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GREEN SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES AS POTENTIAL ANTIMICROBIAL AGENT

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Abstract: **The use of noble metal nano particles as drugs nano carriers presents principal advantages, firstly they are able to transport several therapeutic molecules adsorbed on their surface. Penicillium chrysogenum previously known as Penicillium notatum is widely distributed in nature and is well known for it's β-lactam antibiotics and secondary metabolites, most significantly penicillin. The current research activities synthesized the silver bio-nanoparticles from Penicillium chrysogenum by intracellular method. Nanotechnology is an emerging field, with enabling technology, the creation and application of materials, devices, and systems at atomic and molecular levels and the exploitation of novel properties that emerge at the nanometer scale. The morphological studies of the biosynthesized nano particles are done using UV-vis, SEM, & FTIR techniques. The O.D.value and spectrum was measured through UV-visible spectrophotometer. After confirmation of the formation of silver bio-nanoparticles (Ag-NPs) they were treated against different pathogenic organisms like Pseudomonas aeruginosa, Bacillus cereus, E. coli, and Staphylococcus aureus. The highest sensitive antimicrobial activities were observed in Staphylococcus aureus followed by E. coli, Pseudomonas aeruginosa and Bacillus cereus. These data constitute a preliminary study about the knowledge of the physical-chemistry properties of the drugs adsorbed on noble metal nanoparticles surface that will allow us to deep in the design of new drug delivery systems with potential to improve the clinical efficacy of the therapeutic effect.**

Keywords: **Penicillium chrysogenum, Bio-nanoparticles, UV–Visible Spectrophotometer, Scanning Electron Microscopy, Antimicrobial activity.**

1. INTRODUCTION

The synthesis of silver metal nanoparticles by using several strains of the fungus through intra and extracellular methods achieved more attention for the researchers towards the nanotechnology. The extracellular enzyme present in the fungi like Verticillium and *Fusarium oxysporum* has showed excellent redox properties and it can act as an electron shuttle in metal reduction[1,2]. Biosynthetic methods by using microorganisms are eco friendly in nature[3] and it has been investigated as an alternative to chemical and physical ones. Silver bio-nanoparticles (Ag-NPs) are known to possess bactericidal effects. The intra- or extracellular methods depends on the place where the nanoparticles or nanostructures are created on the microorganisms [4]. Biosynthesis of silver nanoparticles can be produced by using fungi in intra- or extracellularly. The silver nanoparticles synthesized from fungi have become a challenge for the nano-biologists which possess good antimicrobial activity against various multi drug resistant pathogens [5].

From the ancient period it has been found that people are using silver in applications ranging from traditional medicines to culinary items. Silver has disinfecting effect and moreover, several salts of silver and their derivatives are commercially

manufactured as antimicrobial agents[6]. Silver is safe for human cells when it is used in small concentrations but that concentration is lethal for bacteria and viruses [7]. A bactericidal property of metallic silver seems to be promising specified nanosilver drugs as a special class of biocidal agents. It is known that a large number of organisms, both unicellular and multicellular, are able to produce inorganic nanomaterials, either intracellularly or extracellularly[8,9,10&11].The present study focused on the synthesis of silver nanoparticles from *Penicillium chrysogenum* and extraction of its bioactive compounds. As *Penicillium* spp are well known for their antibiotic compounds, the study also comprises the antimicrobial activity of bioactive compounds and silver nanoparticles against bacterial pathogens. The morphological studies of the biosynthesized nanoparticles are done using UV-vis, FESEM, techniques. As *Penicillium* spp are well known for their antibiotic compounds, the study also comprises the antimicrobial activity of bioactive compounds and silver nanoparticles against bacterial pathogens.

