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EFFECT OF SILVER NANOPARTICLES SYNTHESIZED USING AQUEOUS LEAF EXTRACT OF *AMHERSTIA NOBILIS* ON MARINE BIOFILMS

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Abstract

The present study emphasized the synthesis of silver nanoparticles (SNP) from silver nitrate through bioreduction method using *Amherstia nobilis* leaf extract and evaluation of anti-marine biofilm activity of the synthesized particles. The phytosynthesis of silver nanoparticles was demonstrated firstly by visual observation and then by spectral methods : UV-Visible absorption spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR) and Dynamic Light Scattering technique (DLS). The Energy dispersive X-ray spectrum of the colloidal solution confirmed the presence of an elemental silver signal. Transmission Electron Microscopy analysis revealed that the nanoparticles were mostly spherical in shape and the particle size ranges from 3.74nm to 40.43nm. The stability of the silver nanoparticles was checked by ξ-potential measurements. It is found that silver nanoparticles synthesized by aqueous leaf extract of *A. nobilis* were effective against biofilm formation of microbes such as marine *B. subtilis* and marine *E. coli.*

Key words: SNP, *A. nobilis*, Green synthesis, marine biofilm, *E. coli*, *B. subtilis*

Introduction

Silver nanoparticles (SNPs) have attracted intensive research interest because of their important applications as antimicrobials in textile/fabric industry and plastic industry to eliminate microorganisms and as a catalyst Baker *et al.* (2005), Shahverdi *et al*., (2007). Generally, silver nanoparticles are synthesized and stabilized through chemical methods, mechanical methods, electrochemical techniques Patakfalvi *et al.* (2010), Rodriguez-Sanchez *et al*. (2000), Pileni *et al*. (2000), Sun *et al.* (2001), Henglein *et al.* (2001) and nowadays *via* green chemistry methods Mondal *et al.* (2011), Begum *et al.* (2009). Application of green chemistry to synthesize nanomaterials has vital importance in medicinal and technological aspects. It is also gaining importance due to its simplicity and eco-friendliness. Several biological systems including microorganisms Klaus *et al.* (1999), Nair *et al*. (2002), Konishi *et al*. (2007), fungi Vigneshwaran *et al.* (2007) and plants have been used in the synthesis of nanoparticles. The living plants are considerably preferred for biosynthesis of silver nanoparticles due to their rich diversity of phytochemicals that have strong antioxidant properties. Shankar *et al.* (2004), Chandran *et al.* (2006), Jae *et al.* (2009).

Amherstia nobilis, 'The Pride of Burma' is one of the most popular ornamental trees belonging to the family Fabaceae (Leguminosae). It is also known as Tree of heaven, Noble Amherstia and Queen of flowering trees. The present study is the first report on the synthesis of stable silver nanoparticles in ambient conditions using *Amherstia nobilis* leaf extract as a reducing and stabilizing agent. This study is further designed to determine the effect of synthesized silver nanoparticles on the biofilm of marine biofouling organisms such as *E. coli* and *B. subtilis*. Biofilm formation leads to biofouling which is an undesirable accumulation of microbes on damp or moist surfaces. The primary function of a biofilm is protection, adherence or attachment to surfaces which may be of biotic or abiotic origin and resilience of bacteria. Marine biofouling refers to uncontrolled growth of marine

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microflora on immersed structures such as ship hulls, jetty pilling, fishing gear, pipes *etc*. affecting shipping industries and industrial aquatic processes. This paper aims to demonstrate a simple and cost-effective synthesis method of silver nanoparticles using *A. nobilis* leaf extract and to test the ability of the synthesized nanoparticles against the biofilms of marine microbes.

Materials and Methods

Bio Reduction with Plant Extract

In the present study, leaves of *Amherstia nobilis* were collected from University of Mumbai - Fort Campus garden, Mumbai, Maharashtra. The leaves of *Amherstia nobilis* were initially rinsed thrice in distilled water and dried on paper towelling. Samples of 5 g each were cut into fine pieces and boiled with 100 ml of sterile distilled water for 5 min. The crude extract was passed through Whatman filter paper No.1 and the filtrates were stored at 4^0C for further use. Leaf extract of *Amhersia nobilis* was mixed with 10^{-3} M aqueous $AgNO_3$ solution in a ratio 5:45 (v/v) and was maintained at 90^0C for the bioreduction process. The resulting silver nanoparticle preparation [A] was centrifuged at 1500rpm for 10 minutes. The resulting upper light coloured supernatant [B] was separated from the lower deeply coloured suspension [C].

