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Biosurfactants production by some Aspergilli species under solid state fermentation

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BIOSURFACTANTS are active secondary metabolites producing` by certain microorganisms that have the ability to reduce surface and interfacial tension. They have numerous advantages, such as high biodegradability, increased foaming, low toxicity and performance under challenging conditions. Current study was compared between the potential production of biosurfactant by *A. carneus* OQ152507, *A. niger* OQ195934 and *A. fumigatus* OQ195264 using solid state fermentation (SSF) as a low-cost alternative technology. These isolates were cultivated under SSF using different eight agricultural wastes to produce biosurfactants. The optimized bioprocess conditions for the maximum biosurfactant productivity were potato peels waste (20g/l) as a carbon source at 35ºC and pH 7 in glucose peptone medium for *A. carneus* OQ152507, sun flower seed shells (28g/l) at 35ºC and pH 7 in starch nitrate medium for *A. niger* OQ195934 and tea wastes (20g/l) at 30ºC and pH 6 in glucose peptone medium for *A. fumigatus* OQ195264. Their stability was observed at 120°C, 30g/l of NaCl and alkaline pH 10 for *A. carneus* and pH9 for *A. niger* and *A. fumigatus* biosurfactants. The biosurfactants were identified as glycolipid and phospholipids molecules by Phenol-H_2SO_4 and phosphate test. GC-MS and FT-IR analysis confirmed that the tested fungi participate in producing three hydrophobic fatty acid chains: *Cis*-Vaccenic acid, n-Hexadecanoic acid and Hexadecanoic acid 2-hydroxy-1 (hydroxymethyl) ethyl ester in distinct peaks with the highest percentage area. While, each one has at least one specific biosurfactant moiety.

Keywords: biosurfactant, *A. carneus*, *A. niger*, *A. fumigatus*. bioprocess, GC-MS, FT-IR

Introduction

Microbial biosurfactants has potential uses in the agriculture, bioremediation, detergent, petrochemical, cosmetic, pharmaceutical and food industries (Kumari et al., 2023). They are amphiphilic surface-active substances, classified into five major groups including lipopolysaccharides, lipopeptides, phospholipids, glycolipids, and fatty acids (Sobrinho et al., 2014). They are distinctive secondary metabolites, produced non-ribosomal by certain bacteria, fungi (Ndlovu et al., 2017). They are either integrated into the cell membrane or released extracellularly (El-Shahed et al., 2022; Santos et al., 2023). The amphiphilic structure of a surfactant, consists of a hydrophobic moiety which is either a long-chain fatty-acid, α-alkyl-β-hydroxy fatty acid or hydroxyl fatty acid and the hydrophilic moiety can be a carbohydrate, a peptide, an amino acid, a phosphate group, a carboxylic acid, or alcohol, among others (Rufino et al., 2008; Mandal et al., 2013). Most biosurfactants described in the literatures are produced by bacteria such as *Bacillus* sp., *Pseudomonas* sp. and *Acinetobacter* sp. (Geetha et al., 2018) and fungi such as *Candida Antarctica* (Rodrigues et

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al., 2006), *Candida bombicola* (Pinto et al., 2022) *Trichoderma atroviride, T. citrinoviride, T. reesei* and *T. harzianum* (Piegza et al., 2021), *T. viride* (Maheswari & Parveen, 2012), *Fusarium fujikuroi* (Dos Reis et al., 2018), *Aspergillus flavus* (Ishaq et al., 2015), *A. niger* (Asgher et al., 2020) and *Penicillium chrysogenum* (Sanches et al., 2021). The emulsification of hydrophobic substrates by the biosurfactants enhances the availability of nutrients required for their development (Singh *e*t al., 2020). The production of microbial surfactants is influenced by many factors including physical factors such as temperature, pH, agitation, available oxygen as well as nutrient composition, of growing substrate materials including carbon, nitrogen, phosphorus, magnesium, iron and sulfur (Banat et al., 2010; Asgher et al., 2020). In this context, numerous studies have focused on biosurfactant production by certain fungi cultivated on solid state fermentation (SSF) (Banat et al., 2021; Dos Santos et al., 2021). Solid-state fermentation uses industrial or agricultural wastes such as sunflower seed shell, grape wastes or potato peels as renewable cheap in order to reduce costs. Velioglu et al. (2015) succeeded in optimizing biosurfactant production by *Pleurotus_djamor* in solid state fermentation. *Candida bombicola* was grown on soy molasses as a substrate and to produce sophorolipid biosurfactant (Solaiman et al., 2004). The maximum biosurfactant production was obtained from *A. niger* M2 spp. grown solid state fermentation of banana stalks powder (Asgher et al., 2020). The aim of the work is to investigate the biosurfactant potentials of three Eurotiaceae family members and compare their stability at extreme conditions as agents for improved oil recovery.

Materials and Methods

Isolation of biosurfactant producer fungal strains:

Fungi were isolated from crude oil contaminated soil and water samples of Kafr El-Sheikh governorate, Egypt which were collected in sterile glass bottle and sterilized plastic bags in PDA as pure culture. A serial dilution was made up to 10^{-4} using one gram of petrochemical soil sample in a sterile tube containing nine milliliters of sterilized distilled water, and then 100µl of each dilution was plated onto a Petri dish with Sabouraud medium (Da Silva et al., 2019). The colonies that formed were purified in sterile Petri dishes with the same culture after 72hrs of incubation at 29°C. To get monosporic crops, a streaking technique was used. The same culture media was used to retain fungal colonies in slants, and they were then identified by looking at both their macroscopic and microscopic characteristic (Nandhini & Josephine, 2013; Abedi et al., 2023). A total of eighty-nine morphological distinct fungal organisms were obtained**.**

Biosurfactant producing fungi identification

Three different *Aspergillus* species were isolated from crude oil contaminated soil. They were identified using the morphological and microscopical features (color, colony diameter, and textural appearance), microscopic features (mycelium shape, spores color and shape, phialides shape, sterigmata shape as *Aspergillus carneus*, *A. fumigatus* and *Aspergillus niger*. The emulsification index (%) and oil spreading test (cm) were used to evaluate the potential of biosurfactant producing fungi.

Morphological and molecular characterizations of the tested isolate

Identification of the promising three fungal isolates identified morphologically as biosurfactant producers were confirmed by molecular analysis using 28S-rRNA. Comparisons with sequences in the GenBank database were achieved in Blast searches at the National Center for Biotechnology Information (NCBI) site (http://WWW.ncbi.nlMnih.gov). Molecular and evolutionary genetics analysis (MEGA X) software was used to align the sequences of NCBI GenBank data to determine the taxonomic position of the strains and to create the phylogenetic tree (Kumar et al., 2018). The nucleotide sequences of fungal strains were submitted in GenBank to obtain their accession number**.**

Biosurfactant producing fungi cultivation

Spore suspension of each fungus was prepared in glycerol 20% to obtain 1×10^4 spores/ml^{-1}. To study the effect of different hydrocarbon oil on the biosurfactant production, the fungi was cultivated separately in in Erlenmeyer flasks (250ml) containing 100ml of potato dextrose broth medium supplemented with 10ml/l of different oils separately. Each flask was inoculated with one ml of spores of the isolates. Then, the flasks were incubated with shaking at 130 rpm, 29±2ºC for 7 days, followed by filtration with a quantitative filter paper (Ø,12.5cm; pore size, 28μm; Quanty) for removal of fungal cells. The filtrate was separated for use in screening of biosurfactants by oil spreading test (cm) and the emulsification index (%) (Accorsini et al., 2012).

Various cultural conditions like culture media type, different agricultural wastes, temperature of incubation, initial medium pH and cultural media amount were optimized by one factor at a time for the biosurfactant production by *Aspergillus carneus*, *Aspergillus niger* and *A. fumigatus*.

Emulsification index

Emulsification index (E_{24}) was determined according to Asgher et al. (2020), four mL aliquot of the culture filtrate was mixed with 6ml of toluene in a test tube. The mixture was shaken vigorously for 2min on a tube shaker-type vortex. After 24h, the ratio of emulsified toluene was compared with the total volume. The emulsification index of 1% (w/v) SDS was also determined as a control. The emulsification index was calculated using the following formula (Alvarez et al., 2015; Sena et al., 2018):

$$\text{Emulsification index}(E_{24}) = \frac{\textit{Hight of emulsion layer (cm)}}{\textit{Total hight of solution (cm)}} \times 100$$

Oil spreading test

Oil spreading test was performed using the method described by Asgher et al. (2020) with little modifications. Briefly, distilled water (50 ml) was poured in a glass Petri dish. Two mL of sunflower seed oil was added to the water surface to form a thin layer. Cell free supernatant containing biosurfactant (100 µl) was gently poured at the center of Petri plate. The area of displaced oil layer was measured.

