A study to evaluate the effect of phyto silver nano-particles synthesized using *Azadiracta indica* leaf extract on extracellular fungal amylase and cellulase

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**ABSTRACT**

In the present study, an attempt has been made to evaluate the effect of plant mediated silver nano-particles against two enzymes i.e. Amylase and Cellulase produced by *Aspergillus niger* through submerged fermentation under optimum condition and the crude enzyme thus obtained was incubated with the respective substrate in presence of phyto silver nano-particles. The enzyme activity was determined with different concentration of nano-particles. Silver nano-particles were fabricated using leaf extract of *Azadiracta indica*. The bio-reduction was monitored by UV-Vis spectrophotometer, the possible biomolecules responsible for bioreduction, capping and efficient stabilization were identified using Fourier transform infrared spectroscopy, while the size and shape of the synthesized silver nano-particles was determined using scanning electron microscopy (SEM). The synthesized silver nano-particles were found to be potentially active in enhancing the activity of fungal amylases and cellulase. The study thereby concludes utilization of plant material for a rapid, simple, cost-effective and ecofriendly alternative for nano-particles synthesis and these silver nano-particles may have potential application in processes that requires amylolytic and cellulolytic activity at commercial or industrial levels. The “green nanotechnology” thus aims to exploit plant materials to generate products and processes that are energy efficient as well as economically and environmentally sustainable.

**KEYWORDS**

Silver Nano-Particles, Azadiracta indica, Aspergillus niger, Extracellular Enzymes

**HOW TO CITE THIS ARTICLE**


Nanotechnology is one of the most active areas of research that is developing day by day making an impact in all spheres of human life (Singh and Singh, 2014). Nanotechnology deals with the synthesis and stabilization of matters at the nanoscale level ranging from 1 to 100 nm (Song and Kim, 2009) that posses wondrous optical, electronic, magnetic and catalytic properties than their respective bulk material owing to their high surface area to volume ratio (Poulse et al., 2014). Due to all these unique properties nano-structured metals are becoming important in applications as catalysts, sensors, electronics, biotechnology and biomedicine (Bankar et al., 2010). Among all the metal nanoparticles, silver nano-particles have received attention due to their physical, chemical and biological properties due to their catalytic activity and bactericidal effects and hence, finding applications in nano-biotechnology research (Sharma et al., 2009). They are also used as antimicrobial agents in wound dressing (Habibollah et al., 2014) as topical creams to prevent wound infection (Tian et al., 2007) and as anticancer agents (Orlowski et al., 2013). Since silver nano-particles finds a wide applications, currently, scientists focus on the synthesis of nano-particles using various biological agents including bacteria (He et al., 2007), fungus (Verma et al., 2010), enzymes (Willner et al., 2007) and plants (Krishnaraj et al., 2010). These biogenic processes are of low cost and high yield, safe and eco-friendly in comparison with the physical and chemical synthetic procedure (Song et al., 2009). Among all the biological agents, plants provide a better platform for nano-particles synthesis as they are free from toxic chemicals and provide natural capping agents. As well as, use of plant extracts reduces the cost of microorganism isolation and culture media and hence the nano-particles synthesized by plant extract are cost effective as compared to the nano-particles synthesized by microorganisms (Singhal et al., 2011). Enzymes are biological catalysis and the interaction of enzymes with ligands offered stimulating opportunities for a wide variety of applications in the field of...
biotechnology and medicine. Interaction of enzyme and silver nano-particles leads to structural changes of protein molecules and its surface modifications (Iseult and Dawson, 2008). In the presence of silver nano-particles the rate of hydrolysis increases, ensuring that enzyme has successfully immobilized on the surface of nano-particles. This entails an increase in catalytic activity due to stabilization of enzyme on the silver nano-particles thereby interaction of silver nano-particles with amylase facilitated faster hydrolysis of starch, for its efficient use in food industries (Falkowska et al., 2014). Amylase is an enzyme that catalyzes the breakdown of starch into sugar and plays a pivotal role in a variety of areas such as digestives, for production of ethanol and high fructose corn syrup, detergents, desiring of textiles, modified starches, hydrolysis of oil field drilling fluids and paper recycling (Sundarram and Murthy, 2014). The increased rate of reaction with amylase by biosynthesized silver nano-particles has been shown when the degradation of starch digestion kinetics in the presence of silver nano-particles rapidly produced larger amounts of reducing sugar (Rangnekar et al., 2007). Another fungal enzyme cellulase, is a hydrolytic enzyme that is capable of degradation of cellulose to smaller molecules and hence posses potential applications in various industrial areas including pulp and paper, textile, laundry, biofuel production, food and feed, brewing and agriculture (Gupta et al., 2015).

