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Influence of cell immobilization on the production of benzaldehyde and benzyl alcohol by the white-rot fungi *Bjerkandera adusta*, *Ischnoderma benzoinum* and *Dichomitus squalens*

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Abstract Three white-rot basidiomycetes, *Bjerkandera adusta*, *Ischnoderma benzoinum* and *Dichomitus squalens*, were cultivated on a liquid medium supplemented with L-phenylalanine, a precursor for benzaldehyde (bitter almond aroma) and benzyl alcohol. Remarkable amounts of benzaldehyde (587 mg l^{-1}) were found in cultures of *B. adusta*. Immobilization of this fungus on polyurethane foam cubes allowed an 8.3-fold increase of the production of benzaldehyde and a 15-fold increase of the productivity as compared with non-immobilized cells. Aryl-alcohol oxidase activity was only detected in *B. adusta*. This activity was also significantly enhanced in immobilized cells, suggesting that it plays an important role in benzaldehyde biosynthesis. Conversely, consistent amounts of benzyl alcohol (340 mg l^{-1} for *B. adusta* and *I. benzoinum* and 100 mg l^{-1} for *D. squalens*) were produced by the three fungi when immobilized. Laccase activity was found only in the strains *I. benzoinum* and *D. squalens*. This activity was markedly enhanced in free cells cultures. Immobilization of the fungi did not promote benzyl alcohol production by comparison with free cell cultures (500 mg l^{-1}).

Introduction

Because consumers prefer aromas from biological origin to synthetic aromas, synthesis of natural flavour compounds by biotechnological processes plays an increasing role in the flavour industry. Among them, aromatic aldehydes vanillin, cinnamaldehyde and benzaldehyde (bitter almond aroma) have commercial flavour importance. In total amount, benzaldehyde comes in second after vanillin (Welsh et al. 1989). In the food and flavour industry, it is used for apricot, cherry, almond and several other notes.

Aromatic compounds produced by microorganisms can therefore be considered as alternative sources for natural flavours of commercial significance. Mushrooms, particularly white-rot fungi belonging to the basidiomycetes, are responsible for the extensive degradation of lignin (Kirk and Farrell 1987), the most abundant aromatic polymer on earth. This lignin-degrading complex involves a variety of extracellular oxidative enzymes to cleave lignin, giving rise to a wide variety of aromatic compounds (Buswell and Odier 1987).

Among the extracellular aromatic compounds produced by white-rot fungi, veratryl alcohol has received special attention. This compound is generally produced during secondary metabolism and plays diverse roles in white-rot fungal metabolism (Lundquist and Kirk 1978). The production of other aromatic metabolites has also been studied in several white-rot fungi. For instance, the production of flavour compounds by 29 ligninolytic basidiomycetes has been studied by Gallois et al. (1990) under various culture conditions. More than 100 compounds were identified, the most numerous being alcohols (2-phenylethanol, benzyl alcohol, veratryl alcohol, 4-vinylphenol) and aldehydes (veratraldehyde, benzaldehyde). The odorous volatile compounds produced by *Phlebia radiata* have also been investigated on different culture media. Aromatic primary alcohols, such as benzyl alcohol and 2-phenylethanol, were the most abundant metabolites identified (Gross et al. 1989).

The production of anisaldehyde has been studied in liquid cultures of the basidiomycetes of the genus *Pleurotus*. Anisylic and hydroxybenzylic compounds, such as alcohols, aldehydes and acids, were also identified (Gutiérrez et al. 1994) in this fungus. Kawabe and Morita (1993) have reported the production of benzaldehyde and benzyl alcohol by a strain of *Polyporus tuberaster*.

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They found that the most abundant component was benzaldehyde, which accounted for 61% of the total amount of the volatile compounds.

