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ORIGINAL ARTICLE

Production of aroma compounds by *Geotrichum candidum* on waste bread crumb

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After selection from eight yeast commercial or type strains based on their aromatic potential to valorize bread by-products, Geotrichum candidum *ATCC 62217 formed fruity aroma compounds (pineapple-like) on fermented waste bread (35% white bread crumb and 65% water). Fatty acids esters were identified, including ethyl esters of acetic acid, propionic acid, butyric acid and isobutyric acid. Their production corresponded to the stationary growth phase of the strain and, after 48 h, it was improved by agitation and, to a lesser extent, at 30°C compared to 25 or 20°C. Aromatic properties of the strain were linked to its ability to metabolize organic acids.*

Introduction

Flavors and fragrances constitute a world-wide market of US$7 billion a year, with a share of 25% of the food additives market (Armstrong and Yamazaki 1986). The consumer's preference for natural food additives is more important than ever. The use of biotechnology for the production of natural aroma compounds by fermentation or bioconversion using micro-organisms is an economic alternative to the difficult and expensive extraction from raw materials like plants (Janssens et al. 1992).

A fungus with aromatic properties and often referred to as a yeast, *Geotrichum candidum*, has been used for commercial cheese ripening (Jollivet et al. 1994). Some strains may produce fatty acids esters, often related to specific fruit aroma (Koizumi et al. 1982, Latrasse et al. 1987). Ester formation results from enzymatic activities such as alcohol acetyltransferase and carboxylic esters hydrolases, including lipases or esterases. Depending on the strain (Baillargeon et al. 1989), *G. candidum* is highly lipolytic with a whole range of substrate specificity (Jacobsen et al. 1990, Sidebottom et al. 1991). Its proteolytic activity may also form aroma compounds and has been partly characterized by Gueguen and Lenoir (1975a,b, 1976).

About 2–5% of commercial bread production is lost as a consequence of returns or production problems (Fallows and Wheelock 1982). Bakery products are exceptional growth media for micro-organisms, especially in North America where sugar and fat are normally part of their formulation (Gélinas et al. 1999). During bread dough fermentation, bakers' yeast form large quantities of ethanol and, in theory, it might be esterified with fatty acids by cheese ripening yeasts such as *G. candidum*, characterized by their high ability to metabolize organic

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acids. Organic acids are present in variable concentrations as a result of the metabolism of lactic acid bacteria present in flour. Calcium propionate, a mold inhibitor, is present in most commercial breads manufactured in North America and might act as a flavor precursor. The aim of this study was to select aroma-producing yeast strains on waste bread and to determine the effects of fermentation temperature, agitation and time on aroma production.

Materials and Methods

Waste bread analyses

White breads (Multi-Marques, Montreal, Quebec, Canada) and crumbled sweet-type breads (Les Plats du Chef, Pointe-Claire, Quebec, Canada) were used. White breads were ground with a Comil grinder (Quadro Engineering Inc., Waterloo, Ontario, Canada). Both bakery products were vacuum-packed in plastic bags and stored at -30°C. Crumb analyses were performed as described in Gélinas et al. (1999).

Micro-organisms

Eight yeast strains were tested including three strains from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and five commercial cheese ripening starters: (1) *Geotrichum candidum* ATCC 28129, (2) *Geotrichum candidum* ATCC 26321, (3) *Geotrichum candidum* ATCC 62217, (4) *Geotrichum Candidum* (Geo 15; Rhône-Poulenc, Boucherville, Quebec, Canada), (5) *Geotrichum candidum* (called 'Oidium lactis'; Rosell Institute, Montreal, Quebec, Canada), (6) *Debaryomyces hansenii* (DH; Rhône-Poulenc), (7) *Kluyveromyces lactis* (KL 71, Rhône-Poulenc), (8) *Cryptococcus albidus* (B2#3380; Rosell Institute).

Freeze-dried ATCC strains were rehydrated according to ATCC recommendations. Strains were then kept on Potato Dextrose Agar (PDA; BBL, Cockeyville, Maryland, USA) at 4°C under paraffin oil previously sterilized at 160°C for 2 h in an oven. Freeze-dried commercial yeast starters were kept at -20°C, except *Cryptococcus albidus* which was in the liquid form and was stored at 4°C.

