A STUDY OF THE FORMATION OF GUM LEVAN FROM SUCROSE

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A large number of species of bacteria are known to produce gums from sugars. These may be classified into two main classes according to whether they form capsules, like the *Leuconostoc mesenteroides*, and *Semiclostridium commune*, or whether such capsules are absent, as in the case of Kramer's (1889) *Bacillus viscosus-sacchari*, Fritz Glaser's (1895) *Bacterium gelatinosum betae*, or Ritsert's (1891) *Bacterium gummosum*. These gum-forming species of bacteria may be still further classified according to the type of sugars which they are capable of utilizing for gum production. There are those on the one hand which form gum from either sucrose or invert sugar, others only from sucrose; one has been described which forms it from sucrose or maltose, and another from sucrose or raffinose. The species of bacteria comprising these three classes are as follows:

I. Gum formed either sucrose or invert sugar
   Leuconostoc mesenteroides
   Bacterium gelatinosum betae (Glaser)
II. Gum formed only from sucrose
   Bacterium gummosum (Ritsert, 1891)
   Bacillus gummosum (Happ, 1893)
   Bacillus levaniformans (Smith, 1901, 1902a)
   The Ginger Beer plant of Ward's consisting of *Bacterium vermiforme* and *Saccharomyces pyriformis*. (1899)
III. Gum formed from sucrose or maltose
   Micrococcus gummosus (Happ, 1893)
IV. Gum formed only from sucrose or raffinose
   Bacterium eucalypti (Smith, 1902b)

The species of bacteria of the first group are generally supposed to secrete invertase, and hence the gum that they form

is thought to be from the product of the inversion of the sucrose rather than from the sucrose itself. It is quite different with the second class, in which with the exception of the Ginger beer plant, no action of invertase is indicated. Its presence is inferred in the latter case only because of the fact that a true saccharomycete is involved in the transformation of the sucrose into gum.

In studying the gum-forming ability of the species of bacteria which he isolated from raw cane sugars, and which he named *Bacillus levaniformans*, Greig Smith (1901, 1902 a) discovered that it produced this gum only from sucrose. Neither glucose nor levulose, nor any combination of these sugars, was suitable as material for the formation of gum by these organisms. He concluded therefore that gum levan could only be formed from glucose or levulose in their nascent condition, thus assuming the action of invertase, where its presence had never been proved. The proof or disproof of this theory seemed relatively simple to the writer, who several years ago carried out some experiments in which levan formation in the presence and absence of growing yeasts was compared (1911 a and b). The results of these experiments showed that the amount of gum levan formed from the mixed inoculation of yeast and bacteria was never more than one-half, and frequently only one-tenth of that formed by pure cultures of the bacteria. These results showed that the relationship between yeast and bacteria was not a symbiotic one, and suggested that the presence of invertase deprived the bacteria of a suitable form of sugar for gum production. The conditions of those experiments were not entirely free from criticism, and the conclusions drawn from them might be objected to on the possible grounds that the relationship between the yeast and the bacteria were competitive. The decreased production of levan might be regarded as due to the inhibiting effect of the yeast upon the development of the bacteria rather than to the transformation by the former of the sucrose into sugars that the bacteria could not utilize. In order to remove such objections it was decided to use invertase instead of inoculation with yeasts. The amount of invertase added was to be so regulated that the

bacteria would have a slowly diminishing supply of sucrose to act upon, or if, as has been claimed, nascent glucose or levulose is the source of the gum, the microorganisms would be provided with a steady supply of this material in quantities commensurate with their power of assimilation.

PLAN OF THE EXPERIMENTS

The culture solution used for testing the gum-forming power of the bacteria was that used by Greig Smith (1902 a). The formula was modified as regards its sucrose content, as we used a 20 per cent instead of a 10 per cent sucrose solution. This was necessary in order that the period of inversion might be sufficiently prolonged so that it might proceed throughout the entire incubation period. The formula was as follows:

	per cent
Sucrose	20
KCl	0.5
Na_2HPO_4	0.2
Pepton	0.1

Two hundred cubic centimeter portions of this solution were poured into 300 cc. Erlenmeyer flasks, which were plugged with cotton and sterilized for thirty minutes on each of three successive days. The contents of the flasks were then inoculated with a pure culture of the levan-forming bacteria that had been isolated from a raw sugar. The inoculations were always made from forty-eight-hour cultures of the bacteria in the above solution. The invertase used in the experiments had been prepared by Kopeloff (1920) from Fleischmann's yeast. The invertase was added to the flasks with a sterile pipette, and they were then incubated for a period of ten days at 32°C. The analytical determinations were made as follows: The densities were determined by the Abbé refractometer, and expressed in degrees Brix. Sucrose was determined by the polariscope, the Clerget method of double polarization being used, and reducing sugars determined by the volumetric method of copper reduction, using Soxhlet's solution and Ross's (1912) method of testing for unreduced copper. For the determination of gum levan the

method of Kopeloff and Taggart (1920) was used. This method consists in calculating the levan present from the difference between the Clerget values by the acid and invertase method of inversion. The factor of 1.27 is used to convert this difference in values into the per cent of gum. This factor is derived from the change in specific rotation of gum levan from −40 to −92.5 when converted into fructose by hydrolysis with the acid used for inversion. This difference of −52.5 between the Clerget and the invertase values in the specific rotation of gum corresponds to 0.787 of the specific rotation of sucrose, which is 66.5. Hence 1.27 per cent of gum will indicate on hydrolysis by the Clerget method of inversion 1 per cent of sucrose in excess of the amount actually present. In the following tables are given the results of the experiments on the production of gum levan by bacteria in the presence of invertase. The abbreviations used in the tables are as follows:

TABLE 1

The results from Experiment 1 in which invertase was used in the following amounts, 0.1, 0.25, 0.5, 0.75, and 1 cc.

