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Microbiologic Oxidation of Isosafrole into Piperonal

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Abstract

The biotransformation of isosafrole by *Cladosporium sphaerospermum* yielded piperonal, which is a compound of great commercial importance in the flavor and fragrance industries. The experiments were performed in 500-mL conical flasks containing 100 mL of Czapek-modified medium in an orbital shaker with controlled agitation and temperature. Spores of *C. sphaerospermum* were used as inocula, and after 96 h of incubation the substrate was added to the culture. Samples of 2 mL were withdrawn at 24-h intervals and analyzed by gas chromatography, (GC) and/or GC/MS spectroscopy.

Index Entries: Biotransformation; *Cladosporium sphaerospermum*; phenylpropanoids; isosafrole; piperonal.

Introduction

Oxidation is one of the most studied processes in the biologic transformation of organic compounds besides reduction *(1)*. The simplest and most suitable microbiologic process is the direct incorporation of oxygen to an exogenous (or xenobiotic) substrate from molecular oxygen, because dioxygen is the cheapest oxidant in the chemical industry *(2)*. Therefore,

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alcohols can be produced from hydrocarbons, phenol derivatives from benzene, and allylic alcohols and/or epoxides from alkenes. Of course, thermodynamically, these reactions are quite different owing to the different redox potentials involved. Kinetically, considering the same feedstock (e.g., limonene) different catalytic pathways might take place. Therefore, multiple product formation is possible. Although many examples of these biologic oxidations have been described so far *(3)*, not any enzymes responsible for these transformations have been identified, and few have been fully characterized. However, specific investigations have indicated that at least three types of enzymes can be involved in microbiologic oxidation: heme-dependent monooxygenases, W-hydroxylases, and methane monooxygenases *(4)*. Biologic incorporation of one oxygen atom is catalyzed by a group of enzymes—cytochrome P450–dependent monooxygenases. This group of enzymes drives the reactions under mild conditions, and it is one of the most important classes of enzymes for the biotransformation (via oxidation) of xenobiotics. These enzymes, cytochrome P450–dependent monooxygenases, are easily detected in all types of living cells. Many studies have been carried out in mammalian enzymes owing to their involvement in the metabolism of pharmaceuticals, mainly in the liver cells. The greatest feature of these enzymes is the ability to oxygenate lipophilic xenobiotics leading to water-soluble metabolites to facilitate their excretions. Based on this phenomenon, many groups have been working on the oxidation-based biotransformation of lipophilic compounds (xenobiotics) by different microorganisms, living cells, or crude enzymatic preparations.

In addition, it has been vastly demonstrated that this enzymatic or biomimetic system is able to conduct this oxidation enantioselectively *(5–7)*. As extensively emphasized in the literature *(8)*, the screening of microorganisms is a very important tool to find the best microorganism capable of effecting a desired chemical transformation. Furthermore, the best reaction conditions as well as the optimum process operation must be investigated if commercial interest is to be pursued. The empirical basis of such a piece of work is based on the fact that most, if not all, microorganisms have the necessary machinery to effect a given chemical transformation. Of course, whenever working in such a way the substrate is commonly xenobiotic to the microorganism and, consequently, potentially toxic to it. Therefore, very careful choice of the reaction's conditions is needed.

Although oxidations have been known for more than a century, the first systematically studied reaction effected by microorganisms was the (enantioselective) reduction of activated ketones, which has been extensively studied *(9,10)*. When bearing the potential difficulties in mind, the screening of microbiologic transformations of chemical compounds (xenobiotic substrates) certainly seems to be the method of choice to assay a desired biochemical transformation of an organic compound *(4)*. Whole or fractions of microorganisms, animal and plant cells, or crude enzymatic preparations have been used (and extensively tested) as selective (or specific) biocatalysts. The methodology is relatively rapid; is versatile; and, in principle, can be

