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Production of Natural Fruity Aroma by *Geotrichum candidum*

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Abstract

Based on its aromatic potential, *Geotrichum candidum* isolated from olive vegetation water was tested for the production of volatile compounds. When *G. candidum* was cultivated on media with glucose as the carbon source, flavor volatile compounds were produced and accumulated in the broth. Fruity flavoring compounds (pineapple-like) such as esters and alcohols were analyzed by gas chromatography coupled to mass spectrometry, including ethyl esters of acetic acid and butyric acid, methyl-3-butan-1-ol, and methyl-2-propan-1-ol. Their synthesis corresponded to the stationary growth phase of the strain. Production of the volatile compounds reached 9.5 g/L of 2-hexanoic acid ethyl ester and 1.6 g/L of benzaldehyde as the main concentrated molecules. Ethyl alcohol seems to be an intermediate metabolite in this pathway.

Index Entries: Benzaldehyde; ethanol; fruity aroma; *Geotrichum candidum*; 2-hexanoic acid ethyl ester.

Introduction

Flavors and fragrances constitute a worldwide market of several billion dollars a year, with a share of 25% of the food additives market. The consumer's preference of natural food additives is more important than ever. The use of biotechnology for the production of natural flavoring

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compounds by fermentation or bioconversion using micro-organisms is an economic alternative to the difficult and expensive extraction from raw materials such as plants *(1,2)*. Under suitable conditions, many yeasts produce intensive flavors, such as volatile compounds produced by strains of *Geotrichum* sp. *(3)*, *Kluveromyces marxianus (4)*, *Ischnoderma benzoinum (5)*, and others.

Geotrichum candidum is a fungus with aromatic properties, and it is often considered a yeast. The micro-organism has been used for commercial cheese ripening *(6)*. It contains a high lipolytic activity with a whole range of substrate specificity *(7–9)*. Some strains may produce in the culture broth a fruity odor, owing to the production of esters and alcohols. The fungus and its products are present in food and appreciated by consumers, especially in cheese *(10,11)*.

In the present study, production of a flavoring mixture was carried out in a micro-organism culture. Volatile compounds in a culture of *G. candidum* were identified in order to ascertain which molecules are responsible for the fruity odor. To follow the culture development precisely, the growth of *G. candidum* and the production of volatile compounds were studied throughout the culture period. A lipase activity, that could be involved in ester synthesis, has been described in the fungus *(12,13)*. Previous works described the production of volatile compound mixtures by micro-organisms growing on different agricultural products or wastes *(14–16)*.

Materials and Methods

Organism

G. candidum was isolated from sludge produced in an aerated pilot-scale bubble column. The micro-organism was identified as the white-rot fungus *G. candidum* (Central Bureau Voorschimmel cultures [CBS], Netherlands) *(17)*.

Culture Media

Cells of *G. candidum* were stored on a potato dextrose agar slant at 4°C and transferred to fresh YEG plates (20 g/L of glucose, 5 g/L of yeast extract, 15 g/L of agar) before incubating at 30°C for 48 h. The spores formed on the surface of the YEG were suspended in sterile water to prepare the spore suspension *(17)*.

Three media were used for preliminary tests: YM (3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 20 g/L of glucose); Sabouraud (5 g/L of tryptone, 5 g/L of meat peptide digest, and 20 g/L of glucose); and a synthetic medium, MS (20 g/L of glucose, 1 g/L of KH_2PO_4, 5 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, and 3 g/L of tryptone) *(3,4)*. Fifty milliliters of the medium was placed in a 250-mL Erlenmeyer flask and inoculated with 5 mL of the spore suspension. Cultures were placed on a rotary shaker (120 rpm) at 30°C. The initial pH was fixed at 6.0.

Two optimized media were tested for volatile compound production: MOGE and MOG. MOGE was composed of 30 g/L of glucose, 15 g/L of ethyl alcohol, 1 g/L of tryptone, 4 g/L of $(NH4)_2SO_4$, 0.25 g/L of $MgSO_4 \cdot 7H_2O$, and 5 g/L of KH_2PO_4. Seventy-five milliliters of MOGE medium was placed in a 250-mL Erlenmeyer flask, inoculated with 5 mL of the spore suspension, and placed on a rotary shaker (120 rpm) at 30°C. The initial pH was fixed at 7.0.

