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Toxicity and dyeing properties of dyes obtained through laccase-mediated synthesis

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ABSTRACT

The use of enzymes in textile processes has many advantages as far as environmentally friendly processes are concerned. These advantages include water and energy savings, the use of lower amounts of chemicals, and mild and more sustainable production processes. Several orange–red colored bio-dyes were synthesized during laccase-mediated homomolecular or heteromolecular transformations of simple aromatic precursors. Due to the presence of many various substituents in the structure of these precursors, ensuring their laccase-mediated transformation into colored products, the dyes obtained were tested for their toxicity effect against humans and the environment. The evaluation of environmental toxicity employed the bioluminescent marine bacterium (*Vibrio fischeri*) as a test organism according to the Microtox® basic test protocol. The effect of dyes on human cells was tested in culture of normal human colon epithelial cells, using two independent tests. Despite the presence of the reactive amino group in the structure of precursors, a majority of the dyes obtained were non-toxic. The presence of a carboxylic and/or sulfonic group in the formula of the precursors ensures a good solubility of the dyes in water. The dyes obtained possess good dyeing activity towards natural and synthetic fibers. Mild conditions of synthesis thereof, as well as a lack of toxic by-products and effluents, constitute advantages of the application of laccase-mediated biocatalysis in the synthesis of textile dyes. Due to the environmental impact associated with the production of textile dyes, the potential application of fungal laccase as a biocatalyst for the synthesis of valuable colorants has been demonstrated in this paper.

1. Introduction

Bio-products arouse interest among biotechnologists around the world. Cheap, easy to handle and, what is very important, non-toxic products are flags of innovative biotechnology (Sheldon et al., 2007). Together with a wide range of enzymes used in the field of white biotechnology, laccases play an important role as multi-functional biocatalysts with a broad variety of applications (Rodriguez Couto and Toca Herrera, 2006). Fungal and bacterial laccases were used for the synthesis of products with high commercial importance such as biopolymers, bio-dyes, antibiotics etc. (Mikolasch and Schauer, 2009; Polak and Jarosz-Wilkolazka, 2012a). As very efficient biocatalysts, they are involved in four-electron oxidation of different aromatic compounds with concomitant reduction of oxygen, the purest co-substrate, to water (Thurston, 1994). Laccases are generally extracellular and hence they are easy to handle and can be derived from bacteria or fungi on a large scale. Especially fungal laccase has a wide spectrum of action due to the high redox potential of its active center, which determines the oxidation mainly of phenolic compounds and also non-phenolic compounds such as amines as well as heterocyclic and polycyclic aromatic compounds (Giardina et al., 2010). During the action of laccase, very unstable radicals are formed. They can undergo spontaneous coupling reactions to many various coupled products, including colorful dimers, oligomers, and polymers, whose color can be used as dyes (Bruyneel et al., 2008; Forte et al., 2010; Ganachaud et al., 2008). These

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colored products are results of laccase-mediated oxidation of simple aromatic compounds containing reactive substituents such as methoxy, amino, hydroxy, and sulfonic groups (Polak and Jarosz-Wilkolazka, 2012b).

The toxicity of products is mostly determined by the structure of precursors used for synthesis thereof. Due to the very frequent use of harmful and toxic precursors such as phenols, naphthalene derivatives, and aromatic amines, the toxicity of final bio-products must be monitored. They should be non-toxic to both the environment and humans. All commercial dyes, especially food dyes used for coloring bakery goods, sweets, cereals, and drugs, should be monitored for their toxicities, because of the multiple chemical sensitivities following intolerance to artificial food dyes (Inomata et al., 2006). Many dyes like Amaranth entered the market and then they were banned because they were discovered to be carcinogenic (Williams, 2012). Despite their allergenic properties and the potential to cause hyperactivity and histological changes in the thyroid, some dyes like Allura Red and Erythrosine are still permitted as food colorants for example in the USA (Scotter and Castle, 2004; Tanaka, 2001).

Potentially less important for human health is the toxicity of textile dyes, as well as dyes used for coloring human hair, because they are not directly consumed. However, they may cause irritation and sensitization by skin contact (Saunders et al., 2004) and have a great influence on environmental pollution (Chequer et al., 2013; Robinson et al., 2001). Besides the pure dyes, also chemicals used for production thereof, such as aniline, are highly toxic, carcinogenic, and dangerous to work with. Moreover, during the dyeing process and garment finishing, addition of other harmful chemicals such as formaldehyde, dioxin, and toxic heavy metals known as carcinogens, is necessary to obtain a good dyeing effect (Leube, 2003). What is the alternative to synthetic dyes? One of the options is a return to natural dyeing; however, natural dyes will never be able to replace synthetic dyes completely because of their instability (Ferreira and Rodriguez-Amaya, 2008; Najar et al., 1988). Another solution is to provide methods for synthesis of dyes involving oxidizing enzymes without the use of harmful and toxic compounds. Hence, the capability of laccase of eco-friendly transformation of precursors to non-toxic dyes with good dyeing properties might be useful (Polak and Jarosz-Wilkolazka, 2012a).

