

Biodegradation of ciprofloxacin by white rot fungus *Pleurotus ostreatus*

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Abstract Unrestricted and reckless use of antibiotics has resulted in their accumulation in environment. This, in turn, has led to the emergence of multiple drug-resistant microbes. The present study focuses on degradation of ciprofloxacin (CIP) by an edible white rot fungus *Pleurotus ostreatus*. Effect of CIP was determined on radial growth and biomass of *P. ostreatus*. Titrimetric and spectrophotometric assays were carried out to assess the degrading potential of *P. ostreatus* towards CIP. It was found that CIP has a stimulatory effect on growth and enzyme activity of *P. ostreatus*. Maximum enzyme (glucanase, ligninases, laccase) production was observed at the highest concentration of CIP (500 ppm). Antibiotic degradation of about 68.8, 94.25 and 91.34% was estimated after 14 days of incubation at 500 ppm CIP using Titrimetric, Indigo carmine and Methyl orange assay, respectively. Degradation of CIP was further validated by high performance liquid chromatography (HPLC) and microbiological analysis. HPLC analysis revealed 95.07% degradation while microbiological test also exhibited a decreased antimicrobial activity of degraded products against *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes*. To the best of our knowledge, this is the first study wherein *P. ostreatus* was used for the degradation of ciprofloxacin.

Keywords Antibiotic resistance · Bioremediation · CIP · *Pleurotus ostreatus* · HPLC

Introduction

Antibiotics are among the most frequently prescribed medicaments in modern day medicine (Hernandez et al. 2012). Between 2005 and 2009, the units of antibiotics sold in India specifically increased by about 40%. Increased sales of cephalosporins were particularly striking, with sales increasing by 60% though some increase was seen in most antibiotic classes, making India world's largest consumer of antibiotics (Shah et al. 2015). Being non-degradable in nature, most of these antibiotics persist in the environment for long periods. Antibiotics like ciprofloxacin (CIP) degrade very slowly and may persist in soil in its original form for up to 1–4 months, thus creating a microenvironment for the development of antibiotic resistant strains (Laxminarayan et al. 2013). CIP is one of the most widely used second-generation broad spectrum quinolone which has been detected in domestic wastewaters in concentrations of up to 1000–6000 ng/l, that causes the possible occurrence of selective pressures and the consequent selection of resistant bacteria (Batt et al. 2007). This problem of extensive and unrestricted use of antibiotics has aggravated to such magnanimous proportions that it is becoming increasingly difficult to treat diseases caused by such resistant strains. It has now, therefore, become necessary to search for new methods for effective degradation of antibiotics persisting in the environment.

A number of methods including physical and chemical have been adopted for treatment of water contaminated with antibiotics (Hernandez et al. 2012; Hubicka et al. 2013). However, these methods are largely ineffective and end up adding more pollutants into the environment. An alternative lies in the use of living organisms for remediation of these antibiotics. Bioremediation involves the use of microbes to remove or breakdown complex hazardous

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substances into simpler, less toxic or nontoxic substances. The process is generally 60–70% less costly than other technologies (Laxminarayan et al. 2013). Fungi such as *Gloeophyllum striatum* (Wetzstein et al. 1999), *Phanerochaete chrysosporium* (Guo et al. 2014; Martens et al. 1996) and *Trametes versicolor* (Rodríguez-Rodríguez et al. 2012) have been reported for their use in bioremediation of antibiotics. The aim of this study was to evaluate the potential use of mycelium of a basidiomycetous fungus *Pleurotus ostreatus* for degradation of CIP.

Pleurotus ostreatus (*P. ostreatus*), is a temperate edible mushroom which forms oyster shaped fruiting bodies that can be grown on different agricultural wastes in a temperature range of 25–28 °C (Ahmed et al. 2009). The ability of *P. ostreatus* as bioremediation agent has been attributed to the production of various enzymes such as laccase, manganese peroxidase, lignin peroxidases, xylanases, etc. which are important for various metabolic reactions such as substrate utilization as well as degradation of pollutants (Martens et al. 1996; Espindola et al. 2007; Rana and Rana 2011; Jegatheesan et al. 2012; Singh et al. 2012).