Characterization of silver nanoparticle

The reduction of silver ions was monitored periodically at room temperature. Initially Silver naoparticles were characterized using UV-Visible spectroscopy (Varian, Cary 50) operated at a resolution of 1 nm between a 300 and 800 nm range.

Size distribution of bioreduced silver nanoparticles was measured using DLS (ZetasizerVer 6.34, Malvern, UK). This measurement indicates the mean size of particles present in sample.

The ξ-potential measurements of the silver nanoparticles were realized by applying an electric field across the analyzed aqueous dispersions using the appropriate accessory of ZetasizerVer 6.34 (Malvern, UK.). The zeta potential is related to the electrophoretic mobility and to the stability of the of silver nanoparticle suspensions.

Transmission Electron Microscopy (TEM) was performed for characterizing the size and shape of the biosynthesized silver nanoparticles. The aqueous silver nano-particles suspension was first sonicated (Vibronics VS 80) for 15 min. A drop of this solution was loaded on carbon-coated copper grids, and the solvent was allowed to evaporate under Infrared light for 30 min. TEM measurements were performed on Philips model CM 200 instrument operated at an accelerating voltage at 200 KV.

EDX was used mainly to determine the bulk elemental composition of the sample materials. The leaf - extract - reduced silver nanoparticles was drop coated on to carbon film and the analysis was performed on JEOL - MODEL 6390 SEM instrument equipped with a Thermo EDAX attachments.

Fourier Transform Infrared spectroscopy (FTIR) is a technique that detects the vibrations of the atoms within a molecule. The FTIR measurements of biosynthesized silver nanoparticles and leaf extract were carried out to understand the chemical change of the functional group which was involved in the bioreduction. The range of reflection mode used was from $4000cm^{-1}$ to $400cm^{-1}$ with a resolution of $4cm^{-1}$.

Evatution of Synthesized SNPs on Marine Biofilms

Marine microbial cultures such as *E. coli* and *B. subtilis* were obtained from National Institute of Oceanography, Mumbai for this study. The three preparations of silver nanoparticles suspensions were tested for their anti-biofilm activities, namely: silver nanoparticles preparation without centrifugation [A], the pale supernatant [B] and the deeply coloured suspension [C]. Appropriate controls were also included in the study and all the experiments were conducted in test-tubes of dimensions-150mm × 15mm (L × OD). Each test was replicated 15 times.

A 24 hr grown cultures of *B.subtilis* and *E.coli* were mixed with silver nanoparticles preparations in the proportion of 4:1 respectively and incubated in ambient conditions for 24 hrs to determine the effect of siver nanoparticles on biofilm formation.

The effect of silver nanoparticles suspension on preformed bacterial film was determined by incubating 1 ml for the whole silver nanoparticles suspension [A] with the preformed biofilm for 24 hrs followed by analysis for biofilm.

Determination of extent of biofilm formation or destruction of preformed biofilm was achieved by the method described by S. Bhattacharyya *et al.* (2016). The liquid in the culture tubes after incubation was discarded. The tubes were then flooded with saffranine [aqueous, 0.5 % (w/v)] lasting for 1 min to stain the bioflim. The tubes were washed with saline thrice with each rinse lasting 1 min. The saffranine remaining on the tube (an indication corresponding the biofilm present on surface of the tube) was extracted using methanol and its absorbance at 528 nm was recorded using the

spectrophotometer shimadzu UV 1800. The saffranine content was quantified using a standard graph (regression coefficient: $r^2 = 0.991$). The presence of biofilm was expressed as µg of saffranine. The results obtained were statistically analyzed for the following parameters: Mean, standard deviation and Students t test at confidence levels of 95% and 99% was done using Maxstat lite version.

