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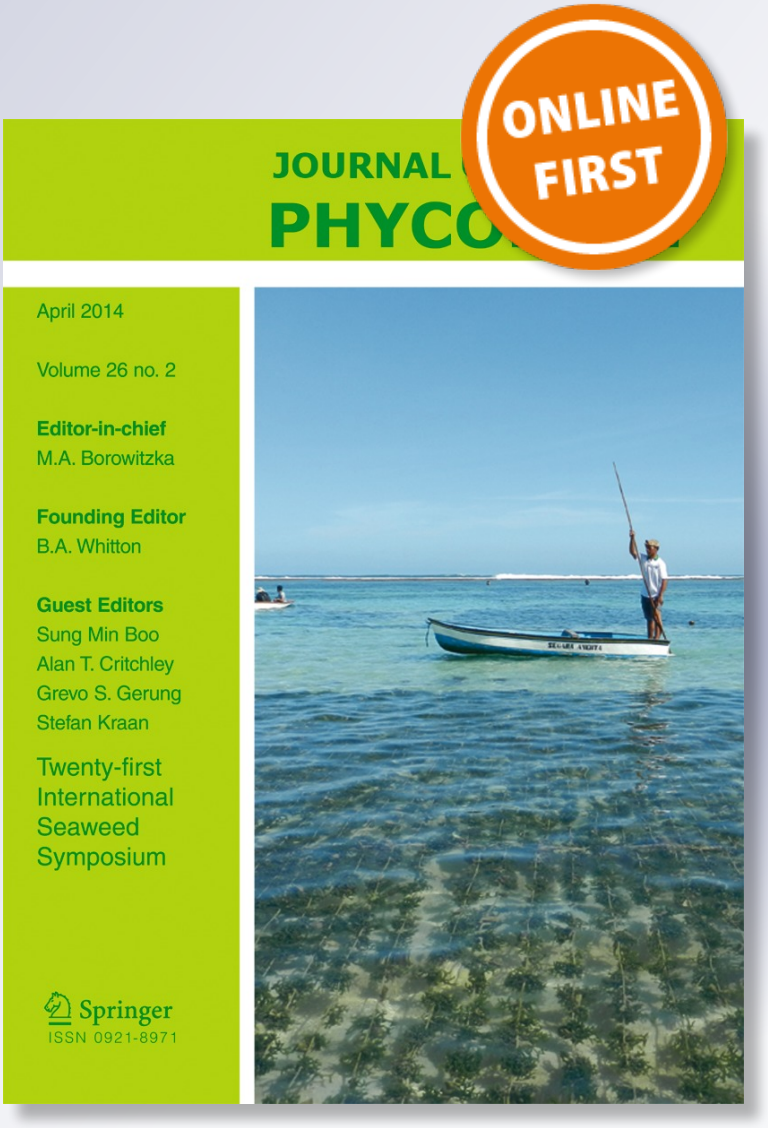

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Abstract Upscaling of metal nanoparticle production through green pathways requires selection of a suitable candidate species, an appropriate and cost-effective bioreactor for the conversion of ionic form of metals to nanomaterials. With this perspective, the potential of a freshwater Chlorococcalean alga, *Chlorella vulgaris*, was investigated for silver nanoparticle production in a continuously stirred non-aerated culture assembly. The findings reveal that the alga can reduce the silver ions and remains viable at 10^{-3} M silver nitrate concentration. The nanoparticles produced were of polydisperse type with size range of 8–20 nm and mean size of 12.62 nm. Zeta potential of the particles was −16.48 mV indicating a moderate stability of nanoparticles in the environment. Fourier transform spectroscopy of the treated biomass showed the presence of carboxylic, alcohol, phenol, and hydroxyl groups other than aromatic functional groups. X-ray diffraction study showed the 2θ values 38.38° and 45.28° which correspond to (111) and (200) planes of face-centered cubic (fcc) crystal structure of metallic silver with d-spacing (A°) of 2.343. Overall information reported here will help in gaining a better insight into the potential of algae as a suitable material for green chemistry of metal nanoparticle synthesis.

