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Isolation and purification of a new enamide biosurfactant from *Fusarium proliferatum* using rice-bran

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ABSTRACT

A new fungus *Fusarium proliferatum* was isolated from the oil contaminated sludge of rice-bran oil industry, which was capable of producing biosurfactants when grown on rice-bran. The dark brown honey colored biosurfactant was recovered using *ultrasonication* as one of the steps in the extraction process. The biosurfactant was purified by column chromatography and structure elucidation of the purified biosurfactant was done with the aid of various spectroscopic techniques *viz.* FTIR, 2D-NMR, and Mass Spectrometry. The purified biosurfactant reduced the surface tension of the distilled water from 71.2 to 36.6 mN/ m and critical micelle concentration was calculated to be 0.33 mg/mL. The biosurfactant was also capable of emulsifying various hydrocarbons *viz.* coconut oil, refined oil, kerosene, and n-dodecane. The crude biosurfactant was checked for its anti-oxidant activity using ascorbic acid as standard and determining 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity. The IC_{50} was calculated and for ascorbic acid it comes out to be 0.056 mg/mL and for biosurfactant it was 18.81 mg/mL.

Keywords: Biosurfactant, cmc, emulsification activity, *Fusarium proliferatum* surface tension, *ultrasonication*

1. Introduction

Biosurfactants are the molecules which contain both hydrophilic and hydrophobic moieties in their chemical structure, therefore, regarded as amphiphilic in nature. The biosurfactants are produced as secondary metabolites in the fermentation media when microbes, instead of using nutrients for their growth utilize them for the production of biosurfactants. Various microorganisms may simultaneously synthesize a variety of biosurfactants by distinct molecular configurations.[1] The microbial surfactants are classified into various classes (*viz.* glycolipids, lipopeptide and lipoprotein, fatty acids, phospholipids and neutral lipids, polymeric biosurfactants and particulate biosurfactants) based upon their wide variety of chemical structures.[2,3] Biosurfactants possess a lot of applications in a variety of areas ranging from microbial enhanced oil recovery, bioremediation, soil remediation, food, cosmetics, antimicrobial and biomedical fields.[4-11]

The most limiting factor in the complete industrialization of biosurfactants is their higher production costs compared to synthetic surfactants. However, when compared with the synthetic surfactants their low toxic nature, functionality under extreme conditions, biodegradability, and production from renewable sources makes them an interesting research area. [12-15] To make them cost effective, the use of various unconventional substrates as the carbon sources have been in progress as it influence both their yield and quality.[16,17] Out of various unconventional sources, the use of oil industry by-products for this purpose is of great importance as the biosurfactants are produced by mainly hydrocarbon degrading microorganisms and show best growth on hydrocarbon substrates.[1,13] Also, the other factor is the higher yields of biosurfactants by various micro-organisms and fungi are known to yield good amount of biosurfactants as compared to bacteria due to the presence of a rigid cell wall.[18,19]

The present work deals with the production of biosurfactants from a fungus *Fusarium proliferatum* which was isolated from the rice-bran oil industry sludge. The fungus was capable of producing biosurfactants in higher yields by using inexpensive substrates of the same industry. The extensive structural elucidation was done using FTIR, 2D-NMR, and Mass Spectrometry techniques.

2. Material and methods

2.1. Materials

All the chemicals used were of reagent grade. The rice-bran and sludge were collected from a local rice-bran oil industry, A.P. Organics Private Limited, Dhuri, Sangrur (India).

2.2. Strain Isolation and preparation of seed culture

The strain was isolated from the rice bran oil industry sludge. 1mL of sludge sample was taken and grown in the minimal salt medium (MSM) containing rice-bran oil for microbial isolation. Serial dilutions with 1mL of MSM were made in the sterilized distilled water (10 mL) up to 10^{-6} dilutions. From each dilution, agar plates were streaked. The dilution numbers

from 10^{-2} to 10^{-6} single colonies were picked and grown in nutrient broth (composition (g/50 mL); peptone, 0.5; beef extract, 0.25; NaCl, 0.25) at 30 °C in a New Brunswick Scientific (Innova 42- Eppendorf) incubator shaker at a shaking speed of 150 rpm for 24-48 h of incubation. The strains were again streaked on nutrient agar slants to check the purity. Isolated pure strains were grown in the fermentation medium for the biosurfactant production and surface tension was monitored to get the biosurfactant producing strains. Finally the strain which reduced the surface tension to maximum was characterized by translation elongation factor gene sequence data, at IMTECH, Chandigarh. TEF gene region was amplified using standard PCR reaction. The primer pair EF-1 (5'-ATGGGTAAGGA(A/ G)GACAAGAC-3') and EF-2 (5'-GA(G/A)GTACCAGT(G/C)ATCATGTT-3') was used in a PCR reaction using annealing temperature 53 ^{0}C.[20] The amplified product was sequenced using a ABI PRISM ® Big Dye Terminator Cycle sequencing.

In the laboratory, the cultures were stored in 20 % glycerol at 4 °C as well as maintained on nutrient agar plates and was transferred to 50 mL of nutrient broth to prepare the seed culture. The cultivation conditions for the seed culture were 30 °C, 150 rpm, and 24-48 h of incubation.

2.3. Fermentation medium

The optimized fermentation media for the production of biosurfactants had composition (g/L): glucose (20.0), rice bran (30.0), fatty acids (25) yeast extract (18.0), NH_4NO_3 (2.0), K_2HPO_4 (2.2), NaCl (0.01), and 0.5 mL of trace element solution containing (g/L): $ZnSO_4.7H_2O$ (2.32), $MnSO_4.4H_2O$ (1.78), H_3BO_3 (0.56), $CuSO_4.5H_2O$ (1.0), $Na_2MoO_4.2H_2O$ (0.39), $CoCl_2.6H_2O$ (0.42), EDTA (1.0), $NiCl_2.6H_2O$ (0.004), KI (0.66) and pH of the medium was adjusted to 6.5 ± 0.2 by 6N HCl and the media was sterilized by autoclaving at 15 psi for 20 min. The reactions were carried out in 500 mL Erlenmeyer flasks containing 150 mL of the culture media. 10% of the inoculum was used for the fermentation reactions. The fermentation was carried out at 30 °C with agitation at 150 rpm for 120 h in a New Brunswick Scientific (Innova 42- Eppendorf) incubator shaker. During fermentation hours no pH adjustments were done. At the end of the fermentation, samples were taken from the media to determine surface tension.

