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THE PRODUCTION OF BIOSURFACTANTS BY LOCAL FUNGI ISOLATED FROM EGYPTIAN SOIL

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ABSTRACT

One of the most important sources of biomolecules is soil fungi which have unique metabolic and physiologic features. The present study investigates the ability of local fungi isolated from Egyptian soil to produce biosurfactants. The oil displacement area (ODA) test was used to study the effect of three different environmental conditions (pH, temperature, and salinity) on the stability of produced biosurfactants. The study aimed also to reuse four vegetable oils (sunflower oil, olive oil, waste frying oil, and corn oil), which are widely used in the daily activities of the Egyptians, for the enhancement of the production and activity of biosurfactants by fungal isolates. The highest production of biosurfactants was observed by *Aspergillus wentii*, *Aspergillus flavus*, and *Fusarium* sp. The results showed that most of the biosurfactants were stable at a wide range of temperatures, alkaline pH, and high salinity. Sunflower oil followed by waste frying oil showed the highest enhancement effects. Olive oil showed moderate enhancement while corn oil showed the lowest effect. Thus, it can be concluded local soil fungi are considered a promising potential source to produce biosurfactants. It can be concluded that our study is the first study that mentioned the ability of *A. wentii* isolated from the soil as a biosurfactant producer.

Keywords: soil fungi, biosurfactants production, environmental conditions, vegetable oils.

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INTRODUCTION

The amphibious unique (contains polar and non-polar compounds) molecules are defined as biosurfactants. Biosurfactants can be used valuably in bioremediation as well as in some industries including pharmaceuticals (e.g. gene delivery agents, inhibitors of pathogens adhesion to solid surfaces, immunological adjuvants, anti-adhesive agents in surgical implants, and respiratory failure agents), food industries (e.g. bakery, lecithin, food additives, meat products, emulsifiers, and ethylene glycol), and oil recovery [1-3].

Surfactants can be produced artificially or naturally (biosurfactants) by different groups of microorganisms as metabolic by-products. Artificial surfactants have some disadvantages such as hard biodegradation and soil deterioration via their residues [4, 5]. Biosurfactants contain multiple and different kinds of chemical structures and functional groups such as glycolipids, lipopeptides, protein-polysaccharide complexes, phospholipids, and neutral lipids [6].

Biosurfactants have some advantages over synthetic surfactants including low toxicity, biodegradability, eco-friendly, foaming properties, biocompatibility, and tolerance to wide ranges of temperatures, pH values, and salinity [7]. One of the major applications of biosurfactants is the enhancement of oil recovery since biosurfactants are effective under a wide range of soil conditions, very selective, and can be used in small quantities [8, 9].

Different kinds of microorganisms (bacteria, fungi, and yeasts) are capable to synthesize multiple types of biosurfactants using water-soluble substrates such as glucose, sucrose, ethanol, and glycerol [1]. The bioremediation process of hydrocarbons can be improved by biosurfactants via the rising of (a) the bioavailability of substrate to microorganisms, and (b) the hydrophobicity through its interaction with cell surface allowing the association between the hydrophobic substrate and microbial cells [10-13].

There are some factors controlling the quantity of produced biosurfactants which are source and type of microorganism, and growth conditions (temperature, pH, pressure, and salinity) [14]. Researchers reported the ability of different microorganisms to produce biosurfactants. Bacteria, especially the genus Pseudomonas can produce a wide range of biosurfactants. In addition, different types of fungal species can produce biosurfactants such as *Yarrowialipolytica* [15], *Candida bombicola* [16], *Penicillium chrysogenum* [17], *Penicillium spiculisporum* [18], *Ustilago maydis* [19], and *Aspergillus versicolor* [20]. The present study investigates the ability of some fungal isolates isolated from Egyptian soil to produce biosurfactants as well as to study the production of biosurfactants using different types of oils such as olive oil, sunflower oil, corn oil, and frying waste oil.

MATERIAL AND METHODS

Isolation and Purification of Fungi

Oil-contaminated soil samples were collected from Al Dakahlia governorate, Egypt. The soil was leaked by oil from a nearby oil company. A 100 gm of soil sample was collected at a depth of 1-5 cm from the soil

surface. The dilution-plate technique [21] was applied to isolate fungi from soil samples. The modified inorganic salt medium (ISM) is used for the screening of fungi that can produce biosurfactants. The pH of ISM was adjusted to 7 for isolation of filamentous fungi [22] with the following composition: KCl (0.1 g l^{-1}), $CaCl_2$ (0.1 g l^{-1}), $MgSO_4$ (0.5 g l^{-1}), $NaNO_3$ (2 g l^{-1}), KH_2PO_4 (1 g l^{-1}), K_2HPO_4 (2 g l^{-1}), and trace salt solution (1 ml l^{-1}). The carbon source of ISM was soybean oil (1.0% *v/v*) which was selected for its low complexity (simple carbon chains). Fungal cultures were kept in 125-ml Erlenmeyer flasks at 30 °C then placed inside an orbital incubator for 7 days under continuous stirring at 120 rpm. Control flasks were used which contain only 50 ml of culture media supplemented with 1% soybean oil (*v/v*). All experiments were carried out in triplicates. After the incubation period, membrane filters (0.45 mm porosity, TPP, Europe/Switzerland) coupled with a 20-ml sterilized syringe were used for culture media filtration. The purified fungal isolates were given the code NRCB as an abbreviation for National Research Centre Biosurfactant.

