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EFFECT OF PROCESS PARAMETERS ON THE SYNTHESIS OF SILVER NANOPARTICLES AND ITS EFFECTS ON MICROBES

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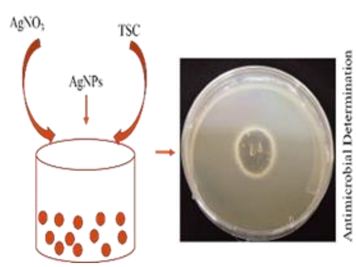
Graphical abstract



Abstract

Metallic silver (Ag) and some Ag compounds have long being used to fight microbial infection even before the emergence of antibiotics in the form of drugs. But lately, one of the problems facing the medical world is the occurrence of drug resistance microbes. Therefore, actions must be taken to overcome the problem, one of which is to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to patients. In this regard, with the advent of nanotechnology, it is now possible to synthesize nano scale Ag which can be used in diverse medical applications. The aim of this study is to investigate the effect of some important parameters towards the production of high yield Ag nanoparticles (AgNPs) from silver salts by making use of reducing agent. One-Factor-at-a-Time (OFAT) design of experiment was conducted, involving five important synthesis parameters. Based on the analyses of its yellow color, absorption by UV-vis in the range of 350 to 420 nm wavelength and surface plasmon resonance (SPR) at 420 nm, AgNPs produced were estimated to be in the range of 5 to 50 nm in diameter. Out of the five parameters tested, such as the concentration of silver nitrate ($AgNO_3$), the initial pH of $AgNO_3$ solution, the concentration of tri-sodium citrate (TSC), the reaction time between $AgNO_3$ and TSC and stirring time (after reduction process), results showed that all factors except stirring time affect the production. Characterization of AgNPs, carried out using Transmission Electron Microscope (TEM), showed that the synthesized AgNPs were spherical with diameters less than 15 nm. Antimicrobial activity assays of AgNPs, carried out against selected microbes, namely *Aspergillus niger*, *Microsporum canis* and *Staphylococcus aureus* showed various degree of potencies.

Keywords: One-Factor-at-a-time (OFAT), $AgNO_3$, Silver nanoparticles (AgNPs), Transmission Electron Microscopy (TEM), Surface plasmon resonance (SPR)

Abstrak

Logam argentumperak (Ag) dan beberapa sebatian Ag telah lama digunakan untuk melawan jangkitan mikrob sebelum munculnya antibiotik dalam bentuk ubat. Tetapi akhir-akhir ini, salah satu masalah yang dihadapi dunia perubatan adalah berlakunya rintangan mikrob terhadap ubat. Oleh itu, tindakan perlu diambil untuk mengatasi masalah, dan salah satunya ialah meneruskan kajian untuk membangunkan ubat-ubatan baru, sama ada sintetik atau semula jadi. Matlamat utama adalah untuk menawarkan ubat antimikrob yang sesuai dan berkesan kepada pesakit. Dalam hal ini, dengan kemunculan teknologi nano, ia memungkinkan untuk mensintesis Ag skala nano yang boleh digunakan dalam aplikasi perubatan yang pelbagai. Tujuan kajian ini adalah untuk mengkaji kesan beberapa parameter penting ke arah pengeluaran hasil nanopartikel Ag (AgNPs) dari garam Ag dengan menggunakan agen penurunan. Reka bentuk eksperimen, Satu-Faktor-Satu-Masa (SFSM), telah dijalankan yang melibatkan lima parameter sintesis penting. Berdasarkan analisis warna kuning produk, penyerapan oleh UV dalam panjang gelombang 350 hingga 420 nm dan resonans plasmon permukaan (SPR) pada 420 nm, AgNP yang dihasilkan dianggarkan berada dalam lingkungan 5 hingga 50 nm. Daripada lima parameter yang diuji, seperti kepekatan $AgNO_3$, pH permulaan $AgNO_3$, kepekatan tri-natrium sitrat (TSC), masa reaksi antara $AgNO_3$ dan TSC dan masa pengadukan (selepas proses penurunan), hasil menunjukkan bahawa semua faktor kecualimasa pengacauan mempengaruhi pengeluaran. Pencirian AgNPs, yang dilakukan menggunakan Mikroskop Elecktron Penghantaran (MEP), menunjukkan bahawa AgNPs yang disintesis adalah berbentuk sfera dengan diameter kurang dari 15 nm. Pemeriksaan aktiviti antimikrob AgNPs, yang dilakukan terhadap mikrob terpilih, iaitu *Aspergillus niger, Microsporum canis* dan *Staphylococcus aureus* menunjukkan pelbagai tahap potensi antimikrob.

