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Abstract

Manganese peroxidases were overproduced by *Phanerochaete chrysosporium* I-1512 immobilized on nylon net in a bubble-column reactor. This study investigates a new design of bioreactor, a compromise between a pneumatic reactor and an immobilized biofilm reactor. The carrier, a sheet of nylon net, was maintained by a cylindrical stainless-steel frame installed vertically. It was characterized by its hydrophilic nature, its surface morphology and its surface roughness. *P. chrysosporium* adhesion was highly efficient; mycelial hyphae invaded the tridimensional structure and strengthened the bonding to the network, as shown by electron scanning microscopy. High levels of Mn peroxidases were produced by strain I-1512 under conditions of glycerol and nitrogen sufficiency when the medium was supplemented with phospholipid and veratryl alcohol. Yields of 3600 U/l Mn peroxidase were produced after 95 h of incubation, indicating significant productivity for industrial purposes (900 U day^{-1} l^{-1}).

Introduction

The wood-rotting fungus *Phanerochaete chrysosporium* is the subject of extensive investigation. The main interest arises from the ability of *P. chrysosporium* to degrade lignin, a wood polymer, and an extremely diverse range of aromatic compounds (Higson 1991; Lin et al. 1990; Mougin et al. 1994). Most of these non-specific degrading mechanisms depended on the ligninolytic system (Tien and Kirk 1983). Some of the ligninolytic enzymes i.e. lignin peroxidases and manganese-dependent

peroxidases, are produced by the fungus in response to low levels of carbon, nitrogen (Keyser et al. 1978) or sulphur (Jeffries et al. 1981) and addition of inositol or phospholipids (Asther et al. 1988, Moukha et al. 1991). These enzymes are able to catalyse a variety of reactions with hydrogen peroxide as the electron acceptor. The role in the degradation of aromatic compounds of either lignin peroxidase or manganese peroxidase is not clearly demonstrated, although the involvement of manganese peroxidase and Mn^{3+} in the biodegradation of chlorolignin has been shown by Lackner et al. (1991). Moreover Michel et al. (1991) showed that Mn peroxidases play a more important role than lignin peroxidases in decolorization of kraft bleach plant effluent by *P. chrysosporium*.

The low yield of manganese peroxidase in large-scale production has become a problem. Several different cultivation methods for peroxidase production are presently known, including the use of shallow cultures and immobilization methods. The different systems and culture conditions used to produce lignin and Mn peroxidase by *P. chrysosporium* were recently summarized by Linko (1992). The sensitivity of pellets of *P.chrysosporium* to agitation have limited lignin peroxidase production to small-scale static or gently agitated cultures. High activities have been reported in some small cultures of less than 100 ml using various mutant strains and culture conditions (Capdevilla et al. 1989; Moukha et al. 1991; Bonnarme et al. 1991).

Several attempts to produce the enzyme were made by Linko (1988a, b) with *P. chrysosporium* immobilized on nylon net and polyurethane (10-l fermentor). Systems recently used include immobilization on porous supports (Cornwell et al.1990). Bonnarme et al. (1993) showed evidence of the superiority of a pneumatic bioreactor over a mechanically agitated bioreactor (2.5-l fermentor). The maximum lignin peroxidase and Mn peroxidase activities obtained were respectively 4500 U/l and 1944 U/l.

The increasing interest in the ligninolytic degradation system (lignin and Mn peroxidases) of *P. chrysosporium* is contrasted by its incapacity to generate important quantities of enzyme on a large scale, therefore the development of new methods of production is necessary. In this regard, a new bioreactor design combining a pneumatically agitated bubble-column reactor and biofilm immobilization has been considered.

We investigated, in this study, an innovative biofilm reactor system with an immobilization carrier made of nylon net positioned in an air-bubble bioreactor. Enhancement of the amount of immobilized mycelium was achieved and closely associated with an increased enzyme synthesis (Asther et al. 1990). This type of immobilization allows a greater contact area with the culture medium and therefore a more efficient mass transfer. Moreover the INRA-patented strain I-1512 (Moukha et al. 1995) was used in this study for Mn peroxidase hypersecretion.

Materials and methods

Fungal strain

P. chrysosporium I-1512 (CNCM, Institut Pasteur, Paris, France) was used in this study. It was maintained at 37° C on MYA2: malt 20 g, agar 16 g, yeast extract 1 g (l^{-1}).

