

Mechanistic approach for fabrication of gold nanoparticles by *Nitzschia* diatom and their antibacterial activity

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Abstract The problem of chemically synthesized nanoproductions motivated scientific community to explore ecofriendly methods of nanosynthesis. Diatoms belong to a group of aquatic, unicellular, photosynthetic microalgae have been scarcely investigated as a source of reducing and capping agents for nanosynthesis of pesticides and antibiotics. The present study reports a novel ecofriendly method for the fabrication of bioactive gold nanoparticles using locally isolated *Nitzschia* diatoms. The diatom-fabricated gold nanoparticles show characteristic ruby red colored with sharp absorbance peak at 529 nm. Electron microscopy confirmed irregular shape of gold nanoparticles, with average size of 43 nm and zeta potential of -16.8 mV. The effects of gold nanoparticles on diatom viability were investigated using light and electron microscopy. The mechanistic approach to shed light on how diatoms reacted after exposure to gold metal salt revealed that exposure to gold chloride triggers elevated levels of catalase and peroxidase (12.76 and 14.43 unit/mg protein, respectively) to relieve reactive oxygen species (ROS) stress induced by gold salt exposure. Investigation studies on mechanisms

behind *Nitzschia*-mediated gold nanoparticles fabrication outlined the role of diatom proteins, polysaccharides in reduction, and stabilization of nanoparticles as confirmed by FT-IR analysis. Bioactivity of gold nanoparticles was accessed by coupling them with antibiotics (penicillin and streptomycin), which increased their antibacterial activity compared to individual nanoparticles and antibiotics (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*). Overall, the present novel phyco-nanotechnological approach is a promising tool to be used as sustainable strategy in green nanotechnology as well as to reduce use of antibiotics in microbial control.

Keywords Catalase · Green nanosynthesis · Peroxidase · Reactive oxygen species · Antibacterial activity

Introduction

Diatoms belong to a group of aquatic, unicellular, photosynthetic microalgae found in river biofilms and have unique structural features [1]. Cell wall of diatoms is made up of nanostructured amorphous polymerized silicic acid termed “frustule” generated by bio-mineralization of silica [2]. *Nitzschia* is dominant genus of diatom found in colder (fresh) water, as well as in marine environments, including tropical areas are also observed in water bodies with heavy organic pollution. Diatoms are found to be useful in several areas such as ecological monitoring, biofuel, and CO₂ sequestration [3].

Gold nanoparticles (AuNPs/GNP) possess peculiar properties such as small size, high surface-to-volume ratio, biocompatibility, and ability to attach different molecules on its surface (i.e., functionalization), biocidal potential, and low toxicity [4, 5]. Above properties made gold

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nanoparticles an ideal candidate in many areas of medicine, environment, and agriculture [6, 7] may be due to this reason; gold nanoparticles are among the six most traded nanomaterials in different consumer applications [8].

The use of hazardous chemicals upsurges the concern to exploit chemically synthesized nanoproducts in area of human health and environment. This problem motivated scientific community to explore ecofriendly methods of nanosynthesis [9]. Biological synthesis of gold and silver nanoparticles from bacteria, plant extracts, and algae had reported in the literature [9–11].

To the best of our knowledge, there is no report available on nanogold synthesis using diatoms of *Nitzschia* species [1, 12]. Trace elements, such as lead (Pb), copper (Cu), aluminium (Al), cadmium (Cd), boron (B), and selenium (Se), are required for growth of diatom culture, but can be toxic at higher concentration with direct influence on physiological, biochemical processes, and also on bioaccumulation [13].

There are many reports on biological synthesis and applications of gold nanoparticles [14–16], but rarely tried to elucidate the mechanism of synthesis. The present investigation is a true effort to shed light on diatom-mediated nanosynthesis, how diatoms reacted after exposure to gold metal salt by monitoring the level of two important enzymes (i.e., catalase and peroxidase) in control and gold exposed diatoms, as both enzymes play a vital role in relieving metal-induced reactive oxygen species stress in plant and algae. Beside, the gold bioaccumulation by diatoms was also studied.

Furthermore, microbial pathogens adopted diverse strategies to overcome lethal effects of antibiotics such as mutations in target site, enzyme modification, etc. [6, 17]. The uses of antibiotics have their own several disadvantages, such as side effects and emergence of microbial resistance. This scenario demands more research on natural products useful in microbial control and combinational therapy using multiple compounds to enhance activity spectrum. Nanoparticles appeared to be potent antimicrobial agent due to their multiple mode of action against microorganisms and efficacy at low doses. These properties demand detailed studies on antimicrobial nano-formulations, including novel nano-sized platforms for clinical and biomedical applications.

In this framework, this research has the following objectives: (1) to analyze the potential of diatom culture of *Nitzschia* species useful for gold nanoparticles synthesis; (2) to investigate consequences of metal stress on diatom viability and changes in antioxidative enzymes profile; and (3) to test the potential of nanoantibiotics (diatom fabricated gold nanoparticles + antibiotics) to inhibit pathogenic bacteria. The study revealed that diatom extracellularly fabricated gold nanoparticles on outer cell

wall. The most interesting fact is that when bacterial species (*E. coli*, *P. aeruginosa*, and *S. aureus*) exposed to the mixture of gold nanoparticles with antibiotics, there was drastic increase in inhibition spectrum if compared to inhibition caused by gold nanoparticles and antibiotics alone. Both the above-discussed aspects (nanosynthesis and nanoantibiotics) added novelty and significance to the present study.

Materials and methods

Chemicals

Gold (III) chloride trihydrate was purchased from Sigma-Aldrich (St. Louis, USA). Chemicals required for the preparation of F/2 modified media, Luria–Bertani (LB) broth, and agar were obtained from HiMedia Ltd. (Mumbai, India). Antibiotics (penicillin and streptomycin) were procured from HiMedia Ltd. (Mumbai, India). Chemicals required for enzymatic assay were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL, Mumbai, India) and S D Fine Chem Ltd. (Mumbai, India).

