AN ABSTRACT OF THE THESIS OF

Beverly Ann Holmes (Name) for the M. S. (Degree)

in Microbiology (Major) presented on April 26, 1967 (Date)

Title: SOME FACTORS INFLUENCING CARBON DIOXIDE PRODUCTION BY LEUCONOSTOC CITROVORUM

Abstract approved: Redacted for Privacy
Dr. Wm. E. Sandine

This study was undertaken to determine the role Leuconostoc citrovorum may play in carbon dioxide production in milk. The ability of L. citrovorum strains to produce gas was studied by two methods. A qualitative method used visual measurement of gas where an agar plug was forced up the neck of a volumetric flask. It demonstrated that 0.25 percent yeast extract in a milk medium stimulated L. citrovorum to produce at least ten ml of gas at 30°C. Studies using a Gilson Differential Respirometer revealed that L. citrovorum produced 500-900 µl of CO_2 in milk and 800-1500 µl of CO_2 in nonfat milk containing 0.33 percent yeast extract. Cell extracts of Streptococcus cremoris, Streptococcus lactis, Lactobacillus lactis, Lactobacillus casei, and Lactobacillus helveticus also enhanced gas production of L. citrovorum 20 to 70 percent. Autolysates of these bacteria present during ripening or aging of certain cheeses may stimulate

L. citrovorum, a common organism in starter cultures, to produce gas causing, for example, the slit-open defect of Cheddar cheese. Yeast extract caused an increase in acid and gas production per cell, but did not cause an increase in growth as measured by counting the cells. Experiments indicated that one metabolic source of the carbon dioxide was the decarboxylation of pyruvate produced during catabolism of citric acid; yeast extract stimulated this reaction 16 percent.

SOME FACTORS INFLUENCING CARBON DIOXIDE PRODUCTION BY *LEUCONOSTOC CITROVORUM*

by

Beverly Ann Holmes

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1967

APPROVED:

Redacted for Privacy

Professor of Microbiology
in charge of major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented April 26, 1967

Typed by Kay Smith for Beverly Ann Holmes

ACKNOWLEDGMENTS

To Dr. Wm. E. Sandine, my major professor and teacher:

"The teacher who walks in the shadow of the temple,
among his followers, gives not of his wisdom but rather
of his faith and his lovingness.
If he is indeed wise he does not bid you enter the house
of his wisdom, but rather leads you to the threshold
of your own mind."

— Kahil Gibran

To the members of my committee: Dr. P. R. Elliker and Dr. L. W. Parks:

"Ask me no questions and I'll tell you no lies."

— Goldsmith

"Facts in isolation amount to mere gossip
Facts in relation become philosophy."

— O. W. Holmes

To my fellow graduate students and friends:

"wotthehell wotthehell
toujours gai toujours gai"

— archy and mehitabel

To my family:

"Kindness in words
  Creates confidence
Kindness in thinking
  Creates profoundness
Kindness in giving
  Creates love."

— Lao-Tse

What
  we have done
  will not
  be lost
  to all eternity
Everything ripens at
  its time
  and becomes fruit
  at its
  hour.

— Divyavadana

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SOME FACTORS INFLUENCING CARBON DIOXIDE PRODUCTION BY *LEUCONOSTOC CITROVORUM*

I. INTRODUCTION

Cultured dairy products are the end result of milk fermentations carried out by various microorganisms. The starter cultures used for these fermentations include such bacteria as the *Leuconostoc* and the lactic streptococci. Carbon dioxide is often an end product of bacterial fermentations and may be desirable or undesirable in dairy products. An appreciable amount of carbon dioxide yields a desirable effervescence to buttermilk but may result in a floating curd defect in cottage cheese or a slit-open defect in cheddar cheese.

Involvement of *Streptococcus diacetilactis* in the latter two defects is well documented (Sandine, 1958). However, the role *Leuconostoc* may contribute to carbon dioxide production during dairy fermentations is less well defined. The present study was undertaken in an effort to determine the role *Leuconostoc citrovorum* may play in carbon dioxide production in milk.

II. HISTORICAL

Taxonomy of the Leuconostoc genus

The Leuconostoc exist abundantly in nature and can be isolated from a variety of sources such as slimy sugar solutions, fermenting vegetables, milk and milk products. Taxonomically, (Breed, Murray, and Smith, 1957) the genus Leuconostoc has been described as hetero-fermentative bacteria whose growth on ordinary culture media can be enhanced by the addition of yeast, tomato, and other vegetable extracts. The features that distinguish these gram positive, chain-forming cocci from the streptococci are their apparent inertness in litmus milk and their production of levo-rotatory lactic acid. Some Leuconostoc grow with the characteristic production of slime in sucrose and the majority produce mannitol from fructose.

The above classification description contains the fundamental physiological characters which justify placing these organisms in one genus for which the name Leuconostoc has priority. The division of this genus into species designations has not been as well-defined as the genus designations. The confusion that exists in the literature is due in part to utilitarian instead of physiologically based nomenclature. Hucker and Pederson, in 1930, made the first comprehensive study of the Leuconostoc genus. In general, their strains produced 45 percent levo-lactic acid from glucose, 20 percent carbon dioxide, and

25 percent volatile products, including acetic acid and ethanol. They also produced mannitol from fructose and sucrose and a levulan or dextran from sucrose. From their biochemical studies, these authors recognized three species: Leuconostoc mesenteroides which fermented pentoses and sucrose, produced slime in sucrose solutions, and was generally found in fermenting vegetable materials and sugar solutions; Leuconostoc dextranicum which fermented sucrose but not pentoses, produced a moderate amount of slime from sucrose and may be associated with either vegetable or dairy products; Leuconostoc citrovorum which fermented neither sucrose nor pentoses, produced no slime from sucrose, and was generally associated with milk or milk products. The authors also included in their study the synonomy of species of Leuconostoc then existing in the literature. Leuconostoc citrovorus, Betacoccus cremoris, and Streptococcus citrovorus were considered synonymous. Leuconostoc mesenteroides, Betacoccus arabinosaceus, Leuconostoc pleofructi, Leuconostoc aller, and Leuconostoc opalanitza were considered to be one species and Leuconostoc dextranicus, Lactococcus dextranicus, Betacoccus bovis, and Streptococcus paracitrovorus also were considered synonymous.

The work of Hucker and Pederson remained unchallenged in the literature until 1961 when Garvie published a study on the genus Leuconostoc and its nomenclature. During this period, much has been published on the individual species and their identification,

nutritional requirements, etc. Garvie based her study on the limits of the genus as described earlier with the additional criterion of no ammonia production from arginine. She divided the genus into six groups. Group I was designated Leuconostoc cremoris (name used in preference to Leuconostoc citrovorum for non-sucrose fermenters) and was characterized by the production of acid and a small amount of gas from glucose, galactose, and lactose, and no growth at 37°C. Group II, called Leuconostoc lactis, was composed of organisms isolated from milk and previously designated as Streptococcus kefir and Leuconostoc mesenteroides. The characteristics of this group were: no fermentation of mannitol, salicin or raffinose, no formation of slime from sucrose, growth at 39°C, survival at 55°C for 30 minutes, and active fermentation of lactose. Group III, Leuconostoc mesenteroides, was comprised of organisms that did not attack salicin, did not form slime, but were active in sugar fermentations. Group IV, Leuconostoc dextranicus contained only five strains. Each strain produced slime, failed to ferment arabinose and xylose, failed to grow at 39°C, was destroyed by heating to 55°C for 15 minutes. Groups V and VI were designated Leuconostoc mesenteroides and separated on their ability to produce acid from pentoses. The author pointed out that groups III, V, and VI contained unwieldy species and were not easily distinguishable. She also explained that the property of slime production was reversibly induced and was therefore not a

justifiable differentiating characteristic. Another problem in the taxonomy of this genus dealt with by Garvie was the close relationship between the Leuconostoc and Pediococcus genus. Several Leuconostoc strains in the American Type Culture Collection as of 1958 have since been designated as pediococci.

A third taxonomic study of the Leuconostoc was published by Sandine, Elliker, and Hays in 1962. These authors were primarily interested in differentiating the lactic streptococci. They concluded that the Leuconostoc genus could be distinguished by the relative inactivity of its members in litmus milk at 21°C and 30°C. The Leuconostoc strains studied seemed to fall into two groups by virtue of their ability or inability to produce dextran on the sucrose medium. These authors also offered a simple utilitarian classification for the genus to be used by the dairy microbiologist: Leuconostoc dextranicum—associatively produce aroma compounds (acetylmethylcarbinol plus diacetyl) in nonfat milk and dextran on sucrose medium; Leuconostoc citrovorum—associatively produce aroma compounds in nonfat milk but do not form dextran on sucrose medium; Leuconostoc mesenteroides—do not associatively produce aroma compounds in nonfat milk but do elaborate dextran on sucrose medium. So-called Leuconostoc having none of these abilities might be placed in the Pediococcus genus.

The present study is concerned with Leuconostoc citrovorum

which was described as early as 1926 by Orla-Jensen, Orla-Jensen, and Spur. They recognized this organism as one important to flavor and aroma development in cultured dairy products. These authors described the organism as a citrate fermenter whose growth was stimulated by the addition of 10-50 percent yeast extract to the milk. These bacteria formed a variety of by-products and, upon primary isolation, were vigorous gas producers. Since the property of gas production was lost upon further propagation, it was considered taxonomically unimportant. Because the acid produced during growth of this organism was levo-rotatory lactic acid, Orla-Jensen et al. assigned it to the genus Betacoccus (Leuconostoc). Knudsen (1931), in his discussion of starters, uses Orla-Jensen's classification for the Betacocci. The primary criteria for identification were: production of levorotatory lactic acid, acetic acid, and carbon dioxide; isolation from ordinary starters; and production of aroma in associative growth. Jesperson (1959) used similar criteria for the identification of Betacoccus cremoris (Leuconostoc citrovorum) in starter cultures.

Carbon Dioxide Production by the Leuconostoc and Its Enhancement

The Leuconostoc are heterofermentative bacteria and therefore, by definition, are producers of carbon dioxide. Orla-Jensen et al., in 1926, recognized this ability in the butter aroma bacteria (Leuconostoc citrovorum), but they found that vigorous gas production

was a variable property. Studies on Streptococcus paracitrovorus [some strains classified as L. dextranicum by Hucker and Pederson (1930) and some considered Streptococcus diacetilactis by Sandine et al. (1962)] by Hammer and Baker (1923) and by Brewer and Werkman (1940) exemplify the capacity of this organism to produce carbon dioxide. Hammer and Baker (1923) noted that their 124 cultures of S. paracitrovorus were all capable of producing considerable carbon dioxide from milk and from milk plus peptone. Brewer and Werkman (1940) quantitated the studies on this organism's gas-producing ability using the Warburg respirometer technique. These authors established that this organism was capable of producing 400 μl of gas in six to eight hours from citrate plus glucose or citrate plus lactose. They concluded that the gas was produced primarily from the citrate, with the glucose acting as a catalyst.

Gibson and Abdel-Malek (1945) also studied carbon dioxide formation by various lactic acid bacteria including Streptococcus kefir, S. citrovorus, L. mesenteroides, and Lactobacillus brevis. Of all the organisms examined, only certain strains of S. citrovorus (L. citrovorum) were variable in the ability to vigorously produce gas. The authors observed that their recently isolated strains of L. citrovorum were quite consistent in their ability to form gas readily from citrate and citrated milk. This observation is in agreement with the findings of Orla-Jensen et al. (1926). The

authors found that yeast preparations had a pronounced stimulatory effect on the formation of carbon dioxide by the heterofermentative lactic acid bacteria in glucose-milk or glucose-gelatin. They found the stimulatory effect to be on the rate of some phase of the fermentation and not due to enhanced growth.

Several authors have noted the stimulatory effect of accessory growth factors on the Leuconostoc. Hucker and Pederson (1930) noted that yeast extract enhanced growth as measured by acid production. Sauberlich and Bauman (1948) also found that yeast extract and Bacto-peptone stimulated growth of L. citrovorum in a complex medium. The effect was measured by acid production and turbidity. The work of Braz and Allen in 1939 supported the observations of Gibson and Abdel-Malek (1945) that yeast extract increased acid production but not cell number. Speck, McAnelly, and Wilbur (1958) noted that growth of L. citrovorum (measured by colony counts on trypticase soy agar) was enhanced by pancreas extract. The influence of autolyzed cells of Streptococcus cremoris and Streptococcus lactis on Betacoccus cremoris (L. citrovorum) in skim milk was studied by Hansen (1941). These cell extracts raised the end point of titration for L. citrovorum. Other experiments exhibited that no proportionality between amount of growth and end point of titration existed. The active principle in these extracts was filtrable through a Seitz filter EK. The authors pointed out that this stimulation may

be of importance in butter and cheese cultures where the two streptococci are found mixed with the aroma bacteria.

