International Biodeterioration & Biodegradation 87 (2014) 109–115

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Screening of *Trametes* strains for efficient decolorization of malachite green at high temperatures and ionic concentrations

Jinping Yan [a], Jiezhen Niu [a], Daidi Chen [a], Yuhui Chen [b], Chagan Irbis [a,*]

[a] Biotechnology Research Center of Life Science and Technology College, Kunming University of Science and Technology, Kunming, Yunnan 650500, PR China
[b] College of Life Science, the Southwest Forest University, Kunming, Yunnan 650224, PR China

ARTICLE INFO

Article history:
Received 17 September 2013
Received in revised form
14 November 2013
Accepted 14 November 2013
Available online 7 December 2013

Keywords:
Trametes trogii
Laccase
Malachite green
Decolorization

ABSTRACT

Trametes (synonym *Coriolous*) species are the most important sources for laccases with attractive properties. Several *Trametes* species were screened for their ability to produce laccase and decolorize malachite green (MG). *Trametes trogii* Berk S0301 was chosen for further study because it showed the highest 2,2′-azino *bis* (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) oxidization ability, which is closely correlated with MG decolorization. The laccase activity of *T. trogii* Berk S0301 was enhanced up to 34.3-fold and 5.6-fold by Cu^{2+} (1.5 mM) and Tween 80 (7 g L^{-1}), respectively. Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} and Na^{+} (100 mM) only slightly (less than 10% inhibition) affected MG decolorization by the crude laccase of *T. trogii* Berk S0301. MG was decolorized effectively at pH 4.5–7.0 and 30–80 °C with an optimum decolorization at pH 6 and 50–70 °C. Phyto- and micro-toxicity tests revealed that less toxic metabolites were formed after laccase treatment. The present work demonstrates that strain *T. trogii* Berk S0301 and its laccase may facilitate novel and more efficient bio-catalytic process applications.

1. Introduction

Malachite green (MG) is a synthetic dye used to color silk, wool and leather, as well as an effective biocide that is widely used to resist fungal, parasitic and bacterial infections, especially in aquaculture (Bergwerff and Scherpenisse, 2003; Srivastava et al., 2004). However, MG is highly toxic to mammalian cells and aquatic organisms even at trace concentrations (Panandiker et al., 1992; Culp and Beland, 1996; Manel et al., 2009). As a consequence, the public may be placed at risk because approximately 10–14% of the total dye used in the dying process may remain in wastewater (Vaidya and Datye, 1982), and MG-treated fish may be eaten without consumer awareness. Therefore, studies aiming to decolorize or detoxify MG and its metabolites are of great interest.

In recent years, the use of microbial biomass and enzymes for decolorization and detoxification of MG-containing wastewater has emerged as a promising solution. Previous studies have demonstrated that many enzymes are involved in MG decolorization and detoxification and those enzymes are often specific to particular species. Biodegradation of MG in yeast has been shown to involve the activities of laccase, lignin peroxidase, aminopyrine *N*-demethylase, NADH–DCIP reductase and malachite green reductase, of which NADH–DCIP reductase and MG reductase are the most important (Jadhav and Govindwar, 2006). Laccase and MG reductase are responsible for MG decolorization and detoxification in *Pseudomonas* bacteria (Kalyani et al., 2012), the non-basidiomycete filamentous fungi *Penicillium pinophilum* and *Myrothecium roridum* (Jasinska et al., 2012). In addition to the enzymes mentioned above, lignin peroxidases, manganese peroxidases (MnP) and laccases in white rot fungi (WRF) are currently the focus of much attention, especially the laccases (Grassi et al., 2011).

Laccase (EC 1.10.3.2, Lac), secreted by most of the WRF, is one of the most important lignin oxidation enzymes that can non-specifically degrade many phenolic and non-phenolic compounds, such as lignin and many environmental pollutants (including synthetic dyes, toxic substances found in industrial effluents, and herbicides and pesticides in the environment) (Rodríguez Couto and Toca Herrera, 2006; Rodgers et al., 2009). However, in terms of the practical applications of laccases to decolorize and detoxify dyes, such as MG and MG-containing wastewater, there are still many difficulties to overcome. Industrial waste generally contains high levels of salt, metal ions and organic compounds, which could cause laccase denaturation (Grassi et al., 2011). Therefore, there is an urgent need for a laccase with tolerance to organic solvents, high temperatures and ionic concentrations, functionality over a wide pH range.