Materials and methods

Growth studies

Mycelium of *P. ostreatus* was maintained on Potato dextrose agar (PDA) medium at 25 ± 2 °C. The effect of different concentrations of CIP (100, 200, 300, 400 and 500 ppm) on growth of *P. ostreatus* mycelium and biomass production was studied by inoculating mycelium bits (5 mm) in PDA and Potato dextrose broth (PDB), respectively. One-way ANOVA was used to determine whether there was any significant effect of concentration of CIP used on the radial growth of *P. ostreatus* with increase in incubation time by calculating Critical Difference (CD) at 5%.

Enzymatic studies

Effect of different concentrations (100–500 ppm) of CIP on Endo-β-D-1,4-glucanase (Miller 1959), Laccase (Jegatheesan et al. 2012), Hemicellulase (Bucht and Erikson 1968), Lignin peroxidase (Tien and Kirk 1984) and Manganese peroxidase (Kuwahara et al. 1983) produced by *P. ostreatus* was determined after 7 and 14 days of incubation. Culture broth devoid of CIP was used as control.

Degradation of CIP

Two different approaches, viz., titrimetric and spectrophotometric were used to estimate the amount of CIP degraded by *P. ostreatus*.

Titrimetric analysis

The amount of CIP degraded by *P. ostreatus* was determined according to Basavaiah et al. (2006). A 10 ml aliquot of standard solution containing 100–500 ppm of CIP was placed in a 100 ml volumetric flask. The solution was acidified by adding 5 ml of 5 M sulfuric acid. 10 ml of 0.025 M cerium sulfate was added to content in the flask, mixed well and kept aside for 15 min. Finally, the unreacted oxidant was back titrated with 0.025 M Ferrous Ammonium Sulfate (FAS) solution using one drop of ferroin indicator. Simultaneously, a blank titration was performed and the amount of drug left in the aliquot was calculated from the amount of cerium sulfate reacted. Standard curve of FAS was used to calculate the amount of CIP degraded.

Spectrophotometric analysis

Spectrophotometric determination of degraded CIP was carried out by methyl orange method and Indigo carmine method (Basavaiah et al. 2006; Nijhu et al. 2011).

Indigo carmine method

20 µg of spent broth was added to 4 ml distilled water. 1 ml of 5 M sulfuric acid was added to each tube followed by addition of 500 µg/ml of cerium sulfate solution (0.025 M). The contents were mixed well and kept aside for 10 min with occasional swirling at room temperature. 1 ml of indigo carmine was added to each tube and absorbance was measured at 610 nm after 5 min. Standard curve was prepared for determination of degraded amount of CIP.

Methyl orange method

10 µg of spent broth was added to 4 ml of distilled water. 1 ml of 5 M sulfuric acid and 250 µg/ml of cerium sulfate (0.025 M) were added to above mixture and kept at room temperature for 10 min with occasional swirling. Finally, 1 ml of methyl orange was added and absorbance was measured at 520 nm. Standard curve was prepared for determination of degraded amount of CIP.

Validation of CIP bioremediation

High performance liquid chromatography (HPLC)

HPLC was carried out to validate the degradation results obtained in the above mentioned assays. AC18 column with acetonitrile as mobile phase was used for chromatography (Singh et al. 2013). A standard run of pure CIP was performed to comparatively assess the samples taken, i.e., 100 and 500 ppm concentrations of CIP.

Antimicrobial activity of degraded products

Antibacterial activity of degraded products formed post incubation with *P. ostreatus* was evaluated against *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes* on Mueller–Hinton agar plates by disc diffusion method and compared with antibacterial activity of different CIP concentrations (Thillaimaharani et al. 2013; Hernandez et al. 2012).