MOG was composed of 30 g/L of glucose, 5 g/L of tryptone, 4 g/L of $(NH4)_2SO_4$, 0.25 g/L of $MgSO_4 \cdot 7H_2O$, and 5 g/L of KH_2PO_4. Seventy-five milliliters of MOG medium was placed in a 250-mL Erlenmeyer flask, inoculated with 5 mL of the spore suspension, and placed on a rotary shaker (120 rpm) at 30°C. The initial pH was fixed at 7.0.

Analytical Methods

Five-milliliter samples from the medium were centrifuged at 4000 rpm (4800*g*) for 30 min, and the biomass was washed twice with water. Biomass evolution was estimated by measuring the dry weight after 24 h at 100°C. The pH of the medium was measured with a pH meter in samples withdrawn at different times. The same samples were used for glucose analysis using dinitrosalicylic acid (DNS) reagent (10 g of DNS in 200 mL of 2 *M* NaOH added to 300 g of potassium–sodium tartrate in 500 mL of water). The final volume was adjusted with water to 1 L.

Analysis of Volatile Compounds

Volatile compounds were analyzed by gas chromatography (GC) and identified by GC-mass spectrometry (MS). Pure volatile standards purchased from Sigma (France) were used. The volatile flavor components were determined by headspace analysis on an HP5890 series II gas chromatograph equipped with an HP 7694 headspace sampler and coupled with a mass spectrometer (HP 5972 series Mass Selective Detector [HS-GC-MS]). Carrier gas He was injected at 1 mL/min. Oven temperature was raised from 50 to 180°C at a rate of 30°C/min. A capillary HP5 fused silica column crosslinked with 5% phenyl ethyl silicone (30 m × 0.2 mm) was used. Positive identification of unknown components was achieved through a library search on an NBS 75 K. I. Mass Spectra Library.

Volatile compounds were quantified using a gas chromatograph HP series II5890 equipped with a flame ionization detector (180°C). A Carbowax capillary column (30 m × 0.32 mm) was used. Carrier gas (nitrogen) was injected at 1 mL/min. The temperature program was 50°C for 1 min increased to 150°C at a rate of 10°C/min. This temperature was maintained at 150°C for 20 min. The major components were quantified using standard solutions.

Qualitative evaluation of the flavors emitted by *G. candidum* cultures was achieved in the laboratory by sniffing the odor of the culture media. This direct sensorial test is a simple method that rapidly evaluates the quality and intensity of the produced flavor mixture.

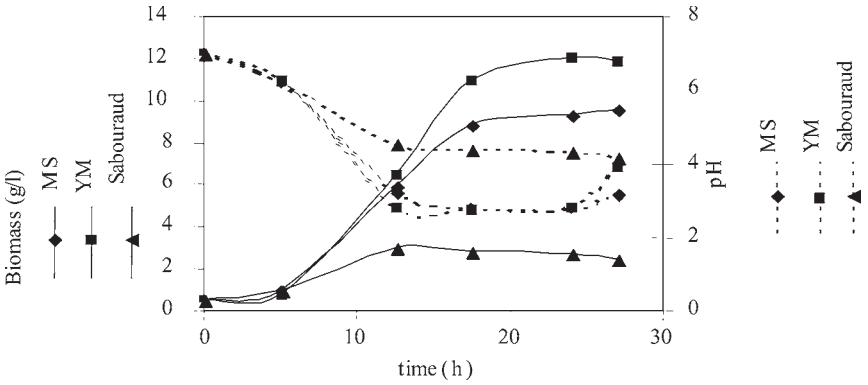

Fig. 1. Biomass production and variation in pH during growth of *G. candidum* on MS, YM, and Sabouraud media.

