FRUITY AROMAS PRODUCTION IN SOLID STATE FERMENTATION BY THE FUNGUS *Ceratocystis fimbriata*

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Abstract

Solid state fermentation (SSF) has been studied for enzymes, antibiotics, alcohol production or for protein enrichment, but few papers report the production of aromas by such a process. In this work, the study of the production of fruity aromas in SSF by the fungus *Ceratocystis fimbriata* is presented, with special interest in the nature of the support/substrate, the importance of added precursors in the medium and the aeration. The aromas were characterised by "sniffing" technique and GC headspace analysis; growth was followed by respirometry.

It was shown that wheat bran, cassava and sugarcane bagasse were adequate supports for growth and a detectable aroma production. Among the nutritive media tested, the synthetic medium previously defined, used with a higher glucose concentration (200 g/l) gave a strong apple aroma while those containing aminoacids precursors such as leucine and valine gave strong banana aroma. It was found that aroma production was growth dependent and the maximum aroma intensity was detected a few hours before or after the maximum respirometric activity, this varying between 0.8 and 1.6 ml CO_2 / h. g dry matter, after 24 hours). Experiments made under various aeration rates (0.05 and 0.005 ml/h. g dry matter) showed that this parameter was not limiting for growth even if the exit gas was very poor in oxygen at the lower aeration rate giving in this case the most intense aroma. For experiments made without forced aeration, the same aromas were also found with higher intensity. Fourteen compounds have been separated by GC headspace and 11 of them such as acetaldehyde, ethanol, ethyl acetate, isoamyl acetate and isoamyl alcohol were identified.

Introduction

Micro-organisms play an important role in the generation of natural flavouring compounds particularly in the field of food aromas. One can refer to the extensive reviews dealing with flavour generation by micro-organisms in the past few years (Latrasse *et al.*, 1985; Welsh *et al.*, 1989; Janssens *et al.*, 1992). As pointed out recently by Bigelis (1992) and Christen (1995), filamentous fungi are especially interesting because they are able to produce flavouring compounds or flavours-related enzymes.

Some recent papers have reported the production of aromas in SSF: Yamauchi *et al.* (1989) obtained a fruity flavour growing a *Neurospora* strain on pregelatinized rice; Gervais and Sarrette (1990) studied the production of coconut aroma by *Trichoderma viride* on agar and Humphrey *et al.* (1990) patented a process where an *Aspergillus* strain grown on cellulose fibres produced methyl ketones from coconut oil. Moreover, the capacity of some moulds from the genus *Ceratocystis* to produce fruit-

like aromas has already been demonstrated (Hanssen and Sprecher, 1981; Senemaud 1988, Christen *et al.*, 1994).

In this work, the ability of *Ceratocystis fimbriata* to produce aromas in SSF was explored. It involved the study of the influences of the substrates/supports used, the aeration flow rates and the presence of precursors, on both growth and aroma production.

Organism and culture media. *Ceratocystis fimbriata* CBS 374-83 was used. It was periodically transferred onto Potato Dextrose Agar (PDA) slants and stored at 4°C. Four substrates / supports were used: wheat bran, sugarcane bagasse, cassava (donated by Pr. C. Soccol, UFPR, Brazil) and an anionic resin (Amberlite IRA-900, Rohm & Haas). There were prepared according to Christen *et al.* (1993). When forced aeration (packed bed) was used, the cultures were carried out in small columns placed in temperature controlled bath. For experiments without forced aeration (surface culture), they were made in 500 ml Erlenmeyer flasks covered with gauze or tight-sealed. They were filled with 7.5 g Initial Dry Matter (IDM) for wheat bran and Amberlite, and 5.25 g IDM for bagasse and cassava. For all experiments, initial conditions were: temperature, 30°C; pH, 6; inoculum size, 1×10^7 spores/g IDM. Aeration rates were 0.05 or 0.005 l/h.g IDM. Initial water content was calculated according to the maximum adsorption capacity of each support (wheat bran, 50%; Amberlite, 58%; sugarcane bagasse, 63% and cassava, 65%). Culture conditions are given in tables 1 and 2. SM (See table 1 and 2) refers to the synthetic medium optimised by Christen and Raimbault (1991). It was used with 200 g/l of glucose. For all cases, an oligoelement solution previously used by these authors was added. Urea, leucine and valine (167 mmol/l) were used as nitrogen source and/or precursor of the aroma.

