EFFECT OF CARBON SOURCE ON RATE OF ACID PRODUCTION BY STREPTOCOCCI[1]

Aurelio Revilla R[2]

The lactic acid fermentation by homofermentative streptococci has received the attention of various workers (3, 4, 5) because of its importance in milk and dairy products, and also because of its preservative action in other food products.

The present study was done in an attempt to determine the effects of lactose, glucose and fructose at different concentrations on the rate of acid production.

The Warburg apparatus (9) was used in this work to detect the amount of carbon dioxide liberated by the reaction of calcium carbonate with the acid produced by either *Streptococcus lactis* or *Streptococcus cremoris*.

Hucker (3) found that low acid producing lactic acid streptococci generally ferment levulose at a faster rate than glucose. According to Rahn, et al. (5), the rate of acid production is independent of the glucose concentration when it is present to the extent of 0.2 percent of the culture medium and if the pH does not decrease. Smith and Sherman (7, 8), inoculated washed cells of *S. lactis* or *S. cremoris* into 0.5 percent glucose and a phosphate buffer medium. The results indicated that the 96.6 percent of glucose was converted to lactic acid by *S. lactis* and 93.7 percent by *S. cremoris*. Mizuno and Jezeski (4) showed an increase in acid production by *S. cremoris* and Leuconostoc species by adding 2 percent glucose to a milk medium.

MATERIALS AND METHODS

Manometric Techniques: The volume from the Warburg reaction flask to the reference point (150 mm.) on the manometer was found with the aid of mercury, which was used as a calibrating fluid, following Umbriet's, et al. (9), instructions.

Preparation of Reagents: Lactose, glucose and fructose levels were: 0.5, 1.0, 2.0, 3.0, and 4.0 percent. The molar concentrations were calculated for each level of the three sugars. (See Table I).

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2. Associate Professor of Dairy Science, Escuela Agrícola Panamericana. (The writer expresses his appreciation to Dr. K. L. Smith for his guidance, suggestions and counsel during the present investigation).

TABLE I

SUGAR CONCENTRATIONS USED FOR ACID PRODUCTION BY *S. CREMORIS* AND *S. LACTIS*

Percent	Moles of Glucose	Moles of Fructose	Moles of Lactose
0.5	2.8×10^{-2}	2.8×10^{-2}	1.4×10^{-2}
1.0	5.6×10^{-2}	5.6×10^{-2}	2.8×10^{-2}
2.0	11.1×10^{-2}	11.1×10^{-2}	5.6×10^{-2}
3.0	16.6×10^{-2}	16.6×10^{-2}	8.3×10^{-2}
4.0	22.2×10^{-2}	22.2×10^{-2}	11.1×10^{-2}

The quantity of calcium carbonate used was 0.0444 moles per liter. This was estimated to be twice the amount needed to neutralize the lactic acid produced.

A solution of calcium chloride was prepared such that the final concentration of salt in the reaction flask was 0.0444 moles per liter.

Preparations of cells: strains of *S. lactis* and *S. cremoris* (See Table II) after being identified as such, (2) were carried in litmus milk. The cultures were transferred daily. A loopful of a 12-hour litmus milk culture of the organism to be used in trials the following day was transferred into a tube containing 5 ml of carbohydrate broth. The carbohydrate broth contained 1.0 percent proteose-peptone, 0.1 percent beef extract, 0.5 percent sodium chloride and 1.0 percent of the test sugar. This medium was sterilized at 121°C for fifteen minutes in a pre-heated sterilizer.

After 12 hours of incubation at 32°C the whole broth culture was transferred into 200 ml of the test sugar medium previously described and after inoculation it was incubated at 32°C for 12 hours.

The cells were harvested in sterile centrifuge tubes by centrifugation at 2,000 rpm for 15 minutes. After removing the supernatant broth, the sediment was resuspended in sterile 0.85 percent sodium chloride solution and the saline suspension of cells was centrifuged at 2,000 rpm for 15 minutes. The washing procedure was repeated.

A stock cell suspension was prepared by resuspending the washed cells in 10 ml of the saline solution. Dilutions for the standard plate count were determined by checking the optical density of the stock cell suspension in a Bausch and Lomb Spectronic 20 at 640 millimicrons.

The dilutions for the stock cell suspension were 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. All dilutions were made using 99 ml of sterile, phosphate-buffered distilled water (1).

