Analysis of Odor-Active Volatiles from *Pseudomonas fragi* Grown in Milk

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Pseudomonas fragi produced a pleasant strawberry-like odor when grown in skim milk at 15 °C. Volatiles from the culture broth were extracted and enriched by using a purge and trap method and analyzed by using sniffing FID-GC. FID revealed the presence of approximately 90 compounds. Concomitant olfactory analysis of the eluted compounds demonstrated that 26 were odor-active. Ethyl butyrate, ethyl 3-methylbutanoate, and ethyl hexanoate were the major contributors to the odor, while other compounds contributed to its complexity and richness.

INTRODUCTION

Pseudomonas fragi contamination can be responsible for the spoilage of milk products and is characterized by the production of a strawberry-like odor. Ethyl esters of butyric, hexanoic (Reddy et al., 1968; Morgan, 1970), and 3-methylbutanoic acid (Pereira and Morgan, 1958) were shown to be the major contributors to the odor on the basis of their relative concentration in the headspace. Although headspace methods for aroma analysis are simple and rapid and measure what is typically presented to the nose, they are suitable for the measurement of only the major volatiles and are not useful for trace analysis (Brunke et al., 1989). The current goal of the aroma analysis is not only to identify major and minor components but also to determine their contribution to a strawberry-like odor of *P. fragi* grown in skim milk. For this reason, we used purge and trap extraction and enrichment of volatiles followed by gas chromatographic sniffing analysis to assess the sensory properties of every separated peak.

MATERIAL AND METHODS

***Pseudomonas* Culture.** Stock cultures of *P. fragi* were maintained immersed under mineral oil on brain heart infusion (BHI) agar (Difco). A fresh culture of *P. fragi* obtained after two passages in BHI broth (Difco) at 30 °C and 200 rpm for 24 h was inoculated at a rate of 0.01% (v/v) in BHI broth and grown under the same conditions for 17 h. For the production of strawberry odor, the resulting suspension was inoculated at a rate of 1% (v/v) in skim milk (12% w/w) that contained 0.2% (v/v) ethanol and incubated for 48 h at 15 °C and 130 rpm. Addition of 0.2% (v/v) ethanol to skim milk has been reported to enhance the production of ethyl esters that are responsible for the fruity aroma (Reddy et al., 1968). The best temperature, agitation, and time of incubation for the production of fruity aroma by *P. fragi* grown in skim milk were determined during preliminary trials on the basis of odor intensity and quality (no rancidity). Ten-milliliter aliquots of the suspension were kept frozen at −60 °C in sealed ampules until they were analyzed.

Extraction and Enrichment of Volatiles. Purge and trap sampling was carried out by using a Hewlett-Packard Model 7675A apparatus under the following conditions. Volatiles from 5 mL of suspension were transferred to a trap (4.5 mm i.d. × 105 mm length) composed of Carbopack B 60/80 mesh (Supelco) and Carbosieve SIII 60/80 mesh (Supelco) (4:1 ratio) by heating the sample to 80 °C for 7 min and purging it with 40 mL·min^{-1} He for 3 min. Volatiles were desorbed from the trap onto the GC column by heating the trap for 3 min to a final temperature of 200 °C. Cryogenic enrichment of the volatiles was performed by keeping the GC oven door open and cooling the anterior portion of the column with ice. An internal standard, heptanoic acid methyl ester, was added to the suspension prior to extraction of the volatiles at a concentration of 1 ppm.

Sniffing FID-GC Analysis. GC separation of the volatiles was performed by using a Perkin-Elmer Model 8320 apparatus under the following conditions. The split injector was set at a ratio of 20:1, and the carrier gas (He) flow was 1.2 mL·min^{-1}. The oven door was closed and the temperature raised to 60 °C at 30 °C·min^{-1}, then at a rate of 3 °C·min^{-1} to 160 °C, and finally to 250 °C at 30 °C·min^{-1}. A 30 m × 0.316 mm (1-μm film thickness) DB-5 fused silica column (J&W Scientific) was used. An effluent splitter (Perkin-Elmer) attached at the end of the column enabled half the sample to be directed to a flame ionization detector while the other half of the sample was directed to a sniffing port. Along the latter line, humidified air was added through a "T" piece at a rate of 100 mL·min^{-1}. Each sample was sniffed three times by three trained panelists. Sniffing sessions lasted 20 min at a time (first and second half of a chromatographic run) and were conducted on separate days. Panelists were asked to assess odor intensity (ordinal scaling from 1 to 5) and to describe odors (Acree et al., 1984). Description and mean intensity are presented for odors that were perceived in more than half of the trials.

GC/MS. For GC/MS a HP Model 5890 chromatograph coupled to a VG Analytical mass spectrometer 7070E-HF were used. GC runs were performed as described above, while mass spectrometric analysis was performed by using electron impact ionization of 70 eV. Resolution was 1000 and scanning range 30–350 m/z acquiring approximately 2 scans·s^{-1}. Acceleration potential was 6 kV.

