Biosurfactant production by *Mucor circinelloides* on waste frying oil and possible uses in crude oil remediation

Parvin Hasanizadeh, Hamid Moghimi and Javad Hamedi

**ABSTRACT**

Biosurfactants are biocompatible surface active agents which many microorganisms produce. This study investigated the production of biosurfactants by *Mucor circinelloides*. The effects of different factors on biosurfactant production, including carbon sources and concentrations, nitrogen sources, and iron (II) concentration, were studied and the optimum condition determined. Finally, the strain’s ability to remove the crude oil and its relationship with biosurfactant production was evaluated. The results showed that *M. circinelloides* could reduce the surface tension of the culture medium to 26.6 mN/m and create a clear zone of 12.9 cm diameter in an oil-spreading test. The maximum surface tension reduction was recorded 3 days after incubation. The optimum condition for biosurfactant production was achieved in the presence of 8% waste frying oil as a carbon source, 2 g/L yeast extract as a nitrogen source, and 0.01 mM FeSO₄.

*M. circinelloides* could consume 8% waste frying oil in 5 days of incubation, and 87.6% crude oil in 12 days of incubation. A direct correlation was observed between oil degradation and surface tension reduction in the first 3 days of fungal growth. The results showed that the waste frying oil could be recommended as an inexpensive oily waste substance for biosurfactant production, and *M. circinelloides* could have the potential to treat waste frying oil. According to the results, the produced crude biosurfactant or fungal strain could be directly used for the mycoremediation of crude oil contamination in oil fields.

**Key words** | bioremediation, biosurfactant, crude oil, *M. circinelloides*, waste frying oil

**INTRODUCTION**

Surfactants are organic compounds with both hydrophilic and hydrophobic parts. They tend to be at the interface of two different phases containing a diverse degree of polarity and different hydrogen bonds and, therefore, reduce the surface tension or cause emulsion. Based on the hydrophilic parts, surfactants are divided into four groups, including anionic, cationic, non-ionic, and amphoteric compounds (Fakruddin 2012). These compounds are important for various applications, such as laundry detergent, emulsion stabilizer, and emulsion disruptive. They are used in different industries, such as those of food, oil, chemical, and cosmetics (Reis *et al.* 2015). The commonly used surfactants are petrochemical products, which have unfavorable environmental impacts. Biosurfactants are biological surface-active agents produced by microorganisms. The advantages of biosurfactants over chemical reagents include sustainability, low toxicity, high efficiency, biodegradability, specificity, and stability in extreme conditions (Bezza & Chirwa 2016). Due to these advantages, they seem potent enough to replace the chemical surfactants in various industries. One of the potential uses of biosurfactants is in the oil industry. These compounds are considered as the key factors in the Microbial Enhanced Oil Recovery (MEOR) process, and the bioremediation of petrochemical contaminations due to their ability to emulsify crude oil hydrocarbons in the water phase and also their high stability in extreme conditions. So, they are useful for oil-spill remediation and for dispersing oil slicks into fine droplets and converting mousse oil into an oil-in-water emulsion (Bustamante *et al.* 2012).

Most of the microorganisms produce biosurfactants as secondary metabolites at the end of the logarithmic growth phase or in the stationary phase as they grow on hydrophobic substrates (Fakruddin 2012). These microorganisms are mainly hydrocarbon consumers. However, some strains can produce biosurfactants only in the presence of...
hydrophilic substrates (Schaechter 2009). Biosurfactants are produced by several microorganisms, including bacteria and some fungi. So far, most of the research in the field of biological active surface compounds has been carried out using bacteria and yeasts. The majority of biosurfactant-producing bacteria belong to the genera *Pseudomonas*, *Bacillus*, *Acinetobacter*, and *Arthrobacter* (Amaral et al. 2010). So far, few fungi have been reported to produce biosurfactants using renewable sources. Fungi are highly efficient in producing biosurfactants, and various studies have introduced them as a suitable choice for application in industry by replacing chemical surfactants (Amaral et al. 2010). In addition to the high yield of biosurfactants, fungi have much potential for degrading organic compounds because of their spreading mode of growth, ability to survive, grow, and reproduce in harsh environmental conditions, and their symbiotic association with other microorganisms and plants (Hosokawa et al. 2009).

