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Utilization of Horticultural Waste for Laccase Production by *Trametes versicolor* Under Solid-State Fermentation

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Abstract Horticultural waste collected from a landscape company in Singapore was utilized as the substrate for the production of laccase under solid-state fermentation by *Trametes versicolor*. The effects of substrate particle size, types of inducers, incubation temperature and time, initial medium pH value, and moisture content on laccase production were investigated. The optimum productivity of laccase (8.6 U/g substrate) was achieved by employing horticultural waste of particle size greater than 500 μm and using veratryl alcohol as the inducer. The culture was at 30 °C for 7 days at moisture content of solid substrate of 85% and initial pH 7.0. The decolorization was also investigated in order to assess the degrading capability of the ligninolytic laccase obtained in the above-mentioned cultures. The decolorization degree of a model dye, phenol red, was around 41.79% in 72 h of incubation. By far, this is the first report on the optimization of laccase production by *T. versicolor* under solid-state fermentation using horticultural waste as the substrate.

Keywords Horticultural waste · *Trametes versicolor* · Laccase · Solid-state fermentation · Decolorization

Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper enzymes that catalyze the oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines, and ascorbate with the concomitant four-electron reduction of oxygen to water [1]. Laccases have received much attention from researchers in last decades due to their broad substrate specificity, which makes them very useful for their application to several biotechnological

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processes. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp [2], textile and petrochemical industries [3], and the used as a tool for medical diagnostics [4, 5] and as a bioremediation agent to clean up herbicides, pesticides, and certain explosives in soil [6, 7]. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs [8] and as ingredients in cosmetics [9, 10]. In addition, their capacity to remove xenobiotic substances and produce polymeric products makes them useful for bioremediation purposes [11].

Many laccases from different sources could be considered for various industrial applications. Among these, laccase from fungus *Trametes versicolor* was reported to have the highest redox potential among laccases, i.e., 785 mV versus the standard hydrogen electrode [12–14], which makes this laccase particularly interesting since high redox potentials correlate with its high oxidative ability [15, 16]. In addition, the ability of this laccase to exert its catalytic activity on many types of aromatic compounds has been demonstrated [17–19]. These characteristics make laccase from *T. versicolor* particularly promising for industrial applications.

Enzyme production is an increasing field of biotechnology. Most enzyme manufacturers produce enzymes by submerged fermentation techniques. However, in the last decade, there has been an increasing trend toward the utilization of the solid-state fermentation (SSF) technique to produce several enzymes [20]. SSF is defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support the growth and the metabolism of the microorganisms [21]. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms. Lignocellulosic residues comprising a broad range of wastes from agricultural and forest industries are known to stimulate lignocellulose-degrading enzyme production. These lignocellulosic wastes provide cheap source of nutrients to the microorganisms and thereby reduce the production costs considerably [22]. Utilization of these wastes in bioprocesses not only provides alternative, cheap, and renewable substrates but also helps in solving pollution problems. In addition, the microorganisms in solid-state cultures grow under conditions closely resembling to their natural habitat. This results in higher yield of certain enzymes and metabolites than those in submerged cultures [23].

Horticultural waste (HW) refers to tree trunks and branches, plant parts, and trimmings generated during the maintenance and pruning of trees and plants. In Singapore, 229,300 tonnes of horticultural waste was generated in 2008. The recycling rate is only 42% for horticultural waste [24, 25]. Until now, various natural lignocellulosic residues, e.g., grape seeds [26], chestnut shell [27], barley bran [27], coconut flesh [28], etc., have been used successfully in laccase production under SSF. To our knowledge, HW has not been tested as a substrate for laccase production. The reutilization of HW could not only help solve pollution problems caused by incineration and land filling but also create their added value. The objective of this study is therefore to optimize the process of SSF to get high-yield laccase production by *T. versicolor* grown on HW.