Results and Discussion

A direct olfactory test with Petri dishes revealed that *G. candidum*, cultivated on agar medium, emitted a fruity aroma. Therefore, the production of fruity compounds was further studied using a variety of culture media.

Volatile Aroma Compounds Related to Culture Media

After *G. candidum* cultivation on YM broth, Sabouraud, and MS media, a direct sensorial test was achieved. It revealed that culture on MS emitted a fruity odor of pineapple and apple. However, on YM it produced first an unpleasant odor followed by a fruity odor. This demonstrated that the behavior of the fungus depended on the composition of the medium and the sources of carbon and nitrogen.

Figure 1 shows that after 18 h culture on YM produced 10.98 g/L of dry biomass, which is more important than the biomass produced on MS (8.87 g/L). The growth on Sabouraud broth was limited to 3 g/L.

The pH of the medium decreased during the exponential phase and reached 3.0 in YM and MS. The decrease in pH was less important in Sabouraud broth, reaching 5.0. This suggests that the fungus produced fatty acids, which seems to represent the precursors for the detected aroma. The organic nitrogen present in the medium did not favor the production of biomass and volatile compounds.

Analysis of the cultures by HS-GC-MS led to the identification of volatile compounds (Table 1). The liberated esters, including ethyl esters and alcohols, were identified as ethyl alcohol, ethyl acetate, 2-methyl-1-propanol, isoamyl alcohol, 2-methyl propyl acetate, ethyl butyrate, ethyl isovalerate, isopentyl acetate, benzaldehyde, and other minor compounds (Table 1). These molecules are known as flavoring compounds in many natural preparations, and their mixture could make them highly suitable for human consumption.

Table 1
Production of Volatile Compounds at Different Times by *G. candidum* in MS Medium as Identified by HS-GC-MS

Peak no.	14 h	18 h	24 h	38 h	Odor
1	Ethanol	Ethanol[a]	Ethanol[a]		Fragrant
2		Ethyl acetate[a]	Ethyl acetate[a]	Ethyl acetate[a]	Pinapple, apple
3		2-Methy-1-propanol			
4		Isoamyl alcohol	Isoamyl alcohol		Fragrant
5		2-Methyl propyl acetate[a]		2-Methyl propyl acetate[a]	
6		Ethyl butyrate[a]	Ethyl butyrate[a]		Banana, pineapple
7		Ethyl isovalerate[a]	Ethyl isovalerate[a]		Intense apple
8		Isopentyl acetate			
9		Ether, *tert*-butyl 3,3-dimethyl butyl			
10		Butanoic acid, 2-methyl propyl ester			
11		Hexane, 2,3-dimethyl			
12		2,3-Methyl, butanoic acid			
13		Butaonic acid, 3-methyl butyl ester			
14		3,3-Methyl butanoate			
15		Carvone (2-cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)			
16		Cyclopentanol, 2-methyl, *trans*			
17		3-Methyl butyl, 2-ethyl hexanoate			

[a]Major compound.
Empty cells in the odor column indicate "not determined."

