Ester Synthesis by Zoogloea ramigera 115 Grown in the Presence of Ethanol

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Received for publication 4 October 1971

The ester ethyl butyrate is produced by Zoogloea ramigera 115, a bacterium isolated from an aerobic waste treatment plant, when ethanol is present in culture media. The cells appear to produce butyric acid which is then esterified with residual ethanol in the culture medium.

Zoogloea ramigera, a floc-forming bacterium indigenous to waste treatment systems and natural waters, is able to metabolize a wide variety of organic compounds, including numerous sugars, amino acids, organic acids and alcohols (2, 3, 6), and can utilize ammonium salts as sole nitrogen sources if supplied with suitable carbon and vitamin sources (8). During growth of Z. ramigera 115 on media supplemented with short-chain alcohols, a fruity odor was detected. This study was undertaken to identify this odor and to characterize its formation.

MATERIALS AND METHODS

Growth conditions. Z. ramigera isolate no. 115 (6, 8; available as ATCC 25935 through the American Type Culture Collection) was stored at 28°C on slants consisting of 0.5% (w/v) proteose peptone (PP, Difco, Detroit, Mich.) plus 0.5% (w/v) yeast extract (YE, Difco) solidified with 1.5% agar (Difco). Cultures were also stored in proteose peptone-yeast extract (PPYE) broth at -20°C.

Short-chain alcohols, C<sub>4</sub> through C<sub>8</sub> (including primary and secondary butyl alcohol), stearyl, and lauryl alcohols were tested in concentrations ranging from 0.1% to 10% (v/v) as substrates for ester production and as sole carbon sources. Culture media ingredients per liter were: (i) PP, 0.5 g; YE, 0.5 g; ethanol (96%), 23.0 ml; (ii) arginine·HCl, 0.5 g; MgSO<sub>4</sub>·7HOH, 2.0 g; K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 2.0 g; KH<sub>2</sub>PO<sub>4</sub>, 1.0 g; vitamin B<sub>12</sub>, 1.5 x 10<sup>-7</sup> g; ethyl alcohol, 23.0 ml; (iii) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g; MgSO<sub>4</sub>·7HOH, 2.0 g; K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 2.0 g; KH<sub>2</sub>PO<sub>4</sub>, 1.0 g; B<sub>12</sub>, 1.5 x 10<sup>-7</sup> g; ethanol 23.0 ml.

Cultures were grown in Erlenmeyer flasks containing liquid volume in volumes equal to 50% of the flasks' capacities, both as stationary and shaken (gyratory shaker, 160 rev/min; New Brunswick Scientific Co., New Brunswick, N.J.) cultures and in 8 liters of medium in an aerated fermentor (New Brunswick Scientific Co.). Ester production was also examined in agar plate cultures incubated at temperatures in the range of 4 to 45°C in a gradient temperature incubator (Lennox Corporation, Columbus, Ohio).

Paper chromatography. Shaken cultures were sampled several times during growth for the production of ester and assayed by the two-dimensional thin-layer chromatography technique of Bleiweiss (1) for intermediary acid compounds. One-dimensional paper chromatography [absolute ethanol-ammonium hydroxide-water (16:1:3)] was also employed. Acidic spots were located with bromphenol blue spray [0.04% (w/v) alcoholic bromphenol blue]. Micro-organic spot tests of Feigle (4) were employed to detect esters and intermediate acids.

Gas-liquid chromatography. Ester in liquid cultures was detected and identified by a combination of infrared spectroscopy and gas-liquid chromatography (GLC). Five GLC column packings were used: a 10-ft (ca. 3 m) column packed with Carbowax 600 (10% w/w) on Chromosorb W (Varian Aerograph, Walnut Hills, Calif.), a 10-ft column packed with Carbowax 1540 (15% w/w) on Chromosorb W (Varian Aerograph), a 3-ft (ca. 0.9 m) column packed with Hercoflex 5% on Chromosorb W (Varian Aerograph), a 23-ft (ca. 7 m) column composed of the latter three columns in sequence, and a 5-ft (ca. 1.5 m) column packed with ethylene glycol succinate (EGS, 15% w/w) on Gas-Chrom P (Applied Science, State College, Pa.). Nitrogen, with a flow rate of 25 ml/min at the exit port, was employed as the carrier gas. Injector and detector temperature was 165°C. The column was operated isothermally at 45°C for ester identification. A linear program with a rise of 4°C/min to 145°C was employed for examination of other culture products. Glass columns [inner diameter, one-eighth inch (ca. 3 mm)] were used in an Aerograph model 204 gas-liquid chromatograph equipped with a hydrogen flame detector (Varian Aerograph). Ester concentration in solution was estimated by comparison of GLC peak area to peak area obtained from stock ethyl butyrate solutions and adjusted for solution volume.

Ester identification. Cell-free culture supernatant fluids were directly injected into the GLC column or concentrated before injection by one of
two methods: (i) the cell-free supernatant material was placed in a dialysis tube and concentrated by water removal with Aquacide II (Calbiochem, Los Angeles, Calif.); (ii) the cell-free supernatant material was continuously extracted in a countercurrent continuous extractor using ethyl ether (anhydrous) and then evaporated at 70 C to one-tenth volume prior to injection into the GLC. The Aquacide concentrate was further extracted with absolute ethanol, and traces of water were removed by adding excess K₂CO₃. Ethanol solution was examined by infrared spectroscopy.

