Research Article

Physicochemical Characterization of Rhamnolipids from Novel Strains of *Acinetobacter boumanii* and *Acinetobacter variabilis*

Mrs. Rutuja Ankulkar*, Dr. Meera Chavan
Walchand College of Arts and Science, Solapur, Maharashtra, India.
***Corresponding author's E-mail:** rutuja.govardhan@gmail.com

Received: 22-08-2017; **Revised:** 28-09-2017; **Accepted:** 15-10-2017.

ABSTRACT

A novel rhamnolipid producing *Acinetobacter boumanii* and *Acinetobacter variabilis* strains were isolated from oil contaminated soil, near refinery area. Biosurfactant compound was separated by solvent extraction of the cell free broth. Semi purified biosurfactant of *Acinetobacter boumanii* and *Acinetobacter variabilis* categorized as rhamnolipid using TLC was able to reduce the surface tension of water from 72 to 43 mN/m^{-1} and 72 to 50 mN/m^{-1} respectively. Shake flask biosurfactant production studies *of Acinetobacter boumanii* and *Acinetobacter variabilis* could produce 1.2g/l and 0.921g/l biosurfactant along with E24 of 44± 0.3 and 40±0.5 at 72 hr. Fourier transform infrared spectroscopy revealed the presence of a highly charged amine, carbonyl, carboxyl and hydroxyl groups. Presence of sixteen rhamnolipid homologues with variation in chain length was revealed from liquid chromatography coupled to mass spectrometry. Further, it elucidated highest relative abundance of Rha-C10:C12 and Rha-C8:2 in both strains, along with unique and major isoform of Rha-Rha-C14: C12 in *Acinetobacter variabilis*.

Keywords: *Acinetobacter spp.*, Biosurfactants, Congeners, FTIR analysis, Glycolipids, LC/ESI-MS analysis, Rhamnolipids.

INTRODUCTION

Biosurfactant is biologically synthesized surfactant produced by microorganisms. It is the secondary metabolite synthesized by *Pseudomonas spp.*, *Acinetobacter spp.*, and *Enterobacter spp.* [1, 2]

In comparison with Petrochemical and oleo-chemical surfactants, biosurfactant are suitable as are environmentally friendly and biodegradable.[3] They carry low or non-toxicity, effective surface activity, reusability, and high specificity. Also, they have a broad range of applications in various areas like hydrocarbons, waste products degradation, bio-remediation, agriculture, health care, cosmetics, food and waste utilization.[4] They are effective at wide range of $_pH$ and temperature.[5, 6] The worldwide average annual growth rate of surfactant is approximately 3% along with its market capture of approximately 15 million tones/annum.[7,8] Biosurfactants have been the subject of increasing attention because of their lower toxicity and higher biodegradability compared to their synthetic chemical surfactants. Despite of their many commercial applications, their large-scale production has not been possible because of the low yields and high production costs.[9]

Rhamnolipids are glycolipid biosurfactant by type.[10] Rhamnolipids may play multiple roles in the life of microorganisms.[11] Particular biosurfactant purpose seems to be connected with environmental surroundings and corresponding regulatory procedures. It is very different subject matter for study. In the view of most current studies, rhamnolipid should be considered as a versatile segment of mechanism which commands the fundamental component of microbial life. The consideration supporting this mechanism is more suitable and more apparent with each sequential scientific study. Moreover, studies emphasis on acquiring a superior assumption and understanding of the proposition of microbial community will surely bring about diverse new possible applications for rhamnolipids.[11]

Acinetobacter spp. is one of the groups; very well studied for biosurfactant and bio emulsifier production.[12]It can produce mono and di-rhamnolipid biosurfactants.[13] Physiologically, they are Gram negative bacilli which are strictly aerobic and non-fermentative in nature[14] and can sustain in a broad range of temperature and environmental conditions.[15] Rhamnolipids are surface-active glycolipid of microbial origin, which have been extensively studied by numerous scientists all over the world.[11]

Biosurfactant belongs to high and low molecular weight class. Glycolipids, lipopeptides, and phospholipids are three subgroups which belong to low molecular weight class. Whereas, polymeric and particulate surfactants fall under high molecular weight class.[16] Bacteria like *Acinetobacter spp.* can produce both classes of biosurfactant, which are rhamnolipidic biosurfactant by type.[13, 17]

This work is aimed at isolation of novel biosurfactant producing bacteria and physicochemical characterization of produced biosurfactant. Qualitative and quantitative evaluation of various strains for biosurfactant production was concluded with an identification of *Acinetobacter variabilis* and *Acinetobacter boumanii*. To our knowledge; this is the first report of the production of rhamnolipid biosurfactant by *Acinetobacter variabilis*. In context to this; ten different congeners were detected in *Acinetobacter variabilis* followed by eight in

Acinetobacter boumanii. The highest relative abundance of Rha-C10: C12 and Rha-C8:2 in both species of *Acinetobacter* along with presence of the unique, major isoform of Rha-Rha-C14: C12 in *Acinetobacter variabilis* expands the scope for future research to unfold deep insights about the same.

MATERIALS AND METHODS

Sample collection and processing

The sample was collected from oil contaminated soil near refinery area, Pune. It was followed by its enrichment in MSM broth containing $NaNO_3$ (Himedia) 15g/l; KCl (Himedia) 1.1g/l; NaCl (Himedia)1.1g/l; $FeSO_4$ (Himedia) 0.00028g/l; KH_2PO_4,$3H_2O$ (Himedia) 3.4g/l; K_2HPO_4 (Himedia) 4.4g/l; $MgSO_4$ (Himedia) 0.5g/l; Yeast Extract (Himedia) 0.5g/l; and 20 g/l soya oil as the sole carbon source. For this, 1g of soil was inoculated into 100 ml of MSM broth and incubated at 37°C for 24 hr.

Screening and Isolation of biosurfactant producing bacteria

1 ml of enriched broth was diluted serially in 0.85% saline and was spread inoculated over nutrient agar. It was followed by incubation at 37 °C for 24 hr. Strains obtained in primary screening were purified using nutrient agar and screened for qualitative detection of biosurfactant production. Oil displacement test, hemolysis test, and blue agar test were used for this evaluation.

Oil displacement test was performed in a petri dish using 20µl oil. To this, 20ml of deionized water was added to maintain layer uniformity. 10µl culture of each of selected strain was suspended over oil layer and observed for clear zone formation which was an indicator used for biosurfactant production.

Hemolysis test was conducted using blood agar medium. Pure culture of each of selected strain was streak inoculated over blood agar, followed by incubation at 37 °C for 24 hr. Alpha and or beta hemolysis was used as a standard for biosurfactant production.

Blue agar test was performed using Cetyltrimethyl-ammonium bromide (CTAB)-methylene blue agar medium. It was prepared by adding CTAB(Himedia) 0.2g/l, methylene blue (Himedia)0.005g/l, and agar-agar (Himedia) 15g/l in Minimal Salt medium (MSM) with 20g/l glucose as a sole carbon source. Activity testing was performed in 5mm well with inoculation of 50µl of 48 hr fermented broth, followed by incubation at 37 °C for 24-72hr. Dark blue color zone formation was used as an indicator for biosurfactant production.

Comparative analysis of secondary screening and its results evaluated the performance of selected strains. Thereby, strains showing consistent, reproducible and highest biosurfactant production were finalized for shake flask fermentation and physicochemical characterization of biosurfactant.

