Biotechnology Letters **24:** 795–800, 2002.

The importance of the morphology and hydrophobicity of different carriers on the immobilization and sugar refinery effluent degradation activity of *Phanerochaete chrysosporium*

Carla Guimarães[1,2], Cláudio Matos[2], Joana Azeredo[2], Manuel Mota[2] & Rosário Oliveira[2,*]
[1]*DID – Sugar Research Department, RAR – Refinarias de Açúcar Reunidas, S.A., Portugal*
[2]*Centro de Engenharia Biológica – IBQF, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
[*]*Author for correspondence (Fax: +351253678986; E-mail: roliveira@deb.uminho.pt)*

Received 25 January 2002; Revisions requested 12 February 2002; Revisions received 5 March 2002; Accepted 8 March 2002

Key words: biodegradation activity, hydrophobicity, immobilization, *Phanerochaete chrysosporium*, sugar refinery effluent

Abstract

There was no direct correlation between the surface hydrophobicity or hydrophilicity of four solid carriers and the amount of immobilized *Phanerochaete chrysosporium*. The immobilized biomass was 1.5–1.8 times higher and the fungal degradation activity was 5–8 and 3 times greater in terms of decolorization and phenolics reduction, respectively, with porous carriers than with non-porous carriers.. Morphology of the carriers was important and governed the amount of immobilized mycelium and specially the fungal biodegradation activity.

Introduction

Sugar refineries generate a highly colored effluent resulting from the regeneration of anion-exchange resins (used to decolorize sugar liquor). This effluent represents an environmental problem due to its high organic load, intense coloration and presence of phenolic compounds. The organic load can be eliminated, at least in part, using traditional biological treatments but the compounds responsible for the intense coloration are poorly degraded by the organisms normally involved in these treatments (Ohmomo *et al.* 1987). The white-rot fungus, *Phanerochaete chrysosporium*, can remove color and total phenols from this effluent (Guimarães *et al.* 1999) and is also widely studied for the treatment of other related wastewaters, such as molasses distilleries wastewaters (Fahy *et al.* 1997), pulp and paper mill effluents (Eaton *et al.* 1982) and olive mill wastewaters (Sayadi & Ellouz 1992).

In order to achieve an effective continuous wastewater treatment with *P. chrysosporium*, a bioreactor has to be developed in which the fungal cells can grow well while keeping a high effluent degrading activity for long periods. A promising method to achieve this goal is the immobilization of fungus in an appropriate solid carrier. Therefore, knowledge of the basic processes of biomass immobilization is important to choose the best solid carrier.

Previous reports on the immobilization of *P. chrysosporium* are contradictory. According to Asther *et al.* (1990) the amount of immobilized mycelium is higher for hydrophobic (polypropylene and polyurethane) than for hydrophilic carriers (stainless steel and grey). Jones & Briedis (1992) found that biomass adhesion is correlated to the surface energy of the carriers, with greater adhesion observed on materials of higher surface energy, that is to say to materials with higher degree of hydrophilicity.

The objective of the present work, besides the selection of the most appropriate carrier, was also to verify the effect of surface physico-chemical properties (hydrophobicity and surface tension) and morphology (porosity) of the carrier on the process of *P. chrysosporium* immobilization and subsequent activity. Accordingly, four different carriers were assayed: two hydrophobic, one porous and one non-porous and two hydrophilic, also porous and non-porous.

Table 1. Solid carriers used in *P. chrysosporium* immobilization tests.

Carrier	Shape	Size (mm)	Porosity[a]
Poly(vinylchloride) (PVC)	Rashig ring	10 × 10	Non-porous
Stainless steel (SS)	Rashig ring	10 × 10	Non-porous
Polyurethane foam (PUF)	Cube	5 × 5 × 7	+++
Scotch-Brite (SB)[b]	Cube	5 × 5 × 7	++

[a]Green scourer – nylon web covered with a phenol/formaldehyde resin (3M Company, Spain).
[b]Porosity was assessed by scanning electron microscopy observation (Figure 1).

Moreover, as in the literature the hydrophobicity is expressed qualitatively in terms of the contact angles formed by a polar and a non-polar liquid, it was decided to determine quantitatively the degree of hydrophobicity, following the approach of van Oss & Giese (1995).

Materials and methods

Microorganism and inoculum

Inoculum consisted of a suspension of homogenized *Phanerochaete chrysosporium* (ATCC 24725) mycelium, grown in liquid culture for 48 h (Tien & Kirk 1988).