II. MATERIALS AND METHODS

1. Intracellular biosynthesis of Ag nanoparticles using Penicillium chrysogenum

The fungal culture was grown aerobically in same liquid medium and incubated on orbital shaker at 25^0C and agitated at 150 rpm up to72 hrs. The biomass was harvested by filtration followed by extensive washing with distilled water. Typically 20gm of biomass (fresh weight) was brought in contact with 200ml of Milli- Q deionized water in an Erlenmeyer flask and followed by sonication. Sonication was done at 100% amplitude by using 30 mm probe for 15 min. The suspension was centrifuged at 12,000 rpm for 10 min at 25^0C. The cell filtrate was subjected to $AgNO_3$ for intracellular synthesis of silver nanoparticles. At different time intervals the absorbance was measured by UV–visible spectrophotometer at a resolution of range 200-800 nm. After confirming the antimicrobial activity the nanoparticles were allowed to characterize by AFM or SEM to know its particle size.

2. Extraction of Bioactive Compounds

Penicillium chrysogenum was isolated from the air of the Sathyabama University campus, Chennai, India during the winter period (November-December) and cultured in the Sabouraud Dextrose Broth at 24^oC and allowed to grow up to 25 days. After the complete color change of the broth, the broth was decanted slowly and subjected to ethyl acetate and chloroform at equal ratio of the filtrate in a separating funnel. With a continuous shaking for five minutes the separating funnel was kept in a stand and slowly the lower part containing the bioactive compounds were collected in a separate conical flask. The lower portion containing the compounds kept in the rotary evaporator for extracting the bioactive compounds.

3. Antimicrobial activity Test

Disc diffusion method and well diffusion method was used for checking the antimicrobial activity of bioactive compounds and silver nanoparticles against bacterial pathogens and also to confirm the dose dependant concentration. The zone of inhibition was measured and compared with the control in its raw form and with the different antibiotic discs to confirm their activity ability.

4. Nitrate reductase assay

Qualitative assessment of the enzyme was determined using Nitrate reductase assay. One hundred mL Nitrate broth (modified) was prepared in 250 mL Erlenmeyer flasks and sterilized. One millilitre of 24 h grown culture isolate was used as inoculum and incubated on a rotatory shaker at 150 rpm for 96 h at 37ºC. Assay reagents: equal volumes of sulphanilic acid and α-naphthylamine in 5N α-acetic acid were prepared freshly and 0.1 mL was added to the culture filtrate and observed for color change.

III. RESULTS AND DISCUSSION

1. Extraction of Bioactive Compounds

Penicillium chrysogenum was cultured in the Sabouraud Dextrose Broth and allowed to grow upto 25 days for secondary metabolites (Fig.2a). The broth was subjected to chloroform and ethyl acetate at equal ratio of the filtrate in a separating

funnel which showed the clear separation of the bioactive compounds (Fig. 2b). Slowly the lower part containing the bioactive compounds was collected in a separate conical flask and kept in the rotary evaporator for extracting the bioactive compounds. Especially fungi, bacteria and actinomycetes are important group of microorganisms in our environment that have served as a rich source of secondary metabolites like bioactive compounds and commercial antibiotics [12]. Among the naturally derived high commercial value bioactive compounds and antibiotics are produced from fungi and actinomycetes[13].

2. Intracellular biosynthesis of silver nanoparticles from Penicillium chrysogenum

The fungal biomass was harvested after 72 hrs of growth by filtration using ordinary filter paper followed by extensive washing. Typically 20gm of biomass (fresh weight) was sonicated at 100% amplitude by using 30 mm probe for 15 min. The suspension was centrifuged at 12,000 rpm for 10 min at 25 ^{0}C. The cell filtrate was subjected to $AgNO_3$ for intracellular synthesis of silver nanoparticles[3]. The cell filtrate when subjected to $AgNO_3$, the reaction was started after four hours and the color of the solution turned to yellowish brown, indicating the formation of AgNPs (Fig. 1). It is well studied by several researchers' investigation that microorganisms have been explored as potential bio-factories for synthesis of metallic nanoparticles such as cadmium sulfide, gold and silver[3,14&13] and the AgNPs exhibit a yellowish brown color in water, arising from excitation of surface plasmon vibrations in the metal nanoparticles. The AgNPs were characterized by UV-vis spectrophotometry. The formation and stability of the reduced AgNPs in the colloidal solution was monitored by using UV–vis spectral analysis. The UV–vis spectra recorded from at different time intervals of reaction were plotted in figure-6 and the curves a, b, c, d, and e correspond to the readings at different time intervals like 6, 12, 24, 48 and 72 hours, respectively and the peak was noted around 420 nm (Fig. 3). It is observed from the spectra that the silver surface plasmon resonance band occurs at 420 nm.

