ELSEVIER

Process Biochemistry 35 (2000) 857–861

PROCESS BIOCHEMISTRY

www.elsevier.com/locate/procbio

Fruity flavour production by *Ceratocystis fimbriata* grown on coffee husk in solid-state fermentation

Marlene Soares [a,1], Pierre Christen [b,*], Ashok Pandey [a], Carlos Ricardo Soccol [a]

[a] *Laboratorio de Processos Biotecnológicos, Departamento de Engenhería Química, Universidade Federal do Paraná, CEP 81531-970, Curitaba, Brazil*

[b] *Laboratoire de Microbiologie, IRD, Université de Provence, 163 Avenue de Luminy, 13288 Marseille cedex 09, France*

Received 30 August 1999; received in revised form 30 September 1999; accepted 14 November 1999

Abstract

Ceratocystis fimbriata was grown for flavour production, on steam treated coffee husk supplemented with glucose. Solid media with 20 and 35% glucose, developed a strong pineapple aroma, resulting in the production of 6.58 and 5.24 mmol/l per gram total volatiles (TV), respectively. Compounds such as acetaldehyde, ethanol, isopropanol, ethyl acetate (representing 80.5 and 75.4% of TV, respectively), ethyl isobutyrate, isobutyl acetate, isoamyl acetate and ethyl-3-hexanoate were identified in the headspace of the cultures. At 46% glucose, only a weak odour of banana was detected, and TV production was poor. The addition of leucine increased TV production (8.29 mmol/l per gram), especially for ethyl acetate and isoamyl acetate and a strong banana odour was detected. The biosynthesis of volatile compounds was not improved by the addition of soybean oil and was reduced by that of mineral salts.

Keywords: *Ceratocystis fimbriata*; Coffee husk; Flavour compounds; Gompertz model; Solid-state fermentation

1. Introduction

Most food flavouring compounds are produced via chemical synthesis or extraction from natural material. However, recent market surveys have demonstrated that consumers prefer foodstuff that can be labeled as natural. Plants have been major sources of essential oils and flavours but their use depends on factors difficult to control, such as weather conditions and plant diseases. An alternative route for flavour production is based on microbial biosynthesis or bioconversion [1,2]. Solid-state reactors offer a high potential for this purpose [3].

Fungi from the genus *Ceratocystis* produce a large range of fruit-like or flower-like aromas (peach, pineapple, banana, citrus and rose), depending on the strain [4] and the culture conditions [5–7]. Among this genus, *Ceratocystis fimbriata* has a great potential for ester synthesis [6]. Moreover, it grows rapidly, has a good ability to sporulate and produces a wide variety of aromas [4,8].

Solid-state fermentation (SSF) has been considered as a useful tool for biomass energy conservation, solid waste treatment and production of added-value molecules such as enzymes, organic acids and biologically active secondary metabolises [9–12]. One of the major advantages that SSF offers is in the utilization of agro-industrial residues, which have no other practical applications [12,13]. One of these residues is coffee husk, which is generated in large quantities during the dry-process of coffee cheries. Due to the presence of anti-nutritional factors such as caffeine and tannins, coffee husk does not find any potential application and its disposal is a serious problem in coffee producing countries. Attempts have been made to use it for edible fungi cultivation [14,15], and microbial production of enzymes [16] or organic acids [17].

In this work, solid-state fermentation of coffee husk with glucose added was tested for production of fruity flavours by *C. fimbriata*. Attempts were made to enhance the yields of aroma compounds by supplement-

* Corresponding author. Fax: +33-491-82-8570.

E-mail address: christen@esil.univ-mrs.fr (P. Christen)

[1] Present address: CEFET-PR UNED, Ponta Grossa, Brazil.

0032-9592/00/$ - see front matter
PII: S0032-9592(99)00144-2

ing the fermentation medium with precursors or saline solution.

2. Material and methods

2.1. Microorganism and inoculum

C. fimbriata CBS 374-83 was grown and periodically transferred onto Potato Dextrose Agar (PDA) medium, and stored at 4°C. A spore suspension was prepared after 5 days of culture at 30°C in 250-ml Erlenmeyer flasks. Spores were collected with sterile distilled water containing a few drops of Tween 80 and small glass beads.

