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ANTIBACTERIAL EFFICACY OF EXTRACELLULAR SILVER NANOPARTICLES BIOFABRICATED FROM CHROMIUM REDUCING BACTERIA

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ABSTRACT

Use of microbes is very promising approach for the synthesis of technology based, ecofriendly, cost effective and biocompatible nanoparticles possessing unique physical and chemical properties is one of the developments in nanobiotechnology. Bacterial flora obtained from the tannery effluent was identified as Pseudomonas putida. Biosynthesis of silver nanoparticles (AgNPs) by using culture supernatant of P. putida was investigated and the formation of silver nanoparticles was confirmed by the change in colour of the culture filtrate from yellow to brown. Furthermore, the silver nanoparticles were characterized by means of UV-Visible spectroscopy and it showed absorption peak at around 440 nm which corresponds to the Plasmon resonance of silver nanoparticles. The XRD pattern showed the presence of sharp reflections at (111), (200), (220), (311) which indicate the presence of biologically synthesis of silver nanoparticles. The Scanning electron micrograph showed the spherical natures of particles have size ranged from 20-100 nm and possess an average size of 60 nm. A highly significant antimicrobial activity against pathogenic bacteria by the bio fabricated Ag NPs was also revealed by the highest zone of inhibition of 20 mm dia. Therefore, this novel bacterial strain could be used for biofabrication of AgNPs and ultimately in the nanoparticle based drug formulation for the treatment of bacterial diseases.

KEYWORDS: *Biofabrication, Bacteria, Chromium, Pseudomonas Putida, Silver Nanoparticle*

INTRODUCTION

One of the most important criteria of nanotechnology is that of the development of clean, nontoxic, and environmentally acceptable “green chemistry” procedures, involving organisms ranging from prokaryotes to eukaryotes. The biosynthetic method employing bacteria, fungi and plant parts is proving as a simple and cost – effective method for the synthesis of nanoparticles.

Microorganisms are recently found as possible eco-friendly nanofactories, even though they have many biotechnological applications such as remediation of toxic metals [1]. Formation of silver nanocrystallites in microorganisms including bacteria, yeasts and fungi has been reported [2,3]. Some of the most commonly used microorganisms for developing microbes based silver nanoparticles (AgNPs) include *Escherichia coli* [4], *Staphylococcus aureus* [5], *Bacillus megaterium* [6], *Bacillus cereus* [7], *Salmonella typhimurium* [8], *Serratianemato diphila* [9], *Pseudomonas fluorescens* [10], *Fusarium* spp. [11] and *Aspergillus* spp.[12]. The ability of bacterial strain to reduce nitrate has been exploited in the reduction of silver nitrate (AgNO3) in to elemental nanomaterial [13].

Due to certain difficulties associated with physicochemical methods adapted for detoxification/remediation of Cr contaminated sites, biological treatments in recent times have received greater attention being an economical and environment friendly as compared to conventional technologies. The bioremediation strategy involves the conversion of Cr(VI) into less toxic and less mobile Cr(III), which consequently is immobilized in the soil matrix [14]. In this context, many microbes have been reported to reduce Cr(VI) under aerobic and anaerobic conditions [15].

Considering the significance of bio-based fabricated nanomaterials, the present study was carried out to find bacterial strain isolated from the heavy metal contaminated sites and to characterize the strain through biochemical approaches. The bacterial strain was further tested for its chromium reducing ability. The bacterial strain was also used to synthesize AgNPs at room temperature in the absence of any reducing agent. The resulting AgNPs were subsequently characterized using some of the standard analytical techniques like, UV–visible, SEM and XRD spectroscopy. Antibacterial activity on both Gram-positive and Gram-negative bacteria of biofabricated AgNPs was evaluated.

MATERIAL AND METHODS

Isolation and Bacterial Characterization

The effluent samples were collected in sterile containers (2 litres capacity) from the raw discharge point of tannery industry located at the outskirts of Vaniyambadi, Tamilnadu, India.

