Dehalogenation and detoxification of 2,4-dichlorophenol with induced laccase

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Abstract

2,4-dichlorophenol is a recalcitrant compound which is used in the production of pesticides. Chlorine removal of 2,4-dichlorophenol with the crude laccase produced *Trametes versicolor* ATCC (200801) in potato dextrose broth including wheat bran was investigated. The optimization parameters for dechlorination such as pH, initial substrate concentration, reaction period, reaction temperature and amount of enzyme were examined. At the end of these studies, pH 4, 400 µM of initial substrate concentration, 7 min of reaction time, 30 ºC of temperature were selected. It was tested that correlation with oxygen consumption and dechlorination processes under the determined optimum conditions before and the decrease of dissolved oxygen was observed. Also, after the dechlorination of this compound, changes in chemical structure of the compound were determined with FTIR analysis and toxicity alterations with Microtox test were studied. Wide and intense peaks at spectra may be an evidence to dechlorination occurring with load transfers and electrostatic interactions. Toxicity experiments showed that 2,4-DCP was detoxified in addition to dechlorination. Besides, statistical analyses were performed for dechlorination experiments.

Keywords: 2,4-DCP, laccase, dechlorination
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İndüklenmiş lakkaz ile 2,4-diklorofenolün dehalojenasyonu ve detoksifikasyonu

Özet

2,4-diklorofenol pestisitlerin üretiminde kullanılan rekalsitrant bir bileşiktir. Buğday kepeği ile indüklenmiş patates dekstroz broth besiyerinde üretilen *Trametes versicolor* ATCC (200801)'den elde edilen ham lakkaz ile 2,4-diklorofenolden klorun uzaklaştırılması değerlendirilmiştir. pH, substrat konsantrasyonu, reaksiyon süresi, sıcaklığı ve enzim miktarı gibi deklorinasyon için optimizasyon parametreleri çalışılmıştır. Bu çalışmaların sonunda pH 4, 400 µM başlangıç substrat konsantrasyonu, reaksiyon süresi 7 dakika, sıcaklık 30 ºC seçilmiştir. Daha once belirlenen optimum şartlarda oksijen tüketimi ile deklorinasyon süreci arasındaki ilişki incelenmiştir ve çözünmüş oksijende azalma gözlemlenmiştir. Bu bileşiğin deklorinasyonundan sonra kimyasal yapısındaki

değişiklikler FT-IR analizi ile, toksisitesindeki değişimler ise Microtox ile belirlenmiştir. Spektradaki yayvan ve şiddetli pikler; yük transferi ve elektrostatik etkileşimlerle meydana gelen deklorinasyona bir kanıt olarak gösterilebilir. Toksisite deneyleri, 2,4-DCP'nin deklorinasyona ilaveten detoksifiye olduğunu da göstermiştir. Bunun yanısıra deklorinasyon deneylerinde istatistik analizler gerçekleştirilmiştir.

Anahtar kelimeler: 2,4-DCP, deklorinasyon, lakkaz.

Introduction

Chlorophenolics, which are widely used in industries for the manufacture of dyes, wood preservatives, disinfectants, insecticides and chemical products are compounds of serious environmental concern owing to their hazardous effects for lives due to their high toxicity and carcinogenicity (Hoos 1978; Exon 1984; Haggblom 1992; Agostini et al. 2003; Ping et al. 2003). These compounds are persistently liable to degradation owing to their chlorine molecule. At this stage, biological processes have been advantageous compared with physiochemical approaches for the reason that they are relatively harmless, eco-friendly and cost-efficient (Gallizia et al. 2003). Some aerobic microorganisms can perform oxidative dechlorination, while some anaerobic organisms can perform reductive dechlorination (Annachhatre and Gheewala 1996; Vroumsia et al. 2005; Gallizia et al. 2003; Lin et al. 2008). However in the literature, enzymatic process was suggested for this procedure in order to shorten time (Tabak et al. 2009).

Laccase, having low substrate specificity, can degrade polyaromatic hydrocarbons (PAHs), textile dyes, lignite, chlorinated compounds and other xenobiotic compounds similar to lignin structure (Arısoy and Kolankaya 1997; Aytar et al. 2010; Mayer and Staples 2002). Industrial applications for laccases such as pulp and paper, textile, organic synthesis, environmental, food, pharmaceutical, and nano-biotechnology have been proposed (Osma et al. 2010).

