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Utilisation of lignocellulosic wastes for lignin peroxidase production by semi-solid-state cultures of *Phanerochaete chrysosporium*

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Abstract

In the present work, the production of ligninolytic enzymes by semi-solid-state cultures of *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725), employing different lignocellulosic wastes as support, was investigated. The waste materials employed were grape seeds, wheat straw and wood shavings. Maximum lignin peroxidase activities of 1620 ± 123 U/l, 364 ± 35 U/l and 571 ± 42 U/l were attained, respectively. Nevertheless, low manganese-dependent peroxidase activities were found, being insignificant in the grape seed cultures. Moreover, the *in vivo* decolourisation of a model dye compound, the polymeric dye Poly R-478 (polyvinylamine sulfonate anthrapyridone), by the above-mentioned cultures was monitored to assess the degrading capability of the extracellular liquid secreted by such cultures. The percentage of biological decolourisation attained by grape seed and wood shaving cultures was around 74% and 63%, respectively, whereas it was rather low (40%) in the wheat straw ones.

Abbreviations: A_{ini}, Initial absorbance; A_{obs}, Observed absorbance; D, Decolourisation (in %); *LiP*, Lignin peroxidase; *MnP*, Manganese-dependent peroxidase; *SSF*, Solid state fermentation.

Introduction

Research on lignin biodegradation has proliferated greatly in the last two decades in response to potential applications in several areas (Odier & Artaud 1992). Nonetheless, most studies on this topic have been performed in liquid cultures, despite lignin degradation in nature occurs in solid-state conditions.

Solid-state fermentation is generally defined as the growth of microorganisms on solid materials in the absence or near-absence of free water (Pandey 1992). As our culture system presents some free liquid, it is called semi-solid-state fermentation. This type of cultivation presents several advantages over liquid-submerged fermentation. Among them, reduction of the production costs, simpler handling and improved product recovery are outstanding (Cannel & Moo-Young 1980; Lonsane et al. 1985).

The organism in SSF not only grows at or near the surface of the substrate but also penetrates deeply into the intercellular and intracellular spaces of the substrate, showing a really close contact or association with the substrate.

The materials that can be employed in SSF may be either inert (synthetic materials) or non-inert (organic materials). The former act only as attachment places whereas the latter also function as a source of nutrients, on account of which they are called support-substrate.

The utilisation of support-substrate has several advantages such as the reduction in production costs, since they supply to the fungus some nutritive substances. Furthermore, this type of support provides to the fungus a similar environment to its natural habitat (wood) and it offers the possibility of reusing solid wastes. Lignocellulosic wastes are a good example

of this kind of materials. They are composed mainly of polysaccharides (cellulose and hemicellulose) and lignin.

Some studies demonstrated a good correlation between biodegradation of aromatic pollutants and decolourisation of polymeric dyes by ligninolytic fungi (Field et al. 1992, 1993). Therefore, the decolourisation of such dyes is a simple method to assess the degrading capability of the extracellular enzymes secreted by these fungi.

The aim of this paper is to evaluate the potential of several lignocellulosic materials (grape seeds, wheat straw and wood shavings) as support-substrate for ligninolytic enzyme production in semi-solid-state conditions by the white-rot fungus *P. chrysosporium.* Moreover, the ability of the ligninolytic complex secreted in such conditions to decolourise the polymeric dye Poly R-478 was also investigated.

Materials and 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 before use (Jäger et al. 1985).

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

The inoculum was produced in submerged cultures in a 1.8-litre Fernbach flask, containing 90 ml of growth medium and 2 ml of spore solution (4.0 ± 108 spores/ml). The cultures were incubated statically under an air atmosphere at 37 °C for 48 hours, in complete darkness. After that, the culture broth was homogenised with a blender for 1 minute. This homogenate was employed to inoculate the cultures.

Carriers

Grape seeds (*Vitis vinifera* cv. Albariño; 1.5 g/Erlenmeyer) were utilised as support-substrate. Grape seeds showed a chemical composition of 43% lignin, 41% carbohydrates and 10.5% oil (Rodríguez 1999).