Keywords *Chlorella* · Green chemistry · Nanoparticle production · Bioreactor

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Introduction

The unique properties of nanoparticles such as large surface area to volume ratio and the ability to catalyze chemical transformation have stimulated a considerable interest in engineered nanostructures (Taniguchi 1974; Krolikowska et al. 2003). Nanoparticles associated with a capping agent are considered as an effective tool in fight against cancer and bacterial or viral pathogens. Silver nanoparticles are incorporated into products that range from biological and chemical sensors to photovoltaics. Due to their antibacterial properties, silver nanoparticles are commonly used as antimicrobial coating on the materials such as textiles, keyboards, wound dressings, and biomedical devices (Gong et al. 2007; Rai et al. 2009; Prasad et al. 2013). Due to their wide-ranging applications, upscaling of nanomaterial production through environment-friendly pathways has a pivotal role in meeting the increasing demand for nanomaterials (Sastry et al. 2004)

Recently, a few attempts were made to upscale the production of metal nanoparticles through a continuous flow tubular microreactor; however, the processes used for the reduction of the metal ions to form nanoparticles were mostly based on synthetic chemicals (Chandrasekharan and Kamat 2000; Peto et al. 2000; Kumar et al. 2003; Shavel et al. 2012). In view of growing concern about the hazards of the chemical-based nanoparticle production systems, the green chemistry-based nanoparticle production systems are considered as a better and more environment-friendly approach for upscaling of the production (Salunkhe et al. 2011). Since the green synthesis does not generate any harmful by-products or waste, many endeavors are in the pipeline to use microorganisms including microalgae as an eco-friendly material for synthesis of nanoparticles (Albrecht et al. 2006; Thakkar et al. 2010; Rajesh et al. 2012). The reduction of Ag^{+} ions in green chemistry occurs either on the cell wall or in intracellular environment through various functional groups in the biomolecules or extracellularly

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by the release of a reducing agent in the medium. Though, the intracellular reduction allows a better maneuvering of the size and shape of the nanoparticle, but harvesting of nanoparticles from the biomass requires cumbersome efforts. In case of extracellular synthesis, the release of a strong reducing agent in the medium facilitates synthesis of metal nanoparticles with a range of sizes and shapes, and the harvesting of nanoparticles is less tedious as compared to intracellular process (Ogi et al. 2010; Ali et al. 2011). In literature, most of the reports on algae-mediated biosynthesis of nanoparticles are based on either intracellular reduction by live cells or by the crude extracts of dried algal biomass (Lengke et al. 2007; Singaravelu et al. 2007). Therefore, there is a considerable gap in the knowledge with regard to green synthesis of nanoparticles through algae-based bioreactors.

In view of the increasing applications of nanoparticles, there is a commensurate increase in demand for the metal nanoparticles in the market. Therefore, upscaling of nanoparticle production is a major requirement for fulfilling the demand (Dahoumane et al. 2010). A key step in upscaling of production through green chemistry is the development of a continuous culture unit where metal ions are reduced in the culture medium without arresting the growth of the organism. There are few reports in literature describing the intracellular reduction of metal ions; however, reports pertaining to species selection and designing of nanobioreactors for upscaling of production are very few. In this context, this study attempts to assess the feasibility of upscaling of the production of silver nanoparticles through a chlorophycean alga *Chlorella vulgaris* (Chlorococcales) grown in a non-aerated (without air-injection device) reactor. The basic properties of silver nanoparticles produced in the bioreactor were studied on the basis of the parameters viz., zeta potential, particle size, UV–visible spectroscopy, transmission electron microscopy(TEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR).

Material and methods

A heterogeneous assemblage of algae collected from the cement tanks of Central Institute of Fisheries Education, Mumbai, was suspended in 10-mL double distilled water. The suspension was observed under microscope to confirm the presence of *Chlorella* cells (Vijayraghavan and Kumari 1995). A drop of the suspension with adequate number of the algal cells was inoculated on nutrient agar (2 %) BG-11 medium (Allen 1968). The petri plates were incubated under photoautotrophic conditions (temperature 24±2 °C; illumination ±54 μmol photons m^{-2} s^{-1}). The light and dark periods were maintained at 12:12 h. The colonies appearing after 72 h of incubation were picked up and resuspended in the medium, and a drop of this suspension was reinoculated on nutrient agar medium. This was repeated until unialgal populations of *C. vulgaris* were raised. The biomass of the organism for further experiments was produced in an air-lift culture assembly consisting of a 20-L aspirator bottle fitted with an air-injection device. The air passing through the culture was decontaminated by a sintered glass air filter. The culture in the reservoir was continuously stirred (200 rpm) with a magnetic stirrer.