In this paper, we focused on the enzyme-mediated synthesis of orange–red–purple colored compounds that were obtained from homo- and heteromolecular transformations of aromatic precursors by high redox potential fungal laccase. The bio-dyes obtained were analyzed in terms of their spectroscopic and dyeing properties and their toxicity effects against humans and the environment.

2. Materials and methods

Dyes were synthesized during homo- or heteromolecular transformations of selected precursors in combinations provided in Tables 1a and 1b, respectively, using fungal laccase as a biocatalyst. All dye precursors of analytical grade were purchased from Sigma–Aldrich–Fluka (USA, Germany, UK) and used without further purification (Fig. 1). 100 mM substrate stock solutions were prepared in distilled water with addition of 1 mM NaOH to improve the solubilization of chemicals. The acronyms of all precursors belonging to benzene (3A4HBA, 3A4HBSA, 2A3MeBA) and naphthalene (2ANS, 4ANS, 5ANS) derivatives with functional substituents are summarized in Fig. 1. Acid Red 299 (Fig. 2) was purchased from Reax (Poland).

2.1. Catalyst

Extracellular laccase (LAC) from the white rot fungus *Cerrena unicolor* (Fungal Collection of the Department of Biochemistry, UMCS, Lublin, Poland) was purified using a previously described procedure (Luterek et al., 1997). LAC was stored frozen at −18 °C until use, and after thawing and diluting with a tartrate buffer solution, it was used to prepare a working LAC solution with precise activity. LAC activity was determined by following the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Wolfenden and Willson, 1982). The reaction mixture contained appropriate activity of LAC and 2.5 mM ABTS in 100 mM Na-tartrate buffer pH 3. Oxidation of ABTS was monitored spectrophotometrically at 414 nm (ε_{414} = 36,048 M^{-1} cm^{-1}) and LAC activity was expressed in U mL^{-1}. One unit of LAC activity (U) corresponds to the amount of enzyme that oxidizes 1 μmol of ABTS per 1 min at 25 °C.

2.2. Synthesis of dyes

Transformations were carried out in a 500-mL volume reaction mixture containing 3 mM of one precursor (in the case of homomolecular transformations) or two precursors (in the case of heteromolecular transformations, 3 mM each, molar ratio 1:1). Buffered conditions have been provided by addition of 100 mM tartaric acid in order to adjust the pH value to 4.5. Both to minimize the concentration of tartrate in the final product (lyophilized powder of dye) and to ensure the stability of the reaction mixture pH value, the concentration of added tartaric acid was as minimal as possible and did not exceed the final values of 3 mM. The transformation of precursors was carried out using LAC in the final activity of 1 U mL^{-1} at 28 °C in shaken conditions (140 rpm). The synthesis of colored products was monitored by a UV–Vis spectrophotometer up to 7 days, until the absorbance of the dye declined. The liquid dye obtained was frozen and lyophilized, and the dye powder was used in the further experiments.

2.3. Screening of dyeing properties

Pure meradiso wool fibers and cotton fibers were dyed with the tested dyes in a bath at a concentration 1 mg mL^{-1}. Acetic acid was added to the dyeing bath (pH 4) and afterward dyeing was carried out at 100 °C during 30 min. After dyeing, the fabrics were washed twice with water and dried at room temperature.

2.4. Evaluation of the dyeing property of AB3

To check the dyeing property of the selected dye (AB3), natural fibers (wool, natural silk) and synthetic fibers (polyamide) were used. These fibers can form ionic bonds with dyes. The parameters of the fibers were as follows: woven fabric of wool (100%) – raw, mass per unit area 158 g/m^2; woven fabric of natural silk (100%) – mass per unit area 70 g/m^2; polyamide knitted fabric (100%) – mass per unit area 144 g/m^2. Acid Red 299 was used as a model of a commercial dye. This dye used in an aqueous solution was characterized by maximum absorption at wavelength λ_{max}, 525 nm, at which the absorbance measurements were made.

2.4.1. The process of dyeing woven fabrics of wool, natural silk, and polyamide knitted fabric

The selected products, the fabric from wool and natural silk fibers as well as the polyamide knitted fabric were dyed with the AB3 and AR299 dyes in a bath at a concentration of 0.2% relative to the fiber mass. Formic acid was added to the dyeing bath in an amount of 1% (pH 3.1–3.4); the maximal dyeing temperature was 100 °C and the whole process lasted 115 min. The polyamide knitted fabric

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