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Research article

BIOSYNTHESIS OF SILVER NANO-PARTICLES BY THE BACTERIUM *MICROCOCCUS LUTEUS*

A.Babu Vimalanathan, Vinita Ernest, K Arumugasamy and Manoj G Tyagi

Department of Pharmacology, Christian Medical College, Vellore, Tamilnadu, India

ABSTRACT: Nanotechnology is the application of science to control matter at the molecular level. It is a field that is burgeoning in the recent times, making an impact in all spheres of human life. Microorganisms play an important role in the eco-friendly synthesis of metal nanoparticles. The use of microorganisms for the synthesis of nanoparticles in the lime light of modern nanotechnology is a novel approach. Biological methods of synthesis have paved the way for the greener synthesis of these nanoparticles which can have application in biomedical sciences. In this study, we report the synthesis of nanoparticles of silver by the reduction of aqueous Ag+ by a culture of ***Micrococcus luteus.*** The formation of silver nano-particles was monitored by X-ray diffraction spectroscopy (XRD) and elucidated by transmission electron microscopy (TEM).

Key words: Silver, nano-particles, Micrococcus luteus, ultraviolet, XRD and TEM.

INTRODUCTION

Nano-biotechnology, is an emerging field of nanoscience, and utilizes nanobased systems for various biomedical applications. This rapidly developing field of nanoscience has raised the possibility of using nanoparticles in the diagnosis and treatment of human cancers and also in therapeutics (Yezhelyev et al 2006). Nanotechnology involves the tailoring of materials at the atomic level to attain unique properties which can be suitably manipulated for the desired applications (Gleiter, 2000). In the last few years several pharmaceutical companies have obtained approval from the US Food and Drug Administration (FDA) for the development of nano-technology based drugs. The global market for medical nano-technology is expected to reach more than $3 billion within the next five years (Sahoo et al 2008). A recent report based on a study by the European Science Foundation has stated that there is a need for large investment in developing new nano-technology based medical tools for diagnostics and therapeutics (Waggner et al 2006).Silver was known only as a metal until the recent advent of the nano-technology era, when it became recognised that silver could be produced at the nanoscale and metallic silver has been subjected to recent engineering technologies, resulting in ultrafine particles, the sizes of which are measured in nanometers (nm) and possess distinctive morphologies and characteristics (Silver et al 2006, Klasen 2006). Nanoscale particles and molecules are a potential alternative for treatment of disease because they have unique biological effects based on their structure and size, which differ from traditional small-molecule drugs (Waggner et al 2006). At the present time, there is a growing need to develop environmentally friendly nanoparticles synthesis processes that do not use toxic chemicals in the synthesis protocol. (Whitesides, 2003). Microorganisms, such as bacteria and fungi, now play an important role in the remediation of toxic metals through the reduction of the metal ions. (Fortin & Beveridge, 2000, Klaus et al 1999). The bacteria, ***Pseudomonas stutzeri*** is interestingly resistant to silver and this property is attributed to the accumulation of silver crystals of dimension 200nm and of a well-defined composition and shape, inside the cell (Klaus et al 1999). Nanoparticles present a higher surface area-to –volume ratio with decrease in the size of the particles. Specific surface area is relevant for catalytic activity and other related properties such as anti-microbial activity of Silver nanoparticles (Gupta & Silver, 1998, Korihara et al 2005, Pal et al 2007). Biologically synthesized silver nanoparticles could have many applications in the form of cosmetics (Peurgini et al 2002), drug delivery systems (Jin and Ye, 2007), therapeutics (Czupyna et al 2006), treatment of Cancers (Arora et al 2008) and in Biosensors (Prow et al 2006).

Silver nanoparticles have been found to have strong antimicrobial activity, because of which they are used in wound dressings, contraceptive devices, surgical instruments and bone prostheses (Kowshik et al 2003, Duran et al 2005, Cho et al 2005, Mukherjee et al 2001),and also coated on the ocular lens for the prevention of microbial activity.(Alt et al 2004) Moreover silver nanoparticles are reported to posses antifungal activity (Kim et al 2009) and, anti-inflammatory effect (Nadworny et al 2008) and anti-viral activity (Rogers et al 2008).Silver nanoparticles could be well applied in therapy when the effects of silver nanoparticles are completely delineated and well understood. It has been demonstrated that, at low concentrations, silver is nontoxic to human cells.(Klaus et al 1999, Shrivastava et al 2007).It has also been reported that Ag+ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the cytoplasmic membrane (Hott & Bard, 2005).The interaction of Ag+ions with bacteria is directly related to the size and shape of the nanoparticles.(Korihara et al 2005, Morones et al 2005).The Green synthesis of silver nanoparticles involves two major steps, which must be evaluated based on green chemistry perspectives, including (1) selection of solvent medium and (2) selection of environmentally benign reducing agent.(Raveendran et al 2003, Sharma et al 2008).

