2. Materials and Methods

2.1 Intracellular biosynthesis of Ag nanoparticles using *Penicillium chrysogenum*

The fungal culture was grown aerobically in liquid medium containing KH_2PO_4, K_2HPO_4, $MgSO_4$. H_2O, $(NH_4)_2SO_4$, Yeast extract and glucose. The flask was inoculated and incubated on orbital shaker at 25^0C and agitated at 150 rpm. The biomass was harvested after 72 hrs of growth by filtration using ordinary filter paper. The biomass was followed by extensive washing with distilled water to remove all possible medium components from the biomass. Typically 20gm of biomass (fresh weight) was brought in contact with 200ml of Milli- Q deionized water for 72h at 25^0C in an Erlenmeyer flask and agitated for further 72 hours. The biomass 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 25^0C. 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.

Antimicrobial activity Test

Disc diffusion method was used for checking the antimicrobial activity of 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.

2.2 Nitrate reductase assay

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

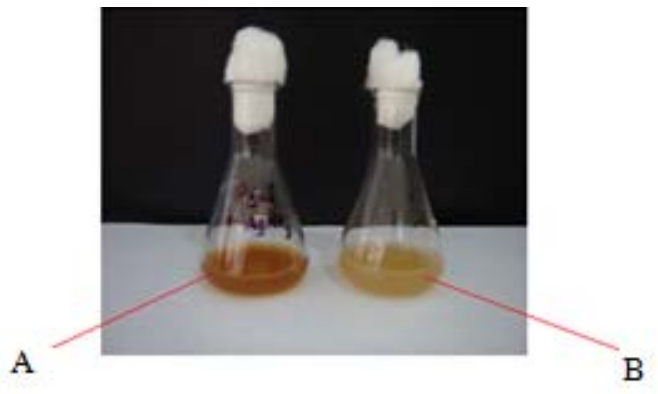
2.3 Experimental wound healing

The animals suffered dorsal surgery as described: after anesthesia (2,2,2-Tribromoethanol, Aldrich, 25mg/100g body weight - intraperitoneal injection) and after removing the hair from the dorsal skin, skin wound was made (4 mm diameter)[21], [22], [23]. Povidone-iodine was used as topical anti-infective in control animal. The wound received topical application of silver nanoparticles daily; iodine solution was applied immediately after surgery in the remainder. All the animals received veterinary pentabiotic, (0.4ml intra-muscular), immediately after surgery. The effectiveness of the drug is studied.

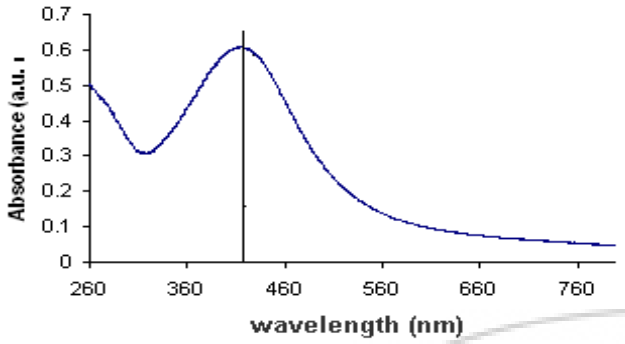
3. Results and Discussion

3.1 Intracellular biosynthesis of silver nanoparticles from *Penicillium chrysogenum*

The fungal culture was grown aerobically in liquid medium containing KH_2PO_4, K_2HPO_4, $MgSO_4$. H_2O, $(NH_4)_2SO_4$, Yeast extract and glucose[15]. The flask was inoculated and incubated on orbital shaker at 25^0C and agitated at 150rpm. The biomass was harvested after 72 hrs of growth by filtration using ordinary filter paper. The biomass was followed by extensive washing with distilled water to remove all possible medium components from the biomass. Typically 20gm of biomass (fresh weight) was brought in contact with 200ml of Milli- Q deionized water for 72h at 25^0C in an Erlenmeyer flask and agitated for further 72 hours. The 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 25^0C. The cell filtrate when subjected to $AgNO_3$ (Fig. 1b). The cell filtrate was subjected to $AgNO_3$ for intracellular synthesis of silver nanoparticles[16], the reaction was started and color of the solution turned to yellowish brown (Fig. 1a), indicating the formation of AgNPs. 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[17], [18]. The AgNPs exhibit a yellowish brown color in water, arising from excitation of surface plasmon vibrations in the metal nanoparticles. The formation and stability of the reduced AgNPs in the colloidal solution was monitored by using UV–vis spectrophotometry spectral analysis. The UV–vis spectra recorded from at different time intervals of reaction were plotted 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. 2). It is observed from the spectra that the silver surface plasmon resonance band occurs at 420 nm.



***A) Penicillium chrysogenum* treated with $AgNO_3$**
B) *Penicillium chrysogenum* after sonication (without AgNO3)
Figure 1: Intracellular biosynthesis of silver nanoparticles by using fungi *Penicillium chrysogenum*



a-6 hrs b- 12 hrs c-24 hrs d-48hrs e-72 hrs
Figure 2: Confirmation of silver nanoparticles by UV-Vis Spectrophotometry

3.2 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.3) 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.

