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Title: Metabolism of Aroma Bacteria as Related to Diacetyl

Abstract approved: Dr. William E. Sandine

Factors influencing diacetyl production by the aroma bacteria *Leuconostoc citrovorum* and *Streptococcus diacetilactis* were investigated. When grown in association with lactic streptococci, *L. citrovorum* strain 91404 decreased in cell numbers from $10^8$ to $10^6$ over two weeks of daily subculturing in sterile non-fat milk incubated at 21°C. The ability to produce diacetyl over this period also was lost by *L. citrovorum*, but the reduction in cell numbers did not appear responsible because it was also shown that $2.5 \times 10^4$ cells were able to produce detectable diacetyl after one transfer. Disk assay procedures did not reveal any antibiotic activity by the lactic streptococci against *Leuconostoc*. The enrichment of milk with 100 µg/ml sodium citrate, 100 µg/ml sodium pyruvate, 10% yeast extract or 50% pea extract did not enhance diacetyl production by *L. citrovorum*. The addition of 0.125 ppm MnSO$_4$ caused enhanced diacetyl production up to 12 hours and enhanced diacetyl reduction from 12 to 24
hours by \textit{L. citrovorum} grown in association with lactic streptococci. The cell-free extracts prepared from these $\text{Mn}^{++}$ supplemented cultures of \textit{L. citrovorum} had 14 times more diacetyl reductase activity than non-treated cultures. Diacetyl production by \textit{S. diacetilactis} 18-16 was not enhanced by $\text{MnSO}_4$ and flavor reduction was increased only slightly; diacetyl reductase activity was not affected. Treatment of the milk with 0.03\% $\text{H}_2\text{O}_2$ and sufficient 0.004\% catalase to decompose the $\text{H}_2\text{O}_2$ resulted in at least twice as much diacetyl production by \textit{S. diacetilactis} 18-16; the resulting flavor also was stable. This treatment did not increase cell numbers, decrease diacetyl reductase activity or enhance respiration as measured by manometry studies. Diacetyl plus acetoin was synthesized by the cell-free extracts of \textit{L. citrovorum} 91404 and \textit{S. diacetilactis} 18-16 when supplemented with 0.1 µmole acetyl CoA plus 0.1 µmole pyruvate in the presence of catalytic amounts of $\text{Mn}^{++}$ and TPP. \textit{S. diacetilactis} also produced diacetyl plus acetoin from a acetolactic acid plus 0.1 µmole acetyl CoA. The synthetic activity of the extracts was not reduced by $\text{MnSO}_4$ addition or stimulated by $\text{H}_2\text{O}_2$-catalase treatment. From the results it was clear that important differences exist between the aroma bacteria with regard to optimum requirements for diacetyl production.
Metabolism of Aroma Bacteria as Related to Diacetyl Production

by

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Metabolism of Aroma Bacteria as Related to Diacetyl Production

I. INTRODUCTION

Bacteria of the Leuconostoc genus, particularly L. citrovorum, are used in mixed strain starter cultures along with the lactic streptococci to initiate many dairy fermentations. The latter are responsible for the acid characteristic of cultured dairy products while the Leuconostoc produce flavor and aroma compounds, especially diacetyl.

There are many reports in the literature of methods which increase acid production or growth of the Leuconostoc in pure culture. However, since these organisms are used industrially in mixed cultures, it would be worth while to study the growth of these bacteria under these conditions and to examine factors which influence their flavor and aroma production in mixed cultures. The present study was undertaken with these objectives in mind. Streptococcus diacetylactis, an acid-producing aroma bacteria, was included in many experiments for comparative purposes.
II. HISTORICAL

Nutritional Requirements of the *Leuconostoc* Genus

Bacteria of the *Leuconostoc* genus are nutritionally fastidious organisms. They have been isolated from a variety of sources such as green plant materials and raw milk where they find the nutrients required to satisfy their many needs. *Betacoccus* (*Leuconostoc*) organisms were described as early as 1926 by Orla-Jensen, Orla-Jensen and Spur. They isolated these bacteria from milk and established their importance in the flavor and aroma of cultured dairy products. In addition, these workers noted that growth of betacocci was stimulated by the addition of 10 to 50% yeast extract. Subsequently, Hucker and Pederson (1930) reported that yeast extract stimulated growth of these organisms as measured by increased acid production. Sauberlich and Bauman (1948) supported earlier reports of these workers by noting enhanced acid production in response to added yeast extract or Bacto-peptone.

Braz and Allen (1939) and Gibson and Abdel-Malek (1945) reported that yeast extract did not cause an increase in the total cell population achieved in *Leuconostoc* cultures. Since then, it has been reported that cell numbers were increased in response to pancreatic extract (Speck, McAnelly and Wilbur, 1958), liver extract (Sauberlich and Bauman, 1948) and lactic and citric acids (Glen and Prouty, 1955).
A number of workers investigated the nutritional requirements of \textit{L. citrovorum} and the number of compounds known to be essential for growth increased. Shankman \textit{et al.} (1945) determined the vitamin requirements for \textit{L. citrovorum} 7013 as nicotinic acid, pantothenic acid and thiamine. They also reported that vitamin requirements varied among the strains. Deoxyribosides and cyanocobalaminine (B$_{12}$) were added to the list of growth requirements by Kitay and Snell (1950). Studies on vitamin requirements by Gonsalves \textit{et al} (1957) were in agreement with those of Shankman and included pyridine, biotin and riboflavin as essential for three strains of \textit{L. citrovorum}. They also found considerable variance among strains concerning requirements for B complex vitamins. The basal medium used for vitamin studies by Whiteside-Carlson and Carlson (1949) included 18 amino acids, five nucleotides and three vitamins.

