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METABOLIC PATHWAYS OF BIOTRANSFORMATION AND BIOSYNTHESIS OF AROMATIC COMPOUNDS FOR THE FLAVOUR INDUSTRY BY THE BASIDIOMYCETE *PYCNOPORUS CINNABARINUS*

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ABSTRACT

Among filamentous fungi, white-rot Basidiomycetes have become a strategic group to generate industrial aromatic flavours. In the course of a basidiomycete screening, the biotechnological potential of *Pycnoporus cinnabarinus* strains was studied in order to produce, by transformation or *de novo*, natural aromatic flavours in liquid cultures. Ferulic acid and L-phenylalanine were found to be suitable substrates for vanillin and benzaldehyde (bitter almond aroma) production, respectively. These strains were also capable of producing *de novo* methylanthranilate, which has been described as the organoleptic note of wood strawberry.

However, strains of *P. cinnabarinus* often expressed laccase activity, which was unfavourable because the pathway to aromatic flavours is bypassed. To overcome this problem, the selection of monokaryotic laccase-defficient strains from basidiospores by classical genetics allowed to obtain more productive and more stable mycelial lines.

Key words: **Basidiomycetes, *Pycnoporus cinnabarinus*, vanillin, benzaldehyde, methylanthranilate.**

INTRODUCTION

An increasing demand of natural aromas by the agri-food industry, as well as by perfumy, cosmetic and pharmaceutical industries, has led the European Union to establish more strict regulations since 1988 for controlling the market. At present, substances from animal and plant cells, or from microorganisms and/or enzymes can only be considered as natural. According to these regulations, biotechnological pathways offer a new promising alternative for the market (Féron *et al.*, 1996). Filamentous fungi (Basidiomycetes) have high potential as a tool for the biotransformation of plant compounds with aromatic structure; however, cellular pathways and metabolic processes involved are yet poorly understood (Tiefel & Berger, 1993).

In this study, a screening of Basidiomycetes was carried out for the production of natural aromas, considering their ability to biotransform or to produce by biosynthesis compounds of aromatic structure. Only strains of *Pycnoporus cinnabarinus* were capable of producing vanillin and benzaldehyde (bitter almond aroma) from ferulic acid and L-phenylalanine, respectively, by biotransformation. At present, these aromas are the most widely used aromatic molecules in the world (Lomascolo *et al.*, 1998). This species can also produce methylanthranilate (wood strawberry aroma) by biosynthesis, another key molecule for the industry (Gross *et al.*, 1990).

MATERIALS AND METHODS

Biological material

Two monokaryotic strains of *Pycnoporus cinnabarinus* (Jacq. : Fr.) P. Karsten were studied: 1) MUCL 39532 used for vanillin production from Belgium, 2) CBS-38466 used for benzaldehyde production from the Netherlands. A dikaryotic strain (I-937) was used for methylanthranilate production from France. Strains were fruited for isolation of monokaryons. Cultures were inoculated in Petri dishes with 2% (w/v) malt agar medium (MA2), kept at 30°C for 15 days in the dark, and exposed to daylight for 2-3 weeks at room temperature. Fruit bodies had an orange-red to cinnabar-red colour. Spores were harvested with sterile water in the upside down covers from incubated Petri dishes. The spore suspension was diluted, and spread on MA2 plates. Monosporous cultures were isolated, and maintained on MA2 medium.

Culture conditions

Strains were grown for vanillin production in a medium containing maltose (20 g/L), ammonium tartrate (1.842 g/L), yeast extract (0.5 g/L), KH_2PO_4 (0.2 g/L), $CaCl_2$ (0.00132 g/L), and $MgSO_4$ (0.5 g/L). Inoculation of Erlenmeyer flasks (100 ml of culture medium) was carried out at 30°C according to Falconnier *et al.* (1994), followed by continuous agitation (120 r.p.m.) and incubation at 30°C. After three days, cellobiose (2.5 g/L) and ferulic acid (0.6 g/L) were added to each flask. A piece of mycelium was deposited in an empty Petri dish, and covered with two drops of a 0.1% syringaldazine ethanolic solution for determination of laccase activity. A change of colour was observed after 15 min; low to high laccase activity ranged from yellow to red, respectively (Harkin *et al.*, 1974). Benzaldehyde production was carried out as previously described, but using glucose (20 g/L) as a carbon source, and thiamine (0.0025 g/L). L-phenylalanine (3 g/L) was added at the beginning of the culture process. Likewise, in the case of methylanthranilate production, maltose was used as a carbon source, whereas spores were used as inoculum (2.10^5 spores/ml culture medium). Experiments were performed in triplicate, two different times (standard deviation < 5%).

