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ORIGINAL ARTICLE

Pigment mediated biogenic synthesis of silver nanoparticles using diatom *Amphora* sp. and its antimicrobial activity

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Abstract Light induced biosynthesis of polycrystalline silver nanoparticles (SNPs) using the aqueous extract of a diatom *Amphora*-46 was studied. Rapid formation of stable SNPs was observed only on exposure of the reaction mixture to light. Strong surface plasmon resonance at 415 nm due to SNP formation was confirmed by spectroscopic analysis. TEM analysis confirmed the formation of polycrystalline spherical SNPs of an average size of 20–25 nm which was further corroborated by XRD, EDAX and SAED results. Compositional analysis using EDAX showed strong characteristic signal for silver. The XRD spectra show four intense diffraction peaks at 38.48°, 44°, 64.74°, and 77.4° which correspond well to (1 1 1), (2 0 0), (2 2 0), and (3 1 1) plane of (fcc) polycrystalline SNP and the intensity of peak at (1 1 1) plane is more than the other peaks, suggesting that this plane is the predominant one. Both XRD and SAED results clearly indicated that the SNPs were polycrystalline in nature and were of high purity. The bio-molecule responsible for the reduction of silver ion was identified to be a photosynthetic pigment fucoxanthin, which is light sensitive and acts as a reducing agent. Furthermore, the synthesized SNPs possess significant antimicrobial activity against gram positive and gram negative bacteria.

This study demonstrates for the first time, the involvement of photosynthetic pigment fucoxanthin isolated from *Amphora*-46 in silver nanoparticle formation through a light dependent reaction.

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1. Introduction

Nanoparticles have drawn significant attention in recent years due to their unique optical, electrical, and catalytic properties that differ from that of bulk materials. All these unique properties are attributed to the variation in characteristics such as size, shape, structure and distribution/composition of the

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particles [1]. Although several physical and more frequently chemical methods are successfully employed for the preparation of various metal nanoparticles, these are considered hazardous owing to the use of non-biodegradable toxic chemicals as reducing and stabilizing agents and are of environmental concern. Many chemically synthesized nanoparticles are not suitable for biological applications due to the chance of chemical contamination [2]. Hence in recent years research efforts are being made to develop simple, benign procedure consisting of environmentally acceptable solvent system, nontoxic reducing and capping agent, resulting in the green synthesis of nanoparticle [3–5]. Among the metal nanoparticles silver nanoparticles (SNPs) have gained importance due to their applications in diverse areas such as catalysts, nanomedicine, biological labeling, sensing, solar cell surface coatings, electronics, surface-enhanced Raman scattering detection, staining pigments for glasses and ceramics, and antimicrobial agents [6]. SNPs are preferred as antimicrobial agent in biomedical applications due to their effectiveness at very low concentration and toxicity against antibiotic resistant bacteria. Various biological resources such as bacteria, actinomycetes, fungus, plant extracts and photosynthetic algae have been exploited for the biosynthesis of SNPs [7–10].

Among the microalgae diatoms are a group of structurally unique aquatic unicellular photosynthetic microalgae (~1–500 μm in length) belonging to the group of brown algae (Bacillariophyceae). They are ubiquitous in nature and have autotrophic mode of metabolism [5,11]. Their key feature is their hydrated amorphous silica exoskeletons of diverse range of size, shape and consisting of micro to nanoscale biosilica, unique for each species. Live diatoms are reported to have biotechnological use like ecological monitoring [12], biofuel production due to high oil content [13] and CO_2 sequestration due to high carbon concentrating mechanism [14]. Diatoms in living or dead conditions have attracted key attention for various nanomaterial formations either through metabolic insertion (gold, germanium, titanium, and cadmium) or through in situ coating/fabrication (SNPs) [15–19]. Diatoms are reported to have many photosynthetic pigments like chlorophyll a (Chl a), chlorophyll c (Chl c) and fucoxanthin (Fuco) which play a major role in light harvesting photosystem [20]. However the amount of fucoxanthin is relatively higher than the amount of chlorophyll in diatoms [21].