In this study, three white-rot fungi, *Bjerkandera adusta*, *Ischnoderma benzoinum* and *Dichomitus squalens*, were compared for their ability to produce benzyl alcohol and benzaldehyde. The influence of the culture technique (free cells or immobilized mycelium) on benzyl alcohol and benzaldehyde production was studied. The activities of different enzymes (aryl-alcohol oxidase, aryl-alcohol dehydrogenase, manganese peroxidase, lignin peroxidase, laccase), able to degrade lignin and/or synthesize aromatic compounds, were also measured.

Materials and methods

The strains

Three strains of white-rot fungi were used in this study, *Bjerkandera adusta* (CBS 595.78), *Ischnoderma benzoinum* (INRA 33 from our INRA collection) and *Dichomitus squalens* (CBS 432.34). All fungi were cultivated at 25 °C on potato dextrose agar slants, and kept at 4 °C until use.

Medium and culture conditions

Inoculum was prepared in a preculture medium containing 0.2 g l^{-1} KH_2PO_4, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 2 g l^{-1} L-phenylalanine, 10 g l^{-1} glucose, 0.01 g l^{-1} $CuSO_4 \cdot 5H_2O$ and 0.5 g l^{-1} yeast extract. The bioconversion medium for aromatic compound production, had the following composition: 0.2 g l^{-1} KH_2PO4, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 3 g l^{-1} L-phenylalanine, 10 g l^{-1} lecithin, 0.01 g l^{-1} $CuSO_4 \cdot 5H_2O$ and 0.5 g l^{-1} yeast extract. For both media, the pH was adjusted to 5.5 with NaOH or HCl before sterilisation.

Mycelium grown (10 days old) on potato/dextrose/agar medium was suspended in sterile water; 1 ml of this suspension was used to inoculate each flask (500 ml) containing 100 ml preculture medium. Incubation was carried out at 25 °C and shaken at 120 rpm (5-cm-diameter stroke). After 10 days, the fungal pellets were decanted. Two-thirds of the supernatant was discarded, and the remaining was collected and homogenized (Ultraturrax T25, IKA Labortechnik, Staufen, Germany).

For free cell cultures, 2 ml the homogenized mycelial pellets were used to inoculate 500-ml flasks containing 100 ml bioconversion medium. Incubation was carried out at 25 °C and 100 rpm.

When cells were immobilized, each 500-ml flask containing 125 ml bioconversion medium was inoculated with 2.5 ml homogenized mycelium. Immobilization supports were polyurethane foam (Filtren T45, Recticel, Belgium) and 12 foam cubes (2 cm × 2 cm × 2 cm) were placed in each flask and sterilized at 120 °C for 20 min. Incubation was carried out at 25 °C and 100 rpm.

HPLC quantitative analysis of aromatic metabolites

Benzaldehyde, benzyl alcohol and L-phenylalanine have been quantified by HPLC (high-performance liquid chromatography). Samples were filtered through 0.2-μm syringe filters (Microgon Inc., DynaGard, Laguna Hills, Calif., USA), then diluted tenfold and analysed every day. Analysis of aromatic compounds was performed with a Waters (Saint Quentin en Yvelines, France) column (μBondaPak C18; porosity 12.5 nm; diameter 10 μm). Operating conditions were 0.6 ml min^{-1}, 35 °C; detection at 200 nm. A solution of water plus methanol (40%) and acetic acid (0.01%) was used as the eluent.

Aromatic compound extraction and analysis

Volatile components were recovered by co-distillation of the complete culture broth with pure dichloromethane by the Likens and Nickerson (1964) method. Samples (100 ml culture broth) were co-distilled at 100 °C with 30 ml dichloromethane for 45 min.

GC analyses were carried out on a DB 1701 column (length 30 m, internal diameter 0.32 mm, film thickness 0.25 μm; J & W Scientific) using a Delsi DI700 chromatograph equipped with a flame ionization detector. The conditions used were as follows: carrier gas helium, 0.75 ml min^{-1}, injection in split/splitless mode (2 μl), linear temperature gradient from 60 °C to 240 °C (5 °C min^{-1}).

GC-MS analyses were performed on a bentch-top mass spectrometer MSD 5970 (Hewlett Packard) coupled to a HP 5890 gas chromatograph (Hewlett Packard) using helium as the carrier gas. The conditions used were as described above. Electron-impact spectra were recorded with an ion source energy of 70 eV.