Isolation and maintenance of diatom culture

Water samples were obtained from river and pond biofilm near Jalgoan, India (21° 00' 24.5" N, 75° 29' 45.5" E, and elevation 218 msl) and diluted with sterile F/2 modified media (S1) and allowed to grow for two weeks. The grown biomass was serially diluted and streaked on F/2 modified agar medium (S1). The isolated colonies were purified and maintained in liquid medium to use for further study. The growth condition provided was as follows: temperature 21 °C and 16/8 h L/D cycle. Microscopic observation confirmed that isolated diatom belongs to *Nitzschia* species. Diatoms were allowed to grow for 2 weeks before initiating nanosynthesis experiment.

Diatom-mediated gold nanoparticles synthesis

Early stage diving culture of diatoms was centrifuged at 1042g pellet which was redispersed in the 50 ml distilled water followed by the incubation of 24 h. After incubation, gold chloride salt was added in such an amount that its concentration in medium will be 0.1 mM followed by further incubation for 24 h. The controls included separate bottles of gold solution and diatoms.

Extraction and purification of GNPs from diatoms

The extraction was done by slight modified method of Kröger et al. [18]. The gold chloride challenged cells were

harvested by centrifugation and suspended in 2 ml buffer solution containing EDTA (0.1 M) and SDS (0.2%) pH 8.0 followed by incubation in water bath at 80 °C for 10 min, and then, the whole sample was sonicated for 10 min. The mixture was centrifuged at 1000 rpm for 10 min and colored supernatant was collected. The procedure was repeated thrice. Then, 1 ml of hexane was added and mixed thoroughly using vortex mixture and 2 ml of distilled water was added and further incubated for 10 min at 30 °C. After incubation, hexane layer was removed using pasture pipette. Then, colored supernatants containing GNP were collected and lyophilized for further use of experiments.

Characterization of diatom synthesized gold nanoparticles

Visual observation of control and test diatoms was done for transformation of yellow color gold salt solution to ruby red color as it is the preliminary indication of nanosynthesis. The test bottle showing ruby red color was used for further nanocharacterization [7, 10, 11].

UV–Visible spectroscopy

UV–Vis spectroscopy is a common tool for preliminary confirmation of gold nanoparticles synthesis. For UV–Vis spectrum measurement, diatom cells containing gold nanoparticles were sonicated for 10 min. To remove cell debris, the solution was centrifuged at 2000g for 10 min. Ruby red color supernatant containing gold nanoparticles was studied for measurement of absorption maxima between 300 and 700 nm at resolution of 1 nm (Shimadzu 1601) [9].

Transmission electron microscopy (TEM)

Morphology (shape and size) of *Nitzschia*—synthesized gold nanoparticles—was analyzed on TEM (Philips Morgagni (268D) operated at 100 kV. For TEM analysis, samples were prepared by inoculating a drop of aqueous solution of gold nanoparticles over the carbon coated copper grids [7]. The presence of elemental gold in sample was analyzed using energy-dispersive spectroscopy (EDX) (Bruker, Germany).

Fourier transform infrared (FT-IR) spectroscopy, particle size distribution (PSD) analysis, and zeta potential

FTIR analysis (IR Prestige 21) was performed to understand the involvement of different diatom metabolites in synthesis and stabilization of gold nanoparticles. PSD revealed percentage of different size nanoparticles in solution and depicted result as average size of nanoparticles, while zeta

potential gives information about stability of nanoparticles in solution (Zeta sizer nano S90).

Extraction of enzymes and enzyme assays

With the purpose of throwing light on mechanism adopted by diatoms after exposure to gold metal salt solution, diatom culture was incubated with gold chloride salt (0.001 mM) for different time periods, i.e., 5, 10, 15, and 30 days (test solution), while in control diatoms were grown in F/2 medium without gold salt. Both test and control diatoms were collected by centrifugation at 1042g for 10 min at 4 °C, and 10 mg of biomass from pellet was resuspended in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) followed by sonication (VCX130, Sonics Material Inc, USA) for 5 min and centrifugation at 2000g for 10 min at 4 °C. The centrifugation process was repeated thrice and collected supernatant was used as enzyme extract for enzyme assays.

Catalase activity was estimated by tracking the reduction of H₂O₂ at wavelength of 240 nm at 20 °C [19]. The reaction mixture contained enzyme extract along with 200 μM H₂O₂ in 50 mM of pH 7.5 potassium phosphate buffer. One unit of enzymatic activity was defined as the amount of enzymes that catalyses the conversion of 1 μmole of substrate per min.

Peroxidase activity was evaluated by analyzing the increase in absorbance at 436 nm of guaiacol (20 mM) substrate in a reaction mixture composed of 0.1 ml enzyme extract, 0.05 ml of guaiacol (20 mM) in 3 ml 50 mM of pH 7.0 phosphate buffer, and 0.03 ml of H₂O₂ (12.3 mM). Increase in absorbance was measured at 25 °C. One unit of enzymatic activity was calculated using extension coefficient of guaiacol dehydrogenation product at 436ε = 6.69 μm/cm and time required for increase in absorbance [20]. Each experiment was carried out in triplicates and results were analyzed with student *t* test for statistical significance (*P* < 0.05).

Antibacterial assay to evaluate combined effect of gold nanoparticles and antibiotics

For assessment of antibacterial potential of diatom-fabricated gold nanoparticles and its combination (gold nanoparticles + antibiotics), microbial cultures of Gram-negative (*Pseudomonas aeruginosa* NCIM 5029 and *Escherichia coli* NCIM 2931) and Gram-positive (*Staphylococcus aureus* NCIM 5021) bacteria were purchased from National Collection of

Industrial Microorganisms (NCIM) (Pune, India) were maintained on Luria–Bertani (LB) agar slants. Cultures were grown in LB broth at 37 °C at 120 rpm for 24 h before using for antibacterial assay [21].

Gold chloride solution (100 µg/ml), gold nanoparticles (100 µg/ml), and standard antibiotics (penicillin, streptomycin) of a known concentration (100 µg/ml) were taken as controls, whereas in test, the gold nanoparticles were impregnated with individual antibiotics at a ratio of 1:10 %v/v and were allowed to incubate for 1 h. All test and control solutions were dipped in sterilized Whatman filter paper (Grade 1) discs (4 mm) for 3 h and dried in oven at 55 °C. Both control and gold nanoparticle-treated antibiotics discs were put on LB agar Petri dishes which spread with 0.1 ml 24-h-old cultures of Gram-negative and Gram-positive organisms. Assays were performed in triplicate to obtain mean value. The diameter of the zone of inhibition caused by control and test compounds was measured, and an increase in the fold area was calculated [22, 23].

