ANADOLU ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ –C
Yaşam Bilimleri ve Biyoteknoloji

ANADOLU UNIVERSITY JOURNAL OF SCIENCE AND TECHNOLOGY –C
Life Sciences and Biotechnology
Cilt/Vol.:1-Sayı/No: 2 : 113-124 (2011)

DECHLORINATION OF 2,4,6-TRICHLOROPHENOL BY FREE AND IMMOBILIZED LACCASE FROM *TRAMETES VERSICOLOR* IN A LAB SCALE BIOREACTOR

Arzu ÜNAL [1], Ahmet ÇABUK [2], Nazif KOLANKAYA [3]

ABSTRACT

Detoxification of a chlorinated phenolic compound, 2,4,6-trichlorophenol through treatment with laccase enzyme produced by a white rot fungus, *Trametes versicolor* was investigated. Enzymatic dechlorination experiments by using free and immobilized laccase have been performed in a lab scale bioreactor. Chlorine ion and dissolved oxygen electrodes mounted to the bioreactor were used continuously to detect the profiles of chlorine ions and oxygen consumption, respectively, in reaction medium. The maximum dechlorination activity of laccase for free and immobilized form was determined as 160 μM of substrate concentration at pH 5.0, 25 °C, and 30 min of incubation time. Also, GC/MS analyses of enzymatic degradation products indicated that chlorine removal was a result of degradation of 2,4,6-trichlorophenol by the laccase under the determined optimum conditions.

Keywords: Laccase, *Trametes versicolor*, 2,4,6-tricholorophenol, Dechlorination.

LABORATUAR ÖLÇEKLI BIYOREAKTÖRDE *TRAMETES VERSICOLOR*'DAN ELDE EDILEN SERBEST VE IMMOBILIZE LAKKAZ ILE 2,4,6-TRICHLOROPHENOL'DEN KLOR UZAKLAŞTIRILMASI

ÖZ

Bu çalışmada, klorlu fenolik bileşiklerden 2,4,6-triklorofenolün *Trametes versicolor* olarak bilinen beyaz çürükçül fungusun ürettiği lakkaz enzimi ile muamele edilerek detoksifikasyonu araştırılmıştır. Serbest ve immobilize enzim kullanılarak yapılan enzimatik deklorinasyon deneyleri laboratuar ölçeğinde bir reaktörde gerçekleştirilmiştir. Biyoreaktöre monte edilmiş klor iyon ve çözünmüş oksijen elektrodları, sürekli olarak reaksiyon ortamında klor ve çözünmüş oksijen değişimlerini belirlemek amacıyla kullanılmıştır. Serbest ve immobilize formda lakkazın en yüksek deklorinasyon aktivitesi; substrat konsantrasyonu 160 mM, pH 5.0, reaksiyon süresi 30 dakika ve sıcaklık olarak da 25 °C'de bulunmuştur. Ayrıca klor uzaklaştırılmasının gösterildiği enzimatik yıkım ürünlerinin GC/MS analizi, belirlenen optimum şartlarda lakkazla 2,4,6-triklorofenolün parçalandığı sonucunu vermiştir.

Anahtar Kelimeler: Lakkaz, *Trametes versicolor*, 2,4,6-tricholorophenol, Klor uzaklaştırılması.

[1,] Ministry of Agriculture and Rural Affairs, General Directorate of Agricultural Research, Yenimahalle, Ankara, TURKEY.
[2,] Eskişehir Osmangazi University, Science and Arts Faculty, Department of Biology, Eskişehir, TURKEY. Faks: 0222 2393578, e-posta: acabuk@ogu.edu.tr
[3,] Hacettepe University, Science Faculty, Department of Biology, Division of Biotechnology, 06532, Beytepe Campus, Ankara, TURKEY.

Bu çalışma kapsamında sunulan verilerin bir kısmı Arzu Ünal tarafından yapılan Doktora Tez çalışmasına aittir.

Recieved: 24 March 2010; **Revised:** 28 June 2010; **Accepted:** 28 October 2010

1. INTRODUCTION

Chlorinated compound contamination of aquatic and terresterial field is one of the significant environmental problems due to their toxicity and persistence in nature. Chlorinated aromatics are known to be more resistant to biodegradation than chlorinated aliphatic compounds. Toxicity of organics depends on the number and position of chlorine substituent in the organic molecules. Therefore bioremediation of these environments which are polluted with such polychlorinated recalcitrant and toxic compounds is a serious problem faced by the environmental scientist. Also, polychlorophenols are one of the most persistent environmental pollutants. Xenobiotic sources of phenolic compounds in environment are industrial wastes derived from fossil fuel extraction, chemical manufacturing processes such as wood processing industry, pharmaceutical industry, pesticide production etc. (Kumaran and Parachur, 1997; Taspinar and Kolankaya, 1998; Majumder and Gupta, 2007).

Studies on the biodegradation of xenobiotics were mainly conducted with bacteria until 1985, however it was demonstrated that white-rot fungus *Phanerocehaete chrysosporium* had the ability to degrade a large spectrum of recalcitrant organopollutants such as chlorinated phenols, polychlorinated biphenyls etc besides lignin (Thomas et al., 1992; Yadav and Reddy, 1992). The ability of various white-rot fungi to degrade a wide range of environmental pollutants such as phenol, 4- chlorophenol, 2,4,6 trichlorophenol (2,4,6-TCP), ppDDT, pentachlorophenol (PCP), cyanide and polycyclic aromatic hydrocarbons has been reported (Barr and Aust, 1994; Pallerla and Chambers, 1998; Toumela et al., 1999; Zouari et al., 2002; Ünal and Kolankaya, 2004; Ehlers and Rose, 2005; Walter et al., 2005; Cabuk et al., 2006; Valentin et al., 2006). Also, they can mineralize xenobiotic materials to CO_2 and H_2O through their non-specific and highly oxidative enzyme systems. Ligninolytic systems in white-rot fungi have been used in improving the degradation of toxic pollutants and bioconversion of lignin into useful organic compounds (Arora et al., 2002; Hofrichter, 2002). Primarily three enzymatic systems, laccase, lignin peroxidase and manganese peroxidase, have been held responsible for the degradation of various pollutants (Hofrichter, 2002; Novotny et al., 2004). Laccase is a multi copper enzyme that catalyzes the oxidation of a variety of phenolic compounds with the concomitant reduction of O_2 to H_2O. In addition laccases have been of potential use in detoxification of environmental pollutants, wine stabilization, paper processing, enzymatic conversion of chemical intermediates, and the production of useful chemicals from lignin (Duran and Esposito, 2000).

