Chapter 4

Microbial Synthesis of Silver Nanoparticles

Green biosynthesis of silver nanoparticles from an actinobacteria *Rhodococcus* sp.
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**RESEARCH ARTICLE**

Green synthesis of silver nanoparticles by microorganism using organic pollutant: its antimicrobial and catalytic application

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“I feel that the greatest reward for doing is the opportunity to do more.”
-Jonas Salk
"Always trust a microbiologist because they have the best chance of predicting when the world will end.” —Teddie O. Rahube

4.1. Introduction

The intense light emission properties of noble metals (gold, silver, etc.) nanoparticles have caught a lot of attention. Nobel metal nanostructures have contributed for the great enhancement of the nanobiology field, which have proven to be highly versatile and tunable materials for a range of bioapplications including biophysical studies, biological sensing, imaging, medical diagnostics, and cancer therapy. Silver has been known, among various metals, since ancient times as effective antimicrobial agent for the treatment of diseases, for food preservation and water purification [1]. The recent advancements in the field of nanotechnology have made AgNPs to be widely used as a novel therapeutic agent as antibacterial, antifungal, antiviral, anti-inflammatory and anti-cancerous agents. The broad-spectrum antimicrobial AgNPs persuade its use in a large number of biomedical and environmental applications as well as cosmetics, clothing and numerous consumer products [2, 3].

Among most of the methods, the physical and chemical methods are energy and capital intensive, employ toxic chemicals, and often yield particles in non-polar organic solutions, precluding their biomedical applications. On the contradictory, biological syntheses are not only a good way to fabricate benign nanostructure materials, but also to reduce the use or generation of hazardous substances to human health and the environment. Thus the focus for nanoparticles synthesis is transferring from physical and chemical processes towards ‘green’ chemistry i.e. bioprocesses. In variety of biological techniques of nanoparticle synthesis, microbial synthesis would help thwart many of the detrimental features by enabling synthesis at mild pH, pressure and temperature and at a substantially lower cost. As described in chapter 2, a number of microorganisms have been found to be capable of synthesizing inorganic nanocomposites either intra- or extracellularly. Some examples of nanoparticle formation by organisms are magnetotactic bacteria synthesizing magnetite
nanoparticles [4]. There are several reports on use of microorganisms such as Lactic acid bacteria [4], *Bacillus subtilis* [5], etc. and fungi such as *Aspergillus flavus* [4], *Fusarium oxysporum* [6], etc. for the biosynthesis of AgNPs and marine alga, *Sargassum wightii Greville* for gold nanoparticles [7].

In the present study, the versatile genus *Rhodococcus* sp is used for the synthesis of monodispersed AgNPs. *Rhodococcus* are non-sporulating, aerobic bacteria classified into mycolate containing nocardioform actinomycetes [8]. Since they are equipped with a large number of enzymatic activities, unique cell wall structure and suitable biotechnological properties, *Rhodococcus* strains may be utilized as industrial organisms, primarily for biotransformations and biodegradation of many organic compounds [9]. The intracellular accumulation of gold nanoparticles with a dimension of 5–15 nm by an alkalotolerant actinomycete, *Rhodococcus* sp. has been demonstrated where the available reductases on the cell wall reduced Au$^{3+}$ and accumulated as Au$^{0}$ on the cell wall and on cytoplasmic membrane with good dispersity [10]. From the current experiment we are predicting that the synthesis of the AgNPs occurred inside the cell i.e. intracellular synthesis. The optimization of the synthesis process was done for synthesis of monodispersed AgNPs by varying the concentrations of AgNO$_3$, carbon sources, pH, temperature and nitrate source. The synthesized nanoparticles were characterized using standard analytical techniques, viz, UV–visible spectroscopy, TEM, EDX, XRD and fluorescence spectroscopy. The particle size and stability of as-synthesized AgNPs in cell free culture broth were analyzed by using DLS and zeta potential respectively. The toxicity of the nanoparticles was analyzed on pathogenic microorganisms.

**4.2. Intra-cellular synthesis of AgNPs using *Rhodococcus* NCIM 2891**

**4.3. Experimental Methods**

**4.3.1. Microorganism and Chemicals**

An actinobacteria *Rhodococcus* NCIM 2891 was obtained from NCIM (National Collection of Industrial Microorganisms), National Chemical
Laboratory (NCL), Pune, India. The culture was maintained on GYEME (Glucose Yeast extract Malt extract) agar containing 1 % glucose, 0.3% yeast extract, 0.5% peptone and 0.3% malt extract and 1.5% agar-agar at 4 °C.

Chemicals were purchased from Lobachemie Pvt. Ltd. of GR grade and media components were obtained from Himedia Laboratories Pvt. Ltd. All chemicals are 100 % soluble in water and used without further purifications.

4.3.2. Synthesis of AgNPs

*Rhodococcus* was aerobically grown in M9 medium containing sodium acetate (0.420%), NaNO₃ (0.3%), K₂HPO₄ (0.3%), Na₂HPO₄ (0.6%), MgSO₄ (0.05%), NaCl (0.05%) in double distilled water for 24 h at 30 °C and agitated at 130 rpm. After 24h, the grown culture was collected by centrifugation at 10000 rpm and then cells were inoculated in slight modified M9 media without NaCl in 500 ml Erlenmeyer flask at pH 7.0 to which 1 mM of AgNO₃ was added. For the TEM studies, some microbial culture was collected centrifugally after 10 h incubation and fixed by 2.5 % glutaraldehyde. The change in the color was observed after 24 h incubation.

4.3.3. Characterization of AgNPs

To demonstrate intracellular synthesis of AgNPs, pellets of freshly harvested Ag-loaded actinobacteria were fixed in 2.5% (w/v) aqueous glutaraldehyde, centrifuged, re-suspended in 1.5 ml of 0.1 M phosphate buffer (pH-7.2) at 4 °C and post fixed in 1% Osmium tetraoxide at 4 °C in 0.1 M phosphate buffer. Samples were dehydrated using a graded series of acetone. After two 15 min washes in acetone, cells were embedded in fresh araldite followed by polymerization at 60 °C for 24 h. Ultrathin sections (90 nm) were cut on a Leica Ultracut R microtome, and mounted on pioloform-coated Cu grids. The sections were stained with 2% aqueous uranyl acetate for 10 min and triple lead stain for 5 min. Also for the determination of the morphology and size of the AgNPs in the colloidal solution, the colloidal solution of the AgNPs was transferred on to a carbon coated copper grid and allowed to air dry. The grids were then scanned using Philips CM200 model Transmission Electron Microscopy, operating voltage 20-200 kV with resolution 2.4 Å. After 24 h of
incubation the change in the color from white to dark brown was observed. For the complete recovery of the AgNPs the cells were sonicated based on a 60 amplitude output, at 4 °C with 30 strokes of 30 s, each at 1 min intervals. The sonicated cells were centrifuged (4 °C, at 9000 rpm for 25 min) and supernatant used as AgNPs colloidal solution. The formation of the AgNPs was monitored by UV–visible spectroscopy of the cell supernatant by recording spectra between wavelength 300 to 600 nm and simultaneously monitoring the appearance of the characteristic peak at 400–430 nm using a Schimadzu (Model No.UV 1800) double beam spectrophotometer at different time intervals from 0 h to 120 h. XRD pattern of AgNPs drop coated and air- dried on the glass substrate was recorded to study the structural and phase analysis by Philips PW-3710 diffractometer using Cu Kα radiation in the 2Θ range from 20° to 80°. The X-Ray diffraction (XRD) patterns were evaluated by X’pert high score software and compared with JCPDS card No. 89-3722. Energy-dispersive analysis of X-ray spectroscopy (EDX, JEOL JSM 6360) of the freeze dried cell free extract. The Fourier transform infrared (FTIR) spectra of were recorded in transmittance mode with Aplpha ATR Bruker (Eco ATR 500-400 cm⁻¹) spectrum of freeze dried cell free extract. TEM study was used to determine the morphology and size of the AgNPs in colloidal solution. For this purpose, the colloidal solution of the AgNPs was transferred on to a carbon coated copper grid and allowed to air dry. The grid was then scanned using Philips CM200 model Transmission Electron Microscopy, operating voltage 20-200 kV with resolution 2.4 Å. DLS measurements and zeta potential of AgNPs in cell free extract were performed using a NICOMP™ 380 ZIS (Santa Barbara, California, USA) for the determination of hydrodynamic diameter (HDD) with the viscosity of the cell free extract of 1.095 cP.