used by any trained chemist (respecting the biosecurity-associated protocols). Several assays of microbiologic transformations have been described in the literature. The different classes of xenobiotic compounds and the different microorganisms are incredibly great and growing rapidly for biotransformation purposes *(1,11–15)*. The main appeal of using microrganisms instead of the traditional chemical transformations is associated with various factors; for example, the solvent is usually water (not very cheap but always friendly), and reaction conditions are generally mild and can be engineered to recycle the biotransformation agent (e.g., immobilized). Consequently, the overall process is normally an energy-saving one, compatible with the new (and justified) claims of society. Based on these facts, the oxidation of isosafrole (1,2-methylenedioxy-4-[1'-propenyl]-benzene) to the corresponding aldehyde, piperonal (4-carboxyaldehyde-1,2-methylenedioxybenzene) was envisaged, although careful perusal of the literature indicated that compounds bearing the methylenedio-xybenzene would behave as cytochrome P450 inhibitors *(16,17)*. On the other hand, the process itself is very attractive from an industrial point of view because it provides an alternative to the commonly used highly energy demanding process—ozonolysis. Additionally, once the product is mainly used both as a fixateur in the fragrance industry and as a flavor additive, good manufacturing practices and processes will be highly desirable. The use of a clean, mild, and energy-saving process to obtain a product of considerable economic importance was therefore the objective of the present work.

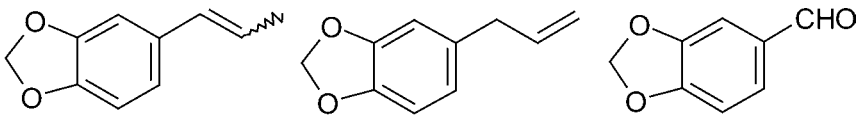
Isosafrole belongs to an important class of natural products, the arylpropenoids. However, reports concerning the microbiologic oxidation of these compounds are not common *(4,18)*. For instance, microbiologic oxidation of safrole (1,2-methylenedioxy-4-[2´-propenyl]-benzene) and the corresponding isomer, isosafrole (1,2-methylenedioxy-4-[1´-propenyl]-benzene), has not been the subject of many biotransformation studies. Evidence pointing to the high toxicity of these types of compounds toward microorganisms and their known properties of acting as cytochrome P450–dependent monooxygenase inhibitors *(19)* are probably the main reasons for the scarce interest in the microbiologic oxidation of arylpropenoids with the methylenedioxyl group. However, metalloporphyrin biomimetic oxidation of arylpropenoids has been reported *(17)*.

The present article reports a biotransformation process of a natural product compound originated from a plant source, isosafrole, as the xenobiotic substrate to be oxidized by *Cladosporium sphaerospermum*. To our knowledge, ours is the first study in which a compound belonging to the arylpropenoid class containing a methylenedioxy group (Fig. 1) has been successfully submitted to a microbiologic oxidation process.

Materials and Methods

Chemicals

The isomeric mixture of Isosafrole (*E*- and *Z*-, 85:15 by high resolution gas chromatography) was kindly supplied from Geroma do Brasil (Paraná,



E- and *Z*-Isosafrole Safrole Piperonal

Fig. 1. Arylpropenoids containing methylenedioxyl group.

Table 1
Compositions of Different Media for First Adaptation of Lyophilized Microorganisms

Compound	Luria-Bertani broth (g/L)	Czapek-Dox agar (g/L)	Sabouraud-dextrose agar(g/L)
$NaNO_3$	—	3.00	—
K_2HPO_4	—	1.00	—
MgSO4·7H2O	—	0.50	—
KCl	—	0.50	—
FeSO4·7H2O	0.30	0.01	—
Sucrose	—	30.00	—
Glucose	—	—	40.00
Agar	—	30.00	30.00
Tryptone	10.00	—	—
NaCl	10.00	—	—
Yeast extract	5.00	—	—
Neopeptone	—	—	10.00

Brazil). The solvents used were of analytical grade and were supplied from VETEC (Rio de Janeiro, Brazil). All other chemicals and those used in the broth medium were of analytical grade and purchased from Sigma/Aldrich (St. Louis, MO).

