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CHARACTERIZATION OF BIOSURFACTANTS PRODUCED BY BACILLUS SUBTILIS USING FRESH AND WASTE COOKING OIL ENRICHED MEDIUM

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(1-hydroxyethyl)amino)methyl heptanoate.

ABSTRACT

This study aims to compare and analyze the chemical structures of bacterial biosurfactants produced by an indigenous *Bacillus subtillis* strain cultured in minimal salt medium (MSM) enriched with fresh cooking oil and used cooking oils that are cheaper and easy to obtained in huge quantities. Optimization of biosurfactants was successfully conducted in two MSMs enriched with 2% (v/v) fresh cooking oil and 2% (v/v) waste cooking oil respectively. The initial pH of growth medium was fixed at pH 7.64 and the whole fermentation process was maintained in a constant temperature of 30 °C. Chemical structures analysis of biosurfactants produced at 1L scale in bioreactor were conducted using spectroscopic methods such as Nuclear Magnetic Resonance (NMR) and Fourier-Transform Infrared spectroscopy (FTIR). The chemical compound isolated from the biosurfactant produced from the waste cooking oil was pentyl 2-ureidobut-3-enoate (1) while (1-hydroxyethyl)amino)methyl heptanoate (2) was isolated from the biosurfactant produced from fresh cooking oil. This study demonstrated the ability of *Bacillus subtilis* to produce a low-cost biosurfactant characterized as lipopeptide.

Contribution/ Originality: This study used cheaper yet less toxic cooking oils as substrate for biosurfactant production. Furthermore, we also successfully identified the impact of used and fresh cooking oils on the rate of bacterial growth, rate biosurfactant productions and chemical structures of biosurfactants produced using FTIR and NMR method.

1. INTRODUCTION

Biosurfactants are common surface-active secondary metabolites of microorganisms, with amphipathic properties due to the presence of both hydrophilic head and lipophilic hydrocarbon tail [1]. Commonly used as emulsifiers and additives, biosurfactants such as lectin have been found to improve the taste and flavor of foods without compromising consumer health [2].

In recent years, some microbial biosurfactants were also successfully being developed into bioactive compounds possessing either antibacterial or antifungal properties.

Both carbon and nitrogen sources used for microbial growth can caused variation within biosurfactants molecular structures produced by the same bacterial strains [3]. To date, majorities of microbial biosurfactants were produced from growth medium that utilizing agro-industrial wastes, including bran, straw of wheat and rice, hull of soy, corn and sugarcane molasses as main carbon source [4-6].

Unlike synthetic surfactants derived from petroleum, microbial biosurfactants are more environmentally friendly due to their low toxicity and biodegradable nature [4, 7].

The main objective of this study is to characterize the molecular structures of biosurfactants produced by *Bacillus subtillis* growing in two different types of cooking oils: fresh cooking oil and waste cooking oil using NMR and FTIR analysis. These data are essential to optimize the subsequent pilot study for larger scale biosurfactant production.

2. MATERIALS AND METHODS

2.1. Bacterial genomic DNA Extraction

For DNA isolation, bacterial cells were obtained from one-day old culture growing in LB broth. Cell pellets were then collected through high speed centrifugation before suspended in 20 mM Tris-HCL, 2mM EDT A, 1% Triton X-00, 20 mg/mL Lysozyme or 0.2 mg/ml lysotaphin, pH 8.0 (10^9 cells add 100 µl). The mixture was then incubated at 37°C for 60 mins. Bacterial genomic DNA was extracted using Genomic DNA Isolation Kit (Protech Technology, Taiwan).

2.2. 16S rDNA Sequencing

PCR amplification for bacterial 16S rDNA was carried out using universal PCR primers, 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-TAC GGT TAC CTT GTT ACG ACT-3') in a reaction mixture that contains 3.0µL of 10X PCR buffer, 0.3µL of dNTP mix, 1.0µL each of PCR primers, 0.3µL of *Taq* DNA polymerase, and 0.3µL of bacterial genomic DNA. Sterile ultrapure water was added to the reaction mixture to achieve a final mixture volume of 30µL. PCR was then conducted in Takara PCR Thermal Cycler with an initial denaturation of 94°C for 5 mins, followed by 40 repeating cycles of denaturation at 94°C for 30 sec, primers annealing at 55°C for 30 sec, and DNA extension at 72 °C for 2.3 mins. The PCR was completed with a final extension of PCR products at 72 °C for 5 mins. PCR product obtained in this study was sent to Mission Biotech for sequencing and DNA sequences were then BLAST with data deposited in NCBI GenBank.

