Effect of Citric Acid Concentration on the Formation of Diacetyl by Certain Lactic Acid Bacteria

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Diacetyl has been associated with the “buttermilk off-flavor” sometimes found in orange juice and in frozen concentrated orange juice. Bacteria of the Lactobacillus and Leuconostoc genera have been identified as causative agents of this type of spoilage (Hays, 1951; Hays and Riester, 1952; Murdock et al., 1952, 1953; and Hill et al., 1954). Growth rates of these spoilage organisms have been investigated by Barreto (1953) and Rushing et al. (1956). Witter (1956) reported a Lactobacillus species capable of diacetyl production at a rate of 2.5 ppm per hr per million organisms per ml in a continuous culture system. This means that it would take 10,000 organisms per ml 4 hr to produce 0.1 ppm. This amount is well below the flavor threshold in orange juice (Hill et al., 1954). In any case, generation times were found to be insufficient for spoilage to occur in the juice or concentrates in the evaporator unless relatively static films or pockets of juice existed (Rushing et al., 1956). Other factors favoring diacetyl accumulations could be high initial populations, especially of efficient diacetyl producers, or recirculation of the juice in the evaporators. Christensen and Pederson (1958) found citric acid to be essential for diacetyl production by some strains of lactic acid bacteria, and in all species studied, found that diacetyl production in low sugar medium was enhanced by citric acid.

The present investigation was undertaken to clarify some of the factors influencing the accumulation of diacetyl in citrus juice products. Conditions were kept within normal limits of pH, concentration of nutrients, and temperatures encountered in the processing of orange juice concentrates.

Materials and Methods

Cultures. Four species of lactic acid bacteria isolated from frozen concentrated orange juice showing buttermilk off-flavor spoilage were used in these studies. Most of the results reported were obtained with Lactobacillus brevis. In certain of the experiments, Lactobacillus plantarum var. mobilis, Leuconostoc mesenteroides, and Leuconostoc dextranicum were also employed. The history of these strains was previously reported (Rushing et al., 1956). Inoculation was usually from orange serum broth, and in the more definitive experiments, inoculation was with washed cells which had been adjusted to 40 per cent transmittance at 530 m\(\mu\) on the Lumetron model 401 colorimeter. The inoculum was 1 per cent of the volume of the medium. In certain experiments, inoculation was from nutrient broth or Micro Inoculum Broth (Difco).

Fermentation media. The media used varied with the objective of the experiment. To determine the relative efficiency of diacetyl accumulation under commercial evaporator conditions in which one species may predominate in a mixed culture, nonsterile partially concentrated orange juice was the medium. Concentrated Valencia orange juice without added oil or cutback juice was diluted to 20° Brix with distilled water and the pH adjusted to 3.8 with NaOH. The titratable acidity before pH adjustment was 2 per cent, expressed as citric acid. This uniform batch of juice was divided into four portions. Three were inoculated with L. brevis, L. plantarum var. mobilis, and L. mesenteroides, respectively. The fourth was retained as a control. The inocula were grown in orange serum broth and the centrifugally sedimented cells used to give an initial population level of about 6 to 8 million per ml of the well mixed medium. Each portion was sealed into 4-ounce cans and incubated at 4 to 5 C. One can of each was removed initially and daily for 11 days for plate counts and diacetyl determinations.

Nutrient broth was used as the basal medium to detect possible diacetyl precursors among the major components of orange juice. This was supplemented with glucose, fructose, sucrose, citric acid, or ascorbic acid for this purpose. It was also supplemented with malic acid and with succinic acid to determine whether or not the Krebs cycle was directly involved in diacetyl synthesis. In an effort to gain some insight into the nature of the enzymes involved in diacetyl synthesis, this basal medium was supplemented with citric acid and treated with certain enzyme inhibitors or with methyl ethyl

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1 One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

2 The mention of trade products does not imply that they are recommended by the Department of Agriculture over similar products not mentioned.

3 Will Corporation, Buffalo, New York.

4 Difco Laboratories, Inc., Detroit, Michigan.
ketone as a possible competitive inhibitor. Comparisons of the relative efficiency of citric acid and pyruvic acid as diacetyl precursors were also made with nutrient broth as the basal medium. Inocula were from nutrient broth or orange serum broth in these experiments.

