

# Decolorization of synthetic melanins by crude laccases of *Lentinus polychrous* Lév.

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Received: 21 November 2011 / Accepted: 10 April 2012 / Published online: 8 June 2012  
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**Abstract** Melanins are complex natural pigments that darken the skin and are difficult to degrade. This study evaluated synthetic melanin decolorization by the crude laccase from fungus *Lentinus polychrous* in the absence and presence of selected redox mediators. The greatest melanin decolorization activity was 87 % at pH 6.5 within 3 h in the presence of 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS), whereas only about 22 % melanin decolorized at pH 5.0 in case of no mediator. The optimum temperatures for melanin decolorization in the absence and presence of ABTS were 55 and 35°C, respectively. Using a natural redox mediator, 1.0 mmol/L vanillin leads to 45 % melanin decolorization. Our results suggest the possibility of applying vanillin for *L. polychrous* laccase-catalyzed decolorization of melanin.

## Introduction

Melanins are complex natural pigments which can be found in many organisms including animals, plants, and microorganisms. Several functions of melanin have been reported. Melanins are believed to offer protection against environmental stresses (Kogej et al. 2006, 2007; Liu and Nizet 2009). Melanins have been reported to increase resistance

to antibiotics in bacteria (Lin et al. 2005), and in fungi, they are involved in fungal pathogenesis (Butler et al. 2005), or survival in extreme environments (Kogej et al. 2006, 2007; Liu and Nizet 2009). In mammals, two types of melanin can be distinguished, a dark eumelanin and a yellow to red pheomelanin. Eumelanin, the more ubiquitous mammalian melanin type, is found in different regions of the human body, including the skin, hair, eye, inner ear, and brain (Clancy and Simon 2001).

Skin depigmentation is of interest in cosmetic skin lightening, and many efforts have been made to find more effective skin-whitening agents (Rendon and Gaviria 2005). Prevention of melanogenesis by using inhibitory agents for tyrosinase can lighten skin color, and these have been studied extensively (Terao et al. 1992; Boissy et al. 2005; Kim and Uyama 2005). An alternative method of skin lightening is direct decolorization of the melanin pigment. Although melanins are very stable compounds, under special conditions, chemical or photochemical degradation is possible as well as biodegradation by fungi (Rättö et al. 2001; Butler et al. 2005). Some fungi have been reported to degrade specific melanins of different origins, and thus, fungal enzymes with melanolytic ability have the potential for use in cosmetics. Woo et al. (2004) reported that lignin peroxidase from *Phanerochaete chrysosporium* was able to decolorize synthetic melanin. Enzymes isolated from *Sporotrichum pruinosum* which could degrade melanin in the human skin have also been reported by Mohorčič et al. (2007).

It has been reported previously that the fungus *Lentinus polychrous* secreted extracellular ligninolytic enzymes mainly laccase and manganese peroxidase. The laccase production on solid-state fermentation and certain synthetic dye decolorization have been successfully studied (Sarnthima et

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al. 2009). In this present work, the application of enzymes of this species in melanin decolorization is firstly reported.

## Materials and methods

### Enzymes preparation

The fungus *L. polychrous* Lév., a kind gift from Rujira Mushroom Farm, Ka La Sin province which is an originate isolate from Phupan, Sakhonnakhon province (northeastern Thailand), was cultured on solid substrates of rice bran and rice husk as previously described (Sarnthima et al. 2009) but with presence of  $\text{Cu}^{2+}$  as a laccase inducer. Crude enzyme from *L. polychrous* was prepared by water extraction from the fungus culture substrate by 1:3 (w/v).

### Enzyme activity assay

Since the fungus mainly secreted laccase into the culture substrate, the crude enzyme was assayed for laccase activity based on the oxidation of 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS; Sigma, USA), according to previously described method (Sarnthima et al. 2009). Oxidation of ABTS was monitored by a UV–vis spectrophotometer at 420 nm ( $\epsilon=3.6 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ ). One unit of laccase activity is defined as the amount of enzyme that catalyzes the oxidation of its substrate into corresponding product 1  $\mu\text{mol}/\text{min}$  at the assay condition.

