Analysis of the Volatile Components of a Bacterial Fermentation That Is Attractive to the Mexican Fruit Fly, *Anastrepha ludens*

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An analysis was performed to identify the volatile components of a supernatant derived from a bacterial (*Klebsiella pneumoniae*, ATCC 13883, American Type Culture Collection) fermentation of a trypticase soy broth. The bacteria-produced supernatant (BPS) was comparable to a 30% solution of NuLure in attractiveness to mixed sexes of adult Mexican fruit flies, *Anastrepha ludens* (Loew), in a laboratory bioassay. The volatile components of BPS, most resulting from bacterial metabolism, were identified by a combination of capillary GC and GC–MS techniques. A total of 21 compounds were identified, including 7 alcohols (2 sulfur containing), 8 alkyl-substituted pyrazines, 2 ketones, 2 acids, 1 ketoalcohol, and phenol. The five most abundant compounds, in decreasing order of abundance, were 3-methyl-1-butanol, phenethyl alcohol, 2,5-dimethylpyrazine, 2-methyl-1-propanol, and 3-(methylthio)-1-propanol. One or more unidentified, low molecular weight amines were also present in the mixture of volatiles.

Keywords: *Lure; bacterial metabolites; Mexican fruit fly; Anastrepha ludens; Klebsiella pneumoniae; analysis of volatiles*

INTRODUCTION

The Mexican fruit fly, *Anastrepha ludens* (Loew), is a major pest of citrus, mango, guava, and other subtropical fruit. Currently, McPhail traps baited with borax-buffered aqueous solutions of 10% NuLure or 2.2% torula yeast are used to survey and monitor populations of this insect (Anonymous, 1993). Despite the widespread use of these lures, they possess disadvantages, i.e., both require portage of water and bulky glass traps to the field, and torula yeast–borax baited McPhail traps appear to have a low trapping efficiency for a related species, *Anastrepha suspensa* (Loew) (Calkins et al., 1984). Due to these problems, it is generally agreed that a better lure is needed for *A. ludens*.

In the search for more effective fruit fly attractants, extensive studies have been conducted to characterize the volatile components of proteinaceous lures (Morton and Bateman, 1981; Buttery et al., 1983; Teranishi et al., 1985; Flath et al., 1989). Among the volatiles derived from proteinaceous baits, ammonia appears to play an important role in attracting fruit flies (Bateman and Morton, 1981; Mazor et al., 1987), and its rate of release may be a critical factor in its effectiveness (Bateman and Morton, 1981).

Bacterial cultures were reported long ago as attractants for fruit flies (Gow, 1954). However, little is known about the chemistry of the principal volatiles. Metabolites 2-butanone and butanol, produced by *Proteus* bacteria, are effective attractants for *Bactrocera tryoni* (Froggatt) (Drew and Fay, 1988). Principal odors that attract *A. ludens*, and which are produced by bacterial (*Staphylococcus*) fermentation of tryptic soy culture, were partially characterized (Robacker et al., 1993). Recent studies showed that a variety of bacteria-produced metabolites were attractive to *A. ludens* in both laboratory and field tests and that autoclaved supernatants were significantly more attractive than whole beer or membrane-filtered supernatants (Martinez et al., 1994). Moreover, metabolites from American Type Culture Collection (ATCC) 13883 captured as many adult *A. ludens* in comparative field tests against NuLure (Martinez et al., 1994).

Pursuant to the reported attractiveness of bacterial supernatant ATCC 13883 (*Klebsiella pneumoniae*), we initiated a study to isolate and identify the volatiles collected from an autoclaved bacteria-produced supernatant (BPS) from that strain. We anticipate that compounds identified in this study will be the focus of subsequent studies to determine their relative attractancy to *A. ludens* and other tephritids and to determine their potential for deployment in dry-type traps.

MATERIALS AND METHODS

Materials. NuLure (Miller Chemical and Fertilizer Corp., Hanover, PA) was diluted to 30%, and this solution was used as a standard for the laboratory bioassay. The bacteria-produced supernatant (BPS) was obtained by culturing *K. pneumoniae* (ATCC 13883) in trypticase soy broth medium (Baltimore Biological Laboratory, Baltimore, MD). The medium consisted of trypticase peptone (17 g), phytone peptone (3 g), sodium chloride (5 g), dipotassium phosphate (2.5 g), and dextrose (2.5 g). The medium was suspended in water and subjected to reverse osmosis and carbon filtration, and 500–800 mL portions were transferred to 1 L Erlenmeyer flasks (pH 6.8). The flasks were autoclaved at 121 °C for 20 min and then allowed to cool (pH 6.8). The medium was seeded with 1 mL of bacterium (1×10^9 cells) and then placed in a rotary shaker for 8 days (shaker speed, 250 rpm). Temperature was maintained at 30 °C. After fermentation, the flasks

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were removed and the whole beer was centrifuged at 10 000 rpm for 7 min. The supernatant was placed in 1 L Erlenmeyer flasks and autoclaved as before (pH 7.5). The flasks were cooled and refrigerated until used.