16srRNA gene sequencing and phylogenetic analysis

Bacterial DNA was extracted using Wizard® Genomic DNA Purification Kit. To amplify the 16S rRNA gene, polymerase chain reaction (PCR) was performed using a pair of 27F and 1492R primers. PCR was performed in 25ul volume using 0.4 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 200 uM each), 0.75 U Taq DNA polymerase, supplied with buffer having 3 mM MgCl2 and water. The PCR mixtures were preheated at 95 ^{0}C for 5 min prior to running the following cycles: 95^{0}C, 30 s; 51^{0}C, 15 sec; 72^{0}C, 2 min. The PCR was run for 30 cycles in a Bio-Rad thermal cycler. At the end of the final cycle, a chain elongation step at 72^0 C for 5 min was programmed. The PCR products were purified by standard methods and directly sequenced with primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing was completed by First Base Malaysia. The 16S rRNA sequence of strains RA3 and RA4 were subjected to a similarity search BLAST on the NCBI website (http://www.ncbi.nlm.nih.gov).The phylogenetic tree was constructed accordingly.

Shake Flask Fermentation studies for biosurfactant production

Inoculum preparation

Nutrient broth was used as a medium for inoculum preparation. 10% culture was inoculated into 100ml nutrient broth followed by its incubation at 37°C for 16 hr. It was observed microscopically for its purity and consistency. Culture density of inoculum was determined at 660_{nm} using UV –visible spectrophotometer (Agilent).

Fermentation

Fermentation studies were performed in 1 lit Minimal Salt Medium (MSM) containing $NaNO_3$ (Himedia) 15g; KCl (Himedia) 1.1g; NaCl (Himedia) 1.1g; $FeSO_4$ (Himedia) 0.00028g; KH_2PO_4,$3H_2O$(Himedia)3.4g; K_2HPO_4 (Himedia) 4.4g; $MgSO_4$ (Himedia) 0.5g; Yeast Extract (Himedia) 0.5g; and 20 g soya oil as the sole carbon source; added before sterilization. 1ml of glycerol stock was used as an inoculum for fermentation and it was performed at 37°C for 96 hr. Sampling was done at the interval of 24hr till 96hr and was analyzed for $_P$H (Figure 3: a) microscopically for purity, and spectrophotometrically (at 660_{nm}) for cell density (Figure 3: b). Biosurfactant production in fermented broth was monitored in a qualitative mode using oil displacement test. In particular, presence of rhamnolipidic biosurfactant in fermented broth and its concentration was estimated using orcinol method. Its details are as described below.

Detection of Biosurfactant production

Qualitative detection

Biosurfactant production was checked with oil displacement test using methodology as described above. Fermented broth of *Acinetobacter sp*. was centrifuged at

12000 rpm for 15 min. 10 µl of supernatant was suspended over the top of oil layer and observed for clear zone formation. Its data is shown in Figure: 4(a) and (b)

Quantitative detection

Quantitative detection was performed with purified rhamnolipids for which; fermented broth was processed as described above. 0.5ml of supernatant was treated with 1ml C: I (2:1v/v) for phase separation. In this, organic phase was evaporated till complete drying and suspended with 0.2ml of water. To 0.1ml of each of the sample, 0.9ml of a solution containing 0.19% orcinol (prepared in 53% H_2SO_4) was added.

100µl water, taken as a blank was also treated with 0.9ml orcinol reagent. Standard solutions of L-rhamnose between 0-50ug/ml concentrations were prepared with water and treated with orcinol reagent. Sample, standard, and blank were boiled at 80 °C for 30 min and cool down to room temperature. Optical density was measured at 421_{nm}. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose and were expressed as rhamnose equivalents.

Characterization of Rhamnolipid biosurfactant

Surface Tension Measurement

The surface tension of cell-free medium was determined at the interval of 24hr till 120hr for strains RA3 and RA4 using stalagmometer at a constant temperature 30 ± 2 °C and calculated by the following formula where σ_{H2O}= surface tension of reference solvent (for water 71.18 ± 0.02 mN/m), σ= surface tension of the test BS solution, *m H2o*= drop numbers of the reference solvent, *m*= drop numbers of the test BS solution. All surface tension values were recorded in triplicates [18,19]. Its data is shown in Figure: 5(a)

$$\sigma = \sigma_{H2O}.m\ /m_{H2O}$$

Emulsification Index (E_{24})

The emulsification index of cell free medium was determined using 2ml of crude oil and an equal volume of cell free supernatant. It was homogenized in a vortex at high speed for 2 min. It was verified using 0.1 % SDS as a positive control and water as a negative control. The emulsification stability was measured at the interval of 24 hr. Emulsification index was calculated as per the formula stated below [20]. Its data is show in Figure: 5 (b)

E_{24} (%) = (Total height of the emulsified layer/ Total height of the liquid layer) × 100

Thin Layer Chromatography

Bio surfactants on crude extract by TLC on precoated silica gel of standard 20 × 20 Kiesel-gel 60 F254 Merck plates using the appropriate solvent system and visualization agent for biosurfactant. In the case of RLs, the solvent system used was Chloroform: Methanol: Acetic acid (65:15:2 v/v/v), the spray agent was anthrone[21]. Its data is shown in Figure: 6.

Fourier Transformation Infrared Spectroscopy

Semi purified rhamnolipids were used for FT-IR analysis. The FT-IR spectra were recorded on a Perkin-Elmer 31725 X FTIR spectrophotometer in a spectral region of 4000-400cm^{-1} using Potassium bromide (KBr) solid cells. Spectra were recorded and analyzed using standard method [22, 23]. Its data is shown in Figure: 7 (a) and (b).

Liquid Chromatography-Mass Spectrometry analysis

Mass spectra of rhamnolipids were recorded on MS system consisting of an HPLC (Agilent 1200 Series, Agilent Technologies) and a 6210 Time-of-Flight LC/MS (Agilent Technologies), using Zorbax Eclipse Plus C18 column and a DAD detector. The mobile phase was a mixture of solvent A (0.2 % formic acid in water) and solvent B (acetonitrile) in a gradient mode: 0–1.5 min 95 % A, 1.5–12 min 95–5 % A, 12–15 min 5 % A, 15–16 min 5–95 % A.Its data is shown in Figure: 8-12.

RESULTS AND DISCUSSION

Screening of biosurfactant producing microorganisms

Among 24 strains obtained, qualitative detection tests for biosurfactant production seen negative for strains RA1, RA2, RA11, and RA12. Strains RA5 to RA7 could only show positive Hemolysis. Whereas, strains RA8 and RA9 could only give positive drop collapse test. RA10 was only strain found positive for oil displacement. Strains RA3 and RA4 were only strains shown positive blue agar test. The activity of RA3 was seen highest for all qualitative detection tests followed by RA4. Irrespective of this, strain RA1 to RA21 showed growth in MSM broth in 24 hr. Only strains RA22 to RA24 neither grown in MSM broth nor shown positive results for any of the qualitative test performed. Here, water was used as a negative control for analysis. Its data is shown in Table: 1.

16srRNA gene sequencing and phylogenetic analysis

Strains RA3 and RA4 were identified using the 16srRNA gene sequence with a 1431 bp and 1440 bp fragment, which was aligned with sequences from the GenBank database and checked manually. It was 97% similar to the 16SRNA gene of species *Acinetobacter boumanii* and *Acinetobacter variabilis.* A phylogenetic tree was constructed using sequence distance method. Based on the above characteristics, strains RA3 and RA4 were identified as *Acinetobacter boumanii* and *Acinetobacter variabilis.* Its details are presented in Figure: 1 and Figure 2.