The organisms were plated in Difco Bacto plate count agar, incubated at 32°C for 48 hours and then counted, using a Quebec colony counter. Smears were made from 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions and stained with a drop

TABLE II

IDENTIFICATION OF LACTIC CULTURES

Culture Number	Action in Litmus Milk	Gas from Glucose	Gas from Fructose	Gas from Lactose	Ammonia from Arginine	Growth in 4% Na Cl	Growth in 40°C	Growth in 0.3% M. B.	Acid from Maltose	Organism
LC-1	+	−	−	−	+	+	+	+	+	S. lactis
LC-2	+	−	−	−	+	+	+	+	+	S. lactis
LC-3	+	−	−	−	+	+	+	+	+	S. lactis**
LC-4	+	−	−	−	+	+	+	+	+	S. lactis
LC-5	+	−	−	−	∓	+	±	−	+	S. cremoris*
LC-6	+	−	−	−	∓	+	∓	−	+	S. cremoris*
8-P	+	−	−	−	+	+	+	+	+	S. lactis
Fl-1	+	−	−	−	−	−	−	∓	+	S. cremoris
Fl-2	+	−	−	−	−	−	−	∓	+	S. cremoris**
Fl-3	+	−	−	−	+	+	−	+	+	S. lactis
Fl-4	+	−	−	−	+	+	−	+	+	S. lactis

* Were not positively identified as **S. cremoris**

** Were used in all fermentation studies.

of diluted crystal violet stain. The number of cells per chain were counted to calculate the frequency of cells per chain. The number of cells in the flask inoculum was estimated by multiplying the average frequency of cells per chain by the number of colonies on the plates.

Lactic Acid Assay: The Warburg flasks and manometers were numbered at random for each trial. Each concentration of sugar was tested in duplicate with each organism. Into each reaction flask, 11 mg. of calcium carbonate were weighed, then 1.5 ml of sterile distilled water containing 16 mg. of calcium chloride were added. To this mixture, 0.5 ml of stock cell suspension and 1.5 ml of the appropriate sugar solution were added, making a total volume of 2.5 ml in each flask. The sugar solutions were prepared so that the final concentration in the Warburg flask would be 0.5, 1.0, 2.0, 3.0 or 4.0 percent carbohydrate.

Four flasks were used as thermobarometers. Each of the control flasks contained 2.5 ml total volume and they were prepared exactly like the test flasks except that one contained no carbohydrate, another no calcium carbonate, a third contained no cells and a fourth contained only distilled water.

The manometers with the flasks attached and stopcock opened were placed on the Warburg apparatus and allowed to equilibrate at 32°C until no further pressure change was noted. The first reading recorded as zero time was made prior to mixing the sugar with the flask fluid, and subsequent readings were made at 15 minute intervals. At the end of each trial the pH of the contents of each flask was determined. The carbon dioxide release rate was calculated using the data obtained during the first 45 minutes of each trial.

Dilutions of the flask contents were plated and incubated at 32°C for 48 hours. Smears of the contents of each flask were made and the number of cells calculated using the procedure described for the stock cell suspension.

RESULTS

Manometric Techniques: The calculated volume of each reaction flask and manometer to the 150 mm mark on the manometer is shown in table III. The volume of fluid in each matched set was held constant at 2.5 ml. After each experiment the pH of the reaction fluid in each flask was used to determine the flask constant.

TABLE III

CALIBRATION OF WARBURG FLASKS AND MANOMETERS

Manometer Number	Volume From Flask to 150 mm. in ml.	95% Confidence Limit	Percent of Error at 95% Confidence Limit
136	18.47743	0.01329	0.072
165	18.39863	0.01216	0.066
141	18.03490	0.00649	0.036
97	18.59973	0.01686	0.085
36	18.78016	0.01282	0.058
149	18.49163	0.00477	0.026
192	22.13600	0.00537	0.024
137	22.64823	0.00973	0.045
171	21.71180	0.00645	0.0297
182	23.05013	0.01257	0.055
8	23.04306	0.01545	0.067
37	22.04626	0.00603	0.027
23	22.73953	0.01425	0.063
39	22.19870	0.01179	0.053
5	22.44920	0.00318	0.014
139	21.31793	0.00956	0.045
40	22.93276	0.00371	0.016
99	23.07936	0.00930	0.040
155	22.59030	0.00795	0.035

Effect of the Different Sugars on the Rate of Acid Production: The carbon dioxide production from the reaction of the acid(s) produced by *S. cremoris*, with calcium carbonate (see table IV) indicated that fructose was utilized at a higher rate than glucose or lactose. The larger amount of carbon dioxide measured per cell per minute from fructose was 7.65 times higher than that of glucose and 10.5 times higher than that of lactose.

