Optimization of Mycoremediation Potential of A Fungi: *Aspergillus Ochraceus* Strain

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Abstract

Mycoremediation is an important process that targets the removal of petroleum hydrocarbons by fungi. Accordingly, colorimetric method was used in the preliminary investigation of petroleum degradation with ten fungal strains as *Aspergillus ochraceus, Aspergillus parasiticus, Aspergillus niger, Fusarium acuminatum, Fusarium graminearum, Fusarium equiseti, Fusarium oxysporum, Paecilomyces lilac, Penicillium crustosum*, and *Penicillium chrysogenum.* Petroleum degradation of spore suspension, live biomass (fungal pellet and disc) and cell-free culture supernatant of the potent *A. ochraceus* strain were determined by gravimetric analysis. It was obtained that the fungal disc (94%) was more successful than the spore suspension (87%) in petroleum degradation under optimized conditions as pH:5.0, 1% of petroleum concentration, %5 (v/v) of inocum concentration, 1 g/100mL of inoculum amount and 7 days of incubation period. The degradation rate constant and half-life period of spore suspension were calculated as 0.291 day^{-1} and $t_{1/2}$ = 0.340 and of fungal disc were 0.401 day^{-1} and $t_{1/2}$ = 0.247. 7.5% and 10% (v/v) concentration of cell-free culture supernatant were achieved more than 80% of petroleum removal. However, the cell-free culture supernatant was not as effective as fungal disc. According to GC/MS analysis, the fungal disc of *A. ochraceus* strain degraded long chain *n*-alkanes such as C_{35} and C_{36} more effectively than *n*-alkanes in the range of C_{22}-C_{34}. Drop-collapse and oil-spreading methods showed that *A. ochraceus* is a good biosurfactant producer. This study clearly pointed out that *Aspergillus ochraceus* NRRL 3174 strain with high its removal capacity can be used as an effective agent in petroleum bioremediation process.

Highlights

- The colorimetric method showed that *Aspergillus ochraceus* was the most potent strain in petroleum removal among 10 fungal strains.
- The fungal disc of potent strain (94%) was more successful than the spore suspension (87%) in petroleum degradation under optimized conditions.
- %7.5 and %10 concentration of cell-free culture supernatant was not as effective as fungal disc.
- The fungal disc degraded long chain *n*-alkanes such as C_{35} and C_{36} more effectively than *n*-alkanes in the range of C_{22}-C_{34}.
- *A. ochraceus* is also a good biosurfactant producer.

Introduction

Petroleum consists of the saturated hydrocarbons, aromatic hydrocarbons and polar organic compounds. Environmental pollution caused by petroleum and petroleum derivatives is important problem of developing countries (Balaji et al. 2014). Especially, polycyclic aromatic hydrocarbons in the structure of petroleum (PAHs) is one of the major environmental pollutants that damage lung, kidney, liver, intestine and other internal organs with its anthropogenic activities, carcinogenic and mutagenic properties. Removal of these pollutants from the environment by traditional physicochemical methods is

expensive and time consuming. In addition, these methods are not effective enough, and their applicability to large areas is very low. In this direction, the most promising approach to treating these areas is biological methods (Deshmukh et al. 2016; Islam et al. 2017). Microbial Enhanced Oil Recovery (MEOR) is a cost-effective and environmentally friendly technology as well as being biologically based (Zhang et al. 2016; Asemoloye et al. 2020). Microbial degradation is one of the most important and fundamental mechanisms in the bioremediation of petroleum hydrocarbons. Biodegradation is defined as the transformation of chemical compounds into energy, mass, $CO_{2,}$ and biological waste products through living microorganisms (Marinescu et al. 2009; Wang et al. 2010; Joutey et al. 2013). Biosurfactants produced by microorganisms in the biodegradation process are very important due to increasing the solubility and usability of petroleum. They are also important biotechnological products with great uses in other industries (Khan and Butt 2016).

Many microorganisms, including bacteria, fungi, and algae, can use petroleum hydrocarbons as a carbon and energy source (Al-Hawash et al. 2018a). They play an important role on environmental pollutants such as hydrocarbons, heavy metals, dyes, and pesticides with their biodegradation and biosorption mechanisms. There are three basic mechanisms in the uptake of hydrocarbons into the cell by microorganisms. In the first mechanism, microorganisms take hydrocarbons dissolved in the aqueous phase directly into the cell and use them as a carbon source. In the second mechanism, the target compound is enzymatically degraded but it is not used as a carbon source (co-metabolism). In the third mechanism, while hydrocarbons larger than the cell cannot be easily metabolized, the target compound establishes a direct relationship with microbial cells, is taken into the cell (biosorption) and concentrated (bioaccumulation).

Mycoremediation is an important process that targets the removal of organic compounds by fungi. Although fungi use all three strategies in the bioremediation process, they are more effective in co-metabolism and bioaccumulation. In addition to the aromatic and aliphatic hydrocarbons in the petroleum, the fungi can also co-metabolically transform many aromatic organic pollutants, including PAHs, biphenyls, dibenzofurans, nitroaromatics, various pesticides and plasticizers. It has been shown that the PAHs, have strong hydrophobicity, are taken into the cells by some fungi and stored in their oil vesicles. The cross-linking mechanism between the PAHs and the cell wall is not fully known (Fritsche and Hofricher 2000; Abruscia et al. 2007; Thion et al. 2012; Xu et al. 2013; Shi et al. 2019). The mycoremediation potential of fungi is directly related to the high amounts of organic acids, chelators, oxidative and extracellular enzymes they produce (Fuad et al. 2015; Zhang et al. 2016). Yeasts can use aromatic compounds as well as transform them co-metabolically. On the other hand, mycelial growing molds can easily colonize to insoluble substrates compared to cellular growing bacteria and yeasts. While they rapidly distribute the substrates among their micelles, they release their extracellular enzymes out of the cell. In addition to chemical destruction, they also provide tolerance to high concentrations of toxic chemicals with their extracellular enzymes and mechanical complementation by the penetration of hyphae (Bennet et al. 2002). They are generally able to convert toxic compounds into low toxic products, thus increasing the susceptibility of bacteria to decomposition of petroleum. In this context, a synergistic

interaction between fungi and bacteria is realized in the mineralization process of petroleum hydrocarbons (Steliga 2012). *Aspergillus sp*., *Penicillium sp*., *Rhizopus sp*. and *Fusarium sp.* have been shown to be able to efficiently metabolize petroleum hydrocarbons (Al-Hawash et al. 2018b). *Cladophialophoria sp. and Aspergillus sp.* play a role in the removal of aliphatic hydrocarbons while *Cunninghamella sp., Penicillimum sp., Fusarium sp. and Aspergillus sp.* are effective in the removal of aromatic hydrocarbons. (Olajire and Essien 2014). Thus, fungi can grow by tolerating petroleum pollution and can be used as mycoremediation agent in the petroleum contaminated ecosystems (Vanishree et al. 2014).

Fungi have become an important focus on mycoremediation due to their high enzymatic activities. In this manner, it is aimed to investigate the petroleum biodegradation abilities of *Aspergillus ochraceus, Aspergillus parasiticus, Aspergillus niger, Fusarium acuminatum, Fusarium graminearum, Fusarium equiseti, Fusarium oxysporum, Paecilomyces lilac, Penicillium crustosum*, and *Penicillium chrysogenum*. In this context, colorimetric method was used in the preliminary investigation of petroleum degradation with the spore suspensions of these fungal strains. Petroleum biodegradation efficiencies of spore suspension, live biomass (fungal pellet and disc) and cell-free culture supernatant of the potent fungal strain (*Aspergillus ochraceus*) were determined by gravimetric analysis. Optimal physiological conditions such as initial pH, petroleum concentration, inoculation concentration and amount, and incubation period were also determined. The petroleum degradation under optimized conditions was also supported by GC/MS analysis. In the last stage, the biosurfactant production of potent fungal strain was also investigated.

Materials And Methods

Fungal strains

Aspergillus ochraceus, Aspergillus parasiticus, Aspergillus niger, Fusarium acuminatum, Fusarium graminearum, Fusarium equiseti, Fusarium oxysporum, Paecilomyces lilac, Penicillium crustosum, and *Penicillium chrysogenum* strains were used in this study. The fungal strains were stored at +4 ℃ in Hacettepe University Culture Collection Laboratory, Beytepe, Ankara, Turkey.

Preparation of inoculum for petroleum degradation

Fungal spore suspension

The fungal strains were inoculated on PDA (potato dextrose agar) (Merck) and incubation was performed at 30 ℃ in static condition for a week. Following the fungal cultures were suspended in 0.9% NaCl (pH 7.0) solution, and the spores were counted on a Thoma slide (Asemoloye et al. 2020).

Fungal disc

The fungal strains were inoculated on PDA and incubation was performed at 30 ℃ in static condition for a week. Following the incubation, 7 mm diameter of fungal discs were cut from the surface of an actively

growing fungi on PDA (Benguenab and Chibani 2020).

Fungal pellet

The fungal strains were inoculated into PDB (Potato dextrose broth) (Merck) and incubation was performed at 30 ℃ in a rotatory incubator for a week (Mipro MCI, Turkey). Then, cultures were filtered using Whatman No:1 filter paper under sterile conditions. Obtained pellets were dried at 30 °C under steril conditions (Tugrul Yucel 2018).

Cell-free culture supernatant

The fungal strain was inoculated into Bushnell Haas (BH) (Sigma-Aldrich) medium containing 1% (v/v) of petroleum and incubation was performed at 30 °C and 150 rpm for a week (Mipro MCI, Turkey) Following this, the culture was filtered using Whatman No:1 filter paper under sterile conditions.

The petroleum biodegradation assay

The BH medium (g/L: 0.2 $MgSO_4$, 0.02 $CaCl_2$, 1 KH_2PO_4, 1 K_2HPO_4, 1 NH_4NO_3, 0.05 $FeCl_3$) containing 1% (v/v) of Triton X:100, 0.1% (w/v) of glucose, 0.1% (w/v) of yeast extract and 0.1% (v/v) of trace element in 50 mL was sterilized at 121 ℃ for 15 minutes. Following the cooling of BH medium to 45 °C, 1% (v/v) of petroleum sterilized with 0.22 µm cellulose acetate syringe filter (Millipore, Sartorius) was added. 5% (v/v) of fungal spore suspension (1.5×10^7 CFU mL^{-1}), 1 g/100 mL of live biomass and 5% (v/v) of sterile culture supernatant were inoculated into BH medium and incubation was performed at 30 °C and 150 rpm under dark condition for 7 days (Mipro MCI, Turkey). All experiments were performed in triplicate (Maddela et al. 2015; Bilen Ozyurek and Avcioglu 2020a).

