JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 3163-3169

Biodegradation of β-lactam Antibiotic 'Ampicillin' by White Rot Fungi from Aqueous Solutions

Ramasamy Rajesh Kumar+, Bong Ju Park1+, Hae Ran Jeong, Jae Taek Lee and Jae Young Cho*

Department of Bioenvironmental Chemistry, Chonbuk National University, Jeonju, Republic of Korea.
[1]Department of Horticultural Science, Chungbuk National University, Cheongju, Chungcheongbuk-do, 361 763, Republic of Korea.

(Received: 08 February 2013; accepted: 25 March 2013)

The worldwide higher usage and release of Pharmaceutical and Personal Care Products (PPCPs) into is an emerging environmental issue as it has adverse effects to humans, animals, plants and microorganisms. Hence it is necessary to find a novel mechanism to degrade and remove these PPCPs from the polluted site. One of the possible methods is employing white rot fungi, as it has the capability of degrading lignin. In this study, use of white rot fungi *Verticillium leptobactrum* KCTC 26026 to degrade a β-lactam antibiotic ampicillin was investigated. The antibiotic was added at a concentration of 0.5 mg L^{-1}, 1.0 mg L^{-1} and 2.0 mg L^{-1} and incubated for 7 days in a laboratory simulated environment. The HPLC-DAD studies have shown 100% depletion of ampicillin. To the best of our knowledge, this is the first study in which white rot fungi *Verticillium leptobactrum* KCTC 26026 was employed for degrading antibiotic.

Key words: Biodegradation, Ampicillin, *Verticillium leptobactrum*, White rot fungi, HPLC.

Antibiotics are generally classified based on their structure or by their mechanism of action including subgroups such as beta lactams, quinolones, tetracycline's, macrolides, sulfonamides, and others1. The production and use of antibiotics increased rapidly worldwide over the last several decades. Numerous pharmacologically active substances are used as human and animal medicines annually for treating and preventing diseases[1]. Approximately 3,000 compounds are used as medicine [2-5] and 100,000-200,000 tons $year^{-1}$ are used globally[6]. After human and animal administration the antibiotics are absorbed, distributed, metabolized and finally excreted across the urine and feces, together with their active compounds and metabolites[7,8]. The main emission sources of these emergent pollutants are hospitals, pharmaceutical industries and veterinary medicine[9]. However, the release of antibiotics into the environment has received attention in recent years.

Mostly antibiotics cannot be easily removed at Waste Water Treatment Plants (WWTPs) and earlier studies detected antibiotics concentrations in the environment in the range of nanogram per liter (μg/L)[10, 7, 11-14]. Various treatment methods has been proposed and adopted for Pharmaceutical and Personal Care Products (PPCPs) removal. Bioremediation using biologically active substances are readily available and cost effective when comparing to other techniques. White rot fungi, a group of basidiomycetes that cause white rot decay of wood materials, are considered the most efficient organisms in mineralizing lignin in nature[15]. There is great potential for using lignin-modifying enzymes in bioremediation as they are able to break down recalcitrant compounds showing partial structural similarities to lignin. Some of the xenobiotic compounds that have already been degraded by

* To whom all correspondence should be addressed.
+Co-first author
E-mail: soilcosmos@jbnu.ac.kr

whole cultures of white rot fungi under laboratory conditions include: chlorinated phenols, polychlorinated biphenyls (PCBs), DDT, dioxins, PAHs, alkyl halides, nitrotoluenes, chloroanilines and dyes16. In this study we evaluated the potential of white rot fungi Verticillium leptobactrum KCTC 26026 to degrade antibiotic ampicillin (Fig. 1) in a laboratory simulated environment.

