Coconut-Like aroma production by *Trichoderma harzianum* in solid state fermentation

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SUMMARY

From the present work, it is now established that, by solid state fermentation (SSF), it is possible to produce 6-pentyl-α-pyrone (6-PP), an unsaturated δ-lactone with a strong odour of coconut, by using *Trichoderma harzianum*. 6-PP has been produced on a solid substrate, impregnated with a culture medium of a very high carbon/nitrogen (C/N) ratio, after an initial biomass production stage in a liquid culture medium at a low C/N ratio. 6-PP is the main component. Other volatile compounds accompanied this biosynthesis, and became more diversified with the progress of fermentation. The amount of 6-PP produced during SSF, following a 5 day culture, was 2.8±0.5 mg/g dry matter. Therefore, the 6-PP concentration produced during SSF is greater than that reported in literature during liquid culture. 6-PP does not inhibit growth during SSF. Moreover, with a nitrogen deficiency, the strain sporulated on a solid substrate. Semi-continuous 6-PP production by medium inoculation may, therefore, be possible.

Keywords: Solid state fermentation, sugarcane bagasse, dessiccated coconut, *Trichoderma harzianum*, coconut-like aroma, 6-pentyl-a-pyrone, physiology, spore production, C/N ratio, criterion, fermentation monitoring, microscopic examination, macroscopic examination.

RESUME

Production d'arôme noix de coco par fermentation solide de Trichoderma harzianum

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En fermentation en milieu solide (FMS), il a été possible de produire la 6-pentyl-α-pyrone (6-PP), un composé à odeur dominante noix de coco, par *Trichoderma harzianum* . La 6-PP a été produite sur un support solide imprégné d'un milieu de culture dont le rapport carbone/azote C/N est très élevé après une étape préalable de production de biomasse dans un milieu de culture liquide dont le rapport (C/N) est faible. La 6-PP est majoritairement présente. D'autres composés volatils ont accompagné cette biosynthèse. Ces derniers se sont diversifiés au cours du temps de culture. La quantité de 6-PP produite en FMS au bout de 5 jours de culture a été de 2,8±0,5 mg/g Matière Sèche (MS). La concentration de 6-PP produite en FMS paraît être plus importante qu'en culture liquide (données bibliographiques). Il semblerait qu'il n'y ait pas d'inhibition de la croissance par la 6-PP en FMS. De plus dans des conditions de carence d'azote, la souche a sporulé sur support solide. Une production semi-continue de 6-PP par ensemencement par pied de cuve est donc envisageable.

Mots clés: Fermentation en milieu solide, bagasse de canne à sucre, coco râpé, *Trichoderma harzianum* , arôme noix de coco, 6-pentyl-α-pyrone, physiologie, production de spores, rapport C/N, critère, suivi de fermentation, examen microscopique, examen macroscopique.

INTRODUCTION

Demand for flavour compounds has increased substantially during the recent years. Extraction from natural raw materials and chemical synthesis are the conventional ways of producing flavour compounds, but they have their drawbacks. For example, agricultural production of aromatic plants is seasonal and of limited quantities. The quality of the essential oils is governed by uncontrollable factors, such as climatic and geographical conditions. Chemical synthesis leads to the so-called artificial compounds, which are not often appreciated by consumers (Gross and Asther, 1989). Volatile compounds production by micro-organisms - yeasts, bacteria and filamentous fungi (Janssens *et al*, 1992) - seems to be an interesting alternative. In

fact, the process is easily manageable, from both a qualitative and quantitative point of view. Moreover, European legislation considers biotechnological aroma as natural substances, on the sole condition that the precursors involved in their synthesis being natural (CEE, 1988).

Micro-organisms are capable of producing aroma, notably fruity aroma, such as aldehydes, terpenes, and lactones. For example, γ-decanolactone, with its peach smell, is produced by different bacteria, yeasts and filamentous fungi (Farbood and Willis, 1983).

