

Treatment of sugar refinery ion exchange resins effluent with *Phanerochaete chrysosporium*

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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 presented to overcome this situation. One process involves anionic colourants precipitation with lime¹. The colourants fraction not removed by this process results from the pre-regeneration stage at low salt concentration (Fig. 1). These colourants possess a low anionic charge and are not efficiently removed with lime. The utilisation of microorganisms to remove these colourants is the purpose of this work.

Phanerochaete chrysosporium is a white-rot wood decaying basidiomycete which secretes a family of lignin-degrading enzymes under nutrient limitation. The major enzymes associated with degradation are lignin peroxidase (LiP), manganese peroxidase (MnP), and hydrogen peroxide-producing enzymes. LiP appears to be the key enzyme leading to polymer fragmentation². MnP oxidizes Mn²⁺ to



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Mn³⁺ in the presence of proper chelating agents which in turn can oxidize phenolic structures in lignin^{3,4}. Glyoxal oxidase, an extracellular copper oxidase which oxidizes glyoxal, methyl glyoxal and other α -hydroxy carbonyl and dicarbonyl compounds produces hydrogen peroxide, a necessary cofactor in lignin oxidation⁵. Besides lignin, ligninases can also degrade a number of

organic pollutants, such as chlorinated aromatics, DDT, lindane, benzo(α)pyrene⁶, etc. Other potential applications include the use of ligninase in the pulp and paper industry⁷.

With this enzymatic capability, this microorganism may prove useful for the elimination of toxic wastes that are otherwise resistant to degradation.

In the sugar refinery huge volumes of intensely coloured waste effluents with a high salt content are released into the environment every year mainly from the regeneration of ion exchange resins. Due to the ability of *P. chrysosporium* to metabolize aromatics and phenolic compounds, polyphenols and melanoidins, which are major contributors to the total colour present in the effluents, may be potential substrates for the fungal growth.

In natural environments, lignin-degrading fungi grow on their lignocellulosic substrates under conditions different from those used in liquid cultures in laboratory studies. Fungal growth in solid-substrate fermentations is different from that in submerged cultures because of the different physical structure, chemical composition of the substrate, moisture content etc.

The ligninolytic system is synthesized without the presence of lignin only in response to nitrogen, carbon, or sulphur⁸ starvation, thereby triggering the secondary metabolism. Its appearance is associated with the accumulation of a secondary metabolite, veratryl alcohol⁹.

Lignin degradation is mainly an oxidative process. The production of the ligninolytic enzymatic system, including the LiPs and MnPs, is greatly enhanced under high oxygen tension^{10,12}.

Previous studies have shown that, in agitated nitrogen limited cultures of *P. chrysosporium*, the ligninase activity was detected in about 4 days and reached a peak in about 5 to 6 days¹¹.

The sugar refinery effluent used in this work still has a rather high salt content (3% w/v. Moreover, the