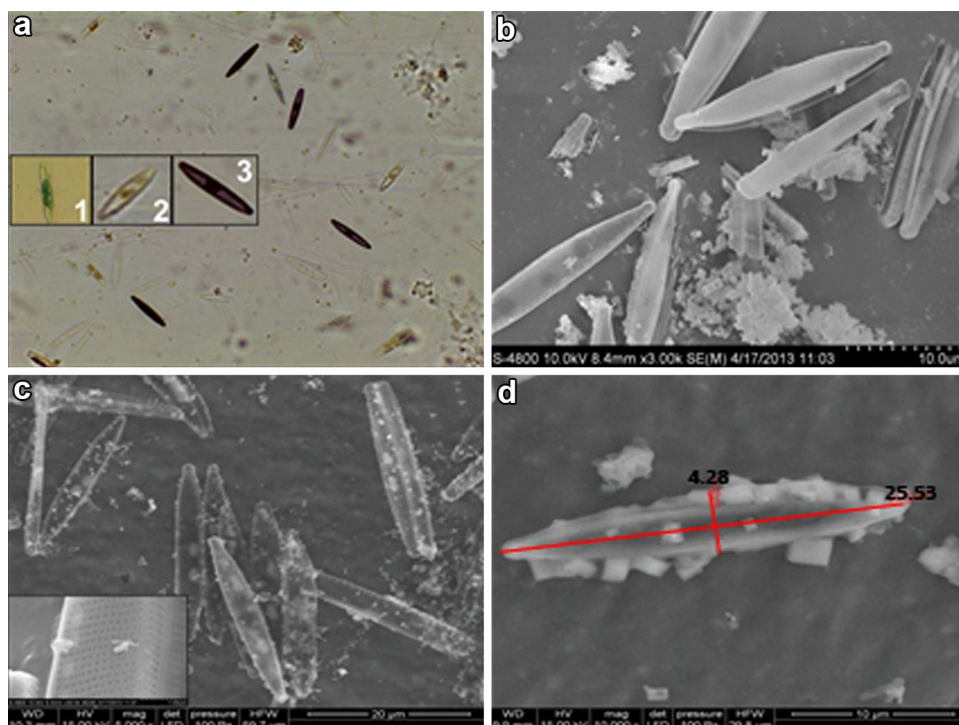
Increase in fold area was calculated by formula = $[(RB^2 - RD^2)]/[(RA^2 - RD^2) + (RC^2 - RD^2)]$, where RA, RB, RC, and RD are zone inhibition radius in mm for antibiotic alone, antibiotic + nanoparticles, nanoparticles alone, and blank discs, respectively.

Results

Microscopic analysis of *Nitzschia* in nano-environment (diatoms–gold interactions)

Nitzschia culture was examined under light and electron microscope. Shape of diatom was found to be bilaterally symmetrical with narrow ends at both sides (Fig. 1).

Fig. 1 **a** Light microscope images of diatoms of *Nitzschia* sp. (inset: 1 diatoms grown in F/2 medium (Motic BA210 Trino, Germany), 2 diatoms exposed to 0.05 mM gold chloride solution and 3 diatoms exposed to 1 mM gold chloride solution). **b** SEM micrograph of diatoms: without gold chloride exposure **c** diatoms exposure to 0.05 mM gold chloride solution showing adherence of gold nanoparticles on body surface and **d** Individual diatom cell surrounded with gold nanoparticles



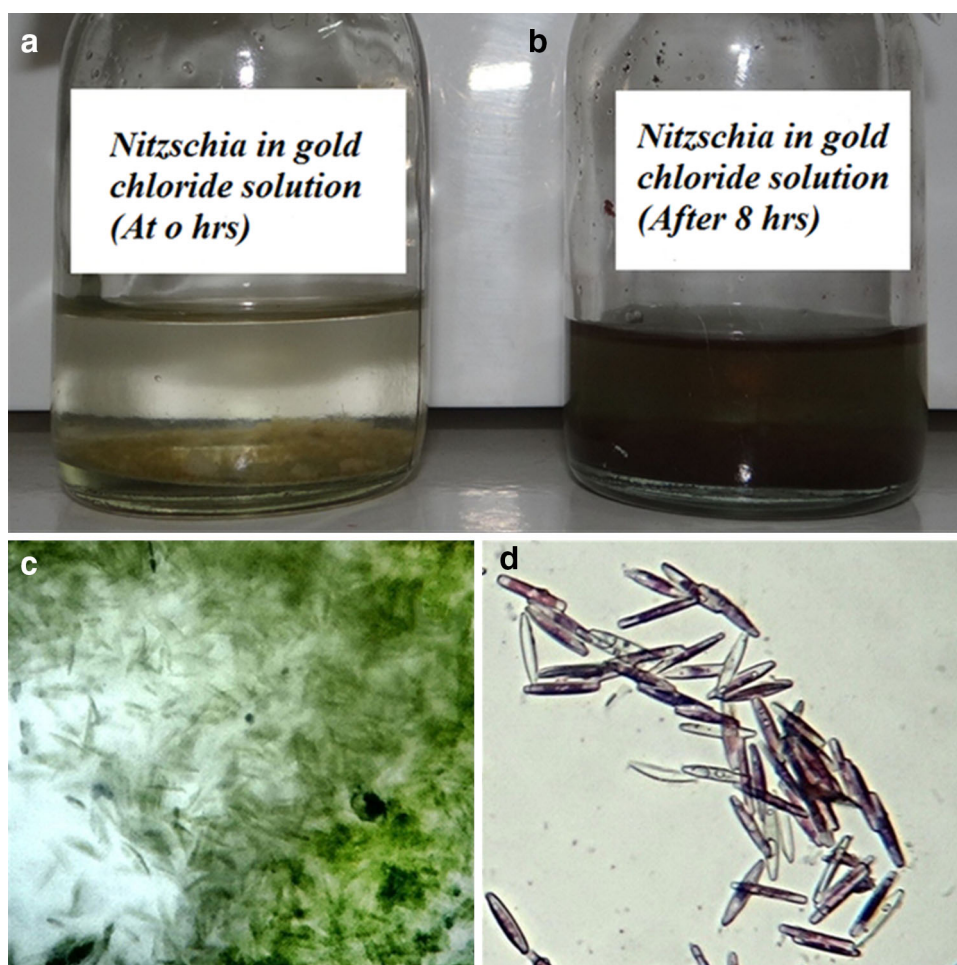
Normally, grown diatoms (without exposure to gold nanoparticles) were showed greenish appearance due to the presence of chlorophyll, while test diatoms (diatoms expose to gold chloride solution and synthesize gold nanoparticles) appeared to be ruby red (Fig. 1a). This color change indicated gold nanoparticles biosynthesis by diatoms. Interestingly, the intensity of ruby red colored diatom cells increased with increase in concentration of gold salt (Figs. 1a, 3). Scanning electron microscopy showed diatom size ranged from 18 to 25 µm in length and 2–4 µm in width (Fig. 1b–d). Adherence of nanoparticles on outer surface of diatom cell was confirmed by electron microscopy (Fig. 1b–d).

