Biosynthesis of silver nanoparticles using *Ulva fasciata* (Delile) ethyl acetate extract and its activity against *Xanthomonas campestris* pv. *malvacearum*

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ABSTRACT

Metallic nanoparticles have been traditionally synthesized using wet chemical techniques, where the chemicals used are quite often toxic and flammable. In this research, we present a simple and ecofriendly biosynthesis of Ag nanoparticles using *Ulva fasciata* crude ethyl acetate extract as reducing and capping agent. The bionanoparticle characterized with UV-vis Spectroscopy, FTIR, XRD, SEM and EDX. Characterization reveals that the nanoparticles are crystalline in nature, spherical in shape and poly-dispersed with size ranging from 28 to 41 nm. The alkyne group (3424.30 cm^{-1}) of *U. fasciata* crude ethyl acetate extract shifted and reduced the $AgNO_3$. GC-MS analysis revealed the presence of 1-(Hydroxymethyl)–2, 5, 5, 8A-tetramethyl decahydro-2-napthalenol as reducing agent and hexadecanoic acid was found to be a stabilizing agent. *Ulva fasciata* based bionanoparticles inhibited the growth of *Xanthomonas campestris* pv. *malvacearum* (14.00±0.58 mm Zone of inhibition), with a Minimum Inhibitory Concentration of 40.00±5.77µg/mL. The study shows that *U. fasciata* crude ethyl acetate extract could be used as a reducing agent for simple ecofriendly synthesis of silver nanoparticles. However, more studies are essential before recommending them for disease management.

Key words: Antibacterial, EDX, FTIR, MIC, silver nanoparticles, SEM, *Ulva fasciata*, *Xanthomonas campestris* pv *malvacearum*, XRD

INTRODUCTION

Silver has been used for many years for its antimicrobial properties. Alexander the Great used silver vessels to store drinking water (Silver *et al.*, 2006). However, the formulation of silver has changed during antiquity, from bulk silver to ionic silver or adsorbed on carrier materials (Zeolite) (Kwakye-Awwah *et al.*, 2008) and now to silver nanoparticles. In order to advance nanotechnology for antimicrobial applications, development of methods to understand and control the behavior of nanomaterials is needed. A nanomaterial may be defined as any material (insulator, conductor or semiconductor), which has been controllably synthesized on the size range of roughly 1 to 100 nm. At this size and dimensional range, essentially any material will exhibit different properties from those it would as an atomic cluster or as the larger bulk materials.

Production of metallic nanoparticles can be achieved through chemical, physical or biological methods. Chemical approaches are the popular mode of synthesis of nanoparticles However; these methods cannot avoid the use of toxic chemicals. Now-a-days biological synthesis of metallic nanoparticles is gaining importance as it is reliable and ecofriendly. Previous literature revealed that the nanoparticle synthesis using algae as source has been unexplored and underexploited. Recently there are a few, reports that algae is being used as a biofactory for synthesis of metallic nanoparticles. Singaravelu *et al.* (2007), Rajasulochana *et al.* (2010) and Vivek *et al.* (2011) reported the synthesis of silver bionanoparticles using *Sargassum wightii*, *Kappaphycus alvarezii* and *Gelidiella acerosa* crude extracts, respectively. Govindaraju *et al.* (2008) reported the synthesis of silver nanoparticles using microalgae, *Spirulina platensis*. However, no report was available about the synthesis of nanomaterials using the ethyl acetate from marine algae. On the other hand there are numerous works related to green synthesis of metallic nanoparticles using higher plants. Gardea-Toroesday *et al.* (2003) first reported the formation of gold and silver nanoparticles by living plants. Shankar *et al.* (2004) reported pure metallic silver and gold nanoparticles synthesis by the reduction of Ag^+ and Au^+ ions using neem (*Azadirachta indica*) leaf broth. There have been recent reports on phytosynthesis of silver and gold nanoparticles by employing lemon grass extract (Shankar *et al.*, 2004, 2005), *Sesbaniadrammandii* (Sharma *et al.*, 2007), green tea (*Camellia*

