

Enhancement with inducers of lacasse production by some strains and application of enzyme to dechlorination of 2,4,5-trichlorophenol

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Abstract Lacasse is one of the extracellular enzymes excreted from white and brown rot fungi, which is involved in ligninolysis. In the present study, the effects of the addition of lacasse inducers to the medium on enhancement of enzyme production under conditions of submerged fermentation were researched. At first, a culture medium was selected suitable for lacasse production. To increase the production of lacasse using different inducers and to examine the ability of dechlorination, this article focuses on screen lacasse activity of 21 basidiomycetes isolates grown in five culture media. All inducers evaluated influenced lacasse activity positively except for gallic acid, mannitol, and malt extract for studied isolates. Our findings showed that lacasse activity of *Trametes versicolor* ATCC (200801) when it was induced with wheat bran reached up to 29.08 U ml⁻¹ and was examined the ability of dechlorination of 2,4,5-trichlorophenol (2,4,5-TCP). The parameters including pH, initial substrate concentration, amount of enzyme, period of reaction, and temperature were tested for dechlorination process. Correlation between oxygen consumption and dechlorination processes under the determined optimum conditions was analyzed. Toxicity of 2,4,5-TCP before and after enzymatic treatment was evaluated by Microtox test. The results demonstrated that toxicity of intermediates formed 2,4,5-TCP did not change.

Keywords: 2,4,5-trichlorophenol, basidiomycetes, dechlorination, inducer, lacasse

INTRODUCTION

Lacasse (benzenediol: oxygen oxidoreductase; E.C. 1.10.30.2) is a multi-copper containing protein using molecular oxygen to oxidize various phenolic and non-phenolic compounds by a radical-catalyzed reaction mechanism (Thurston, 1994).

Lacasse is involved in lignin degradation and its low substrate specificity allows it to oxidize a wide range of compounds (Saparrat et al. 2002). Compounds involved in the natural degradation of lignin by white-rot fungi may be derived from oxidized lignin units or directly from fungal metabolism (Johannes and Majcherczyk, 2000). Besides, most of phenolic compounds are oxidized by lacasse, the mediators can diffuse far away from the mycelium to sites that are difficult to reach by the enzyme itself; therefore, these nonphenolic compounds have been oxidized through lacasse mediated system (Camarero et al. 2005; D'Acunzo et al. 2006).

This enzyme has been distributed among plants, insects, bacteria and fungi (Gianfreda et al. 1999). However, lacasse activity has mainly been demonstrated in basidiomycetes, ascomycetes and deuteromycetes (Sadhasivam et al. 2008).

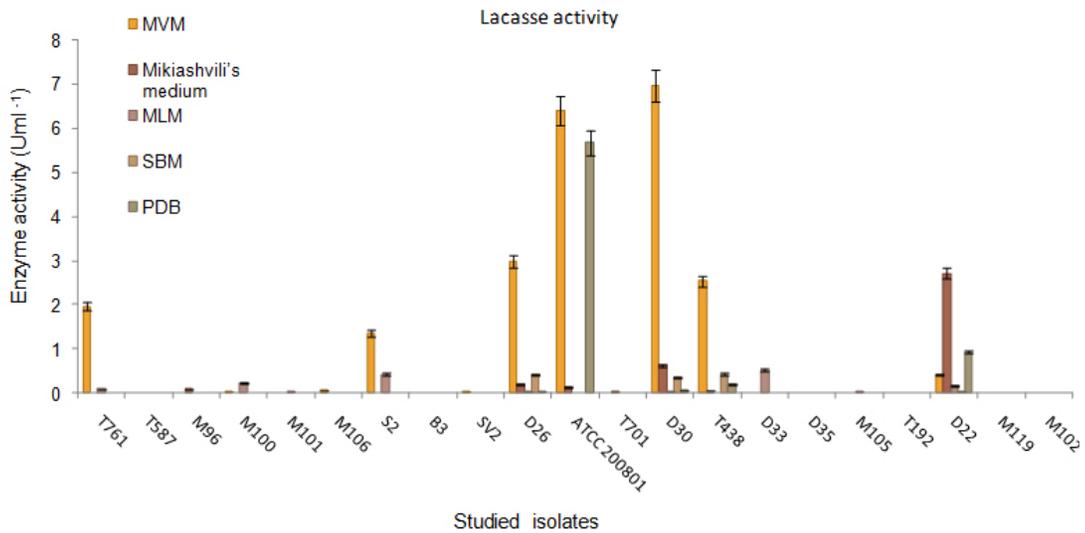


Fig. 1 Laccase activities of isolates in different five media (see Materials and Methods for species names of fungal train).

White rot fungi belong to basidiomycetes are known to produce extracellular enzymes which are laccase, manganese peroxidase, lignin peroxidase oxidizing phenolic and non-phenolic substrates. These fungi were grown on dead and/or live trees by degrading compounds such as cellulose and lignin. Enzymes excreted white rot fungi have recently taken attention (Ko et al. 2001).

Laccase activity depends on the type of fungal strain used, growth conditions employed, the presence and the absence of inducers, incubation time, nature and composition of culture medium and genetic manipulation. Among these factors, corresponding to the presence of an inducer, its chemical structure, the amount added and the time of its addition influence laccase production. Several compounds like phenolics strictly related to lignin or its derivatives induce this enzyme activity (Gianfreda et al. 1999). Generally, xyloidine, copper sulphate, wheat bran, veratryl alcohol, ethanol, solid lignin were used for inducing laccase activity (Rodriguez Couto et al. 2002; Papinutti et al. 2003; Cavallazi et al. 2005; Birhanli and Yesilada, 2006; Xavier et al. 2007). Besides, Flores and co-workers (Flores et al. 2010) used biological inducer and studied production of laccases by *Pleurotus ostreatus* in submerged fermentation in co-culture with *Trichoderma viride*.