3. Fourier Transform Infra-red Spectroscopy

FTIR spectroscopy is a useful tool for quantifying secondary structure in the metal nanoparticles –protein interaction by the absorption of infra red (IR) radiation through resonance of non-centro symmetric (IRactive) modes of vibration. The FTIR spectrum (Fig. 4) displayed a peak at 3807 cm-1 for free hydroxyl O-H stretch, small peak at 2929 cm -1 corresponds to the stretching vibrations of primary and secondary amines between P-H was reported at 2343 cm -1 with a bending vibration peak at 1896 cm -1 with N-H vibration. The peaks at 655 cm -1, 510 cm -1 and 497 cm -1 correspond to C-S disulfide stretching vibration. Similarly, the peak at 402 cm -1 showed -S-S (polysulfide) stretching vibration indicating the frequent occurrence of thiols and its substituted compounds constituting the backbone of the interacting protein.

4. Antimicrobial Activity Test

Disc diffusion method was used for checking the antimicrobial activity of bioactive compounds and silver nanoparticles synthesized by intracellular methods against Bacillus cereus, Staphylococcus aureus, E. coli and Pseudomonas aeruginosa. The bioactive compounds was treated against all the pathogens and zone of inhibition measured and compared with antibiotic penicillin G. The dose dependant concentration confirm that 15 µl showed the zone of inhibition was more in Bacillus cereus followed by E. coli, Staphylococcus aureus and Pseudomonas aeruginosa (Table-1). Similarly the intracellular silver nanoparticles were treated against all the pathogens and zone of inhibition measured. But there is not much difference about the bioactive compound and intracellular silver nanoparticles (Table-2). When Bacillus cereus was treated with bioactive compounds the zone of inhibition measured more (17mm) than compared to intracellular silver nanoparticles. Nanda and Saravanan (2009, 2010) also proved that silver nanoparticles synthesized extracellularly showed positive response against multidrug resistant pathogens. The maximum zone of inhibition was recorded by Staphylococcus aureus (18mm) followed by E. coli (15mm), and Pseudomonas aeruginosa (10mm) and the least Bacillus cereus (8mm)[16]. We have not studied the mechanism perhaps the silver nanoparticles do not respond to Bacillus cereus. Marcato et al, (2003)[17] showed that silver nanoparticles, like its bulk counterpart, are an effective antimicrobial agent against various pathogenic microorganisms. Though various chemical and biochemical methods are being explored for silver nanoparticles production, microbes are very much effective in this process. New enzymatic approaches using bacteria and fungi in the synthesis of nanoparticles both intra- and extra cellularly have been expected to play a key role in many conventional and emerging technologies.

