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ARAŞTIRMA MAKALESİ/**RESEARCH ARTICLE**

BIODEGRADATION OF OILS BY *Citrobacter freundii*

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ABSTRACT

A number of industrial organizations create oil-containing wastewater. As of yet, a valid method for biologically treating these wastewaters has not been developed. The development of microbial cultures for use in a bioreactor could, therefore, provide effective treatment of these wastewaters. In this study, the degradation of a mixture consisting of machine, olive, sunflower and soybean oil by microorganisms with lipolytic activity was studied.

Firstly, bacterial cultures were isolated from wastewater containing oil. The effectiveness of these cultures was assessed in terms of their efficiency to remove machine oil. *Citrobacter freundii* was found to be the most effective in removing 68% of the soybean oil, 55% of the olive oil, 47.5% of the machine oil with an initial concentration of 0.8% (w/v), and 51.2% of the sunflower oil with an initial concentration of 1% (w/v). The total initial inoculum concentration was approximately $20x10^3$ cfu/ml and the shaking rate was 120 rpm for each oil.

This culture removed oil from both the oil mixture prepared in the laboratory and wastewater collected from a meat combine plant, at rates of 68.5% and 69%, respectively.

Keywords: Wastewater treatment, Biodegradation, Plant oils, Mineral oils, *Citrobacter freundii*

Citrobacter freundii İLE YAĞLARIN BİODEGREDASYONU

ÖZ

Çeşitli endüstriyel kuruluşlar yağ içeren atık sular oluştururlar. Bu yağ içeren atık suların biyolojik arıtımı için henüz tam bir arıtım teknolojisi geliştirilmemiştir. Bir biyoreaktörde kullanılmak üzere mikrobiyal kültürlerin geliştirilmesi bu tip atık suların etkili bir şekilde arıtımını sağlayacaktır. Bu çalışmada atık sularda bulunan makina yağı, zeytin yağı, ayçiçek yağı ve soya yağından oluşan yağ karışımının lipolitik aktiviteye sahip mikroorganizmalar tarafından parçalanması incelenmiştir.

Bakteri kültürleri, yağ içeren atık sulardan izole edildi ve bu kültürlerin etkinlikleri makina yağını parçalama yeteneklerine göre belirlendi. Saf kültürler arasında en etkili olan *Citrobacter freundii*, %0.8 başlangıç konsantrasyonuyla soya yağını %68 oranında, zeytin yağını %55 oranında, makina yağını %47.5 oranında, ayçiçek yağını ise %1 başlangıç konsantrasyonuyla %51.2 oranında parçalamıştır. Her yağ için kültürün toplam başlangıç konsantrasyonu $20x10^3$ cfu/ml ve çalkalama hızı 120 rpm idi.

Bu kültür, laboratuvarda hazırlanmış yağ karışımını ve et kombinası fabrikasının yağ içeren atık suyunu sırasıyla %68.5 ve %69 oranında parçalamıştır.

Anahtar Kelimeler: Atık su arıtımı, Biodegredasyon, Bitkisel yağlar, Mineral yağlar, *Citrobacter freundii*

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1. INTRODUCTION

Fats and oils are essential triglycerides consisting of straight-chain fatty acids attached, as esters, to glycerol. The fatty acid components of edible fats and oils vary considerably. They can differ in chain length, may be saturated or unsaturated and contain an odd or even number of carbon atoms. Fats and oils, typical components of food-processing wastewater, tend to clump together and clog drain lines and grease traps, causing frequent problems. If present in excessive amounts, they may interfere with microbiological processes at wastewater treatment plants and lead to decreased efficiency, or even complete failure of treatment. When discharged into the environment, they may cause the formation of surface films and shoreline deposits and lead to environmental pollution (Wakelin and Forster, 1997; Keenan and Sabelnikov, 2000; Scholz and Fuchs, 2000).