Table 1: Qualitative detection of biosurfactant production by 24 shortlisted strains

Shortlisted Strains	Growth in MSM	Oil Displacement Test	Hemolysis Test	Blue Agar Test	Drop Collapse Test
RA1	+	-	-	-	-
RA2	+	-	-	-	-
RA3	+	+++	+	++	++
RA4	+	+	+	++	+
RA5	+	-	+	-	-
RA6	+	-	+	-	-
RA7	+	-	+	-	-
RA8	+		-	-	+
RA9	+	-	-	-	+
RA10	+	+	-	-	-
RA11	+	-	-	-	-
RA12	+	-	-	-	-
RA13	+	+	+	-	+
RA14	+	-	+	+	+
RA15	+	+	-	-	+
RA16	+	+	+	-	-
RA17	+	+	-	-	+
RA18	+	+	-	-	+
RA19	+	+	-	-	+
RA20	+	+	+	-	+
RA21	+	+	+	-	-
RA22	-	-	-	-	-
RA23	-	-	-	-	-
RA24	-	-	-	-	-
Negative Control	-	-	-	-	-

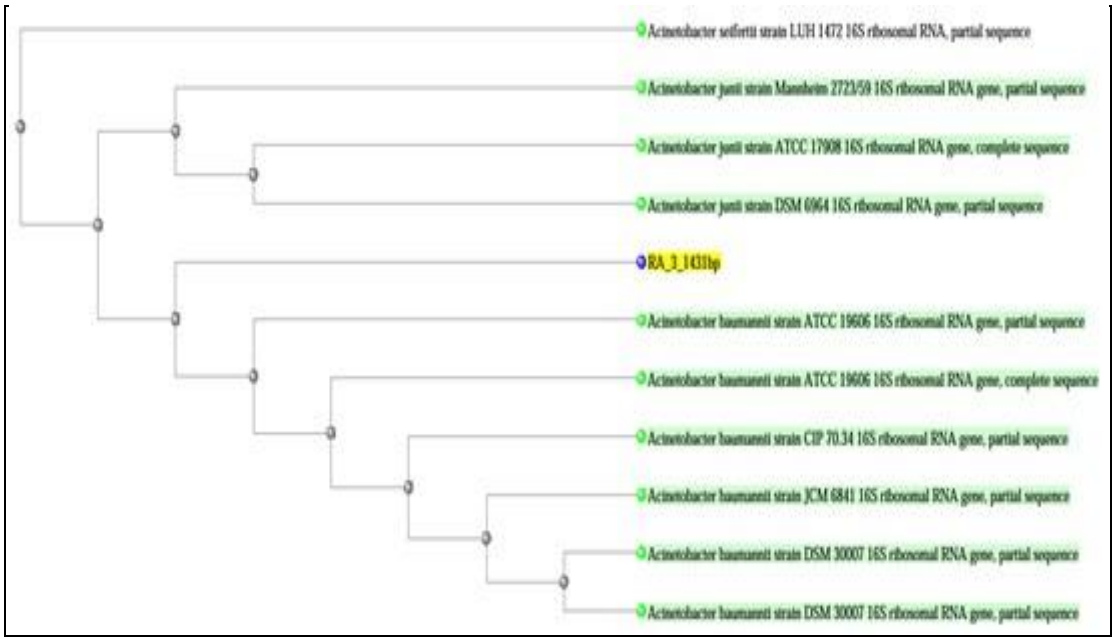


Figure 1: 16srRNA gene sequencing and Identification analysis for RA3

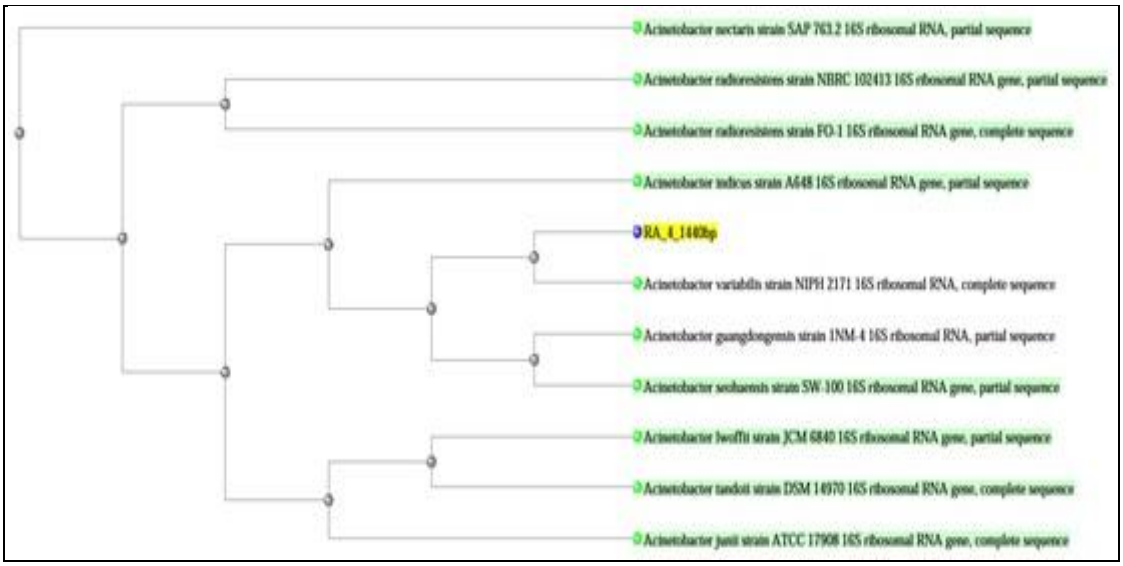
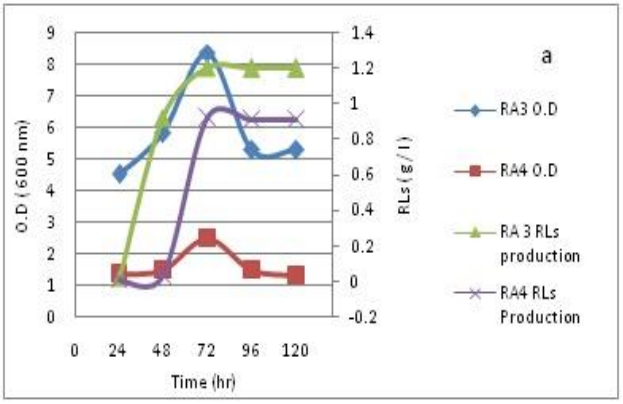


Figure 2: 16srRNA gene sequencing and Identification analysis for RA 4

Fermentation studies with *Acinetobacter boumanii* and *Acinetobacter variabilis*

Acinetobacter boumanii and *Acinetobacter variabilis* were cultivated in MSM with 2% soya oil as a sole carbon source; biomass growth (determined as O.D. at 660_{nm}), $_{p}$H and rhamnolipid production were measured during the cultivation (Figure: 3a and 3b). Rhamnolipid production was determined at five cultivation times with 24hr interval. Highest rhamnolipid production was observed at 72hr for both strains with (1.2 g/l) for *Acinetobacter boumanii* and (0.921g/l) *for Acinetobacter variabilis* (Figure: 3a).The kinetics of growth had a different progress in both cases where highest O.D. obtained at 72hr for both strains was 8.3 and 2.5 respectively. Both strains showed a gradual decrease in $_{p}$H at the end of the exponential phase (72hr) (Figure: 3b). Fermentation broth (72hr) of both strains showed the large diameter of clear zone in oil displacement activity which is an indirect indicator of the high surface activity of rhamnolipid against soya oil (Figure: 4a and 4b).