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE I.R.	INVERTASE S.C.	PER CENT GUM	PER CENT R.S.
Inoculated, 1 cc. bacteria, 1 cc. invertase	23.19	−5.1	−5.2	0.48	−5.0	0.28	0.25	22.00
Inoculated, 1 cc. bacteria, 0.75 cc. invertase	22.00	−4.6	−5.7	1.28	−4.7	0.44	1.06	21.17
Inoculated, 0.5 cc. invertase	22.08	−3.1	−5.5	2.30	−4.1	1.09	1.53	20.00
0.25 cc. invertase	22.08	−2.0	−5.6	3.24	−2.5	1.40	2.28	16.40
0.1 cc. invertase	23.19	−2.5	−5.5	3.91	−3.5	1.04	2.18	16.40
Control	22.14	21.0	−5.7	21.27	−5.4	20.75		

S.P. = single polarization
I.R. = invert reading
S.C. = sucrose clerget
R.S. = per cent reducing sugars

The results in table 1 show that the production of gum by the bacteria is in inverse proportion to the amount of invertase added. In only one case did this inverse relationship between invertase added, and gum produced, fail to exist, and that was in the case of the addition of 0.25 and 0.1 cc. These results agree fairly well with those we obtained in our earlier work (1911 a, 1918) in which we used joint inoculations of yeast and bacteria. It is obvious that in both cases the transformation of sucrose, either by invertase added direct, or secreted by the yeast, tends to deprive the bacteria of the material from which they produce gum levan. The following table shows the results

TABLE 2

Results of experiment in which gum production in the presence of yeast and bacteria was determined

SERIES	CULTURE	ACIDITY—20 CC. USED; CC. N/10 K.OH REQUIRED	REDUCING SUGAR	SINGLE POLARIZATION	SUCROSE (CLERGET)	GUM
	Control	0.7		9.3	9.73	
A. (1)	B. IV	4.1	4.25	4.5	5.89	2.69
B. (1)	B. IV and yeast	8.5	7.02	−0.8	2.08	1.47
C. (1)	Yeast	6.6	7.28	−0.6	2.32	
	Control	0.8		9.3	9.44	
A. (2)	B. IV	3.8	6.78	−0.3	1.88	4.85
B. (2)	B. IV and yeast	9.1	2.27	−2.8		0.39
C. (2)	Yeast	5.4	6.25	−4.5		

of one of the earlier experiments in which gum production in the presence of yeast and bacteria was determined.

In table 2 it will be seen that the presence of the yeast in the sucrose solution decreased the amount of gum produced by the bacteria by approximately 50 per cent in one case and almost 90 per cent in another.

In studying the production of gum levan by bacteria in the presence of invertase, it is of great importance to so regulate the amount of invertase added that the inversion will proceed at a regular rate throughout the entire incubation period. If the process is too rapid and glucose and levulose are formed faster than the bacteria can utilize them while in the nascent con-

dition, then the addition of the invertase might be regarded as a disadvantage to the bacteria, even in the light of Greig Smith's theory. In the following experiment the rate of action of the varying amounts of invertase added was measured, and the results are given in table 3.

It will be noted from the results given in table 3 that while the addition of 1 cc. of invertase resulted in an inversion of

TABLE 3

The results of daily analyses showing the rate of action of invertase in experiment 2

SAMPLE	FIRST 24 HOURS		48 HOURS		72 HOURS		96 HOURS		120 HOURS		168 HOURS	
	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.	S.P.	S.C.
1 cc. invertase....	14.7	15.87	11.5	13.37	8.3	10.99	6.0	8.5	4.2	7.92	0.3	4.72
0.5 cc. invertase..	17.1	17.82	15.3	16.27	9.7	12.05						
0.25 cc. invertase.	18.3	18.75	17.3	18.13	14.8	16.1	12.6	14.42	10.8	13.07	6.4	8.5
0.1 cc. invertase..	19.0	19.38	18.2	18.65	17.4	18.04	16.1	17.20	15.0	16.3	12.6	14.46

TABLE 4

The results of experiment 2 on the formation of gum levan in the presence of invertase

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
No invertase, 1 cc. bacteria.....	21.9	−8.6	2.9	11.92	−2.1	9.37	3.23	8
0.1 cc. invertase.....	21.9	−3.4	−3.3	2.92	−2.5	1.60	1.67	18
0.25 cc. invertase.....	21.9	−4.0	−3.0	1.96	−2.3	0.7	1.60	17
0.5 cc. invertase.....	21.9	−6.0	−3.2	0.7	−3.1	0.62	0.1	20
1 cc. invertase.....	21.9	6.6	−3.0	0	−3.2			20
Control.....	21.3	19.0	−5.8	19.27	−3.3	20		

sucrose at the rate of 2.5 per cent per day, the addition of 0.5 per cent caused inversion at the rate of 1.5 per cent of sucrose daily, and the smaller additions at correspondingly slower rates. While the addition of 1 cc. would have resulted in the exhaustion of the sucrose before the end of the ten-day incubation period, the smaller additions should have furnished ideal conditions for the maintenance of a constant supply of nascent glucose and levulose. The results of the experiment are given in table 4.

The results in table 4 tend still further to confirm the theory that sucrose, rather than the nascent products of its inversion, is the source of gum levan. If we compare the rate of action of the various additions of invertase as shown in the above table, with those shown in the previous one, where the invertase was acting in the absence of bacteria, we find that the rate of action was much more rapid in the present experiment. For example, where 1 cc. of invertase had been used alone, only about 16 per cent of the sucrose was inverted in one week, while the same amount when used with the bacterial inoculation resulted in the almost complete inversion of the sucrose in a period of ten days. The more rapid action under the latter conditions is due to the production of acids by the bacteria, which makes the condition more favorable for invertase action. The rate of action of invertase in the presence and absence of bacteria was compared in the next experiment, in which larger inoculations of bacteria were used. Instead of a platinum loop, 1 cc. portions of a 48-hour old bouillon culture were employed, in order that the bacteria might have a better opportunity to utilize the invert sugar as rapidly as it is formed. The results are given in table 5.

