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Photocatalytic degradation of ampicillin using silver nanoparticles biosynthesised by *Pleurotus ostreatus*

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Abstract

The past few decades have witnessed a tremendous increase in the consumption of antibiotics worldwide. This rampant and unregulated use of antibiotics and their improper disposal has led to the accumulation of these drugs in the environment. This in turn has led to the emergence of antibiotic resistance in microbes and has become one of the most pressing global concerns in medicine, with highly resistant pathogens of many species proving difficult to treat. The aim of the study was to synthesise silver nanoparticles (Ag-NPs) using white rot fungus, *Pleurotus ostreatus* and assess its potential to carry out photocatalytic degradation of ampicillin. UV–Vis spectroscopy, Fourier transform infrared spectroscopy and transmission electron microscope have been used to characterize biosynthesized Ag-NPs. The photocatalytic degradation of ampicillin in aqueous solution by Ag-NPs was evaluated under natural sunlight. The effect of the operating conditions (contact time, Ag-NP concentration and initial ampicillin concentration) on the photocatalytic degradation was also investigated. The highest ampicillin degradation of 96.5% was observed after exposure of the solution (antibiotic + nanoparticles) for 4 h in sunlight. The maximum degradation was observed at an Ag-NP concentration of 5 ppm at pH 6. To the best of our knowledge, photocatalytic degradation of ampicillin using Ag-NPs synthesised by *P. ostreatus* has not been reported earlier.

Key words: ampicillin, FTIR, photocatalysis, silver nanoparticles (Ag-NPs), TEM

Introduction

In the past few years, global antibiotic consumption has increased to more than 70 billion standard units (SU), to which India alone contributes 13 billion SU (Gelband et al., 2015). The use of antibiotics is not just confined to human drugs alone; they are being extensively employed in animal breeding, analysis experiments, crop production, fish farming, and cultivation (Thurman et al., 2002). This excessive and unregulated usage of antibiotics has led to increased release of these drugs into the environment. Most antibiotics, once administered to animals or humans are not metabolized completely and eventually end up accumulating in the environment (Roose-Amsaleg and Laverman, 2016). Being recalcitrant in nature, they persist in the environment for a long time, thus creating conditions for micro-organisms to develop mechanisms to evade their antimicrobial actions. Antibiotic resistance occurring in bacterial pathogens has been recognized as a major public health concern affecting humans worldwide. The span of multidrug-resistant organisms is not only restricted to hospitals, but they are also often present in community settings (Munita et al., 2016). For instance, Indian water bodies have been reported to contain various antibiotics such as ampicillin, ciprofloxacin, erythromycin, and enrofloxacin (Balakrishna et al., 2016). Ampicillin belongs to the penicillin group of betalactam antibiotics and is capable of penetrating both Gram-positive and Gram-negative bacteria. Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase, thereby affecting cell wall synthesis (Sharma et al., 2016).

The rampant and unregulated use of antibiotics and their improper disposal has become a significant challenge to the environment. Therefore, a two–pronged approach, based on a controlled use of antibiotics and their effective removal from contaminated sites, is needed. Although a number of methods have been reported for the degradation/removal of antibiotics, their efficiency

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varies significantly. Readers may refer to the review article by Homem and Santos (2011), which provides a comparative analysis of different conventional methods like adsorption, coagulation, filtration, flocculation, sedimentation, and advanced oxidation processes used to remove antibiotics from the environment. However, the existing treatment methods for these contaminants focus only on the removal of the chemicals present and do not turn them into non-toxic wastes. The bottlenecks associated with these conventional methods of antibiotic degradation have led to an increased interest in the use nanoparticles (NPs) in the degradation of antibiotics (Guzmán et al., 2008). Photocatalytic detoxification is a process which amalgamates heterogeneous catalysis with solar technologies. Thus, the prime advantage of the photocatalytic process over other prevailing technologies is that it eliminates the need for further disposal. Furthermore, the application of NPs in phototocatalysis enhances photoredox chemistry, which in turn leads to increased photoactivity (Beydoun et al., 1999). The semiconductor material used in photocatalysis helps in the degradation of various organic pollutants present in water by the photoexcitation of electrons. Compared with traditional synthetic methods, biological systems employ novel techniques for the efficient production of nanomaterials. Fungi are valuable biological agents for synthesizing metal NPs. The use of fungi is potentially exciting as they secrete bulky amounts of enzymes and their biomass is easy to manage (Yehia and Al-Sheikh, 2014).