Collection of Volatiles. A 50 mL aliquot of BPS was charged in a three-neck, round-bottom flask. As the solution was stirred at ambient temperature, prepurified nitrogen (i.e., passed through an activated charcoal bed) was swept at 300 mL/min over the BPS headspace, and volatiles were collected in a glass tube packed with 300 mg of activated charcoal (Darco, 20–40 mesh, Aldrich). Charcoal used in the collection trap was prepurified by continuous extraction (Soxhlet extractor) with methylene chloride and then benzene (caution: benzene and methylene chloride, cancer suspect agents, should be handled with care and adequate ventilation). After collecting volatiles for 15 h, the charcoal trap was removed and eluted with ca. 1 mL of freshly distilled diethyl ether. The ether extract was analyzed without concentration.

Gas Chromatography (GC). A Shimadzu Model GC-14A (Shimadzu, Columbia, MD) equipped with a flame ionization detector (FID) and a bonded SPB-1 (Supelco, Bellefonte, PA) fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used to analyze volatile components. GC peak areas were quantified using a Shimadzu CR-501 integrator. GC operating conditions: injector/detector temperature, 250 °C; helium carrier, ca. 1 mL/min (1.5 kg/cm^2 head pressure), injector operated in split mode, 50:1; temperature program, 50 °C (5 min) then ramped at 5 °C/min to 250 °C.

Gas Chromatography–Mass Spectrometry (GC–MS). A Hewlett-Packard 5890A GC–MS equipped with a 5971A MSD and a HP5 (Hewlett-Packard, Avondale, PA) bonded fused-silica capillary column (25 m × 0.2 mm i.d., 0.11 μm film thickness) was used. GC conditions were the same as those described for GC analysis on the Shimadzu instrument except the injection port was operated in the splitless mode. MS conditions (EI mode): ionization voltage, 70 eV; mass range, m/z 30–550; ion source temperature, 180 °C. The mass spectra of the unknown compounds were compared with those in the Wiley/NBS spectral data base. Identifications were also made by comparing Kovats indices (Kovats, 1966) of unknowns with those determined for authentic samples.

Bioassay. The laboratory bioassay was a modified version of a previously described cage-top bioassay (Robacker and Hart, 1984). Briefly, a 50–200 μL aliquot of BPS at the desired concentration was applied to a 12.7 mm diameter filter paper disk attached to the bottom of a glass Petri dish. After application of the test solution, the dish (inverted) was placed on top of a large (2 m^3) wire-screened cage containing 1000 mixed-sex adult *A. ludens* (3–7 days old) that were maintained, since emergence, on a 6% sucrose diet. The night before tests, the sucrose was removed and replaced with water. Tests were conducted in a room held at 25 °C and 70 ± 5% relative humidity. Generally, four different concentrations of a BPS sample were bioassayed in combination with a standard (30% NuLure) and water control. Five replicates of each of these treatments were placed on the cage in a randomized complete block design. Response determinations were made as follows: counts of flies found under the area of Petri dishes were taken every 5 min for a total of six recordings. Response means, standard deviations (SD), and standard errors (SE) were calculated by using a SuperAnova (Accessible General Linear Modeling) program (Abacus Concepts, Inc., Berkeley, CA).

RESULTS AND DISCUSSION

The application of purge and trap techniques to collect the volatile components of BPS seemed appropriate since results from these methods quite often accurately represent the composition of the volatiles continuously being emitted from the liquid matrix. Activated charcoal was selected as the trapping agent since its capacity to absorb organic compounds, even from dilute aqueous solutions, is appreciable (Heinz et al., 1966).

Table 1. Comparison of the Attractancy of Different Concentrations of BPS[a] to Mixed Sexes of Adult *A. ludens*

test material	mean fly response index[b]	SD	SE
water	1.000		
10% BPS	4.124	1.102	0.493
30% BPS	4.228	0.483	0.216
50% BPS	4.298	0.488	0.218
70% BPS	4.224	0.509	0.228
30% NuLure[c]	2.900	0.762	0.341

[a] Eight-day fermentation of ATCC 13883 in a trypticase soy broth. [b] Analysis performed using the means of two tests (five replicates/test). Response index = no. of fly responses for test material/no. of responses for water. Total responses, n = 1198. [c] Unbuffered, pH 4.1.

To confirm the activity of BPS volatiles, a series of freshly prepared solutions (i.e., 10–70%) were evaluated in comparison tests against a water control and a 30% NuLure standard (Table 1). Data show that all BPS solutions were ca. 4-fold more attractive than water, while NuLure (unbuffered) was only ca. 3-fold more attractive. Despite the wide range of concentrations, the observed level of attractiveness remained fairly constant.

