Purification and Identification of Surface Active Amphiphilic Candidates Produced by *Geotrichum candidum* MK880487 Possessing Antifungal Property

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Abstract

The present study was held to optimize production, purify and identify biosurfactant from yeast strain *Geotrichum candidum* MK880487. Biosurfactant production was done on modified Hua medium and optimized in shake-flask method. Soybean oil (8%, v/v), KNO₃ (0.75 g l⁻¹) and yeast extract (0.3 g l⁻¹) were the best carbon and nitrogen sources, respectively, with C:N ratio of 200:1, pH 8 at 30°C for 168 h. Production scale-up was achieved on 2.5 L bioreactor as batch method. The resulting crude biosurfactant extract with yield of 1.75 g l⁻¹ was purified by reversed phase column preparative HPLC. The resulting most active fraction F47 significantly reduced water surface tension by 51.92%. Chemical characterization using TLC, GC-MS, FTIR and LC-MS/MS methods revealed the biosurfactant to contain mainly glycolipid structure existing as mixture of Icariside F2, Cardenolide Di-Hexopyranoside and Di Galactosyl Di Acyl Glycerol glycolipid that collectively showed potential antifungal activity towards *Macrophomina phaseolina*.

1. Introduction

Biosurfactants (BSs) are known to be moistening agents, dispersing agents, anti-adhesive agents, emulsifiers, solubilizers, thickeners and foaming agents that can be applied in many industrial sectors including chemical, petrochemical, metallurgy, mining, polymer, plastic, coal, textiles, leathers, pharmaceutical, cosmetic, food, paper, ceramic processing and uranium ore-processing, added to that in biological control (Makkar et al., 2011; Sachdev and Cameotra, 2013; Jha et al., 2016; Santos et al., 2016; Karlapudi et al., 2018). Moreover, Kiran et al. (2010) reported that glycolipid BSs were used as potential “green” stabilizers for the synthesis and stabilization of silver nanoparticles, compared to synthetic surfactants.

BS antifungal activities open up possibilities for development of efficient and eco-friendly
ideal therapeutic products with diverse agricultural, industrial and biomedical applications. Thus, they are considered the multifunctional biomolecules of the twenty-first century.

BSs had been used as biopesticides for controlling plant diseases and protecting stored products. They had antifungal activities towards phytopathogenic fungi beside others were found to have larvicidal and/or mosquitocidal potencies, thus permitting the preservation of plant crops from pest invasion (Mnif and Ghribi, 2016). Nevertheless, Sivapathasekaran and Sen (2017) stated that BSs from many microorganisms that had demonstrated antimicrobial properties had been investigated for stem the incidence of antibiotic resistance plaguing the world today.

Depending on their microbial sources, the produced BSs are of structural diversity that makes them differ in their actions and related application fields as biocontrol agents. For instance, Yoo et al. (2005) reported two glycolipid BSs including a rhamnolipid and a sophorolipid under test differed in inhibition efficiency against mycelial growth of Phytophthora sp. and Pythium sp.

Rhamnolipid BS produced from Pseudomonas sp. GRP3 were applied successfully as biocontrol agents against zoosporic plant pathogens and damping-off disease in chilli and tomato plants (Sharma et al., 2007), while rhamnolipid Bs from Candida tropicalis had broad range antimicrobial activity against both bacterial and fungal pathogens (Thenmozhi and Boominathan, 2016).

In another field of pathogens, Sen et al. (2017) found that Rhodotorula babjevae produced sophorolipid BS (SL) of antifungal activity against a broad group of both plant and human pathogenic fungi including Colletotrichum gloeosporioides, Fusarium verticilliiodes, F. oxysporum, Corynespora cassiicola and Trichophyton rubrum. Added to that, Balan et al. (2019) isolated marine yeast Cyberlindnera saturnus that produced new glycolipid BS,
cybersan which inhibited growth of the clinical bacterial pathogens.

Nevertheless, Bhardwaj et al. (2013) stated that any small alteration in the nutrients’ composition lead to modification of the resulting biosurfactant. Remarkably, Dengle-Pulate et al. (2014) compared inhibitory action of sophorolipid BSs (SL) produced by Candida bombicola grown on different substrates. The lauryl alcohol SL showed inhibition lower than that of oleic acid SL and linolenic acid SL against gram negative bacteria Escherichia coli and Pseudomonas aeruginosa, beside gram positive bacteria Staphylococcus aureus and Bacillus subtilis and yeast Candida albicans, as the three SLs types were different in their lipophilic moiety structure.

BSs production is influenced by various physicochemical factors that greatly affect its type and quantity, including components of production media such as the nature of the carbon and nitrogen sources, C:N ratio, salts and operational culture conditions such as temperature, pH, incubation period, agitation speed and aeration. Thus, optimizing these factors with potential for commercial exploitation is of paramount importance (Adamczak and Bednarski, 2000; Cortés-Sánchez et al., 2013; Ekpenyong et al., 2017).

Scaling up through bioreactor systems facilitated manipulation of control parameters to optimize and maximize BS production, while introducing more alternatives making it possible to reduce production costs and be economically feasible compared to benchtop techniques (Chikere et al., 2012; Marti et al., 2014; Luna et al., 2015; Almeida et al., 2017).

2. Materials And Methods

2.1. Strain and Inoculum preparation

The yeast strain Geotrichum candidum MK880487, previously isolated, identified and tested successfully for its ability to produce BS by Ahmed et al. (2019), was used in
present study. The inoculum was prepared by transferring a loopful of G. candidum cells from a 24 h cultured YM agar slants to flasks containing 50 mL of YM broth medium (Atlas, 2010). Cultures were incubated at 28°C with agitation speed of 110 rpm for 48 h on a rotary shaker.

2.2. Optimization of biosurfactant production

Biosurfactant production by G. candidum was optimized on Hua broth medium (Hua et al., 2004) with following ingredients (g l⁻¹): KNO₃ 2.5, KCl 0.1, KH₂PO₄ 3.0, K₂HPO₄ 7.0, CaCl₂ 0.01, MgSO₄·7H₂O 0.5, yeast extract 1.0, supplemented with soybean oil (8%, v/v), with pH 7.0. Optimization was done on the structure of the media including variation in carbon source types and concentrations, nitrogen source type, C:N ratio, pH initial value and incubation temperatures. Inoculated flasks (6%, v/v) were incubated on a rotary shaker incubator (110 rpm) at 30°C for 168 h and the culture broth was centrifuged (3000 rpm at 4°C for 15 min) off which the residual oil and cell pellets were discarded. SUP was considered as crude source for BS test assays and that of non-inoculated culture medium was considered the negative control. Surface tension was measured by the capillary rise method (Wang et al., 2007) and its reduction (STR%) was calculated according to Pornsunthorntawee et al. (2008).