Results and discussion

Radial growth studies

Radial growth studies of *P. ostreatus* on PDA at different concentrations of CIP revealed that there was no inhibitory effect of CIP on the growth of the fungus. On the contrary, as the concentration of the antibiotic was increased, a subsequent increase was observed in the radial growth. It was also seen that with the increase in incubation period, there was an increase in the radial growth of the fungus, with maximum growth being observed on the 6th day at all concentrations. However, 4th day onwards, there was no substantial change in the growth rate even when the concentration of CIP was increased (Table 1). Maximum

growth was observed at an antibiotic concentration of 500 ppm, exhibiting a 27% increase as compared to the control. CD at 5% showed a significant effect of concentrations of CIP used on the radial growth of *P. ostreatus*. Such significant increase in the growth of the fungi in presence of antibiotics has not been reported before. Use of antibiotics as growth promoters in animal feeds has been well documented (Dibner and Richards 2005; Butaye et al. 2003; Miles et al. 2006; Wegener et al. 1999) but the stimulatory effect of antibiotics specifically ciprofloxacin on the growth of *P. ostreatus* has not been reported before. The stimulatory effect of CIP on the fungus can be supported by the fact that increased fungal microbiota growth is a common side effect of antibiotic therapy (Noverr et al. 2004). This can be probably due to decrease in competition with bacterial species for food and space and reduction in microbial metabolites that depress growth (Dibner and Richards, 2005), there by stimulating fungal growth. Further, work needs to be carried out to understand the mechanism behind the growth stimulatory effect of ciprofloxacin on *P. ostreatus*.

Biomass studies

Biomass studies showed that after 7 days of incubation, maximum growth (3.703 gm wet weight/1.436 gm dry weight) was observed in medium having concentration of 500 ppm of CIP while lowest biomass production (2.448 g wet weight/1.194 g dry weight) was seen at 100 ppm. The trend continued up to 14 days of incubation where maximum growth was observed at 500 ppm (3.965 g wet weight/3.965 g dry weight) and lowest at 100 ppm (3.128 g wet weight/1.398 g dry weight). CD (5%) revealed a significant increase in mycelial growth at increasing concentrations of CIP (Table 2). Biomass studies also exhibited a pattern similar to the one observed in radial mycelium growth studies.

Table 1 Effect of different concentrations of CIP on radial growth of *P. ostreatus*

Concentration of CIP used (ppm)	Radial Growth of <i>P. ostreatus</i> (mm) ^a					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0 (control)	7.5 ± 0.228	9.0 ± 0.000	19.0 ± 0.577	34.0 ± 0.000	46.0 ± 1.154	55.0 ± 1.154
100	8.0 ± 1.154	11.0 ± 0.000	22.5 ± 1.443	34.0 ± 3.464	50.0 ± 4.612	74.0 ± 0.000
200	8.5 ± 0.866	11.5 ± 0.866	23.0 ± 1.410	36.0 ± 1.732	50.0 ± 1.154	74.0 ± 0.577
300	9.5 ± 0.288	12.0 ± 1.154	26.0 ± 1.154	39.0 ± 0.577	53.0 ± 0.577	75.0 ± 0.000
400	11.0 ± 0.000	13.0 ± 0.000	26.5 ± 1.443	40.0 ± 0.000	55.0 ± 5.196	75.5 ± 0.288
500	11.5 ± 0.288	14.0 ± 0.000	26.5 ± 1.154	40.5 ± 4.33	55.5 ± 0.288	75.5 ± 0.288
CD 5%	1.967	1.816	3.559	NS	NS	1.703

NS not significant

^a Average of three replicates with standard error; Medium used-PDA; Temperature: 25 ± 2 °C

Table 2 Effect of different concentrations of CIP on wet and dry weight of *P. ostreatus* after 7 and 14 days of incubation