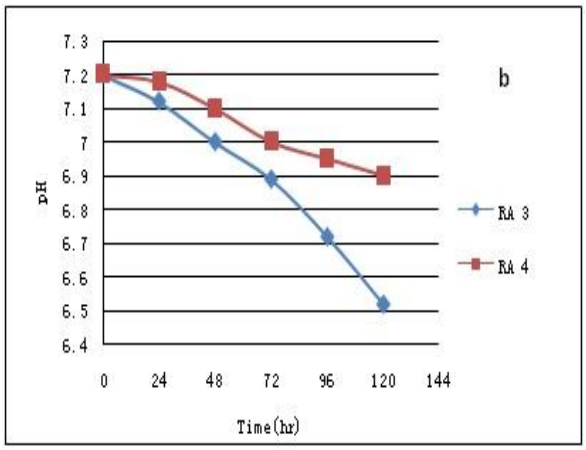


Figure 3: (a) O.D. and RLs production profile studies (b)$_{p}$H profiling of fermentation studies with *Acinetobacter boumanii* (RA3) and *Acinetobacter variabilis* (RA4)

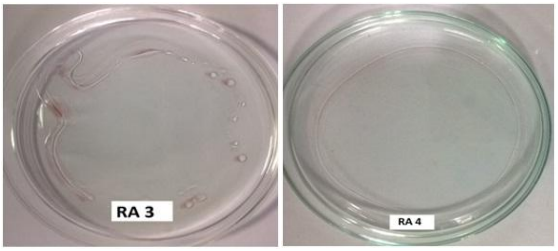


Figure 4: (a) Qualitative detection of biosurfactant production by *Acinetobacter boumanii* (RA3) **(b)** Qualitative detection of biosurfactant production by *Acinetobacter variabilis* (RA4)

Characterization of Rhamnolipid biosurfactant

Rhamnolipid biosurfactant production of *Acinetobacter boumanii* and *Acinetobacter variabilis* was studied in MSM with 2% soya oil as a sole carbon source. Surface tension (mN/m^{-1}) was determined at five processing times with 24hr interval; equivalent and highest surface tension reduction were observed at 120hr for both strains. In comparison, *Acinetobacter boumanii* showed highest surface tension reduction up to (43 mN/m^{-1}) (Figure: 5a).

Emulsion indices tested with crude oil (of 72hr) were observed as highest and equivalent for *Acinetobacter boumanii* (E24=44) and *Acinetobacter variabilis* (E24=40) (Figure: 5b).

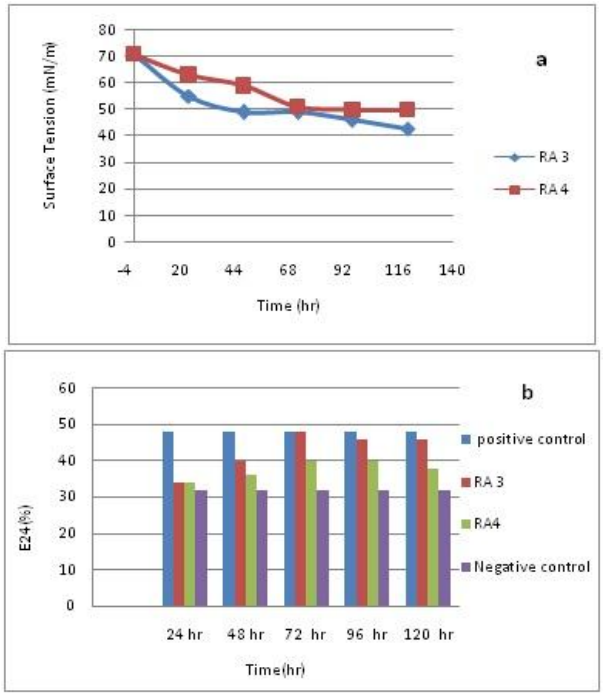


Figure 5: (a) Surface tension reduction **(b)** Emulsification index analysis by *Acinetobacter boumanii* (RA3) and *Acinetobacter variabilis* (RA4)

Thin Layer Chromatography

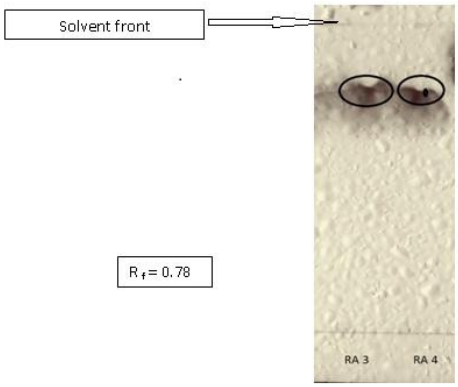


Figure 6: Rhamnolipids TLC detection

Biosurfactant extracted from cell-free broth was analyzed by thin-layer chromatography (TLC) and visualized with specific reagents. Spots with similar Rf (retention factor) obtained were as shown in Figure: 6. Single spot with Rf value of 0.78 obtained for *Acinetobacter boumanii* and *Acinetobacter variabilis* indicated about presence of rhamnolipid having monorhamnolipidic composition.[21]

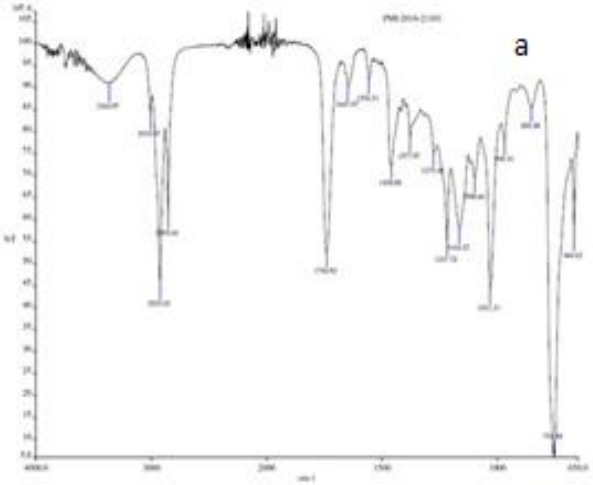
Fourier Transformation Infrared Spectroscopy

The functional groups of biosurfactant were confirmed by FT-IR spectra of *Acinetobacter boumanii* (RA3) and *Acinetobacter variabilis* (RA4) revealed that a heterogeneity evidenced by different characteristic peaks; was in agreement with the possible presence of amino, carboxylic, hydroxyl and carbonyl groups.

The FT-IR range of *Acinetobacter boumanii* (RA3) has the specific stretching vibration band of –OH, throughout 3364cm^{-1}. The potent peaks at 3010cm^{-1} and 1647cm^{-1} were originated by the bending and stretching of –CH and -C=O groups respectively. Immersion around 2925 cm^{-1} is specified to the asymmetric C-H stretch of CH_2 and CH_3 groups of aliphatic chains. The corresponding uniform stretch is seen at 2854cm^{-1}. Also, a weak symmetric stretching peak at 1740cm^{-1} demonstrated the existence of ester carbonyl group (C=O in COOH) in the biosurfactant. The band at 1458 cm^{-1} be compatible to C-O-H in the plane binding of carboxylic acid (-COOH). The ester carbonyl group was demonstrated by the band at 1377cm^{-1} which corresponds to C-O deformation vibrations, although different groups also absorbed in this region. Absorption bands at 1275cm^{-1} and 1217cm^{-1} represent O-H deformation. The peak at 1164cm^{-1}, 1098cm^{-1} and 1031cm^{-1} and 968 cm^{-1} present to C-O stretching and CH_3 rocking.

