International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org || Volume 3 Issue 11 || November 2014 || PP.16-23

Production of Amphiphilic Surfactant Molecule From Saccharomyces Cerevisiae Mtcc 181 And Its Protagonist In Nanovesicle Synthesis

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ABSTRACT **:** *Microbial expel nurture medicinal sciences in various dimensions.Though surface active compounds from microbes are being applied in several areas,their biodegradability and amphiphilic propertiesoutspread greatly in therapeutic sector especially in improving drug delivery.The current work transmits on applicability of biosurfactant produced by Saccharomyces cerevisiae in nanovesicle synthesis.The organism was proficient in producing biosurfactant using groundnut oil as a carbon substrate and the chemical structure of the surfactant moiety was predicted using FTIR, HPTLC and GCMS analysis.Biosurfactants were further employed in nanovesicular synthesis in composition with cholesterol and the vesicles were further characterized for shape, surface charge, size, using SEM and Zeta potential measurements. The surfactant from Saccharomyces cerevisiae displays emulsification property and its amphiphilic structure was proficient in nanovesicleswith desirable shape, size and stability for drug delivery.The study was effectively made on the vesicle forming ability of the biosurfactant from Saccharomyces cerevisiae. Glycolipid of this yeast appears to support nanovesicle formation and this formulation can be exploited for better drug entrapment and release kinetics in the field of Pharmaceutical sciences. This is the first prospect reported on extracellular secretion and nano application of biosurfactant from Saccharomyces cerevisiae.*

KEYWORDS*: Biosurfactant, Nanovesicle, Rhamnolipids,Saccharomyces cerevisiae,Sophorolipid Send correspondence to Department of microbiology, Vel's University, velan nagar, P.V vaithalingam road, Chennai 600117, India*

I. INTRODUCTION

Biosurfactant are organic biomolecule which possess both hydrophilic and lipophilic group and this peculiar nature of this molecule keeps demanding in many areas where it can be applied vitally.Biosurfactant molecules were produced majorly by bacterial community followed by yeast and other fungi that also extends its vital part. Based on the nature of the producer, the biosurfactant differs in their property chemically and physically e.g.Glycolipid, lipoprotein, glycoprotein etc.[1,2].Biosurfactant has better proficiency in compare to chemical surfactant in terms of low toxicity, low molecular weight with better degradation nature and also they do have the potency to reduce the interfacial tension between the surface of two immiscible liquid or between a solid and a liquid[3, 4]. Amphiphilic nature of this molecule was used in several areas like remediation, textile, cosmetics etc.,but now has gained attentions with special focus on aggregation behaviour and vesicle formation tendencies to imply their usage in drug delivery and drug targeting.Currently chemical surfactant likes polysorbate 80 is being used for vesicle preparation like liposome and noisome. However, these microbial monomers of amphiphilies have the ability to self- assemble into nanovesicle which can be efficient in drug delivery. Aggregation behaviour of rhamnolipids was reported and this glycolipid class of rhamnolipid has the tendency of vesicle and micelle formation in aqueous phase environment [5, 6,7]. Encapsulation of drug with rhamnolipid based vesicle can be improved in addition with cholesterol was reported [8]. Alike rhamnolipids, phospholipids like phosphatidylcholine were used as vesicle for bacterocin delivery against microbial infections [9].Though bacterial surfactants were studied along the years, surfactant of fungi and yeast also has better functionality as surfactant. Surfactant from *Saccharomyces lipolytica* was extracted and it shows high emulsification activity [10]. Potent application of membrane surfactant from *Saccharomyces cerevisiae*in the field of bioremediation was reported [11]. Sophorose sugar based glycolipids are majorly identified surfactants in several yeast and fungi [12]. Though yeast surfactants equally supported in various application there is less knowledge on aggregation and vesicle formation ability of glycolipids of yeast. This present study is about to

identify and characterise the surfactant produced from *Saccharomyces cerevisiae* MTCC 181 and evaluating the aggregation and vesicle forming ability.

