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Original Research

Screening of Biosurfactants Producing Fungi from Infected *Citrus sinensis* (L.)

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Abstract

The possibility of using the diseased *citrus sinensis* plant as a feedstock for the synthesis of biosurfactants by various species was investigated in this study. Four fungal species were identified, namely *Penicillium glabrum*, *Alternaria alternata, Alternaria solani*, and *Rhizopus stolonifer.* Preliminary screening of biosurfactants was carried out by dye binding assay. About 5.75cm ring was noted for *Penicillium glabrum.* In the blood hemolysis assay, about halo diameter of clear zone 3.85cm was noted for *Rhizopus stolonifer.* The highest emulsification (E24) and oil displacement ability were noted for *Penicillium glabrum* and *Rhizopus stolonifer.* Maximum drop collapsing ability was observed for *Alternaria alternata.* Maximum drop sliding was observed for *Alternaria solani.* In the Phenol-Sulfuric acid and foaming activity assay, all four fungal species showed good activity, which indicates

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the presence of biosurfactants. In the microplate assay, four fungal species showed small results and minor distortion. In the penetration assay, only three species, namely, *Penicillium glabrum*, *Alternaria alternata*, and *Rhizopus stolonifer,* showed positive results and were able to move through hydrophilic fluids to reach the oil layer, resulting in a color change from red to hazy white.

Keywords: biosurfactants, fungal species, isolation, identification, spoiled Citrus sinensis

Introduction

Biosurfactants are amphipathic biomolecules that are formed on the surfaces of microbial cells (extracellularly) and contain both hydrophobic and hydrophilic moieties. Surfactants can be microbial or synthetic; synthetic surfactants are formed by chemical synthesis, while microbial surfactants are produced by microorganisms. Synthetic surfactants are widely used in medical, pharmaceutical, agricultural, and oil industries [1]. A cyclic peptide containing a short order of amino acids is found in the hydrophilic domain of biosurfactants, and the conservative fatty acid particle is found in the hydrophobic moiety. Enzymes of four subunits catalyze the synthesis of the peptide process, which culminates in covalent attachment and cyclization towards a straight chain of hydrophobic domains to produce biosurfactants [2]. Microbiological surfactants are divided into two types: lower molecular weight biosurfactants (lipopeptides and glycolipids) and higher molecular weight.

Biosurfactants such as lipoproteins and polysaccharides [3]. Glycolipids are a well-known biosurfactant. Plants, animals, bacteria, and fungi all produce these molecules in their cells. Such molecules improve the absorption of nutrients, increase motility, and therefore are commonly elaborated in the microbial biofilm's formation. Glycolipids are classified into four subgroups depending on their structural carbohydrate: sophorolipids, rhamnolipids, mannosyl-erythritol, and cellobiose lipids [4]. Liposaccharides, glycolipids, phospholipids, neutral lipids, fatty acids, and some polymers are some of the natural bioactive compounds found in such materials [5]. Biosurfactants have a more difficult chemical structure that is associated with distinct surface properties. Compared to synthetic surfactants, they do not have a clear polarity distribution and have ring or branch structures [6]. The ability of biosurfactants to reduce surface rigidities at the boundary of phase between substrates and liquids permits these bacteria to gain access to nutrients, allowing for metabolism and absorption of nutrients in addition to growth on the water-immiscible substances. Surfactants, in general, function as emulsifiers, dispersants, and formers [7]. Biosurfactants are used to improve of the hydrophilic abilities of agricultural soils as additives to pesticides and fertilizers, showing increased activity and solubility. They are utilized in the food industry as food products, such as in the production of sweets or the clarification of beer and juices. They had a positive effect on food texture [8]. Biosurfactants are biodegradable amphipathic infective compounds with low critical micelle concentration (CMC), nontoxicity, resistance to dangerous acidity and basicity and acidity, osmotic pressure, and antimicrobial and anti-inflammatory potential that can be derived from renewable sources, substrates, and industrial by-products [9]. Biosurfactants as emulsifiers can function as antivirus biomolecules, therapeutically, and as immunomodulator molecules in dealing with immunological disorders [10]. Heavy metals form metallic or ionic complex precipitates on the surface of soil. They are removed from polluted areas with surfactant-mediated formation of complexes and ion exchange [11].