MATERIALS AND METHODS

Antibiotics and Chemicals

The antibiotic Ampicillin (017-10381) was procured from Wako Pure Chemical Industries Ltd., Japan. The following chemicals were used potassium phosphate monobasic P5655 – 500G (Sigma, USA) and potassium phosphate dibasic S7907-500G (Sigma-Aldrich, USA). The following HPLC solvents were used, acetonitile (J.T. baker, 99.8%), hexane (Burdick and Jackson, 95 %), methanol (J.T. Baker) and water (J.T. baker)

Fungi

White rot fungi *Verticillium leptobactrum* (KCTC 26026) was purchased from Korean Collection for Type Cultures, Republic of Korea. The strains were grown in slants with malt extract agar at 30C for a week and then transferred to plates with agar (15g/L), glucose (10g/L) and malt extract (3.5g/L) and incubated at 30ºC for a week.

Mycelium growth inhibition assay

White rot fungi *V. leptobactrum* resistance to different concentrations of ampicillin (0.5, 1.0 and 2.0 ppm) in malt extract agar plates were studied according the protocol from Soo-Min *et al*., [17] Also the plates with no ampicillin were used as controls. The plates were incubated for 7 days at 30ºC in dark and the hyphae growth extension was measured daily from the center of the colony to the edge of the plate, considering that the maximal growth correspond to a hyphae extension of 4 cm[18].

Freeze-drying of V.leptobactrum

Before start of the experiments, white rot fungi *V. leptobactrum* was inoculated into conical flasks containing 250 ml of malt extract broth and incubated for a week at 30ºC in a shaker incubator at 120 rpm. The well gown mycelium was transferred to falcon tube and freeze dried in Ilshin Freeze dryer FDS series, Ilshin Lab Co ltd., Republic of Korea. The freeze dried live mycelium was stored in -20ºC for future use.

Ampicillin removal assay

The ampicillin removal experiment was carried out conical flasks (100ml) containing 25 ml of malt extract broth. The different concentrations of ampicillin (0.5, 1.0 and 2.0 ppm) was added to three conical flaks containing each 25 ml broth and 2 gm of freeze dried active fungus *V. leptobactrum* was inoculated into each flaks. This experiment was performed in triplicates. The control flasks with 0.5, 1.0, 2.0 ppm of ampicillin in 25 ml of broth without fungus were also kept. The conical flasks were incubated at 30ºC for a week in an orbital shaker of 120 rpm.

High performance liquid chromatography analysis

After extraction with acetonitrile, 4 ml were taken from each flask and filtered using Biofil Syringe filter 0.45ym (Jet Bio-Filtration Products Co. Ltd., China). Thereafter 2 ml from each concentration was used to determine the concentration of ampicillin in HPLC-DAD (Hitachi Elite LaChrome D 2000, Japan) by using TSKgel ODS – 100V column (TOSOH, Japan). The elution conditions were 90:10 potassium phosphates (0.01 M, pH 4.8): acetonitrile, injection volume 10il, retention time of 10 min and wavelength of 229 nm.

RESULTS AND DISCUSSION

Fungal resistance to ampicillin

The white rot fungus *V. leptobactrum* resistant to ampicillin was conducted with three different concentrations of ampicillin (0.5, 1.0 and 2.0 ppm). These concentrations did not affect the fungal growth (Fig 2-5) and the hyphae growth extension was 4 cm similar to the growth of white rot fungi used by Rodarte-Morales *et al*. [18]. Hence these three different concentrations such as 0.5, 1.0, 2.0 ppm of ampicillin were used for degradation assay.