In order to isolate the bacterial strain, a serial dilution assay was carried out in normal saline solution and 10 mL of diluted suspension was spread plated on nutrient agar (NA) medium. Plates were incubated at 30±2 °C in the dark for 48 hrs. Subsequently, eight isolates were selected according to their morphological shapes and characterized. Biochemical activities were tested through Mae Conkey agar test, IMViC test whereas, physiological activities were tested by Starch hydrolysis, Urea hydrolysis, Nitrate reduction, Hydrogen sulfide production, Cytochrome oxidase and Catalase tests for the identification of bacteria [16].

Optimization of Growth and Hexavalent Chromium Reduction Conditions

The effect of viable bacterial populations and pH on the reduction of Cr(VI) was assessed using nutrient broth (NB) containing 100 µg/ml of Cr6+. The sterilized medium was adjusted to pH 2 to 12 with 1 M HCL or 1 M NaOH. A 100 ml of exponentially grown culture of *Pseudomonas putida* was inoculated into NB medium containing 100 µg/ml of Cr(VI) and incubated at 35±2°C in an orbital shaking incubator at 120 rpm upto 48 h. For Cr6+ reduction, 1 ml culture from each flask was centrifuged (6000 rpm) for 10 min at 20°C, and Cr6+ in the supernatant was determined by Atomic Absorption Spectroscopy [17].

Medium and Growth Conditions for Supernatant Preparation

The bacterial isolate *Pseudomonas putida* was inoculated in sterile NB medium (pH 7.2). Bacteria were allowed to grow at 35±2°C for 24 h in a 500 ml Erlenmeyer flask with working volume of 300 ml with agitation at 120 rpm on orbital shaking incubator. Culture medium was then centrifuged at 5000 rpm to obtain cell-free supernatant [18].

Preparation and Characterization of AgNPs

To obtain AgNPs, 2 ml supernatant extracted from exponentially grown bacterial culture was added to 98 ml of 1 mMAgNO3 solution [19]. The reaction mixture was incubated in dark at room temperature.

Purification of AgNPs

Bacterial supernatant was used for the synthesis of AgNPs. Bioreduction of silver was monitored in the wavelength ranging from 200-1100nm. UV-Vis spectroscopy measurements of silver nanoparticles were recorded on Shimadzu dual beam spectrophotometer (model UV-1650 PC) operated at a resolution of 1nm. Since AgNPs are soluble in water, the change in colour was observed. A yellowish brown colour formation was noticed during the synthesis phase.

The synthesized particles were washed four times by centrifugation and redispersed in double distilled water to remove the remaining unconverted silver ions.

XRD (X-Ray Diffraction) Analysis

X-ray diffraction pattern was measured in the scanning mode on an X' PERT-PRO analytical instrument operated at 40KV and a current of 30 mA with cu Kα radiation in BHAT Biotech Pvt Ltd, Bangalore. The diffraction intensities were recorded in 2θ range from 100 to 790°. The diffraction intensities were compared with the standard JCPDS files. The software gave the information about the crystal structure of the particles. The average size of the particle can be estimated using the Debye Scherrer equation.

The size of the silver nanoparticles was made from the line broadening of the (111) reflection using the following Debye-Scherrer's formula.

$D = K\lambda/\beta Cos\theta$

Where D = thickness of the nanocrystals,

K = Constant,

λ = Wave length of x-rays

β = Width of half maxima of reflection at Bragg's angle 2θ,

θ =Bragg's angle.

Scanning Electron Microscopy Analysis

Morphology of the Ag NPs was examined using scanning electron microscopy (JEOL - 6390 SEM). The sample was prepared on a carbon coated grid by dropping a small amount of the sample and then allowed to dry prior measurements. The synthesized silver nanoparticles can be calculated by using the scale provided in the micrograph.

Antibacterial Activity of Silver Nanoparticle Against Urinary Tract Infection (UTI) Pathogens

Antibacterial Assay

The biofabricated AgNPs were tested for bactericidal activity by agar disc-diffusion method against Gram positive *Staphylococcus aureus* and Gram negative *Klebsiella pneumonia*, *E.coli* and *Proteus vulgaris.* The pure culture of each bacterium was sub-cultured in in nutrient broth for 12h at 37^{o}C. Each bacterial strain was spread uniformly onto the individual plates by using sterile glass rod spreader.