4.3.4 Optimization AgNPs synthesis

For the optimization of AgNPs synthesis, effect of different parameter such as AgNO₃ concentration, temperature, pH, growth phase, nitrate source and carbon sources was investigated. Culture was subjected to different AgNO₃ concentrations from 1 mM to 10 mM after 24 h of growth and further incubated
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for 24 h with respect to concentrations at pH 7 and 30 °C under agitation at 130 rpm in the dark. The effect of pH was evaluated by changing pH of the media from 4 to 10 adjusted by 1 N NaOH and 1 N HCl. The organism was incubated with 1 mM AgNO₃ at 30 °C for 24 h. To study effect of temperature on synthesis, the organism was incubated in presence of 1 mM AgNO₃ at 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C for 24 h at pH 7. The microorganism was fed with 1 mM AgNO₃ at different growth phases as lag phase, late lag phase, early log phase, mid log phase, stationary phase and late stationary phase to study effect of age of the culture on synthesis of AgNPs. The effect of source of nitrate was observed by growing organisms on different nitrate source as NaNO₃ and KNO₃ and in absence of nitrate source. To observe effect of carbon sources on synthesis of the AgNPs, microorganism was grown on different carbon sources as simple compounds *viz* glucose, fructose, sucrose, maltose, lactose, sodium acetate and aromatic compounds *viz* benzene, phenol, toluene and xylene. The effects of all parameters were analyzed using UV-vis spectroscopy.

4.3.5. **Antimicrobial activity**

The antibacterial activity of AgNPs was studied by both growth curve method and agar diffusion method with different concentrations of AgNPs (5, 10 and 30 μg/mL). Pathogenic Gram positive bacteria (*Staphylococcus aureus* NCIM 2654, *Enterococcus faecalis* NCIM 2403), and Gram negative bacteria (*Klebsiella pneumoniae* NCIM 2957, *Proteus vulgaris* NCIM 2813, *Pseudomonas aeruginosa* NCIM 5032, and *Escherichia coli* NCIM 2066) were chosen for the assay. The above strains (10⁵–10⁷ CFU/mL) cells were inoculated in nutrient broth and the growth was monitored at OD 600 nm at every hour interval for 50 h using a Bio-Rad iMARK micro plate reader. The same concentrations of AgNPs were also tested by agar diffusion method and the zone of inhibition was noted.

4.4. **Results and Discussion**

4.4.1. **UV-Vis spectroscopy study**
The formation of AgNPs by reduction of aqueous AgNO₃ by Rhodococcus sp. was indicated by change in the color from white to yellow. As the process continue the color changes to dark yellow after 24 h incubation. The UV-Vis spectra of the suspension showed surface plasmon resonance (SPR) around 405 nm (figure 4.1) which is characteristic SPR of the AgNPs. The SPR spectra suggest these AgNPs are spherical in shape [11]. The synthesis of the AgNPs continued for 72 h as the SPR spectra continued to increase for 72 h and no further increase was observed. The organism required more than 11 h for the synthesis of detectable amount of AgNPs.

![UV-Vis spectra of AgNPs synthesized by Rhodococcus sp. at various times](image)

**Figure 4.1.** UV-Vis spectra of AgNPs synthesized by *Rhodococcus* sp. at various times

### 4.4.2. XRD analysis

XRD pattern taken using Cu Kα target in the range 30–80 ° of AgNPs is shown in figure 4.2. The all peaks were well match with JCPDF Card No-89-3722 which exhibits the characteristic peaks of silver crystallites observed at 2θ values of 37.8, 44.1, 62.9 and 75.9 °. The obtained pattern is for fcc cubic crystal structure. The crystallite size was calculated from the full-width at half-maximum (FWHM) of the diffraction peaks using the Debye–Sherrer formula;
D = 0.9 \lambda/\beta \cos\theta, \text{ where } D \text{ is the mean grain size, } \lambda \text{ is the X-ray wavelength for Cu target, } \beta \text{ is FWHM of diffraction peak and } \theta \text{ is diffraction angle. In order to measure the size of nanoparticles accurately each peak was Gaussian fitted and also the instrumental broadening was subtracted using Si standard sample broadening. The size of nanoparticles from value measured for (111) plane of reflection is } \sim 9 \text{ nm which approximately similar to actual size by TEM.}

![XRD Pattern](image)

**Figure 4.2.** XRD pattern of AgNPs synthesized by *Rhodococcus* sp

### 4.4.3. FT-IR study

The interaction of biomolecules present in cell free extract with AgNPs was evaluated using FTIR measurement of the freeze dried cell free extract containing AgNPs which may be responsible for the stability (capping material) of nanoparticles in medium. The biomolecules may be the peptides, proteins, carbohydrates present in cell free extract. The amide linkages between amino acid residues in proteins give rise to well known signatures in the infrared region of the electromagnetic spectrum. FTIR spectrum reveals two bands at 1650 and 1550 cm\(^{-1}\) that corresponds to bending vibrations of the amide I and amide II bands of the proteins respectively; while their corresponding stretching vibrations were seen at 3310 and 2903 cm\(^{-1}\).
respectively (figure 4.3). The protein–nanoparticle interactions can occur either through free amine groups or cysteine residues in proteins and via the electrostatic attraction of negatively charged carboxylate groups in enzymes [12]. The two bands observed around 1381 and 1038 cm\(^{-1}\) can be assigned to the C–N stretching vibrations of the aromatic and aliphatic amines, respectively [13]. These results indicated that the carbonyl group of proteins adsorbed strongly to metals, demonstrating that proteins could have also formed a layer along with other bio-organic molecules, securing nanoparticles from aggregation and subsequently adds advantage for the stabilization of AgNPs.

