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BIOSYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES BY A MARINE STRAIN *ESCHERICHIA COLI* SJ101

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The present study is on characterization of silver nanoparticles synthesized by an *E.coli* SJ101 strain isolated from Uppanar estuary of Cuddalore coastal waters. The study proved that the above bacterial strain was capable of producing nanoparticles of <10µm size in abundance. Both free proteins capped and aggregated nanoparticles which were spherical in shape and varied in size were observed. Nanoparticles synthesize started by 4-10hrs in various strains tested and in the present strain used, it was continued for 10days at which the experiment was terminated due to want of time. UV spectral study indicated this. The nanoparticles produced were inhibitory to 8 pathogens among 10 tested. They were most inhibitory to *Salmonella typhi* followed by *Salmonella paratyphi* and *Staphylococcus aureus* which are proven pathogens and prevalent in Indian community. FTIR spectral analysis showed bands at at 3410 and 2940 Cm^{-1} indicating the roles of proteins (primary and secondary amines and other functional groups) in synthesis and stabilization of nanoparticles. Thus the present study revealed many facts about silver nanoparticles synthesized by *E.coli* SJ101 strain.

INTRODUCTION

Microbial contamination of water poses a major threat to public health. With the emergence of microorganisms resistant to multiple antimicrobial agents there is an increased demand for improved disinfection methods. The antimicrobial properties of silver ions were known since ancient times and silver ions are widely used as bactericide in catheters, burn wounds and dental work. Researchers have also recommended the use of silver and copper ions as superior disinfectants for wastewater generated from hospitals containing infectious microorganisms. However, residual copper and silver ions in the treated water may adversely affect human health. The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution[1].

Metal nanoparticles with bactericidal activity can be immobilized and coated on to surfaces, which may find application in various fields, i.e., medical instruments and devices, water treatment and food processing. Metal nanoparticles may be combined with polymers to form composites for better utilization of their antimicrobial activity. Metal nanoparticles are also finding application in various other fields, i.e., catalysis and sensors. However, it is also recognized that nanoparticles may have many undesirable and unforeseen effects on the environment and in the ecosystem[2].

It is established that nanosized and nanocrystalline calcium orthophosphates can mimic the dimensions of constituent components of calcified tissues[3]. Thus, they can be utilized in biomineralization and as biomaterials due to their improved biocompatibility. Further development of calcium orthophosphate-based biomaterials will obviously stand to benefit mostly from nanotechnology, which offers a unique approach to overcome the shortcomings of many conventional materials. For example, nanosized ceramics can exhibit significant ductility before failure contributed by the grain-boundary phase. In 1987, Karch *et al*[4] reported that, with nanograin dimensions, a brittle ceramic could permit a large plastic strain up to 100%. In addition, nanostructured ceramics can be sintered at a lower temperature; thereby problems associated with high

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temperature sintering processes are also decreased. Thus, nanosized and nanocrystalline bioceramics clearly represent a promising class of orthopedic and dental implant formulations with improved biological and biomechanical properties[5].

Many other advances have been made in the biomaterials field due to the rapid growth observed in nanotechnology. For example, the recent theory of ‘‘aggregation-based crystal growth’’ and the new concept of ‘‘mesocrystals’’ have highlighted the roles of nanoparticles in biological crystal engineering. In this respect, the study of calcium orthophosphates is a specific area in nanotechnology, because calcium orthophosphates may be applied readily to the repair of hard skeletal tissues of mammals. Concept of ‘‘mesocrystals’’ have highlighted the roles of nanoparticles in biological crystal engineering. In this respect, the study of calcium orthophosphates is a specific area in nanotechnology, because calcium orthophosphates may be applied readily to the repair of hard skeletal tissues of mammals[6].

Although it is widely accepted that the prefix ‘‘nano-’’ specifically refers to 10^{-9} m, in the context of nanosized and nanocrystalline materials, the units should only be those of dimensions, rather than of any other unit of scientific measurement. Besides, for practical purposes, it appears to be unrealistic to consider the prefix ‘‘nano-’’ to solely and precisely refer to 10^{-9} m, just as it is not considered that ‘‘micro-’’ specifically and solely concerns something with a dimension of precisely 10^{-6} m[7].
Recent advancement in technology has introduced silver nanoparticles into the medical field. As studies of silver nanoparticles improve, several silver nanoparticles based medical applications have been developed to prevent the onset of infection and promote faster wound healing.

