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A Biotechnological Process Involving Filamentous Fungi to Produce Natural Crystalline Vanillin from Maize Bran

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Abstract

A new process involving the filamentous fungi *Aspergillus niger* and *Pycnoporus cinnabarinus* has been designed for the release of ferulic acid by enzymic degradation of a cheap and natural agricultural byproduct (autoclaved maize bran) and its biotransformation into vanillic acid and/or vanillin with a limited number of steps. On the one hand, the potentialities of *A. niger* I-1472 to produce high levels of polysaccharide-degrading enzymes including feruloyl esterases and to transform ferulic acid into vanillic acid were successfully combined for the release of free ferulic acid from autoclaved maize bran. Then vanillic acid was recovered and efficiently transformed into vanillin by *P. cinnabarinus* MUCL39533, since 767 mg/L of biotechnologic vanillin could be produced in the presence of cellobiose and XAD-2 resin. On the other hand, 3-d-old high-density cultures of

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P. cinnabarinus MUCL39533 could be fed with the autoclaved fraction of maize bran as a ferulic acid source and *A. niger* I-1472 culture filtrate as an extracellular enzyme source. Under these conditions, *P. cinnabarinus* MUCL39533 was shown to directly biotransform free ferulic acid released from the autoclaved maize bran by *A. niger* I-1472 enzymes into 584 mg/L of vanillin. These processes, involving physical, enzymic, and fungal treatments, permitted us to produce crystallin vanillin from autoclaved maize bran without any purification step.

Index Entries: Aroma; maize bran; enzyme; ferulic acid; vanillic acid; vanillin; *Aspergillus niger*; *Pycnoporus cinnabarinus*.

Introduction

Vanillin is one of the most universally used flavors in food, pharmaceutical, and cosmetic industries. It occurs in the *Vanilla* pods at a level of 20 g/kg dry wt, where it is associated with many other flavoring compounds, and can be estimated in the pure form at US$ 4000/kg. Approximately 12,000 t/yr of vanillin are consumed, essentially in the form of synthetic vanillin at a price of US$ 12/kg *(1)*. However, nowadays, because of increasing consumer demand for natural ingredients, it is more and more essential that the flavor compounds be designed as "natural." According to European and U.S. legislations, this means that the compound has to be obtained by physical, enzymic, or microbiologic processes and only from materials of plant or animal origin (EC Directive 88/388, OJ no. L 184 15/07/88).

Ferulic acid, the most abundant hydroxycinnamic acid in plant cell walls, is widely distributed in higher plants where it is ester-linked to polysaccharide compounds *(2)* and is largely reported to be a suitable precursor for vanillin *(3)*. Bioconversion of ferulic acid by white-rot basidiomycetes, an important group of fungi that generate aromatic flavoring molecules *(4)*, has been investigated. Among basidiomycetes, *Pycnoporus cinnabarinus* has been shown to biotransform ferulic acid into vanillic acid, which is further metabolized into vanillin, vanillyl alcohol, and methoxyhydroquinone *(5)*. A two-step process for the production of vanillin from ferulic acid has been developed *(6)*. In the first step, *Aspergillus niger* transformed ferulic acid into vanillic acid. In the second step, vanillic acid was metabolized into vanillin by *P. cinnabarinus*. The addition of cellobiose and resin XAD-2 in the culture medium of *P. cinnabarinus* was also reported to channel the transformation of vanillic acid into vanillin *(7–9)*. Efficient enzymic removal of ferulic acid from plant cell-wall materials (sugar-beet pulp, wheat bran) has been demonstrated and will allow this acid to be exploited to produce high-added-value aromatic compounds *(10,11)*.