Laccase has attracted much interest in recent years owing to its degradation ability to lignin related compounds as well as highly recalcitrant pollutants which makes it suitable for biotechnological and environmental applications. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, being used as biosensor, a tool for medical diagnostics, and bioremediation agent to clean up herbicides, pesticides and certain explosives in soil and water (Rodriguez Couto and Toca, 2006). Laccases are also used as catalysts for the manufacture of anti-cancer drugs (Unyayar et al. 2006).

White rot fungi degrade a wide variety of environmental pollutants such as chlorophenolic compounds, polycyclic aromatic hydrocarbons (PAHs), cyanide as well as desulphurizing coal (Sedarati et al. 2003; Baborová et al. 2006; Çabuk et al. 2006; Aytar et al. 2008).

Chlorinated phenols, recalcitrant compounds, are discharged from many chemical industries (Rubilar et al. 2008), especially pentachlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol, lindane, aldrin, 2,4-dichlorophenol, dichlorodiphenyltrichloroethane (DDT) have a widespread environmental concern because of toxicity and persistence (Dercova et al. 2003; Furukawa, 2003; Sedarati et al. 2003; Kargi and Eker, 2004).

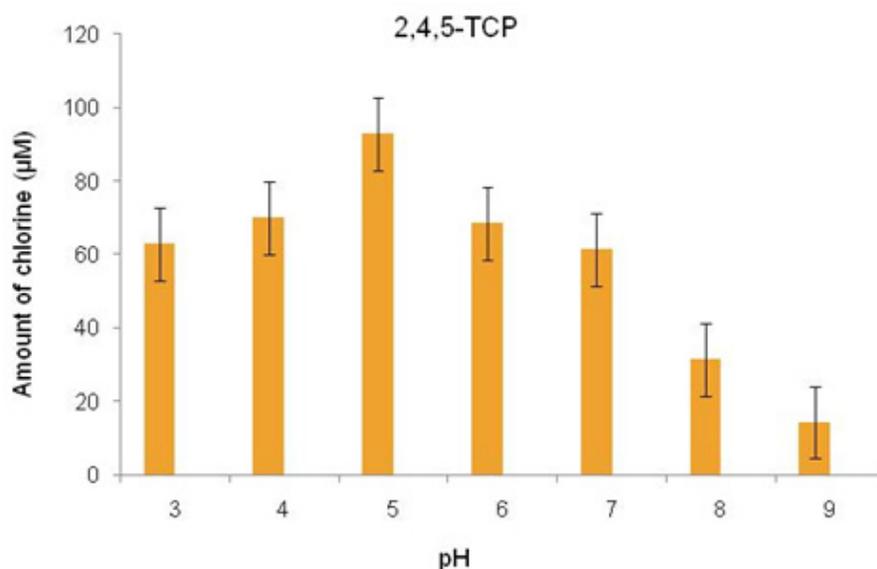


Fig. 2 Effect of pH on enzymatic dechlorination for 2,4,5-TCP (Substrate concentration: 150 µM; enzyme amount: 1 ml; incubation time: 30 min; temperature 30°C).

2,4,5-trichlorophenol (2,4,5-TCP) has been an intermediate in the manufacture of industrial and agricultural chemicals, such as the phenoxy acid herbicides 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), [2-(2,4,5-trichlorophenoxy) propionic acid, 2,4,5-TP] (Silvex), *o,o*-dimethyl-*o*-2,4,5-trichlorophenyl phosphorothionate (Ronnell), and sodium 2,4,5-trichlorophenolate. Additionally, 2,4,5-TCP and its salts have been used to preserve to microorganisms for adhesive products, synthetic textiles, rubber, wood, paints, in the leather industry and in pulp and paper mills (Nichkova et al. 2002). 2,4,5-TCP is considered to have significant toxicological effects and potential carcinogenicity (IARC, 1986).

Toxicity, persistence and biodegradability of chlorophenols depend on the number and position of chlorine substituents on the phenolic ring. Chlorinated aromatics are known to be more recalcitrant than chlorinated aliphatics and biodegradability decreases with increasing number of chlorine groups (Kolankaya, 2006).

Biological treatment of chlorophenolics has been studied with different fungi including *Phanerochaete chrysosporium*, *Candida maltosa*, *Pleurotus ostreatus*, *Lentinus edodes*, *Trametes versicolor*, *Pycnoporus cinnabarinus*, *Pleurotus sajor-caju* (Cameron et al. 2000; Schultz et al. 2001; Rodriguez et al. 2004; Ünal and Kolankaya, 2004).

The aim of this study was to investigate the abilities of laccase production of new white and brown rot fungi which had isolated from Eskişehir province. In this way, the production of laccase of 21 microorganisms in five different media for up to 15 days of incubation period was explored. Then, the laccase production of 5 microorganisms was evaluated in the presence of different inducers such as CuSO₄, gallic acid, ammonium tartrate, malt extract, barley bran, wheat bran, Tween 80, veratryl alcohol, mannitol, cone biomass of *Pinus nigra* by adding to potato dextrose broth (PDB) as the selected medium. Dechlorination of 2,4,5-TCP was done with the highest activity of laccase obtained. The toxicity of this treated chlorophenolic compound was examined by means of the Microtox toxicity test.

MATERIALS AND METHODS

Microorganisms and chemicals

Formerly isolated from province of Eskişehir, Turkey and identified macrofungi *Polyporus arcularius* (T438), *Schizophyllum commune* (T701), *Clavariadelphus truncatus* (T192), *Pleurotus eryngii* (M102),

