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Ligninolytic enzyme production and the ability of decolourisation of Poly R-478 in packed-bed bioreactors by *Phanerochaete chrysosporium*

S. Rodríguez Couto, I. Rivela, M. R. Muñoz, A. Sanromán

Abstract The production of ligninolytic enzymes by the fungus *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) in packed-bed tubular bioreactors, operating in semi-solid-state conditions, was studied. Three types of carriers were assayed: cubes of polyurethane foam, cubes of nylon sponge and chopped corncob, in order to determine the more suitable one to produce ligninolytic enzymes by this fungus. The cultivations were carried out in discontinuous and in continuous mode.

For discontinuous cultivation, maximum individual manganese-dependent peroxidase (MnP) activities of 1593, 1371 and 346 U/l were achieved in the bioreactors filled with cubes of nylon sponge, cubes of polyurethane foam and with corncob, respectively. On the other hand, lignin peroxidase (LiP) activities about 100 U/l were found in the two former and around 200 U/l in the latter. Moreover, laccase, was detected in all cultures, with average values of 30 U/l. Nonetheless, continuous mode cultivation led to lower ligninolytic enzyme activities than those produced in discontinuous, except in the case of the corncob.

Furthermore, the decolourisation of the dye Poly R-478 by the above-mentioned cultures was investigated. The percentage of biological decolourisation reached was about 70% in the bioreactor filled with cubes of nylon sponge whereas it was rather low in the others (around 30%).

List of symbols

SSF	Solid state fermentation
MnP	Manganese-dependent peroxidase
LiP	Lignin peroxidase

1 Introduction

The production of ligninolytic enzymes by *Phanerochaete chrysosporium* has been thoroughly studied in liquid [1, 2] and immobilised [3–5] cultures.

Solid-state fermentation (SSF) is undergoing a renewed surge of research interest, primarily because of the opportunities SSF affords for increased productivity and product concentration compared to submerged fermentations and the prospect of using a wide range of agro-industry residues [6].

Semi-solid-state conditions are defined as the growth of microorganisms on solid materials in presence of small quantities of free water [7]. Two types of materials can be used: inert (e.g. plastic foams) and non-inert (e.g. crop wastes). The former acts only as an attachment place whereas the latter functions also as source of nutrients.

The main advantage of SSF systems is that they simulate the fermentation reactions occurring in nature [8]. However, there are several major problems in the development of SSF on an industrial scale, including the mass- and heat-transfer limitations and difficult solids handling inherent in the process as run in existing reactors and the lack of kinetic and design data on various fermentations [9].

Several workers have suggested forced air convection in packed-bed bioreactors as, at least, a partial solution to the mass and heat-transfer difficulties [10–14] noted in even shallow tray fermentors [15].

Glenn and Gold [16] first established that ligninolytic cultures of *P. chrysosporium* are able to decolourise several polymeric dyes. In addition, *P. chrysosporium* is also capable of degrading various azo and heterocyclic dyes [17, 18].

In the present work, the production of ligninolytic enzymes by *P. chrysosporium* in packed-bed tubular bioreactors, operating with polyurethane foam, nylon sponge or corncob as carriers, was studied. The results obtained were compared to those achieved in static-bed bioreactors and in static flasks. Furthermore, the continuous productions of ligninolytic enzymes in the above-mentioned bioreactors were also investigated, which presents a great interest, since there are few studies on continuous production of ligninolytic enzymes.

On the other hand, the decolourisation of the polymeric dye Poly R-478 was monitored to assess the efficiency of the ligninolytic complex secreted, since the decolourisation of this dye is correlated with the ability to degrade several lignin model compounds [19, 20]. Moreover, to our knowledge dye decolourisation by *P. chrysosporium* in semi-solid-state bioreactors has not been reported.

2 Materials and methods

2.1 Microorganism and growth medium

Phanerochaete chrysosporium BKM-F-1767 (ATCC 24725) was maintained at 37 °C on 2% malt agar slants and plates.