Integration of the GC peaks was carried out by a multichannel chromatography work station "Coconut" (R. Almanza & P. Mielle, INRA, F-Dijon).

Enzymatic assays

All the enzymatic activities were determinated extracellularly at 25 °C. Lignin peroxidase activity was measured by the method of Tien and Kirk (1983). The manganese peroxidase activity was measured as described by Gold and Glenn (1988) using ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] as the substrate. The laccase, aryl-alcohol oxidase (AAO) and aryl-alcohol dehydrogenase (AAD) activities were measured as previously described by Gutiérrez et al. (1994). Activities were expressed in U l^{-1} (1 U = 1 μmol min^{-1}).

Dry weight measurement

Dry weight was measured by filtering the mycelium on glass-fibre filters (GF/D Whatman, diameter 4.7 cm). Mycelium was rinsed twice with bidistilled water and dried at 60 °C until constant weight.

For immobilized cell cultures, the dry weight of the cubes was predetermined and subtracted from the weight of foam plus mycelium. The dry weight is expressed in g l^{-1}.

Protein measurement

The culture supernatant was sampled regularly. The insoluble fraction (soybean phospholipids and proteins) was eliminated by centrifugation (9000 *g*, 10 min) and the supernatant was filtered through a 0.4-μm filter (Dynagard, Microgon, Laguna Hills, Calif.). The protein concentration was then determined according to the method of Bradford (1976), using bovine serum albumin as the standard, and expressed in mg ml^{-1}.

Data analysis

All data were obtained from triplicate assays and repeated at least once. The reported values are means ± standard deviation.

Results

Mycelial growth, extracellular protein and substrate consumption

The time course of changes in mycelial dry weight was followed together with L-phenylalanine consumption.

