International Journal of Scientific Research in Biological Sciences **Research Paper**
Vol.6, Issue.5, pp.05-11, October (2019) **E-ISSN:** 2347-7520
DOI: https://doi.org/10.26438/ijsrbs/v6i5.511

In Situ Production of Silver Bio Nanoparticles from Marine Biosurfactant Bacteria and Evaluation of Its Antibacterial Activity

M. Indhuja[1], D. Kavitha[2]*, P.Selvamaleeswaran[3], A. Palanisamy[4], M. Sureshkumar[5]

[1,2,3,4]Dept. of Biotechnology, Muthayammal College of Arts & Science, Rasipuram, Tamil Nadu, India-637 408
[5]Dept. of Zoology, Muthayammal College of Arts & Science, Rasipuram, Tamil Nadu, India-637 408

**Corresponding Author: ibtkavitha.d@gmail.com*

Available online at: www.isroset.org

Received: 25/Sept/2019, Accepted: 15/Oct/2019, Online: 31/Oct/2019

***Abstract*- BIOSURFACTANTS FROM MICRORGANISMS** Biosurfactants are the surface-active molecules synthesized by microorganisms. With the advantage of environmental compatibility, the demand for biosurfactants has been steadily increasing and may eventually replace their chemically synthesized counterparts. Marine biosurfactants produced by some marine microorganisms have been paid more attention, particularly for the antimicrobial activity against various microbes in medicinal field. In this study, the screening of biosurfactant-producing marine microorganisms, the determination of biosurfactant activity as well as the recovery of marine biosurfactant and the antibacterial activity of the biosurfactant were done. The uses of silver nanoparticles with marine biosurfactants for the antibacterial activity also discussed. The marine *Serratia sp*. had the ability to produce the biosurfactant and shows high amount of antibacterial activity against some pathogenic bacteria.

Keywords: Biosurfactant, marine microorganisms, Silver nanoparticles

I. INTRODUCTION

Marine environment is the largest habitat as compared to other habitat in the biosphere. About 70% of the earth surface is covered by salt water. It is believed that the life is originated first from ocean. Biosurfactants are amphiphilic agents which, by accumulating at interface between immiscible phases, can reduce surface and interfacial tensions produced by bacteria, fungi and yeast, which either adhere to the cell surfaces or are excreted extracellular in the growth medium. They belong to various classes including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopolysaccharides. The best biosurfactants can lessen the surface tension of water from 72 to 30 Nm^{-1}. The large surface-to-volume ratio enables many bacterial species to produce several types of structurally diverse surface-active compounds, which having antimicrobial, antiviral, haemolytic, and anti tumour activity [1]. During the last decades, there has been a growing interest in isolating microorganisms that produce surface active molecules with good surfactant characteristics such as low critical micelle concentration (CMC) and high emulsification activity, simultaneously presenting low toxicity and good biodegradability. Generally the type and amount of the biosurfactants, produced by microbes depends on the producer organism, factors like carbon and nitrogen, trace element, temperature. They are also classified into two different categories on the basis of their molecular weight: Low molecular weight biosurfactants are lipopeptides, glycolipids and phospholipids (having lower surface and interfacial tension) and high molecular weight biosurfactants are polymeric and particulate surfactants (more efficient as emulsion stabilizing agents) [2].

The silver nanoparticles coated textiles with enhanced functionalities, such as antibacterial, antistatic and UV protection, are greatly appreciated by a more discerning and demanding consumer market for high-value-added products. Silver nanoparticles can be exploited in medicine for burn treatment, dental materials, coating stainless steel materials, textile fabrics, water treatment, sunscreen lotions, etc. and possess low toxicity to human cells, high thermal stability and low volatility [3]. Antibacterial finishes are applied to gauge cloth for the major reasons is to control the spreading of disease through microorganism and avoid the danger of injury-induced infections.

II. MATERIALS AND METHODS

Sample collection:
Marine water samples were collected from different locations Marina beach, Chennai, Tamilnadu, India. The samples were collected in different sterile glass bottles, and immediately transported to laboratory for further analysis.