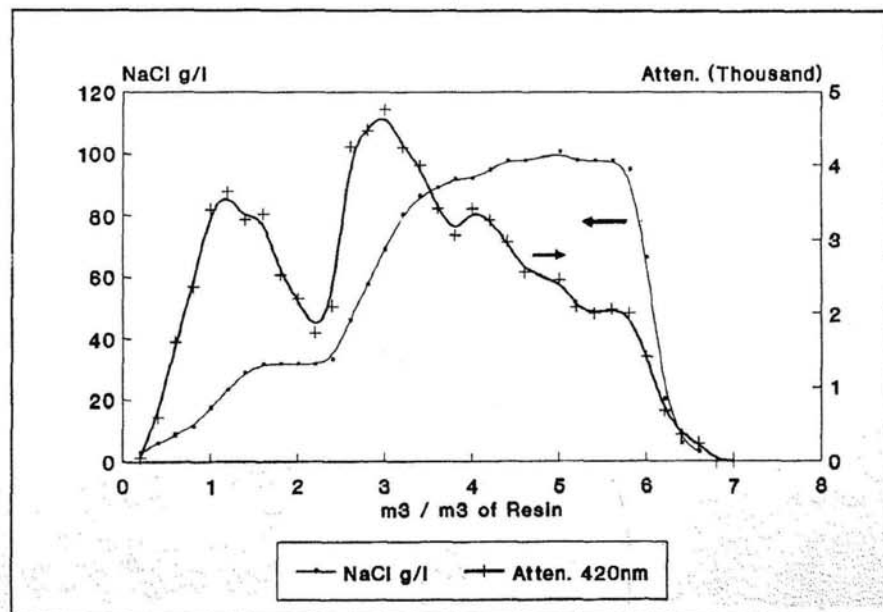


Fig. 1. Ion exchange resin regeneration with two salt concentrations

inhibitory power of polyphenolics impairs the traditional biological waste water treatments. Therefore, we intend to investigate in this work whether the fungus is able to perform colour removal even under such drastic conditions and, if so, what kind of modifications should be made in order to improve the fungus performance.

Materials and methods

Phanerochaete chrysosporium was kindly supplied by J. Lema (University of S. Compostela, Spain) and was maintained on 2% malt extract agar slants, pH 4.5 containing (per litre): 10 g of glucose, 10 g of malt extract, 2 g of yeast extract, 2 g of peptone, 1 g of asparagine, 2 g of KH_2PO_4 , 1 g of $MgSO_4 \cdot 7H_2O$ and 20 g of agar. This medium was also used for spore production. Spores were obtained by suspension in 10 ml of distilled water.

The highest level of enzyme production^{11,12} is achieved in a medium with glucose as the carbon source and with a low-nitrogen content as ammonium tartrate. This medium contained (per litre) the following: 10 g of glucose, 2 g of KH_2PO_4 , 0.5 g of $MgSO_4$, 0.132 g of $CaCl_2 \cdot 2H_2O$, 1.2 mmol of ammonium tartrate, 20 mmol of sodium acetate (pH 4.5), and 1 mg of thiamine hydrochloride. The following trace elements were also added (per litre): 0.14 g of nitrilotriacetate, 0.10 g of NaCl, 0.007 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4$, 0.013 g of $CoCl_2 \cdot 6H_2O$, 0.007 g of $ZnSO_4 \cdot 7H_2O$, 0.011 g of $CuSO_4 \cdot 5H_2O$, 0.0007 g of $AlK(SO_4)_2 \cdot 12H_2O$, 0.0007 g of H_3BO_3 and 0.0007 g of $Na_2MoO_4 \cdot 2H_2O$.

The medium (10 ml) was

dispensed into sterile 125 ml cotton-stoppered Erlenmeyer flasks and inoculated with a 10% (vol/vol) suspension of spores inoculum which was grown in shallow stationary culture as referred and incubated at 39°C.

Some authors have stated that agitated cultures in hyperbaric oxygen are good conditions to obtain high levels of ligninase production. However culture agitation, according to some authors¹², can almost completely suppress lignin degradation while others reported that degradation of lignin by submerged pellets in agitated cultures was achieved by using a mutant strain^{13,14} or by adding detergent¹⁴. Veratryl alcohol could be also used as inducer of secondary metabolism.

The fungus has the ability to produce the enzyme both in carbon and nitrogen limited cultures. In the first experiments we used glucose 1% (w/v) as the only carbon source, in order to fulfil all the requirements of the microorganism and to obtain a rapid growth. This amount was later reduced in order to determine the lowest level of glucose which still allowed the same enzyme production.

Most of the experiments were made in nitrogen starvation conditions, at a concentration of 2.58 mM L-asparagine (100 mg/litre) and NH_4NO_3 (50 mg/litre).

The volume of the effluent used in the early cultures was 10 ml (13.6 after complementation) and 100 ml after medium optimization (in 1000 ml cotton-stoppered Erlenmeyer flasks). Fungus performance was evaluated by measuring the effluent colour after incubation at 420 nm, pH 9 with a

Perkin-Elmer LCC-55B spectrophotometer. Phenolics content in the effluent, before and after incubation, was determined with the Folin-Ciocalteu method.

