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A comparative study of biosurfactant preparation by *Pseudomonas aeruginosa* MTCC 424 using rice bran oil and soybean oil substrates

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Researchers world over are trying to find the low cost feed stocks for cost-effective production of biosurfactants in order to compete with the chemically synthesized traditional surfactants. In this study rhamnolipid biosurfactant was prepared by *Pseudomonas aeruginosa* MTCC 424 culture. The rice bran oil and soybean oil were the two substrates taken in the study, which were extracted from spent bleaching earth for use as low cost carbon source. Spent bleaching earth is a waste of vegetable oil processing industries and is usually disposed of in the landfills or waste dumps. *Pseudomonas aeruginosa* culture was preferred over other microorganisms due to its nutritional and biochemical versatility along with simple culture conditions. The two biosurfactants were screened using oil displacement, drop-collapse, cetyltrimethyl ammonium bromide agar plate, emulsification index and surface tension measurement tests. The chemical structure and composition were analyzed by Fourier transform infrared, nuclear magnetic resonance and thin layer chromatography spectroscopic test methods. The study proved residual rice bran oil based rhamnolipid biosurfactant as the better one compared to residual soybean oil based biosurfactant. Since the residual rice bran oil extracted from spent bleaching earth is a very cheap raw material, the present study is hoped to contribute to some extent in solving the issue of high production cost of biosurfactants.

Keywords: *Pseudomonas aeruginosa*, rhamnolipid biosurfactant, spent bleaching earth, rice bran oil, soybean oil.

Introduction

Surf**ace **act**ive **a**ge**nts abbreviated as surfactants for ease of communication and expression are amphiphilic compounds of high relevance. Being amphiphilic compounds surfactants possess both hydrophilic and hydrophobic (lipophilic) characteristics with strong surface activity[1]. The polar hydrophilic part has strong affinity towards polar solvents like water, acetic acid, methanol, etc., whereas non-polar hydrophobic part has affinity towards non-polar substances such as oils, fats, and greases[2]. Surfactants are extensively used in the agricultural sector, foodstuff, cosmetics, medicine as well textile industries and for enhanced oil recovery from the rocks because of their ability to reduce overhead or interfacial tensions[3,4].

Nearly all commercial surfactants available today are chemically synthesized from hydrocarbon feedstock[5]. These surfactants are inherently toxic and non-degradable and often result in an additional source of pollution to already depleting natural habitat[1]. Since the beginning of 21st century, increasing environmental awareness among consumers, more stringent environmental regulations across the globe, and advances in biotechnology have motivated the researchers and drawn the attention of industries as well to develop environmentally friendly surfactants as potential alternative to existing chemical surfactants[6,7].

Biological surfactants often called as biosurfactants are actually the biomolecules produced by a variety of microorganisms on various carbon sources. Biosurfactants do have both hydrophilic and hydrophobic affiliations just like conventional chemical surfactants. The hydrophilic component contains the carboxyl group of fatty acids or amino acids, the hydroxyl group of saccharides, the phosphoryl group of phospholipids, and peptides or proteins, whereas the hydrophobic part is comprised of hydrocarbon chain of saturated or

unsaturated fatty acids or sterol ring[3,8,9].

The active research on biosurfactants started in the 1960s that gained momentum over successive decades[6]. There is no satisfactory reason known to researchers till today for the microbial production of biosurfactants. Though conditions favoring biosurfactant production by microorganisms have been extensively studied and identified as carbon substrate, pH, temperature, incubation time, air flow, salt, and the concentration of minerals such as nitrogen and phosphorus[10].

Biosurfactants are highly versatile compounds with multifunctional properties such as dispersion, foaming and detergency, emulsification and de-emulsification, moisturization, penetration, thickening, wetting and coating, coagulation, metal sequestration and corrosion inhibition[1,9]. Due to these peculiar properties biosurfactants find potential applications in agriculture, cosmetics, fertilizers, foods, beverages, pharmaceuticals, textile processing, enhanced oil recovery, bioremediation of organics and metals[11,12]. The growing interest in biosurfactants is mainly due to their ability to offer an alternative to chemically synthesized surfactants in terms of properties and performance. A partial list of advantages extended by biosurfactants include improved biodegradability[13], environmentally friendly[14], low toxicity, non-hazardous[9], higher selectivity, mild production conditions using renewable materials and industrial waste/by-products as substrates[6,15], ability to function at extreme temperatures, pH and salt concentrations[11], and modification by biotechnology and genetic engineering[5].

There is lack of consistency in available data on value of global biosurfactant market. Reis *et al.*[7] reported the actual worth of inclusive biosurfactant market place at USD 1.7 billion in year 2011. Now one market research group reported the actual worth of global biosurfactant market in 2018 as USD 1.6 billion[16], whereas another research group reported it as USD 4.7 billion for the same year[17]. The difference between the two values is quite big. One possible reason for this anomaly might be involuntary ignorance of the terms 'actual' and 'estimated'. Most market research groups have predicted a composite twelve-monthly enlargement rate of 3.5–5.6% for global biosurfactant market for the period 2018-2026. It would be worthwhile at this point to mention the size of global surfactant market for the sake of comparison, which was at USD 43.655 billion in 2017[18]. It is evident from these data that biosurfactants, despite their numerous advantages, have not been yet able to seize an appreciable chunk of surfactant market. The major factors affecting the commercialization of biosurfactants are their high production cost (three to ten times over that for chemical surfactant)[4] and low product yield[5]. All research efforts are now focused on reducing the cost for raw material and downstream processing in addition to increasing the product yield.

Mukherjee *et al.* (2006) suggested a three prong strategy to perk up the economy of biosurfactant manufacture. This includes exploiting high yielding strains, using cheap agro industrial wastes with the right nutrient balance, development of more efficient biochemical processes under optimum process conditions, and cost-effective downstream processing for maximum product recovery[3]. The intend of current exertion was to make a comparative swot of biosurfactant preparation by *Pseudomonas aeruginosa* MTCC 424 by means of rice bran oil and soybean oil extracted from spent bleaching earth discarded in a vegetable oil refinery. Different nutritional and environmental parameters including concentration of carbon, pH of media, incubation time and temperature were also studied for high biosurfactant yield. The biosurfactants thus produced were first screened using oil displacement method (ODM), the drop collapse method (DCM), the cetyltrimethyl ammonium bromide (CTAB) agar plate method, emulsification index (EI24) and the surface tension (SFT) measurement and then characterized by various techniques such as Electro spray ionization-mass spectrometry (ESI-MS), Fourier transform infrared (FTIR) spectroscopy, Nuclear magnetic resonance (NMR) and Thin-layer chromatography (TLC).

Materials and methods

Chemicals:

All chemicals were of analytical or highest available grade purchased from Qualikems Fine Chem Private Limited, Gujarat and were used without further purification. Standard rhamnolipid biosurfactant (95% purity) was procured from Sigma Aldrich.

Agro-industrial feedstocks:

Two spent bleaching earth samples, one containing rice bran oil and the other containing soybean oil in range of 14–25%, were received as a generous gift from M/s Kanpur Edibles Private Limited, Kanpur, India. Residual oil in the spent bleaching earth was extracted by soxhlet extraction

method using hexane as solvent. The optimum extraction temperature was 68°C and the optimum time was 2 h. The residual oil was used as sole carbon resource or substrate in this study.

Organism:

Pseudomonas aeruginosa MTCC 424 culture was acquired from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was preserved on nutrient agar tilt at 4°C for 24 h prior to further investigation.

Cultivation of seed culture:

The strain from 24 h culture on nutrient agar tilt was relocated to untainted Erlenmeyer flask containing 250 mL nutritive broth to get ready the seed ethnicity for biomass preparation. The nutritive broth was prepared as per following composition: beef extract (1.07 g/L), yeast extort (2.05 g/L), peptone (5.04 g/L), NaCl (5.02 g/L), agar (15.10 g/L) and distil water (1.0 L) at pH 7.0. The microbial cultures were grown-up in this broth under orbital agitation at 100 rpm and 32°C for 16 to 18 h. The optical-density of the broth was measured at regular intervals by a spectrophotometer at wavelength of 600 nm (OD_{600}) until it reached a value of 0.7 equivalent to the inoculum of 10^7 colony form units (CFU)/mL. The cell concentration of 3% (v/v) was used as inoculum for biomass preparation[15,19–21].