The synthetic medium of Lyman et al. (1956) was used to determine the effect of added citric acid at levels up to 4 per cent on diacetyl accumulation. In each case, glucose was added as required to maintain total nutrients at the same level. This medium was prepared double strength, adjusted to pH 3.9, and sterilized in bulk. The cooled medium was inoculated with washed cells in distilled water at a level of 2 ml cell suspension per 100 ml double strength medium. Cell density in the inocula was controlled turbidimetrically as indicated above. After mixing, the inoculated double strength medium was dispensed in 5-ml portions into sterile screw-capped tubes. Dilution was with 5 ml per tube of sterile mixtures of glucose and citric acid, adjusted to pH 3.9 with NaOH. Citrate concentrations were 0.1, 1.0, 2.0, 3.0, and 4.0 per cent. Incubation was at 21 C. Diacetyl determinations and plate counts were made initially and at 8-hr intervals for at least 5 days.

Other experiments designed to determine the effect of citric acid concentrations on diacetyl accumulation were carried out in filtered orange juice. This orange serum was prepared by filtration with diatomaceous filter aid of a single lot of freshly extracted Valencia orange juice. Total organic acids was determined to be 1.0 per cent by titration between the limits pH 2.0 to 7.8. This was considered to be entirely citric acid in adjusting total citrate levels, since it is known that only minor amounts of other acids occur in the juice of mature oranges (Braverman, 1949). Citrate levels were adjusted to 1.0, 2.0, 3.0, and 4.0 per cent. Appropriate amounts of sucrose were added in each case to keep the total available nutrients at the same level. The pH was adjusted to 3.8 with NaOH and the supplemented juice sterilized in flowing steam at 100 C for 30 min. The cooled media were inoculated with washed cells of L. brevis, mixed thoroughly, and dispensed in 10-ml portions into sterile, screw-capped tubes. Incubation was at 21 C. Diacetyl determinations and plate counts were made initially and daily.

Plate count medium. Commercial orange serum agar was used to determine all population levels. Lactobacillus colonies were counted after 48 hr incubation at 30 C and Leuconostoc colonies after 24 hr at 21 C. In a few cases when counts could not be made at the proper time, the plates were stored in a refrigerator overnight and counted the next day.

Diacetyl determinations. When the volume of medium was sufficient, diacetyl was concentrated from 100 ml of fermentation medium by the distillation method of Byer (1954) and diacetyl determined in an appropriate dilution of the distillate by the method of Hill et al. (1954). A micro modification was devised for the test tube scale experiments. In these cases, 5 ml of fermentation mixture were introduced into a micro-Kjeldahl still, diluted with 10 ml distilled water, 10 ml distillate collected, and diacetyl determined as above. The diacetyl content of the medium was determined in both cases from calibration curves prepared in the same manner from known concentrations in water of freshly distilled diacetyl.

Results

Marked differences in the ability of the different organisms to accumulate diacetyl were observed in 20° Brix orange concentrate which had not been sterilized prior to inoculation. In all cases, 11 days of incubation were sufficient to permit populations to rise to a maximum and begin to decline. In figure 1, it is shown that under these conditions, L. brevis accumulated more diacetyl (360 ppm) than either of the other two species or the control. Diacetyl reached 50 ppm with L. mesenteroides and 43 ppm in the nonsterile inoculated control. With L. brevis, L. mesenteroides, and the control, the diacetyl peak corresponded with the midpoint of the logarithmic phase of the growth curve. Both population and diacetyl accumulation curves were atypical with L. plantarum. Since L. brevis produced more diacetyl than did the other species, it was selected for more detailed study.

It was found that nutrient broth would support the growth of L. brevis without the production of more than traces of diacetyl. Addition of glucose, fructose, or sucrose did not increase diacetyl production ap-

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Footnote:

5 Products from both Difco and Baltimore Biological Laboratory, Inc., Baltimore, Maryland, were used in this investigation.
preciable. Ascorbic acid was also inactive. The addition of either citric or pyruvic acid to nutrient broth supplemented with one of the sugars resulted in substantial diacetyl accumulation. When supplied at equal carbon levels, these two acids were equivalent as diacetyl precursors, with the peak of the diacetyl curve coming slightly earlier with pyruvic acid. Since no diacetyl accumulated in unsupplemented nutrient broth, no amino acids were tested as diacetyl precursors.

