WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 5, 2562-2576. Research Article ISSN 2277– 7105

HPLC-MS, HRMS ANALYSIS OF MICROBIAL ACID FREE, SHORT CHAIN ALKYL SOPHOROSIDES

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Article Received on
15 March 2015,

Revised on 06 April 2015,
Accepted on 30 April 2015

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ABSTRACT

Sophorolipids (SL) are glycolipid biosurfactants abundantly produced from different feedstocks by yeasts and have been widely developed for various applications. The amphipathic structures of sophorolipids imparts to them surfactant type properties. These biosurfactants are readily isolated in high yield, are nontoxic and biodegradable, which makes them industrially interesting as surfactants or emulsifiers. Sophorolipid production was done using glucose as hydrophilic source and lauryl alcohol, as hydrophobic source using *Candida bombicola* ATCC 22214. Zeta Potential measurements were performed at different pH values to determine the surface charges. Initial identification of structures was performed by MALDI-TOF/TOF. A reverse phase HPLC method combined with electrospray ionization mass detection (HPLC-MS) system was used to identify the structures of individual SL components. Further confirmation of the structures was done using HRMS. Acid free and short chain sophorose lipids were synthesized using primary alcohol. Glycosidic linkage was incorporated directly between the sophorose and hydroxyl function of the lauryl alcohol. Terminal oxidation of the non-functionalized end resulted in novel sophorolipids.

KEYWORDS: Biosurfactants, Sophorolipids, *Candida bombicola*, lauryl Alcohol, Zeta Potential, HPLC-MS. (SLLA - Sophorolipid obtained using lauryl alcohol).

INTRODUCTION

Sophorolipids are surface-active compounds synthesized by a selected number of yeast species. They have been known for over 40 years, but because of growing environmental awareness, they recently regained attention as biosurfactants due to their biodegradability,

low ecotoxicity, and production based on renewable resources. Several types of molecules can act as hydrophobic carbon source: oils, fatty acids, their corresponding esters, alkanes, etc. Despite the abundance of reports on sophorolipid fermentations with various substrates, it is hard to compare them as often different culture conditions or medium compositions are used. Furthermore, oils (especially those of vegetable origin) are widely used as lipidic carbon source. The most common vegetable oils are comprised of saturated or unsaturated fatty acids with chain lengths of 16 or 18 carbon atoms, making them an ideal substrate for direct incorporation and the consequent high sophorolipid production and yield.[1]

The term new-to-nature sophorolipids, refers to all sophorolipid molecules different from the ones found in nature; i.e. the ones synthesized de novo microbially or based on the conventional substrates such as described above.[2] The hydroxylation step governs the sophorolipid fatty acid moiety. Brakemeier et al. (1995, 1998a) developed a method to circumvent the length-dependent and restricted incorporation of substrates. When secondary alcohols were used (C_{12} to C_{16}) as the lipophilic carbon source their direct incorporation was observed.[3,4] The resulting compounds displayed better surface active properties as compared to native sophorolipids. Similarly 12-hydroxy dodecanoic acid and dodecanediol were also applied.[5] Secondary alcohols applied by Brakemeier et al. were infact a racemic mixture. Ketones were found to bypass the configuration problem as apparently the yeast reduces 2-, 3- or 4-dodecanones to their corresponding S-alcohol, resulting in higher yields when compared to the corresponding racemic alcohol mixture despite the additional reduction.[6]

Primary alcohols too were utilized for SL synthesis where these compounds were metabolized in β-oxidation pathway in large degree. This was partially avoided by applying higher glucose and yeast extract concentrations of 150 and 4 g/L, respectively.[4] Yet, in order to completely redirect the primary alcohols towards sophorolipid synthesis, the competing β -oxidation pathway was blocked at the genome level of the yeast by targeting the multifunctional enzyme 2 gene. The deletion strains showed a 2 to 3-fold higher production of sophorolipids compared to the wild-type when grown on substrates such as 1-dodecanol, 1-tetradecanol and 1, 12-dodecanediol.[7,8] Dengle Pulate et al., reported use of lauryl alcohol as a potential lipidic feed stock for sophorolipid production using *C. bombicola*. Modification of the lipophilic portion of the sophorolipid structure has shown to alter the physicochemical properties.[9]

As per previous reports, applications potentials of SL's can be expanded if the lipophilic moiety being shortened rendering molecule more hydrophilic and consequently better water solubility. In this study SL production was done using medium chain fatty alcohol lauryl alcohol. Lauryl alcohol is a medium chain primary alcohol derived from natural fats and oils. Major application is in the manufacture of chemical intermediates and surfactants. As discussed previously by Brakemeier et al. (1995, 1998a), the hydroxylation step governs the sophorolipid fatty acid moiety. Yet, the length-controlling action can be circumvented by supplying the yeast with suitable hydroxylated substrates. From an economical and structural point of view, primary alcohols are ideal substrates; they are cheaper than the previously described secondary alcohols, ketons, and diols, and their lack of stereospecificity allows full incorporation. Also, without blocking the β-oxidation pathway at the genome level of the yeast new to nature alkyl sophorosides were synthesized. This restricted length and new to nature SL's have broaden up applications range as it has resulted in good surface tension lowering capacities. Also found to be potent antimicrobial agent SL's showed complete inhibition of some of the gram positive as well as gram negative microorganisms.[10] A germicidal composition suitable for cleaning fruits and vegetables was formulated using SLLA and its effectiveness was determined on the shelf life of the sprayed vegetables.[11] This is the first report where without blocking the β -oxidation pathway 1-alkanol was used to synthesize SLs. Complete surface activity and structural characterization of this new to nature sophorolipids was done using various techniques.

EXPERIMENTAL

All chemicals used for this investigation were of analytical grade and were used as received unless otherwise noted. Glucose was procured from Qualigens, India. Yeast extract, malt extract and peptone were procured from Hi-media, India. Lauryl alcohol (C_{12}) was a kind gift from Galaxy Surfactants LTD India.

***Fermentative procedure for production of Lauryl alcohol derived Sophorolipids*:** 10 mL inoculum was developed by growing the *C. bombicola* ATCC 22214 cells in medium consisting of g/L: Glucose, 100; Yeast extract, 1.0; $MgSO_4.7H_2O$, 0.3; Na_2HPO_4, 2; NaH_2PO_4, 7; $(NH_4)_2SO_4$, 1; for 24 h at 30°C and 180 rpm orbital shaking.[12]

Starter culture was prepared by transferring the inoculums in 50 mL medium followed by incubation at 30°C for 24 h with 180 rpm orbital shaking. The fermentative production was initiated by transferring the starter culture into 250 mL of the respective medium in 1000 ml

conical flask followed by incubation at 30^{o}C with 180 rpm orbital shaking. The medium was supplemented with lauryl alcohol (1mL/100mL of medium) dissolved in 1mL of ethanol.