Culture medium

The medium (pH 4.5) used in the immobilization tests contained (per liter): 10 g glucose; 2 g KH_2PO_4; 1.06 g $MgSO_4 \cdot 7H_2O$; 0.064 g NH_4Cl; 10 ml mineral solution; 10 μg thiamin and 880 ml effluent (collected in the sugar refinery RAR, Porto, Portugal), and was sterilized by filtration (0.45 μm). The mineral solution composition is described elsewhere (Guimarães *et al.* 1999).

Solid carriers

The four carriers tested are described in Table 1, together with some of their relevant physical characteristics.

Surface characterization

The surface tension was calculated from the contact angles formed with liquids having different polarities (van Oss *et al.* 1987). The contact angles were measured (25 determinations for each sample) using the sessile drop technique (Busscher *et al.* 1984). The liquids used were: water, diiodomethane, glycerol, α-bromonaphthalene and formamide. For polyurethane, contact angles were measured on the non-porous film of the corresponding polymer and for Scotch-Brite were measured on the non-porous solid film of the phenol/formaldehyde resin. Mycelium from the inoculum (before being homogenized) was used directly for contact angle measurements.

The morphology of the solid carrier surface and *P. chrysosporium* colonization was observed using a Leica Cambridge S360 scanning electron microscope. Before examination, samples were dried and sputter-coated with gold.

Immobilization tests

Each carrier (dried at 150 °C for 24 h and weighed) was placed in a 250 ml flask. After sterilization at 121 °C for 30 min, culture medium (36 ml) plus 4 ml inoculum were added to each flask. A net volume of 0.75 l solid carrier per l medium was used. A non-inoculated control was prepared for each carrier studied. Cultures with free cells were run in parallel in the same conditions. All tests were made in triplicate and repeated at least once.

After 8 days of incubation (38 °C, 100 rpm), the solid carriers with immobilized cells were carefully drained and gently washed with water in order to eliminate all non-adhering mycelium. Immobilized and non-immobilized mycelia were measured in terms of dry weight (105 °C for 24 h). The carrier dry weight was subtracted from the total weight of carrier plus mycelium to determine the immobilized biomass.

Analytical procedures

Total phenols were determined using the Folin and Ciocalteau reagent, based on the method described by Singleton & Rossi (1965). Color was measured at 420 nm after pH adjustment to pH 9 with 0.012 M borate buffer. Reduction of total phenols was expressed as the percentage in concentration decrease against the control (non-inoculated cultures). Decolorization was expressed as the percentage in color decrease against the control.

Table 2. Surface tension components (γ_s^{LW} – apolar; γ_s^+ – electron acceptor parameter; γ_s^- – electron donor parameter; γ_s^{AB} – polar) and interfacial free energy of interaction between material surfaces (s) immersed in water (w) (ΔG_{sws} – hydrophobicity) of mycelium and carriers (mJ m^{-2}).

Material	γ_s^{LW}	γ_s^+	γ_s^-	γ_s^{AB}	ΔG_{sws}
Mycelium	34.4	2	32.5	16.1	6.6
PVC	37.5	0	17.6	0	−21.5
SS	36.1	0	65.6	0	58
PUF	41.4	0	7.3	0	−53.6
SB	39.1	0	41	0	22.3

PVC – poly(vinylchloride); SS – stainless steel; PUF – polyurethane foam; SB – Scotch-Brite.

Table 3. Immobilized mycelium dry weight and percentage of immobilized mycelium of *P. chrysosporium* in the immobilization tests with different carriers, after 8 days of incubation.

Carrier	Immobilized mycelium (mg/flask)	Immobilized mycelium (%)
PVC	222 ± 30	85 ± 5
SS	250 ± 20	88 ± 7
PUF	326 ± 16	94 ± 2
SB	440 ± 24	97 ± 2

Values are means of three replicates ± standard deviation. PVC – poly(vinylchloride); SS – stainless steel; PUF – polyurethane foam; SB – Scotch-Brite.

Results and discussion

The surface tension of the materials tested and of *P. chrysosporium* mycelium was calculated from contact angle measurements using the approach of van Oss *et al.* (1987). Accordingly, the surface tension of a given substance comprises a component arising from Lifshitz-van der Waals interactions (γ^{LW}) and a component related to polar interactions (γ^{AB}) of the electron acceptor-electron donor type (or acid-base of Lewis), γ^+ and γ^-, respectively ($\gamma^{AB} = 2[\gamma^+\gamma^-]^{1/2}$). The values obtained are presented in Table 2. With the values of the components of surface tension it is possible to calculate the free energy of interaction between two moieties of a solid (s) in the presence of water (w) − ΔG_{sws} (van Oss & Giese 1995).

