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Effect of veratryl alcohol and manganese (IV) oxide on ligninolytic activity in semi solid cultures of *Phanerochaete chrysosporium*

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Abstract

An inert carrier (nylon sponge), a non-inert carrier (barley straw) and the addition of veratryl alcohol or manganese (IV) oxide to the cultures were used to study the production of ligninolytic enzymes by *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) during semi solid state fermentation conditions. By supplementing the medium with these compounds we could stimulate the ligninolytic system of this fungus.

The different carriers employed and the effect of adding veratryl alcohol or manganese (IV) oxide to the cultures were compared in order to determine the best system to produce high activities of ligninolytic enzymes. Lignin peroxidase (LiP) activities higher than 500 U/L and manganese-dependent peroxidase (MnP) activities about 1100 U/L were achieved.

In recent years the possibility of using white rot fungi for bioremediation has initiated considerable research effort in academic, industrial and government institutions. The interest in this subject arises from the ability of white rot fungi to degrade an extremely diverse range of very persistent or toxic environmental pollutants. This ability sets the use of white rot fungi apart from many of the existing methods of bioremediation (Barr & Aust 1994). White rot fungi are organisms that are able to degrade lignin, the structural polymer in woody plants. Lignin is a very complex three-dimensional polymer consisting of non repeating phenyl propanoid units linked by various carbon-carbon and ether bonds (Sarkanen & Ludwig 1971). The stereo irregularity of lignin makes it very resistant to attack by enzymes. Thus, the white rot fungi have developed very nonspecific mechanisms for degrading lignin (Barr & Aust 1994).

White rot fungi comprise a heterogeneous collection of hundreds of species of Basidiomycetes. The white rot fungus *Phanerochaete chrysosporium* has become the most commonly used model organism in lignin biodegradation studies. The reasons for the popularity of this organism for lignin degradation studies include rapid growth and metabolism of lignin; ability to grow optimally at relatively high temperatures of 40 °C; ability to produce conidia (asexual spores) and basidiospores (sexual spores); ability to grow on chemically defined media; existence of an already considerable knowledge base on its ecology, physiology, biochemistry, molecular biology, and genetics; and low phenol oxidase activity (Buswell 1987; Kirk & Farrell 1987).

Despite the fact that lignin is rich in carbon, it is not a growth substrate for microorganisms which are reported to degrade lignin. Lignin-degrading fungi metabolize various lignin preparations only in the presence of an alternate energy/carbon source (Buswell & Odier 1987; Buswell 1987; Kirk & Farrell 1987). To degrade lignin, the fungus demands easily metabolizable nutrients such as sugars from the polysaccharides of wood. These sugars are also necessary for the production of hydrogen peroxide, which is an absolute prerequisite for the organism to degrade lignin (Eriksson et al. 1986; Reddy & Kelley 1986). Lignin is degraded only during secondary metabolism. Secondary metabolism in *P. chrysosporium* is triggered

by nitrogen, carbon or sulfur limitation but not by phosphorus limitation (Jeffries et al. 1981).

When *P. chrysosporium* is cultivated under ligninolytic conditions it produces two types of extracellular glycosylated heme proteins: lignin peroxidases and manganese-dependent peroxidases (Tien & Kirk 1983; Glenn et al. 1983; Gold et al. 1984; Tien & Kirk 1984; Renganathan et al. 1985; Jäger et al. 1985; Leisola et al. 1987; Tien & Kirk 1988; Troller et al. 1988; Farrell et al. 1989; Gold et al. 1989; Dass & Reddy 1990; Odier & Delattre 1990). Several efforts have been made to provide sufficient amounts of lignin peroxidases for basic research as well as supply for applications in the technical field. Different modes of cultivation have been used to improve the production of ligninases by *P. chrysosporium* grown under N- or C- limitation in media of varied composition (Janshekar & Fiechter 1988).

Jäger et al. (1985) showed that addition of non-ionic surfactants such as sorbitol polyoxyethylene monooleate (Tween 80), sorbitol polyoxyethylene monolaurate (Tween 20) or 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate to the culture medium permitted LiP production with agitation. Kirk et al. (1986) showed that addition of veratryl alcohol, a secondary metabolite of *P. chrysosporium*, and excess trace elements improved LiP activity. According to Kern (1989) addition of solid manganese (IV) oxide to cultures of *P. chrysosporium* at the beginning of ligninolytic activity improved production, enzymatic activity and stability of LiP isozymes produced.

