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Physicochemical and analytical characterization of biosurfactant produced by *Serratia rubidaea* KAP

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***Abstract*-** In an attempt to replace the chemical compounds that may potentially disrupt the environment, biological sources has been exploited extensively for characteristic recovery of their metabolic products. The present study was carried out with a similar attempt to characterize the biosurfactant produced by *Serratia rubidaea* KAP isolated from oil polluted soil sample. The biosurfactant sample was analyzed qualitatively by the Thin-layer chromatographic (TLC) technique which indicated the presence of proteins, carbohydrates (with rhamnose as major sugar) and lipids. This was further confirmed by High Performance-TLC technique. The concentration of proteins, carbohydrates and lipids were also quantified using suitable colorimetric and gravimetric techniques. They were estimated to be 11%, 37% and 51.09% respectively in the biosurfactant sample. It was further characterized to be ionic in nature, on the basis of agar double diffusion method. The analytical tests including Fourier Transformed Infra-Red spectrum and Gas Chromatography-Mass Spectroscopy confirmed the presence of rhamnolipids. The biosurfactant further showed a critical micelle concentration of 62%. Moreover, the stability studies indicated considerable biosurfactant activity at a wide range of temperature (from 4°C -100°C), alkaline pH and in an environment of salinity up to 12%. Thus, these results strongly suggest that the biosurfactant produced by *S. rubidaea* KAP can be useful in industries which operate at extreme conditions of pH, temperature and salinity e.g., pharmaceutical, cosmetics, food industries, and for bioremediation in the marine environment.

Keywords—*Biosurfactant, TLC, Rhamnolipid, Serratia rubidaea, Stability*

I. INTRODUCTION

Surfactants are compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface. Biosurfactants are a heterogeneous group of such surface-active moieties produced by a number of microorganisms [1]. The biosurfactant molecule consists of low molecular weight polymers which possess both hydrophobic and hydrophilic moieties that partition preferentially at the interface between fluid phase with different degrees of polarities and hydrogen bonding such as oil/water or air/water interfaces [2]. Thus, they are capable of reducing the surface and interfacial tension. In addition, it also contains high molecular weight moieties that have highly efficient emulsification capabilities [3].

The effectiveness of a biosurfactant is determined by its ability to lower the surface tension, which is a measure of the surface free energy per unit area required to bring a molecule from the bulk phase to the surface [4]. In addition, the biosurfactant enhances solubility and possesses a low critical micelle concentration, which is a measure of the lowest stable surface tension required to maintain the molecular assemblies, known as micelles [5]. Altogether, these characteristics confer excellent detergency, emulsification, foaming and dispersion properties; thereby making the market of biosurfactants extremely competitive [6]. The characteristics of biosurfactants that have attracted industrial applications are attributed to features like low toxicity, action specificity, higher biodegradability and functionality at variable pH, temperature and salinity.

The increasing industrialization and consequential pollution demand the exploitation of compounds like biosurfactants that are capable of degrading hydrocarbon contaminants; making it a perfect product of commercial value. Also, the considerations for sustainable industrialization and the eco-friendly nature of biosurfactants mark its recommendation as a substitute to chemical surfactants [7]. Moreover, since India is a developing country with agriculture being the main occupation; proper utilization of agro-waste can be diverted towards biosurfactant production. Given the fact that the raw material consumption in biosurfactant production accounts for 10-30% of total production cost; using cheaper raw materials will increase the feasibility in scale-up of biosurfactants [8].

Apart from production, the characterization of a biosurfactant that contributes to its unique physicochemical properties is extremely important. Since, the high molecular weight biosurfactants are structurally

complex polymers, they are analyzed by colorimetric assays (Lowry's method, Bradford assay), mass spectrometry (MS) and sequencing techniques. Fatty acid content and peptide sequence is determined with the help of automated Edman degradation sequencing and mass spectrophotometry techniques. The combination of all these different methods is important to predict the complete structure of biosurfactants. Following the characterization, based on our requirement for emulsification, surface tension reduction, bioremediation or even food and pharmaceutical applications, a class of biosurfactant may be selected. Biosurfactants with higher lipid content may be suitably chosen for bioremediation of oil-contaminated sites [9]. The anionic glycolipid biosurfactants may be employed to remove toxic heavy metals from agricultural sites [10]. Phospholipids may be used extensively in agriculture owing to its characteristic stability observed against several environmental factors [3].

Due to the above mentioned facts, currently, the production and characterization of biosurfactants, for various industrial applications is a prime consideration for a number of organizations. Biosurfactants also make a strong appearance as a valuable green product which is necessary for sustainable industrial development by maintaining a balance between nature and human life. Moreover, the biodegradable nature of biosurfactants along with low toxicity makes it a reasonable candidate for trials in the sectors of food production, health, chemical, agriculture and cosmetic industries. In the current study, biosurfactant produced by *Serratia rubidaea* KAP has been characterized using analytical techniques, and its stability studies are presented.

In the current study, Section I contains a brief introduction on biosurfactants and the importance of its characterization studies. Section II explains the methodology used for characterization of biosurfactant obtained from *S. rubidaea*. Section III describes the results and discussion, and section IV concludes our research work with future directions.

II. METHODOLOGY

Separation of biosurfactant components using chromatographic techniques

The preliminary detection of biosurfactant components was carried out using Thin Layer Chromatography (TLC). For this purpose, pre-coated silica gel aluminium plates (G60, F254 Merck, Germany 7 x 3cm) were used. The sample was prepared by dissolving 0.1g of the partially purified biosurfactant, obtained after optimization in our previous study [11], in 1mL of analytical grade methanol. A 20µL volume of this sample was spotted twice, with intermittent drying, at a point which was 2cm away from the bottom of the plates. The mobile phase used for the development of plates consisted of chloroform, methanol and acetic acid in the ratio 65:15:2 (v/v/v). Three different plates were prepared for detection of lipids, carbohydrates and amino acids, and were developed using iodine vapours, Molish's reagent and 1% ninhydrin solution respectively. On detection of carbohydrates, another plate was prepared similarly for specific detection of sugars, using 1% rhamnose, and it was developed with anthrone reagent [12]. The plates were then kept at 110°C until the appearance of definite spots [13]. The plates were observed for development of light violet and yellow spots for the detection of carbohydrates and sugars respectively. Yellowish brown spots were indicative of lipids, and purple spots for amino-acids. The Rf value for sugars was calculated according to the method suggested in another study [14].

The results of TLC were further confirmed using automated HPTLC system (CAMAG, Switzerland) at Anchrom laboratory, Mulund (E). The mobile phase used in our study was specific for glycolipids and comprised of toluene: ethyl acetate (9.3:0.7 v/v) [15]. The plates were derivatized using phosphomolybdic acid and visualized under visible light, UV-254nm and UV-366nm light intensity and the images were captured for documentation.

Estimation of components of the partially purified biosurfactant for its carbohydrates, proteins and lipid content

The estimation of carbohydrate and protein concentration of the biosurfactant was carried out by the phenol-sulphuric acid method [12] using a standard curve of rhamnose sugar and Lowry's method [16] using Bovine Serum Albumin (BSA) standard curve respectively. The estimation of lipid concentration in the biosurfactant was carried out by the solvent extraction (Folch) method using organic solvents viz., chloroform and methanol, and the weight of the lipids was determined gravimetrically [17].

Determination of the ionic nature of the partially purified biosurfactant

The ionic charge of the biosurfactant molecule was determined using the agar double diffusion technique [18]. For this purpose, 1% agar was prepared in sterile D/W, molten and poured in a clean and dry large-sized sterile plate. The appropriate concentration of the agar was chosen to allow proper diffusion of the solutions from the well. The plate was then allowed to set at refrigeration temperature for 30min and four equidistant wells of 6mm diameter were made in the plate. A 50µL volume of the following content was added to the wells;

1. Standard Sodium Dodecyl Sulphate (SDS, 20mM) as the anionic surfactant.
2. Cetyl-Trimethyl-Ammonium Bromide (CTAB, 20mM) as the cationic surfactant.