Ampicillin degradation by V. leptobactrum

Various investigations on degradation of pharmaceutical products present in the effluents of the waste water treatment plants, as well as the degradation mechanisms[7, 9, 10, 12, 13, 19-23] (Carballa *et al*., 2004; Carballa *et al*., 2005; Clara *et al*., 2005; Suarez *et al*., 2005; Joss *et al*., 2006; Kosjek *et al*.,

2007; Esplugas *et al*., 2007; Ikehata *et al*., 2007; Reif *et al*., 2008; Suarez *et al*., 2008). The results of this experiment, after 14 days of incubation of ampicillin with *V. leptobactrum*, MnP (Microbial Manganese Peroxidase) activity was measured and approximately 80 U/l was detected (data not shown). Also the HPLC analysis of the samples was carried out and the results suggested the complete depletion of ampicillin (0.5-2.0 ppm) in all test samples, when compared with controls (Fig. 6-11). The complete degradation was achieved because of the white rot fungi, *V. leptobactrum* which synthesis oxidative enzymes that are capable of degrading lignin, a highly recalcitrant polymer with an aromatic structure similar to many pollutants.

White rot fungus synthesizes and secretes a substrate for its own peroxidase enzymes: veratryl alcohol (3,4-dimethoxybenzyl alcohol). The partially oxidized veratryl alcohol (the cation free radical) then oxidizes other chemicals that are not directly oxidized by the peroxidase enzymes. To complete further degradation, the fungus produces organic acids that also inhibit veratryl alcohol oxidation (data not shown).

The researchers [24-29] demonstrated that oxalate, the major organic acid synthesized by white rot fungi, is also an excellent inhibitor of lignin peroxidases. Oxalate is easily oxidized to carbon dioxide, thus its oxidation is essentially irreversible. However, the oxidation of oxalate is a two-electron oxidation, whereas the reduction of the veratryl alcohol cation radical consumes only one electron. Therefore, the odd electron left in oxalate is available for other reductions. A number of electron acceptors can thus be reduced by lignin peroxidases provided with hydrogen peroxide, veratryl alcohol (which is now called a "mediator"), and oxalate (which has been termed the 'donor"). The reductive dechlorination of carbon tetrachloride (a highly oxidized chemical) to the trichloromethyl radical was demonstrated with this system. The reductive dechlorination process was accomplished using a peroxidase, thus opening up a new field of investigation into the role of lignin peroxidases in the metabolism of chemicals requiring reductive dechlorination. Molecular oxygen can also be reduced by the oxalate radical to give superoxide, a species of oxygen that is useful for either oxidations or reductions. At low pH, superoxide is an excellent oxidant. Alternatively, superoxide is a good reductant, especially at higher pH, for reductive dechlorinations. Superoxide is also useful to generate another powerful oxidant, the hydroxyl radical. In the presence of transition metals, such as iron, superoxide can catalyze the generation of hydroxyl radicals by a sequence of reactions called the Haber- Weiss reaction. Because white rot fungi also produce hydrogen peroxide, it may not be necessary to dismutate

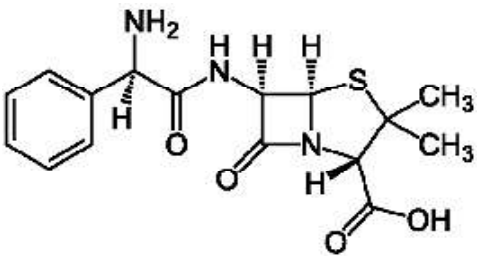

Fig. 1. (2S, 5R, 6R)-6-([(2R) - 2- amino-2-phenylacetyl] amino)-3, 3-dimethyl-7-oxo - 4-thia-1 azabicyclo [3.2.0] heptane-2- carboxylic acid

