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Mycogenic Silver Nanoparticle Biosynthesis And Its Pesticide Degradation Potentials

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ABSTRACT: Chemical pesticides are resistant to biodegradation beside carcinogenic in nature even at trace levels. Effort to remove pesticide by conventional methods has several inherent disadvantages of longer time or cost and this bottleneck can be avoided by utilizing nanoparticles as it can mineralise at a faster rate. Nanoparticles are syntheised by reduction of metal ions using physical, chemical and biological process either alone or in combination. In the present study biosynthesis of silver nanoparticle (AgNPs) using hydrophilic fungal secretes (HFS) of Penicillium pinophilum and degradation of chlorpyrifos pesticides in different pH environment has been reported. The AgNPs formed were characterized by using UV-Visible spectrum, FTIR and XRD. The HFS is the supernant of 72h incubated 10 g of mycelial mat in 100 ml deionized water at 28°C. AgNPs were synthesized by the reaction of HFS with 1 mM silver nitrate solution at 1:5 ratio. The spectrophotometric absorption peak at 420 nm is due to the Surface Plasmon response (SPR) properties of AgNPs. FTIR anlysis of HFS revealed functional groups are 1096 cm^{-1} (C-O & C-C Stretch), 1664 cm^{-1} (C=C Stretch), 2907 cm^{-1} (C-H Stretch), 2931 cm^{-1} (C-H Stretch), 3475 cm^{-1} (O-H, N-H Stretch). The outranking of functional groups in Ag^{+} reduction using depletion percent of FTIR bands before and after reaction revealed the O-H (Alcohols, phenols) and C-H (Alkenes) functional groups showed highest (>40%) whereas functional group esters were the lowest (<10%). AgNPs was examined by the XRD pattern of diffraction peaks showed at 2θ values of 32.23°, 46.18°, 64.82° and 77.21° assigned to the planes of (111), (200),(220) and (311) faced centre cubic (fcc) of silver nanoparticles. The chlorpyrifos degradation by AgNPs showed highest in mild acidic (pH-6) followed by alkaline (pH 9) and astrong acidic (pH 0.3) environment.

Keywords : Penicillium pinophilum, extracellular fungal secrete, Silver nanoparticles, pesticide degradation, Chlorpyrifos, FTIR, XRD