Results & Discussion

The addition of *A.nobilis* leaf extract to silver nitrate solution resulted in a color change of the solution from transparent to a dark brownish yellow due to the production of silver nanoparticles (fig-1). It is well known that silver nanoparticles exhibit yellowish brown colour in aqueous solution due to excitation of Surface Plasmon vibrations of silver nanoparticles Krishnaraj *et al.* (2010).

UV-Visible absorption spectrophotometer is commonly used to investigate the Surface Plasmon Resonance. The UV-Visible spectrum shows no evidence of absorption in the range of 400-800nm for the leaf extract and the 1mM $AgNO_3$ solution individually. However the silver nanoparticles preparation which is a combination of the leaf extract solution and $AgNO_3$ solution heated to 90^0 for 10 min shows a distinct absorption at around 422 nm (fig-2). The broad peak obtained indicated that the particles are poly dispersed in size.

The size of the synthesized silver nanoparticles was determined by dynamic light scattering measurements and the physical stability of the silver nanoparticles was evaluated in terms of ξ-potential. The hydrodynamic diameter (z-average), the polydispersity index (PdI) and the zeta potential values of the phytonanosilver suspension are presented in table 1.

Z-average represents the hydrodynamic diameter of the particle. The hydrodynamic diameter is affected by the environment surrounding the particle and is calculated assuming isotropic spherical particle. Therefore, it is important to determine the real particle size by other characterization techniques like High resolution transmission electron microscopy (HRTEM). The polydispersity index is the measure of the distribution of nanoparticle population and a higher value indicates a high size distribution with multiple silver nanoparticles population.

Concentration of the silver nanoparticle in the suspension is calculated using the equation:

$$C = N_{Total} / NVN_A$$

Where N_{Total} is the total number of silver atoms added to the reaction solution, N ($N = 31\ d^3$ where d is the mean particle size of the nanoparticle in nm) is the number of silver atoms present in each nanoparticle, V is the volume of the reaction solution in litres and N_A is the Avogadro's constant Liu *et al.* (2007), Mariam *et al.* (2011). Using the above equation the concentration of silver nanoparticle was calculated to be 0.9038×10^{-9} molL^{-1}.

Fig–3 shows the TEM Micrograph of the synthesized silver nano particles. It is observed that most of the silver nanoparticles were spherical in shape and the particle size ranges from 3.74nm to 40.43nm. fig–4 shows the selected area electron diffraction (SAED) Pattern with bright circular rings indicating that silver nanoparticles are highly crystalline in nature

The DLS measured size is larger than the particle size measured from TEM micrographs, because dynamic light scattering (DLS) method measures the hydrodynamic radius Kasture *et al.* (2008). High hydrodynamic diameter value obtained by DLS indicates that the silver nano particles are protected by a thick layer of compounds present in leaf extract.

Fig–5 shows the EDX spectrum of silver nano particles prepared with this bio-reduction method. The peak observed around 3 KeV predicts the binding energy of AgL which proves the confirmation of pure silver due to Surface Plasmon Resonance Kalimuthu *et al.* (2008), Prathna *et al.* (2011). Some weaker atoms like C and O appeared suggestive of capping components or minor impurities.

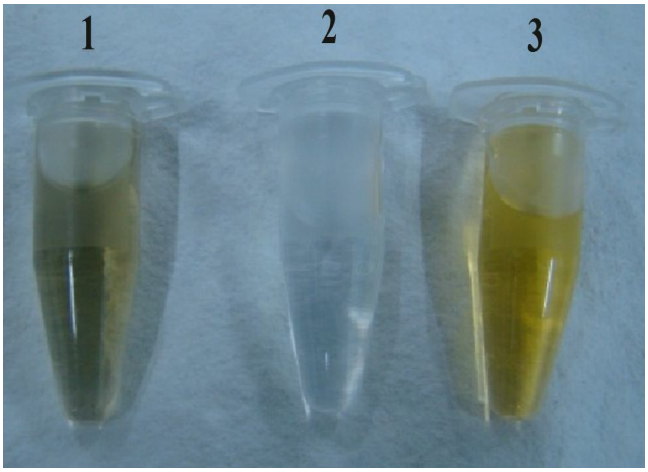
FT-IR analysis was used for characterization of the extract and resulting nanoparticles. The nature of interaction between the extract and silver nanoparticles can be studied using FT-IR spectra. Absorbance bands were observed at 618.25, 716.85, 1384.82, 1440.36, 1483.82, 1592.94, 1664.14, 1723.09, 1811.01, 2234.86, 2854.19, 2923.91, 3535.06, 3703.51, 3780.93cm^{-1} in the

Table 1: DLS data of AgNPs and the ξ-potential values of AgNPs.