* Corresponding author. Tel.: +86871 65952573.
E-mail address: sunflower201201@gmail.com (C. Irbis).

0964-8305/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2013.11.009

Previous studies have demonstrated that laccases produced from various microorganisms have unique advantages and disadvantages, which makes it possible to search for laccases with special properties from different strains. Among the WRF, the *Trametes* (synonym *Coriolous*) species are the most important sources for laccases with attractive properties such as thermotolerance and higher resistance to high alkalinity, extreme acidity, organic solvents or heavy metals (Boonlue et al., 2003; Hildén et al., 2009; Grassi et al., 2011; Daâssi et al., 2013).

The main objectives of this study were (i) to investigate MG decolorization and laccase production by *Trametes* (synonym *Coriolous*) strains identified by the morphological characters, from Yunnan, China; (ii) to enhance the laccase production of the potential strains; and (iii) to assess the effects of metal ions, pH and temperature on the MG decolorization by laccase.

2. Materials and methods

2.1. Chemicals and cultures

ABTS was obtained from Sigma (USA), while Malachite green (MG), Gentian Violet and Congo Red were obtained from Merck (USA). Reactive Black, Reactive Green, Reactive Blue and Reactive Red were supplied by the Environmental Monitoring Station of Dali, Yunnan province, China.

Strains used in this study are routinely maintained on 2% malt extract agar (MA) slants at 4 °C. The mycelia from the slant are transferred to the MA plates and incubated at 30 °C for 7 d. Fungi are stored at the strain collection of Biotechnology Research Center of Life Science and Technology College, Kunming University of Science and Technology.

2.2. MG decolorization and localization of laccase activity on the solid medium

MG decolorization screening was performed on MA medium supplemented with MG at a final concentration of 15 mg L^{-1}. The plates were inoculated with agar disks (5 mm diameter) of growing mycelia and incubated at 30 °C. MG decolorization was estimated after 2, 4, 8 and 15 d of incubation by measuring the diameters of the colony and the coloration zone of each strain. Localization of laccase activity was determined according to the method of Hiscox et al. (2010). A mycelial block of diameter 5 mm from the 2% MA plate was transferred to a 9 cm Petri dish with 250 mg L^{-1} ABTS, and the diameter of colony and the green/violet zone was recorded at 2, 4, 8 and 15 d, respectively.

2.3. Laccase production

The mono-factorial experiment was conducted using the GYP as the base medium (Galhaup and Haltrich, 2001). For the carbon source tests, 2% (*w*/*v*) CMC, wheat bran, xylose, maltose, glucose, fructose, lactose or sucrose were added to replace glucose in GYP medium. For the nitrogen source tests, 2% (*w*/*v*) glucose was supplied as the carbon source, and 2% (*w*/*v*) $(NH_4)_2SO_4$, peptone, yeast extract, malt extract, or ammonium tartrate were used to replace the mixture of peptone and yeast extract, or the concentration of the peptone/yeast extract mixture was doubled. Cu^{2+} was added at increasing concentrations from 0 to 3.0 mM to explore the optimal concentration.

