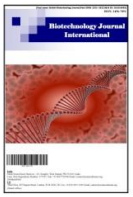

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# Optimization of Selected Process Parameters Affecting Yield of Green Synthesized Silver Nanoparticles and Their Antibacterial Activity

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author COA designed the study, wrote the protocol and jointly conducted the study. Author FAE jointly conducted the study, carried out statistical analysis and wrote the first draft of the manuscript. Author SIU managed the analyses of the study and proofreading of manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** To optimize effects of selected process parameters affecting yield of green synthesized silver nanoparticles and their antibacterial activity.
**Study Design:** Study was designed with 3 factors Box Behnken Design (Minitab 17) and Response optimizer (Minitab 17) was used to determine optimum values of the factors.
**Place and Duration of Study:** Department of Microbiology, Federal University of Technology, Owerri, Nigeria, from March to November, 2020.
**Methodology:** After extraction by boiling, qualitative phytochemical analysis of leaves' extracts of *Ipeoma batatas*, *Commelina africana* and *Manihot esculenta* was carried out. Following synthesis of silver nanoparticles as prescribed by Box Behnken design, yield of AgNPs was optimized with Response optimizer (Minitab 17). Then antibacterial activity of resulting AgNPs was tested against isolates of *P. aeruginosa* and *E. coli.*
**Results:** Extracts contained alkaloids, tannins, proteins and amino acids, flavonoids and phenolic

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compounds, but no sterols and cardiac glycosides. Optimum pH, temperature and time obtained with Response Optimizer resulted in 62.6%, 55.8% and 54.9% increase in yield of AgNPs, with leaf extracts of *C. africana, M. esculenta* and *I. batatas* respectively, compared to un-optimized conditions. Absorbance for resulting AgNPs peaked between 380 to 400 nm. Zones of inhibition (mm) of *P. aeruginosa* with AgNPs synthesized using extracts of *C. africana, I. batatas* and *M. esculenta* were 10, 10 and 9 respectively, under un-optimized condition, and 12, 10 and 8 respectively, for optimized conditions. Against *E. coli,* they were 11, 11 and 12 for AgNPs synthesized with extracts of *C. africana, I. batatas* and *M. esculenta* respectively, under un-optimized condition, and 13, 9 and 11 respectively, for optimized conditions.

**Conclusion:** Leaf extracts of *C. africana, I. batatas* and *M. esculenta* can be used in synthesizing AgNPs, with marked antibacterial activities. Box Behnken design is useful for optimization of effects of process parameters.

*Keywords: Optimization; nanoparticles; parameters; temperature; Box Behnken design; antibacterial; yield.*

## 1. INTRODUCTION

Nanoparticles with dimensions of about 100 nm or less are characterized with remarkably unusual properties, which make them suitable for diverse applications, unlike their bulkier counterparts [1,2]. Observed unique properties are due to differences in physiochemical properties and surface to volume ratio between nanoparticles and their bulkier counterparts [3]. During the last few decades, the application of nanoparticles has grown to include vital areas like consumer products [4]. Consequently, different methods of synthesizing nanoparticles include chemical, biological, physical, as well as hybrid methods [5,6]. Different types of strong and weak chemical reducing and protective agents, such as sodium citrate, borohydride, alcohols sodium, etc. are usually employed in physical and chemical methods for synthesis of silver nanoparticles. Some of these reagents are toxic, flammable and environmentally unfriendly [7]. Conversely, biological method, which uses mostly microorganisms or plant extracts is more eco-friendly. However, it is limited by its time-consuming nature, requiring about 24 to 120 hours for completion of synthesis [8].

The use of plant extracts is due to primary and secondary metabolites found in plants which include fats, proteins, carbohydrates, various enzymes, flavonoids, polyphenols, alkaloids, coenzymes, tannin, terpenoids, gum, lignin etc. These are reportedly capable of reducing silver ions to silver nanoparticles. However, various factors, such as temperature, pH, time of incubation, species and concentrations of active ingredients in plant extracts, etc are known to affect the size, shape and quantity of resulting nanoparticles [9]. During green synthesis, the colourless mixture of silver ion ($Ag^+$) and plant extracts (which provides natural capping and reducing agents) turns yellow to dark brown, or reddish yellow to deep red in colour on synthesis of silver nanoparticles [10].

Plant-mediated synthesis of nanoparticles is more advantageous than use of microorganisms because it involves use of fewer chemicals, eliminates the need for maintenance of microbial cultures, less expensive [11,12], less time-consuming and non-interference of toxic chemicals, as well as ease of scale up [7,13]. Silver nanoparticles have been known to possess antimicrobial activity against a wide range of microorganisms, hence their integration in medicine for the treatment of burns and infections [14]. Their antifungal, antiviral and larvicidal activities have been widely reported [15].

