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Biodegradation of Methyl red by *Galactomyces geotrichum* MTCC 1360

S.U. Jadhav[b], S.D. Kalme[a], S.P. Govindwar[a,*]

[a]*Department of Biochemistry, Shivaji University, Vidyanagar, Kolhapur 416004, India*
[b]*Department of Microbiology, Shivaji University, Vidyanagar, Kolhapur 416004, India*

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Abstract

Galactomyces geotrichum MTCC 1360 can decolorize triphenylmethane, azo and reactive high exhaust textile dyes. At shaking condition this strain showed 100% decolorization of a toxic azo dye Methyl red (100 m gl^{-1}) within 1 h in deionized water at 30 °C. The degradation of Methyl red was possible through a broad pH (3–12) and temperature (5–50 °C) range. Glucose and mycelium concentration had increased the decolorization rate, but the addition of 1 gl^{-1} molasses in deionized water made decolorization possible in only 10 min. Induction in the NADH–dichloro phenol indophenol (NADH–DCIP) reductase, Malachite green reductase, laccase and lignin peroxidase (Lip) activities were observed in the cells obtained after complete decolorization, showing that there is direct involvement in the degradation of Methyl red. The absence of *N-N′*-dimethyl-p-phenylenediamine (DMPD) in 5 °C, 2-aminobenzoic acid (ABA) in 50 °C and both the compounds in 30 °C sample have shown the differences in the metabolic fate of Methyl red at different temperatures. The untreated dye at 300 mg l^{-1} concentration showed 88% germination inhibition in *Sorghum bicolor*, whereas it was 72% in *Triticum aestivum*. There was no germination inhibition for both the plants by Methyl red metabolites at 300 mg l^{-1} concentration.

The scientific relevance of the paper: The azo dye Methyl red (100 mg l^{-1}) was decolorized by *G. geotrichum* MTCC 1360 within 1 h at shaking condition in deionized water. This organism could decolorize Methyl red at wide pH and temperature ranges. Decolorization time was reduced to 10 min by the addition of molasses to deionized water. There was induction in laccase and Lip, NADH–DCIP reductase and Malachite green reductase activities. The metabolic fate of Methyl red changes with temperature which can be evidenced by the formation of 2-ABA at 5 °C, *N-N′*-DMPD at 50 °C and both the compounds were absent at 30 °C. Phytotoxicity showed that metabolites of dye had induced shoot and root length of both the tested plants.

Keywords: Azo dye; Decolorization; *Galactomyces geotrichum*; Methyl red; Molasses

1. Introduction

Dyes are recalcitrant molecules, which are difficult to degrade biologically. With the increasing use of a wide variety of dyes, pollution by dye-waste water is becoming increasingly alarming. Color removal, in particular, has recently become a major scientific interest. Azo dyes are important colorants and are characterized by the presence of one or more azo groups (−*N* = *N*–) and constitute the largest class of dyes having extensive applications in textiles, papers, leathers, gasoline, additives, foodstuffs and cosmetics (Stolz, 2001). Microbiological decolorization and degradation is an environmental-friendly and cost-competitive alternative to the chemical decomposition process (Verma and Madamwar, 2003). Most studies on azo dye biodegradation have focused on bacteria and fungi, where bacteria are found to be more efficient (Eichlerová et al., 2006; Stolz, 2001). Chang et al. (2000) found that mutant strain of *Escherichia coli* was able to decolorize azo dye C.I. Reactive red 22. *Rhodopseudomonas palustris* could decolorize azo dye Acid red B and *Rhodobacter sphaeroides* was able to decolorize Methyl orange (Liu et al., 2006; Song et al., 2003).

Very little work was devoted to the decolorization ability of yeast. It was observed that few ascomycetous yeast species such as *Candida zeylanoides*, *C. tropicalis*, *Debaryomyces polymorphus* and *Issatchenkia occidentalis* perform a putative enzymatic biodegradation and concomitant

*Corresponding author. Tel.: +91 231 2609153; fax: +91 231 2691533.
E-mail address: spg_biochem@unishivaji.ac.in (S.P. Govindwar).

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decolorization of different azo dyes (Martins et al., 1999; Ramalho et al., 2002, 2004).

In our study *Galactomyces geotrichum* MTCC 1360, an industrially important strain was found to decolorize various textile dyes. One of the dyes, Methyl red is mutagenic in nature (Wong and Yuen, 1998). Most of the studies on Methyl red degradation dealt only with optimization of physicochemical parameters for decolorization (Moutaouakkil et al., 2004; Sun-Young et al., 2002; Vijaya and Sandhya, 2003), but it is important to select these parameters based on the end products of biodegradation which should not be toxic in nature. Most of the Methyl red degradation studies reveled in the formation of DMPD which remains undegraded in the culture and mutagenic in nature (So et al., 1990; Wong and Yuen, 1998). The untreated dyeing effluents that are straightly used in agriculture have a serious impact on environment and human health (Pourbabaee et al., 2006). So, it becomes necessary to study the effects of dyes and their metabolites on important agricultural plants.

The present work is planned to study various physicochemical parameters for optimization of decolorization processes, involvement of different enzymes during degradation and effect of temperature on the metabolic fate of Methyl red by *G. geotrichum*.

