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VOLATILE CONSTITUENTS OF *PLUS* AND *MINUS* STRAINS OF *BLAKESLEA TRISPORA*

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Key Word Index—*Blakeslea trispora*; Mucorales; xylene; isoamyl alcohol; 3-hydroxy-3-methylbutanal; dimethyl allyl alcohol.

Abstract—Identification of the predominant constituents produced by the *plus* and the *minus* strains of *Blakeslea trispora* is described. The occurrence of xylenes in the volatiles produced by the *plus* strain is reported. Additionally, production of 3-hydroxy-3-methylbutanal by the *plus* strain and dimethyl allyl alcohol by the *minus* strains were confirmed. Isoamyl alcohol, 1-octen-3-ol, 3-octanol and β-phenethyl alcohol were identified in volatiles from both strains.

INTRODUCTION

Blakeslea trispora, a heterothallic fungi belonging to the order Mucorales, is a carotenoid-producing microorganism. Biosynthesis of carotenoids in *B. trispora* is stimulated by the action of trisporic acids whose production is the result of an interaction between the *plus* and *minus* strains by means of substances, permeable through membrane filters [1, 2]. In general, the morphogenic phenomena (zygophore formation) [2, 3], the zygotropism [4], carotene synthesis [1] in the order Mucorales are regulated by sex hormones. Some of these regulators were speculated to be gaseous organospecific compounds [4, 5]. For example, the volatile compound from *Mucor mucedo*, which is the precursor of trisporic acids, showed chemotropic activity to zygophores in a sex-specific way [4].

Thus, it became of interest to compare the volatiles from the *plus* strain of *B. trispora* with that from the *minus* strain of the same organism. In this paper, the comparison of the constituents in the volatile fractions and the identification of the predominant components is described.

RESULTS AND DISCUSSION

Two strains of *B. trispora*, NRRL 2895 (*plus* mating type) and NRRL 2896 (*minus*) were used for all experiments. Cultures of both strains were grown separately in a medium containing yeast extract–glucose–thiamine hydrochloride for subcultures, and then transfered to Czapek–Dox medium. Both strains were incubated at 28.5° on a rotatory shaker. Fig. 1 indicates the typical growth of both the *plus* and *minus* strains in Czapek–Dox medium expressed on a dried cell basis. In the Czapek–Dox medium, the length of the lag period is *ca* 3 hr; rapid growth of both strains was then maintained until 24 hr after inoculation. According to the growth profile, the culture filtrates were separated from the culture broths after 3, 12, 24, 48, 72 and 130 hr. The volatile compounds were obtained from the filtrates by CH_2Cl_2 extraction followed by steam-distillation at atmospheric pressure. The components in the volatiles were analysed by GLC on Carbowax 20 M and LAC 446 columns, and GC–MS (27 eV).

GLC of the constituents of the *plus* volatile from 3, 12 and 24 hr-old cultures markedly differed from those of the *minus* volatile. Peaks 1–3, 5 and 6 were observed as predominant components in the *plus* volatile. In contrast, peaks 8 and 9 were specifically contained in the *minus* volatile. On the other hand, the volatiles from 48, 72 and 130 hr-old cultures of both strains were similar to each other. Peaks 4 and 12 were observed in both volatiles at all growth stage. It is notable that peaks corresponding to 1–3 and 5 were not observed in the *plus* volatile from the 72 hr-old culture. The peaks, 10 and 11, however, appeared in both volatiles from both strains at this stage.

