gave the highest growth mass 9.018 and 9.962 g/l, respectively after 15 days of incubation.

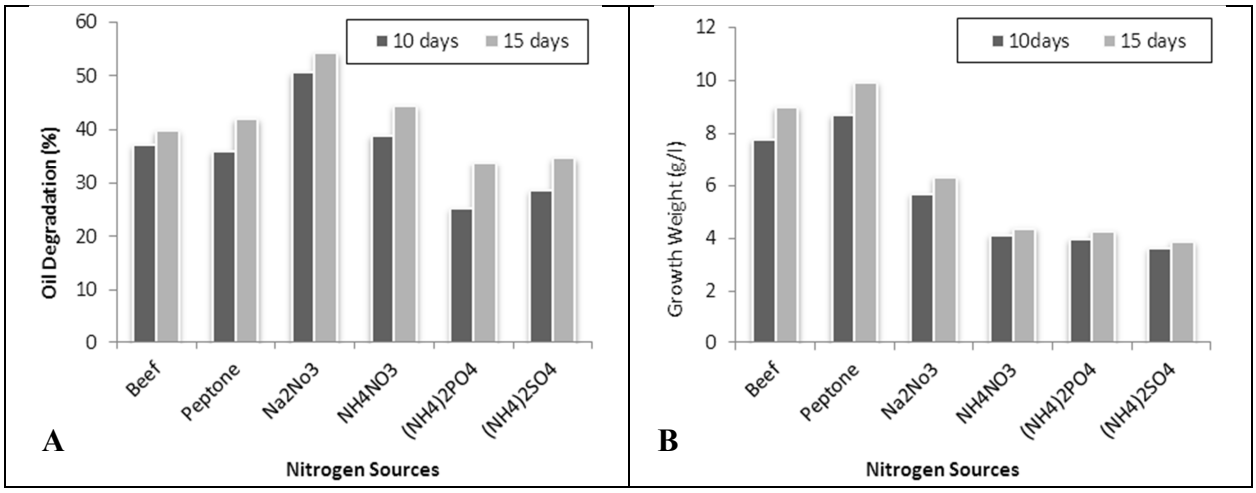


Fig. 7. Effect of additive nitrogen sources on (A) oil degradation (%) and (B) bacterial growth mass (g/l) by *Pseudomonas reinekei* MT1 after 10 and 15 days of incubation at 30ºC and initial pH 7

Raymond *et al.,* (1976) and Odu (1978) observed an increase in crude oil biodegradation with addition of nitrogenous fertilizers after several months to year of delay.

On the other hand the addition of nutrients such as nitrogen or phosphorus salts may be washed out in open aquatic systems, so it will be more effective in enclosed systems (Atlas & Bartha 1973; Atlas & Busdosh 1976; Dibble & Bartha 1976 and Horowitz & Atlas 1978).

Effect of oil derivatives on oil degradation

Many of crude oil degrading microorganisms able to degrade oil derivatives such as gasoline and diesel. Kulkarni and Wani (2016) mentioned that Kerosene and gasoline have been used in decreasing oil viscosity due to their good solvent properties. Yaghi and Al-Benami (2002).used mixture of 15% kerosene and heavy crude oil to reduce oil viscosity. Data represented in Fig. (8) show the effect of oil derivatives on oil degradation and growth mass of *Pseudomonas reinekei* MT1 after 10 and 15 days of incubation. Data show that addition of 10% gasoline to crude oil increased the oil degradation by *Pseudomonas reinekei* MT1, where reached 51.26 and 64.98% after 10 and 15 days, respectively. Also addition of gasoline

enhanced bacterial growth mass, it reached 5.631 and 6.698 g/l after 10 and 15 days, respectively. Data also show that other oil derivatives such as diesel, acetone and petroleum ether decreased oil degradation and bacterial growth mass in varying proportions. Vieira *et al.,* (2009) reached 75.9% bio-removal of diesel and gasoline using bacterial mixed culture after 3 days of incubation with agitation speed at 110 rpm. Lee *et al.,* (2011) used *Pseudomonas putida* AY-10 in degrading benzene, toluene, ehthylbenzene and xylene in different concentrations. Where *P. putida* completely degraded all hydrocarbons pollutants after 15 hours of incubation.

