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OPTIMIZATION OF CULTURE MEDIUM FOR AROMA PRODUCTION BY *CERATOCYSTIS FIMBRIATA*

Pierre CHRISTEN[1]* and Maurice RAIMBAULT[2]

1 - ORSTOM México. Homero 1804#1002. Col. Los Morales. CP 11510 México D.F. MEXICO

2 - ORSTOM Montpellier. 2051 Av.Val de Montferrand. 34032 Montpellier Cedex. FRANCE

SUMMARY

The fungus *Ceratocystis fimbriata* is known to produce among others a banana-like aroma. With a fractional factorial experimental design a culture medium was optimized to produce characteristic volatile metabolites of this aroma. HPLC was used to separate and quantify some of the metabolites. This showed the influence of the composition of the basal culture medium. It is not yet possible to correlate these results with sensorial evaluation.

INTRODUCTION

The flavour of many foods comes from the natural action of microorganisms and/or enzymes. These produced aromas belong to well identified chemical groups such as : aldehydes, ketones, lactones, terpenes, alcohols and esters, all characterized by their high volatility.

In this area, filamentous fungi display an important potential for the production of natural aromas. Many have been studied since the beginning of the century (OMELIANSKI, 1922 ; BADCOCK, 1939). More recently, several authors have shown that a great diversity of aromas can be produced by different fungal species : coconut aroma by *Trichoderma viride* (COLLINS and HALIM, 1972 ; YONG and LIM, 1986) , pine, rose, fruity aromas by *Penicillium decumbens* (HALIM and al., 1975) or the characteristic "mushroom" flavour by *Aspergillus oryzae* (KAMINSKY and al., 1980).Due to its economic interest, *Penicillium roquefortii* has been studied (DESFARGES and al., 1987; REVAH and LEBEAULT, 1989) for the production of blue cheese aroma which is related to the methyl ketones obtained through ß-oxidation and decarboxylation of fatty acids (KINSELLA and HWANG, 1976). The widely studied genus *Ceratocystis* produced a large diversity of fruit-like aromas:i.e.peach, banana, pear, rose or citrus, depending on the strain and environmental conditions (COLLINS and MORGAN, 1962 ; LANZA and PALMER, 1977 ; HANSSEN and SPRECHER, 1981). Among this genus, *Ceratocystis fimbriata* seems to be particularly interesting because of its relatively rapid growth, the variety of aromas synthetized and its good ability for spore production (SENEMAUD, 1988).

In this study, growth and banana-like aroma production conditions were investigated by optimizing a modified BEEVER medium (BEEVER, 1969) already employed for *Ceratocystis* cultures (HUBBAL and COLLINS, 1978). To optimize the culture medium a fractional factorial design was employed. An HPLC method, developed in this laboratory, allowed quantification of residual glucose and of metabolites simultaneously.

* *To whom all correspondence should be addressed.*

MATERIALS AND METHODS

Organism and culture medium : *Ceratocystis fimbriata* CBS 374-83 (ELLIS and HALST.) donated by Pr. BENSOUSSAN (ENSBANA Dijon - FRANCE) was periodically transfered and kept on potato dextrose agar slants. The basal growth medium (BEEVER, 1969) was modified to increase the "buffer" capacity. It consisted of : glucose 20 g/l; urea 0.75 g/l ; $(NH_4)_2SO_4$, 2.25 g/l ; KH_2PO_4 , 1 g/l ; $Ca(NO_3)_2.4H_2O$, 0.5 g/l ; $MgSO_4.7H_2O$, 0.5 g/l ; trace element solution, 2 ml/l. The trace element solution contained the following : $Fe(NO_3)_3.9H_2O$, 723.8 mg/l; $ZnSO_4.7H_2O$, 439.8 mg/l ; $MnSO_4.4H_2O$, 203 mg/l. The medium was supplemented with chloramphenicol 0.5 g/l to inhibit bacterial contamination and the pH adjusted to 6.0 with NaOH 0.5 N before autoclaving at 121°C during 20 mn.