![FT-IR spectra of AgNPs](image)

**Figure 4.3.** FT-IR spectra of AgNPs synthesized by *Rhodococcus* sp

### 4.4.4. Compositional analysis and TEM

The compositional analysis was carried out by EDX, showed characteristic signals of crystalline AgNPs at 3 keV (figure 4.4 (a)) [14]. The TEM analysis confirmed the spherical AgNPs with average size of 15 nm (figure 4.4 (b)). The rings observed in SAED are corresponded to planes, (111), (200), (220) and (311) of face-centered cubic (fcc) structure of elemental silver (figure 4.4 (b) inset).
Figure 4.4. (a) Compositional analysis by EDX, (b) TEM AgNPs synthesized by *Rhodococcus* sp (SAED pattern inset)

4.4.5. Optimization AgNPs synthesis process

4.4.5.1. Effect of AgNO₃ concentration

The effective concentration of AgNO₃ is essential for the optimum and maximum production of the AgNPs. To study the effect of AgNO₃ concentration, microorganism was exposed to different concentrations of AgNO₃ from 1 to 10 mM and incubated for 24 h. Figure 4.5 shows SPR spectra of AgNPs colloidal solution synthesized by *Rhodococcus* sp grown in different AgNO₃ concentration. The high yield of AgNPs synthesis was occurred at 1 mM as the maximum SPR intensity at 405 nm was observed at this concentration. Further increase in the concentrations of AgNO₃ led to decrease in SPR intensity. The AgNO₃ concentrations more than 4 mM completely ceased the synthesis of AgNPs. The obtained results were very contradictory to results obtained in extracellular synthesis of AgNPs from *Morganella* sp. and *E. coli* where SPR intensity increased as the concentration of AgNO₃ increased up to 5 mM [15, 16]. So for synthesis of AgNPs from *Rhodococcus* sp, intracellular enzyme system was essential. Here high concentrations of AgNO₃ inhibited growth of the microorganism which affected synthesis of
AgNPs. The result clearly indicates that 1 mM AgNO₃ was required for optimum synthesis of AgNPs.

![UV-Vis spectra of AgNPs](image)

**Figure 4.5.** Effect of AgNO₃ concentration on synthesis of AgNPs. UV-Vis spectra of AgNPs synthesized in presence of different AgNO₃ concentrations from 1-10mM at pH 7 and 30 °C

### 4.4.5.2. Effect of pH

In general, the reduction reaction of metallic ions is sensitive to the pH of the solution as it may affect the morphology of product via the formation of certain species as demonstrated [17]. To observe effect of pH, the organisms were grown in pH range of 4 to 10 where the microorganisms could effectively grow. After 36 h of incubation microorganisms were exposed to 1 mM AgNO₃ at 30 °C and further incubated for 24 h. The SPR intensity (figure 4.6) showed gradual increase as pH increased from acidic to alkaline. The maximum SPR intensity was found for the pH 8. Though there was decrease in the synthesis of AgNPs at more than pH 8, the synthesis of AgNPs was found for the pH 9 and pH 10 also. So it is clear that *Rhodococcus* is alkalotolerant actinomycete and the enzyme system of *Rhodococcus* reducing AgNO₃ may be active in slightly alkaline environment.
4.4.5.3. Effect of temperature

The behavior of nanoparticle synthesis over a range of temperatures was determined by exposing Rhodococcus sp to AgNO₃ for 24 h at pH 7 at different temperatures 30, 35, 40, 45, 55 and 60°C (figure 4.7). The optimum temperature range required for the synthesis of the AgNPs was 30-35 °C. The decrease in the synthesis of AgNPs was observed with increase in the temperature. Rhodococcus showed sufficient growth up to 60 °C as Rhodococcus sp contains high mole% G+C which provide them high stability towards the high temperature. SPR intensity of AgNPs decreased as the there was temperature rise from 35 to 45 °C. The activity of the enzyme required to convert AgNO₃ to AgNPs went on decreasing as temperature mounted. There was complete inhibition of AgNPs synthesis for the temperatures 50, 55 and 60 °C. The result showed contradiction to the extracellular synthesis of AgNPs from E. coli where maximum synthesis occurred at 60 °C [16].

**Figure 4.6.** UV-Vis spectra of AgNPs synthesized at different, pH values from 4-10 for 30 °C
Figure 4.7. UV-Vis spectra of AgNPs synthesized at different temperature values from 30-60 °C for pH 7

4.4.5.4. Effect of age of microorganism

The age of microorganism is essential parameter for the maximum synthesis of the AgNPs. Enzyme activities of the microorganisms at log phase are much higher than lag phase and stationary phase. So to investigate the age effect, microorganisms were fed with 1 mM AgNO₃ at different growth phases at pH 7 for 24 h incubation. Figure 4.8 shows effect of the age on the synthesis of the AgNPs and inset displays growth curve of *Rhodococcus* demonstrating various growth phases as lag phase at 0 h, late lag phase at 12 h, early log phase at 24 h, mid log phase at 36 h, stationary phase at 48 h and late stationary phase at 72 h. The SPR intensity is maximum for the log phase and mid log phase as the enzyme activity is higher at these phases. The synthesis of AgNPs did not occur at lag phase and late lag phase as the enzyme required for the reduction of AgNO₃ could not synthesized by microorganisms as AgNO₃ may have inhibited the growth of organisms. The synthesis of AgNPs was found less active in stationary phase and late stationary phase as compared to log
phase. So the activity of AgNO$_3$ reducing enzyme decreased when microorganisms matured from log phase to stationary phase.

**Figure 4.8.** Effect of growth phase of *Rhodococcus* sp at lag phase 0 h (i), late lag phase at 12 h (ii), early log phase at 24 h (iii), mid log phase at 36 h (iv), stationary phase at 48 h (v), late stationary phase at 72 h (Inset: Growth curve of *Rhodococcus* sp)

Here it is cleared that there was no role of extracellular biomolecules in the reduction of AgNO$_3$. Hence from current experiment, it can be said that the synthesis of AgNPs was enzyme mediated. Also the microorganisms at active state were essential for transformation of AgNO$_3$ to AgNPs.

### 4.4.5.5. Effect of nitrate source

The source of nitrate is essential for induction of the enzymes required to reduce nitrates. In case of *Rhodococcus* RB1, the nitrate reductase enzyme greatly enhanced both by nitrate and nitrite [18]. Figure 4.9 shows effect of presence of nitrate source as KNO$_3$, NaNO$_3$ and absence of nitrate source. The reduction of AgNO$_3$ occurred in all the experiment but broadening of SPR peak was observed in synthesis process without nitrate source. Though the SPR
intensity is greater for KNO₃ than NaNO₃, the synthesis of AgNPs was occurring. So there may be involvement of enzyme in the reduction of the AgNO₃ but the role of nitrate reductase enzyme need to be analyzed further.

![Figure 4.9](image)

**Figure 4.9.** Effect of nitrate source as NaNO₃ and KNO₃ and absence of nitrate source

### 4.4.5.6. Effect of carbon sources

The substrate variation is crucial parameter in the synthesis of nanoparticles with respect to cost of the fermentation process. Figure 4.10 (a) is showing the SPR peaks of the AgNPs synthesized using different carbon sources *viz* glucose (G), mannitol (Mn), fructose (F), sucrose (S), sodium acetate (N), maltose (Ma) and lactose (L). There is an increase in the intensity of the absorbance for glucose so the concentration of silver particle increases with reaction time of 24 h. There was little or no detectable amount AgNPs was observed in presence of lactose and maltose. Furthermore there was no significantly different wavelength shift in the absorption spectra for all carbon sources. The absorbance band at lower wavelength with a good symmetry indicates that the mean diameter of AgNPs is very small with a uniform size distribution. The rate of synthesis of AgNPs in presence glucose was
considerably more as compared to other carbon sources after incubation. It might be due to glucose which acts as electron donor had positive effects on nanoparticle production. Unutilized glucose molecules present on the microorganisms or in the medium may reduce the silver ions before the microorganisms do and hence there may be high rate of formation of AgNPs [19]. The exact mechanism behind the variation was not understood clearly.

The synthesis of AgNPs was also carried using aromatic compounds as carbon sources. Fig. 4.11(a) showing SPR spectra of AgNPs synthesized on benzene (B), phenol (P), toluene (T) and xylene (X). After incubation of 120 h when the culture attained OD 600 of 0.2, the AgNO₃ was added to the respective medium. And further organisms were incubated for 24 h at 30 °C. The synthesis was observed for benzene, phenol and toluene. There was no detectable amount of synthesis observed for xylene as carbon source although the color change in the cell free extract was noticed (Fig. 4 Inset).