There exists in the literature many reports relating the Leuconostoc to the slit open defect in Cheddar cheese. Sherwood (1939) isolated organisms from "open" cheese and found them to be primarily lactobacilli. Out of eleven strains, he found one that was L. citrovorum and two that were L. dextranicum. Galesloot in 1950 isolated cultures of Betacoccus arabinosaceus and Betacoccus bovis from defective cheese. The author pointed out that cheese-making tests showed that these betacocci were able to produce early gas defects. However, the defect was greatly reduced by the presence of active streptococci. Galesloot also stated that Betacoccus species normally occurring in starters (L. citrovorum) were not dangerous in this respect. Overcast and Albrecht (1952) isolated L. citrovorum as the causative organism from slit-open cheese. The isolation medium was glucose milk containing filtered tomato juice, yeast extract, and sodium citrate. The identification was made using the properties of gram stain, morphology, inertness in litmus milk, gas from glucose-milk, and fermentation of sugars. The authors pointed out that the solubility of carbon dioxide at various temperatures may influence the appearance of this defect even though the causative organism was present. Jarvik and Kjell (1956) found that L. dextranicum and L. mesenteroides but not L. cremoris produced

abnormal amounts of gas in Swedish cheese. Robertson (1957) studied the carbon dioxide content of New Zealand Cheddar Cheese. He found that the cheese made from commercial starters (containing betacocci and Streptococcus diacetilactis) in contrast to single strain starters was characterized by the rapid increase of carbon dioxide in the two weeks following manufacture. The high carbon dioxide content of these cheeses may be attributable to S. diacetilactis alone (Sandine, Elliker, and Anderson, 1959).

Galesloot and Hassing (1961) examined starters as to the nature of the aroma-bacterial flora. Their results helped differentiate the often confused Leuconostoc cremoris and Streptococcus diacetilactis. The organisms were plated out on selective media, propagated at 35°C and tested qualitatively for citric acid. The authors found the following differences: S. diacetilactis, in contrast to L. cremoris, grew better in milk, grew equally well in milk of all seasons, had a higher optimum temperature, did not reduce acetoin and diacetyl to butylene glycol, and needed less calcium for growth. The organisms exhibit these differences in dairy products. For example, S. diacetilactis produces more gas which is advantageous in Gouda and Edam cheese but disadvantageous in Cheddar and cottage cheese (floating curd defect). The diacetyl flavor in butter is subject to the seasonal influence on L. cremoris whereas the yoghurt flavor is due to the presence of S. diacetilactis. In cultured milk products, the rate of

citric acid fermentation, carbon dioxide production, and reduction of aroma compounds are all important flavor and texture determining factors.

Hamamoto, in 1966, performed several experiments with Leuconostoc species isolated from blown Gouda cheese. He demonstrated that these organisms did not affect the appearance, texture, and body of the cheese even when yeast extract had been added to the pasteurized milk used in the cheese making process.

Carbohydrate Metabolism of Leuconostoc citrovorum

The heterolactic organisms utilize carbohydrates via the 3,2 cleavage of pentose. In this fermentative pathway, glucose is converted to 6-phospho-gluconate which in turn is decarboxylated to ribulose-5-phosphate. This molecule is rearranged to xylulose-5-phosphate and cleaved to yield acetyl phosphate and glyceraldehyde-3-phosphate. Acetyl phosphate can transfer the terminal high energy phosphate bond (~P) to adenosine diphosphate (ADP); then adenosine triphosphate (ATP) is formed and acetate accumulates. Under anaerobic conditions, however, acetyl phosphate is reduced to ethanol, in order to reoxidize reduced nicotinamide adenine dinucleotide phosphate (NADP), then the ~P is not conserved. Glyceraldehyde-3-phosphate passes via glycolysis to pyruvate and pyridine nucleotides are regenerated by reduction of pyruvate to lactate. Thus, there is a net

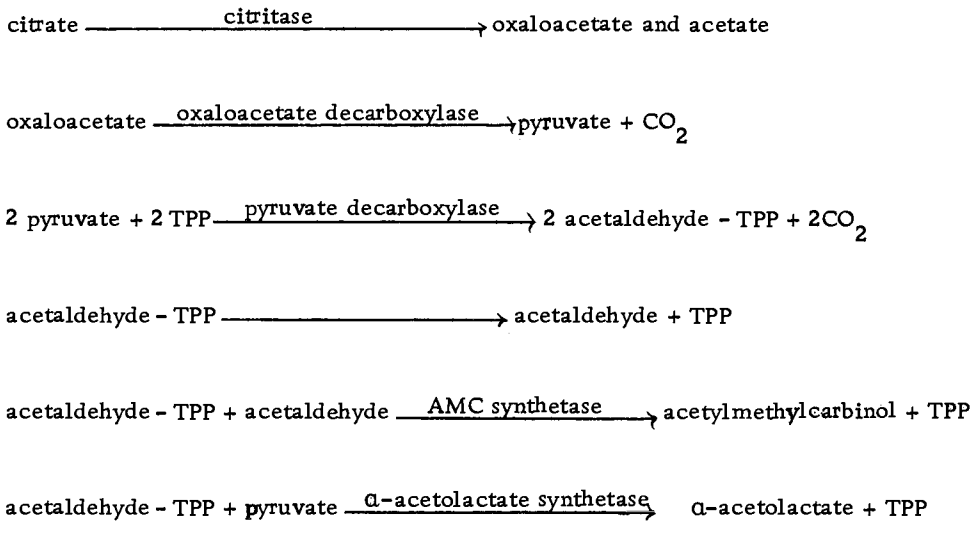
gain of one ATP/glucose metabolized; ethanol, carbon dioxide, and lactate are the end products. Marth (1962), at the Symposium on Lactic Starter Cultures, reviewed the above mechanism of carbohydrate utilization. He pointed out that L. citrovorum was able to convert glucose to carbon dioxide, lactic acid, and alcohol or acetic acid under both aerobic and anaerobic conditions.

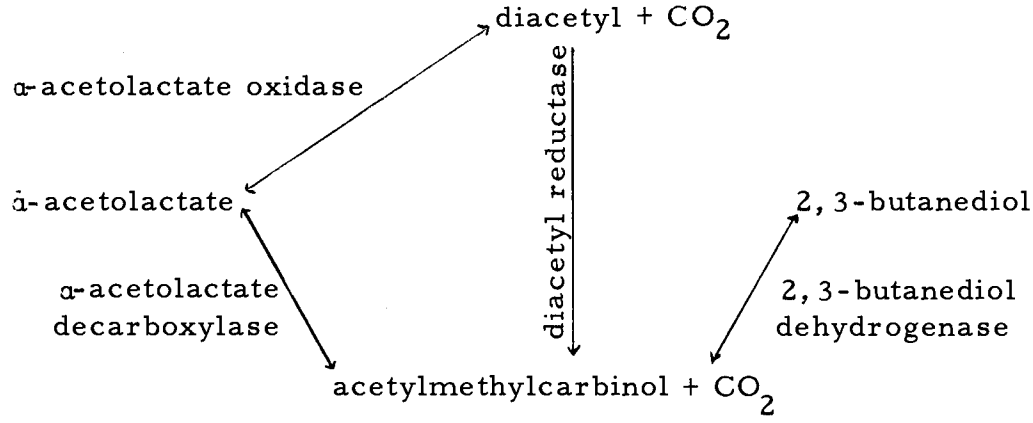
Marth also discussed the controversial subject of citrate metabolism by these bacteria. This pathway is particularly important in the dairy industry because of the aroma and flavor compound (diacetyl) produced. Marth explained the variability in the data concerned with this pathway: (a) pyruvic acid is the key intermediate in the fermentation of lactose and citrate to acetoin and diacetyl; (b) in the fermentation of lactose to pyruvate by the Embden-Meyerhof-Parnas (EMP) scheme, sufficient nicotinamide adenine dinucleotide (NAD) is produced to reduce pyruvate to lactic acid; (c) fermentation of citrate results in production of pyruvate without a simultaneous supply of reduced NAD and, hence, products other than lactic acid are formed.

Many investigators have studied the citrate utilization reaction. The end products of this reaction, i.e. the volatile acids, have long been associated with the activity of S. citrovorus and S. paracitrovorus (Hammer, 1920; Hammer and Sherwood, 1923). In 1939, Van Beynun and Pette examined more closely the decomposition of citric

acid by Betacoccus cremoris. These authors found that 1.0 mole of citric acid yielded 2.0 moles carbon dioxide, 1.5 moles of acetic acid, and 0.0-0.5 moles of acetylmethylcarbinol at an acid pH. Slade and Werkman (1941) confirmed the fact reported by Knudsen and Sorensen (1929) (as cited in Slade and Werkman, 1941, p. 676) and Hucker and Pederson (1930) that S. paracitrovorus (L. mesenteroides) was unable to utilize citrate unless a fermentable carbohydrate was present. These authors did note, however, that cells grown up in the presence of citrate plus lactose could, when suspended in a nitrogen-free medium, ferment citric acid in the absence of a carbohydrate. They considered the presence of citrate necessary for the formation of enzymes involved in its fermentation.

The more recent studies using labeled hexoses and citrate (Anderson, 1959; Mizuno and Jezeski, 1961; Sandine et al. (1961) supported the scheme of citrate metabolism proposed by Seitz et al. (1962):





The work of Harvey and Collins (1961; 1962 a; 1962 b; 1963) using cell-free extracts of S. diacetilactis and L. citrovorum support the above scheme. These authors pointed out that citrate fermentation proceeded better at pH 6.0 or below, that citrate metabolism was necessary for acetoin production (need pyruvate in excess of that obtained from glycolytic scheme), and that the entry of citrate into the cell was mediated by an inducible transport system. The acid pH increased the activity of the permease system responsible for transporting the citrate through the cell permeability barrier and increased the proportion of metabolized citrate that was converted to acetoin. The citrate transport system of S. diacetilactis required a source of metabolic energy for operation even when extracellular concentration of citrate exceeded the intracellular concentration. These authors felt that the citrate utilization pathway of S. diacetilactis and L. citrovorum was the same.

The most recent work by Speckman and Collins (1966 a; 1966 b)

contradicted their previous conclusions and the above diacetyl-synthesizing scheme. The authors suggested that diacetyl was not formed from α-acetolactate or acetoin but that it was formed by the acetaldehyde-TPP complex from pyruvate attacking the carbonyl carbon of acetyl-Co-A to give an addition product that can rearrange to yield diacetyl and regenerate the TPP carbanion. In their studies, the authors used dialyzed cell-free extracts which were treated with EDTA to remove metal ions and with anion exchange resin to remove coenzyme A.

III. EXPERIMENTAL METHODS

Cultures Used

The sixteen strains of Leuconostoc citrovorum used in this study were obtained from the culture collection of the Department of Microbiology, Oregon State University. These cultures were originally isolated from commercial lactic starter cultures using sodium azide agar and characterized by their inactivity in litmus milk. Each strain is designated by a number: L_2, L_3, L_4, L_5, L_6, L_7, L_8, L_9, L_{14}, L_{15}, L_{17}, L_{21}, L_{28}, L_{31}, L_{91404}, DN_3. The other lactic cultures used in cell-extract preparations and as controls were obtained from the above collection. They include: Streptococcus diacetilactis 18-16, Streptococcus lactis C_2F, Streptococcus cremoris 3, Lactobacillus lactis, Lactobacillus casei, and Lactobacillus helveticus.

Culture Propagation

All cultures were maintained by weekly transfer at the rate of one percent inoculum in 10 ml of sterile (121°C for 12 min.) 11 percent reconstituted nonfat dry milk. The cultures were incubated at 21°C for 36 to 40 hours and then held at 5°C until the next transfer. Strains L_2, L_7, L_{31}, L_8, L_{91404}, L_9, and L_{21} were also maintained in Leuconostoc Broth Medium (LBM, see Table 1) by weekly transfer

Table 1. Composition of Leuconostoc Broth Medium (LBM).

Ingredient	Grams/liter
Yeast extract	10.0
Tryptone	10.0
Citrated whey powder[1]	2.0
Sodium citrate	5.0
Sodium acetate	1.5
Glucose	10.0
Ascorbic acid	0.7
Tap water	33.0 ml
NaCl	2.0
K_2HPO_4	2.0
KH_2PO_4	2.0
$MgSO_4$	2.0

[1] Provided by Erik Lundstedt, H. P. Hood and Sons, Inc., Boston, Mass.

at the rate of one percent inoculum in 10 ml of sterile broth (121°C for 15 min.). The cultures were incubated 24 hours at 27°C and then held at 5°C until the next transfer. The other lactic organisms used in this study were propagated in lactic broth (Table 2) at the following incubation temperatures: 37°C - L. helveticus and L. lactis; 32°C - S. diacetilactis 18-16 and L. casei; 27°C - S. lactis C_2F and S. cremoris 3.

Table 2. Composition of lactic broth.

Ingredient	Grams/liter
Tryptone	20.0
Yeast extract	5.0
Gelatin	2.5
Lactose	5.0
NaCl	4.0
Na acetate	1.5
Ascorbic acid	0.5

Culture Preparation for Gas Production Studies

Forty ml of LBM were inoculated at the rate of one percent with 24 hour broth culture, incubated at 27°C for 20 to 24 hours, and harvested in a sterile centrifuge tube in an RC-2 refrigerated Servall centrifuge at 6,000 rpm for five minutes. The cells were washed once with sterile physiological saline and resuspended to the desired volume in saline (0.85 percent NaCl). The saline suspension was either counted by the Most Probable Number (MPN) technique or adjusted to a desired optical density at 650 mμ on a Bausch and Lomb Spectronic 20. The suspension was used to inoculate volumetric flasks or in manometry flasks.