II. MATERIALS

Saccharomyces cerevisiae MTCC181 was used in this study. It was obtained from Microbial Type Culture Collections (MTCC), Chandigarh, India. The organism was maintained on YPD agar slant at 4°C and it was sub cultured periodically. Chemicals used in the analysis were of laboratory grade. It was purchased from Loba Chemie Pvt Ltd, Mumbai, India and Merck chemicals, India.

III. METHODOLOGY

3.1.1 Cultivation of microorganism

25ml of yeast extract peptone dextrose broth was prepared, sterilized, inoculated with fresh culture of *Saccharomyces cerevisiae* MTCC181and incubated at 30°Cfor 24 hrs and further used as the seed culture for the production of surfactant.

3.1.2 Production of biosurfactant [12]

Mineral salt medium was prepared with ground nut oil as carbon source, Sterilized, inoculated with 5% seed culture of *Saccharomyces cerevisiae* MTCC181 and it was kept for shaking at 150 rpm for 4 days. The biomass was separated by centrifugation at 8,000 rpm for 15 minutes; the supernatant was collect for screening of surfactant.

3.1.3 Screening of surfactant by oil displacement test [13]

50 ml of distilled water was added to a large petri dish (15 cm diameter) followed by the addition of 20 µl of crude oil to the surface of water over which 10 µl of sample was added. The diameter of clear zone was measured.

$$ODA = \frac{22}{7}(Radius)^2 Cm^2$$

3.1.4 Emulsification index (e24) [14]

The emulsifying capacity was evaluated by an emulsification index (E24). The E24 was determined by adding 2 ml of mustard oil and 2 ml of the crude surfactant in a test tube. It was vortex at high speed for 2 min and allowed to stand for 24hours.

The percentage of emulsification index was calculated by using the following equation.

$$E24 = \frac{\text{Height of emulsion}}{\text{Total height of Solution}} \times 100$$

3.1.5 Purification of Biosurfactant by Solvent Extraction [15]

The culture was centrifuged at 12000 rpm and the cells were removed. The supernatant was extracted with a mixture of the extraction solvents with the following ratio methanol/chloroform/1-butanol, 1:2:1 by Volume. The mixture was kept in magnetic stirrer for 6hrs at 200 rpm. After stirring, lower layer was separated and its pH was converted into 2 using 6N hydrochloric acid and kept refrigerated at 4°C for 12 hrs. Precipitate was separated by centrifugation at12000 rpm for 15min, further the precipitate was dried overnight.

3.1.6High performance thin layer chromatography

HPTLC were performed on CAMAG Linomat 5 "Linomat 5_ 150423" using HPTLC silica gel 60F254 plate as stationary phase with plate size of 8x 10 cm, chloroform and methanol (2:1) used as the solvent system.

3.1.7Fourier Transform Infrared Spectroscopy (FTIR) [16]

One milligram of purified biosurfactant was ground with 100mg of Potassium bromide and pressed with 7500 kg for 30 sec to obtain translucent pellet. The infrared spectra were recorded on Bruker Optics FT-IR system within the range of 500 to 4000 cm^{-1} wave number.

3.1.8Gas chromatography and Mass spectroscopy

Surfactant sample meant to acid hydrolysis, further it is dissolved in 80% ethanol and then injected(1µl) in to GC-MS-QP 2010 [Shimadzu] equipped with the capillary inlet and the mass selective detector, set scan from 40 – 1000 m/z at a scan rate of 1.2 scans per second. The capillary column used was a VF-5ms of length 30m, diameter 0.25mm and full thickness 0.25um. Column oven temperature was 70°C with the injector temperature of 240°C.The oven temperature was programmed from 70°C to 300°C for 3min.The ion

source temperature was set to be 200^{o}C and the interface temperature was 240^{o}C. The carrier gas helium at a flow rate of 1.5ml/min with spilt ratio set as 10.

3.1.9Preparation of Nanovesicle in Hand shaking method [17]

Equal volume of surfactant and cholesterol was dissolved in 10ml of ether; evaporate the solvent under 60°C after which add 10ml of phosphate buffer for vesicle formation.

3.2.0 Scanning Electron Microscopy analysis of Nanovesicle

The dehydrated Biosurfactant were dispersed in ethanol before analysis. The morphology of the synthesized biosurfactant was characterized by SEM (Hitachi S4800, Japan) using an accelerating voltage of 0.1–30 kV.