Biosurfactant extraction

For extraction, content of each flask (1000ml) has agricultural waste materials received 100ml of sterilized distilled water and was agitated for 1h at 29ºC and 150rpm (Derguine-Mecheri et al., 2018). The suspension was centrifuged at 4ºC and 5,000rpm for 15 min. Cells were removed from the cultivation medium by re-centrifugation at 5,000rpm and 4°C for 30min. The supernatant was removed; then, 10ml of chloroform and methanol (2:1, v/v) was added to the precipitated pellet and incubated in a rotary shaker at 30°C and 200rpm for 20min. The content was centrifuged at 5,000rpm and 4°C for 30min and the supernatant was evaporated by air drying (Chander et al., 2012).

Biosurfactant characterization

Phenol-H_2SO_4 test

One ml of cell free supernatant was mixed with 1ml of 5% phenol, then 3-5ml of conc. H_2SO_4 was added drop wise until an orange color was developed, shows the presence of glycolipids in the crude biosurfactant (Dubois et al.,1956).

Biuret test

Two ml of cell free supernatant was heated to 70ºC and mixed with 1M NaOH solution and then $CuSO_4$ solution was added to it drop wise. Formation of violet or pink color ring indicates the presence of lipopeptides (Feigner et al., 1995; Jamal et al., 2014).

Phosphate test

Two mL of cell free supernatant was mixed with 6-10 drops of 5M HNO_3 and heated to 70ºC, then 5% ammonium molybdate was added drop wise until the appearance of yellow colored precipitate, indicates the presence of phospholipids (Okpokwasili & Ibiene, 2006).

Gas-Mass chromatography

The gas chromatograph coupled to the mass spectrometer detector was a GCMS-QP2010 Ultra. The composition was elucidated by comparison with an analytical standard of methylated fatty acid ester-FAME mix standard. Individual components were identified using their relative retention indices with the Wiley Registry of Mass Spectral Data (Thavasi et al., 2007; El-Housseiny et al., 2020).

Fourier transform infrared spectroscopy (FT-IR):

The characterization of produced biosurfactant was performed using FTIR analysis (Yan et al., 2012). The column-purified biosurfactant was analyzed using a TENSOZ 27 (serial no 2887) FTIR-Spectrophotometer at 500 to 4000 cm^{-1} in ATR mode for the present functional groups and bond type detection (Patowary et al., 2017).

Stability of the biosurfactants

The obtained biosurfactants from solid state cultures were subjected to different factors in order to study its stability such as temperature, pH, salt concs and different hydrocarbons.

Effect of temperature

Two mL of supernatant were exposed to various temperatures for 1hr and then allowed to cool at room temperature. The activity of the biosurfactant was measured by E_{24}.

Effect of pH value

The supernatant of each culture was adjusted at different pH values (2-14). The activity of each was tested using E_{24}.

Effect of salt stress

Different concentrations of NaCl (10, 20 and

30%, w/v) were prepared using the supernatant of each culture and left for 20 min, after which the activity of each was tested using E_{24} (El-Sheshtawy & Doheim, 2014; Fawzy et al., 2023).

Effect of different hydrocarbons

In this study, the effect of different hydrocarbons on the stability of biosurfactant produced by the tested fungi illustrated, the used hydrocarbon are three types vegetable oils, motor oils and aromatic oils.

Statistical analysis

The SPSS program (version 16 for Windows) was used for all statistical analyses. After a one-way ANOVA, Student–Newman–Keuls multiple range test was used to compare the means at $p \leq 0.05$.

Characterization and molecular identification of fungal strains

Aspergillus carneus morphologically characterized by a white color at the beginning turns to buff (Brownish) with diameter of 1.2cm, a reverse color pale yellow to pale brown and brown droplets as extra cellular exudates. Microscopically, conidial head is Large globose biserrate, brown conidia, thick-walled hyphae and smooth, brownish conidiophore that carrying a globose vesicle. The initial growth of *Aspergillus niger* is white in color but after few days, it converts into black with production of conidial spores. Microscopically, *A niger* is characterized by smooth colored conidia (with radial heads and biseriate) and conidiophores (hyaline and septate). However, *A. fumigatus* characterized by hyaline, septate, branched, multicellular mycelium and faint green conidia appear in chains in basipetal succession carried on round vesicle.

Molecular identification of the isolated biosurfactant producing fungi was carried out by 28S rRNA gene sequence analysis. The 28S-rRNA genes of *A. carneus*, *A. niger* and *A. fumigatus* have been sequenced and entered into GenBank with accession number OQ152507, OQ195934 and OQ195264, respectively. The studied strains belong to the fungal class Ascomycetes. The result represented in **Fig.1** after multiple sequence alignment between the obtained sequences indicated that the tested sequences had 100% similarity PCR sequences with their respective species *Aspergillus carneus*, *A. niger* and *A. fumigatus*.

Optimization of biosurfactant production

Effect of different hydrocarbon oils

Ten ml per liter of different hydrocarbon oils were added into potato dextrose broth medium separately for the selected fungi which was cultivated separately. The supernatant of all the tested fungi showed an emulsification activity with the emulsification index ranging from 16.67 to 65.42% **(Table 1).**

Emulsification index for each fungus varied with the hydrocarbon oil used. Kerosene had the highest emulsification index of *A. carneus* (65.42%) followed by petrol 80 (62.33%) for *A. niger* and diesel (59.39 %) for *A. fumigatus***.** Oil spreading test mentioned that the kerosene was the favorable substrate for biosurfactant activity of *A. carneus* with the oil spreading 4.27cm, while the highest oil spreading of *A. niger* was 4.13 cm when using petrol 80 as a substrate. Diesel oil gave the maximum oil spreading of *A. fumigatus* surfactant (3.93 cm). The oil spreading of biosurfactant of *A. fumigatus* supernatant that produced by using diesel as a substrate was higher than those obtained with the other tested oils. It represents more than seven folds compared to petrol 80 (**Fig. 1**).

TABLE 1. Effect of different hydrocarbon oils on the biosurfactant production potential of *A. carneus, A. niger* and *A. fumigatus* depending upon their emulsification index (E_{24}) and oil spreading test.

Hydrocarbon oils	Emulsification index (%)			Oil spreading test (cm)		
	A. carneus	*A. niger*	*A. fumigatus*	*A. carneus*	*A. niger*	*A. fumigatus*
Sun flower seed	53.33^{c}	42.33^{c}	37.00^{d}	2.00^{d}	2.60^{d}	2.00^{e}
Paraffin	55.67^{bc}	31.67^{d}	46.82^{c}	2.60^{c}	1.50^{e}	2.87^{d}
Olive	29.18^{d}	45.45^{c}	54.78^{b}	1.93^{d}	3.00^{c}	3.43^{b}
Diesel	59.06^{b}	28.00^{e}	59.39^{a}	3.33^{b}	1.00^{f}	3.93^{a}
Petrol 80	24.36^{d}	62.33^{a}	16.67^{e}	1.50^{e}	4.13^{a}	0.50^{g}
Kerosene	65.42^{a}	57.33^{b}	33.48^{d}	4.27^{a}	3.70^{b}	1.37^{f}
Soy bean	24.33^{d}	22.22^{f}	51.21^{b}	1.00^{f}	0.60^{g}	3.20^{c}

Different superscript letters in the same column indicate significant differences ($p \leq 0.05$)