In this work the production of ligninolytic enzymes by *P. chrysosporium* in stationary cultures operating with barley straw or nylon sponge as carriers was studied. Supplementing the culture medium with veratryl alcohol or adding MnO_2 to the cultures was intented to improve both production and stability of the enzymes produced.

Methods

Microorganism and growth medium

P. chrysosporium BKM-F-1767 (ATCC 24725) was maintained at 37 °C on 2% malt agar slants and plates. Spores were harvested, filtered through glass-wool, and kept at −20 °C until used (Jäger et al. 1985).

The growth medium was prepared according to Tien & Kirk (1988) with 10 g glucose per litre as carbon source, except dimethylsuccinate was replaced by 20 mM acetate buffer (pH 4.5) (Dosoretz et al. 1990) and 2.6 mM ammonium acetate as nitrogen source.

Carriers

Chopped barley straw (0.72 g/bottle, particle length between 3–7 mm) and 0.5 cm cubes of fibrous nylon sponge (Scotch Brite ^{TB}, 3M Company, Spain) (0.95g/bottle) were used as carriers.

Before immobilization, the nylon sponge was pretreated according to Linko (1992) by boiling for 10 min and washing thoroughly three times with distilled water. The carriers were dried at 60 °C (Ferrer & Solá 1992).

Culture conditions

The production medium composition was the same as the growth medium except in the case of straw in which the content of glucose was only 2 g per litre.

Erlenmeyer flasks (250 mL) containing 12 mL of production medium and 0.5% (vol/vol) Tween 80 were inoculated with 10% (vol/vol) homogenized mycelium. Barley straw or cubes of nylon sponge were employed as carriers.

Some bottles were supplemented with veratryl alcohol at the time of inoculation to a final concentration of 2 mM (Tonon & Odier 1988). After the first day of incubation solid manganese (IV) oxide was added (1g/L medium) to other bottles (Kern 1989; Kern 1990).

The culture bottles were loosely capped for passive aeration and incubated statically under an air atmosphere at 37 °C in complete darkness.

Analytical methods

Lignin peroxidase activity for nylon sponge cultures
This was determined spectrophotometrically at 310 nm according to Tien & Kirk (1984) because the samples from the nylon cultures were clear. One unit (U) was defined as 1 μmol of veratryl alcohol oxidized in 1 min, and the activities were reported as U L^{-1}.

Lignin peroxidase activity for barley straw cultures
Since the liquid obtained had a dark brown colour due to the products of straw degradation, the standard veratryl alcohol method previously adopted for assaying LiP activity in colourless fungal cultures was not suitable (Vares et al. 1995). As an alternative method, the method described by Archibald (1992) was employed except that the dye Azure B was replaced by

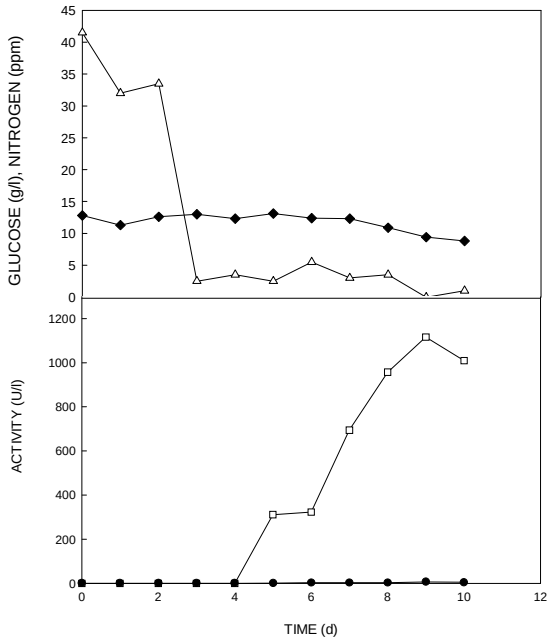

Figure 1. Ligninolytic activity by supplementing the medium with veratry alcohol in nylon sponge cultures. Symbols: ● LiP and □ MnP activities (U/L) ♦ Glucose (g/L) and Δ Nitrogen (ppm) concentrations during the fermentation.

