Myco-degradation pesticides using newly isolated strain of *Pleurotus ostreatus* from Tree Trunk of *Spathodia campanulata* and its bio-silver nanoparticles

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ABSTRACT

In this current work, biodegradation of pesticides by silver nanoparticles were studied was executed using new strain isolate edible mushroom *Pleurotus ostreatus* and its biologicaly made nano-Silver (AgNPs). AgNPs prepared using the extracts from mushroom were subjected to UV–VIS spectral analysis, FTIR, XRD and SEM. The results of UV-Vis Spectral analysis show SPR peak around at 473 nm, indicates the reduction silver to nanoparticles. FTIR spectrum shows the presence of functional groups such as amine, carboxyl, and hydroxyl corresponds to the silver nanoparticle decrease caused by the mushroom extract. The SEM image of bio-AgNPs prepared from mushroom extract shows the presence of spherical to elliptical shaped agglomerate ranging from 50-200 nm in diameter, with an average size of about 90 nm. Biodegradation potential of *Pleurotus ostreatus* and silver nanoparticles were evaluated against quinalphos and cypermethrin under three different pH conditions 5, 7 and 9 were investigated. The highest percentage of degradation of 96% was recorded in neutral condition (pH7) in both pesticides by *Pleurotus ostreatus* and their silver nanoparticles.

Keywords: myco-remediation, *Pleurotus ostreatus*, pesticide, silver nanoparticle

Introduction

Wheat, rice, pulses, sugarcane, and cotton are among the crops that India produces in the largest quantities. India is an agronomic country and its majority of the population relies on agriculture for their survival. Food grains produce of India has been increasing every year, the one of top producer of milk and second largest producer of vegetables & fruits. Pesticides and agrochemical plays an important role in increase in the crop yield and food production and became essential component in agriculture systems during the last century worldwide (Alexandratos and Bruinsma, 2012). The pesticides are array of chemicals used as insecticides, fungicides, rodenticides, weedicide nematicides etc., worldwide in order to destroy pests, weeds and cure diseases in crops. Many studies showed that Occupational

exposure is one of the major concerns in farm workers and pesticide applicators cause serious health effects. Moreover, few farmers in agriculture practice got touch and breathe in pesticides, suffers from acute and chronic poisoning and few got severe side effects such as pesticide induced cancers. Improperly processed fruits, vegetables and food grains are the most common methods for pesticide exposure by infants, children, and adult human beings. Residual pesticides in soil and water are recognized as caricinogenic pollutants causing significant threat to environment in many countries. (Dich J et al.,1997, Bressa G et al.,1997). Therefore, enormous application of these recalcitrant pesticides over past 50 years turned in to serious health hazard to the human population (Kolpin DW et al.,1998). These become threat to water systems to become polluted are now well agreed and steps are necessary for the protection of quality of our water.

Bio remediation of the soils is one of the novel approach in recent years, detoxification and remediation studies using spent mushroom substrate (SMS) on its application to soil, has reduced the toxic elements and other residue due to its laccase activity, makes as attractive organic nutrients. (Chiu, S.W. et al., 2000). Purnomo et al., (2010) examined the bioremediation potentials of DDT-polluted soil using Pleurotus *ostreatus*, where he used the SMS. The results showed that SMS efficiently degrades 50% of DDT within 28 days of incubation of which 5.1% of the DDT was completely mineralized after 56 days. In another study significant dissipation pesticide such as chlorothalonil and imidacloprid and significant improvement in the microbial activity was noted after application of biomixtures containing different SMS from *Pleurotus eryngii*, *Flammulina velutipes*, and *Lentinula edodes* (Gao et al., 2015). To ensure the availability of food for the world's expanding population, pesticides are still used. Quinalphos (Organophosphorous) and Cypermethrin (Synthetic pyrethroid) are wide range insecticides routinely used for killing and their mode of action is disruption of nervous system. Due to hydrophobic nature of organophosphate pesticide it adsorbs to soil particles and reduces its run off 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 Quinalphos (Organo phosphorous) and Cypermethrin (Synthetic pyrethroid) on environmental quality and public health. Though the conventional bioremediation is the one of best method degradation of Quinalphos and Cypermethrin, however it has many operation difficulties such as slower biodegradation efficiency, sometimes results in incomplete degradation yielding more harmful products.

Myconanotechnology is applied as alternative and highly effective solutions for the environmental clean-up and remediation. Mycogenic nanoparticle are efficient nanoparticles having wide range of application and helps in bioremediation of environment using zero-valent nano particle as a chemical reductant. Realizing impacts of pesticides in our water systems, humans, animals and environment, an attempt has been made to study the effects of mushroom and their mycogenic nanoparticles in the bioremediation of pesticide.

