

PHYSIOLOGICAL CHARACTERISTICS OF LACTIC ACID BACTERIA NEAR THE MAXIMUM GROWTH TEMPERATURE

I. GROWTH AND ACID PRODUCTION^{1, 2}

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Little information is available on the growth and activity of bacteria at temperatures above the optimum, despite the fact that in many commercial processes the organisms used are often subject to temperatures higher than those most favorable for growth. In the manufacture of Swiss cheese, for example, starter cultures must not only survive the high temperatures used during cooking of the curd, but also must grow at temperatures close to their maximum for several hours. Preliminary investigations have indicated that rates of enzyme production and destruction and of enzyme activity were affected differently by growth at optimum temperatures and at those near the maximum for the organism.

It was believed that investigations of the physiological activities of bacteria at near their maximum growth temperatures would add significant information to that regarding these activities at optimum temperatures.

Barber (1908), using single cell isolations, studied the rates of multiplication of *Escherichia coli* at different temperatures. From the minimum temperature of about 10°C., the rate of multiplication increased rapidly as the temperature was raised to 37.5°C.,

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where a minimum generation time of less than 17 minutes was reached. From 37.5 to 45°C. the rate remained relatively constant, then fell rapidly, until at 50°C. no growth was evident. Lane-Clayton (1909), working with *Escherichia coli*, *Salmonella enteritidis*, and *Eberthella typhosa*, studied their growth from 20 to 50°C. The generation time decreased as the temperature rose to 42°C., and then increased from 42 to 50°C. Above 50°C. a diminution in numbers of organisms instead of an increase was noted.

Slator (1906) studied the ratio between constants of rate of growth at two temperatures and recorded these quotients for every 10°C. rise in temperature (Q_{10}). With yeasts the values for Q_{10} ranged from 5.6 at 10°C. to 1.3 at 40°C. Similar results were reported by Sherwood and Fulmer (1926) working with yeasts, and by Woodruff and Baitsell (1911) with *Paramecium aurelia*.

The acclimatization of an organism to temperatures far above those to which it is normally accustomed, was reported by Dallinger (1887), who stated that in seven years he was able to acclimatize to 70°C. a flagellate which initially was unable to survive at 23°C. Casman and Rettger (1933), however, had little success in raising the maximum growth temperature of members of the *Bacillus subtilis* group by the gradual increase of the incubation temperature over a period of a year.

The effect of temperature on the length of the latent and lag phases has been observed by numerous investigators. Penfold (1914), noted that the lag phase of *Escherichia coli* was shortened as the incubation temperature was raised, Müller (1903) reported that *Pseudomonas fluorescens* exhibited at 30°C. a six-hour lag phase before entering the logarithmic phase, while at 12°C. the lag phase was 12 to 24 hours, at 6°C. 32 hours and at 0°C. 124 hours. Barber (1908), Lane-Clayton (1909), and others confirmed these observations.

Burkey and Rogosa (1940) grew cultures of *Streptococcus thermophilus* and of *Lactobacillus bulgaricus* for several months under "temperature shocking" conditions, or at maximal growth temperatures, or in a medium of unheated milk filtrate. They claimed

that each treatment enabled the cultures to survive high temperatures, and to grow at temperatures several degrees above the maximum for the parent laboratory stock cultures.

Goodner and May (1927), studying the production of gas by strains of *Salmonella pullorum*, observed gas production at 27, 30, 34.5, and 37.5°C. At 40°C. no gas was produced, although there was no decrease in growth. Dorn and Rahn (1939) found that the rate of fermentation of *Streptococcus lactis* and *Streptococcus thermophilus* increased greatly at temperatures above their optimum. On the other hand, efficiency of acid production was greatest at temperatures slightly below the optimum.

GROWTH AND ACTIVITY OF LACTOBACILLUS BULGARICUS NEAR THE MAXIMUM GROWTH TEMPERATURE

Before specific physiological mechanisms were studied, a comparative study of some of the factors influencing the growth of *Lactobacillus bulgaricus* at optimum temperatures and at temperatures near the maximum was made. The influence of age of culture and size of inoculum on growth at 37 and at 49.5°C. is herein reported.

Experimental methods

Following preliminary experiments, 37°C. was chosen as the optimum since maximum growth and acid production, with no apparent degeneration of the culture during continued transfer, were obtained at this temperature. For the temperature near the maximum for growth, 49.5°C. was chosen, since this was a degree or two below the maximum, and the results obtained at this temperature could be readily duplicated.

Since preliminary observations indicated that plate counts and direct microscopic counts could not be applied accurately to the problems under consideration, an Evelyn photoelectric colorimeter with a 7,200 Å filter was employed for all determinations of growth. All tubes used gave identical readings with sterile medium. Values in turbidity were recorded by a galvanometer on a scale reading from zero to 100, and were reported as logarithms, so that when plotted against time, a typical growth curve

was obtained. However, since an increase in turbidity was reflected by a lower galvanometer reading, the logarithm of the readings was subtracted from the logarithm of 100 to obtain values that increased as the turbidity of the culture increased. The expression adopted for recording growth was $\log 100 - \log$ galvanometer reading or $2 - \log G$.

