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STUDY ON CIPROFLOXACIN DEGRADATION BY EDIBLE FUNGI *Pleurotus florida.*

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT

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CERTIFICATE

This is to certify that **Sushil Kumar Singh (11306517)** has completed Dissertation project report (BTY 731), entitled "**Study On Ciprofloxacin Degradation By Edible Fungi *Pleurotus florida*"** under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study. No part of the report has ever been submitted for any other degree at any university.

This report is fit for submission and the partial fulfilment of the conditions for the award of M. Tech. in Biotechnology**.**

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DECLARATION

I hereby declare that this thesis entitled **"Study On Ciprofloxacin Degradation By Edible Fungi *Pleurotus florida*"** is an authentic record of my own work carried out at School of Biotechnology and Biosciences, **Lovely Professional University, Phagwara,** for the partial fulfillment of the award of Master of Technology in Biotechnology under the guidance of Dr. Loveleen SIngh, School of Biotechnology and Biosciences, Lovely Professional University, Phagwara.

This work is my original and has not been submitted for any degree/diploma in this or any other University. The information furnished in this dissertation is genuine to the best of my knowledge and belief.

Place: Sushil Kumar Singh

Date: (11306517)

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ABSTRACT

Antibiotics are the metabolites that secreted microbes under the stressed conditions to suppress the growth of other microbes in its habitat so that it can only utilize the nutrition for its own growth. We have isolated many of the antibiotics and have classified it into various classes according to its action of inhibition of growth of microbes. The reckless consumption of antibiotics have led to its accumulation in environment as our body is not capable of metabolizing all of the consumed antibiotics, most of the antibiotics is excreted out without being metabolized and this is getting accumulated in nature. This accumulation of antibiotics have led to the development of new antibiotics resistant microbial strains as microbes have potency of developing resistance against antibiotics if exposed to same type for prolonged durations. These resistant strains are a global threat as in present times we don't posses antibiotics that can be used to treat infections caused by resistant strains.

Therefore we must take measures either to identify and eventually killing of these developed resistant strains and preventing further development of resistant strains.

Present study focuses on degrading ciprofloxacin, with the help of an edible white rot fungi *Pleurotus ostreatus*. *P. ostreatus* is known for production of enzymes responsible for bioremediation and that is why it is being assumed that it may be helpful in simplifying the complex ciprofloxacin into non toxic substances.

Various analytical and enzymatic assays carried out to explore the degrading potentials of *Pleorotus ostreatus* gave a significant result for the degradation of antibiotics. For enzymatic studies it was observed a maximum increase in production of enzymes (glucanase, ligninase, laccases) at highest concentration of ciprofloxacin, 500 ppm. Comparative assessments of FTIR and HPLC results confirms the degradation of different concentrations of ciprofloxacin

Keywords: Antibiotics, Antibiotic resistance, Bioremediation, Ciprofloxacin, Global threat,

Pleurotus ostreaus(earlier know as *florida*).

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Chapter 1

INTRODUCTION

An antibiotic is a therapeutic agent that either kills or inhibits the growth of a microorganism. The term antibiotic was first introduced by Selman Waksman in year 1942. Antibiotics are a class of antimicrobials, including anti-fungal, anti- viral, and anti-parasitic drugs. They are among the most frequently prescribed medicaments in modern day medicine and can be either bactericidal or bacteriostatic in action. Antibiotics that can be used to treat a wide range of infections are known as broad-spectrum antibiotics. Others are effective against only a few types of bacteria and are called narrow-spectrum antibiotics (Bayarski 2007).

Antibiotics can be classified on the basis of their mechanism of action, chemical structure, or spectrum of activity. They can be classified as: aminoglycosides, cephalosporins, fluoroquinolones, macrolides, penicillins and tetracyclines. The present research focuses on the bioremediation of Ciprofloxacin, a broad spectrum antibiotic which is a frequently used fluoroquinolone. Antibiotics vary in their action. Fluoroquinolones in particularly inhibit the growth of bacteria by interfering with their ability to produce DNA, making it difficult for them to multiply. Some of the commonly-prescribed fluoroquinolones are ciprofloxacin, gatifloxacin, moxifloxacin, norfloxacin, ofloxacin, gemifloxacin, levofloxacin and trovafloxacin (Shah 2011).

Ciprofloxacin, a second generation flouroquinolone is used for the treatment of a number of bacterial infections (Kassab *et al* 2005). Ciprofloxacin hydrochloride is a mono hydrochloride monohydrated salt of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid. It is a crystalline substance which is faintly yellowish to light yellow in color and having molecular weight of 385.8 D.

The bactericidal action of ciprofloxacin results because of the inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for DNA replication, repair, transcription and recombination by a bacterium. Its spectrum of activity includes most strains of bacterial pathogens which are responsible for causing respiratory, urinary tract, gastrointestinal, and abdominal infections, that includes gram-negative (*Escherichia coli*, *Legionella pneumophila*, *Klebsiella pneumoniae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*) and also gram-positive (*Streptococcus pneumoniae* , *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*) bacterial pathogens. Ciprofloxacin can be used alone or in

combination with other antibacterial drugs for the treatment of infections in which the bacterial pathogens have not been identified (Manoranjan *et al* 2012).

Since the antibiotics have proved to be effective antimicrobial agents, this had led to rampant increase in their utilization. However, the extensive and unrestricted usage of antibiotics in our daily life has posed a major threat of accumulation of antibiotics in nature which has led to the development of antibiotic resistant strains of bacteria. This problem has aggrarated to such magnanimous proportions that it is increasingly becoming impossible to treat many diseases caused by these resistant strains. Therefore, degradation of antibiotics is an area that has been attracting attention in order to prevent the development of resistant strains (Martens *et al* 1996).

Different methods including physical, chemical and biological methods are being adopted for treatment of waste water contaminated with antibiotics adding more pollutants to environment and being marginally effective. Bioremediation involves the use of microbes to remove or breakdown complex hazardous substances into simpler less toxic and non toxic substances. Bioremediation is an ecologically safe and cost effective natural process. The process is generally 60 -70% less costly than other technologies (Laxminarayan *et al* 2013).

Therefore an alternative lies in the use of living organism for remediation of these antibiotics. However, these methods have their own limitations in being expensive. Apart from bacteria, fungi *Gloeophyllum striatum* (Martens *et al* 1996), *Phanerochaete chrysosporium* and *Trametes versicolor* (Collins et al 1997) has earlier been used to carry out degradation of antibiotics. The present study focuses on the use of mycelium of an edible fungus *Pleurotus ostreatus* (earlier known as *Pleurotus florida*) to degrade ciprofloxacin.

Pleurotus ostreatus, is a temperate edible mushroom which forms fan or oyster shaped fruiting bodies, ranging from white to gray or tan or dark brown. It can be grown on different agricultural wastes namely paddy straw, soyabean straw, wheat straw and their combinations in a temperature range of 25-28°C. The ability of *Pleurotus ostreaus* as bioremedifying agent has been attributed to production of various enzymes such as laccase, manganese peroxide, lignin peroxidases, xylanases, etc. which are important for various metabolic reactions such as substrate utilization and degradation of pollutants. The present study tends to investigate the biodegradation and/or bioaccumulation potential of *Pleurotus ostreaus* against ciprofloxacin.

Chapter 2

TERMINOLOGIES

Antibiotics: An antibiotic is an agent that either kills or inhibits the growth of a microorganism. Antibiotics can be classified as: aminoglycosides, cephalosporins, fluoroquinolones, macrolides, penicillins and tetracyclines.

Ciprofloxacin: Ciprofloxacin is a second generation fluoroquinolone. It is a broad spectrum antibiotic that can used against a number of bacterial infections. It is mono hydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid.

Antibiotic Resistance: Ability of any microbe to get resistant against any antibiotic is known as antibiotic resistance**.**

Mycoremediation: Mycoremediation is the process of using fungi for degradation or sequestering contaminants in the environment.

Photodegradation: It is a process of degrading larger compounds into smaller components with the help of radiations (Infrared, Visible or Ultra Violet).

Titrimetry: Titrimetry is an analytical technique used in laboratories for quantitative chemical analysis of unknown concentration or an identified analyte. It is also known as volumetric analysis as volume plays a key role.

Spectrophotometry: Spectrophotometry is a quantitative measurement technique which uses property of reflection or transmission of a material as a function of wavelength. Spectrophotometry can use wavelengths of light of visible, near ultra violet or Infra red range.

FTIR: Fourier transform infrared spectroscopy (FTIR) is a technique that uses infrared spectrum of light for absorption, emission, photoconductivity or raman scattering of a solid, liquid or gaseous sample. An FTIR spectrometer collects high spectral resolution data over a wide range of spectral lines. FTIR spectrometer gives a significant advantage over a dispersive spectrometer as it measures intensity over a narrow range of wavelengths at a time.

The term "Fourier transform infrared spectroscopy" originated from Fourier transform (a mathematical process) which is required to convert the raw data obtained from FTIR into the actual spectrum.

HPLC: High Performance Liquid Chromatography (HPLC) is an analytical chemistry technique which used to separate, to identify, and to quantify each component from a mixture. Pressurized liquid solvent containing the sample mixture is pumped through a column filled with a solid adsorbent. Each component in the sample interacts slightly differently with the adsorbent material, which causes a difference in flow rates of different components, leading to the separation of the components as they flow out the column.

TLC: Thin Layer Chromatography (TLC) is a chromatographic technique which is used to separate non-volatile mixtures. Thin-layer chromatography is performed on Sheets of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose. This layer of adsorbent is known as the stationary phase

CHAPTER 3

REVIEW OF LITERATURE

Waksman introduced the term “antibiotic” in 1942. Antibiotics are natural drugs secreted by several fungi or bacteria which have an inhibitory action against other microorganisms. On the other hand chemotherapeutic drugs are man-made substances. The difference between the two were realized when the process of chemical synthesis of some antibiotics along with new drugs were developed from the natural products by binding various side chains to the basic structures.

Bayarski (2007) and Shah (2011), separately explained the different types, uses and side effects of each of the antibiotic. Antibiotics have been classified into different groups based upon their structure and mode of action: aminoglycosides, cephalosporins, macrolides, penicillins, tetracyclines and fluoroquinolones. The following paragraphs deals with different classes of antibiotics in detail.

Aminoglycosides

Aminoglycoside are mainly used to cure infections caused by gram-negative bacteria. These can also be used in combination with penicillins and cephalosporins to have prolonged effect bacteria. The aminoglycosides inhibit the growth of bacteria by inhibiting protein making pathways. Side effects caused by aminoglycosides include possible damage to kidneys and ears. Though aminoglycosides work fairly significantly, but bacteria may become resistant to them if exposed to higher and frequent usage. The most commonly prescribed aminoglycosidic drugs includes Amikacin, Gentamicin, , Tobramycin, Kanamycin , Neomycin, Streptomycin (Leclercq *et al*, 1999).

Cephalosporins

Cephalosporins are closely related to the penicillins. Cephalosporins have been categorized chronically, and therefore these are divided into generations viz first, second, third generations, according to their antimicrobial properties. Each next generation of cephalosporins have greater gram negative antimicrobial property than the preceding generation. Cephalosporins show a bactericidal effect by inhibiting the synthesis bacterial cell

wall, making it difficult to multiply. Cephalosporins are used to treat of infections like strep throat, pneumonia, otitis media, staphy infections, bronchitis, tonsillitis, various types of skin infections, such as gonorrhea, etc. Cephalosporins are also used for surgical prophylactic treatment (Yusof *et al*, 2011).

Macrolides

Macrolides belong to the polyketide class of natural products. Macrolides bind with ribosomal unit of susceptible bacteria inhibiting protein production in it. This is mainly bacteriostatic action, but can also be bactericidal if high concentrations of macrolides are used. Macrolides are used for the treatment of respiratory tract infections, soft tissue infections, genital and gastrointestinal tract infections caused by bacteria of specific strains. The most concerning issue of these antibiotics is that they can irritate the stomach. The most commonly prescribed macrolides are Clarithromycin, Erythromycin, Roxithromycin and Azithromycin (Zuckerman MJ, 2004).

Penicillins

Penicillin was the first antibiotic that was discovered by Alexander Fleming in year 1929. Mode of action of penicillins includes blocking the cell wall construction in bacteria and causing the breaking down of walls which eventually leads to killing of the bacteria. Penicillins are prescribed for the treatment of infections of skin, ear respiratory tract, urinary tract, dental problems and gonorrhea. Penicillins are generally safe in use but the greatest risk is occurrence of allergic reaction, which can be severe in some people. The most commonly given penicillins are Penicillin, Ampicillin, Bacampicillin, Oxacillin, and Amoxicillin (Myers W G, 1944).

Tetracyclines

Discovery of Tetracyclines were done in late 1940s and these were extremely popular in use when these were first discovered. These are inhibitors of protein synthesis in bacteria. They act by inhibiting the binding of aminoacyl tRNA with bacterial ribosome. These are broad spectrum antibiotics targeting for the treatment of diseases like rocky mountain spotted fever, mild acne, upper respiratory tract infections, lyme disease, urinary tract infections, typhus and

sexually transmitted diseases. Commonly given tetracyclines are Minocycline, Doxycycline and Tetracycline (Zakeri and Wright, 2008).

Fluoroquinolones

Fluoroquinolones are broad-spectrum antibiotics that inhibit bacterial growth by interfering with their DNA making ability. This interference does not allow the bacteria to synthesize DNA or to carry out repair mechanism which eventually leads to killing of bacteria as well as prevent them from multiplying. This mode of action is bacteriocidal. Fluoroquinolones are used for the treatment of infections of skin, common urinary tract, and respiratory infections (such as sinusitis bronchitis, pneumonia). Side effects caused by fluoroquinolones include problems related to the digestive system such as mild stomach pain or stomach upset, vomiting and diarrhea (Somasundaram and Manivannam, 2013, Sharma *et al*, 2009).

The most commonly prescribed fluoroquinolones are Gatifloxacin, Ciprofloxacin, Ofloxacin, Gemifloxacin, Moxifloxacin, Ofloxacin, Norfloxacin, Trovafloxacin (Goldman and Kearns, 2011).

Use of Antibiotics

Antibiotics is not only used as a medicament for humans but it also used for agricultural purpose as herbicides against various herbs, insecticides for insects that infect crops, pesticides for pests control in fields. Antibiotics are also used for veterinary purpose for the treatment of bacterial infections in animals. Antibiotics are also used for the treatment of water to make it available for drinking purpose. Antibiotics are also used in food processing industries to so that the packaged food remains free from microbial contamination for longer durations (Perlinski and Mudryk, 2009).

The following paragraphs discuss on various aspects of ciprofloxacin and its degradation.

