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GROWTH AND AROMA PRODUCTION BY *Ceratocystis fimbriata* IN VARIOUS FERMENTATION MEDIA

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ABSTRACT

The ability of *Ceratocystis fimbriata* to generate aroma notes from different carbon and nitrogen sources was studied in liquid culture. The medium that gave the best sensory results produced a strong banana aroma. Other notes such as pineapple, apple, pear and nuts, with varied, intensities were obtained from other culture media. Biomass and metabolite productions are reported. In solid state fermentation, the mould was grown on wheat bran, sugar cane bagasse and a synthetic resin (Amberlite IRA 900) imbibed with a nutritive solution. Mould respirometry was used as growth indicator. While growth was regular, the aromas were not strong as those obtained in liquid culture.

INTRODUCTION

Microorganisms play an increasing role in the generation of natural flavour compounds particularly in the field of food aromas. One can refer to the extensive reviews dealing with flavour generation by bacteria, yeasts and fungi, in the past few years (Sprecher and Hanssen, 1985; Latrasse *et al.*, 1985; Gatfield, 1988; Welsh *et al.*, 1989; Janssens *et al.*, 1992). As pointed out recently by Bigelis (1992), filamentous fungi can be very useful in this field because they are able to produce a great number of flavouring compounds and also to release aroma modifying enzymes. The ability of some moulds from the genus *Ceratocystis* to produce a wide variety of fruity-like aromas has been already demonstrated (Hanssen and Sprecher, 1981; Lanza *et al.*, 1976; Hubbal and Collins, 1978; Senemaud, 1988). A nutritive medium has been optimized for flavour production with *C. fimbriata* (Christen and Raimbault, 1991).

Also, moulds are known to grow easily and to produce with high yields metabolites of interest like enzymes and other compounds in solid state fermentation (SSF) (Raimbault, 1988; Doelle *et al.*, 1992). Nevertheless, few papers reported the study of aroma production in solid state cultures (Revah and Lebeault, 1989; Gervais and Sarrette, 1990). A practical application has been patented for the industrial production of methyl ketones from coconut oil by *Aspergillus niger* in solid state fermentation (Humphrey *et al.*, 1990).

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The aim of this work is to study the effect of carbon and nitrogen sources on the biomass and metabolites production by *C. fimbriata* in liquid fermentation and then, with a defined medium, explore the ability of the microorganism to grow and produce aromas on solid substrate (wheat bran) and solid supports (sugar cane bagasse and an anionic polymeric resin) complemented with a nutritive medium.

EXPERIMENTAL AND ANALYTICAL PROCEDURES

Organism and culture media. *Ceratocystis fimbriata* CBS 374-83 was periodically transferred on Potato Dextrose Agar (PDA) slants and stored at 4°C. The basal growth medium was the one previously optimized by Christen and Raimbault (1991). This medium was adjusted to pH 6 with NaOH 0.5 M and then autoclaved at 121 C during 15 min. For inoculum, a spore suspension was prepared from 5 days old PDA cultures. For liquid cultures, the volume of inoculum was calculated to yield a final concentration of $1x10^7$ spores/ml and then transferred into a 250 ml Erlemeyer flask containing 100 ml of culture medium. For solid state experiments, inoculum concentration was $1x10^7$ spores/g Initial Dry Matter (IDM).

In liquid culture, six carbon sources and six nitrogen sources (defined and complex) complemented with a mineral medium were used to study the influence of the substrate on the growth and the nature and intensity of the aromas produced. The carbon sources were : glucose, oleic acid, ethanol, glycerol, sodium citrate and sucrose while the nitrogen sources were leucine, urea, ammonium sulfate, asparagine, casein peptone and fish hydrolyzate. A concentration of 10 g/l of carbon was employed and the relation C/N was maintained at 10. The combinations used are displayed in Table 1. Moreover, a laboratory made potato broth was tested as complex medium. It was prepared with 300 g of cut potatoes in 1l of distilled water. Initial conditions were : temperature, 30°C; pH, 6 and agitation speed, 180 rpm. The fermentation time was 4 days. Each experiment was duplicated.

In solid state culture, 3 supports were used: wheat bran, sugar cane bagasse and an anionic resin : Amberlite IRA 900 (Rohm & Haas) and prepared as described by Christen *et al.* (1993). For wheat bran, only water was added, except in one experiment where urea solution (3.8 g/l) was used up to final water content of 50%. For the case of Amberlite and bagasse, nutritive media (glucose 100g/l) was added to the dried supports to complete 58% and 70% final water content, respectively. The cultures were carried out in small columns placed in temperature controlled bath. Initial conditions were: temperature, 30°C; pH, 6 and aeration rate, 0.05 l/h.g IDM.