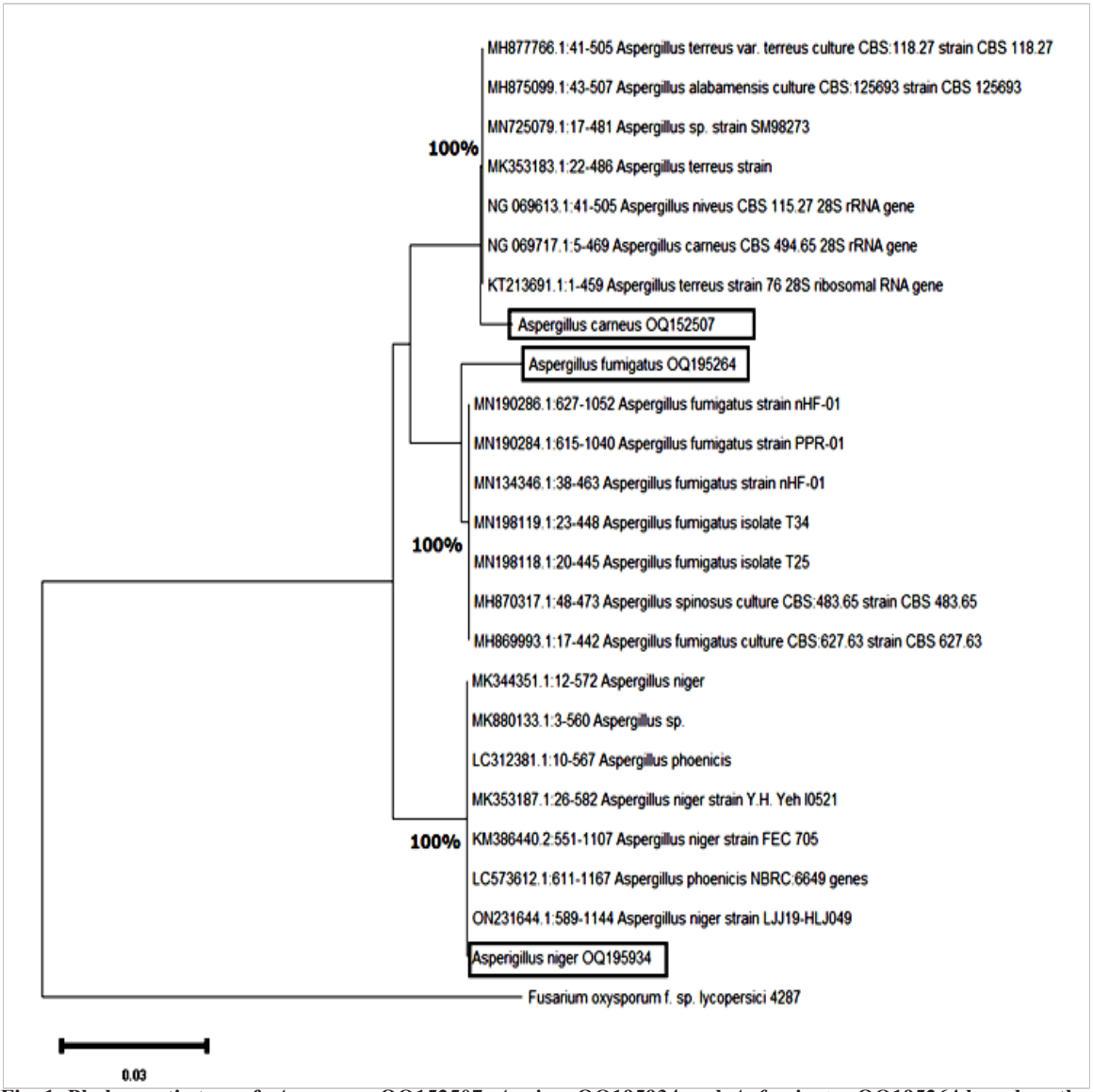

Fig. 1. Phylogenetic tree of *A. carneus* OQ152507, *A. niger* OQ195934 and *A. fumigatus* OQ195264 based on the outcomes of PCR amplification of the 28S-rRNA gene.

Effect of different culture media

In this experiment, flasks containing different culture media were incubated with the best oil for each fungus, so Kerosene was added for *A. carneus*, Petrol 80 for *A. niger* and diesel for *A. fumigatus* at 30°C for 10 days and shaked at 120rpm. The cultivation of *A. carneus, A. niger* and *A. fumigatus* separately showed that the isolates can efficiently produce biosurfactant under submerged culture conditions. Both the emulsification index and oil spreading test of *A. carneus* and *A. niger* biosurfactant varied significantly with the type of the culture media used **(Table 2).** Concerning of the emulsification index, the highest value (62.7%) obtained by *A. niger* surfactant using starch nitrate medium. While both *A. carneus* and *A. fumigatus* preferred the glucose peptone medium to produce their biosurfactant with emulsification index (57.2 and 58.7%, respectively). The oil spreading test conferred that the starch nitrate was the best for *A. niger,* while the glucose peptone medium was preferred by *A. carneus* and *A. fumigatus* to produce their best biosurfactant. The highest oil displacement (3.63cm) was observed using glucose peptone medium for *A. fumigatus,* followed by 3.47cm for *A. carneus* using the same medium. While the oil displacement was 3.30cm by *A. niger* supernatants collected Bennett´s and Starch nitrate media.

TABLE 2. Effect of different culture media on the biosurfactant potential of *A. carneus, A. niger* and *A. fumigatus* depending upon their emulsification index (E_{24}) and oil spreading test.

Culture media	Emulsification index (%)			Oil spreading test (cm)		
	A. carneus	*A. niger*	*A. fumigatus*	*A. carneus*	*A. niger*	*A. fumigatus*
Glucose liquid	44.79^{b}	32.50^{d}	54.33^{b}	3.20^{b}	2.10^{c}	3.00^{b}
Glucose peptone	57.27^{a}	50.40^{b}	58.70^{a}	3.47^{a}	2.50^{b}	3.63^{a}
Yeast-malt extract	40.19^{c}	45.53^{c}	46.67^{d}	2.00^{d}	2.13^{c}	2.00^{d}
Bennett´s	41.26^{c}	52.26^{b}	49.67^{c}	2.00^{d}	3.30^{a}	2.37^{c}
Starch nitrate	42.11^{c}	62.7^{a}	33.33^{e}	2.53^{c}	3.30^{a}	1.07^{e}
Potato dextrose	42.70^{c}	31.2^{d}	44.15^{d}	2.00^{d}	1.92^{c}	2.02^{d}

Means with different letters in the same column differ significantly at $p \leq 0.05$.

Effect of Solid state fermentation using different agricultural wastes as substrate

To study the effect of different agricultural wastes on the biosurfactant activity, the identified isolates were cultivated separately in their preferred solid media inoculated with different agricultural wastes (Wheat bran, Wheat straw, Rice straw, Sugarcane bagasse, Potato peels, Banana peels, tea waste and sunflower seed shells) as a carbon source to determine the best substrate for biosurfactant production.

The biosurfactant potential expressed by emulsification index (E_{24}) and oil spreading test varied significantly according to the type of agricultural wastes. The highest emulsification indexes were obtained by potato peels (62.44%) for *A. carneus* and sunflower seed shells (60.33%) for *A. niger,* while *A. fumigatus* preferred tea wastes to registered 60% of emulsification index **(Table 3).** The oil displacement of the tested fungi had relatively the same pattern of the emulsification index, with the maximum value at potato peels, Sunflower seed shells and tea wastes for *A. carneus*, *A. niger* and *A. fumigatus*, respectively**.**

Effect of agricultural waste concentrations

To study the effect of different agricultural wastes concentrations on the biosurfactant activity, the tested fungi; *A. carneus, A. niger* and *A. fumigatus* were cultivated separately in their preferred culture media supplemented with different concentrations of previously agricultural wastes. The data obtained in the **Table 4** conferred that the biosurfactant activity measured by emulsification index and oil spreading varied significantly with the concentration of the wastes in the microbial culture medium. The highest emulsification index (75.57%) was observed by *A. niger* using 28g/l sunflower seed shells, while it was 69.22% for *A. carneus* when using 20g/l of potato peels. On the other hand, the highest biosurfactant activity expressed by E_{24} was detected using 20g/l ml of tea wastes for *A. fumigatus*.

The highest oil spreading test value (4.5cm) was obtained by *A. niger* using 28g/l of sunflower seed shells followed by 4cm for *A. fumigatus* at 20g/l of potato peels, and 3.67cm at 40g/l of tea wastes for *A. carneus*.

Influence of temperature

This experiment was carried out in different temperature using potato peels, sunflower seed shells and tea wastes as a substrate for *A. carneus, A. niger and A. fumigatus*, respectively. Temperature of the culture media was one of the main environmental conditions for the microbial growth and their biosurfactant production. The results showed that the emulsification index and oil spreading test of the tested fungi varied significantly according to the temperature **(Table 5).**

The emulsification index reached the highest value when *A. carneus* and *A. niger* grown at 35°C. It was 64.99% and 65.73% for *A. carneus and A. niger*, respectively, while it was 62.22% at 30°C for *A. fumigatus*. Concerning on oil spreading test, the highest value obtained (3.7cm) at 35°C and 30°C for *A. carneus* and *A. fumigatus*, respectively, while it was 3.5cm for *A. niger* at 35°C. The results indicated that the highest biosurfactant activity was observed at 30°C for *A. fumigatus* and at 35°C *A. carneus* and *A. niger*.

TABLE 3. Effect of different agricultural wastes as substrate on biosurfactant potential by *A. carneus, A. niger* and *A. fumigatus.*

Substrate	Emulsification index (%)			Oil spreading test (cm)		
	A. carneus	*A. niger*	*A. fumigatus*	*A. carneus*	*A. niger*	*A. fumigatus*
Tea waste	22.67^{e}	43.33^{d}	60.00^{a}	0.50^{e}	2.53^{e}	3.47^{a}
Rice straw	30.00^{d}	16.67^{f}	16.67^{f}	1.00^{c}	0.70^{f}	0.43^{e}
sun flower seed shells	10.67^{g}	60.33^{a}	57.42^{b}	0.47^{e}	4.58^{a}	3.20^{b}
Wheat straw	16.67^{f}	39.86^{e}	20.00^{e}	0.40^{e}	2.20^{e}	0.50^{e}
Sugar cane bagasse	40.00^{b}	46.88^{c}	15.17^{f}	1.70^{b}	3.30^{d}	0.50^{e}
potato peels	62.44^{a}	59.35^{a}	10.00^{g}	2.70^{a}	4.33^{b}	0.50^{e}
banana peels	16.67^{f}	52.11^{b}	38.46^{d}	0.80^{d}	3.73^{c}	2.00^{d}
Wheat bran	33.33^{c}	0.00^{g}	54.29^{c}	1.60^{b}	0.00^{g}	3.13^{c}

Different superscript letters in the same column indicate significant differences ($p \leq 0.05$).