The increasing cases of bacterial resistance to available antibiotics pose great threat to public health and medical operations. This has increased the search for alternative approaches and methods for finding new compounds against bacteria and fungi [14]. Besides, there is need to optimize process parameters which affect the quality and quantity of resulting nanoparticles, so as to maximize productivity of the method and efficacy of the products.

## 2. MATERIALS AND METHODS

### 2.1 Collection and Preparation of Plant Materials

Samples of fresh leaves of sweet potato (*Ipeoma batatas*), commelina (*Commelina africana*) and cassava (*Manihot esculenta*) were collected from

the premises of the Federal University of Technology, Owerri (FUTO) and identified by a competent plant taxonomist at the Department of Biology, FUTO. The samples were thoroughly washed three times with distilled water to remove dust particles, and then sundried to constant weight. Then the dried leaves were in turn ground using clean mortar and pestle. According to the method of Yadav et al. [16], with minor modifications, 10 g of each ground sample was boiled in 100 ml of deionized water for 20 minutes. The aqueous extracts were then separated by filtration with Whatman No. 1 filter paper and then centrifuged at 1200 rpm for 5 minutes to remove heavy biomaterials. The extracts were stored at 4°C until further use.

## 2.2 Qualitative Phytochemical Analysis of Extracts

Qualitative analysis of each of the plant extracts was carried out for the following phytochemicals; phlobatannins, saponins, sterols and triterpenoids as described by Ejikeme et al. [17], alkaloid, anthraquinone glycosides, phenolic compounds, cardiac glycoside, tannins, fixed fat and oil, as described by Banu and Catherine [18], carbohydrates, proteins and amino acids, as described by Raaman [19], flavonoids as described by Saxena et al. [20].

## 2.3 Synthesis of AgNPs under Un-optimized Conditions

The method of Akujobi et al. [21] was adopted. Using separately labeled test tubes, 10 ml of leaf extract was mixed with 90 ml of 1 mM $AgNO_3$ solution. Each mixture was left under ambient conditions and colour changes which indicated reduction of $Ag^+$ to $Ag^0$ nanoparticles (NPs) was visually monitored. After 6 hours of incubation, yield of silver nanoparticles was determined by measuring the absorbance of each solution at 400 nm using LABMAN Spectrophotometer at a resolution of 1 nm. The mixture was centrifuged at 4000 rpm for 20 minutes. After discarding the supernatants, the AgNPs were dispersed again in distilled water and washed. They were then stored at 4°C until used.

## 2.4 Design of Experiment and Optimization of Factors of Synthesis

In line with the method of Akujobi et al. [21], three factors that were optimized include; pH, temperature and time of incubation, at three levels, including; 6, 7 and 8; 25, 30 and 35°C and 2, 4 and 6 hours respectively. Therefore, 3 x 3 design of Box-Behnken plot (Minitab 7®) was adopted. This gave a total of 15 non-randomized runs, each with specific combinations of the factors as shown in Table 1. For each of the plant extracts, fifteen conical flasks were labeled 1 – 15. Then 90 ml of 1 mM silver nitrate was mixed with 10 ml each plant extract in each test tube, after the pH was adjusted using either 0.1 M HCl or 0.1 M NaOH solution [22] and buffered with 10 ml 0.1M phosphate buffer. Each mixture was left to synthesize AgNPs for a period of time and at temperature as defined in Table 1. At the end of time, yield of silver nanoparticles was obtained by measuring the absorbance at 400 nm using LABMAN Spectrophotometer, at a resolution of 1 nm, and results were recorded.

**Table 1. Design of experiment for non-randomized 15 runs in Box-Benken**

| Std order | Pt type | Blocks | pH | Temperature (°C) | Time (hours) |
|---|---|---|---|---|---|
| 1 | 2 | 1 | 6 | 25 | 4 |
| 2 | 2 | 1 | 8 | 25 | 4 |
| 3 | 2 | 1 | 6 | 35 | 4 |
| 4 | 2 | 1 | 8 | 35 | 4 |
| 5 | 2 | 1 | 6 | 30 | 2 |
| 6 | 2 | 1 | 8 | 30 | 2 |
| 7 | 2 | 1 | 6 | 30 | 6 |
| 8 | 2 | 1 | 8 | 30 | 6 |
| 9 | 2 | 1 | 7 | 25 | 2 |
| 10 | 2 | 1 | 7 | 35 | 2 |
| 11 | 2 | 1 | 7 | 25 | 6 |
| 12 | 2 | 1 | 7 | 35 | 6 |
| 13 | 0 | 1 | 7 | 30 | 4 |
| 14 | 0 | 1 | 7 | 30 | 4 |
| 15 | 0 | 1 | 7 | 30 | 4 |

Using Response Optimizer (Minitab® 17), various yield of silver nanoparticles (absorbance of solution) recorded for each set of the 15 setups were optimized for each plant extract. Then the resulting optimum pH, temperatures and times of incubation were used to synthesize AgNPs following the methods of Akujobi et al. [21]. Yield of silver nanoparticle was measured by reading absorbance at 400 nm, at a resolution of 1 nm, before centrifuging at 4,000 rpm for 20 minutes. The AgNPs were washed and stored at 4°C till used.

