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Nanocatalytic Application of the Green Synthesized Silver Nanoparticles for Enhancement of the Enzymatic Activity of Fungal Amylase and Cellulase

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Abstract

The present study aims to evaluate the effect of silver nanoparticles (AgNPs) on the enzymatic activity of fungal amylase and cellulase. The AgNPs were synthesized using aqueous fresh leaf extract of Camellia sinensis of AgNPs. The synthesis of nanoparticles was initially observed by a visible colour change and further confirmed by UV-Vis spectrum analysis. Fourier transform infrared spectroscopy (FTIR) identified the functional groups and their relevant biomolecules such as amide, alkene, carbonyl, and hydroxyl groups present in the aqueous leaf extract of C. sinensis. These biomolecules were responsible for the synthesis, capping, and stabilization of the AgNPs. The field emission scanning electron microscope (FESEM) image showed spherical and polydispersed AgNPs with a diameter of 22-55 ±2 nm. The energy dispersive X-ray (EDX) analysis illustrates 91.19% silver in the synthesized AgNPs. The effect of synthesized AgNPs on the enzymatic activity of fungal amylase and cellulase was evaluated using the 3,5-dinitrosalicylic acid (DNSA) method. The enzymatic activity of fungal amylase and cellulase increased significantly with increased concentration of AgNPs. The enhancement in the amylase and cellulase activity achieved through nanoparticles may be further explored for its industrial applications.

Keywords: *Green synthesis, Camellia sinensis, silver nanoparticles, nanocatalyst First, Second, Third.*

1. INRODUCTION

Green nanotechnology is an emerging field within smart materials science, that has modified the center of attention from physical and chemical processes to the 'green' synthesis of nanoparticles [1]. Green synthesis is an eco-friendly, eco-sustainable, non-toxic, and cost-effective method for large-scale synthesis of nanoparticles [2, 3]. This new alternative process counteracts the limitations of physicochemical processes, such as the high energy cost of the process and the use of harmful radiation or toxic chemical reagents that can generate hazardous by-products [4]. Green synthesis uses the metabolic capabilities of living beings to synthesize nanoparticles, which have proven to be more biocompatible than their counterparts obtained by traditional methods [5]. Green synthesis of nanoparticles due to their unique physio-chemical properties possesses various applications in the field of catalysis, antimicrobial agents, fertilizers, pesticides, diagnostics, sensing devices, and drug delivery [6, 7]. Among the wide range of

nanomaterials, metallic nanoparticle may be synthesized using biological methods ranging from plants to microorganisms [8, 9]. The green synthesis relies on plant extract. However, the biological synthesis depends on the microorganisms for nanoparticles formation. Green synthesis involving plant extract is significantly advantageous over microbial mediated synthesis [10]. Plants are free from toxic chemicals and provide a single-step economical, rapid, non-pathogenic, and non-toxic process for the synthesis of metal nanoparticles unlike, microbe-mediated synthesis which requires the maintenance of an elaborative process of maintaining cell cultures and highly aseptic conditions, hence, limit its feasibility for industrial applications [11, 12]. Utilizing plant extracts for nanoparticle synthesis reduces the costs associated with microorganism isolation and culture media, thereby enhancing the competitiveness of this approach over microbial mediated methods [13]. Plant extract contains various antioxidants and secondary metabolites such as terpenoids, flavones, ketones, aldehydes, amides, carboxylic acids, etc. which are responsible for the synthesis, capping, and stabilization of metal nanoparticles [14, 15]. Although among the microorganisms, fungi are explored as potential nano-factories for the mycosynthesis of nanoparticles [16]. Fungi possess extracellular biomass for the reduction of silver ions to AgNPs [17]. Mycosynthesized AgNPs possess wide applications in areas such as catalysts, antimicrobials, optics, biomaterial production, etc. [18]. *Camellia sinensis* commonly known as tea, boasts an array of phenolic compounds viz., flavonoids including catechins, catechin gallates, and proanthocyanidins. Its composition comprises caffeine (3.5%), theophylline (0.02–0.04%), and other methyl xanthines, lignin (6.5%), organic acids (1.5%), chlorophyll (0.5%) and amino acids (1-6%), in addition to the unique amino acid theanine (4%) [19]. Catechin a potent antioxidant found in *C. sinensis*, plays a vital role in combating diseases such as diabetes, inflammatory diseases, cardiovascular diseases, as well as fungal and bacterial infections [20]. The phenolic compounds present in *C. sinensis* play an important role in the reduction, capping, and stabilization of the nanoparticles through green route mediated synthesis [21, 22]. The efficacy of green synthesized silver nanoparticles (AgNPs), in biological activities, is influenced by several critical factors, including size, distribution, morphology, surface charge, surface chemistry, particle composition, capping agents, agglomeration, dissolution rate, etc. [23, 24]. These significant physic-chemical properties make them highly suitable as nanocatalyst [25]. Compared to organic nanoparticles like liposomes, dendrimers, carbon-based, and protein-based nano-particles, green synthesized AgNPs have been reported with notable advantages [26]. Moreover, the unique physic-chemical properties of green synthesized AgNPs have led to their utilization in drug delivery, pharmaceutical, etc. [27, 28]. Gol et al. (2020) reported the green synthesis of AgNPs using plant extract of *C. sinensis*. The synthesized AgNPs exhibited a spherical morphology with particle sizes ranging from 10 to 20 nm and demonstrated enhanced antibacterial activity against both gram-positive and gram-negative bacteria [29]. Following Gol et al. (2020) with some modifications, this study presents the green synthesis of AgNPs using *C. sinensis* to enhance the enzymatic activity of extracellular fungal enzymes viz., amylase and cellulase. Amylase and cellulase are the industrial enzymes that significantly play a central role in biotechnological and agricultural applications spanning food, chemical, detergent, cosmetics, pulp, and paper. These applications often encounter challenges due to the limited thermal and storage stability of these enzymes. Inadequate reusability and product