Keywords: Satu-Faktor-Satu-Masa (SFSM), $AgNO_3$, Nanopartikel perak (AgNPs), Mikroskop Elecktron Penghantaran (MEP), Resonans plasmon permukaan (SPR)

1.0 INTRODUCTION

The production of metal nanoparticles has been widely reported due to their distinctive chemical and physical properties, which have many potential applications [1, 2]. However, silver (Ag) is one of the most studied metals due to its properties and areas of use. There is a lot of interest in controlling the process parameters of Ag nanoparticles (AgNPs) synthesis to yield particles of different properties (stability, morphology, particle size distribution and surface state charge/modification) intended for different applications. Various methods of synthesis have been used to prepare AgNPs, such as by chemical reduction in aqueous and non-aqueous media, in soft matrices and in solid matrices (e.g., mesoporous silicate) [3-5], by applying physical processes/various types of irradiation [1, 6], by electrochemical processes [7] and in emulsion systems [8]. The AgNPs are widely used as photo-catalysts [9], catalysts [10], antibacterial [11], in biosensor [12] and in bioimaging [13]. Subsequently, AgNPs synthesis by chemical mediated method has been reported by many researchers, for example, Kim *et al*. (2007) reported on the synthesis of spherical AgNPs [14]. In addition, the effect of various process parameters for AgNPs synthesis has been carried out to gain better particles stability, high yield and to prevent product aggregation [15]. In one study, stirring time was found to have effect on the surface plasma resonance (SPR) of the produced AgNPs [16].

In the biomedical world, Ag and some Ag compounds have long being used to fight microbial infections even before the emergence of antibiotics in the form of drugs. But lately, one of the problems faced in this field is the occurrence of drug resistance microbes. Therefore, actions must be taken to overcome the problem, one of which is to continue the work in the development of new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial cure to patients. Although high concentration of Ag is toxic to mammals, many reports suggest a beneficial effect to human cells if it is used in low concentration [17, 18]. Kora and Sashidhar [19] recently reported an antibacterial activity of AgNPs against both gram-positive and gram-negative bacteria, which was as a result of cell surface damage which occur during the bacteria and nanoparticle interaction. This is apparently due to the fact that the AgNPs affect cells at several levels, such as with the bacterial cell wall, protein synthesis and DNA metabolism [20]. Antibacterial activity of Ag containing material has also been reported to help to reduce infection during burn treatment [21]. There are many reports on the antimicrobial property of AgNPs, for instance, Panáček *et al*. (2009) [20] reported that AgNPs were effective against *Candida spp.*, while Kim *et al*., (2009) [14] documented the mode of action of AgNPs against *Candida albicans*. Similarly, AgNPs have inhibitory effect on different plant fungi including *Alternaria alternata, Sclerotinia sclerotiorum, Macrophomina phaseolina, Rhizoctonia solani, Botrytis cinerea and Curvulari alunata, as* reported by Krishnaraj *et al*. (2012) [22].