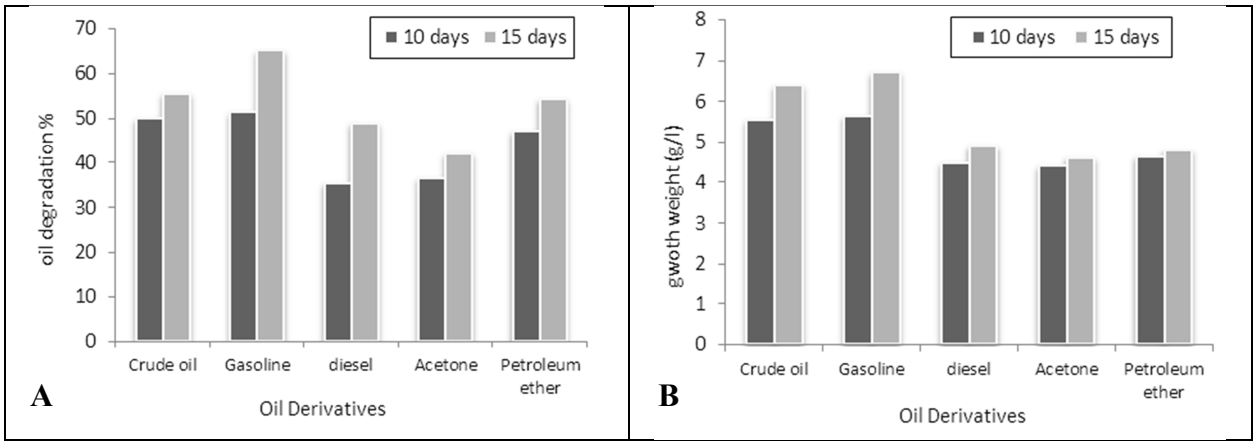
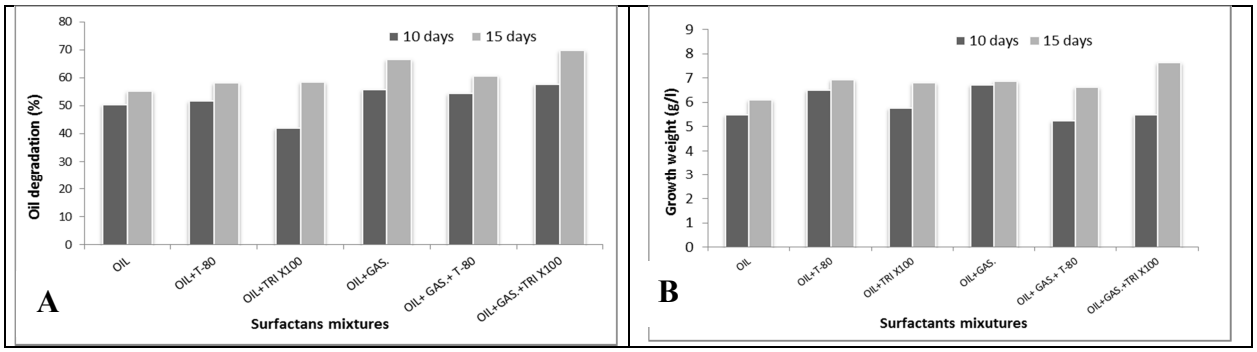


Fig. 8. Effect of oil derivatives on (A) oil degradation (%) and (B) bacterial growth mass (g/l) by *Pseudomonas reinekei* MT1 after 10 and 15 days of incubation at 30ºC, initial pH 7 and sodium nitrate as nitrogen source.

Effect of tween 80 and triton X100 on oil degradation

Surfactant considered as a major factor affecting oil biodegradation (Mahjoubi *et al.,* 2018). Tween 80 and triton X100 were among the most known surfactants. Where, adding surfactants reduce the surface tension of crude oil causing increases in the bioavailability of the oil to microbial action. Bioavailability is defined as the substrate mass transferred into microbial cells (Ghosal *et al.,* 2016).

Obtained data represented in Fig. (9) reveal that addition of surfactants increased oil degradation and growth mass of *Pseudomonas reinekei* MT1. Addition of triton X100 in mixture with crude oil and gasoline gave highest oil degradation and bacterial growth mass. Oil degradation with the addition of triton X100 to crude oil and gasoline reached 57.61 ad 69.92 % after 10 and 15 days, while bacterial mass growth reached 5.486 and 7.648 g/l after 10 and 15 days, respectively. On the other hand the addition of tween 80 enhanced oil degradation but to a lesser degree than triton X100. Similar results were obtained by Celik *et al.,* (2008), they found significant increases in oil degradation by *Pseudomonas stutzeri* G11 with addition of tween 80 and triton x100 to crude oil. *Pseudomonas stutzeri* G11 gave 69% of oil degradation with oil concentration of 1% on mineral salt medium, with the addition of 1% triton X100, oil degradation increased to 76%, while addition of 1% tween 80 enhanced oil degradation to 96%. With raising oil concentration to 2.5%, oil degradation decreased to 59% without addition of tween 80 and triton X100, while addition of triton X100 and tween 80 in concentration 2.5% gave 61 and 48% of oil degradation, respectively.