2. Materials and methods

2.1. Microorganisms used

G. geotrichum MTCC 1360 was obtained from Microbial Type Culture Collection, India. Pure culture of *G. geotrichum* was maintained on malt yeast agar slants at 4 °C. The composition of malt yeast medium used for decolorization studies was (gl^{-1}) malt extract (3.0), yeast extract (3.0), peptone (5.0), and glucose (10.0) (pH 7.0).

2.2. Dyestuff and chemicals

2,2′-Azinobis (3-ethylbezthiazoline-6-sulfonate) was purchased from Sigma-Aldrich. Tartaric acid and *n*-propanol were purchased from Sisco Research Laboratories, India. The azo dyes Malachite green, Amido black 10B and Methyl red were purchased from Hi-media, India. Scarlet RR and Orange HE 2R were kindly gifted by Manpasand Textile Industry, Ichalkaranji, India. All chemicals used were of the highest purity available and of analytical grade.

2.3. Decolorization of different industrial dyes

G. geotrichum MTCC 1360 was grown for 24 h at 30 °C in 250 ml Erlenmeyer flasks containing 100 ml malt yeast medium. After 24 h, different industrial dyes, Malachite green, Scarlet RR, Orange HE 2R and Amido black 10B (except Methyl red which was added at 100 mg l^{-1})were added at 50 mg l^{-1} concentration in each flask and incubated at 30 °C for 150 rpm in an orbital-shaking incubator. At different time intervals, an aliquot (3 ml) of the culture media was withdrawn and centrifuged at 5000*g* for 15 min. Decolorization was determined by measuring the absorbance of culture supernatant at their λ_{max} (Methyl red:530, Malachite green:620, Scarlet RR:530, Orange HE 2R:482 and Amido black 10B:618 nm). The percentage decolorization was calculated according to the following formulation:

$$\text{Percentage of decolorization} = \frac{A_b - A_a}{A_b} \times 100,$$

where A_b is the absorbance at the maximum absorption wavelength of the dye before decolorization and A_a is the absorbance at the same wavelength after decolorization. All decolorization experiments were performed in three individual sets. Abiotic controls (without microorganisms) were always included.

2.4. Decolorization of methyl red

G. geotrichum was grown for 24 h at 30 °C in 250 ml Erlenmeyer flasks containing 100 ml malt yeast medium ((gl^{-1}): malt extract (3.0), yeast extract (3.0), peptone (5.0), and glucose (10.0) (pH 7.0)). Biomass was collected by filtration through Whatman filter paper no.1 and suspended in 100 ml deionized water containing 100 mg l^{-1} concentration of Methyl red. The time required for complete decolorization of Methyl red was noted down. Decolorization of Methyl red by *G. geotrichum* was studied at different temperatures (5, 30 and 50 °C) at pH 3 and at various pH conditions (3, 5, 7, 9 and 12 pH) at 30 °C. The effects of mycelium concentration (20, 40 and 60 gl^{-1}), various glucose concentrations (10, 30 and 60 gl^{-1}) and various molasses concentrations (1, 2 and 3 gl^{-1}) on decolorization performance of *G. geotrichum* were studied at 30 °C (pH 3).

2.5. Preparation of cell free extract

G. geotrichum was grown in malt yeast medium at 30 °C for 24 h and biomass was collected by filtration through Whatman filter paper no.1. This mycelium was suspended in 0.1 mM sodium phosphate buffer (pH 7.4) for sonication (sonics-vibracell ultrasonic processor) keeping sonifier output at 60 amplitude, maintaining temperature at 4 °C and giving 9 strokes each of 30 s with 4 min intervals. This extract was then centrifuged at 13,000*g* for 30 min at 4 °C and the resulting supernatant was used as an enzyme source.

2.6. Enzyme assays

Lignin peroxidase (Lip) and laccase activities were assayed in cell-free extract as well as in culture supernatant. Lip activity was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 ml containing 100 mM *n*-propanol, 250 mM tartaric acid, 10 mM H_2O_2 (Kalme et al., 2006). Laccase activity was determined in a reaction mixture of 2 ml containing 0.1% ABTS in 0.1 M acetate buffer (pH 4.9) and measured increase in optical density at 420 nm (Hatvani and Mecs, 2001). Enzyme assays were carried in triplicate at 30 °C, where reference blanks contained all components except the enzyme. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg $protein^{-1}$. Azoreductase activity was determined by monitoring the decrease in the Methyl red concentration at 440 nm in a reaction mixture of 2.2 ml containing 152 μM Methyl red, 50 mM sodium phosphate buffer (pH 5.5) and 20 μM NADH. One unit of enzyme activity was defined as a microgram of Methyl red reduced min^{-1} mg $protein^{-1}$. NADH–dichloro phenol indophenol (NADH–DCIP) reductase activity was determined using a procedure reported earlier by Salokhe and Govindwar (1999). Malachite green reductase activity was determined using a procedure reported earlier by Jadhav and Govindwar (2006).

2.7. Extraction and analysis of metabolites

Decolorization was qualitatively analyzed using a UV-visible spectrophotometer (Hitachi U-2800), whereas biodegradation was monitored by high performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR). After complete decolorization (pH 3) at 5 and 30 °C and color change at 50 °C, culture broth was centrifuged at 13,000 rpm for 30 min and an equal volume of ethyl acetate was used to extract metabolites from clear supernatant. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. HPLC analysis was carried out (Waters model no. 2690) on a C_{18} column (symmetry, 4.6 × 250 mm^2). The rotary vacuum evaporated samples were dissolved in HPLC grade methanol. The mobile phase was acetonitrile and