Identification of Fungi

Fungal isolates were subjected to morphological and microscopic identification. The morphological identification depends on some features and characteristics including the diameter and color of conidia, the color and pigmentation of reverse mycelium, and extracellular exudates. The microscopic identification was carried out using an optical light microscope [(10 x 90) Olympus CH40]. The microscopic identification of fungi depends on some characteristics including sporulation degree, heads of conidia, homogeneity characters of conidiogenous cells, and fruiting bodies [23-29]. The fungal isolates were cultured on Malt extract agar (MA) at 28 °C for 7-10 days, then the fungal cultures were kept at 4 °C for further experiments.

Production of Biosurfactants

Oil displacement activity (ODA) test

ODA test was carried out according to Datta *et al*. [30] with a minor modification to determine the production of biosurfactants by fungal isolates. A 100 µl of used engine oil was placed on the surface of Petri dishes containing 40 ml of distilled water. A 10 µl of cell-free broth was then dropped on an oil-coated thin film. The circle clear zone diameter of displaced oil was measured.

Emulsification index (EI 24%)

EI 24% test was used for the screening of fungal isolates capable to produce biosurfactants. A mixture (2 ml cell-free supernatant and 2 ml kerosene) was vortexed for 2 min, then left for 24 h at room temperature. The following equation [31] was used for the calculation of results:

EI 24 % = (emulsified layer height after 24 h / total height of liquid) × 100

CTAB agar test

CTAB (cetyltrimethylammonium bromide) agar test was carried out to detect anionic surfactants according to Siegmund and Wanger [32]. ISM media was supplemented with 0.2 mg ml-1 methylene blue and 0.5 mg ml-1 CTAB [33], then 10 mm wells were made inside agar dishes using sterile cork and filled with the culture supernatant. The dishes were incubated for 48 h at 30 °C then left for 12 h at room temperature. The occurrence of bluish or bluish-green colors around the wells was observed.

Formation of foam

A graduated caped cylinder was used supplemented with a known volume of each supernatant then closed tightly and shaken vigorously for 1 min. The formation of foam was observed and recorded.

Stability of produced biosurfactants

Most important three environmental conditions (temperature, pH, and Salinity) were used for studying the stability of produced biosurfactants by fungal isolates. First, 10 ml of the supernatant was subjected to different temperatures (50, 70, 90, 110, and 121 ºC) for 30 min then left for cooling at room temperature. ODA test was used then to detect the activity of biosurfactants. Second, the supernatants were adjusted at different pH values (2, 3, 6, 10, 12, and 15), then the ODA test was carried out to detect the activity of biosurfactants. Third, the supernatants were supplemented with multiple saline concentrations (5, 10, 15, 20, and 25 % NaCl, *w/v*) and left for 20 min, the ODA test was used to study the activity of biosurfactants.

Vegetable oils for the production of biosurfactants

Four different vegetable oils (sunflower, waste frying, olive, and corn, 1 %) were used separately as carbon sources instead of soybean oil in ISM (50 ml), then inoculated with fungal isolates, and incubated under shaking (135 rpm) for 7 days at 30 °C. After the incubation period, an ODA test was carried out to examine the production of biosurfactants.

RESULTS

Isolation and Purification of Fungi

Soil samples (contaminated with oil) were subjected to isolation and identification of filamentous fungi depending on the growth on the modified ISM (with oil), the isolates (seventeen) which belonged to five different genera and twelve species were then identified morphologically. Table-1 summarizes the scientific names of fungal isolates after identification.

Table-1. Scientific names and code numbers of the different fungal isolates.

Fungal isolate number*	Scientific name
NRCB1	*Aspergillus niger*Van Tieghem
NRCB2	*Penicillium cyaneum* (Bainier&Sartory) Biourge ex Thom
NRCB3	*Trichoderma* sp. (Persoon) Horz
NRCB4	*Aspergillus niger*Van Tieghem
NRCB5	*Gibberella* sp.Schwabe
NRCB6	*Penicillium frequentans*Westling
NRCB7	*Aspergillus niger*Van Tieghem
NRCB8	*Aspergillus flavus* Link
NRCB9	*Aspergillus niger*Van Tieghem
NRCB10	*Aspergillus wentii*Wehmer
NRCB11	*Fusarium* sp. Link
NRCB12	*Aspergillus versicolor* (Vuill.) Tiraboschi
NRCB13	*Aspergillus glaucus* Link
NRCB14	*Aspergillus ornatus*Raper, Fennell and Tresner
NRCB15	*Aspergillus glaucus*Link
NRCB16	*Aspergillus glaucus* Link
NRCB17	*Aspergillus terreus*Thom

*NRCB: National Research Centre Biosurfactant.

Morphological Identification of Fungal Isolates

A total of 17 fungal isolates belonging to 5 genera and 12 species were identified as shown in Figure-1. The fungal isolates were subjected to identification depending on macroscopic and microscopic features (Figure-1). Table-2 summarizes the main macroscopic and microscopic features of identified fungal isolates.

Table-2. The main features of fungal isolates.