Amongst lactones, 6-pentyl-α-pyrone (6-PP), an unsaturated δ-lactone, has an interesting aromatic potential. It has a predominantly coconut-type fruity smell, but 6-PP is not naturally found in the coconut meat. It is one of the aroma compounds found in peaches and nectarines (Sevenants and Jennings, 1966). It is produced chemically, after seven reaction steps, under a high working temperature (Pittet and Klaiber, 1975). Hence, its biotechnological production is a good alternative.

It was Rifai (1969), who first described and classified the different *Trichoderma* species, including *T. harzianum*, which produce a characteristic coconut aroma. 6-PP was first detected in *Trichoderma viride*, and its presence was correlated to the coconut smell (Collins and Halim, 1972). Considerable work has been carried out on the optimization of production of this lactone in a liquid medium (Zeppa *et al*, 1990; Sastry *et al*, 1985). However, Serrano-Carreon (1992) was the first to study the metabolic pathways involved in the formation of this unsaturated lactone. Similar to the saturated lactones, 6-PP seems to stem from lipid metabolism by successive β-oxidation of fatty acids. Serrano-Carreon *et al* (1992) showed that limited *T. viride* growth promotes 6-PP production by enabling lipid accumulation in the cell. Serrano-Carreon *et al* (1993) proposed a hypothetical diagram for 6-PP formation: under the effect of a lipoxygenase, linoleic acid seems to be converted into 6-PP through successive steps involving β-oxidation.

Lozano *et al* (1995) described a new aroma preparation procedure, notably for coconut aroma, by solid state fermentation (SSF) of various agricultural substrates. These results were used as guidelines in the present study of 6-PP production in SSF.

The aim of the work was to monitor 6-PP production by *T. harzianum* cultured in SSF system, using 80% sugarcane bagasse as support, 20% dessicated coconut followed by impregnation of the solids with the liquid medium at a C/N ratio of 60. In this first stage of the work, the interest was focused on utilizing dessicated coconut as a substrate to develop the process (Lozano *et al*, 1995). The second stage involved the quantification of the volatile compounds formed and comparison of the results with those reported for liquid culture. In the next stage, the interest

concentrated on the physiology of the fungus growth, particularly the evolution of pH changes, the dry matter and spore counts.

MATERIALS AND METHODS

MICROORGANISMS

Trichoderma harzianum (IMI 206040) was used. The strain was cultured and maintained on potato dextrose agar (PDA), in Petri dishes. The cultures were incubated at 22°C. The 5-day-old mycelium obtained under these conditions was used to inoculate the biomass production medium. The strain was maintained on PDA at 4°C.

BIOMASS PRODUCTION IN LIQUID MEDIUM (STAGE 1)

As *T. harzianum* strain used does not sporulate on solid media, it was impossible to inoculate the solid medium using a spore suspension. A liquid medium was, therefore, used for mycelium production. The liquid medium (50 ml), containing malt extract (20 g/l) and glucose (10 g/l) was poured into 250-ml Erlenmeyer flasks. The medium was autoclaved at 115°C for 20 min, before inoculation with 1 cm^2 of mycelium-impregnated gel took place. It was incubated at 22°C for 48 h on a rotary shaker (100 rpm). The mycelium formed was separated from the medium by decantation and rinsed twice with physiological saline (0.9% NaCl). It was then suspended in 14 ml of the same saline solution, and the suspension (2 ml) was used to inoculate two solid media.

AROMA COMPOUNDS PRODUCTION IN SSF (STAGE 2)

The solid substrate, comprising of 3.6 g bagasse and 0.9 g dessicated coconut, was placed into 250-ml flasks. It was impregnated with 13.5 ml of a medium, which promoted lipid accumulation (high C/N ratio, glucose 30 g/l and $(NH_4)_2SO_4$ 0.94 g/l). The solid culture medium was autoclaved at 115°C for 20 min. Following cooling, the flasks were inoculated with 2 ml of mycelial cell suspension. The flasks were incubated at 22°C.