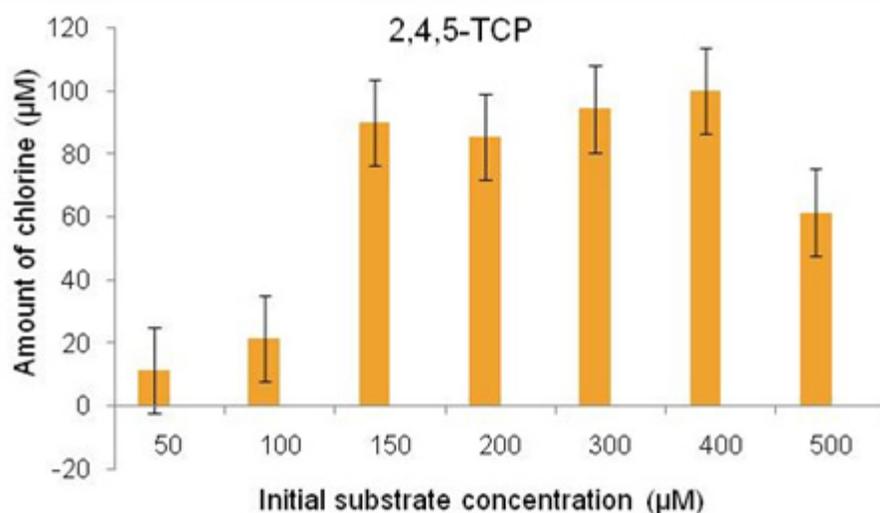


Fig. 3 Effect of initial substrate concentration on enzymatic dechlorination for 2,4,5-TCP (Enzyme amount: 1 ml; incubation time: 30 min; temperature: 30°C; pH: 5).

Ganoderma applanatum (M105), *Trametes versicolor* (D22), *Cerrena unicolor* (D30), *Schizophyllum commune* (D35), *Ganoderma lucidum* (D33), *Pleurotus ostreatus* (M119), *Trametes hirsuta* (T587), *Polyporus arcularius* (T761), *Trametes versicolor* (M96), *Fomes fomentarius* (M100), *Antrrodia serialis* (M101), *Inonotus hispidus* (M106), *Lenzites betulina* (S2), *Stereum hirsutum* (B3), *Tapinella panuoides* (SV2), *Lentinus strigosus* (D26) were maintained in potato dextrose agar (PDA) at +4°C in Fungal Culture Collection of Biology Department, Eskişehir Osmangazi University. *Trametes versicolor* ATCC (200801) was provided from ATCC.

Culture conditions and screening of lacasse activities

Lacasse production abilities of basidiomycetes isolates were tested during the growth under agitated culture conditions. After incubation at 30°C on PDB (Acumedia) for 4 days, obtained fungal pellets were homogenized and used as inoculum. Four ml of homogenate was transferred into 100 ml liquid medium in a 250 ml flask for agitated culture conditions (150 rev min⁻¹). To determine the best lacasse production medium, five media were evaluated including stock basal medium (SBM) (Forney and Reddy, 1979), modified Vogel medium (MVM) (Aktas et al. 2001), potato dextrose broth (PDB), mycological liquid medium (MLM) (Paice et al. 1989) and medium proposed by Mikiashvili et al. (2006). All cultures were incubated at 30°C for 15 days and lacasse activities were measured on 5, 10, and 15 days of incubation period.

To determine the lacasse activities of the tested isolates, 0.1 ml of culture supernatant was added to 4.9 ml sodium acetate buffer (50 mM, pH 4.5), which contains 1 mM guaiacol as substrate and incubated at 37°C for 15 min. 1 U of enzyme activity was defined as the amount of enzyme that elicited an increase in A_{465} of 1 absorbance unit per min. Absorbance was measured with a UV-Visible spectrophotometer (Schimadzu 2550 UV-Visible), (Ünal and Kolankaya, 2001).

Addition of lacasse inducers in growth medium

Effects of some inducers on lacasse activities of selected five isolates: *L. strigosus*, *C. unicolor*, *T. versicolor* (D22), *T. versicolor* ATCC (200801) and *S. commune* (D35) were investigated. *T. versicolor* ATCC (200801), known to produce lacasse (Birhanli and Yesilada, 2006) was used to enhance the activity changing of medium and adding inducers. At this stage of experiment, *S. commune* was selected as negative control. In this way, *S. commune* normally not a lacasse producer, was investigated to determine whether it acquired the ability of lacasse production with inducers. Then, CuSO₄ (1 mM), gallic acid (1 mM), ammonium tartrate (55 µM), malt extract (5 g l⁻¹), mannitol (1 g l⁻¹),

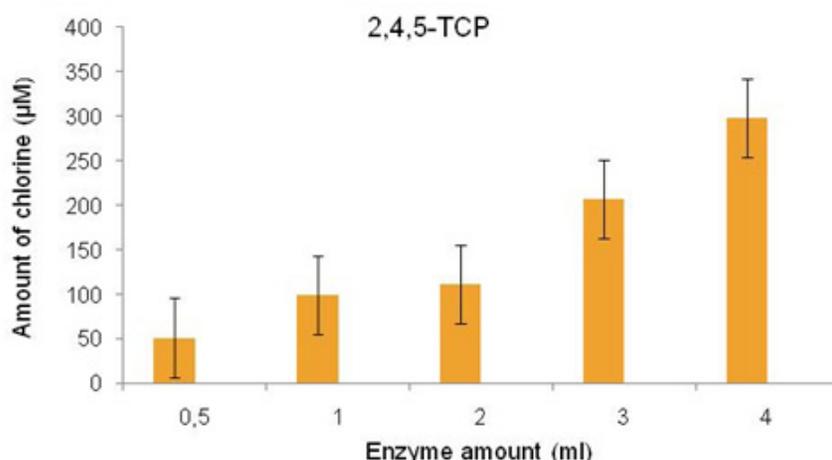


Fig. 4 Effect of enzyme amount on enzymatic dechlorination for 2,4,5-TCP (Substrate concentration: 400 µM; reaction time: 30 min; temperature: 30°C; pH: 5).

Tween 80 (0.005%), veratryl alcohol (0.2 mM), *Pinus nigra* cone biomass (2.5 g 100 ml⁻¹), barley bran (2.5 g 100 ml⁻¹), and wheat bran (3 g 100 ml⁻¹) were used as inducers.

CuSO₄, gallic acid, ammonium tartrate, malt extract, mannitol, Tween 80, veratryl alcohol as laccase inducers, also 2,4,5-TCP were purchased from Merck and barley bran, wheat bran were supplied from local market.