Phenol red dye belongs to the phenolsulfonephthaleinic class. It is a phenol and therefore a natural substrate for phenoloxidase enzymes. The decolorization of phenol red is considered a way for assessing the aromatic degrading capability of ligninolytic enzymes [29]. In this study, phenol red decolorization was performed to evaluate the aromatic compound degrading capabilities of the crude laccase samples prepared by *T. versicolor* on HW.

Materials and Methods

Solid Substrate

HW was collected from Environmental Landscape Pte Ltd, Singapore. The collected HW was immediately dried at 80 °C overnight. They were then cooled down to room temperature and stored in sealed plastic bags until use. For solid-state fermentation, the dried HW was mechanically milled with a lab mill (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Germany) and sieved through standard mesh sieves using an electronic sieve shaker model RP09 (Barcelona, Spain) to obtain a powder of 200 to 500 μm particle sizes.

Microorganism and Inoculum Preparation

T. versicolor ATCC 20869 was used in this study. It was purchased from American Type Culture Collection. The strain was routinely maintained and sporulated on potato dextrose agar plate. After 4 to 5 days of fungi incubation at 30 °C, three 3-mm disks of fungal mycelium were excised from agar plates and were transferred to 250 ml Erlenmeyer flasks containing 100 ml of the following standard medium: glucose, 10.0 gl^{-1}; NH_4NO_3, 1.0 gl^{-1}; KH_2PO_4, 0.8 gl^{-1}; Na_2HPO_4, 0.2 gl^{-1}; $MgSO_4{\cdot}7H_20$, 0.5 gl^{-1}; and yeast extract, 2.0 gl^{-1}. The medium was adjusted to pH 6.0–6.2 with 2 M NaOH. To prepare the inoculums, the 4- to 5-day fungal mycelia pellets were harvested and homogenized using a laboratory blender. Homogenate was inoculated for the solid-state fermentation.

Optimization of Fermentation Process Under SSF

Five grams of dried HW was added in 250-ml cotton-plugged Erlenmeyer flasks with certain amount of mineral salt medium to attain the predesigned moisture content. The basal mineral salts solution used for the experiment had the following composition: NH_4NO_3, 1.0 gl^{-1}; KH_2PO_4, 0.8 gl^{-1}; Na_2HPO_4, 0.2 gl^{-1}; $MgSO_4{\cdot}7H_20$, 0.5 gl^{-1}; and yeast extract, 2.0 gl^{-1}. The initial pH value of the medium was adjusted to 6.0. The flasks with substrate were autoclaved at 121 °C for 1 h. Each flask was inoculated with 3-ml homogenized mycelia pellets at 30 °C under static conditions. The contents were mixed thoroughly and incubated under controlled temperature and humidity.

Various process parameters influencing enzyme production during SSF were investigated. One-factor-at-a-time approach was adopted in this study. The following process parameters were optimized: particle size (>500, 200–500, <200 μm), types of inducers ($CuSO_4$, veratryl alcohol, guaiacol, pyrogallol, syringic acid, caffeic acid, and ethanol), temperature (24 °C, 27 °C, 30 °C, 34 °C, 37 °C), initial pH (4, 5, 6, 7, 8, and 9), and initial moisture content (60%, 70%, 75%, 80%, 85%, and 90%). All experiments were conducted independently in duplicates, and the data presented here are the mean values ± SD.

Enzyme Extraction

At the end of incubation period, the enzyme was recovered by extraction. Five grams (dry weight) of the fermented substrate was extracted with 100 ml 0.05 M sodium acetate buffer (pH 4.8), by shaking at 150 rpm for 2 h at 30 °C. The suspended materials and fungal biomass were separated by centrifugation at 12,000×*g* and 4 °C for 20 min. The supernatant was used for enzyme activity assay.

Enzyme Assays

Laccase activity was determined spectrophotometrically according to what described by Vladimir et al. [30] using 2,2′-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS; Boehringer) as the substrate with some modifications. The reaction mixture (total volume 1 ml) contained 100 μl enzyme extract and 900 μl ABTS solution (1 mM) in sodium acetate buffer pH 5.0 (50 mM). Oxidation was followed via the increase in absorbance at 420 nm (ε=3.6×10^4 cm^{-1}M^{-1}). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 μmol of ABTS per minute and the activities were expressed in units per liter. Laccase yield was expressed as units per gram dry substrate. All enzyme analyses were carried out in duplicates. The soluble protein content, an indicator of the enzyme released, was measured by the Lowry protein assay using bovine serum albumin as the standard [31].