Pleurotus ostreatus is a temperate edible mushroom that can be grown on different agricultural wastes in a temperature range of 25–28 °C. *P. ostreatus* is known to produce various enzymes such as laccase, manganese peroxide, lignin peroxidases, xylanases etc. which catalyze various metabolic reactions such as substrate utilization and degradation of pollutants (Singh et al., 2017).

The aim of the study was to develop a suitable method with the potential to overcome all of the challenges associated with the improper disposal of antibiotics.

Materials and methods

Maintenance of culture

P. ostreatus was procured from ICAR, Directorate of Mushroom Research, Solan, India, and maintained on malt extract agar (at 25 ± 2 °C). The composition of malt extract agar includes maltose (12.75 g/l), dextrin (2.75 g/l), glycol (2.35 g/l), peptone (0.78 g/l), and agar (15.0 g/l). For suspension culture, mycelium Plugs (6 mm) were excised using a sterile plug borer, and were inoculated in malt media. The culture was incubated at 25 ± 2 °C for 72 h at 100 rpm.

Preparation of the cell filtrate

The cell filtrate was prepared according to the modified method of Abdel-Hafez et al. (2016). Fungal biomass was harvested by centrifugation at 5000 rpm for 5 min followed by washing with sterile distilled water to eliminate any traces of the media. The fungal biomass (about 2 g) was then inoculated into sterile distilled water, incubated for 72 h at 25 ± 2 °C, and shaken at 100 rpm for 72 h. Whatman filter paper no. 1 was used for the filtration of the biomass and the obtained filtrate was stored at 4 °C till further use.

Green synthesis of silver nanoparticles

Silver nitrate was added to 100 ml of cell filtrate to obtain a final concentration of 4 mM, followed by incubation for 72 h at 25 ± 2 °C with shaking (100 rpm) under dark conditions. Silver nitrate solution without the cell filtrate was used as control and subjected to similar treatment. Nanoparticles were garnered by centrifugation at 5000 rpm for 15 min and the pellets were freeze dried.

UV-Visible spectroscopy

The reduction of silver ions during silver nanoparticles (Ag-NP) synthesis was observed using UV–visible spectroscopy. The dried Ag-NPs were resuspended in sterile distilled water and subjected to a wavelength scan in the range of 300–700 nm.

Fourier-transform infrared spectroscopy (FTIR)

Infrared measurements of Ag-NPs were carried out at room temperature on a Shimadzu 8400S FTIR spectrometer, armed with a potassium bromide (KBr) beam splitter. A thin layer of samples was dispersed in KBr in 100 : 1 ratio. This solid base was positioned in the sample holder and FTIR spectra were scanned in the range of 4000–500 cm^{-1}.

Transmission electron microscopy (TEM)

The sample was sonicated for 15 min and the aqueous suspension of Ag-NPs was loaded on a carbon-coated copper grid. The solvent was permitted to evaporate

under infrared light for 30 min (Devika et al., 2012). TEM measurements were performed at an accelerating voltage at 200 KV.

Photocatalytic degradation of ampicillin

Effect of contact time

Ampicillin (10 ppm) was mixed with freeze-dried Ag-NPs and incubated in sunlight (in March) for 6 h (between 10:00 am and 5:00 pm). Ampicillin solution without NPs served as control and was subjected to similar treatment. The samples were withdrawn after a an interval of 1 h. Ampicillin assay was carried out every h to optimize the time for maximum degradation. It is worth specifying that all ampicillin assays in this paper were carried out according to the method described by Ahmed et al. (2004). Three ml of Folinciocalteu phenol reagent were added to 5 ml of the sample, and incubated in a water bath at 95°C for 20 min. The mixture was allowed to cool down and the absorbance was measured at 750 nm. All experiments were carried out in triplicate. In addition, kindly note that in this paper the percentage of degradation has been calculated for every concentration of NPs using the method developed by Nosrati et al. (2012).

$$\text{Percentage degradation} = ((Co - Ct)/Co) \times 100$$

where, Co – initial absorbance, Ct – final absorbance after incubation.