HPLC measurements of compounds having aromatic structures

Separation of aromatic compounds was carried out in a C18 column (Merck,

Darmstadt, Germany) at 30°C (lichrospher 100R-P18, 125 x 4 mm), using a HPLC 1050 apparatus (Hewlett-Packard, U.S.A.). Methods used were previously described by Lesage-Meessen *et al.* (1997).

RESULTS AND DISCUSSION

It was found that strains of *Pycnoporus cinnabarinus*, from 200 filamentous fungi (Basidiomycetes) screened, were capable of producing vanillin and benzaldehyde (almond aroma) by biotransformation of ferulic acid and L-phenylalanine. Methylanthranilate (wood strawberry) can also be produced by biosynthesis from this species (**Fig. 1**). However, at industrial level, it is

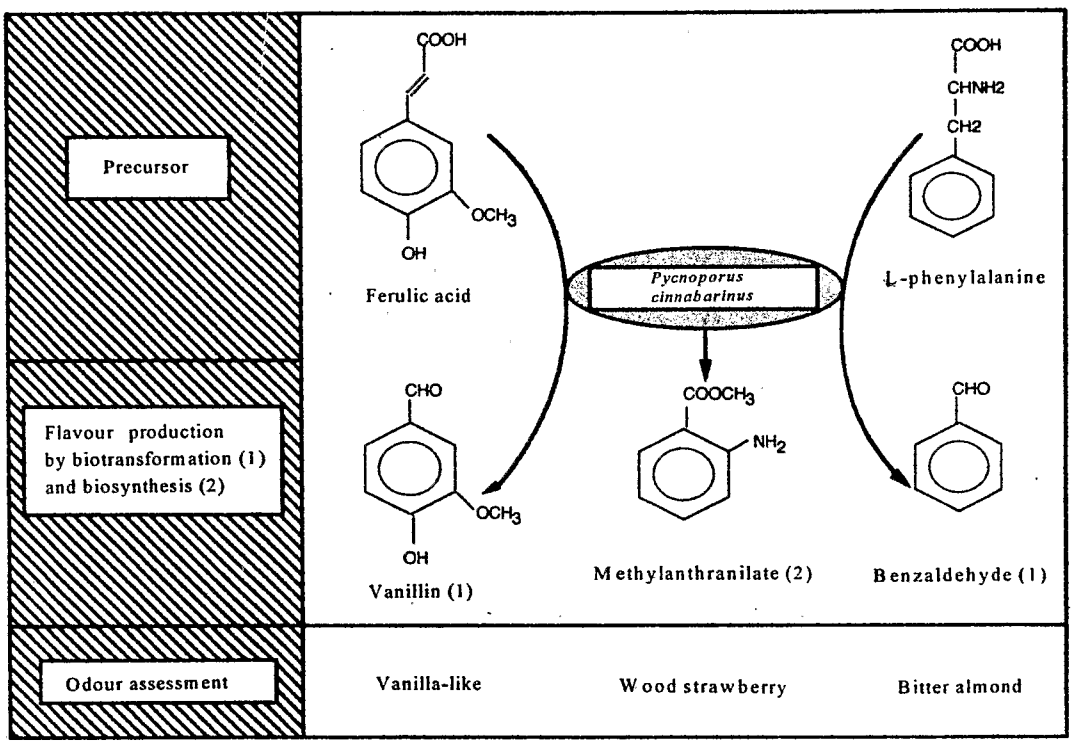

Fig. 1. Metabolic pathways for the production of vanillin and benzaldehyde by biotransformation of ferulic acid and L-phenylalanine, as well as the biosynthesis of methylanthranilate, in *Pycnoporus cinnabarinus*.

often difficult to maintain these metabolic abilities of Basidiomycetes for long periods, as a number of sexual and asexual processes may lead to recombination events which affect the genetic potential of strains.

