APPLIED MICROBIOLOGY, Jan. 1968, p. 56-61

Vol. 16, No. 1
Printed in U.S.A.

Some Factors Influencing Carbon Dioxide Production by *Leuconostoc citrovorum*[1]

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Received for publication 14 August 1967

This study was undertaken to determine whether *Leuconostoc citrovorum* plays a role in carbon dioxide production in milk. The ability of *L. citrovorum* strains to produce gas was studied by two methods. A qualitative method, in which an agar plug was forced up the neck of a volumetric flask, measured gas visually. This method demonstrated that 0.25% yeast extract, in a milk medium at 30 C, stimulated the production of at least 10 ml of gas. Studies using a Gilson Differential Respirometer revealed that *L. citrovorum* produced 500 to 900 μliters of CO_2 in 6.5 hr, whereas 800 to 1,500 μliters of CO_2 was produced in nonfat milk which contained 0.33% yeast extract. Cell extracts of *Streptococcus cremoris*, *S. lactis*, *Lactobacillus lactis*, *L. casei*, and *L. helveticus* also enhanced gas production of *L. citrovorum* from 20 to 70%. Autolysates of these bacteria, present during the ripening or ageing 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. Experiments indicated that one metabolic source of carbon dioxide was the decarboxylation of pyruvate produced during catabolism of citric acid. Yeast extract stimulated this reaction by 16%.

Cultured diary products are the end result of milk fermentations 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 CO_2 gives buttermilk a desirable effervescence but it may also 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 (13). However, the role of *Leuconostoc* in CO_2 production during dairy fermentations is less well defined. Orla-Jensen et al. (11) recognized *L. citrovorum* as an important organism in the development of flavor and aroma in cultured dairy products. They also found *L. citrovorum* to be a vigorous gas producer upon primary isolation, but this property was lost with further propagation. Gibson and Abdel-Malek (4) studied CO_2 formation by various lactic acid bacteria and found, as did Orla-Jensen et al. (11), that recently isolated strains of *L. citrovorum* are quite consistent in their ability to form gas readily from citrate and citrated milk. However, other strains were quite variable in this respect, and yeast preparations had a pronounced stimulatory effect on the formation of CO_2 by the heterofermentative lactic acid bacteria in glucose-milk or glucose-gelatin. Gibson and Abdel-Malek found that this stimulatory effect was manifested in the rate of some phase of the fermentation and was not due to enhanced growth.

Several authors have noted the stimulatory effect of accessory growth factors on the *Leuconostoc*. Hucker and Pederson (6) noted that yeast extract enhanced growth, as measured by acid production. Sauberlich and Bauman (14) also found that yeast extract and Bacto-peptone stimulated growth of *L. citrovorum* in a defined medium. This effect was measured by acid production and turibidity. The work of Braz and Allen (1) supported the observations of Gibson and Abel-Malek (4) that yeast extract increased acid production but not cell number. Speck et al. (17) 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 *S. cremoris* and *S. lactis* on *Betacoccus cremoris* (*L. citrovorum*) in skim milk

[1] Technical paper no. 2342, Oregon Agricultural Experiment Station.

was studied by Hansen (5). These cell extracts raised the end point of titration for *L. citrovorum*. Other experiments revealed that no proportionality existed between the amount of growth and the end point of titration.

There have been many reports relating the *Leuconostoc* to the slit-open defect in cheddar cheese. Sherwood (16) isolated organisms from "open" cheese and found that they were primarily lactobacilli. From a total of 11 strains, he found one that was *L. citrovorum*. Galesloot (3) isolated cultures of *Betacoccus arabinosaceus* and *Betacoccus bovis* from defective cheese, but he stated that the *Betacoccus* species which normally occur in starters (i.e., *L. citrovorum*) are not dangerous. Overcast and Albrecht (12) isolated *L. citrovorum* as the causative organism of slit-open cheese. The authors emphasized that the solubility of CO_2 at various temperatures may influence the appearance of this defect even though the causative organism was present. Hamamoto et al. (Dairy Sci. Abstr., p. 31, 1966) performed several experiments with a *Leuconostoc* species isolated from blown Gouda cheese. They demonstrated that these organisms did not affect the appearance, texture, or body of the cheese even when yeast extract had been added to the pastuerized milk used in the cheese-making process. The present study was undertaken in an effort to determine whether *L. citrovorum* plays a role in CO_2 production in milk. Environmental conditions necessary for gas production and the effect of various substances on CO_2 evolution were examined. A secondary objective was to determine the metabolic source of the evolved CO_2.