Conditions of experiments were stable at 30°C, 150 rev min⁻¹ and laccase activities were measured at 5, 8, 12 and 15 days of incubation period. To understand the effects of inducers, control cultures without inducers were run under similar conditions. Three parallel experimental media were prepared for each experiment.

Optimization of studies of dechlorination

The bioreactor used for enzymatic dechlorination studies, consisted of reactor vessel, stirred-heating system and waterbath with temperature control. On this study, the bioreactor designed by Ünal and Kolankaya (2004) was used.

Incubation time (1-120 min), pH (3-9), initial substrate concentration (50-500 µM), temperature (10-50°C) enzyme amount (0.5-4 ml) were the parameters tested for this purpose in the bioreactor with constant working volume at 100 ml.

At pH optimization, for pH 3-5, sodium acetate-acetic acid buffer; for pH 6-8, Na₂HPO₄-NaH₂PO₄ buffer; for pH 9-10, Tris-HCl buffer were used.

Reaction volume, amount of chlorophenolic compound, medium temperature were kept constant as 100 ml, 1 ml and 30 ± 1°C, respectively. While enzyme activity was 29.08 U ml⁻¹ for dechlorination, 1% ethanol was added to the bioreactor medium for dissolving 2,4,5-TCP. Control experiments were also performed containing all reactants but no active enzyme. All the experiments were carried out in triplicate.

Measurement of free chlorine and dissolved oxygen

The variations in the amount of dissolved oxygen were detected by oxygen electrode (WTW). Free chloride ions produced upon oxidation of the chlorinated substrates were analyzed spectrophotometrically by the mercury thiocyanate method (Greenberg et al. 1992).

Table 1. Effects of different inducers on lacasse activity in liquid cultures and PDB medium at shaking conditions.

	<i>Schizophyllum commune</i> D35				<i>Trametes versicolor</i> ATCC200801				<i>Trametes versicolor</i> D22				<i>Cerrena unicolor</i> D30				<i>Lentinus strigosus</i> D26			
Days inducers	5	8	12	15	5	8	12	15	5	8	12	15	5	8	12	15	5	8	12	15
Control (U ml ⁻¹)	0	0	0.05	0.08	2.87	4.27	4.32	5.67	0	0.01	0.02	0.03	0	0	0.35	0.08	0	0	0	0
CuSO ₄ (U ml ⁻¹)	0	0.08	0.05	0.08	17.79	23.68	16.4	13.76	6.08	22.06	18.03	20.06	4.71	12.93	24.06	16.1	0	0	0	0
Gallic acid (U ml ⁻¹)	0	0	0	0	2.85	3.77	3.97	5.53	0	0	0	0.16	0	0	0	0	0	0	0	0.16
Ammonium Tartrate (U ml ⁻¹)	0	0	0	0	2.86	3.99	5.35	7.3	0	0	0	0.24	0	0	0	0	0	0	0	0
Malt extract (U ml ⁻¹)	0	0	0	0	3.06	3.76	4.91	4.50	0.02	0	0	0.17	0	0	0	0	0	0	0	0
Barley bran (U ml ⁻¹)	0	0.03	0	0	17.93	18.15	15.54	8.44	15.06	12.8	20.33	7.86	4.05	3.9	1.76	0.69	0	0	2.19	0.69
Wheat bran (U ml ⁻¹)	0	0.10	0	0	12.83	25.78	29.0	22.00	18.76	17.46	18	10.24	17.2	19.28	16.22	6.44	0	2.3	7.84	4.66
Tween 80 (U ml ⁻¹)	0	0	0	0	2.99	3.73	2.5	4.10	0.04	0.03	1.82	0.14	0	0	0	0	0	0	0	0
Veratryl alcohol (U ml ⁻¹)	0	0	0	0	14.17	21.35	13.65	10.49	5.90	4.27	2.42	11.48	2.8	4.49	2.62	1.23	0	0	0	0
Mannitol (U ml ⁻¹)	0	0	0	0	2.90	3.67	4.87	4.95	0	0	0	0.08	0	0	0	0	0	0	0	0
Cone biomass of <i>Pinus nigra</i> (U ml ⁻¹)	0	0	0	17.5	3.19	5.41	8.91	18.59	9.42	7.56	6.04	15.7	0	0	0	17.5	0	0	0.05	0

Toxicity measurements

Acute toxicity was investigated by determining the luminescence inhibition of the *Vibrio fischeri* NRRL number B-11177, formerly known as *Photobacterium phosphoreum*. Sodium thiosulphate (1%) was used to retain chlorine. A vial of commercial toxicity testing bacterium was purchased in freeze-dried form from Strategic Diagnostics Inc. (SDI, Newark, DE, USA) and activated by reconstitution solution (Azur). The light emission of this bacterium in contact with different samples such as the chlorinated solution including denatured enzyme as the control group and the dechlorinated solution including active enzyme as the experiment group and 5, and 15 min of exposure times was measured using the Microtox® 500 analyzer according to Basic Test Protocol. The luminescence was recorded at 490 nm. The data were processed using the Microtox Omni Software. The concentration of the sample producing 50% decrease in light after exposure for 15 min was defined as the effective concentration (EC₅₀).

RESULTS AND DISCUSSION

Selection of potent laccase producer

Twenty isolates and *T. versicolor* ATCC (200801) were investigated in the five different media in respect of abilities of laccase production. The most effective microorganisms and media were selected at screening stage. In the result of screening study, isolates of *S. commune* (D35) as negative control, *C. unicolor* (D30), *T. versicolor* (D22) and *L. strigosus* (D26) and *T. versicolor* ATCC (200801) and medium of PDB were chosen for determining effects of inducers on activity. The values of enzyme activities were shown in Figure 1.