In Fig. 4.10; (b), (c), (d), (e), and (f) are HDD distribution of AgNPs, synthesized using carbon sources as glucose (G), mannitol (Mn), fructose (F), sodium acetate (N) and sucrose (S) respectively. There are large distributions of AgNPs produced by *Rhodococcus* sp for all carbon sources. The polydispersity was found for glucose, fructose, mannitol and sucrose. Whereas compared to other, mannitol is showing less polydispersity.
Figure 4.10: Effect of the different simple carbon sources. UV-Vis spectra of AgNPs synthesized using different simple carbon sources, glucose [G], fructose [F], mannitol [Mn], sodium acetate [N], sucrose [S], maltose [Ma] and lactose [L] (a); HDD of the AgNPs synthesized using, glucose (b); mannitol (c); fructose (d), sodium acetate (e) and sucrose (f).

The monodispersed AgNPs were produced when sodium acetate used as carbon source where the majority nanoparticles were of 34 nm and fewer
concentrations of large nanoparticles. The presence of the biomolecules in the cell free extract may inhibit the aggregation of the AgNPs, consequently the size and the distribution of the nanoparticles. Fig 4.11; (b), (c) and (d) is showing HDD distribution of AgNPs, synthesized using aromatic compounds as carbon sources; benzene (B), phenol (P) and toluene (T) respectively.

Figure 4.11. Effect of the different aromatic compounds as carbon sources. UV-Vis spectra of AgNPs synthesized using different aromatic carbon sources, benzene [B], phenol [P], toluene [T] and xylene [X] (a); HDD of the AgNPs synthesized using, phenol (b); phenol (c); toluene (d).

Table 4.1 illustrates different parameters associated with size, stability and aggregation of AgNPs in colloidal solution. The SPR spectra for all the carbon sources were around 405 nm as there was no effect on the shape of the nanoparticles due to change in carbon sources. But there was broad distribution of nanoparticles in the colloidal solution. The size of the nanoparticles obtained from DLS is around 30 nm for glucose, sucrose, mannitol and fructose but the variation in particles distribution given by variance or polydispersity index
(P.I.) was more than 0.5 which demonstrated large particle distributions. In case of sodium acetate as carbon source, AgNPs size was around 34 nm but the variance is less than 0.5. The zeta potential of the AgNPs in the colloidal solution is -37.6 mV at pH 9 which is more than other carbon sources used for synthesis. Though the size of the AgNPs is around 20 nm for phenol as carbon source, the variation (P.I.) is more than 0.6 and the zeta potential is around -17.3 mV at pH 9. The zeta potential is an indication of the stability of colloidal aqueous dispersions and usually particles with zeta potential ($\xi > \pm 30$ mV) are considered to be stabilized due to electrostatic repulsion [20].

**Table 4.1.** Parameters associated with AgNPs synthesized using different carbon sources

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Carbon sources</th>
<th>Protein (µg/mL)</th>
<th>SPR spectra (nm)</th>
<th>DLS (nm)</th>
<th>Zeta (mV)</th>
<th>Variance (P.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>295.2</td>
<td>408</td>
<td>22.9</td>
<td>-30.16</td>
<td>0.598</td>
</tr>
<tr>
<td>2</td>
<td>Fructose</td>
<td>257.2</td>
<td>404</td>
<td>11.7</td>
<td>-27.76</td>
<td>0.758</td>
</tr>
<tr>
<td>3</td>
<td>Mannitol</td>
<td>270.2</td>
<td>402</td>
<td>44.8</td>
<td>-28.36</td>
<td>0.566</td>
</tr>
<tr>
<td>4</td>
<td>Sodium acetate</td>
<td>425.2</td>
<td>405</td>
<td>34.8</td>
<td>-37.76</td>
<td>0.381</td>
</tr>
<tr>
<td>5</td>
<td>Sucrose</td>
<td>235.2</td>
<td>413</td>
<td>37.4</td>
<td>-20.47</td>
<td>0.568</td>
</tr>
<tr>
<td>6</td>
<td>Phenol</td>
<td>208.2</td>
<td>400</td>
<td>19.1</td>
<td>-17.83</td>
<td>0.618</td>
</tr>
</tbody>
</table>

The polydispersity might be due to the biomolecules present in the cell free extract. The presence of protein on nanoparticles may inhibit the aggregate formation of AgNPs [21] which ultimately contribute to the stability of the nanoparticles. From the FTIR results, it is evident that there was presence of protein on nanoparticles. So the concentration of the protein in the silver colloidal solution was calculated using the Lowry method whose sensitivity is 100 µg/mL. The protein concentration found was almost identical for all simple carbon sources except for sodium acetate and phenol. The concentrations of
proteins were 425.2 µg/mL and 208 µg/mL respectively for sodium acetate and phenol.

![Figure 4.12. The TEM images of AgNPs synthesized using, sodium acetate (a) and (b); phenol (c) and (d) as carbon source at different magnifications.](image)

The distribution variation was confirmed by TEM of the AgNPs (figure 4.12), synthesized using sodium acetate and phenol. Figure12; (a) and (b) are showing the TEM of the AgNPs synthesized from sodium acetate as carbon source at different magnifications. It is clear that there was formation of the distinct, disaggregated and uniform size (~15 nm) i.e. monodispersed AgNPs. From figure 12; (c) and (d), it is evident that the AgNPs synthesized using phenol as carbon source are in cluster form. There was aggregation and variation in the size of AgNPs i.e. polydispersed. So effect of different protein concentration had shown on the distribution and stability of nanoparticles. The presence of other biomolecules may have also affected the aggregation of AgNPs. Thus from these results it is clear that the presence of biomolecules
have effect on size and distribution of the nanoparticles in the colloidal solution.

The biosynthesis of AgNPs is possible in two ways viz enzymatic and non-enzymatic synthesis. One of the disadvantages of the enzymatic reduction of silver is the slow rate of the reaction. The time required for reduction ranges has varied between 24 and 120 h. The non-enzymatic reduction is often fast, taking only a couple of minutes. The non-enzymatic reduction of silver is based on the chemical reduction where the reducing and stabilizing compounds are produced by microorganisms or plants [22, 23]. Within 5 min the supernatant of Klebsiella pneumoniae, Escherichia coli, and Enterobacter cloacae turned brown, indicating the formation of nanosilver. Mukherjee et al. first time suggested that enzymes might be responsible for the reduction of silver ions using Verticillium sp [24]. The NADPH-dependant reductase was involved in the reduction of the AgNO₃ with fungus Fusarium oxysporum [25]. Form the current experiments it is evident that there is role of enzyme in the reduction of Ag⁺ ions. The viability and the age of the microorganisms are essential for the transformation. Rhodococcus RB1 and Bacillus subtilis are carrying NADH-dependant nitrate reductase inside the cell [18]. The Bacillus sp has been demonstrated for the intracellular synthesis of AgNPs [25]. From the optimization process it was predicted that synthesis of AgNPs is occurring inside the cell. To confirm the results, TEM images of the Rhodococcus sp were taken after 10 h of incubation of microorganism with AgNO₃ were taken. Figure 4.13 shows the TEM images of Rhodococcus sp showing the AgNPs are well inside the cell cytoplasm of microorganisms. More than cell membrane the synthesis is appeared to be occurring in the cytoplasm. SAED pattern confirmed theses nanoparticles are crystalline in nature.
Figure 4.13. TEM micrograph of the *Rhodococcus* sp and SAED pattern of the AgNPs synthesized inside the microorganism