2.3. Initial Biosurfactant Production

Initially, bacterial isolates were cultured in a 100 mL minimal salt medium (MSM) containing (w/v) 0.2 g/L NaCl, 2.0 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.1 g/L $CaCl_2.2H_2O$, 2.5g/L $NaNO_3$, 3.0 g/L yeast extract and 10.0 g/L CMC. Following that, another six replicates of these 100 mL MSM were prepared for biosurfactant production. For the first three MSM, 1%(v/v) fresh cooking palm oil was added as main carbon source while the remaining three MSM were added with 1%(v/v) waste cooking oil, also serving as main carbon source for the bacteria isolate.

Subsequently, 10 mL of bacterial culture from previous MSM were added into each of these six MSM before incubated at 30°C for 4 hours. Bacterial growths in all the MSM were monitored at every 1-hour interval using spectrophotometer.

2.4. Upscale Production of Biosurfactants

Following the initial production, a total of 200 mL of bacteria culture were collected and transferred into two new 1L of MSM, each containing 2% (v/v) fresh cooking oil and 2% (v/v) waste cooking oil respectively. All these

culture mixtures were then transferred into two separate 5 L bioreactors with constant temperature set at 30°C, 200 rpm and pH of 7.64. Bacterial growth was maintained and observed based on OD readings of spectrophotometry. Fermentation was stopped once the OD value has stop rising.

2.5. Oil Spreading Assay

Oil spreading assay was performed to detect the presence of crude biosurfactants during the upscaling production. This assay was carried out by adding one drop of the crude biosurfactant onto the surface of distilled water covered with 20 µL cooking oil in a Petri dish. The biosurfactant was detected by observing the presence of clear zones produce on the cooking oil.

2.6. Extraction of Crude Biosurfactants

Following the upscale fermentation, culture medium within the 5L bioreactor were then collected. At first, growth mediums were centrifuged at 12000 rpm at 25 °C for 10 mins to remove unwanted bacterial cells. The biosurfactants were then precipitated by acidification process, until the pH of mixture reached 2.0. Then, the precipitates were further collected through centrifugation. Eventually, these 200 mL of the cell-free broth from both bioreactors were collected and extracted twice with an equal volume of ethyl acetate. The solvent was further removed by reduced-pressure distillation and the dried product was washed with distilled water.

2.7. Structural Characterization of Biosurfactants

The infra-red light was used for the irradiation of molecules that gives the characteristic frequencies of every molecule for the identification of chemical compound. Infra-red spectra give information about functional groups of a molecule [8]. Perkin Elmer FTIR spectroscopy analysis was performed to evaluate if the biosurfactant induces modifications on cell surface functional groups [9].

Structural elucidation was performed using FT-NMR Bruker 400 MHz Nuclear Magnetic Resonance (NMR) spectroscopic analysis. It was based on transitions in atoms and chemical shifts in their frequency of absorption. It will allow more accurate structure and purity analysis than IR spectroscopy. Each sample of biosurfactant was dissolved in deuterated solvents. The supernatant was inserted into NMR tube after centrifugation 400 µL for the analysis [10].

3. RESULT AND DISCUSSION

3.1. Identification of Bacterial Strain

In this study, 16S rRNA PCR fragments with molecular sizes of 1500 bp Supplementary Figure S1 was successfully amplified by using primers 8F and 1492R. No smears were detected in the agarose gel indicating high purity of PCR product obtained from the reaction protocols used in this study.

