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# Antimicrobial Properties of Diacetyl†

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Diacetyl preparations from three commercial sources were found to be essentially similar when tested primarily against a set of 40 cultures, including 10 of lactic acid bacteria, 4 of yeasts, 12 of gram-positive non-lactic acid bacteria, and 14 of gram-negative bacteria. The compound was effective at pH ≤7.0 and progressively ineffective at pH >7.0. The lactic acid bacteria were essentially unaffected by concentrations between 100 and 350 μg/ml over the pH range of 5.0 to 7.0. Of the 12 gram-positive non-lactic acid bacteria, 11 were inhibited by 300 μg/ml at pH ≤7.0. The three yeasts and the 13 gram-negative bacteria that grew at pH 5.5 were inhibited by 200 μg/ml. Diacetyl was ineffective against four clostridia under anaerobic conditions. It was lethal for gram-negative bacteria and generally inhibitory for gram-positive bacteria. Nongrowing cells were not affected. The effectiveness of diacetyl was considerably less in brain heart infusion broth, Trypticase soy agar, and cooked-meat medium than in nutrient broth or plate count agar. The antimicrobial activity was antagonized by glucose, acetate, and Tween 80 but not by gluconic acid. As an antimicrobial agent, diacetyl was clearly more effective against gram-negative bacteria, yeasts, and molds than against gram-positive bacteria.

Diacetyl (2,3-butanedione, biacetyl) is perhaps best known as the compound responsible for the characteristic aroma and flavor of butter. It is produced by some species and strains of the genera *Streptococcus, Leuconostoc, Lactobacillus*, and *Pediococcus*, as well as by other organisms. In addition to butter and other dairy products, it is found in red and white wines, brandy, roasted coffee, ensilage, and many other fermented foods.

Although diacetyl was identified as a flavor compound in butter around 1929 (22), its antibacterial action was noted at least two years earlier by Lemoigne (15), who found that *Bacillus globigii* and *Bacillus subtilis* are inhibited by one concentration, whereas the organisms utilize or destroy the compound at lower concentrations. At least four reports on the antibacterial action of this compound appeared in 1936 (7, 9, 16, 17). The interest in diacetyl during this time stemmed from a folk medicine practice in Finland of inhaling wood tar as treatment for tuberculosis. Several investigators demonstrated the in vitro effectiveness of diacetyl against *Mycobacterium tuberculosis* (1, 3, 6, 7, 9, 18). Other organisms shown to be inhibited or killed by this compound include *Corynebacterium diphtheriae* (17), *Enterobacter aerogenes* (16), *Escherichia coli* (6, 7, 9, 17, 18, 20), *Erysipelothrix* sp. (7), *Klebsiella pneumoniae* (17), lactic acid bacteria (6, 16), *Mycobacterium phlei* (18), *Neisseria gonorrhoeae* (17), *Pseudomonas* spp. (9, 17, 18), *Salmonella* spp. (17), *Staphylococcus aureus* (6, 7, 9, 17, 20), and *Streptococcus* spp. (6, 7, 17, 20).

In his studies on the effect of bacteria on diacetyl in skimmed milk, Elliker (4) used 44 μg/ml and noted that 150 μg/ml inhibits some bacteria not affected by 44 μg/ml. Hedgecock and Jones (L. Hedgecock and L. Jones. Bacteriol. Proc., p. 127–128, 1950) found the compound at concentrations of 500 to 2,500 μg/ml (0.05 to 0.25%) to be effective against bacteria. They found it more effective against gram-negative bacteria than gram-positive bacteria, with lactobacilli being the most resistant. Among the first to assess the antibacterial activity of diacetyl against food-borne bacteria were Pinheiro et al. (19), who tested the compound and 13 others against *Pseudomonas fragi* by a disk assay procedure and found that although no zones of inhibition occur around disks dipped in 0.1 M solutions, 3-cm zones occur when disks are treated with a 1.0 M preparation. In their study of factors affecting the survival of *Salmonella* spp. in cheddar and colby cheese, Hargrove et al. (8) tried various compounds, including diacetyl, and noted that high levels slowed the growth of both salmonellae and lactic starters. The exact concentration used was not stated. Using

