DETERMINATION OF SOME POLLUTANTS FROM TEXTILE INDUSTRY USING AMPEROMETRIC BIOSENSORS

Florentina-Daniela Munteanu, B.I. Pancan
University "Aurel Vlaicu" of Arad, Faculty of Food Engineering, Tourism and Environment Protection, Elena Dragoi, No 2, 310330, Arad, Romania
florentina_daniela@yahoo.com

Abstract

In this paper are presented the results obtained on azo dyes decolourization, using two different enzymes, a laccase and a peroxidase. The ability of Trametes villosa laccase to degrade an azo dye (Methyl orange) and of a novel peroxidase to decolourize Orange IV was studied in terms of Michaelis-Menten constants.
***Keywords**: biosensor, laccase, versatile peroxidase, methyl orange, orange IV.*

Introduction

Azo dyes, the largest chemical class of dyes with the greatest variety of colors, have been used extensively in the latest years for the textile dyeing and paper printing. The release of azo dyes into the environment is a concern due to coloration of natural waters and due to the toxicity, mutagenicity and carcinogenicity of the dyes and their biotransformation products. Therefore, considerable attention has been given to evaluating the fate of azo dyes during wastewater treatment and in the natural environment.

The elimination of coloured effluents in wastewater treatment systems is mainly based on expensive physical or chemical procedures such as adsorption, concentration, chemical transformation and incineration(Moreira et al., 2000).

Several processes for enzymatic decolourization of textile dyes have been suggested.

The extracellular ligninolytic enzyme system of white-rot fungi can degrade a wide variety of recalcitrant compounds, such as xenobiotics, lignin, and various types of dyes(Paszczynski et al., 1995). The major enzymes associated with the lignin-degrading ability of white-rot fungi are lignin peroxidase (EC 1.11.1.14) (Macarena et al., 2005), manganese peroxidase (EC 1.11.1.13) (McCue et al., 2005) and laccase (1.10.3.2)(Sigoillot et al., 2005).

Laccases are a multi-copper phenol oxidase, which reduces oxygen to water and simultaneously catalyze the oxidation of aromatic pollutants like anilines and phenols (Bourbonnais et al., 1997; Robles et al., 2000). Several methods using laccase, immobilized laccase and laccase/mediator system have been developed for the treatment of the textile effluents (Blanquez et al., 2004; Kirby et al., 2000; Martins et al., 2003; Maximo et al., 2004; Novotny et al., 2004; Peralta-Zamora et al., 2003; Robinson et al., 2001; Soares et al., 2002; Wesenberg et al., 2003). This enzyme decolorizes some azo dyes without direct cleavage of the azo bond through a highly non-specific free radical mechanism, thereby avoiding the formation of toxic aromatic amines (Chivukula et al., 1995). However, the substrate specificity of laccase limits the number of azo dyes that can be degraded (Chivukula et al., 1995; Chung et al., 1992; Chung et al., 1993).

Versatile peroxidase (VP) is a novel heme peroxidase type described in fungi from the genera *Pleurotus* and *Bjerkandera*, whose biochemical, molecular and structural aspects are being thoroughly investigated (Camarero et al., 1999; L. Banci et al., 2003; Martínez et al., 1996; Meunier et al., 1998; Ruiz-Dueñas et al., 1999.). The most intriguing characteristics of this new enzyme is its ability to use a variety of electron donor substrates that were previously considered as characteristic of other peroxidase types, such as manganese peroxidase (MnP) lignin peroxidase (LiP) and horseradish peroxidase (HRP) (Heinfling et al., 1998; Martinez, 2002).

The bioelectrocatalytical properties of these two enzymes open the perspectives for a possible further development of biosensors for azo dyes detection/degradation.