Non-aerated bioreduction unit

The bioreduction of silver ions was carried out in a culture assembly (Fig. 1) consisting of a culture/bioreduction vessel, ammonium chloride reservoir, a magnetic stirrer for continuous stirring of the culture, and a collection tank for the withdrawal of cell-free suspension containing silver nanoparticles. The outlet of the bioreduction vessel was fitted with a filter paper (0.45-μm mesh size) to obtain cell-free suspension in the collection tank. Fifteen liters of exponentially growing population of *C. vulgaris* (density 38-mg dry weight L^{-1} of algal culture), cultured in the unit described above, was harvested by centrifugation (3,600×*g*, 20 min) and washed with deionized water to remove the metal ions present in the culture medium. The washed algal biomass was resuspended in deionized water (15 L). An appropriate volume of ammonium chloride stock

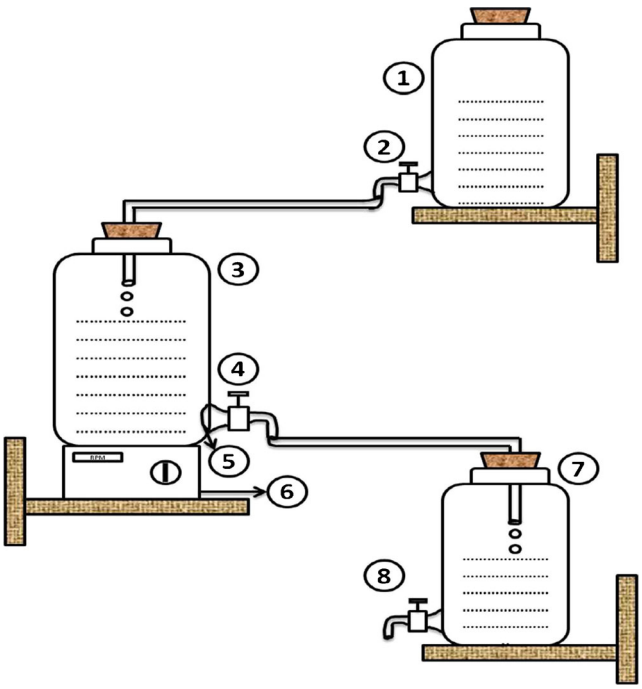

Fig. 1 Culture assembly for silver nanoparticle production through *Chlorella vulgaris* (*1* ammonium chloride (1 %) reservoir, *2* flow regulator, *3* culture tank with *Chlorella* cells in deionized water supplemented with 1 % ammonium chloride every third day, *4* flow regulator, *5* filter paper mounted at the inlet of the flask, *6* magnetic stirrer with Teflon-coated bar (3″), *7* collection tank for silver nanoparticle, *8* flow regulator)

(100 mM) was added to this to maintain 1-mM ammonium chloride concentration in the culture tank, followed by silver nitrate stock solution (100 mM) to maintain the concentration at 10^{-3} M. The algal suspension was stirred with a magnetic stirrer at 200 rpm. The conditions maintained for the bioreduction unit were similar to those maintained for batch cultivation (temperature 24±2 °C; illumination ±54 μmol photons m^{-2} s^{-1}, alternating 12-h light and dark periods).

Monitoring of nanoparticle production

The conversion of silver ions into metallic silver was monitored by ultraviolet–visible (UV–Vis) absorption measurement in the range of 200–750 nm using a spectrophotometer (Thermospectronic, UK; UV-1 model). The formation of silver nanoparticles was observed by measuring the absorption maxima of the silver nitrate-algae solution which showed a peak absorbance at 400 nm due to shift in plasmon resonance imparting a brown color to the solution. For the characterization of silver nanoparticles, the brown color solution was concentrated at 60 °C to reduce the volume. The concentrated solution was centrifuged at 16,000×*g* for 30 min using a high-speed centrifuge (Elektrocraft, India; Model MP400R). The pellet obtained was washed with deionized water and centrifuged again for 15 min. The process was repeated to remove water soluble biomolecules from AgNP suspension.

Cell viability assay

Algal cells exposed to silver nitrate were harvested after centrifugation (3,600×*g*, 30 min), and the pellet was washed with deionized water and resuspended in 50-mL deionized water. Oxygen evolution was measured in the above suspension using a Clark-type electrode connected to an amperometric detector. Rate of oxygen evolution was measured in the treated and untreated (control) cells after dark and illumination periods of 1 min (data not presented).