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S. Rodríguez Couto, I. Rivela, M. R. Muñoz, A. Sanromán (✉)
Department of Chemical Engineering,
University of Vigo, E-36200 Vigo, Spain

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Spores were harvested, filtered through glass wool, and kept at −20 °C before use [21].

The growth medium was prepared according to Tien and Kirk [22] with 10 g glucose per litre as carbon source, except dymethylsuccinate was replaced by 20 mM acetate buffer (pH 4.5) [23].

2.2 Carriers

The bioreactors were filled with 5-mm cubes of polyurethane foam, with 5-mm cubes of fibrous nylon sponge (Scotch Brite^{TB}, 3M Company, Spain) or with chopped corncob (particle length about 7 mm). These carriers will act as a supporting matrix on which the mycelium can be bound. The latter will also function as source of nutrients.

Prior to use, the cubes of nylon sponge were boiled for 10 min and thoroughly washed three times with distilled water. Polyurethane foam cubes were washed once in methanol, then three times with distilled water. Both carriers were dried at 60 °C and autoclaved until used.

2.3 Bioreactor configuration and operating conditions

The production medium composition was the same as the growth medium except in the case of corncob in which the content of glucose was only 2 g per litre. Moreover, sorbitan polyoxyethylene monooleate (Tween 80, 0.5% v/v) and veratryl alcohol (3,4-dimethoxybenzyl alcohol; 2 mM final concentration) were added at the beginning of the cultivations. The medium was inoculated with 10% (vol/vol) homogenised mycelium.

The bioreactors employed consisted of a jacketed glass column, which is 4.5 cm in internal diameter and it is 20.0 cm high (working volume: 300 ml). Temperature was maintained at 37 °C by circulation of temperature controlled water. Air was supplied to the bioreactors in a continuous way at 0.5 vvm, occasioning a gentle agitation of the culture medium.

The bioreactors were operated in discontinuous and in continuous mode. The discontinuous operation lasted for twelve days whereas the continuous operated was maintained for thirty days. For the continuous process, the feeding medium was supplied in up-flow mode at an average rate of 0.05 ml/min without recycling. Tree samples of different parts of each bioreactor (top, middle and outlet stream) were collected every day.

2.4 Studies on biological decolourisation

To the above-mentioned cultivations, the polymeric dye Poly R-478 (polyvinylamine sulfonate anthrapyridone) was aseptically added, after twenty-nine days of continuous operation, as an aqueous solution to a final concentration of 0.02% (w/v).

2.5 Analytical determinations

Reducing sugars: They were measured by the dinitrosalicylic acid method using D-glucose as standard, according to Ghose [24].

Nitrogen ammonium content: It was assayed by the phenol-hypochlorite method described by Weatherburn [25], using NH_4Cl as standard.

Mn(II)-dependent peroxidase activity: It was assayed spectrophotometrically by the method of Kuwahara et al. [26]. The reaction mixture contained 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 starting by adding 0.4 mM H_2O_2. One activity unit was defined as the amount of enzyme that oxidised 1 μmol of dimethoxyphenol per minute and the activities were expressed in U/l.

Lignin peroxidase activity: It was determined spectrophotometrically according to Tien and Kirk [27]. 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.

Laccase activity: It was determined spectrophotometrically as described by Niku-Paavola et al. [28] with ABTS (2,2′-azino-di-[3-ethyl-benzothiazolinsulphonate]) as substrate. The laccase reaction mixture contained 2.3 ml enzyme diluted to buffer (0.025 M succinic acid, pH 4.5) and 0.7 ml 0.02 M ABTS. The reaction was monitored by measuring the change in A_{436} for 2 min. One activity unit was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute. The activities were expressed in U/l.

Estimation of decolourising activity: Decolourisation was measured by removing 0.1 ml of extracellular culture medium and diluting 10-fold in distilled water. Samples were 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 [29].

Decolourisation percentage was calculated according to Sani et al. [30]. Biological decolourisation was determined by subtracting the decolourisation due to the physical adsorption of the dye to the carrier from the total decolourisation.