Whatman No. 1 filter paper disc of 6mm diameter were prepared and sterilized. Sterilized discs were soaked with different concentrations such as 25 mg/ml, 50mg/ml and 100 mg/ml of silver nanoparticles. The bactericidal activity was

determined by a clear inhibition zone around the sample loaded wells after incubation of plates overnight at 37^{o}C.

`Filter paper discs with impregnated solution of synthesized silver nanoparticles and control discs (without silver nanoparticles) were carefully dispensed at uniform distance over the agar surface and were pressured for correct implantation. Incubation period of 48-72 hours at 35°C was maintained for observation of antibacterial activity of the silver nanoparticles. The antibacterial activity was evaluated by measuring zones of inhibition of bacterial growth surrounding the silver nanoparticles. The complete antibacterial analysis was carried out under strict aseptic conditions. The zones of inhibition were measured with antibiotic zone scale in mm dia and the experiment was carried out in triplicates.

RESULTS

Characterization of Bacterial Strain

In the present study, Cr(VI) resistant bacterium was isolated from tannery effluent water. Out of the eight bacterial isolates, S03 strain was selected especially due to its ability to tolerate high level of most toxic form of chromium and was characterized morphologically and biochemically (Table1).

Strain S03 grew well on nutrient agar plates mixed with 435mg K2Cr2O7/ ml. The chromium resistant bacteria strain was found to be Gram-negative and rod shaped. The freshly grown cultures showed a positive reaction for Mac Conkey agar, citrate utilization, hydrogen sulphide production, cytochrome oxidase, catalase, ammonia production, IAA, phosphate solubilisation and dextrose tests.

Strain S03 was presumptively identified as ***Pseudomonas putida*** **o**n the basis of the biochemical and physiological characteristics compared with those listed in Bergey`s Manual of Determinative Bacteriology,

Chromium Reduction Assay

The effect of pH on chromium reduction by *Pseudomonas putida* was shown in the Figure 1. The bacterial strain grew well at pH 7 and found to remove hexvalent chromium by 94% after 48 h of growth. However, with increase or decrease in pH, there was a corresponding decrease in bacterial growth which subsequently affected the reduction of Cr(VI) very negatively (Figure 3). A cent percent decrease in chromium reduction was observed at pH 2 compared to those recorded at pH 7.

Characterization of Biofabricated AgNPs

The colour of reaction mixture changed from colourless to brown in 2hrs when the supernatant prepared from strain S03 was added to solution of AgNO3 (Figure 2). The intensity of colour further increased with increasing incubation periods. NADPH-dependent nitrate reductase enzyme function as a reducing agent to reduce the silver nitrate solution to produce the AgNPs.

The synthesis and stability of the AgNPs synthesized in the solution was confirmed by UV– vis spectral analysis with aliquots of the reaction mixture. The formation and stability of the reduced silver nanoparticles was analysed by UV-Vis spectrum. In biological method, the UV-Vis spectrum of the silver nanoparticles showed at 420 nm (Figure 3) which indicated the formation of silver nanoparticles.

X-Ray Diffraction

An XRD pattern obtained for the silver nanoparticles is shown in Figure 4. A number of Bragg’s reflections

corresponding to (111), (200), (220), (311) sets of lattice planes are observed. With the obtained XRD pattern, the crystalline nature of the synthesized silver nanoparticles was confirmed. The diffraction peak at 2θ value of 31.70^0 forms the lattice plane and three additional broad bands are observed at 45.43^0 (2θ), 66^0 (2θ) and 75.25^0 (2θ) which corresponds to the (200), (220), and (311) planes of biologically synthesized silver nanoparticles respectively. All the Bragg's peaks and their intensities matched well with the standard JCPDS of spherical AgNPs.

Scanning Electron Microscope

Scanning electron microscope of silver nanoparticles synthesized by *Pseudomonas putida* was shown in Figure 5. SEM image observed in the present study was spherical, pseudo spherical, and some undefined morphology. The particle size ranges from 20-100 nm and possesses an average size of 60 nm.

