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Co-Utilization of Motor Oil Waste and Sunflower Oil Cake on the Production of New Sophorolipids by *Candida bombicola* NRRL Y-17069.

Mona Mohamed Rashad[1*], Amr Soliman Al-kashef[1] , Mohamed Usama Nooman[1] , Abeer Essam El-Din Mahmoud[1].

[1]Biochemistry Department, Division of Genetic Engineering and Biotechnology, National Research Center, Cairo, Egypt.

ABSTRACT

Production of *Candida bombicola* sophorolipid (SL) was studied using a mixture of motor oil waste and sunflower oil cake as a substrate. In this work, submerged and solid state fermentation (SSF) techniques were used. The highest yield was achieved with SSF giving two fractions methanol fraction (F I) of 4.75 g% and ethyl acetate fraction (F II) of 41.77g%. To optimize the production, time course was studied. The minimum surface tension and critical micelle concentrations (CMC) were obtained at the 12th day of fermentation for both fractions, giving 36.2 mN/m and 45.2 mN/m at CMC of 68.5 mgl^{-1} and 391.4 mgl^{-1}, respectively. Emulsification activity with different hydrocarbons and the stability against pH, temperature, and salinity studies revealed that both fractions showed high tolerance for these environmental factors, suggesting their potential applications in extreme industrial and environmental conditions. The efficiency of the produced SLs for microbiologically enhanced oil recovery (MEOR) was 71.43% and 72.73%, respectively. FTIR and NMR spectroscopy analyses confirmed the structure of the produced compounds.

Keywords: Sophorolipids, *Candida bombicola*, Sunflower oil cake, Motor oil waste, Characterization.

Corresponding Author

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INTRODUCTION

Sophorolipids (SLs) a glycolipids type of biosurfactant that have been produced by yeasts [1]. Each of them is comprised of one sophorose molecule, hydrophilic part, linked to one hydroxyl fatty acid, lipophilic part, by one or two cross lines [2]. The numerous advantages of SLs, such as mild production conditions, lower toxicity, higher biodegradability, better foaming properties, higher selectivity and environmental compatibility have prompted applications not only in the food, cosmetic and pharmaceutical industries but also in petroleum production, oil formulations, environmental protection, and energy-saving technology. SLs found to be useful in hard surface cleaning, automatic dishwashing rinse aid formulations and are thus suitable for use in commercial household products. [3-5].

Although SLs exhibit such important advantages, they have not been yet employed extensively in industry because of relatively high production costs and low produced amount. Moreover, the amphiphilic characteristic of these molecules increased the difficulty of recovering. One possible strategy for reducing costs and polluting effect at the same time is the utilization of alternative substrates such as agro-industrial as well as petroleum industrial wastes [6-11].

The main problem related to use of alternative substrates as culture medium is to find a waste with the right balance of nutrients that permits cell growth and product accumulation [12], in such case SLs can provide the large quantities that is particularly needed in petroleum and environmental applications, which, due to the bulk use, may be expensive [6].

Oil cakes as agro-industrial wastes are byproducts obtained after oil extraction from oil industry processes; they also can serve as cheap media for the production of SLs. Edible oil cakes have a high nutritional value due to their contents of starch, proteins and little amount of lipids therefore; they are generally fed to animals [13]. Another example for the polluting wastes are petroleum industrial wastes, such as waste oil. It is considered as the worst environmental impact of all automotive products because during use, new oil picks up toxic chemicals, carcinogenic hydrocarbons, and heavy metals which harm the environment and public health when used oil is disposed of improperly.

Oil waste poured into storm drains often ends up in streams, lakes, and bays and one pint of oil can produce a slick covering approximately one acre of water. When, pouring on the ground or deposited in landfills it can leach into groundwater. However, oil waste leakage into waterways or in salt water threatens the aquatic and marine life, and if burned it can pollute the air we breathe with chemicals which are potentially harmful to human health [14].

Candida bombicola is known to produce SLs when grown on glucose and lipophilic substrates, such as oleic acid [15], palmitic acid [16] and vegetable oils [17-19, 11] frying oil [20] and oil cake [11]. No references have been reported for the utilization of motor oil waste by *Candida bombicola.*

Researchers have focused on optimization of SL production in submerged fermentation, [21, 22] but some efforts have also investigated the possibility of its production using solid state fermentation (SSF) [10, 23, 11]. Many reports of solid-state fermentation systems (SSF) have been published in recent years supporting the application of SSF in upgrading agricultural byproducts and in the production of fine-chemicals and enzymes [24]. Solid state processes are therefore of special economic interest for countries with an abundance of biomass and agro-industrial residues, as these can be used as cheap raw materials.

This study was aimed to produce high yield of SL from non-conventional sources through bioconversion of mixed substrates of agro-industrial and petroleum wastes seeking for economic production process with reduction of environmental pollutants, as well as studying the physico-chemical properties of the produced SL to investigate its potential industrial applications.