3 Results and discussion

3.1 Batch cultivation

3.1.1 Bioreactor filled with cubes of polyurethane foam

As it is shown in Fig. 1, glucose consumption, measured, as reducing sugars, was very low and nitrogen was depleted in two days.

MnP activity first appeared on the 3rd day (760 U/l), peaked on the 4th day (1371 U/l), after that it decreased and then, from days 6 to 8, activity levels were maintained around 500 U/l. As for LiP activity, it began very early on the 1st day (90 U/l) and reached its maximum value on the 7th day (197 U/l). These enzymatic activities are higher

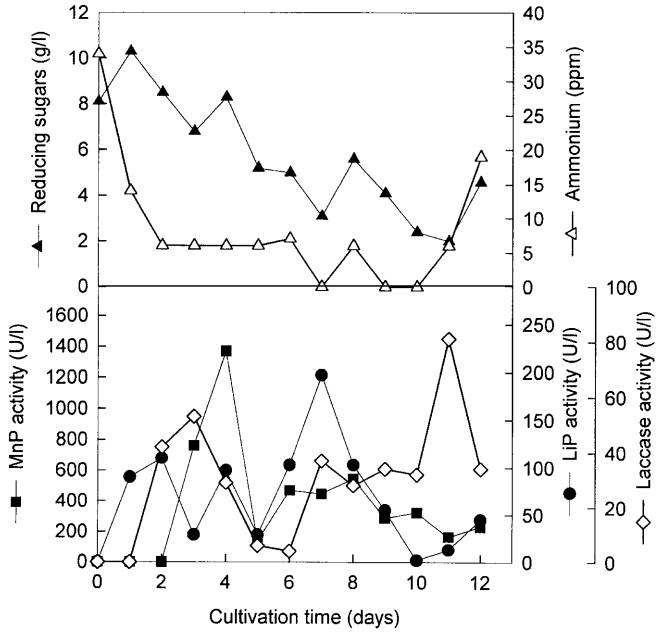

Fig. 1. Glucose and nitrogen consumption and ligninolytic activities obtained by *P. chrysosporium* in a tubular bioreactor filled with polyurethane foam, operating in batch: ▲ Total reducing sugars, △ Ammonium nitrogen, ■ MnP activity, ● LiP activity, ◇ Laccase activity

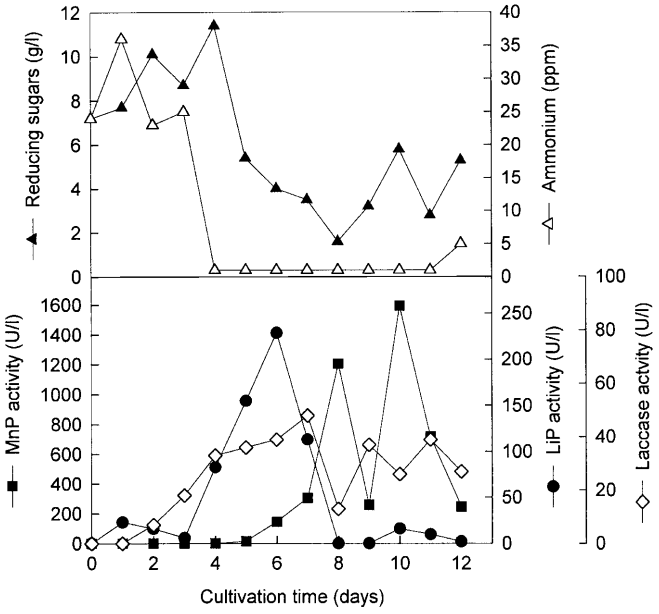

Fig. 2. Glucose and nitrogen consumption and ligninolytic activities obtained by *P. chrysosporium* in a tubular bioreactor filled with nylon sponge, operating in batch: ▲ Total reducing sugars, △ Ammonium nitrogen, ■ MnP activity, ● LiP activity, ◇ Laccase activity

than those obtained both in a static-bed bioreactor [31] and in static flask cultures (Table 1).

