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Effect of added amino acids on the production of a fruity aroma by *Ceratocystis fimbriata*

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RÉSUMÉ Effet de l'ajout d'acides aminés sur la production d'un arôme fruité par *Ceratocystis fimbriata*.

Parmi neuf acides aminés ajoutés à des cultures en milieu solide de *Ceratocystis fimbriata*, il a été démontré que la leucine et la valine favorisent la production de CO_2 par le micro-organisme, ainsi que la production de composés volatils. Ces deux paramètres ont été corrélés à la concentration de l'acide aminé ajouté à l'aide d'une équation de type Monod (modèle de saturation). Vingt quatre composés ont été séparés par chromatographie en phase gazeuse, parmi lesquels l'acétaldéhyde, l'éthanol, l'acétate d'éthyle, le propionate d'éthyle et l'acétate de 3-méthyl butanol, sont présents à des concentrations significatives.

Mots clés : *bioconversion, précurseur, arôme, fermentation en milieu solide.*

SUMMARY

Among nine amino acids added to solid state cultures of *Ceratocystis fimbriata*, leucine and valine were found to promote growth and volatile compound production. Both parameters could be correlated with the concentration of the amino acid added, according to a Monod-like equation. Twenty four compounds were separated by GC analysis. Among them, acetaldehyde, ethanol, ethyl acetate, ethyl propionate and 3-methylbutyl acetate were found in significant amounts.

Key-words: *bioconversion, precursor, flavour, solid state fermentation.*

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1 - INTRODUCTION

Microorganisms, especially fungi, can be an alternative for producing flavouring agents (JANSSENS *et al.*, 1992) and, as outlined by BERGER (1995) and SEITZ (1995), solid state systems can be suitable for this purpose. Among the different natural solid substrates that can be used to cultivate microorganisms, cassava bagasse, an agro-industrial residue available in large amounts in the inter-tropical area, has previously been reported for the production of single cell protein (RAIMBAULT *et al.*, 1985) or lactic acid (SOCCOL *et al.*, 1994). In a previous report, the ability of the fungus *Ceratocystis fimbriata* to produce, in liquid culture, a large number of volatile compounds with a fruity flavour, has been shown (CHRISTEN *et al.*, 1994). Further studies demonstrated that this strain could grow and produce aromas on other substrates such as wheat bran (CHRISTEN *et al.*, 1997). Moreover, amino acids have been reported as potential precursors to overproduce flavouring metabolites (SPINNLER et DJIAN, 1991; FABRE *et al.*, 1996).

The aim of this work is to investigate the influence of the addition of various amino acids on both growth and flavouring compound production by *Ceratocystis fimbriata* in solid state culture. An attempt to correlate the amounts of volatiles produced to the concentration of the amino acids added was carried out.

2 - MATERIAL AND METHODS

2.1 Organism and culture media

Ceratocystis fimbriata CBS 374-83 (ELLIS and HALSTED) was used as a flavour producing fungal strain. It was periodically transferred onto Potato Dextrose Agar (PDA) slants and stored at 4°C. Spores were produced on PDA after 5 days of culture at 30°C. They were collected in sterile water solution containing 0.1% Tween 80 and small glass beads. After cell counting in a Neubauer chamber, the solution provided 1×10^7 spores$\cdot g^{-1}$ initial dry matter (IDM).

The substrates used were wheat bran and cassava bagasse (donated by Pr. C.R. SOCCOL, UFPR, Brazil), a high starch-content tuber (more than 63% according to CEREDA (1994)). They were milled and sieved through –20+40 mesh screens to obtain particles of 0.42 to 0.82 mm in diameter and then sterilized at about 1 bar for 15 min. Experiments were performed in 250 mL-Erlenmeyer flasks covered with six layers of gauze, without forced aeration (surface culture). These were filled with 5.25 g IDM. For all experiments, initial conditions were as follows: temperature, 30°C; pH, 6.0; inoculum size, 1×10^7 spores$\cdot g^{-1}$ IDM. Initial water content was calculated according to the maximum absorption capacity of the substrates (wheat bran 50% (w:w), and cassava bagasse 65% (w:w)). These substrates were complemented with an oligoelement solution having the following composition: $Fe(NO_3)_3.9H_2O$, 723.8 mg$\cdot L^{-1}$; $ZnSO_4.7H_2O$, 439.8 mg$\cdot L^{-1}$;

$MnSO_4.4H_2O$, 203 mg·L^{-1}. Amino acids were sterilized separately and added to the solid medium at the same time as the oligoelement solution. The concentrations reported refer to litre of water added.