Considering the definition of hydrophobicity proposed by van Oss & Giese (1995), a solid is hydrophobic when the free energy of interaction between its surface molecules in the presence of water is negative ($\Delta G_{sws} < 0$); otherwise it will be hydrophilic.

Table 4. Decolorization activity and phenolic compounds reduction activity of *P. chrysosporium* immobilized on the carriers and of free cells, after 8 days of incubation in sugar refinery effluent.

Carrier	Decolorization (%)	Phenolics reduction (%)
PVC	12 ± 1	21 ± 1
SS	7 ± 1	21 ± 2
PUF	60 ± 2	71 ± 3
SB	59 ± 2	69 ± 2
Free cells	7 ± 1	2 ± 2

Values are means of three replicates ± standard deviation. PVC – poly(vinylchloride); SS – stainless steel; PUF – polyurethane foam; SB – Scotch-Brite.

From the results obtained (Table 2), polyurethane foam (PUF) and poly(vinylchloride) (PVC) are considered to be hydrophobic, while stainless steel (SS) and Scotch-Brite (SB) are hydrophilic, with PUF displaying the higher degree of hydrophobicity and SS displaying the higher degree of hydrophilicity.

As regards the effect of hydrophobicity, comparing the supports with the same type of morphology (PVC *vs* SS and PUF *vs* SB), a simple observation of the values in Table 3 could lead to the conclusion that a higher degree of hydrophilicity would favor biomass immobilization. However, the difference in the amount of immobilized biomass in the non-porous carriers (SS and PVC), besides being small, cannot be considered very significant taking into account the associated experimental errors. The mycelium of *P. chrysosporium* is expected to have a particular behavior in terms of its interaction with other surfaces because it has a $\gamma^+ \neq 0$ (Table 2), meaning that it has a non-oriented hydration layer (van Oss 1997). This fact may be responsible for the difficulty in determining its affinity towards the support hydrophobicity/hydrophilicity. Moreover, in the case of porous supports part of the biomass may be retained only by entrapment or can develop more easily, on account of being protected from the effects of hydrodynamic shear stress.

There are many reports in the literature about the advantages of using porous and rough supports for biofilm development. Apart from displaying a high surface area, a rough surface and/or internal pore space may provide a more hydrodynamically quiescent environment thereby reducing the detachment of immobilized cells by hydraulic shearing forces (Quirynen & Bollen 1995). In fact, in this case, the morphology of the carrier seems to be an important parameter governing the amount of immobilized *P. chysospo-*

