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# Solid State Fermentation: Extraction of Enzyme from Fungus and its use in the synthesis of Silver Nanoparticles

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**ABSTRACT:** Solid State Fermentation technique has been used to obtain crude enzyme from the pure culture of *Aspergillus niger* by inoculating a uniform suspension of the fungal spores in autoclaved fermentation media. A six-day long session of its incubation at optimum ambient was followed by the hydration, stirring and macro-filtration to obtain crude extract. After two rounds of its centrifugation at low temperature, it was stored and its microfiltration was executed to acquire cell-free supernatant full of enzymes stemming from the fungus. Once the neutrality of its pH was ensured, it was retained on PDA slants, sub-cultured periodically and preserved at 4 °C. This clear supernatant containing the enzyme extracted from the fungus *A. niger* was characterized using UV-visible spectroscopy. It was also used to synthesize silver nanoparticles from two different concentrations of the precursor  $\text{AgNO}_3$ . The enzyme was found to have successfully bio-reduced the silver ions to nanoparticles. Two reaction mixtures and a control sample of the supernatant were incubated for 36 hours at  $29 \pm 1$  °C in dark to avoid any photochemical reaction. After incubation, the color of the reaction mixtures got changed, indicating the formation of silver nanoparticles. These were centrifuged to obtain purified silver nanoparticles, whose characteristics were experimentally examined.

**KEYWORDS:** *A. niger*, Bioextract, Enzyme, Extracellular synthesis, Fourier Transform Infrared spectroscopy, PDA, Nanoparticles, Solid state fermentation, Surface Plasmon Resonance, toxicity, UV-visible spectroscopy

## I. INTRODUCTION

Nanoparticles (NPs) have been fetching a steady growth of interest in Physics, Chemistry, Biology, Biomedicine, etc. (Slawson *et al.*, 1992). Molecules or atoms bonded together in tiny ensembles, whose one or more dimensions is less than a few hundred nanometers, are called nanoparticles. Being so light in weight that they are often found naturally suspended in air, liquids, powders etc. Their miniscule dimension dramatically enhances their surface to volume ratio, which in-turn modifies their physico-chemical properties like conductivity, surface scattering, catalytic activity and optical behavior forcing the NPs to behave vastly different from their bulks. (Sharma *et al.* 2009). These new or improved properties exhibited by NPs also depend upon their size, shape and their distribution among the ensemble. (Mukunthan *et al.*, 2011). Lately, the fabrication of silver nanoparticles has been the focus of developments since they are endowed with antimicrobial properties and these contribute to protect the environment and hygiene (Fabrega *et al.* 2011). Silver nanoparticles could be spun into fibres or spread as a layer that protects against the pathogens. They find immense other applications such as in food, textiles, cosmetics, medicine and electronics industries. Various physical, chemical and biological techniques have been in vogue for their synthesis, particularly for the synthesis of metal nanoparticles (Vaidyanathan *et al.*, 2010). Majority of these processes entail large budget of finance and energy and if these are used for large throughput production of NPs, their impact on the environment is rather severe. Consequently, with the evolution of newer industrial forays of the NPs, devising efficient avenues of their production has become a mission and it has attracted a flurry of innovations.



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Obviously, consistent efforts have been made all over the globe to synthesize, stabilize and achieve a control on the dispersion in the size and morphology of the nanoparticles, and also towards bringing down the cost of their synthesis. These efforts are upheld and bolstered by the emerging applications of nanoparticles in drug delivery, environmental remediation, nano-electronics and in the catalysis *etc.* The physical methods of synthesis of NPs such as thermal decomposition, ablation, sputtering, arc-discharge may offer a morphological bonus. But these methods consume lots of energy and also raise the temperature of the environment. And the chemical methods used for their synthesis and stabilization involve the chemicals such as ethylene glycol, tri-sodium citrate and sodium borohydride, which often have a toxic footprint. These actualities necessitate the search for alternate routes of synthesizing the nanoparticles. Thus there is a quest for novel ingredients and processes that could be executed without using major capital equipment and tedious ambient. Especial yearning is towards developing those procedures, which could alleviate the chemicals that harm the humans or the environment. The impetus of innovation for ongoing research is to develop processes that are simple to execute, consume minimum amount of energy and whose effluents are least unfriendly to the environment.

