Effects of glucose and NH$_4^+$ concentrations on sequential dye decoloration by *Trametes versicolor*

J. Swamy, J.A. Ramsay*

Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada K7L 3N6

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Abstract

Mycelial pellets of *Trametes versicolor*, grown at initial glucose and ammonium tartrate concentrations of 10 g/l (56 mM) and 0.442 g/l (2.4 mM) respectively, decolorized successive additions of the same dye (Amaranth) and of different dyes and dye mixtures. Decoloration commenced by Day 3 and all dyes were decolorized at high rates during the next 12 days. Glucose was consumed at a steady rate and was depleted by Day 15. Dyes added after this time were decolorized at decreased rates and decoloration ceased at Day 18. Replenishing glucose levels to 5 g/l restored the decoloration rate. Above about 0.13 g/l of glucose, the rate of decoloration was constant and when less than 0.13 g/l, the rate of decoloration decreased as glucose concentration decreased. Decolorizing cultures used glucose at a higher rate than when no dye was added. Ammonium was depleted at Day 4 whether dye was initially present or absent. Rapid decoloration was maintained at 0.086 g/l NH$_4^+$, but was inhibited at 0.86 g/l NH$_4^+$. When NH$_4^+$ was increased from 0.086 to 0.86 g/l in actively decolorizing cultures, decoloration ceased. This requirement for nitrogen-limitation has been reported during decoloration by ligninolytic cultures of *Phanerochaete chrysosporium* and suggests that decoloration by *T. versicolor* may be a similar process. However, the culture conditions used in this study repress ligninolysis and dye decoloration by *P. chrysosporium*, indicating that dye decoloration by *T. versicolor* may be distinct from lignin and pollutant degradation by *P. chrysosporium*. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

Over the past decade, the white rot fungi have been extensively studied for their ability to degrade a broad range of xenobiotic organo-pollutants [1,2]. Biodegradation of most xenobiotics has been ascribed to extracellular oxidation by the nonspecific, lignin-degrading enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and the laccases. The ligninolytic enzymes of the white rot fungi are thought to be expressed during secondary metabolism following growth when carbon (C) and/or nitrogen (N) becomes limiting [2–6]. Neither lignin nor any pollutants degraded by these enzymes has been shown to be utilized as growth substrate, and a separate C source is required for the metabolic activity [2].

To date, most of the research on lignin and organopollutant degradation has focused on *Phanerochaete chrysosporium*. The expression of LiP and MnP by this organism can be triggered by C or N limitation [2,3,4,6,7]. Dye decoloration by *P. chrysosporium* has been shown to occur only after N depletion and to be poor in N-rich cultures, with substantial adsorption of the dyes to the mycelium [9–11]. Since purified *P. chrysosporium* LiP and MnP can decolorize several dyes [12,13] and inhibitors of lignin degradation prevent decoloration [9], the ligninolytic enzymes are implicated in this process.

The expression of LiP by *T. versicolor*, like that by *P. chrysosporium*, requires N limitation, stationary cultures, high O$_2$ partial pressure, and the addition of detergents and veratryl alcohol [14–17]. However, studies of pulp biobleaching indicate that *T. versicolor* also produces MnP and laccase in N-rich, agitated cultures without addition of any detergent or veratryl alcohol [19]. It has been proposed that lignin degradation and pulp biobleaching by *T. versicolor* occur during primary metabolism and are distinct from the secondary, metabolic, lignin-degrading system [19–21].

To date, there are no studies characterizing dye decolor-
ation by \textit{T. versicolor}. In previous work in our laboratory, batch cultures of \textit{T. versicolor} decolorized a number of dyes individually and upon repeated additions [22]. This paper investigates the effects of glucose and ammonium concentrations on the sequential decoloration of textile dyes by \textit{T. versicolor} and addresses whether decoloration requires conditions of secondary metabolism.