Microorganisms

The strains of lyophilized microorganisms belonging to different genera were obtained from the different institutions: *Aspergillus flavus*, *C. sphaerospermum*, *Aspergillus niger*, *Pseudomonas putida*, and *Pseudomonas aeruginosa* were generously supplied by the Fundação Instituto Oswaldo Cruz (Rio de Janeiro, Brazil). *Bacillus subtilis* and *Saccharomyces cerevisiae* were obtained from the Instituto de Microbiologia of the Universidade do Brasil (Rio de Janeiro, Brazil).

Bacteria were cultivated in Luria-Bertani broth *(20)*, and fungi and yeast in Czapeck-Dox agar and Sabouraud-dextrose agar *(21)*, respectively. Bacteria and yeast were cultivated at 26°C for 48 h and fungi at 26°C for 120 h. We used this procedure in the first inoculum condition (Table 1).

Screening of Biotransformation Assay

Glass tubes (1 × 20 cm) were filled with 5 mL of the different broth media. Each strain was inoculated in an individual tube, by a platinum loop, containing its respective culture medium. Only spores were transferred from fungi. Bacteria and yeast were grown normally. The tubes were properly closed with a wad of hydrophilic cotton and maintained in an orbital shaker (150 rpm, 26°C). Under these conditions bacteria and yeast were grown for 12 h and filamentous fungi for 96 h. After these periods, 0.2 mL of substrate emulsified with a 1% tween-80 suspension was added to each respective tube. These cell cultures were incubated in the same initial cultivation conditions. After 24 h, two tubes of each strain were removed from the culture and submitted to the extraction procedure. All experiments were carried out in parallel with controls, in the same conditions, without the presence of microorganism.

Fungal Growth and Culture Medium

The strain of lyophilized spores of *C. sphaerospermum* 2950 was suspended in 1 mL of 1% NaCl solution. This suspension was used to inoculate Czapeck-modified agar plates. The growth and sporulation of mycelia was maintained at 26°C for 5 d. Cultivation conditions were accomplished in Czapeck-modified broth medium, containing 20 g/L of sucrose, 2.0 g/L of $NaNO_3$, 1.0 g/L of KH_2PO_4, 1.0 g/L of $MgSO_4 \cdot 7H_2O$, 0.010 g/L of $(NH_4)SO_4$ and 0.50 g/L of $FeSO_4 \cdot 7H_2O$. The salts and carbon source were dissolved in distilled water, made up to 100 mL, and transferred to 250 mL Erlenmeyer flasks. The flasks containing the medium were sterilized at 121°C (1 atm) for 15 min.

Several strains of different filamentous fungi microorganisms were inoculated in a slant test tube containing 20 mL of Czapeck-modified agar (30 g/L) by a platinum loop. Only spores were transferred from the agar plate. They were cultivated at 26°C for 5 d. After this period, the spore suspensions were prepared with 10 mL of 1% NaCl solution. One milliliter of the microbial suspension was used to inoculate 100 mL of sterilized medium in a baffled 250-mL Erlenmeyer flask and maintained in a shaker at 150 rpm and 26°C.

Addition of Substrate

After 4 d, 2 mL of substrate emulsified in a 1% Tween-80 and 1 mL of 30% (v/v) H_2O_2 were added to the Erlenmeyer flask, and the pH was adjusted to 3.0 with glacial acetic acid. The flask was returned to the initial incubation conditions. Samples of 2 mL were withdrawn at 24-h intervals from the medium and submitted to an empty test tube for extraction of the product, as described next.

Extraction Procedure

The addition of 2 mL of ethyl acetate to the cell cultivation allowed extraction. In each experiment, the mixture was submitted to agitation in

a vortex shaker (without glass beads) for 20 s. Then it was left to decant for 3 min, and after organic phase separation, a 1.5-mL aliquot was withdrawn, filtered (through a Millipore 0.45-μm membrane), and transferred to a 4-mL glass septum capped vial. The samples were then submitted to gas chromatography (GC) and/or GC/mass spectroscopy (MS) analyses.