MATERIALS AND METHODS

Cultures. The *L. citrovorum* strains 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 by use of sodium azide agar (8) and were classified according to the criteria described by Sandine et al. (13). The lactic cultures used in cell-extract preparations and as controls also were obtained from the above collection. The *Leuconostoc* strains were maintained by weekly transfers of a 1% inoculum in 10 ml of sterilized (at 121 C for 12 min) 11% reconstituted nonfat dry milk. The cultures were incubated at 21 C for 36 to 40 hr and then held at 5 C until the next transfer.

Culture preparation for gas production studies. Forty ml of *Leuconostoc* broth medium (LBM), containing 10 g of yeast extract, 10 g of tryptone, 2 g of citrated whey powder, 5 g of sodium citrate, 1.5 g of sodium acetate, 10 g of glucose, 0.7 g of ascorbic acid, 2 g of NaCl, 2 g of K_2HPO_4, 2 g of KH_2PO_4, 2 g of $MgSO_4$, and 1,000 ml of distilled water, was inoculated into a 24-hr broth culture, at the rate of 1%, and the culture was incubated at 27 C for 20 to 24 hr. Cells were harvested in a sterile centrifuge tube using an RC-2 refrigerated Servall centrifuge operated at 4,340 $\times$ *g* for 5 min. The cells were washed once with sterile physiological (0.85% NaCl) saline and resuspended to the desired volume in saline. The saline suspension was either counted by the most probable number (MPN) technique (9) or adjusted to a desired optical density at 650 mμ by use of a Bausch and Lomb Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). The suspension was used to inoculate volumetric flasks or used directly in manometric flasks.

One-liter broth cultures, inoculated and incubated as stated above, were used to prepare cell-free extracts for manometric studies. The harvested cells were washed twice with saline and resuspended in 40 ml of 0.1 M potassium phosphate buffer, *p*H 7.0. This suspension was sonically treated in a Raytheon model DF 101, 10-kc sonic oscillator (Raytheon Co., South Norwalk, Conn.) for 15 min. The cellular debris was removed by centrifugation at 27,000 $\times$ *g* for 25 min. The supernatant fluid was frozen at -20 C until used. It was not held, however, for more than 2 weeks.

Preparation of cellular extracts used as additives. One liter of lactic broth (2) cultures (1% inoculum from an 18-hr culture) were harvested at 4,340 $\times$ *g* for 20 min, washed twice with distilled water, and resuspended in 50 ml of distilled water. The suspension was sonic-treated for 30 min and then recentrifuged at 12,000 $\times$ *g* for 15 min. The supernatant fluid was autoclaved for 15 min at 121 C. The percentage of solids of each extract was determined quantitatively (in triplicate) by weighing a dried (at 110 C, for 24 hr) 1-ml sample.

Gas production in volumetric flasks. Graduated 110-ml glass volumetric flasks (VC 570, Aloe Scientific, St. Louis, Mo.) containing 5 ml of prepared cells (final saline suspension of 40 ml gave 10^3 cells per ml), 5 ml of sterile 10% yeast extract (Difco) or other additives, and sterile 11% reconstituted nonfat dry milk, for a final volume of 100 ml, were sealed with 1 ml of Vaspar as a sealing plug. The Vaspar was composed of 50 g of Vaseline, 2 g of agar, 1 ml of Tween 80, and 50 ml of distilled water. Control flasks contained 5 ml of cells, 95 ml of milk, and 1 ml of Vaspar. All flasks were incubated at 21 or 30 C. Gas production was measured visually by observing the rise of the plug in the calibrated neck of the flask.

