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Production of a Recombinant Laccase from *Pichia pastoris* and Biodegradation of Chlorpyrifos in a Laccase/Vanillin System

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The recombinant strain *P. pastoris* GS115-lccC was used to produce laccase with high activity. Factors influencing laccase expression, such as pH, methanol concentration, copper concentration, peptone concentration, shaker rotate speed, and medium volume were investigated. Under the optimal conditions, laccase activity reached 12,344 U/L on day 15. The recombinant enzyme was purified by precipitating and dialyzing to electrophoretic homogeneity, and was estimated to have a molecular mass of about 58 kDa. When guaiacol was the substrate, the laccase showed the highest activity at pH 5.0 and was stable when the pH was 4.5~6.0. The optimal temperature for the laccase to oxidize guaiacol was 60°C, but it was not stable at high temperature. The enzyme could remain stable at 30°C for 5 days. The recombinant laccase was used to degrade chlorpyrifos in several laccase/mediator systems. Among three synthetic mediators (ABTS, HBT, VA) and three natural mediators (vanillin, 2,6-DMP, and guaiacol), vanillin showed the most enhancement on degradation of chlorpyrifos. Both laccase and vanillin were responsible for the degradation of chlorpyrifos. A higher dosage of vanillin may promote a higher level of degradation of chlorpyrifos, and the 2-step addition of vanillin led to 98% chlorpyrifos degradation. The degradation of chlorpyrifos was faster in the L/V system (k_{obs} = 0.151) than that in the buffer solution (k_{obs} = 0.028).

Key words: Recombinant laccase, *Pichia pastoris*, laccase/vanillin system, chlorpyrifos, biodegradation

Chlorpyrifos (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is a broad-spectrum and moderately toxic pesticide with a long half-life in the environment and very low solubility in water [14]. Large-scale manufacture and usage of chlorpyrifos have led to the contamination of soil, air, surface, and groundwater by this pesticide in many parts of the world. In view of its toxicity, it is critical to have efficient means to remove it from the environment. It is generally considered that biodegradation is a harmless and effective process, and it is therefore desirable to find an enzymatic system for environmentally safe degradation of organophosphorous pesticide including chlorpyrifos. It was previously noted that a fungal lignin-degrading system is able to transform pesticides including chlorpyrifos and its degrading products such as TCP (3,5,6-trichloro-2-pyrifinol) [5]. Laccase is one of the main enzymes comprising the fungal lignin-degrading system [17, 22].

Laccase can oxidize phenolic aromatic compounds and has been studied for depletion of various xenobiotics [4, 17, 22]. The presence of mediator compound extends the spectrum of laccase to various substrates [21, 24]. Amitai *et al*. [1] showed that laccase from the white rot fungus *Pleurotus ostreatus* together with ABTS [2,2'-azino*bis*(3-ethylbenzthiazoline-6-sulfonate)], a synthetic mediator, displayed complete and rapid oxidative degradation of the organophosphorus nerve agents and insecticide containing a P-S bond. The mechanism of laccase/mediator system was described as laccase-induced mediator oxidation to a radical form, which subsequently is able to oxidize a bulky or high-redox potential substrate *via* a non-enzymatic pathway [3]. Therefore, it is conceivable that enzymatic oxidative biodegradation of chlorpyrifos will lead to new efficient detoxification. In contrast, the application of synthetic mediators such as ABTS, HBT (1-hydroxy-benzotriazole), and VA (violuric acid) was not ideal because of their high price and toxic properties, whereas natural mediators, obtained from natural and renewable sources, have the advantage of low cost, environmental safety, and non-toxicity [13]. In our study, vanillin was selected as the mediator for its ability to enhance the degradation of chlorpyrifos. The

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biodegradation of chlorpyrifos in the L/V (laccase/vanillin) system was studied to better understand the possible contribution of laccases to pesticide degradation in the environment.

At the same time, the successful application of laccase requires large production amounts at a low cost and a short time. However, the production level from parent strains is usually too low for commercial use. One possible solution is to produce the recombinant protein in a heterologous host. The yeast *Pichia pastoris* has been successfully used to express *Coriolus versicolor* laccase [8]. In this work, *Trametes versicolor* laccase was expressed in *P. pastoris* and conditions were further optimized to obtain a high expression of laccase. The recombinant laccase was purified in simple steps and characterized for its application of degrading chlorpyrifos.