### Protein determination

Soluble proteins in the crude enzyme were measured by a method described by Bradford (1976) using Bio-Rad Protein Assay Reagent (Bio-Rad, USA) and using bovine serum albumin to create a standard curve.

### Melanin decolorization experiments

Tyrosine plays a key precursor of human melanin synthesis. Therefore, the melanin product of Sigma which was prepared by oxidation of tyrosine with hydrogen peroxide (product number M8631, Sigma, USA) was used as a model melanin for enzymatic decolorization experiments. The fungus was firstly grown on a PDA plate containing 0.02 % (w/v) melanin for 1 week at room temperature. Melanin decolorization ability was observed on the culture plate.

#### *Effect of pH*

Melanin decolorization reactions were performed at pH conditions ranging from 3.0 to 7.0 using the crude enzyme 0.2 U/mL in citrate–phosphate buffer containing 0.02 % (w/v)

melanin. The reactions were done in the absence or presence of a redox mediator ABTS (0.1 mmol/L). The reactions were incubated at 32°C for 3 and 5 h. Control sets of enzymatic reaction were performed in parallel using denatured crude enzyme (boiled at 95°C for 10 min). Control redox mediator was set up in a melanin-containing tube without any crude enzyme. The decolorization of melanin was monitored by a spectrophotometer at 540 nm. Decolorization was expressed as percentage using the following equation:

$$\text{Decolorization efficiency(\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where  $A_0$  is absorbance at 540 nm measured immediately after addition of enzyme and  $A_t$  is absorbance at 540 nm at the final time.

#### *Effect of enzyme activity*

The reactions were performed at the optimum pH condition (pH 5.0) using the crude laccase at different amounts (0.2, 0.4, and 0.8 U/mL) in the absence or presence of ABTS redox mediator (0.1 mmol/L) at 32°C for 3 h.

#### *Effect of temperature*

Melanin decolorization reactions were carried out under pH 5.0 using the crude laccase of activity 0.2 U/mL in the absence or presence of ABTS redox mediator (0.1 mmol/L) at different temperatures ranging from 30 to 60°C for 3 h.

#### *Effect of redox mediator type*

The reactions were performed at pH 5.0 using the crude laccase of activity 0.2 U/mL in the absence or presence of ABTS, vanillin, or vanillic acid (0.1 mmol/L) at 32°C for 3 h.

#### *Effect of selected redox mediator concentration*

The melanin decolorization reactions were performed at pH 5.0 using the crude laccase of activity 0.2 U/mL in the absence or presence of vanillin or vanillic acid at concentrations ranging from 0 to 10 mmol/L at 32°C for 3 h. All experiments were performed triplicate. Values shown in result parts are presented as mean values  $\pm$  standard deviation.

#### *Cytotoxicity of treated melanin*

To test whether the enzymatic degradation products of melanin are nontoxic to environment, the treated reaction mixture was added to a culture of *Escherichia coli* in Luria–

Bertani (LB) broth. In a 5-mL culture, 150  $\mu$ L of untreated or treated melanin was added (final concentration of melanin  $\sim$ 0.0006 %, w/v). The cultures were grown at 37°C and 140 rpm shaking, and growth curve of the bacteria was observed periodically until early stationary phase was reached. Control conditions of untreated melanin as well as a normal growth were done in parallel. The toxicity reduction percentage (%TR) of the melanin was calculated by the following equation:

$$\text{TR}(\%) = \frac{A_{600,\text{tm}} - A_{600,\text{m}}}{A_{600,\text{ctrl}}} \times 100$$