One liter broth cultures inoculated and incubated as stated above, were used to prepare cell-free extracts for manometer studies. The harvested cells were washed twice with saline and resuspended in 40 ml of 0.1 M potassium phosphate buffer, pH 7.0. This suspension was sonicated in a Raytheon Model DF 101, 10KC sonic oscillator for 15 minutes. The cellular debris was removed by centrifugation at 15,000 rpm for 25 minutes. The supernatant was kept frozen at -20°C until used, but not longer than two weeks.

Preparation of Cellular Extracts Used as Additives

One liter lactic broth cultures (one percent inoculum from an 18 hour culture) were harvested at 6,000 rpm for 20 minutes, washed

twice with distilled water, and resuspended in 50 ml of distilled water. The suspension was sonicated 30 minutes and then recentrifuged at 10,000 rpm for 15 minutes. The supernatant was autoclaved for 15 minutes at 121°C. The percent solids of each extract was determined quantitatively (in triplicate) by weighing a dried (110°C, 24 hours) one ml sample.

Most Probable Number Counting Technique

The technique described by Meynell and Meynell (1965) was used. Each of three consecutive saline dilutions was inoculated into five test tubes, each containing ten ml of LBM. The tubes were incubated at 27°C for 48 hours and then read visually on the basis of turbidity. The cell number was determined from a standard table (Table 3). This technique was verified statistically for Leuconostoc citrovorum by plotting the proportion of turbid tubes (P) against the log of the mean number (m) of organisms per tube (determined by counting the undiluted saline suspension in a Petroff-Hauser counting chamber - chain of cells was considered one). The curve obtained is compatible with the equation $P = 1-e^{m}$ or $S = e^{-m}$ (S = proportion of tubes remaining clear, e = natural log of ten).

Cell Dry Weight vs. Optical Density

A standard curve was constructed for three strains: L_2, L_7,

Table 3. Values of the M. P. N. for five tubes inoculated from each of three successive ten-fold dilutions.

Numbers of turbid tubes observed at three successive dilutions			M. P. N. (per inoculum of the first dilution)	Numbers of turbid tubes observed at three successive dilutions			M. P. N. (per inoculum of the first dilution)
0	1	0	0.18	5	0	0	2.3
1	0	0	0.20	5	0	1	3.1
1	1	0	0.40	5	1	0	3.3
2	0	0	0.45	5	1	1	4.6
2	0	1	0.68	5	2	0	4.9
2	1	0	0.68	5	2	1	7.0
2	2	0	0.93	5	2	2	9.5
3	0	0	0.78	5	3	0	7.9
3	0	1	1.1	5	3	1	11.0
3	1	0	1.1	5	3	2	14.0
3	2	0	1.4	5	4	0	13.0
4	0	0	1.3	5	4	1	17.0
4	0	1	1.7	5	4	2	22.0
4	1	0	1.7	5	4	3	28.0
4	1	1	2.1	5	5	0	24.0
4	2	0	2.2	5	5	1	35.0
4	2	1	2.6	5	5	2	54.0
4	3	0	2.7	5	5	3	92.0
				5	5	4	160.0

L_{91404}. A cell suspension of each strain was prepared from a 24-hour culture in 250 ml of LBM. The cells were harvested by centrifugation at 6,000 rpm for 20 minutes, washed twice in 0.05 M Tris Buffer (pH 7.2), and resuspended in 10 ml of buffer. The dry weight of the final suspension was determined using quantitative analytical technique. Triplicate one ml samples were dried at 110°C for 24 hours on planchets, cooled, and weighed. The constant weights obtained were corrected for the constant dry weight of the buffer. Dilutions of the cell suspension were made in buffer and their optical density (OD) read at 650 mμ on a Bausch and Lomb Spectronic 20. The data obtained were plotted as shown in Figure 1 for each strain.

Gas Production in Volumetric Flasks

Graduated 110-ml glass volumetric flasks (VC 570, Aloe Scientific) containing five ml of prepared cells (final saline suspension of 40 ml gave 1 x 10^8 cells/ml), five ml of sterile ten percent Difco yeast extract or other additives, and sterile 11 percent reconstituted nonfat dry milk for a final volume of 100 ml were sealed with one ml of vaspar as a sealing plug; the vaspar had the following ingredients: 50 gm Vaseline, 2 gm agar, 1 ml Tween 80, 50 ml dH_2O. Control flasks contained five ml of cells, 95 ml of milk, and one ml of vaspar. All flasks were incubated at 21°C or 30°C. Gas production was measured visually by observing the plug rise in the

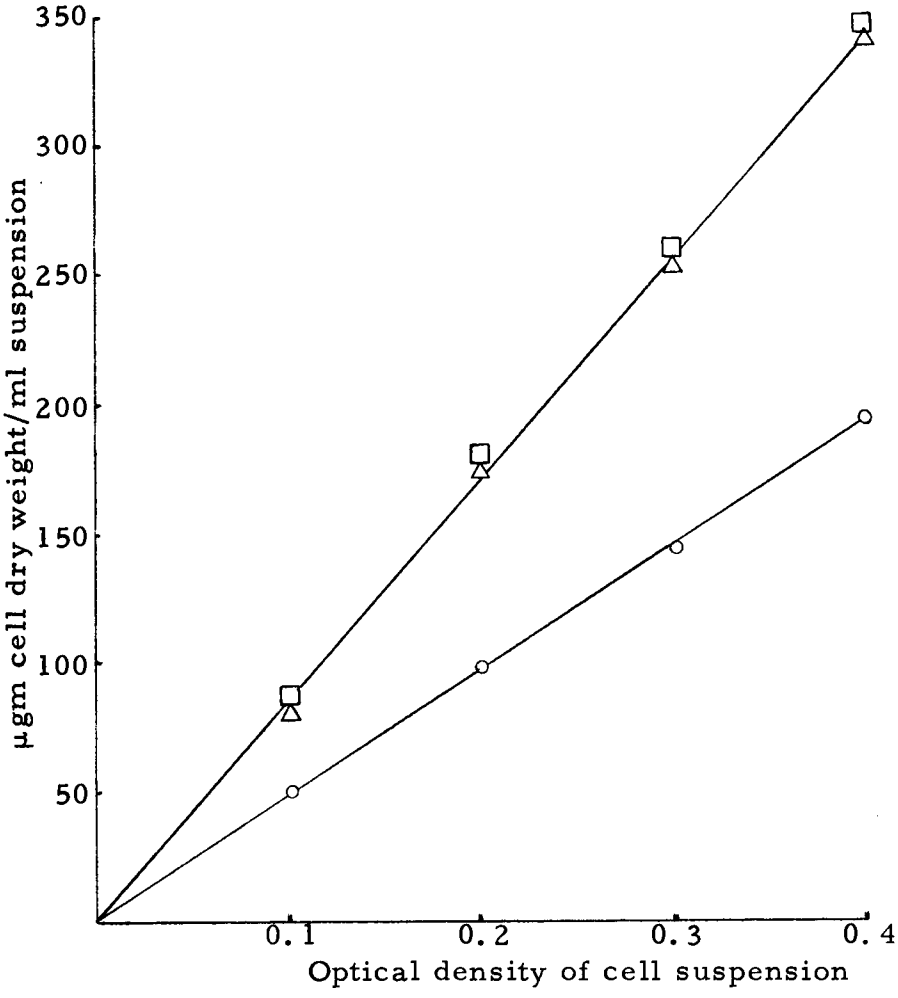


Figure 1. Standard curve of optical density vs. cell dry weight for Leuconostoc citrovorum strains 2 (o-o), 7 (△-△), and 91404 (□-□).

calibrated neck of the flask.

Measurement of Carbon Dioxide Production by Manometry

A Gilson Differential Respirometer (Model G8, Gilson Medical Electronics) and the direct manometric method of Umbreit, Burris and Stauffer (1957) were used in these studies for determining carbon dioxide production. Seven double-armed flasks were used in each trial run. The thermobarometer flask contained 2.5 ml of the appropriate liquid in the main compartment, 0.2 ml of distilled water in the center well, 0.5 ml distilled water in one side arm and 0.5 ml of 0.5 N HCl in the other side arm. Two each of the control and experimental flasks were prepared; one flask, with 0.2 ml of 20% KOH in the center well, was used to measure the oxygen uptake. The other, with 0.2 ml distilled water in the center well, was used to measure oxygen uptake plus carbon dioxide evolution. In the experiments with whole cells, 0.5 ml of cells were placed in one side arm and 0.5 ml of 0.5 N HCl in the other. The ten ml saline suspension of cells used as the source of inoculum gave an OD reading of 0.13 to 0.16 or a population cell count of 4×10^8/ml. The substrate and additive were placed in the main compartment. In the cell-free extract experiments, 2.5 ml of the extract were placed in the main compartment, the substrate and cofactors in one side arm, and acid in the other side arm.

The flasks were equilibrated for 10 to 15 minutes in the 30°C water bath. The system was then closed to atmospheric pressure and the cells or substrate tipped into the main compartment. The flasks were shaken continuously throughout the experiment. Readings were taken every hour for six hours by adjusting the fluid in the manometer with the reference line. Microliters of gas were read directly from the gauge. The acid was tipped in at six hours to release any dissolved carbon dioxide and the experiment terminated at 6.5 hours. The flasks were soaked in a Haemo-sol cleaning solution for 15 minutes, rinsed six times with water, and twice with distilled water.

Carbon dioxide production was calculated by difference. The hourly readings of each flask were corrected for the thermobarometer. The oxygen uptake values were then subtracted from the combined oxygen uptake plus carbon dioxide evolution values and totaled. The totals obtained for the control flasks were subtracted from the totals of the experimental flasks and a percent difference calculated. The 6.5-hour period of measuring gas production was arbitrary. Preliminary experiments indicated that gas production did not reach a plateau even after 12.5 hours.

Growth Curve Procedure

The growth curves of strain L_2 in milk with and without yeast extract were designed to measure cell numbers, pH, titratable

acidity, and lactic acid. Four 500 ml Erlenmeyer flasks were prepared containing 290 ml of sterile (121°C, 15 minutes) 11 percent reconstituted nonfat milk. Five ml of a sterile 10 percent yeast extract solution were added to two of the flasks. Five ml of a 1×10^6 saline dilution of a 10 ml saline suspension of cells were inoculated into one flask containing milk and one containing milk and yeast extract. Samples were taken every four or eight hours. Cell numbers were determined by MPN and by plate counts using Nickels and Leesment Agar (see Table 4 for composition). All dilutions were made in sterile (121°C, 30 minutes) 99 ml saline dilution blanks. The pH of a nine ml sample diluted to 50 ml with distilled water was recorded directly using a Beckman zeromatic pH meter. The titratable acidity of this same diluted sample was then measured using a Fisher Automatic Titrimeter, dispensing 0.1 N NaOH, to the end point of pH 8.2. The lactic acid determination was made by a modified Ling Method (Ling, 1951). The determination was run on ten ml samples within 24 hours of sampling (sample held at 5°C until used). The sample was diluted to 25 ml with distilled water in a 125 ml Erlenmeyer flask. The following reagents were added to the flask with agitation: ten ml of $BaCl_2$ reagent; ten ml of 0.66 N NaOH; five ml of $ZnSO_4$ reagent. The resulting mixture was filtered by gravity through Whatman #40 filter paper. To ten ml of filtrate, an appropriate amount of $FeCl_2$ reagent was added (compare titratable acidity

Table 4. Composition of Nickels and Leesment Agar[1].

Ingredient	Grams/liter
Tryptone	20.0
Yeast extract	5.0
Gelatin	2.5
Glucose	5.0
Lactose	5.0
NaCl	4.0
Na citrate	2.0
Ca lactate	10.0
Nutrient agar	15.0

[1] Before use, to 85 ml of agar add 5 ml of a 1.5 percent carboxymethylcellulose solution that contains 10 gm of calcium citrate and add 10 ml of a sterile filtrate of Streptococcus cremoris (48 hour milk culture).

of sample with standard chart in reference article). The sample was read within 20 minutes on a Spectronic 20 at 450 mμ (wavelength chosen from an absorption spectra of a 0.085 percent lactic acid sample). A standard curve (Figure 2) was prepared using known concentrations of lactic acid in sterile 11 percent reconstituted nonfat milk.