contamination further hinder economic efficiency. Immobilizing these enzymes on AgNPs can address these issues by improving their stability, reusability, and catalytic efficacy [30]. The use of nanoparticles for enzyme immobilization is a novel technological advancement that supersedes earlier methods such as cross-linked enzyme aggregates, microwave-assisted immobilization, mesoporous supports, etc. This new approach enhances enzyme loading, activity, and stability, thereby reducing the cost of enzyme biocatalysts in industrial biotechnology [31]. The high surface-to-volume ratio of nanoparticles, compared to conventional 2D surfaces, results in higher concentrations of immobilized bio-molecules, leading to enzymes with broader working temperature ranges, pH tolerance, and thermal stability compared to free enzymes [32, 33]. Previous studies have demonstrated that nanocatalytic approaches significantly enhance enzymatic activity and protein digestion using a variety of nanomaterials, including metallic nanoparticles, magnetic nanoparticles, nonporous materials, and polymer nanofibers [34]. The interaction between enzymes and metal nanoparticles, such as AgNPs, occurs through adsorption, forming bonds via hydrogen, hydrophobic, electrostatic, ionic, and van der Waals forces. These interactions maintain the enzyme's tertiary structure and ensure high catalytic activity [35, 36]. Green synthesized AgNPs, for instance, interact with amylase, leading to the faster degradation of starch complexes due to enzyme immobilization on the surface of AgNPs, resulting in increased production of reducing sugars compared to the free enzyme form [37]. Similarly, AgNPs interact with cellulase, facilitating the rapid breakdown of cellulose through enhanced digestion kinetics, yielding higher amounts of reducing sugars [38]. Consequently, immobilizing enzymes on AgNPs ensures a higher catalytic rate, positioning AgNPs as potential nanocatalyst for expediting the hydrolysis of complex molecules in various food industries by leveraging the enzymes' affinity for nanoparticle surfaces [39].

2. MATERIALS AND METHODS

2.1. Chemicals

Silver nitrate ($AgNO_3$) was purchased from Merk, India. Whatman No. 1 filter paper was purchased from Sigma-Aldrich, USA. Starch, lactose, ammonium, sodium chloride, sodium nitrate, potassium chloride, magnesium sulphate, dipotassium hydrogen sulphate, ferrous sulphate, carboxy methyl cellulose, monobasic sodium phosphate, dibasic sodium phosphate, 3,5-dinitrosalicylic acid (DNSA), potato dextrose agar (PDA) was obtained from Himedia Laboratories Pvt Ltd., India. All chemicals were used as received without further purification. Double-distilled water was used in all experiments.

2.2. Preparation of *Camellia Sinensis* Extract

Fresh and healthy leaves of *Camellia sinensis* were collected from the Temi Tea Garden, south Sikkim, India. The collected leaves were washed thoroughly 3-5 times in tap water followed by double distilled water to remove impurities such as debris, dirt, and particulate matter from the leaf surfaces. The leaves were shade-dried at room temperature for 10-15 days, finely chopped, and ground into a fine powder. The standard filtration method was used to prepare the aqueous leaf extract. 200 mg of powdered leaves were boiled in 100 ml of double-distilled water at 100°C for 2 hours with continuous agitation and mixing at 250-300 rpm. After boiling, the leaf extract was cooled down, and the obtained crude extract was filtered through Whatman No. 1 filter paper to remove particulate matter, yielding an aqueous solution of the leaf extract. The extract was stored at 4°C and further used for the green synthesis of AgNPs.

