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Design of a Fungal Bioprocess for Vanillin Production from Vanillic Acid at Scalable Level by *Pycnoporus cinnabarinus*

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The biotechnological process of vanillin production from vanillic acid by *Pycnoporus cinnabarinus* was scaled-up at the laboratory level. Vanillin production was studied in two types of bioreactors, a mechanically agitated and an air-lift bioreactor. In the mechanically agitated bioreactor where vanillin was produced in greater quantities, oxygen availability was studied during the growth and production phases. A maximal aeration rate (90 *l*/h equivalent to 0.83 volume of air/volume of medium/min or vvm) during the growth phase and a minimal aeration rate (30 *l*/h equivalent to 0.28 vvm) during the production phase were necessary to increase vanillin production to 1260 mg/*l*. Vanillic acid bioconversion to vanillin occurred under the conditions of reduced dissolved oxygen concentration, gentle agitation, high carbon dioxide production and low specific growth rate. However, under these conditions, vanillin production was accompanied by a significant amount of methoxyhydroquinone. Vanillin over a concentration of 1000 mg/*l* was shown to be highly toxic to the growth of *P. cinnabarinus* on agar medium. The application of selective XAD-2 resin led to a reduction of vanillin concentration in the medium, thus limiting its toxicity towards the fungal biomass as well as the formation of unwanted by-products such as methoxyhydroquinone and allowed the concentration of vanillin produced to reach 1575 mg/*l*.

[**Key words:** vanillin, fungal conversion, vanillic acid, bioreactor, aeration, adsorbent]

Vanillin is one of the most widely used flavour chemicals in the food industry. Two commercial types exist, (i) pure vanillin obtained by chemical synthesis from guaiacol, with an annual world market of 12,000 t and a price of approximately US $ 13.5/kg and (ii) natural vanillin from vanilla beans (1). Vanillin is present in vanilla beans at a level of about two percent by weight. Based on the current prices for cured vanilla beans, the extracted vanillin from vanilla beans would cost approximately US $ 3200/kg and its annual consumption is estimated to the approximately 20 t (2). The high price of extracted vanillin and the trend towards natural flavours have stimulated the use of biotechnological tools for the production of natural vanillin (3).

Microbial transformation of natural precursors could provide the desired bio-vanillin, which may offer the "natural" status required by the European legislation (European Directive 88/388/CEE, JO No. L184, 22nd of June 1988) for flavours produced from biological sources using living cells, as compared to the "nature-identical" status of synthetic vanillin. The production of a natural biotechnological vanillin requires a natural precursor such as ferulic acid. This derivative of cinnamic acid, extremely abundant in nature (4), occurs mainly as esters linked to carbohydrates in the plant cell wall, from which it can be enzymatically released (5, 6). Filamentous fungi and especially the white-rot basidiomycetes, have been largely reported to metabolize ferulic acid to vanillic acid and vanillin, but always with low yields of vanillin (7, 8).

A recent two-step process, with the ascomycete *Aspergillus niger* transforming ferulic acid into vanillic acid, and basidiomycetes, *Pycnoporus cinnabarinus* or *Phanerochaete chrysosporium*, transforming vanillic acid into vanillin, allowed vanillin production to reach 237 mg/*l* (9). Two major divergent pathways leading to a decrease in the yield of vanillin from vanillic acid were observed. The first one leads to the production of methoxyhydroquinone via oxidative vanillic acid decarboxylation and the second yields vanillyl alcohol as a result of vanillin reduction (8). The addition of cellobiose to fungal cultures channeled the flow of vanillic acid through the reductive pathway, leading to a concentration of more than 500 mg/*l* vanillin (10). This two-step process using filamentous fungi exhibiting complementary abilities of bioconversion opens up new prospects to scale-up the production of vanillin in bioreactors.

Little is known about white-rot fungi cultures in bioreactors. Most of the recent studies have centered around the use of *P. chrysosporium*. Shear stress, bioreactor design, aeration and agitation and the physiological state of the fungus were described as key parameters for the control of lignin peroxidase production by *P. chrysosporium* (11, 12). In contrast, a few investigations have been reported for *P. cinnabarinus* grown in bioreactors, but only for laccase production (13, 14).

In this paper, we describe for the first time the use of *P. cinnabarinus* cultures on a bioreactor scale to improve the efficiency of vanillic acid transformation to vanillin. Regarding process control, specific attention

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was given to aeration and the use of selective resin.

MATERIALS AND METHODS

Microorganism The strain used in this study was *Pycnoporus cinnabarinus* MUCL 39533, a monokaryotic laccase-deficient strain obtained from the Mycothèque de l'Université Catholique de Louvain (Louvain-La-Neuve, Belgium). The strain was maintained in petri dishes on 20 g/*l* malt agar medium.

Chemicals The phenolic compounds used as substrates in the incubation experiments or as controls in the high performance liquid chromatography (HPLC) studies were: vanillin (Prolabo, Paris, France); vanillic acid and 2-methoxyhydroquinone (Fluka, Saint-Quentin Fallavier, France) and vanillyl alcohol (Aldrich, Saint-Quentin Fallavier). The solvents were of HPLC grade.