Collaborations among various laboratories (EC contract FAIR CT 96-1099) allowed the establishment of a complete process from European agroindustrial byproducts such as sugar-beet pulp and wheat bran (less than US$ 0.1/kg dry wt) to produce vanillin. This involved altogether the release of free ferulic acid, using a combination of commercial polysaccha-

ride-degrading enzymes and feruloyl esterases originated from fungi, its extraction, purification, and bioconversion into vanillin either by *P. cinnabarinus* alone or by *A. niger* and *P. cinnabarinus* successively *(12,13)*. Under these conditions, this process of biotechnologic vanillin production was priced at very high levels because of the expensive steps of ferulic acid purification and, consequently, was not economically feasible.

Aspergillus niger was recently shown to produce high levels of polysaccharide-degrading enzymes, including feruloyl esterases, when sugar-beet pulp was used as a carbon source *(14)*. Moreover, by combining thermal and enzymic treatments, the release at high extent of free ferulic acid from maize bran was recently reported *(15)*.

In the present study, cultures of *A. niger* I-1472 grown on sugar-beet pulp were tested for their ability to release ferulic acid from maize bran and to transform it into vanillic acid. Thus, a new process for the production of pure vanillin from maize bran has been designed with a limited number of steps involving *A. niger* and *P. cinnabarinus*. Biotechnologic vanillin was characterized by X-ray diffraction.

Materials and Methods

Fungal Strains

Two strains were used: *A. niger* I-1472 (Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris, France); and *P. cinnabarinus* MUCL39533, a monokaryotic laccase-deficient strain obtained from the Mycothèque de l'Université Catholique de Louvain (Louvain-La-Neuve, Belgium). The strains were kept at 4°C on 20 g/L malt-agar slants.

Chemicals

The commercial phenolic compounds used as substrates in the incubation experiments or as controls in high-performance liquid chromatography (HPLC) studies were provided by Prolabo (Paris, France), Fluka (Saint-Quentin Fallavier, France), and Aldrich (Saint-Quentin Fallavier, France). The solvents were of HPLC grade.

Autoclaved Fraction of Maize Bran

The autoclaved fraction of maize bran used was produced by Agro-Industrie Recherche et Développement (Pomacle, France), according to a process adapted from ref. *15*. The process has been applied to maize bran in order to obtain feruloyled oligosaccharides, more accessible substrates for feruloyl esterases.

Maize bran, containing 3.1% ferulic acid (w/w) *(16)*, was diluted with water to obtain 10% dry matter (w/v), heat treated by autoclaving for 3 h at 140°C, and pressed. The aqueous extract was recovered, filtered, and concentrated by spray-drying to reach 24 g of total ferulic acid as linked form/kg of dry matter. The yield of linked ferulic acid production reached 30.1%.

Table 1
Specific Activities of Polysaccharide-Degrading Enzymes and Feruloyl Esterases Synthesized by *A. niger* I-1472 Grown on Sugar-Beet Pulp *(14)*

Enzyme activities	nkat/mg proteins
Arabinanase	142.6
Xylanase	163.3
Galactanase	50.4
Rhamnogalacturonase	23.3
Carboxymethylcellulase	54.2
Galacturonase	178.7
p-Nitrophenyl rhamnosidase	16.2
p-Nitrophenyl galactosidase	53.1
p-Nitrophenyl arabinosidase	211.3
Feruloyl esterase activity[a]	
Using FA as substrate	24
Using FAX as substrate	11
Using FA_2 as substrate	0

[a]FA, (5-*O*-*trans*-feruloyl)-L-arabinofuranose, and FAX, [5-*O*-(*trans*-feruloyl)-α-L-arabino-furanosyl]-1,2-β-D-xylopyranose, were isolated from maize bran *(17)*. FA_2, [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-1,5-L-arabinofuranose, was isolated from sugar-beet pulp *(18)*.

For use as a ferulic acid source, the autoclaved maize bran was solubilized at a concentration of 7.5 g/L of total ferulic acid and sterilized at 110°C for 30 min.