Effect of antibiotic concentration

To assess the influence of the antibiotic concentration on the ability of nanoparticles to degrade the antibiotics, different concentrations of antibiotic (10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, 70 ppm, and 80 ppm) were incubated in sunlight for an optimized time with freeze-dried Ag-NPs. Antibiotic solutions without NPs served as controls and were subjected to similar treatment. Ampicillin assay was carried out and the percentage of degradation was calculated for every concentration. It is important to highlight that different concentrations of the antibiotic were evaluated to determine the optimized concentration at which maximum degradation occurred.

Effect of NP concentration

The optimized ampicillin concentration was treated with different concentrations of freeze-dried Ag-NPs (1 ppm, 3 ppm, 7 ppm, 9 ppm, 11 ppm, 13 ppm, 15 ppm, and 17 ppm) and subjected to photocatalytic degradation for an optimized contact time. The antibiotic solution without NPs served as control and was subjected to similar treatment. Ampicillin assay was carried out and the percentage of degradation was calculated for every concentration of NPs. It is important to highlight that different concentrations of Ag-NPs were used to determine the optimized concentration at which maximum degradation of antibiotics occurred.

Effect of pH

Ampicillin solutions at the optimized concentration were prepared for different pH values ranging from 4 to 9. The samples were treated with the optimized NP concentration and subjected to photocatalytic degradation for the optimized contact time. The antibiotic solution without NPs served as control and was subjected to similar treatment. Ampicillin assay was carried out and the percentage of degradation was calculated at every pH value.

Statistical analysis

A statistical analysis of the obtained results was performed to evaluate the significance of the variables over the range of antibiotic degradation. One-way and two-way ANOVA were used with the statistical software package Graphpad PRISM 5.0.

Results and discussion

The purpose of the present study was to assess the influence of different parameters, such as the reaction time, the concentration of antibiotics and NPs, and the pH of the solution on the degradation of ampicillin. An in-depth analysis of the degradation mechanism revealed that photocatalytic activity plays a prominent role in the reduction of toxic wastes, as holes (H^+) in aqueous media get trapped by water molecules and hydroxyl radicals (OH^-) are generated. These radicals act as strong oxidizing agents and oxidize organic pollutants to water and gaseous products (Anjum et al., 2016).

Green synthesis of NPs

The preliminary indication of NP synthesis was the alteration in fungal suspension color. In the present study, a change in color to dark brown was observed within 72 h of incubation of silver nitrate with cell filtrate of *P. ostreatus* (Fig. 1). However, no color change

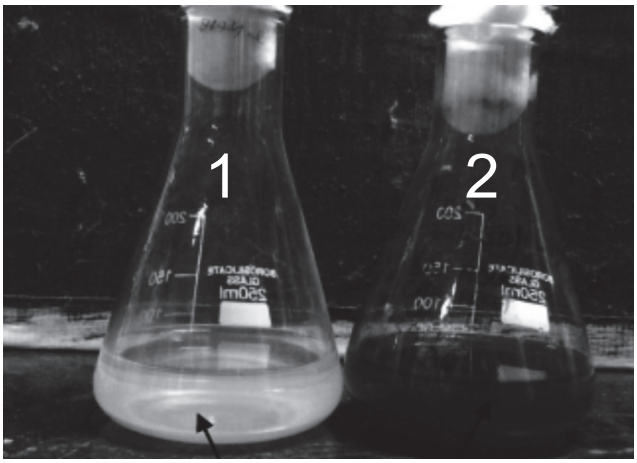

Fig. 1. The synthesis of AgNPs by *Pleurotus ostreatus* resulting in a change of the color of the suspension 1) before and 2) after synthesis

