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ORIGINAL RESEARCH ARTICLE

Biosynthesis of Silver Nanoparticles by Rhamnolipid produced by *Pseudomonas fluorescens* MFS-1 from Mangrove Soil

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ABSTRACT

Biosurfactant are emerging as a potential nanoparticles stabilizing agent, the chemically synthetic surfactants are not eco-friendly. Therefore, microbial surfactants are emerging as alternative processes for the chemical surfactant. In this study rhamnolipid was isolated from crude oil enriched mangrove soil by *Pseudomonas fluorescens* (MFS-1). Based on the emulsification index, biochemical composition, the chromatogram, FTIR analysis, the surface active compound produced by MFS-1 was concluded as rhamnolipid. The purified biosurfactant was 19 g/l. It was used for the synthesis of silver nanoparticles and elucidated as rhamnolipid as a stabilizing agent.

The characteristic study of the silver nanoparticle was studied by UV-Vis spectrophotometery and TEM analysis. The TEM analysis revealed the average size of the particles as 50 nm. The silver nanoparticles synthesis in this study was uniform and stable for 3 months. Considering this, the study concluded that the biosurfactant mediated synthesis is a green processing stabilizer of nanoparticles.

Key words: Biosurfactant, Rhamnolipid, Nanoparticles, *Pseudomonas fluorescence.*

1. INTRODUCTION

Biosurfactants are amphiphilic compounds which are produced mainly by hydrocarbon degrading microorganisms. They shown wide range of applications in environmental protection and management, crude oil recovery, as antimicrobial agents in health care and food processing industries. These compounds are capable of reducing the surface tension of the culture broth and emulsification of insoluble carbon sources in the culture medium [1, 2]. Biosurfactant are high biodegradability, low toxicity and stable in extreme pH and temperature. These unique properties of biosurfactant allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial operations. Recent studies suggest that biosurfactant are a good alternative for synthesizing and stabilizing nanoparticles. Selection of biosurfactant as a stabilizing agent of synthesizing silver nanoparticles is one of the best and emerging method in nanotechnology. The size controlled synthesis still remaining as a challenge in material science [3].

Biosurfactant can be used for high performance nano material production, since they easily form a variety of liquid crystals in aqueous solution. Considering the need of greener bioprocess and novel enhances for the synthesis using microbial process. Biosurfactant and biosurfactant producing microbes are emerging as an alternate source of rapid synthesis of nanoparticles [4]. [3] Showed that synthesis of silver nanoparticles could be stabilized by surfactin. In the present study we aimed to develop the green synthesis of silver nanoparticles by the surface active compound which was extracted from the microbial origin.

2. MATERIALS AND METHODS

Isolation, screening and identification of biosurfactant producing bacteria

The soil sample was collected from mangrove ecosystem (Pitchavara, Tamil Nadu). 100 g of freshly collected soil samples was enriched with 100 ml of crude oil to enrich the biosurfactant producers, mixed thoroughly and incubated at room temperature 28 ± 2°C for 30 days [5]. The

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crude oil was obtained from Indian Oil Corporation, Chennai, Tamil Nadu, India. The homogenized enriched soil sample was serially diluted according to [6] technique and the aliquot was placed on various isolation media and incubated at room temperature until the colonies are visible. Biosurfactant producing bacteria were screened using the following techniques. (i) Blue agar plate [7] method, (ii) Hemolytic activity [8], (iii) drop collapsing test [9], (iv) Oil displacement test [10], (v) emulsification activity [11]. All the assays were prepared in triplicate with distilled water as control. The biosurfactant producing bacteria was identified based on the morphological and biochemical analysis and the isolate was used for the present studies.

Production media of biosurfactant

The mineral salt medium (MSM) supplemented with 2% glycerol was used as a production medium. The composition of this medium are (g/L) KH_2PO_4 0.2, K_2HPO_4 0.2, NH_4Cl 0.25, KCl 0.5, $CaCl_2.2H_2O$ 0.15, NaCl 10, $MgCl_2.6H_2O$ 0.6, Na_2SO_4 2.84, yeast extract 0.05, peptone 0.05, trace element solution 1 ml with the following composition (mg/l) $CaCl_2.2H_2O$ 2.0, $MnCl_2.4H_2O$ 0.4, $FeCl_3.6H_2O$ 0.2, $ZnSO_4.7H_2O$ 0.4, $NiCl_2.6H_2O$ 0.4, $Na_2MOO_4.2H_2O$ 0.2. The production of biosurfactant was performed in triplicate 250 ml Erlenmeyer flasks. The flasks were mixed properly and incubated at room temperature for 5 days. Growth was monitored by spectrophotometer at 620 nm.