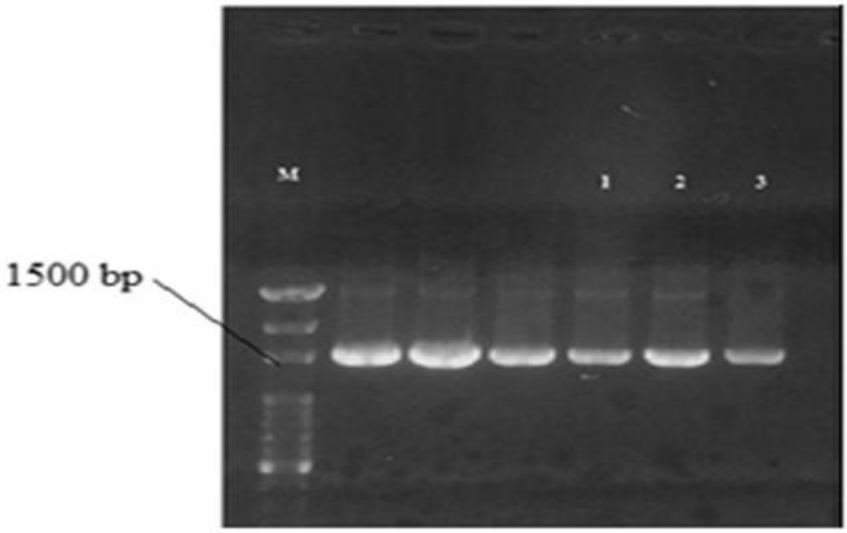


Figure-S1. PCR amplicons amplified with 8F and 1492R primers.

3.2. Bacterial Growth Rate Calculation

Prior to fermentation in bioreactors, OD readings was collected to monitor the bacterial growth in initial MSM culture. This essential stage will provide crucial information for optimizing the fermentation duration for maximizing biosurfactant production in later stages. Growth data Supplementary Table S1 indicated an exponential growth of bacteria only after 5 hours of initial fermentation. The total amount of biomass (in gram) produced during this stage is approximately 0.4440 g.

Meanwhile, for bacterial growth in the bioreactors, enriched with either fresh cooking oil or waste cooking oil, it was observed that there was continuous bacterial growth even after four hours of incubation period Supplementary Table S2. The amount of biomass obtained from the fermentation in the bioreactors were approximately 0.2456g. Growth data suggested at least four hours of incubation period are required for *Bacillus subtilis* used in this study to produce biosurfactant.

Besides the continuous OD readings, pH of growth medium in the bioreactors were also being continuously monitored Supplementary Table S3. An initial 3-day (Day-1 to Day-3) pH monitoring indicated a stable decrement of pH, suggesting the presence of fatty acid and biosurfactants within fermentation medium.

Table-S1. OD Value of bacterial growth taken per hour.

Time (hours)	1	2	3	4	5
OD Value	0.397	0.384	0.836	3.22	9.760

Table-S2. OD Value of biosurfactants taken per hours during four hour scaling up process.

Time (hours)	1	2	3	4
OD Value	0.108	0.141	0.237	0.418

Table-S3. pH readings recorded during biosurfactant production (day-basis).

Time (days)	1	2	3
pH Value	7.64	6.87	6.86

3.3. Effect of Different Carbon Sources for Biosurfactant Production

Interestingly, it was observed that the used of waste cooking oil as main carbon source did yielded much higher amount of biosurfactant (3.4887g) as compared to fresh cooking oil (0.4440g). This finding is due to the presence of contains higher amount of saturated fat in the waste cooking oil that serve as carbon source for bacterial growth.

3.4. Effect of Additional Nitrogen Sources in Biosurfactant Production

Nitrogen sources is also another important supplement for bacterial growth and enhancing the production rate of biosurfactants. In fermentative processes, the C:N ratio will affect the build-up of metabolites [11]. High C:N ratio (low nitrogen levels) will limit the bacterial growth, leading to production of secondary metabolites. In contrast, excessive nitrogen leads to the synthesis of cellular material used in biosurfactant production [12]. In this study, $NaNO_3$ was used and proven to be an effective limiting agent for enhancing the cellular metabolism for biosurfactant production.