Medium and culture conditions Fungal cultures were grown on a basal medium containing maltose as the carbon source (20 g/*l*), diammonium tartrate (1.842 g/*l*) as the nitrogen source, yeast extract (0.5 g/*l*), KH_2PO_4 (0.2 g/*l*), $CaCl_2 \cdot 2H_2O$ (0.0132 g/*l*) and $MgSO_4 \cdot 7H_2O$ (0.5 g/*l*). The liquid preculture was prepared as follows: the mycelium was grown for 10 d in 500 ml flat-shaped bottles on a medium containing 2.5 g/*l* cellobiose as the carbon source and then collected and mixed with sterile water using an Ultra-Turrax T25 blender (Janke & Kunkel, GMBH & Co. KG, Staufen, Germany) as described by Falconnier *et al.* (8). Two hundred milliliters of this suspension was inoculated into the bioreactor containing 1.6 *l* of basal medium, representing an inoculum of between 0.5 to 0.7 g/*l* mycelial dry weight. Cultures were carried out in two types of bioreactors, a mechanically agitated bioreactor of standard geometry (Biolafitte, France) and an air-lift bioreactor, as shown in Fig. 1. The mechanically agitated bioreactor was equipped with a marine propeller (5 mm in diameter) composed of three blades. The agitation rate was maintained at 120 rpm during the growth phase (the three first days of cultivation) to avoid the adhesion of the mycelium to the propeller and was decreased to 100 rpm during the production phase (from the third day until the end of incubation) to limit shear stress. Pure air was injected through a perforated pipe sparger. Several levels of aeration were tested for the mechanically agitated bioreactor: 30, 40, 60 and 90 *l*/h (this last value being the maximal flow rate attainable with our equipment) with air (respectively, 0.28, 0.37, 0.56 and 0.83 vvm: volume of air/volume of medium/min) and 15 *l*/h with pure oxygen (0.14 vvm), while the level of aeration was kept constant at 30 *l*/h in the air-lift bioreactor. The core temperature was maintained at 30°C with a temperature-control jacket.

In order to improve vanillin production, 2.5 g/*l* cellobiose was added to the culture medium on day 3, 2 h before the addition of 0.6 g/*l* vanillic acid, added as a 40 g/*l* filtered salt solution at pH 7.2 (9). Then, from days 4 to 6, the culture medium was replenished daily with a total of 0.6 g/*l* vanillic acid added in aliquots of 0.025 g/*l* each hour for 24 h (total vanillic acid concentration of 2.4 g/*l* for 4 d). Each experiment was run at least in duplicate and the standard deviation for each measured parameter was less than 5% of the mean.

Each bioreactor was connected to a process computer via a process interface. The pH, dissolved oxygen and carbon dioxide (monitored by gas analyzer NGA 2000, Fischer-Rosemount, France) were measured.

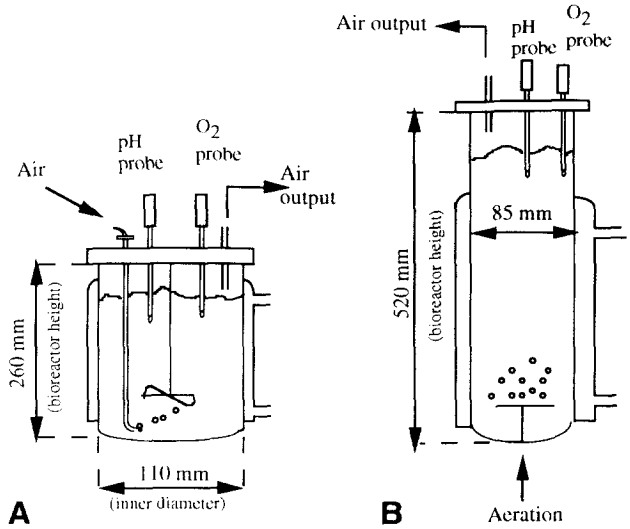

FIG. 1. Bioreactor geometry. (A) Mechanically agitated bioreactor; (B) air-lift bioreactor.

Adsorbent material Amberlite resin XAD-2 (a polystyrenic resin) was purchased from Rohm et Haas (France). It was chosen as the most suitable resin to adsorb vanillin on the basis of experiments conducted by Stentelaire *et al.* (15). On day 4 of cultivation, wet XAD-2 resin was added directly to the culture medium as free particles (100 g/*l*). Three days after the resin addition, the XAD-2 resin was separated (including the mycelium) from the culture medium by filtration, washed with water and eluted with pure ethanol. The amounts of phenolic compounds recovered in each step were quantified by HPLC as described below; more than 80% of the total amount of vanillin was recovered by the resin. The vanillin concentration in the medium was calculated from these data.

Growth measurements The fungal growth was measured in terms of the dry weight of the mycelia after filtration of a 5 ml sample (in duplicate) through GF/A glass-fibre filters (Whatman, Maidstone, England) and drying overnight at 105°C.

Phenolic metabolite analyses Samples were filtered through 0.2 μm syringe filters (Microgon Inc., DynaGard, Laguna Hills, CA, USA) and analysed by HPLC (25 μl injected) on a C18 column (Merck, Darmstadt, Germany) maintained at 30°C (Lichrospher 100 RP-18, 5 μm, 125 × 4 mm). A HPLC model 1050 (Hewlett-Packard, Rockville, MD, USA), equipped with a variable UV/VIS detector set at 280 nm and a 34-position autosampler-autoinjector, was used. The mobile phase, at a flow rate of 0.75 ml/min, comprised a mixture of two degassed solvents: A, 0.01% (v/v) acetic acid in water and B, methanol (8). The quantification was performed using external standards.