Experimental

The samples were collected from different aerial parts of infected *Citrus sinensis* (L.) Osbeck plants like fruit, stem, and leaves from orange fields. The species were grown on the SYPGA medium containing starch 20 g, yeast extract 3.0 g, peptone 5.0 g, glucose 1.0 g, and agar 18.0 g at 27°C [12]. Cultures were stored at 4°C and refreshed fortnightly. Cultures were preserved in a glycerol-water mixture and stored at 20°C. The fungal species identified after [13].

CTAB-MB Agar Medium

The development of a blue ring around the colony shows the production of biosurfactants in the CTAB-MB agar method after [14] was used.

Blood Hemolysis Assay

Blood agar was prepared by dissolving (2.4 g) of nutrient agar in (100 mL) of distilled water. The liquid was sterilized for 15 min at 121°C, then left to cool down before aseptically adding (5 mL) of sheep blood. The diameters of hemolysis-induced clearing zones around colonies on agar dishes were calculated [15].

Preparation of Culture Medium for Biosurfactant Production

The (GPSY) medium was prepared for biosurfactant production, containing l.0 g glucose, 5.0 g peptone, 20 g starch, and 3.0 g yeast extract. Replicates were prepared to get fine results. After two days, surface activity was tested daily by taking samples (1 mL) from each flask in Eppendorf tubes [16].

