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BIOSYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES USING *ENTEROBACTER AEROGENES*: A KINETIC APPROACH

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The focus of the study has been made for synthesis, characterization and kinetic parameters estimation of silver nanoparticles (AgNPs) from *Enterobacter aerogenes*. The resulting nanoparticles were examined using UV-visible spectroscopy, Scanning Electron Microscopy(SEM),Transmission Electron Microscopy(TEM), and Energy Dispersive X-ray Spectroscopy(EDX).The absorption spectra showed a progressive surface plasmon resonance (SPR) in the wavelength of 410-420nm corresponding to the silver SPR. The size of the nanoparticles produced was approximately in the range of 25-35 nm. The temperature and pH optimum of the reaction was found to be 80 °C and 7.0 respectively.The reaction parameters were grouped and analyzed to understand the kinetics of the nanoparticle synthesis. The kinetic parameters such as K_m and V_{max} were calculated and the reaction rate kinetics for the production of nanoparticles were found. The value of V_{max} was found to be 3.46 mmol/min and K_m was 8.65 mmol.

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1. Introduction

Nanobiotechnology, a new field of research gives rise to the emerging future of science for the past decade. It covers a large number of technological fields like biotechnology, nanotechnology, chemical processing, physical methodology and system engineering [1]. Synthesis, processing and utilization of nanoparticles are the basic aspect of nanotechnology. Recently nanoparticles have been the subject of focus for research due to their unique applications. AgNPs synthesized by various routes find many application in the fields such as electronics which includes nanoelectronics and optics, coatings for solar energy absorption and electronics applications, intercalation material for electrical batteries, as optical receptors, and biological application such as biosensors, biolabelling and new carriers for targeted drug delivery [2, 3, 4]. Therefore, producing nanoparticles in an economical way is essential for the progressive advancement in nanotechnology. Although chemical and electrochemical methods are being applied for the synthesis of nanoparticles, the cost involved in such method is prodigious. As a result the prospect of producing metallic nanoparticles by a cheaper way and eco-friendly technology has turned its importance to biological systems [5, 6]. A more feasible and better way for nanoparticle synthesis is by biological process. At present biosynthesis of metallic nanoparticles is gaining popularity among the research community [7, 8]. Among the different types of nanomaterials such as copper, zinc, titanium, magnesium, gold and silver, AgNPs have proved to be the more effective antimicrobial agent against bacteria, viruses and other eukaryotic micro-organisms [9, 10].

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The antibacterial activity of AgNPs is being exploited and deeply searched for future use. The determination of kinetic parameter is necessary in order to design and scale up for industrial production. AgNPs can be produced by enzyme reduction of silver nitrate ($AgNO_3$) [11]. The molecular property of SPR exhibited by AgNPs can be tailored depending upon the shape and size of the nanoparticles synthesized [12]. This study reports *Enterobacter aerogenes* as a model organism to produce AgNPs and tries to estimate the kinetics of the production reaction using simple analytical tools such as UV-visible spectrophotometer, SEM-EDX, TEM. With changes in the kinetic parameters, the kinetics of the formation of the AgNPs was monitored.

2. Materials and methods

2.1 Materials

The culture *Enterobacter aerogenes* (MTCC 111) was obtained from Microbial Type Culture Center (MTCC), Chandigarh, India. All chemicals used in the analysis are of analytical grade and were procured from Sigma Aldrich & Hi Pure chemicals, India.

2.2 Culturing of *Enterobacter aerogenes*

The culture biomass used for biosynthetic experiments were grown aerobically in liquid medium containing (g/l) beef extract, 1.0; yeast extract,2.0; peptone, 5.0; and NaCl, 5.0. Erlenmeyer flasks were inoculated with the culture inoculum and incubated at 30 ºC with shaking (150 rpm) for 48 h. After the incubation, the biomass was centrifuged using a refrigerated centrifuge (Eltek RC4100D) at 10,000 rpm for 15 min at 4 °C. After centrifugation the tubes were removed and the supernatant was collected in a plastic container and was stored at -20 °C to preserve all the ingredients intact, until further use.