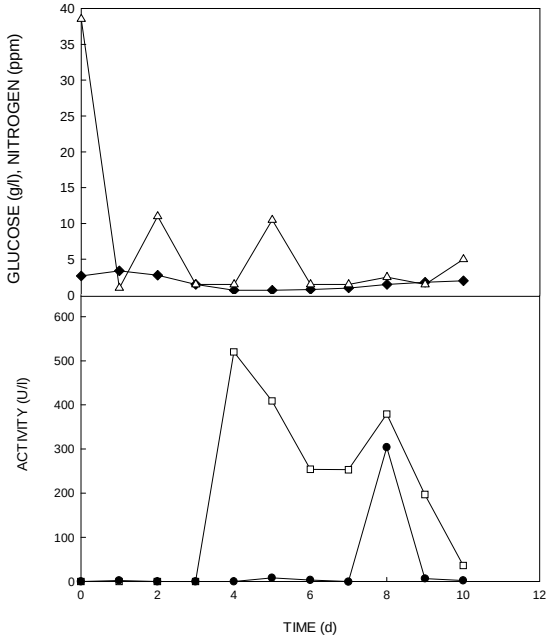

Figure 2. Ligninolytic activity by supplementing the medium with veratryl alcohol in barley straw cultures. Symbols: ● LiP and □ MnP activities (U/L) ♦ Glucose (g/L) and Δ Nitrogen (ppm) concentrations during the fermentation.

the dye Remazol Blue R. This assay, as developed in this work, contained (final concentrations) 32 μm Remazol Blue R and 100 μm H_2O_2 in 50 mM Na tartrate buffer (pH 4.5, 25 °C). Measurement occurred at 620 nm, where the visible brown colour present in the samples interferes very little (Archibald 1992). One unit (U) was defined as 1 μmol of Remazol R oxidized in 1 min, and the activities were reported as U L^{-1}.

Mn (II)-dependent peroxidase activity

This was assayed spectrophotometrically by the method of Kuwahara et al. (1984). The reaction mixture contents 50 mM sodium malonate (pH = 4.5), 1 mM 2,6 dimethoxyphenol, 1 mM $MnSO_4$ and 600 μL of diluted culture fluid (200 μL of enzyme sample plus water) in a final volume of 1 mL. The reaction was started by adding 0.4 mM H_2O_2. One unit was defined as 1 μmol of dimethoxyphenol oxidized per minute and the activities were expressed in U L^{-1}.

Reducing sugars

They were measured spectrophotometrically at 640 nm by the dinitrosalicylic acid method using D-glucose as a standard, according to Ghose (1987).

Nitrogen ammonium content

This was determined spectrophotometrically at 635 nm by the phenol-hypochlorite method described by Weatherburn (1967).

Microscopic examination

For observation by scanning electron microscopy nylon sponge and barley straw particles were fixed with 5% glutaraldehyde. After dehydration in ethanol the samples were dried, coated with gold and observed with a Philips XL30 microscope belonging to CACTI (Centro de Apoyo Científico y Tecnológico a la Investigación) of the University of Vigo.

Results and discussion

Effect of veratryl alcohol on ligninolytic activity

It has been shown that veratryl alcohol, added as a supplement to cultures of *P. chrysosporium*, stimulates ligninolytic activity (Faison & Kirk 1985; Kirk et al. 1986; Leisola et al. 1985). It has been proposed that veratryl alcohol protects ligninases against inactivation by hydrogen peroxide produced by this fungus in cultures (Tonon & Odier 1988) or against