Carbohydrate determination Isocratic HPLC analysis on an ion-exchange column maintained at 80°C (Bio-Rad, Richmond, CA, USA; Aminex ion-exclusion HPX-87P, 300 × 7.8 mm), using a HPLC model 1050 (Hewlett-Packard, Rockville) equipped with a refractive index detector and a 34-position autosampler-autoinjector, was carried out directly on culture filtrates for maltose, cellobiose and glucose determination. The eluant was water at a flow rate of 0.4 ml/min. A chromatograph was con-

with the production of 1219 mg/*l* (with a molar yield of 60%) and 1260 mg/*l* (with a molar yield of 66.2%), respectively; in addition, the total recovered metabolites from fungal vanillic acid metabolism constituted 83.7% at 60 *l*/h aeration and 90.7% at 90 *l*/h aeration, respectively. Therefore, large amounts of methoxyhydroquinone and vanillyl alcohol were detected under these conditions.

Aerating the medium at 15 *l*/h (0.14 vvm) with pure oxygen during the growth phase led to the production of 831 mg/*l* vanillin (amounting to a molar yield of 48.6%) and lowered the total concentration of the identified phenolic compounds produced from vanillic acid bioconversion, as compared with those under conditions where pure air at a 30 *l*/h aeration rate was used during the growth phase. However, increasing the aeration rate above 30 *l*/h during the production phase led to the reduction of vanillin concentration and to the production of up to seven times more methoxyhydroquinone.

Vanillin production in a bioreactor under optimum aeration conditions As previously shown, a maximal aeration rate of 90 *l*/h during the growth phase and a minimal aeration rate of 30 *l*/h during the production phase were defined as the optimum aeration conditions to produce high amounts of metabolites, in particular of vanillin, from vanillic acid by *P. cinnabarinus*. Vanillic acid metabolism, carbohydrate and nitrogen consumption, mycelial growth, pH evolution and gas analysis were determined in these cultures and were compared to those under standard conditions of aeration, *i.e.*, 30 *l*/h during the entire period of cultivation. The results are shown in Fig. 2. Aerating the culture at 30 *l*/h during the growth phase and the production phase led to a vanillin production rate of 145 mg/*l*/d (Fig. 2A) and to a maximal specific production rate of 0.23 g/g/d between days 4 and 5. Less than 150 mg/*l* methoxyhydroquinone and vanillyl alcohol was detected in the culture medium.

When the maximum air flow rate permitted by our equipment (90 *l*/h) was applied to the culture medium during the growth phase followed by decrease to 30 *l*/h during the rest of the cultivation period, the vanillin production rate reached 180 mg/*l*/d and the maximal specific production rate reached 0.26 g/g/d between days 4 and 5.

Maltose, glucose and nitrogen consumption is shown in Fig. 2B. Under both conditions of aeration and during the three first days of incubation (growth phase), carbon and nitrogen were slowly consumed; thereafter, they were quickly dissimilated by the end of the incubation period. However, they were more slowly consumed at the aeration rate of 30 *l*/h. At the end of these experiments, 12 mM nitrogen was present, while less than 5 g/*l* maltose and about 6 g/*l* glucose (released from maltose metabolism) were detected in the culture medium. Vanillic acid bioconversion therefore occurred without carbon