1 INTRODUCTION

Environmental pollution due to pesticides is an important problem in developing countries due to indiscriminate utility for pest and diseases management. The awareness about the risk associated with pesticide contamination is increasing and the allowable limits are revised and the permissible limits are expected to reach molecular levels in the coming years. However, pesticides are still utilized to ensure the food supply for the ever growing population "[1]". Chlorpyrifos (CP) (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothionate) is a broad-spectrum insecticide act as a cholinesterase inhibitor and used to kill a wide variety of insects by disrupting their nervous system. Due to hydrophobic nature of organophosphate pesticide it adsorbs to soil particles and reduces its runoff to natural water system. Its abundant use poses a serious threat to environmental quality and public health. The potential risk associated with the use of these pesticide necessities to find out the ways based on biological and biotechnologies approaches to mitigate the ill-effects of Chlorpyrifos on environmental quality and public health. Though the conventional bioremediation is a good option for the degradation of Chlorpyrifos however it has several operational constraints like slow rate of biodegradation, requiring long time, and incomplete degradation resulting more harmful degradation products. Nanotechnology is applied as alternative and highly effective solutions for the environmental cleanup and remediation. This is mainly due to their chemical reduction properties, high surface area and surface reactivity of nanoparticles. There are a large number of physical, chemical, biological, and hybrid methods available to synthesize different types of nanoparticles "[2],[3]". Although physical and chemical methods are more popular in the synthesis of nanoparticles, the use of toxic chemicals greatly limits their biomedical applications. Compared with the traditional synthetic methods, biological systems provide a novel idea for the production of nanomaterials "[4]". Physical and chemical methods limitation on nanoparticle synthesis increases the interest in the use of biological agents as tools for synthesizing nanoparticles "[5]". The properties of nanoparticles depend on chemical composition, size, shape, composition and their environment including their spatial distribution "[6]". Thus the synthesis techniques can affect considerably the properties of the nanoparticles. Nanoparticles are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymes generated by the cell activities. Mycogenic nanoparticle provides highly effective solutions to environmental cleanup and remediation by using zero-valent nano particle as a chemical reductant. Nanoparticles are the clusters of atoms in the size range of 1–100 nm and metal nanoparticle in the nanometre size exhibit unique physical properties due to higher surface to volume ratio which results in increased catalytic activity due to morphologies with highly active facets. The enhanced reactivity of nanomaterials is mainly due to their large number of edges, corners, and high-energy surface defects. Both intracellular and extracellular production of nanoparticles from inoganic materials is a well known attribute of many microorganisms including fungi. Fungi are excellent secretors of protein resulting into higher yield of nanoparticles. Mostly fungi are regarded as the organisms that produce nanoparticles extracellularly because of their enormous secretary components, which are involved in the reduction and capping of nanoparticles resulting stabilization. Fungi provide a broad variety of bioactive secondary metabolites with unique structures they could be the explored for their ability to biosynthesis of silver nanoparticles to developed an efficient environment friendly process. Many of the proteins secreted by fungi are capable of hydrolyzing metal ions quickly and through non-hazardous processes. In addition, nanoparticles of high monodispersity and dimensions can be obtained from fungi "[7]". The main reaction occurs during biosynthesis of nanoparticles involves reduction/oxidation of substrates, giving rise to colloidal structures. Microbial enzymes with antioxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles. Mycogenic nanoparticle production have been achieved by different workers using various fungal species like extracellular synthesis

and stabilization of silver nanoparticle (AgNPs) using Aspergillus flavus "[8]", Aspergillus tamarii "[9]", Cladosporium cladosporioides [10], Penicillium citrinum "[11]", extracellular production of gold, silver and bimetallic Au-Ag alloy nanoparticles by the fungus Fusarium oxysporum "[12], [13]", etc. Recent research investigates the use of fungi for their potential redox systems using silver nitrate as the source of silver ions "[14]". There has been considerable interest in the potential use of fungi and fungal systems as nanofactories for the synthesis of metal nanoparticles "[15]". These particles are typically produced as a result of reactions between fungal biomass or its metabolites and aqueous metallic salt solutions. Biosynthesis of silver nanoparticles using fungal species like Amylomyces rouxii, Penicillium sp., "[16]", P. janthinellum, Pestalotia sp., Aspergillus clavatus "[17]", A. concius, Epicoccum nigrum and Phomopsis sp. have been reported by various workers "[18], [19]". Fungi have the potential to produce nanoparticles faster than some chemical synthesis methods. Many of the proteins secreted by fungi are capable of hydrolyzing metal ions quickly and through non-hazardous processes. These particles are typically produced as a result of reactions between fungal biomass or its metabolites and aqueous metallic salt solutions. Nanopaticle biosynthesis mainly depends on optimum concentration ratio of metal salt and reducing agent. Silver nanoparticles are the nanocrystals grown from Ag+ solutions after reduction to atoms by means of a reducing agent and these atoms then nucleate in small clusters that grow into particles which depend on the availability of atoms in the reaction mixture. There are many possible uses for biologically produced nanoparticles in diverse applications in different fields "[20]". In the present study, an attempt has been made to investigate the potentiality of Penicillium pinophilum for their ability to synthesize silver nanoparticles, identification of active compound responsible for biosynthesis by FTIR, and the size distribution of the biosynthesized nanoparticles were studied by using XRD techniques and finally the mineralization of chlorpyrifos pesticide in different pH environment.