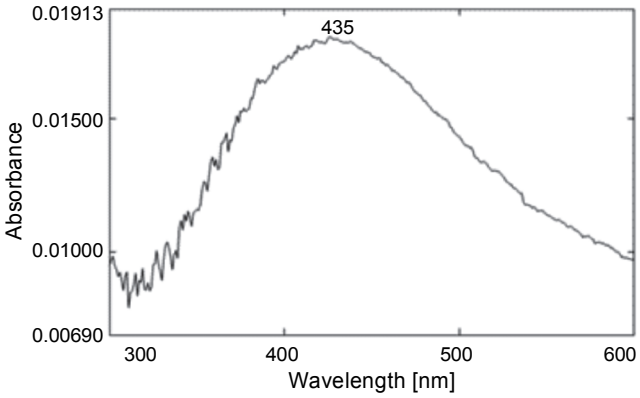

Fig. 2. UV-visible absorption spectra of Ag-NPs

was observed in the case of negative control (silver nitrate solution alone). A similar change in color, from a colorless solution to dark brown, indicating the synthesis of Ag-NPs by the fungus *Verticillium* was reported by Mukherjee et al. (2001). Similarly, the synthesis of Ag-NPs by *Aspergillus fumigatus, Candida albicans, Penicillium italicum, Syncephalastrum racemosum, Fusarium oxysporum,* and *Aspergillus ochraceus* resulted in a dark brown colour of the suspensions (Magdi et al., 2014). Kathiresan et al. (2009) also reported a change in color to dark brown as a result of the synthesis of Ag-NPs by *Penicillium fellutanum* isolated from mangrove root-soil. This change in color is attributed to the surface plasmon resonance of the deposited Ag-NPs. Ag-NPs absorb radiation in the visible region of the electromagnetic spectrum; the excitation of surface plasmons (vibrations) is responsible for the prominent yellow-brown color of Ag-NPs in various media (Vigneshwaran et al. 2007).

UV-Visible spectroscopy

To validate the formation and evaluate the stability of Ag-NPs, UV-Visible spectroscopy of the colloidal solution was carried out. A typical surface plasmon absorption band was observed around 435 nm in the tested sample with a corresponding band gap of 2.85 eV; no absorption band was observed in the control (Fig. 2). The formation of Ag-NPs from the fungus biomass can be attributed to the trapping of silver ions at the surface of fungal cells followed by their reduction by the enzymes present in the fungal system. Devaraj et al. (2013) also reported a silver surface plasmon resonance band of 430 nm for NPs synthesised using cannonball leaves. A 414 nm resonance peak of the surface plasmon has been reported for Ag-NPs synthesised by *Urtica dioica* linn leaves. Previous studies suggest that an SPR peak located between 410 nm and 450 nm has been observed for Ag-NPs and might be attributed to spherical NPs (Jyoti et al., 2016). Ahmad et al. (2004) reported a sharp SPR peak at 413 nm for Ag-NPs synthesised by *Fusarium oxysporum.* Ag-NPs from *Schizophyllum radiatum* exhibited an SPR peak at 435 nm (Metuku et al., 2013). Similarly, Yehia and Al-Sheikh (2014) reported a maximum absorbance at 435 nm for Ag-NPs synthesised by *P. ostreatus.*

FTIR spectroscopy

FTIR analysis was done to characterize the functional groups associated with NPs. As shown in Figure 3, the spectrum band at 1645 cm^{-1} can be attributed to –C=O stretch vibrations and these stretch vibrations are present in the amide linkages of the proteins. The peaks at 3441.2 cm^{-1} and 2926.1 cm^{-1} pertain to the stretching vibrations of primary and secondary amines, respectively (Vigneshwaran et al., 2007). The two bands observed near 1342 cm^{-1} and 1037 cm^{-1} can be assigned to the C–N stretching vibrations of aromatic and aliphatic amines, respectively. The molecular vibrational positions of these bands are in agreement with earlier studies on native proteins by Labrenz et al. (2000). FTIR measurements indicate (data not shown) that the secondary structures of proteins are not affected by their interaction with silver ions or NPs. However, there are no data to confirm/prove this.

The carbonyl groups present in amino acids, peptides and proteins residues can strongly bind to metals. There-