Materials and Methods

Strains and Medium

The recombinant strain *P. pastoris* GS115-lccC was constructed and preserved in the Microbial Technology Research Laboratory, Nanjing Forestry University. While *P. pastoris* GS115-lccC was constructed, PCR primers for cDNA amplification of *lcc*C were designed based on the sequence of lac4 (GenBank Accession No. 212734). The PCR product *lcc*C was digested with *Eco*RI and *Xba*I, ligated with the digested expression vector pPICZαB, and then transformed into the recombinant *P. pastoris* GS115 carrying pPICZαB competent cells.

For production of laccase, the strain was transferred to YPD (yeast extract-peptone-dextrose) plates and incubated at 28°C for 3 days.

BMGY (buffered glycerol complex) and YPD media were prepared according to the manual of the Easy Select *Pichia* Expression Kit (Invitrogen).

Assay of Laccase Activity

During the production of laccase, the activity was determined at 50°C using 1 mmol/l ABTS as the substrate in 100 mmol/l tartaric acid buffer, pH 4.5. The oxidation of ABTS was detected by measuring the absorbance increase at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) after 3 min using a spectrophotometer (Purkinje TU-1810).

At the same time, guaiacol, a natural phenol, was used as the substrate to determine the activity, when its characteristics related to degradation of chlorpyrifos were studied. The oxidation of guaiacol was detected by measuring the absorbance increase at 465 nm ($\varepsilon_{465} = 1.2 \times 10^4\ M^{-1}\ cm^{-1}$) after 3 min at 30°C in citrate buffer, pH 5.0.

For every sample, the activity was measured three times and the average result was used to determine the laccase activity.

Optimization of the Recombinant Laccase Production in *P. pastoris* GS115-lccC

BMGY was used as the basic medium for the production of laccase in *P. pastoris* GS115-lccC. The cell pellets were resuspended in BMGY medium to an OD_{600} of 1.0 in 250 ml baffled shake flasks. The flasks were cultivated at 28°C in a shaking incubator and methanol was added every 24 h to maintain final concentrations of 0.4% to 1.2%.

In order to optimize the laccase expression, the effects of key factors on the recombinant laccase production were investigated. These factors and the variation ranges were as follows: initial pH values of cultures 3.0~8.0, final methanol concentration 0.4%~1.2% (v/v), copper concentration 0.1~0.7 mmol/l, peptone concentration 2%~6% (v/v), shaker rotate speed 120~210 rpm and medium volume 30~70 ml. For the level of each factor, the experiment was done in triplicate and the mean and SD of the relative activity for each factor were determined.

Purification and Characterization of the Recombinant Laccase

The liquid culture was purified by ammonium sulfate precipitation and dialyzed against 50 mmol/l sodium citrate buffer 3 times. SDS-PAGE was carried out with 5% stacking gel and 10% resolving gel using the Electrophoresis System 301 (Amersham Biosciences). The molecular mass of the recombinant laccase was estimated by calculating the relative mobility of standard protein markers (Takara, China).

Some characteristics of the recombinant laccase were investgated using guaiacol as the substrate. The effect of pH on laccase activity was studied over a pH range of 2.5–7.0 in 50 mmol/l citrate buffer. To determine the pH stability, enzyme was kept at 30°C in different citrate buffers (pH 2.5–7.0) for 48 h and the residual activity was determined periodically. The impact of temperature was examined for a range of temperatures (25–70°C), and laccase activity was determined at the optimal pH value. Thermal stability was investigated by determining the residual activity of laccase after it was kept at a certain temperature for different pre-incubation times (12 h at 25–70°C and 5 days at 25–35°C). Every experiment was performed in triplicate and the relative activity mean and SD were calculated.

Degradation of Chlorpyrifos by Laccase/Mediator Systems

Chlorpyrifos was dissolved in a citrate buffer (50 mmol/l, pH 5.0) to a final concentration of 25 mg/l. The system contained 2 mmol/l of mediators, unless otherwise stated. The degradation of chlorpyrifos was initiated by the addition of laccase with an original activity of 0.05 unit/l based on the guaiacol oxidation. The vials were placed on a rotary shaker (120 rpm) at 25°C.

When the effects of vanillin dosage were tested, vanillin was added according to the molar ratio of vanillin to chlorpyrifos (M_1 = 0~40) when adding only once. The supplementary amount M_2 was tested at a range over 0~80 when M_1 = 40.

Samples were periodically withdrawn and extracted by 5-fold volume petroleum ether. The extraction was dried by N_2 and dissolved with methanol and filtered with a 0.22-μm-pore filter. The filters were subjected to HPLC analysis. The reaction was monitored by an HPLC system (Agilent 1100) equipped with an Eclipse XDB-C18 column (4.6 mm × 150 mm × 5 μm) eluted with 90% methanol in water at a flow rate of 1.0 ml/min at 25°C. The detection of eluted compounds was carried out using a UV detector at 293 nm.