Biosurfactant preparation, recovery and purification

Production medium:

Biosurfactant preparation was accomplished in two identical 3 L Erlenmeyer flasks each containing 1 L mineral salt medium of following composition[22]: KH_2PO_4 (3.0 g/L), K_2HPO_4 (2.5 g/L), $NaNO_3$ (2.5 g/L), $MgSO_4.7H_2O$ (1.0 g/L), KCl (1.0 g/L), $CaCl_2$ (0.02 g/L), and trace elements (1 ml/L). The trace element solution concentration was $FeSO_4.7H_2O$ (0.120 g/L), H_3BO_3 (0.227 g/L), $CoCl_2.6H_2O$ (0.39 g/L), $CuSO_4.5H_2O$ (0.018 g/L), $MnSO_4.H_2O$ (0.015 g/L), $[NH_4]_6Mo_7O_{24}$ (0.027 g/L) and $ZnSO_4$ (0.177 g/L). The bacterial broth (3% v/v) was inoculated into the mineral salt medium supplemented with 4% residual rice bran oil (RRBO) and 6% residual soybean oil (RSBO) in respective flasks as sole carbon source for a comparative study. The medium had pH synchronized at 7.2 by addition of HCl or NaOH[23] and an air supply of 3 Lpm was maintained[24]. Incubation for biosurfactant preparation was carried out in incu-shaker at 32°C and 100 rpm for a seven days.

Biosurfactant recovery and purification:

Most researchers have followed the acid precipitation-cum-solvent extraction method to recover biosurfactant from broth culture[4,5,15,20,22,25–28]. After seven days of incubation, the broth customs containing RRBO as sole carbon source was centrifuged at six thousand rpm for 30 min to attain the cell free supernatant. The cell free supernatant was then acidified with 6 *N* HCl to pH 2.0 and held at 4°C overnight with effervescent stirring in magnetic stirrer until rudimentary biosurfactant pellets appeared. The pellets were recovered from acidified broth by centrifugation at 8000 rpm for 20 min. The pellets were then dissolved in distilled water and modified to pH 7.0 with 1 *N* NaOH. Now the organic layer of the solution containing biosurfactant was separated by solvent extraction method. To do this, an equal volume of chloroform and methanol solvent mixture in 2:1 (v/v) proportion was mixed together. The resulting solution was vigorously stirred for 20 min and allowed to settle until phase separation. The organic layer was separated and the process was further repeated twice. Any remaining solvent from biosurfactant was removed by rotary evaporator. The biosurfactant thus obtained was dried in an oven at 105°C for 24 h and a brownish biomass product was obtained. The biosurfactant concentration was dictated by seperating the weight of the dried biosurfactant with the absolute volume of unrefined biosurfactant solution and expressed in terms of rhamnose mg/mL (dry weight)[14]. Same procedure was followed to recover biosurfactant from the broth culture containing residual soybean oil as sole carbon source.

Test methods for biosurfactant screening and characterization:

The cell free supernatants from two broth cultures were initially screened for successful preparation of biosurfactant using ODM, DCM, CTAB agar plate method, EI24 and SFT measurement. ESI-MS was used to analyze the RRBO and RSBO used in the study as sole carbon sources. The substance design and biosurfactant product composition was measured with the help of FTIR, NMR and TLC methods.

Screening for biosurfactant production

Oil displacement method (ODM):

Oil displacement activities of two cell free supernatants

were determined by using the now widely recognized classic method described by Morikawa *et al.*[8]. The tests were performed in 150 mm diameter Petri dish filled with 40 ml of distilled water. Further 20 microliter of diesel oil was put onto the surface of the distilled water to form a thin film. 10 μL of supernatant sample was softly put in the centre of the oil film. The formation of a clear zone by displacement of oil indicated the successful preparation of biosurfactant and the diameter of this circle gave a measure of biosurfactant activity. Tests were run in triplicate and the average clear zone diameters were reported[1].

Drop-collapse method (DCM):

The qualitative drop-collapse tests were performed in standard 96-well (8 mm i.d.) microtiter plate (12.7×8.5 cm) polystyrene lids. The lids were carefully cleaned using hot water and ethanol, and was further dried before use. Each well was layered with 2 μL of HP Milcy Turbo 15W-40 engine oil and left for 24 h at ambient conditions to confirm a consistent covering. 5 μL cell free supernatant was carefully transferred into the center of each well using a glass syringe held at an angle of 45°. The drops were observed after 1 min with the help of a magnifying glass[23]. If the drop collapsed and spread out completely over the surface of oil, the result was considered as positive (+) for biosurfactant preparation. If the drop remained beaded, the result was considered as negative (–) indicating lack of biosurfactant preparation[29].

Cetyltrimethyl ammonium bromide (CTAB) agar plate method:

Mineral salt medium (MSM) supplemented with 4% RRBO and 6% RSBO as carbon source and 0.5 $mg.ml^{-1}$ CTAB and 0.2 $mg.ml^{-1}$ methylene blue were used for determination of rhamnolipid biosurfactant. Different wells each of 4 mm dia and equally distant apart were made in blue agar plate using a cork borer. 30 μL of culture extract were charged into specified wells and incubated at 32°C for 48–72 h. Development of dark blue halos around the bacterial strains is an indication of presence of rhamnolipid biosurfactant[1,30]. CTAB agar plate method is basically a colorimetric technique in which colour reactions are carried out by binding a dye to the rhamnolipid biosurfactant. Here the cationic surfactant CTAB and the basic dye methylene blue form an insoluble ion pair with anionic rhamnolipid biosurfactant[12].

Emulsification index (EI_{24}):

Emulsification activities of the two cell free supernatants were analyzed by using the time honored Cooper and Goldenberg method[15]. According to which, 2 mL of HP Milcy Turbo 15W-40 engine oil as a liquid hydrophobic compound was added to approach volume of cell free culture stock in a graduated screwcap test tube. The blend was twirled at high velocity for 2 min and afterward permitted to make due with 24 h at room temperature. EI_{24} was then dictated by isolating the tallness of the emulsified layer by the absolute blend stature and duplicating this outcome by 100[5,19,20,31,32].

Emulsification index (%EI_{24}) = [Height of the emulsified layer/Total height of the mixture]×100 EI_{24} is used to determine the ability of biosurfactant to emulsify the non-polar hydrophobic phase in polar hydrophilic phase. A good emulsifier should be able to retain at least 50% of initial emulsion volume after 24 h of its formation. The good emulsification activity of a biosurfactant is of paramount importance for its successful environmental and industrial application[31].

Surface tension (SFT) measurement:

SFT measurements for two cell free supernatants with different carbon sources were made at the intervals of 24 h using a surface tensiometer (Usha Instruments, Kolkata) at room temperature following du Nuoy methodology as discussed by Bodour and Miller-Maier[23]. 20 mL volume of each decontaminated surfactant arrangement was moved into a spotless 50 mL measuring utencil and set onto the tensiometer stage.

A 6-cm du Nuoy platinum wire ring was drenched into the solution and the power needed to get it through the fluid air interface was recorded from the graduated dial as surface pressure (dyn/cm). Between every estimation, the platinum wire ring was flushed multiple times with water, multiple times with CH_3COCH_3 and was permitted to dry. The alignment was done before every estimation with ultrapure refined water (ST = 71.5 mN/m±0.5) and all estimations were made in three-fold[5,21].

Tensiometers utilize an ideally wettable ring swinging from an exactness equilibrium to gauge the surface strain. The test liquid placed on the tensiometer platform is elevated by a platform adjusting screw until the ring reaches below the surface of the liquid. The stage is currently brought down again so the fluid film created underneath the surface is extended. The power needed by the ring to break the surface

is estimated and used to figure the surface tension[15,27]. Tensiometric measurements are widely applied in rhamnolipid research due to their simplicity[12].