3.5. Oil Spreading Assay

Oil spreading assay is important for detecting the presence of biosurfactants. The oil displacement area is directly proportional to the surface-active compound in the solution [13]. The assay conducted in this study indicated a positive result for biosurfactant presence in culture medium using both fresh and waste cooking oils. Presence of clear zones in the oil droplets spread on the water surface, together with emulsification effects Supplementary Figure S2 suggested that the biosurfactants produced by *Bacillus subtilis* used in this study were highly effective in dispersing hydrocarbon-based contaminants.

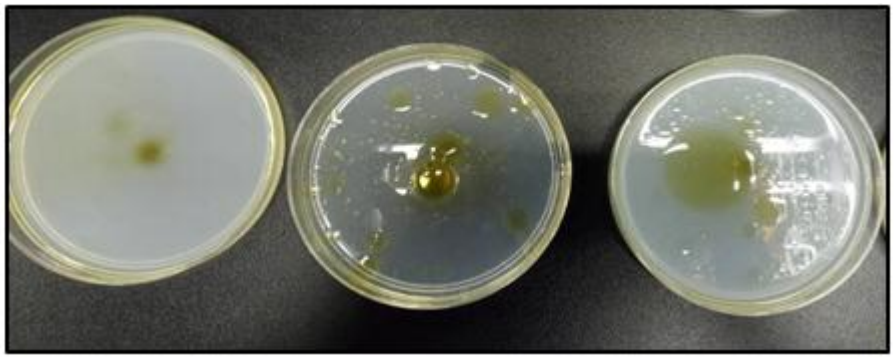
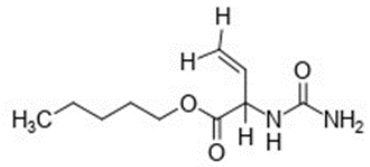


Figure-S2. Positive results of oil spreading assay showing clear zone formation and emulsifying effects on the oil droplets spread on distilled water in Petri dishes.

3.6. Physical Properties of Biosurfactants

The biosurfactants Figure 1 obtained from waste cooking oil has slightly darker brown colour compared to that from fresh cooking oil but both have strong smell and sticky in texture. The biosurfactants are very polar and fully dissolved in water but partially dissolved in acetone-methanol.



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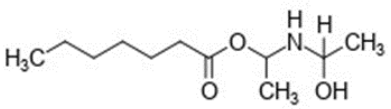

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Figure-1. Suggested biosurfactant **1–2** obtained from the waste and fresh cooking oil.

3.7. Structural Characterization of Suggested Biosurfactant Obtained from the Waste Cooking Oil

The FTIR absorption spectra Supplementary Figure S3, the strong absorption bands in the region 3291.38 cm^{-1} was due to the stretching vibrations of –OH group. The absorption peaks at 2921.43 cm^{-1} and 2852.25 cm^{-1} indicated the presence of methylene groups confirming the presence of aliphatic chains. The absorption peaks 1744.51 cm^{-1} confirmed the presence of carbonyl (-C=O) group. The peak at 1093.38 cm^{-1} revealed the presence of amine (C-NH_2) group and the absorption peak at 1160.49 cm^{-1} was assigned to the stretching vibrations due to C-O in ester linkage. The bands at 1464.61 cm^{-1} and 1415.70 cm^{-1} are due to aliphatic chains.

The ^{1}H NMR spectrum Supplementary Figure S4 revealed obtained from the **1** Figure 1 from waste cooking oil indicated that the purified surfactant was a possible lipopeptide due to the presence of a long aliphatic chain (CH_2 at 1.43-0.75 ppm). The chemical shift at 5.19 ppm was consistent with a proton attached to the C-3 of the hydroxyl fatty acid (3-HFA) residue and indicated that this carbon was attached to amino acid residue by an ester bond Supplementary Table S4. The intense singlet at 3.85 ppm, is similar to ^{1}H NMR spectrum of lipopeptide monoesters reported by other researchers, which suggests the existence of a methoxy group on the Glu or Asp amino residues [14]. The ^{1}H NMR analysis detected an ester carbonyl group at 5.19 ppm, indicating the presence of a lactone ring in the structure of the **1** Figure 1 [15].

Table-S4. The ^{1}H NMR signals and the functional groups of the biosurfactant component (WCO).