One such avenue emanates from Biotechnology, which offers the ways to produce chemicals by the microbes such as bacteria and fungi using intra- as well as extra-cellular routes. Some microorganisms survive and grow in metal ion ambient. For instance the yeast MKY-3 is resistant to silver ions as its redox reactions alter the toxicity and its extracellular complex formation helps precipitate the metals (Fang *et al.* 2005). These chemicals are known to interact (Fortin and Beveridge, 2000) with metals such as in corrosion, mineralization, bleaching and bioremediation, through their ability to reduce the metal ion (Bruins *et al.*, 2000). Incidentally, the attempts made to synthesize metallic nanoparticles by harnessing the chemicals produced by these microbes have shown success (Ahmad *et al.* 2003). These have opened up the biological ways of generating nanoparticles, esp. the nanoparticles of metals (Sastry *et al.*, 2003).

Such biological synthesis using microorganisms has the potential to synthesize nanoparticles of various sizes, shapes and morphology. Shivaji *et al.* extracted cell-free culture supernatants of five psychrophilic bacteria and made it execute extracellular synthesis of silver nanoparticles at room temperature. The NADPH-dependent enzyme nitrate reductase extracted from the fungus *Fusarium oxysporum* and a shuttle quinine extracellular process were observed to convert  $Ag^+$  to  $Ag^0$  (Torres *et al.*, 2007) in presence of NADPH. This route of using enzymes present in the bio-extract offers a vast potential to create NPs through a process, whose projected imprint of toxicity is far smaller than associated with the chemical routes and which uses far less energy than used by the physical processes. Saravanan *et al.* (2011) have reported that the extracellular biosynthesis using supernatants from microorganisms has the potential for large-scale production of silver nanoparticles.

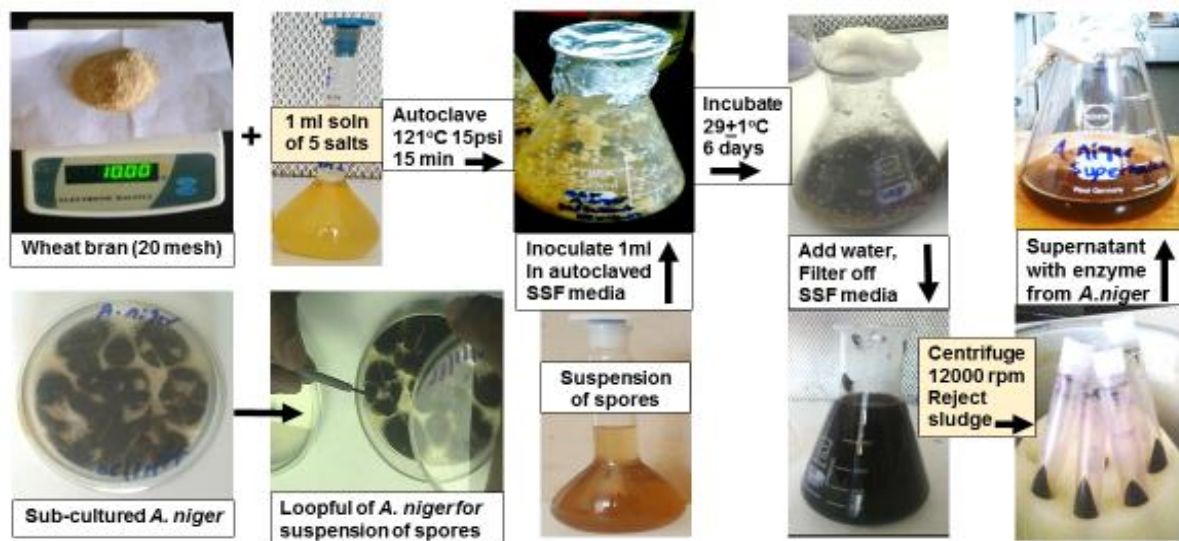
Bio-extracts can be obtained from the microbes using one of the three prevalent methods of fermentation. These are submerged fermentation (SmF), surface fermentation (SF) and solid state fermentation (SSF). Submerged state fermentation (SmF) executed in alcohol, oil or nutrient broth requires a control on pH, a constant supply of nutrients as well as oxygen. However, the filaments generated by the fungi during the fermentation enhance the viscosity of its medium and the stirring needed for the absorption of oxygen can disrupt its cell network. Thus the submerged state fermentation suits the growth of unicellular organisms like bacteria or yeasts but not the fungi. On the other hand, in Solid State Fermentation (SSF), a solid culture substrate, for instance moistened rice or wheat bran or beet pulp along with suitable nutrient additives is deposited on flatbeds. Microorganisms are seeded in it and it is allowed to incubate at the optimum ambient for a period as long as a week. The growing filaments of fungi decompose the flat substrate complementing its natural ventilation. The composition of growth medium initially added to the substrate is selected in such a way as to stimulate the release of desired metabolites. SSF is often used in fruit, vegetable, animal feed, bio ethanol and brewing industries. In the present work we have harnessed SSF to produce relatively inexpensively a high yield of metabolites with single enzyme or a complex having multiple enzymes (Singhania *et al.*, 2009). The potential of such enzymes in breaking down difficult-to-transform macromolecules such as cellulose, hemicelluloses, pectin and proteins is well known (Christen *et al.*, 1995). We have tried to use the extracted metabolites to reduce the ions present in silver salt solution into the neutral nanoparticles.

