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Enhanced crude oil biodegradation and rhamnolipid production by *Pseudomonas stutzeri* strain G11 in the presence of Tween-80 and Triton X-100

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Abstract: *In this study, the growth of sixty-one bacterial strains in crude oil were determined spectrophotometrically at 620 nm. Pseudomonas aeruginosa G1, Pseudomonas fluorescens G6, Pseudomonas stutzeri G11 and Pseudomonas putida G15 were chosen for the study based on the efficiency of crude oil utilisation. At 1% (v/v) crude oil concentration, P. stutzeri G11 strain degraded a maximum of 69%. The percentage of degradation by the P. stutzeri G11 strain decreased from 69% to 59% as the concentration of crude oil was increased from 1% (v/v) to 2.5% (v/v). Strain G11 was selected to determine the effects of surfactants (Tween- 80 and TritonX-100) on the biodegradation of crude oil. While strain G11 showed 76% degradation at mineral salts medium (MSM) containing 1% (v/v) crude oil + 1% (v/v) TritonX-100, it showed 61% degradation at MSM containing 2.5% (v/v) crude oil + 2.5% (v/v) TritonX-100. Also, degradation rate of this strain was 96% in the presence of 1% (v/v) crude oil + 1% (v/v) Tween-80, while degradation rate was 48% in the presence of 2.5% (v/v) crude oil + 2.5 % (v/v) Tween-80. Additionally, we investigated the rhamnolipid production of P. stutzeri G11 strain both in crude oil and in crude oil + two different surfactants (TritonX-100 and Tween-80, separately). These results suggest that surfactants have improved both crude oil degradation and rhamnolipid production and the degradation rates have depended very much on the chemical structure of surfactants*

Key words: *Biodegradation, Crude oil, Pseudomonas sp, Rhamnolipid, Surfactant*
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Introduction

Petroleum hydrocarbon continues to be used as the principle source of energy and hence an important global environmental pollutant (Rahman *et al*., 2003). Environmental pollution with petroleum and petrochemical products (complex mixtures of hydrocarbons) has been recognised as one of the most serious current problem, especially when associated with accidental spills on the large scale (Plohl and Leskovsek, 2002, Ghosh *et al.,* 2006).

Crude oil is composed of a wide range of hydrocarbons. Microbial utilization of these compounds as sole carbon sources is highly dependent on the chemical nature of the compounds within the petroleum mixture and on the environmental determinants (Atlas, 1981; Bharathi and Vasudevan, 2001; Chukwu and Odunzeb, 2006). Under ideal conditions, the hydrocarbons are completely mineralized to carbon dioxide and water, with some biomass production. Biodegradation efficiency depends on microorganisms, capability of producing enzymes and that will degrade the target compounds. Factor such as temperature, pH and nutrient status are of importance as moderators (Alexander, 1994). Degradation of these hydrocarbon by microorganisms has been assessed by a variety of strategies including the seeding of the environment with mixed oil-utilizing bacteria (Dave *et al*., 1994). Numerous microorganisms, including bacteria, fungi, and yeasts are known for their ability to degrade hydrocarbons (Chaillan *et al*., 2006).

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces (Desai and Banat, 1997). Therefore chemical surfactants are widely used in industrial applications; biosurfactants are biological molecules with similar properties to their chemical counterparts. Probably the most important advantage of biosurfactants over chemical surfactants is their ecological acceptability. Moreover, biosurfactants are non-toxic, natural biodegradable products and thus, essentially compatible with the biogeochemical cycle (Haba *et al*., 2000). There have been recent many reports on using them in enhanced oil biodegradation (Mulligan *et al.,* 2001; Rahman *et al*., 2002a; Urum *et al*., 2003). Biosurfactants can be produced with high yield by some microorganisms, especially *Pseudomonas* sp. These microorganisms can use the various renewal resources, especially agro industrial wastes, as the potential carbon sources (Maneerat, 2005). Among the best studied biosurfactants are rhamnolipids that belong to the glycolipid class (Tuleva *et al*., 2002). Rhamnolipids have been identified predominantly from *Pseudomonas aeruginosa* (Burger *et al.,* 1963; Tuleva *et al.,* 2002).

