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Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment

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Abstract Laccases are enzymes belonging to the group of oxidases. Laccases catalyze the oxidation of a variety of phenolic compounds, diamines and aromatic amines. Twenty-four fungal isolates were isolated from biodeteriorated ancient paper and parchment. These isolates were identified and found to belong to six genera: *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Rhizopus* and *Trichoderma*, and were tested for producing laccase enzyme. *Trichoderma harzianum* have the ability of secreted laccase enzyme. The maximum production of laccase enzyme by *T. harzianum* was observed at 35 °C and pH 5 after 6 days. The highest activity of laccase achieved at 35 °C and pH 5 during the reaction. FTIR analysis revealed that the structure of extracted fungal pigments has aromatic ring and phenols group. Crude laccase was capable to decolorize different pigment structures. The enzyme showed great decolorization efficiency toward the extracted yellow pigment produced from *Asp. terrus* and *Asp. ochareceous* treated by 200 µl of partially purified enzyme. Laccase enzyme was used to decolorization pigment secreted from deteriorated pigmented fungi on paper and parchment during 30 days by using a pieces of paper and parchment inoculated by spore suspension. The results indicated that a high removal effect of fungal pigment on paper (71.21%) was recorded comparing to parchment samples (32.39%).

Introduction

The use of enzyme in the diverse field of industrial application is of greater importance in recent years. Many of such potential enzymes are widely distributed in nature; laccase is one among them which is oldest and most studied enzymatic system. Laccase is currently the focus of much attention because of its diverse applications such as dye decolorization, waste detoxifications and bioremediation applications. Laccases catalyze the oxidation of a broad range of substrates such as ortho and para-diphenols, methoxy-substituted phenols, aromatic amines, phenolic acids and several other compounds

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coupled to the reduction of molecular oxygen to water with one electron oxidation mechanism (Atallah et al., 2013).

Laccase is most widely distributed in a wide range of higher plants, fungi and bacteria (Benfiled et al., 1964; Diamantidis et al., 2000). Laccases are secreted out in the medium extracellulary by several fungi during the secondary metabolism but not all fungal species produce laccase such as Zygomycetes and Chytridiomycetes (Morozova et al., 2007). Fungi belong to Deuteromycetes, Ascomycetes as well as Basidiomycetes are known producers of laccase (Gochev and Krastanov, 2007; Sadhasivam et al., 2008).

Archives, museums and libraries worldwide conserve many historical document collections of important cultural value for all humankind. These documents may be composed of paper but oldest may be of parchment (Kraková et al., 2012). Many microorganisms that cause deterioration to ancient paper and parchment have been exploited for the production of color, because the microbial colors have the advantage of being climate independent, do not require large area for their growth and can be produced in any quantity in shorter period (Sanjay et al., 2007). Fungal degradation of library materials and paintings causes different kinds of damage depending on the species of organism responsible for the attack and the characteristics of the substratum. Damage can occur because of mechanical stress, production of staining compounds or enzymatic action (Blyskal, 2009; López-Miras et al., 2013; Pinzari et al., 2010; Santos et al., 2009; Sterflinger, 2010).

Microbial pigments are typically composed of many complex chemical substances that are formed during metabolic process (Szczepanowska and Lovett, 1992). The fungal species are found on paper and produce characteristic stains: *Alternaria solani*, *Pencillium notatum*, *Fusarium oxysporium* and *Chaetomium globosum* (Szczepanowska and Lovett, 1992). Among the bacteria, *Bacillus*, *Micrococcus* and *Pseudomonas* were commonly found on paper and leather and *Actinomycetes* were also present such as *Streptomyces*. Yeasts, such as *Candida*, were isolated (Valentin, 2010(.

Chemical compounds were effective in removing fungal stains present varying degree of toxicity and should be handled with extreme care (Tavzes et al., 2013; Szczepanowska and Lovett, 1992). The enzyme laccase has recently been employed in bioremediation, biofuel, biosensor, and organic synthesis applications (Dubé et al., 2008) as well as in textile industries to decolorize a variety of pigments and dyes (Ramsay and Goode, 2004) and may have cultural heritage applications (Konkol et al., 2009). The use of laccase in decolorization of unwanted pigment on ancient paper or parchment instead of using chemicals is considered a safety solution. Ramsay and Nguyen (2002) found that after decolorization by laccase enzyme, toxicity of few dyes remained the same while some became nontoxic.

The current work was designed to optimize the conditions of laccase enzyme activity and its use for decolorizing the fungal pigment on ancient paper and parchment.

Materials and methods

Isolation and identification of fungal isolates

All fungi were isolated from biodeteriorated paper from Kasr Abdin, Cairo, Egypt, and biodeteriorated parchment from Othman's manuscript, Cairo, Egypt, on Czapek's agar medium (Difco, 1984).

Identification of fungal isolates was accomplished depending on colonial characters of the pure culture, microscopic characters and dimensions of informative character of each fungal isolate using a specific program (Axio Vision 4.7) for measurement (with help of computerized Carl Zeiss microscope Axioplane 2) and comparing it with that are present in the identification references (Gilman, 1969; Traute et al., 1980; Alexopoulos et al., 1985).

Screening of laccase producing fungi

All collected fungal isolates were cultured on Petri plates containing sterilized potato dextrose agar (PDA) (ATCC, 1982) supplemented with 0.04% guaiacol (Sigma, USA) and 0.01% (w/v) chloramphenicol (to avoid bacterial growth) adjusted at pH 5.5. These Petri plates were incubated at 28–30 °C for 72 h and then screened for the formation of reddish brown zones around the fungal colonies (Kalra et al., 2013).

Fermentation process for growth and enzyme production

Standard inoculum (disk of fungal growth 2 mm) of the most potent fungus was cultivated into 250 ml flasks containing 100 ml of productive liquid medium (Kalra et al., 2013) which contained the following: 3 g peptone, 10 g glucose, 0.6 g KH_2PO_4, 0.001 g $ZnSO_4$, 0.4 g K_2HPO_4, 0.0005 g $FeSO_4$, 0.05 g $MnSO_4$ and 0.5 g $MgSO_4$ per L, pH 5.5, and then incubated at 30 °C for 12 days on rotary shaker (150 rpm). Fungal growth and enzyme activity were assayed periodically.

Guaiacol assay method for laccase assay

Oxidation of guaiacol has been reported for laccase assay by Kalra et al. (2013). The reddish brown color developed due to oxidation of guaiacol by laccase is used to measure enzyme activity at 450 nm. The reaction mixture can be prepared as follows:

(a) Guaiacol (2 mM) 1 ml.
(b) Sodium acetate buffer (10 mM) 3 ml.
(c) Enzyme source 1 ml (fungal supernatant).

A blank was also prepared that contains 1 ml of distilled water instead of enzyme. The mixture was incubated at 30 °C for 15 min and the absorbance was read at 450 nm using UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 μmol of guaiacol per min. The laccase activity in U/ml is calculated by this formula:

$$\text{E.A} = A \times V/t \times e \times v$$

where
E.A = Enzyme activity
A = Absorbance
V = Total mixture volume (ml)
v = enzyme volume (ml)
t = incubation time
e = extinction coefficient for guaiacol (0.6740 μM/ cm).