Concentration of CIP used (ppm)	Wet weight(gm) ^a		Dry weight (gm) ^a	
	7 Days	14 Days	7 Days	14 Days
0 (control)	2.448 ± 0.002	2.686 ± 0.000	1.194 ± 0.000	1.389 ± 0.002
100	2.503 ± 0.000	3.128 ± 0.000	1.254 ± 0.000	1.596 ± 0.000
200	2.521 ± 0.000	3.143 ± 0.000	1.291 ± 0.000	1.604 ± 0.000
300	2.901 ± 0.000	3.457 ± 0.000	1.400 ± 0.001	1.609 ± 0.000
400	3.094 ± 0.000	3.816 ± 0.000	1.415 ± 0.001	1.614 ± 0.000
500	3.703 ± 0.000	3.965 ± 0.000	1.436 ± 0.001	1.647 ± 0.001
CD 5%	0.008	0.003	0.003	0.002

Medium used-PDB; Incubation temperature-25 ± 2 °C

^a Average of three replicates with standard error

Endo-β-D-1,4-glucanase, hemicellulase, lignin peroxidase, manganese peroxidase and laccase activity of *Pleurotus ostreatus*

P. ostreatus produces different extracellular enzymes to utilize the substrate. The effect of various concentrations of CIP on production of endo-β-D-1,4-glucanase, hemicellulase, laccase, lignin peroxidase and manganese peroxidase was determined. It was observed that with the increase in antibiotic concentration, the enzymatic activity of the fungus also increases. In case of endo-β-D-1,4-glucanase, hemicellulase, laccase, lignin peroxidase and manganese peroxidase, maximum activity was observed at 500 ppm CIP after 14 days of incubation. *P. ostreatus* exhibited a maximum endo-β-D-1,4-glucanase specific activity 392.94 μmol/min/mg as compared to 58 μmol/min/mg of specific activity observed in the absence of antibiotic in the control. Similarly, maximum specific activity of 331.40, 0.0256 and 23.864 μmol/min/mg, was observed for hemicellulase, lignin peroxidase and manganese peroxidase, respectively, after 14 days of incubation at 500 ppm antibiotic concentration (Table 3). Enzymatic activities were determined after 7 and 14 days only so as to evaluate the profile of enzymes being produced by the fungus. The work was not designed to optimize the conditions for enzyme production. A similar pattern was observed in the enzyme activities of laccase with maximum enzyme activity at 500 ppm CIP after 14 days of incubation (Data Not Provided). CD at 5% revealed significant increase in the activities of all the enzymes at different concentrations of CIP.

Sandhu and Arora (1985) reported the induction of laccase production in *Polyporus sanguineus* in the presence of different phenolic compounds and protein synthesis inhibitors. They further proposed that laccase thus represents a mechanism to eliminate these toxic compounds by enzymatic transformation. Hence the possibility that the white rot fungi might sense the antibiotic as phenolic

substrates and detoxify them, cannot be ruled out. Similarly, *Phlebia radiata* has been shown to produce lignin-modifying enzymes for detoxification purposes when toxic compounds are present in its environment (Rogalski et al. 1991) Froehner and Eriksson (1974) have reported that inhibition of extracellular protein synthesis in *Neurospora crassa*, stimulated laccase production in the presence of cycloheximide, owing to normal protein turnover. Dhawan et al. (2005) also reported the stimulatory effect of Apramycin sulfate (200 mg/l) on laccase production (18.2 U/ml) in *Pycnoporus cinnabarinus*. Similarly, Praveen et al. (2012) reported an increase in Lac, LiP and MnP production by *Stereum ostrea* and *Phanerochaete chrysosporium* in media supplemented with Tetracycline at a concentration of 200 ppm. The stimulation of ligninolytic enzyme production by certain antibiotics could be attributed to the fact that the fungi might be treating antibiotics as phenolic compounds and adopting the mechanism used for their detoxification for degradation antibiotics.