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a disk assay method, Kulshrestha and Marth (10) tested 27 milk-associated volatile compounds, including diacetyl, against bacteria and found that at 1,000 μg/ml, diacetyl is ineffective against *Escherichia coli* and *Staphylococcus aureus* but effective against *Salmonella typhimurium* and one strain of *Streptococcus diacetylactis*. Later, these investigators, in a series of reports (11–14), showed that diacetyl is one of the most effective of 25 volatile and nonvolatile milk-associated compounds tested against *Escherichia coli, Salmonella typhimurium*, and *Staphylococcus aureus*.

In the studies cited above, diacetyl was evaluated against only a few bacteria, and in most cases, concentrations of >400 μg/ml were found to be necessary for inhibition. The parameters that affect the antimicrobial activity of the compound have not been reported. In the present report, some of the conditions necessary for the maximum activity of this compound, in addition to the use of a larger number and broader range of organisms, are investigated. Under certain conditions, diacetyl has been found to be an effective inhibitor of microorganisms, with gram-negative bacteria and fungi being more sensitive than gram-positive bacteria.

## MATERIALS AND METHODS

**Diacetyl.** Diacetyl was obtained in pure form from three commercial sources (Eastman Kodak Co., Rochester, N.Y.; Sigma Chemical Co., St. Louis, Mo.; U.S. Biochemicals Corp., Cleveland, Ohio). Working solutions of either 1 M or 10% (vol/vol) were made up in absolute alcohol and kept at 2 to 5°C. Initially, the stock solutions were filter sterilized with 0.45-μm membranes, but it was later determined that sterilization was unnecessary. New stock solutions were prepared monthly. We achieved the desired medium concentration by adding diacetyl to sterile cooled agar, which was then poured into petri dishes. Agar plates were inoculated after overnight incubation at room temperature.

**Cultures and inoculation procedures.** A selected set of 40 cultures was used for most studies. Lactic acid bacteria were represented by the following strains: *Lactobacillus fermentans* ATCC 9338, *Lactobacillus plantarum* ATCC 8014, *Leuconostoc dextranicum* ATCC 19255, *Leuconostoc lactis* ATCC 19256, *Pediococcus acidilacticus* ATCC 8081, *Streptococcus cremoris* ATCC 19257, *Streptococcus faecalis* ATCC 19433, *Streptococcus faecalis* ATCC 19434, *Streptococcus faecium* ATCC 14432, and *Streptococcus lactis* ATCC 11454. Yeasts were represented by the following strains: *Candida lipolytica* WSU 8313, *Debaryomyces cantarellii* ATCC 24172, *Rhodotorula rubra* ATCC 9449, and *Torulopsis candida* ATCC 12790. Gram-positive non-lactic acid bacteria were represented by the following strains: *Bacillus cereus* WSU 60, *Bacillus cereus* 39 Benz, *Bacillus subtilis* ATCC 9799, *Bacillus megaterium* WSU 63, *Corynebacterium striatum* ATCC 6940, *Corynebacterium* sp. strain WSU 280, *Brevibacterium taipei* ATCC 13744, *Micrococcus luteus* WSU 82, *Micrococcus varians* WSU 202, *Staphylococcus aureus* 196E and ATCC 6538, and *Staphylococcus epidermidis* ATCC 155. Gram-negative bacteria were represented by the following strains: *Acinetobacter calcoaceticus* ATCC 9036, *Citrobacter freundii* WSU 600, *Escherichia coli* ATCC 11229, *Moraxella osloensis* ATCC 19955, *Serratia marcescens* WSU 120, *Salmonella kumasii* WSU 224, *Salmonella schottmuelleri* WSU 223, *Pseudomonas fluorescens* ATCC 17400, *Pseudomonas geniculata* ATCC 14150, *Pseudomonas alcaligenes* ATCC 14909, *Pseudomonas dimuta* ATCC 11568, two fluorescent *Pseudomonas* spp. isolated from spoiled meats, and *Yersinia enterocolitica* ATCC 27739. Other cultures employed are given in Results.