Ammonia treatment for desorption of silver nanoparticles

Ammonia solution (25 % GR, Merck) was used for the recovery of the nanoparticles from the algal biomass. The dried biomass (10 g/100 mL ammonia solution) was suspended in ammonia solution (2 M) and shaken for 2 h at 150 rpm on a rotary shaker at room temperature (24±2 °C). The biomass was separated by centrifugation at 3,600×*g* for 30 min, and supernatant was collected. This procedure was repeated till the biomass became almost colorless. The desorbate supernatant was passed through 0.22-μm filter assembly and used for further analysis after evaporation of ammonia solution.

FTIR analysis

For FTIR analysis, solution cast films of the liquid samples were used. Samples were prepared by drop-coating method on smooth NaCl crystal, and the spectra were recorded using PerkinElmer spectrometer (Model 100 FT-IR) in the range of 4,000 to 400 cm^{-1}. Zeta potential was measured using a Beckman Coulter DelsaNano C particle analyzer.

Measurement of size

The size of the particles was measured by transmission electron microscopy. A drop of nanoparticle solution after sonication in a sonicator bath (5 min) was placed on a carbon-coated copper grid and allowed to dry. The grid was scanned using a Phillips Tecnai-20 model transmission electron microscope operated at a voltage of 100 kV.

X-ray diffraction analysis

X-ray diffraction measurements were carried out after preparing a colloidal solution of silver nanoparticles by repeated centrifugation (16,000×*g*, 15 min) and redispersion in deionized water (Millipore, USA). The samples were analyzed using X'Pert PRO diffractometer (PANalytical, The Netherlands) operated at 45 kV, 40 mA generator settings. The start and end positions for 2θ were 2.0134° and 49.9874°, respectively. The other specifications of the instrument were anode material copper, K-Alpha1 [Å] 1.54060, K-Alpha2 [Å] 1.54443, K-Beta [Å] 1.39225, and K-A2/K-A1 Ratio 0.50000.

SEM-EDAX

Scanning electron and energy dispersive X-ray analyses of the surface of the algal cells were carried out by capturing the images through FEI Quanta-200 scanning electron microscope fitted with energy dispersive X-ray analysis (EDAX) spectroscope at an operating voltage of 15 kV. Samples were prepared by drop-coating method on smooth surface. A small amount of solution was dropped on smooth carbon surface and dried with a critical point dryer (critical point 31 °C; 73.8 bar). Different portions of the image were selected, and EDAX was done for the confirmation of the presence of silver nanoparticles on the cell surface.

Results

The unialgal population of *C. vulgaris* when treated with 10^{-3} M silver nitrate turned deep brown due to change in plasmon resonance after 48 h of exposure. In order to rule out the reduction of silver ions due to chemical constituents of the

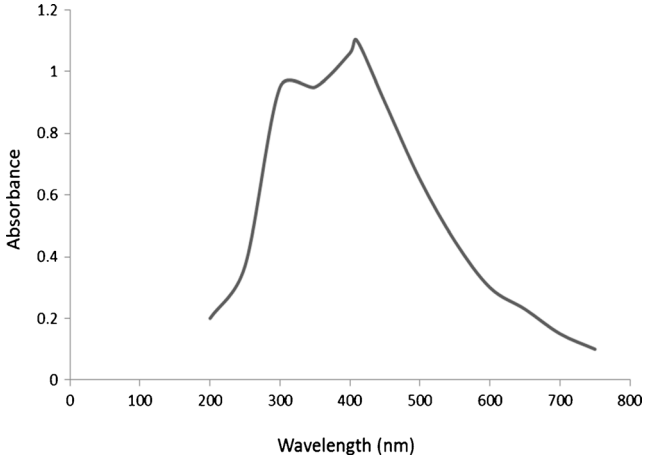

Fig. 2 Absorption spectrum of the cell-free suspension of *C. vulgaris* after silver nitrate (10^{-3} M) treatment

medium, a parallel experiment without algal cells (medium+ 10^{-3} M, silver nitrate) was also carried out (data not presented). There was no color development by cell-free culture medium, indicating that reduction of silver ions was solely a biological phenomenon.