Diatom-mediated gold nanoparticle synthesis

Visual observation

Approximately after 8 h of challenging the diatom cell mass with gold chloride solution, the yellow color gold chloride solution transformed into ruby red color (Fig. 2b, d). Formation of ruby red color is primary indication of gold nanoparticles synthesis. However, in the control set, i.e., gold chloride solution without diatoms and diatoms without gold solution did not show ruby red coloration after incubation at similar conditions (Figs. 2a, c, 3c). It was observed that although diatoms synthesized gold nanoparticles, but the ruby red color of the solution was observed only after vigorous shaking and when we put down the bottle containing diatom cell with gold

Fig. 2 **a** Glass bottle showing *Nitzschia* cells at 00 h after addition of gold salt solution. **b** Ruby red color formation after 08 h incubation of gold salt solution with diatoms cells. **c** Light microscopic observation of diatoms grown in absence of gold salt, and **d** after synthesizing gold nanoparticles



nanoparticles for stabilizing the content, the supernatant remains colorless, and ruby red precipitation at the bottom was observed (Fig. 3a); this gives a hint that the gold nanoparticles synthesized by diatom remain adhered on proximate surrounding of the diatom cells and are not released in solution. This preliminary visual result was further confirmed by light microscopy observations showing deep ruby red diatom cells exposed to gold chloride as compared to normally grown diatoms showing the absence of ruby red color (Fig. 2c, d). The colloidal solution of ruby red color gold nanoparticles along with controls (gold chloride salt solution and diatom cells) is shown in Fig. 3.

UV-Visible spectroscopy

It was observed that gold nanoparticles exhibit characteristic absorption maxima peak centered at 529 nm in UV-Vis spectroscopy (Fig. 4a). While this characteristic peak was not observed in 0.1 mM gold salt solution (Data not shown). This typical absorption maxima is arising due to property of metal nanoparticles termed 'surface plasmon resonance'. The gold nanoparticle solution was further

transferred in screw cap glass tube and kept for 30 days to check stability; after this period, there was no nanoparticles aggregation and the color intensity remains the same. Stability studies were performed by UV-Vis spectroscopy (S2). The stable gold nanoparticle solution was lyophilized and used for further analysis.

FT-IR of gold nanoparticles

FT-IR spectrum of gold nanoparticles suggested involvement of different metabolites produced by *Nitzschia* sp. in reduction and stabilization of nanoparticles. Peaks at 3254 cm^{-1} for N-H stretch vibration in peptide linkages, while the peak at 2956 cm^{-1} for C-H stretch vibration in aliphatic and alicyclic rings and peak at 1367 cm^{-1} for C-O-C ether linkages present in polysaccharides. Other peaks at $1447, 814\text{ cm}^{-1}$ indicated presence of aromatic bending vibrations (Fig. 4b). Besides the peak at $1259, 1128\text{ cm}^{-1}$ for Si-O-Si linkage present in *Nitzschia* sp. fabricated nanoparticles, indicating the formation of gold-silica nanoconjugates. Earlier reports the proposed sugar and proteins as important biomolecules in diatom-mediated

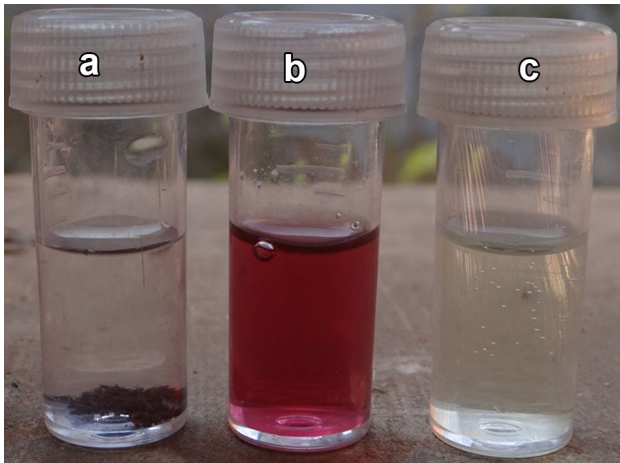
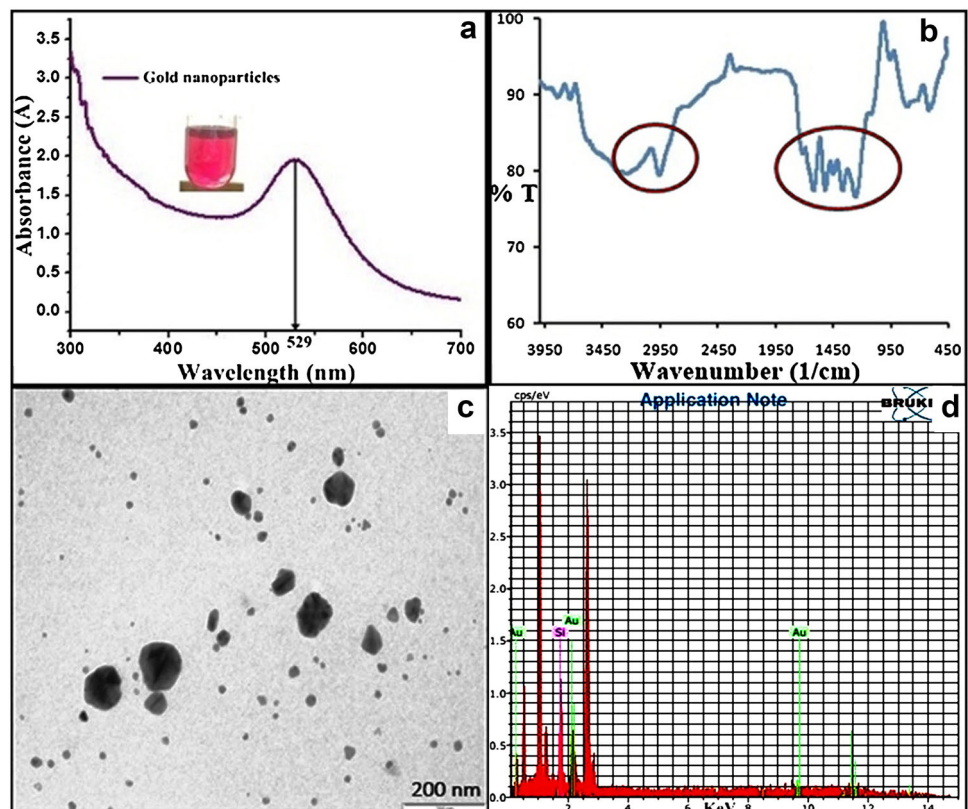