S. lactis fermented glucose at the highest rate. The maximum amount of carbon dioxide liberated per cell per minute from glucose was 4.05 times more than that from fructose and 6.15 times more than that from lactose.

TABLE IV

RATE OF CARBON DIOXIDE PRODUCTION
PER CELL PER MINUTE

	% Sugar	Microliters	
		S. cremoris	S. lactis
Glucose	.5	1.33 x 10^{-9}	17.7 x 10^{-9}
	1.0	1.42 x 10^{-9}	18.46 x 10^{-9}
	2.0	1.43 x 10^{-9}	18.58 x 10^{-9}
	3.0	1.37 x 10^{-9}	17.97 x 10^{-9}
	4.0	1.33 x 10^{-9}	17.72 x 10^{-9}
Fructose	0.5	10.95 x 10^{-9}	4.58 x 10^{-9}
	1.0	10.33 x 10^{-9}	3.89 x 10^{-9}
	2.0	6.7 x 10^{-9}	3.53 x 10^{-9}
	3.0	6.6 x 10^{-9}	3.80 x 10^{-9}
	4.0	7.3 x 10^{-9}	3.37 x 10^{-9}
Lactose	0.5	0.97 x 10^{-9}	2.94 x 10^{-9}
	1.0	0.99 x 10^{-9}	3.01 x 10^{-9}
	2.0	1.02 x 10^{-9}	3.02 x 10^{-9}
	3.0	1.04 x 10^{-9}	2.72 x 10^{-9}
	4.0	0.98 x 10^{-9}	2.83 x 10^{-9}

Effect of the Different Concentrations of Sugars on the Rate of Acid Production: Table IV also shows the general results expressed in microliters per cell per minute. *S. cremoris* showed a slight increase of its fermentation rate when the concentration of glucose was increased from 0.5 percent to 2.0 percent. At concentrations of 3.0 and 4.0 percent, the fermentation rate decreased gradually. The fructose fermentation rate was highest at the 0.5 percent concentration of sugar. It decreased slightly at the 1.0 percent level and dropped rapidly at the 2.0 percent level. The changes were very small at the 3.0 and 4.0 percent of fructose concentrations. From the 0.5 percent to the 3.0 percent concentration of lactose, *S. cremoris* showed a slight increase of its fermentation rate. At the 4.0 percent level the fermentation rate decreased.

The rate of fermentation by S. lactis and S. cremoris increased at the 0.5, 1.0 and 2.0 percent levels of glucose, but at the 3.0 and 4.0 percent levels, the rate of fermentation decreased.

When testing the 0.5, 1.0 and 2.0 percent levels of lactose, there was a gradual increase by both organisms, followed by a decrease at the 3.0 percent level and an increase at the 4.0 percent level.

Interactions Between Organisms, Sugars, and Levels of sugar: S. cremoris and *S. lactis* were similarly affected by 0.5, 1.0 and 2.0 percent of glucose. However, the rate of acid production by *S. lactis* was much higher than that

of *S. cremoris*. The highest amount of carbon dioxide per minute per cell of *S. lactis* was 18.58 x 10^{-9} microliter in 2.0 percent of glucose. *S. cremoris*, under the same conditions, liberated 1.43 x 10^{-9} microliters. In fructose the highest fermentation rates of both organisms occurred in the lowest sugar concentration.

In both lactose and glucose the highest fermentation rates occurred at the middle (1.0, 2.0, or 3.0 percent) carbohydrate concentration. There was also interaction between the organism and the carbohydrate: *S. cremoris* exhibited its highest rate of fermentation in fructose but *S. lactis* exhibited its highest rate in glucose.

DISCUSSION

Manometric Techniques: The percent of error at 95 percent confidence limit was no greater than 0.085 (see Table III). An accuracy of 1.0 percent is considered satisfactory in manometry, according to Umbreit, et al. (9).

Effect of Different Carbohydrates on the Rate of Acid Production: No information was found relating to the metabolic products resulting under the conditions of this investigation. It was assumed that lactic acid constituted the main end-product; however, the author preferred to express the rate of fermentation by means of carbon dioxide per cell per minute.