The compounds released from the cells were characterized on the basis of UV–Vis spectrum of the cell-free suspension obtained after centrifugation. Measurement of the absorbance (range 190–400 nm) showed peaks at 200 and 265 nm, which is attributed to aromatic amino acids such as tyrosine and tryptophan in the protein residue (Ali et al. 2011). The observations indicated the proteinaceous nature of the secreted compounds from *Chlorella* cells which reduced silver ions to silver nanoparticles.

To further confirm that the reduction of silver ions occurs in the extracellular medium by the compounds excreted from *Chlorella* cells, cell-free suspension was prepared by removing *Chlorella* cells from the medium by centrifugation (15,000×*g*, 30 min). The cell-free suspension was treated with 10^{-3} M silver nitrate, and the absorbance of the solution was monitored in the range 200−750 nm for 72 h (Fig. 2). The color of the suspension gradually changed from light to deep brown during 36–72 h of exposure time. This provided the evidence for the release of biomolecules from *Chlorella* cells in the extracellular medium which reduced silver ions to silver nanoparticles. The algal cells after exposure to silver nitrate showed 10–15 % lower photosystem II (PSII) (oxygen evolution) activity compared to control (untreated cells), confirming the viability of cells after the treatment.

It was noticed that the recovery of the silver nanoparticles in the supernatant obtained after centrifugation (15,000×*g*, 30 min) was much less as the precipitated cells showed a dark brown color, indicating that most of the colloidal silver remained adsorbed on the cell surface. The procedure followed for the ammonia treatment resulted in desorption of most of the colloidal silver from the cell surface. Though the magnitude of the recovered silver nanoparticles after each ammonia treatment cycle was not quantified, three repeated cycles were found adequate for recovering almost all the silver nanoparticles.

FTIR analysis of cell-free liquid samples with and without silver nitrate treatment showed the presence of alcohol (3,426.72 cm^{-1}), amide (C=O) stretch (1,642.70 cm^{-1}), and aromatic groups (799.99–499.07 cm^{-1}) (Fig. 3, upper spectrum). However, after silver nitrate treatment, the spectrum (lower) showed that the silver ions bind with the above groups except with the alcohol groups.

The TEM images (Fig. 4) of the cell-free suspension after treatment with silver nitrate showed that the size of the particles ranged from 8 to 20 nm with a mean size of 12.62 nm. The zeta potential of nanoparticles was −16.48 mV with conductivity of 0.7804 mS cm^{-1} (supplementary figure).

The FTIR spectrum of isolated nanoparticles showed bands at 3,280 and 2,924 cm^{-1} corresponding to stretching vibrations of primary and secondary amines, respectively (supplementary figures). The bands observed at 1,648.84, 1,533.80,

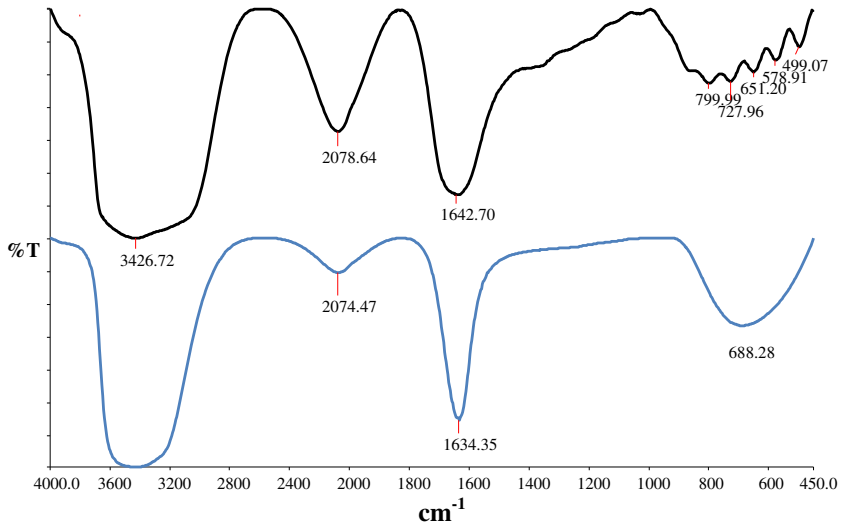

Fig. 3 FTIR spectra of the cell-free suspension of untreated (*upper spectrum*) and treated (*lower spectrum*) cultures of *C. vulgaris*