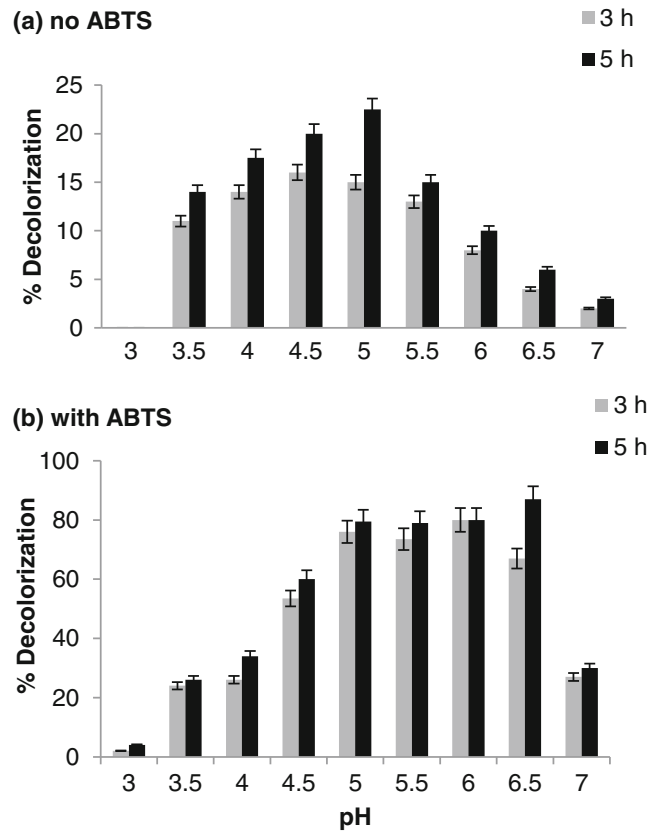
where  $A_{600,\text{tm}}$  is the absorbance at 600 nm at early stationary phase (9 h) of a bacterial suspension grown in the presence of treated melanin,  $A_{600,\text{m}}$  is the absorbance of the bacterial suspension grown in the presence of untreated melanin, and  $A_{600,\text{ctrl}}$  is the absorbance of the bacterial suspension grown in absence of melanin. Experiments were performed in triplicate.

## Results

The fungus *L. polychrous* grown on PDA plates containing synthetic melanin (0.02 %, w/v) at room temperature showed melanin decolorization ability as it appeared slightly lighter in color than uninoculated plate (data not shown), suggesting the possibility of the fungus itself or its extracellular enzymes. Therefore, the fungus was cultured on solid-state fermentation as state to produce its crude enzymes for study of melanin decolorization. The crude enzyme had a specific laccase activity 6.99 U/mg protein.

### Effect of pH

To investigate decolorization of melanin in vitro by the crude extracellular enzyme from *L. polychrous*, the optimum pH was evaluated first. Reactions were performed at different pH conditions ranging from 3.0 to 7.0 at 32°C. The results found that in the absence of a synthetic redox mediator, ABTS, similar percentages of melanin decolorization were obtained at pH 4.0–5.5 (around 13–16 %) within 3 h of treatment, where the highest yield was approximately 22 % at pH 5.0 within 5 h (Fig. 1a). In the presence of ABTS, melanin decolorization percentage clearly increased under all pH conditions. The results in Fig. 1b show that high levels of melanin decolorization could be observed in a broad range of pH conditions (pH 4.5–6.5) with around 80 % decolorization at pH 5.0–6.0 occurring within 3 h. The highest value was 87 % at pH 6.5 within 5 h. The results of control experiments, with no enzyme or with denatured enzyme, showed no melanin decolorization (data not shown).