MATERIALS AND METHODS

Microorganism

C. bombicola NRRL Y- 17069 was obtained from the Agricultural Research Service, Peoria, Illinois, USA. The culture was maintained on a stock slant medium [25].

Substrates

Sunflower seeds (Giza 1) were obtained from the local market (Cairo, Egypt). The seeds were pressed under 10.000 Ib/in^2 pressure for 1 h at room temperature using laboratory-type of Carver hydraulic [26], then the sunflower oil cake residue was collected, freezed and kept at - 4°C for the experiment. Motor oil waste was obtained from local motor oil change service center, Giza, Egypt. All chemicals and reagents used were of analytical grade.

Inoculum and Cultivation conditions

The inoculum was prepared by transferring a loopful of a stock culture of *C. bombicola* (7 days old) to a 50 ml sterile inoculum medium [25], then incubated at 30°C, 180 rpm for 24h.

Submerged fermentation

An aliquot of 1 ml with cell density 1x10^8 cells/ml of the inoculum was transferred to Erlenmeyer flask (250 ml) containing 50 ml of the modified medium [27] consists of (g/l) sunflower oil cake waste, 50; motor oil waste, 50; NH_4NO_3, 1.0; K_2HPO_4, 2.55; NaH_2PO_4, 0.15; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $MnSO_4.H_2O$, 0.02; peptone, 1.0, final pH 7.8 and incubated for 96h, 180 rpm on controlled incubator shaker (New Brunswick Scientific, USA) at 30°C.

Solid state fermentation

A modification has been done in the medium of Ohno et al [28] for solid state fermentation (SSF) as follows: 1 ml of the overnight culture ($1x10^8$ cells/ml density) was mixed with a 5 g of sunflower oil cake waste, 5 g of motor oil waste and 4 ml of previously mentioned nutrients, then incubate for 96 h in a static incubator at 30°C.

Isolation of sophorolipids (SLs)

Extraction from submerged medium

The crude SL was isolated from the submerged medium according to Kim et al[27]. The fermented product was centrifuged at 15000 rpm for 15 min. and the supernatant was then twice extracted by 2 volumes of ethyl acetate. The extracts were collected, then treated with anhydrous sodium sulfate, finally the ethyl acetate was evaporated by rotary evaporator, and the residue was collected.

Extraction from solid medium

The crude SL was isolated from solid medium by adding 45ml of methanol to one volume of the solid medium and the mixture was shaken at 92 strokes/min for 60 min with a reciprocal shaker (New Brunswick Scientific, USA), the crude extract was filtered through a 0.20 µm membrane filter (GELMAN sciences, USA), to obtain the methanol fraction (F I) [28, 29]. Re-extraction was conducted according to the new technique for the residual fermented substrates as modified by Rashad et al [11] using 45ml of ethyl acetate and shaking at 92 strokes/min for 60 min with a reciprocal shaker, filtered through a 0.20 µm membrane filter then vacuum-dried at 40 °C to remove the solvent and finally obtain the ethyl acetate second fraction (F II).

Functional Characterizations of SL

Surface tension and CMC measurement

The measurement of the surface tension was carried out on the obtained SL fractions by the ring method using a Du-Nouy tensiometer (Kruss type 8451) at room temperature. The critical micelle concentration (CMC) was determined by measuring the surface tensions of serial dilutions of the isolated SLs in distilled water up to a constant value of surface tension. The value of CMC was obtained from the plot of surface tension against SL concentrations [30].

Emulsification activity with different hydrophobic compounds

The emulsification index was measured for the produced SLs, where 2 ml of hydrocarbons (hexane, hexadecane and motor oil) or vegetable oils (soybean, sunflower and safflower oils) were added to 2 ml of each of the SLs solution (0.1%) [F I and F II] in a graduated screw cap test tube, then mixed and vortexed at high speed for 2 min. The emulsification index (E) was calculated by dividing the measured height of the

emulsion layer by the total mixture height and multiplying by 100. The emulsion stability was determined after 24 h and was observed during consecutive7 days [31].

Stability characterization

Stability studies were done on F I and F II compounds using motor oil as follows

Heat stability was investigated by heating 2ml of 0.1% F I or F II at 10, 25, 55, 70 and 100°C for 15 min then cooled to room temperature, after which the emulsification index were measured. To study the pH stability, the pH of 0.1% F I or F II were adjusted to different pH values (2.0–10.0) using NaOH or HCl and the emulsification activity were measured. The effect of NaCl concentration (2.0–10.0 %) was also determined in the same way after addition of the salt to the samples. The assays were carried out in triplicate and did not vary more than 5 % [32].

Structural characterization of isolated SLs

Fourier transform infrared spectroscopy (FTIR)

The infrared (IR) spectrum (from 400 to 4000 wave numbers, cm^{-1}) of SLs extracts were recorded using KBr pellet in Nicolet Impact 6100 FTIR spectrophotometer JASCO, USA.