Further characteristic golden brown color of diatoms is due to the presence of photosynthetic pigment fucoxanthin, belonging to the group of xanthophylls. Several recent reports suggest that fucoxanthin has several biological properties such as antioxidant, antimicrobial, antiobesity, anticancer and other use in various biomedical applications [22,23]. Fucoxanthin has several oxygenic functional groups such as hydroxyl, epoxy, carbonyl and carboxyl moieties (Fig. 1) which may impart the antioxidant property [24]. Although several studies were carried out on biosynthesis of metal nanoparticle in living diatom, the mechanism/biomolecules responsible for reduction is largely unclear. The free hydroxyl group on the aromatic ring is reported to be responsible for the antioxidant properties [24,25]. According to existing reports carbohydrates, proteins and pigments rich in the hydroxyl group could be responsible for metal nanoparticle synthesis in algae [18] and extensive studies need to be focused on. This implies that fucoxanthin molecule may act as a reducing agent. However, to the best of our knowledge, till date no such reports on biosynthesis of SNPs in diatom either intracellular or extracellular system are available.

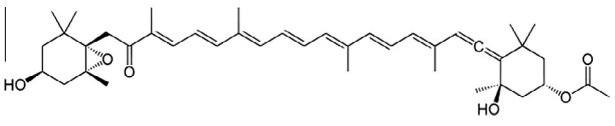

Figure 1 Structure of fucoxanthin.

The present work reports for the first time the biosynthesis of silver nanoparticle by a light dependent reaction in aqueous cell extract of a ubiquitous diatom *Amphora* sp. Further experiments were carried out to identify the biomolecule responsible for silver ion reduction and biosynthesized SNPs are tested for the antimicrobial activity against some pathogenic bacteria. All the experiments were carried out at the beginning of December 2013 at the CSIR-Institute of Minerals and Materials Technology, Bhubaneswar, Odisha.

2. Materials and methods

2.1. Diatom strain and sample preparation for SNP synthesis

Diatom culture *Amphora* sp. (IMMTCC-46) denoted as *Amphora*-46 was obtained from the culture collection center and repository at the CSIR-Institute of Minerals and Materials Technology, Bhubaneswar, India. The diatom cells were grown in Erlenmeyer flask containing f/2 medium [26] made with filter sterile brackish water (salinity 3%, pH 8.2, collected from Chilika Lake, Odisha, India). The flasks were incubated at temperature of 30 °C with 16:8 h photoperiod provided by cool white fluorescent light having intensity of 3500 lux inside an orbital incubator shaker (INNOVA 44R) with shaking at 130 rpm. The full grown diatom cells were collected and homogenized to obtain aqueous cell extract. Aqueous $AgNO_3$ solution was added to fresh diatom extract to get a final concentration of 2×10^{-3} M $AgNO_3$. The mixture was exposed to light to initiate the reaction (room temperature about 35–40 °C). Control experiments of the diatom extract without $AgNO_3$ and another with $AgNO_3$ solution without diatom extract were also run simultaneously. Another set of experiments were kept in dark for comparison. To obtain acetone extract the cell pellet was collected and homogenized in the presence of acetone instead of water. TLC was done on silica gel plates with Hexane:Ethylacetate (60:40) as eluent. The pure yellow compound on TLC plate was removed from plate and extracted in acetone. The UV–Vis spectrum of this compound was obtained. After complete evaporation of acetone the compound was dissolved in water. This solution containing pure fucoxanthin extracted from *Amphora*-46 was further used for SNP synthesis.

2.2. Characterization and antimicrobial activity of SNPs

2.2.1. UV–Visible spectral analysis

The initial characterization of silver ion reduction to SNP was monitored by a Cecil double beam UV–Visible spectrophotometer with a resolution of 1 nm between wavelength range of 190 and 1100 nm at different reaction times i.e. 5, 10, 15, and 30 min.