The amino acid requirements for \textit{Leuconostoc} reported by Dunn \textit{et al.} (1947) were obtained by determining those which stimulated acid production. The strains again varied widely in this regard: one needed glutamine and valine while another required glutamine, valine, isoleucine, leucine, arginine, tryptophan, cysteine, histidine, threonine, phenylalanine, glycine and alanine. Prouty (1961) listed arginine, histidine, isoleucine, lysine, valine, phenylalanine and tryptophan as amino acid requirements for \textit{L. citrovorum} isolated from dairy starter cultures and cultured milk products.
Diacetyl Biosynthesis

Diacetyl was first determined in 1928 by Schmalfuss. His identification was confirmed and other aroma-flavor compounds were discovered. Hammer and Bailey (1919) had previously isolated organisms capable of flavor and aroma production. These organisms were designated Streptococcus citrovorus and S. paracitrovorus, but later became known as L. citrovorum and L. dextranicum respectively (Hucker and Pederson, 1930).

Early workers considered lactose as the source of diacetyl (Marth, 1962). However, the observations by Hammer and Bailey (1932) that citrate enhanced diacetyl production directed attention to citrate as the carbon source for diacetyl. In 1930 Hucker and Pederson reported that Leuconostoc could not utilize citrate without another carbon source present. Bang (1943) concluded that citric acid, in the presence of glucose, was converted to acetic acid, ethanol, acetoin (acetyl methylcarbinol) and carbon dioxide with diacetyl and pyruvic acid as intermediates.

Pette (1949) proposed that pyruvate formed an undetermined intermediate which was oxidized to prodiacetyl and then decomposed to diacetyl. DeMan (1956) identified the intermediate as α acetolactic acid. Juni (1952) isolated enzymes from Aerobacter aerogenes which acted on pyruvic acid forming α acetolactic acid which was
then decarboxylated to acetoin. Krampitz (1948) concluded that acetaldehyde condensed with pyruvic acid, forming a acetolactic acid, which subsequently decarboxylated to yield acetoin.

Radioactive isotope studies by Mizuno and Jezeski (1961) supported Krampitz and revealed that part of the acetoin carbon skeleton was derived from glucose. They concluded the acetoin carbons originated from glucose plus citric acid, each of which was metabolized to the common precursor pyruvate. Carbons two and three of pyruvate were found in acetoin while carbon one appeared as carbon dioxide. They proposed that citrate was split, forming oxaloacetic acid, which was decarboxylated to pyruvic acid. Active acetaldehyde was formed from the pyruvic acid and condensed with another molecule of pyruvic acid, yielding a acetolactic acid. This product was decarboxylated to form the acetoin. The active acetaldehyde was not
actually demonstrated in this work, but free acetaldehyde would not substitute in the reaction.

These same workers also demonstrated that the pyruvate produced from citric acid appeared in greater amounts than the glucose derived pyruvate. Further attention was given to pyruvic acid in the diacetyl pathway by Harvey and Collins (1961). They proposed that growth on carbohydrates alone would not yield diacetyl because insufficient pyruvate was available to maintain both acetoin synthesis and glycolysis: the *Lactobacillus* growing under near anaerobic conditions must reoxidize the reduced nicotinamide adenine dinucleotide (NAD) with the available pyruvate, forming lactic acid. Therefore, without citrate, the amount of pyruvate was too low to supply the needs of both reactions. Marth (1962) and Busse and Kandler (1961) agreed and stated that in the presence of a favorable hydrogen donator (glucose), the pyruvate was reduced so quickly to lactic acid that the condensation reaction would not occur. The citrate fermentation yielded pyruvate without reduced NAD and consequently diacetyl was formed.

The most recent studies on diacetyl biosynthesis in the literature by Speckman and Collins (1966) refuted the above scheme. Dialyzed cell-free extracts of *S. diacetylactis* 18-16 and *L. citrovorum*, treated to remove acetyl coenzyme A (acetyl CoA), produced acetoin from acetolactic acid or pyruvate in the presence of manganese and
thiamine pyrophosphate (TPP). No diacetyl was produced unless acetyl CoA was added. The acetaldehyde TPP complex was formed from pyruvate and condensed at the carbonyl carbon of acetyl CoA. This addition product rearranged yielding diacetyl plus TPP.

\[ \text{pyruvic acid} \rightarrow \text{TPP acetaldehyde} \rightarrow \text{diacetyl + TPP} \]

Enhancement of Diacetyl Production

The work of Hammer and Baker (1923) showed an increase in diacetyl production could be achieved by the addition of citrate to the starter culture. Michaelian, Hoecker and Hammer (1938) reported no diacetyl production by \textit{L. citrovorum} before acidification of the milk; the most rapid appearance occurred at pH 4.0 to 4.3. Glen and Prouty (1955) found that citric acid stimulated while lactic acid decreased diacetyl production by \textit{L. citrovorum}. Harvey and Collins (1962) proposed a mechanism of citrate transport which explained the pH effect; citrate uptake in \textit{L. citrovorum} and \textit{S. diacetilactis} was increased rapidly below pH 6, due to a citrate permease with a low pH requirement for optimum activity.

Pack (1966) treated milk with hydrogen peroxide and catalase
prior to inoculation with *S. diacetilactis* 18-16. He obtained 100% increase in diacetyl production and the resulting flavor was stable over eight days at 21°C. Pack suggested that the enhancement may be due to oxidative effects of the liberated oxygen. However, the treatment effect could not be duplicated by bubbling oxygen into the cultures.