The activity of two compounds related to the Krebs cycle suggested that other acids associated with this system might also be effective precursors of diacetyl. However, addition of neither male nor succinic acid resulted in diacetyl accumulation in nutrient broth medium. Inhibitors of Krebs cycle enzymes (or of other enzymes), among them malonate, fluoride, iodoacetate, cyanide, and \(\beta\)-propiolactone failed to inhibit preferentially either growth or diacetyl accumulation. Methyl ethyl ketone added to nutrient broth failed to act as a competitive inhibitor either of diacetyl synthesis or catabolism. The ability of the organism to couple aromatic analogues of pyruvic acid or of acetaldehyde to form acetoin or diacetyl or analogues thereof was not tested.

Although nutrient broth was a suitable fermentation medium for these experiments, it was not suitable as a medium for carrying stock cultures, since the organism lost its capacity to produce diacetyl from citrate after several transfers on this medium. This ability was regained on transfer through a citrate containing medium.

*Lactobacillus plantarum* also grew acceptably on nutrient broth, but produced large amounts of diacetyl in the absence of both preformed citrate and pyruvate. Similar results were obtained on Lyman's medium. In fact, no medium was found during this investigation in which *L. plantarum* would grow without the production of substantial amounts of diacetyl. Nutrient broth failed to support the growth of the *Leuconostoc* species at the pH of orange juice.

The synthetic medium of Lyman et al. (1956) recommended for *L. mesenteroides* failed to support the growth of either *L. mesenteroides* or *L. dextranicum* when used at the pH of orange juice (3.8 to 4.0), although both grew well at pH 6.8. *Lactobacillus plantarum* grew well, but also produced substantial amounts of diacetyl, whereas *L. brevis* grew well, but produced no diacetyl in the absence of citrate or pyruvate. When *L. brevis* was grown on this medium supplemented with citric acid and glucose, and maximal diacetyl accumulation was plotted against citrate concentration, it was found that a straight line resulted (figure 2). At 0.1 per cent citrate, maximal diacetyl was found at 40 hr. At the higher citrate concentrations, maximal diacetyl occurred at 80 to 88 hr. In all cases, the time required to reach maximal population was about 50 per cent longer than that required to reach maximal diacetyl. Maximal population also showed a straight line relationship to concentration of citrate.

In the early stages of incubation of *L. brevis* on all media, the pH tended to rise about 0.2 units at the higher citrate levels, apparently due to metabolism of citric acid. In the later stages, the pH decreased to values as low as 3.5 or 3.6 probably because of lactic acid synthesis, although lactic acid was not actually determined.

The apparent utilization of citric acid for growth by *L. brevis* was checked by an additional experiment with Lyman's synthetic broth as the basal medium. In this case, the citrate content was held at 2 per cent and the total sugar content was increased from the 4 per cent glucose of the basal medium to approximate that of single strength orange juice by adding an additional 7 per cent of sucrose. The pH was adjusted to 3.9. It was anticipated that some inversion of sucrose would occur during autoclaving, but the amount was not determined. Sucrose was added instead of glucose to more nearly approach the approximately 1:1 reducing sugar to sucrose ratio of orange juice. Plate counts with the 4 per cent glucose broth indicated a maximal population of 430 million per ml. At 11 per cent total sugar the maximal population was 390 million. With 4 per cent sugar the maximal diacetyl was 202 ppm, and at 11 per cent total sugar it was 220 ppm. The deviations were within experimental error.

When filtered orange juice as the basal medium was supplemented with citric acid and sucrose, a straight line relationship was observed between citrate concentration and both diacetyl accumulation and plate counts, similar to results with synthetic medium, but with different slopes. The results are presented in figure 3.

A comparison of the results obtained with the synthetic medium and with the supplemented orange
juice medium (table 1) indicates that the growth of *L. brevis* was much better in the synthetic medium. At 1 per cent citrate level, this organism caused the accumulation of less diacetyl in the orange juice medium than in the synthetic medium. At the 4 per cent level, which is approximately that of frozen concentrated orange juice, diacetyl accumulation was greater in the orange juice medium. Thus, in orange juice medium, diacetyl accumulation is more affected by citrate level than in the synthetic medium.

**DISCUSSION**

It is apparent from the results of this investigation that the mechanism of spoilage of frozen concentrated orange juice through the synthesis of diacetyl by lactic acid bacteria is complicated. The mechanism of synthesis and the maximal diacetyl accumulation in a mixed population is dependent upon the species of bacteria which predominates. *Lactobacillus brevis* was an efficient producer of diacetyl, but was unable to produce it in the absence of citric acid. Pyruvic acid, an alternate precursor, is absent in orange juice. *Lactobacillus plantarum* var. *mobilis*, on the other hand, was able to produce diacetyl in substantial amounts in any medium in which it would grow.

