THE PHYSIOLOGICAL CHARACTERS OF BACILLUS COAGULANS (BACILLUS THERMOACIDURANS)[1]

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The "flat sour" organism, *Bacillus thermoacidurans* Berry (1933), is important to the canning industry because it is one of the few sporeforming organisms capable of growing in an acid food product, such as tomato juice. The seriousness of this type of spoilage lies in the fact that its occurrence is sporadic and as yet unpredictable. Furthermore, because little or no gas is produced to reduce the vacuum in the container, detection of the spoilage is impossible without first opening the container. The organism, a heat-resistant, sporeforming mesophile, was described by Berry (1933), who noted its tolerance to acid. In general, he observed that it produced only moderate growth in culture media and produced no gas in carbohydrate media, although acid was produced in a variety of sugars.

Smith, Gordon, and Clark (1946) demonstrated from comparative cultural studies that *Bacillus thermoacidurans* Berry and the more recently described *Bacillus dextrolacticus* Anderson and Werkman (1940) are identical with *Bacillus coagulans* Hammer (1915), described some time before either of these names was proposed. The identity of these three organisms is also accepted by Breed, Murray, and Hitchens (1948). Although *Bacillus coagulans* is therefore the correct name for the species here discussed, so far as is shown by the studies made thus far, it has proved convenient to use the name *Bacillus thermoacidurans* in this paper for those cultures that were isolated from tomato products. The name *Bacillus coagulans* is used for the culture isolated by Hammer from evaporated milk.

Although little work has been done on the physiology of *Bacillus thermoacidurans*, several papers of practical importance dealing with control measures designed to destroy the resistant spore have been presented. The organism is acid-tolerant to a degree. Pederson and Becker (1949) found that although the vegetative cells of some strains could grow in tomato juice of pH 4.15 to 4.25 the heated spores could not germinate and grow in tomato juices that had been adjusted to a pH lower than 4.32. During the normal processing of juice the non-heat-resistant vegetative cells are readily destroyed. Further physiological studies have been undertaken to learn more of the nature of the organism in the hope of developing better measures for controlling its growth in foods.

Cultures studies. The 23 cultures of *Bacillus thermoacidurans* and 1 culture of *Bacillus coagulans* studied included cultures isolated in the laboratory and others received from the laboratories of the National Canners' Association, Continental Can Company, American Can Company, and the American Type

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Culture Collection. All were isolated from spoiled tomato juice except two cultures from soil and the one culture of *Bacillus coagulans*.

Methods. Detailed comparative morphological, cultural, and physiological studies were made upon all cultures. These included all the usual spore and vegetative cell measurements and flagella staining, cultural and growth characteristics, and physiological reactions such as oxygen relations, temperature of growth, reduction of nitrates, gelatin liquefaction, and growth on sugars or related carbon compounds. However, since it was felt that certain of these determinations might have economic significance, particular attention was given to the temperature for growth, the limiting hydrogen ion concentration for the germination of spores and the growth of vegetative cells, oxygen relationships, carbon dioxide production, and the end products of sugar metabolism.

The effect of acidity and pH upon growth was studied in broth as well as tomato juice. Tomato juices were used particularly for building up the viability of cultures. Stern, Hegarty, and Williams (1942) had noted that *Bacillus thermoacidurans* often failed to grow in tomato juice when it was previously grown in broth. The reaction of the tomato juice was adjusted by the use of standard solutions of inorganic and organic acids, such as hydrochloric and citric acids, and sodium hydroxide. Eldredge tubes were used to determine the effect of pH and oxygen upon carbon dioxide production both in tomato juice and in broth. End products from glucose metabolism were determined from growth in a medium composed of tryptone 0.5 per cent, yeast extract 0.25 per cent, glucose 3.0 per cent, and mineral salts A and B, adjusted with excess calcium carbonate. The medium was incubated at 37 C for 21 days.

RESULTS

The various cultural and physiological results are in close agreement with those of Gordon and Smith (1949). Vegetative cells of young cultures grown on nutrient media are typically gram-positive rods occurring singly, in pairs, and occasionally in short chains. Five-day-old cultures on nutrient agar, stained with malachite green spore stain using safranine as the counterstain, yielded cells ranging in size from 0.5 by 2.0 μ to 0.9 by 8.0 μ, with an average of 0.7 by 4.1 μ. In tomato juice or other unfavorable media filaments similar to those described by Anderson and Werkman (1940) for *Bacillus dextrolacticus* are often produced. In synthetic media, in old cultures, and in very acid tomato juice the cells are usually thinner, becoming gram-variable and often exhibiting granulation. Newly germinated cells are commonly more deeply stained at the poles. This appearance is retained after several cell divisions, so that short chains occur with deeply stained ends.