CIPROFLOXACIN

Ciproflaxacin, a fluoroquinolone is most commonly given in the form of ciprofloxacin hydrochloride, which is a mono hydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1,4 dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid. It is a crystalline substance

which is faintly yellowish to light yellow in color having a molecular weight of 385.8. Its empirical formula is $C_{17}H_{18}FN_3O_3.HCl.H_2O$ and its chemical structure is as follows:

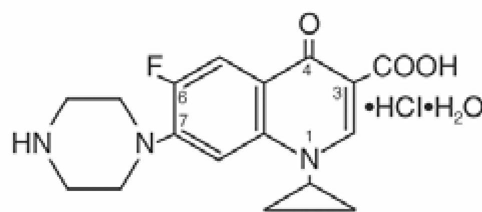

Fig 2.1. Structure of ciprofloxacin

Ciprofloxacin is used in the treatment of acute uncomplicated cystitis in females, urinary tract infections, chronic bacterial prostatitis, respiratory tract infections, complicated intra-abdominal infections, acute sinusitis, infectious diarrhea, uncomplicated cervical and urethral gonorrhea, typhoid fever (enteric fever) and many more diseases (Amin A S, 2000, Sarkozy G, 2001).

Mode of action

It is one of the most commonly prescribed antibiotics which have *in vitro* activity against a wide range of gram-negative as well as gram-positive bacteria. The mode of action of ciprofloxacin is of bactericidal type and it affects the enzymatic activity of enzymes topoisomerase II and IV (DNA gyrases), that help in DNA replication, transcription, repair and recombination mechanism by any bacteria. The mode of action of ciprofloxacin differs from other types of antibiotics therefore microbes resistant to any of the above mentioned classes of drugs may be susceptible to ciprofloxacin and other fluoroquinolones. There is no reported cross-resistance between ciprofloxacin and antimicrobials of other class antibiotics (Hassib *et al*, 2007, Razuq *et al,* 2010).

Antibiotic Resistance

The reckless and unmonitored use of antibiotics has been a major cause of concern in the recent past. Due to the excessive use of antibiotics microorganisms are becoming increasingly resistant to them and higher doses or novel antibiotics are required to prevent infection. Antibiotic resistance is developed when bacteria are exposed to the same type of drugs over and over again with time. The bacteria develop resistance due to these continuous

exposures and become resistant to the drugs that were used earlier to stop its growth. There are number of ways in which bacteria may transform to antibiotic-resistant strain. For example, bacteria have an internal mechanism with the help of which it can modify its structure so that the antibiotic cannot interact with its outer membrane. Similarly they develop different pathways to neutralize or inactivate the antibiotic. In addition, bacteria may transfer the genes encoding for antibiotic resistance to similar strains making it possible for other bacteria who was never exposed to an antibiotic to gain resistance from those who have. Development of resistance to antibiotics poses a serious and growing global threat, because some infectious diseases are now becoming more difficult to be treated. Resistant bacteria don't respond to the presently given antibiotics and continue to infect. Some of these resistant bacterial strains can be treated with even more powerful medicines, but there are some infections that cannot be treated even with new or experimental drugs.

Need of antibiotic bioremediation

Reckless use of antibiotics containing drugs is the single most important factor leading to development of antibiotic resistant microbial strains around the world. Up to 50% of the antibiotics prescribed to people are not needed or are not optimally effective as prescribed. Antibiotics are also used in veterinary to prevent, control and treat disease, and to promote the growth of animals. The use of antibiotics for promoting growth is not at all required, and this practice of use of antibiotics for this purpose must be phased out. According to data provided by the Department of Health and Human Services, 20,49,442 cases of illness and 23,000 deaths have been reported in US only in year 2013 only due to antibiotics resistance. An estimated minimum number of cases of illnesses and death due to *Clostridium difficile* in US are about 2, 50, 000 and 14, 000.

The other major factor responsible for the rise in antibiotic resistance is spread of the resistant strains from person to person, or by non-human sources in the environment. There are few core actions that are needed which may help us to fight with these deadly infections: first could be preventing infections and the spread of resistance, second tracking down the resistant strain of bacteria, third, limiting the use of antibiotics and promoting the development of new antibiotics as well as developing new diagnostic tests for detection of resistant strains of bacteria. Bacteria will inevitably find ways of developing resistance against antibiotics we develop, that is why an aggressive action is now needed to keep a check on development of new resistant strains and to prevent the resistance from spreading.

Degradation of Antibiotics

a. Physical and Chemical Methods

Use of an advanced process of oxidation for detoxification, mineralization of waste water and recovery of water mainly contaminated by antibiotics (mainly ciprofloxacin) was considered for degradation of antibiotics. Methods such as oxidation and mineralization can be employed alone or in combination with one another or even by complementing the traditionally used methods, allowing the disinfection of bacteria and viruses. Using experimental systems like $UV/H_2O_2/O_3$ or UV/H_2O_2, total mineralization of the compound can be achieved. Ciprofloxacin solutions were used at concentrations of 50, 100, 200, 300, 400 and 500 ppm for study and degradation of 100% of antibiotics was achieved and it was confirmed microbiologically (with the presence or absence of inhibition zone) showed that loss of biological activity of antibiotic is inversely proportional to the time it was exposed for (Hernandaz *et al* 2012).

According to Razuq *et al* (2010), ciprofloxacin can be photochemically degraded with the use of UV light but understanding the kinetic of the photodegradation under different conditions is needed. They irradiated samples with UV Vis spectra for about 20 mins. Tandem arrangement was observed using diode array detectors. The fusion of UV-Vis and fluorescence data by Multivariate Curve Resolution Alternating Least Squares (MCR-ALS) made it possible to study the kinetic of ciprofloxacin degradation. In results, a degradation profile of ciprofloxacin was observed and also photodegradation products were observed. From photodegradation results, it was deduced that degradation was effective at higher pH.

Ratpukdi (2014), carried out process of antibiotic degradation (paracetamol and norfloxacin) using vacuum ultraviolet (VUV) process using contaminated water. The effects of initial concentrations of antibiotics (1, 5 and 10 mg/L), initial pHs (5, 7 and 9), VUV powers (30, 60 and 120 W) and bicarbonate concentrations (100 and 200 mg/L as CaCO3) were studied. The experiments under ultraviolet (UV) condition were performed for comparative assessment. The results displayed that the VUV process exhibited superior degradation performance than that by the UV process. The rates for both paracetamol and norfloxacin degradation were of second order reaction. Higher concentrations resulted in the decrease in degradation rate. The initial pH had low impact on pharmaceuticals removal efficiency but the neutral pH showed the highest degradation efficiency. Bicarbonate decreased the pharmaceutical removal. In the last it was concluded that with increase in VUV power efficiency of degradation increased.

Deshpande *et al*, (2004) gave a review on degradation of β lactam antibiotics. β lactam antibiotics are characterized by susceptibility to various nucleophiles, acidic or basic reagents, metal ions, oxidizing agents or even solvents like water and alcohol. Degrdation of penicillins takes place at various conditions viz. alkaline or acidic, in the presence of enzyme *b*-lactamase or treatment of weak nucleophiles like water and metal ions. Under strong UV absorbtion at 322nm, it has been reported that penicillin breakdown intolightercomponents products, including penilloic acid, penicillamine and penilloaldehyde. It has also been stated that if penicillins are exposed under UV radiations at 320 nm under acidic conditions,it gives a complete degradation.
Enzymes such as β lactamase, acylase cephalosporin C and penicillinase can also be used for degradation of β lactams.

Homem and Santos, 2011 together, gave a review on methods of degradation and removal of antibiotics from aqueous matrices. Different techniques of remediation were evaluated and compared, such as conventional techniques (biological processes, filtration, coagulation, flocculation and sedimentation), advanced oxidation processes (AOPs), adsorption, membrane processes and combined methods. In their study, they found that ozonation, Fenton/photo-Fenton and semiconductor photocatalysis were the most tested methodologies. Combined processes seemed to be the best solution for the treatment of effluents containing antibiotics, especially those using renewable energy and by products materials.

Tifenbacher *et al*, (1994), studied the photo stability of different flouroquinolones (ciprofloxacin, ofloxacin and fleroxacin) against ultra violet radiations and visible light in dilute aqueous solutions. Photoproducts were observed by high performance liquid chromatography (HPLC) for all the three drugs. An exposure of about 1 hr, was sufficient to detect photoproducts of ciprofloxacin. The major ciprofloxacin products was characterized as a dimer by liquid secondary ion mass spectrometry but its structure was not determined.

b. Biological Degradation

In addition to the photolytic degradation, microbial degradation can also be employed for ciprofloxacin bioremediation.

Wetzstein *et al* (1999) carried out degradation of ciprofloxacin using basidiomycetous fungus *Gloeophyllum striatum* by keeping a check on production of $^{14}CO_2$ from [^{14}C] which was supplemented with ciprofloxacin in liquid cultures. Sixteen species inhabiting soil, wood, animal dung or humus produced up to 35% $^{14}CO_2$ in incubation period of 8 weeks. Despite low rates of formation of $^{14}CO_2$, all species that were isolated were tested and were found to reduce the antibacterial activity of ciprofloxacin in supernatants in between 0 and 33% after an incubation time of 13 weeks. Although more than half of ciprofloxacin (applied at 10 ppm) was transformed into metabolites after 90 h. The structures of 11 products were elucidated by high performance liquid chromatography combined with electrospray ionization mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy.

Martens *et al* (1996), worked on the degradation of floroquinonolone enrofloxacin by wood rotting fungi. They isolated different fungal strains viz. *Gloephyllum striatum, Stropharia rugosonnulata, Phanerochaete chrysosporium, Irpex lacteus and Phellicnus gilvis* on wet straw containing carbonyl C^{14} labeled enrofloxacin. They observed a maximum production of $^{14}CO_2$, about 17% per week using the brown rot fungus *Gloephyllum striatum*, resulting in increase of yield upto 53% after 8 weeks. If enrofloxacin was preadsorbed on native or gamma ray sterilized soil, then the rates reached 0.2 and 0.9 % per week which was lower then observed rate of degradation (17% per week).

Enzymes produced by white rot fungi

Singh *et al* (2012), studied the production of extracellular enzyme xylanase using three species of *Pleurotus* viz. *P. flabellatus, P. florida and P. sajor caju* under lab conditions. Pretreated lignocellulosic waste was used for their growth. Pretreatment of paddy straw and wheat straw was done using oil extracted from neem (*Azadirachta indica*) and ashoka (*Saraca indica*) leaves. Paddy straw pretreated with neem oil, supported better xylanase production than wheat straw. Initial, production of xylanase was less but in subsequent days it increased and reached its peak on 25*th* day of cultivation. Thereafter, decrease was observed in the activity of the enzyme. Among the three species, *P. florida* showed maximum enzyme activity followed by *P. flabellatus and P. sajor caju.*

Jegatheesan *et al* (2012), suggested laccase as the major ligninolytic enzyme secreted by the white-rot fungi that has many biotechnological applications such as dye degradation, removal of toxic organo pollutants and melanin synthesis. In presence of PAH (Anthracene) and by using media engineering techniques and statistical design experiments involving Response Surface Methodology they tried to increase laccase production activity in *Pleurotus florida,* Central composite design was used to optimize media components as well as to study the effect of malt extract, peptone and $CaCl_2$ on laccase activity. The optimized amounts of nutrients (Peptone, Malt Extract and $CaCl_2$ was 1: 1: 0.1gm respectively) were used as supplements to basal salt medium, resulting in an increase in activity of laccase to ten folds (from 2.20 IU/ml to 22.6 IU/ml) over the controlled treatment using optimised basal salt medium.

Tellez *et al* (2013) discussed the importance of *Pleurotus* species are considered as white rot fungi for their ability to degrade lignocellulosic residues due to their enzymatic complex. The most studied enzymes in these fungi are lignin-degrading, such as laccases, Manganese peroxidases and veratryl alcohol oxidases; however, these fungi also have the ability to degrade cellulose, hemicellulose and other carbohydrates present in agro-industrial waste; so these fungi are a potential source of production of hydrolytic enzymes, which have been studied a little in these organisms.

Study on the enzyme activity *of Pleurotus tuber-regium* fruit bodies and sclerotia cultivated using various types of agro-wastes was carried out by Ahmad *et al* (2009). Higher activity of amylase, proteinase and glucose-6-phosphatase were observed in the sporophores when compared to the sclerotia. Cellulase, lipase and carboxymethylcellulase values were higher in fruit bodies that were grown on cotton waste, sawdust of *Khaya ivorensis* and rice straw (2.4, 0.4 and 3.0 mg/h protein, respectively). Sclerotia started on shells of ground nut and cocoyam peels, gave lipase and phenoloxidase levels of 5.8 and 2.6 mg/h protein, respectively.

Pleurotus florida, *Pleurotus ostreatus* and *Pleurotus sajor-caju*, were screened by Das *et al* (2012) for cellulolytic enzyme production under submerged fermentation conditions. Of these, *Pleurotus florida*, was studied for optimizing medium composition, incubation period, initial pH and incubation temperature to maximize cellulolytic enzyme production. Malt extract at 0.5%, 12 day of incubation period and 1% carboxymethylcellulose as carbon source supported maximum production of cellulases. The optimum temperature and pH for

maximum production of enzymes were found to be 35 to 40°C and 5.0 respectively for exo- and endoglucanases and 30°C and 4.5 for β-glucosidase.

Study on *Pleurotus ostreatus* by Singh *et al* (2012) suggested doubling in production of lignin biodegradation when of sawdust (36%) was used as substrate as compared with rice straw (18%). However, degradation of hemicelluloses and cellulose was observed to be faster in rice straw (66.6%, 31.2%) than sawdust (60.0%, 18.2%). On the contrary, xylanase showed an early increase in production of enzyme activity on sawdust (21.0I U/g) during mycelial growth which sharply decreased when fruiting stage (11.0I U/g) arrived.

Rana and Rana (2011), obtained crude enzyme extracts of *Agaricus* and *Pleurotus species,* and carried out assays for cellulases namely CMCase, cellobiohydrolase, α-glucosidase and filter paperase. For ligninase enzyme assay, guaiacol-oxidizing peroxidase activity and laccase activities were determined. All the strains of *Agaricus* showed elevated level of cellulases activities as compared to *Pleurotus* strains. In contrast to this *Pleurotus* species exhibited high ligninase activity.