2,4-dichlorophenol (2,4-DCP), utilized for the production of germicides, is recognized as a priority pollutant by the US Environmental Protection Agency (Vroumsia et al. 2005). Diverse bacteria such as *Micrococcus* sp., *Chrysosporium* sp., *Achromobacter* sp. (Xiangchun et al. 2004) and *Mucor* sp. (Gallizia et al. 2003; Vroumsa et al. 2005) and fungi could degrade 2,4-DCP.

The target of this experiment was to determine the abilities of dechlorination and investigate the changes of chemical structure of 2,4-DCP through FT-IR analysis and the alterations of toxicity of this compound by Microtox after treatment of laccase. The effects (pH, initial substrate concentration, enzyme amount, reaction time, and temperature) of various operating parameters on dechlorination were tested and each parameter studied was evaluated statistically.

Materials and Methods

Laccase production

Trametes versicolor ATCC200801 was grown on submerged cultures in potato dextrose broth (PDB) using wheat bran as inducer (Gedikli 2008). After 12 days of culture, supernatant was filtered and used as crude laccase source for all of this study.

0.1 mL culture supernatant was added to 4.9 mL sodium acetate buffer (50 mM, pH 4.5), containing 0.1 mM guaiacol as substrate and incubated at 37 °C for 15 minutes. One activity unit was defined as the amount of enzyme that oxidized and increase in A_{465} of 0.1 absorbance units per minute. Incubations with denatured laccase served as a control. Absorbance was measured with a UV-Vis spectrophotometer (Schimadzu 2450) (Coll et. al., 1993).

Optimization of studies of dechlorination

2,4-DCP had 98% purity and was *purchased* from Merck, Germany. Dechlorination experiments were performed in a 100 mL

volume of lab scale bioreactor designed by Ünal and Kolankaya (2004). The studied parameters were reaction time, pH, reaction temperature, initial substrate concentration, and enzyme amount

To pH range of 3.0-10.0 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. Initial substrate concentrations of 50-500 μM were tested to determine the initial chlorophenolic concentration. The experiment was carried out in 120 minutes to determine the reaction period. To determine the optimum temperature value, the experiment was performed at temperatures varying between 10 – 50±1 °C. Finally, the determination of enzyme concentration was made through addition of laccase (0.5-4 mL) with varying activity between 22.50 and 23.0 U mL^{-1}. Amounts of chlorine ions released were detected by chlorine ion electrode (Jenway 3205) while consumption of O_2 during the reactions was determined by O_2 electrode (Jenway DO_2 meter 9071). All the experiments were carried out at least in triplicate. Control experiments were also carried out containing all reactants and denatured enzyme instead of active enzyme.

Catalase experiment

To inspect activities of peroxidase group enzymes on dechlorination, catalase (Sigma) was added to culture supernatant.

Statistical and FT-IR analyses

One way analysis of variance (ANOVA) was used to investigate the significant differences between factors (pH, initial substrate concentration, enzyme amount, reaction time, and temperature) and the Tukey test was also used for multiple comparisons. $\alpha = 0.05$ was the selected significance level in all analysis. Analyses were performed in SPSS 13 software.

FTIR analyses were performed before and after dechlorination with enzyme. Liquid samples of chlorophenolic compound and potassium bromide were prepared 10 μl samples and KBr in liquid cell were carried out with Perkin Elmer 100 FT-IR spectrometer having 4000-400 cm^{-1} wavelength, one of scan number, and 4 cm^{-1} of resolution.

Results and Discussion

Dechlorination Experiments

Since 2,4-DCP is an important chlorophenolic compound, treatment of this chlorophenolic has increasingly received more attention. The structure of 2,4-DCP was shown in Fig. 1.