Sample	Z-average (nm)	PDI	ξ-potential (mV)
Phyto silver nano suspension	93.38	0.333	-26.0

Table 2: Effect of SNPs on biofilms of *B. subtilis* and *E. coli* isolated from marine habitats

Treatment	*B. subtilis* (µg saffranine)	*E. coli* (µg saffranine)
Biofilm control	57.05±24.63 µg	58.40±15.41 µg
SNP [A] before biofilm formation	15.59±6.04 µg	20.50±14.78 µg
SNP [A] after biofilm formation	36.15±36.06 µg	27.65±7.90 µg



Legend: 1: *A. nobilis* Leaf extract 2: Leaf extract + $AgNO_3$ (before reduction) 3: Leaf extract + $AgNO_3$ (after reduction)

Fig.-1: Bioreduction of silvernitrate (transparent) into silvenanoparticles (brownish yellow color)

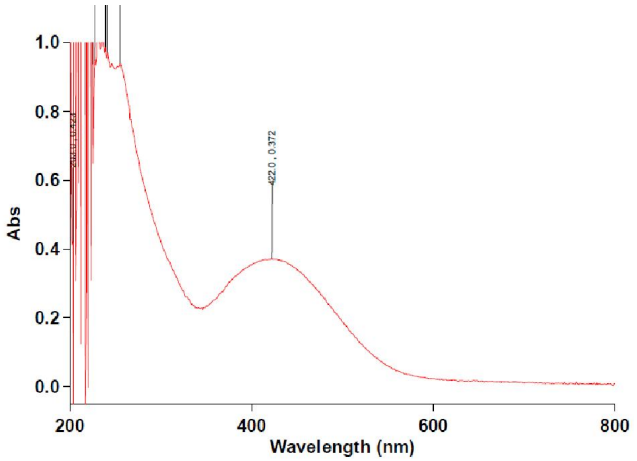


Fig-2: UV-Visible spectra of silver nanoparticles synthesized by treating 1mM aqueous $AgNO_3$ solution with 5% *A.nobilis* leaves extract.

colloidal solution after bioreduction and reduction and stabilization may be due to the biomolecules derived from carbonyl group in the plant extract. Thus, FTIR analysis confirmed that bioreduction of Ag+ ions to silver nanoparticles are due to the reduction by capping material of plant extract.

The effect of *A. nobilis* leaf extract, $AgNO_3$ (1 mM), silver nanoparticles preparations [A], [B] and [C] on biofilm formation of *B. subtilis* and *E. coli* is depicted in figure-8. The effect of $AgNO_3$ (1 mM) and whole silver nanoparticles suspension [A] on the preformed biofilms of *B. subtilis* and *E. coli* is reported in table–3. The whole silver nano particles preparation was found to be more effective in prevention of biofilm formation than the disruption of the preformed biofilm. The lower deep coloured silver nanoparticles fraction obtained after