TABLE 4. Effect of different agricultural wastes concentrations on biosurfactant production potential (Emulsification index and oil spreading test) of *A. carneus, A. niger* and *A. fumigatus.*

Agricultural waste concentration (g/l)	Emulsification index (%)			Oil spreading test (cm)		
	A. carneus	*A. niger*	*A. fumigatus*	*A. carneus*	*A. niger*	*A. fumigatus*
4	33.33^{e}	54.22^{c}	27.67^{d}	1.00^{e}	3.13^{d}	0.50^{a}
12	40.00^{d}	53.67^{c}	53.33^{b}	1.50^{d}	3.00^{d}	2.67^{b}
20	69.22^{a}	66.27^{b}	67.17^{a}	3.47^{a}	4.20^{c}	4.00^{a}
28	66.67^{b}	75.57^{a}	54.60^{b}	3.00^{b}	4.50^{a}	2.60^{b}
40	51.33^{c}	74.29^{a}	43.81^{c}	3.67^{c}	4.00^{b}	2.20^{c}

Different superscript letters in the same column indicate significant differences ($p \leq 0.05$).

TABLE 5. Effect of different temperature on biosurfactant production potential (Emulsification index and oil spreading test) of *A. carneus, A. niger* and *A. fumigatus.*

Temperature °C	Emulsification index (%)			Oil spreading test (cm)		
	A. carneus	*A. niger*	*A. fumigatus*	*A. carneus*	*A. niger*	*A. fumigatus*
25	49.64^{c}	40.91^{c}	53.89^{b}	3.23^{b}	2.40^{c}	3.00^{b}
30	59.78^{b}	54.54^{b}	62.22^{a}	3.40^{b}	3.03^{b}	3.70^{a}
35	64.99^{a}	65.73^{a}	33.33^{c}	3.70^{a}	3.50^{a}	1.33^{c}
40	33.33^{d}	38.13^{c}	16.67^{d}	1.06^{d}	1.6^{d}	0.33^{d}

Different superscript letters in the same column indicate significant differences ($p \leq 0.05$).

Influence of the initial pH

This experiment was carried out in different pH values using potato peels, sunflower seed shells and tea wastes as a substrate for *A. carneus, A. niger and A. fumigatus*, respectively. The solid cultures incubated at the favored temperature for each fungus. The results showed significant variation in both emulsification index and oil spreading test depending on the initial pH for all the tested fungi. The highest emulsification activity was recorded at pH =7 for *A. carneus* (65.56 %) and *A. niger* (69.90%), while it was 66.41% at pH =6 for *A. fumigatus*.

The results indicated that the optimum pH was 7 for the emulsification activity and oil spreading of *A. carneus* and *A. niger*. While, *A. fumigatus* preferred pH=6 to produce the highest emulsification index and oil displacement **(Table 6).**

The highest oil spreading test was observed for *A. carneus* and *A. niger* was 3.37cm and 3.87cm, respectively at pH 7. On the other hand it was 3.97cm for *A. fumigatus* at pH 6. Accordingly, the optimum pH for biosurfactant production was 7 for *A. carneus* and *A. niger*, while it was 6 for *A. fumigatus*.

Effect of cultural media amount

This experiment was carried out to evaluate the activity of *A. carneus*, *A. niger* and *A. fumigatus* to produce their surfactants depending upon the amount used of the cultural media. All the microbial cultures were grown at the previously optimum condition of temperature, substrate, pH and amount of substrate. The fungal biosurfactants collected from the solid state of *A. carneus, A. niger* and *A. fumigatus* exhibit excellent biosurfactant activity.

All the variables (E_{24} and oil spreading test) varied significantly according to media amount. The highest emulsification indexes were recorded at 10ml for *A. carneus* and *A. fumigatus*. They were 63.78 and 70.02%, respectively. While, the highest E_{24} (65%) was recorded using 100ml of culture of *A. niger* **(Table 7).**

The results indicated that the optimum media amount was 10ml for the emulsification activity and oil spreading of *A. carneus* and *A. fumigatus*. While, *A. niger* preferred 25ml of media amount to produce the highest emulsification index and oil spreading test. The highest oil spreading test was 3.73, 4.30 and 3.90cm for *A. carneus, A. niger* and *A. fumigatus,* respectively.

Chemical properties of biosurfactant

The chemical nature of biosurfactant determined by Phenol-H_2SO_2 test to detect glycolipids, biuret test to detect lipopeptides and phosphate test to detect phospholipids. Both glycolipids and lipopeptides were detected in *A. fumigatus* biosurfactants, while glycolipids and phospholipids were detected in *A. carneus* and *A. niger* biosurfactants **(Table 8).**

TABLE 6. Effect of different pH on biosurfactant production potential (Emulsification index and oil spreading test) of *A. carneus, A. niger* and *A. fumigatus.*

pH	Emulsification index (%)			Oil spreading test (cm)		
	A. carneus	*A. niger*	*A. fumigatus*	*A. carneus*	*A. niger*	*A. fumigatus*
5	38.22^{d}	16.67^{c}	49.81c	1.50^{d}	0.50^{c}	1.80^{d}
6	44.89^{c}	56.39^{b}	66.41^{a}	2.13^{c}	2.67^{b}	3.97^{a}
7	65.56^{a}	69.90^{a}	62.17^{b}	3.37^{a}	3.87^{a}	3.47^{b}
8	53.33^{b}	41.78^{c}	58.16^{b}	2.87^{b}	1.43^{c}	2.00^{c}

Different superscript letters in the same column indicate significant differences ($p \leq 0.05$).

TABLE 7. Effect of cultural media amount on biosurfactant potential of *A. carneus, A. niger* and *A. fumigatus.*

Media amount (ml)	Emulsification index (%)			Oil spreading test (cm)		
	A. carneus	*A. niger*	*A. fumigatus*	*A. carneus*	*A. niger*	*A. fumigatus*
10	63.78^{a}	63.89^{b}	70.02^{a}	3.73^{a}	3.63^{b}	3.90^{a}
25	42.22^{b}	65.00^{a}	66.67^{b}	3.20^{b}	4.30^{a}	3.60^{b}
50	20.89^{c}	16.67^{c}	22.00^{c}	1.77^{c}	1.50^{c}	0.47^{c}

Different superscript letters in the same column indicate significant differences ($p \leq 0.05$).

TABLE 8. Chemical characterization of biosurfactant produced by *A. carneus, A. niger* and *A. fumigatus* in solid-state fermentation (SSF).

Test	*A. carneus*	*A. niger*	*A. fumigatus*
Phenol-H_2SO_4 test	+	+	+
Biuret test	-	-	+
Phosphate test	+	+	-

Stability of biosurfactant:

Influence of temperature

The stability of the fungal biosurfactants was evaluated under different physical conditions. Supernatants of the cultured fungi were submitted to various temperatures. The results of their E_{24} showed a wide range thermal stability from 4°C to 100°C **(Fig. 2).** In case of *A. carneus* and *A. fumigatus*, not only a thermal stability of biosurfactant was observed until 100°C but also significate increases in E_{24} was obtained. Biosurfactant produced from *A. niger* showed different emulsification index with a peak at 80°C. The thermal stability of *A. niger* was relatively reduced at 100°C. *A. fumigatus* biosurfactant was more sensitive to lower temperature than those of *A. carneus* and *A. niger.*

Influence of pH

The supernatant of each culture was adjusted at different pH values (2-14). E_{24} stability of all biosurfactants varied significantly according to the adjusted pH. Both the lower and higher pHs decreased the E_{24} of all biosurfactants. *A. niger* biosurfactant was more sensitive to alkaline pH than those of *A. carneus* and *A. fumigatus* **(Fig. 3).** The activity of *A. niger* biosurfactant was nil al pH 14.

Influence of salts (NaCl)

The biosurfactants were subjected to three different concentrations of NaCl (10, 20 and 30g/l) before measuring their emulsification activity. The biosurfactant activity of *A. carneus, A. niger* and *A. fumigatus* statistically increased with increasing NaCl concentration. The highest E_{24} values were 75, 60 and 66.67% at 30g/l NaCl for *A. carneus, A. niger* and *A. fumigatus*, respectively **(Fig. 4).**

Influence of different hydrocarbons

The effect of different hydrocarbons on the stability of biosurfactant produced by *A. carneus*, *A. niger* and *A. fumigatus* was determined by measuring E_{24}. The presence of motor oils (kerosene, diesel and benzene) or aromatics (hexane, toluene and xylene) induced the emulsification activity of *A. carneus* biosurfactant. It reached 83.33% using benzene, followed by 76.67% using hexane, toluene, xylene or kerosene **(Fig. 5).** While, soybean and olive oils reduced the E_{24} to 26.67 and 20%, respectively. The emulsification activity of biosurfactant produced by biosurfactant produced by *A. niger* OQ195934 was relatively low compared to those of *A. carneus* and *A. fumigatus*. The emulsification activity of *A. niger* biosurfactant reached to 66.67% with diesel oil followed by 63.33% using soybean oil and 62.5% using sunflower seed oil. A notably reduction in E_{24} (23.33%) was observed using olive oil **(Fig. 5).** While the highest emulsification activity obtained by *A. fumigatus* using kerosene (84%), followed by hexane (80%) and toluene and xylene (74.07%). The lowest emulsification activity was observed by olive oil (16.76%).