The following medium supported abundant growth, and was clear, as was necessary for use in the photometer:

Peptonized milk (Difco).....	10 gms.
Neopeptone (Difco).....	1 gm.
Glucose.....	5 gms.
Monopotassium phosphate (KH_2PO_4).....	6 gms.
Sodium acetate.....	6 gms.
Carrot extract*.....	100 ml.
Liver extract*.....	100 ml.
Distilled water.....	800 ml.

Adjust pH to 6.6 for lactobacilli and 6.9 for streptococci.

* One lb. of finely ground carrots or liver extracted for 1 hr. at 80°C. in a liter of water.

The cultures were carried in tubes of the medium, and unless otherwise indicated, one per cent of inoculum was used. Stock cultures were incubated at 37°C. for eight hours and then kept at 10°C. and transferred weekly. Mother cultures also were transferred with a one-per-cent inoculum and incubated at 37°C. for eight hours, were then placed at 21.5°C. for four hours, after which they were again transferred; and then a similar inoculation was made into 100 ml. of medium in a six-ounce bottle. This culture, known as an inoculating culture, was incubated for eight hours at 37°C.

Liter Erlenmeyer flasks, each containing 500 ml. of medium, were placed in a water bath at the desired temperature for 30 minutes and then inoculated. Incubation of all flasks was carried out in thermostatically controlled water baths. Samples were removed and determinations for pH, titratable acidity, and dry weights were made at specified intervals. Rates of lactic acid production per milligram of dry weight per half hour were calculated by the Buchanan formula as modified by Bayne-Jones and Rhees (1929) and Walker and Winslow (1932). The form

in which the equation was used in these experiments is given below:

$$M = \frac{S2.303 \log \frac{h}{B}}{t(b - B)}$$

M = amount of product per milligram dry weight per unit time

S = increment of product in time t

t = duration of production in hours

b = milligram of dry weight at end of time t

B = milligrams of dry weight at start of time t

2.303 = modulus of common logarithms

Results

Growth and acid production by Lactobacillus bulgaricus at 37°C. and at 49.5°C. The growth and acid production by *Lactobacillus bulgaricus* at 37 and at 49.5°C. are shown in figure 1. As was expected the culture entered the logarithmic phase of growth earlier, had a greater rate of increase during this period, and left the phase sooner at 49.5 than at 37°C. Likewise a greater maximum population in the culture grown at 37°C. was to be anticipated. The rates of production of acid at the two temperatures, however, are worthy of special attention. In figure 1 the curve representing acid production at 37°C. is similar to that of growth. This is indicative of a close relationship between the rates of growth and acid production. This relationship was not apparent at 49.5°C., for at this temperature about as much acid was produced after reproduction had ceased as during the period of growth.

The data in table 1 show a very rapid rate of metabolism during the early hours of growth at 49.5°C. Up to the fourth hour the rate of increase in dry weight and in lactic acid, and of decrease in pH were greater at the higher temperature than at the lower. After this time there was a decided decrease in activity at the higher temperature and an increase at the lower temperature. Of equal significance are the figures for the production of acid

per milligram of dry weight per half hour. During the early hours of growth the rate of production of acid per milligram of dry weight was considerably higher at 49.5 than at 37°C., especially during the first and second hours, after which the rate of acid production decreased rapidly at 49.5°C. until after four

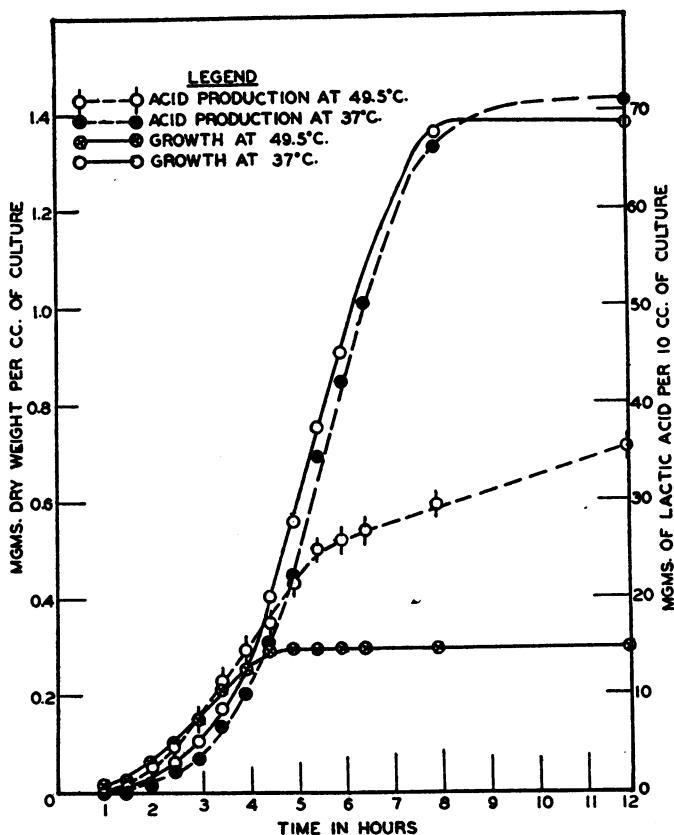


FIG. 1. GROWTH AND ACID PRODUCTION BY *LACTOBACILLUS BULGARICUS* IN CARROT-LIVER EXTRACT MEDIUM AT 37°C. AND AT 49.5°C.

and one-half hours when growth had practically ceased. At 37°C. the production of acid per milligram of dry weight per half hour proceeded at a gradually decreasing rate from 60.8 milligrams at two and one-half hours to 5.63 mgm. at 12 hours.