The aim of this study is to determine (a) biodegradation of crude oil by bacterial strains which were previously isolated from soils in laboratory, (b) effect of surfactants (Tween- 80 and TritonX-100) on crude oil degradation by *P. stutzeri* strain G11 which was selected due to its high crude oil degradation (c) rhamnolipid production both in crude oil and crude oil + surfactant by *P. stutzeri* strain G11.

Table - 1: Different growth levels of sixty-one bacterial strain in crude oil [1% (v/v)]

Genera	OD[a] at 620 nm						
	Total no.	0.0-0.2	0.21-0.4	0.41-0.6	0.61-0.8	0.81-1.0	1.01-1.2
Pseudomonas sp	33	18	4	5	2	1	3
Bacillus sp	28	28	-	-	-	-	-
Total	61	46	4	5	2	1	3

[a] = Number of strains reaching this OD, ND = None detected, Low growth = 0.0-0.4, Moderate growth = 0.41-0.6, High growth = 0.61-1.0, Excellent growth = 1.01-1.2

Table - 2: Biodegradation of two concentrations [1 %(v/v) and 2.5 %(v/v)] of crude oil by bacterial strains after 7 days incubation at 30°C

Strain*	Crude oil	
	1% ; 7 days**	2.5%; 7 days**
Pseudomonas aeruginosa G1	50[a]	48
Pseudomonas fluorescens G6	46	42
Pseudomonas stutzeri G11	69	59
Pseudomonas putida G15	58	57

* = Strain were previously isolated and identified, ** = Oil degradation (%)

Materials and Methods

Bacterial strains: The strains of 33 *Pseudomonas* spp. and 28 *Bacillus* spp. used in this study were obtained from the culture collection of the Biotechnology Laboratuary at Gazi University (Ankara, TURKEY). These strains were previously identified by the Analytical Profile Index (API 20 NE for *Pseudomonas* isolates and API 20E and API 50CHB for *Bacillus* isolates).

The growth determination: The composition of the MSM used in this study was as follow (g l^{-1}): $NaNO_3$ 4.0, NaCl 1.0, KCl 1.0, $CaCl_2 \cdot 2H_2O$ 0.1, KH_2PO_4 3.0, $Na_2HPO_4 \cdot 12H_2O$ 3.0, $MgSO_4$ 0.2, $FeSO_4 \cdot 7H_2O$ 0.001; 2 ml trace element stock solution composed of (g l^{-1}): $FeCl_3 \cdot 6H_2O$ 0.08, $ZnSO_4 \cdot 7H_2O$ 0.75, $CoCl_2 \cdot 6H_2O$ 0.08, $CuSO_4 \cdot 5H_2O$ 0.075, $MnSO_4 \cdot H_2O$ 0.75, H_3BO_3 0.15, $Na_2MoO_4 \cdot 2H_2O$ 0.05. The initial pH was adjusted to 6.8. The bacterial cultures (12 hr) were inoculated MSM with 1% (v/v) crude oil (Kiriqkkale Refinery-National Oil Company of Turkey) as carbon source. They were kept in a shaker at 200 rpm at 30 °C for a period of 7 days. The growth was monitored through culture densities, measuring absorbance spectrophotometrically (HitachiUV-VIS) at 620 nm wavelength.

Biodegradation of crude oil: The overnight culture at the log phase of growth were transferred to 250 ml flasks, each containing 100 ml of sterile defined mineral salts medium (MSM) with [1% (v/v) and 2.5% (v/v)] two concentrations of crude oil. The flasks were incubated in a shaker at 200 rpm at 30°C for 7 days. The total hydrocarbons in the treatments were determined spectrophotometrically at 420 nm wavelength. Degradation was estimated as the difference between the initial and the final concentrations of total hydrocarbons (Rahman *et al*., 2002b).