Experimental

The methyl orange dye (3-(4-dimethylamino-1-phenylazo) benzene sulfonic acid sodium salt) (see Figure 1A) was synthesized by the conventional method of coupling the diazonium salt of methanilic acid with either N,N-dimethyl-p-phenylenediamine(Furniss et al., 1989). The minimum dye content was 90%. The structures of the isolated dye, as sodium salts, were confirmed by 1H NMR spectroscopy in dymethylsulfoxide (DMSO).

Orange IV (C.I. 13080; Acid Orange 5) (an azo dye) (Figure 1B) was purchased from Sigma, St. Louis, MO, USA.

1-hydroxybenzotriazole and the salts were purchased from Sigma, St. Louis, MO. All chemicals were of high purity and used as received. Citrate buffer

salts were of analytical grade and obtained from Merck, Darmstadt, Germany. All aqueous solutions were prepared using distilled water.
Recombinant VP was obtained by expressing the cDNA of the allelic variant VPL2 of *P. eryngii* in *E. coli* followed by protein refolding in the presence of heme, as previously reported (Perez-Boada et al., 2002).
Laccase (EC 1.10.3.2) from *Trametes villosa* (5.3 mg protein/mL, 600 U/mL) was kindly provided by Novo Nordisk, Denmark.

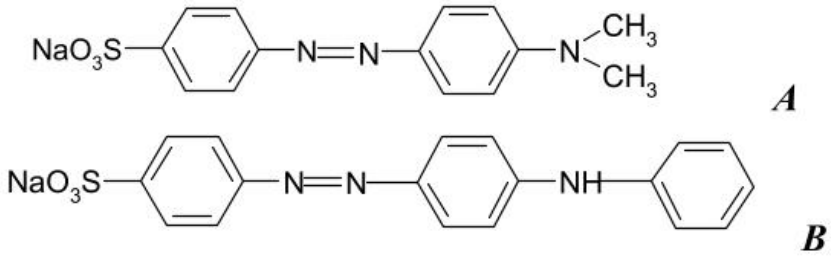

Fig. 1: Structure of (A) 3-(4-dimethylamino-1-phenylazo) benzene sulfonic acid sodium, and (B) Orange IV

The enzymes modified electrodes were prepared using rods of solid spectroscopic graphite (SGL Carbon, Werke Ringsdorff, Bonn, Germany, type RW001, 3.05 mm diameter). The graphite rods were first polished on wet fine-structured emery paper (grit size: P1200) and then additionally polished on paper to obtain a mirror-like surface. The electrode rods were carefully rinsed with deionised water and allowed to dry at room temperature. A 5 µl aliquot of the enzyme solution was added to each of the polished ends of the graphite rods and the adsorption was allowed to proceed for 1 h at 4°C. The enzyme electrodes were then thoroughly rinsed with buffer, and if not immediately used, they were stored in buffer at 4°C.

Weakly adsorbed enzyme was desorbed before measurements, by rotating the electrode in buffer for at least 30 min.

The enzyme electrode was fitted into a rotating disk electrode holder (RDE; Radiometer Analytical, Villeurbanne, France, model EDI101), which was placed in a three-electrode cell with an Ag|AgCl (saturated KCl) reference electrode (BAS, Bioanalytical Systems, West Lafayette, IN, USA) and a platinum wire auxiliary electrode. The electrodes were connected to a Voltalab 30 Potentiostat (Radiometer Analytical) controlled by the Voltamaster 4 (version 5.6) electrochemical software. All measurements were performed at an applied potential of -50 mV versus Ag|AgCl (Lindgren

et al., 1998; Ruzgas et al., 1995). The current was registered using various rotation speeds (200 rpm). Prior to experiments, the solution was bubbled with nitrogen for 15 min. Nitrogen was passed over the solution during the experiments.