2.3 Preparation of $AgNO_3$ solution

To 100 ml of de-ionized water, $AgNO_3$ of weight equivalent was added to bring the concentration to 1millimol. It was seen that the solution was a clear liquid and shows mild lustrous appearance.

2.4 Preparation of Silver nanoparticles (AgNPs)

To 50ml of 1mM $AgNO_3$ solution 10 % (v/v) of *Enterobacter aerogenes* culture supernatant sample was added slowly and a colour change was noticed (colouress to yellow-brownish) which indicates the formation of AgNPs [2, 11]. The colour is due to the phenomenon called SPR.

2.5 Monitoring the reaction

The reaction was constantly monitored by recording the SPR of AgNPs produced for every 30 min and by performing a Nitrate reductase assay (NED assay) at regular time intervals to ascertain the reaction [11].

2.6 Measuring the SPR

The SPR of AgNPs was measured using a UV-vis double beam spectrophotometer (Systronics 2201), in the wavelength range of 300 – 600 nm. The SPR for AgNPs was observed at a wavelength range of 400 -430 nm.

2.7 Characterization of AgNPs

The surface structure was visualized by SEM (Hitachi S-3400W) at an accelerating voltage of 10kV and TEM (Philips TECNAI 10). Elemental analysis measurement was done using EDX (Thermo EDX) at an accelerating voltage of 10kV.

2.8 Buffer System and their pH range

Buffer solutions of pH range from 3.0 to 11.0 were prepared accordingly [13]. The use of HCl and NaOH was avoided since the AgNPs may react with HCl and NaOH instantaneously. The

$AgNO_3$ prepared in all these pH's were taken in aliquots of 10 ml, to it 10% (v/v) of broth was added to each aliquot. After incubating it for one hour at room temperature, the solutions were analyzed for Nitrite.

2.9 Kinetic study

The kinetic parameters involved in the formation of AgNPs were monitored at regular time intervals by estimating the amount of nitrite formed using NED assay. This assay is based on the stoichiometry of the reaction that for every ion of Ag^+ reduced there was formation of one nitrite molecule. Therefore, the amount of nitrite formation from time to time was monitored for more than 5 hours and the amount of nitrite produced was then estimated. [14]

2.10 Effect of initial Substrate concentration

For finding out the effect of initial concentration of the substrate, experiments were carried out using different concentration of $AgNO_3$ (10, 20, 30, 40 and 50mM). NED assay was performed accordingly for all the collected samples.

3. Results and discussion

3.1 Extracellular production of silver nanoparticles

The $AgNO_3$ solution was reduced to AgNPs due to the addition of the supernatant containing extracellular material produced by *Enterobacter aerogenes*. The change of color from colorless to yellow-brownish (Fig. 1) clearly indicates the formation of AgNPs in the reaction mixture [15, 16]. The characteristics color of the colloidal silver solution is due to the excitation of surface plasmon vibrations in the nanoparticle and provides a convenient spectroscopic signature of their formation [17, 18, 19]. The principle behind this process is the activity of a most abundantly found enzyme nitrate reductase which reduces nitrate to nitrite and there by reducing Ag^+ ions to AgNPs. Several hydroquinones with excellent redox properties were reported that could act as electron shuttle in metal reductions. Thus, it was evident that electron shuttle or other reducing agents released by *Enterobacter aerogenes* are capable of reducing silver ions to AgNPs [2].

Fig. 1. Color change of silver nitrate 1mM solution from colorless to yellow-brownish as the reaction proceeds on addition of the culture supernatant Enterobacter aerogenes.