3.2.1Measurement of zeta potential

Nanovesicle sample was set at a power level of +200 V by an ultrasonic generator (Sonics-Vibracell ultrasonic processor). Size and surface charge of vesicle were measured at room temperature (25^{0}c) with a Malvern zeta analyser.

IV. RESULTS AND DISCUSSION

4.1.1Culture condition:

Saccharomyces cerevisiae is a species of yeast having more beneficial role in fermentation industry. As per the instruction of Microbial type culture condition (MTCC), YEPD medium were used for the cultivation, the most commonly used rich medium for growing *Saccharomyces cerevisiae* when special conditions are not necessary. YEPD medium contains rich amount vitamins, precursor molecules and necessary metabolites for cell growth. Yeast cells divide every ~90 min when grown in YPD during the exponential phase of the growth cycle [18]

4.1.2 Surfactant production:

Surfactant is a significant molecule for microorganism for its various physiological activities [19]. Mineral salt medium is commonly used for the production of biosurfactant which contains carbon, nitrogen and electrolytes as the major sources of nutrients. Mineral salt medium containing crude oil as a carbon source can be used for surfactant production [20]. In this work 2% ground nut oil was used as the carbon source for the production of surfactant [21] Hydrophobic sources such as ground nut oil, crude oil etc. were used in the mineral salt medium as carbon sources was found to enhance the synthesis of surfactant molecules [20, 21]. It also considered as an indirect screening method as the growth on hydrophobic carbon source confirms the production of surfactant like molecules which helps the solubilization of hydrophobic materials **[**22].

4.1.3 Screening of surfactant:

Screening of surfactant is commonly based on its interaction with hydrophobic molecules because of amphiphilic nature. Oil spreading test is simple and reliable method for screening of surfactant molecule [22]. 10µl culture supernatant was added on oil surface, shows a clear halo zone of diameter was measured as 5.9cm and the oil displacement area was found to be 27.34 cm^{2}. Increase in the area of the circle signifies the surfactant activity. Emulsification test is the first method used for screening for surfactant it will just confirm the presence of surfactant. In our study groundnut oil is used as carbon sources for surfactant production and Saccharomyces cerevisiae showed emulsification index of 57.5 %. Biosurfactant isolated from yeast species exposed emulsification activity over a pH range from 2 to 10 hence it is very useful in both acidic and alkaline condition and showed emulsification activity of 60% in the temperature range of 10 – 100^{0}C. This increases the scope of surfactant from yeast in the applications which involves various pH and higher temperature [23].

4.1.4 High performance thin layer chromatography (HPTLC)

Surfactant sample exposed different patterns in HPTLC with R.f values 0.03 - 0.05, 0.08 – 0.09, 0.09 – 0.14, 0.15 – 0.17, 0.29 – 0.33, 0.33 – 0.41, 0.47 – 0.50, 0.53 – 0.72, 0.74 – 0.80, 0.81 – 0.89, 0.90 – 0.92 and 0.93 – 0.94. The peak 0.09 – 0.14 and 0.15 – 0.17 is relates to glycolipid class of acidic sophorolipid. The peak 0.29 – 0.33, 0.33 – 0.41, 0.47 – 0.50 and 0.53 – 0.72 is relates to glycolipid class of lactone sophorolipid and fatty acid derivatives. The peaks 0.74 – 0.80, 0.81 – 0.89, 0.90 – 0.92 and 0.93 – 0.94 are relating to sophorolipid surfactant [24]. The purified fraction of surfactant from *Saccharomyces cerevisiae* comes under glycolipid class of biosurfactant and its peaks typically bear a resemblance with sophorolipid. Similar study was observed from lactonic and acidic class of sophorolipid which exhibited five to six patterns of R.f 0.59, 0.48, 0.37, 0.22, 0.07 in thin layer chromatography [25]. In addition Copper et al., 1983 also identified glycolipid fractions of surfactant using thin layer chromatography and obtained R.f value of 0.31, 0.21, and 0.07 from yeast organism.