It should be noted that a one-per-cent inoculum from an eight-

hour-old culture grown at 37°C. was used to inoculate the medium in the flasks. Preliminary work had indicated that an increase or decrease in the size of inoculum might change materially the results at the two temperatures.

The influence of size of inoculum on the growth of Lactobacillus bulgaricus at 37 and at 49.5°C. The growth curves in figures 2

TABLE 1

Growth and acid production by Lactobacillus bulgaricus in carrot-liver extract medium at 37°C. and at 49.5°C.

TIME	ORGANISMS GROWN AT 37°C.				ORGANISMS GROWN AT 49.5°C.			
	Dry wgt. per ml. of culture	Lactic acid per 10 ml. of culture	Lactic acid per mgm. dry wgt. per unit time	pH	Dry wgt. per ml. of culture	Lactic acid per 10 ml. of culture	Lactic acid per mgm. dry wgt. per unit time	pH
hours	mgm.	mgm.	mgm.		mgm.	mgm.	mgm.	
0	0.0000	0.00		6.47	0.0000	0.00		6.45
1	0.0000	0.00		6.44	0.0135	0.00		6.44
1½	0.0130	0.00		6.39	0.0270	0.90	92.10	6.36
2	0.0280	0.90	46.1	6.36	0.0665	2.70	82.30	6.28
2½	0.0625	2.25	60.8	6.31	0.1030	4.95	54.40	6.24
3	0.1070	3.60	37.6	6.26	0.1520	7.65	43.30	6.02
3½	0.1730	6.95	42.4	6.11	0.2190	11.70	44.20	5.90
4	0.2580	10.20	30.6	5.95	0.2540	14.85	26.75	5.76
4½	0.4050	15.60	31.0	5.70	0.2960	17.55	19.69	5.61
5	0.5590	22.50	27.6	5.36	0.3020	21.60		5.42
5½	0.7520	34.65	? (37.4)	5.15	0.3020	25.20		5.36
6	0.9030	42.30	18.55	4.85	0.3020	26.10		5.35
6½	1.0500	50.40	16.60	4.77	0.3020	27.00		5.31
8	1.3600	66.60	9.89	4.64	0.3020	29.70		5.22
12	1.3840	71.10		4.42	0.3020	35.55		5.10
24	1.3840	71.55			0.3090	36.90		

and 3 show that an increase in the size of inoculum within the limits used in the experiment produced an increase in the maximum population of cultures grown at 49.5°C. while at 37°C. differences as a result of variations in the size of inoculum were apparent only during the early stages of growth. An increase in the number of organisms in the inoculum tended to shorten the lag period, but eventually the maximum amount of growth was approximately the same in all of the cultures at 37°C. Increasing

the size of the inoculum also shortened the lag phase at 49.5 as at 37°C. However, as growth progressed at the higher temperature, the increase in turbidity was markedly more rapid where the larger inocula were employed, and the maximum growth obtained apparently depended on the number of cells originally added. In all of the cultures, growth ceased at approximately the same time, regardless of the size of inoculum used, except

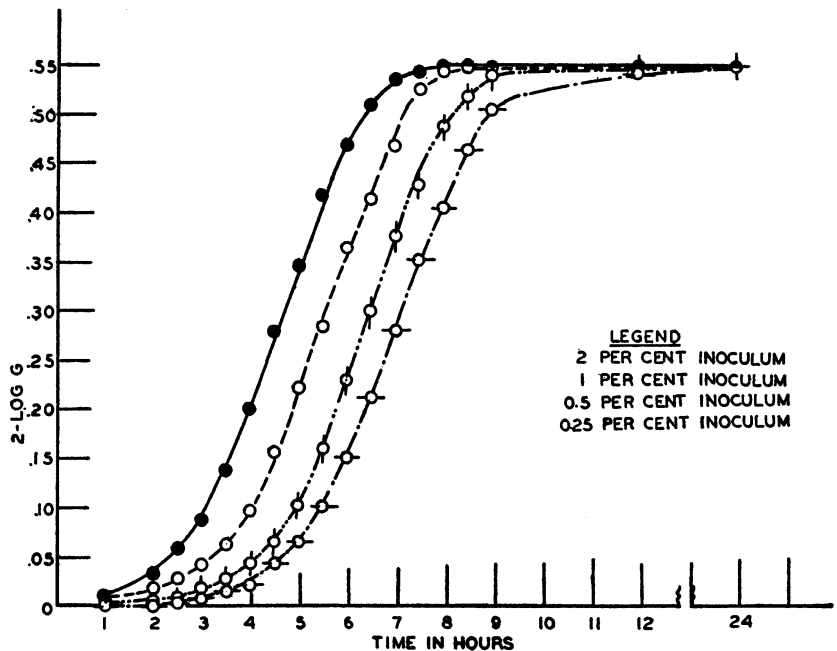


FIG. 2. THE INFLUENCE OF SIZE OF INOCULUM ON THE GROWTH OF *LACTOBACILLUS BULGARICUS* IN CARROT LIVER EXTRACT MEDIUM AT 37°C.

in the flask containing the 0.25 per cent inoculum where little growth was observed during 24 hours of incubation at 49.5°C. Then the cultures were incubated farther at 37°C. and amounts of growth were determined after 8, 12, and 24 hours. In all cultures a large increase in turbidity was observed, and the maximum growth obtained with the 0.25 per cent and the 0.5 per cent inocula closely approximated the normal at 37°C. Less growth was observed in the flasks with one and two-per-cent inocula.