δ ,ppm	Functional groups
5.19	Representing ester carbonyl group
3.85	Chemical shifts of methoxy group
3.57	R-C=O-O-C-H
3.48	R-N-H
1.77	R-C=C-C-H
1.43	CH_2 long aliphatic chain
1.16	The proton signals in the region belong to saturated R-CH_2-R chains
1.03	The proton signals of the methyl groups, R-CH_3
0.93	Terminal branching in the fatty acyl chain [-$(CH_3)_2$-CH-].
0.75	The proton signals of the methyl groups, R-CH_3

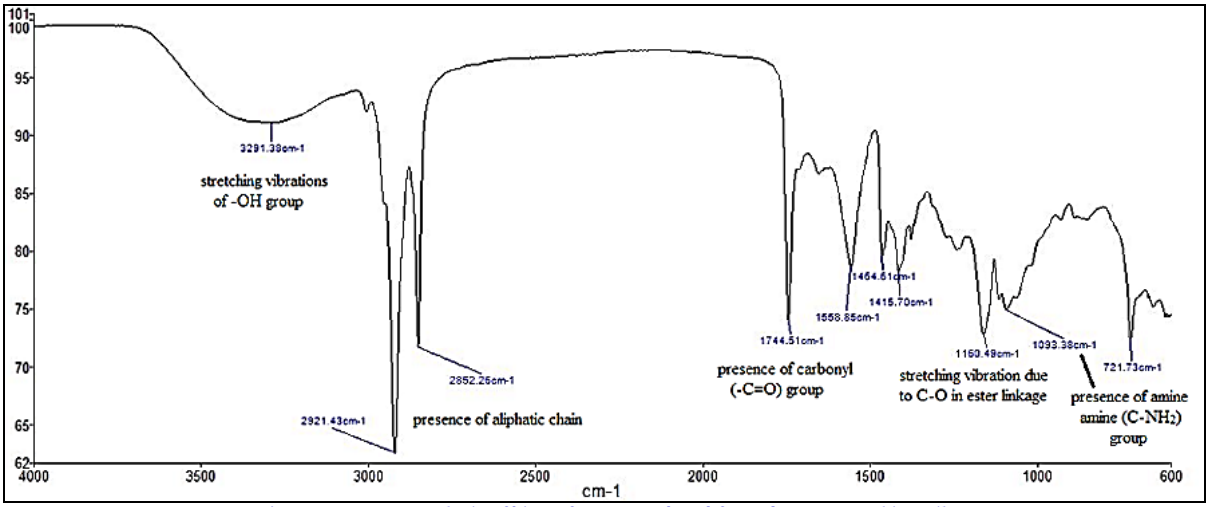

Figure-S3. FTIR analysis of biosurfactant produced from the waste cooking oil.

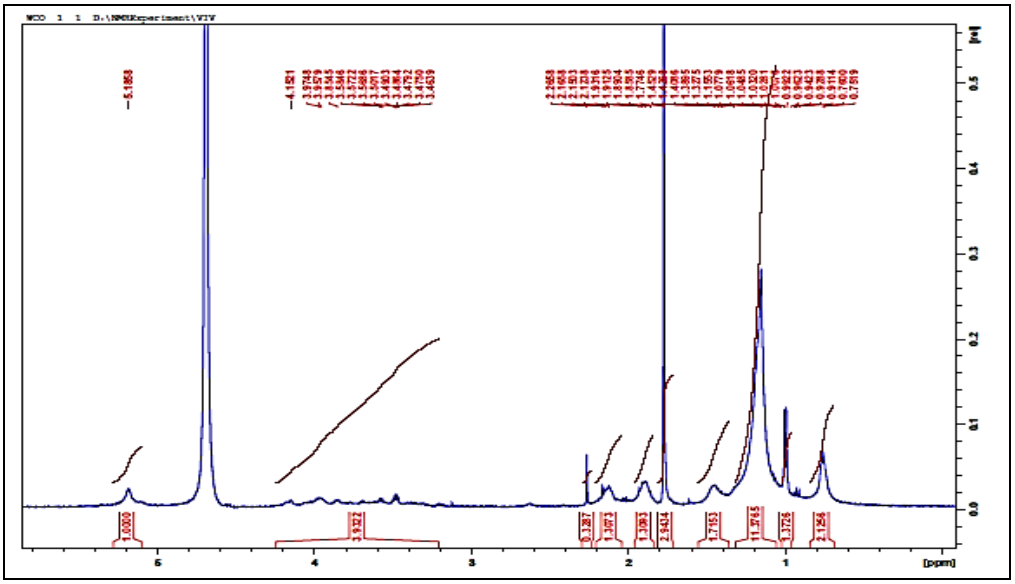

Figure-S4. The 1H NMR signals of biosurfactant from waste cooking oil.