## II. MATERIALS AND METHODS

In Solid state fermentation (SSF) process, several factors crucially govern the extraction of enzymes. These are the quality of microbe, the quantity of inoculum, the surface and porosity of the substrate, the control of humidity,

temperature, and oxygenation and the duration of incubation (Abubakar and Oloyede, 2013). In order to produce enzymes using SSF, there is a wide choice of materials which can be used to prepare the substrates. These are cane bagasse, bran, straw, husk, flour of cereals, coconut coir, fruit pulp, oilcake *etc.* From amongst these materials, we restricted to the bran of wheat, though the bran of maize, rice and gram have also been used successfully (Bhattacharya *et al.*, 2012). The amount of fibres and proteins secreted by the fungi are more copious than those secreted by the bacteria and since the profusion of enzyme is the key ingredient for the conversion of ions to nanoparticles, we selected the former for our experiments. It is understood that the extracellular enzyme secreted by the fungal cell walls reduces the positive ions of metals into the neutral nanoparticles (Bhainsa *et al.*, 2006).

For our SSF process, we picked up commercially available wheat seeds, washed them, dried at 40°C, micro-grinded, and sieved with a 20-mesh (840 µm) sieve. 10gm of this bran of uniform size was weighed and sprinkled with 1ml solution of five salts to moisturize it to 70%. The salt solution was prepared by dissolving ammonium sulphite, sodium hydrogen phosphate, potassium hydrogen phosphate, magnesium sulphate, calcium chloride in sterile distilled water. Then it was autoclaved at 120°C and 15psi for 15min. The autoclaved SSF media was aseptically inoculated with 1ml of uniform suspension of repeatedly sub-cultured *A. niger* spores in sterile distilled water and allowed to incubate at 29±1°C for 6 days to secrete the crude enzyme. 50ml deionized water was added to it and stirred for 15 min before the SSF media was filtered off with a muslin cloth. The residue was rehydrated as above and the remaining enzyme was also extracted. Then it was centrifuged in two steps at 12000 rpm for 15 min each at 4°C.



**Fig. 1 Steps of Solid State Fermentation process to obtain enzyme from the fungus *Aspergillus niger***

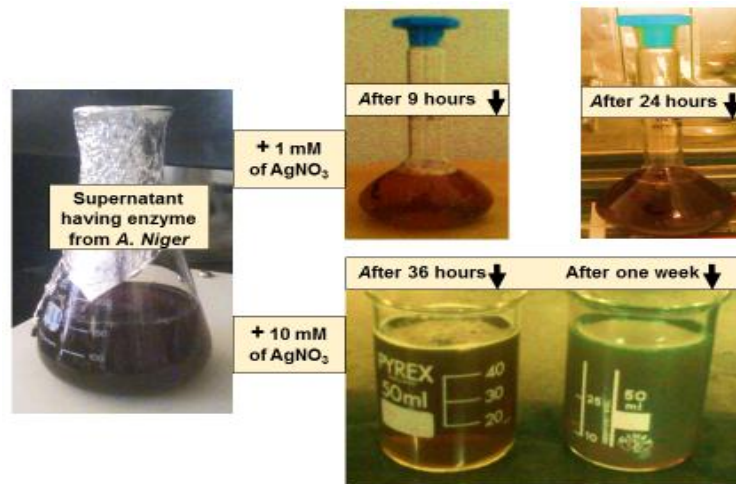
The pellets of sludge were rejected and the cell-free crude extract was thus obtained. This supernatant was filtered with 0.45 µm Millipore membrane filter and a further filtering was done with 0.2 µm filter. Two chemicals viz. NaOH and HCl were used to adjust the pH of this clear supernatant containing enzyme secreted by the fungus *A. niger* to a neutral value. Then it was maintained on PDA slants, sub-cultured every 7 days and stored at 4°C. The sequence of this SSF process is depicted pictorially in Fig.1.

