This chapter describes biological synthesis of anisotropic gold and silver nanoparticles using bacterial metabolites. A number of primary or secondary metabolites like siderophores, pigments and enzymes were explored for the shape directed synthesis of gold and silver nanoparticles. The ferric ion chelating siderophore–pyoverdin from the bacterium *Pseudomonas aeruginosa* was shown to be capable of synthesizing triangular gold nanoparticles. The edge length of the triangular gold particles could be controlled by varying the concentration of siderophore. Pyocyanin, a blue–green pigment produced by *Pseudomonas aeruginosa* was capable of synthesizing nearly uniform sized gold nanoparticles and wire-like silver nanoparticles. This chapter also illustrates the accelerated synthesis of small triangular gold nanoparticles employing protease enzyme synthesized by *Actinobacter* spp. in the presence of bovine serum albumin (BSA).

Part of the work presented in this chapter has been communicated in the following journals:
6.1 Introduction

Development of new synthetic protocols for the synthesis of metallic nanoparticles with different size and shapes constitutes an ever-growing field of nanoscience. Among various metallic nanoparticles, noble metal nanoparticles show exciting optical properties such as wavelength selective plasmon absorption resonances, conductivity and catalysis [1]. These properties of noble metal nanoparticles depend on their size and shape which can be exploited for numerous applications ranging from catalysis [2] to biosensing [3] to optics [4]. Hence, it is highly desirable to produce gold and silver nanoparticles with different morphology and size in high yield. A large volume of work on the synthesis of gold and silver nanoparticles with myriad shapes is based primarily on the chemical synthesis methodologies developed until now [5].

Research in the past few years has demonstrated that biological synthesis of inorganic materials can be equally important due to the environmental hazards caused by chemical synthesis methods. Many biological systems exert exquisite control over formation of the metal nanoclusters through concerted mechanisms. The means of nanocluster formation involves bioaccumulation [6], reduction and mineralization processes [7]. Recent work in this field has inclined towards biological methods owing to their environmental friendly and economically viable nature [8].

So far, biological synthesis of gold and silver nanoparticles has relied on the systematic use of micro-organisms like bacteria [9] and fungi [10] or plants/plant extracts [11]. Several studies have demonstrated that proteins isolated from biological organisms can be used as enzymes or templates for material synthesis in vitro. This includes silica and silicones synthesis by silicateins [12], silaffins [13] and silica precipitating peptides [14], calcium carbonate by specialized biological macromolecules [15], cadmium sulfide by glutathione and phytochelatins [16] etc. Though these reports show formation of inorganic materials, similar protocols for the synthesis of metallic nanoparticles have not been explored extensively. Nevertheless, there are a few reports on biomimetic synthesis of metallic nanoparticles. Small peptide molecules, called as dodecapeptides derived by phage display library have been implemented for the synthesis of gold nanoparticles [17]. A small peptide isolated from a combinatorial phage display peptide library associated
with silver binding and reduction has been used to synthesize silver nanoparticles with a variety of crystal morphologies [18].

This chapter illustrates the biological synthesis of gold and silver nanoparticles using bacterial metabolites, which are intermediates or products of metabolism. Metabolites like a ferric ion chelating compound siderophore and pigment from \textit{Pseudomonas aeruginosa} have been used to synthesize anisotropic gold and silver nanoparticles. Furthermore, the accelerated synthesis of small triangular gold nanoparticles by protein hydrolyzing enzyme protease from \textit{Actinobacter} spp. has been illustrated.

\textit{Pseudomonas aeruginosa} is a gram negative aerobic bacterium known to synthesize a number of primary and secondary metabolites with physiological significance. Under the limiting conditions of ferric ion, \textit{P. aeruginosa} produces two types of siderophores, pyochelin and pyoverdin [19]. Pyochelin is a salicylic acid derivative [20], while pyoverdin is a small linear octapeptide bound to 2,3-diamino-6,7 dihydroxyquinoline [21]. The structure of pyoverdin is as follows:

\begin{center}
\includegraphics[width=0.5\textwidth]{pyoverdin_structure.png}
\end{center}

\textit{Chemical structure of the siderophore pyoverdin synthesized by the bacterium \textit{P. aeruginosa} used in the present work.}

Pyochelin and pyoverdin show strong fluorescence when exposed to UV light [22]. Though the primary function of siderophores synthesized by \textit{P.aeruginosa} is iron acquisition under iron limiting conditions, many physiological implications for these molecules are reported. Pyochelin and pyoverdin can act as an excellent biocontrol agent in plant growth promoting pseudomonads [23]. The biosynthesis of these siderophores is
governed by the genes \textit{pchDCBA} and \textit{pchEF}, which are clustered in the chromosome of \textit{P.aeruginosa} [19].

\textit{Pseudomonas aeruginosa} is also known to synthesize a blue-green coloured pigment called pyocyanin. Structurally, pyocyanin is 1-hydroxymethyl-5-methyl phenazine, synthesized in the late logarithmic or stationary phase of the bacterial life cycle [24]. Pyocyanin exerts a strong antimicrobial action against the closely related species of pseudomonas [25] and also has cytotoxic effects against mammalian cells [26]. In addition, pyocyanin acts as a redox shuttle and can undergo a reversible two electron reduction to form a colorless product leucopyocyanin, which in turn, is readily oxidized by molecular oxygen [27]. Further, pyocyanin is speculated to play a role in bacterial energy metabolism under non-optimal growth conditions [27]. Biosynthesis of pyocyanin, an offshoot of shikimic acid pathway, is controlled by the enzymes involved in the synthesis of phenazine [28]. Phenazine biosynthesis is governed by an operon consisting of seven genes in \textit{P. aeruginosa} that encodes seven enzymes generally named as Phz A–G [28]. The structure of pyocyanin synthesized by \textit{P. aeruginosa} is as follows:

![Chemical structure of pyocyanin molecule synthesized by the bacterium P. aeruginosa.](image)

Many micro-organisms extracellularly synthesize protein hydrolyzing enzymes known as proteases. Proteases are divided into the four subclasses; cysteine, serine, aspartic and metallo-proteases depending on the amino acid residues present in their active sites [29]. Aspartic protease contains aspartic acid in their active site triad and show structural and functional variation from species to species. Proteases are essential for the growth and metabolism of all living cells, hence they are considered as primary metabolites. There are few reports on the interaction of protease and gold nanoparticles

\textit{Ph.D. Thesis} \hspace{1cm} \textit{Atul Bharde} \hspace{1cm} \textit{University of Pune}
[30]. Recently, enzymatic catalysis has been used for the synthesis of nanoparticles [31]. However, in this case, nanoparticles were not synthesized by the direct action of enzymes.

In pursuing new “green chemistry” methods for the synthesis of metallic nanoparticles, the use of bacterial primary and secondary metabolites like siderophores, enzymes and pigments respectively have been demonstrated in this chapter. Numerous metabolites are at the center of redox or electron transfer reactions occurring inside the cell fulfilling various physiological demands. Many biological molecules have their redox potential lower than ionic gold and silver and hence can be used for the synthesis of metallic nanoparticles. Use of bacterial metabolites as a reducing and shape directing agent for the synthesis of anisotropic gold and silver nanoparticles can be considered as an important development in the steadily evolving biological synthesis methods. Biological synthesis methods described here are rapid and are comparable with existing chemical synthesis methods.

**Outline of the present work:**

The current chapter describes biological synthesis of the anisotropic gold and silver nanoparticles using a siderophore and pigment from Gram negative bacterium *P. aeruginosa*, which was isolated from Arabian sea, Goa, India. Siderophores show higher affinity towards Au$^{3+}$ ions and rapidly reduces them to Au$^{0}$, eventually resulting in the formation of the triangular gold particles. It is also capable of reducting Ag$^{+}$ ions and results in the subsequent synthesis of silver nanoparticles. However, the rate of silver ion reduction was found to be lower indicating the lower affinity of the siderophore molecules towards silver ions in comparison with gold ions. On the other hand, reduction of Ag$^{+}$ ions with pyocyanin, a pigment from *P. aeruginosa* is very rapid and the synthesis of silver nanoparticles could be observed in 60 minutes resulting in ribbon like structures. Pyocyanin could also synthesize gold nanoparticles with a narrow size distribution. However, the synthesis of gold nanoparticles was slower in comparison with silver nanoparticles. The last part of this chapter describes the accelerated synthesis of small gold nanoparticles by induction of the enzyme protease in *Actinobacter* spp. The presence of atmospheric oxygen seems to be a crucial factor in controlling the rate of nanoparticle synthesis.
6.2 Biological synthesis of gold and silver nanoparticles by the siderophore pyoverdin

This part of the chapter describes biological synthesis of gold and silver nanoparticles from gold and silver ions using pyoverdin as a reducing agent.