^{1}H NMR spectra analysis

The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. ^{1}H spectra were run at 300 MHz in deuterated chloroform ($CDCl_3$). Chemical shifts are quoted in δ and were related to that of the solvents.

Application of the produced SLs in oil recovery enhancement

Microbial Enhanced Oil Recovery (MEOR) was evaluated using sand pack technique [33]. A glass column (40×2.5 cm) was packed with 100 g of acid-washed sand. The column was then saturated with 100 ml of kerosene. The activity of the isolated SLs in oil recovery was estimated by pouring 100 ml of aqueous solution of F I or F II (1.0 mg ml^{-1}) onto the column. The amount of oil released was measured. The assay was repeated three times with three replications for each treatment.

Statistical Analysis

The results are reported as Mean ± Standard error (S.E.) for at least four times experiments. Statistical differences were analyzed according to one way ANOVA test followed by student's t test wherein the differences were considered to be significant at $p < 0.05$.

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RESULTS AND DISCUSSION

Production of sophorolipids (SLs)

This work was conducted to find an appropriate production process and suitable economic substrates, besides finding new strategies to increase the productivity of SLs. Therefore, two fermentation techniques (submerged and solid state) were employed with a mixture of two different agricultural and industrial wastes (sunflower oil cake and motor oil waste, respectively) for maximum SL production.

On comparing SLs yield obtained from the two fermentation techniques at the 4th day of growth, the results (data not shown) revealed that the yield obtained from submerged technique was 26.4 g/l (13.2 g/100 g mixed substrates). By adopting the extraction technique for SSF [11] we attained two fractions of SLs using two steps of extraction, F I (4.75 g/100g substrates) and F II (41.76 g/100g substrates) which their sum will be 45.76 g/100g substrates. Subsequently, SSF technique was favorable as it is more economic and gave much higher yield of SLs. So, the following studies will be done using SSF technique. This yield (45.76 g%) was higher than that obtained previously [34], when *C. starmerella was* cultivated on mango kernel olein by SSF (17.48 g%). While it was in the range (49.5 g%) of that reported previously by the same authors Rashad et al [11] using the same organism with sunflower oil cake supplemented with soy bean oil. However, much lower yields (1.2 g% and 0.33 g%) were observed for bacterial surfactants (*B. subtilis and B. pumilus* respectively) produced by SSF technique using media based on okara [35, 36].

In order to optimize the production of SLs derived from SSF, time course (4, 8, 12 and 16 days) was studied and presented in the figure (1). The results showed that there was no significant change in the SLs yield during the time study for methanol fraction (F I). The yield obtained ranged from 3.5 g% on the 8th day of fermentation to 5 g% on the 12th day of fermentation. These results of methanol fraction F I was disagree with that reported before [11], as the yield of methanol fraction was directly proportional with fermentation time and the optimum one was achieved at the 16th day of growth (28.75 g%) using a mixture of sunflower oil cake and crude soy bean oil as carbon sources.

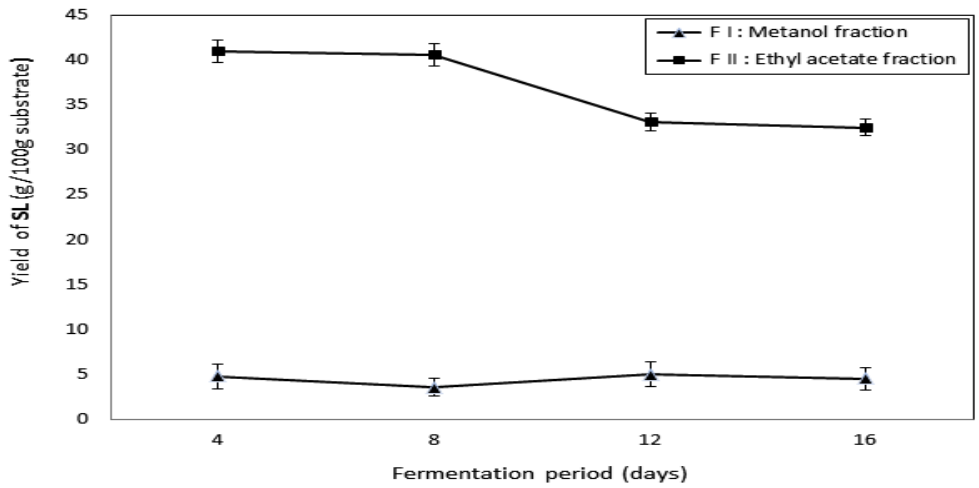

Figure 1: Time course of *C. bombicola* SLs (F I and F II) using SSF of mixed sunflower oil cake and motor oil waste. (data were expressed as mean ± S.E. of 4 experiments).

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However, the maximum production was obtained for fraction (F II), as it represents the untraditional extraction method (re-extraction by ethyl acetate) whereas, the highest yield (41.01 g %) has been noticed on the 4th day, followed by 40.6 g% on the 8th day of fermentation. Lower yields obtained on 12th and 16th days were 33.11 and 32.5 g%, respectively. Therefore, it can be observed that the yield obtained from ethyl acetate fractions (F II) decreased over incubation time which agrees with that stated by Rashad et al [11] for the yield of the same fraction, which was inversely proportional with fermentation time using a mixture of sunflower oil cake and crude soybean oil.