The inoculum was prepared by growing the fungus on potato dextrose agar in 100-ml Erlenmeyer flasks for 4 days. Spores were harvested with distilled water containing a few drops of Tween 80 and some small glass beads. The inoculum was then aseptically introduced in a 250-ml Erlenmeyer flask containing 100 ml of the basal medium. The volume of inoculum transferred was calculated to reach a final concentration of 1.2×10^6 spores/ml in the culture medium, typically between 9 and 10 ml . The 250-ml flasks were then incubated at 30°C during 5 days on a rotary shaker (150 rpm) . Each experiment was duplicated and the average values are reported.

Analysis :

. *Growth determination* : Mycelia were collected after centrifugation (5000 rpm for 20 mn at 0°C) . The mycelia was then filtered through Whatman # 1 filter paper and washed twice with distilled water.Biomass was measured by dry weight determination (24 hours in a drying oven at 100°C) .

. *Residual glucose and metabolites produced* : These were determined by HPLC using a Chromatem 380 pump, a Jones Chromatography oven and two detectors in serial : a Knauer refractometer detector and a Shimadzu UV detector (210 nm) . Eluent was 6 mmol.sulphuric acid with a flow rate of 0.8 ml/mn and oven temperature was 65°C. The separation was made with an Aminex HPX 87H column (Bio-Rad) .The total peak area of metabolites (M) is expressed arbitrarily in millivolts.seconds (Mv.s) irrespective of metabolite identities.

. *Sensorial evaluation* : A panel consisting of three members of this laboratory characterized the aroma of the fungal cultures. The panel members were not restricted to any list of descriptive terms.

RESULTS AND DISCUSSION

Two experimental designs were performed:the first one to determine which components have a significant effect on growth and aroma production and the second to improve the medium composition and to test the influence of other parameters.

In both cases, a 2^{7-4} experimental design was performed (De MEO and al.,1985) , allowing the study of the influence of 7 parameters with only 8 experiments (See the matrix of the experimental design in Table 1) . The level of each constituent in each run is given in Table 2. For each experimental design, coefficients - **Bx** for biomass and **Bm** for the metabolites produced- were calculated in order to determine the effect of each constituent: If **X** is the biomass produced and **M** the area of the peaks corresponding to the metabolites detected, the coefficients **Bx** and **Bm** are given by:

$$\mathbf{Bxj} = 1/4 \; . \; [\; \Sigma^{8}_{i=1} \; \mathbf{Aj \; Xi}] \qquad \text{and} \qquad \mathbf{Bmj} = 1/4 \; . \; [\; \Sigma^{8}_{i=1} \; \mathbf{Aj \; Mi}]$$

were **Aj** is the level of the constituent **j** (+ or -), **Xi** the biomass and **Mi** the total area of the peaks for the run **i** expressed in millivolts.seconds (Mv.s) .

RUN	A	B	C	D	E	F	G
1/9	-	-	-	+	+	+	-
2/10	+	-	-	-	-	+	+
3/11	-	+	-	-	+	-	+
4/12	+	+	-	+	-	-	-
5/13	-	-	+	+	-	-	+
6/14	+	-	+	-	+	-	-
7/15	-	+	+	-	-	+	-
8/16	+	+	+	+	+	+	+

Table 1 : Eight run experimental design (ED) for the optimization.

Constituent	Concentration (g/l) at the level	
	-	+
Glucose (A)	10	30
Urea (B)	0,375	1,125
(NH4)2SO4 (C)	1,125	3,375
KH2PO4 (D)	0,5	1,5
Ca(NO3)2 (E)	0,25	0,75
MgSO4 (F)	0,25	0,75
Trace elem. sol.(ml/l) (G)	1	3

Table 2 : Experimental field for ED 1.