Isolation of biosurfactant producing bacteria:
The marine water sample was spread on nutrient agar plate by using the serial dilution process. The dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}dilutions were spread on the nutrient agar plate with salt. Then it had been incubated for 28 hours. After incubation individual colonies had been grown in different morphology.

Screening of biosurfactant producing bacteria:
Cetyltrimethyl ammonium bromide (CTAB) methylene blue agar method
The bacterial isolates were inoculated on to MSM agar plates supplemented with 2 % (w/v) glucose, 0.25% w/v yeast extract and containing 200 mg l^{-1}cetyltrimethylammonium bromide (CTAB), a cationic surfactant and 5 mg l^{-1}methylene blue. The plates were incubated at 30°C for24-48 h to observe dark blue halo. The clear zones are due to accumulation of methylene blue around the colonies of cells producing anionic surfactant.

Determination of the Emulsification Index
A mixture of 2 mL supernatant and 3 mL kerosene (or diesel) was vertically stirred for 2 min and the height of the emulsion layer was measured after 24 hr to determine the emulsification index. The equation used to determine the emulsification index (E_{24} (%)) is as follows:

The E_{24} (%) = (height of emulsion layer/ height of total solution) *100%

Blood haemolysis test:
Blood agar haemolysis method was used to screen biosurfactant producing strain. The method was based on the fact that biosurfactants were able to haemolysis the red blood cell present in blood. The selected isolates were incubated on blood agar plates incubated for 24- 48 hours at 37°C. The zone of haemolysis indicated the production of biosurfactant.

Biochemical characterization of isolated bacteria:
Extraction of biosurfactant
The culture was centrifuged at 5000rpm at 4°C for 30 minutes to obtain the supernatant. To the supernatant, equal volumes of chloroform: methanol in the ratio 2:1 was added and the mixture was acid to pH 2.0 using 6 N HCl and kept overnight for evaporation. Finally white colour precipitation was seen between the two liquid layers i.e., supernatant and chloroform: methanol. The same was then collected and lyophilized and characterized using FTIR.

Characterization of biosurfactant by FTIR analysis
For FTIR measurements, the Ag nanoparticles solution was centrifuged at 10,000 rpm for 30 min. The pellet was washed three times with 20 ml of de-ionized water to get rid of the free proteins/ enzymes that are not capping the silver nanoparticles. The samples were dried and grinded with KBr pellets and analyzed on a Shimadzu IR-IR Affinity1 model in the diffuse reflectance mode operating at a resolution of 4 cm−1.

GC-MS (Gas Chromatography Mass Spectrometry)
GC-MS technique was used in the study to identify the components present in the sample. GC-MS analysis of this sample was performed using a Perkin Elmer GC Clarus 500 systems and chromatography interfaced to a silica capillary column (30mx 1µl was Mdf. Composed of 100% Dimethyl poly siloxane) for GC-MS detection. An electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1ml/min and an injection volume of 2µl was employed (split ratio of 10:1). Injector temperature was 250°c. The oven temperature was programmed from 110°c (isothermal for 2 mins) with an increase of 10°c/min to 200°c, then 5°c/min to 280°c ending with 9min isothermal at 280°c. Mass spectra were taken at 70eV, a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36mins. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbomass Version 5.2.0. Compound identification was obtained by comparing the retention times with those of authentic compounds and the spectral date obtained from library data of the corresponding compounds. The given sample was extracted by ethyl acetate and analysed in GC-MS for different components.

Estimation of biosurfactant
The biosurfactant activity had been estimated by using the orcinol method. The rhamnose had been prepared for 100 mg and it had estimated by using our sample, water and orcinol reagent. Then it would keep at 80^{o} C for 10 mins in the water bath. Then the nm had been identified in the UV-spectrophotometer. The precipitated silver nano particles were isolated by centrifugation at 15,000 rpm. This procedure results in the production of silver nano sized particle. The particles where then sonicated in 10ml.