3.2 SPR of Silver

The AgNPs were characterized using UV–visible spectroscopy (Fig. 2), which shows the UV-vis double beam spectrometer results. A strong, broad peak located between 410 and 420 nm was observed for the AgNPs prepared using the *Enterobacter aerogenes*. Observation of this peak assigned to a surface plasmon is a strong evident for the formation of silver metal nanoparticles [20, 21]. The strong resonance centered at about 420 nm is clearly observed and increases with

increase in time which denotes the high concentration of the AgNPs. The sharper and more symmetrical peaks in Fig. 2 reflect the uniformity in particle size distribution.

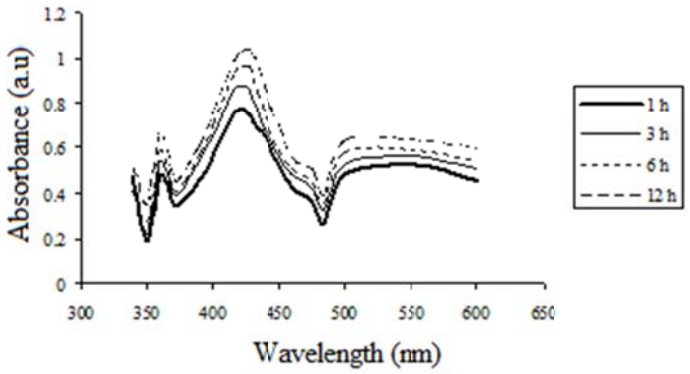

Fig.2. UV–visible spectra recorded from the aqueous silver nitrate-culture supernatant of the Enterobacter aerogenes reaction medium as a function of time of the reaction.

3.3 Particle Size

Fig. 3 and Fig. 4 shows the SEM and TEM micrograph recorded from AgNPs respectively. In this micrograph, AgNPs in the range of size 25-35nm were observed. The morphology of the nanoparticles was found to be spherical, monodispersed and uniformly distributed [6]. The particles are also not aggregated, which might be an indication to the presence of a caping agent[11]. As discussed earlier, the AgNPs solution synthesized by the reaction of Ag^+ ions with *Enterobacter aerogenes* culture supernatant is exceptionally stable and the stability is likely to be due to capping agents secreted by the bacteria. The separation between the AgNPs seen in the SEM image could be due to capping by proteins and would explain the UV-vis spectroscopy measurements, which is characteristic of well dispersed AgNPs.

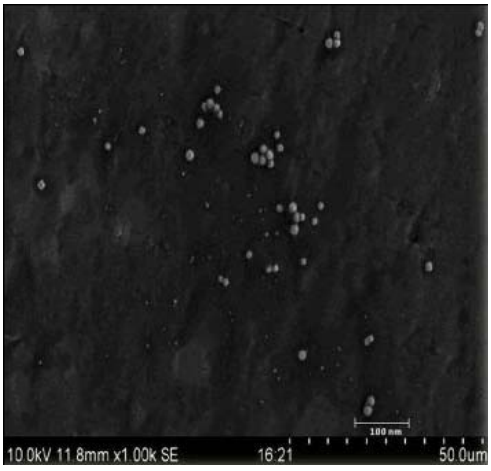

Fig.3. SEM micrograph of the silver nanoparticles(scale bars correspond to 50 µm).

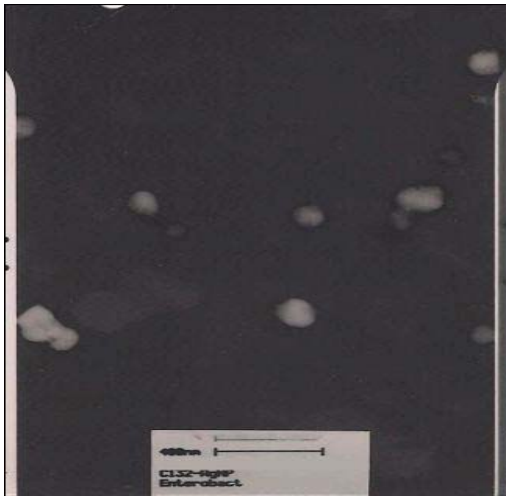

Fig.4.TEM micrograph of the silver nanoparticles (scale bars correspond to 400 nm).