A. niger *Culture Medium*

Previous studies found that a large spectrum of polysaccharide-degrading enzymes including feruloyl esterases could be produced when *A. niger* I-1472 was grown on sugar-beet pulp (Table 1). On the basis of these studies, *A. niger* I-1472 was grown in a basal medium containing maltose (2.5 g/L) and sugar-beet pulp (15 g of dry matter/L) as carbon sources, diammonium tartrate (1.842 g/L) as 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). Under these conditions, 0.5 g/L of Tween-80 was added to this medium to favor contact between fungus and sugar-beet pulp. The cultures were inoculated with 2×10^5 *A. niger* I-1472 conidiospores/mL *(6)*. Incubation was carried out at 30°C in 500-mL baffled flasks containing 100-mL of medium and shaken at 105 rpm.

In one case, the culture broth of *A. niger* I-1472 rich in cell wall–degrading enzymes was recovered on d 3 before the addition of ferulic acid, filtered on glass filters (GF/D, then GF/F; Whatman, Maisdone, UK), and kept at 4°C in order to be used as enzyme source in the culture medium of *P. cinnabarinus* MUCL39533.

In another case, the autoclaved maize bran fraction, equivalent to 300 mg/L of total ferulic acid, was added to *A. niger* culture medium as ferulic acid source on d 3 of incubation. Then, the culture medium was regularly supplemented with 300 or 600 mg/L of total ferulic acid according to consumption. Two or three additions per day of total ferulic acid could be performed. The cultures of *A. niger* I-1472 grown with the autoclaved fraction of maize bran as ferulic acid source were stopped when a significant amount of vanillic acid (>1.5 g/L) was produced and a minimum quantity of ferulic acid (<200 mg/L) remained in the medium. The culture broth of *A. niger* I-1472 was harvested, filtered on Miracloth paper (Carbochem®; France Biochem, Meudon, France), concentrated under low pressure at 50°C (Rotavapor) up to 23 g/L of vanillic acid (with residual 2.3 g/L of ferulic acid), and kept, after filtration, at 4°C no more than 1 wk.

P. cinnabarinus *Culture Medium*

The cultures of *P. cinnabarinus* MUCL39533 were grown in the same basal medium as *A. niger* except that 20 g/L of maltose was used as the carbon source *(19)*. When autoclaved maize bran was added as the ferulic acid source in *P. cinnabarinus* culture broth, high-density cultures were induced as previously described *(20)*: the yeast concentration was increased 10-fold and 20 g/L of maltose was replaced by 30 g/L of glucose and 5 g/L of Nat 89 (commercial phospholipids from Natterman Phospholipids, Germany). All the cultures were inoculated with mycelial fragments representing an inoculum of about 0.5 g/L mycelial dry wt and incubated at 30°C under shaking at 120 rpm. Cellobiose (5 g/L) was added to the culture medium on d 3 *(7)*.

In one case, the cultures of *P. cinnabarinus* MUCL39533 were fed with 300 mg/(L·d) of vanillic acid, from the culture filtrate of *A. niger* I-1472, from d 3 until the end of incubation. In another case, 100-mL cultures of *P. cinnabarinus* MUCL39533 (which corresponded to 6.5 g/L dry wt) were mixed, on d 3 of incubation, with 50 mL of the *A. niger* I-1472 culture filtrate rich in cell wall–degrading enzymes (corresponding to 1.1, 14, and 16 nkat/mL of feruloyl esterase, galacturonase, and arabinofuranosidase, respectively). Then, *P. cinnabarinus* cultures were fed with the autoclaved fraction of maize bran as the ferulic acid source (500 mg/[L·d] of total ferulic acid), from d 3 until the end of incubation. Each experiment was run at least in duplicate.

Adsorbent Material

Amberlite resin XAD-2, a polystyrenic resin purchased from Rohm and Haas (France), was added to 3-d-old cultures of *P. cinnabarinus* MUCL39533 as previously described *(9)*. At the end of cultivation, the culture medium and resin were separated by filtration. The compounds adsorbed on the resin were extracted twice with pure ethanol and analyzed by HPLC as

described next, as were the remaining compounds present in the culture medium.