The FT-IR spectra of semi-purified rhamnolipid of *Acinetobacter variabilis* (RA4) revealed bands at 3366cm^{-1} which corresponds to O-H stretch while asymmetric stretching and symmetric stretching of methylene occurs at 2925 cm^{-1} and 2855 cm^{-1} respectively. The strong peak at 3010cm^{-1} was caused by the bending and stretching of –CH group. The absorption band at 1742cm^{-1} contribute to C=O stretching from lactone ester or acids. The band at 1456 corresponds to C-O-H in the plane binding of carboxylic acid (-COOH). Similarly, absorption bands at 1375 cm^{-1} and 1218 cm^{-1} represents C-H and O-H deformation. Bands at 1160 cm^{-1}, 1094 cm^{-1} and 1029cm^{-1} contribute to C-O stretching. CH3 rocking and C-H deformations exist at 966cm^{-1} and 912 cm^{-1} respectively.

Distinguishing structural spectra was observed for *Acinetobacter boumanii* (RA3) as alpha-pyronyl II sorption band at 850cm^{-1} which indicates the presence of di-rhamnolipid in the mixture.[24,]



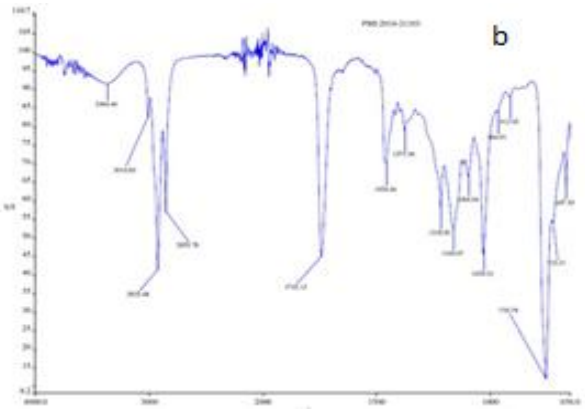


Figure 7: (a) FT-IR spectra of *Acinetobacter boumanii* (RA3) and **(b)** FT-IR spectra of *Acinetobacter variabilis* (RA4)

Liquid Chromatography-Mass Spectrometry analysis

LC/ESI-MS revealed presence of sixteen rhamnolipid homologues in *Acinetobacter boumanii* and *Acinetobacter variabilis* with the pseudo molecular ions between m/z 302 and 734. The pseudo molecular ions and comparative target score (%) for all rhamnolipid congeners are summarized in Figure: 8. Mono rhamno di lipidic horologes were found to be the major constituent in the mixture with Rha-C10: C12 as a predominant component in *Acinetobacter boumanii* (94.65%) and *Acinetobacter variabilis* (93.91%) and its m/z is 532.3831 and 532.3826 respectively. Mono-rhamno- monolipid with m/z of 285.1323 and 285.1336 is the isoform within mono-rhamno- monolipid homologs which correspond to Rha-C8.2 in *Acinetobacter boumanii* (95.85%) and *Acinetobacter variabilis* (98.4%) respectively. Di-rhamno-di-lipid with m/z of 716.4723 was observed as unique and major isoform in *Acinetobacter variabilis* (95.61%), within di-rhamno-di-lipid homologs which correspond to Rha-Rha-C14-C12.

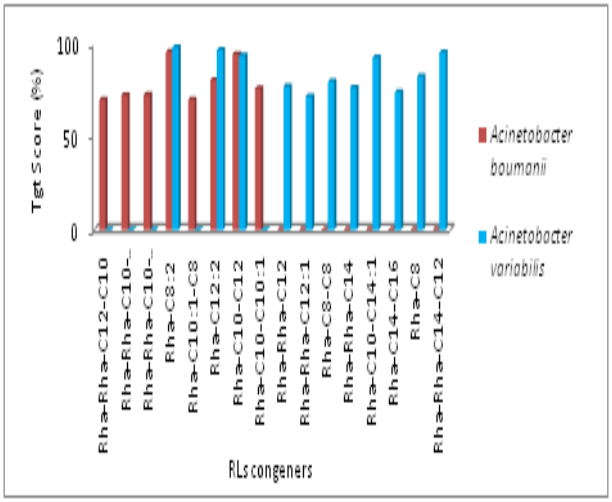
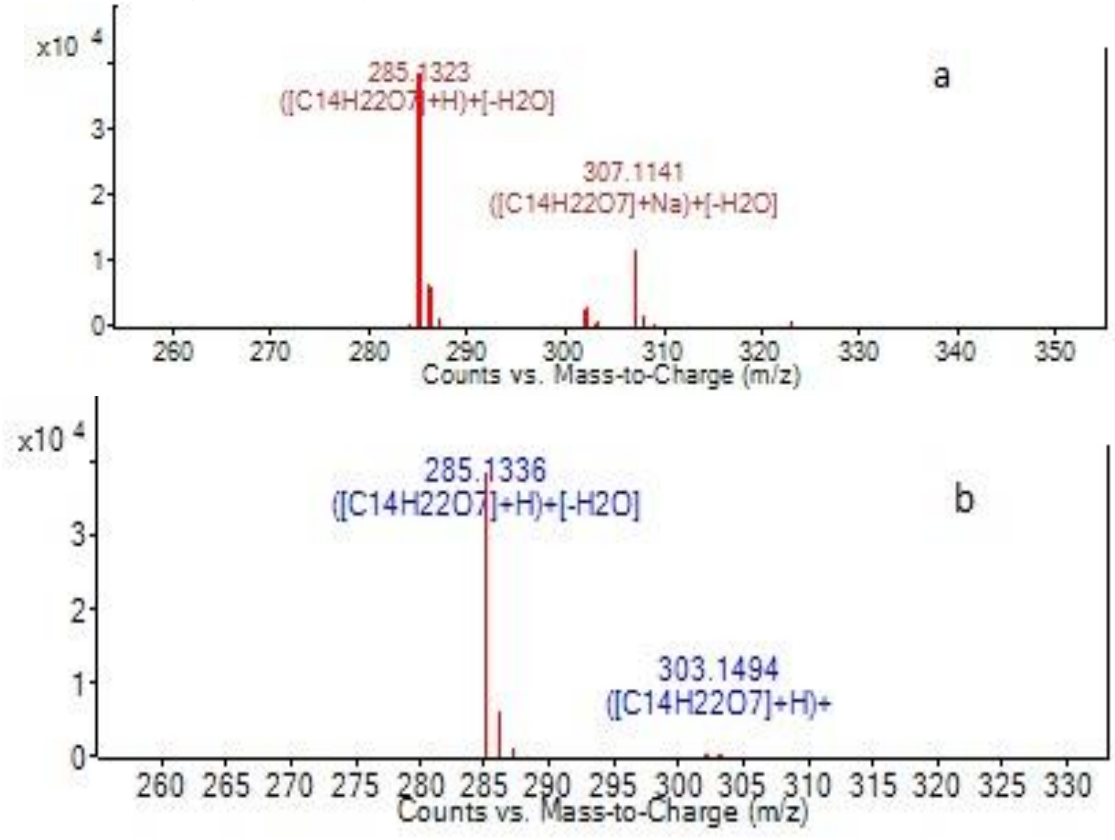


Figure 8: Comparative abundance (Target score) of RLs congeners and their unique distribution pattern in *Acinetobacter boumanii* (RA3) and *Acinetobacter variabilis* (RA4)