Currently, the use of biosurfactants is limited because of their high production cost. An alternative to making the process economically appealing is to optimize the culture condition (Makkar et al. 2011). Using low-cost materials, such as industrial waste, is also a useful and promising strategy to reduce the cost of biosurfactant production. This can lead to a greater possibility of economical production of these compounds (Haba et al. 2000). Generally, the presence of hydrophobic substrates in the microbial medium induces the production of the biological surfactants, and the utilization of vegetable oils, animal fats, and petrochemical hydrocarbons is the most common precursor and inducer for biosurfactant production. A raw material associated with the vegetable oil industry is residual cooking or frying oil. The longer the food is cooked and the higher the temperature, the more these harmful compounds are produced. But these oils can be considered as high-energy and low-cost fermentative waste sources for microbial growth and can be transformed into high-value products like biological surfactants. Thus, these wastes can be better managed by recycling (Makkar et al. 2011).

In this study, we introduced a fungal strain, *Mucor circinelloides*, as a potent biosurfactant producer. The effect of carbon and nitrogen sources and iron (II) concentration on biosurfactant production was studied and optimized. We introduced waste frying oil as the best carbon source and low-cost substrate for biosurfactant production. The relationship between the reduction of surface tension and crude oil remediation by *M. circinelloides* was investigated and this strain was a candidate for the remediation of hydrocarbon-contaminated environments and waste frying oil treatment.

**MATERIALS AND METHODS**

**Microorganism**

During previous studies (unpublished data), *M. circinelloides* was isolated from the oil-contaminated area of the Marun oilfield (E 30° 51’ 13” N, 49° 50’ 19”) as a biosurfactant-producing strain, and was revived on the potato dextrose agar medium. The plate was incubated at 28°C for 5 days.

**Assessment of different culture media for biosurfactant production**

To achieve an efficient base culture medium for biosurfactant production, five different culture media were selected following the literature (Adamczak & Bednarski 2000; Konishi et al. 2007; Sarubbo et al. 2007; Ilori et al. 2008; Rufino et al. 2014). Each medium’s ability to produce biosurfactants by *M. circinelloides* was investigated. The compositions of the five studied media are summarized in Table 1. The following steps were performed for all five culture media. The experiments were performed in 250-mL flasks containing 50 mL of each medium. The media were sterilized at 121°C for 20 min. Fungal spores of 5×10⁶ were added into each flask and incubated at 28°C and 180 rpm for 7 days. The cells were harvested by centrifugation at 4,000 g for 20 min. The reduction in surface tension activity in cell-free supernatants was evaluated using the oil-spreading test and the Du Nouy ring method. All the experiments were performed in triplicate in three different biological runs.

**Do Nouy ring method**

The surface tension was measured using a Kruss K7 tensiometer (Atension 700 Germany) (Qazi et al. 2014). The sample temperature was adjusted to 25°C. A 20-mL volume of each sample was poured into the sample container of the tensiometer and the surface tension was measured in triplicate for each sample in three different biological runs to increase the accuracy of the measurements. Distilled water and an uninoculated medium were used as controls.
Oil-spreading method

Fifty milliliters of distilled water was added to a petri dish with a 15-cm diameter. Twenty microliters (μL) of crude oil was added to the water surface. Subsequently, 10 μL of fermentation broth was added to the oil surface. The diameter of the visually detectable clear zone on the oil surface was measured after 30 s. Each medium was measured in three different runs. Distilled water and an uninoculated medium were used as the negative controls (Sekhon et al. 2012).

Time-course profile of biosurfactant production by M. circinelloides

The fungal spores (5 × 10⁶) were inoculated in a 250-mL flask containing 50 mL of the selected medium (Medium 1). The flasks were incubated at 28 °C and 180 rpm. Five-milliliter samples were taken at 24-h intervals and the surfactant production was evaluated. The experiments were performed in triplicate.