2.2. Preparation of the substrate

Two hundred grams of milled coffee husk (0.4–0.8 mm diem.) in 1 l of distilled water were steam treated at 100°C during 40 min. After filtration through Whatman paper no. 1, the liquid fraction and the solid residue were separated. A previous study showed that the solid residue was the most appropriate substrate for growth and aroma production as compared with liquid fraction and the untreated coffee husk, probably because some inhibitory compounds (caffeine) present in the coffee husk were eliminated by the steam treatment [18].

2.3. Fermentation procedure

Experiments were conducted in 250-ml Erlenmeyer flasks, containing 15 g of substrate (dry weight basis) and covered with eight layers of gauze. For all experiments, initial conditions were: temperature, 30°C; pH, 6; inoculum size, 1×10^7 spores/g dry matter (d.m.), and initial water content, 70% (v/w) (maximum absorption of the substrate). Experiments were performed to study the effect of glucose addition (20, 35 and 46 g/100 g d.m.) on the production of volatile compounds, and also the effect of addition of some possible precursors: leucine (10 mmol), soya bean oil (10% v/w)) and a saline solution, previously optimized [19]. All experiments were conducted in three sets.

2.4. Analytical procedures

The dynamics of volatile compound production was followed by sensorial evaluation (olfactometry) with a four member non-trained panel and by gas chromatography (GC). The compounds in the headspace of the cultures were identified by comparing their retention time with those of standards. GC analysis was performed with a Hewlett-Packard 6890 apparatus equipped with a flame ionization detector and a 30 m Megabore HP-5 column. The injector and detector temperature was 250°C. The oven temperature program was 40°C for 6 min followed by a rise of 25°C/min up to 150°C, maintained for 2 min. The helium gas flow rate was 1.5 ml/min and the split ratio was 1:50. Total volatiles (TV), aldehydes, alcohols, and ketones were expressed as micromole ethanol equivalent per litre of headspace per gram of d.m. (μmol/l per gram) and total esters as micromole ethyl acetate equivalent (μmol/l per gram), both from standard curves. The data reported are the balance between accumulated production and losses by passive diffusion out of the flask. For this reason, samples were taken precisely 4 cm below the gauze layers. Each injection was duplicated.

2.5. Data analysis

Raw data were integrated to calculate the TV accumulated during fermentation. The Gompertz model, a logistic-like equation, was used to fit the integrated data, as previously reported [6]. This model described the dynamics of the production with respect to time as follows:

$$TV = TV_{max} \exp\{-b \exp(-kt)\}$$

where:

TV: total volatile production at time t (mmol/l per gram)

TV_{max}: maximum production of total volatile when $t \rightarrow \infty$ (mmol/l per gram)

b: fitting parameter. It is used to obtain the time of maximum production rate (t_{max}) as follows:

$$t_{max} = (\ln b)/k$$

k: production rate (/h)

Data integration and non-linear Gompertz regression were made with a KaleidaGraph program (Abelbeck Software, USA).

3. Results and discussion

3.1. Effect of glucose concentration

Table 1 shows the results of SSF under different experimental conditions. The odour detected in the headspace of the culture depended on the amount of glucose added. At the highest glucose concentration, a limiting effect was observed in terms of aroma intensity.

Fig. 1a shows the TV dynamics in the headspace. At the lowest glucose concentration (run 1), the production was faster, attaining a maximum around 28 μmol/l per gram after 40 h, which was maintained at same level for >140 h. At intermediate glucose concentration (run 2), the increase in TV was slower and the

Table 1
Sensorial evaluation of culture headspace and kinetic constants of the Gompertz model for different culture conditions with *C. fimbriata* grown on coffee husk

Run	[Glucose] (g/100 g d.m.)	Addition[a]	Aroma and intensity[b]	TV_{max} (mmol/l per gram)	k (/h)	t_{max} (h)	R^{2c}
1	20	–	Pineapple +++	6.58	0.0123	123	0.997
2	35	–	Fruity, pineapple +++	5.24	0.0105	214	0.996
3	46	–	Banana, sweet +	1.13	0.0080	169	0.997
4	35	ss	Banana +	2.71	0.0067	288	0.994
5	35	so	Banana ++	5.7	0.0085	234	0.996
6	35	leu	Banana +++	8.29	0.0098	225	0.997

[a] ss, salt solution; so, soybean oil; leu, leucine.
[b] Aroma intensity: weak (+), medium (++), strong (+++).
[c] R^2, correlation coefficient.