Fermentation conditions

Commercial starters were used directly to seed media. For ATCC strains, a spore suspension was prepared from a sporulated culture on PDA (Essers et al. 1994), centrifuged for 15 min at 2800 *g* then washed twice with water and kept in water at 4°C until use. Colony forming units (cfu) were determined on PDA. One day before the experiments, bags containing bread crumbs were thawed at room temperature (about 22°C). Crumb was diluted to 35% in water and homogenized for 2 min with a mixer (model K45SS; Kitchen-Aid, St. Joseph, Michigan, USA, then 150 g of medium were placed in 250-ml Erlenmeyer and inoculated with 10^6 cfu g^{-1} of medium. In duplicate, two samples for each condition tested were incubated at 30°C and 300 rpm (Lab-Line Orbit Instruments Inc., Melrose Park, Illinois, USA) for 24 or 48 h, except where indicated.

Volatile compounds analyses

In duplicate, three extraction methods were used: headspace sampling, cold-finger trapping and solid-phase trapping. Headspace sampling was carried out over 5 g of fermented medium in a vial, using a Headspace sampler HP7694 (Hewlett-Packard, Avondale, Pennsylvania, USA). Identification was performed with a gas chromatograph (Model 3400; Varian Associates, Palo Alto, California, USA) coupled with a mass spectrometer Finnigan INCOS 50. Carrier gas (He) was injected at 1 ml min^{-1} and injection was in a split mode (50 : 1). Oven temperature was raised from 40 to 90°C at a rate of 3°C min^{-1}, up to 120°C at 10°C min^{-1} and up to 240°C at 20°C min^{-1}. A DB-WAX column (30 m × 0·25 mm i.d.) was used (J&W Scientific, Folsom, California, USA). For mass spectrometry, ionization was performed at 70 eV with 384 cycles s^{-1}.

Cold-finger trapping was performed on 150 g of medium in a 250-ml flask heated at about 60°C on a magnetic stirrer. A glass stick from a thermos containing liquid nitrogen was placed a few millimetres over the medium for

Table 1. Typical bread crumb composition (%, w/w)

Crumb type	Dry matter	Protein	Fat	Glucose	Fructose	Sodium chloride
White	62·5	14·4	3·6	1·09	1·79	2·3
Sweet	77·2	12·3	13·5	3·94	4·71	1·6

30 s. Condensate was recovered with 2 ml of methanol. The extraction process was performed 20 times with the same solution which was then filtered. Identification of aroma compounds was performed as described above for headspace sampling.

Solid-phase trapping was carried out on 10 g of medium diluted in 90 g of 0·05 M potassium phosphate buffer (pH 7·5) in a 250-ml Drechsel gas washing bottle (Quickfit; Pegasus, Agincourt, Ontario, Canada) with inlet made of No. 3 frittered glass. Sample was heated at 80°C for 7 min followed by a purge with 100 ml of air min^{-1} for 60 min. Volatile compounds were then trapped on a solid-phase silica cartridge (Silica Sep-Pak; Waters, Milford, Massachusetts, USA) previously conditioned with 6 ml of hexane. Trapped compounds were then recovered with 2 ml of cold acetone followed by filtration through a 0·2-μm filter. Identification was performed as described above for headspace sampling.

Quantification of volatile compounds was performed with a gas chromatograph (Model 6890; Hewlett Packard, Avondale, Pennsylvania, USA) equipped with a flame ionization detector (FID). A DB-FFAP column was used (30 m × 0·25 mm i.d.). Flow rate of gas was 2 ml min^{-1} with a split ratio of 30 : 1. Temperature was raised from 40 to 140°C at a rate of 15°C min^{-1}, up to 150°C at 1°C min^{-1} and up to 220°C at 30°C min^{-1}.

Organic acids analyses

Method described by Gélinas and Lachance (1995) was used except that HPLC analyses were performed with a Waters system (Model 510; Waters, Milford, Massachusetts, USA) equipped with an automatic injector (WISP Model 710B) and a UV detector (Model 286) at 210 nm. Tests were performed in duplicate on two samples.