Colorimetric method

The redox solution was prepared by dissolving 1 g (w/v) of 2,6-dichlorophenol indophenol (DCPIP) in 1 L of distilled water. Following the inoculation of the fungal spore suspensions into BH medium containing 1% (v/v) of petroleum and 1% of redox indicator, the cultures were incubated at 30 °C and 150 rpm under dark condition for 7 days (Benguenab and Chibani 2020). Accordingly, the potent fungal strain in petroleum degradation will be determined with this method. The change of the DCPIP redox indicator from blue (oxidized form) to colorless (reduced form) indicates that the fungi are effective in hydrocarbon degradation (Lima Souza et al. 2016).

Gravimetric method

Following the incubation period, extraction was carried out with dichloromethane (DCM) (CH_2Cl_2) (1:2) (Sigma-Aldrich). The flasks containing petroleum + DCM were left in the water bath (Memmert, Schwabach, Germany) at 90 °C for 1 hour and DCM was removed. The degradation of petroleum was also calculated as:

$D\,(\%) = (p0 - p1 - p2)/p0 \times 100$

where $p0$ and $p1$ show the initial and remaining concentrations of petroleum at different incubation periods, $p2$ indicates the abiotic loss (Barnes et al. 2018; Benguenab and Chibani 2020).

Petroleum degradation kinetics

The degradation data applied to first order kinetic model according to Maletić et al (2009):

$ln_{ct} = ln_{co} - k_{t,}$

where c_t is the residual petroleum concentration at time; c_o is the initial petroleum concentration; k is the first-order kinetic degradation constant (day^{-1}), and t is time (day). The half-life period of petroleum was calculated as follows:

$t_{1/2} = ln2/k$

Investigation of optimal physiological conditions on petroleum degradation with fungal spore suspension

To optimize physicological conditions, initial pH (3.0 - 8.0), petroleum concentration (0.5% - 4%), inoculum concentration (2.5% - 10%) (v/v) and incubation period (7, 14, 21 and 28 days) parameters were investigated. The degradation of petroleum was obtained by gravimetric analysis.

Investigation of optimal physiological conditions on petroleum degradation with fungal live biomass

By comparing the efficiencies of fungal pellets and fungal discs in petroleum biodegradation, the most effective fungal live biomass was determined and used in further optimization studies. Accordingly, initial pH (3.0 - 8.0), petroleum concentration (0.5% - 5%), inoculum amount (0.5 - 2.5%) (g/100mL) and incubation period (7, 14, 21 and 28 days) parameters were investigated. The degradation of petroleum was obtained by gravimetric analysis.

GC/MS analysis

The petroleum was extracted with DCM from the culture with the highest degradation ratio under optimized conditions. The analysis was carried out by the Petroleum Research Center at Middle East Technical University (Turkey) using TRB-1 GCMS-QP-2020 (Shimadzu, Tokyo, Japan) to obtain the removal of *n*-alkane fractions in petroleum with fungal disc of potent strain. The procedure was performed according to Bilen Ozyurek and Seyis Bilkay (2020b).

Screening assay for biosurfactant production

The assay was aerobically carried out with 50 mL of sterile BH medium supplemented with 1% (v/v) of petroleum and 1% (w/v) yeast extract (Merck). 1 g (w/100mL) of live biomass was inoculated into BH medium and incubation was performed at 30 °C and 150 rpm for a week. Following the incubation period,

the whole broth was centrifuged at 4650 ×*g* for 10 min (Eppendorf 5810R) and at 10752 ×*g* for 10 min (Eppendorf 5417C, Sigma-Aldrich, USA). Then, the supernatant was also filtered with 0.45µm pore size filter paper (Millipore, Sigma-Aldrich). The cell-free culture supernatants were transferred to a clean test tube and used for biosurfactant screening assays (Parthipan et al. 2017).

Drop-collapse method

The drop-collapse method was carried out as described by Bodour et al. (1998). 5µL of petroleum was added into 96-microwell plate and left in room temperature for 2 h. 5 µL of cell-free culture supernatant was added on petroleum in 96-microwell plate. The change in the drop size was observed after 1 min. Deionized water and Triton X:100 (a chemical surfactant) was used as negative and positive controls, respectively (Parthipan et al. 2017).

Oil-spreading method

The oil spreading method was carried out as described previously Hassanshahian (2014). 50 mL of distilled water was added into petri plate followed by addition 20 µL of petroleum. Then, 10 µL of culture supernatant was added on petroleum-coated water surface. The diameter of the clear zone on petroleum surface was measured after 30 s. Deionized water and Triton X:100 was used as negative and positive controls, respectively.

Results

Petroleum removal of fungal strains by DCPIP redox indicator

When the petroleum degradation efficiencies of the fungi were evaluated by the colorimetric method, it was determined that oil biodegradation ranged from 73–90% with 10 different fungal strains. Furthermore, the highest petroleum removal was obtained with *Aspergillus ochraceus* NRRL 3174 strain (Fig. 1).

Optimal physiological conditions on petroleum degradation by fungal spore suspension

The optimal physiological conditions for the maximum petroleum degradation by spore suspension of *Aspergillus ochraceus* strain were determined as pH: 5.0, 1% of petroleum concentration, 5% (v/v) of inoculum concentration and 7 days of incubation period (Fig. 2, Fig. 3, Fig. 4, Fig. 5). Accordingly, it was also determined that there was a decrease in petroleum degradation at pH values below and above pH 5. The petroleum degradation efficiencies of fungal spore suspension was above 80% even at high concentrations of petroleum and the maximum degradation occurred at 1% of petroleum concentration. There was no significant increase in petroleum degradation at values above 5% (v/v) of spore suspension concentration. The increase in incubation period caused a decrease in petroleum degradation, while the highest degradation was obtained in 7 days of incubation period.

Optimal physiological conditions on petroleum degradation by fungal live biomass

When the efficiencies of fungal disc and fungal pellet of *A.ochraceus* strain in petroleum degradation were compared, it was clearly observed that the fungal disc is more effective than fungal pellet as a live biomass in petroleum removal (Fig. 6).

The optimal physiological conditions for the maximum petroleum degradation by fungal disc of *A. ochraceus* strain were determined as pH: 5.0, 1% of petroleum concentration, 1 g/100mL of inoculum amount and 7 days of incubation period. In addition, the highest degradation was obtained with 10% (v/v) of cell-free culture supernatant concentration (Fig. 7, Fig. 8, Fig. 9, Fig. 10, Fig. 11). Accordingly, it was also determined that there was a decrease in petroleum degradation at pH values below and above pH 5. The petroleum degradation efficiencies of fungal disc was 60% and above at high concentrations of petroleum and the maximum degradation occurred at 1% of petroleum concentration. There was no significant increase in petroleum degradation at values above 1 g/100 mL of fungal disc amount. The increase in incubation period caused a decrease in petroleum degradation, while the highest degradation was obtained in 7 days of incubation period. Moreover, 7.5% and 10% (v/v) concentration of cell-free culture supernatant removed more than 80% of petroleum. It was clearly obtained that the cell-free culture supernatant containing only extracellular enzymes was not as effective in petroleum degradation as fungal disc.

GC/MS analysis

When the results of GC/MS analysis were evaluated, *n*-alkanes were degraded by *A. ochraceus* strain as; C_{10} to C_{20} were 90% and above, C_{23} to C_{28} were 60%, C_{29} to C_{34} and C_{22} were around 70%; C_{21} and C_{35} were 85% and above; C_{36} was 75%. It was also clearly shown that the potent fungi degraded long-chain *n*-alkanes such as C_{35} and C_{36} more effectively than *n*-alkanes in the range of C_{22}-C_{34} (Fig. 12).

Biosurfactant production with *A. ochraceus* strain

When the results of the drop-collapse and oil spreading methods were examined, the presence of biosurfactant was determined with *A. ochraceus* strain (Fig. 13, Fig. 14). The drop-collapse method showed a partially positive result in the production of biosurfactant. So, the results of drop-collapse method was also supported by oil spreading method.

Discussion

Petroleum hydrocarbons, being the most important raw materials for energy sources and industrial chemicals used in daily life, are also one of the most important environmental pollutants. Some microbial groups, including bacteria, fungi, and algae, use petroleum hydrocarbons as a source of carbon and energy, breaking them down into carbon dioxide and water, or less toxic or non-toxic substances. Biodegradation is known as a highly effective, economical and environmentally friendly alternative method in removing of the petroleum pollutants from the environment and preventing damage caused by petroleum spills (Al-Hawash 2018a; Benguenab and Chibani 2020). Because of playing an important role in breaking down organic materials or recalcitrant hydrocarbons into smaller pieces with their

extracellular enzyme systems, the roles of fungi as bioremediation agents need to be further developed (Barnes et al. 2018; Odili et al. 2020).

In the study, DCPIP redox indicator was used in the preliminary investigation of petroleum degradation abilities of 10 fungal strains (Fig. 1). According to Fig. 1, it was determined that the highest degradation ability obtained with *A. ochraceus* NRRL 3174 strain. In a similar study, the petroleum degradation efficiencies of *Penicillium sp* RMA1 and RMA2 strains were determined with the DCPIP redox indicator (Al-Hawash et al. 2018b). Benguenab and Chibani (2020) showed that 4 fungal strains were efficient in the degradation of hydrocarbons. The colorimetric assay performed with DCPIP redox indicator is an effective method for screening microbial strains that degrade petroleum (EL-Hanafy et al. 2017). In the microbial oxidation process of petroleum hydrocarbons, electrons are transferred to the final electron acceptors such as nitrates, sulfates and O_2. In this case, the capacity of microorganisms to metabolize hydrocarbons is interpreted by the discoloration / discoloring of the DCPIP blue-colored redox indicator, which is the final electron acceptor. This mechanism is similar in fungi (Hanson et al. 1993).