2.4. Extraction of biosurfactant

In the extraction process of biosurfactants, *ultrasonication* (Ultrasonic bath, LINCO, 40 MHz) was also performed in the procedure to check the yield difference with and without this step. After 120 h of incubation the flasks were withdrawn and kept in an ultrasonic bath for 30 min and then centrifuged at 5800 rpm for 20 min. the supernatant was filtered for the next step and the settled rice bran was discarded. The supernatant served the source of crude biosurfactant. The pH of the supernatant was adjusted to 2.0 with 6N HCl and kept overnight for precipitation. The biosurfactant was extracted thrice with various solvent systems *viz.* acetone, chloroform, ethyl acetate, methanol, varying ratios of ethyl acetate to methanol (ranging from 1:4 to 4:1) at room temperature and the organic layer was transferred to a rotary evaporator at 40 °C. The

dried residues were washed with hexane to remove unutilized fatty acids and re-dissolved in methanol and sodium sulfate was added to remove the traces of water. After one hour the organic layer was filtered with Whatman filter paper no. 1, again concentrated in the rotary evaporator, the yield was noted and the product obtained was purified using column chromatography.

2.5. Surface tension and critical micelle concentration (CMC) measurements

The surface tension of the purified biosurfactant was measured in an Easy Dyne tensiometer (KRUSS, Germany) using the du Nouy ring method at room temperature. Critical micelle concentration (CMC) was calculated by plotting surface tension of serially diluted biosurfactant solutions as a function of surfactant concentration. Tensiometer determines surface tension readings with the help of an optimally wettable platinum orii suspended from a measuring probe. In the ring method, the liquid is raised until contact with the surface is recorded. The sample is then brought down again so that the film produced beneath the liquid is stretched for the maximum force which is used to estimate the surface tension of the liquid. The instrument was calibrated against de-ionized water and after each reading, washed with de-ionized water, acetone and heated till red hot in a Bunsen burner. The readings were taken in triplicates and average was considered.

2.6. Emulsification activity

The emulsification activity was checked with the cell free supernatant obtained after the 120 h of the fermentation. To check the emulsification activity, 2 mL of the hydrocarbons (kerosene, refined oil, coconut oil, and n-dodecane) were added to 2 mL of the cell free supernatant in a test tube and vortexed for 2 min at high speed. The emulsification activity was measured by the height of the emulsion layer divided by the height of the total solution.[21]

2.7. TLC-Column Chromatography

The crude biosurfactant was analyzed by thin layer chromatography (TLC) using silica gel plates (Silica gel 60, F_{254}, Merck, Germany). The chromatograms were developed by using chloroform, methanol (9:1) solvent system and visualized with the reagents (i) iodine vapors and (ii) 5% sulfuric acid. Further the crude biosurfactant was purified using column chromatography. For this purpose, column (60×2.5 cm) was packed with 500 g silica gel (60-120) with wet packing using chloroform. 500 mg of the recovered biosurfactant was dissolved in the minimum amount of chloroform and loaded into the column, dry silica and cotton was placed after pouring the sample into the column, in order to avoid the disturbance of silica while running the solvent. The compounds were eluted in a stepwise fashion with chloroform: methanol in varying ratios (100, 99:1, 98:2, 97:3…..90:1, 80:20, 70:30, 60:40, and 50:50). In order to obtain the purified fractions, TLC of each fraction was performed by developing in the solvent system chloroform: methanol (9:1) and visualizing with iodine vapors and 5% sulfuric acid. All the fractions with the same pattern of spots were pooled and dried by rotary evaporation to obtain the purified fractions.

2.8. FTIR

The purified biosurfactant was analyzed by FTIR spectroscopy and the spectrum was recorded in an FTIR system (Nicolet, USA) using KBr disc.

2.9. NMR

The biosurfactant was subjected to further analysis with the help of NMR spectroscopy. All the ^{1}H, ^{13}C NMR, HSQC, COSY, HMBC spectra was recorded on Ultrashield 400 NMR spectrometer (Bruker, Germany) equipped with a 5 mm multinuclear inverse probe head. Preliminary data processing was carried out with Bruker software, TOPSPIN 3.2. The NMR samples were prepared immediately prior to NMR analysis by dissolving the purified biosurfactant (15 mg/ 0.6 mL) in deuterated chloroform.

2.10. LC-ESI-HRMS Analysis

The purified biosurfactant was analyzed using a Thermo-Finnigan LCQ ion-trap MS (Arcade, New York, USA) using the syringe-infusion pump. The collected samples were dissolved in acetonitrile and infused in the LC-ESI source. An ESI spectrum (positive mode) was recorded from *m/z,* 50-1500. The MS/MS profiles were obtained by collision-induced dissociation (CID) using argon as collision gas. The positive ions of compounds were obtained with capillary at 4200 V, cone 45 V, spray voltage 4.03 kV, spray current 5.2 µA, dry heater temperature 250 °C, nebulizer pressure 1.2 Bar and sheath gas flow rate 7.0 mL/min. The fragmentation was achieved with the collision energy at 18 and 20 V.

2.11. 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity

The crude biosurfactant was observed for its anti oxidant properties by performing DPPH scavenging activity. The DPPH scavenging activity was determined with the help of method given by Yalcin and Cavusoglu[22] with slight modifications. In this method, the stable DPPH radical was used as a reagent and ascorbic acid was used as standard. The aliquots of different concentration *viz.* 100 µL from 1-5 mg/mL, of biosurfactant and standard ascorbic acid were added to 3.0 mL of the 0.004% (w/v) methanolic solution of DPPH. The samples were incubated for 30 min in dark at the room temperature and absorbance at 517 nm was determined against a blank in a UV-Visible Spectrophotometer (SHIMADZU, UV 1800). The percentage inhibition (I %) of free radical DPPH was evaluated using the following formula.

$$I\ \%\ [\text{DPPH radical}] = [(A_c - A_s / A_c)]$$

Where, A_s= absorbance of the sample and A_c= absorbance of the control. IC_{50} (the half maximal inhibitory concentration) was also determined. IC_{50} denotes to the concentration of sample required to scavenge 50% of the DPPH free radicals.