In Vitro Decolorization of Phenol Red

In vitro decolorization of phenol red was conducted according to Lorenzo et al. [29] with some modifications. The reaction was carried out in test tubes at 30 °C and the reaction mixture contained sodium acetate (50 mM; pH 5.0), phenol red (75 μM), and laccase (120 U/l) in a total volume of 2.5 ml. Commercial laccase from *T. versicolor* ATCC 38429 (Fluka, Neu-Ulm, Germany) was used for comparison. Reaction mixtures using distilled water in place of the enzyme solution was used as the control.

Absorbance at 431 nm (the maximum visible absorbance of phenol red [26]), an indication of the dye concentration, was monitored periodically (at 12, 24, 48, 72, and 96 h). All assays were carried out in duplicates. The decolorization degree was calculated according to Sani et al. [32] using the following formula:

$$D = 100\left(A_{\text{in}_\text{i}} - A_{\text{obs}}\right)/A_{\text{ini}},$$

where D is the decolorization degree (in percent), A_{ini} the initial absorbance, and A_{obs} the observed absorbance.

Results and Discussion

Effect of Particle Size on Laccase Production

The substrate particle size is a very important factor that influences the enzyme production. Generally speaking, smaller particle size could provide larger surface area for microbial growth, and it is also advantageous for heat transfer and mass transfer, e.g., exchange of oxygen and carbon dioxide between the air and the solid surface. However, too small particles may result in substrate agglomeration, which may interfere with microbial respiration and thus cause poor cell growth. In the mean time, larger particles provide better aeration efficiency (due to the increased inter-particle space), however, present limited surface area for microbial colonization. Consequently, a compromised particle size is necessary for high-yield enzyme production [33].

HW of three different particle sizes (particle size >500 μm; particle size between 200 and 500 μm; particle size <200 μm) was used in this experiment. Fermentation was carried out at a moisture level of 80% and initial pH 6.0. Enzymes were extracted after 7-day

incubation at 30 °C. Results are displayed in Table 1. It was found that laccase activity on HW of particle size >500 μm was higher than those obtained on HW of other particle sizes. It was 1.4-fold higher than that obtained using HW of particle size <200 μm. In addition, HW of particle size >500 μm favored the soluble protein production. This suggests that SSF containing larger particles (particle size >500 μm) exhibit high porosity and consequently better heat and mass transfer efficiency. Laccase production was therefore enhanced under such condition. HW of particle size >500 μm was selected for subsequent studies.

Effects of Various Inducers on Laccase Production

One of the most effective approaches to increase the yield of ligninolytic enzymes is the supplementation of the nutrient medium with an appropriate inducer. Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms [34, 35], and the nature of the compound that induces laccase activity differs greatly with the species. Hence, in order to enhance laccase activity, the addition of different inducers to cultures of *T. versicolor* under SSF was investigated. Among the various laccase inducers reported in the literature, copper sulfate, veratryl alcohol, guaiacol, pyrogallol, syringic acid, caffeic acid, and ethanol were selected in this study. The inducer concentration in the basal medium was kept at 1 mM. A culture without the addition of an inducer was used as the control. All cultures were incubated at 30 °C for 7 days at 80% water moisture and an initial pH of 6.0.