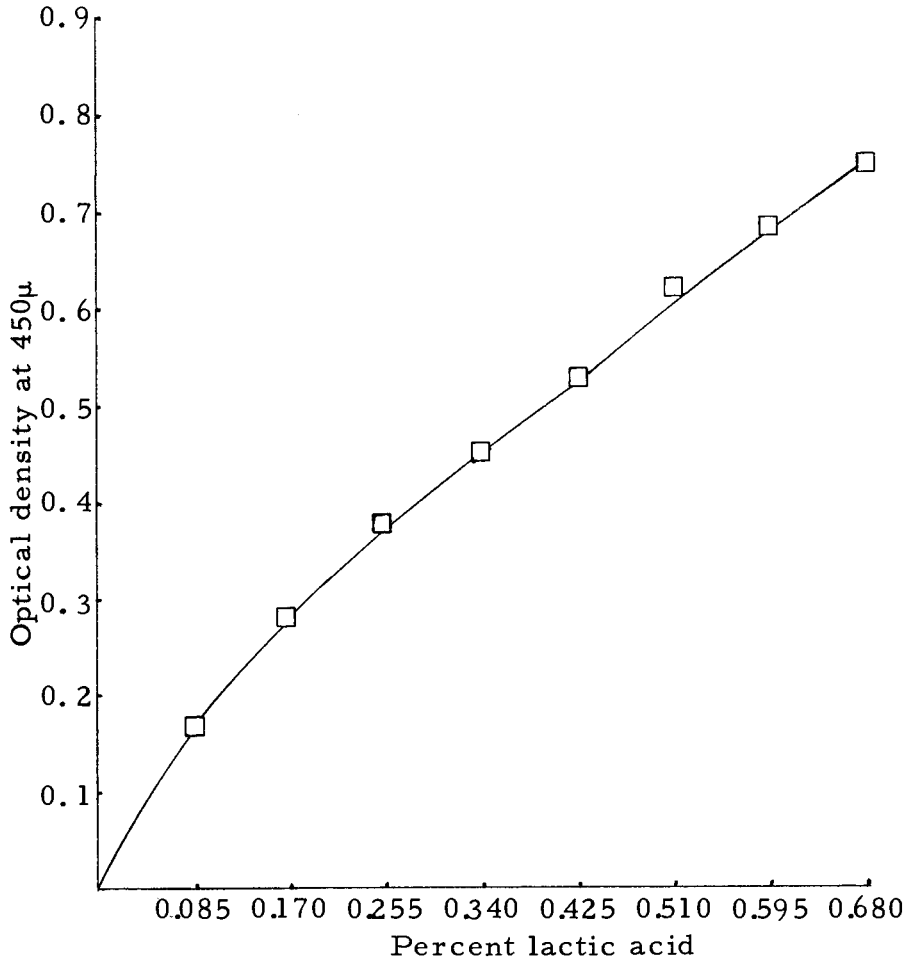


Figure 2. Standard curve for modified Ling determination of lactic acid. Each point represents the average of three trial determinations.

IV. RESULTS

Gas Production in Volumetric Flasks

The less refined quantitative technique of using sealed graduated volumetric flasks demonstrated the effect of yeast extract on gas production by L. citrovorum. Figures 3, 4, 5, 6, and 7 illustrate this technique. The cells in milk without the addition of yeast extract did not normally produce any visible gas even after incubation for ten days; whereas the cells in milk plus yeast extract produced gas within four days. Sixteen strains of L. citrovorum were examined in this manner and classified as fast, medium, and slow gas producers. At 21°C, the fast exhibited gas production in 40-50 hours, the medium in 50-70 hours, and the slow in 70-100 hours. The fast strains were L_{31}, L_{21}, L_{9}, L_{8}, L_{2}; the medium were DN_{3}, L_{14}, L_{7}, L_{6}, L_{5}; the slow strains were L_{91404}, L_{28}, L_{17}, L_{15}, L_{4}, L_{3}.

The effect of temperature on gas production under these conditions was documented using a fast gas-producing strain, L_{2}. The data in Table 5 illustrate that gas production was maximum at 30°C; 27°C and 21°C were both suitable but gas production lagged 6-12 hours. Several yeast extract concentrations were tested to determine the range of this stimulatory effect. It can be seen from the data in Table 6 that a concentration of yeast extract as small as 0.05 percent was still effective.

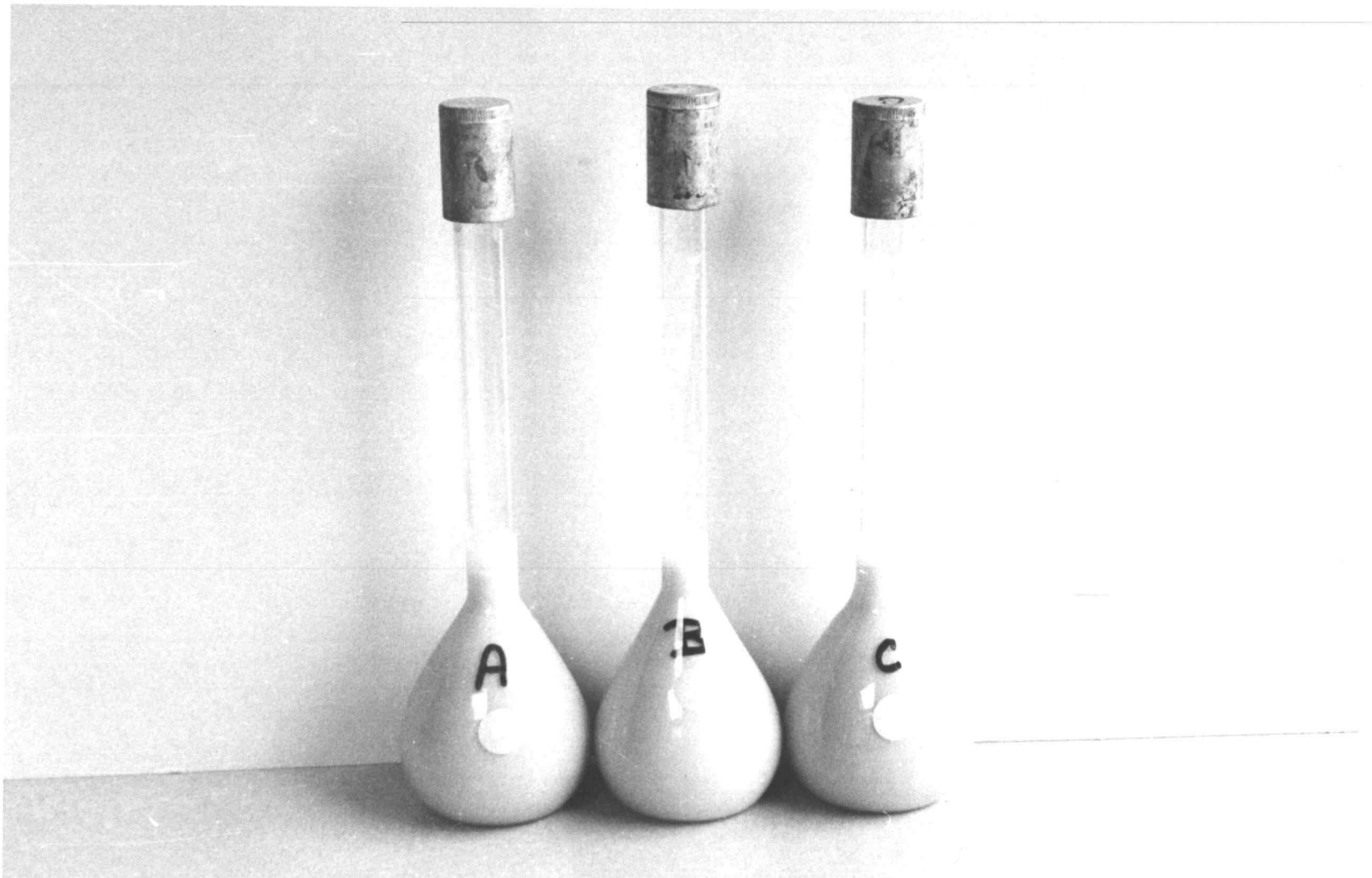


Figure 3. Sealed volumetric flask technique exhibiting visual gas production at 21 C by Leuconostoc citrovorum, strain 31, in the presence and absence of 0.5% yeast extract at zero time. Flask A contains uninoculated milk (control); flask B contains inoculated milk; flask C contains inoculated milk plus yeast extract.

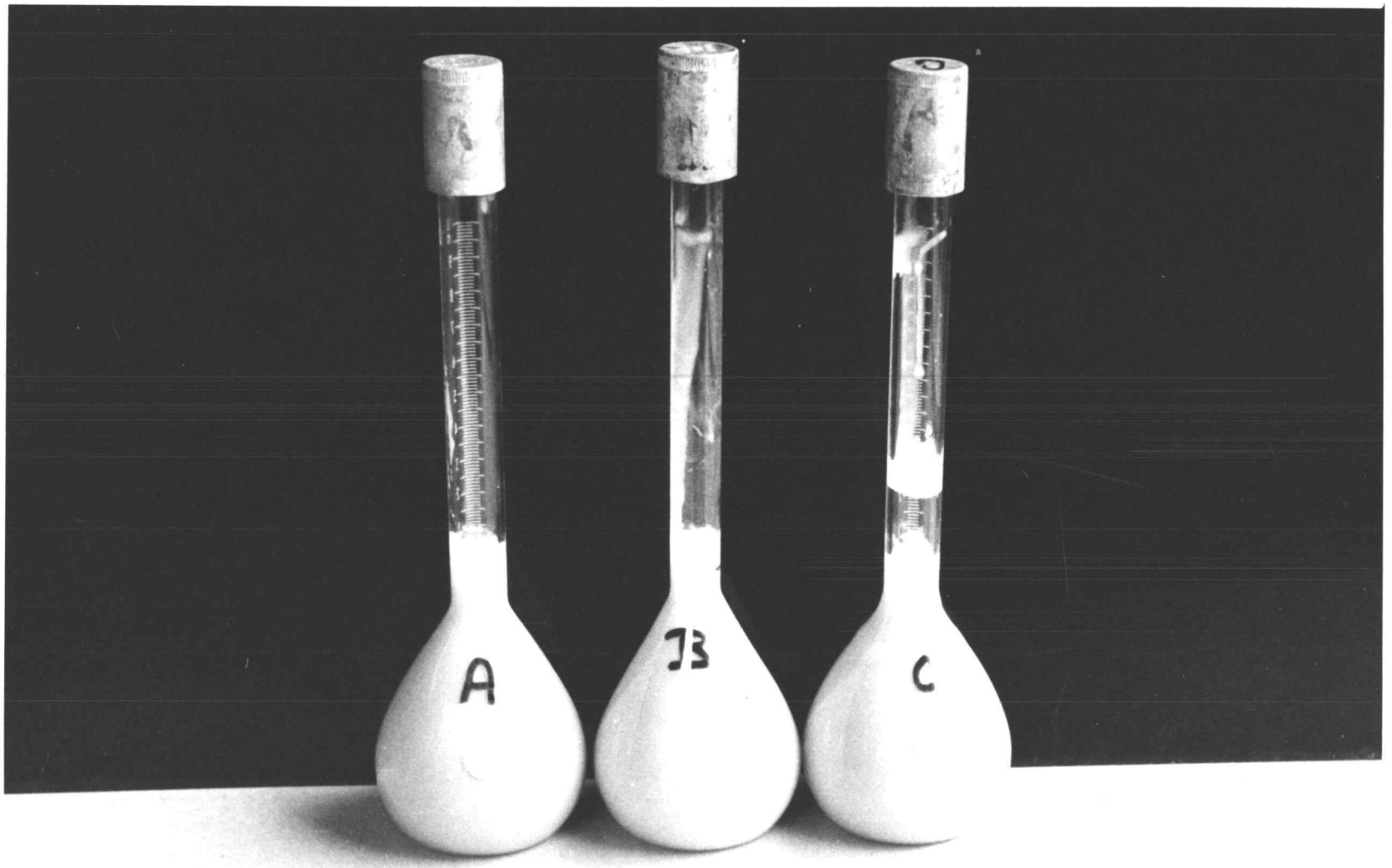


Figure 4. Sealed volumetric flask technique exhibiting visual gas production at 21 C by Leuconostoc citrovorum, strain 31, in the presence and absence of 0.5% yeast extract at 44 hr. Flask A contains uninoculated milk (control); flask B contains inoculated milk; flask C contains inoculated milk plus yeast extract.