Silver nano-particles and cellulose after interaction with cellulase were capable of breaking down the cellulose complex with the attachment of the enzyme over its surface thereby being immobilized and degrading cellulose even much faster as compare to free cellulose. As the collision frequency between the free enzyme and the substrate molecule and their steric orientation forms the basis of the enzyme activity, the constraint was overcome by the support of solid nano-particles while this was not the case with free cellulose (Rangnekar et al., 2007) and therefore the reaction rate of hydrolysis of cellulose to smaller molecules like monosaccharides and disaccharides was high in case of silver nano-particles (Deka et al., 2008). Thus, keeping above point in mind current study was conducted to evaluate effect of silver nanoparticles synthesized from Azadirachta indica leaf extract on fungal amylase and cellulase.

**MATERIALS AND METHODS**

**Biological Material**

The fungal culture (*Aspergillus niger*) was procured from culture collection of Department of Biotechnology and Microbiology, M.I.E.T. Meerut. Leaves of *Azadirachta indica* were collected, washed, dried and powdered for preparation of aqueous extract.

**Preparation of the Aqueous Plant Extract**

Powdered leaves (200 mg) was dispensed in 100 ml of sterile distilled water and boiled for one hour at 80°C. Then the leaves extract was collected in separate conical flasks by standard filtration method.

**Plant Mediated Synthesis of Silver Nano-Particles**

Silver nitrate ($10^{-3}$ M) and aqueous plant leaves extract were mixed in a ratio 95:5 ml. The time of addition of leaves extract into the aqueous silver nitrate solution was considered as the start of the reaction. Then the solution was kept at 80°C at shaking condition for 24 hrs and colour change was observed. This reaction mixture was used for further study. The reaction mixture was centrifuged using Centrifuge (C-24BL) at 10,000 rpm for 15 minutes in order to obtain the pellet which was used for further study.

**Detection and characterization of phyto-silver nanoparticles**

**Visual Observation**

The reaction mixture containing 95 ml of 0.001 M silver nitrate and 5 ml of aqueous plant leaves extract was examined after every 1 hour upto 24 hours and the change in colour was observed with respect to time for the detection of silver nanoparticles.

**UV-Visible Spectroscopy Analysis**

For the UV-visible spectrum analysis the aliquots of reaction mixture were subjected to the measurement of the absorbance by UV-Visible
Spectrophotometer (239/Gen/01) at the wavelength 300 to 550 nm for the detection of silver nanoparticles and the baseline was always set with a relevant blank.

Fourier Transform Infrared Spectroscopy Analysis

Fourier transform infrared measurement was recorded by FT-IR instrument (Shimadzu FTIR-840S) in a range of 400 to 4000 cm⁻¹. For FT-IR analysis of silver nanoparticles, the solution of silver nano-particles was converted to powdered form by lyophilizer (Macflow FD3C) to determine the variation of the functional groups present in the synthesized silver nano-particles.

Scanning Electron Microscopy Analysis

Scanning electron microscopy was done with Scanning Electron Microscope (LEO 435 VP). SEM analysis was employed to characterize the size, shape, surface morphology and distribution of synthesized silver nanoparticles.

Enzyme Production

Inoculum Preparation

The spores obtained from 5 days old Sabroud's agar slants culture of Aspergillus niger were scrapped off from the slant surface by adding sterile distilled water in it. The spores were dispensed in autoclaved distilled water and optical density of the inoculums was set upto 0.12-0.15 at 530 nm that correspond 10⁶ CFU/m.

Crude Enzyme Preparation

The prepared production media (200 ml) for amylase and cellulase was transferred to two separate 250 ml of conical flasks which were plugged and sterilized in an autoclave then cooled to room temperature. 4 ml of inoculum was transferred to each flask. The flasks were placed in shaking condition for 8 days at 30°C temperature. The extract of each flask were then filtered through whatmann filter paper No. 1 and the filtrate was used as crude enzyme extract for further study and store at 4°C temperature until further used.

Enzymatic Assay

To study the effect of silver nanoparticles on enzymes, the enzymatic activity of fungal amylase and fungal cellulase was checked in presence and absence of silver nanoparticles (Rangnekar et al., 2007; Falkowska et al., 2014) One unit of enzyme activity was defined as the amount of enzyme causing the release of 1 mg of reducing sugar in 1 minute under the assay condition.

Fungal Amylase

To study activity of fungal amylase a reaction mixture was prepared comprising of 0.1 ml of crude enzyme, 0.5 ml of 0.5% w/v soluble starch solution in 0.2 M of phosphate buffer solution (pH 7.0) and in this reaction mixture 100 μl of phyto silver nano-particles were added and then the incubation was done at 28°C for 30 minutes followed by the termination of reaction mixture by adding 2 ml of dinitro salicylic acid in the reaction tube. Then the reaction tubes were immersed in water bath at 100°C for 10 minutes and finally absorbance was measured at 540 nm. A positive control containing enzyme and substrate (in absence of silver nano-particles) and a negative control i.e. enzyme blank was also made.