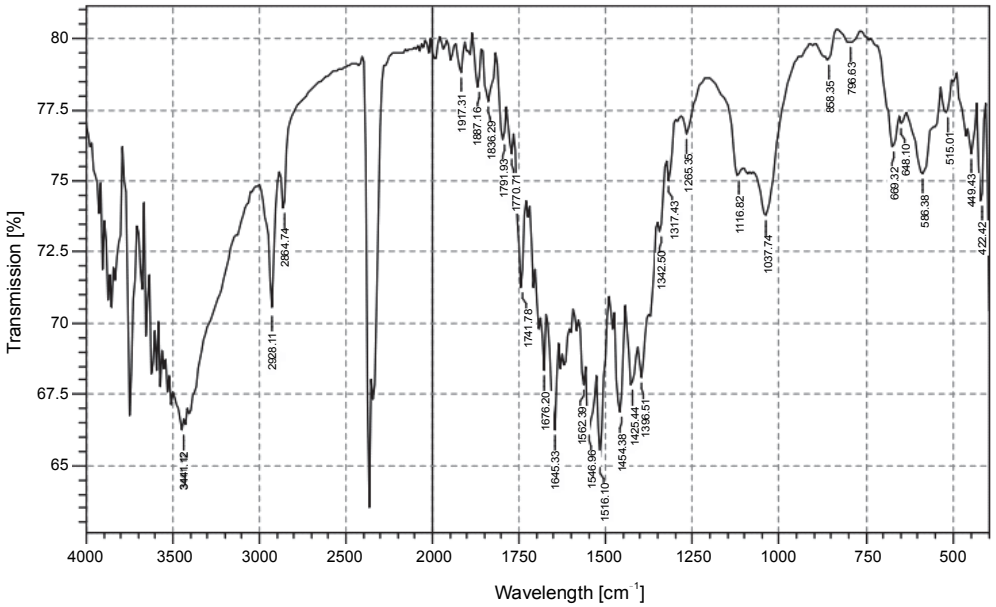

Fig. 3. FTIR spectra of Ag-NPs

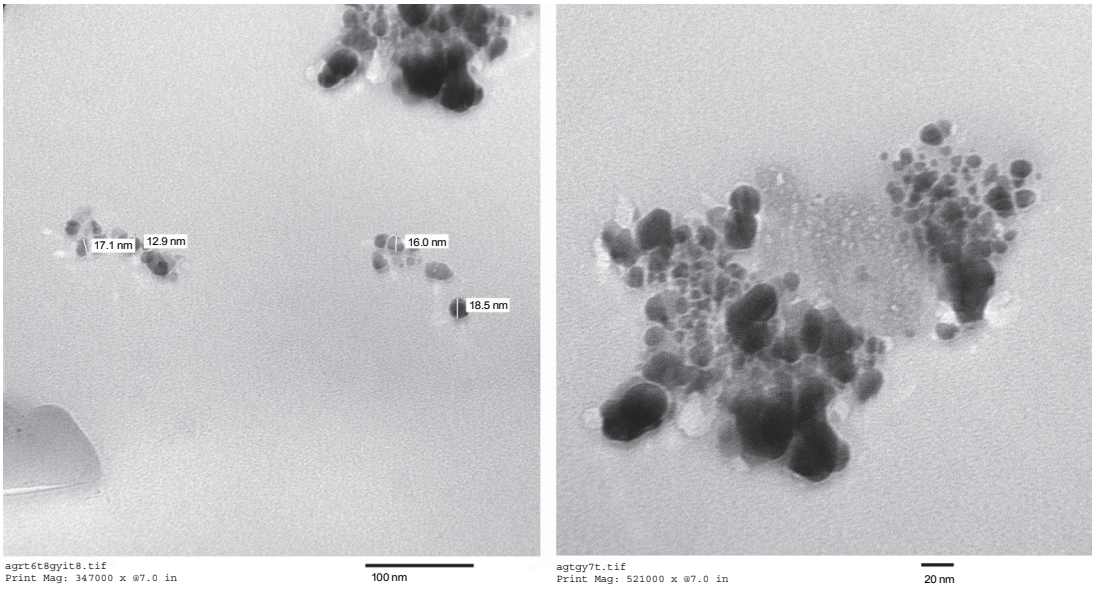

Fig. 4. TEM images of biogenic silver nanoparticles

fore, proteins may act as capping agents of Ag-NPs, preventing agglomeration and stabilizing the particles within the medium (Abdel-Hafez et al., 2016). Proteins can bind to NPs either via free amine groups or cysteine residues or by the electrostatic interactions of negatively charged carboxylate groups of enzymes present in the cell wall of mycelia and therefore, stabilize the NPs (Magdi et al., 2014).

Transmission electron microscopy (TEM)

The TEM analysis allowed further understanding of the size and shape of Ag-NPs. TEM images recorded from the Ag-NPs, placed on carbon-coated Ag-NPs, revealed their polydispersed and spherical nature (Fig. 4). Most of the NPs shown in the micrograph are in the range of 12.9 nm to 18.5 nm.