3. Results and Discussion

3.1. Characterization of the strain

The fungal strain was characterized by translation elongation factor (TEF) and was found to be *Fusarium proliferatum*. The strain was found to reduce the surface tension to 36.6 mN/m. The TEF gene sequence data of the *Fusarium proliferatum* strain was sequenced using a ABI PRISM ® Big Dye Terminator Cycle sequencing. The TEF gene sequence data is as follows:

GTCGACTCTGGCAAGTCTGATGATCTGTGAGTACTACCCTGGACGATGAGCTTATCTGCCATCGTGATCCTGACCAAGATCTGGCGGGG
TACATCTTGGAAGACAATATGCTGACATCGCTTCACAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCATCGAG
AAGTTCGAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTCTGCCCACCGATTTCACTTGCGATTCGAAACGTGCCTGCTACCCCGCT
CGAGACCAAAATTTTTGCGATATGACCGTAATTTTTTTGGTGGGGCATTTACCCCGCCACTCGAGCGATGAGCGCGTTTTTGCCCTTTCCT
GTCCACAACCTCAATGAGCGCATTGTCACGTGTCAAGCAGCGACTAACCATTCGACAATAGGAAGCCGCTGAGCTCGGTAAGGGTTCCT
TCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGCTCTCTGGAAGTTCGAGACTCCTC
GCTACTATGTCACCGTCATTGGTATGTTGTCGCTCATACCTCATCCTACTTCCTCATACTAACACATCATTCAGACGCTCCCGGTCAC

3.2. Biosurfactant characteristics

The effect of *ultrasonication* on enhancement of the production of biosurfactant was studied and it was found that the yield of biosurfactants increased up to 30% on ultrasonication of the media for 30 min before solvent extraction. This enhancement in the production of biosurfactant explained from the fact that biosurfactant produced might be intracellular in nature and is liberated into the medium after the cell rupture during sonication.[23,24] Further, ethyl acetate and methanol was the best solvent system for the optimal extraction of biosurfactants by *Fusarium proliferatum.* The biosurfactant collected was dark brown honey like in color and purified by column chromatography.

3.3. Surface tension and CMC measurements

The surface tension and CMC measurements were determined for the pure biosurfactant. The de-ionized water used for calibration purposes had the surface tension, 71.2 mN/ m. The surface tension and CMC of the purified biosurfactant were calculated to be 36.6 mN/m and 0.33 mg/mL respectively. The CMC value was determined by the plot of surface tension with varying concentration of purified biosurfactant as given in Fig. 1. The biosurfactant produced by *Candida lipolytica* grown on industrial residue lowered the surface tension to 32 mN/m, while the biosurfactant produced by *Nocardiopsis* sp. and *Candida lipolytica* grown on olive oil and canola oil reduced the surface tension to 29 and 30 mN/m respectively.[8,25,26]

3.4. Emulsification Activity

The biosurfactant produced was able to emulsify various hydrocarbons *viz.* coconut oil, refined oil, kerosene, n-dodecane. Among all the hydrocarbons, coconut and refined oil were the best substrates and n-dodecane was the poorest. The ability of biosurfactants to emulsify coconut and refined oil suggests its potential applications as cleaning and emulsifying agents in the food industry. The emulsification order of hydrocarbons by the culture broth of *Fusarium proliferatum* was in the order coconut oil= refined oil (95%) > kerosene (90%) > n-dodecane (43%). The results show the biosurfactant could

emulsify different hydrocarbons which confirmed their applicability against different hydrocarbon pollution, such that it enhances the availability of the recalcitrant hydrocarbons.[21]

3.5. TLC- Column Chromatography Analysis

The TLC analysis performed for the crude extract showed the presence of many compounds after charring with 5% sulfuric acid and iodine vapors. The product obtained was purified using column chromatography as detailed in section 2.7 TLC-Column Chromatography. Light brown colored viscous compound was isolated in 15 mg amount. The pure biosurfactant thus isolated showed surface active properties. The purity of the compound was checked by performing TLC analysis which showed a single spot at R_f value 0.66 (chloroform: methanol, 9:1).

3.6. FTIR analysis

The functional groups present in the purified biosurfactant were identified on the basis of FTIR spectra recorded in the spectral region of 4000-400 cm^{-1}. In Fig. 2, a strong band at 3409 cm^{-1} occurred due to stretching vibration of the N-H group. The peak at 3077 cm^{-1} corresponded to the presence of vinyl =C-H- group. The symmetric stretching vibrations of -CH, $-CH_2$ and $–CH_3$ occurred in the region 2923-2852 cm^{-1} in line with the previous results reported by Biria et. al.[1] The band at 1718 and 1508 cm^{-1} indicated the stretching and bending vibrations of secondary amide group. The band at 1641 cm^{-1} could be assigned to the stretching vibrations of the imine group (-C=N-). The presence of bending modes in the region 1463- 1377 cm^{-1} indicates the presence of methyl and methylene groups which is similar to the results reported by Hazra et. al.[16] The strong bands at 992 and 909 cm^{-1} show the bending vibrations of monosubstituted double bonds (vinyl) of alkenes. The absorption band at 720 cm^{-1} indicates the presence of *cis* substitution of the double bonds. The above FTIR spectral data demonstrate the presence of functional groups amide, imine, alkane and alkene (wave numbers 3409, 1641, 2923-2852 and 992-909 cm^{-1} which is similar to the results reported by Maneerat et. al.[27]

3.7. NMR analysis

The structure of the purified biosurfactant was elucidated using ^1H, ^{13}C NMR, HSQC, ^1H -^1H COSY, and HMBC experiments. The ^{13}C NMR (Fig. 3) showed 22 peaks, in which seven signals appeared in the downfield region of the spectrum which indicated olefinic carbons (δ_c 114.0, 115.9, 123.5, 124.0, 135.1, 139.2, 151.8) and fifteen signals in the upfield region of the spectrum which indicated the methyl and methylene carbons (δ_c 14.1, 22.7, 28.6, 29.1, 29.3, 29.5, 29.6, 29.6, 29.7, 30.2, 31.6, 31.9, 33.8, 34.2, 34.7). Combined analysis of ^1H, ^{13}C NMR, and HSQC indicated the presence of seven signals in the olefinic region, one was quaternary of imine moiety, and remaining six signals indicated the presence of three double bonds.