The aim of this work was to determine the optimal conditions for 2,4,6-TCP biodegradation by laccase enzyme produced by *T. versicolor* in a lab scale bioreactor with chlorine ion and dissolved oxygen electrodes. Also, GC/MS analyses of enzymatic degradation products have been investigated under the determined optimum conditions.

2. MATERIALS AND METHODS

2.1 Culture Conditions and Screening of Microorganisms

Stock cultures of the microorganisms were maintained on malt extract agar slants at +4 °C. The microorganisms were firstly grown on agar slants using malt extract agar (Merck).They were subcultured and incubated at 30 °C for 7 days. Mycelia and spore suspension of stock cultures were used as inoculum source. 1 ml of inoculant prepared by suspending the stock culture was added into the 250 ml Erlenmeyer flask containing 100 ml of Modified Vogel's medium (Aktaş et al., 2001). Inoculated flasks were incubated at 30 °C in an incubator shaker rotating at 150 rpm up to 10 days. After this, supernatant and biomass were separated by filtering through Whatman No:1 filter paper.

The white-rot fungal strains used in the experiments were obtained from different sources. While *Trametes versicolor* ATCC 200801 was provided from ATCC, *Phanerochaete chrysosporium* ME 496 and *Pleurotus sajor-caju*, *Pleurotus ostreatus*, *Pleurotus florida*, *Pleurotus sapidus*, *Pleurotus eryngii* were provided from Dr. Kirk (U.S. Dept. of Forest product Agriculture Lab., Madison, Wisconsin, 53705, U.S.A) and Dr. Zadrazil (Weisdrangveg 4, 3300 Braunschweing, Germany). For preliminary tests, six strains of white-rot fungi were investigated for their high laccase production ability. To compare the laccase production features of these microorganisms and to determine the most effective strain, cultivations were carried out in the Modified Vogel's medium, as was mentioned above.

2.2 Laccase Assay

To assay the laccase enzyme activity, 0.1 ml enzyme source was added into test tube containing 4.9 ml of 0.1 M acetate buffer at pH 4.6 and 1 Mm guaiacol as substrate. The reaction mixture so prepared was allowed to incubation at 37 °C for 5 min. Blanks contained inactive enzyme prepared by boiling enzyme source. Enzyme activity in the tubes was measured by reading optical density in Jenway 6105 UV/VIS spectrophotometer adjusted to 465 nm wavelength. One unit activity was defined as enzyme activity that elicited an increase in A_{465} of 0.1 absorbance unit per minute (Taspinar and Kolankaya, 1998).

2.3 Optimization

The parameters including incubation time, pH, temperature, initial substrate concentration, and enzyme concentration were tested to find the effective strain in a 100 ml volume of lab scale bioreactor.

The experiment was performed in 45 minutes to determine incubation period, achieving chlorine and O_2 measurements via electrodes at certain intervals. To determine pH value, values of 3.0-10.0 pH were investigated. Acetate buffers (0.2 M) were used for pH 3.0-5.0, phosphate buffers (0.2 M) for pH 6.0-8.0 and Tris-HCl buffers (0.2 M) for pH 9.0 and 10.0. To determine the optimum temperature value, the experiment was performed at temperatures varying between 10 - 45±1 °C. Concentrations of 50-300 μM were tested to determine the initial 2,4,6-TCP concentration. Finally, determination of enzyme concentration was made through addition of laccase with varying activity between 0.59 and 6.1 U/ml. Amounts of chlorine ions released and consumption of O_2 during reactions were detected by chlorine ion electrode (Jenway 3205) and O_2 electrode (Jenway DO_2 meter 9071).

All the experiments were carried out at least in triplicate and experimental errors were estimated and are depicted with error bars and standard deviations are indicated wherever necessary.

2.4 Enzyme Immobilization

The method described by Rozie et al. (1988) for alginate beads immobilization was employed. Alginate beads were maintained in 0.03 M $CaCl_2$ at +4 °C until for use.

2.5 Determination of Degradation Products

GC/MS analysis of enzymatic degradation products indicated that chlorine removal from 2,4,6-TCP was a result of the degradation of these chlorinated substances by laccase.

3. RESULTS

T. versicolor was the most potent laccase producer organism among the ones examined (Fig. 1). 30 min of contact time was found to be necessary for the maximum dechlorination of 2,4,6-TCP. The degradation rate was quite fast up to 30 min and then showed no significant variation (Fig. 2).

Furthermore, the increasing pH was found to favor increase in chlorine removal up to pH 5.0 and then did not considerably change (Fig. 3). Also, 25 °C was found to be the optimum temperature as an another parameter affected the chlorine removal of 2,4,6-TCP by laccase. The results are presented in Fig. 4.

There was a proportionality between the initial 2,4,6-TCP concentration and the extent of chlorine removal up to 150 μM of 2,4,6-TCP (Fig. 5). Also, parallel results were found out in chlorine removal data of laccase for various values of enzyme activity are represented in Fig. 6.

Fig. 7 showed the chlorine removal efficiency of immobilized laccase in a lab scale bioreactor for 2,4,6-TCP. These results were similar to free enzyme studies under the determined optimum conditions.

The GC/MS analyses of pure 2,4,6-TCP and treated by laccase of 2,4,6-TCP presented in Fig. 8 and 9.

4. DISCUSSION

Since 2,4,6-TCP is an important chlorophenolic compound, it has attracted more attention. Michizoe et al. (2001) studied reversed mycelles inducing laccase activity in organic solvents and determined optimum conditions. Under these conditions, environmental pollutants such as 2,4,6-TCP was degraded in 3 h.

