Metabolic potentialities of white rot fungi to degrade recalcitrant aromatic compounds in waste waters and polluted soils

J.C. Sigoillot, C. Laugero, Christian Mougin, R. Rama-Mercier, L. Sohier, Véronique Chaplain, M. Asther

BayFORREST

Bayerischer Forschungsverbund Abfallforschung und Reststoffverwertung

INRA

Laboratoire de Biotechnologie de l'Environnement

REGION LANGUEDOC ROUSSILLON

Bayerisches Staatsministerium
für Landesentwicklung und Umweltfragen

Freistaat Bayern

TREATMENT OF SOLID WASTE AND WASTEWATERS

TRAITEMENT DES DECHETS SOLIDES ET LIQUIDES

BEHANDLUNG FLÜSSIGER UND FESTER ABFÄLLE

1st INTERNATIONAL CONFERENCE

NARBONNE

28. - 29. April 1997

Conference Proceedings
and
6th BayFORREST Report

published
by

P. A. WILDERER
J.-P. DELGENES
D. C. TARTLER
S. GRAJA

Metabolic Potentialities of White Rot Fungi to Degrade Recalcitrant Aromatic Compounds in Waste Waters and Polluted Soils

J.-C. SIGOILLOT[1], C. LAUGERO[1], C. MOUGIN[2], R. RAMA - MERCIER[3], L. SOHIER[3], V. CHAPLAIN[2] ET M. ASTHER[1].

[1] INRA. Laboratoire de Biotechnologie des Champignons Filamenteux. ESIL -CESB. CP 925. 163 avenue de Luminy, F-13288 Marseille cedex 09.

[2] INRA. Unité de Phytopharmacie et médiateurs chimiques. Route de Saint-Cyr, F-78026 Versailles cedex.,

[3] Krebs réseau Eurysis, Division rehabilitation des sites, 8 Rue Jean-Pierre Timbaud, BP 67, F-78184 Saint Quentin en Yvelines.

Abstract

Filamentous fungi, particularly white-rot, are potentially very useful to degrade aromatic compounds from industrial wastes. Basidiomycetes like *Phanerochaete chrysosporium* are strongly resistant to toxic chemicals and possess adapted enzymatic activities (*i.e.* lignolytic system) that can be surexpressed using well defined culture conditions in bioreactors. Since some years, the laboratory of biotechnology of filamentous fungi (INRA Marseilles) and the phytopharmacy and chemical mediators unit (INRA Versailles) cooperate to study the degradation of various aromatic compounds like pentachlorophenol or PAH in aqueous systems, and more recently, in association with KREBS corporation (Eurisys network, COGEMA), to remediate polluted industrial sites.

Résumé

Les champignons filamenteux et particulièrement les basidiomycètes de la pourriture blanche offrent des potentialités intéressantes pour la dégradation de composés aromatiques polluants rejetés par l'industrie. Ces basidiomycètes tels que P*hanerochaete chrysosporium* présentent une forte résistance aux composés toxiques et sont dotés d'équipements enzymatiques adaptés (système lignolytique en particulier) qui peuvent être surexprimés dans des conditions de culture en bioréacteur bien définies. Depuis plusieurs années, les laboratoires de biotechnologie des champignons filamenteux (INRA Marseille) et l'unité de phytopharmacie et des médiateurs chimiques (INRA Versailles) se sont associés pour étudier la dégradation de composés aromatiques tels que le pentachlorophénol ou les HAP dans des milieux aqueux ou plus récemment, en association avec la société KREBS (Réseau Eurisys, COGEMA) pour la réhabilitation de sols industriels pollués.

Zusammenfassung

Filamenteuse Pilze, insbesondere Weißfäulepilze, können beim Abbau aromatischer Verbindungen aus Industrieabfällen von großem Nutzen sein. Basidomyceten wie z.B. *Phanerochaete chrysosporium* sind äußerst widerstandsfähig gegenüber toxischen Chemikalien. Sie besitzen angepaßte enzymatische Eigenschaften, die unter genau definierten Wachstumsbedigungen in Bioreaktoren überexprimiert werden. Seit einigen Jahren arbeitet das Laboratorium für Biotechnologie der Fadenpilze (INRA Marseilles) mit der Abteilung für Phytopharmacie und Chemische Mediatoren (INRA Versailles) zusammen um den Abbau verschiedenster aromatischer Verbindungen wie z.B. Pentachlorphenol oder PAH's in wäßrigen Systemen zu untersuchen. In letzter Zeit wurde in Zusammenarbeit mit der Fa. KREBS (Eurisys Netztwerk, COGEMA) an der Sanierung kontaminierter Industriestandorte gearbeitet.