Cells grown in the presence and absence of ethanol were harvested at mid-log growth stage and examined for ability to produce ester. Butyric acid was added to a 1% concentration in cell-free supernatant fluids. Portions of this solution were treated in groups of 10 in the following manner: (i) held 5 min at 45 C, (ii) held 5 min at 60 C, (iii) held 5 min at 100 C, (iv) autoclaved 15 min at 121 C, (v) filter-sterilized, (vi) unheated control. Ethanol was then added (2% by volume), and duplicate samples were held at each temperature: 0, 4, 20, 28, and 37 C. The samples were examined after 72 hr for presence of ester. The above procedure was repeated using phosphate buffer (pH 7.0)-washed cells (washed eight times) in place of cell-free supernatant fluid, except for filter-sterilization. In this case, cells were suspended in filter-sterilized supernatant fluid.

RESULTS

Growth on alcohol-supplemented media. Growth was obtained within 24 hr at 28 C on all media supplemented with C-1 through C-5 alcohols at concentrations below 5%. Higher concentrations appeared inhibitory. Alcohols of length C-6 through C-8 also permitted growth, but the growth was considerably slower, requiring as long as 1 week to reach the levels obtained with shorter chain alcohols. Lauryl and stearyl alcohol inhibited growth completely. Short-chain alcohols (C 1 through C 5) also could serve as the sole source of carbon in the synthetic medium (5) containing ammonium salts and cyanocobalamin.

Ester identification. A fruity odor typical of esters was detected only during growth in alcohol-supplemented media. In the case of ethanol-supplemented media, an odor like that of fresh pineapple was noted. An infrared spectrum of an absolute alcohol extract of an Aquacide culture supernatant concentrate is shown in Fig. 1A. Figure 1B represents the spectrum of a control ethyl butyrate ester (1% v/v). Both spectra show doublet absorption bands at 1,720 and 1,730 cm⁻¹ and a band at 1,180 cm⁻¹ (carbonyls of ester groups).

Table 1 lists the retention times of known esters and the unknown ester produced in the Z. ramigera culture system after passage through an ethylene glycol succinate column at 75 C. Ethyl butyrate was the only ester with a retention time identical to that of the culture ester. Similar results were obtained with other columns tested and at lower temperatures.

The response to direct injection of cell-free culture supernatant fluids from PPY E- and PPYE-alcohol-grown cells into an EGS column at 45 C is shown in Fig. 2. It can be seen that the only response to the supernatant fluid from the nonsupplemented media (A) occurred after 360 sec as a broad peak or series of peaks. The alcohol-supplemented culture (B) yielded peaks with maxima at 104 and 149 sec and a broad response from 483 to 720 sec. The response at 104 sec corresponds to ethanol, whereas the response at 149 sec represents the culture ester. The responses occurring after 360 sec in both curves may be due to fatty acids [as suggested by the response to injection of an aqueous mixture of unmethylated (formic, acetic, propionic, butyric, and lactic) acids] or to as yet unidentified esters present in low concentrations. Fig. 3A illustrates the response to injection of an Aquacide concentrate of alcohol-supplemented culture supernatant material. Peaks corresponding to ethanol and the ester are seen. Addition of ethyl butyrate (0.5% v/v) reinforced the ester peak, as seen in Fig. 3B, whereas addition of 2% (v/v) ethanol reinforced the alcohol peak (Fig. 3C).

Nature of ester production. A comparison of the times required for production of ethyl butyrate, in quantities detectable with GLC, by spontaneous esterification of ethanol and butyric acid in the presence and absence of sulfuric acid and in the presence of alcohol grown cells of Z. ramigera 115 is shown in Table 2. The data indicate that the formation of ester in the presence of alcohol-grown cells occurs more rapidly than in the absence of cells (spontaneous esterification). Reddy (10) reported the formation of ethyl butyrate by Pseudomonas fragi in milk to be quantitatively enhanced by the inclusion of ethanol and butyric acid in the system. In the case of Z. ramigera, inclusion of butyric acid did not speed or quantitatively increase the production of the ester.

The pH (6.7 to 8.5) of the culture system favored the ester formation, while the chemical esterification occurred at levels below pH 6.5. Figure 4 illustrates the pH changes occurring during growth of isolate 115 in media, both alcohol-supplemented and nonsupplemented. The pH of the nonsupplemented and supplemented media dropped from 7.0 to approximately 6.5 during the first 4 hr. The nonsup-
Table 1. Retention times of selected esters on a 15% EGS column at 75°C

| Ester, % | Time (sec) |
|---------|------------|
| Methyl propionate | 98         |
| Methyl butyrate   | 98         |
| Ethyl propionate  | 105        |
| Ethyl butyrate    | 126        |
| Butyl propionate  | 210        |
| Allyl caproate    | 371        |
| Capryl acetate    | 315        |
| Culture ester     | 126        |

Supplemented cultures remained slightly acidic for 40 hr when the pH rose, probably reflecting the formation of ammonium ions due to the protein or amino acid degradation (7). The pH of the alcohol-supplemented media rose after the initial drop, reaching a maximum of 8.5 after 48 hr. This pH rise corresponds in time to the detection of the ester in the culture medium. The pH of the alcohol-supplemented media dropped to approximately 6.8 after 72 hr, which may reflect a decreasing alcohol content of the medium and loss of ability to remove acid via ester formation. Growth at this point is in the late stationary or early decline phase.

