FACTORS INFLUENCING THE RATE OF FERMENTATION OF STREPTOCOCCUS LACTIS

OTTO RAHN, C. P. HEGARTY AND R. E. DEUEL

Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca, N. Y.

Received for publication November 19, 1937

The rate of lactic fermentation can be measured readily with centrifuged cells in glucose solution. Virtanen and associates have done this with cells of *Streptococcus lactis* and *Lactobacillus casei*, mostly with dried or toluene-treated cells, but one series (1927) deals with living cells. Their results in regard to the optimal pH differ widely from ours. Several other factors are reported here which Virtanen and his associates did not study.

METHOD

Our studies are limited to strains 14 (isolated from plants) and 125 (from milk) of *S. lactis*. Virtanen grew his bacteria in whey, i.e., in a lactose medium. We used a broth made of 5 grams peptone, 5 grams tryptone, 10 grams glucose, 36.6 grams $Na_2HPO_4 \cdot 12H_2O$ and 4.2 grams KH_2PO_4 per liter. This same medium plus 15 grams agar was used for plate counts. Inoculation was heavy, 5 to 10 cc. of a young culture per liter. The cells were centrifuged when the culture was 12 to 14 hours old. The sedimented cells were re-suspended in a phosphate buffer of the same concentration as given above. This suspension was added to the test medium in such quantity that the cells from 250 cc. of culture were condensed in 50 cc. of the test medium. Thus, we hoped to avoid growth.

The test medium consisted of the same 2 per cent buffer (36.6 grams $Na_2HPO_4 \cdot 12H_2O$ = 15.8 grams anhydrous, plus 4.2 grams KH_2PO_4) to which 3 or 5 grams peptone and 20 grams glucose per liter were added. The peptone is essential. All cultivation and all fermentation tests were carried out at 30°C.

We measured the lactic acid by titration of 5 cc. of the cell suspension with N/10 NaOH and phenolphthalein. Virtanen states that this method is not accurate when the phosphate concentration is 2 per cent or higher. By using a color standard for titration, the determinations were fairly accurate even with 8 per cent phosphates, although the endpoint becomes less sharp as the phosphate concentration increases. All experiments except the first one were made with a 2 per cent buffer. Since titration permitted 8 to 10 determinations in half-hour intervals, it had great advantages over one or two determinations after 2 to 8 hours. The curve of acid formation was quite useful in the interpretation of the results.

The basis of comparison was always the fermenting capacity, i.e., the milligram lactic acid produced per cell per hour. This was computed from titration and plate counts. Usually, the bacteria did not multiply during the experiment, and the fermenting capacity remained constant until enough acid had been formed to change the pH.

EFFECT OF pH AND OF BUFFER CONCENTRATION

An extensive experiment is most apt to show what kind of results could be obtained by our method. In this set of data, an acetate phosphate buffer was used since phosphate alone did not buffer sufficiently at pH 4.5. The mineral concentration was so arranged that it was very nearly 8 per cent throughout the entire pH range. Of these buffers, dilutions were made to contain 6, 4 and 2 per cent of buffering substances. All buffers contained 2 per cent glucose and 0.3 per cent peptone. The bacteria were obtained by means of a super-centrifuge and their concentration in the final experiments was 4.0×10^9 cells per cubic centimeter.

Table 1 shows the acid produced, and table 2 the fermenting capacities computed from the periods of constant rates. Slight irregularities are unavoidable in these strongly buffered solutions. Fermentation was most rapid at pH 7.0, and almost as rapid at pH 7.5 and 8.0. Even at pH 9.0, the rate was faster than at pH 6.0. This result differs greatly from Virtanen's who found