3. Biosurfactant solution (10mg/mL prepared in phosphate buffer pH7.0) was added in the remaining two wells.

The plate was kept at room temperature and observed for the appearance of zones of precipitation between the wells after 24h incubation. This was indicative of the ionic nature of the bio-surfactant.

Characterization of biosurfactant using FTIR and GCMS analytical techniques

The functional groups of the biosurfactant molecule were analysed using FTIR technique. For carrying out the analysis, 1mg of the biosurfactant was ground with 100mg KBr pellet and pressed with 7500kg weight for 30sec to obtain a translucent pellet. The FTIR spectrum was recorded over the frequency range of 4000-400cm^{-1} on Jasco6600 Model (Tokyo, Japan) with a spectral resolution and wave number accuracy of 4 and 0.01cm^{-1} respectively. The IR spectrum of the sample was the average of 500 data scanning over the entire range of wave numbers. A KBr pellet was used as background reference and all data were corrected for the background spectrum.

After determination of the functional groups, the specific compounds in the biosurfactant sample were detected using GC-MS analysis using GCMS QP2010 Plus Shimadzu, Japan. The model was equipped with capillary column Rtx-5MS. The column oven temperature was held at 0-150°C for 0min, then programmed at 150-200°C for 10min hold, and then again at 200-270°C for 10min hold. A selective detector (AOC-20i) was used which was set to scan from m/z 20 to m/z 310 at a scan rate of 1.5 scans per second with an initial column temperature of 45°C and injection temperature of 270°C for three minutes. The flow rate of the carrier gas (helium) was maintained at 5.5 lb/in2 and a split ratio of 25:1 was used.

Both FTIR and GC-MS analysis was carried out at Sai Biosystems Private Limited, India.

Determination of critical micelle concentration

The Critical Micelle Concentration (CMC) was reported by observing the surface tension values, of various dilutions (0-1600mg/L) of the dried biosurfactant product, with the help of Du Nouy ring-type tensiometer [19]. The surface tension measurement was carried out at 30°C, after calibrating the instrument with Milli Q water, until a constant value was observed. The CMC value was obtained by plotting a graph of surface tension values against the biosurfactant concentration [20]. CMC is defined as that concentration of the biosurfactant necessary to initiate micelle formation. At this point, the surface tension value remains constant with increasing biosurfactant concentration.

Stability study

To carry out the stability study of biosurfactant with respect to temperature, pH and effect of sodium chloride concentration, its solution was prepared by dissolving 1% (w/v) of the dried product in 0.2M phosphate buffer (pH 7.0). The effect of heat, pH and salinity, on the stability of the biosurfactant solution, was studied by incubating 40mL of the biosurfactant solution (prepared as indicated above) at various temperatures (4°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 100°C and 121°C), pH (2, 4, 5.5, 6.5, 7.0, 7.5, 8.5, 9.5, 10.5, 11.5 and 12) and salt concentration (2%-20%) [21], [22], [23].

All the tests were carried out in triplicates, in our study.

III. RESULTS AND DISCUSSION

Detection of biosurfactant components using chromatographic techniques

The TLC detected four major components (carbohydrates, sugars, lipids and proteins) in the biosurfactant sample. The Molisch's reagent confirmed the presence of carbohydrates (violet spots), and anthrone reagent further helped in the identification of rhamnose (yellow) as a part of carbohydrate components of the biosurfactant molecule. The specificity of this test was higher than the Molisch's test since the Rf value of the yellow spot developed by the test sample (0.22) was close to the Rf value of the standard sugar rhamnose (0.24). Similarly, the development of yellowish-brown spots, on TLC plates, on exposure to iodine vapours indicated the presence of lipids in the biosurfactant sample. A considerable size, as well as the intensity of the colour observed, indicated the presence of lipids in higher proportion as compared to other components. The ninhydrin test showed very faint purple colour spots indicating considerably less concentration of proteins in the biosurfactant sample. However, proteins are often present as contaminants extracted along with biosurfactants from the growth media. Hence, combined with the above observations, it was suggested that the biosurfactant produced by *S. rubidaea* KAP was glycolipid in nature.

The findings of TLC were further confirmed by HPTLC results. The Figure 1 represents five distinct spots observed as dark blue to light blue colour on derivatization with phosphomolybdic acid which confirmed the presence of glycolipids. The HPTLC analysis was carried out using a mobile phase i.e. toluene: ethyl acetate (9.3:0.7 v/v) that was specifically formulated for optimum separation of glycolipid components, in our study. To the best of our knowledge, this solvent mixture has not been used, as mobile phase, for the separation of biosurfactant components.

A literature survey of biosurfactants produced by Serratia sp. indicates that they principally produce biosurfactants that are lipopeptide or glycolipid in nature [24], [25], [26]. The serrawettins and stephensiolides are the common lipopeptides reported among *Serratia* sp. whereas rubiwettins and rhamnolipids are the majorly identified glycolipids [27].

Similar to our findings, the glycolipid nature of biosurfactant produced by *S. marcescens* NSK-1 was confirmed on the basis of positive chromogenic reactions observed on TLC plates [28]. A biosurfactant produced by another *S. marcescens* strain showed a single spot with an Rf value of 0.72 on TLC plates which was confirmed to be glycolipid in nature by further analysis using analytical techniques like GC-MS [29].

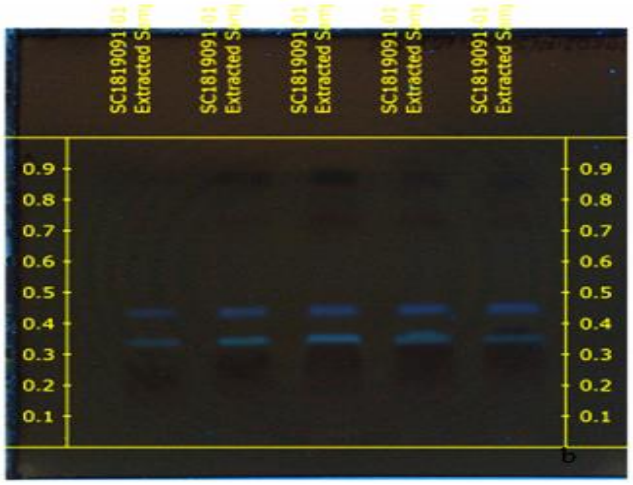

Figure 1. HPTLC analysis of plates after derivatization with phosphomolybdic acid showing blue spots at R 366nm

In contrast to our findings, the lipopeptide nature of biosurfactant was reported in *S. marcescens* sp. which was characterised on the basis of Rf value (0.75), on TLC plates, that corresponds to lipids. The protein moiety was observed as a red spot developed by ninhydrin test [30]. The crude biosurfactants obtained from isogenic strains of *S. marcescens* SM3 and SMRG-5 were subjected to HPLC analysis which confirmed the lipopeptide nature of the molecule [31]. Another study reported a cyclopeptide moiety in biosurfactant produced by Serratia sp. ZS6. The cyclic nature of the biosurfactant was suggested since ninhydrin staining was apparent only after acid hydrolysis. No reaction was observed prior to the acid hydrolysis of the biosurfactant sample [32].

Among other organisms, a study reported the presence of carbohydrate, protein and lipid moieties in biosurfactants produced by *Corynebacterium* sp. DDv1, *Micrococcus roseus* DDv3, *P. aeruginosa* DDv4 and *S. cerevisiae* DDv5 [33]. The lipopeptides and glycolipids in *B. licheniformis* and *Bacillus safensis* PHA3 respectively, were reported on the basis of Rf value on TLC plates [34, 35]. Biosurfactants from *P. aeruginosa* PBS showed two spots on TLC plates with Rf values of 0.43 and 0.76 which directly corresponded to mono-rhamnolipids and di-rhamnolipids [36]. Similarly, the biosurfactants from *P. aeruginosa* SWP-4 was also characterised [37].