Methods of Product Identification

Gas Chromatography

An HP5890 II gas chromatograph equipped with an HP5 WCOT capillary column (25 m × 0.32 mm ID), with a film thickness of 0.52 μm, was used. H_2 was used as carrier gas (1 mL/min, 32 cm/s). The oven programming temperature started from 100 to 250°C at 3°C/min. The injector was held at 150°C and detector at 240°C. Injection was operated in the splitless mode (1-μL injection). In addition to mass spectra, retention times (and literature retention indexes) of authentic standards were used as identification parameters *(22)*.

Gas Chromatography/Mass Spectroscopy

A 5973HP gas chromatograph–mass spectrometer equipped with an HP5 capillary column (as just described) was used. H_2 was used as carrier gas a flow rate of at 1 mL/min (32 cm/s) under the chromatographic conditions just described. Mass spectra were obtained under electron impact at 70 eV (scan mode: 1 scan/min; acquisition *m/z*: 40–400 units). Chromatographic analysis revealed the presence of a peak at 8.85 min, evidencing the presence of 4-carboxaldehyde-1,2-methylenedioxybenzene through comparison with an authentic sample, confirmed by comparison of the respective retention index and mass spectrum.

Results and Discussion

Several strains of microorganisms including filamentous fungi, yeast, and bacteria were submitted to assay for the biotransformation of the isosafrole into piperonal in the presence and absence of H_2O_2. *A. niger* (IOC 4222), *A. flavus* (IOC 3974), *C. sphaerospermum* (IOC 2950), *P. aeruginosa* (INCQS 00311), or *P. putida* (INCQS 00113) were able to oxidize the substrate, whereas *S. cerevisiae* and *B. subtilis* were not. Among these microorganisms, fungi presented the better capacity to oxidize isosafrole. Therefore, one strain of each specie of fungi was selected for further study, and *C. sphaerospermum* showed the highest selectivity and conversion yield. The biotransformation of isosafrole was made possible through the action of *C. sphaerospermum*, which had the ability to oxidize this substrate into piperonal with 100% selectivity and 9.8% conversion, after 7 d of incubation. Figure 2 represents the schematic reaction. Chromatographic analysis revealed the presence of a peak at 8.85 min (Fig. 3), evidencing the presence of 4-carboxaldehyde-1,2-methylenedioxybenzene through comparison with an authentic pattern, in addition to comparison with the result of the analysis and sample control

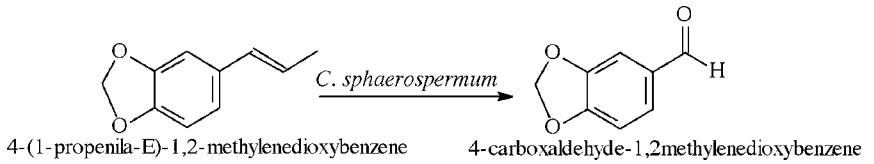


Fig. 2. *C. sphaerospermum* as oxidizer catalyst agent in biotranformation process.

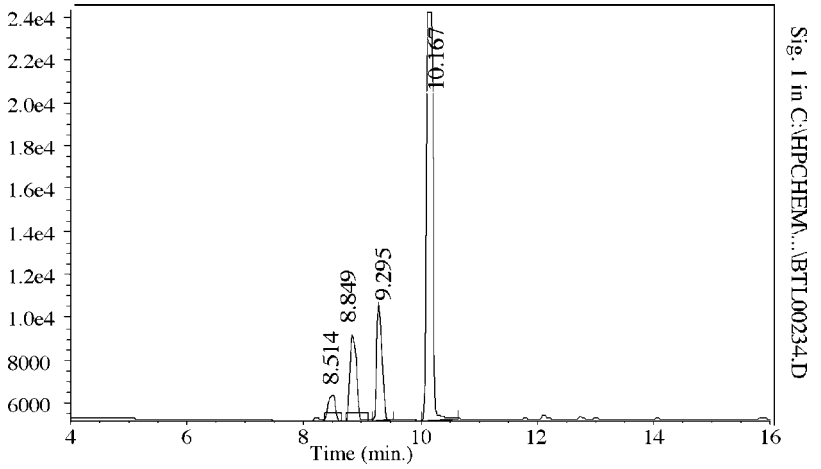


Fig. 3. GC analysis showing the biotransformation product peak at 8.84 min.