2.2.1. Effect of different carbon sources

The production medium was supplemented with carbon sources including soybean oil 8% (v/v) as hydrophobic and glucose 14.01% (w/v) as hydrophilic, individually, whose quantities were equivalent to same carbon concentration of 56.0459 g l⁻¹ medium, in presence of nitrate salt either as sodium or potassium ions.

2.2.2. Effect of different concentrations of soybean oil
The production medium was supplemented with different concentrations of soybean oil as a sole carbon source including 2, 4, 6 and 8% (v/v), individually.

2.2.3. Effect of different nitrogen sources

The production medium was supplemented with different nitrogen sources including KNO₃ (2.5), NH₄Cl (1.3229), NH₄NO₃ (0.9898), yeast extract (0.5773) and peptone (2.5659), individually, equivalent to same nitrogen concentration of 0.3464 g l⁻¹ medium.

2.2.4. Effect of different C:N ratios

The production medium was supplemented with different concentrations of KNO₃ and yeast extract, while concentration of soybean oil was fixed at 8% (v/v), representing C:N ratios of 15, 30, 60, 90, 120, 200, 300, 400 and 500:1, individually.

2.2.5. Effect of pH values

The initial pH value of the production medium was adjusted to cover a range of 3 up to 8, before sterilization using the potassium phosphate buffering system of 0.1M for pH 6, 7 and 8 while citrate buffer 0.1M for pH 3, 4 and 5.

2.2.6. Effect of different incubation temperatures

The production medium was incubated at different temperatures including 30 and 40°C.

2.3. Growth curve and specific growth rate

The yeast inoculum was introduced at a concentration of (6%, v/v) in a bioreactor containing 2.5 L of optimized Hua broth medium with agitation speed of 150 rpm and aeration of 1.6 lpm at 30°C for 240 h. Samples were taken regularly at 24-hour intervals after 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h of incubation and cell count was determined as colony forming unit (Log₁₀cfu/ml).

The specific growth rate was calculated as noted by Ginovart et al. (2018)
\[
\text{specific growth rate (log10 cfu/ml hr}^{-1}) = \frac{(\log_{10} \text{cfu/ml}_T)_T - (\log_{10} \text{cfu/ml}_T)_{T1}}{T2 - T1}
\]

Where

\((log_{10} \text{cfu/ml hr}^{-1})\): is the specific growth rate

\((log_{10} \text{cfu/ml})_T\): is the growth cell count measured at time T

\(T_1\) and \(T_2\): are the two successive periods at which growth rates were measured

cfu: is the cell count measured as colony forming unit

2.4. Scaling up of biosurfactant production in bioreactors

The bioreactor of 2.5 L capacity containing the optimized Hua broth medium was
inoculated with inoculum at 6\% (v/v) and supplemented with soybean oil 8\% (v/v), \(\text{KNO}_3\)
(0.75 g l\(^{-1}\)) and yeast extract (0.3 g l\(^{-1}\)) with C:N ratio of 200:1, pH 8 and incubated at
30\(^\circ\)C, aeration 1.6 lpm and agitation 150 rpm for 168 h.

2.5. Isolation, purification and structural characterization of the
biosurfactant

2.5.1. Isolation of biosurfactant

The culture broth was centrifuged at 4\(^\circ\)C using 3000 rpm for 15 min to remove the cells as
well as debris. The obtained SUP was extracted with an equal volume of ethyl acetate,
shaking vigorously each time and allowed the two layers to stand until phase separation in
a separation funnel. Transfer bottom aqueous layer and the top ethyl acetate layer to
separate flasks.

Re-extract the aqueous portion twice more or until no further color persists in the ethyl
acetate layer to insure complete product recovery. Add 0.5 g of anhydrous sodium sulfate
per 100 ml of ethyl acetate fraction, to remove the traces of water present, filter to remove materials other than biosurfactant, and then the organic phase was vacuum-dried by using a rotary evaporator at 40°C to remove the solvent and obtain a sticky brown gum extract. Biosurfactant yield was expressed in g l⁻¹ (Hewald et al., 2005). The crude extract was obtained, purified and analyzed for the structural characteristics of the biosurfactants present.

2.5.2. Thin Layer Chromatography (TLC)

Biosurfactant aliquots of 5 µl were applied to pre-coated silica gel (60; E. Merck, Darmstadt, Germany, Art. 5748) plastic sheets without fluorescent indicator (20 × 20 cm, layer thickness 0.2 mm). A mixture of the solvent system chloroform: methanol: water (65:15:2, v/v/v) was used as the mobile phase to separate any possible glycolipids present and then after developing, the plates were dried under a stream of fast moving air for an hour to remove traces of solvent on the plates. Spot visualization was performed by spraying individually with anthrone reagent of 0.3% (w/v), acridine orange fluorescent dye of 0.02% (w/v) and ninhydrin reagent of 0.2% (w/v) for staining polysaccharides, lipids and free amino groups, respectively, followed by heating at 90°C for 30 min or until the detection of the definite spots, according to Kitamoto et al. (1990).

2.5.3. Gas chromatography–mass spectrometry (GC-MS) for Fatty acids analysis

The fatty acid profiles of the crude biosurfactant were first examined by the method previously described by Kitamoto et al. (1998). The methyl ester derivatives of fatty acids were prepared by mixing the above-mentioned purified glycolipids (10 mg) with 5% HCl-methanol reagent (1 mL). After heating at 80 °C for 30 min, the reaction was quenched
with water (1 mL) and the methyl ester derivatives were extracted with n-hexane (2 mL) and then analyzed by GC-MS.

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m, 0.25 mm, 0.25 µm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1 mL min$^{-1}$ and a split ratio of 50:1. Mass Selective Detector (MSD) was set to scan from m/z 50 to m/z 650 at a scan rate of 1.2 scans per second. The injector port and MS detector transfer line temperature was set at 280°C. The oven temperature was programmed at an initial temperature 150°C (hold 4 min) to 280°C as a final temperature with an increasing rate of 5°C min$^{-1}$ (hold 4 min).

The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of NIST, WILEY and MAINLIB online library data of the GC/MS system.