sinensis) (Vilchis – Nestor *et al.,* 2008), *Aloe vera* plant extract (Chandran *et al.,* 2006), coriander leaves (Narayanan and Sakthivel, 2008), sundried *Cinnamomium camphora* leaves (Huang *et al.,* 2007), *Cinnamon zeylanicum* (Sathishkumar *et al.,* 2009), Phyllanthin extract (Kasthuri *et al.,* 2009a), purified apilin compound extracted from henna leaves (Kasthuri *et al.,* 2009b), *Acalypha indica* (Krishnaraj *et al.,* 2010), *Curcuma longa* (Sathishkumar *et al.,* 2010), *Hibiscus rosasinensis* (Philip, 2010a), Honey (Philip, 2010b), *Rosa rugosa* (Dubey *et al.,* 2010a), *Solanum torvum* (Govindaraju *et al.,* 2010), *Ficus benghalensis* (Saxsena *et al.,* 2012), *Crossandrain fundibuliformis* (Kaviya *et al.,* 2012). A review has been published by Sahayaraj and Rajesh (2011) in the proposed aspect.

The genus *Xanthomonas* (Proteobacteria) is a diverse and economically important group of Gram negative bacterial pathogens. Bacterial blight, caused by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye [= *Xanthomonas axonopodis* pv. *malvacearum* (Vauterin *et al.,* 1995, 2000)] has become an increasing problem to cotton production world-wide. Very few studies are available for the applicability of silver nanoparticles to control plant diseases. Antifungal effectiveness of colloidal nano silver solution against rose powdery mildew caused by *Sphaerothe capannosa* var. *rosae* (Kim *et al.,* 2008) is already known. Sclerotium-forming *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *S. minor* (Min *et al.,* 2009); *Bipolaris sorokiniana* and *Magnaporthe grisea* (Jo *et al*. (2009); *Colletotrichum gloesporioides* (Aguilar-Me´ndez *et al*., 2010); *Escherichia coli* (Li *et al.,* 2010). But there is no report about the antibacterial activity and mechanism of biosynthesized silver nanoparticles on the cotton bacterial pathogen, *Xanthomonas campestris* pv. *malvacearum*. Raghavendra *et al*. (2007) challenged cotton seedlings with *X. campestris* pv. *malvacearum* and found that the cotton seedlings developed considerable resistance to the bacterial pathogen if the seeds were soaked prior to germination for 12 hrs in a 1:500 solution of Dravya, an aqueous formulation of *Sargassum wightii*.

The terrestrial plant-based extract used for the control of this bacterial pathogen is reported and were found to have promising activity (Khan *et al.,* 2000; Goutam Brahmachari, 2008; Mahesh and Satish *et al.,* 2008; Dey *et al.,* 2010; Bai *et al.,* 2011; Govindappa *et al.,* 2011). This current study investigates the biosynthesis of silver nanoparticles using the *Ulva fasciata* crude ethyl acetate extract and its mechanism of inhibition towards *Xanthomonas campestris* pv. *malvacearum* in the sub-cellular level.

MATERIALS AND METHODS

Materials

Silver nitrate was purchased from HiMedia, India. All glasswares were washed thrice with deionized water and dried in oven before use. The marine algae, *Ulva fasciata* was collected by hand picking from the submerged marine rocks at Kuthenkuzhi, Tirunelveli district of Tamil Nadu, India during low tide. Samples was washed thrice with tap water and once with distilled water to remove sand, salt and partially powdered using domestic blender and stored in air-tight container until use.

Extraction

For extraction, the dried material (1 gm) was macerated with 30 ml of 2N Hcl. It was incubated in an oven for 4 hrs at 100^{o}C, subsequently extracted with equal volume of ethyl acetate and this step was repeated thrice. The ethyl acetate fractions were pooled and dried over anhydrous Sodium Sulphate (Suresh Chand and Muralirangan, 2010). The residue was dissolved in Dimethyl Sulphoxide (DMSO) for biosynthesis. The chemical nature of the crude ethyl acetate extract was analyzed using GC-MS.

Biosynthesis of nanosilver

Typically 3 mL of 0.1 % *U. fasciata* ethyl acetate extract was added to 100 ml of 10^{-3} M aqueous silver nitrate solution. To this reaction mixture 30 µL of 1N NaOH was added for reduction of Ag^{+} ions at room temperature. The colour of the solution changed from light yellow to brown indicating the formation of silver nanoparticles.