As a control, the headspace analysis of a ripe banana was performed under the same conditions as the solid state culture experiments. For this purpose, 20 g of sliced ripe banana were equilibrated with air for 1 h at 30°C.

For both CO_2 and volatile analysis, samples were manually taken from the headspace above the culture at 4 cm below the gauze layers. Each determination was duplicated and average values were reported.

2.2 Analytical methods

The odours of the cultures were qualitatively estimated by sensorial evaluation with a non-trained panel consisting of four members, with no restriction in descriptive terms for the quality of the aroma. For aroma intensity, panel members were asked to choose between the following options ("scoring test"): – none, + weak, ++ medium, +++ strong. Before each evaluation, a comparison with a non-inoculated control flask was made.

As reported previously by MITCHELL (1992) and AURIA *et al.* (1995), CO_2 production in the headspace above the culture is a good indicator for growth. Its evolution was followed by gas chromatography according to CHRISTEN *et al.* (1994).

Volatile compounds in the headspace of the cultures were analysed by injecting 2-mL sample in a Hewlett-Packard 5890 gas chromatograph equipped with a Megabore HP-1 column (length: 5 m, inner diameter: 0.53 mm) and with a flame ionization detector. Conditions were: temperatures, injector and detector: 210°C, oven held at 40°C for 2 min and then programmed at + 10°C·min^{-1} to 150°C. The nitrogen gas flow rate was 1.5 mL·min^{-1} and the split ratio was 1:32. The compounds were identified according to the retention time of the pure compound. Total volatiles produced were expressed as µmol ethanol equivalent per liter of air.

For both CO_2 and volatile production, the raw data were integrated with respect to time, corrected with the initial dry matter (5.25 g) and the real volume of the headspace (0.375 L) and results were expressed as L·g^{-1} IDM and mmol·g^{-1} IDM for CO_2 and total volatiles, respectively.

3 - RESULTS AND DISCUSSION

3.1 Screening of amino acids

Wheat bran was used for this part of the experiment because, as previously demonstrated, no aroma was detected when *C. fimbriata* was cultivated on this subtrate (CHRISTEN *et al.*, 1997). The diversity of aromas detected according to the amino acids added can be seen in *table 1*.

Table 1

Sensorial evaluation of the aromas detected in 2-day cultures of C. fimbriata *grown on wheat bran complemented with amino acids*

Amino acid	–[2]	Arg	His	Leu	Lys	Met	Phe	Thr	Trp	Val
Aroma & intensity[1]	–	–	–	Banana +++	Sour +, Rancid +	Fruity +	Floral +, Grassy +	Fruity +, Sweet ++	Floral +, Grassy +	Banana +++

1. – none, + weak, ++ medium, +++ strong. 2. Control without addition.

Arg: Arginine; His: Histidine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Thr: Threonine; Trp: Tryptophan; Val: Valine.

Only arginine and histidine did not promote the production of aroma. Aromatic amino acids (tryptophane and phenyl alanine) gave a floral/grassy aroma of low intensity. Those derived from pyruvate (leucine and valine) gave a strong banana aroma attaining its maximum after 2 d of culture. With those derived from aspartate, a weak fruity aroma (methionine and threonine) or sour/rancid aroma (lysine) was obtained. Hence, the origin of the amino acid added has an influence on the aroma detected. Moreover, it was found that leucine and valine were the most efficient for flavour production. Consequently, the influence of the concentration of these two amino acids on both growth and volatile compound production is discussed below.

3.2 Effect of leucine and valine concentration

Different concentrations of these amino acids from 1.1 to 166.5 mmol·L^{-1} were tested. The solid substrate used for these experiments was cassava bagasse because it allowed a better fungal growth than wheat bran (CHRISTEN *et al.*, 1997). Results for aroma perception are presented in *table 2*.