Separation of SLLA from culture broth: Cells were harvested by centrifugation at 5000 rpm, 10^{o}C for 20 min. The supernatant was transferred to a separating funnel and extracted twice with equal volume of ethyl acetate. Bottom aqueous layer and the top ethyl acetate layer were transferred to separate flasks. The aqueous portion was re-extracted until no further colour persists in the ethyl acetate layer. 0.5 g of sodium sulfate per 100 mL of ethyl acetate portion was added, to remove the traces of water present, filtered and rotary evaporated to yield a faint brown product. This product was washed twice with n-hexane to remove unreacted fatty alcohol (lipophilic substrate) and was stored at 4^{o}C. Removal of this residual lipophilic substrate will not influence the various measurements of surface activity.

Zeta Potential: Zeta potential of SLLA was determined with a Brookhaven 90+ zeta analyzer (Brookhaven Instrument, Long Island, NY, USA). 1% SLLA samples at different pH ranging from 3-9 were prepared. Prior to measurements the instrument was calibrated. Samples were injected directly into the quartz capillary with a 10mL syringe. Prior to the injection of sample 100mL of pure water was injected to wash the quartz capillary. Each injection was read seven times and the average value adopted. Every sample in the above experiment was conducted in triplicate.

Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS): MALDI-TOF MS screening was accomplished using a AB SCIEX TOF/TOF 5800 instrument in linear mode with positive ion detection. 1mg of SLLA was dissolved in 1mL of acetonitrile. Further, 5 μL of the sample was mixed with 20 μL of dithranol matrix. Determinations were performed in duplicate.

HPLC-MS: Sophorolipids mixtures were separated by HPLC with a Waters Acquity UPLC using a 2.1* 100mm Symmetry C18 column coupled in series. The gradient solvent elution profile used was as follows: water/acetonitrile (0.1% formic acid) (90:10 v/v) holding for 5 min; to a final composition of water/acetonitrile (0.1% formic acid) (10:90 v/v) with a linear gradient over 20 min and holding for 5 min. The flow rate was 0.3 ml min^{-1}. The mixtures were scanned by PDA detector from 210nm to 400nm. The effluent was connected to SQ mass detector with an ESI probe set to positive or negative mode and to scan from m/z 100 to

900 at 0.5 seconds per scan. Capillary voltage was tune to 2.98 kV, sample cone 33.46V and extraction cone 1V for detection of fragments and molecular ion.

HR-MS: SLLA synthesized as a mixture was resolved into its individual components based on the derived molecular formula using LC-HRMS. This technique enables detection of molecular weight to the nearest 0.001 atomic mass units. The mass resolution of a mass spectrometer is defined mathematically in parts per million (ppm): [MW measured - MW theoretical]/MW theoretical X 10^6. The high-resolution mass spectrometer (Thermo Scientific) used in the study was Hybrid Quadrapole Q-Exactive orbitrap. For chromatographic separation of SLLA, Thermo Scientific Hypersil ODS C18 column of dimensions, 150mm X 4.6 mm, having 8µm particle size was used. The sample was prepared by dissolving SLLA in methanol to a final concentration at 100 µg/mL. The gradient solvent elution profile used was as follows: starting with water/acetonitrile in ratio of 99:1 v/v, followed by 95:5 v/v upto 2.41 minutes, then increasing the water/acetonitrile gradient to 70:30 v/v upto 10.21 minutes and finally water/acetonitrile in ratio of 60:40 v/v uptil 15 minutes. Flow rate was set as 500µL/min. The mass spectrometer was operated in positive electrospray ionization mode in 70,000 full-width at half-height maximum resolution with mass range *m/z* 300 to 800. The operation conditions were as follows: spray voltage at 3.6 kV, capillary temperature at 320 °C, S-lens RF level at 50, automatic gain control (AGC) at 1×10^6, and maximum injection time at 120ms.[13] Nitrogen was used as the sheath gas, auxiliary gas, and sweep gas, set at 45, 10, and 2, respectively (arbitrary units). A volume of 5 µL of sample was injected and full HR-MS scan was performed using positive polarity. Data were analyzed with Thermo Scientific Xcalibur software. The exact mass-to-charge ratios of molecular ions of the predicted SLLA mixture components were calculated and used to extract the ions from the full-scan total ion current (TIC). The mass spectra associated with chromatographic peaks were analyzed. The peaks with highest relative abundance values were correlated with [M]/ [M+$NH3^+$]/ [M+Na^+] i.e. native molecular mass, ammonium adduct and sodium adduct. Thus based on the m/z (mass to charge ratio); the structures were putatively identified.

RESULTS AND DISCUSSIONS

Zeta Potential: The zeta potential, ξ, also called the electro kinetic potential, is defined as the value of the electrical potential at the "shear plane" of the particle. For typical colloids this point is close to the actual surface of the particle. It is the potential difference between the

dispersion medium and stationary layer of fluid attached to the dispersed particle. The significance of zeta potential is in the stability of colloidal dispersions as seen in the case of SLLA solution. Higher the zeta potential, higher is the stability of the dispersion. A zeta potential of ± 20 to ±40 is moderate stability and ±40 to ± 60 is considered a good stability measure of the colloidal dispersion. The pH of the suspension strongly influences the net charge of the colloidal particles and therefore their stability against aggregation. Zeta potential of SLLA solution was measured at different pH ranging from 3.0 to 9.0. **Fig 1**. SLLA at pH 5.0 had a mean zeta potential of -20.45 which assigns moderate stability. Lack of charged (polar) group such as –COOH results into weak surface charge hence lesser repulsion between the group resulting into aggregation and lower zeta potential. Thus, at pH 5.0 the SLLA solution is stable and could be used for germicidal formulations and skin lotions along with shampoos.