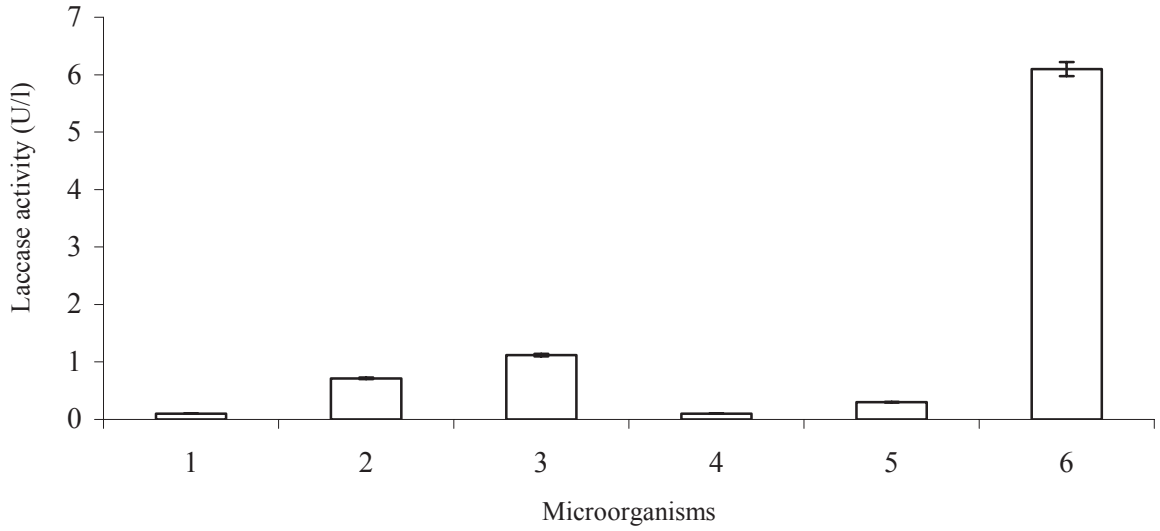

Figure 1. Comparison of the laccase activity level of white rot fungi for *Phanerochaete chrysosporium* (1), *Pleurotus sapidus* (2), *Pleurotus florida* (3), *Pleurotus sajor-caju* (4), *Pleurotus ostreatus* (5), *Trametes versicolor* (6). Error bars represent deviations.

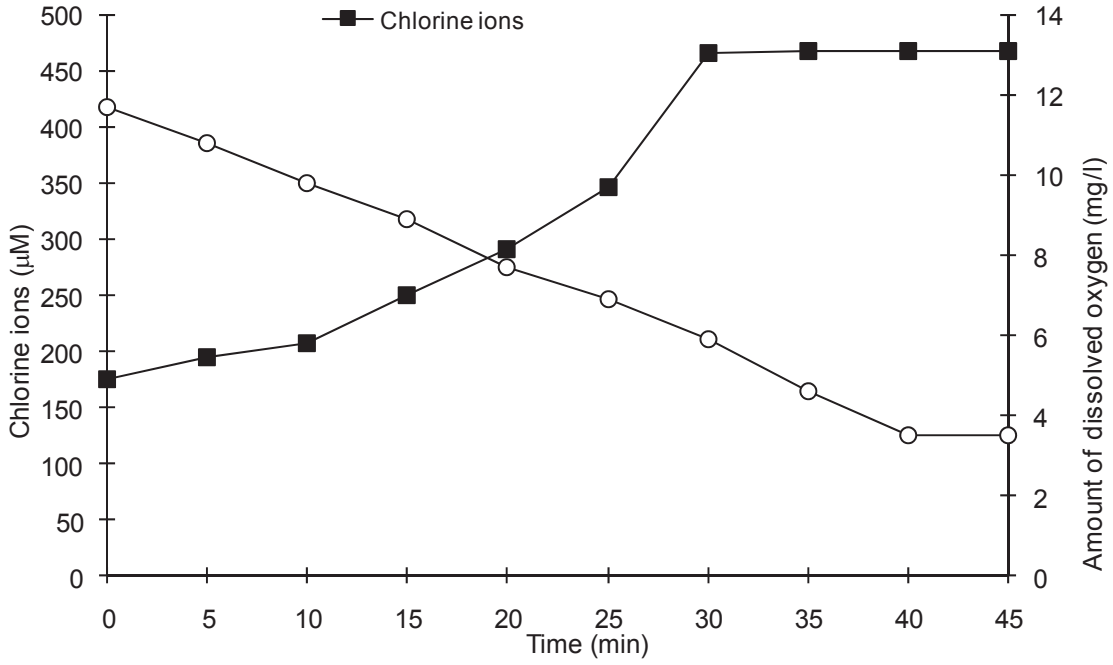

Figure 2. The effect of incubation period on the enzymatic dechlorination of 2,4,6- trichlorophenol by laccase from *T. versicolor*.

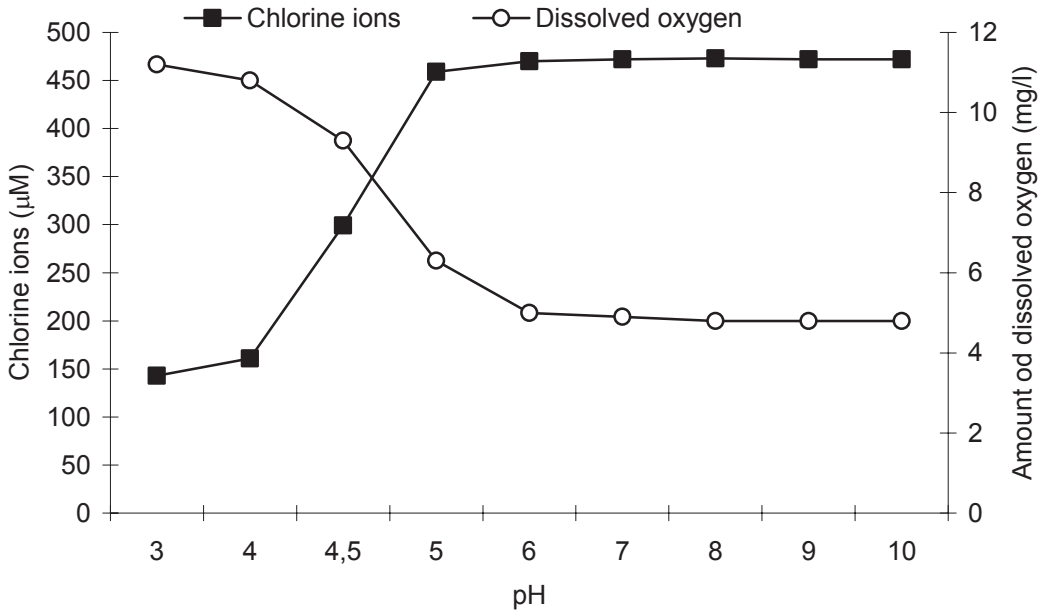

Figure 3. The effect of pH on the enzymatic dechlorination of 2,4,6-trichlorophenol by laccase from *T. versicolor*.