Results and Discussion

Selection of microbial starter and bakery product

Table 1 presents the composition of the two bakery products tested for screening microbial starters. On the whole, sweet-type breads contained much more fat and sugar than white bread, but less salt. Especially with *G. candidum* starters, white bread gave more intense aroma than sweet-type bread, suggesting that high concentrations of sugar and fat in the growth media did not stimulate aroma production with this microbial species (data not shown). Under the tested conditions (35% bread crumb in water), most of the cultures containing *Geotrichum candidum* gave more pleasant aroma in both growth media, compared to the other strains. Starters not containing *G. candidum* formed bland or off-flavors and were discarded. Because *G. candidum* ATCC 62217 formed a very typical and intense fruity aroma under the tested conditions, it was retained for further study.

Identification of volatile aroma compounds

Using three extraction methods, 14 major aroma compounds formed during white bread crumb fermentation with *G. candidum* were identified (Table 2). Coupled with mass spectrometry, headspace analysis let to the identification of ten of these aroma compounds; solid-phase trapping/FID and coldfinger trapping/FID permitted to identify four and three volatile compounds, respectively.

Alcohols obtained by headspace sampling or cold-finger trapping are normally found in bread aroma (Hironaka 1986) and were less typical of fruity aroma formed by *G. candidum*. Because solid-phase trapping permitted to extract high concentrations of short-chain ethyl esters (ethyl acetate, ethyl propionate,

Table 2. Profile of volatile aroma compounds formed in white bread medium (35% solids) after fermentation for 48 h with *G. candidum* ATCC 62217 at 30°C and 300 rpm according to extraction method

Compound	Headspace	Cold-finger	Solid-phase	Aroma[a]
Ethanol	x			
Ethyl acetate	x		x	Pineapple, ethereal
Ethyl 2-butenoate	x			
Ethyl butyrate			x	Fruity, ethereal, banana, pineapple
Ethyl caproate	x			Fruity, wine, apple, banana, brandy
Ethyl isobutyrate			x	Citrus
Ethyl isovalerate	x			Fruity, apple on dilution
Ethyl propionate	x		x	Sweet, fruity, ethereal, rum
Ethyl valerate	x			Fruity, apple
2-hexenoic acid	x			Fatty, acid musty odor, fruity
Methyl-2 propanol-1	x	x		
Methyl-3 butanol-1		x		
Propanol-1		x		Alcohol, sweet
Propyl propionate	x			Oily, sherry

[a]As described in Anonymous (1997).

ethyl butyrate and ethyl isobutyrate), all typical fruity aroma, this extraction method was used for the rest of the study. These four esters were monitored to determine the effects of fermentation conditions on the activity of *G. candidum*.

Effect of fermentation conditions on the production of fruity aroma compounds

Production of aroma compounds was optimal after about 48 h (Fig. 1), which corresponded to the stationary phase of growth because, under these conditions, stationary phase of growth occurred between 16–24 h (data not shown). Among the four esters monitored during the course of these experiments, ethyl propionate was produced in the highest amounts with about 150 mg kg^{-1} of medium (Fig. 1(a)), followed by ethyl acetate (Fig. 1(b)) with about 50 mg kg^{-1}. About 30–50 and 10–25 mg kg^{-1} respectively of ethyl isobutyrate (Fig. 1(c)) and ethyl butyrate (Fig. 1(d)) were produced after fermentation for 48–72 h. These yields were considered very high and superior, for example, to the results of Koizumi et al. (1982) who reported the production of about 5 mg kg^{-1} of ethyl butyrate. In general, the production of volatile compounds was more rapid at 30°C than 20°C. These results confirm results obtained by Latrasse et al. (1987) and stress the effect of the strain on aroma production, considering that *G. candidum* ATCC 62217 formed more esters than alcohols compared to typical dairy strains (Jollivet et al. 1994).