The study on the synthesis parameters of AgNPs is vital in getting superior quality and quantity product. The aim of this research is to study the effect of various synthesis parameters toward the yield of AgNPs by using the traditional One-Factor-At-a-Time (OFAT) design of experiment. The OFAT experimental results are important to determine the optimal range of each selected process parameters, whereby the optimal range of these values can then be employed in the next phase of process optimization experiments using Response Surface Methodology (RSM). Five process parameters were evaluated in this study, namely, $AgNO_3$ concentration, initial pH of $AgNO_3$ solution, concentration of reducing agent (tri-sodium citrate, TSC), the reaction time between $AgNO_3$ and TSC and lastly, the stirring time of the product, after the reduction process. Characterization of the produced AgNPs was carried out via Transmission Electron Microscopy (TEM) and the test of antimicrobial potency was carried out against three microbes, namely, *Aspergillus niger, Microsporum canis* and *Staphylococcus aureus.*

2.0 METHODOLOGY

2.1 Synthesis and Characterization of Silver Nanoparticle

In a typical experimental run, 20 mL of 1 mM $AgNO_3$ (AR Grade ≥ 99.00%, Sigma Aldrich, USA) was kept stirring at 90°C for 5 minutes on a hot plate, after which 2.5 mL of 1% w/v tri-sodium citrate (TSC) (FG Grade ≥ 99.00%, Sigma Aldrich, USA) was added drop by drop. Reduction process started when the solution started turning into pale yellow, and became darker yellow as the reduction progressed. The reduction time was kept at 3.5 minutes as the standard time period. The solution was then placed on a magnetic stirrer at ambient temperature for another 15 minutes stirring time. The absorbance of the product was scanned between 300 nm to 600 nm wavelength, using Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA). The characterization of AgNPs was carried out using TEM (200 KV FEI TECNAI G2 20S TWIN, FEI, USA) and Energy Dispersive X-Ray (EDX).

2.2 Design of Experiments by OFAT

Five process parameters have been selected in the design of experiment by One-Factor-at-a-time (OFAT), namely, $AgNO_3$ concentration, initial pH of $AgNO_3$ solution, TSC concentration, reaction time between $AgNO_3$ and TSC and stirring time after the reduction process. The effects of each parameter were conducted, while keeping the other four parameters constant. The responses were quantitated as 'Yield of AgNPs', whereby the yield is represented by the estimated area under the curve from 350 to 420 nm wavelength [15].

2.3 Antimicrobial activity of AgNPs

Antimicrobial activity assays were carried out against three microbes, namely, *Aspergillus niger* (ATCC: A121), *Microsporum canis* (ATCC: M96) and *Staphylococcus aureus* (IMR: S822). There was no basis for this selection, only base on their availability in the laboratory. Poisoned agar assay [23, 24] was performed to determine the antifungal activity, whereas, well diffusion assay [25] was done to determine the antibacterial activity. All medium were purchased from OXOID, UK, through a local supplier. All analytical tests were performed in triplicates. The responds for the different parameters were expressed as the Mean ± SD (Standard Deviation.

3.0 RESULTS AND DISCUSSION

3.1 Effect of Selected Parameters on AgNPs Yield and Characterization of AgNPs

Before the statistical optimization of process parameters for maximum AgNPs synthesis can be carried out, it is very important to find the optimum range of values that can be used in the design of experiment. For that purpose, OFAT design of experiment was imposed on the five important process parameters. OFAT experimental runs will provide the effect of each selected process parameters by varying the values of one parameter, while keeping the other four parameters constant. The reaction products were submitted to a scanning UV-vis absorbance from 300 nm to 600 nm wavelengths, and the responses are recorded as 'Yield of AgNPs', whereby the 'Yield of AgNPs' is represented by the estimated area under the curve from 350 to 420 nm wavelength [15]. The observed absorbance of product at 420 nm indicates the presence of spherical AgNPs measuring approximately 5-50 nm in diameter [15].