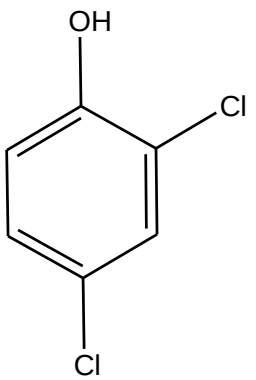

Figure 1. 2D structures of 2,4-DCP

It is known that, *T. versicolor* is a good laccase source (Arcand and Archibald, 1991; Limura et al., 1996; Taspinar and Kolankaya, 1998; Novotny, 2004). Hence, culture supernatant of *T. versicolor* was used as a crude laccase source in dechlorination of 2,4,-DCP studies. The dechlorination parameters such as pH, initial substrate concentration, enzyme amount, reaction period, and reaction temperature were tested.

As shown in Fig. 2; pH 4 was selected. Following the results of Tukey Multiple Comparison tests, an unreasonable dissimilarity was found according to the difference of the studied pH values. We have obtained higher yield for 2,4-DCP as 85.72 when dechlorination tests were performed in pH 4. This value appears to be close to the pH of laccases of other fungi, which are usually between pH 3.5

and 4.5 (Bollag and Leonowicz 1984). The free chlorine measurement results obtained above of the values of pH 8 lead to the consideration that the increase may depend on chemical oxidation rather than a biological process.

In the case of initial substrate concentration experiments at dechlorination optimization, 400 μM was determined as the optimum value as far as chlorine analyses and statistical analyses were concerned. Also, a free chlorine amount was found 144.28 μM (0.01<0.05) for 2,4-DCP. According to Tukey Multiple Comparison tests, the dechlorination process is significantly different as it relates to the initial substrate concentration parameter. Matafonova et al. (2006) isolated 2,4-DCP degrading bacterium and found that 2,4-DCP degradation rates by *B. cereus* GN1 could be determined at concentrations up to 400 μM. However, higher concentrations of 2,4-DCP (560 μM) were inhibitory to cell growth.

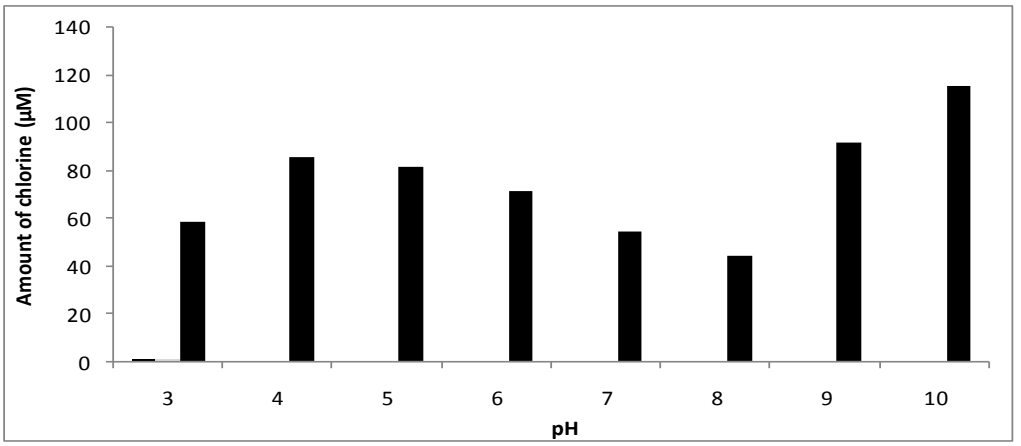

Figure 2. Effect of pH on enzymatic dechlorination of 2,4-DCP (Initial substrate concentration: 150 μM; enzyme amount:1 mL; reaction time: 30 min, reaction temperature: 30 °C)

According to Fig. 3, the rate of dechlorination was enhanced through an increase in the enzyme amount from 1 mL to 4 mL. The result of statistical analyses with ANOVA, p value for enzyme amount were found reasonable because of being p<0.05.