For the determination of the antimicrobial properties of diacetyl, a 0.5-in (1.27-cm) line of each of eight 24-h broth cultures was swabbed onto the surface of individual agar plates containing diacetyl and control plates (without diacetyl). The streaked plates were incubated at 30°C for 48 h and read for growth or inhibition. For inoculation into broths containing diacetyl, 24-h broth cultures were diluted in sterile saline and then inoculated to give a final concentration of $\sim 10^3$ cells per ml. After incubation at 30°C for 48 h, the tubes were observed visually for the presence or absence of turbidity. Tubes of the same medium without diacetyl were used as reference.

**Culture media.** All standard culture media used were manufactured by Difco Laboratories (Detroit, Mich.), except for Trypticase soy agar (TSA), which was manufactured by BBL Microbiology Systems (Cockeysville, Md.). The media were prepared in accordance with the directions of the manufacturers. Each solid medium contained 1.5% agar. To adjust the pH, we used 1 N HCl or NaOH.

**Effects of additives.** To test the effects of acetate, gluconic acid, glucose, and Tween 80 on the activity of diacetyl, we added various concentrations of these compounds to nutrient broth before it was autoclaved. Diacetyl was added to the sterile cooled broth, which was then added aseptically to sterile tubes.

**Effect on nongrowing cells.** Physiological saline was prepared and dispensed in 20-ml quantities into test tubes (20 by 150 mm). To one set of five tubes was added 344 ppm of diacetyl (0.004 M), whereas the other five tubes were used as controls. Both sets were inoculated with cells suspended in cold saline and were incubated at 3 to 5°C for 4 days. After incubation, the tubes were observed for turbidity, and then 1 ml of the contents of each tube was transferred to 19 ml of brain heart infusion (BHI) broth and incubated at 30°C for 48 h.

**Determining whether diacetyl was inhibitory or lethal.** Fifteen organisms were exposed to various levels of diacetyl for 7 days in BHI adjusted to pH 5.5. Broth from tubes with the lowest concentration that completely inhibited growth was diluted 1:5 in BHI broth containing 1% (vol/vol) Tween 80 and incubated at 30°C for 48 h. Growth in these tubes indicated that the compound only inhibited growth or rendered it static, whereas organisms which did not grow after direct exposure to the compound were considered to be killed.

**Effect on anaerobes.** Plate count agar (PCA) plates with various concentrations of diacetyl, along with control PCA plates, were streaked with four clostridia