6.2.1 Experimental details

The siderophore pyoverdin was isolated from *P. aeruginosa* by a previously described method [32]. Briefly, bacterial seed culture was grown in LB medium for a period of 12 h. This seed culture was then inoculated in 100 ml of King’s B medium in a 500 ml Erlenmeyer flask for the induction of siderophore formation. The flask was incubated on the shaker (150 rpm) for 48 h at 37 °C. Next, the siderophore was isolated from the aqueous medium by solvent extraction described as follow. The supernatant containing siderophore was collected by centrifugation at 7000 rpm (5533 X g) and the pellet containing the bacterial cells was discarded. The supernatant was acidified with concentrated hydrochloric acid until pH of the solution became 2. The acidified supernatant was mixed with ethyl acetate in 1:1 ratio and incubated overnight. The organic layer was separated from the aqueous layer. The organic layer of ethyl acetate was evaporated and the dried and the powdered residue containing siderophore was dissolved in a small volume of methanol. Thin layer chromatography (TLC) was performed to assess the production and purity of pyoverdin by applying to Silicagel 60F254 TLC plate (Merck). The plate was developed using chloroform-acetic acid-ethanol (90:5:2.5 vol/vol/vol) solvent system and then visualized under UV light (λ ~ 305 nm). For the synthesis of gold and silver nanoparticles, 10^{-3} M of aqueous HAuCl\textsubscript{4} and 10^{-3} M AgNO\textsubscript{3} was reacted with 0.05%, 0.1% and 0.2% of pyoverdin in the final reaction volume of 10 ml respectively. Gold and silver nanoparticles were characterized by TEM, AFM, UV-vis-NIR spectroscopy, and FTIR spectroscopy.

6.2.2 Isolation and purification of Pyoverdin

Synthesis of siderophore by *P. aeruginosa* is induced by the iron limiting conditions, which are generally developed in the stationary phase of bacterial growth due to the depletion of nutrients in the growth medium. Growth of *P. aeruginosa* in King’s B medium promotes the synthesis of pyoverdin and suppresses the synthesis of pyochelin [33]. Pyoverdin was secreted extracellularly in the surrounding medium as indicated by
the yellow–green colour of the medium. Bacterial cell-free supernatant when acidified with concentrated HCl became pale brown in the colour due to the presence of pyoverdin. The acidified supernatant was mixed with ethyl acetate and kept overnight. The organic layer showed a brown colour due to the presence of pyoverdin, which was later isolated by evaporation of ethyl acetate.

The presence of pyoverdin is indicated by the UV spectroscopic analysis, where pyoverdin shows a strong absorption at 402 nm. Purity of the pyoverdin is checked by TLC analysis. The TLC plate is irradiated with UV light (λ ~ 305 nm) and shows the presence of a single spot, which exhibits blue–green fluorescence. Figure 6.1A shows a photograph of TLC plate after irradiation with UV light showing the presence of a single spot. The R_F values calculated for pyoverdin is found to be 0.4. Pyoverdin forms a highly stable brown coloured complex with ferric iron under acidic conditions [32]. Figure 6.1B shows a photograph of the TLC plate after spreading with 0.1 M FeCl_3 in 0.1 M HCl indicating the brown colour due to the formation of complex with ferric ions.

**Figure 6.1** (A) Photograph of TLC plate showing the migration of pyoverdin in chloroform: acetic acid: ethanol solvent system. A single spot of pyocyanin showing bluish green fluorescence in UV-light (λ~305 nm) was observed. “*” indicates the origin, at which pyoverdin was spotted, while “•” indicates the final position of the spot attained by migration in the solvent system as mentioned in the text. (B) Photograph of TLC plate after reaction with 0.1 M FeCl_3 in 0.1 M HCl.
6.2.3 Synthesis of anisotropic gold particles by Pyoverdin

6.2.3.1 TEM analysis

Representative TEM images of pyoverdin reduced gold ions solution show a number of nanoparticles with the triangular and hexagonal morphology. The edge length of the triangular and hexagonal particles varied between 0.2 μm to 5 μm depending on the concentration of pyoverdin used for reduction of chloroaurate ions. It was observed that the edge length of triangular and hexagonal nanoparticles synthesized using pyoverdin depends on the concentration of pyoverdin used for the reduction of gold ions. At lower concentration of pyoverdin i.e. 0.05 % (V/V), large, flat triangular and hexagonal particles are formed with the edge lengths up to ~ 6 μm (Figure 6.2A). The size range for gold particles varied between 3 to 5 μm. On the other hand, at a concentration of 0.1 % (V/V) of pyoverdin, the edge length of triangular and hexagonal particles varied between 0.5 μm to 3 μm (Figure 6.2 B&C).

![Representative TEM images of gold nanoparticles synthesized by reaction between HAuCl₄ and pyoverdin. The edge length of triangular particle increases with the decreasing concentration of pyoverdin. Triangular and hexagonal gold particles of edge length between 100 nm to 5 μm are synthesized using 0.05 % (A), 0.1 % (B & C) and 0.2 % (D & E) concentrations of pyoverdin. The inset in (E) shows the AFM image of one of the triangular gold particles synthesized using 0.2 % pyoverdin. The scale bar in AFM image is 250 nm. (F) SAED pattern obtained from a single triangular gold particle along with the lattice planes.](image)
Triangular and hexagonal gold particles with nearly uniform edge length of 200 nm to 250 nm are synthesized at 0.2 % (V/V) concentration of pyoverdin. However, a number of small spherical particles are also observed along with triangular and hexagonal particles at this concentration of pyoverdin (Figure 6.2 D & E). AFM analysis carried out on one of the triangular gold particle synthesized using 0.2 % pyoverdin indicates that the thickness of the triangular gold particle is ~ 25 nm (inset in image E, Figure 6.2). The selected area diffraction pattern obtained from one of the triangular particle indicates that each particle is a single crystal and the diffraction pattern was indexed on the basis of FCC structure of gold. The corresponding \( d \) values for the respective crystal planes are as follows: 2.04 (200), 1.45 (220), 1.25 (311) [34].

### 6.2.3.2 UV-vis-NIR spectroscopic analysis

After the addition of pyoverdin to \( 10^{-3} \) M HAuCl\(_4\), the originally yellow colour of the reaction medium gradually changed to a dark brown within 4 h of reaction, indicating the formation of gold nanoparticles. Figure 6.3A shows UV-vis spectra of gold nanoparticles synthesized using 0.05 % and 0.1 % solution of pyoverdin respectively. A strong absorption band at 580 nm is observed due to the formation of gold nanoparticles by 0.05 % and 0.1 % of pyoverdin respectively (curves 1 and 2, Figure 6.3A). The absorption band shows a red shift in comparison with the surface plasmon resonance of spherical gold nanoparticles, which is normally centered at 520 nm. Further, the absorption band at 580 nm corresponding to gold particles synthesized using 0.05 % of pyoverdin shows broadening (curve 1 in Figure 6.3A), due to the aggregation of small spherical particles. The SPR band at 580 nm is accompanied by gradually increasing absorption in the near infra-red (NIR) region of the electromagnetic spectrum. The absorption in the NIR region is due to the in-plane collective electronic oscillation indicating the anisotropic nature of gold particles [35]. The absorption observed in the NIR region is higher in intensity for gold particles synthesized using 0.1 % of pyoverdin in comparison with gold particles synthesized using 0.05 % of pyoverdin (Figure 6.3, curves 1 and 2). The rise in intensity of the absorption in the NIR region could be due to increasing population of anisotropic gold particles. Figure 6.3B shows UV-vis spectra as a function of time of reaction between aqueous solution of gold ions and 0.2 % of pyoverdin. The surface plasmon resonance band recorded after 1 h of reaction shows a
weak absorption at 560 nm (curve 1, Figure 6.3B), which gradually red shifts and increases in intensity after 4 h (curve 4, Figure 6.3B).

![Figure 6.3](image)

*Figure 6.3* (A) UV-vis-NIR spectra of gold nanoparticles synthesized by reaction between gold ions and 0.05 % (curve 1) and 0.1 % (curve 2) of pyoverdin. (B) UV-vis-NIR spectra recorded as a function of time of reaction between 0.2 % of pyoverdin and aqueous gold ions. Curves 1 to 4 correspond to the progress of reaction after 1, 2, 3 and 4 h respectively.

