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EFFECT OF MANGANESE AND VERATRYL ALCOHOL ON THE COLOUR REMOVAL FROM SUGAR REFINERY EFFLUENTS BY *PHANEROCHAETE CHRYSOSPORIUM*

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ABSTRACT

At RAR - Refinarias de Açúcar Reunidas - ion exchange resins are used to remove colourants present in sugar liquor after carbonatation. The pre-regeneration of these resins is made with 50 g/l NaCl, giving rise to a heavily coloured effluent exhibiting a high phenolics content. The compounds present in this effluent are poorly degraded by the organisms normally involved in waste water treatments.

Previous studies made in our laboratory demonstrated the possibility of using *Phanerochaete chrysosporium* to treat this effluent. This organism has an enzymatic system related with lignin degradation, and is also able to degrade xenobiotics present in wastes from several chemical and food industries. The major components of the ligninolytic system are two families of extracellular peroxidases, namely lignin peroxidase (LiP) and manganese-dependent lignin peroxidase (MnP), along with the H_2O_2 generation system.

In this work the effect of manganese, essential for MnP activity, and veratryl alcohol, a normal inducer of LiP, on the removal of the sugar colourants from the resins regeneration effluent by *Phanerochaete chrysosporium*, was studied.

It was observed that the presence of veratryl alcohol (2 mM) did not improve the decolourization. To test the influence of manganese, concentrations of 0; 30; 60; 120; 165 and 727 μM of added Mn(II) were assayed. The concentration of 60 μM of Mn(II) was shown to be the optimal concentration for this cation, as far as colour and phenolics removal is concerned - about 63% of colour and 73% of phenolics removal was achieved.

INTRODUCTION

Ion exchange resins have proved to be excellent sugar liquor decolourizers. Effluents resulting from salt regeneration are a disadvantage of this process as they represent an environmental problem. Different processes have been proposed to overcome this situation. One process involves anionic colourants precipitation with lime (2). The colourants not removed by this process result from the pre-regeneration stage at low salt concentration (50 g/l). This fraction of effluent still represents 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 (6,13). 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 as the growth of primary consumers as well as secondary and tertiary consumers is affected.

The presence of phenolic compounds and caramels in the effluent impairs the traditional biological waste water treatment. Several authors reported that phenolic compounds (3,16,26) and caramels, such as furfurals (1), are inhibitory to methanogenic bacteria involved in anaerobic waste treatments. On the other hand, several phenolics are well known as disinfectants for many microorganisms and inhibit, at least partially, aerobic treatment processes (18). Another problem is the fact that most microorganisms normally involved in biological treatments are not able to degrade a broad spectrum of structurally diverse compounds, as it occur in the pre-regeneration effluent, thus restricting their use to situations where only a limited number of pollutants are present, or forcing the use of sometimes hard-to-maintain consortia of microorganisms, each of which possess the requisite enzymes to degrade one or, at best, a few compounds (8).

The white-rot fungus *P. chrysosporium* is a potentially useful microorganism in waste treatment systems because it is able to degrade a broad spectrum of structurally diverse organic compounds (9), such as DDT, lindane, ferulic acid, benzo(α)pyrene, chlorinated aromatics, etc (8). Indeed, previous studies made in our laboratory demonstrated the possibility of using *Phanerochaete chrysosporium* to treat the effluent from the pre-regeneration of decolourizer resins (28).

Evidence suggests that the unique ability of *P. chrysosporium* to degrade those persistent compounds is due, at least in part, to the lignin degrading enzymatic system of this microorganism (8,9), that is non-specific and partially extracellular.

The major components of the ligninolytic system are two families of peroxidases, namely lignin peroxidase (LiP) (30) and manganese-dependent lignin peroxidase (MnP) (24), along with the H_2O_2 generation system (22). LiP's catalyse H_2O_2-dependent one-electron oxidation of a variety of lignin-related aromatic compounds resulting in the formation of unstable aryl cation radicals. These radicals undergo various non-enzymatic reactions yielding a multiplicity of end products (5,22,29). The reactions involve carbon-carbon bond cleavage, aromatic ring opening, demethoxylation, hydroxylation, decarboxylation and phenol coupling reactions (5,22,29). MnP's catalyse the H_2O_2-dependent oxidation of Mn(II) to Mn(III) as well as some phenolic substances. Mn(III) can then oxidise other compounds (10). These enzymes could not efficiently complete the catalytic cycle in the absence of Mn(II), suggesting the absolute requirement of Mn(II) (31). In the degradation of lignin, Mn(III) can oxidise phenolic lignin substructures, leading to phenoxy radical intermediates (29). Subsequently, Ca-Cb cleavage or alkyl-phenyl cleavage would yield the depolymerised fragments, including quinones and hydroquinones.