Graph 1: HPLTLC peaks of partially purified surfactant

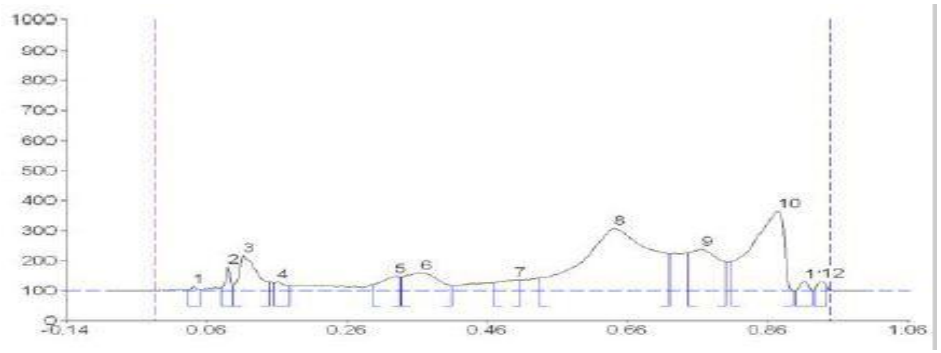


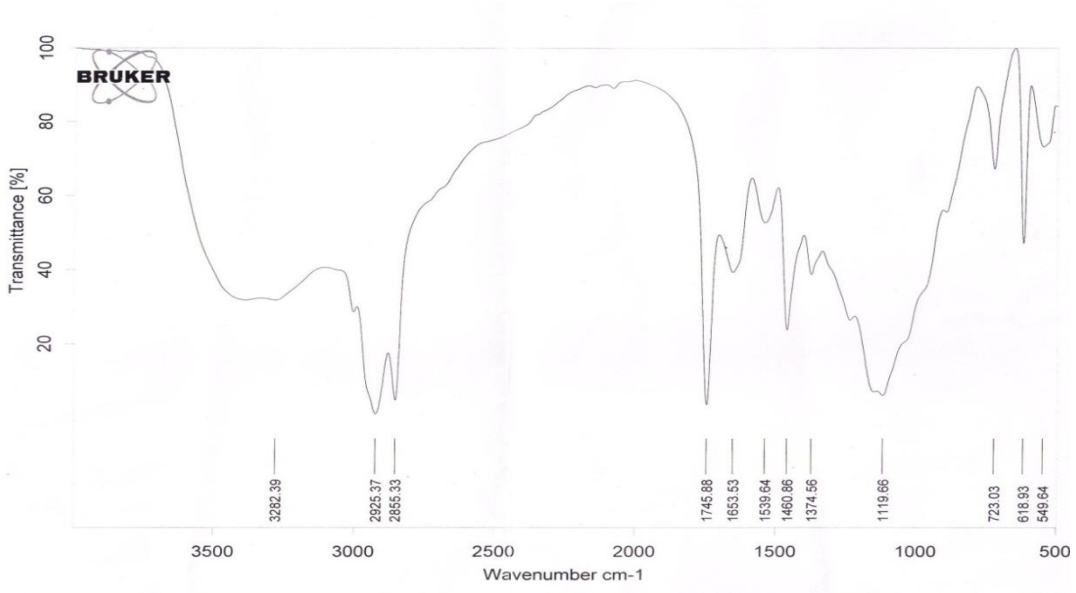
Table 1: Table showing surfactant peak values

PEAK	START RF	START HEIGHT	MAX RF	MAX HEIGHT	MAX %	END RF	END HEIGHT	AREA	AREA %
1	0.03	2.6	0.04	14.6	1.37	0.05	3.5	113.0	0.24
2	0.08	9.4	0.09	80.8	7.59	0.09	15.7	525.0	1.12
3	0.09	17.9	0.11	117.4	11.03	0.14	30.7	2765.1	5.91
4	0.15	26.3	0.16	30.9	2.91	0.17	19.4	470.0	1.01
5	0.29	21.0	0.33	48.4	4.55	0.33	48.1	1262.3	2.70
6	0.33	47.9	0.36	59.9	5.63	0.41	19.2	2778.7	5.94
7	0.47	27.3	0.50	37.7	3.55	0.50	36.6	1104.4	2.36
8	0.53	42.4	0.64	207.9	19.53	0.72	122.7	19647.6	42.02
9	0.74	126.3	0.76	138.3	13.00	0.80	96.0	5618.4	12.02
10	0.81	97.0	0.87	264.1	24.82	0.89	0.6	11776.4	25.19
11	0.90	0.8	0.91	30.5	2.87	0.92	0.7	386.4	0.75
12	0.93	2.0	0.96	33.6	3.16	0.94	18.1	386.5	0.75

