RESEARCH BULLETIN 805 NOVEMBER, 1962

UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE
AGRICULTURAL EXPERIMENT STATION
ELMER R. KIEHL, *Director*

Studies on the Heat Responses of Bacterial Spores Causing Flat Sour Spoilage in Canned Foods

I. Effects of Heating Menstrua, Spore Age, and Suspension Preparation on the Heat Activation of *Bacillus Coagulans* Spores

M. L. FIELDS AND R. C. JENNE

(Publication authorized August 23, 1962)

COLUMBIA, MISSOURI

CONTENTS

ACKNOWLEDGMENT

This research bulletin reports on Department of Horticulture research project 434, Thermal Activation of Bacterial Spores in Processed Foods.

Studies on the Heat Responses of Bacterial Spores Causing Flat Sour Spoilage in Canned Foods

I. Effects of Heating Menstrua, Spore Age, and Suspension Preparation on the Heat Activation of *Bacillus Coagulans* Spores

M. L. FIELDS AND R. C. JENNE

In the canning of tomato juice, there are sporadic outbreaks of flat sour spoilage (can remains flat or not bulged and the bacteria produce acid making it sour) caused by *Bacillus coagulans var. thermoacidurans.* Various workers (Pearce, 1940; Williams, 1940; Wessel and Benjamin, 1941; Bohrer and Reed, 1949) have shown that within the canning plant there may be an increase in the number of spores which may be found after an overnight interval. Until recently, the litera-

ture was lacking to explain how these increases might come about in these short periods in possible canning plant environments. Wildman and Clark (1947) have shown that *Oidium lactis* produces a slime on machinery in tomato canning factories where strict sanitary conditions are not maintained. Fields (1962) showed that *O. lactis* can raise the pH of tomato juice in thin layers to a pH range optimum for sporulation of *B. coagulans* in 24 hours at 25°C and in 48 hours at 35°C. As high as 61.7 percent sporulation occurred within 24 hours in tomato juice in which *O. lactis* had grown when this juice was incubated at 52°C. When the tomato juice was adjusted to pH 5.0 and *B. coagulans* or a mixture of *O. lactis* and the vegetative cells of *B. coagulans* were added as an inoculum, only in the juice in which *O. lactis* was growing were spores produced within 72 hours at 35°C.

Much of the previous research on endospore germination of aerobic, thermophilic and thermotolerant organisms has been done using various concentrations of phosphate buffer in the menstruum to maintain the desired pH. Phosphate buffer has also been used in thermal death time studies. Recently, researchers have observed the effect of phosphate on the heat activation of bacterial spores. Brachfeld (1955), working with spores of *Bacillus stearothermophilus*, found that phosphate buffers in concentrations of M/20, M/80, M/200, M/800, and M/2000 failed to stimulate or inhibit germination. He also noted that in the case of the M/20 phosphate buffer there was a significant decrease in the average plate count of the heat-activated spores. Other buffer solutions yielded slightly lower counts than the control but these were insignificant.

Amaha and Sakaguchi (1952) compared tenfold differences in phosphate concentrations as they affected the thermal death rate of spore-forming bacteria. They found that the spores exhibited a more rapid death rate in the higher buffer concentrations. According to Ordal and Lechowich (1958), the maximum resistance to thermal destruction occurred when the bacterial spores were suspended in M/40 phosphate buffer. When the phosphate concentration was increased or decreased, the death rate was accelerated. Levenson and Sevag (1953) reported that the spores of *Bacillus megatherium* required a proper balance of ions in the nutritive medium for rapid germination and respiration. They found that M/50 potassium phosphate had a depressing effect on germination and respiration if there was unfavorable balance provided by monovalent anions. The effect was much less when M/5000 phosphate was used. Amaha and Ordal (1957) reported that an increase in buffer strength from the standard M/15 buffer (used in thermal death time studies with bacterial spores) to M/40 caused a significant increase in the thermal death rate of the spores of *B. coagulans*. According to Williams and Hennessee (1956), the spores of *B. stearothermophilus* when heated in phosphate buffers exhibited an apparent increase in resistance with decreasing molar concentrations over a range of M/15 to M/120 molar. There were no observed differences between M/120 and distilled water.

In addition to the problem of phosphate buffer strength and pH in heat activation and thermal death time studies, the researcher is also faced with the problem of producing vegetative cell-free suspension without the use of heat.

Desrosier and Heiligman (1956) used an ultrasonic radiation to destroy the vegetative cells. Others have used lysozyme, an enzyme, to lyse the vegetative cells (Brachfeld, 1955, Brown *et al.,* 1957, and Guse and Hartsell, 1959). Guse and Hartsell showed that lysozyme treatment had a depressing effect upon *Bacillus sublilis var. niger* while it had an enhancing effect upon *Bacillus cereus.*

Since tomato juice is one of the media in which the spores of *B. coagulans* germinate and grow, heat activation studies in this food would seem desirable. Pederson and Becker (1949) and Knock *et al.* (1959) have heated spores of different strains in tomato juice and determined growth after an incubation time. Growth was determined, however, by measuring pH of the tomato juices. Desrosier and Heiligman (1956) did not perform heat activation in tomato juice. Since it has been shown that *O. lactis* can raise the pH of tomato juice in films to allow *B. coagulans* to sporulate (Fields, 1962), it would seem that activation in tomato juice in which *O. lactis* has grown is also desirable.

Specifically, this research was instituted to determine the following: 1, the effect of phosphate buffer strength and pH on 2 strains of *Bacillus coagulans var. thermoacidurans;* 2, the effect of heat shocking "old" (stored) spores and "new" spores in tomato juice and Oidium tomato juice at various pH units; 3, the effect of spore preparation on the degree of spore activation.