1 Introduction

Water and soil contamination by xenobiotic compounds such as PCP (Pentachlorophenol) or PAH (Polycyclic aromatic hydrocarbons) is to day a general problem. In that occurrence, the wood-rotting fungus *Phanarochaete chrysosporium* is the subject of extensive investigation. The main interest arises from the ability of *Phanarochaete chrysosporium* to degrade lignin, a wood polymer, and an extremely diverse range of aromatics compounds (1, 2, 3). Most of these nonspecific degrading mechanisms depended on the ligninolytic system (4). Part 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 (5) or sulfur (6) and addition of inositol or phospholipids (7).

Several industrial applications of ligninolytic enzymes have been proposed in pulping, bleaching, pollutant degradation and waste treatment but it has been hindered for a long time by the difficulty to find convenient methods to cultivate this fungus.

The low yield of manganese peroxidase in large scale production has become a problem. Several different cultivation methods for the peroxidases production are presently known, including the use of shallow cultures and immobilization methods. The sensitivity of pellets of *Phanarochaete chrysosporium* to the agitation had limited the lignin peroxidase production to small scale static or gently agitated cultures. High activities were reported in some small cultures of less than 100 ml using various mutant strains and culture conditions.

The increasing interest for the degrading ligninolytic system (LiP and MnP) of *Phanarochaete chrysosporium* is contrasted by the incapacity to generate important enzyme quantities in large scale, therefore development of new methods of production is necessary. In this regard, a new bioreactor design combining bubble column reactor pneumatically agitated and biofilm immobilization has been considered.

We investigated in this study innovative biofilm reactor system with an immobilization carrier made of stainless steel mesh positionned in an air bubble bioreactor. 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 (8) was used in this study for MnP hypersecretion.

As concern PCP degradation, comparative study between static culture and immobilized cells on stainless steel mesh with agitated medium was developped at laboratory scale level in order to determine metabolic changes between the two conditions and the applicability of the method to large scale degradation system.

2 Culture conditions

Culture medium

Phanarochaete chrysosporium I-1512 was grown in a synthetic medium containing: KH_2PO_4 (1.33 $g.l^{-1}$), $CaCl_2 \cdot 2\,H_2O$ (0.1 $g.l^{-1}$), $MgSO_4 \cdot 7\,H_2O$ (0.46 $g.l^{-1}$), $FeSO_4 \cdot 7\,H_2O$ (0.05 $g.l^{-1}$), $ZnSO_4 \cdot 7\,H_2O$ (0.03$g.l^{-1}$), $MnSO_4 \cdot H_2O$ (0.023 $g.l^{-1}$), $CuSO_4 \cdot 5\,H_2O$ (4.66 10^{-3} $g.l^{-1}$), glycerol (6.66 $g.l^{-1}$), diammonium tartrate (1.22 $g.l^{-1}$), yeast extract (0.66 $g.l^{-1}$), 1 ml of the vitamin solution described by Tatum *et al.* (9), commercial soybean phospholipids (NAT 89, 0.5 $g.l^{-1}$), supplied by Natterman Phospholipid GmbH (Koln, Germany). The culture medium was buffered to pH 6.5 with 1.53 $g.l^{-1}$ of disodium tartrate.

Flask cultures

In agitated flasks, the fungus was immobilized on 4 stainless steel mesh rings (20 mm ID, 30 mm height) disposed vertically in 250 ml erlenmeyer flasks containing 100 ml of culture medium. Flasks were incubated on a rotary shaker operating at 120 RPM, 37 °C.

Cultures were grown under oxygen atmosphere and flushed with pure oxygen at each sampling. Labelled pentachlorophenol (^{14}C PCP) and pentachloroanisole (^{14}C PCA) in acetone (100 ml or less) were added to cultures since the begining of the incubation at 1 mg/l concentration (40 kBq) and then after 3 days of growth (0,5 mg/l, 20 kBq). At the same time, the culture medium was also supplemented with NAT89 (0,1 g/l) and veratryl alcohol (0,42 g/l).

Static culture were performed in the same condition without stainless steel rings and agitation.

Bioreactor geometries

Cultures were carried out in a 2,5 l glass fermentor (Fig. 1). Bioreactor vessel was cylindrical (8 cm Ø, H/D = 5) with a temperature control jacket. Gas was injected through a perforated pipe sparger. For immobilization, the carrier (stainless steel mesh) was maintained by a stainless steel frame installed vertically in the bioreactor ensuring a good circulation of gas bubbles. Bioreactors were connected to a process computer via a process interface. pH, dissolved oxygen and temperature were monitored.