Phenolic Metabolite Analysis

Samples were filtered through 0.2-µm syringe filters (Microgon, DynaGard, Laguna Hills, CA) and analyzed by HPLC *(6)*. The quantification was performed using external standards.

Crystallization and Optical Microscopy

An ethanolic solution of vanillin recovered from XAD-2 resin was purified by solvent extraction, vacuum distillation, and crystallization several times. A few crystals of biotechnologic vanillin were observed between a slide and a cover glass in polarized light with a crossed analyzer.

X-Ray Diffraction

Samples of commercial and biotechnologic vanillin (about 4 mg) were placed in glass capillaries (2-mm diameter) for measurement. Diffraction diagrams were recorded using a transmission technique with an XRG 3000 X-ray generator (Inel Orléans, France) operating at 40 kV and 30 mA. CuK_{a1} radiation (λ = 0.15405 nm) was selected using a quartz monochromator *(21)*. A curved-position sensitive detector (Inel CPS120) was used to monitor the diffracted intensities using 3-h exposure periods. The recorded diagrams were normalized at the same total scattering between 3 and 35° (2θ).

Results

Use of A. niger *I-1472 to Release Ferulic Acid from Maize Bran and to Biotransform It into Vanillic Acid*

A. niger I-1472 was grown on sugar-beet pulp for 3 d, and then the autoclaved fraction of maize bran was regularly added as the ferulic acid source. As shown in Fig. 1, the free ferulic acid released was immediately biotransformed into vanillic acid. Between d 3 and 6 of incubation, about 1 g/L of free ferulic acid/d was consumed by *A. niger* I-1472. After 7 d of incubation, vanillic acid production reached a maximum of 1770 mg/L with a molar yield of 60.5% and a productivity of 253 mg/(L·d). From the autoclaved fraction of maize bran corresponding to 3800 mg/L of added ferulic acid, the whole ferulic acid was released and 89% of it was consumed.

Vanillin Production by P. cinnabarinus *MUCL39533 from Vanillic Acid–Enriched Culture Filtrate of* A. niger *I-1472*

P. cinnabarinus MUCL39533 was grown in the presence of culture filtrate of *A. niger* I-1472 enriched in vanillic acid. A significant production of vanillin was observed with a maximum of 503 mg/L on d 7, a molar yield of 48.5%, and a productivity of 72 mg/(L·d) (Fig. 2). Ferulic acid present in the vanillic acid solution from *A. niger* I-1472 was slightly consumed during

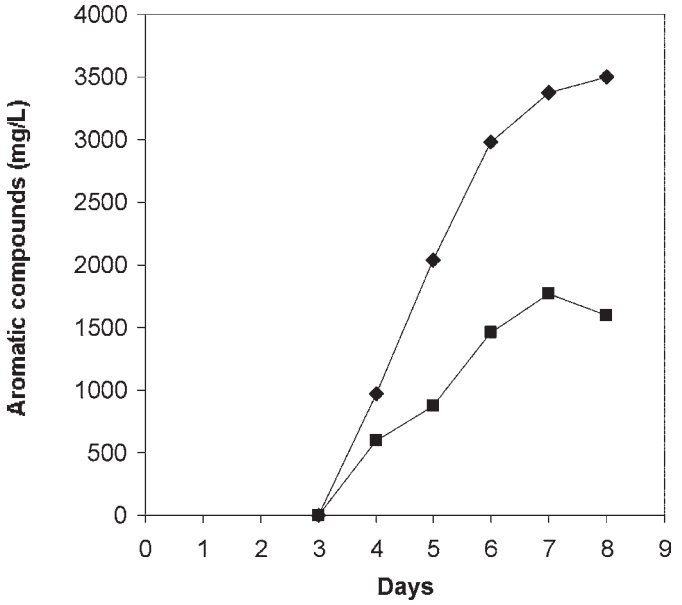

Fig. 1. Free ferulic acid consumption and vanillic acid production in cultures of *A. niger* I-1472 grown with sugar-beet pulp as carbon source and autoclaved fraction of maize bran as ferulic acid source. (◆) Consumed free ferulic acid; (■) produced vanillic acid.

