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Assessment of the decolourization ability of *Phanerochaete chrysosporium* towards different sugar colourants

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SUMMARY

The ability of *Phanerochaete chrysosporium* to degrade the colourants present in a sugar refinery effluent : caramels, melanoidins and alkaline degradation products of reducing sugars (ADP's), was studied. The fungus was incubated with each type of colourant and decolourization was evaluated. Little or no decolourization was observed in high-nitrogen cultures, in which no LIP and MNP activities were detected. All of the cultures grown under nitrogen limiting conditions showed a high decolourization ability for the different colourants, caramels being the most degraded ones. MNP, but not LIP, activity, was detected in these cultures, indicating that MNP plays a key role in decolourization.

INTRODUCTION

Colour in the sugar industry consists of a complex mixture of different types of colourants, the most important being : 1) phenolic compounds, coming from the cane plant, 2) caramels, which are produced by thermal degradation and condensation reactions of sugars, 3) melanoidins, formed from sugar-amino acid reactions via the Maillard reaction and 4) alkaline degradation products of reducing sugars (ADP's) (6). During the refining process one fraction of the colourants is removed from the sugar liquor by anion-exchange resins. The pre-regeneration of these resins is made with 50 g/l NaCl, giving rise to an effluent containing those types of colourants. This effluent presents an environmental problem due to the presence of phenolic compounds, intense colouration and high organic load (COD). The high toxicity of phenolic compounds to living organisms is well reported in literature (2,5). The brown colour of the effluent is not only aesthetically unacceptable but also inhibits the natural process of photosynthesis in natural waters leading to a chain of adverse effects on the aquatic ecosystem, especially affecting the growth of primary consumers.

The organic load can be eliminated, at least in part, using traditional biological treatments but the compounds responsible for the intense colouration are poorly degraded by the organisms normally involved in these treatments (7).

The white-rot fungus *Phanerochaete chrysosporium* is a potentially useful microorganism in waste treatment systems because it is able to degrade a broad spectrum of structurally diverse organic compounds. Evidence suggests that the unique ability of *P. chrysosporium* to degrade those compounds is due, at least in part, to the lignin degrading enzymatic system of this microorganism (3,4).

Previous studies made in our laboratory demonstrated that *P. chrysosporium* was able to degrade the phenolic compounds present in the pre-regeneration effluent. In this work we intended to investigate the ability of *P. chrysosporium* to degrade the other colourants existing in the effluent - caramels, melanoidins and ADP's - and to investigate the possible involvement of the ligninolytic system in the decolourization performed by the fungus.

MATERIALS AND METHODS

Organism and inoculum. *P. chrysosporium* (ATCC-24725) was maintained on agar medium containing 2% glucose, 2% malt extract and 0.1% peptone. This medium was also used for spore production. Inoculum consisted of filtered (glass wool) conidial suspension (A_{650nm} = 0.500/cm).

Colourants. Caramels, melanoidins and ADP's, were prepared in the laboratory as described by Shore et al. (9). Each colourant was passed through an anion-exchange resin and the pre-regeneration fraction was collected. The colourant solution used in the experiments consisted of the respective pre-regeneration fraction diluted with distilled water in order to have a colour of 1200 in the culture media. When necessary, NaCl was added to the colourant solution to adjust the salt concentration to 16 g/l.

Decolourization experiments. Colourant solution at pH 4.5 (27.4 ml) was supplemented with 4.6 ml 0.22 M Na acetate buffer (pH 4.5), 5 ml basal medium, 2.5 ml glucose (20%), 0.5 ml thiamine (100 mg/l) and 5 ml ammonium tartrate. The basal medium was based on that described by Tien and Kirk (10), but contained double the concentration of $MnSO_4$. The supplemented colourant solution (45 ml) was dispensed into sterile cotton-stoppered 500 ml flasks and inoculated with 5 ml of the conidial suspension mentioned above. This inoculum was replaced with sterile water in the control flask. The organism was grown in shallow stationary cultures at 39 °C, for 22 days. Two nitrogen concentrations, 2.4 mM and 24 mM, corresponding, respectively, to limiting and non-limiting conditions, were tested.

Analytical procedures. Colour was measured at 420 nm after pH adjustment to pH 9.0. To determine the colour adsorbed, the biomass was removed by filtration and washed twice with 0.1 N NaOH. The colour released was measured and reported to the initial colour. Gel filtration chromatography was performed in a Pharmacia FPLC system equipped with a Superose-12 column. The UV/Vis detector was a L-4500 Merck-Hitachi Diode Array. 200 µl of sample (filtered with 0.45 µm Gelman filter) was eluted with acetonitrile 30% (v/v) + NaAcetate 0.1 M (pH 8.0), at a flow rate of 0.50 ml/min. Nitrogen ammonia content was determined by the Nesslerization method according to Standard Methods (1).

Enzymatic assays. LIP activity was measured as described by Tien and Kirk (10). MNP was measured by a method modified from that of Paszczynski et al. (8). The reaction mixture contained 50 mM Na malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM $MnSO_4$, and 600 µl of culture broth in a total volume of 1 ml. The reaction was started by adding 0.4 mM H_2O_2. 1 U of activity was defined as 1 µmol of 2,6-dimethoxyphenol oxidised per min.