As observed with gold particles synthesized by 0.05 % and 0.1 % of pyoverdin (curves 1 & 2, Figure 6.3A), the SPR band at 560 nm is accompanied by an absorption at 850 nm (curve 4, Figure 6.3B). However, the intensity of the observed in plane SPR band at 850 nm is lower than that of out of plane SPR band at 560 nm probably indicating that the population of anisotropic gold particles is less than that of spherical gold nanoparticles (curve 4, Figure 6.3B) [36].

### 6.2.3.3 FTIR spectroscopic analysis

FTIR measurements were carried out to identify the binding of gold nanoparticles with various functional groups present in pyoverdin. The mechanism of reduction of chloroaurate ions by pyoverdin can also be studied by FTIR spectroscopic analysis carried out on pyoverdin before and after the reaction with chloroaurate ions. As mentioned earlier, chemically pyoverdin is a octapeptide bound to dihydroxyquinoline [21]. Figure 6.4 shows the FTIR spectra of purified pyoverdin molecules (curve 1) and pyoverdin–chloroaurate ions reaction mixture (curve 2) after the synthesis of gold nanoparticles. The different vibrational frequencies shown in the FTIR spectra are given in the Table 6.1. A prominent absorption band at 1144 cm\(^{-1}\) in the spectrum
corresponding to pyoverdin originates due to the stretching vibrations of C–N bonds
(curve 1, Figure 6.4A), which are not observed in the spectrum corresponding to
pyoverdin – chloroaurate ion reaction mixture after the formation of gold nanoparticles
(curve 2, Figure 6.4A). The complete disappearance of a small vibrational band at 1544
\( \text{cm}^{-1} \), assigned to amide II (N–H) stretching vibrations from curve 2, which corresponds
to the reduction of chloroauric acid indicates that it could be involved in the formation of
gold nanoparticles (curve 2, Figure 6.4A). This observation is consistent with the
disappearance of the C–N bond absorption and supports the idea that amide or amine
groups could be responsible for the reduction of chloroaurate ions or the interaction with
gold nanoparticles.

\[
\begin{align*}
\text{Figure 6.4 (A)} & \quad \text{FTIR spectra obtained from pyoverdin in powdered KBr before (curve 1) and after}
\text{reaction with chloroauric acid (curve 2). Symbol “*” indicates vibrational bands that could be}
\text{involved in the reduction of chloroauric acid and binding to the surface of gold nanoparticles. (B)}
\text{Enlarged view of the same spectra in the amine and hydroxyl groups vibrational regions.}
\end{align*}
\]

The presence of a weak absorption band centered at 1438 cm\(^{-1}\) in curve 1 could be
assigned to the CH\(_2\) group stretching vibrations that is not observed in the spectrum
equivalent to pyoverdin–chloroaurate ion reaction mixture (curve 2 Figure 6.4A). The
strong absorption at 1663 cm\(^{-1}\) due to amide I (C=O) vibrational band in pyoverdin (curve
1, Figure 6.4A) is shifted towards lower wavenumber at 1642 cm\(^{-1}\) (curve 2, Figure 6.4A)
after reaction with chloroauric acid. This observation further suggests that amine or
amide groups play an important role in the formation of gold nanoparticles. A broad and strong absorption band at 3140 cm\(^{-1}\) could be assigned either to the stretching vibrations of amine or hydroxyl groups present in pyoverdin as shown in the curve 1 and 2 in Figure 6.4B. Amine and hydroxyl groups have overlapping absorption (3200–3600 cm\(^{-1}\)) in the FTIR spectrum and therefore it is very difficult to assign the bond vibrations in this region to a particular functional group. The absorption band at 3140 cm\(^{-1}\) observed in the curve 1 in Figure 6.4B, which corresponds to pyoverdin before reaction with chloroaurate ions is considerably shifted to 3400 cm\(^{-1}\) after reaction with chloroaurate ions and the formation of gold nanoparticles (curve 2, Figure 6.4B).

**Table 6.1** Vibration frequency assignment for the peaks observed in the FTIR spectra of pyoverdin before and after reaction with chloroaurate ions represented by the curves 1 and 2 in Figure 6.3, respectively [36].

<table>
<thead>
<tr>
<th>Vibration modes</th>
<th>Pyoverdin (cm(^{-1}))</th>
<th>Pyoverdin treated with chloroaurate ions (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C – O ((\nu))</td>
<td>1067</td>
<td>1048</td>
</tr>
<tr>
<td>C – N ((\nu))</td>
<td>1144</td>
<td>1142</td>
</tr>
<tr>
<td>C – O ((\nu))</td>
<td>1221</td>
<td>– –</td>
</tr>
<tr>
<td>CH(_3) ((\delta))</td>
<td>1402</td>
<td>1400</td>
</tr>
<tr>
<td>O – C – H ((\delta)), CH(_2) ((\delta))</td>
<td>1438</td>
<td>– –</td>
</tr>
<tr>
<td>N – H ((\delta)) of amide group</td>
<td>1545</td>
<td>– –</td>
</tr>
<tr>
<td>C = O ((\nu)) of amide group</td>
<td>1663</td>
<td>1641</td>
</tr>
<tr>
<td>C = O ((\nu)) of aldehyde and ketone</td>
<td>1735</td>
<td>1730</td>
</tr>
<tr>
<td>C – H</td>
<td>2929</td>
<td>2930</td>
</tr>
<tr>
<td>N – H ((\nu)) of amine group or O – H ((\nu))</td>
<td>3140</td>
<td>3400</td>
</tr>
</tbody>
</table>

(\(\delta\)) bending vibrations, (\(\nu\)) stretching vibration

This shift could be because of the interaction of amine groups with the surface of gold nanoparticles. However, it is very difficult to draw such a conclusion at this stage.
due to the overlapping absorption of amine and hydroxyl group in the FTIR spectrum. Nevertheless, the disappearance or shift in amide stretching vibrations strongly suggests that this group of pyoverdin is possibly responsible for the synthesis of gold nanoparticles and their stabilization. Thus the FTIR analysis reveals considerable changes in the vibrational frequencies of different functional groups present in pyoverdin after reacting with aqueous chloroaurate ions. However, due to the complex structure of pyoverdin it is not possible to precisely monitor the exact functional group that donates the electrons and reduces gold ions by FTIR spectroscopy.

6.2.4 Biological synthesis of silver nanoparticles by Pyoverdin

6.2.4.1 TEM analysis

Figure 6.5 shows TEM images of silver nanoparticles synthesized by reaction between $10^{-3}$ M AgNO$_3$ and 0.1% pyoverdin after 6 h of reaction. A large number of silver nanoparticles are observed to be distributed throughout the grid surface (images A & B in Figure 6.5).

![TEM images of silver nanoparticles synthesized by reaction between 0.1% pyoverdin and 10^{-3} M AgNO$_3$ solution after incubation for 6 h. (A-C). SAED pattern obtained from silver nanoparticles indicates their crystalline nature and the diffraction pattern was indexed on the basis of FCC structure of silver (D).](image)

The nanoparticles are irregular in shape with a broad particle size distribution. The overall size of silver nanoparticles appears to be in between 50 to 100 nm. The nanoparticles are not regularly separated and tend to form the aggregated structures. The
nanoparticles are observed to be embedded in a continuous matrix consisting of the organic siderophore molecules as indicated by the lighter contrast at the periphery of silver nanoparticles (image C, Figure 6.5). Therefore, the overall structure appears as an interconnected network with high degree of irregularity. SAED analysis obtained from silver nanoparticles shows well defined diffraction rings indicating the crystalline nature of silver nanoparticles (image D, Figure 6.5). The diffraction pattern was readily indexed on the basis of FCC structure of silver. The corresponding $d$ values for the respective crystal planes are as follows: 2.372 (111), 2.05 (200), 1.44 (220), 1.24 (311) [34].

6.2.4.2 UV-vis spectroscopic analysis

Figure 6.6A illustrates the UV-vis spectroscopic analysis of silver nanoparticles formation using 0.1% of pyoverdin solution as a function of time of reaction. Following the addition of 0.1% pyoverdin to $10^{-3}$ M AgNO$_3$ and subsequent incubation for 2 h, a weak absorption hump at 430 nm was observed (curve 1, Figure 6.6A). This absorption arises due to the surface plasmon resonance of silver nanoparticles, which is responsible for the bright yellow colour of the solution of silver nanoparticle. The absorption at 430 nm gradually increases in intensity with time (curve 2 and 3 in Figure 6.6A).

![Figure 6.6](image)

**Figure 6.6** (A) UV-vis spectroscopic analysis of silver nanoparticle synthesis by 0.1% pyoverdin as a function of time of reaction. Curve 1–3 corresponds to pyoverdin – silver ions reaction mixture after 2, 4 and 6 h. (B) UV-vis spectrum recorded after 6 h of reaction from the solution of silver nanoparticles synthesized using 0.2% pyoverdin.