Characterization of bionanomaterial

Biogenic synthesis of nanosilver was monitored using UV-Vis spectrophotometer (UV-1601 Shimadzu spectro photometer) at regular intervals with samples in Quartz cuvette operated at a resolution of 1 nm. After the complete reduction of Ag+ ions by the *U. fasciata* ethyl acetate extract. It was analyzed by FTIR spectrophotometer in the range of 4000-400 cm^{-1}. The FTIR spectra of *U. fasciata* ethyl acetate extract was also analyzed for knowing the possible functional groups responsible for the formation of silver nanoparticles.

XRD pattern of dry nanosilver powder was acquired by Cu Ká radiation (1.5406 Å; 45 kV, 30 mA). It was analyzed to determine peak intensity, position and width. The particle size was calculated using the Scherrer formula,

$$d = 0.9? / ßcos?$$

where, d is the mean diameter of the nanoparticles, ?, the wavelength of X-ray radiation source and â, the angular

FWHM of the XRD peak at the diffraction angle ß (Culity, 1978). Powder samples for analysis were prepared by centrifugation at 13,000 rpm for 15 min, redispersed in sterile distilled water to get rid of any uncoordinated biological molecules. Centrifugation and redispersion were repeated thrice in order to ensure better separation.

The size and shape of the biosynthesized nanoparticles were observed by Scanning Electron Microscope (SEM) (JSM-6390 Scanning electron microscope). Samples were prepared by drop coating the Ag nanoparticles solutions onto carbon copper grid. The films on the grids were allowed to dry prior to measurement. The presence of elemental silver was confirmed through energy-dispersive spectroscopy (EDS).

Antimicrobial assay

For dose-dependent assay, *Xanthomonas campestris* pv. *malvacearum* was isolated from infected cotton plants and were used for the experiment. The pathogen was isolated, sub-cultured on Nutrient Agar and identified using standard protocol (Schaad *et al.*, 2001). Well diffusion assay was used to record the dose-dependent assay of nanosilver on test bacteria. Bacterial inoculum was prepared by growing a single colony overnight in nutrient broth and adjusting the turbidity to 0.5 McFarland standard. Mueller Hinton agar plates (9 cm*) were prepared and wells were made using sterile cork borer under aseptic condition. The U. fasciata* ethyl acetate extract and biosynthesized nanosilver with various concentrations (10, 20, 40, 80 and 160 µg/mL) was added to the respective wells. Streptomycin (0.1%) and DMSO were used as positive and negative control respectively. After incubation for 24 hrs at 37^0C, a clear zone around the well was an evidence of antibacterial activity. Diameter of the zones of inhibition was measured in millimeter using HiMedia ruler. Each test was performed in triplicate.

Minimum Inhibitory Concentration (MIC)

Broth micro dilution assay was used to determine the MIC (Eloff, 1998). Test sample (75 µl) of various concentrations (10-320µg/ml) was added into sterile microtitre plates. Bacterial cell suspension (75 µL) corresponding to 1×10^8CFU/mL was added in all wells except those in control well. Control well consisted of sterile distilled water and broth to check sterility while those in negative control well were filled with nutrient broth and bacterial suspension to check for adequacy of the broth to support bacteria growth. The plates placed in sterile petriplate and incubated at 37°C for 24 hrs. To indicate bacterial growth, 40 µl of 0.2 mg/mL P-Iodonitroterazolium chloride (INT, HiMedia) was added to each well and incubated for another 30 min. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms. Inhibition of bacterial growth was visible as a clear colourless well and the presence of growth was detected by the presence of pink-red color. The lowest concentration showing no colour change was considered as the MIC.

Determination of bacterial growth kinetics

Mueller Hinton broth was used for the aerobic cultivation of bacteria at 37 °C with shaking at 150 rpm. Different concentrations of nanosilver (10, 20, 40, 80 and 160µg/mL) was supplemented in broth. Growth curve of bacteria exposed (test) and unexposed (control) to nanosilver were determined based on the absorbency value of OD_{600nm}.