Functional Characterization of the produced SLs:

CMC values are important in all of the petroleum industry surfactant applications. For example, a number of improved or enhanced oil recovery processes involve the use of surfactants including micellar alkali/surfactant/polymer and gas (hydrocarbon, N_2 , CO_2 or steam) flooding [37]. CMC is a widely used index to evaluate surface activity, which is defined as the surfactant concentration at which a sharp decrease in surface tension is observed [38]. Surface tension at CMC value were determined for the isolated SL fractions (F I and F II) during the fermentation period.

The methanol fractions (F I) exhibited a good surface tension reducing activity ranged from 36.2 mN/m to 37.6 mN/m at the CMC range (68.55 – 80.51 mg/l), during the time course study. The results revealed that the reduction of surface tension had convergent values. The lowest CMC of F I recorded 68.5 mg/l which is attributed to the 12th day of fermentation.

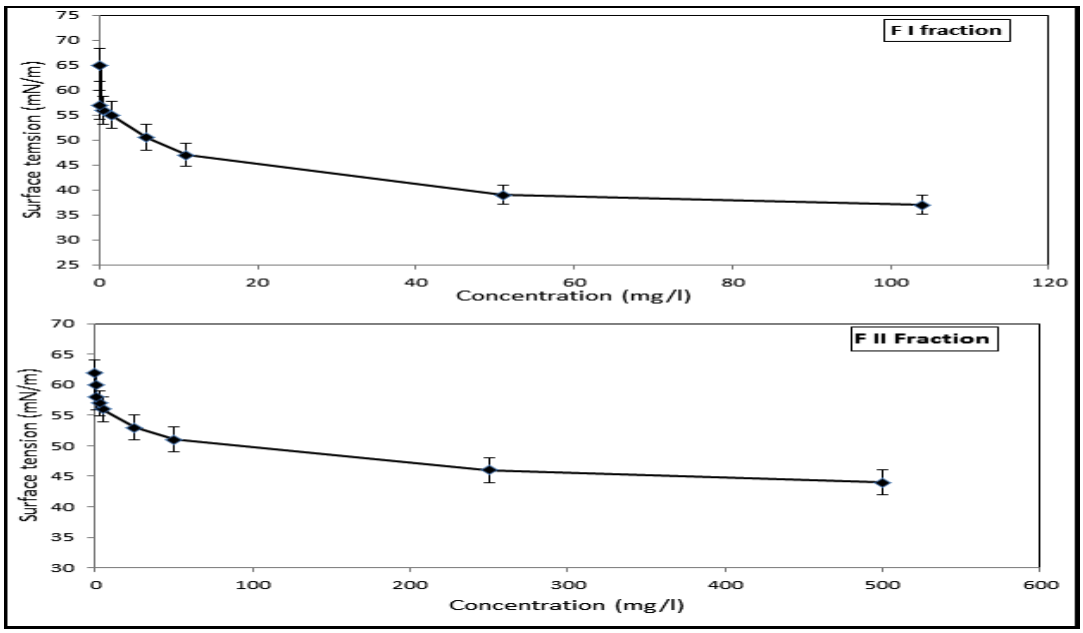

Figure 2: Critical micelle concentration (CMC) and minimum surface tension of the isolated SL fractions on the 12th day of fermentation (data were expressed as mean±S.E. of 4 experiments).

On the other hand, the ethyl acetate fractions (F II) were found to have the ability to reduce the surface tension of water from 72 mN/m to a range of 55 – 45.2 mN/m at the CMC values of 292.69 – 470.13 mg/l during the time course investigation. The highest

efficiency to reduce the surface tension (45.2 mN/m) at the CMC of 391.37 mg/l, was observed on the 12th day of fermentation. Thus the 12th day of fermentation for both fractions was selected for the following characterization (Fig. 2).

The CMC of the methanol fraction (F I) was lower than that previously observed (130 mg/l) at minimum surface tension 39 mN/m for a mixture of SLs produced in a two-stage process using *C. curvatus* and *C. bombicola* grown on deproteinized whey and rapeseed oil as the carbon sources [39]. The results for F II fractions were in the range of the SL biosurfactant produced by *Rhodotorula muciliginosa* and *C. rugosa* grown on diesel oil which reduced the surface tension of water from 72 mN/m to 33 and 34 mN/m at CMC of 250 and 300 mg/l, respectively [32]. The authors also found that Tween 80 and SDS reduced the surface tension but with high CMC values of 1250 and 380 mg/l, respectively.