DeMan and Galesloot (1961) observed that manganese levels as low as 0.250 ppm would increase diacetyl production in milk. Swartling and Lindgren (1966) disagreed and reported no change in diacetyl levels with additional manganese. In studies with diacetyl reductase, Overby and Vigh-Laren (1966) reported manganese to be a necessary co-factor for enzymatic reduction of the aroma compound to acetoin.
III. EXPERIMENTAL METHODS

** Cultures **

The cultures used in this project were obtained from the culture collection maintained in the Department of Microbiology, Oregon State University. The strains of *L. citrovorum* were designated 2 and 91404. *S. diacetilactis* 18-16 and mixed strain starter cultures 40, 41 and 43 were also used. *S. cremoris* strain 3 was used to prepare the filtrate involved in Nickels-Leesment agar.

All cultures were maintained by weekly transfers in 10 ml of sterile (121°C for 11 minutes) reconstituted 11% nonfat dry milk and incubated at 21°C for 36 to 48 hours. After growth the cultures were held at 5°C until the next transfer.

** Enhancement of Diacetyl Production**

Experiments were designed to compare the amount of diacetyl produced by *Leuconostoc* strains 91404 and 2 grown in association with mixed strain lactic streptococcus cultures in the presence and absence of several additives. Substances tested were 50% pea extract, 10% yeast extract, 10% monosodium glutamate, 100 µg/ml sodium citrate, 2.5 µg/ml MnSO₄, 100 µg/ml sodium pyruvate, 10% citric acid and 85% lactic acid.

Screw-capped bottles containing 100 ml, or flasks containing
500 ml, of sterile (121°C for 12 minutes) nonfat dry milk were inoculated with one ml of a 20-hour culture of strain 91404 or 2 and with one ml of a 20-hour mixed strain lactic culture. Sterile pea extract, yeast extract or monosodium glutamate was added over the range of 0.1 to 1.5 ml per 100 ml of milk. Sodium citrate was added at the rate of 20-200 µg per 100 ml of milk. Sodium pyruvate (50 to 200 µg per 100 ml) and MnSO₄ (0.125 ppm, 0.250 ppm or 0.375 ppm) also were used in several experiments. Incubation was at 21°C. Twenty ml samples were taken every one or two hours for diacetyl measurement and 10 ml samples were removed for citrate and pyruvate determinations.

When the mixed strain lactic cultures were not used, citric acid or lactic acid was added before inoculation to lower the pH of the milk to 4.5. Twenty ml samples were removed for diacetyl and 10 ml samples for citrate determinations.

The hydrogen peroxide-catalase treatment process of Pack (1966) for enhancement of diacetyl production was studied in several experiments. Hydrogen peroxide (0.03%) was added to 100 ml of sterile milk followed by sufficient 0.004% catalase to decompose the oxidant. The milk was then inoculated with one ml of strain 91404 or 2 and one ml of mixed strain starter culture, or with *S. diacetilactis* 18-16 followed by incubation at 21°C for 18 to 24 hours. Samples were removed at various times to determine diacetyl
production and citrate and pyruvate utilization.

The amount of catalase required to destroy hydrogen peroxide was determined colorimetrically by adding five drops of 40% KI to 10 ml of the treated milk. A yellow color resulting from the liberation of free iodine from the oxidized KI indicated excess \( \text{H}_2\text{O}_2 \); samples to which sufficient catalase was added were free of the iodine-yellow color.

**Growth Curve Studies**

Separate growth experiments with *Leuconostoc* strains 2 and 91404 and *S. diacetilactis* 18-16 were conducted with added MnSO\(_4\), sodium pyruvate, sodium citrate or \( \text{H}_2\text{O}_2 \)-catalase to determine whether the various additives increased or decreased growth. The cells were grown in 250 ml of sterile (121 °C for 12 minutes) nonfat dry milk plus the specific additive; 0.125 ppm MnSO\(_4\), 200 µg sodium citrate, or 200 µg sodium pyruvate was used. Flasks were incubated at 21 °C and the samples plated using Nickels and Leesment agar (Table 1) for *Leuconostoc* counts and lactic agar (Table 2) for *Leuconostoc* plus lactic streptococci counts.

To determine the effect of the individual strains of *S. lactis* on associative growth of *Leuconostoc* strains 2 and 91404, a disk assay method was employed. A cell-free extract of each lactic strain was prepared from 250 ml of 24-hour milk cultures. The milk solids
Table 1. Composition of Nickels and Leesment agar.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>20.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>2.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0</td>
</tr>
<tr>
<td>Na citrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Ca lactate</td>
<td>10.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Add to the agar before use, sterile calcium citrate solution (10 grams calcium citrate suspended in 100 ml 1.5% solution of carboxymethyl cellulose) and sterile culture filtrate (48-hour milk culture of *S. cremoris*) in the ratio 85:10:5 (by volume) of agar:calcium citrate:culture filtrate.

Table 2. Composition of lactic agar.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/liter</th>
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<tbody>
<tr>
<td>Tryptone</td>
<td>20.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>2.5</td>
</tr>
<tr>
<td>Lactose</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.0</td>
</tr>
<tr>
<td>Na acetate</td>
<td>1.5</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>
were removed by centrifugation at 17,300 x G for 15 minutes in an RC-2 Servall refrigerated centrifuge. The supernatant was filtered through Whatman #1 filter paper and sterilized with a Millipore filter apparatus.