Determination of effect of SNP's on membrane leakage in XCM

To detect the leakage of reducing sugars and proteins through membrane, different volumes of SNP's were added to Mueller Hinton broth so as to reach final test concentration (10, 20, 40, 80 and 160µg/mL), and *Xanthomonas campestris* pv. *malvacearum* cells (10^9 cfu/mL) were added. Control experiments were conducted without SNP's. The cultures were incubated at 37°C with shaking at 150 rpm. 1 ml of culture was sampled out at different time intervals (1, 2, 6 and 12 hrs). The sample was centrifuged at 12,000 rpm, the supernatant liquid was frozen at -20°C immediately, and then the concentrations of reducing sugars and proteins were determined as soon as possible (Lowry *et al.*, 1951; Miller, 1959).

RESULTS

A detailed investigation on the extracellular synthesis of nanosilver by *U. fasciata* extract was carried out and its antibacterial effect on *X.campestris* pv. *malvacearum* ascertained in this work.

Synthesis of SNP's

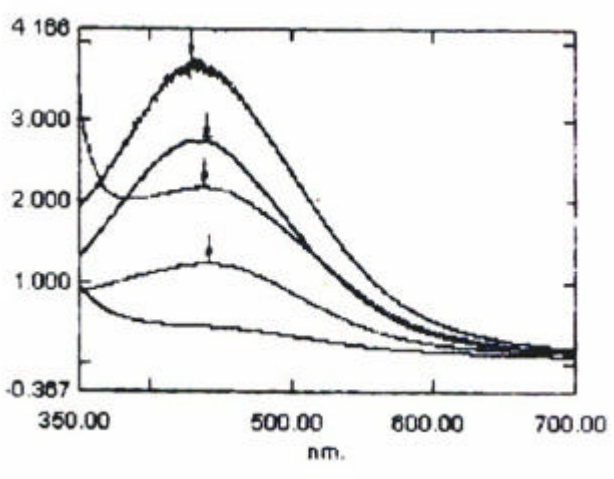
After the addition of the extract to the silver nitrate solution, the solution changed from colourless to pale yellow within 2 min, the final colour deepening to brown within 30 min. Figure 1 shows the *U. fasciata* ethyl acetate extract with silver nitrate at initial point of time and after 30 minutes reaction end point.

Characterization of SNP's

UV-Vis spectra were recorded as a function of time of reaction, Surface Plasmon Resonance (SPR) of nanosilver was obtained at 440nm with brown-yellow (Fig. 2). Fourier Transform Infrared spectroscopy (FTIR) measurements are carried out to identify the possible biomolecules responsible for the reduction of the Ag^+ ions and capping of the bio-reduced SNP's synthesized by *U. fasciata*. The FTIR spectra of *U.*

Figure 1. Biosynthesis of silver nanoparticles using *U. fasciata* ethyl acetate extract. a) 10^{-3}M $AgNO_3$ b) initial point c) final point of time

Figure 2.UV-Visible spectra of silver nanoparticles synthesized by treating *U. fasciata* ethyl acetate extract with 10^{-3}M $AgNo_3$ solution



fasciata ethyl acetate and biosynthesized nanosilver are depicted in Fig. 3a and 3b respectively.

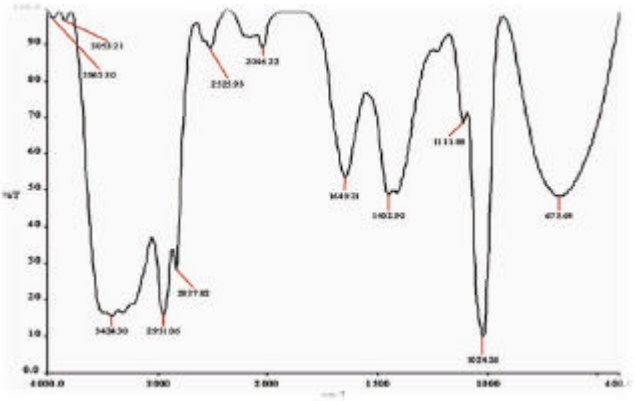
Figure 4 shows the XRD patterns of the nanosilver synthesized using *U. fasciata* ethyl acetate extract. A number of Bragg reflections with 2q values of 38.4æ%, 46.5æ%, 64.8æ%, 77.7æ% sets of lattice planes are observed which may be indexed as (111), (200), (220) and (311) facets of nanosilver. This clearly illustrates that the nanosilver formed in the green synthesis are crystalline in nature. Average crystal size of AgNP's was calculated by applying Scherrer equation and found to be of size 33nm. SEM analysis of nanosilver is shown in Figure 5.