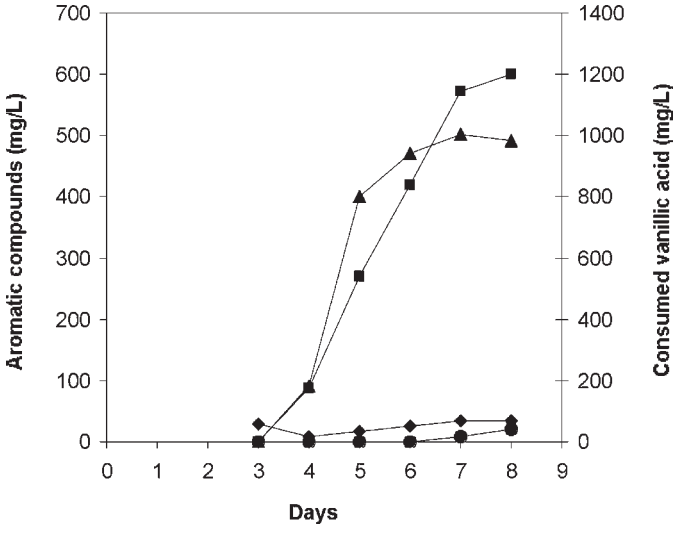

Fig. 2. Vanillin production by *P. cinnabarinus* MUCL39533 from concentrated culture filtrate of *A. niger* I-1472 rich in vanillic acid. (■) Consumed vanillic acid; (◆) residual free ferulic acid; (▲) vanillin; (●) methoxyhydroquinone.

the cultivation. Only very low amounts of methoxyhydroquinone (20 mg/L) were detected. Using XAD-2 resin in the cultures of *P. cinnabarinus* MUCL39533 led to an increase in vanillin concentration up to 767 mg/L

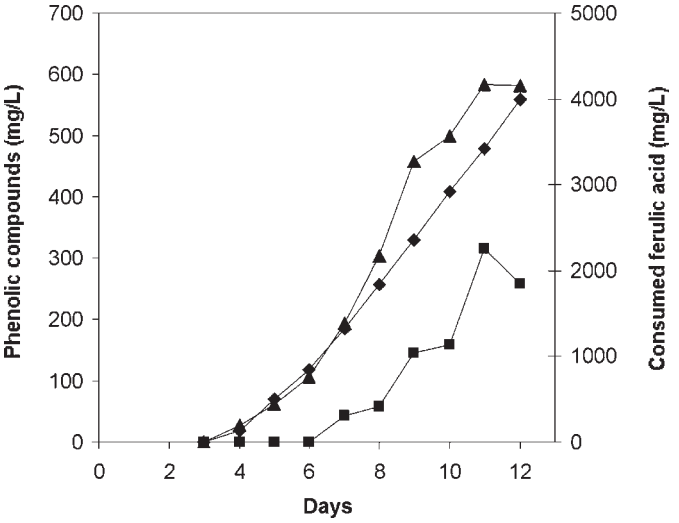

Fig. 3. Vanillin production by *P. cinnabarinus* MUCL39533 grown in presence of autoclaved fraction of maize bran as ferulic acid source and culture filtrate of *A. niger* I-1472 rich in polysaccharide-degrading enzymes and feruloyl esterases. (◆) Consumed free ferulic acid; (▲) vanillin; (■) vanillic acid.

(on d 7) with a molar yield of 71%. The other phenolic compounds present in the cultures were not significantly affected by the addition of resin (data not shown).