EXTRACTION OF AROMA COMPOUNDS

Samples (5 g) were removed from solid state *T. harzianum* cultures and placed into 250-ml flasks, with 50 ml distilled water. The aroma compounds were extracted from the samples with 10 ml pentane for one hour by simultaneous distillation-extraction using Likens-Nickerson apparatus, modified by Godefroot *et al* (1981). After extraction, the mixture was evaporated and then dissolved in 1 ml solvent.

ANALYTICAL METHODS

pH measurement

The pH was determined directly on a suspension of 2.5 g fermented substrate in 100 ml distilled water, using a Knic digital pH meter.

Dry matter (DM) measurement

The dry weight was determined by the weight difference. Around 2-3 g fermented substrate was weighed on a Sartorius R160 D precision balance and then dried to constant weight at 105°C.

Macroscopic and microscopic description of Trichoderma

The macroscopic description was carried out using a binocular microscope, and the state of the culture was recorded in terms of substrate colonization (white filaments, bagasse set in a mass) and culture colour. The latter provided an idea of the state of strain sporulation (*Trichoderma* spores are green). The microscopic examination of the mycelium was carried out under a microscope (magnification x 400). The physiological state of the strain (spores, germination, mycelium, conidiophores) was recorded.

Spores counting

The spores were counted in the suspension of 2.5 g fermented substrate in 100 ml distilled water, after blending for 2 min in an Ultraturax at 20,000 rpm. They were either counted in the direct fermented substrate suspension or after dilution, using a Malassez cell. The results are expressed as the number of *T. harzianum* spores produced/dry matter.

Qualitative analysis of aroma compounds

The aroma compounds were identified, using a quadripolar mass spectrometer (Fisons Trio 1000), with ionization by electronic impact (70 eV). The apparatus was connected to a Carlo Erba GC 8000 gas chromatograph, fitted with a DB Wax column (internal diam: 0.32 mm, length: 60 m, film thickness: 0.25 μm). The splitless injector and detector temperatures were 200 and 300°C, respectively. Oven temperature was increased from 100 to 200°C, at a rate of 10°C/min.

Quantitative analysis of aroma compounds

A Hewlett Packard gas chromatograph, fitted with a flame ionization detector, was used. The constituents were separated on a Carbowax capillary column (internal diam: 0.32 mm, length: 30 m, film thickness: 0.25 μm) using a split injection mode. The temperature was increased from 40 to 180°C, at an initial rate of 10°C/min for 14 min, followed by a rate of 3°C/min to 200°C. Quantitative analysis of 6-PP was carried out using the internal calibration method, with γ-undecanolactone (99%, Aldrich) as the internal standard.

RESULTS AND DISCUSSION

EVOLUTION OF AROMA COMPONENTS

The compounds, produced by culturing *T. harzianum* for seven days on a solid medium, were extracted by simultaneous distillation-extraction using the Likens-Nickerson apparatus, modified by Godefroot *et al* (1981). The compounds found in the samples were identified by GC/MS coupling. Fig. 1A is the chromatogram for the aroma extract of non-inoculated dessicated coconut. Figs. 1B, C and D are the chromatograms of *T. harzianum* cultures, fermented for 2, 5 and 7 days, respectively.

Fig. 1A shows that 6-PP is not naturally found in the non-inoculated substrate. It did not appear clearly until the substrate had been fermented by the filamentous fungi for 2 days (Fig. 1B). The next chromatograms (Figs. 1B, C and D) obtained after 2, 5 and 7 days, show the presence of 6-PP, in addition to the molecules, such as alcohols, ketones, fatty acids, etc. These molecules led to additional aroma categories arised during culture.