The experiments were performed in triplicate and the percent of chlorpyrifos degradation mean and SD were determined.

Results and Discussion

Optimization of the Recombinant Laccase Production in *P. pastoris* GS115-lccC

The expression conditions used for *P. pastoris* are key to improving the productivity of a correctly processed protein

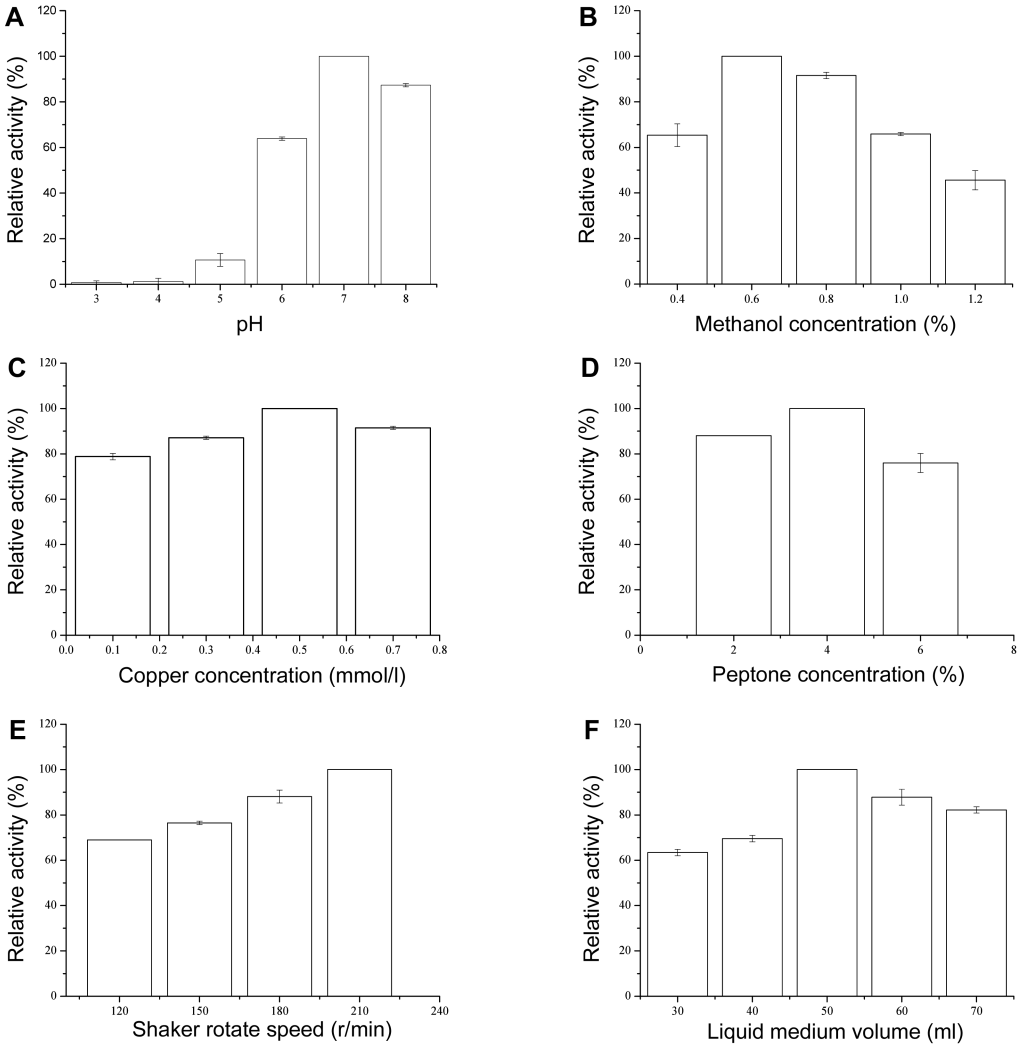

Fig. 1. Effects on pH (**A**), methanol concentration (**B**), copper concentration (**C**), peptone concentration (**D**), shake rotate speed (**E**), and liquid medium volume (**F**) on the production of laccase.

Other cultivation conditions: (**A**) 0.5% methanol, 0.5 mmol/l Cu^{2+}, 2% peptone, 180 rpm, 30 ml liquid medium; (**B**) pH 6.0, 0.5 mmol/l Cu^{2+}, 2% peptone, 180 rpm, 30 ml liquid medium; (**C**) pH 6.0, 0.5% methanol, 2% peptone, 180 rpm, 30 ml liquid medium; (**D**) pH 6.0, 0.5% methanol, 0.5 mmol/l Cu^{2+}, 180 rpm, 30 ml liquid medium; (**E**) pH 6.0, 0.5% methanol, 0.5 mmol/l Cu^{2+}, 2% peptone, 30 ml liquid medium; (**F**) pH 6.0, 0.5% methanol, 0.5 mmol/l Cu^{2+}, 2% peptone, 180 rpm.