TABLE 1. Effect of pH on the antimicrobial activity of diacetyl in PCA

| Diacetyl concn and type of organism | No. of strains that grew/no. tested at pH: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 | 7.7 | 8.1 | 8.6 |
| 100 μg/ml | | | | | | | | |
| Lactic acid bacteria | 9/9 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | —[a] | — |
| Gram-positive non-lactic acid bacteria | 4/4 | 8/8 | 11/11 | 11/12 | 11/12 | 12/12 | — | — |
| Yeasts | 3/3 | 3/3 | 3/4 | 4/4 | 4/4 | 4/4 | — | — |
| Gram-negative bacteria | 7/13 | 7/13 | 10/14 | 8/14 | 9/14 | 14/14 | — | — |
| 200 μg/ml | | | | | | | | |
| Lactic acid bacteria | 9/9 | 9/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| Gram-positive non-lactic acid bacteria | 1/4 | 3/8 | 7/11 | 4/12 | 2/12 | 11/12 | 12/12 | 12/12 |
| Yeasts | 1/3 | 0/3 | 0/4 | 1/4 | 0/4 | 4/4 | 4/4 | 4/4 |
| Gram-negative bacteria | 0/13 | 0/13 | 0/14 | 1/14 | 0/14 | 5/14 | 13/14 | 13/14 |
| 300 μg/ml | | | | | | | | |
| Lactic acid bacteria | 9/9 | 9/10 | 10/10 | 10/10 | 9/10 | 10/10 | 10/10 | 10/10 |
| Gram-positive non-lactic acid bacteria | 1/4 | 2/8 | 1/11 | 1/12 | 0/12 | 6/12 | 12/12 | 12/12 |
| Yeasts | 0/3 | 0/3 | 0/4 | 0/4 | 0/4 | 2/4 | 4/4 | 4/4 |
| Gram-negative bacteria | 0/13 | 0/13 | 0/14 | 0/14 | 0/14 | 1/14 | 5/14 | 10/14 |
| 350 μg/ml | | | | | | | | |
| Lactic acid bacteria | 9/9 | 8/10 | 9/10 | 10/10 | 7/10 | 10/10 | 10/10 | 10/10 |
| Gram-positive non-lactic acid bacteria | 1/4 | 1/8 | 0/11 | 1/12 | 0/12 | 5/12 | 11/12 | 12/12 |
| Yeasts | 0/3 | 0/3 | 0/4 | 0/4 | 0/4 | 1/4 | 4/4 | 4/4 |
| Gram-negative bacteria | 0/13 | 0/13 | 0/14 | 0/14 | 0/14 | 0/14 | 3/14 | 5/14 |
| 400 μg/ml | | | | | | | | |
| Lactic acid bacteria | — | — | — | — | — | — | 10/10 | 10/10 |
| Gram-positive non-lactic acid bacteria | — | — | — | — | — | — | 7/12 | 11/12 |
| Yeasts | — | — | — | — | — | — | 4/4 | 4/4 |
| Gram-negative bacteria | — | — | — | — | — | — | 2/14 | 3/14 |

[a] —, No data.

and five facultative anaerobes and incubated in a GasPak jar (BBL) at 30°C for 48 h. Freshly prepared thioglycolate broth in screw-capped tubes was inoculated with the same organisms and incubated in an open incubator at 30°C for 48 h. In both instances, growth was assessed visually.

## RESULTS

**Comparison of the three diacetyl preparations.** In regard to antimicrobial spectra, the three commercial diacetyl preparations were essentially similar. Each preparation was tested at 172 and 344 μg/ml, using the set of 40 cultures in PCA at pH 6.5, and the results were essentially similar at 172 μg/ml and identical at 344 μg/ml. The gram-positive non-lactic acid bacteria not inhibited by 344 μg/ml (*Corynebacterium striatum, Bacillus subtilis, Staphylococcus epidermiditis*) were the same for each preparation. At 172 μg/ml, the Sigma preparation inhibited *Yersinia enterocolitica*, whereas the other two preparations did not. The other gram-negative bacteria inhibited by this concentration were pseudomonads. The Eastman Kodak preparation was used to gather most of the data in this study. This preparation was assayed by the company at 97% by gas-liquid chromatography, and this concentration was factored into the stock solutions employed. Preparations freshly distilled in a water bath at 88 to 90°C showed the same antimicrobial spectrum shown by undistilled preparations. The infrared and proton nuclear magnetic resonance spectra revealed undistilled preparations, distilled preparations, and distillate residue to be identical (data not shown). The infrared spectrum for each matched that in the Aldrich Library of Infrared Spectra (Aldrich Chemical Co., Milwaukee, Wis.) for diacetyl. By nuclear magnetic resonance, the 3% impurity was shown to be water.

**Effect of pH.** The effects of diacetyl on microorganisms in PCA at different pH values are shown in Table 1. The data are from an experiment in which Eastman Kodak diacetyl was