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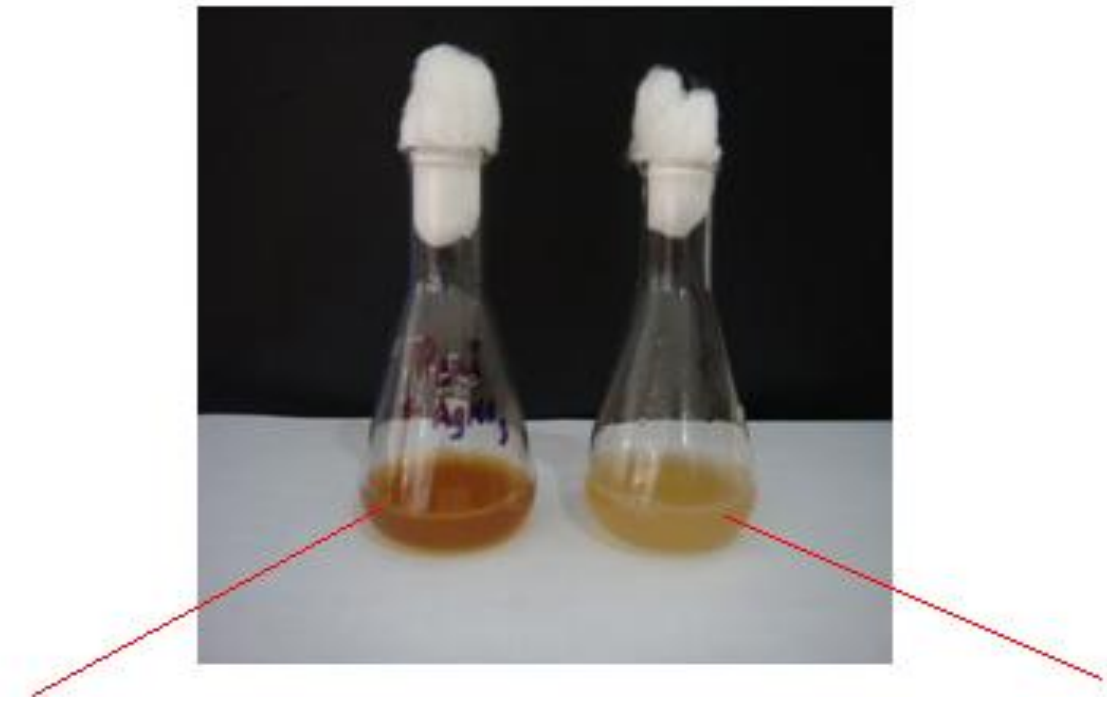
Figure- 1: Intracellular biosynthesis of silver nanoparticles by using fungi *Penicillium chrysogenum*



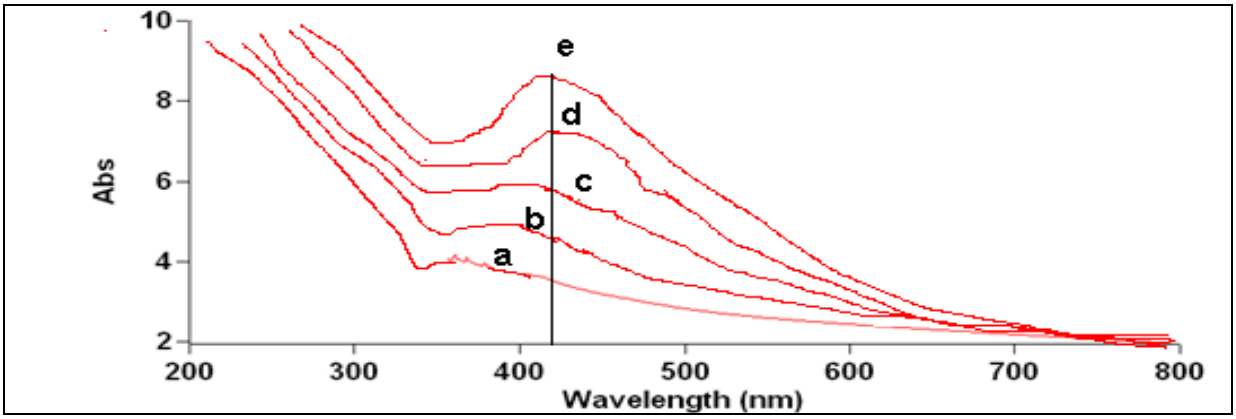
A) *Penicillium chrysogenum* treated with AgNO3

B) *Penicillium chrysogenum* after sonication (without AgNO3)

Figure- 2: Formation of secondary metabolite and extraction of bioactive compounds from *Penicillium chrysogenum*