Bioremediation potentials of *Pleurotus ostreatus*

Espindola *et al* (2007), worked on the area of degradation of (Red 40 dye) with the help of edible mushroom *Pleurotus florida*. *Pleurotus florida* was grown on nutrient rich media and poor liquid media in different concentration of dye and with varying pH. Spectrophotometric analysis indicated, that the dye had biodegraded in concentrations of 1.1 - 5 and 2.1 - 5 $Moldm^{-3}$, with the best results obtained at a pH of 4.5. It was also found that the mycelia grown in both nutrient-rich and nutrient-poor media presented Red 40 biodegrading activity. From this experiment it was presumed that the cultivation of mushrooms in liquid medium increase biodegradation, as it favors hexosamine and laccase activity

Methods of detection of ciprofloxacin after degradation

Microbiologically

Cazedey and Salgado (2013) worked on developing a rapid turbidometric assay for determination of ciprofloxacin hydrochloride in ophthalmic solutions. The validation method

used yielded good results including excellent linearity, precision, accuracy and specificity. The bioassay method was based on the inhibitory effect of ciprofloxacin on the strain of *Staphylococcus epidermidis* ATCC 12228 which was used as the test microorganism. The results were found to be linear (*r*=0.9994, in the range of 14.0–56.0 µg/mL), precise (intraday RSD%=2.06; interday RSD%=2.30) and accurate (recovery=99.71%). The turbidimetric method was compared to the UV spectrophotometric and HPLC for the same drug.

Hernandaz *et al* (2012) used microbiological method for the confirmation of loss of biological activity of ciprofloxacin after irradiating it with UV/H_2O_2/O_3, UV/H_2O_2. Disk diffusion method was employed for the same by saturating the disks with different solutions of ciprofloxacin having different concentrations (50, 100, 300, 400 and 500 ppm) and results were evaluated on Mueller Hinton media against *Klebsiella pneumonia.* The plates were incubated for 24 hrs at room temperature and reading of inhibion diameter was taken.

Titrimetry

Basaiah *et al* (2006), assayed ciprofloxacin titrimetrically using cerium sulphate. The method involved treating ciprofloxacin with cerium sulphate in presence of acidic conditions and the unreacted ciprofloxacin was then back titrated using ammonium ferrous sulphate using ferroin indicator.

Spectrophotometry

Nijhu *et al* (2011) developed an assay method for the determining ciprofloxacin and naproxen using UV spectrophotometer. Both the drugs obey beer's law in the concentration range of about 1-5 $\mu g ml^{-1}$. Phosphate buffer and distilled water were used to plot the standard curve for ciprofloxacin and naproxen. Maximum absorbance for ciprofloxacin in distilled water was observed at wavelength of 278 nm and for naproxen wavelength was 228 nm. In 0.1N HCl maximum absorbance for ciprofloxacin was at 277 nm and for naproxen at 228 nm.

Basavaiah *et al* (2006) also worked on developing spectrophotometric methods for determining ciprofloxacin and naxopren in pharmaceutical formulations. They used two spectrophotometric methods one using cerium sulphate as an oxidimetric agent and methylorange as chromogenic agents while the other method used cerium sulphate along with

indigo carmine dye. Using methyl orange, maximum absorbance was obtained at 520 nm and maximum absorbance was found to be at 610 nm when indigo carmine was used.

Chromatographic Methods

The conventional methods of separation and analysis have been taken over chromatographic methods. Other then separation from a mixture having many different components, chromatographic methods have an advantage of greater accuracy and sensitivity for even small quantity of degraded product produced. Various chromatographic types and methods such as TLC, HPTLC, GC, HPLC, RPHPLC, Up flow HPLC have been used for assaying products.

Nandipati *et al* (2013) worked on developing a sensitive and selective method for rapidly quantifying the ciprofloxacin present in pharmaceutical formulations by using ultra high performance liquid chromatography. They carried out the ciprofloxacin assay using octadecryl C18 column having particle size of 1.8µm connected to water acquity UPLC system. Buffer solution was prepared using 2.9 ml of phosphoric acid (85%) in 1000ml of water. Mobile phase was prepared by using buffer and acetonitrile in the ratio of 88:12 v/v respectively. The flow rate was set at 0.3ml min^{-1}, column oven temperature was kept at 50ºC and detection was made at wavelength of 278 nm. During % RSD calculation peak areas of ciprofloxacin was found to be 0.18, the tailing factor and theoretical plates for ciprofloxacin were found to be 1.20 and 17341 respectively. The resolution between ethylenediamine and ciprofloxacin was found to be 10.41.

Jansari *et al* (2012) also worked in developing and validating the stability method for estimation of tinidazole and ciprofloxacin using RP-UPLC. They analyzed the sample by using reverse phase C18 column (Purospher Star 100x2.1 mm, 2 µm) as stationary phase and used buffer of phosphate and acetonitrile (80:20) as mobile phase. pH was adjusted to 3.0 using orthophosphoric acid and flow rate was maintained at 0.3ml min^{-1} .Quantification of ciprofloxacin hydrochloride was achieved at 278.5 nm and tinidazole at 317.5 nm with PDA detector. Retention time for ciprofloxacin hydrochloride was 1.71 and for tinidazole it was 2.22 min.

Hubicka *et al* (2013) carried out chromatographic separations using the Acquity UPLC BEH C18 column (2.1x100mm, 1.7 µm particle size). The column was maintained at 40ºC and the

following gradient was used: 0 min, 95% of eluent A: 0.1% (v/v) formic acid in water and 5% of eluent B: 0.1% formic acid in acetonitrile, 10min: 0% of eluent A and 100% of eluent B at a constant flow rate 0.3μgml^{-1}.

Kassab *et al* (2005) worked on quantitative determination of ciprofloxacin and norfloxacin in the pharmaceutical preparations using high performance liquid chromatography. For HPLC they used LiChrospher® 100 RP-18 column (5 μm, 125x4 mm). A mixture of water : acetonitrile : triethylamine in ratio of 80:20:0.3 v/v/v was used as mobile phase. Phosphoric acid was used to adjust the final pH of the mixture to 3.3. 1.0 mlmin^{-1} of flow rate was maintained constantly and UV detection was made at 279 nm. The analysis was performed at room temperature (24 ± 2ºC). ciprofloxacin and norfloxacin were eluted out with in 5min.

CHAPTER 4

RATIONALE AND SCOPE OF THE STUDY

Medical practitioners throughout the World are prescribing antibiotics more frequently and many times in higher than required dose. Our body cannot metabolise the entire consumed drug. Most of the drug is excreted out by the body which gets accumulated in nature. Use of fertilizers, pesticides, insecticides, etc containing antibiotics in fieldsand veterinary practices is also one of the major causes of accumulation of antibiotics in nature. This accumulation of large amount of antibiotics have led to development of various antibiotic resistant microbial strains as bacteria have a potential to develop resistance against antibiotics, if exposed for longer durations. Some examples of antibiotic resistant strains of bacteria are *Clostridium dificile, Pseudomonas aeruginosa*, etc.

The purpose of the current research work is to study the degradative effect of edible fungus *Pleurotus ostreatus* on ciprofloxacin, which is one of the most prescribed antibiotics for both human and veterinary use. *Pleurotus ostreatus* has been found to be an effective biodegradation agent, therefore, the use of this fungus for bioremediation of ciprofloxacin is being explored.

CHAPTER 5

OBJECTIVES OF THE STUDY

The broad objective of the study is to study to evaluate the potential of *Pleurotus ostreatus* for biodegradation of antibiotic ciprofloxacin.The study was planned in order tomeet the following ojectives:

1. To study the degradation of antibiotic ciprofloxacin with the help of edible fungi *Pleurotus ostreatus.*
2. To study various enzymes produced by *Pleurotus ostreatus.*
3. To study the degraded products of ciprofloxacin.

Chapter 6

MATERIALS AND RESEARCH METHODOLOGY

EXPERIMENTAL WORK

Experiment 1: Procurement and maintenance of *Pleurotus florida* culture.

Place of work: Department of Biotechnology, LPU.

Methodology: The mycelium of *Pleurotus ostreatus* was procured and maintained by frequent sub culturing on Potato Dextrose Agar medium in laboratory from the culture obtained from Department of Microbiology, PAU, Ludhiana Each culture was separately checked for its fruiting ability and was maintained on potato dextrose agar (PDA) slants.

39 gms of the medium was suspended in 1000ml of distilled water (media composition given below). It was then heated to boiling to dissolve the components completely. The medium was dispensed into test tubes to $1/4^{th}$ of their capacity. The culture tubes were then plugged with non-absorbent cotton, autoclaved at 15 psi for 20 mins (at 121^0 C) and allowed to solidify on support to make slants.

Components	Composition (gm/litre)
Potato infusion form	200
Dextrose	20
Agar	15
Final pH at 25^0 C	5.6 ± 6.2

Maintenance of culture

Sub-culturing of *Pleurotus ostreatus* strains was done routinely after every 4 weeks. From the mycelia slants, small bits of mycelial agar was picked and transferred to freshly prepared agar slants by placing the side of bit in such a way that mycelium end touches the agar. The slants were then maintained at 25±2 °C for 8-10 days to let the mycelium run on agar surface. The mycelia run slants were then stored in refrigerator for four weeks before next sub culturing.

Statistical analysis – Nil

Experiment 2: To study the effect of different concentrations of antibiotic ciprofloxacin on mycelial growth of *Pleurotus ostreatus*.

Place of work: Department of Biotechnology, LPU.

Methodology: Mycelium of *Pleurous ostreatus* was inoculated in sterile flasks containing PDB with different concentration of ciprofloxacin @ 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm and were kept subsequently for 7 and 14 days for biomass production and degradation of ciprofloxacin. The flasks were maintained at 25 ± 2^0C for 7-14 days. A control was also prepared for each set devoid of ciprofloxacin for further assays and analytical studies.

Pleurotus ostreatus mycelium was inoculated on PDA, supplemented with different concentration of ciprofloxacin (@ 100, 200, 300, 400, 500 ppm) and its effects on radial growth of *Pleurotus ostreatus* were studied. Sterile PDA medium containing different concentration of ciprofloxacin will be dispersed into sterile glass petriplates. The plates were then inoculated at the centre of plate with 8mm bit of agar culture of *Pleurotus ostreatus* and were incubated at 25 ± 2^0C for 6 days. The colony diameter was measured and noted after every 24 hours until the petri plates are covered with white fluffy *Pleurous ostreatus*. Medium devoid of antibiotic was taken as control.

Statistical analysis – CD @ 5%

Experiment 3: To study changes in enzymatic activity of *Pleurotus ostreatus* in the presence of ciprofloxacin.

Place of work: Department of Biotechnology, LPU.

Methodology: *Pleurotus ostreatus* was grown in broth culture in presence of different concentrations of ciprofloxacin. After 7 and 14 days wet weight as well as dry weight of biomass produced and enzymatic activity were checked and determined. For estimating quantitative enzyme activity, wet weight and dry weight studies were carried out by collecting and weighing the fungal mycelium after 7 and 14 days of incubation.

For this, 50ml of PDB was prepared in 100ml conical flasks and was supplemented with different concentrations of ciprofloxacin (100-500ppm). The medium was then inoculated

with equal sized (8 mm) bits of culture of *Pleurotus ostreatus*. After 7 and 14 days of incubation at 25 ± 2^{0}C the culture broth was centrifuged and the supernatant was taken in order to quantify the enzyme activity in the terms of cellulase (endo β-1,4 glucanase), hemicellulase (xylanase) and ligininase (laccase, lignin peroxidase, manganese peroxidase).

Estimation of Endo-β-D-1,4-glucanase (Miller 1959)

Reagents

1) 0.1M citrate buffer (pH 4.8)

   A) 0.1M citric acid
   B) 0.1M tri sodium citrate

23 ml of A and 27 ml of B were mixed and volume was made to 100ml with distilled water

2) Carboxymethyl cellulose (CMC)- 1g of CMC was dissolved in 90 ml of 0.1M citrate buffer of pH 4.8 and volume was made to 100 ml.
3) Dinitrosalicylic acid (DNS) solution- 10 gms of DNS and 0.5g sodium sulphite in 500 ml of 2% NaOH solution already prepared in distilled water was added. The solution was allowed to cool down, 2g of phenol was dissolved in that and final volume was made to 1000ml. The solution was then filtered and stored in dark bottles in refrigerator.
4) Sodium potassium tartrate ((Na-K-tartrate) solution (40%)- 40g of Na-K-tartrate was dissolved in 100ml distilled water. The solution was filtered and stored at room temperature.

Assay

Test tubes containing a mixture of 0.5 ml of CMC solution and 0.5 ml of culture filtrate were kept for incubation at 50° C for 30 mins in water bath. Controls devoid of enzyme extract were also run simultaneously.

Reducing sugar produced during this reaction was measured by using DNS method. 3 ml of DNS was added to each tube and kept in boiling water bath for 15 mins. While still hot

1 ml of Na-K-tartrate solution was added, the contents were allowed to cool down to room temperature followed by addition of 2 ml of distilled water in each test tube. Enzyme extract was added to controls after adding DNS. The per cent light absorbance by the resulting solution was recorded at 575nm by using Systronics UV- VIS spectrophotometer 108. The corresponding enzyme activity was calculated from standard curve.

Laccase

Laccase estimatation was carried out according to the method of Turner (1974) with some modification as described by Singh *et al* (1988).

Reagents

1) 0.1M phosphate buffer (pH 6.0)

   a) 0.2M KH_2PO_4

   b) 0.2M K_2HPO_4

   87.7ml of A and 12.3ml of B and diluted to 200ml.

2) 0.22M Guaiacol (pyrocatechol monoethyl ether $C_7H_8O_2$) solution- 0.22M solution of guaiacol was prepared in 0.1M phosphate buffer (pH 6.0) and lept in dark colored bottle and stored in refrigerator.

Assay

1 ml of enzyme extract and 3 ml of buffered guaiacol was added to each test tube containing enzyme extract, solution was mixed and tubes were placed in colorimeter immediately. The change in absorbance was recorded for every 15 sec upto 120secs at 495nm. The constant change in absorbance was plotted for laccase activity.

Enzyme units were expressed as an increase in OD by 0.01 in 60 sec. Enzyme activity was expressed as units/ ml of culture filtrate.

Hemicellulase (Endoxylanase): Bucht and Erikson (1968)

Reagents

1) Xylan solution- 1 gm of xylan was dissolved in 0.05M citrate buffer, pH 4.8 and volume made 100ml
2) Dinitrosalicylic acid reagent
3) Sodium potassium tartrate solution (40%)

Assay

One ml of sample of approximately diluted culture was mixed with 1ml of 1% xylan solution and incubated for 30 mins at 50° C. Reducing sugar was measured as xylose equivalents by DNS method. Units of enzyme activity were taken as one unit of endoxylanase that catalysed the formation of 1µmol of xylose/min/ml of culture filtrate.

Lignin peroxidases: Tien and Kirk (1983)

The assay is based on the oxidation of veratryl alcohol to veratraldehyde in the presence of hydrogen peroxide. Reaction mixture contained 0.25ml of enzyme solution, 0.25ml of 1mM veratryl alcohol, 0.2mM hydrogen peroxide and 0.5ml of 0.1M citrate buffer. The absorbance was measured at 310nm.