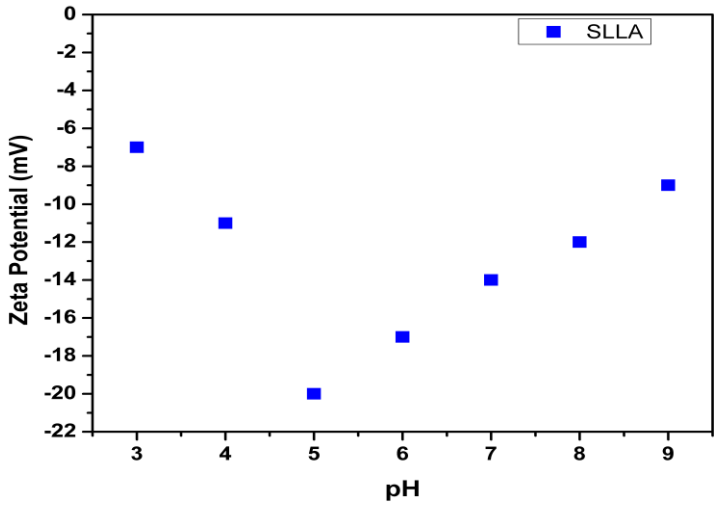


Figure 1: **Zeta potential of SLLA solution at different pH**

MALDI-TOF/TOF: MALDI is a soft ionization mass spectrometry technique that allows the identification of intact compounds. The recovered SLLA was initially screened for the presence of sophorolipids by MALDI-TOF/TOF. The spectra were characterized by molecular adduct ions for SLLA in the mass range 100 to 800 Da **(Fig 2)**. Major ions at m/z 575, 533, 617 are attributed to the $[M + Na]^+$ for alkyl sophorosides while m/z 633.

$[M + Na]^+$ corresponds to structure of sophorolipids hydroxylated at both (ω-1) and α (ω-1) positions.

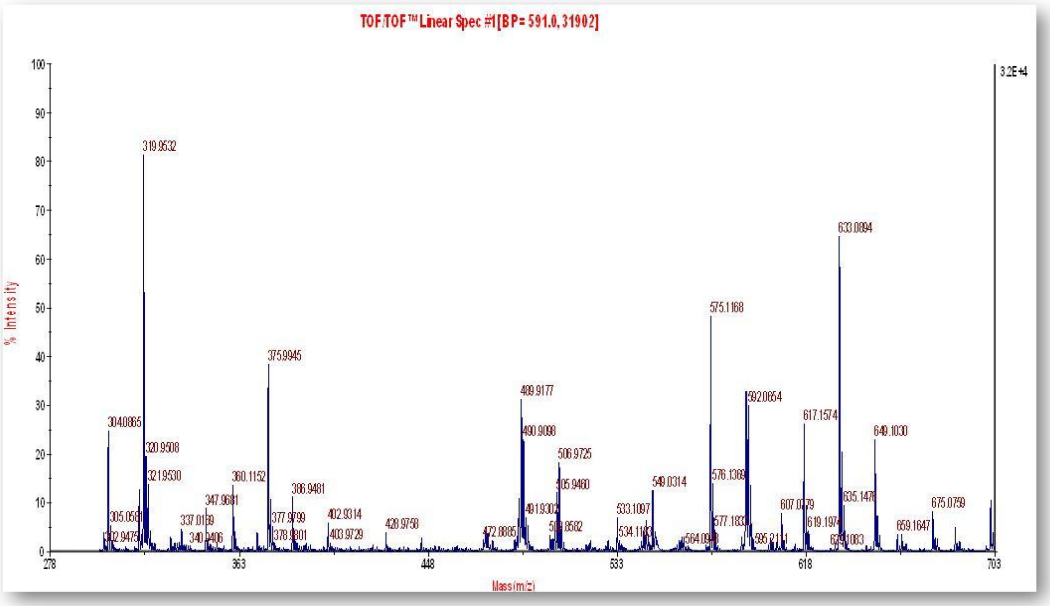
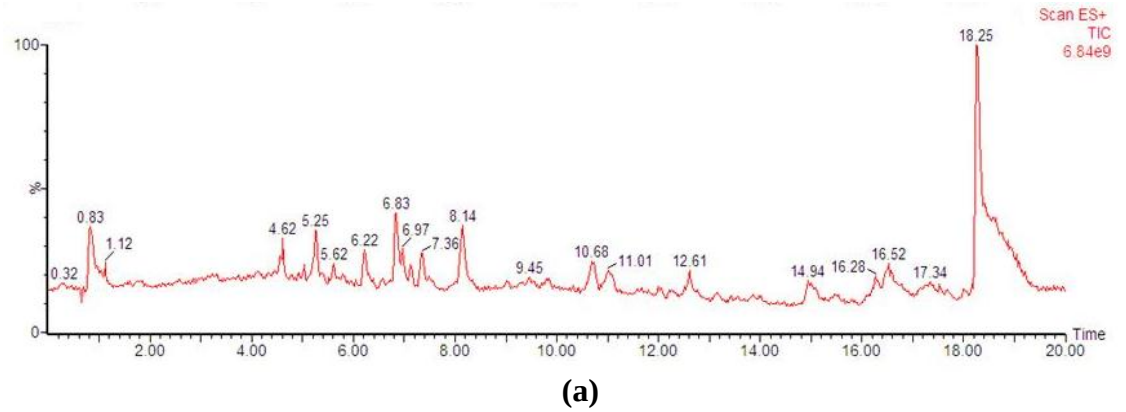
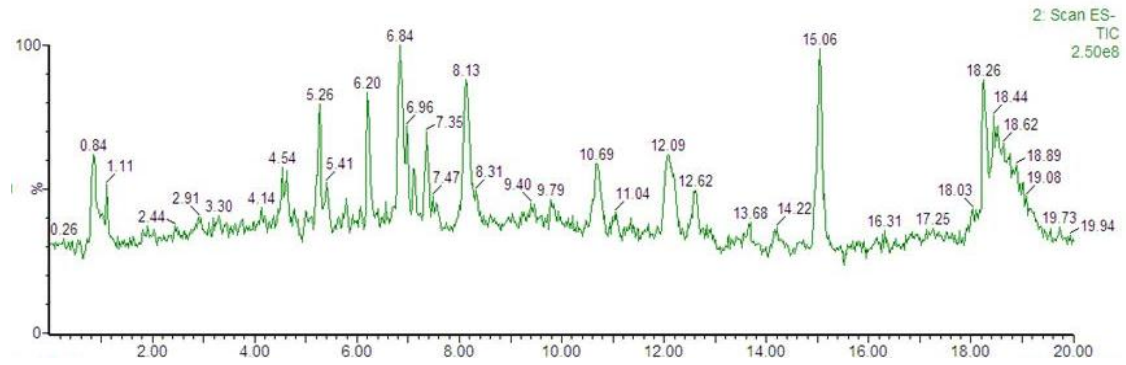


Figure 2: MALDI TOF/TOF of SLLA

LC-MS: Samples were separated inline by using the above mentioned HPLC method and ionized by electrospray both in positive and negative mode. Different forms of SLLA were obtained, in the mixture. Chromatogram for the formed SLLA is shown in the **Fig. 3 (a,b)**.