Fig. 3 **a** Ruby red color diatoms cell mass due to gold nanosynthesis **b** purified gold nanoparticles solution from diatoms, and **c** gold chloride solution (control)

nanosynthesis [12, 24]. Flavonoids, polyphenol, carboxylic acid, aldehyde, ketone, and other biomolecules have carbonyl groups playing an important role in reducing bulk gold salt into nanogold, while proteins were showed to be attached over the surface of nanoparticles and maintain them in stable colloidal form. There are several mechanisms proposed behind diatom-mediated nanosynthesis such as oxidation of OH, SH groups by polysaccharides, and fucoxanthins present abundantly in cell wall [1, 25].

Fig. 4 **a** UV–Vis absorption spectra of *Nitzschia* sp. fabricated gold nanoparticles (Inset: test tube showing ruby red color gold nanoparticles solution). **b** FT-IR spectrum of gold nanoparticles **c** representative TEM image of *Nitzschia* sp. fabricated gold nanoparticles and **d** EDS spectrum gold nanoparticles



Biofunctionalization refers to the binding of organic moieties on nanosurface leading to stabilization against aggregation forces.

Electron microscopy

In TEM, irregular shape nanoparticles (grain shape, triangular, and spherical) were visualized with varying size around range from 15 to 90 nm. The nanoparticles were in stable, colloidal state without any sign of aggregation (Fig. 4c).

Energy-dispersive X-ray spectroscopy (EDS), particle size distribution (PSD), and zeta potential (ZP) of gold nanoparticles

In the EDS spectrum of gold nanoparticles, characteristic peaks indicating the presence of gold in nanoparticles solution were found (Fig. 4d). Interestingly, it was observed that along with peak of gold, there is peak of silica. From the EDS results, we could hypothesize synthesis of gold–silica nanoconjugate. Schrofel et al. (2011) [12] also pointed out similar observation of nanocomposite formation with diatom species *N. atomus* and *D. gallica*.

In particle size distribution analysis, the average size of gold nanoparticles fabricated by *Nitzschia* sp. was found to be around 43 nm (Fig. 5). ZP value of gold nanoparticles

before extraction was found to be -4.92 and -16.8 mV after extraction (S2).

Enzyme-mediated mechanism in gold nanoparticle synthesis by *Nitzschia* sp

Diatoms are also known to have a range of antioxidative enzymes such as catalase, ascorbate peroxidase, and glutathione peroxidase and some nonenzymatic oxidants such as beta-carotene and α -tocopherol, which can breakdown H_2O_2 [26]. It was previously identified that metal elements at elevated level affect variety of biochemical changes in diatoms and plants [27, 28]. Vital consequences of metal exposure are the enhanced synthesis of reactive oxygen species (ROS), which could damage cell membranes, nucleic acids, and chloroplast [29]. The higher production of ROS may be lead to further induction of the antioxidative activity, which is majorly composed of enzymatic antioxidants such as catalase, peroxidase, and superoxide dismutase [30] and several nonenzymatic scavengers such as glutathione, carotenoids, and ascorbates [31]. During study on diatom-mediated synthesis of gold nanoparticles, diatoms were exposed to gold salt at different time intervals and it was found that 15-day-old gold salt exposed culture showed high synthesis of gold nanoparticles as evidenced from high absorbance at 529 nm (Fig. 6a). Similarly, when we analyzed peroxidase and catalase level in diatoms as a function of time with gold salt containing medium and without gold salt medium (diatoms grown in F/2 medium only), it revealed that cell mass of 15-day-old gold salt exposed culture had high catalase and peroxidase level (12.76 and 14.43 unit/mg), while as compared to 15-day culture, low level of these enzymes was found in 5, 10, and 30 day gold salt exposed diatoms (Fig. 6a). Diatoms without gold salt exposure (control) exhibited low level of peroxidase and catalase during entire study period with complete absence of gold nanoparticles (Fig. 6b). The results were statistically significant ($P < 0.001$ for Fig. 6a and $P < 0.008$ for Fig. 6b) when analyzed for student

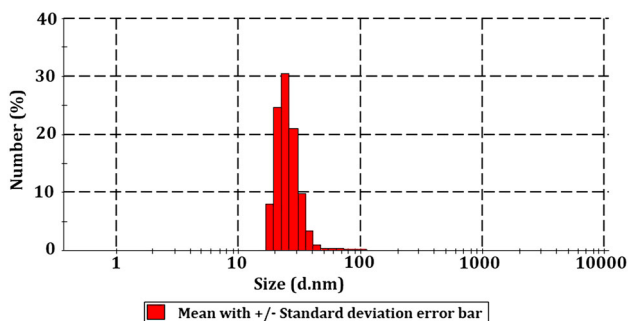


Fig. 5 Particle size distribution histogram *Nitzschia* sp. fabricated gold nanoparticles

t test. Hence, it is possible that the presence of gold salt may induce ROS stress which leads to activation of the antioxidant defense strategies, i.e., induction of catalases and peroxidases enzymes. Such conditions correlate with previous observation by Asada et al. [32] which reported that in stress condition, the free radical species of active oxygen increased, which enhances the activities of these enzymes involved in antioxidant mechanism, while in normal circumstances, the concentration of these antioxidative enzymes remains low [32].