Temperature, humidity, presence of surfactants, soil pH, mineral composition, and organic matter content are among the most important factors affecting biological degradation. The effectiveness of bioremediation depends on the selection of the appropriate microorganism and the combination of suitable environmental conditions (Kumari et al. 2016). In this context, the effect of differential physiological conditions such as initial pH, petroleum concentration, inoculum concentration and amount and incubation period on petroleum degradation with fungal spore suspension and fungal disc were investigated. It was found that the maximum petroleum degradation with fungal spore suspension and fungal disc of *A. ochracerus* strain was at pH: 5.0 (Fig. 2, Fig. 7). A decrease in petroleum degradation was observed at values below and above this pH. Since the pH range for better growth for the yeast and molds is 4.5–5.5, the optimal petroleum degradation is considered to be in the range of pH 6.0–8.5 (Kumari et al. 2016). Rahman et al. (2002) reported that excessive pH values on both sides may have a negative effect on the degradation ability of microbial populations.

In the selection of fungal strains to be used in the bioremediation process, it is extremely important to know the ability of the tested fungi to withstand high concentrations of contaminants (Mrozik and Piotrowska-Seget 2010). However, the fungal spore suspension of *A. ochraceus* was found to tolerate higher concentrations of petroleum than the fungal disc, petroleum degradation efficiencies of fungal spore suspension and fungal disc were 6o% and above even at high concentrations of petroleum. The maximum degradation occurred at 1% of petroleum concentration for both (Fig. 3, Fig. 8). In accordance with this study, Kota et al. (2014) showed that *A. flavus* and *A. versicolor* were resistant to petroleum contamination within the range of 5–1%. Although it was found that *A. oryzae* and *M. irregularis* can tolerate varying concentrations of engine oil, their growth rate and degradation abilities differ (Asemoloye et al. 2020). The removal of 3% petroleum concentration with *Saccharomyces sp.* was 39.5% while it was 41.8% with *Actinomyces sp*. and *Sachharomyces sp*. Strains (Shahaby 2014). Kumari et al. (2016) emphasized that the degradation rate decreases with the increasing petroleum concentration. In addition, the composition of the petroleum as well as the its concentration plays an important role in fungal

growth and degradation (Elshafie et al. 2007). The high tolerance of fungi to these complex hydrocarbons is not due to their ability to degrade but to enzyme secretions that increase biodegradation. (Aemoloye et al. 2017, Asemoloye et al. 2020).

This study was clearly indicated that there was no significant increase in petroleum degradation at values above 5% (v/v) of spore suspension concentration and 1 g/100 mL of fungal disc amount. It was also determined that 1 g/100 mL of fungal disc amount was more effective than 5% (v/v) of spore suspension in petroleum degradation (Fig. 4, Fig. 9). In similar studies, *Aspergillus sp.* strains have been shown to play an effective role in degradation of complex hydrocarbons (Harms et al. 2011; Ye et al. 2011; El Hanafy et al. 2015; Banerjee et al. 2016; Barnes et al. 2018). Furthermore, determining the appropriate incubation period is of great importance in terms of increasing the degradation efficiency. Ajani et al. (2017) reported that incubation period has a strong effect on petroleum degradation. In this study, petroleum degradation of the spore suspension was 87% while the fungal disc was 94% at the end of the seven-days incubation period (Fig. 5, Fig. 10). A decrease in petroleum degradation was observed with the increasing of incubation period. Al-Hawash et al. (2018a) reported that %51.8 of maximum petroleum removal was determined by live micellar pellets of *Aspergillus* RFC-1 strain within 7 days of incubation period. Kumari et al. (2016) showed that the highest degradation (30.8%) of TPH in petroleum was obtained within 10 days of incubation. *Penicillium sp.* RMA1 and RMA2 degraded the petroleum by 57% and 55%, respectively within 14 days of incubation at 30°C (Al-Hawash et al. 2018b). Al-Jawhari (2015) showed that 95% of petroleum removal by mixed culture of *A. niger* and *A. fumigatus* after 28 days. Odili et al. (2020) indicated that maximum petroleum removal (98.42%) was obtained at pH = 6.5, 1% of crude oil, 3 spores/mL of inoculum concentration for 14 days of incubation. However, minimum removal (55.81%) was obtained at pH = 7.5, 4 spores/mL of inoculum concentration for 4.61 days of incubation period. Accordingly, the petroleum degradation varies depending on the type of pollutants, the metabolic capabilities of the microbial population, and environmental conditions (Ustun Kurnaz and Buyukgungor 2016). The degradation rate also varies according to the chemical structure of the petroleum containing different types of hydrocarbons, the exposure dose, the structure of the microorganism and the duration of exposure (Benguenab and Chibani 2020). According to the degradation of fungal spore suspension and fungal discs under optimized conditions, the degradation rate constant and half-life period of spore suspension of *A. ochraceus* were calculated as 0.291 day^{-1} and $t_{1/2}$ = 0.340 and of fungal disc of A. *ochraceus* were calculated as 0.401 day^{-1} and $t_{1/2}$ = 0.247. Benguenab and Chibani (2020) showed that the petroleum removals were 44.55% and 30.43%; the rate constant (K) and half-lives ($t_{1/2}$) were 0.02 day^{-1}, 34.66 day and 0.015 day^{-1}, 46.21 day for *P. lilacinium* and *A. ustus*, respectively. So, it has been clearly demonstrated that the fungal disc with high degradation capacity has a low half-life period with high degradation rate constant. This can be explained with that the fungi can reach the petroleum hydrocarbons more easily by penetrating the contaminated area faster through fungal hyphae (Barnes et al. 2018).

Enzyme systems that play an important role in the hydrocarbon degradation process are generally extracellular and non-specific (Zhang et al. 2016; Al-Hawash et al. 2018a). The most important reason

why filamentous fungi are important potential agents in degradation of petroleum hydrocarbons is that they rapidly bind to the substrate and secrete extracellular enzymes (Steliga 2012). In this study, 7.5% and 10% (v/v) concentration of cell-free culture supernatant achieved more than 80% of petroleum removal (Fig. 11). However, the cell-free culture supernatant containing only extracellular enzymes was not as effective in petroleum degradation as fungal disc containing both of intracellular and extracellular enzymes. It was determined that the effect of extracellular enzymes in the petroleum biodegradation process was quite high, but not sufficient alone. Al-Hawash et al. (2018a) reported that the degradation efficiency of petroleum by extracellular enzymes is slightly higher than intracellular enzymes. In contrary, Deive et al. (2009) found that extracellular enyme activity is lower than intracellular activity. In the literature, it has been shown that fungal enzymes belonging to six different *Aspergillus sp* strains are capable of degrading *n*-alkanes in petroleum to lower fractions even under anaerobic conditions (Zhang et al. 2016; Al-Hawash et al. 2018a). With all this, it is clear that the combination of extracellular and intracellular enzymes play a significant role in the degradation process of petroleum hydrocarbons.

Because of its complex structure consisting of non-water-soluble compounds such as different chain lenghts of *n*-alkanes, the degradation of petroleum is lower than other hydrocarbons (Okoh 2006). Due to the rapid volatilization of low molecular weight of *n*-alkanes, they do not remain in the contaminated area for a long time, so high molecular weight of *n*-alkanes are more toxic than short chain ones (Barnes et al. 2018). The long-chain *n*-alkanes in the petroleum are solid and have low solubility, so it is known that the percentage of degradation decreases with increasing of *n*-alkane chain length (Al-Hawash et al. 2018b). However, it was clearly shown in this study that *A. ochraceus* degraded long-chain *n*-alkanes such as C_{35} and C_{36} more effectively than *n*-alkanes in the range of C_{22}-C_{34} (Fig. 12). Compatible with this study, Barnes et al. (2018) showed that *Penicillium citrinum* NIOSN-M126 strain was efficient in degradation of the higher chain mutagenic *n*-alkanes. The findings in the present study were close to Wang et al. (2011), Covino et al. (2015), Asemoyoloe et a (2020). On the other hand, different studies have shown that short-chain *n*-alkanes are degraded by fungal strains at a higher rate compared to long-chain *n*-alkanes (Bento et al. 2005; Simister et al. 2015). This can be explained that not all fungal strains have a stable physiological character in their ability to use petroleum as a carbon source (Elshafie et al. 2007). However, it is an undeniable fact that the fungi generally have high tolerance to complex hydrocarbons, rapid adaptation to different environmental conditions and the mechanisms required for petroleum removal. (Barnes et al. 2018).

Biosurfactants have significant effect on degradation of petroleum hydrocarbons, mainly by two basic mechanisms. In the first mechanism, the emulsification of hydrophobic compounds to micellar pellet structures is promoted. In the second mechanism, it induces high cell surface hydrophobicity. This increases direct physical contact between cells and low solubility of substrates (Al-Hawash et al. 2018a). Two different methods, drop-collapse and oil-spreading, were used to determine the presence of biosurfactant. The first method showed a partially positive result in the production of biosurfactant (Fig. 13). So, this method should be supported by oil spreading, as it is not effective in determining the low level of biosurfactant production (Youssef et. al. 2004; Mahjoubi et al. 2013; Al-Hawash et al. 2018a).

When the results of oil-spreading method compared with negative and positive controls, it was determined that *A.ochraceus* strain is a good biosurfactant producer (Fig. 14). Biosurfactants produced by micellar pellets play an important role in limiting the surface tension in petroleum (Hassanshahian et al. 2012).

Fungi play an important role in degradation process of petroleum hydrocarbons with their high enzymatic capacity. In this manner, the degradation abilities of 10 fungal strains were determined by DCPIP redox indicator with colorimetric method. Petroleum degradation efficiencies of spore suspension, live biomasses (fungal pellet and disc) and culture supernatant of the potent fungal strain of *A. ochraceus* were determined by gravimetric analysis. Optimal physiological conditions such as initial pH, different petroleum concentrations, inoculation concentration and amount, and incubation period were compared between fungal spore suspension and fungal disc of *A. ochraceus* strain. Under optimized conditions, it was determined that the fungal disc had better penetration on the petroleum and was more successful in degradation process than spore suspension. Moreover, high concentration of cell-free culture supernatant were achieved more than 80% of petroleum removal. GC/MS analysis indicated that the fungal disc of *A. ochraceus* degraded long-chain *n*-alkanes such as C_{35} and C_{36} more effectively than *n*-alkanes in the range of C_{22}-C_{34}. It was also determined that *A. ochraceus* is a good biosurfactant producer. As a conclusion, the combination of extracellular and intracellular enzymes of fungi plays an important role in the degradation process of petroleum hydrocarbons. With this study, it was clearly emphasized that the *A. ochraceus* NRRL 3174 strain, which was not encountered in a similar study in the literature, can be used as an potent agent in different petroleum bioremediation processes with its high enzymatic capacity.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability statement The authors can confirm that all relevant data are included in the article.

