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**Araştırma Makalesi / Research Article**

# Screening of Lipid Production Capacities of *Bacillus* sp. Strains Isolated from Soil and Lipid Staining with Different Staining Techniques

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***Keywords***

*Bacillus*; Lipid; Staining; Sudan Black B

## Abstract

Microbial lipids have been attracting more and more attention in recent years as promising raw materials for the production of biodiesel and value-added compounds. In the current economic and environmental situation, finding new renewable sources of lipids will be crucial. Recent research has necessitated the search for new efficient microbial producers with lipid production efficiency. For this purpose,50 *Bacillus* sp. strains previously isolated from the soil were screened for their lipid production capacity. As a result of the production using a single-cell oil production medium, only two *Bacillus* spp. strains showed growth. These bacteria were named as *Bacillus* sp. E40 and *Bacillus* sp. E226. Bacteria were then characterized in terms of their biomass, lipid yields, and lipid contents. The biomass of *Bacillus* sp. E40 and E226 bacterial isolates, were 0,28 and 0,22 $g.L^{-1}$, respectively. The highest lipid content was reached by E226 with 54.5%. *Bacillus* sp. E40 lipid content was determined as 46.4%. In addition, both intracellular lipid staining and colony staining demonstrated the presence of lipids. With this study, it was reported for the first time that local *Bacillus* sp. strains isolated from soil have lipid producing capacity.

# Topraktan İzole Edilen *Bacillus* sp. Suşlarının Lipid Üretim Kapasitelerinin Taranması ve Farklı Boyama Teknikleri ile Lipid Boyama

***Anahtar kelimeler***

*Bacillus*; Lipid; Boyama; Sudan Siyahı B

## Öz

Mikrobiyal lipitler, biyodizel ve katma değerli bileşiklerin üretimi için umut verici hammaddeler olarak son yıllarda giderek daha fazla dikkat çekmektedir. Mevcut ekonomik ve çevresel durumda, yeni yenilenebilir lipit kaynakları bulmak çok önemli olacaktır. Son araştırmalar, lipit üretim verimliliğine sahip yeni verimli mikrobiyal üreticilerin araştırılmasını gerekli kılmıştır. Bu amaçla daha önce topraktan izole edilen 50 *Bacillus* sp. suşu lipid üretme kapasiteleri açısından taranmıştır. Tek hücreli yağ üretimi ortamında yapılan üretim sonucunda bu bakterilerden sadece iki *Bacillus* sp. suşları üreme göstermiştir.

Bu bakterilerden sadece 2 *Bacillus* sp. suşu tek hücreli yağ üretimi ortamında üremiştir. Bu bakteriler *Bacillus* sp.E40 ve *Bacillus* sp. E226 olarak adlandırıldı. Bakteriler daha sonra biyokütleleri, lipid verimleri ve lipid içerikleri açısından karakterize edildi. *Bacillus* sp. E40 ve E226 bakteri izolatlarının biyokütlesi sırasıyla, 0,28 ve 0,22 $g.L^{-1}$ idi. En yüksek lipid içeriğine %54.5 ile E226'da ulaşılmıştır. *Bacillus* sp. E40 lipid içeriği %46.4 olarak belirlendi. Ek olarak, lipid varlığı hem hücre içi lipid boyama hem de koloni boyama ile gösterilmiştir Bu çalışma ile topraktan izole edilen yerel *Bacillus* sp. suşlarının lipit üretme kapasitesine sahip olduğu ilk kez rapor edilmiştir.

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## 1. Introduction

Oils produced from microorganisms are called as microbial oils or unicellular oils (SCOs). The composition of these oils is important because of their similarity to the composition of edible plants, animal oils and fats (Kyle and Ratledge 1992, Boswell *et al*. 1996). Lipids have been interested great in recent years due to their great potential for biotechnological uses, as well as their important properties such as sustainability and renewability. They are used in many products such as solvents, biosurfactants, food supplements, lubricants and nutraceuticals (Steen *et al*. 2010, Patel *et al*. 2020). The most important application area is the use of biodiesel, which can be used instead of petroleum-based diesel fuel. Biodiesel is considered an environmentally friendly alternative to fossil fuels as it contains zero or fewer emissions of gases such as sulfur oxides (SOx) and carbon monoxide (CO) (Gufrana *et al*. 2022).