Photocatalytic degradation of ampicillin

Effect of contact time

To assess the optimal time for the antibiotic (ampicillin) degradation, Ag-NPs were incubated with ampicillin (10 ppm) in sunlight for 6 h with an average light intensity of 940-1000 Lux. The samples were taken regularly at an interval of 1 h. The antibiotic solution without NPs served as control and was subjected to similar treatment. Although antibiotic degradation was observed in both controls and samples, the presence of Ag-NPs accelerated the degradation process, with a maximum degradation of 96.5% observed after 4 h of incubation as compared to 69.3% in the control. A substantial degradation of 75.5% was observed after just 1 hour of incubation as compared to 35% degradation in the control. Although there was a gradual increase in the antibiotic degradation with increase in contact time, no significant increase was observed after 4 h of incubation. This can be attributed to the decreased intensity of sunlight with time (Fig. 5). Exposure to UV rays in sunlight leads to the excitation of electrons in Ag-NPs from the valence band to a conduction band. The holes generated in the valence band of the semiconductor, along with hydroxyl radicals, may lead to direct or indirect oxidation of ampicillin. Indirect oxidation may be the result of the generation of hydroxide reactive radicals (OH) via the reaction of holes in the valence band with water or hydroxide anions (OH^-). Primary photo-products formed due to the electron-hole transfer undergo further transformational steps to form a final photo-product, thereby leading to complete antibiotic degradation. The rate of degradation therefore, decreases with the decrease in light intensity, which leads to the decrease in the energy of the photon, moreover the overall energy introduced into the photocatalytic process is reliant on light intensity (Jodat and Jodat, 2013). It is worth mentioning here that the extent of degradation achieved during 4 h of incubation in natural sunlight is of great significance for developing an efficient and quick method for antibiotic remediation. In a work reported by Nosrati et al., (2012), 41% ampicillin degradation was achieved after 120 min of incubation in sunlight with ZnO/polyamine nanocomposites. In another report by Elmolla and Chaudhuri (2010), 96% ampicillin degradation was observed upon incubation with ZnO NPs for 300 min under UV lamp (365 nm). The biggest advantage of using NPs as photocatalysts is the reduction in treatment time compared to that with conventional biological methods which have been reported to take around 7–14 days (Singh et al., 2017; Kumar et al., 2013; Prieto et al., 2011).

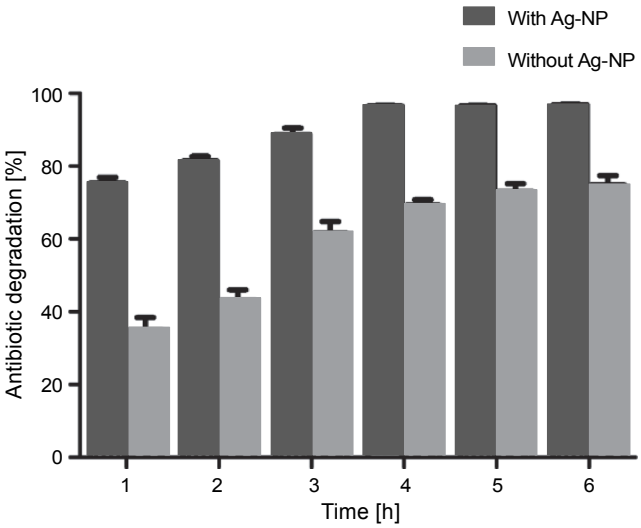

Fig. 5. Effect of contact time on ampicillin degradation

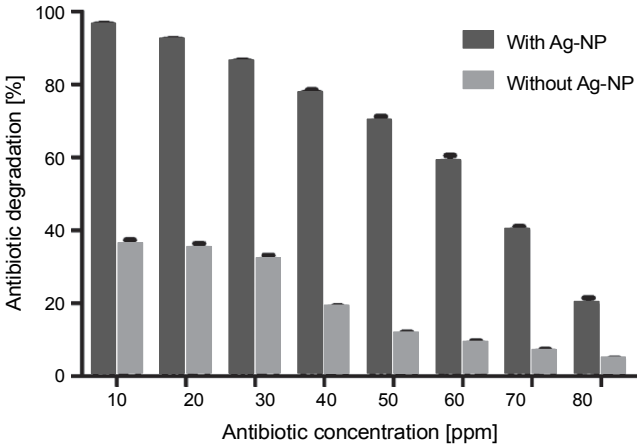

Fig. 6. Effect of initial ampicillin concentration on degradation

Two way ANOVA revealed that both contact time and NPs have a significant effect on antibiotic degradation. This effect was found to be in synergy as revealed by the interaction p value ($P < 0.0001$).