Characterization of surface active compound

Biochemical characterization

The presence of macromolecule in the surface active component, the extract was estimated for protein, lipid and carbohydrates. For protein [12], carbohydrate [13], lipid [14] and glycolipid [15].

Purification and chemical analysis of the surface active compounds

For the purification of the biosurfactant, the extract was subjected to column chromatography on reverse phase silica gel (240 -400 mesh size). The step - wise elution using methanol from 55 per cent to 100 per cent under room temperature (28°C) at a flow rate of 0.5 ml/min. The active fraction was confirmed by the emulsification activity and the purity was checked by the thin layer chromatography. TLC was performed for the protein (n-butanol:acetic acid: water, 4:3:2), carbohydrates (Chloroform: acetic acid: water, 60:30:10) and lipids (chloroform: methanol: water, 65:25:4). The active fraction was used for the FT-IR analysis. To check the surfactant activity of the TLC purified compound, emulsification index (E_{24}%) the surface tension measurement with a tensiometer using the duNovy procedure (Sigma) used to determine the CMC value of the active fraction [16].

Synthesis of silver nanoparticles

A silver nanoparticles was synthesized by in situ in the water-in-oil micro-emulsion phase as described by [4]. For the synthesis of silver nanoparticles 1 ml of biosurfactant solution was mixed with 1 mM of 1ml silver nitrate solution double distilled water was used. They were vigorously stirred for 5 min. Then 1mM of 20µl sodium borohydrate (Sigma) solution was added under vigorous stirring control maintained without adding biosurfactant solution. The synthesized silver nanoparticles were characterized by UV-Vis spectrophotometer and TEM analysis.

Antimicrobial activity of silver nanoparticles

The antimicrobial activity of silver nanoparticles was investigated against 5 different pathogens by well diffusion method [17]. In this procedure, the nutrient agar plates were swabbed with the pathogens *K. pneumonia, Staphylococcus aureus, Vibrio cholera, Candida albicans* and *Proteus mirabilis* as well, was created using well cutter. 100 µl of the synthesized silver solution was added to each well. The positive control ($AgNO_3$) and the negative control were maintained. All the plates were incubated at 37° for 1 day. The growth inhibition zone was measured. Finally, MIC was calculated by agar diffusion assay.

3. RESULTS AND DISCUSSION

Isolation and identification of biosurfactant producer MFS-1

Based on the screening test and biochemical analysis, the isolate MFS-1 was considered as a potential biosurfactant producer among the 21 isolates which are screened. The isolate MFS-1 shows formation of dark blue zone on blue agar plate, haemolytic activity (9 mm), oil displacement (9 mm), lipase activity (8 mm), drop collapsing tests positive and emulsification activity (E_{24}%), 70%. The isolate MFS-1 was gram negative, rod, motile green pigment producer, hydrolyses catalase, utilize glucose but not mannitol. Based on the morphological and biochemical characteristic the isolate MFS-1 was confirmed as biosurfactant producing bacteria.

Biosurfactant production

The isolate MFS-1 produced biosurfactant with highest emulsification index (E_{24}%), 70% which was obtained during the 3rd day of the growth (**Fig 1**). In this study we found that the growth kinetics of the cultures and the biosurfactants production was different. The maximum production was found at the third day of incubation time. It reveals that the stationary phase culture secretes biosurfactant as a secondary metabolites. By this kinetics were selected the 3rd day culture for the further extraction and purification. Emulsification activity enhances the biodegradation of hydrocarbons by increasing their bioavailability of hydrocarbons by increasing their bioavailability to the microbes involved. Emulsification activity of crude oil by an alkane-oxidizine *Rhodococcus* sp. isolated from sea water reported by [18] also supported the study on emulsification of hydrocarbons.