Lipid-producing microorganisms have been isolated from soil, marine environments, sewage sludge and decomposing plant material in forests (Liu *et al*. 2010, Neema and Kumari 2013, Pan *et al*. 2009, Wang *et al*. 2014). While the main producers of lipids are yeasts, fungi and algae, it has been stated that bacteria do not produce enough lipids (Wynn and Ratledge 2005, Bellou *et al*. 2016, Li *et al*. 2008).

The most important fatty microorganisms are yeast (*Rhodotorula glutinis, Yarrowia lipolytica, Lipomyces starkeyi, Cryptococcus curvatus*), fungi (*Mucor rouxii, Alternaria* spp., *Aspergillus oryzae*), bacteria (Actinobacteria, Proteobacteria, *Streptomyces, Nocordia* spp.) and microalgae (*Scenedesmus*, *Nannochloropsis, Chlorella vulgaris)* (Gufrana *et al*. 2022). In oily microorganisms, the oil content can reach 70% of the biomass under suitable production conditions. A medium containing high concentrations of glucose and low concentrations of nitrogen is required to induce lipid production in microorganisms. Lipid accumulation occurs when nitrogen is depleted. (Ratledge 2004, Dzurendova *et al*. 2020). In such a nutrient medium, carbon is directed directly to lipid synthesis, and discrete oil droplets of triacylglycerols are formed inside the cells. If nitrogen is depleted, biomass production decreases and lipid accumulation begins (Ratledge 2004, Wynn and Ratledge 2005). The main form of SCO is Triglycerides (TAG) and consists mainly of long-chain fatty acids with significant added value. The most general fatty acids produced by microorganisms are palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2) acids (Llamas *et al*. 2020). In addition, polyunsaturated fatty acids (PUFA) or saturated (SFA) and monounsaturated (MUFA) fatty acids have gained importance. This is because they are used as a renewable energy source for the biodiesel industry (Patel *et al*. 2020).

The search for efficient microbial producers for microbial lipids, which has gained importance in recent years, is aimed at increasing the commercial potential of biological production of SCOs by using alternative low-cost renewable substrates (Bettencourt *et al*. 2020) .

Studies are carried out on yeast and molds, which are generally eukaryotic in the production of microbial oil. The fact that these organisms produce large amounts of oil is related to cell size and structure. They have larger cell structures than prokaryotes. This makes them stand out more than bacteria (Denli and Tekin 2000). However, scientists are also working with bacteria as alternative sources, since bacteria can reproduce in a shorter time and even in simple fermentation conditions. In the literature, there is no source on lipid production by *Bacillus* strains. *Bacillus* sp. strains are extremely valuable bacteria in terms of producing important enzymes in the biotechnological field. In this study, it was aimed to reveal the productive *Bacillus* strains by determining the lipid production capacities of 50 *Bacillus* sp. strains isolated from different provinces soils in our previous studies.

## 2. Materials and Methods

### *2.1 Materials*

In the present study, 50 previously isolated from different provinces soils *Bacillus* sp. strains in our culture collection were used (Demirkan *et al*. 2020). The localities where the *Bacillus* sp. strains used in the study were isolated and *Bacillus* spp. codes are given in table 1.