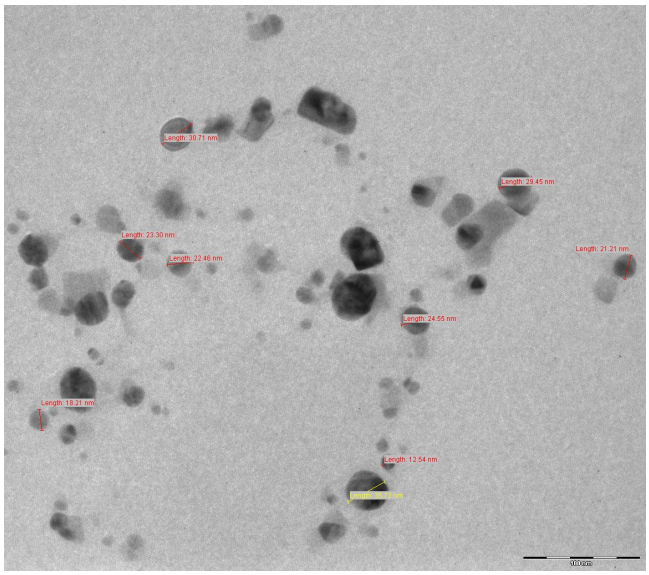


Fig.-3: TEM image of SNPs

Fig.- 4: SAED image of SNPs

centrifugation [C] was the best among all the silver nanoparticles preparations tested against the biofilm formation of *B. subtilis* and *E. coli* (reduction by 79% in *B. subtilis* and 80.5% in *E. coli*). Whole silver nanoparticles preparation without centrifugation [A] and upper light coloured supernatant of silver nano particles suspension after centrifugation [B] were equally effective against *B. subtilis* and *E. coli* biofilm formation.

There was a significant reduction in biofilm formation when culture was incubated with leaf extract or $AgNO_3$ or silver nanoparticles.

At confidence level 95% and 99% although Leaf Extract (30.54 ±9.10 µg saffranine for *B.subtilis* 42.99 ±21.88µg saffranine for *E.coli*) and $AgNO_3$ (20.69 ±10.13µg saffranine for *B.subtilis* and 23.42±16.75 µg saffranine for *E.coli*) have significant effect on biofilm formation, the SNP [C] (12.10±7.30 µg saffranine for *B.subtilis* and 11.38 ±7.86 µg saffranine for *E.coli*)

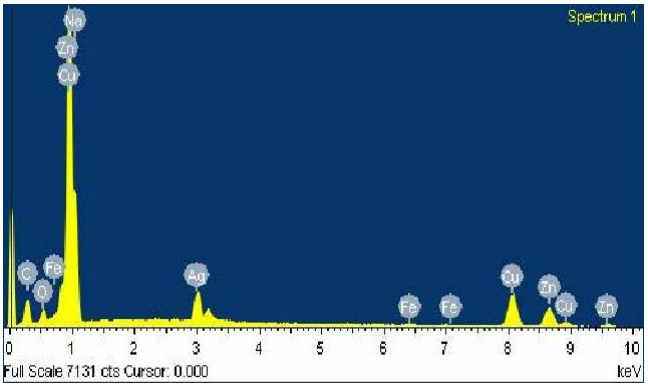


Fig.-5 : EDAX spectrum of the silver nanoparticles synthesized using *A. nobilis* leaf extract