The mobile phases in chromatographic techniques are known to influence the separation of components in a natural compound. A lipopeptide (similar to surfactin) biosurfactant produced by *B. subtilis* B30 showed better separation of components when solvent system containing butanol: acetic acid: water (8:2:2, v/v/v) was used, as compared to chloroform: methanol: water (65:25:4, v/v/v) [38]. In another study, butanol: acetic acid: water (4:1:1, v/v/v), proved to be most effective in the separation of components from biosurfactant produced by *B. licheniformis* W16. It helped in effective separation of components and multiple spots were obtained that could be used for further characterization using analytical techniques. The other solvent systems viz., chloroform: methanol: ammonium hydroxide (65:25:4, v/v/v) and chloroform: methanol: acetone: acetic acid (90:10:6:1, v/v/v/v) produced only two spots. The chloroform: methanol: water (65:25:4, v/v/v) solvent system, on the other hand, showed 4 diffused and well-separated spots. It was identified as lipopeptide, similar to lichenysin-A [39]. In another study, two different solvent systems containing methanol: acetic acid (95:5, v/v) and chloroform: methanol: water (65:15:2, v/v/v) were used for the separation of sophorolipids components produced by *Candida bombicola* ATCC 22214 [40]. An interesting modification was used in the existing HPTLC technique for separation as well as quantification of cyclic lipopeptides viz., surfactin, iturin A and fengycin isolated from *B. subtilis* DSM 10T, *B. amyloliquefaciens* DSM 7T and *B. methylotrophicus* DSM 23117. The separation was carried out using a two-step development step using different mobile phases. Here, the first development was conducted with chloroform: methanol: water (65:25:4, v/v/v) until the sample reached 60mm distance from the spot. After this, the second development was conducted with butanol: ethanol: acetic acid (1:4:1, v/v/v) until the next 60mm distance on the TLC plate. The use of this method along with a threefold extraction of the cell-free broth of the above mentioned cultures using chloroform: methanol (2:1, v/v) proved to be most effective for biosurfactant estimation for all three isolates [41].

Estimation of components of the partially purified biosurfactant for its carbohydrates, proteins and lipid content

The concentration of carbohydrates, proteins and total lipids was calculated to be 37%, 11% and 51.09% respectively. The statistical significance of the standard curves was confirmed by the adequately high value of the coefficient of determination (R2) i.e. 0.9922, obtained from the graphs. As expected by the observation on TLC plates and the fact that the test organism thrived on 5%

rice-bran oil as carbon source, it was confirmed that lipids form a major proportion of the biosurfactant molecule.

The glycolipid biosurfactant, produced by *P. cepacia* CCT6659 revealed the presence of 75% lipids and 25% carbohydrates. The high lipid content made this biosurfactant a suitable candidate for its application in the biodegradation of hydrophobic compounds in soil [18]. A recent study reported the production of a light brown water-soluble precipitate of biosurfactant by *S. marcescens* UCP 1549. This polymeric biosurfactant was composed of 43% lipids, 32% proteins and 11% carbohydrates. This isolate was capable of biosurfactant production on the utilization of low-cost medium containing cassava flour wastewater indicating its application in the management of agro-industrial wastes and bioremediation [10]. Another biosurfactant obtained from *Cronobacter sakazakii* isolated from oil-contaminated wastewater comprised of total sugars (73.3%), reducing sugars (1.464%), protein (11.9%), uronic acid (15.98%) and sulphate (6.015%). It was reported to be suitable for bioremediation of oil and hydrocarbons [42]. The emulsan biosurfactant produced by *A. calcoaceticus* and *Acinetobacter* RAG-1 was reported to contain 11.2% and 20% of protein respectively [43], [44]. Similarly, the lipopeptide biosurfactant produced by *A. chroococcum* showed the presence of lipid and protein content in the ratio 31.30: 68.69 [45], and *S. marcescens* NSK-1 showed presence of 55.2% lipid, 38.8% protein and 0.43% carbohydrate [25]. Both studies indicated their suitable application in environmental bioremediation of toxins, crude oil recovery, food and pharmaceutical industries.

Determination of ionic nature of the partially purified biosurfactant

The purified biosurfactant was found to be anionic in nature as indicated in Figure 2. It showed the presence of an arc of precipitation between CTAB (a cationic surfactant) and the test biosurfactant sample; whereas no zone was observed between sodium lauryl sulphate (an anionic surfactant) and the test biosurfactant sample. Since the working of this test is based on a simple reaction of the precipitate formation when a positively charged cationic and negatively charged anionic molecule react with each other, it can be concluded that the test biosurfactant molecule was an anionic surfactant which is rhamnolipid in nature.

The ionic nature of surfactants is determined on the basis of charge on individual polar moieties in a molecule. As the name suggests, the anionic and cationic surfactants are negatively and positively charged molecules respectively. The anionic surfactants have carboxylate, sulphonate or sulphur groups in the polar region that contributes to the negative charge of the compound. Similarly, the cationic surfactants are characterised by a quaternary ammonium group in the polar region of the compound that confers a positive charge on the molecule. The non-ionic surfactants lack ionic constituents. Majorly they appear as polymers of 1, 2-epoxyethane. The amphoteric surfactants, on the other hand, have both positively and negatively charged moieties in the same molecule [46]. The anionic surfactants find extensive application in commercial products like household cleaners and cosmetics. The ionic nature is also one of the contributing factors for adequate removal of heavy metals from sediment and soil [47].

The anionic nature of several glycolipid biosurfactant viz., rhamnolipids and sophorolipids, produced commonly by Pseudomonas sp. and Torulopsis sp. respectively have also been reported [36], [48]. The anionic character of biosurfactants produced by Serratia sp. is also reported in many studies [28], [29], [49]. On the other hand, *R. erythropolis*, and various *Mycobacterium* and *Arthrobacter* sp. are reported to produce nonionic trehalose corynomycolate biosurfactants [50], [51], [52], [53].

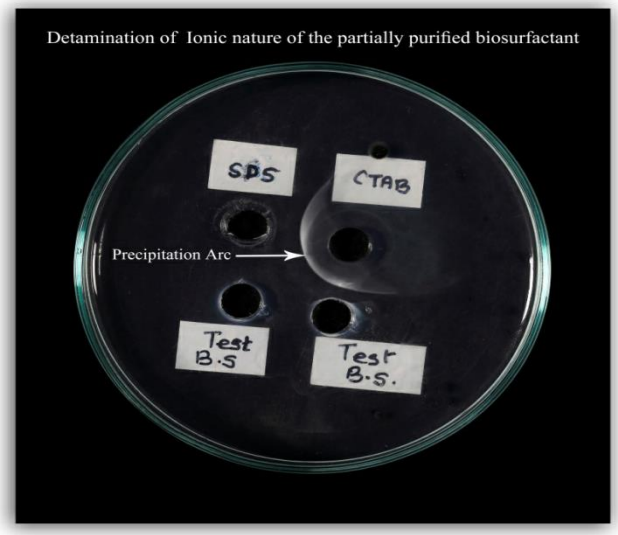

Figure 2. Ionic nature of the partially purified biosurfactant confirmed by CTAB method

Several techniques have been used for determining the ionic nature of biosurfactants. In a study, the analysis of biosurfactants produced by *S. marcescens* using a Zeta Potential Meta 3.0+ indicated an anionic character with Pz = − 20.13 mV [10]. The agar gel immunodiffusion assay indicated that biosurfactant from *Serratia* sp. ZS6 was non-ionic [47]. Similar to our study, the anionic nature of extracted biosurfactants from *O. anthropi* HM-1, *C. bombicola*, *C. freundii* HM-2 and another Candida sp. was determined using the agar double diffusion method with the help of sodium dodecyl sulphate (SDS, 20 mM) as the anionic surfactant, cetyltrimethylammonium bromide (CTAB, 20 mM) and barium chloride (BaCl2, 50 mM) as the cationic surfactants [48], [54], [55]. In another study, the amphoteric nature of the biosurfactant was suggested on the basis of its interaction with resin, studied for the duration of 30min. It was observed that both cationic and anionic resins were able to remove and entrap the

biosurfactant from the aqueous solution achieving a surface tension similar to the pure water [56].