[7] and a large number of strategies are used to optimize the expression level of heterologous proteins [7, 10, 19]. Therefore, the expression of laccase in *P. pastoris* GS115-lccC under various cultivation conditions was investigated first.

Medium with initial pH values of 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 were prepared to study the effect of pH on laccase production. A higher laccase activity was observed in cultures of initial pH 7.0 (Fig. 1A). pH was very important for transformants to produce laccase [10] and sometimes special strategies were needed to control the pH value during the cultivation [18]. The pH value was stable during the fermentation in this work, so it was not necessary to deploy other strategies.

Methanol was added every 24 h during the cultivation to induce the expression of laccase ranging from 0.4% to 1.2%. It was determined that the optimal final methanol concentration was 0.6% (Fig. 1B). When methanol was 0.4%, the laccase expression level was low. Using a methanol concentration of 1.2%, the high level of methanol was toxic to the cells and resulted in lower laccase expression. These results were similar to those seen for recombinant *P. pastoris* to produce laccase [12, 18].

Copper atoms are also important for the activity of laccases [4] and responsible for the correct folding and assembly of protein at the post-transcriptional step [9]. Different amounts of Cu^{2+} were added to the BMGY medium

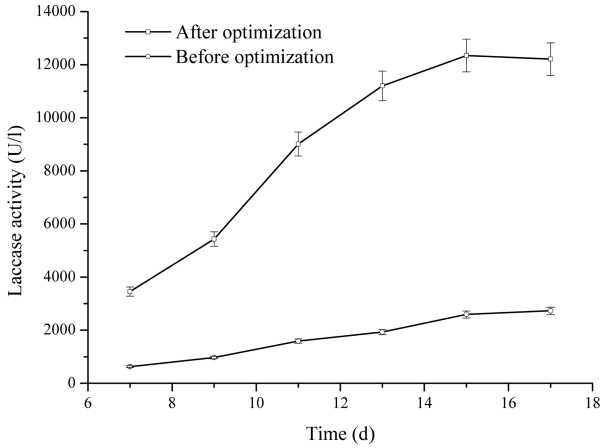

Fig. 2. The activity of recombinant laccase in BMGY medium before and after optimization.
Before optimization: 30 ml BMGY medium in 250 ml flask with initial pH 6.0, containing 0.5 mmol/l Cu^{2+}, 2% peptone, final 0.5% methanol by adding daily, 28°C, 180 rpm. After optimization: 50 ml BMCY medium in 250 ml flask with initial pH 7.0, containing 0.5 mmol/l Cu^{2+}, 4% peptone, final 0.6% methanol by adding daily, 28°C, 210 rpm.

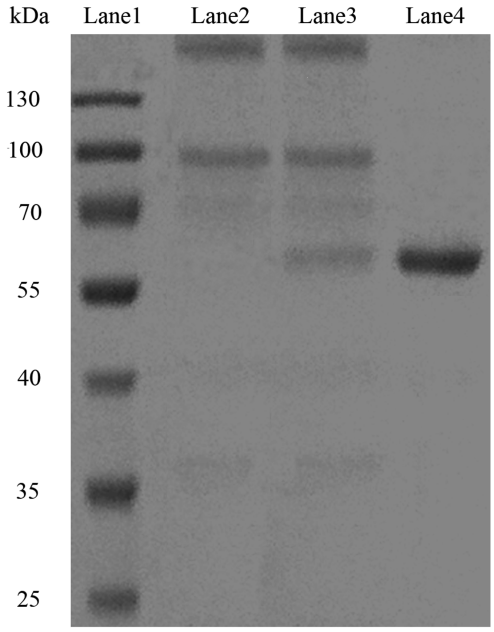

Fig. 3. SDS-PAGE of the recombinant laccase.
Lanes: 1, Molecular weight markers (10–170 kDa); 2, Control (Culture filtrates from strain *P. pastoris* GS115); 3, Crude laccase from recombinant strain *P. pastoris* GS115-lccC; 4, purified laccase.

to final concentrations of 0.1, 0.3, 0.5, and 0.7 mmol/l. Laccase activity increased as the Cu^{2+} concentration increased from 0.1 to 0.5 mmol/l, indicating the requirement of copper by the enzyme. However, when the Cu^{2+} concentration reached 0.7 mmol/l, the laccase activity decreased, indicating that excessive Cu^{2+} inhibited laccase production. The optimal Cu^{2+} concentration for inducing laccase expression was 0.5 mmol/l (Fig. 1C).

