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

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The worldwide higher usage and release of Pharmaceutical and Personal Care Products (PPCPs) into 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 loptobactrum* 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 loptobactrum* KCTC 26026 was employed for degrading antibiotic.

Key words: Biodegradation, Ampicillin, *Verticillium loptobactrum*, 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 others. 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. Approximately 3,000 compounds are used as medicine and 100,000-200,000 tons year⁻¹ are used globally. 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. The main emission sources of these emergent pollutants are hospitals, pharmaceutical industries and veterinary medicine. 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)²⁻⁵,⁷⁻¹⁴. 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. 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
whole cultures of white rot fungi under laboratory conditions include: chlorinated phenols, polychlorinated biphenyls (PCBs), DDT, dioxins, PAHs, alkyl halides, nitrotoluenes, chloroanilines and dyes. 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 30°C 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) was studied according the protocol from Soo-Min et al., Also the plates with no ampicillin were used as controls. The plates were incubated for 7 days at 30°C 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. **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 30°C 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 -20°C for future use.

**Ampicillin removal assay**

The ampicillin removal experimental 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 flasks 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 30°C 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 10ìl, 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. 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 (Carballa et al., 2004; Carballa et al., 2005; Clara et al., 2005; Suarez et al., 2005; Joss et al., 2006; Kosjek et al.,...
The results of this experiment, after 14 days of incubation of ampicillin with \textit{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, \textit{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 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. 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

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**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.² employed *Phanerochaete chrysosporium* to degrade the antibiotics tetracycline and oxytetracycline and reported 95% degradation of both the antibiotics. Using *Trametes versicolor* Rodriguez-Rodriguez et al.³ depleted the naproxene completely after 72 h incubation and 48% of carbamazepine.

CONCLUSION

In this study white rot fungus *V. leptobactrum* depleted completely the ß-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.

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