TABLE 2. Relative effectiveness of diacetyl against microorganisms in various substrates[a]

| Determination for[b]: | No. that grew on diacetyl in: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PCA[c] | NA[c] | AOAC-YE[d] | TSA[d] | BHIA[d] | NBG | Skimmed milk | Cooked-meat medium with diacetyl concn of: | |
| | | | | | | | | 344 μg/ml | 860 μg/ml |
| Lactic acid bacteria (10).. | 10 | 9 | 10 | 10 | 10 | 10 | 10 | 5/5[e] | 5/5 |
| Gram-positive non-lactic acid bacteria (12)..... | 2 | 9 | 7 | 12 | 12 | 5 | 7 | 7/7 | 7/7 |
| Yeasts (4)..... | 0 | 3 | 3 | 4 | 4 | 0 | 2 | 1/2 | 0/2 |
| Gram-negative bacteria (14) | 0 | 3 | 5 | 11 | 14 | 1 | 3 | 10/10 | 4/10 |
| Total no. inhibited..... | 28 | 26 | 15 | 3 | 0 | 24 | 17 | 1 | 8 |

[a] All substrates were adjusted to pH 6.5 except for cooked-meat medium, which was pH 7.2. NA, Nutrient agar; AOAC-YE, Difco synthetic AOAC broth supplemented with 1% yeast extract; BHIA, BHI agar; NBG, nutrient broth supplemented with 0.1% glucose.

[b] Numbers in parentheses are the numbers of organisms tested.

[c] Diacetyl concentration, 350 μg/ml.

[d] Diacetyl concentration, 344 μg/ml.

[e] Number that grew/number tested.

used and are typical of those obtained in two other experiments in which U.S. Biochemicals diacetyl was used. The 10 lactic acid bacteria were essentially unaffected by any concentration of the compound. At pH 5.0, the *Streptococcus faecium* strain did not grow on control plates. This strain and *Streptococcus lactis* were the two lactic acid bacteria most sensitive to diacetyl.

In regard to the gram-positive non-lactic acid bacteria, all were inhibited by 300 μg/ml at pH 7.0. *Bacillus subtilis* was not inhibited by this concentration at pH values of 6.0 and 6.5. This organism was not completely inhibited by 350 μg/ml at pH values of 5.0, 5.5, and 6.5. Thus, of this group of organisms, it was the most resistant to diacetyl. The most sensitive of this group were *Bacillus cereus* 60, *Micrococcus luteus*, and *Corynebacterium* sp.

The three yeasts that grew at pH 5.5 were inhibited by 200 μg/ml. All four yeasts were inhibited by 300 μg/ml. The most sensitive was *Rhodotorula rubra*.

In regard to the gram-negative bacteria, the 13 that grew at pH values of 5.0 and 5.5 were inhibited by 200 μg/ml. All 14 were inhibited at pH values of 6.0 and 7.0, but one (*Acinetobacter calcoaceticus*) grew at pH 6.5. The most sensitive gram-negative bacteria were *Pseudomonas alcaligenes*, *Pseudomonas dimuta*, and *Pseudomonas fluorescens*, and they were consistently inhibited by 100 μg/ml at pH ≤7.0. The gram-negative bacteria were the most sensitive to diacetyl, followed by the yeasts, the gram-positive non-lactic acid bacteria, and the lactic acid bacteria (in that order).

In regard to the effect of diacetyl under alkaline conditions, the compound was less effective against each of the three groups affected at pH ≤7.0, but the effect was most pronounced on the gram-positive non-lactic acid bacteria. Although 300 μg/ml was inhibitory to these 12 organisms at pH 7.0, it was ineffective at pH ≥8.1. The greater sensitivity of gram-negative bacteria to diacetyl was further manifested by the observation that these organisms were inhibited at pH ≥7.7 to a greater extent than were either of the other groups.

**Influence of growth substrate.** In regard to antimicrobial acitivity on eight substrates, diacetyl was most effective on PCA and least effective on BHI agar and cooked-meat medium (Table 2). In terms of diacetyl effectiveness, the eight substrates ranked as follows: PCA > nutrient agar > nutrient broth with 0.1% glucose > skimmed milk > ADAC broth with 1% yeast extract > TSA > cooked-meat medium > BHI agar. In cooked-meat medium, only 1 of 24 organisms (a yeast) was inhibited by 344 μg/ml. Even at 860 μg/ml, only 8 of 24 organisms were inhibited: the 2 yeasts and 6 gram-negative bacteria. This medium was used at pH 7.2.