Direct Transformation of Autoclaved Fraction of Maize Bran into Vanillin by P. cinnabarinus *MUCL39533 Cultures Owing to Extracellular Enzymes of* A. niger *I-1472*

P. cinnabarinus MUCL39533 was grown on phospholipid medium for 3 d to induce high-density cultures *(20)*. Then the autoclaved fraction of maize bran was added to the culture medium as the ferulic acid source in the presence of the culture filtrate of *A. niger* I-1472 as the source of polysaccharide-degrading enzymes and feruloyl esterases. Under these conditions, ferulic acid originated from the autoclaved fraction of maize bran was totally released by the enzymes of *A. niger* I-1472 to be biotransformed into vanillin by *P. cinnabarinus* MUCL39533 (Fig. 3). A maximum of 584 mg/L of biotechnologic vanillin was produced on d 11 with a productivity of 53 mg/(L·d) and a molar yield of 22%. Total added ferulic acid was consumed. Vanillic acid accumulated up to 300 mg/L in the culture medium. Applying XAD-2 resin to the culture medium did not improve vanillin production (data not shown).

Vanillin Structure and X-Ray Analysis

The crystalline structure of biotechnologic vanillin is shown in Fig. 4. It consists of an array of parallel long needles. The X-ray diffraction dia-

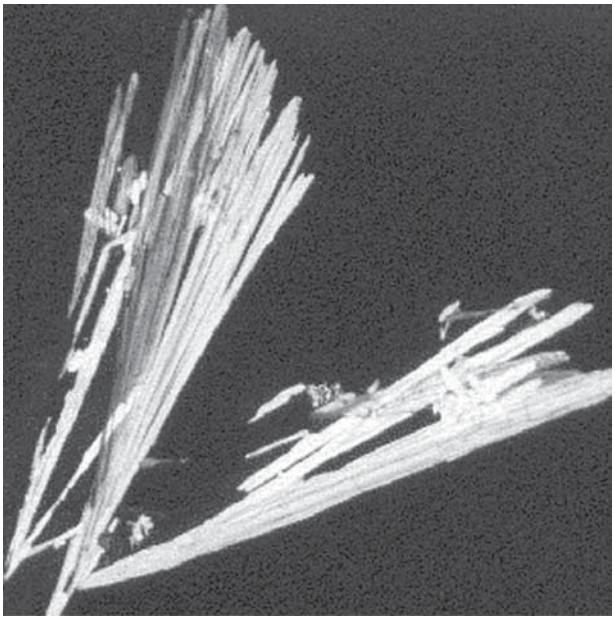

Fig. 4. Crystals of biotechnologic vanillin in polarized light (×200).

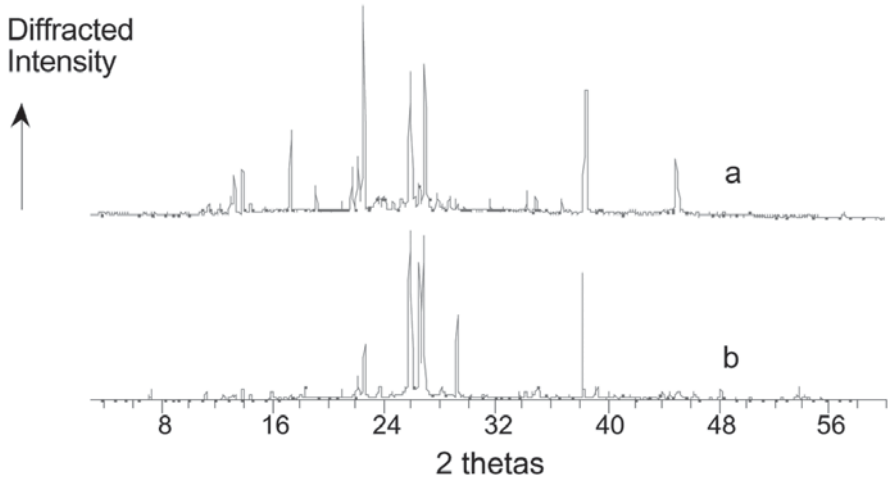

Fig. 5. Powder X-ray diffraction diagrams of biotechnologic (a) and commercial (b) vanillins.

grams of commercial and biotechnologic vanillins were examined (Fig. 5). The main peaks were present on both diagrams, showing that both vanillins have the same crystalline structure. The discrepancies in intensity were owing to the morphology of crystals, inducing preferred orientations, and, therefore, different relative intensities.