4.1.5 Fourier Transform Infrared Spectroscopy (FTIR)

Surface active compounds from *Saccharomyces cerevisiae* were absorped between 500 – 3500 wavenumber cm^{-1} and the transmittance are 3282.39 cm^{-1}, 2925.37 cm^{-1}, 2855.33 cm^{-1}, 1745.88 cm^{-1}, 1653.53 cm^{-1}, 1539.64 cm^{-1}, 1460.86 cm^{-1}, 1374.56 cm^{-1}, 1119.66 cm^{-1}, 723.03 cm^{-1}, 618.93 cm^{-1} and 549.64 cm^{-1}. All transmittance peak data's were measured using standard IR values. The peak 3282.39 cm^{-1} relates to broad spectrum of O-H stretch of phenolic or alcoholic group [26] and also it may corresponds to N-H medium stretch which can be secondary amide structure [27]; further this peak has a strong C-H stretch of Alkynes group. The peak 2925.37 cm^{-1} and 2855.33 cm^{-1} corresponds to asymmetrical (CH_2) and symmetrical (CH_2) stretching of methylene group [26]and also matches with aliphatic chain of C-H stretch which is usually less useful in determining structure. Carbonyl stretching (C=O) of Ketone, Ester and Carboxylic group were measured in the peak of 1745.88 cm^{-1} and the peak 1653.53 cm^{-1} correspond to C=O stretching of Amide group, these carbonyl stretch is one of the strongest absorption which is very useful in structure determination and this absorption may bea contribute from lactones, ester or acidic forms of sophorolipid [26]. Aromatic C=C bending correspond to the peak 1539.64 cm^{-1} which has strong asymmetrical stretch of nitro (NO2) compound and also N-H bend of amines and amide groups. The band peak at 1460.86 cm^{-1} corresponds to C-O-H plane bending of carboxylic acid (COOH) and also C-H=O of aliphatic aldehyde [26, 27] This peak may further relate to scissoring and bending of C-H alkanes group. The transmittance peak of 1374.56 cm^{-1} was matches with C-H plane bend and vibration at CH_3 bends. It also corresponds to strong symmetrical stretch of –NO2 compound. The band peak 1119.66 cm^{-1} relates with C-O, C-O-C stretch of alkyl aryl esters [27] and also medium stretches in C-N amine group. The 723.03 cm^{-1} peak corresponds to aromatic C-H bending, phenyl ring substitution. C-Cl stretches of acid chloride and alkyl halide relates to the peak 723.03 cm^{-1} and 618.93 cm^{-1}. The peak 549.64 cm^{-1} corresponds to R –Cl stretch of alkyl halides. From FTIR pattern, it is manifest that lactonic forms of surfactant are common among yeast species.

Graph 2: FTIR peaks of partially purified surfactant



4.1.6 Gas chromatography and Mass spectroscopy

Hydrolysed surfactant sample showed 13 GC/MS peak, peak 3, 5, 8, 12 and 13 relates to similar base peak at 88 (m/z) comes under fatty acid esters class of decanoic acids and their structure were identified and partitioned using MS library (NIST08s, WILEY8 &FAME). The peak 1- Hexadecanol and dodecanones of peak 6, 3, 11 and 13 have also been successfully incorporated into sophorolipid. Alcohol such as 1- dodecanol, 2- dodecanol, 1- hexadecanol, 1- tetradecanol, 2,13- tetradecandiol, 2,15- hexadecandiol and ketones such as 2-dodecanones , 3- dodecanones have all can be successfully incorporated into sophorolipids[28, 29].9th peak of retention time 20.910 and mass peak 161(m/z) is found to be 12- hydroxyoctadecanoic acid is pretend to be hydroxy fatty acid esters of sophoroside for a reason that it can bind glycosidically to several residues of sugar molecule to form glycosidic acid[30]. The peak 7 was ethyl oleate an exosecreted fatty acid molecules by yeast organism. Individual fatty acid molecules canaslobe used by yeast to produce novel sophorolipid. A similar study was accomplished with hydrocarbons from tetradecane to docosane (14 – 20 carbon) molecule into sophorolipid [25]. From the data surfactant of *Saccharomyces cerevisiae* has fictitious to have sophoroside of hydroxy fatty acid derivatives.