A portion of this clear supernatant was put in quartz cuvette and its optical absorption spectra was observed by using UV-Visible spectrometer. After that its ability to bio-reduce the silver ions and synthesize Ag NPs was tested. For this purpose, identical samples of the supernatant were mixed with silver nitrate solutions of different concentrations. These reaction mixtures were incubated in dark to prevent any photochemical process. When these mixtures were examined after different durations of elapsed time, they showed up a gradual formation of silver nanoparticles, evidenced by the changing color of the reaction mixtures. This time taken by this supernatant containing the enzyme to accomplish a complete reduction of the salt solution into the NPs was found to increase with the concentration of silver nitrate. While 1mM solution of AgNO<sub>3</sub> got completely reduced in a time duration between 9 and 24 hours, the 10 mM solution required 36 hours to exhibit significant level of formation of NPs.

The above suspension containing silver nanoparticles was centrifuged at 12,000 rpm for 15 min. The resulting pellets thus obtained were dried, powdered, dispersed in water and centrifuged again to remove the traces of chemicals adhering on it and obtain pure stabilized silver nanoparticles. The optical absorption characteristics of these NPs were analyzed over a wide range of wavelength through a UV-Visible spectrometer. The bonding between the biological capping and the metal of these nanoparticles was investigated by analyzing their Fourier transform Infrared (FTIR) spectral analysis.

### III. RESULTS AND DISCUSSIONS

The cell-free supernatant containing enzyme extracted from the fungus *A. niger* grown in the SSF medium appears brown in colour. Optical absorption through it was studied using UV-Visible spectrometer to find out its molecular structure. It exhibited a major peak in absorption at a wavelength of 223 nm and a minor peak at 267 nm. While the former corresponds to Ethyl ether/Isopropyl alcohol, the later denotes bioactive molecules, which could participate in the biosynthesis of NPs through redox reactions. 5 ml each of  $\text{AgNO}_3$  solutions having concentration 10 mM and 1mM respectively was mixed with two different samples of volume 10 ml of this supernatant. When these two reaction mixtures and a third control sample of virgin supernatant were allowed to incubate at controlled ambient in dark, the colour of the first two reaction mixtures changed gradually from brown to dark brown. This process was slower for the mixture in which the  $\text{AgNO}_3$  had higher concentration. The sequence of this process of synthesizing silver nanoparticles from the fungal enzymes is depicted pictorially in Fig.2.



**Fig. 2 Synthesis of Silver NPs by the fungal enzyme present in supernatant extracted from *Aspergillus niger***

This result resembles with the observations of Gade *et al.* (2008), who reported that the color of the crude enzyme of *A. niger* got changed to brown after treatment with 1mM  $\text{AgNO}_3$ . This change in the colour of two reaction mixtures is an initial indicator of the formation of silver nanoparticles through the bio-reduction of  $\text{Ag}^+$  to  $\text{Ag}^0$  (Devaraj *et al.*, 2013) caused by the enzyme present in supernatant prepared from the SSF process. It is notable that an identical control sample of the virgin supernatant showed no change of colour, as it lacked any precursor to synthesize the NPs.

To further verify the capability of the enzyme extracted through SSF in synthesizing Ag NPs, we did the UV-Vis spectroscopy of the reaction mixture, where the nanoparticles are conjectured to be present. The mixture exhibited a peak in absorption at the wave length of 425 nm, which is similar to the maxima in the UV-Vis absorption was reported by Singhet *et al.* (2014) for the Ag NPs synthesized by endophytic *Penicillium* species. Our findings are parallel to the results reported by Gade *et al.* (2008), who observed a similar change in the color of the *A. niger* enzyme when it bio-reduces  $\text{AgNO}_3$ . The occurrence of absorption peak near 425 nm is attributed to have been produced by the Surface Plasmon Resonance caused by the nanoparticles of silver metal. Longoria *et al.* (2011) also obtained a similar absorption peak arising from surface plasmons for silver nanoparticles fabricated by using *Neurospora crassa*. Such absorption peaks are known to shift to longer wavelengths as the size of nanoparticles increases (Haiss *et al.*, 2007).

The virgin supernatant which had enzyme but no silver precursor and which had not undergone any change in its colour, also did not exhibit any peak in the UV-Vis absorption in this range of wavelength.