A seed lawn of strains 2 or 91404 was prepared with freshly washed cells from one liter of Leuconostoc broth (Table 3). The cells were suspended in sterile saline and 0.5 ml pipetted onto dried (34°C for 24 hours) lactic agar plates. Sterile disks were dipped into the S. lactis culture extracts and placed on the seeded lawns. Plates were incubated at 21°C and readings taken at 8, 12, 16, 20, 24, and 48 hours.

Table 3. Composition of Leuconostoc broth.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Citrated whey powder</td>
<td>2.0</td>
</tr>
<tr>
<td>Na citrate</td>
<td>5.0</td>
</tr>
<tr>
<td>Na acetate</td>
<td>1.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.7</td>
</tr>
<tr>
<td>Tap water</td>
<td>33.0 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.0</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>2.0</td>
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Preparation of Cell-free Extracts

Extracts of cells were prepared from culture samples taken hourly for citric and pyruvic acid analyses. Equal amounts of 10% trichloroacetic acid and sample were thoroughly shaken and allowed to stand at 21°C for two hours. After centrifugation at 12,000 rpm for ten minutes, the supernatant was filtered through Whatman #1 filter paper. The filtrate was used in the citrate and pyruvate determinations.

Colorimetric Assays

Citric acid was determined by the method of Balad and Shtrikman (1951). Cell-free extracts (0.2 ml) were placed in glass-stoppered test tubes. Seven ml of reagent grade acetic anhydride were added and the tubes placed at 65°C. After exactly ten minutes, one ml of reagent grade pyridine was added and the tubes shaken immediately. The reaction proceeded for 40 minutes, and the color resulting was measured on a Bausch and Lomb Spectronic 20 at 420 mµ. The standard curve (Figure 1) was determined using differing amounts of sodium citrate in 0.2 ml quantities.

The method of Juni and Heym (1962) was used for pyruvate analysis. One ml of cell-free extract was diluted with two ml distilled water. The clear extract was reacted with
Figure 1. Standard curve for Marier and Boulet method of citric acid determination. Each point represents the average of two determinations.
p-nitrophenylhydrazine for 45 minutes at 30°C. The pyruvate-nitrophenylhydrazine complex was extracted into reagent grade ethyl acetate and then into 0.5 M tris [tris (hydroxymethyl) amino methane] buffer. The color was read at 390 mµ in the Bausch and Lomb Spectronic 20. The standard curve (Figure 2) was made using sodium pyruvate at various concentrations. Each sample was made to a 3-ml volume for the assay.

In early experiments diacetyl was determined by the Kings test (King, 1948). This was later replaced by the more accurate quantitative method of Owades and Jakovac (modified by Pack et al., 1964). Twelve tubes, each containing 20 ml of sample, were immersed in a 65°C water bath and purged with nitrogen for 1.5 hours. The diacetyl was swept into buffered hydroxylamine where it was converted to dimethylglyoxime. A color complex was formed with ferrous sulfate, the intensity of which was determined by reading at 530 mµ on the Spectronic 20. The standard curve (Figure 3) was made using freshly distilled diacetyl added to reconstituted 11% nonfat dry milk to give a final concentration of 0.1 to 6 ppm.

Westerfeld's (1945) method of measuring diacetyl plus acetoin was used in the enzymatic studies. One ml of the enzyme reaction mixture was diluted to 15 ml with distilled water. Three ml each of 0.5% creatine and 5% anaphthol in 2.5 N NaOH were added and the mixture incubated at 25°C for 60 minutes. After centrifugation
Figure 2. Standard curve for pyruvic acid using the method of Juni and Heym. Each point represents the average of two determinations.
Figure 3. Standard curve for diacetyl using the modified Owades and Jakovac method. Each point represents the average of three determinations.
at 12,000 rpm for ten minutes, the color intensity in the supernatants was measured at 540 nm on the Beckman Model DU spectrophotometer. Reagent blanks contained the enzyme as a cell-free extract without added substrate. The reference standard curve of Seitz (1962) was used.

enzyme Procedures

The crude cell-free extracts for enzyme analysis were prepared from cells grown 20 to 24 hours at 30°C in one liter of Leuconostoc broth (Table 3). The cells were washed in 0.1 M potassium phosphate buffer at pH 7.2 and sonicated in a Raytheon Model DF 101, 10 KC sonic oscillator. Strains 2 and 91404 were treated for 30 minutes and S. diacetilactis 18-16 was treated for 20 minutes. Cellular debris was removed by centrifugation at 12,000 rpm at 3°C for 15 minutes and the clear supernatant stored at 0°C until used (less than one week). Protein determinations were made by the method of Lowry et al. (1951).

Diacetyl reductase activity was followed by observing absorbancy changes at 340 nm using the Gilford 2000 continuous recording spectrophotometer under standardized conditions. Cell-free extract (one ml) plus 0.2 ml (2 mg per ml) of NADH and 0.1 ml of diacetyl was diluted to three ml with 0.1 M potassium phosphate buffer at pH 7.2 and placed in a cuvette. The changes in absorbancy were
caused by the oxidation of NADH; one unit of enzyme was defined as the amount of enzyme to cause an absorbancy change of 0.05 per minute under standard assay conditions.

Enzymatic production of diacetyl was studied by the method of Speckman and Collins (1966). Six ml of crude extract plus 0.1 µmole of acetyl CoA, 0.1 µmole of pyruvate, 0.01 µmole of Mn^{++} and 0.01 µmole of TPP were placed at 30°C for three to four hours. One ml samples were withdrawn and tested for diacetyl plus acetoin using the Westerfeld method previously described.