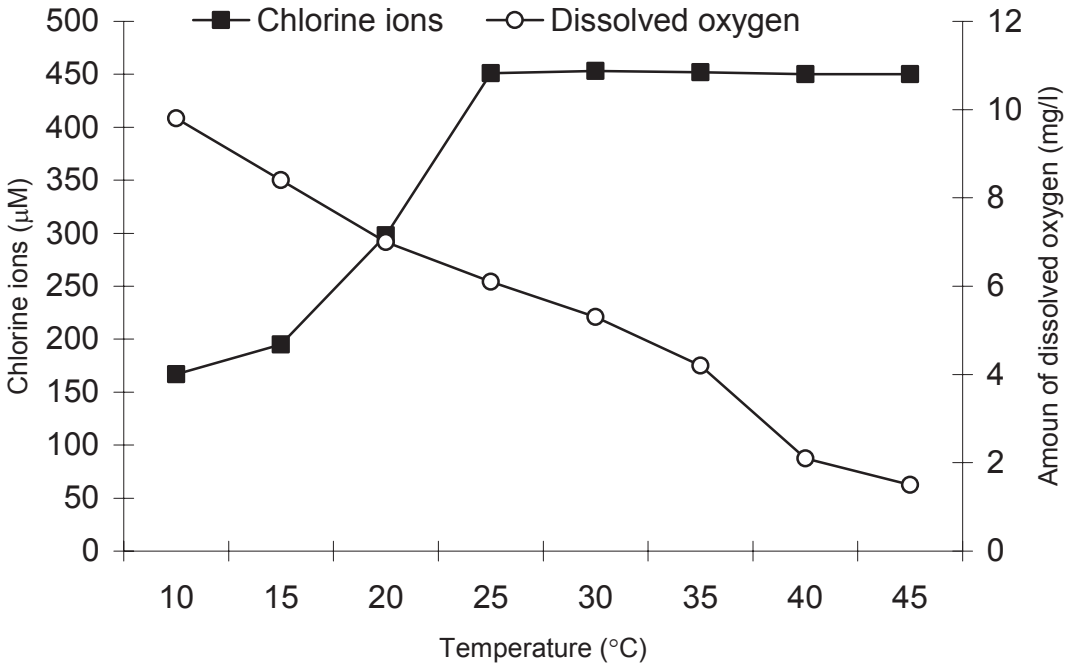

Figure 4. The effect of incubation temperature on the enzymatic dechlorination of 2,4,6-trichlorophenol by laccase from *T. versicolor*.

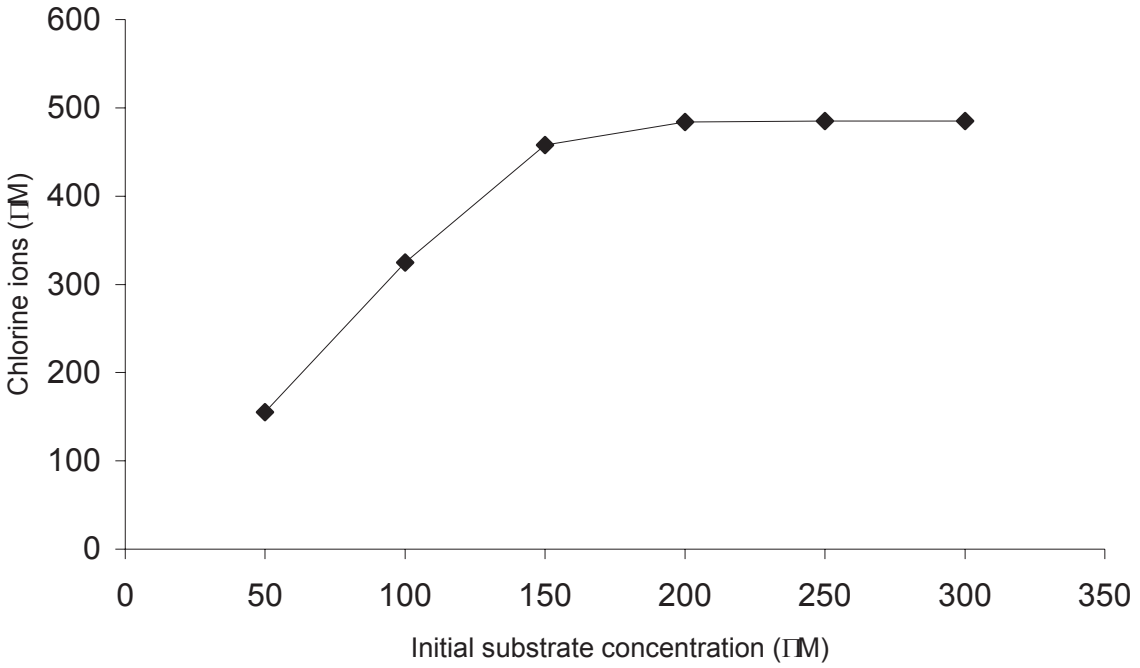

Figure 5. The effect of initial substrate concentration on the enzymatic dechlorination of 2,4,6-trichlorophenol by laccase from *T. versicolor*.

