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Controllable biosynthesis of silver nanoparticles using actinobacterial strains

Abstract: We report the effect of various parameters,
namely substrate concentration, time, pH and tem-
perature, on the biosynthesis of silver nanoparticles
(AgNPs) by using the extract of actinobacterial strains,
which were isolated from the sediments of Lonar Cra-
ter Lake in India. It was found that the formation of
AgNPs and its morphology depended on synthesis
conditions. Visual observation of the reaction mixture,
ultraviolet-vis spectroscopic analysis and mass of syn-
thesized AgNPs indicated that 25°C, pH 7 and 3 days of
incubation time were optimal for its efficient synthesis.
The transmission electron microscopy (TEM) analysis
revealed aggregation and irregular shape of AgNPs both
at acidic pH and below 25°C. It was found that alkaline
pH and temperature higher than optimal fostered the
formation of nanoparticle aggregates. Based on the
obtained results, it was concluded that the efficiency
of biological synthesis by using actinobacteria as well
as the size and shape of fabricated nanoparticles can
be manipulated by controlled conditions of synthesis
process. The use of desired nanoparticles increases its
potential for medical applications.

Keywords: actinobacteria; AgNPs; biological synthesis;
pH; synthesis conditions; temperature.

Abbreviations

AgNPs   silver nanoparticles
ISP2   yeast extract-malt extract medium
NPs   nanoparticles
SCA   starch casein agar
SPR   surface plasmon resonance

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1 Introduction

Nanotechnology is a fast-growing branch of science that
deals with the synthesis and development of different
nanomaterials [1]. Silver nanoparticles (AgNPs) are the
main focus of intensive study because of their wide appli-
cations, such as catalysts, optics, antimicrobials, antican-
cer agents, and for biomaterial production [1–4]. There is
enormous interest in metal nanoparticles (NPs) because
of their unique physical and chemical properties revealed
at the nanoscale level [5].

The microbial recovery of noble metals with the for-
mation of their NPs is a green alternative to physical and
chemical methods, which use high amounts of energy or
toxic solvents and reagents. A biological synthesis process
provides a wide range of environmentally acceptable
methodology, low-cost production and minimum time
required [6].

The biosynthesis of AgNPs using various bacte-
ria, fungi and plants is already well documented [7–11].
However, the exploration of actinomycetes has recently
gained interest for efficient biological synthesis of metal-
lic NPs [6].

The bio-fabrication of NPs, which is a "bottom-up"
approach, as well as the shape, size, distribution and
composition of NPs, depends upon physicochemical
properties such as temperature, time, pH, concentration
of the substrate and the presence of biomolecules acting
as reducing agents (amino acids, proteins, vitamins) and
enzyme sources [12–19]. NPs of desired shape and size
can be obtained by controlling the above physical and
chemical parameters, which influence the rate of nuclea-
tion, growth and atomic/molecular precursor [15, 20].
Hence, the demand for the controllable synthesis of NPs
is growing day by day.

In the present study, the effect of substrate con-
centration, time, temperature and pH on the synthe-
sis, morphology, size distribution and surface charge
of AgNPs synthesized from actinobacterial strains was
evaluated.

2 Materials and methods

2.1 Actinobacterial strains

Four actinobacterial strains (OT1, OF1, OF2 and IF19) were isolated from the sediments of Lonar Crater located in Buldhana district, Maharashtra, India, by the dilution plate procedure described by Rathod et al. [21] on the starch casein agar [22] supplemented with 5% (w/v) sodium chloride (NaCl), at pH 8.5. The identification of actinobacterial strains to the closest phylogenetic neighbors based on the nucleotide sequence analysis of 16S rRNA gene was made according to method described previously by Golińska et al. [23] and Rathod et al. [21].

In the preliminary studies, the growth of actinobacterial strains for the synthesis of AgNPs was previously estimated by using different media, namely starch casein broth [22], yeast extract-malt extract broth (ISP2) [24] and halophilic nutrient broth [25], all with 5% NaCl (w/v) and pH 8.5. The most abundant growth of actinobacterial strains was recovered in halophilic nutrient broth, which was further used for the cultivation of actinobacteria.