Synthesis of Silver Nanoparicles:
The synthesis of silver nano particles in the water in oil micro emulsion phase was performed by the addition of synthesized two reverse micelles in the presence of reducing agent.The synthesis involves mixing up to 0.5 m/l aqueous silver nitrate solution, 3.0g of biosurfacatant, 1.5g of butanol together and stirred thoroughly at room temperature until homogeneous reverse micelles were formed.

Characterization of silver nanoparticles:
UV-VIS spectra analysis
The bio reduction of Ag+ ions in solution was monitored in optimal measurement, which carried out by using UV-Visible spectrophotometer and scanning the spectra between 200-800 nm at the reduction of 1mn.

EDAX (ENERGY DISPENSIVE X RAY SPECTRA ANALYSIS)

EDX pattern shows the crystalline and elemental composition of silver nanoparticles synthesized from the biosurfactant producing organism. The strong signal in the silver region was observed at 3 keV for silver nanoparticles due to the surface Plasmon resonance.A weak signal from 'O' is recorded it may due to the presence of organic moieties from the enzymes or proteins in the extract.

SEM (Scanning Electron Microscope)

The SEM image showing the high intensity of silver nanoparticles synthesized by biosurfactant producing organism further confirmed the development of silver nanostructures. SEM provided further insight into the morphology and size details of the silver nanoparticles. SEM analysis showed the particle size of about 100 nm as well the crystal structure of the nanoparticles. The silver nanoparticles synthesized via green route are highly toxic to multidrug resistant bacteria hence has a great potential in Biomedical applications.

Antimicrobial activity

The well diffusion method had been used for the antimicrobial activity studies. The Muller Hinton Agar had been prepared. The sample supernatant had been added in the wells. Incubate the plates at 37°c for 24 hours. After incubation the zone of inhibition had been formed and the zones had been measured in millimeters using the scale.

III. RESULT AND DISCUSSION

Isolation of Bacteria

Five strains observed different colony morphology were streaked on nutrient agar plate to obtain pure culture and they were taken for further study. Out of five, isolates 5 produced pink coloured non diffusible pigment producing colonies and the colony morphology.

Staining characters

All the five bacterial isolates reviewed in this study were stained by Gram's stain. All the five isolates were Gram negative. The morphology details of the bacteria.

Isolation of biosurfactant producing organisms

5 Bacterial isolates were screened as a biosurfactant producer and then the bacterial isolates were identified based on biochemical tests and Bergy's Manual classifications. The isolates are as followed,

- *E.coli*
- *Pseudomonas sp.*
- *Klebsiella sp.*
- *Vibrio sp.*
- *Serratia sp.*

Table 1: Results of colony morphology and Biochemical tests of bacterial isolation

Isolates/characters	1	2	3	4	5
Type of bacteria	Aerobic	Aerobic	Aerobic	Aerobic	Facultative Anaerobic
Colonies	White	White	White	Yellowish white	Pink colour
Gram's staining	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Morphology	Rods in chains with terminal spores	Rods in chains with terminal spores	Rods in chains	Straight rods in single	Straight rods in single or in pairs
Straight rods in single or in pairs	-	-	-	+	-
Methyl red	-	-	-	+	
VogesProskauer	-	-	-	-	+
Citrate utilization	+	+	+	+	+
H_2S production	-	-	-	-	-
Urease	+	+	+	-	-
O-nitrophenyl-β-D-galacto pyranoside (ONPG)	-	-	-	+	+
Phenylalanine deamination	-	-	-	+	-
Nitrate reduction	+	+	+	+	+
Ornithine utilization	+	+	+	+	+

Screening of Biosurfactant

CTAB methylene blue agar method

In the CTAB, methylene blue agar medium after the 24 hours of incubation the biosurfactant activity of blue color zone had been formed. In the CTAB agar plate the organism Pseudomonas sp, Serratia sp, E. coli and Klebsilla sp, had shown the positive result *Serratia sp*. shows high zone and it was used for further studies. Nwaguma *et al*., [4] reported that the selected isolates were performed with different screening test to check the biosurfactant production ability and to find the efficient biosurfactant producer by CTAB plate assay