The disposal of waste oils from commerce and industry has traditionally involved incineration or consolidation. The environmental impact of these methods has prompted the investigation of bioremediation technologies as an alternative form of treatment. Bioremediation has been shown to be both more environmentally acceptable and economically competitive than established methods of waste disposal (Benntham et al, 1997). Currently, two primary bioremediation approaches are used to degrade fats and oils in wastewater. The first uses enzyme preparations, primarily lipases that can hydrolyze fats and oils to fatty acids and glycerol. However, fatty acids tend to form micelles-colloidal particles that may aggregate and precipitate from solutions during environmental changes. These then again cause slogging in drains, grease traps, and treatment tanks. The second approach, biological augmentation, utilizes the addition of live microbial cells to wastewater, which not only hydrolyze fats and oils to fatty acids and glycerol, but also metabolize them further to carbon dioxide and water (Wakelin and Forster, 1997; Keenan and Sabelnikov, 2000).

Microbial cells are protected from the environment by their cell envelopes and thus have a greater tolerance to extreme environmental changes encountered in food-processing facilities compared with enzyme preparation. In addition, microorganism preparations are less expensive and more stable than enzymes and they can also reproduce themselves at the waste sites (Keenan and Sabelnikov, 2000).

This paper reports the results of a biological treatment approach employed to treat oils in wastewater by a bacterial isolate. With a properly devised treatment process and the appropriate selection of bacterial strains, it will be possible to not only reduce grease and oil content in wastewater to a level acceptable to local wastewater management authorities, but also offer the complete elimination of them.

2. MATERIAL AND METHOD

2.1. Isolation, Selection and Identification of Oil-Degrading Microorganism

In order to isolate a bacterium degrading oil, samples from the municipal wastewater treatment plant were passed aseptically through a tenfold dilution of distillated water to yield final concentrations of 10^{-3}-10^{-6}. Plates were then prepared from each dilution (1 ml) in PCA (Oxoid) medium and incubated at 28±1°C for 24 h. Discrete colonies were picked from each plate and stored on nutrient agar (Oxoid) slopes.

Bacterial isolates were selected according to their ability to grow on machine oil as carbon and energy sources. Conical flasks (250 ml) containing the growth medium (100 ml) and 0.7 g machine oil were prepared. In all the studies, a growth medium consisting of the salts; $(NH_4)_2SO_4$ 0.2%, K_2HPO_4 0.3% and KH_2PO_4 0.1 % and adjusted to pH 7 were used. The flasks were autoclaved at 121°C and 1.1 atm pressure for 15 min prior to inoculation. The flasks were inoculated (approximately 10^3 cfu/ml) with pure cultures. The flasks were then placed on an orbital shaker (150 rpm) at 28±1 °C for 7 days.

The identification of microorganisms was carried out using the Biomeriux (Vitek GN Microplates) and Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

2.2. Determination of Oil Quantity

Oil analysis was carried out by partition-gravimetric method. A 100 ml sample was acidified to pH 2.0 with 1:1 diluted HCl and then transferred to a separatory funnel. Oil was then repeatedly extracted with 30 ml portions of n-hexane (Merck) / methyl-tert-butyl ether (Merck) [80/20 (v/v)], until the aqueous phase showed no oil layer and the solvent phase was clear. Following this, the combined solvent extracts were evaporated at 85 °C and cooled in a desiccator for at least 30 min before being weighed. The amount of oil present in the sample was calculated using the following equation: mg oil and grease/L=(A-B) .1000/mL sample, where A= total gain in weight, mg, B= flask tare, mg (APHA, 1992).

2.3. Determination of Lipase Activity

The lipase activity of the bacterial isolates were determined by the "Opacity Capacity in Deep Culture Technique" modified for bacteria (Topal et al., 2000). To determine lipolytic activity in cultures a lipase medium containing 0.5% peptone, 0.3% yeast extract and 1% agar was used. The medium was autoclaved at 121 ^{o}C for 15 min under 1.1 atm pressure. Tributyrin (Sigma) sterilized by membrane filtration was added (0.1 % v/v) to the medium after first cooling to 60 °C. The tributyrin medium was then homogenized for 8 min and distributed into test tubes, which were left to permit solidification. Agar blocks were aseptically cut from the nutrient agar cultures with a sterile drill and added to the tubes. After incubation at 30 °C for 5 days the tubes were evaluated according to their opacity depths (mm).