Discussion

Natural flavors are increasingly required by the food industry. In our study, a new process, involving the filamentous fungi *A. niger* and *P. cinnabarinus*, was designed for the release of ferulic acid by enzymic degradation of autoclaved maize bran, and its biotransformation into vanillic acid and/or vanillin with a limited number of steps (Fig. 6).

Aspergillus species have been largely investigated for the production of various polysaccharide-degrading enzymes such as arabinanases *(22)*, xylanases *(23)*, and feruloyl esterases, the latter being specific for the release of ferulic acid *(24)*. The efficient degradation of plant cell-wall polysaccharides by *Aspergillus* depends on a complex system of synergistic interactions between enzymes cleaving the different bonds in these structures *(25)*. In maize bran, ferulic acid is ester linked to heteroxylans *(16)*. Thus, to release ferulic acid from such polymers, it is necessary to break down the heteroxylan core using cell wall–degrading enzymes (i.e., xylanases and osidases) to provide feruloyled oligosaccharides that are substrates for feruloyl esterases *(26)*. Generally, the release of ferulic acid from plant cell-wall polysaccharides requires a combination of commercial enzyme preparations, such as pectinases or xylanases, often very poor in esterase activities, with purified feruloyl esterases *(27,28)*. As previously related *(14)*, a large spectrum of cell wall–degrading enzymes could be induced in the cultures of *A. niger* I-1472 by using sugar-beet pulp as the carbon source. In our study, the culture filtrate of *A. niger* I-1472 was thus clearly demonstrated as a very efficient substitute for commercial enzymic mixtures, to release free ferulic acid from plant cell polysaccharides, further available as vanillin precursor without any expensive purification step. The autoclaved maize bran, an industrial material (US$ 30–40/kg dry wt), composed of feruloyled oligosaccharides *(15)*, was used as the ferulic acid source. Under these conditions, the plant cell wall–degrading enzymes of *A. niger* I-1472 could totally release as a free form the ferulic acid present in autoclaved maize bran.

A. niger I-1472 is known for use in the bioconversion of ferulic acid into vanillic acid *(6)* and *P. cinnabarinus* MUCL39533 for the bioconversion of vanillic acid *(19)* and ferulic acid *(20)* into vanillin. The high biotechnological potential of these fungi was used to generate a process of vanillin production from the autoclaved fraction of maize bran. On the one hand, the potentiality of *A. niger* I-1472 to free ferulic acid from autoclaved maize bran was combined with its ability to transform it into vanillic acid. Then, *P. cinnabarinus* cultures could be fed with natural vanillic acid to produce vanillin. On the other hand, the cultures of *P. cinnabarinus* were directly fed with the enzymes of *A. niger* and the autoclaved fraction of maize bran as the ferulic acid source to generate vanillin.

Even if very high-yield microbial processes of vanillin production from ferulic acid (>10 g/L) exist in the aroma industries such as Haarman and Reimer (European patent 0761 817 A2) or Givaudan-Roure