Bacillus thermoacidurans will produce spores on solid media and, under certain conditions, in liquid cultures. Spore production on nutrient agar is rather meager. Sarles and Hammer (1932) noted some spore production for *Bacillus coagulans* in coagulated milk and on beef infusion agar slants. Stern, Hegarty, and Williams (1942) used an acidified protein peptone agar that proved more favorable for spore production than nutrient agar.

In this study profuse spore production has been obtained with a sugar-deficient tryptone, glucose, yeast extract agar. On the theory that spore production may be initiated by healthy, actively growing cells facing starvation, young broth cultures were inoculated onto slants containing 0.01 per cent glucose. Profuse spore production was nearly always attained on this medium within 8 days at 37 C, and usually within 2 to 3 days. The *Bacillus coagulans* culture and strain 32G of *Bacillus thermoacidurans* were less active in this respect. Neither of these cultures was an active flat sour organism. Spores produced were typically oval and terminal, and, when stained with malachite green, averaged 0.7 by 0.9 μ. Spores normally do not swell the cell. Sporulation occurs over a wide range in pH with the best between pH 5.15 and 6.95. A few spores have been produced in media with a pH as low as pH 4.5 or as high as pH 7.8, but sporulation has not been observed below or above these limits.

Bacillus thermoacidurans is an actively motile organism. With the Hofer-Wilson modification of Gray's flagella stain, frequently as many as 10 flagella per cell were revealed. The organism is peritrichous, but the flagella tend to be clustered at the poles. When cells are in filaments, tufts of flagella can often be seen coming from the filament at fairly regular intervals. Flagella are usually at least three times as long as the cells.

After 48 hours at 37 C colonies on agar plates are normally grayish white, flat, and translucent, and range in diameter from 1 to 5 microns. In general, two types of colonies are formed. One type has a dark-brown granular center with a finely granular periphery and entire lobed margin. The other type is fairly granular throughout, with a darker center and mycelial margin.

Growth on agar slants is smooth, moderate, and white to light gray in color. Growth in stabs is moderate, slightly granular, and most abundant beneath the surface. After growth has occurred along the length of the stab, some surface growth will usually develop. In making a comparative study of cultures received as possible strains of the organism under study, a few cultures were found to be strict aerobes. None of these were as acid-tolerant as typical strains or produced the typical flat sour spoilage when inoculated into tomato juice.

No visible pigmentation was produced on agar slants. However, an ether extraction of 750-ml quantities of broth cultures yielded a small quantity of an ether-soluble yellow-orange pigment from two cultures.

Normally, young broth cultures were uniformly turbid, becoming clear with sedimentation. The two soil isolates produced a light adherent ring at the surface.

In litmus milk at 37 C many strains exhibit a slight to moderate reducing power in 2 to 4 days. Reduction occurs from the bottom, progressing upward. Curdling occurs later, usually requiring 4 to 8 days. The curd is rennetlike, occasionally contracting and thus leaving a layer of clear whey. As the culture ages, the coagulum becomes more like a typical rigid acid curd. Hammer (1915) noted that *Bacillus coagulans* curdled well before sufficient acid had been produced to give a typical acid curd. Hammer found no evidence of caseolysis although there was an increase in both soluble and amino nitrogen. Evidence

of caseolysis occurred with only one culture, no. 712. This strain also liquefied gelatin. Berry (1933) reported no liquefaction of gelatin but found a variation among strains in regard to the curdling of milk.

A moderate, butyrous, tan-colored growth was produced on potato slants, and three strains produced a slight darkening of the potato. Scant growth was obtained in gelatin incubated at 21 C for 4 weeks, but only one strain liquefied the gelatin. With the more sensitive Frazier technique, two other strains showed slight liquefaction.

Growth was poor in synthetic media using ammonium salts or nitrates as sources of nitrogen. Urea was quite readily utilizable. Nitrates were reduced slowly by some strains when these were added to the tryptone sugar broth.

The ability of all strains to grow at high temperatures was shown by the fact that growth occurred both at 45 and 55 C, and most strains grew at 63 to 65 C (water bath temperature). These organisms are not obligately thermophilic, however, since growth occurred at as low a temperature as 18 C. Best growth occurred between 37 and 45 C, as shown by the final pH attained in neutral tryptone, glucose, yeast extract broth. Berry (1933) states that the optimum growth temperature is between 40 and 60 C and that 37 C is optimum for the development of flat sours.