Chopped wheat straw (0.72 g/Erlenmeyer; particle length of about 3-7 mm) was employed as support-substrate. The chemical composition of the wheat straw was 12.4% lignin, 42.3% cellulose, 30% hemicellulose and 9.3% protein.

Wood shavings (0.72 g/Erlenmeyer; particle length around 3–7 mm) were used as support-substrate. The chemical composition of the wood shavings was 30% lignin, 45% cellulose and 20% hemicellulose.

Prior to use, all the supports were autoclaved at 121 °C for 20 minutes.

Culture conditions

The cultivations were carried out in semi-solid-state conditions, which are defined as the growth of microorganisms on solid materials in the presence of small quantities of free liquid (Rodríguez Couto et al. 1998b).

The production medium composition was identical to the growth medium, except that glucose was at 2 g per litre.

Erlenmeyer flasks (250 ml), containing the above-mentioned supports and 12 ml of production medium, were inoculated with 10% (v/v) homogenised mycelium. The cultures were supplemented with both veratryl alcohol (2 mM) and Tween 80 (0.5% v/v), in order to stimulate ligninolytic enzyme production (Rodríguez Couto et al. 1999a).

The Erlenmeyer flasks were loosely capped with cellulose stoppers, which permitted a passive aeration, and incubated statically under an air atmosphere at 37 °C, and 90% humidity to avoid evaporation, in complete darkness.

Two experiments for each support were conducted in parallel and triplicate samples were analysed.

Analytical methods

Reducing sugars

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

Ammonium nitrogen content

This was determined spectrophotometrically at 635 nm by the phenol-hypochlorite method (Weatherburn 1967), using ammonium chloride as a standard.

Carbohydrates

They were analysed as described in Rodríguez (1999).

Lignin peroxidase activity

This was determined spectrophotometrically at 310 nm according to Tien & Kirk (1984). One unit (U) was defined as the amount of enzyme that oxidised 1 μmol of veratryl alcohol in 1 min, and the activities were reported as U/l.

Mn (II)-dependent peroxidase activity

It was assayed spectrophotometrically by the method of Kuwahara et al. (1984). One activity unit (U) was defined as the amount of enzyme that oxidised 1 μmol of dimethoxyphenol per minute, and the activities were expressed in U/l.

In vivo Poly R-478 decolourisation

Poly R-478 was aseptically added to three-day-old cultures, as an aqueous solution, to a final concentration of 0.02% (w/v). Culture broth was collected immediately after dye addition and every day thereafter. It was centrifuged (8000 g, 10 min) to eliminate suspended particles and the residual dye concentration was measured spectrophotometrically at 520 nm, which is the maximum visible absorbance of Poly R-478 (Kim et al. 1995).

Two control tests were conducted in parallel: a biotic control (without dye) and an abiotic control (without fungus). In the former ligninolytic activities were determined and in the latter the physical adsorption of the dye to the support was evaluated.

Decolourisation percentage was calculated according to Sani et al. (1998), by means of the formula: $D = 100\ (A_{ini} - A_{obs})/A_{ini}$. The absorbances were corrected by subtracting the absorbance of culture broth without dye.

Biological decolourisation was determined by subtracting the decolourisation due to the physical adsorption of the dye to the support from the total decolourisation.

Dye adsorption to the mycelia was also tested. Spectrophotometric examinations of methanol extracts of the fungal mats showed that the dye bound to the mycelium of *P. chrysosporium* was insignificant.

In vitro Poly R-478 decolourisation

This was monitored at 520 nm, which is the maximum visible absorbance of the Poly R-478 (Kim et al. 1995), according to Ollika et al. (1993). The reaction mixture consisted of 0.002% Poly R-478, extracellular liquid containing 0.1 U of lignin peroxidase and 0.4 mM H_2O_2 in 50 mM sodium tartrate (30 °C; pH 3), in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2 and the absorbance was measured immediately after adding the H_2O_2 and 15 minutes later.

Results and discussion

In a previous report (Rodríguez Couto & Rättö 1998), the superiority of barley straw for LiP production by semi-solid-state cultures of *P. chrysosporium* over nylon sponge was demonstrated. Therefore, in the present study the potential of other lignocellulosic materials for ligninolytic enzyme production was investigated.