Table 1. Culture conditions with forced aeration. *SM: synthetic medium

Run	Substrates/Supports	Nutritive media	Aeration rate (l/h.g)
1	Amberlite	Potato broth	0.05
2	Amberlite	Potato broth	0.005
3	Amberlite	SM*	0.005
4	Wheat bran	Urea	0.05
5	Wheat bran	-	0.005
6	Wheat bran	Urea	0.005
7	Wheat bran	Leucine	0.005
8	Wheat bran	Valine	0.005
9	Sugarcane bagasse	SM*	0.005
10	Sugarcane bagasse	Potato broth + glucose	0.005

Analytical procedures.

The odors of the cultures were determined by sensorial evaluation with a non-trained panel consisting of six members, with no restriction in descriptive terms.

Growth was characterised by respirometry measured by gas chromatography. For packed column experiments, this allowed the calculation of the carbon dioxide production rate (CDPR), the oxygen uptake rate (OUR) and the respiratory quotient (RQ) (Christen *et al.*, 1993). For surface cultures, O2 and CO2 concentrations evolu-

tion was followed. Water activity and pH were also determined at the end of the fermentation.

Table 2: Culture conditions without forced aeration. Each experiment was made with both gauze and tight-sealed.

Run	Substrates/Supports	Nutritive media
11	Wheat bran	-
12	Wheat bran	SM
13	Wheat bran	Leucine
14	Wheat bran	Urea
15	Cassava	-
16	Cassava	SM
17	Cassava	Leucine
18	Cassava	Urea
19	Sugarcane bagasse	SM + Leucine

Volatiles produced during the fermentation were characterised by gas chromatography (Hewlett-Packard 5890 equipped with a Megabore HP-1 column (length, 5m) and with a flame ionisation detector) of headspace vapour from the cultures (only for experiments without forced aeration). Conditions were: Temperatures, injector and detector: 210°C, oven held at 40°C during 2 minutes and then programmed at 10°C/min to 150°C. The nitrogen gas flow rate was 1.5 ml/min and split ratio 1:32.

Table 3: Results of aroma production in packed cultures and forced aeration. * - none, + weak, ++ medium, +++ strong. # tmax: time of maximum aroma perception. § in ml/h.g IDM.

Run	Aroma & Intensity *	tmax (h)#	CDPR max§	Aw final	pH final
1	-	-	2.58	0.996	6.76
2	banana +, then apple/pear +	67 / 91	0.95	0.973	3.05
3	banana +	91	0.89	0.990	2.65
4	pear/apple +	42	2.45	0.983	8.76
5	pear/apple ++	17	1.30	0.988	9.06
6	pear/apple ++	39	1.04	0.985	8.95
7	banana +++	17	0.72	0.989	9.03
8	banana +++	17	0.79	0.990	9.06
9	pear/apple +, then peach +++	20 / 91	0.84	0.993	2.71
10	pear/apple ++, then peach ++	68 / 116	0.70	0.998	6.24

Results are presented in two parts according to the modes of culture and aeration used.

Packed cultures experiments with forced aeration.

From the results presented in table 3, it can be seen that at the higher aeration rate (0.05 l/h.g IDM in runs 1 and 4), no or poor aroma was detected. It can be assumed that this rate swept away the volatiles produced and/or oxygenated conditions reduce the synthesis of such molecules. It is why a very low aeration was then used (0.005 l/h.g IDM). In that case, the overall aromas detected (pear/apple and banana) were stronger. In particular, it was clearly shown that leucine and valine, when added to the medium, played a precursor role for the development of the banana aroma (runs 7 and 8). This fruity aroma appeared very rapidly (before the first 24 hours) in these cases. When no precursor was used, pear/apple aroma was also detected (runs 4, 5, 6, 9 and 10). It must be pointed the observations made in runs 9 and 10 when 2 successive kinds of aromas were detected, first the pear/apple one and then at 5 days a strong peach one. With this aeration rate, lower CDPR max were observed (less than 1.3 ml/h.g IDM) which indicates maybe that, in poorly aerated media, growth was limited and volatile metabolites production favoured.

In terms of support evaluation, wheat bran (supplemented or not) and supplemented bagasse gave better result than Amberlite. In all cases, water activity was maintained at a satisfactory level, but pH sometimes at the end of the fermentation were alkaline (for wheat bran) or acid (for bagasse). In all cases, no compounds were detected in headspace analysis of the cultures.

As a conclusion, wheat bran and bagasse are adequate substrates and supports, very low aeration is recommended (0.005 l/h.g IDM), precursors like leucine or valine can shift fermentation to a particular aroma, although fruity aroma was also produced without them. From table 3 and figures 1 and 2, it can be seen that in both cases, the maximum aroma was detected just after the maximum in CDPR was attained. Seemingly, aroma production was found to be growth related.