Emulsification activity and stability studies

Emulsification activity

In addition to surface and interfacial tension, stabilization of an oil and water emulsion is commonly used as a surface activity indicator for industrial applications. The emulsifying activity of F I and F II fractions (isolated on the 12th day of fermentation) with various short and long-chain hydrocarbon substrates at room temperature is represented in Figure (3). The results revealed that the produced SLs have a high emulsification specificity towards motor oil (E_{24}=70% & 62.5% for F I and F II, respectively), followed by Soybean oil (E_{24}= 57.5% & 56.25% for F I and F II, respectively). Sunflower and Safflower oils also gave a good emulsification activity (50-52.5%) with both fractions. In contrary, the lowest emulsions values have been obtained with short-chain hydrocarbon substrates namely hexadecane followed by n-hexane for F I fraction, while F II showed the lowest activities with the n-hexane followed by hexadecane hydrocarbons. These results suggests that the emulsification activities of the SLs (F I and F II) are effective for the long-chain hydrocarbon substrates and consequently, could be used in the industrial applications. Similar results were obtained by using *Corynebacterium kutscheri, C. guilliermondii* and *B. subtilis* biosurfactants, respectively [40, 10].

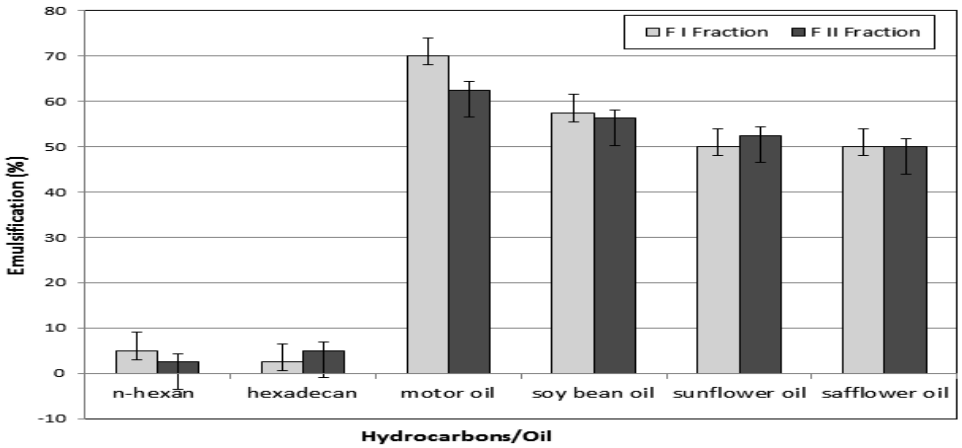

Figure 3: Efficiency of emulsifying activity of the produced SL fractions against different hydrocarbons and oils (data were expressed as mean±S.E. of 4 experiments).

Stability studies

The emulsification stability of the F I and F II fractions obtained at the 12th day of fermentation with various short and long-chain hydrocarbon substrates during 7 days is summarized in figure (4). The stability with long chain hydrocarbons substrates (motor oil followed by vegetable oils) was much more favorable than short chain ones for both fractions. Stability results obtained were in agreement with the emulsification stability conducted on *C. guilliermondii* and *B. subtilis* biosurfactants [10]. On contrast with our results, the short chain hydrocarbon (1-hexadecene) exhibited the highest emulsion stability in the study conducted on SL from *C. bombicola* [1].

Environmental factors such as pH, Salinity and temperature also affect biosurfactant activity [41]. Consequently, the studies of the influence of these parameters are of basic importance if considering the possibility of specific applications for these biomolecules. So, the emulsification index values of SLs (F I and F II) isolated on the 12th day of fermentation were measured at these influence factors (Fig. 5a, b and c). The effect of thermal treatment on the emulsifier activity indicated that both fractions showed emulsification activities from 10-100 °C, whereas the optimum temperatures for F I were at 55 and 70 °C, while the optimum for F II were at 25 and 55 °C (Fig. 5a). However, F I fraction retained 83 % of its activity after boiling for 15 min which, suggests that the produced SLs may be useful in extreme temperature in industrial systems. Previous reports have also shown thermal stability at high temperatures for SLs produced from different yeast species [42, 32].

Regarding the influence of the pH on the emulsification activity, it was observed that the optimum pH for fractions F I and F II was at pH 2, while the lowest emulsification activity was at pH 4 for both fractions. Despite of the obvious inhibition in the emulsification activity of both fractions (specially F II) at pH 4, the F II fraction kept 80 % of its emulsifier activity over the tested pH range (Fig. 5b). Thus, it can be concluded that the isolated fractions may be useful in the extreme acidic and alkaline environmental conditions. It was reported that extremes of pH could possibly transform less surface-active species into more active emulsifiers by increased ionization [42]. The activity of the biosurfactants produced from bacterial and yeast species was also pH stable [43, 32].