Results and Discussion

Laccase catalyzes the oxidation of organic substrates such as phenolic compounds by molecular oxygen in homogeneous solutions. When laccase is adsorbed on graphite, bioelectrocatalytic reduction of oxygen occurs and is observed as a reduction current caused by direct (mediatorless) electron transfer (DET) from the electrode to the immobilized laccase and then further to molecular oxygen in solution. In the presence of soluble electron donors, laccase can be reduced in a mediated electron transfer (MET) mechanism. In this mechanism the electron donor (substrate) penetrates the active site of the enzyme where it is oxidized in a single electron oxidation step often producing an electrochemically active compound (possibly a radical) that in turn can be re-reduced at the electrode surface in a mediated electron transfer (MET) step.

Initial experiment (applied potential -50mV versus Ag|AgCl) showed that these electrodes have low noise/background current while upon the injection of the solution of methyl orange a reduction current will be generated. Such a response is relatively well understood and it is usually ascribed to electrochemical reduction of laccase oxidation products. The responses are dependent on the concentration of the azo dye in the solution of interest. At higher azo dye concentrations the current-concentration dependence gradually reached saturation (Figure 2). The apparent Michaelis–Menten constants (K_m^{app}) and maximal currents (I_{max}) have been calculated by fitting the variation of current–concentration dependencies of the analyzed compounds to the electrochemical Michaelis–Menten equation (Equation 1)(Shu et al., 1976). K_m^{app} is an indicator of the affinity that an enzyme has for a given substrate, and hence the stability of the enzyme-substrate complex.

$$I = \frac{I_{max}[S]}{[S] + K_M^{app}} \qquad (1)$$

where S is the substrate concentration, I_{max} the maximum current and K_m^{app} the apparent Michaelis–Menten constant. The calculated values of K_m^{app} and the catalytic efficiencies are presented in Table 1.

Table 1: Michaelis Menten constants

	I_{max} (μA)	K_m^{app} (μM)	I_{max}/K_m^{app} ($A/M^{-1} cm^{-2}$)
Methyl Orange and laccase	0.793±0.002	31.497±0.075	0.025±0.003
Azo dye and laccase in presence of HBT	1.510±0.009	0.699±0.004	2.160±0.008
Orange IV and versatile peroxidase	0.249±0.009	24.559±2.635	0.141±0.012

The kinetics of mediated laccase catalyzed reactions is firstly affected by the affinity between enzyme and the mediator. An estimation of this influence can be done by amperometric measurements in terms of I_{max}/K_m^{app} ratio. These parameters are often calculated in the design of enzymatic sensors to evaluate the sensitivity of the system proposed, which is related to the low or high affinity of the enzyme towards a specific substrate. Lower K_m^{app} values at similar catalytic currents involved higher effectiveness of the enzyme at lower mediator concentrations.

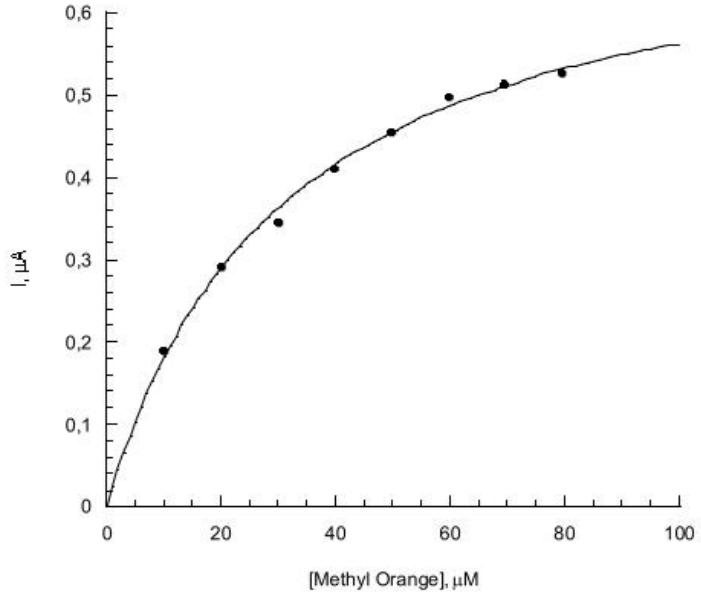

Fig 2: Calibration graph for methyl orange obtained with a laccase modified graphite electrode in 0.1 M citrate buffer pH 5.0, at -50mV vs. Ag|AgCl.