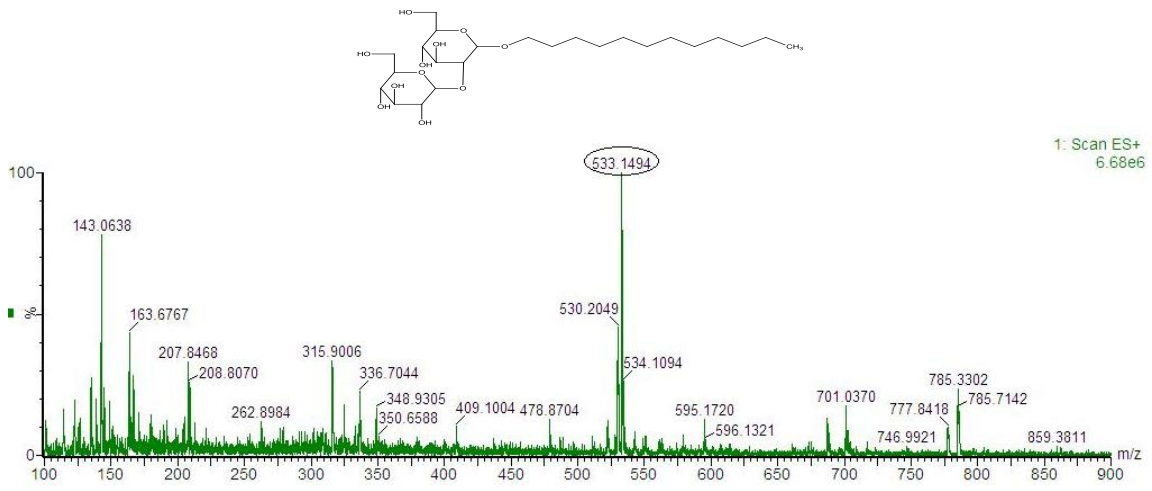
(a)



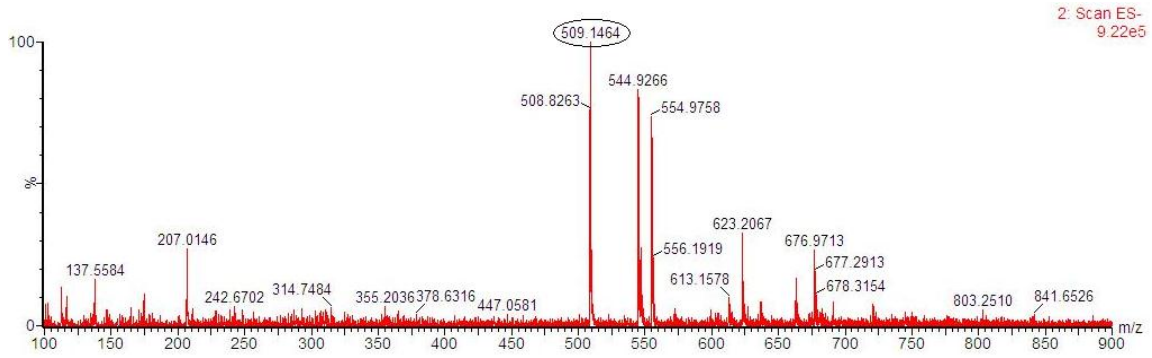
(b)

Figure 3: Chromatogram of SLLA acquired in (a) Positive mode (b) Negative mode

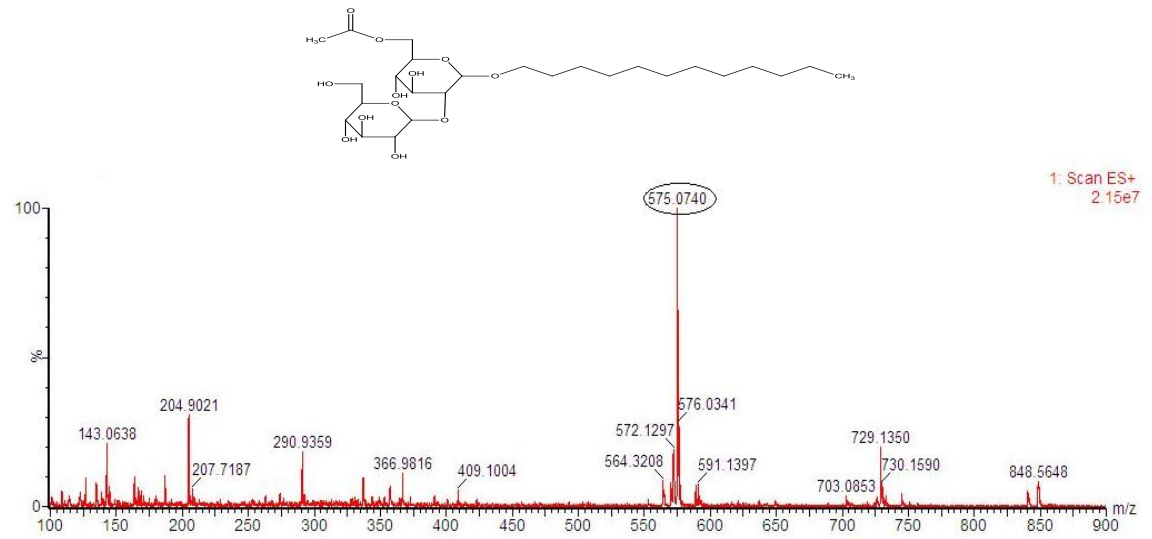
Four distinct molecular masses were determined at retention time concurrent with the applied chromatogram. They varied in the degree of acetylation at 6' and 6" position and terminal group (lipidic group). Acid free and short chain sophorose lipids was synthesized using primary alcohol. Glycosidic linkage was incorporated directly between the sophorose and hydroxyl function of the lauryl alcohol. As shown in **Fig. 4(a,b)** m/z 509 (M-H) signifying nonacetylated CH_3 end group, with (M+Na) m/z 533 was obtained in positive mode. Also m/z 551, and m/z 593 representing monoacetylated and diacetylated CH_3 end group, with (M+Na) m/z 575 and m/z 617 were determined **Fig. 4(c-f)**. Novel sophorolipids were synthesized were terminal oxidation of the nonfunctionalized end resulting in a more polar sophorose structure. Structure having a free hydroxyl group and acetylated at both the positions were noticed. This mode of metabolism was first reported by Kester and Foster who obtained ω-hydroxyl and α ω di-carboxylic acids from corynebacterium species growing on C10-C14 alkanes [14]. The structures are shown in **Fig.4 (g,h)** were m/z 609 (M-H) and m/z 633 (M+Na] represents formation of more polar sophorose moieties.