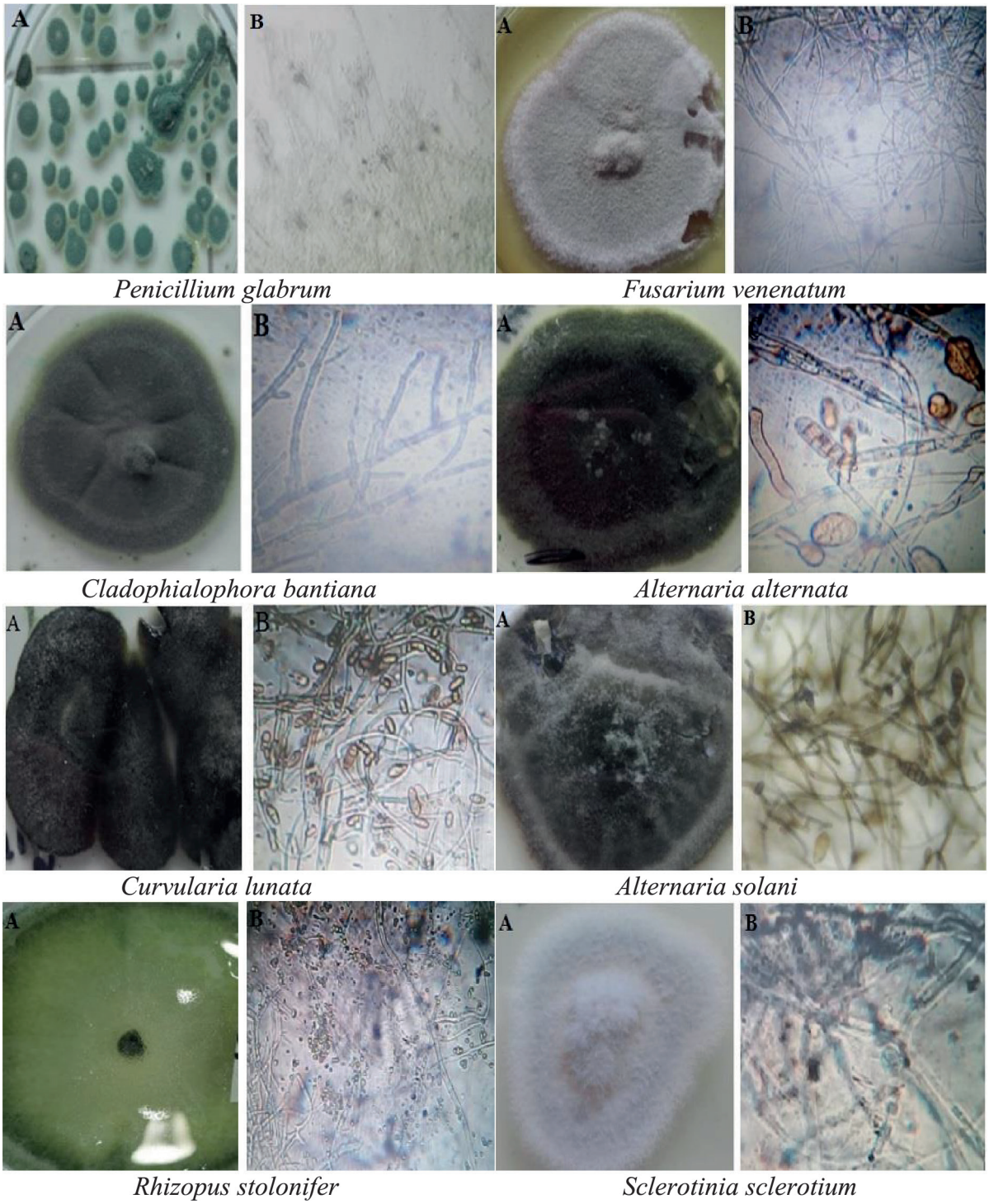

Fig. 1. Macro & Micrographs of fungal isolates.

Surface Activity Test

Quantitative Analysis

The oil displacement assay was performed by pouring 50 mL of distilled water into a Petri plate topped with 20 µL of kerosene oil and 10µL of crude extract, which exhibited the oil-spreading ability of biosurfactants [17]. The drop collapsing method was performed by a drop of the culture extract (about 10 µL) placed on a glass slide that was already coated with kerosene oil [18]. The Emulsification Index was carried out by the following protocol. An equal volume (1 mL) of kerosene oil and culture extract was taken in a test tube and vortexed for 2 minutes at rapid speed before resting at 20ºC for 24 h [19]. In the drop sliding assay, a drop of (0.9%) NaCl was placed on one corner of the glass slide that was tilted at an angle of 45º to which 10 µL of crude extract was in it [20].

Qualitative Analysis

In the phenol: H_2SO_4 method, approximately (1mL) of 5 percent phenol was added to (1mL) of supernatant. 2-5 mL conc. H_2SO_4 was added to this mixture drop by drop, till an orange shade developed [21]. In the foaming assay, cultures and nutrient broth were cultured in a shaker incubator for 96 h at 30°C. In a graduated cylinder, 10 mL of culture was stirred forcefully for 2 minutes [22]. In the microplate assay, about 100µL samples of each supernatant were obtained and placed in a microwell on a 96-well plate. A backing paper with a grid was used to view the plate [23]. A 96-well plate with 150 µL of a hydrophobic paste consisting of oil and silica gel was used for the penetration assay. The mixture was covered with 20 µL of oil. Afterward, 90 µL of supernatant was made, supplemented with 10 µL of 1% safranin, of which 20 µL was carefully spread on the top of the preparation [24].

Results

Identification of Fungi

On the (PDA) potato dextrose agar medium, selected high-yielding species were grown in plates as described in the methods. Morphological and microscopic features and their growth patterns were examined. Fungal species belonged to *Penicillium* sp., *Alternaria* sp., and *Rhizopus* sp. (Table 1)

Preliminary Screening for Biosurfactant Production

Biosurfactant production by fungi was determined by measuring the diameter of the turquois ring formed around the microbial colonies growing on CTAB-MB plates after 120 h. The biggest ring was formed by CSFS2 (5.75±1.65 cm) and CSLS8 (4.55±0.75 cm) (Table 2). Results are shown in the following macrographs (Fig. 1).

Blood Hemolysis Assay for Biosurfactant Production

Biosurfactant production by fungi was determined by measuring the diameter of the turquois ring formed around the microbial colonies growing on nutrient agar and sheep blood after 48 h. As clear from the data in Table 3, the biggest ring was formed by *Rhizopus stolonifer* (3.85±3.25) and *Penicillium glabrum* (3.65±0.15).