Manganese peroxidases: Kuwahara *et al* (1984)

This assay is based on using phenol red as substrate by measuring the optical density spectrophotometrically at 610nm. The reaction mixture consists of enzyme extract (250µL), 0.1% phenol red, 250mM sodium lactate, 2mM manganese sulphate, 0.5% BSA, 0.2mM hydrogen peroxide in 0.1M citrate buffer pH 5 (0.5ml).

Statistical analysis – CD @ 5%

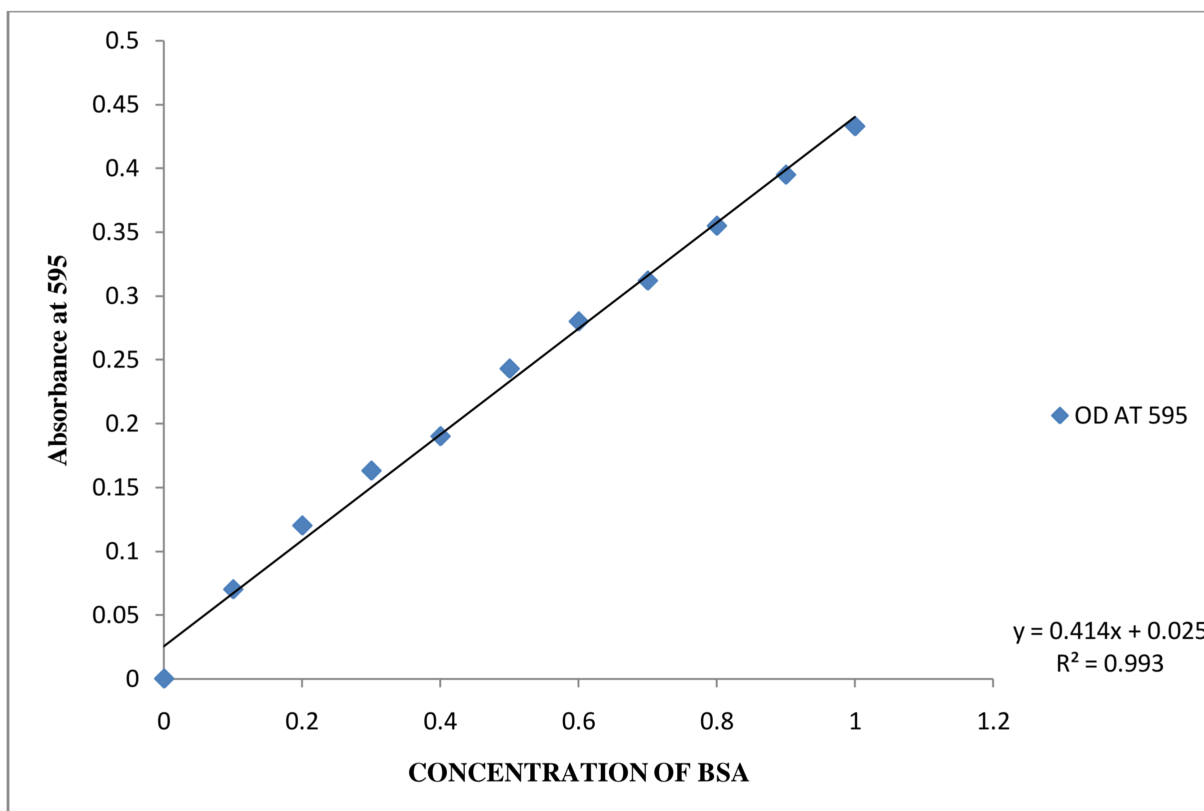

Fig 6.1. Standard Curve of BSA for total protein estimation.

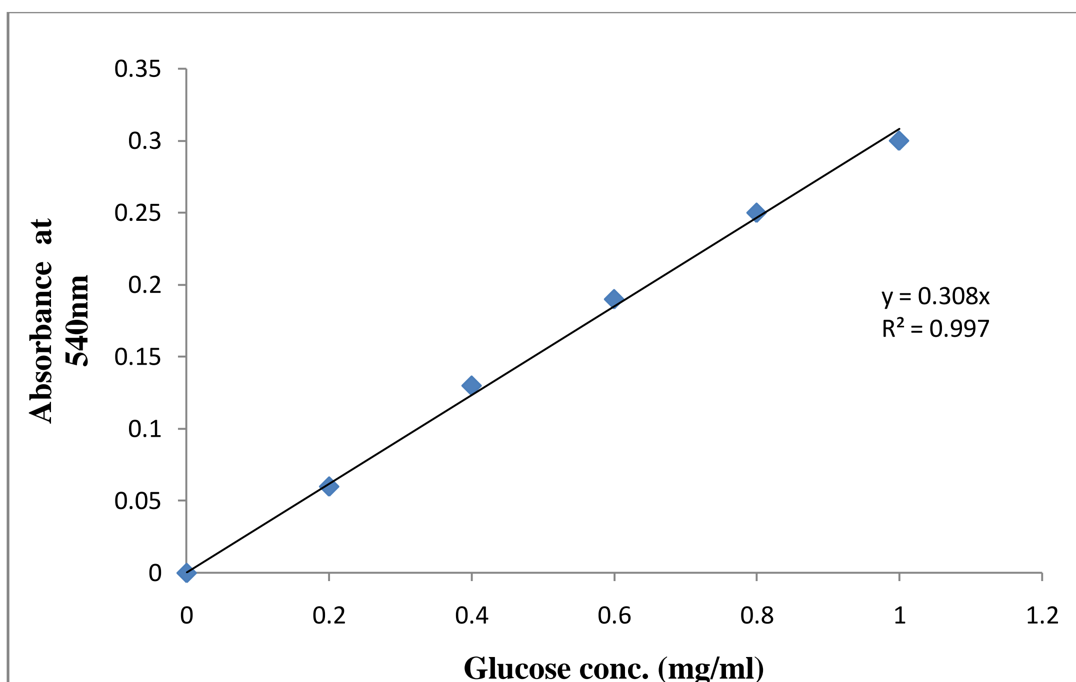

Fig 6.2. Glucose standard curve for enzyme activity estimation.

<u>Experiment 4:</u> To study the biodegradation of ciprofloxacin and its degradation products.

Place of work: Department of Biotechnology, LPU.

Methodology:

Titrimetric Assay

For titrimetry, a 10 ml aliquot of standard drug solution containing 100-500 ppm of ciprofloxacin is placed in a 100 ml titration flask and solution was acidified by adding 5 ml of 5M sulphuric acid. Then 10 ml of 0.025 M cerium sulphate was added. The content of the flask were mixed well and was kept aside for 15 min. Finally, the unreacted oxidant is back titrated with 0.025 M FAS solution using one drop of ferroin indicator. Simultaneously a blank titration is performed and the amount of drug in the aliquot is calculated from the amount of cerium reacted.

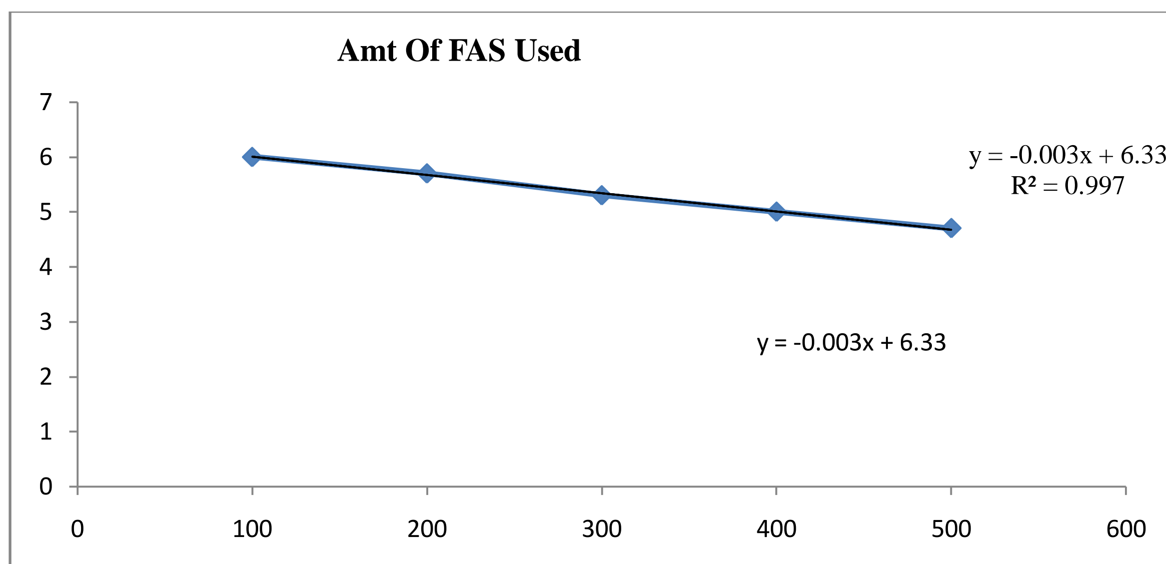

Fig 6.3. Standard curve prepared by using standards concentrations of ciprofloxacin to determine degraded concentration of ciprofloxacin.

Spectrophotometrical analysis :

A. By Methyl orange

In spectrophotometrical analysis to study the degradation of ciprofloxacin by *Pleurotus ostreatus,* the filtered extract was used against the standard. For spectrophotometric analysis 10 µg of the enzyme extract was added to 4 ml of distilled water. 1 ml of 5M sulphuric acid and 250 $\mu g ml^{-1}$ of cerium sulphate solution was added to above mixture and was kept at room

temperature for 10 min with occasional swirling. 1 ml of methyl orange was added in the last and absorbance was measured at 520 nm against water blank after 5 min.

B. **By Indigo Carmine**

In this method of estimation of degradation of ciprofloxacin, 20µgml^{-1} of the enzyme extract was added to 4ml of distilled water. 1 ml of 5M sulphuric acid was added to each tube followed by addition of 500 µgml^{-1} of cerium sulphate solution. The contents were mixed well and was kept aside for 10 min's. Then 1 ml of indigo carmine solution was added to each tube and absorbance was measured at 610 nm after 5 min.

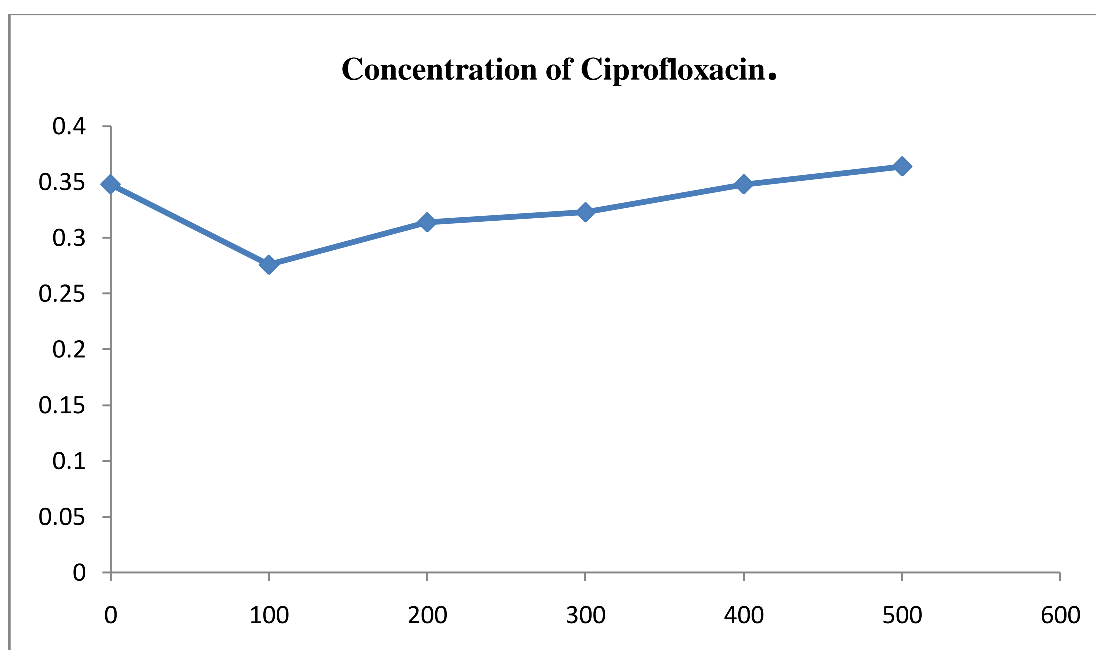

Fig 6.4. Standard curve for Methyl orange method for determination of ciprofloxacin left after incubation period

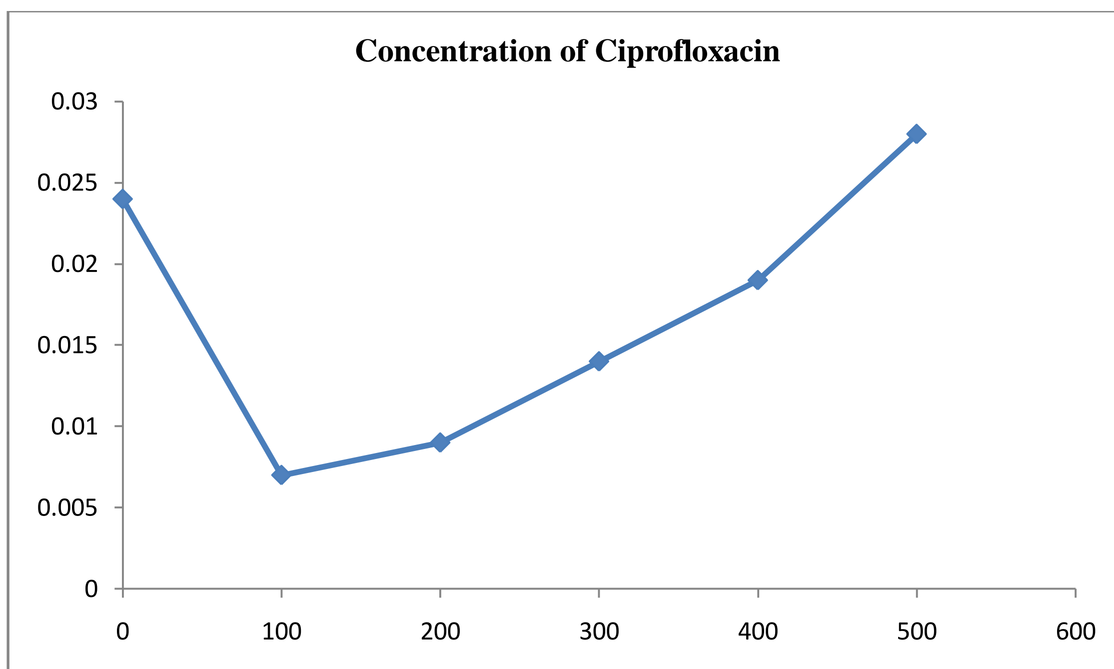

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Fig 6.5. Standard curve for indigo carmine method determination of ciprofloxacin left after incubation period.