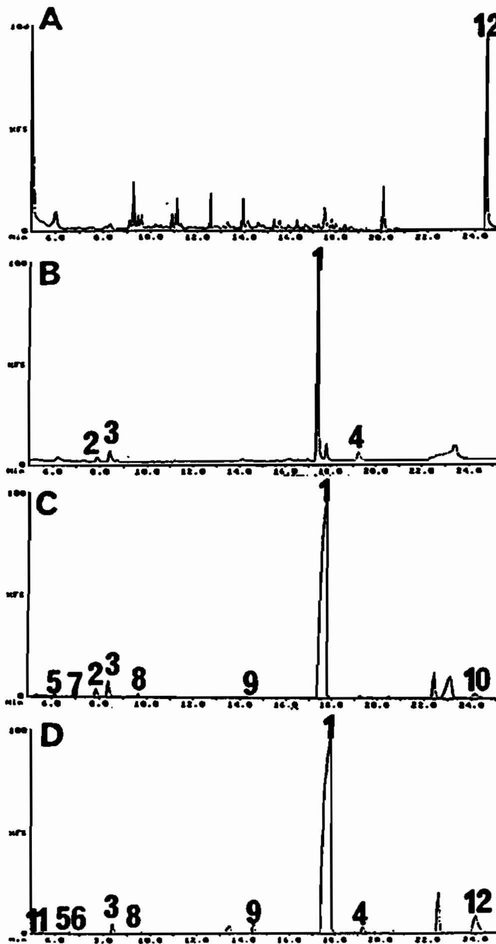

Fig. 1. Chromatografic profile (GC/MS coupling) of aroma extracts of *T. harzianum* cultures grown in SSF on a mixture of bagasse and desiccated coconut for two days (B), five days (C) and seven days (D). The chromatogram for the aroma extract of non-inoculated desiccated coconut is shown in A. (The sensitivity of the photomultiplier is the same for the 4 samples: 500V). List of some compounds identified by CPG/SM: **1.** 6-pentyl-α-pyrone, **2.** 2-decanone, **3.** 2-undecanone, **4.** decanoic acid, **5.** 2-nonanone, **6.** 1-octen-3-ol, **7.** 2-nonanol, **8.** 2-undecanol, **9.** octanoic acid, **10.** tetradecanoic acid, **11.** 2-heptanol, and **12.** δ-lactone.

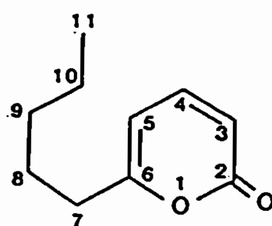

Fig. 2. Mass spectra of 6-PP from fungal solid state culture and structure of the molecule

6-PP was identified by its mass spectra obtained from liquid and solid fungal cultures (Fig. 2). Data were compared to the previous studies on the production of 6-PP in liquid culture (Collins and Halim, 1972; Kikushi *et al*, 1974; Sevenants and Jennings, 1974). Electron impact mass spectral data indicated a molecular ion peak (M+) at m/z 166. The formula is $C_{10}H_{14}O_2$. The biosynthesis was accompanied by the production of other compounds, and the range of compounds widened in number with culture time. However, 6-PP remained by far the main component. More than twenty components were identified, using the mass spectra in the files of the GC/MS apparatus. There might be some discrepancies for certain peaks, which could result from unresolved groups of compounds. Anyhow, this part of the work will be continued during a further stage of the project.

The compounds produced by culturing *T. harzianum* for 5 days were extracted and quantified by GC, using the internal calibration method. The response factor of C11/C10 (γ-undecanolactone/ γ-decanolactone) was used, which is 0.8. The amount of 6-PP recovered was 2.8±0.5 mg/g dry matter (DM). Assuming that the fermented product contained 75% moisture, it could be estimated that about 930 mg of 6-PP was produced per litre of liquid solution adsorbed on the substrate. In liquid culture, the maximum concentration recorded, using Amberlite XAD-2 as an adsorbent, was 248 ppm, which allows to overcome growth inhibition by 6-PP (Prapulla *et al*, 1992). A 6-PP concentration of 90 to 110 mg/l would be enough to inhibit growth and prevent further 6-PP production. The 6-PP concentration obtained in SSF was, therefore, greater than that in liquid culture. Moreover, there was no evidence of growth inhibition by 6-PP in SSF.