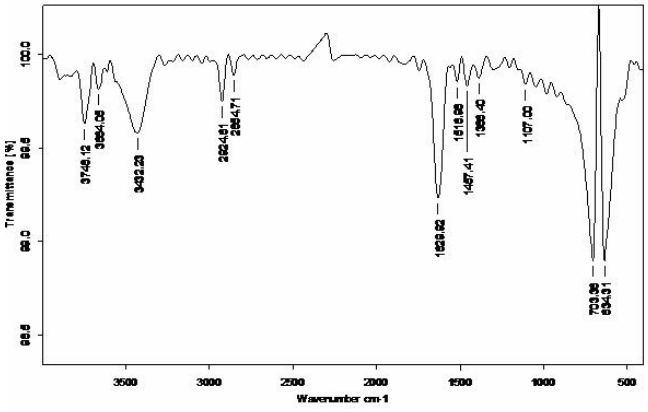


Fig.-6: FT-IR spectrum of crude leaf extract of *A. nobilis*

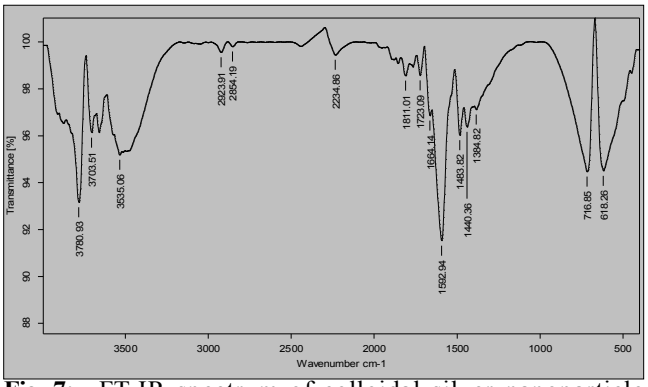


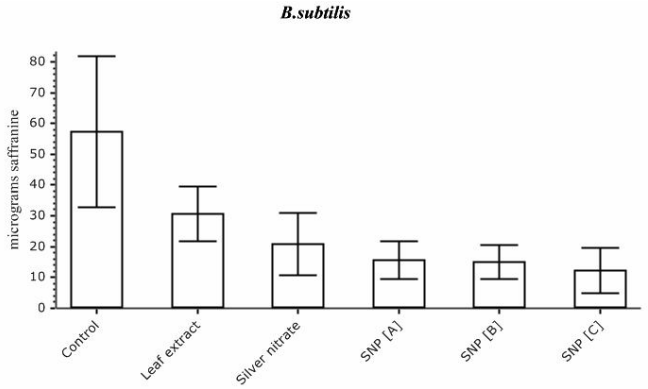
Fig.-7: FT-IR spectrum of colloidal silver nanoparticle suspension after bioreduction using *A. nobilis* leaf extract

preparation was significantly more effective in the prevention of biofilm formation.

Conclusion

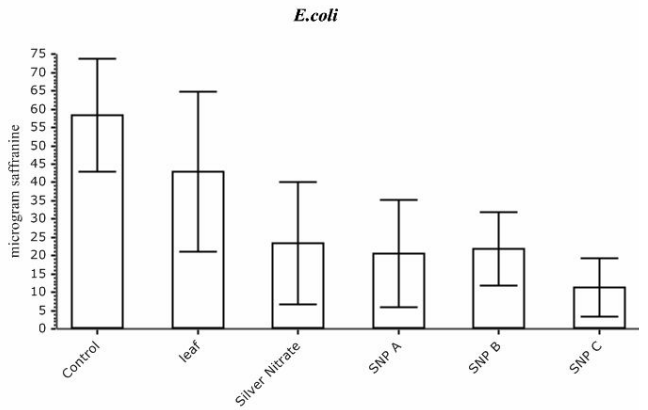
From the experiment results, it can also be concluded that *A.nobilis* leaf extract can be used as an effective capping as well as reducing agent for the synthesis of stable silver nanoparticles. This is the first report using *A.nobilis* extract as a bio reducing agent to synthesize silver nanoparticles to the best of our knowledge.

A] Effect of SNP preparations on biofilm formation of *B.subtilis*



Legend : The presence of biofilm expressed in µg saffranine
Control : 57.20 ±24.63 µg; Leaf Extract : 30.54 ±9.10 µg ;
Silver Nitrate / $AgNO_3$: 20.69 ± 10.13 µg ; SNP [A] : 15.59 ± 6.04 µg;
SNP [B] : 15.15 ± 1.35 µg; SNP [C] : 12.10 ±7.30 µg

B] Effect of SNP preparations on biofilm formation of *E.coli*



Legend : The presence of biofilm expressed in µg saffranine
Control : 58.40 ±15.41 µg; Leaf Extract : 42.99 ±21.88 µg
Silver Nitrate/ $AgNO_3$: 23.43 ±16.75 µg; SNP [A] : 20.50 ±14.78 µg
SNP [B] : 21.80 ±10.01 µg; SNP [C] : 11.38 ± 7.86 µg

Fig.-8: Effect of SNP preparations on biofilm formation

Synthesized nanoparticles were crystalline in nature and their size ranged from 3.74nm to 40.43nm. They were predominantly spherical in shape and were active against biofilm formation of two biofouling marine microbes namely *B.subtilis* and *E.coli* This result encourages studies on the efficacy of silver nanoparticles against formation of biofilms or retardation of marine biofilm formation and the best possible mode of its application . It also found that the possible biomolecules responsible for the reduction and stabilization process may be carbonyl group from the leaf extract.

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