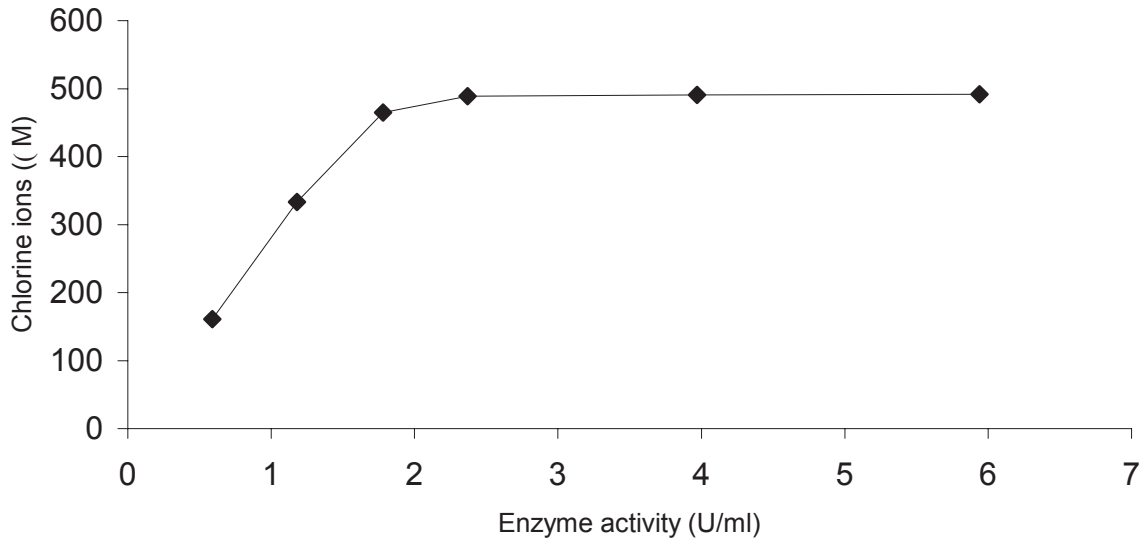

Figure 6. The effect of enzyme activity on the enzymatic declorination of 2,4,6-trichlorophenol by laccase from *T. versicolor*.

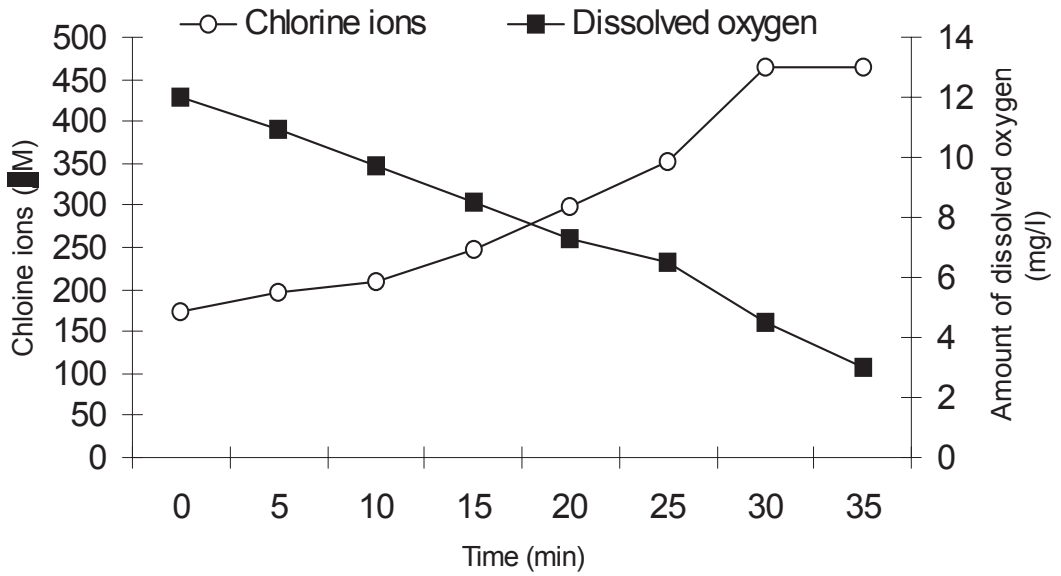

Figure 7. Dechlorination of 2,4,6-trichlorophenol by immobilized laccase enzyme in alginate beads.