Effect of initial antibiotic concentration

To evaluate the extent of photocatalytic degradation at higher concentrations of ampicillin, antibiotics of different concentrations (10–80 ppm) with Ag-NPs were exposed to sunlight for 4 h. The antibiotic solution without NPs served as control and was subjected to similar treatment. As observed previously, ampicillin degrada-

Table 1. Two way ANOVA for effect of time and presence of NPs on antibiotic degradation

Source of variation	Df	Sum of squares	Mean square	*F*	*P*-value	Significant?
Interaction	5	445.2	89.04	21.38	< 0.0001	yes
Presence o NPs	1	7989	7989	1918	< 0.0001	yes
Time	5	4961	992.3	238.2	< 0.0001	yes
Residual	24	99.97	4.166			

Table 2. Two way ANOVA for antibiotic concentration and presence of NPs on antibiotic degradation

Source of variation	Df	Sum of squares	Mean square	*F*	*P*-value	Significant?
Interaction	7	2733	390.4	337.9	< 0.0001	yes
Presence of NPs	1	28290	28290	24490	< 0.0001	yes
Antibiotic concentration	7	16130	2304	1994	< 0.0001	yes
Residual	32	36.97	1.155			

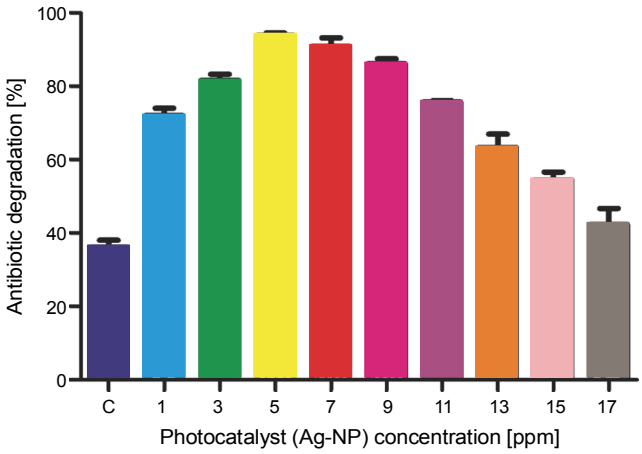

Fig. 7. Effect of photocatalyst concentration on antibiotic degradation

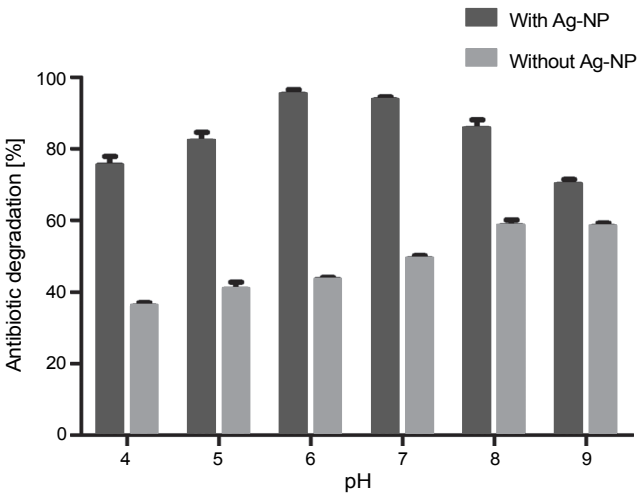

Fig. 8. Effect of pH on the photocatalytic degradation of antibiotics

tion was observed in both cases, though the presence of Ag-NPs enhanced the process. The maximum degradation of 96.5% was observed at 10 ppm of ampicillin; however, a considerable amount of degradation, 69.9% and 58.8%, was observed at 50 ppm and 60 ppm, respectively, in contrast to 11.2% and 8.9% degradation in controls (Fig. 6). A similar degradation pattern has been reported for the degradation of ampicillin by ZnO/polyamine nanocomposites by Nosrati et al. (2012). The decrease in the degradation level at high concentrations can be attributed to the fact that the adsorbed reactant molecules lodge in all the active sites of the catalyst. Moreover, with an increase in the target pollutant (antibiotic) concentration, more antibiotic molecules become adsorbed on the surface of the catalyst. Therefore, the requirement of the reactive species (OH) for the degradation of the pollutants also increases. As the concentration of the target pollutant increases, the pollutant molecules absorb more light, and therefore, photons never reach the catalyst surface, and so the photodegradation rate decreases (Jodat and Jodat, 2013).