The final hydrogen ion concentrations produced depended upon the medium and the temperature of incubation, but the minimum pH attained at 37 to 40 C ranged from 4.5 to 4.1. Above or below these temperatures the final pH attained was usually higher. At 55 C the lowest pH observed was 4.14, and at 18 C, 4.22. If, instead of 1.0 per cent glucose, 0.1 per cent or less was used, an interesting reversal of pH often occurred. The minima attained in this case were about pH 4.5 to 5.0, after which a gradual rise occurred until the final pH attained after 4 to 5 days would be as high as pH 7.0 to 8.0. This reversal was variable with individual cultures. A change in the growth characteristics of the organism occurred almost invariably with this rise in pH. It becomes more strongly aerobic, usually producing surface growth in the form of a thin pellicle. Whereas in acid broth no spores are produced, abundant sporulation occurs at the same time in a sugar-deficient medium. The reversal in pH might be explained on the basis of a preliminary utilization of glucose followed by the breakdown of protein and a possible utilization of the acids first produced. In 1.5 per cent glucose broth, sufficient acid is produced to inhibit further development and the pH remains at the minimum value.

Although *Bacillus thermoacidurans* is of importance because of the losses to the tomato juice industry, tomato juice itself does not appear to be a favorable growth medium. Many of the strains studied grew very slowly and seemed to require a period of acclimatization before producing the typical flat sour condition. Stern, Hegarty, and Williams (1942) found that their strain had to be acclimated to tomato juice. Growth in tomato juice of pH 4.2 to 4.3 is very slow even at 37 C. Pederson and Becker (1949) discuss this relationship much more fully from a practical standpoint. Meager macroscopic growth is manifested by granular white specks in the juice and sometimes by a very

fine ring at the surface. The final pH tends to be lower in tomato juice than in broth, often as low as pH 3.9 to 4.0. The organism tends to kill itself by the acid produced in normal tomato juices. In the naturally less acid juices or juice adjusted to pH 4.5 or above, growth is more prolific and in some cases may produce a creamy, thick pellicle accompanied by the production of spores. Spores have never been observed in the more acid juices, that is, at pH 4.3 or lower. In the adjusted tomato juice a reversal of reaction sometimes occurs, but this was variable as in broth. Culture 710, for example, brought the reaction back to pH 9.02, whereas the highest pH recorded for culture 4 was 5.32. As in broth cultures, these tubes exhibited a striking, sweet, nauseating odor.

All strains fermented glucose, fructose, galactose, mannose, maltose, sucrose, raffinose, dextrin, salicin, amygdalin, and *alpha*-methylglucoside, normally producing a final hydrogen ion concentration from pH 4.25 to 4.75 and 1 to 2 ml of 0.1 N acid in 10 ml of the tryptone, yeast extract, basic salt culture medium. A few strains in some sugar broths produced a lower pH. Two strains failed to ferment arabinose; 11 failed to ferment lactose and mannitol; the results with xylose and rhamnose were variable; and all strains failed to ferment inulin. Berry (1933) also noted a variable fermentation of arabinose, lactose, and mannitol.

The determination of the end products of fermentation is apparently complicated by the relation of the organism to oxygen. In several preliminary determinations it was observed that considerable quantities of carbon dioxide were produced in Eldredge tubes. It was felt that the amount of carbon dioxide might be related to the amount of growth. Since the amount of growth can be partially regulated by the acidity of the medium, a series of cultures was inoculated into broth adjusted to various pH levels from 4.58 up to 7.90. Obviously there was less growth in the tubes of the higher acidity, but in all cases an abundance of carbon dioxide was produced. The amount of carbon dioxide produced was related to, but was not directly proportional to, the acidity of the broth and thus to the amount of growth. Less carbon dioxide was produced with the medium at pH 4.58 than at pH 6.07, but the differences were not so great as the differences in acid produced at these two levels. It seemed entirely possible that this amount of carbon dioxide was governed to some extent by the amount of oxygen present. With this in mind, two series of tubes were filled with tomato juice adjusted to pH 4.48. In one series 20 ml of juice were used with 20 ml of barium hydroxide, leaving approximately 40 ml of air space. In the second series 35 ml of juice and 35 ml of barium hydroxide solution were placed in the tubes. These amounts practically filled the tubes, and cotton plugs saturated with alkaline pyrogallol to absorb oxygen were inserted into the necks of the tubes just below the paraffined stopper.