2.5.4. Further Purification of the biosurfactant

The crude extract was purified by preparative HPLC instrument using a semi preparative Kromasil 100 – 10 C18 reversed phase column (250 × 10 mm, 5 µm particle size, Shimadzu, Japan) operating with the total pump flow rate of 4 ml min$^{-1}$ for 20 min and maximum pressure of 15 MPa, at room temperature with a SPD-M20A photodiode array detector (PDA) set at 220–400 nm. LC system equipped with a CBM-20A System Controller, LC-20AP Pump A, SIL-10AP Auto Sampler and FRC-10A Fraction Collector.

The acetonitrile was filtered through a 0.2 µm membrane filter and used to wash the column. The HPLC-methanol extract was filtered through a 0.2 µm membrane filter before
injection. The filtrate (700 µl per run) was injected into the column automatically. The elution system consisted of solvent A (Nanopure water) and solvent B (HPLC grade acetonitrile, CARLO ERBA Reagents S.A.S.).

The solvents were degassed by purging with helium before use. The solvents were used in a gradient system by which the concentration percentage of solvent B was linearly increased from 10–100% for 17 min, whereas the solvent A concentration was gradually decreased from 90 – 0.0%. After this time, the solvents were returned to the initial point of their concentrations as the concentration of the solvent B was 10% with solvent A constituting the remaining 90% maintained for an additional 3 min. After which, the controller was stopped.

The purified biosurfactant fractions were collected according to their retention times and tested for their surface activities in the reduction of surface tension. Then the positive highly active effluents were combined, concentrated, lyophilized and finally subjected to the molecular mass analysis.

2.5.5. Fourier Transform Infrared Spectroscopy (FTIR) analysis

One milligram of dried purified biosurfactant was ground with 100 mg of a potassium bromide salt (KBr) in the mortar to remove scattering effects from large crystals and the powder mixture was compressed with 7500 g for 30 s to obtain translucent pellets in a dry atmosphere through which the beam of the spectrometer can be passed.

The infrared absorption spectra were recorded by Nicolet-iS10 (Thermo Scientific, USA) FT-IR spectrometer and plotted within the frequency range of 4000 – 400 cm\(^{-1}\) wavenumber. Spectral region was performed at a resolution and wave number accuracy of 4 and 0.01 cm\(^{-1}\), respectively. All measurements consisted of 500 scans, and KBr pellet was used as background reference as described by Smith (2011).
2.5.6. Liquid chromatography–mass spectrometry (LC-MS) analysis

The purified biosurfactant sample (fraction 47) 7 µl was injected directly into the LC-QTOF 5600 (Sciex, USA) detection system equipped with two columns, In-Line filter disks pre column (Phenomenex, 0.5 µm x 3.0 mm) and Xbridge C18 reversed phase column (Waters, 3.5 µm particle size, 2.1 x 50 mm) operating at 40 °C with the pump flow rate of 0.3 ml min⁻¹ for 28 min. Sample mobile phase was prepared by filtering using a 0.2 µm filter membrane disc and degassed by sonication before injection.

Mobile phase gradient elution system was comprised of two eluents: [A] was DI-Water containing 0.1% formic acid and [B] was 100% acetonitrile. The [A/B] gradient % mixtures were of 90/10, 90/10, 10/90, 10/90, 90/10 and 90/10 which were pumped at timing of 0, 1, 21, 25, 25.01 and 28 min, respectively. ESI-MS positive ion IDA acquisition mode was carried out on a Sciex Triple TOF 5600 + instrument and chromatographic separation was done by Sciex Exion LC. Mass spectra were detected in the ESI between m/z 100–1100.

The peaks and spectra were processed using the MS-DIAL 3.52 software for data analysis and tentatively identified by comparing its retention time (RT) and mass spectrum with the reported data in the database used; such as global natural product (GNPS).

2.6. Evaluation of antifungal activities of the efficient biosurfactant producing yeasts

In vitro screening for antifungal effect of the biosurfactant compounds was performed on sabouraud dextrose (SD) agar medium (Atlas, 2010) with the following composition (ingredients g l⁻¹): mycological peptone 10, dextrose 40, with pH 5.6, using filter paper disc diffusion method (Balouiri et al., 2016) against the under test phytopathogenic fungi. Each under test fungal culture disc (10 mm diameter) was transferred over the media using sterile cork borer. The filter paper (Whatman No.1, 5 mm diameter) was sterilized
and impregnated with the tested biosurfactant as either crude or purified form, while the bioreactor optimized production medium was the negative control. The saturated discs were placed on the surface of the SDA medium and the plates were incubated at 30°C for 1-3 weeks. After the incubation period, the plates were observed for the formation of clear inhibition zone around the paper discs.

2.7. Statistical analysis

The obtained data were statistically analyzed using one-way analysis of variance (ANOVA) supported by Duncan’s multiple range test (Duncan, 1955) running on Co-Stat statistical software (1990). Means were compared using L.S.D. (5% significance level). The standard deviation (SD) and the standard error (SE) of mean for triplicates were done using Excel program software (Microsoft office 2010).

3. Results

3.1. Optimization of culture conditions for maximum biosurfactant production using shake-flask method [all presented in Figure (1)]

3.1.1. Effect of organic carbon source type and nitrate salt type

The produced BS activity using oil as sole carbon source was superior to that using glucose by STR of 59.27% than 40.35% with KNO3 and by 56.70% than 38.91% with NaNO3 achieving 1.5 fold increase. Added to that, BSs produced from oil and glucose source tests using KNO3 increased by 5% and 4% than using NaNO3, respectively.

3.1.2. Effect of soybean oil concentration

Apparently, increasing the oil concentrations 2, 4, 6 up to 8% while KNO3 concentration was fixed led to variation in C:N ratios to be 60, 45, 30 and 15:1, respectively, resulting in increasing STR from 39.75, 46.58, 54.40 to 59.34%, respectively.
3.1.3. Effect of different organic and inorganic nitrogen sources

Among all, BS produced with KNO$_3$ as main N source achieved highest STR of 59.384% followed by NH$_4$NO$_3$ with 56.135%, while the two organic nitrogen sources yeast extract and peptone achieved STR of 50.352% and 44.469%, respectively, but with NH$_4$Cl achieved the lowest STR of 35.535%.

3.1.4. Effect of C:N ratio

BS production used optimized soybean oil concentration of 8% with variable KNO$_3$ concentrations to test C:N ratio effect. The results for STR% were distributed in a pyramid shape where statistically C:N ratios of 60:1, 90:1, 120:1 and 200:1 achieved the highest STR to be 59.787, 61.222, 62.577 and 63.922%, respectively, at the top of the pyramid. Up to those C:N ratios the STR increased gradually in a positive correlation of +0.788, while descending STR% values in reversible strong negative correlation of -0.996 parallel to increment in C:N ratios above 200:1 could be observed statistically.