Thus, grape seeds, wheat straw and wood shavings were selected as support-substrate, mainly due to their low cost, since the former are agricultural wastes and the latter is a by-product of the forest industry.

Lignin peroxidase production

Grape seeds

As can be observed in Figure 1, ammonium was totally depleted in one day. Nonetheless, glucose consumption, measured as reducing sugars, was very low, remaining a residual level around 2 g/l in the course of the cultivation. It could indicate that the fungus is able to metabolise some of the carbohydrates contained in the grape seeds. This hypothesis is corroborated by the fact that a reduction in carbohydrates of 49.73% in this support was found at the end of the cultivation.

LiP activity began on the 4th day (930 U/l) and peaked on the 8th day (1620 U/l). The values achieved are much higher than those attained employing corncob as support (Rodríguez Couto et al. 2000b), which was considered the best carrier for LiP production among several support tested by our research group (Rivela et al. 1999; Rodríguez et al. 1997, 1998; Rodríguez Couto & Rättö 1998; Rodríguez Couto et al. 1998a).

These high values of LiP detected are likely due to the high lignin content in the grape seeds (43%), which may stimulate ligninase production. It agrees with the investigations of 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).

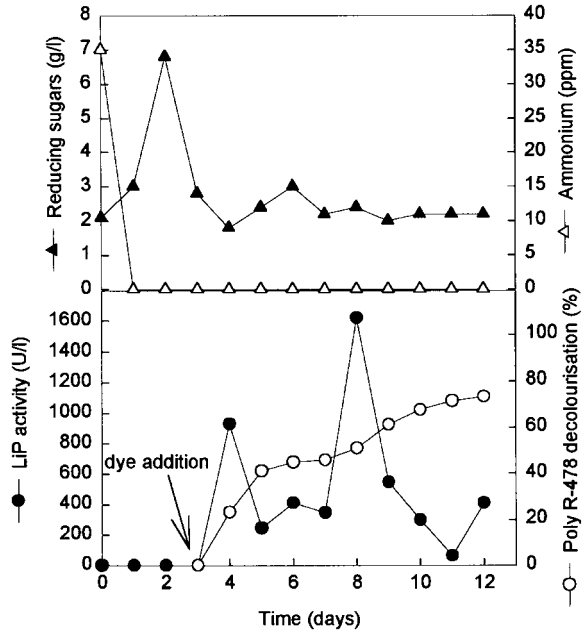

Figure 1. Reducing sugars and ammonium consumption, lignin peroxidase activity and percentage of Poly R-478 decolourisation from grape seed cultures of *P. chrysosporium*.

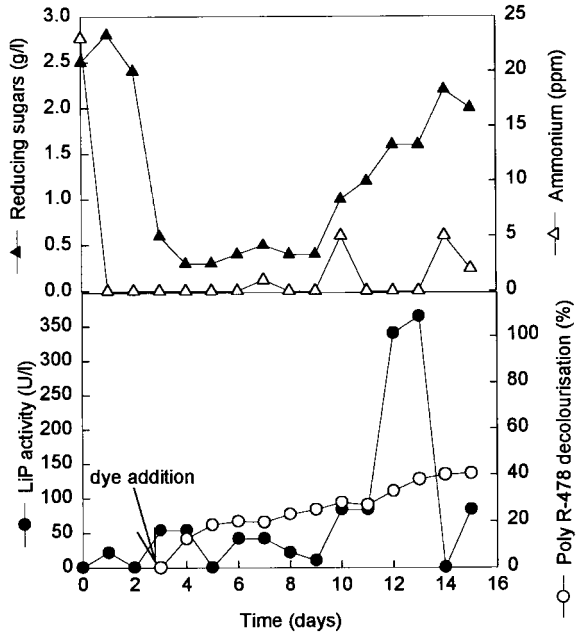

Figure 2. Reducing sugars and ammonium consumption, lignin peroxidase activity and percentage of Poly R-478 decolourisation from wheat straw cultures of *P. chrysosporium*.

