Biodegradation of colorants in refinery effluents

Potential use of the fungus *Phanerochaete chrysosporium*

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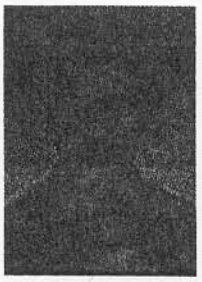
Technical summary

The degradative ability of *Phanerochaete chrysosporium* towards each of the four main types of colorants present in regeneration effluents from ion exchange resins was investigated. The fungus was able to decolorise melanoidin, caramel and HADP (hexose alkaline degradation product) solutions by 74%, 87% and 80%, respectively, and to reduce levels of phenolic compounds by 72%. Gel permeation chromatography studies showed that decolorisation was accompanied by effective degradation of the colorants. As well as transformation of the visible chromophores, minor degrees of polymerisation of phenolic compounds and depolymerisation of melanoidin occurred during the incubation. No significant adsorption of colorants onto the biomass was observed, indicating that colorants were biochemically degraded and not physically adsorbed.



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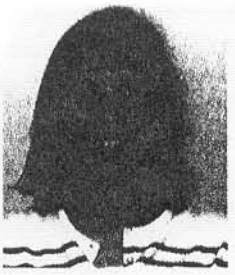

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Introduction

Colour in cane sugar process streams arises from a complex mixture of colorants, the most important being:

(1) phenolic compounds, derived 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) hexose alkaline degradation products (HADPs)[1].

During the refining process, some of the colorants are removed from the sugar liquor by anion-exchange resins. These resins are regenerated with sodium chloride solutions in two steps: at 50 g/l and 100 g/l NaCl, giving rise to an effluent with a high pollution load. The effluent from the second phase of the regeneration (100 g/l NaCl) can be treated by lime precipitation, followed by ultra-filtration, after which it may be recycled[2]. However, the remaining effluent, from the pre-regeneration step (50 g/l NaCl), still presents an environmental problem due to the presence of phenolic compounds, intense coloration and high organic load (COD). The toxicity of phenolic compounds to living organisms has been well reported[3,4]. Furthermore, the brown colour of the effluent is not only aesthetically unacceptable, but it also inhibits the natural process of photosynthesis in natural waters, leading to a chain of adverse effects on the aquatic ecosystem. Therefore, environmental pressures are compelling the industries to reduce substantially the colour of their effluents.

The organic load can be eliminated, at least in part, using traditional biological treatments, but compounds responsible for the intense coloration, such as melanoidins, are poorly degraded by the organisms normally involved in these treatments[5,6]. Furthermore, the inhibitory power of some phenolic compounds[7-9] and some caramels, such as furfurals[10], impair the traditional biological wastewater treatment. Another problem is that most microorganisms normally involved in biological treatments are not able to degrade a broad spectrum of structurally diverse compounds. Thus, their use is restricted to situations where only a limited number of pollutants are present, unless complex consortia of microbes (which are often difficult to maintain) are utilised, each of which generally possesses the requisite enzymes to degrade one or, at best, a few compounds[11].

Previous studies undertaken in our laboratory[12,13] demonstrated that the white-rot fungus *Phanerochaete chrysosporium* is able to decolorise sugar refinery effluents. This fungus has been widely studied as a potentially useful organism for wastewater treatment systems because it is able to degrade a broad spectrum of structurally diverse organic compounds such as lignin, chlorinated aromatics, polycyclic aromatics, pesticides, and textile and general dyes. Evidence suggests that the unique ability of *P. chrysosporium* to degrade these compounds is due, at least in part, to its lignin degrading enzymatic systems, which are non-specific and partially extracellular[11,14]. The very non-specific nature of the mechanisms used by the fungus allows it to completely degrade even complex mixtures to water and carbon dioxide[15].

Four main types of colorants are known to be present in the pre-regeneration effluent: phenolics, melanoidins, caramels and HADPs[16]. The relative proportions of these colorants in the effluent may vary considerably, which is typical of an industrial operation. Thus, in order to understand and predict the effluent decolorising ability of *P. chrysosporium*, we have investigated the activity of the fungus towards each of these four types of colorants.