Analysis of the functional groups of the biosurfactant molecule using FTIR technique

The FTIR study showed the existence of 15 peaks as observed in Figure 3. The corresponding molecular vibrations of functional groups are represented in Table 1.

All the peaks obtained, on FTIR analysis of biosurfactant produced by *S. rubidaea* KAP, demonstrated the presence of fatty acids and carbohydrates.

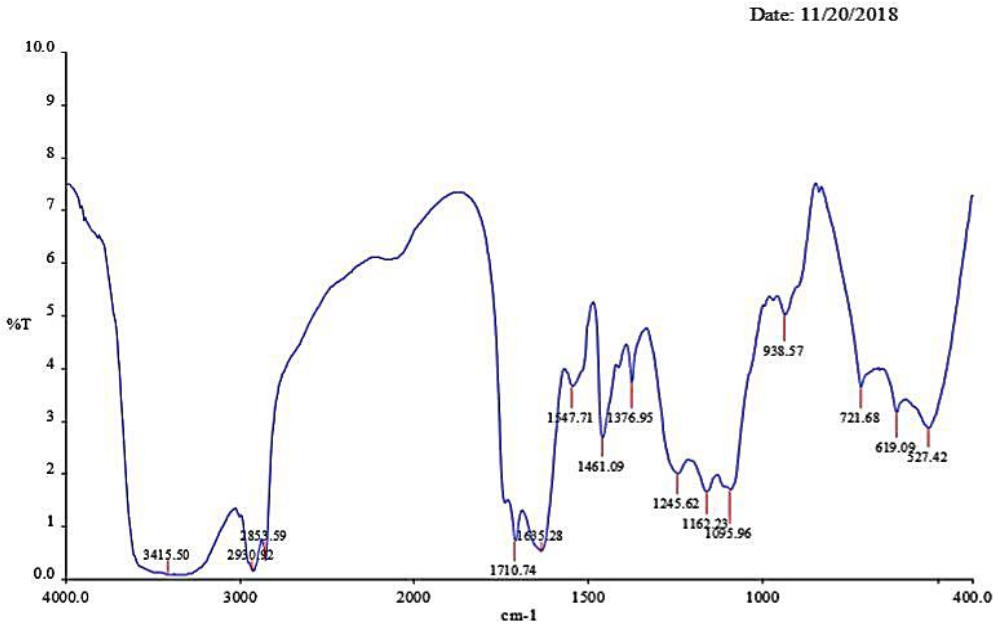

Figure 3. FTIR spectrum of the biosurfactant produced by *S. rubidaea* KAP

Table 1. FTIR analysis of biosurfactant produced by *S. rubidaea* KAP

Sr. no.	Approximate frequency (cm^{-1})	Molecular vibrations of functional groups
1	3415.50	Stretching vibration of O-H bond
2	2853.59	Stretching vibration of the C–H bond of constituent sugar residues
3	2930.92	The occurrence of C-H stretching vibrations of hydrocarbon chain of alkyl (CH2-CH3) / aliphatic chain
4	1635.28	carbonyl functionality present in carboxylate or amide moieties of protein and peptide amines
5	1710.74	>C=O stretching of ester
6	1547.71	The presence of bands at the regions of 1400–1600 cm^{-1} show the presence of C=C stretching vibrations
7	1461.09	The presence of bands at the regions of 1400–1600 cm^{-1} show the presence of C=C stretching vibrations
8	1376.95	The characteristic band present in glycolipids
9	1245.62	The broad stretch of C–O–C, C–O at 1000–1200 cm^{-1} exhibited the presence of carbohydrates.
10	1162.23	The broad stretch of C–O–C, C–O at 1000–1200 cm^{-1} exhibited the presence of carbohydrates.
11	1095.96	The broad stretch of C–O–C, C–O at 1000–1200 cm^{-1} exhibited the presence of carbohydrates.
12	938.57	The fingerprint region below 1200 cm^{-1} represents different kinds of C-H, C-O, and CH3 vibrations.
13	721.68	C-H rocking of >CH2
14	619.09	(for CH2 group)
15	527.42	Indicates sulphides

The functional groups of carbohydrates and lipids were found in combination in the biosurfactant molecule. The characteristic stretching for C-H group was observed between 2800-3000 cm^{-1} indicating vibrations of hydrocarbon chain of an alkyl group (CH2-CH3) / aliphatic chain. Other significant band observed in our study between 3200cm^{-1} and 3500cm^{-1}, with a peak occurring at 2930.92 cm^{-1}, indicated the presence of O-H group in constituent sugar residues. The C=O stretching between 1690-1735cm-1 indicated the presence of esters. The broad stretch observed between 1000-1200cm^{-1} were characteristic of C–O–C and C–O bonds indicating the presence of carbohydrates. In addition, the peak obtained at 1376.95 is a characteristic of a glycolipid band.

The above observations obtained from FTIR analysis, and estimation of components in biosurfactant sample that showed presence of lipids (51.09%) and carbohydrates (37%) as major components suggested the glycolipid nature of the biosurfactant sample.

A recent study reported characteristic similarity between our findings and that observed for standard rhamnose sugar. In this study, the free -OH group stretching occurred at 3430cm^{-1}, the C-H stretching vibrations of CH2 and CH3 groups at 2938cm^{-1}, C=O from ester and carboxylic groups were observed at 1739cm^{-1} and 1629 cm^{-1} respectively. They also reported the C-O and C-O-C stretching at 1042cm^{-1} to be a characteristic of rhamnose sugar [57]. In another study, the absorption peak obtained at 1107cm^{-1} was reported to be similar to the stretching vibrations of –C–O–C–, a group of ether linkage of rhamnose [37]. Similarly, other authors have also attributed findings similar to our observations as a characteristic of rhamnolipid [36], [58], [59]. Similarly, the lipopeptide biosurfactant was reported to be a surfactin, arthrofactin and lichenysin produced by *B. subtilis*, Arthrobacter sp. strain MIS38 and *B. licheniformis* respectively on the basis of FTIR analysis [53], [60]. Therefore, it was reasonable to summarize that the biosurfactant obtained in our study was similar to those of previously reported rhamnolipids. In addition, the results of TLC, HPTLC analysis, and the ionic nature of the glycolipid biosurfactant molecule obtained from *S. rubideae* KAP, further postulated a high probability of the test biosurfactant to be a rhamnolipid.