A 15 l. airlift bioreactor was also tested with stainless steel mesh as immobilization carrier with the same geometry (15 cm Ø, H/D = 5).

Culture conditions for bioreactors

During the first 40 hours, aeration flow rate was kept at 0,25 VVM using pure air. After 40 hours of growth, the culture medium was supplemented with NAT 89 (0.1 $g.l^{-1}$) and veratryl alcohol (0.42 $g.l^{-1}$) and aeration flow rate was kept at 0,15 VVM using pure oxygen.

The bioreactor was innoculated with 4 days old cultures grown on the same medium at 37 °C starting with 5 mm agar disc of mycelium (without NAT 89 and additives). Two mats were harvested and pounded with an Ultra Turax (10000 rpm) in 250 ml distilled water. The pounded mats was used to inoculate bioreactor.

LiP and MnP assays

Lignin peroxidase activity was determined spectrophotometrically at 30°C by the method of Tien and Kirk (4) using veratryl alcool as substrate.

Manganese peroxydase was determined spectrophotometrically at 30°C by the vanillylacetone method of Paszczynski *et al* (10).

One unit of enzyme activity is equivalent to 1 µmol of product formed per min.

3 Results and discussion

3.1 Lignolytic enzyme production in immobilized conditions

Results still obtained on mechanically agitated bioreactors have shown that this type of stirring is defavourable to the production of MnP. Immobilisation is clearly the most efficient process for the production of MnP by *Phanerochaete chrysosporium* and we have compared productions in two immobilised bioreactors of different shape (2, 5 L and 15 L). On the 2,5 L bioreactor, we obtained a production about 3600 U/L.

We transposed that geometry to a 15 L bioreactor, but for enhancing the rigidity of the draft tube, we used a mesh made with a 0,25 mm stainless steel wire instead of the 0,16 mm used in the 2,5 L bioreactor.

A very rapid fixation of the biomass on the mesh was observed in both cases, but the fixation seemed less efficient in the 15 L bioreactor. Experiments with the same mesh made of the 0,25 mm wire on 2,5 L bioreactors gave the same result (slight removal of the biomass from the mesh after 24 hours) and the diameter of the wire seems to be very important for a good fixation of the biomass.

However, in spite of the slight removal of the biomass, production of MnP in the 15 L bioreactor was close similar to that obtained in 2,5 L.

Experiments performed in 15 L bioreactor show that aeration and oxygenation of the culture are the main difficulties for scaling up airlift bioreactors. In that device, oxygen transfer coefficient (K_La) is very low, and there is an equilibrium to search between aeration (which give shear stress and removal of the biomass) and the large amount of dissolved oxygen needed by *Phanerochaete chrysosporium* to produce MnP.

3.2 Mineralization of PCP and PCA

Erlenmeyer flasks (6 for each experiments) were incubated in one-liter sealed flasks in the presence of vials containing 10 ml of 1N NaOH and 10 ml of water, respectively. The headspace of the flasks were flushed with pure oxygen. The amount of $^{14}CO_2$ trapped in NaOH was determined every day by liquid scintillation counting. Results are reported in Fig 2. Mineralization levels apparead quite different according to chemicals and the culture conditions tested. PCP mineralization started after 1 day incubation under both conditions, whether 13 and 23 % of the total radioactivity were respectively converted to CO_2 in static and agitated cultures after 30 days incubation. No $^{14}CO_2$ could be trapped from uninoculated sterile controls.

Mineralization also occured in PCA experiments, but at lower rates. It was 1,2 and 3,2% of the total radioactivity at day 15, in static and agitated cultures respectively.

Total radioactivity recovered in cultures incubated in the presence of PCP or PCA are shown in Fig 3. This comprised radioactivity found in extracellular fluid, in mycelium, and released as $^{14}CO_2$. The recovery was calculated throughout the experiments at day 3, 10, 15 and 30 for agitated cultures and everyday for static cultures.

With regard to PCP degradation, the radioactivity recovered represents between 91,7 and 101,5 % (standard deviation comprised) of the initial radioactivity for agitated cultures. In static culture, it decreased until 48,7% at the end of the incubation. In the uninoculated sterile controls, PCP content remained constant in agitated cultures and decreased to approximately 70% in static cultures.