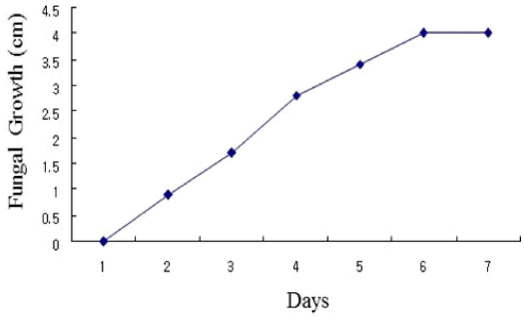

Fig. 2. Growth of fungi *V. leptobactrum* in absence of ampicillin

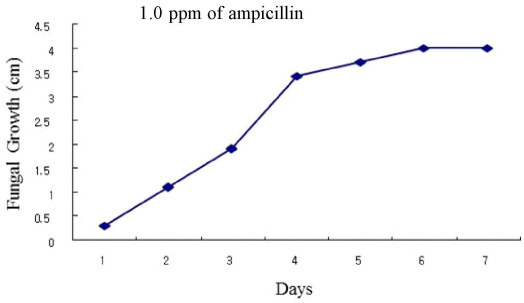

Fig. 3. Growth of fungi *V. leptobactrum* in 0.5 ppm of ampicillin

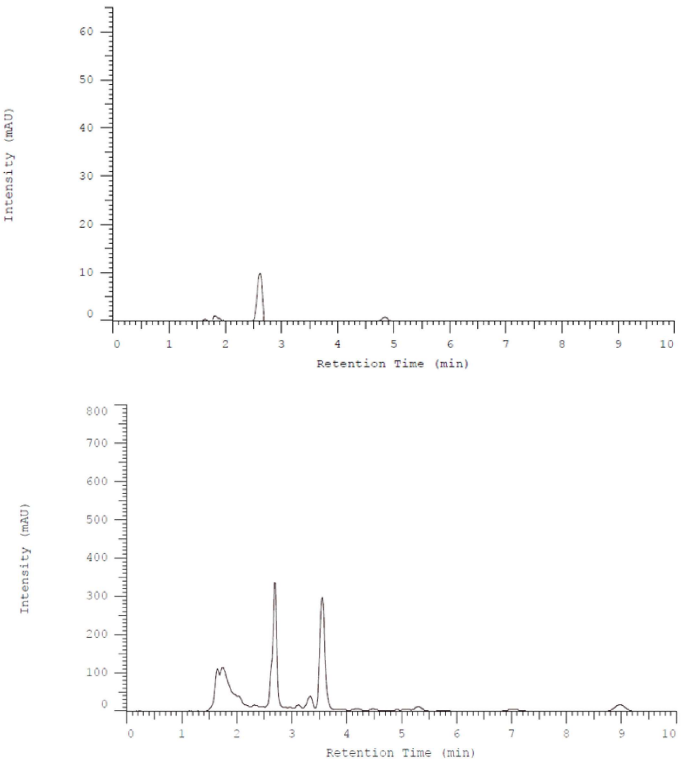

Fig. 4(a-b). HPLC elution profile of control and test sample of ampicillin 0.5 ppm in broth

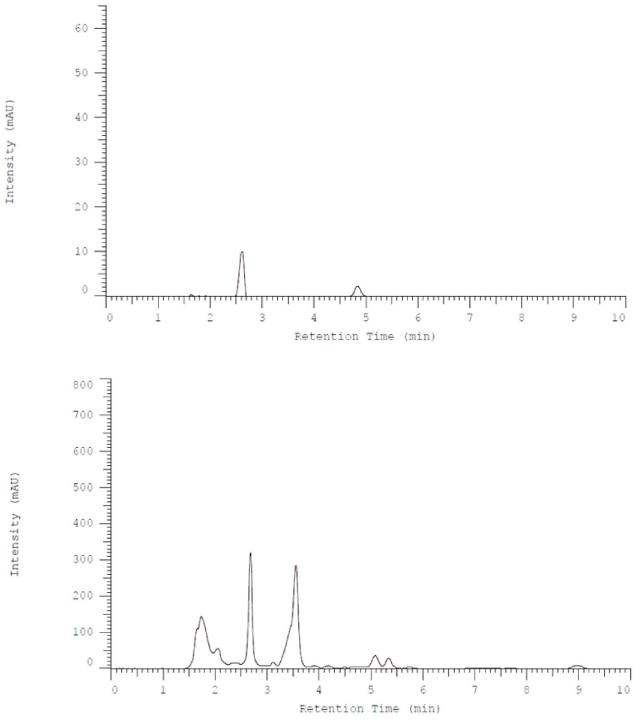

Fig. 5(a-b). HPLC elution profile of control and test sample of ampicillin 1.0 ppm in broth