The results (table 1) are very striking in that they show the production of a considerable quantity of carbon dioxide in the tubes with air present, but only a small quantity in the absence of oxygen. In all tubes containing 20 ml of juice in which growth occurred, all the barium hydroxide solution (equivalent to 16.6 ml N/10) was utilized. On the other hand, only a small amount (0.9 to 4.7 ml N/10 barium hydroxide) was used to absorb the carbon dioxide produced

from the 35-ml quantities of juice. In fact, it is doubtful whether all of this was produced by fermentation since the control tubes as well as those in which no growth had occurred showed some carbon dioxide, possibly evolved from the alkaline pyrogallol solution. In general, a slightly higher acidity was produced in the tubes containing only 20 ml of medium. This relationship of oxygen to carbon dioxide production was confirmed by two additional series of tests.

TABLE 1

Carbon dioxide production in Eldredge tubes

(Medium: tomato juice adjusted to pH 4.48)

CULTURE NUMBER	CARBON DIOXIDE PRODUCTION IN			
	35 ml medium anaerobic conditions		20 ml medium aerobic conditions	
	Final pH	Carbon dioxide ml N/10	Final pH	Carbon dioxide ml N/10
Control	4.49*	2.8	4.48	0
Control	4.49	3.0	4.48	0
4	4.48	3.5	4.47	0
43P	4.00	4.7	3.88	16.6
N.J.5	4.17	3.5	3.90	16.6
57G	4.18	4.3	4.20	16.6
711	4.14	3.5	4.08	16.6
712	4.13	3.6	4.05	16.6
713	4.15	0.9	4.00	16.6
714	4.10	4.5	4.04	16.6
710	3.99	1.6	3.97	16.6
27-1	4.23	3.8	4.18	16.6
27-2	4.47	3.4	4.48	0
27-5	4.49	3.3	4.47	0
27-6	4.18	4.3	4.21	16.6
27-7	4.48	2.9	4.47	0
C2253	4.02	3.8	3.97	16.6
C2273	4.06	3.6	3.98	16.6
720-17	4.49	3.5	4.47	0
717-24	4.05	4.2	4.14	16.6
7050	4.45	3.8	4.47	0
821	4.49	3.3	4.47	0

* A final pH of 4.48 to 4.49 would indicate that there had been no growth.

Eight of the cultures were selected for a determination of other end products of glucose metabolism. After 3 weeks of incubation at 32 C analyses were made according to those outlined by Pederson and Breed (1928). From the results (table 2), one may see that the major end product of the glucose fermentation was *dextro*-rotatory lactic acid, i.e., the L (+) form. Smaller amounts of volatile acid were produced. These consisted primarily of acetic and formic acids according to the Duclaux constants (table 3). However, a small amount of a higher volatile fatty acid was present in one instance (culture 710). Traces of alcohol were present and four cultures gave a weak but positive test for acetylmethyl-

carbinol. The alcohol solubility of the barium salts apparently indicates that there was a trace of succinic acid present in the nonvolatile acid fraction. There was undoubtedly some carbon dioxide produced in these fermentations since they were not conducted under anaerobic conditions. The results are essentially

TABLE 2

End products of glucose fermentation by strains of Bacillus thermoacidurans

CULTURE NUMBER	WEIGHT OF GLUCOSE FERMENTED	VOLATILE ACID AS ACETIC		FORMIC ACID	ALCOHOL	NONVOLATILE ACID AS LACTIC		WATER OF CRYSTALLIZATION OF ZINC LACTATE	TYPE OF ACID
	g	g	%	g	g	g	%		
712	10.71	0.63	5.9	0.104	0.14	8.34	77.9	12.97	L(+)
N.J.5	10.85	0.25	2.3	0.024	trace	8.87	81.7	13.00	L(+)
4	10.70	0.48	4.5	0.024	trace	7.56	70.7	12.97	L(+)
C2273	10.64	0.38	3.6	0.033	trace	8.54	80.3	—	L(+)
7050*	10.64	0.35	3.3	0.013	trace	7.72	72.6	12.95	L(+)
27-1	10.67	0.26	2.4	0.048	0.05	8.99	84.3	12.98	L(+)
700	10.64	0.20	1.9	0.018	0.15	8.59	80.7	13.10	L(+)
43P	10.61	0.49	4.6	0.019	0.08	7.69	72.4	12.93	L(+)

Acetylmethylcarbinol was produced from cultures 712, N.J.5, 710, and 43P.
Traces of succinic acid were present.
* *Bacillus coagulans* no. 7050 came from A.T.C.C.