maximum (24 μmol/l per gram) was attained after 280 h. This could be due to a decrease in water activity in relation to the high glucose concentration. It could also be partly explained by a catabolic repression due to the carbon source. This phenomenon was amplified at the highest glucose concentration (run 3), where the TV remained very low throughout the experiment. This correlated with the olfactometric observations (Table 1). As reflected by the nature and the intensity of the odours detected, it seems that glucose concentration had a direct influence on the metabolic pathways and thus on the nature of the volatile compounds produced. This was confirmed by the distribution of each compound in the headspace, reported for the four most productive experiments (Table 2). In run 1, one aldehyde, two alcohols and seven esters, from acetic, butyric, isobutyric and hexanoic acids, were identified. Among these, ethanol (12.7%) and ethyl acetate (80.5%) were the most abundant. Ethyl acetate is known to be responsible for the 'fruity' note, and is present in most fruits. For run 2, the proportion of ethanol was higher and that of ethyl acetate was lower, probably due to a repression of the esterifying enzyme system. The number of esters found in the headspace was also lower than in run 1, but a ketone (2-octanone) was detected in significant amounts, maybe coming from the oxidation of fatty acids present in the substrate. In both cases, *C. fimbriata* showed a high potential for ester production.

The application of the Gompertz model to the raw data confirmed that maximum TV production and highest k were observed for run 1 (Table 1 and Fig. 2). As the amount of glucose added increased, TV_{max} and k decreased. In all cases, the fitting of the model to the experimental data was excellent ($R^2 > 0.994$)

When compared to other solid wastes or substrates, coffee husk amended with glucose appears to be a better material for aroma production in solid-state fungal cultures. For example, using sugar cane bagasse and the same conditions, it was found that the production was much faster ($k = 0.041$ /h), but the TV_{max} only reached values of 0.3 mmol/l per gram [6]. The same authors also reported TV_{max} values of 0.09 and 0.32 mmol/l per gram on wheat bran and cassava bagasse, respectively [6].

3.2. Effect of the addition of salt solution, soybean oil or leucine

Studies on the effects of addition of a salt solution, soybean oil and leucine were undertaken with a view to

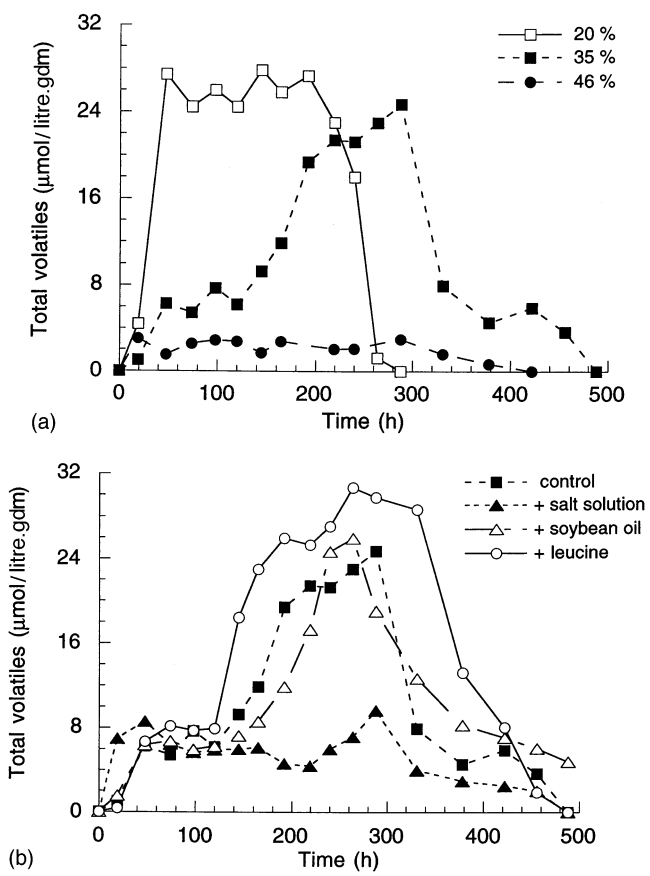

Fig. 1. (a) Dynamics of TV compounds measured in the headspace of the cultures supplemented with different glucose concentrations. (b) Dynamics of TV compounds measured in the headspace of the cultures supplemented with different compounds.

Table 2
Production of flavour compounds in the most productive experiments. Values reported represent the percentage of TV.