EXPERIMENTAL DESIGN N°1 (ED 1)

The concentration of each constituent at level - and + is given in Table 2. An example of HPLC chromatograms obtained with *C. fimbriata* culture is given in Figure 1. Three compounds were identified on the basis of their retention times : glucose, acetic acid and ethanol, but other compounds are still unidentified. Nevertheless, all peaks correspond to metabolites produced during the fermentation since only glucose appeared on chromatograms of the initial medium (See Figure 1). Although it can be supposed that not all the metabolites were separated and detected, it has been assumed that the measured compounds could be taken as reference for the evaluation of the metabolites produced.

RUN	[Gluc.]i	pH final	X(g/l)	M(mV.s)	[Ethanol](g/l)	Aroma (a)	Intensity (b)
1	10	3.03	3.11	198	0	banana	+
2	30	2.18	3.36	1032.3	6.33	banana	++
3	10	7.83	3.46	421.2	0	ammonia	+
4	30	3.01	4.31	1075.6	5.92	banana	+++
5	10	2.87	2.81	131.4	0	banana	+
6	30	2.16	3.98	1338.7	5.61	banana	+++
7	10	8.15	3.04	561.8	0	ammonia	+
8	30	3.06	3.79	1478.2	6.19	banana	+++

Table 3 : Experimental results for ED 1. (a) : detected by sniffing. (b) : +++, strong ; ++, medium; +, weak.

Constituent	Glucose	Urea	(NH4)2SO4	KH2PO4	Ca(NO3)2	MgSO4	Trace elements sol.
Bx	+0,73	+0,34	-0,16	+0,04	+0,2	-0,32	-0,26
Bm	+903	+209	+195,8	-117,8	+158,8	+76,0	-27,6

Table 4 : **Bx** and **Bm** coefficients for ED 1.Average values : Xo = 3,48 ; Mo = 780

Table 3 displays the experimental results of ED 1 and in Table 4 the **Bx** and **Bm** coefficients are given.At low glucose and high urea concentrations an unpleasant ammonia smell was detected and final pH was slightly alkaline (runs 3 & 7). The other runs were characterized by a banana-like aroma and low final pH. Ethanol, total metabolites production and detected aromas were more intensive when glucose was present at high concentration; this carbon source being strictly used for biomass production when runs were performed with initial glucose concentration of 10 g/l.The secondary metabolism only begins when the concentration is higher than 10 g/l. For all the eight runs no residual glucose was detected. On

Figure 2, the respective areas of each compound for the eight runs is represented.

According to **Bx** and **Bm** presented in Table 4, and assuming that there is no interaction between each factor studied, it can be concluded for this first experiment that: glucose, urea and calcium nitrate had a positive effect on growth and production. Ammonium sulphate had a positive effect on production and nearly none on growth. Magnesium sulphate had a slightly positive influence on production and slightly negative on growth. Potassium phosphate exhibited a negative effect on production and slightly positive on growth. The trace element solution had nearly no effect on either growth or production.

For the following experimental design (ED 2), five parameters were kept (glucose, urea, calcium nitrate, ammonium sulphate and potassium phosphate) with increased concentrations. Magnesium sulfate and trace elements solution were maintained constant at 0,75 g/l and 2 ml/l respectively. Consequently with the 2^{7-4} experimental design it is possible to study the influence of two new parameters. In order to increase biomass production and subsequently the aroma production, the influences of thiamin and biotin were studied.

EXPERIMENTAL DESIGN N°2 (ED 2)

For ED 2, the same matrix was employed (Table 1). The experimental field for ED 2 is given in Table 5. The temperature, pH and agitation speed were kept identical to those used for ED 1.

Constituent	Concentration (g/l) at the level	
	−	+
Glucose (A)	30	50
Urea (B)	1,125	1,875
(NH4)2SO4 (C)	3,375	5,625
KH2PO4 (D)	0,75	1
Ca(NO3)2 (E)	0,75	1,25
Thiamin (F)	5.10^{-5}	15.10^{-5}
Biotin (G)	0	10^{-5}

Table 5 : Experimental field for ED 2.