The results are summarized in Fig. 1. It is notable that almost all the inducers listed in this study were capable of enhancing laccase production by *T. versicolor*. The highest laccase activity was obtained in the presence of veratryl alcohol (2.8-fold of that of the control) followed by copper sulfate (1.9-fold) and ethanol (1.5-fold). Other potential inducers such as caffeic acid, syringic acid and pyrogallol did not significantly increase the total laccase yields. Copper sulfate and veratryl alcohol have been reported to be strong laccase inducers for many fungal species [36, 37]. Lee and his coworkers found that ethanol could also induce laccase production by *T. versicolor* [38]. According to Gianfreada et al. [39], the presence of inducers, their chemical nature, the amount added, and the time of their addition influenced laccase production by many fungal species. Interestingly, the addition of veratryl alcohol did not yield the highest soluble protein concentration in this study, indicating that the higher amount of laccase was produced under such condition. Based on the above results, the subsequent experiments were conducted in the medium supplemented with 1 mM veratryl alcohol.

Effects of Incubation Temperature and Fermentation Time

Probably the most important factor among all the physical variables affecting the SSF performance is the incubation temperature because both cell growth and the production of enzymes and metabolites are usually sensitive to temperature. A range of incubation

Table 1 Effects of particle size on laccase yield and soluble protein concentration.

Particle size (μm)	Laccase activity (U/g substrate)	Soluble protein (mg/g substrate)
>500	1.89±0.13	15.39±0.39
200–500	1.37±0.01	14.13±0.04
<200	1.34±0.04	15.17±0.14

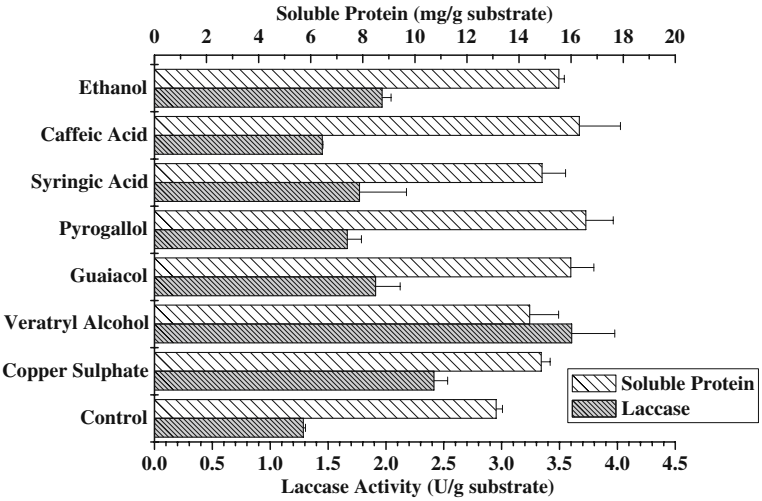

Fig. 1 Effect of inducers on laccase yield and soluble protein concentration

temperatures, viz., 24 °C, 27 °C, 30 °C, 34 °C, 37 °C were investigated to optimize laccase production. The fermentation was carried out for 7 days with 80% water moisture and at initial pH of 6.0. The results are presented in Fig. 2. The maximum laccase production (1.6 U/g substrate) and soluble protein concentration (18.3 mg/g substrate) were attained at 30 °C. Decreases in the yield of laccase and soluble protein concentration were observed when the incubation temperature was higher or lower than the optimum incubation temperature. Higher temperature has some adverse effects on the metabolic activities of the microorganisms by denaturing the key enzymes, and the metabolic activities of the microorganisms become slow at lower temperature [40]. As a result, incubation temperature and its control in SSF process is crucial. The heat evolved during SSF processes is accumulated in the medium due to poor heat dissipation in the solid medium, and this may result in reduced microbial activity, thereby decreasing the product yield [41].