It was apparent that the factors responsible for the cessation of growth at 49.5°C. were effective only at the high temperature, for when the organisms were placed at optimal temperatures, growth was readily initiated.

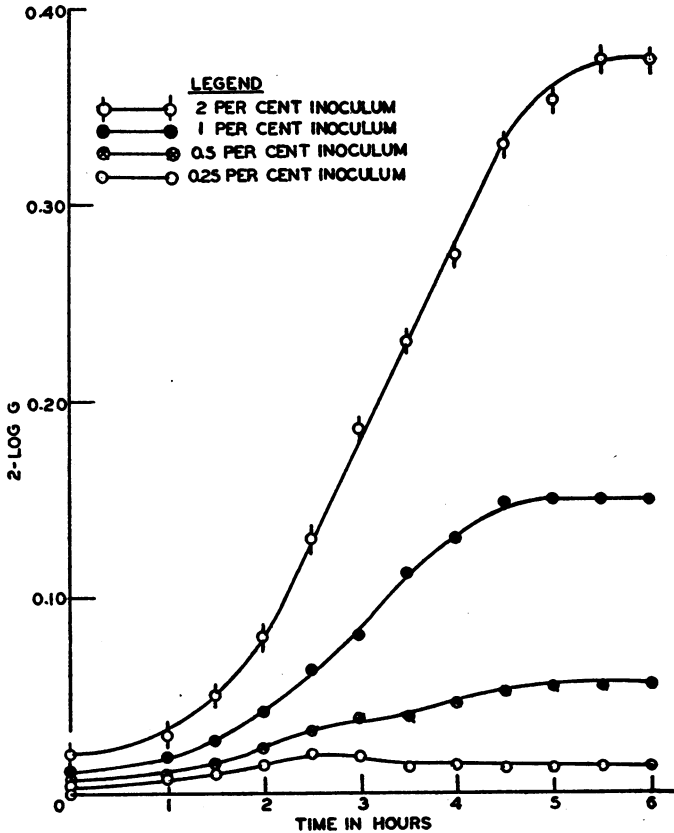


FIG. 3. THE INFLUENCE OF SIZE OF INOCULUM ON THE GROWTH OF *LACTOBACILLUS BULGARICUS* IN CARROT-LIVER EXTRACT MEDIUM AT 49.5°C.

Since differences in the size of inoculum were found to affect the growth at 49.5°C., it was expected that differences in the age of the inoculating cultures also might have an effect on growth.

Influence of age of inoculating culture on growth and acid production by Lactobacillus bulgaricus at 37 and at 49.5°C. A modifica-

tion of the technique previously described was required for the preparation of the cultures to be used in these experiments. The organisms were grown in 50 ml. centrifuge cups containing 30

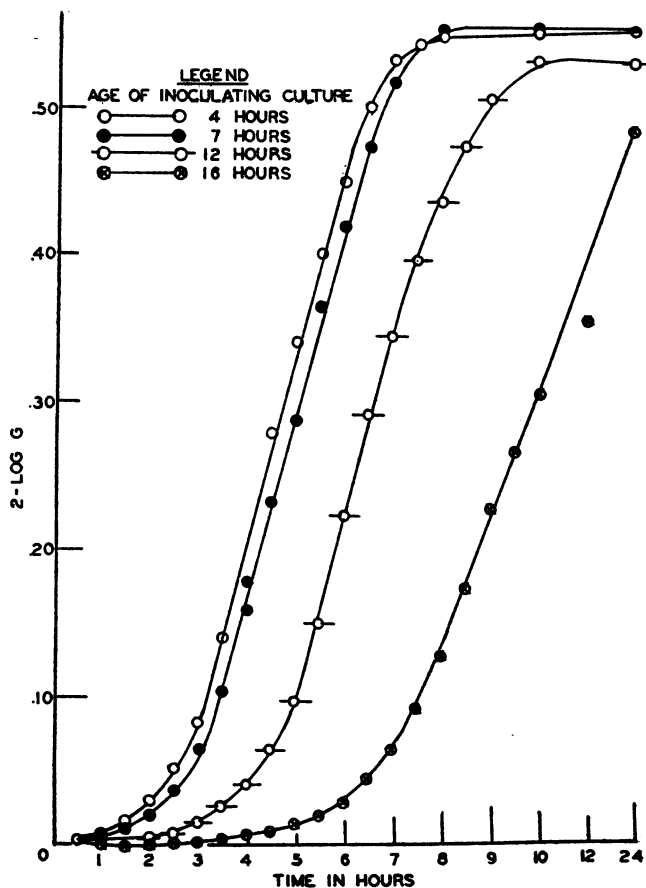


FIG. 4. THE INFLUENCE OF AGE OF INOCULATING CULTURE ON THE GROWTH OF *LACTOBACILLUS BULGARICUS* IN CARROT-LIVER EXTRACT MEDIUM AT 37°C.

ml. of carrot-liver extract medium. Cultures, inoculated by the usual method, were grown for 4, 7, 12, and 16 hours, respectively, at 37°C., and immediately after incubation were centrifuged for 10 minutes. The organisms were resuspended in the sterile medium and the turbidities brought to the same reading in the

Evelyn photometer. Flasks of medium were then inoculated with the standardized cultures and growth and acid production observed as in the previous experiments.