	Glu 20%	Glu 35%	Glu 35% +so	Glu 35% +leu
Aldehyde	1.8	2.4	2.3	1.4
Acetaldehyde	1.8	2.4	2.3	1.4
Alcohols	14	17.8	24	16.2
Ethanol	12.7	17.6	24	15.9
Isopropanol	1.3	0.2	nd[b]	0.3
Esters	84.2	77.1	73.7	81.4
Ethyl acetate	80.5	75.4	72.6	79
Ethyl isobutyrate	0.4	0.4	0.3	0.4
Isobutyl acetate	0.7	0.8	0.6	1
Ethyl butyrate	0.3	0.1	0.1	0.6
Isoamyl acetate	0.1	0.4	nd[b]	0.4
Propyl acetate	0.2	nd[b]	nd[b]	nd[b]
Ethyl-3-hexanoate	2	nd[b]	0.1	0.1
Ketones	nd[b]	2.7	nd[b]	0.9
2-Heptanone	nd[b]	nd[b]	nd[b]	0.1
2-Octanone	nd[b]	2.7	nd[b]	0.8

[b] nd, not detected.

examine if the solid substrate lacked some minerals, or if TV production could be enhanced by precursors like fatty acids of soybean oil (that can be transformed into methyl ketones by some *Penicillium* species [20]) or leucine (direct precursor of some volatile esters [7]). This study was conducted at an initial glucose concentration of 35%.

A banana aroma was detected in the headspace of the cultures for each experiment (runs 4, 5 and 6) unlike the control made without any complement (run 2). The intensity of the aroma depended on the compound added (Table 1). The dynamics of TV in the headspace presented similar shape for each run (Fig. 1b). After a short lag phase, the concentration increased for 40 h, then remained constant around a value of 7 μmol/l per gram for ≈ 100 h and increased again to reach its maxima around 280 h. The maxima attained also depended on the complement. Soybean oil did not display any effect on the volatile production (run 5) when compared with the control, but leucine increased it slightly (run 6) and the salt solution displayed a strong negative effect (run 4). This was confirmed by the fitting parameters of the Gompertz model (Table 1). The addition of the salt solution decreased k and the TV_{max}. As this mineral medium solution was especially optimized for aroma production by this fungus [19], this decrease could be attributed to an inhibition by a cumulative effect of the salts present in the coffee husk and those added. After the steam treatment, the coffee husk lost 30% salts and, as previously observed, this treatment improved the TV production [14]. However, this study does not allow a conclusion that this negative effect influenced growth or the metabolic pathways involved in the generation of the volatile compounds. The addition of soybean oil produced k and TV_{max} values close to those obtained for the control. There was apparently no effect of this amendment, which means that the fungus could not use this substrate for its primary metabolism, nor as a precursor of methyl ketones synthesis [20]. On the contrary, leucine increased significantly the production of volatiles in the headspace, but has no effect on k, which indicated that it was not used as carbon or nitrogen source, but only as precursor for aroma synthesis. Leucine favoured the synthesis of esters, particularly ethyl acetate and to a lesser level isoamyl acetate (Table 2). In contrast to experiments with other solid substrates amended with leucine [6,7], the increase in other ester production was not significant.

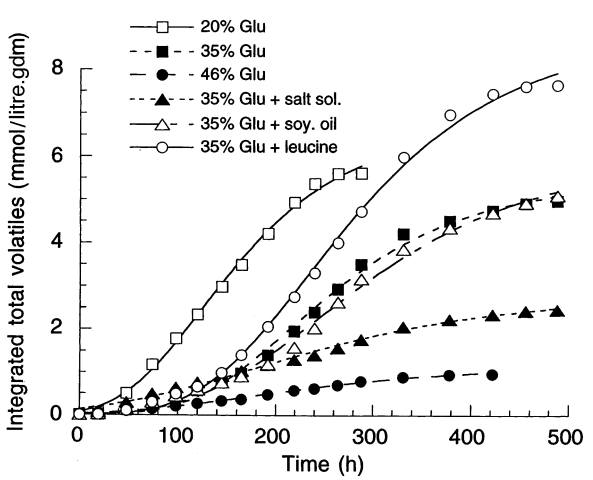

Fig. 2. Dynamics of integrated TV data. Symbols represent experimental data and curves, the Gompertz model.