(a)



(b)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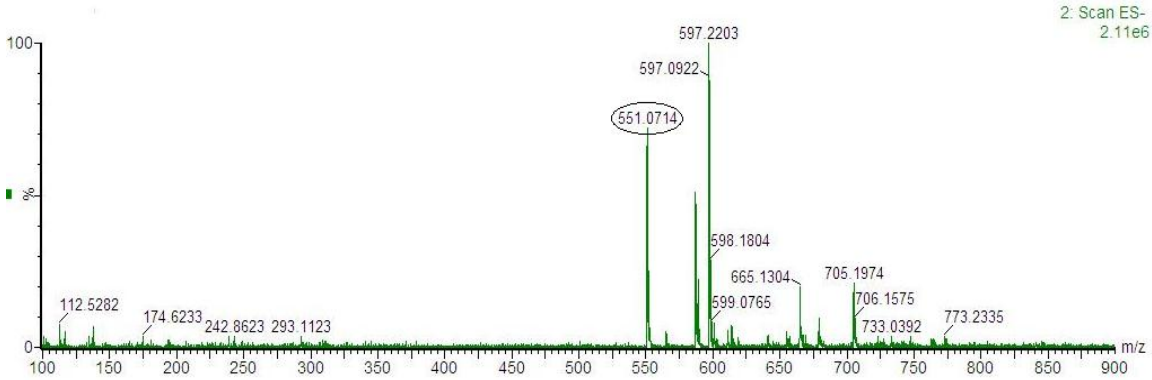
575.0740
576.0341
204.9021
143.0638
290.9359
207.7187
366.9816
409.1004
572.1297
564.3208
591.1397
729.1350
730.1590
703.0853
848.5648
100
%
0
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900

(c)


2: Scan ES-
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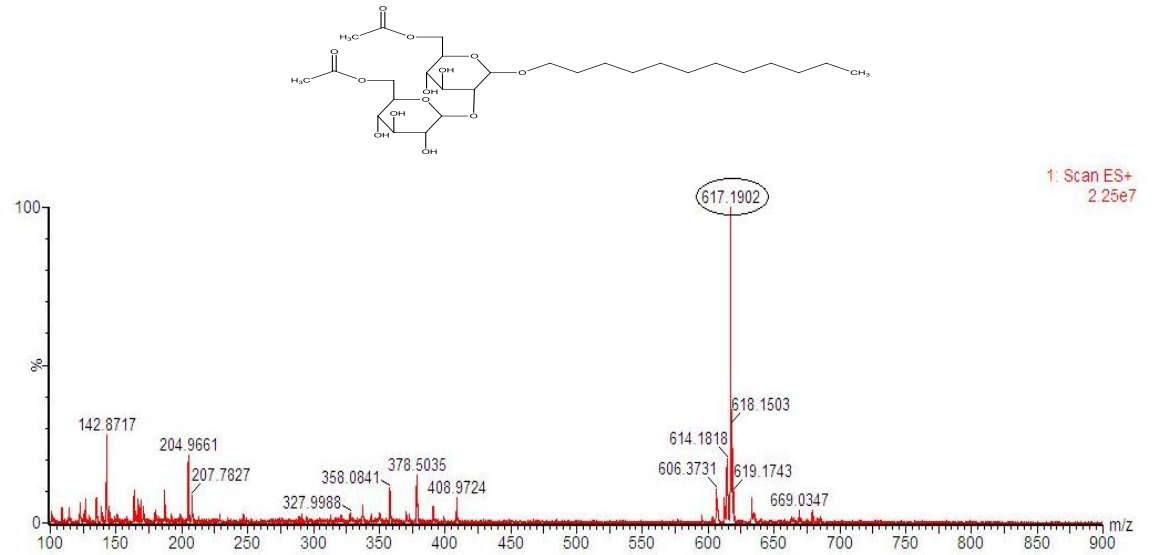
597.2203
597.0922
551.0714
598.1804
665.1304
705.1974
599.0765
706.1575
112.5282
174.6233
242.8623
293.1123
733.0392
773.2335
100
%
0
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900

(d)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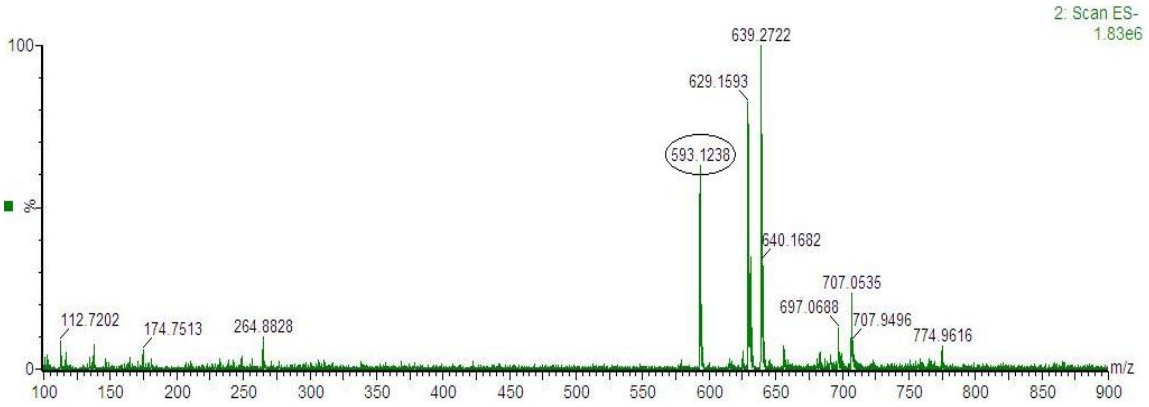
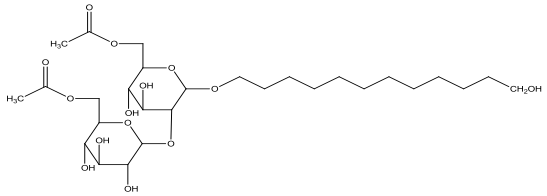
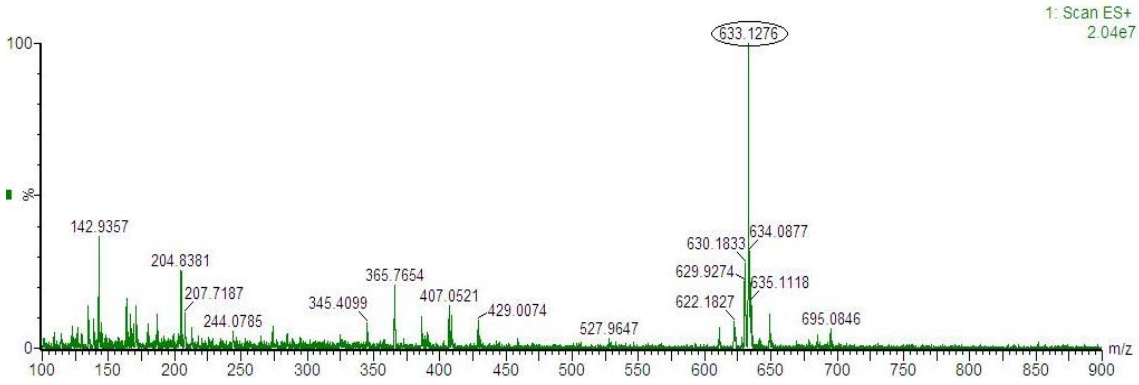
2.25e7
617.1902
618.1503
614.1818
142.8717
204.9661
207.7827
358.0841
378.5035
408.9724
327.9988
606.3731
619.1743
669.0347
100
%
0
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900