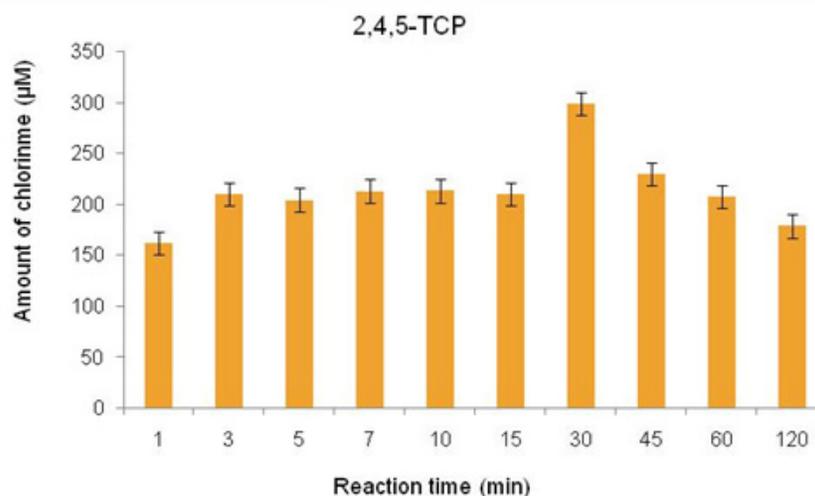


Fig. 5 Effect of reaction time on enzymatic dechlorination for 2,4,5-TCP (Substrate concentration: 400 µM; enzyme amount: 4 ml; temperature: 30°C; pH: 5).

Effects of laccase inducers

Various inducers were tested to increase activities by laccase of *S. commune* (D35), *C. unicolor* (D30), *T. versicolor* (D22), *L. strigosus* (D26) and *T. versicolor* ATCC (200801). As it can be deduced from Table 1, the best results were observed in *T. versicolor* (D22) grown on medium with CuSO₄ (22.06 U ml⁻¹), barley bran (20.33 U ml⁻¹) and wheat bran (18.76 U ml⁻¹), in *C. unicolor* (D30) grown on medium with CuSO₄ (24.06 U ml⁻¹), wheat bran (19.28 U ml⁻¹) and *P. nigra* cone biomass (17.50 U ml⁻¹) in *T. versicolor* ATCC (200801) was grown on CuSO₄ (23.68 U ml⁻¹), barley bran (18.15 U ml⁻¹), wheat bran

(25.78 U ml⁻¹), veratryl alcohol (21.35 U ml⁻¹), *P. nigra* cone biomass (18.59 U ml⁻¹) according to Table 1.

Dechlorination of 2,4,5-TCP

During the experiments related to dechlorination, pH, initial substrate concentration, enzyme amount, reaction time, and temperature were selected as 5, 400 µM, 4 ml, 30 min, 50°C, respectively (Figure 2, Figure 3, Figure 4, Figure 5 and Figure 6). Furthermore dissolved oxygen reduced from 3.57 mg l⁻¹ to 3.21 mg l⁻¹ under the determined optimization conditions.

Evaluation of toxicity values

Bioassays for acute toxicity to *V. fischeri* showed that the bacterial EC₅₀ for the 2,4,5-TCP was not significantly changed. While EC₅₀ concentrations for 15 min before and after enzymatic treatment were 61.0% and 62.0% respectively. Toxicity values of 2,4,5-TCP for 5 and 15 min before and after enzymatic treatment, was given as % effect, as shown in Figure 7 and Figure 8.

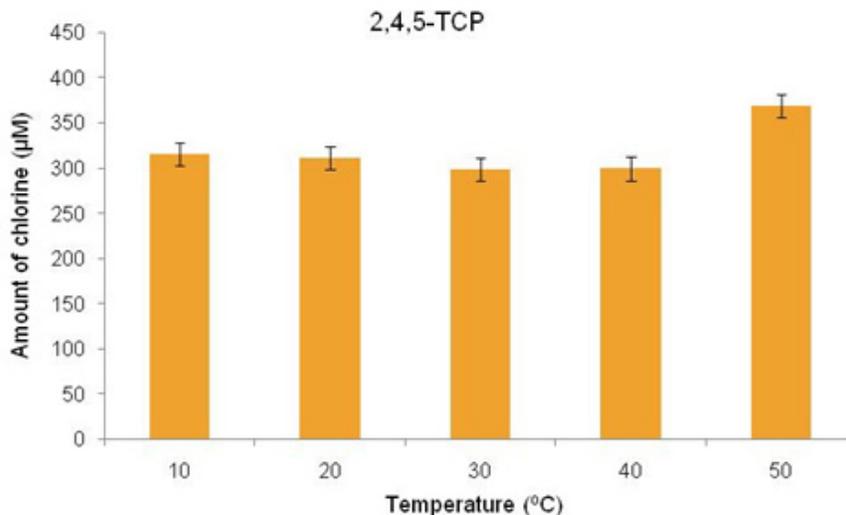


Fig. 6 Effect of temperature on enzymatic dechlorination for 2,4,5-TCP (Substrate concentration: 400 µM; enzyme amount: 4 ml; reaction time: 30 min; pH: 5).

DISCUSSION

Lacasse, one of the enzymes of phenol oxidase group, has high potential for industrial biotechnology. Many studies have been devoted to identify the most efficient lacasse-producing source; to select the most suitable culture medium; to developing appropriate, reproducible, and inexpensive isolation procedures; and mainly to optimize the enzyme production (Gianfreda et al. 1999; Arora and Gill, 2001; Mikiashvili et al. 2006). Also, there are many studies engaged with the type, the time of addition inducers and the concentration of the inducers to enhance the lacasse production. Most of the used inducers are phenolic or aromatic compounds related to lignin or lignin derivatives (Klonowska et al. 2001; Myasoedova et al. 2008).