So in this study, an attempt has been made to evaluate biodegradation potentials of *Pleurotus ostreatus* and its mycogenic silver nanoparticle against Quinalphos and Cypermethrin pesticides in different pH environment

2. Materials and Methods

The mushroom was collected from the premise of college, and cultured on Potato Dextrose Agar under aseptic conditions and maintained at 25 ± 1^0 C. The strain is isolated and the species of the mushroom is identified by using DNA sequencing method.

2.1 Molecular identification of fungus by rDNA –ITS region amplification and sequencing

Isolation of genomic DNA

Fungal cultures (5to7 days Old) grown in Potato dextrose broth were subjected for Genomic DNA extraction (Doyle and Doyle, 1987). Isolation of DNA was done by preheated cetyltrimethyl ammonium bromide buffer (CTAB) at 60°C in a water bath (100 mMTris, 1.4 M NaCl, 1.11% EDTA and 2% CTAB). After harvesting the Mycelium, 3 volumes of CTAB buffer was added and ground sterile mortar and pestle. The mixture was incubated at 65^{o}C for 30 min. Following incubation, the mixture was added with equal volumes of Phenol: Chloroform mixed vigorously for 10 minutes and kept at RT. The aqueous layer was separated and re-mixed with equal volumes of chloroform: isoamyl alcohol (24:1) vigorously and kept at RT. The aqueous layer was transferred to new Eppendorf and extracted with 100% of ethanol overnight centrifuged at 4°C for 10 minutes and the pellet was washed with 75% ethanol and resuspended in TE buffer. Purity and quality of DNA was determined using UV- Vis Spectrophotometer. Isolated at DNA was kept at -25 °C until use.

Amplification and sequencing of ITS region of genomic r-DNA

The fungal ribosomal r-DNA (ITS1, 5.8S, and ITS2) gene regions were amplified using universal primers (White *et al*., 1990). The ITS1 region forward primer sequence was 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2-reverse primer sequence was 5'-GCTGCGTTCTTCATCGATGC -3' was used and the genomic DNA was amplified using Thermal cycler (Thermo scientific). The amplified DNA was subjected to agarose gel electrophoresis and examined through Gel doc system. The amplified ITS region fragment was sequenced using Sangers method (Eurofins Scientific, Bangalore) using ITS primers. The sequence was blast using GenBank Nucleotide Database the algorithm Blast N (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990).

Phylogenetic analysis and Evolutionary relationships of taxa

Version 7 of MEGA software was used for the evolutionary analysis. The Neighborhood-Joining approach was used to examine the evolutionary history (Tamura K. et al., 2016; Nei M. et al., 1987). The Maximum Composite Likelihood technique was used to calculate the evolutionary distances.

2.2 Preparation silver nanoparticles

About 20g (wet weight) mycelium was harvested and washed with deionized water to remove any remnant medium. The harvested fungal biomass was homogenized with deionized water and it was filtered using Whatman No: 4 filter paper and stored at 4^0C until use. 1mM of

Silver nitrate ($AgNo_3$) solution was prepared and utilized for the synthesis of silver nanoparticle. The supernatant containing the proteins and other materials was used as stabilizing agent. To synthesis silver nanoparticles, mushroom filterate was added to the 50ml 1mM $AgNO_3$ solutions and kept under constant stirring at room temperature for 24 hours (Madhavi *et al*., 2013).

2.5 Characterization of silver nanoparticles

UV–Visible Spectroscopy Analysis

The preliminary indication of AgNPs production was examined by the color change from off white to dark brown within 1hr. Further, it has been characterized by UV-Visible Spectroscopy (Thermoscientific). The process of reaction between $AgNO_3$ and fungal biomass extract was monitered by UV-Visible Spectra at 200-600 nm.

Fourier Transmission Infrared Spectroscopy (FTIR)

Prepared AgNPs sample was completely air dried at room temperature; the powdered AgNP's were collected and taken for FTIR analysis using Perkin Elmer Spectrum FT-IR Spectroscopy in the range of 450 to 4500 cm^{-1}.

Scanning Electron Microscope imaging of AgNps

Bio synthesized silver nanoparticle was prepared and dried completely into a fine powdered sample. The sample was placed in a copper grid and coated using carbon support film. The copper grid was allowed to dry and Scanning electron microscopic imaging was carried out using (SEM) JEOL model JEM-2000EX

XRD measurement

The XRD analysis of the AgNPs crystalline powder was performed using Philips PW 1830 X-Ray diffraction spectrophotometer. The AgNps were subjected to 40 kV operating voltage and 30mA current, Cu Kα radiation of 0.1541 mm wavelength, 2θ angle was set at a range 10- 80º, with an interval of 0.02/θ.