By means of GC–MS and co-chromatography on GLC with authentic samples, the individual peaks were identified: peak 1 [*p*-xylene, RR_t to cyclohexanol; on LAC 446: 0.51, on Carbowax 20 M: 0.41, MS *m/e*: 106 (M^+), 91, 77, 65 and 43], 2 [*m*-xylene, RR_t (0.51, 0.42), MS *m/e*: 106 (M^+), 91, 77, 65 and 43], 3 [*o*-xylene, RR_t (0.58, 0.50), MS *m/e*; 106 (M^+), 91, 77, 65 and 43], 4 [isoamyl alcohol, RR_t (0.51, 0.53), MS *m/e*: 70 ($M^+ - H_2O$), 56, 42, 31], 8 [*iso*butanol, RR_t (0.33, 0.33), MS *m/e*: 74 (M^+), 73, 57, 56, 55, 43, 42 and 41], 9 [*n*-butanol, RR_t (0.42, 0.41), MS *m/e*: 74 (M^+), 73, 57, 56, 55, 43, 42 and 41], 10 [3-octanol, RR_t (1.05, 1.0). MS *m/e*: 101 ($M^+ - Et$), 83, 59, 57 and 55], 11 [1-octen-3-ol, RR_t (1.17, 1.16), MS *m/e*: 99 ($M^+ - Et$), 85, 72 and 57, 12 [β-phenethyl alcohol, RR_t (2.61, 2.41), MS *m/e*: 122 (M^+), 104, 91, 77, 65 and 43].

As mentioned above, isoamyl alcohol was one of the predominant components in the *plus* and *minus* strains at all growth stage. Sex-specific 5-carbon unit compounds, however, were observed in both volatiles.

The component peculiar to the *plus* volatile (peak 7) was observed as an isolated peak in the GLC

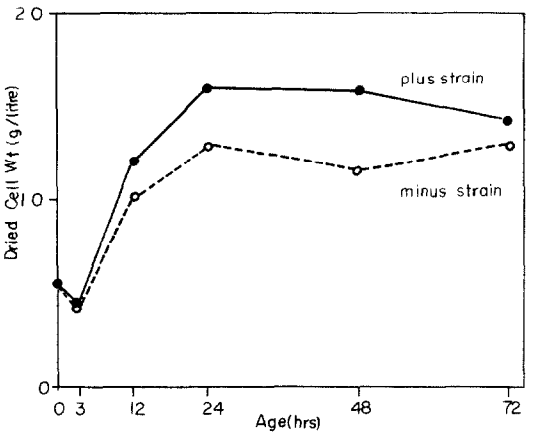

Fig. 1. Growth of the *plus* and *minus* strains of *B lakeslea trispora*.

chromatogram on Carbowax 20 M column (RR_t 0.71). The MS is simple, giving the predominant fragment ions at *m/e* 59, 44, 43, 41 and 31. These ions indicated the possible structures **1** and **2**. The structure of peak 7 was determined by the direct comparison of GC–MS and GLC of the synthesized samples. Compound **1** (3-hydroxy-3-methyl-1-butanal) was prepared by Schroeter's method [7]. Compound **2** (3-hydroxy-3-methyl-butan-2-one) was prepared by the bromination of 3-methyl-butan-2-one with Br_2 in aqueous HOAc, followed by the hydroxylation with Na_2CO_3. **1** gave the same MS as peak 7, but compound 2 also gave the fragment ions at *m/e* 59, 43 and 41. R_ts on Carbowax 20 M were 0.72 for **1** and 0.60 for **2**. From these data, peak 7 was identified as 3-hydroxy-3-methylbutanal.

In *minus* volatiles, dimethyl allyl alcohol peak 13 (RR_t: on LAC 446: 0.83, on Carbowax 20 M: 0.72; MS *m/e*: 71, 68, 57, 53, 44 and 41, **3**) was specifically contained. The structure was confirmed by direct comparison with authentic sample on GLC and GC–MS.

The amounts of *o*-xylene and isoamyl alcohol in the *plus* volatile were estimated on the basis of GLC peak area using cyclohexanol as internal standard. The amounts of *o*-xylene in the *plus* volatile from 1 l. of culture broth were as follows; 1.39 mg from 3 hr-old culture broth, 1.55 mg from 12 hr and 1.15 mg from 24 hr, respectively. No xylene peak was observed in the volatile separated after 48 hr. Correlating information on the xylene production of *plus* strain with the growth showed that xylene was produced during the lag and rapid growth stages. In contrast, the amounts of isoamyl alcohol from 3, 12, 24, 48 and 72 hr-old cultures were 0.25, 1.31, 2.05, 3.77 and 3.93 mg per l. culture broths. The amount of isoamyl alcohol increased at all growth stages.