Table 1. Microscopic and Morphological characteristics of the fungal species isolated.

Species names & sources	Genus	Species	Morphological characters	Microscopic features
CSFS2 (Fruit)	*Penicillium* sp.	Penicillium glabrum	Colonies are flat and gray,	Hyaline, smooth, or rough-walled, flask-like shape.
CSFS4 (Fruit)	*Fusarium* sp.	Fusarium venenatum	Colonies are brightly colored.	Cottony aerial mycelium is coenocytic.
CSFS6 (Fruit)	*Cladophialophora* sp.	Cladophialophora bantiana	Black colony, velvety texture,	Wavy chains of conidia, and absence of conidiophores. Hyphae are septate, unbranched conidial chains,
CSLS8 (Leaf)	*Alternaria* sp.	*Alternaria alternata*	Colonies are fast-growing, greyish, velvety	Acropetal branch chains of conidia. Conidia are obclavate, pale brown, smooth-walled.
SLS10 (Leaf)	*Curvularia* sp.	Curvularia lunata	Colony is rapidly growing, velvety	Matures septate brown hyphae, simple & branched conidiophores
CSLS12 (Leaf)	*Alternaria* sp.	*Alternaria solani*	Dark green velvety colony,	Mycelia consist of branched septate brown hyphae and dark-colored conidia.
CSSS16 (Stem)	*Rhizopus* sp.	Rhizopus stolonifer	Colony is a grayish fluffy mass of black	Sporangia contain spores and have rhizoids cotton-like white growth spotted with black color.
CSSS18 (Stem)	*Sclerotina* sp.	Sclerotina sclerotium	Round to irregular shape white colony water drops observe	Cup-shaped apothecia Aerial mycelium, septate hyphae.

Table 2. Halo production on CTAB-MB agar medium; ND: not detected, ± indicates standard deviation.fungal species isolated.

Species	Halo diameter(cm)
CSFS2	5.75±0.5
CSFS4	3.95±0.6
CSFS6	3.35±0.95
CSLS8	4.55±0.75
CSLS10	2.85±0.15
CSGH22	ND
CSSS16	4.15±0.75
GHSA65	ND
ASSA80	ND
CSSS18	3.85±0.35
CSSS20	ND
CSLS12	3.05±0.85

Table 3. Halo production on nutrient agar medium; ND: not detected, ± indicates standard deviation.

Fungal Species	Halo diameter(cm)
Penicillium glabrum	3.65±0.15
Fusarium venenatum	1.35±0.3
Cladophialophora bantiana	ND
Alternaria alternata	2.45±0.35
Curvularia lunata	1.35±0.25
Alternaria solani	ND
Rhizopus stolonifer	3.85±0.75
Sclerotina sclerotium	1.25±0.8

Surface Activity Test

Oil Displacement Test

Biosurfactant production in culture extract isolated from the spent medium was confirmed by an oil displacement test after incubation for a period of 216 h (9 days). As clear from Fig. 2, the maximum oil displacement was observed for *Penicillium glabrum* and *Rhizopus stolonifer,* i.e., 6.75 cm after 144 incubation periods (6 days); *Curvularia lunata* showed maximum production of 6.75 cm after 168 incubation times (7 days); ND: not detected.

Drop Sliding on Tilted Glass Slide

Culture extract was examined to detect the presence of biosurfactant production by drop sliding ability on a tilting glass slide. Maximum drop sliding was observed for *Alternaria solani,* showing maximum production, i.e., 7.75 cm, followed by *Fusarium venenatum,* showing 7.55 cm after 168 h of incubation of time on 7 days of culture extract (Fig. 3).

Drop Collapsing Ability

Biosurfactant production in culture extract was confirmed by drop collapsing ability. Maximum drop collapsing ability was observed for *Alternaria alternata,* i.e., 2.4 cm after 168 h of incubation on 7 days of culture extract; ND: not detected (Fig. 4).

Emulsification Index Ability

The culture extract was tested for the presence of biosurfactants using an emulsification assay. The highest emulsification (E24) was noted for *Penicillium glabrum* and *Rhizopus stolonifer,* i.e., 72% after 24 h of incubation on 7 days of culture extract. ND: not detected (Fig. 5).