(e)

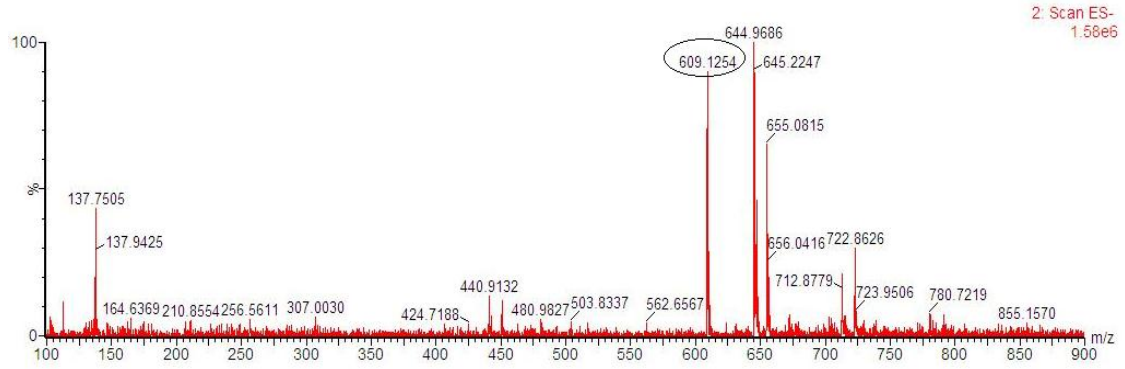


(f)





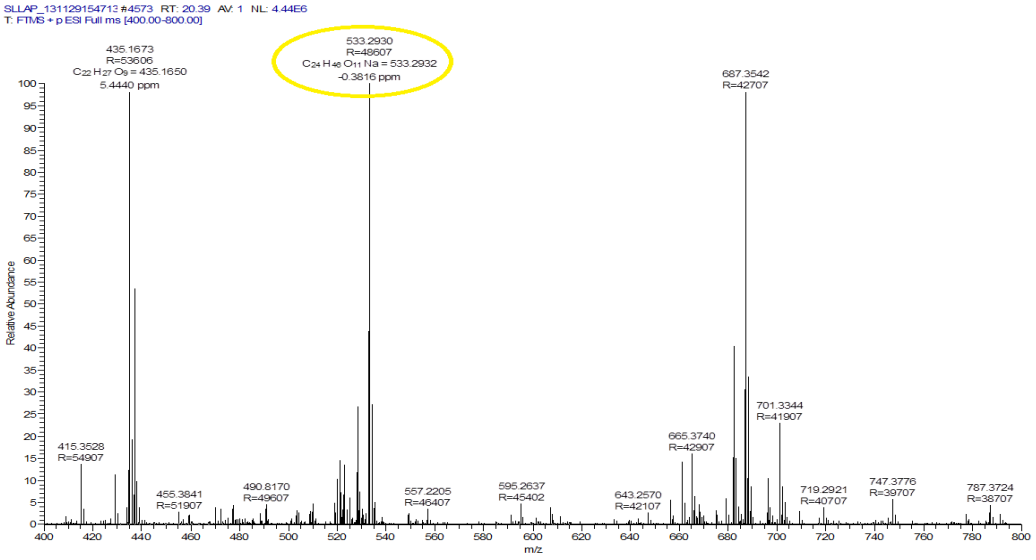
(g)



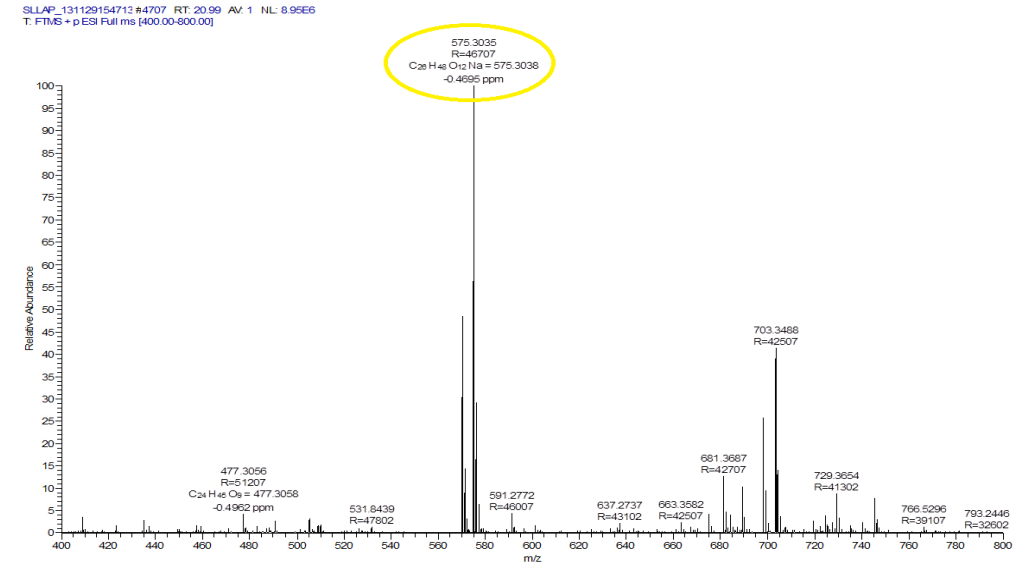
(h)

Figure 4: Positive and negative ion spectra of different SLLA components eluted at 6.240 min (a,b), 6.806 min (c,d), 8.159 min (e,f), 5.252 min (g,h) respectively