As mentioned above, the constituents of the *plus* and *minus* volatiles, separated at the lag and rapid growth stages, were different. Xylene, specifically observed in the *plus* volatile as the prominent component, were obtained from a micro-organism for the first time, although these are widely distributed in higher plants (e.g. [8, 9]). Xylenes are thought to inhibit the growth of *B. trispora* [10]. The production of xylene in *B. trispora* might be a cause for the cessation of growth of the organism. Formation of *n*-butanol, *iso*butanol, *iso*amyl alcohol and β-phenethyl alcohol are often seen in fermentation. 3-Octanol and 1-octen-3-ol have already been isolated as the musty, fungal odorous compound from several *Aspergillus* species [11]. The 5-carbon unit compound, 3-hydroxy-3-methyl-butanal, specifically produced by the *plus* strain, is related to 3-hydroxy-3-methyl butanoic acid isolated from the urine of a patient with isovaleric acidemia [12]. This compound might be biosynthesized from mevaldic acid by decarboxylation. The alternative formation from isoprenyl alcohol (or dimethyl allyl alcohol) by hydration and oxidation is also possible as shown in Fig. 2. Instead of the production of carotenoids, this compound might be a waste

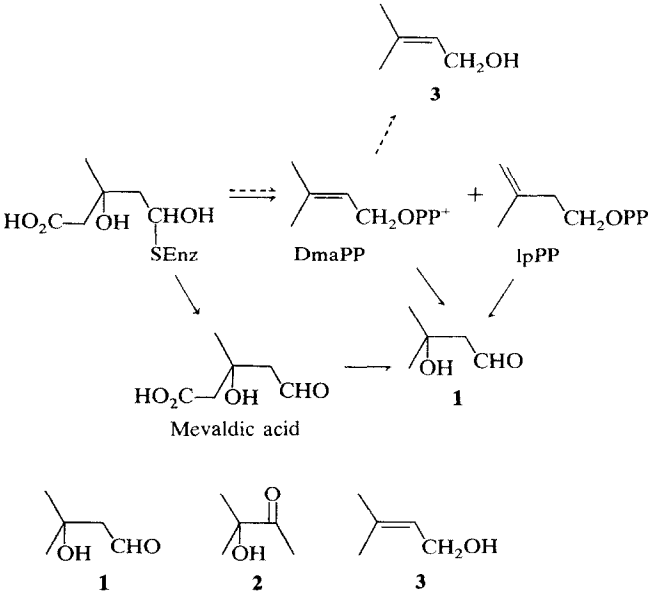

Fig. 2. Possible biosynthetic pathway of 3-hydroxy-3-methylbutanal. ——, *Plus* strain; ----, *minus* strain.

product from isoprenoid biosynthesis during single-strain culture.

Further work is in progress aimed at clarifying the roles of sex-specific substances on the carotenogenesis, growth and morphogenic phenomena in *B. trispora*.

EXPERIMENTAL

GLC analysis was carried out on stainless columns 5 m × 2 mm packed with 5% LAC 446 and 3 m × 3 mm packed with 5% Carbowax 20 M with temp. programming 70–160° at 2°/min. Carrier gas was N_2 at 20 ml/min. Detector used was FID. GC–MS was carried out on the same columns with the same column temp. programming with those for GLC. Carrier gas was He at 20 ml/min.

Micro-organisms. The *plus* (NRRL 2895) and *minus* (NRRL 2896) strains of *B. trispora* were gifts from Dr. Ellis of the Northern Regional Research Laboratory, USDA. Both strains were maintained on agar slopes consisting per l., glucose 20 g, potato extract (250 g per l. desalted H_2O) and NaOH to adjust to pH 6.8. Stock cultures were maintained at 28.5°. Transfer was made once a month.