4.4.6. Antimicrobial activity

Silver is known for its antimicrobial properties and has been used for years in the medical field for antimicrobial applications. The different mechanisms of the bactericidal effect of AgNPs have been proposed from the several studies but the exact mechanism remains to be understood. Many literatures stated that AgNPs may attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell. It is also possible that AgNPs not only interact with the surface of membrane, but can also penetrate inside the bacteria [26]. Smaller AgNPs having the large surface area available for interaction would give more bactericidal effect than the larger AgNPs [27]. The shape of AgNPs has been shown very essential for the effective antibacterial activity [28]. A triangular nanoplate has predominately desirable activity than spherical and rod-shaped AgNPs [29].
Figure 4.14. Antimicrobial activity of AgNPs on a) Escherichia coli, b) Enterococcus fecalis, c) Staphylococcus aureus, d) Klebsiella pneumoniae, e) Pseudomonas aeruginosa and f) Proteus vulgaris with different concentration as — control, — 5 µg mL⁻¹, ▲ 10 µg mL⁻¹, ▼ 30 µg mL⁻¹.

Here, the antibacterial activity was studied on Gram positive bacteria (Staphylococcus aureus NCIM 2654, Enterococcus faecalis NCIM 2403), and Gram negative bacteria (Klebsiella pneumoniae NCIM 2957, Proteus vulgaris NCIM 2813, Pseudomonas aeruginosa NCIM 5032, and Escherichia coli NCIM 2066). The bactericidal and bacteriostatic effects of AgNPs were studied by observing the effect on the different growth phases of these pathogenic microorganisms using different concentrations of AgNPs (5, 10 and 30 µg mL⁻¹ of medium). Figure 4.14 demonstrates the antibacterial activity of the AgNPs. The lowest concentration i.e. 5 µg mL⁻¹ was found most effective for Klebsiella pneumonia (figure 4.14(d)) where organism showed growth after 48 h of incubation. For other organisms this low concentration of AgNPs affected log phase causing delay. Staphylococcus aureus (figure 4.14(c)) and Enterococcus faecalis (figure 4.14(b)) attained the stationary phase earlier than control one.
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So for these two organisms, 5µg mL\(^{-1}\) of AgNPs acted as bacteriostatic concentration for these microorganisms. *Escherichia coli* (figure 4.14(a)), *Staphylococcus aureus* and *Pseudomonas aeruginosa* (figure 4.14(e)) showed some growth after 50 h incubation for 5µg mL\(^{-1}\) of AgNPs. The higher concentration (30µg mL\(^{-1}\)) of AgNPs completely arrested the growth of the all organisms.
4.5. Extracellular synthesis of silver nanoparticle with degradation of phenol by microorganisms with its antimicrobial and catalytic activity

The extracellular synthesis by comparison is more adaptable to the synthesis of a wider range of nanoparticles system. There are plenty of reports which quoted the use of *Rhodococcus* sp for degradation of phenol and phenolic compounds [30]. So here in this study the cell free extract of the phenol degraded broth is used for the extracellular synthesis of AgNPs. Formed AgNPs were characterized by various analytical techniques. The stability of the AgNPs in colloidal solution was also analyzed in different environmental conditions. The antimicrobial against human pathogen and catalytic activity in reduction of 4-NP are also demonstrated in this study.

4.6. Experimental Methods

4.6.1. Microorganism and Chemicals

An actinobacteria *Rhodococcus* NCIM 2891 was obtained from NCIM (National Collection of Industrial Microorganisms), National Chemical Laboratory (NCL), Pune, India. The culture was maintained on MGYP agar containing 1% glucose, 0.3% yeast extract, 0.5% peptone, 0.3% malt extract and 1.5% agar-agar at 4 °C.

Chemicals were purchased from Lobachemie Pvt. Ltd. of GR grade and media components were obtained from Himedia Laboratories Pvt. Ltd. All chemicals are 100 % soluble in water and used without further purifications.

4.6.2. Preparation of microorganism and biodegradation of phenol

The actinobacterial cells were grown in 500 mL Erlenmeyer flask containing 100 ml MGYP medium (malt extract 0.3 g, glucose 1 g, yeast extract 0.3g and peptone 0.5 g) containing 100 mgL⁻¹ phenol under condition of pH 5.0, 220 rpm, 28 °C for 24 h. After 24 h of incubation 10% volume of cells were centrifuged at 8000 rpm at 4 °C for 20 min and used for biodegradation study.
For the biodegradation of the phenol, the mineral salt medium consisted of (gL\(^{-1}\)): \(\text{Na}_2\text{HPO}_4\cdot 12\text{H}_2\text{O}\), 3.80; \(\text{KH}_2\text{PO}_4\), 1.00; \(\text{NaCl}\), 1.00; \(\text{MgSO}_4\), 0.20; \(\text{NH}_4\text{Cl}\), 0.10 in deionized water, adjusted to pH 7.2 with 800 mgL\(^{-1}\) of phenol used. The experiment was carried under 140 rpm agitation and 28 °C. The small aliquots were drawn for the analysis of growth of microorganisms and estimation of residual phenol in broth. Phenol was estimated by direct photometric method based on rapid condensation with 4-aminoantipyrine, followed by oxidation with potassium ferricyanide under alkaline conditions to give a red color product. The estimation was carried out according to standard methods (APHA–AWWA–WPCF 1993).

4.6.3. Synthesis of AgNPs

The cell free extract of phenol degraded broth was used for the synthesis of AgNPs. After complete degradation of phenol from above experiment, the broth was centrifuged for 8000 rpm to separate cell mass. To the cell free extract, 3 mM aqueous \(\text{AgNO}_3\) was added and further incubated for 24 h at room temperature under dark condition with agitation at 130 rpm. The change in the color of cell free extract was observed during incubation. The aliquots were analyzed by UV-Vis spectroscopy to check the synthesis of AgNPs. The formed AgNPs were characterized by various analytical techniques.

4.6.4. Characterizations of AgNPs

The formation of the AgNPs was monitored by UV–visible (UV-Vis) spectroscopy of the cell supernatant by recording spectra between wavelength 300 to 600 nm and simultaneously monitoring the appearance of the characteristic peak at 400–430 nm using a Schimadzu (Model No. UV 1800) double beam spectrophotometer at different time intervals from 0 h to 120 h. XRD pattern of AgNPs drop coated and air- dried on the glass substrate was recorded to study the structural and phase analysis by Philips PW-3710 diffractometer using Cu K\(_\alpha\) radiation in the 2\(\Theta\) range from 20° to 80°. The X-Ray diffraction (XRD) patterns were evaluated by X’pert high score software.
and compared with JCPDS card No. 89-3722. Energy-dispersive analysis of X-ray spectroscopy (EDX, JEOL JSM 6360) of the freeze dried cell free extract. The Fourier transform infrared (FTIR) spectra of were recorded in transmittance mode with Alpha ATR Bruker (Eco ATR 500–400 cm\(^{-1}\)) spectrum of freeze dried cell free extract. TEM analysis was carried out to study morphology and size of the AgNPs. For this purpose, the colloidal solution of the AgNPs i.e. the supernatant of the culture was transferred on to a carbon coated copper grid and allowed to air dry. The grid was then scanned using Philips CM200 model Transmission Electron Microscopy, operating voltage 20-200 kV with resolution 2.4 Å. DLS measurements and zeta potential of AgNPs in cell free extract were performed using a NICOMP™ 380 ZIS (Santa Barbara, California, USA) for the determination of hydrodynamic diameter (HDD). The fluorescent spectrum of the colloidal AgNPs was studied using fluorescent spectrometer (model JASCO) at 23 kV. Emission spectra of AgNPs were observed for the different excitation wavelengths at 390 nm, 400 nm, 420 nm and 440 nm.