Determination of emulsification index

The height of the emulsion layer was measured after 24 hr to determine the sample emulsification index. The equation used to determine the emulsification index (E_{24} (%)) is as follows

E_{24} (%) = (height of emulsion layer/ height of total solution) *100%

Table 2: Emulsification Activity of Bacterial Isolates

S.No	SAMPLE	TOTAL LENGTH (E_{24}%)
1	*Pseudomonas sp.*	68%
2	*E. coli*	68%
3	*Vibrio sp.*	64%
4	*Klebsiella sp.*	72%
5	*Serratia sp.*	76%

The emulsification index of the organism *Pseudomonas sp, Serratia sp, E. coli* and *Klebsilla sp* had shown above 50 %. *Serratia sp*. shows high emulsification index and it was used for further studies.

Blood haemolysis test

In the blood haemolysis test, the organism had get haemolysed in the blood agar medium. The blood agar had get haemolysed by *Serratia sp*. cell free supernatant. Dhail and Jasuja [5] studied that haemolytic activity assay, and emulsification activity measurement was used to screen the biosurfactant producer.

Estimation of biosurfactant

The surface tension of the culture medium decreased to a maximum extent of 26.5 mN/m after 72 h of incubation. Furthermore, the CMC of the crude biosurfactant produced by *Serratia sp*. was estimated at 60.13 mg/l.

Extraction and characterization of biosufactant

The biosurfactant was extracted by acidification method from cell free supernatant of *Serratia sp.* This was used for FTIR and GCMS studies.

FTIR analysis of biosurfactant

The functional group of the partially purified biosurfactant was investigated for the chemical nature of the biosurfactant using FTIR. The IR spectrum was collected in the range of 4000 – 428 cm^{-1}. The absorption peaks are observed at 1641, 1506, 1352, 1217, 1055 and 695 cm^{-1}. An intense broad absorbance at 3259 cm^{-1} results due to the strong broad stretch H-bonded of O-H of alcohol group. The band at 454 and 480 cm^{-1} assigned to the approximate C≡C stretching modes of alkynes. The band at 428 cm^{-1} can be assigned to the N-H stretch vibrations of primary amines. The band at 554 and 600 cm^{-1} represents C-H-OOP and Potassium Bromide strong stretch of aromatic anhydrides and alkyl halide respectively. Rahman *et al*. [6] studied the molecular structure of the rhamnolipids with the help of FTIR spectroscopy. Strong and broad bands of the hydroxyl group free(-OH) stretch due to hydrogen bonding were observed in the region (3368 cm-1). The presence of carboxylic acid functional group in the molecule was confirmed by the bending of the hydroxyl (O-H) of medium intensity bands in the region of 1455-1380cm-1. The aliphatic bonds CH3, CH2 and C-H stretching with strong bands are shown in region of 2925 -2856 and 1455-1380 cm–1.

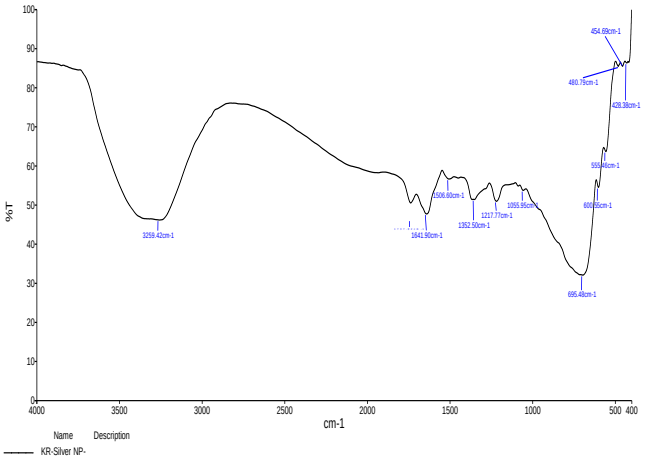


Fig 1: FTIR analysis of biosurfactant from *Serratia sp*.