Culture conditions

P. chrysosporium was grown in a synthetic medium containing: KH_2PO_4 (1.33 g l^{-1}), $CaCl_2$ $2H_2O$ (0.1 g l^{-1}), $MgSO_4$ $7H_2O$ (0.46 g l^{-1}), $FeSO_4$ $7H_2O$ (0.05 g l^{-1}), $ZnSO_4$ $7H_2O$ (0.03 g l^{-1}), $MnSO_4$ H_2O (0.023 g l^{-1}), $CuSO_4$ $5H_2O$ (4.66 mg l_{-1}), glycerol (6.66 g l^{-1}), diammonium tartrate (1.22 g l^{-1}), yeast extract (0.66 g l^{-1}), 1 ml vitamin solution described by Tatum et al. (1950) and commercial soybean phospholipids (NAT 89, 0.5 g l^{-1}), supplied by Natterman Phospholipid GmbH (Koln, Germany) (Capdevilla et al. 1990). The culture medium was buffered to pH 6.5 with 1.53 g l^{-1} disodium tartrate. During the first 40 h the aeration flow rate was kept at 30 l h^{-1} using pure air. After 40 h of growth, the culture medium was supplemented with NAT 89 (0.1 g l^{-1}) and veratryl alcohol (0.42 g l^{-1}) and the aeration flow rate was kept at 15 l h^{-1} using pure oxygen.
The bioreactor was inoculated with 4-day-old cultures grown on the same medium at 37° C, starting with a 5-mm agar disc of mycelium (without NAT 89 and additives). Two mats were harvested and pounded with an Ultra Turrax homogeniser (10000 rpm, green position) in 250 ml distilled water. The pounded mats were used to inoculate the bioreactor.
Each experiment was repeated at least three times. Standard deviations did not exceed 5% of the average values.

Determination of surface morphology

The morphology of the solid carrier surface and *P. chrysosporium* adhesion was observed using a JEOL JSM35 scanning electron microscope. For observation, solids were frozen, dried and coated with gold.

Roughness of the solid carrier

The roughness of the solid-carrier surface was studied with a surface- tracing instrument, the Diavite DT15 (Asmeto), equipped with a stylus of 5 mm radius. The surface roughness was quantified by the

average roughness R_a:

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$R_a = 1/l_m \int \backslash y \backslash dx$

where l_m is the measuring length and y is the distance of the stylus roughness profile R to the centre line (Asther et al. 1990).

Contact-angle measurements

The surface free energy of the solid carrier was measured according to Asther et al. (1990). Contact-angles were measured using the sessile-drop technique at 25° C with distilled water and α-bromonaphthalene (Merck).
For nylon net, contact angles were determined on non-porous solid film of the corresponding polymer.

Bioreactor geometry

Cultures were carried out in a 2.5-l glass fermentor (Fig. 1). The bioreactor vessel was cylindrical (8 cm diameter) with a temperature-control jacket. The carrier was maintained by a stainless-steel frame installed vertically in the bioreactor ensuring a good circulation of gas bubbles. Gas was injected through a perforated pipe sparger.
The bioreactor was connected to a process computer via a process interface. pH, dissolved oxygen and temperature were monitored.
The volumetric oxygen transfer coefficient (k_{1a}) was determined by the gassing-out method in water.

Lignin and manganese peroxidase assays

Lignin peroxidase activity was determined spectrophotometrically at 30° C by the method of Tien and Kirk (1984).
Manganese(II)-dependent peroxidase activity was determined spectrophotometrically at 30° C by the method of Paszczynski et al. (1986).
One unit of enzyme activity is equivalent to 1 µmol product formed/min.

Protein determination

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin (Sigma) as standard.

Residual glycerol determination

Glycerol analyses were performed on a high-pressure liquid chromatography (HPLC) ORH 801 column (Interaction chemicals). Sulphuric acid (2.5 mM) was used as the eluant.

Residual nitrogen determination

The residual nitrogen concentration was determined with the indophenol blue method, which is specific for the analysis of ammonia in distillates of sea water (Riley 1953).

Residual phospholipid determination

Phospholipids were extracted with methanol/chloroform mixture (1:2 v/v) following the Folch method (Folch et al. 1957). Extracts were evaporated to dryness and mineralized using 5 M H_2SO_4 and H_2O_2 (30% w/v) at 160° C. Mineralized phosphorus was then titrated using Fiske and SubbaRow reagent, as reported by Bartlett (1959).