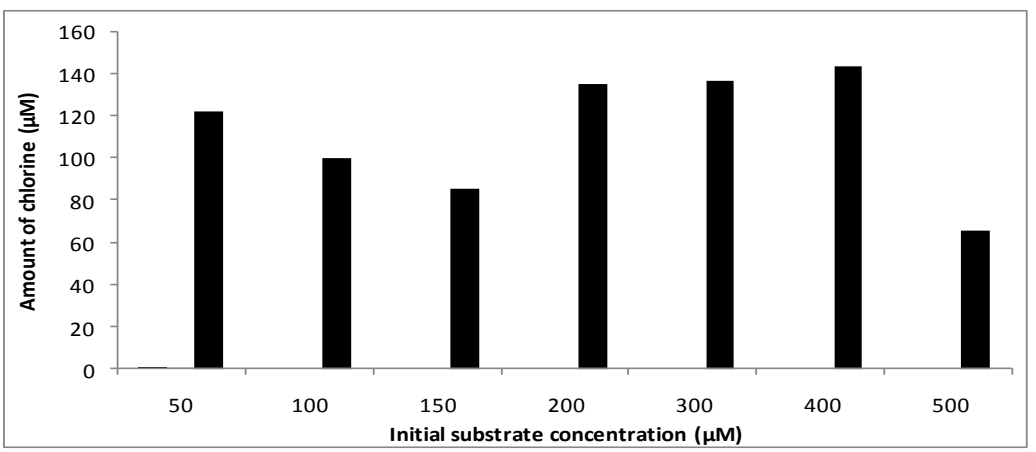

Figure 3. Effect of initial substrate concentration on enzymatic dechlorination of 2,4-DCP (Enzyme amount: 1 mL; pH 4; reaction time: 30 min, reaction temperature: 30 °C).

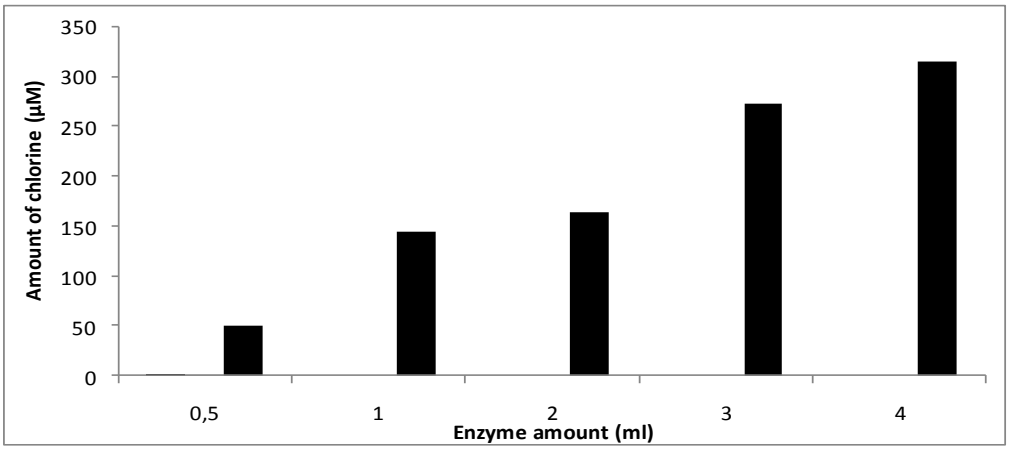

Figure 4. Effect of enzyme amount on enzymatic dechlorination of 2,4-DCP (pH 4; initial substrate concentration: 400 µM, reaction time: 30 min, reaction temperature: 30 °C)

332.86 µM of free chlorine amount ($p<0.05$) at 7 min was found for this experiment duration for reaction time experiments (Fig. 5) and this period was selected due to being a shorter time. We concluded that removal of chlorine in different reaction times was significantly different. Zhang et al. (2008) reported 94% removal efficiency of 2,4-DCP within 10 h at pH 5.5 while exploring the idea of a promotion in the rate of degradation with the increase in laccase concentration or an increase in temperature. Bhattacharya and Banerjee (2008) showed a maximum degradation efficiency of ~98% at pH 6, temperature 40 ºC, time 9 h and an enzyme concentration of 8 IU mL^{-1}. In our study however, dechlorination was performed within a short period. According to the result of a 2,4,6-TCP dechlorination study to determine the effect of contact time, it was observed that there was nearly a linear increase in dechlorination up to 30 min of the incubation period (Ünal, 2004) and then, the amount of dechlorination did not significantly change with contact time.