A typical gas chromatogram of the BPS volatiles is shown in Figure 1; chemical identification, Kovats indices (KI), and relative concentrations are provided in Table 2. Twenty-three peaks in the chromatogram were identified on the basis of a comparison of their KI values or GC–MS spectra with those obtained for authentic samples. Two of the peaks, *d* and *e*, were identified as benzene and acetal, respectively, and were inadvertently introduced contaminants (see footnotes in Table 2). Of the 21 identified components, 7 were alcohols (two sulfur containing), 2 ketones, 1 ketoalcohol, 8 alkyl-substituted pyrazines, 2 acids, and phenol. 3-Methyl-1-butanol was the most abundant, comprising ca. 97% of the volatiles of BPS. In addition to the 21 identified compounds, low molecular weight amines were also detected in the BPS volatiles using ammonia test paper (Laboratory Supplies Co., Hicksville, NY). However, due to the nonspecific nature of the test, these amines could not be characterized, although ammonia release is commonly associated with protein degradation (Schönberg and Moubasher, 1952) and bacterial metabolism (Drew and Fay, 1988). 3-Methyl-1-butanol, furfuryl alcohol, phenethyl alcohol, 3-methylpyrazine, 2,5- (and 2,3)-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, trimethylpyrazine, isovaleric acid, and 2-heptanone, identified in the BPS volatiles, were previously identified as volatiles in NuLure, formerly PIB-7 (Morton and Bateman, 1981; Buttery et al., 1983; Matsumoto et al., 1985; Flath et al., 1989). Other compounds that we detected in an ether extract of the liquid phase of BPS were identified by GC–MS as benzyl alcohol, hexanoic acid, octanoic acid, and phenylacetic acid (none of these were detected in the BPS volatiles). It is noteworthy that five of the alcohols, 2-methyl-1-propanol, 3-methyl-1-butanol, furfuryl alcohol, 3-(methylthio)-1-propanol, and phenethyl alcohol, were an oxidation state removed from similarly structured aldehydes previously identified in NuLure/PIB-7 (Buttery et al., 1983; Flath et al., 1989).

The presence of pyrazines in volatiles of proteinaceous lures is well documented (Buttery et al., 1983; Matsumoto et al., 1985; Flath et al., 1989), and their concentrations both in the liquid and in the gas phase appear to be a function of pH (Matsumoto et al., 1985). In this