Moreover, laccase was also obtained. It started on the 2nd day (42 U/l) and peaked on the 11th day (81 U/l). This shows that this fungus is able to produce laccase, despite always held belief that it was not a laccase producer, which is in agreement with Dittmer et al. [32] and with a previous report [33]. These values are higher than those obtained in a static-bed bioreactor, although they are lower than those achieved in static flask cultivations (Table 1).

3.1.2
Bioreactor filled with cubes of nylon sponge

As it could be observed in Fig. 2, nitrogen was not consumed until the 3rd day, which caused a delay in the appearance of the ligninolytic activities, since the depletion of this nutrient activates the ligninolytic system of this fungus. Glucose consumption, measured as reducing sugars, was also very low and it was consumed at an average rate of 0.7 g/l day.

MnP activity started on the 6th day (145 U/l) and it increased from there onward, reaching a maximum value of 1593 U/l on the 10th day. On the other hand, LiP activity first appeared on the 1st day (23 U/l) and the highest value was achieved on the 6th day (229 U/l). These activities are higher than those obtained both in a static-bed bioreactor [31] and in static flask cultures [34] (Table 1).

Laccase was also produced under these conditions, starting on the 3rd day (18 U/l) and reaching its maximum value on the 7th day (48 U/l).

3.1.3
Bioreactor filled with chopped corncob

The utilisation of support-substrates like corncob presents several advantages such as the reduction in production costs, due to their double function as an attachment place and as source of nutrients. Furthermore, this type of support provides to the fungus a similar environment to its natural habitat (wood) and it offers the possibility of reusing a waste by-product from the agricultural industry.

Table 1. Maximum individual activities obtained in semi-solid-state cultures of *P. chrysosporium* grown on polyurethane foam, nylon sponge and chopped corncob in different types of cultivation

Support	Type of cultivation	MnP (U/l)	LiP (U/l)	Laccase (U/l)
Polyurethane foam	Static flasks	577	58	95
	Static-bed bioreactor	478	50	62
	Tubular bioreactor	1371	197	53
Nylon sponge	Static flasks	1116	32	0
	Static-bed bioreactor	571	203	114
	Tubular bioreactor	1593	229	48
Corncob	Static flasks	1780	901	295
	Static-bed bioreactor	72	187	58
	Tubular bioreactor	346	232	63

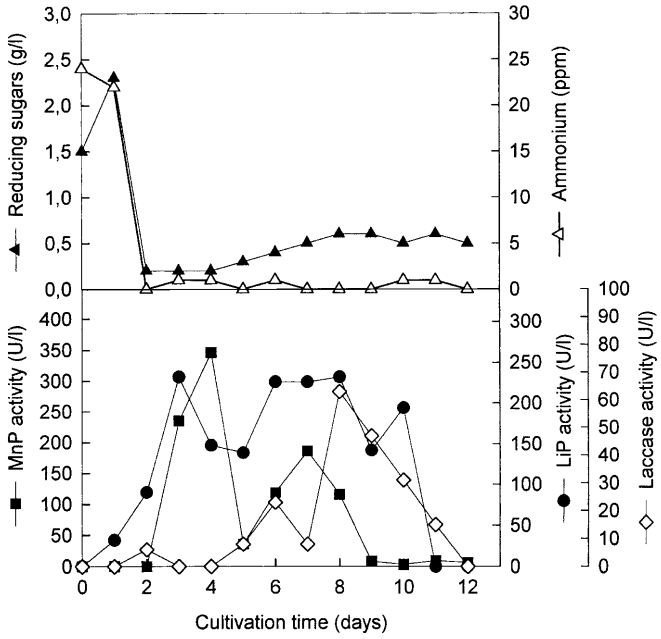

Fig. 3. Glucose and nitrogen consumption and ligninolytic activities obtained by *P. chrysosporium* in a tubular bioreactor filled with corncob, operating in batch: ▲ Total reducing sugars, △ Ammonium nitrogen, ■ MnP activity, ● LiP activity, ◇ Laccase activity

As it is shown in Fig. 3, glucose, measured as reducing sugars, was almost totally consumed on the 2nd day. However, from day four onward it increased, which can be due to either products from corncob degradation or the fungus releases some sugars contained in the corncob depending on its energetic requirements. On the other hand, nitrogen was totally depleted in two days.

