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Optimization of Rapid Green Synthesis of AgNPs using *Citrus sinensis* peel extract for antibacterial activity

Santhini S. Nair[1*], Syed Tahira Rizvi[2], P.D. Anthappan[3]

[1,2]Department of Microbiology, VES College of Arts, Science & Commerce, Chembur, Mumbai -400089, India

[1,3]Department of Microbiology, Bhavan's College, Andheri West, Mumbai – 400058, India

Abstract:

Green synthesis of silver nanoparticles was carried out using *Citrus sinensis* peel extract which acted as a reducing and capping agent. Process parameters like volume ratio of *Citrus sinensis* peel extract: 1mM $AgNO_3$, incubation temperature during synthesis and initial peel extract pH, were optimized. The synthesis was confirmed by determination of the surface plasmon resonance pattern using absorption spectra obtained through UV-visible spectroscopy with λ max in the range of 429-432 nm. Electron micrographic observations using TEM analysis of nanoparticles revealed their size in the range of 30-45nm. Post optimization, the produced silver nanoparticles were tested for their storage stability, confirming retention of their above characteristics, over a period of 15 days. Further, the antibiogram of the AgNPs synthesized, indicated them to be effective against both Gram-positive and Gram-negative bacteria tested viz *Escherichia coli* ATCC 9022 NCTC, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 P, *Bacillus subtilis* ATCC 6633 and two environmental isolates viz *Staphylococcus xylosus* and *Pseudomonas pseudoalcaligenes*. Thus, the ecofriendly green synthesis method of AgNPs from orange peel extract used in our investigation, is indicative of gainful exploitation for cost effective alternate antibacterial therapy for restoration of human health.

Keywords: *AgNPs green synthesis, Antibacterial activity, Citrus sinensis peel extract, Process optimization, TEM*

1. Introduction:

The use of silver nanoparticles is constantly on the rise due to their unique physical and chemical properties with a broad range of applications as antibacterial agents in industrial, household, and health care-related products, in consumer products and cosmetics, medical device coatings etc. (Chernousova, S.*et al*, 2012) [2]. The synthesis of Ag nanoparticles is carried out by physical, chemical and biological methods. However due the use of toxic solvents, the generation of hazardous byproducts, and high energy consumption in physical and chemical methods, there is an increasing demand for biological methods for synthesis of nanoparticles. (Kim *et al*,2015) [6]. Also, biologically-prepared AgNPs show high yield, solubility, and high stability and biological methods seem to be simple, rapid, non-toxic and dependable, which after proper optimization, enable the synthesis of Ag nanoparticles of well-defined size and morphology (Zhang *et al*, 2016). [17], (Priti Ranjan *et al*,2013) [10]. (Saifuddin *et al*,2009) [13].

Biosynthesis of Ag nanoparticles usually involves the use of plant extracts, microbial cell biomass (bacteria or fungi) (Bhainsa *et al*,2006) [1] or cell free growth medium and biopolymers. Medicinally important plants like *Boerhaavia diffusa, Ocimum tenuiflorum, Azadirachta indica, Tinospora cordifolia, Aloe vera,*

Terminalia chebula, *Emblica officinalis*, *Cocos nucifera*, common spices *Piper nigrum*, *Cinnamon zeylanicum* etc. have been exploited for Ag nanoparticle production (Khalil *et al*,2014) [5], (Srikar *et al*, 2016) [16]. More recently lesser utilized plant parts like bark, stem, root and fruit peel extracts have also been tested for their potential to synthesize Ag nanoparticles. However, there is a significant variation in chemical compositions of plant extract of same species when it is collected from different parts of world and may lead to different results in different laboratories. This is the major drawback of syntheses of silver nanoparticles using plant extracts as reducing and stabilizing agents and there is need to resolve this problem. (Shakeel Ahmed *et al* ,2016) [14]. Citrus species contain a wide range of active ingredients and are rich in vitamin C, flavonoids, acids and volatile oils (Kavya S *et al*, 2011) [4]. The current work deals with biosynthesis of Ag nanoparticles using *Citrus sinensis* peel extract, optimization of the volume ratio of *Citrus sinensis* peel extract: 1mM$AgNO_3$, temperature during process and pH of the extract for rapid yield of nanoparticles and storage stability of synthesized AgNPs and testing their antibacterial activity against standard bacterial cultures and representative environmental isolates.