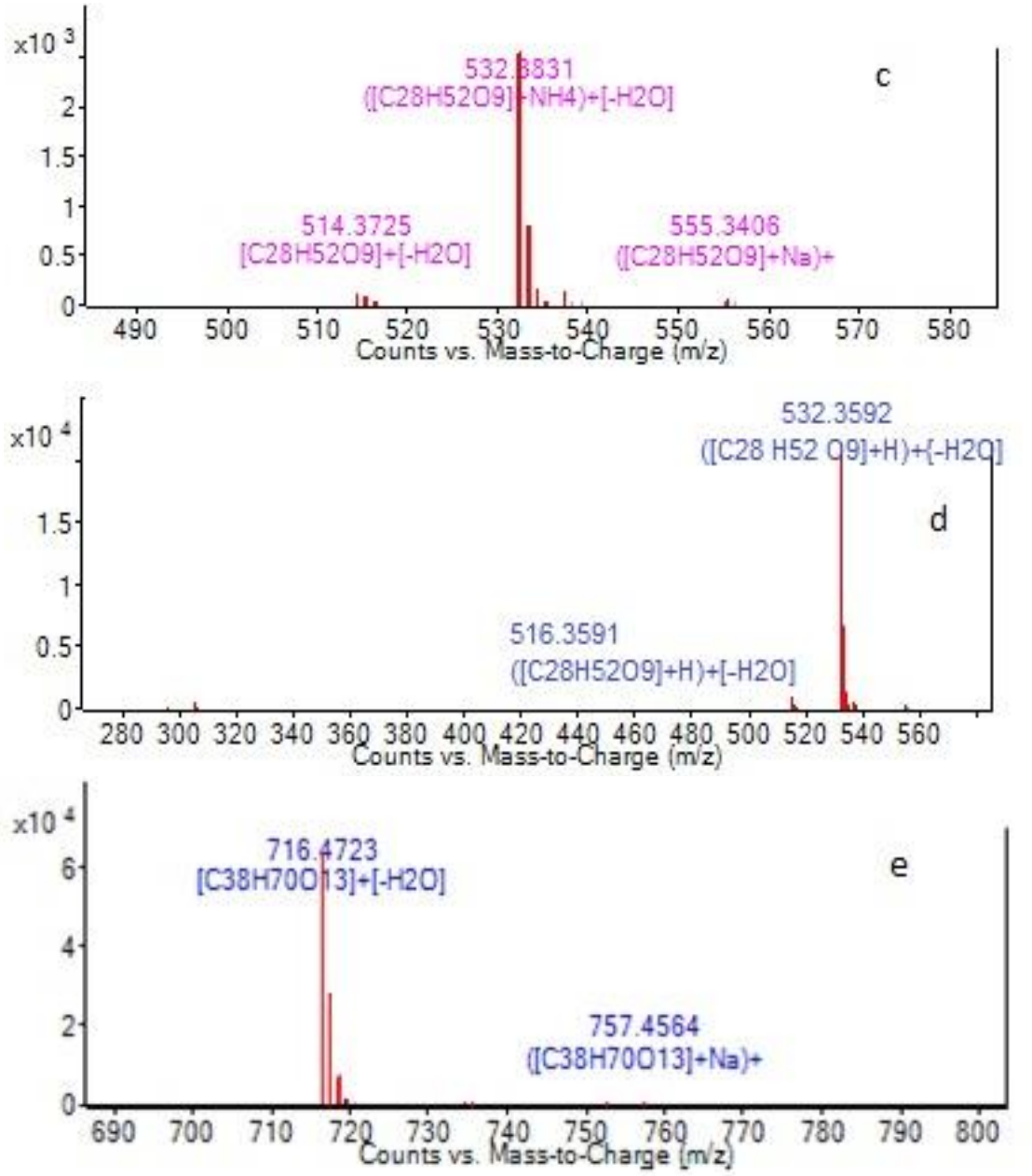


Figure 9: MS spectrum of highly abundant rhamnolipid congeners of *Acinetobacter boumanii* (RA3) and *Acinetobacter variabilis* (RA4) **(a)** & **(b)** mono-rhamno-mono-lipidic Rha-C8.2 rhamnolipid congener detected in both strains **(c)** &**(d)** mono-rhamno-di-lipidic Rha-C10-C12 detected only in both strains and **(e)** di-rhamno-di-lipidic Rha-Rha-C14-C12 detected only in *Acinetobacter variabilis* (RA4)

This research has focused onto screening of novel rhamnolipidic biosurfactant producing bacteria followed by structural characterization of its biosurfactant. It pertains to the increase in environmental awareness about use of renewable-based, biodegradable and more environmentally friendly surfactants.[26] In comparison with oil spills and marine environments, oil refinery area may have lower degrees of extreme environments which possibly reduce the stress of simulation ways while screening of novel biosurfactant producing microorganisms. In this study, 24 bacterial strains obtained had shown growth in MSM broth irrespective of their performance in qualitative tests including hemolysis, drop collapse test, blue agar test, and oil displacement test. This may suggest that strains capable of growing in a minimal salt medium may not have the ability for functional biosurfactant production. In context with this, only strains RA3 and RA4 with a remarkable performance in described qualitative tests have shown promising biosurfactant activity.

Through 16SrRNA gene sequencing and identification, shortlisted strains RA3 and RA4 were identified as *Acinetobacter boumanii* and *Acinetobacter variabilis*. Though 97% sequence similarity with NCBI GeneBank database concludes about their earlier existence, very few reports are available on *Acinetobacter spp.* biosurfactant production. The literature describes genera *Pseudomonas* and *Bacillus* as great biosurfactant producers and is very well studied by the researchers. The

current study for isolation of *Acinetobacter spp*. from hydrocarbon-contaminated soil was done by Wong and Zhao [27] and Nevarez-Moorillonet al.[28] *Acinetobacter spp*.were discovered as biosurfactant producers very earlier.[29, 30] Furthermore many strains from this genus were studied and reported. For example, *Acinetobacter calcoaceticus* BD4 and BD413 was reported by Kaplan et al. [31] and Koket al.[32] respectively. Torenet al.[33] had studied *Acinetobacter radioresistens* KA53 for rhamnolipids production. *Acinetobacter venetianus* RAG-1was reported by Bach et al.[34], *Acinetobacter haemolyticus* TA77 and *Acinetobacter lwoffii* TA38 was isolated by Jagtap et al.[35] from human skin for rhamnolipids production. Novel microorganisms producing biosurfactant is potent and helpful from a biotechnological point of view and to avoid uses of infective *P. aeruginosa* strains which ultimately ensure industrially safe production of Rhamnolipids.

Shake flask biosurfactant production studies of *Acinetobacter boumanii* and *Acinetobacter variabilis* showed their highest performance at 72hr with the production of 1.2g/l and 0.921g/l biosurfactant at O.D. of 8.3 and 2.5 respectively. It clearly inclines the scope towards highly efficient *Acinetobacter variabilis* for future biosurfactant production studies. Apart from this, LenkaKrizova et al.[36] isolated *Acinetobacter variabilis sp*.nov. from clinical specimens of humans and animals. However, this work reveals the potential application of *Acinetobacter variabilis* in the field of biosurfactant production for the first time. Baoet al.[37] could get 0.52g/l biosurfactant production after 7 days of fermentation using MSM with 0.5% crude oil. It illustrates the need for media optimization studies with *Acinetobacter variabilis* to unfold its true potential. In continuation with this, our study with *Acinetobacter boumanii* and *Acinetobacter variabilis* showed a gradual drop in $_{p}$H from 7.2 to 6.4± 0.5 throughout the fermentation, suggesting that; minor $_{p}$H range near neutral $_{p}$H affected neither the synthesis nor the yield of biosurfactant.[38.39] These results are in accordance with previous reports which revealed a direct relationship between the production of surface-active compounds with a reduction in the surface tension values.[40]