2.3. Green Synthesis of AgNPs

1mM $AgNO_3$ and aqueous leaf extract of *C. sinensis* were mixed in a ratio of 19:1 ml at 80℃ for 24 hours with stirring at 250-300 rpm. The colour change was observed from 0-24 hours for initial confirmation of AgNPs synthesis. The reaction mixture was then left still for 120 hours with occasional stirring to complete the synthesis process. This reaction mixture was subsequently centrifuged (REMI C24BL) at 10,000 rpm for 15 minutes. The supernatant was discarded, and the resulting pellet was washed 3-4 times with 70% ethanol. The synthesized AgNPs were lyophilized and stored in an airtight container for further characterization [40].

2.4. Characterization of AgNPs

An optical analysis of the colloidal solution of synthesized AgNPs was measured using a Shimadzu spectro-photometer at wavelengths from 200 to 800 nm at different time intervals from 0 to 144 hours. The lyophilized AgNPs were investigated by Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer L1600300) in the range of 4000–400 cm^{-1}. FTIR identify various functional groups and different biomolecules within the aqueous leaf extract that interacted with the silver ions, subsequently reducing them and facilitating the synthesis, capping, and stabilization of AgNPs. The morphology of the synthesized AgNPs was determined through field emission scanning electron microscope (FESEM) (Carl Zeiss, Ultra Plus series). Elemental analysis was carried out using energy dispersive X-ray analysis (EDX) attached to the aforementioned FESEM at the Institute Instrumentation Center, IIT Roorkee, India [41]. The particle size of the synthesized AgNPs was calculated using Image J software (version 1.8.0).

2.5. Production and Assay of Amylase

The pure fungal culture of *Aspergillus niger* was procured from the Department of Microbiology, Meerut Institute of Engineering and Technology, Meerut, U.P., India, and maintained on a PDA slant. Spores of *Aspergillus niger* were collected from the slant and transferred to double-distilled water. The optical density was set to 0.12-0.16 at a wavelength of 530 nm, corresponding to 106 CFU ml^{-1}. The amylase production media contained starch (15 gm l^{-1}), lactose (10 gm l^{-1}), ammonium sulphate (5 gm l^{-1}), and sodium chloride (2 gm l^{-1}) for amylase production. The production media with the *Aspergillus niger* spore inoculum was incubated in a microbial incubator shaker (MSW 231) at 28℃ for 7 days at 50 rpm. The recovered filtrate obtained after filtration was used as a crude enzyme. To determine the effect of AgNPs on fungal amylase, the enzymatic activity was examined in the presence of different concentrations of AgNPs. The reaction mixture contained crude amylase (0.1 ml), 0.5% w/v soluble starch (0.5 ml) in 0.2 M of phosphate buffer at pH 7.0. This reaction mixture was supplemented with 0, 50, 100, 150, 200, 250, and 300 µg ml-1 of AgNPs and incubated at 28℃ for 30 minutes. Then, 2 ml DNSA was added, and the reaction mixtures were kept in a water bath at 100℃ for 10 minutes to terminate the reaction. Finally, the absorbance was measured at 540 nm at room temperature [42].

2.6. Production and Assay of Cellulase

Spores of *Aspergillus niger* were collected from the slant and transferred to double-distilled water, and the optical density was set to 0.12-0.16 at a wavelength of 530 nm, corresponding to 106 CFU ml^{-1}. The cellulase production media contained sodium nitrate (2 gm l^{-1}), lactose (10 gm l^{-1}), potassium chloride (0.5 gm l^{-1}), magnesium sulphate (0.5 gm l^{-1}), dipotassium hydrogen sulphate (1 gm l^{-1}), ferrous sulphate (0.01 gm l^{-1}) and carboxyl methyl cellulose (10 gm l^{-1}) for cellulase production. The inoculums of *Aspergillus niger* spores with the production media were incubated in a microbial incubator

shaker at 28℃ for 7 days at 50 rpm. The recovered filtrate obtained after filtration was used as the crude enzyme. The enzymatic activity was determined to observe the effect of AgNPs on fungal cellulase in the presence of different concentrations of AgNPs. The reaction mixture contained crude cellulase (0.1 ml), 0.5% w/v soluble cellulose (0.5 ml) in 0.2 M of phosphate buffer at pH 7.0. This reaction mixture was supplemented with 0, 50, 100, 150, 200, 250, and 300 µg ml^{-1} of AgNPs and incubated at 28℃ for 30 minutes. To cease the reaction, 2 ml DNSA was added to the reaction mixture and kept in a water bath at 100℃ for 10 minutes. Finally, the absorbance was examined at 540 nm at room temperature.