2. Materials and Methods:

2.1 Source and preparation of *Citrus sinensis* peel extract

Orange peel was procured from a local fruit juice stall at Chembur. These were washed and rinsed with tap water and later with Double Distilled water. 10 percent [w/v] peel was boiled in Double Distilled water for 5 mins, strained and the extract was filtered through Whatman filter paper no 1 and stored at 5°C until further use.

2.2 Green synthesis of Ag nanoparticles

Ag nanoparticles were initially synthesized by adding 3 ml of the prepared *Citrus sinensis* peel extract in 40 ml of 1mM $AgNO_3$ (Himedia) solution made previously with Double Distilled water. This reaction mixture was then incubated in dark and periodically checked for the development of brown color in the solution indicating the synthesis of Ag nanoparticles (R. Konwarh *et al*, 2011) [12]. A negative control was also set up with 40 ml aliquot of 1mM AgNO3 containing 3 ml Double Distilled water. Spectral analysis with ultraviolet spectrophotometry (Systronics) in the range of 300-500nm was done for confirmation of silver nanoparticle formation. Nanoparticle solutions were diluted 1:4 with distilled water and distilled water served as a blank.

2.2.1 Optimization of volume of *Citrus sinensis* peel extract: volume of 1mM $AgNO_3$ solution.

Different proportions of orange peel extract and 1mM $AgNO_3$ solution were tested for rapid synthesis of Ag nanoparticles which are as follows. Color change of the reaction mixtures were monitored to determine nanoparticle formation which is indicated by appearance of dark brown color.

Table 1. Different proportions of volume

No	*Citrus sinensis* Peel extract (ml)	1mM $AgNO_3$ solution (ml)
1	2	20
2	2	75
3	2	100
4	3	20
5	3	30
6	3	40

2.2.2 Optimization of temperature for synthesis - AgNPs were synthesized by adding the optimized proportion of the reaction mixture of extract and $AgNO_3$ as determined above, and then maintaining the flasks at temperatures of 5°C,37°C ,60°C, 80°C and 100°C for a period of 15 mins. Appearance of brown color in all the flasks was monitored and suitable aliquots were drawn and measured spectrophotometrically. All the flasks were then kept at RT to ascertain the stability of prepared Ag nanoparticles solution after storage at RT for 15 mins, 24 hrs, 72 hrs and 15 days by monitoring the UV-Visible spectra of all the solutions in the range of 300-500 nm.

2.2.3 Optimization of pH for synthesis- The effect of alkaline pH on synthesis of AgNPs was next demonstrated. Originally the pH of the extract was measured as 4.56. 50 ml of the extract was taken in five different flasks each, and pH of each extract adjusted to pH 6,7,8,9 and 10 using pH meter, to see the effect of pH change on the rate of synthesis. The stability of the nanoparticles post synthesis after storage for 15 mins, 24 hrs, 72 hrs and 15 days at RT was monitored by analyzing the UV Visible spectra.

2.3 Characterization of Ag nanoparticles

The synthesized nanoparticles were characterized by using UV-visible spectroscopy and TEM. The bio reduction of Ag+ ion in solution was monitored using UV–visible spectrometer (Systronics). Further the morphology of the AgNPs was determined using transmission electron microscopy (TEM) having a resolution of 0.2nm at 100kV. (TECNAI) (Shrivastava S. *et al*,2007) [15].