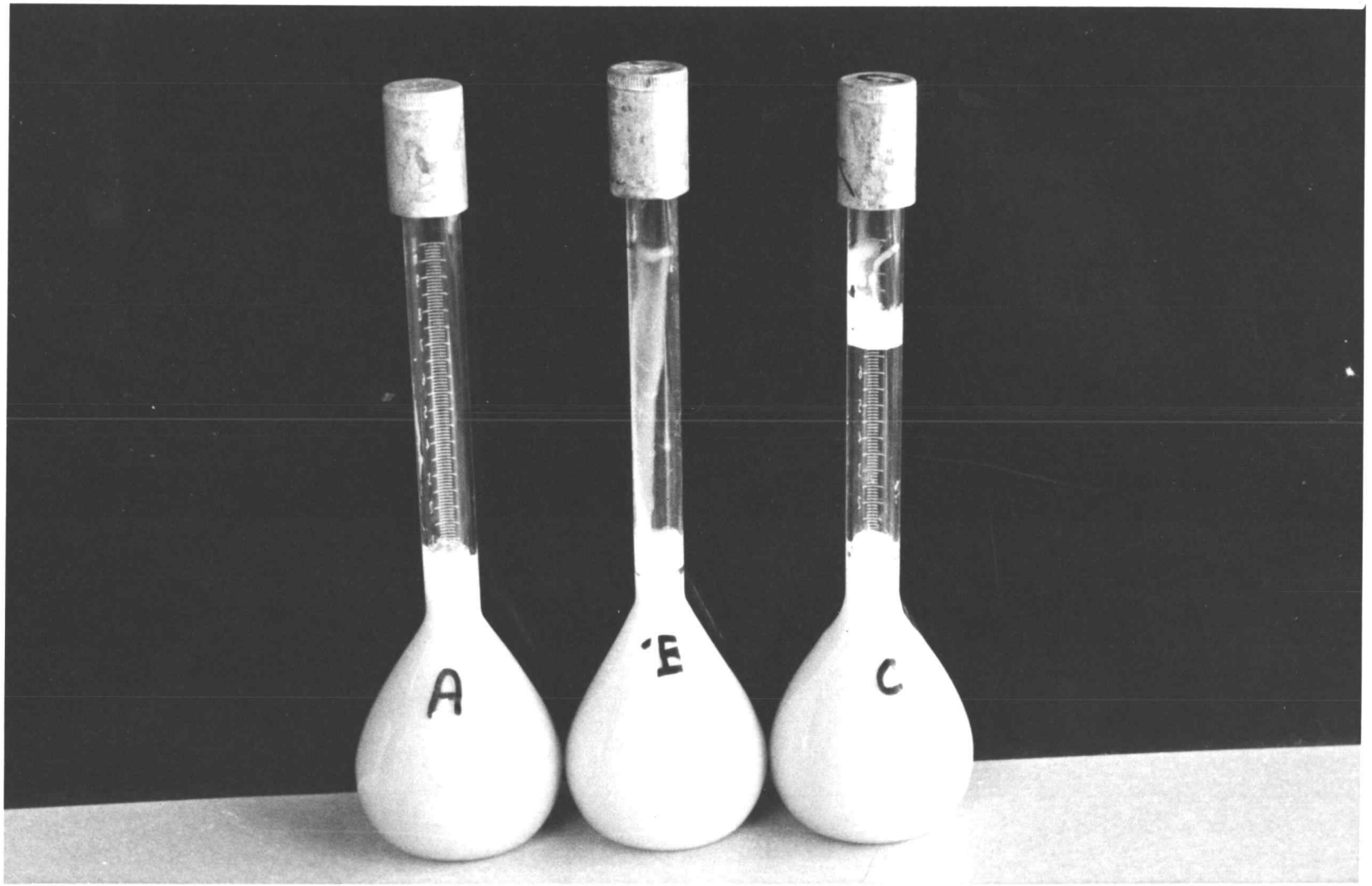


Figure 5. Sealed volumetric flask technique exhibiting visual gas production at 21 C by Leuconostoc citrovorum, strain 31, in the presence and absence of 0.5% yeast extract at 65 hr. Flask A contains uninoculated milk (control); flask B contains inoculated milk; flask C contains inoculated milk plus yeast extract.

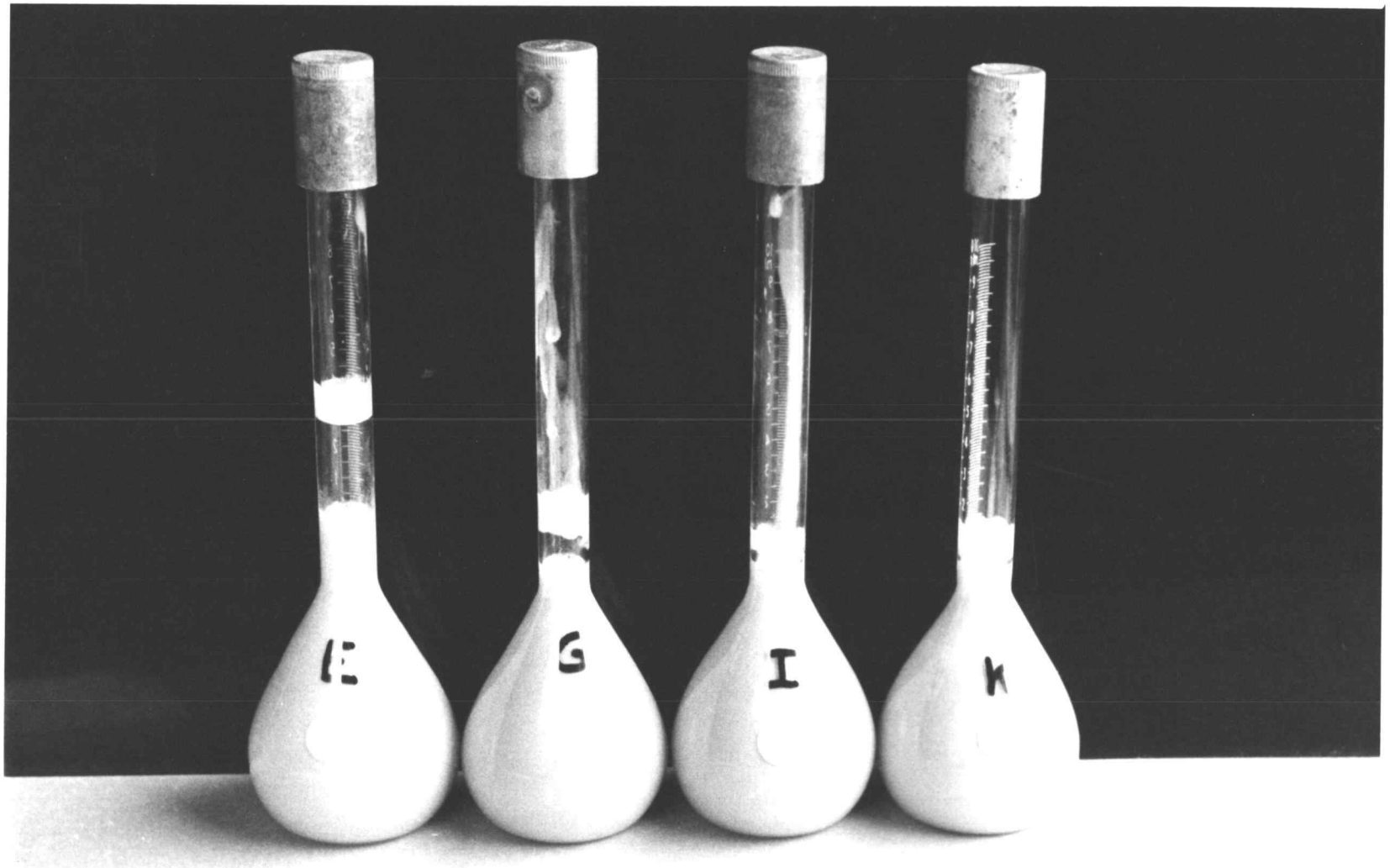


Figure 6. Sealed volumetric flask technique exhibiting visual gas production at 21 C by Leuconostoc citrovorum strains in the presence of 0.5% yeast extract at 44 hr. Flask E contains strain 21; flask G contains strain 2; flask I contains strain 9; and flask K contains strain 8.

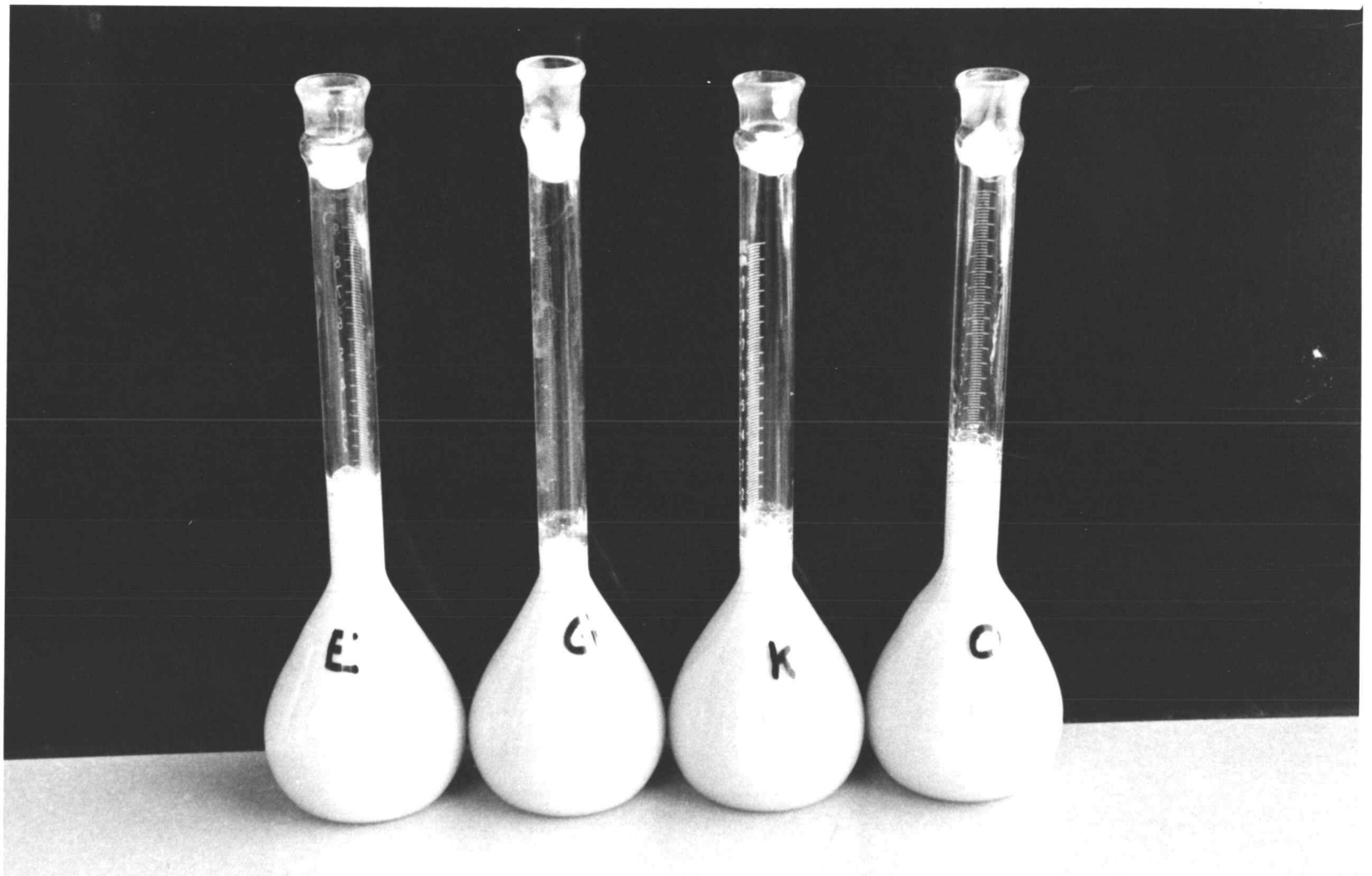


Figure 7. Sealed volumetric flask technique exhibiting visual gas production at 21 C by Leuconostoc citrovorum strains in the presence of 0.5% yeast extract at 65 hr. Flask E contains strain 21; flask G contains strain 2; flask K contains strain 9; and flask O contains strain 7.

Table 5. Effect of temperature on gas production by Leuconostoc citrovorum (1.7×10^6 cells/ml), strain 2, in milk plus 0.5 percent yeast extract.

Temperature	Gas produced
5°C	No gas at 240 hours
10°C	No gas at 240 hours
21°C	Bubble at 57 hours; 10 ml at 72 hours
27°C	Bubble at 42 hours; 10 ml at 56 hours
30°C	10 ml at 42 hours
37°C	Bubble at 72 hours; 3 ml at 120 hours
45°C	No gas at 240 hours

Table 6. Effect of various concentrations of yeast extract on gas production by Leuconostoc citrovorum (2.7×10^7 cells/ml), strain 2, at 21°C and at 30°C.

Percent yeast extract	Gas Produced at 21°C	Gas Produced at 30°C
0.5 %	5 ml at 48 hours 10 ml at 72 hours	10 ml at 24 hours
0.25%	2.5 ml at 48 hours 10 ml at 72 hours	10 ml at 24 hours
0.1 %	0.2 ml at 48 hours 10 ml at 72 hours	3.5 ml at 24 hours 10 ml at 30 hours
0.05%	2 ml at 72 hours 10 ml at 96 hours	0.5 ml at 30 hours 10 ml at 48 hours
0.01%	Bubble at 96 hours	1 ml at 72 hours 2.8 ml at 96 hours

This technique was used to screen a variety of compounds as potential stimulatory agents. None of the agents tested exhibited a stimulatory effect on gas production. Table 7 lists the substances

Table 7. Substances screened for possible stimulatory effect on carbon dioxide production by Leuconostoc citrovorum.