### 2.5 Characterization of Synthesized AgNPs

Silver nanoparticles synthesized under both optimized and un-optimized conditions were characterized using UV-visible spectrophotometer (LABMAN UV-vis spectrophotometer) at wavelength of 340 – 820 nm. Analysis was done at room temperature operated at a resolution of 1 nm, immediately the time of incubation elapsed for each sample as designated by design in Table 1. Silver nanoparticles are known to exhibit yellowish brown colour in water due to surface plasmon vibration [23].

### 2.6 Collection of Bacterial Isolates

Clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa* used as test microorganisms were obtained from Anthony Van Leuwenhoek's Research Centre, Nekede, Owerri, Imo State, Nigeria. Viability test and identities of the isolates were confirmed using routine biochemical screening and identification methods as described by Cheesbrough [24].

### 2.7 Antimicrobial Activity Assay for AgNPs

The antibacterial activity of AgNPs synthesized under optimized and un-optimized conditions was studied, following the Kirby-Bauer disc diffusion method described by Cheesbrough [24], with slight modifications. The turbidity of each inoculum in nutrient broth was adjusted to 0.5 McFarland's standard (≈1.5 x $10^8$ CFU/ml), and 0.1 ml of each isolate was inoculated onto prepared Mueller Hinton agar (MHA) plates. McFarland standard (0.5 ml) was prepared by mixing 0.05 ml 1% $BaCl_2$ and 9.95 ml 1% $H_2SO_4$. For each extract, 5 mm diameter paper discs were labeled 1 – 5 and soaked with AgNPs synthesized under un-optimized and optimized conditions. Raw extracts, 1 mM $AgNO_3$ and distilled water were also impregnated on paper discs and used as controls. The discs were then deposited at equidistance on each inoculated plate, before incubating at 37°C for 24 hrs. The diameters of zones of inhibition (ZI) were measured to the nearest millimetre.

## 3. RESULTS AND DISCUSSION

### 3.1 Phytochemical Analysis

Results of phytochemical analysis of plant extracts confirmed the presence of different concentrations of alkaloids, tannins, flavonoids, phenolic compounds, proteins and amino acids in the three extracts, but none of them contains sterol and cardiac glycosides. Extracts of *Commelina africana* (Co) and *Manihot esculenta* (Ca) are more similar in their phytochemicals contents than that of *Ipeoma batatas* (Po), as shown (Table 2). Thiyagarajan and Suriyavathana [25] had reported the presence of alkaloids, tannins, saponins, flavonoids, glycosides and phenols in extracts of *Manihot esculenta*. Similarly, our results favourably compares to the report of Apagu et al. [26] that *Manihot esculenta* and *Ipeoma batatas* had glycoside, tannin, saponin, terpenoid and flavonoids, while alkaloids was only found in *M. esculenta*.

### 3.2 Optimization of Conditions and Synthesis of Silver Nanoparticles

In both optimized and un-optimized synthesis of AgNPs, following addition of silver nitrate to leaf extracts studied, it was observed that their colours overtime increased in intensity. This is an indication of reduction of silver ions and formation of silver nanoparticles. At the end of period of synthesis, as prescribed in the design, yield of silver nanoparticles in each case was determined by measuring absorbance at 400 nm, using UV-visible spectrophotometer, and results obtained are shown (Fig. 1). After optimization experiment, optimum pH, temperature and time of incubation obtained using Response Optimizer are 8, 35°C and 5 h for extract of *Manihot esculenta;* 7.7, 35°C and 3 h for *Commelina africana* extract; and 8, 35°C and 2 h for extract of *Ipeoma batatas,* with predicted maximum yield of 0.7008, 0.414 and 0.726 respectively. Synthesis of AgNPs using these optimum conditions produced 62.6%, 55.8% and 54.9% increase in yield of AgNPs, than was obtained under un-optimized conditions, using leaf extracts of *Commelina africana, Manihot esculenta* and *Ipeoma batatas* respectively.

