Metabolism of azo dyes, methyl red and methyl orange by plants

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Abstract

Azo dyes like methyl red and methyl orange are known to be major human carcinogens besides being water pollutants. These dyes are still a cause of concern in the developing nations due to their unrestricted usage. Laccases and peroxidases isolated from bacteria and fungi are presently being explored for decolorizing dyes. Whole plants have rarely been employed in degrading dyes. The goal of our work is to identify and characterize the groups of enzymes involved in the breakdown of dyes. *Arabidopsis thaliana* is chosen as the model organism as its entire genome is sequenced ensuring accurate gene identification. Hydroponically cultivated *Arabidopsis thaliana* were treated with 20 mg/L solutions of methyl red and methyl orange prepared at two pH values, 4.6 and 6.3 in the presence or absence of external hydrogen peroxide. Presence of peroxide at pH 4.6 or 6.3 does not accelerate the decolorization of the dyes. Plants assayed at pH 4.6 (methyl red-4.5 nmoles/hr; methyl orange-3.9 nmoles/hr) were found to degrade the dyes at the same rate as that observed for pH 6.3 (methyl red-4.2 nmoles/hr; methyl orange-3.5 nmoles/hr). Within three days the plants were able to decolorize 60% of both the dyes. A strong salt, 0.1M magnesium sulphate, has been found to extract nearly 30% of the total enzyme activity measured by ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] and peroxide at either pH. ABTS color formation is a one electron oxidation process. The peroxidase activity as measured by ABTS color reaction is in ~1000 fold excess over observed degradation of methyl red or methyl orange. This suggests that the enzymes involved in the dye uptake might have low substrate affinity, or low reactivity with the dyes, which may require two electrons, compared to ABTS.

Introduction

Azo dyes are non-natural color compounds which posses an azo group, (-N=N-) (1). Their stable structures and high water solubility not only makes them resistant to microbial attack but also are responsible for them being carcinogenic and genotoxic to the living biota (2,3). Indeed, conventional physicochemical techniques employed for dye decolorization such as aerobic & anaerobic sewage treatment, adsorption by activated carbon, ultrafiltration, coagulation, and irradiation have proved to be quite inefficient in completely degrading azo dyes from the effluents due to them being expensive and the toxic byproducts generated after the treatment (3-6). Bioremediation processes using oxidoreductase enzymes such as laccases and peroxidases isolated and immobilized mainly from bacteria and/or white rot fungi have been found to be highly successful in degrading and decolorizing of various azo dyes (2-8). Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are blue multicopper oxidases that catalyze the oxidation of an array of aromatic substrates concomitantly with the reduction of molecular oxygen to water (4) while peroxidases (EC 1.11.1.7; donor: hydrogen-peroxide oxidoreductases) are enzymes that catalyzes the oxidation of a large number of aromatic structures at the expense of hydrogen peroxide (5). However, extracting and immobilizing the enzymes from fungal or bacterial sources is a very expensive process and leads to a lot of unwanted biomass (6,7). Recent research has shown that peroxidases isolated from leaves (6) and plant roots (7) also bring about significant degradation of azo dyes. Thus, whole plants may serve as cheap and sustainable reservoirs for producing peroxidases which can decolorize and detoxify azo dyes Our research focuses on determining the extent of decolorization of two commonly available azo dyes, methyl red and methyl orange using hydroponically cultivated whole plants.

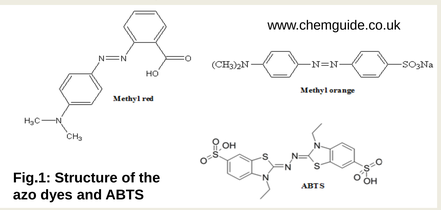

Fig.1: Structure of the azo dyes and ABTS

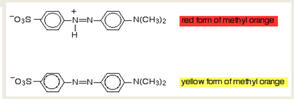

Figure 3: Red (pH-3.5 or below) and yellow (pH-4.5 or above) form of methyl orange .