Degradation of CIP

Titrimetric analysis

Titrimetric analysis was used as one of the methods for the determination of degradation of CIP by *P. ostreatus*. On determining the concentration of FAS remaining after oxidation by cerium sulfate and calculating the % degradation thereof, it was found that the maximum degradation (68.8%) was obtained after 14 days of incubation at 500 ppm CIP (Table 4). This can be attributed to the fact that with the increase in CIP concentration, an increase in radial growth and enzyme production was observed, which in turn may have led to the increased biodegradation of the antibiotic. These results also suggest that the concentration of CIP in environment may significantly affect the biodegradation of antibiotic by the fungus.

Table 3 Effect of different concentrations of CIP on Enzyme activity of *P. ostreatus* after 7 and 14 days of incubation

Concentration of CIP used (ppm)	Enzyme activity (in $\mu\text{mol}/\text{min}/\text{mg}$) ^a							
	Endo- β -D-1,4-glucanase		Hemicellulase		Lignin peroxidase		Manganese peroxidase	
	7 Days	14 Days	7 Days	14 Days	7 Days	14 Days	7 Days	14 Days
0 (control)	35.53	58.00	43.40	62.84	0.0117	0.0129	4.295	13.779
100	51.93	99.73	58.68	105.99	0.0134	0.0159	4.704	13.863
200	53.81	164.86	65.02	144.10	0.0161	0.0184	4.805	15.454
300	94.20	185.52	71.78	164.82	0.0184	0.0212	5.426	15.909
400	94.30	269.95	82.73	235.97	0.0208	0.0222	6.231	21.786
500	112.87	392.94	93.69	331.40	0.0244	0.0256	7.441	23.864
CD 5%	0.101	0.162	0.121	0.181	0.001	0.001	0.029	NS

Medium used-PDB; Incubation temperature- 25 ± 2 °C

NS non significant

^a Average of three replicates with standard error

Table 4 Percent degradation of CIP by *P. ostreatus* after 7 and 14 days of incubation by Titrimetric and Spectrophotometric Analysis

Concentration of CIP used (ppm)	Percent degradation of CIP					
	Titrimetric analysis		Spectrophotometric analysis			
			Indigo carmine method		Methyl orange method	
	7 Days	14 Days	7 Days	14 Days	7 Days	14 Days
0 (control)	ND	ND	ND	ND	ND	ND
100	12.0	46.0	81.60	86.50	79.20	85.80
200	41.0	52.0	83.40	90.90	81.05	88.00
300	57.3	61.3	87.13	91.00	85.37	90.26
400	60.5	63.0	90.87	93.00	88.97	90.30
500	65.4	68.8	93.50	94.25	88.92	91.34
CD 5%	4.847	4.691	0.55	0.75	0.168	0.161

ND not detected

Spectrophotometric analysis

Indigo carmine assay

Spectrophotometric determination of CIP using Indigo carmine method also exhibited a pattern similar to that obtained in titrimetric analysis. Percent degradation increased with the increasing concentration of CIP. 81.50% degradation of CIP was carried out by *P. ostreatus* after 7 days of incubation when 100 ppm CIP was initially taken. This increased to 86.50% after 14 days of incubation. A maximum degradation of 94.25% was achieved at CIP concentration of 500 ppm, after 14 days of incubation (Table 4).

Methyl orange assay

Showed that after 14 days of incubation, about 86.50% of CIP was degraded when 100 ppm of CIP was originally

supplemented in the broth. As observed above, degradation increased with the increase in antibiotic concentration with highest degradation (91.34%) obtained at 500 ppm of CIP, after 14 days of incubation (Table 4).