Suitable amounts of peptone reduced the degradation of laccase by protease during the cultivation. Addition of 4% peptone produced the highest level of laccase activity (Fig. 1D).

Higher shaker rotation speeds affected the amount of dissolved oxygen in the fluid medium, and the laccase expression level increased with the shaker rotation speed from 120 to 210 rpm after incubating for 15 days (Fig. 1E).

The volume of liquid medium affected the laccase production. As shown in Fig. 1F, the optimal liquid medium was 50 ml in the 250 ml flasks.

Under the optimal conditions, laccase activity reached 12,344 U/l on day 15 (Fig. 2), 4.6 times higher than that expressed in the initial medium.

Purification and Characterization of the Recombinant Laccase

P. pastoris produced relatively low levels of native secreted proteins and pigments, which would be beneficial for the purification of recombinant protein [18, 19]. Here, the laccase was simply purified only by precipitating and dialyzing. The homogeneity of the purified laccases was checked on SDS-PAGE (Fig. 3). The enzyme showed a single band of approximately 58 kDa. So the production cost, the main bottleneck for application of laccase, will be dramatically reduced because of the simpler purification steps.

For the pure recombinant laccase, the characteristics related to the oxidation of natural phenol, taking guaiacol as the example, were investigated. The pH profile showed a peak of maximum activity at pH 5.0 (Fig. 4A). At the same time, the enzyme was stable when the pH was maintained between 4.5 and 6.0. The activity maintained 82% after 48 h when the pH was 5.0 at 30°C (Fig. 4B).

The optimal temperature for the guaiacol oxidation was observed at 60°C (Fig. 4C), but there was dramatic loss of activity during pre-incubation at 60°C and only 14% activity remained after 4 h (Fig. 4D). The loss was also high when the enzyme was pre-incubated at 50°C. When the temperature was controlled at 30°C, the recombinant laccase could maintain its activity for guaiacol oxidation for a long time, and about 88% activity remained even after 5 days (Fig. 4E). It is therefore possible for the recombinant laccase to be used for a long time under mild conditions.

Degradation of Chlorpyrifos in Several Laccase/Mediator Systems

The degradation of chlorpyrifos was tested in several laccase/mediator systems. The three synthetic mediators (ABTS, HBT, and VA) were the common mediators when laccase was used to degrade xenobiotics [1, 2, 15]. For the