The effect of added NaCl concentrations (2-10%) on emulsification capacity for F I and F II Fractions was also studied (Fig. 5c), the F I fraction showed a high emulsification activity at the highest concentration (10 %), while the optimum NaCl concentration for F II was at 8%. However, both fractions showed a relative tolerance over the tested salt concentrations (2-10%) with activity retention of 46.66 % for F I and 77.14 % for F II, which suggests that the produced SLs especially fraction F II might be useful in marine environments and other systems where salt concentration is above physiological level. The activity of the biosurfactant produced by the yeast species *C. glabrata, Rhodotorula muciliginosa* and *C. rugosa* also showed stability in presence of high salt concentration up to 10% [42, 32]. Other reports indicated that concentrations above 2% of NaCl were enough to inactivate a synthetic surfactant [44].

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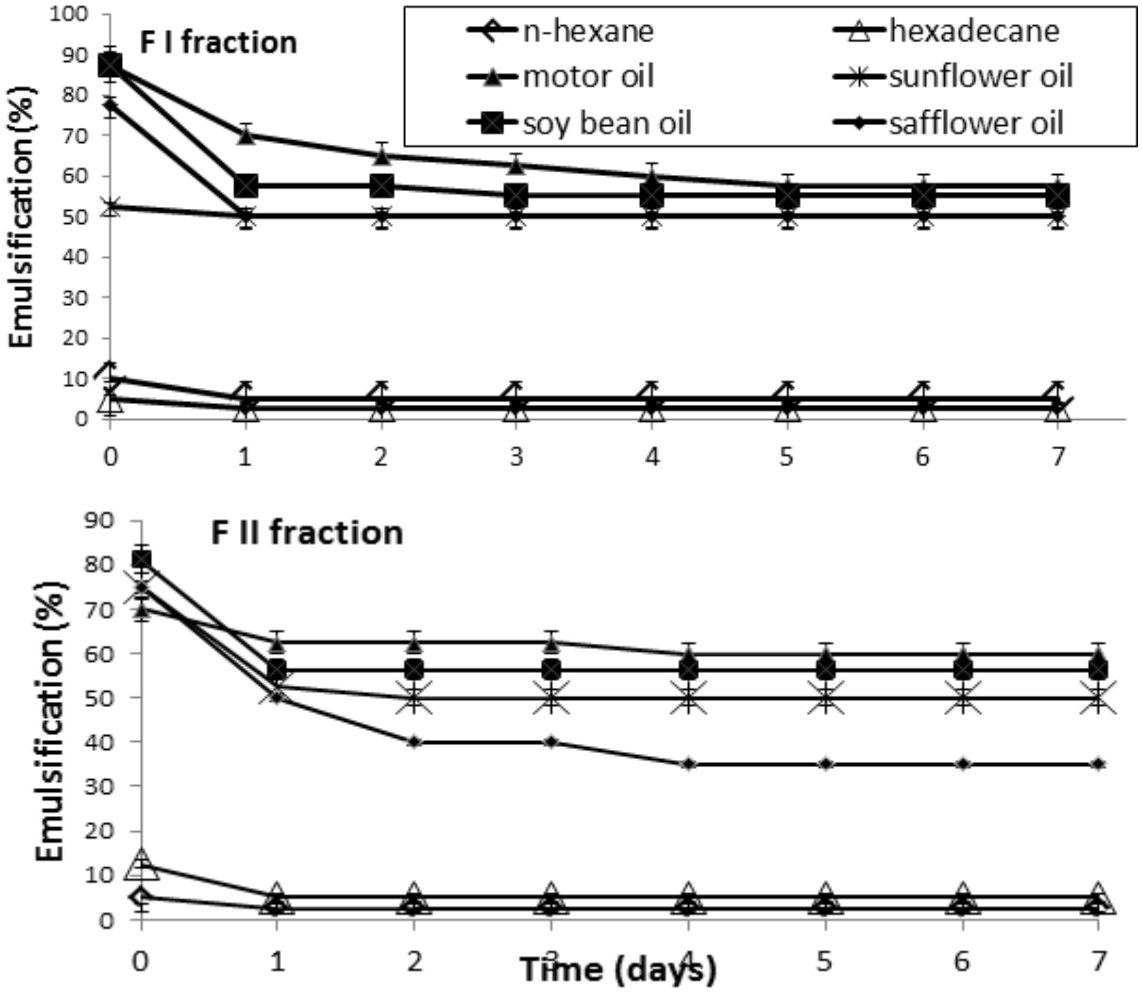

Figure 4: Emulsification stability of the produced SL fractions during 7 days at room temperature (data were expressed as mean±S.E. of 4 experiments).