The fermentation rate exhibited by *S. cremoris* was higher with fructose than with glucose. This agreed with the general statement made by Hucker(3). Lactose appeared to be utilized at a lower rate than was glucose, but the difference was small. The difference between the fermentation rate of fructose and that of glucose and lactose was considerable. This is shown in Figure I. During the propagation of cells in fructose broth, *S. cremoris* exhibited a lower optical density at 640 millimicrons, than when grown in glucose or lactose possibly, this was due to its rate of fermentation which in a short time made possible the accumulation of enough acid to force a decline in its growth.

The preference for sugar by *S. cremoris* was not the same as that of *S. lactis* (See Table IV). The rate of fermentation of glucose by *S. lactis* was the fastest, and fructose was fermented more rapidly than lactose. The difference between the glucose fermentation rate and that of fructose or lactose was very large (See figure 2). The rate of fermentation of these organisms appeared to be related to the enzymes synthesized during the propagation of the cells because the number of cells did not increase during the fermentation period. The higher rate of fermentation of glucose compared with the rate of fermentation of fructose and lactose disagreed with the finding of Shahani and Vakil (6) who concluded that glucose, galactose, and lactose were metabolized at almost the same rate. They also suggested that *S. lactis* utilized the carbohydrates partly through the Embden-Meyerhof pathway and partly through the hexose mono-phosphate pathway. This might explain the difference in the fermentation rates of glucose, fructose and lactose.

Effect of Different Levels of Each Carbohydrate on the Rate of Acid Production: The gradual increase of the rate of fermentation of *S. cremoris* and *S. lactis* at 0.5, 1.0 and 2.0 percent levels of glucose was followed by a decline at the 3.0 and 4.0 percent level. (See figures 1 and 2). This disagreed with the report of Rahn et al. (5). However, the difference between the fermentation rate in the 0.5, 1.0, and 2.0 percent levels was very small. When fructose was used, the fermentation rate of *S. cremoris* reached its maximum at 0.5 percent level, then a slight decrease was registered at the 1.0 percent level of this sugar. When the concentration of fructose was 2.0 and 3.0 percent the rate of fermentation dropped sharply. It appeared that the increasing levels of fructose were slightly inhibitory to the cells.

The difference in the rate of fermentation between *S. cremoris* and *S. lactis* at 0.5, 1.0,, 2.0, 3.0 and 4.0 percent levels of lactose was negligible. Nevertheless, the 3.0 percent level exhibited the larger fermentation rate for *S. cremoris* while at the 2.0 percent level *S. lactis* showed the larger fermentation rate.

Interactions between Organisms, Sugar and Levels of Sugars: The analysis of variance indicated that there was significant ($P < .05$) interaction between organisms, carbohydrates and levels of carbohydrates. (See Table V).

The figures 1 and 2 show that the changes in the rate of carbon dioxide liberation were different between the two organisms when metabolizing the different carbohydrates at various levels studied. The significant interaction indicated that the main effects were not independent and therefore would not justify testing of the main effects for significance.

TABLE V

ANALYSIS OF VARIANCE

Source	Df	SS	MS	F. Ratio
Organisms	1	329.5144	329.5	
Sugar	2	605.8424	302.9	
Levels of sugar	4	8.1718	2.0	
Organisms x sugar	2	1188.8709	549.4	
Organisms x levels	4	4.0514	1.0128	
Sugar x Levels	8	17.1285	2.141	
Organisms x sugars x levels	8	8.679	1.085	2.7608*
Error	30	11.7918	.393	
T o t a l	59	2174.502		

* Significant at five percent level.