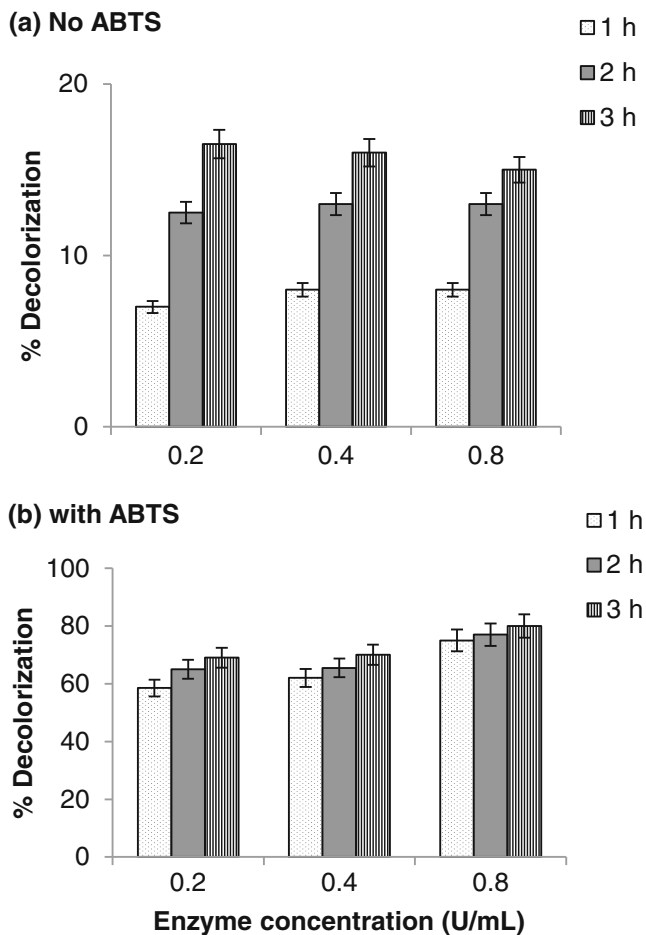
**Fig. 1** pH profiles for melanin decolorization by the crude enzyme secreted by *L. polychrous* in the absence of ABTS redox mediator (a), and in the presence of ABTS (0.1 mmol/L) (b) at 3 and 5 h

### Effect of laccase quantity

To study the effect of laccase activity on melanin decolorization by the crude enzyme, reactions with three different laccase amounts (0.2, 0.4, and 0.8 U/mL) were set up in the absence or presence of ABTS at pH 5.0 at 32°C. In the absence of ABTS, no difference between results was observed (Fig. 2a). Meanwhile, in the presence of ABTS, the higher laccase activities increased only slight decolorization percentages by approximately 10 %. The maximum percentage decolorization was about 80 % within 3 h.

### Effect of temperature

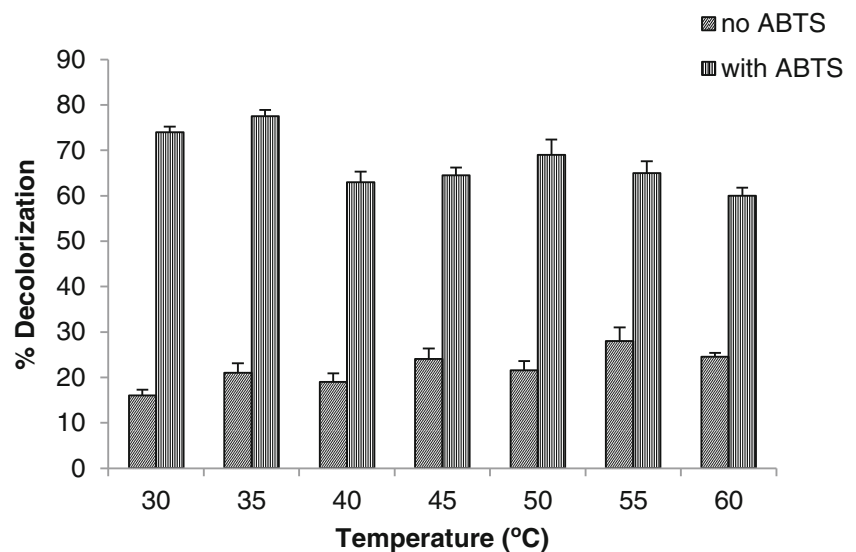
To observe whether temperature had any effect on melanin decolorization, the reactions were performed under temperatures ranging from 30 to 60°C. The results show that in the absence of a redox mediator, the increase in temperature could not improve much the melanin decolorization. The temperature yielding the highest decolorization (28 %) was 55°C. However, at the highest tested temperature (60°C), the decolorization efficiency decreased slightly (Fig. 3). In the presence of ABTS redox mediator (0.1 mmol/L), the increase in temperature seemed not improve the decolorization ability.



**Fig. 2** Effect of laccase activity on melanin decolorization by the crude enzyme secreted by *L. polychrous* in the absence (a) and in the presence of ABTS redox mediator (0.1 mmol/L) at various times (b)

However, in this case, the optimum temperature is 35°C where the highest amount of melanin was decolorized (77.5 %) as shown in Fig. 3.