Antibacterial Activity of Biologically Synthesized Silver Nanoparticles against UTI Pathogens

The bactericidal activity of biologically synthesized silver nanoparticles was investigated against UTI pathogens such as *Staphylococcus aureus, E. coli, Klebsiella pneumonia* and *Proteus vulgaris.* Interestingly, different concentrations of the biofabricated AgNPs exhibited significant antibacterial activity against both Gram-negative and Gram-positive UTI pathogen. The diameters of zone of inhibition was observed to be high for all the test pathogens against control. The zone of inhibition in diameter (mm) of silver nanoparticles against UTI pathogens is represented in Table 2. The AgNPs produced could inhibit four different typical pathogenic bacteria with maximum effect against *K. pneumonia* (inhibition zone 20 mm dia) compared to the other UTI pathogens (Figure 6 and 7). The antibacterial activity of AgNPs increased considerably with increase in concentrations which ranged from 25 to100 µg /ml.

DISCUSSIONS

Exploitation of several physical and chemical methods in nanoparticle synthesis not only threatens the environment, but also limits the uses of these biofabricated materials in biological applications. However, recent studies have recommended the integeration of the green chemistry principles in the synthesis of silver nanopartilces. Fungi were widely used for the synthesis of AgNo3 as the use of fungi is more advantageous in processing and handling the bio-mass [20]. Reduction of aqueous silver nitrate ions were observed when they were exposed to the *Fusariumoxysporum*cell filtrate [21]. The change of colour of the reaction mixture from pale yellow to brown indicated the formation of silver nanoparticles**. The silver nanoparticles exhibit yellowish brown in colour due to the excitation of surface Plasmon vibration in metal nanoparticles.**

The reduction of silver ions into silver nanoparticles by bacterial cultures was evidenced by the visual change of colour from yellow to reddish brown [22].

In the present experiment, the appearance of yellow to brown colour observed in the reaction mixture indicates the rapid formation of silver nanoparticles. This could be justified that certain reducing agents released in the cultures of the tested bacteria might have been involved in the reduction of Ag+ to silver nanoparticles Ag-NPs. Thus, it was evident that electron shuttle or other reducing agents released by *P. putida* which are capable of reducing silver ions to silver nanoparticles. On the other hand, the reduction of silver ions did not occur in the absence of bacterial cells. This clearly indicates that reducing agents released into the cultures of *P. putida* were involved in the reduction process [23].

The mechanisms of biosynthesis of Ag-NPs has been hypothesized that silver ions required NADPH-dependent nitrate reductase enzymes for their reduction, which was secreted by the bacteria in its extracellular environment [24,25]. The biosynthesis of Ag-NPs by *P. aeruginosa* strain showed the same result with extracellular process [26,27].

A strong peak (maximum absorbance) at 420 nm was observed for the silver nanoparticles prepared using *P. putida*, while no absorption band was observed for AgNO3 solution. This event clearly indicates that the reduction of silver ion to metallic silver took place extracellular through the reducing agent released into the solution [28]. Observation of this peak, assigned to surface Plasmon. The surface Plasmon resonance (SPR) band of silver nanoparticles remain in the range of (380 to 440 nm) till the end of the reaction period and this suggests that the particles were dispersed in the aqueous solution with no evidence for aggregation after completion of the reaction [29].

A band corresponding to the surface plasmon resonance at 410 to 420 nm was reported by [22]. UV-Vis spectral band corresponding to the surface plasmon resonance at 410 to 430 nm was also reported [9]. **In the present experiment, the peak of UV-Vis spectrum at 420 nm indicated the formation of silver nanoparticles.** Many authors reported that for various metal nanoparticles with sizes ranging from 2 nm to 100 nm, the peak at 420nm indicated a surface plasmon resonance (SPR) [3,31]. From the previous reports, it is evident that the presence of single SPR peak indicates spherical shape of AgNPs which was further confirmed by scanning electron microscopy [32].

XRD analysis with three distinct diffraction peaks at 38.28^0, 44.38^0, 64.54^0, and 77.64^0, and indexed with 2θ values of (111), (200), (220), (311) crystalline planes of cubic Ag was observed by [21]. Absorption peak observed at 2θ values of 38.28^0, 44.27^0, 64.64^0, and 77.66^0[33]. Biosynthesized AgNPs were confirmed by using XRD and the silver peaks noted at 2θ values of 37.8^0, 44.1^0, 62.9°, and 75.9°[34]. In the present study, the diffraction peak at 31.70°. From the lattice plane and three additional broad bands observed at 45.43^0(2θ), 66^0(2θ) and 75.25^0(2θ), they corresponds to (200), (220), and (311) planes of biologically synthesized silver nanoparticles.