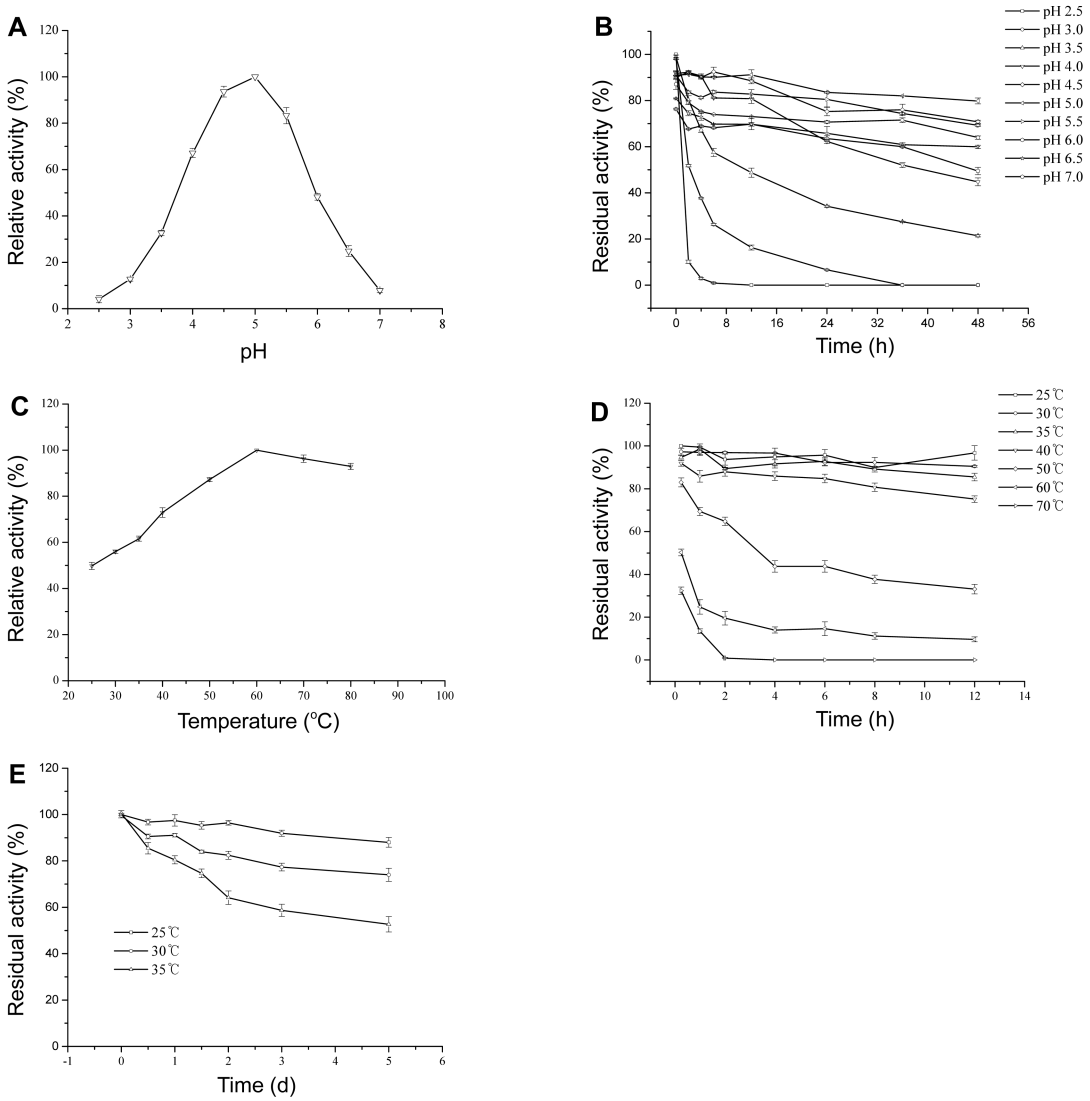

Fig. 4. Effects of pH (**A**) and temperature (**C**) on the activity, and the pH stability (**B**) and thermal stability (**D**, **E**) of the recombinant laccase.

three mediators in our tests, however, there was no improvement in chlorpyrifos conversion after 24 h as shown in Fig. 5. On the contrary, degradation was even lower than laccase alone. If ABTS, $MnSO_4$, and Tween 80 were added together, chlorpyrifos could be degraded a little more than with the laccase alone, but not as high as the degradation of glyphosate by this system reported by Pizzul *et al*. [23]. The mediators such as ABTS, HBT, and VA would contribute to laccase from *Trametes versicolor* inactivation in certain instances, as reported by Kurniawati *et al.* [15]. Thus, the exploration and determination of the optimal conditions for the use of mediators for laccase applications was important.

In contrast, for 3 natural mediators [2,6-DMP (2,6-dimethoxyphenol), guaiacol, and vanillin], chlorpyrifos was successfully degraded in the laccase/mediator system. However, the percent degradation varied depending on the kind of mediators used. In particular, the use of 2 mmol/l vanillin resulted in over 70% degradation of chlorpyrifos after 24 h (Fig. 5). That is to say, the presence of vanillin in the reaction mixture significantly enhanced the conversion of chlorpyrifos relative to the laccase alone and the control (with inactive laccase) system.

Kang *et al*. [11] reported that vanillin and guaiacol were very effective when used to mediate the oxidation of the fungicide cyprodinil, whereas 2,6-DMP showed little effect. Similarly, 2,6-DMP showed the weakest enhancing effects on degradation of chlorpyrifos among the three natural mediators in our study. The different effects may be related to the structures of mediators. According to the analysis of Barneto *et al*. [2] and Tanaka *et al*. [25], 2,6-DMP is the most likely to yield a radical cation and the

radicals are most stable in the three natural mediators. Thus, the radicals coming from 2,6-DMP showed greatest tendency of polymerization themselves, rendering it inactive as a mediator. This character of 2,6-DMP inhibited its enhancement for degradation of chlorpyrifos compared with the vanillin. The structure–effect relationships of natural phenols in the degradation of chlorpyrifos requires further study, which we are pursuing.

Effect of Vanillin on the Degradation of Chlorpyrifos

The degradation percent of chlorpyrifos in the L/V system was much higher than that in the control, which contained vanillin and inactivated laccase (Fig. 6). Taking the results in Fig. 5 into account, it was concluded that both laccase and vanillin were responsible for the degradation of chlorpyrifos. The dependence of chlorpyrifos degradation on both vanillin and laccase suggests that the enzymatic oxidative degradation of chlorpyrifos comprises both the enzymatic oxidation of vanillin by laccase and the non-enzymatic reaction.