Measurement of CO_2 production by manometric methods. To determine CO_2 production quantitatively, a Gilson Differential Respirometer (Model GS, Gilson Medical Electronics, Middleton, Wis.) and the direct manometric method of Umbreit et al. (18) were used. The double-armed flasks, containing a total volume of 3 ml, were shaken continuously throughout the experiment. After 6 hr, 0.5 ml of 0.5 N HCl was tipped in to release any dissolved CO_2, and the experiment was terminated 30 min later. The 6.5-hr period was arbitrary. Preliminary experiments indicated that gas production did not reach a plateau even after 12.5 hr.

RESULTS

Gas production in volumetric flasks. The less refined quantitative technique, using sealed grad-

uated volumetric flasks, demonstrated the effect of yeast extract on gas production by *L. citrovorum*. Figure 1 illustrates this technique. The cells in Flask B (milk without the addition of yeast extract) did not usually produce any visible gas, even after incubation for 10 days, whereas the cells in milk plus yeast extract produced gas within 4 days. The effect of temperature on gas production under these conditions was documented with strain L_2. The data in Table 1 illustrate that gas production was maximal at 30 C; 27 and 21 C were both suitable temperatures, but gas production lagged from 6 to 12%. Several yeast extract concentrations were tested to determine the range of this stimulatory effect. It can be seen from the data in Table 2 that a concentration of yeast extract as small as 0.05% was still effective.

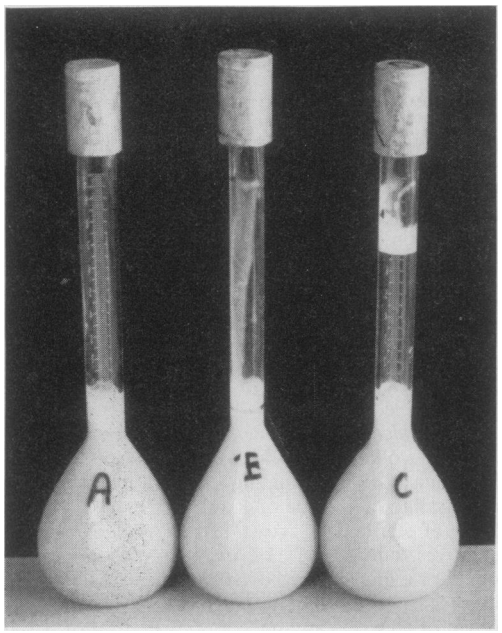

FIG. 1. *Gas production by Leuconostoc citrovorum strain 31 at 65 hr. Flask A contains milk alone (control); flask B contains milk and cells; flask C contains milk, cells, and 0.5% yeast extract.*

TABLE 1. *Effect of temperature on gas production by Leuconostoc citrovorum strain 2 (1.7 × 10⁶ cells/ml) in milk plus 0.5% yeast extract*

Temp	Time required for accumulation of at least 10 ml of CO_2
C	*hr*
5	>240
10	>240
21	72
27	56
30	42
37	240
45	>240

TABLE 2. *Effect of concentrations of yeast extract on gas production by Leuconostoc citrovorum strain 2 (2.7 × 10⁷ cells/ml) at 21 and 30 C*

Concn of yeast extract	Gas produced at 21 C		Gas produced at 30 C (24 hr)
	48 hr	72 hr	
%	*ml*	*ml*	*ml*
0.5	5.0	10.0	10.0
0.25	2.5	10.0	10.0
0.1	0.2	10.0	3.0
0.05	—	5.0	0.5
0.01	0.5	0.5	None[a]

[a] At 72 hr, 1.0 ml.