The average size of the bionanoparticles were found to be 40.05 nm.EDX spectra reveal strong signals in the silver region and confirm the formation of nanosilver and its elemental nature (Fig. 6).

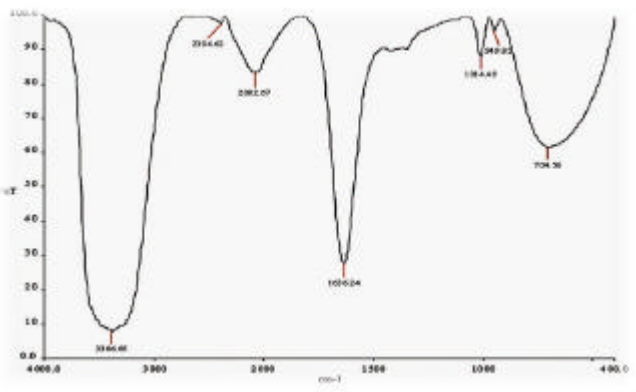
GC-MS analysis of crude ethyl acetate extract

The GC-MS analysis of Crude ethyl extracts (CEAE) is shown in Figure 7. It contains Hexadecanoic acid (46.97%), 5-Azulenemethanol, 1,2,3,3a,4,5,6,7-octahydro-à,à,3,8-tetramethyl-, [3s-(3à,3aá,5à)]- (16.82%), 1,1,4,7-Tetramethyldecahydro-1h-cyclopropa[e]azulen-4-ol (12.66%),

Figure 3. FTIR spectra of *Ulva fasciata* ethyl acetate extract (a) and silver nanoparticles synthesized using *U. fasciata* ethyl acetate extract with 10^{-3}M $AgNo_3$ solution (b)



(a)



(b)

Figure 4. XRD pattern of silver nanoparticles synthesized by treating*U. fasciata* ethyl acetate extract with 10^{-3}M $AgNo_3$ solution

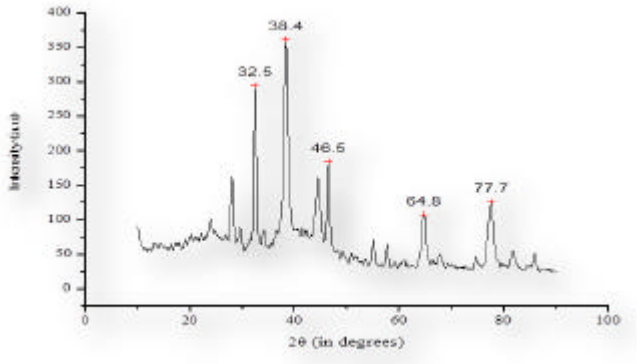


Figure 5. SEM images of silver nanoparticles synthesized by treating *U. fasciata* ethyl acetate extract with 10^{-3}M $AgNo_3$ solution

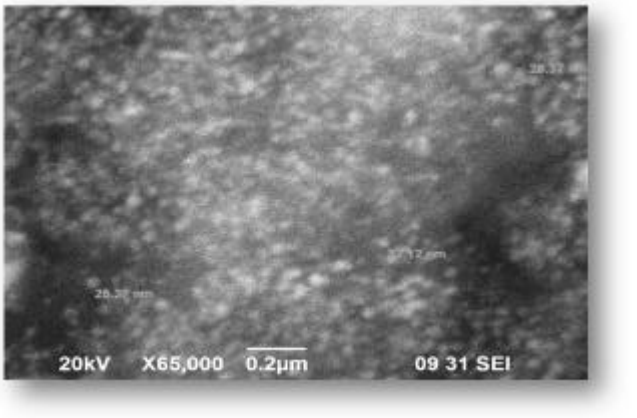


Figure 6. EDAX spectrum of silver nanoparticles synthesized by treating *U. fasciata* ethyl acetate extract with 10^{-3}M $AgNo_3$ solution