2.4 Antibacterial activity /Antibiogram

Antibacterial activity of the obtained isolates was checked against both Gram Positive and Gram-negative organisms. For this purpose, we used four standard bacterial cultures obtained from HiMedia, viz *Escherichia coli* ATCC 9022 NCTC, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 P, *Bacillus subtilis* ATCC 6633 named H1, H2, H3 and H4 respectively and two environmental isolates, viz *Staphylococcus xylosus* and *Pseudomonas pseudoalcaligenes* named H5, and H6 respectively. The method used for the same was agar cup method (Kaviya.S *et al*, 2011) [4]. Positive control used was 1mM $AgNO_3$ solution and negative control was the Double Distilled water extract of the orange peel.

3.Results and Discussion

3.1. Synthesis and optimization of Ag nanoparticles.

AgNPs were synthesized from *Citrus sinensis* peel extract by the methods described above (Kaviya.S *et al*, 2011) [4]. On addition of the aqueous extract (pH 4.56) to 1 mM solution of $AgNO_3$, color changed from colorless to yellowish brown in about 4 hrs. The color of the solution deepened with increase in time. Previous reports have stated the presence of AgNPs exhibiting yellowish brown color in solution due to excitation of surface plasmon vibrations. Metal nanoparticles such as silver and gold have free electrons, which give rise to surface plasmon resonance (SPR) absorption band (Rai.A *et al*, 2006) [11].

Formation of AgNPs using 1mM solution of $AgNO_3$was confirmed using UV–visible spectral analysis with an absorption maximum of 425 nm. The AgNPs formation at room temperature occur within 4hrs.

In a study conducted, orange peel has been reported to be used to prepare biopolymer-templated "green" silver nanoparticles. Aqueous extract of orange peel at basic pH was exploited to prepare starch supported nanoparticles under ambient conditions. The compositional abundance of pectins, flavonoids, ascorbic acid, sugars, carotenoids and myriad other flavones may be envisaged for the effective reductive potential of orange peel to generate silver nanoparticles effectively. (R. Konwarh *et al*,2011) [12]

3.1.1 Optimization of volume of *Citrus sinensis* peel extract: volume of 1mM $AgNO_3$ solution

Among all the proportions tested (Table 2.1) Color change was observed in all ratios tested within 1h of addition. However, agglomeration was seen in all ratios except in proportion no 5 & 6 within four hours of observation. Synthesis of Ag nanoparticles occurred beyond twenty-four hours observation, for proportion No. 6 (i.e. **3 ml extract: 40 ml $AgNO_3$** solution) optimally and this proportion was used for further optimization studies. Formation of AgNPs using this proportion No.6, was confirmed using UV–visible spectral analysis with an absorption maximum of 432 nm.

3.1.2 Optimization of Temperature for synthesis

AgNPs were synthesized by maintaining the reaction mixtures, (i.e. 3 ml extract: 40 ml 1mM $AgNO_3$ solution) at different temperatures following which, UV-Visible spectra of all the solutions were measured. The solutions changed color from colorless to brown within 5 mins as shown (Fig 1)

Fig.1 Optical analysis of AgNPs synthesized at different temperatures within 5 mins.

Stability of the synthesized AgNPs was tested by UV visible analysis after 15 mins, 24 hrs, 72 hrs and 15 days of storage at RT (Table 2). The results indicated that AgNPs synthesis occurred within 5 mins when temperature was maintained at 60℃, 80℃ and 100℃. Further when the flasks were kept at RT, AgNPs synthesis occurred within 24 hrs for all other temperatures except 5℃. This indicates that slow rate of AgNPs synthesis at RT can be accelerated by increasing the temperature of reaction mixture. AgNPs synthesized at 80℃ were found to retain their stability even up to 15 days of storage at RT. Jerushka S Moodley *et al*,2018[3] also have reported that nanoparticle synthesis can be enhanced to yield nanoparticles with smaller sizes by increasing the temperature of synthesis.