Materials and methods

Microorganism and inoculum

P. chrysosporium (ATCC-24725) was maintained at 4°C on 2% malt extract agar. The inoculum consisted of a conidial suspension collected from 6 - 8 day-old cultures, filtered through glass wool (A_{650nm} = 0.500/cm).

Colorants

Caramel, melanoidin and HADPs were synthesised in the laboratory as described by Shore *et al.*[17]. To obtain the colorant fraction present in the effluent, each

colorant was passed through an anion-exchange resin and the pre-regeneration fraction (eluted with 50 g/l NaCl) was collected[16]. The phenolic compound solution consisted of the effluent collected before the regeneration of the resins loaded with sugar refinery liquor, since it has a large load of phenolic compounds. All the solutions were adjusted to the same initial colour (A_{420} = 1200) and NaCl concentration in the culture medium.

Culture conditions

The solutions with each colorant (at pH 4.5) were supplemented with Na acetate buffer, pH 4.5 (20 mM); basal III medium; glucose (1%); thiamine (1 mg/l) and diammonium tartrate. The limiting nitrogen concentration added to the cultures was 2.4 mM N-NH_4 for experiments with caramels and HADPs, and 2.1 mM N-NH_4 for experiments with melanoidins and phenolic compounds, since the solutions containing these colorants already provide some nitrogen to the cultures (approximately 0.3 mM N as free amino nitrogen). The basal medium was based on the medium described by Tien and Kirk[18], but contains 60 µM Mn(II). Supplemented colorant solutions (9 ml) were dispensed into sterile cotton-stoppered 100 ml flasks, after being sterilised by filtration (pore size, 0.45 µm), and inoculated with 1 ml of the above mentioned conidial suspension. This inoculum was replaced with sterile water in the control flasks. Cultures were incubated statically under an air atmosphere at 39°C.

Analytical procedures

Phenolics were determined as "phenol-reacting substances" using the Folin and Ciocalteu reagent[19]. Colour was measured as absorbance at 420 nm, after pH adjustment to pH 9.0 with borate buffer. Reduction of phenolic compounds was expressed as the percentage decrease in the concentration compared to the control (noninoculated cultures). Decolorisation of caramels, melanoidins and HADPs in cultures was expressed as the percentage decrease in colour compared to the control. Growth was measured in terms of dry weight, at 105°C, of mycelium after vacuum filtration of cultures (10 ml) through a tared filter. Glucose content was determined spectrophotometrically as the formation of NADPH in a reaction mixture containing hexokinase, ATP, NADP, glucose-6-phosphate dehydrogenase and culture filtrate sample (D-glucose assay, Boehringer Mannheim GmbH). Nitrogen content in cultures containing caramels or HADPs was expressed as the concentration of ammonia nitrogen (N-NH_4). Nitrogen content in cultures containing melanoidins or phenolic compounds was expressed as ammonia nitrogen + free amino nitrogen. N-NH_4 concentration was determined spectrophotometrically as the oxidation of NADH to NAD+ in a reaction mixture containing NADH, glutamate dehydrogenase and culture filtrate sample (Ammonia assay, Boehringer Mannheim GmbH). Free amino nitrogen concentration was measured by the ninhydrin method[19]. Interference by N-NH_4 in the ninhydrin method were taken into account in all determinations. Gel permeation chromatography was performed in a Pharmacia FPLC system equipped with a Superose-12 column and a L-4500 Merck-Hitachi Diode Array UV/Visible light detector. A sample volume of 200 µl (filtered through a 0.45 µm Gelman filter) was eluted with acetonitrile 30% (v/v) + 0.1 M Na acetate (pH 8.0), at a flow rate of 0.50 ml/min.

Results and discussion

P. chrysosporium was incubated with each type of colorant solution, under N-limited conditions. These conditions are favourable for the expression of the lignin degrading system by the fungus[20].

The reduction of phenolic compounds (in incubations with phenolic compounds) or decolorisation (in incubations with melanoidins, caramels or HADPs) was monitored during the incubation period in parallel with fungal growth and nutrient consumption (glucose and nitrogen). The results are shown in Figure 1.