After 6 h of the reaction no appreciable rise in the absorption intensity at 430 nm was observed indicating that the reaction is completed. Figure 6.6B shows the UV-vis spectrum obtained from the aqueous solution of silver nanoparticles synthesized using
0.2% pyoverdin. The SPR band of silver nanoparticles is centered at 430 nm as observed with the solution of silver nanoparticles synthesized using 0.1% pyoverdin. However the SPR band is prominent and sharper in comparison with silver nanoparticles synthesized using 0.1% pyoverdin (curve 3, Figure 6.6A).

### 6.2.4.3 FTIR spectroscopic analysis

Biological synthesis of silver nanoparticles can also be easily monitored by FTIR spectroscopic analysis as demonstrated in the case of gold particles. FTIR analysis was carried out on pyoverdin in powdered KBr before and after reaction with silver ions. FTIR analysis also enables following the interaction of pyoverdin molecules with the surface of silver nanoparticles, as indicated by the change in the positions of vibrational bands arising due to the various functional groups present in pyoverdin.

![FTIR spectra](image)

**Figure 6.7** (A) FTIR spectra of pyoverdin molecules before (curve 1) and after reaction with silver ions. Symbol “*” indicates the vibrational bands which are not observed after reaction with silver ions. (B) Enlarged view of the same spectra in amine and hydroxyl bonds vibration region.

Figure 6.7 shows the FTIR spectra obtained from pyoverdin molecules before and after reaction with silver ions. Considerable variation is observed in the vibrational bands in the FTIR spectra of pyoverdin molecules before and after reaction with silver ions. Two distinct bands in the 1540 to 1670 cm\(^{-1}\) are assigned to vibrations of amide I and II bands that originates from the amide bonds present in the structure of pyoverdin. As
observed earlier with gold particles, a prominent peak at 1663 cm\(^{-1}\) due to the amide I vibrational band in pyoverdin (curve 1, Figure 6.7A) is shifted towards lower wavenumbers to 1633 cm\(^{-1}\) (curve 2, Figure 6.7A) after reaction with \(10^{-3} \text{ M AgNO}_3\), which is reduced to metallic silver. The amide II vibrational band at 1544 cm\(^{-1}\) observed from the FTIR spectrum obtained from unreacted pyoverdin molecules (curve 2, Figure 6.7A). This indicates that \(-\text{NH–}\) group of peptide bond, which is responsible for amide II vibrations bands, may be involved in the reduction of silver ions. The shift in the amide I band vibration after reaction with silver ions could be due to the binding of \(-\text{CO–}\) group of pyoverdin on the surface of silver nanoparticles. The disappearance of the amide II vibrational band along with the shift in the amide I vibrational band indicates strong interaction between pyoverdin and silver ions.

6.2.5 Discussion

This part of the chapter describes the biological synthesis of gold and silver nanoparticles by the siderophore – pyoverdin isolated from \(P.\text{aeruginosa}\). It is observed that flat triangular gold particles with edge length between 0.2 \(\mu\text{m}\) to 6 \(\mu\text{m}\) can be synthesized using pyoverdin as a reducing agent. The edge length of triangular and hexagonal gold particles was strongly dependent on the concentration of pyoverdin used for the reduction of gold ions. At lower concentration of pyoverdin (0.05%), triangular and hexagonal gold particles with edge length between 3 \(\mu\text{m}\) to 6 \(\mu\text{m}\) were predominantly formed. When 0.1% pyoverdin was used for the reduction of gold ions, triangular particles with the edge length in between 0.5 \(\mu\text{m} – 2 \mu\text{m}\) are synthesized. At higher concentration of pyoverdin, the formation of triangular gold particles with nearly uniform edge length of 200 nm to 250 nm were readily observed. The FTIR spectroscopic analysis performed on pyoverdin before and after reaction with gold ions indicated that amine groups of pyoverdin are likely to be involved in the formation of gold nanoparticles. FTIR analysis also indicated the possibility of some interaction between the surface of gold nanoparticles and pyoverdin molecules. Silver nanoparticles were also synthesized using pyoverdin as a reducing agent. However, no anisotropy in silver nanoparticles is observed as compared to gold. A large number of silver nanoparticles with irregular morphology were observed with dimension of 20 to 100 nm. In contrast to gold nanoparticles, no change in the size and shape of silver nanoparticles was observed.
when the concentration of pyoverdin was varied. FTIR analysis of pyoverdin molecules before and after reaction with silver ions indicated that carbonyl and amide groups are likely to be involved in either formation of silver nanoparticles or binding on the surface of silver nanoparticles.

6.3 Biological synthesis of gold and silver nanoparticles by the pigment – pyocyanin

6.3.1 Experimental details

Bacteria from the genus *Pseudomonas* are known to synthesize a number of different pigments, which give characteristic appearance to the bacterial colony [32]. *P. aeruginosa* synthesizes a blue – green pigment pyocyanin during the stationary phase of growth. Pyocyanin was isolated from *P. aeruginosa* using a broth culture as described previously [38]. Freshly inoculated bacterial culture was grown overnight in 2 ml of LB medium, which was further used as a seed culture for bacterial propagation in 100 ml LB in a 500 ml Erlenmeyer flask. The flask was incubated at 30 °C on the rotary shaker (200 rpm) and incubated for 72 h until the medium showed a dark green colour. The bacterial biomass was then separated from the rest of the medium by centrifugation at 7000 rpm. The colored supernatant was isolated in another sterilized conical flask and the pH of the supernatant was adjusted to 2 with concentrated hydrochloric acid (HCl). This acidified supernatant was repeatedly treated with the equal volume of chloroform until all the blue – green colored part was transferred to the chloroform phase. The chloroform layer was then subjected to alternative extractions with acidified and neutral water layers to allow the separation of red, acid form, and blue – green forms of pyocyanin. After five such conversions the pyocyanin was crystallized by evaporation of chloroform, washed with the deionised water and dried to a powder. Pyocyanin was solubilized in deionised water before use for the synthesis of gold and silver nanoparticles. Thin layer chromatography (TLC) was performed to assess the formation and purity of pyocyanin by applying the extracted pyocyanin to Silicagel 60F254 TLC plates (Merck). The plate was developed using chloroform-acetic acid-methanol (90:5:2.5 vol/vol/vol) solvent system and visualized under UV light (λ 305 nm) for detection of fluorescence signal. Gold and silver nanoparticles were synthesized by reacting $10^{-3}$ M of HAuCl₄ and AgNO₃ respectively with 0.1% of purified pyocyanin in the final reaction volume of 10 ml. Gold
and silver nanoparticles so obtained were characterized by TEM, UV-vis-NIR spectroscopy, X-ray diffraction, and FTIR spectroscopy.

**6.3.2 Isolation and purification of pyocyanin**

After growth of *P. aeruginosa* in LB medium for 48 h, the colour of the growth medium became dark and blue–green as observed visually. When the cell free supernatant was repetitively extracted with equal volume of chloroform, pyocyanin was transferred to the chloroform phase as indicated by the blue–green coloration of the organic layer. After evaporation of the chloroform layer, pyocyanin was dissolved in deionised water.

*Figure 6.8* A photograph of TLC plate showing the migration of pyocyanin in chloroform: acetic acid: ethanol solvent system. A single spot of pyocyanin showing bluish green fluorescence in the UV-light (*λ* 305 nm) was observed. “•” indicates the origin, at which pyocyanin sample is spotted, while “∗” indicates the final position of the spot after migration in the solvent system mentioned above.

Presence of pyocyanin was indicated by UV spectroscopic analysis, where a strong absorption at 360 nm was observed. Purity of the pyocyanin was checked by TLC analysis. Figure 6.8 shows a picture of TLC plate after irradiation with UV light that shows the presence of a single spot. The TLC plate irradiated with the UV light (*λ* 305
nm) showed the presence of single spot, which emitted bluish – green fluorescence. The \( R_f \) value calculated for pyocyanin was found to be 0.62 [38].

### 6.3.3 Biological synthesis of gold nanoparticles by Pyocyanin

#### 6.3.3.1 TEM analysis

Representative TEM images of gold nanoparticles obtained from the reaction of 0.1% pyocyanin with \( 10^{-3} \) M aqueous chloroaurate acid solution at different magnifications are shown in Figure 6.9. A highly dense population of gold nanoparticles was obtained after 7 h of reaction. At low magnification (image A & B, Figure 6.9) large population of spherical gold nanoparticles can be observed. Uniformity in the size of gold nanoparticles with a narrow size distribution is a significant achievement of the biological synthesis method described here.