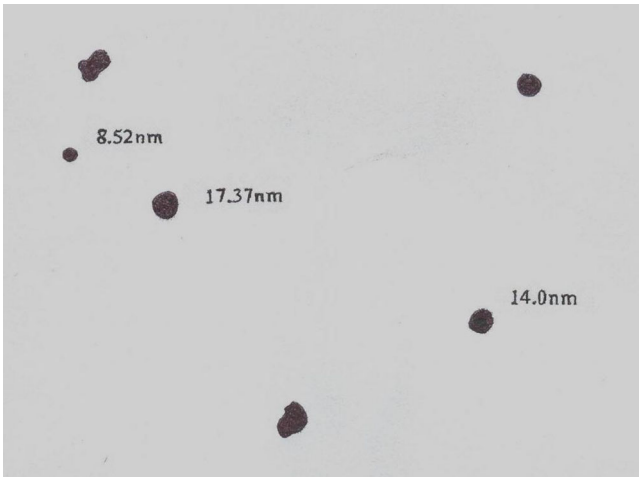

Fig. 4 TEM image of the silver nanoparticles produced by bioreduction through *C. vulgaris*

and 1,243 cm^{-1} indicated the presence of amide I–III bands of polypeptide/proteins. The band observed at 827.59 cm^{-1} indicated the presence of aromatic groups in the protein moiety. The overall observations confirmed the involvement of proteins in silver ion reduction in the extracellular medium.

SEM images (Fig. 5) of the *C. vulgaris* cells with and without silver nitrate treatment showed that the cells maintained their integrity after silver nitrate treatment. This was evident from the comparable cell size and absence of any bulge or pores on the cell wall. The AgNPs formed layers of variable shape and size on the surface of algal cells. The AgNPs aggregated on the outer surface of the wall were dislodged during stirring and harvested through centrifugation. The EDXA spectrum (Fig. 6) of the pre-washed cells confirmed the presence of Ag particles on the cell surface.

The XRD spectrum (Fig. 7) of the silver nanoparticles showed two well-defined characteristic peaks at 2θ values, i.e., 38.38° and 45.28° which corresponded to (111) and (200) planes of face-centered cubic (fcc) crystal structure of metallic silver with d-spacing (A°) of 2.343. This is in agreement with the standard silver values (JCPDS PDF card 04-0783).

Discussion

It is established by earlier reports that the charged biomolecules with diverse functional groups are capable of reducing silver ions to silver nanoparticles (Govindaraju et al. 2008; Sharma et al. 2009). A change in color of algal suspension to brown indicates the conversion of silver ions to silver nanoparticles. Our observations show that the algal suspension turned brown after 36 h of exposure to 10^{-3} M silver nitrate. However, the color changed to deep brown with almost black appearance, and this change in the color of cells was due to the intracellular and membrane-bound silver nanoparticles. This investigation shows that *C. vulgaris* has a potential to synthesize silver nanoparticles intracellularly and extracellularly. The latter phenomenon is rare as few algal species have shown the potential to synthesize silver nanoparticles by releasing biomolecules which can reduce ionic silver to silver nanoparticles (Ali et al. 2011). It was interesting to note that *C. vulgaris* strain used in the present investigation showed the tolerance to 10^{-3} M silver nitrate confirming that cells remained viable in the presence of silver ions. Though, there was 10 % decrease in the PSII activity (photosynthetic oxygen evolution), but the cells maintained the viability to almost 90 % level after the treatment with 10^{-3} M silver nitrate (data not presented).

It was observed that after 72 h onward, the algal cells appeared dark brown in color, and it was difficult to recover silver nanoparticles embedded in the algal biomass. However, a pretreatment with ammonia solution was found effective in desorption of silver nanoparticles from the biomass after repeated washing with ammonia solution. Silver nanoparticles were recovered after evaporation of ammonia solution. These nanoparticles were found free of any contamination and were suitable for FTIR, TEM, XRD, and other analyses. The XRD pattern corroborates the highly crystalline nature of nanoparticles. It is evident from a clear peak at 2θ value 38.38°, which indicates that (111) lattice plane is the preferred orientation which is also known for its high antibacterial activity.