2. Materials and methods

2.1. Cultures and growth conditions

\textit{T. versicolor} ATCC 20869 (American Type Culture Collection) was maintained on 2\% malt agar plates at 4\(^\circ\)C. All experiments were performed in autoclaved Kirk’s medium [23] by using 20 mM 2,2-dimethyl succinate buffer [22] at a pH of 5.0, with an initial glucose concentration of 10 g/l (56 mM glucose).

2.2. Preparation of inoculum

\textit{T. versicolor} from a malt agar plate was used to inoculate 200 ml of Kirk’s medium in 500-ml Erlenmeyer flasks, which were incubated at 30\(^\circ\)C and 200 rev./min. Four-day-old pellets from these cultures were used as inoculum for decoloration studies.

2.3. Dyes and monitoring of decoloration

The dyes in this study are commonly used in the textile industry and represent different dye classes. Dye was added to the cultures as aliquots of concentrated stock solutions. Decoloration in liquid medium was measured spectrophotometrically at the maximum visible wavelength of absorbance for each dye. Complete decoloration was taken to be the total decoloration of the medium, with no visible sorption to the biomass. The dyes studied, their dye class and their maximum visible wavelengths of absorbance were: Amaranth (monoazo; 523 nm), Remazol Black (reactive diazo; 597 nm), Remazol Brilliant Blue (reactive anthraquinone; 497 nm), Reactive Blue 15 (reactive phthalocyanin; 675 nm), Tropaeolin O (monoazo; 397 nm), Remazol Orange (reactive monoazo; 473 nm), and Remazol Brilliant Red BB (reactive monoazo; 532 nm).

2.4. Sequential, batch-decoloration studies

One-liter Erlenmeyer flasks, containing 400 ml of medium, were inoculated with 30-ml portions of inoculum and incubated at 30\(^\circ\)C and 200 rev./min. On Day 3, the first aliquot of dye was added to all flasks. After the thorough decoloration of each dye aliquot, a subsequent aliquot was added to the flask. Inoculated flasks, to which no dye was ever added, served as biotic controls. There was no decoloration in the abiotic controls.

The decoloration of repeated additions of (1) a single dye (50 ppm Amaranth), and (2) of different dyes was examined. The dyes were added in the following order for the concentrations shown: (A) 50 ppm Amaranth, (B) 60 ppm Remazol Black, (C) 60 ppm Remazol Orange, (D) 20 ppm Tropaeolin O, (E) 60 ppm Reactive Blue, and a mixture of (F) 30 ppm Remazol Orange, and 30 ppm Remazol Black.

2.5. Sequential, glucose-fed, batch-decoloration studies

One-liter Erlenmeyer flasks, containing 400 ml of medium, were inoculated with 30-ml portions of inoculum and incubated at 30\(^\circ\)C and 200 rev./min. Glucose concentrations were monitored daily, and replenished to 5 to 10 g/l on Days 8, 15, and 24 with aliquots of a 1 M glucose stock solution. The dyes were added in the same order and concentrations as for the batch experiments. Following the complete decoloration of these dyes, four mixtures of dyes were also added: (G) 30 ppm Remazol Black, 20 ppm Remazol Orange, 25 ppm Amaranth and 8 ppm Tropaeolin O, (H) 60 ppm Remazol Black and 20 ppm Reactive Blue, (I) 20 ppm Reactive Blue and 20 ppm Remazol Orange, and (J) 60 ppm Remazol Black, 20 ppm Remazol Orange and 8 ppm Tropaeolin O. After Day 26, glucose was no longer replenished.

2.6. Decoloration by N-rich and N-limited cultures

One-liter Erlenmeyer flasks, containing 400 ml of either N-limited (0.442 g/l) or N-rich (4.42 g/l ammonium tartrate) medium, were inoculated with 30-ml portions of inoculum and incubated at 30\(^\circ\)C and 200 rev./min. Glucose and NH\textsubscript{4}\textsuperscript{+} concentrations were monitored daily, and maintained approximately at initial concentrations by replenishing with aliquots of 1 M glucose and 0.5 M ammonium tartrate solutions. On Day 4, the first aliquot of Amaranth was added to all flasks. A second aliquot of 50 ppm was added to N-rich cultures on Day 5, after which dye addition was stopped. Aliquots of 50 ppm Amaranth were added daily to the N-limited cultures from Day 4 to Day 9. On Day 10, the concentration of NH\textsubscript{4}\textsuperscript{+} was increased from 0.086 to 0.86 g/l in one set of N-limited flasks (set B). The concentration of NH\textsubscript{4}\textsuperscript{+} in the other set of N-limited flasks (set A) was maintained at 0.086 g/l. A mixture of 10 ppm of each dye was added to both sets of flasks.