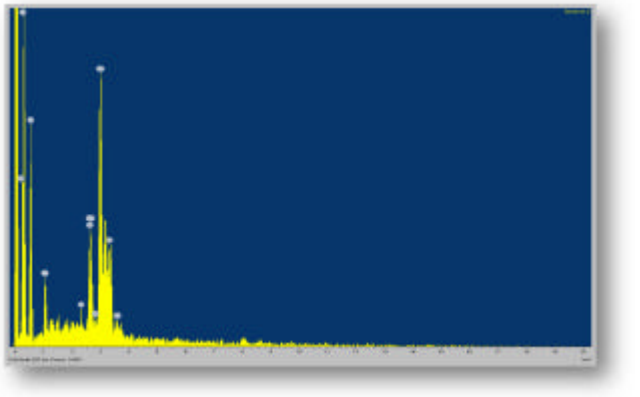


Figure 7. GC-MS analysis of crude ethyl acetate extract of *U. fasciata*

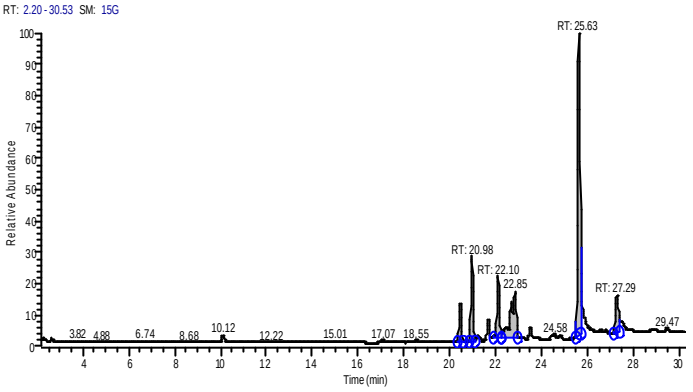
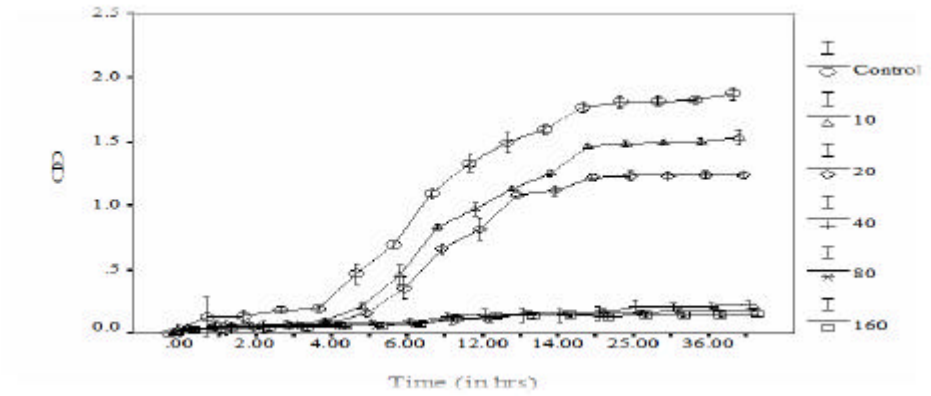


Figure 8. Growth kinetics of *X. campestris* pv. *malvacerum* exposed to different concentrations



5-Azulenemethanol, 1,2,3,3a,4,5,6,7-octahydro-à,à,3,8-tetramethyl-, [3s-(3à,3aá,5à)]- (10.28%), 9,12-Octadecadienoic acid (7.52%) and 1-(Hydroxymethyl)-2,5,5,8a-tetramethyldecahydro-2-naphthalenol (5.74%). The presence of 1-(Hydroxymethyl)-2,5,5,8a- tetra methyl deca- hydro-2-naphthalenol is found to be responsible for the reduction of the $AgNO_3$ and hexadecanoic acid acts as a stabilizing agent and thus prevents the aggregation of silver nanoparticles. Any one of the fatty acids, hexadecanoic acid (palmitic acid) ($C_{16}H_{32}O_2$) is used to protect the formed silver nanoparticles. Initially, the addition of sodium hydroxide to the ethyl acetate extracts containing hexadecanoic acid results in the formation of Sodium Palmitate which is found to be a stabilizer of formed nanosilver.