**Table 2. Phytochemicals in leaf extracts of *C. africana* and *M. esculenta* and *I. batatas***

| Phytochemicals | Leaf extracts | | |
|---|---|---|---|
| | ***M. esculenta*** | ***C. africana*** | ***I. batatas*** |
| Phlobatannins | - | + | - |
| Saponin | +++ | ++ | - |
| Alkaloids | +++ | + | + |
| Carbohydrates | + | - | ++ |
| Sterols | - | - | - |
| Triterpenoids | - | - | + |
| Tannins | +++* | +++** | +++* |
| Proteins and amino acids | ++ | + | +++ |
| Flavonoids | +++ | + | ++ |
| Fixed fats and oils | - | - | +++ |
| Anthraquinone glycosides | - | - | ++ |
| Phenolic compounds | +++ | +++ | +++ |
| Cardiac glycosides | - | - | - |

*- = Absent; + = slightly present; ++ = moderately present; +++ = highly present; * = catecholic tannins; ** = condensed tannins*

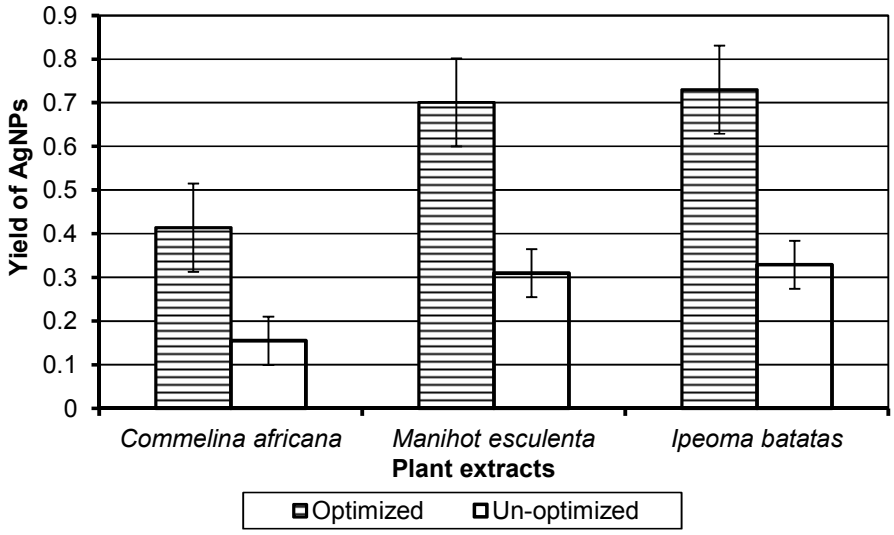


**Fig. 1. Yield of silver nanoparticles under optimized and un-optimized conditions**

Jain and Mehata [27] indicated that biomolecules, such as flavonoids, terpenoids, phenolic compounds present in plant extracts are responsible for reduction of silver ions to AgNPs. They reported that after few hours of reaction, there was no further change in colour of the solution indicating that the whole silver salt present in the solution had been reduced. Shekhawat et al. [28] reported that when mixed with 1 mM $AgNO_3$, color change in extract from pale yellow to dark brown, which attained maximum intensity after 10-12 h, was indicative of formation of silver nanoparticles.

### 3.3 Surface Plot of Interaction of Parameters

Interactions exhibited by pH, time and temperature showed that when time is held constant at 4 h, yield of AgNPs with *Manihot esculenta* extract, was originally high at 25°C at pH 6. However, increase in pH to 7 at 25°C reduced yield of AgNPs. Interaction at 35°C and pH 8 produced highest yield of AgNPs. Similarly, yield was highest when temperature, pH and time interacted at 30°C, 8 and 4 h respectively. At pH 7, yield was highest when interaction was

at 35°C and 4 h. The surface plots of these interactions are shown (Fig. 2). These interactions were best described by Regression equation, in uncoded unit stated as;

Response = 2.511 - 0.190 pH - 0.0767 Temperature - 0.0392 Time + 0.0058 pH*pH + 0.001033 Temperature*Temperature - 0.00792 Time*Time+ 0.00200 pH*Temperature + 0.01375 pH*Time + 0.000250 Temperature*Time.

With *Commelina africana* extract, at 4h, interaction of pH and temperature at 7.5 and 35°C produced the maximum yield of AgNPs. At 30°C, interaction at pH 7 for 2 h produced the highest yield. Also, at pH 7, reacting medium interacting at 25°C for 2 h gave the highest yield of AgNPs. Their surface plots are shown (Fig. 3), with interactions which obeyed Regression equation in uncoded units, expressed as;

Response = 0.922 + 0.153 pH - 0.0767 Temperature + 0.0477 Time - 0.0142 pH*pH + 0.000933 Temperature*Temperature - 0.00292 Time*Time + 0.00250 pH*Temperature - 0.00875 pH*Time + 0.00100 Temperature*Time

With *Ipeoma batatas* extract at 4h, yield of AgNPs was highest when pH and temperature interacted at 8 and 35°C respectively. When temperature is constant at 30°C, interaction at pH 8 for 2 h produced highest yield of AgNPs. At pH 7, yield was highest when interaction was at 35°C for 6 h. The surface plots of interaction of factors are shown in Fig. 4. Observed interactions followed Regression equation in uncoded units expressed as;

Response = 3.90 - 0.287 pH - 0.183 Temperature + 0.075 Time + 0.0083 pH*pH + 0.00193 Temperature*Temperature + 0.01083 Time*Time + 0.01000 pH*Temperature - 0.0275 pH*Time + 0.00100 Temperature*Time