Cultures. The culture medium was composed of yeast extract 5 g, glucose 50 g, thiamine HCl 2 mg and NaOH to adjust to pH 6.8. The whole mycelia of both *plus* and *minus* strains, obtained from the surface of slopes, were separately inoculated to 100 ml of the subculture medium in 500 ml conical flasks and cultured on a gyrotatory shaker at 28.5° for 2 days. Mycelial mats from two 2-day-old culture broths of both strains were separately combined and homogenized in 100 ml of Czapek–Dox medium and grown on a gyrotatory shaker at 28.5°.

Extraction and separation of volatiles. Mycelia and culture filtrates were separated 3, 12, 24, 48, 72 and 130 hr after transfer. Growth of both strains was measured from the dry wt of harvested mycelia. The culture filtrates (2 l.) was extracted ×3 with 200 ml of Et_2O. The Et_2O extract was dried (Na_2SO_4) and concd to *ca* 1 ml. The conc soln was transferred to 20 ml of Et_2O and H_2O and subjected to steam distn for 10 min at atm. pres. The distillates were extracted ×3 with 70 ml of Et_2O, extract was dried (Na_2SO_4) and concd to *ca* 1 ml *in vacuo* below 20°. Cyclohexanol was added to the concentrate as int. standard. (Recovery of cyclohexanol by this separation procedure from the culture medium was 61%.)

Preparation of 3-hydroxy-3-methyl-2-butanone. Br_2 (25 mg) was added at a rate such that it was continuously decolorized to a stirred mixture of 3-methyl-2-butanone (12.7 g), HOAc (8.1 ml) and H_2O (35 ml) at 60–80°. The reaction mixture was diluted with H_2O (17 ml) and neutralized with Na_2CO_3. The product was separated, dried ($CaCl_2$) and distilled (bp 110° at atm. pres.), giving 6.4 g (43% yield) of 3-bromo-3-methyl-2-butanone; IR $\nu_{max}^{liquid\ film}$ cm^{-1}: 1730 (α-haloketone), ^1H NMR (90 MHz, $CDCl_3$): δ 1.86 [6H, *s*, —C(Br)$(Me)_2$], 2.46 (3H, *s*, —Ac). 3-Bromo-3-methyl-2-butanone (2 g) was refluxed for 12 hr with 1.08 g Na_2SO_4. Distillation of the conc Et_2O extract at 150–180° at 160 mm Hg gave 19.8 mg of pure 3-hydroxy-3-methyl-2-butanone (yield 1.7%): IR $\nu_{max}^{liquid\ film}$ cm^{-1}: 3500 (OH), 1720 (α-hydroxy C=O), ^1H NMR (90 MHz, $CDCl_3$): δ 1.41 [6H, *s*, C(OH)$(Me)_2$], 2.27 (3H, *s*, —Ac], GC–MS (27 eV) *m/e* (rel. int.): 87 (M^+–Me–H_2O, 21), 59 (M^+ – Ac, 88), 57 (15), 43 (Ac^+, 100), 41 (97), 40 (27), 39 (8), 32 (34), 31 (36). GLC: (RR_t to cyclohexanol: 0.60 on Carbowax 20 M).

Preparation of 3-hydroxy-3-methyl butanal. 3-Hydroxy-3-methylbutanal was prepared from ethyl vinyl ether and Me_2CO_3 by the method of ref. [7]. Irradiation was carried out for 48 hr with a 100 W Wako Denki mercury lamp. The reaction mixture containing 3-hydroxy-3-methylbutanal was analysed directly by GLC (RR_t to cyclohexanol: 0.72 on Carbowax 20 M) and GC–MS (27 eV) *m/e* (rel. int.): 59 ($M^+ - CH_2CHO$, 68), 44 (36), 43 (CH_2CHO^+, 100), 41 (64), 39 (25), 31 (24).

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