Physicochemical characterization of biosurfactant is a direct way to understand the efficiency and commercial potential of new biosurfactant. For industrial scale, there needs to be an improved development of fermentation process and downstream separation techniques for effective biosurfactant production.[41, 14] The ability to form and stabilize emulsions is one of the most important features to be considered for the practical application of a surfactant. Chen J et al. and Bao M et al. [42,37]isolated *Acinetobacter sp. YC-X 2* and *Acinetobacter sp.*D3-2 from petroleum contaminated soil. Here, *Acinetobacter sp. YC-X 2* showed E24 and surface tension of 54.87 ± 1.23 and 32.5±0.51 mN/m $^{-1}$ at 20^{0}C. Whereas, *Acinetobacter sp.*D3-2 decreased surface tension from 48.02 to 26.30 mN/m^{-1}. In our study, *Acinetobacter boumanii* and *Acinetobacter variabilis* showed highest E24 of 44± 0.3 and 40±0.5 at 72hr. And, their surface tension was seen to be 43 mN/m^{-1} and 50 mN/m^{-1} respectively. Soil belongs to petroleum contamination area and oil refinery area can have variable proportions of saturated and or unsaturated fatty acids which in turn changes the emulsification index (E24) and surface tension potential of isolated strains.

In the present research, advanced structural characterization of a biosurfactant produced by *Acinetobacter boumanii* and *Acinetobacter variabilis* was evaluated using thin -layer chromatography (TLC), LC/ESI-MS and FT-IR. Thin layer chromatography[21] suggests that; isolated surface–active product from *Acinetobacter boumanii* and *Acinetobacter variabilis* is composed of glycolipid and more specifically it belongs to monorhamnolipidic type. This indicates that, both *Acinetobacter sp.* produce a similar category of biosurfactant though their thin layer chromatography analysis using orcinol-sulphuric acid method [43] may reveal different biosurfactant composition. In detail FT-IR characterization of semi purified biosurfactant of *Acinetobacter boumanii* and *Acinetobacter variabilis* predicted about their glycolipid structure. Unfolding of alpha-pyronyl II as a di-rhamnolipid moiety in *Acinetobacter boumanii* through FT-IR indicates a higher sensitivity of this technique than TLC [21]which could detect the presence of only monorhamnolipidic biosurfactant. Also, FT-IR showed the presence of highly charged groups such as carboxyl; amine and hydroxyl moieties in both strains of *Acinetobacter sp*. which needs to get evaluated for their antibacterial potential against negatively charged surfaces.[25]

Among 16 different congeners as detected by LC/ESI-MS, three belongs to monorhamnolipidic nature which can be co-related to the thin layer chromatography analysis revealing the presence of monorhamnolipidic components in both strains of *Acinetobacter sp.* In contrast to this, four congeners representing di-rhamno-di-lipidic moieties in LC/ESI-MS correspond to FT-IR absorption band at 850cm^{-1} suggesting the presence of di-rhamnolipids in *Acinetobacter boumanii* only. This can be evidenced by the presence of single predominant Rha-Rha-C14-C12 in *Acinetobacter variabilis* in 95.61% vs. three different congeners of Rha-Rha-C12-C10, Rha-Rha-C10-C10-1 and Rha-Rha-C10-C10-1 as detected in *Acinetobacter boumanii*. LC/ESI-MS is more comprehensive to understand about quantitative compositional analysis. In context with this, ten different congeners were detected in *Acinetobacter variabilis* followed by eight in *Acinetobacter boumanii*. It can be said that platforms of structural characterization gradually increased their analytical sensitivity from TLC to LC/ESI-MS. Though our study reported existing rhamnolipid congeners, it's the first report to unfold about biosurfactant production, growth, fermentation characteristics and structural characterization of rhamnolipid biosurfactant from *Acinetobacter variabilis*.

CONCLUSION

Rhamnolipid biosurfactant producing strains of *Acinetobacter boumanii* and *Acinetobacter variabilis* elucidated the true potential of oil refinery area for isolation of a novel, non-pathogenic microorganisms for biosurfactant production. Physico-chemical characterization of their biosurfactant with FT-IR revealed its glycolipid nature with the presence of a highly charged amine, hydroxyl and carboxyl moieties which may have an association with surface tension and emulsification index potential of microorganisms. Highest abundance noticed for Rha-Rha-C10-C12 and Rha-C8.2 in *Acinetobacter boumanii* and *Acinetobacter variabilis* interestingly noted the existence of congener Rha-C8.2 in *Acinetobacter variabilis* which have been unfolded first time in our study. Accuracy and sensitivity of diligent analysis seen expanded from TLC to LC/ESI-MS. To our knowledge, this is the first report of isolation of *Acinetobacter variabilis* from oil refinery area with detailed characterization of its rhamnolipid biosurfactant. In this regard, extensive optimization studies with *Acinetobacter variabilis* and its biosurfactant production is necessary to step up towards its new applications and commercialization.

REFERENCES

1. Desai JD, Banat IM., Microbial production of surfactants and their commercial potential, MicrobiolMolBiol, Rev 61(1), 1997, 47-64.
2. Abdel-Mawgoud AM, Lépine F, Déziel E., Rhamnolipids. Diversity of structures, microbial origins and roles, Appl Microbiol Biotechnol, 86(5), 2010, 1323-36.
3. Benincasa M, Abalos A, Oliveira I, Manresa A, Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock,Antonie Van Leeuwenhoek, 85(1), 2004, 1-8.
4. DanyelleKhadydja F. Santos, Raquel D. Rufino, Juliana M. Luna, Valdemir A. Santos., Biosurfactants. Multifunctional Biomolecules of the 21st Century, Int J MolSci, 17(3), 2016, 401.
5. Urum K, Pekdemir T., Evaluation of biosurfactants for crude oil contaminated soil washing, Chemosphere, 57(9), 2004, 1139-50.
6. Rodrigues L, Banat IM, Teixeira J, Oliveira R., Biosurfactants, potential applications in medicine, J Antimicrob Chemother, 57(4), 2006, 609-18 .
7. Laurence S. Romsted, Part IV Bio-surfactants in Surfactant Science and Technology, Retrospects and Prospects, CRC Press, 2014.
8. Caroline Edser, Focus on Surfactants, 1, 2017, 1-8.
9. Fariq A, SaeedA., Production and Biomedical Applications of Probiotic Biosurfactants, Curr Microbiol, 72(4), 2016, 489-95.
10. Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R., Microbial biosurfactants production, applications and future potential, Appl Microbiol Biotechnol, 87(2), 2010, 427-44.
11. Chrzanowski Ł, Ławniczak L, and KatarzynaCzaczyk., Why do microorganisms produce rhamnolipids, World J Microbiol Biotechnol, 28(2), 2012, 401–419.
12. Foght JM, Gutnick DL, Westlake DW.,Effect of emulsan on biodegradation of crude oil by pure and mixed bacterial cultures,Appl Environ Microbiol, 55(1), 1989, 36-42.
13. Rooney AP, Price NP, Ray KJ, Kuo TM., Isolation and characterization of rhamnolipid-producing bacterial strains from a biodiesel facility, FEMS MicrobiolLett, 295(1), 2009, 82-87.
14. Patil JR, Chopade BA., Distribution and in vitro antimicrobial susceptibility of Acinetobacter species on the skin of healthy humans,Natl Med J India, 14(4), 2001, 204-2-8.
15. Doughari HJ, Ndakidemi PA, Human IS, BenadeS.,The ecology, biology and pathogenesis of Acinetobacter spp. an overview, Microbes Environ, 26(2), 2011, 101-12.
16. Milena Rikalovic G., Gojgic-Cvijovic Gordana, VrvicMiroslav M., Karadzic Ivanka., Production and characterization of rhamnolipids from *Pseudomonas aeruginosa*, san ai Journal of the Serbian Chemical Society, 77(1), 2012, 27-42.
17. J. Chen, P.T. Huang, K.Y. Zhang and F.R. Ding., Isolation of biosurfactant producers, optimization and properties of biosurfactant produced by *Acinetobacter sp*. from petroleum-contaminated soil, Journal of Applied Microbiology, 112, 2012, 660–671.
18. Mishra RK, Soni GC, Mishra R., Manoemulsion. A novel drug delivery tool,Int J Pharma, Res Rev 3(7), 2014, 32-43.
19. Dilmohamud B, Seeneevassen J, Rughooputh S., Surface tension and related thermodynamic parameters of alcohols using the Taube Stalagmometer, European Journal of Physics, 26(6), 2005, 1079-1084.
20. Lai C.C., Huang Y.C., Wei Y.H., Chang J.S., Biosurfactant-enhanced removal of total petroleum hydrocarbons from contaminated soil, J Hazard Mater, 167, 2009, 609–614.
21. Smith TJP, Perfumo A, Marchant R, Banat IM., Isolation and analysis of low molecular weight microbial glycolipids, In. Kenneth N. Timmis (ed) Handbook of hydrocarbon and lipid microbiology, Chapter28, Springer-Verlag, Berlin Heidelberg, 5(2), 2010, 3705–3723.
22. Yin H, Qiang J, Jia Y, Ye J, Peng H, Qin H, Zhang and He B., Characterization of biosurfactants produced by *Pseudomonas aeruginosa* S6 isolated from oil-containing wastewater, Process 7, 2008, 262-266.
23. Pornsunthorntawee O, Maksung S, Huayyai O, Rujiravanit R amdChavadej S., Biosurfactant production by *Pseudomonas aeruginosa* SP4 using sequencing batch reactors, Effects of oil loading rate and cycle time, Bioresour Technol, 100, 2009, 812-818.
24. Moussa T. A. A., M. Mohamed S., Samak N., Production and characterization of di-rhamnolipid produced by *Pseudomonas aeruginosa* TMN, Braz. J. Chem. Eng, 31 (4), 2014.
25. Emine, A.L.P., M. Yerer, S. Kocagö, G. Metan, and D. Esel ., The risk factors and spread of multidrug-resistant *Acinetobacter baumannii* in intubated patients in a medical intensive care uni, Turk J Med Sci, 39 (5), 2009, 761-769.
26. Gudina EJ, Rangarajan R, Sen R, Rodrigues LR., Potential