Analytical procedures. In liquid culture, two types of analysis were made : sensorial (sniffing) for aroma determination with a panel of six non-trained members and instrumental. The following were determined at the end of the fermentation:

- Reducing sugars with the dinitrosalicylic method (Miller, 1959).

- Biomass by dry weight after centrifugation and filtration on Whatman paper n 2.
- Total volatiles were detected by gas chromatography (GC). Samples from the fermentation medium were centrifugued and injected to a Shimadzu GC mini 2 FID chromatograph equipped with a 1/8" Por apak Q (80/100 mesh) column at an oven temperature of 100 C. Chromatograms were run for 30 minutes and the total area of the produced peaks was obtained. This area is reported as the equivalent ethanol concentration (g/l) from an ethanol external standard. This measure does not necesarily reflect the total amount of volatiles that are related to the aroma but is related to metabolites production and might be complementary to the sensorial evaluation.

In solid state culture, total sugars were determined by phenol sulfuric method with previous acid hydrolysis (Dubois *et al.* 1956). Water activity (Aw) was measured with an Aqualab CX-2 apparatus (Decagon, USA) and pH from an homogeneate of the sample in deionized water. Respirometry was followed by gas chromatography (Christen *et al.*, 1993) in order to calculate carbon dioxide production rate (CDPR), oxygen uptake rate (OUR) and respiratory quotient (RQ), where:

CDPR = (% CO_2 produced x F) / (100 x W) in ml/h.g IDM

OUR = (%O_2 consumed x F) / (100 x W) in ml/h.g IDM

where F is the air flow rate (ml/h) and W the initial dry matter load (g IDM)

RQ = CDPR / OUR

RESULTS and DISCUSSION

The results of the sensorial evaluation of the liquid cultures of *C. fimbriata* after 5 days are listed in Table 1. Aroma production from the mould was detectable from 24 to 48 hours and increased in intensity up to the 4th or 5th day and decreased thereafter. The more characteristic and intensive notes (banana or nut) were obtained with glucose or sucrose as carbon source (#1, #2 and #17). With respect to the nitrogen source, leucine, urea and asparagine and at a lesser level ammonium sulfate are adequate for producing fruity notes (#1, #2, #3, #8 and #9). Leucine is known to be a precursor of isoamyl alcohol and isoamyl acetate, major volatile constituents of the banana aroma. Moreover, it can be observed that the fungus is able to assimilate ethanol or glycerol as sole carbon sources. Potato broth seemed to be a convenient culture medium given the numerous and intensive fruity notes obtained (#19).

In Table 1, it can also be observed that the maximum biomass production (more than 11 g/l) was obtained with potato broth (#19). Glucose, oleic acid, glycerol and sucrose allowed biomass concentrations between 4 and 6 g/l according to the nitrogen sources used. For this purpose, fish hydrolyzate has a potential interest (#18), and at a lesser level, asparagine and peptone casein (#10 and #17). Only when used with glucose, urea and ammonium sulfate (#2 and #3) allowed concentrations superior to 4 g/l.

Table 1: Sensorial evaluation of the fermentation broths aromas, biomass and metabolites produced: * Intensity : - none, + weak, ++ medium, +++ strong. **a** : apple, **ap** : apricot, **o** : orange, **pi** : pineapple, **p** : peach, **pe** : pear, **s** : strawberry. † expressed as equivalent ethanol (g/l). ** from Christen and Raimbault (1991). *** not determined.

Run #	Carbon and Nitrogen Sources	Aroma(s) and Intensity (ies) *	Biomass (g/l)	Volatiles † (g/l)
1	Glucose / Leucine	Banana +++/ Solvent ++	2.26	2.07
2	Glucose / Urea	Banana +++/ Fruity (**a,pe,pi**) ++	5.20	4.27
3	Glucose / Ammonium sulfate	Fruity, alcohol (**pi**) +++	5.12	2.44
4	Oleic acid / Asparagine	Rancid ++/ Lactic +	3.89	0.69
5	Oleic acid / Casein Peptone	Amines ++	2.23	0.74
6	Oleic acid / Fish hydrolyzate	Fish ++/ Amines ++	4.99	1.34
7	Ethanol / Leucine	Lactic ++/Alcohol+++	1.26	2.11
8	Ethanol / Urea	Fruity, alcohol (**o,a**) +++ / Wine +	1.07	7.92
9	Ethanol / Ammonium sulfate	Fruity, alcohol (**o**) +++	0.94	5.62
10	Glycerol / Asparagine	Sweet, fruity ++	5.91	2.40
11	Glycerol / Casein Peptone	Nut +++/Sweet +	3.37	0.97
12	Glycerol / Fish hydrolyzate	Fish ++	5.59	0.13
13	Sodium citrate / Leucine	Cheese +/Banana ++/Yogurt ++	1.05	1.52
14	Sodium citrate / Urea	Cheese / Culture medium (Agar) ++	1.01	0.81
15	Sodium citrate /Ammonium sulfate	- / Oil +	1.03	0.67
16	Sucrose / Asparagine	Fermented fruit (alcohol) ++	2.30	4.26
17	Sucrose/ Casein Peptone	Nut ++/ Banana +++	4.18	2.93
18	Sucrose / Fish hydrolyzate	Fish +++	10.21	1.35
19	Potato Broth	Fruit, sweet (**ap, p, s**)+++/ Flowery +	11.24	1.96
20 **	Glucose/ Urea + Ammonium sulfate	Banana +++	5.02	n.d.***

