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Original Article

Application of response surface methodology to optimize the extracellular fungal mediated nanosilver green synthesis

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ABSTRACT

This study aims to optimize the biosynthesis of nanosilver particles mediated by *Trichoderma viride* ATCC36838 using response surface methodology (RSM). Silver nanoparticles (AgNPs) were biosynthesized effectively in terms of the factors impacting silver ion (Ag^{+}) reduction to metallic nanosilver (Ag^{0}) using culture filtrate under shaking condition. The results of statistics calculations revealed that 2 mM silver nitrate and 28% (v/v) of culture filtrate at pH 7.0 for 34 h were the optimum values for AgNPs biosynthesis. The characterization of the produced AgNPs was conducted using electron microscopy, energy dispersive X-ray analysis, UV/visible spectrophotometry, and Fourier transform infrared spectroscopy. Round to oval AgNPs were detected with aspects of TEM within diameter range of 4–16 nm. The results of this study could help in developing a reliable ecofriendly, simple, and low cost process for microbial assisted AgNPs green synthesis especially with the continuous increase in its application fields.

1. Introduction

Due to the daily increase in nanotechnology applications fields, the scientific community gives a great attention to develop a low cost, easy and ecofriendly approach for nanoparticles production, especially the biosynthesis of stable, well defined shape and size nanoparticles [1,2]. Traditional methods for nanoparticles synthesis have many circumstances due to its environmental hazards and expensive costs [3]. The main gate for nanoparticles formation is via reduction of metal salts through the aid of proper reducing agents. Specific attention was directed toward nanosilver particles (AgNPs) due to its wide range of applications in different areas like biomaterial production, optics, catalysis, and antimicrobials [4,5]. In addition, it expose excellent improved characters as a result of its unique morphology and particles distribution [6,7].

Green synthesis of nanoparticles using microorganisms is a promising research area for developing simple, inexpensive, and ecofriendly approach. The high capacity of fungi to produce diverse quantities of enzymes able to reduce the metal salts makes it the good choice for biosynthesis of metal nanoparticles [8]. The extracellular pathway through using cell free filtrate (CFF) for AgNPs biosynthesis has many advantages over the intracellular one, as it avoid the need for harvesting, purification, and product recovery techniques which are expensive, time consuming, and cumbersome techniques [9]. As the reductase enzyme which is required for silver ion reduction, secreted into the medium in case of extracellular choice, so there is no need for these separation techniques [3,10].

Experimental optimization using classical methods through changing one factor per time and fixing the other factors has many disadvantages, where it illustrates the impact of each variable individually via a huge number of experiments, however it doesn't consider the effect of interaction between different factors under study [11]. We previously described the biosynthesis of AgNPs using a culture supernatant of *Penicillium politans* NRC510 [3] as well as many previous studies which used these classical methods for AgNPs biosynthesis [12–18].

On the other hand, usage of statistical methods solves the problems of effective variables selection among many case affecting factors. In addition, it helps in understanding the interaction between different important parameters [19,20]. Response surface methodology (RSM) is a mathematical and statistical analysis method which improves and optimizes the process settings through involving the interactions between different process parameters. RSM has been widely applied to get the optimum con-

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ditions for many different biotechnological processes through evaluation of the interaction effects between process variables [21].

In the current study, *Trichoderma viride* ATCC36838 CFF has been used to mediate the biosynthesis of AgNPs through the reduction of silver ions to metallic nanosilver. Characterization of the biosynthesized AgNPs was conducted using means of spectrophotometry, Fourier Transform Infrared spectroscopy (FTIR), Transmission Electron Microscopy (TEM), Scanning Electron Microscope (SEM), and Energy Dispersive X-ray (EDX) examination. The biosynthesis process was optimized through application of RSM - central composite design (CCD) technique in order to get the proper biosynthesis conditions and understand the interactions effect.

2. Materials and methods

2.1. Microorganism

The fungus *Trichoderma viride* ATCC36838 has been maintained at 4 °C on modified Czapek-Dox's solid medium at the culture collection of Microbial Chemistry Dept, NRC, Egypt. Then it has been re-cultivated before use in silver nanoparticles biosynthesis.