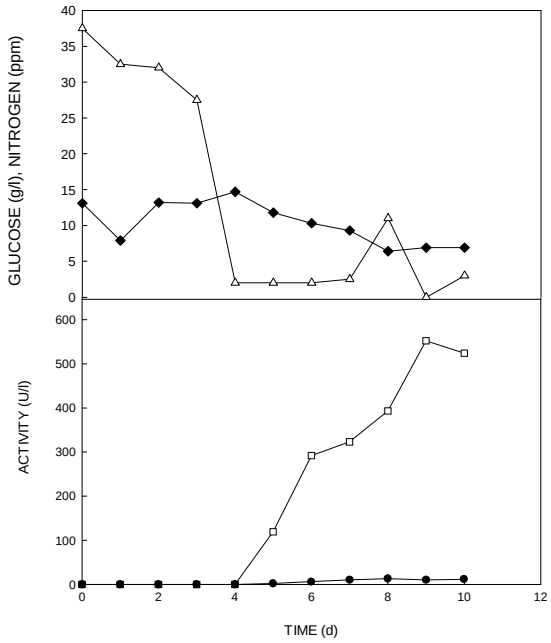

Figure 3. Ligninolytic activity by supplementing the medium with solid MnO_2 in nylon sponge cultures. Symbols: ● LiP and □ MnP activities (U/L) ♦ Glucose (g/L) and Δ Nitrogen (ppm) concentrations during the fermentation.

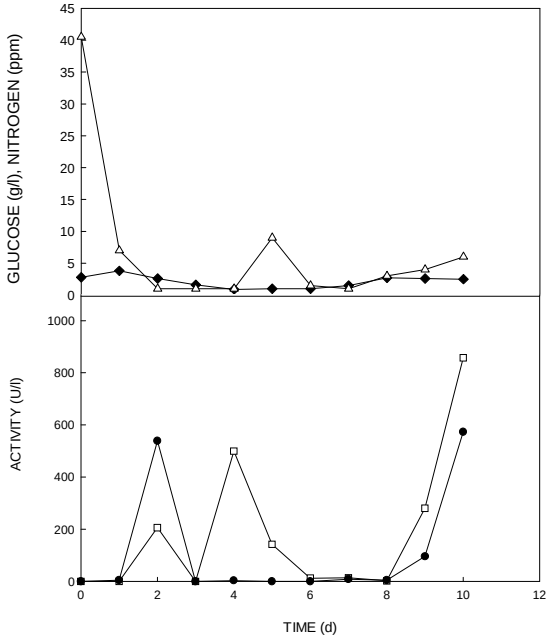

Figure 4. Ligninolytic activity by supplementing the medium with solid MnO_2 in barley straw cultures. Symbols: ● LiP and □ MnP activities (U/L) ♦ Glucose (g/L) and Δ Nitrogen (ppm) concentrations during the fermentation.

proteolytic decay (Faison et al. 1986). However, the mechanism of stimulation by veratryl alcohol has not been determined, yet.

When veratryl alcohol was added to cultures with nylon sponge as a carrier, nitrogen was consumed at 3^{rd} day. The amount of glucose consumed, measured as reducing sugars, was very low and MnP activity appeared at day 5. The highest MnP activity (1116 U/L) was reached at day 9 (Figure 1). This activity is higher than obtained by Feijoo (1994). He reported a MnP activity of 175 U/L in a free liquid system (pellets) with shaking (150 rpm), assayed according to Kuwahara et al. (1984). However, we found low lignin peroxidase activities.

In veratryl alcohol supplemented cultures with barley straw as a carrier, nitrogen was consumed faster (at day 1). The amount of glucose measured as reducing sugars decreased to 0.7 g/L (4^{th} and 5^{th} day) and then increased to a maximum value of 2 g/L (10^{th} day). This is probably due to some reducing products of straw degradation. MnP activity appeared at day 4 (520 U/L) and then went down. One peak of high lignin peroxidase activity (304 U/L) was noticed (Figure 2).

Effect of manganese (IV) oxide on ligninolytic activity

Addition of solid manganese (IV) to cultures of *P. chrysosporium* at the beginning of ligninolytic activity has been known to improve the activity and stability of the ligninases produced. The addition of MnO_2, probably mimicking the naturally occurring deposition of MnO_2 on the mycelia of some white rot fungi, was intended to protect ligninases against inactivation and damage by hydrogen peroxide via catalytic decomposition of H_2O_2 by MnO_2 (Kern 1989). MnO_2 precipitates accumulate in wood after decay by several white rot fungi (Blanchette 1984). Furthermore, lignin degradation by several white rot fungi is strongly dependent on the presence of manganese (Pèrié & Gold 1991).