**Table 1.** The localities where *Bacillus* sp. strains are isolated and *Bacillus* spp. no.

| No. | Locality isolated | *Bacillus* spp. No |
|---|---|---|
| 1 | Adana (Kozan) | E46 |
| 2 | Adana (Merkez) | E291 |
| 3 | Antalya (Merkez) | E283 |
| 4 | Antalya (Alanya) | E302 |
| 5 | Amasya (Merkez) | E5 |
| 6 | Amasya (Suluova) | E8 |
| 7 | Ankara (Haymana) | E228 |
| 8 | Artvin (Arhavi) | E216 |
| 9 | Balıkesir (Merkez) | E274 |
| 10 | Balıkesir (Bigadiç) | E226 |
| 11 | Bartın (Merkez) | E101 |
| 12 | Bilecik (Merkez) | E182 |
| 13 | Bilecik (Bozüyük) | E271 |
| 14 | Burdur (Bucak) | E70 |
| 15 | Burdur (Gölhisar) | E313 |
| 16 | Bursa (Nilüfer) | E264 |
| 17 | Bursa (Orhangazi) | E316 |
| 18 | Denizli (Merkez) | E171 |
| 19 | Denizli (Çal) | E168 |
| 20 | Edirne (Merkez) | E109 |
| 21 | Edirne (Havsa) | E321 |
| 22 | Eskişehir (Merkez) | E154 |
| 23 | Hatay (Merkez) | E89 |
| 24 | İstanbul (Çatalca) | E224 |
| 25 | İstanbul (Şile) | E312 |
| 26 | Kastamonu (Merkez) | E114 |
| 27 | Kayseri (Merkez) | E185 |
| 28 | Kırklareli (Merkez) | E16 |
| 29 | Kırklareli (Kofçaz) | E21 |
| 30 | Kocaeli (Merkez) | E166 |
| 31 | Konya (Merkez) | E179 |
| 32 | Konya (Akşehir) | E297 |
| 33 | Kütahya (Merkez) | E40 |
| 34 | Malatya (Merkez) | E129 |
| 35 | Malatya (Yeşilyurt) | E207 |
| 36 | Manisa (Merkez) | E35 |
| 37 | Mersin (Merkez) | E212 |
| 38 | Mersin (Anamur) | E315 |
| 39 | Muğla (Merkez) | E261 |
| 40 | Niğde (Merkez) | E190 |
| 41 | Niğde (Bor) | E295 |
| 42 | Ordu (Merkez) | E195 |
| 43 | Sakarya (Merkez) | E94 |
| 44 | Sakarya (Sapanca) | E230 |
| 45 | Sinop (Merkez) | E205 |
| 46 | Sivas (Merkez) | E132 |
| 47 | Tokat (Merkez) | E151 |
| 48 | Trabzon (Merkez) | E200 |
| 49 | Tunceli (Merkez) | E63 |
| 50 | Tunceli (Hozat) | E340 |

### *2.2. Screening and Culture Conditions*

*Bacillus* sp. isolates were screened for single-cell oil production using of the medium composition described by Pan *et al*. (2009). The same amount of glucose was used instead of only xylose in the medium. *Bacillus* sp. strains from stocks were first grown in pre-incubation medium. The contents of the preincubation medium are (g/L) glucose 20.0, yeast extract 0.5, $(NH_4)2SO_4$ 5.0, $MgSO_4.7H_2O$ 0.5 and $KH_2(PO_4)$ 1.0 (250 ml Erlenmeyer flasks with 50 ml medium). Inoculated bacteria were grown in a shaker incubator at 150 rpm for 48 hours at 37°C. Then, 5 mL of the growth cultures with $OD_{600}$ = 0.3 were inoculated into a nitrogen-limited medium. This medium contains (g/L) Glucose 40.0, and yeast extract 1.0, $(NH_4)2SO_4$ 2.0, $MgSO_4.7H_2O$ 1.5, $KH_2PO_4$ 7.0, $NaH_2PO_4$ 2.0 (250 ml Erlenmeyer flasks with 45 ml medium). Bacteria were incubated for 5 days at 37°C in a 150 rpm shaker.