Many reports have shown the stimulative effects of surfactants on enzyme production by microorganisms. To further improve the laccase activity, the effects of Tween 80, sodium dodecylsulfate (SDS), polyethylene glycol 4000 (PEG 4000) and cetyl-trimethyl ammonium bromide (CTAB) (0.05 g L^{-1}) on laccase production were determined. Based on the results of the single factor experiments, a modified GYP medium containing 2% glucose (*w*/*v*), 2% peptone and yeast extract mixture (1:1, *w*/*v*), 1.5 mM $CuSO_4$ and 8.3 mM $MgSO_4$ was used to determine the effects of surfactants on laccase production. Tween 80, sodium dodecylsulfate (SDS), polyethylene glycol 4000 (PEG 4000) and cetyl-trimethyl ammonium bromide (CTAB) (0.05 g L^{-1}) were added to treat the 3-d-old fungal cultures incubated at 30 °C and shaken at 200 rpm. To assay optimal concentration, Tween 80 was tested at various concentrations (0–12 g L^{-1}). Laccase activity was measured daily for 14 d. Crude enzyme solutions were obtained via centrifugation (10,000*g*, 10 min) at 4 °C and the supernatants were used for further study.

2.4. Enzyme assays

Laccase activity was determined with ABTS ($\varepsilon_{420} = 36\,(\text{mM cm})^{-1}$) as the substrate (Bourbonnais and Michael, 1990). A mixture of 0.5 mL of appropriately diluted crude enzyme and 1.1 mL of 2 mM ABTS in phosphate citrate buffer (100 mM, pH 4.0) was used to determine the activity. The increase in absorbance was monitored at 420 nm in 3 min. One unit of the enzyme activity was defined as the amount of the enzyme that oxidized 1 μmol of the ABTS per minute. MnP activity (E.C:1.11.1.13) was measured using phenol red as the substrate in 0.1 M sodium dimethyl-succinate buffer (pH 4.5) at room temperature ($\varepsilon_{610} = 22\,(\text{mM cm})^{-1}$) (Glenn and Gold, 1985).

2.5. Effects of pH, temperature and metal ions on MG decolorization

Unless otherwise indicated, all experiments were performed as follows: 3 mL of reaction mixture was prepared containing 10 mM acetate buffer (pH 4.5), 25 mg L^{-1} MG and 0.5 U mL^{-1} laccase from the crude enzyme extracts. The reactions were carried out at 30 °C in the dark. The absorbance of the mixture at the maximum wavelength of MG (λ_{max} 620 nm) was recorded at 0.5, 1–6 and 8 h. Decolorization was calculated according to the following formula: decolorization (%) = $A_0 - A/A_0{}^*100\%$, where A_0 was the initial absorbance and A was the final absorbance. Control samples were run in parallel containing the reaction mixture with the same amount of heat-denatured laccase.

The effect of pH on MG decolorization was determined using citrate–phosphate buffer (100 mM) at pH 4.5, 6.0 and 7.0. The effect of temperature on decolorization was tested in a range from 30 to 80 °C at pH 6.0. To determine the effect of metal ions on the decolorization of MG by laccase, $MnSO_4$, $MgSO_4$, $NiCl_2$, $CaCl_2$, $CuSO_4$, $ZnSO_4$, $CoSO_4$, $FeSO_4$, $FeCl_3$ or NaCl were added to the reaction mixture, which consisted of 10 mM acetate buffer (pH 4.5), 25 mg L^{-1} MG and 0.5 U mL^{-1} crude laccase enzyme. All metal ions were used at 100 mM, except for $FeSO_4$ and $FeCl_3$, which were used at 1 mM.

2.6. Dye decolorization at high temperature

The dye decolorization reaction was performed by adding appropriately diluted crude enzyme (with a final concentration of 0.5 U mL^{-1} laccase) to a 3-mL reaction mixture containing different dyes in 100 mM citrate–phosphate buffer (pH 6.0) and incubated at 60 °C. The concentration of each dye was as follows: MG (150 mg L^{-1}), Reactive Black (50 mg L^{-1}), Reactive Green (50 mg L^{-1}), Gentian Violet (20 mg L^{-1}), Congo Red (60 mg L^{-1}), Reactive Blue (50 mg L^{-1}) and Reactive Red (50 mg L^{-1}). The absorbance of the mixture at the maximum wavelength of each dye was recorded at 0.5, 1–6 and 8 h. The maximum wavelengths of Reactive Black, Reactive Green, Gentian Violet, Congo Red, Reactive Blue and Reactive Red are 585, 420, 590, 488, 610 and 520 nm,