The accelerative action of the growing bacteria upon the inversion of sucrose by the added invertase is very clearly shown in table 5. While 0.25 cc. of invertase had only inverted about 16 per cent of the sucrose in the 20 per cent solution in one week, the addition of an equal quantity of invertase had inverted 85 per cent of the sucrose when acting in the presence of bacteria for the same length of time. While larger inoculations of bacteria had resulted in an increase in gum production as compared with previous experiments, the ratio between the amounts formed in the presence and absence of invertase was similar to that found in the previous experiments.

The additions of the small amounts of invertase used in the previous experiments could scarcely be expected to inhibit the development of bacteria. However, this possibility was tested in the following experiment, in which the solutions were plated out on a 10 per cent sucrose agar at the end of the incubation period.

TABLE 5

The results of experiment 3 showing the rate of action of invertase in the inoculated and uninoculated sucrose solutions

SAMPLE	48 HOURS			72 HOURS			96 HOURS			144 HOURS			7 DAYS		
	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.
A. invertase only	17.3	−6.3	18.4	15.9	−6.4	17.42	13.8	−5.5	15.20	9.9	−6.2	12.89	9.0	−6.7	12.38
B. 0.25 cc. invertase, 1 cc. bacteria	16.4	−6.3	17.69	12.3	−6.3	14.59	6.2	−5.9	9.71	−2.4	−6.1	3.31	−3.0	−6.3	2.97
C. bacteria only	20.0	−6.4	20.5	19.3	−6.2	19.7	17.7	−5.7	18.3	9.4	−5.8	11.23	6.0	−6.3	9.78

	8 DAYS			10 DAYS			PER CENT R.S.	INVERTASE			PER CENT GUM	SOLIDS
	S.P.	I.R.	S.C.	S.P.	I.R.	S.C.		S.P.	I.R.	S.C.		
A. invertase only	7.0	−6.5	10.74	5.4	−3.5	9.96	12.9		−6.0	9.2		23.02
B. 0.25 cc. invertase, 1 cc. bacteria	3.6	−6.4	2.61	−4.6	−3.3	2.02	20.0		4.4	0.18	2.33	23.05
C. bacteria only	3.4	−6.6	8.01	−8.0	−3.4	5.11	14.9		−0.8	0.74	5.54	22.75

It will be observed from table 6 that the numbers of bacteria in the solutions containing invertase are higher in some cases than where no invertase is present. Since the largest number of bacteria occurred in one case where the largest amount of invertase had been added, the apparent decreases in the other cases where smaller amounts of invertase had been added, cannot be attributed to the inhibiting action of the invertase. Even in the flasks where the bacteria occurred in the smallest numbers,

TABLE 6

The results of experiment 4 showing the development of bacteria and the production of gum levan in the presence and absence of invertase

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	NUMBER PER CUBIC CENTIMETER	S.P.	I.R.	S.C.	PER CENT R.S.	I.R.	S.C.	PER CENT GUM
Control	23.12		20.8	−6.1	21.15	Trace	−5.5	20.77	
Inoculated, 1 cc. bacteria only	22.92	11,800,000	−0.8	−6.0	4.42	15.3	−2.2	1.24	4.03
Inoculated, + 1 cc. invertase	23.12	14,200,000	−2.6	−6.3	3.3	16.9	−1.9	1.21	2.65
Inoculated, + 0.25 cc. invertase	23.16	9,320,000	−4.9	−6.3	1.54	20.0	−5.0	0.57	1.27
Inoculated, + 0.5 cc. invertase	23.46	11,400,000	−5.8	−6.3	1.06	20.4	−5.5	0.1	1.21
Inoculated, 1 cc. invertase	23.49	7,710,000	−6.5	−6.4	0.41	20.6	−5.5	0	0

they were sufficiently numerous to have produced large amounts of gum levan, had other conditions been favorable.

The larger inoculations of bacteria used in this experiment resulted in the production of gum levan in the solution containing 0.5 cc. of invertase, whereas in a previous experiment only a trace of gum had been formed by the bacteria in the presence of that amount of the enzyme.

The results of the previous experiments show that not only does invertase decrease the amount of gum formed, in proportion to the amount of sucrose inverted, but that the remaining

sucrose is utilized to a smaller extent than would be the case if this same amount of sucrose had been available to the bacteria, with no invertase present. This would indicate that the products of the inversion of sucrose hinder rather than aid the bacteria in the production of gum. This fact was observed in our earlier investigations in which various mixtures of sucrose and glucose and levulose were used. The results from those experiments showed that gum production was decreased by the presence of invert sugars, or in other words, that a high ratio between sucrose and invert sugar is most favorable for this type of fermentation. In order to test this point further, the following experiment was conducted. A 20 per cent sucrose solution was inverted by the addition of 4.5 cc. of invertase to 850 cc. of the solution, and allowing it to stand in an incubator at 32°C. for two days. At the end of this period its sucrose content was 2.24, and it was then mixed with an equal volume of an uninverted 20 per cent Greig Smith solution, containing all of the nutrient material in such proportions that after mixing the two solutions, the mixture would conform to the original formula. This solution was then flasked and inoculated, and compared with

TABLE 7

The results of experiment 5 showing the production of gum levan in the presence of reducing sugars

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	PER CENT R.S.	INVERTASE		PER CENT GUM
						I.R.	S.C.	
Inoculated bacteria	22.53	7.8	−6.0	10.97	10.8	−2.86	8.5	3.13
0.25 cc. invertase, inoculated, bacteria	22.82	−5.3	−6.2	1.15	19.5	−4.18		1.55
Half inverted solution, inoculated, bacteria	17.19	+3.2	−4.7	6.38	10.0	−5.06	6.3	
Control sucrose solution	21.70	18.8	−6.0	19.29	Trace	−6.6	19.24	
Control, half inverted solution	17.25	8.4	−4.6	10.26	5.7	−5.2	10.50	
Half inverted solution, inoculated, bacteria 0.25 cc. invertase	17.22	−4.8	−4.7	0.28	16.4	−4.62	0	

a 20 per cent sucrose solution inoculated in a similar manner, and kept under the same conditions.