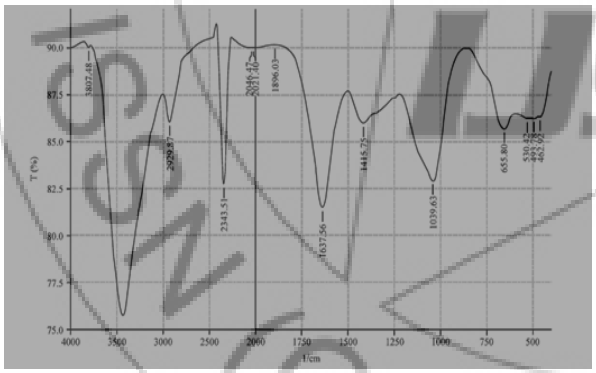


Figure 3: FTIR spectra of AgNps synthesized using the intracellular filtrate

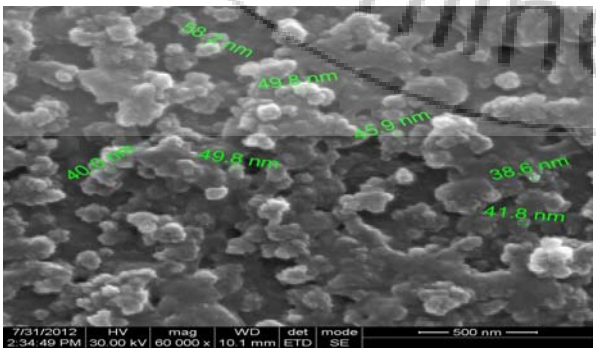


Figure 4: SEM micrograph of silver nanoparticle

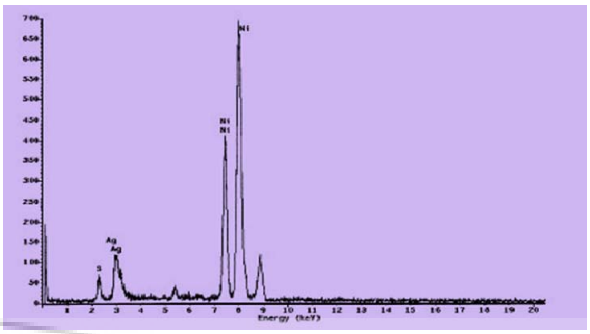
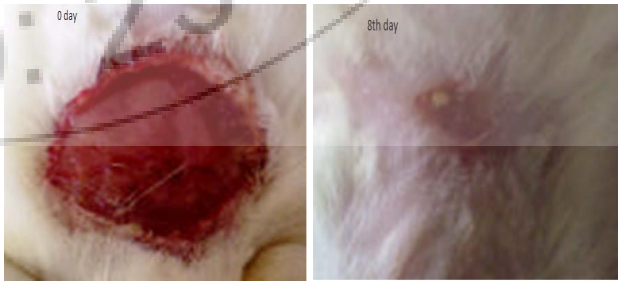


Figure 5: EDAX spectra of silver nanoparticles

3.3 Experimental wound

Three set of animals were taken for experiment. Group 1: animals not disturbed, fed with normal food and water –not wounded, Group 2: animals wounded and treated with nanoparticle sample and Group 3: animals wounded and treated with Povidone-iodine. The animals suffered dorsal surgery as described: after anesthesia (2,2,2-Tribromoethanol, Aldrich, 25mg/100g body weight - intraperitoneal injection) (Fig. 6) and after removing the hair from the dorsal skin, wound was made (15mm diameter). Povidone-iodine was used as topical anti-infective in control animal. The wound received topical application of silver nanoparticles. All animals received veterinary pentabiotic, (0.4ml intra-muscular), immediately after surgery. The effectiveness of the drug is studied. The diameter of the wound in nanoparticle treated animal was reduced to 7mm whereas it is 8mm in providone-iodine treated animal. During 18th day, the wound was completely cured and it is 1mm in providone-iodine treated animals. The comparative study shows that natural healing will take natural time[21]. Effective drug like nanoparticle will reduce the healing time of the wound. Jun Tian et al., investigated that the wound-healing properties of silver nanoparticles in an animal model and found that rapid healing and improved cosmetic appearance occur in a dose-dependent manner[22], also his studies showed that silver nanoparticles exert positive effects through their antimicrobial properties, reduction in wound inflammation, and modulation of fibrogenic cytokines. Heydarnejad M. S. et al., studies shows that nanoparticle interaction with components of the immune system and suggests that Ag-NPs have strong anti-inflammatory effects on skin wound healing and reduce scarring[23]. Hence the results have given the insight actions of silver and have provided a novel therapeutic direction for wound treatment in clinical practice.



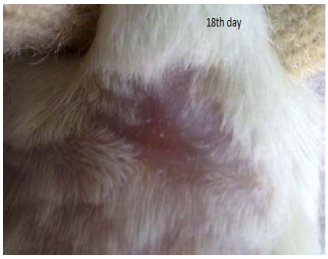


Figure 6: Silver nanoparticles synthesized from *Penicillium chrysogenum* accelerate wound healing

3.4 Antimicrobial Activity Test

Disc diffusion method was done for checking the antimicrobial activity of silver nanoparticles synthesized by intracellular methods against *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. The antimicrobial activity of intracellular nanoparticles and the raw culture filtrate was compared and it shows that the activity was more bio-nanoparticles (Table-1). The silver may enhance the activity. The bioactive compounds was treated against all the pathogens and zone of inhibition measured. 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*. Similarly the intracellular silver nanoparticles were treated against all the pathogens and zone of inhibition measured. Nanda and Saravanan (2009, 2010)[9&19] proved that silver nanoparticles synthesized extracellularly showed positive response against multidrug resistant pathogens. Our findings correlating with others findings showed good result when treated with intracellular silver nanoparticles. 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)[15]. Marcato et al, (2003)[20] 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.

The data constitute the study that noble metal nanoparticle 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.

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

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