During these experiments, strains of *P. cinnabarinus* showed extracellular laccase activity, in which transformation pathways of ferulic acid and L-phenylalanine are inactivated (Falconnier *et al.*, 1994). For this reason, stable monokaryotic strains lacking this activity were isolated and selected. Several laccase deficient monokaryons, out of more than 300, were shown (*e.g.* MUCL-39532) to be capable of accumulating vanillin (155 mg/L) when ferulic acid is added after three days of continuous culture (**Fig. 2**). It was observed that the propanoic chain of ferulic acid was prevented, involving

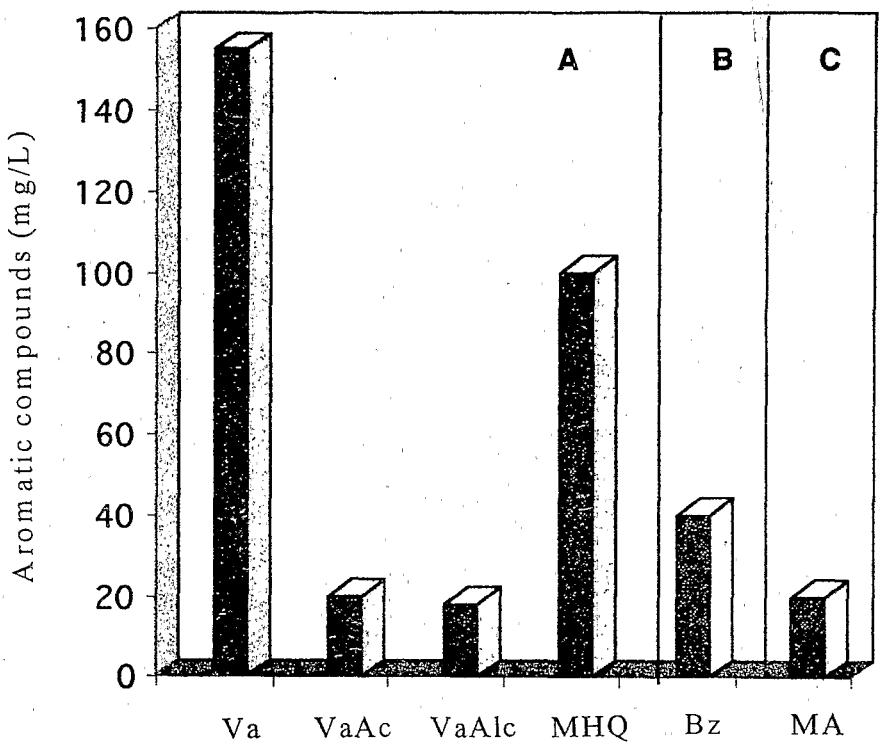

Fig. 2. Aromatic compounds of *Pycnoporus cinnabarinus*. A: Production of vanillin (Va), vanillic acid (VaAc), vanillic alcohol (VaAlc), and methoxy-hydroquinone (MHQ) by strain MUCL-39532. B: Production of benzaldehyde (Bz) by strain CBS-38466. C: Production of methylanthranilate (MA) by strain I-937.

the loss of two carbons and formation of vanillic acid. The reduction of this acid leads to vanillin which may be transformed, after a second reduction, to vanillic alcohol (Falconnier *et al.*, 1994; Lesage-Meessen *et al.*, 1997). Vanillic acid can also be decarboxylated to form methoxy-hydroquinone by the action of intracellular vanillate-decarboxylase (Buswell *et al.*, 1981). This research work has led to an European patent (Lesage-Meessen *et al.*, 1994).

The ability of the monokaryotic strain CBS-38466 to biotransform L-phenylalanine into benzaldehyde was assessed. When glucose is used as carbon source, 3 g/L of L-phenylalanine are transformed into 40 mg/L after eight days of culture (Fig. 2). This is the first observation reported from *P. cinnabarinus*, in which natural benzaldehyde is produced, by biotransformation, from L-phenylalanine. Similar results have been recently obtained in other Basidiomycetes, such as *Bjerkandera adusta*, *Ischoderma benzoinum*, and *Dichomitus squalens* (Lapadatescu *et al.*, 1997).

When maltose is used as carbon source and the nitrogen content is kept at a low level, strain I-937 was capable of producing *de novo* 20 mg/L of methylanthranilate after five days of culture (Fig. 2). Only those strains having orange pigmentation produced methylanthranilate (**Fig. 3**) [Gross *et al.*, 1990]. The synthesis of these pigments was carried out by Sullivan & Henry (1971), showing that cinnabarin appears to be related to the production of methylanthranilate. In general, this research has shown that *P. cinnabarinus* is capable of producing, by biotransformation or by biosynthesis, the following aromas of industrial interest: vanillin, benzaldehyde, and methylanthranilate. Further studies on tropical species of Basidiomycetes, as well as on the cellular metabolic pathways involved, will certainly bring about new prospects for the industrial production of these aromas.

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Fig. 3. Wild fruit bodies of *Pycnoporus cinnabarinus* growing on wood.

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