2.2. Supernatant preparation and AgNPs biosynthesis

For getting the *T. viride* ATCC36838 supernatant, the fungus was grown on malt glucose yeast peptone (MGYP) broth [18] contained (g/L): 3.0, yeast extract; 3.0, malt extract; 10.0, glucose; and 5.0, peptone, at 28 °C and 100 rpm for 72 h. After that, culture was filtered via Whatman filter paper No.1 and the filtrate was used for the extracellular formation of silver nanoparticles. Silver nitrate ($AgNO_3$) dissolved in distilled water was used as silver source for AgNPs biosynthesis. Reaction mixtures with a total volume of 50 mL contained different *T. viride* ATCC36838 cell free filtrate (CFF) (%), and silver nitrate concentrations under different pH values for different incubation times according to the central composite design (CCD) described in the next section. Reaction mixtures were incubated at 100 rpm under dark conditions to avoid the photo activation of silver nitrate. The effect of temperature on the AgNPs biosynthesis was determined through incubation of reaction mixtures at 30, 40, 50, 60, 70, 80 or 90 °C for 1 h at pH 5.0. All experiments were done in triplicates and the mean values were presented.

2.3. Experimental design and optimization by RSM

RSM using CCD was applied in order to optimize the levels of the most effective variables in AgNPs biosynthesis and to analyze their relationships. Based on the one-factor experimental results, four critical variables selected were reaction incubation time, silver nitrate concentration, CFF volume, and reaction pH value. Every parameter was studied at different rational five coded levels (−2, −1, 0, 1, 2), and the actual values for these codes was indicated in Table 1.

Depending on the previous design, the total experimental runs were calculated as: $2^k + 2k + x_0$, where k is variables number and x_0 is the repetitions number of the experiments at the center point. Thus, for this design, a total of 30 runs of experiments were performed according to CCD given in Table 2.

The following second order polynomial equation was used to calculate the relationship between different variables and the response.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y is the predicted response, X_i, X_i^2, X_j are variables in coded values; β_0 is the constant; β_i is linear effect; β_{ii} is squared effect and β_{ij} is interaction effect. The analysis of results was performed with statistical and graphical analysis software (Design Expert®, Version 7.0.0). Design Expert software was used for regression analysis of the data obtained and to estimate regression equation coefficient.

2.4. Characterization of synthesized AgNPs

The spectra (UV–Visible) of AgNPs were measured as a wavelength function using UV/Vis spectrophotometer (Cary 100 UV–Vis; Agilent Technologies, Germany) at 1.0 nm of data intervals. Elemental analysis of the biosynthesized AgNPs was studied using SEM (Quanta FEG250) operated at an accelerating voltage of 20 kV and coupled with energy dispersive X-ray analysis (EDX) for compositional analysis and the conformation of presence of elemental silver. AgNPs solution was centrifuged for 20 min at 10,000 rpm and drop coated on a carbon coated copper grid and dried. AgNPs shape and size were determined by TEM (JEOL JEM-HR-2100) operating at 160 kV, where a drop of aqueous AgNPs was loaded on a carbon coated copper grid, and allowed to dry at room temperature. For FTIR measurements, dry powder of the AgNPs was obtained according to Othman et al. [3] and then used for FTIR spectroscopy measurements on a JASCO FTIR (Japan) instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets.

2.5. Effectiveness of the biosynthesized AgNPs as antimicrobial agent

The assessment of AgNPs as antimicrobial agent was explored using agar well diffusion assay [3]. The microorganisms (*Bacillus mycoides, Escherichia coli,* and *Candida albicans*) were seeded in the plates of nutrient agar, and then different concentrations (12.5, 25, 50, and 100%) of the biosynthesized AgNPs solution were added to the agar wells (15 mm) which were made previously using sterile cork borer. The inoculated plates were incubated for 3 h at 37 °C after that; the inhibition zone diameter was measured.

3. Results and discussion

3.1. Explanation of regression analysis

As a result of multiple regression analysis validation for the investigational records, the results obtained from the central composite design were integrated with a full polynomial equation in a second-order. The experiential correlation between the absorbance

Table 1
Coded and actual values of the experimental variables.

Parameter	Symbol	−2	−1	0	1	2
Reaction time (h)	A	4.00	14.00	24.00	34.00	44.00
Silver nitrate conc. (mM)	B	0.50	1.00	1.50	2.00	2.50
Cell free filtrate (%)	C	12.50	28.13	43.75	59.38	75.00
Reaction pH value	D	4.00	5.00	6.00	7.00	8.00

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