2. METHODS

2.1 Nanoparticle Biosynthesis

Fungal isolates of sporadic, fast growing and average random fungal species were isolated according to the dilution –plate method as described by Jonson and Curl "[21]" for isolation of soil fungi. All chemicals used were of analytical grade. For the UV-vis calibration curves, we have used Silver nitrate ($AgNO_3$, 99%) was purchased in Sigma-Aldrich. We have used de-ionized water for silver nanoparticle biosynthesis. Fungi Penicillium pinophilum was identified based on the morphological and microscopical observations. These cultures were maintained in PDA (potato dextrose agar) medium and the pH was adjusted to 6.5. The pure culture established in PDA media and these plates were incubated 3-5 days for proper sporulation. For the synthesis of AgNP(silver nanoparticles), the biomass of the fungal isolate was grown aerobically in potato dextrose broth containing infusion of 200 g potato and 20 g dextrose per litre of de-ionized water. Potato Dextrose (PD) broth was prepared, and inoculated with the conidial suspensions. The inoculated flasks were incubated on incubated shaker at 25 ± 2°C and agitated at 120 rpm for 96 h. The biomass was harvested after incubation by filtering through filter paper followed by repeated washing with de-ionized water to remove any medium component from the biomass. 10 g (wet weight) of fungal biomass dispensed in 100 ml of pre-sterilized de-ionized water and incubated for 48 h at 28 ± 2°C in a 250 mL Erlenmeyer flask and agitated at 120 rpm. After the incubation, the cell filtrate i.e. extracellular fungal secretes was obtained by filtering it through Whatman filter paper No. 1. The filtrates were again centrifuged at 10000 rpm for 10 min to remove all the cell debris and conidia from the extracellular fungal secretes. The fungal secrete obtained were treated with 1 mM $AgNO_3$ solution (1:5 vol/vol) in an Erlenmeyer flask and incubated at 25°C in dark. A control was maintained where equal volume of deionized water added in lieu of fungal secretes.

Fig. 1 *Silver nanopartical bio-synthesis using extracellular fungal secretes (a) Penicillium pinophilum culture in PDA media, (b) Fungal secretes after synthesis of AgNPs (c) AgNP suspense, (d) AgNP palates after centrifugation at 12000rpm (d) Ag nanoparticles.*

2.2 Characterization techniques

2.2.1. UV–visible spectroscopy analysis: Change in colour from light yellow to dark yellow in fungal secretes with $AgNO_3$ reaction mixture during incubation showed the formation of AgNPs. The reaction was monitored at different time intervals by taking absorption spectrum in UV-Visible spectrophotometer (Simadzu UV1700) at 200-800 nm.

2.2.2 FTIR analysis of fungal extract: The cell free fungal secretes were concentrated by centrifugation at 12,000 rpm for 25 min and then freeze-dried in lypholizer (Modulyo D230, Thermofisher) for crystalline powder. FTIR (Fourier transform infrared) spectra was analyazed by KBr pellets methods using FTIR spectrophotometer [Perkin Elmer, Spectrum two FTIR, Standard DTC (Dithiocarbamates) KBr (Potassium bromide)] to investigate the functional groups present in the MIR (Mid Infrared) region of 400 – 4000 cm^{-1} in the fungal secrete. The fuinctional groups were assigned referring FTIR data available in published literature. FTIR analysis was carried for HFS samples before and after Ag^+ reduction reaction. Thereafter the depletion of percent of fuctional groups were caluclated as follows Functional group depletion = (I-R)/A x 100 where I is the initial and R is residual percent recorded in FTIR spectrum. The obtaioned data were classified as >40%, 30-40%, 20-30%, 10-20% and <10% depletion of functional groups.