3.1.5. Effect of different pH values

Greatest STR% was achieved by BS produced at higher pH levels, recording 63.951% and 64.914% at pH 7 and 8, respectively. An ascending strong positive correlation of +0.951 was verified statistically between pH values and STR%. The STR of 35.943, 26.037, 23.355 and 17.672% were recorded by BSs produced at initial pH values of 6, 5, 4 and 3, respectively.

3.1.6. Effect of different temperatures

Superiority of BS produced at 30°C tan 40°C was statistically confirmed as it exhibited significantly higher STR of 65.22% which amazingly comprised an increment of 4.5 fold that STR of 14.372% for BS produced at 40°C.
3.2. Growth profile and biosurfactant production scaling up using 2.5 L batch bioreactor

After revealing the optimum conditions for maximum BS produced activity, the profile of the cell growth and BS production by G. candidum in a 2.5 L batch bioreactor was evaluated using optimized Hua broth medium containing soybean oil (8%, v/v), potassium nitrate (0.75 g l$^{-1}$) and yeast extract (0.3 g l$^{-1}$) as pivotal C and N sources with ratio of 200:1, with initial pH adjusted at 8 by phosphate buffer (recording 7.7 after sterilization). The medium was inoculated with cell culture (6%, v/v), agitation 150 rpm and aeration 1.6 lpm at 30°C for 240 h.

The first log phase stage recognized up to 48 hrs. where specific growth rate was the highest recording $0.321 \text{ Log}_{10} \text{ cfu/ml.hr}^{-1}$ after which it decreased at 72hr to begin a slower second log phase stage up to 96hrs recording $0.159 \text{ Log}_{10} \text{ cfu/ml.hr}^{-1}$, as shown in Table (1) and illustrated in Figure (2).

During those two log phase stages and according to data correlations noted in table (1), it could be noticed that the there was a positive progress in BS production parallel to continuous decrease in pH from 7.7 down to 6.6 after which the specific growth rate entered the stationary phase where pH became stable at 6.5 up to 168 hrs. Finally, the specific growth rate decreased dramatically after 192 hrs. recording the beginning of net death phase where BS decreased in its STR% values appeared as strong negative change in rates and dramatic increase in pH values up to 7.5 that became constant all over the death phase.

Therefore, the incubation period after 144 to 168 hrs was target timing for maximal BS production recording STR of 66% in the bioreactor.

3.3. Isolation, purification and structural characterization of biosurfactant
3.3.1. Isolation of biosurfactant

As noted before, the ethyl acetate extract containing the organic phase isolated from culture SUP was rotary evaporated at 40°C until we obtained a brownish sticky gum and considered as the crude BS extract which recorded productivity yield of 1.7482 g l⁻¹.

3.3.2. TLC

According to TLC results shown in Figure (3), the existence of the glycosyl moiety and lipid moiety spots having similar Rf value of 0.9290, suggested the glycolipid nature of the produced BS. On the other hand, negative reaction towards ninhydrin solution indicated the absence of detectable amino groups.

3.3.3. GC-MS Spectrum

The resulting peak chart shown in Figure (4) revealed presence of different separated organic compounds from hydrophobic moiety characterized further by their mass spectra into structural categories found to be related to fatty acids, ergosterols and flavones which were denoted by colored arrows in the presented chart.

Methyl esters of three fatty acid forms that appeared in the GC-MS analysis result included a saturated hexadecanoic (palmetic, MW 270), a longer double unsaturated octadecadienoic (Linolelaidic, MW 294) and a single unsaturated octadecenoic (Oleic, MW 296) fatty acids, having peak area of 5.86%, 20.55% and 30.10%, respectively.

Beside fatty acids, other hydrophobic compounds of basic structures related to ergosterols having MW range 408–520 were present, forming collectively peak area of 0.7% relative to that of fatty acids. The squalene of MW 410 appeared at RT 34.16. The seven ergosterol like compounds appeared at RT 6.84, 12.80, 22.22, 22.35, 25.30, 25.62 and 36.71, of MW 408, 448, 362, 366, 520, 418 and 408, respectively, beside three lanosterol like compounds noticeably appeared at RT 15.58, 19.45 and 37.34 with MW of 502, 430 and
Flavone core structures included 3 compounds of same formula \( \text{C}_{27}\text{H}_{30}\text{O}_{16} \) named Lucenin 2 and one compound of formula \( \text{C}_{27}\text{H}_{30}\text{O}_{14} \) named Luteolin 6,8-di-C-glucoside, collectively of a peak area 0.2% relative to that of fatty acids.

3.3.4. Preparative HPLC

Due to large variation in hydrophobic moieties revealed in GC-mass analysis, the crude BS needed more dependable procedure to purify and identify the constituent target surfactant mainly responsible for its action.

The fractionation of the raw BS was done using the preparative HPLC and the resulting fractions separated in accordance to mobile phase gradient polarity (water/acetonitrile). Fractions had characterized peaks in accordance to their absorbance (mAU: milli absorbance unit) detected by SPD-M20A photodiode array detector (PDA) set at 254 nm, 220–400 nm, 235 nm and 280 nm as shown in Figure (5).

Peaks with considerable mAU were detected at 220–400 nm between RT 15 up till 23 min, as shown in Figure (5). Fractions collected under those peaks were tested for their surface tension reduction activity (STR%) as presented in Figure (6). Six fractions out of preparative HPLC resulting fractions were found to have reasonable STR% activities. Fraction designated no 47 (F47) emerging between RT 20.2 up to 20.8 min exhibited maximum STR% activity of 51.919 out of the six fractions followed by no 48, based on statistical analysis (LSD = 5.184).

3.3.5. FT-IR Spectrum

The purified fraction F47 of BS of significant surface tension reduction (STR%) activity (51.9%) was subjected to FT-IR spectrum analysis to reveal its characterized functional groups. Based on the presented data in Figure (7), 2 dip peaks at 2856.93 and 2958.90 cm\(^{-1}\) with a % transmittance of 43.61 and 36.84, respectively, characterizing single bond
stretch range (2500-4000 cm\(^{-1}\)).

One major dip peak at 1728.31 cm\(^{-1}\) of 34.33% and minor dip peak at 1600.47 cm\(^{-1}\) of 71.43% transmittance were present in double bond stretch region (1500–2000 cm\(^{-1}\)). In the fingerprint region (500–1500 cm\(^{-1}\)) three pair dip peaks were worthy to recognize at [1379.76–1463.96], [1247.58–1272.89] and [1072.50–1123.41] cm\(^{-1}\) with transmittance of 56.69% – 59.29%, 42.11% – 41.42% and 53.84% – 54.24%, respectively.