The morphology of the SEM images having spherical, pseudo spherical, and some undefined morphology with traces of agglomeration was due to binding of biological molecules with nanoparticles present in the bacteria [9]. In the present experiment, biosynthesized silver nanoparticles are spherical in nature and some undefined morphology with traces of agglomeration.

The silver nanoparticles have found widespread applications in biomedicine, and other industries including numerous household products [35]. Several mechanisms such as direct damage to the bacterial cell membrane, the release of silver ions and subsequent generation of reactive oxygen species (ROS) which finally lead to the increased membrane permeability and DNA damage, have been reported for antibacterial activity of AgNPs [36].

Antimicrobial activity of silver nanoparticles against gram-negative bacteria was dependent on the concentration of Ag nanoparticles [37]. Accumulation of Ag nanoparticles in the bacterial membrane facilitated the permeability, resulting in cell death [38]. Nanosilver may have the capacity to penetrate inside the bacteria and causes damage by interacting with DNA [39]. In this study to evaluate the antibacterial effects against various microorganisms, four representative of UTI organisms, such as *E. coli*, *Staphylococcus aureus*, *Proteus vulgaris* and *Klebsiella pneumonia* were used. There were distinct differences among them. When the AgNPs were tested, they effectively inhibited the bacterial growth. Biosynthesized silver nanoparticles showed high inhibitory effect (20) on the growth of *K. pneumonia* and the moderate inhibitory effect of 15, 12 and 7 mm dia on the growth of *E. coli*, *Staphylococcus aureus*and *Proteus vulgaris*

respectively.

CONCLUSIONS

The usage of bacteria is good approach for the production of silver nanoparticles eco-friendly and costs effective. This study suggested that the aqueous extract of *Pseudomonas putida* is the best candidate for green synthesis of high quality AgNPs with promising antibacterial effect. This result spotlights the research work on metal resistant bacteria-assisted synthesis of AgNPs. New insights about the various pharmacological applications could be gleaned with these AgNPs.

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APPENDICES

Table 1: Biochemical Characteristics of Isolated Bacteria

Sl No	Characteristics	Isolate 1
Morphology		
1	Gram Reaction	-
2	Cell shape	Rod
Biochemical Tests		
1	Mac Conkey agar test	+
2	Indole test	-
3	Methyl red test	-
4	VogesProskauer test	-
5	Citrate utilization test	+
6	Starch hydrolysis test	-
7	Urea hydrolysis test	-
8	Nitrate reduction test	-
9	Hydrogen sulfide production test	+
10	Cytochrome oxidase test	+
11	Catalase test	+
12	Ammonia production	+
13	IAA	+
14	Phosphate solubilization	+
15	Dextrose	+
16	Mannitol	-
Species Name		***Pseudomonasputida***

Table 2: Zone of Inhibition in (mm) of Silver Nanoparticles Synthesized by *Pseudomonas putida*Against UTI Pathogens

UTI Pathogens	Zone of Inhibition(mm dia)		
	25µl	50µl	100µl
Proteus vulgaris	3.0 ± 0.27	6.0 ± 0.48	7.0 ± 0.77
Klebsiella pneumonia	2.0 ± 0.2	8.0 ± 0.88	20 ± 1.80
Escherichia coli	4.0 ± 0.48	6.0 ± 0.54	15 ± 1.35
Staphylococcus aureus	2.0 ± 0.16	8.0 ± 0.96	12 ± 1.08