4. Conclusion

Steam treated coffee husk is an adequate substrate for aroma production by *C. fimbriata*. Glucose addition was necessary for this purpose and the optimal concentration is ≈20 g/100 g d.m. The production maintained six days at its maximum, which was a significant improvement when compared with results reported for other solid substrates [6]. The addition of leucine improved TV production by 58%, especially ester production, but the addition of a mineral salt solution was unnecessary. Steam treatment applied to the coffee husk probably brought all the minerals needed by the *C. fimbriata*. It could not utilize the fatty acids of soybean oil. The Gompertz model confirmed its accuracy for describing the dynamics of flavouring compounds production in SSF.

Acknowledgements

Financial assistance from the European Union (grant no. INCO DC: IC18*CT 970185) and IRD, France is gratefully acknowledged. CRS thanks CNPq for a scholarship under Scientific Productivity Scheme. We thank Dr P. Roger (IRD, France) for his helpful comments.

References

[1] Welsh FW, Murray WD, Williams RE. Microbiological and enzymatic production of flavor and fragrance chemicals. Crit Rev Biotechnol 1989;9:105–69.
[2] Janssens L, de Pooter HL, Vandamme EJ, Schamp NM. Production of flavors by microorganisms. Process Biochem 1992;27:195–215.
[3] Berger RG. Aroma Biotechnology. Berlin: Springer, 1995.
[4] Senemaud C, Les champignons filamenteux producteurs d'arômes fruités: etudes de faisabilité sur substrats agro-industriels. Ph.D Thesis, Université de Bourgogne, 1988.
[5] Lanza E, Ko KH, Palmer JK. Aroma production by cultures of *Ceratocystis moniliformis*. J Agric Food Chem 1976;24:1247–50.
[6] Christen P, Meza JC, Revah S. Fruity aroma production in solid-state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors. Mycol Res 1997;101:911–9.
[7] Meza JC, Christen P, Revah S. Effect of added amino acids on the production of a fruity aroma by *Ceratocystis fimbriata*. Sci Aliment 1998;18:627–36.
[8] Christen P, Villegas E, Revah S. Growth and aroma production by *Ceratocystis fimbriata* in various fermentation media. Biotechnol Lett 1994;16:1183–8.
[9] Pandey A. Recent developments in solid-state fermentation. Process Biochem 1992;27:109–17.
[10] Pandey A. Solid-state fermentation. In: Pandey A, editor. Solid-state Fermentation. New Delhi: Wiley, 1994:3–10.
[11] Pandey A, Soccol CR. Bioconversion of biomass — a case study of lignocellulosics bioconversions in solid-state fermentation. Braz Arch Biol Technol 1998;41:379–90.
[12] Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid-state fermentation for the production of industrial enzymes. Curr Sci 1999;77:149–62.
[13] Pandey A, Soccol CR, Nigam P, Soccol VT. Biotechnological potential of agro-industrial residues: sugarcane bagasse, Bioresour Technol 1999 (accepted).
[14] Martinez-Carrera D, Morales P, Sobal M. Cultivation of several Mexican strains of *Pleurotus ostreatus* on coffee pulp and barley straw. Rev Mex Mycol 1988;4:153–60.
[15] Leifa F, Pandey A, Soccol CR. Solid-state fermentation and fructification of *Pleurotus ostreatus* on the residues of coffee. Paper presented at the 3rd International Seminar on Biotechnology in the Coffee Agroindustry, Londrina, PR, Brazil, 24–28 May 1999.
[16] Boccas F, Roussos S, Gutierrez M, Serrano L, Viniegra G. Production of pectinase from coffee pulp in solid-state fermentation system: selection of wild fungal isolate of high potency by a simple three step screening technique. J Food Sci Technol 1994;31:22–6.
[17] Vandenberghe LPS, Pandey A, Lebeault JM Soccol CR. Citric acid production by *Aspergillus niger* in solid-state fermentation on coffee husk. Paper presented at the 3rd International Seminar on Biotechnology in the Coffee Agroindustry, Londrina, PR, Brazil, 24–28 May 1999.
[18] Soares M. Biossintese de metabolitos volateis frutais por *Pachysolen tannophilus* e *Ceratocystis fimbriata* pela fermentaçao no estado sólido da casca de café. Ms.C thesis, Paraná Federal University, 1999.
[19] Christen P, Raimbault M. Optimization of culture medium for aroma production by *Ceratocystis fimbriata*. Biotechnol Lett 1991;13:521–6.
[20] Gatfield IL. Production of flavor and aroma compounds by biotechnology. Food Technol 1988;42:110–20.