3.3.6. LC-MS/MS

The LC-MS/MS spectral analysis for F47 separated 163 compounds of which three distinctive ones with functional characteristics were observed having the highest intensities among all compounds, as summarized in their data Table (2) and illustrated in Figure (8).

The “Icariside F2” (C\(_{18}\)H\(_{26}\)O\(_{10}\)) as adducted [M + Na]\(^+\) of MW 425.1341 at RT 13.9742 min formed of phenyl methyl as the hydrophobic moiety and a Glucopyranoside 6-O-[(2R,3R,4R)-tetrahydro-3,4-dihydroxy-4-(hydroxymethyl)-2-furanyl] as the hydrophilic moiety, having InChIKey: NJMQSVWMCODQIP-UHFFFAOYNA-N.

The “Cardenolide Di-Hexopyranosyl” (C\(_{35}\)H\(_{54}\)O\(_{14}\)) as adducted [M + Na]\(^+\) of MW 721.388 at RT 18.86957 min formed of a type of steroid as the hydrophobic moiety and the dihexopyranoside structure as the hydrophilic moiety, having InChIKey: RIPMDUQTRRLJTE-UHFFFAOYNA-N.

Finally the “Di Galactosyl Di Acyl Glycerol” (C\(_{49}\)H\(_{86}\)O\(_{15}\)) as adducted [M + NH\(_4\)]\(^+\) of MW 932.633 at RT 23.90967 min was formed of di-galactoside hydrophilic moiety and di-acyl glycerol hydrophobic moiety, having InChIKey: WVVINZZVFAFVMJ-SVNLWEDNA-N. Worthy to notice the detailed structure of DGDG that was revealed in MS2 as illustrated by LC-
MS/MS analyses detailed chart in Figure (9) and shown in Table (3) which discussed the fractions loss during DGDG fragmentation. The deduced structure revealed the two acyl groups to be palmetic (16:0) of MW 256.42 and linolenic (18:3) fatty acids of MW 278.436.

3.4. Application of the biosurfactant

Generally, the crude glycolipid BS extract showed significant antimicrobial activities against all the tested pathogenic fungi with varying diameters of the inhibition zones recording 19, 17.5, 8.5, 6 and 5.5 mm against Rhizoctonia solani, Phytophthora sp., Macrophomina phaseolina, Fusarium solani and Alternaria alternata, respectively, as all illustrated in Figure (10) and partially illustrated in Figure (11). Also, the serial dilutions of this extract including 5, 4 and 2% effectively inhibited fungal growth of Macrophomina phaseolina with zone diameters of 5, 4.5 and 3 mm, respectively, while dilution of 1% inhibited Alternaria alternata with 2 mm diameter. Moreover, the pure glycolipid BS (F47) displayed activity against Macrophomina phaseolina with 3 mm inhibition zone diameter.

4. Discussion

Depending on the finding that the yeast strains were considered BS producers when this difference was equal or higher than 8 mN m⁻¹ as reported by Gudiña et al. (2015). The present study introduced G. candidum which succeeded to produce BS that reduced surface tension of culture media from 95.623 down to 38.890 mNm⁻¹ by a difference of 56.73 mNm⁻¹ (STR was 59.27%) before optimization and down to 30.612 mNm⁻¹ by a difference of 65.01 mNm⁻¹ (STR 66.447%) after optimization and scaling up. Many parameters such as C:N ratio, pH, T°C, agitation, and aeration interact with each other in a complex way that apparently affect BS production kinetics as reviewed by Banat et al. (2014).

Effect of C source type
This study aimed to distinguish the capability of G. candidum to produce efficient BS by metabolizing hydrophobic and hydrophilic substrates, of which soybean oil hydrophobic substrate proved to be the best choice.

It was important to consider that the substrates act differently for a specific BS producing microorganism, as clarified by Haba et al. (2000). For instance, Nitschke et al. (2005) found that Pseudomonas bacterial strains were favoring the hydrophobic carbon sources such as soybean oil gave higher levels of rhamnolipid BSs when compared with hydrophilic substrates as carbohydrate rich ones. Also, Thaniyavarn et al. (2008) found that soybean oil was the best carbon source for optimal sophorolipid BS production by thermostable yeast Pichia anomala PY1 compared with the other sources such as glucose, starch, palm oil and coconut oil. Furthermore, Accorsini et al. (2012) found that seven yeast isolates produced BSs in higher concentrations after 168 h of cultivation by using soybean oil as a substrate than those grown on glycerol.

Effect of soybean oil concentration

Several studies previously reported the ability of yeasts to produce BS compounds using soybean oil as a sole carbon source at different concentrations. Pseudozyma shanxiensis produced new extracellular MELs BS from 4% soybean oil as the best carbon source according to Fukuoka et al. (2007). Sari et al. (2014b) evaluated three yeast isolates belonging to the genus Pseudozyma as potent novel BS producers from 4% soybean oil as a sole substrate. Thaniyavarn et al. (2008) found that 4% (w/v) soybean oil was the most effective concentration for optimal sophorolipid BS production by a thermotolerant yeast Pichia anomala PY1.

In the present work, G. candidum gave its highest BS production activity when 8% (w/v) of soybean oil was used. Similarly, highly efficient mannosylerythritol lipids BS (MELs) was produced by yeasts Pseudozyma antarctica T-34 and Pseudozyma hubeiensis Y10BS025
when grown on media containing 8% (w/v) soybean oil (Morita et al., 2006; Sari et al., 2014a).

Effect of N source type

The present study confirmed that using KNO₃ leveled up the BS production activity than other inorganic and organic N sources. Similarly, Johnson et al. (1992) stated that NaNO₃ was an important nitrogen source for maximum bioemulsifier production by yeast Rhodotorula glutinis IIP-30. Also, Thaniyavarn et al. (2008) found that sodium nitrate supported highest BS production by Pichia anomala PY1 than other acidic nitrogen sources such as (NH₄)₂SO₄, NH₄NO₃ and NH₄Cl.

On the other hand, Guerfali et al. (2019) and Sharma et al. (2019) stated that yeasts Rhodotorula babjevae Y-SL7 and Meyerozyma guilliermondii YK32 maximum BS production was achieved when yeast extract was the sole nitrogen source, respectively, compared to other sources as (NH₄)₂SO₄ and urea. However, these findings were clarified by the differential role of nitrate and ammonium salts in BS production by yeasts as stated by Amaral et al. (2010).