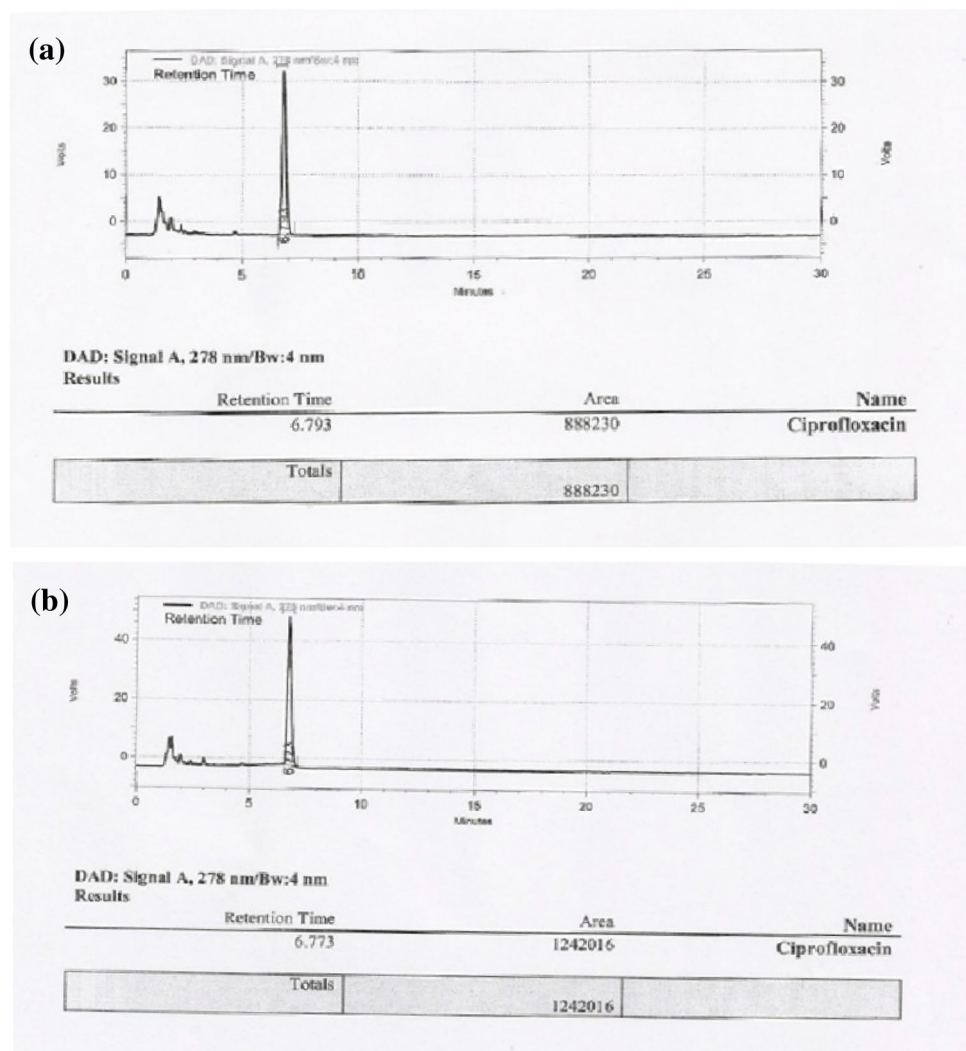
These results suggest that spectrophotometric methods are more sensitive in determination of CIP as compared to titrimetric method and Indigo carmine assay was a more sensitive method than methyl orange assay.

Validation of CIP bioremediation

High performance liquid chromatography (HPLC)

Validation of the results obtained by titrimetric and spectrophotometric methods was carried out by HPLC. On comparison with CIP standard, a degradation of 82.3% of 100 ppm CIP and 95.07% for 500 ppm CIP was observed after 14 days of incubation with *P. ostreatus* (Fig. 1).

Fig. 1 Chromatogram for CIP a 100 ppm b 500 ppm after treatment with *P. ostreatus* for 14 days



These results are in agreement with the results obtained by titrimetric and spectrophotometric analysis.