Surface cultures experiments without forced aeration

In this case 9 combinations were tested, and in each case, experiments were made with gauze cover (static aerated culture) and tightly-sealed (without aeration). Sensorial evaluation was only possible in the case of aerated cultures. In the second case, respiration was characterised by O2 (%) consumption and CO2 (%) accumulation in the flask. Results are presented in Table 4.

All of the three substrates/support were found to allow growth and aroma production in aerated conditions. Aroma detection was more important than in experiments made with forced aeration. The strongest aroma detected (banana) corresponded to the media in which leucine was added (runs 13, 17, 19) while pear/apple aroma, with a lower intensity, was obtained with wheat bran completed with synthetic medium and urea (runs 12 and 14). These aromas were detected with major intensity between the first and the second day.

Table 4: Results of aroma production in surface cultures without forced aeration. * - none, + weak, ++ medium, +++ strong. # tmax: time of maximum perception of the aroma. § in ml/h.g IDM. 1 refers to aerated cultures and 2, to tight-sealed flask cultures.

Run	Aroma & Intensity *1	tmax (h)#1	CDPRmax §1	CO2 max (%)2
11	-	-	1.15	11.3
12	apple/pear ++	44.3	0.06	81.3
13	banana +++	35.8	0.16	31.5
14	apple/pear ++	41.3	1.23	22.3
15	banana ++	40	0.20	36.7
16	-	-	0.90	62.5
17	banana +++	40	0.20	29.6
18	-	-	0.45	7.3
19	banana +++	39.3	0.08	46.9

For tight-sealed flask cultures, growth was also observed. As growth and substrate fermentation evolved, CO_2 was produced and the internal pressure increased. This pressure was released during sampling which provoked the increase in CO_2 concentration up to 81%. These values of CO_2 were coupled with low O_2 (less than 2 % of residual oxygen) which channelled the metabolism toward the fermentative route. No sensorial evaluation was made for tight-sealed flask cultures but it can be seen in figure 3 that large amounts of volatile were also produced.

Separation and identification of GC detected compounds

For headspace chromatograms of aerated and tight-sealed cultures of run 19, fourteen compounds were detected. Eleven compounds were identified through retention time comparison with a standard and can be classified according to their relative quantity (peak area): ethanol, ethyl acetate, ethyl propionate and isoamyl acetate are important; acetaldehyde, isoamyl alcohol and isobutyl acetate are intermediate and 1-propanol, 2-propanol, 1-butanol and amyl acetate are in small amounts. Among them, isoamyl acetate and isoamyl alcohol are known to be important compounds in the aroma of banana, while acetaldehyde, ethanol and ethyl acetate are always present in fruit aromas. Other minor compounds like ethyl propionate and isobutyl acetate are also reported to participate in fruit aromas.

Some differences can be observed between aerated and tight-sealed cultures. Acetaldehyde peak is bigger, ethyl propionate peak is smaller and unknown peak #13 is absent in the second case. Unfortunately, it is not possible to evaluate directly the impact of these differences on the aroma.

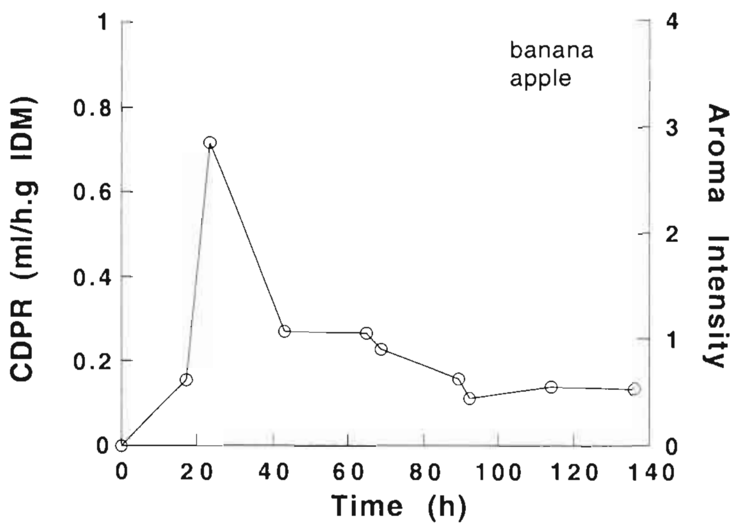

Figure 1. Evolution of Carbon Dioxide Production Rate (CDPR) during production of banana/apple aroma.