Optimization of surfactant production by M. circinelloides

Four major parameters were used to optimize biosurfactant production conditions, including the carbon source type and concentration, the nitrogen source, and the concentration of Fe (II) (Table 2). The operational parameters were initially maintained at pH 7, 28 °C, and an agitation speed of 180 rpm in 100-mL Erlenmeyer flasks containing 20 mL basal salt medium. The values of these parameters varied according to the scheme given in Table 2. Surfactant production was calculated in terms of the oil-spreading zone diameter as previously described. To have an equal amount of inoculum in each experiment, each flask was inoculated with 5 × 10⁶ spores. All experiments were carried out in triplicate.

Evaluation of the crude-oil-removal efficiency in M. circinelloides

The ability of M. circinelloides to degrade crude oil was evaluated using total petroleum hydrocarbon assay (TPH) by spectrophotometry (Rahman et al. 2002). Fungal spores (5 × 10⁶) were inoculated into the minimal salt medium (MSM) supplemented with 1% crude oil as a sole carbon source. The uninoculated medium was considered as the control to confirm the amount of oil removed. The inoculated flasks and the control were incubated at 28 °C and 180 rpm for 12 days. Every 3 days, the fermentation broth was evaluated for the extraction of the total hydrocarbon.

Table 1 | The compositions of five different studied media for the cultivation of biosurfactant producer M. circinelloides

<table>
<thead>
<tr>
<th>Order</th>
<th>Media constitutes, pH</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soybean oil 5 g/L, Yeast extract 0.1 g/L, NaNO₃ 0.3 g/L, KH₂PO₄ 0.025 g/L, MgSO₄(H₂O)₇ 0.025 g/L (pH:7)</td>
<td>Konishi et al. (2007)</td>
</tr>
<tr>
<td>2</td>
<td>Soybean oil 6 g/L, NH₄NO₃ 0.1 g/L, KH₂PO₄ 0.02 g/L, MgSO₄(H₂O)₇ 0.02 g/L, Glutamic acid 0.1 g/L (pH:7)</td>
<td>Rufino et al. (2014)</td>
</tr>
<tr>
<td>3</td>
<td>NaNO₃ 0.25 g/L, MgSO₄(H₂O)₂ 0.02 g/L, KH₂PO₄ 0.02 g/L, Yeast extract 0.1 g/L, Glucose 8 g/L (pH:6.5)</td>
<td>Adamczak &amp; Bednarski (2000)</td>
</tr>
<tr>
<td>4</td>
<td>NaNO₃ 0.2 g/L, CaCl₂ 2H₂O 0.01 g/L, KH₂PO₄ 0.3 g/L, MgSO₄ 0.02 g/L, FeSO₄ 7H₂O 0.001 g/L, Na₂HPO₄ 12H₂O 0.3 g/L, KCl 0.1 g/L, Yeast extract 0.002 g/L, Crude oil 1 g/L (pH:7)</td>
<td>Sarubbo et al. (2007)</td>
</tr>
<tr>
<td>5</td>
<td>NH₄NO₃ 0.1 g/L, KH₂PO₄ 0.02 g/L, MgSO₄ 7(H₂O) 0.02 g/L, Yeast extract 0.2 g/L, Glucose 5 g/L, Canola oil 5 g/L (pH:6.7)</td>
<td>Ilori et al. (2008)</td>
</tr>
</tbody>
</table>

Table 2 | Evaluated factors in the optimization process and their studied values

<table>
<thead>
<tr>
<th>Case study factor</th>
<th>Condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon source</td>
<td>Sunflower oil, Waste frying oil, Soybean oil, Canola oil, Diesel, Glycerol, Glucose (5% w/v)</td>
<td>Makkar et al. (2011)</td>
</tr>
<tr>
<td>Oil concentration</td>
<td>2%, 4%, 6%, 8%, 10% (v/v)</td>
<td>Andrade Silva et al. (2014)</td>
</tr>
<tr>
<td>Nitrogen source</td>
<td>Urea, Yeast extract, beef extract, Peptone, Ammonium nitrate (2 g/L)</td>
<td>Qazi et al. (2014)</td>
</tr>
<tr>
<td>Iron concentration</td>
<td>0, 0.1, 0.02, 0.1, 0.2 (mM)</td>
<td>Gudiña et al. (2015)</td>
</tr>
</tbody>
</table>
content with toluene. Subsequently, to determine the amount of the remaining oil, the absorption of the sample was measured at 420 nm (Rahman et al. 2002). In addition, surfactant production during the fungal growth on MSM with crude oil was evaluated for each sample. All the experiments were done in triplicate.