The ^1H NMR (Fig. 4) indicated the presence of six methyl signals at δ 0.8 (t, *J*=6.64) which appeared as triplets suggesting their attachment with methylene protons. Another five proton signal was overlapped with the methylene

signals at δ 1.2. Forty three methylene signals appeared in the region δ 1.2-1.4 suggesting the long chain hydrocarbons. Ten methylene signals appeared in the region δ 1.5 and 2.0 suggesting their allylic nature. A doublet at δ 7.3 (*J*=2.4) suggested the attachment of protons with an electron withdrawing functional group. At δ 7.1 (*J*=8.2) and 6.6 (*J*= 8.2) doublet of doublet and a doublet showed the presence of -HC=CH- and its attachment somewhere in the proximity of the electron withdrawing group. Two signals at δ 5.8 and 4.9 suggested the presence of isolated olefinic protons in long hydrocarbon chain.

The protons and the carbons present in the ^{1}H, ^{13}C NMR spectrum were correlated with the help of HSQC experiment. Fig. 5 represents the HSQC spectrum and the lines connecting proton and carbon represents the respective proton to carbon ($^{1}H \rightarrow ^{13}C$) attachments.

The correlations between the protons were observed from the ^{1}H-^{1}H COSY experiments (Fig. 6). The ^{1}H-^{1}H COSY spectrum presented correlations between proton signals δ 7.3/ 7.1 (19-H/18-H) and at δ 7.1/ 6.6 (18-H/17-H) and at δ 5.8/4.9 (21-H/ 16-H) and δ 2.0/5.8 (13-H/21-H) which suggested the presence of 19-18, 18-17, 16-21, and 13-21 proton connectivities.

The HMBC spectrum showed three types of proton to carbon ($^{1}H \rightarrow ^{13}C$) 19-H/18-H, 13-H/16-H, 20-H/17-H correlations. Fig. 7 represents the HMBC spectrum and lines drawn shows the connections.

Combined analysis of the FTIR, ^{1}H, ^{13}C NMR, HSQC, ^{1}H -^{1}H COSY, and HMBC experiments have led to the tentative structure represented in Figure 8. The IUPAC name of this compound generated by ChemBioDraw Ultra 12.0 was (Z)-5,9,18-trimethyl-N-((Z)-N'-((Z)-5-methyltetradec-2-en-1-yl)-N-((2Z,4Z,9Z,11Z,17Z)-5,11,18-trimethyltriaconta-2,4,9,11,17-pentaenoyl)carbamimidoyl) dotriacont-7-enamide. The proposed structure correlates to the composition of fatty acid chains of rice-bran which gets utilized in the biosynthesis of biosurfactants by the fungus *Fusarium proliferatum.* The rice-bran contained protein, fat and crude fibre contents.[28] The major fatty acids present in rice-bran oil were palmitic, oleic, and linoleic acids, which were in the ranges of 13.9–22.1, 35.9–49.2, and 27.3–41.0%, respectively.[29]

3.8. LC-ESI-HRMS Analysis

Further, support to the proposed structure was obtained through MS studies. The ESI/HRMS of the compound (Fig. 9) gave peaks at *m/z* 871.1974, 701.4782, 634.4374, 605.2707, 475.3173, 453.3367, for the possible fragments as shown in Fig. 10. The exact mass of the tentative structure as calculated in ChemBioDraw Ultra 12.0 was 1236.1963. The fragmentation of the proposed structure was also done in ChemBioDraw Ultra 12.0 and respective peaks were matched with the molecular ion peaks in the ESI-HRMS spectrum (Fig. 9). The parent ion peak was not observed in the ESI-HRMS spectrum due to longer fatty acid chains and higher molecular weight. The fragment ion m/z 870.7816 appeared when m/z 365.4147 gets removed from the molecular weight 1236.1963, which gave M+1 peak at m/z 871.1974 (Fig. 9). Further, the molecular ion

fragment m/z 169.1956 gets removed from m/z 870.7816 and gave another fragment of m/z 701.5859, which showed M+ peak at m/z 701.4782 (Fig. 9). This fragment ion m/z 701.5859 further dissociated into m/z 633.5233, by the removal of the m/z 68.0626 and gave M+1 peak at m/z 634.4374 (Fig. 9). The fragment ion m/z 633.5233, again dissociated into m/z 605.4920 by the elimination of m/z 28.0313, which gave M+ peak at 605.2707 (Fig. 9). Consequently, the fragment ions m/z 26.0157 and 127.1487 gets removed from the m/z 605.4920 to produce another fragment m/z 452.3277 which gave M+1 peak at m/z 453.3367 (Fig. 9). The base peak at m/z 475.3173 (Fig. 9) is supposed to be due to the removal of one chain in the structure of the purified biosurfactant as shown in Fig.10.

3.9 DPPH assay results

The anti-oxidants are capable of seizing the free radical chain reactions. If the biosurfactant possess the anti-oxidant activity the absorbance of the solution decreases and thus IC_{50} (the half maximal inhibitory concentration) was determined. The DPPH activity of the crude biosurfactant was checked using ascorbic acid as standard. The IC_{50} of the standard was 0.056 mg/mL and that of the biosurfactant produced was 18.81 mg/mL. The results suggest that the biosurfactant produced by *Fusarium proliferatum* grown on rice-bran possessed anti-oxidant activity to a lesser extent as compared to the surfactin biosurfactant produced by *Bacillus subtilis.* The IC_{50} of the surfactin biosurfactant reported was 0.25 mg/mL when BHT (butylated hydroxyl toluene) was used as the standard.[22]

4. Conclusions

The fungus *Fusarium proliferatum* isolated from rice-bran oil industry sludge was capable of producing biosurfactant when grown on the residues of the same. The biosurfactant was purified using column chromatography and determined to be a enamide. This new biosurfactant can be explored for its more applications in the future as it was capable of reducing the surface tension of the distilled water to a good extent as well as possessed excellent emulsification activities. Even the crude biosurfactant possessed antioxidant potential and it increases as the concentration of the biosurfactant increases in the solution.