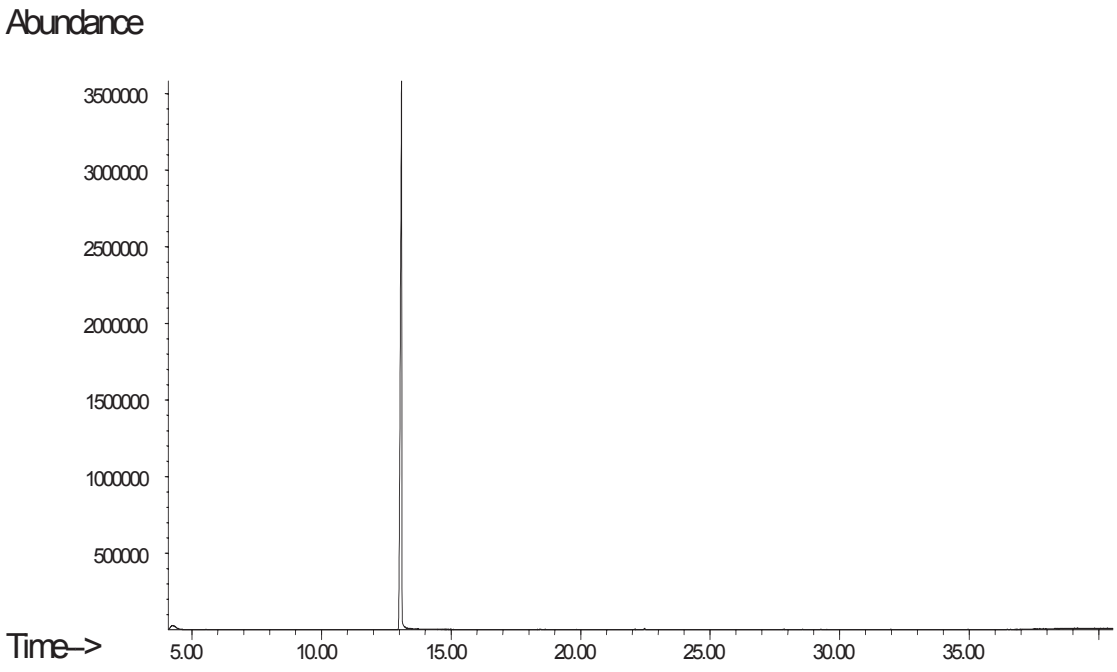

Figure 8. The peaks of 2,4,6-trichlorophenol of GC/MS chromatogram

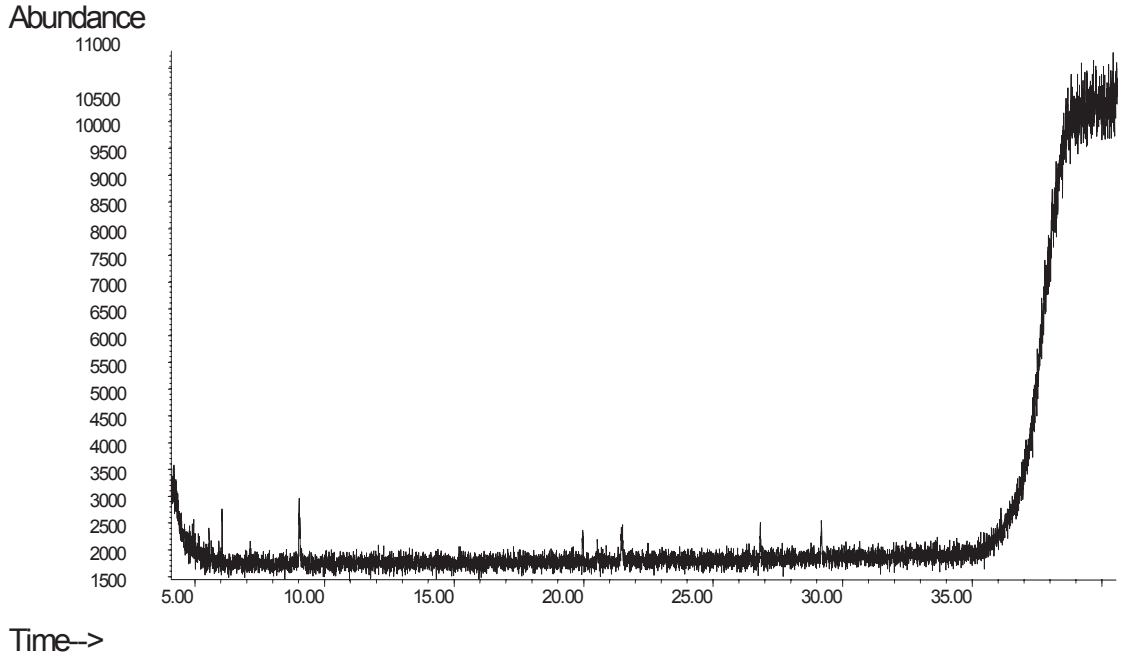

Figure 9. The peaks of 2,4,6-trichkorophenol after treated with laccase of GC/MS chromatogram

Leontievsky and coworkers (2001) demonstrated the transformation of 2,4,6-TCP; therefore, laccase from the *Coriolus versicolor* VKM F-116 immobilized on Celite R-637 by covalent binding with glutaraldehyde. According to the results obtained, after a sharp primary decline in activity (up to 50%), the retained enzyme activity was stable over a storage period of 33 days at 4°C. A comparative study of soluble and immobilized laccases revealed the increased resistance of immobilized enzyme to the unfavourable effects of alkaline pH, high temperature and the action of inhibitors. A combination of these properties of immobilized laccase resulted in the ability to oxidize 2,4,6-TCP at 50°C and pH 7.0. The reactions of soluble and immobilized laccase with 2,4,6-TCP were examined in the presence and absence of redox mediators. 3,5-dichlorocatechol, 2,6-dichloro-1,4-benzoquinone and 2,6-dichloro-1,4-hydroquinone were found to be the primary products of 2,4,6-TCP oxidation by laccase; oligo- and polymeric compounds were also found. Furthermore in this study, it was examined that the white rot fungus *P. tigrinus* could be acclimatized to higher concentrations of 2,4,6-TCP by the addition of increasing concentrations into the culture medium over a certain period of time (Leontievsky et al., 2001). For 2,4,6-TCP, the discrepancy between our results and that of Leontievsky et al. (2001) could lie in the difference in fungal strains from which the laccase was obtained, in laccase activity provided, in culture conditions and immobilization agent used.

The most recent study of Lisov et al. (2007) investigated the action of purified laccase from *Cerrena unicolor* and *Trametes* sp. The study with 2,4,6-TCP was including reactions involving 1-hydroxybenzotriazole as a mediator. The oxidation of 2,4,6-TCP by laccase without the mediator yielded 2,6-dichlorobenzoquinone as a primary conversion product.

In another study, the reductive dechlorination and biodegradation of 2,4,6-TCP was investigated in a laboratory-scale sequential barrier system consisting of a chemical and biological reactive barrier (Choi et al., 2007). Palladium coated iron (Pd/Fe) was used as a reactive barrier medium for the chemical degradation of 2,4,6-TCP, and a sand column seeded with anaerobic microbes was used as a biobarrier following the chemical reactive barrier in this study. Only phenol was detected in the effluent from the Pd/Fe column reactor, indicating that the complete dechlorination of 2,4,6-TCP was achieved. The residence time of 30.2 h was required for the complete dechlorination of 2,4,6-TCP of 100 mg/l in the column reactor. Moreover, the sequential permeable reactive barriers were improved consisting of iron barrier and biobarrier.

Martinez-Ruiz et al. (2009) studied the biodegradation of 2,4,6-TCP catalysed by the me-

thylsyringate–laccase mediator system and found the best assay conditions for the biodegradation in 6 min of 1 mM TCP are 300 nM of enzyme concentration, 0.7 mM of methylsyringate concentration, and pH 4.0 at 25 °C.

As seen in Fig. 1, *T. versicolor* had maximum laccase production (6.1 U/ml) capacity when compared to the other examined white rot fungus. According to some researchers, *T. versicolor* has been known as a good laccase source (Arcand and Archibald, 1991; Limura et al., 1996; Taspinar and Kolankaya, 1998; Novotny, 2004). Therefore culture supernatant of *T. versicolor* was used as a crude laccase source in dechlorination of 2,4,6-TCP studies.

According to the result of the experiment to determine the effect of contact time, it was observed that there was nearly a linear increase in dechlorination up to 30 min of incubation period (Fig. 2) and then, the amount of dechlorination did not significantly change with contact time. As known clearly, laccase use oxygen as an electron acceptor during the laccase dependent oxidation reactions (Kirk and Farrel, 1987; Yarapalov et al., 1994). The amount of dissolved oxygen decreased first with increasing of the free chlorine ion concentration and reached to a saturation value at 40 min and then the value did not change (Fig. 2). The data also suggest that the dechlorination of 2,4,6-TCP reaction is catalyzed by the laccase activity of the culture supernatants of *T. versicolor*.