HRMS: Full-scan HR-MS analysis, followed by postacquisition ion extraction, was used to identify SLLA mixture. The ion spectra of SLLA (non-acetylated, -CH_3 end group), is illustrated in **Fig. 5a**. The measured accurate mass (*m/z* 533.2930 (elemental composition $C_{24}H_{46}0_{11}$)) of the peak at retention time (RT) 20.39 mins matched the calculated exact mass (*m/z* 533.2932) of its sodiated form. The corresponding ion spectra of the mono-acetylated (1-Ac-CH_3end group) and diacetylated (1-Diac-CH_3 end group) forms (non-acetylated, -CH_3 end group) are shown in **Fig. 5(b,c)**. The measured accurate mass (*m/z* 575.3035) of the peak at RT 20.99 mins matched the calculated exact mass (*m/z* 575.3038) of sodiated mono-acetylated whereas, accurate mass (*m/z* 595.3323) of the peak at RT 22.62 mins matched the calculated exact mass (*m/z* 595.3324) of protonated di-acetylated form of SLLA. Its presence in SLLA mixture was confirmed by formation of its sodium adduct at *m/z* 617.3134.



(a)



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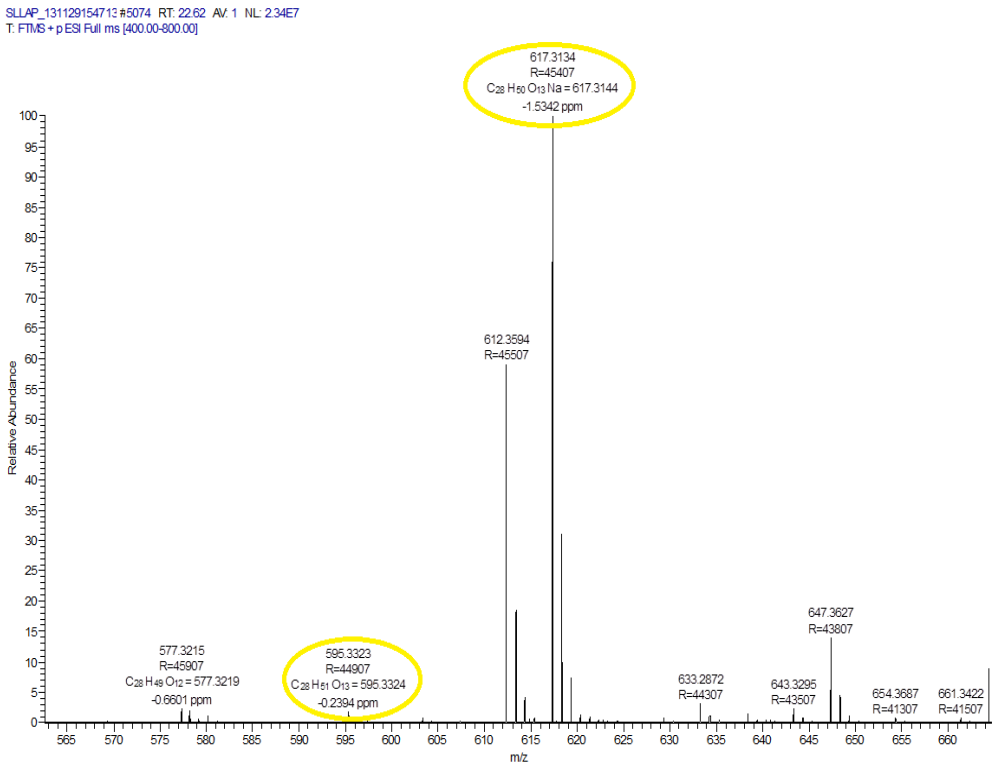
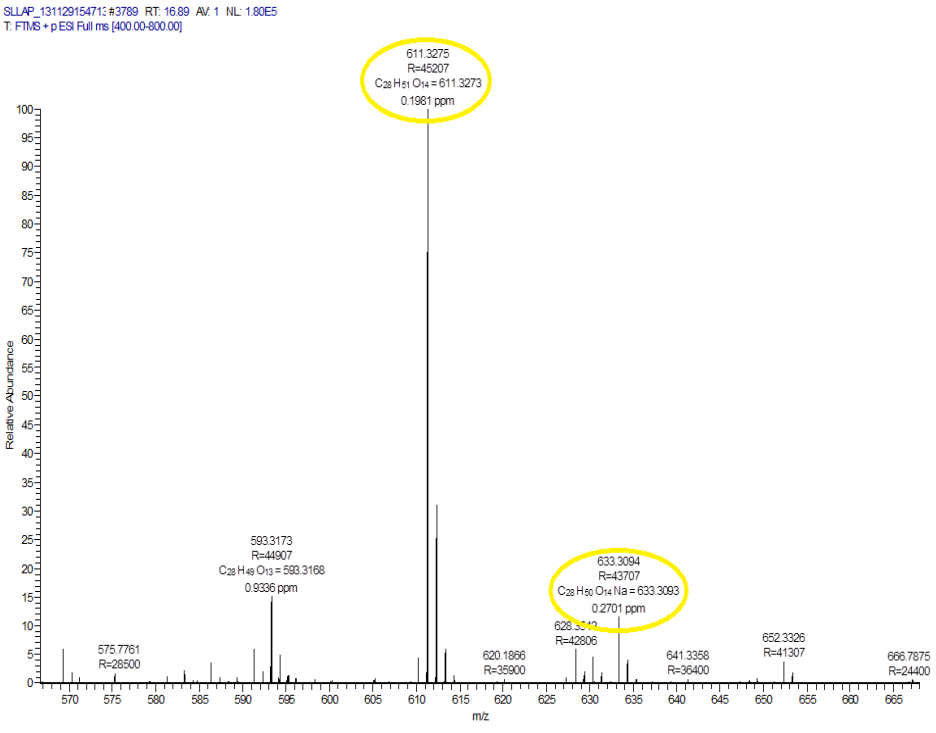


(c)