As vanillin played the key role in the L/V system, we investigated the dosage and adding method for improving the degradation of chlorpyrifos.

As shown in Fig. 7A, the use of higher dosage of vanillin gave higher levels of degradation of chlorpyrifos. The improved degradation yield of pesticide imazalil was also observed when the natural mediator concentration was improved [20].

During the analysis of chlorpyrifos in HPLC, it was found that the peak of vanillin determined by HPLC became weaker over time. At 2 mmol/l concentration of vanillin, the peak became dramatically weaker at 8 h and disappeared at 24 h. The results suggested that the vanillin may be

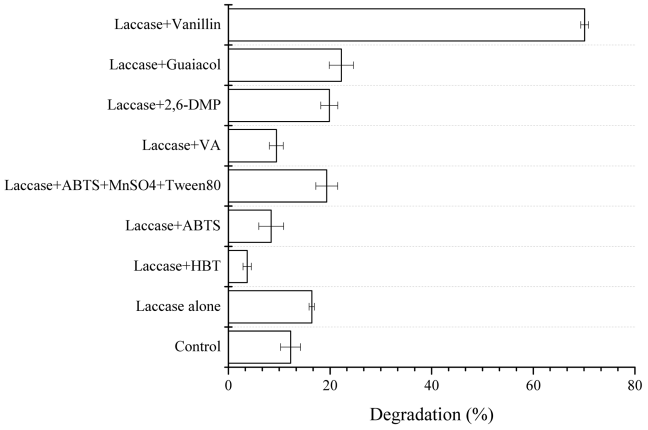

Fig. 5. Degradation of chlorpyrifos by laccase in several laccase/mediator systems.
In "Laccase + ABTS + $MnSO_4$ + Tween80", the $MnSO_4$ was 2 mmol/l and Tween80 was 1% (w/v); the control was done same with "Laccase alone" unless laccase was inactivated.

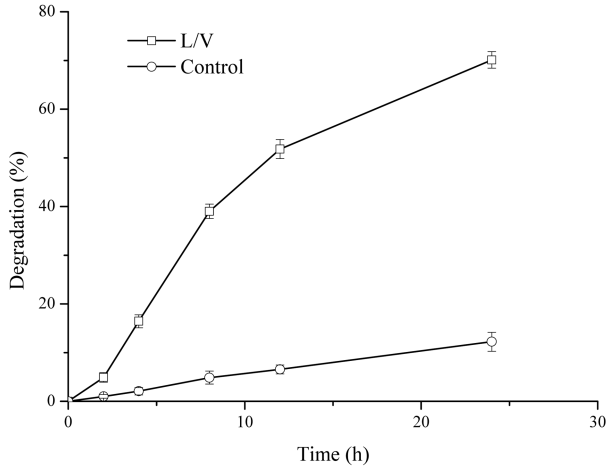

Fig. 6. Degradation of chlorpyrifos in L/V system.
Initial chlorpyrifos concentration was 25 mg/l, pH 5.0, vanillin 2 mmol/l; the control means laccase was inactivated in L/V system.

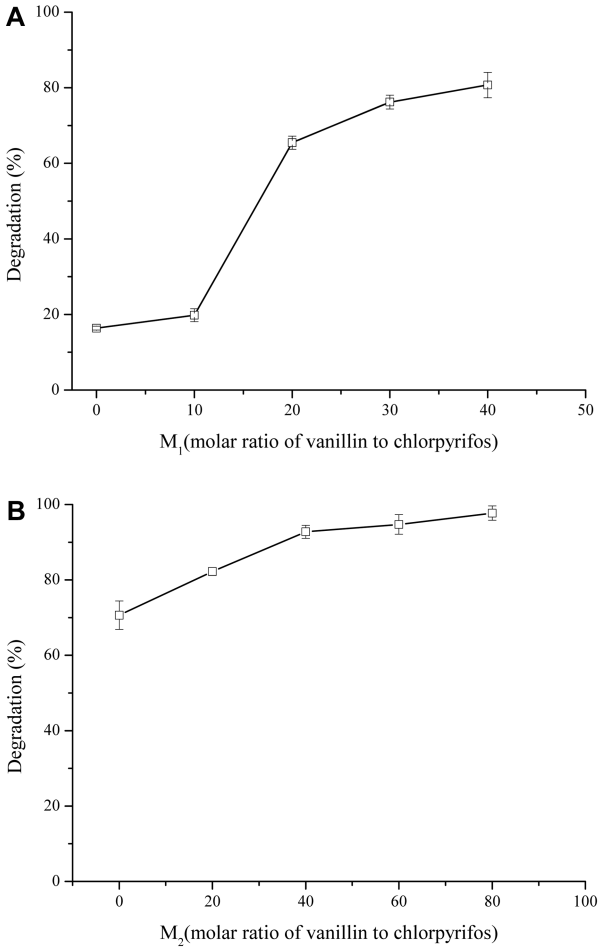

Fig. 7. Effects of the usage of vanillin on degradation of chlorpyrifos.
(**A**) Adding vanillin only once. (**B**) Supplementing vanillin at 8 h.