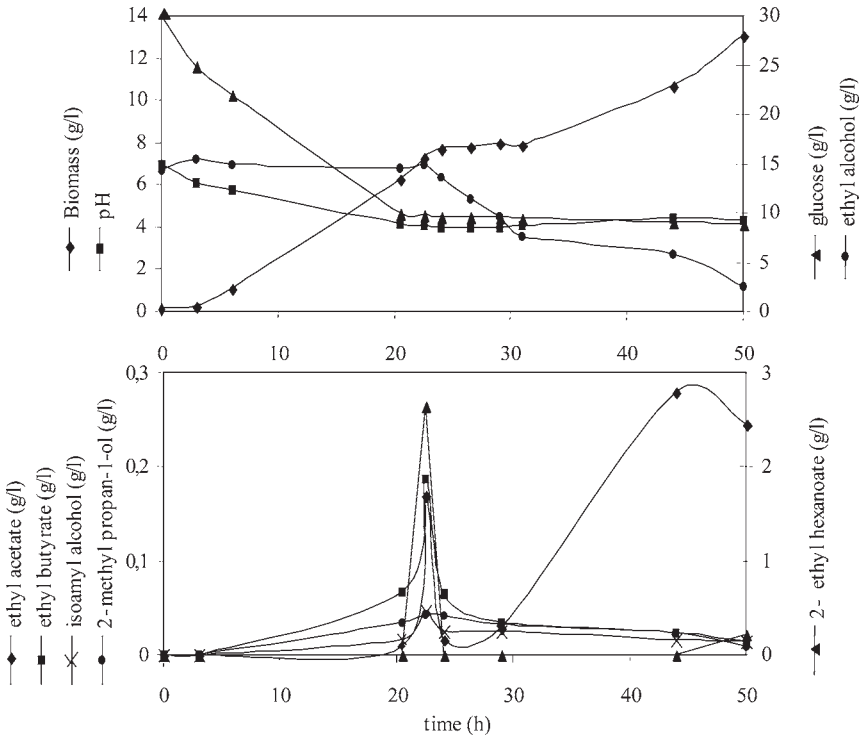

Fig. 2. Evolution of growth and production of volatile compounds by *G. candidum* on MOGE liquid culture.

The medium MS was selected for optimization experiments of volatile compound production. Sensorial tests exhibited the production of a very strong fruity aroma.

Kinetics of Production of Volatile Compounds by G. candidum

Experiments with MOGE and MOG liquid broths were carried out to study the production kinetics of volatile compounds. The liquid cultures gave a fruity odor, owing to the synthesis of several volatile molecules. The most concentrated compounds were identified and quantified by GC-MS and GC, respectively.

Figure 2 shows the variation in the levels of glucose, ethyl alcohol, biomass, and pH during incubation of *G. candidum* on MOGE medium. The evolution of the production of volatile compounds throughout the culture period was followed.

The glucose concentration of the medium decreased whereas ethyl alcohol was virtually constant during the exponential phase. The latter compound was consumed during the stationary phase although the glucose was not exhausted.

Analysis of the data summarized in Fig. 2 revealed an early production of ethyl acetate, isoamyl alcohol, ethyl butyrate, 2-ethyl hexanoate, and

isobutanol (2-methyl propan-1-ol) after 22 h of culture, giving a fruity odor. After 44 h of incubation, ethyl acetate reached its maximum level. Optimal production of these esters with the exception of ethyl acetate coincided with the end of the exponential phase and the first hours of the stationary phase. No production of benzaldehyde was observed in the medium. The decrease of pH during the culture seems to be owing to the production of fatty acids, intermediates in the synthesis of volatile compounds.

Among the esters with flavoring properties that appeared during the culture, 2-ethyl hexanoic acid ester was produced in the highest amount (2.64 g/L) in the medium, followed by ethyl acetate (0.281 g/L) and ethyl butyrate (0.188 g/L). Ethyl acetate and ethyl butyrate concentrations were considered very high and were more important than those described by Daigle et al. *(11)*. They reported the production of about 50 mg/kg of ethyl acetate and 25 mg/kg of ethyl butyrate after 48–72 h of fermentation. In the same work, the used *G. candidum* strain also produced 0.174 g/L of benzaldehyde (aroma of bitter almond), 0.044 g/L of 2-methyl propan-1-ol, and 0.047 g/L of isoamyl alcohol.

The quantitative analysis in the present work shows the high concentrations of esters produced by our strain of *G. candidum* cultivated in the shake culture with MOGE broth. In addition, this study permitted us to understand further the correspondence between the perceived odor and the chemical compounds produced by the fungus.

Similarly, the same culture was achieved in MOG medium. Figure 3 shows the relationship among the growth of *G. candidum* in MOG medium, consumption of glucose, change in pH, and production of volatile compounds. After 20 h of incubation, the rapid growth of *G. candidum* in MOG medium led to a quick exhaustion of the available glucose followed by a synthesis of new volatile compounds (Fig. 3).