TABLE 1

Effect of pH and of buffer concentration upon the rate of fermentation of Streptococcus lactis 125

pH	PER CENT OF LACTIC ACID FORMED AFTER									pH AFTER	
	0.5 hour	1.0 hour	1.5 hours	2.0 hours	2.5 hours	3.0 hours	4.0 hours	5.0 hours	6.0 hours	4 hours	6 hours
In 2 per cent buffer concentration											
4.5	0.022	0.047	0.050	0.074	0.085	0.085	0.090	0.095		4.3	4.26
5.0	0.036	0.072	0.108	0.135	0.153	0.162	0.216	0.216	0.243	4.62	4.65
6.0	0.054	0.126	0.180	0.216	0.253	0.280	0.342	0.360	0.378	4.65	4.80
7.0	0.072	0.144	0.198	0.262	0.315	0.360	0.405	0.450		5.34	
7.5	0.072	0.144	0.207	0.280	0.350	0.414	0.513	0.530	0.585	5.66	5.28
8.0	0.072	0.135	0.207	0.270	0.342	0.396	0.477	0.522		5.80	5.28
9.0	0.072	0.126	0.216	0.288	0.342	0.414	0.495	0.558	0.602	5.70	5.44
In 4 per cent buffer concentration											
4.5	0.018	0.018	0.032	0.045	0.054	0.054	0.054	0.054	0.072	4.45	4.48
5.0	0.018	0.054	0.090	0.099	0.081	0.172	0.172	0.180	0.190	4.77	5.00
6.0	0.018	0.126	0.162	0.216	0.270	0.315	0.342	0.424	0.486	5.16	5.16
7.0	0.126	0.216	0.306	0.396	0.450	0.540	0.675		0.722	5.9	
7.5	0.090	0.171	0.254	0.334	0.414	0.506	0.640	0.730	0.745	6.32	5.85
8.0	0.063	0.135	0.207	0.342	0.433	0.522	0.666			6.5	6.02
9.0	0.063	0.126	0.198	0.270	0.360	0.450	0.576	0.702	0.722	6.7	6.11
In 6 per cent buffer concentration											
4.5	0.014	0.018	0.040	0.090	0.090	0.090	0.090	0.090	0.072	4.45	4.55
5.0	0.009	0.036	0.090	0.108	0.081	0.099	0.099	0.108	0.134	4.82	5.05
6.0	0.018	0.144	0.234	0.243	0.306	0.360	0.426	0.504	0.575	5.40	5.50
7.0	0.054	0.126	0.288	0.350	0.450	0.531	0.655	0.755	0.827	6.22	
7.5	0.081	0.144	0.216	0.300	0.423	0.476	0.621	0.746	0.818	6.8	6.64
8.0	0.090	0.153	0.234	0.306	0.414	0.495	0.630	0.772	0.910	6.8	6.63
9.0	0.036	0.108	0.198	0.234	0.306	0.360	0.495	0.639	0.700	7.0	6.72
In 8 per cent buffer concentration											
4.5	0.009	0.070	0.110	0.110	0.110	0.110	0.110		0.110	4.52	4.50
5.0	0.054	0.090	0.126	0.144	0.099	0.099	0.144	0.090	0.153	4.90	5.00
6.0	0.072	0.072	0.144	0.207	0.243	0.306	0.396	0.441	0.512	5.54	5.65
7.0	0.126	0.144	0.234	0.288	0.396	0.476	0.592	0.655	0.772	6.45	
7.5	0.036	0.099	0.144	0.225	0.234	0.387	0.531	0.621	0.729	6.77	6.47
8.0	0.063	0.090	0.153	0.207	0.261	0.288	0.396	0.512	0.549	7.13	6.95
9.0	0.009	0.081	0.099	0.117	0.153	0.189	0.225	0.288	0.306	7.55	7.50

the rate to be highest at pH 6.2, and 40 to 50 per cent lower at pH 7.8. His test temperature was 20°. Probably, this and the difference in strains accounts for the difference in results.