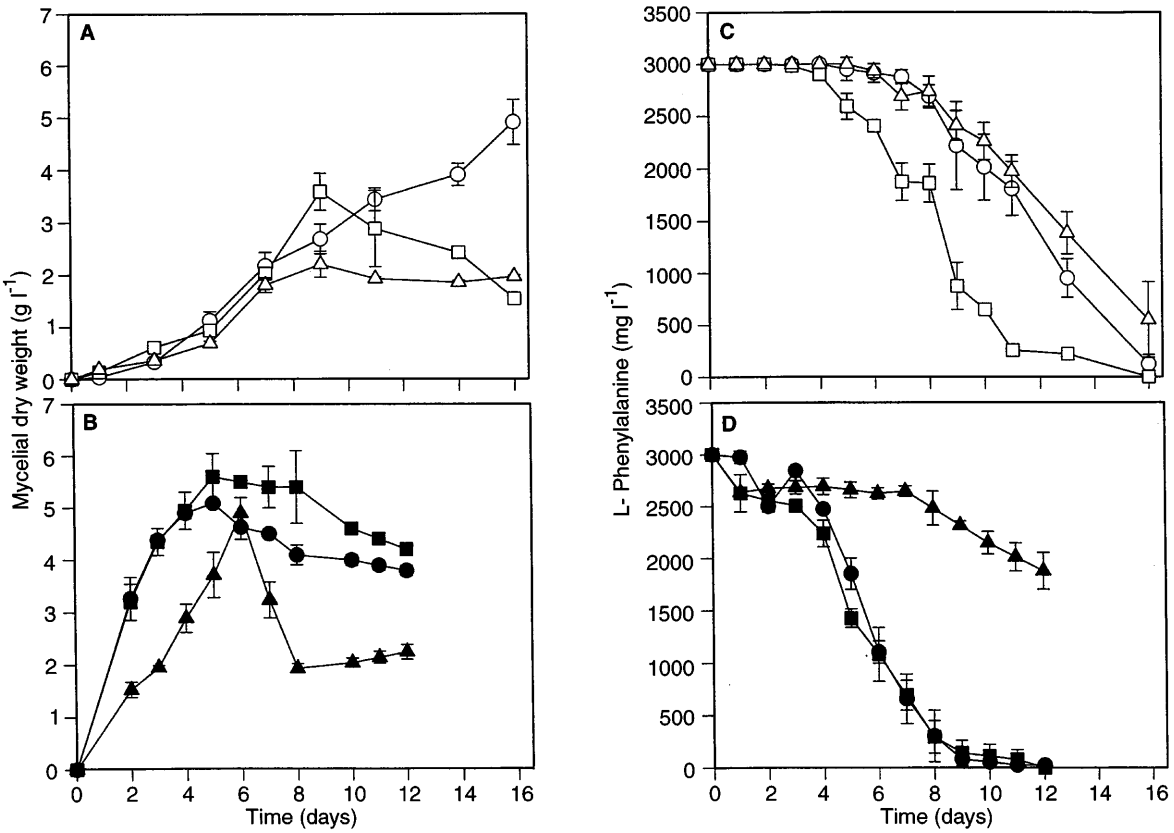

Fig. 1 Biomass concentration (**A**,**B**) and L-phenylalanine consumption (**C**, **D**) for free cell cultures (□ ○ △) or immobilized cell cultures (■ ● ▲) of *Bjerkandera adusta* (● ○), *Ischnoderma benzoinum* (■ □) and *Dichomitus squalens* (▲ △)

The initial biomass production accelerated when the mycelium was immobilized (Fig. 1B). In this case, the maximum biomass concentration was 5 g l^{-1}, 5.6 g l^{-1} and 4.9 g l^{-1} respectively with *B. adusta*, *I. benzoinum* and *D. squalens* (Fig 1B). This maximum was reached after 5 days with *B. adusta* and *I. benzoinum*, and after 6 days with *D. squalens*, after which the biomass declined. With non-immobilized cells (Fig. 1A), maximum biomass concentrations were respectively 5 g l^{-1}, 3.7 g l^{-1} and 2.2 g l^{-1} with *B. adusta*, *I. benzoinum* and *D. squalens* after 9 days or 16 days (for *B. adusta*). Biomass steadily increased during the whole duration of the culture for *B. adusta*, but decreased (*I. benzoinum*) or remained constant (*D. squalens*) for the other species after 9 days of cultivation. Both culture techniques gave better mycelial growth for *B. adusta* and *I. benzoinum* than for *D. squalens*.

For the three strains tested, the initial extracellular protein concentration increased to reach a peak between 2 days and 6 days of incubation, and declined rapidly thereafter (data not shown). This initial increase in the extracellular protein concentration is most probably due to the insoluble protein fraction contained in soybean lecithin, which is solubilized under the action of the fungi then depleted. Elevated proteolytic activities that could participate in solubilizing soybean protein were detected in the culture broth of the three fungi (data not shown).

The L-phenylalanine concentration was also measured against time (Fig. 1C and 1D). In free cell cultures, initial L-phenylalanine consumption was delayed (Fig. 1C). Precursor consumption rates (0.3 g l^{-1} day^{-1}) were nearly identical for *D. squalens*, *B. adusta* and *I. benzoinum* but its depletion started sooner in the latter. With the exception of *D. squalens* (0.14 g l^{-1} day^{-1}), the L-phenylalanine consumption rate was much higher (0.5 g l^{-1} day^{-1}) when the fungus was immobilized on polyurethane foam (Fig. 1D). It was totally depleted after 10–12 days (Fig. 1D) as compared to 16 days in non-immobilized cells cultures (Fig. 1C).

Production of benzyl alcohol and benzaldehyde

The presence of both benzyl alcohol and benzaldehyde in the culture broth of the three white-rot fungi was confirmed by a set of analyses including HPLC and GC-MS. Their identity was definitely assigned by mass spectrometry. The mass spectra of benzaldehyde and benzyl alcohol were found to be identical to those of commercial products.