Author contribution statement SBO and NHA conceived and designed research. SBO and NHA conducted experiments. SBO and NHA contributed new reagents or analytical tools. SBO, NHA and ISB analyzed data. SBO wrote the manuscript. All authors read and approved the manuscript.

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Figures

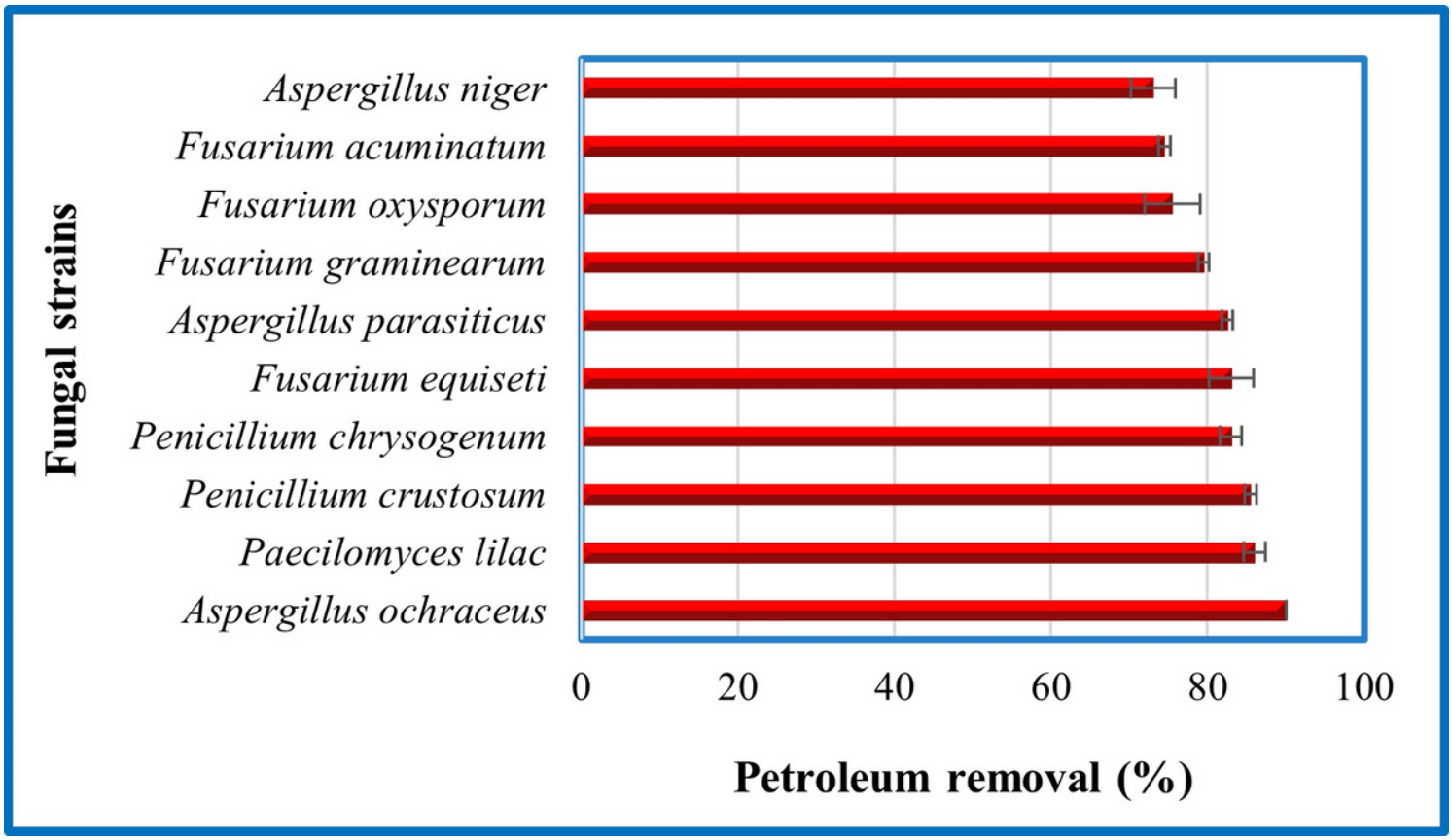

Figure 1

The petroleum removal of fungal strains by colorimetric method

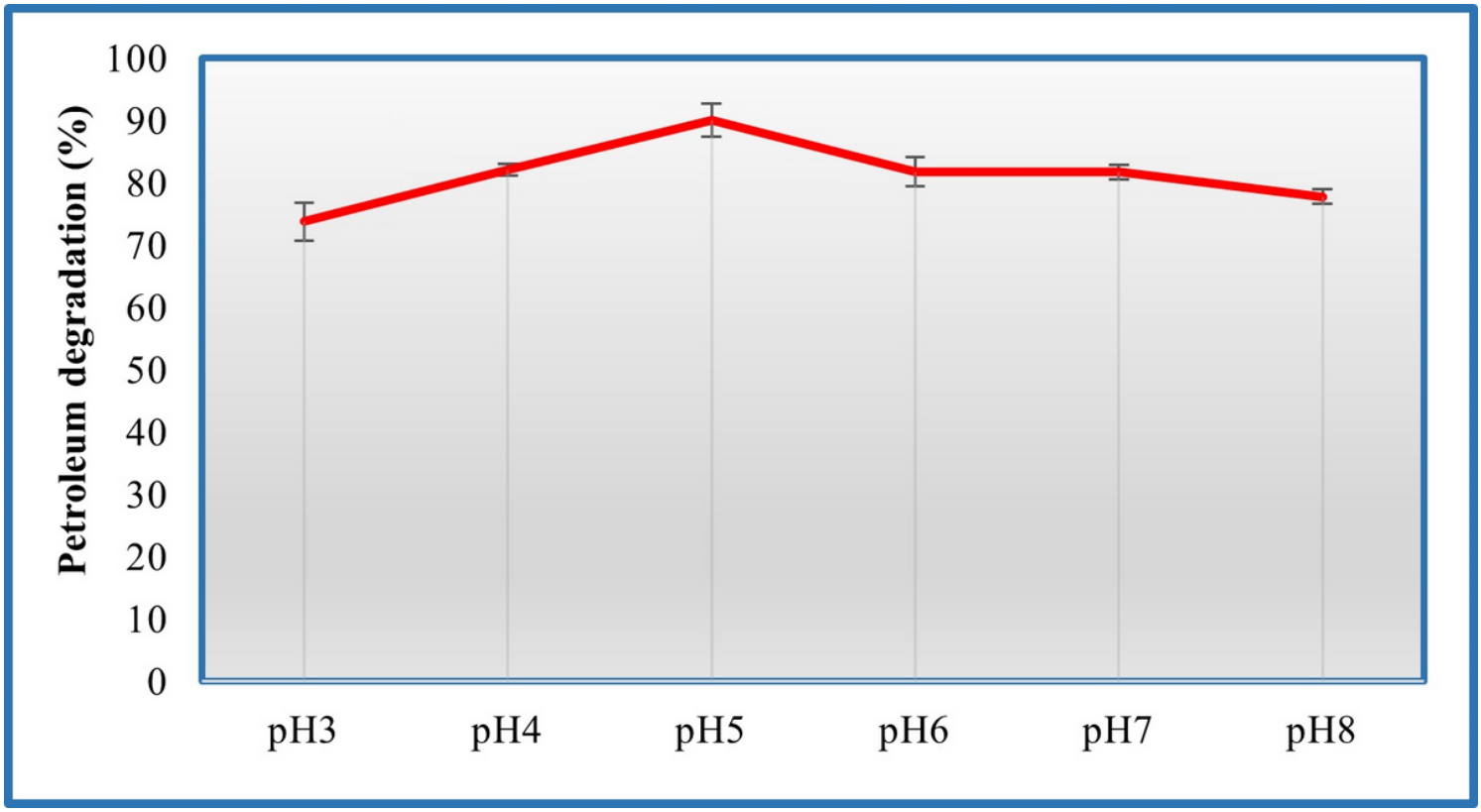

Figure 2

The effect of initial pH on petroleum degradation by fungal spore suspension

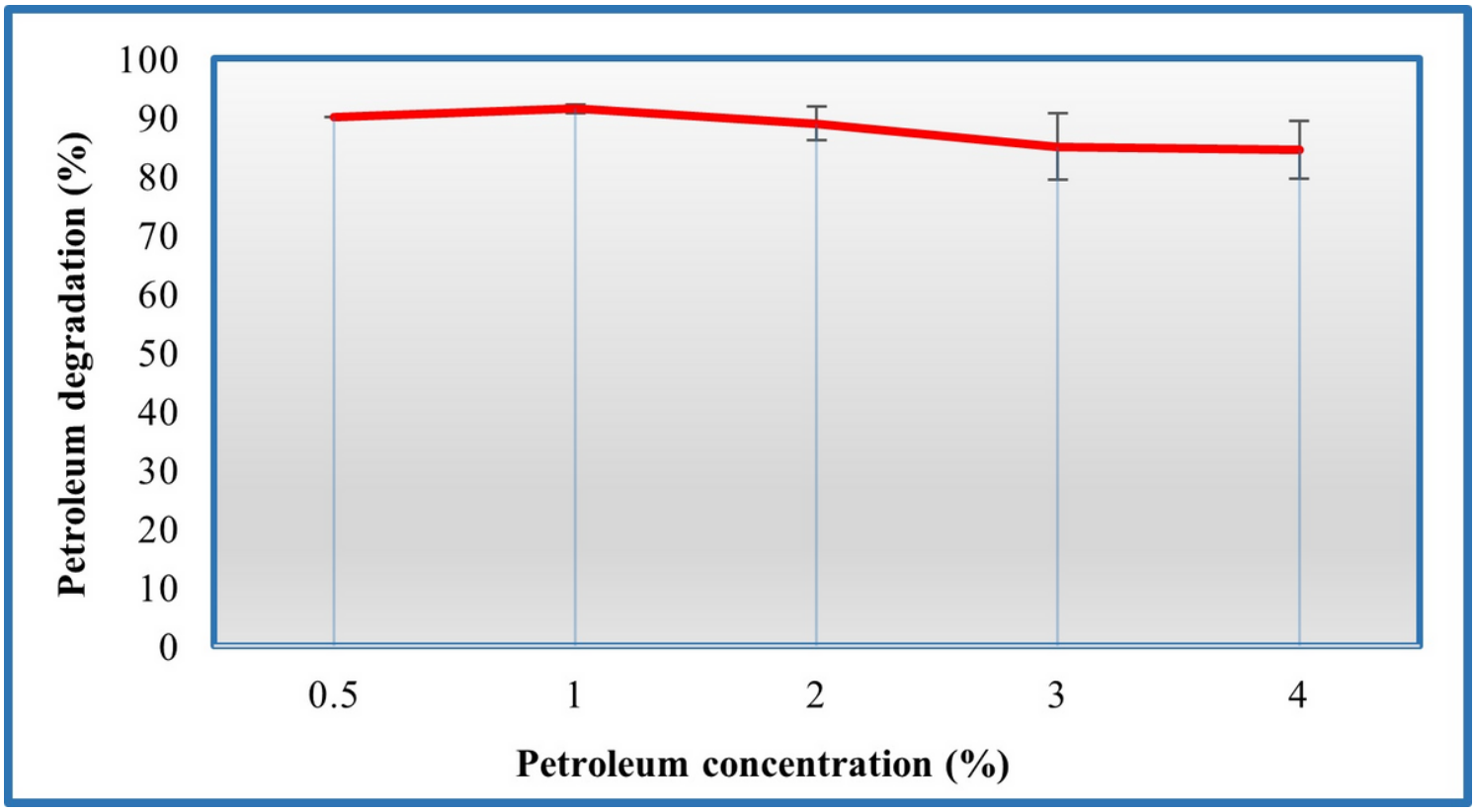

Figure 3

The effect of concentration of petroleum on degradation by fungal spore suspension