In cultures treated with PCA, the total radioactivity recovered was relatively low. In agitated cultures, only 47% of total radioactivity was quantified after 2 days of incubation. Total radioactivity felt also below 20% in static cultures. In uninoculated sterile controls PCA content decreased during incubation. The loss of 30 and 70 % of the initial PCA in agitated and static cultures could be attributed to volatilization, because radioactivity could be trapped in traps for organic volatils (Data not shown).

The results obtained in our experiments showed some difference in the biodegradation pathway between the two culture conditions. In PCP degradation by agitated cultures, a higher percentage of radioactivity was recovered than with static cultures, in which important loss accompanied PCA accumulation (10%, data not shown). PCP transformation to PCA and subsequent volotilization was still reported by Lamar et al. (11). In our experiments, the mineralization of PCA was not very important as indicated by the low amount of $^{14}CO_2$ trapped. So, it seems that immobilisation technique with agitation oriented the metabolism of the strain, and gave better mineralization without significant accumulation of PCA, as indicated by the absence of PCA in the culture medium and the total recovery of radioactivity in PCP experiments.

4 Conclusion

Immobilization of white-rot fungi in airlift bioreactors seems to be very efficient for the treatment of wastes containing toxic xenobiotics. The good fixation of the fungus to the carrier is favourable to continuous flow operations. PCP metabolism, for instance, seems to be improved with immobilized biomass and medium agitation. Oxygen consumption seems to be most important parameter and must be strictly monitored. Important dissolved oxygen concentration needed for enzyme production and degradation could be achieved by absorption of CO2 with NaOH and recycling pure oxygen under pressure.

White rot fungi are also be very efficient for the degradation of xenobiotics in soils (11). Experiments performed in collaboration with KREBS corporation (Eurysis network, COGEMA) are giving promising results for industrial soil remediation contaminated by PAH. Large scale experiments will be conducted by inoculation of white rot fungi in batch of 10 tons or more of contaminated soils.

References

[1] HIGSON, F.K. (1991) Degradation of xenobiotics by white rot fungi. *Rev. Environ. Contamination and Toxicology* : 122-123.

[2] LIN, J.E., WANG, H.Y., HICKEY, R.F. (1990) Degradation kinetics of pentachlorophenol by *Phanerochaete chrysosporium.* Biotech. Bioeng. **35** : 1125-1134.

[3] MOUGIN, C., LAUGERO, C., ASTHER, M., DUBROCA, J., FRASSE, P., ASTHER, M. (1994) Biotransformation of the herbicide atrazine by the white rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **60** : 705-708.

[4] TIEN, M., KIRK, T.K. (1983) Lignin degrading enzyme from hymenomycete *Phanerochaete chrysosporium Burds. Science* : 661-663.

[5] KEYSER, P., KIRK, T.K., ZEIKUS, J.G. (1978) Ligninolytic enzyme system of *Phanerochaete chrysosporium* synthesized in the absence of lignin in response to nitrogen starvation. *J. Bacteriol.* **135** : 790-797.

[6] JEFFRIES, T.W., CHOI, S., KIRK, T.K. (1981) Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **42** : 290-296.

[7] ASTHER, M., LESAGE, L., DRAPRON, R., CORRIEU, G., ODIER, E. (1988) Phospholipid and fatty acid enrichment of *Phanerochaete chrysosporium* INA 12 in relation to ligninase production. *Appl. Microbiol. Biotechnol.* **27** : 393-398.

[8] MOUKHA, S., SIGOILLOT, J.C., FRASSE, P., ASTHER, M. (1995) Definition of a new process for peroxidases production by free or immobilized fungal cells : use of new hypersecretory strains. INRA French Patent n° 9500002.

[9] TATUM, E.L., BARRAT, R.W., FRIES, N., BONNER, D. (1950) Biochemical mutant strains of *Neurospora* produced by physical and chemical treatment. *Am. J. Bot.* **37** : 38-46.

[10] PASZCZYNSKI, A., HUYNH, V.B., CRAWFORD, R. (1986) Comparison of ligninase-1 and peroxidase-M2 from the white rot fungus *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **244** : 750-765.

[11] LAMAR, R.T., GLASER, J.A., KIRK , T.K. (1990) Fate of pentachlorophenol (PCP) in sterile soils inoculated with the white rot basidiomycete *Phanerochaete chrysosporium* : mineralization, volatilization and depletion of PCP. *Soil. Biol. Biochem.* **22**, 433-440.

Session 4

Biological Treatment of Wastewater by Anaerobic Processes