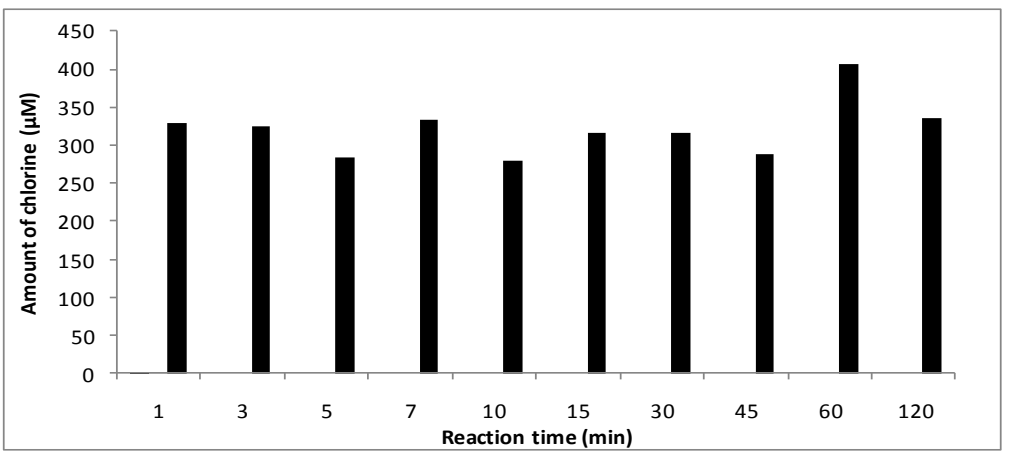

Figure 5. Effect of reaction time on enzymatic dechlorination of 2,4-DCP (pH 4 for; initial substrate concentration: 400 µM, enzyme amount: 4 mL; reaction temperature: 30 °C)

In the temperature related experiments another important factor influencing dechlorination procedure was observed and 30 ºC was determined as the optimum value (Fig.

6). Following the results of statistical analyses with ANOVA, p value for temperature was found unreasonable because of being p>0.05. According to this, chlorine removal at different temperatures is in the same homogenous group. Similarly, it was found that the maximum rate of degradation was between 36 and 40 ºC in the study of Bhattacharya et al. (2009). At higher temperatures, there was a sharp decline in the activity. In our study, dechlorination efficiency enhanced while temperature increased at moderate conditions. Chlorine removal at 30 °C may be a significant point for a practical application of in situ bioremediation for chlorophenolic compound in the environment.

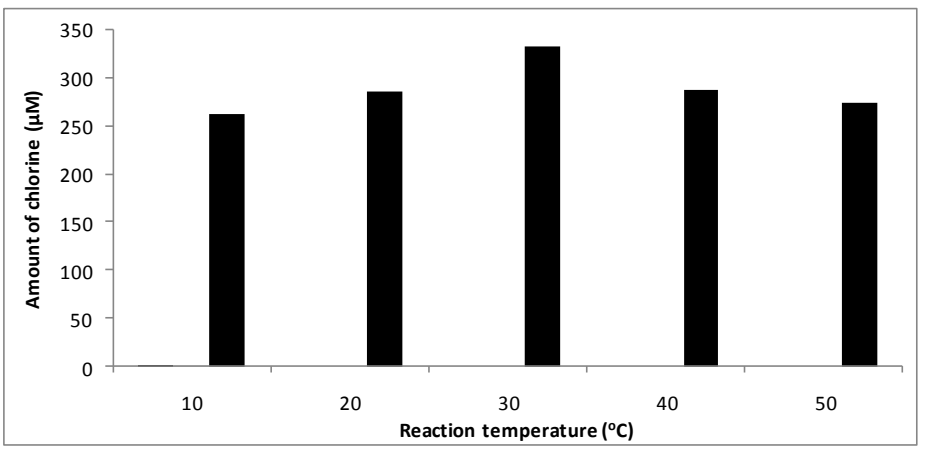

Figure 6. Effects of reaction temperature on enzymatic dechlorination of 2,4-DCP (pH 4; initial substrate concentration: 400 µM, enzyme amount: 4 mL; reaction time: 7 min)

According to the literature, laccase use oxygen as an electron acceptor during the laccase dependent oxidation reactions, therefore the oxygen amount in the environment decreases alongside this enzyme reaction (Kirk and Farrel, 1987; Yarapalov et al.; 1994). For 2,4-DCP, the decrease of dissolved oxygen was observed under the determined optimization conditions (Fig. 7).