Fungal isolate	Macroscopic and microscopic features
Aspergillus niger	Initial growth is white then converts to black after a few days with the production of conidial spores. Smooth-colored conidia (with radial heads and biseriate). Conidiophores (hyaline and septate).
Aspergillus flavus	Thick mycelial mat (3 to 6 µm size). Colorless and rough-textured conidiophores.
Aspergillus wentii	Dense fluffy to cottony white colonies with a reddish reverse. Slow to moderate growth rate.
Aspergillus glaucus	Grayish turquoise to deep green cleistothecial. Cleistothecial has yellow central areas with a reverse color (pale yellow to pale brown).
Aspergillus ornatus	Very sparingly growth of colonies. Conidial heads radiate and are borne on conidiophores. No production of cleistothecia.
Aspergillus terreus	The rapid growth of colonies. Shallow radial furrows and velvety colonies.
Penicillium cyaneum	Mycelium consists of highly branched networks of multinucleated. Usually colorless hyphae.
Penicillium frequents	Rapid growth. Green color colony with yellowish border and valvate appearance. The reddish backside of the colony with yellowish border on PDA media.
Trichoderma sp.	Fast-growing colonies. Colonies appear white and downy at first then develop in small areas (or in concentric ring-like zones). Yellowish green to deep green compact tufts on the surface of the agar.
Gibberella sp.	Rapidly grown with white aerial mycelium then changes to purple with discrete orange sporodochia. *Gibberella* sp. is a teleomorph of *Fusarium moniliforme*.