2.2.3 XRD measurement: The freshly synthesized AgNPs were concentrated by centrifugation of the reaction mixture at 12,000 rpm for 15 min; thereafter the pellet was again re-dispersed in deionized water to remove unwanted contaminants. The washed pellets were then freeze-dried in lyophilizer for crystalline powder. XRD analysis of the AgNPs crylster powder was carried out using Philips PW 1830 maintaining operating voltage of 40 kV and current of 30mA with Cu Kα radiation of 0.1541 mm wavelength, in the 2θ range 10- 80º, step size 0.02/θ.

2.2.4 Chlorpyrifos degradation efficiency of mycogenic nanoparticles: Chlorpyrifos degradation by mycogenic AgNps was investigated at three pH conditions (strong acidic, mild

acidic and basic adjusted by NaOH and HCl). The reaction matrix for AgNPs, Chloropyrifos and pH (3, 6 and 9) are presented in Table-1. Residual Chloropyrifos was measured using UV-visible Spectrophotometer (Shimadzu UV 1700, Japan) at 298 nm different at time interval``[22]'',``[23]"against a standard curve. The Chloropyrifos degradation percent was calculated as follows

Chloropyrifos degradation percent= (Ci-Cr)/Ci*100

Where Ci and Cr are the intial and residual Chloropyrifos content. The Chloropyrifos degradation percent value was plotted against time and calcuated the R^2 values for linear equation.

3. Results and discussion

The hydrophilic fungal secretes (HFS) after freeze drying obtained dried pellet and the yield was 0.3 mg ml^{-1}. The reaction between Ag ions in AgNO3 solution with HFS was noticed with change in colour from yellowish green tinge to yellowish brown and the intensity increased with incubation time where control with Ag ions with out HFS reaction the color remain unchanged. The generation of yellowish-brown colour in aqueous Ag solution is due to the surface Plasmon resonance (SPR) exhibited by the nanoparticles and is typical of the silver nanoparticles "[24]".

TABLE 1
REACTION MIXTURE FOR CHLORPYRIFOS AND MYCOGENIC NANOPARTICLE MATRIX

Treatment	AgNPs (ml)	Chloropyrofos working stock (ml)	Chloropyrofos PPM	H_2O ml	Total (ml)	Incubation temperature °C
+ve control	0	2	2	23	25	25
pH6						
pH3	5	2	2	18	25	25
pH6	5	2	2	18	25	25
pH9	5	2	2	18	25	25

3.1 UV-Visible analysis:

UV-visible spectroscopy is one of the most widely used techniques for structural characterization of AgNPs. The progress of Ag^+ reduction to Ag NPs was be monitored in UV-Vis spectrophotometer. The absorption spectrum after 72h of treatment is presented (Fig.2). The hydrophillic secretes (HS) of Penicillium pinophilum reacted with Ag^+ showed a characteristic surface Plasmon absorption band with a peak absorption at 420 nm, indicating the presence of Ag NPs. The wavelength of the plasmon absorption maximum in a given solvent can be used to indicate particle size.The typical absorption spectrum of AgNPs is in the band of 350 nm to 450 nm "[25]". In general with the increasing particles size the plasmon peak shifts to longer wavelengths and broadens. Our findings correlate with Singh et al "[26]" and Banu et al., "[27]"who used 1.0 mM AgNO3 for the production of AgNPs using endophytic Penicillium sp. and fungus Rhizopus stolonifera respectively. The absorption spectrum AgNPs colloids show surface plasmon absorption band with a maximum of ~420 nm, indicating the presence of lone spherical or roughly spherical AgNPs "[28]". The size of metallic nanoparticles ensures a significantly large surface area of the particles. Considering a hypothetical case with spherical particles of uniform size, a reduction in the particle size from ~10 µm to 10 nm will increase the surface area by 10^9. We observed strong absorption band in the UV range after 72h of incubation of the mycogenic AgNPs reaction mixture, the peak at 210 nm indicating presence of peptide bonds and at 280 nm due to tryptophan, tyrosine and/or phenylalanine present in the HFS. This observation indicates the release of proteins into filtrate that suggests a possible mechanism for the reduction of the Ag^+ present in the reaction mixture. The repeated washing of the AgNPs pelete with deionized water the residual metabolites was removed successfully for getting a clearer resolution of AgNPs.