GC-MS Analysis

GC-MS is an analytical method comprising of gas chromatography coupled to mass spectroscopy for identification of different substances in the sample. GC-MS chromatogram of the biosurfactant producing organism was performed, showing six peaks indicating the presence of different compounds (Figure 2). Major peak compounds at the retention time of 11.59, 12.47, 13.84, 14.48, 18.16, 19.78 minutes were identified from the standard library compound as 5- methyl-Z-5 docosene, hexadecanoic acid, 3-hydroxydecanoic acid, trans-2-dodecenoic acid, N-hexadecanoic acid, 2(1H)-benzocyclooctenone respectively. Therefore, the study indicates rhamnolipid biosurfactant.

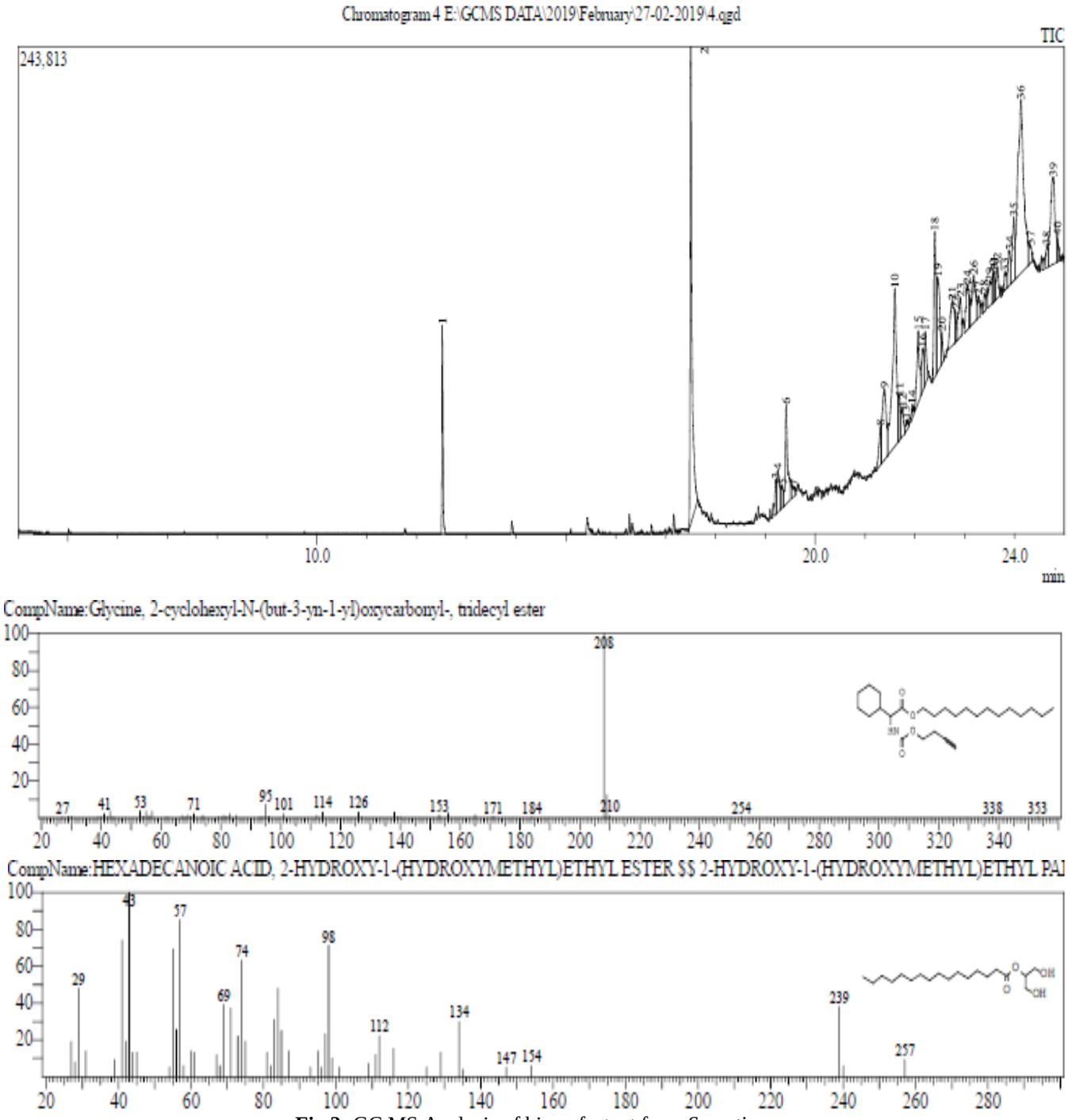


Fig 2: GC MS Analysis of biosurfactant from *Serratia sp.*

Synthesis of silver nano particles

UV–visible spectra of the marine water biosurfactant are synthesised by Silver nanoparticles. The contaminants present in the marine water biosurfactants absorb at higher wavelengths (300–700 nm) while the pure biosurfactants absorb only in the far UV region of the spectrum. Das *et al*. [7] reported the extracellular synthesis of silver nanoparticles by the *Bacillus* strain CS 11. The size of silver nanoparticles obtained was in 42 - 92 nm range. Jaffat, *et al*., [8] confirmed the nanoparticles synthesis from *Lactobacillus* Mixtures.