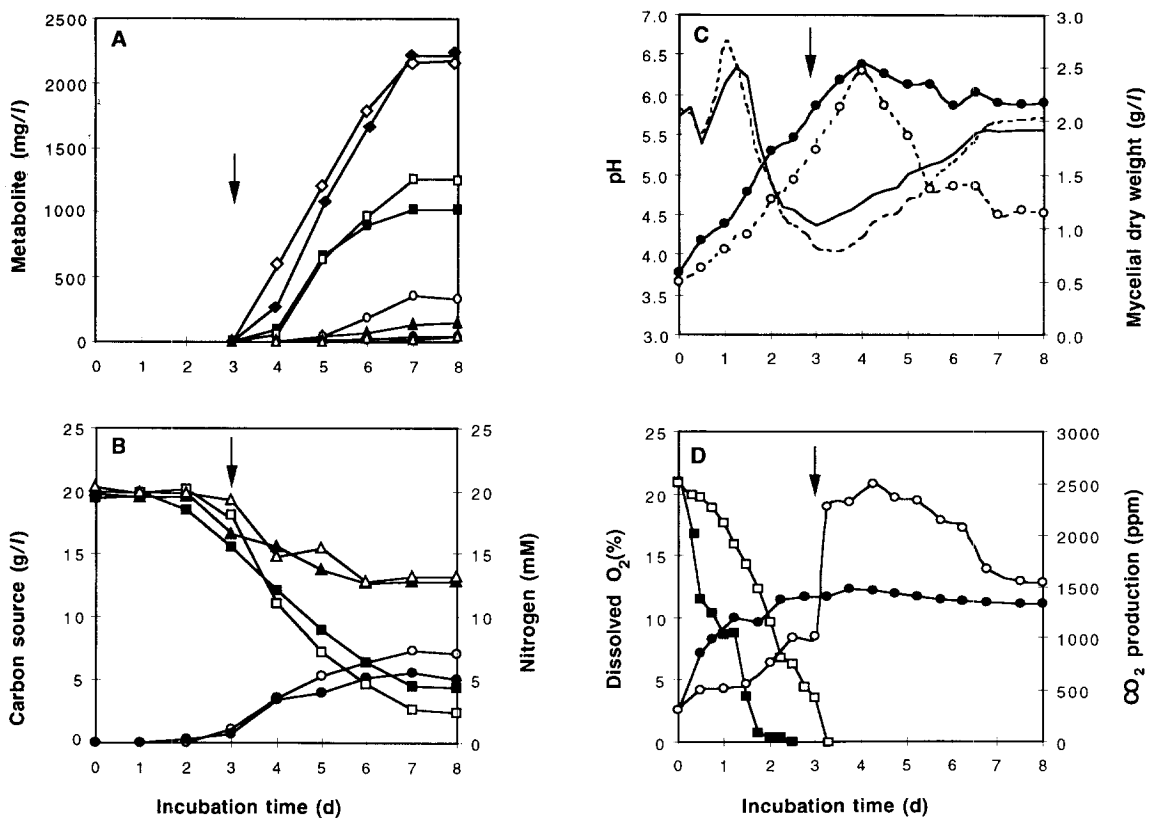

FIG. 2. Vanillic acid metabolism by *P. cinnabarinus* in a mechanically agitated bioreactor as a function of aeration, in relation to carbon and nitrogen consumptions, fungal biomass formation and variations in the values of pH, dissolved oxygen and carbon dioxide. (A) Consumed vanillic acid (◆, ◇), vanillin (■, □), methoxyhydroquinone (●, ○), vanillyl alcohol (▲, △); (B) maltose (■, □), glucose (●, ○), nitrogen (▲, △); (C) fungal biomass (●, ○), pH (—, ----); (D) dissolved O_2 (■, □); and output CO_2 (●, ○). Closed symbols or solid lines correspond to the aeration rate of 30 *l*/h during the growth and production phases. Open symbols or dotted lines correspond to the aeration rate of 90 *l*/h during the growth phase and that of 30 *l*/h during the production phase. Arrows correspond to vanillic acid addition on day 3.

nected to the HP 3365 Chem Station for chromatographic data processing and quantification was performed using external standards. Maltose, glucose and cellobiose were determined and quantified.

Ammonium determination Ammonium was measured quantitatively using the Spectroquant^R 14752 ammonium method (Merck, Darmstadt, Germany). In alkaline medium, ammonium reacted with hypochloride and thymol to form blue indophenol. The reaction was monitored at 690 nm. Ammonium chloride was used as the standard. Nitrogen consumption was calculated from the ammonium data.

Effects of vanillin and vanillic acid concentrations on radial growth of *P. cinnabarinus* Twenty grams per liter sodium vanillate (pH 7.2) and 10 g/*l* vanillin solutions were prepared in water and sterilized by filtration (0.22 μm). Malt agar medium was autoclaved at 120°C for 20 min, cooled and the necessary amount of vanillate or vanillin was added to achieve the following concentrations: 300, 600, 900 and 1200 mg/*l*. The media were then poured into sterile petri dishes (150 mm in diameter). Duplicate petri dishes for each concentration were centrally inoculated with agar disks of 5 mm diameter from cultures of *P. cinnabarinus*. Petri dishes without phenolic compounds served as controls. The inoculated petri dishes were incubated at 30°C and examined regularly. The increase in culture diameters was plotted as a function of the incubation time, and the radial growth rate was obtained from the slope by linear regression of the linear phase of growth (16, 17).

Calculation of the kinetics parameters Specific production rate (q_p) is the concentration of vanillin (g/*l*) produced per g/*l* biomass per day (*d*). It was calculated daily, from day 3, as follows:

for day n:

$$q_p=(V_n-V_{n-1})/(d_n-d_{n-1})\cdot[(X_n+X_{n-1})/2]$$

with V_n and V_{n-1} representing vanillin concentration in g/*l* at day n and n−1, X_n and X_{n-1} representing biomass in g/*l* at day n and n−1 and d_n and d_{n-1} representing day n and day n−1, respectively.

Vanillin production rates (r_p) during the cultivation were calculated as follows:

$$r_p=(P_f-P_i)/\Delta t$$

with P_f and P_i representing g/*l* vanillin at the end and at the beginning of the cultivation, respectively, and Δt for change in time.

Specific growth rates (μ) were calculated during the fast-growth period according to the following equation (18):

$$X=X_0\,e^{\mu t}$$

with X for g/*l* biomass at time t and X_0 for g/*l* biomass at time t_0.

The growth yield ($Y_{X/S}$) in relation to the carbohydrate source consumption was calculated during the cultivation as follows:

$$Y_{X/S}=(X_f-X_i)/S_i-S_f$$

with X_f and X_i for g/*l* biomass, and S_f and S_i for g/*l* carbohydrate at the end and at the beginning of the cultivation, respectively.

RESULTS

Vanillin production as a function of the bioreactor design and the aeration rate As a first step, vanillic acid metabolism by *P. cinnabarinus* was studied in two types of bioreactors (mechanically agitated and air-lift bioreactors) under standard conditions of 30 *l*/h (0.28 vvm) aeration as already used for basidiomycetes (19). In each assay, vanillic acid metabolism was followed by quantification of the phenolic compound produced during the cultivation and the results at the optimum of vanillin production (on day 7) are summarized in Table 1.

Cultivation in the air-lift bioreactor led to the production of 770 mg/*l* vanillin (with a molar yield of 38%) and a large amount of unwanted by-products such as methoxyhydroquinone (252 mg/*l*) and vanillyl alcohol (165 mg/*l*). Increasing the aeration rate did not improve these results (data not shown) and consequently, the mechanically agitated bioreactor was chosen as the optimal bioreactor for enhanced fungal vanillin production from vanillic acid.