With *L. brevis*, maximal diacetyl accumulation was found to show a linear relationship to citrate level in media in which total nutrients were kept constant by the appropriate addition of either glucose or sucrose, further demonstrating that citric acid is the diacetyl precursor in orange juice for this organism. The observation that maximal cell populations of this organism also bear a linear relationship to citrate concentration suggests that the strains used in this investigation can use citrate in preference to sugars for growth. This hypothesis was confirmed by the observation that maximal populations were not changed by increasing the sugar level, while keeping the citrate level constant. That citrate is consumed is suggested by the fact that the pH of the rather strongly buffered media used rises in the first few days of incubation, and then falls. The drop in pH would be expected, due to the production of lactic acid by these organisms.

The results of this investigation suggest that there may be a fundamental difference in the mechanism of diacetyl synthesis by *L. brevis* and by *L. plantarum*. There are considerable experimental data in the literature which suggest a probable mechanism of synthesis of diacetyl (or of its precursor, acetylmethylcarbinol) from pyruvic acid, for example Kobayashi and Kalnitsky (1954). It may be that *L. plantarum* is able to synthesize a sufficiently high concentration of pyruvic acid from a wide variety of carbohydrate or proteinaceous substrates to satisfy its metabolic requirements while leaving an excess for diversion to diacetyl. Diacetyl may function as a stored energy food since it is utilized in the later stages of the growth of the culture. It may be also that diacetyl synthesis is part of the defensive mechanism of the organism to minimize competition from other species, since it has been shown by Hedgcock and Cohn (1954) that diacetyl has an inhibitory effect on certain other organisms. *Lactobacillus brevis*, on the other hand, is able to use only citric acid among the major components of orange juice for diacetyl synthesis. Whether or not this organism produces diacetyl from citrate by way of pyruvic acid, which is also an effective substrate, is not known. Pyruvic acid can arise by decarboxylation of oxalacetic acid. That oxalacetate does not arise by the normal functioning of the Krebs cycle as part of the mechanism of diacetyl synthesis is indicated by the fact that neither succinic acid nor malic acid were observed to serve as diacetyl precursors. Another possibility has been suggested by Bartley *et al.* (1959) for the formation of oxalacetate from citrate with acetyl coenzyme A as the other product. If this reaction can occur in cultures of *L. brevis* as well as in rat liver, it appears possible that two of these acetyl radicals may couple to produce diacetyl directly, or one may couple with pyruvate.
from another source to yield diacetyl by way of α-acetolactate as an intermediate. These possible mechanisms have not yet been investigated. The possibility that pyruvic acid is not involved is suggested by the observations that such sources of pyruvate as carbohydrates and the glycogenic amino acids were not precursors of diacetyl under the experimental conditions reported. This is also suggested by the fact that the diacetyl synthesizing enzyme system is adaptive. When _L. brevis_ is grown for several transfers in a medium lacking citrate, the culture loses its capacity to accumulate diacetyl. This ability is regained on transfer through citrate-containing medium.

The results of this investigation suggest that there may be unidentified growth factors or accessory factors in orange juice. This is suggested by two observations: the inability of the _Leuconostoc_ to grow at the pH of orange juice (3.8 to 4.0) in the synthetic medium tested or in nutrient broth, although they grow reasonably well in orange juice; and the increased ability of _L. brevis_ to accumulate diacetyl in orange juice medium as contrasted with synthetic medium at the same pH and citrate level.

**Summary**

Marked differences were found in the capacity of certain lactic acid organisms to produce buttermilk off-flavor (diacetyl) spoilage in frozen orange concentrates. _Lactobacillus brevis_ produced diacetyl and growth in direct relationship to the amount of citric acid in the medium when total nutrients were maintained constant. The enzyme system producing diacetyl was adaptive, and diacetyl accumulates only when the strain is carried on citrate-containing medium. Of the major constituents of orange juice, only citric acid was found to be a precursor of diacetyl for this species. No medium was found in which _Lactobacillus plantarum_ var. _mobilis_ would grow without producing substantial amounts of diacetyl. Both _Leuconostoc mesenteroides_ and _Leuconostoc dextranicum_ produced diacetyl in orange juice medium, but would not grow in synthetic or semisynthetic media at the pH of orange juice (3.8 to 4.0).

**REFERENCES**


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