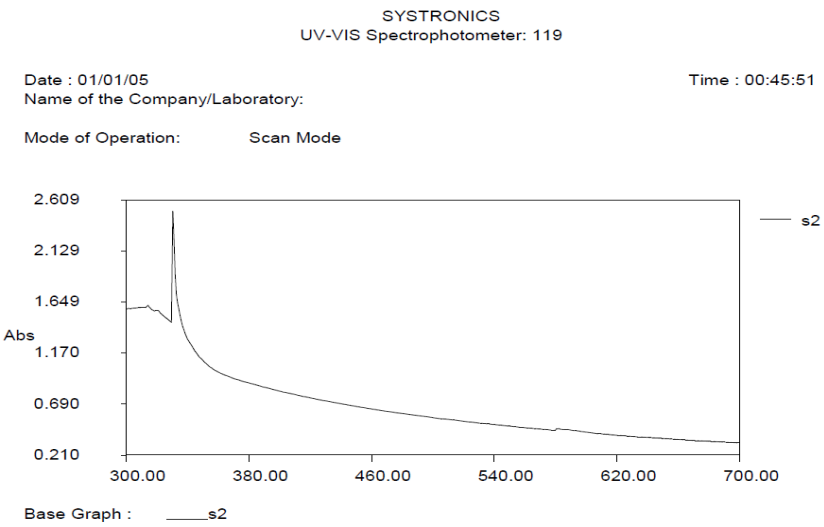


Fig 3: The UV-Visible absorbance spectrum of silver nanoparticles which the peak hbeen formed in 350mm

EDAX result

Determine the elemental composition of synthesized nanoparticles EDAX analysis was performed. In the EDAX spectra of Ag Nanoparticles, the most intense peak around 3 key corresponds to binding energies of Ag. Hence it was confirmed the formation of AgNPs. The remaining elements were due to presence of biosurfactant producing organism *serratia sp.* Silver Nano crystallites display an optical absorption band peak at approximately 3 keV, which is typical of the absorption of metallic silver Nano crystals due to surface Plasmon resonance. Corresponding band peak was obtained in same AgNPs synthesized from the organism *serratia sp*. EDS confirmed the Presence of the signal characteristic of elemental silver. Silver Nano crystallites display an optical absorption band peak at approximately 3 keV, which is typical of the absorption of metallic silver Nano crystals due to surface Plasmon resonance [9].