Statistical analysis revealed that the initial antibiotic concentration had a profound effect on the antibiotic degradation rate ($P < 0.0001$). Also, the presence of NPs affected antibiotic degradation in a significant way ($P < 0.0001$). Antibiotic degradation was affected by the presence of NPs over the entire range of antibiotic concentration as indicated by a small interaction *P*-value (Table 2).

Table 3. One-way ANOVA for nanoparticle concentration on antibiotic degradation

One-way analysis of variance			
P-value	< 0.0001		
Are means significantly different? ($P < 0.05$)	yes		
ANOVA Table	SS	df	MS
Varying NP concentration (between columns)	7160	8	895.0
Residual (within columns)	103.3	18	5.739
Total	7263	26	

Table 4. Two-way ANOVA for pH and the presence of NP on antibiotic degradation

Source of variation	Df	Sum of squares	Mean square	*F*	*P*-value	Significant?
Interaction	5	1520	303.9	109.3	< 0.0001	yes
Presence of nanoparticles (NP)	1	11650	11650	4189	< 0.0001	yes
pH	5	1236	247.2	88.90	< 0.0001	yes
Residual	24	66.73	2.780			

Effect of photocatalyst concentration

To observe the effect of Ag-NP concentration on ampicillin degradation efficiency, a range of concentrations (1–19 ppm) was tested. Antibiotic degradation increased with increase in NP concentration, with maximum degradation (93.8%) achieved at 5 ppm compared to 36% in the control sample without Ag-NPs (Fig. 7). Interestingly, a further increase in photocatalyst concentration was associated with a decrease in the degradation level of ampicillin. Initially, with increase in photocatalyst concentration, an increase in antibiotic degradation was observed, and it can be attributed to the increase in the available catalyst surface. However, increasing the amount of catalyst beyond a threshold concentration led to decreased antibiotic removal due to the increased turbidity of the solution which caused scattering of light, thus inhibiting its path through the solution. Similar antibiotic degradation efficiencies have been reported by various researchers (Nosrati et al., 2012; Jodat and Jodat, 2013; Elmolla and Chaudhuri, 2010).

One-way ANOVA revealed a strong correlation between photocatalyst concentration and antibiotic degradation ($P < 0.0001$) as depicted in Table 3.

Effect of pH

The incubation of ampicillin with Ag-NPs in sunlight for 3 h at different pH values revealed a gradual increase in the percentage of degradation with increase in pH value. The maximum degradation (95.2 %) was achieved at pH 6, while at higher pH values a decrease in antibiotic degradation was observed (Fig. 8). Generally, the effect of pH on antibiotic degradation depends on the properties of both the antibiotic and the NPs. When pH increased, the overall surface charge of Ag-NPs changed from highly negative to less negative. A positive surface charge is typically not seen on Ag-NPs at any pH unless engineered with a surface coating for this purpose (Badawy et al., 2010). On the other hand, ampicillin exists in cationic form at acidic pH and in an anionic form at alkaline pH. Since at acidic pH, ampicillin has a positive charge and the photocatalyst has a negative charge, it favours the absorption of ampicillin molecules on the photocatalyst surface, thereby exhibiting high degradation efficiency. At alkaline pH, both ampicillin and the Ag-NPs are negatively charged, which leads to the generation of repulsive forces between the antibiotic and the catalyst. However, a substantial degradation is still observed at alkaline pH, and it can be attributed to the hydrolysis of the antibiotic due to the instability of the β-lactam ring at pH >7.3 (Rozas et al., 2010).

Two-way ANOVA suggested a significant effect of pH and the presence of NPs, individually, on the degradation of the antibiotic ($P < 0.0001$). Also, the *P*-value of interactions ($P < 0.0001$) revealed that NPs were effective in the degradation of the antibiotic in the entire range of pH under study (Table 4).

Conclusions

Cell filtrate of *P. ostreatus* mediated Ag-NPs were synthesized in the range 12.9–18.5 nm. The synthesized NPs exhibited a maximum ampicillin degradation of 96.5% after a 4 h incubation in sunlight. Likewise, other optimized parameters for maximum degradation included Ag-NP concentration of 5 ppm, ampicillin concentration of 10 ppm and pH 6. Additionally, to the best of our knowledge, photocatalytic degradation of ampicillin using Ag-NPs produced by *P. ostreatus* has not been reported before.

Acknowledgments

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