Statistical analysis

The results obtained from the different tests were analyzed using SPSS Version 22. The statistical tests used in this study were analyzed by the Tukey’s test, and the one-way analysis of variance (ANOVA) test. A significance level of results was reported at $P < 0.05$.

RESULTS AND DISCUSSION

Fungal isolation from contaminated soil

Marun area is located in the southwest of Iran and consists of two oil reservoirs that have been producing sweet oil and gas since 1964. The Marun oil field is estimated to contain recoverable oil reserves of 22 billion barrels, making it the world’s sixth largest oil field. In December 2009, an oil well in Marun leaked around 20,000 barrels of crude oil, contaminating around 100 hectares of land and the Jarahi River, which supplies water to three cities in Iran. In addition, petrochemical hydrocarbons leaked into the soil and water because of oil and gas exploration and extraction, worsening environmental pollution. In this study, *M. circinelloides* was isolated from the crude-oil-contaminated soil of Marun. Isolation and bio-augmentation of indigenous organisms from oil-contaminated sites is of great interest because these organisms will be more adapted to prolonged exposure to contaminants compared with exotic microorganisms (Pasumarthi et al. 2013). The presence of hydrophobic substrates in contaminated Marun soil greatly increases the chance of finding microorganisms with biosurfactant production activity. There are some reports about the pathogenicity of some *M. circinelloides* strains in humans, and their relationship with some mycosis (Iwen et al. 2007; Khan et al. 2009). On the other hand, there are scientific papers related to the biotechnological uses of this strain in different fields, such as biodiesel production (Carvalho et al. 2017), extracellular proteases production (Andrade et al. 2002), wastewater treatment, and bio-oil production (Bhanja et al. 2014). These studies mentioned that environmental strains of this fungus are saprophytic and lack virulence factors (Bhanja et al. 2014; Carvalho et al. 2017).

Assessment of surfactant production in different media

Biosurfactant production was evaluated in five different media. The maximum production of surfactant occurred in Medium 1 containing 5 g/L soybean oil as the carbon source (Figure 1). Results of the surface tension test showed that, after 7 days, *M. circinelloides* could reduce the surface tension to 29 mN/m in Medium 1, compared with distilled water (70 mN/m) as the control (Figure 1). In addition, the results of the oil-spreading test demonstrated that, after 7 days, a clear zone of 9-cm diameter was achieved by the medium containing soybean oil and yeast extract as a carbon and nitrogen source, respectively (Figures 1 and 2).

![Figure 1](https://iwaponline.com/wst/article-pdf/76/7/1706/450318/wst076071706.pdf)
The effect of the carbon and nitrogen sources and Fe (II) concentration on biosurfactant production

Despite the numerous advantages of biosurfactants over chemical surfactants, operational problems and high cost of large-scale production are the main obstacles to increasing the feasibility of biosurfactant production (Zhu et al. 2010). To minimize the production costs, various studies have focused on increasing the productivity of surface active agents (Saharan et al. 2014). The optimization of culture conditions is one of the most proposed strategies to increase the production efficiency and reduce the costs (Sekhon et al. 2015). Despite the variety of biosurfactant structures, there are factors that generally affect biosynthesis of all the biosurfactants.

In this study, the effect of different carbon sources, including different types of vegetable oils, waste frying oil, glycerol, glucose and diesel on biosurfactant production was assessed by M. circinelloides. The maximum biosurfactant production (11.7 cm oil spreading) occurred in the presence of waste frying oil as a carbon source. Also, biosurfactant production was very low (<6 cm oil spreading) in the presence of glycerol and glucose as hydrophilic substrates (Figure 3(a)). Some studies have reported biosurfactant production by bacterial and fungal strains in the presence of hydrophilic substrates (Schaechter 2009), whereas many related studies have stated that the presence of hydrophobic substrates is necessary for the production of biosurfactants (Cooper & Paddock 1983; Karanth et al. 1999). A former study using Arthrobacter paraffineus showed the lack of biosurfactant production when glucose was used instead of hexadecane (Karanth et al. 1999). Cooper & Paddock (1983) reported that Turupsis petrophilium could not produce glycolipid using hydrophilic substrates.