**Effects of additives.** The effects of 350 μg of diacetyl per ml on the 40 microorganisms in nutrient broth containing glucose and gluconic acid at pH 6.5 was determined, and the results (Table 3) indicate that ≤5% (wt/vol) glucose was more antagonistic to diacetyl for all groups except the lactic acid bacteria. Gluconic acid at ≤5% had no effect on the antimicrobial properties of the compound. Acetate was more antagonistic than glucose or Tween 80, but a combination of 1% acetate and 1% Tween 80 was slightly less antagonistic than either compound alone (Table 4). The reason for this is unclear.

**Effect of anaerobes.** Of four clostridia exposed

TABLE 3. Effects of glucose and gluconic acid on the antimicrobial activity of 350 μg of diacetyl per ml in nutrient broth at pH 6.5

| Determination for[a]: | No. that grew on indicated % of: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Glucose | | | | Gluconic acid | | |
| | 0 | 0.5 | 1.0 | 5.0 | 0.5 | 1.0 | 5.0 |
| Lactic acid bacteria (10) | 9 | 10 | 10 | 10 | 9 | 9 | 8 |
| Gram-positive non-lactic acid bacteria (12) | 9 | 9 | 9 | 7 | 0 | 0 | 0 |
| Yeasts (4) | 3 | 2 | 2 | 2 | 0 | 0 | 0 |
| Gram-negative bacteria (14) | 3 | 3 | 3 | 4 | 0 | 0 | 0 |
| Total no. inhibited | 16 | 16 | 16 | 17 | 31 | 31 | 32 |

[a] Numbers in parentheses are numbers of organisms tested.

to 344 and 688 μg of diacetyl per ml in GasPak jars containing PCA, none was inhibited by either concentration (Table 5). On the other hand, when the same organisms were exposed to 344 μg/ml in thioglycolate broth, one was inhibited and three were not. The latter three were inhibited, however, by 688 μg/ml. Why diacetyl was more inhibitory in thioglycolate than in PCA is unclear. These organisms were not inhibited in cooked-meat medium by 860 μg/ml (0.01 M) under the same incubation conditions, but the latter medium had a pH of 7.2, whereas thioglycolate broth had a pH of 6.5.

The five facultative anaerobes which were treated in similar ways were all inhibited by 344 μg/ml on PCA, but only one (*Yersinia enterocolitica*) was inhibited by this concentration in thioglycolate broth.

**Effect on nongrowing cells.** Diacetyl was not inhibitory to four nongrowing cultures which were susceptible to the level of compound used under growth conditions. Although growing *Streptococcus faecalis* was not inhibited by 344 μg/ml, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella schottmuelleri*, and *Torulopsis candida* were. None grew in saline at 2 to 4°C for 4 days but all grew when subcultured to BHI broth, indicating that in cold saline, they were not affected by diacetyl. That the lack of effect was not due to low temperature alone was demonstrated by the observation that three organisms which grew in control nutrient broth at 5°C in 7 days they did not grow in duplicate sets of broth which contained 344 μg of diacetyl per ml.

**Inhibitory or lethal effects.** In regard to whether diacetyl was inhibitory or lethal to microbial cells, data obtained from 15 cultures indicated that the gram-negative bacteria were killed upon exposure to 258 or 344 μg/ml, with the exception of one strain of *Escherichia coli*. The results from four yeasts indicated that two were inhibited and two were killed, whereas the one gram-positive bacterial strain (*Bacillus cereus*) was inhibited. Additional studies involved the subculturing of inocula from inhibitory plates to BHI broth. Repeated subculturing of *Bacillus cereus*, *Corynebacterium* sp., *Staphylococcus aureus*, and *Micrococcus varians* resulted in growth in BHI broth, thus indicating that the