In this study, 20 basidiomycetes isolates such as *P. arcularius* (T438), *S. commune* (T701), *C. truncatus* (T192), *P. eryngii* (M102), *G. applanatum* (M105), *T. versicolor* (D22), *C. unicolor* (D30), *S. commune* (D35), *G. lucidum* (D33), *P. ostreatus* (M119), *T. hirsuta* (T587), *P. arcularius* (T761), *T. versicolor* (M96), *F. fomentarius* (M100), *A. serialis* (M101), *I. hispidus* (M106), *L. betulina* (S2), *S. hirsutum* (B3), *T. panuoides* (SV2), *L. strigosus* (D26) and *T. versicolor* ATCC (200801) were examined to investigate their effects on the lacasse production under a variety of culture conditions. Types and

amounts of carbon and nitrogen content are fundamental to laccase formation. Among of the five media, PDB and MVM showed maximum laccase activity (Figure 1). MVM composed of many components and it was a costly medium. That is why further experiments were continued to use PDB though MVM was an effective medium. Besides, in comparison to MVM, PDB is a basic medium; thereby, investigating effects of inducers would be easier.

After screening the study of media and isolates, different inducers were added to PDB for understanding the effects of laccase production of the five examined fungi (Table 1). Gallic acid, malt extract and mannitol were shown that they did not affect laccase activity positively compared to control groups for all isolates studied.

The experimental results from the screening studies show that limited production of laccase enzyme from *S. commune* (D35). In relevant literatures, it is possible to show the studies to production of laccase by using *S. commune* without any inducer (Leonard and Phillips, 1973). However, in this study, laccase was efficiently produced by *S. commune* (D35) once, in the presence of pinus cone biomass increasing laccase production in batch cultivation. On the other hand, we can not find any study in literature concerned with *P. nigra* cone biomass as laccase inducer. According to Micales et al. (1994), the chemical composition of pinus cone was investigated and composed of lignin derivatives. Nevertheless, detailed research relevant to this matter needs to be carried out and components of cone biomass to induce activity are required for determination.

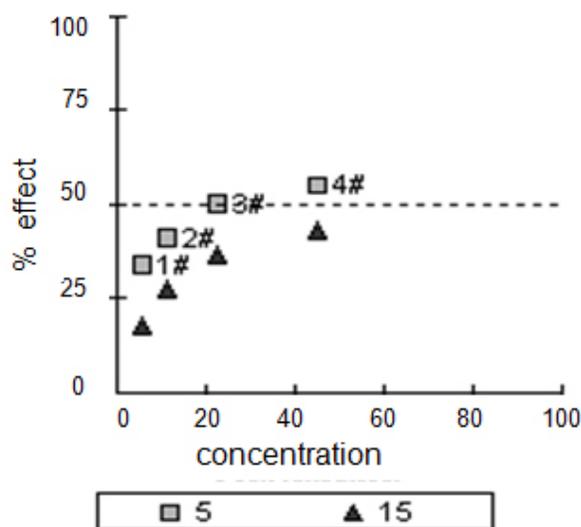


Fig. 7 Toxicity values of 2,4,5-TCP before enzymatic treatment.

It is well documented that both the type and concentration of nitrogen in culture media for growing white rot fungi are essential to laccase production (Galhaup et al. 2002). Our study demonstrated that ammonium tartrate as inducer affected only *T. versicolor* ATCC (200801) laccase positively, from 5.67 U ml⁻¹ to 7.30 U ml⁻¹ after 15 days. Other isolates tested were not induced with ammonium tartrate. It was reported that *Lentinula edodes* cultures with ammonium tartrate exhibited laccase activity higher than control cultures, reaching 251 U ml⁻¹ of extract after 30 days by Cavallazi et al. (2005).

A nonionic surfactant, Tween 80 only showed an effect on *T. versicolor* (D22) laccase and while control group had 0.02 U ml⁻¹ of enzyme activity, broth with Tween 80 reached to 1.82 U ml⁻¹ on 12 days of incubation period.

Veratryl alcohol is oxidized by laccase and it is thought to be involved in lignin degradation. Thereby, an affirmative effect shows at enzyme production of many fungi. The inductive effect of veratryl alcohol on laccase production by *Polyporus sanguineus*, *Daedalea flavida*, *Phlebia brevispora*, and *Phlebia*

radiata has been reported in the earlier studies (Arora and Gill, 2001). In our experiments, lacasse activities reached at 4.49 U ml^{-1} , 11.48 U ml^{-1} and 21.35 U ml^{-1} in the presence of veratryl alcohol for *C. unicolor* (D30), *T. versicolor* (D22) and *T. versicolor* ATCC (200801), respectively.

A waste biomass of agriculture, barley bran containing lignin, was evaluated in respect of induction. We observed that activities of enzyme increased except for lacasse synthesized from *S. commune* (D35). In the presence this inducer, 4-fold increase was performed for *C. unicolor* (D30) after five days while *T. versicolor* (D22) lacasse activity increased 20-fold after 12 days of incubation period. Activity of lacasse produced from *T. versicolor* ATCC (200801) enhanced 6-fold thanks to barley bran after 5 days of the incubation period. Rosales et al. (2002) studied lacasse production of *Trametes hirsuta* with barley bran in solid conditions and enzyme production of this culture began on the day 4 (230 U l^{-1}). Then, it peaked on the day 8 (2247 U l^{-1}) and hereafter it decreased. According to reports of Moldes et al. (2004) barley bran cultures gave the highest lacasse activities, showing values of $2500\text{-}3200 \text{ U l}^{-1}$ from the day 20 to the day 35, that of barley straw cultures exhibited values of about $300\text{-}500 \text{ U l}^{-1}$ at the same days. Schlosser et al. (1997) proposed the mechanism for these materials to induce the lacasse production is the presence of little quantities of phenolic soluble compounds. Similarly, in a previous work (Lorenzo et al. 2002), the production of lacasse by *T. versicolor* in submerged cultures was optimized by addition of different lignocellulosic residues. Of those, barley bran gave the highest activities (639 U l^{-1} , 12-fold to control culture).