Chemical characteristics of biosurfactant

The surfactine extract of *Pseudomonas fluorescens* MFS-1 contained 0.3 mg/ml protein, 1.8 mg/ml carbohydrates, 4.856 mg/ml lipid and glycolipid 20.52 g/l. [3] reported that the concentration of lipopeptide compound from *B. amylolipuifaciens* S13 was 452 mg/l and *B. subtilis* ATCC 21332 was 109.5 mg/l. The biosurfactant was further purified by the column chromatography packed with silica (mesh size 240 - 400nm). The biosurfactant as eluted in the 75-85% of the methanol extraction. The FT-IR spectral analysis revealed the important adsorption bands at 3450.26, 2925.06, 2856.20, 1745.34 and 1407 - 1064 cm^{-1} (**Fig 1**) indicating the fact that all of them have chemical structures identical to those of rhamnolipids, which are composed of rhamnose rings and long hydrocarbons chains. The FT-IR analysis confirmed that the surface active compound produced by *Pseudomonas fluorescens* MFS-1 as rhamnolipid. The FT-IR results obtained in this study was further corroborates with the findings of [19 - 21]. Based on the biochemical composition, the chromatogram of glycolipids showed Rf value of 0.81 which was compared with results predicted by [22]. Based on the comparison the spot obtained was revealed as rhamnolipid. The purified biosurfactant concentration in the extract was 19 g/L.

Biosurfactant mediated synthesis of silver nanoparticles

The synthesis and stabilization of silver nanoparticles was monitored by UV-V is absorption spectrum analysis and TEM analysis. A strong broad peak at about 420 – 440 nm was observed for the silver nanoparticles (**Fig 2**). UV-Vis absorption spectrum is sensitive to the formation of silver nanoparticles and shows intense absorption peak around 400 nm originating from the surface Plasmon of nanosized silver particles [23, 24]. This result evidenced that the nano-scale silver can be synthesized in reverse micelles using rhamnolipid as stabilizer. To determine the stability the synthesized silver nanoparticles. The silver nanoparticles synthesis in the presence of rhamnolipid was kept at room temperature for different days intervals; it was stable for 3 months. In the control experiments, instead nanoparticles, aggregated clumps of black colour were observed. This indicates that the biosurfactant presents the aggregation and it enhance the production and stability of the silver nanoparticles. TEM Image (Fig - 3) determines the morphological and shape of the silver nanoparticles, TEM observation shows the spherical shape of the silver nanoparticles with the average size is 50 nm. [25] revealed the shape of silver nanoparticle that are reduced and stabilized by various sugars. The silver nanoparticles appeared to be almost spherical and the average size in 20nm.

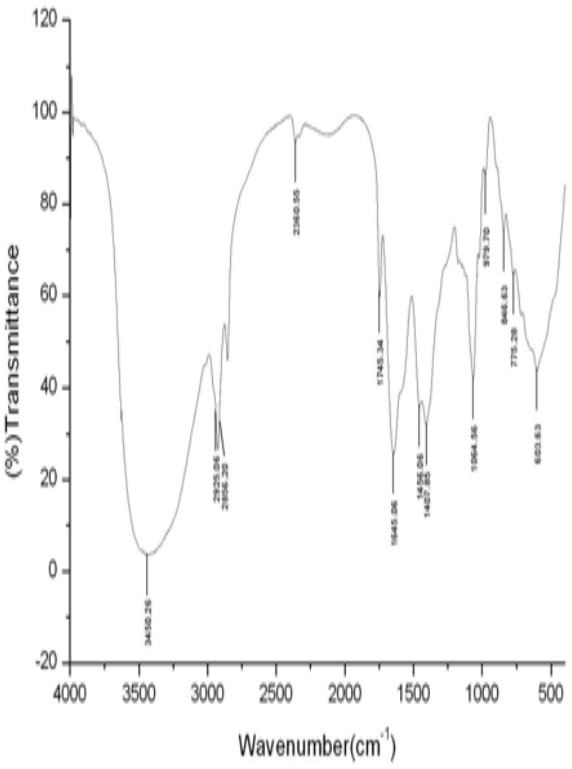


Fig 1: FTIR spectrum of biosurfactant produced by *Pseudomonas fluorescens MFS-1*