Alpha-acetolactic acid, a possible diacetyl precursor, was similarly tested. The ethyl ester was saponified for four hours at 25°C with two equivalents of NaOH. The sodium salt then was added to the crude cell-free extract along with TPP, Mn^{++} and pyruvate or acetyl CoA. The reaction was allowed to proceed three to four hours at 30°C. Diacetyl plus acetoin was determined by the Westerfeld method.

The effect of H_2O_2-catalase treatment on the activity of these enzyme systems was studied by adding measured amounts of each to the crude cell-free extract and determining enzyme activity.

**Manometric Procedures**

To determine oxygen uptake (metabolic rate) a Gilson Differential Respirometer Model G-8 was used. The oxygen was measured
directly (Umbriet et al., 1957) and read directly as microliters (µl) from the respirometer. Each double-armed flask contained two ml of a four-hour *S. diacetylactis* 18-16 milk culture in the main compartment and 0.2 ml of 20% KOH on a filter paper in the center well. H$_2$O$_2$ (0.03%) was placed in one sidearm and sufficient 0.004% catalase for breakdown of the oxidant in the other sidearm. Thermo-barometers contained two ml milk plus one ml of water or H$_2$O$_2$-catalase. The flasks were tipped to mix the additives and equilibrated for 15 minutes at 30°C. Readings were taken every hour for eight hours.
IV. RESULTS

Increasing Diacetyl Production

Pea extract and yeast extract did not increase the level of diacetyl produced by the aroma bacteria. Low levels (0.1 to 0.5 ml of 50% pea extract or the same amount of 10% yeast extract per 100 ml of milk) did not alter diacetyl production as observed throughout 24 hours of growth. Amounts greater than 0.5 ml actually caused a decrease in the level of diacetyl observed after 24 hours.

After seven days of daily subculturing of L. citrovorum strains 2 or 91404 in association with a mixed strain lactic culture fortified with pea or yeast extract, diacetyl was not detected using King's testing method. However, diacetyl plus acetoin was detected each day up to 11 days in control flasks without added extracts. Monosodium glutamate also did not enhance the level of diacetyl production under these conditions.

Trisodium citrate and sodium pyruvate added to milk cultures of the aroma bacteria did not influence diacetyl biosynthesis; furthermore, the rate of citrate utilization by L. citrovorum 91404 or S. diacetilactis 18-16 was not altered by these additives. The normal rate of citrate use by these organisms is seen in Figure 4; citrate began disappearing at 12 hours for 91404 and at four hours for
Figure 4. The utilization of milk citrate by \textit{L. citrovorum} 91404 (○○) and \textit{S. diacetilactis} 18-16 (■■). Incubation was at 21°C.
S. diacetilactis 18-16. Diacetyl produced by strain 91404, as measured using the modified method of Owades and Jakovac, in a freshly combined mixed strain culture, first appeared within four to five hours when the cell population was $2.4 \times 10^4$; peak production of the flavor compound, as indicated in Figure 5, was not realized until 18 hours. Diacetyl produced by S. diacetilactis 18-16 was first detected at three hours and peak production (Figure 5) occurred by 12 hours. The pyruvate level measured in growth supernatants remained constant in strain 91404, but increased at 12 hours with S. diacetilactis 18-16.

Additional MnSO$_4$ (0.125 ppm) in nonfat milk resulted in a decrease in the final diacetyl level attained after 24 hours of growth for strains 91404 and 2 (Figure 6); the citrate utilization pattern, however, was not altered during such experiments. Addition of greater amounts of MnSO$_4$ (or MnCl$_2$) caused a complete disappearance of diacetyl by 24 hours, whereas Mg$^{++}$, Ca$^{++}$ or ethylenediaminetetraacetate (EDTA) chelated Mn$^{++}$ did not affect diacetyl production in these strains. S. diacetilactis 18-16, however, was only slightly affected by the MnSO$_4$ treatment as seen in Figure 6.

Hydrogen peroxide-catalase treatment of milk greatly increased and stabilized the diacetyl produced by S. diacetilactis 18-16 (Figure 7) without altering the utilization pattern of either citrate or pyruvate. Strain 91404 of L. citrovorum, however, was
Figure 5. Diacetyl production in nonfat milk by *L.* *citrovorum* 91404 (●●) and *S.* *diacetilactis* 18-16 (▲▲). No additives were used and incubation was at 21° C.
Figure 6. Diacetyl production by L. citrovorum 91404 in nonfat milk in the presence (●●) and absence (■■) of 0.125 ppm MnSO₄ and by S. diacetilactis 18-16 in the presence (□□) and absence (▲▲) of MnSO₄. Incubation temperature was 21°C.
Figure 7. Diacetyl production by \( L. \) citrovorum 91404 with (●●) and without (■■) the \( \text{H}_2\text{O}_2 \) - catalase treatment and by \( S. \) diacetilactis 18-16 with (▲▲) and without (△△) the \( \text{H}_2\text{O}_2 \) - catalase treatment. Incubation temperature was 21\(^\circ\) C.
not stimulated under the same condition; catalase or hydrogen peroxide alone did not affect diacetyl production or stability.

Lactic acid added to milk resulted in decreased diacetyl production by _L. citrovorum_ strains 91404 and 2 (Figure 8). Citrate utilization also decreased under these conditions. Added citric acid, however, increased diacetyl production in these same strains, as shown in Figure 8.

In all experiments during these studies, diacetyl production by the aroma bacteria was detected prior to citrate or pyruvate utilization and before the pH was lowered to the point believed required (pH 4.5) for maximum citrate transport activity. It also was found that _S. diacetilactis_ 18-16 used citrate and synthesized diacetyl more rapidly than _Leuconostoc_ and was affected differently by the H\textsubscript{2}O\textsubscript{2}-catalase treatment and Mn\textsuperscript{2+} addition (Figures 6 and 7).