FTIR

FTIR was performed on FTIR SHIMADZU- 8400s manufactured by Shimadzu, Japan. For FTIR analysis, KBr cells/pellets were used as a base on which a thin layer of samples were dispersed This solid base was kept on wedge for FTIR under Infra red rays. Peaks were observed on display screen for each sample.

HPLC

High Performance Liquid Chromatography/ High Pressure Liquid Chromatography is an analytical technique, which is used to separate the components present in a mixture, to identify and quantify each of the component present. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material eg- silica, PEG, etc. Each component of the sample interacts differently with adsorbent material causing differences in flow rates for the different compounds present in mixture which leads to separation of the mixed components as they flow out of the column. Acetonitrile was used as mobile phase. A standard run of ciprofloxacin was performed to asses the comparative assessment of samples taken i.e control, 100 ppm and 500 ppm of concentration of ciprofloxacin.

Thin Layer Chromatography

Thin layer Chromatography (TLC),is a chromatographic techniques which is used to separate non volatile mixtures. TLC is performed on a sheet of glass or plastic which is coated with thin layer on adsorbent material, usually silica gel, aluminium oxide, or cellulose. This thin layer of adsorbent is known as stationary phase. After the sample has been applied on the plate, solvent or solvent mixture (mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

Silica 60 was used to prepare the stationary phase on glass sheet, a thickness of about 250 µm was maintained. The stationary phase was air dried for 30 min's. after air drying the plate was activated by keeping it for 60 min at 120 ^{o}C. For mobile phase a mixture of chloroform, methanol and ammonia (70:30:10) was used. Samples were applied on activated plates and plate was kept in mobile phase for 45 min. After run duration the plate was viewed under UV transilluminator.

Antimicrobial Activity of Degraded Products.

To check the activity of degraded products of different concentration of ciprofloxacin that was used earlier, antimicrobial experiment was performed. Activity of degraded products were checked against three bacterial strain namely, *Escherichia coli, Staphylococcus aureus* and *Streptococcus pyogenes.* Spread plate method of plating was adopted to check the antimicrobial activity. Medium used for this was Mueller Hinton Agar and temperature was maintained at 37 ± 2 ^{o}C

Chapter 7

Results and Discussion

7.1 Procurement and maintenance of cultures

Pure culture of *Pleurotus ostreatus* was obtained from Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab. Cultures were maintained by periodic sub-culturing in culture tubes and Petri plates. Plate 1 shows sub culturing of *Pleurotus ostreatus* in slants and plate 2 shows growth fungal mycelium on petri plates which was later used for obtaining equal sized bits for enzyme assays and degradation studies. The culture obtained after subculturing showed white, cottony and fluffy fungal mycelium. Plates devoid of any contamination were used for further studies.

Plate 7.1. Petriplates containing mycelium of *Pleurotus ostreatus*.

7.2 Biomass studies in presence of different concentrations of ciprofloxacin

7.2.1 Study of radial growth of *Pleurotus ostreatus* under the effect of different concentration of ciprofloxacin.

Table 1 gives an overview of the radial growth of *Pleurotus ostreatus* on petri plates at different concentration of ciprofloxacin.

The effect of different concentrations of ciprofloxacin on radial growth of *Pleurotus ostreatus* was found that it showed an increase in the radial growth of *Pleurotus ostreatus* per day with increase in concentration as after day 1 it was observed that plate of concentration of 100

ppm, the radial growth was of 8 mm when compared to 11.5 mm for 500 ppm. Similar trends were observed on daily basis as on day 6 final growth readings for 500 ppm plate was 75.5 mm against 74.0 mm for 100 ppm plate whereas radial growth of control was of about 68 mm.

Table 1. Effect of different concentrations of ciprofloxacin on radial growth of *Pleurotus ostreatus*.

Days / Conc.	Radial Growth of *Pleurotus ostreatus* (mm)					
	1	**2**	**3**	**4**	**5**	**6**
0 (ppm)	7.5±0.228	9.0±0.000	19.0±0.577	34.0±0.000	46.0±1.154	68.0±1.154
100 ppm	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 ppm	8.5±0.866	11.5±0.866	22.5±1.443	36.0±1.732	50.0±1.154	74.0±0.577
300 ppm	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 ppm	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 ppm	11.5±0.288	14.0±0.000	26.5±1.154	40.5±4.33	55.5±0.288	75.5±0.288

All experiments were performed in replicates.

Medium used was PDA

Temperature - 25±2 ^{o}C

7.2.2 Effect on biomass production of *Pleurotus osteeatus* under different concentrations of ciprofloxacin

For biomass production and enzyme assay, equal sized bits were inoculated in flasks containing PDB and different concentration of ciprofloxacin. Fig 3a and 3b give an account of the growth of

Plate 7.2a. Results: Flasks showing growth of P*leurotus ostreatus* mycelium in broth supplemented with different concentration of ciprofloxacin (100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm) after 7 days of incubation.

Plate 7.2b. Results: Flasks showing growth of P*leurotus ostreatus* mycelium in broth supplemented with different concentration of ciprofloxacin (100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm) after 14 days of incubation

Pleurotus ostreatus mycelium in flasks inoculated at 25±2 ^{O}C for 7 and 14 days, respectively. After 7 days of incubation it was found that maximum growth was observed in flask no. 5 having conc. of 500 ppm of ciprofloxacin with wet biomass of 3.703 gm while lowest biomass production was found to be in flask having conc. of 100 ppm having 2.448 gm of biomass produced. After, 14 days the results were similar i.e. maximum growth was observed

in flask having conc. of 500 ppm having biomass weight of about 3.965 gm whereas lowest in flask having conc. 1ppm weighing 3.127 gm of biomass produced.

Table 2 gives an overview of the fungal biomass produced after 7 and 14 days of incubation in medium supplemented with different concentrations of ciprofloxacin.

Table 2. Wet and dry weight obtained from *P. ostreatus* grown in broth culture in presence of ciprofloxacin after 7 and 14 days of incubation.

Concentration (ppm)	Wet weight (gm)		Dry weight (gm)	
	7 Days	14 Days	7 Days	14 Days
0	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

Medium used was PDB

Temperature conditions- 25±2 ^{O}C

7.3 Enzymatic Assays of *Pleurotus ostreatus* grown in presence of ciprofloxacin after 7 and 14 days of incubation.

Pleurotus ostreatus produces different extracellular enzymes in order to utilize the substrate. The effect of various concentrations of ciprofloxacin on enzyme production was determined with respect to endo-β-1,4-glucanase, hemicellulase, laccase, lignin peroxidase and manganese peroxidase.

7.3.1 Endo-β- 1,4- Glucanase enzyme activity of *Pleurotus ostreatus*

Table 3 outlines the Endo-β- 1,4- Glucanase enzymatic assay carried out after 7 and 14 days of incubation. It was observed that maximum specific activity after 7 days of incubation was 112.87 μmol $min^{-1}mg^{-1}$ whereas after 14 days it was 392.94 μmol $min^{-1}mg^{-1}$ and minimum

enzymatic activity was seen in control of about 35.53 µmol $min^{-1}mg^{-1}$ after 7 days and after 14 days 58.00 µmol $min^{-1}mg^{-1}$ in control

Table 3. Endo-β- 1,4- Glucanase enzyme activity of *Pleurotus ostreatus* grown in broth culture in presence of ciprofloxacin after 7 and 14 days of incubation.

Concentration	Enzyme Activity(units/ml)					
	7 Days			14 Days		
	EA	TPC	SA	EA	TPC	SA
0 (Control)	70.71	1.99	35.53	101.50	1.75	58.00
100 ppm	74.26	1.59	51.93	104.72	1.05	99.73
200 ppm	85.57	1.59	53.81	129.42	0.785	164.86
300 ppm	130.00	1.43	94.20	129.87	0.7	185.52
400 ppm	130.14	1.38	94.30	133.63	0.495	269.95
500 ppm	137.71	1.22	112.87	140.28	0.357	392.94

EA- Enzyme Activity, Unit- Units/ml

TPC- Total Protein Concentration, Unit- µg/ml

SA- Specific Activity, Unit- µmol $min^{-1}mg^{-1}$

7.3.2 Laccase enzyme activity of *Pleurotus ostreatus*

From graphs it was observed, maximum laccase activity was found in flask containing highest amount of ciprofloxacin i.e 500 ppm for incubation period of 7 and 14 days. It was found that with increase in incubation time duration the activity of laccase also increased.

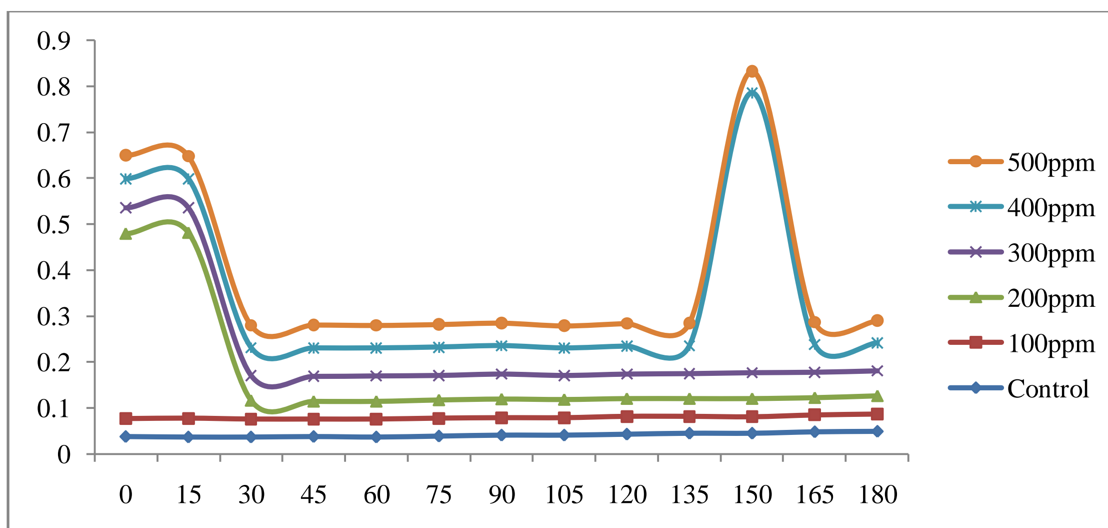


Fig 7.1a: Laccase enzyme activity of *Pleurotus ostreatus* grown in broth culture in presence of ciprofloxacin after 7 days of incubation.

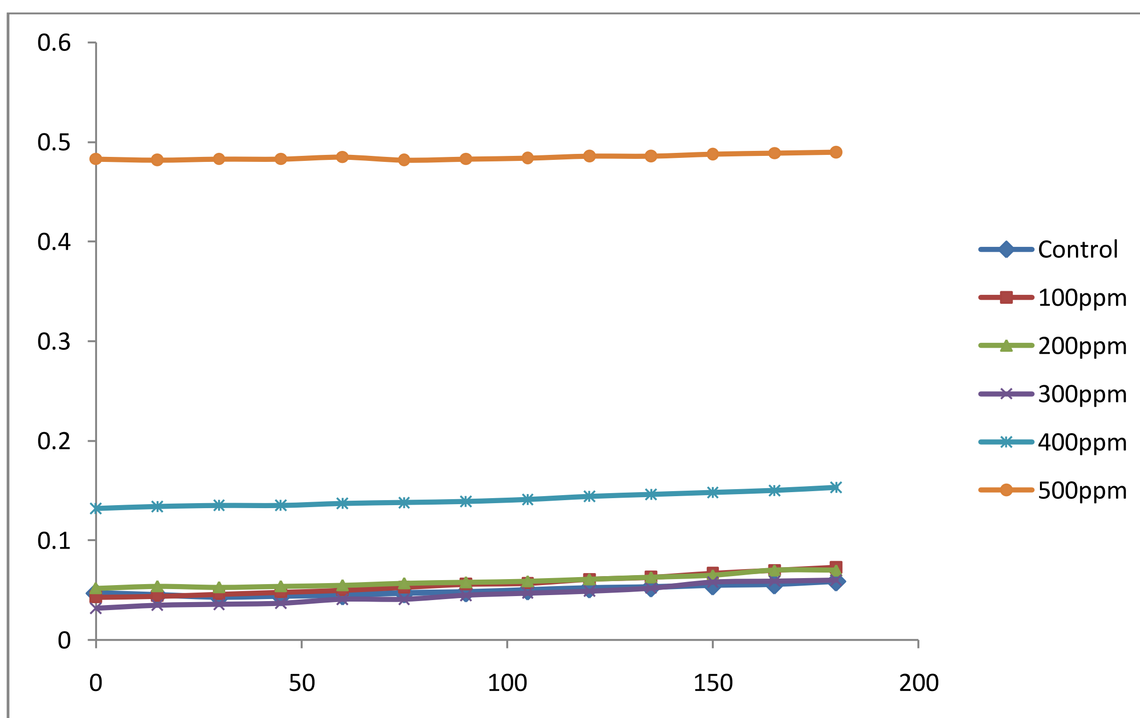


Fig 7.2b: Laccase enzyme activity of *Pleurotus ostreatus* grown in broth culture in presence of ciprofloxacin after 14 days of incubation.

7.3.3 Hemicellulase enzyme activity of *Pleurotus ostreatus*.

Table 4 outlines the hemicellulase enzymatic assay carried out after 7 and 14 days of incubation. It was observed that maximum enzyme activity after 7 days of incubation was

93.69 µmol $min^{-1}mg^{-1}$ whereas after 14 days it was 331.40 µmol $min^{-1}mg^{-1}$ and minimum enzymatic activity was seen in control of about 43.40 µmol $min^{-1}mg^{-1}$ after 7 days and after 14 days 62.84 µmol $min^{-1}mg^{-1}$ in control.

Table 4. Hemicellulase enzyme activity of *Pleurotus ostreatus* grown in broth culture in presence of ciprofloxacin after 7 and 14 days of incubation.

Concentration (ppm)	Enzyme Activity(units/ml)					
	7 Days			14 days		
	EA	TPC	SA	EA	TPC	SA
0	86.37	1.99	43.40	109.98	1.75	62.84
100	92.99	1.59	65.02	111.29	1.05	105.99
200	93.31	1.59	58.68	113.12	0.785	144.10
300	114.14	1.43	71.78	115.38	0.7	164.82
400	114.18	1.38	82.73	116.81	0.495	235.97
500	114.31	1.22	93.69	118.31	0.357	331.40

*EA- Enzyme Activity, Units- units/ml

*TPC- Total Protein Concentration, µg/ml

*SA- Specific Activity, Units- µmol $min^{-1}mg^{-1}$

7.3.4 Lignin peroxidase enzyme activity of *Pleurotus ostreatus.*

Table 5 outlines the lignin peroxidase enzymatic assay carried out after 7 and 14 days of incubation. It was observed that maximum enzyme activity after 7 days of incubation was 0.0256 µmol $min^{-1}mg^{-1}$ and after 14 days it was 0.0244 µmol $min^{-1}mg^{-1}$ for 500 ppm concentration of ciprofloxacin whereas and minimum enzymatic activity was seen in control of about 0.0117 µmol $min^{-1}mg^{-1}$ after 7 days and after 14 days 0.0129 µmol $min^{-1}mg^{-1}$ in control. Here, a dip in enzyme activity was observed after 7 and 14 days of incubation.