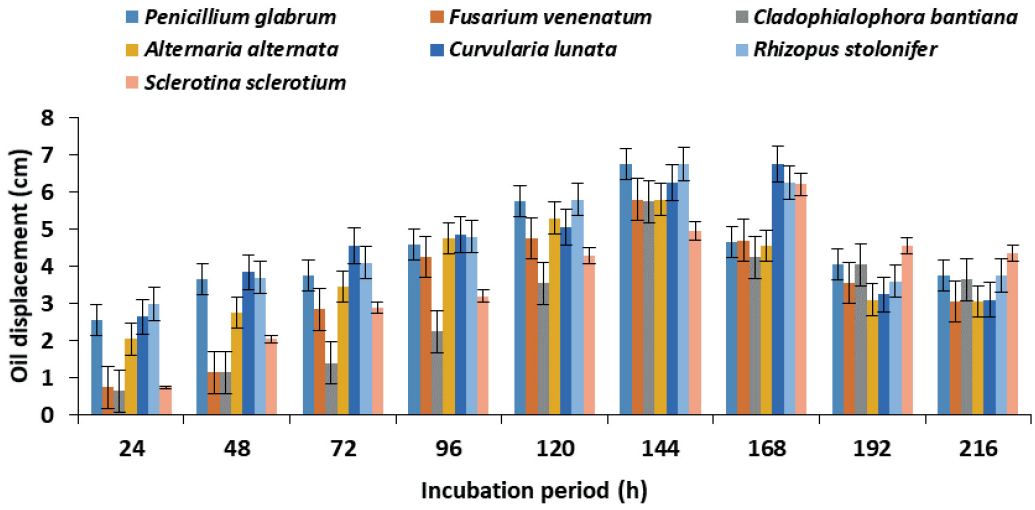

Fig. 2. Oil displacement ability of crude extract of fungal species.

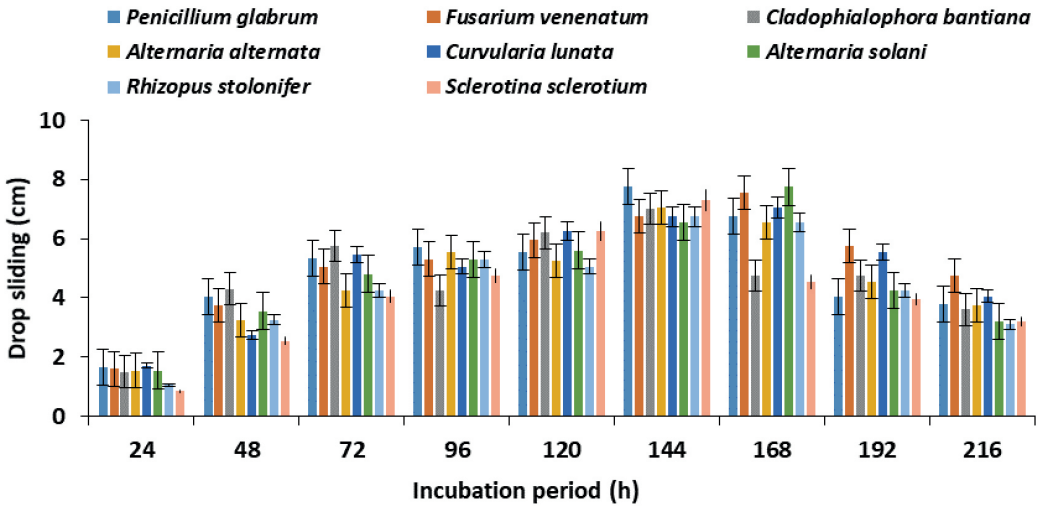

Fig. 3. Drop sliding ability of crude extract of fungal species.