The coating of medical instruments with silver nanoparticles is under study. A combination of the bacteriolytic action of lysozyme and biocidal activity of silver nanoparticles were synthesized together as a form of antimicrobial coating on medical instruments. This coating is done on surfaces of stainless surgical steel blades and needles through an electrophoretic process. The efficacy of the antimicrobial coatings was tested in an environment that mimics the normal use of the surgical instruments. The environment was an *in vitro* lytic assay, in which punctures and incisions were done into an agar that has been inoculated with bacteria. Bacterial cell destruction was reported on the areas where the surgical instruments coated with silver nanoparticles came in contact with. This means that the antibacterial properties of the silver nanoparticles were transferred to the surface where the surgical instruments came in contact with, while the same antibacterial properties were retained in the blades and needles.

Infections due to catheter use are common in hospitals, especially in-dwelling catheters. Use of nanopartices in catheters is another example of a medical application for silver nanoparticles that is gaining popularity. A study entitled “Antimicrobial Surface Functionalization of Plastic Catheters by Silver Nanoparticles” was published in, “The Journal of Antimicrobial Chemotherapy”[8] supported the value of using silver as a coating on catheters. A silver coating was proven to be effective against methicillin-resistant *Staphylococcus aureus* (MRSA), a common pathogen that causes infections in surgical sites. The cited study observed the action of microbial and biofilm formations in catheter-related infections. A layer of sustained-release of silver nanoparticles was applied on the catheters that exhibited antimicrobial properties for a 10-day period. Major *in vitro* antimicrobial activity was noted in the coated catheters, as well as inhibition of biofilm formation of several strains of bacteria including *Escherichia coli*, *Enterococcus* and *Staphylococcus aureus.* This finding is quite significant as patients who have in-dwelling catheters are at high risk in developing urinary tract infections due to prolonged use.
Several studies and experiments are being conducted with the use of silver nanoparticles to promote wound healing and prevent infection through topical applications. Wound dressings are now made with silver nanoparticles for local treatment of ulcers of the limbs as well as treatment of deep second degree and third degree burns. The dressings can either be used in a hospital setting or applied in an ambulatory procedure. The nanoparticles work by coagulating and dissecting intracellular compounds, resulting in cellular breakdown and destruction. Significant levels of antibacterial activity were observed even in the presence of low silver nanoparticle concentration.

There are many different synthetic routes to produce silver nanoparticles. They can be divided into three broad categories: physical vapor deposition, ion implantation, or wet chemistry. Biosynthesis of nanoparticles is a recent development and that route was attempted in the present study.

Over the last decades silver nanoparticles have found applications in catalysis, optics, electronics and other areas due to their unique size-dependent optical, electrical and magnetic properties. Currently most of the applications of silver nanoparticles are in antibacterial/antifungal agents in biotechnology and bioengineering, textile engineering, water treatment, and silver-based consumer products. Silver is also promoted within alternative medicine in the form of *colloidal silver*, although its use is controversial. Hippocrates, the "father of medicine", wrote that silver had beneficial healing and antidisease properties[9]. Samsung has created and marketed a material called Silver Nano that includes silver nanoparticles on the surfaces of household appliances. Silver nanoparticles have been used as the cathode in a silver-oxide battery.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from Uppanar estuary, Cuddalore. Samples were transferred in to sterile plastic bags kept at 4°C up to further processing.

Isolation of bacterial cultures

Isolation of soil bacteria was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-3} to 10^{-6}. A volume of 0.1 ml

of each dilution was transferred aseptically to Zobell marine agar plates. The sample was uniformly distributed by using a sterile glass spreader. The plates were incubated at 37°C for 24hrs. The bacterial isolates were further subcultured on the nutrient agar plates in order to obtain pure culture. Pure isolates were maintained at 4ºC in a refrigerator for further studies.

Biosynthesis of silver nanoparticles

Colonies were selected based on their morphology, ensured for their purity and inoculated into nutrient broth. The flasks were incubated at 37°C for 24h in a rotary orbital shaker at a speed of 150 rpm. The biomass was harvested after 24hrs of growth by centrifugation. The biomass was washed with sterilized distilled water to remove any medium component. 8g of biomass (fresh weight) was mixed with 200 ml of deionized water in a 500 ml Erlenmeyer flask and agitated in the same condition for 24h at 37°C. After the incubation, the cell filtrate was obtained by passing it through 0.45µ filter. Filtrate was collected and used further for nanoparticle synthesis.

For the synthesis of silver nanoparticles, 50 ml of 1mM AgNO3 solution was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 150rpm at 37°C in dark. Control (without the silver ion, only biomass) was also run along with the experimental flask .