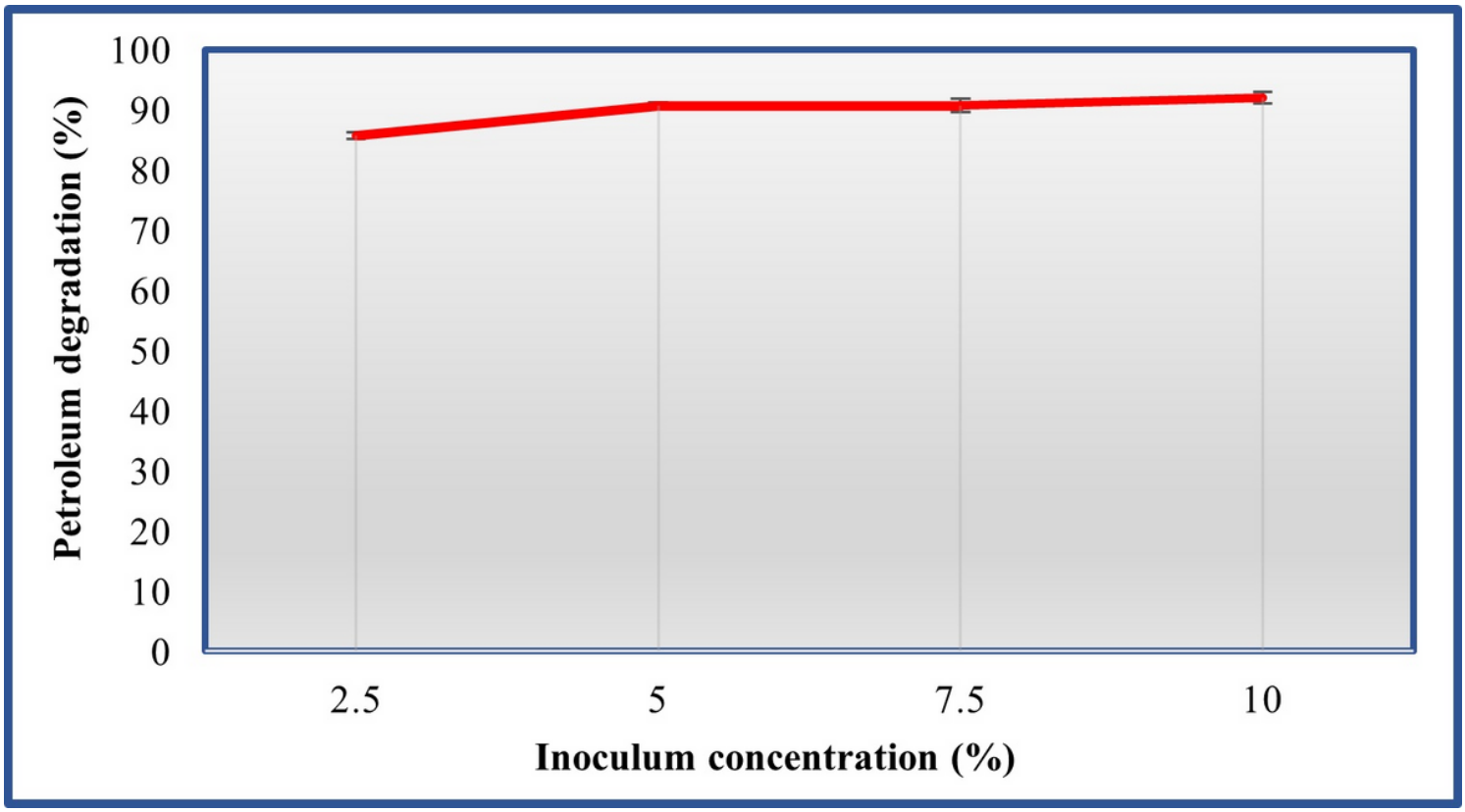

Figure 4

The effect of inoculum concentration on petroleum degradation by fungal spore suspension

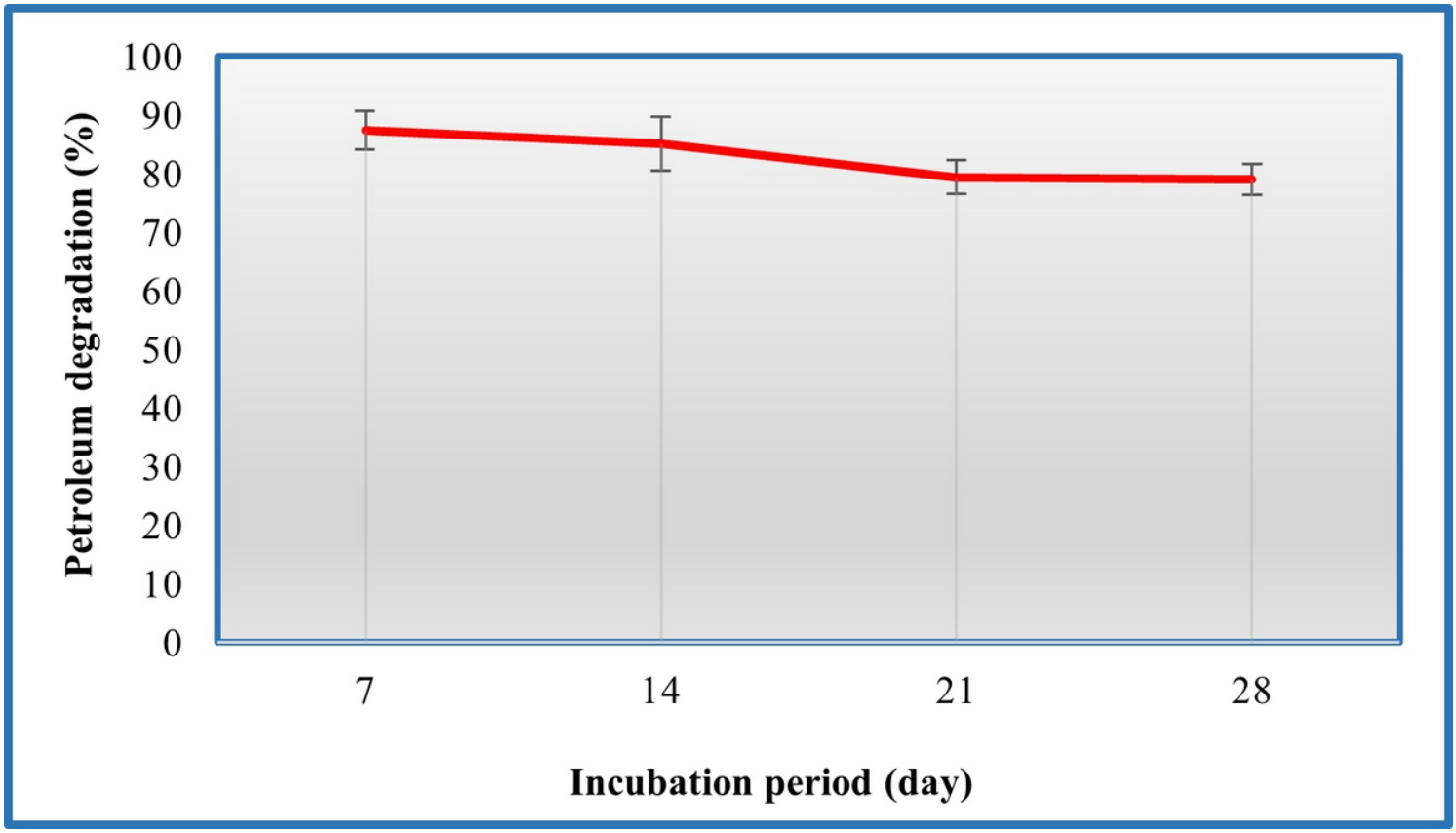

Figure 5

The effect of incubation period on petroleum degradation by fungal spore suspension