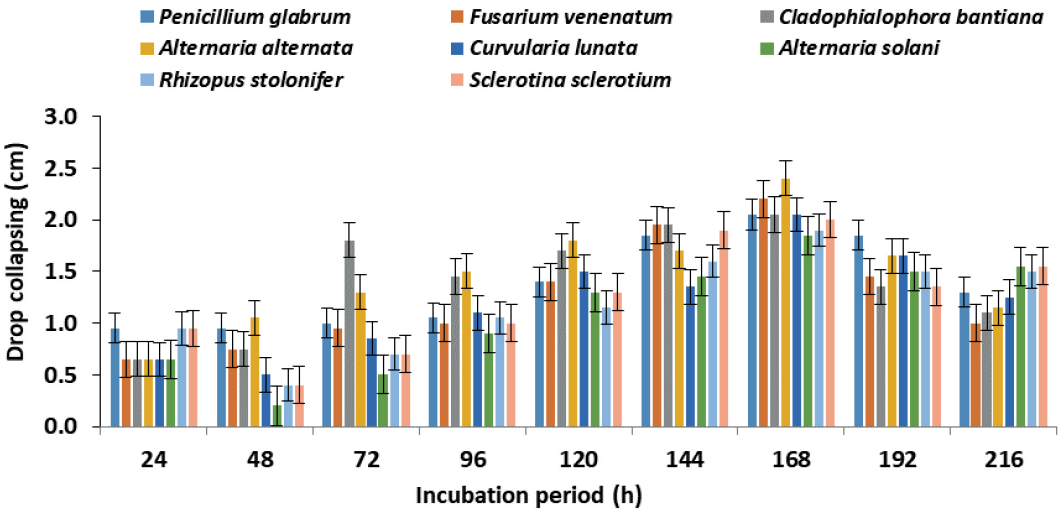

Fig. 4. Drop collapsing ability of crude extract of fungal species.

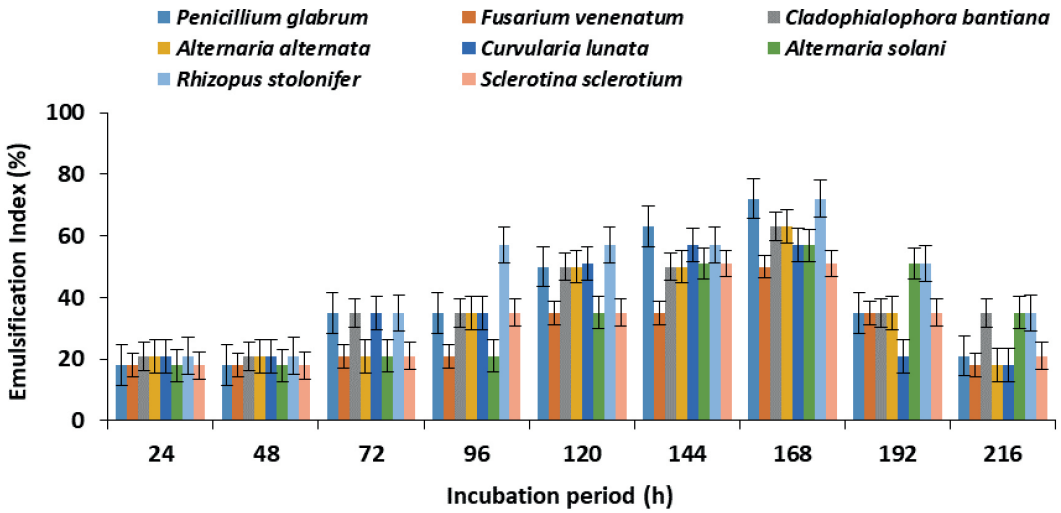

Fig. 5. Emulsification index ability of crude extract of fungal species.

Discussion

The fungal species were collected from different aerial parts of infected *Citrus sinensis* (L.) Osbeck plants like fruit, stems, and leaves. Fungal species were identified by macro and microscopy, belonging to *Penicillium* sp, *Fusarium* sp, *Cladophialophora* sp, *Alternaria* sp., *Curvularia* sp, *Rhizopus* sp, and *Sclerotinia* sp. For preliminary screening of relatively high-producing organisms, the dye-binding ability of different species was studied. Maximum halo production, i.e., 5.75cm ring diameter, was observed in *Penicillium glabrum*, followed by 4.55cm for *Alternaria alternata* and 3.55 cm for *Fusarium venenatum*. On CTAB-MB agar, medium dark blue ring formation around the colony reveals the biosurfactant production [14]. Biosurfactants especially rhamnolipids, are anionic and form complexes with cationic surfactants CTAB and dye methyl blue [25].