TABLE 4. Effects of 1% acetate and 1% Tween 80 on the antimicrobial activity of 344 μg of diacetyl per ml in PCA at pH 6.5

| Determination for[a]: | No. that grew on: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Diacetyl | Acetate | Acetate + diacetyl | Tween 80 | Tween 80 + diacetyl | Acetate + Tween 80 | Tween 80 + acetate + diacetyl |
| Lactic acid bacteria (10) | 8 | 10 | 10 | 10 | 10 | 10 | 10 |
| Gram-positive non-lactic acid bacteria (11)[b] | 0 | 11 | 11 | 6 | 5 | 11 | 6 |
| Yeasts (4) | 0 | 4 | 3 | 4 | 2 | 4 | 1 |
| Gram-negative bacteria (14) | 0 | 14 | 7 | 14 | 4 | 13 | 6 |
| Total no. inhibited | 31 | 0 | 8 | 5 | 18 | 1 | 16 |

[a] Numbers in parentheses are the numbers of organisms tested.
[b] *Corynebacterium striatum* was not used.

TABLE 5. Effect of diacetyl on four anaerobes and five facultative anaerobes under anaerobic conditions at pH 6.5 in GasPak jars containing PCA and in thioglycolate broth

| Organism[a] | Growth in PCA + diacetyl at (μg/ml): | | | Growth in thioglycolate broth + diacetyl at (μg/ml): | | |
|---|---|---|---|---|---|---|
| | 0 | 344 | 688 | 0 | 344 | 688 |
| *Clostridium sporogenes* WSU 480 | +[a] | + | + | + | + | −[b] |
| *Clostridium bifermentans* ATCC 19299 | + | + | + | + | + | − |
| *Clostridium perfringens* ATCC 3624 | + | + | + | + | + | − |
| *Clostridium sordellii* WSU 491 | + | + | + | + | − | − |
| *Staphylococcus aureus* ATCC 6538 | + | − | − | + | + | − |
| *Escherichia coli* ATCC 11229 | + | − | − | + | + | − |
| *Citrobacter freundii* WSU 600 | + | − | − | + | + | − |
| *Salmonella schottmuelleri* WSU 223 | + | − | − | + | + | − |
| *Yersinia enterocolitica* ATCC 27739 | + | − | − | + | − | − |

[a] +, Growth.
[b] −, No growth.

viability of these organisms was not lost upon exposure to diacetyl.

When diacetyl was dialyzed overnight at 5°C against distilled water, the antimicrobial activity was lost as expected. Concentrations as high as 3,400 μg/ml (0.04 M) in PCA at pH 6.5 did not inhibit any of the 40 organisms.

## DISCUSSION

Diacetyl is a greenish-yellow liquid with a boiling point of 88°C and the sharp odor of butter. The three commercial preparations used in this study were quite similar in antimicrobial spectra. Although not used in this study, diacetyl preparations from four other companies were evaluated against the 40 cultures and found to be similar in activity to the preparations from the three sources we used.