Figure 1 shows the effect of different concentration of $AgNO_3$ (0.5, 0.75, 1.0, 1.25 and 1.5 mM) on the synthesis of AgNPs. The scanning profile of absorbance of each concentration tested is plotted and the inset of Figure 1 shows the estimated area under each spectrum from 350 to 420 nm, recorded as the 'Yield of AgNPs'. Results showed that there is a sharp increase in yield from 0.5 to 1.0 mM whereby it peaked at 1.0 mM. Increasing the concentration of

$AgNO_3$ further was of no advantage, as the yield decreased, implying that less AgNPs were produced. The presence of surface plasmon resonance (SPR) peak at 420 nm indicates the presence of spherical AgNPs, whereby the maximum synthesis was observed at 1 mM, implying that the concentration of $AgNO_3$ plays an important role in the synthesis of AgNPs. This observation is also supported by the visual evaluation as pictured in Figure 2, which shows a darkest yellow solution for product using 1 mM $AgNO_3$.

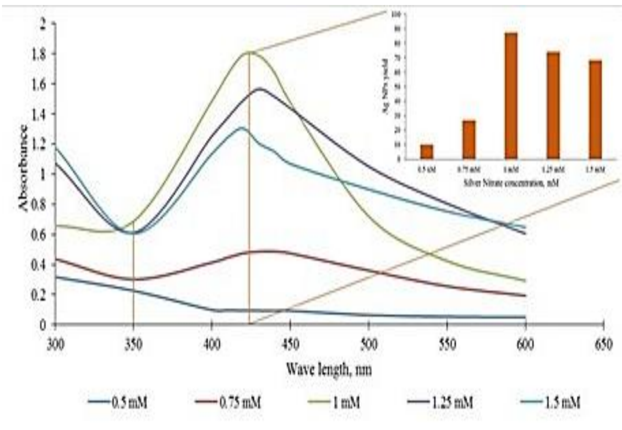


Figure 1 UV-vis absorbance profile of AgNPs synthesized product at different $AgNO_3$ concentration. Inset: Plot of 'Yield of AgNPs' at different $AgNO_3$ concentration

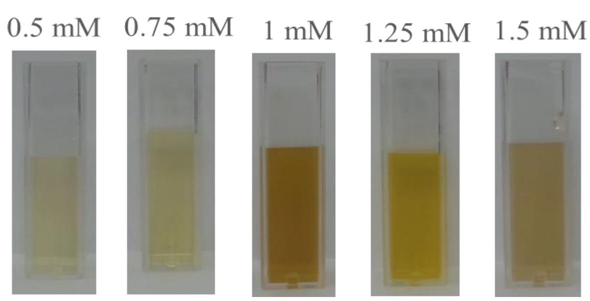


Figure 2 Visual appearance of AgNPs synthesized product at different $AgNO_3$ concentration showing the darkest yellow when using 1 mM $AgNO_3$ indicating the highest presence of spherical AgNPs

The effect of initial pH of $AgNO_3$ solution (pHs 4, 5.5, 6, 7, 7.5, 8 and 11) on the synthesis of AgNPs was also studied and the results are given in Figure 3. As shown by the inset in Figure 3, the 'Yield of AgNPs' increased as pH was increased from 4 to 7 and the yield peaked at pH 7 and then decreased as the alkalinity is increased to pH 7.5 until pH 11. The observation on the color of the products showed a change from pale yellow to green (results not shown). These results showed that the initial pH of $AgNO_3$ solution is important, and for high yield spherical AgNPs, keeping the solution at pH 7 is vital. These results also confirms the report by Krishnaraj *et al*. [22] that agglomeration of product can happen at highly acidic pH [22], thus lowering the production of spherical AgNPs. The formation of green solution at alkaline pH (>7) implied the formation of triangular AgNPs [24].