Antibacterial assay

Emergence of microbial resistance to antibiotics has negative effects on human health such as decreased in effectiveness of drug molecules, side effects due to intake of higher doses, prolonged illness period, etc. Green synthesized nanoparticles from plant extract, microbial, and marine organisms were postulated as promising solution in drug resistance management [5, 6, 9]. In the present study, it was found that diatom-fabricated gold nanoparticles inhibited *E. coli* and *S. aureus* with zone of inhibition 6.10 and 6.03 mm, respectively, while it is ineffective against *P. aeruginosa*. Gold chloride solution did not have any inhibitory effect on tested bacterial species. Penicillin had zone of inhibition of 6.33, 13.2 mm on *E. coli* and *S. aureus* with no effect against *P. aeruginosa*, while streptomycin had 12.4, 13.16, and 14.45 mm zone of inhibition against *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively. The most interesting part of study is drastic increase in inhibitory spectrum of antibiotics (penicillin and streptomycin) when they were applied by mixing with gold nanoparticles (nanoantibiotics). Gold nanoparticle-penicillin complex showed 17.16, 9.20, and 16.16 mm zone corresponding to 3.81, 5.29, and 1.24 increase in fold area against *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively (Fig. 7 and S3). Similar synergistic increase in biocidal potential was obtained with gold nanoparticle-streptomycin complex with 1.43, 1.38, and 1.28 fold area increments (Fig. 7 and S3). The results found to be statistically significant with two-way ANOVA ($P < 0.0001$)

Nanoparticles reported to be act as mediators of drug release for enhancing the selectivity and efficiency of drugs. Moreover, because of their extremely small size and high surface-to-area ratio, their surfaces can be modified with different chemical moieties for targeted drug delivery [17].

Discussion

In the era of nanotechnology, green synthesis of nanoparticles having its own importance and many researchers tried different biological sources for the synthesis of metal

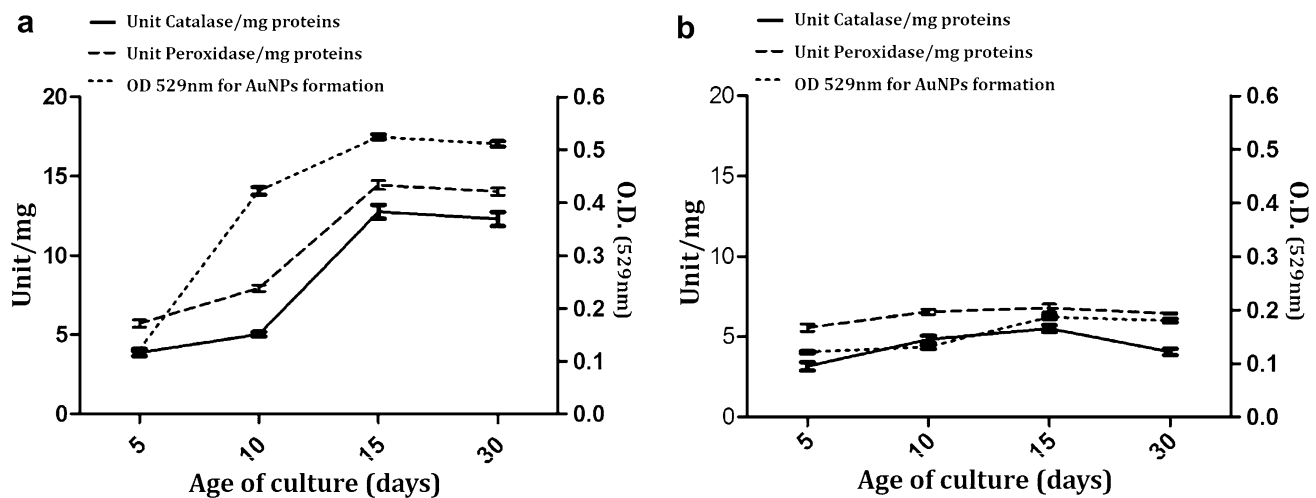


Fig. 6 Catalase and peroxidase enzymes and gold nanoparticles production as function of exposure time with **a** ($P < 0.0017$) and **b** without gold chloride ($P < 0.008$)

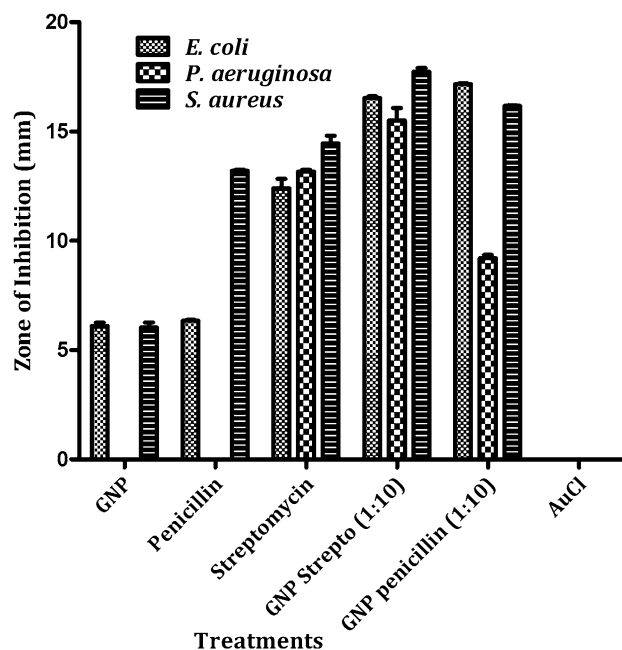


Fig. 7 Zone of inhibition of gold nanoparticles and antibiotics in the presence and absence of gold nanoparticles against Gram-positive and Gram-negative bacterial species

nanoparticles and its applications in diver fields [14–16]. Biological systems are a source of complex biomolecules which affect synthesis of metal nanoparticles. The complete understanding of mechanism of synthesis with biological sources will facilitate the control synthesis of metal nanoparticles and also helpful for its application in overlook areas. Hence, the present study explored novel diatom *Nitzschia* species-mediated gold nanoparticles synthesis, its mechanistic study, and biomedical application.