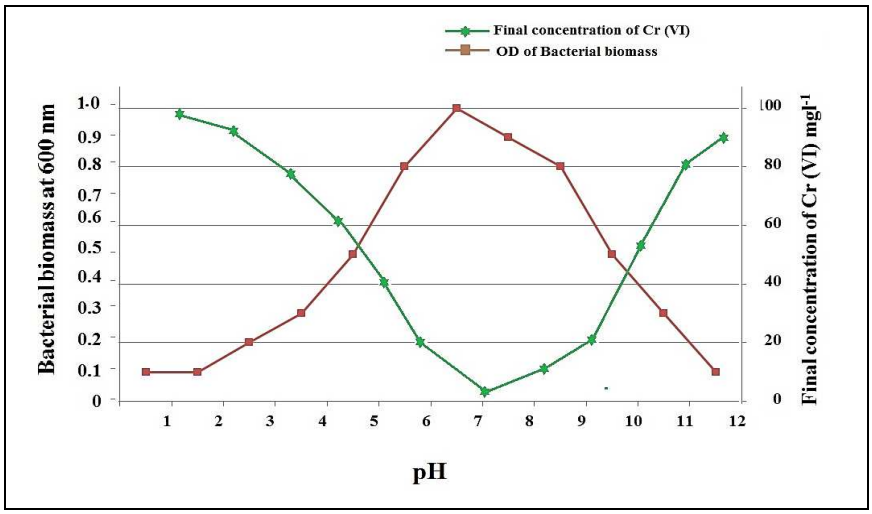


Figure 1: Determination of Bacterial Growth by Taking the Absorbance at 600 nm and Observation of Chromium Reduction by *pseudomonas putida* (S03) in Response to Various ph at 35±2°C Temperature After 48 h Incubation

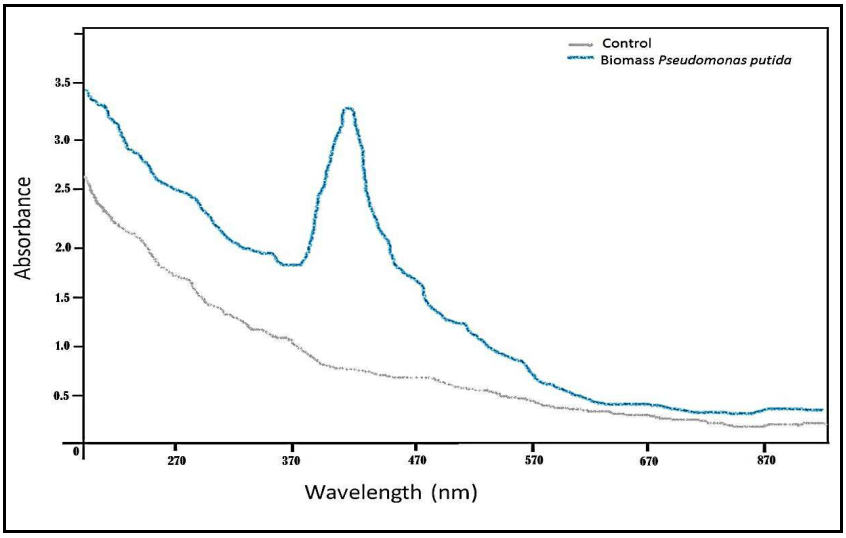
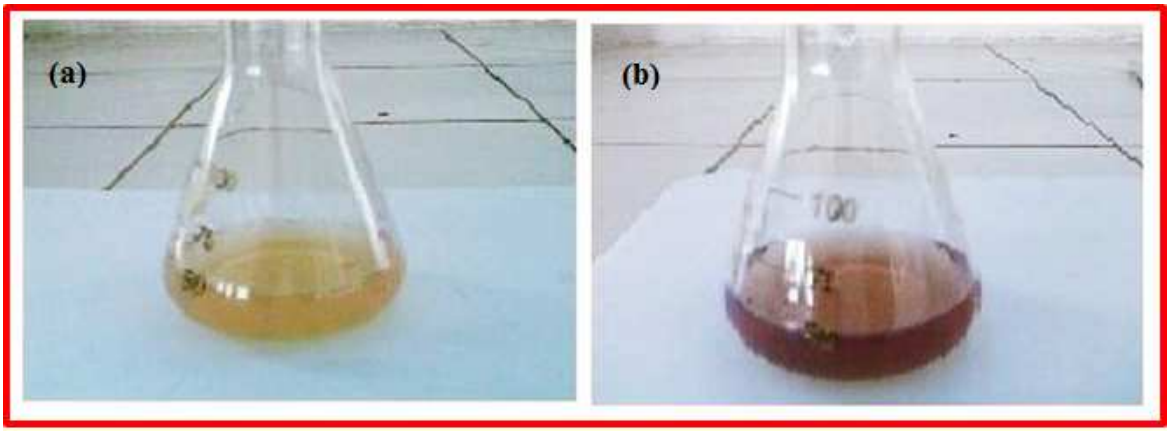