Ethyl butyrate was produced by cultures in ethanol-supplemented media only in the temperature range of 14 to 37°C, with an optimum of 28°C. Z. ramigera could grow over a range of 4 to 40°C. Transfer of cells grown at 4°C in alcohol media to 28°C resulted in ester production.

Fig. 1. Infrared spectra of (A) alcohol extract of concentrated culture supernatant fluid and (B) authentic ethyl butyrate. Doublet bands at 1,720 and 1,730 cm⁻¹ and a band at 1,180 cm⁻¹ (stars) are in the regions assigned to the carboxyls of ester groups.
Fig. 2. Gas-liquid chromatography response to injection of supernatant fluid of culture grown in proteose peptone-yeast extract (PPYE) (A) and in PPYE-ethanol (EtOH) (B).

Fig. 3. Gas-liquid chromatography response to injection of concentrated proteose peptone-yeast extract-ethanol culture supernatant fluid. (A), Culture; (B), culture supernatant fluid containing added ethyl butyrate (0.5%); (C), culture supernatant fluid containing added ethyl alcohol (2%).
Transfer of cells grown in alcohol-free media for extended time periods (30 days) to fresh alcohol-supplemented media did not immediately yield ester. However, after several transfers in alcohol-supplemented media, ester production was again detected.

While the supernatant fluids from cells grown in the absence of ethanol produced no ester when supplemented with 2% of the alcohol and incubated at 20, 28, and 37 C, the supernatant fluids from cells grown with ethanol yielded the expected ester under the same conditions. The enzymatic esterification was indicated, since heating of the supernatant fluids abolished the reaction.

Addition of unwashed or washed alcohol-grown cells to filter-sterilized PPYE culture supernatant fluids heated to 45 or 60 C did not result in the formation of ethyl butyrate. However, addition of these cells to heated PPYE culture filtrate supplemented with 2% ethanol resulted in the formation of the ester with a 0.5% maximum yield. Addition of large quantities of these cells to phosphate buffer, pH 7.2, containing 2% alcohol resulted in the formation of the ester in very low concentrations (less than 0.1%); in this case the ester was lost very rapidly, probably due to volatization. Non-alcohol-grown cells did not cause ester formation under similar conditions.

DISCUSSION

The ability of Z. ramigera 115 to grow on organic acids and alcohols had been demonstrated (8). Further, the organism is known to produce poly-β-hydroxybutyric acid (PHB) granules which are observed to be depleted when the organism (9) is placed in a starvation system. This implies that short-chain acids, including butyric acid, are being formed as the result of PHB degradation.

During growth of Z. ramigera in alcohol-free media, no esters were detected by either gas chromatography or examination of an alcoholic extract of the culture supernatant fluid by infrared spectroscopy. Addition of alcohol to the medium, however, resulted in the production of esters by the organism. Ethyl butyrate was the only detectable ester formed during culturing in ethyl alcohol.

We believe this esterification may permit the rapid removal of the acid which is formed.
as a metabolic by-product and may explain why the organism has been reported not to have an acidic reaction on organic substrates in spite of its rapid oxidation of organic compounds without CO₂ production. It is also possible that an ester-forming enzyme is tightly adsorbed to the extracellular polysaccharide strands that are produced by this organism, since it has been shown that the extracellular polymer has a high affinity for adsorption of protein and amino acids (7, 8). This could explain the association of the ester producing factor with cells and also media supernatant fluid.

Although the concentrations of organic acids and alcohols present in waste waters are generally low, the supply would augment due to the metabolic activities of other microorganisms. Removal of acid products could thus be of survival value to the organisms. We have observed the formation of ethyl butyrate in a mixed culture system containing Z. ramigera 115 and a saccharolytic yeast (unpublished data), suggesting that the ethanol formed by the yeast was esterified by the bacterium. Although no data are presented, there is no a priori reason why other alcohols would not also be esterified if present in the growth medium. A variety of alcohols would be expected to be present in the natural habitat of the organism (e.g., in sewage).

Furthermore, Z. ramigera appears to be related to Acetomonas and Acetobacter, organisms associated with C₂ metabolism (Schmidt et al., Bacteriol. Proc. 70:44). We believe this esterifying type of metabolic activity could be responsible for the formation of bouquets in wines and vinegars and could also aid in converting undesirable sewage odors to less objectionable odors.

ACKNOWLEDGMENTS

This investigation was supported by grant no. WP00713 from the Environmental Protection Agency, U.S. Department of Interior.

We wish to express appreciation for the technical assistance of Faith Reilly.

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