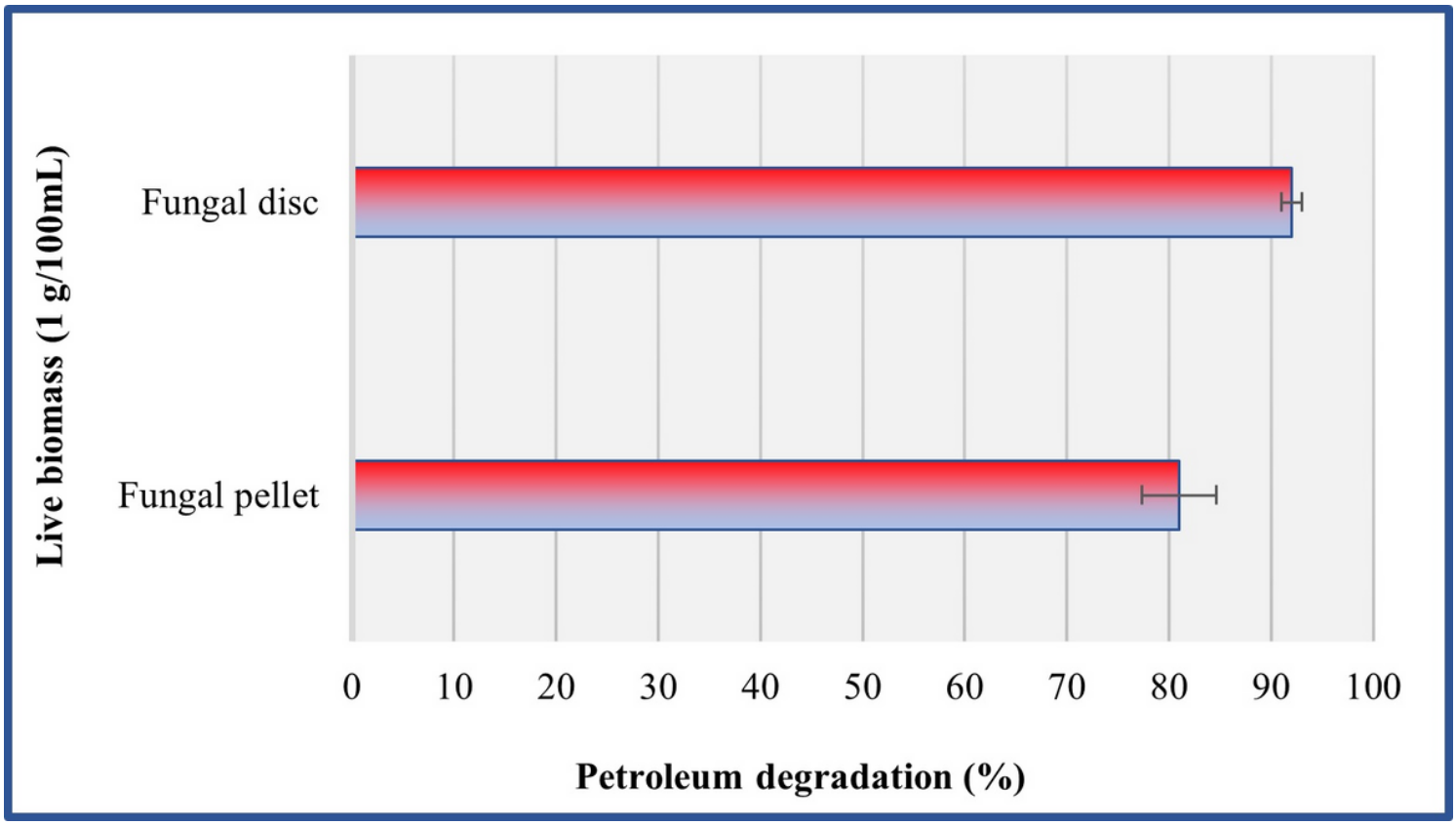

Figure 6

Comparison of degradation efficiencies of fungal disc and fungal pellet of A.ochraceus strain