Dose-dependent assay and MIC determination

The antibacterial activity of *U. fasciata* ethyl acetate and nanosilver against *X. campestris* pv. *malvacearum* showed dose dependent activity. CEAE does not show any antibacterial activity. However, CEAE at 0.4 and 0.8% showed 9.67±0.33 and 11.67±0.67 respectively. The MIC was found to be 40±5.77 µg/ml.

Growth kinetics

The growth curves of XCM exposed to different concentrations of SNP's are shown in Figure 8 by measuring optical density at 600 nm. In the presence of SNP's, the growth curves of XCM consisted of three phases lag, log and stationary phase. However, decline phase could not be revealed as we assayed the total number of bacteria including live and dead ones, based on the measurement of turbidity at OD_{600} nm. Under the absence of SNP's the XCM reached exponential phase rapidly. But exposure to SNP's halted the growth of XCM. The complete inhibition of growth was detected at the concentration of 40 µg/ml; indicating the Minimum Inhibitory Concentration of SNP's towards XCM cells.

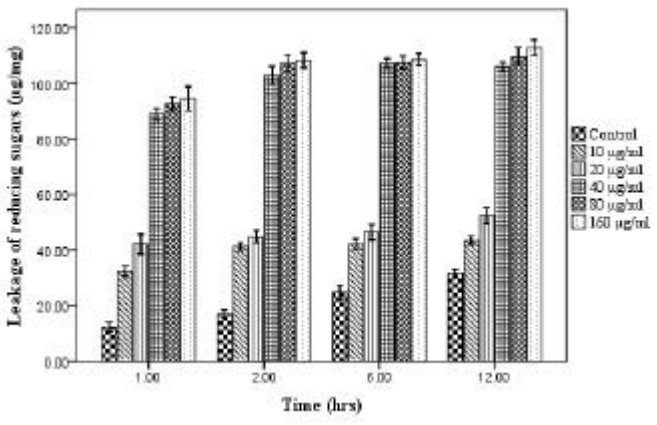
Cytolysis

Figure 9a reveals that SNP's could enhance the membrane leakage of reducing sugars. After 1 hr of treatment almost no reducing sugars could be detected in control experiment (12.5± 0.23 µg/mg of bacterial dry weight), while the leakage amount of reducing sugars from cells treated with 160 µg/ml of SNP's was 92.8± 0.36 µg/mg. After treatment with SNP's for 6 hrs the leakage was more evident, suggesting SNP's may accelerate the reducing sugars leakage from bacterial cytoplasm. Similarly, SNP's also elevated the leakage of proteins through the membrane of XCM (Figure 9b). After 1 hr of treatment, the leakage of proteins from cells in control cells was 6.8± 0.40 µg/mg of bacterial dry weight, while leakage from cells treated with SNP's was 17.6±0.35 µg/mg.

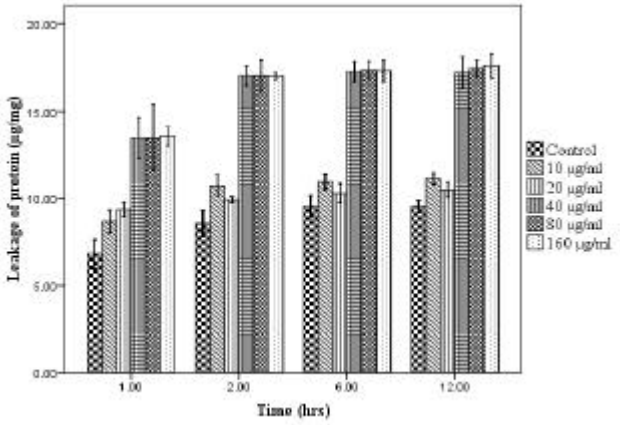
Discussion

It is generally recognized that UV–vis spectroscopy could be used to examine size- and shape-controlled nanoparticles in aqueous suspensions (Wiley *et al.,* 2006). SNP's have free electrons, which give rise to an SPR absorption band (Noginov *et al.,* 2006), due to the combined vibration of electrons of metal nanoparticles in resonance with the light wave (Nath *et al.,* 2007; Dubey *et al.,* 2010b). Due to the