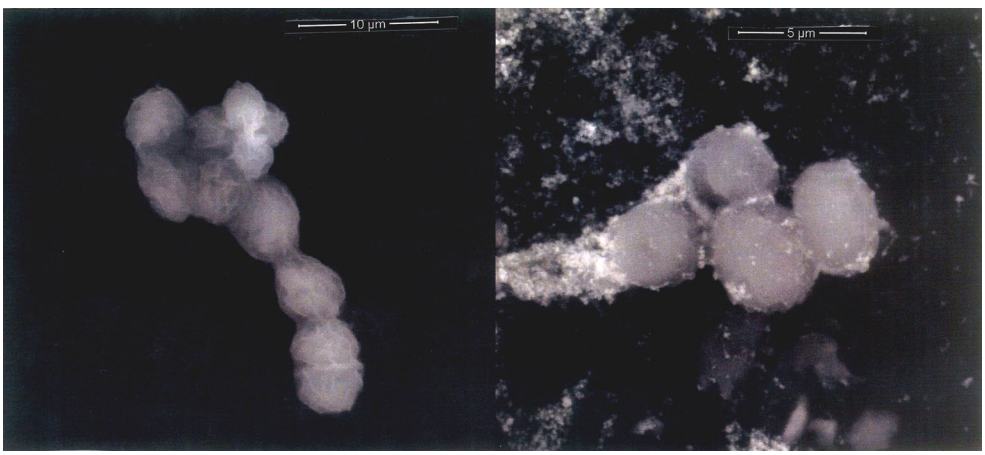

Fig. 5 SEM image of untreated cells (*left*) and silver-nitrate-treated cells (*right*) of *C. vulgaris*

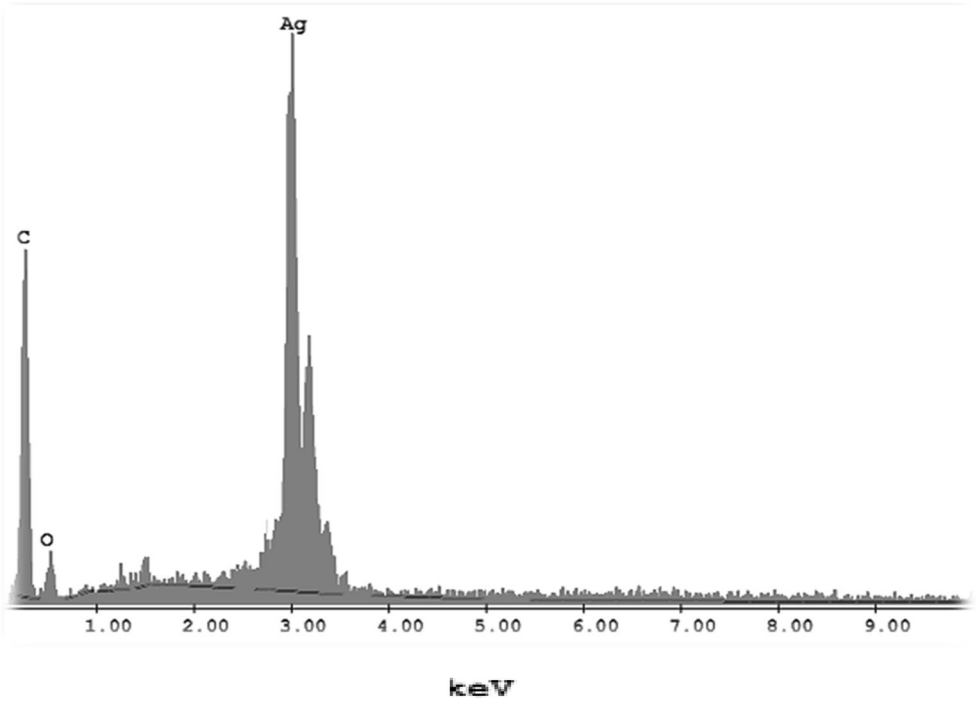

Fig. 6 EDXA spectrum of *C. vulgaris* cells after 10^{-3} M silver nitrate treatment

A moderate value of zeta potential of silver nanoparticles (−16.48 mV) was recorded for the particles synthesized by *C. vulgaris.* This value is higher than the value (−26.3 mV) recorded by Sadowski et al. (2008). A comparatively lower value of zeta potential for the silver nanoparticles suggests that AgNPs have a tendency to aggregate to form bigger sized particles due to low zeta potential.