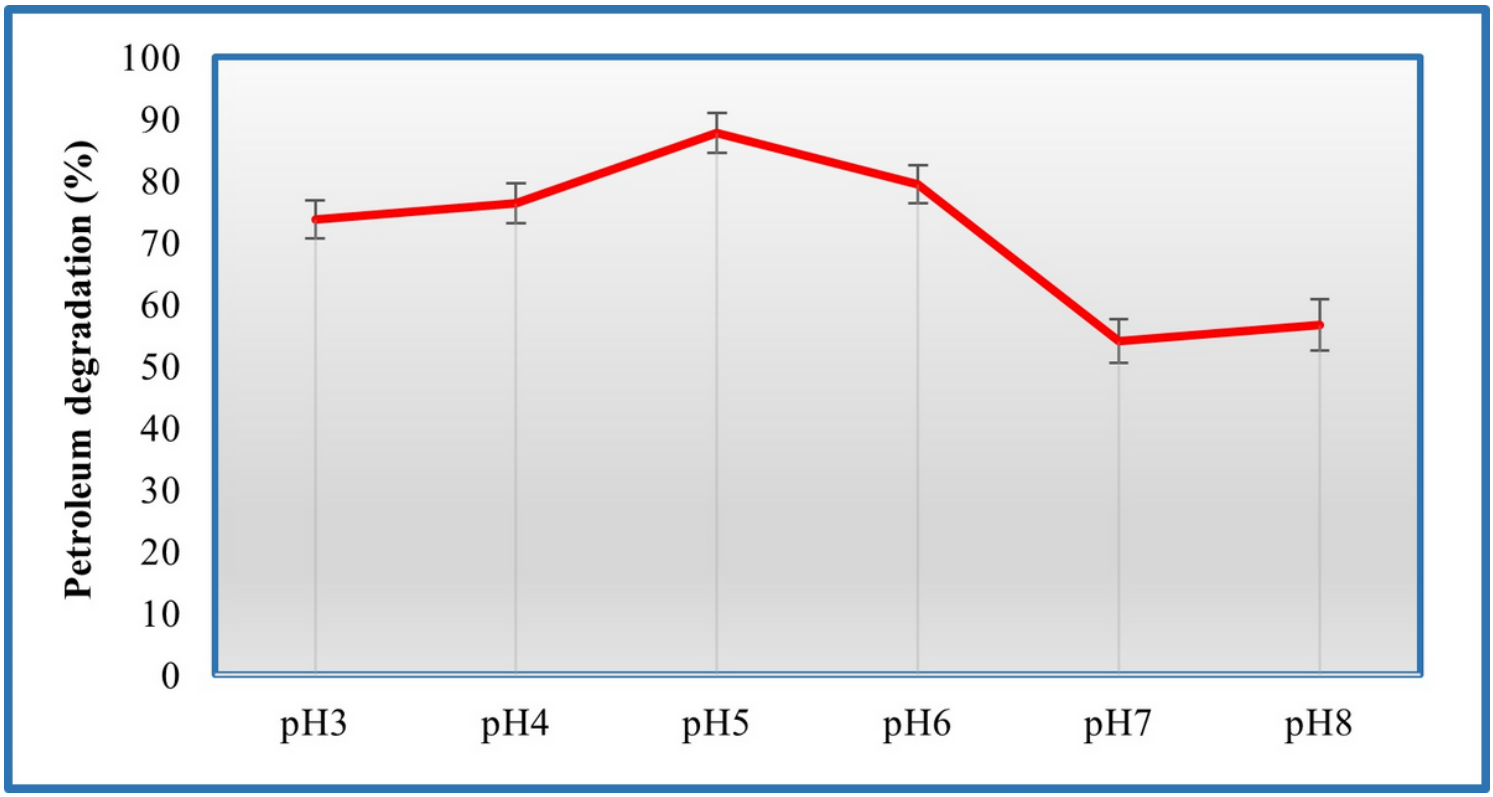

Figure 7

The effect of initial pH on petroleum degradation by fungal disc

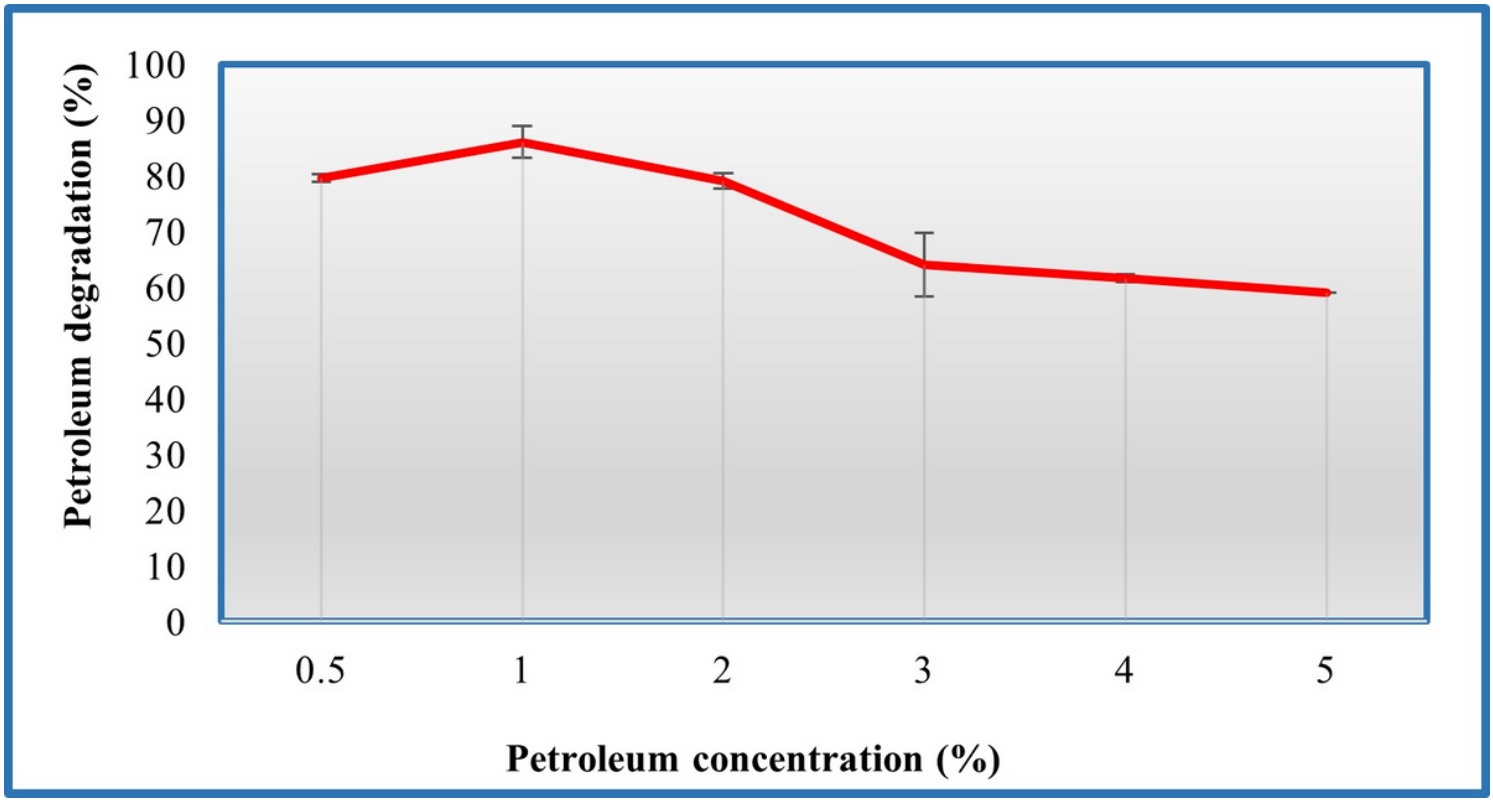

Figure 8

The effect of concentration of petroleum on degradation by fungal disc

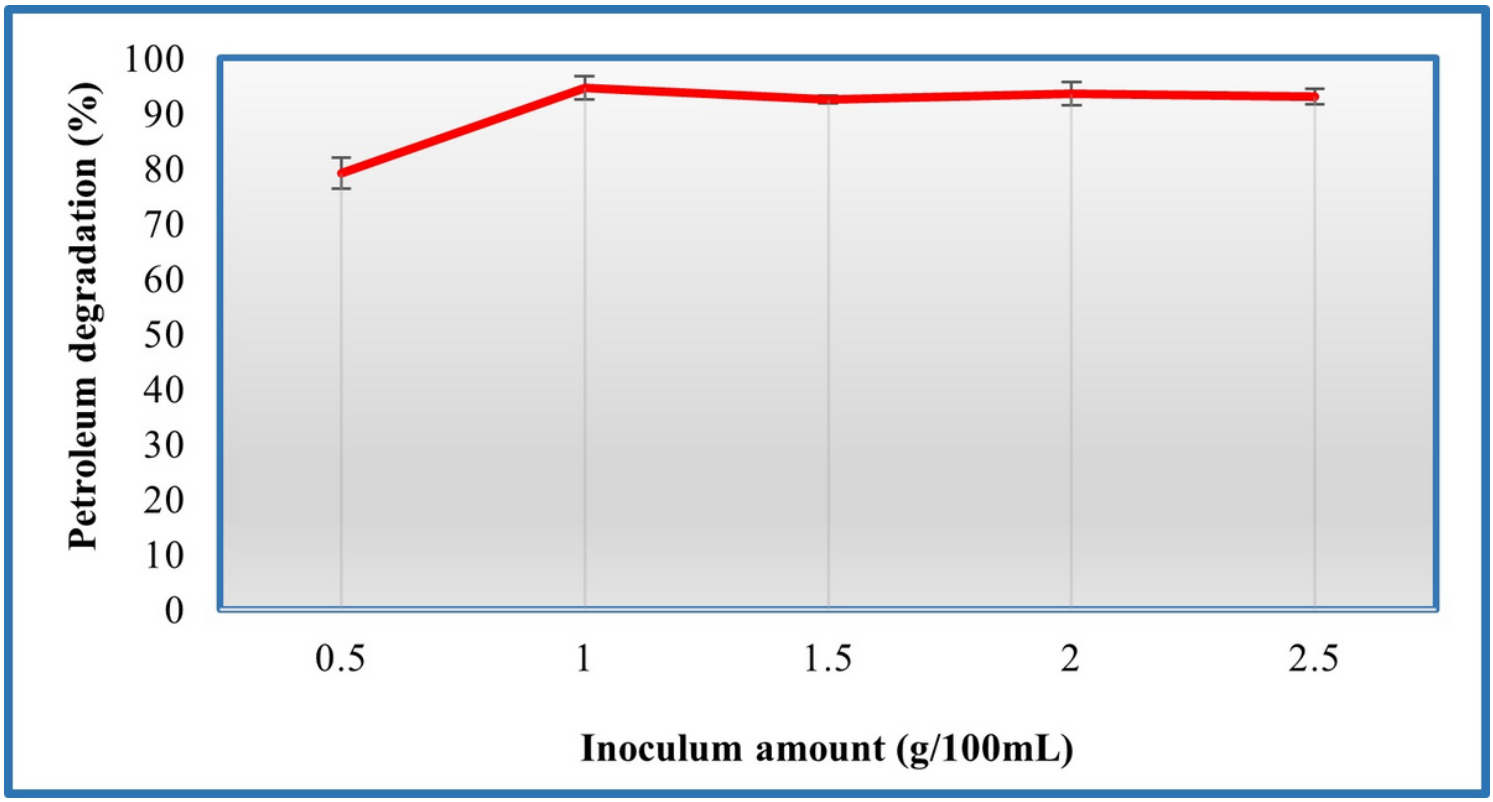

Figure 9

The effect of inoculum amount on petroleum degradation by fungal disc

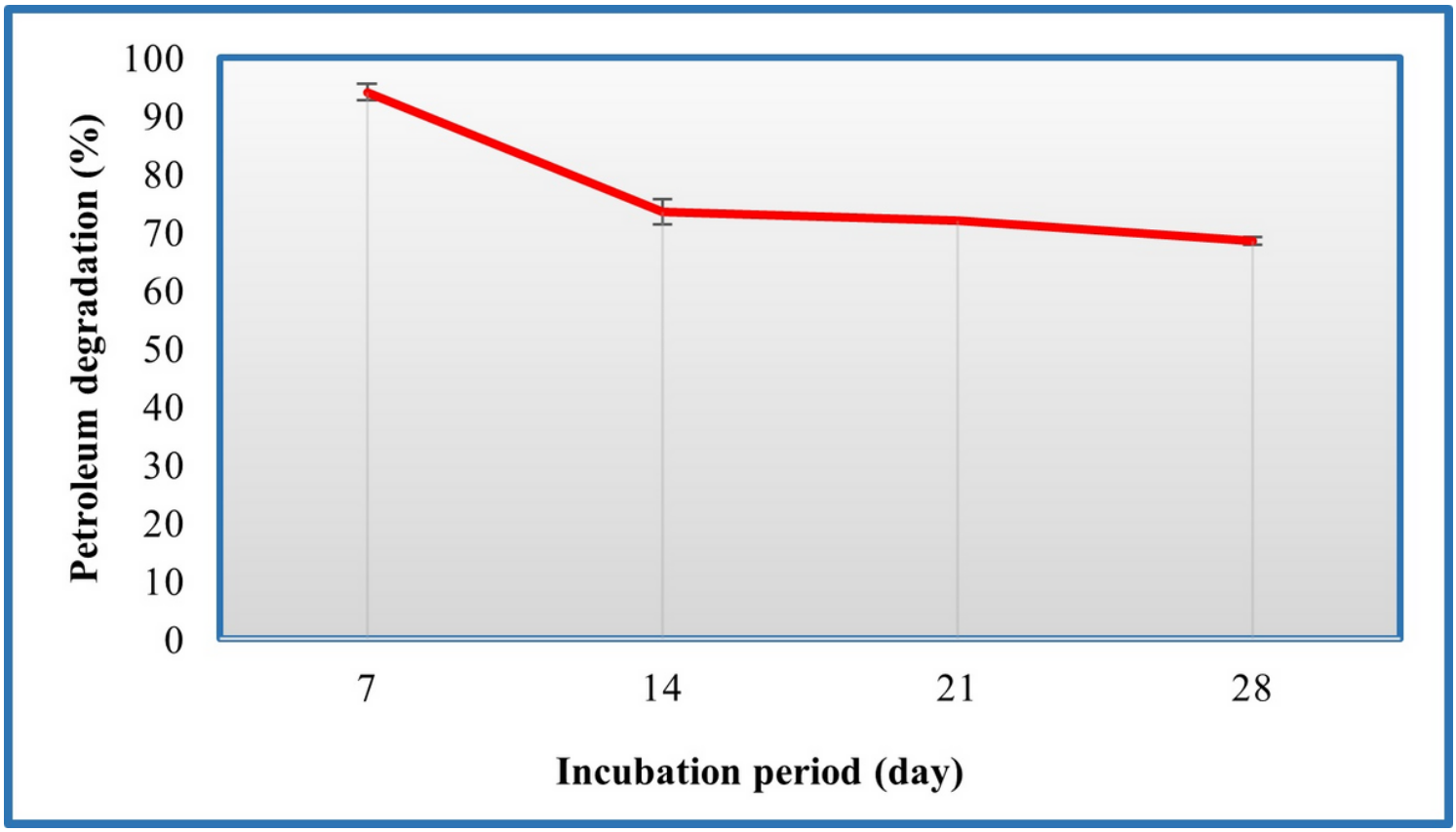

Figure 10

The effect of incubation period on petroleum degradation by fungal disc

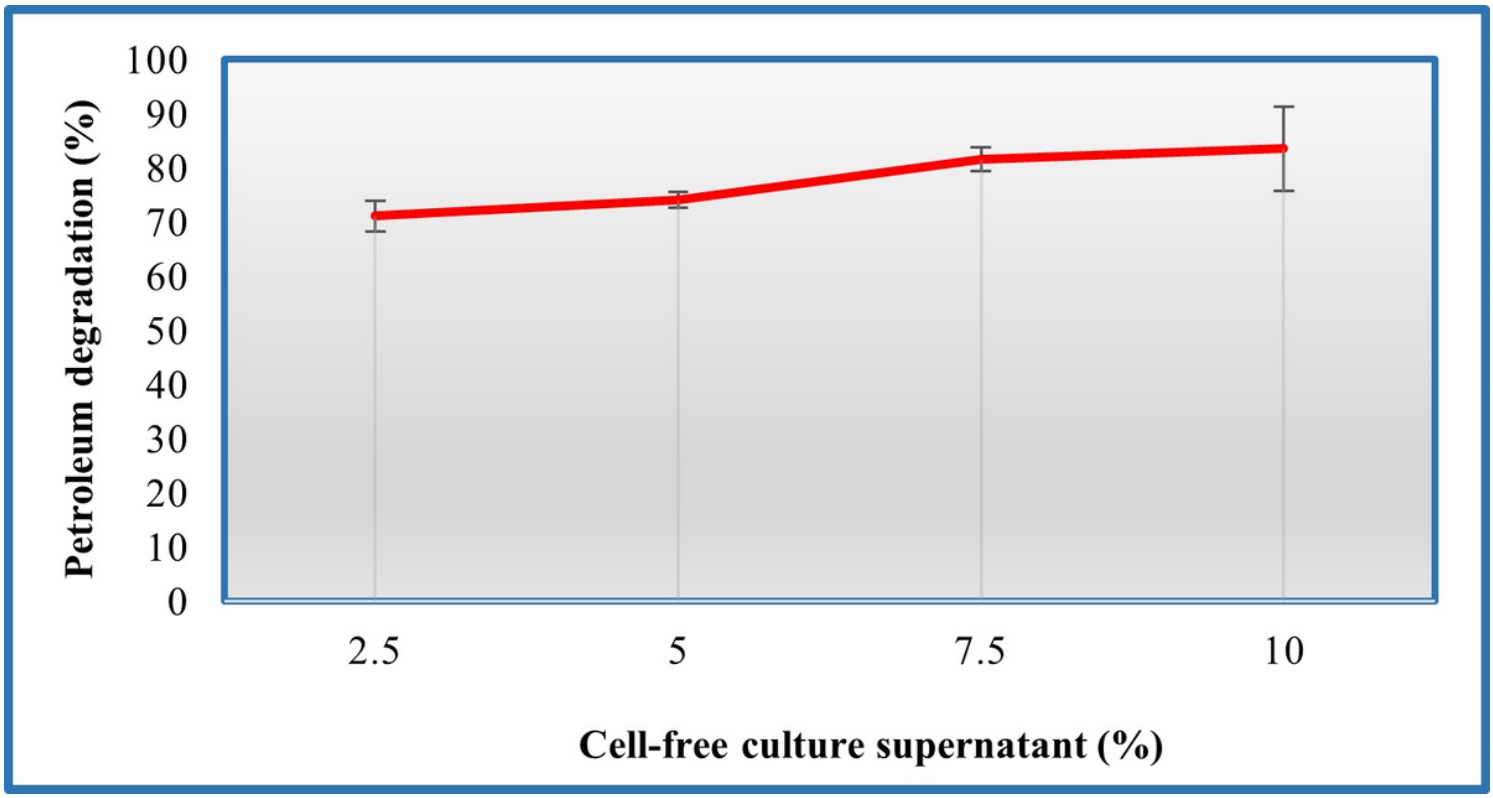

Figure 11

Petroleum degradation by cell-free culture supernatant

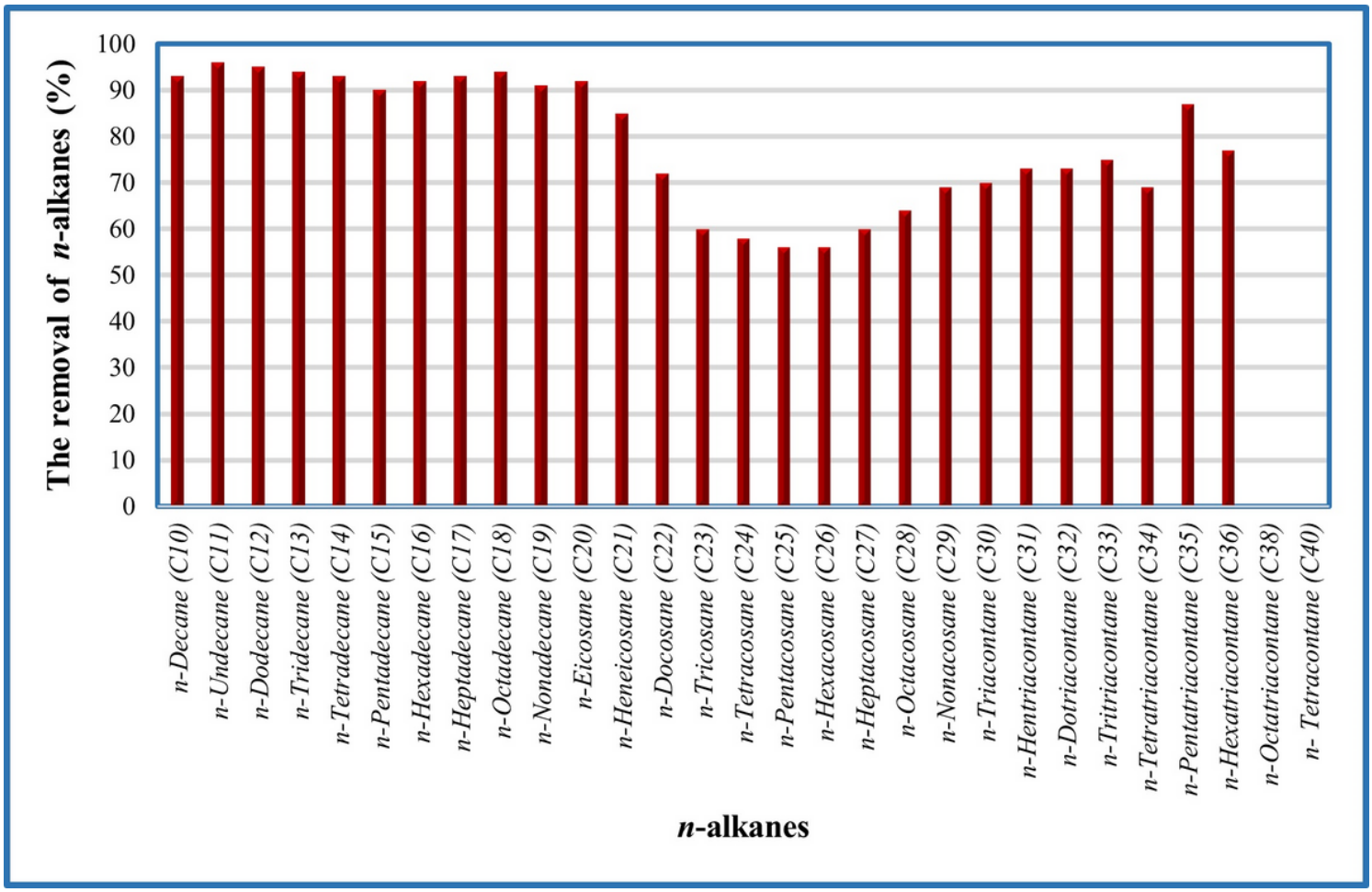