A) Growth of *Penicillium chrysogenum* in SDB B) Extraction of Bioactive compounds

Figure- 3: Confirmation of silver nanoparticles by UV-Vis Spectrophotometry



a- 6 hrs b- 12 hrs c-24 hrs d-48hrs e-72 hrs

Figure- 4: FTIR spectra of AgNps synthesized using the intracellular filtrate

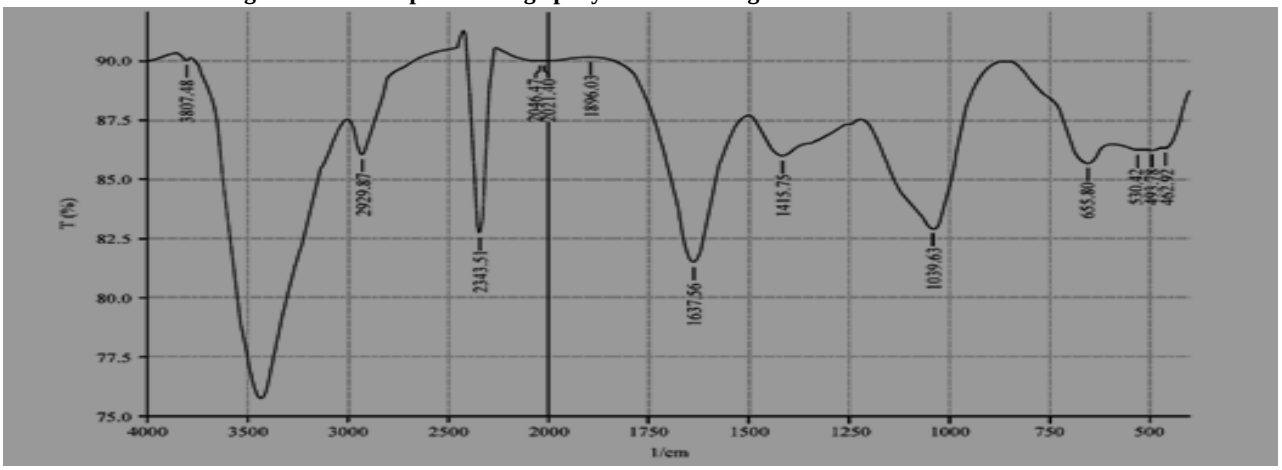


Figure- 5: SEM picture showing silver nanoparticles

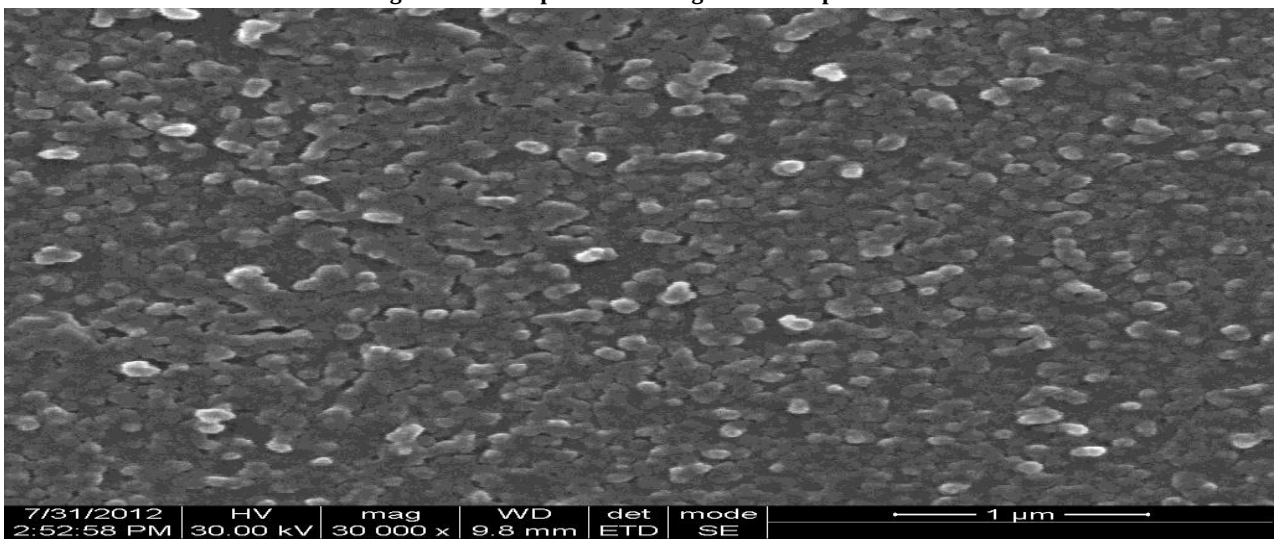


Figure- 6: X-ray Energy Dispersive spectra (EDS) of prepared silver nanoparticles

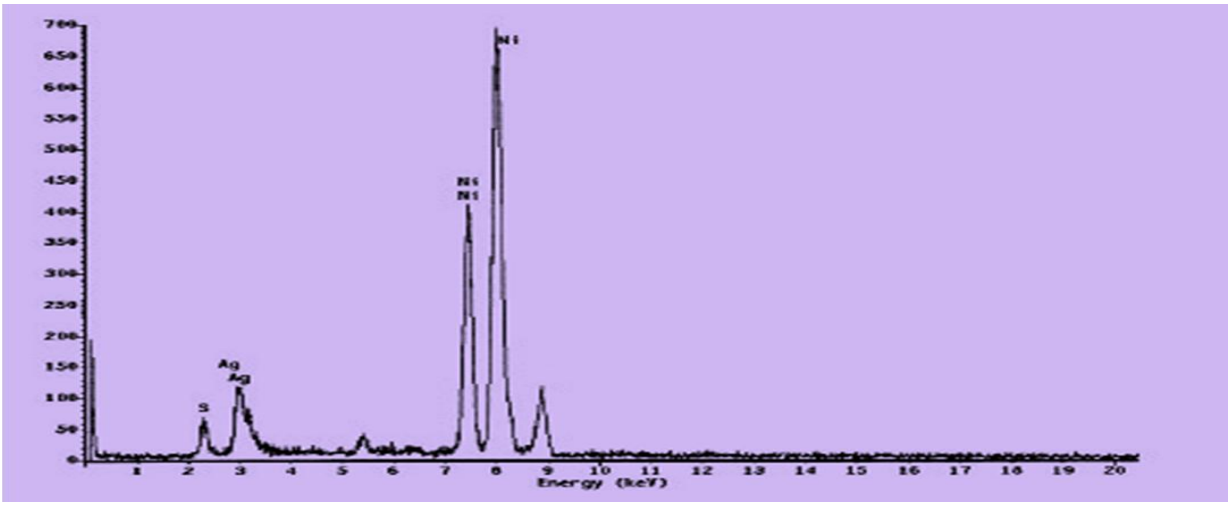


Table-1: Comparative analysis of antimicrobial activity of raw culture and silver nanoparticles synthesized intracellularly

Sl. No.	Name of the pathogens	Zone of inhibition in mm	
		Raw culture (mm)	Silver nanoparticles (Intracellular) (mm)
1.	*E. Coli*	9	13
2.	*Pseudomonas aeruginosa*	8	10
3.	*Bacillus cereus*	11	14
4.	*Staphylococcus aureus*	10	13

Table-2: Comparative analysis of antimicrobial activity of bioactive compounds and silver nanoparticles Intracellularly

Sl. No.	Name of the pathogens	Zone of inhibition in mm against different compounds	
		Bioactive Compounds (mm)	silver nanoparticles (Intracellular) (mm)
1.	*E. Coli*	13	13
2.	*Pseudomonas aeruginosa*	8	10
3.	*Bacillus cereus*	16	14
4.	*Staphylococcus aureus*	10	12