**Figure 6.9** Representative TEM images of gold nanoparticles obtained by the reaction between 0.1% pyocyanin and aqueous chloroaurate ions at different magnifications (A-E). The inset in E shows SAED pattern obtained from gold nanoparticles. Rings 1, 2 and 3 correspond to (111), (200) and (220) set of lattice planes respectively. (F) A histogram of particle size distribution in gold nanoparticles synthesized using pyocyanin.

The high magnification image (image E, Figure 6.9) obtained from gold nanoparticles also shows a small number of triangular particles with edge length of \(~15\) nm. Particle size distribution analysis performed on 100 particles in different micrographs indicated that the average diameter of gold nanoparticle was centered at 12.7 nm with a
standard deviation of 1.57 nm (image F, Figure 6.9). SAED analysis indicates the crystalline nature of gold nanoparticles and the diffraction pattern was indexed on the basis of FCC structure of gold (inset in image C, Figure 6.9). The corresponding $d$ values for the respective crystal planes are as follows: 2.36 (111), 2.06 (200), 1.44 (220) [34].

6.3.3.2 UV-vis spectroscopic analysis

Reduction of gold ions and the formation of gold nanoparticles by pyocyanin can be easily followed by UV-vis spectroscopy. Figure 6.10 shows UV-vis spectroscopic analysis performed on gold nanoparticles synthesized using 0.1% pyocyanin as a reducing agent. UV-vis spectroscopic analysis of chloroaurate ion reduction indicates that reaction is completed in six hours.

It is observed that the formation of gold nanoparticles begins after 1 h of reaction between 0.1% pyocyanin and aqueous chloroaurate ions as indicated by a weak absorption at 545 nm, which is due to the surface plasmon resonance of gold nanoparticles (curve 1, Figure 6.10). As the reaction progresses, the population of gold nanoparticles increases with time. Curves 1-4 indicate the progress of the reaction after 1, 2, 4 and 6 h respectively.

![UV-vis spectroscopic analysis](image)

*Figure 6.10* UV-vis spectroscopic analysis of the formation of gold nanoparticles using pyocyanin. Gradual rise in the absorption at 540 nm shows that the number of gold nanoparticles increases with time. Curves 1-4 indicate the progress of the reaction after 1, 2, 4 and 6 h respectively.
nanoparticles increases steadily. During the 4 h of reaction large number of gold nanoparticles is accumulated in the solution as indicated by the sharp and intense SPR band (curve 3, Figure 6.10). This is indicated by the sharp rise in the SPR absorption band, which is stabilized after 6 h, where no further increase in SPR absorption band intensity is observed (curve 4, Figure 6.10). A sharp SPR peak after complete reduction of chloroaurate ions may be due to the narrow size distribution of gold nanoparticles.

### 6.3.3.3 FTIR analysis

FTIR analysis of pyocyanin before and after the reduction of chloroaurate ions was done in KBr powder and the spectra are shown in Figure 6.11. The FTIR analysis shows a number of vibrational bonds, which corresponds to the various functional groups present in pyocyanin molecules (curve 1, Figure 6.11A and B).

![FTIR spectra](image)

**Figure 6.11 (A)** FTIR spectra obtained from pyocyanin before (curve 1) and after reaction with aqueous chloroaurate ions (curve 2). Symbol “*” indicates the vibrational bands that are absent from curve 2, which corresponds to the FTIR spectrum of gold nanoparticles obtained by reaction of pyocyanin and chloroauric acid. (B) Enlarged view of the same spectra in amine and hydroxyl groups absorption region.

All the major vibrational bands corresponding to the respective functional groups are listed in Table 2. A substantial variation is observed in the 1500 cm\(^{-1}\) to 1700 cm\(^{-1}\) region of the spectrum after reaction with chloroaauric acid (curve 2, Figure 6.11A). A region of the spectrum in between 1450 cm\(^{-1}\) to 1650 cm\(^{-1}\) corresponds to the stretching
vibration bands of C = C of aromatic rings. The disappearance of a sharp absorption band at 1509 cm\(^{-1}\) from the spectrum corresponding to pyocyanin treated with chloroaurate ions (curve 2, Figure 6.11A) could be due to the loss of the aromatic character of pyocyanin ring structure. Also, a prominent absorption band at 1603 cm\(^{-1}\) is shifted at 1657 cm\(^{-1}\) indicating the possibility of alteration in the aromatic character of pyocyanin after reacting with chloroaurate ions (curve 2, Figure 6.11A).

Table 6.2 Vibration frequency assignment for the peaks observed in the FTIR spectra of pyocyanin before and after reaction with chloroaurate ions represented by the curves 1 and 2 in Figure 6.10 respectively [37].

<table>
<thead>
<tr>
<th>Vibration modes</th>
<th>Pyocyanin (cm(^{-1}))</th>
<th>Pyocyanin treated with chloroaurate ions (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C – H of aromatic ring</td>
<td>896</td>
<td>--</td>
</tr>
<tr>
<td>C – O ((\nu))</td>
<td>1056</td>
<td>1060</td>
</tr>
<tr>
<td>C – O ((\nu))</td>
<td>1120</td>
<td>1132</td>
</tr>
<tr>
<td>CH(_3) ((\delta))</td>
<td>1387</td>
<td>1400</td>
</tr>
<tr>
<td>O – C – H ((\delta)), CH(_2) ((\delta))</td>
<td>1458</td>
<td>1470</td>
</tr>
<tr>
<td>C = C of aromatic ring</td>
<td>1508</td>
<td>--</td>
</tr>
<tr>
<td>C = C of aromatic ring</td>
<td>1603</td>
<td>1657</td>
</tr>
<tr>
<td>CH(_3) ((\nu))</td>
<td>2895</td>
<td>2930</td>
</tr>
<tr>
<td>C – H</td>
<td>3058</td>
<td>--</td>
</tr>
<tr>
<td>O – H</td>
<td>3344</td>
<td>3345</td>
</tr>
</tbody>
</table>

(\(\delta\)) bending vibrations, (\(\nu\)) stretching vibration

Out of plane C–H bond vibration of the aromatic ring present in pyocyanin was not observed in the spectrum of pyocyanin after reaction with chloroauric acid (curve 2, Figure 6.11A). This observation also indicates that the aromaticity from pyocyanin ring structure could be altered after reaction with chloroaurate ions. The ring nitrogen of pyocyanin can donate one electron and get oxidized. This feature is observed in the FTIR spectrum, where 3058 cm\(^{-1}\) absorption band disappears from the spectrum corresponding
to pyocyanin after the reaction with chloroauric acid (Curve 2, Figure 6.11B). Thus from FTIR spectrum it can be concluded that the pyocyanin can be also involved in the binding on the surface of gold nanoparticles.

**6.3.4 Biological synthesis of silver nanoparticles using Pyocyanin**

Pyocyanin can also mediate the extracellular electron transfer reaction during the metabolism of *P. aeruginosa* and thus is capable of reduction of many metal ions. Anisotropic silver nanoparticles were obtained after reduction of silver ions with pyocyanin. We have developed here a new protocol for biological synthesis of silver nanoparticles.

**6.3.4.1 TEM analysis**

Pyocyanin shows greater affinity towards silver ions, as the mixing of pyocyanin with the solution of silver ions readily results in their reduction and the formation of silver nanoparticles. Figure 6.12 shows representative TEM images of pyocyanin reduced silver ion solution after 30 min of the reaction. Large number of nanoparticles with different morphologies like wires, triangles, pentagons, hexagons and spindles are observed after reaction of 0.1 % pyocyanin and the aqueous solution of 10^{-3} M AgNO_3. The wire-like structures with high aspect ratio are predominantly present on the grid surface (image A-E, Figure 6.12). The wire-like structures are found to be continuous and with interconnecting junctions. The thickness of silver nanowire is found to be around 30 nm, while length is up to 3 μm. Silver nanoparticles appears to be aggregated at the junction point from where the nanowires branch out.

Along with the wire-like structures, triangular and pentagonal silver nanoparticles with edge length of 100 nm to 300 nm are observed. However, the overall population of these structures was less in comparison with the wires. Insets in C and F shows the representative images of such triangular and pentagonal silver nanoparticle present along with the wire-like structures. The triangular and pentagonal structures show multiple twinning. A high magnification image of one of such pentagon clearly shows multiple twin boundaries in five-fold symmetry (Inset in Figure 6.12B). Reduction of silver ions by pyocyanin also results in the formation of spindle shaped silver nanoparticles, which are 100 nm to 200 nm long (image F, Figure 6.12). Inset in image E shows the SAED
pattern obtained from silver nanoparticles, which indicates the crystalline nature of silver nanoparticles.