TABLE 3. *Summary of Gilson Respirometer studies on CO_2 production by Leuconostoc citrovorum strains 91404, 2, and 7, in milk and in milk plus 0.33% yeast extract, after 6.5 hr at 30 C*

Strain	CO_2 in milk	CO_2 in milk plus yeast extract	Increase	Avg
	μliters	*μliters*	%	%
L_{91404} (8.0 × 10⁷ cells/flask)	780	1,069	37	43
	742	1,141	54	
	720	995	38	
L_2 (3.2 × 10⁸ cells/flask)	868	1,226	41	38
	939	1,189	27	
	797	1,167	46	
L_7 (4.0 × 10⁸ cells/flask)	985	1,520	54	47
	878	1,337	41	
	561	822	46	

Growth curve of L. citrovorum strain 2. A growth curve of strain L_2 in milk, with and without yeast extract, was run to determine whether or not the additive increased cell numbers or acid production per cell. The cells were counted by both the MPN and plate count methods (10). The *p*H, titratable acidity, and lactic acid concentration (7) of the samples also were followed throughout the course of the experiment. An increase in acid production was noted when a cell concentration of approximately 10^8 cells per ml was reached. Yeast extract enhanced this acid production but did not affect growth. For example, at zero time the *p*H of the samples was 6.7; at 48 hr, the *p*H of the sample without yeast extract was 4.7, whereas the sample which contained yeast extract was 3.6.

Manometric studies. The stimulatory effect of

yeast extract was quantitatively measured by a Gilson Differential Respirometer on three strains of *L. citrovorum*. Table 3 summarizes triplicate determinations of gas production by these organisms. The data illustrate that the cells in milk alone produced a measurable amount of gas which was enhanced 38 to 47% by the addition of 0.33% yeast extract. It was noted in these studies that the cell concentration had to be at least 10^8 cells per ml 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 revealed no stimulatory effect with the volumetric flask technique, they proved to be potent stimulatory agents when measured by the respirometer. Owing to the different concentrations of the prepared extracts, the data in Tables 4 and 5 can be compared horizontally but not vertically. The percentage of change obtained from five trial runs did not always compare as closely as is indicated in the tables. The stimulatory effect among different experiments ranged from 10 to 150%. Extracts of *L. lactis* and *L. helveticus* affected the two strains equally, whereas the other extracts stimulated gas production of L_2 to a greater degree than that of L_{91404}. From the data, one can conclude that these extracts were as effective as yeast extract in enhancing gas production and may even be more effective under proper conditions.

TABLE 4. *Summary of Gilson Respirometer studies on CO_2 production by Leuconostoc citrovorum strain 2 (0.45 mg of cell/flask, dry weight) in milk and in milk plus cell extracts, after 6.5 hr at 30 C*

Extract	CO_2 in milk	CO_2 in milk plus extract	Increase	Avg
	µliters	*µliters*	%	%
Lactobacillus casei 0.12%	578	934	61	73
	638	1,187	86	
	588	1,011	72	
Streptococcus cremoris and 0.46%	701	1,044	49	64
	655	1,079	58	
	655	1,035	64	
L. lactis 0.52%	512	659	28	29
	578	777	36	
	533	660	24	
S. lactis 0.58%	795	1,082	36	52
	638	1,058	66	
	512	797	55	
L. helveticus and 0.1%	533	653	22	31
	701	916	30	
0.1%	588	826	40	

TABLE 5. *Summary of Gilson Respirometer studies on CO_2 production by Leuconostoc citrovorum strain 91404 (0.62 mg of cell/flask dry weight) in milk and in milk plus cell extracts after 6.5 hr at 30 C*

Extract	CO_2 in milk	CO_2 in milk plus extract	Increase	Avg
	µliters	*µliters*	%	%
Lactobacillus casei 0.12%	332	463	39	35
	558	757	35	
	558	732	31	
Streptococcus cremoris and 0.46%	593	864	45	38
	638	915	43	
	948	1,184	25	
L. lactis 0.52%	638	796	24	26
	410	521	27	
	631	811	28	
S. lactis 0.58%	554	761	37	33
	593	845	42	
	948	1,140	20	
L. helveticus and 0.1%	554	712	28	19
	573	636	10	
	631	747	18	