Earlier studies on dechlorination of some phenolic compounds showed that pH was an important factor affecting the dechlorination process by laccase enzyme (Arcand and Archibald, 1991; Taspinar and Kolankaya, 1998). In the experiments performed to find out to effect of pH on the laccase depended dechlorination of 2,4,6-TCP, it was observed that there was a sharp increase in chlorine removal up to pH 5.0 and then amount of chlorine removal did not significantly change with the increasing pH values up to 10.0 (Fig. 3). Some researchers reported similar results at pH 5.0 as an optimum pH value for laccase enzyme isolated from *T. versicolor* (Arcand and Archibald, 1991; Taspinar and Kolankaya, 1998).

The effect of temperature on the chlorine removal of 2,4,6-TCP was investigated at a constant value at pH 5.0 and 45 min incubation time. The variation of the chlorine removal and consumption of dissolved oxygen with temperature was presented in Fig. 4. As seen in this figure, the chlorine removal by laccase enzyme from *T. versicolor* appears to be temperature dependent in the temperature range studied. When the temperature was increased from 10 to 25 °C, removal increased from 167 μM to 451 μM. This chlorine removal at 25 °C may be an important parameter for a practical application of in situ bioremediation for chlorophenolic compounds in the environment.

In order to determine the effect of the initial substrate concentration on the chlorine removal efficiency of 2,4,6-TCP the amounts of substrate into reaction medium were varied from 50 μM to 300 μM and the results are presented in Fig 5. With increase in the initial 2,4,6-TCP concentration from 50 to 150 μM the amount of free chlorine ions was increased from 155 μM to 458 μM. Further increase up to 300 μM did not significantly change the amount of free chlorine ion concentration in the reaction medium and it almost stayed constant.

Similar trend which was observed in chlorine removal data of laccase for different values of enzyme activity are represented in Fig 6. When the laccase activity increased from 0.59 to 5.94 (U/ml) the amount of free chlorine concentration in the reaction medium significantly increased from 161 μM to 492 μM. Further increase in the laccase enzyme activity did not change the free chlorine ions concentration in the medium. The constant trend was observed with the laccase activity between 1.78 to 5.94 U/ml.

The experimental results from the optimization studies show that laccase enzyme from *T. versicolor*, can be effectively used as a promising enzyme for the dechlorination process of 2,4,6-TCP with the advantage of practical application. The maximum yield was obtained at pH 5.0 and 150 μM initial substrate concentration, 1.78 U/ml laccase activity and at 25 °C of reaction temperature. In relevant literatures, it is possible to meet studies that reduce the toxicity of chlorophenols by using white rot fungi and also the related enzyme (Reddy et al., 1998; Taspinar and Kolankaya, 1998; Ehlers and Rose 2005).

Free and immobilized enzymes can be utilized in the dechlorination and detoxification process for a lot of pollutant. However, immobilized form of an enzyme with various carrier is ideal for use in reactor applications with the advantages of improved mechanical strength, online matrix isolation, self supporting rigidity, excellent durability, easy regeneration

and continuous processes (Ehlers and Rose, 2005). For this purpose, laccase enzyme from *T. versicolor* was immobilized with alginate beads in this study. Batch and continuous bioreactor experiments were carried out under the determined optimum conditions by using the free laccase described above. As can be seen in Fig 7, chlorine removal efficiency of immobilized laccase was similar to free enzyme studies in a lab scale bioreactor for 2,4,6-TCP. However, it was observed that chlorine removal from 2,4,6-TCP by immobilized laccase relatively occur rapidly and after the equilibrium was reached in 30 min differing from using free laccase. Also, the dechlorination efficiency of immobilized laccase did not change significantly and only a maximum 5% decrease was observed after three cycles in a continuous experiment. These results showed that the alginate immobilized laccase has a good potential for the removal of chlorine ions repeatedly from 2,4,6-TCP without any detectable loss in total removal capacity in the lab scale bioreactor. The results demonstrate that the maximum dechlorination level occurred in comparatively short time. The rapid dechlorination is a significant parameter for large scale application in industrial processes. The rate of declorination is of great significance for developing a microbial origin enzyme-based water-treatment technology and practical application of process.

The GC/MS analyses were conducted with appropriate conditions in order to determine the possible degradation products indicated that chlorine removal from 2,4,6-TCP was a result of the degradation of these chlorinated substances by laccase. The diagrams obtained from GC/MS analyses with pure 2,4,6-TCP and treated by laccase of 2,4,6-TCP were shown in Fig 8 and 9. These findings indicated that degradation processes for the removal of 2,4,6-TCP by laccase from *T. versicolor* which is confirmed by the peaks obtained from GC/MS analyses. Also, some researchers reported similar findings in the literature (Yadav et al., 1995; Krcmar, et al., 1999).

In this study, the laccase activities of white rot fungi examined were firstly compared. Then, optimal conditions of dechlorination in order of incubation period, pH, incubation temperature, initial substrate concentration, and enzyme activity. Furthermore, removal efficiency of 2,4,6 trichlorophenol by immobilized laccase enzyme in alginate beads was investigated. The results obtained showed that immobilized laccase had reaction stability without losing activity provided that the method is desirable for the reuse of laccase. GC-MS analysis demonstrating degradation was also performed.

REFERENCES

Aktaş, N., Çiçek, H., Ünal Taşpınar, A., Kibarer, G., Kolankaya, N. and Tanyolaç, A. (2001). Reaction kinetics for laccase-catalyzed polymerization of 1-naphthol. *Bioresource Technol* 80**,** 29–36.

Arcand, R.L. and Archibald, F.S. (1991). Direct dechlorination of chlorophenolic compounds by laccases from *Trametes (Coriolus) versicolor*. *Enzyme Microb. Technol* 13, 194–203.

Arora, D.S., Chander, M. and Gill, P.K. (2002). Involvement of lignin peroxidase,manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. *Int. Biodeter. Biodegr* 50, 115–120.

Barr, D.P. and. Aust, S.D. (1994). Enzyme degradation of lignin. *Rev. Environ. Contam. Toxicol* 138, 49–72.

Cabuk, A., Unal, A.T. and Kolankaya, N. (2006). Biodegradation of cyanide by a white rot fungus, Trametes versicolor. *Biotechnol Lett* 28, 1313–1317.