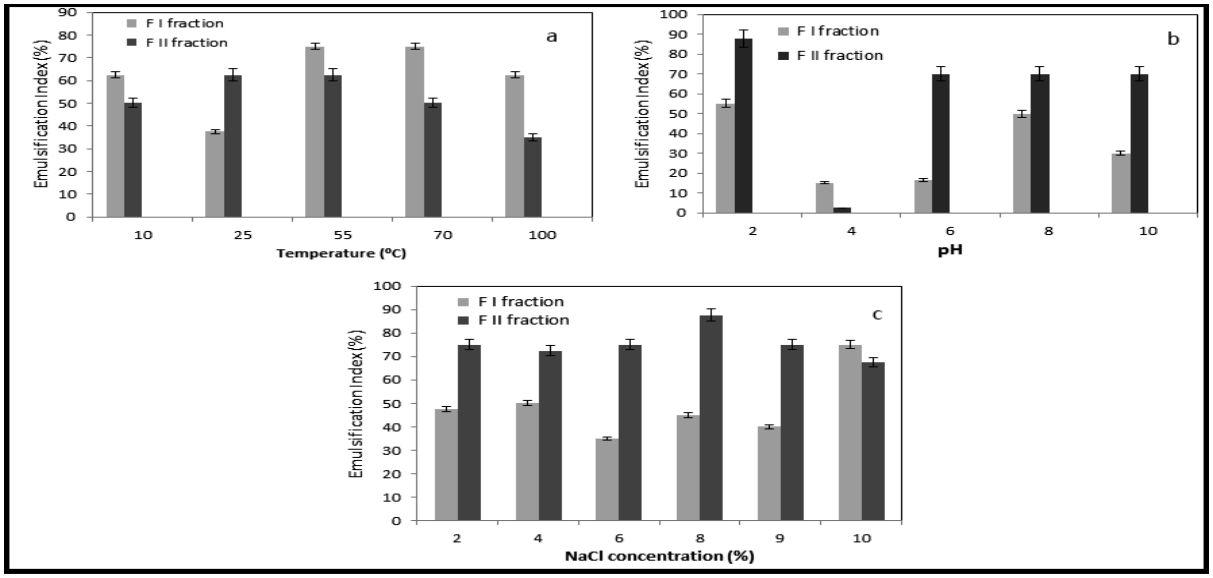

Figure 5: (a) Heat stability of SL fractions for emulsifying activity towards motor oil .
(b) Influence of pH on the emulsifying activity of produced SL fractions towards motor oil.
(c) Effect of NaCl concentrations on the emulsifying activity of produced SL fractions towards motor oil (data were expressed as mean±S.E. of 4 experiments).

Structural characterization of isolated SLs

FTIR spectroscopy

The isolated SLs (F I and F II) produced at the 12th day of fermentation were identified and characterized by FTIR spectroscopy. The FTIR spectra of the fractions reveal broad bands at 3425.92 and 3410.49 cm^{-1} corresponding to the O-H stretch in its structure, respectively. The spectra also reveals that asymmetrical stretching (v_{as} CH_2) and symmetrical stretching (v_s CH_2) of methylene groups occurred at 2925.48 & 2854.13 cm^{-1} for F I and 2924.52 & 2855.1 cm^{-1} for F II. While the absorption bands at 1741.41 and 1745.26 cm^{-1} contribute due to C=O stretching from lactones esters or acids in F I and F II, respectively. The bands at 1459.85 and 1460.81 cm^{-1} corresponded to the C-O-H in plane binding of carboxylic acid (-COOH) in the structure of the products. The stretch of C-O band of C(-O)-O-C in lactones exists at 1166.72 and 1163.83 cm^{-1} in F I and F II fractions, respectively. The absorption band for C=C at 720.28 cm^{-1} was observed only in ethyl acetate fraction (F II). These structural details of the crude fractions were similar to the results reported by many authors [1, 45, 46].

^{1}H NMR analysis

^{1}H NMR spectra of the produced SLs (F I and F II) were assigned to a typical glycolipid-type structure (Fig. 6). The protons of two glucose were resonated at 3.4 – 4.25 ppm, while a weak signal was observed for poly (SL) assigned to trans vinyl protons (-CH=CH-) at 5.31 ppm for F I and 5.33 ppm for F II. Peaks from 1.2 to 1.4 ppm indicate the presence of fatty acid chain moiety in both fractions. Signals at 2.53 ppm confirm the existence of $–CH_2$-COO- group. Similar ^{1}H NMR spectra for SL structure were formerly reported by other researches [47, 48, 49, 46]. Both FTIR and ^{1}H NMR analyses indicated that the isolated fractions have a SL group of compounds (acidic and lactone ring form) in its structure.

Application of the produced SLs in oil recovery enhancement

To investigate the efficiency of the produced SLs (F I and F II) sand pack studies were conducted at room temperature to check if the biosurfactant can be applied for commercial use in microbiologically enhanced oil recovery (MEOR). The SLs (F I and F II) were highly effective in recovering kerosene oil from the sand as fraction F I recovered 71.43% while, F II recovered 72.73% from the tested Kerosene indicating their potential application in microbiologically enhanced oil recovery. kerosene oil was recovered from the sand pack column with the percentage of 62% using a thermophilic *B. subtilis* biosurfactant [50]. While, 41% of the engine oil was recovered using biosurfactant produced from *B. subtilis* sp. over a period of 48 h when the column was maintained at 30˚C and the recovery was enhanced when the column was maintained at higher temperatures giving a recovery of 55% and 64% at 50˚C and 70˚C, respectively [51]. Higher recovery percentages (86% and 84%) were achieved for the biosurfactants produced by *Rhodococcus* and *C. glabrata* sp, respectively [33, 52].