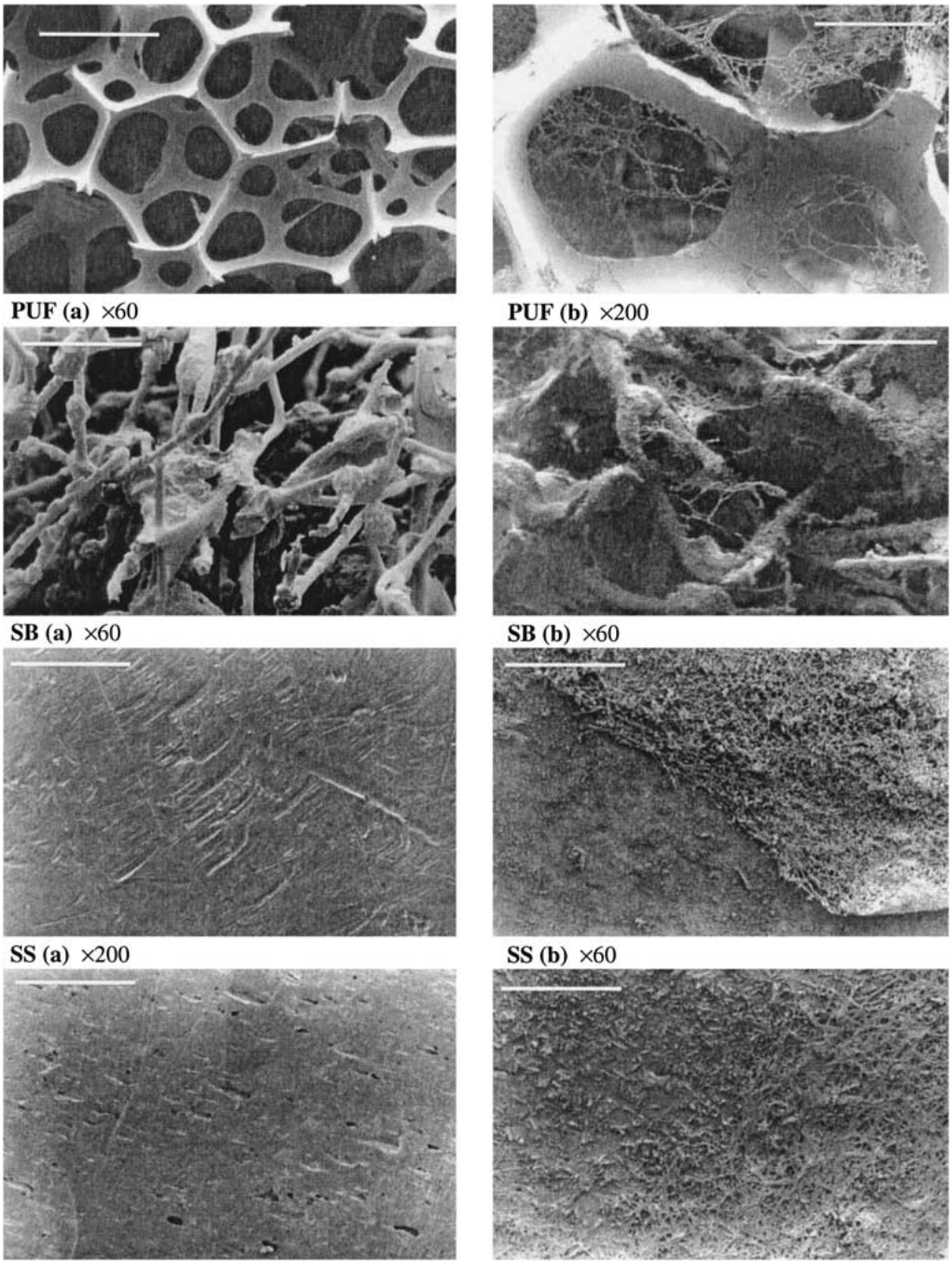

Fig. 1. SEM photographs of polyurethane foam (PUF), Scotch-Brite (SB), stainless steel (SS) and poly(vinylchloride) (PVC). Before (a) and after (b) fungal colonization. Scale bar denotes 500 μm for PUF(a), SB(a), SB(b) and SS(b), and 150 μm for PUF(b), SS(a), PVC(a) and PVC(b).

rium. By SEM observation of the carriers, it was clearly seen that the porous structures of PUF and SB were well colonized by mycelia after the period of incubation (Figure 1). In these types of carriers, the biomass seems not to be simply entrapped inside the porous structure, because some of the mycelial hyphae are visibly attached to the surface of the support. A similar observation was reported by Laugero *et al.* (1996) when studying the immobilization of this fungus on nylon net. In the non-porous materials (PVC and SS) the distribution of the immobilized mycelium was spotty and seemed to be more loosely attached (Figure 1). Jones & Briedis (1992) refer to a better colonization of supports with textured surfaces, whereas no biomass growth could be measured on smooth discs of the same materials.

The biodegradation activity of *P. chrysosporium*, expressed as the capacity of decolorization and phenolic compounds reduction, when immobilized in the different types of materials and as free cells, is summarized in Table 4. Biodegradation activity was significantly higher with PUF than PVC for hydrophobic carriers and with SB rather than SS for hydrophilic carriers, i.e., activity was higher with porous carriers. In these conditions, and particularly in the case of decolorization activity, the immobilized biomass on the two non-porous supports (PVC and SS) had a very low activity, of the same order of the one found with free mycelium. Sayadi *et al.* (1996) also observed that *P. chrysosporium* grown as free cells could not promote the decolorization of olive mill wastewater, while it performed efficiently when it was immobilized in PUF.

A previous study (Guimarães *et al.* 1999) presented some evidence of a possible involvement of the ligninolytic enzymatic system of the fungus in the biodegradation of sugar refinery effluent. This system has also been implicated in the decolorization of pulp and paper mill effluents (Eaton *et al.* 1982) and olive mill wastewaters (Sayadi & Ellouz 1992). Ligninolytic enzymes of *P. chrysosporium* are very sensitive to shear stress (Faison & Kirk 1985). Immobilization in porous supports may protect the enzymatic system against the detrimental effect of shear forces thereby improving the fungus activity. This is the most probable explanation for the high activity observed in cultures containing mycelium immobilized on PUF and SB. Due to this very special characteristic of *P. chrysosporium*, its biodegradation activity cannot be directly related to the amount of biomass, because biomass activity is strongly dependent on environmental conditions. The amount of immobilized biomass on non-porous supports was only 1.5 to 1.8 times lower than the amount of biomass present in the porous carriers (Table 3). However, the degrading activity of the biomass immobilized in non-porous supports was 5 to 8 times lower in terms of decolorization, and 3 times lower in terms of phenolics reduction (Table 4). These results elicit the conclusion that the morphology of Rashig rings *per se* does not offer enough protective effect in terms of shear stress.