As the second step, vanillic acid metabolism by *P. cinnabarinus* was studied under various aeration rates in the mechanically agitated bioreactor (Table 1). A constant standard aeration rate of 30 *l*/h, during the entire period of cultivation, led to the production of 1018 mg/*l* vanillin, with a molar yield of 51%. When the aeration rate was increased to 60 *l*/h (0.56 vvm) and to a maximum of 90 *l*/h (0.83 vvm) during the growth phase, a significant increase in vanillin formation was observed

TABLE 1. Phenolic compound production from vanillic acid by *P. cinnabarinus* grown in a mechanically agitated or air-lift bioreactor under various aeration rates. Values are obtained at the optimum of vanillin production (on day 7)

Culture conditions	Vanillin (mg/*l*)	Methoxyhydroquinone (mg/*l*)	Vanillyl alcohol (mg/*l*)	Molar yield[a]	
				Vanillin (%)	Total recovered metabolites (%)
Air-lift bioreactor					
30/30[b]	770	252	165	38.0	68.6
Mechanically agitated bioreactor					
30/30[b]	1018	34	136	51.0	59.6
60/30[b]	1219	120	358	60.0	83.7
60/40[b]	1143	192	300	58.7	84.7
60/60[b]	1050	250	142	54.0	75.1
90/30[b]	1260	350	88	66.2	90.7
O_2/30[c]	831	72	200	48.6	64.7

[a] Molar yield was calculated by the ratio of moles of vanillin or moles of total recovered metabolites/mol of consumed vanillic acid after 7 d of cultivation.

[b] Aeration rate (*l*/h) during the growth phase followed (/) by the aeration rate (*l*/h) during the production phase.

[c] Pure oxygen (15 *l*/h) during the growth phase followed (/) by the aeration rate (*l*/h) during the production phase.

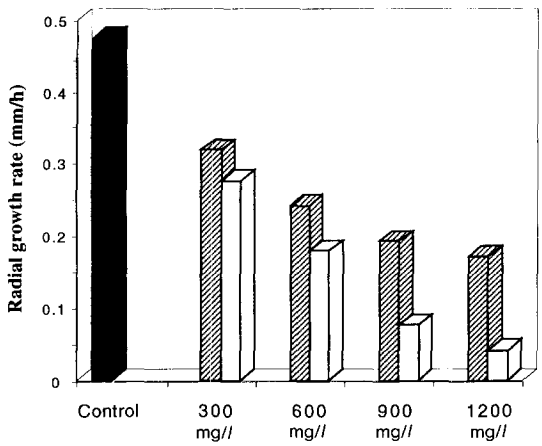

FIG. 3. Effects of vanillic acid (▨) and vanillin (□) concentrations on the radial growth rate of *P. cinnabarinus* after 12 d of incubation (optimum growth under these conditions for the control).

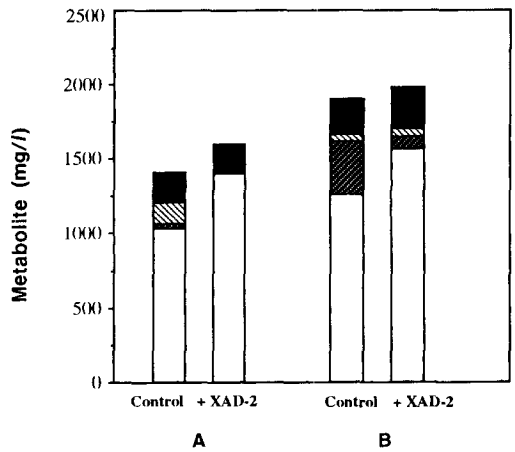

FIG. 4. Phenolic compound production from vanillic acid by *P. cinnabarinus* grown in a mechanically agitated bioreactor with or without the addition of XAD-2 resin under different conditions of aeration. A: Aeration rate of 30 *l*/h during the growth and the production phases; B: aeration rate of 90 *l*/h during the growth phase and that of 30 *l*/h during the production phase. (■) Remaining vanillic acid, (▩) vanillyl alcohol, (▨), methoxyhydroquinone, (□) vanillin. Values are obtained at the time of optimum vanillin production (on day 7).

and nitrogen starvation.

Biomass production, (Fig. 2C), improved under a high dissolved oxygen concentration during the growth phase (*i.e.*, 90 *l*/h), and reached a maximum of 2.5 g/*l*. This biomass remained constant during the rest of cultivation period in the assay with 30 *l*/h aeration, while it fell dramatically from day 4 to the end of the incubation period in the assay with 90 *l*/h aeration.

During the fast-growth period, the specific growth rates reached maximal values of 0.014/h and 0.016/h, respectively, at aeration rates of 30 and 90 *l*/h. Experiments performed without the addition of vanillic acid led to a twofold increase in biomass of *P. cinnabarinus* with a maximum value on day 5 (data not shown).

Variations in dissolved oxygen concentrations, pH and carbon dioxide values were measured during the experiment and data are shown in Figs. 2C and 2D. During the growth phase, the pH increased to a maximum of 6.4 and 6.7 after 1 d and then significantly decreased to values of 4 and 4.3 after 3 d, respectively, for aeration rates of 30 *l*/h and 90 *l*/h. Concomitantly, the dissolved oxygen concentration dropped significantly from 21% (air saturation) to concentration near zero, while the carbon dioxide concentration increased gradually as a consequence of intensive growth of the fungus during this period.

In the both assays, the aeration rate was maintained at 30 *l*/h during the production phase. Under these conditions, the dissolved oxygen concentration remained at a level near zero and the pH increased to a maximum of 5.5. Carbon dioxide production stabilized at 1400 ppm in the culture aerated at 30 *l*/h during the growth phase, while under the aeration rate of 90 *l*/h, it abruptly increased approximately 6 h after the addition of vanillic acid, remained at a high level (about 2400 ppm) until day 6 and then slowly decreased. This intense production of carbon dioxide coincided with a high carbon consumption rate (Fig. 2B). Interestingly, higher aeration rates for *P. cinnabarinus* cultures during the growth phase enhanced vanillic acid biotransformation into other phenolic metabolites, particularly vanillin.