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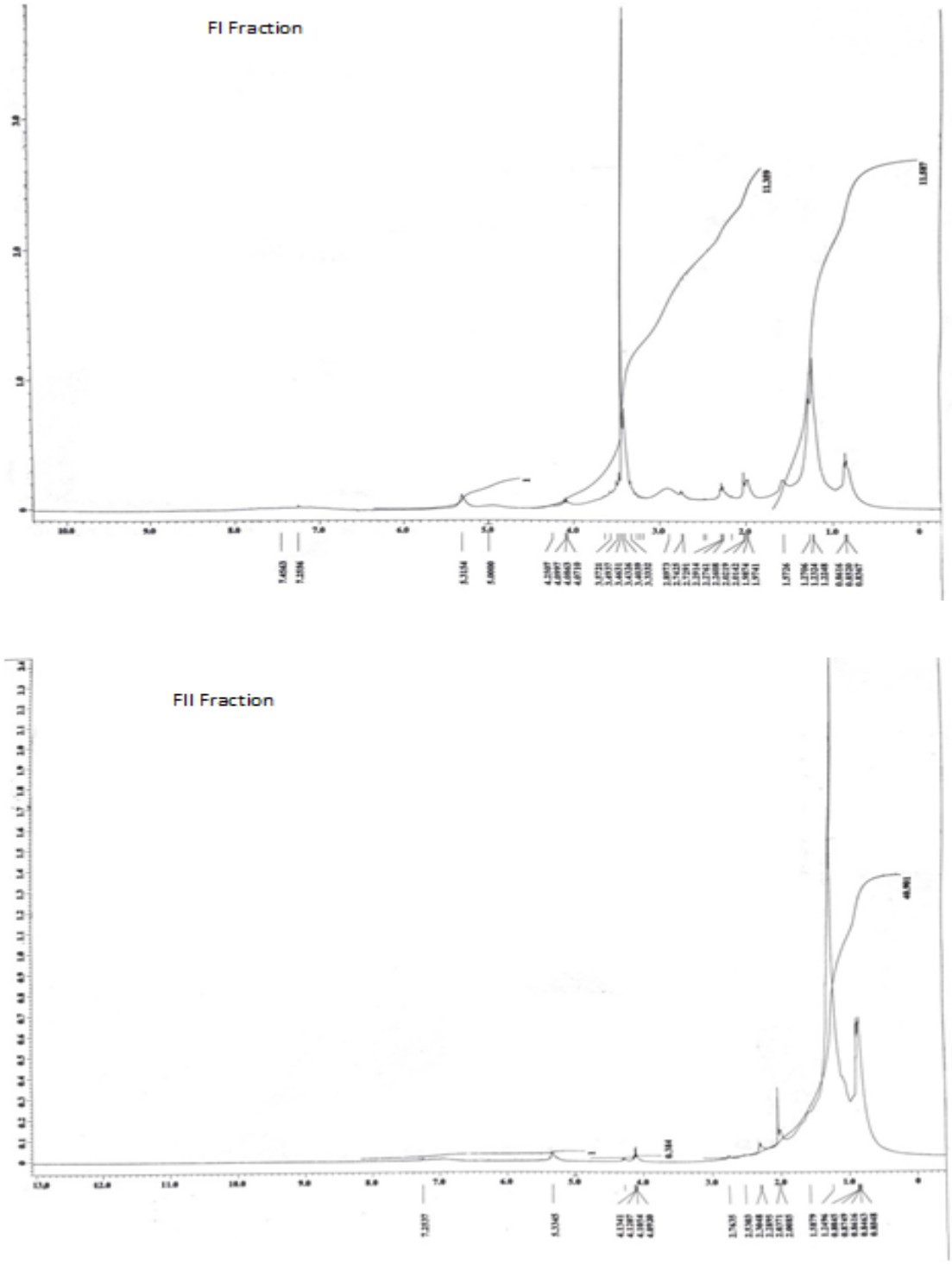

Figure 6: ^{1}H NMR spectra of the produced SL fractions (FI & FII).

CONCLUSION

Based on the experimental results described in this study, it can be concluded that the motor oil waste, which is one of the worst environmental pollutants and sunflower oil cake (food processing waste), considered to be valuable sources for novel SLs production when utilized by *C. bombicola*. The significant amount of SLs produced by using the new extraction technique described above contribute to solve the requirement of the large quantities

particularly needed for petroleum and environmental applications. The produced SLs exhibited high emulsification activity and surface tension reduction. The stability of SLs in wide range of pH, temperature and salinity also enable the produced compounds to be used in extreme environments.

The results of oil recovery from sand for the produced SLs reflect their possible application for commercial use in microbiologically enhanced oil recovery (MEOR). Moreover, preliminarily studies showed the possibility of the residual media (culture) to be reused as another source for additional amounts of SLs (need for further research).

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