Figure 9. Leakage of cytoplasmic reducing sugars (a) and proteins (b) from *X. campestris* pv. *malvacerum* treated with silver nanoparticles of *Ulva fasciata* crude ethyl acetate extract at different concentrations (10, 20, 40, 80 and 160 ìg/ml)



(a)



(b)

excitation of plasma resonances on inter-band transitions, some metallic nanoparticles dispersions exhibit unique bands/ peaks (Creighton and Eadont, 1993). The broadness of the peak is a good indicator of the size of the nanoparticle. As the particle size increases, the peak becomes narrower with a decreased bandwidth and increased band intensity (Petit *et al.,* 1993; Kong and Jong, 2006). The stabilizing properties of fatty acids such as palmitic acid of hexadecanoic acid were reported earlier (Chepuri *et al.,* 2006; Uznanski and Bryszewska, 2010).

The growth curves of *X. campestris* pv. *malvacearum* exposed to SNPs indicated that SNPs could inhibit the growth and reproduction of this bacteria. At low concentrations SNP's could prolong the lag phase until the concentration of SNP's was up to 40 ìg/ml, that was found to be its MIC. The inhibitory effect of silver is probably the sum of distinct mechanisms of action. Some studies reported that silver ions react with SH groups of proteins (Liau *et al.,* 1997; Feng *et al.,* 2000) and play an essential role in bacterial inactivation (Morones *et al.,* 2005). It is also reported to uncouple respiratory electron transport from oxidative phosphorylation which inhibits respiratory chain enzymes or interferes with membrane permeability to protons and phosphate (Feng *et al.,* 2000). The presence of silver ions and sulphur in the electron dense granules observed after silver ion treatment in the cytoplasm of bacterial cells suggests an interaction with nucleic acids that probably results in the impairment of DNA replication (Feng *et al.,* 2000). Thus it is reasonable to infer that the biosynthesized nanosilver can be used to manage the disease caused by *X. campestris* pv. *malvacearum* in cotton plant; there is a high probability of generating a new antibacterial agent. Li *et al*. (2010) reported the antibacterial mechanism of SNP's towards *Escherichia coli* as a model organism. They reported the leakage of proteins and reducing sugar at 2 hrs of exposure to 100 µg/ml of SNP's. However, in our studies we recorded the cytolysis of *X. campestris* pv. *malvacearum* cell at different time intervals ranging between 2, 4, 6 and 12 hrs and using different concentrations of SNP's (10, 20, 40, 80 and 160 µg/mL). The protein and reducing sugar leakage was maximum at 40 µg/mL even after 1 hr of exposure which clearly explains the "formation of pits" in the cell membrane of *X. campestris* pv. *malvacearum* resulting in the "oozing out" of the proteins and reducing sugars from the cytoplasm of bacterial cell.

Recently proteomic analysis revealed that even a short exposure of nanosilver to *Escherichia coli* cells resulted in alterations in the expression of a panel of envelope and Heat Sock Proteins (HSP) (Lok *et al.,* 2006). Therefore these particles can penetrate and can disrupt the membranes of bacteria. A massive loss of intracellular potassium was induced by nanosilver. Furthermore the nanosilver decreased the ATP levels. The possible molecular targets for the nanosilver could be protein thiol groups (respiratory enzymes). The phospholipids portion of the bacterial membrane may also be the site of action for the nanosilver.

The development of reliable, economical and eco-friendly process for the synthesis of metallic nanoparticles is a critical need in the field of nanotechnology. In this investigation, we reported natural, biological, low cost *U. fasciata* ethyl acetate extract as a reducing and capping agent. This process eliminates the usage of hazardous chemicals and solvents. The biogenic nanosilver showed promising antibacterial activity against *X. campestris* pv. *malvacearum* an economically important pathogen of cotton plant causing severe yield loss throughout the cotton growing regions all over the world.

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