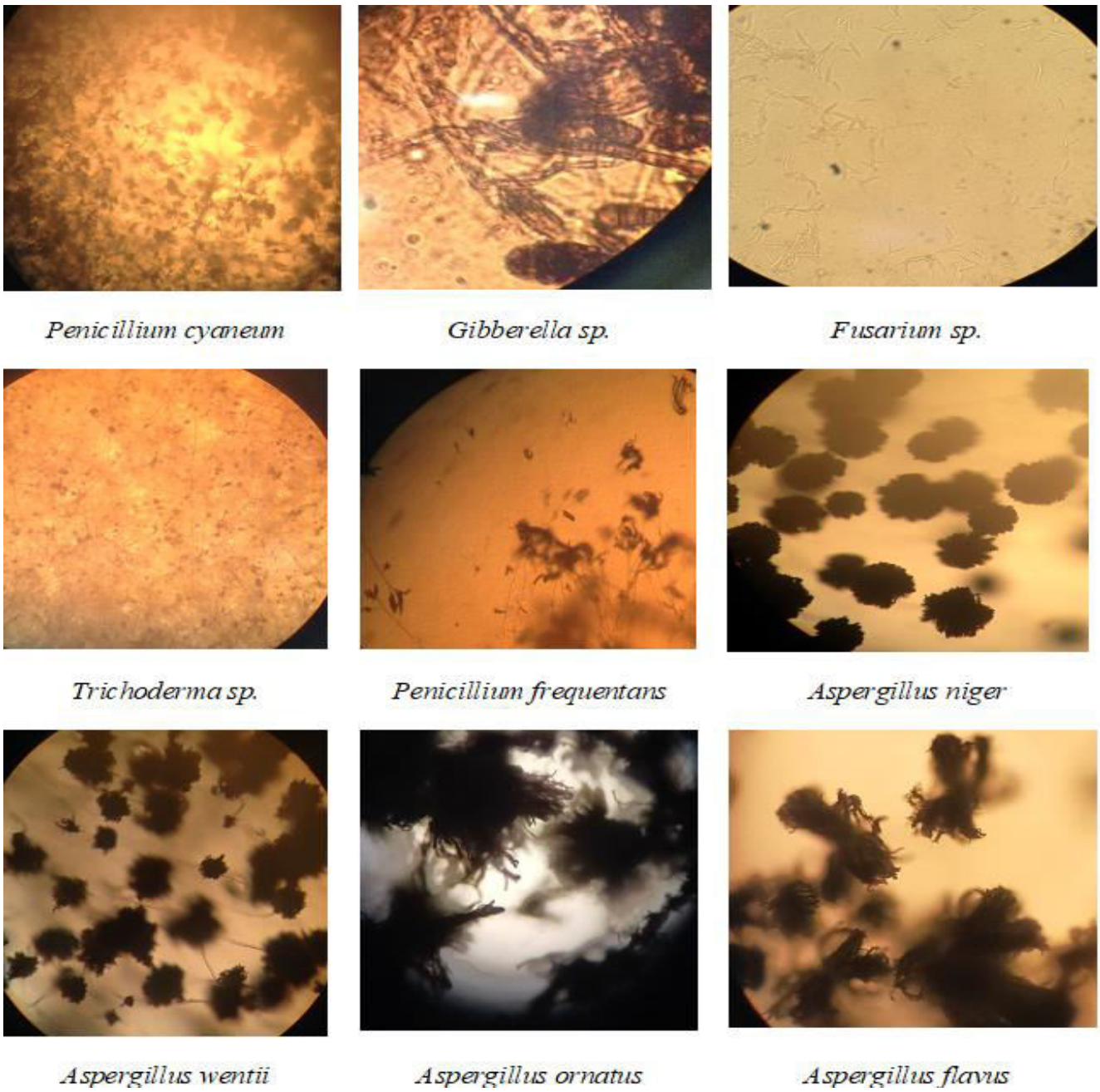

Figure-1. Microscopic observations of the isolated fungi; (A) *Gibberella* sp.: 1- microconidia, 2- conidiophore, 3- macroconidia, 4- sporodochia. (B) *Fusarium* sp.: 1- curved macroconidia, 2- pointed microconidia. (C) *P. cyaneum*: 1- long conidiophore, 2- a branched network of hyphae. (D) *P. frequentans*: 1- conidiophore, 2- rami, 3- phialides, 4- metula. (E) *Tricoderma* sp.: 1- phialospores, 2- conidiophore, 3- sterile hyphae. (F) *A. flavus*: 1- conidial head, 2- conidial chain. (G) *A. wentii*: 1- conidiophore, 2- conidial head, 3- conidial chain. (H) *A. niger*: 1- rotated conidial head. (I) *A. ornatus*: 1- conidial head, 2- conidial chain, 3- ascospores with additional crests. (J) *A. glaucus*: 1- conidiophore, 2- conidial head, 3- conidial chain, 4- cleistothecium. (K) *A. versicolor*: 1- conidiophore, 2- conidial head. (L) *A. terrus*: 1- conidiophore, 2- conidial head.

Ability to Produce Biosurfactants

Depending on some parameters, including the CTAB agar test, ODA, EI24%, and foam formation, a survey was carried out to detect the ability of fungal isolates to produce biosurfactants (Table-3). The results in Table-3 indicated the capability of all fungal isolates for the production of biosurfactants. A remarkable ODA (8 cm diameter) and high EI 24 % value (57.14 %) was observed for *Fusarium* sp. (isolate NRCB11). *A. flavus* (NRCB8) and *A. wentii* (NRCB10) showed slightly higher EI 24 % of 61.76 % and 60.0 %, respectively. Regarding the CTAB agar test, the positive biosurfactants production fungal isolate shows a bluish color. *Trichoderma* sp., *A. wentii*, and *Fusarium* sp. showed dark bluish color, while *A. niger*, *P. frequentans*, *A. flavus*, and *A. glaucus* showed faint bluish color. In addition, the results showed that ten

isolates of fungi could foam formation, while the other seven fungal isolates showed no ability to foam formation (Table-3). *Aspergillus ornatus* and *A. glaucus* showed the highest foam formation. Moreover, there was no observed correlation between the gained results from the foam formation test and the gained results from other used tests.

Table-3. Screening of tested fungi for biosurfactant production.

Fungal Isolate number*	ODA diameter (cm)	CTAB agar test	Foam formation	EI 24%
NRCB1	0.5	Very Faint bluish color	Good	54.28
NRCB2	2.5	Bluish color	Good	52.94
NRCB3	7.0	Very bluish color	Negative	37.5
NRCB4	3.5	Bluish color	Good	47.36
NRCB5	3.0	Bluish color	Good	44.11
NRCB6	1.0	Very faint bluish color	Negative	50.0
NRCB7	4.5	Bluish color	Negative	42.85
NRCB8	0.2	Very faint bluish color	Negative	61.76
NRCB9	4.5	Bluish color	Negative	45.45
NRCB10	2.5	Very bluish color	Good	60.0
NRCB11	8.0	Very bluish color	Negative	57.14
NRCB12	2.5	Bluish color	Good	51.11
NRCB13	5.5	Bluish color	Good	40.0
NRCB14	4.0	Bluish color	Excellent	52.63
NRCB15	2.5	Bluish color	Excellent	54.28
NRCB16	2.0	Very faint bluish color	Negative	50.0
NRCB17	1.0	Faint bluish color	Good	45.0

*NRCB: National Research Centre Biosurfactant.

Stability of Biosurfactants

The stability of biosurfactants at a wide range of different environmental conditions such as temperature, pH, and salinity play a major role in the application of biosurfactant in industry. ODA test was carried out to detect the stability of produced biosurfactants. Three environmental conditions were applied to study the stability degree of the different types of biosurfactants produced by fungal isolates. The first condition, different values of pH (2, 3, 6, 10, 12, and 15), the second condition, a wide range of temperatures (50 ℃, 70 ℃, 90 ℃, 110 ℃, and 121 ℃), and lastly the third condition, different saline concentrations (5 %, 10 %, 15 %, 20 %, and 25 %, as NaCl conc. (*w/v*)).

The biosurfactants produced by *A. niger* and *A. glaucus* showed the maximum ODA of eight cm at pH value 12, while at pH values 2, 6, and 10, the ODA results were retracted, however, at pH value 15, the ODA results were the lowest (Figure-2). It was clear that the alkalinity, especially at pH 12, strongly affected the stability and activity of the biosurfactants' emulsification.

Figure-3 represents the effect of different temperature degrees on the produced biosurfactants depending on the results of the ODA test. The biosurfactants produced by *Trichoderma* sp., *P. frequentans*, *A. flavus*, *A. niger*, *A. wentii*, and *A. glaucus* (NRCB15) showed good stability at a wide range of temperatures. The stability of the rest of produced biosurfactants is affected clearly by a change in temperature degree. In addition, it was observed that some of the biosurfactants showed stability at a fixed temperature degree, for example, biosurfactants produced by *A. versicolor* and *A. glaucus* (NRCB13) were stable at 110 ℃, and biosurfactants produced by *A. glaucus* (NRCB16) was stable at 90 ℃.