The time course of benzyl alcohol (Fig. 2A, B) and benzaldehyde (Fig. 2C) concentration was followed for the three basidiomycetes. Benzyl alcohol production increased when fungal biomass was non-immobilized (Fig. 2A) as compared with immobilized cell cultures

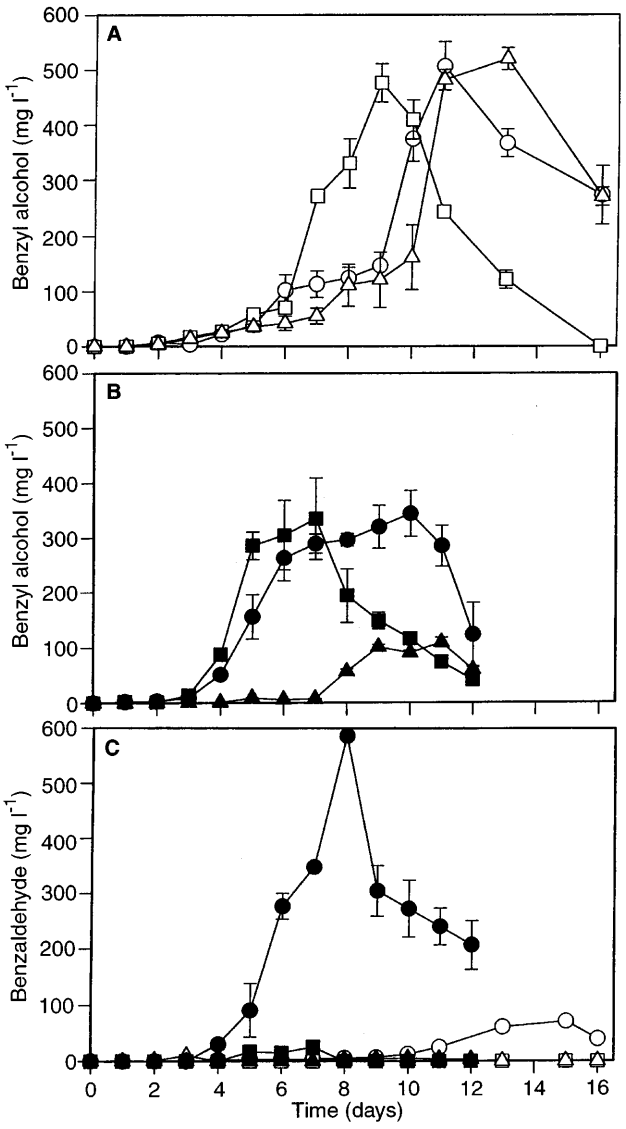

Fig. 2 Benzyl alcohol (**A**, **B**) and benzaldehyde (**C**) production by free (□ ○ △) or immobilized cell cultures (■ ● ▲) of *B. adusta* (● ○), *I. benzoinum* (■ □) and *D. squalens* (▲ △)

(Fig. 2B). In immobilized cell cultures, maximum benzyl alcohol concentrations were 344 mg l^{-1}, 335 mg l^{-1} and 101 mg l^{-1} with *B. adusta*, *I. benzoinum* and *D. squalens* respectively, the best productivity (48 mg l^{-1} day^{-1}) being obtained with *I. benzoinum* (Table 1). Free cell cultures promoted benzyl alcohol biosynthesis for every strain. Maximum benzyl alcohol concentrations of 510 mg l^{-1}, 477 mg l^{-1} and 520 mg l^{-1} were reached respectively with *B. adusta*, *I. benzoinum* and *D. squalens* (Table 1). The best productivity (53 mg l^{-1} day^{-1}) was reached with *I. benzoinum* free cell cultures. In addition, conversion yields were enhanced in free cell cultures as compared with immobilized cell cultures (Table 1). The highest conversion yield (66%) was obtained with the fungus *B. adusta*. In comparison, yields of 49% and 34% were obtained with *D. squalens* and *I. benzoinum*. In immobilized cell cultures, bioconversion yields did not exceed 27%.