RUN	Aroma (a)	Intensity (b)
9	ripe banana	++
10	banana / solvent	++
11	fermented banana	+++
12	banana / rancid smell	+++
13	banana	+++
14	banana / solvent	+++
15	banana / solvent	+++
16	banana / rancid smell	+++

Table 6A : Experimental results for ED 2 : Sensorial determination. (a) : detected by sniffing. (b) : +++, strong ; ++, medium.

RUN	[Gluc.]i	pH final	X (g/l)	M (mV.s)	[Ethanol] (g/l)	[Acetic ac.] (g/l)	[Gluc.]r
9	30	6.52	6.12	1456	8.38	0.2	0.3
10	50	4.78	3.82	2195	19.23	0	0.5
11	30	6.47	4.64	2132	8.64	2.16	0.19
12	50	6.41	4.97	5387	16.86	1.15	0.43
13	30	6.76	4.98	1645	8.43	0.36	0.45
14	50	4.87	4.38	1973	17.7	0	0.5
15	30	6.69	3.44	2434	8.52	2.26	0.49
16	50	6.47	7.78	4423	17	1.87	0.52

Table 6B : Experimental results for ED 2 : Instrumental determination

The second experimental design allowed a significant improvement in both biomass and metabolites production i.e. with Xo = 5,02 g/l and Mo = 2706 mV.s, an increase of 44% for biomass and 247% for metabolites. As in ED 1, the glucose was nearly all consumed. At the same time, the global sensorial determination showed an increased aroma detection (Table 6 A). As in ED 1, it can be observed the importance of initial glucose concentration on primary and secondary metabolism

(Table 6B). This effect is especially evident for ethanol production since the average ethanol produced (by Pasteur effect or secondary metabolism) is far superior in this case (13 g/l) compared to that obtained in ED 1 (3 g/l). Moreover, it can be pointed out that:

- an increase of initial glucose concentration from 30 g/l to 50 g/l corresponds to an increase of approximately 100% of ethanol production.
- a substantial improvement of the culture composition was reached since with the same initial glucose concentration (30 g/l), a higher biomass content and more metabolites production were obtained. However, this increase was not correlated with aroma intensity. This might be explained by a partial evaporation during the fermentation. This hypothesis could be verified by carrying out the time course of the fermentative process.
- a new secondary metabolite (absent in ED 1) was produced and identified as acetic acid.

These results can be partly explained by a better pH stability (final pH : 6,5 except for runs 10 and 14) improving the ambient conditions for the fungus. This was probably due to the global increase of urea and salts concentation which increased the buffering capacity of the medium.

Constituent	Glucose	Urea	$(NH_4)_2SO_4$	KH_2PO_4	$Ca(NO_3)_2$	Thiamin	Biotin
Bx	+0,44	+0,38	+0,26	+1,89	+1,43	+0,55	+0,58
Bm	+1578	+1777	-174	+1044	-419	-158	-214

Table 7 : **Bx** and **Bm** coefficients for ED 2. (Average values : X0 =5,02 ; M0 =2706).

According to **Bx** and **Bm** listed in table 7, it may be observed that: glucose, urea and potassium phosphate have an important and positive effect on both primary and secondary metabolism . They should be maintained at the upper level. Ammonium sulphate and calcium nitrate do not have an evident effect (positive for growth and slightly negative for metabolite production) and for this reason should be maintained at their intermediate value (i.e. 4,5 g/l and 1 g/l respectively). Although thiamin and biotin do not have a positive influence on metabolites production, they stimulate growth (**Bx** of 0,55 and 0,58 respectively) so they will be incorporated in the medium at their upper level.

CONCLUSION

The initial Beever medium was improved for the production of banana-like aroma using a 2^{7-4} factorial design. The composition of the optimized medium is the following : glucose 50 g/l; urea 1.9 g/l; $(NH_4)_2SO_4$, 4.5 g/l; KH_2PO_4, 1 g/l; $Ca(NO_3)_2.4H_2O$, 1 g/l; $MgSO_4.7H_2O$, 0.75 g/l; trace element solution (with identical composition) 2ml/l; thiamin 150 µg/l; biotin 10 µg/l. It should be emphasized that, except for glucose, urea and ammonium sulfate, the concentration of the constituents are rather close to the initial ones, this probably means that these first three constituents are essentially involved in the aroma production, while all the constituents are involved in the growth of the fungus.