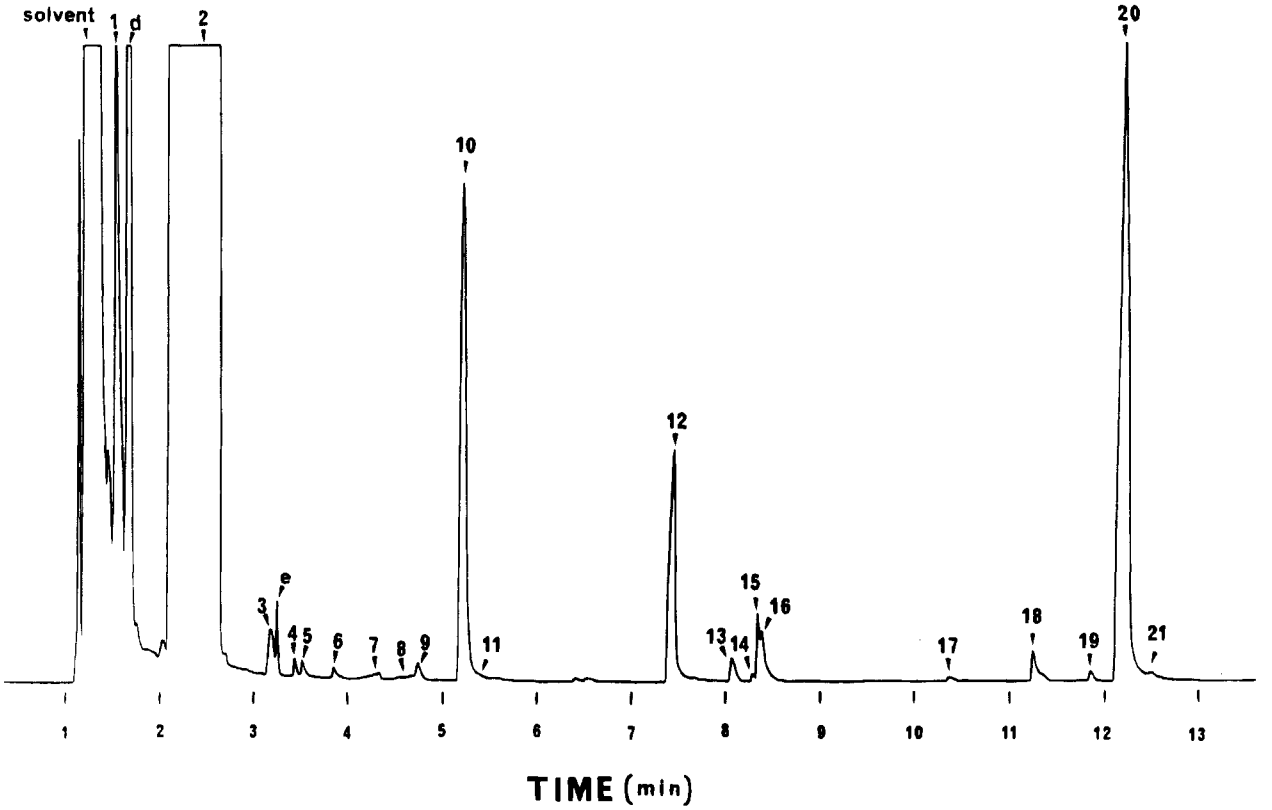

Figure 1. Gas chromatogram of volatile components in bacteria-produced supernatant (ATCC 13883). Peak numbers correspond to peak numbers in Table 1.

Table 2. Identification and Composition of Volatiles Derived from a Bacterial (ATCC 13883) Fermentation of Trypticase Soy Broth

GC peak no.[a]	compound	Kovats index[b]	ID[c]	rel area %
1	2-methyl-1-propanol	612	MS, KI	0.509
d	benzene		MS	
2	3-methyl-1-butanol	717	MS, KI	97.0
3	2-methylpyrazine	799	MS, KI	0.081
e	acetal		MS	
4	2-(methylthio)ethanol	810	MS, KI	0.024
5	4-(hydroxymethyl)-2-pentanone	813	MS, KI	0.022
6	furfuryl alcohol	827	MS, KI	0.010
7	isovaleric acid	827	MS, KI	0.020
8	2-methylbutyric acid	838	MS, KI	0.020
9	2-heptanone	867	MS, KI	0.028
10	2,5-dimethylpyrazine	884	MS, KI	0.606
11	2,3-dimethylpyrazine	892	MS, KI	0.006
12	3-(methylthio)-1-propanol	944	MS, KI	0.285
13	phenol	959	MS, KI	0.027
14	2-ethyl-6-methylpyrazine	969	MS, KI	0.004
15	2-ethyl-5-methylpyrazine	973	MS, KI	0.044
16	trimethylpyrazine	974	MS, KI	0.061
17	2-methyl-5-isopropylpyrazine	1031	MS	0.008
18	2,5-dimethyl-3-ethylpyrazine	1055	MS, KI	0.040
19	2-nonanone	1069	MS, KI	0.009
20	phenethyl alcohol	1081	MS, KI	1.12
21	2-nonanol	1087	MS, KI	0.005

[a] Peak number shown in chromatogram in Figure 1. [b] Kovats indices calculated from retention time data obtained on a SPB-1 capillary column. [c] Identification methods. [d] Impurity introduced from charcoal washings. [e] Impurity in diethyl ether solvent.

context, the presence of a variety of pyrazines in the volatiles of BPS was not entirely surprising. Interestingly, a GC analysis of volatiles collected from a BPS check (i.e., sample treated identically to BPS except medium was not inoculated) showed the presence of all pyrazines identified from the inoculated sample and in approximately the same relative concentrations. Aside from the pyrazines, the only other compound detected in the GC was benzaldehyde. Benzaldehyde's presence was likely due to the fact that an appreciable quantity of it was detected in the solution of raw materials used for the fermentation. Moreover, laboratory tests showed that a BPS check was significantly less attractive than 30% NuLure or BPS ATCC 13883. Mean fly response indices (±SEM, from five replicates) were as follows: BPS check = 3.475 ± 0.183; 30% NuLure = 5.066 ± 0.159; BPS ATCC 13883 = 4.517 ± 0.336; water = 1.000.

The most direct way to form pyrazines is through the Maillard reaction of α-dicarbonyls (from sugars) and amines generated from the Strecker degradation (de Rijke et al., 1981; Hwang et al., 1993). Also, pyrazine formation may result from thermal (Maga, 1992), chemical (Maga, 1992), or photochemical processes (Sheldon and Shibamoto, 1987). Exactly how pyrazines were formed in the BPS is a matter of conjecture, and further experiments will be required to determine their source.

In summary, we have identified 21 volatile constituents derived from bacterial fermentation of a trypticase soy broth. New studies are in progress to determine the attractiveness of compounds already identified and to further characterize and elucidate the role of the low molecular weight amines. Information gleaned from these and future studies should serve as a guide in the development of more efficacious, persistent, and conveniently deployable lures needed for detection and population monitoring of *A. ludens* and other tephritids.

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