When MnO_2 was added to cultures with nylon sponge as a carrier, the amount of glucose consumed, measured as reducing sugars, was very low. Nitrogen was consumed at 4^{th} day and lignin peroxidase activity was low. MnP activity appeared at day 5 and the highest activity (552 U/L) was reached at day 9 (Figure 3). This activity as mentioned previously, is higher than that obtained by Feijoo (1994).

Also in MnO_2 supplemented cultures with barley straw as a carrier glucose consumption was slow. The

Table 1. Maximum manganese-dependent peroxidase and lignin peroxidase activities obtained

	MnP maximum (U/L)	LiP maximum (U/L)	
		Veratryl method	Dye method
Nylon (control)	673	0	
Nylon + MnO_2	552	13	
Nylon + veratryl alcohol	1116	6	
Straw (control)	302		300
Straw + MnO_2	857		573
Straw + veratryl alcohol	520		304

amount of glucose measured as reducing sugars decreased to 0.9 g/L (4^{th} day) and then started to increase reaching the initial concentration. This can be due to some reducing products of straw degradation. Nitrogen was consumed faster (day 1) than it was with nylon sponge. MnP activity appeared at day 2 and the highest value (857 U/L) at day 10. In this case, two peaks with high lignin peroxidase activities were found: one at day 2 (539 U/L) and the other one at day 10 (573 U/L) (Figure 4).

The manganese-dependent peroxidase and lignin peroxidase production could be enhanced by adding solid MnO_2 to the cultures (1g/ L medium) after 1 day of incubation, employing barley straw as a carrier.

In spite of the low consumptionof glucose by the fungus high ligninolytic activities were obtained. This means that the ligninolytic system of the fungus is triggered by nitrogen starvation, which is not surprising given the low levels of nitrogen found in wood.

By supplementing the medium with veratryl alcohol high MnP activities were achieved in both cultures. Nevertheless, by adding MnO_2 both MnP and LiP activities could only be increased in barley straw cultures (Table 1). So, it seems that the addition of MnO_2 has only a possitive effect on lignocellulosic carriers (Table 2). The reason for this has not been determined yet, and further experiments are necessary.

Table 2. Effect of veratryl alcohol and MnO_2 on nylon and barley straw grown cultures: ▼ effect on lignin peroxidase; ♦ effect on manganese peroxidase

Type of carrier employed	Veratryl alcohol cultures	MnO_2 cultures
nylon	♦	
barley straw	♦	♦ ▼

Effect of the carrier

Natural as well as synthetic substances can be used as substrates in solid state fermentation processes. The major organic materials available in nature are polymeric in structure, e. g. polysaccharides, proteins and lignins. In general, all these can be used by microorganisms as substrate (carbon source). Solid substrates used in solid state fermentation are insoluble in water. Fungal mycelia penetrate into the particles of the substrate (Pandey 1992).

The utilization of solid substrates by the microorganisms is affected by several physical and chemical factors. Among the physical factors, accessibility of substrate to microbes, film effects and mass effects are important (Knapp & Howell 1985). The physical morphology, especially porosity and particle size of the substrate, governs the accesible surface area to the organism. Among the chemical factors, the chemical nature of the substrate is an important criterion (Pandey 1992).

Knapp & Howell (1985) reviewed the literature on the effects of alteration of substrate particle size on solid state fermentation. It was reported that substrates with finer particles showed improved degradation due to an increase in surface area (Molony et al. 1984; Pandey et al. 1988).

The difference between nylon and straw is that nylon is only utilized by the fungus as an attachment place, whereas straw is utilized both as an attachment place and as a source of nutrients.