Table 2

Evaluation of the intensity of the banana aroma detected in cultures of C. fimbriata *complemented with different concentrations of leucine and valine. Comparison with real banana*

Amino acid conc. (mmol·L^{-1})	No	Leucine			Valine			Banana[4]
		1.1	41.6	166.5	1.1	41.6	166.5	
Intensity[2]	–	++	+++	+++	++	+++	+++	+++
T max (h)[3]	–	61	60	58	71	61	56	–

1. Control without addition.
2. – none, + weak, ++ medium, +++ strong.
3. T max: time of maximum perception of the aroma.
4. Refers to the experiment achieved with 20 g of sliced banana.

The aromas detected were qualified by the panel as banana-like for both amino acids. They were detected with increasing intensity when increasing amounts were added, from weak, when no amino acid was added, to strong for a concentration of 41.6 mmol·L^{-1}. No gain in aroma intensity was noticed when more amino acid was added.

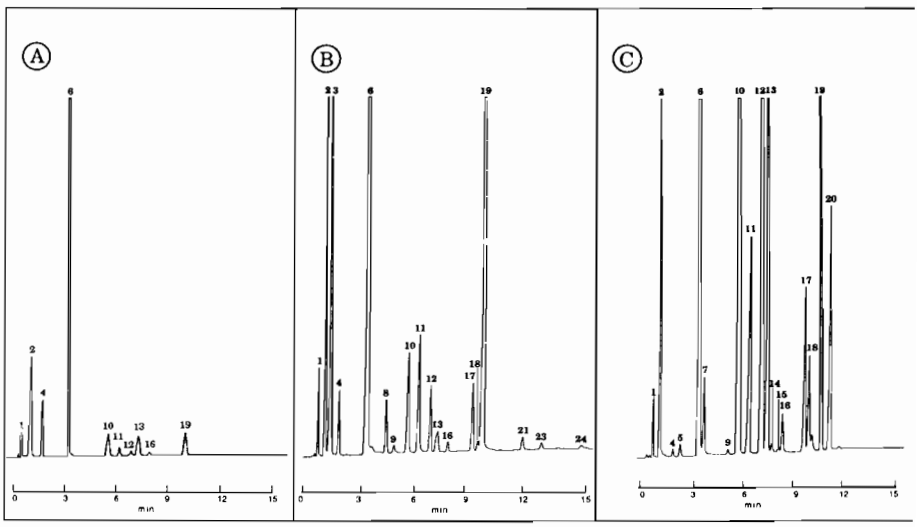

Figure 1

Headspace chromatograms of 2-day culture of C. fimbriata *on cassava bagasse (A), cassava bagasse + 166.5 mmol·L^{-1} leucine (B), cassava bagasse + 166.5 mmol·L^{-1} valine (C)*

The compounds are: 1, acetaldehyde; 2, ethanol; 3, acetone; 4, 2-propanol; 5, 1-propanol; 6, ethyl acetate; 7, 2-methyl propanol; 8, 1-butanol; 9, unknown; 10, ethyl propionate; 11, 3-methyl butanol; 12, 2-hexanone; 13, 2-hexanol; 14, 2-methylpropyl acetate; 15, unknown; 16, ethyl butyrate; 17, 2-heptanone; 18, unknown; 19, 3-methylbutyl acetate; 20, ethyl pentanoate; 21, 2-octanone; 22, 2-octanol; 23, ethyl hexanoate; 24, unknown

When leucine *(figure 1b)* or valine *(figure 1c)* was added, there was an increase in the number of compounds detected (18 and 19 respectively) against 10 compounds with the non supplemented cassava *(figure 1a)*. Among the compounds separated in this case, acetaldehyde, ethanol, 2-propanol, ethyl acetate, ethyl propionate, 3-methylbutanol (3-MB) and 3-methylbutyl acetate (3-MBA) were identified. Their maximum concentrations are reported in *table 3*.

Table 3

Maximum concentrations (in µmol·L^{-1} air) of some compounds in the headspace of cultures of C. fimbriata

Compound	Cassava	Cassava + leucine*	Cassava + valine*
Acetaldehyde	1.0	20.0	16.5
Ethanol	4.0	57.9	83.6
Ethyl acetate	23.2	131	155
Ethyl propionate	0.6	3.4	16.1
3-MB	0.3	5.3	6.4
3-MBA	0.5	47.6	43.4

* Added at a concentration of 166.5 mmol·L^{-1}.