Using low-cost materials like industrial wastes is a useful strategy to reduce the cost of biosurfactant production, which can lead to a significant decrease (10–30%) in the cost of upstream processes (Makkar et al. 2011). Many types of vegetable oils are produced annually (Haba et al. 2013). Much of the oil is discarded as waste after use and only a small amount is recycled (Yaakob et al. 2013). Waste frying oil causes several issues with sewer and septic systems. That is because when vegetable oil cools and settles, it congeals. This can clog up pipes and corrode certain materials, and also, pose problems for wildlife (Chhetri et al. 2008). In this study, waste frying oil was selected as the best substrate for biosurfactant production by M. circinelloides. The optimization of waste frying oil concentration demonstrated that increasing it up to 8% led to the maximum biosurfactant production. The total amount of oil (8% v/v) added to the culture medium was consumed by the fungal strain during 7 days of incubation. Biosurfactant production decreased at a concentration of 10% (Figure 3(b)). Zhu et al. (2007) showed that oil concentration and hydrophobic substrate are effective factors in biosurfactant production by Pseudomonas aeruginosa. According to their results, increasing the oil concentration up to 4% caused an increase in biosurfactant production, and at concentrations above 4%, biosurfactant production was reduced. The results of this study showed that increasing the concentration of frying oil waste as a potential low-cost substrate up to 8% leads to an increase in biosurfactant production by M. circinelloides. Taking into account strategies in the scaling-up process and with the knowledge of waste...
frying oil consumption in the laboratory scale experiments, *M. circinelloides* could be offered for microbial waste frying oil treatment in an aerobic, submerged fermentation process. In this condition, we expect all the waste frying oil to be consumed in 7 days and the biosurfactant to be produced as a valuable product so that it could be recommended in MEOR or spill oil remediation.

The evaluation of the effect of different nitrogen sources on biosurfactant production showed that maximum production (12.5 cm oil-spreading zone) occurred in the presence of 2 g/L yeast extract. Urea was a more efficient inorganic nitrogen source for biosurfactant production by this strain compared with ammonium nitrate (Figure 3(c)). Ammonium nitrate and urea were reported to be the best nitrogen sources for biosurfactant production by *Artherobacter paraffineus* whereas the best nitrogen source reported for biosurfactant production by *P. aeruginosa* and *Rhodococcus* sp. was nitrate (Karanth *et al.* 1999). The best concentration of Fe (II) added in the form of FeSO₄ for biosurfactant production by *M. circinelloides* was 0.01 mM, and the production decreased at higher concentrations (Figure 3(d)). Iron concentration plays an important role in the production of biosurfactants (Gudiña *et al.* 2015). Iron deficiency can cause an overproduction of biosurfactants by *P. aeruginosa* and *P. fluorescens* (Glick *et al.* 2010). Karadi *et al.* (2013) stated that the biosurfactant production of *Flavobacterium* increased in low concentrations of iron and decreased in high concentrations. Biosurfactant production by *M. circinelloides* increases in low iron concentration but our results showed that Fe (II) has an inhibitory effect on biosurfactant production at concentrations up to 0.01 mM (Figure 3(d)). Finally, in the optimal condition of carbon, nitrogen, and iron concentrations, the surface tension was reduced to 26.6 mN/m by *M. circinelloides*.