3.8. Structural Characterization of Suggested Biosurfactant Obtained from the Fresh Cooking Oil

In the FTIR spectrum Supplementary Figure S5, the strong absorption bands in the region 3368.82 cm-was due to the stretching vibrations of –OH and –NH groups. The absorption peaks 1587.23 cm^{-1} confirmed the presence of carbonyl (-C=O) group in **2** Figure 1. The absorption peaks at 1351.70 cm^{-1} was appeared due to the presence of alkyl (-CH_3 and –CH_2) groups. The sharp peak at 1050.94 cm^{-1} revealed the presence of amine (C-NH_2) group.

The result obtained with ^{1}H NMR Supplementary Figure S6 further suggested the lipopeptide nature of 2 Figure 1. The alpha-hydrogen (Hαs) of the amino acids showed resonance from 4.7-3.8 ppm. A δ = 1.20 ppm was observed, which indicated a terminal branching in the fatty acyl chain [-$(CH_3)_2$-CH-] Supplementary Table S5. Other multiplets in the upfield region arise as a result of the side chain protons of the amino acids, and remaining spectra confirmed the presence of β-hydroxy fatty acid [16].

In this study, potential biosurfactant bacterial strain *Bacillus subtillis* was identified. The biosurfactant production were optimized using waste and fresh cooking oil. The study aimed at comparing the effect of various carbon and nitrogen sources to the biosurfactant production. The optimization of biosurfactant production was done through upscaling of *Bacillus subtillis* in bioreactor at specific parameter to produce large scale of biosurfactant.

The oil spreading assay was done to test the presence of the biosurfactant. The result showed a positive result for both biosurfactant produced from waste and fresh cooking oil. The pH had shown to give effect to the biosurfactant production which the pH between 5.5 and pH 6.0 gave the best result for the biosurfactant production. Besides, the use of vegetable oils added with molasses gave the highest yield of biosurfactant. On the other hand, sodium nitrate proved to be more effective as nitrogen sources to biosurfactant.

The chemical structure of biosurfactants produced in different substrates were characterized and compared using spectroscopic methods such as Nuclear Magnetic Resonance (NMR) and Fourier-Transform Infrared spectroscopy (FTIR). The chemical compound isolated from the biosurfactant produced from the waste cooking oil was pentyl 2-ureidobut-3-enoate **(1)** Figure 1 while (1-hydroxyethyl)amino)methyl heptanoate **(2)** Figure 1 was isolated from the biosurfactant produced from fresh cooking oil. This study demonstrated the ability of *Bacillus subtilis* to produce a low-cost biosurfactant characterized as lipopeptide.

Table-S5. The ^{1}HNMR signals and the functional groups of the biosurfactant component (FCO).

δ ,ppm	Functional groups
4.15	$(CH_2)_2CH(OH)$
4.01	R-C=O-O-C-H
3.88	R-N-H
3.10	HO-C-H
1.20	The proton signals in the region belong to saturated R-CH_2-R chains