Glucose and sucrose promoted the production of volatiles (#2 and #16). Ethanol must be considered carefully because it was used as substrate. Urea, ammonium sulfate and asparagine, when combined with glucose or sucrose (#2, #3 and #16) produced high amounts of metabolites. It must be observed that there was not a clear correlation between the aroma detected by sniffing and the volatiles detected by chromatography. Some literature exists on aroma production from *Ceratocystis* species from defined media. Lanza *et al.* (1976) reported that cultures of *C. moniliformis* grown on various carbon and nitrogen sources, gave banana, citrus or "fruity" notes according to the medium. They did not determined biomass product nor the volatiles produced. Nevertheless, some compounds such as acetate esters of C2 to C5 alcohols were identified.and established that the more intensive aroma was observed after 6 days. Senemaud (1988) observed that this same mould starts to produce banana- like flavors after 5 days of growth. As can be seen in Table 1, *C. fimbriata* has a great potential as aroma producer not only from defined media but from natural substrates as well. From the data shown below and the results previously obtained by Christen and Raimbault

(1991), the medium composed of glucose and urea / ammonium sulfate was chosen to continue the studies in solid state fermentation. This combination gave a strong banana aroma.

For the growth of *C. fimbriata* under SSF conditions, results are presented in Table 2.

Table 2: Final results of *C. fimbriata* solid state cultures performed on four different media. * As in Table 1.

	Amberlite	Bagasse	Wheat bran	Wheat bran with urea
Elapsed time (h)	48	66	96	96
Final Aw	0.975	0.944	0.983	0.984
Final pH	7.24	4.83	9.7	9.63
RQ (Range)	1.4 / 1.5	0.8 / 1.3	0.8 / 1.1	0.7 / 1.1
CDPR max (ml/h.g)	1.69	2.58	6.43	3.66
Aroma and intensity *	-	-	Solvent +	Solvent +

In all cases, maximum respirometric activity, represented by CDPR, was observed after 24 hours of fermentation. It was not clearly demonstrated that urea had a positive influence on growth. On the contrary, a higher value for CDPR was obtained without urea. It must be pointed out the high pH final value (probably inhibitory) for wheat bran due to the accumulation of ammonia resulting for the hydrolysis of wheat proteins. Final Aw values were in an acceptable range for growth.

Aroma production on SSF was low as compared to the submerged culture. A slight solvent odour was detected from 24 hours to 48 hours, just after the maximum CDPR was observed, in the columns filled with wheat bran (with or without urea). No odour was found on bagasse or Amberlite. Other authors (Gervais and Sarrette, 1990) have found that age and water activity (Aw) play an important role in aroma formation in *Trichoderma viride*. While these parameters have been measured, they have not been optimized for *C. fimbriata*.

CONCLUSION

The studies made in liquid media, varying carbon and nitrogen sources, showed the ability of the fungus to grow and produce a great variety of fruity aromas from different substrates such as that fish hydrolyzate, potato broth and synthetic compounds. Specially, the glucose/urea and glucose/ammonium sulfate media gave a characteristic and intensive banana aroma. It was found that potato broth and fish hydrolyzate were efficient for biomass production while the former was also found to be adequate for aroma production.

In solid state cultures, it was observed that growth was possible on the three substrates studied with best results with wheat bran. The production of a slight solvent

aroma was observed when wheat bran was used as solid substrate, but it was not possible to reproduce in SSF the aroma detected with the same nutritive medium in liquid fermentation. Amberlite was not found to promote growth or aroma production. Further studies are carried with other natural substrates on SSF and on conditions that may alter the Aw of the SSF system to amplify the aroma production.

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