Several studies made with synthetic culture media demonstrated that lignin peroxidase (LiP) activity of *P. chrysosporium* is stimulated by incubating cultures with veratryl (3,4 - dimethoxybenzyl) alcohol, a secondary metabolite of this fungus (11,14,15,23,25). The increase in lignin peroxidase activity can be attributed to the ability of veratryl alcohol to protect lignin peroxidase from H_2O_2 inactivation (11,19,20,21) and to function as low molecular weight electron mediator between substrate and peroxidase (19-22). It was also demonstrated that the addition of veratryl alcohol to the cultures did not alter the Mn peroxidase activity (11).

Recent studies showed that LiP or MnP production can be selectively affected by regulating manganese levels in the culture medium of *P. chrysosporium* in presence of veratryl alcohol (4,7,27). LiP titres varied as an inverse function of and MnP titres varied as a direct function of the Mn(II) concentration. Addition of 727 μM of manganese to the medium resulted in no LiP production and a great enhancement in MnP production.

In this work we intended to investigate the effect of veratryl alcohol and manganese on the removal of sugar colourants from the effluent by *P. chrysosporium*.

METHODS AND MATERIALS

Organism and inoculum

Phanerochaete chrysosporium (ATCC-24725) was kindly supplied by Prof. J. Lema (Univ. Santiago Compostela, Spain) and was maintained on agar medium containing 1% glucose, 1% malt extract, 0.2% peptone, 1% yeast extract, 0.1% asparagine, 0.2% KH_2PO_4 and 0.1% $MgSO_4.7H_2O$. This medium was also used for spore production. Inoculum consisted of filtered (glass wool) conidial suspension in sterile water ($A_{650\,nm}$ = 0.500/cm, approximately 2.5 x 10^6 spores/ml).

Effluent

The effluent was collected in the Refinery during the regeneration of the decolourizing resins with 50 g/l NaCl. The final salt concentration of the collected effluent was 20 g/l. As it had been previously shown (28) that this concentration reduces the fungal growth rate, the effluent was diluted to a final salt content of 15g/l.

Biological treatment

Effluent at pH 4.5 was supplemented with Na acetate buffer at pH 4.5, basal medium, glucose (0.2%) and thiamine (1 mg/l). The basal medium was composed of several minerals as described in (28). Effluent, basal medium, glucose and thiamine were filter sterilised. Na acetate buffer was autoclaved (121°C, 15 min). Nitrogen was not added because the effluent contained enough nitrogen (4-6 mM) to sustain growth. The supplemented effluent (50 ml) was dispensed into sterile cotton-stoppered 500 ml flasks and inoculated with 5 ml of the above mentioned conidial suspension. This inoculum was replaced with sterile water in the control flask (inoculated control). The organism was grown in shallow stationary cultures at 39 °C, during 26 days.

Mn(II) was added at the time of inoculation, as part of the basal medium. The concentration of added manganese was as follows : 0 µM, 30 µM, 60 µM, 120 µM, 165 µM and 727 µM.

Veratryl alcohol (2 mM) was added at the time of inoculation. Control cultures received an equivalent volume of water.

Each assay was done in triplicate. Standard deviations were always less than 10%.

Colour

Colour, expressed as attenuation*, was measured at 420 nm, pH 9, with a Perkin-Elmer LCC-55B spectrophotometer.

*Attenuation = (Absorbance x 1000) / cell length, cm

Phenolic compounds

Phenolics were determined as "phenol-reacting substances" using the Folin and Ciocalteu reagent (12,17).

Gel filtration chromatography

A Pharmacia FPLC system was used, equipped with a Superose-12 column. The detector was a L-4500 Merck-Hitachi Diode Array and the software was model D-6500 Merck-Hitachi. 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.

RESULTS AND DISCUSSION

Mn(II) concentration is considered as the concentration of manganese added to the cultures, not to the total Mn(II) concentration, since effluent has always manganese traces (3-4 µM).

Effect of manganese in the absence of veratryl alcohol

All previously reported experiments about the effect of manganese in LiP and MnP were made using veratryl alcohol. A study on the effect of manganese without veratryl alcohol would however be interesting, and that was one of the aims of this work.

Various concentrations of Mn(II) were added to the effluent prior to treatment by *P. chrysosporium*. To evaluate the fungus performance the colour and phenolic compounds were measured after fungal treatment.