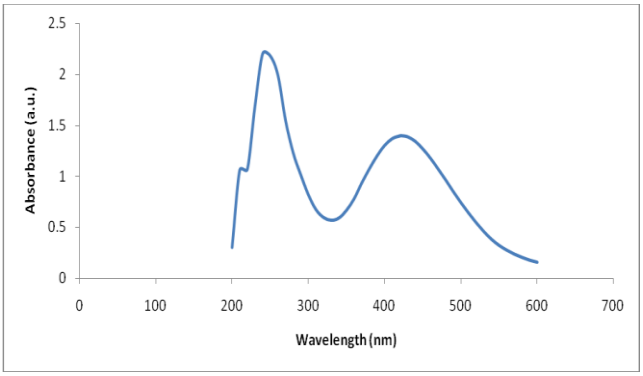

Fig. 2 *UV visible absorption spectra of bio-synthesized silver nanoparticles with absorption spectra obtained for Penicillium pinophilum derived silver nanopartilces. A characteristic peak at 420 nm wavelength is clearly observed, confirming the formation of Ag nanoparticles.*

3.2 FT-IR analysis:

FT-IR measurements of the dried samples were carried out to identify the potential functional groups present in the HFS of Penicillium pinophilum and their role in Ag+ reduction process (Fig.3). The transmitance spectrum showed 5 major zones and assigned the corresponding functiongroups such as i) 1250-1050 cm^{-1} Carboxylic Acid C-O, ii) 1680-1450 cm^{-1} Alkene C=C Aromatic ring pseudo double bonds, iii) 1850-1650 cm^{-1} Carbonyl (C=O) iv) 3200-2700 cm^{-1} Aryl. Aldehyde, carboxilic acid Alkyl C-H and v) 3200-3700 cm-1 Alcohol OH, amine N-H, Alkyne C-H. It was found that all the functional groups present in HFS does not involve equally in Ag+ reduction. The involvement of functional groups calculated based on its percent exhaustion during Ag+ reduction were categorized into >40%, 30-40%, 20-30 %, 10-20% and <10% (Table-2). In the Ag^+ reduction process the O-H (Alcohols, phenols) and C-H (Alkenes) functional groups get depleted highest (>40%) that essentially indicates their significant role in AgNP formation. This is in agreement with Obreja et al "[29]" for synthesis of platinum nanoparticles and Alagumuthu and Kirubha, "[30]" for silver nanoparticle using alcoholic media. From the wavenumber involvement study it is observed that the functional group esters are least depletion in Ag+ reduction process. However, esters, secondary and tertiary amides of Catharanthus roseus flower are reported for synthesis and stabilization of selenium nanoparticles "[31]". Probably this is the first report to outrank the functional groups of HFS in Ag^+

reduction and mycogenic nanoparticle formation. The FT-IR spectrum of HFS shows the presence of broad absorption band between 3647 and 3048 cm^{-1} due to the O–H stretching and H- bonded alcohols and phenol groups (Figure 3). The bands at the representative mid spectra of nanoparticles obtained absorption peak located at about 3475cm^{-1} (NH group of amines, -OH group of phenols), 2931cm^{-1} (C-H stretch), 2907 cm^{-1} (aromatic-CH stretch), 1664cm^{-1} (-C=C stretch) and 1096 cm^{-1} (C-O, C-C & C-N stretch) (Table-2). Similar results also reported earlier by Naveen et al., "[14]". The bands at 1664 cm^{-1} correspond to primary amine NH band, and those at 1096 cm^{-1} correspond to secondary amine NH band and primary amine CN stretching vibrations of the proteins respectively.