The organism grew rapidly during the first 2 - 4 days of incubation with phenolic compounds, caramel and HADPs (Figures 1A, C and D). During incubation with melanoidin (Figure 1B) the initial growth was somewhat slower. However, the final dry weight obtained was similar for all cultures, at around 13 - 15 mg/10 ml (Figs. 1A, B, C and D).

Although the maximum biomass obtained was the same for the different cultures, the remaining glucose in the end of the experiments was different, as we can observe in Figures 1A, B, C and D. Glucose was gradually consumed, to a final content of 3.8 g/l (Figure 1A), 1.8 g/l (Figure 1B), 2.2 g/l (Figure 1C) and 4.4 g/l (Figure 1D) in the corresponding incubations. This indicates that there was, probably, an additional carbon source in cultures containing phenolic compounds or HADPs. Since there was some glucose remaining at the end of the experiments, we may consider adding less in the future, thus reducing overall costs.

Nitrogen was depleted within two days, coinciding with the onset of fungal activity towards the different colorants (Figures 1A, B, C and D). Between days 2 and 6 there were rapid losses of phenolic compounds, and rapid decolorisation of caramel and HADPs. Rates of these changes slowed after 63%, 74% and 65%, of the phenolics, caramel and HADPs had been degraded, respectively (Figures 1A, C and D). Final values were 72%, 87% and 80% for phenolic conversion, caramel decolorisation and HADP decolorisation, respectively, after 14 days of incubation. Decolorisation of melanoidin was delayed for about 2 days (Figure 1B), probably due to the slower initial growth on this substrate. In this case, decolorisation was rapid between days 4 and 8 (up to 62%) and slower during the following days, reaching a final decolorisation of 74%, after 14 days.

For each colorant studied, the molecular weight distribution, obtained by gel permeation chromatography (GPC), was examined before and after incubation with the fungus.

The phenolic compound solutions, before and after incubation with the fungus, were eluted through a gel permeation column. In order to monitor