Figure 2: UV-VIS Absorption Spectrum of Silver Nanoparticles Synthe Sized by the Fresh Culture of *Pseudomonas Putida*



(a) Cell Filtrate of *Pseudomonas putida*Without Silver Nitrate (Control)
(b) Extract with $AgNO_3$ After 16 h Incubation

Figure 3: Biosynthesis of Silver Nanoparticles

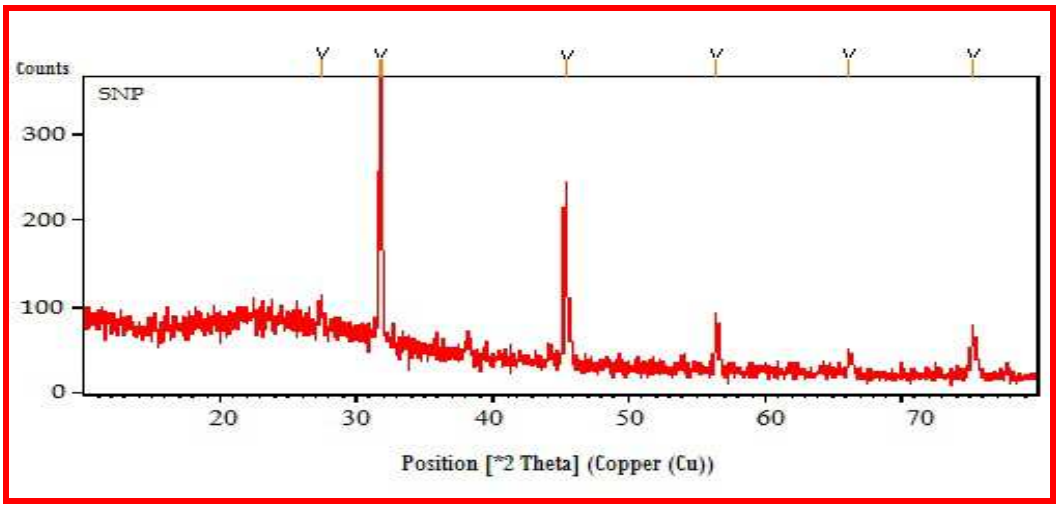


Figure 4: XRD Peaks of Silver Nanoparticles Synthesized by theFresh Culture of *Pseudomonas putida*