Graph 3: chromatogram showing peaks of surfactant molecule.

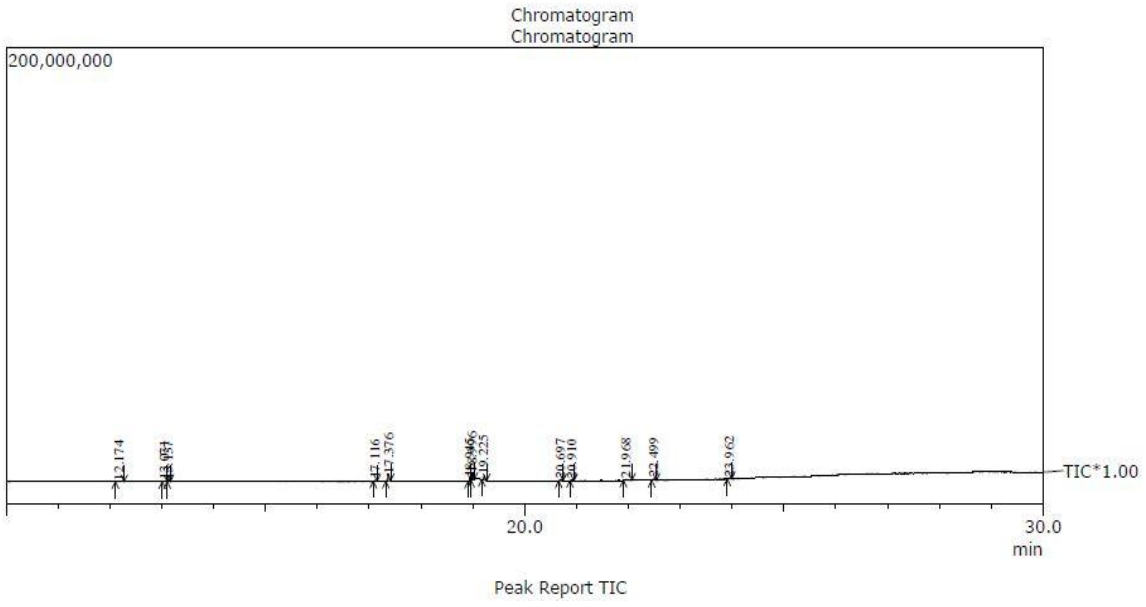
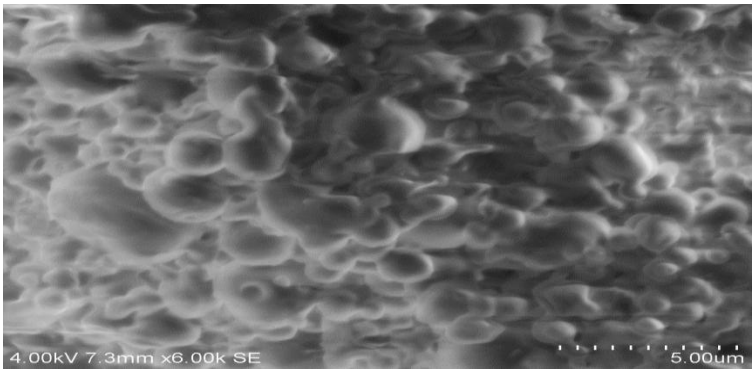