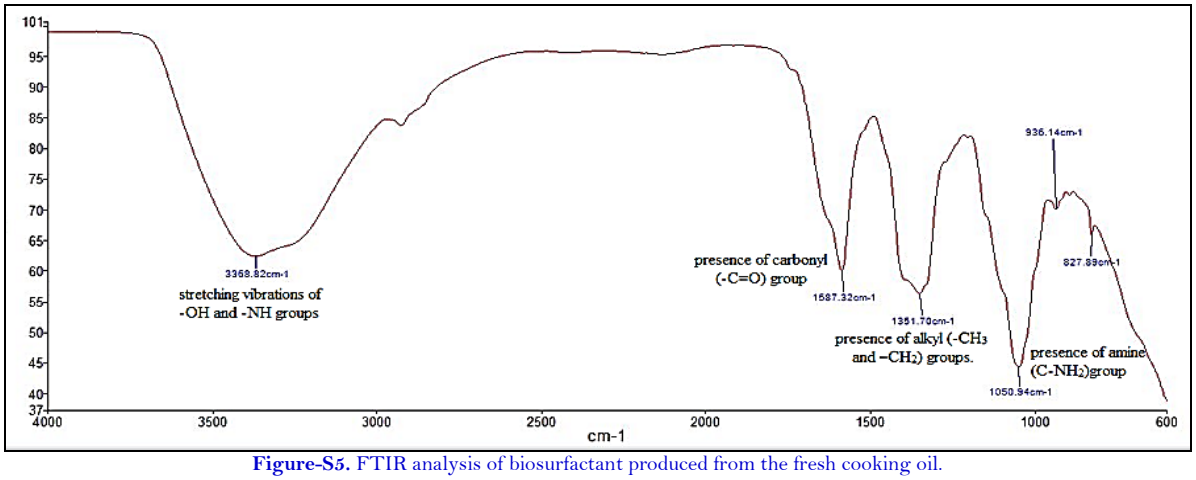

Figure-S5. FTIR analysis of biosurfactant produced from the fresh cooking oil.

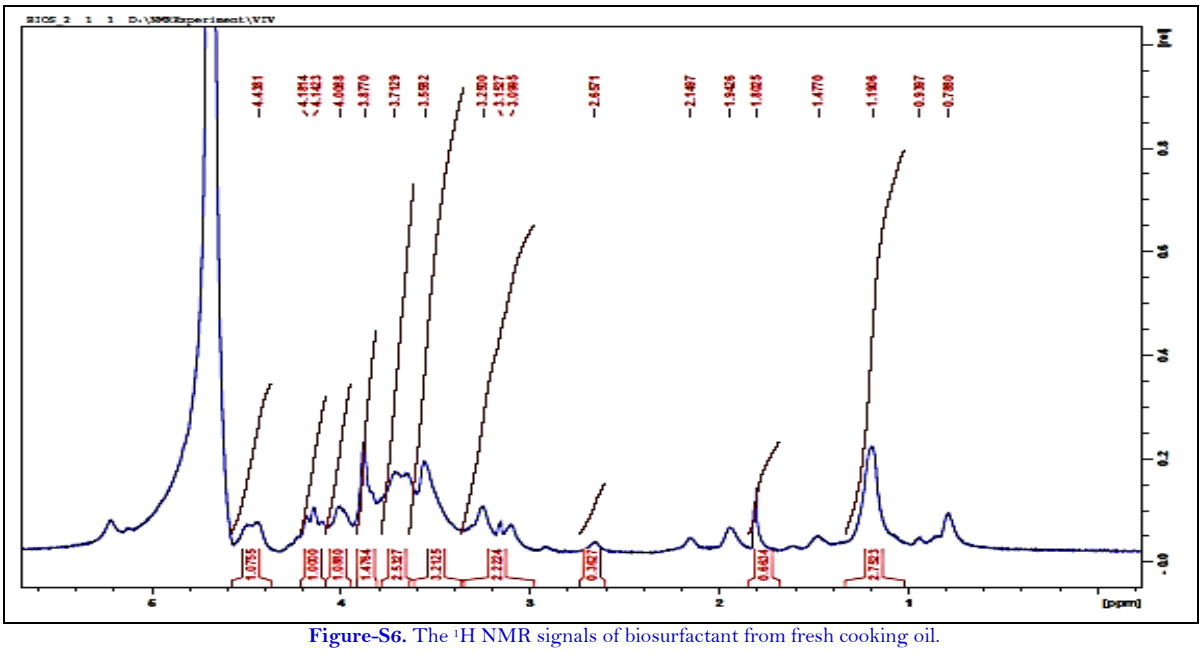

Figure-S6. The ^{1}H NMR signals of biosurfactant from fresh cooking oil.

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