2.6 Pesticides degradation Potential of *Pleurotus ostreatus*

At three pH levels—pH 5, pH 7, and pH 9—*Pleurotus ostreatus* was observed degrading pesticides (acidic, basic and neutral adjusted by NaOH and HCl). To inoculate 150 ml of media in 250 ml Erlenmeyer flasks, P. ostreatus mycelium was cultured in Erlenmeyer flasks with Potato Dextrose medium. The flasks are filled with a pesticide like Quinalphos (2PPM) and incubated at 25°C. For a further 15 days, Pleurotus ostreatus's pesticide degradation potential was assessed. Similar to this, three different pH settings were used to test how pesticides degraded mycogenic AgNps (acidic, basic and neutral adjusted by NaOH and HCl). The HPLC method was used to quantify residual pesticides in the samples. A centrifugal tube was filled with 10 ml of the sample, followed by 40 ml of a 99:1 hexane and acetone combination, which was then incubated for 10 minutes. For three minutes, the refining was extracted. Anhydrous sodium sulfate was used to filter the mixture, and 1 mL of hexane was used to wash the residue. The filter liquor was assembled and nearly dried in the evaporating dish at N2. The material was then eluted with 80.0 mL of a petroleum ether and ethyl acetate solution (9:1). The entire eluent was collected, concentrated entirely in a water bath at 40°C, and reduced to 5.0 mL with hexane after a small amount of hexane was added,

spin evaporated, and added. HPLC was then used to evaluate the material. Used for the study was chromatographic column C18 (250 mm x 4.6 mm). Hexane and tetrahydrofuran were combined to form the mobile phase, which was then ultrasonically degassed (99:1). The flow rate was 1.0 mL/min, and the detector wavelength was 254 nm. According to the aforesaid process, a 10.00 L aliquot of this solution was injected into the HPLC and detected by UV. Identification and measurement. Using calibration curves, quantitation was performed, and identification was based on the retention time of the pesticide.

$$\text{Pesticides degradation (\%):} \quad \frac{\text{Initial Concentration} - \text{Final Concentration}}{\text{Initial concentration}} \times 100$$

The Pesticides degradation percent value was plotted against time.

2.7. Statistical Analysis

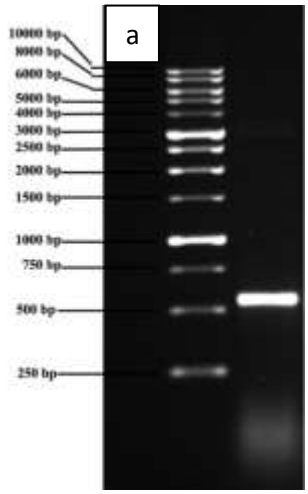
The results were compared using one-way ANOVA. SPSS software was used for the analysis P value < 0.05 was taken as least significant.

3. Results

3.1 Phylogenetic analysis of *Pleurotus ostreatus*

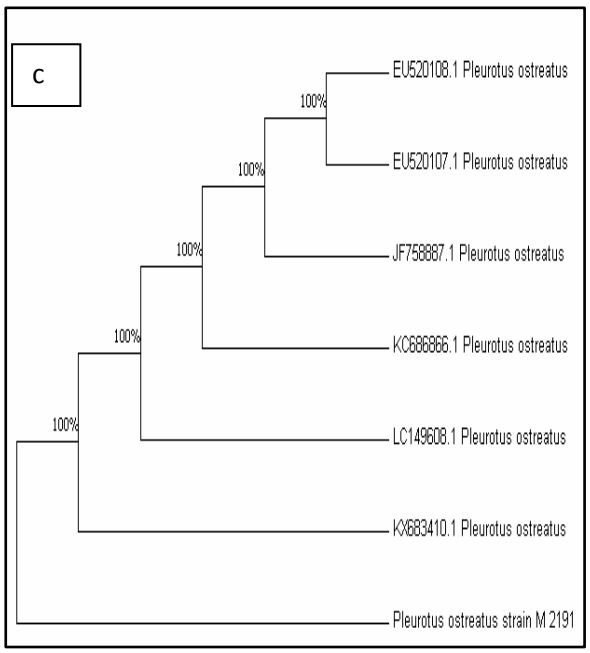
A 5.8S fragment of the r-DNA ITS region was amplified with the molecular weight 600bp (Figure 1a) of which 293 base pairs were sequenced, the results were shown in the figure 1b and submitted to NCBI gene bank database. The phylogenetic tree was made by aligning the sequences of Agaricomycetes organisms (figure1c). Based on the sequences, phylogenetic analysis the organism isolated from tree Trunk of *Spathodia campanulata* belongs to the genus of *Pleurotus* and species ostreatus.