Figure 12

The removal of n-alkane fractions of petroleum by Aspergillus ochraceus strain

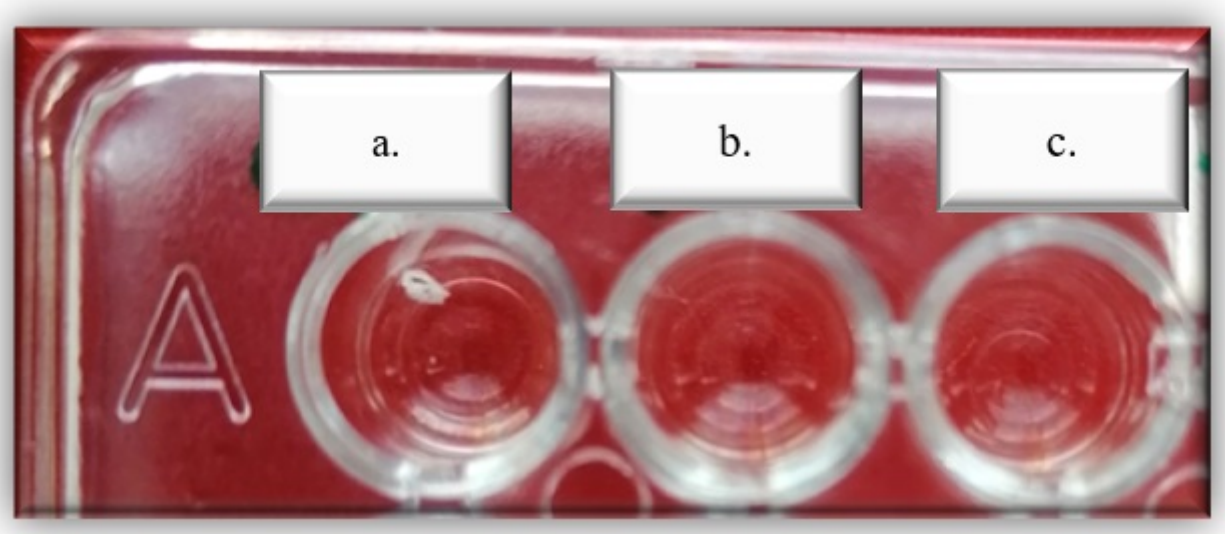

Figure 13

Determination of biosurfactant presence by drop-collapse a. Cell-free supernatant of fungal strain, b. Positive control (Triton X:100), c. Negative control (Distilled water)

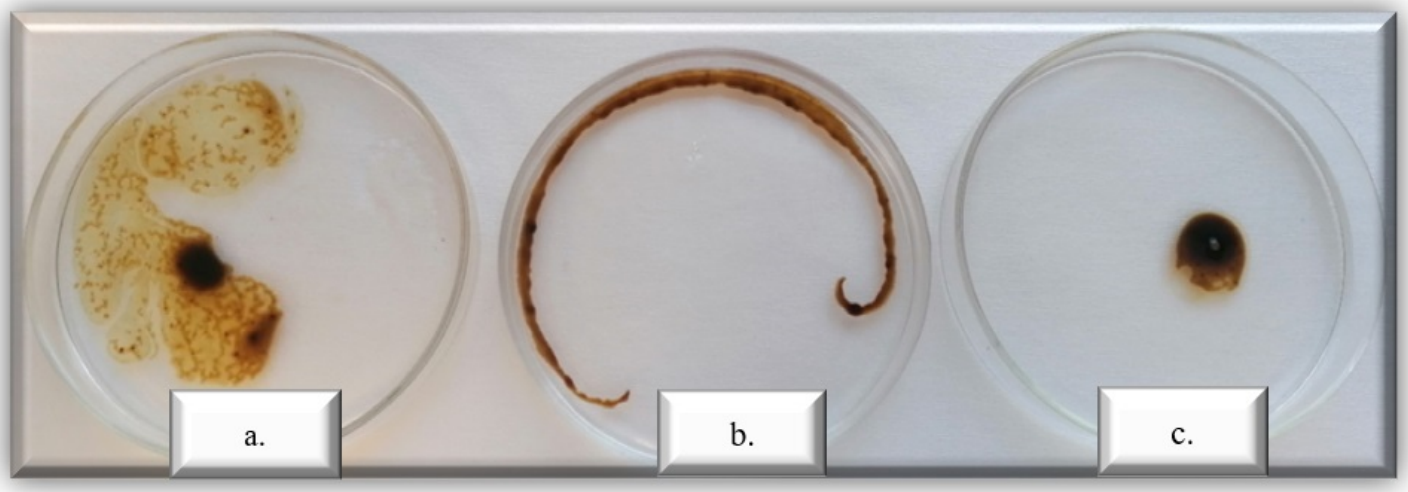

Figure 14

Determination of biosurfactant presence by oil spreading a. Cell-free supernatant of fungal strain, b. Positive control (Triton X:100), c. Negative control (Distilled water)

Supplementary Files

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