On the other hand, insignificant levels of MnP (<16U/l) were detected. Therefore, the use of this support allows for obtaining a ligninolytic fluid composed mainly of lignin peroxidases. This is very interesting for the subsequent application of this enzymatic complex to biotechnological processes, since LiP purification would be much easier, making the process more economical.

Wheat straw

As is shown in Figure 2, ammonium was totally consumed in one day. As for glucose consumption, measured as reducing sugars, it decreased up to a value of 0.3 g/l on the 5th day and from there onwards, it increased reaching similar values to the initial ones. This could be due to either products from straw biodegradation or to the fungus releases some of the nutritive substances contained in the straw to grow.

LiP activity first appeared on the 1st day (22 U/l), reaching its maximum values on the 12th and 13th days (340 U/l and 364 U/l, respectively). These values are much lower than those obtained employing grape seeds as support, and they are very similar to those encountered utilising barley straw as support (Rodríguez Couto et al. 1999b). As for MnP, two peaks around 100 U/l were found.

Wood shavings

Operating with wood shavings as support, glucose consumption, measured as reducing sugars, was rather low and it was not totally depleted until the 5th day, although from there onwards a residual level around 0.2 g/l was maintained. On the other hand, ammonium was totally consumed on the 3rd day (Figure 3).

As for LiP activity, it started on the 1st day (571 U/l), which was its maximum value. In addition, several peaks with LiP activities around 300 U/l were detected along the whole cultivation. These values are higher than those attained with wheat straw but lower than those achieved with grape seeds. In this cultivation MnP activities about 100–150 U/l were found.

The differences in the activity levels of LiP obtained in each case could be due to the different composition of the support employed. Thus, according to our results (Table 1), the support with higher lignin content produced the highest LiP activities. This suggests that lignin or its biodegradation products induce ligninase production in *P. chrysosporium*. This is in agreement with the studies of Ulmer et al. (1984) and Faison & Kirk (1985), as it has already described above.

The high levels of reducing sugars found during the fermentation processes are likely due to the degra-

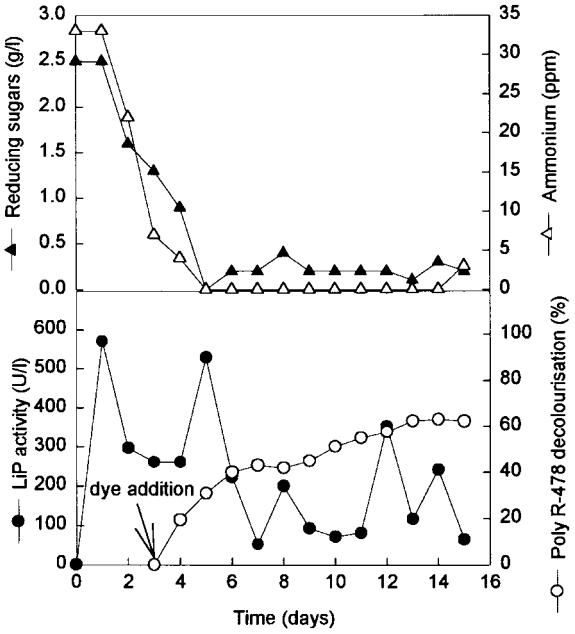

Figure 3. Reducing sugars and ammonium consumption, lignin peroxidase activity and percentage of Poly R-478 decolourisation from wood shaving cultures of *P. chrysosporium*.

Table 1. Maximum LiP activities obtained by the different cultures and percentage of lignin content of the support employed

Support	Maximum LiP activity (U/l)	% lignin content
Grape seeds	1620 ± 123	43
Wheat straw	364 ± 35	12.4
Wood shavings	571 ± 42	30

dation of the carbohydrates contained in the support. Furthermore, it was found that the different support employed lost about 40% of their weight at the end of the cultivation, which confirms the idea that support degradation is occurring.

On the other hand, LiP has not previously been detected in fungi when grown on lignocellulosic substrate during SSF, 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 (Corticiaceae s. lato) as *P. chrysosporium*.