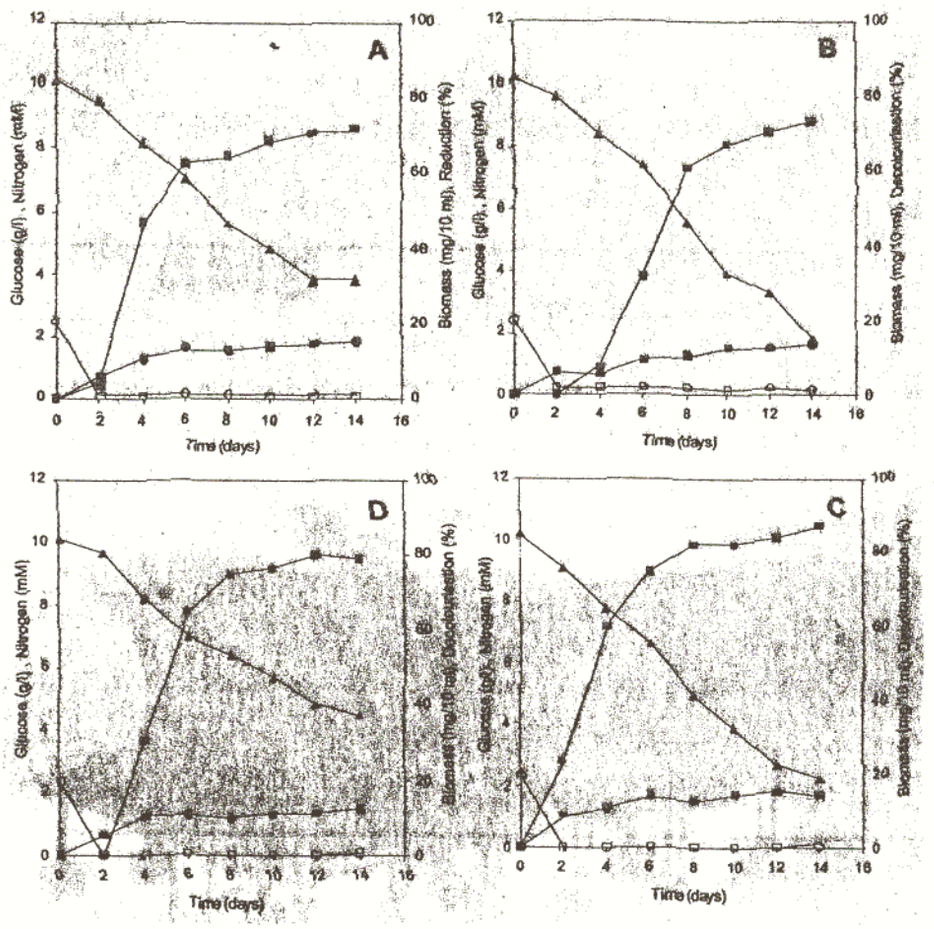

Figure 1. Time course study of reduction in phenolic compounds (graph A), or decolourisation (graphs B, C and D), together with growth and nutrient consumption, in cultures of *P. chrys.* containing: (A) phenolic compounds; (B) melanoidin; (C) caramel and (D) HADPs. Symbols: ■, phenolic compound reduction or decolourisation; ●, biomass; ▲, glucose concentration; ○, nitrogen concentration. Results are the average of triplicates. Standard deviations were lower than 10%.

only the phenolic compounds, 0.5 ml fractions of the effluent eluting from the column were collected and the concentration of phenolic compounds was measured. The chromatography profiles obtained are shown in Figure 2A. We can see that the fungal treatment eliminated the peaks corresponding to lower molecular weight phenolic compounds, and reduced the peak corresponding to the higher molecular weight species. The slight polymerisation observed after incubation could be due to the polymerisation of some of the lower molecular weight compounds which disappeared, possibly via coupling of free radicals generated in the compounds by enzymes of the ligninolytic system. In support of this hypothesis, ligninases of *P. chrysosporium* have been reported to polymerise lignin[21,22] and lignite[23]. GPC elution profiles of melanoidin, caramel and HADPs were monitored by UV absorbance (Figures 2B, C and D).

Gel permeation chromatograms of melanoidin (Figure 2B), showed a slight reduction in the average molecular weight and an accumulation of lower molecular weight species, following incubation, suggesting that some depolymerisation occurred. This is in agreement with the findings of Blondeau[24].

By comparing the chromatograms obtained for caramel (Figure 2C), it is apparent that the fungus was able to attack all the caramel constituents.

The chromatographic profiles of the HADPs (Fig. 2D) show that there was a reduction in the size of the two main peaks, indicating that both high and low molecular weight constituents of HADPs were modified by the action of the fungus, especially the low molecular weight constituents.

Thus, GPC elution profiles monitored by UV absorbance indicate that *P. chrysosporium* was able to effectively degrade melanoidin, caramel and HADPs, rather than just transform the visible chromophores.

At the end of the experiments, the mycelium was removed by filtration and washed with 0.1 N NaOH in order to quantify the colour and phenolic compounds adsorbed onto the biomass. Only 1 - 2% of the colour, and 5% of the phenolic compounds, were recovered from the mycelium surface. This indicates that colorants were predominantly degraded and not physically adsorbed.

It is well established that the lignin degrading system is expressed upon nutrient (N, C or S) limitation[20]. Since little or no degradation was observed until nitrogen was limiting, the lignin degradation system might be involved in the degradation. This is currently being investigated.

In summary, we demonstrated that *P. chrysosporium* is able to degrade all the four main types of colorants present in the pre-regeneration effluent: phenolic compounds, caramels, melanoidins and HADPs. Thus, *P. chrysosporium* seems to be a potentially useful microorganism for the treatment of effluents from sugar industries (cane and beet) and other, related, industries (e.g., fermentation industries using molasses). Similarly, Kumar *et al.*[25] recently reported that white-rot fungi were able to decolorise effluents resulting from a cane molasses based distillery.