Analysis of the data shown in Fig. 3 reveals an early production of ethyl alcohol in parallel with glucose consumption. The production of volatile compounds was optimal after 20 h, which corresponded to the stationary phase of growth. This production seems to be related to the consumption of ethyl alcohol, which could play the role of intermediate in the metabolism. When the ethyl alcohol was used initially as raw material as in MOGE medium, its excess led to a rapid but limited production of volatile compounds. This phenomenon was observed with *Monascus purpureus* initially grown with controlled ethanol formation by glucose consumption in order to increase the production of red pigment *(18)*.

Unlike the culture in MOGE, the production of volatile compounds in MOG broth was observed at different periods of incubation. Consequently, the fruity aroma changed at every measurement and corresponded to one or two major compounds. This could be useful for the optimization of component extraction, because a specific flavor could be selectively extracted at a definite phase of the culture. All the components reached a maximum level before decreasing and disappearing in the medium.

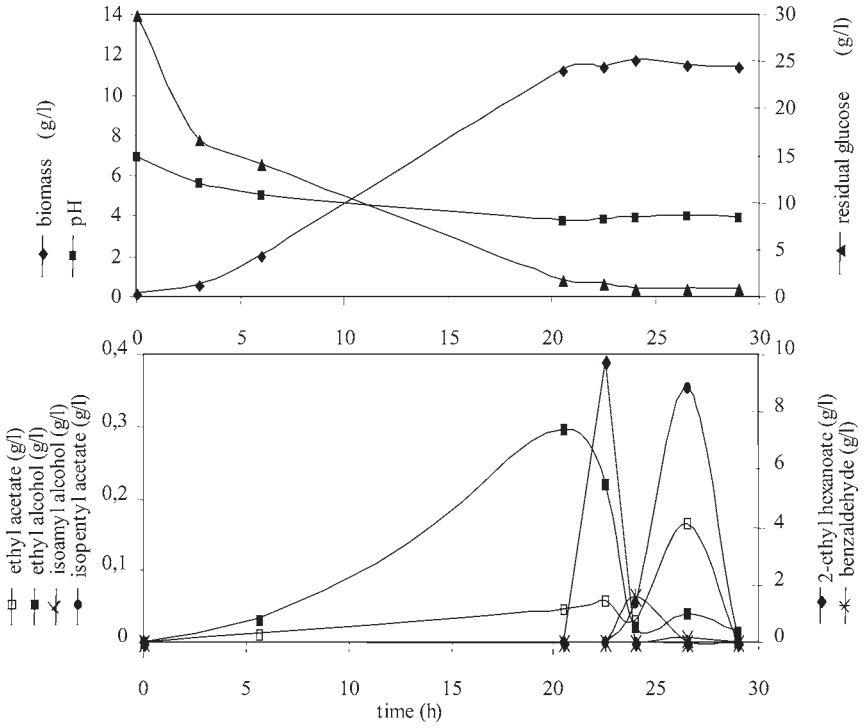

Fig. 3. Evolution of growth and production of volatile compounds by *G. candidum* on MOG liquid culture.

G. candidum produced in MOG medium 9.5 g/L of 2-hexanoic acid ethyl ester, which resulted, in theory, from the esterification of ethyl alcohol with 2-hexanoic acid. The fungus also produced 1.6 g/L of benzaldehyde. This concentration was very important compared with concentrations described in the literature *(5)*. Fabre et al. *(5)* reported the production by a strain of *Ischoderma benzoinum* of about 0.158 g/L of benzaldehyde, 0.353 g/L of isopentyl acetate, and 0.164 g/L of ethyl acetate. Benzaldehyde was not detected in MOGE medium. These results demonstrate the importance of our strain in the production of volatile compounds.

Conclusion

The *G. candidum* strain that we used formed high concentrations of fruity aroma in a simple synthetic medium. High concentrations of specific ethyl esters were produced during the stationary phase of growth and were related to the assimilation of ethyl alcohol. *G. candidum* seems to be a potential source of natural fruity aroma. Scale-up and the use of fed batch are necessary to determine the economic potential of *G. candidum* for specific production of volatile compounds.

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