In a similar study, the biosurfactant produced by *S. marcescens* showed characteristic peaks and functional groups of sewarratin on FTIR analysis. It showed a strong band in the region with wave number 2908.65cm^{-1} and 1105.25cm^{-1} indicating the presence of alkanes, carboxylic acids and their derivatives. Weak bands were observed at 2400.85cm^{-1} and 1662.69cm^{-1} confirming the presence of phosphines and amines. In addition, the presence of alcohols and phenols suggested from the broad bands stretching at 978.74cm^{-1} and weak bands that stretched at 500.34cm^{-1} indicated disulphides as a characteristic of sewarratin [31]. Similarly, the biosurfactant produced by *L. helveticus* showed most significant bands located at 3456cm^{-1} and 3286cm^{-1} (O–H stretching), 1720cm^{-1} (C=O ester bond), 1273cm^{-1} (C–O stretching vibration in sugars), 1041cm^{-1} (polysaccharides), 702cm^{-1} and 648cm^{-1} (for CH2 group) confirming the presence of glycolipid moieties [36]. The FTIR analysis of biosurfactant obtained from *A. chroococcum* revealed its lipopeptide nature. The wavenumbers 2,852, 2,923, 1,421, and 1,465cm^{-1} resulting from the C–H stretching mode suggested the presence of aliphatic chain [45]. The biosurfactant produced by an alkaliphilic bacterium *C. sakazakii* showed the presence of several functional groups in the FTIR spectra. In addition to the similar vibration frequencies observed for C-H, O-H and C=O bonds, they also reported specific peaks for uronic acid (1078cm^{-1} indicating cyclic C-O), carbohydrates (851cm^{-1} indicating glycosidic linkage), proteins (1645cm^{-1} indicating amide moieties) and sulfates (1251cm^{-1}). It was hence classified as polysaccharide-protein complex [61]. The biosurfactant showing band features of lipopeptide compound i.e., surfactin and lichenysin were observed from biosurfactant obtained from *B. licheniformis*. It showed strong bands, indicating the presence of a peptide component at 3280.92cm^{-1} resulting from the N–H stretching mode. It also showed vibrational stretching at 1627.92cm^{-1} (for CO–N bond) and 1535.34cm^{-1} (resulting from the deformation mode of the N–H bond combined with the C–N stretching mode). The bands at 2958–2856cm^{-1} and 1384cm^{-1} resulting from the C–H stretching mode suggested the presence of an aliphatic chain. These results were strong evidence that lipopeptide contains aliphatic and peptide likes moieties. The band at 1735cm^{-1} was also observed due to lactone carbonyl absorption [34].

The presence of sulphides was also detected in our study. Similarly, a lipopeptide biosurfactant produced by *P. stutzeri* NA3 showed a peak at 598cm^{-1} and 638cm^{-1} corresponding to C–I (Carbon–Iodine) and C–Br (Carbon–Bromine) bond respectively [62]. In another study, *A. baumannii* MN3 also showed analogous peaks for C–I and C–Br [63].

The analytical tests carried out earlier in this study suggested that the molecule was composed largely of glycolipid moiety. FTIR results also consistently indicated presence of bonds specific to C-H, C=O, C=C and O-H of the hydrocarbon chain. Hence the GCMS analysis was further carried out to confirm the presence of specific fatty acids.

GCMS analysis of the partially purified biosurfactant

Figure 4 represents the GC-MS chromatogram obtained for the partially purified biosurfactant sample. It showed the presence of 20 peaks with the highest peak obtained at

Rt 15.813. This compound was identified as 9-Octadecenoic acid (Oleic acid). The second highest peak was obtained at Rt 13.909, which was identified as Hexadecanoic acid (Palmitic acid). The detailed results for compounds identified using GCMS analysis are outlined in Table 2.

It clearly shows an abundance of long-chain fatty acids including the above compounds found to be present in highest concentrations. The 9-Octadecenoic acid is an unsaturated fatty acid with an aliphatic tail containing 13-21 carbon atoms while hexadecanoic acid a saturated long-chain fatty acid with 16 carbon backbone which is conjugate acid of hexadecanoate. The biosurfactant molecule also showed the presence of simple as well as complex molecules in addition to palmitic and oleic acid.

Simple alcohols like 1-Dodecanol (CAS) n-Dodecanol which is a primary alcohol (fatty alcohol) and a member of dodecanols derived from a hydride of dodecane was identified in relatively less concentration. On the other hand, complex compounds like Hexadecanoic acid, 1-(hydroxymethyl)-1, 2-ethanediyl ester (CAS), 1, 2-Dipalmitin and 3- Ethoxy-1, 1, 1, 7, 7, 7- hexamethyl -3, 5, 5-tris (trimethylsiloxy) tetrasiloxane were also observed. The GCMS study, showing an abundance of oleic acid and palmitic acid, thus co-relates with our FTIR findings that confirmed the presence of various functional groups like C-H, C=O, C=C and O-H observed in their structures. The GCMS analysis thus helped us in further confirmation of lipid moieties in the biosurfactant sample as detected by analytical techniques like TLC, HPTLC and FTIR.

Previous reports confirm that the biosurfactant molecules are composed of low molecular weight compounds or high molecular weight polymers. Among the low molecular weight compounds, glycolipids, flavolipids and phospholipids are commonly known. The high molecular weight polymers like lipoproteins, lipopolysaccharide-protein complexes and polysaccharide-protein-fatty acid complexes are also reported. The hydrocarbon chain of a fatty acid forms a major part of the lipophilic (hydrophobic) moiety of the known biosurfactants. In contrast, the other groups like esters, alcohols, carboxylates as well as organic acids form the hydrophilic moiety of the biosurfactants [64], [65], [66].

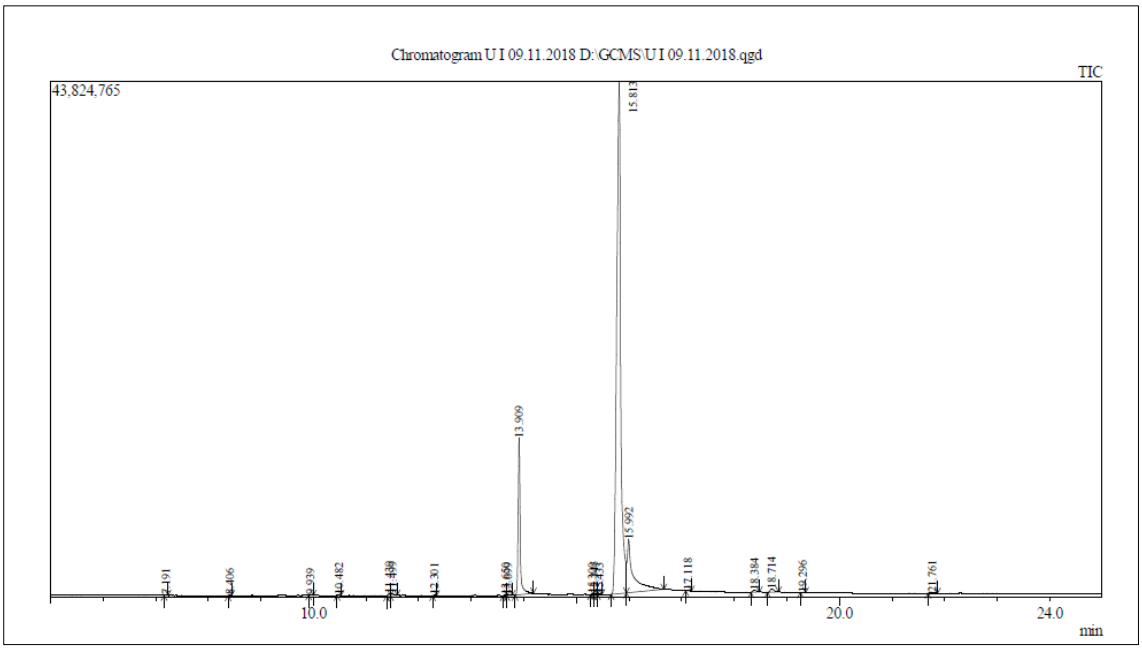

Figure 4. GC-MS chromatogram obtained for the partially purified biosurfactant sample

Table 2. Compounds identified by GC-MS in partially purified biosurfactant sample

Peak	Retention time	Area (Area %)	Height (Height %)	A/H	Name of compounds	Formula	Molecular Weight	CAS No.	SI
1.	7.191	97343 (0.03)	49464 (0.08)	1.97	1- Dodecanol (CAS) n-Dodecanol	$C_{12}H_{26}O$	186	112-53-8	84
2.	8.406	100694 (0.03)	57313 (0.09)	1.76	3- Ethoxy-1, 1, 1, 7, 7, 7-hexamethyl -3, 5, 5-tris	$C_{17}H_{50}O_7SI_7$	562	72439-79-3	79