Peroxidases are important enzymes in analytical biochemistry. The common feature of all heme peroxidases is that their active sites contain very similar prosthetic groups, which for a majority of known plant peroxidases it is a heme b, or ferriprotoporphyrin IX.

The peroxidase electrodes were used to determine the mediators in the RDE mode. Each experiment started having just the buffer in the system. When a stable baseline was achieved, the enzyme substrate, H2O2 (100 µM final concentration), was added to the system and a steady-state current due to the direct ET was registered. If the sample containing a donor substrate is added in the peroxidase system, enzymatic oxidation of the mediators results in radicals that can be electrochemically rereduced at the electrode (-50 mV vs. Ag|AgCl), resulting in a reduction current proportional to the substrate concentration. A representative calibration curve for Orange IV is presented in figure 3.

In all cases the VP-electrodes displayed Michaelis-Menten like kinetics. The kinetics of mediated laccase catalyzed reactions are firstly affected by the affinity between enzyme and the mediator.

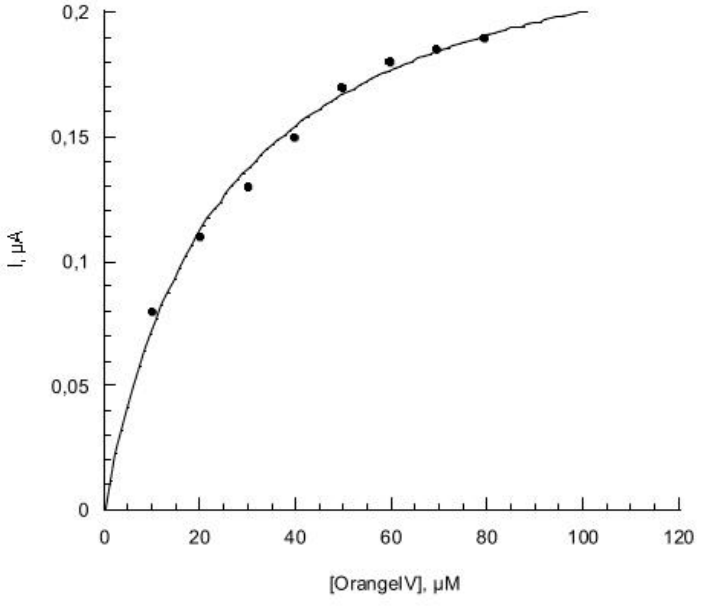

Fig. 3: Calibration graph for Orange IV obtained with a peroxidase modified graphite electrode in 0.1 M citrate buffer pH 3.0, at -50mV vs. Ag|AgCl

Conclusions

The present study reveals that these two enzymes, laccase and versatile peroxidase, have the potential for analytical applications.

This versatile peroxidase, was shown to possess optimal bioelectrocatalytical properties for a possible further development of peroxidase-modified biosensors for azo dyes detection/degradation.

With the addition of the HBT as a mediator it was possible to improve the degradation of the Methyl orange using laccase. Beside, the good results obtained when the laccase is adsorbed onto the electrode surface gives us excellent promises for usage of these systems in online monitoring of the enzyme activity.

Acknowledgments

This work was done under the frame of National Authority for Scientific Research projects, CEEX 3179/2005 and CEEX 77/2006

References

Blanquez, P., Casas, N., Font, X., Gabarrell, X., Sarra, M., Caminal, G. and Vicent, T. (2004) Mechanism of textile metal dye biotransformation by Trametes versicolor, Water Research, Vol. 38, No. 8, pp. 2166-2172.