Regarding the effect of salinity, Figure-4 represents the results of the effect of different salt concentrations on the stability of the produced biosurfactants depending on ODA test results. The results indicated that all biosurfactants were stable at a salt concentration of 10 % NaCl. The stability of biosurfactants was decreased at a salinity of 15 % and 20%. Moreover, it was clear that both lower salt concentration (5 %), as well as highest salt concentration (25 %), had a negative effect on the stability of biosurfactants.

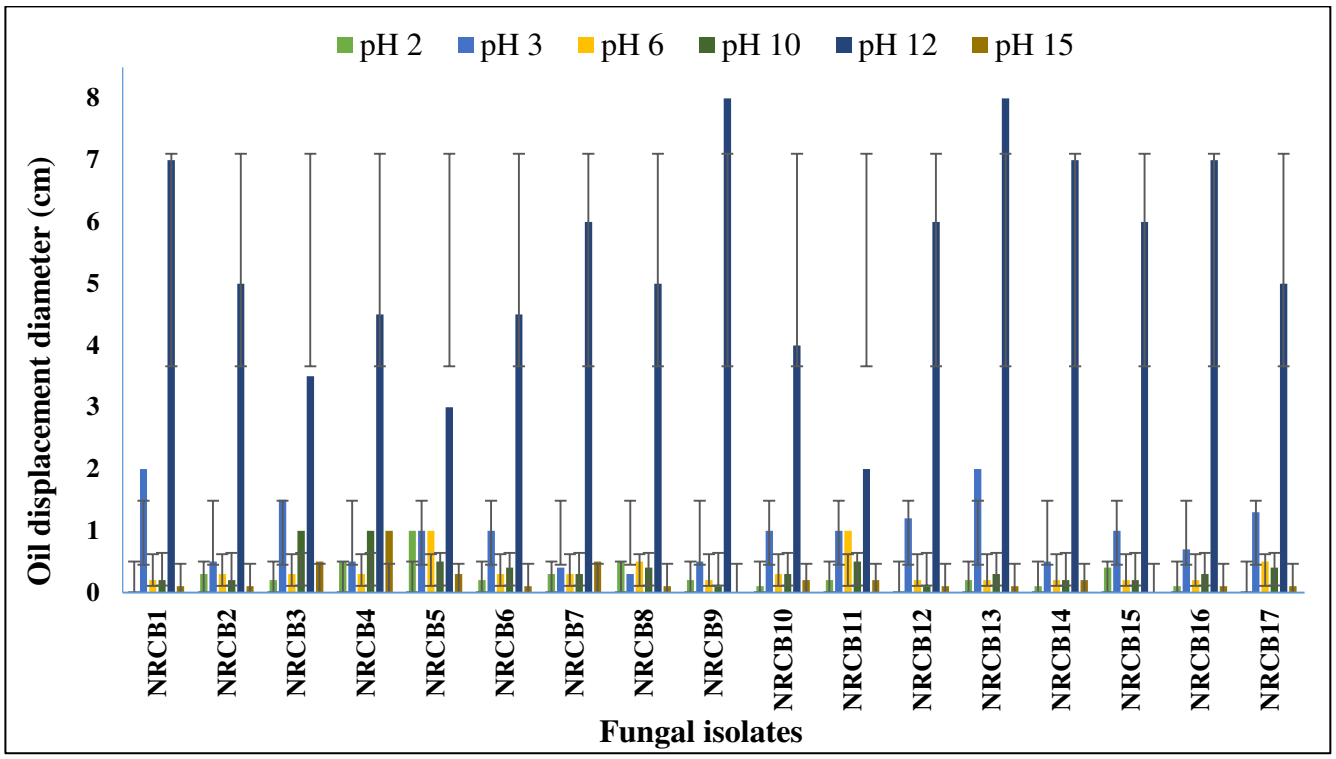

Figure-2. Effect of different pH values on biosurfactants stability. Data were expressed as mean (n = 3) ± SD.