Figure: 1 Molecular and Phylogenetic analysis of *Pleurotus ostreatus* a) PCR amplified DNA b) Amplified gene sequence of ITS region c) Phylogenetic Tree based on the amplified gene sequence of *Pleurotus osteratus*



b

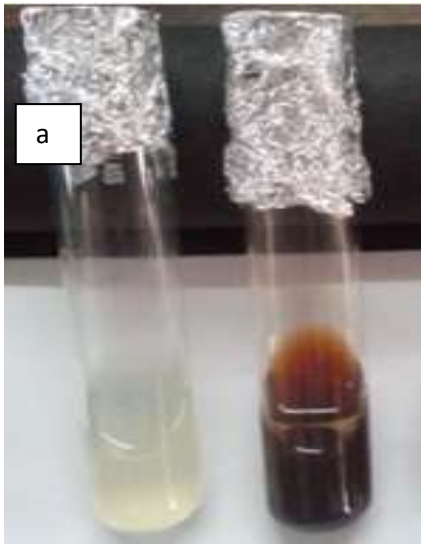
TAGGTGAACCTGCGGAAGGATCATTAATGAATTCACTATGGAGTTGTTGCT
GGCCTCTAGGGGCATGTGCACGCTTCACTAGTCTTTCAACCACCTGTGAAC
TTTTGATAGATCTGTGAAGTCGTCTCTCAAGTCGTCAGACTTGGTTGCTGG
GATTTAAACGTCTCGGTGTGACTACGCAGTCTATTTACTTACACACCCCAA
ATGTATGTCTACGAATGTCATTTAATGGGCCTTGTGCCTTTAAACCATAAT
ACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGA

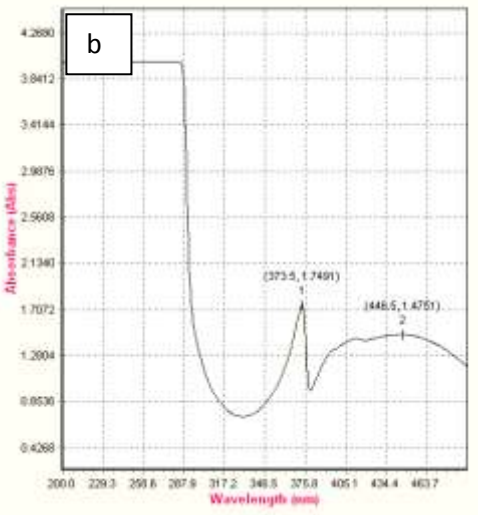


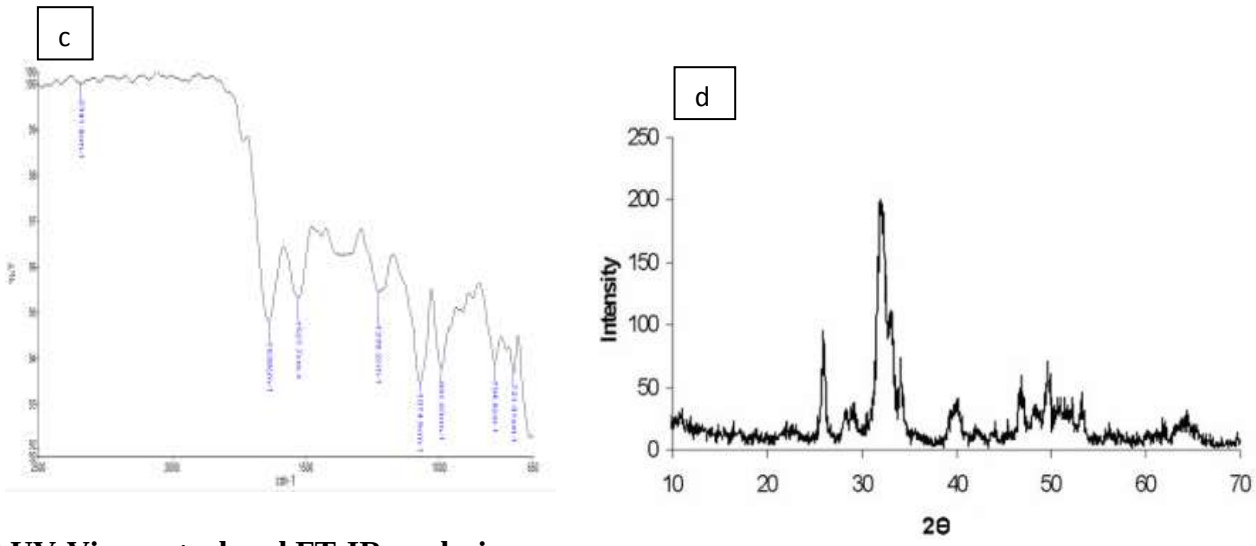
3.2 Biosynthesis of Silver Nanoparticle

When the mycelial extract was subjected to aqueous solution of 1mM silver nitrate, a gradual variation in color was noted in 24 hours of incubation at room temperature, and changed its colour from pale yellow to dark brown (Figure 2a). This change of colour could be due to the typical formation of silver nanoparticles of varying shape and size exhibited (Swapan Kumar Sinha et al.,1998).