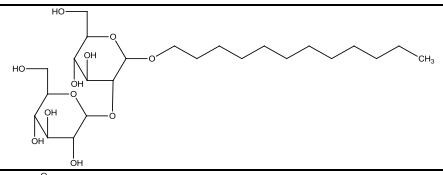
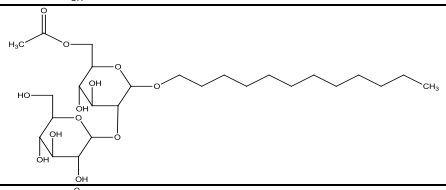
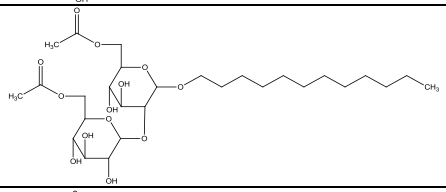
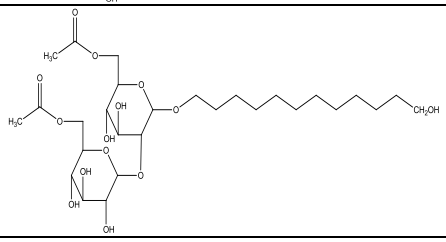


(d)

Figure 5: Ion spectra for SLLA a.(non-acetylated, -CH3 end group) b. mono-acetylated (1-Ac- CH3 end group) c. diacetylated (1-Diacend CH3 group) d. di-acetylated (2-Diac-CH2OHend group) forms acquired using full-scan liquid chromatography high-resolution accurate mass spectrometry

Similarly, terminal oxidation of the nonfunctionalized end resulted in novel sophorolipids di-acetylated, -CH_2OH end group) is illustrated in **Fig.5d**. The measured accurate mass (*m/z* 611.3275, (elemental composition $C_{28}H_{50}O_{14}$)) of the peak at RT 16.89 min matched the calculated exact mass (*m/z* 611.3273) of its protonated form, its sodium adducts at *m/z* 633.094 was also determined. The mass accuracy for each compound was within 2 ppm of the corresponding theoretical *m/z* value. Different forms of SLLA synthesized by *Candida bombicola* when supplemented with Lauryl alcohol is represented in **Table 1**.

Table 1: Different forms of SLLA synthesized by *Candida bombicola* when supplemented with Lauryl alcohol

SLLA components	Assigned Structure	Elemental Composition	Theoretical Mass (Na^+ Adduct)	Practical Mass (Na^+ Adduct)	PPM error
1-end CH_3		$C_{24}H_{46}O_{11}$	533.2932	533.2930	-0.3816
1-Ac-end CH_3		$C_{26}H_{48}O_{12}$	575.3038	575.3035	-0.4695
1-Diac-end CH_3		$C_{28}H_{50}O_{13}$	617.3144	617.3134	-1.5342
2-Ac-end CH_2OH		$C_{28}H_{50}O_{14}$	633.3093	633.3094	0.2701

CONCLUSIONS

We have successfully demonstrated the use of lauryl alcohol as a potential lipidic feed stock for sophorolipid production by *C. bombicola* ATCC 22214. Also, without blocking the β-oxidation pathway at the genome level of the yeast new to nature alkyl sophorosides were synthesized. Acid free and short chain sophorose lipids were synthesized using primary alcohol. Glycosidic linkage was incorporated directly between the sophorose and hydroxyl function of the lauryl alcohol. Monoacetylated and diacetylated CH_3 end group sophorosides were determined**.** Novel sophorolipid structure with a free hydroxyl group and acetylation at

both the positions were noticed. These terminal oxidation of the non-functionalized end resulted in a more polar sophorose structure. Modification of the lipophilic portion of the sophorolipid structure has shown to alter their physicochemical properties. This restricted length and new to nature SL's have broaden up applications range as it had resulted in good surface activities such as improved surface lowering capacities and contact angle.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of this work by UGC-SAP, India, the Institute of Chemical Technology, Mumbai, India for technical support provided and the National Chemical Laboratory, Pune, India for allowing us to perform the experimental work.

REFERENCES

1. 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, 2010; 87: 427-444.
2. Van Bogaert IN, Zhang J, Soetaert W. Microbial synthesis of sophorolipids. Process Biochem, 2011; 46: 821-833.
3. Brakemeier A, Lang S, Wullbrandt D, Merschel L, Benninghoven A, Buschmann N, Wagner F. Novel sophorose lipids from microbial conversion of 2-alkanols. Biotechnol. Lett., 1995; 17: 1183-1188.
4. Brakemeier A, Wullbrandt D, Lang S. *Candida bombicola*: production of novel alkyl glycosides based on glucose/2-dodecanol. Appl. Microbiol. Biotechnol., 1998; 50: 161-166.
5. Van Bogaert IN, Fleurackers SJJ, Van Kerrebroeck S, Develter D, Soetaert W. Production of new-to-nature sophorolipids by cultivating the yeast *Candida bombicola* on unconventional hydrophobic substrates. Biotechnol. Bioeng., 2010; 108: 734-741.
6. Brakemeier A, Wullbrandt D, Lang S. Microbial alkyl-sophorosides based on 1-dodecanol or 2-, 3- or 4-dodecanones. Biotechnol. Lett., 1998; 20: 215-218.
7. Van Bogaert IN, Sabirova J, Develter D, Soetaert W, Vandamme EJ. Knocking out the MFE-2 gene of Candida bombicola leads to improved medium-chainsophorolipid production. FEMS Yeast Res., 2009; 9: 610-617.
8. Fleurackers SJJ, Van Bogaert IN and Develter D. On the production and identification of medium-chained sophorolipids. Eur. J. Lipid Sci. Technol., 2010; 112: 655-662.

9. Dengle-Pulate V, Bhagwat S and Prabhune A. Microbial Oxidation of Medium Chain Fatty Alcohol in the Synthesis of Sophorolipids by Candida bombicola and its Physicochemical Characterization. J. Surfact. Deter., 2013; 16: 173-179.
10. Dengle-Pulate V, Chandorkar P, Bhagwat S and Prabhune A. Antimicrobial and SEM Studies of Sophorolipids Synthesized Using Lauryl Alcohol. J. Surfact. Deterg., 2014; 17: 543-552.
11. Dengle-Pulate V, Joshi J, Bhagwat S and Prabhune A. Application of sophorolipids synthesized using lauryl alcohol as a germicide and fruit-vegetable wash. World J. Pharm. Pharmaceut. Sci., 2014; 3: 1630-1643.
12. Prabhune A, Fox S R and Ratledge C. Transformation of arachidonic acid to 19-hydroxy and 20-hydroxy- eicosatetraenoic acids using *Candida bombicola*. Biotechnol. Lett., 2002; 24: 1041-1044.
13. Dubey P, Selvaraj K and Prabhune A. Physico-chemical, analytical and antimicrobial studies of novel sophorolipids synthesized using cetyl alcohol. World J. Pharm. Pharmaceut. Sci., 2014; 3: 993-1010.
14. Kester A S and Foster J W. Diterminal oxidation of long-chain alkanes by bacteria. J Bacteriol., 1963; 85**:** 859-869.