The results on growth at 37°C. are summarized in figure 4. Little difference was noted between the growth curve obtained with the four-hour-old culture and that with the seven-hour-old culture. With a four-hour inoculum there was a slightly shorter lag phase than with a seven-hour inoculum, but the maximum

TABLE 2

The influence of age of inoculating culture on the production of lactic acid by Lactobacillus bulgaricus in carrot-liver extract medium at 37°C.

Time	TITRATABLE ACIDITY AS LACTIC ACID PER 10 ML. OF CULTURE				pH			
	4 hour culture as inoculum	7 hour culture as inoculum	12 hour culture as inoculum	16 hour culture as inoculum	4 hour culture as inoculum	7 hour culture as inoculum	12 hour culture as inoculum	16 hour culture as inoculum
hours	mgm.	mgm.	mgm.	mgm.				
0	0.00	0.00	0.00	0.00	6.49	6.49	6.50	6.48
1	1.35	0.90	0.45	0.45	6.45	6.47	6.49	6.47
2	3.15	1.80	0.90	0.90	6.35	6.40	6.46	6.46
3	9.00	6.30	1.80	1.35	6.11	6.26	6.38	6.45
4	18.90	15.30	4.50	2.25	5.66	5.80	6.31	6.43
5	38.70	31.95	10.35	2.70	4.98	5.14	6.04	6.39
6	56.25	53.10	22.95	4.05	4.63	4.70	5.41	6.33
7	68.85	65.70	39.60	5.85	4.42	4.48	4.94	6.17
8	73.35	73.35	57.15	13.95	4.42	4.42	4.65	5.87
9	73.35	73.35	67.95	22.95	4.42	4.42	4.54	5.44
10	73.35	73.35	71.55	35.10	4.42	4.42	4.42	5.09
11	73.35	73.35	73.35	44.10	4.42	4.42	4.42	4.87
24	73.35	73.80	73.35	73.35	4.42	4.42	4.42	4.42

amount of growth was the same for both cultures. A 12-hour inoculum caused a marked lengthening of the lag phase and a slight decrease in maximum amount of growth as compared with growth of the younger inocula. A 16-hour culture gave a longer lag phase than did any of the other three cultures.

The decreases in pH and the increases in acidity, shown in table 2, were relatively greater during the early hours of growth than in the later hours. With all cultures, however, the final acidity was the same in spite of the fact that less growth was ob-

tained in the medium inoculated with the 12- and 16-hour cultures.

The lengthening of the lag phase, so evident following inoculation with the two older cultures, might be attributed to a smaller number of viable cells in the inocula. The observation that these cultures showed less growth but as much acid as the younger cultures indicated that acid production was an important factor in determining maximum population.

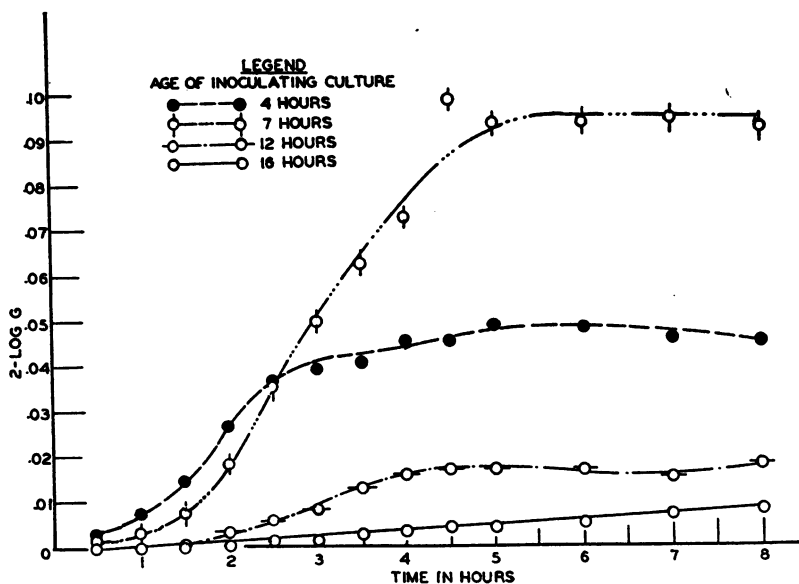


FIG. 5. THE INFLUENCE OF AGE OF INOCULATING CULTURE ON THE GROWTH OF *LACTOBACILLUS BULGARICUS* IN CARROT-LIVER EXTRACT MEDIUM AT 49.5°C.