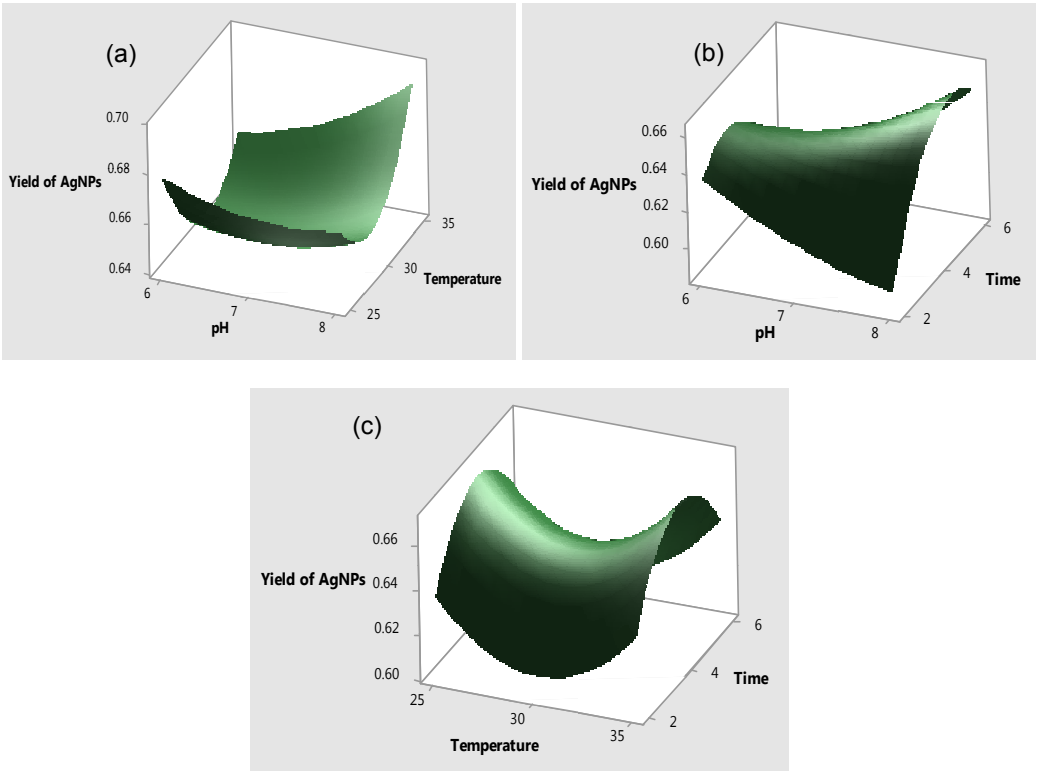


**Fig. 2. Surface plots of interactions of (a) Temperature and pH (Hold Time 4h) (b) Time and pH (Hold temperature; 30°C and (c) Temperature and time (Hold value, pH 7) using cassava (*Manihot esculenta)* leaf extract**