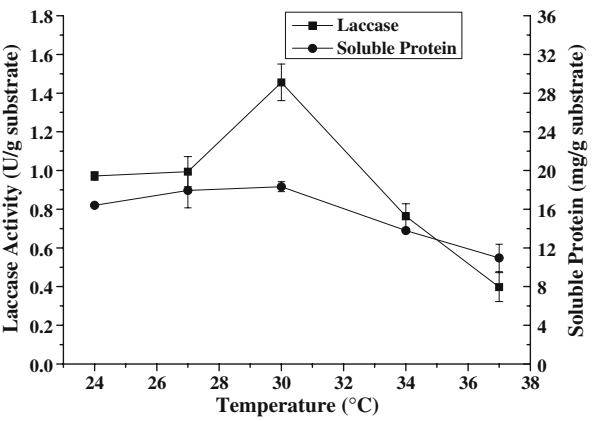

Fig. 2 Effect of incubation temperature on laccase yield and soluble protein concentration

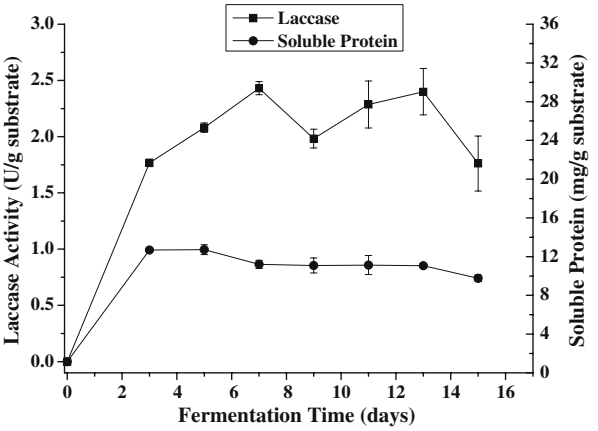

Fig. 3 Effect of fermentation time on laccase yield and soluble protein concentration

Varied fermentation time (3–15 days) was employed to study their effect on laccase production. The fermentation was carried out at 30 °C, 80% water moisture, and initial pH 6.0. The optimal fermentation time for the production of laccase was found on day 7. The decline in enzyme activity was observed after 7 days of incubation. A prolonged time beyond this period did not help to further increase the enzyme yield (Fig. 3).

Effects of Initial pH on Laccase Production

Each microorganism possesses a pH range suitable for its growth and metabolic activity. To evaluate the effects of initial pH value on laccase production under SSF, experiments were conducted at varied initial pH of 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. Water moisture was kept at 80% and incubation was conducted at 30 °C for 7 days using veratryl alcohol as the inducer. The results of six batches of experiments are shown in Fig. 4. It can be observed that laccase yield was comparable in the initial pH range of 5.0 to 9.0. Laccase yield at initial pH value of 7.0 was 1.2-fold of that obtained at initial pH 4.0. Meanwhile, the soluble protein concentration at initial pH 7.0 was also 1.2-fold of that obtained at initial

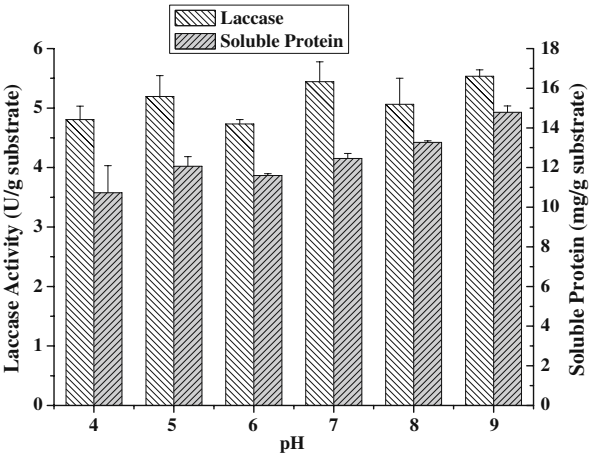

Fig. 4 Effect of initial pH on laccase yield and soluble protein concentration

pH 4.0, indicating that *T. versicolor* ATCC 20869 secreted more effective enzymes under initial pH value 7.0. Filamentous fungi generally have reasonably good growth over a broad range of pH, 2–9, with an optimal range of 3.8 to 6.0 [42]. However, our observations demonstrated that pH higher than 5 was favorable to laccase production.