2.7. Statistical Analysis

The statistical analysis was carried out using SPSS (Version 25) software and R studio (4.3.1) software. A one-way analysis of variance (ANOVA) was employed to compare the means, further the post hoc test, Tukey's was applied, with the evaluation of significant differences at a significance level of 5%.

3. RESULTS AND DISCUSSION

3.1. Green Synthesis of AgNPs

The green synthesis of AgNPs involves the phytochemicals, particularly phenolic compounds present in the aqueous leaf extract of *C. sinensis*, which, when mixed with silver nitrate, play a role in the reduction of silver ions and the subsequent synthesis, capping, and stabilizing of the AgNPs. The synthesis of AgNPs was initially indicated by the color change, i.e., the transparent $AgNO_3$ solution rapidly turned pale yellow shortly after the addition of the aqueous leaf extract of *C. sinensis*. Within 30 minutes, the color of the reaction mixture transformed into brown, and over the next 24 hours, it intensified to a dark brown, providing the reduction of Ag^+ ions and synthesis of AgNPs, evident through the surface plasmon resonance (SPR) phenomenon (Figure 1) [43].

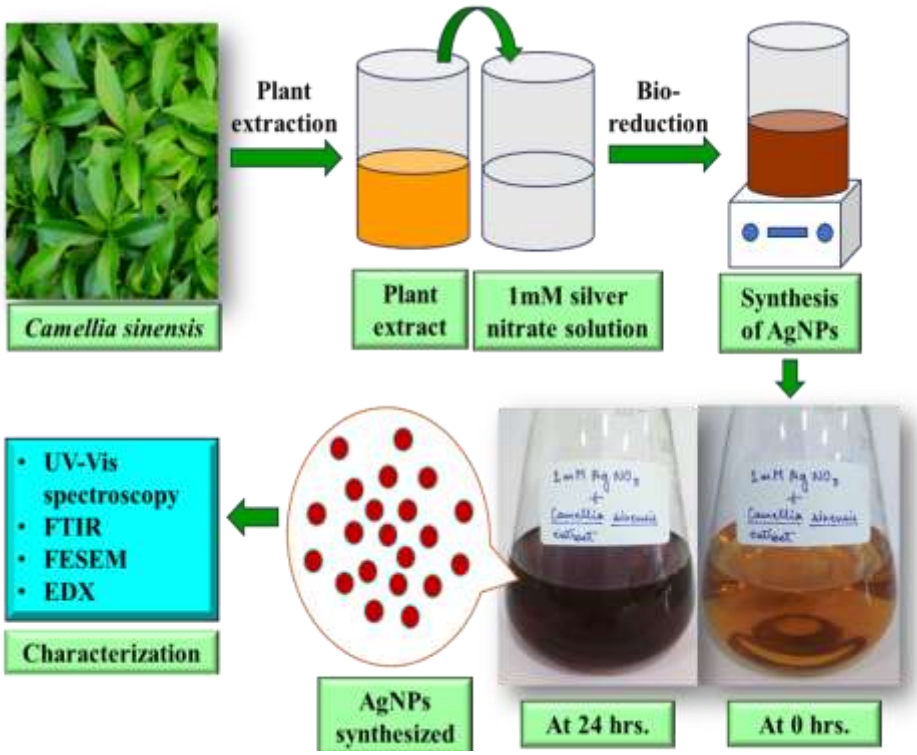

***Figure 1**. Schematic illustration of the green synthesis of AgNPs using aqueous leaf extract of Camellia sinensis.*

3.2. UV-vis Spectroscopic Analysis

The formation and stability of the AgNPs in the colloidal solution were confirmed by a UV-Vis spectrophotometer. The UV-Vis spectra exhibited absorbance within the range of 385 to 425 nm, with a maximum absorption peak of 415 nm at 144 hours (Figure 2, Table 1). Over time, this peak absorbance increased, indicating the complete reduction of silver ions and the synthesis of AgNPs. The broadened spectral peak suggests polydispersity of the synthesized AgNPs [44]. This characteristic absorption peak within the 390-480 nm range is consistent with the reported literature on noble metal nanoparticles, including AgNPs, thus providing further validation of the successful synthesis [45, 46, 47].