Microscopic analysis of *Nitzschia* in nano-environment (diatom–gold interactions)

Results obtained in the present investigation on gold nanoparticle synthesis from *Nitzschia* species can be corroborated with earlier reports of nanosynthesis using diatoms [1, 12]. According to Feurtet-Mazel et al. [2015], after exposure of lower concentration of gold chloride solution (20 $\mu\text{g/ml}$) to diatom species *Eolimna minima*, the gold nanoparticles were found in frustules, while after exposure to higher concentration (100 $\mu\text{g/ml}$), nanoparticles occupied intracellular space including vital internal organs of diatoms. Conversion (reduction) of macro (bulk) gold salt to nanoparticles of gold appeared to be a protection mechanism to detoxify toxic effects of gold salt as gold salt reported to cause defects in metabolism [33]. Similarly in our investigation, it was observed that diatoms remain viable after fabricating nanoparticles at 0.1 mM gold salt concentration. A report suggested that an exposure to gold ions decreased level of chlorophyll, protein and carbohydrate in green alga *Rhizoclonium fontinale*, pointing toxicity to algal cells due to gold exposure. On the other hand, there was an elevation in stress-related enzymes such as catalase, ascorbate peroxidase, and superoxide dismutase indicating enzyme-mediated action by algae to relieve metal-induced toxicity [34]. Liang et al. [2016] also observed perturbations of antioxidant enzyme activities (superoxide dismutase, catalase) and reactive oxygen species (ROS) after exposing earthworm *Eisenia fetida* to nanoscale zerovalent iron (nZVI) and concluded that ROS (antioxidant enzyme) is most profound strategy to tackle nZVI stress and could be utilized as biomarkers in ecotoxicity studies [35].

Mode of action of antibiotic-gold nanoparticles complex against bacteria

Potential of antibiotic-gold nanoparticles complex as promising biocidal agent is evaluated first time in the present report and a drastic synergistic increase in antibacterial potential of antibiotic-nanoparticle complex was observed as compare to nanoparticles and antibiotics alone. Antibiotics have different mode of action on bacteria such as inactivation of enzymes, cell wall inhibition, etc. On the other hand, nanoparticles known to inhibit pathogens by binding and inhibition of various enzymes, DNA, electrostatic attraction, formation of pits in the cell wall, change in membrane permeability, and cytoplasmic leakage [21]. Nanoparticles have protective effect toward antimicrobial drugs and also ability to overcome resistance in conjugation with antibiotics [36]. From above multiple biological targets of both candidates (antibiotics and gold nanoparticles), it is interesting to study in depth regarding actual mechanism taking place leading to inhibition of bacteria as such a type of study can lead to development of new and more potent drug agent capable of tackling new threats display by pathogenic microbes such as resistance to antibiotics.

Currently, very few reports are available on nanoparticles synthesis using marine organisms. They include *Navicula atomus* [12], *Diademsis gallica* and *Eolimna minima* [1]. In the present study, an increase in antibacterial effect of gold nanoparticles after antibiotics combination suggested synergistic mode of action. The possible mechanism for the increase activity of antibiotic with gold nanoparticles might be easy entry of antibiotic due to pit formation in the cell wall and increase in cell permeability facilitated by gold nanoparticles but it needs to be confirmed with further detailed study. Present approach of combining nanoparticles with drug molecules present itself as an approach to tackle resistance problem due to the fact that nanoparticles and antibiotics have different and diverse mode of action against pathogenic bacteria. However toxicity studies merits further research.

Conclusion

Diatoms mediated nanosynthesis is advantageous over chemico-physical methods due to simple nanosynthesis protocol, easy mass scale production of diatoms, no use of toxic chemicals and biocompatibility. In present study, potential of natural resources (diatom) in nanobiotechnology and microbial control is put forward for the first time. Present study fulfills ecofriendly nanosynthesis protocol which is major demand and current trend in nanotechnology. It was found that diatoms synthesize and accumulates

gold nanoparticles while maintaining cellular viability. This is of particular interest in ecological point of view as they can behave as storage hub of nanoparticles and can enter in food chain. Elevated level of catalase and peroxidase found to play role in metal-induced ROS stress. In the other aspect of study, it was found that diatoms fabricated gold nanoparticle-antibiotics complex showed promising inhibitory action against pathogenic bacteria. Study on chemical aspects behind diatoms mediated nanoparticles synthesis and mode of action of nanoantibiotics complex on pathogens will be an interesting research path with future applications in area of nanosynthesis and its applications in diverse areas of science, engineering, medicine to name a few.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

- Feurtet-Mazel A, Mornet S, Charron L, Mesmer-Dudons N, Maury-Brachet R, Baudrimont M (2015) Biosynthesis of gold nanoparticles by the living freshwater diatom *Eolimna minima*, a species developed in river biofilms. *Environ Sci Pollut Res* 23:4334–4339
- Van den Hoek C, Mann DG, Jahns HM (1995) *Algae. An introduction to phycology*. Cambridge University Press, Cambridge, p 623
- Jena J, Pradhan N, Bishnu BP, Panda PK, Mishra BK (2015) Pigment mediated biogenic synthesis of silver nanoparticles using diatom *Amphora* sp. and its antimicrobial activity. *J Saudi Chem Soc* 19:661–666
- Zheng M, Huang X (2007) Biofunctionalization of gold nanoparticles. *Nanotechnol Life Sci*. doi:10.1002/9783527610419.nls0004
- Singh P, Singh H, Kim YZ, Mathiyalagan R, Wang C, Yang DC (2016) Extracellular synthesis of silver and gold nanoparticles by *Sporosarcina koreensis* DC4 and their biological applications. *Enzyme Microb Technol* 86:75–83
- Salata OV (2004) Applications of nanoparticles in biology and medicine. *J Nanobiotechnol* 2:1–6
- Patil CD, Borase HP, Suryawanshi RK, Patil SV (2016) Trypsin inactivation by latex fabricated gold nanoparticles: A new strategy towards insect control. *Enzyme Microb Technol* 92:18–25
- Corti CW, Holliday RJ, Thompson DT (2007) Progress towards the commercial application of gold catalysts. *Top Catal* 44:331–343
- Borase HP, Salunke BK, Salunke RB, Patil CD, Hallsworth JE, Kim BS, Patil SV (2014) Plant extract: A promising biomatrix for ecofriendly, controlled synthesis of silver nanoparticles. *Appl Biochem Biotechnol* 173:1–29
- Luangpipat T, Beattie IR, Chisti Y, Haverkamp RG (2011) Gold nanoparticles produced in a microalga. *J Nanopart Res* 13:6439–6445