**Time course of biosurfactant production and evaluation of the oil removal efficiency**

Bioremediation is an attractive strategy to remediate crude oil contaminations (Banat *et al.* 2010). A major problem with the bioremediation of hydrophobic compounds is the low availability of these compounds for microorganisms. The larger the surface area of the oil accessible to bioremediating microorganisms, the faster the oil spill can be degraded. Biosurfactants reduce the interface tension and, as a result, the solubility of hydrophobic compounds increases (Bezza & Chirwa 2016). In this study, the biosurfactant produced by the fungal strain was measured at regular intervals (72 h) over 12 days.
According to Figure 4, the surface activity increased rapidly after inoculation, reaching its highest value (12.9 cm oil-spreading zone) after about 72 h. To determine the crude oil degradation efficiency and biosurfactant production, crude oil removal was measured with specific time frequency. The maximal oil removal (66.13%) was achieved in the first 72 h of the incubation (Figure 4). The slope of the oil-removal diagram decreased considerably since the third day in such a way that until the 12th day, the strain removed only 21.47% of the remaining oil (Figure 4). Finally, *M. circinelloides* could utilize 87.6% of the petroleum compounds in a basal salt medium for 12 days. Reis et al. (2013) reported a relationship between biosurfactant production and the removal of hydrophobic compounds. The maximum biosurfactant production occurred in 72 h after inoculation and it corresponded to crude oil biodegradation (Figure 4). So, the biosurfactant production and the oil removal were coincidental (Figure 4). To determine the statistical correlation between biosurfactant production and oil removal, Pearson’s correlation coefficient was calculated for these two variables. The value was –0.77, indicating a strong relationship. *M. circinelloides* was isolated from the oil-contaminated soil of Marun area. So, this indigenous strain may be naturally capable of crude oil degradation in such oil-contaminated areas, and biosurfactant production by this strain could help solubilize and utilize hydrophobic hydrocarbons in such polluted environments.

The addition of biosurfactants, bio-emulsifiers, and/or biosurfactant-producing microorganisms can be used in soil biodegradation techniques, for soil washing, and for wastewater treatment (in situ and ex situ) (Urum & Pekdemir 2004; Zhou & Zhou 2008). According to our results, *M. circinelloides* could be considered as a potent candidate to bio-remediate crude-oil contamination in two different ways: bio-stimulation of the environment by adding the required mineral nutrients and bio-augmentation of the environment by fungal spores to grow the strain and produce biosurfactants and degrade petrochemical hydrocarbons. In the second way, biosurfactants could be produced in the presence of waste frying oil as an inexpensive carbon source by the fungal strain. Then, the produced fermentation broth or the crude biosurfactant could be recommended to recover oil from the oil-contaminated sand (soil washing). Moreover, the crude or partially purified biosurfactant could be used to increase the bioavailability of hydrophobic pollutants to enhance and accelerate the degrading process in contaminated soil and waters.

**CONCLUSION**

Our results revealed that the optimal condition for biosurfactant production by this strain was 8% waste frying oil as a carbon source, 2 g/L yeast extract as a nitrogen source, and 0.01 mM FeSO₄. The observed surface tension...
reduction was very satisfactory (26.6 mN/m), especially when considering that the substrate was composed of waste that would otherwise pose an environmental hazard. *M. circinelloides* could use up 8% (v/v) waste frying oil in 7 days of incubation. These results indicated the possibility of waste frying oil to be used as an inexpensive alternative substrate for the economic production of biosurfactants by *M. circinelloides* and a useful strategy in solving environmental problems resulting from this dumped waste. The evaluation of the oil removal showed that *M. circinelloides* could remove 87.6% of the crude oil in the MSM medium during 12 days. The Pearson’s correlation coefficient revealed a strong coincidence between the biosurfactant production rate and oil removal by *M. circinelloides*. It would be possible to develop approaches to use this strain to remove hydrocarbons from polluted water by providing the required nutrients in those sites or using the produced biosurfactant in the washing process of oil-contaminated soil. To our knowledge, this is the first report of *M. circinelloides* as a potent biosurfactant-producing fungal strain by the utilization of waste frying oil and its strong capability of oil hydrocarbon removal.

**ACKNOWLEDGEMENT**

This project was financially supported by the University of Tehran under grant No. 321265/04/6.

**REFERENCES**


Konishi, M., Morita, T., Fukuoka, T., Imura, T., Kakugawa, K. & Kitamoto, D. 2011 Production of different types of mannosylerythritol lipids as biosurfactants by the newly isolated yeast strains belonging to the genus Pseudozyma. Applied Microbiology and Biotechnology 75 (3), 521–531.


First received 11 February 2017; accepted in revised form 23 May 2017. Available online 15 June 2017