Table 5. Lignin peroxidase enzyme activity of *P. ostreatus* grown in broth culture in presence of ciprofloxacin after 7 and 14 days of incubation.

Concentration (ppm)	Enzyme Activity(units/ml)					
	7 Days			14 days		
	EA	TPC	SA	EA	TPC	SA
0	23.28	1.99	0.0117	8.72	1.75	0.0129
100	21.30	1.59	0.0134	10.29	1.05	0.0159
200	29.25	1.59	0.0184	12.64	0.785	0.0161
300	30.32	1.43	0.0212	12.88	0.7	0.0184
400	30.64	1.38	0.0222	16.69	0.495	0.0208
500	31.23	1.22	0.0256	22.57	0.357	0.0244

*EA- Enzyme activity, units- Unit/ml

*TPC- Total Protein Concentration, Units- µg/ml

*SA- Specific activity, Units- µmol $min^{-1}mg^{-1}$

7.3.5 Manganese peroxidase enzyme activity of *Pleurotus ostreatus.*

Table 6 outlines the manganese peroxidase enzymatic assay carried out after 7 and 14 days of incubation. It was observed that maximum enzyme activity after 7 days of incubation was 7.441 µmol $min^{-1}mg^{-1}$ and after 14 days it was 23.864 µmol $min^{-1}mg^{-1}$ for 500 ppm concentration of ciprofloxacin whereas minimum enzymatic activity was seen of about 4.925 µmol $min^{-1}mg^{-1}$ after 7 days and after 14 days 13.779 µmol $min^{-1}mg^{-1}$ in control. Here, a dip in enzyme activity was observed after 7 and 14 days of incubation.

Table 6. Manganese peroxidase enzyme activity of *P. ostreatus* grown in broth culture in presence of ciprofloxacin after 7 and 14 days of incubation.

Concentration (ppm)	Enzyme Activity(units/ml)					
	7 Days			14 days		
	EA	TPC	SA	EA	TPC	SA
0	74.79	1.99	4.295	85.19	1.75	13.779
100	76.39	1.59	4.704	107.84	1.05	13.863
200	77.59	1.59	4.805	111.36	0.785	15.454

300	85.47	1.43	5.426	121.31	0.7	15.909
400	87.23	1.38	6.231	145.56	0.495	21.786
500	90.78	1.22	7.441	241.13	0.357	23.864

*EA- Enzyme Activity, Units- units/ml

*TPC- Total Protein Concentration, Units- µg/ml

*SA- Specific Activity, Units- µmol $min^{-1} mg^{-1}$

7.4 Titrimetric determination of degraded products of ciprofloxacin obtained after the incubation period of 7 and 14 days..

Titrimetry was performed to determine the degraded concentration of ciprofloxacin after the incubated time period of 7 and 14 days. Titration was performed against FAS solution (0.025 M). A color change from green to orange was observed upon addition of FAS to reaction mixture after addition of 1 drop of ferroin indicator.

Table 3. Titrimetric results for the determination of post incubated degraded products of ciprofloxacin after 7 and 14 days.

Concentration (Ppm)	Amount of Ciprofloxacin left (ppm)	
	7 days	**14 days**
Control	ND	ND
100	88	48
200	118	108
300	128	116
400	158	148
500	173	156

ND- Not Determined.

7.6 Thin Layer Chromatography to determine the post incubated results of different concentration of ciprofloxacin.

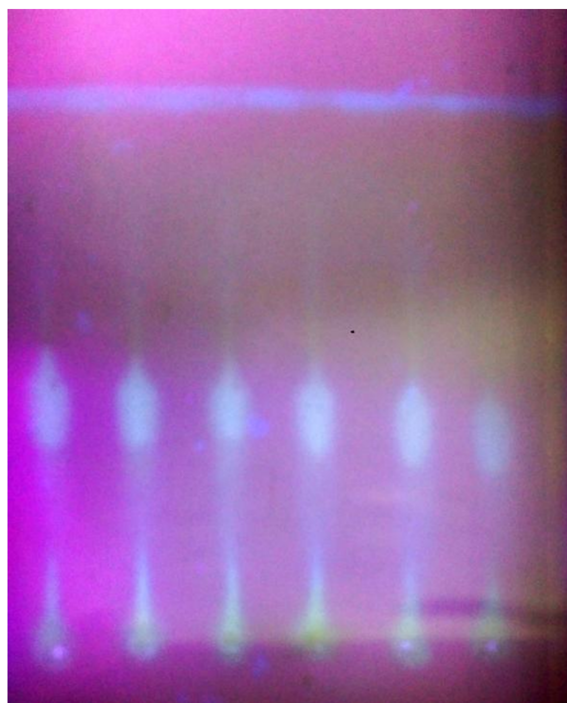


Plate 7.3 . TLC plate for ciprofloxacin for 14 days

No Concrete results could be obtained from TLC plates.

Table for TLC , to determine the retention factor for different concentrations of ciprofloxacin.

Concentrations	Retention Factor	
	7 Days	**14 Days**
Standard	0.615	0.556
Control	0.923	0.391
100	0.750	0.565
200	0.769	0.521
300	0.807	0.513
400	0.846	0.504
500	0.884	0.478

7.7 Per cent degradation of Ciprofloxacin by Pleurotus ostreatus after 7 and 14 days of incubation.

7.7.1 Spectrophotometeric analysis by Methyl Orange method

Concentration	Amount of ciprofloxacin left (ppm)	
	7 Days	14 days
100 ppm	18.2	1.2
200 ppm	27.9	2.0
300 ppm	43.9	2.2
400 ppm	44.1	2.8
500 ppm	55.4	3.3

By spectrophotometric determination of ciprofloxacin using Methyl Orange method it was found after 7 and 14 days of incubation that the amount of ciprofloxacin was degraded by *Pleurotus ostreatus*. After 7 days of incubation it was found that per cent of ciprofloxacin was left against 100 ppm which was initially taken similarly after 14 days of incubation it was further degraded down to 1.2 ppm. Similarly, at concentration of 500 ppm the degraded ciprofloxacin detected was about 55.4 ppm after 7 days of incubation later on which further reduced down to 3.3 ppm after 14 days.

7.7.2 Indigo carmine Indicator

Concentration	Undegraded Ciprofloxacin left	
	7 Days	14 days
100 ppm	6.4	2.5
200 ppm	9.2	3.5
300 ppm	11.6	6.0
400 ppm	16.5	8.0
500 ppm	17.5	10.5

By spectrophotometric determination of ciprofloxacin using Indigo carmine method it was found after 7 and 14 days of incubation that the amount of ciprofloxacin was degraded down by *Pleurotus ostreatus*. After 7 days of incubation it was found 6.4 ppm of ciprofloxacin was left against 100 ppm which was initially taken similarly after 14 days of incubation it was further degraded down to 2.5 ppm. Similarly, at concentration of 500 ppm the degraded

ciprofloxacin detected was about 17.5 ppm after 7 days of incubation later on which further reduced down to 10.5 ppm after 14 days.

7.8 Antimicrobial Activity of degraded products against standard ciprofloxacin concentrations

7.8.1 Antimicrobial Activity of degraded products ciprofloxacin against *Escherichia coli*

Antimicrobial activity was carried out on *Escherichia coli*, to check the activity of degraded ciprofloxacin. It was observed that the control devoid of ciprofloxacin, showed no inhibitory action on *E.coli,* whereas for other plates it was observed that the inhibitory action of ciprofloxacin degradation products was less then standards used. For example, standard of 100 ppm showed a larger zone of inhibition, 2.5 cm where as degraded concentration of same showed an inhibition zone of about 1.5 cm only. Similarly, for 500 ppm, standard showed 4.0 cm but degraded product for same concentration gave a zone of inhibition of about 2.95 cm, which is lower than standard used.

7.8.2 Antimicrobial Activity of degraded products ciprofloxacin against *Staphylococcus aureus*

Antimicrobial activity was carried out on *Staphylococcus aureus*, to determine the activity of degraded ciprofloxacin. It was observed that the control i.e devoid of ciprofloxacin, showed no inhibitory action on *S. aureus,* whereas for other plates it was observed that the inhibitory action of degraded ciprofloxacin was less then standards used. The standard of 100 ppm showed a larger zone of inhibition, 2.0 cm where as degraded concentration of same showed an inhibition zone of about 1.7 cm only. Similarly, for 500 ppm, standard showed 3.6 cm but degraded product for same concentration gave a zone of inhibition of about 2.65 cm, which is lower than standard used.

7.8.3 Antimicrobial Activity of degraded products ciprofloxacin against *Streptococcus pygenes*

Antimicrobial activity was carried out on *Streptococcus pygenes*, to check the activity of degraded ciprofloxacin. It was observed that the control i.e devoid of ciprofloxacin, showed no inhibitory action on *S. pyogenes,* whereas for other plates it was observed that the

inhibitory action of degraded ciprofloxacin was less then standards used. For example, standard of 100 ppm showed a larger zone of inhibition, 2.8 cm where as degraded concentration of same showed an inhibition zone of about 2.35 cm only. Similarly, for 500 ppm, standard showed 3.4 cm but degraded product for same concentration gave a zone of inhibition of about 2.60 cm, which is lower than standard used.

Thus, from the using above data we can compare the antimicrobial activity of our degraded ciprofloxacin using 3 strains of microbes and it was found that the with time the activity of ciprofloxacin was reduced as it showed a smaller zone of inhibition for all the 3 strains of bacteria against the standards of individual concentrations.

Table. Table showing the zone of inhibition obtained on plates of *Escherichia coli* by post degraded products against standards.

Concentration	Zone Of inhibition (cm)	
	Post treatment broth	Ciprofloxacin
Distilled water	ND	ND.
0 (Control)	1.5 ± 0.057	3.0
100 ppm	1.5 ± 0.005	2.5
200 ppm	1.5 ± 0.057	3.0
300 ppm	2.3 ±0.057	3.4
400 ppm	2.6 ± 0.000	3.6
500 ppm	2.95 ± 0.016	4.0

ND- Not defined

Medium Used- Mueller Hinton Agar

Temperature conditions- 37±2 ^{o}C

Table. Table showing the zone of inhibition obtained on plates of *Staphylococcus aureus* by post degraded products against standards.

Concentration	Zone of inhibition(cm)	
	Post Treated Broth	Ciprofloxacin
Distilled water	ND	ND
0 (Control)	1.6 ± 0.115	3.8
100 ppm	1.7 ± 0.057	2.0
200 ppm	2.5 ± 0.057	2.8
300 ppm	2.5 ± 0.00	3.0
400 ppm	2.65 ± 0.028	3.4
500 ppm	2.65 ± 0.028	3.6

ND- Not defined

Medium Used- Mueller Hinton Agar

Temperature conditions- 37±2 ^{o}C

Table. Table showing the zone of inhibition obtained on plates of *Streptococcus pyogenes* obtained by post degraded products against standards.

Concentration	Zone of Inhibition (cm)	
	Post treatment broth	Ciprofloxacin
Distilled water	Not determined	Not determined
0 (Control)	2.0 ± 0.057	3.6
100 ppm	2.35 ± 0.086	2.8
200 ppm	2.5 ± 0.057	3.0
300 ppm	2.55 ± 0.028	3.2
400 ppm	2.1 ± 0.057	3.2
500 ppm	2.6 ± 0.00	3.4

ND- Not defined

Medium Used- Mueller Hinton Agar

Temperature conditions- 37±2 ^{o}C

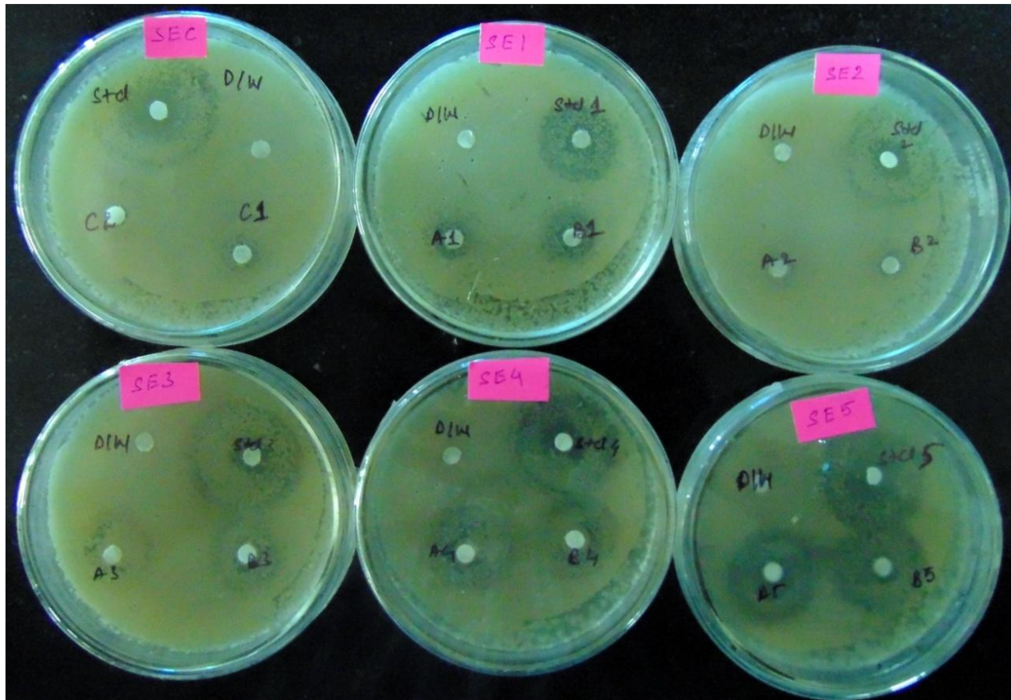


Plate. Plate showing zone of inhibition against *E coli*, using different concentration of degraded ciprofloxacin against standards.

