Volatile Compounds Secreted by the Oyster Mushroom (*Pleurotus ostreatus)* and Their Antibacterial Activities

Miguel J. Beltran-Garcia,[†,‡] Mirna Estarron-Espinosa,[§] and Tetsuya Ogura*[,†]

Departamento de Quimica, Universidad Autonoma de Guadalajara, Av. Patria 1201, Lomas del Valle, Guadalajara, Jal., Mexico, and Departamento de Ingenieria de Procesos, Centro de Investigacion y Asistencia en Tecnologia y Diseño del Estado de Jalisco, Av. Normalistas 800, Guadalajara, Jal. CP 44270, Mexico

The oyster mushroom (*Pleurotus ostreatus*) is widely cultivated, but the volatile compounds it produces are little known. Several methods of isolation of volatile components were compared: extraction by carbon tetrachloride, vacuum distillation, and conveyance by nitrogen flow. The observed order of extraction efficiency was CCl_4 > vacuum > N_2. Compounds extracted by CCl_4 were 3-octanone, 3-octanol, 1-octen-3-ol, benzaldehyde, 1-octanol, benzoic acid, and an unidentified trace component. The extracted mixture of compounds demonstrated strong antibacterial activity against some bacterial strains. Addition of a facsimile mixture containing these volatile compounds to a culture broth resulted in complete inhibition of the eight bacteria used for antibacterial assay. Benzaldehyde, which does not contribute notably to the inhibition of bacterial growth, is formed as a reaction to the stress of CCl_4 contact or vacuum dehydration. The amount of benzaldehyde produced may be an indicator of the degree of stress imposed.

Keywords: *Oyster mushroom; Pleurotus ostreatus; volatile compounds; 3-octanone; 3-octanol; 1-octen-3-ol; benzaldehyde; 1-octanol; benzoic acid; minimum inhibitory concentration, MIC; antibacterial activity*

INTRODUCTION

The oyster mushroom (*Pleurotus ostreatus*) is one of the most widely cultivated mushrooms (Bano and Rajarathnam, 1987); however, little is known about the volatile compounds formed by this mushroom. Rajarathnam and Bano (1988) showed a gas chromatogram of a methanol extract of *Pleurotus flabellatus*, which demonstrated the presence of 2-pentanone, 3-pentanone, 2-methyl-3-pentanol, 2-pentanol, 3-octanone, 1-octen-3-one, and 1-octen-3-ol. Recently, Lizarraga-Guerra *et al.* (1997) studied odor causative compounds in austern pilzen (*Pleurotus* sp.) and huitlacoche (*Ustilago maydis*).

The aroma compounds from the mushroom (*Agaricus bisporus*) are well documented (Mau *et al*., 1993). Loch-Bonazzi and Wolff (1994) described 1-octen-3-ol, 1-octen-3-one, 3-octanol, 3-octanone, benzyl alcohol, and benzyl aldehyde as the most important flavor components among more than 150 volatile compounds found in the mushroom. Particularly, 1-octen-3-ol is well-known as the cause of characteristic mushroom odor.

Dried shiitake mushroom (*Lentinus edodes*) has a pleasing unique flavor. Chen *et al.* (1984, 1986) have studied the effect of pH on the formation of volatile compounds and shown that the aroma results from abundant sulfurous compounds which are formed during submersion processing of the mushrooms at room temperature just before cooking.

The C_8 volatile compounds are formed by oxidation of linoleic or linolenic acid in the presence of enzymes such as lipoxygenase and hydroperoxide lyase (Assaf *et al*., 1995; Mau *et al*., 1992; Chen and Wu, 1984; Tressl *et al*., 1982).

The volatile compounds of edible mushrooms have been studied from the consumer's viewpoint to understand the origin of their various flavors; however, mushrooms secrete them to meet specific needs. In this study we compared some methods of extraction of volatile compounds from *P. ostreatus*: vacuum distillation, nitrogen flow conveyance, and extraction by carbon tetrachloride. The compounds isolated were mainly C_8 compounds that exhibited strong antibacterial activity against some bacteria. The addition of a mixture of volatile compounds similar to that found in the mushroom completely inhibited growth of the eight bacteria examined. It is reasonable to conclude that these compounds provide a defense against bacterial overgrowth.

MATERIALS AND METHODS

Reagents. 1-Octen-3-ol, 3-octanol, octanol, 3-octanone, 2-octanone, benzyl alcohol, benzaldehyde, benzoic acid, and carbon tetrachloride were purchased from Sigma Chemical Co., St. Louis, MO. Sugar and trypticase soy were obtained from Merck, Naucalpan de Jesus, Mexico. Malt extract broth was purchased from Difco, Detroit, MI.