TABLE 3

Duclaux distilling constants of volatile acid produced from glucose

CULTURE NUMBER	FRACTIONS (ML)									
	10	20	30	40	50	60	70	80	90	100
27-1	6.2	13.1	20.5	25.6	36.3	45.4	55.8	67.0	80.4	100
712	6.3	13.2	20.6	28.6	37.2	46.3	56.2	67.5	81.2	100
7050	6.4	13.4	20.8	28.4	37.1	46.3	56.5	68.0	81.0	100
N.J.5	6.8	13.9	21.6	29.7	38.3	47.6	57.9	69.3	82.2	100
43P	7.0	14.3	22.0	30.1	38.3	47.4	57.2	68.2	81.9	100
C2273	7.0	12.7	22.1	30.5	39.0	48.2	58.4	69.9	83.0	100
4	7.2	15.0	23.1	31.7	40.6	49.8	59.8	71.0	83.6	100
710	9.6	18.0	26.6	34.5	42.2	50.7	59.6	69.7	82.1	100
Authors' constants										
Acetic acid	7.4	15.3	23.5	32.2	41.3	51.0	61.3	72.6	85.3	100
Formic acid	4.9	10.8	17.4	24.6	33.0	42.1	52.5	64.6	79.7	100

the same as those obtained by Sarles and Hammer (1932) and Anderson and Werkman (1946), with strains of *Bacillus coagulans* and *Bacillus dextrolacticus.*

A consideration of the various cultural data, particularly growth characteristics as controlled by factors such as acidity and oxygen, provides an explanation for the sporadic nature of the flat sour spoilage of tomato juice. Spoilage of foods is ordinarily accompanied by marked changes in the food as well as by the

presence of large numbers of the causative microorganisms. Here is a case in which the spoilage is often difficult to detect by any change in the food and one in which plate counts are always low and often zero. It is difficult, and sometimes impossible, to isolate the organism from spoiled juice. This is due to the fact that the organism produces acid and, when growing in tomato juice, quickly produces acid enough to attain the limiting hydrogen ion concentration for growth and thus eventually to cause the death of the organism.

Although the species is described as acid-tolerant, acid tolerance should always be considered in relation to the degree of tolerance. In this case it has been observed that the limiting hydrogen ion concentration for the growth of the vegetative cells is higher than that for the germination of the spores as well as for the production of spores. The limiting hydrogen ion concentration for spore germination is actually lower than the hydrogen ion concentration of much of the tomato juice processed, particularly in New York State. Thus, even the less acid tomato juices cannot be considered as suitable media for growth, and some strains acclimate themselves only with difficulty to this medium of low pH. Spores are not produced readily in either liquid or acid media, but rather on solid neutral media under aerobic conditions. Spore production rarely occurs in tomato juice. The acidity is almost immediately raised to an amount that kills the organisms and thus sporulation cannot occur.

The sporadic nature of the spoilage can be explained on the basis of a combination of these circumstances: low-acid tomatoes, the presence of the organism, and an opportunity and sufficient time for the organism to grow and acclimate itself to an abnormally low pH and then to produce the resistant spores. This may occur under such circumstances as in the mixture of soil and tomato juice so often present on the surface of tomatoes in crates, or in decaying tomatoes (whether it be around machinery or in crates) in which a miscellaneous flora of yeasts and molds may raise the pH to a point at which growth and spore production may occur.

The organism is a prolific carbon dioxide producer under aerobic conditions. Although cans of spoiled juice never swell, Stern, Hegarty, and Williams (1942) noted a slight loss of vacuum. Since there is usually very little, if any, oxygen in a can of juice, carbon dioxide is not produced in sufficient quantities to cause a swelled can. Sarles and Hammer (1932) also reported carbon dioxide production under aerobic conditions with the closely related *Bacillus coagulans*.

There is no justification for considering *Bacillus thermoacidurans* as a species distinct from *Bacillus coagulans*, and the latter name has priority. The various morphological and physiological characteristics as well as the end products of growth are all identical or within the degree of variability of different strains of *Bacillus thermoacidurans*. One of the minor differences observed was the greater difficulty in obtaining the growth of the *Bacillus coagulans* culture in many of the samples of tomato juice. However, it was found necessary to acclimate other cultures to juice. Stern, Hegarty, and Williams (1942) stress the necessity of repeated transfers from juice to juice in order to develop cultures that cause spoilage.

SUMMARY

Twenty-three strains labeled *Bacillus thermoacidurans* and one strain of *Bacillus coagulans* have been compared morphologically, physiologically, and biochemically and have been found to be essentially alike. The limiting factors for the growth of the organism offer an explanation for the sporadic nature of the spoilage of tomato juice. Tomato products are not good media in which to grow these sporeforming organisms, and they grow only when conditions are sufficiently favorable. All growth might be stopped by a slight acidification of these products.

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