With leucine, the concentration of these compounds increased drastically, especially ethanol, ethyl acetate, ethyl propionate, 3-MB and 3-MBA, the last two being of great importance in the banana aroma. Compounds such as acetone, 1-butanol and heptanone were also detected. In spite of the similar aroma detected in both cultures, the headspace chromatogram obtained with valine presented some differences *(figure 1c)*. Some compounds such as acetone, or 1-butanol were not found but others such as 1-propanol, 2-methylpropanol (2-MP) or ethyl pentanoate appeared. Moreover, a strong increase in ethyl propionate, 3-MB, 2-hexanone and 2-hexanol was observed. As for the bioconversion of amino acids into alcohols and esters by *Erwinia carotovora* (SPINNLER and DJIAN, 1991), it can be assumed that 2-MP and 3-MB come from the Erlich's pathway (decarboxylation and deamination) of valine and leucine, respectively. These two alcohols are then esterified with acetic acid to form 2-methylpropyl acetate (2-MPA) and 3-MBA, respectively. However, some 3-MB and 3-MBA are present in the headspace of cultures made with valine, which indicates that other metabolic routes are probably involved in the biosynthesis of these compounds.

Accumulated (*i.e.* integrated) CO_2 and volatile productions were correlated with the amino acid concentration by a Monod-like equation as follows:

$$[P] = m_0 + \frac{m_1 \cdot [A]}{m_2 + [A]}$$

where:
[P]: concentration of accumulated CO_2 ($L \cdot g^{-1}$ IDM) or volatile compounds ($mmol \cdot g^{-1}$ IDM)
[A]: concentration of amino acid (leucine or valine) added ($mmol \cdot L^{-1}$)
m_0, m_1, m_2 are the fitting coefficients

The results are given in *figures 2* and *3* for CO_2 and volatile production, respectively.

The fitting of the model with the experimental data is satisfactory, as can be seen with the R^2 coefficients *(table 4)*. In both cases, growth, as indicated by CO_2 production is highly promoted by the addition of these N-containing compounds. This model was also successfully applied to the production of volatile compounds. Valine promoted the production more than leucine for both CO_2 and volatiles (see m_1 coefficients).

Relation between growth and volatile metabolite production

For each concentration of amino acid, the maximum of total volatile metabolites [V] is plotted against maximum of CO_2 production *(figure 4)*.

A linear relation exists between these two parameters:

For leucine:

$$[V] = -0.97 + 29.3\,[CO_2] \quad (R^2 = 0.986)$$

For valine:

$$[V] = -0.65 + 38.5\,[CO_2] \quad (R^2 = 0.975)$$

The higher value of the slope observed for valine means that this amino acid promotes the volatile production more than leucine does. The negative values of

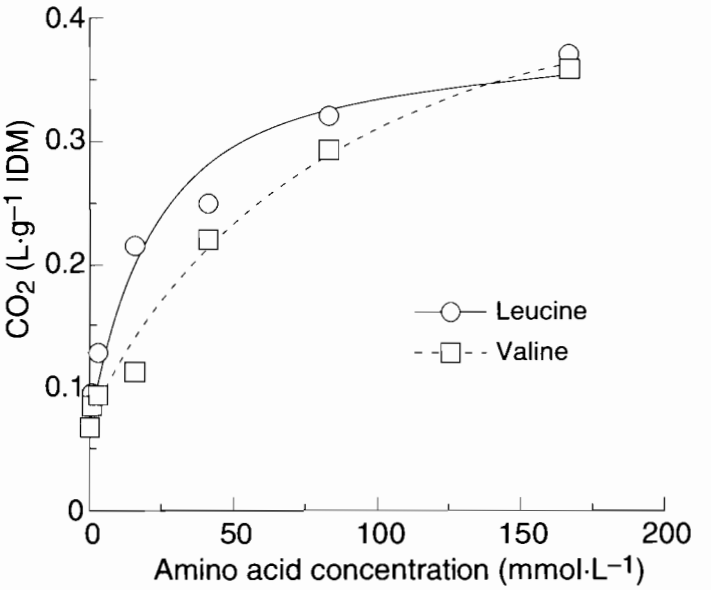

Figure 2

Accumulated CO_2 versus amino acid concentration in solid state cultures of C. fimbriata