Determination the critical micelle concentration of biosurfactant produced from A. carneus, A. niger and A. fumigatus in solid state fermentation.

The critical micelle concentration (CMC) of the purified biosurfactant was determined in aqueous solution. The sample of the fungal biosurfactant was diluted in different concentrations. The CMC is the biosurfactant concentration at which the lowest value of surface tension is attained. All the fungal biosurfactants exhibit excellent surface tension reducing activity. Increasing the biosurfactant concentration of the tested fungi decreased the surface tension about 53.5 and 56.9 and 45.9 % reduction in the surface tension using the crude biosurfactant of A. *carneus,* A. *niger* and *A. fumigatus*, respectively. The surface tension of water (71.57 mN/m) decreased to 33.3, 30.86 and 38.73mN/m by using 120 mg/l of biosurfactant of *A. carneus*, *A. niger* or *A. fumigatus*, respectively. The critical micelle concentration of biosurfactant produced by *A. carneus* biosurfactants was 30mg/l that reduce the surface tension of water to 36.70mN/m. In case of *A. niger*, it was 40mg/l that reduced the water surface tension to 32.73mN/m, and *A. fumigatus* was 60mg/l that reduce water surface tension to 40.75mN/m **(Fig. 6).**

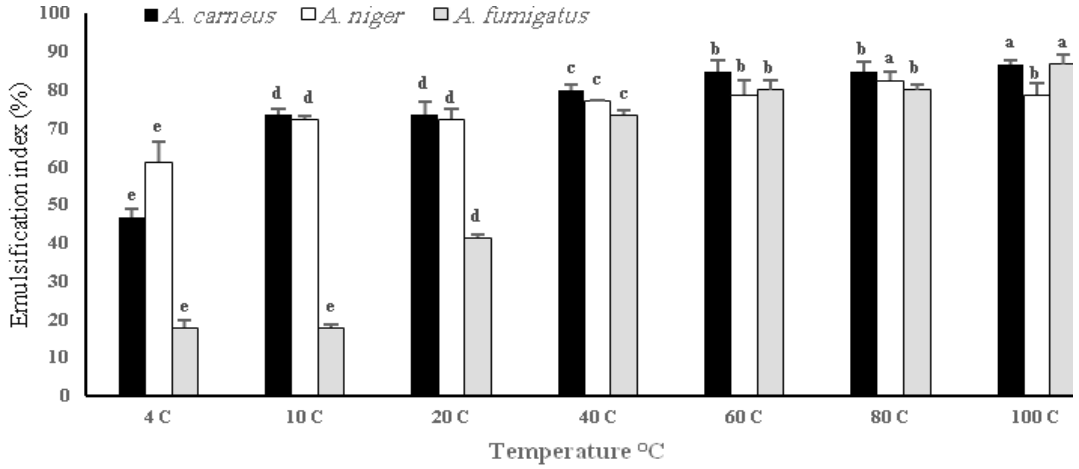

Fig. 2. Effect of temperature on *A. carneus*, *A. niger* and *A. fumigatus* biosurfactant stability.

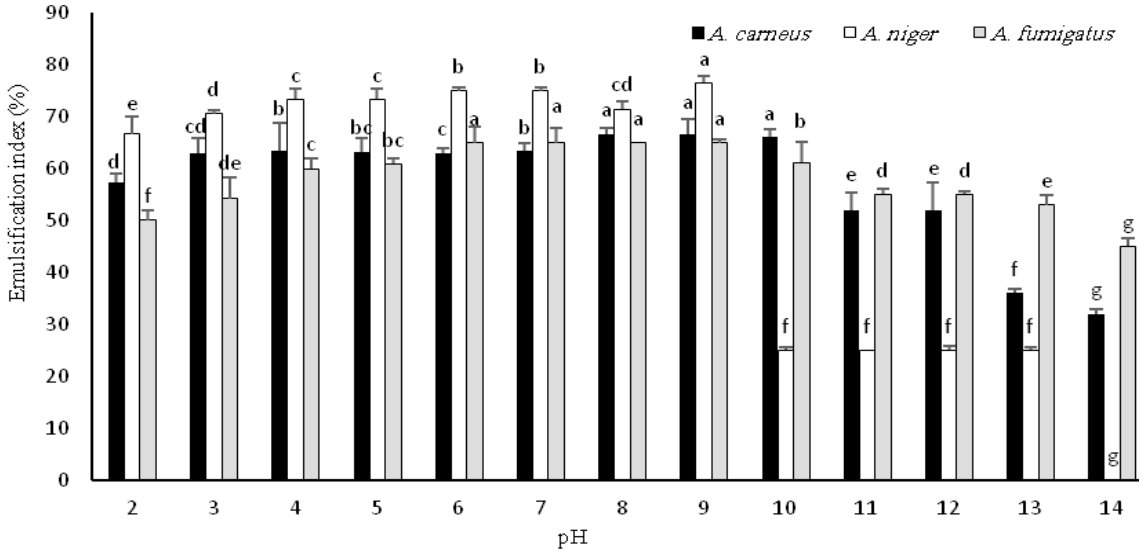

Fig. 3. Effect of pH on A. carneus, A. niger and A. fumigatus biosurfactant stability.

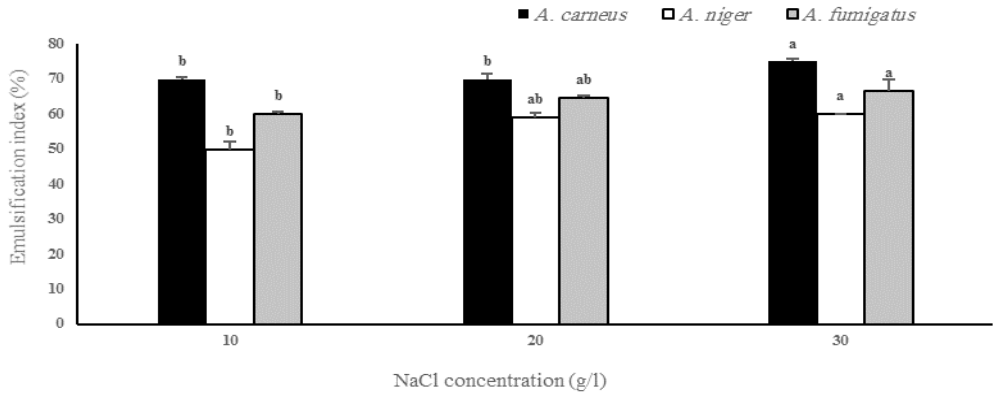

Fig. 4. Effect of different NaCl concentrations on *A. carneus*, *A. niger* and *A. fumigatus* biosurfactant stability.

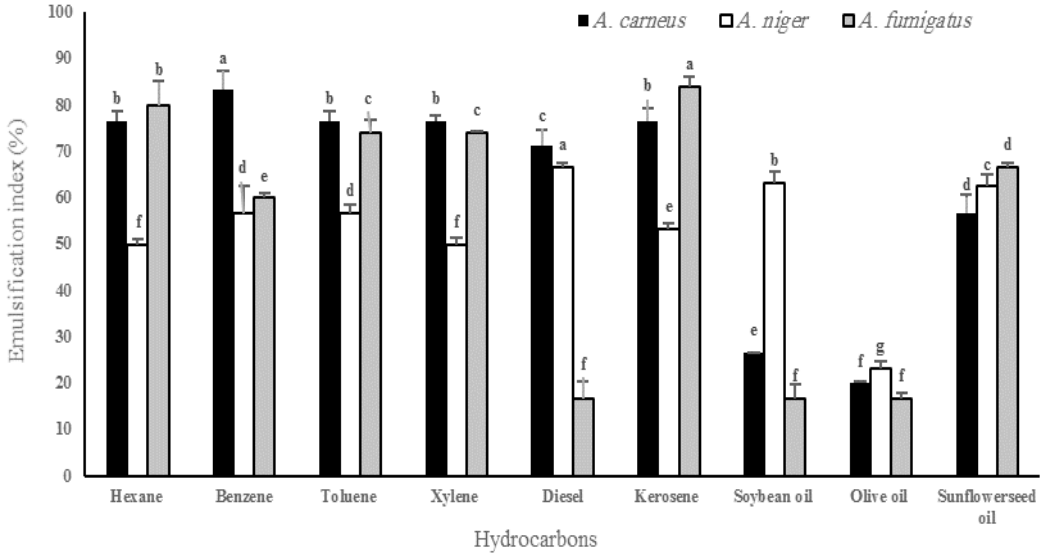

Fig. 5. Effect of different hydrocarbon on *A. carneus, A. niger* and *A. fumigatus* biosurfactant stability.