Substance tested	Concentration	Incubation Temperature	Strains used
Casamino acids	0.5%	21°C	L_2, L_9, L_{14}
Vitamin-free Casamino acids	0.5%	21°C	L_2, L_9, L_{14}
Adenine, Guanine, Cytosine, Thymine	0.002 mg/ml of each	21°C	L_2, L_9, L_{14}
Pea extract	0.5%	21°C	L_2, L_7, DN_3
$MnCl_2$	2-4 ppm	21°C	L_2, L_7, DN_3
DNA solution	4.8 μg/ml 16 μg/ml	21°C	L_2
RNA solution	5.0 μg/ml 25 μg/ml	21°C	L_2
DNA solution and RNA solution	4.8 μg/ml + 5.0 μg/ml 16 μg/ml + 25 μg/ml	21°C	L_2
Cell extracts of:			
L. casei	0.006%	30°C	L_2, L_{91404}
L. helveticus	0.005%		
L. lactis	0.026%		
S. lactis	0.029%		
S. cremoris	0.023%		

tested, their concentrations, the strain(s) used, and the incubation temperature. A control of milk plus 0.5 percent yeast extract was always included in the screening tests. The casamino acids (Difco) and vitamin-free casamino acids were tried in hopes that a substance less complex than yeast extract would produce the same effect. The pea extract had been found by Vedamuthu (1966) to enhance diacetyl production by L. citrovorum and thus was tried to determine its effect, if any, on gas production. Galesloot and Hassing (1961) reported the growth enhancement of L. citrovorum by manganese and thus this compound was screened. The DNA, RNA, and bases were tried because of a report by Eysser et al. (1965) that hydrolyzed nucleic acids could be substituted for yeast extract as a source of growth factors for Lactobacillus acidophilus. Cell extracts of various lactic organisms commonly associated with L. citrovorum in dairy products were tested. Their stimulatory nature was not demonstrable in the volumetric flasks. This is most likely due to the small concentrations used.

The volumetric flask technique was also used with cells in LBM instead of milk. The cells were capable of producing considerable gas in this medium alone. Consequently, this medium was not of any value in illustrating the stimulatory effect of various agents on gas production.

Growth Curve of Leuconostoc citrovorum, strain 2

A growth curve of L_2 in milk with and without yeast extract was run to determine the nature of the yeast extract effect, i.e., increase in cell number or increase in acid production per cell. The cells were counted by both MPN and by plate count methods. Figures 8 and 9 illustrate that yeast extract did not enhance growth by increasing cell numbers. The parallel nature of the pH, titratable acidity, and lactic acid curves in Figures 10, 11, and 12 revealed that the cells increased their acid production when they reached a concentration of approximately 1×10^8 cells/ml. Yeast extract enhanced this acid production.

Manometry Studies

The stimulatory effect of yeast extract was quantitatively measured using a Gilson Differential Respirometer. Three strains of L. citrovorum, a fast, a medium, and a slow gas-producer, were used in these studies. Table 8 summarizes triplicate determinations of gas production by these organisms. Figure 13 is a plot of the data in Table 8 for strain 2; the rate of CO_2 evolution is representative of the manometry data obtained in the various studies. The data illustrate that the cells in milk alone produced a measurable amount of gas which was enhanced 38-47 percent by the addition of 0.33 percent

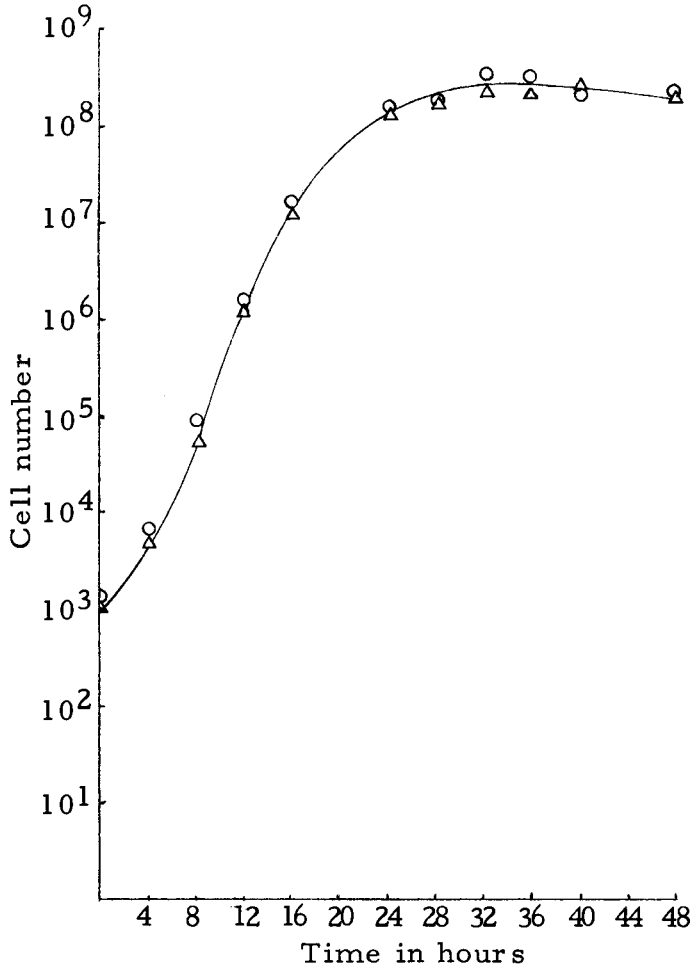


Figure 8. Determination of growth rate of Leuconostoc citrovorum in milk in the presence (o-o) and absence (Δ-Δ) of 0.16 percent yeast extract using plate count technique. Incubation temperature was 21°C.

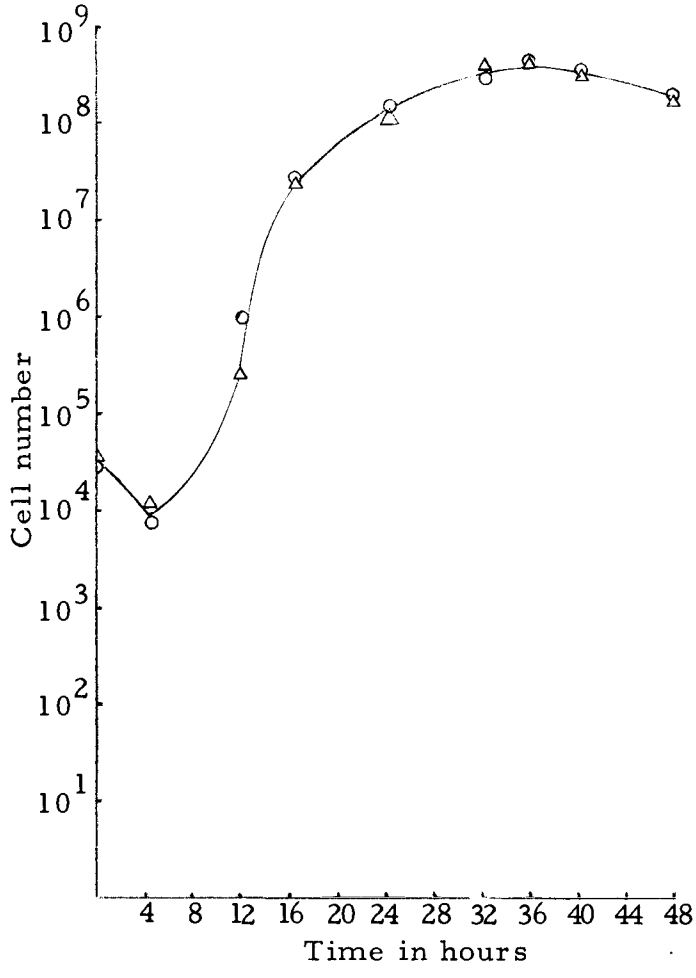


Figure 9. Determination of growth rate of Leuconostoc citrovorum in milk in the presence (o-o) and absence (Δ-Δ) of 0.16 percent yeast extract using Most Probable Number technique. Incubation temperature was 21°C.

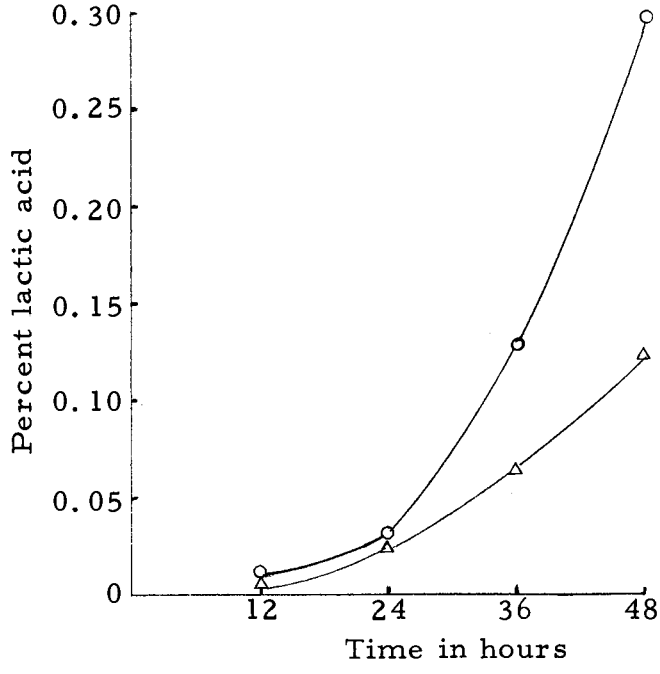


Figure 10. The rate of production of lactic acid by Leuconostoc citrovorum, strain 2, in milk in the presence (o-o) and absence (Δ-Δ) of 0.16 percent yeast extract. Incubation temperature was 21°C.

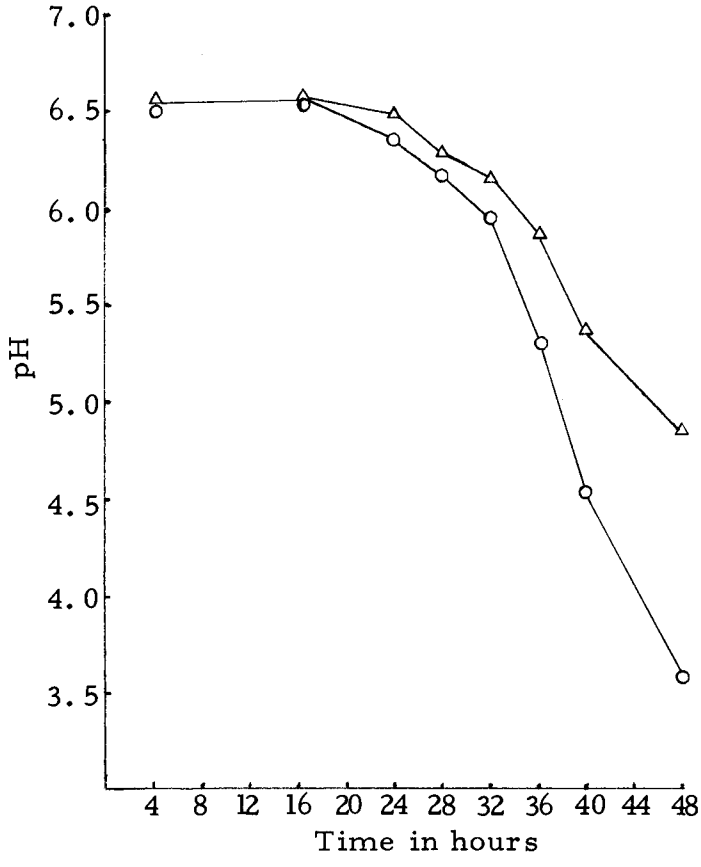


Figure 11. Change in pH with time of incubation by Leuconostoc citrovorum, strain 2, in milk in the presence (o-o) and absence (Δ-Δ) of 0.16 percent yeast extract. Incubation temperature was 21°C.

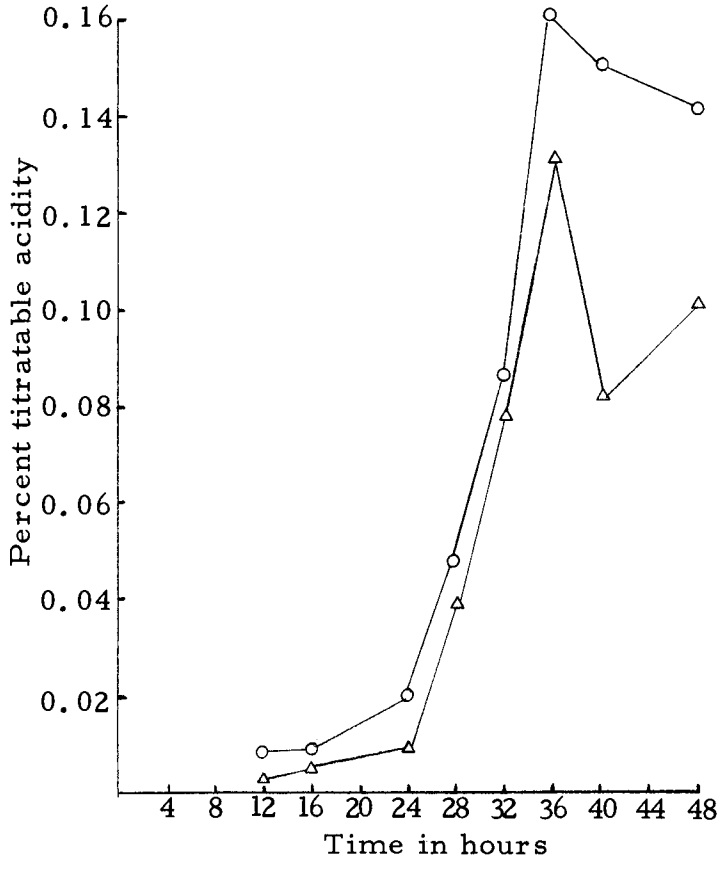


Figure 12. Change in titratable acidity with time of incubation by Leuconostoc citrovorum, strain 2, in milk in the presence (o-o) and absence (Δ-Δ) of 0.16 percent yeast extract. Incubation temperature was 21°C.