In conclusion, the morphology of the carrier is more important than the physico-chemical properties of the surface on to which *P. chrysosporium* is attached, as far as immobilization and biodegradation activity are concerned, with special relevance for the biodegradation activity. No correlation could be established between support surface hydrophobicity or hydrophilicity and the amount of immobilized biomass. Polyurethane foam and Scotch-Brite appear to be suitable carriers for the immobilization of *P. chrysosporium* in sugar refinery effluent treatment.

Acknowledgements

The authors thank PEDIP program (Portugal) for the financial support through the Project RESINAS, JNICT (Portugal) for the Ph.D. grant accorded to C. Guimarães and 3M Company (Spain) for supplying the phenol/formaldehyde resin.

References

Asther M, Bellon-Fontaine M-N, Capdevila C, Corrieu G (1990) A thermodynamic model to predict *Phanerochaete chrysosporium* INA-12 adhesion to various solid carriers in relation to lignin peroxidase production. *Biotechnol. Bioeng.* **35**: 477–482.

Busscher HJ, Weerkamp HA, van der Mei HC, van Pelt AWJ, de Jong HP, Arends J (1984) Measurements of the surface free energy of bacterial cell surfaces and its relevance for adhesion. *Appl. Environ. Microbiol.* **48**: 980–983.

Eaton DC, Chang H–M, Joyce TW, Jeffries TW, Kirk TK (1982) Method obtains fungal reduction of the colour of extraction stage kraft bleach effluents. *Tappi J.* **65**: 89–92.

Fahy V, FitzGibbon FJ, McMullan G, Singh D, Marchant R (1997) Decolourisation of molasses spent wash by *Phanerochaete chrysosporium. Biotechnol. Lett.* **19**: 97–99.

Faison BD, Kirk TK (1985) Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **9**: 299–304.

Guimarães C, Bento LSM, Mota M (1999) Biodegradation of colorants in refinery effluents – Potential use of the fungus *Phanerochaete chrysosporium. Int. Sugar J.* **101**: 246–251.

Jones SC, Briedis DM (1992) Adhesion and lignin peroxidase production by the white-rot fungus *Phanerochaete chrysosporium* in a rotating biological contactor. *J. Biotechnol.* **24**: 277–290.

Laugero C, Sigoillot J-C, Moukha S, Frasse P, Bellon-Fontaine M-N, Bonnarme P, Mougin C, Asther M (1996) Selective hyperproduction of manganese peroxidases by *Phanaerochaete chrysosporium* I-1512 immobilised on nylon net in a bubble-column reactor. *Appl. Microbiol. Biotechnol.* **44**: 717–723.

Ohmomo S, Kaneko Y, Sirianuntapiboon S, Somchai P, Atthasampunna P, Nakamura I (1987) Decolorization of molasses wastewater by a thermophilic strain, *Aspergillus fumigatus* G-2-6. *Agric. Biol. Chem.* **51**: 3339–3346.

Quirynen M, Bollen CML (1995) The influence of surface roughness and surface free energy on supra- and subgingival plaque formation in man. *J. Clin. Periodontol.* **22**: 1–14.

Sayadi S, Ellouz R (1992) Decolourization of olive mill wastewaters by the white-rot fungus *Phanerochaete chrysosporium*: involvement of lignin-degrading system. *Appl. Microbiol. Biotechnol.* **37**: 813–817.

Sayadi S, Zorgani F, Ellouz R (1996) Decolorization of olive mill waste-waters by free and immobilized *Phanerochaete chrysosporium* cultures. *Appl. Biochem. Biotechnol.* **56**: 265–276.

Singleton VL, Rossi A (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **16**: 144–158.

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium. Meth. Enzymol.* **161**: 238–249

van Oss CJ (1997) Hydrophobicity and hydrophilicity of biosurfaces. *Curr. Opinion Coll. Int. Sci.* **2**: 503–512.

van Oss CJ, Giese RF (1995) The hydrophilicity and hydrophobicity of clay minerals. *Clay Minerals* **43**: 474–477.

van Oss CJ, Chaudhury MK, Good RJ (1987) Monopolar surfaces. *Adv. Coll. Int. Sci.* **28**: 35–64.