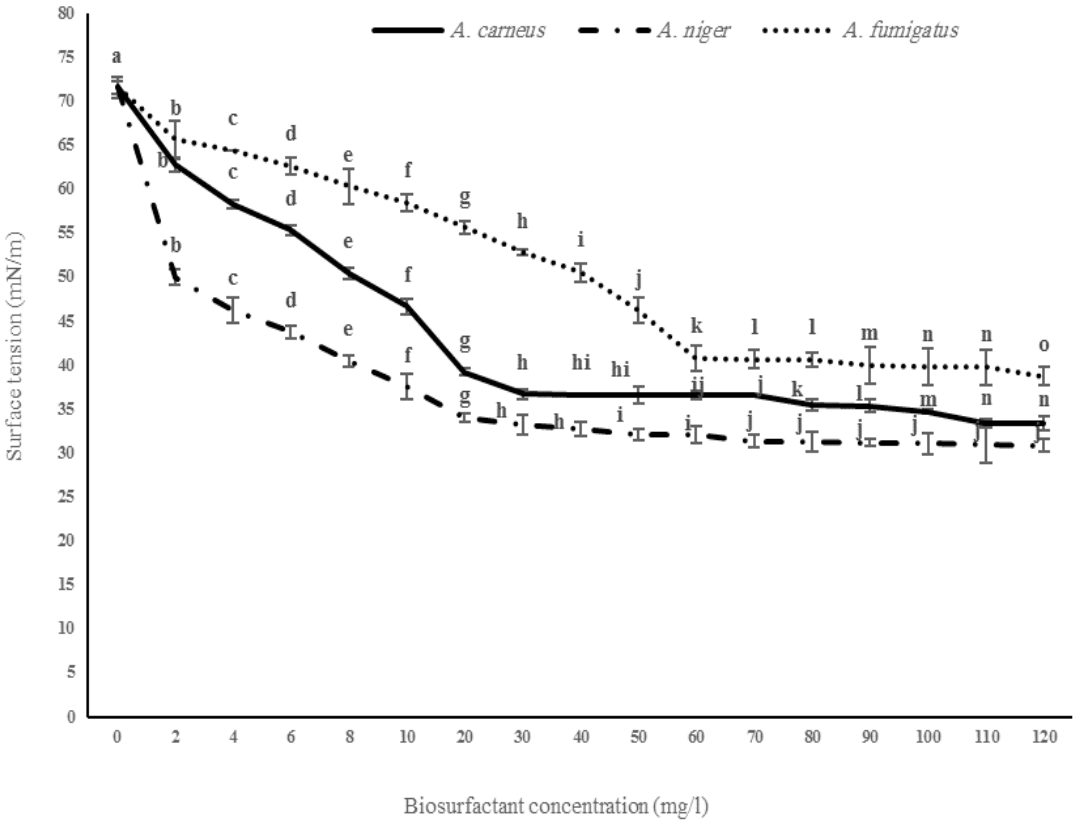

Fig. 6. Critical micelle concentration of biosurfactant produced from *A. carneus, A. niger* and *A. fumigatus* in solid state fermentation.

GC–MS analysis

One of the important aims in the present study was to compare the bioactive surfactant compounds produced by *A. carneus* OQ152507, *A. niger* OQ195934 and *A. fumigatus* OQ195934 in solid state culture using Gas Chromatography-Mass Spectroscopy. On comparing the biosurfactant compounds produced by tested fungi, GC-MS analysis detected the presence of six hydrophobic compounds in all the tested fungi. They are Trans-13-Octadecenoic acid ($C_{18}H_{34}O_2$), Myristic acid ($C_{14}H_{28}O_2$) Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester ($C_{19}H_{38}O_4$), Oleic Acid ($C_{18}H_{34}O_2$), *Cis*-Vaccenic acid ($C_{18}H_{36}O_2$) and n-Hexadecanoic acid ($C_{16}H_{32}O_2$) **(Fig. 7).** Out of these compounds, *Cis*-Vaccenic acid, n-Hexadecanoic acid, Hexadecanoic acid and 2-hydroxy-1 (hydroxymethyl) ethyl ester have a distinct peak with the highest percentage content (area %) **(Fig. 7).** They represented in varied area percentage as follow; 30.92, 15.56 and 7.47% for *Cis*-Vaccenic acid extract in *A. fumigatus*, *A. niger* and *A. carneus*, respectively; 15.99, 7.88 and 11.66 for n-Hexadecanoic acid in *A. fumigatus*, *A. niger* and *A. carneus*, respectively and 5.17, 14.48 and 2.29 for Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester in *A. fumigatus*, *A. niger* and *A. carneus*, respectively **(Fig. 7).** On the other hand, each fungus characterized by specific hydrophobic fatty acid chains. Focusing on the *A. carneus* extracted filtrate, It was showed that the presence of Stearic acid ($C_{18}H_{36}O_2$), 9,17-Octadecadienal, (Z) ($C_{18}H_{32}O$) and Cis-13-Octadecenoic acid ($C_{18}H_{34}O_2$) with conservable percentage area as the follows: 26.316, 13.991 and 7.295, respectively.

The GC-MS analysis of *A. fumigatus* filtrate showed also the presence specific hydrophobic fatty acids chains; Tridecane ($C_{13}H_{28}$)(RT,16.88), Oleic acid, 3-hydroxypropyl ester ($C_{21}H_{40}O_3$) (RT,31.43), Octadecanoic acid, 3-oxo, methyl ester ($C_{19}H_{36}O_3$) (RT,32.33) and Erucic acid ($C_{22}H_{42}O_2$) (RT,29.84) (Fig. 8). While, Hexadecenoic acid, Z11 ($C_{16}H_{30}O_2$) (RT, 26.52) was detected in *A. niger* culture filtrate in low percentage (0.108%) **(Fig. 8).**

The GC-MS analysis showed that both *A. niger* and *A. carneus* participate in producing Undecane ($C_{11}H_{24}$) and Dodecane ($C_{12}H_{26}$) at the relatively retention times (14.65 and 1688, respectively). But they produce Octadecanedioic acid ($C_{18}H_{36}O_2$), collected in two different retention times, 28.86 and 7.45 for *A. niger* and *A. carneus*, respectively.

On the other hand, fatty acids; 7-Hexadecenal, (Z) ($C_{16}H_{30}O$), Undecane, 2-methyl ($C_{12}H_{26}$), Octadecanoic acid, methyl ester ($C_{19}H_{34}O_2$), and 9,12-Octadecadienoic acid (Z,Z) ($C_{18}H_{32}O_2$) were observed in *A. fumigatus* and *A. niger* supernatants (Fig. 8). While, *A. carneus* and *A. fumigatus* characterized by 9,12,15-Octadecatrienoic acid, 2 [(trimethylsilyl)oxy]1 [[(trimethylsilyl) oxy]methyl]ethyl ester, (Z,Z,Z) ($C_{18}H_{30}O_2$) and Octadecanoic acid ($C_{18}H_{36}O_2$) in their culture filtrates.

FT-IR analysis

The depicted in **Fig. 9c** showed characteristic peaks at 3418, 1740 and 144cm^{-1} which may assign for -OH, COOH and C=C stretching frequency. Biosurfactant compounds of *A. fumigatus* show relatively similar IR (KBr) data except the beak at 3418, which is broad and intense **(Fig.9b).** This can be ascribed to the presence of extra -OH groups. This further support the GC-MS data. Focusing on *A. carneus* culture extract, peak at 3418cm^{-1} is assigned for the hydroxy group of the carboxylic moiety. Peaks at 1725 and 1636cm^{-1} are assigned to ester carboxylic function present in these compounds **(Fig. 9a).** Finally, the data are in line with that obtained above for the GC-MS data. FT-IR spectrum of all tested fungal surfactants may confirm the presence of all compounds showed in **Fig. 8**.