Effect of C:N ratio

Gradual increase in STR% of produced BS was parallel to the increase in C:N ratio up to 200:1 obviously causing nitrogen limitation. In some cases, reported the limitation in nitrogen had enhanced BS production (Abu-Ruwaida et al., 1991; Patel and Desai, 1997). On the same trend, Wei et al. (2005) observed that high nitrogen content in a fermentation medium had limited the BS production. Worthy to notice that further increase in C:N ratio beyond 200:1 led to the decrease in STR% values due to the nitrogen severe scarcity that was a crucial factor for cell growth and consequently BS biosynthesis.

Effect of initial pH value
The results in the present study proved that pH 7 and 8 were much preferable by G. candidum yeast than lower pH levels to maximize BS production activity. Yeasts Candida bombicola and Trichosporon asahii produced BS at pH values of 8 and 7.5 characterized by maximum emulsification index (Daverey and Pakshirajan, 2009; Chandran and Das, 2010), respectively, similarly supporting those results in the present study. Furthermore, Sarubbo et al. (2007) and Campos et al. (2019) found that the BS produced by Candida lipolytica and Candida utilis showed better emulsification and STR at alkaline pH values of 12 and 10, respectively.

Worthy to notice that soybean oil hydrolyzed by lipases produced by yeasts resulting in free fatty acids that lead to decrease in pH of the culture medium, directly correlated to the efficacy of their produced glycolipid BS (Bednarski et al., 2004), which might explained the drop in culture pH at the end of production period in the present study.

**Effect of temperature**

Kim et al. (1999) found that the most favorable temperature for the production of BS by Candida sp. SY16, was at 30°C. Similarly, maximum BS production by Pichia anomala was obtained at 30°C, as reported by Thaniyavarn et al. (2008).

The BS produced by Meyerozyma guilliermondii YK32 exhibited a better performance at 30°C than 25, 35 and 40°C. Interestingly, a complete elimination of BS production was at 40°C similar to our reports at this temperature after 7 days of incubation, (Sharma et al., 2019).

**BS production on batch bioreactor**

Compared to the present results, in which G. candidum entered its exponential phase after 24 hr for 72 hr after which stationary phase began at 120 hr for 48 hr, Monteiro et al. (2010) stated that BS producing yeast strains, Trichosporon loubieri CLV20, Geotrichum sp.
CLOA40 and T. montevideense CLOA70 exhibited similar growth rates in the exponential growth phase, with a clear acceleration after 24 h and stabilization of the activity after 144 h.

Similarly, found that where there was a simultaneous relationship between growth, BS production and substrate consumption and thus, the production of BS compounds was associated with growth kinetics (Desai and Banat, 1997; Amaral et al., 2006). Added to that, Ishaq et al. (2015) found that Aspergillus flavus AF612 produced highest yield of a novel BS glycolipid after 196 h of incubation, indicating that stable BS production had occurred during stationary phase.

Worthy to notice variation in exponential growth rates appeared as two log phases during batch bioreactor. On the same trend, Silva et al. (2010) stated that BS production by P. aeruginosa was associated with growth shortly after inoculation forming a two phase profile, the first up to 24 h and remaining constant until 48 h, while in the second phase, production increased at a slower rate up to 96 h.

On the other hand, maximum BS production activity achieved in the stationary growth phase coincided with stable acidity formed in the medium that might be due to release of lipase degradation products of the soybean oil substrate such as monoacylglycerols, diacylated glycerols and nonesterified fatty acids into the culture broth as those findings reported before by Morita et al. (2012).

The results obtained by Adamczak and Bednarski (2000) and Santos et al. (2014) indicated that the BSs production increased with enough aeration facilitating oxygen transfer from gas to aqueous phase as a vital role of aeration and agitation system in batch bioreactor in the present study. That was why the complete its omission significantly decreased the BS production by Meyerozyma guilliermondii YK32, as recommended by Sharma et al. (2019).
BS isolation and identification

The crude BS of G. candidum analyzed by the GC-MS revealed the presence of hexadecanoic (16:0) and octadecanoic (18:2 and 18:0) fatty acids in the form of methyl esters at concentration of 5.86, 20.55 and 30.10%, respectively. There was a great probability that those fatty acids had a structural role as hydrophobic moieties of a glycolipid BS which was primarily detected by TLC results previously mentioned. Similarly, Souza et al. (2012) reported that their yeast strain which produced a BS that was analyzed by GC-MS and was found to be formed of monosaccharide moiety attached to both octadecanoic acid and hexadecanoic acids at concentrations of 48.01% and 43.16%, respectively and were considered sharing in the BS structure as glycolipids. According to Sokolov et al. (2019), many intermediates in yeasts shared in ergosterol biosynthesis as water insoluble compounds included in successive steps. Those intermediates resemble in their structures to what was presented by GC-MS spectral analysis in the present work. For example, the squalene at RT 34.16 considered as a precursor for lanosterol like compounds at RT 15.58, 19.45 and 37.34. Mostly, ergosterols are not considered as extracellular BS produced by yeasts but are majorly located in the cytoplasmic leaflet of yeast plasma membrane (about 80%) as reported by Solanko et al. (2018) and being released after cell death, as reported by Headley et al. (2002).

In the present study, both Lucenin 2 and luteolin 6,8-di-C-glucoside were present too as flavone compounds. Flavone compounds with several types were known for known antifungal, antibacterial and antiviral activities as stated by Cushnie and Lamb (2005). Bitencourt et al. (2014) demonstrated 4 types of flavone structures that act as fatty acid synthase inhibitors against several fungal types. Also, Meenupriya and Thangaraj (2011) separated three bioactive compounds from marine fungi Aspergillus ochraceus of which Lucenin 2 of structural formula C27H36O16 having MW of 610 and was found to possess
antibacterial activity and was identical to that in the present work. On the other hand, Materska (2015) studied luteolin 6,8-di-C-glucoside which was similar to that in our present study. He confirmed that the presence of C-glucosides attached to luteolin structure increased available hydroxyl groups which made it bioactive in both lipophilic and hydrophilic systems. Depending on this, the luteolin 6,8-di-C-glucoside could have its own surfactant behavior in the present work.

In preparative HPLC results, fraction separation depending on gradient polarity by the mobile phase (water/acetonitrile) so that the more hydrophobicity of the separated compound the later the retention time RT it appeared. Notice that the highest fractions in STR% activity including F47 (51.919%) and F48 (38.991%) separated at later RT considering their lower polarity than the other fractions of less STR% activities regardless of their relative area concentrations. This might spot on the large hydrophobic moiety in their structure and/or the less polar groups in the hydrophilic moiety.