Antimicrobial activity of degraded products

Antimicrobial activity of degraded CIP was determined against *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes*. It was observed that the inhibitory action of CIP degradation products produced after incubation with *P. ostreatus* for 14 days was less as compared to standard CIP (100 and 500 ppm CIP solution). When 100 ppm CIP was used, a zone of inhibition of 2.5, 2.0 and 2.8 cm for *E. coli*, *S. aureus* and *S. pyogenes*, respectively, was observed whereas when degradation product obtained after incubation of 100 ppm CIP with *P. ostreatus* for 14 days was used, an inhibition zone of about 1.9, 1.7 and 2.3 cm for *E. coli*, *S. aureus* and *S. pyogenes* was observed. This corresponds to a 24, 15 and 17% decrease in the antimicrobial activity against *E. coli*, *S. aureus* and *S. pyogenes*, respectively. Similarly, for 500 ppm CIP used, a

zone of 4.0, 3.6 and 3.4 cm for *E. coli*, *S. aureus* and *S. pyogenes* was obtained while with degradation product obtained after incubation of 500 ppm CIP with *P. ostreatus* for 14 days, a zone of inhibition of 2.9, 2.6 and 2.6 cm for *E. coli*, *S. aureus* and *S. pyogenes* was observed, exhibiting an average decrease of 26.3% in the antimicrobial activity of CIP (Table 5).

When these results were compared by calculating CD5% using one-way ANOVA, they suggested a significant decrease in the antimicrobial activity of degraded products produced when CIP was incubated with *P. ostreatus*. These results are supported by Wetzstein et al. (1999) who found that another fungus *Gloeophyllum striatum* reduced the activity of CIP in liquid culture to 0–33% after 13 weeks.

These results are also in agreement with the growth and enzymatic studies of *P. ostreatus* in presence of CIP. The stimulatory effect of CIP concentration on the growth of *P. ostreatus* can be attributed to an increase in amount of enzyme production in the presence of CIP. Extracellular

Table 5 Antimicrobial activity of degradation products of ciprofloxacin against *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes*

Concentration of CIP used (in ppm)	Zone of inhibition (in cm) ^a								
	<i>E. coli</i>			<i>S. aureus</i>			<i>S. pyogenes</i>		
	CIP degradation products	CIP	CD 5%	CIP degradation products	CIP	CD 5%	CIP degradation products	CIP	CD 5%
D/w	ND	ND	–	ND	ND	–	ND	ND	–
0(Control)	1.5	3.0	0.226	1.6	3.8	0.358	2.0	3.6	0.358
100	1.9	2.5	0.226	1.7	2.0	0.226	2.3	2.8	0.240
200	1.9	3.0	0.226	2.5	2.8	0.226	2.5	3.0	0.226
300	2.3	3.4	0.358	2.5	3.0	0.321	2.5	3.2	0.330
400	2.6	3.6	0.321	2.6	3.4	0.330	2.1	3.2	0.160
500	2.9	4.0	0.179	2.6	3.6	0.330	2.6	3.4	0.320

Medium used—Mueller Hinton Agar; Temperature conditions 37 ± 2 °C

D/w distilled water, CIP ciprofloxacin, ND not detected

^a Average of triplicates

enzymes of *P. ostreatus* have been found to have a degradative effect on various environmental pollutants in past studies (Tellez et al. 2013; Collins et al. 1997) and can possibly be involved in the degradation of CIP using similar metabolic pathways. These results are of much significance since they indicate that treatment of CIP containing effluent with *P. ostreatus* might effectively decrease antimicrobial activity of the antibiotic, thereby leading to a negative effect on the development of antibiotic resistant bacteria in nature.

Conclusion

1. *Pleurotus ostreatus*, a basidiomycetous fungus has shown a considerable biodegradation potential towards antibiotic ciprofloxacin. Not only was *P. ostreatus* found to have a high tolerance to CIP, but it was also observed that CIP has a stimulatory effect on the fungus as observed by increased mycelial growth and enzyme activity.
2. Spectrophotometric methods were better at detection of CIP than titrimetric methods with Indigo carmine assay being the most sensitive assay in detection of CIP.
3. The products produced after degradation of CIP were found to have a reduced antimicrobial activity against test microorganisms as detected from their zones of inhibition.
4. To the best of our knowledge, the effect of ciprofloxacin on growth of *P. ostreatus* and its subsequent degradation by the fungus has not been reported before.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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