Compounds were identified by comparing the retention time with those of known standards and/or by GC/MS. Quantity of compounds was determined from peak area relative to that of the internal standard.

RESULTS AND DISCUSSION

Preliminary trials using sensory analysis of headspace and sniffing FID-GC demonstrated the need to conduct extraction of volatiles at a temperature that did not generate off-flavors. Hence, a temperature of 80 °C was deemed adequate to carry out purge and trap extraction and enrichment of volatiles from the culture broth.

GC analysis of the volatile fraction of strawberry-smelling cultures of *P. fragi* revealed a complex profile that consisted of approximately 90 peaks (Figure 1). The gas chromatographic sniffing technique permitted the description of odor qualities and assigning of relative intensities of the odor-relevant components in the complex mixture. Simultaneous sensory analysis at the sniffing port revealed that some major components made little or no contribution to the aroma, whereas other components present in low concentrations caused strong perceptions, e.g., peaks 5, 8, 12, 14, 19, and 23–25. Most odors were of

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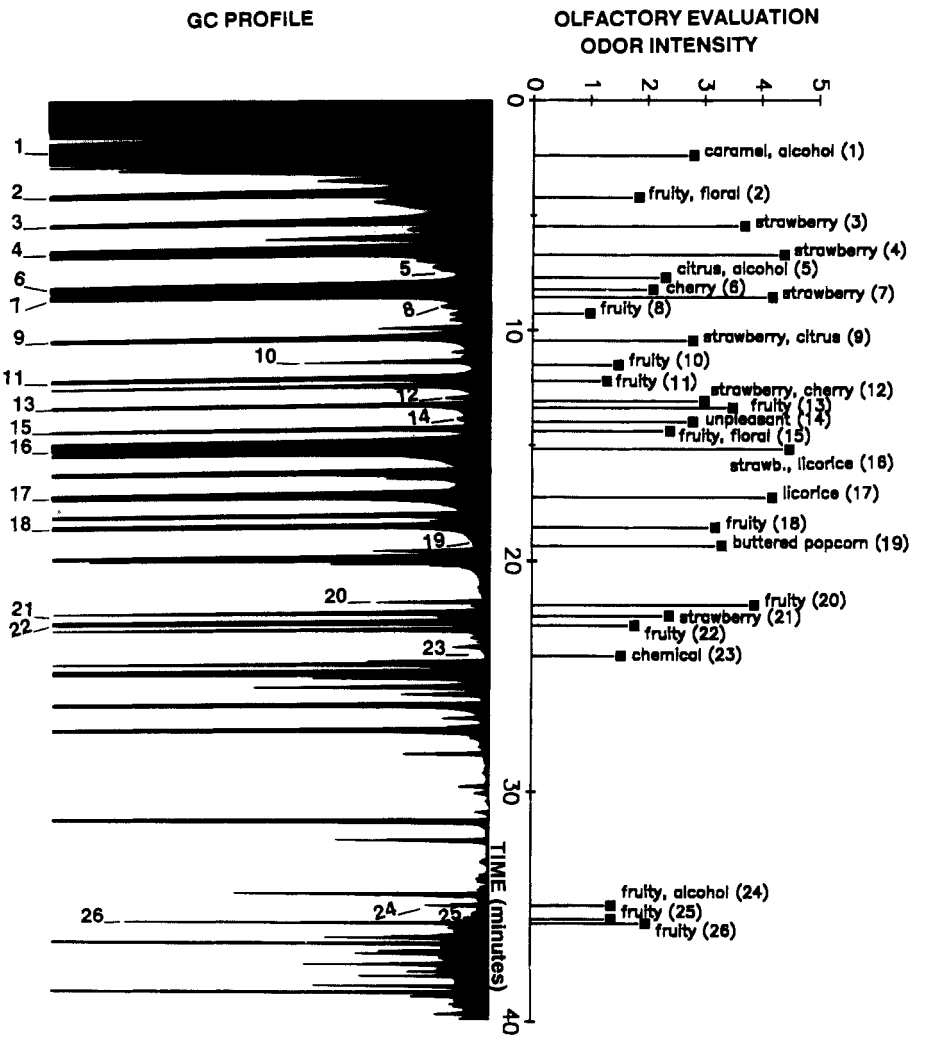

Figure 1. Gas chromatogram and odor-description and intensity (average) at the sniffing port of volatiles from *P. fragi* grown in skim milk. Numbering of the aroma compounds as in Table I.