Effects of vanillin and vanillic acid on the radial growth rate of *P. cinnabarinus* The effects of vanillin and vanillic acid concentrations (from 300 to 1200 mg/*l*) on the growth of *P. cinnabarinus* were evaluated using petri dishes containing malt-agar. The radial growth rates were calculated for each condition after 12 d of incubation, which is the optimum time of growth under these conditions for the control cultivated without phenolic compounds. The results are shown in Fig. 3. The radial growth rates of *P. cinnabarinus* decreased five- and tenfold when 900 and 1200 mg/*l* vanillin, respectively, was added to the culture medium. The addition of vanillic acid affected the growth of *P. cinnabarinus* to a lower extent.

Effect of XAD-2 resin addition XAD-2 resin was added directly to the bioreactor containing a 4-day-old culture of *P. cinnabarinus* grown in the presence of vanillic acid and aerated at 30 *l*/h or 90 *l*/h during the growth phase and at 30 *l*/h during the production phase. After 7 d of incubation, the phenolic compounds were eluted and analysed. The concentrations of phenolic compounds in the medium are shown in Fig. 4 as compared to those in the culture without resin addition but under the same aeration conditions. Under each condition of aeration in the presence of XAD-2 resin, vanillin production markedly increased to 1398 and 1575 mg/*l*, corresponding to a 1.4 and 1.3-fold increase, respectively, for aeration rates of 30 *l*/h and 90 *l*/h as compared to that in the corresponding cultures without resin. Under these conditions, molar yields of 69.6 and 82.1% were obtained (with 92.4 and 88.1% vanillic acid consumed), respectively, for aeration rates of 30 and 90 *l*/h and the recovered metabolites represented 69.7 and 90% of the consumed vanillic acid. At the aeration rate of 90 *l*/h, the addition of resin led to a significant fourfold decrease in the amount of methoxyhydroquinone.

DISCUSSION

As previously reported (10), *P. cinnabarinus* is able to produce more than 500 mg/*l* vanillin from vanillic acid, in a medium containing maltose as the carbon source and supplemented with cellobiose on day 3 of cultiva-

tion. This work has opened new prospects to scale-up the production of vanillin on a laboratory scale.

Our results concern the scaling-up of this biotechnological process of vanillin production which involves biotransformation by *P. cinnabarinus* of vanillic acid to vanillin. For the first time, this process has been described in laboratory-scale bioreactors. It has long been known that scale-up involves the control of many parameters such as aeration, agitation and pH (20). In our process, a suitable bioreactor design is required in order to provide sufficient oxygen supply. Our choice was oriented towards two types of bioreactors, mechanically agitated and air-lift bioreactors, with the expectation of a different oxygen transfer as the aeration rates were kept similar. Although mechanically agitated bioreactors are commonly used in industry, alternative configurations such as the air-lift bioreactor have found applications (21). Using a mechanically agitated bioreactor in this process markedly improved vanillin production and lowered that of unwanted by-products, such as vanillyl alcohol and methoxyhydroquinone, as compared to the air-lift bioreactor. Experiments were performed in mechanically agitated bioreactors after this point was determined.

Thereafter, particular attention was given to the aeration rate, related to the dissolved oxygen concentration which has been observed to be a key parameter for maximizing cell density in bioreactors (22, 23). In a recent review (24), oxygen transfer enhancement was shown to improve the productivity of cephalosporin C by *Cephalosporium acremonium*. By satisfying the oxygen demand of the *P. cinnabarinus* cultures, the vanillin yield and specific production rates were expected to be significantly enhanced. When a large amount of oxygen was provided to *P. cinnabarinus* cultures during the growth phase, a high vanillin yield and a maximal specific production rate were determined, but a significant increase in the concentration of by-products, particularly of methoxyhydroquinone, was also detected. However, aerating the cultures with pure oxygen during the growth phase significantly diminished the total amount of recovered metabolites, suggesting an inhibition of vanillic acid metabolism. In addition, a higher rate of aeration during the production phase progressively channeled fungal vanillic acid toward methoxyhydroquinone production. As previously reported (8), vanillic acid can be oxidatively decarboxylated into methoxyhydroquinone. The enzyme involved in the oxidative decarboxylation, vanillate hydroxylase, is now well known, particularly in *P. chrysosporium* (25) and has been reported to be stimulated by high levels of oxygen (26, 27).

The estimation of cell growth via biomass production allowed for the determination of specific growth rates, with a maximum 10-times lower than those generally observed during the fungal growth phase, *i.e.*, *Penicillium chrysogenum* in fed-batch penicillin fermentation (18). In the same manner, use of low concentrations of carbohydrate (maltose) as carbon source is associated with a low growth yield ($Y_{X/S}$) of less than 0.20 dry cell weight/g carbohydrate for any aeration rate during the growth phase. This value was very low as compared to that observed for *P. chrysogenum* in penicillin production (18) but was on the same order as the value obtained for *P. chrysosporium* grown in a bioreactor for manganese peroxidase production (19).

The inhibitory effect of vanillin on fungal growth has been previously demonstrated, particularly on the growth of *Aspergillus* species (16). As observed with *P. cinnabarinus* grown on agar medium, a concentration of over 1000 mg/l vanillin was shown to be highly toxic for the growth of *P. cinnabarinus*. The presence of a toxic level (more than 1200 mg/l) of vanillin during the production phase of the process inhibited biomass production. This antimicrobial activity of vanillin, generally used as a flavouring agent in foods, suggests its potential for use as a preservative (28). In contrast, vanillic acid did not show clear toxic effects on *P. cinnabarinus* when added to agar medium. Vanillic acid is known to be assimilated by many species of micromycetes (29). Under the conditions employed by us, vanillic acid was sequentially added at low concentrations to prevent its eventual inhibitory against *P. cinnabarinus* biomass production.