In vivo decolourisation of Poly R-478

The decolourisation of polymeric dyes by fungi has led to the development of simple, rapid and quantitative spectrophotometric assays for the ligninolytic system in microorganisms (Glenn & Gold 1983). Several studies showed that only ligninolytic fungi are able to decolourise some polymeric dyes and that efficiency of decolourisation is correlated with the ability to degrade several lignin model compounds (Chet et al. 1985; Platt et al. 1985; Podgornik et al. 1995).

In this work, the decolourisation of the polymeric dye Poly R-478 (polyvinylamine sulfonate anthrapyridone) was monitored to assess the degrading capability of the ligninolytic complex secreted by the different cultures of *P. chrysosporium* investigated in the present study. The assay with PolyR-478 was chosen due to its simplicity and reliability (Field et al. 1992, 1993)

As is observed in Figure 1, when the waste material employed as support was grape seeds, the biological decolourisation percentage of Poly R-478 obtained, was around 23%, after one day of having added the dye in the culture medium, and it gradually increased up to a value of about 73.4% in the last day of the cultivation.

Figure 2 shows the biodegradation of Poly R-478 in wood shaving cultures. A percentage of biological decolourisation of about 63% after ten days of dye incubation was found, whereas wheat straw (Figure 3) showed a rather lower percentage, around 40% in the same culture time.

The highest percentage of Poly R-478 decolourisation was obtained in grape seed and wood shaving cultures, which could be related to the highest LiP activities attained in such cultures, or it may also indicate that the LiP isoenzymes obtained in such cultures are more powerful in dye degradation.

The results obtained show that the ligninolytic complex produced by *P. chrysosporium* cultures is able to fade a synthetic dye. This agrees with a recent report by Manimekalai & Swaminathan (2000), who found that liquid cultures of *P. chrysosporium* efficiently removed the dyes congo red and crystal violet.

In vitro decolourisation of Poly R-478

In the present section, some studies on the *in vitro* decolourisation of the polymeric dye Poly R-478 by the extracellular fluid from the different cultures of *P. chrysosporium* studied in the present report were

Table 2. Percentage of Poly R-478 decolourisation obtained by LiP (0.1 U/ml) from the cultures studied

Source of the extracellular liquid	% Poly R-478 decolourisation
Grape seeds	5.04%
Wheat straw	2.25%
Wood shavings	5.86%

performed, in order to assess the degrading capability of the ligninolytic complex secreted by such cultures. Extracellular liquid from *P. chrysosporium*, containing mainly LiP, cultivated under the conditions assayed in the present work, was employed. The decolourisation was carried out directly in the spectrophotometer cubette as it is indicated in materials and methods.

The percentages of dye decolourisation obtained after 15 minutes of dye incubation are indicated in Table 2.

To exclude the possibility that dye decolourisation was due to a non-biological oxidation, the dye was incubated with 0.4 mM H_2O_2 in the absence of the enzyme. The dye did not show any change in absorption after a 15-min incubation with H_2O_2.

The highest percentage of Poly R-478 decolourisation was obtained by the extracellular liquid from grape seed and wood shaving cultures, as it occurred in the *in vivo* decolourisation. This clearly indicates that the enzymatic complex secreted by the above-mentioned cultures are more efficient in dye bleaching.

The results obtained in this section are preliminary, since like in the *in vitro* decolourisation of Poly R-478 by MnP from *P. chrysosporium* cultures (Rodríguez Couto et al. 2000a), it is essential to optimise the amount of reagents involved in the reaction in order to maximise the percentage of decolourisation reached. Consequently, more studies to obtain the optimal conditions of the process are underway.

Conclusions

In view of the results obtained, it can be concluded that from the different supports assayed, grape seeds appear to be the most adequate one for ligninase production. This could be related to the high lignin content of this support.

On the other hand, extracellular fluid (composed mainly of LiP) from grape seed and wood shaving cultures exhibited high ability to decolourise the polymeric dye Poly R-478 both *in vivo* and *in vitro*, showing the efficiency of the enzyme produced.

These promising results suggest the application of this system to large-scale processes in order to produce high amounts of enzyme, which would subsequently be utilised to degrade different recalcitrant compounds.

Acknowledgements

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