Effects of Moisture Content on Laccase Production

An optimum moisture level has to be maintained in SSF, as lower moisture level tends to reduce microbial growth, enzyme stability and secretion, and substrate swelling [43]. Higher moisture level leads to particle agglomeration, gas transfer limitation, and competition from bacteria. As oxygen transfer affects both the growth and the metabolism of fungi, the solid substrate should contain suitable amount of water to enhance mass transfer [42]. As a general rule, the moisture levels in SSF processes vary between 30% and 85%. For bacteria, the moisture of the solid matrix must be higher than 70%, and in the case of filamentous fungi, it could be as wide as 20–70% [44].

To investigate the influence of the initial total moisture content of the substrate, the fermentation was carried out under various initial moisture contents (60%, 70%, 75%, 80%, 85%, and 90%). The particle size was greater than 500 μm and the initial pH was 7.0. HW was dried at 105 °C overnight to fully remove the free water. Five grams of the dried HW was added to 250-ml flasks, and varying amount (9.3, 11.7, 15.0, 20.0, 28.3, and 45 ml) of basal medium supplemented with veratryl alcohol was added to the substrate to maintain the moisture levels of 60, 70, 75, 80, 85 and 90, respectively. The flasks were then autoclaved and inoculated. They were then incubated at 30 °C for 7 days. Maximum laccase yield (8.6 U/g substrate) was obtained at the moisture level of 85–90% (Fig. 5). In addition, fungus *T. versicolor* secreted more soluble protein within these moisture contents. Moisture below 85% influenced laccase yield negatively. It is worth noting that laccase yield was more sensitive to the moisture change than soluble protein content, indicating that moisture of 85–90% was favorable to laccase-related protein production and secretion.

Niladevi et al. used various industrial residues to identify the suitable substrate for laccase production in solid-state fermentation by *Streptomyces psammoticus* [45]. The substrates used were wheat bran, rice bran, rice straw, coffee pulp, coir pith, and sugarcane bagasse. They yielded laccase of 10, 5, 17.5, 16.5, 12, and 9 U/g substrate, respectively.

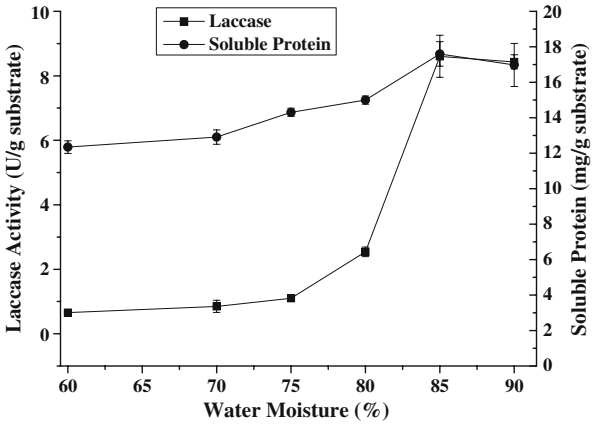

Fig. 5 Effect of water moisture on laccase yield and soluble protein concentration

Vikineswary et al. used agro-residues by *Pycnoporus sanguineus* to produce laccase under SSF [46]. Laccase productivity was the highest during degradation of sago "hampas" and oil palm frond parenchyma tissue. A range from 7.5 to 7.6 U/g substrate was obtained on the 11th day of fermentation, while a maximum laccase productivity of 5.7 U/g substrate was obtained by the degradation of rubberwood sawdust. Rakrudee Sarnthima et al. also found that enzyme production by the white-rot fungus *Lentinus polychrous* Lév under SSF was markedly different according to the substrate used [47]. Rice bran yielded the highest laccase activity of 4.4 U/g substrate after 21 days of cultivation. Rice bran supplemented with rice husk (2:1 by wt) showed highest laccase activity of 10.0 U/g substrate (after 17 days of culture). In our study, the highest laccase activity of 8.6 U/g substrate was obtained after 7 days of cultivation using HW as the substrate under SSF. This is the first report using horticultural waste as the substrate for laccase production by *T. versicolor*. Further improvement is likely by applying proper substrate pretreatment methods or by the supplementation of other substrates, such as rice bran or rice husks.