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Fig. 1.

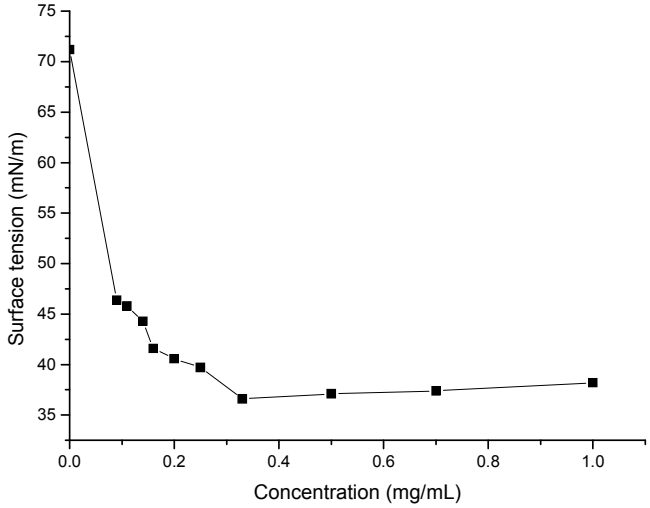
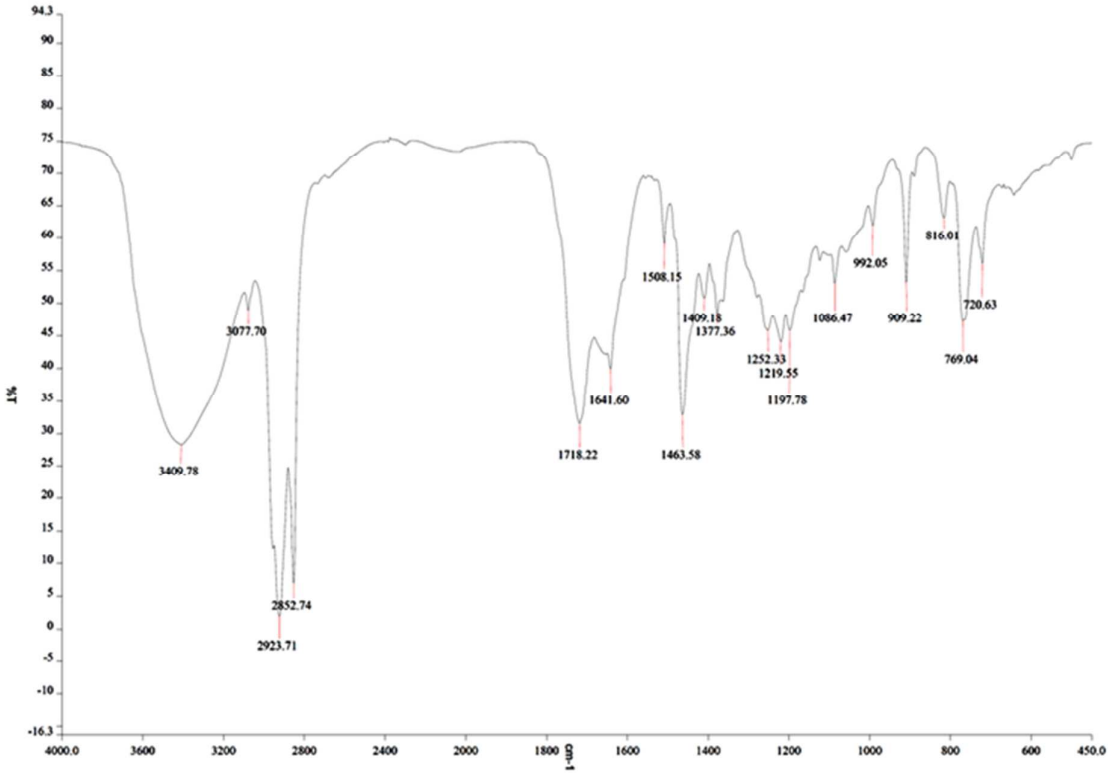


Fig. 2.