					(trimethylsiloxy) tetrasiloxane				
3.	9.939	94365 (0.03)	39039 (0.06)	2.42	1, 2- Benzoldicarbonsaeure, di-(hex-1-en-5-yl-ester)	$C_{20}H_{26}O_4$	330	0-00-0	85
4.	10.482	117289 (0.04)	64256 (0.10)	1.83	3, 4- Dihydroxymandelic acid-tetratms	$C_2H_{40}O_5SI_4$	472	0-00-0	82
5.	11.439	476178 (0.15)	181990 (0.29)	2.62	Cyclododecyne (CAS)	$C_{12}H_{20}$	164	1129-90-4	89
6.	11.499	571272 (0.18)	179152 (0.28)	3.19	9- Octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	282	112-80-1	88
7.	12.301	95321 (0.03)	51511 (0.08)	1.85	Cyclohexasiloxane, dodecamethyl- (CAS) Dodecamethylcyclohexasiloxane	$C_{12}H3_6O_6SI_6$	444	540-97-6	76
8.	13.650	182256 (0.06)	65195 (0.10)	2.80	Cycloheptene, 1- methyl-	C_8H_{14}	110	1453-25-4	81
9.	13.699	266940 (0.08)	83936 (0.13)	3.18	9- Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1	93
10.	13.909	35434464 (11.23)	13336632 (21.14)	2.66	Hexadecanoic acid (CAS) Palmitic acid	$C_{16}H_{32}O_2$	256	57-10-3	94
11.	15.300	135233 (0.04)	48237 (0.08)	2.80	9, 12- Octadecadienoic acid (Z, Z)- (CAS) Linoleic acid	$C_{18}H_{32}O_2$	280	60-33-3	78
12.	15.345	202743 (0.06)	91875 (0.15)	2.21	Methyl 9- octadecenoate	$C_{19}H_{36}O_2$	296	0-00-0	85
13.	15.433	107909 (0.03)	50260 (0.08)	2.15	3, 4- Dihydroxymandelic acid-tetratms	$C_{20}H_{40}O_5SI_4$	472	0-00-0	68
14.	15.813	237419175 (75.26)	43540909 (69.02)	5.45	9- Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1	93
15.	15.992	37207304 (11.79)	4565903 (7.24)	8.15	9- Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1	93
16.	17.118	192314 (0.06)	66396 (0.11)	2.90	1H- Purin-6-amine, [(2-fluorophenyl)methyl]- (CAS)	$C_{12}H_{10}FN_5$	243	74421-44-6	71
17.	18.384	777474 (0.25)	183132 (0.29)	4.25	9- Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1	92
18.	18.714	1377594 (0.44)	298708 (0.47)	4.61	9- Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1	92
19.	19.296	134435 (0.04)	42180 (0.07)	3.19	Eicosamethyl cyclo-deca-siloxane	$C_{20}H_{60}SI_{10}$	740	18772-36-6	66
20.	21.761	460557 (0.15)	84870 (0.13)	5.43	Hexadecanoic acid, 1-(hydroxymethyl)- 1, 2- ethanediyl ester (CAS) 1, 2- Dipalmitin	$C_{35}H_{68}O_5$	569	761-35-3	85

In another study, the phospholipid biosurfactant obtained from, *S. marcescens* UEO15 showed the presence of 8 peaks. The phospholipid nature of the biosurfactant was confirmed on the basis of observed phosphine P-H3 stretch in FTIR analysis and peak 7 identified as a hydroxyphosphinyl group by GC-MS [31]. Similarly, the GC-MS analysis confirmed the lipopeptide nature of biosurfactants obtained from *S. marcescens* NSK-1 and *S. marcescens* UCP 1549 respectively [10], [25]. The isogenic strains of *S. marcescens* i.e. SM3 and SMRG showed the presence of octadecanoic and hexadecanoic acid respectively as major compounds in the biosurfactant [30]. Similarly, the glycolipid nature of biosurfactant obtained from *S. marcescens* was reported with glucose and palmitic acid as the hydrophilic and hydrophobic moiety respectively [29].

Interestingly, in another study, Serratia sp. ZS6 showed secretion of both serrawettin-type biosurfactant as well as lipase production that was confirmed by LC-MS analysis [32]. Similar to our findings, it was also reported octadecanoic acid and 9-octadecenoic acid as the major components of the lipopeptide biosurfactant isolated from *B. subtilis* [67]. The presence of hexadecanoic acid was also reported in the biosurfactant produced by Rhodococcus sp. TW53 [68]. Another study also reported the presence of palmitic acid in *Candida bombicola* [69]. The biosurfactant from *P. stutzeri* and *A. baumannii* MN3 also showed presence of hexanedioic acid and bis (2-ethylhexyl) ester ($C_{22}H_42O_4$) respectively [63].

The GCMS analysis of glycolipid biosurfactant produced by *Bacillus safensis* PHA3 indicated that the lipid moiety of the sample was majorly comprised of hexadecanoic acid. tetradecanoic acid and octadecanoic acid was also identified in low concentrations. The presence of mannose was confirmed using other analytical techniques. Hence, the GCMS analysis strongly indicated that the glycolipid (mannolipid) was comprised of d-Mannose as the hydrophilic moiety and hexadecanoic acid as the hydrophobic moiety [35]. In another study, 14 different

components (mostly lipids) were detected in a hydrocarbon-degrading *P. aeruginosa* NGB4 [70].

Determination of Critical micelle concentration (CMC)

Figure 5 represents the CMC value determined for the biosurfactant produced by *S. rubidaea* KAP. It was capable of reducing the surface tension of distilled water from 72.05-27.28 dynes cm^{-1} i.e. a surface tension reduction of 62% was observed. In general, the molecules capable of reducing the surface tension by more than 50% are considered good [71, 72]. In this study, the reduction in surface tension was directly proportional to the concentration of the biosurfactant until it reached a concentration of 1000mg/L, after which it remained constant.

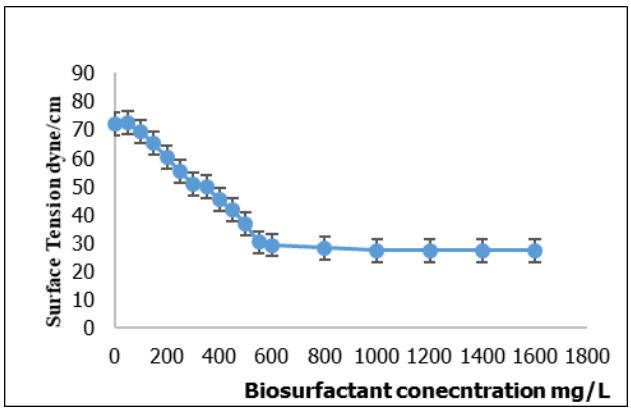

Figure 5. Determination of CMC value of the biosurfactant produced by *S. rubidae*a KAP

The concentration of biosurfactant at which the molecules are organised into micelles, and achieve the lowest stable surface tension is characterised as the critical micelle concentration [72]. The biosurfactants are more effective and efficient and their CMC is several times lower than chemical surfactants [2]. Literature survey reflects a higher efficacy of the bacterial biosurfactants in reducing surface tension [71], [73], [74]. Among the bacterial species, *P. aeruginosa* is most studied for its biosurfactant producing potential. It shows a considerable reduction of surface tension to values around 28–27 mN/m [75], [76].

Studies have reported surface tension reduction to 27mN/m and 26.5mN/m by different strains of *S. marcescens* [30, 77]. A CMC value of 29mg/L (0.03%) was reported in *S. marcescens* NSK-1 isolate [33]. Similarly, biosurfactant produced by *S. marcescens* UCP 1549 exhibited a CMC of 1.5%, with the surface tension reduction to 25.92mN/m [10]. The crude extract of biosurfactant produced by *Serratia* sp. ZS6 exhibited the CMC of 19g/L that reduced surface tension to 35mN/m [32].