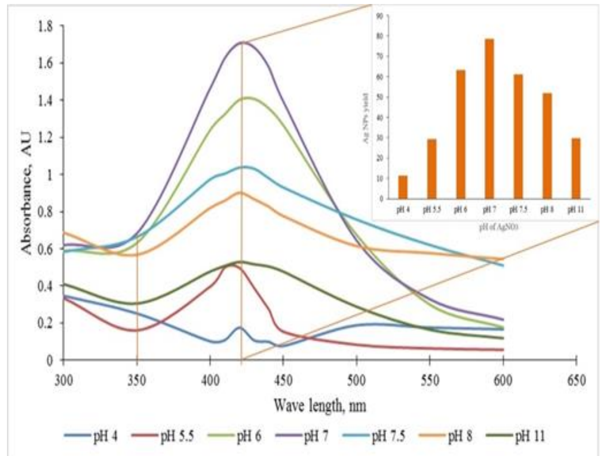


Figure 3 UV-vis absorbance profile of AgNPs synthesized product at different pHs of $AgNO_3$ solution. Inset: Plot of 'Yield of AgNPs' at different pHs

The reaction time between $AgNO_3$ and TSC, the reducing agent, was varied (1.5, 2.5, 3.5, 4.5 and 7.5 minutes) while keeping them stirred on hot plate at 90°C. UV-vis absorbance scanning profile of products, as given in Figure 4 and the inset showed that very low yields were obtained for 1.5 and 2.5 minutes, however the yield was maximum at 3.5 minutes. However, lengthening the period of reaction to 4.5 minutes and 7.5 minutes were of no advantage, as the production of spherical AgNPs were much lowered as given in the inset. High AgNPs in the solution was indicated by dark yellow solution, but as time of reaction was increased the resultant solution turned green (results not given) which may imply the formation of triangular NPs.

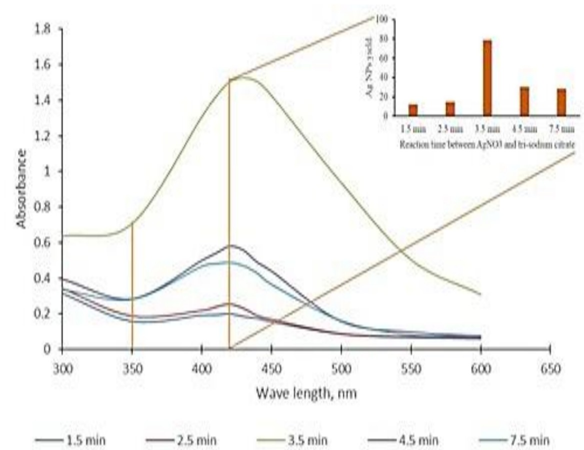


Figure 4 UV-vis absorbance profile of AgNPs synthesized product at different reaction time between $AgNO_3$ and TSC, the reducing agent. Inset: Plot of 'Yield of AgNPs' at different reaction time between $AgNO_3$ and TSC

Figure 5 shows the effect of TSC concentration (0.5, 0.75, 1.0, 1.25 and 1.50 %) on the synthesis of AgNPs. The UV-vis profiles of the products using different TSC concentrations are given in the figure, with an inset showing the 'Yield of AgNPs'. There was a small increase in peak from 0.5 to 0.7% TSC, however the product is maximum at 1% TSC. Increasing TSC concentration to 1.25 and 1.5% is of no advantage as the product was shown to decrease thereon.

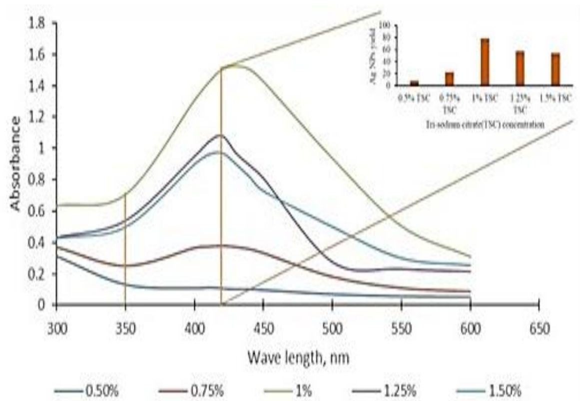


Figure 5 UV-vis absorbance profile of AgNPs synthesized product at different TSC concentration. Inset: Plot of 'Yield of AgNPs' at different TSC concentration