In regard to some of the previous reports on the antibacterial activity of diacetyl, Myrvik and Volk (18) found that 3 μg/ml inhibits three strains of *Mycobacterium tuberculosis* in a liquid medium that contains 5% bovine serum, whereas 31 μg/ml is required to inhibit *Escherichia coli* and a pseudomonad. These are the lowest inhibitory concentrations reported, especially for *Mycobacterium tuberculosis*, and the results are somewhat surprising since the cultures were incubated at 37°C and observed after 7 and 14 days. The finding by Hano (7) that a diacetyl concentration of 1:50 is necessary to inhibit *Mycobacterium tuberculosis* was based upon a 15-day incubation at 37°C with Petroniani medium. The relative lack of activity against *Pseudomonas fragi* in the study by Pinheiro et al. (19) was probably due to the ineffectiveness of the disk assay procedure for this compound. When Kulshrestha and Marth (10) used a similar method, 1,000 μg/ml was found to be ineffective against *Escherichia coli* and *Staphylococcus aureus*. The volatility of diacetyl may be responsible for the relative ineffectiveness observed when this test method was used. Gupta et al. (6) evaluated the compound in raw milk and found that 250 μg/ml increases methylene blue reduction time from 3 to 5 h. With 500 to 1,500 μg/ml, the reduction time is extended only to 6 h. Why these investigators did not find that four strains of *Escherichia coli* are inhibited by 500 μg/ml is not clear. Although the investigators exposed the organisms to diacetyl for 24 h, neither the medium employed and its pH nor the incubation temperature was stated. Kulshrestha and Marth exposed bacteria to diacetyl in nutrient broth and incubated the cultures at 37°C for up to 14 h in aerosal cans (11). To determine the effect of diacetyl, the researchers performed plate counts with PCA and compared the results to control plate counts in order to determine the degree of inhibition. They found that for *Escherichia coli*, statistically significant reduction in numbers occurs 2 h after exposure to 1, 10, or, 100 μg/ml, and complete inhibition occurs 2 h after exposure to 1,000 μg/ml. For *Salmonella typhimurium*, significant reduction occurs 2 h after exposure to 100 μg/ml and complete inhibition occurs 2 h after exposure to 1,000 μg/ml (12). At 1 or 10 μg/ml, significant reduction does not occur until after 5 h of exposure, indicating that *Salmonella typhimurium* has a greater resistance to diacetyl than does *Escherichia coli*, as determined by the methods used. On the other hand, the lowest concentration that produces statistically significant reduction of *Staphylococcus aureus* is 10 μg/ml, and 8 h are required (13). After 5 h, similar results are obtained with 100 μg/ml, and complete inhibition is obtained with 1,000 μg/ml, indicating that this gram-positive bacterium is more resistant than the two gram-negative bacteria. When *Streptococcus lactis* is employed, 5 h are required for 1 or 10 μg/ml to cause significant reduction and for 1,000 μg/ml to cause

complete inhibition (14). Significant reduction after 2 h requires 100 μg/ml.

There are three primary reasons why in most of the previous studies diacetyl concentrations above 400 to 500 μg/ml were found necessary for the inhibition of bacteria: (i) the exposure of bacteria to the compound at pH ≥7.0, (ii) the use of culture media that contained antagonistic substances, and (iii) the exposure of organisms to diacetyl generally at 37°C. Preliminary studies on the effect of incubation temperature indicate that the compound is even more effective at 10 and 20°C than at 37°C (data not shown).

The lack of effectiveness of diacetyl in BHI broth was probably due to one or all of the following factors: (i) the nutrient composition of the medium, (ii) the size of inoculum used, and (iii) the medium pH. When BHI broth at pH 5.5 was inoculated with $\sim 10^3$ cells per ml, 344 μg of diacetyl per ml prevented the growth of all but 3 of the 36 non-lactic acid organisms, including 5 yeasts and 2 molds. At 258 μg/ml, only 13 of the 36 organisms were not inhibited. Of the 10 fluorescent pseudomonads, 6 were inhibited by 172 μg/ml. Under all conditions studied, the latter group of organisms was the most sensitive to diacetyl.

Although concentrations of several hundred micrograms per milliliter have been shown to be necessary for inhibition of most organisms under the conditions tested, this does not obviate the use of the compound as a broad-spectrum antimicrobial agent. In certain foods, nitrite at ≤200 μg/ml, sulfur dioxide at ≤200 to 300 μg/ml, benzoate at ≤0.1%, and sorbate and propionates at ≤0.2 and ≤0.32%, respectively, are permitted. Unlike most of these compounds, diacetyl imparts a sharp aroma of butter, but because of its volatility, its use as an antimicrobial dip for utensils and surfaces has potential application in the food industry. The uniqueness that it displays as an antimicrobial agent (most effective against gram-negative bacteria and fungi) is of such importance that other related compounds that lack its aroma should be sought and developed. It is generally regarded as safe (GRAS), but whether it lacks mutagenic activity is unclear. It does not produce lung tumors in strain A mice (21), but when subjected to the Ames test, it showed dose-related activity against strain TA 100 and produced 400 revertant colonies per plate in one study (2), whereas in another, it was found to be nonmutagenic when tested at 3 μmol per plate against strain TA 100 (5).

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