2.4. Determination of Bacterial Protein

Bacterial protein in cultures was determined according to the "Stickland Method" (Gürgün and Halkman, 1988). For the purpose of this, a 30 mL sample was centrifuged at 4500 rpm for 15 min. Supernatant

was then removed and the pellet was suspended in 6.6 ml water. 1.2 ml of 20% NaOH was added and boiled for 5 min. After cooling 0.2 ml of 25% $CuSO_4$., $5H_2O$ was added, thoroughly mixed and centrifuged (3000 rpm for 15 min) after being kept at room temperature for 30 min. The absorbans of supernatant was measured at 545 nm in an UV visible spectrofotometer (Cecil 4000, England). The protein quantities equivalent to the values of the absorbans measured were determined via standard curve obtained using 20% human albumin immuno (Baxter AG, Austria) as standard protein.

2.5. Optimization of Degradation Conditions

Fat and oil biodegradation were examined using different initial concentrations of inoculum ($4x10^3$-$20x10^3$ cfu/ml) and concentrations of oils (4-12 g/L), at different pH (4-9), temperature (20-35°C) and shaking rates (static-180 rpm).

2.6. Application for Biodegradation of Oil in an Industrial Wastewater

Industrial wastewater sample was obtained from a meat combine plant's wastewater. The wastewater had an oil concentration of 92 mg/L. Under the optimal conditions determined, the microorganism was inoculated ($20x10^3$ cfu/ml) in 250 ml conical flasks containing 100 ml of wastewater and the oil biodegradation process was repeated.

3. RESULT AND DISCUSSION

Thirty-eight strains were isolated from wastewater treatment pools, including a variety of oils. Since the degradation of machine oil is more difficult to achieve than the degradation of vegetable and animal oils, all isolates were evaluated in respect of their ability to degrade machine oil. Twenty-six of the isolates were unable to degrade machine oil. A comparison of performance of the other twelve cultures, using machine oil, is shown in Table 1. H1 and A10 had significantly higher growth or higher oil degrading capacities than those of the others. These bacteria were identified according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). These isolates had the properties of *Citrobacter freundii* (Table 2). It was found that *C. freundii* had lipolytic activity and its lipase activity was determined as 30 mm opacity depth by the "Opacity Capacity in Deep Culture Technique". In similar studies, *Acinetobacter* sp., *Acetobacter calcoaceticus* L_{009}, *Alcaligenes* sp., *Bacillus subtilis* B_{304}, *Corynebacterium* sp., *Pseudomonas* sp., *Pseudomonas aeruginosa* L_{602}, *Rhodococcus* sp., *Rhodococcus rubra*, *Nocardia amarae and Microthrix parvicella*, *Caseobacter* sp., *Candida rugosa*, *Aeromonas/Vibrio* spp. have been used as biodegrading cultures for corn, olive, linseed, coconut, rapeseed and sunflower oils (Del Rio et al. 1990; Wakelin and Forster, 1997; Keenan and Sabelnikov, 2000; Mongkolthanaruk and Dharmsthiti, 2002). The most consumed oil types in Turkey are sunflower, soybean and olive oil, as well as machine oil. These oils in wastewaters have created problems in wastewater treatment plants. Thus, in this study, different types of oils (sunflower, soybean, olive and machine oil) were selected.

Table 1. A comparison of performance of the bacterial isolates, using machine oils.

Isolates	Gram reaction	Morphological properties	Degraded oil (%)
A2	+	coccus	37
A7-1	-	rod	8
A8	-	coccus	30
A10	-	rod	42
A11	-	rod	22
A22	-	rod	2
Ç2	+	rod	20
Ç4-1	-	rod	13
Ç5-2	-	rod	16
H1	-	rod	43
H3-1	-	rod	8
H4	-	rod	13

Initial oil quantity: 7 g/L

Initial inoculum concentrations of between $4x10^3$ - $20x10^3$ cfu/ml were studied. As a result, all of the oils were degraded over 45% at the inoculum quantity of $20x10^3$ cfu/ml. These rates were found to be 47.5%, 51.2%, 55%, 68% for machine, sunflower, olive and soybean oil, respectively (Figure 1). The results indicated that the degradation rate of oils increased with the augmentation of the inoculum concentration.