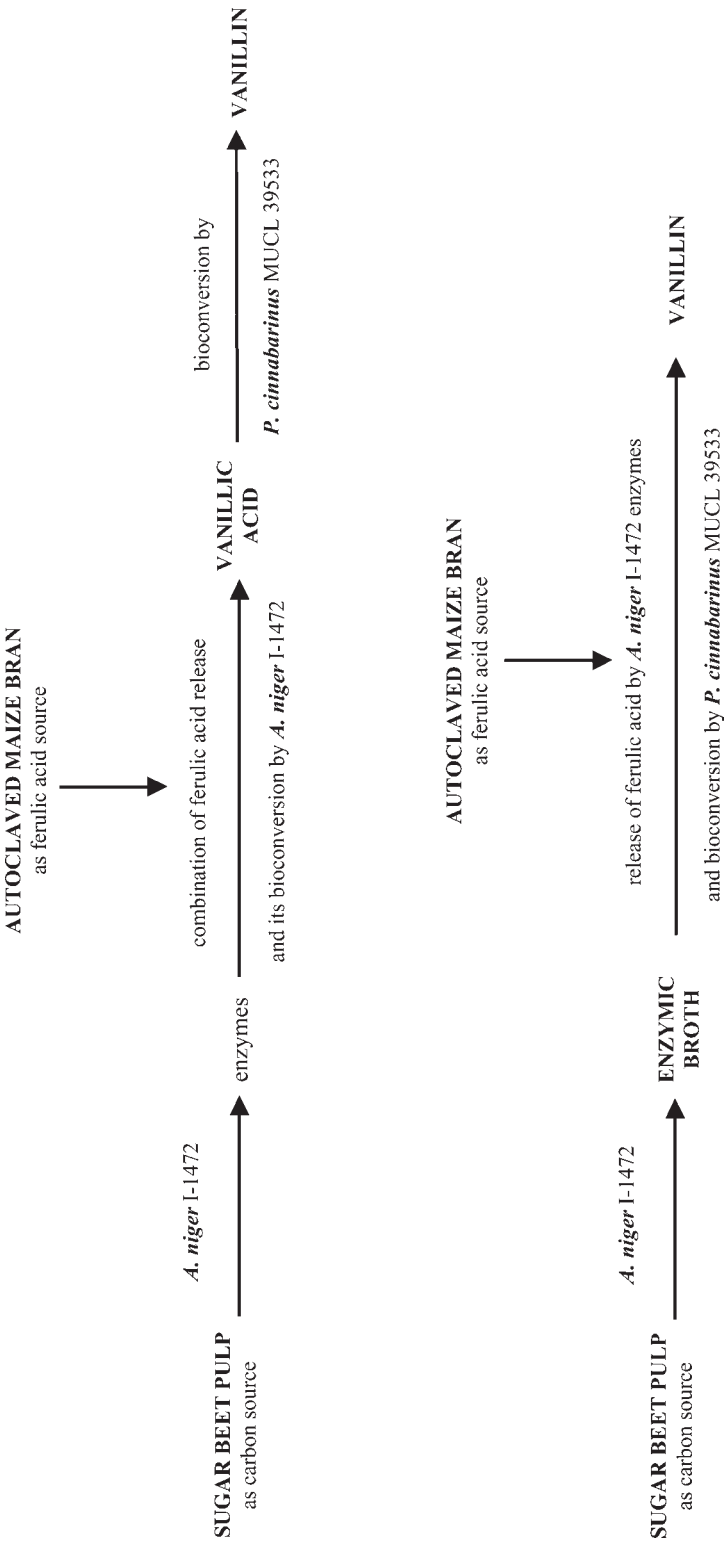

Fig. 6. Different biotechnological processes involving *A. niger* and *P. cinnabarinus* for production of pure vanillin from autoclaved maize bran with limited number of steps.

(European patent 0 885 968 A1), it is also necessary to ensure safety. Nowadays, genetically modified organisms are very difficult to introduce into the market because of consumer apprehension and the lack of legislation. Thus, the production of natural vanillin through biotechnological processes needs clearance, especially by using safe precursors, food-grade organisms, defined media for culture of cells, and downstream processing with allowed solvents *(3)*.

It is obvious that, in order to increase their importance in the flavor industry, biotechnological processes involving fungi must compete economically with processes involving bacteria and with traditional processes, to lead to natural aromas at least fivefold cheaper than those extracted from plants. Since biotechnologic vanillin is regarded as a natural source of vanilla flavor, it can still be sold at US$ 2000/kg *(3)*. By involving soft physical and enzymic treatments, and by using nongenetically modified filamentous fungi, the process that we developed permitted production of natural crystallin vanillin (estimated at about US$ 600–1000/kg) from renewable and cheap agroindustrial byproducts, rich in ferulic acid, such as autoclaved maize bran, in a limited number of steps.

Acknowledgments

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