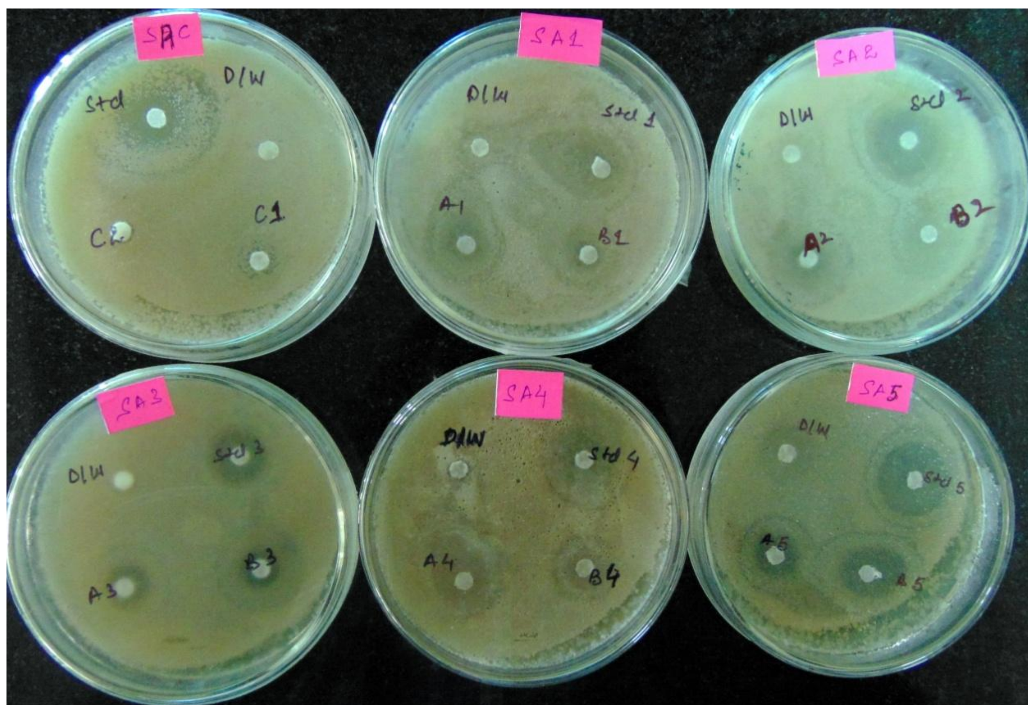


Plate. Plate showing zone of inhibition of different concentration of degraded ciprofloxacin against standards.

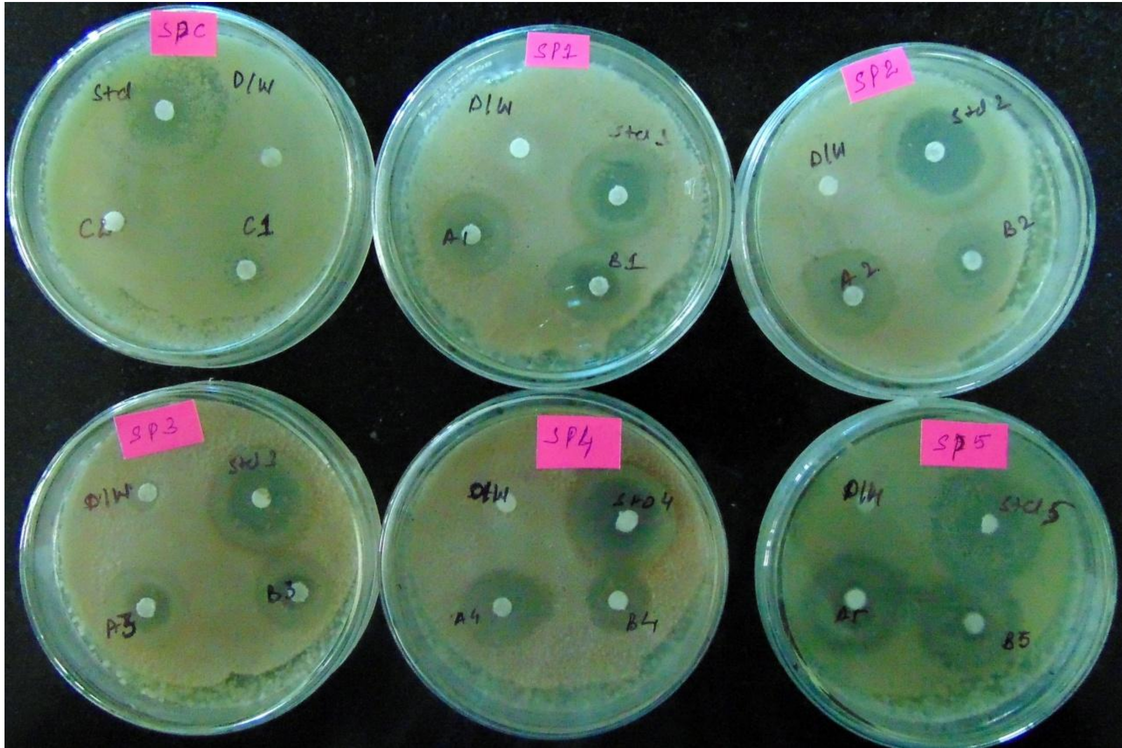


Plate. Plate showing zone of inhibition of *Streptococcus pygenes* using different concentration of degraded ciprofloxacin against standards.

7.9 FTIR Graphs

7.9.1 Standard.

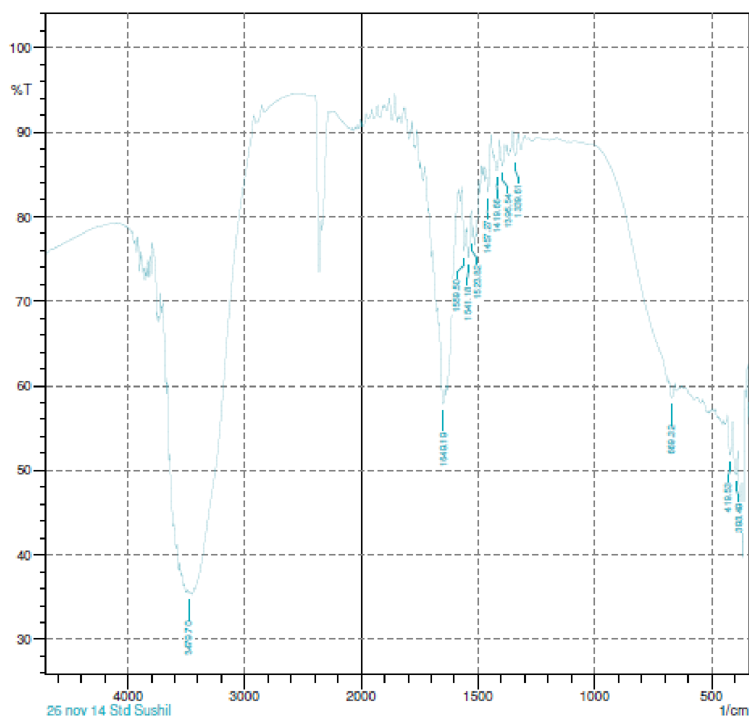
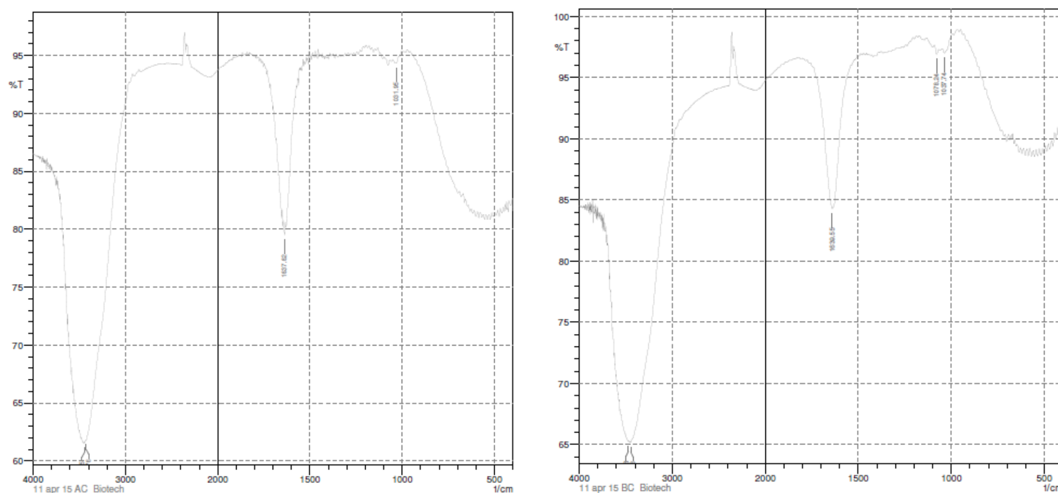


Fig. IR Graphs showing peaks of standard ciprofloxacin used.

Peaks observed in FTIR of Standard i.e ciprofloxacin gave the information for the presence of bonds like Alkyl halides (corresponding peak is 669.32), Aromatic amines (corresponding peak is 1339), Alkanes (corresponding peak is 1395.54), Aromatics (corresponding peak is 1457), Nitro Compounds (corresponding peak is 1541), aromatics (corresponding peak is 1559), alcohols and phenols (corresponding peak is 3489).

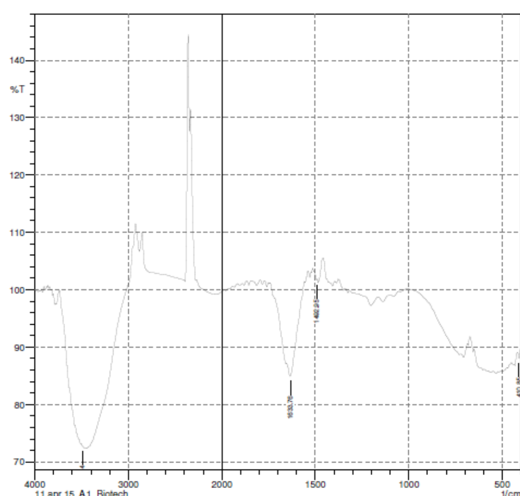
7.9.2 Controls.

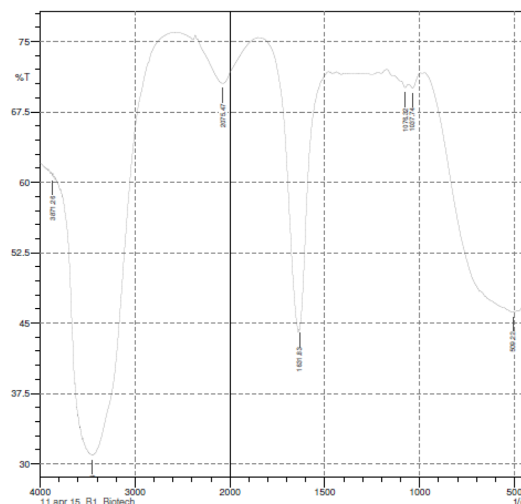


AC BC

Bonds present in both the controls AC (Control after incubation of 7 Days) and BC (Control after 14 days of incubation) were found to be aliphatic amines (corresponding peak is 1031, 1037, 1071), 1 ^{o} amine (corresponding peak is 1637, 1639), alkenes (corresponding peak is 1637, 1639) and alcohols and phenols (corresponding peak is 3423, 3443, 3444, 3471).

7.9.3 100 ppm

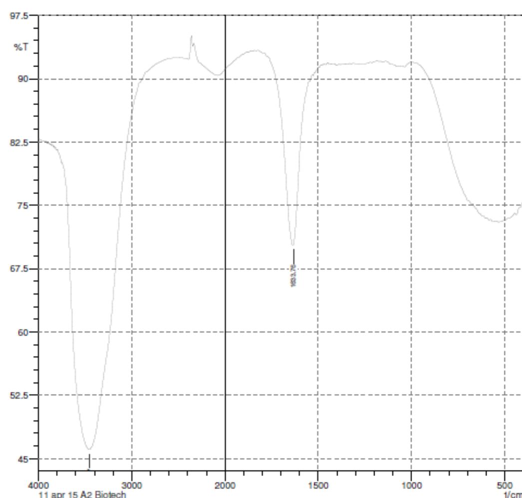


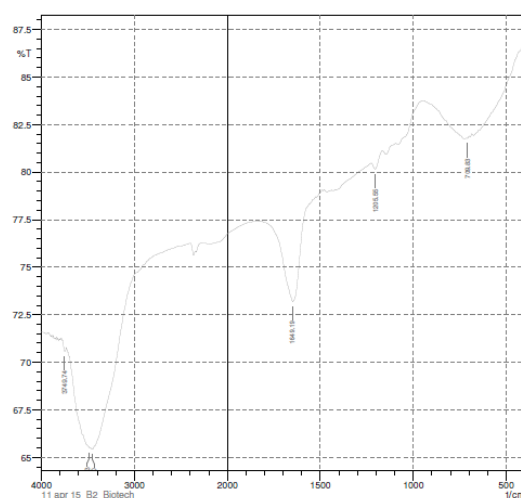


A1 B1

Bonds present Aliphatic amines (corresponding peak is 1037, 1076), 1° amines (corresponding peak is 1631, 1633), aromatics (corresponding peak is 1492), alcohols, phenols (corresponding peak is 3448, 3489).

7.9.4 200 ppm

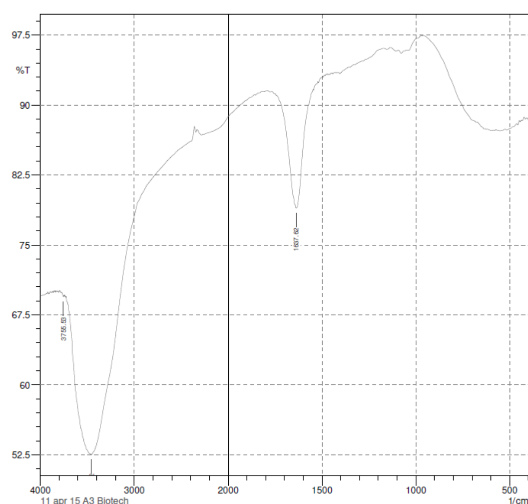




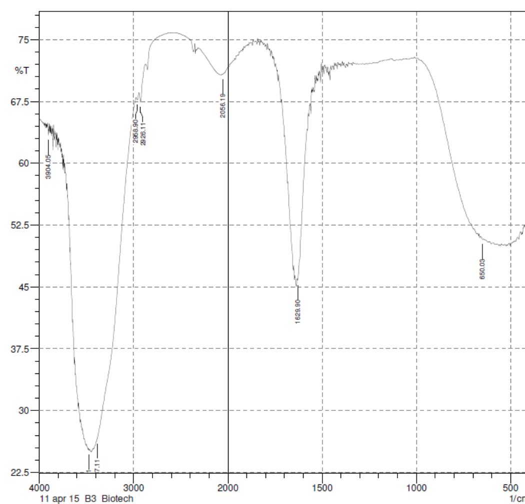
A2 B2

Bonds present aliphatic amines (corresponding peak is 1205), 1° amines (corresponding peak is 1633, 1649), alkenes (corresponding peak is 1649) alcohols and phenols (corresponding peak is 3450, 3452, 3481).