### 4.6.5. Stability study of colloidal AgNPs

The stability of so-formed AgNPs was studied for different physiological parameters like ionic strength, pH and temperature. Ionic strength of AgNPs colloidal solution was varied from 1 to 1500 mM by addition of NaCl. The pH was adjusted using 1 N NaOH for alkaline pH and 1 N HCl for acidic pH. The AgNPs colloidal solution was heated from 30 °C to 90 °C for half an hour to measure the temperature stability. The AgNPs colloidal solution was then analyzed by UV-Vis spectroscopy and DLS. The aggregation parameter (AP) was calculated by measuring the variation of the integrated absorbance between 395 and 700 nm, and it was defined as AP = (A - A\(^0\))/ A\(^0\), where A is the integrated absorbance of the sample at the given moment and A\(^0\) is the integrated absorbance of the initial solution of AgNPs colloidal solution.
4.6.6. Antimicrobial activity

The antimicrobial activity of AgNPs was evaluated using agar diffusion method against Gram positive species viz Staphylococcus aureus NCIM 2654, Bacillus cereus NCIM 2155, Enterococcus faecalis NCIM 2403, Gram negative species viz Escherichia coli NCIM 2066, Pseudomonas aeruginosa NCIM 5032, fungi viz Candida albicans NCIM 3466, Candida tropicalis NCIM 3118, Candida krusei NCIM 3515, Candida guilliemondii NCIM 3126 and Candida lusitaniae NCIM 3484 used for the antimicrobial study were purchased from NCIM (National Collection of Industrial Microorganisms), National Chemical Laboratory (NCL), Pune, India. Log phase bacterial cultures containing 10^8 cfu mL⁻¹ of each bacterial strain grown in nutrient broth were swabbed on the surface of Mueller–Hinton agar plates. Candida species were grown in MGYP broth and for the antimicrobial test were spread on the MGYP agar plates.

4.6.7. Homogenous and heterogeneous catalytic activity

The catalytic activity of AgNPs was studied using borohydride reduction of 4-nitrophenol (4-NP). In this for homogeneous catalytic experiment, a freshly prepared sodium borohydride (NaBH₄) solution was added, to generate a final concentration of 10⁻² M, to a 3 mL reaction mixture containing 10⁻⁴ M 4-NP, and 0.0101 mM AgNPs. For heterogeneous catalytic activity, sodium alginate was mixed in colloidal solution of AgNPs to prepare 2% alginate solution. To this alginate solution, 500 mg combustion synthesized super paramagnetic Mg-ferrite nanoparticles was added [31]. Then the mixture was added using 5 mL syringe with approximately same quantity of the drop in 0.1 M solution of CaCl₂ under continuous magnetic stirring to form magnetic silver-alginate beads of diameter of approximately 2 mm. After gelling for 1 h, the beads were kept for 10 min in CaCl₂ for hardening of beads. Above mentioned concentrations of 4-NP and NABH₄ with 2 mg magnetic silver-alginate beads were used for the heterogeneous catalytic activity. The conversion of 4-NP to 4-aminophenol (4-AP) was then monitored.
spectrophotometrically by the disappearance of the peak at 400 nm with time. The \( \text{H}_2 \) evolved during reaction helped in stirring the mixture of solution.

4.7. Results and discussion

4.7.1. Phenol degradation and growth kinetics

The *Rhodococcus* sp NCIM 2891 was grown on 800 mg L\(^{-1}\) and residual phenol was measured spectroscopically by measuring absorbance at 590 nm. Simultaneously the growth kinetics was of *Rhodococcus* during phenol degradation was derived by measuring absorbance at 530 nm (figure 4.17). It was observed that the *Rhodococcus* was able to grow on phenol as a sole source of carbon. The maximum phenol degradation was observed after 72 h. i.e., in log phase. The residual phenol concentration was 420 mg L\(^{-1}\) at 72 h that is nearly half of initial phenol concentration (500 mg L\(^{-1}\)). In figure 4.15, it can be seen that there was complete degradation of phenol within 120 h.

![Degradation curve and growth curve](image)

**Figure 4.15.** Phenol degradation and growth kinetics of *Rhodococcus* sp grown on 800 mgL\(^{-1}\) concentration of phenol at room temperature
4.7.2. Synthesis of AgNPs

The above phenol degraded broth was used for the synthesis of AgNPs. After addition of 1 mM of AgNO₃, the cell free extract of phenol degraded broth was incubated for 24 h. The change in the color of cell free extract was observed in 10 h of incubation from pale yellow (figure 4.16(a)) to dark brown (figure 4.16(b)).

![Figure 4.16. The change in the color of cell supernatant of phenol degraded broth (a) before addition of AgNO₃, (b) after addition of AgNO₃](image)

The culture supernatant was further incubated for 24 h. The UV-Vis spectra of the suspension after 12 h of incubation showed surface plasmon resonance (SPR) around 410 nm (figure 4.17 (a)) which is characteristic SPR of the AgNPs. The SPR spectra suggest these AgNPs are spherical in shape [8]. The synthesis of the AgNPs continued for 24 h as the SPR spectra continued to increase for 24 h. Yet, only the following bacteria viz, B. cereus, B. subtilis, B. licheniformis, E. coli, E. cloacae, K. pneumonia, L. acidophilus, S. aereus, and Pseudomonas aeruginosa [32, 33, 34] have been shown to produce extracellular AgNPs. It has been suggested that DNA [35], sulfur containing proteins [26], NADH-dependent nitrate reductase [36] are involved in the synthesis of AgNPs by the bio-reduction of silver ion to metallic silver. Extracellular synthesis of AgNPs was observed with the fungus Fusarium oxysporum and the reducing agent responsible for synthesis of AgNPs was found to be proteins, mainly NADH-dependent reductase [36]. Thus, it is
implied that the factors involved in the biosynthesis of AgNPs may be present in the supernatants of actinobacteria used in the present study.

4.7.3. Characterizations of AgNPs

XRD pattern taken using Cu Kα target in the range 30–80 ° of AgNPs is shown in figure 4.17 (b). The all peaks were well match with JCPDF Card No-89-3722 which exhibits the characteristic peaks of silver crystallites observed at 2θ values of 37.8, 44.1, 62.9 and 75.9 °. The obtained pattern is for fcc cubic crystal structure. The crystallite size was calculated from the full-width at half-maximum (FWHM) of the diffraction peaks using the Debye–Sherrer formula; 

\[ D = \frac{0.9 \lambda}{\beta \cos \theta} \]

where D is the mean grain size, λ is the X-ray wavelength for Cu target, β is FWHM of diffraction peak and θ is diffraction angle. In order to measure the size of nanoparticles accurately each peak was Gaussian fitted and also the instrumental broadening was subtracted using Si standard sample broadening. The crystallite size of nanoparticles from value measured for (111) plane of reflection is approximately of 5 nm which approximately similar to actual size by TEM.