2.2.2. Transmission Electron Microscopy (TEM)

The size and morphology of the biosynthesized SNPs were examined using TEM (FEI TECHNAI 20 G2 model) operated at an accelerating voltage of 200 kV equipped with SAED and EDAX. In order to obtain TEM micrographs, a diluted sample was sonicated for 15 min and a drop of the sonicated sample was placed on the carbon-coated copper grid and dried under infrared lamp prior to examination.

2.2.3. X-ray diffraction study (XRD)

The XRD analysis was carried out using X-ray powder diffractometer (Philips X'pert Pro, Panalytical) having Cu Kα (λ = 1.54 Å) radiation and a programmable divergent slit with scanning range of 2θ = 20–80° operating at a voltage of 40 kV and a current of 30 mA. Sample prepared on silica plate was obtained by applying many layers of sample on the plate with intermittent drying.

2.2.4. Antimicrobial activity test

The antimicrobial activity of biosynthesized SNPs was assessed by the agar well diffusion method against gram negative bacteria *Escherichia coli* and gram positive bacteria *Bacillus stearothermophilus* and *Streptococcus mutans* grown in nutrient agar (NA) medium at 35 °C for 24 h. Active bacterial culture was prepared one day prior to the experiment. For bacterial suspension, a single colony was picked from the mother plate and transferred to nutrient broth medium (NB) incubated at 35 °C for 24 h. On the day of experiment both the cultures were diluted with sterile NB medium to get equal bacterial population in both sets. The bacterial suspension was inoculated in NA plates using sterile cotton swab. Two wells of 2 mm per plate were made. Using sterile pipette, wells were filled with 25 µL of biosynthesized SNPs and fresh cell free diatom extract as control, incubated at 35 °C for 24 h. After incubation, zone of inhibition was measured around each poured well. Diameters of the clear zone known as bacterial inhibition zones were measured and represented as antimicrobial activity of the test sample.

3. Results and discussion

In the present study, a green synthesis route for SNP synthesis was demonstrated using the extract of the diatom *Amphora*-46. Aqueous extract of the diatom was light yellow in color as mostly yellow colored pigment got extracted but not the chlorophyll. In comparison the acetone extract was green in color which later was demonstrated to extract most of the pigments including fucoxanthin and chlorophyll. The aqueous extract was used for silver nanoparticle biosynthesis from silver nitrate solution. The gradual change in color of reaction mixture from light yellow to brown within few seconds indicated the initiation of SNP formation. It was interesting to note that the SNP formation occurred only in the presence of light. An increase in intensity of the color was observed which ultimately became red–brown within 30 min. No change in color was observed in case of control flasks as well as in the mixture of diatom extract and silver nitrate kept in dark. This indicated that the formation of SNPs in the present case was light dependent. Visual observation was further confirmed by UV–Vis spectroscopy. An intense peak at 413 nm was observed due to surface plasmon resonance (SPR) and attributed to the excitation of free electrons in the silver nanoparticles (Fig. 2). The intensity of SPR band increased exponentially with time without any shift in the peak position up to 30 min. Thereafter it attained a constant absorption value indicating the completion of the reaction. Increase in peak intensity at 413 nm with respect to time of reaction, indicated increase in number of SNPs in the mixture. Appearance of peak at 413 nm is indicative of relatively small monodispersed and spherical SNPs. Further no precipitation or agglomeration was observed during the reaction and up to three months after the experiment suggesting that the biosynthesized SNPs are stable. No such SPR peak was observed in the mixture kept in dark indicating that the SNP synthesis is completely light dependent.