superoxide to produce hydrogen peroxide. In such a case, the hydroxyl radical is simply produced by the last reaction in the sequence using Fe^{2+} and hydrogen peroxide, Fenton's reagent. The production of oxygen radicals by white rot fungi had been suggested earlier by several investigators[24-29]. However, the source of oxygen radicals remained unknown. Now the involvement in the biodegradation of environmental pollutants must be elucidated. Polyaromatic hydrocarbons present a case in point. Some of these chemicals, such as in coal tar and creosote, have oxidation potentials too high to be oxidized by the lignin peroxidases. However, polyaromatic hydrocarbons are oxidized by the fungi, and there is evidence that the lignin peroxidases are indeed involved in their oxidation. Perhaps polyaromatic hydrocarbons are being oxidized by the hydroxyl radicals. These mechanisms may also explain why the peroxidases catalyze the depolymerization of lignin rather than polymerization, as well as dehalogenation rather than halogenation. The presence of reductants would help form reduced, not polymerized, products of radicals[24-29].

Similar studies using white rot fungi *Bjerkandera* sp R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium* were used for the removal of 11 different PPCPs [15] . To our knowledge this is the first study of employing white rot fungi *V. leptobactrum* for the removal of β – lactam antibiotic ampicillin in a laboratory simulated environment. Prieto *et al*. [30] reported that more than

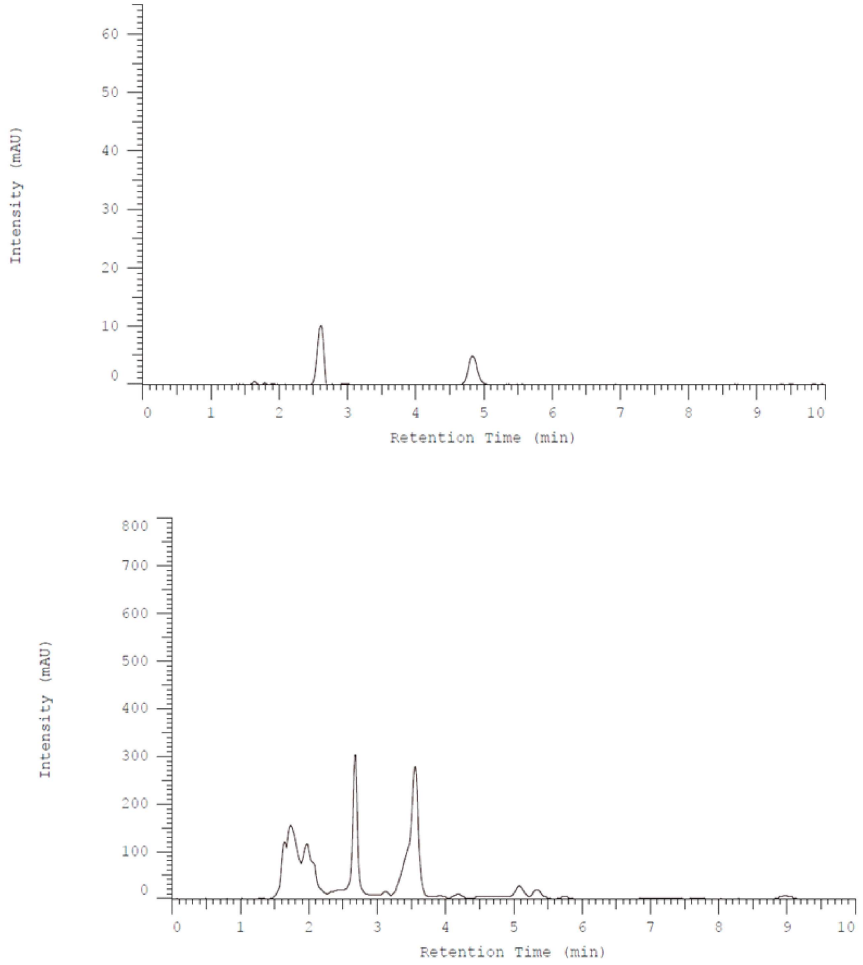

Fig. 6. HPLC profile of control sample of ampicillin 0.5 ppm in broth without *V. leptobactrum*