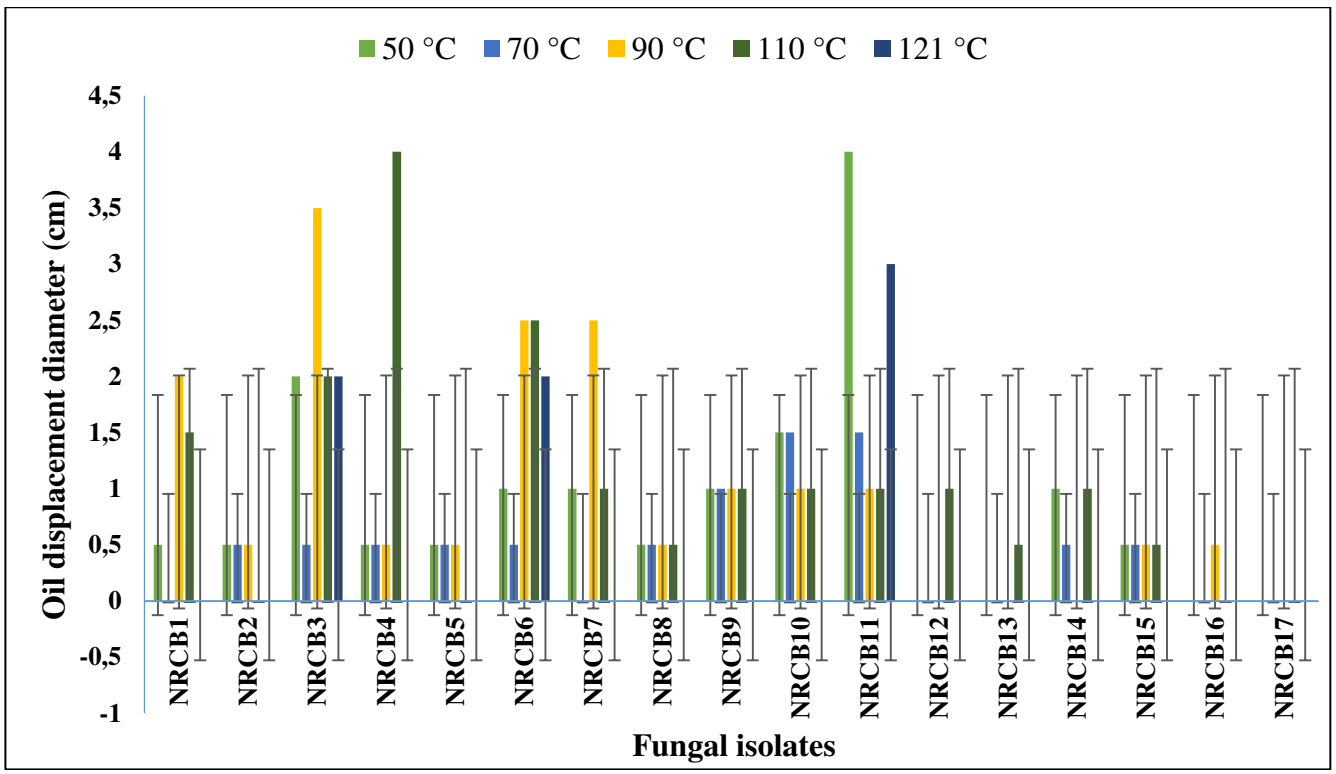

Figure-3. Effect of different temperature degrees on biosurfactants stability. Data were expressed as mean (n = 3) ± SD.

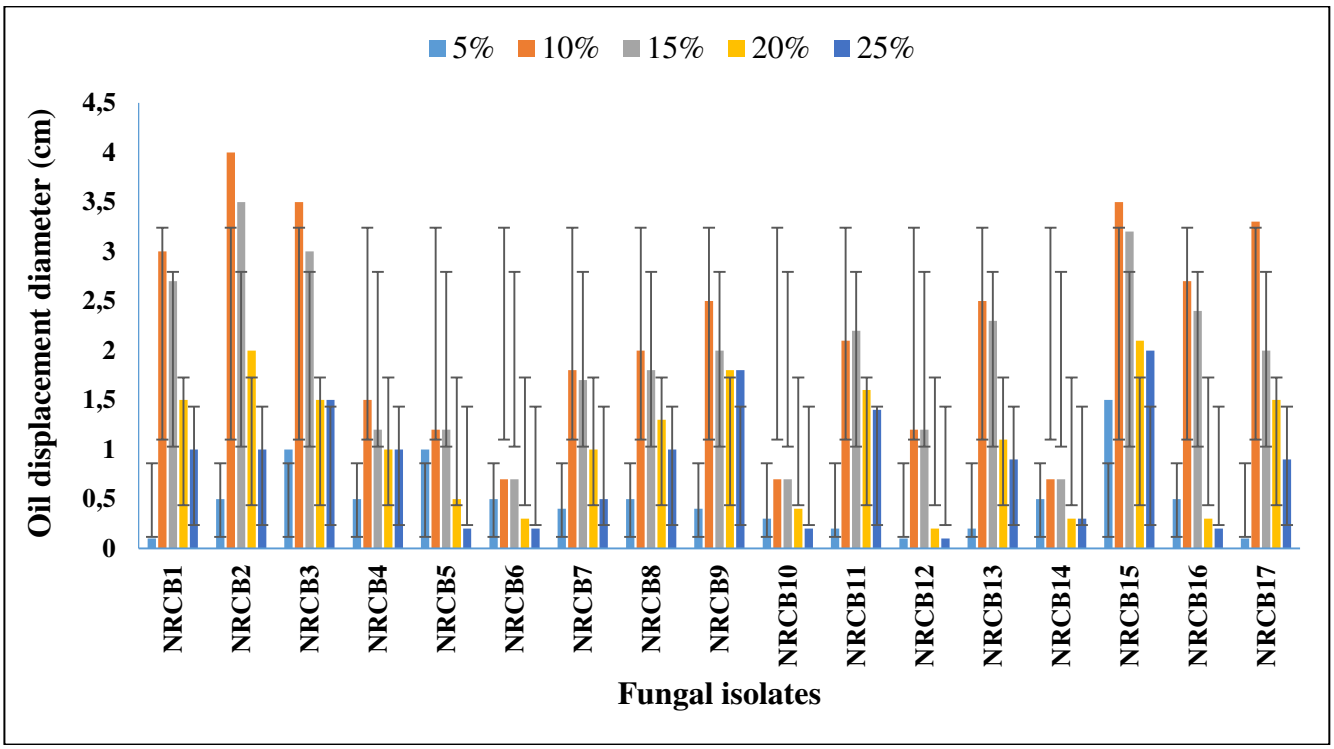

Figure-4. Effect of different salt concentrations on biosurfactants stability. Data were expressed as mean (n = 3) ± SD.