### *2.3. Extraction of Single Cell Oil (SCOs)*

Single-cell oil (SCOs) content was determined by the methods of Pan *et al*. (2009) and Andeden (2021). After five days of incubation, 50 mL of growth medium was centrifuged at 6000 rpm for ten minutes. The pellets obtained after centrifugation were washed twice with distilled water.

Different lysis methods have been used for cell lysis. Of these, in the freezing and thawing method, the pellets were kept in the freezer at -5°C for ten

minutes. The mixture was then exposed to boiling water for ten minutes. This process was repeated three times to allow complete cell lysis. As with the other lysis method, 300 mg glass beads of 0.5 mm diameter were used. Pellets were vortexed vigorously with glass beads for ten minutes. After both cell lysis methods, 10 mL of chloroform/methanol/water (1:1:0.8) mixture was added to the samples and stirred at room temperature for two-three hours. It was then centrifuged at 4000 rpm for five min. to allow complete separation of the upper and lower phases. Since there were unwanted parts such as cell debris in the lower phase after centrifugation, the lipids in the lower phase were carefully removed with a Pasteur pipette and transferred to a clean tube (Pan *et al*. 2009). The tubes were left open in the air overnight to evaporate all of the chloroform. Then the tubes were weighed, and the amount of lipid was calculated. Lipid productivity (1) and lipid content (2) were calculated with the following formula as gravimetrically (Akın 2017).

$$\text{Lipid productivity } (\text{g.L}^{-1}.\text{d}^{-1}) = \frac{\text{weight of extracted oil (g)}}{\text{volume of culture x time(day)}} \quad (1)$$

$$\text{Lipid content } (\%) = \frac{\text{lipid productivity (g/L)}}{\text{biomass (g/L)}} \text{x100} \quad (2)$$

### *2.4. Determination of culture dry weight*

50 ml of culture (five days of production under production media and production conditions above) was collected by centrifugation (6000 rpm, ten min.) and washed with distilled water and centrifuged again. It was then dried to constant weight at 110°C. The weight of the biomass was determined gravimetrically (Wynn *et al*. 2001). Cell dry weight was calculated using the following equation (3) (Zainuddin *et al*. 2022).

$$\text{Cell dry weight } (\text{g.L}^{-1}) = \frac{\text{Weight of the dried cell (g)}}{\text{Volume of biomass (L)}} \quad (3)$$

All values derived are the means of triplicate measurements.

### *2.5. Lipid Staining*

Lipid-producing *Bacillus* strains were selected and staining of both cells and colonies were performed to show the presence of lipids.

Sudan Black B and Nile red are generally used to stain intracellular lipids, but Sudan III staining was also performed in the study. For this purpose different dyeing methods were used. The staining was performed by using the Burdon (1946) method, bacterial cells were fixed on the slide, and 0.3 g Sudan Black B dye solution prepared in 70% ethanol was poured on the slide and stained for 15 minutes. The dye was then poured off and the slide was air-dried. It was washed with xylol and dried again. It was stained with 0.5% saffranin for five-ten seconds. Dye was spilled and washed with water. The slide was dried in air and examined under a light microscope (Nikon Eclipse E1000) with using immersion oil (Arda 2000).

On the other hand, in our study, a dye test was also performed with Sudan III staining for trial purposes by ourselves. After the bacterial cells were fixed on the slide, 0.08% g Sudan III dye solution prepared in 95% ethanol (Gogte *et al*. 1989) was dripped and waited for 15-20 minutes. The slide was washed with water, dried and examined under a microscope with using immersion oil. In addition, after Sudan III staining, the dye was poured and the slide was washed with water and stained with 0.5% safranine for five-ten seconds. It was then washed with water, dried and examined under a light microscope (Nikon Eclipse E1000, x100x) with oil immersion. The appearance of lipid granules in the cell in blue-black or blue-gray color under the microscope was evaluated as positive (Arda 2000).