2.7. Sampling

Four-milliliter samples were taken daily from each flask and replaced with 4 ml of Kirk’s medium without glucose or NH\textsubscript{4}\textsuperscript{+}, resulting in only a 1\% change in the overall medium constitution. Samples were centrifuged to remove suspended biomass and the concentrations of dye, glucose, and NH\textsubscript{4}\textsuperscript{+} in the supernatant were determined.

Glucose was quantified by using the 3,5-dinitrosalicylic acid test [25], and NH\textsubscript{4}\textsuperscript{+}, by the phenol hypochlorite reaction [26].
All experiments were performed in duplicate, and all readings were performed in triplicate.

3. Results

3.1. The effects of glucose on sequential dye decoloration

3.1.1. Sequential batch decoloration of Amaranth

*T. versicolor* decolorized 14 successive additions of 50 ppm Amaranth in batch culture (Fig. 1) with no visible sorption to the biomass. Following the first addition of Amaranth on Day 4, there was a lag of approximately 24 h, during which only about 35% of the dye was decolorized. The remainder of the dye was rapidly decolorized during the next 24 h. Five subsequent aliquots of Amaranth were each rapidly decolorized within 6 h of being added. However, decoloration of the sixth aliquot, which was added on Day 15, required 24 h. Less than 50% of the seventh aliquot, added on Day 17, was decolorized in 3 days. This decrease in decoloration rate coincided with glucose depletion by Day 15.

When glucose was replenished to 5 g/l on Day 20, the remaining dye was rapidly decolorized and high decoloration rates were restored (Fig. 1). Three further additions of Amaranth were rapidly decolorized, each within 6 h. However, only 60% of the aliquot added on Day 25 was decolorized in 4 days. Because sufficient glucose (2.98 g/l) was available, this decrease in decoloration rate may have been due to the exhaustion of other essential nutrients or trace elements.

The measured decrease in dye concentration was not related to changes in pH. The 2,2-dimethylsuccinate buffer was used in these studies because it was previously shown to maintain a constant pH upon successive decoloration of various dyes [22]. When pH was periodically measured, it was always found to be around 5.0. Furthermore, the absorbance of individual dyes were found to be similar at pHs 3, 5, 7 and 10.

3.1.2. Comparing sequential decoloration of glucose-fed and unfed batch cultures

*T. versicolor* decolorized sequential additions of the different dyes and a dye mixture in batch culture (Fig. 2). The first dye—Amaranth—was decolorized in 48 h, with a lag period of about 24 h. Each of the five subsequent additions—Remazol Black (B), Remazol Orange (C), Tropaeolin O (D) and Reactive Blue (E)—was completely decolorized within 24 to 72 h. However, only approximately 25% of the mixture of Remazol Black and Remazol Orange (F) added on Day 17 was decolorized by Day 20. As before, decreased decoloration corresponded to glucose depletion by Day 16 (Fig. 2) and was restored by replenishing glucose to 5 g/l on Day 20. The next dye mixture G (Remazol Black, Remazol Orange, Amaranth and Tropaeolin O), added on Day 23, was also decolorized within 48 h. However, less than 30% of mixture H (Remazol Black and Reactive Blue) was decolorized in 6 days, coinciding again with glucose depletion by Day 24.