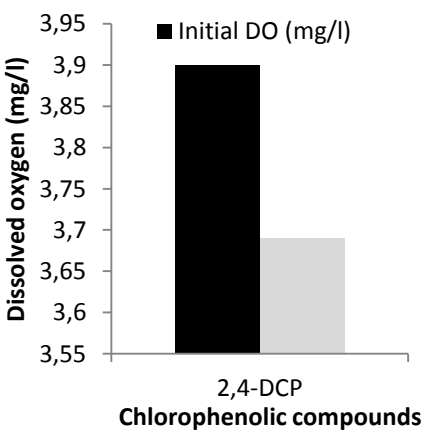

Figure 7. Change of oxygen concentration of laccase during chlorine removal from 2,4-DCP under optimized conditions for each compound.

Culture supernatant with high laccase activity as the enzyme source was treated with catalase. Thereby supporting the hypothesis that laccase was the enzyme responsible for dechlorination.

FT-IR analyses of chlorophenolic compounds before and after enzymatic treatment

FT-IR spectra of 2,4-DCP before and after enzymatic dechlorination are shown in Fig. 8. Intense and broad peak at 3351 cm^{-1} at ethanol spectrum of 2,4-DCP molecule belongs to O-H stretching vibration. C-H stretching vibrations at 2975, 2928 and 2887 cm^{-1} were observed. C=C stretching vibration was observed at 1425 cm^{-1} and C-C stretching mode at 1421, 1381 and 1331 cm^{-1}. Besides, C-O stretching vibration at 1275 cm^{-1}, in plane C-H bending vibrations at 1090 and 1050 cm^{-1}, and extra plane C-H bending vibrations at 804 and 662 cm^{-1} were observed.