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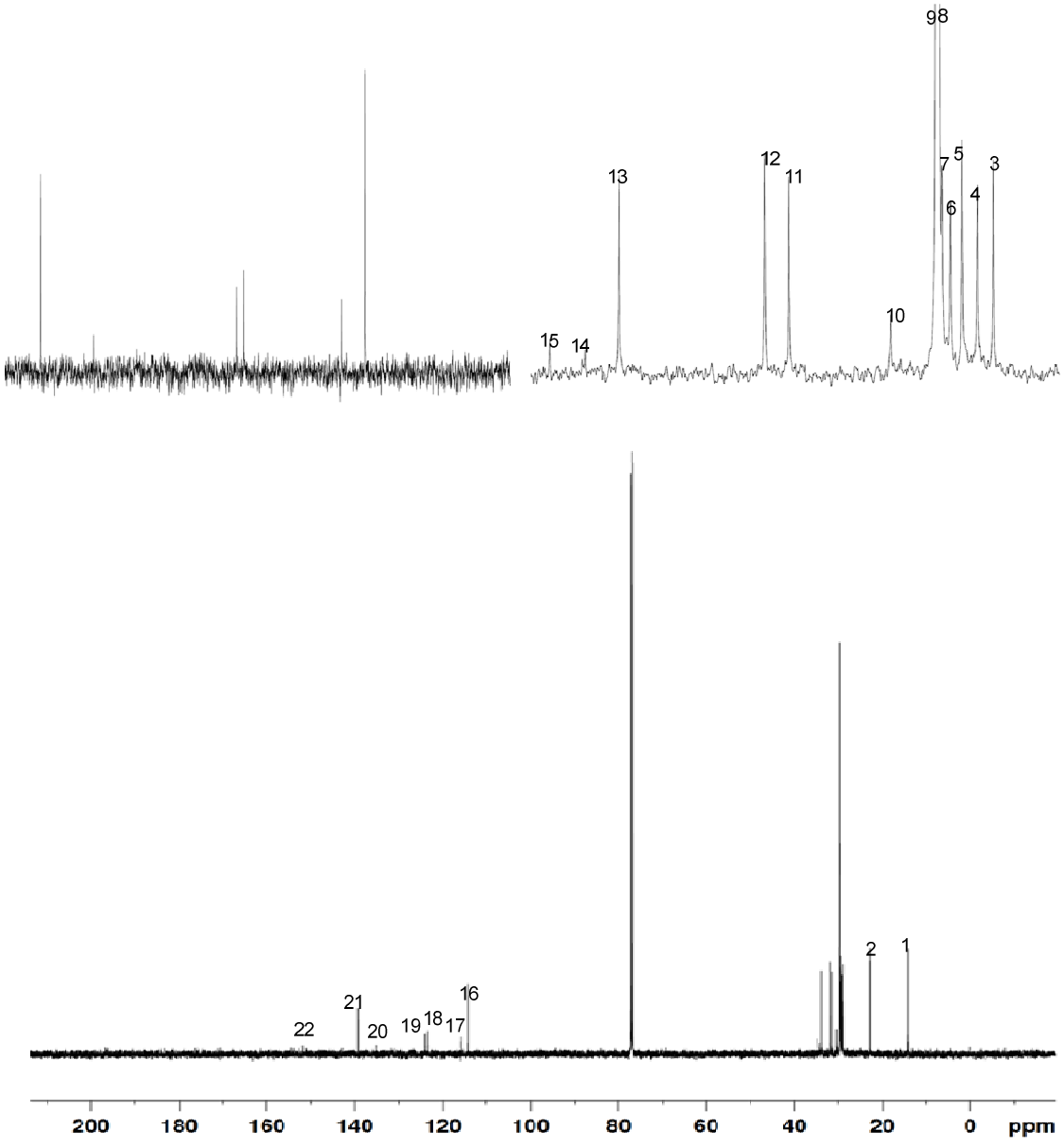

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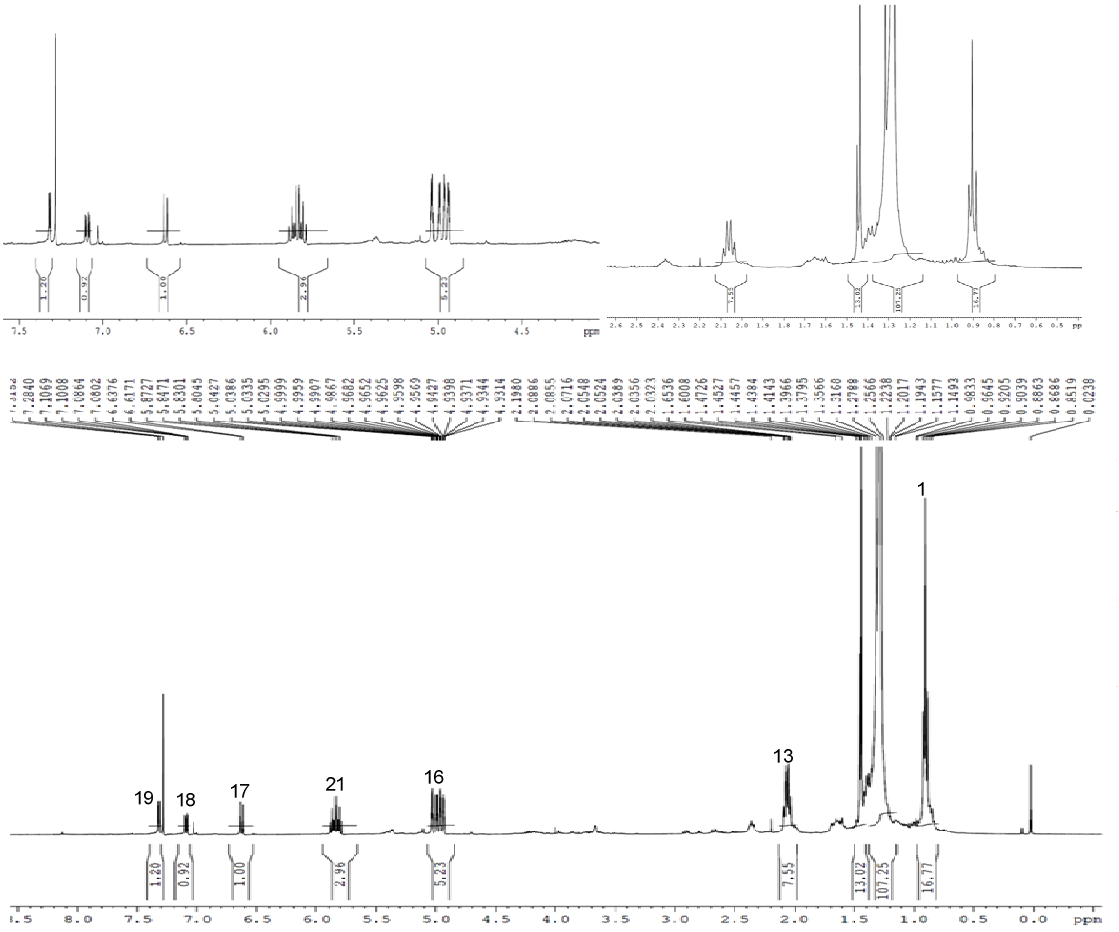