Calculation of kinetic parameters and yields

Mean production rates of biomass rx during the growth are calculated as follows:

$$r_x = \frac{x_f - x_i}{\Delta t}$$

where $x_ʃ$, x_i are biomass concentrations, respectively at the end and the beginning of the growth. Δt is the time variation. r_G, r_N, r_P, r_{LIP}, r_{MnP}, are calculated in the same way, taking into account the corresponding data (x is the fungal biomass, G glycerol, N ammonium expressed as nitrogen, P proteins, LiP lignin peroxidase, MnP manganese peroxidase)

Results

Surface characterization of nylon net and colonization of the carrier

The surface roughness Ra of the nylon net was quantified with a surface-tracing instrument. The value obtained, 21.05 $\pm$ 4.82 µm was consistent with the tridimensional structure of the network examined by scanning electron microscopy (Fig. 2A).

The hydrophobic/hydrophilic nature of the solid carrier surface was characterized by contact angles of water and α-bromonaphthalene. Results showed that the nylon net surface is hydrophobic. Moreover, the hydrophobic nature of the surface and its roughness are close to the data obtained by Asther et al. (1990) for reticulated polyurethane foam (Table1). In this study, the authors reported that the relative amount of immobilized biomass is higher for hydrophobic than for hydrophilic carriers and that an increase of the surface roughness enhances in parallel the total biomass immobilization. This result was confirmed in our work by following the colonization of the support (Fig. 2B, C, D) using scanning electron microscopy.

The electron microscopy performed at the start of growth (Fig. 2B; first 12 h) showed an attachment of the mycelial fragment by adhesion to the carrier rather than by entrapment into the network, probably be- cause of the hydrophobic nature of the carrier. After 24 h (Fig. 2C), mycelial hyphae invaded the tridimensional structure and strengthened the bonding to the network. Therefore, the considerable roughness of the nylon net seems to allow an efficient immobilization of *P. chrysosporium* on the carrier after 96 h (Fig. 2D).

Enzymatic production and substrate consumption

Dissolved oxygen and pH were monitored during the experimentation and data records are shown in Fig. 3. Two relative steps might be distinguished on the basis of these data: phase I (0-40 h), corresponding to the invasive growth of the fungus in the nylon net and phase II (40-120 h) corresponding to the peroxidase production.

During phase I, the aeration flow rate was kept at 30 l h^{-1}, using pure air. This phase corresponded to the first 40 h of incubation during which the fungus produced mainly biomass, as shown by comparison bet- ween the volumic rate of production of fungal biomass r_x during phase I (0.0725 g l^{-1} h^{-1}) and phase II (0.0135 g l^{-1} h^{-1}) (Table 2). The dry weight after the first 40 h was 2.90 g and 3.98 g at the end of incubation. O_2 consumption was maximum, corresponding to a low level of dissolved oxygen (Fig. 3A). The oxygen consumption then decreased and dissolved oxygen reached a stable value of approximately 12% saturation, indicating that growth was maintained at a low level. At the beginning of growth, the pH (Fig. 3B) remained stable around 5.3 during the first 12 h of incubation; this period corresponds to the increase of oxygen consumption. The pH increased slowly after 20 h and reached a maximum (5.6) after 36 h. At the end of phase I (from 36 h to 40 h) the pH decreased dramatically and reached 4.2. This event coincided with the stabilization of oxygen consumption.

During phase II, after supplementation (40 h) with phospholipid (NAT89) and veratryl alcohol, air was replaced by pure oxygen at a flow rate of 15 l h^{-1}. The dissolved oxygen rose rapidly to 60% saturation and then decreased slowly, showing a rise of respiration corresponding to a relatively slow

increase of the pH. After 80 h, respiration was maintained at a low level and pH increased rapidly. The time course of Mn peroxidase and lignin per- oxidase production was followed, together with those of extracellular protein, glycerol and ammonium consumption. Results showed a good correlation between Mn and lignin peroxidase activities and extracellular protein concentration (Fig. 4). Lignin peroxidase activity is six times lower than Mn peroxidase activity. The maximum lignin peroxidase activity was 600 U/l as compared to the maximum Mn peroxidase activity of 3600 U/l. The volumic rate of production of Mn peroxidase (37.9 U l^{-1} h^{-1}) was four times higher than the volumic rate of lignin peroxidase (9.38 U l^{-1} h^{-1}) (Table 2). Enzymatic production appeared during phase II after nylon-net colonization and accompanied the increase of oxygen consumption, the maximum activity being obtained at 96 h, corresponding to an increase of pH and a decrease of respiration, suggesting that secretion stopped.