We used a Pharmacia FPLC system to verify the removal of particular compounds, formed by two LKB P-500 pumps, a LCC 500 Plus controller, a Superose 12 column, a MV-7 valve and a VWM-2141 UV/ VIS detector. Samples before application were eluted through a non-polar Amberlite XAD-2 ion exchange resin at pH 3 to eliminate the NaCl. The compounds retained on the resins were later eluted with a blend of water, methanol and ammonium (56/40/4) concentrated and dissolved in a 0.15 M NaCl solution.

Results and discussion

The high initial concentration of NaCl in the effluent was a problem because high levels of salt inhibit life. We made several assays at concentration of 5, 10, 15, 20, 25, 30, 35 g/litre of NaCl and verified that *P. chrysosporium* grew better at 5, 10 and 15 g/litre, the growth rate being smaller at 20 and 25 g/litre. At 30 and 35 g/litre no growth was observed.

Carbon

When the effluent was incubated with no carbon complementation, the microorganism showed very reduced growth even after 22 days. Glucose supply induced a fast growth with abundant production of mycelium in 3 days. These results have shown that the original carbon sources in the effluent cannot sustain fungal growth, probably

Table I. Influence of culture parameters in the decolorization of the effluent

Sample	Complementation			% of decolorization 4 days	% of decolorization 22 days	Final growth
	Glucose	Nitrogen	Minerals			
2.1	1.0%	No	No	+	++	+++
2.2	0.2%	No	1	+++	+++++	+
2.3	0.2%	No	No	+	+	++
2.4	0.5%	No	0.5	++	++++	+++
2.5	0.2%	No	0.5	+++	++++	+

because the complex structure of the colourants make them inaccessible to the fungus in the early stages of growth when the enzymatic system is not fully developed. In a later phase when ligninolytic activity appeared, the colourants were used by the fungus with a consequent decrease in the effluents colour. The initial amount of glucose (1% w/v) could be reduced to lower levels (0.2% w/v) with no change in the decolourization obtained (Table I).

Nitrogen

In the first experiments with a total supplementation in carbon and nitrogen the fungus showed a rapid growth and a great production of mycelium, but no decolourization of the effluent. With nitrogen-limited cultures the rate of growth was unchanged but the final amount of mycelium decreased, and the decolourization increased corresponding always to a reduction of more than 48 % of the initial colour (Fig. 2). Non-nitrogen supplementation did not affect these results. This fact can

be probably due to the presence in the effluent of some colourants, such as melanoidins, having amino groups which can act as nitrogen sources. Therefore the colour fraction of the melanoidins responsible can be eliminated.

Some authors reported⁸ that when using lignin as substrate, the ligninolytic activity appeared earlier in carbon limited cultures than in nitrogen starved conditions, but the fungus are better adapted to nitrogen-deficient environments. That is the reason why we have chosen in our experiments to limit the nitrogen availability.

Mineral elements

Trace elements are fundamental in regulating ligninolytic activity and ligninase production⁸. In our assays we found that reducing by more than 50% the total initial concentration, the efficiency of the fungus was remarkably affected. However some of them, like Mg^{2+} , Na^+ , Zn^{2+} and Cu^{2+} could be reduced or even eliminated with no great

losses in *P. chrysosporium* activity with our effluent. This can be explained by the presence in the effluent of some inorganic salts coming from the sugar factory and sugar refinery. Mn^{2+} however proved to be essential² and its reduction affected both growth and colour removal.

Veratryl alcohol, a secondary metabolite in lignin degradation and a normal inducer of the ligninolytic system¹⁵, was used in some experiments at a final concentration of 1 - 2 mM. Its addition to our medium did not improve the previous experiments.