B. adusta demonstrated the best capabilities of producing benzaldehyde (Fig. 2C). The highest benzaldehyde concentration obtained with this fungus was 587 mg l^{-1} when immobilized on polyurethane foam (Table 1). The productivity was 73 mg l^{-1} day^{-1} and the conversion yield was 33%. In comparison, only 71 mg l^{-1} benzaldehyde was produced by free cell cultures with a conversion yield of 5.5%. Therefore, the production increased 8.3-fold and productivity 15-fold when the biocatalyst was immobilized. Although immobilization promoted benzaldehyde production in *I. benzoinum* and *D. squalens*, maximum concentrations remained fairly low. The maximum amounts of benzaldehyde produced were 25 mg l^{-1} and 5 mg l^{-1} respectively with *I. benzoinum* and *D. squalens*.

Enzymatic activities

The time course of extracellular lignin and Mn peroxidase, AAO, AAD and laccase activities is presented on Fig. 3. No extracellular lignin or Mn peroxidase or AAD activities were measured for any strain.

Table 1 Effect of culture conditions on the production of benzaldehyde and benzyl alcohol by the white-rot fungi *Bijerkandera adusta*, *Ischnoderma benzoinum* and *Dichomitus squalens*. Maximum concentrations are shown; numbers in parentheses show the day of maximum concentration. *ND* not detected The bioconversion yield is the ratio (total moles of metabolite formed/total moles of phenylalamine consumed) × 100

Strain	Culture conditions	Benzaldehyde			Benzyl alcohol		
		Concentration (mg l^{-1})	Productivity (mg l^{-1} day^{-1})	Yield (%)	Concentration (mg l^{-1})	Productivity (mg l^{-1} day^{-1})	Yield (%)
Bjerkandera adusta	Free cells	71 ± 0 (15)	4.7	5.5	510 ± 40 (11)	46	66
	Immobilized cells	587 ± (8)	73	33	344 ± 42 (10)	34	18
Ischnoderma benzoinum	Free cells	ND	–	–	477 ± 35 (9)	53	34
	Immobilized cells	25 ± 7 (7)	3.6	1.7	335 ± 74 (7)	48	27
Dichomitus squalens	Free cells	ND	–	–	520 ± 20 (13)	40	49
	Immobilized cells	5 ± 3 (10)	0.5	0.9	101 ± 5 (9)	11	22

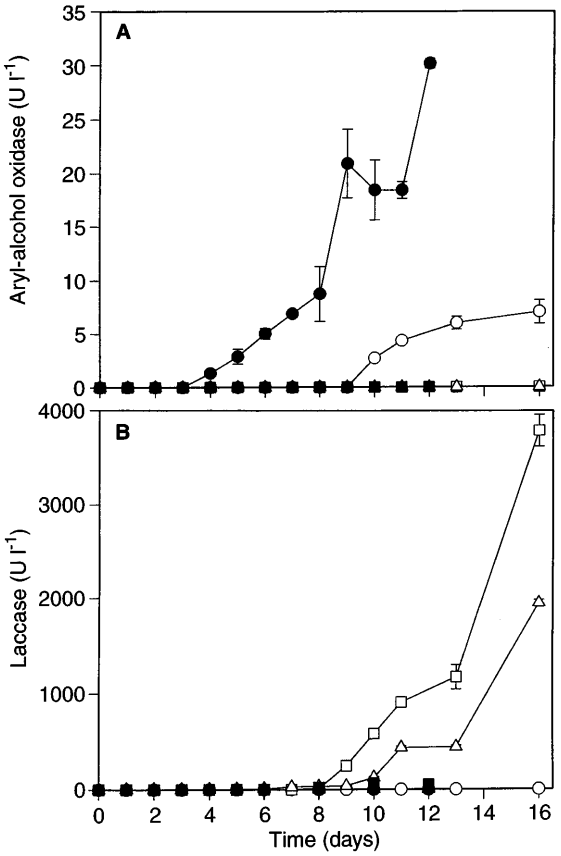

Fig. 3 Aryl-alcohol oxidase (**A**) or laccase (**B**) activity produced by free (□ ○ △) or immobilized (■ ●) cell cultures of *B. adusta* (● ○), *I. benzoinum* (■ □) and *D. squalens* (▲ △)