Characterization of biosurfactant

Electro spray ionization-mass spectrometry (ESI-MS):

ESI-MS has been effectively applied in analysis of triglycerides, fatty acids, and rhamnolipids[33]. This technique is based on the ionization of primary molecules, which are then picked by a mass analyzer as per their mass-to-charge ratio (*m/z*) and identified accordingly[12]. The mass spectrometry of residual rice bran and soybean oils and of purified rhamnolipid biosurfactants were recorded on Waters Q-TOF Micromass Mass Spectrometer.

ESI-MS of vegetable oils were completed as followed. 250 µL of RRBO was taken in a flagon and dissolved in water-methanol (1:1 v/v) making the last volume to 1 mL. The entire arrangement was permitted to get comfortable two unmistakable layers. The top hydroalcoholic layer was disposed of and 10 µL formic corrosive was added to answer for positive particle mode examination. The arrangement was then infused with a needle siphon at a stream pace of 10 µL min^{-1} and filtering was done preposterous 1200 *m/z* range. Regular ESI-MS conditions were: disintegration temperature 100°C, warmed slim temperature 100°C, hairlike voltage 3000 V, and cone voltage ±40 V. ESI-MS of chose particles was accomplished by low-energy (15–30 V) collision instigated separation[4,34]. Same method was applied for analysis of RSBO.

ESI-MS of purged rhamnolipid biosurfactants were proceeded as examined further. 2 mg of rhamnolipid combination was dissolved in 1 mL methanol:water (9:1, v/v) and blended altogether. The blend was sifted and aliquots of 0.1 mL were diluted to 1.9 mL of acetonitrile-water (7:3, v/v). The sample was then brought into the source with a syringe pump at a flow rate of 10 µL min^{-1} keeping up the stock of nitrogen and auxiliary gas at 50 and 5 ml min^{-1}, individually. The checking was done more than 100–750 *m/z* range in negative ion mode. Typical ESI-MS conditions were: heated capillary temperature 250°C, mass analyzer temperature 100°C, capillary voltage 3000 V, and cone voltage 40 V. ESI-MS of selected ions was attained with low-energy (15–30 V) collision-induced dissociation[4,14,28,35].

Fourier transform infrared spectroscopy (FTIR):

FTIR has a proven capacity to identify chemical bonds in a molecule by producing a spectrum of characteristic infrared absorption bands that can be used as fingerprint to identify and characterize the sample[14]. FTIR absorption spectrums of purified biosurfactants were obtained with Perkin-Elmer spectrophotometer in a dry atmosphere over region of wave numbers 400–4000 cm^{-1} with 20 scan speed by the KBr pellet method. 10 mg freeze-dried pure biosurfactant was milled with 100 mg spectral grade KBr and compressed by a bench-top hydraulic press applying 7500 kg load for 20 min to obtain thin translucent pellets[4,5,36]. KBr was used as a background reference[31].

Nuclear magnetic resonance (NMR) spectroscopy:

NMR is a leading spectroscopy technique available to chemists to determine the precise structure and purity analysis of newly synthesized chemicals. The technique is based on transitions in atoms along with magnetic moment under effect of an external magnetic field[12]. ^{1}H and ^{13}C NMR spectrum of biosurfactants were obtained with JEOL JNM-ECS400 NMR spectrometer operating at 400 mHz. The purified biosurfactant was re-dissolved in chloroform-deuterated methanol (2:1 v/v) and spectra were determined at 30°C using tetramethylsilane (TMS) as internal standard[35].

Thin layer chromatography (TLC):

TLC of biosurfactants was conducted out following work of George and Jayachandran[37]. The biosurfactants were dissolved in 1 ml chloroform. 100 µL of each biosurfactant was applied to a 10 cm×10 cm pre-covered silica gel TLC plate utilizing glass slim. The versatile stage utilized for the partition and investigation of biosurfactants included chloroform/methanol/acidic corrosive (65:15:2 v/v/v) solvents. The plate improvement chambers were soaked with the versatile stage for thirty minutes before advancement. The plates were put vertically in these improvement chambers. After advancement the biosurfactant spots were deliberately rejected off from the plates and suspended in a chloroform/methanol (1:2 v/v) blend in a glass tube. The dissolvable scratching combination was then vortexed at fast for 1 min and saved for extraction. After extraction, the silica was eliminated by centrifugation and the dissolvable was taken out by evaporation. The weight of the biosurfactant extricates were recorded[11,37].

Results and discussion

Biosurfactant activity analysis:

Purified biosurfactants based on RRBO and RSBO substrates were analyzed for their biosurfactant activity and found positive for oil displacement test, CTAB agar plate test, drop-collapse test, EI_{24} and SFT measurement (Table 1). RRBO based biosurfactant was found quite effective in oil displacement test with average clear zone diameter of 2.1 cm than RSBO based biosurfactant having average clear zone diameter of 1.2 cm. The test result indicated the presence of strong surface active properties in RRBO based biosurfactant. Rath *et al.* (2016) recommended that the lipase movement of *Pseudomonas aeruginosa* culture is fundamentally liable for corruption of triacylglycerol of two vegetable oils to free unsaturated fats, di and mono acyl glycerol which thus go about as forerunner for biosurfactant synthesis[38].

DCM registered complete spreading for RRBO based biosurfactant and partial spreading for RSBO based biosurfactant over the hydrophobic oil film. Both results were considered positive for biosurfactant production though with difference in their concentrations. Most researchers like the current one have applied the DCM for qualitative screening of biosurfactant production on account of its being a sensitive and easy method[1,21,25,29–31,39]. However, according to Bodour *et al.* (1998), the procedure can be applied to quantitative screening of biosurfactant preparation as well. They also gave a reasonable account of the principle underlying in the method. A plain water drop bearing no surfactant will form a bead on a hydrophobic surface because such a surface repels the polar water molecules. On the contrary, when the water drop containing surfactant is placed on the hydrophobic surface, the interfacial tension among the drop and the surface is decreased and the water drop is spread over the hydrophobic surface. The drop spread area depends on concentration of surfactant in drop and its ability to reduce surface and interfacial tension[23]. Youssef *et al.* (2004) found that the DCM is not as sensitive as the ODM in detecting small amounts of biosurfactant production; though its ability to be performed in a microtiter plate makes it possible to carry out multiple analyses simultaneously[29].

CTAB methylene blue agar plate test was found positive for both rhamnolipid biosurfactants as confirmed by creation of dark blue halos around the bacterial colonies. Heyd *et al*. (2008) reported that spot diameter is a function of several key parameters such as rhamnolipid concentration, cell growth of bacteria culture, cultivation time, migration of rhamnolipids and loading level of agar plates[12].

The RRBO and RSBO substrate based two biosurfactants possessed good emulsion stabilizing capacity for HP Milcy Turbo 15W-40 engine oil as can be seen with their $\%EI_{24}$ values of 74.30 and 62.60, respectively. The RRBO based biosurfactant was found having better surface tension reducing ability than RSBO based biosurfactant in 96 h test. The results are satisfactory based on comparison with value of 28 to 27 mN/m reported for *Pseudomonas aeruginosa* in available literature[15,40]. The lowering of surface tension of biosurfactant over a period of time is now a standard test method for selection of biosurfactant producing bacteria and to analyze the ability of biosurfactant produced in lowering of surface tension to an acceptable value[27].