Figure 2. (a) Formation of Silver nanoparticles using mushroom extract (b) UV-visible spectroscopy of synthesized silver nanoparticles (c) FTIR spectrum of silver nanoparticles biosynthesized from *Pleurotus ostreatus* (d) XRD pattern of *Pleurotus ostreatus* biosynthesized AgNPs







3.3 UV-Vis spectral and FT-IR analysis

UV Vis analysis showed a strong absorption band in the UV range after five days of incubation of the mycogenic AgNPs reaction mixture, the peak at 373 nm (Figure 2b). FT-IR measurements was carried out in the AgNPs sample to identify the possible compounds present in the fungal extract of *Pleurotus ostreatus* and their role in Ag+ reduction process (figure 2c). The transmittance spectrum exhibited five major peaks and corresponds to the functional groups i) 2341 cm-1- Aldehyde stretching ii) 1638 cm-1 - C=C stretching, Alkene iii) 1527cm-1 N-H Bend, Amides iv) 1230-1074 cm-1 C-O stretch , Esters v) 991-721cm-1 C-H Bending, Aromatic. The band at 1527 cm^{-1} was belongs to the N–H vibration of amide linkages existing in the protein.

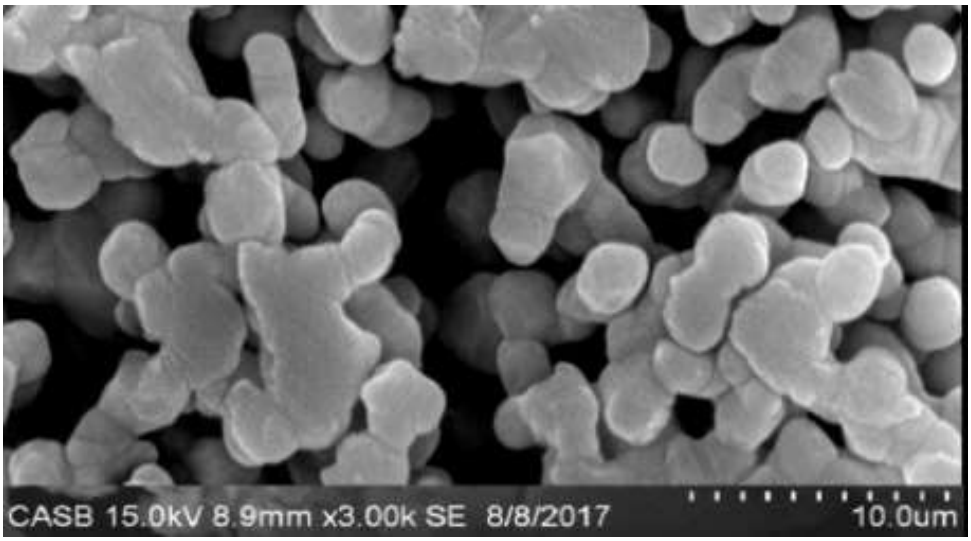
3.4. XRD analysis

XRD pattern of silver nanoparticles produced from *Pleurotus ostreatus* was recorded at 2θ angles ranging from 10° to 70°. The result of intensity of the diffraction was found to be much stronger than other diffractions pattern. In this study XRD diffraction showed intense peaks as shown in the figure 2d. The 2θ values about 27°, 35°, 40° and 50° was found to be characteristic peak of silver Nanoparticles.

3.7. Scanning Electron Microscope (SEM)

The myco-synthesized AgNps were examined using SEM. The Scanning electron micrograph of Silver nanoparticles clearly shows that they are spherical in shape ranging from 50-200 nm, and the average dimension of about 90 nm to 110 nm. Hence from SEM result it is clearly evident that the maximum particles are found to be in spherical shapes (figure 3) and found to be responsible for variation in sizes.

Figure 3: Scanning electron micrograph of Silver nano particle synthesized from *Pleurotus ostreatus*



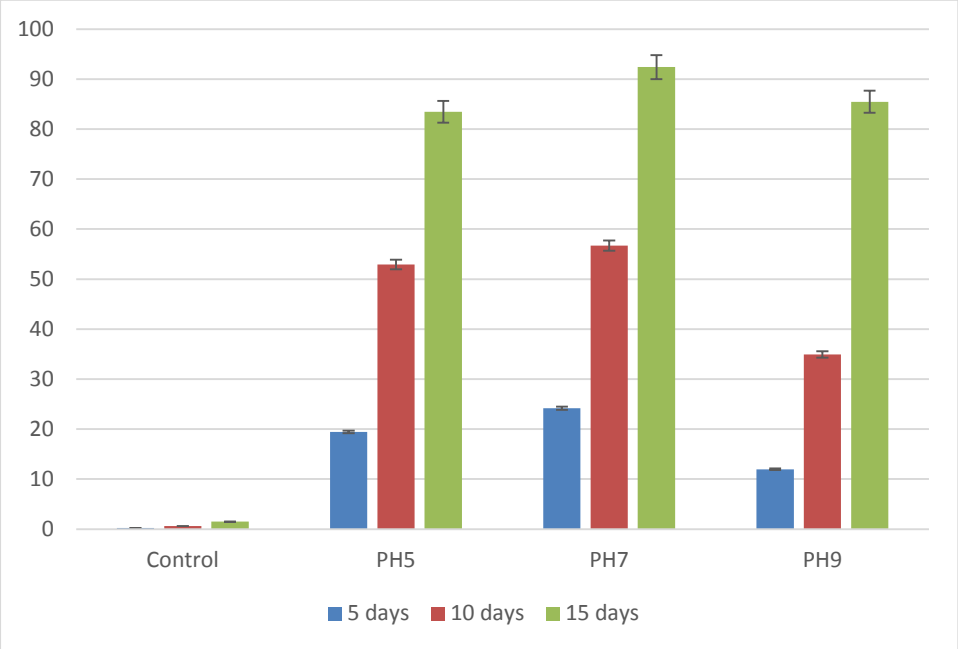
3.8. Pesticide degradation efficacy of *Pleurotus ostreatus*