The reduction of colour and phenolic compounds, obtained after treatment, increased with increasing Mn (II) and reached a maximum at 60 µM (Figure 1). Above 60 µM the removal was maintained at a constant level, even when the concentration of Mn (II) was increased to 727 µM (Figure 2).

Gel filtration chromatography confirmed the above results (Figure 3). At 0 µM Mn (II) the removal of colourants was low whilst at 60µM as well as at 165 µM the removal was higher.

The results obtained are consistent with the fact that Mn(II) is essential for MnP activity. The Mn(II) added to the cultures was probably used by MnP to oxidise substrates existing in the effluent, thus increasing the removal of colourants as the Mn(II) concentration was increased. Above 60 μM a "plateau" was reached, the Mn(II) added had no effect on the removal probably because there was no need for more Mn(II).

Effect of veratryl alcohol addition

In order to study the effect of manganese in the presence of veratryl alcohol, 2 mM of veratryl alcohol and various concentrations of Mn(II) were added to the effluent before the inoculation with *P. chrysosporium*.

As the concentration of Mn (II) was increased from 0 μM to 165 μM (Figure 4), there was a decrease in the fungus performance, as far as removal of colour and phenolic compounds was concerned. This decrease was even more pronounced when the concentration of Mn(II) was increased to 727 μM (Figure 5).

The results obtained in gel filtration chromatography (Figure 6) showed that in fact the removal of colourants was inhibited at high Mn(II) concentrations, confirming the above results.

Bonnarme et al. (4) and Perez et al. (27) showed that in the presence of veratryl alcohol the LiP titres varied as an inverse function of, and MnP titres varied as a direct function of, the Mn (II) concentration. Furthermore, a concentration of 727 μM Mn(II) totally repressed LiP and stimulated MnP. Therefore the decrease in fungus performance observed at high concentrations of Mn(II) could be due to inhibition of LiP and the reduction of colour and phenolic compounds observed at 727 μM would be due only to the action of MnP.

For the low Mn(II) concentrations (0, 30 and 60 μM), not inhibitory to LiP, we expected to see an increase in removal of colourants when veratryl alcohol was added, due to stimulation of LiP, as it was reported in literature. Surprisingly, we observed that the removal was always better in the absence of veratryl alcohol (Figure 1 and 4, Figure 2 and 5, and Table 1).

There is not a simple explanation for these results. Indeed, if the fungus was not producing LiP or if veratryl alcohol, in the experimental conditions used, did not stimulate LiP, the simple addition of veratryl alcohol should have a neutral, not a negative effect.

It seems that there is a synergic negative effect of manganese and veratryl alcohol.

The best results were obtained in the absence of veratryl alcohol and with a Mn(II) concentration of 60μM or higher (Figure 7). In these conditions we obtained 63% colour removal and 73% phenolics removal. The results from gel filtration chromatography showed an effective removal of some compounds.

CONCLUSIONS

Manganese had a positive effect on the removal of sugar colourants from the effluent by *P. chrysosporium*, but only in absence of veratryl alcohol. When veratryl alcohol was present the effect of manganese was negative.

The addition of veratryl alcohol gave rise to a decrease in the fungus performance. In terms of industrial application this is a positive result because this alcohol is very expensive.

The best results were obtained in the absence of veratryl alcohol and with 60 μM of Mn(II). In these conditions *P. chrysosporium* was able to reduce colour by 63% and phenolic compounds by 73%.

In a near future, pilot scale experimentation will be initiated.

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Table 1. Colour reduction in presence or absence of veratryl alcohol (V.A.)

Conc. Mn (II) (μM)	0	30	60
Without V.A.	51.0	57.7	62.6
With V.A.	46.5	41.7	36.9

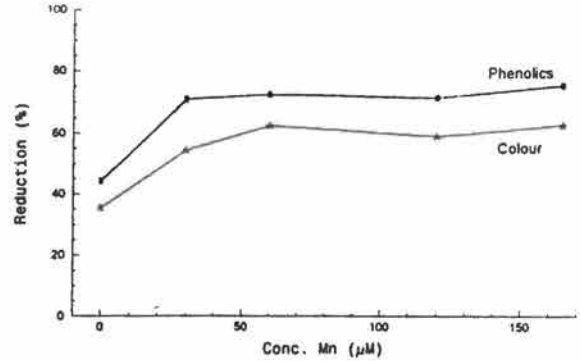

Figure 1 - Reduction of colour and phenolic compounds obtained after treatment with *P. chrysosporium* in presence of different Mn(II) concentration (0, 30, 60,120 and 165 µM), without veratryl alcohol.