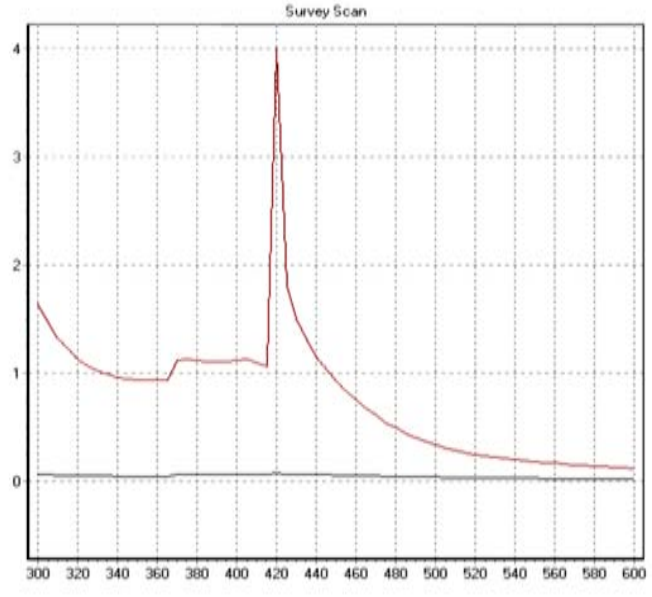
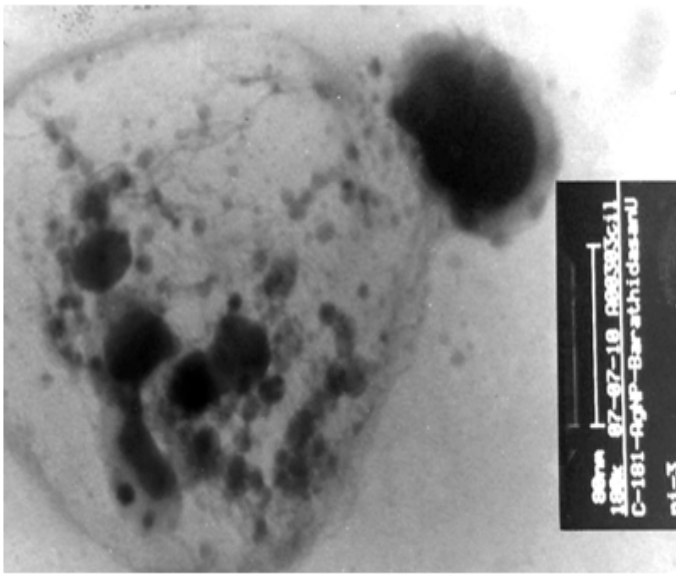


Fig 2: UV-Vis spectroscopy analysis of silver nanoparticles at 420 nm



Considering the need of green synthesis using microbial processes, biosurfactant and biosurfactant producing micro organisms are emerging as a alternative source for the synthesis of silver nanoparticles [3] reported a lipopeptide biosurfactant used to stabilize the formation of silver nanoparticles. Although the chemical surfactants are highly promising these chemicals are toxic to the environment. The focus on biosurfactant increases the potential implications on the synthesis of silver nanoparticles. [26, 27] reported biosurfactant isolated from marine *Actinobacteria* was used for the biosurfactant mediated synthesis of silver nanoparticles and it was stable for 2 months. In the present study, we demonstrate that rhamnolipids mediated synthesis of silver nanoparticles would be an effective processes over the chemical surfactants.

Antimicrobial activity of silver nanoparticles

The purified silver nanoparticles have showed highest antimicrobial activity against various human pathogens. In this study the highest activity was measured against *S. aureus* and *C. albicans*. When compared to silver nitrate solution the purified silver nanoparticles showed highest activity. The MIC results represents the concentration of purified Ag nanoparticles has required to control the growth of pathogens tested. The stability of the particles monitored using three months olden purified nanoparticles. It shows the same antimicrobial activity against the pathogens. Thus we concluded that the stability of the silver nanoparticles have been brought by the rhamnolipid. Recent literature data reports encouraging results about the bactericidal activity of silver nanoparticles of either a simple or composite nature. [28] revealed considerable changes in the cell membranes upon treatment, resulting in cell death. The nanoparticles can either directly interacts with the microbial cells, e.g., Interrupting transmembrane electron transfer, disrupting/penetrating the cell envelope or oxidizing cell components or produce secondary products (e.g., reactive oxygen species (ROS) or dissolved heavy metal ions) that cause damage.

CONCLUSION

In this present investigation, we studied that the "green synthesis of biosurfactant-mediated-synthesis of silver nanoparticles." We found that the biosurfactant produced by *P. fluorescens* MFS-1 may enhance the synthesis of silver nanoparticles and acting as a stabilizing agent. The biosurfactant produced form the microbial origin has high degrading, low toxicity and excellent biological activities. In this study, the Ag nanoparticles produced by the *P. fluorescens* MFS-1 isolated from the crude oil enriched mangrove soil are stable for 3 months. Therefore, the biosurfactant mediated synthesis of silver nanoparticles can considered as green processor for stabilization of silver nanoparticles.

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