**Fig. 3** Melanin decolorization by the crude enzyme secreted by *L. polychrous* in the absence and in the presence of ABTS (0.1 mmol/L) at various temperatures for 3 h



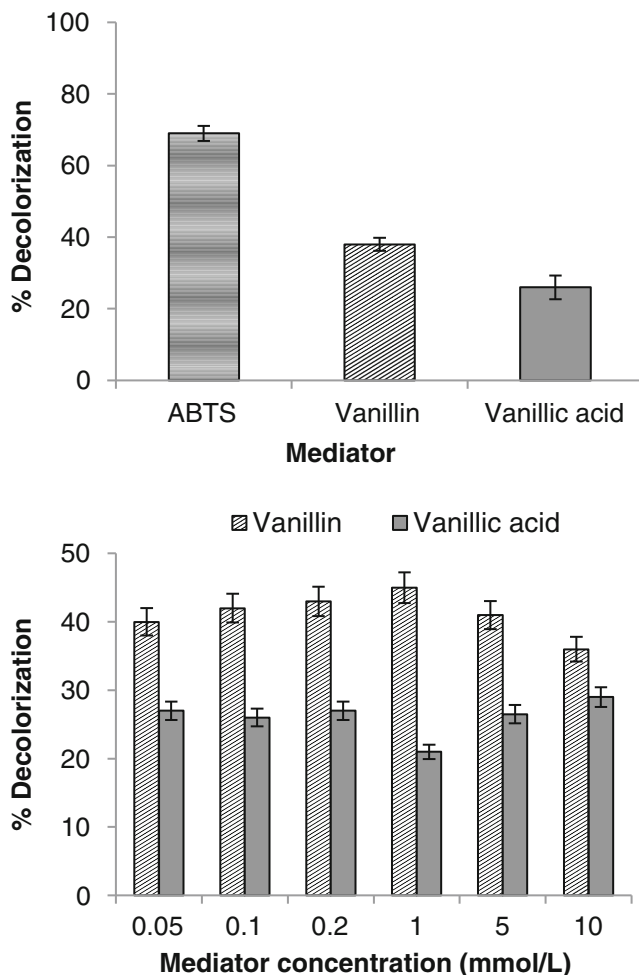
Because using ABTS as a redox mediator might not be safe for the consumer even in the small amounts used in this study (0.1 mmol/L), enzymatic reactions with the natural redox mediators, vanillin, and vanillic acid were conducted to determine their ability to decolorize melanin. The results of this test were compared with the results from enzymatic reactions using ABTS. The results showed that in the presence of ABTS, melanin could be decolorized by approximately 70 % within 3 h, whereas only about 38 and 26 % decolorization were obtained using vanillin and vanillic acid as redox mediators, respectively (Fig. 4a).

When the reactions were carried out at different concentrations of redox mediators ranging from 0 to 10 mmol/L at pH 5.0, the results showed that the redox mediator concentration had little effect of melanin decolorization ability. Vanillin at concentrations of 0.05–1.0 mmol/L resulted in melanin decolorization by approximately 40 %. The concentration with the highest amount of decolorization was 1.0 mmol/L vanillin, which yielded 45 % melanin decolorization (Fig. 4b).

In the presence of vanillic acid at different concentrations, melanin decolorization ability was not significantly different between 0.05 and 0.2 mmol/L with about 27 % decolorization. However, a lower decolorization percentage was obtained at higher concentrations as shown in Fig. 4b, and if the concentrations of both redox mediators were higher than 1.0 mmol/L, turbid precipitations occurred but did not improve melanin decolorization efficiency.