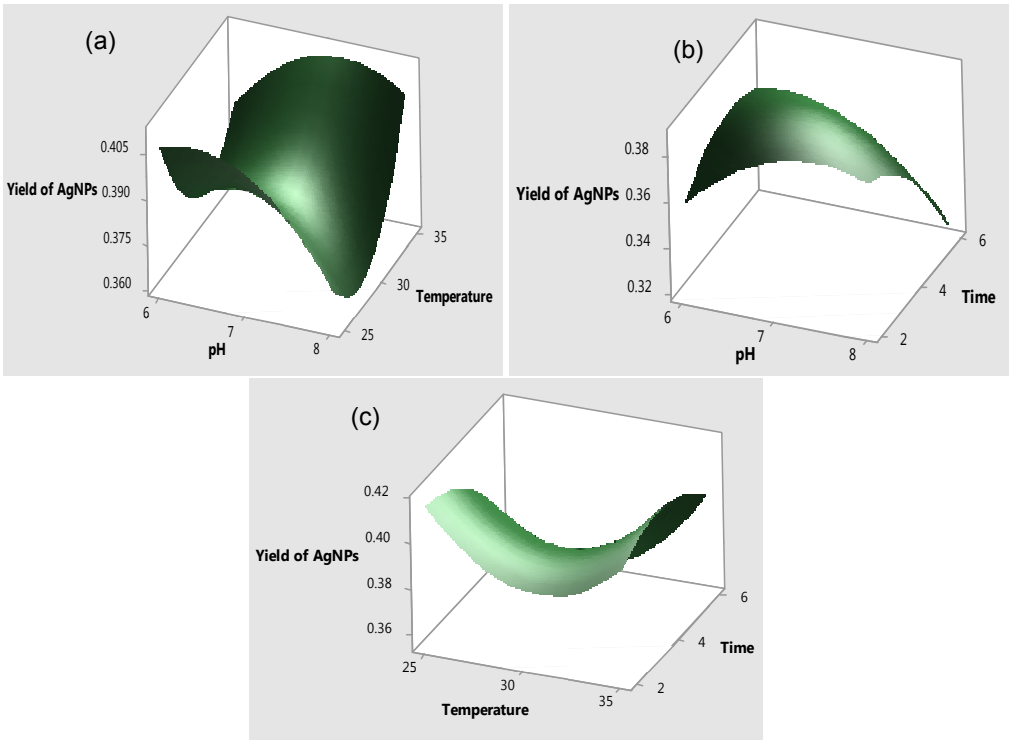


**Fig. 3. Surface plots of interactions of (a) Temperature and pH (Hold time at 4h) (b) Time and pH (Hold temperature at 30°C and (c) Temperature and time (Hold value, pH 7) using cassava (*Commelina africana)* leaf extract**

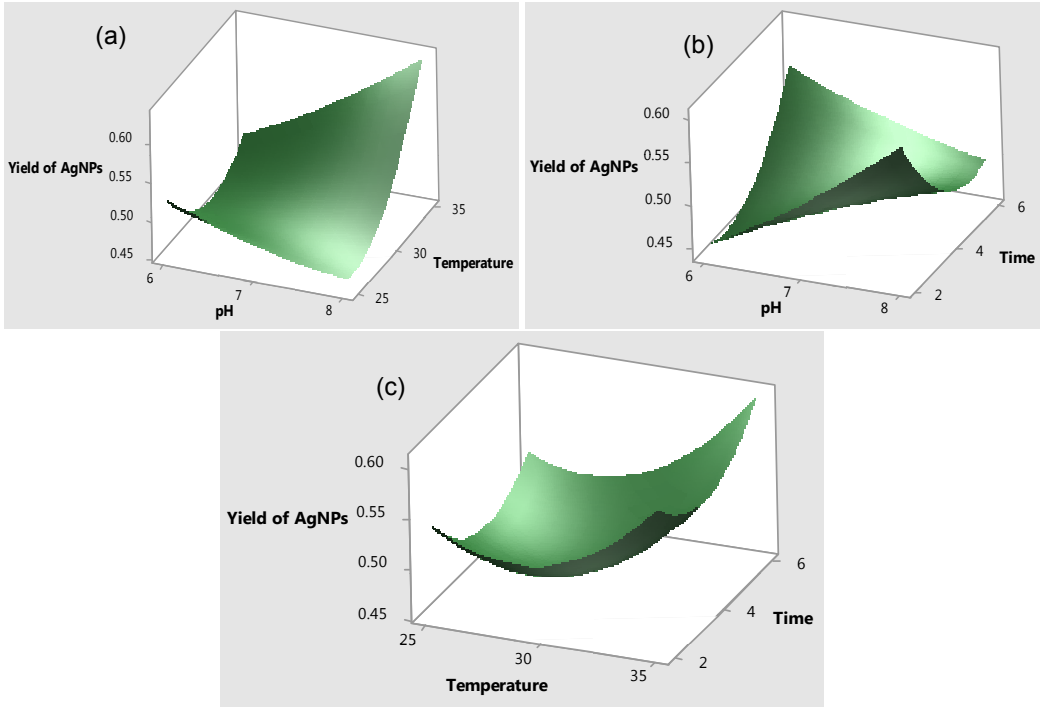


**Fig. 4. Surface plots of interactions of (a) Temperature and pH (Hold time; 4h) (b) Time and pH (Hold temperature; 30°C and (c) Temperature and time (Hold value, pH 7) using cassava (*Ipeoma batatas)* leaf extract**

According to Jain and Mehata [27], with an increase in reaction temperature, shift in absorption peak towards lower wavelength was observed, which indicated a decrease in particle size with increase in temperature [29]. This shift in absorption peak was attributed to localization of surface plasmon resonance of AgNPs. This implies that size of synthesized AgNPs reduces with increasing temperature, maybe due to faster reaction rate at a higher temperature. At high temperature, the kinetic energy of molecules increases and silver ions get consumed faster leaving less possibility for particle size growth. Thus, smaller particles of nearly uniform size distribution are formed at higher temperature [30]. The major influence of reaction pH is its ability to change the electrical charges of the biomolecules, which might change their reducing and capping ability and the subsequent growth of nanoparticles [31]. It was observed that higher pH enhances the rate of reduction as the colour of the solution turned colloidal brown more quickly compared to a solution of lower pH. Hence alkaline pH is favorable for the synthesis of AgNPs [27].