Fungal Cellulose

Fungal cellulase activity was studied using a reaction mixture comprises 0.1 ml of crude enzyme, 0.5 ml of 0.5% w/v soluble starch solution in 0.2 M of Phosphate buffer solution (pH 7.0). In this reaction mixture 100 μl of phyto silver nano-particles were added. The reaction mixture incubated at 28°C for 30 minutes and the reaction was then terminated by adding 2 ml of dinitro salicylic acid in the reaction tube. Then the reaction tubes were immersed in water bath at 100°C for 10 minutes. The absorbance was then measured at 540 nm. A positive control containing enzyme and substrate (in absence of silver nano-particles) and a negative control i.e. enzyme blank was also made.

Effect of Varying Amount of Silver Nano-Particles on Fungal Amylase Activity

To study the effect of varying amount of silver nano-particles on fungal amylase, different
amount of phyto silver nano-particles \((i.e. 50 \mu l, 100 \mu l, 150 \mu l, 200 \mu l, 250 \mu l \text{ and } 300 \mu l)\) were added to the reaction mixture containing 0.1 ml of crude enzyme, 0.5 ml of 0.5% w/v soluble starch solution in 0.2 M of Phosphate buffer solution (pH 7.0). In this reaction mixture 100 \mu l of phyto silver nano-particles were added. The reaction mixtures were then incubated at 28°C for 30 minutes and the reaction was terminated by adding 2 ml of dinitro salicylic acid in the reaction tube. Then the reaction tubes were immersed in water bath at 100°C for 10 minutes and then the absorbance was measured at 540 nm. A positive control containing enzyme and substrate (in absence of silver nano-particles) and a negative control \(i.e.\) enzyme blank was also made. One unit of amylase activity was defined as the amount of enzyme causing the release of 1 mg of reducing sugar in 1 minute under the assay condition.

RESULTS AND DISCUSSION

Plant Mediated Synthesis of Silver Nano-Particles

On treatment of plant leaves extract of \textit{Azadirachta indica} with 0.001M silver nitrate and incubation in dark at room temperature, the colour changes from transparent to pale yellow within one hour and were further enhanced to dark brown within two hours and became stable after three hours indicating the synthesis of silver nano-particles. It is an efficient and rapid synthesis which corroborates with the result obtained by other researchers who had worked with different plant systems (Krishnaraj \textit{et al.}, 2007; Sathyavathiet \textit{et al.}, 2010; Bonde \textit{et al.}, 2011). Colour change was due to excitation of surface plasmon vibrations in the synthesisedphyto silver nano-particles.

Detection and Characterization of Phyto Silver Nano-Particles

Visual Observation

The change in colour of 0.001M silver nitrate solution after the addition of aqueous plant leaves extract of \textit{Azadirachta indica} from transparent to pale yellow and then to dark brown was observed and with time the intensity of the colour was enhanced and became stable hours which concludes that silver nitrate solution was reduced by the organic moiety present in the plant extract to silver ions and silver nano-particles was formed (Fig1).

UV-Visible Spectroscopy Analysis

UV-Visible absorption spectra had proved to be quite sensitive for the detection of silver nano-particles and the absorption band in visible light region of 300-550 nm is typical for silver nanoparticles. The present study shows the absorbance peak due to the excitation of surface plasmon vibrations at 427 for the synthesized silver nanoparticles that confirmed the synthesis of silver nanoparticles using plant leaves extract of \textit{Azadirachta indica} (Figure 2).

Fourier Transform Infrared Spectroscopy Analysis

FT-IR is a unique quantitative analysis technique that identifies the types of chemical bonds in a molecule by producing an infrared absorption spectrum. It is used for the characterization of the synthesized silver nano-particles to study the presence of the different functional group, their characteristic absorption and the type of vibrations shown by these functional groups to identify the possible biomolecules responsible for reducing, capping and efficient stabilization of the synthesisedphyto silver nano-particles. The FT-IR analysis of the synthesized silver nano-particles using \textit{Azadirachta indica} shows the different functional group, their characteristic absorption in the range of 400-4000 cm\(^{-1}\) and the type of vibrations shown by these functional groups present in the prepared complex (Figure 3, Table 1). FT-IR analysis revealed that the protein molecules not only act as reducing agent but also can act as stabilizing agent by binding to silver nano-particles through hydroxyl groups or carboxyl groups of plant leaves extract that were mainly involved in the fabrication of silver nano-particles and the chemical constituents present in plant leaves extract such as flavonoids, alkaloids or fatty acids were responsible for the reduction of silver ions to silver nanoparticles due to their capping and reducing capacity.
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Fig. 1. Synthesis of silver nano-particles by *Azadirachta indica*