For screening of biosurfactant formation, a hemolytic blood agar assay has been applied [26]. Biosurfactants cause erythrocyte lysis through the colloid-osmosis process. Biosurfactant production is typically detected using a blood hemolysis test, which produces translucent or yellow/green halos surrounding colonies in blood agar [27].

A blood agar lysis method was applied to the preliminary screening procedure. However, none of this research addresses the idea of producing biosurfactants without hemolytic action. Many biosurfactant producers were excluded from using this strategy. Surface tension does not correlate with blood lysis agar. While the concentration of biosurfactant increased, the diameter of lysis on agar plates increased linearly. Eight fungal species were isolated; six were significant for the production of biosurfactants, but two could not lyse the agar plates. The diameter of the zone formed around microbial colonies growing on nutrient agar and sheep blood after 48 h. The biggest diameter was formed by *Rhizopus stolonifer,* i.e., 3.85 cm, and *Penicillium glabrum,* i.e., 3.65 cm [28] also reported the same result of the blood agar plate.

The emulsion ability of the supernatant isolated from different species was detected for screening. Emulsification ranged from 18% to 72%. The existence and quantity of biosurfactants can be determined using oil emulsification. An emulsification test was performed in the current study using crude oil, kerosene, and olive oil. Surfactant emulsification qualities are measured using emulsification index activity. The capacity of biosurfactants to produce an emulsion is inversely proportional to the volume of a droplet; the slighter the droplet, the greater the activity [29]. As a result, kerosene had the most excellent emulsification index (EI) with biosurfactants because it had the smallest oil droplet size. The emulsification index seems to have a strong relationship with pH, indicating that pH affects biosurfactant stability. Maximum emulsification ability (E24) of crude biosurfactants from *Penicillium glabrum* and *Rhizopus stolonifer* was 72% after 24 h of incubation on 7 days of culture extract, which was closely related to the 10% of standard CTAB and greater than SDS. This value is higher than those reported by other workers [30]. The crude extract was used to test the oil displacement ability. Oil displacement was directly related to biosurfactant concentration, i.e., the greater the extent of oil drop dispersal, the higher the biosurfactant concentration. Biosurfactants are non-ionic molecules that reduce the interaction between oil and water [31]. Maximum oil displacement was observed for *Penicillium glabrum* and *Rhizopus stolonifer,* i.e., 6.75cm after 144 incubation periods on 6 days of culture extract. *Curvularia lunata* showed the maximum production of 6.75 cm after 168 incubation times on 7 days of culture extract, which was equivalent to 4 g/L of CTAB (6.6cm) and 10 g/L of SDS (6.5cm), respectively. This value is higher than those reported by other workers [32].

The drop-collapse method is used to estimate the reduction in surface tension. Water molecules' surface tension is caused by cohesive forces between them. The positioning of the hydrophilic and hydrophobic moieties of the biosurfactants influences surface and interfacial activity. Maximum drop collapsing ability was observed for *Alternaria alternata,* i.e., 2.4 cm after 168 h of incubation on 7 days of culture extract, which was close to the maximum value of 12g/L of SDS (2.5 cm) and greater than CTAB. Drops collapsed due to a reduction in surface tension and the breaking of cohesive interactions between water molecules. This property of biosurfactants is essential for their use in detergents. This value is higher than those reported by other workers [23].

A drop sliding test was used to determine the anti-adhesive characteristics of biosurfactants from various species. Drop sliding decreases cohesion, adhesion, and hydrophobicity despite raising the contact angle. Biosurfactants alter the balance of adhesive and cohesive forces, causing liquid droplets to fall to the ground [33]. Drop sliding ranged from 0.8-7.7 cm after 24 h of incubation. Maximum drop sliding was observed for *Alternaria solani,* showing maximum production, i.e., 7.75 cm, followed by *Fusarium venenatum,* shown at 7.55 cm, which was close to the 20 g/L of SDS (7.5cm) standard and greater than CTAB. The biosurfactant's anti-adhesiveness is essential for its antiviral and biofilm-removing abilities [34].