The fungus mycelium developed a dark brown colour after a few days of incubation. This could be the result of high levels of trace elements in the effluent² despite the reduction we have made in the initial medium composition or could result from the assimilation of the coloured compounds. Another possibility could be the physical adsorption of the colourants on the mycelial surface. To assess this hypothesis we tried to extract the colourants with several organic solvents (methanol, acetone and isopropyl alcohol) and with a 10% (w/v) sodium chloride solution. No colour was transferred to the solvent phase.

With the FPLC we intended to separate and identify groups of colourants existing in the effluent which disappeared after metabolic activity. Moreover we found important to verify if the colourants degradation was complete or if they were transformed into non-coloured compounds. The results (Fig. 3) showed that an effective removal of some compounds occurred while others were not affected.

As it may be seen in Table II, high levels of decolourization (76%), and 81% removal of phenolic compounds could be achieved with few corrections in the effluent composition.

Conclusion

The white rot fungus *Phanerochaete chrysosporium* presents large potential utilisation in the food and

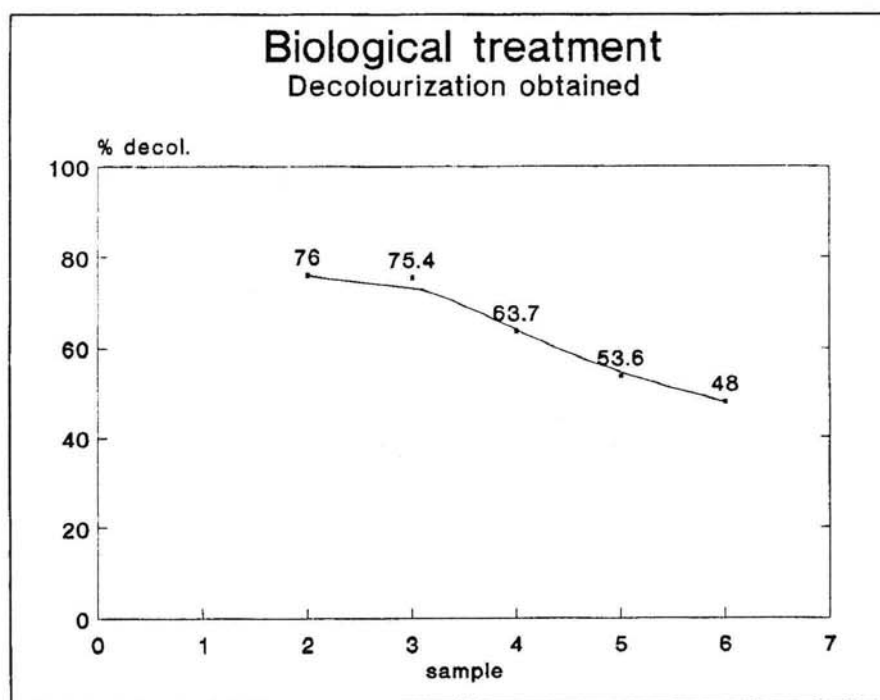


Fig. 2. Decolorization of effluent samples after fungal growth

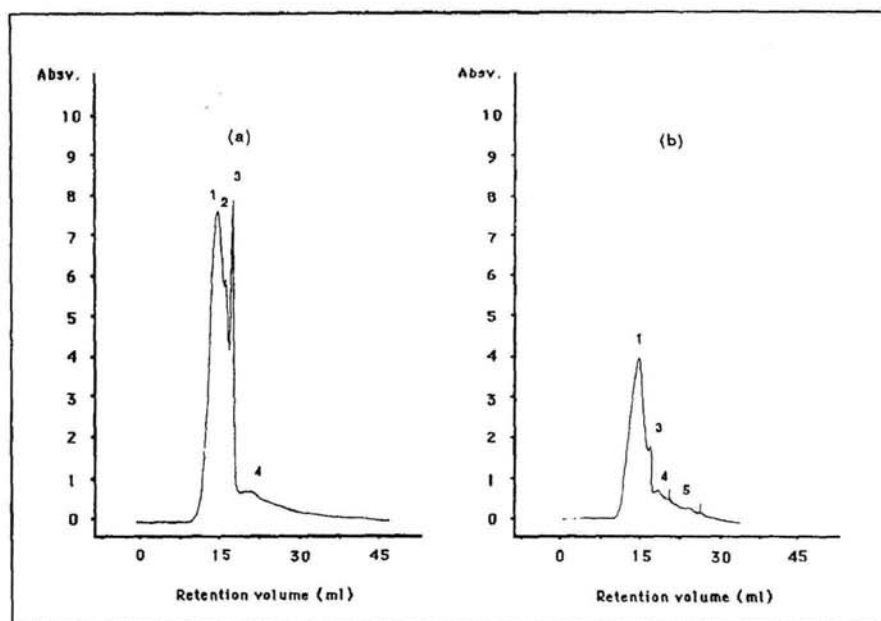


Fig. 3. Comparison between the FPLC chromatograms of the effluent before (a) and after (b) biological treatment

chemical industry. Due to its complex and non-specific enzymatic system it produces, a great number of organic compounds namely aromatics and phenolics can be degraded and eliminated.

The level of decolourization achieved seems relevant since before the treatment the effluent had a very dark brown colour.

P. chrysosporium also proved to be efficient in metabolising phenolic compounds with a final reduction of 81%.

Growth conditions were optimized showing that the addition of low amounts of glucose and some trace elements are necessary and enough to support fungal growth. Mn^{2+} appeared to be fundamental. The reduction of the initial values of complementation seems

important because the process becomes less expensive and suitable for an industrial application.

NaCl at high concentrations decreases fungus activity and completely inhibits it above 30 g/litre.