According to FT-IR spectrum reference (Sherman Hsu, 1997), the dip peaks which appeared at 2958.90 cm\(^{-1}\) corresponded to sp\(^3\) C-H stretch in alkane and 2856.93 cm\(^{-1}\) corresponded to C-H stretch in aldehyde group, while both dip peaks corresponded to O-H stretch in carboxyl group. On the other hand, dip peaks at 1728.31 cm\(^{-1}\) corresponded to sp\(^2\) C = O in aldehyde group and ester bonding, while at 1600.47 cm\(^{-1}\) corresponded to sp\(^2\) C = C stretch with weak probability.

Finally, dip peak pairs at 1379.76-1463.96, 1247.58-1272.89 and 1072.50-1123.41 cm\(^{-1}\), corresponded to ester - alkane, C-O in acyl as with aldehyde in aldohexoses or with ester of glycerides and C-O in alkoxy as with ether bond between sugar and glucose or ester bond in glyceride or even the O-H group in alcohols, respectively.

Depending on this knowledge, the fatty acid moiety mainly as an alkane (2958 cm\(^{-1}\) with
possible double bonding (at minor dip peak 1600.47 cm\(^{-1}\)) sharing its carboxyl group in glyceride ester bonding (1247.58–1272.89 cm\(^{-1}\)) with glycerol moiety (1072.50–1123.41 cm\(^{-1}\)) could be confirmed. The 2958.90, 2856.93 and 1379.76 cm\(^{-1}\) pointed to long chain aliphatic fatty acid. The sugar moiety would be characterized by the alkane main body (2958 cm\(^{-1}\)) with its aldehyde group (2856.93 and 1728.31 cm\(^{-1}\)). Ether bonding (glycoside) confirmed between glycerol and sugar (1072.5-1123.41 cm\(^{-1}\)). This was briefly pointing to glycolipid structure with long chain fatty acid, as demonstrated before by Mani et al. (2016).

The previously three elucidated structures in the F47 LC-MS/MS analyses are considered structurally as emulsifiers, due to possessing both hydrophobic and hydrophilic moieties, as stated by Goodarzi and Zendehboudi (2019). The Cardenolide di hexopyranosyl known as steroidal glycoside was nearly similar to ergosteryl 3-beta glucoside produced by baker’s yeast that was found to be a weak basic to a neutral compound based on its pKa as stated by Herrgard et al. (2008). On the other hand the ergosteryl 3-beta glucoside had a polar surface area 99.38\(\text{A}^\circ\) while in the present study Cardenolide di-hexopyranoside was found to possess a bigger polar surface area of 225\(\text{A}^\circ\) due to its extra hexopyranosyl molecule as computed by Cactvs 3.4.6.11 (PubChem release 2019.06.18) referred to in (https://pubchem.ncbi.nlm.nih.gov/compound/45360041#section=Computed-Properties).

The Cardenolide di hexopyranoside was found to have a net charge of “1” as mentioned in (https://mona.fiehnlab.ucdavis.edu/spectra/display/CCMSLIB0000855692) and (http://gnps.ucsd.edu/ProteoSAFe/gnpslibraryspectrum.jsp?SpectrumID=CCMSLIB0000855692) instead of being zero net charge as in case of ergosteryl 3-beta glucoside.

The three structures are closely related to BS structures and collectively were responsible
for the surfactant action measured in F47, pointing especially to DGDG as a distinctive glycolipid previously noted by Alves et al. (2019).

As the DGDG 34:3 fragmentations revealed the presence of 11 molecules having m/z ranging from 932.62708 to 259.16699. The recorded loss in the DGDG of m/z 932.62708 was by 341.16 m/z corresponding to di-galactoside and consequently the resulting di-acyl glycerol hydrophobic of m/z 591.49744 lost 256.24 and 278.22 m/z corresponding to palmitic saturated fatty acid (16:0) and linolenic unsaturated fatty acid (18:3), respectively. The structural property of this glycolipid as a distinctive BS was nearly related to glycolipid BS produced by yeast Wickerhamomyce anomalus when grown on oil as studied by Souza et al. (2017).

**Antifungal effect of crude BS and F47**

The results indicated that G. candidum produced bioactive glycolipid BS agents with antimicrobial activities against broad spectrum of phytopathogenic fungi when applied as crude extract at different concentrations including Fusarium solani, Rhizoctonia solani, Alternaria alternata, Macrophomina phaseolina and Phytophthora sp., while when F47 was applied it exhibited antimicrobial effect only against Macrophomina phaseolina. The hydrophobic content of crude extract analyzed by GC-MS suggested the major existence of ergosterol intermediate structures, Lucenin 2 and luteolin-6,8-di-C-glycoside beside fatty acids, in which both of flavonoid structures including Lucenin 2 and luteolin glycosides were known for their antimicrobial effects as stated by Basile et al. (1999) and thereby were possible agents responsible for the strong antifungal effect exhibited in the present study. Górniak et al. (2019) pointed in their study to the flavonoids as antimicrobial agents too.

While the F47 LC-MS/MS analysis suggested the major contents to be Icariside F2 as a type of flavone like structure, Cardenolide di hexopyranosyl as a structure of ergosteroid,
beside the glycolipid DGDG. Cushnie and Lamb (2005) stated that flavonoids had antifungal activities against Candida albicans and Aspergillus flavus. Also, Bitencourt et al. (2014) evaluated four compounds of flavonoid structures that had antifungal activities. Added to that, glycolipids BS had structures and properties similar to that of detergents and thus intercalated into the phospholipid bilayer of the cell membrane thereby facilitating the membrane permeability and metabolites flow. Consequently, both structure and function of the phospholipid bilayer were altered, effectively interrupting the protein conformation, transport and energy generation, which eventually led to the cell death, according to Sotirova et al. (2008), thus corroborating the results obtained in the current study. Ndlovu et al. (2017) succeeded to apply the crude surfactin and rhamnolipid BS extracts produced by Bacillus amyloliquefaciens ST34 and Pseudomonas aeruginosa ST5, respectively as antimicrobial agents against pathogenic fungi Candida albicans.

On the other hand, crude biosurfactant produced by Wickerhamomyces anomalus yeast had glycolipid structure that when applied exhibited antimicrobial activity against pathogens including Candida albicans, Escherichia coli, Staphylococcus epidermis and Streptococcus galacticae, by affecting fatty acid synthase as stated by Souza et al. (2017).

Finally a concluded explanation on the antifungal activity of BS produced by G. candidum in the present study could be deduced, that using crude BS form applied Ergosterol structures, Lucenin 2 and Luteolin 6, 8 di C-glucoside had wide antifungal spectrum on all phytopathogens under test, while after fractional purification the chosen F47 fraction contained part of effective compounds including Icariside F2, Cardenolide di hexopyranosyl and DGDG glycolipid that exerted antifungal action only on Macrophomina phaseolina.