Table 2. A comparison of the H1 and A10 with *C. freundii* according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

Properties	H1 and A10	*C. freundii*
Gram reaction	-	-
Citrat (simmons)	+	+
Lactose fermentation	+	D
Metil red	+	+
Voges proskauer test	-	-
H_2S production	+	[+]
Urease	-	D
Inositol	-	-
Arabinose	+	+
D-glucose	+	+
Maltose	+	+
Xylose	+	+
Esculin hydrolysis	-	-
D-sorbitol	+	+
Ornithin decarboxilase	-	[-]
D-mannitol	+	+
Adonitol	-	-
Arginine	-	D
Sucrose	+	D
L-rhamnose	+	+
Oxidase	-	-
Raffinose	+	D
Lysine	-	-

+: Positive; -: Negative; D: % 26-75 positive; [+]: %76-89 positive; [-]: % 11-25 negative

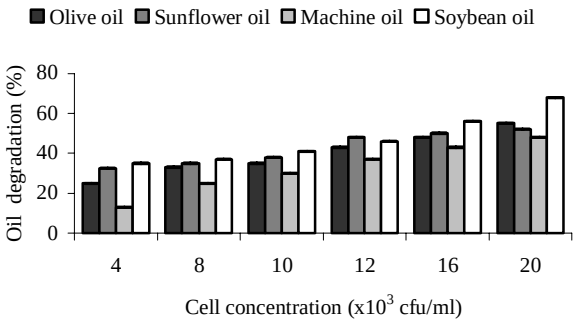

Figure 1. The effect of initial inoculation concentrations on oil degradation by *C. freundii* [Oil concentration 7 g/L, pH 7, temperature 28±1 °C, shaking rate 120 rpm, time 7 days].

The types and amount of oil in wastewater differ depending on the industry**.** However, the determination of the oil concentration with the highest biodegradation rate is important in order to maintain treatment processes. Therefore, the range of oil concentration was selected as 4-12 g/L through consulting other studies. When oil concentration is over 8 g/L, reduction at the biomass is usually observed. There is no documented evidence available on oil's direct toxic effect on the microorganism. However, we do know that oil covers the water's surface and can decrease the entry of oxygen. Thus, bacterial growth decreases when oxygen concentration decreases in wastewater (Qasm, 1994; Mongkolthanaruk and Dharmsthiti, 2002). In our study, the optimal initial oil concentration was determined as 8 g/L. After a 7-days incubation, the biomass quantity in the medium with soybean oil was higher than that of the other oils. As a result, it was concluded that *C. freundii* could degrade soybean oil more easily than other biodegrading cultures (Figure 2).

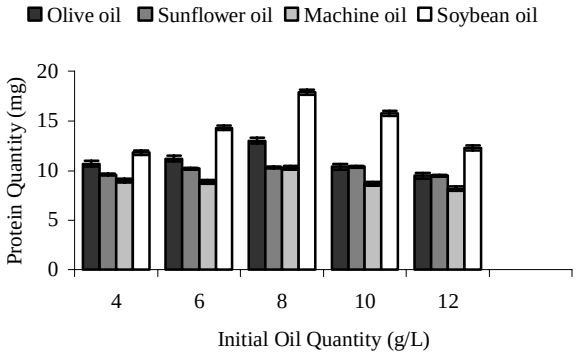

Figure 2. The effect of initial oil concentration on growth of *C. freundii* [Cell concentration $20x10^3$ cfu/ml, pH 7, temperature 28±1 °C, shaking rate 120 rpm, time 7 days].

The medium pH plays an important role during oil degradation. Keenan and Sabelnikov (2000) reported that the pH of a bakery plant's wastewater was 4.7 before treatment process and after adjusted to pH 7.2, an important reduction was observed at the COD. Tan and Gill (1987) declared that neutral pH was the best condition for biomass increase and oil degradation. In this study, the pH values were set between 4-9 in the experiments so as to best determine the effect of pH on the oil degradation. The results showed that at the near neutral pH values, as was previously mentioned, the oil degradation was at the highest degree. The optimum pH values for oil degradation were determined as pH 7 for soybean and sunflower oil, while pH was 6 for olive and machine oil (Figure 3).