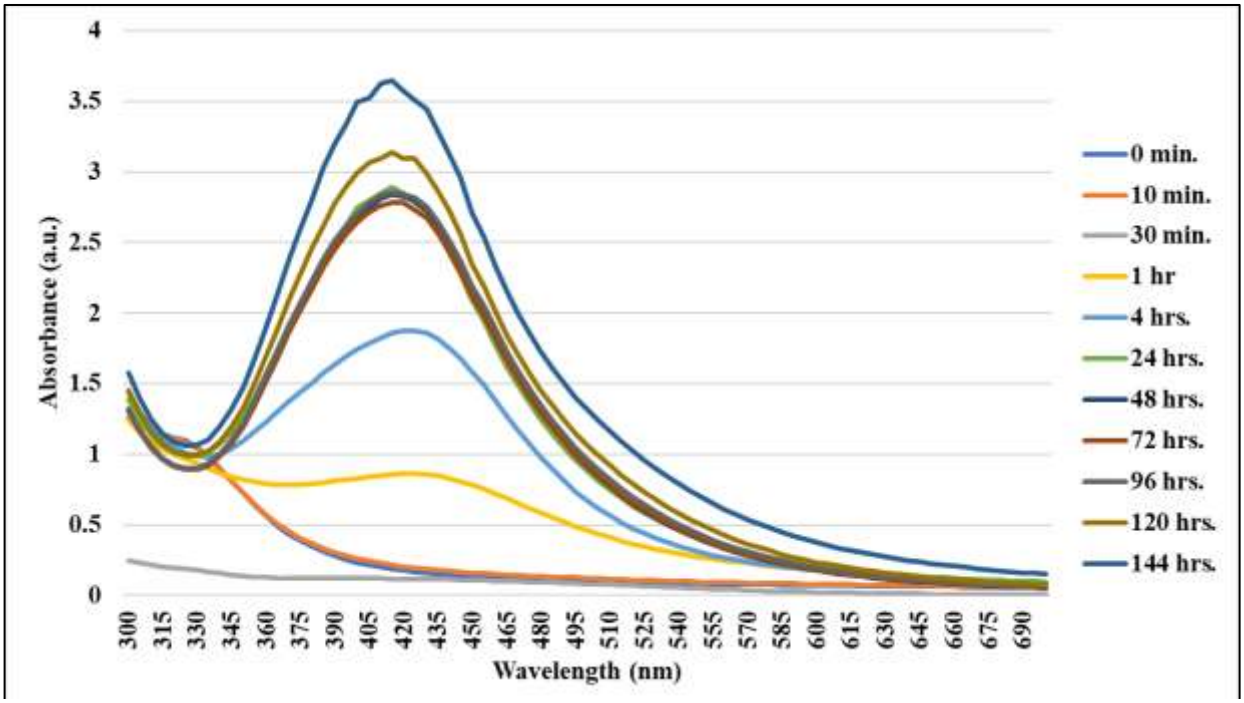

Figure 2. *UV-Visible spectra of AgNPs.*

Table 4. *UV-Visible spectra analysis of AgNPs.*

S. No.	Time	Peak of Ag NPs (nm)
1	0 min.	-
2	10 min.	-
3	30 min.	385
4	1 hr.	420
5	4hrs.	425
6	24 hrs.	415
7	48 hrs.	415
8	72 hrs.	415
9	96 hrs.	415
10	120 hrs.	425
11	144 hrs.	415

3.3. Fourier Transform Infrared Spectroscopy Analysis

FTIR analysis was employed to identify the bio-reducing biomolecules present in the leaf extract of *C. sinensis* that interacted with silver ions to synthesize AgNPs. FTIR showed that the synthesized AgNPs have the characteristic absorption within the range of 400-4000 cm^{-1} due to the presence of different functional groups. The broad peaks at 3201, 2669, and 1320 cm^{-1} were attributed to the O–H stretching vibration of the alcohol group while the peaks at 2941, 2158, and 1036 cm^{-1} were corresponding to N–H, S–C=N, and S=O stretching in the amide, thiocyanate, and sulphoxide groups respectively. Furthermore, intense bands at 2112 and

1614 cm^{-1} were indicative of –C=C and C=C stretching in alkyne and alkene groups, while the peaks at 1842, 1693, and 823 cm^{-1} were characteristic of C–H bending and C=O stretching in the aromatic and anhydride moieties respectively. Additionally, the peaks at 1236, 761, and 485 cm^{-1} were associated with S=O, C–Cl, and C–Br stretching of the alkyl and halo compounds respectively. These overlapping stretching and bending vibrations confirmed the presence of different phytochemicals viz. phenols, amines, carboxylic acids, aliphatic, and aromatic and halo compounds, etc., contributing to the green synthesis of AgNPs in the present study (Figure 3). The interaction between the metallic AgNPs and the organic moieties, specifically phytochemicals present in the prepared complex, facilitated the reduction, capping, and stabilization of the AgNPs on the molecular level. This was further corroborated by the FTIR analysis, which confirmed that hydroxyl, amide, ether, aromatic, alkene, and carbonyl groups within the phytochemicals served as strong binding sites for AgNPs synthesis. The functional groups identified in the analysis suggested the involvement of potential biomolecules such as proteins, terpenoids, and flavonoids, creating an electron-rich environment crucial for the reduction, capping, and stabilization of AgNPs [48, 49, 50].