STUDY OF THE GROWTH PHYSIOLOGY OF *T. HARZIANUM* CULTURED ON A SOLID MEDIUM WITH A VIEW TO PRODUCE 6-PP

T. harzianum growth on a solid medium was monitored under a microscope and by determining the pH, moisture content and sporulation. The morphological appearance of the mycelium and data on the different parameters are presented in Table 1.

Table 1. Morphological appearance and parameters of *T. harzianum* development on a solid substrate under nitrogen deficiency conditions (C:N = 60)

Time (days)	pH	DM (%)	No. of spores/g DM	Macroscopic description	Microscopic description
0	4.8	24.3	0		Uniform inoculum
2	4.6	22.5	0	Substrate set in one mass	Long, tangled filaments
5	4.6	23.7	4.0×10^8	Substrate set in one mass. White and green clumps	Long filaments with phialides and spores
7	4.6	22.6	2.1×10^9	Green clumps	Spores
14	4.5	22.3	4.0×10^9	Green surface	Spores

DM : Dry Matter

Macroscopic description of the cultures provided a picture of the physiological state of *T. harzianum* growth. Following a 2 day culture in SSF, the mycelium was well developed, which meant that the substrate got set in one mass. Between 4 and 5 day cultures, phialides developed and conidiospores were released into the culture medium. The surface turned green, reflecting the release of conidiospores. This microscopic observation enabled the monitoring of culture development during SSF and correlation of these results with coconut aroma production. The pH was hardly varied during SSF, remaining constant at around 4.6. However, DM decreased slightly, from 24.3 to 22.3%.

The *T. harzianum* strain, used for aroma production, did not sporulate on solid media, and its choice for aroma production in a liquid medium was based on this criterion. On a solid medium containing bagasse, the strain sporulated after 5 days. As the medium used had a C:N ratio of 60, a nitrogen deficiency must have led to sporulation of the strain. Once enough spores were available, semi-continuous culture by inoculating the medium was possible. The number of spores produced after 5 day culture was around 4×10^8/g DM. The number of spores stabilized at around 4×10^9 between 7 and 14 days. As a result, inoculation of the culture medium

could be considered, so as to obtain 10^7 spores/g DM, which is the usual inoculation rate for SSF (Roussos, 1985).

CONCLUSIONS

6-PP production has only been demonstrated and optimized by liquid culture recently. The present study demonstrates that 6-PP production is possible by SSF in two stages, i.e., biomass production in a liquid culture medium at a C/N ratio of 14, followed by biosynthesis on a solid medium impregnated with a culture medium at a C/N ratio of 60.

A study of the aroma profile showed that 6-PP was produced once the medium was inoculated. The amount of 6-PP produced, after 5 day culturing under nitrogen deficiency conditions, was four times greater than that in the liquid culture. The absence of the growth inhibition by 6-PP, seen in liquid culture, may be the cause for this more substantial production. SSF, therefore, seemed to be appropriate for 6-PP production.

As the strain used did not sporulate on solid media, the mycelial inoculum had to be produced first in a liquid medium. The solid medium used for 6-PP production was then inoculated with a mycelium suspension, and not a spore suspension, as it is the case in SSF. The study of the physiological development of the strain during culture showed that the strain sporulated on bagasse, whereas it dit not sporulate on other solid media. Environmental stress (nitrogen deficiency) enabled strain sporulation. Semi-continuous culture by inoculating the medium may therefore be possible. The recovery of the product in a lesser quantity of extract in SSF made this technique even more interesting for an industrial scale production.

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