No laccase activity was detected in *B. adusta* culture supernatant. Conversely, both *I. benzoinum* and *D. squalens* produced this enzymatic activity. Laccase activity was significantly increased in non-immobilized cell cultures of these fungi (Fig. 3B). The onset of laccase activity occurred after 8–9 days of total incubation in free cell cultures. When immobilized, fungus *I. benzoinum* produced a maximum laccase activity of 65 U l^{-1} after 11 days, whereas the maximum activity was 3800 U l^{-1} after 16 days when non-immobilized. Further measurements showed that laccase activity was stable (3900 U l^{-1}) after 18 days and 20 days of total incubation. Therefore, the maximum activity increased 58-fold in pellet cultures. With *D. squalens*, laccase activity was affected in the same way by the culture technique. The maximum laccase activity was 10 U l^{-1} on the 6th day of incubation in immobilized cell cultures, and reached 2000 U l^{-1} after 16 days of cultivation in free cell cultures, remaining stable thereafter (data not shown), which means a 200-fold increase in laccase activity.

The aryl-alcohol oxidase was only detected in cultures of *B. adusta* (Fig. 3A), the only strain that produced high concentrations of benzaldehyde (Fig. 2C). Immobilization of the microorganism promoted AAO activity. For both culture techniques (immobilized and non-immobilized), benzaldehyde production started concomitantly with the onset of AAO activity (Figs. 2C and 3A). The maximum activity was 30 U l^{-1} after 12 days in immobilized cell cultures, and 7 U l^{-1} after 16 days in free cell cultures. No further increase in AAO activity was observed for immobilized or non-immobilized cell cultures after 12 or 16 days respectively.

Discussion

Three white-rot fungi, *B. adusta*, *I. benzoinum* and *D. squalens* have been compared for their ability to produce benzyl alcohol and benzaldehyde from L-phenylalanine.

These fungi were all able to produce benzyl alcohol and/or benzaldehyde, the respective amounts being strongly influenced by the culture technique employed (free or immobilized biocatalyst). It was also observed that the biomass production was enhanced and accelerated in immobilized cell cultures. This is consistent with previous studies done on pellets or immobilized cells of *Phanerochaete chrysosporium*, showing a 1.4-fold increase in mycelial dry weight for immobilized cells (Bonnarme et al. 1991).

Out of the three fungi tested, only *B. adusta* was capable of producing remarkable amounts of benzaldehyde. When cultivated immobilized on polyurethane foam, this fungus produced 8.3 times more benzaldehyde, and productivity increased 15-fold, as compared to non-immobilized cells cultures.

The white-rot fungi are well known for their enzymatic complex capable of degrading lignin in wood. The best characterized of these enzymes is lignin peroxidase (Kirk and Farrell 1987), which is common to many white-rot basidiomycetes. It participates in lignin degradation by *P. chrysosporium* (Tien and Kirk 1983) and other fungi (De Jong et al. 1994). In addition to lignin peroxidase, white-rot fungi can also produce extracellular Mn peroxidase under ligninolytic conditions. Both peroxidases are glycosylated haem proteins, containing protoporphyrin IX (Kirk and Farrell 1987). Another enzyme, laccase, is also produced by numerous fungi (De Jong et al. 1994), and is thought to play a role in lignin degradation. However, this point still remains unclear, since using purified enzyme during in vitro experiments causes lignin polymerisation reactions to predominate. All laccases are glycoproteins and are multi-copper-containing enzymes (Sariaslani 1989). Whereas Mn peroxidase and laccase oxidize only phenolic lignin subunits, lignin peroxidase is also able to attack non-phenolic lignin subunits.