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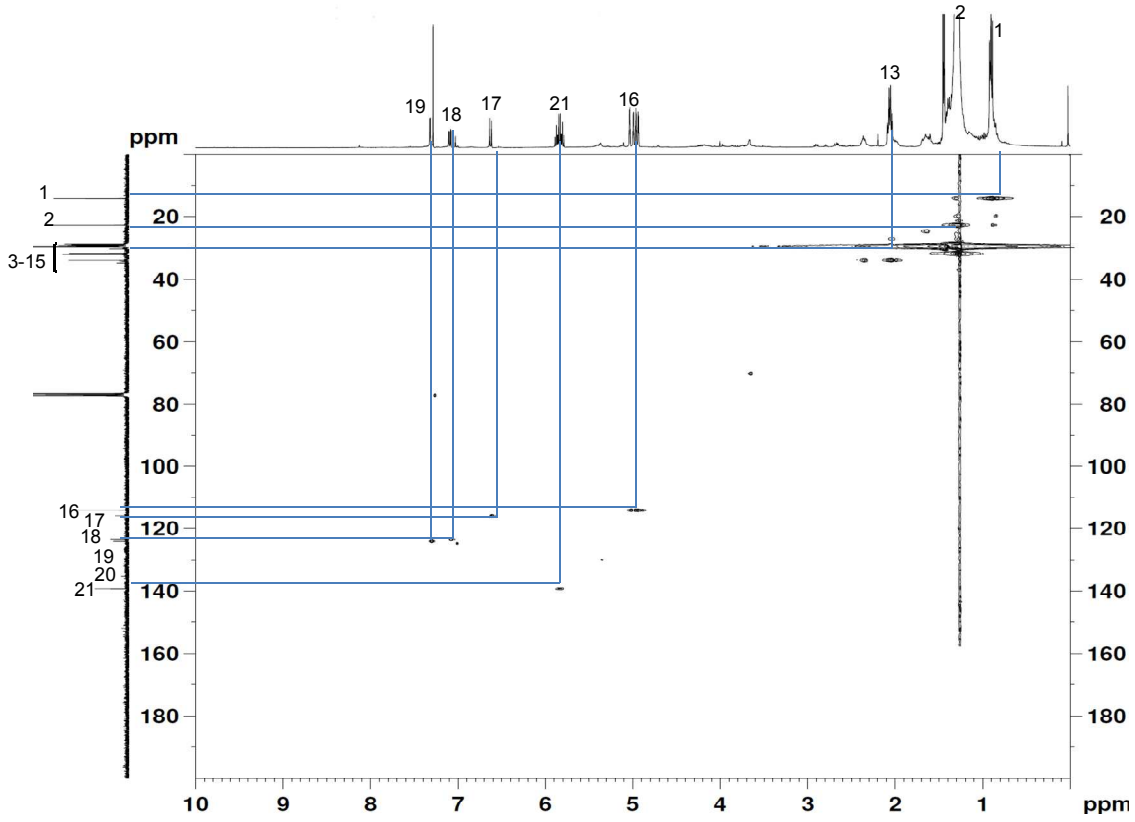

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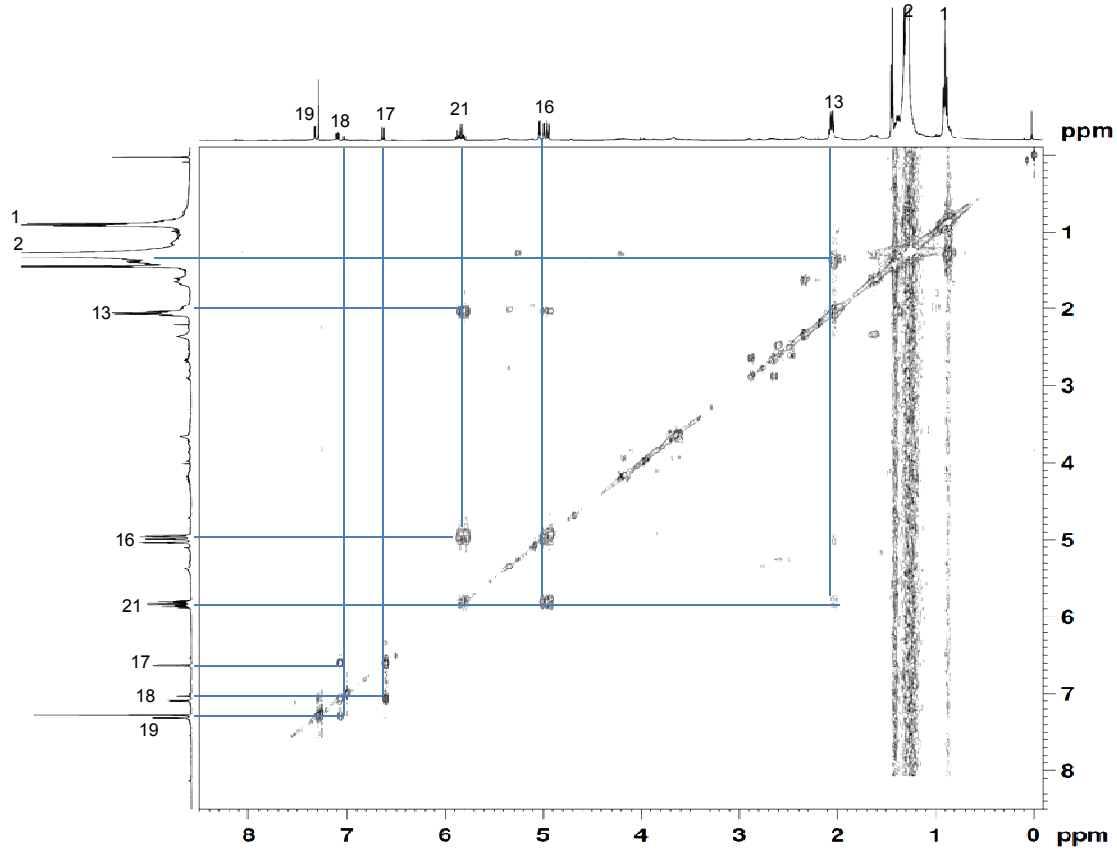