We may therefore conclude that, in a sugar refinery and according to our results at a laboratory scale, *Phanerochaete chrysosporium* is a microorganism that can be effectively used in the treatment of effluents resulting from the regeneration of ion exchange resins.

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Table II. Percentage of decolorization and phenolics removal after fungal growth

Sample	NaCl (g/litre)	% decolorization	% of Phenolics removal
2	2.2	76	81
3	4.1	75	81
4	10.7	64	81
5	18.0	54	-
6	29.0	48	71

Summary

Ion exchange resins produce a salt effluent with a high colour content and a high salt concentration. Some of the colourants can be removed by precipitation with lime. Colourants which are not removed can be degraded by microorganisms.

The white rot fungus *Phanerochaete chrysosporium* has been widely studied, due to its capacity of metabolising xenobiotics coming from several chemical and food industries. This organism has an enzymatic system related with lignin degradation, which was also shown to be able to degrade such compounds as DDT, lindane, pentachlorophenol, melanoidins, etc.

In the sugar industry, melanoidins and polyphenols are among the most important coloured compounds in the process, and appear in great quantities in the effluents from the regeneration of ion-exchange resins. The study was made at laboratory scale to investigate the possibility of using *Phanerochaete chrysosporium* in the removal of these compounds.

Growth studies performed in the effluent were made in order to optimize the colour removal (and the culture medium), varying several conditions - micronutrients, pH, salt content, carbon and nitrogen source, etc. In ideal conditions, 76% colour reduction and 81% polyphenolic compounds removal was obtained.

Tratamiento del efluente de las resinas de intercambio iónico en una refinería de azúcar

Las resinas de intercambio iónico producen un efluente salino con alto contenido de color y alta concentración salina. Algunos de los colorantes pueden removerse por precipitación con cal. Los colorantes que no son removidos pueden ser degradados por microorganismos.

El hongo blanco de la descomposición *Phanerochaete chrysosporium* ha sido estudiado ampliamente, debido a su capacidad de metabolización xenobiótica proveniente

de varias industrias químicas y de alimento.

Este organismo tiene un sistema enzimático relacionado con la degradación de la lignina y a su vez es capaz de degradar compuestos tales como el DDT, pentaclorofenol, melanoidinas, etc.