Youssef *et al*.[29] reported to find an inverse linear relationship between the diameter of the clear zone in oil displacement test and the surface tension value of test biosurfactant. As evident from the Table 1, the RRBO based biosurfactant with large clear zone diameter has low surface tension. Similarly, Soltanighias *et al.*[1] found inverse linear

Table 1. Qualitative analysis of biosurfactant produced by *Pseudomonas aeruginosa*

Sr. No.	Name of test	Biosurfactant based on two oil substrates	
		RRBO	RSBO
1.	Oil displacement method	+++ (2.1 cm)	++ (1.2 cm)
2.	Drop-collapse method	+++ (complete spreading)	+ (partial spreading)
3.	CTAB agar plate method	+ (dark blue)	+ (dark blue)
4.	Emulsification index ($\%EI_{24}$)	74.30%	62.60%
5.	Surface tension after 96 h	30.25 mN/m	46.46 mN/m

+ efficient / + + very efficient

relationship between the drop size in drop-collapse test and surface tension of test biosurfactant. The drop-collapse test performed in present study was qualitative only and diameters were not measured, still the results obtained were in general agreement with the observations made in available literature.

Characterization of biosurfactant

Fourier transform infrared spectroscopy:

Figs. 1(A) and 1(B) show FTIR spectrums of dried biosurfactants synthesized using *Pseudomonas aeruginosa* grown on 4% RRBO and 6% RSBO substrates, respectively. FTIR spectrum of the RRBO based biosurfactant disclosed a peak at 3417.22 cm^{-1} which is characteristic of N-H bond stretching vibrations of amine groups[14,22,41]. The adsorption peak ~2926.91 cm^{-1} is supposed to be the asymmetric vibration of CH_2 and CH_3 groups of aliphatic chains. The accompanying symmetric stretch can be noticed at 2856.08 cm^{-1} [5,14,31,35,41,42]. The two characteristic peaks at 1722.59 cm^{-1} and 1651.38 cm^{-1} of C=O in COOH specified the presence of ester carbonyl group in the biosurfactant[4,5,22,30,35,36,41,42]. The strong absorption band at 1572.77 cm^{-1} indicated the vibrations of C-O and C=O bonds in carboxyl esters[30]. The absorption peak at 1402.03 cm^{-1} appeared due to the in plane bending of C-O-H corresponding to carboxylic group[42]. The deformation vibration at 1377.86 cm^{-1} confirmed the presence of unsaturated alkyl group in aliphatic chain[30], whereas the C-O-C stretching vibrations at 1124.47 cm^{-1} marked the ether linkages in the chemical structures of the rhamnose rings[4,30,36]. The vibration at wave number 1068.14 cm^{-1} can be assigned to C-O bonds[5,14]. The absorption peak at 1050 cm^{-1} is indicative of the presence of polysaccharide or polysaccharide-identical substances in the biosurfactant[43]. The prominent peak at 982.65 cm^{-1} was attributed to C-H bonding[31]. Further the absorption at 722.05 cm^{-1} indicated the presence of alkyl groups[4].

FTIR spectrum of the RSBO biosurfactant can be described on similar lines. The absorption peaks at 3392.86 cm^{-1} and 1457.16 cm^{-1} indicated O-H bond stretching vibrations of -OH groups[4,30,35,42]. The peaks at 2956.25 cm^{-1}, 2926.01 cm^{-1}, and 2855.90 cm^{-1} are attributed to C-H bond stretching of CH, CH_2, and CH_3 groups in aliphatic chains[4,5,14,31,35,41,42]. The two peaks at 1723.84 cm^{-1} and 1651.71 cm^{-1} implied the stretching vibrations of C=O in COOH suggesting the presence of ester compound in the biosurfactant[4,5,22,30,35,36,41,42]. The strong absorption band at 1572.45 cm^{-1} expressed the stretching vibrations of C-O and C=O bonds in carboxyl esters[30]. The absorption peaks at 1403.06 cm^{-1} and 1317.14 cm^{-1} were assigned to the C-O bending of ester carbonyl group[35,42]. The C-O-C stretch-

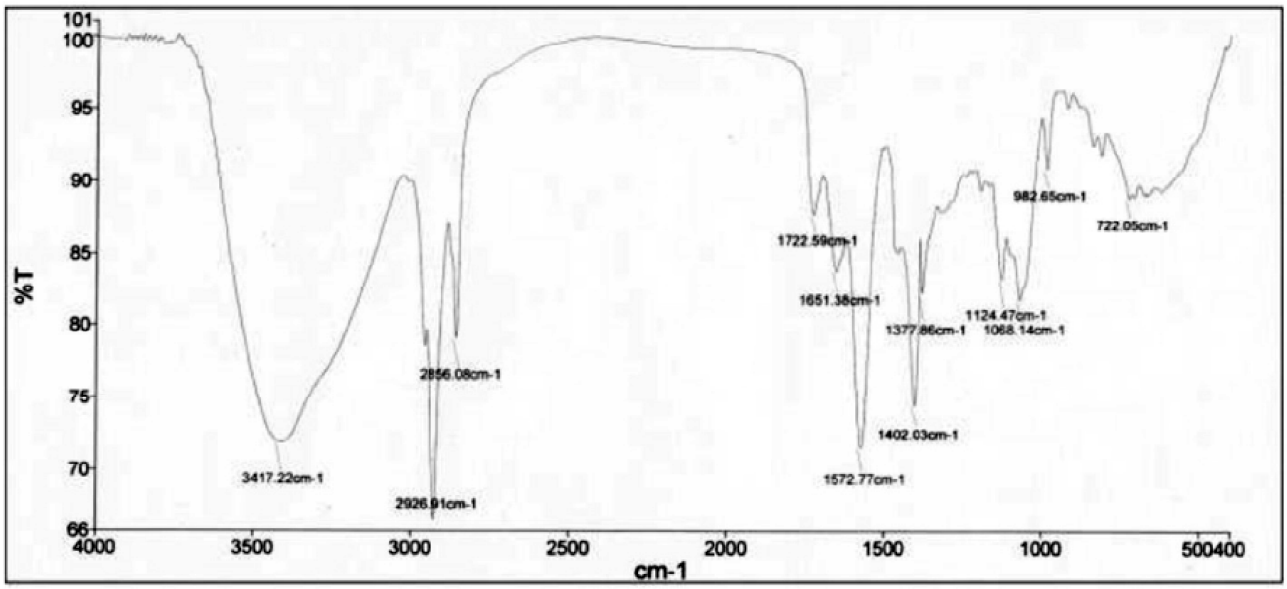

Fig. 1(A). FTIR spectrum of RRBO substrate based biosurfactant produced by *Pseudomonas aeruginosa.*

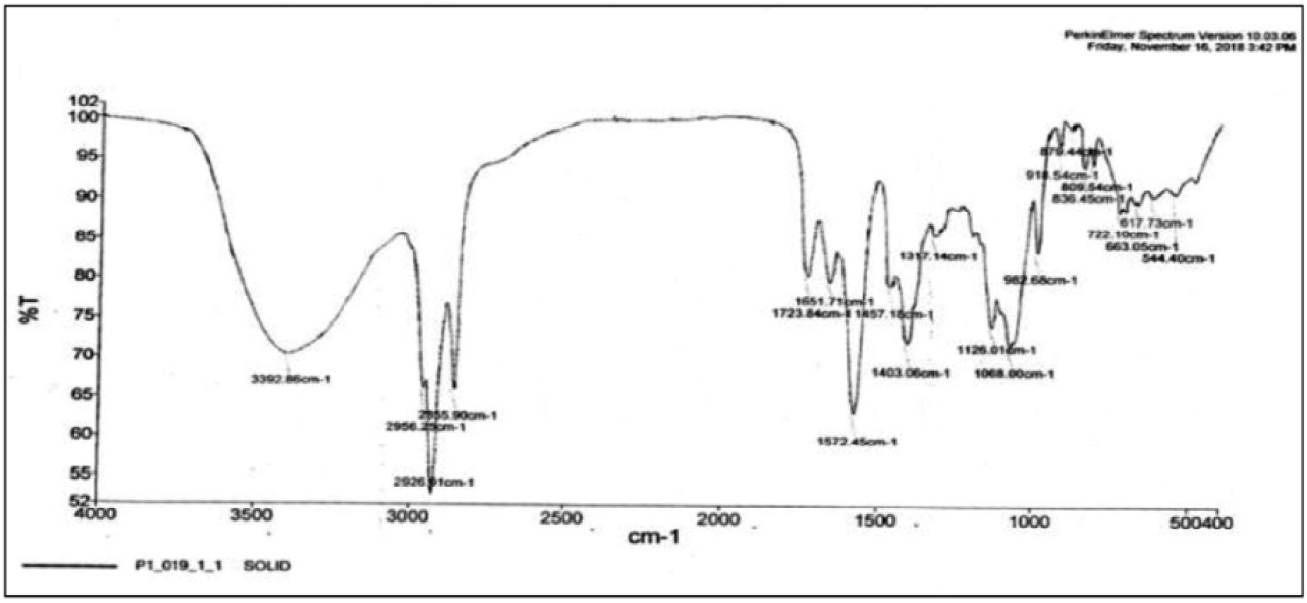

Fig. 1(B). FTIR spectrum of RSBO substrate based biosurfactant produced by *Pseudomonas aeruginosa.*