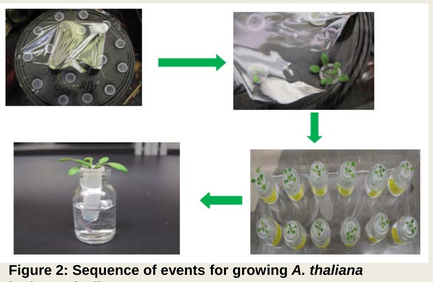

Figure 2: Sequence of events for growing *A. thaliana* hydroponically.

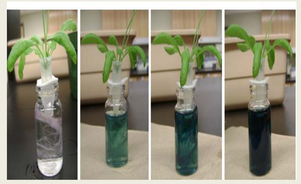

Fig.4: ABTS assay for laccase and peroxidase.

Objectives

- Measure the rate of decolorization of azo dyes, methyl red and methyl orange at two different pH 4.6 and 6.3 by **whole plants** in presence or absence of peroxide.
- Compare rates of azo dyes decolorization by whole plants with those observed for ABTS to estimate the substrate specificity.
- Determine how much of the total enzyme is **extractable** when a strong salt like 0.1M magnesium sulphate is used as an extractant.
- Evaluate the amount of dye decolorization at pH 4.6 and 6.3 in the presence of the extractable enzyme extract.

Methods and Materials

- Plant model: *Arabidopsis thaliana* Columbia ecotype. Entire genome is sequenced, short life cycle, genetic manipulation and transgenic plants generated with ease.
- Hydroponics (8): Wildtype *A.thaliana* C seeds were surface sterilized and placed on microcentrifuge tubes without lids containing 0.6% solid agar plugs. The seeds were allowed to germinate under light in ½ strength Hoagland's solution. Plantlets which had a significant amount of roots penetrating through the agar and visible in the nutrient solution were selected for analysis (Figure 2).
- Dyes selected: Two representative azo dyes, methyl red and methyl orange were selected. Both are quite similar in structure in that they both contain the azo group but the functional group in methyl red is carboxylic acid while sulphonic acid is present in methyl orange (Figure 1). Both exist as two distinct species depending on the pH (Figure 3). 20mg/L concentrations of methyl red and methyl orange in acetate buffer (pH-4.6) and phosphate buffer (pH-6.3) were prepared with or without 1mM hydrogen peroxide to check the effect of peroxide on the rate of dye degradation.
- ABTS assay for peroxidase and laccase: ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] is commonly used as a substrate to monitor the enzyme kinetics of laccases (without hydrogen peroxide) and peroxidases (in presence of hydrogen peroxide). Laccases and peroxidases, if present facilitates the reaction of colorless ABTS (Figure 1) with or without hydrogen peroxide respectively in a one-electron oxidation process turning it into a soluble green end-product read at 414nm (Figure 4).
- Extraction of the enzymes from the roots of whole plants was carried out using a mixture containing a strong salt, 0.1Mmagnesium sulphate with 1mM calcium chloride in the respective buffers for one hour. Equal volumes of the enzyme extract was mixed with the buffered dye and the dye decolorization was monitored.
- Decolorization of both the dyes with the whole plants as well as the enzyme extract were monitored for seven days by measuring the amount of residual dye remaining behind using a Hitachi-U2300 double beam UV-Visible spectrophotometer over 230-630nm wavelength range.

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Results

Figure 5: Methyl red and methyl orange disappearance with or without hydrogen peroxide using whole plants