In fungi producing extracellular peroxidases, H_2O_2 is required for activity. Several enzymes generating H_2O_2 have been identified in white-rot fungi including glyoxal oxidase, glucose oxidase and AAO (Kirk and Farrell 1987; De Jong et al. 1994). Several extracellular enzymatic activities were also measured in our fungal

cultures. Among them, AAO was only detected in *B. adusta*, the only fungus that produced high concentrations of benzaldehyde out of the three fungi tested. Recent studies have also described a survey for enzymes produced by ligninolytic fungi, carried out in 90 strains of 68 species of different groups of basidiomycetes (Peláez et al. 1995). AAO activity was found in 40% of species, highest activities being obtained with *B. adusta* and *Pleurotus ostreatus*. Two strains of *D. squalens* were also tested, but they exhibited very low or no AAO activity. AAO has been purified in several basidiomycetes including *B. adusta* (Muheim et al. 1990), *Pleurotus eryngii* (Guillén et al. 1992) and *P. chrysosporium* (Asada et al. 1995). It can oxidize in vitro a wide variety of arylalcohols like veratryl, anisyl, cinnamyl or benzyl alcohols to their corresponding aldehyde (Muheim et al. 1990; Guillén et al. 1992). This makes it of importance in the biosynthesis of aromatic compounds by white-rot fungi. Our results show that AAO activity was significantly enhanced with immobilized *B. adusta*, which correlates with an increase in benzaldehyde production by this strain. Maximum AAO activity increased 4.3-fold when the biocatalyst was immobilized. Similar behaviour was noticed for the production of lignin and Mn peroxidases by *P. chrysosporium* (Bonnarme et al. 1991). Two strains of *P. chrysosporium* BKM-F-1767 and INA-12 were compared as free cells or under immobilized cell culture conditions. When the fungus was immobilized, lignin and Mn peroxidase production was increased 2- to 3-fold and productivity 3- to 4-fold. This can be attributed to the effect of shear forces and/or culture techniques on fungal morphology (Omar et al. 1992; Van Suijdam and Metz 1981) and on fungal metabolism (Bonnarme et al. 1993, 1991). For instance, determination of marker enzymes in *P. chrysosporium* cultures revealed that the enhancement in peroxidase secretion observed with immobilized cells was correlated with a proliferation of endoplasmic reticulum and mitochondrial marker enzymes (Bonnarme et al. 1991). An important increase in gene expression of lignin and Mn peroxidase isoenzymes (Bonnarme 1993) was also observed in fixed cultures as compared to pellet cultures.

Extracellular lignin and Mn peroxidases, AAD and laccase activities have also been assayed in the cultures of the three fungi tested. Among these enzymatic activities, only laccase was found in *I. benzoinum* and *D. squalens*, the strains that produce low amounts of benzaldehyde but large quantities of benzyl alcohol. Unlike AAO, laccase activity was greatly enhanced in free cells cultures. Laccase activity is produced by numerous lignin-degrading basidiomycetes including *D. squalens* and *Trametes versicolor* but not *B. adusta* (Peláez et al. 1995). Its production is dependent on the culture conditions and both constitutive and inducible forms are known, the former generally having a higher activity (De Jong et al. 1994). However, its role in lignin biodegradation is a question that has given rise to much controversy. Therefore, the involvement of laccase in aromatic compound biosynthesis, if any, still needs to be clarified.

So far, AAD has only been detected intracellularly in white-rot fungi such as *P. chrysosporium* (Muheim et al. 1991) or *P. eryngii* (Guillén and Evans 1994). This enzyme can reduce aryl aldehydes, including veratraldehyde and benzaldehyde, to their corresponding alcohols, which are oxidized by AAO, producing H_2O_2 (Guillén and Evans 1994). Intracellular AAD activity has already been measured in *B. adusta* cultures (data not shown). Therefore, the possible involvement of an intracellular AAD activity in the benzaldehyde/benzyl alcohol balance should be studied, and is under current investigation.

Future studies will try to clarify the biosynthetic pathway of benzyl alcohol and benzaldehyde from L-phenylalanine, as well as the importance of enzymes such as AAO, AAD, laccase and peroxidases in this process.

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