RESULTS AND DISCUSSION

Degradation of sugar colourants by *P. chrysosporium*. After 22 days of incubation, in nitrogen limiting conditions, it was observed that the fungus promoted a high decolourization of the three colourants (Table 1). At the end of the experiment, the biomass was separated from the liquid medium, and washed twice with 0.1 N NaOH solution. The colour recovered was measured and compared with the initial colour. With all of the colourants, the colour adsorbed onto the biomass was between 1 and 2 % of the initial colour, showing that the decolourization was almost totally due to the fungal activity. Controls and samples, from the experiments made under N limiting conditions, were also analysed by gel filtration chromatography, using a Diode Array as detector, obtaining three dimensional chromatograms.

Comparing the chromatogram volume between the sample and the control we can evaluate the extension of the degradation performed by *P. chrysosporium* at all wavelengths (Table 2). These results show that *P. chrysosporium* degrades caramels more efficiently.

Table 1 - Reduction of colour obtained after incubation of colourants with *P. chrysosporium*.

COLOURANT	COLOUR RED. (%)
Melanoidins	69.5
Caramels	81.7
ADP's	76.9

Table 2 - Volume reduction of the 3D gel filtration chromatograms, obtained after incubation with *P. chrysosporium*.

COLOURANT	VOLUME RED. (%)
Melanoidins	34.3
Caramels	59.1
ADP's	43.6

Involvement of Lignin Degrading System in the degradation of sugar colourants by *P. chrysosporium*. There was little or no decolourization in high-nitrogen cultures (Fig. 1 - **d**, **e**, and **f**), in which neither LIP nor MNP activity were detected. When the fungus was grown under nitrogen limiting conditions, there was no significant decolourization before day four (Fig. 1 - **a**, **b**, and **c**), in which, according to the Nesslerization method, the nitrogen was already limiting. In these conditions MNP, but not LIP, was detected, indicating that MNP plays a key role in decolourization. The onset and time-course for decolourization and MNP activity coincides (Fig. 1 - **a**, **b**, and **c**). Decolourization occurred only when MNP appeared in the extracellular fluid and reached a maximum level soon after MNP activity reached its highest level. These results suggest the involvement of the lignin degrading system, especially MNP, in the degradation of the three tested colourants.

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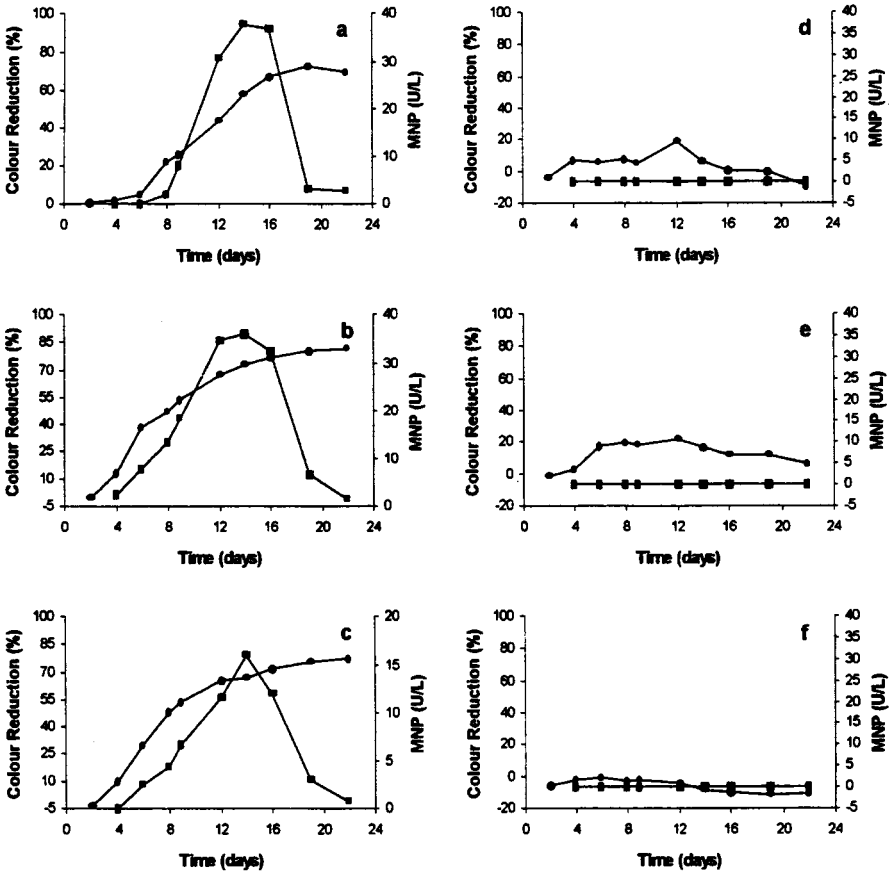

Figure 1 - Time course study of the decolourization and MNP activity in N limiting conditions (2.4 mM) (**a**, **b** and **c**) and in N sufficient conditions (24 mM) (**d**, **e** and **f**). (**a** and **d**) Melanoidins. (**b** and **e**) Caramels. (**c** and **f**) ADP's. Symbols : ●, colour reduction; ■, MNP activity.