11. Thakkar KN, Snehit S, Mhatre Parikh RY (2010) Biological synthesis of metallic nanoparticles. *Nanomed NBM* 6:257–262
12. Schröfel A, Kratošová G, Bohunická M, Dobročka E, Vávra I (2011) Biosynthesis of gold nanoparticles using diatoms—silica gold and EPS-gold bionanocomposite formation. *J Nanopart Res* 13:3207–3216
13. Rietzler AC, Fonseca AL, Lopes GP (2001) Heavy metals in tributaries of Pampulha reservoir. *Minas Gerais Brazil J Biol* 61:363–370
14. Pani A, Yun SI (2016) Biosynthesis of gold nanoparticles by biotechnologically important active and inactive form of the Ascomycetes *Pichia membranifaciens* and *Debaryomyces hansenii*. *J Food Chem Nanotechnol* 2:92–96
15. Ramachandran R, Krishnaraj C, Sivakumar AS, Prasannakumar P, Kumar VKA, Shim KS, Song CG, Yun SI (2017) Anticancer activity of biologically synthesized silver and gold nanoparticles on mouse myoblast cancer cells and their toxicity against embryonic zebrafish. *Mater Sci Eng C Mater Biol Appl* 73:674–683
16. Mishra A, Tripathy SK, Yun SI (2011) Biosynthesis of gold and silver nanoparticles from *Candida guilliermondii* and their antimicrobial effect against pathogenic bacteria. *J Nanosci Nanotechnol* 11:243–248
17. Roshmi T, Soumya KR, Jyothis M, Radhakrishnan EK (2015) Effect of biofabricated gold nanoparticle-based antibiotic conjugates on minimum inhibitory concentration of bacterial isolates of clinical origin. *Gold Bull* 48:63–71
18. Kröger N, Bergsdorf C, Sumper M (1994) A new calcium binding glycoprotein family constitutes a major diatom cell wall component. *EMBO J* 13:4676–4683
19. Aebi A (1984) Catalase in vitro. *Methods Enzymol* 105:121–126
20. Putter J (1974) In: Bergmeyer HU (ed) *Methods of enzymatic analysis*, Vol 2, Verlag Chemie-Academic Press, New York, pp 685–690
21. Patil SV, Borase HP, Patil CD, Salunke BK (2012) Biosynthesis of silver nanoparticles using latex from Few Euphorbian plants and their antimicrobial potential. *Appl Biochem Biotechnol* 107:776–790
22. Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK (2009) Fabrication of silver nanoparticles by *Phoma glomerata* and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Lett Appl Microbiol* 48:173–179
23. Borase HP, Patil CD, Salunkhe RB, Suryawanshi RK, Salunke BK, Patil SV (2014) Catalytic and synergistic antibacterial potential of green synthesized silver nanoparticles: Their ecotoxicological evaluation on *Poecillia reticulata*. *Biotechnol Appl Biochem* 61:385–394
24. Subramaniam S, Subashchandrabose SR, Thavamani P, Megharaj M, Chen Z, Naidu R (2015) Chlorococcum sp. MM11—a novel phyco-nanofactory for the synthesis of iron nanoparticles. *J Appl Phycol* 27:1861–1869
25. Brayner R, Barberousse H, Hemadi M, Djedjat C, Yéprémian C, Coradin T, Livage J, Fiévet F (2007) Cyanobacteria as bioreactors for the synthesis of Au, Ag, Pd and Pt nanoparticles via an enzyme-mediated route. *J Nanosci Nanotechnol* 7:2696–2708
26. Hernando M, Schloss LR, Malanga G, Almandoz GO, Ferreyra GA, Aguiar MB, Puntarulo S (2015) Effects of salinity changes on coastal antarctic phytoplankton physiology and assemblage composition. *J Exp Mar Biol Ecol* 466:110–119
27. Manimaran K, Karthikeyan P, Ashokkumar S, Ashok Prabu V, Sampathkumar P (2012) Effect of copper on growth and enzyme activities of marine diatom, *Odontella mobiliensis*. *Bull Environ Contam Toxicol* 88:30–37
28. Siedlecka A, Tukendorf A, Skórzynska-Polit E, Maksymiec W, Wojcik M, Baszynski T, Krupa Z (2001) Angiosperms (Asteraceae, Convolvulaceae, Fabaceae and Poaceae: other than brassicaceae). In: Prasad MNV (ed) *Metals in the environment*. Marcel Dekker Inc, New York, pp 171–215
29. Tewari RK, Kumar P, Sharma PN, Bisht SS (2002) Modulation of oxidative stress responsive enzyme by excess cobalt. *Plant Sci* 162:381–388
30. Li M, Hu C, Zhu Q, Chen L, Kong Z, Liu Z (2006) Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae). *Chemosphere* 62:565–572
31. Mallick N (2004) Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris* response of the antioxidant system. *J Plant Physiol* 161:591–597
32. Asada K (1984) *Chloroplasts* formation of active oxygen and its scavenging. *Methods Enzymol* 10:422–429
33. Robinson MG, Brown LN, Hall BD (1996) Effect of gold (III) on the fouling diatom *Amphora coffeiformis*: uptake, toxicity and interactions with copper. *Biofouling* 11:59–79
34. Parial D, Pal R (2015) Biosynthesis of monodisperse gold nanoparticles by green alga *Rhizoclonium* and associated biochemical changes. *J Appl Phycol* 27:975–984
35. Liang J, Xia X, Zhang W, Zaman WQ, Lin K, Hu S, Lin Z (2016) Biochemical and toxicological responses of earthworm (*Eisenia fetida*) following exposure to nanoscale zerovalent iron in a soil system. *Environ Sci Pollut Res* 24(3):2507–2514
36. Santos-Magalhães NS, Mosqueira VC (2010) Nanotechnology applied to treatment of malaria. *Adv Drug Deliv Rev* 62:560–575