Wheat bran was also used as lacasse inducer. Isolates used apart from *S. commune* (D35) had high lacasse activity when wheat bran was added into culture broth. Findings showed that lacasse activities of *T. versicolor* ATCC (200801), *T. versicolor* (D22), *C. unicolor* (D30) and *L. strigosus* (D26) reached up to 29.08 , 18.76 , 19.28 and 7.84 U ml^{-1} , respectively. Literature screening indicated that wheat bran was frequently used as one of the inducers (Arora and Gill, 2001; Lechner and Papinutti, 2006; Murugesan et al. 2007). Wheat bran as well as barley bran has more advantage than chemical inducers due to the fact that it is natural biomass. Nyanhongo et al. (2002) studied the effects of the carbon and nitrogen sources, initial pH and incubation temperature on lacasse production by *Trametes modesta*. They evaluated using the one-factor-at-a-time method. Response-surface analysis showed that 7.34 g l^{-1} wheat bran, 0.87 g l^{-1} glucose, 2.9 g l^{-1} yeast extract, 0.25 g l^{-1} ammonium chloride were the optimal amounts for lacasse production.

Copper was used as stimulating agent for the production of lacasse. Our study demonstrated CuSO_4 (1 mM) as inducer affected all isolates studied apart from *L. strigosus* (D26) and *S. commune*. The activity of lacasse produced from *C. unicolor* (D30) reached 24.06 U ml^{-1} within 12 days that of *T. versicolor* (D22) reached 22.06 U ml^{-1} after 8 days and that of *T. versicolor* ATCC (200801) increased up to 23.68 U ml^{-1} in a batch cultures. A previous report showed the effect of copper on lacasse activity (Birhanli and Yesilada, 2006). They observed that lacasse activities of growing cells of *Funalia trogii* and *T. versicolor* in copper-supplemented in repeated-batch cultures were increased to 40.29 and 12.09 U ml^{-1} , respectively while in the Cu-free repeated-batch cultures of *F. trogii* and *T. versicolor* pellets, maximum lacasse activities were found 0.83 and 0.61 U ml^{-1} , respectively. Also, the induction of lacasse by copper has already been documented in *P. ostreatus*. Baldrian and Gabriel (2002) indicated that the addition of copper ($0.5\text{-}5 \text{ mM}$) to the white rot fungus *P. ostreatus* cultivated in liquid nitrogen-limited medium for 12 days increased the activity of lacasse and the addition of 1 mM copper led to eight fold increase of activity. The results show that the presence of copper as a metal activator plays a key role in the regulation of extracellular enzyme activities, especially in oxidase and superoxidase group enzyme activities. Because copper is a component which is in the lacasse prostetic group presenting four cupric ions each associated with one single polypeptide chain, it is therefore an important component of active lacasse structure (Thurston, 1994). Besides, it has been proved that this ion may play an important role in lacasse genes regulation at transcription level as showed in *T. versicolor* (Collins and Dobson, 1997), *Pleurotus sajor-caju* (Soden and Dobson, 2001), *P. ostreatus* (Palmieri et al. 2000).

According to results of this study, different lacasse activity was obtained for both *T. versicolor*. Difference of lacasse activities between *T. versicolor* ATCC (200801) and *T. versicolor* (D22) may depend on the type and localization of fungal strain used.

Potentials of lacasse production of *S. commune* and *C. unicolor* without adding an inducer were reported in previous studies (De Vries et al. 1986; Kim et al. 2002; Stepanova et al. 2003). Myasoedova et al. (2008) studied with *L. strigosus* by inserting inducer such as CuSO_4 and 2,6-dimethylphenol and found 186.5 U ml^{-1} . This high lacasse activity may result from localization of

isolation and type of inducers used. The effect of inducers on the laccase production of these fungal biomasses were investigated under identical conditions. As can be seen in Table 1, laccase activity reached to 24.06 U ml^{-1} for *C. unicolor* with using CuSO_4 as an inducer in PDB medium. Also, laccase activity was obtained at 17.50 U ml^{-1} for both *S. commune* and *C. unicolor* with *P. nigra* cone biomass in the PDB medium. Besides, laccase activity was induced up to 7.84 U ml^{-1} with wheat bran for *L. strigosus*. These results indicated that *S. commune* and *C. unicolor* cells may be used as an inexpensive, effective and easily induced alternative laccase producer.

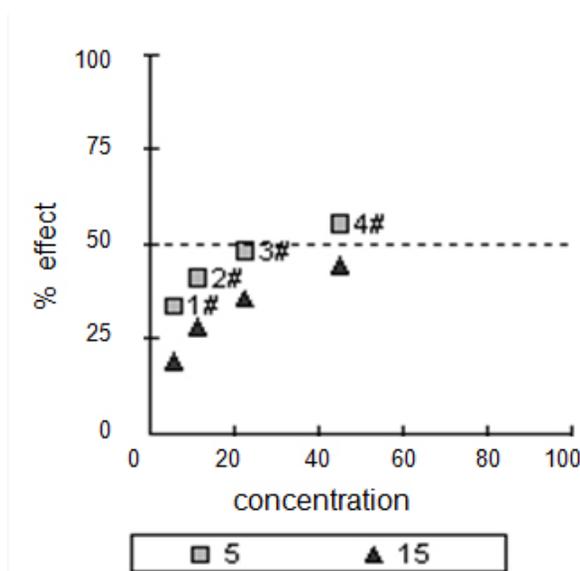


Fig. 8 Toxicity values of 2,4,5-TCP after enzymatic treatment.

In our study, chlorine removal from 2,4,5-TCP was examined as an environmental application. This application was performed with the highest activity of laccase using wheat bran as an inducer. According to these results, 79.74% of dechlorination of 2,4,5-TCP was obtained on optimum conditions which were pH 5, $400 \mu\text{M}$ of initial substrate concentration, 30 min of reaction period, 4 ml of enzyme amount, and 50°C of temperature (Figure 2, Figure 3, Figure 4, Figure 5 and Figure 6). Madsen and Aamand (1992) studied transformation of TCPs and they rapidly resumed after heating of the culture at 80°C for 1 hr, suggesting that the dechlorinating bacteria are spore-forming anaerobes. 2,4,5-TCP was dechlorinated to 3,4-dichlorophenol at initial concentrations of $\leq 40 \mu\text{M}$. Stazi et al. (2001) dechlorinated 2,4,6-TCP, a trichlorophenol compound, by mixture of peroxidase isozymes from *Vaccinium myrtillus* and investigated the effect of pH, temperature, reaction time, enzyme amount and initial pollutant concentration on the treatment efficiency in order to optimize the reaction conditions for TCP removal. According to these results, at the extreme values of the range considered, at 0, 1 and $20 \mu\text{mol dm}^{-3}$ of TCP, the removal efficiency of 2,4,6-TCP was about 96 and 50%. Besides, in the pH range 3.0 ± 9.0 , the optimal value was found to be 5.0. The effect of temperature on the extent of TCP transformation was also studied between 20 and 60°C . At earlier incubation periods, peroxidase was most active at 30°C but the relative differences observed between the various temperatures were less prominent when evaluating longer reaction times. Furthermore, the amount of substrate removal was increased with increasing amounts of peroxidase.