A different picture was obtained when cultures of different ages were grown at 49.5°C. During the first two and one-half hours (fig. 5) most growth was obtained with the four-hour inoculum, and, as indicated in table 3, the most acid was produced. However, the rate of growth of these organisms decreased rapidly and remained low until the fifth hour when growth ceased. During the first two and one-half hours after inoculation with the seven-hour culture, less growth and less acid were produced than with the four-hour inoculum. Growth, however, continued at a rapid

rate, and by the time it had ceased at five hours, the culture had produced twice as much turbidity measured by the colorimeter, as had the four-hour culture. Table 3 indicates that a similar relationship existed between the growth of the organism and the increase of acidity or the decrease in pH. Considerably less growth and acid production were evident for the culture inoculated with the twelve-hour inoculum, and the 16-hour inoculum showed only about one-half the turbidity produced by the 12-hour inoculum.

TABLE 3

The influence of age of inoculating culture on the production of lactic acid by Lactobacillus bulgaricus in carrot-liver extract medium at 49.5°C.

TITRATABLE ACIDITY AS LACTIC ACID PER 10 ML. OF CULTURE					pH			
Time	4 hour culture as inoculum	7 hour culture as inoculum	12 hour culture as inoculum	16 hour culture as inoculum	4 hour culture as inoculum	7 hour culture as inoculum	12 hour culture as inoculum	16 hour culture as inoculum
hours	mgm.	mgm.	mgm.	mgm.				
0	0.00	0.00	0.00	0.00	6.55	6.55	6.55	6.55
1	1.35	0.90	0.00	0.00	6.51	6.53	6.55	6.55
2	3.60	2.70	0.90	0.45	6.41	6.46	6.53	6.53
3	5.40	6.75	1.80	0.90	6.31	6.30	6.50	6.52
4	8.10	12.60	3.15	1.80	6.19	6.05	6.45	6.51
5	12.60	17.10	4.50	2.25	6.09	5.98	6.40	6.50
6	13.50	22.05	5.40	2.70	5.99	5.61	6.38	6.48
7	16.20	24.30	6.30	2.70	5.93	5.51	6.33	6.47
8	17.10	26.10	6.30	3.15	5.87	5.43	6.28	6.47
9	17.10	27.45	6.30	3.15	5.84	5.38	6.28	6.47
12	18.10	30.15	6.30	3.15	5.84	5.13	6.28	6.47

In the 49.5°C. cultures inoculated with the four- and seven-hour cultures, the production of acid, unlike that at 37°C., continued throughout the entire twelve-hour period during which determinations were made. The total amount of acid produced was 18 and 30.5 mgm. of acid, respectively. An inoculum of organisms 12 hours old produced 63 mgm. of acid, while the 16-hour culture produced 3.15 mgm. It was evident that for the organisms grown at 49.5°C., production of acid could not be considered as a major limiting factor for growth.

There were, then, marked differences between the growth and

activity of *Lactobacillus bulgaricus* at 37 and at 49.5°C. Variations in the size and age of inoculum had a distinct influence on growth and acid production at 49.5°C. and little influence at 37°C. Therefore it seemed that studies on some of the specific physiological mechanisms of the organisms should lead to a fuller understanding of reasons for the variations in growth and activity, so apparent during growth at the two temperatures.

THE PRODUCTION OF VOLATILE ACIDS AT NEAR THE MAXIMUM GROWTH TEMPERATURE

Besides large quantities of lactic acid, small quantities of volatile acids are produced by lactic acid bacteria during growth. Most of the volatile acid produced under so-called optimal conditions has been found to be acetic, but small amounts of propionic and traces of formic acid have also been found. The influence of temperatures near the maximum for growth on the production of volatile acids by *Lactobacillus bulgaricus* and by *Streptococcus thermophilus* (C₃) was studied.

Experimental methods

The reconstituted skim-milk medium used contained 90 grams of dry skim-milk and one gram of Difco Neopeptone in one liter of city water. The medium was heated for 28 minutes at fifteen pounds.

Mother cultures and inoculating cultures were prepared as previously described except that the skim-milk medium was used instead of carrot-liver-extract medium.

For the determination of total and volatile acidity, two 800 ml. Kjeldahl flasks, each containing 210 ml. of skim-milk medium, received a one per cent inoculum from an inoculating culture. Two uninoculated flasks of medium were used as controls. In the case of *Lactobacillus bulgaricus*, one inoculated and one sterile flask of medium were placed at 37°C. and one each at 49.5°C., with *Streptococcus thermophilus* at 37 and 50°C. All cultures were analyzed for total acidity and volatile acidity after five days' incubation.

Total acidity was determined by titration with N/10 sodium

hydroxide of a 10 ml. aliquot, to which had been added 10 ml. of distilled water and 10 drops of one per cent phenolphthalein.

The remainder of the culture was made acid to Congo red with phosphoric acid and steam-distilled until one liter of distillate was obtained. A 500 ml. aliquot then was titrated for volatile acidity using N/10 sodium hydroxide with 10 drops of phenolphthalein as indicator. Titrations obtained with the control milk were subtracted from those of the inoculated milks.

TABLE 4

Production of volatile acid by Streptococcus thermophilus at 37 and 50°C. and by Lactobacillus bulgaricus at 37 and 49.5°C.