In this work, it was noted that the fungus grew better and faster in nylon sponge cultures than in barley straw cultures. In Figure 5, one can observe a higher biomass in nylon sponge than in barley straw in spite

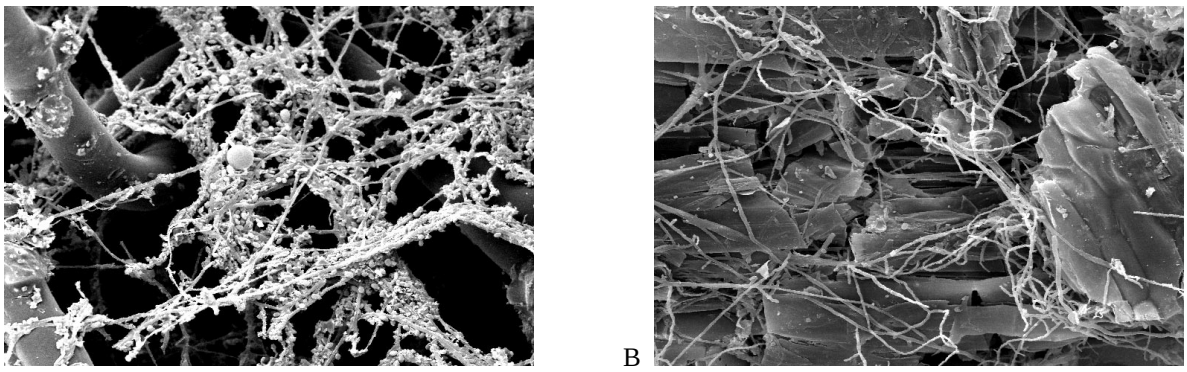

Figure 5. Scanning electron microphotographs of *Phanerochaete chrysosporium* in the maximum production day grown on (A) nylon sponge cultures (two days old), (B) barley straw cultures (three days old).

of the straw culture is one day older. This is due to the higher roughness and porosity of the nylon carrier, which offers a higher surface area to the microorganism and this makes easier the attachment of the fungus to the carrier. Asther et al. (1990) also reported that carrier surface roughness may contribute to the adhesion. Hence, it appears that nylon sponge is a better carrier for microorganism growth than barley straw.

In the cultures supplemented with veratryl alcohol to a final concentration of 2mM higher MnP activities were obtained with the nylon carrier. However, when MnO_2 was added after 1 day of incubation higher activities were achieved with barley straw. High lignin peroxidase activity was found in the cultures with straw (Table 1). This is probably due to the lignin content in the straw, which can stimulate ligninase production. This is agreement with Ulmer et al (1984), who reported an apparent induction of the total ligninolytic system after incubation of the cultures with high concentrations (2 g/L) of a dioxane-HCl lignin from wheat straw. Faison & Kirk (1985) also showed that the activity of the ligninase was markedly increased by a 12-h preincubation of idiophasic cultures with either synthetic or natural lignins (38 mg/L).

Furthermore, LiP has not previously been detected in fungi when grown on lignocellulosic substrate during solid state fermentation, probably due to the interference of coloured compounds derived from ligninocellulosic carriers (Vares 1996). Nevertheless, Vares (1996) detected LiP in purified culture extract from *Phlebia radiata*, a fungus that belongs to the same family as *P. chrysosporium*.

Conclusions

Upon supplementing the production medium with veratryl alcohol to a final concentration of 2 mM, high MnP activities were achieved. The addition of manganese (IV) oxide to the cultures after the first day of incubation also had a very positive effect on the production of ligninolytic enzymes, when barley straw was used as a carrier.

On the other hand, when barley straw was used as a carrier nitrogen was consumed faster. Maybe because of this, MnP appeared earlier than with the nylon sponge. In the medium supplemented by MnO_2, MnP activity was higher with barley straw whereas in the medium with veratryl alcohol higher activities were reached with nylon sponge. This means that the addition of veratryl alcohol and manganese (IV) oxide has different effects depending on the nature of the carrier employed (Table 2). So, the best way to improve enzymatic activity seems to be by adding veratryl alcohol to the medium when one uses an inert carrier and by adding MnO_2 if one employs a lignocellulosic carrier. According to our results, although highest MnP activities were reached by employing nylon sponge as a carrier in a 2 mM veratryl alcohol medium, the best system to produce both MnP and LiP enzymes is by using barley straw as a carrier in a medium containing 1 g/L of MnO_2.

The fact that lignin peroxidase was only found in barley straw cultures is probably due to the content in lignin of the straw, which can act as an activator of this enzyme. So, barley straw seems to be a better carrier for lignin peroxidase production than nylon sponge.

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