#### Cytotoxicity of treated melanin

After treating melanin with the crude enzyme for 2 days in the absence of ABTS redox mediator, the reaction mixture

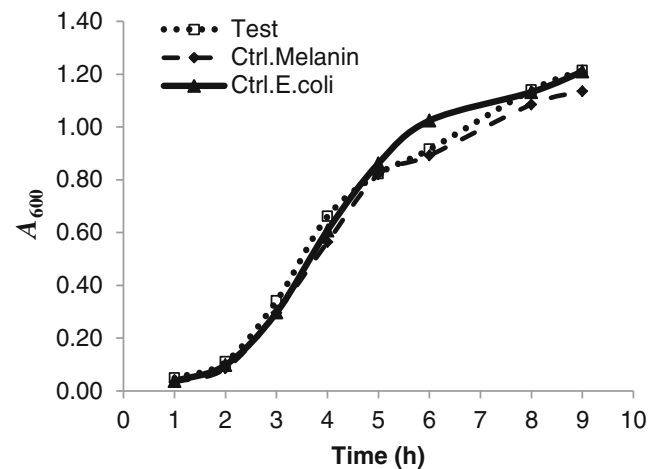


**Fig. 4** Effect of redox mediator type (a) and concentration (b) on melanin decolorization by the crude enzyme secreted by *L. polychrous* at 3 h

was tested for cytotoxicity using *E. coli* as a microorganism model. The result appeared that the culture containing treated melanin mixture showed intently higher growth compared to the condition containing untreated melanin. The toxicity reduction percentage was approximately 6.5 %. The growth curves are as shown in Fig. 5.

## Discussion

Melanin is a complex stable polymer, difficult to degrade. In this study, the fungus *L. polychrous* and its extracellular enzymes were able to decolorize synthetic melanin made from the human melanin precursor L-tyrosine. The fungus was grown and decolorized PDA plates containing melanin (0.02 %, w/v), suggesting that this fungus is able to decolorize melanin. Partial melanin bleaching on PDA plate was also reported in *P. chrysosporium* (Woo et al. 2004) and some other fungal strains (Rättö et al. 2001). The fungus secreted extracellular enzymes containing both laccase and



**Fig. 5** Growth curve of *E. coli* cultured in LB medium containing treated and untreated melanin by crude enzyme of *L. polychrous* at 37°C, 9 h

Mn peroxidase activities (Sarnthima et al. 2009). Melanin decolorization by fungi has been reported in *Phlebia radiata* strain BP-11-2 (Kaneko et al. 2009) and *S. pruinosum* (Mohorčič et al. 2007). The main enzymes responsible for that ability are laccases (Kaneko et al. 2009) and other ligninolytic enzymes (Butler and Day 1998; Woo et al. 2004).

By catalytic reaction of recalcitrant compounds by oxidoreductase, laccase in the presence of certain small molecule acts as a redox mediator can widen its substrate (Morozova et al. 2007). The crude enzyme from this fungus also showed ability to decolorize melanin in vitro in the absence or presence of a synthetic redox mediator ABTS. However, in the presence of ABTS, melanin decolorization was more effective. This may occur because ABTS is a good laccase substrate that easily loses its electron to become a cation radical. ABTS radicals can react with melanin, therefore inducing melanin decolorization. Redox mediators thus indirectly help the enzyme catalyze the oxidation of substrates, and are why higher melanin decolorization was obtained in the presence of ABTS. Many investigations have used ABTS as a laccase redox mediator for decolorization of dyes such as Reactive Black 5 (Chhabra et al. 2008) and Acid Violet 17 (Camarero et al. 2005). Melanin decolorization by the crude enzyme from this fungus was highly efficient at pH conditions around 4.0–6.5. This might be because the laccase enzymes are stable and active in this pH range for this substrate.

The effect of laccase amount on melanin decolorization by the crude enzyme in the absence or presence of ABTS at pH 5.0 at 32°C showed unclear increase in decolorization efficiency. Normal enzymatic catalysis efficiency or reaction rate is influenced by the quantity of biocatalyst. For example, the decolorization of RB-5 and Remazol Brilliant Blue R (RBBR) by laccase of *Ganoderma lucidum* KMK2



increased with increase in enzyme quantity (Murugesan et al. 2007). Our results showed not much increase in melanin decolorization when the enzyme amount was increased. This might be because melanin is complex and difficult to degrade and enzymatic oxidation is only one in a series of reactions. Moreover, using the much higher amount of crude enzyme or high specificity of purified enzyme might improve the overall percentages. In some dye decolorization experiment, to obtain maximal decolorization amounts, as high as 25 U/mL enzyme was used (Murugesan et al. 2007). This also agrees in another report such as in RBBR decolorization by laccase of *Trametes pubescens* (Rodríguez-Couto 2011).