On the other hand, to investigate the effects of surfactants (Tween- 80 and TritonX-100) on the biodegradation of crude oil, *P. stutzeri* G11 strain which was chosen due to the high crude oil degradation was cultivated in the sterile defined MSM containing 1% (v/v) crude oil +1% (v/v) Tween -80 and in the sterile defined MSM containing 2.5% (v/v) crude oil + 2.5% (v/v) Tween- 80. Also, strain G11 was cultivated in the sterile defined MSM containing 1% (v/v) crude oil + 1% (v/v) TritonX-100 and 2.5% (v/v) crude oil + 2.5% (v/v) TritonX-100. The cultivations were conducted in 250 ml shaking (200rpm) flasks containing 100 ml medium for a period of 7 days. The temperature was controlled at 30°C. Then, biodegradation determination was conducted as above.

Determination of rhamnolipid: Strain G11 was inoculated on 100 ml of sterile defined MSM containing different concentrations of crude oil [1% (v/v) and 2.5% (v/v)] for biosurfactant production, followed by incubation on a rotary shaker (130 rpm) at 30ÚC. To investigate the effect of crude oil + surfactants (Tween-80 and TritonX-100) on the biosurfactant production (rhamnolipid), strain G11 was cultivated in sterile defined MSM containing 1% (v/v) crude oil+ 1% (v/v) Tween -80 and 2.5 % (v/v) crude oil +2.5 % (v/v) Tween- 80. Also, strain G11 was cultivated in the sterile defined MSM containing 1% (v/v) crude oil + 1% (v/v) TritonX-100 and in the sterile defined MSM containing 2.5 % (v/v) crude oil + 2.5 % (v/v) TritonX-100. Bacterial cells were removed from biosurfactant containing medium by centrifugation at 6000 rpm for 10 min. This crude extract was dried with the aid of a rotary evaporator under vacuum. Rhamnolipid concentration was determined according to Dubois *et al.* (1956) by the colorimetric phenolsulphuric acid method at 480 nm using the spectrophotometer.

Results and Discussion

Biodegradation of crude oil: The growth of sixty-one bacterial strains was determined spectrophotometrically at 620 nm(Table 1). Bacterial strains (*P. aeruginosa* G1, *P. fluorescens* G6, *P. stutzeri* G11, *P. putida* G15) with highest growth in crude oil [1% (v/v)] were chosen. Previous observations have identified the *Pseudomonas* genus among hydrocarbon degrading microorganisms (Lal and Khanna, 1996; Banat *et al*., 2000, Bharathi and Vasudevan, 2001; Saadoun, 2002). Prevalence of members of the genus *Pseudomonas* in all soils tested confirms previous reports about the widespread distribution of such bacteria in hydrocarbon-polluted soils and reflects their potential in utilizing these hydrocarbon contaminants for growth (Cork and Krueger, 1991). Also, Nwachukwu *et al*. (2001) have reported that treatment of oil-impacted agricultural soil with *P. putida* as a bioremediation agent does produce soil which is capable of growing larger and healthier plants than where without *P. putida* inoculation has not taken place.

Table - 3: The biodegradation and the rhamnolipid production crude oil and crude oil+surfactant of *P stutzeri* strain G11

Strain	*P. stutzeri* G11					
Concentration	Crude oil (v/v)		Crude oil + tritonX-100 (v/v)		Crude oil + tween-80 (v/v)	
	1 %	2.5 %	1 %	2.5 %	1 %	2.5 %
Degration rate (%)	69	59	76	61	96	48
Rhamnolipid* (g l^{-1})	0.003±0.1	0.009±0.0	0.4±0.2	0.5±0.1	0.2±0.0	0.1±0.2

* = Values are the means±SD of triplicate measurements

Fig. 1: Biodegradation from 0-7 days of crude oil [1 %(v/v)] by *P. stutzeri* G11 strain in flasks