Microbial identification

Identification was done based on morphological, cultural, biochemical and physiological characteristics based on Bergey's Manual of Determinative Bacteriology.

Characterization of synthesized silver nanoparticles Antibacterial activity

(Schillinger and Lucke[10])Nutrient broth was prepared and sterilized. Ten different human pathogens such as *Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi, Klebsiella oxytoca, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Lactobacillus vulgaris, Vibrio cholerae* and *Klebsiella pneumoniae* were inoculated separately and kept for incubation.

Each bacterial suspension was spread over the surface of Muller-Hinton agar Plates. Then 100µl of sample was filled in 8mm diameter wells cut in the plates. The inoculated plates were incubated for 24hrs at 37°C, and the diameter of the inhibition zone was measured with calipers in millimeter from the edge of zone to the edge of the well.

UV–visible spectroscopic analysis

The reduction of silver ions was confirmed by qualitative testing of supernatant by UV–visible spectrophotometer. 1 ml of sample supernatant was withdrawn after 24 hrs and absorbance was measured by using UV–visible spectrophotometer on 420 nm and scanning the spectra between 250 and 800 nm at the resolution of 1 nm.

Fourier Transform Infrared (FTIR) Spectroscopy analysis

The lyophilized sample was subjected to FTIR Spectroscopy analysis. Two milligrams of the sample was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4000-450 cm^{-1} in FT-IR spectroscopy at a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION

Bacterial cultures were isolated from the soil samples collected from Uppanar estuary, Cuddalore. The bacterial isolates were characterized on the basis of colony morphology and biochemical methods.

Table 1 Antibacterial activity of *E.coli*

S.No.	Name of the organism	Zone of inhibition (mm)
1.	*Staphylococcus aureus*	14
2.	*Salmonella typhii*	21
3.	*Salmonella paratyphii*	13
4.	*Klebsiella oxytoca*	12
5.	*Pseudomonas aeruginosa*	10
6.	*Escherichia coli*	5
7.	*Proteus mirabilis*	6
8.	*Lactobacillus vulgaris*	4
9.	*Vibrio cholerae*	-
10.	*Klebsiella pneumoniae*	-

Table 2 Production of silver nanoparticles by *E.coli*

Hours	Optical density at 420nm
24	0.737
48	1.043
72	1.201
96	1.300
120	1.578
144	1.802
168	2.041
192	2.284
216	2.351
240	2.407

Cell free filtrate of *E.coli* was mixed with silver nitrate solution and incubated in dark in a rotary shaker. Samples showed change in colour from almost colourless to brown, which is a clear indication of the formation of silver nanoparticles in the reaction mixture. The intensity of the colour was increased during the period of incubation. The appearance of brown colour was due to the excitation of surface plasmon vibrations. Control showed no change in colour of the mixture when incubated in the same conditions.

Table 3 FTIR peaks and their respective assigned functional groups

S.No.	FTIR peak	Assigned functional groups
1	3410	N-H Stretch-medium
2	2940	C-H Stretch-strong
3	2304	C≡N Stretch-medium
4	2344	C≡N Stretch-medium
5	2106	C≡N Stretch-medium
6	1654	C=O Stretch-medium
7	1637	C=C Stretch-medium
8	1629	C=C Stretch-medium
9	1618	C=C Stretch-medium
10	1611	C=C Stretch-medium
11	1437	C-H Bend in plane
12	1406	C-H Bend in plane
13	1084	C-N Stretch

Kathiresan *et al*[11] have reported the biosynthesis of silver nanoparticles by a marine fungus, *Penicillium fellutanum.* The fungal culture was isolated from the rhizosphere soil of *Rhizophora annamalayana*. The synthesis of silver nanoparticles was controlled with respect of pH, temperature, silver ion concentration and exposure time to silver nitrate.