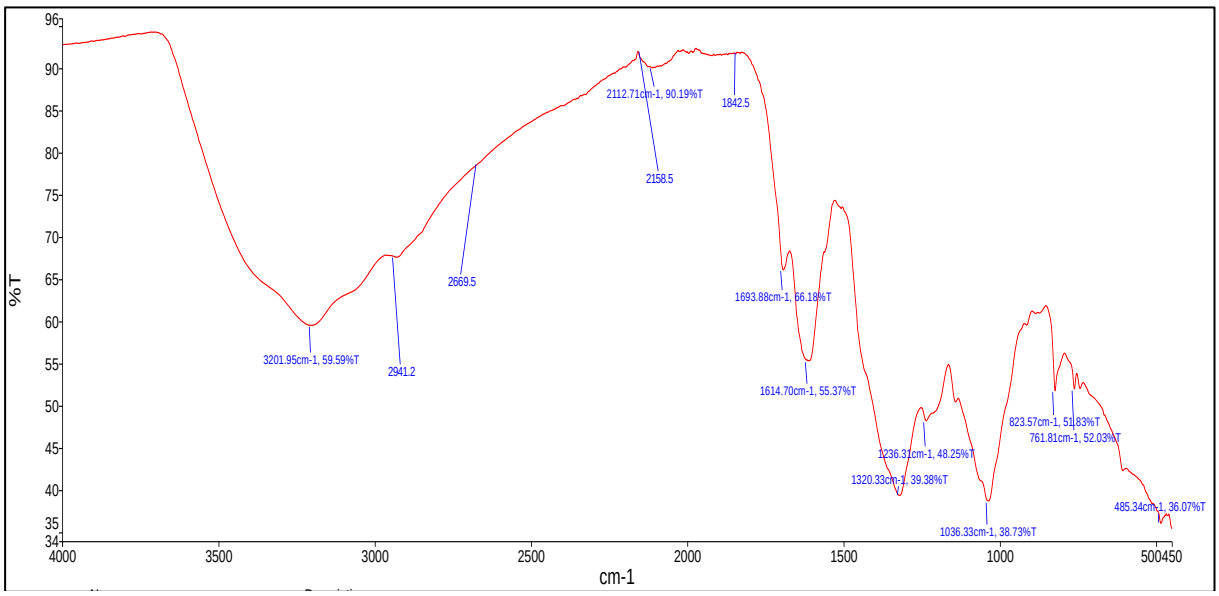

Figure 3. *Fourier transform infrared analysis of AgNPs.*

3.4. FESEM and EDX Analysis

The morphology of the synthesized AgNPs was examined using FESEM. The FESEM image illustrated that the synthesized AgNPs were spherical and exhibited polydispersity. Further analysis of the FESEM image using Image J software revealed that the diameter of the synthesized AgNPs ranged from 22 to 55 ±2 nm. EDX image of the synthesized AgNPs demonstrated an intense signal at 3 KeV, attributed to the presence of elemental silver (Ag). This observation serves as strong evidence for the crystalline nature of the synthesized AgNPs. The EDX analysis revealed that the synthesized AgNPs contain 91.19% of Ag. The remaining 8.81% of impurities, primarily consisting of carbon, can be attributed to factors such as the carbon-coated copper grid used in the analysis or the emission of X-rays from the organic phytochemicals present in the leaf extract, which served as capping agents for the AgNPs (Figure 4).