The effect of stirring time after the reduction process need also be evaluated. The product is typically stirred on magnetic stirrer placed at ambient temperature, for 15 mins. To find the effect of stirring time on the synthesis of AgNPs, several stirring time was evaluated which are at 5, 10, 15, 20 and 25 mins. Figure 6 shows the UV-vis absorption profile of the product at different stirring time, with an inset showing the 'Yield of AgNPs'. There was a very small gradual increase in peak from 5 to 25 minutes, implying that there is some advantage in increasing the time of stirring confirming a previous report [16]. However in this case, the effect of lengthening the stirring time towards the production of AgNPs is very small and insignificant. The increasing of absorbance peak at 420 nm also can be explained by multilayer Mie theory model, in short, chemical interaction occurred in lower electron conductivity containing outer most layer of atom and cause the red shift of absorbance peak [26].

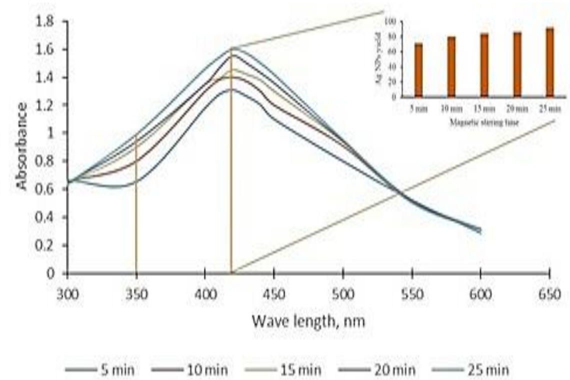


Figure 6 UV-vis absorbance profile of AgNPs synthesized product at different stirring time. Inset: Plot of 'Yield of AgNPs' at different stirring time

The presence of AgNPs in the reaction product was shown by the observed presence of 'yellow colour' solution and the absorbance of SPR at 420 nm. In addition to the analyses, morphological characterization of the product was conducted via TEM analysis. Figure 7 confirms the existence of small uniform spherical shaped AgNPs, which are measuring less than 15 nm in diameter. Energy dispersive X-ray (EDX) spectroscopy displayed a strong silver peak between 2.7 and 4 keV in term of Energy (results not shown), and this was similarly reported by Chowdhury *et al*. [27].

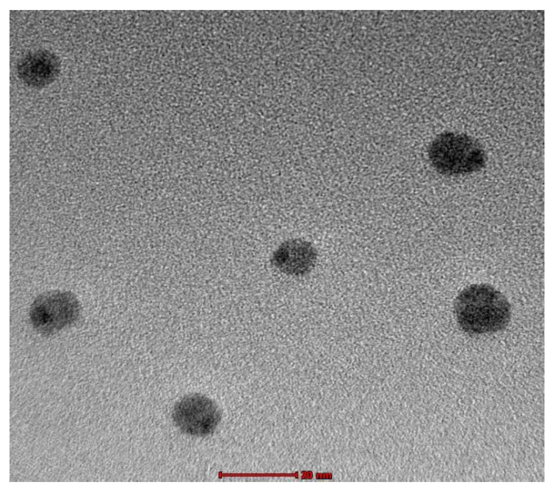


Figure 7 TEM image of AgNPs taken from product with the highest surface plasmon resonance (SPR) peak at 420 nm

3.2 Antimicrobial activity of AgNPs

The antimicrobial activity of the best synthesized AgNPs, based on the highest surface plasmon resonance (SPR) peak at 420 nm, was tested for its potency as antimicrobial agents, against two fungi, namely, Aspergillus niger and Microsporum canis and one bacteria, Staphylococcus aureus. The results are presented in Figure 8 and tabulated in Table 1. By poisoned agar assay, the percentage growth inhibitions for the two fungi were 72.22% and 74.4% for Aspergillus niger and Microsporum canis respectively.