As far as substrate consumption is concerned, the rate of glycerol utilization was identical during phases I and II. The volumic rate of consumption r_G was 0.037 g l^{-1} h^{-1} during phase I and 0.038 g l^{-1} h^{-1} during phase II (Table 2). Only 50% of the glycerol was consumed at the end of the culture.

The nitrogen supply mostly derived from ammonium salts and, to a lesser extent, from yeast extract. r_N was respectively 0.475 mg l^{-1} h^{-1} and 0.574 mg l^{-1} h^{-1} during phases I and II (Table 2). The ammonium consumption was relatively low and the final concentration represented 80% of the initial quantity, indicating that this strain was able to produce peroxidases when the nitrogen source was non-limiting and in the presence of glycerol as carbon source.

Discussion

Immobilization of *Phanerochaete chrysosporium* I-1512 on nylon web in a laboratory-scale bubble-column bioreactor led to hyperproduction of Mn peroxidase as compared with previously published results. The highest reported Mn peroxidase production in a bioreactor had been 1812 U/l and was achieved by an airlift reactor using free cells of *P. chrysosporium* (Bonnarme et al. 1993). Consequently, 3600 U/l of Mn peroxidase obtained in our bioreactor is a very promising production for potential industrial use.

Previous efforts at producing high manganese peroxidase titres in an agitated fermentor have been unsuccessful because of the sensitivity of the fungus and its enzyme-production system to agitation. Inactivation of ligninolytic enzymes was attributed to mechanical inhibition (Venkatadri and Irvine 1990). In order to avoid problems mainly due to shear forces, different immobilized bioreactor designs have been considered using several immobilization carriers (Linko and Zhong 1987; Cornwell et al. 1990). Fungus immobilization on cylindrical carriers combined with an airlift bioreactor design (Fig. 1) has never been considered, but the immobilization of *P. chrysosporium* on

nylon net allowed an increase in the mycelium/culture fluid interfacial contact area and a decrease of the shear forces. In addition the design, which is similar to a bubble-column reactor (Bonnarme et al. 1993) with a cylindrical carrier, allowed an efficient medium circulation and mass transfer compared to carriers in bulk, like polyurethane foam cubes (Asther et al. 1990). Moreover, the immobilization fungal cells was reported to stimulate both the respiration (mitochondria) and the secretion pathway (endoplasmic reticulum, Golgi apparatus) compared to the situation in a stirred-tank reactor and production with pellets (Bonnarme et al. 1991, 1993). Therefore, this bioreactor is thought to be a good compromise between an airlift reactor and an immobilized-biofilm reactor.

In these culture conditions, *P. chrysosporium* appeared to be well attached and to grow quite well on the nylon-net carrier. Nevertheless, the dry weight at the end of experiment was relatively low (1.6 g/l) compared to nutrient availability and consumption. These results indicate that a low biomass production is not incompatible with high peroxidase production. The choice of the carbon source is also an important factor in the enzyme production (Buswell et al. 1984). It has already been suggested that a low rate of glycerol consumption should favour the appearance of carbon limitation (Roch et al. 1989) and the transition to secondary metabolism but should preserve sufficient carbon metabolism for enzyme synthesis. This limited growth was confirmed by the low ammonium consumption, which is for the most part available for synthesis of extracellular proteins. In this paper, high levels of Mn peroxidase were produced by strain I-1512 under conditions of glycerol as well as nitrogen sufficiency.

In agreement with Bonnarme et al. (1993) and Bar-Lev and Kirk (1981), we also observed that oxygen is an important factor in peroxidase production. The use of pure oxygen enhanced enzyme production whereas e periments performed without oxygenation and in the same conditions showed much lower peroxidases titres (data not shown). In the same way, experiments per- formed without supplementation with phospholipid (NAT 89) and veratryl alcohol did not allow Mn peroxidase activities higher than 2000 U/l. Phospholipids are known to increase the amount of mitochondria and endoplasmic reticulum, and consequently the peroxidase secretion of *P. chrysosporium* (Capdevilla et al. 1990). The residual phospholipid determination showed that NAT 89 was entirely consumed during the first 24 h of incubation (data not shown).