In this process, when the concentrations of phenolic compounds produced from vanillic acid metabolism (in particular vanillin) started to increase and to accumulate, biomass production decreased dramatically, while a low level of dissolved oxygen and a high level of carbon dioxide, indicating intense fungal metabolic activity, were detected. *P. cinnabarinus* probably directed its metabolism and energy towards vanillic acid degradation, as part of a detoxication system, as already observed in *Sporotrichum pulverulentum* cultures (30). This metabolism could be considered as a secondary metabolism since it is not linked to the cell maintenance (*i.e.*, to the generation of ATP for growth cell).

The application of selective styrene-divinylbenzene resins for adsorption of aroma compounds has recently become well developed (31). The use of XAD-2 polystyrenic resin has already been reported to show a good ability to recover and increase the yields of microbial aromatic compounds generated by biotechnological processes (32, 33). Recently, *in situ* adsorption by XAD-2 resin was found to be a suitable method to recover vanillin before its transformation into vanillyl alcohol by *P. chrysosporium* (15). In our process of vanillin production, the addition of XAD-2 resin to the culture medium of *P. cinnabarinus* allowed the trapping of produced vanillin to bypass vanillic acid transformation into methoxyhydroquinone and prevent vanillin toxicity towards fungal metabolism and consequently, to yield a concentration of 1575 mg/l vanillin.

The successful scale-up of vanillin production from vanillic acid by *P. cinnabarinus* results in a better control of key parameters, particularly of the aeration rate during the growth and production phases and in the use of selective adsorbent materials. As a result of these experiments, vanillin production has been increased threefold as compared to the most recent conditions published (10). Productivity and yields were also significantly enhanced.

The development of biotechnological processes using microorganisms or their enzymes for the production of natural aromatic flavours thus presents a strategic challenge to the flavour industry (4). The elucidation of biosynthetic pathways involved in microbial transformation of biological sources for flavour production could allow the overexpression of these pathways by genetic engineering. At this time, no pathway has been clearly enzymically characterized in fungi. However, information has been reported on the enzymic and genetic character-

ization of the pathway responsible for ferulic acid metabolism by *Pseudomonas fluorescens* (34). The gene coding for a key enzyme involved in vanillin formation from ferulic acid was discovered in *P. fluorescens* and the authors considered the engineering of a pathway into food-grade organisms, such as *Saccharomyces cerevisiae*, for modified *in situ* flavour production in dairy products or the expression of the gene in plants in order to generate vanilla-related flavours of industrial interest. However, genetically modified organisms are very difficult to introduce to the market because of consumer apprehension and the lack of legislation.

In order to increase their importance in the flavour industry, biotechnological processes involving fungi must be optimized to compete economically with processes involving bacteria which are able to produce high levels of aroma in a few hours (Eur. patent, no. 076817A2, 1997) and with traditional processes, leading to the production of natural aromas at least five times cheaper than for the case of flavors extracted from plants.