En la industria azucarera, las melanoidinas y los polifenoles son entre otros los compuestos más importantes en la generación de color en el proceso y aparecen en grandes cantidades en los efluentes de la regeneración de las resinas de intercambio iónico. El estudio se realizó a escala de laboratorio para investigar la posibilidad de utilizar el *Phanerochaete chrysosporium* en la remoción de estos compuestos.

Amplios estudios se hicieron en el efluente para optimizar la remoción del color variando algunas condiciones, los micronutrientes, pH, contenido de sal, fuente de carbón y nitrógeno, etc. En condiciones ideales, se obtuvo un 76% de reducción de color y un 81% de remoción de compuestos polifenólicos.

Le traitement de l'effluent à l'échange des ions de la raffinerie sucrière avec *Phanerochaete chrysosporium*

Les résines d'échange d'ion produisent un effluent de sel avec un contenu de couleur élevé et une concentration de sel élevée. Il y a des colorants qui puissent être enlevés par la précipitation des chaux. Les colorants qui ne sont pas élevés puissent être dégradés par des microorganismes.

Le champignon de pourriture blanche *Phanerochaete chrysosporium* a été largement étudié, dû à sa capacité de métaboliser xenobiotiques venant de quelques industries chimiques et alimentaires. Cet organisme a un système enzymatique lié à la dégradation ligninique qui peut aussi dégrader des composés tels que DDT, le lindane, le pentachlorophénole, les melanoidins, ainsi de suite.

Dans l'industrie sucrière, des melanoidins et des polyphenoles sont parmi des composés colorés les plus importants dans le processus et ils apparaissent en grande quantité dans les effluents de la régénération des résines d'échange d'ion. Cette étude était effectuée à l'échelle laboratoire afin d'enquêter la possibilité d'utiliser *Phanerochaete chrysosporium* dans l'enlèvement de ces composés. Les études de la croissance effectuées dans l'effluent avait pour but d'optimiser l'enlèvement de couleur (et le medium de culture) en variant plusieurs conditions - des micronutrients, le pH, le contenu de sel, le carbon et le source de nitrogène, ainsi de suite. Dans les conditions idéales, on a réalisé une réduction de couleur d'environ 76% et l'enlèvement de 81% des composés polyphénoliques.

Aufbereitung von Ionenaustauschabwasser in einer Zuckerraffinerie mittels *Phanerochaete chrysosporium*

Ionenaustauschharze rufen ein Salzabwasser von hohem Farbgehalt und hoher Salzkonzentration hervor. Einige der Farbstoffe sind mit Kalk niederschlagbar. Farbstoffe, die nicht entfernt werden, lassen sich durch Mikroorganismen abbauen. Der Weissfäulepilz *Phanerochaete chrysosporium* ist beträchtlich studiert worden, weil er Xenobiotika metabolisieren kann, die von mehreren chemischen und Lebensmittelindustrien ausgehen. Zu diesem Organismus gehört ein in Zusammenhang mit Lignin-Abbau stehendes, enzymatisches System, das sich zu Abbau von solchen Zusammensetzungen wie z.B. DDT, Lindane, Pentachlorophenol, Melanoidinen, usw. fähig erwies. In der Zuckerindustrie gehören Melanoidine und Polyphenole zu den wichtigsten Farbstoffen im Verfahren, und sie kommen in grossen Mengen in den Abwässern von Ionenaustauschharz-Regenerierung vor. Die Studie wurde im

Labor durchgeführt, um die Möglichkeit der Verwendung von *P. chrysosporium* zur Entfernung dieser Zusammensetzungen zu untersuchen. Studien von Wachstum im Abwasser wurden durchgeführt, um die Farbeentfernung (auch den Nährboden) zu optimieren unter verschiedenen Bedingungen mit Bezug auf Mikronährstoffe, pH-Wert, Salzgehalt, Kohlenstoff- und Azotquelle, usw. Unter besten Verhältnissen wurde die Farbe um 76% vermindert und die Polyphenole um 81% entfernt.

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