therapeutic applications of biosurfactants Trends, PharmacolSci, 4, 2013, 667–675.

27. Wong, Jonathan W.C, Zhao, Zhenyong and Zheng, Guanyu., Biosurfactants from *Acinetobacter calcoaceticus* BU03 Enhance the Bioavailability and Biodegradation of Polycyclic Aromatic Hydrocarbons, Proceedings of the Annual International Conference on Soils, Sediments, Water and Energy, 15(5), 2014.
28. Nevarez-Moorillon, G.V., Viramontes-Ramos, S., Portillo-Ruiz, M.C., Ballinas-Casarrubias, M.D., Torres-Munoz, J.V. and Rivera-Chavira, B.E., Selection of Biosurfactant / Bioemulsifier-producing bacteria from hydrocarbon-contaminated soil, Braz J Microbiol, 41, 2010, 668–675.
29. Zuckerberg, A., Diver, A., Peeri, Z., Gutnick, D.L. and Rosenberg, E., Emulsifier of *Arthrobacter*RAG-1. Chemical and physical properties, Applied and Environmental Microbiology, 37, 1979, 414-420.
30. Zosim Z, Gutnick DL, Rosenberg E., Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacte r*RAG-1 emulsan, Biotechnol Bioeng, 24, 1982, 281.
31. Kaplan N, Zosim Z, Rosenberg E., Reconstitution of emulsifying activity of *Acinetobacter calcoaceticus* BD4 emulsan by using pure polysaccharide and protein,Appl EnvironMicrobiol,53,1987, 440–6.
32. Kok, R.G., Christoffels, V.M., Vosman, B. and Hellingwerf, K.J., Growth-phase-dependent expression of the lipolytic system of *Acinetobacter calcoaceticus* BD413, cloning of a gene encoding one of the esterases. J Gen Microbiol, 139, 1993, 2329–2342.
33. Toren, A, Orr, E., Paitan, Y., Ron, E.Z., and Rosenberg, E., The active component of the bioemulsifier alas an from *Acinetobacter radio resistens* KA53 is an OmpA-like protein, J Bacteriol1, 84(1), 2002, 165–170.
34. Bach, H., Berdichevsky, Y. and Gutnick, D., An exocellular protein from the oil-degrading microbe *Acinetobacter venetianus* RAG-1 enhances the emulsifying activity of the polymeric bioemulsifieremulsan, Appl Environ Microbiol, 69, 2003, 2608–2615.
35. Jagtap S., Yavankar, S., Pardesi, K. and Chopade, B., Production of bioemulsifier by *Acinetobacter* species isolated from healthy human skin,Ind J Exp Biol,48, 2010, 70–76.
36. LenkaKrizova, Jana McGinnis, Martina Maixnerova, Matej Nemec, Laurent Poirel, Lisa Mingle, Ondrej Sedo, William Wolfgang, and Alexandr Nemec., *Acinetobacter variabilis sp.nov.* (formerly DNA group 15sensu Tjernberg &Ursing), isolated from humans and animals, Int J SystEvol Microbiol, 65(3), 2015, 857–863.
37. Bao M, Pi Y, Wang L, Sun P, Li Y, Cao L., Lipopeptide biosurfactant production bacteria *Acinetobacter sp*. D3-2 and its biodegradation of crude oil, Environ Sci Process Impacts,16(4), 2014,897-903.
38. Abu-Ruwaida, A.S., Banat, I.M., Haditirto, S. and Khamis, A., Nutritional requirements and growth characteristics of a biosurfactant producing *Rhodococcus* bacterium, World J Microbiol Biotechnol, 7, 1991, 53–60.
39. Kiran, G.S., Thomas, T.A., Selvin, J., Sabarathnam, B. and Lipton, A.P., Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture, Bioresour Technol, 101, 2010, 2389–2396.
40. Verma M, Seto-Poon M, Wheatley JR, Amis TC, Kirkness JP., Influence of breathing route on upper airway lining liquid surface tension in humans, J Physiol, 574, 2006, 859–866.
41. Milena Rikalovic G., Gojgic-CvijovicGordana, VrvicMiroslav M., Karadzic Ivanka., Production and characterization of rhamnolipids from *Pseudomonas aeruginosa*, san ai Journal of the Serbian Chemical Society, 77(1), 2012, 27-42.
42. Chen, J., Yang, Q.Y., Huang, T.P., Zhang, Y.K. and Ding, R.F., Enhanced bioremediation of soil contaminated with viscous oil through microbial consortium construction and ultraviolet mutation, World J Microbiol Biotechnol, 27, 2011, 1381–1389.
43. Sharma D, Mohammad Javed Ansari, Ahmed Al-Ghamdi, Nuru Adgaba, Khalid Ali Khan, Vikas Pruthvi, Noori Al-Waili., Biosurfacatant production by *Pseudomonas aeruginosa* DSVP20 isolated from petroleum hydrocarbon-contaminated soil and its physicochemical characterization, Environ SciPollut Res, 2015.

Source of Support: Nil, **Conflict of Interest:** None.