2.2 Effect of temperature and time on the formation of AgNPs using actinobacterial strains

The biosynthesis protocol of AgNPs was described in detail by Golińska et al. [23], Rathod et al. [21] and Wypij et al. [26]. Briefly, actinobacterial strains grew in halophilic nutrient broth [25] for 7 days at 26°C. The bacterial biomass was then collected by centrifugation ($6000 \times g$ for 10 min) and washed thrice with sterile, distilled water. Cell pellet was then resuspended in sterile, distilled water for 3-day autolysis at 25°C. The supernatant, after centrifugation at $6000 \times g$ for 15 min, was combined with silver nitrate (AgNO$_3$; final concentration 0.001 mol l^{-1}) and incubated for 3 days at different temperatures of 10, 15, 20, 25 and 35°C ($\pm2°C$) for 3 days in the darkness. The formation of AgNPs was monitored visually at the interval of 24 h by color change of the reaction mixture from colorless to yellowish-brown and ultraviolet (UV)-visible spectroscopy (Nano Drop ND2000, Thermo Scientific, USA) in a wavelength range of 200–800 nm. The efficiency of AgNP synthesis was evaluated based on their dry mass (mg 100 ml^{-1}). Meanwhile, the effect of time on the synthesis of AgNPs synthesized from actinobacterial strains was recorded at the interval of 24 h up to 7 days.

A final concentration of AgNO$_3$ of 0.003 mol l^{-1} was also used for the synthesis of biogenic AgNPs. However, the use of higher concentration of AgNO$_3$ demonstrated an inhibitory effect on the synthesis of AgNPs.

2.3 Effect of pH on the formation of AgNPs

The actinobacterial strain was cultivated as described above. An autolysis of cell biomass was made by suspending the cells in sterile, distilled water of different pH (4, 5, 6, 7 and 8) and incubated at 25°C for 3 days. The pH of distilled water was set up at 4, 5, 6, 7 and 8 with sterile 0.2 mol l^{-1} HCl and 0.2 mol l^{-1} NaOH using a pH meter (Hanna), sterilized by filtration and used for testing. After autolysis, the cell biomass was centrifuged ($6000 \times g$, 10 min) and the supernatant was combined with silver nitrate (final concentration of 0.001 mol l^{-1}).

The reaction mixture was then incubated for 3 days in the darkness. Visual observations and UV-vis spectroscopy analysis of the biosynthesized AgNPs were performed.

The autolysis of actinobacteria was also performed in the buffers, namely citrate and phosphate, to maintain appropriate pH value (4, 5, 6, 7, and 8). However, the use of buffers had an inhibitory effect on AgNP synthesis.

The physical properties of AgNPs biosynthesized under various conditions of pH and temperature were estimated for AgNPs from the OT1 strain.

2.4 Transmission electron microscopy analysis

The size and morphology of the AgNPs from *Nocardiopsis valliformis* OT1 actinobacterial strain, synthesized in different pH and temperatures, were analyzed by using transmission electron microscopy (TEM) (FEI Tecnai F20 X-Twintool, Fei, Hillsboro, OR, USA) operating at an acceleration voltage of 100 kV. The samples for analysis were prepared by dropping a small amount of solution of AgNPs on a carbon-coated copper grid (400 μm mesh size). The samples were then allowed to dry at room temperature prior to measurements. The obtained data were assessed by Statistica Software (StatSoft, USA).

2.5 Nanotracking analysis

The average particle size and size distribution of AgNPs synthesized from *N. valliformis* OT1 strain were analyzed by using a nanotracking analysis (NTA) system LM20 (NanoSight Ltd., UK) after AgNP dilution with the nuclease-free water. 0.5 ml of the sample was injected into the sample chamber and observed through LM20.

2.6 Zeta potential analysis

The surface charge of AgNPs synthesized from *N. valliformis* OT1 was evaluated in a colloidal suspension of AgNPs by zeta potential measurement. The NP liquid sample was diluted 10 times with water and sonicated at 20 Hz for 15 min (Sonic Ruptor 250, Omni International, USA) to break down NP aggregates. The mixture was then filtered through a 0.22-μm millipore filter and analyzed by using Malvern Zetasizer 90 (ZS 90; Malvern Instruments Ltd., Malvern, UK).