The compositional analysis was carried out by EDX, showed characteristic signals of crystalline AgNPs at 3 keV (Data not shown) [14]. The TEM analysis confirmed the spherical AgNPs with average size of 15 nm (figure 4.17 (c)). The rings observed in SAED are corresponded to planes, (111), (200), (220) and (311) of face-centered cubic (fcc) structure of elemental silver (Inset figure 4.17 (c)).
Figure 4.17. (a) UV-Vis spectroscopy of AgNPs, (b) XRD of AgNPs, (c) TEM image of AgNPs (In inset, SAED pattern), (d) fluorescence spectroscopy of AgNPs

Fluorescence spectra of AgNPs stabilized in aqueous solutions and exhibiting maximum emission at 299 and 315 nm were reported by Siwach et al. [37] and Jian et al. [38] respectively. As to the excitation at the surface plasmon resonance, i.e. $\lambda_{ex} = 380–440$ nm, we found a weak and broad fluorescence band centered at $\sim$490 nm (figure 4.17 (d)) in line with the study reported by Boyd et al. [39]. There is a decrease in the intensity and shifting of the emission wavelength for excitation wavelength 440 nm. This decrease in intensity suggests that due to the close proximity of emissive species with nanoparticles, quenching of emission takes place through energy transfer process. Hydrodynamic diameter (HDD) distribution of AgNPs in colloidal solution was found to be $30 \pm 5$ nm (figure 4.18 (a)). The zeta potential is an indication of the stability of colloidal aqueous dispersions and usually particles with zeta potential ($\zeta > \pm 30$ mV) are considered to be stabilized due to
electrostatic repulsion (14). Here the zeta potential of as-formed AgNPs in colloidal solution was -40 mV which shows the excellent stability of the AgNPs in the colloidal solution (figure 4.18 (b)).

**Fig. 4.18.** (a) DLS, (b) zeta potential of AgNPs in colloidal solution

### 4.7.4. Stability studies

The general mechanism for stabilization of colloidal material in water has been described in the Derjagnin-Landau-Verwey-Overbeek (DLVO) theory which collectively reveals the effects of Van der Waals attraction and electrostatic repulsion due to double layer of counter ions [40, 41]. The theoretical implication of the DLVO theory in case of silver conjugates has been extensively studied by Panigrahi *et al.* [42] and Xuan Li *et al.* [43] who measured the stability of colloidal dispersion at different pH values and ionic strength respectively. The rate of aggregation of the colloidal particles can be quantified in terms of a semiempirical aggregation parameter. The definition of this parameter has been modified by Sastry *et al* and applied to the problem of aggregation of 4-CTP-capped gold particles [44] as well as avidin-induced aggregation of biotinylated silver and gold particles [45]. Aggregation plays an important role in determining the toxicity of AgNPs as dispersed AgNPs provide enhanced bactericidal effects over aggregated particles. The generation of aggregates leads to a reduction in the antibacterial activity of AgNPs [46].
Here for this study, the SPR spectra and HDD were recorded using UV-Vis spectroscopy and DLS respectively.

The electrostatic stability of AgNPs conjugates to ionic strength was analyzed by increasing concentration of the NaCl from 0 to 1500 mM. Figure 4.19 (a) shows the SPR spectra for AgNPs colloidal solution at pH 9 with increase of NaCl concentration which exhibit DLVO type behavior. At low NaCl concentration (0 to 300 mM), each increase in the electrolyte leads to a corresponding increase in the aggregation rate (figure 4.19 (b)). The SPR peak was increased with NaCl concentration from 0 to 700 mM. Further increase in the NaCl concentration from 700 to 1500 mM caused decrease in the SPR peak. The DLS measurement showed increase in the HDD from 20 to 179 nm as NaCl concentration is increased from 0 to 1500 mM. At low NaCl concentration (<300mM), the aggregation of the AgNPs occur at very slow rate and depend on the NaCl concentration. Increasing the electrolyte concentration leads to corresponding increase in the aggregation rate by diminishing the electrostatic barrier to aggregation that exist between negatively charged AgNPs [43]. When electrolyte concentration reach and exceed the critical coagulation concentration (>300mM) resulting in rapid aggregation. Thus the electrostatic interaction stabilizes the AgNPs in colloidal solution.

Figure 4.19 (c) shows SPR spectra of the AgNPs colloidal solution with change in the pH from 2-11. The biomolecules capped AgNPs showed stability in alkaline, neutral and slightly acidic condition. The SPR absorption was stable from pH 6-11. At alkaline pH carboxylic groups are fully charged leading to maximum electrostatic repulsive interactions and maximum stabilization. Further decrease in the pH from 6 to 2, the intensity of SPR absorption strongly decreases. The presence of a longer wavelength component arises due to aggregation of the clusters in quasi-linear strands [45]. AgNPs agglomerated due to neutralization of negatively charge of the acidic group and subsequent decrease of electrostatic repulsion between particles as the pH of the solution was below the pKa (~5) of the terminal carboxylic group [44]. As a result of this agglomeration, DLS showed decrease in the HDD and
aggregation parameter below pH 4 (figure 4.19 (d)). So the surface charge which leads to electrostatic property is responsible for the stability of the AgNPs in the colloidal solution.

The temperature stability of the AgNPs in the colloidal solution was studied by incubating AgNPs colloidal solution for half an hour from temperature 30 °C to 90 °C. The SPR absorption of the AgNPs colloidal solution (figure 4.19 (e)) showed that AgNPs were stable for temperature range from 30 °C to 60 °C. The biomolecules attached to AgNPs were intact up to 60 °C. Further rise in the temperature from 60 °C to 90 °C, may have denatured biomolecules attached to the AgNPs. As the capping of AgNPs was removed, the aggregation of the AgNPs occurred. So there was rise in the SPR absorption of the AgNPs in the colloidal solution as temperature increases. DLS shows the increase in the HDD of AgNPs from 20 to 230 nm and so as the AP (figure 4.19 (f)). So the proteins or other biomolecules attached to the AgNPs may have contributed to stability of AgNPs in the colloidal solution as mesophilic proteins denature at high temperature.
Figure 4.19. Stability studies of AgNPs in colloidal solution: effect of ionic strength (a–b), pH (c–d) and temperature (e–f) on stability of AgNPs. UV-vis spectra of AgNPs suspension at different NaCl (a), in presence of different amount of pH values (C) at pH 9.0; and following incubation at different temperature (e). Right panels (b, d, and f) show aggregation parameter (AP) and size of AgNPs (HDD)
4.7.5 Antimicrobial activity

Since ancient times, silver and its compounds have demonstrated for their strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities for bacteria, fungi, and virus [46]. Currently there is large use of AgNPs in portable water filters, clothing, medical devices and coating for washing machines and food containers for its antimicrobial property [46]. Herein, the antimicrobial activity of cell free extract, AgNO$_3$ (3mM), and as-formed AgNPs is shown in table 4.2.

**Table 4.2.** Antimicrobial activity of cell free extract, AgNO$_3$ and as-formed AgNPs

<table>
<thead>
<tr>
<th>Test microorganisms</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell free extract</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. lusitaniae</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. guilliemondii</em></td>
<td>0</td>
</tr>
</tbody>
</table>

The cell free extract did not show any antimicrobial activity on test microorganisms. The average zone of inhibition for Gram negative microorganisms (*E. coli, P. aeruginosa*) is more than that of Gram positive microorganisms (*B. cereus, S. aureus and E. faecalis*). The difference in
antimicrobial activity between gram positive and gram negative could be due to their difference in membrane structure.

The gram positive bacteria have a thick peptidoglycan layer, whereas peptidoglycan layer in the gram negative bacteria is thin but surrounded by a lipid layer outside. The *candida* species used for the study showed complete resistance to the aqueous AgNO₃. The as-formed AgNPs showed excellent antimicrobial activity against these microorganisms. Comparatively, the zone of inhibition obtained for AgNPs was found more than that of aqueous AgNO₃ for all test microorganisms. So it is clear from the study that the as-formed AgNPs can be used for therapeutic purpose against bacteria and candidial infections.