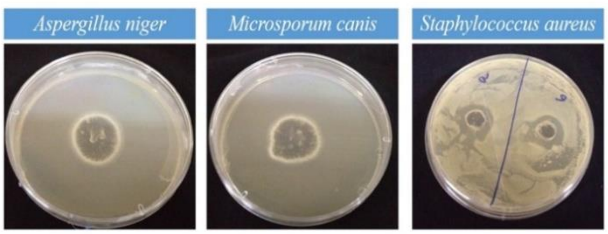


Figure 8 Antimicrobial activity of synthesised AgNPs indicated by growth/zone of inhibition of microbes.

Table 1 The values of the zone of inhibition of the sample

Name of microbes	Growth/zone of inhibition size in diameter, mm
Aspergillus niger	25 ± 1.3[a,c]
Microsporum canis	23 ± 1.5[a,c]
Staphylococcus aureus	17 ± 1.2[b,c]

[a]Plate contains media without AgNPs solution was served as control with 5 mm in diameter mycelial plug of both fungi.
[b]From solidified surface with bacterial strain of medium, 5mm in diameter hole were cut.
[c]Data is represented as mean value ± SD (Standard Deviation).

The results in Figure 8 and Table 1 supported the fact that AgNPs have promising antimicrobial activity and may appear as an alternative for conventional antibiotics. The mode of action of AgNPs against microbes is not well understood. Some study have documented that the positive charge of Ag ion is the pivotal reason of the antimicrobial activity through the electrostatic attraction between positive charged AgNPs and negative charged microorganism cell membrane [14]. Panáček *et al*. [20] has reported on AgNPs having antifungal activity against Candida spp. at around 1 mg/ml concentration. Some studies have shown that AgNPs may have kill fungal microorganisms by destructing the membrane integrity, which is similar to the mode of antifungal action of peptide as suggested by Faruck *et al*. [28], whereas other studies indicated that AgNPs may have interacted with phosphorus and sulphur containing compounds and this interaction may cause damage to DNA and proteins resulting in cell death [22]. Kim *et al*. [14] has reported that AgNPs break down the membrane permeability barrier of Candida albicans, thus disturbing the membrane lipids as well as forming pores and dissipating the electrical potential of membrane. They also confirmed that AgNPs have no effect or has weakly cytolytic effect on human erythrocytes.

Similarly, some literatures also documented on the antibacterial activity of AgNPs are due to the interaction of the nanoparticles with bacterial cell membrane, whereby the bacterial cell membrane will then loses its integrity, releasing the intracellular content, namely, nucleic acid, nucleotide and protein [19]. In short, the interaction of nanoparticles with bacteria causes the structural and morphological damage on the bacterial cell surface. Panáček *et al*. [29] mentioned in their study that the antibacterial effect of AgNPs not only result in inhibiting the bacterial growth but also in killing the bacteria. However, it is believed that the mode of action of antibacterial activity of AgNPs is more complex than mentioned above; perhaps involving more than one mechanism or a series of steps which finally leads to cell death.

4.0 CONCLUSION

The effect of five process parameters on the production of AgNPs using a chemical mediated technique has been investigated. The responses of the synthesis were monitored by scanning the absorbance of the product from 300 and 600 nm, and the quantitation of synthesis was captured by observing the total absorbance from 350 to 420 nm wavelength, which is considered as 'Yield of AgNPs'. It is assumed that the higher the total absorbance between 350 and 420 nm, the better is the synthesis. AgNPs that absorb light maximally at 420 nm are considered superior and according to TEM and EDX analysis, most of them are spherical nanoparticles, having diameters less than 15 nm. AgNPs synthesis peaked when $AgNO_3$ concentration is at 1 mM, initial pH of $AgNO_3$ at 7, TSC concentration of 1%, 3.5 minutes reaction time between $AgNO_3$ and TSC and a stirring time of 15 minutes. Antimicrobial activities test showed that the synthesized AgNPs were potent against the microbes tested. These OFAT generated results are important, and this will be later used in the design of experiments to optimize the process parameters under RSM, for maximal AgNPs production, in terms of quantity and quality.

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