Glycolipid biosurfactant production was also determined by the Phenol-Sulfuric test. The existence of glycolipids was indicated by the formation of an orange color in the supernatant from a yellow color [35]. All eight species showed the appearance of an orange color, indicating the production of glycolipids containing surfactants, which was a positive result [36]. They also reported the same positive result of the Phenol-Sulfuric test.

Foaming power is among the most important properties that biosurfactants must have [37]. The

Table 4. Qualitative test.

Qualitative test	Penicillium glabrum	Alternaria alternata	Alternaria solani	*Rhizopus stolonifer*
Phenol: H_2SO_4 test	++	+	_	+
Foaming activity	++	+++	++	+
Microplate assay	+	+	_	_
Penetration assay	++	++	+	_

qualitative measure of biosurfactant formation is the existence of stable foaming in combination with the lower surface tension of the solution [38]. All eight species showed good foaming activity (Table 4), which indicates the presence of biosurfactants in the medium [39], and also reported the same results of foaming activity.

Biosurfactant production was also determined by microplate assay. Distilled water in a well also has a smooth surface that touches the well's sides at a 90° angle. The surfactant in the water provides some wetting at the well's edge, causing the fluid surface to become concave. When viewed from above, the fluid resembles a single diverging lens, distorting the picture of the grid beneath the well [40]. When the view of the grid was seen through the baking sheet of the paper, all eight fungal species that were placed in the hydrophobic media of the microplate showed small results and minor distortion in the image as compared to the workers, whose results showed more biosurfactant production [41].

The existence of biosurfactants was confirmed in the penetration assay since the hydrophilic solution broke through oil film barriers into the mixture. Within 15 minutes, the silica entered the hydrophilic phase, and the top side changed from clear red to cloudy white [42]. From eight culture extracts, only four samples (*Penicillium glabrum*, *Fusarium venenatum*, *Alternaria alternata, and Rhizopus stolonifer*) were able to move through hydrophilic fluids to reach the oil layer, resulting in a color change from red to hazy white. In a previous study, the screening of biosurfactants by penetration assay was performed in which two fungal species gave positive results, while in this research, four gave positive results and four gave negative results [43].

Conclusions

In this study, different parts of the infected *Citrus sinensis (L.) Osbeck* plants were used to examine fungi and screened to contain powerful biosurfactant producers, which were easily detected using screening tests such as oil drop collapse, blood hemolysis assay, microplate assay, oil displacement test, emulsification index, CTAB microplate assay, and foaming activity test. The selected species were identified by macro and microscopy. In CTAB-MB agar medium, maximum halo production i.e., 5.75 cm ring diameter was observed in *Penicillium glabrum*. In the blood hemolysis essay, the biggest diameter was formed by *Rhizopus stolonifer,* i.e., 3.85 cm. Maximum emulsification ability (E24) of crude biosurfactant from *Penicillium glabrum* and *Rhizopus stolonifer* was 72% after 24 h of incubation on 7 days of culture extract. Maximum oil displacement was observed for *Penicillium glabrum* and *Rhizopus stolonifer,* i.e, 6.75cm after 144 incubation periods on 6 days of culture extract; *Curvularia lunata* showed maximum production of 6.75 cm after 168 incubation periods on 7 days of culture extract. Maximum drop collapsing ability was observed for *Alternaria alternata,* i.e., 2.4 cm, after 168 h of incubation on 7 days of culture extract. Among the isolates, *Penicillium glabrum* and *Rhizopus stolonifer*, it has been discovered to be a great performer as a biosurfactant manufacturer. Reaction between phenol and sulfuric acid. It is necessary to confirm the existence of an anionic biosurfactant if the biosurfactant is of the glycolipid type (rhamnolipid).

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Conflict of Interest

The authors declare no conflict of interest.

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