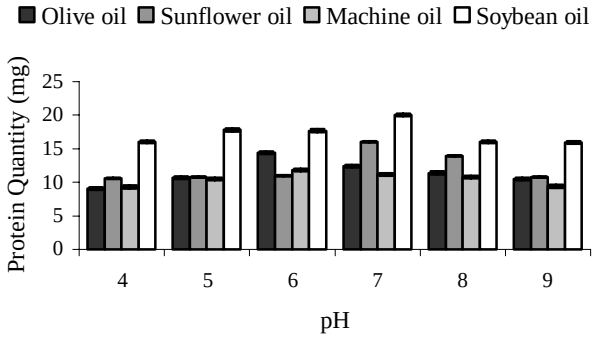

Figure 3. The effect of pH on growth of *C. freundii*. [Cell concentration $20x10^3$ cfu/ml, oil concentration 10 g/L (sunflower oil); 8 g/L (olive, machine, soybean oil), temperature 28±1 ^oC, shaking rate 120 rpm, time 7 days].

In most studies up to the present, a 30 °C incubation temperature has frequently been chosen for oil biodegradation (Tan and Gill, 1987, Wakelin and Forster, 1997). For the purposes of this study, it was decided to investigate the effect of temperatures between 20 °C and 35 °C. As a result, the best biomass increase and oil degradation were estimated at 30 °C temperature (Figure 4). *C. freundii* is a mesophylic bacterium, therefore it shows optimal enzymatic activity at ambient temperatures.

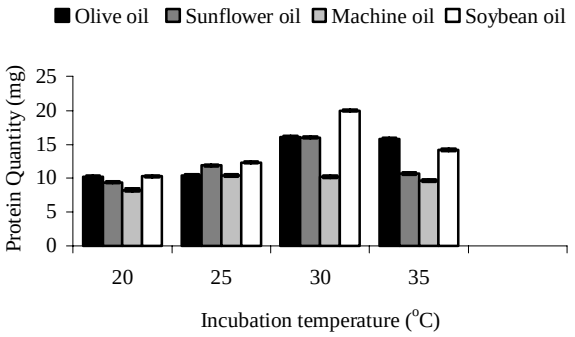

Figure 4. The effect of incubation temperature on growth of *C. freundii*. [Cell concentration $20x10^3$ cfu/ml, oil concentration 10 g/L (sunflower oil); 8 g/L (olive, machine, soybean oil), pH 6 (machine, olive oil); pH 7 (sunflower, soybean oil), shaking rate 120 rpm, time 7 days].

The shaking process homogeneous dispersion of the oils in the water, and thus increases the likelihood of contact occurring between the oil and the organism. As a result of experiments being conducted at different shaking rates (0, 120, 150, 180 rpm), while 120 rpm shaking rate was seen to be the most suitable for all the oils, the other shaking rates were found to be inefficient for biomass increase (Figure 5). At shaking rates of over 120 rpm, foam forming may prevent the dissolving of oxygen. In aerobic biodegradation of organic substances, the amount of dissolved oxygen is of importance. However, because *C. freundii* is a facultative aerobe bacterium, a low shaking rate was sufficient for the oxygen requirements of this study.

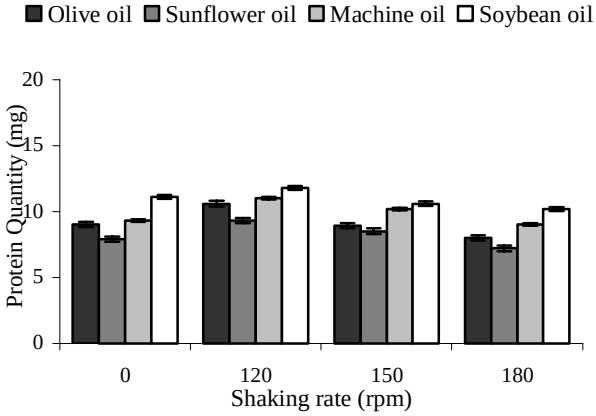

Figure 5. The effect of shaking rate on growth of *C. freundii*. [Cell concentration: $20x10^3$ cfu/ml, oil concentration: 10 g/L (sunflower oil); 8 g/L (olive, machine, soybean oil), pH: 6 (machine, olive oil); pH: 7 (sunflower, soybean oil), temperature 30 ^oC, time 7 days].