Bourbonnais, R., Paice, M.G., Freiermuth, B., Bodie, E. and Borneman, S. (1997) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds, Appl. Environ. Microbiol., Vol. 12, pp. 4627-4632.

Camarero, S., Sarkar, S., Ruiz-Dueñas, F.J., Martínez, M.J. and Martínez, A.T. (1999) Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites, J.Biol.Chem., Vol. 274, pp. 10324-10330.

Chivukula, M. and Renganathan, V. (1995) Phenolic Azo Dye Oxidation by Laccase from Pyricularia oryzae, Appl. Environ. Microbiol., Vol. 61, pp. 4374-4377.

Chung, K.T. and Cerniglia, C.E. (1992) Mutagenicity of azo dyes: structure activity relationship, Mutat. Res., Vol. 277, pp. 201-220.

Chung, K.T. and Stevens, J.S.E. (1993) Decolorization of azo dyes by environmental microorganisms and helminthes, Environ. Toxicol. Chem., Vol. 12, No. 11, pp. 2121-2132.

Furniss, B.S., Hannaford, A.J., Smith, P.W.G. and Tatchell, A.R. (1989) Vogel's Textbook of Practical Organic Chemistry (5th Edition): Vogel's Textbook of Practical Organic Chemistry (5th Edition), Longman Group UK, Ltd, Harlow, Essex, p. 951.

Heinfling, A., Ruiz-Dueñas, F.J., Martínez, M.J., Bergbauer, M., Szewzyk, U. and Martínez, A.T. (1998) A study on reducing substrates of manganese-oxidizing peroxidases from Pleurotus eryngii and Bjerkandera adusta, FEBS Lett., Vol. 428, pp. 141-146.

Kirby, N., Marchant, R. and McMullan, G. (2000) Decolourisation of synthetic textile dyes by Phlebia tremellosa, FEMS Microbiology Letters, Vol. 188, No. 1, pp. 93-96.
L. Banci, Camarero, S., Martínez, A.T., Martínez, M.J., Pérez-Boada, M., Pierattelli, R. and Ruiz-Dueñas, F.J. (2003) NMR study of Mn(II) binding by the new versatile peroxidase from the white-rot fungus Pleurotus eryngii, J.Biol.Inorg.Chem., Vol. 8, pp. 751-760.
Lindgren, A., Munteanu, F.-D., Gazaryan, I.G., Ruzgas, T. and Gorton, L. (1998) Comparison of rotating disk and wall-jet electrode systems for studying the kinetics of direct and mediated electron transfer for horseradish peroxidase on a graphite electrode, J. Electroanal. Chem., Vol. 458, pp. 113-120.
Macarena, S., Fernando, L.L., Monica, V., Rafael, V. and Bernardo, G. (2005) Incomplete processing of peroxidase transcripts in the lignin degrading fungus Phanerochaete chrysosporium, FEMS Microbiology Letters, Vol. 242, No. 1, pp. 37-44.
Martinez, A.T. (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases, Enzyme and Microbial Technology, Vol. 30, No. 4, pp. 425-444.
Martínez, M.J. and Martínez, A.T. (1996) Characterization of MnP isoenzymes of Pleurotus eryngii exhibiting Mn-independent activities on 2,6-dimethoxyphenol and veratryl alcohol., in Srebotnik, K.M.a.E. (Ed.): Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research, Vienna: Facultas-Universitätsverlag, pp. 417-420.
Martins, M.A.M., Lima, N., Silvestre, A.J.D. and Queiroz, M.J. (2003) Comparative studies of fungal degradation of single or mixed bioaccessible reactive azo dyes, Chemosphere, Vol. 52, No. 6, pp. 967-973.
Maximo, C. and Costa-Ferreira, M. (2004) Decolourisation of reactive textile dyes by Irpex lacteus and lignin modifying enzymes, Process Biochemistry, Vol. 39, No. 11, pp. 1475-1479.
McCue, P.P. and Shetty, K. (2005) A model for the involvement of lignin degradation enzymes in phenolic antioxidant mobilization from whole soybean during solid-state bioprocessing by Lentinus edodes, Process Biochemistry, Vol. 40, No. 3-4, pp. 1143-1150.
Meunier, B., Rodriguez-Lopez, J.N., Smith, A.T., Thorneley, R.N.F. and Rich, P.R. (1998) Redox- and anion-linked protonation sites in horseradish peroxidase: analysis of distal haem pocket mutant, Biochem. J., Vol. 330, pp. 303-309.
Moreira, M.T., Feijoo, G. and Lema, J.M. (2000) Evaluation of different fungal strains in the decolorisation of synthetic dyes, Biotechnol. Lett., Vol. 22, pp. 1499-1503.
Novotny, C., Svobodova, K., Kasinath, A. and Erbanova, P. (2004) Biodegradation of synthetic dyes by Irpex lacteus under various growth conditions, International Biodeterioration & Biodegradation, Vol. 54, No. 2-3, pp. 215-223.
Paszczynski, A. and Crawford, R.L. (1995) Potential for bioremediation of xenobiotic compounds by the white rot fungus Phanerochaete chrysosporium., Biotechnol. Prog., Vol. 11, pp. 368-379.
Peralta-Zamora, P., Pereira, C.M., Tiburtius, E.R.L., Moraes, S.G., Rosa, M.A., Minussi, R.C. and Duran, N. (2003) Decolorization of reactive dyes by immobilized laccase, Applied Catalysis B: Environmental, Vol. 42, No. 2, pp. 131-144.
Perez-Boada, M., Doyle, W.A., Ruiz-Duenas, F.J., Martinez, M.J., Martinez, A.T. and Smith, A.T. (2002) Expression of Pleurotus eryngii versatile peroxidase in Escherichia coli and optimisation of in vitro folding, Enzyme and Microbial Technology, Vol. 30, No. 4, pp. 518-524.