Discussion

Fungal strains were assessed for its ability for bio-surfactant production in solid state culture. In this study, three Eurotiales fungi were isolated and identified as *A. carneus* OQ152507, *A. niger* OQ195934 and *A. fumigatus* OQ195264. The results agree with those obtained by Gmoser et al. (2017) who confirmed the production of certain biosurfactants by *Aspergillus, Penicillium,* and *Fusarium* species. Biosurfactant production of *A. carneus* OQ152507, *A. niger* OQ195934 and *A. fumigatus* OQ195264 was analyzed using common screening tests, such as E_{24}%, oil spreading, and surface tension tests. The biosurfactant production of many fungal microorganisms have already been studied e.g., *A. flavus* (Adekunle et al., 2015; Ishaq et al., 2015), *Aspergillus ustus* (Kiran et al., 2009), *A. versicolor* (Gul & Donmez, 2012), *A. niger* (Superb *et al*., 2018), and *Piper hispidum* (Da Silva et al., 2019), and various factors affecting the total biosurfactant yield and its activity during fermentation process have been studied in previous few decades (Hema et al., 2019). Generally, the microbial

cultures grown on hydrocarbons or other water immiscible substrates yield the majority of known biosurfactants (Rane et al., 2017). In the present work, the highest biosurfactant activity was observed using oil hydrocarbon; Kerosene for *A. carneus*, Petrol 80 for *A. niger* and diesel for *A. fumigatus* at 30°C of incubation. While soybean and olive oils reduced the biosurfactant activity. It was confirmed by Sodagari & Ju (2014) who stated that the soybean oil able to decrease the maximum foam volume by reducing foam stability of *Pseudomonas aeruginosa* surfactant. On the contrary, soybean oil was described as a suitable carbon source for yeast (Accorsini et al., 2012) and *Penicillium* 8CC2 (Sena et al., 2018) biosurfactant production.

Exploring affordable materials to utilize as substrates for the synthesis of biosurfactants has become more and more important in the last few years. In the same context, this work interested in studying the biosurfactant production by fungi grown on many wastes as a substrate. The results showed that, about 60% of emulsification indexes were obtained when used potato peels, sunflower seed shells and tea wastes as solid substrates for *A. carneus* for *A. niger* and *A. fumigatus*, respectively**.** Different renewable feedstocks as substrates in fermentation bioprocesses have been studied. These include rice straw (Panjiar et al., 2020), sugarcane bagasse (Marcelino et al., 2019) corncobs (Sari et al., 2020), corn husk (Saimmai et al., 2018), banana stalks (Asgher et al., 2020), pineapple peels (Vieira et al., 2021), orange peels (Sari et al., 2020), beetroot peels (Dos Santos et al., 2018) and others. Using *Achromobacter* sp. BP(1)5 and *Penicillium citrinum*, Sari et al. (2020) synthesized biosurfactant from lignocellulosic waste of corncobs and rice straw. Another work by Saimmai et al. (2018) used maize husk powder as a raw material for the fermentation bioprocess to obtain biosurfactants from 51 bacterial isolates.

The bioprocess and nutritional conditions factors directly affect the fungal growth and biosurfactant production. The highest biosurfactant activity for the fungal strains obtained in the narrow range of pH (6-7), so also, the temperature range for production was from 30 to 35°C. Rane et al. (2017) stated that incubation temperature of 30°C is the optimum temperature for maximum biosurfactant production by *B. subtilis* ANR 88. The maximum yield of *A. niger* M2 was obtained by Asgher et al. (2020) at 35°C temperature and 7 pH.

A. niger biosurfactants tolerate the high temperature of 80°C, while a thermal stability of both *A. carneus* and *A. fumigatus* biosurfactant was observed at 100°C. In the same context, Sen et al. (2017) showed a good stability for *Rhodotorula babjevae* YS3 biosurfactant after heating at 120°C for different time intervals. The activity of *A. carneus, A. niger* and *A. fumigatus* biosurfactant increased with increasing NaCl concentration. They showed a significant stability at 3% NaCl. This contradicts with data obtained by Sena et al. (2018) who confirmed that a high NaCl concentration reduces the efficiency of *Penicillium* 8CC2 biosurfactant. While, these findings bear conformity with those reported by Sen et al. (2017), where they reported stability of *Rhodotorula babjevae* YS3 biosurfactant over a wide range of salinity (2–10% NaCl).

Haba et al. (2003) stated that the minimal surfactant concentration (CMC) at which surfactant monomers begin to form micelles. At this point, surfactant molecules totally fill the medium's solution interface in the surfactant-dispersed form. In our study, the surface tension reduction is directly proportional to biosurfactant concentration in the medium. The best surface tension reduction obtained at 120mg/l of our tested biosurfactant confirmed their importance. Many authors believe that a reduction in the surface tension to 50mN/m already qualifies the microorganism for further testing (Lima et al., 2016), while the value of 40mN/m indicates a possibility deserving more examination (Meneses et al., 2017).

Furthermore, Aparna et al. (2012) decided that the best biosurfactant that reduce the surface tension to 30mN/m or less. In this study, the lowest value of 30.86mN/m was recorded *A. niger* biosurfactant. Banat (1995) stated that the prospective biosurfactant-producing microorganisms should cause the culture media's surface tension to drop to nearly 35 mN/m. Accordingly, *A. niger* OQ195934 was able to significant reduce the surface tension below this value indicating successful biosurfactant activity. GC-MS analysis of extracted biosurfactant of all tested fungi showed the presence various hydrophobic moieties. The main moieties found in the culture filtrates of these fungi was *Cis*-Vaccenic acid, n-Hexadecanoic acid, Hexadecanoic acid and 2-hydroxy-1 (hydroxymethyl) ethyl ester with the highest percentage content.

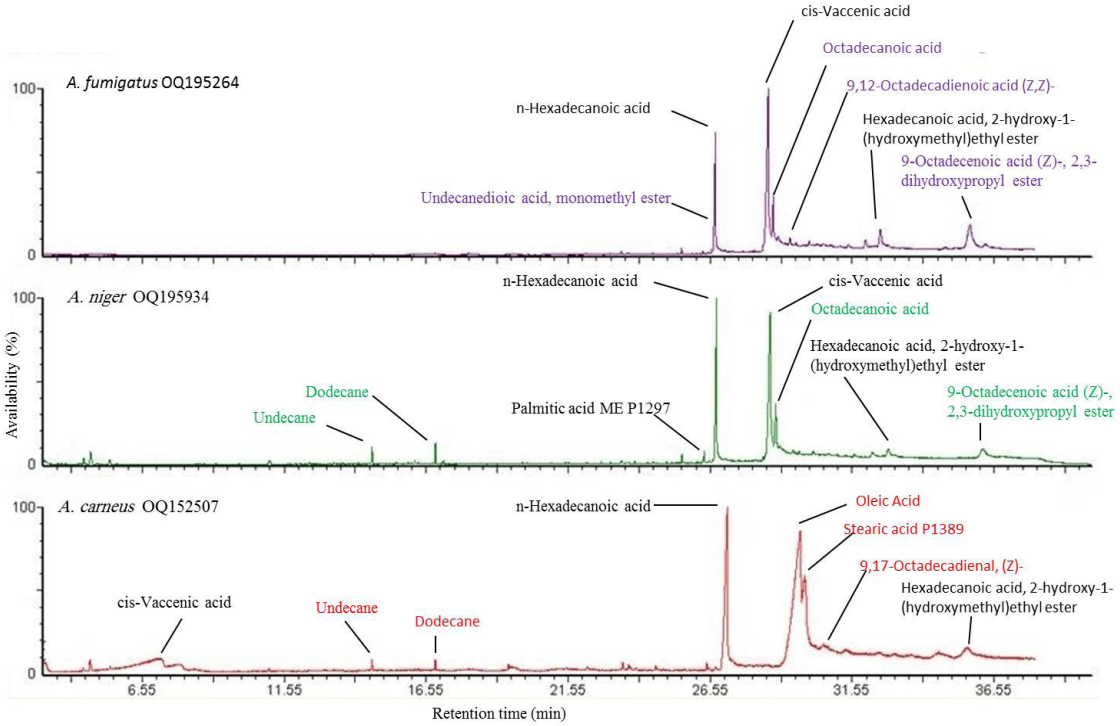

Fig. 7. GC-MS analysis of *A. carneus* OQ152507, *A. fumigatus* OQ195934 and *A. niger* OQ195934 biosurfactants showing the major chemical compounds.