ing vibrations at 1126.01 cm^{-1} marked the ether linkages in the chemical structures of the rhamnose rings[4,30,36]. The wave number 1068.00 cm^{-1} was assigned to C-O bonds[5,14], while the wavenumber 982.68 cm^{-1} was attributed to C-H bond stretching[31]. The α-pyranyl II sorption band at 836.45 cm^{-1} demonstrated the presence of di-rhamnolipid in the biosurfactant[35]. The band at 809.54 cm^{-1} represented C-H-OOP stretch of aromatic anhydride[25]. Further the absorption at 722.10 cm^{-1} indicated the presence of alkyl groups[4].

Hisatsuka *et al.*[44], da Rosa *et al*.[45], Mahalingam and Sampath[30], and Shekhar *et al.*[25] have reported that the *Pseudomonas aeruginosa* mostly produce lipid-carbohydrate compounds classified as rhamnolipid biosurfactants. FTIR analyses of the two biosurfactants in present study confirmed the findings of earlier studies.

Thin layer chromatography (TLC):

The purified rhamnolipid biosurfactants obtained with RRBO and RSBO carbon substrates were examined using TLC on silica gel plates. The first plate synthesized for RRBO substrate based biosurfactant exhibits the existence of two major spots having R_f values of 0.62 and 0.26 (Fig. 2(A)) indicating the presence of two major homologues in the biosurfactant sample. Similarly, the second plate prepared with RSBO carbon source based biosurfactant confirmed the presence of two spots at R_f values of 0.55 and 0.19 (Fig. 2(B)). These results were then compared with the R_f values of mono- and di-rhamnolipid as mentioned in the available literature. According to Schenk *et al.*[46], Arino *et al.*[47], and Jadhav *et al.*[48], the R_f values of 0.74.0.55 and 0.40.0.19 relate to mono- and di-rhamnolipids, respectively. TLC analysis was thus successful in confirming further that *Pseudomonas aeruginosa* MTCC 424 strain has actually generated and developed rhamnolipid biosurfactant in present study.

Nuclear magnetic resonance (NMR) spectroscopy:

The ^1H and ^{13}C NMR spectroscopic analyses of the two biosurfactants were performed to confirm their rhamnose ring and long hydrocarbon chain structure[12]. The ^1H and ^{13}C NMR spectra of RRBO substrate based rhamnolipid biosurfactants are shown in Figs. 3(A) and 3(B). The characteristic chemical shifts obtained in ^1H NMR spectra were 0.857 ppm for $-CH_3$, 1.232 ppm for $-(CH_2)_5$, 2.742 ppm for -CH(O)-CH_2-COO-, 3.536 ppm for -O-CH- lipid moiety, 4.101 ppm for -OH, and 5.308 ppm for -CH-O-C- on rhamnose moiety. The ^{13}C NMR displayed chemical shifts of 76.773 ppm (characteristic of RL1) and 77.412 ppm (characteristic of RL2). All these results indicated the molecular structure of L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL1) and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxy-

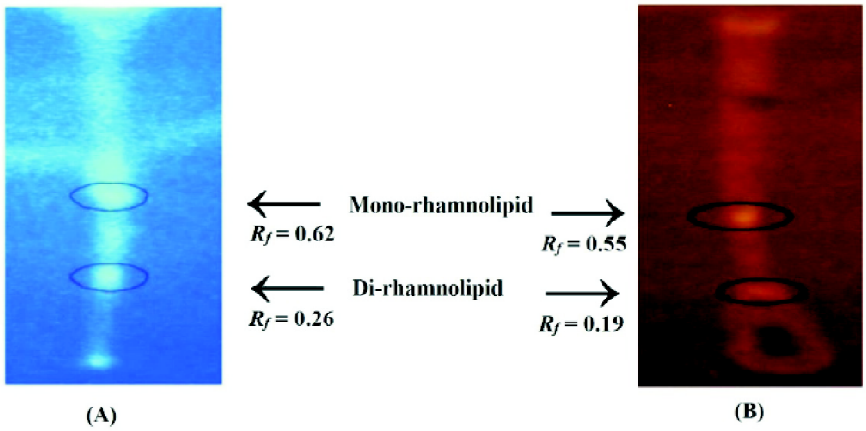

Fig. 2. TLC analysis of RRBO (A) and RSBO (B) substrate based rhamnolipid mixture synthesized by *Pseudomonas aeruginosa* MTCC 424 with chloroform/methanol/acetic acid (65:15:2, v/v/v) developing solvent system.

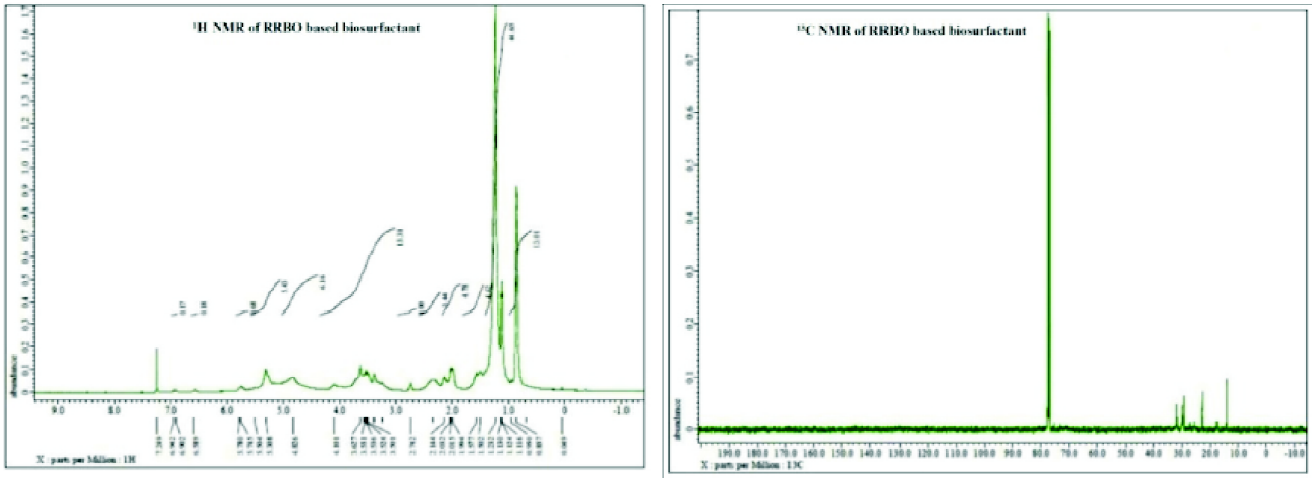

Fig. 3. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of RRBO based biosurfactant.

decanoate (RL2), which are the principal glycolipids synthesized using *Pseudomonas aeruginosa*[12,27]. The ^{1}H and ^{13}C NMR spectra of RSBO substrate based rhamnolipid biosurfactants are shown in Figs. 4(A) and 4(B). The distinguished chemical shifts appeared in two spectra were almost similar to that obtained with RRBO substrate based biosurfactant.