3.4 Elemental analysis

In the analysis of the silver nanoparticles by energy dispersive spectroscopy (EDX), the presence of elemental silver signal was confirmed (Fig. 5). The Ag nanocrystallites display an optical absorption band peaking at ~3 keV, which is typical of the absorption of metallic Ag nanocrystallites due to the SPR [16].The other peaks like Na and Si are quoted for the media components in supernatant. The presence of peak for Al is due to the aluminium foil base on which the particles are coated. The presence of carbon indicates the presence of stabilizers . [4]

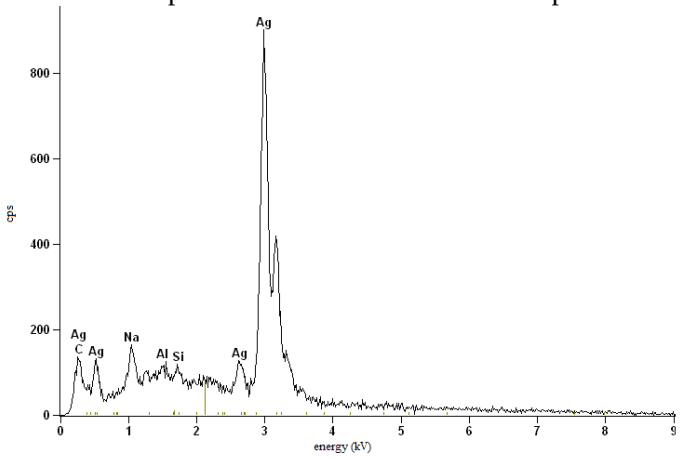

Fig.5. EDX spectrum of silver nanoparticles.

3.5 Effect of temperature

The effect of temperature on the production of the AgNPs was studied by placing aliquots of reaction mixture in different test tubes and placing them in various temperatures ranging from 0 °C to 110 ºC. After 15 min the amount of nitrite reduced was quantified using NED assay. Then the nitrite reduced at a time duration of after one hour at the respective temperature were also quantified, this is to find out the effect of denaturation of the protein at these temperatures and to find the temperature of maximum activity (Fig. 6). The effect of temperature was measured and the maximum activity found to be around 80 °C for 15 min, which coincides with the optima pH of *P. aerophilum* nitrate reductase. [22]

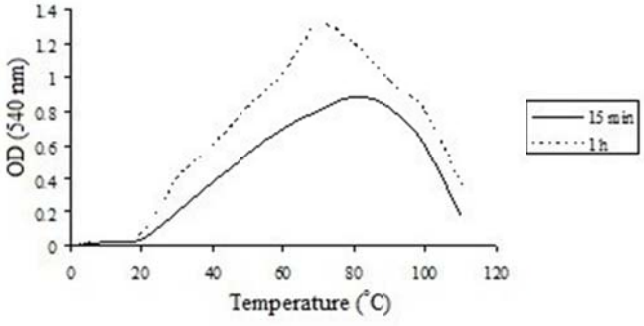

Fig.6. UV–vis spectra of $AgNO_3$ recorded at different temperatures.