In our study, oil degradation test is subjected to these strains to different concentrations of crude oil [1% (v/v) and 2.5%(v/v)] in MSM medium. The efficiency of different oil concentrations on the crude oil degradation of bacterial cultures was tested. The results showed that at 1% (v/v) crude oil concentrations, *P. stutzeri* G11 strain could exhibit a maximum of 69% of degradation after 7 days incubation. *P. stutzeri* G11 strain is followed by *P. putida* G15, *P. aeruginosa* G1 and *P. fluorescens* G6 strains with 58%, 50% and 46% respectively. Also at 2.5% (v/v) crude oil, *P. stutzeri* G11 strain showed 59% degradation after 7 days incubation followed by *P. putida* G15, *P. aeruginosa* G1 and *P. fluorescens* G6 strains (57%, 48%, 42%, respectively) again (Table 2). Similarly, several researches have reported that crude oil degradation was inversely proportional to the concentration of oil (Rambeloarisoa *et al.,* 1984; Rahman *et al.,* 2002b). Rahman *et al.* (2002b) have described that at 1% (v/v) crude oil, this strain could carry out of 66% degradation while 2.5% (v/v) crude oil, the *Pseudomonas* sp DS10-129 strain could carry out of 54% degradation.

Effect of surfactants on crude oil degradation: Microorganisms growing on petroleum usually produce potent emulsifiers and these surfactants help to degrade petroleum (Rosenberg, 1993). In this study *P. stutzeri* G11 strain was selected due to the high crude oil degradation (Table 2 and Fig. 1). The effect of TritonX-100 and Tween-80 on crude oil degradation after 7 days incubation is showed in Table 3. While strain G11 showed 76% degradation at MSM containing 1% (v/v) crude oil + 1% (v/v) TritonX-100, it showed 61% degradation at MSM containing 2.5% (v/v) crude oil + 2.5% (v/v) TritonX-100. Also, degradation rate of this strain was 96% in the presence of 1% (v/v) crude oil + 1% (v/v) Tween-80 while degradation rate was 48% in the presence of 2.5% (v/v) crude oil + 2.5 % (v/v) Tween-80. Similarly, several researches have reported that the use of surfactants have enhanced degradation of crude oil (Balba *et al.,* 2002; Urum *et al*., 2003). In our study, the degradation at Tween-80 decreased at a higher rate compared to the degradation at TritonX-100. Some researchers reported that synthetic surfactants were effective on biodegradation of diesel oil and cell growth was inhibited at high concentrations of Tween 80 (Lee *et al*., 2006). These reports have confirmed our results. Finally, these results have shown that the surfactants were able to stimulate the biodegradation of crude oil, but the degradation rates have depended on the chemical structure (Table 3).

Biosurfactants can be produced with high yield by some microorganisms, especially *Pseudomonas* sp (Maneerat, 2005). Water soluble carbon sources such as glycerol, glucose, mannitol and ethanol were all used for rhamnolipid (biosurfactant) production by *Pseudomonas* sp (Desai and Banat 1997). In this study we investigated the production of rhamnolipid of strain G11 in defined MSM containing different concentrations of crude oil [1 % (v/v) and 2.5% (v/v)]. Due to the emulsifying properties of surfactants we have also investigated the rhamnolipid production in crude oil + surfactants. Table 3 shows the production of rhamnolipid biosurfactant of this strain. Rhamnolipid production in MSM supplemented with 2.5% (v/v) crude oil (0.009 g/l) was higher than in MSM supplemented with 1% (v/v) crude oil (0.003 g/l) (Table 3). We determined the production of rhamnolipid (0.4% and 0.5%, respectively) of strain G11 in MSM containing 1% (v/v) crude oil + 1% (v/v) TritonX-100 and in MSM containing 2.5% (v/v) crude oil + 2.5% (v/v) TritonX-100. Also the production of rhamnolipid biosurfactant of this strain was found (0.2 % and 0.1%, respectively) in MSM containing 1% (v/v) crude oil + 1% (v/v) Tween-80 and in MSM containing 2.5% (v/v) crude oil and 2.5% (v/v) TritonX-100 (Table 3). To our knowladge, this is the first report describing the rhamnolipid production of *Pseudomonas* genus in crude oil + surfactant.

In summary, the result of these experiments indicates following important points, (a) these four *Pseudomonas* strains capable of degrading crude oil, (b) our results suggest that the additions of surfactants could facilitate the biodegradation of crude oil by strain G11, (c) rhamnolipid production of G11 strain in MSM containing crude oil + surfactant was higher than MSM with only

crude oil. However, further research is needed to determine the maximum crude oil degradation and the rhamnolipid production by strain G11 under different environmental conditions.

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