In the buffer solutions of pH 8 and 9, acid formation tends to reduce the pH and to bring it nearer to the optimum of 7. The concentrated buffers will prevent this by their strong buffering effect, and therefore retard the rate of fermentation. The opposite is true at pH 4.5 and 5. Acid production decreases the pH and removes it still further from the optimal range. The more concentrated buffers prevent this further drop, and therefore show the higher rate of fermentation. For pH 7 to 8, the optimal buffer concentration is 4 per cent.

That the rate is constant for a longer time in the concentrated solutions, need hardly be mentioned. In the 8 per cent buffer solutions, however, the fermentation mechanism seems to be

TABLE 2

Rates of fermentation from table 1 expressed in 10^{-10} mgm. lactic acid per cell per hour

pH	BUFFER CONCENTRATION			
	2 per cent	4 per cent	6 per cent	8 per cent
4.5	0.8	0.5	1.1	1.3
5.0	1.7	1.2	1.5	1.8
6.0	2.7	2.7	3.0	2.6
7.0	3.3	5.0	4.3	4.0
7.5	3.5	4.2	4.0	3.2
8.0	3.3	4.4	4.1	2.6
9.0	3.5	3.8	3.0	1.6

damaged to quite an extent because the rate falls off quickly, and the final amount of acid produced is decidedly lower than in the 6 per cent buffers at any pH except the two lowest.

EFFECT OF CELL CONCENTRATION

If we are dealing here with the zymase alone, i.e., uninfluenced by other cell functions, the rate must be strictly proportional to the cell number. Several experiments were made to test this.

Strain 125

cells per cc.	*mgm. per cell per hour*
3,600,000,000	6.0×10^{-10}
1,800,000,000	6.4×10^{-10}
900,000,000	8.0×10^{-10}

Strain 14

cells per cc.	*mgm. at 30°*	*mgm. at 45°*
1,000,000,000	23.2×10^{-10}	35.5×10^{-10}
500,000,000	28.8	34.6
250,000,000	32.4	38.4
125,000,000	37.8	40.4
62,500,000	50.4	46.0

A parallel experiment with the strain 14 was made where the cells were killed during washing by a too high temperature of the centrifuge. Fermentation was weaker, but measurable. The cell ratio was 8:4:2:1 while the acid formed showed the ratio 7.85:4:2.00:1.28.

The experiments with living cells show an increasing rate of fermentation when fewer cells are present. This is due to multiplication of the cells if the concentration is less than 10^9 per cubic centimeter. At 45° where multiplication becomes extremely slow, the increase in rate of fermentation by the lower cell concentrations is comparatively small.

EFFECT OF GLUCOSE CONCENTRATION

Slator (1906) has shown that alcoholic fermentation by yeast depends upon the sugar concentration only if less than 1 per cent is present. Above 1 per cent, the rate is practically independent of the sugar concentration. Our method made the study of this question quite simple, and the result is shown in table 3. The data are averages of 10 complete experiments made by 10 different workers with the same bacterial suspension in 2 per cent buffer solution of pH 6.4, with varying amounts of glucose. For some unknown reason, the fermentation did not start until about 5 or 10 minutes after the cells were placed in contact with the glucose, and therefore, the rate for the first half hour is low throughout.

The buffer concentration was 2 per cent, and the pH remained constant at 6.4 until about 0.35 per cent of lactic acid had been formed. This occurred between 1.5 and 2 hours at the higher glucose concentrations. After that, it decreased rapidly, and, with it, decreased the rate of fermentation.

The amount of sugar available at any time can be roughly estimated by subtracting the total acid formed from the original glucose concentration. Thus, after 0.5 and 1 hour, the glucose of the second flask had decreased from 0.25 to 0.15 and 0.07 per cent respectively.