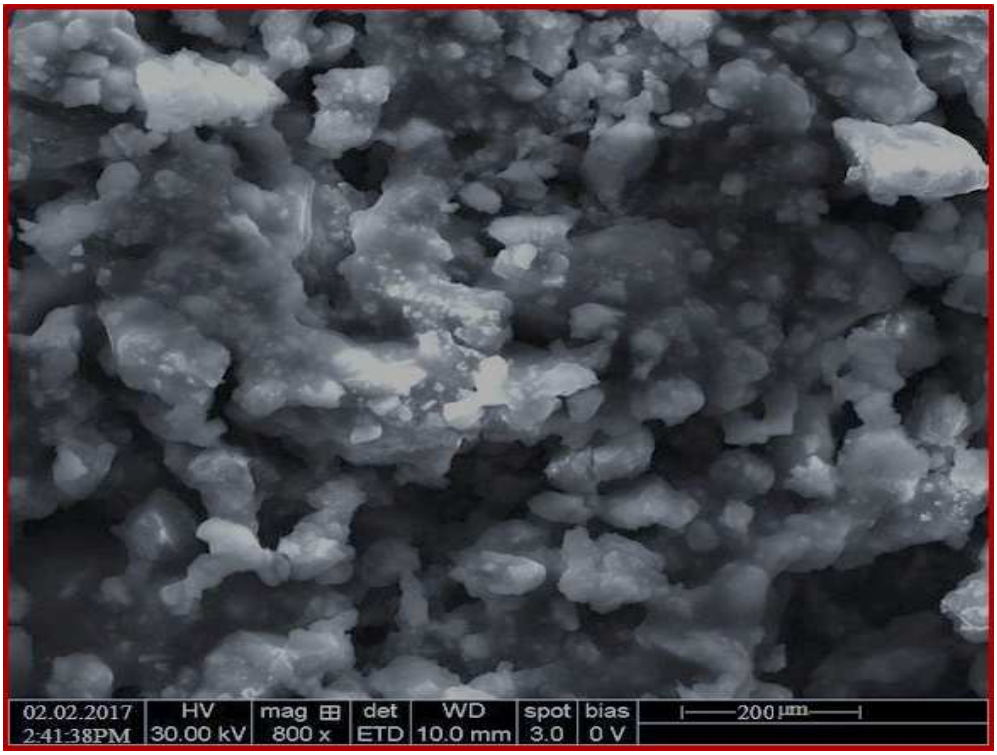


Figure 5: SEM Micrograph of Silver Nanoparticles Synthesized by the Fresh Culture of *Pseudomonas Putida*

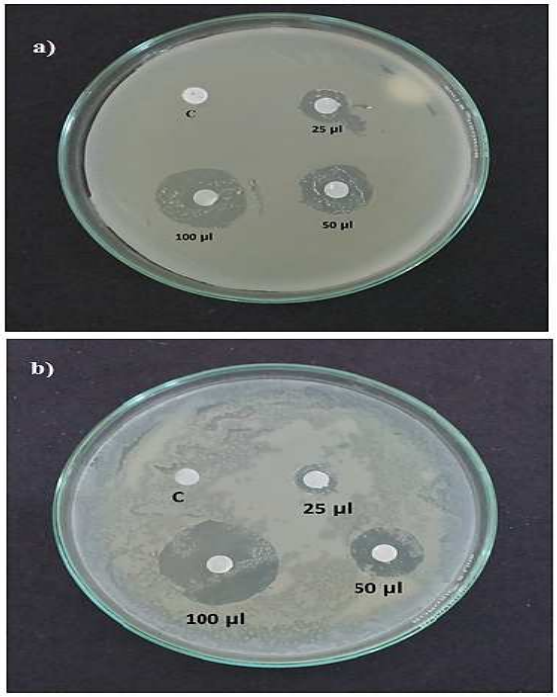


Figure 6: Antimicrobial Activities of Silver Nanoparticles Synthesized by *Pseudomonas Putida* Against (a) *E. coli* and (b) *Klebsiella Pneumonia*

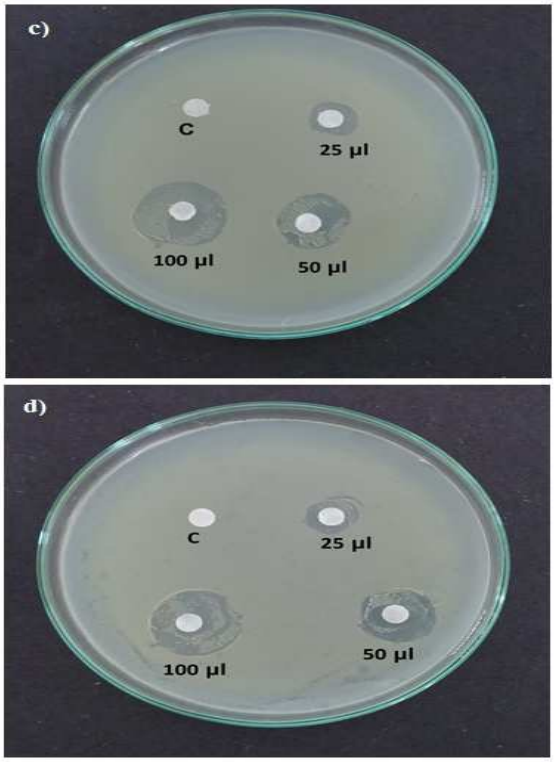


Figure 7: Antimicrobial Activities of Silver Nanoparticles Synthesized by *Pseudomonas Putida* Aagainst(c) *Proteus Vulgaris* and (d) *Staphylococcus Aureus*