Choi, J., Kim, Y. and Choi, S.J. (2007). Reductive dechlorination and biodegradation of 2,4,6-trichlorophenol using sequential permeable reactive barriers: Laboratory studies, *Chemosphere* 67, 1551–1557.

Duran, N. and Esposito, E. (2000). Potantial applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment, *Review. Appl. Catal. B- Environ* 28, 83–99.

Ehlers, G.A. and Rose, P.D. (2005). Immobilized white-rot fungal biodegradation of phenol and chlorinated phenol in trickling packed-bed reactors by employing sequencing batch operation. *Bioresource Technol* 96, 1264–1275.

Hofrichter, M. (2002). Review: lignin conversion by manganese peroxidase (MnP). *Enzyme Microb. Tech* 30, 454–466.

Kirk, T.K. and Farrel, R.L. (1987). Enzymatic "Combustion": The microbial degradation

of lignin. *Annu. Rev. Microbiol* 41, 465–505.

Krcmar, P., Kubatova, A., Votruba, J., Erbanova, P. and Novotny, C. (1999). Degradation of polychlorinated biphenyls by extracellular enzymes of *Phanerochaete chrysosporium* produced in a perforated plate bioreactor. *World J. Microb. Biot* 15, 249–276.

Kumaran, P. and Paruchuri, Y.L. **(**1997). Kinetics of phenol biotransformation. *Water Res* 31,11–22.

Leontievsky, AA., Myasoedova, NM., Baskunov, BP., Golovleva, LA., Bucke, C. and Evans, C.S. (2001). Transformation of 2,4,6-trichlorophenol by free and immobilized fungal laccase, *Appl. Microbiol. Biotechnol* 57, 85–91.

Limura, Y., Hartikainen, P. and Tatsumi, K. (1996). Dechlorination of tetrachloroguaiacol by laccase of white rot basidomycete *Coriolus versicolor*. *Appl. Microbiol. Biot* 45, 434–439.

Lisov, A.V., Pozhidaeva, Z.A., Stepanova, E.V., Koroleva, O.V. and Leontievsky, AA. (2007), Conversion of Polychlorophenols by Laccases with 1-Hydroxybenzotriazole as a Mediator, *Appl. Biochem. Microbiol* 43, 616–619.

Majumder, P.S. and Gupta, S.K. (2007). Removal of chlorophenols in sequential anaerobic–aerobic reactors. *Bioresource Technol* 98,118–129.

Martinez-Ruiz, J., Parra, M., Tomás, V., Martinez-Gutiérrez, R., García-Canovas, F. and Tudela, J. (2009). Biodegradation of 2,4,6-trichlorophenol catalysed by the methylsyringate–laccase mediator system, New Biotechnol. 25, 160.

Michizoe, J., Goto, M. and Furusaki, S. (2001). Catalytic activity of laccase hosted in reversed micelles, J. Biosci. Bioeng 92, 67–71.

Novotny, C., Svobodova K., Erbanova, P., Cajthamla, T., Kasinatha, A., Lang, E. and Sasek, V. (2004). Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biol. Biochem* 36, 1545–1551.

Pallerla, S. and Chambers, R.P. (1998). Reactor development for biodegradation of pentachlorophenol. *Catal. Today* 40, 103–111.

Reddy, G.V.B., Sollewijn Gelpke, M.D. and Gold, M.H. (1998). Degradation of 2,4,6-Trichlorophenol by *Phanerochaete chrysosporium*: Involvement of Reductive Dechlorination. *J. Bacteriol.* 180, 5159–5164.

Rozie, H., Somers, W., Bonte, A., Visser, J., Van't Riet, K. and Rombouts, F.M. (1988). Adsorption Characteristics of Endo-polygalacturonase on Aljinate Beads. *Biotechnol. Appl. Bioc.* 10, 346–358.

Taspinar, A. and Kolankaya, N. (1998). Optimization of enzymatic chlorine removal from Kraft pulp. *B. Environ. Contam. Tox.* 61, 15–21.

Thomas, D.R., Carswell, K.S. and Georgiou, G. (1992). Mineralization of biphenyl and PCBs by white rot fungus *Phanerochaete chrysosporium*. *Biotechnol. Bioeng* 40, 1395–1402.

Tuomela, M., Lyytikainen, M., Oivanena, P. and Hatakka, A., (1999). Mineralization and conversion of pentachlorophenol (PCP) in soil inoculated with the white-rot fungus Trametes versicolor . *Soil Biol. Biochem* 31, 65–74.

Ünal, A. and Kolankaya, N. (2004). Chlorin removel from pp' DDT by laccase enzyme produced from *Trametes versicolor*. *Turkish Electronic J. Biotechnol* 2, 17–21.

Valentin, L., Feijooa, G., Moreirab, M.T. and Lema, J.M., (2006). Biodegradation of polycyclic aromatic hydrocarbons in forest and salt marsh soils by white-rot fungi. *Int. Biodeter. Biodegr* 58, 15–21.

Walter, M., Boyd-Wilsona, K.S.H., McNaughtonb, D. and Northcott, G. (2005). Laboratory trials on the bioremediation of aged pentachlorophenol residues. *Int. Biodeter. Biodegr 5*5, 121–130.

Yadav, S. and. Reddy, C.A. (1992). Noninvolvement of lignin peroxidases and manganese peroxidases in 2,4,5-trichlorophenoxyacetic acid degradation by *Phanerochaete chrysosporium*. *Biotechnol. Lett* 14, 1089–1092.

Yadav, J.S., Ouensen, J.F., Tiedje, J.F.Q.J.M. and Reddy, C.A. (1995). Degradation of Polychlorinated Biphenyl Mixtures (Aroclors 1242,1254, and 1260) by the White Rot Fungus *Phanerochaete chrysosporium* as Evidenced by Congener-Specific Analysis. *Appl. Environ. Microbiol* 2560–2565.

Yarapolov, A.I., Skorobogatko, O.V., Vartanov, S.S. and Varfolomeyev, S.D. (1994). Laccase properties, catalytic mechanism and applicability. *Appl. Biochem. Biotech* 49, 257–279.

Zouari, H., Labat, M. and Sayadi, S. (2002). Degradation of 4-chlorophenol by the white rot fungus *Phanerochaete chrysosporium* in free and immobilized cultures. *Bioresource Technol* 84,145–150.