The lower half of the table shows the rate of fermentation to be uniform for the second and third half-hour period, from 0.5

TABLE 3

Effect of the concentration of glucose

TIME	GLUCOSE CONCENTRATION					
	0.125 per cent	0.25 per cent	0.50 per cent	1.00 per cent	2.00 per cent	4.00 per cent
	Total lactic acid formed					
hours						
0.5	0.073	0.100	0.090	0.084	0.084	0.090
1.0	0.091	0.179	0.202	0.204	0.206	0.206
1.5	0.093	0.207	0.321	0.333	0.326	0.312
2.0	0.093	0.210	0.354	0.415	0.394	0.380
2.5	0.093	0.210	0.358	0.469	0.459	0.428
3.0	0.093	0.210	0.360	0.489	0.480	0.464
3.5	0.093	0.210	0.361	0.502	0.489	0.478
Final pH....	6.4	6.4	6.1	4.4	4.4	4.4
	Per cent lactic acid formed per half hour					
half hours						
1st	0.073	0.100	0.090	0.084	0.084	0.090
2nd	0.018	0.079	0.113	0.120	0.122	0.116
3rd	0.002	0.027	0.118	0.129	0.120	0.106
4th	0	0.003	0.034	0.083	0.068	0.068
5th	0	0	0.004	0.054	0.065	0.048
6th	0	0	0.002	0.020	0.022	0.036
7th	0	0	0.001	0.013	0.008	0.014

to 4 per cent sugar. With 0.125 per cent sugar, the initial rate was fairly high, and with 0.25 per cent, almost as high as the highest during the first half hour, but in both cases, the glucose concentration during the second half hour fell below 0.1 per cent. With 0.5 per cent initial sugar, fermentation was constant for the first 1.5 hours (0.18 per cent glucose remaining), but dropped decidedly during the next half-hour. It seems safe to state from these results that the rate of lactic fermentation is independent

of the glucose concentration when it amounts to at least 0.2 per cent of the culture medium, provided, of course, that the pH does not decrease.

EFFECT OF Na-LACTATE

Two experiments with strain 14 showed very little effect. Even the highest concentrations corresponding to 3.2 per cent lactic acid still permitted very noticeable fermentation though the rate had been reduced to about one-third. Rogers and Whittier (1928) had found in milk 0.1829 molar acid (= 2.2 per cent Na-lactate) to be completely inhibiting; but in an artificial medium with 0.5 K_2HPO_4, no constant relation could be found.

TABLE 4

EXPERIMENT I		EXPERIMENT II	
Na-lactate	Fermenting capacity	Na-lactate	Fermenting capacity
per cent	*10^{-10} mgm. per cell per hour*	*per cent*	*10^{-10} mgm. per cell per hour*
0	22.9	0	25.6
0.2	22.9	1	19.2
0.4	21.9	2	18.7
0.8	20.4	3	12.9
		4	9.6

This latter experiment corresponds more closely to ours than the one with milk. It must be expected that our strongly buffered test medium would give results different from those obtained in milk or whey.

EFFECT OF AERATION

Air, oxygen and nitrogen were bubbled through cell suspensions of strain 125 in wide test tubes as rapidly as this could be done without losing some of the culture by foaming. The effect in a preliminary test was slight, 19 per cent retardation by oxygen and 17 per cent by air. In the final experiment, the fermenting capacities were

Quiet controls	8.65 × 10^{-10} mgm.	
Agitated with current of air	6.7	= 22 per cent reduction
Agitated with current of oxygen	4.8	= 44 per cent reduction
Agitated with current of nitrogen	10.3	= 20 per cent increase
In vacuum	8.60	

The number of viable cells was not changed materially during the 4.5 hours of the experiment. We must conclude that the enzyme action as such is in some way inhibited by too much oxygen. The great reducing power of streptococci as shown by their effect upon litmus and methylene blue seems to be sufficient to eliminate the harmful effect of oxygen in a quiet suspension. Oxygen is removed more rapidly than it can diffuse into the culture, and therefore, no difference was observed between vacuum and regular control. However, the increase by nitrogen does not fit into this picture.