ORGANISM	TEMPERATURE OF INCUBATION	TOTAL ACID	VOLATILE ACID	AMOUNT OF VOLATILE ACID AS COMPARED WITH TOTAL ACID
	°C.	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
<i>Streptococcus thermophilus</i>	37	1.008	0.0275	2.73
		1.010	0.0270	2.67
		0.998	0.0272	2.72
	50	0.540	0.0201	3.72
		0.524	0.0200	3.80
		0.567	0.0206	3.63
<i>Lactobacillus bulgaricus</i>	37	1.460	0.0429	2.93
		1.500	0.0430	2.83
		1.480	0.0428	2.89
	49.5	1.008	0.0436	4.32
		1.030	0.0439	4.26
		1.060	0.0443	4.18

Results

Table 4 contains a summary of results on three determinations with *Streptococcus thermophilus* (C₃). In all cases both the total acidity and the volatile acidity were less at 50 than at 37°C., but the total acidity showed a considerably greater decrease than the volatile acidity. This fact is emphasized further by the observation that the volatile acidity, when expressed as a percentage of the total acidity, was greater at the higher temperature than at the lower temperature. Results obtained with *Lactobacillus*

bulgaricus also are summarized in table 4. The same general picture was evident with this organism as with *Streptococcus thermophilus*. At 49.5°C. *Lactobacillus bulgaricus* produced slightly more volatile acidity than at 37°C., but only about two-thirds as much total acidity. Consequently the volatile acidity, when expressed as a percentage of the total acidity, was considerably greater at 49.5°C. than at 37°C.

It is evident that when the organisms were grown at temperatures near their maximum, the total production of acid was considerably less than that at optimum temperatures. On the other hand the production of volatile acid was affected only slightly and, in the case of *Lactobacillus bulgaricus*, an increase instead of a decrease at the high temperature was observed.

THE FORM OF LACTIC ACID PRODUCED NEAR THE MAXIMUM GROWTH TEMPERATURE

Most of the energy for the so-called "true lactics" is obtained from the fermentation of a variety of substrates with the production of lactic acid. Since lactic acid possesses an asymmetric carbon atom it is optically active, and during the course of a fermentation it is possible to have produced levo-acid, dextro-acid, or varying mixtures of both forms. It is generally considered that the type of acid produced is constant for a bacterial species and is unaffected by the nature of the substrate or the conditions of the fermentation. Investigations were undertaken to determine the form of lactic acid produced by *Streptococcus thermophilus* (C₃) and by *Lactobacillus bulgaricus* when grown at optimum temperatures and at temperatures near the maximum.

Experimental methods

Both the mother cultures and the inoculating cultures used for the determinations of the form of lactic acid were prepared similarly and in the same medium as those for the volatile acid determinations.

Two 1,000 ml. Erlenmeyer flasks, each containing 500 ml. of sterile milk, were seeded with a one per cent inoculum. When

the determinations were to be made on *Streptococcus thermophilus*, the cultures were placed at 37 and at 50°C., while in the case of *Lactobacillus bulgaricus* they were placed at 37 and at 49.5°C. All cultures were incubated for 14 days.

After incubation the contents of each flask were filtered through cheese cloth to remove most of the casein. The filtered medium was acidified to pH 2.0 with sulfuric acid and extracted with ethyl ether in a Kutscher-Steudel extractor for 48 hours. After the addition of a small volume of water the ether was distilled off and the extract boiled slowly with the constant addition of zinc carbonate and a small quantity of Norite until all of the acid had been converted to zinc lactate. The contents then were filtered and evaporated almost to dryness over a steam bath. A small quantity of 95 per cent alcohol was added, and the zinc lactate was allowed to crystallize out over night in the ice box. To insure complete removal of all of the zinc lactate, two additional crystallizations were made on the original filtrate. All fractions were incorporated into one sample and allowed to air dry. The optical rotation and the amount of water of crystallization then were determined.

For the determination of water of crystallization, the sample was brought to constant weight, and placed in a 110°C. oven until all of the water of crystallization had been driven off. The percentage of inactive salt then was determined from the loss of weight of the sample according to the following formula:

$$\text{per cent inactive salt} = 100 \times \frac{\text{per cent H}_2\text{O} - 12.89}{18.18 - 12.89}$$

For the determination of specific rotation, four-per-cent solutions of the anhydrous salts of zinc lactate were prepared, clarified with Norite and infusorial earth, and filtered through quantitative filter paper. Care was taken during the filtration and subsequent washing to insure complete recovery of the salts. The volume then was adjusted to that of the original four-per-cent solution and the rotation read at room temperature on a saccharimeter in degrees Vetzke with the use of a four-decimeter tube. The

specific rotation in angular degrees was calculated from the following formula:

$$[\gamma]_{\text{D}}^{23^{\circ}} = 0.3462 \times \frac{100 \times \text{degrees Ventzke}}{l \times d \times p}$$

l = length of tube

d = density of solution

p = per cent concentration of solution

γ = angular degrees

0.3462 = conversion factor from degrees Ventzke

From such data the percentage of dextro- or levo-salt can be determined, since the rotation obtained from any sample, unless it is 100 per cent dextro- or levo-rotatory is in reality the result of an excess of one type of salt over the other.