Microorganisms and Culture Conditions. All microorganisms used in this study were purchased from American Type Culture Collection (ATCC, Rockville, MD). The *P. ostreatus* strain was maintained on malt extract−sugar−agar (10−40−20 g/L) (MSA). *Bacillus cereus* ATCC 11778 (Bc), *Bacillus subtilis* 6633 (Bs), *Escherichia coli* 10536 (Ec), *Klebsiella pneumoniae* 10031 (Kp), *Pseudomonas aeruginosa* 27853 (Pa), *Salmonella typhimurium* 6539 (St), *Staphylococcus aureus* 6538P (Sa), and *Staphylococcus epidermidis* 12228 (Se) were cultured using trypticase soy culture broth (30 g/L) maintained at 37 °C for 18−24 h to obtain a stationary phase culture.

* Author to whom correspondence should be addressed [telephone Mexico (52) 3-641-5051, ext. 32433; fax Mexico (52) 3-642-5427; e-mail togura@uagunix.gdl.uag.mx].

[†] Universidad Autonoma de Guadalajara.

[‡] Present address: Departamento de Morfologia, Escuela de Nacional de Ciencias Biologicas, Carpio y Plan de Ayala S/N, Santo Tomas, CP 11340, Mexico DF.

[§] Centro de Investigacion y Asistencia en Tecnologia y Diseño del Estado de Jalisco.

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Fruiting Body Formation. *P. ostreatus* mycelia were grown in a liquid medium (100 mL) of malt extract−sucrose (10−40 g/L) supplemented with corn stalk powder (1 g/L of medium) and incubated for 20 days at room temperature.

Solid medium for fruiting body formation was prepared as follows: One kilogram of corn stover treated with 5% NaOH was supplemented with 400 g of wheat bran, 1 L of an aqueous solution containing 5.1 g of $FeSO_4 \cdot 7H_2O$, 4,9 g of Na_3PO_4, 5.4 g of KCl, and 3.1 g of $MgSO_4 \cdot 7H_2O$, and the resultant slightly basic mixture was adjusted to pH 4.5 by H_3PO_4. The mixture was kneaded with addition of 1.4 L of water, divided into thirds, and placed into glass bottles (1 L) with cotton plugs, which were sterilized at 120 °C for 60 min.

The liquid culture of *P. ostreatus* mycelia was poured into the solid medium and kept at room temperature (25 + 4 °C). Primordios were formed within 35 days. The contents of each bottle were transferred to a growing box (1.5 m long × 1 m wide × 1 m high) ventilated by humidified air at room temperature. Mushrooms were harvested within 7−14 days.

Collection of Volatile Compounds. A total of approximately 50 g of young oyster mushrooms (each fruiting body weighed about 1 g) was harvested from three corn stover-based solid cultures. Three similar 10-g portions were collected from the combined produce to assay for volatile compounds as follows:

(1) Nitrogen Flow. Ten grams of young oyster mushrooms was cut into 1.5-cm lengths and placed in a 50-mL Büchner flask equipped with a rubber cup penetrated by a glass tube that reached to the bottom of the flask. A nitrogen flow of 50 mL/min was passed through the sample via the glass tube at 35 °C for 4 h, the volatile compounds being condensed into 30 mL of ethanol kept in a dry ice−acetone cold trap.

(2) In Vacuo. Ten grams of young oyster mushrooms was cut into 1.5-cm lengths, which were placed in a 50-mL Büchner flask kept at 35 °C. The volatile compounds were distilled *in vacuo* into a glass cold trap kept in a dry ice−acetone mixture. The condensate was dissolved into 30 mL of ethanol.

(3) Extraction by CCl_4. Ten grams of young oyster mushrooms was cut into 1.5-cm lengths and submerged in 30 mL of CCl_4. The pieces were broken into smaller fragments using a spatula. The mixture was kept stirring slowly by use of a magnetic bar for 24 h.

Gas Chromatography. GC/MS analyses were carried out using a Hewlett-Packard (Wilmington, DE) gas chromatograph (Model 5890 Series II) fitted with a mass selective detector (Model 5972) and a data operator 5972 MS ChemStation (Mustang). The volatile compounds were separated using a 50-m glass capillary column (0.20-mm i.d., 0.20-mm film thickness) coated with Carbowax 20M. The gas chromatograph was programmed such that the oven temperature was kept at 70 °C for the first 5 min and then increased to 210 °C at a rate of 3 °C/min. The mass spectra were recorded at 70 eV and compared with spectral database Wiley 138. Tentative assignments were confirmed by GC/MS employing pure compounds. Nonanol was used as the internal standard for quantification, in which each compound was determined by comparison with a standard solution composed of a known composition of the components and nonanol.