AGE OF CELLS

In the most extensive of our experiments, the age varied from 2 to 48 hours. By age is meant the time from inoculation until the beginning of centrifugation. For centrifugation and re-suspension, 35 more minutes were required before the first titration could be made. In order to avoid lag, the inoculum always consisted of young cells. Thus, to obtain the 2-hour-old cells, 1,500 cc. were inoculated with 60 cc. of a 4-hour-old culture which had been obtained from a 9-hour culture. The cells from 250 cc. of culture were re-suspended in 50 cc. of the test medium, with the exception of the 2- and 4-hour-old cultures. We expected rather small numbers of cells, and hence, the cells from 500 cc. of the 4-hour culture, and from 1500 cc. of the 2-hour culture were concentrated in 50 cc. of the test medium.

Of each suspension, a plate count was made at once, and again after 4 hours when the titrations were finished. No increase in cells beyond the limits of error was observed in any case.

Acid formation proceeded in a very regular way, the only abnormal set being the two 8-hour-old cultures which had an initial delay, and later showed further inconsistencies. The fermenting capacities showed a surprising uniformity.

Age of cells in hours.	2	4	6	8	10	12	14	16	18	24	36	48
10^{-10} mgm. per cell per hour.....	7.4	6.4	8.8	[6.0]	8.2	6.8	5.5	5.2	6.3	6.3	6.8	6.5

The fermenting capacity is surprisingly uniform. The very short but very greatly increased rate of metabolism observed by

Bayne-Jones and Rhees (1929) and by Walker, Winslow, Huntington and Mooney (1934) was not evident. These authors observed that cells just coming out of lag, before the plate count showed a doubling of the cells, had a rate of metabolism 4 to 8 times as high as a few hours later, during the logarithmic phase. While our cells had not become old, on account of frequent transfers, they had not recently come out of lag. To induce this condition, 1.5 liters of a 36-hour-old culture were transferred to 13.5 liters of fresh medium. Every hour, part of this culture was centrifuged, and the fermenting capacity tested by using the cells of large volumina, e.g., the cells of 2 liters for the 1- and 2-hour culture. The plate count showed that the cells of

TABLE 5

Effect of the age of cells on the rate of fermentation

AGE OF CULTURE	MILLION CELLS PER CUBIC CENTIMETER		LACTIC ACID PRODUCED	
	Of culture, before centrifugation	Of final cell suspension	In 3 hours	10^{-10} mgm. per cell per hour
hours			*per cent*	
12	379	1,720	0.387	7.5
24	308	1,330	0.279	7.0
24	308	1,220	0.270	7.4
36	142	325	0.108	11.1
48	83	370	0.099	8.9

the culture had not multiplied after 2 hours, and had doubled after 3 hours. There was a definite maximum in the 3-hour culture, but the difference was not nearly as large as that found by the above-cited authors.

Age of cells in hours.....	1	2	3	4	5	6	8	10	36
10^{-10} mgm. per cell per hour...	10.5	16.3	17.7	10.3	9.3	9.2	8.7	6.8	7.4

There seems to be a difference between cells kept young by continuous transfering and those made young by rejuvenation of old cultures.

The values for the cultures older than 12 hours are probably a little too high because the cell numbers begin to decrease, due to aging of the culture. The cells which lose the power to produce colonies may still be able to produce acid (Rahn and Barnes, 1933)

and the computation would ascribe this acid to the viable cells. This is perhaps more plainly visible from table 5 where the number of cells had been determined before and after centrifugation. An estimate of this error does not seem possible.

LONGEVITY

The following experiment was made to test how soon the centrifuged cells lose their vitality. Cells from a 12-hour culture were re-suspended as usual, and the initial plate count of the suspension (3 determinations) was 2500 million per cubic centimeter. After $2\frac{1}{2}$ and $4\frac{1}{2}$ hours at 30°C., the counts showed 1860 and 1890 million per cubic centimeter (6 determinations each).