Mass cultivation of the alga for obtaining sufficient biomass for experiments was accomplished in a continuously stirred culture unit with an air-injection device, under photoautotrophic conditions. However, the unit used for bioreduction of silver ions through *Chlorella* cells was a continuously stirred non-aerated culture unit (Fig. 1), and the organism in this unit was grown under similar photoautotrophic conditions as maintained for the mass cultivation of the alga (temperature 24±2 °C; fluorescent light intensity 54±1.35 μmol photons m^{-2} s^{-1}; pH of the medium after sterilization 7.1). In the latter unit, deionized water was used instead of growth medium, and 1 mM ammonium chloride was spiked every 72 h to provide nitrogen source to growing cells. Removal of aeration process and spiking of the medium with ammonium chloride indicate that *C. vulgaris* can maintain its growth by utilizing ambient carbon dioxide as carbon source; however, the growth cannot be sustained if ammonium chloride is not spiked every third day.

The cell-free suspension of the culture medium when treated with silver nitrate also showed a shift in plasmon resonance of silver ions indicating bioreduction, but the quantity of such

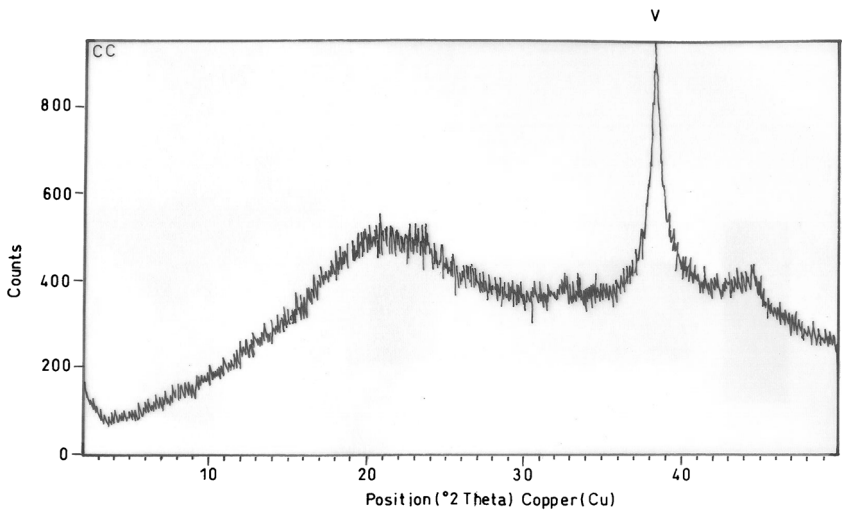

Fig. 7 X-ray diffraction spectrum of silver nanoparticles produced by *Cnn vulgaris*

particles was very small as compared to the particles attached to the cell wall of the algal cells. SEM pictures clearly showed that most of the silver nanoparticles produced extracellularly form a layer on the external surface of the cell wall. Continuous stirring of the medium dislodges the clumps of nanoparticles after some time, and they can be recovered at the outlet fitted with 0.45-μm filter at the mouth of the culture vessel. FTIR spectra of treated and untreated algal biomass showed that carboxylic and aromatic functional groups mainly contribute in the bioreduction process. This observation corroborates the earlier findings where the functional groups viz., carboxyl, polyphosphate, and amino acids present in proteins are attributed for the binding and subsequent reduction of metal ions. The XRD analysis of the biomass washed with ammonia to remove the silver nanoparticles adhered to the external surface of the cell wall showed the presence of fcc silver particles.

The reduction of silver ions is lower in the suspension-free algal biomass. The reason is the low quantity of free biomolecules excreted by the algal cells to the external medium. The light microscopy (supplementary files) and SEM images clearly showed that most of the silver ions aggregate on the outer surface of the wall suggesting that reduction on the wall surface was higher than that in intracellular environment. Though a little speculative, there is a possibility of aggregation of the nanoparticles (reduced intracellularly, extracellularly, and on the cell wall) on the external surface of the wall. The particles adhered to the outer surface of the wall may act as the nucleus for further aggregation of the silver nanoparticles.

Taken together, the data of present investigation indicates that algae in general, and the algal strains such as *C. vulgaris* with a potential for extracellular reduction of silver ions in particular, have an immense potential for continuous production of silver nanoparticles by a green synthesis pathway. It is evident from the study that the algal strain *C. vulgaris* with a capacity to reduce silver ions to nanoparticles is a suitable candidate species for metal nanoparticle production and upscaling of the process. However, considering the ubiquitous distribution of algae in diverse ecosystems from tropics to poles, further investigations with larger number of algal strains will facilitate bioprospecting of algae for green chemistry-based nanoparticle production.

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