Absorption spectra obtained on characterization of resulting AgNPs synthesized under optimized and un-optimized conditions showed peak of absorption of UV between 380 – 400 nm, as shown (Figs. 5 and 6). UV-visible spectrophotometry is used for analysis for pH dependency, silver nitrate ion concentration, the formation of AgNPs [9]. Shekhawat et al. [28] reportedly observed sharp peak in between 422 nm to 447 nm, which indicated formation of silver nanoparticles.

### 3.4 Antibacterial Activity of AgNPs Synthesized under Optimized and Un-optimized Conditions

Result of antibacterial activity of AgNPs synthesized under optimized and un-optimized conditions, as well as other substances used as control are presented in Fig. 7. AgNPs were effective against test isolates. There was no substantial variation in antibacterial activities of AgNPs synthesized under optimized and un-optimized conditions, irrespective of increased yield recorded when optimized. This suggests that optimum conditions at which there was increase in yield of nanoparticles had no significant effects on size or morphology of resulting nanoparticles. However, antibacterial activities of both AgNPs were higher than limited activity of 1 mM $AgNO_3$ solution against *E. coli.* Distilled water and the three raw extracts did not produce antibacterial activity against the isolates, except for extract of *Ipeoma batatas,* which

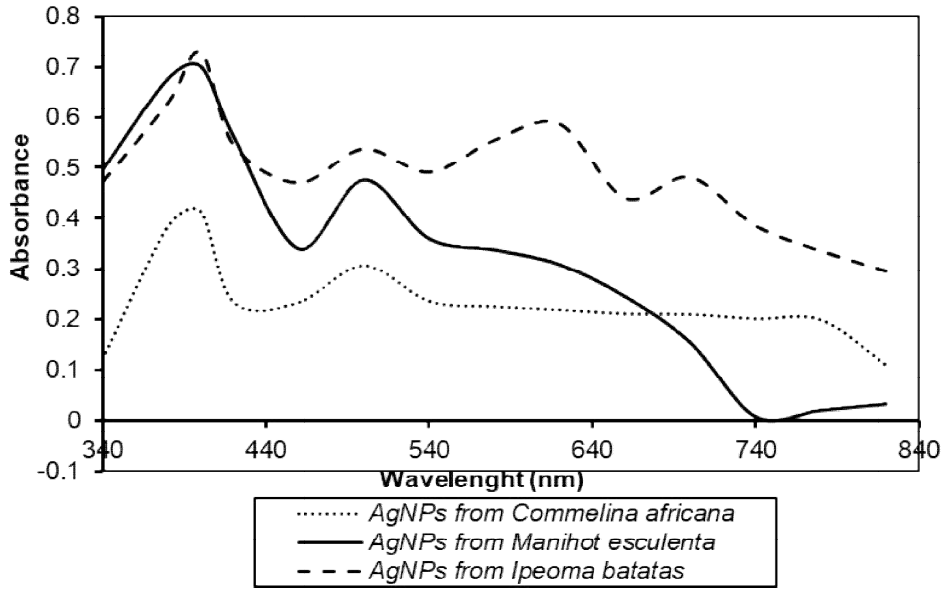


**Fig. 5. Absorption spectra of AgNPs synthesized under optimized conditions using different leaf extracts**

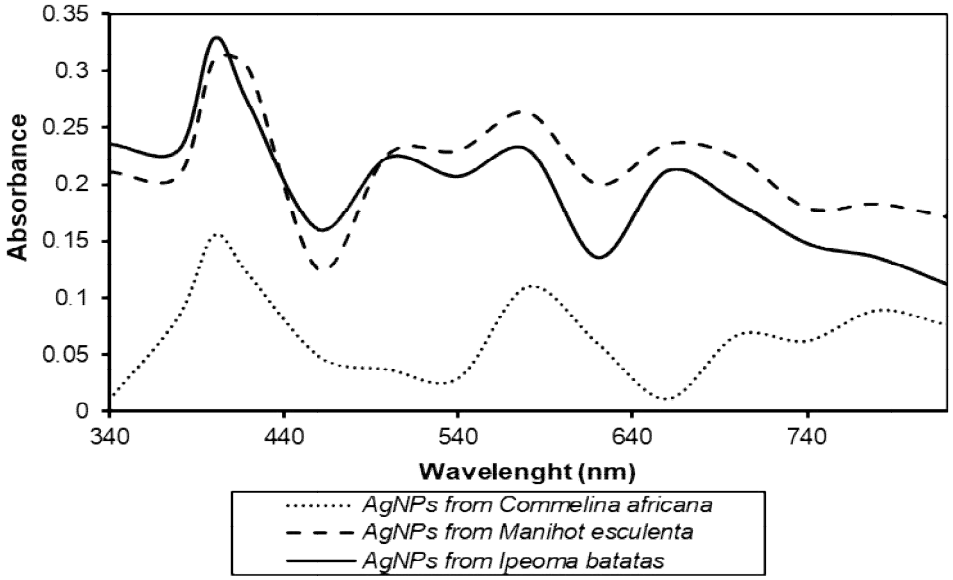


**Fig. 6. Absorption spectra of AgNPs synthesized under un-optimized conditions using different leaf extracts**

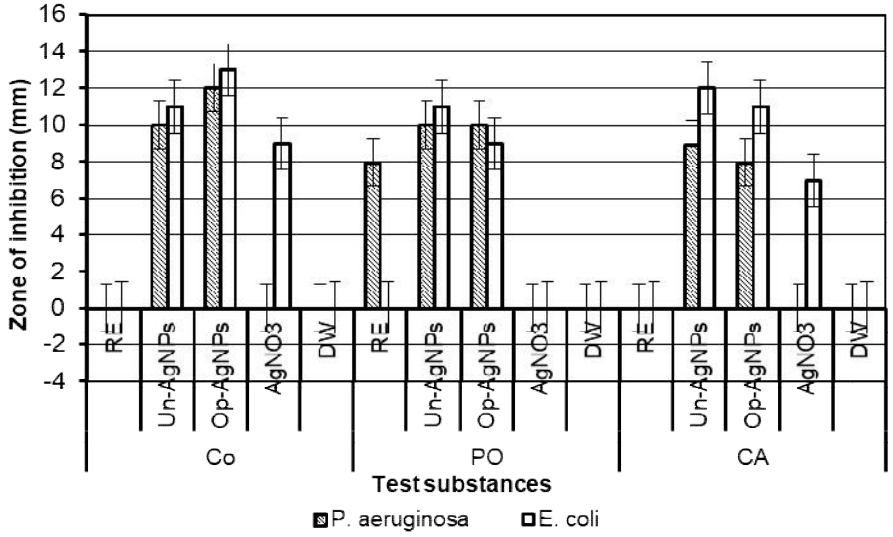


**Fig. 7. Antibacterial activity of AgNPs and other control agents. (RE = Raw extract; Un-AgNPs = AgNPs synthesized under un-optimized conditions; Op-AgNPs = AgNPs synthesized under optimum conditions, DW = Distilled water)**

showed limited activity against *P. aeruginosa*. This may be attributed to the low concentrations of raw extracts used in this study. Generally, *Escherichia coli* were observed to be more susceptible to both AgNPs than *Pseudomonas aeruginosa*. This supports the finding by [32] which indicated that results *Sambucus ebulus* extract based zinc oxide nanoparticles showed more activity against Gram negative bacteria, which may be attributed to existence of a thick rigid layer that surrounds their cells.