_Growth Curves_

Growth curves of _L. citrovorum_ strains 91404 and 2 with and without yeast extract, pea extract and monosodium glutamate were run to determine the influence of these additives on cell numbers. None of the three materials altered the growth rate or final cell concentration. Therefore, lowered diacetyl was not due to a decrease in cell population. During the daily subculturing of _Leuconostoc_ 91404 or 2 in association with the mixed strain
Figure 8. The rate of diacetyl production by \textit{L. citrovorum} 91404 in nonfat milk with no additives (▲▲), with lactic acid (●●) and with citric acid (■■). Lactic and citric acids were added as 85% and 10% solution respectively until a pH of 4.5 was attained.
lactic culture, however, the numbers of *Leuconostoc* did decrease both in the presence and absence of these additives. Results of the disk assay test revealed the decrease was not the result of antibiotic production by the lactic streptococci. Furthermore, while this decrease in *Leuconostoc* occurred from subculture to subculture, the numbers of lactic streptococci remained constant in mature cultures (18 hours).

The effect of added sodium pyruvate, MnSO₄ or treatment of the milk with hydrogen peroxide and catalase on growth of *L. citrovorum* 91404 is shown in Figure 9. It may be seen that none of these influenced the growth of this bacterium. Similar results were obtained with *S. diacetilactis* 18-16 (Figure 10).

**Enzyme Studies**

Diacetyl reductase activity (Table 4) measured in the crude cell-free extracts of *L. citrovorum* 91404 was only slightly greater when the cells were grown with additional manganese. The same strain grown in association with the lactic streptococci and MnSO₄, however, revealed 14 times the level of diacetyl reductase activity as compared to the control. *S. diacetilactis* 18-16 extract did not have increased diacetyl reductase activity in the presence of MnSO₄. Also, though not shown in the table, this enzyme activity was not affected in either case by H₂O₂-catalase treatment of the extract.
Figure 9. Growth rate of *L. citrovorum* 91404 in nonfat milk in the presence of 200 µg Na pyruvate (●●), 0.125 ppm MnSO₄ (▲▲), after treatment of the milk with H₂O₂-catalase (■■) and with no additives (□□). Incubation was at 21°C.
Figure 10. Growth rate of *S. diacetilactis* 18-16 in nonfat milk in the presence of 0.125 ppm MnSO₄ (▲▲), after treatment of the milk with H₂O₂-catalase (■■) and with no additives (●●). Incubation was at 21°C.
Table 4. Diacetyl reductase activity (units per ml of protein) found in extracts of cells grown in Leuconostoc broth with and without 0.250 ppm MnSO₄ enrichment.

<table>
<thead>
<tr>
<th>Extract</th>
<th>units/mg¹</th>
<th>No added MnSO₄</th>
<th>MnSO₄ added</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. diacetilactis 18-16</td>
<td>0.5</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>L. citrovorum 91404</td>
<td>1.5</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>L. citrovorum 91404 plus</td>
<td>0.1</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>lactic streptococci</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹One unit equals the amount of enzyme necessary to cause a change of 0.05 in absorbancy under standard assay conditions.

Enzymes for diacetyl plus acetoin biosynthesis (Table 5) were active in all extracts when the pyruvate and acetyl CoA were provided, supporting the findings of Speckman and Collins (1966a, b) that the aroma bacteria produce diacetyl by condensation of acetyl CoA and active acetaldehyde. During these experiments it also was observed that the S. diacetilactis 18-16 extract could utilize acetolactic acid plus acetyl CoA to synthesize diacetyl plus acetoin; L. citrovorum could not accomplish this. The biosynthetic activity was not enhanced by the H₂O₂-catalase treatment.
Table 5. Diacetyl plus acetoin biosynthetic activity (µg per mg of protein) found in cell extracts supplemented with various additives as described by Speckman and Collins (1966 a, b). Effect of the \( \text{H}_2\text{O}_2 \)-catalase treatment on the activity of the extract also was measured.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>acetoin + diacetyl(^1)</th>
<th>acetolactate (\alpha)</th>
<th>acetolactate (\alpha)</th>
<th>acetyl CoA</th>
<th>pyruvate</th>
<th>acetyl CoA</th>
<th>pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. diacetilactis 18-16</td>
<td>1.2</td>
<td>0.95</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. diacetilactis 18-16</td>
<td></td>
<td></td>
<td></td>
<td>0.95</td>
<td>1.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>plus ( \text{H}_2\text{O}_2 )-catalase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. citrovorum 91404</td>
<td>0.72</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. citrovorum 91404</td>
<td></td>
<td></td>
<td></td>
<td>0.80</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>plus lactic streptococci</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. citrovorum 91404</td>
<td>0.72</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus ( \text{H}_2\text{O}_2 )-catalase</td>
<td></td>
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</tr>
</tbody>
</table>

\(^1\) Acetoin plus diacetyl was detected by the Westerfeld method and expressed as µg per mg of protein.