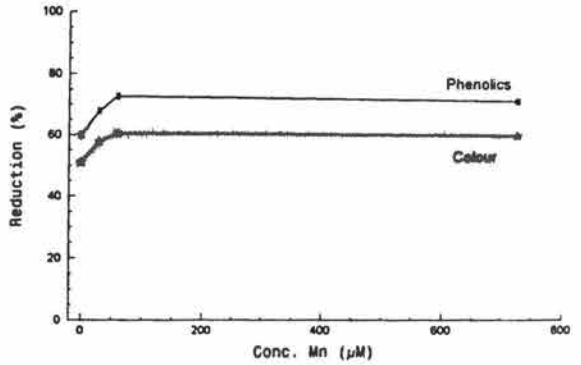

Figure 2 - Reduction of colour and phenolic compounds obtained after treatment with *P. chrysosporium* in presence of different Mn(II) concentration (0, 30, 60 and 727 µM), without veratryl alcohol.

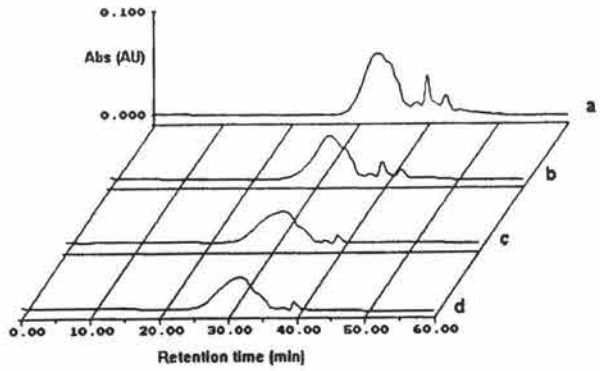

Figure 3 - Gel filtration chromatograms, at 420 nm, of the effluent not treated (a) and treated by *P. chrys.* in presence of different concentrations of Mn(II) : b) 0 µM; c) 60 µM and d) 165 µM.

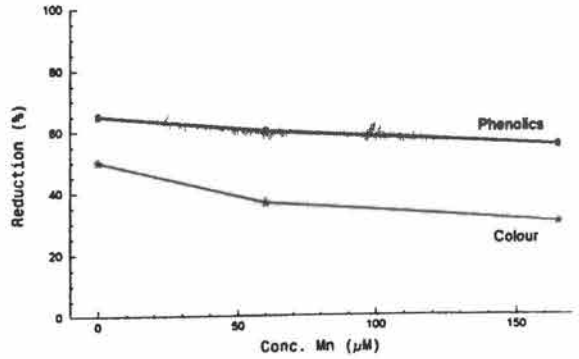

Figure 4 - Reduction of colour and phenolic compounds obtained after fungal treatment in presence of 2 mM veratryl alcohol and various Mn(II) concentration (0, 60 and 165 µM).

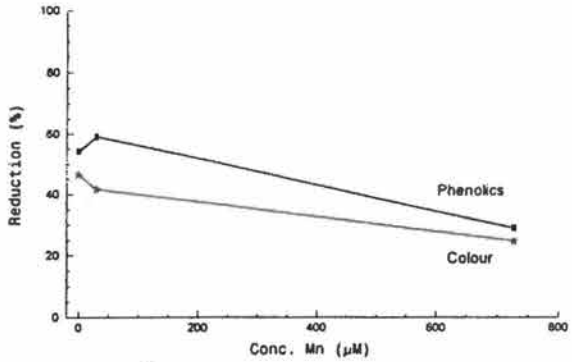

Figure 5 - Reduction of colour and phenolic compounds obtained after fungal treatment in presence of 2 mM veratryl alcohol and various Mn(II) concentration (0, 30 and 727 µM)

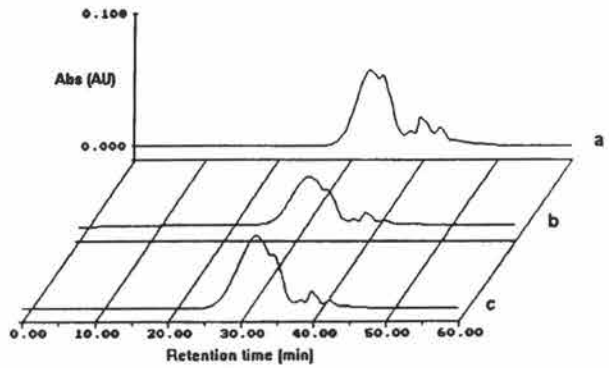

Figure 6 - Gel filtration chromatograms, at 420 nm, of the effluent not treated (a) and treated by *P. chrys.* in presence of 2 mM veratryl alcohol and 0 µM (b) or 727 µM (c) of Mn(II).