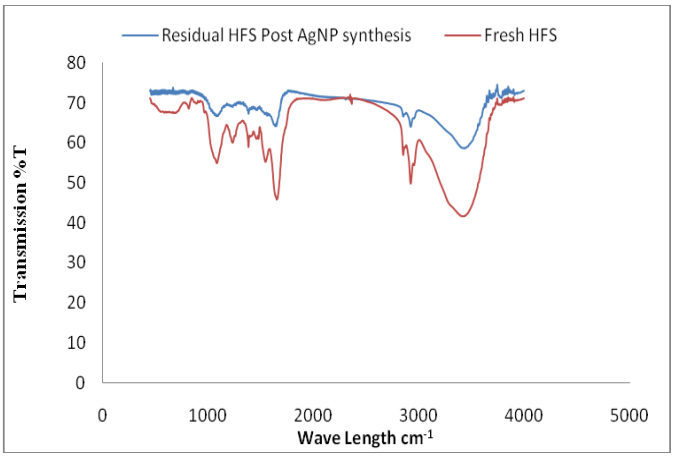

Fig. 3. *FT-IR absorption spectra of HFS of Penicillium pinophylium before and after Ag+ reduction process.*

3.3 XRD analysis:

The dry powders of the silver nanoparticles were used for XRD analysis. The diffracted intensities were recorded from 20° to 80° at 2 theta angles. The intensity of the diffraction was much stronger than those of the other diffractions. The XRD diffraction measured in this case result-ed in four intense peaks shown in figure 4. For confirmation of synthesized AgNPs was examined by the XRD pattern of diffraction peaks showed at 2θ values of 32.23°, 46.18°, 64.82° and 77.21° assigned to the planes of (111), (200),(220) and (311) faced centre cubic (fcc) of silver were obtained ranging from 10 to 80 (Fig.- 4). The values agree well with those reported for silver (face centric cubic) by Joint Committee on Powder Diffraction Standards File No. 04-0783. Similar results were also reported by other worker on different species "[32], [33]". The high peaks in the analysis indicated the active silver composition with the indexing. The intensity of peaks reflected the high degree of crystallinity of the silver nanoparticles. The diffraction peaks are broad indicating the very small crystallite size of nanoparticles "[23]".

TABLE 2
ROLE OF FUNCTIONAL GROUPS PRESENT IN PENICILLIUM PINOPHYLIUM EXTRACELLULAR HYDROPHILIC SUBSTANCES IN AG+ REDUCTION TO NANOMETAL

Role in Ag+ reduction (%)	FT-IR wave-number, cm^{-1}		Bond	Functional group and class
> 40	3455	3296	OH stretch	Alcohols, phenols
	1667	1651	C=C stretch	Alkenes
30-40	3558	3456	NH stretch	Primary amines & amides
	3295	3212	O-H, N-H	Carboxylic acid, primary amines & amides
	1684	1668	C=C	Imines & oximes
	1650	1624	C=C	alkene
	3621	3559	OH Stretch	Alcohols, phenols
20-30	3211	3126	N-H	Primary & Secondary amines
	2964	2910	C-H	Aldehyde
	1704	1685	–C=C– stretch	Alkenes
	1623	1608	N–H bend	Primary amines
	1557	1534	N-H Bend	Amides
	1093	1068	C–N stretch	Aliphatic amines
	3674	3622	N-H stretch	Amide
	3125	2965	C-H stretch	Alkenyl
10-20	2909	2841	C-H stretch	Alkyl
	1754	1705	C=O	Ester
	1607	1558	C=C	Aromatic
	1533	1506	C=C	Aromatic
	1485	1435	N-H Bend	Amines—Secondary
	1431	1374	N=O Bend	Nitro Groups
	1275	1202	C-O stretch	Esters
	1153	1094	C-O stretch	Esters
	1067	1009	C-O stretch	Esters
	2840	2644	C-H	Aldehyde
	1886	1755	C=O	Carboxylic Acid
	1505	1486	C-C=C Asymmetric stretch	Aromatic Rings
2-10	1434	1432	O-H	Carboxylic Acid
	1373	1276	C–N stretch	Aromatic amines
	1201	1154	C–N stretch	Aliphatic amines
	1008	450	C-H	Aromatic

The observed peak broadening and noise were probably related to the effect of nanosized particles and the presence of various crystalline biological macromolecules in the fungal secretes. The obtained results illustrate that silver ions had indeed been reduced to AgNPs by Penicillium pinophilum secretes under reaction conditions.