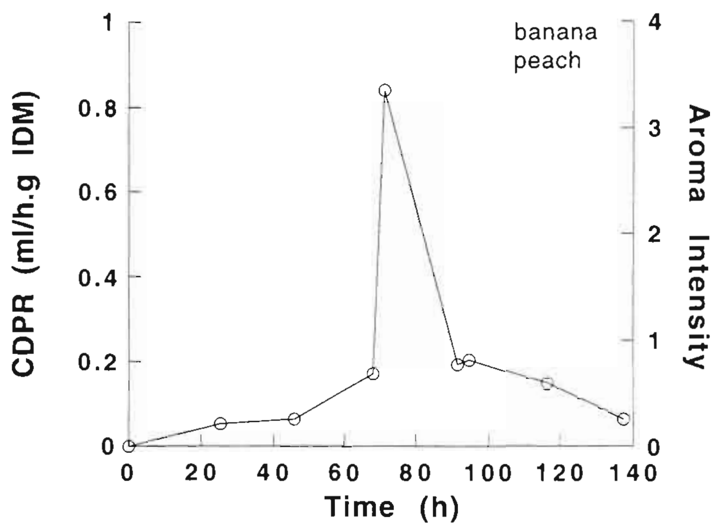

Figure 2. Evolution of Carbon Dioxide Production Rate (CDPR) during production of banana/peach aroma.

Conclusion

Wheat bran, cassava and sugarcane bagasse were found to be adequate substrates/support for aroma production by *C. fimbriata*. Amino acids like valine or leucine seemed to be direct precursors of banana-like aroma. Other aromas (peach, apple) were also detected without adding any precursor. The corresponding compounds of banana aroma (isoamyl alcohol and isoamyl acetate) were detected in the headspace of the culture at relatively important amounts. A total of 14 compounds were separated by GC and among them 11 were identified (1 aldehyde, 5 alcohols and 5 esters). Work is currently continued to identify the unknown peaks and to quantify the identified compounds.

Very low aeration (0.005 l/h.g IDM or passive diffusion) favoured the detection of strong aromas. Results were highly improved in these conditions in comparison with those obtained by Christen *et al.* (1994) at higher aeration rates. In the tight-sealed experiments, it was shown that the fungus was able to ferment the carbohydrates present in the medium (glucose in the case of bagasse, derivatives of starch in the case of wheat bran and cassava). The fact that very low or no aeration is required opens interesting technological perspectives for the production of fruity aromas by *C. fimbriata.*

References

Bigelis, R. 1992. Flavor metabolites and enzymes from filamentous fungi. *Food Technol.* 46 (10) : 151-161.

Christen, P., Raimbault, M. 1991. Optimization of culture medium for aroma production by *Ceratocystis fimbriata*. *Biotech. Lett.* 13 (7) : 521-526.

Christen, P., Auria, R., Vega, C., Villegas, E., Revah, S. 1993. Growth of *Candida utilis* in solid state fermentation. *Biotech. Adv.* 11 : 549-557.

Christen, P., Villegas, E., Revah, S. 1994. Growth and aroma production by *Ceratocystis fimbriata* in various fermentation media. *Biotech. Lett.* 16 (11) : 1183-1188.

Christen, P. 1995. Producción de aromas en fermentación sólida. *Tópicos de Investigación y Docencia*. Submitted.

Gervais, P., Sarrette, M. 1990. Influence of age of mycelium and water activity on aroma production by *Trichoderma viride*. *J. Ferment. Bioeng.* 69 (1) : 46-50.

Hanssen, H.P., Sprecher, E. 1981. In: Schreier P. (ed.), *Flavour'81*, 547-556, W. de Gruyter, West Germany.

Humphrey, M., Pearce, S., Skill, B. 1990. Biotransformation of coconut fat to methyl ketones. A commercial scale solid-state fermentation. In: *Abst. Symp. Bioformation of Flavours*. London.

Janssens, L., de Pooter, H.L., Schamp, N.M., Vandamme, E.J. 1992. Production of flavours by microorganisms. *Process Biochem.* 27 : 195-215.

Latrasse, A., Degorce-Dumas, J.R., Leveau, J.Y. 1985. Production d'arômes par les microorganismes. *Sci. Alim.* 5 : 1-26.

Senemaud, C. 1988. Les champignons filamenteux producteurs d'arômes fruités. Etudes de faisabilité sur substrats agro-industriels. Ph. D. Thesis, Université de Bourgogne (France), 170p.

Welsh, F.W., Murray, W.D., Williams, R.E. 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. *Crit. Rev. Biotech.* 9 (2) : 105-169.

Yamauchi, H., Akita, O., Obata, T., Amachi, T., Hara, S., Yoshizawa, K. 1989. Production and application of a fruity odor in a solid-state culture of *Neurospora sp.* using pregelatinized polish rice. *Agric. Biol. Chem.* 53 (11) : 2881-2886.