The size of SNPs was confirmed by TEM analysis (Fig. 3a and b) which illustrates that, SNPs were spherical in shape with size ranging from 5 to 70 nm (average particle size of 20–25 nm) (Fig. 3c). Synthesized SNPs appeared polydispersed and well scattered. The compositional analysis using EDAX showed strong characteristic signal of silver (Fig. 3d). All the peaks except Cu may be attributed to proteins and other biomolecules associated with the biomass extract. SAED showed diffraction pattern directed toward (1 1 1), (2 0 0), (2 2 0), and (3 1 1) planes which correspond to the face-centered cubic (fcc) structure of elemental silver thus confirming the polycrystalline nature of SNPs (Fig. 3d (inset)). The XRD spectra showed four intense diffraction peaks at 2θ values of 38.48°, 44°, 64.74°, and 77.4° which correspond to (1 1 1), (2 0 0), (2 2 0), and (3 1 1) plane of (fcc) crystalline SNP and in agreement with the standard JCPDS card no 89-3722 (Fig. 4). The intensity of peak at (1 1 1) plane was more than the other peaks, suggesting that this plane was the predominant one. Both XRD and SAED results clearly indicated that the SNPs were polycrystalline in nature. This clearly showed that aqueous extract of diatom was very much capable of reducing silver ions in the medium and converting them into well dispersed silver nanoparticle.

The aqueous extract is a mixture of many biomolecules present inside the cell. Some biomolecules may be capable of reducing metal ions to metal nanoparticles. Algae and diatoms are known to contain different molecules rich in the –OH group [18] which get oxidized, simultaneously causing the

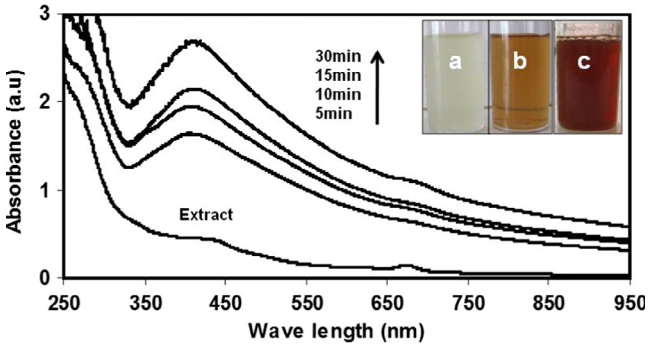

Figure 2 UV–Vis spectra of sunlight exposed silver nanoparticle synthesis using diatom *Amphora*-46 extract. Inset image (a) Diatom extract (control), (b) & (c) after 5 min & 30 min of silver exposure.