Under the optimum conditions determined, during the degradation experiments of seven days, biomass increase became the greatest after five days for each type of oil, with the exception of machine oil. The degradation process for machine oil was determined at the highest degradation ratio after four days, but generally the degradation ratio was low when compared with that of the other oils (Figure 6). The lipids present in the wastewater, such as machine oil, show significant differences in physical and chemical properties when compared with common edible fats and oils such as olive, sunflower and soybean oils.

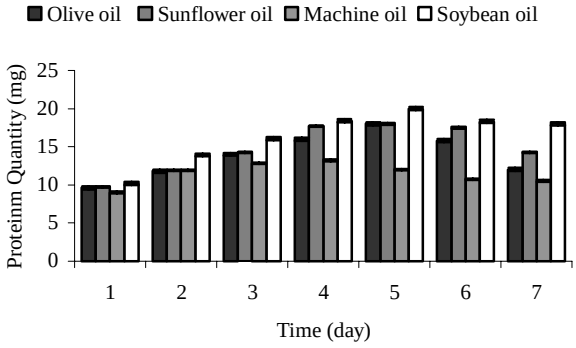

Figure 6. Determination of appropriate time for oil removal with *C. freundii*. [Cell concentration $20x10^3$ cfu/ml, oil concentration 10 g/L (sunflower oil); 8 g/L (olive, machine, soybean oil), pH 6 (machine, olive oil); pH 7, (sunflower, soybean oil), temperature 30 ^oC, shaking rate: 120 rpm, time 7 days].

One of the methods commonly used to monitor bacterial growth is to determine the protein content of bacterial mass. Therefore, the protein content of bacterial mass was established throughout the incubation period, and an inverse ratio was found to exist between the bacterial growth and oil degradation (Figure 7). This finding indicates that the bacterium used oils as a carbon source. A variety of oils are present in waste-

water, each having different properties. Under the optimal conditions, a comparison of the mixed oils [machine oil (0.2%), sunflower oil (0.2%), soybean oil (0.2%) and olive oil (0.2%)] degradation performance is seen in Figure 7. Initial oil quantity decreased according to the day of incubation. At the end of twelve days, 68.5% of the initial oil was degraded. However, after six days the degradation ratio was seen to be less. It was found that under the optimum conditions (initial inoculum concentration $20x10^3$ cfu/ml, pH 7, temperature 30 ºC, shaking rate 120 rpm) the wastewater from the meat combine plant containing 92 mg/L initial oil concentration was degraded by 69% within five days.

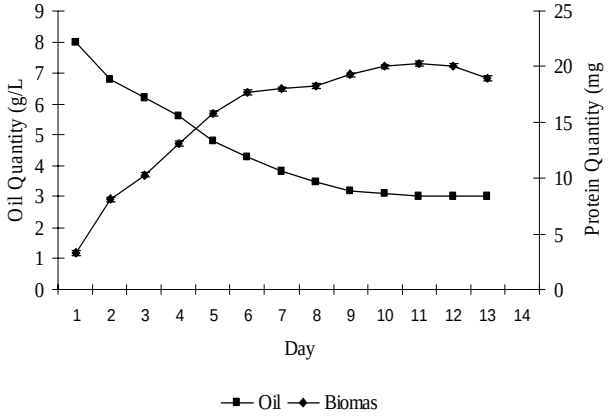

Figure 7. Determination of appropriate time for mix oil removal with *C. freundii*. [Cell concentration $20x10^3$ cfu/ml, mixed oil concentration machine oil (0.2%), sunflower oil (0.2%), soybean oil (0.2%), olive oil (0.2%) temperature 30 °C, shaking rate: 120 rpm, time 13 days].

4. CONCLUSION

The use of *C. freundii* in the treatment of wastewater containing lipid was formulated under the determined optimal conditions, with the results showing that bacterium degraded soybean oil at the high ratio. It reduced the oil-mix and the wastewater from a meat combine plant by 68.5% and 69% respectively. These results show that *C. freundii* can be applied to wastewater containing miscellaneous oils, as a recourse for its treatment.

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