Among other isolates, *B. amyloliquefaciens* MO13 displayed a CMC of 36mg/L and was able to reduce the water surface tension from 71.2mN/m to 27mN/m. Isolates *B. gibsonii* ODW12, *B. subtilis* ODW02, *B. amyloliquefaciens* MO4B and *B. amyloliquefaciens* MO13 showed water surface tension reduction from 71.2mN/m to values below 40mN/m at concentrations between 1 and 200mg/L [78]. In other studies, the surfactin and rhamnolipid biosurfactant produced by *B. subtilis* and *P. aeruginosa* were capable of reducing the water surface tension to 25mN/m and 26mN/m respectively. Both strains exhibited a CMC of 1.5% (w/v) and reduced the interfacial tension water/hexadecane to less than 1mN/m [79], [80]. Similarly, the CMC of the glycolipid produced by Acinetobacter sp. ACMS25 was determined as 3.25% from the constant value of surface tension of 25.8mN/m. Amongst the well-studied yeast *Candida glabrata* UCP 1002, the extracellular biosurfactant showed a CMC of 1% and the surface tension at that point was 24mN/m [81]. The CMC value of crude biosurfactant was found to be 40mg/L with high emulsification activity of 72.45 ± 0.85% for the lipopeptide biosurfactant, surfactin [82]. In another report, the biosurfactant from *O. sagaranensis* AT18 showed a lower minimum surface tension of 25.0mN/m at a CMC value of 8mg/L [83].

Recent studies reported a CMC of 0.12mg/mL, 23.6mg/mL and 21.6mg/L exhibited by *P. aeruginosa* HAK01, *L. acidophilus* NCIM 2903 and Pseudomonas sp. PDD-14b-2 respectively [84], [85], [86].

The distinct variations noted in CMC values reported by many authors is due to several factors like ratio and composition of homologues, the presence of unsaturated bonds, the branching and length of the aliphatic chain of the biosurfactant. These variations may be avoided by using pure biosurfactants for analytical studies [87].

Stability study

Figure 6 represents the effect of temperature on the surface tension reduction and emulsion forming capacity of the biosurfactant isolated from *S. rubideae* KAP. As observed from the figure, our results were very significant. The surface tension reduction values were recorded from 28.4 to 40.9 dynes/cm at 4°C to 100°C. The surface tension and emulsification activity were also studied at a temperature maintained for sterilization of medium and glassware in microbiology laboratory i.e. 121°C (autoclaving temperature). It is important to note that the biosurfactant retained its ability to reduce surface tension and emulsify hydrocarbons from 4°C – 45°C up to 28.1dynes/cm and 80-87.5% respectively, between 55°C - 100°C it was 30-40 dynes /cm and 72-62% respectively. Even at very high temperature, there was little reduction in these values indicating thermal stability.

Figure 7 represents the effect of various pH on the surface tension reduction and emulsion forming capacity of the biosurfactant isolated from *S. rubideae* KAP. It showed a

significant reduction in surface tension as well as emulsification activity at acidic pH i.e. between 2- 5.5. Considerable biosurfactant activity was observed with an increase in pH i.e. between pH 6.5- 8.5. It further declined with an increase in pH towards the alkaline conditions i.e. pH of 9.5 -12. However, this reduction was much less compared to that in the acidic range where a considerable loss of biosurfactant activity was observed. This may be due to partial precipitation of the molecule at acidic pH which leads to change of conformation of the molecule [33], [88].

Figure 8 represents the effect of various concentrations of NaCl on the surface tension reduction and emulsion forming capacity of the biosurfactant isolated from *S. rubidaea* KAP. The biosurfactant molecule retained its ability to reduce the surface tension of distilled water and showed good E24 index, till a concentration of 10% of NaCl. Beyond 12% NaCl concentration, the ability of biosurfactant to reduce the surface tension was retained, however, significant reduction in emulsification activity was observed. Thus the stability studies indicate considerable biosurfactant activity at a wide range of temperature (from 4°C -100°C), alkaline pH and in an environment of salinity up to 12%. These results strongly suggest that this product can be useful in industries which operate at extreme conditions of pH, temperature and salinity e.g., pharmaceutical, cosmetics, food industries and for bioremediation in the marine environment.

One of the major reasons due to which, biosurfactants are preferred over chemical surfactants is their observed stability towards parameters such as temperature, pH and salinity. Most of the industrial activities revolve around these three parameters. Hence stability studies are carried out as important criteria for biosurfactant characterization. Some biosurfactants have been found to be less stable over extreme pH range [22], [89]. However, further screening and isolation of organisms and biotechnological approaches may help in overcoming this hurdle. Also, biosurfactants showing tolerance to around 12-15% NaCl concentration is reported which makes it superior to chemical surfactants that are deactivated at 2-3% salt concentration [88].

Similar to our findings, the biosurfactant produced by *S. marcescens* UCP 1549 evidently remained stable at extreme values of pH, temperature and NaCl concentration. However, better surface tension reduction of 25.1-26.8mN/m was observed at all tested conditions as compared to our study [10].

Another *S. marcescens* strain was reported to produce stable biosurfactants which were unaffected by extremes of pH, temperature and salinity [67]. The biosurfactant produced by *S. marcescens* NSK-1 was relatively heat-stable. Even at 100°C, it retained over 80% of its activity. It also showed tolerance up to 12% sodium chloride concentration [25]. Similarly, biosurfactant produced by *Virgibacillus salaries* was found to be thermostable and showed tolerance up to 12% NaCl. As observed in their study, its biosurfactant activity was also strongly affected at low pH values from 2 to 5 [90].

BS-FLU5 produced by a new hydrocarbonoclastic marine bacterium, *Bacillus stratosphericus* FLU5, showed a stable surface tension of the solution at about 34mN/m from pH 2.1–12 [91]. Another study reported the stability of biosurfactant between pH 6.0 and 12.0 (28 to 29 mN/m), and increased slightly below pH 6.0, reaching 32mN/m at pH 2.0 [92]. This could be attributed to the denaturation of protein components or the increase in the ionisation of the medium which can cause a change in surface structure at extreme pH values [93]. During a study with *B. subtilis* R1, it was found that the surface-active agent was stable up to 80°C, pH range of 4.0–12.0, and 10% salt concentration till 10th day which was a significant result [94].

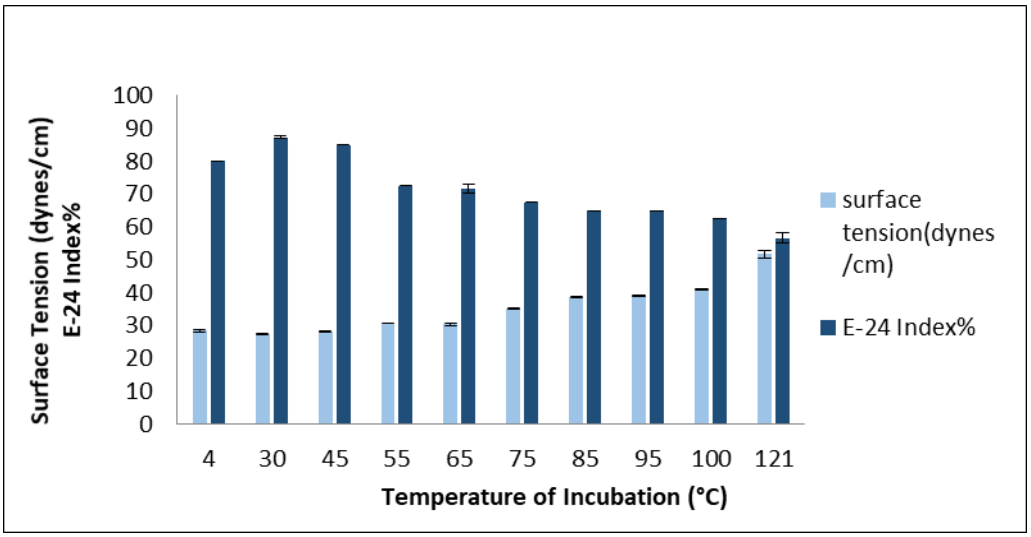

Figure 6. Effect of temperature on the stability of biosurfactant produced by *S. rubidaea* KAP