3 Results

3.1 Molecular identification of actinobacterial strains

Nearly complete 16S rRNA gene sequences (1412, 1418, 1413 and 1402 nt) of OT1, OF1, OF2 and IF19 strains, respectively, were determined. Based on the EzBioCloud analysis [27], OT1 strain was most closely related to *N. valliformis* DSM

45023[T] (99.4%) as previously described by Rathod et al. [21]. Isolates OF1 and OF2 were found to be most closely related to *Streptomyces palmae* CMU-AB204[T] (98.23 and 98.17%, respectively), while strain IF19, to *Streptomyces alkaliphilus* DSM 42118[T] (99.71%).

3.2 Effect of time, temperature and pH on the formation of AgNPs

The absorption spectra of AgNPs biosynthesized after various incubation times are presented in Figure 1, those biosynthesized at various temperatures (10, 15, 20, 25, 35), in Figure 2, while those synthesized at different pH (4, 5, 6, 7, 8), in Figure 3.

Time, pH and temperature were found to influence AgNP synthesis significantly. With the increase in duration of synthesis, temperature and pH, the reduction of silver salt was enhanced, as indicated by a change in the color intensity of the experimental samples (Figures 1–3).

The effects of time on the synthesis of AgNPs using *N. valliformis* OT1, *S. palmae* OF1 and OF2 and also *S. alkaliphilus* IF19 strains were studied visually and by UV-vis spectroscopy at an interval of 24 h. The color change in the reaction mixture and specific absorbance peaks in the range of 380–450 nm were observed after 48 and 72 h of incubation at 25°C. Moreover, absorption peak intensity increased with the increase in duration of biosynthesis process as a result of the continuous formation of AgNPs in the reaction system. The optimum time required for the completion of synthesis was found to be 72 h (Figure 1). Further extension of incubation time (up to 7 days) did not affect the synthesis process.

Generally, a broad peak of less intensity was observed after analysis of AgNPs synthesized at lower temperatures (10, 15 and 20°C). With an increase in temperature value to 25°C, the surface plasmon resonance (SPR) peaks (at wavelength about 400 nm) became sharper, which suggests that this temperature is optimal for the biosynthesis of AgNPs. Biosynthesis at a higher temperature (35°C) affected lower intensity peaks for AgNPs from OF1, OF2 and IF19 strains but not from OT1, which was similar to that recorded at 25°C (Figure 2).

The highest intensity of absorbance peaks was observed for AgNPs synthesized at alkaline pH, namely 7 and/or 8 (Figure 3).

The bands were observed in the wavelength range of 380–450 nm, which is typical for AgNPs (Figures 1–3).

Moreover, the efficiency of AgNP synthesis from *N. valliformis* OT1 strain after various incubation periods
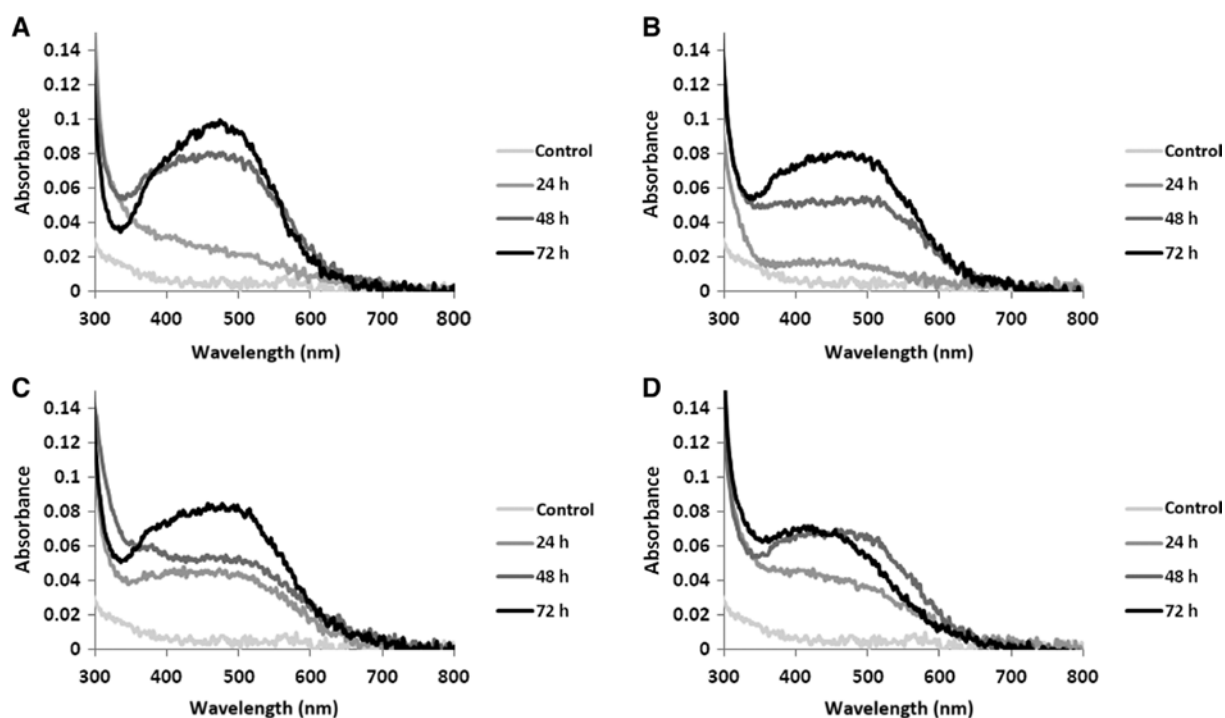