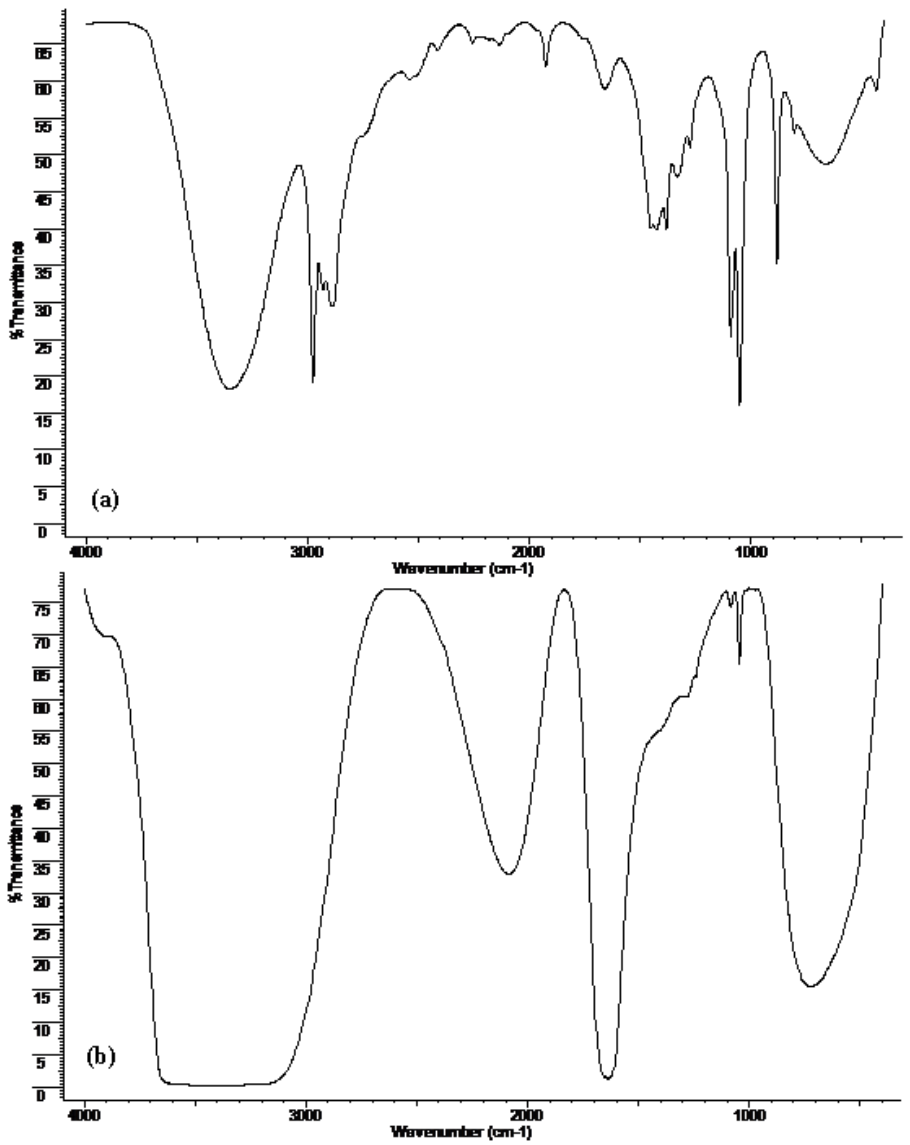

Figure 8. FTIR spectra of 2,4-DCP (a) Spectrum of 2,4-DCP in ethanol solvent (b) Spectrum of 2,4-DCP after dechlorination with laccase

Wide and intense peaks may be an evidence of the dechlorination reaction occurring with load transfers and electrostatic interactions (Fig. 8). On account of the electronegative property of the chlorine atom, degradations in molecule are expected to occur on this atom. As a result of changes of peak might be a sign of cleavage of chlorine atom and the rest molecule was less persistent compound. Moreover, the electronegativity of the oxygen atom in the OH group will cause previously dechlorination of chlorine atom being the closest in position to this group. The decreasing intensity of the C–Cl stretching vibration after treatment with laccase may be also evidence of dechlorination. Parallel results were observed in the study of Yoshioka et al. (2008) and Kameda et al. (2010).

Toxicity changes of chlorophenolic compounds with enzymatic treatment

Acute toxicity experiments with *V. fischeri* showed that the bacterial EC_{50} for the 2,4-DCP was surprisingly changed and detoxification was simultaneously carried out with dechlorination. While EC_{50} concentrations for 5–min before and after enzymatic treatment were 28.0% and 66.0%, respectively (Fig. 9a, b).

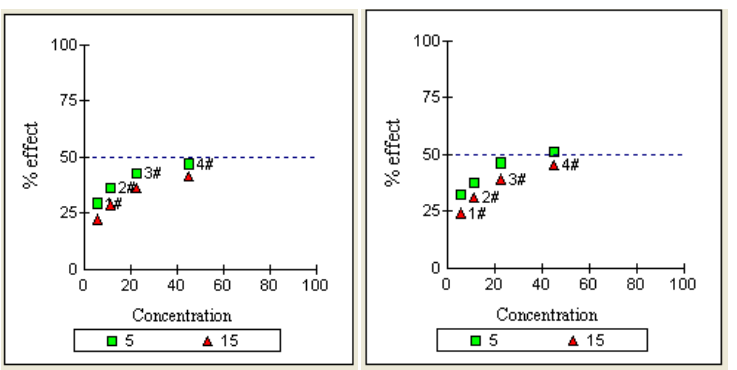

Figure 9. Toxicity values of chlorophenolic compounds before and after enzymatic treatment under optimized conditions for each compound. (a) and (b) indicate toxicities of 2,4-DCP before and after enzymatic treatment.

In our study, this enzymatic procedure suggested that 2,4–DCP dechlorination by *T.versicolor* laccase was especially an eco-friendly approach, there was a contribution to the reduction of total toxicity. Gan (2002) dechlorinated chloroacetanilide herbicides including alachlor, acetachlor, metolachlor, and propachlor by thiosulphate salts. Dissipation of 200 µM of alachlor was performed in 100 h through sodium thiosulphate and affirmative toxicological alterations were observed. In related literature, you will find studies that reduce the toxicity of chlorophenols by using white rot fungi or the related enzyme (Reddy et al. 1998; Taspinar and Kolankaya 1998; Ehlers and Rose 2005).

Induced crude laccase from a locally isolated *T. versicolor* ATCC 200801 was used for the dechlorination of 2,4-DCP. Enzymatic treatment of 2,4-DCP may be used for both dechlorination and detoxification.

Acknowledgements

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