90% of the antibiotics ciprofloxacin and norflaxacin at 2 mg L^{-1} were degraded by white rot fungus *Trametes versicolor* after 7 days incubation. The researchers Wen *et al*.[31] employed *Phanerochate chrysosporium* to degrade the antibiotics tetracycline and oxytetraycline and reported 95% degradation of both the antibiotics. Using *Trametes versicolor* Rodriguez-Rodriguez *et al*.[32] depleted the naproxene completely after 72 h incubation and 48% of carbamazepine.

CONCLUSION

In this study white rot fungus *V. leptobactrum* depleted completely the â- lactam antibiotic ampicillin after a week of incubation in a laboratory simulated environment. Further researches can be carried out to optimize the degradation parameters for white rot fungus *V. leptobactrum* and the possibility of degradation of other PPCPs.

ACKNOWLEDGMENTS

This research was supported by "Cooperative Research Program for Agriculture Science & Technology Development" Rural Development Administration, Republic of Korea.

REFERENCES

1. Kumar, R. R., Lee, J.T., Cho, J.Y. Fate, occurrence, and toxicity of veterinary antibiotics in environment. *J Korean Soc. Appl. Biol. Chem*., 2012; **55**, 701-709
2. Díaz-Cruz, M.S., López de Alda, M.J., BarcelóD. Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. *Trend. Anal. Chem.,* 2003; **22**, 340–51.
3. Sarmah, A.K., Meyer, M.T., Boxall, A.B.A. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*, 2006; **65**, 725–59.
4. Calisto V and Esteves V.I. Psychiatric pharmaceuticals in the environment. *Chemosphere,* 2009; **77**, 1257–74.
5. Kümmerer K. Antibiotics in the aquatic environment. *Chemosphere*. 2009; **75**, 417–34.
6. Wise R. Antimicrobial resistance: priorities for action. *J Antimicrob. Chemothera.,* 2002; **49**, 585–6.
7. Ikehata, K., Naghashkar, N.J., EI-Din, M.G. Degradation of aqueous pharmaceuticals by ozonation and advanced oxidation processes: A review. *Ozone Sci Eng*., 2006; **28**, 353–414.
8. Zuccato, E., Castiglioni, S., Fanelli, R., Reitano, G., Bagnati, R., Chiabrando, C., Pomati, F., Rossetti, C. Calamari, D. Pharmaceuticals in the environment in Italy: Causes, occurrence, effects and control. *Environ. Sci. Pollut. Res.,* 2006; **13**(1), 15-21.
9. Kosjek, T., Heath, E., and Kompare, B. Removal of pharmaceutical residues in a pilot wastewater treatment plant. *Anal. Bioanal. Chem.,* 2007; **387**, 1379-1387.
10. Clara, M., Strenn, B., Gans, O., Martínez, E., Kreuzinger, N., Kroiss, H. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. *Water Res.,* 2005; **39**, 4797-4807.
11. Kim, I., Yamashita, N., Tanaka, H. Photodegradation of pharmaceuticals and personal care products during UV and UV/$_{H2O2}$ treatments. *Chemosphere,* 2009; **77**:518–525
12. Larsson, J.D,G,, De Pedro, C., Paxeus, N. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *J Hazar. Mater.,* 2007; **148**:751–755
13. Reif, R., Suárez, S., Omil, F., Lema, J, M. Fate of pharmaceuticals and cosmetic ingredients during the operation of a MBR treating sewage. *Desalination*, 2008; **221**:511–517
14. Suárez, S., Carballa, M., Omil, F., Lema, J.M. How are pharmaceutical and personal care products (PPCPs) removed from urban wastewaters? *Rev. Environ. Sci. Biotechnol.* 2008; **7**:125–138
15. Buswell, J.A., Odier, E. Lignin biodegradation. CRC *Crit. Rev. Microbiol.,* 1987; **15**:141–168
16. Field, J. A., de Jong, E., Feijoo-Costa, G., de Bont J. A. M. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. *Trends in Biotechnology*, 1993, **11**, pp.44-48.
17. Soo-Min, L., Bon-Wook, K., Joon-Weon, C., Don-Ha, C., Beum-Soo, A., Eui-Bae, J., In-Gyu, C. Degradation of bisphenol A by white-rot fungi, Stereum hirsutum and *Heterobasidium insulare*, and reduction of its estrogenic activity. *Biol. Pharm. Bull.,* 2005; **28**(2):201–207
18. Rodarte-Morales, A.I., Moreira, M.T., Feijoo, G., Lema, J.M. Degradation of selected pharmaceutical and personal care products (PPCPs) by white-rot fungi. *World J Microbiol. Biotechnol.,* 2011; **27**, 1839–46.