It will be seen from table 7 that the bacteria formed no gum in the solution containing invert sugar and sucrose, although there was 10 per cent of sucrose present. The relative amounts of gum formed in the sucrose solution with and without invertase were approximately the same as in the previous experiments.

THE PRODUCTION OF GUM LEVAN UNDER VARYING CONDITIONS OF H-ION CONCENTRATION

In the preceding experiments the solution used was approximately neutral in reaction. In previous investigations it had been found that a neutral reaction was most favorable for the gum levan fermentation, but the optimum H-ion concentration had not been determined. In order to determine this point, and to learn whether the optimum for gum production and invertase action coincided, the following experiment was conducted. A 10 per cent sucrose solution (Smith) was prepared and flasked in 200 cc. portions. These were sterilized by the intermittent method, and after the third sterilization and while the contents of the flasks were still warm, measured portions of N/1 NaOH and H_2SO_4 were added. The flasks were then inoculated the invertase added as before. At the end of the ten-day period and analyses were made and the pH values determined on the controls by the potentiometer. The results are given in table 8.

The pH range in this series (table 8) varied from 6.7 to 9.5, and the greatest gum production occurred in the more acid series, when invertase was absent, but in the presence of invertase the amount of gum produced in that series was less than one-tenth of that produced in its absence. In the series containing 0.5 cc. of N/1 NaOH, the gum produced in the presence of invertase was approximately half as great as that produced in the absence of invertase, and as the NaOH was increased a point was reached where the invertase was inactive, and the gum produced in its presence actually greater than where no invertase was added. This beneficial effect of the invertase was probably due to the fact that it protected the bacteria against the action

TABLE 8

The results of experiment 6 showing the development of gum in the presence and absence of invertase in solutions of varying H-ion concentration

SAMPLE	pH	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
						I.R.	S.C.		
Blank, inoculated *B. vulgatus*	7.4	11.49	−1.0	−2.8	1.66	−1.6	0.4	1.98	7.75
Blank, inoculated culture XI.	7.4	11.27	−1.2	−2.8	1.00	−1.6	0.28	1.80	8.29
Blank, 0.5 cc. invertase inoculated, culture XI.	7.4	11.23	−2.8	−3.0	0.38	−2.0		0.48	8.82
0.5 NaOH, inoculated, culture XI	8.6	11.27	−1.2	−2.4	1.10	−1.6	0.36	1.34	7.86
0.5 NaOH, 0.5 cc. invertase, Culture XI	8.6	11.66	−2.0	−2.4	0.49	−1.6		0.62	8.54
0.5 NaOH, inoculated, *vulgatus*	8.6	11.60	−1.6	−2.8	1.15	−1.6		1.77	8.77
O.5 NaOH, control, 0.5 cc. invertase	8.6	11.66	8.0	−3.6	9.18	−2.4	8.3		2.00
1.2 cc. NaOH, inoculated, culture XI	9.5	11.60	1.6	−3.6	1.81	−1.2		2.29	7.24
1.2 cc. NaOH, inoculated, 0.5 cc. culture XI, invertase	9.5	11.63	−1.6	−3.8	1.98	−1.6		2.51	
1.2 NaOH, inoculated, *vulgatus*	9.5	11.33	9.2	−4.0	10.44	−2.8	9.65	1.00	
1.2 NaOH, control, 0.5 cc. invertase	9.5	11.66	8.8	−3.8	9.97				
2.4 NaOH, inoculated, culture XI		11.63	10.0	3.6	10.72	−3.2	10.6		
2.4 NaOH, 0.5 cc. invertase.		11.37	10.0	3.6	10.72	−3.6	10.9		
2.4 NaOH, 0.5 cc. invertase, control		11.70	10.0	−3.6	10.72	−2.8	10.2		
4.8 NaOH, inoculated, culture XI		11.37	10.0	−4.0	11.06	−2.8	10.2		
4.8 NaOH, 0.5 cc. invertase, control		11.70	9.6	−3.6	10.41	−3.2	10.27		
1.2 cc. N/2H_2SO_4, inoculated, culture XI	6.7	11.40	−1.2	−3.6	2.12	−1.2		2.57	9.26
1.2 N/2 H_2SO_4, inoculated, *B. vulgatus*	6.7	11.40	−1.6	−3.6	1.81	−1.2		2.29	9.26
1.2 N/2 H_2SO_4, 0.5 cc. invertase, inoculated, culture XI	6.7	11.63	−3.2	3.2	0.41	−1.6		0.17	8.33
1.2 N/2 H_2SO_4, 0.5 cc. invertase, control	6.7	11.66	−2.8	−4.0	1.22	−2.8	0.2	0.129	9.2
Control	6.7	11.40	10.4	2.0	11.3	−0.7	10.5		
Control	6.7	11.70	10.0	2.6	11.06	−0.7	10.24		

of the alkali. In the next experiment a series of solutions were prepared having a pH range from 4.8 to 9.5.[1] The results are given in table 9.