Table 2: λ max for various Temperature used during synthesis over different duration of storage at RT

Temperature (℃)	Maximum wavelength			
	15mins	24hrs	72hrs	15days
5℃	403	377	438	313
37℃	596	431	430	385
60℃	413	429	464	377
80℃	420	421	425	400
100℃	420	420	422	393

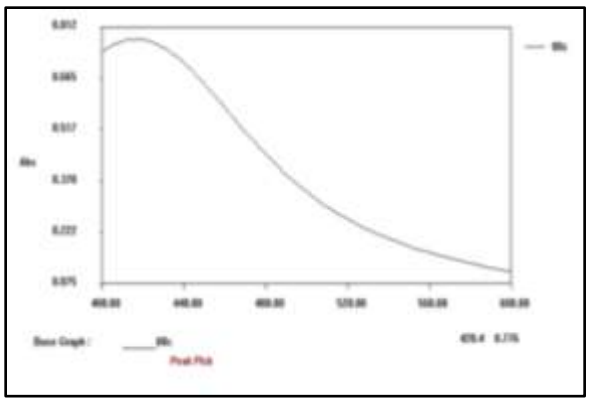

Fig.2 Absorption Spectra analysis of AgNPs synthesized at 80 ℃ within 5 mins

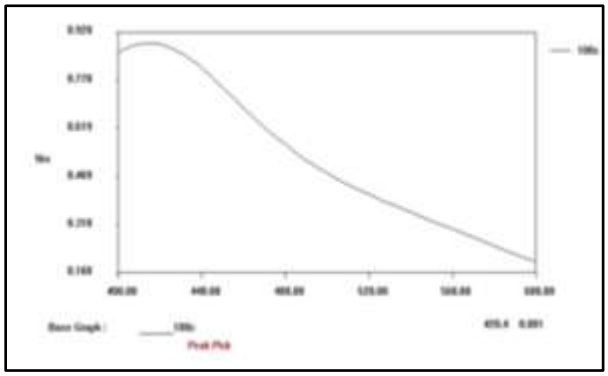

Fig 3 Absorption Spectra analysis of AgNPs synthesized at 100 ℃ within 5 mins.

3.1.3 Optimization of pH for synthesis

AgNPs were synthesized from *Citrus sinensis* peel extract having different pH values of 6, 7, 8, 9 and 10. It was observed that AgNPs were synthesized rapidly within two mins using all the extracts adjusted to alkaline pH (Fig 4). In order to monitor the formation and stability of silver nanoparticles, the absorption spectra of the synthesized silver nanoparticles were recorded after 15 mins, 24hrs ,72 hrs and 15 days of storage at RT (Table 3). It was seen however that AgNPs formed at **pH 8 and 9** maintained stability even after 15 days of storage at RT, with λ max of 429 and 432 respectively. (Table 3). This finding ascertains that pH of the reducing agent plays an important role in synthesis.

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Fig 4: Color change within two mins on addition of extract adjusted to different pH (6, 7, 8, 9 & 10) in 1mM $AgNO_3$

Table 3: - λ max for pH at different time intervals

pH	Maximum wavelength			
	15mins	24hrs	72hrs	15days
6	401	421	439	345
7	433	428	466	385
8	439	433	425	429
9	439	424	436	432
10	420	420	414	401