Figure 1: The absorption spectra of AgNPs biosynthesized after incubation time (24, 48 and 72 h) by *N. valliformis* OT1, *S. palmae* OF1, OF2 and also *S. alkaliphilus* IF19 strains. *N. valliformis* OT1 strain (A); *S. palmae* OF1 strain (B); *S. palmae* OF2 strain (C); *S. alkaliphilus* IF19 strain (D).
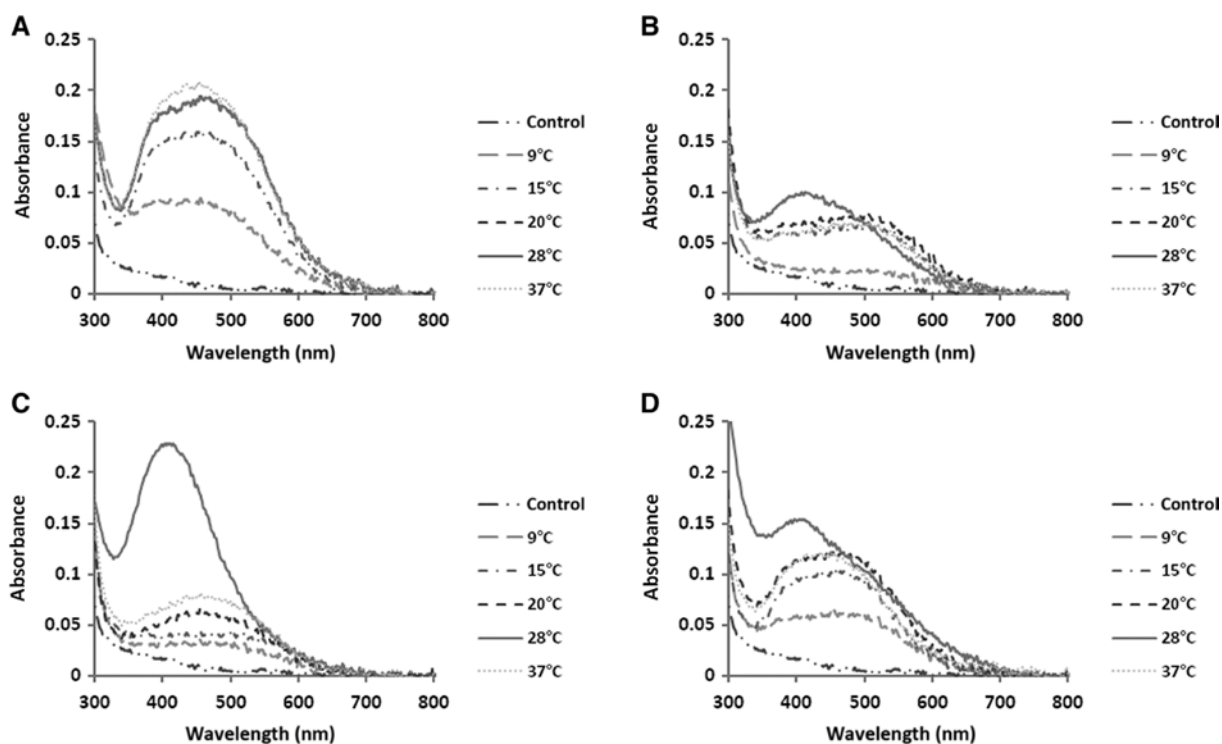
Figure 2: The absorption spectra of AgNPs biosynthesized under various conditions of temperature. *N. valliformis* OT1 strain (A); *S. palmae* OF1 strain (B); *S. palmae* OF2 strain (C); *S. alkaliphilus* IF19 strain (D).
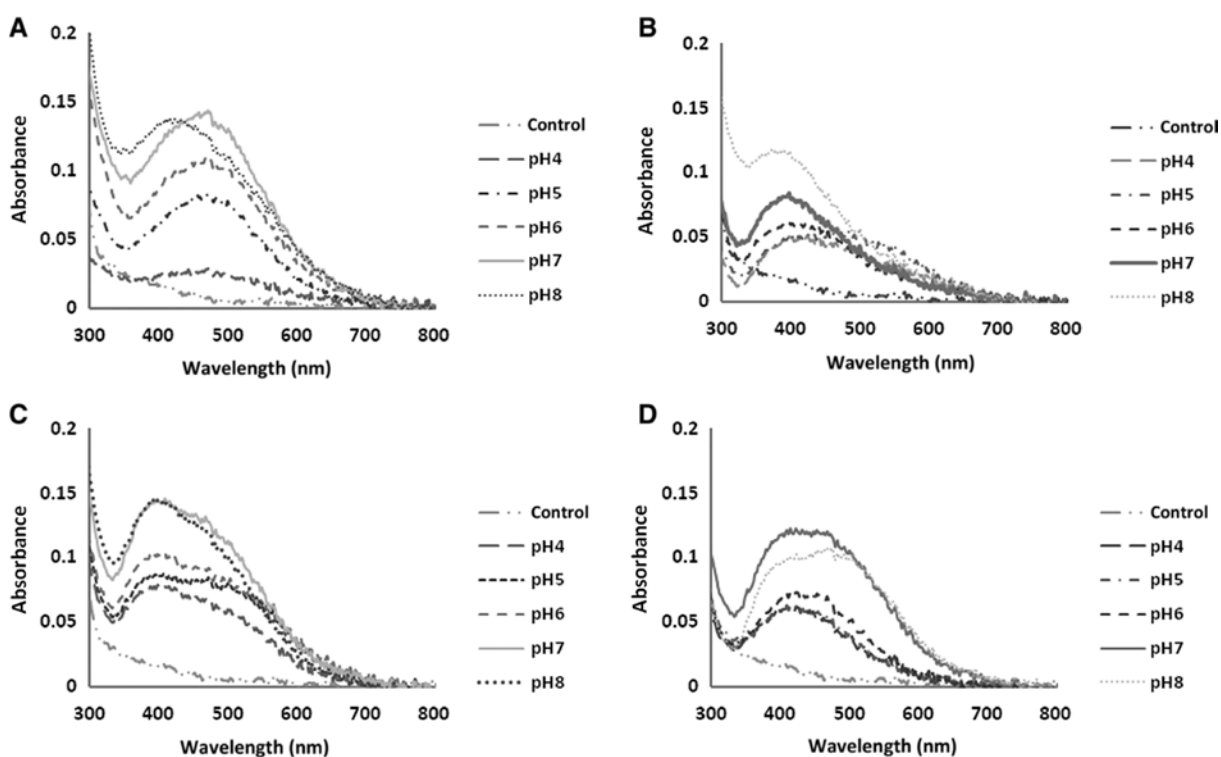