The reduction of silver ions (Ag+) in aqueous solution generally yield colloidal silver with particle diameter in the range of nanometers (Wing et al 2005, Kapoor et al 1994). Initially, the reduction of various complexes with Ag+ ions leads to the formation of silver atoms (Ag◦),which is followed by agglomeration into oligomeric clusters (Henglein, 1989).These clusters eventually lead to the formation of colloidal Ag+ nanoparticles (Sharma et al 2008, Kapoor et al 1994).The band exhibited during the absorption spectrum is attributed to collective excitation of the electron gas in the particles, with a periodic change in electron density at the surface (Gutierrez & Henglein, 1993). Size control during synthesis of particles is an important criterion in the area of silver nanoparticles biosynthesis. Depending on the size of the nanoparticles, their applications branch out. Although silver nanoparticles are synthesized both intra- and extracellularly, the later method of biosynthesis of nanoparticles is highly advantageous, because of the ease of control over the environment, large-sacle synthesis and easy downstream processing steps. (18). Many Microorganisms synthesize silver nanoparticles extra-cellularly, among which Fusarium oxysporum (Anilkumar et al 2007), Bacillus licheniformis (Kalimuthu et al 2008), Klebsiella pneumonia (Shahverdi et al 2007) have been studied extensively. Hence, in this article, biosynthesis of Silver nanoparticles was investigated using the bacterium, *Micrococcus luteus* isolated from sewage water samples supplied by the local municipality. This study used microscopic characterization using TEM-Transmission electron microscopy and X-ray diffraction spectroscopy (XRD) analyses of the silver nanoparticles. In this study we report the use of the non-pathogenic culture of *Micrococcus luteus* for the synthesis of silver nanoparticles from the stand point of ease mass production and safety in handling the organism. Apart from its eco-friendliness, compared with fungi, *Micrococcus luteus* as a bio-manufacturing unit has the added advantage of ease of handling.

MATERIALS AND METHODS

Isolation of the Bacterium:

Sewage samples were collected from municipal wastes in sterilized falcon tubes. They were serially diluted and spread on the nutrient agar plates. The plates were then incubated at 37◦C for 23 h.

Characterization of the isolates

The morphological and physiological characterization of the aerobic isolate was performed according to the methods described in Bergey's manual of determinative bacteriology (Baird Parker, 1974).

Biosynthesis of Silver nano-particles

The characterized isolate was inoculated into sterile nutrient broth (NB) and 2g of wet biomass (*Micrococcus luteus*) was taken in an Erlenmeyer's flask. 1 mM silver nitrate solution was prepared using deionised water and 100 ml of the solution mixture was added to the biomass. Then the conical flask was in a shaker at 37◦C (200rpm) for 48h for the synthesis of nanoparticles (36).Finally resulting solution was filtered through a 0.22µm filter (Millipore), and used for further purposes. The size distribution of the nanoparticles was evaluated using UV Spectrophotometers (ELICO) with a resolution of 1nm.

Sonication of the samples

The cells from each Erlenmeyer flasks were washed twice with 60mM Phospahte buffer (pH 7.0) and resuspended in 1ml of the same buffer. Ultrasonic disruption of cells was carried out with an ultrasonic processor (sonic vibra cell VC-505/220, Newston, USA).Over three 15 periods and with an interval of 45s between periods. The sonicated samples were centrifuged at 15,000 rpm for 30 min at 4◦C to remove cell-debris. The supernatants were then used for the characterization of silver nanoparticles.

Evaluation of maximum nano-particles synthesis

To determine the time-point of maximum production of silver nanoparticles, the absorption spectra of the supernatant were taken using a UV spectrophotometer (ELICO) with a resolution of 1nm.

Characterization of Silver nanoparticles

To check phase formation and purity, powder XRD patterns were recorded using XDL3000 powder X-ray diffractometer.The supernatant from the maximum time point of production (of silver nanoparticles) was air-dried and subjected to XRD using XDL 3000 powder X-ray diffractometer.The micrograph were recorded by focusing on clusters of particles. The XRD spectrum of silver nanoparticles exhibited two theta values**.** The synthesized silver nanoparticles were confirmed by XRD. X-ray diffraction spectroscopy measurements of the bio-reduced silver nitrate solution drop-coated onto class substrates were done for the determination of the formation of Silver nanoparticles.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) (JEOL model 1200 EX) is a microscopy technique; where by a beam of electron is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through it. The supernatant from the maximum time point of production (of silver nanoparticles) was subjected to TEM using (JEOL model 1200 EX).Silver nanoparticles image was formed from interaction of the electron transmitted through the specimen; image of silver nanopartilces was magnified and focused onto an imaging device. The micrograph was recorded by focusing on clusters of particles. TEM measurements were performed on a JEOL model 1200 EX instrument operated at an accelerating voltage of 120kv and dried powder was used for TEM analysis.