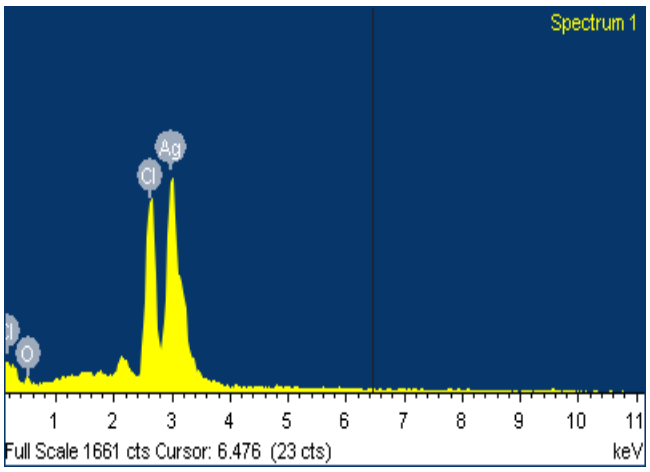


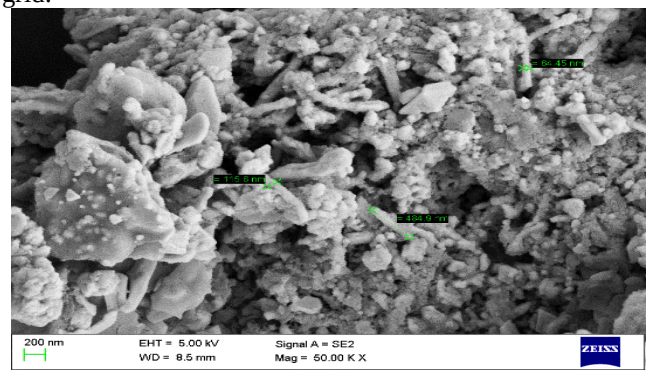
Fig 4: EDAX analysis shows the elemental composition of synthesized nanoparticles. The elemental composition of synthesized nanoparticles by EDAX analysis

Table 3:

Element	Weight%	Atomic%
O K	-0.07	-0.31
Cl K	24.69	50.07
Ag L	75.38	50.24
Totals	100.00	

SEM Result

SEM analysis was carried out to understand the topology of Ag nanoparticles, which showed the synthesis of mono dispersed spherical Ag nanoparticles. In the family of electron microscopy techniques the sample is exposed to a high energy focused beam of electrons. In scanning electron microscopy (SEM) the interaction of the beam with the particle surface are scanned over the sample and measured as secondary electrons (most common), or backscattered electrons or X-ray photons. Due to the high depth of field in SEM a three dimensional appearance can be obtained. The sample needs to be conductively coated with gold or graphite and maintained under ultrahigh vacuum in order not to have the secondary electrons interact with gas molecules. The substrate is typically a filter membrane or a conducting grid.



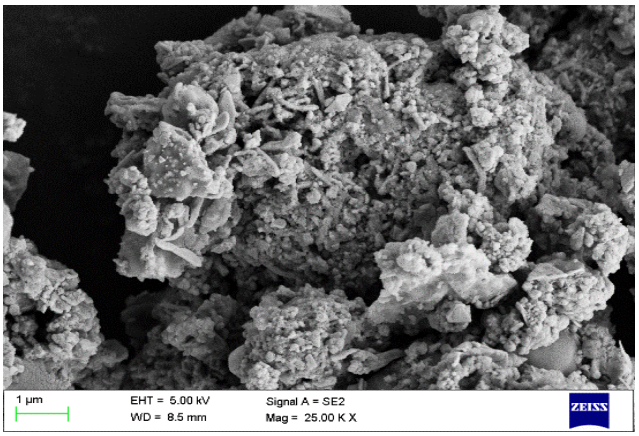


Fig 5: SEM analysis of Ag nanoparticles from biosurfactant of *Serratia sp*.

Antimicrobial activity of biosurfactant by well diffusion method

The antimicrobial activity of organism *serratia sp.* which was synthesized by silver nanoparticles was studied in different concentrations (5, 25, 50, 100 and 250) against the organism *E.coli* (gram negative bacteria). The antimicrobial potential of the organism were assessed in terms of Zone of inhibition of bacterial growth [10]. A number of studies exist which reports the Antimicrobial activity of extracellular synthesized biosurfactant silver nanoparticles isolated from various bacterial strains [11].