19. Carballa, M., Omil, F., Lema, J.M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez M., Ternes, T. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.,* 2011; **38**:2918–2926
20. Carballa, M., Omil, F., Lema, J.M. Removal of cosmetic ingredients and pharamaceuticals in sewage primary treatment. *Water Res.,* 2005; **39**: 4790-4796.
21. Suárez, S., Ramil, M., Omil, F., Lema, J.M. Removal of pharmaceutically active compounds in nitrifying-denitrifying plants. *Water Sci. Technol.,* 2005; **52**(8):9–14.
22. Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., McArdell, C.S., Ternes, T.A., Thomsen. A., Siegrist, H. Biological degradation of pharmaceuticals in municipal wastewater treatment: proposing a classification scheme. *Water Res.,* 2006; **40**:1686–1696.
23. Esplugas, S., Bila, D.M., Krause, L.G.T., Dezotti, M. Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents. *J Hazar. Mater.,* 2007; **149**:631–642
24. Aust, S.D. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. *Microbial Ecol.,* 1990; **20**: 197-204
25. Bumpus, J. A., Aust S.D. Biodegradation of environmental pollutants by thw white rot fungus *Phanerochaete chrysosporium*: involvement of lignin degrading system. *BioEssays.,* 1987; **6**: 166-190.
26. Crawford, R.L. Lignin biodegradation and transformation. New York: John Wiley, 1981.
27. Kirk, T.K., Farrell, R.L. Enzymatic "combustion": the microbial degradation of lignin. *Annu Rev Microbiol.,* 1987; **41**: 465-505.
28. Kirk, T.K., Hiquchi, T, Chnag, H.M. Lignin biodegradation, microbiology, chemistry and potential applications. Boca Raton, Florida: CRC Press, 1980.
29. Tien, M. Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. *Crit Rev Microbiol.,* 1987; **15**: 141-168.
30. Prieto, A., Moder, M., Rodil, R., Adrian, L., Marco-Urrea, E. Degradation of norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products. *Biores Technol.,* 2011; **102**; 10987-10995.
31. Wen, X., Jia, Y., Li, J. Degradation of tetracycline and oxytetracycline by crude lignin peroxidase prepared from *Phanerochaete chrysosporium*-A white rot fungus. *Chemosphere,* 2009; 1003-1007.
32. Rodriguez- Rodriguez, C.E., marco-Urrea, E., Caminal, G Degaradation of naproxen and carbamazepine in spiked sludge by slurry and solid phase *Trametes versicolor* systems. *Biores Technol.,* 2010; **101**, 2259-2256.