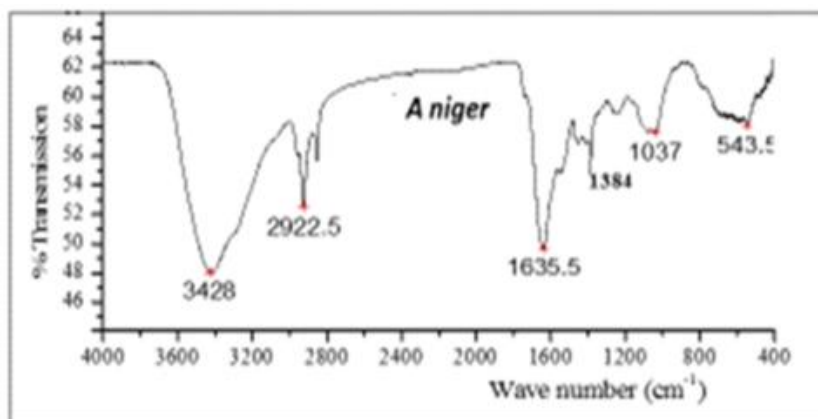


Fig. 3 FTIR spectra of silver nanoparticles synthesized by enzymes extracted from *A. niger*

Purified powder of silver nanoparticles obtained by repeating the sequence of centrifugation, dispersion in distilled water and drying was mixed with an IR transparent material and moulded into pellets. Its Fourier Transform Infrared (FTIR) spectra analysis was done to explore the stabilizing chemicals present on its surface. We executed this experimental study of IR absorption in the range  $400\text{ cm}^{-1}$  to  $4000\text{ cm}^{-1}$ . Several peaks in the absorption of IR by this suspension of nanoparticles were observed. These are shown in the FTIR transmission spectra (Fig.3) as various troughs. The six different wavenumbers at which they occur could be correlated to identify the functional group present on the surface of these nanoparticles. Starting from the lowest wavenumber, the first two peaks correspond to IR transparent halides and amine bonds. The third emanates from C=O bond contributed by the proteins which has contributed to the stabilization of nanoparticles preventing its agglomeration (Loo *et al.*, 2012). The last three peaks are apparently caused by the alkanes and O-H bonds of the alcohols.

Thus the proteins and enzymes present in the supernatant extracted from the fungus *A. niger* by the SSF process have contributed to the reduction of silver ions to silver nanoparticles. Our FTIR analysis confirms that these have also executed the capping of the nanoparticles and thus stabilizing them from joining together and forming a bulk. Similar results have been reported by (Jeevan *et al.*, 2012). The carbonyl groups of the amino acid residues and the peptides have strong affinity to bind with the silver. These biomolecules occurring in the enzyme extracted by SSF have been responsible for the synthesis and stabilization of Ag NPs.

#### IV. CONCLUSIONS

Silver nanoparticles have been synthesized biologically using a bottom-up approach that is primarily governed by the redox reactions. We have used wheat bran for the solid state fermentation process and extracted the enzymes secreted by the fungus *A. niger*. The extracted enzyme exhibited its antioxidant or reducing properties and it could successfully bio-reduce the specific precursor compound to convert it to the nanoparticles of silver (Rout *et al.*, 2012; Singhet *et al.*, 2015). The solvents and chemicals utilized in this biosynthesis process of reduction are water and fungi, which are environment-friendly. The execution of stabilization of the synthesized silver nanoparticles preventing their agglomeration into bulk has also been done by a nontoxic stabilizing agent. This primary constituent of this environment friendly process of biosynthesizing the silver nanoparticles was solid state fermentation, which could deliver the bio-extract from the fungi in an efficient and economical way. Solid state fermentation based microbial method of synthesis is simple, economical, reproducible and its energy budget is far smaller than that of the chemical methods.



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Vol. 4, Issue 5, May 2017

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Tizazu Habte comes from Harbucho village of Shurmo Dubancho Kebel in Haldiya zone of Ethiopia. He graduated from Awassa, taught Physics in Homecho and got B.Ed. from Mekelle University. The research work reported in this publication is a part of the experimental work done by him for his Masters thesis in the stream of Nanoscale Physics under the guidance of Professor U.S. Tandon at Haramaya University. His research interests include chemical and green synthesis of nanoparticles and their experimental characterization. He has been doing experiments to analytically evaluate the antimicrobial characteristics of silver nanoparticles. He aspires to synthesize nanomaterials through environment-friendly process and apply them for improving the prevailing hygiene and health.