Results of NMR study for both surfactants are summarized in Table 2 and were found comparable with earlier studies of Ramana *et al.*[49], Wei *et al.*[50], Moussa *et al.*[35] and Sharma *et al.*[4].

Electro spray ionization–mass spectrometry (ESI–MS):

Scanning of the two biosurfactants over 100 to 750 *m/z* in the negative ion mode allows for the selection of the different rhamnolipids. The two mass spectrums of targeted rhamnolipid mixtures are shown in Fig. 5. RRBO substrate based rhamnolipid biosurfactant (Fig. 5A) typically produced by *Pseudomonas aeruginosa* was consist of mono-rhamnolipid fragment ions at *m/z* 141.01 (RhaC8) and *m/z* 195.02 (RhaC10C12:1). The spectrum at *m/z* 333.57, 447.23, 475.36, and 503.23 represented mono-rhamnolipid pseudomolecular ions RhaC10, RhaC8C8, RhaC8C10/

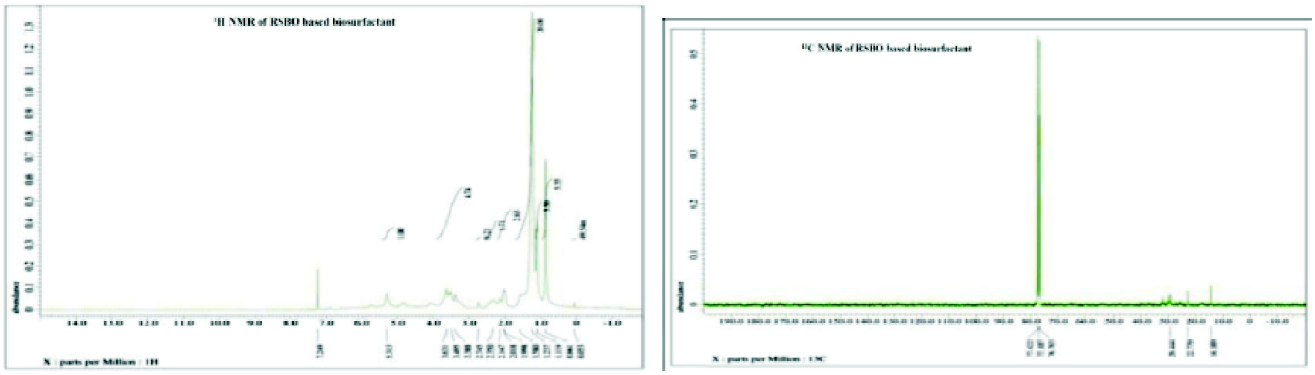

Fig. 4. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of RSBO based biosurfactant.

Table 2. NMR analysis of the two biosurfactants

RRBO substrate based biosurfactant	RSBO substrate based biosurfactant	Analysis
^{1}H chemical shift (ppm)		
0.857	0.861	$-CH_3$ (on β-hydroxy fatty acids)[49,35]
1.232	1.237	$-(CH2)_5-$ (on β-hydroxy fatty acids)[35]
2.742	2.745	$-CH(O)-CH_2-COO-$ (on β-hydroxy fatty acids)[35]
3.536		-O-CH- lipid moiety/C-2′ and C-5′ sugar moiety[5]
4.101	4.100	-OH (D_2O exchange)[49]
4.826		-CH-OH (on rhamnose moiety)[35]
5.308	5.313	-CH-O-C (on rhamnose moiety)[35]
^{13}C chemical shift (ppm)		
76.773	76.783	L-Rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL1)[12]
77.412	77.422	L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxy-decanoate (RL2)[27]

RhaC10C8, and RhaC10C10, respectively. Spectrum at *m/z* 393.19 was detected to be di-rhamnolipid fragment ion (RhaRhaC12:1C12). The two peaks at *m/z* 621.78 and 649.27 were identified as di-rhamnolipid pseudomolecular ions RhaRhaC8C10/RhaRhaC10C8 and RhaRhaC10C10, respectively[4,12,28].

RSBO substrate based biosurfactant showed somewhat complex structure (Fig. 5B). The peak at *m/z* 141.03 was identified that of mono-rhamnolipid fragment ion (RhaC8)[12]. The two adjacent peaks at *m/z* 353.99 and 354.07 represented rhamnolipid homologues with weak molecular ions[42]. The peak at *m/z* 621.86 was attributed to di-rhamnolipid pseudomolecular ion (RhaRhaC8C10/RhaRhaC10C8)[12], whereas the peak at *m/z* 680.66 designated dirhamnolipid homologue (RhaRhaC10C12)[35]. Spectrum at *m/z* 663.45 indicated the presence of carbohydrate moiety in biosurfactant structure[42].

Mass spectrometric examination of two biosurfactants substantiated the existence of mono- and di-rhamnolipid structures in agreement with numerous previous studies. The opinion among researchers is divided on whether the mono-rhamnolipids are predominant components or the di-rhamnolipids are predominant components in rhamnolipid surfactant mixtures[35]. In our study, the RRBO substrate based biosurfactant was found rich in di-rhamnolipid species as opposed to RSBO substrate based biosurfactant enriched in mono-rhamnolipid species. However, many peaks in RSBO substrate based biosurfactant were left unidentified poten-

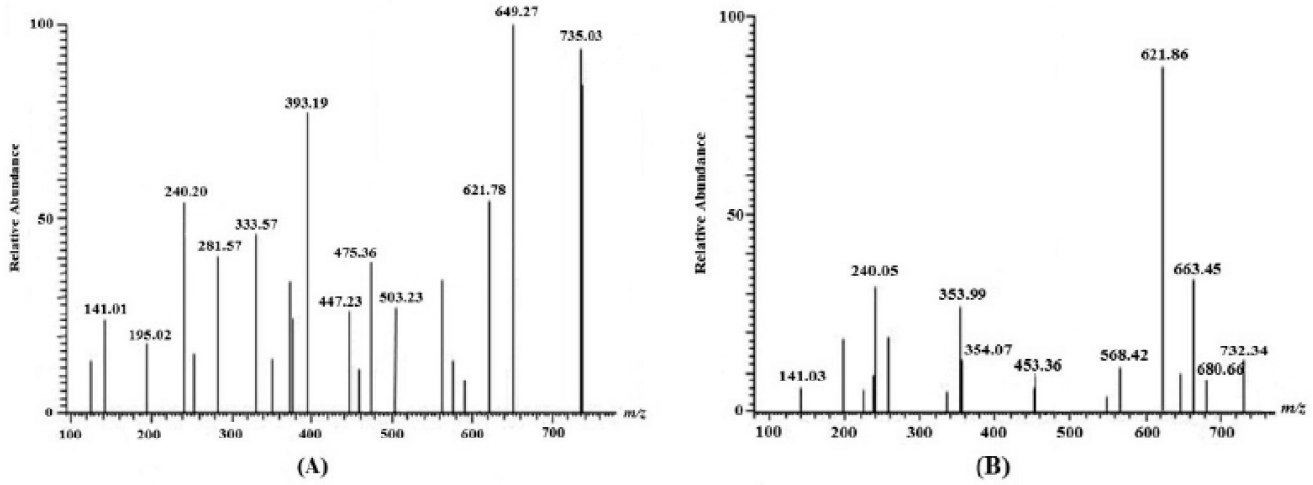

Fig. 5. ESI-MS spectrum of purified rhamnolipid mixtures produced by *Pseudomonas aeruginosa* cultures using RRBO (A) and RSBO (B) carbon substrates.

tially indicating towards poor surfactant structure, a fact already proved by previous tests in present study.