The maximum gum production in this experiment (table 9) occurred in the series with a pH of 7.4. The gum production in

TABLE 9

The results of experiment 7 showing the development of gum in the presence and absence of invertase in solutions of varying pH

SAMPLE	pH	TOTAL SOLIDS BY REFRACTOMETER	S.P.	ACID INVERSION		INVERTASE		PER CENT GUM	PER CENT R.S.
				I.R.	S.C.	I.R.	S.C.		
Blank, inoculated, *B. vulgatus*	7.4	12.44	−2.8	−7.2	3.90	−4.0	1.2	3.43	9.00
Blank, inoculated, *B. vulgatus*, 0.5 cc. invertase	7.4	13.09	−4.4	−4.8	0.67	−1.1	0.33	0.42	12.5
Blank, not inoculated, 0.5 cc. invertase	7.4	13.09	−4.0	−7.2	2.99	−6.4	2.35		11.76
Blank, control	7.4	12.44	9.6	−4.8	11.38	−4.0	10.7		
1.2 cc. NaOH, 200 cc., inoculated, *B. vulgatus*	9.5	12.69	6.0	−5.6	9.30	−4.0	8.08	1.54	2.74
1.2 cc. NaOH, inoculated, culture XI, 0.5 cc. invertase	9.5	12.48	−2.4	−5.6	2.87	−4.0	1.5	1.60	7.14
1.2 NaOH, inoculated, culture XI	9.5	12.48	9.6	−6.4	10.07	−4.4	11.1		
0.5 cc. invertase, 1.2 cc. NaOH, not inoculated	9.5	12.48	8.4	−5.6	8.78	4.8	10.5		1.37
2.4 cc. N/2H_2SO_4, inoculated, culture XI	4.8	12.48	4.4	−4.8	7.40	−4.4	5.25	2.73	3.80
2.4 cc. N/2H_2SO_4, 0.5 cc. invertase inoculated, culture XI	4.8	13.13	−3.2	−4.0	0.91	−4.4	1.21	0.88	12.12
2.4 cc. N/2 H_2SO_4, not inoculated, 0.5 cc. invertase	4.8	12.69	−4.4	−4.0	0	−4.4	0.3		11.76

the series with a pH of 4.8 was greater than in the series having a pH of 9.5. These results show that while the bacteria forming

[1] The writer is indebted to Dr. J. F. Brewster and Mr. W. G. Raines, Jr., of the research Chemical Department, for their kind coöperation in making the pH determinations in the above experiments.

levan from sucrose have a wide range for their activities, their optimum pH does not coincide with the optimum for invertase action. In the presence of invertase, the maximum gum production occurs under conditions where the pH most completely restrains inversion, and the minimum gum production occurs where the conditions are most favorable for it.

THE PRODUCTION OF GUM LEVAN IN RAW AND CLARIFIED SUGAR CANE JUICE

The gum fermentation of cane juices is frequently a problem of considerable economic importance, since it renders the product much more difficult to clarify, and decreases the yields of sugar obtainable from it. An experiment was carried out to determine the relative susceptibility of raw, sulphured, and limed juice to this type of fermentation. A large supply of raw juice was obtained, and divided into two parts, one of which was sulphured to 5.9 cc. (10 cc. of the juice requiring 5.9 cc. of N/10 NaOH to neutralize it). A portion of this sulphured juice was then treated with milk of lime until the acidity had been reduced to 0.5 cc. The original untreated juice made up the third series of the experiment. The pH range was from 3.8 to 6.9. The samples were sterilized on three successive days at 30°, inoculated and incubated as in previous experiments.

The results of table 10 show that the production of gum levan by all three of the cultures used was greater in the limed than in either the raw or the sulphured juice. While the pH of the raw juice was not determined, the average for Louisiana juices is about 5. The acidity of this sample was 1.85 cc., which would indicate a normal juice. From these data it is clear that a clarified juice is much more favorable in its reaction for the production of gum levan than either raw or sulphured juices.

THE SPECIES OF BACTERIA CONCERNED IN THE PRODUCTION OF LEVAN FROM SUCROSE

We have already referred to the fact that Greig Smith (1901, 1902 a), who was one of the first to investigate the bacterial

flora of sugars, gave the name of *Bacillus levaniformans* to the gum forming bacteria that he isolated therefrom. He pointed out the marked similarities between this species and the potato bacillus, especially in regard to the great resistance of its spores to heat, and the characteristic mesentery like growth upon potatoes. The writer in his previous investigation of this sub-

TABLE 10

The results of experiment 7, showing the formation of gum levan in raw, sulphured, and limed cane juice

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	PER CENT R. S.	ACID INVERSION SUCROSE			INVERTASE SUCROSE		PER CENT GUM
			S.P.	I.R.	S.C.	I.R.	S.C.	
1.85 cc. Acid, raw juice, control	16.50	4.00	9.82	4.20	11.09	4.4	11.3	
Raw juice, inoculated, culture XI	15.64	10.4	−0.2	−2.8	2.21	−1.2	1.8	0.6
Raw juice, inoculated, *B. vulgatus*	14.81	10.0	−0.2	−0.4	0.52	−0.6	0.86	
Raw juice, inoculated, potato culture	14.51	7.5	1.4	−4.4	2.65	−1.6	1.6	1.33
Sulphured to 1 cc., 5.9 cc. acid, control	16.96	14.28	2.2	1.61	1.01	1.8	1.2	
Sulphured, inoculated, potato culture	16.96	13.7	2.00	1.80	1.51	2.0	1.7	
Sulphured, inoculated, *B. vulgatus*	16.96	13.2	2.2	2.05	1.85	2.0	1.7	
Sulphured, inoculated, culture XI	16.70	12.7	2.2	1.60	1.01	2.0	1.7	
Limed, 0.5 cc. acid, cold, control.	16.70	3.14	10.6	−1.8	11.18	−1.6	11.40	
Limed, inoculated, *B. vulgatus*	16.70	11.4	2.6	2.2	5.72	−1.6	3.37	2.98
Limed, inoculated potato culture	14.85	7.28	1.4	4.8	5.14	−1.6	2.4	3.47
Inoculated, culture XI	16.70	10.6	2.2	−2.2	5.41	−3.2	4.42	1.28