RESULTS

Pure colonies were obtained and characterized as *Micrococcus luteus* based on the results described in Bergey's manual of determinative bacteriology, 8th ed. (Baird-Parker, 1974).

UV-Vis Spectra analysis

Aqueous silver ions were reduced to silver nanoparticles when added to the biomass of *Micrococcus luteus*. This was indicated by the change in colour from whitish yellow to brown (Henlein, 1993, Ahmad et al 2002) (Fig.1). The Brown colour remained stable and further characterization was done by UV-Spectrophotometer, XRD, TEM. The spectra for supernatants from all harvested biomass samples (after incubation with silver nitrate and subsequent sonication) showed maximum absorbance at 440 nm, which increased with the time of incubation of silver nitrate with the biomass (data not shown).The intensity of the colour obtained from cells harvested at the stationary phase was the maximum and also stable. Silver nanoparticles are known to exhibit a characteristic surface plasma resonance band at 430nm that can be measured using UV Spectrophotometer for silver nanoparticles. (Rajesh et al 2002). In UV-Vis spectrum, a strong, broad peak, located between 420nm and 440 nm was observed for silver nanoparticles prepared using the culture supernatant. Observation of this peak, assigned to a surface Plasmon, is well-documented for various metal nanoparticles with sizes ranging from 2nm to 100 nm (Ahmad et al 2003, Rajesh et al 2002, Sastry et al 1997, Sastry, 1998).The nanoparticles exhibited maximum absorbance at 440nm in UV Spectrophotometer.

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Figure 1: Aqueous silver ions were reduced to silver nanoparticles when added to the biomass of *Micrococcus luteus* .This was indicated by the change in colour from whitish yellow to brown

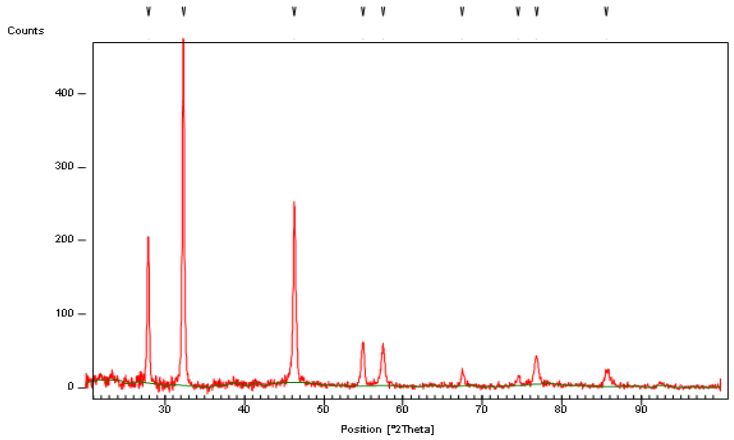

Figure 2: Confirmation of silver nano-particles by X-ray diffraction studies

Transmission electron microscopy

Transmission electron microscopy was used to determine the morphology and shape of the nanoparticles. Purified silver nanoparticles from extra cellular of bacterial culture –*Micrococcus luteus* using centrifugation was characterized by TEM. TEM revealed the average size of particles (53nm) as less than 100nm (average particle size 53nm).TEM characterization showed a uniform distribution of nanoparticles with an average size (particle size 53nm) less than 100nm and have spherical shape.(Fig .3).

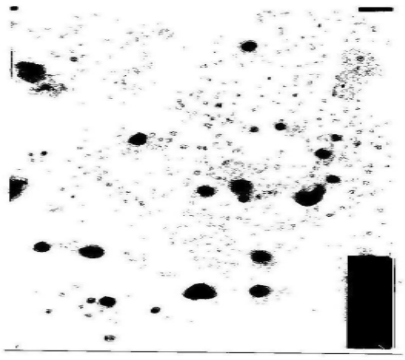

Figure 3: Transmission electron microscopy characterization of synthesized silver nano-particles with an average particle size of 53nm.