To demonstrate the presence of lipids in the colonies, Gogte *et al*. (1989)'s method was used. For this purpose, bacteria were inoculated on nitrogen-limited agar medium by smear method and grown for five days at 37°C. Whatman no:1 filter paper was placed on the colonies that grew in the petri dish and the colonies were transferred to the filter paper. The filter paper containing the colonies was dried at 50°C for 30 minutes. Then, it was stained with 0.08 g Sudan Black B dye prepared in 95%

ethanol for 20 minutes. The dye was removed with 95% ethanol and the filter paper was washed again in 95% ethanol for five-ten minutes and then air-dried. Colonies with lipid content were considered as lipid positive with their blue or grayish appearance.

## 3. Results and Discussion

The use of microbial lipids in various biotechnological applications is a promising alternative. In this study, *Bacillus* sp. strains that have not been studied on lipid production before were included in the trial. It was screened whether *Bacillus* sp. strains, which are industrially good producers of extracellular enzymes, produce lipids. In the study, 50 *Bacillus* sp. strains previously isolated from different provinces soils were used. Of these bacteria, 48 bacteria were not found to grow in the single-cell oil production medium, and two bacteria were found to grow. These bacteria were *Bacillus* sp. E40 isolated from Kütahya soil and *Bacillus* sp. E226 isolated from Balıkesir soil. In the study, two different methods were applied for cell lysis, which was the first step in lipid extraction, and it was seen that the best lysis method was performed with beads (Fig. 1A and 1B).

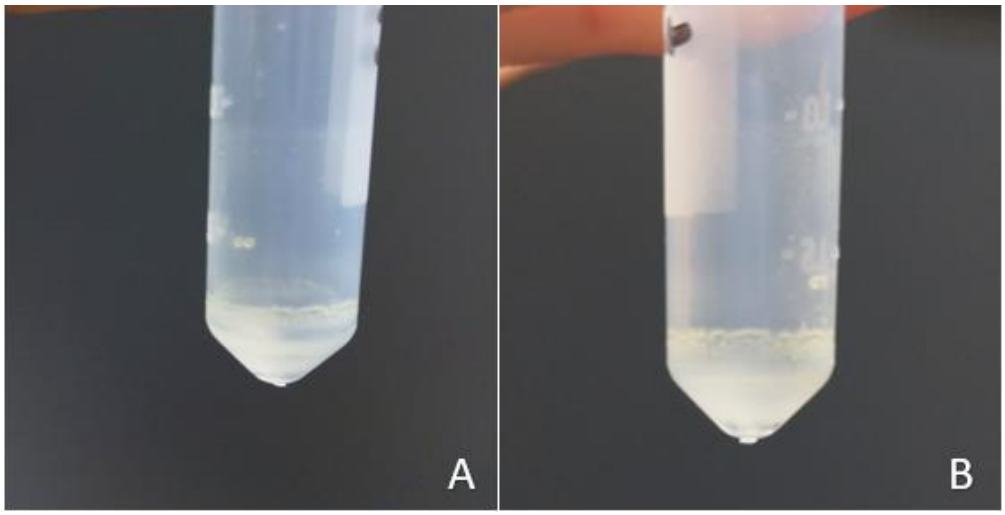


**Figure 1.** (A) Lipid extraction after cell lysis by freze-thaw method, (B) Lipid extraction after cell lysis with glass beads.

Bacteria were then characterized for their biomass, lipid yields, lipid contents as listed in Table 2. The biomass of *Bacillus* spp. E40 and E226 bacterial isolates were relatively low, 0.28 and 0.22 $g.L^{-1}$, respectively. However, the highest lipid content was reached by E226 with 54,5%. *Bacillus* spp. E40 lipid content was determined as 46,4%.