MnP first appeared on the 3rd day (235 U/l) and peaked on the 4th day (346 U/l). After that, it decreased abruptly and then it increased again from day 6 to 8, reaching values of about 100 U/l and then it sharply decreased until the end of the cultivation. On the other hand, LiP activity began on the 2nd day (90 U/l) and then it increased, reaching values around 200 U/l up to the 10th day. In addition, laccase production was detected. It first appeared on the 6th day (23 U/l) and peaked on the 8th day (63 U/l).

These values are much higher than those obtained in a static-bed reactor, although they are much lower than those achieved in a previous experiment in static flask cultures [35]. It is due to the agglomeration of the corncob particles, which knitted together with fungal mycelia, causing aeration problems into the bed. Therefore, the utilisation of this support implies the design of a bioreactor that permits a good oxygen transfer into the corncob particles as well as avoids the aggregation of such particles.

On the other hand, it was found that the corncob lost about 40% of its weight at the end of the cultivation, which suggests that the lignocellulosic components contained in it are degraded by the fungal enzymes secreted.

The results obtained clearly show that under the conditions studied, nylon sponge is the most suitable support for ligninolytic enzyme production in bioreactors from the supports assayed in the present work. This is due to the physical features of this carrier (high roughness, hydrophobic nature and high porosity), which permit a good attachment of the fungus to the carrier as well as a proper oxygen and nutrients diffusion into the reactor bed.

3.2
Continuous cultivation

The attempt to continuously produce ligninolytic enzymes during secondary metabolism implies to overcome several problems due to the physiology and morphology of filamentous fungi. There are several factors to take into account for continuous production of ligninolytic enzymes in a semi-solid-state bioreactor. Among them, C/N ratio, which is a critical factor to induce ligninolytic enzyme secretion, oxygenation levels, and the nature of the support employed are outstanding.

To maintain the stability of the enzymes produced is necessary to avoid the secretion of proteases by the fungus into the medium, since according to Dosoretz et al. [23], they are the main reason for the ligninolytic enzyme desactivation. It has been show that a residual glucose concentration minimises protease production [23, 36].

The bioreactors operated for twenty-nine days, of which eleven days were employed in adjusting the equipment. Fresh medium was supplied by means of a peristaltic pump at an average rate of 0.05 ml/min.

3.2.1
Bioreactor filled with cubes of polyurethane foam

The profile of the ligninolytic enzymes produced was rather irregular, which indicates that the enzymes obtained are easily deactivated. This could suggest that proteases are secreted, despite of residual glucose levels were maintained at 1–5 g/l along the cultivation. Maybe, these values are not high enough to avoid protease secretion (Fig. 4).

Maximum MnP activities of 650 and 782 U/l on the 21st and 28th days were obtained, respectively. As for LiP activities, maximum values of 168 and 255 U/l on the 22nd and 23rd days were attained. On the other hand, laccase production was kept at an average value of 30 U/l along the cultivation (Fig. 4).

3.2.2
Bioreactor filled with cubes of nylon sponge

As it is shown in Fig. 5, the profiles of the enzymes obtained were also very irregular, with the typical "roller coaster"-like behaviour. Maybe, proteases are produced under these conditions, in spite of a high residual glucose concentration (about 5 g/l) was kept during the cultivation.

Several peaks of both MnP and LiP activity were detected with values around 300–400 U/l and about 80–100 U/l, respectively. On the other hand, laccase levels about 40 U/l were found (Fig. 5).