Nevertheless, these results must be completed by an evaluation of the interactions of the contituents by the determination of **bij** coefficient using for example a surface-response method and by more substantive correlation of the aroma characteristics with the level of individual metabolites.

Currently, work is being continued on the identification and quantification of the metabolites (especially the peak detected by UV with retention time of 16.7 mn) and in the study of the kinetics of both growth and production.

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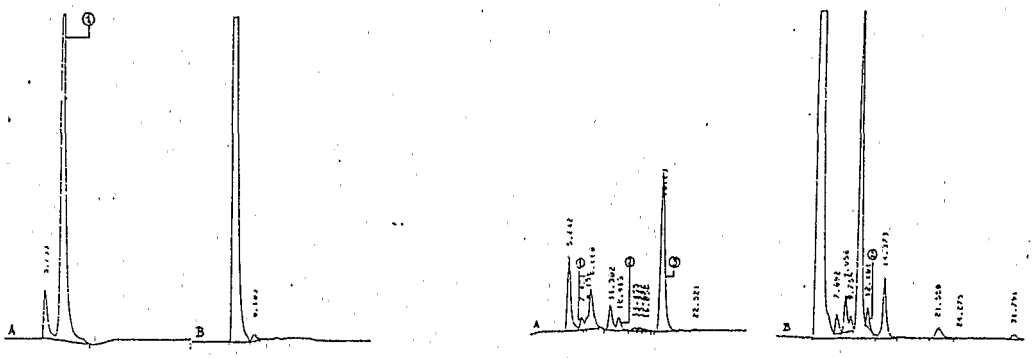
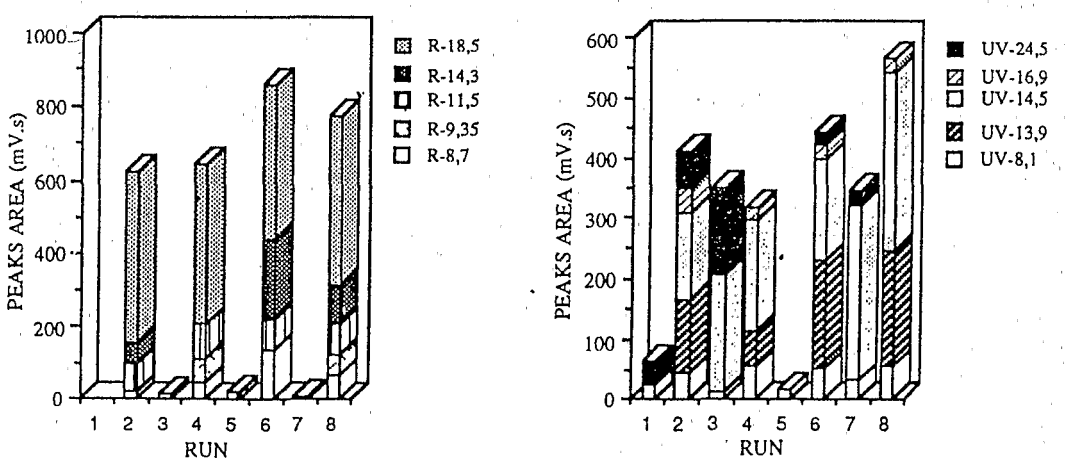


1: Initial culture medium 2:Fermentation broth after 5 days

Figure 1: HPLC chromatograms.(A:Refractometer detector, B:UV detector). Peaks 1:glucose, 2:acetic acid, 3:ethanol. (Run 12 of ED 2).



A:Compounds detected by refractometry B:Compounds detected by UV (210 nm)

Figure 2 : Metabolites produced by cultures of *Ceratocystis fimbriata* and separated by HPLC (R-18,5 was identified as ethanol) . Results obtained from ED 1.

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