Moreover, strain I-1512 and the process favoured Mn peroxidase production rather than lignin per- oxidase production. Thus far, lignin peroxidase has been reported to be the main enzyme produced. Few publications considered the Mn peroxidase production in a bioreactor (Bonnarme and Jeffries 1990; Bonnarme et al. 1993). In our work the maximum Mn peroxidase activity found was five times higher than the maximum lignin peroxidase activity. This would simplify the following purification steps. The high specific activity (185.8 U/mg) due to the low level of extracellular proteins in the medium should provide the same advan-age. This is in agreement with the INRA patent 95.00002

(Moukha et al. 1995) concerning peroxidase production with hyperproductive strains, where I-1512 is reported to favour Mn peroxidase production over lignin peroxidase production as compared to the wild- type strain BKM F1767 (Bonnarme et al. 1993). Indeed, the maximum activity (3600 U/l) obtained with I-1512 was reached after 95 h and provided a significant productivity (900 U day l^{-1}) for industrial purposes.

The different advantages of the immobilization, the pneumatic bioreactor design, the defined medium and the specific strain previously outlined allowed us to increase and select significantly for Mn peroxidase production. This type of bioreactor offers a good com- promise between the space occupied by the fungi and the surface available for exchange with the medium, allowing efficient mass transfer within the bioreactor. The optimization of the ratio of biofilm surface to fermentor space (under investigation in our laboratory) should lead to an improvement in the performance of this system.

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Table 1. Surface free energy and surface roughness of nylon net (NN) and polyurethane (PU)

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Solid carriers	Contact angle (degress)		Surface free energy ($mJ.m^{-2}$)			Surface roughness, R_a (μm)
	Distilled water	α-bromo-naphthalene	γ_s^d	γ_s^p	γ_s	
NN	65	17	40.0	17.0	57	21.05 ± 4.82
PU[a]	70	17	42.0	6.9	48.9	17.80 ± 3.50

[a] Asther et al. (1990)

Table 2. Volumic rate of consumption of substrates and volumic rate of production of enzymes during phase I and II of the fermentation process. Phase I before supplementation (0-40 h); phase II after supplementation (40-120 h).LiP lignin peroxidase, MnP manganese peroxidase, x fungal biomass, G glycerol, N ammonium (nitrogen), P proteins

Volumic rate of production or consumption r	Phase I	Phase II
r_x ($g\,l^{-1}\,h^{-1}$)	0.0725	0.0135
r_{LiP} ($U\,l^{-1}\,h^{-1}$)	0	9.38
r_{MnP} ($U\,l^{-1}\,h^{-1}$)	0	37.9
r_G ($g\,l^{-1}\,h^{-1}$)	0.037	0.038
r_N ($g\,l^{-1}\,h^{-1}$)	0.475×10^{-3}	0.574×10^{-3}
r_P ($mg\,l^{-1}\,h^{-1}$)	0.17	0.70

Fig. 1 Arrangement and dimensions of the bubble-column reactor

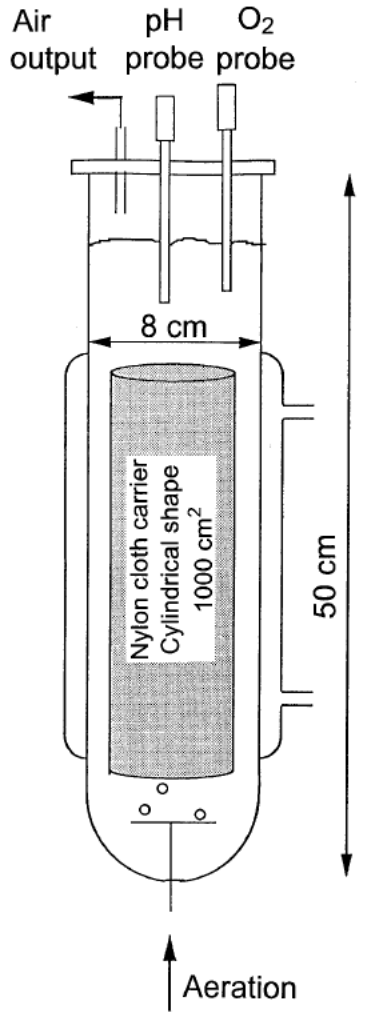

Fig. 2A–D Scanning electron microscopy of nylon net in absence (A), or presence of *P. chrysosporium* after 12 h (B), after 24 h (C) and after 96 h (D) of incubation