Figure 3: The absorption spectra of AgNPs biosynthesized under various conditions of pH. *N. valliformis* OT1 strain (A); *S. palmae* OF1 strain (B); *S. palmae* OF2 strain (C); *S. alkaliphilus* IF19 strain (D).
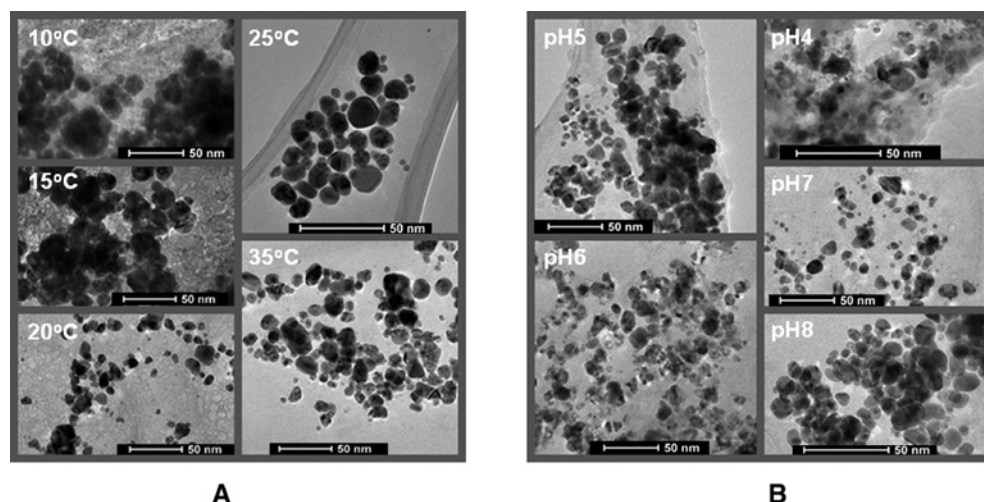
Figure 4: TEM analyses of AgNPs synthesized at different pH and temperature by using *N. valliformis* OT1 strain. Synthesis at different temperatures (A), synthesis at different pH (B).