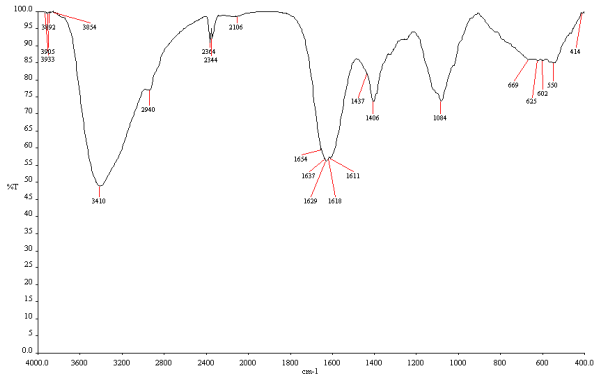

Figure 1 FTIR spectra recorded from powder of silver nanoparticles

The study also showed that among 10 pathogens tested it was inhibitory to 8 pathogens. It was noteworthy that *S. typhi* and *S. paratyphi* and *Staphylococcus aureus* were inhibited to a greater extent than the other pathogens tested (Table 1). As these three pathogens are developing resistance to most of the antibiotics in use, the present study suggests silver nanoparticles as an alternative to this problem. Nithya and Ragunathan[12] reported that silver-nanoparticles were most inhibitory to *Pseudomonas aeruginosa* (14mm) followed by *E.coli* and *Staphylococcus aureus* (11mm). However Kathiresan *et* al[13] reported greatest antibacterial activity of silver nanoparticles to *K. pneumoniae* and lowest to *Micrococcus luteus.* The results indicated that size and concentration of nanoparticles synthesized might play role in exhibiting antimicrobial activity.

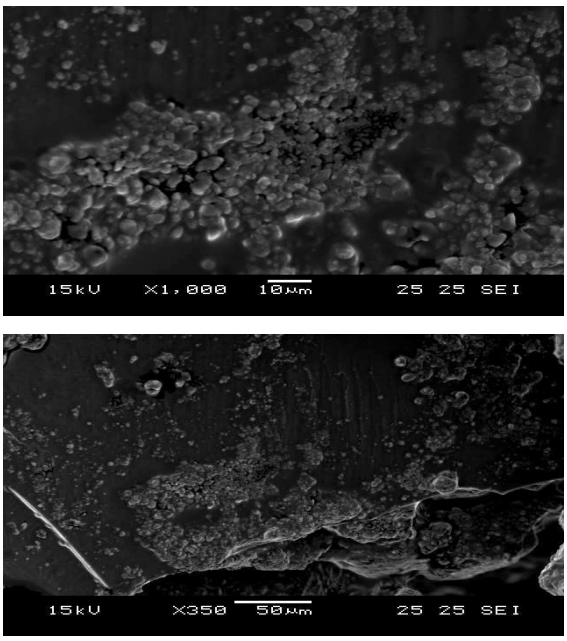

Figure 2 Scanning electron micrograph of silver nanoparticles

Though in the present study after 4-10hrs of incubation visual colour change observed showing the Ag-nanoparticle synthesis, the UV-spectrophotometer reading confirmed that even after 10 days the synthesis continued (Table 2). This observation was contradictory to that of Kathiresan *et* al[13] who observed a decline in nanoparticle synthesis after 4hrs of incubation. The FTIR spectra of *E.coli* filtrate was very different from that reported by them. This along with other reported insisted that various proteins and diverse enzymes might be involved in nanoparticle synthesis. This observation made in the present study deserves further research.

FTIR showed two stretching vibrations at 3410 and 2940 Cm^{-1} referring to primary and secondary amines. Bands at 2304, 2344, 2106 and 1084 Cm^{-1} can be assigned to C≡N stretching. 1629, 1637 and 1654 Cm^{-1} referred the presence of Amide carbonyl stretching (Table 3 & Figure 1). Thus the FTIR spectrum signatures showed that proteins are present in the lyophilized sample. It is well known that protein is involved in binding and stabilizing silver nanoparticles. The role of proteins in plant extracts as reducing and capping agents forming stable and shape-controled silver nanoparticles had been reported by Sharma *et al*[14].

Amide carbonyl stretching was observed in 1640-1670 Cm^{-1} region 1629, 1637 and 1654. FTIR measurements of the freeze-dried samples were carried out to identify the possible interactions which may be responsible for synthesis and stabilization (capping material) of silver nanoparticles. The amide linkages between amino acid residues in proteins give rise to well known signatures in IR region of electromagnetic spectrum.

The Scanning Electron Micrograph revealed the presence of <10µm particles in the sample (Figure 2). The SEM analysis also showed the presence of both protein-capped free and aggregated nanoparticles.
Earlier Shaligram *et* al[15] have reported the biosynthesis of silver nanoparticles using aqueous extract of *Penicillium brevicompactum* WA 2315. The size of synthesized nanoparticles was found to be 58.35±17.88 nm by SEM and TEM analysis.

The SEM photograph also showed almost all the particles produced were of perfect round in shape. The present study thus reaffirm the fact that free amine groups, cysteine residues or negatively charged carboxylate groups are involved in stabilization of metal nanoparticles. Thus the present study clarified many facts regarding the silver nanoparticles synthesized by *E. coli* SJ 101 strain isolated from Uppanar estuary.

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