**Table 2.** Lipid characterization of *Bacillus* sp. strains.

| Bacteria | Biomass ($g.L^{-1}$) | Lipid yield (output) ($g.L^{-1}.d.^{-1}$) | Lipid content (%) |
|---|---|---|---|
| *Bacillus* spp. E40 | 0.28 | 0.13 | 46.4 |
| *Bacillus* spp. E226 | 0.22 | 0.12 | 54.5 |

When the results obtained in this study were compared with the results of studies with oily yeasts in the literature, it was found that the percentage of oil content of both Bacillus strains was higher. For example, *C. tropicalis* V139 had 27% oil content (biomass 3.56 $g.L^{-1}$), while *M. pulcherrima* V213 (biomass 3,5 $g.L^{-1}$ ) was 29%. The highest lipid content (64%) was reported to be obtained from *P. kudriavzevii* V194 (Bettencourt *et al*. 2020). Pan *et al*. (2009) found that the lipid content in yeasts varied between 38,94% to 17,32% in their studies with fatty yeast cells. On the other hand, it was stated that the same yeast species had different lipid content. The persentage of lipid content of *Prunus domestica* PD D2 and *Prunus domestica* PD F1 was found to be 34.8 and 27.3, respectively. Their biomasses were 8.0 and 7.3 (g/L), respectively (Maina *et al*. 2017).

Lipid contents of *Yarrowia lipolytica* JCM 232 different media were examined and 78,3% lipid content was reported in one medium (biomass 8,29 g/L) (Zainuddin *et al*. 2022).

Comparing the biomass of oily yeasts with *Bacillus* sp. biomass, *Bacillus* biomass remained relatively low. This can be explained by the size and cell structure of the cells. This is because of the yeasts are larger in size than bacteria.

Although several oily filamentous fungi have been identified and investigated for SCO production, high production costs and technical difficulties still make the process less attractive compared to traditional lipid sources for biodiesel production (Mhlongo *et al*. 2021).

Lipid production was investigated in the presence of different carbon sources. 43% lipid yield from *Trichoderma viride* NRC 314 in the presence of dextose at five days (Ali and El-Ghonemy 2014), 26% lipid yield from *Mucor circinelloides* URM4182 in the presence of sugarcane molasses at five days (Bento *et al*. 2020), 25% lipid yield from *Mortierella alpina* NRRL-A-10995 in the presence of glucose at 14 days (Mironov *et al*. 2018), and 32% lipid yield from *Mucor circinelloides f. lusitanicus* ATCC 1216B in the presence of hydrolyzed whey permeates at five days (Chan *et al*. 2020) has been obtained. It was determined that some molds were good lipid producers, among them 60% of *Cunninghamella japonic*a and 86% of *Mortierella isabellina (*Denli and Tekin 2000*)*.

It was stated that the main lipid producers are yeasts, fungi and algae, while bacteria are bad producers (Wynn and Ratledge 2005, *Li et al*. 2008, Bellou *et al*. 2016). But, marine bacteria, particularly the genus of Photobacterium, Shewanella, Colwellia, Photobacterium, Psychromonas, Moritella, Alteromonas and Vibrio, are found to be one among the major microbial producers of polyunsaturated fatty acids (Moi *et al*. 2018). On the other hand, it was observed that three bacteria from a total of 27 isolates obtained from both marine and freshwater sources were strongly positive for lipid accumulation. Among these three, highest percentage i.e. 27,5% of lipid was produced by the isolate No. PM2 (*Brevibacillus laterosporus*), around 14.4% lipid content was noted for the isolate No. PM5 (unidentified organism) and about 3.06% lipid content for isolate No. AlkM3 (*Sphingomonas paucimobilis*) (Masurkar *et al*. 2023). In this study, *Bacillus* sp. E226 was found to be more efficient with 54,5% lipid production. Although yeasts and molds have an important place in microbial oil production, it is important that the *Bacillus* strains we used in

our study produced higher oil than some oily yeast and fungi.