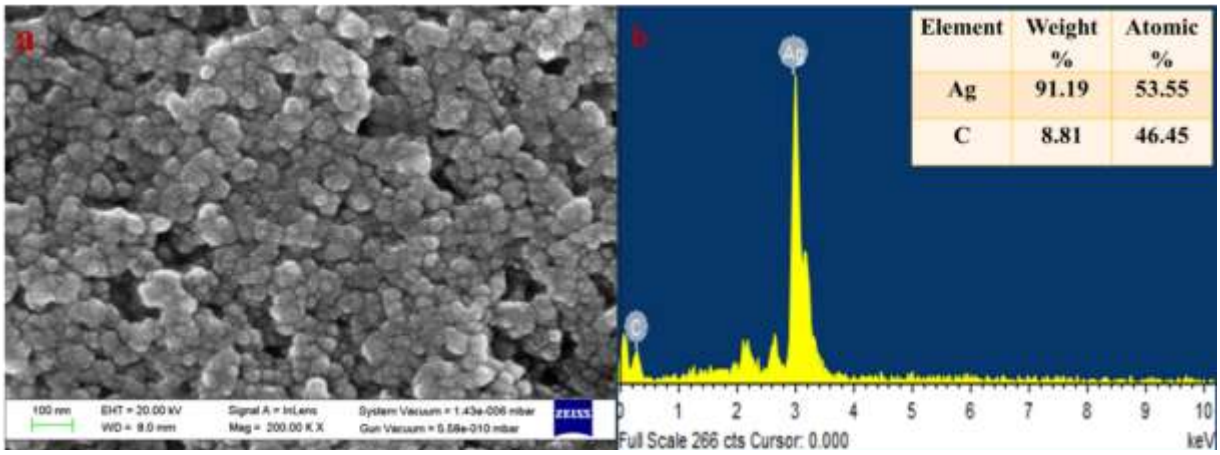

Figure 4. (a) *Field emission scanning electron microscope image of AgNPs (b) Energy dispersive X-ray analysis of AgNPs.*

3.5. Effect of AgNPs on the Enzymatic Activity of Amylase

The effect of AgNPs on the enzymatic activity of fungal amylase, during the degradation of starch into glucose (reducing sugar), was studied across different concentrations of AgNPs utilizing the DNSA method. The quantification of the sugar produced by fungal amylase enabled the measurement of enzymatic activity in µg $ml^{-1}min^{-1}$ [51]. The enzymatic activity of fungal amylase increased cumulatively from two to three folds at 0 to 300 µg ml^{-1} of the synthesized AgNPs. The obtained results establish a positive correlation between the concentration of the synthesized AgNPs and the enhanced enzymatic activity of fungal amylase (Figure 5 a, c). This augmentation in enzymatic activity can be attributed to the immobilization of the fungal amylase on the surface of AgNPs through physical adsorption [52]. The phenomenon of immobilization leads to increased activity of biomolecules, such as enzymes. In the case of enzyme immobilization on the surface of AgNPs, the resultant complex exhibits significantly higher enzymatic activity compared to the free enzyme. Previous studies suggest that carboxylic and amino groups primarily serve as the anchor for enzyme attachment on the surface of AgNPs through weak interactions, viz., adsorption as confirmed by Khan et al. (2013) [53] and Krishnakumar et al. (2018) [54]. The presence of thiol and amino bonds contributes to the thermo-stabilization of the AgNPs, facilitating the immobilization of fungal enzymes onto the surface of AgNPs. This phenomenon ultimately results in a more efficient amylase, in contrast to its free counterpart, making the synthesized AgNPs highly effective as nanocatalyst [55].

3.6. Effect of AgNPs on the Enzymatic Activity of Cellulase

The DNSA method was used to investigate the effect of AgNPs on the enzymatic activity of fungal cellulase, specifically during the degradation of cellulose into its reducing sugar counterpart. Similar to the assay of amylase assay, the released sugar by fungal cellulase was also quantified to measure the enzymatic activity in µg $ml^{-1}min^{-1}$. The enzymatic activity of fungal cellulase increased significantly, ranging from three to ten folds across various concentrations of AgNPs, from 0 to 300 µg ml^{-1}. Hence, the outcome unequivocally establishes a positive correlation between the concentration of the synthesized AgNPs and the augmented enzymatic activity of fungal cellulase (Figure 5 b, c). This increase in enzymatic activity is primarily attributed to the immobilization of fungal cellulase on the surface of AgNPs, resulting in notably higher activity

levels, particularly when compared to the free enzyme. The presence of metal nanoparticles viz., AgNPs, as a solid support significantly enhances the efficiency of the enzyme, surpassing the free enzyme [56]. The hydroxyl group within cellulase plays a pivotal role in facilitating the affinity for immobilization onto AgNPs [57]. The immobilization leads to a more controlled degradation of cellulose at an accelerated rate, as the collisions generated by the immobilized AgNPs are fewer than those observed in the case of free cellulase. Consequently, the synthesized AgNPs function as nanocatalyst, significantly augmenting the reaction rate [37].