TABLE 6

Effect of cold storage on viability and fermenting capacity

	MILLION CELLS PER CUBIC CENTIMETER		LACTIC ACID	
	Cells not washed	Cells washed	Cells not washed	Cells washed
			10^{-10} mgm. per cell per hour	*10^{-10} mgm. per cell per hour*
Fresh	1,890	1,750	11.6	11.8
1 day at 2°C	1,690	1,720	10.6	11.5
2 days at 2°C	1,650	1,620	8.7	11.4
4 days at 2°C	1,140	1,300	12.6	13.5

The decrease is probably not caused by death, but by partial agglutination of the very slimy culture. One of these cultures was kept at 30°, and the number of viable cells after 24 hours was 1000 million, after 48 hours 760 million per cubic centimeter.

When the original cell concentrate was held at +2°C., and at some later time was warmed to 30° and suspended in the same manner as the above cells, the cells showed very little decrease in viability, and no loss of fermenting capacity for at least 4 days, as may be seen from table 6. The only noticeable effect of cold storage was a delay of 10 to 30 minutes in starting the fermentation. It is therefore feasible to centrifuge the cells on one day and use them for experiments at some later date. All experi-

ments mentioned in this paper have been done with fresh cells, immediately after centrifugation.

EFFECT OF OTHER CARBOHYDRATES

Experiments with carbohydrates other than glucose have shown no acid formation in most cases. The experiments are being continued.

NECESSITY OF PEPTONE

Centrifuged cells show some fermentation without peptone, but when the cells have been thoroughly washed, fermentation in the absence of peptone is very slow or entirely lacking. An investigation on the rôle of peptone has been started.

SUMMARY

The rate of lactic fermentation by streptococci can be measured simply by titration when the cells are obtained by centrifugation and suspended in a buffered glucose solution of pH 7.0. It is important that the cells are young (12 to 24 hours) and more concentrated than normal growth would make them. All cells in these experiments were tested in 2 per cent phosphate buffer + 2 per cent glucose + 0.3 to 0.5 per cent peptone.

The rate of fermentation had its optimal pH at 7.0. Deviation to the alkaline side retarded less than deviation to the acid side. Fermentation took place most rapidly in a buffer containing 4 per cent of phosphates, but the largest amounts of final acidity were produced with 6 per cent.

As long as more than 0.2 per cent of glucose were present, the rate of fermentation was not affected by the sugar concentration.

Sodium lactate in concentration of 1 per cent retarded slightly; 4 per cent reduced the rate to less than half the normal rate.

Continued agitation by a current of air or oxygen retarded fermentation, while nitrogen increased the rate.

Very little difference was found between cells of different age, ranging from 2 hours to 2 days, if the cells were kept young by very frequent transfers. However, if old cells were transferred to a fresh medium, the fermenting capacity was doubled when

the cells came out of lag, and decreased again after the number of cells had doubled.

The centrifuged cells can be stored in a phosphate buffer without glucose at a temperature of about +2°C. for 4 days without loss of fermenting capacity, and with very little loss of viability.

REFERENCES

BAYNE-JONES, S., AND RHEES, H. S. 1929 Bacterial Colorimetry. IV. Relationship of heat production to phases of growth of bacteria. Jour. Bact., **17**, 123–140.

RAHN, OTTO, AND BARNES, M. N. 1933 An experimental comparison of different criteria of death in yeast. Jour. Gen. Physiol., **16**, 211–229.

SLATOR, A. 1906 The chemical dynamics of alcoholic fermentation by yeast. Jour. Chem. Soc., **89**, 128–139.

VIRTANEN, A. I., WICHMANN, E., AND LINDSTRÖM, B. 1927 Ueber die Milchsäuregärung, IV. Z. Physiol. Chemie, **166**, 21–38.

WALKER, H. H., WINSLOW, C.-E. A., HUNTINGTON, E., AND MOONEY, M. G. 1934 The physiological youth of a bacterial culture as evidenced by cell metabolism. Jour. Bact., **27**, 303–324.