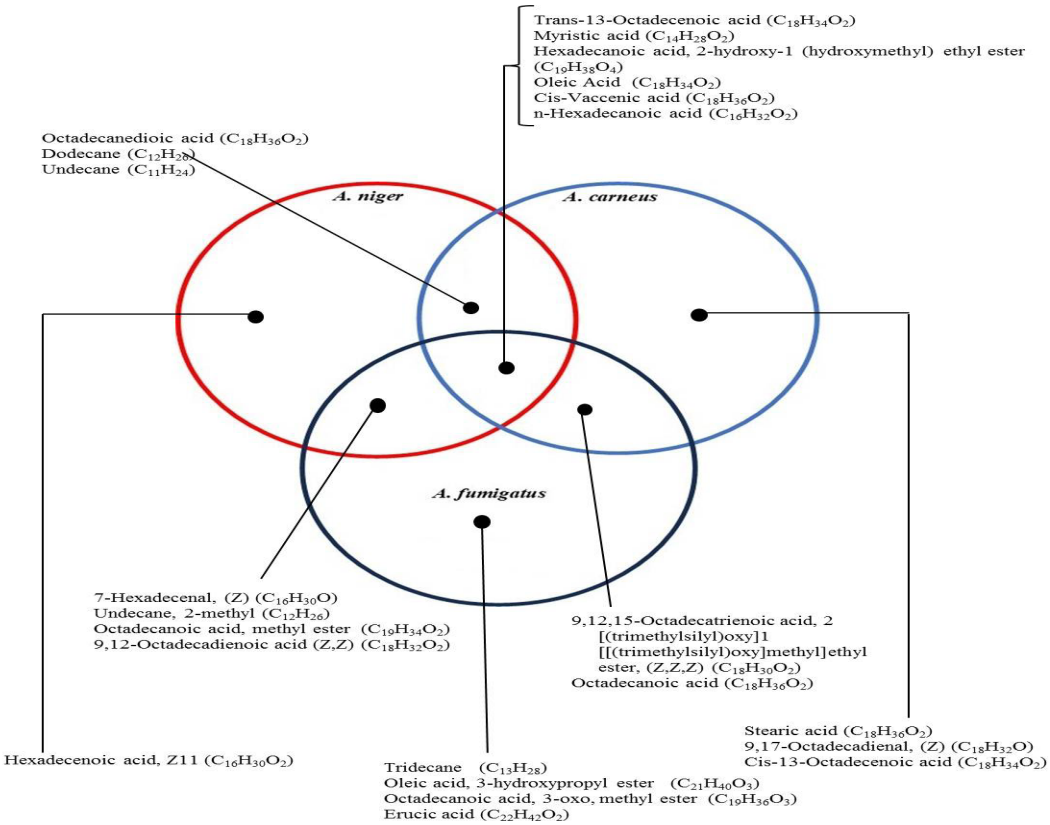

Fig. 8. Venn diagram of the main compounds of hydrophobic compound in *A. carneus* OQ152507, *A. fumigatus* OQ195934 and *A. niger* OQ195934 biosurfactants.

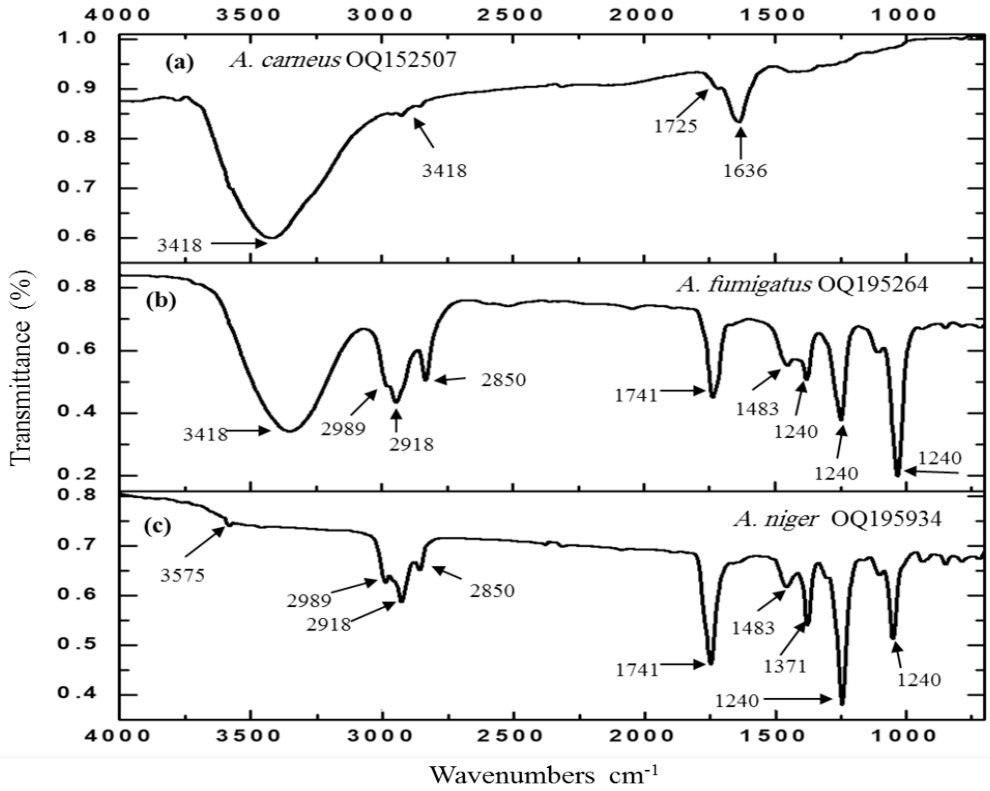

Fig. 9. FT-IR spectrum of *A. carneus* **OQ152507,** ***A. fumigatus*** OQ195934 and ***A. niger*** **OQ195934 biosurfactants.**

Results of FT-IR confirmed that the biosurfactant contained aliphatic chain fatty acid moieties. These findings bear conformity with those obtained by Piegza et al. (2021), where they reported the main components of fatty acids, including 3-hydroxy-stearic acid (C18:0), 3-hydroxypalmitic acid (C16:0), acid octadecene (C18:1, double bond in position 2) represent the biosurfactant moiety in *Trichoderma citrinoviride* HL and C1 supernatants. Also, fatty acids; n-hexadecanoic acid, methyl linoleate, ethyl palmitate, 8-octadecenoic acid, methyl stearate, methyl ester, ethyl stearate, dodecanoic acid, methyl palmitate, linoleic acid, 9,17-octadecadienal and 2,4-ditert-butylphenol were showed in culture filtrate of *Pseudomonas guguanensis* Strain Iraqi ZG.K.M by Ghazi et al. (2023).

Conclusion

In this study, cheap unconventional agro-industrial wastes were used to optimize the bioprocess for biosurfactant production *in vitro* from certain Eurotiales genera. GC-MS and FT-IR analysis of *A. carneus* OQ152507, *A. fumigatus* OQ195934 and *A. niger* OQ195934 filtrate showed the presence of specific hydrophobic fatty acids chains which may act as moieties of their biosurfactants. Each fungus characterized by certain biosurfactant moiety/moieties. Furthermore, Fatty acids; *Cis*-Vaccenic acid, n-Hexadecanoic acid, Hexadecanoic acid and 2-hydroxy-1 (hydroxymethyl) ethyl ester have a distinct peaks within all tested fungi. On the other hand, each fungus characterized by the production of specific hydrophobic fatty acids chains.

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إنتاج المواد النشطة سطحيا بواسطة بعض أنواع الاسبرجلس تحت تخمر الحالة الصلبة

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تعتبر المواد النشطة سطحيا مواد ثانوية تنتجها بعض الكائنات الحية الدقيقة، ولدى هذه المواد القدرة على تقليل التوتر السطحي، حيث تمتاز بالعديد من المزايا مثل قابلية التحلل البيولوجي العالية، وزيادة الرغوة، والسمية المنخفضة وتتحمل الظروف القاسية. في الدراسة الحالية تمت المقارنة بين الإنتاج المحتمل للمواد النشطة سطحيا بواسطة ثلاث أنواع من جنس الاسبرجلس (اسبرجلس كارينز OQ152507 *A. carneus* واسبرجلس نيجر A. *niger* OQ195934 **واسبرجلس** فيوميجاتس OQ195264 *A. fumigatus*) باستخدام تخمير الحالة الصلبة (SSF) كتقنية بديلة منخفضة التكلفة. وقد تمت زراعة هذه العزلات تحت ظروف الحالة الصلبة باستخدام ثماني مخلفات زراعية مختلفة لإنتاج المواد النشطة سطحيا، وكانت مخلفات قشور البطاطس (20 جم/ لتر) كمصدر للكربون عند 35 °م ودرجة الحموضة 7 في وسط ببتون الجلوكوز هي الأمثل لتحقيق الحد الأقصى من إنتاجية هذه المواد لـفطر اسبرجلس كارينز، وقشور بذور نبات عباد الشمس (28 جم/ لتر) عند 35°م ودرجة الحموضة. 7 في وسط نترات النشا الأمثل لـفطر اسبرجلس نيجر، بينما كانت مخلفات الشاي (20 جم / لتر) عند 30 °م ودرجة الحموضة 6 في وسط ببتون الجلوكوز الأمثل لـفطر اسبرجلس فيوميجاتس. وعند دراسة ثبات هذه المركبات لوحظ ثباتها عند 120°م، وتركيز 30جم/لتر من كلوريد الصوديوم ودرجة الحموضة القلوية 10 لـفطر اسبرجلس كارينز ودرجة الحموضة القلوية 9 لكل من اسبرجلس فيوميجاتس واسبرجلس نيجر. تم تعريف المواد النشطة سطحيا على أنها جزيئات جلايكوليبيد وفوسفوليبيدات بواسطة اختبار الفينول- حمض الكبريتيك، والفوسفات. أكدت تحليل GC-MS و FT-IR أن الفطريات التي تم اختبارها تساهم في إنتاج ثلاث سلاسل من الأحماض الدهنية الكارهة للماء بنسب مرتفعة، وهي حمض Cis-Vaccenic، وحمض n-Hexadecanoic، وحمض Hexadecanoic 2-hydroxy-1 (hydroxymethyl) ethyl ester.