3.2.3
Bioreactor filled with corncob

The enzymatic production achieved shows a very irregular profile as it occurred in the above-mentioned cultivations. Nevertheless, the remaining glucose concentration was considerably lower (0.2 g/l) than in the other cultivations, which is likely that causes protease production (Fig. 6).

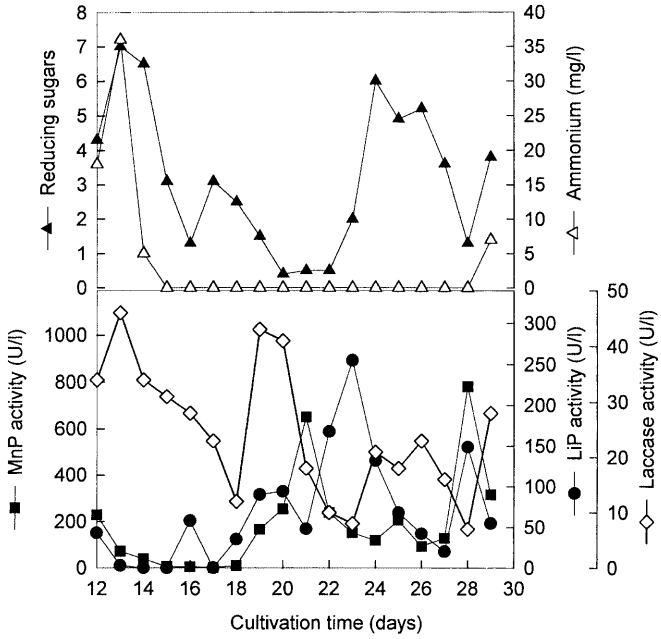

Fig. 4. Glucose and nitrogen consumption and ligninolytic activities obtained by *P. chrysosporium* in a tubular bioreactor filled with polyurethane foam, operating in continuous mode: ▲ Total reducing sugars, △ Ammonium nitrogen, ■ MnP activity, ● LiP activity, ◇ Laccase activity

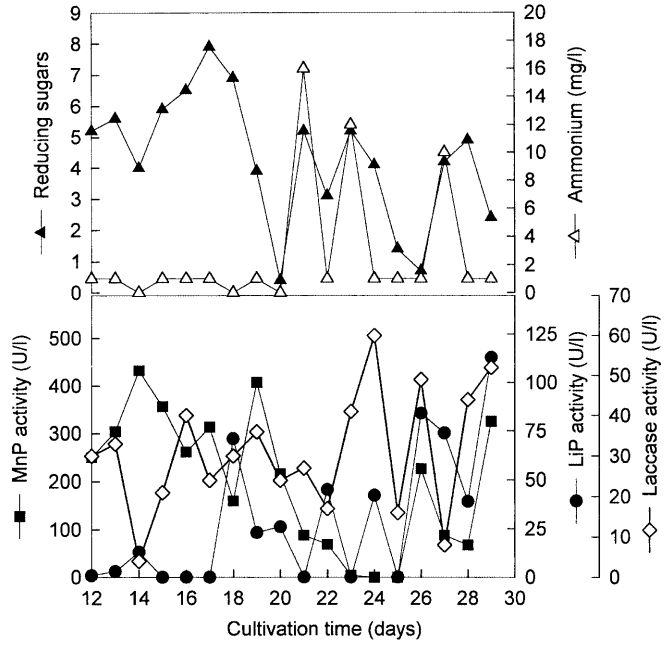

Fig. 5. Glucose and nitrogen consumption and ligninolytic activities obtained by *P. chrysosporium* in a tubular bioreactor filled with nylon sponge, operating in continuous mode: ▲ Total reducing sugars, △ Ammonium nitrogen, ■ MnP activity, ● LiP activity, ◇ Laccase activity

Two peaks with high MnP activity were attained on the 20th and 22nd days (412 U/l and 417 U/l, respectively). These values are higher than those achieved in the discontinuous operation.