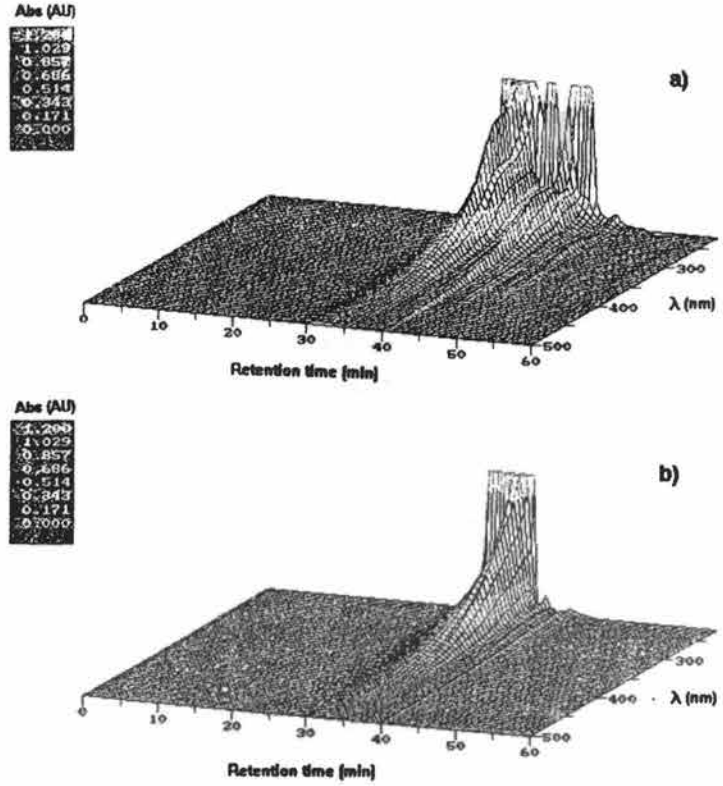

Figure 7 - Diode array gel filtration chromatogram of the effluent before (a) and after (b) treatment with *P chrysosporium*, in presence of 60 µM of Mn(II), without veratryl alcohol.

DISCUSSION

Question: Two questions: one, how long do these fermentations take to get to, say, 60% color removal.

Guimaraes: A long time - about 25 days. We are now trying to get this in 15 days. However, I would like to emphasize that an anaerobic treatment lasts about 30 days.

Question: This is with a strain of *Phanerochaete chrysosporium* that produces the lignin peroxidase (LiP) enzymes only under nutrient stress. There are other strains available that will produce the enzyme before nutrient stress occurs.

The other question is, do you think that this positive effect of magnesium and negative effect of veratryl alcohol will be the same in all strains of *P. chrysosporium*?

Guimaraes: I really don't know. All the published papers say that veratryl alcohol stimulates production of LiP. But in our experiments, the addition of veratryl alcohol gives poorer results. The experimental conditions we use are quite different, because we use an effluent, and literature references report the use of a completely defined synthetic medium.

Question: Thank you for this interesting work. Are you aware, I wonder, of some work carried out in the early 1980's by Prof. Carl-Eric Ericsson at the Swedish Forest Products Research Institute (STFI) and by Kent Kirk of the U.S. Dept. of Agriculture, Madison, Wisconsin. They got to the point where they had a pilot scale system for decolorizing the effluent from paper mills. I see that you know this work. Would you like to comment on how your results compare with theirs?

Guimaraes: Yes, we know this work. The colourants profile of the two effluents is quite different. They did use veratryl alcohol.

Question: A comment: the application of the *P. chrysoporium* to the wood industry is obvious, because the product is lignin. The white rot fungi grow on wood. The application of the system to the remediation of chemical spills and to the sugar industry is, in fact, novel.

Question: What is the percent color in the first portion of regeneration which is not treatable by Bento's process?

Bento: About thirty percent. As it is observed on the special regeneration curve, we have 30% of the total colourants in the salt effluent that are separated at low salt concentration. This part of the effluent is not treated with lime.

Question: Two short questions: one comment, have you looked at any other co-factors as well as manganese - cobalt, for example, and number two, why did you measure the color at pH9?

Guimaraes: To answer the second first, because SPRI's papers recommend a pH higher than 7 as the most stable pH to measure color. On the question of other co-factors, we published a study on the effects of pH, temperature and other factors, (Int. Sugar J., 95: 339-343 (1993)) but we did not look at other co-factors - manganese is well known as a co-factor for these peroxidases.

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