7.9.5 300 ppm



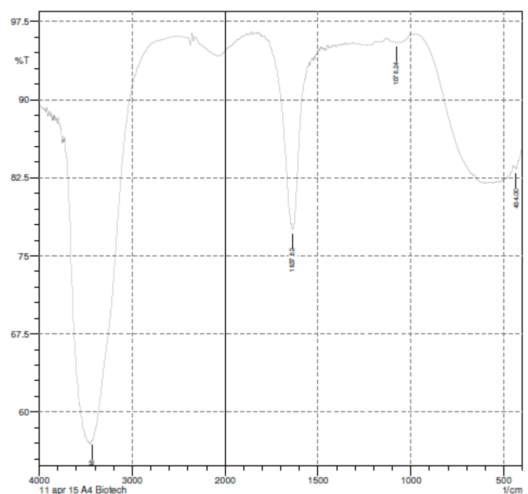
A3



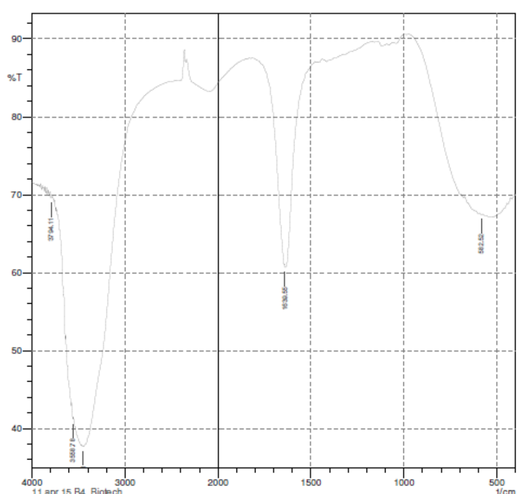
B3

Bonds 1” amines (corresponding peak is 1629, 1637), alkyl halides (corresponding peak is 650), alkanes (corresponding peak is 2926, 2958, 1° 2° amines, amides (corresponding peak is 3387), alcohols and phenols (corresponding peak is 3454, 3473).

7.9.6 400 ppm



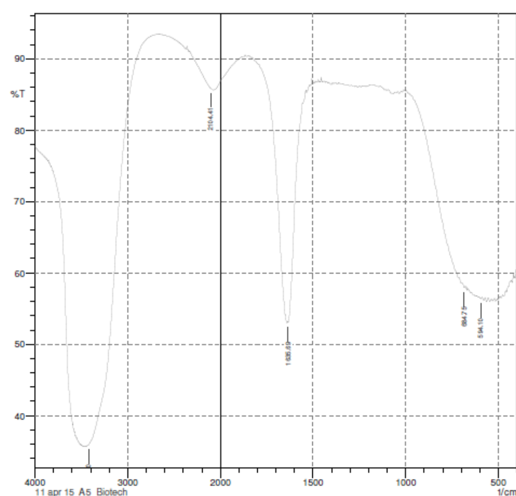
A4



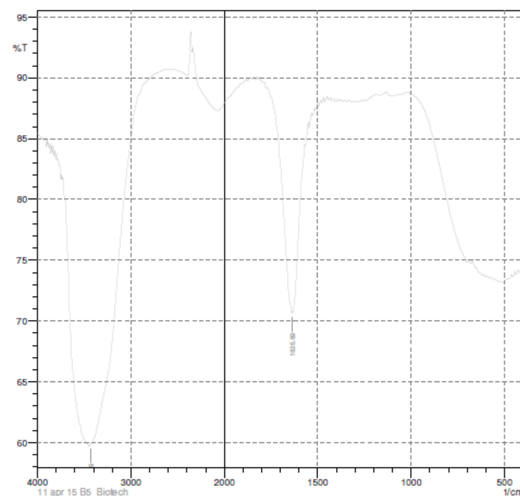
B4

Aliphatic amines (corresponding peak is 1078), 1” amines (corresponding peak is 1637, 1639), alkyl halides (corresponding peak is 582), alcohols and phenols (corresponding peak is 3431,3446,3558) are present in both.

7.9.7 500 ppm



A5

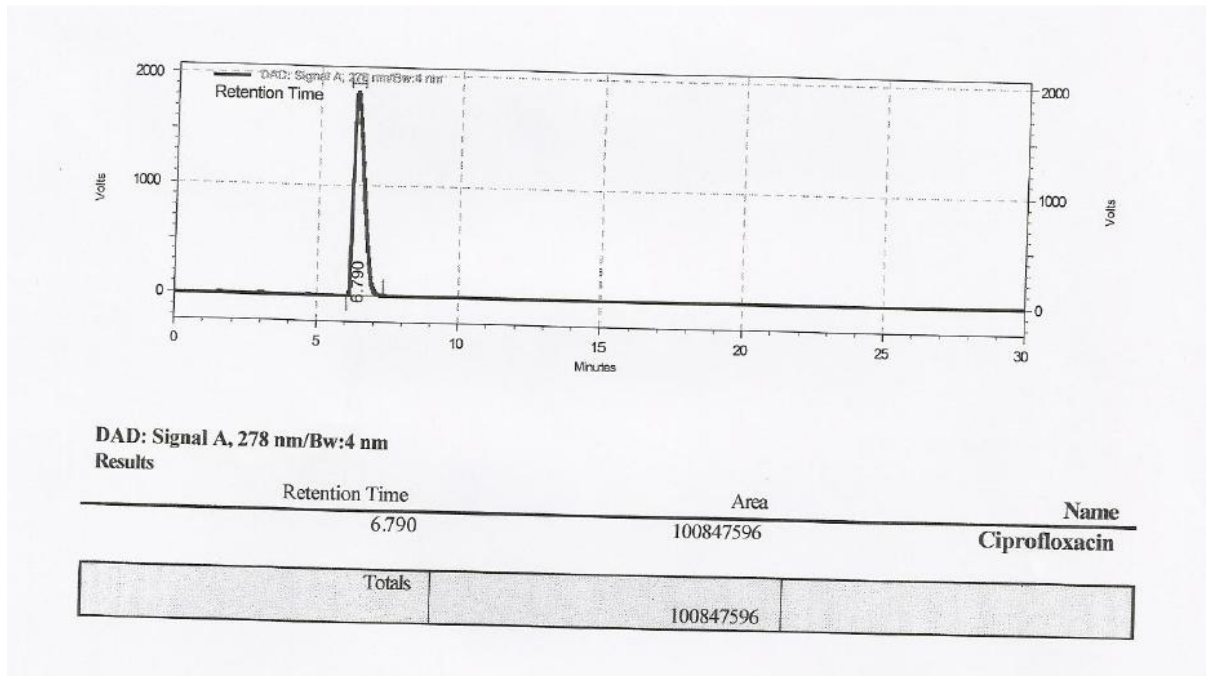


B5

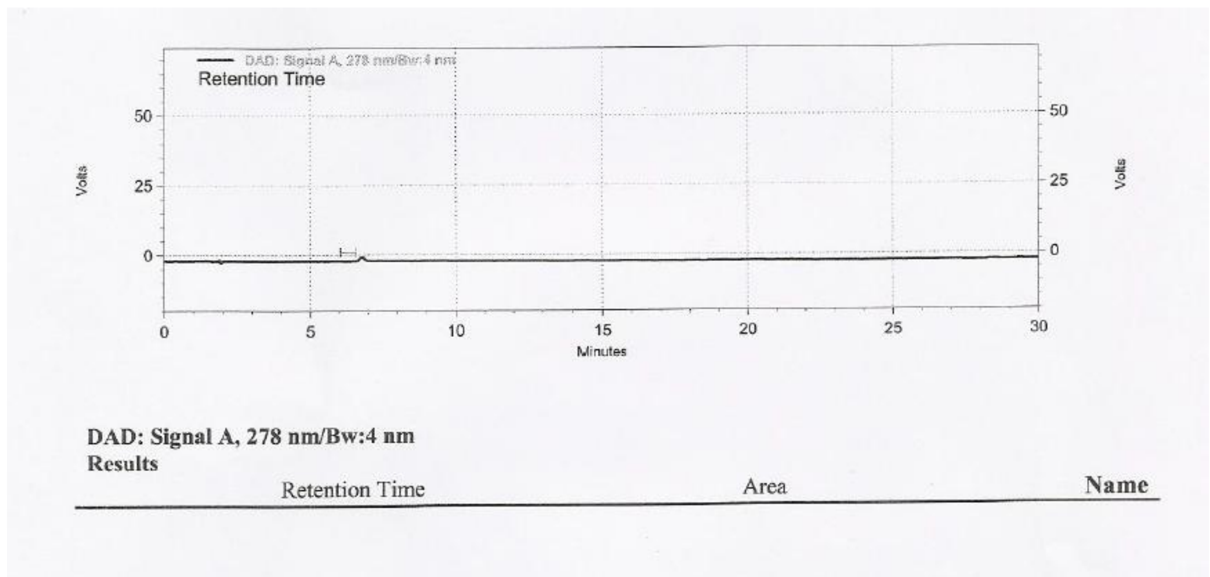
Bonds present are 1” amines (corresponding peak is 1635 , alkyl halides (corresponding peak is 591,684), alkynes (corresponding peak is 2104) alcohols and phenols (corresponding peak is 3419, 3425).

7.10 HPLC

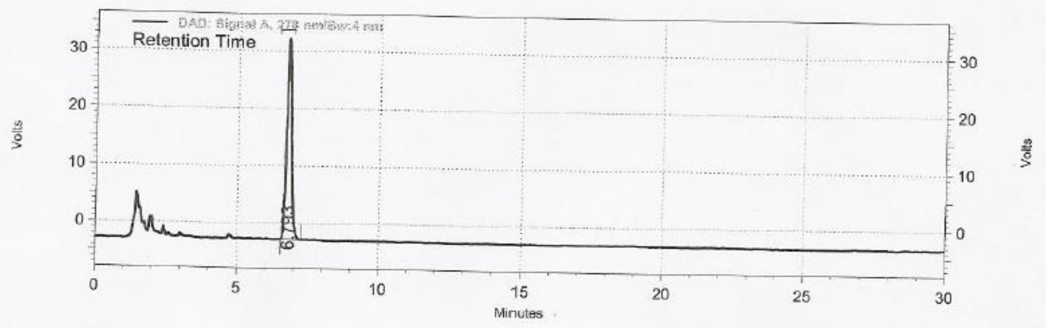
7.10.1 Standard



7.10.2 Control



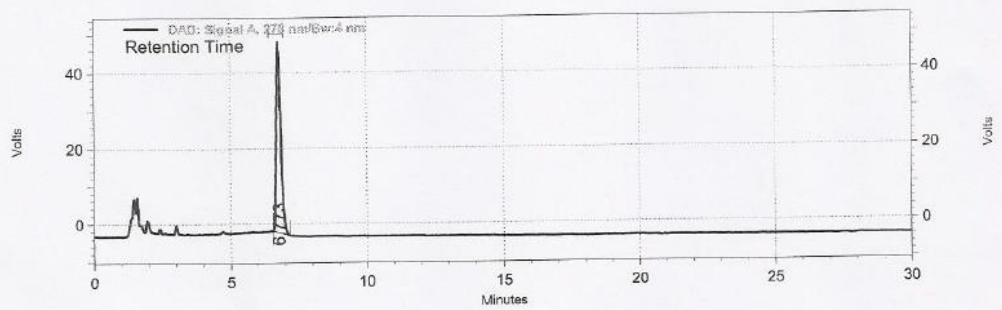
7.10.3 100 ppm



DAD: Signal A, 278 nm/Bw:4 nm
Results

Retention Time	Area	Name
6.793	888230	**Ciprofloxacin**
Totals	888230	

7.10.4 500 ppm



DAD: Signal A, 278 nm/Bw:4 nm
Results

Retention Time	Area	Name
6.773	1242016	**Ciprofloxacin**
Totals	1242016	

Chapter 8

CONCLUSION

Due to consumption of heavy amount of antibiotics in our day to day life these antibiotics are getting accumulated in nature as our body is not capable of metabolizing all of the antibiotics that we take. Sources of accumulation of antibiotics are not only human consumption but it is also used for veterinary purpose, in fields by farmers these antibiotics are present as a component of pesticides, insecticides, etc. This heavy consumption and accumulation of antibiotics have led to development of new antibiotic resistant microbial strains which is now a global threat thus we need to remediate the accumulated antibiotics from our environment so that there is no more development of resistant strains.

This study is being performed on bioremediation ciprofloxacin using edible fungi *Pleurotus ostreatus*. Methodology includes maintenance and procurement of culture after every 4 weeks. Equal sized bits of *Pleurotus ostreatus* was used for radial growth and biomass studies on different concentrations of ciprofloxacin. Enzymatic assay for various enzymes such as cellulose, ligninanse, laccase were performed. Comparative assessment of degraded products of ciprofloxacin was done using various analytical techniques such as FTIR, HPLC, TLC, Titroimetry, Spectrophotometry.

During radial growth and biomass studies it was analyzed that ciprofloxacin was acting a stimulating agent for the growth of *Pleurotus ostreatus* as with increase in concentration 500 ppm it the radial growth was maximum, 11.5mm to 7.5mm on day and on 6th day it was 75.5 mm to 74.5mm. Similarly in biomass studies it was observed wet as well as dry weight of biomass was maximum for concentration of 500 ppm, 3.965gm (wet weight) and 1.647 (dry weight). Results of Enzyme assays were, specific activity of glucanase was 392.94 μmol $min^{-1}mg^{-1}$, lignin peroxidase was 0.0244 μmol $min^{-1}mg^{-1}$, hemicellulase 331..40 μmol $min^{-1}mg^{-1}$ and Mn. Perxidase was 23.864 μmol $min^{-1}mg^{-1}$for 500 ppm of concentration of ciprofloxacin. Methyl orange test gave a result of 3.3 ppm of left from original concentration of 500 ppm after 14 days of incubation, where as indigo carmine method gave 10.5 ppm concentration of degraded ciprofloxacin. Antimicrobial activity of different concentrations degraded products of ciprofloxacin was assessed against different bacterial strains *E coli, S.aureus* and *S pyogenes.* A decrease in zone of inhibiton of degraded products were observed in all three microbes when compared with standard concentration of ciprofloxacin. Titrimetry was done to find out the exact amount of degraded ciprofloxacin

after the incubation of 14 days. FTIR analysis was done for the comparative assessment of degraded products of ciprofloxacin against standard ciprofloxacin. Changes in peaks of standard ciprofloxacin against degraded products and it's comparative assessment showed that the taken concentrations of ciprofloxacin have been degraded to some extent. A difference in area of absorbance in results of HPLC confirmed that taken concentration of ciprofloxacin has been degraded to some extent when it was compared with standard concentration of ciprofloxacin.

Chapter 9

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