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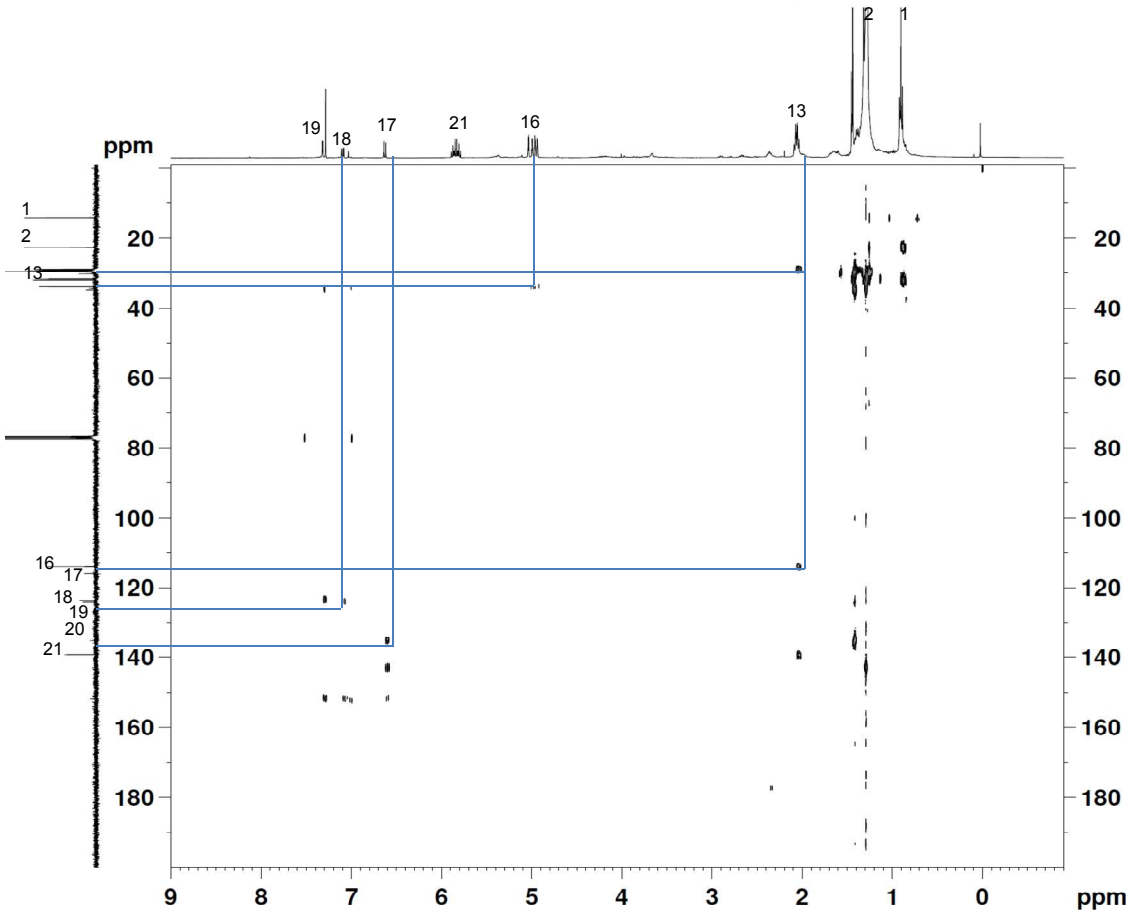

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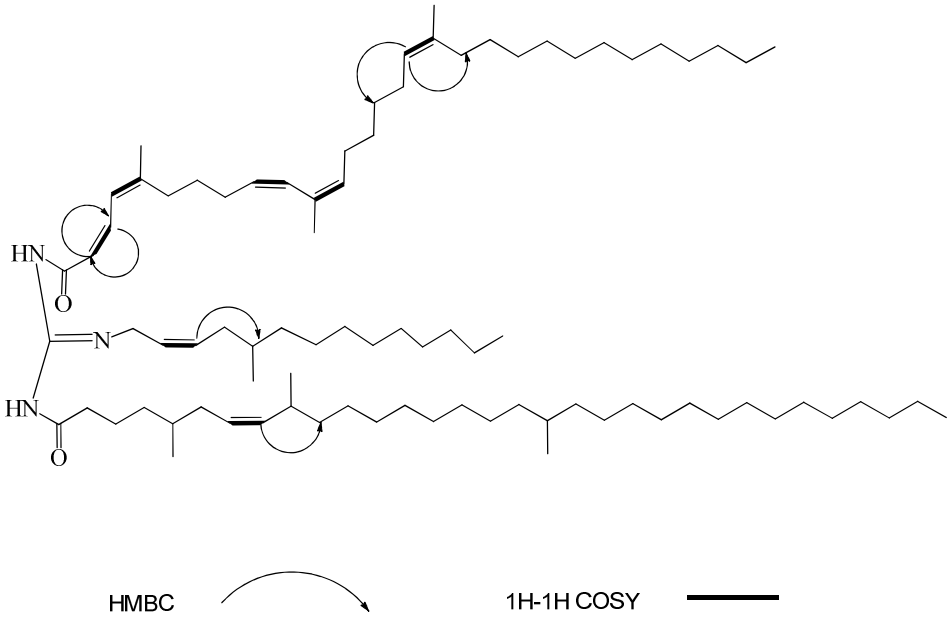

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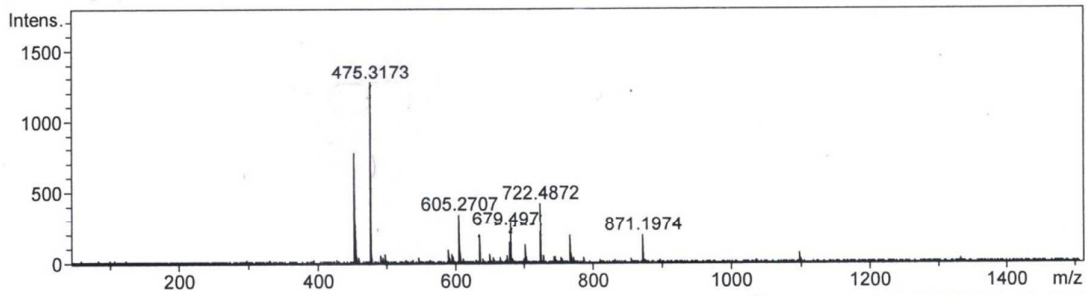

Fig. 10.