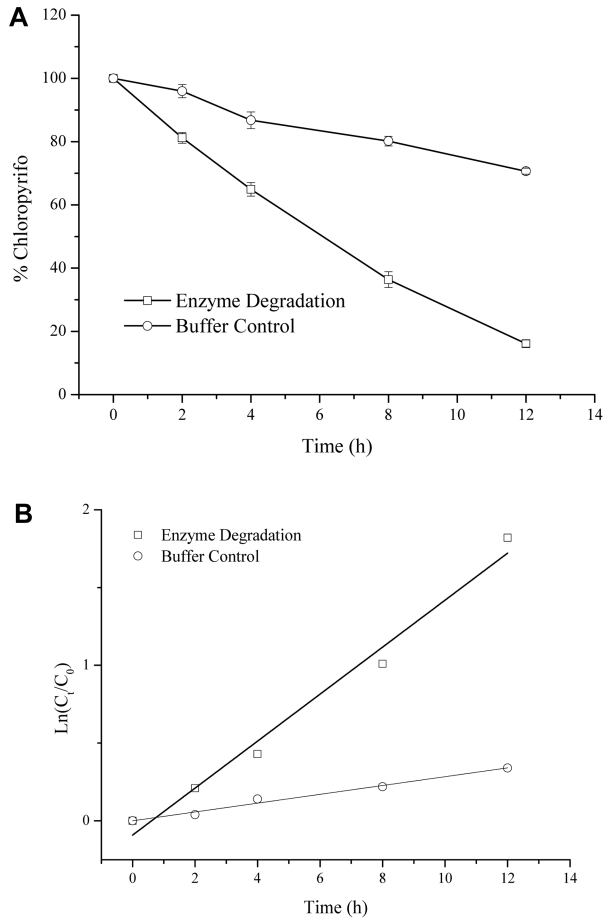

Fig. 8. Degrading kinetics of chlorpyrifos in the L/V system. (**A**) Time-course of chlorpyrifos degradation; (**B**) $Ln(C_t/C_o)$ as a function of degradation time.

consumed over time. These findings are similar to studies with other mediators such as ABTS, HBT, and VA, which are known to be degraded or consumed over time [13, 15, 16]. It was proposed that the compounds should be denominated as laccase enhancers rather than laccase mediators [6]. However, little has been done to explore and optimize the use of mediators. Thus, in order to satisfy the requirement for vanillin and not to affect the catalytic stability of laccase, a certain amount of vanillin was added at 8 h once more (Fig. 7B). The degradation of chlorpyrifos was enhanced to over 90% when a large amount of vanillin ($M_2 \geq 40$) was supplemented at 8 h.

Degradation Kinetics of Chlorpyrifos in L/V System

The time course of enzymatic degradation of chlorpyrifos in the L/V system under optimal conditions (optimal chlorpyrifos transformation and laccase stability) and in the buffer is described in Fig. 8A. Fig. 8B presents the semilogarithmic plots of $Ln(C_t/C_0)$ as a function of time.

The linear curves show that the degradation of chlorpyrifos can be expressed by the first-order exponential decay expression. According to the curves, the first-order rate constants of chlorpyrifos [k_{obs} (h^{-1})] in the L/V system and in the buffer are 0.151 and 0.028, respectively. The calculated $t_{1/2}$ for chlorpyrifos in the L/V system is 6.6 h, and $t_{1/2}$ is 34.6 h in the buffer. Compared with the natural degradation in buffer, chlorpyrifos displays relatively rapid degrading rates in the L/V system.

In conclusion, the recombinant strain *P. pastoris* GS115-lccC could be used to produce heterologous laccase at a high level under the optimizing conditions. The recombinant laccase could be purified by simple steps including precipitation and dialysis. This enzyme showed excellent degradation capacity toward chlorpyrifos in the presence of a natural mediator, vanillin. The L/V system allowed the degradation of chlorpyrifos in mild conditions without negative effects on the ecological system.

Acknowledgments

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