Table 8. Summary of Gilson Respirometer studies on carbon dioxide production by Leuconostoc citrovorum, strains 91404, 2, and 7, in milk and in milk plus 0.33 percent yeast extract after 6.5 hours at 30°C.

Strain	µl CO_2 in milk	µl CO_2 in milk plus yeast extract	Percent change	Average percent
L_{91404}	780	1069	37	
(8.0×10^7 cells/flask)	742	1141	54	43
	720	995	38	
L_2	868	1126	41	
(3.2×10^8 cells/flask)	939	1189	27	38
	797	1167	46	
L_7	985	1520	54	
(4.0×10^8 cells/flask)	878	1337	41	47
	561	822	46	

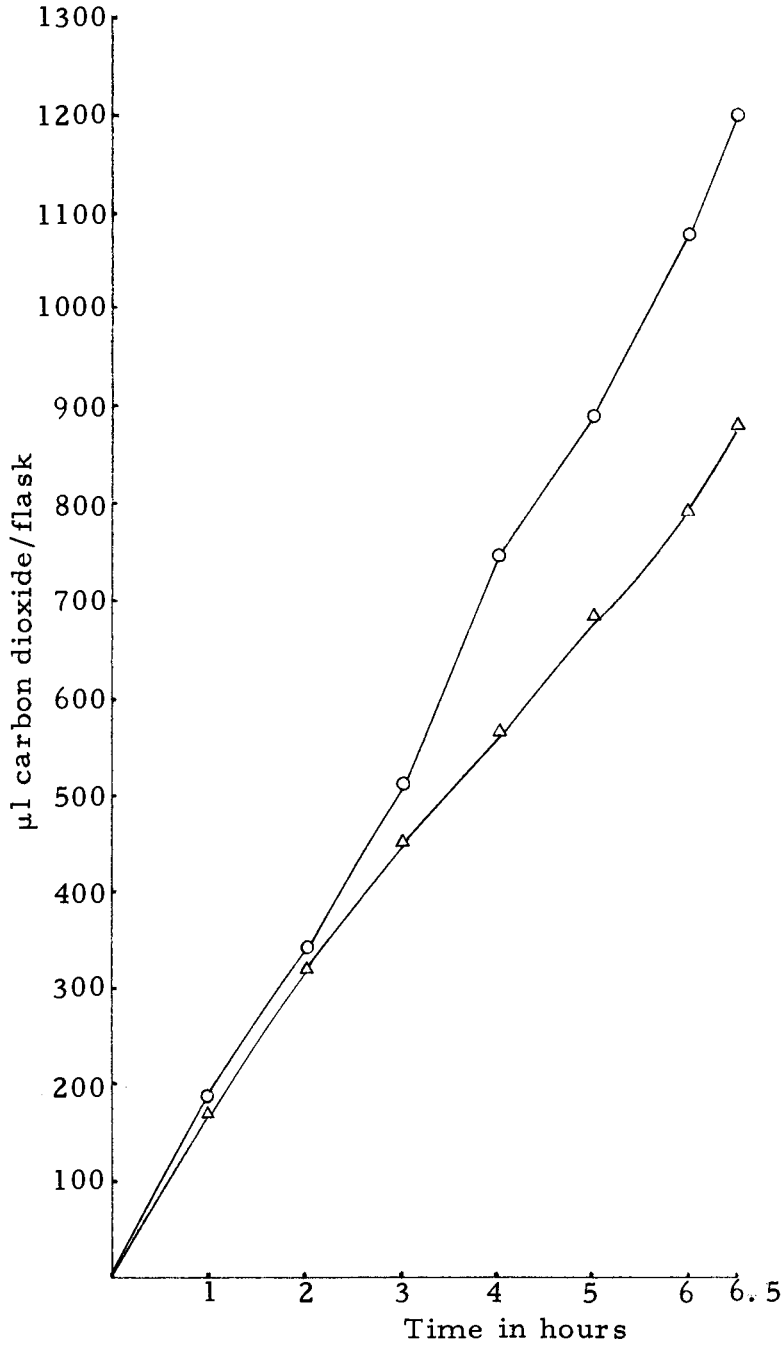


Figure 13. Rate of carbon dioxide evolution in µl by Leuconostoc citrovorum, strain 2, in milk in the presence (o-o) and absence (Δ-Δ) of 0.33 percent yeast extract. The data plotted are averages of the values shown in Table 8.

yeast extract. The difference in the gas producing ability of the three strains observed using the volumetric flasks was not evident under these controlled conditions. It was noted, however, that the cell concentration had to be at least 1 x 10^8 cells/ml in order to have measurable gas production.

Cell extracts of various lactic acid bacteria were tested respirometrically to determine their effect upon gas production by L. citrovorum. Although these extracts exhibited no stimulatory effect using the volumetric flask technique, they proved to be potent stimulatory agents. The data in Tables 9 and 10 can be compared horizontally but not vertically due to the different concentrations of the prepared extracts. The percent change obtained from five trial runs did not always compare as closely as is indicated in the tables. The stimulatory effect often ranged from 10 percent to 150 percent. The three closest values were chosen for the tables. Extracts of L. lactis and L. helveticus appear to affect the two strains equally; whereas the other extracts seem to stimulate gas production by L_2 to a greater degree than that by L_{91404}. From the data one can conclude that these extracts are as effective as yeast extract in enhancing gas production and may even be more effective.

Respirometer studies were also conducted in LBM using resting and growing cells. Because the organisms could produce visible gas in LBM alone, this medium was chosen for analysis.

Table 9. Summary of Gilson Respirometer studies on carbon dioxide production by Leuconostoc citrovorum, strain 2 (0.45 mg cell dry weight/flask) in milk plus cell extracts after 6.5 hours at 30°C.

Extract	µl CO_2 in milk	µl CO_2 in milk plus extract	Percent change	Average percent
Lactobacillus casei 0.12%	578	934	61	
	638	1187	86	73
	588	1011	72	
Streptococcus cremoris (0.35%) and 0.46%	701	1044	49 (64)	
	655	1079	58	62
	655	1035	64	
Lactobacillus lactis 0.52%	512	659	28	
	578	777	36	29
	533	660	24	
Streptococcus lactis 0.58%	795	1082	36	
	638	1058	66	52.5
	512	797	55.6	
Lactobacillus helveticus (0.17%) and 0.1%	533	653	22 (13)	
	701	916	30	22
	588	826	40 (23)	

Table 10. Summary of Gilson Respirometer studies on carbon dioxide production by Leuconostoc citrovorum, strain 91404 (0.62 mg cell dry weight/flask) in milk and in milk plus cell extracts after 6.5 hours at 30°C.

Extract	µl CO_2 in milk	µl CO_2 in milk plus extract	Percent change	Average percent
Lactobacillus casei 0.12%	332	463	39	
	558	757	35	35
	558	732	31	
Streptococcus cremoris (0.35%) and 0.46%	593	864	45	
	638	915	43	40
	948	1184	25 (33)	
Lactobacillus lactis 0.52%	638	796	24	
	410	521	27	26
	631	811	28	
Streptococcus lactis 0.58%	554	761	37	
	593	845	42	33
	948	1140	20	
Lactobacillus helveticus (0.17%) and 0.1%	554	712	28 (17)	
	573	636	10	18
	631	747	18	

The resting cell flasks contained 2.5 ml of pH 6.0, 0.1 M phosphate buffer plus the carbon source(s) tested in a percent proportional to that in the complete LBM (Table 1). The flasks with growing cells contained LBM salts solution (i.e. ascorbic acid, NaCl, K_2HPO_4, KH_2PO_4, $MgSO_4$ in amounts proportional to the complete medium) plus the appropriate amounts of substrate. The substrate combinations had been screened first using sealed test tubes containing ten ml of LBM salts plus the substrates. Only those that exhibited growth (visual turbidity) were tried in the respirometer flasks. The organisms could not grow in LBM salts plus only yeast extract, tryptone, sodium acetate, sodium citrate, or glucose. Table 11 contains data for a single determination of gas production for each combination of substrates. The data complements the data of earlier researchers (Slade and Werkman, 1941) i.e. L. citrovorum can utilize citrate as a carbon source only in the presence of a fermentable carbohydrate. Yeast extract cannot be used alone as an energy source but its presence enabled the organism to utilize citrate. Yeast extract also enhanced the utilization of glucose dramatically. Only one respirometer run was performed for each set of conditions reported in Table 9, therefore, the data should be analyzed for the general phenomena and not for specific μl of gas produced.

Several other substances were tested in milk respirometrically for their effect on gas production. The CO_2 production of strain 2

Table 11. Summary of Gilson Respirometer studies on carbon dioxide production by Leuconostoc citrovorum, strain 2 (2×10^8 cells/flask) from various carbon sources after 6.5 hours at 30°C.

Substrate	µl CO_2 by resting cells	µl CO_2 by growing cells
glucose + Na citrate	384	636
glucose + tryptone	159	436
glucose + tryptone + Na citrate	533	1522
tryptone + citrated whey powder	163	241
tryptone + Na citrate	---	---
yeast extract + glucose	170	434
yeast extract + Na citrate	52	104
yeast extract + Na acetate	--	---
yeast extract + glucose + Na citrate	70	1478

was enhanced 24 percent by 0.25 ppm $MnSO_4$ and 81 percent by 0.5 ppm. Pea extract (1 percent) enhanced the CO_2 production of L_2 by 53 percent and of L_7 by 34 percent. Milk treated with hydrogen peroxide (0.03 percent) and liver catalase (0.006 percent) enhanced gas production of L_2 by 26 percent, but decreased acid production.

Cell-free extracts of L_2 and S. diacetilactis 18-16 were used to study two decarboxylation reactions in the citrate catabolic pathway. S. diacetilactis has been shown to decarboxylate oxaloacetic acid and pyruvic acid (Seitz, 1963) and was thus used as a control. The cell-free preparation of L_2 did not exhibit any activity when provided with ten μmoles of oxaloacetate containing 20 μmoles of $MnCl_2$ at pH 7.0 or at pH 6.0. The preparation did produce 300 μl of CO_2 from 250 μmoles of pyruvate containing 35 μmoles of thiamine-pyrophosphate (TPP) and ten μmoles of $MnCl_2$ at pH 6.0. The addition of 0.33 percent yeast extract to the pyruvate, TPP, $MnCl_2$ mixture enhanced CO_2 production by 16 percent.

V. DISCUSSION

An important consideration in this study of carbon dioxide production by Leuconostoc citrovorum was that the organisms did not produce appreciable amounts of acid or gas until the cell population reached 1 x 10^8/ml. A comparison of Figures 8, 9, 10, 11, and 12 revealed that yeast extract did not stimulate cell division but rather acid production/cell, particularly lactic acid. This stimulatory effect was not noticeable at a cell concentration of less than 1 x 10^8/ml. Manometer studies indicated that gas production was also negligible below this cell concentration. Thus, carbon dioxide production and acid production can be considered directly proportional to cell concentration. This critical concentration of cells may be an explanation for the variable nature of gas production by L. citrovorum as reported in the literature.

The differences in the gas producing ability of various strains noted in the volumetric studies, but not in the manometric studies, may be attributed to the generation time of the particular strain (how fast the population reaches 1 x 10^8/ml). The inoculum of the manometer flasks was always at least 1 x 10^8/ml whereas that of the volumetrics was usually 1 x 10^6 or 1 x 10^7/ml. The variability in gas production by the same strain of L. citrovorum which can be observed in Tables 5 and 6 is also due to differences in cells/ml of the inocula. The technique of using sealed volumetric flasks for

determining gas production was useful to illustrate the general phenomena of yeast extract stimulation, optimum temperature, etc. But the differential respirometer studies pointed out its limitations. The volumetrics did not illustrate gas production in milk alone by L. citrovorum, or the stimulatory nature of manganese, pea extract, and the various cellular extracts upon gas production. One explanation for the observed lack of gas might be that these stimulatory factors enhanced CO_2 production but not acid production. Thus, the gas produced remained soluble in the milk and was not observable. The yeast extract, on the other hand, enhanced both acid and gas which then pushed the plug up the neck of the flask. This same phenomenon was also noticeable when the acid was tipped into the manometer flasks; there was usually more dissolved gas in the flask without yeast extract. In the cell extract, pea extract, and Mn experiments, the addition of acid usually released approximately equal amounts of gas from the control and the experimental flasks.