![Figure 6.12](image)

**Figure 6.12** (A–F) Representative TEM images of silver nanoparticles obtained by reaction between silver ions and pyocyanin. Various anisotropic structures like wires, triangles and pentagons were observed. Insets in B, C and F show a pentagon, triangle and hexagon nanoparticles respectively. The scale bar for the images in the inset is 50 nm. The inset in E shows the SAED pattern obtained from silver nanoparticles shown in the main images.

The diffraction pattern is in good agreement with the FCC structure of silver. Respective $d$ values for the corresponding crystal planes are as follows: 2.374 (111), 2.05 (200), 1.44 (220), 1.24 (311) [34]

### 6.3.4.2 UV-vis spectroscopic analysis

When silver ions were reacted with pyocyanin, the originally colorless reaction medium changed to yellow after 10 min of incubation at 37 °C, which eventually became yellowish brown after 30 min and finally dark brown after 60 min of reaction. Time dependent UV-vis spectra recorded from the aqueous silver nitrate-pyocyanin reaction mixture are shown in Figure 6.13. The reaction between pyocyanin and silver ions is considerably faster and was complete within 1 h in comparison with the reduction of gold ions by pyoverdin as discussed earlier. The faster reduction of silver ions could be due to the greater affinity of pyocyanin molecules toward silver ions. A characteristic absorption at 440 nm is observed after 15 minutes of the reaction as observed from the curve 1 in
Figure 6.13. This absorption in the region of 420-440 nm is due to the surface plasmon resonance (SPR) of silver nanoparticles.

The SPR absorption at around 440 nm is accompanied by a small sharp absorption band at 390 nm after 15 min of the reaction, which further shifts to 395 nm after 30 min of reaction (curve 2, Figure 6.13). However, this absorption band is not observed after 60 min of reaction (curve 3, Figure 6.13). The SPR band at 440 nm shows gradual increase in the intensity with time. After 60 min of reaction, the SPR absorption band at 440 nm red shifts with the absorption maxima centered at 475 nm (curve 3, Figure 6.13). The shift in the SPR absorption band at 440 nm indicates some degree of aggregation in silver nanoparticles. TEM images obtained from the reaction mixture after 60 min of reaction indeed showed the aggregation in silver nanoparticles (images A-C, Figure 6.12). After 30 min of reaction the SPR band at 440 nm is accompanied with a weak absorption at 680 nm (curve 2, Figure 6.13). This absorption band shows increase
in intensity after 60 min of reaction and can be either due to aggregation of nanoparticles or presence of anisotropic nanoparticles or a combination of both [5] (curve 3, Figure 6.13). UV-vis spectroscopic analysis is in good agreement with the TEM analysis, which indicated the presence of aggregated silver nanoparticles as well as anisotropic structures like wires, triangles, and spindles. The SPR band at 440 nm can be assigned to the transverse component of silver nanoparticles and occurs due to the out of plane collective electronic oscillations, while the SPR band at 680 nm corresponds to the longitudinal component and originates due to the in-plane electronic oscillations [39]. No further increase was observed in intensity of the SPR absorption band after 60 min of the reaction indicating that all silver ions have been converted into silver nanoparticles (curve 3, Figure 6.13).

6.3.4.3 FTIR analysis

The reduction of silver ions and the formation of silver nanoparticles can be analyzed by FTIR spectroscopy. FTIR analysis carried out on pyocyanin before and after reaction with silver ions indicates a number of vibrational bands characteristic of the functional groups present in the structure of pyocyanin (Figure 6.14). Curve 1 in Figure 6.14 corresponds to the spectrum obtained from pure pyocyanin molecules while curve 2 in Figure 6.14 indicates the spectrum of pyocyanin – AgNO₃ reaction mixture after the reduction of silver ions and formation of silver nanoparticles. Most of the features in curve 2 of Figure 6.14 are similar to the FTIR spectrum obtained from pyocyanin – chloroaurate ion reaction mixture.

Considerable variation is observed in the 1500 cm⁻¹ to 1700 cm⁻¹ region of the spectrum after reaction with silver ions (curve 2, Figure 6.14A). Loss of a sharp absorption band at 1509 cm⁻¹ initially present in curve 1 of Figure 6.14A could be due to the loss of aromatic character of pyocyanin ring structure. Further, the wide absorption band at 605 cm⁻¹, which corresponds to C = C bond vibrations in aromatic ring is found to be reduced in the intensity (curve 2, Figure 6.14A). A sharp absorption band at 1032 cm⁻¹ in pyocyanin molecule corresponds to the C–O bond stretching vibrations (curve 1, Figure 6.14A). After reaction with silver ions, this absorption band shows shift towards higher wavenumber with the loss in intensity (curve 2, Figure 6.14A). This observation
indicates that pyocyanin molecules may be involved in the capping of the surface of silver nanoparticles.

![Figure 6.14](image)

**Figure 6.14** (A) FTIR spectra obtained from the purified pyocyanin (curve 1) and pyocyanin-silver ion reaction mixture after the reduction of silver ions and the formation of silver nanoparticles (curve 2). Symbol “*” indicates the vibrational bands not observed in the curve 2. (B) Enlarged view of the same spectra in the 2900 cm\(^{-1}\) to 3800 cm\(^{-1}\).

A sharp absorption band in the region 2850 cm\(^{-1}\) to 2970 cm\(^{-1}\) corresponds to the asymmetric and symmetric stretching vibrations arising due to the C–H bond in CH\(_3\) group and the aromatic ring of pyocyanin (Figure 6.14B). In pyocyanin – silver ion reaction mixture the CH\(_3\) band vibrations appear as a weak shoulder (curve 2, Figure 6.14B). Figure 6.14B shows the enlarged view of the same spectra and corresponds to the amine and hydroxyl group vibrations in pyocyanin molecule (curve 1, Figure 6.14B). Not much change in the amide and hydroxyl vibrational bands are observed after the reaction of pyocyanin molecules with silver ions (curve 2, Figure 6.14B).

**6.3.5 Discussion**

In this section of the chapter, synthesis of gold and silver nanoparticles using the pigment molecule, pyocyanin, from *P. aeruginosa* has been described. Pyocyanin is considered as a secondary metabolite since it is synthesized in the stationary phase of the bacterial growth. Gold nanoparticles with nearly uniform size were obtained by the reaction between an aqueous solution of chloroaurate ions and pyocyanin. The mean diameter of gold nanoparticles was found to be 12.7 nm with a standard deviation of 1.6
nm. Pyocyanin was also capable of synthesizing silver nanoparticles when reacted with silver ions. A number of anisotropic nanoparticles such as wires, triangles, pentagons and spindles were obtained after 60 min of the reaction between silver ions and pyocyanin. The edge length of triangular, pentagonal and spindle-like silver nanoparticles was observed to be around 100 to 300 nm. Nanowires of silver were measured to be 30 nm in diameter and were up to 3 μm in length. UV-vis spectroscopic analysis performed on silver nanoparticles further showed the characteristic feature of anisotropic structures. The FTIR spectroscopic analysis performed on the pyocyanin–chloroaurate ions and pyocyanin–silver ions indicated that pyocyanin can interact with gold and silver nanoparticles.

6.4 Bacterial enzyme mediated biosynthesis of gold nanoparticles

This section of the chapter illustrates the bacterial enzyme as a mean to synthesize gold nanoparticles. It has been conclusively shown that presence of protein hydrolyzing enzyme-protease accelerates the rate of gold nanoparticle biosynthesis by *Actinobacter spp*. Also, the oxygen deficiency in the experimental conditions is shown to have a drastic effect on the morphology of gold nanoparticles. Presence of the protein Bovine serum albumin (BSA) triggers the induction of protein hydrolyzing enzyme protease which can accelerate the synthesis of gold nanoparticles.