ject found that *Bacillus vulgatus* obtained from the Museum of Natural History of New York City, had the ability to produce gum levan from sucrose, and was in all other respects identical with the cultures isolated from sugars. The other species belonging to the potato group of bacteria, viz., *Bacillus mesentericus-fuscus* and *Bacillus liodermos*, seemed to have little if any such ability. It was found that this levan-forming ability on the

part of *Bacillus vulgatus* could be rapidly increased by frequent transfers in sucrose solutions. After once acquiring this ability, the microörganism retains it apparently, with but little loss. As the gum determinations in the previous investigation were made simply by a measure of the viscosity of the solution, it was decided to repeat them in connection with this investigation, using a more reliable method of gum determination. For this purpose cultures of Bacillus *vulgatus-mesentericus fuscus* and *B. liodermos* were obtained from the Museum of Natural History New York, through the courtesy of Dr. C.-E. A. Winslow. These cultures were used to inoculate flasks containing 200 cc. of a 10 per cent sucrose solution. The inoculations were made from tubes of the same solution, transfers from which were made to fresh sterile solution every forty-eight hours. The cultures that were obtained from sugar were transferred similarly to tubes of sterile bouillon containing no sucrose, or other sugar. These transfers were kept up for a week, at the end of which time a second set of flasks were inoculated. Table 11 shows the production of gum in the first experiment.

The results in table 11 show that the production of gum levan by *Bacillus vulgatus* is much less than that produced by the cultures obtained from sugars. An old culture that had been kept in the form of a spore preparation on a cover glass, produced almost twice as much gum as the former. The latter culture had been repeatedly used in sucrose solutions.

After transplanting the culture as above described, a second inoculation was made with the results indicated in table 12.

From the results of this experiment (table 12), it will be seen that the production of gum by *Bacillus vulgatus* is exactly three times as great as in the former case. There was considerable variation in the effect of the repeated propagation upon the gum-forming powers of the cultures from sugar. As the first experiment might not have been entirely representative, a comparison with the following experiment (table 13) will probably offer a more reliable indication of the effect of these transplantations in a substratum containing no sugars.

TABLE 11

The results of experiment 8, showing the production of gum from sucrose by Bacillus vulgatus when first grown in sucrose solution

CULTURE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	PER CENT R.S.	INVERTASE		PER CENT GUM
						I.R.	S.C.	
VII	13.16	−3.0	−2.15	1.36	5.4	−1.5	0.2	1.39
XI	12.17	−2.8	−1.85	0.95	7.6	−1.4	0.2	0.88
XVI	13.19	−2.2	−2.0	1.65	7.09	−1.1	0.01	2.08
XVII	13.94	−1.6	−2.4	2.76	7.4	−1.2	0.07	3.41
Old culture, *B. vulgatus*	13.19	−3.4	−2.2	1.08	9.0	−1.2	0	1.37
Control	13.84	8.6	−2.6	10.83		−3.3	11.5	
B. vulgatus	13.74	7.6	−2.4	9.70	0.9	−2.0	9.1	0.7
Culture I, old series, spore preparation	14.09	1.0	−2.4	4.73	6.9	−1.5	1.7	3.84
Culture II	13.74	−3.0	−2.2	1.38	8.7	−1.5	0.23	1.46
Culture IV	13.51	−0.8	−2.35	3.3	8.6	−1.7	2.2	1.39
Culture V	14.16	−3.4	−2.2	1.08	8.7	−1.6	0.09	1.25
Culture VI	15.70	−4.0	−2.6	1.29	8.54	−1.6	0	1.63

TABLE 12

The results of experiment 9, showing the increased ability to produce gum levan by Bacillus vulgatus after repeated transfers in sucrose solutions

SAMPLE CULTURE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
Culture I	11.69	+0.6	−4.0	3.8	−1.4	1.65	2.73	7.5
Culture II	10.36	−1.2	−3.2	1.78	−1.2		2.27	7.27
Culture IV	11.60	−1.8	−2.8	0.9	−2.4	0.6		9.0
Culture V	10.36	0.8	−4.8	4.58	−2.8	3.00	2.00	5.8
Culture VI	11.67	−1.2	−4.8	3.04	−2.8	1.45	1.91	7.5
Culture XI	11.08	−0.4	−4.8	3.74	−2.2	1.39	2.98	8.7
Culture XIII	11.45	2.6	−4.4	5.69	−2.8	4.3	1.86	5.9
Culture XIV	11.45	−1.0	−5.4	3.78	−2.0	0.9	3.75	8.5
Culture XVI	11.45	−2.4	−3.6	1.19	−2.0	0	1.51	8.7
Vulgatus	11.01	2.0	−4.8	5.57	−2.8	3.91	2.10	3.5
Mes. fuscus	10.50	7.8	−3.2	8.66	−3.4	8.8		
Old vulgatus	10.43	−1.6	−4.4	2.48	−2.8	1.18	1.65	7.4
Liodermos	10.22	8.6	−3.0	9.14	1.7	9.55		

It is particularly interesting to note from the results in table 13 that six of the cultures isolated from sugars had decreased in their levan-forming power, and one seemed to have lost it entirely. The old *Bacillus vulgatus* produced only one-fourth as much gum as in the first experiment, and no further increase resulted from the transfers of the new *Bacillus vulgatus* culture.