3.6 Effect of pH

The effect of pH was characterized by preparing $AgNO_3$ in the respective buffer systems each of 1mM concentration. The maximum activity was observed at around pH 7.0 (Fig. 7). The reaction takes place actively around the range of 5.0-9.0 and the activity is more in basic pH than in acidic pH. [23,24]

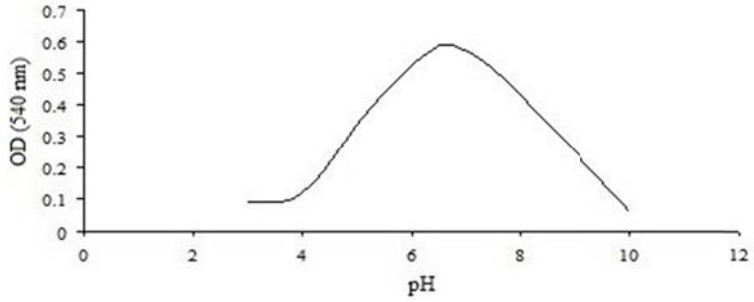

Fig. 7. UV–vis spectra of $AgNO_3$ recorded for various pH at temperature of 80 °C.

3.7 Rate of the reaction

The initial concentration of $AgNO_3$ was plotted against the rate of the reaction observed after 30 min of time interval. It is very well seen that the saturation is achieved at around 10 mM concentration of $AgNO_3$ and this is attributed to the formation of [ES] complex or other wise the number of active sites are completely blocked by the nitrate ion at this concentration. Thus the product formation rate depends on the enzyme concentration as well as on the substrate concentration, which resembles a bimolecular reaction.

The lineweaver – Burk plot was used to determine the enzyme kinetics and it was found that the michelis-menton constant (K_m) value to be 8.65 mmol and maximal velocity(V_{max}) value to be 3.46 mmol/min (Fig. 8). V_{max} represents the initial velocity at saturating levels of all substrates as determined by extrapolation of the reciprocal plots, while K_m gives the substrate binding capacity in the reaction. A high K_m value relative to the physiological concentration of the substrate, is not normally saturated with substrate, and its activity will vary as the concentration of the substrate varies, so that the rate of formation of product will depend on the availability of substrate.

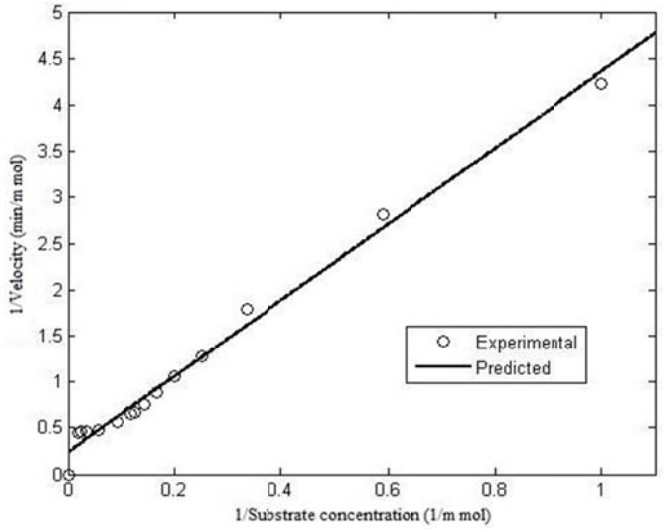

Fig. 8.Lineweaver-Burk plot of kinetic data, showing the significance of the axis intercepts and gradient.

4. Conclusions

The prospect of producing silver nanoparticles using *Enterobacter aerogenes* was one of the most sought. The production of AgNPs with the *Enterobacter aerogenes* was found to be much faster and relaible.There was a progressive SPR observed in the UV-vis spectra of AgNPs at 410-420nm range, which corresponds to AgNPs. The particle size of the AgNPs was found to be 25-35 nm of average size which was measured by SEM/TEM. The EDX data suggests that the particle is actually silver and the presence of some C atoms suggests that some of the media components acts as a capping agent for stabilizing the nanoparticles.

The optimum parameters such as pH and temperature were reported and the value was found to be 7.0 and 80 °C. Further more the kinetic data clearly shows that the reaction proceeds in accordance to Michelies Menten model and is a enzyme mediated process. The V_{max} and K_m from the kinetic plot show that the saturation concentration is as high as 10mM, which suggest that the protein may have multiple active sites.

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