High rates of decoloration were maintained in glucose-fed cultures for a considerably longer period. Fig. 3 shows the decoloration of sequential additions of the different dyes and dye mixtures by a fed-batch culture, to which aliquots of glucose were added on Days 8, 15, and 24 to prevent
glucose depletion. The rates of decoloration for the individual dyes were similar to those in unfed culture when glucose was not limiting (Figs. 2 and 3). However, dye mixture F added on Day 18 was completely decolorized within 48 h, without any decrease in decoloration rate as observed in unfed batch culture. Three additional dye mixtures (G, H, I) were also rapidly decolorized (Fig. 3). Glucose was not replenished after Day 24 and was depleted by Day 33. Correspondingly, less than 25% of the of the final mixture (J), added on Day 33, was decolorized by Day 40.

3.2. The effects of N on sequential decoloration

3.2.1. Comparison of decoloration in N-limited and N-rich cultures

The depletion of NH$_4^+$ in batch and fed-batch cultures by Day 6 (Figs. 1–3) did not visibly inhibit decoloration. To determine the effect of N concentrations, sequential decoloration was examined in N-limited (0.086 g/l) and N-rich (0.86 g/l) batch cultures, with glucose levels maintained at approximately 10 g/l. Aliquots of 50 ppm Amaranth were added to both sets of cultures on Day 4. The first aliquot was completely decolorized by both N-limited and N-rich cultures within 48 h, with the characteristic 24-h lag period. However, in N-rich cultures, even at 8 days following addition of the second Amaranth aliquot, there was only 27% color removal from the medium, with substantial mycelial sorption. In contrast, 6 Amaranth aliquots and a mixture of 10 ppm of each dye were rapidly decolorized in N-limited cultures. Increasing NH$_4^+$ levels from 0.086 to 0.86 g/l on Day 10 quickly inhibited decoloration of Remazol Red. There was strong mycelial sorption of the dye, with no decoloration even after 10 days. Cultures maintained at 0.086 g/l NH$_4^+$ rapidly decolorized 4 subsequent additions of 40 ppm Remazol Red with no mycelial sorption.

3.2.2. Comparison of glucose and NH$_4^+$ consumption in decolorizing and nondecolorizing cultures

Glucose and NH$_4^+$ consumption in unfed, decolorizing batch cultures was compared to cultures to which no dye was ever added. Although the rate of NH$_4^+$ consumption was the same, glucose was consumed at a higher rate by decolorizing cultures (Fig. 4). Glucose was depleted by Day 15 in cultures decolorizing repeated additions of Amaranth. When no dye was present, glucose, consumed for growth and metabolic activity alone, was not depleted until Day 20. This indicates additional consumption of the primary growth substrate during decoloration.

4. Discussion

It is conventionally accepted that C and/or N limitation triggers ligninolytic activity in white rot fungi and is required for pollutant degradation [2–6]. The present study examines the effects of glucose and NH$_4^+$ concentrations on sequential dye and dye mixture decoloration by T. versicolor.
Decoloration was not stimulated by C limitation. Several factors indicate that dye decoloration required a minimal amount of glucose. The cessation of decoloration after successive dye decoloration corresponded to glucose depletion. Decoloration was restored by replenishing glucose. Glucose-fed batch cultures maintained high rates of decoloration for more than twice as long as unfed batch cultures. Furthermore, decolorizing cultures consumed glucose at higher rates than those to which no dye was added.

A critical glucose concentration between 0.1 and 0.3 g/l was required for decoloration (Figs. 1 and 2). At concentrations below this value, decoloration rates rapidly declined with no decoloration at 0 g/l glucose (Fig. 1, Fig. 2).

Decoloration followed first-order kinetics with respect to Amaranth concentration (Table 1). Plots of ln (C_{Amaranth}/C_{Amaranth, initial}) (where C_{Amaranth} = concentration of Amaranth (ppm)) as a function of time (h) were straight lines with correlation coefficients of 0.99. The rate of decoloration did not increase with glucose levels above the critical concentration as reflected by similar rate constants (Table 1). Below the critical glucose concentration, the first order rate constant declined with decreasing glucose concentrations.