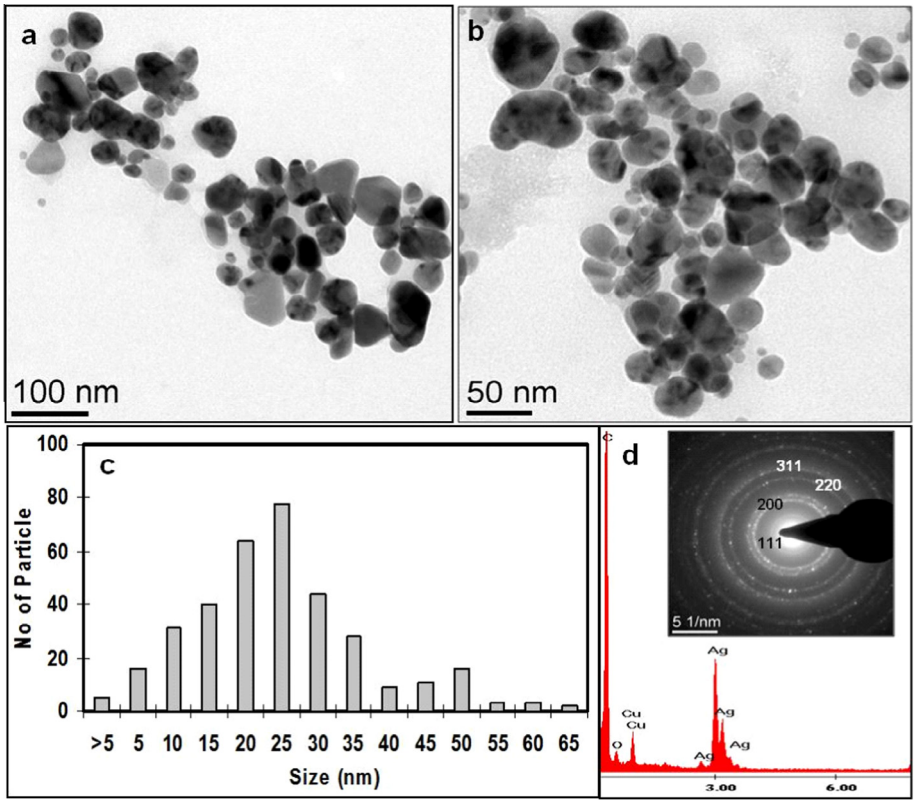

Figure 3 (a, b) TEM image, (c) Particle size distribution, (d) EDAX spectrum and SAED pattern of biosynthesized SNPs.

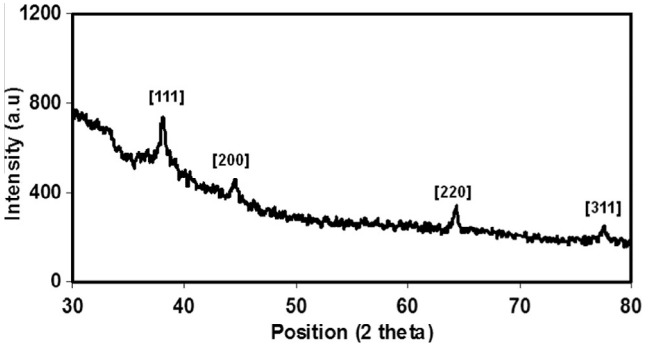

Figure 4 XRD pattern of biosynthesized SNPs in *Amphora*-46.

reduction of metal ions. In the present study reduction of the Ag^+ ion to Ag^0 occurred only in the presence of light. This indicates the involvement of some light sensitive bio-molecules of *Amphora*-46 in bioreduction of the metal. Diatoms are known to be rich in photosynthetic pigments which are sensitive to light [27]. Besides the pigments, silaffin polypeptides (protein responsible for silica nanoparticle formation) have also been suggested to play a role in reduction and stabilization of SNP biosynthesis [18]. To understand the role of responsible molecule attempts were made to purify these molecules and study its role in pure form.

In present study the aqueous cell extract of *Amphora*-46 when subjected to TLC showed the presence of only one yellow colored band. The UV spectra also revealed a small but visible absorbance band around 410 nm thus suggesting the presence of some pigments in the aqueous extract. Further TLC analysis of the acetone extract of cells showed many bands visible with naked eye as well as under UV light of wavelength 366 nm (Fig. 5a lane-Ac and Fig. 5b). Fig. 5c shows UV–Vis spectra of acetone extract in the wavelength range of 200–700 nm. The bright yellow band with $R_f - 0.239$ present in both aqueous (Aq) and acetone (Ac) extracts was purified with preparatory TLC and re-extracted in acetone. Fig. 5d shows the absorbance pattern of the UV–Vis spectra of the pure yellow fraction with peaks at 416, 442, and 466 nm which matched with that of fucoxanthin, a yellow colored photosynthetic pigment found in brown algae and diatoms [27,28]. This pigment is reported to be light sensitive [28] and in the present study it probably acts as a reducing agent in the presence of light. To confirm this all the bands seen in acetone extract (Fig. 5a, lane-Ac) were purified separately from the TLC plate. These bands after reconstitution in water (aqueous medium) were used for SNP synthesis. The solution containing pure fucoxanthin (yellow band), extracted from *Amphora*-46 was further used for SNP synthesis. Formation of light brown color with broad absorbance band from 410 to 470 nm indicates SNPs of various sizes (Fig. 5e). This confirms that fucoxanthin was responsible for SNP formation in *Amphora*-46. Structure of fucoxanthin shows that it has unusual double allenic carbon at C7' and two hydroxyl groups may make it a reducing agent and therefore an efficient antioxidant (Fig. 1) [28]. But in the experiment with pure fucoxanthin, size control and stability were not possible due to the absence of stabilizing molecules like proteins or some other molecules that were present in the original cell extract. This is the first report regarding the direct involvement of a diatom pigment fucoxanthin in SNP formation.