Further research has to be done in order to improve the biodegradation process, and to make it viable for practical applications. Among other issues to be considered are: increases in the treatment rate; reduction of the amount of added nutrients; utilisation of molasses as C, N and mineral sources;

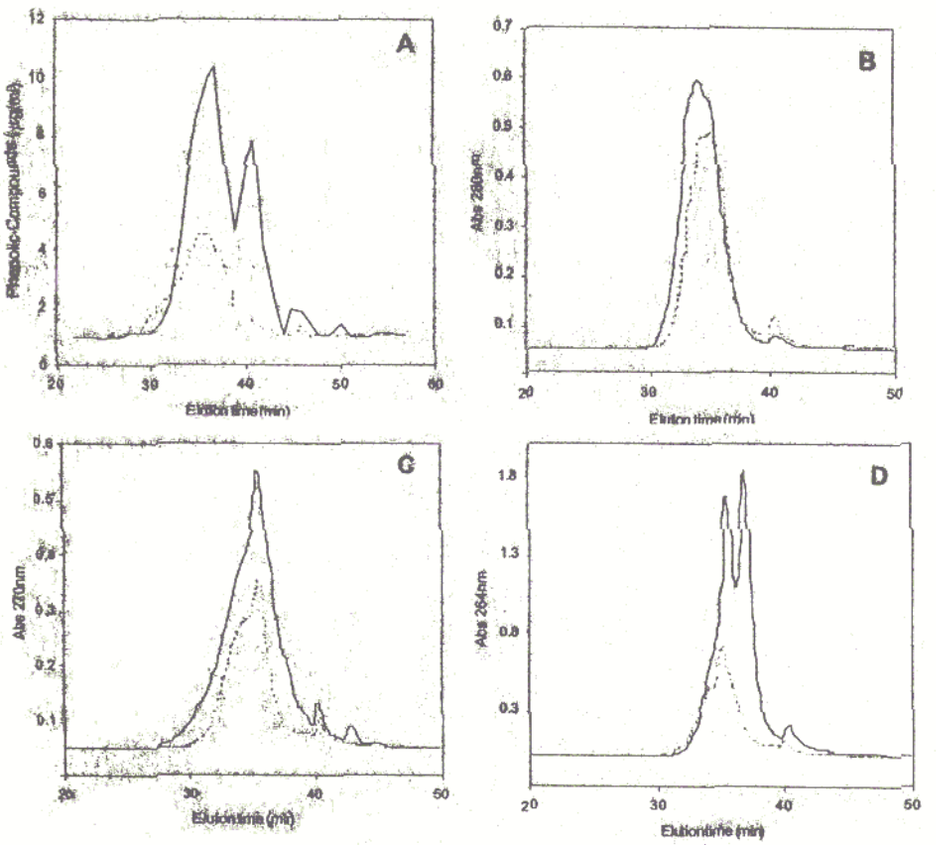

Figure 2. Gel permeation chromatography profiles of the colorant solutions before (—) and after (- -) incubation with *P. chrys.*: (A) phenolic compounds; (B) melanoidin; (C) caramel and (D) HADPs.

fungus immobilisation and design of an appropriate bio-reactor.

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Biodegradación de colorantes en efluentes de refinerías: el uso del hongo *Phanerochaete chrysosporium* (Resumen)

Se investigó la capacidad de degradación del hongo *Phanerochaete chrysosporium* sobre cada uno de los tipos principales de colorantes presentes en los efluentes de regeneración de resinas de intercambio iónico. El hongo fue capaz de decolorar soluciones de melanoidina, caramelo y HADP (producto de la degradación de hexosa alcalina) en un 74%, 87% y 80%, respectivamente, y de reducir los niveles de compuestos fenólicos, en un 72%. Estudios cromatográficos de impregnación en gel mostraron que la decoloración fue acompañada por una efectiva degradación de los colorantes. Al mismo tiempo que se vieron transformaciones de los cromóforos visibles, también se observó un mínimo grado de polimerización de compuestos fenólicos y despolimerización de la melanoidina durante incubación. No se observó adsorción significativa de colorantes sobre la biomasa, lo que indica que los colorantes fueron degradados bioquímicamente y no absorbidos físicamente.

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