Manometry Studies

The oxygen uptake by \textit{S. diacetilactis} 18-16 was not significantly enhanced by \( \text{H}_2\text{O}_2 \)-catalase treatment of the nonfat milk growth medium (Figure 11). The treated culture initiated a slightly faster oxygen uptake rate and maintained that constant rate, whereas the untreated culture, beginning at a slightly slower rate, became equal to the treated culture by six hours.
Figure 11. The rate of oxygen uptake by S. diacetilactis in nonfat milk with (●●) and without (○○) the H$_2$O$_2$ - catalase treatment. Each point represents the average of three determinations.
V. DISCUSSION

One of the most perplexing problems facing the dairy fermentation industry today is the lack of uniform flavor in cultured dairy products (e.g. buttermilk and sour cream). A number of dairy microbiologists have attempted to overcome this problem by using more *S. diacetilactis* strains in mixed strain starter cultures because this bacterium produces more diacetyl than *Leuconostoc* species due to its vigorous citric acid fermenting ability. Soon after mixed strain lactic starter cultures containing *S. diacetilactis* became distributed throughout the United States, another flavor defect appeared in these products; the green or "yogurt-like" flavor. Lindsay, Day and Sandine (1965) showed that acetaldehyde produced by *S. diacetilactis* was responsible for the green flavor which became pronounced when the diacetyl to acetaldehyde ratio became less than 3:1. Subsequently, Keenan, Lindsay and Day (1966) found that *Leuconostoc* could utilize the acetaldehyde formed by *S. diacetilactis* and eliminate the green flavor defect. Fifty percent by volume of *Leuconostoc* cells, however, was required for acetaldehyde utilization and thus the practicability of this approach in industry was questionable. The use of cell concentrates as an alternative was suggested by the authors.

From these research data, the role that *Leuconostoc* organisms
may play in proper flavor development in cultured dairy products assumes great importance. Consequently more information is needed on factors that regulate and contribute to diacetyl production by and growth of *Leuconostoc*, especially in association with lactic streptococci. The present study is only a beginning in this regard.

During the present studies, the decrease in numbers of *Leuconostoc* cells with concomitant loss of diacetyl production during subculturing with lactic streptococci was unexpected. It was believed that conditions for growth and aroma production by the *Leuconostoc* in milk were favorable. Adequate nutrients were available, a pH necessary for citrate transport was attained and the organisms were transferred during the log phase and held under optimum growth conditions. Under these conditions, however, the *Leuconostoc* cell numbers dropped from $10^8$ to $10^6$ cells per ml. Nevertheless, the observed decrease in apparent ability to synthesize diacetyl was not entirely due to a lower cell population since it also was shown that detectable diacetyl was produced by $2.5 \times 10^4$ *Leuconostoc* cells per ml grown associatively for one subculture.

It has been well documented that certain strains of *S. lactis* and *S. cremoris* are capable of producing nisin or diplococcin, respectively, which inhibit growth and acid production by other lactic streptococci in mixed cultures. Colicins, toxic compounds which
act like antibiotics against some members of the Enterobacteriaceae family, have been studied as well. Furthermore, Kooy and Pette (1952) reported similar antibiotics produced by S. lactis active against strains of Leuconostoc. In light of these reports, it seemed reasonable to conclude antibiotic formation by the lactic streptococci inhibited the growth of Leuconostoc. No such inhibition was found in these studies; however, the inability to detect such antibiotics with the disk method did not prove their absence as many factors affect the demonstration of an antibiotic. Some of these are solubility in the agar, concentration and the age of the cells during filtrate extraction.

Although citric acid is known to be the carbon source for diacetyl, sodium citrate enrichment did not increase diacetyl production. These results also were unexpected because it has been common practice in the dairy industry to add as much as 0.2% sodium citrate to milk for buttermilk manufacture in order to enhance flavor. The free acid was found stimulatory for diacetyl production perhaps because this form lowered the pH to the level for maximal citrate transport. The free acid also may be more readily used by the citrate permease system than the sodium salt.

Lactic acid used as an acidogen, however, did not enhance diacetyl biosynthesis although it did lower the pH to the point where natural milk citrate would be rapidly assimilated. This finding may
explain the decrease in diacetyl production observed when *Leuconostoc* were grown in association with the lactic streptococci. A possible explanation may be that the acid alters the surface of the cell, causing a breakdown in the citrate transport system. In support of this theory, it was found that citrate was not significantly utilized in the presence of lactic acid. In this regard, it is interesting that *S. diacetilactis* strains, potent diacetyl producers, synthesize very little lactic acid during their growth and fermentation of milk citrate.

Pyruvate would be expected to increase diacetyl production if it were a direct precursor of the flavor compound as proposed by Pette (1949). Since pyruvate enrichment did not enhance diacetyl, this compound either was not a direct precursor, or it must have combined, as active acetaldehyde, with a substance (i.e. acetyl CoA) present in limited quantity within the cell. Also, before the pyruvate could be utilized it must enter the cell, and if the pyruvate transport mechanism were not operable or if the system were saturated with milk pyruvate, then exogenous pyruvate would not increase diacetyl biosynthesis. Whether any of these possibilities were involved was impossible to determine from the enrichment experiments.

The present investigation revealed that MnSO₄ stimulated diacetyl production up to 12 hours and diacetyl destruction from 12 to 24 hours for *Leuconostoc* and slightly for *S. diacetilactis*
(Figure 6). Previous studies by DeMan and Galesloot (1961) revealed increased diacetyl, gas production, cell numbers and fermentation of citrate by *Leuconostoc* in the presence of 2 to 4 ppm added MnSO$_4$. A recent study by Overby and Vigh-Laren (1966) revealed that manganese stimulated the reduction of flavor and aroma compounds of *S. diacetilactis*, presumably by enhancing diacetyl reductase activity. The present results did not resolve the differences between the two reports; both stimulated production and stimulated destruction of flavor were observed. Further work with purified enzymes will be necessary to remove the confusion.