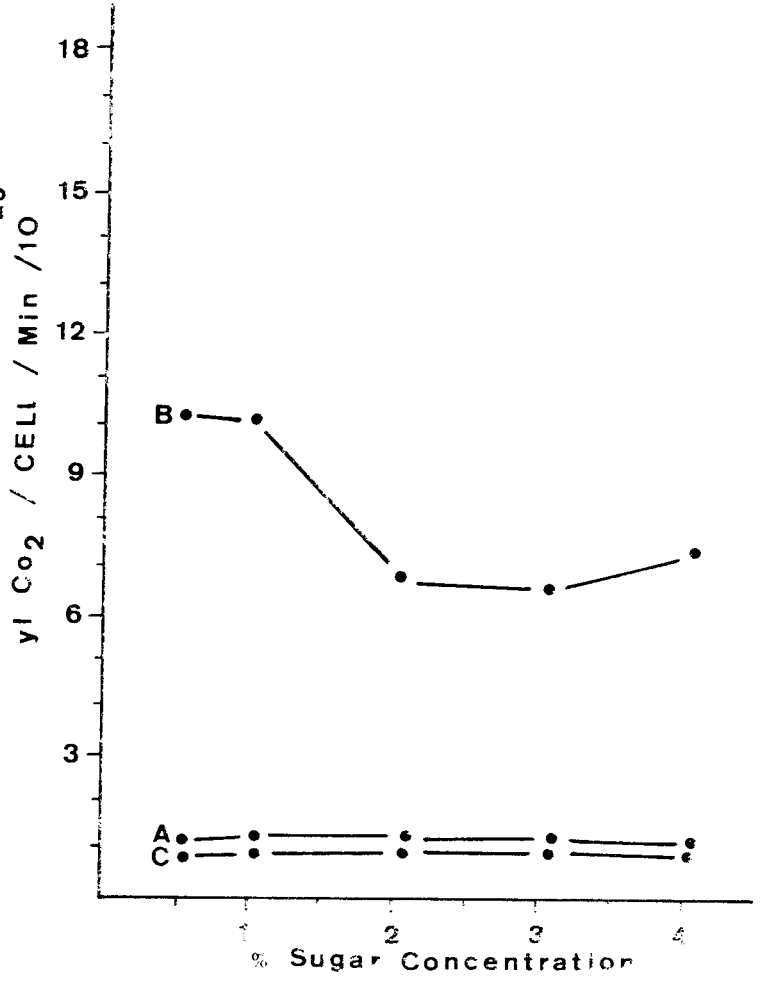

Figure 1.—Rate of carbon dioxide liberation of [illegible] cremoris. A. Glucose. B. Fructose. C. Lactose.

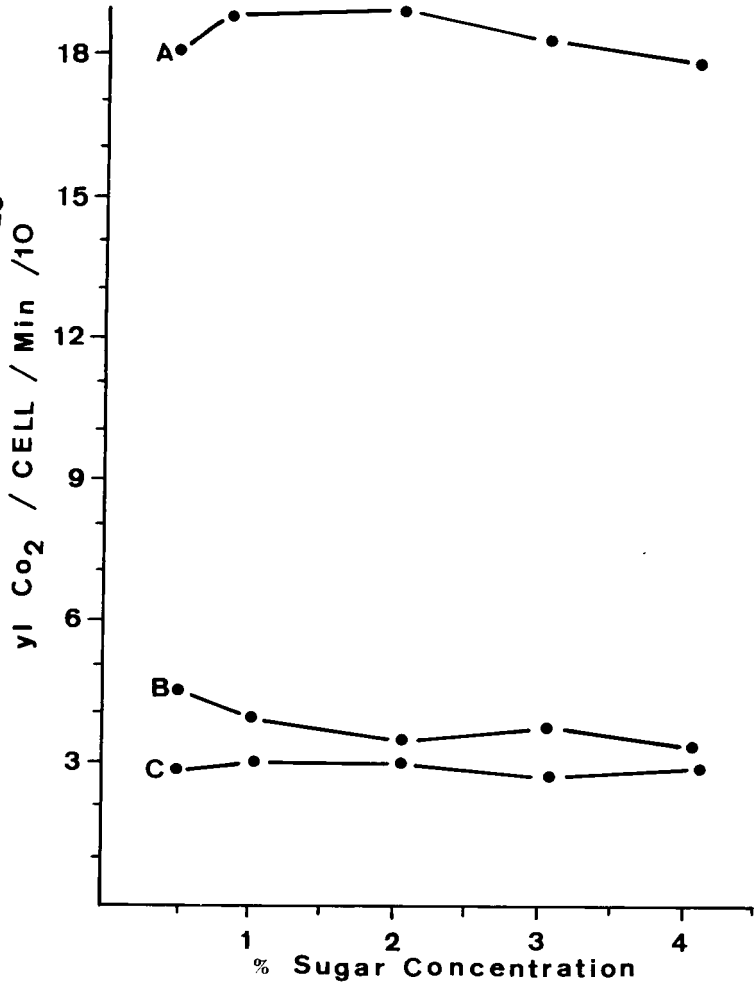

Figure 2.—Rate of carbon dioxide liberation by **S. lactis**
A. Glucose B. Fructose C. Lactose.

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