TABLE 5

Optical properties of the lactic acid produced by Streptococcus thermophilus when grown at 37 and 50°C., and by Lactobacillus bulgaricus when grown at 37 and 49.5°C.

ORGANISM	TEMPERATURE OF INCUBATION	QUANTITY OF INACTIVE SALT (FROM WATER OF CRYSTALLIZATION)	SPECIFIC ROTATION	QUANTITY OF EACH ISOMER	
				Levo-	Dextro-
	°C.	per cent	$[\alpha]_{\text{D}}^{23^{\circ}}$	per cent	per cent
<i>Lactobacillus bulgaricus</i>	37	12.91	-7.96	98.20	1.80
	49.5	12.80	-8.35	100.00	0.00
<i>Streptococcus thermophilus</i>	37	16.40	+6.92	8.05	91.95
	50	16.06	+6.96	7.80	92.20

Results

The investigations on *Lactobacillus bulgaricus* (table 5) revealed that at 37°C. about 98.2 per cent of the lactic acid was levo-rotatory, while at 49.5°C. all of the acid was of the levo-type. The small amount of dextro-rotatory acid reported to have been produced at 37°C. probably was not really dextro-acid and should be attributed to experimental error, especially since the results of the water of crystallization determinations indicated that salts obtained at the two temperatures were both 100 per cent active.

Results with *Streptococcus thermophilus* also are reported in table 5. This organism at both temperatures produced approximately 92 per cent dextro-rotatory acid and eight per cent levo-acid. This was in contrast to the levo-character of the acid produced by *Lactobacillus bulgaricus*. The percentage of inactive salt obtained also indicated that the distribution found for the two isomers was correct.

Therefore for both of the organisms studied growth at temperatures near the maximum had no apparent effect on the kind of lactic acid produced.

DISCUSSION

The results indicate that rates of enzyme production and destruction as well as rates of enzyme activity are different in cultures of lactic acid bacteria grown at temperatures near the maximum and in cultures grown at the optimum temperature. Apparently at temperatures near the optimum there is a satisfactory balance between these processes for a considerable length of time until, as is probably the case with many of the lactic acid bacteria, the enzymes responsible for growth are inactivated largely by the low pH that has resulted from the production of acid. Indications are that, at temperatures near the maximum, enzymatic activity is affected differently from the way it is at optimum temperatures. For instance it has been pointed out that although growth ceased by the fourth hour when *Lactobacillus bulgaricus* was cultivated at 49.5°C., the production of lactic acid continued for some time after growth had stopped, and as much acid was produced after growth had ceased as was produced during growth. In this case it seemed that the enzyme system responsible for the production of acid was inactivated to a lesser degree than were the enzymes involved in the multiplication of the organisms.

On the other hand, the production of volatile acids by *Streptococcus thermophilus* and by *Lactobacillus bulgaricus* was affected only slightly when the organisms were grown at temperatures near the maximum for growth. However, *Lactobacillus bulgaricus* produced more volatile acid at 49.5 than at 37°C., in spite of the

fact that fewer organisms and less total acidity were produced at the higher temperature. It may be that the inactivating influence of the high temperatures was greater for the enzymes responsible for the production of lactic acid than for those responsible for the production of volatile acids, and so the organisms in an effort to obtain sufficient energy to carry on their life processes, utilized the volatile acid mechanisms to obtain this energy.

SUMMARY

1. With *Lactobacillus bulgaricus* there was a close relationship between the rate of production of lactic acid and the rate of growth when the organisms were cultivated at 37°C. This similarity was not observed at 49.5°C. since as much acid was produced after reproduction had ceased as was produced during growth.

2. Differences as a result of variations in the size of inoculum of *Lactobacillus bulgaricus* were apparent only during the early stages of growth at 37°C. An increase in the number of organisms present in the inoculum tended to shorten the lag phase, but had no apparent effect on the maximum amount of growth obtained. At 49.5°C., however, when larger inocula were employed, the increase in growth was considerably more rapid than when smaller inocula were used, and the maximum population obtained depended on the number of cells added at the start of the experiment.

3. When flasks of medium were inoculated with cultures of *Lactobacillus bulgaricus* 4, 7, 12, and 16 hours old, respectively, there was an increase in the length of the lag phase coincident with an increase in the age of the inoculum. The total amount of acid produced at the end of 24 hours was the same regardless of the age of the inoculating culture employed, although somewhat less growth was observed in the medium that had been inoculated with the older cultures. At 49.5°C. most growth was obtained when a seven hour old inoculum was used, and less growth was observed when the medium was inoculated with cultures 4, 12, and 16 hours old. A similar relationship was evident with respect to acid production when measured as titratable acidity and as pH.

4. With *Streptococcus thermophilus* (C_3) and with *Lactobacillus bulgaricus* the production of volatile acids was affected only slightly when the organisms were grown at temperatures near the maximum for growth. However, *Lactobacillus bulgaricus* produced more volatile acid at 49.5°C. than at 37°C.

5. When *Lactobacillus bulgaricus* was grown at temperatures near the maximum there was no difference in the distribution of the two isomers of lactic acid from that found at optimal temperatures. Similar results were obtained with *Streptococcus thermophilus*.

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