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REFERENCES

1. **Clark, G. S.:** Vanillin. Perfumer and Flavourist, **15**, 45–54 (1990).
2. **Webster, T. M.:** New perspectives on vanilla. Cereal Foods World, **40**, 198–200 (1995).
3. **Hartley, R. D. and Harris, P. J.:** Phenolic constituents of the cell walls of dicotyledons. Biochem. Syst. Ecol., **9**, 189–203 (1981).
4. **Lomascolo, A., Stentelaire, C., Asther, M., and Lesage-Meessen, L.:** Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the industry. TIBTECH, **17**, 282–289 (1999).
5. **Rombouts, F. M. and Thibault, J. F.:** Enzymic and chemical degradation and the fine structure of pectins from sugar-beet pulp. Carbohydr. Res., **154**, 189–203 (1986).
6. **Micard, V., Renard, C., and Thibault, J. F.:** Studies on enzymic release of ferulic acid from sugar-beet pulp. Lebensm. Wiss. u. Technol., **27**, 59–66 (1994).
7. **Gupta, J. K., Hamp, S. G., Buswell, J. A., and Eriksson, K. E.:** Metabolism of trans-ferulic acid by the white-rot-fungus *Sporotrichum pulverulentum*. Arch. Microbiol., **128**, 349–354 (1981).
8. **Falconnier, B., Lapierre, C., Lesage-Meessen, L., Yonnet, G., Brunerie, P., Colonna Ceccaldi, B., Corrieu, G., and Asther, M.:** Vanillin as a product of ferulic acid biotransformation by the white-rot fungus *Pycnoporus cinnabarinus* I-937: identification of metabolic pathways. J. Biotechnol., **37**, 123–132 (1994).
9. **Lesage-Meessen, L., Delattre, M., Haon, M., Thibault, J. F., Colonna Ceccaldi, B., Brunerie, P., and Asther, M.:** A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. J. Biotechnol., **50**, 107–113 (1996).
10. **Lesage-Meessen, L., Haon, M., Delattre, M., Thibault, J. F., Colonna Ceccaldi, B., and Asther, M.:** An attempt to channel the transformation of vanillic acid into vanillin by controlling methoxyhydroquinone formation in *Pycnoporus cinnabarinus* with cellobiose. Appl. Microbiol. Biotechnol., **47**, 393–397 (1997).
11. **Bonnarme, P., Delattre, M., Drouet, H., Corrieu, G., and Asther, M.:** Toward a control of lignin and manganese hypersecretion by *Phanerochaete chrysosporium* in agitated vessels: evidence of the superiority of pneumatic bioreactors on mechanically agitated bioreactors. Biotechnol. Bioeng., **41**, 440–450 (1993).
12. **Bosco, F., Ruggeri, B., and Sassi, G.:** Experimental identification of a scalable reactor configuration for lignin peroxidase production by *Phanerochaete chrysosporium*. J. Biotechnol., **52**, 21–29 (1996).
13. **Lonergan, G., Schliephake, K., Jones, C. L., and Mainwaring, D. E.:** The growth of the white-rot fungus, *Pycnoporus cinnabarinus*, in a packed-bed bioreactor. Aust. Biotechnol., **3**, 290–294 (1993).
14. **Lonergan, G., Panow, A., Jones, C. L., Schliephake, K., Ni, C. J., and Mainwaring, D. E.:** Physiological and biodegradative behaviour of the white-rot fungus, *Pycnoporus cinnabarinus* in a 200 litre packed-bed bioreactor. Aust. Biotechnol., **5**, 107–111 (1995).
15. **Stentelaire, C., Lesage-Meessen, L., Delattre, M., Haon, M., Sigoillot, J. C., Colonna Ceccaldi, B., and Asther, M.:** By-passing of unwanted vanillyl alcohol formation using selective adsorbents to improve vanillin production with *Phanerochaete chrysosporium*. World J. Microbiol. Biotechnol., **14**, 285–287 (1998).
16. **Lopez-Malo, A., Alzamora, S. M., and Argaiz, A.:** Effect of natural vanillin on germination time and radial growth rate of molds in fruit based agar system. Food Microbiol., **12**, 213–219 (1995).
17. **Lopez-Malo, A., Alzamora, S. M., and Argaiz, A.:** Effect of vanillin concentration, pH and incubation temperature on *Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus* and *Aspergillus parasiticus* growth. Food Microbiol., **14**, 117–124 (1997).
18. **Mou, D. G. and Cooney, C. L.:** Growth monitoring and control through computer-aided on-line mass balancing in a fed-batch penicillin fermentation. Biotechnol. Bioeng., **25**, 225–255 (1983).
19. **Laugero, C., Sigoillot, J-C., Moukha, S., Frasse, P., Bellon-Fontaine, M-N., Bonnarme, P., Mougin, C., and Asther, M.:** Selective hyperproduction of manganese peroxidase by *Phanerochaete chrysosporium* I-1512 immobilized on nylon net in a bubble-column reactor. Appl. Microbiol. Biotechnol., **44**, 717–723 (1996).
20. **Reisman, H. B.:** Problems in scale-up of biotechnology production processes. Crit. Rev. Biotechnol., **13**, 195–253 (1993).
21. **Merchuk, J. C.:** Why use air-lift bioreactors ? TIBTECH, **8**, 66–71 (1990).
22. **Kobayashi, T.:** High cell density cultivation in fed-batch systems, p. 267–280. *In* Aïba, S. (ed.), Horizons of biochemical engineering, University of Oxford Press, London, UK (1988).
23. **Ozturk, S. S.:** Engineering challenges in high density cell culture systems. Cytotechnology, **22**, 3–16 (1996).
24. **Chisti, Y. and Moo-Young, M.:** Bioprocess intensification through bioreactor engineering. Trans. Inst. Chem. Eng., **74**, 575–583 (1996).
25. **Buswell, J. A., Pettersson, B., and Eriksson, K. E.:** Purification and partial characterization of vanillate hydroxylase from *Sporotrichum pulverulentum*. J. Chromatogr., **125**, 99–108 (1981).
26. **Buswell, J. A., Ander, P., Pettersson, B., and Eriksson, K. E.:** Oxidative decarboxylation of vanillic acid by *Sporotrichum pulverulentum*. FEBS Lett., **103**, 98–101 (1979).
27. **Yajima, Y., Enoki, A., Mayfield, M. B., and Gold, M. H.:** Vanillate hydroxylase from the white-rot basidiomycete *Phanerochaete chrysosporium*. Arch. Microbiol., **123**, 319–321 (1979).
28. **Cerrutti, P. and Alzamora, S. M.:** Inhibitory effects of vanillin on some spoilage yeast in laboratory media and fruit purees. Int. J. Food Microbiol., **29**, 379–386 (1996).
29. **Guiraud, P., Steiman, R., Seigle-Murandi, F., and Benoit-Guyod, J.-L.:** Metabolism of vanillic acid by micromycetes.

World J. Microbiol. Biotechnol., **8**, 270–275 (1992).
30. **Ander, P., Hatakka, A., and Eriksson, K. E.:** Vanillic acid metabolism by the white-rot fungus *Sporotrichum pulverulentum*. Arch. Microbiol., **125**, 189–202 (1980).
31. **Krings, U. and Berger, R. G.:** Porous polymers for fixed bed adsorption of aroma compounds in fermentation processes. Biotechnol. Tech., **9**, 19–24 (1995).
32. **Klingenberg, A. and Hanssen, H. P.:** Enhanced production of volatile flavour compounds from yeasts by adsorber techniques. I. Model investigations. Chem. Biochem. Eng. Q., **4**, 222–224 (1988).
33. **Kühne, B. and Sprecher, E.:** Enhancement of the production of fungal volatiles employing different adsorbents. Flav. Fragr. J., **4**, 77–79 (1989).
34. **Narbad, A. and Gason, M. J.:** Metabolism of ferulic acid via vanillin using a novel CoA-dependant pathway in a newly-isolated strain of *Pseudomonas fluorescens*. Microbiol., **144**, 1397–1405 (1998).