IV. CONCLUSION

The *Serratia sp.* was isolated from the marine water which had been collected from marina beach, Chennai, Tamilnadu. The isolated organism was screened for the production of biosurfactant. The screening process had been used by CTAB method, blood haemolysis method, emulsification activity. Then the silver nanoparticles get synthesized by using the organism *serratia sp.* and the wavelength had been measured in the graphical representation in the UV- visible spectrophotometer. Then the characterization process had been done by using the methods of FTIR, SEM, GCMS and EDAX. These methods clearly show about the compounds present in the organisms and it shows the graphical representation.

ACKNOWLEDGEMENT

The authors owe a debt of gratitude to the Vanetra Muthayammal College of Arts and Science for the Research support.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCE

[1] S.K. Satpute, "Assessment of different screening methods for selecting biosurfactant producing marine bacteria", Ind. J. Mar. Sci., Vol. **3,** Issue. **7,** pp. **243-250, 2008.**

[2] K. K Gautam, and V. K. Tyagi, "Microbial surfactants: A review". J. Oleo. Sci., Vol. **55,** pp.**155-166, 2005.**

[3] P. C. Nagajyothi, and K.D. Lee, "Synthesis of Plant-Mediated Silver Nanoparticles Using Dioscoreabatatas Rhizome Extract and Evaluation of Their Antimicrobial Activities", J. Nanomaterials, pp. **1-7, 2011.**

[4] I.V. Nwaguma, C.B. Chikere, and G.C. Okpokwasili, "Isolation, characterization, and application of biosurfactant by *Klebsiella pneumoniae* strain IVN51 isolated from hydrocarbon-polluted soil in Ogoniland, Nigeria", Bioresour. Bioprocess, Vol.**3,** Issue. **40, 2016.**

[5] S. Dhail, and N.K. Jasuja, "Isolation of biosurfactant producing marinebacteria", African journal of environmental science technology. Vol. **6,** Issue. **6,** pp. **263-266, 2012.**

[6] P.K.S.M. Rahman, G. Pasirayi, V. Auger, and Z. Ali, "Production of rhamnolipid biosurfactants by Pseudomonas aeruginosa DS10-129 in a microfluidic bioreactor", Biotechnology and Applied Biochemistry, Vol. **55,** Issue. **1,** pp.**45-52, 2010.**

[7] A. J. Das, R. Kumar, S. P. Goutam, and S. S. Sagar, "Sunlight irradiation induced synthesis of silver nanoparticles using glycolipid bio-surfactant and exploring the antibacterial activity", Journal of Bioengineering and Biomedical Science, Vol. **6,** Issue. **5, 2016.**

[8] H. S. Jaffat, N. H. Aldujaili, and A. J. Abdul Hassan, "Antimicrobial Activity of Silver Nano Particles Biosynthesized by Lactobacillus Mixtures", Research Journal of Pharmaceutical, Biological and Chemical Sciences, Vol. **8,** Issue. **1,** pp. **1911-1924, 2017.**

[9] E. Ranganath, V. Rathod, and A. Banu, "Screening of Lactobacillus spp. for Mediating the Biosynthesis of Silver Nanoparticles from Silver Nitrate", IOSR Journal of Pharmacy, Vol. **2,** Issue. **2,** pp. **237-241, 2012.**

[10] T. Deepa Singh, P. Sravani, S. P. Sreedhar Bhattar, and K. Anuradha, "Green synthesis of iron, copper and silver nanoparticles and their antibacterial activity on animal pathogens – A Comparative study", Int. J. Sci. Res. in Biological Sciences, Vol. **6,** Issue. **1,** pp.**19-24, 2019.**

[11] P. R. Chaudhari, S. A. Masurkar, V. B. Shidore, and S. P. Kamble, "Antimicrobial activity of extracellularly synthesized silver nanoparticles usingvLactobacillus species obtained from VIZYLAC Capsule", Journal of Applied Pharmaceutical Science, Vol. **2,** Issue. **3,** pp. **25-29, 2012.**