From growth curves for *Geotrichum candidum* ATCC 62217 according to temperature and time, shaking was necessary for rapid cell growth which was much slower at 20°C than 25 or 30°C, showing the importance of air incorporation in the growth medium (data not shown). During fermentation, concentration of most of the organic acids tested dropped according to time and, in general, followed production of the ethyl esters (data not shown). After 72 h, the concentration of lactic acid and acetic acid increased much, probably as a result of the activity of contaminating bacteria in the non-sterile growth medium (data not shown).

In conclusion, *Geotrichum candidum* ATCC 62217 was selected among cheese ripening strains and other *G. candidum*-type strains and formed high concentrations of fruity aroma in a medium prepared with waste bread (35% solids). High concentrations of specific ethyl esters were produced after fermentation for 48–72 h, which corresponded to the stationary phase of growth and was related to the assimilation of organic acids. Aeration of the growth medium was essential and better results were obtained at 30°C compared to 20°C. Scale-up of the process is necessary to determine the economic potential of *G. candidum*

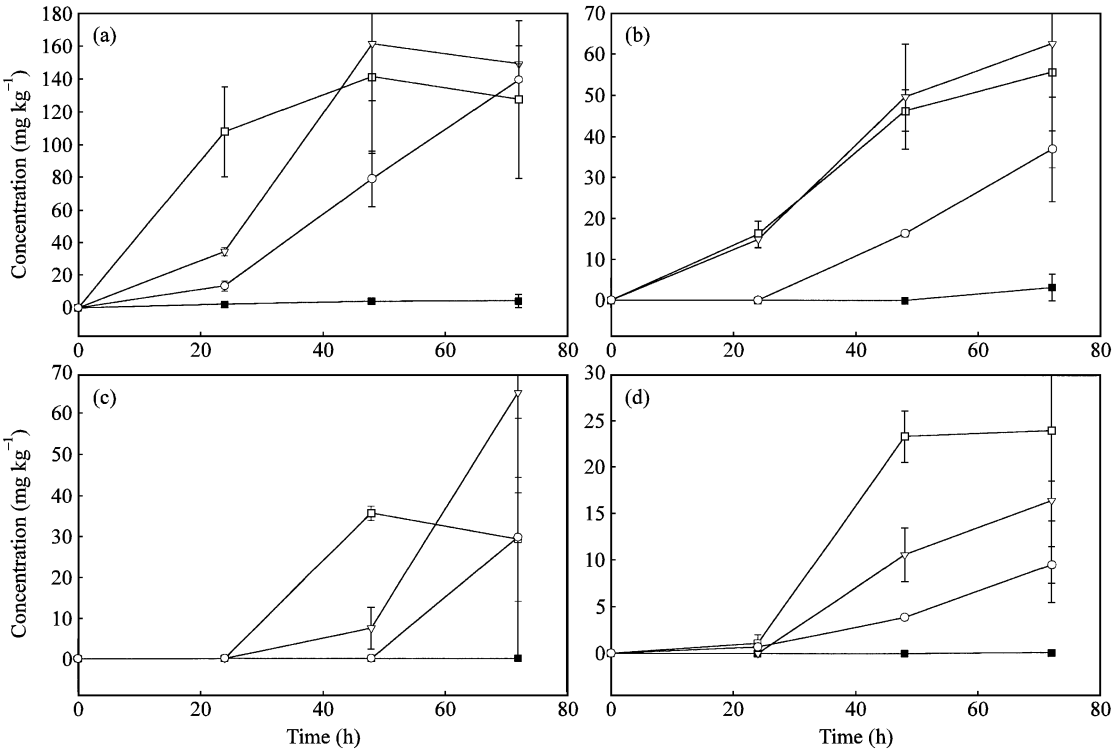

Figure 1. Effect of temperature, agitation and time on (a) ethyl propionate, (b) ethyl acetate, (c) ethyl isobutyrate, and (d) ethyl butyrate formed in white bread medium (35% solids) after fermentation with *G. candidum* ATCC 62217. 30°C, 0 rpm(■); 30°C, 300 rpm(□); 25°C, 300 rpm(▽); 20°C, 300 rpm (○).

for specific aroma production from waste bread.

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