6.4.1 Experimental details

The biosynthesis of gold nanoparticles was carried out at 37 °C under aerobic as well as anaerobic conditions using *Actinobacter spp*. In all the experiments bovine serum albumin (BSA) was added to the reaction mixture leading to final concentration of 3 mg/ml. Soil bacterium *Actinobacter Spp.* was grown and cultured as follows. The seed culture was inoculated in 100 ml of LB medium. The flask was incubated at 37 °C for 48 h on a shaker (150 rpm). For the synthesis of gold nanoparticles at 37 °C, 0.5 g of the bacterial biomass was harvested from the culture medium by centrifugation at 5000 rpm and inoculated in 500 ml Erlenmeyer flask containing 100 ml of autoclaved aqueous solution of 10⁻³ M HAuCl₄ . Filter sterilized BSA (Bovine serum albumin) was added to the above solution leading to the final concentration of 3 mg/ml as an inducer of the enzyme protease. The flask was then kept on a shaker (150 rpm) at 37 °C and reaction
was carried out for 12 h. Biologically reduced gold nanoparticles were collected from the reaction mixture under sterile conditions by centrifugation at 5000 rpm and used for characterization. Aliquots were taken at different time intervals from the reaction medium to monitor the progress of the reaction. For the reduction of HAuCl₄ under anaerobic conditions, the bacterial biomass was harvested and processed as described above. The bacterial biomass was placed in a surface sterilized rectangular glass container containing 100 ml of autoclaved 10⁻³ M HAuCl₄ and filter sterilized BSA (3mg/ml). Bioreduction of HAuCl₄ was carried out under anaerobic conditions generated by passing nitrogen gas at fixed flow rate (5 psi). The reaction was carried out for 4 h and aliquots containing gold nanoparticles were periodically removed for characterization. Bioreduction of HAuCl₄ was monitored by recording the UV-vis spectra as a function of time of reaction.

To establish the role of enzyme protease in the synthesis of gold nanoparticles, 100 μg of commercial fungal protease (source- Aspergilus saitoi, sigma chemicals) was reacted with 10⁻³ M HAuCl₄ in glycine – HCl buffer (0.05 M, pH 3.0). Final volume of the reaction mixture was kept 10 ml and the reaction was carried out for the period of 8 h at 37 °C. The bacterial reaction supernatant was analyzed for protease activity. In all the experiments the reaction mixture containing the bacterial biomass, BSA and HAuCl₄ were subjected to the centrifugation at 5000 rpm. In all the experiments, where the presence of protease activity was expected from the culture supernatant, protease assay was performed using hemoglobin as a substrate. The standard reaction mixture contained 1ml of 5 % (w/v) hemoglobin in glycine – HCl buffer (0.05 M, pH 3.0) and 200 µl of the supernatant containing enzyme. After the incubation at 37 °C for 30 minutes, 1 ml of 1.7 mM perchloric acid (PCA) was added to terminate the reaction. Then the mixture was left undisturbed for 30 minutes at room temperature. The undigested material was removed by filtration (Whatman number 1). Proteolytic activity was measured as the increase in the absorbance at 280 nm of PCA soluble fraction. The appropriate blanks were made by adding PCA before the enzyme. One unit of proteolytic activity was defined as the amount of enzyme required to increase the absorbance by 0.001 under the conditions previously described [40]. To verify the presence of aspartic protease in solution, pepstatin was used as a standard inhibitor at 2 μM concentration from a concentrated stock of 1 mg/ml. To elucidate the role of protease in the synthesis of gold nanoparticles,
Actinobacter spp. was grown in minimal medium. 10 ml of culture supernatant of the minimal medium containing protease was mixed with $10^{-3}$ M HAuCl$_4$ in the absence and presence of the protease inhibitor pepstatin at a final concentration of 2 μM. The reaction was carried out for 8 h. Different control experiments were performed to show the role of protease enzyme in the synthesis and shape control of gold nanoparticles. In the first control experiment, bacterial reduction of HAuCl$_4$ was carried out in absence of BSA under aerobic as well as anaerobic conditions as discussed above. In another control experiment, the aqueous HAuCl$_4$ was mixed with BSA in the absence of Actinobacter spp. and incubated under the aerobic and anaerobic conditions with similar reaction conditions.

**6.4.2 TEM analysis**

Figure 6.15 shows TEM images obtained from gold nanoparticles reduced using Actinobacter spp. in the presence of BSA under the aerobic and anaerobic conditions. TEM images of gold nanoparticles synthesized under aerobic conditions show mixed population of flat nanoparticles with irregular morphology along with the few triangular particles (images A & B, Figure 6.15). A fair degree of aggregation is also observed within the nanoparticles. The edge length of the triangular particles varied between 250 – 500 nm. Insets in image A and B shows the representative TEM images of triangular and hexagonal gold particles that appear with the aggregated spherical gold nanoparticles. SAED pattern obtained from one of the triangular gold particles is shown in image C. SAED pattern indicates that each gold nanotriangle is a single crystal and the diffraction spots are in good agreement with the FCC structure of gold.

A considerable variation occurs in the morphology of gold nanoparticles when synthesized under anaerobic conditions. Images D, E and F in Figure 6.15 correspond to the TEM micrographs of gold nanoparticles synthesized under anaerobic conditions. Nearly uniform size spherical gold nanoparticles of 10 nm diameter are observed. Further, almost half the population of gold nanoparticles shows triangular morphology. The gold nanotriangles observed here are small with edge lengths measuring 30 – 50 nm. The vertices of the triangles are not truncated or sharp, but instead appear blunt unlike in previous studies [11]. Inset in F shows linear arrangement of triangular and spherical nanoparticles. Inset in image E shows the SAED pattern obtained from gold nanoparticles.
shown in image E. The SAED pattern indicates the crystalline nature of gold nanoparticles and the diffraction pattern is in well agreement with the FCC structure of gold.

![Figure 6.15](image)

**Figure 6.15** TEM images of gold nanoparticles obtained by the reaction between aqueous chloroaurat ions and Actinobacter spp. under aerobic conditions (A and B). The SAED pattern obtained from gold nanoparticles shown in A and B indicates that the flat gold particles are single crystalline (C). TEM images of gold nanoparticles synthesized under anaerobic conditions (D to F). The inset in E shows SAED pattern of gold nanoparticles synthesized under anaerobic conditions. Crystal planes are shown with different symbols as follows: □ (111), ◊ (200), ○ (220) and Δ (311) while inset in F shows the presence of small triangular gold nanoparticles.

The corresponding $d$ spacing for the respective crystal planes are as follows: 2.357 (111), 2.063 (200), 1.442 (220) and 1.23 (311) [34]. From TEM images it is clear that presence or absence of atmospheric oxygen drastically alters the morphology of gold nanoparticles synthesized in presence of BSA.

### 6.4.3 UV-vis spectroscopic analysis

Figure 6.16 shows the UV-vis spectroscopic analysis of gold nanoparticles synthesized using Actinobacter spp. in presence of aerobic and anaerobic conditions. Figure 6.16A corresponds to the time dependent UV–vis spectroscopic analysis indicating the progress of the synthesis of gold nanoparticles under aerobic conditions. The SPR band originating from gold nanoparticles appears at 550 nm after 3 h of reaction (curve 1, Figure 6.16A) and gradually increases in intensity with time. No additional
absorption band corresponding to anisotropy in the shape of nanoparticles is observed and this may be due to very small population of triangular particles present in the solution. At the initial stage of reaction, SPR shows broad absorption band (curve 1 and 2, Figure 6.16A), which became sharper with the progress of reaction (curve 3–5, Figure 6.16A).

The reaction is completed after 12 h as indicated by the saturation of SPR absorption band, which do not rise in intensity after 12 h (curve 5 corresponding to 15 h, Figure 6.16A). Figure 6.16B shows the UV-vis spectroscopic analysis of gold nanoparticle synthesis under anaerobic conditions as a function of time. Under anaerobic conditions, the formation of gold nanoparticles is faster as compared to the aerobic conditions. The SPR absorption band due to the formation of gold nanoparticles appears at 550 nm at the initial stages of the reaction (Curve 1 and 2, Figure 6.16B). Complete reduction of the aqueous chloroaurate ions occurs in 3 h and no further increase in the intensity of SPR band occurs (Curve 6 and 7, corresponding to 3 and 3.5 h, Figure 6.16B). A weak absorption band with intensity maxima centered at 650 nm is developed after 1 h of reaction. This absorption band also increases in intensity with time and corresponds to the longitudinal SPR absorption. This additional plasmon band can be attributed to the in-plane plasmon resonance of the triangular gold nanoparticles. These
time-dependent features are characteristic of formation of gold nanoparticles that aggregate with time or formation of anisotropic particles whose aspect ratio increases with time, or a combination of both processes [4]. This observation is in agreement with the TEM images which showed the presence of number of small triangular and hexagonal gold nanoparticles.

6.4.4 Induction of protease from Actinobacter spp. and its role in the accelerated synthesis of gold nanoparticles

The presence of biological molecules like proteins in the growth medium is known to induce hydrolytic enzymes proteases that hydrolyze proteins/peptides extracellularly [29]. Keeping this fact in mind, we hypothesize here that extracellular protease secreted by the bacterium Actinobacter spp. could be involved in the synthesis of gold nanoparticles. To check this hypothesis we carried out various experiments where the enzyme activity was assayed for protein hydrolysis. When chloroauroate ions were reduced by Actinobacter spp. in the presence of BSA under aerobic as well as anaerobic conditions, it was found that the reaction supernatant containing gold nanoparticles indeed had intrinsic proteolytic activity.