(24, 48 and 72 h) and under different temperatures (10, 15, 20, 25 and 35°C) and pH (4, 5, 6, 7 and 8) was evaluated based on their dry mass. The dry mass of AgNPs collected from 100 ml of experimental sample was found to be 0.0, 3.2, 6.6 and 1.0, 2.5, 3.2, 7.0, 6.0 and 2.0, 2.3, 4.0, 8.0, 8.7 mg, respectively.

3.3 Physical properties of AgNPs synthesized from *N. valliformis* OT1 strain under various synthesis conditions

The results of TEM measurements of NPs synthesized from *N. valliformis* OT1 strain under various conditions of temperature are presented in Figure 4. It was found that at temperatures below 25°C, NPs of irregular shape and bigger size were formed and showed a tendency to form

aggregates. The higher aggregation of biosynthesized AgNPs was also observed after synthesis at 35°C. The spherical and smaller NPs were formed when synthesis process was performed at 25°C. The average size of AgNPs biosynthesized under different temperatures is presented in Table 1.

The TEM analyses of AgNPs synthesized at different pH revealed that an increase in pH value resulted in the fabrication of smaller and more spherical NPs. The results of the TEM imaging confirmed those obtained from the absorption spectra. AgNPs synthesized at pH below and above neutral were irregular in shape and able to form numerous aggregates (Figure 4, Table 1). The formation of aggregates was also confirmed by NTA analysis when the biosynthesis process was performed at a pH value other than 7 (Table 1). The average size of AgNPs fabricated at different pH is presented in Table 1.

Table 1: Effect of different pH and temperatures on size of AgNPs synthesized from *N. valliformis* OT1 strain.

Influent factor	Mean size of AgNPs by TEM (nm)	Mean size of AgNPs by NTA (nm)	Zeta potential (mV)
pH			
4	23.2	194.4 ± 48.6	−26.6
5	20.3	157.7 ± 54.1	−14.6
6	18.7	83.3 ± 39.0	−21.4
7	13.9	62.0 ± 51.0	−26.2
8	25.4	168.8 ± 66.5	−28.7
Temperature (°C)			
10	32.6	153.3 ± 39.3	−21.4
15	35.1	143.8 ± 73.0	−24.6
20	17.1	145.4 ± 136.4	−15.0
25	16.5	62.0 ± 51.0	−17.1
35	29.8	179.3 ± 57.8	−25.0

The zeta potential values of AgNPs synthesized from *N. valliformis* OT1 strain showed their varied stability and are presented in Table 1.

4 Discussion

The biological synthesis of nanomaterials offers better manipulation and control over crystal growth and their stabilization. This has led to an upsurge in research on the synthesis routes that allow better control of size and shape for a wide variety of nanotechnological applications [13, 28, 29].