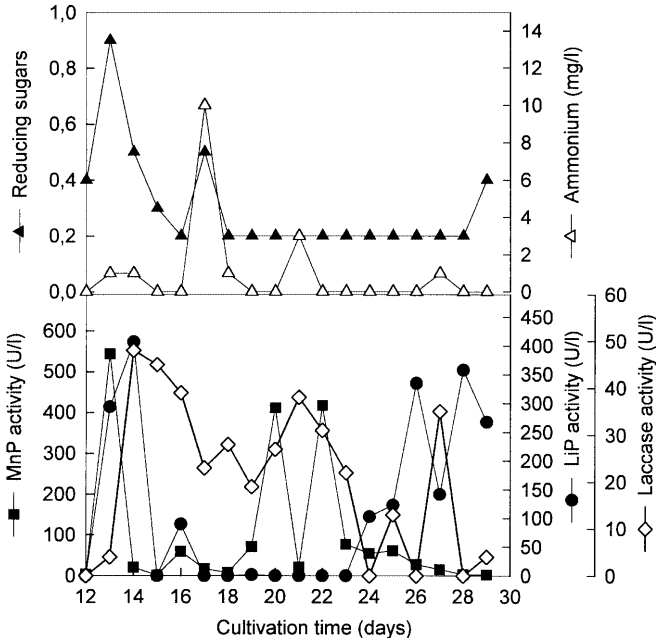

Fig. 6. Glucose and nitrogen consumption and ligninolytic activities obtained by *P. chrysosporium* in a tubular bioreactor filled with corncob, operating in continuous mode: ▲ Total reducing sugars, △ Ammonium nitrogen, ■ MnP activity, ● LiP activity, ◇ Laccase activity

On the other hand, maximum LiP activities of 407, 358 and 268 U/l on the 14th, 28th, and 29th days were obtained, respectively. These activities were about twice higher than those achieved when the fungus grew both on polyurethane and on nylon supports. This is probably due to the lignin content in the corncob, which can stimulate ligninase production. This agrees with the investigations made by Faison and Kirk [37], who showed that ligninase activity was markedly increased by a 12-h preincubation of idiophasic cultures with either synthetic or natural lignins (38 mg/l). As for laccase, activity levels around 30 U/l were detected.

Like in the discontinuous cultivation, it was found that the corncob lost about 40% of its weight at the end of the cultivation, which suggests that the lignocellulosic materials contained in the corncob are degraded by the enzymes secreted by the fungus. This could explain the levels of reducing sugars encountered along the cultivation.

3.3 Decolourisation of Poly R-478

The ability of decolourisation of this dye by fungi is correlated with their ability to degrade several lignin model compounds [19, 20]. Therefore, the decolourisation of the polymeric dye Poly R-478 was monitored to assess the degrading capability of the ligninolytic complex secreted by this fungus in the conditions investigated. The assay with Poly R-478 was chosen due to its simplicity and reliability.

Poly R-478 was added as an aqueous solution to a final concentration of 0.02% (w/v) when high activity levels in the bioreactors were detected. It was observed a rapid decrease in absorbance after 24 h in the nylon cultivation.

A percentage of biological decolourisation about 70% was reached in the bioreactor filled with cubes of nylon sponge. Nonetheless, such percentage was rather low (around 30%) in the other cultivations. This suggests that the ligninolytic complex secreted in the nylon cultivation is more efficient in dye degradation.

4 Conclusions

In view of the results obtained, it can be concluded that semi-solid-state tubular bioreactors seem to be very adequate for ligninolytic enzyme production, operating with both polyurethane foam and nylon as carriers. Nonetheless, more studies are necessary to optimise the conditions that allow to obtain a continuous and stable production of ligninolytic enzymes.

Employing corncob as support makes the process more economical, but it is necessary to assay more bioreactor designs, which facilitate oxygen transfer into the bed and avoids corncob accretion.

The percentage of the biological decolourisation of Poly R-478 was higher in the nylon cultivation, which indicates that the ligninolytic complex secreted under these conditions is more powerful.

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