Electrospray ionization-mass spectrometry (ESI-MS) of RRBO and RSBO substrates:

Several well defined groups were identified in ESI-MS fingerprints of RRBO and RSBO through comparison with data reported by other researchers. In ESI-MS spectra of RRBO (Fig. 6A), the *m/z* 149.0249 and *m/z* 319.1689 were attributed to cinnamic acid and 4-norlempein aglicone, respectively[34]. The ions at *m/z* 575.5056, 599.5067, and 601.5195 conformed to diacylglycerol fragments of PL^+, LL^+ or OLn^+, and LO^+, respectively[51]. The spectra at *m/z* 853 reciprocated to PPL (C50:2), *m/z* 855 to PPO (C50:1), *m/z* 877 to PLL (C52:4), *m/z* 879 to PLO (C52:3), *m/z* 899 to LLLn or OLnLn (C54:7), *m/z* 901 to LLL or OLLn (C54:6), and *m/z* 915 to LLLn or OLnLn. Here O stands for oleic acid, L for linoleic acid, Ln for linolenic acid, and the P stands for palmitic acid[52]. In ESI-MS spectra of RSBO (Fig. 6B), the *m/z* 149.0259 was attributed to cinnamic acid[34]. The *m/z* 263.2376 was considered to be RCO^+ ion reflecting linoleic fatty acid[51]. The ions at *m/z* 279.2329 and 377.2672 were regarded to be deprotonated linoleic acid (C18:2)[33] and hydroxytyrosillenoate[34], respectively.

One important deduction can be made from this interpretation of ESI-MS data of the two substrate oils. For RRBO, it became possible to identify multiple groups with the help of reports of previous studies by other researchers, but for RSBO, hardly a few chemical groups were identified possibly indicating towards non-lipid character of soybean oil extracted from spent bleaching earth in this study. This might also be the principal reason for smaller production of biosurfactant even with higher percentage of soybean oil (6%) in comparison to ricebran oil (4%).

Factors affecting biosurfactant production

Various physicochemical factors like carbon and nitrogen source, variations in pH and temperature of the growth medium, oxygen flow rate, agitation speed, etc. affect biosurfactant production. Santos *et al*. (2016) have reported 30°C, in general, as the most favorable temperature for biosurfactant production by different bacterial strains[6]. The temperature range of 32.36°C is found to be most favorable particularly for *Pseudomonas aeruginosa* culture. The agitation speed of the growth medium has a decisive effect on biosurfactant production possibly by application of strain that provides an effective phase mixture and oxygen transfer[15]. In this study, the temperature in incu-shaker was maintained at 32°C, the agitation at 100 rpm, and the air flow at 3 Lpm. Other parameters like carbon source concentration, pH of the medium, and incubation time were varied in order to study their effect on rhamnolipid biosurfactant production.

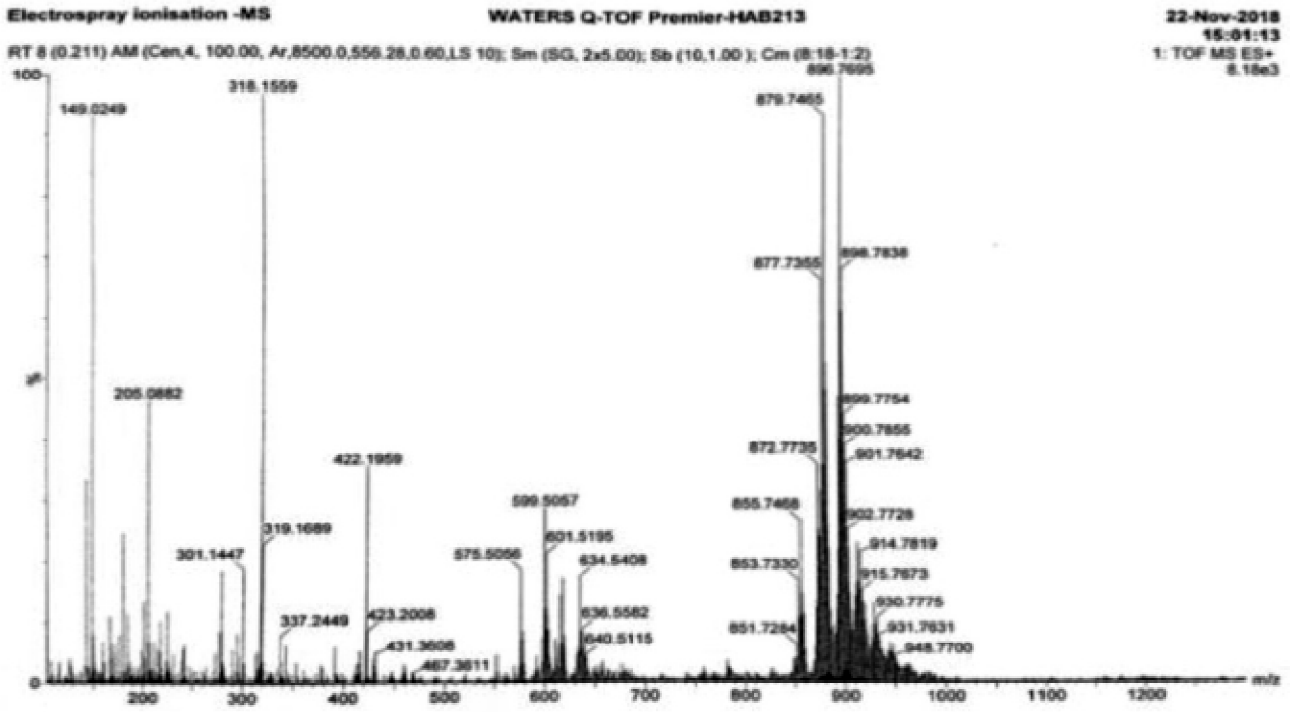

Fig. 6(A). ESI-MS spectra of RRBO substrate.

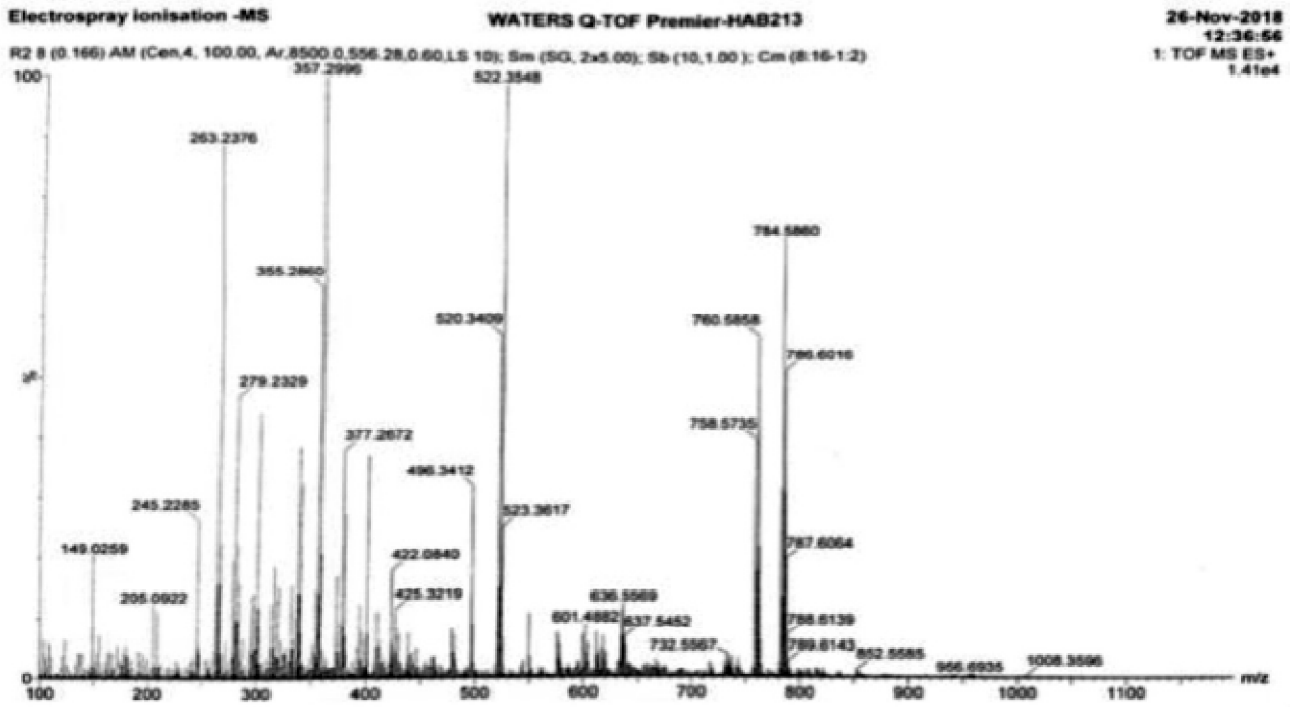

Fig. 6(B). ESI-MS spectra of RSBO substrate.