In the present study, the effect of temperature, time, pH, incubation time and substrate concentration on the formation of NPs with desired properties such as the shape, size and distribution was considered.

The solution of experimental samples, in contrast to the control samples, was found to be intensified along with the increase in temperature, pH and incubation time that was associated with an increase in the yield of the synthesized NPs. The change in color occurs due to the excitation of free electrons in NPs, which generates the SPR. Due to SPR, a strong absorption of electromagnetic waves is exhibited by metal NPs in the visible range [14, 30]. The SPR phenomenon arises when NPs are irradiated with visible light, because of the collective oscillations of the conduction electrons [31]. It is well known that AgNPs exhibit a yellowish-brown color in aqueous solutions due to the excitation of the SPR band in the region [32]. An increase in the intensity of the absorbance peak with time indicates the continued reduction of the silver ions and an increase in the concentration of AgNPs [1, 33]. In the present study, an effect of duration on the synthesis of AgNPs was noticed. The longer duration of time for the synthesis resulted in a higher yield of fabricated NPs. Similar observations were reported by Amin et al. [34] and Darroudi et al. [35], who studied the synthesis of AgNPs by green approach. These authors showed that the intensity of an absorption band increased along with the duration of synthesis.

One of the interesting criteria is increasing bandwidth of resonance with the decrease in the dimensions of the particles as a result of electron scattering induction at the surface. Resonance shifting and the variation of its bandwidth are important information for NP characterization [1]. The position of the absorption peak in the visible absorption spectrum of colloidal solutions can be related to the particle size and shape due to the resonance through SPR [36, 37]. It is claimed that the sharp narrow absorption peaks at lower wavelength region indicates the formation of smaller NPs [37].

Temperature is the basic physical factor that affects the formation of NPs as well as their shape and size [13]. In the present study, the intensity of peak maximum increases with increasing temperature, along with the shift in peak wavelength. The temperature of 25°C was the optimal temperature for the biosynthesis of AgNPs by test strain, which was confirmed by estimation of dry mass of AgNPs. Along with the decline in temperature, the absorption peaks decreased. It suggests the low concentration of biosynthesized NPs in solutions. The smallest particles were formed at optimal temperature (25°C), while below and above this value, a tendency to form bigger NPS and aggregates was observed. The increase in the average size of gold and iron oxide NPs, along with the increase in temperature, was reported by other researchers [14, 38]. They suggested that it can be due to the higher activity of reducer enzymes at high temperature [14, 38]. In contrast, Verma and Mehata [39] reported that the size of the synthesized NPs decreases with an increase in temperature value. These authors claimed that it may be due to the faster reaction rate at higher temperatures. At high temperatures, the kinetic energy of the molecules increases and silver ions are consumed faster; thus, there is a possibility of decreased particle size growth. Quester et al. [40] studied the biosynthesis of AgNPs using the fungal extract of *Neurospora crassa* and observed the formation of smaller NPs (1–6 nm) when both temperature and pH values were low (4°C and pH 3), while at 25°C and pH 6 or 10, bigger NPs were formed (1–10 and 1–13 nm, respectively).

The important parameter that affects the synthesis, shape and size of NPs is the pH of the reaction solution. This factor has the ability to alter the charge of biomolecules, which may have effect on their capping as well as stabilizing properties [39].

It was found that as the pH value increased, the surface plasmon peak shifted to the left, indicating a decrease in the size of the prepared NPs. Moreover, this shift in the peak accompanied the decrease in the width of the peak, indicating size uniformity [12]. However, Verma and Mehata [39] showed that the shift in the peak wavelength indicates that the size of the particles increases with increasing pH of the solution. As the diameter of the particles get larger, the energy required for excitation of surface plasmon electrons decreases, as a result the absorption maximum shifts toward the longer wavelength region.