In conclusion, the extracellular BS produced by yeast Geotrichum candidum MK880487 in
the 2.5 L bioreactor was identified as glycolipid with highest yield of 1.75 g l$^{-1}$. The produced BS in its crude form was promising as eco-friendly bioagent with inhibitory activities against phytopathogenic fungi. Besides, it could have considerable role in sustainable agriculture and industrial processes compared with the synthetic surfactants.

Declarations

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Ethical approval**

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

**Consent for publication**

All authors agree the manuscript submission for publication.

**Availability of data and material**

'Not applicable' for that section.

**Author contributions**

Experiments were conducted by both Ahmed M. Eldin and Nermeen Hossam, equally contributed new analytical tools, analyzed all data and wrote the whole manuscript.

Zeinat Kamel conceived and designed the research. All authors read and approved the manuscript.

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**Tables**

**Table (1):** *G. candidum* grown in Hua broth medium on batch Bioreactor at 30°C for 240 h (agitation speed 150 rpm / aeration 1.6 lpm). Cell count, specific growth rate, produced BS activities (STR% and ODA cm²) and medium pH were measured. Pearson’s correlations between measured parameters are presented below.
Table (2): LC-MS/MS data sheet for A) Icariside F2, B) Cardenolide di-hexopyranoside and C) DGDG (Di Galactosyl Di Acyl Glycerol) glycolipid in F47 from 263 BS.

| Compound | NCGC00385106-01 / C_{18}H_{26}O_{10} / Icariside F2 / Phenylmethyl 6-O-[(2R,3R,4R)-tetrahydro-3,4-dihydroxy-4-(hydroxymethyl)-2-furanyl]-beta-D-Glucopyranoside/ | RT(min) | Precursor m/z | Area | Adduct Ion | InChIKey | MSMS spectrum |
|----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|---------------|------|------------|-----------|----------------|
|          |                                                                                                                                                                                                | 13.9742 | 425.1341      | 25387370 | [M+Na]^+  | NJMQSVWMCODQJP-UHFFFAOYNA-N |                |
|          |                                                                                                                                                                                                |         |               |       |            |           | MSMS spectrum |
|          |                                                                                                                                                                                                | 721.388 |               | 47317.88 | [M+Na]^+  | RIPMDUQTRRLJTE-UHFFFAOYNA-N | 721.3427:294/ 721.39581:1688/ 721.63098:341 |
|          |                                                                                                                                                                                                | 23.90976 | 932.633       | 96154.1 | [M+NaH4]^+ | WWVINZZVFAFMVJ-SVNQLWEDNA-N |                  |
|          |                                                                                                                                                                                                | 261.2215:143/ 313.27728:358/ 325.10852:72/ 335.25861:358/ 573.49036:322/ 591.49744:478/ 915.5946:107/ 932.54083:218/ 932.62708:450 |      |               |       |            | InsilicoMSMS-Lipids- |                  |

Table (3): DGDG 34:3; DGDG 16:0-18:3; [M+NH4]^+ LC-MS/MS fragments spectrum and the
predicted compound corresponding to m/z.

| No. | Predicted name                              | M/Z           | REL INT |
|-----|--------------------------------------------|---------------|---------|
| 1   |                                            | 259.16699     | 7.531   |
| 2   |                                            | 261.2215      | 29.916  |
| 3   |                                            | 309.10748     | 7.531   |
| 4   | Monoacyl glycerol with FA (16:0)           | 313.27728     | 74.895  |
| 5   |                                            | 325.10852     | 15.063  |
| 6   | Monoacyl glycerol with FA (18:3)           | 335.25861     | 74.895  |
| 7   | Diacyl glycerol                            | 573.49036     | 67.364  |
| 8   |                                            | 591.49744     | 100.000 |
| 9   |                                            | 915.5946      | 22.385  |
| 10  |                                            | 932.54083     | 45.607  |
| 11  | DGDG                                       | 932.62708     | 94.142  |

Figures

Figure 1

Comparative study of culture conditions on glycolipid biosurfactant production by G. candidum in shake flask evaluated by surface tension reduction %. Each test had its individual LSD recorded in its colored rectangle, while arrows pointed to correlation range and strength.
Figure 2

G. candidum plot for measured cell count (log10cfu), produced BS activities (STR% and ODA cm2) and medium pH during its growth in Hua broth medium on batch Bioreactor at 30°C for 240 h (agitation speed 150 rpm / aeration 1.6 lpm).
Biochemical characterization of the isolated biosurfactant by TLC: (A) pink spot on spraying with acridine orange indicated the incorporation of lipid to the biosurfactant, (B) brown spot upon spraying with anthrone indicated the presence of carbohydrate in the biosurfactant, and (C) no color development on spraying with ninhydrin indicated the absence of protein in the biosurfactant.
GC-mass analysis peak area chart for crude BS [RT chart]. Relative abundance peaks of separated compounds in accordance to their RT (min) are marked colored arrows. Later mass spectra revealed presence of a) fatty acids [red arrows], B) ergosterol related compounds [blue arrows] and squalene [purple arrow], C) flavone like compounds [green arrows].
Crude BS fractionation by preparative HPLC with SPD-M20A photodiode array detector (PDA) set at 220-400 nm in RT range of 12-26 min (showing maximum plot for the resulting fractions). Red arrows with numbers point to (fraction no./relative area concentration %) of the six most STR% active fractions.
Testing STR% activity for six most promising fractions purified from (263) crude BS by preparative HPLC.
Figure 7

FTIR spectrum graph for F47 separated from 263 BS. The red brackets point to important bonds and groups useful in inspecting the structure.
Figure 8

The structure of the three abundant structures having emulsifying and surfactant properties found in F47 LS-MS/MS analysis of fractionated 263 BS. A) Icariside F2, B) Cardenolide di hexopyranosyl and C) DGDG (Di Galactosyl Di Acyl Glycerol) glycolipid in F47.
LC-MS/MS analyses for DGDG (digalactosyldiacylglycerol 34:3) at RT 23.90967 in positive mode indicating digalactoside loss from the (M+NH4)+ adduct precursor ion as well as m/z differences of the product ions pointing to the fatty acid side chains including palmetic 16:0 and linolenic 18:3 fatty acids.
Figure 10

Antifungal effect of crude BS and F47 produced by G. candidum.
Figure 11

Phytopathogenic fungi inhibition zones against crude BS produced by G. candidum.

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