Dye Decolorization

The decolorization of a typical model dye, phenol red, is a simple approach to assess the aromatic compound degrading capability of ligninolytic enzymes. In order to test the decolorization potentials of the laccase produced by *T. versicolor* ATCC 20869, crude enzyme mixture was prepared by SSF at 30 °C, initial pH value of 7.0, and 85% moisture content using HW of particle size greater than 500 μm as the substrate and veratryl alcohol as the inducer. Enzyme was extracted on day 7. The decolorization was carried out in 10-ml test tubes, and the degradation of phenol red was monitored by measuring the absorbance at 431 nm.

The decolorization results are displayed in Fig. 6. No decolorization was observed for the control, indicating that the decolorization was biological and it was caused by laccase in the samples as no activities of other ligninolytic enzymes such as lignin peroxidase and manganese peroxidase were detected in the enzyme extract. For both the prepared laccase sample and the commercial laccase, the decolorization degree increased with the increase of

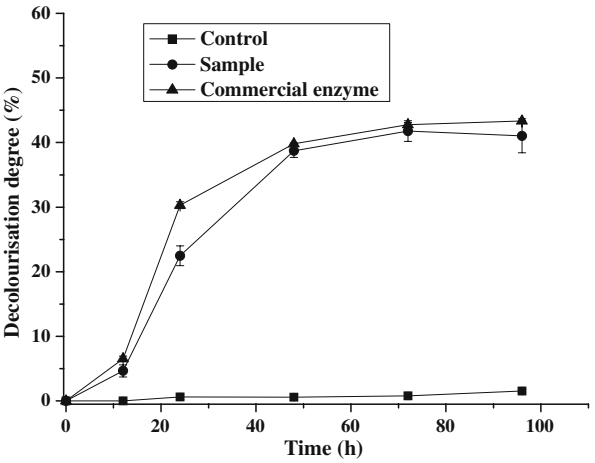

Fig. 6 Decolorization potentials of the prepared laccase samples and the commercial laccase

incubation time. The initial decolorization rate for commercial laccase was higher, indicating that commercial laccase has better decolorization performance than our own enzyme samples. However, such difference became insignificant when the decolorization time reached 48 h. The highest decolorization degree was reached at 72 h for both enzyme samples, being 42.78% for the commercial laccase and 41.79% for our sample. This result suggests that laccase produced under SSF using HW as the substrate is much comparable with the commercial laccase, having a high capability in decolorization. It may have great potentials in aromatic compound degradation and wastewater treatment.

Individual dye structures influence the decolorization extent obtained by laccase. Moldes et al. used the extracellular liquids from the solid-state cultures of *Trametes hirsuta* on grape seeds to degrade different dyes [26]. Indigo carmine and bromophenol blue were completely decolorized in 24 h, whereas phenol red was only 36% decolorized at 24 h, being much more resistance to degradation. When laccase from submerged cultures of *T. versicolor* was employed (using barley bran as the substrate), decolorization of phenol red was limited with a maximum degree of around 40% after 48 h [48]. In our study, around 42% decolorization degree was obtained for both the commercial laccase and our crude laccase samples. Such results were very much consistent with what reported in the literature [26, 48, 49]. However, further improvement is possible by the supplement of laccase mediators [49].

Conclusion

The results obtained in this work have illustrated that horticultural waste collected in Singapore could be a potential substrate for laccase production by *T. versicolor* under solid-state fermentation. Optimization of the enzyme production demonstrated clearly the impact of the process parameters on the gross yield of laccase. Furthermore, the enzyme crude produced by *T. versicolor* in the present study was able to decolorize the dye phenol red effectively, reaching a degree of decolorization higher than 41.79% in 72 h. This result is comparable with that of the commercial laccase, and it suggests the possibility of using such approach to produce large amounts of laccases with high degrading capabilities. Such laccases would subsequently be useful in the degradation of a variety of recalcitrant compounds from industrial wastes. Further characterization of the ligninolytic enzyme complex is necessary in order to enhance its application potentials.

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