Based on the results, it is unlikely that increased glucose consumption during decoloration indicates heightened metabolic activity. The requirement for N limitation is characteristic of secondary metabolism when metabolic activity is low. The need for glucose may be associated with a rate-limiting step. Possible roles for glucose in decoloration may include (i) the generation of H_2O_2 required for extracellular peroxidase activity and/or (ii) the generation of Mn^{3+} complexing agents necessary for MnP activity.

The activity of the extracellular peroxidases, such as LiP and MnP, requires H_2O_2 as a cosubstrate [4,26]. In many species of white rot fungi, enzymes such as glucose-1-oxidase and glucose-2-oxidase are responsible for H_2O_2 generation and are expressed as a part of the lignin degrading system [27]. Glucose-2-oxidase (pyranose oxidase) is a constitutive enzyme in T. versicolor [28,29]. The preferred substrate for this enzyme is D-glucose, the primary C source in this study. It is possible that glucose is the substrate for H_2O_2 generation during decoloration.

The key role played by MnP in the decoloration of bleach plant effluent (BPE) by T. versicolor may extend to dye decoloration. Purified MnP from P. chrysosporium has been shown to decolorize several dyes [30]. The Mn^{3+} ions generated by MnP bind to simple organic compounds to form complexes which, in the presence of H_2O_2, perform nonspecific oxidations. Roy and Archibald reported certain intermediates of T. versicolor glucose metabolism, such as oxalate, glyoxylate, and fumarate, to be highly effective Mn (III) ion-complexing agents [18]. In this study, the first Amaranth aliquot was not decolorized when there was no Mn^{2+}. At an initial concentration of either 3 or 200 μM Mn^{2+}, the same rate of decoloration and the same number of successive dye additions was decolorized (data not shown).
shown). If MnP is involved in dye decoloration, glucose may be required as a precursor to such intermediates. Because small amounts of these intermediates are sufficient for MnP activity, this may explain the low minimum glucose concentration to maintain rapid decoloration.

The requirement for N limitation suggests that decoloration occurs during secondary metabolism. Because there was adequate glucose in N-rich, decoloration-inhibited cultures, the lack of decoloration was not due to glucose depletion. The expression of critical decoloration enzyme(s) or factors may be inhibited in N-rich cultures.

N limitation is a key requirement for ligninolytic enzyme production and degradative activity by *P. chrysosporium* [2-4,6,7]. As observed with *T. versicolor* in this study, substantial biomass sorption of dye has been reported with decoloration-inhibited, N-rich cultures of *P. chrysosporium* [9-11]. However, other than requiring N limitation, similarities between dye decoloration by *P. chrysosporium* and *T. versicolor* were limited. Under the culture conditions employed in this study, N-limited, batch cultures of *P. chrysosporium* did not decolorize the range of dyes decolorized by *T. versicolor* and had limited ability to decolorize successive dye additions (data not shown). Dye decoloration by *P. chrysosporium* requires static culture conditions, high O₂ partial pressure, and the addition of veratryl alcohol and detergents, all of which favor the expression and activity of the ligninolytic enzymes in this organism [14,15].

Despite certain similarities, dye decoloration and BPE decoloration by *T. versicolor* appear to be governed by distinct processes. Like dye decoloration, BPE decoloration by *T. versicolor* also requires adequate supplies of C with no decoloration in the absence of a primary C source [21,31,32,33]. Mixing and aeration conditions used in this study were similar to those used in BPE decoloration and pulp biobleaching studies [18,21,32,34,35]. However, an important difference is the requirement of N limitation for dye decoloration, which implies differences in the enzymatic mechanisms governing the two processes.

5. Conclusions

Sequential dye decoloration by *T. versicolor* required adequate supplies of glucose. However, above a low critical concentration, the rate of decoloration was unaffected by increase in glucose levels. The requirement for glucose appears to be associated with a rate-limiting step in the decoloration mechanism, rather than heightened metabolic activity. The inhibition of decoloration in N-rich cultures
suggests that decoloration occurs during secondary metabolism. The requirement for N limitation distinguishes dye decoloration from the well-studied process of \textit{T. versicolor} BPE decoloration.

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References