The dechlorination of 2,4-dichloro, 2,4,5-trichloro, 2,4,6-trichloro and pentachlorophenol by the lignin peroxidase of *Phanerochaete chrysosporium* has been reported (Hammel and Tardone, 1988). In this process, the release of chloride ions from single polychlorophenols was accompanied by both oxygen consumption and substrate disappearance. In addition to the fact that the chlorine removal from chlorophenols depended on the chemical structure and concentration of the substrate, pH of the reaction mixture, activity of the enzyme, length of incubation, and temperature (Stazi et al. 2001).

Bollag et al. (1988) studied laccase of the fungus *Rhizoctonia praticola*, to detoxify phenolic pollutants such as *o*-chlorophenol, *p*-chlorophenol, 2,4-dichlorophenol, 2,4,5-TCP, 4-chloro-2-methylphenol.

However, toxicity of *p*-chlorophenol and 2,4,5-TCP could not be overcome by a lacase mediated system.

According to the experiments of Joshi and Gold (1993) *P. chrysosporium* mineralized 2,4,5-TCP through its enzymes of LiP and MnP. The multistep pathway involves cycles of peroxidase-catalyzed oxidative dechlorination reactions followed by quinone reduction reactions to yield of the key intermediate 1,2,4,5-tetrahydroxybenzene, which is presumably ring cleaved with subsequent degradation to CO₂. In this pathway, the substrate is oxidatively dechlorinated by LiP or MnP in a reaction which produces a quinone. The quinone intermediate is recycled by a reduction reaction to regenerate an intermediate which is again a substrate for peroxidase-catalyzed oxidative dechlorination. This pathway apparently results in the removal of all three chlorine atoms before ring cleavage occurs.

Itoh et al. (2000) reported phenolic acids affect transformations of chlorophenols by a *T. versicolor* lacase (IFO 30340). 3- and 4-chlorophenols, 2,5-, 3,4- and 3,5-dichlorophenols, 2,4,5-TCP and pentachlorophenol, recalcitrant compounds to the lacase could have transformed through phenolic acids such as sinapinic acid, ferulic and *p*-cumaric acid.

According to the study of Roper et al. (1995), addition of 50 mM guaiacol as a reactive cosubstrate enhanced the dechlorination of 4-chlorophenol, 2,4-dichlorophenol, and 2,4,5-TCP with peroxidase by 12, 32 and 65%, respectively, and with lacase by 20, 32 and 80%, respectively.

Similar toxicity values were observed in our study after dechlorination experiments compared to the parent compound. Our results showed that only the denatured control containing 2,4,5-TCP, was sufficiently toxic to the bacterium *V. fischeri*, resulting in a 15 min EC₅₀ of 61.00%. The dechlorination samples were serially diluted before being tested with the bioluminescent bacterium because the bacterium only shows a response to toxic compounds when these compounds are present by a concentration below the sub-lethal concentration (Gu and Choi, 2001). As expressed in Figure 7 and Figure 8, the given % effects demonstrated that the toxicities of the parent compound and the intermediates were similar. This enzymatic procedure suggested that 2,4,5-TCP dechlorination metabolites by *T. versicolor* lacase was an environmental friendly strategy, there was no contribution to total toxicity. Evgenidou et al. (2005a) and Evgenidou et al. (2005b) investigated the removal of dichlorvos and dimethoate mediated photocatalytic process. They proved that photocatalytic treatment was not always capable of reducing the toxicity of dimethoate or dichlorvos solutions. Moreover, a slight increase could sometimes be observed. Marco-Urrea et al. (2010) studied degradation of the drug sodium diclofenac by *T. versicolor* pellets and evaluated toxicity by the Microtox bioassay. They found that uninoculated control including at 10 mg l⁻¹ was toxic to the luminescence bacterium, resulting in a 15 min EC₅₀ of 34.0%. Choi and Gu (2003) degraded 2,4,5-TCP by *Phanerochaete chrysosporium* assessed toxicity, and detected reduction of toxicity. These results compared to our results showed that degradation products of 2,4,5-TCP was lesser toxic than parent compound, while dechlorination products of 2,4,5-TCP were similar toxicity values. Gan et al. (2002) examined dechlorination of chloroacetanilide herbicides including alachlor, acetachlor, metolachlor, and propachlor by thiosulphate salts and toxicity after this process. Dissipation of 200 µM of alachlor was performed in 100 hrs through sodium thiosulphate and observed toxicological alterations, increasing EC₅₀. However, the more recalcitrant compound, 2,4,5-TCP was dechlorinated in 30 min in our study.

In conclusion, the lacase formation potential of the isolates studied can be enhanced several fold on supplementing the basal media with various natural or chemically synthesized materials and obtained lacase can be utilized in several biotechnological applications. In this study, the induced lacase obtained has shown usability at dechlorination of 2,4,5-TCP; besides, toxicity was tested. Enzymatic treatment with lacase would not always lead to complete detoxification in a previous stage probably requiring higher quantities of biocatalyst or sequential enzymatic treatment after the dechlorination process. Further research is needed to investigate different inducers in this process and mechanisms of induction. Also, it can be determined that lacase inducers based on lignin derivative compounds cause whether the amount of lignin change.

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