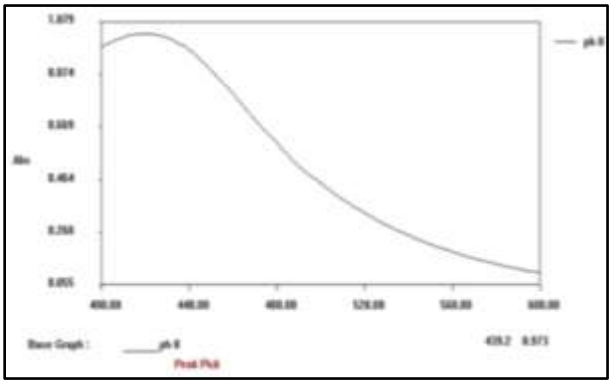


Fig 5: Absorption Spectra analysis of AgNPs synthesized at pH 8 within 2 mins

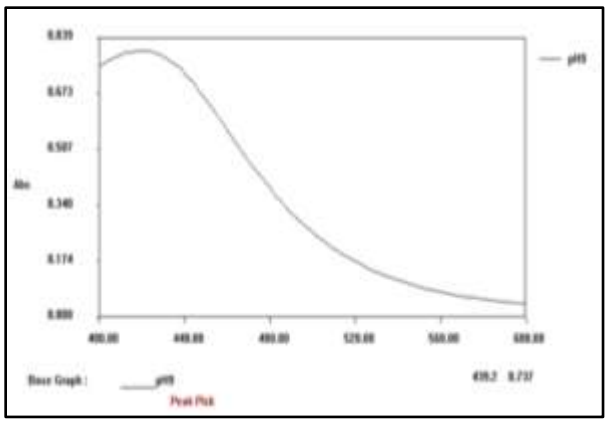


Fig 6: Absorption Spectra analysis of AgNPs synthesized at pH 9 within 2 mins

The role of pH in the green synthesis of AgNPs has been reported by Manjeet Singh *et al*, 2009. [7] They have investigated the effect on synthesis by the addition of NaOH in the reaction mixture.

3.2 Characterization of synthesized AgNPs

The AgNPs were then synthesized using all the optimized conditions as follows:

a) **Volume of *Citrus sinensis* extract: volume of 1mM $AgNO_3$ solution** -proportion No. 6 (i.e. 3 ml extract: 40 ml 1mM $AgNO_3$ solution)

b) **Temperature for synthesis:** 80°C

c) **pH of *Citrus sinensis* extract:** 9

Synthesis of AgNPs using the above conditions occurred within 2 mins and the synthesized AgNPs had an absorption maximum in the range of 429-432nm, which was maintained over the period of storage upto 30 days. The color of the synthesized AgNPs was retained without any agglomeration under observation for a period of six months. The AgNPs synthesized was characterized by Transmission Electron Microscopy (TEM) (Figures 7a and 7b). TEM results showed poly-dispersed particles with spherical shape, and size in the range of 38-44nm.

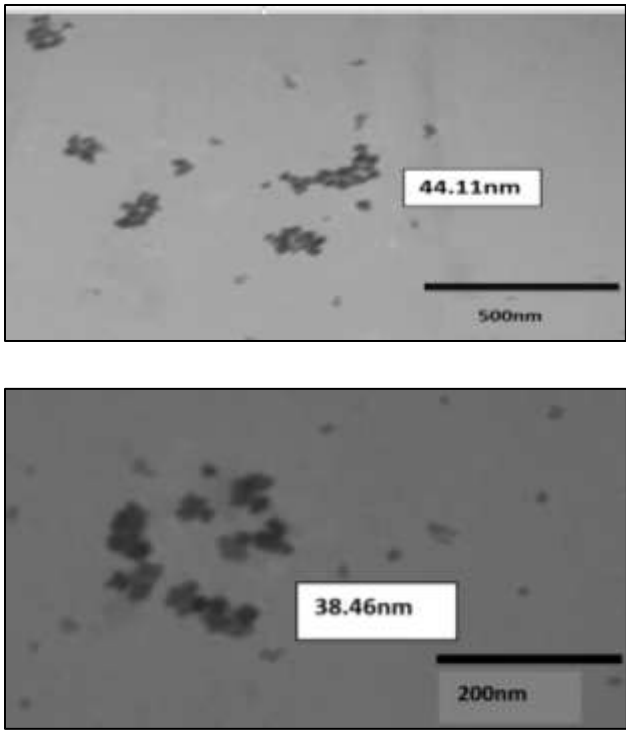


Fig 7a and 7b: TEM electron micrographs of AgNPs

Michael Ndikau *et al*,2017[8] also reported that there is a need for the development of new methods of synthesizing AgNPs that use environmentally safe reagents and solvents. They had developed a similar green method where silver nanoparticles (AgNPs) were synthesized using silver nitrate and the aqueous extract of *Citrullus lanatus* (Water melon) fruit rind as the reductant and the capping agent. The optimized conditions for the AgNPs synthesis were a temperature of 80°C, pH 10, 0.001M AgNO3, 250g/L watermelon rind extract (WMRE), and a reactant ratio of 4:5 (AgNO3 to WMRE)