Antimicrobial Assay. For each test compound (1-octen-3-ol, 3-octanol, octanol, 3-octanone, and 2-octanone), a fresh test solution was prepared for each assay in the following manner: One milliliter of test compound was dissolved in 9.00 mL of dimethyl sulfoxide (DMSO) and passed through a 0.45-μm membrane filter. Forty microliters of the DMSO solution was added to 4.00 mL of sterilized trypticase soy culture broth (30 g/L) to achieve a maximum test compound concentration of 1000 μg/mL. It was shown that the growth of the bacteria employed in this study was not inhibited by an addition of DMSO up to 10%.

Minimum inhibitory concentration (MIC) was determined by adding 30.0 μL of solution containing a total of 300 000 colony forming units (CFU) to each growth test tube. All bacteria were kept in stationary incubation at 37 °C.

A mixture of 3-octanone (0.140 g), 3-octanol (0.220 g), 1-octen-3-ol (0.560 g), and 1-octanol (0.260 g) was diluted by DMSO to 10.0 mL using a volumetric flask and filtered through a 0.45-μm membrane filter. Five test solutions were prepared aseptically by mixing 96, 98, 99, 100, and 100 mL of pasteurized trypticase soy culture broth (30 g/L) with 4.00, 2.00, 1.00, 0.500, and 0.250 mL of the DMSO solution, respectively. From each test solution, a 4-mL portion was placed in eight test tubes. Each tube was inoculated with a bacteria species, 30 μL of solution containing a total of 300 000 CFU. Each run was duplicated and gave reproducible result.

Table 1. Volatile Compounds Found in Fruit Body of *P. ostreatus* by Various Extraction Methods

compd	retention time (min)	concn in mushroom (μg of compd/g of fruit body)		
		CCl_4	*in vacuo*	N_2
3-octanone	9	136 [1.00][a]	56.1 [0.41]	75.3 [0.55]
3-octanol	12	217 [1.00]	163 [0.75]	125 [0.58]
1-octen-3-ol	14	558 [1.00]	294 [0.53]	255 [0.46]
benzaldehyde	16	2800 [1.00]	7.08 [0.0025]	ND[b] [0.000]
1-octanol	17	256 [1.00]	140 [0.55]	117 [0.67]
unidentified	27	*c*	ND	ND
benzoic acid	44	483	ND	ND

[a] The extraction efficiencies relative to CCl_4 extraction are shown in brackets. [b] ND, not detected. [c] Approximately 30 ppm.

RESULTS AND DISCUSSION

Table 1 shows seven peaks detected in the CCl_4 extract, six of them identified as 3-octanone, 3-octanol, 1-octen-3-ol, benzaldehyde, 1-octanol, and benzoic acid. Benzoic acid was probably formed by air oxidation of benzaldehyde in CCl_4. One component (retention time of 27 min) could not be identified. The peak did not correspond to that of benzyl alcohol. The peak area of the unknown compound was 0.6% of all peak areas (4440 ppm), corresponding to a concentration of approximately 30 ppm.

3-Octanone, 3-octanol, 1-octen-3-ol, and 1-octanol were found in the vacuum distillate at room temperature at 41, 75, 53, and 55% of their respective concentrations in the CCl_4 extract, and they were found in the N_2 condensate at 55, 58, 46, and 46% of their respective concentrations in the CCl_4 extract. Therefore, the extraction efficiencies were in the order CCl_4 > vacuum > N_2.

Benzaldehyde was found in the vacuum distillate at only 0.25% of its concentration of the CCl_4 extract and was not found at all in the N_2 condensate. Had benzaldehyde been present in the vacuum distillate and N_2 condensate at levels commensurate with those of the other compounds detected, it would have registered similarly under GC/MS examination. Therefore, benzaldehyde must be formed as a response to stress suffered during CCl_4 extraction or vacuum dehydration, with the degree of stress being indicated by the amount of the aldehyde.

Table 2 shows the MIC of the eight bacteria to 1-octen-3-ol, 3-octanol, octanol, 3-octanone, and 2-octanone. It is seen that the growth of *B. cereus*, *B. subtilis*, *E. coli*, and *S. typhimurium* was inhibited by these compounds at concentrations found in the fruit body. On the other hand, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis* were not inhibited.