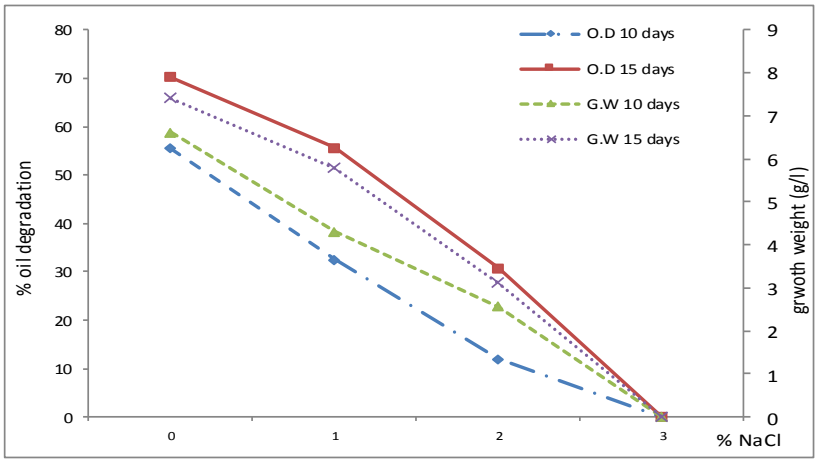


T-80: tween 80, TRI x100: triton x100, GAS: gasoline

Fig. 9. Effect of tween 80 and triton X100 on (A) oil degradation (%) and (B) bacterial growth mass (g/l) by *Pseudomonas reinekei* MT1 after 10 and 15 days of incubation at 30ºC and initial pH 7 and sodium nitrate as nitrogen source.

Effect of salinity on oil degradation

Data represented in Fig. (10) show that oil degradation decreased at 1% and 2% of NaCl. There was no growth of *Pseudomonas reinekei* MT1at 3% of NaCl. Kerr and Capone (1988) reported a positive relationship between oil degradation and salinity in estuarine sediments, while Ward and Brock (1978) found a great reduction of oil degradation with the increases of salinity in range between 3.3-28.4%, as a result of general decline in microbial metabolism.



O.D: oil degradation, G.W: growth weight

Fig. 10. Effect of NaCl concentrations (%) on oil degradation (%) and bacterial growth mass (g/l) by Pseudomonas reinekei MT1 after 10 and 15 days of incubation at 30ºC and initial pH 7 and sodium nitrate as nitrogen source with addition of gasoline and triton X100.

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تحسين التحليل الحيوى للنفط الخام بواسطة العزلة البكتيرية سودوموناس رينيكى م ت 1

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يعتبر التلوث البيئي الناجم عن تسريب زيوت البترول من أخطر المشاكل البيئية. لذلك كان من الضروري البحث عن طريقة بيولوجية لمعالجة تلك الملوثات. وفى هذه الدراسة تم عزل وتعريف السلالة البكتيرية سودوموناس رينيكى م ت 1 من عينات تربة ملوثة بالنفط الخام. وقد أظهرت تلك السلالة القدرة على تحليلها للنفط من خلال نموها على بيئة أملاح معدنية وتحتوى النفط الخام كمصدر وحيد للكربون. من ناحية أخرى فقد أظهرت السلالة القدرة على إنتاج مواد تقلل النشاط السطحي حيث ثبت أن لهذه المواد القدرة على زيادة قابلية النفط للتحليل من خلال تحسين درجة لزوجة النفط الخام وبالتالى زيادة إتاحته للبكتيريا المحللة. ومن خلال التجارب أظهرت النتائج أن بكتيريا السودوموناس رينيكى م ت1 تعطى أكثر نمواً وتحليلاً للنفط على درجة حرارة 30ºم، أس هيدروجينى 7 مع إضافة نترات الصوديوم كمصدر نيتروجين والبنزين والترايتون إكس. وقد أعطت السلالة البكتيرية سودوموناس رينيكى م ت 1 نسبة تحلل للنفط الخام وصلت إلى 62,69% خلال 15 يوم. من ناحية أخرى فقد استطاعت السلالة النمو وتحليل النفط الخام خلال درجات ملوحة وصلت إلى 2% فى حين توقف النمو وتحليل البترول عند نسبة الملوحة 3%.