3.3 Antimicrobial activity of AgNPs against bacterial cultures

The antibacterial activity of the synthesized AgNPs was determined against six bacterial cultures as described and named H1, H2, H3, H4 H5, H6. Agar cup method was used and the zone of inhibition was measured (Fig 8). Inhibition was not seen with the Distilled water extract of orange peels. (negative control). (Fig 9). The synthesized AgNPs were effective against H1, (*Escherichia coli* ATCC 9022 NCTC), H3 (*Staphylococcus aureus* ATCC 6538 P), H4 (*Bacillus subtilis* ATCC 6633), and H5(*Staphylococcus xylosus*). The zone of inhibition was maximum for H1(Gram negative) as compared to Gram positive organisms.

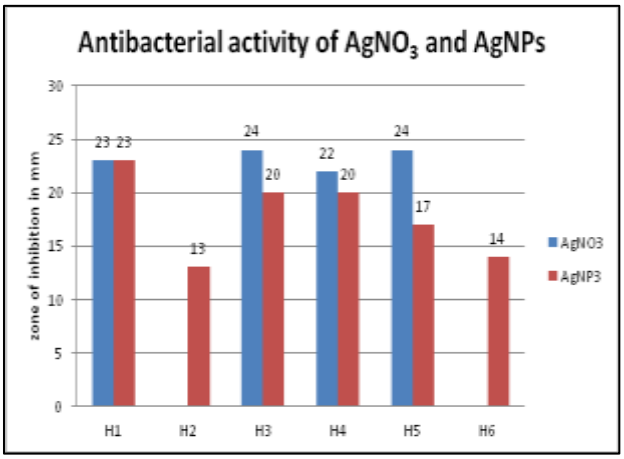


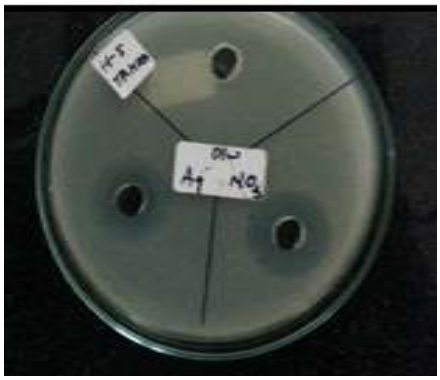
Fig 8: Antibacterial activity of AgNPs

H1

H2

H3

H4



H5

H6

Fig 9: Antibacterial activity of AgNPs against isolates H1, H2, H3, H4, H5 and H6.

Several studies have reported that silver nanoparticles synthesized through green method have been reported to have biomedical applications as well as in controlling the pathogenic microbes. (Prabhu S. *et al* ,2012) [9]

4. Conclusions:

This study has explored an innovative method for green synthesis of AgNPs rapidly using organic wastes like *Citrus sinensis* peel extract in the proportion of 3ml of extract, that is adjusted to pH 9 and adding to 40 ml of 1mM $AgNO_3$ solution and kept at 80℃ within a short period of two minutes. Synthesis of AgNPs was confirmed by determining the surface plasma resonance at 439nm using UV spectral analysis. The synthesized AgNPs were found to be evenly dispersed and spherical in shape measuring 38-44 nm as determined using TEM imaging. The AgNPs showed antibacterial activity against both Gram positive and Gram-negative bacterial organisms. The synthesized AgNPs showed a long storage stability. This research study puts forth a rapid, simple, eco-friendly, safe, reproducible and yet efficient method for synthesis of AgNPs that has a broad antimicrobial spectrum and has the potential for alternate antibacterial therapy to combat the harm being caused by life threatening pathogens.

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