Table 2: Table showing respective compounds of chromatogram

PEAK	R.TIME	AREA	AREA%	NAME
1	12.174	753643	3.06	Phenol,2,4-Bis (1,1-Dimethylethyl)
2	13.071	370999	1.51	1-Hexadecanol
3	13.137	433406	1.76	Dodecanoic acid, ethyl ester
4	17.116	892233	3.62	Dibutyl phthalate
5	17.376	6169722	25.06	Hexadecanoic acid, Ethyl ester
6	18.945	1095659	4.45	Ethyl(9Z,12Z)-9,12-Octadecadienoate
7	18.996	4873416	19.80	Ethyl oleate
8	19.225	3665015	14.89	Octadecanoic acid, Ethyl ester
9	20.697	1387390	5.64	9-octadecenoic acid, 12-hydroxy-
10	20.910	767272	3.12	Octadecanoic acid, Ethyl ester
11	21.968	1764373	7.17	Cyclododecasiloxane, Tetracosamethyl
12	22.499	1545133	6.28	Nonadecanoic acid, ethyl ester
13	23.962	898883	3.65	Ethyl tetracosanoate

Figure 1:Scanning electron microscope image of nanovesicle



4.1.7 Zeta potential analysis of nanovesicle

Table 3 – Table showing standard value of the zeta potential

Zeta potential (mv)	**Stability of the particle**
0 to +/- 5	Rapid coagulation or floculation
10 to +/- 30	Incipient stability
30 to +/- 40	Moderate stability(Thatipamula *et al*, 2011)
40 to +/- 60	Good stability
More than +/-61	Excellent stability

Graph 4: zeta measurement of particle size analysis

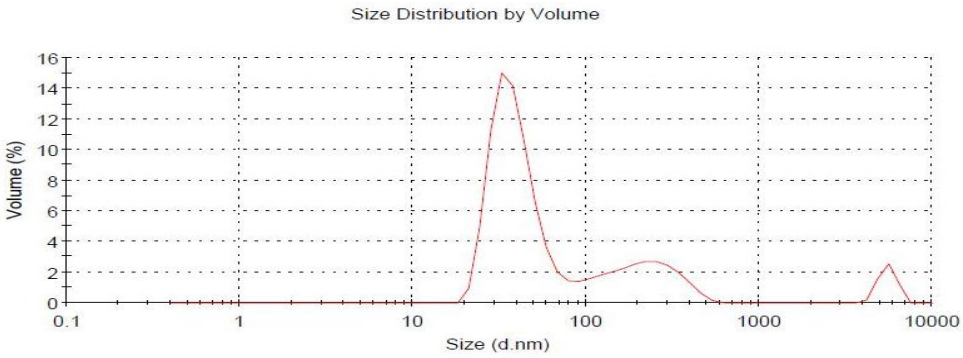


Table 4: Zeta measurement of nanovesicle

Z-Average(d.nm): 152.0
Pdi: 0.457
Intercept: 0.85
Result quality: Good

Peak	Diam.(n.m)	% Volume	Width(nm)
1	223.1	23.5	98.52
2	39.94	71.1	13.81
3	5477	5.5	629.8

Surface active compound of sophorolipidhas the tendency of forming nanovesicle and the addition of cholesterol found to decrease the size of vesicles to nano range. A similar study of rhamnolipid surfactant in the concentration of 2.6 mM used for vesicle synthesis and using transmission electron microscopy the size of nanovesicle has been reduced when addition of cholesterol was implemented; this vesicle has 80% of drug entrapment efficiency was reported [31]. Scanning electron microscopy image of sample showed uniformity in shape but size of larger and smaller vesicles were seen , 150nm of vesicle size were observed in Zeta analyser and negative charge of the vesicle shows incipient stability.The general dividing line between stable and unstable suspensions is generally taken at either +30mV or -30mV Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable. Vesicle stability plays vital role in drug loading capacity but anionic charge of the surfactant vesicle may delay better delivery of drug molecules. Concentration and pH is important in controlling vesicle or micelles formation of surfactant like rhamnolipids [8], hence size and charge of surfactant is crucial in vesicle formation, drug loading and delivery.

V. ACKNOWLEDGEMENT

We thank BioLim Biosolutions Pvt Ltd for their funding support , the authoracknowledge BioLim center for life Science, Microbiology and fermentation research group, played crucial part in nanovesicle analysis and also in revision of the paper.

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