The present study demonstrated that the absorption intensity and amount of biosynthesized AgNPs increase along with the increase in pH value. Similar observations were reported by Sathishkumar and colleagues [41], who observed that a higher amount of AgNPs from *Cinnamon*

zeylanicum bark extract was produced when the pH value of plant extract was higher than pH 5. Verma and Mehata [39] observed that pH 13 was the most favorable for the synthesis of AgNPs by extract of neem (*Azadirachta indica*) leaves. However, at such high pH, the NPs became unstable and agglomerated. In the present study, the AgNPs were slightly bigger in size and irregular in shape and exhibited greater tendency to form aggregates when the pH of the solution was lower than neutral. The tendency of AgNP agglomeration at pH > 7 was also recorded in our research. Alqadi and coauthors [12] who studied the pH effect on the aggregation of AgNPs synthesized by chemical reduction observed that NPs prepared under lower pH (7, 8 and 8.5) were mostly irregular in shape, while those formed at higher pH (10 and 11) were more regular and smaller. It was suggested that the irregularity in the shape of particles could be attributed to the slow reduction rate of the silver ions. Similar observations were also noted by other authors, for example Barabadi et al. [14] and Honary et al. [38], who studied fungal mediated preparation of gold and iron oxide NPs by using *Penicillium crustosum* and *Penicillium waksmanii*, respectively, and optimization of synthesis process by using mathematical methodology. The authors reported that the increase in the pH value of the solution significantly affected the average size of iron oxide and gold NPs. With the increase in the pH of the solution, the average size of NPs decreased [14, 38].

Moreover, the reducing and stabilizing agents such as diastase [16], tyrosine [15] and polyphenols of plant extracts [18] may affect the synthesis and size of the NPs of noble metals such as silver and gold. The biosynthesis process and size of NPs can be controllable by the change in pH of the enzyme or amino acid. It is well known that alkaline pH favors to improve the reduction potential of molecules containing several carboxylic groups [42]. Thus, the use of varied pH of diastase and tyrosine affects the synthesis and size of NPs. The authors showed that the smallest NPs of silver and gold were formed at pH 12, and the use of pH of diastase below 10 influences the aggregation of AgNPs with complete precipitation at pH 8 [17]. Similar results were observed when tyrosine mediated synthesis of AgNPs was performed [15].

The concentration of salt used for biological synthesis of NPs is an important parameter for the optimization of NP formation [19]. The authors found that out of the different concentrations of $AgNO_3$ that they tested (0.0004, 0.0008, 0.0012, 0.0016 and 0.0024 mol l^{-1}), 0.0016 mol l^{-1} $AgNO_3$ was the optimum concentration required for the synthesis of AgNPs. In the present study, the use of 0.001 mol l^{-1} concentration of $AgNO_3$

was found to be more efficient than 0.003 mol l^{-1} for biological synthesis of AgNPs. Other authors also found that on increasing concentration of salts used for the synthesis of gold ($AuCl_4$) and iron oxide ($FeCl_3$) NPs, the average size decreased but began to increase at higher concentrations. The authors suggested that the increased concentration of salt level allowed particle growth at a faster rate and that particles may aggregate in higher salt concentrations [14, 38]. Maddinedi et al. [16] also found that a reduction in the size of gold NPs (AuNPs) was obtained with an increase in the quantity of diastase as a reducing agent. The authors explained that at a low quantity of diastase, the rate of nucleation was much higher, while the rate of coating of AuNPs with diastase was less, which resulted in the formation of bigger NPs. As the quantity of diastase increased, the rate of coating also increases when compared with nucleation rate, resulting in the formation of smaller NPs [16]. Maddinedi et al. [18] found that a higher volume of water extract of *Cinnamonium tsoi* leaf rich in polyphenols as a reducing agent for the preparation of AgNPs resulted in a higher reduction rate of $AgNO_3$ to AgNPs formation.

It is well known that the stability and size of NPs are the main properties that affect their antimicrobial efficacy. Non-agglomerated and smaller NPs are desired for antimicrobial applications as they interact better with pathogenic bacteria and fungi [43].

5 Conclusions

The present study demonstrated the effect of different parameters on the biosynthesis of NPs synthesized from acinobacterial strains. The formation of AgNPs as well as their physical properties depended on reaction time, temperature pH and reactant concentration. It was found that the size and shape of the synthesized NPs can be altered by changing these parameters. Moreover, optimal temperature (25°C) and pH (7.0) required for synthesis of AgNPs were confluent with the optimal growth conditions of actinobacteria used for the synthesis of NPs, suggesting that bacterial enzymes could be involved in this process.

Conflict of interest statement: The authors have no commercial or financial conflict of interest to declare.

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