2-Hexenol and 2-hexenal are produced by most plants. Lyr and Banasiak (1983) showed that these volatile compounds have a broad bactericidal spectrum. They

Table 2. MIC of the Volatile Compounds Found in the Fruit Body of *P. ostreatus*

	MIC (μg of compd)/(g of broth)				
bacteria	1-octen-3-ol	3-octanol	octanol	3-octanone	2-octanone
Bc	3.9	62.5	15.6	62.5	62.5
Bs	125	250	125	125	125
Ec	500	125	125	125	125
Kp	1000	1000	1000	1000	1000
Pa	1000	1000	1000	1000	1000
Sa	1000	1000	250	500	500
Se	1000	1000	1000	500	500
St	250	500	125	500	500

Table 3. Negative (−) and Positive (+) Bacterial Growth in the Presence of Imitative Mixtures of Volatile Compounds Found in the Fruit Body of *P. ostreatus*

	volatile compd in culture broth[a]				
bacteria	4 times	2 times	1 times[a]	1/2 times	1/4 times
Bc	−	−	−	−	+
Bs	−	−	−	−	+
Ec	−	−	−	−	(+)[b]
Kp	−	−	−	(+)	+
Pa	−	−	−	(+)	+
Sa	−	−	−	+	+
Se	−	−	−	(+)	+
St	−	−	−	(+)	+

[a] 3-Octanone (140 mg/L), 3-octanol (220 mg/L), 1-octen-3-ol (560 mg/L), and 1-octanol (260 mg/L). [b] (+), notably less growth than control.

considered these compounds to be gaseous phytoalexins used to defend against to microbial attack. Deng *et al.* (1993) found that (*E*)-2-hexen-1-ol and (*E*)-hexenal inhibited the growth of *E. coli* and *Pseudomonas syringae*. Kubo *et al.* (1993) showed octanol as a flavor component of mate tea and demonstrated inhibitory effect of the alcohols on the proliferation of many microorganisms. Sterner (1995) has proposed that the cytotoxic terpenoids found in some mushrooms are used in chemical defense systems.

A culture broth containing 3-octanone (140 mg/L), 3-octanol (220 mg/L), 1-octen-3-ol (560 mg/L), and 1-octanol (260 mg/L) (the compounds and concentrations that were determined to exist in the fruit body on the basis of CCl_4 extraction) was prepared and called broth 1 as shown in Table 3. Broth 4, broth 2, broth 1/2, and broth 1/4 are culture broths having concentrations of the same compounds which are 4, 2, 1/2, and 1/4 times those of broth 1, respectively. Thus, the composition of broth 1 was an imitation of that from the fruit body of *P. ostreatus*.

Each of the volatile compounds possessed antibacterial qualities, but no single compound was present in a concentration sufficient to inhibit the growth of all bacterial strains tested. When the compounds were combined in concentrations that mimic those found in the fruiting body of *P. ostreatus*, the resulting mixture was found to inhibit completely all bacterial strains tested.

Similar assays were done with the inclusion of benzaldehyde (2800 mg/L) as a component of each broth mixture. Only a small increase in toxicity was observed in *S. aureus*, *P. aeruginosa*, *S. tiphymurium*, and *B. subtilis* cultures. The other bacterial strains were not affected by this addition.

Guillen *et al.* (1994) showed that *Pleurotus eryngii* produced benzaldehyde and benzoic acid in lesser extent from benzyl alcohol by an action of aryl-alcohol oxidase induced by H_2O_2. Kawabe and Morita (1994) reported that benzaldehyde and benzyl alcohol were produced by a white-rot fungus, *Polyporus tuberaster*, by reduction of benzoic acid, phenylalanine, 3-phenylpropionic acid, and 3-phenylpyruvic acid. The maximum concentration of benzaldehyde reached 90 mM with the addition of phenylalanine but was only 8 mM at the most without phenylalanine. In our case, the benzaldehyde concentration was approximately 30 mM in the fruit body but no benzyl alcohol was detected. It is worth noting that the aldehyde was not detected in N_2 conveyance and only at 0.25% relative to the CCl_4 extraction in vacuum distillate as shown Table 1.

Benzaldehyde was formed as a result of stimulation of the fruiting body of *P. ostreatus* by CCl_4. However, benzaldehyde does not contribute notably to the inhibition of bacterial growth. It is well documented that CCl_4 causes oxidative stress to animal tissues (Aguilar *et al.*, 1996; Allis *et al.*, 1996; Castro *et al.*, 1996; Kumaraveluu *et al.*, 1996; Padururu *et al.*, 1996; Parasakty *et al.*, 1996; Wang *et al.*, 1996). It is interesting to study the role of the aldehyde. Care must be taken when using solvents to study plant constituents. Wasowicz (1974) reported vacuum distillate of the fruiting body of *A. bisporus* as containing benzaldehyde to 0.2%. On the other hand, Pyysalo (1976) and Chen and Wu (1984) reported 1 order of magnitude higher concentration of the aldehyde in an ether extract of the same mushroom. Picardi and Issenberg (1973) studied the change in concentration of volatile compounds during cooking. They showed a notable increase of benzaldehyde in the heating process, which indicated the formation of the aldehyde in response to thermal stress.

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