Thiyagarajan and Suriyavathana [25] had reported 24 and 16 mm zones of inhibition against *P. aeruginosa* and *E. coli* by extracts of *Manihot esculenta.* Difference observed with results of present study may be due to higher concentrations they used. Apagu et al. [26],

reported 7.5 mm and 14 mm zones of inhibition against *E. coli* and 4.5 mm and 7 mm zones of inhibition against *P. aeruginosa* by aqueous extracts of *Ipeoma batatas* and *Manihot esculenta.* Moreover, Donda et al. [33] have reported zones of inhibition of 8 mm against *P. aeruginosa* and 8 mm against *E. coli* by 5 µl aqueous extracts of *Securinega leucopyrus*. Besides, other types of nanoparticles, such as zinc oxide nanoparticles (ZnONPs), biosynthesized with *Sambucus ebulus* leaf extract have reportedly shown antibacterial activity against *B. cereus, S. aureus,* and *E. coli* [32]. Use of Ag as dopant has also been reported to enhance antibacterial activities of resulting doped nanoparticles. Example is the case of Ag–$TiO_2$ NCs which was reported to have shown significantly higher antibacterial activity against *Bacillus subtilis, Escherichia coli*, and *Aspergillus niger,* than $TiO_2$ nanoparticles (NPs) [34].

## 4. CONCLUSION

Results obtained showed that silver nanoparticles can be easily synthesized using aqueous extracts of sweet potato *(Ipeoma batatas)* leaf, Commelina *(Commelina africana)* leaf and Cassava *(Manihot esculenta)* leaf. Response Optimizer indicated that optimum pH, temperature and time for synthesis of AgNPs were 8, 35°C and 5 h using *M. esculenta* extract, 7.7, 35°C and 3 h using *C. africana* extract and 8, 35°C and 2 h using *I. batatas* extract, with expected maximum yield of 0.7008, 0.414 and 0.726 respectively. These optimum values amounted to 62.6%, 55.8% and 54.9% increase in yield of AgNPs, compared to synthesis under un-optimized conditions with leaf extracts of *C. africana, M. esculenta* and *I. batatas* respectively. Peak of absorption of resulting AgNPs as shown by UV-visible spectra ranged from 380 – 400 nm. The synthesized AgNPs were effective against test bacterial isolates. However, there was no major difference in antibacterial activities of AgNPs synthesized under optimized or un-optimized conditions.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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