Pesticides degradation potential of Pleurotus *ostreatus* was investigated under three different pH conditions (5, 7 and 9) Figure 4. The Quinalphos and cypermethrin was incubated with *Pleurotus ostreatus* and residual pesticides was calculated, the results show gradual decrease in the residual pesticides were noted with time duration for both Quinalphos (Figure 5a) and Cypermethrin (figure 5b). The maximum pesticide removal was observed in neutral pH ie pH7 conditions (Figure 5a,b). The degradation percentage of Quinal phos at pH7 was 31 %, 77.5% and 96.9% respectively at 5, 10 and 15 days after incubation. In Case of Cypermethrin the Percentage of degradation at pH 7 was 24.16%, 56.67% and 92.42% respectively at 5,10 and 15 days after incubation. Lowest degradation rate was recorded in alkaline pH for both pesticides (figure 5a). Acidic pH showed better removal rate when compared to alkaline pH (Swapan Kumar Sinha *et al.*, 2015).

Figure 4.a- Pesticide degradation at pH5 (cypermethrin, Quinalphos, Control),b- Pesticide degradation at pH7 (cypermethrin, Quinalphos, Control) c- Pesticide degradation at pH9 (cypermethrin, Quinalphos, Control)

Figure 5 - Percentage of degradation of pesticides by *P.ostreatus*

a-Cypermethrin Degradation



b- Quinalphos Degradation

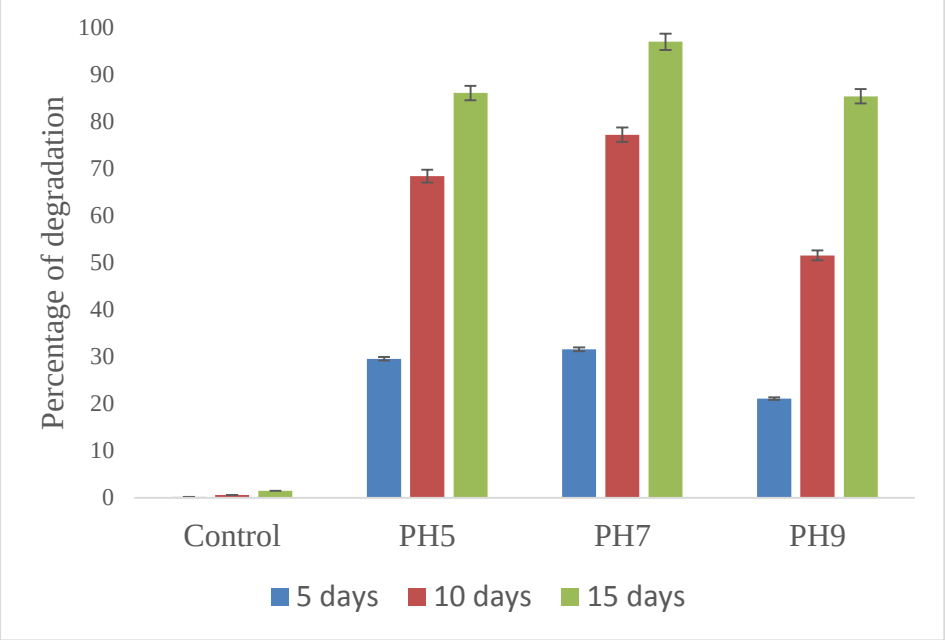
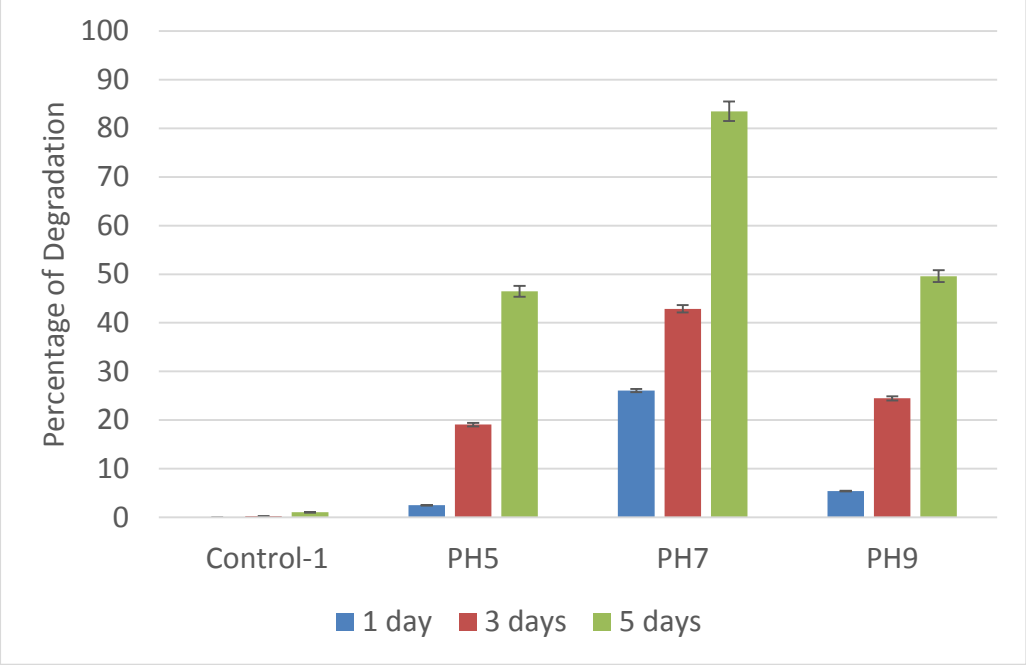
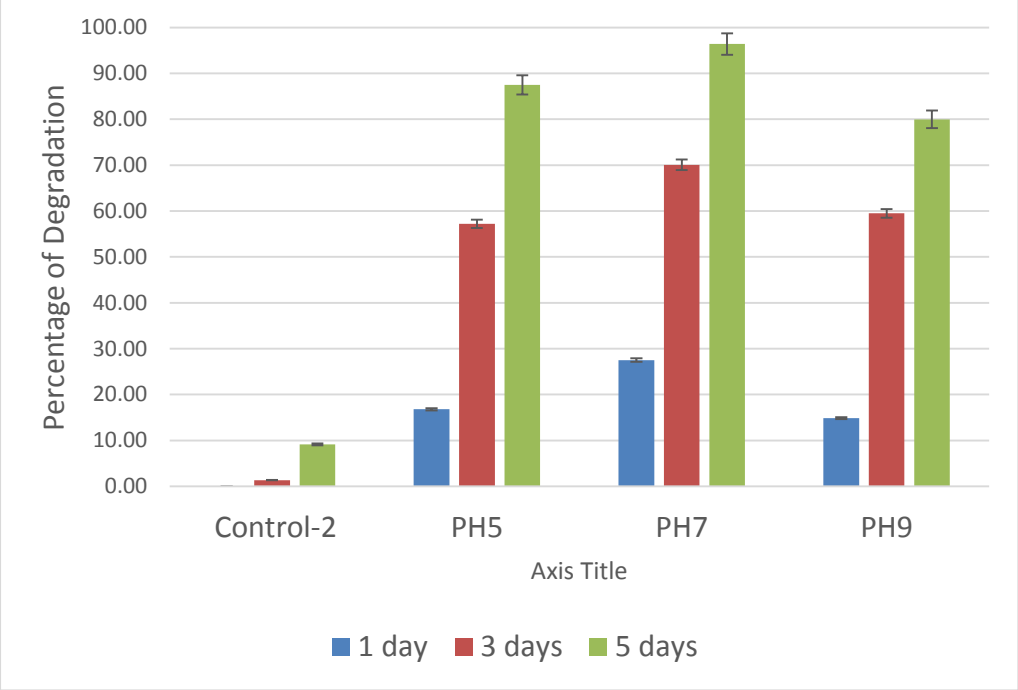


Figure 6- Percentage of degradation of pesticides by AgNps from *P.ostreatus*
a-Cypermethrin Degradation.



b- Quinalphos Degradation



3.9 Pesticide degradation potentiality of AgNPs

Pesticides degradation by mycogenic AgNPs was investigated under three different pH conditions (5, 7 and 9). The residual pesticides decrease as duration of incubation with AgNps increases. Better degradation rate was achieved in Quinal phos and maximum degradation rate was noted in the neutral conditions (Figure 6). Percentage of degradation of Quinal phos at pH7 was 27.5%, 70% and 96.39% respectively at 1,3 and 5 days of incubation (Figure 6a). Percentage of degradation of Cypermethrin at pH7 was 26.1%, 42.8% and 83.5% respectively at 1, 3 and 5days of incubation. Alkaline pH suppresses the degradation rate of both the pesticides by AgNps. Acidic pH showed hindrance in degradation potential of AgNPs, however better than alkaline pH conditions (Figure 6a,b)