In another experiment, Actinobacter spp. was grown in M9 minimal medium containing BSA (3 mg/ml) as a sole source of nitrogen for the cellular growth and metabolism. The bacterial cells are usually grown in minimal medium in the presence of inducer for induction of desired hydrolytic enzymes. The culture supernatant was then assayed for the protease activity after 12 hrs of incubation using hemoglobin as a substrate, which is one of the standard assay substrate for protease [40]. This culture supernatant when reacted with $10^{-3}$ M HAuCl$_4$ and incubated for about 8 h was found to yield gold nanoparticles readily as shown by the TEM analysis. Images A and B in Figure 6.17 correspond to the TEM analysis of gold nanoparticles synthesized by the culture supernatant of Actinobacter spp. grown in M9 medium supplemented with BSA. Spherical gold nanoparticles of $\sim$ 10 nm diameter are observed along with the triangular nanoparticles with edge lengths around 20 nm (image B, Figure 6.17). The UV-vis spectroscopic analysis performed on gold nanoparticles synthesized by the culture supernatant of Actinobacter spp. grown in M9 medium and supplemented with BSA shows characteristic SPR absorption band at 540 nm (curve 2, Figure 6.17C).
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Figure 6.17 TEM images of gold nanoparticles synthesized using crude protease enzyme secreted by Actinobacter spp. in M9 medium supplemented with BSA (A and B). (C) UV-vis spectrum obtained from gold nanoparticles synthesized by crude protease (curve 2). Synthesis of gold nanoparticles was not observed when crude protease was reacted with chloroaurate ions (curve 1) in the presence of protease inhibitor pepstatin. Images D and E shows gold nanoparticles synthesized by commercial protease. Curve 2 in the graph F corresponds to the UV-vis spectrum obtained from gold nanoparticles shown image D and E, while curve 1 represent UV-vis spectrum of commercial protease – chloroaurate ions reaction mixture in the presence of pepstatin.

However no proteolytic activity was detected from the culture supernatant obtained from the growth medium of Actinobacter spp. when grown in M9 medium without supplemented BSA. The synthesis of gold nanoparticles was not observed at all when this supernatant was reacted with chloroaurate ions. This result indicates that the enzyme protease synthesized in the presence of BSA is responsible for the biological synthesis of gold nanoparticles. The role of protease as a reducing agent in the formation of gold nanoparticles can be illustrated by using the protease inhibitor, which is known to block the biological activity of the enzyme. Protease inhibitor binds to the active site or substrate binding site of protease and renders them inactive. Protease activity was not detected from the culture supernatant of M9 medium, in the presence of pepstatin. Further, the synthesis of gold nanoparticles was not observed when the pepstatin mixed culture supernatant was reacted with chloroaauric acid as indicated by the UV-vis analysis.
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of this solution, which do not show the SPR absorption band originating from gold nanoparticles (Curve 1, Figure 6.17C).

To further elucidate the role of protease in the synthesis of gold nanoparticles, commercial protease from the fungus *A. saitoi* (Sigma chemicals) was mixed with $10^{-3}$ M HAuCl$_4$ in the presence of glycine – HCl buffer (0.05 M, pH 3.0). After incubation of about 8 h the colour of solution turned pink from original pale yellow indicating the formation of gold nanoparticles. Images D and E from Figure 6.17 correspond to the TEM analysis of gold nanoparticles synthesized using commercial protease and aggregated gold nanoparticles along with triangular and hexagonal particles are observed. Most of the grid area was covered by aggregates of gold nanoparticles measuring 50 to 200 nm in size and it was difficult to observe the size of individual nanoparticles. The edge length of the triangular and hexagonal gold particles is measured to be around 200 nm. SAED pattern arising from these particles showed single crystalline hexagonal diffraction pattern that could be indexed on the basis of FCC structure of gold (inset in Figure 17D). UV-vis spectroscopic analysis performed on the solution of gold nanoparticles reveals the presence of the SPR absorption band at 580 nm (curve 2 in Figure 6.17F). The SPR absorption band shows broad curve with a small red shift as SPR band originating from spherical gold nanoparticles is usually centered on 520 nm. The observed red shift could be due to the aggregation of individual nanoparticles or the presence of anisotropic nanoparticles. This observation is in well agreement with the TEM analysis (Images D and F in Figure 6.17), which shows the presence of aggregated nanoparticles together with triangular and hexagonal particles. Curve 1 in Figure 6.17F corresponds to the UV-vis analysis of reaction mixture containing the aqueous chloroauroate ions and commercial protease in the presence of protease inhibitor pepstatin. Even in this case the formation of gold nanoparticles was not observed at all as indicated by the absence of SPR absorption band in the range of 520-560 nm (Curve 1 in Figure 6.17F).

In yet another experiment chloroauroate ion were reacted with BSA under aerobic and anaerobic conditions in the absence of *Actinobacter* spp. Synthesis of gold nanoparticles was not observed even after seven days of reaction, indicating that the reduction of aqueous chloroauroate ions was mediated by *Actinobacter* spp. Experiments
carried out using M9 minimal medium supplemented with BSA suggest that protease enzyme was induced in the presence of BSA, which mediates the reduction of aqueous chloroaurate ions and the formation of gold nanoparticles. Also the use of protease inhibitor-pepstatin confirmed that the synthesis of gold nanoparticles is mediated by protease. Thus, from the experimental results it can be inferred that the synthesis of gold nanoparticles by \textit{Actinobacter} spp. in the presence of BSA is resulted due to the bacterial protease.

\textbf{6.4.5 Discussion}

This part of the chapter describes biological synthesis of gold nanoparticles under experimental conditions that favored the induction of proteolytic enzyme protease from \textit{Actinobacter} spp. The presence of protease was found to be responsible for reduction of aqueous chloroaurate ions and the formation of gold nanoparticles. Also the synthesis of gold nanoparticles was accelerated in the presence of BSA. The size and shape of gold nanoparticles was affected due to the presence or absence of molecular oxygen in the reaction medium. Gold nanoparticles with irregular morphology were found to be synthesized in the presence of aerobic conditions along with few triangles and hexagons with the edge length measuring 250–500 nm. Under anaerobic conditions, gold nanoparticles with nearly uniform spherical morphology with 10 nm diameter are observed. A fairly good population of small triangular nanoparticles with edge length around 20 nm is observed together with spherical nanoparticles. Protease activity was detected from solutions of gold nanoparticles synthesized by \textit{Actinobacter} spp. in the presence of BSA under aerobic as well as aerobic conditions as indicated by enzyme assays. The presence of protease inhibitor in reaction mixture inhibited the protease activity and the formation of gold nanoparticles. This observation indicates that the active site of protease enzyme may be involved in reduction of chloroaurate ions. Reaction of chloroaurate ions with commercial and purified protease also resulted in the synthesis of gold nanoparticles. The nanoparticles formed aggregates of 50 to 200 nm along with the triangular and hexagonal particles with \textasciitilde 200 nm edge length. Further the formation of gold nanoparticles was inhibited when commercial protease was reacted with the aqueous chloroaurate ions in the presence of pepstatin, as observed earlier.
6.5 Conclusions

This chapter describes new biological methods for the synthesis of gold and silver nanoparticles. Primary and secondary metabolites like siderophore – pyoverdin and a fluorescent blue – green pigment, pyocyanin, from the bacterium *P. aeruginosa* are used for biological synthesis of gold and silver nanoparticles. Also, protease enzyme mediated biosynthesis of gold nanoparticles using *Actinobacter* spp. has been demonstrated. The control over morphology and size of gold and silver nanoparticles can be achieved by various biological synthesis methods described here. Triangular gold nanoparticles with tunable edge lengths can be synthesized by varying the concentration of pyoverdin used for the synthesis of gold nanoparticles. Reduction of chloroaurate ions and silver ions by pyoverdin resulted in uniform spheroid of gold nanoparticles and wire-like silver nanostructures. Protease mediated reduction of chloroaurate ions by *Actinobacter* spp. resulted in the small triangular gold nanoparticles and nearly uniform spherical gold nanoparticles. The possibility of achieving shape control of metallic nanoparticles at a level equal to chemical routes by purely green chemistry approach makes biological synthesis protocols promising for the future development. The biological procedures for the synthesis of metallic nanoparticles described in this chapter can be important in this regard.
6.6 References


Chapter VI

[34] The XRD pattern was indexed with reference to FCC gold and silver structure from JCPDS – International Center for Diffraction Data PCPDFWIN version 1.30, 04-0784 for gold and 04-0783 for silver.