TABLE 13

The results of experiment 10, showing the effect of repeated transfers in sucrose and nonsucrose media upon the ability of Bacilus vulgatus to produce gum levan

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	INVERTASE		PER CENT GUM	PER CENT R.S.
					I.R.	S.C.		
Culture I, no phosphate	12.85	2.4	−2.8	4.09	−0.8	2.5	2.01	8.5
Culture II, phosphate media	12.95	3.2	−4.0	5.72	1.2	3.48	2.84	9.09
Culture II, phosphate	13.22	1.6	−5.2	5.51	−2.0	2.94	3.26	8.11
Culture II, no phosphate	13.23	2.4	0.9	4.79	−2.0	3.56	1.56	7.33
Culture IV	13.23	1.6	1.1	4.85	−2.8	3.62	1.56	8.69
Culture V	13.23	2.4	−1.2	5.78	−2.8	4.24	1.95	7.84
Culture XI	13.23	1.6	1.1	4.85	−1.2	2.26	3.30	8.69
Culture XIII	13.23	11.2	0.9	11.4	−3.2	11.39		
Culture XIV	12.27	1.6	1.1	5.78	−2.4	3.28	3.17	9.09
Culture XVI	11.66	10.4	1.1	10.53	−2.8	10.39		
Old culture, vulgatus	11.66	2.4	0.9	4.81	−3.2	4.56	0.32	6.94
Culture, *B. vulgatus*	12.31	3.2	1.1	5.32	−1.6	3.82	1.90	7.89
B. liodermos	11.66	11.2	1.1	11.8	−3.2	11.3		
B. mesentericus	11.36	11.2	1.0	11.8	−3.6	11.6		
Culture from potato, 2 transfers	12.56	3.2	1.1	6.08	−2.8	4.84	1.44	7.24
Control	11.67	2.6	1.3	10.49	−3.2	10.6		

From these results one is led to conclude that the production of gum levan by the *Bacillus vulgatus* is a power that is easily acquired and fairly easily lost, until it has become thoroughly established, when it appears that it is held rather tenaciously. This would explain why the old culture of *Bacillus vulgatus* almost entirely lost its gum-producing power, when grown in sugar free media, and why some of the cultures from sugar reacted in a similar way, and why others retained their power

under these same conditions. In addition to being a test of acquiring and losing gum-producing power, this experiment is also a test of the value of phosphates in promoting this type of fermentation. The first four lines of the table show a comparison of gum formation by culture no. 1 and 11 in a solution with and without phosphates. It will be observed that the presence of phosphates greatly promotes gum formation. In the latter part of the table it will be noted that a culture freshly isolated from potato was used, and that it produced 1.44 per cent of gum. The method used to isolate the culture was as follows: A potato was thoroughly cleaned on the outside with a brush. The peelings were then put in a 300 cc. Erlenmeyer flask containing some sterile sucrose solution. The flask was then placed in the incubator, and after growth had taken place, transfers were made to tubes of the same medium. Sucrose agar plates were then made and a pure culture obtained.

It will be noted that neither in this nor in the previous experiment was any gum formed by *Bacillus liodermos* or *Bacillus mesentericus-fuscus*. This is approximately the same conclusion that we reached in the previous investigation to which we have referred.

In order to determine whether the acquisition of gum-producing power is more rapid when the bacteria are transferred to sucrose solutions of higher densities than the 10 per cent that we had been using, a comparison was made between a 10 per cent and a 20 per cent solution.

The cultures used in this experiment (table 14) were the *Bacillus vulgatus* and the culture isolated from a potato, which we had used in the preceding experiment. It will be noted that the gum-producing power of the former culture was much greater after being grown in the 20 per cent sucrose solution. The other culture showed a slightly greater gum-forming power after being grown upon the 10 per cent solution. The results of our earlier investigation had very strongly indicated that the gum-forming power is acquired more rapidly by these species of bacteria, when they are grown in concentrated sucrose solutions.

The conditions for acquiring this property are to be found in cane sugar factories. The gum-forming bacteria are brought to the mill in the trash and particles of soil adhering to the cane. When the cane is crushed they are carried into the juice, and their spores survive the temperatures attained during the boiling and evaporating of the juice, and the crystallization of the sirup.

TABLE 14

The results of experiment 11, showing the relative rate at which gum-forming ability is acquired in 10 and 20 per cent sucrose solutions

SAMPLE	TOTAL SOLIDS BY REFRACTOMETER	S.P.	I.R.	S.C.	PER CENT GUM	INVERTASE		PER CENT R.S.
						I.R.	S.C.	
Control	12.16	11.0	−3.2	11.02		−2.0	10.43	
Control	11.82	10.8	−2.8	10.54		−2.0	9.9	
Inoculated, *B. vulgatus*, 10 per cent sucrose solution	11.50	−0.8	−2.4	1.38	1.25	−1.2	0.39	7.35
Inoculated, *B. vulgatus*, 20 per cent sucrose solution	11.82	−0.8	−4.0	2.70	2.92	−1.2	0.4	7.8
Potato culture, 10 per cent sucrose solution	11.83	−0.4	−5.2	3.99	4.35	−1.2	0.4	7.1
Potato culture, 20 per cent sucrose solution	11.58	+0.4	−5.0	4.4	3.49	−1.6	1.65	6.94
B. mesentericus, 10 per cent sucrose solution	11.07	10.2	−2.6	9.91		−3.6	10.7	
B. mesentericus, 20 per cent sucrose solution	11.58	10.8	−2.9	10.62		−3.2	10.92	
B. liodermos, 10 per cent sucrose solution	11.58	9.8	−2.6	9.61		−2.8	9.8	
B. liodermos, 20 per cent sucrose sulution	11.58	10.6	−2.6	10.22		−2.8	10.4	

The great resistance of the spores of these bacteria to heat, and their ability to withstand the osmotic pressure of the high density sirups and molasses to which they are subjected, fit them admirably for this environment. The potato group of bacteria are enabled to withstand this osmotic pressure, owing to the fact that they have permeable (Fischer, 1903) cell membranes, and hence their cell walls are not easily plasmolyzed. It is on this

account that we find some of these species capable of producing gum levan in sucrose solutions of 50 to 60 per cent. We conclude therefore that the soils surrounding cane sugar factories will gradually become inoculated with potato bacteria which have acquired the ability of forming gum levan from sucrose, owing to the practice of returning the filter press cake to the soil. The final molasses from cane factories, when fed to the stock on the plantation, would probably result in infecting the manure with these gum-forming species, which would again find their way back to the soil in the manure. Hence there will be a continuous cycle of acclimatization and distribution of these organisms on a sugar plantation.