Hydrogen peroxide-catalase treatment of milk has been used in cheddar cheese manufacture as a pasteurization procedure and was recently found useful as a treatment to enhance diacetyl production (Pack, 1966). Pack's work showed that diacetyl biosynthesis by *S. diacetilactis* 18-16 was stimulated 100-fold by H$_2$O$_2$-catalase treatment. The present study revealed that the enhanced diacetyl production was not due to increased oxygen uptake, stimulated activity of diacetyl biosynthetic enzymes or decreased activity of diacetyl reductase. Also, neither pyruvate nor citrate were more rapidly metabolized. The remaining possibility, that a form of oxygen released in the H$_2$O$_2$-catalase reaction enhanced the oxidation of NADH-H$^+$ and thus stimulated general oxidative enzyme activities, could not be demonstrated.
The pattern of diacetyl appearance in relation to citrate disappearance was unexpected; diacetyl was detected before citrate or pyruvate utilization was initiated. Studies by Mizuno and Jezeki (1961) showed that diacetyl carbons originated in part from citrate. However, the initial diacetyl detected in the present study could have been derived from the milk lactose or from an internal pool of citric acid which could not be measured in these experiments. In this regard, intracellular pools of amino acids have been detected in bacteria and yeasts before incorporation into protein. Such a situation may also be true with citric acid. Another possibility is that the methods employed for citrate and pyruvate detection were not sufficiently sensitive to measure minute changes in the available substrate.

The Balad and Shtrikman citrate determination method used in these studies revealed that S. diacetilactis 18-16 began citrate utilization earlier than Leuconostoc strain 91404 and at a pH of 6.9 as also found by Seitz (1962). This may explain in part the better diacetyl-producing ability of S. diacetilactis. The pH at the point of maximum citrate utilization was not the optimum reported for citrate transport by Harvey and Collins (1962 a, b); however, the transport activity was sufficient to utilize all the available substrate.

The utilization of exogenous milk pyruvate could not be detected, nor did pyruvate enrichment enhance diacetyl synthesis. An increase
in external pyruvate noted at 15 hours was undoubtedly an excreted cell metabolite. Sufficient pyruvate, formed through glycolysis and the citrate fermentation, would preclude the need for an external source of pyruvate.

Several important differences between _L. citrovorum_ and _S. diacetilactis_ have been noted in these studies, even though both are aroma bacteria. Their reactions to _H_2O_2_-catalase treatment were extremely different; diacetyl production by _S. diacetilactis_ 18-16 was stimulated, while _Leuconostoc_ strain 91404 was not affected. The MnSO_4_ treatment caused complete diacetyl destruction by 24 hours in _Leuconostoc_ strain 91404, but only slight loss in _S. diacetilactis_ 18-16 during the same period. Cell-free extracts from MnSO_4_ treated cultures showed greater diacetyl reductase activity in the case of _L. citrovorum_, but not for _S. diacetilactis_. When the cell-free extracts were supplied with acetyl CoA and pyruvate, both _Leuconostoc_ strain 91404 and _S. diacetilactis_ 18-16 synthesized acetoin plus diacetyl. However, when acetalactate plus acetyl CoA were present, only _S. diacetilactis_ 18-16 was able to produce the flavor and aroma compound.

It is interesting to note in this regard that differences between these two genera have been recorded by Lundstedt (1962). He found that _L. citrovorum_ produced a distinctly different growth pattern from _S. diacetilactis_, and also, different acid and gas production
patterns. The causes of differences noted in the present study are worthy of further investigation to determine how and why they occur and what metabolic implications these differences might have.
VI. SUMMARY

Diacetyl production by \textit{L. citrovorum} grown in association with lactic streptococci was not enhanced by the addition of sodium citrate, sodium pyruvate, yeast extract, pea extract or by treatment of the milk with hydrogen peroxide-catalase; growth of the \textit{Leuconostoc} also was not affected under these conditions. Citric acid, however, stimulated diacetyl production, apparently by precursor enrichment and by lowering the pH to the optimal point for citrate transport; lactic acid used as an acidogen in a similar manner inhibited diacetyl production. Daily subculturing of \textit{L. citrovorum} in association with lactic streptococci led to decreased diacetyl production and a reduction in \textit{Leuconostoc} cell numbers. MnSO\textsubscript{4} stimulated the rate of diacetyl destruction over the period of 12 to 24 hours of incubation at 21°C in nonfat milk. Diacetyl reductase in crude cell extract from \textit{L. citrovorum} strain 91404 had slightly greater activity when the cells were grown with additional manganese. The crude cell-free extracts produced diacetyl plus acetoin from acetyl CoA and pyruvate in the presence of Mn\textsuperscript{++} and TPP, but not from acetalactic acid and pyruvate or acetyl CoA. Extracts of cells grown with additional manganese were not inhibited in these reactions.

\textit{S. diacetilactis} 18-16 was only slightly affected with regard to
diacetyl production. Hydrogen peroxide-catalase treatment of the milk, however, was very effective in enhancing diacetyl production and stability. This treatment did not influence the metabolism of the cells as revealed by manometry studies. Furthermore, neither the rate of citrate or pyruvate utilization, the time of appearance of diacetyl, nor the level or activity of diacetyl reductase were altered by the treatment. Crude cell-free extracts of \textit{S. diacetilactis} produced diacetyl plus acetoin from acetyl CoA plus pyruvate in the presence of Mn$^{++}$ and TPP and from acetyl CoA plus a-acetolactic acid. Biosynthesis of the flavor compound, however, was not increased by the H$_2$O$_2$-catalase treatment.

The different response by \textit{L. citrovorum} and \textit{S. diacetilactis} to various additives with regard to diacetyl production suggested that these two aroma bacteria were quite different with respect to requirements for optimum flavor production.
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