The manometer studies conducted at 30°C, optimum temperature for gas production but not cell growth, elucidated the nature of gas production by L. citrovorum. This organism is capable of producing 500-900 µl CO_2 in milk and 800-1500 µl CO_2 in 0.33 percent yeast extract milk. The average percent stimulation, under these conditions, was close for the three strains tested. The addition of other cell extracts enhanced gas production 20-70 percent. There

was a wide range of percent increase of CO_2 for the various trial runs using the cell extracts. The concentration of the extract was not proportional to the amount of CO_2 produced (see S. cremoris and L. helveticus in Tables 9 and 10). The effect of the extracts on the two strains tested is quite different. For example, L. casei extract enhanced gas production by L_2 73 percent and by L_{91404} 35 percent. Even extracts of approximately the same concentration had different effects; i.e. L. lactis (0.52 percent) enhanced CO_2 production of L_2 by 29 percent and S. lactis (0.58 percent) by 52.5 percent. These two extracts had approximately the same effect on L_{91404} (i.e. 26 percent and 33 percent respectively). This variable nature of stimulation may reflect the organism's fastidiousness with respect to this property as evidenced by the existing confusion in the literature concerned with gas production by L. citrovorum. This stimulatory effect of extracts of organisms commonly associated with L. citrovorum in starter cultures may have practical application in the dairy industry. It is feasible to envisage a decreasing pH during a dairy fermentation causing lysis of some lactic streptococci of the starter cultures. This cellular lysate could, in turn, stimulate gas production by L. citrovorum resulting in the slit-open defect in Cheddar or other cheeses. The gas-producing potential of L. citrovorum should be considered when selecting organisms to compose a starter culture. The concentration of inoculum and the temperature

of the fermentation should also influence this choice.

The differential respirometer was also used in studies designed to exemplify the metabolic nature of carbon dioxide production by L. citrovorum. The data in Table 11 illustrated that L. citrovorum could utilize citrate in the presence of glucose, glucose plus tryptone, yeast extract, and glucose plus yeast extract. Since L. citrovorum can utilize citrate as an energy source only in the presence of a fermentable carbohydrate, the yeast extract must contain either a fermentable carbohydrate or some other substance which allows citrate metabolism. The citrated whey powder contains lactose as well as citrate and thus can be utilized alone or in the presence of tryptone, etc. These studies also illustrate the stimulatory nature of yeast extract. The gas produced from glucose plus sodium citrate plus yeast extract was considerably greater than that produced from glucose plus sodium citrate. The enhancement of gas production by tryptone under these conditions was similar to that of yeast extract. This might suggest that the stimulatory factor is a protein containing amino acids which can be transaminated or oxidatively deaminated to an α-keto acid which, in turn, can be decarboxylated. The gas production observed in these media was generally less than that observed in milk.

Two decarboxylation reactions were studied in an attempt to understand the source of the CO_2 produced by L. citrovorum.

An extract of L. citrovorum failed to decarboxylate oxaloacetate. Under the same experimental conditions, however, S. diacetilactis 18-16 extract exhibited oxaloacetate decarboxylase activity. This indicated that L. citrovorum had a different mechanism for citrate utilization or an enzyme which required additional cofactors; a third possibility is that the enzyme was labile and became inactivated during extraction. The latter explanation is more plausible considering the work of other researchers. The extract of L. citrovorum did exhibit activity when given pyruvate and cofactors as substrate. The quantity of CO_2 produced was significant but less than that theoretically expected. The addition of yeast extract to this substrate enhanced CO_2 production by 16 percent. Because the yeast extract increased the acidity of a milk culture greatly, the decarboxylation reaction in the 2-3 cleavage pathway i. e. 6-phosphogluconate $\longrightarrow$ pentose-PO_4 + CO_2 may also have been enhanced. This would increase the formation of lactic acid as well as CO_2. The reaction was not experimentally studied.

Pea extract and manganese mimicked the effect of yeast extract. The addition of these substances might reflect the potential raw milk, used in cultured dairy products, possesses with respect to enhancing gas production. The cow's diet influences the composition of its milk and could very easily contain the substances found in

the pea extract. The seasonal variation of the manganese concentration of raw milk and its influence on the growth of Leuconostoc has been reported by Galesloot (1961).

VI. SUMMARY

The ability of Leuconostoc citrovorum to produce carbon dioxide was studied by two methods. A qualitative method using visual measurement of gas (agar plug forced up the neck of a volumetric flask) demonstrated that 0.25 percent yeast extract stimulated gas production. The optimum temperature for observing this phenomenon was 30°C. A differential respirometer was used for quantitative measurement of the CO_2 under a variety of conditions. L. citrovorum produced 500-900 µl of CO_2 in milk and 800-1500 µl of CO_2 in 0.33 percent yeast extract milk. The addition of yeast extract enhanced gas production by 40 percent. Cellular extracts of L. casei, S. cremoris, L. lactis, S. lactis, and L. helveticus mimicked the effect of yeast extract on gas production. These stimulatory factors enhanced gas production 20 to 70 percent. Their effect was not consistent i.e. the concentration of extract was not proportional to the percent stimulation and the percent stimulation was quite variable. The addition of manganese and pea extract also increased gas production. These studies illustrated the potential of L. citrovorum to produce large amounts of carbon dioxide. This property is important to the dairy industry and should be considered in the selection of starter cultures for the various cultured dairy products. The production of large volumes of gas may result in the floating curd defect

of cottage cheese or the slit-open defect of the hard cheeses.

The stimulatory effect of yeast extract was on acid and CO_2 production. The yeast extract addition increased acid and gas per cell but not cell number. The yeast extract also appeared to stimulate the organism's ability to utilize the citrate and the fermentable carbohydrate present as energy sources. Experiments revealed that the decarboxylation of pyruvate was stimulated by the addition of yeast extract. The decarboxylation of oxaloacetate was not demonstrable using a cell extract of L. citrovorum. The yeast extract may stimulate decarboxylation reactions in the 2, 3 cleavage pathway as well as in the citrate metabolic pathway. L. citrovorum can utilize citrate in the presence of yeast extract undoubtedly because a fermentable carbohydrate is provided.

BIBLIOGRAPHY

Aiba, Shuichi, Arthur E. Humphrey and Nancy F. Mills. 1965. Biochemical engineering. New York, Academic Press. 333 p.

Anderson, Viggo Brandstrup. 1959. Formation of diacetyl and acetoin by certain starter organisms. Milchwissenschaft 14: 429-432.

Braz, M. and L. A. Allen. 1939. Protein metabolism and acid production by the lactic acid bacteria in milk. Influence of yeast extract and chalk. Journal of Dairy Research 10:20-34.

Breed, Robert S., E. G. D. Murray and Nathan R. Smith. 1957. Bergey's Manual of determinative bacteriology. 7th ed. Baltimore, Williams and Wilkins Company. 1094 p.

Brewer, C. R. and C. H. Werkman. 1940. Dissimilation of citric acid by Streptococcus paracitrovorus. Antonie van Leeuwenhoek Journal of Microbiology and Serology 6:110-120.

Eyssen, H. et al. 1965. Nucleotide requirements of Lactobacillus acidophilus variants isolated from the crops of chicks. Antonie van Leeuwenhoek Journal of Microbiology and Serology 31:241-248.

Galesloot, Th. E. 1950. An early gas defect in cheese caused by Betacoccus (Leuconostoc) species. The Netherlands Milk and Dairy Journal 4:274-287.

Galesloot, Th. E. and F. Hassing. 1961. Some differences in behavior between starters containing as aroma bacterium either S. diacetilactis or Betacoccus cremoris. The Netherlands Milk and Dairy Journal 15:223-247.

Garvie, Ellen I. 1961. The genus Leuconostoc and its nomenclature. Journal of Dairy Research 27:283-292.

Gibson, T. and Y. Abdel-Malek. 1945. The formation of carbon dioxide by lactic acid bacteria and Bacillus licheniformis and a cultural method of detecting the process. Journal of Dairy Research 14:35-44.

Hamamoto, M., T. Kanauchi, and K. Mino. 1966. Gas production by leuconostoc organisms in cheese. Dairy Science Abstracts 28:31.

Hammer, B. W. 1920. Volatile acid production of S. lacticus and the organisms associated with it in starters. Ames. p. 1-96c. (Iowa. Agricultural Experiment Station. Research Bulletin no. 63)

Hammer, B. W. and M. P. Baker. 1923. Studies on Streptococcus paracitrovorus group. Ames. p. 17-36. (Iowa. Agricultural Experiment Station. Research Bulletin no. 81)

Hammer, B. W. and F. F. Sherwood. 1923. The volatile acids produced by starters and by organisms isolated from them. Ames. p. 1-15. (Iowa. Agricultural Experiment Station. Research Bulletin no. 80)

Hansen, P. Arne. 1941. A study in cheese ripening. The influence of autolyzed cells of Streptococcus cremoris and Streptococcus lactis on the development of Lactobacillus casei. Journal of Dairy Science 24:969-976.

Harvey, R. J. and E. B. Collins. 1961. Role of citritase in acetoin formation by Streptococcus diacetilactis and Leuconostoc citrovorum. Journal of Bacteriology 82:954-959.

_____. 1962a. Citrate transport system of Streptococcus diacetilactis. Journal of Bacteriology 83:1005-1009.

_____. 1962b. Factors responsible for the relationship between pH and acetoin formation by Streptococcus diacetilactis. In: Abstracts of the VIII International Congress for Microbiology, Montreal. p. 46.

_____. 1963. Roles of citrate and acetoin in the metabolism of Streptococcus diacetilactis. Journal of Bacteriology 86:1301-1307.

Hucker, G. J. and Carl S. Pederson. 1930. Studies on the coccaceae. The genus Leuconostoc. Geneva. p. 1-80. (New York. Agricultural Experiment Station. Technical Bulletin no. 167)

Järvik, M. and S. Kjell. 1956. Preliminär rapport över betakockundersökningen (Leuconostoc) i svensk ost. Mejeritekniska Meddelanden 17:59-64.

Jesperson, N. J. Tofte. 1959. Identification of lactic streptococci from starters. In: [Papers] of the 15th International Dairy Congress, London. Vol. 3. p. 1376-1381.

Knudsen, Soncke. 1931. Starters. Journal of Dairy Research 2: 137-163.

Ling, E. R. 1951. The determination of lactic acid in milk. Journal of the Science of Food and Agriculture 2:279-288.

Marth, E. H. 1962. Certain aspects of starter culture metabolism. Journal of Dairy Science 45:1271-1281.

Meynell, G. G. and Elinor Meynell. 1965. Theory and practice in experimental bacteriology. Cambridge, University Press. 288 p.

Mizuno, W. G. and J. J. Jezeski. 1961. Starter metabolism. The mechanism of acetoin formation as determined with C^{14}-labeled substrates. Journal of Dairy Science 44:579-588.

Orla-Jensen, S., A. D. Orla-Jensen and Bernhard Spur. 1926. The butter aroma bacteria. Journal of Bacteriology 12:333-342.

Overcast, W. W. and T. W. Albrecht. 1952. Gas production in cheddar cheese by Leuconostoc citrovorum. Journal of Dairy Science 35:554-558.

Robertson, P. S. 1957. Carbon dioxide content of New Zealand cheddar cheese. Journal of Dairy Research 24:235-241.

Sandine, William E. 1958. The physiology and taxonomy of the carbon dioxide-producing lactic acid streptococci. Ph. D. thesis. Corvallis, Oregon State University. 123 numb. leaves.

Sandine, W. E., P. R. Elliker and A. W. Anderson. 1959. Taxonomic study of high carbon dioxide-producing lactic acid streptococci isolated from mixed strain starter cultures. Journal of Dairy Science 42:799-808.

Sandine, W. E., P. R. Elliker and Helen Hays. 1962. Cultural studies on Streptococcus diacetilactis and other members of the lactic streptococcus group. Canadian Journal of Microbiology 8:161-174.

Sandine, W. E. et al. 1961. Studies on the mechanism of diacetyl synthesis by Streptococcus diacetilactis. Journal of Dairy Science 44:1158.

Sauberlich, H. E. and C. A. Bauman. 1948. A factor required for the growth of Leuconostoc citrovorum. Journal of Biological Chemistry 176:165-173.

Seitz, Eugene W. et al. 1963. Studies on diacetyl biosynthesis by Streptococcus diacetilactis. Canadian Journal of Microbiology 9:431-441.

Sherwood, I. R. 1939. The relation of certain lactic acid bacteria to open texture in cheddar cheese. Journal of Dairy Research 10:326-335.

Slade, H. D. and C. H. Werkman. 1941. The anaerobic dissimilation of citric acid by cell suspensions of Streptococcus paracitrovorus. Journal of Bacteriology 41:675-684.

Speck, M. L., J. K. McAnelly and Jeanne D. Wilbur. 1958. Variability in response of lactic streptococci to stimulants in extracts of pancreas, liver, and yeast. Journal of Dairy Science 41:501-508.

Speckman, R. A. and E. B. Collins. 1966a. Biosynthesis of diacetyl by Leuconostoc citrovorum. Journal of Dairy Science 49:701.

_____. 1966b. Biosynthesis of diacetyl by Streptococcus diacetilactis. Bacteriological Proceedings 66:6.

Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1957. Manometric techniques. 3d ed. Minneapolis, Burgess. 338 p.

Van Beynum, J. and J. W. Pette. 1939. The decomposition of citric acid by Betacoccus cremoris. Journal of Dairy Research 10:250-266.

Vedamuthu, Ebenezer. 1966. Assistant Professor, Iowa State University, Department of Dairy and Food Industry. Personal communication. Ames, Iowa.