Table I. List of Odor-Active Compounds Produced by *P. fragi*

peak[g]	ethyl ester	ret time, min	major peaks in the EI spectra (m/z)	concn, ppm	odor at sniffing port[a]
1	ni[e]	2.41[b]		nq[f]	caramel, alcohol
2	propanoic acid	4.33[c]	102, 75, 57	0.113	fruity, floral
3	ni	5.48		0.104[d]	strawberry
4	butanoic acid	6.72[c]	116, 101, 88, 71	1.424[d]	strawberry
5	ni	7.20[b]		nq	citrus, alcohol
6	2-butenoic acid	8.24[c]	114, 99, 86, 69	1.084	cherry
7	3-methylbutanoic acid	8.57[c]	130, 115, 102, 88, 85	0.309	strawberry
8	ni	9.19[b]		nq	fruity
9	pentanoic acid	10.46[c]	130, 101, 88 85	0.062	strawberry, citrus
10	3-methyl-2-butenoic acid	11.52[c]	128, 113, 100, 83	0.024	fruity
11	3-methyl-3-butenoic acid	12.23	99, 83	0.337[d]	fruity
12	ni	13.10		0.009[d]	strawberry, cherry
13	3-methylpentanoic acid	13.39	115, 101, 99, 88	0.032[d]	fruity
14	ni	14.00[b]		0.007[d]	unpleasant
15	ni	14.40		0.023[d]	fruity, floral
16	hexanoic acid	15.17[c]	115, 99, 88	0.942	strawberry, licorice
17	2-hexanoic acid	17.26	142, 114, 99, 97, 73	0.179[d]	licorice
18	2-methylhexanoic acid	18.56	129, 113, 101, 88	0.059[d]	fruity
19	ni	19.36[b]		nq	buttered popcorn
20	cyclohexanecarboxylic acid	21.90	156, 141, 128, 111, 101	0.007[d]	fruity
21	2-heptenoic acid	22.37	156, 127, 111, 99	0.019[d]	strawberry
22	ni	22.79[b]		nq	fruity
23	ni	24.24[b]		nq	chemical
24	ni	34.96		0.002[d]	fruity, alcohol
25	ni	35.56[b]		0.006[d]	fruity
26	ni	35.74[b]		0.006[d]	fruity

[a] Compound need not be responsible for the observed odor. [b] Approximate retention time determined at the sniffing port. [c] Similar to the retention time of a standard. [d] Quantified as equivalents of butyric acid, ethyl ester; underlined m/z are M − 45 (loss of ethoxyl group). [e] ni, not identified. [f] nq, not quantifiable. [g] Numbering of the aroma compounds as in Figure 1.

the fruity type, some of which were reminiscent of specific fruits (strawberry, cherry, and citrus). As shown in Table I, several of the 26 odor-active components were ethyl esters of short-chain (C_3–C_7) fatty acids. Similarly to Reddy et al. (1968), we found ethyl acetate to occur in large quantity but it was not considered to participate significantly in the odor (results not shown). All compounds that were analyzed by GC/MS showed a strong m/z for M − 45, corresponding to the typical loss of an ethoxy group of ethyl esters. While tentative identification of compounds **11**, **13**, **17**, **18**, **20**, and **21** was made from EI spectra, formal identification of compounds **2**, **4**, **6**, **7**, **9**, **10**, and **16** was possible from EI spectra and by comparison of retention time to that of standards. Although quite large, peaks 1 and 3 were not sufficiently resolved during GC/MS analysis to enable their identification. From the relative position in the chromatogram and EI spectral peaks (m/z 142, 114, 97, and 88), compound **15** might be a branched monounsaturated C_6 ethyl ester. A component, tentatively identified by mass spectrometry (m/z 142, 114, 97, 88) as 3-hexenoic acid ethyl ester, was eluted immediately after hexanoic acid ethyl ester (peak 16) and may be responsible for the licorice odor associated with the latter. Multidimensional chromatography would be required to further resolve the two components (Nitz et al., 1989). As reported by others (Pereira and Morgan, 1958; Reddy et al., 1968; Morgan, 1970), we found ethyl butyrate, ethyl 3-methylbutanoate, and ethyl hexanoate (peaks 4, 7, and 16, respectively) to be among the most concentrated compounds and major contributors to the bacterial strawberry aroma. However, a mixture of only these three compounds resulted in an artificial candy-like aroma. It is thus thought that the complexity, richness, and natural qualities of the aroma depend on the harmony and balance of many aroma compounds. Ethyl esters of *trans*-2-butenoic acid and 3-methyl-3-butenoic acid (peaks 6 and 11, respectively) were also among the most concentrated esters but were not major contributors to the aroma (their intensity did not exceed 2).

Purge and trap extraction and enrichment of volatiles followed by sniffing GC analysis have enabled us to identify several odor-active components of a complex mixture of volatiles produced by *P. fragi* in skim milk. In doing so, we have laid a foundation upon which physiology and biochemistry studies will be undertaken.

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Registry No. Ethyl propanoate, 105-37-3; ethyl butanoate, 105-54-4; ethyl *trans*-2-butenoate, 623-70-1; ethyl 3-methylbutanoate, 108-64-5; ethyl pentanoate, 539-82-2; ethyl 3-methyl-2-butenoate, 638-10-8; ethyl 3-methyl-3-butenoate, 1617-19-2; ethyl 3-methyl pentanoate, 5870-68-8; ethyl hexanoate, 123-66-0; ethyl 2-hexenoate, 1552-67-6; ethyl 2-methylhexanoate, 32400-29-6; ethyl cyclohexanecarboxylate, 3289-28-9; ethyl 2-heptenoate, 2351-88-4.