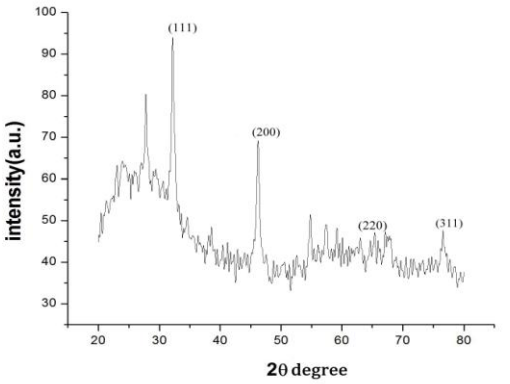

Fig. 4. *A typical XRD pattern of Ag-NPs synthesized from Penicillium pinophilum secretes*.

3.4 Chlorpyrifos degradation potentiality of AgNPs:

Chlorpyrifos degradation by mycogenic AgNPs was investigated under three different pH conditions (3, 6 and 9). The residual chlorpyrifos decreased with increasing duration 0, 30 min, 60 min, 90 min, 48 hrs, 72 hrs and 86 hrs (Fig. 5). Chlorpyrifos degradation followed linear equation with significant R^2 value 0.949, 0.973, and 0.974 for pH3, pH6 and pH9 respectively. The cosistantly highest degradation was recorded in mild acidic condtion (pH 6) irrespective of incubation time.

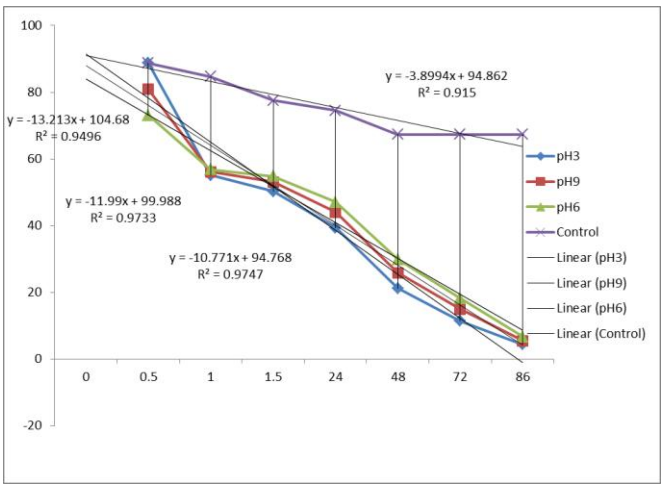

Fig. 5. *UV visible absorption spectra of degradation of chlorpyrifos using Penicillium pinophilum derived AgNPs.*

4 Conclusion

The Penicillium pinophilum could be utilized as sustainable source of hydrophilic secretes for synthesis of silver nanoparticles (AgNPs), which are simple and reproducible. In the Ag^+ reduction process the O-H (Alcohols, phenols) and C-H (Alkenes) functional groups get depleted highest (>40%) whereas functional group esters the least (<10%). This study has also shown that the excess pesticide can be removed from the crop field using Penicillium pinophilum derived AgNPs. The chlorpyrifos degradation by AgNPs showed cosistantly highest degradation in mild acidic condtion (pH 6) irrespective of incubation time. Considering the wide array of biotechnological tools available these indigenous strains may be consider for future development of other pesticide and metal removal technologies.

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