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X-ray diffraction spectroscopy (XRD)

The XRD pattern of the silver nitrate –treated sample correspond to that of silver nanoparticles. The XRD pattern shows intense peaks in the whole spectrum of two theta values ranging from 25 to 90. It is important to know the exact nature of the silver particles formed and this can be deduced from the XRD spectrum of the sample. XRD spectrum confirmed that the silver particles formed in our experiments were in the form of nanoparticles, as evidence by the peaks at two theta values and planes for silver respectively. The reflections were used with the Debye-Scherrer equation to calculate the size of the nanoparticles. The particle sizes obtained from XRD line broadening agreed well with those obtained from TEM. From these the average particle size was found to be around 53nm as less than 100nm. X-ray diffraction pattern of the silver nanoparticles. Silver nanoparticles were synthesized from 1mM Silver nitrate – treated *Micrococcus luteus cells* at 37° C.The samples were harvested at 48h ,sonicated, air-dried and XRD pattern was observed. The synthesized silver nanoparticles were confirmed by XRD. (Fig.2). The results show that *Micrococcus luteus* could be used for the production of silver nanoparticles from silver nitrate.Moreover, biomass that is harvested at the stationary phase result in the maximum production of nanoparticles for the given incubation period.

DISCUSSION

Antibiotic resistance by the pathogenic bacteria has been observed since last decade; hence, the researchers are focusing on the development of new novel anti-bacterial agents. In the current scenario, Silver nano-particles as antimicrobial agents have come up as a promising candidate in the medical field. (Duran et al 2007).

The extremely small size of nanoparticles exhibit enhanced or different properties when compared with the bulk material. There are different physical and chemical methods for the synthesis of nanoparticles, but there is always a need for the development of eco-friendly route for the synthesis process. (Kaliswarlal et al 2008). Therefore, our current study proves to be an important step in this direction. Formation and stability of silver nanoparticles in aqueous colloidal solution were confirmed using UV-Vis spectral analysis. It is now well known that silver nanoparticles exhibit yellowish brown colour in aqueous solution due to excitation of surface Plasmon vibrations.(46). As the biomass (bacterial culture-*Micrococcus luteus*) was mixed with aqueous solution of the silver nitrate, it started to change the colour whitish yellow to brown due to reduction of silver ion, when indicated the formation of silver nanoparticles. It is generally recognized that UV-Vis spectroscopy could be used to examine size and shape-controlled nanoparticles in aqueous suspensions. (Henglein, 1993, Sastry et al 2002). The UV-Vis spectra recorded from the reaction medium. Absorption spectra of silver nanoparticles formed in the reaction media has absorbance peak at 440nm, which correspond to silver and broadening of peak indicated that the particles are polydispersed. Silver nitrate which is readily soluble in water has been exploited as an antiseptic agent for many decades. Lee et al investigated the antibacterial effect of nanosized silver colloidal solution against S.aureus and Klebsiella pneumoniae after padding the solution on textile fabrics. (Lee et al 2003). Shrivastava *et al* studied antibacterial activity against Escheria coli (ampicillin resistant), Escheria coli, Staphylococcus aureus, and Salmonella typhi (multi-drug resistant).They reported that the effect was dose dependent and was more pronounced against gram-negative organisms than gram –positive ones. They found that the major mechanism through which silver nanoparticles manifest antibacterial properties was either by anchoring or penetrating the bacterial cell wall, and modulating cellular signalling by dephosphorylating putative key peptide substrate on tyrosine residues. (Chun Nam et al 2006). Biosynthesis of Silver nanoparticles by the bacterium, aqueous silver ions were reduced to silver nanoparticles when added to the culture supernatant of bacteria. This was indicated by the change in colour from whitish-yellow to brown. (Kaliswarlal et al 2008).Thus it was evident that the metabolites excreted by the culture of the bacterium exposed to silver could reduce silver ions, clearly indicating that the reduction of the ions occur extracellular through reducing agents released into the solution by the bacterium. Silver nanoparticles were synthesized by the biomass of the bacterium, *Micrococcus luteus*. We report a green chemistry approach using *Micrococcus luteus* in the synthesis of silver nanoparticles at room temperature without using any harmful reducing agents. This is a reliable, economical, efficient, eco-friendly, simple and easy process. In conclusion, it has been demonstrated that the Micrococcus luteus bacteria is capable of producing silver nanoparticles extracellularly and these silver nanoparticles are quite stable in solution. This biosynthesis of silver nanoparticle proves to be potential candidate for medical applications, where ever antimicrobial activity is essential. A potential role for silver nano particles in the food industry is envisaged (Ernest et al 2012).

CONCLUSION

We report a green chemistry approach using ***Micrococcus luteus*** in the synthesis of silver nanoparticles at room temperature without using any harmful reducing agents. The average particle size of silver nanoparticles was found (average particle size 53nm) to be less than 100nm.This is an economical, reliable, and efficient, eco-friendly and simple process. This single–step greener approach is generally simple and cost effective. The silver nanoparticles are synthesized highly stable and this method has advantages over other methods as the organism used here is a non-pathogenic bacterium. This study would therefore lead to an easy procedure for producing silver nanoparticles with the added advantage of bio safety.

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