Production of Biosurfactants using Different Vegetable Oils

Four different types of vegetable oils (corn, sunflower, olive, and waste frying) were used, instead of soybean oil, as different carbon sources to study their enhancement effect on biosurfactants' production by fungal isolates. These four vegetable oils were selected for recycling purposes since these oils are used in daily activities in Egyptian houses and restaurants.

The results in Figure-5 summarize the effect of these vegetable oils on the production of biosurfactants depending on ODA test results. Sunflower oil showed a high enhancement effect on the production of biosurfactants followed by waste frying oil. The maximum ODA (8.5 cm) was observed for the biosurfactant produced by *A. wentii*.

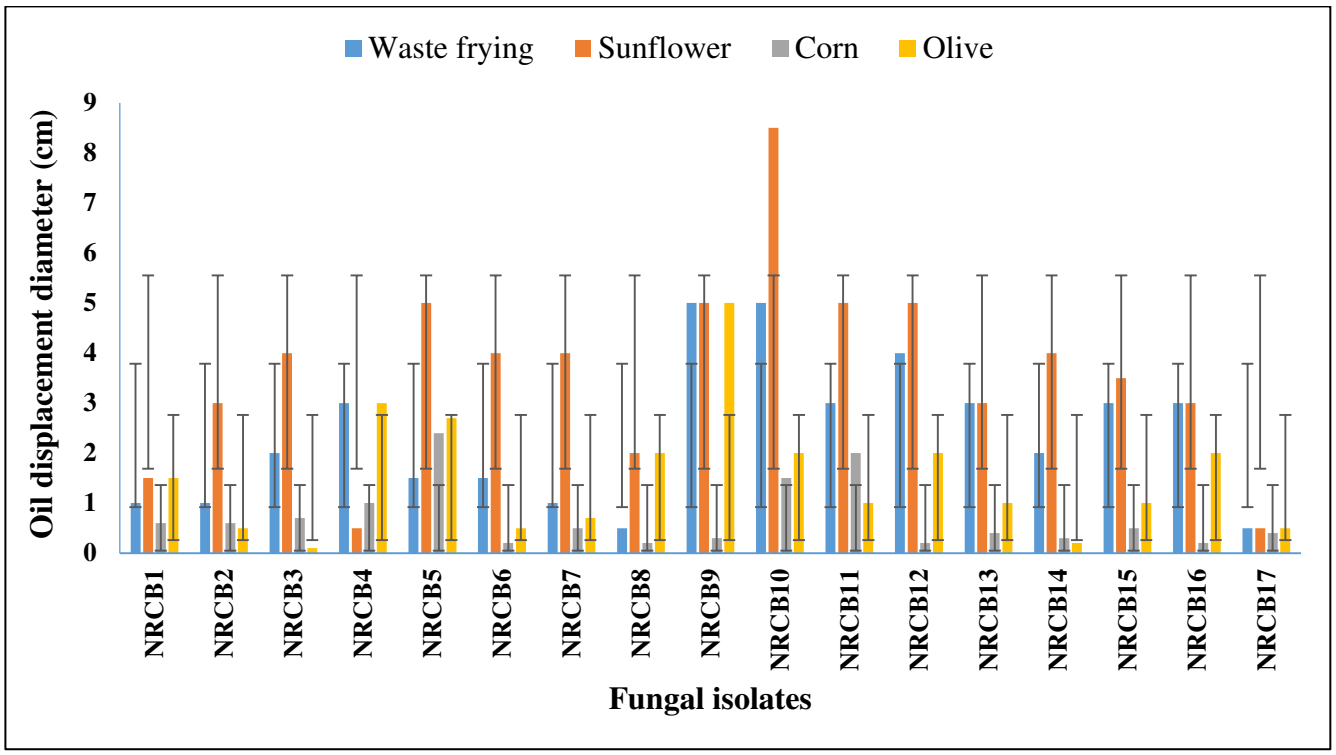

Figure-5. Effect of vegetable oils as carbon sources to produce biosurfactants. Data were expressed as mean (n = 3) ± SD.

DISCUSSIONS

The modified ISM (with oil) was used for the selection of fungal isolates depending on their growth, then identified morphologically and microscopically. The identification results were summarized in Table-1.

Depending on the growth ability of fungal isolates on ISM contain soybean oil; it was clear that all fungal isolates could produce biosurfactants. Previous studies reported that some species of *Aspergillus* sp. can produce biosurfactants from different sources such as *A. terrus* from oil spills [36, 37], *A. flavus* from citrus fruits [34], and *A. niger* from banana stalks powder during solid-state fermentation [35].

All available published literature reported the ability of *Aspergillus* sp. to produce biosurfactants from different sources, not including soil. Moreover, some species of the genus Aspergillus such as *A. wentii*, *A. glaucus*, and *A. ornatus* were not proven yet for their ability to produce biosurfactants. The present study proved their ability to produce biosurfactants. Lima *et al.* [38] reported the ability of *Eichhornia crassipes* (*Fusarium* sp.) isolated from oil-contaminated water to produce biosurfactants. However, another teleomorph of *Fusarium* sp., *Gibbrella* sp., has not yet been reported as a biosurfactant producer. Spina *et al.* [36], Pitocchi *et al.* [37], and Sena *et al.* [39] reported that different *Penicillium* sp. and *Trichoderma* sp. can produce biosurfactants.