THE PRODUCTION OF GUM LEVAN FROM SUCROSE IN ITS RELATION TO THE POTATO GROUP OF BACTERIA

We have already referred to the fact that in morphological and physiological characteristics the bacteria isolated from sugars are, with the exception of the ability to form gum levan from sucrose, identical with the potato species. The potato group of bacteria comprise the following species: *Bacillus vulgatus* (Trevisan), (*Bacillus mesentericus vulgatus* (Flugge) and potato bacillus of various authors), *Bacillus mesentericus fuscus* (Flugge), (*Bacillus mesentericus* (Trevisan) and *Bacillus liodermos* (Flugge), (Chester, 1901)). No data are given by Chester on the behavior of these species in sucrose solutions, although a closely related species *Bacterium panis* (*Bacterium mesentericus, panis-viscosus*), which was first isolated from stringy dough is said to produce a viscous fermentation of bread dough. Migula (1897) classes *Bacillus liodermos* as identical with the gum bacillus of Loeffler. Chester (1903) who studied *Bacillus mesentericus*, mentions only the production of acid in sucrose solutions. However, he also describes a culture of *Bacillus mesentericus fuscus* obtained from the Bacteriological Laboratory of the Johns Hopkins University, which he named *Bacillus subtilis* var. *viscosus*. The following description of the growth of this species on agar very strikingly suggests the species we have under investigation. "*Agar cultures have the same thick glassy viscid appearance and the growth*

can be drawn out into long threads, or behaves under a needle-like thick mucus." Lohnis (1913) refers to the activities of certain gum-forming varieties of the potato bacillus, in the viscous fermentation of the diffusion juice in beet sugar factories. From a review of the literature on the physiological characteristics of the potato group of bacteria it is apparent that but little attention has been paid to their ability to induce the gum fermentation in sucrose solutions. In view of this fact it can be readily understood how easy it would be to overlook this characteristic of these species, particularly since it seems to be largely an acquired ability. Under these circumstances it is very probable that many of the gum-forming species of bacteria have been given specific names, when they are really only derived types of the potato group of bacteria, which have been developing for successive generations upon sucrose solutions.

CONCERNING THE NATURE OF THE TRANSFORMATION OF SUCROSE INTO GUM LEVAN

The results of this investigation have shown that sucrose is the substance from which gum levan is formed, by the bacteria that we have been studying. It has been shown further that sucrose can supply the needs for levan-forming bacteria only when it exists as such in the culture solution. The presence of invertase decreases gum formation in the exact proportion in which it hydrolyzes the sucrose present. A study of the tables shows also that, in the absence of invertase, the amount of gum formed is always in proportion to the sucrose that has been utilized by the bacteria in the solution. In other words, we have here a true fermentation in which definite products are formed, and which is not to be regarded as the result of an abnormal condition of the bacterial cells. We have already referred to the fact that the species of bacteria concerned in the gum levan fermentation do not have a demonstrable capsule. This indicates that the gum is formed extracellularly. Evidence of the existence of an extracellular gum-forming enzyme in connection with this type has been presented by Beijerinck (1910), who succeeded in producing gum levan extracellularly by

the use of extracts prepared from these same species. The gum fermentation of sucrose is, in the light of our present knowledge, of a two-fold advantage to the species of bacteria inducing it; enabling them in the first place to transform sucrose into an assimilable form, and in the second place reducing the osmotic pressure of the sucrose solution, rendering the environment more favorable for their continued activities. For these purposes this fermentation is of greater advantage to the microörganism than the secretion of invertase would be, because the inversion of the sucrose would offer no protection to the bacteria from the osmotic action of the solution, while its conversion into gum would reduce it in proportion to the amount of this colloid formed. It is not easy to explain how this transformation of the disaccharide sucrose into the polysaccharide gum takes place. Browne (1912) has suggested the possibility of the action being a combined assimilative and enzymic action, the sucrose being assimilated into a higher saccharide, which is broken up by an enzyme into glucose and levan.

CONCLUSIONS

The formation of gum levan from sucrose does not depend upon the action of invertase, as implied in Greig Smith's theory of the nascent glucose and levulose origin of the gum, but on the contrary is entirely prevented by the rapid inversion of sucrose by this enzyme.

The action of invertase not only decreases the production of gum levan to the extent to which it inverts sucrose, but retards the production of this gum from the sucrose remaining in the solution.

Decreasing the ratio of sucrose to invert sugars decreases the production of gum levan.

The optimum pH for the development of gum levan is between 6.7 and 7. The relative differences in the amounts of gum produced by the bacteria in the presence and absence of added invertase, was greatest in the solutions whose pH values were nearest the optimum for the action of invertase, and least where the pH values were least favorable for invertase action.

A culture of *Bacillus vulgatus* was found to have the ability to form gum levan from sucrose, and by repeatedly growing it in the presence of a suitable sucrose solution this ability was greatly increased. It is concluded from our study of the species of levan-forming bacteria occurring in sugars, that they are all derived types from the potato group of bacteria. Many of the similar species described in the literature as distinct species are thought also to be derived types.

The gum fermentation of sucrose is believed to be a distinct type of fermentation, probably acquired as a means by which an organism secreting no invertase may convert the unassimilable disaccharide sucrose into assimilable forms, and into products whose combined osmotic pressure value is lower than that of the original sucrose. In this manner the material for supplying the energy for the organism is provided, and the environment, if its osmotic value is too high, is rendered more favorable for the continued growth and development of the organism.

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