The experiment with higher amount of crude enzyme or using purified laccase of this fungal species later might be performed to extend information. Many fungal laccases show high temperature optima and thermostability which are one of the important parameters for enzymatic catalysis including dye decolorization. Typically, the enzyme reaction is increased according to temperature rising before it reaches the point of denaturation. The optimum temperature for dye decolorization by *G. lucidum* laccase was 60°C (Murugesan et al. 2007). However, in this study, the optimum temperature for melanin decolorization by *L. polychrous* crude laccase was observed at 35°C.

The efficiency of melanin decolorization, by the extracellular enzyme from *L. polychrous* in the absence of ABTS, was lower (less than 30 % melanin decolorization) than in the presence of ABTS even though there were increased enzyme activity and temperature. Melanin was highly decolorized by the enzyme from this fungus when the redox mediator was present (about 70–80 % decolorization). The high efficiency shows there is no need to increase the enzyme activity or performance at higher temperatures.

Previously, it has been reported that vanillin and vanillic acid could be used as redox mediators in Rhodamine B decolorization by laccase enzyme from *Trametes versicolor*, but they were not good redox mediators for this reaction (Khamuang and Sarnthima 2009). Other reports have studied vanillin and vanillic acid as redox mediators in dye decolorization, for instance in decolorization of Reactive Black 5 by laccase from *Pycnoporus cinnabarinus* (Camarero et al. 2005) and decolorization of disperse dye (Satar and Husain 2009). Vanillin and vanillic acid can be used as redox mediators for melanin decolorization by the crude enzyme from *L. polychrous*, but the efficiency is less than ABTS's. Because these two natural compounds are regarded as safer than synthetic ABTS, higher concentrations were tested to see if improved decolorization efficiency could be achieved.

There are very limited reports about fungal extracellular laccase being involved in melanin degradation. For instance, laccase from *P. radiata* strain BP-11-2 could decolorize fungal melanin (Kaneko et al. 2009). However, laccase of

this strain did not act on synthetic melanin, which is synthesized from L-tyrosine, unlike the crude enzyme from *L. polychrous* used in this work. Melanin decolorization by peroxidase has been reported in human hair melanin by peroxidase from *Ceriporiopsis* sp. strain MD-1 (Nakasaka et al. 2008) and by lignin peroxidase from *P. chrysosporium* (Woo et al. 2004). Decolorization of melanin by the crude enzyme from *L. polychrous* is initially believed to be involved with laccase, since no hydrogen peroxide has been added into the decolorization reactions. However, more investigations have to be performed to reveal what is exactly responsible. Apart from skin-whitening agents or safe hair-coloring products, enzymatic melanin decolorization might also be applied to other areas such as a biological control approach of phytopathogenic fungi (Butler et al. 2005) or protection of blue stain fungi on wood (Rättö et al. 2001).

The melanin decolorization ability is less effective in the absence of a redox mediator. The results also suggest that the optimal type and concentration of redox mediator may differ depending on the source of laccase and dyes or color substrates to be decolorized. The optimum conditions for melanin decolorization by the enzyme were determined. Vanillin could be used as an alternative safer redox mediator for melanin decolorization by the crude enzyme from this fungus.

The enzymatic degradation products of melanin by crude enzyme of this fungus showed less toxicity towards *E. coli* compared to the untreated condition. However, only a small TR percentage was observed here. This might be because of the very low amount of melanin content in the reactions (~0.0006 %, w/v). A higher amount of melanin might give more clear toxicity on the bacterial growth and in the similar reason for the case of treated melanin. Studies on human hair melanin decolorization by the crude enzyme from this fungus as well as skin-whitening experiments have been designed and are underway in our research group.

**Acknowledgments** This work was financially supported by Mahasarakham University (fiscal year 2009). The authors thank P. Suwannawong for the laboratory assistance and thank Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education and Department of Chemistry, Faculty of Science, Mahasarakham University for the partial supports.

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