Robinson, T., Chandran, B. and Nigam, P. (2001) Studies on the production of enzymes by white-rot fungi for the decolourisation of textile dyes, Enzyme and Microbial Technology, Vol. 29, No. 8-9, pp. 575-579.
Robles, A., Lucas, R., De Cienfuegos, A.G. and Galvez, A. (2000) Phenol-oxidase (laccase) activity in strain of the hyphomycete Chalara paradoxa isolated from olive mill wastewater disposal ponds, Enzyme Microb. Technol, Vol. 26, pp. 484-490.
Ruiz-Dueñas, F.J., Martínez, M.J. and Martínez, A.T. (1999.) Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus Pleurotus eryngii, Mol. Microbiol., Vol. 31, pp. 223-236.
Ruzgas, T., Gorton, L., Emnéus, J. and Marko-Varga, G. (1995) Kinetic models of horseradish peroxidase action on a graphite electrode, J. Electroanal. Chem., Vol. 391, pp. 41-49.
Shu, R. and Wilson, G.S. (1976) Rotating ring-disk enzyme electrode for surface catalysis studies, Anal. Chem., Vol. 48, p. 1679–1686.
Sigoillot, C., Camarero, S., Vidal, T., Record, E., Asther, M., Perez-Boada, M., Martinez, M.J., Sigoillot, J.-C., Asther, M., Colom, J.F. and Martinez, A.T. (2005) Comparison of different fungal enzymes for bleaching high-quality paper pulps, Journal of Biotechnology, Vol. 115, No. 4, pp. 333-343.
Soares, G.M.B., Amorim, M.T.P.e., Hrdina, R. and Costa-Ferreira, M. (2002) Studies on the biotransformation of novel disazo dyes by laccase, Process Biochemistry, Vol. 37, No. 6, pp. 581-587.
Wesenberg, D., Kyriakides, I. and Agathos, S.N. (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnology Advances, Vol. 22, No. 1-2, pp. 161-187.