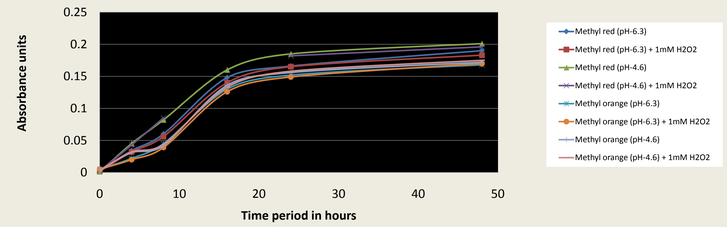


Table 1: Dye decolorization by whole plants

Azo dye	pH-6.3			pH-4.6		
	+ H_2O_2*	- H_2O_2*	Dye lost in 3 days	+ H_2O_2*	- H_2O_2*	Dye lost in 3 days
Methyl red	**4.2 ± 0.054**	**4.1 ± 0.126**	**65.37%**	**4.5 ± 0.114**	**4.4 ± 0.028**	**67.85%**
Methyl orange	**3.5 ± 0.085**	**3.3 ± 0.033**	**62.5%**	**3.9 ± 0.006**	**3.9 ± 0.043**	**65.41%**
ABTS	**8800 ± 92**	**5.2 ± 0.046**	**-**	**5600 ± 108**	**4.8 ± 0.073**	**-**

* Dye decolorization rate is measured in nanomoles per hour. Peroxide when used was 1 mM.

Table 2: Dye decolorization by enzyme extract

Azo dye	pH-6.3			pH-4.6		
	+ H_2O_2*#	- H_2O_2*#	Dye lost in 3 days	+ H_2O_2*#	- H_2O_2*#	Dye lost in 3 days
Methyl red	**1.8 ± 0.037**	**1.6 ± 0.052**	**20.57%**	**2.5 ± 0.037**	**2.3 ± 0.093**	**30.5%**
Methyl orange	**1.1 ± 0.015**	**1.1 ± 0.081**	**18.5%**	**0.9 ± 0.063**	**0.8 ± 0.074**	**12.5%**

*Dye decolorization rate is measured in nanomoles per hour. Peroxide when used was 1 mM
#Enzyme extraction using 0.1M magnesium sulphate: At pH-6.3 ~30% of the enzyme was extractable and activity as measured using ABTS and 1 mM H_2O_2 was estimated to be ~2000 nanomoles per hour while at pH-4.6 ~ 32% of the enzyme was extractable and activity was found to be ~1300 nanomoles per hour which is ~1000 fold higher than those observed for the azo dyes.

Figure 6: Representative assay for laccase and peroxidase using ABTS as substrate

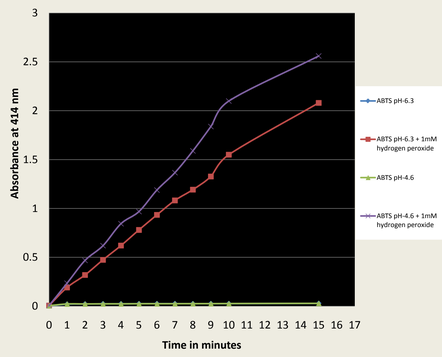
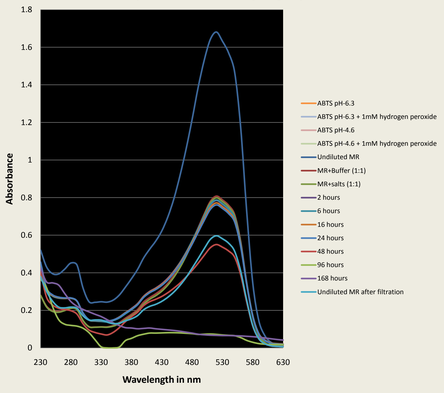


Figure 7: Decolorization of methyl red (pH-4.6) by extractable root enzymes in absence of hydrogen peroxide



Summary

- Whole plants could successfully decolorize around 60-65% of the azo dyes, methyl red and methyl orange at either pH 4.6 or 6.3 over three days (Figure 5, Table 1).
- Presence of external peroxide does not affect the rate or amount of dye decolorization at either pH (Table 1) although it does increase the rate of degradation of ABTS rapidly by over thousand fold over the azo dyes (Figure 6, Table 1) confirming the low affinity of the decolorizing enzymes towards the azo dyes which may require two electrons for their oxidation as opposed to one electron for ABTS oxidation.
- Using a strong salt, 0.1M magnesium sulphate, along with calcium chloride (1mM) we could extract 25-30% of the total enzyme within the roots of the plant at either pH and the extract could still decolorize both the dyes at either pH (Table 2, Figure 7).

Thus, whole plants can act as a good medium for metabolism of azo dyes.