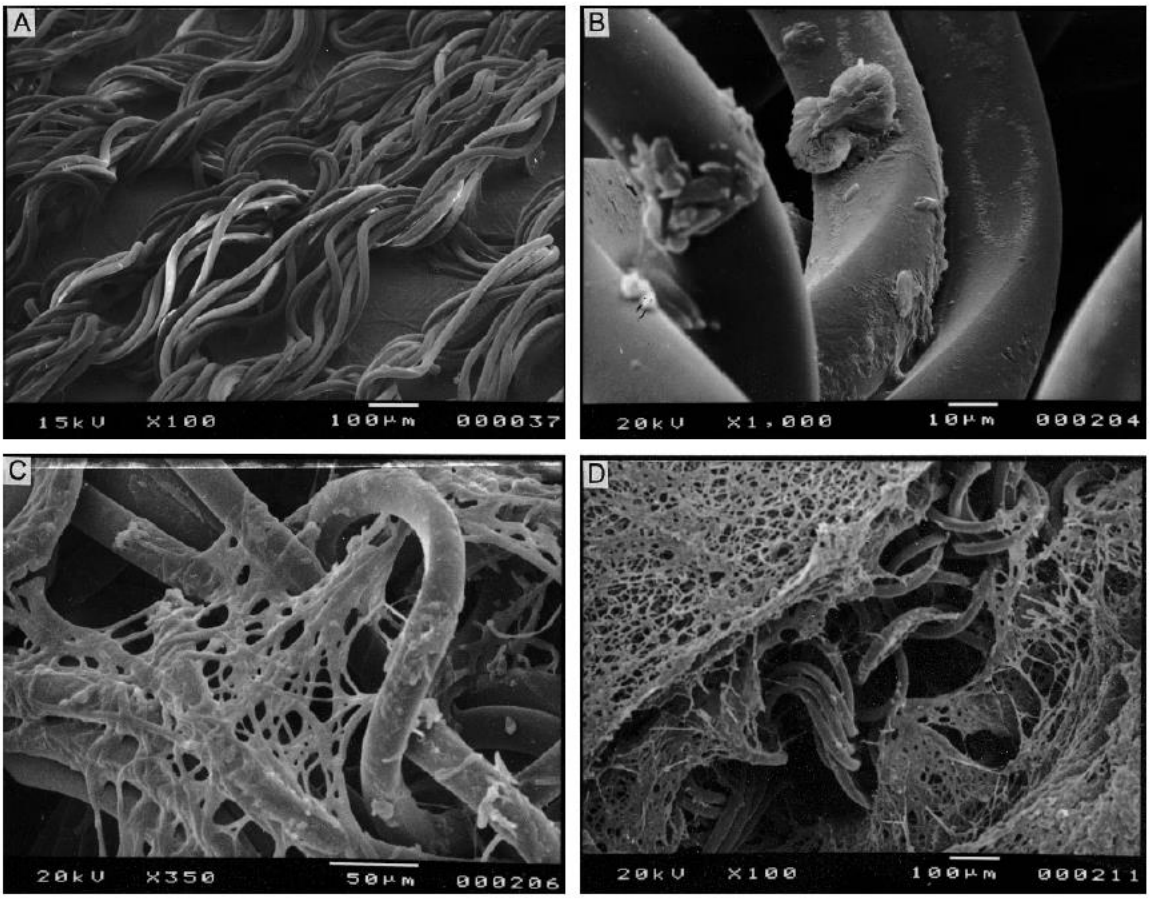

Fig. 3A,B Dissolved O (A) and pH (B) variations in the bubble-column reactor as a function of culture age

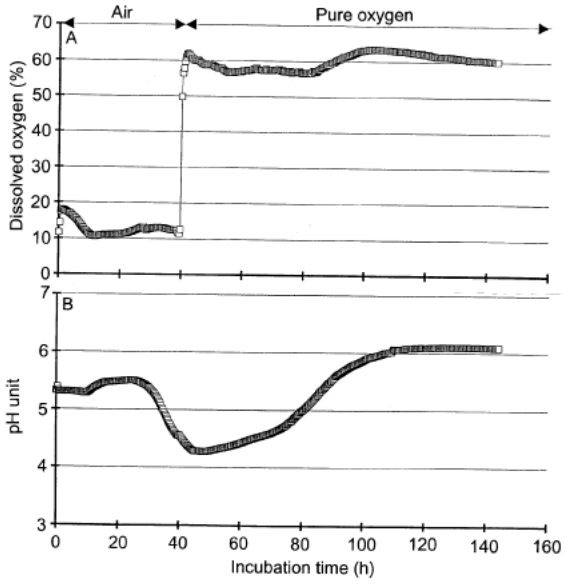

Fig. 4A–D Manganese (△) and lignin(▲) peroxidase activity (A), extracellular protein (B), residual glycerol (C) and residual ammonium (D) in bubble-column reactor