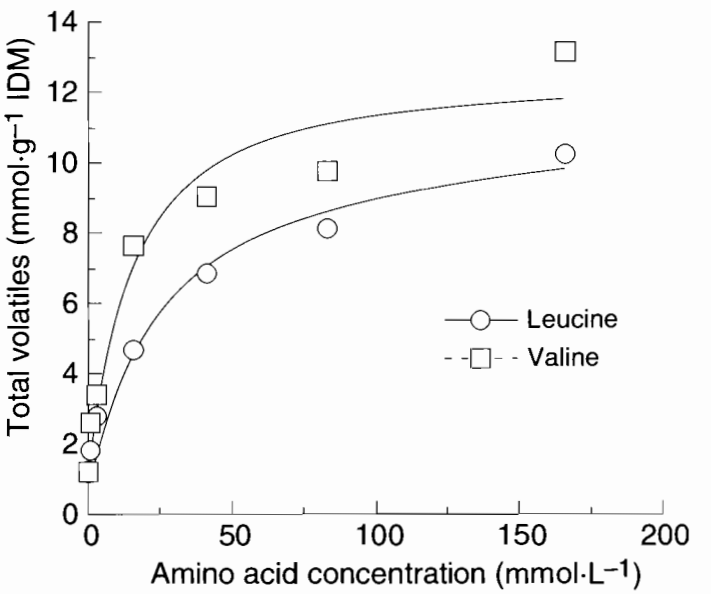

Figure 3

Accumulated total volatiles versus amino acid concentration in solid state cultures of C. fimbriata

Table 4

Fitting parameters of the Monod-like equation for CO_2 and volatiles production

	CO_2 production				Volatiles production			
	m_0	m_1	m_2	R^2	m_0	m_1	m_2	R^2
Leucine	0.067	0.325	21.8	0.976	1.22	10.31	32.8	0.986
Valine	0.067	0.450	86.7	0.987	1.22	11.60	15.3	0.961

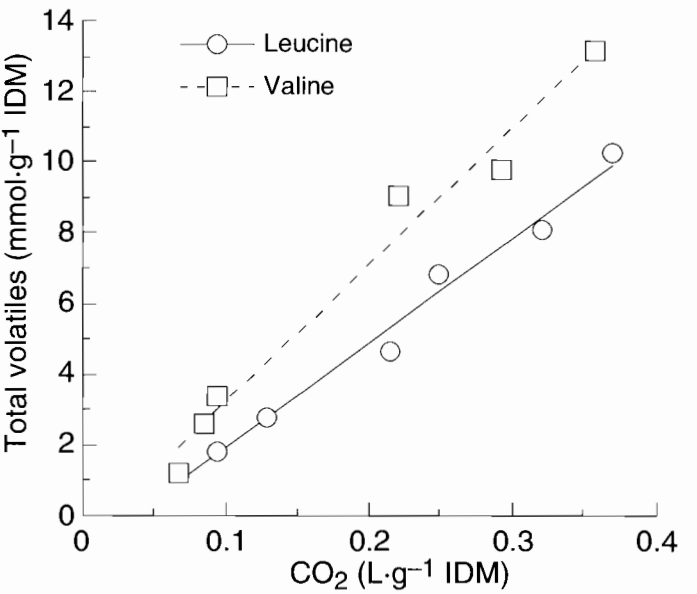

Figure 4

Relationship between carbon dioxide and volatiles metabolite production

the abscisse at the origin correspond to the fact that the volatile production cycle begins after the CO_2 production. The fitting correlation between volatile compounds and CO_2 production means that the amount of volatiles produced can be predicted from the data of CO_2 production. This was also reported by BÖRJESSON *et al.* (1990) for a *Penicillium* strain grown on solid substrates.

4 - CONCLUSION

Valine and leucine promoted CO_2 production which arose from respiratory activity and probably enhance the bioconversion of these amino acids into flavouring compounds. They increased the production of volatile compounds mainly the C4-C5 alcohols and their corresponding acetate esters, which in turn gave strong banana flavours. The relation between both CO_2 and volatiles produced and the concentration of added amino acid can be successfully described by a simple saturation model. As a consequence, it was shown that volatile production was proportional to the amount of CO_2 produced.

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