On the other hand, both intracellular lipid staining and colony staining showed the presence of lipids in two *Bacillus* strains. In microbial lipid dyeing, Sudan Black B is recommended because it is superior to other Sudan dyes 8 (Hartman 1940, Published online: 12 Jul 2009). In the experiments, the presence of lipids could not be demonstrated in Sudan III cell staining. In the study performed with Sudan Black B staining, the cells were seen as blue-black or blue-grey in the examination with using immersion oil under the light microscope (100x) in intracellular staining for both bacteria (Figures 2A and 2B).

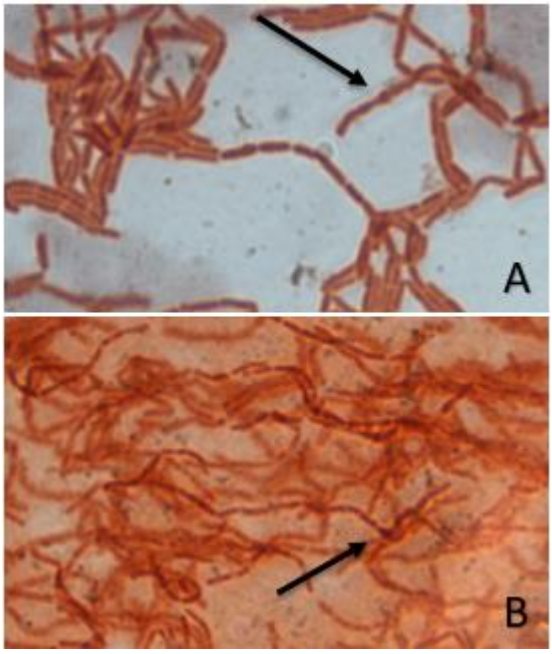


**Figure 2.** Sudan Black B staining in *Bacillus* spp. E226 (A) and *Bacillus* spp. E40 (B) cells.

On the other hand, bacterial colonies that grew on the petri dish for five days were also stained. Colonies on the Petri dish were transferred to Whatman No:1 paper and after staining on the paper, it was observed that the colonies were grayish in color (Figures 3A and 3B).

When the results of both stainings were also examined, in this study, it was reported that the bacteria were lipid producers.

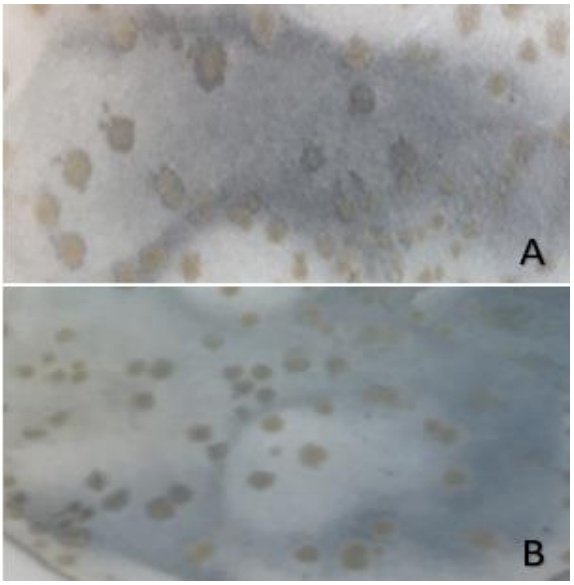


**Figure 3.** Lipid staining of *Bacillus* spp. E226 (A) and *Bacillus* spp. E40 (B) colonies on Whatman no:1 filter paper.

This study was the first to report that two *Bacillus* bacteria isolated from different provinces soils were also single-celled oil producers. As a result of detailed studies on this subject (fermentation temperature, pH, degree of aeration, sugar type and concentration as carbon source, determination of C/N ratio and fat content), these bacteria could be included in the class of oily organisms. Because, microorganisms that can lipid accumulate more than 20% of their biomass, such as bacteria (*Bacillus*), yeasts, molds and algae, are known as oleaginous microorganisms (Madani *et al*. 2017). That can be used in various biotechnological processes could have potential.

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