**4.7.6. Catalytic activity**

As 4-NP is a carcinogenic, mutagenic, and cyto and embryonic-toxic, there is necessity to develop the advanced and effective methods for the degradation of 4-NP. Though, different physical and chemical methodologies as advanced oxidation processes including ozonation, photocatalysis, UV irradiation, sonolysis, microwave, electrocatalysis, and Fenton reaction have been mentioned for removal of 4-NP from industrial waste water and ground water, it is of great need of eco-friendly, efficient and robust technique to degrade 4-NP. The catalytic reduction of 4-NP to 4-AP by NaBH₄ in presence of noble metal as catalyst has turn out to be of great concern. Various metals like Pd, Au, Ag, Cu and Pt in different substrate such as polyelectrolyte, dendrimers and biological cells have been used for this catalytic activity [47, 48]. Here the catalytic activity of as-synthesized AgNPs and magnetic alginate silver beads. As borate is low toxic, the borohydrate is eco-friendly. Though the reduction of 4-NP by NaBH₄ is thermodynamically possible reaction, without catalyst it is kinetically limited.

The ease in the monitoring the catalytic reaction using UV–Vis spectroscopy is the major advantage of this reaction. There is shift of absorbance of 4-NP from 317 nm to 400 nm upon addition of 4-NP in borohydrate due to formation of 4-nitrophenolate anions in presence of
borohydride under alkaline condition. These ions remain stable for weeks in absence of any other reagents. When the reduction of 4-NP was initiated, the absorption peak at 400 nm gradually decreased in intensity along with the increase of a new absorption peak at 300 nm, indicating the formation of 4-AP [49]. After addition of AgNPs in 4-nitrophenolate ion solution, the absorption intensity of 4-NP at 400 nm decreased rapidly with time accompanied by the appearance of the peak at 300 nm of 4-AP demonstrating the conversion of 4-NP to 4-AP and rapid decolorization of yellow-green solution is observable (figure 4.20(a)).

![Figure 4.20](image)

**Figure 4.20.** (a) Color change of pale yellow to colorless in course of 4-NP degradation, (b) Time dependant successive UV-Vis absorption spectra of the reduction of 4-NP by NaBH₄ in the presence of AgNPs colloidal solution, (c) Plot of ln (C/C₀) against the reaction time for AgNPs in colloidal solution

The concentration of 4-NP in the reaction is proportional to its absorbance at 400 nm. Therefore the rate of reaction can be calculated using formula; ln C/C₀ = -kt where, k is apparent rate constant, t is the reaction time. C and C₀ are concentration of 4-NP at time t and 0, respectively. The borohydrate concentration used for entire study was 0.1 M, which is a large
excess compared to that of 4-NP, to consider the reaction as pseudo first order reaction so that the rate of reaction depend on 4-NP concentration.

The time require for the complete reduction of the 4-NP by homogeneous catalytic reaction was about 8 min (figure 4.20(b)) and the rate of reaction (k) obtained from the slope of the straight line was found to be 0.39 min\(^{-1}\) (figure 4.20 (c)). Whereas for the heterogeneous catalytic activity, the time require was 11 min (figure 4.21 (a)) and rate of reaction was about 0.30 min\(^{-1}\) (figure 4.21 (b)). The availability of the nanoparticles for the catalytic reaction is more in the homogeneous catalytic reaction than that heterogeneous catalytic reaction so the rate of reaction is more in case of homogeneous catalytic reactions. The major advantage of the polymer immobilized AgNPs is the reusability in the heterogeneous catalytic reaction as in homogeneous catalytic reaction AgNPs get dispersed in the reaction mixture which is very much difficult to recover. Here in this experiment the magnetic alginate silver beads could be recovered using magnet used for next catalytic reaction (figure 4.21 (c)). As shown in the figure 4.21 (d), it is clear that the as-formed beads can be recycled for successive three catalytic reactions. These rate of reaction obtained in both catalytic reactions were higher than that of Ag@AMH (0.27 min\(^{-1}\)) \[50\], alginate/Ag composites (0.007 min\(^{-1}\)) \[51\], AgNPs derived from *Breynia rhamnoides* (0.24 min\(^{-1}\)) \[52\], and Ag-chitosan composite (0.106 min\(^{-1}\)) \[53\].
Figure 4.21. (a) Time dependant successive UV-Vis absorption spectra of the reduction of 4-NP by NaBH₄ in the presence of magnetic alginate silver beads, (b) Plot of ln (C/C₀) against the reaction time for magnetic alginate silver beads, (c) Photographic images of magnetic alginate silver beads, (d) Plot of ln C/C₀ versus reaction time for 4-NP reduction in the presence of magnetic alginate silver beads by NaBH₄ for consecutive 3 cycles

4.8. Summary

The green microbial synthesis of AgNPs using versatile actinobacteria Rhodococcus sp is demonstrated. The synthesis process was optimized to obtain monodispersed AgNPs by varying AgNO₃ concentration, pH, temperature, age of microorganism and nitrate source. The high yield was observed when microorganism at its active stage i.e. log phase and when microorganism was supplied with 1mM of AgNO₃ at 30-35 °C in slightly alkaline pH. From experiments, it is evident that the AgNPs synthesis was occurring inside the cell. The synthesis of monodispersed and stable AgNPs
were achieved by growing the organisms in different carbon sources for the growth of the microorganisms. The synthesis was observed in both simple carbon sources i.e. glucose, fructose, sucrose, sodium acetate and aromatic compounds i.e. benzene, phenol, toluene. *Rhodococcus* synthesized good stable and monodispersed AgNPs when grown on sodium acetate. The synthesis of polydispersed AgNPs was possible using aromatic compounds with f aire stability. The monodispersity and stability of AgNPs was found depend on concentration of proteins present in colloidal solution produced using different carbon sources. The As-synthesized AgNPs showed excellent bacteriostatic and bactericidal activity against pathogenic Gram positive and Gram negative microorganism. To make use of these AgNPs for biomedical application there is necessity to study the behavior of nanoparticles towards human cell line. From the results it is clear that more than 5 µg mL⁻¹ AgNPs concentrations are inhibitory for microorganisms but 18 µg mL⁻¹ concentration required to kill half of cancerous cells. So it is clear that concentrations of As-synthesized AgNPs needed to inhibit the growth of pathogenic microorganisms were less effective to affect human cells.

Also in next half of the chapter described about the synthesis of AgNPs using cell supernatant of phenol degraded broth. The AgNPs were characterized by TEM and DLS confirming their nanoparticles size less than 30 nm. The AgNPs in colloidal solution showed excellent stability, determined using zeta potential. The presence of extracellular proteins on the AgNPs was analyzed by stability studies. The pH and the ionic strength studies demonstrated that AgNPs are stabilized by electrostatic repulsion due to the negative charge of protein molecules. The protein present on nanoparticles provided the stability of AgNPs against high ionic strength and over a wide range of pH 5–12 and temperature 20–90 °C. The as-prepared AgNPs showed very good antimicrobial activity against human pathogens, two Gram positive, Gram negative bacteria and four fungal species. The rate of reaction in the homogeneous and heterogeneous catalytic property of colloidal AgNPs and magnetic alginate silver beads respectively for the reduction of 4-NP by NaBH₄.
was found more than that of earlier reported. So here highly antimicrobial and catalytic active AgNPs can be synthesized using phenol or phenolic compound degraded by microorganisms and further advancement in the process may lead to use these AgNPs in industries and water disinfection.
References

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