Depending on the obtained results (Table-3), the best fungal isolates for the production of biosurfactants were *A. flavus*, *A. wentii*, and *Fusarium* sp. which was comparable with previous studies [17, 40, 41] reported that ascomycetes genera *Penicillium* sp., *Aspergillus* sp., and *Fusarium* sp. could produce biosurfactants with a wide range of spectrum and variety. For the application of produced biosurfactants in industry, Figures 2-4 represent the stability of produced biosurfactants under different three major environmental conditions including pH, temperature, and salinity. The alkaline environment (pH 12) positively affected the stability and emulsification capability of the produced biosurfactants. Khopade *et al.* [42] explained the effect of alkaline pH and attributed it to the high stability of fatty acid surfactant micelles, especially in the presence of NaOH, and the precipitation of secondary metabolites at alkaline pH values. In contrast, by increasing the alkalinity to 15, there was a notable and clear decrease in the emulsion activity and stability of all produced biosurfactants. This phenomenon may be attributed to the chemical changes and modifications in some functional groups present in biosurfactants as a result of very strong alkalinity, which led to changes in the chemical structure and consequently in its activity and characteristics. The same results were reported about the stability of two types of biosurfactants, at pH 12, produced by *Candida lipolytica* [43] and *Cunninghamella echinulate* [44]. Andrade Silva *et al.* [44] stated that environmental factors such as pH, salinity, and temperature are well known to affect the activity and stability of biosurfactants produced by microorganisms.

The produced biosurfactants showed an observed variation in temperature stability as shown in Figure-3. This variation may be due to the different chemical structures of the produced biosurfactants [45]. Another possible reason for this variation was reported by Udoh and Vinogradov [46] who revealed this variation to the strength of hydrogen bonds between hydroxyl groups

(OH) preventing any significant dehydration relative to high temperatures. High temperatures have adverse effects on biosurfactant activity. Ahmad *et al*. [47] studied the adverse effect of high temperatures up to 100 ℃ on the activity of biosurfactant produced from *Klebsiella* sp. which affected negatively the biosurfactant's surface tension. Many previous studies [48, 49, 50] reported that temperature degrees ranging from 30 ℃ to 120 ℃ have positive effects on the stability of biosurfactants produced by microorganisms such as *Bacillus subtilis* [53], *Candida glabrata* [52], and *C. lipolytica* [51].

Furthermore, salinity is considered one of the main environmental conditions affecting the activity and stability of biosurfactants. In the present study, different salt concentrations were used to study the effect of salinity on the activity and stability of produced biosurfactants by fungal isolates (Figure-4). It was clear that salinity of 10 % to 20 % (NaCl) showed the highest effect on the activity and stability of biosurfactants. The maximum activity occurred at 10 % salt concentration. These results agreed with the results reported by researchers who studied the effect of salinity on the stability and activity of biosurfactants produced by *Fusarium* sp. [55], *Candida tropicalis* [54], and *C. sphaerica* [56]. It was clear that most of the produced biosurfactants during this study showed considerable stability at high salt concentrations and high pH values making them appropriate for the biodegradation of oil spills in marine environments [42].

In a try to enhance the production of biosurfactants by fungal isolates depending on the reuse of four different vegetable oils (corn, sunflower, olive, and waste frying) which are widely used in Egyptian daily activities in both houses and restaurants. The obtained results in Figure 5 showed that sunflower oil, followed by waste frying oil, enhanced the production of biosurfactants more than other used vegetable oils. This may be because sunflower oil contains a high percentage of linoleic acid (60 %). Ferraz *et al*. [57] reported the enhancement of linoleic acid on the production of biosurfactant by *Serratia marcescens*. Waste frying oil was used by Zadeh *et al*. [58] for increasing the biosurfactant production by *Mucor circinelloides*. In contrast, olive oil showed a moderate enhancement effect on the production of biosurfactants by fungal isolates. Qazi *et al*. [55] and Fontes *et al*. [59] reported that olive oil failed to increase the production of biosurfactant by *Yarrowialipolytica* and attributed that to the adhesion of surfactant with *Yarrowialipolytica* cell wall. Finally, we could not explain the lowest enhancement effect of corn oil on the production of biosurfactants by fungal isolates. In a future study, the chemical structure of produced biosurfactants will be carried out which will help for deep understanding and gives more explanations.

CONCLUSIONS

The present study proved the ability of local soil fungi as biosurfactants producers. Most of the biosurfactants produced by soil fungi had notable stability at a wide range of pH values (2-15), temperatures (50 ℃-121 ℃), and salinity (NaCl; 5-25 %). The maximum enhancement of biosurfactants activity was observed by using sunflower oil followed by waste frying oil. Locally isolated fungi from soil can be considered a proper source for the production of biosurfactants. Depending on our results and the published literature, the present study proved for the first time that the following soil fungi: *A. wentii*, *A. glaucus*, *A. ornatus*, *A. versicolor*, and *Gibberella* sp. are recorded as producers of biosurfactants. Also, the present study proved, for the first time, the ability of *A. wentii* to produce biosurfactants.

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