7. Discussion

In the present study, biodegradation potential of Pleurotus ostreatus and silver nanoparticles against Quinalphos and Cypermethrin were studied and compared. The strain which is isolated from the Tree Trunk of Spathodia campanulata was identified using molecular anaylsis. The results of molecular analysis show that the isolate belongs to the Pleurotus genus of the family Pleurotaceae and have a very close evolutionary distance. However, it is also revealed that great genetic differences within the Pleurotus genus according to ITS sequences based evolutionary distances, and it was feasible to identify the fungal species P. ostreatus using a pair of specific primers within ITS region. Many studies were carried out in silver nano particles, these study show that silver ion and silver based nanoparticles have stronger and wide range of biological activity. Large quantities of proteins found in

mushrooms are abundant in amino acids such glutamic acid, tryptophan, lysine, and aspartic acid, which are crucial in the reduction of silver to AgNps. The reduction of the silver ions in the reaction mixture is observed in this study to be caused by the release of proteins into an aqueous suspension. UV-visible spectroscopy is the best widely used methods for physical characterization of AgNPs. The fungal extract of *P.ostreatus* reacts with silver ion to form a reduced AgNPs. AgNps having a typical surface Plasmon absorption band with peak absorption at 373 nm, representing the formation of AgNPs and it correlates with the work of Narasimha et al., (2011). Absorption maximum of plasmon resonance peak of a specific solvent can be used to predict the size of the AgNps. Silver nanoparticles have typical absorption spectrum between 350 nm to 450 nm of UV- visible region. In general, as the size of nanoparticles increases, there is a shift in plasmon resonance peak from shorter to longer wavelengths with broader absorption region. The results findings of the present study correlates and where he used 1.0 mM AgNO3 for the fabrication of AgNPs from bread mould *Rhizopus stolonifer* and endophytic fungus *Penicillium sp*. respectively. Their results showed that absorption spectrum silver nano colloids having surface plasmon resonance band at 420 nm, demonstrating the presence of long spherical to elliptical shaped AgNPs. The repeated washing of the AgNPs pellet with deionised water the residual metabolites was removed successfully for getting a clearer resolution of AgNPs. Moreover, the FTIR spectrum shows the presence of aldehyde, amides esters, plays a major role in capping and stabilization of silver nanoparticles as previously reported (Niraimathi et al., 2013; Prakash et al., 2013).

X-ray diffraction analysis of the silver nanoparticles synthesized from *Pleurotus ostreatus* shows a crystalline nature. Analogous results were also demonstrated by most of the scientists on different species of mushrooms where the high peaks in the investigation showed the presence of active silver composition. The intensity pattern of peaks of XRD represents high degree of crystalline nature of the silver nanoparticles synthesized from *Pleurotus ostreatus*. The observation of broader diffraction peaks specifying the smaller crystalline size of the synthesized AgNps. The peak broadening and noise occurrence were probably due to the presence of nano particles and crystalline nature of the biological macro-molecules present in the filtrate. The obtained results explain that the silver ions had definitely been converted into AgNPs by *P.ostreatus* under specific environmental conditions.

In this study, *Pleurotus ostreatus* was found to show significant ability to carry out the degradation of cypermethrin and quinalphos pesticides. *Pleurotus ostreatus* has shown high degradation effect on cypermethrin and quinalphos at pH7. Bioremediation was formerly found to be the most positive technique for the eradication of pesticide residues from the environment. To date, the degradation process information about cypermethrin and quinalphos is very limited. Very few workers have characterized the mushroom-based degradation of quinalphos, Cypermethrin. *P. ostreatus* capable of producing laccase and manganese peroxidase responsible for the effective degradation of natural lignin and

synthetic polymers. These specific characteristic of lignolytic enzyme and their detoxification mechanism may help to degrade recalcitrant molecules and xenobiotics . Similar work was reported by Sangeetha, et al., (2016), Ayda Maadani Mallak, et al., (2020), Pankaj Bhatt, et al., (2020), Alak Chandra Deka, et al., (2015). These discoveries propounded P. ostreatus has an atypical potential to degrade cypermethrin and quinalphos in different ecological niches, which makes it a dynamic strain for various applications.

8. Conclusion

The newly isolated macrofungal strain Pleurotus ostreatus could be employed as feasible Source for synthesis of silver nanoparticles (AgNPs). Myco-degradation persistent pesticide cypermethrin and quinalphos using *Pleurotus ostreatus* strain its myco-synthesized silver nanoparticles was found effectively and become a scope for future myco degradation of recalcitrant pesticides. Considering these native strains may be consider for future development of other pesticide and metal removal technologies.

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