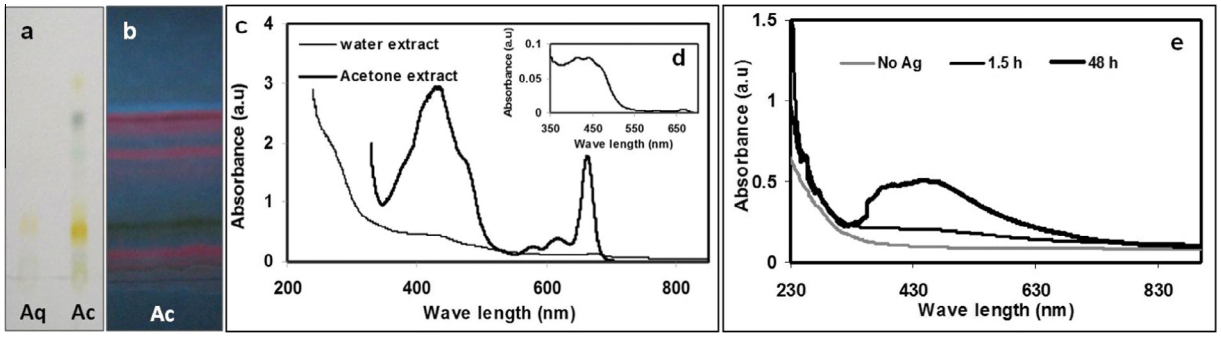

Figure 5 (a) TLC of aqueous (Aq) and acetone (Ac) extracts, (b) Visualization of bands of acetone extract under UV light (366 nm), (c) Characteristic UV–Vis spectra of Aq and Ac extracts; (d) TLC purified compound from yellow spot in Ac extract, (e) SNP synthesis using pure fucoxanthin extracted from diatom *Amphora*-46.

Figure 6 Antimicrobial activity of biosynthesized SNPs against pathogenic bacteria (a) *E. coli*, (b) *B. stearothermophilus* and (c) *S. mutans* [Left-only extract; right-biosynthesized SNPs (25 μL) for each figure].

Table 1 Antibacterial activity of SNPs (zone of inhibition).

Bacterial strain	Zone of inhibition (mm)	
	Biosynthesized SNPs	Diatom extract
E. coli	17	Nil
B. stearothermophilus	16	Nil
S. mutans	12	Nil

The antimicrobial activity of the biosynthesized SNPs was tested by the disk diffusion method. Fig. 6(a–c) clearly indicates that the biosynthesized SNPs using diatom extract exhibited good antibacterial effect due to the formation of significant zone of inhibition of 17 mm, 16 mm and 12 mm for *E. coli*, *B. stearothermophilus* and *S. mutans*, respectively. On the other hand a prolific growth of test bacteria was observed with no inhibition in case of only *Amphora*-46 cell extract (Table 1). Due to the pronounced bactericidal activity, the biosynthesized SNPs can act as an effective antimicrobial agent for various biomedical applications.

4. Conclusion

Amphora sp. is being reported for the first time for a light dependent biosynthesis of SNPs using its aqueous extract and $AgNO_3$. The synthesized SNPs were mostly polycrystalline spherical with an average particle size of 20–25 nm. The light sensitive photosynthetic pigment fucoxanthin was demonstrated to be the molecule responsible for the reduction of Ag^+ ion to Ag^0. Further the biologically synthesized metal nanoparticles showed significant bactericidal activity against gram positive and gram negative bacteria. This can find a lot of application in biomedical field. In view of the above remarkable properties, the present study for the first time, conclusively demonstrates the feasibility of green biosynthesis of SNPs using fresh cell extract of a ubiquitous diatom, *Amphora* sp. Owing to its microscopic size, ubiquitous nature and modest nutritional requirement the diatom *Amphora* may prove to be biotechnologically important in future for green technology.

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