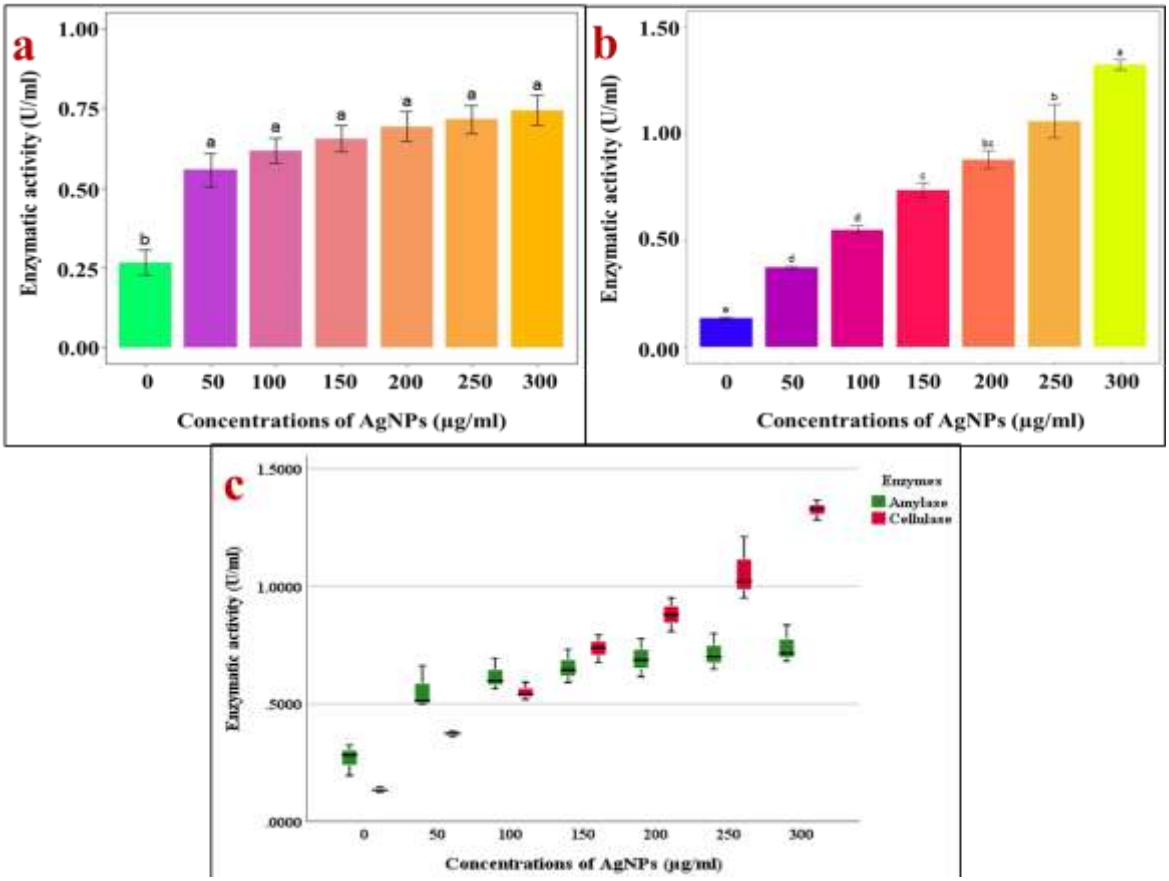

Figure 5. *Effect of synthesized AgNPs on extracellular fungal enzymes (a) amylase (b) cellulase. The effect of different concentrations of synthesized AgNPs on fungal enzymes which were significantly different from each other (indicated by the compact letter based on Tukey's test. Groups with different letters were significantly different, while groups with the same letter were not significantly different). (c) Box plot illustrate that the enzyme activity of amylase and cellulase varies significantly across different concentrations. The upward trend in the median values (represented by the horizontal line within each box) exhibits that with the increasing concentration of AgNPs enzyme activity of both the enzyme increases, while enzyme activity of cellulase increased exponentially with increasing concentrations of AgNPs as compared to amylase. A Positive correlation was observed between the enzymatic activity and different concentrations of AgNPs. (Experiments were repeated in triplicates)*

5. CONCLUSION

The present study showed the successful synthesis of AgNPs through the green route using an aqueous leaf extract of *C. sinensis*. The synthesized AgNPs were confirmed through UV-Vis spectrophotometer, FTIR, FESEM, and EDX. The synthesized AgNPs was

evaluated for their nanocatalytic property in fungal amylase and cellulase in their respective production media. The green synthesized AgNPs upon interaction with fungal amylase and cellulase, facilitate their immobilization, leading to a significantly accelerated hydrolysis of starch and cellulose complexes compared to their free enzyme counterparts. AgNPs demonstrated remarkable nanocatalytic properties and can act as act nanocatalyst in a wide range of applications including pharmaceuticals, paper, food, bakery, fermentation industries, etc.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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