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 oxalacetic acid and pyruvic acid (15) and was thus used as a control. The cell-free preparation of L_2 did not exhibit any activity when mixed with 10 µmoles of oxalacetate which contained 20 µmoles of $MnCl_2$ at *p*H 7.0 or at *p*H 6.0. The preparation did, however, produce 300 µliters of CO_2 from 250 µmoles of pyruvate, which contained 35 µmoles of thamine-pyrophosphate (TPP) and 10 µmoles of $MnCl_2$ at *p*H 6.0. The addition of 0.33% yeast extract to the pyruvate, TPP, $MnCl_2$ mixture enhanced CO_2 production by 16%.

DISCUSSION

An important consideration in this study of CO_2 production by *L. citrovorum* was that the organisms did not produce appreciable amounts of acid or gas until the cell population reached 10^8 cells/ml. The growth curve data revealed that yeast extract did not stimulate cell division but instead stimulated acid production per cell, particularly lactic acid. This stimulatory effect, however, was not noticeable at a cell concentration of less than 10^8/ml. Manometric studies

indicated that gas production was also negligible below this cell concentration. This critical concentration of cells may be an explanation for the variable nature of gas production by *L. citrovorum*. The volumetric technique for determining gas production was useful in illustrating the general phenomena of yeast extract stimulation, optimal temperature, etc. However, the respirometer studies revealed its limitations. The volumetric technique did not illustrate gas production in milk by *L. citrovorum* alone, nor did it illustrate the stimulatory nature of the various cellular extracts upon gas production. One explanation for the observed lack of gas may be that the cellular extracts enhanced only CO_2 production, whereas yeast extract enhanced both CO_2 production and acid production. Therefore, unless the *p*H dropped low enough to liberate the dissolved CO_2, the gas produced remained soluble in the milk and was not observable.

The manometric studies conducted at 30 C elucidated the nature of gas production by *L. citrovorum*. In 6.5 hr, this organism was capable of producing 500 to 900 μliters of CO_2 in milk, whereas 800 to 1,500 μliters of CO_2 were produced in 0.33% yeast extract milk. The average percentage of stimulation, under these conditions, was close for the three strains tested. The addition of other cell extracts enhanced gas production from 20 to 70%. With the cell extracts, there was a wide range of per cent increase of CO_2 for the various trial runs. The concentration of the extract was not proportional to the amount of CO_2 produced (*see S. cremoris* and *L. helveticus* in Tables 4 and 5). The effect of the extracts on the two strains tested was quite different. For example, *L. casei* extract enhanced gas production of L_2 by 73% and gas production of L_{91404} by 35%. Even extracts of approximately the same concentration had different effects; i.e., *L. lactis* (0.52%) enhanced CO_2 production of L_2 by 29%, whereas *S. lactis* (0.58%) enhanced CO_2 production by 52.0%. These two extracts had approximately the same effect on L_{91404} (i.e., 26% and 33%, respectively). This variable nature of stimulation may reflect the organisms fastidious nature with respect to this property, as evidenced by the existing confusion concerning 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 possible that a decrease in *p*H during a dairy fermentation may cause 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 for a starter culture. The concentration of inoculum and the temperature of the fermentation should also influence this choice.

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 oxalacetate, perhaps because the enzyme was labile and was inactivated during extraction. Under the same experimental conditions, however, *S. diacetilactis* 18-16 extract exhibited oxalacetate decarboxylase activity. The extract of *L. citrovorum* did exhibit activity when given pyruvate and necessary co-factors 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%. Since the yeast extract increased the acidity of a milk culture greatly, the decarboxylation reaction in the 2–3 cleavage pathway (formation of pentose phosphate and CO_2 from phosphogluconate) may also have been enhanced. This would increase the formation of lactic acid as well as CO_2.

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