![Graph showing the synthesis process over time](image)

Fig. 2. UV-Visible spectra of silver nano-particles synthesized by plant leaf extract of *Azadirachta indica*

![Graph showing UV-Visible spectra](image)

Fig. 3. FT-IR analysis of silver nano-particles synthesized by *Azadirachta indica*

![Image of FT-IR analysis](image)

Fig. 4. Scanning electron micrograph of silver nano-particles synthesized by plant leaves extract of *Azadirachta indica*

![Image of scanning electron micrograph](image)

Note: SNP- Silver nano-particles synthesized by *Azadirachta indica*

Fig. 5. Effect of silver nano-particles on fungal amylase activity

![Graph showing the effect on fungal amylase activity](image)
Fig. 6. Effect of different amount of silver nano-particles on fungal amylase activity

Table 1. FT-IR analysis of silver nano-particles synthesized by *Azadirachta indica*

<table>
<thead>
<tr>
<th>S. N.</th>
<th>Characteristics</th>
<th>Absorption (cm⁻¹)</th>
<th>Functional Group</th>
<th>Type of Vibrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 2 0 0</td>
<td>– 3 6 0 0</td>
<td>O – H</td>
<td>Stretch</td>
</tr>
<tr>
<td>2</td>
<td>1 6 0 0</td>
<td>– 1 6 8 0</td>
<td>C = C</td>
<td>Stretch</td>
</tr>
<tr>
<td>3</td>
<td>1 3 8 3</td>
<td>– 8 3</td>
<td>C – H</td>
<td>Bending</td>
</tr>
<tr>
<td>4</td>
<td>1 0 4 3</td>
<td>– 4 2</td>
<td>C – O</td>
<td>Stretch</td>
</tr>
<tr>
<td>5</td>
<td>8 2 4</td>
<td>– 5 1</td>
<td>= C – H</td>
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<tr>
<td>6</td>
<td>7 8 0</td>
<td>– 1 5</td>
<td>= C – H</td>
<td>Bending</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy Analysis

Scanning electron microscopy had proved to be a quite sensitive and powerful technique for the characterization of silver nano-particles. SEM analysis the shape, size and distribution of the sample particles and form their three dimensional image. Scanning electron micrographs reveals that the synthesized phyto silver nano-particles were spherical and monodispersed in nature having a size range of 6-8 nm (Figure 4). The resulting silver nano-particle solutions were found to be stable for more than two months without agglomeration of particles.

Enzymatic Assay

Fungal Amylase

The effect on enzyme activity of fungal amylase was measured in presence of synthesized phyto silver nano-particles during the hydrolysis of starch into reducing sugar by dinitosalicylic method. The released sugar by fungal amylase was quantified and the enzyme activity was calculated (in mg/µl/minute). The fungal amylase activity was measured in the presence of 100 µl of silver nanoparticles by dinitosalicylic acid method. The enzyme activity of fungal amylase was increased by the synthesized silver nano-particles (Figure 5).

Fungal Cellulose

The effect on enzyme activity of fungal cellulase was measured in presence of synthesized phyto silver nano-particles during the hydrolysis of starch into reducing sugar by dinitosalicylic method. The released sugar by fungal cellulase was quantified and the enzyme activity was calculated (in mg/µl/minute). The fungal cellulase activity was measured in the presence of 100 µl of silver nanoparticles by dinitosalicylic acid method. The enzyme activity of fungal cellulase was increased by the synthesized silver nanoparticles (Figure 5).

Effect of Varying Concentration of Silver Nano-Particles on Fungal Amylase Activity

The effect on enzyme activity of fungal amylase was measured in presence of increasing amount of synthesized phyto silver nano-particles on enzyme activity of fungal amylase during the
hydrolysis of starch into reducing sugar by dinitrosalicyclic method. The released sugar by fungal amylase was quantified and the enzyme activity was calculated (in mg/µl/minute). Enzyme activity of fungal amylase was increased at all the increasing amount of 50 µl, 100 µl, 150 µl, 200 µl, 250 µl and 300 µl of silver nano-particles and the highest enzyme activity enhancement was observed at 300 µl while the least enzyme activity enhancement was observed at 50 µl of synthesized silver nano-particles (Figure 6).

**CONCLUSION**

Our results conclude that the plant under investigation is able to synthesize silver nano-particles in a simple, rapid, cost effective and eco-friendly manner. Synthesized phyto silver nano-particles could be used at commercial scale for enhancing the activity of industrially important enzymes like amylase and cellulase. Therefore the present study concludes that the nano-particle may have a significant effect in the field of nanocatalysis, promising their potential use in industries for rapid degradation of the complex molecules to simpler ones by immobilizing the enzymes onto the surface of nano-particles.

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**REFERENCES**


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