Effect of carbon source on biosurfactant production:

The need of a carbon source for successful growth and production of biosurfactants by microorganisms is beyond question[6]. Previous studies have found that biomass does not grow in absence of carbon source[53]. In present study, RRBO and RSBO were used as cheap carbon sources in order to economize the large scale biosurfactant production[5]. The RRBO concentration of 4% obtained maximum rhamnolipid yield of 8.5 g/L by *Pseudomonas aeruginosa*. When RSBO was used as carbon source, the maximum rhamnolipid yield of 3.6 g/L was obtained at 6% carbon source concentration (Table 3). Though RRBO proved to be a better carbon source than RSBO in this study, the results validated the possibility of industrial scale biosurfactant synthesis using both RRBO and RSBO as low cost carbon sources.

Effect of pH on biosurfactant preparation:

The ideal pH of the growth medium for highest biosurfactant yield by *Pseudomonas aeruginosa* MTCC 424 using RRBO was reported to be 6.8. The biosurfactant production at this pH was 8.5 g/L. There was a sharp decrease in rhamnolipid concentration beyond this pH. The ideal pH for maximum biosurfactant generation with RSBO was reported to be 7.0 and the biosurfactant concentration was 5.0 g/L (Fig. 7).

Table 3. Amount of rhamnolipid produced at different concentration of RRBO and RSBO substrates

RRBO concentration (%)	Rhamnolipid concentration (g/L)	RSBO concentration (%)	Rhamnolipid concentration (g/L)
1	1.0	1	2.0
2	3.9	2	2.4
3	6.0	3	2.8
4	8.5	4	3.0
5	5.2	5	3.2
–	–	6	3.6
–	–	7	3.5

Effect of incubation time on biosurfactant production:

The ideal incubation time for maximum rhamnolipid production using *Pseudomonas aeruginosa* MTCC 424 was found to be 7 days for both RRBO and RSBO carbon source based growth mediums. The maximum biosurfactant yield with RRBO was 8.0 g/L and 7.6 g/L with RSBO at optimum incubation time. Thereafter there was a reduction in biosurfactant concentration in both samples (Fig. 8).

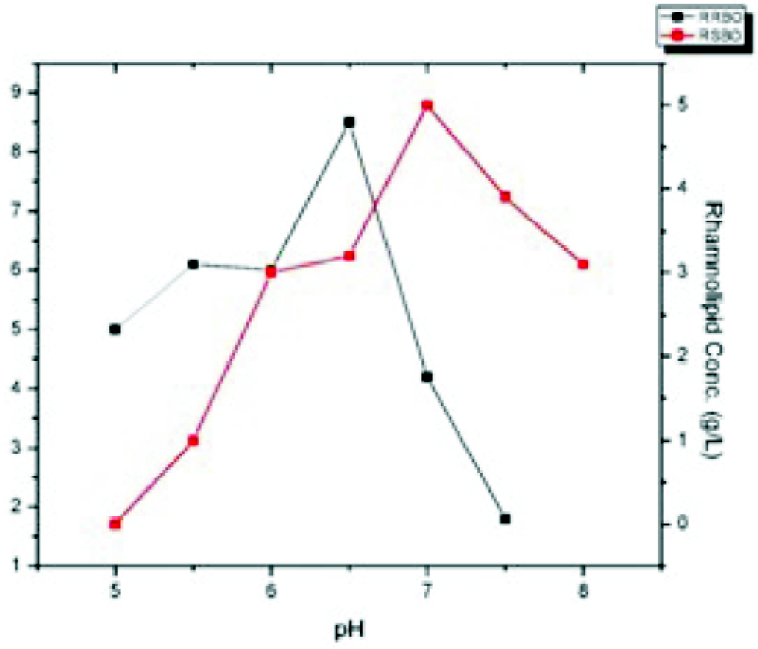

Fig. 7. Effect of pH on biosurfactant preparation using RRBO (4%) and RSBO (5%) as carbon source for incubation period of 7 days.

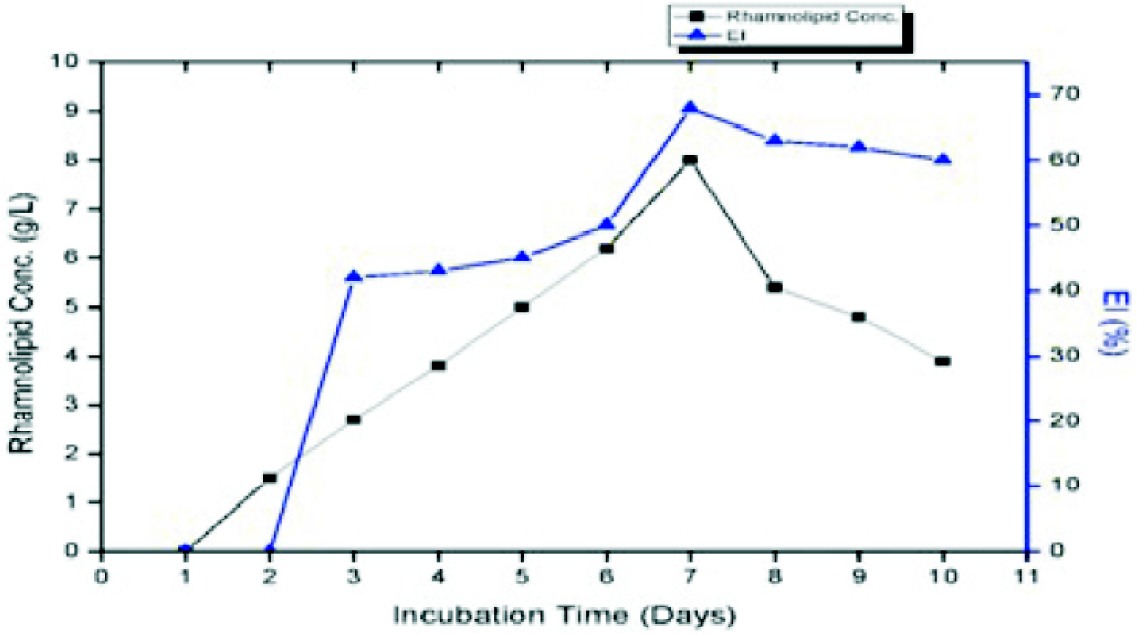

Fig. 8. Effect of incubation time on rhamnolipid production and emulsification index (%) with RRBO (4%) and RSBO (6%) as carbon source.

Conclusion

The two rhamnolipid biosurfactants were synthesized using *Pseudomonas aeruginosa* strain using RRBO and RSBO as low-cost substrates. Following conclusions were made out of this study:

(i) RRBO based biosurfactant was more effective than RSBO based biosurfactant in oil displacement test with larger clear zone diameter.

(ii) RRBO based biosurfactant displayed complete spreading over the hydrophobic film in drop-collapse test, whereas the RSBO based surfactant displayed partial spreading under the same conditions.

(iii) CTAB methylene blue agar plate test was positive for both biosurfactants.

(iv) RRBO based biosurfactant posted better emulsion stabilizing capacity than RSBO derived biosurfactant with same mineral engine oil.

(v) RRBO based biosurfactant registered larger reduction in surface tension than RSBO based biosurfactant in standard 96 h test.

The RRBO was found to be a better source of nutrition than RSBO for biosurfactant production. The oil used in this study is very low-priced as it was extracted from the spent bleaching earth discarded in the edible oil processing industries.

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