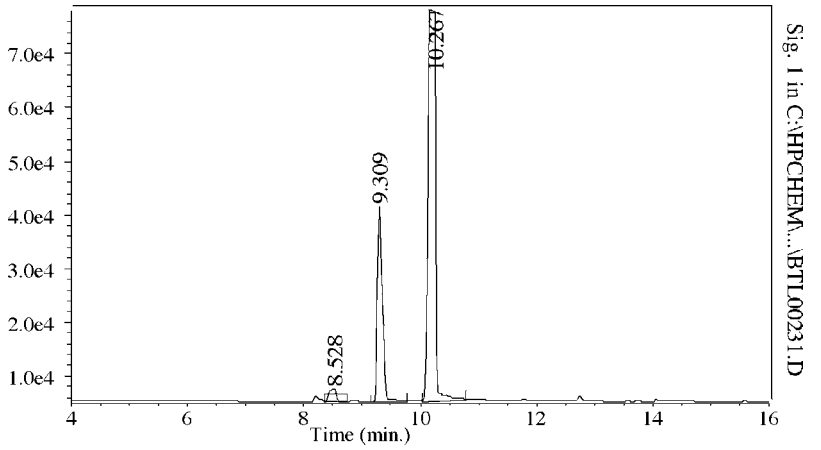


Fig. 4. GC analysis of control sample: 85.34% of 4-(1´-propenyl-*E*)-1,2-methylenedioxybenzene at 10.26 min; 13.32% of 4-(1´-propenyl-*Z*)-1,2-methylenedioxybenzene at 9.30 min; 1.34% of 4-(2´-propenyl)-1,2-methylenedioxybenzene at 8.52 min.

(Fig. 4). Results of the GC/MS analysis confirmed the presence of the product, which was characterized through the mass spectrum, compared with standards in the Wiley/NBS database libraries.

Table 2
Results from Screening of Biotransformation

Microorganism[a]	Strain	Product yield (%)
P. aeruginosa	INCQS 00311	3.01
P. putida	INCQS 00113	1.38
Control C1	—	0.00
A. niger	IOC 4222	3.71
A. flavus	IOC 3974	3.53
C. sphaerospermum	IOC 2950	4.74
Control C2	—	0.65

[a]C1 was the control for bacteria and C2 was the control for fungi.

However, *C. sphaerospermum* was not able to oxidize isosafrole without the addition of H_2O_2. Therefore, the supply of dioxygen by atmospheric air was not enough to conduct the desired biotransformation, which points to the action of a peroxidase instead of a cytochrome P450–dependent monooxygenase. On the other hand, the formation of artifacts was prevented since experiments in the absence of the microorganism did not show any chemical transformation of the substrate. The other microorganisms displayed the potential to oxidize the substrate (Table 2).

The literature provides strong evidence that substances containing the methylenedioxyl group act as cytochrome P450 inhibitors *(19,23)*. Accordingly, the biomimetic oxidation of compounds containing the methylenedioxyl group suggests that these kinds of compounds are potential substrates for lignin peroxidase in appropriate conditions *(15)*. Recent publications summarize the important advances achieved in the last few years on peroxidase-catalyzed transformations with major emphasis on preparative applications. In one of these works, a chloroperoxidase-catalyzed epoxidation of a synthetic methylenedioxybenzene compound is described *(15,24)*. It is worth pointing out that the addition of $H_2O_2$to the reaction medium to promote the desired oxidation with *C. sphaerospermum* has not yet been described in the literature.

Conclusion

Our results show that *C. sphaerospermum*, in the presence of H_2O_2, effected the formation of 4-carboxaldehyde-1,2-methylenedioxybenzene with 100% selectivity. This microorganism is a promising biotransformation agent capable of reaching high conversion yields of isosafrole in the desired product. The need of for H_2O_2 indicates the action of a peroxidase instead of a monooxygenase, wich should be specifically involved since it is normally expressed in conditions of stress.

Acknowledgments

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