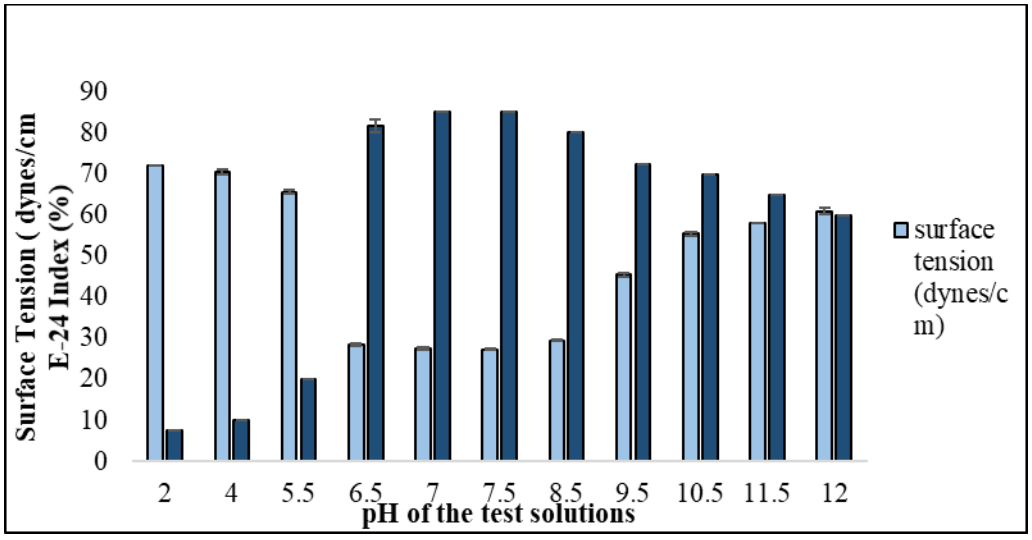

Figure 7. Effect of pH on the stability of biosurfactant produced by *S. rubidaea* KAP

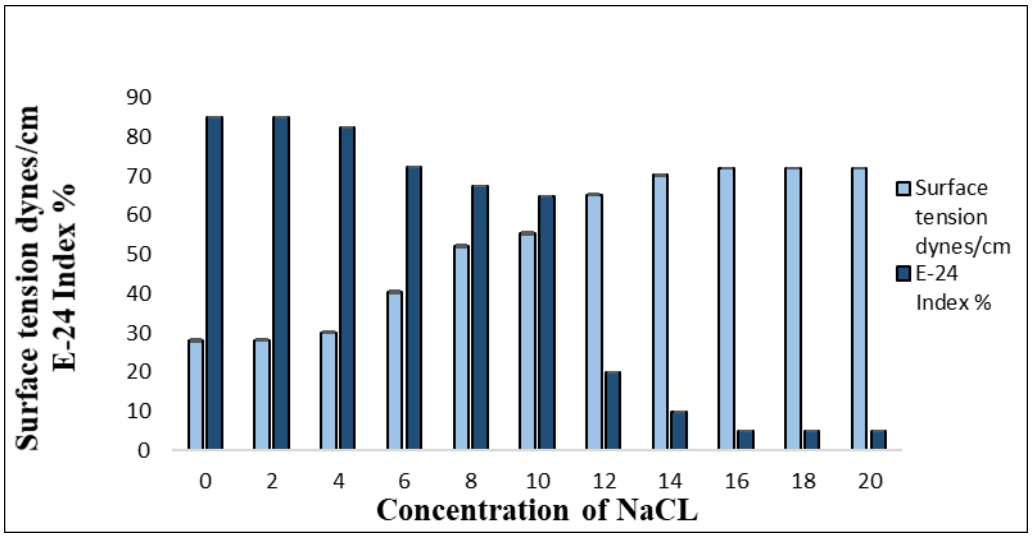

Figure 8. Effect of NaCl concentration on the stability of biosurfactant produced by *S. rubidaea* KAP

In another report, the biosurfactant functioned well at 90°C, basic pH and at 5% salt concentration [95]. At concentrations of sodium chloride above 5%, carboxylate groups of the bio-surfactant (rhamnolipid) is ionized which induces the repulsion between biosurfactant molecules. The Na^+ ions form a compact monolayer around it which decreases the performance of the biosurfactant [96]. The electrolytes present in NaCl affect the interface between solution and air, and acquires negative charge (due to the carboxylic acid groups at saline pH). The biosurfactant activity of *P. fluorescens* was also seen to be unaffected up to 10% sodium chloride concentration in another study [97]. The biosurfactant produced by *P. aeruginosa* BS20 showed similar findings which exhibited excellent stabilities at high temperatures of 100°C for 1h and at 121°C for 10min, salinities up to 6% NaCl and pH values up to 13 [98]. The biosurfactant produced by Paenibacillus sp. exhibited unusual stability at a concentration of 300g/L of NaCl [99].

The thermal stability of oil degrading *B. subtilis* MG495086 was analysed by another method of thermal gravimetric assay and it showed that the strain could resist very high temperature of 150°C [82]. Another study reported the thermal stability of biosurfactants in terms of thermal degradation carried out by gravimetric analysis. Approximately 1% of weight loss was recorded from an increase in temperature from 50°C to 220°C possibly due to loss of solvents and moisture molecules. Complete loss of biosurfactants was observed after 27.5°C. It was previously reported that the biosurfactant produced from an alkalophilic strain of Klebsiella sp. showed maximum degradation at 35–40°C [100]. Similar findings were also reported for rhamnolipids produced by *P. aeruginosa* MA01 [101].

The biosurfactant (lichenysin) produced by *B. licheniformis* showed stability in the pH range of 4.5-9.0 and temperature up to 50°C. Its activity was also least affected in presence of NaCl as well as calcium concentrations of 50 and 25gL^{-1} [102]. A lipopeptide biosurfactant produced by *B. subtilis* was found to be stable at high (121°C) as well as low (15°C) temperatures even after storage for 180 days [103]. Another biosurfactant produced by *Arthrobacter protophormiae* was also found to be stable to high temperature (30-100°C) and pH (2-12) [104]. The biosurfactant produced by *P. aeruginosa* SCMU106 was reported to be non-cytotoxic and stable to high temperature, neutral to alkaline pH (pH 6-10) and tolerated moderate concentration of NaCl (up to 8% NaCl) [105].

IV. CONCLUSION AND FUTURE SCOPE

The significance of biosurfactants is increasingly recognised in industries preferably working on the principles of green chemistry. However, competing with the current industrial demand requires enhanced production of biosurfactants that can be achieved through strain improvement by genetic engineering. Monitoring of the interrelated metabolic bioprocesses using Response Surface Methodology (RSM) and other statistical methods like Taguchi and Plackett–Burman Design (PBD) can help in enhanced biosurfactant production. More efforts are required to enhance economic feasibility by using a variety of waste products from agricultural fields such as bran, hull of rice and corn, potato or orange peel etc. The downstream process employed for recovery and separation of the specific compound can also be tested by newer methods of whole broth processing. In order to increase the applicability of the biosurfactant, it is necessary to gain more information about the structure of the test compound which can be achieved using newer and modern bioanalytical tools such as Nuclear magnetic resonance technique or Fast atom bombardment mass spectrometry.

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