MATERIALS AND METHODS

Organisms used

Two strains of *Bacillus coagulans var. thermoacidurans,* which were originally isolated from tomato juice, were obtained from J. F. Folinazzo, Continental Can Company. These strains, 25 and 27, were maintained on thermoacidurans slants and were stored at 4°C. Both strains grew and sporulated when adjusted to sporulating conditions in tomato juice and Oidium tomato juice (O.T.J.) (Fields, 1962). The vegetative cells of both strains grew as low as pH 4.2.

Spore Production

Spore production was obtained by transferring a small amount of the spores to a test tube containing 10 ml of thermoacidurans broth. After 24 hours' incubation at 52°C, these broth cultures were transferred aseptically to Roux flasks containing thermoacidurans agar, pH 7.0, that was fortified with 10 parts per million of manganese sulfate. After the transfer was complete, the Roux flasks were gently agitated to insure even distribution of the bacteria over the surface of the agar. The flasks were incubated at 52°C, and spore stains were made of the cultures at various times during incubation. The malachite green was used to stain spores while vegetative cells were stained with saffron. After 3 days' incubation, the cultures contained 20-40 percent spores, an amount considered adequate for use in this study.

Cleaning Procedures

The same general cleaning procedure was used in preparing the two-year-

old, non-lysozyme-treated spores; the new, non-lysozyme-treated spores and the new, lysozyme-treated spores. This was accomplished as follows: the spore and vegetative suspensions were transferred from the Roux flasks to sterile 99-ml bottles by pipetting sterile water over the surface of the agar to loosen the bacteria or, if needed, scraping gently the remaining part of the suspension by using a sterile glass rod. Twenty ml of the bacterial suspension were transferred to sterile water bottles containing 80 ml of water and were stored until cleaned by repeated centrifuging and resuspending in sterile water.

The two-year-old, non-lysozyme-treated spores and the new, non-lysozyme-treated spores were washed 10 times to remove vegetative cells, were heated at 80°C for 10 minutes to destroy vegetative cells and were stored at 4°C until used.

The lysozyme-treated spores were prepared as follows: after the initial separation of the spores from the vegetative cells by centrifugation, the suspension was transferred to sterile Erlenmeyer flasks containing magnetic stirrers. Lysozyme in a concentration of 0.5 mg/ml was added, and suspension was stirred continuously during the 2-hour incubation period at 52°C. The adequacy of the lysis was determined by spore stains. All lysis of the vegetative cells was complete after 2 hours; however, to rid the suspension of large amounts of debris, the spores were filtered through a column of tightly packed glass wool that was 1 inch in diameter and 6 inches long.

The suspensions were then transferred to sterile 40-ml centrifuge tubes with rubber caps and centrifuged for 15 minutes at 5,000 rpm and the supernatant was discarded. The tubes were refilled to the 40-ml level with sterile distilled water and shaken thoroughly to wash the spores. The spores were centrifuged for 12 minutes at 3,000 rpm and the supernatant discarded. This procedure was repeated for 9 more times. After the last centrifugation, the spores and distilled water were transferred to a sterile microwaring blender and blended 1 hour at 0°C. This temperature was used to insure no heat activation of the spores due to temperature of the blending procedure. The samples were stored in sterile medicine bottles at 4°C.

Preparation of the Suspending Menstrua

Mono- and di-basic sodium phosphate were used in preparing buffers having molarities of M/120, M/500, and M/1000 with pH units of 5.5 and 6.5 respectively. These pH units were selected since these are pH values which can be produced by *O. lactis* in tomato juice films in 24 hours (Fields, 1962). In studies in which tomato juice and O.T.J. were used, these same units, as well as others, were prepared. Nine-ml amounts of the buffers were prepared, autoclaved at 250°F for 15 minutes and stored at room temperature.

Preparation of the Tomato Juice Serum

The tomato juice serum was prepared from tomatoes which had been carefully washed, trimmed, put through a pulper, heated to 190°F, cooled and stored at 0°C until used. As needed, the tomato juice was thawed, filtered through cheesecloth, and centrifuged for 12 minutes to remove suspended solids.

The tomato juice was adjusted with a Beckman zero-matic pH meter to the desired pH using 3N NaOH. Nine ml were pipetted into test tubes, sterilized for 15 minutes and stored at room temperature until used.

Preparation of Oidium Tomato Juice Serum

O.T.J. was prepared by placing 150 ml of tomato juice in Roux flasks and inoculating the juice with a culture of *O. lactis* that had been grown on malt agar slants. After three days of incubation, the juice was filtered and heated to 212°F and then stored in a gallon jug at 34° until used. When required, the juice was removed from storage, pH adjusted, pipetted in 9-ml quantities into test tubes, plugged and autoclaved at 250°F for 15 minutes.

Heat Activation Treatments

Test tubes containing 9 ml of the appropriate menstrua were inoculated with 1 ml of the properly diluted spore suspension (500 to 1000 spores/ml). Duplicate tubes were used in the heat treatments and for the non-heated controls.

Heat activation treatments were performed at 80°, 90°, and 100°C. The 80° and 90°C heat treatments were performed in a constant temperature water bath while the 100°C treatments were carried out in a 10-liter steam-jacketed kettle.

Timing was begun as soon as the desired temperature was reached. Tubes were removed at appropriate intervals and immersed in a cold-water bath at 50°F. Data in this report are based on counts after heating for 13 or 15 minutes at the designated temperatures. The suspensions were cooled to room temperature in about 2 minutes, depending on the temperature of heat shock, and were plated out immediately after the heat shock.

Counting of Spores

Viable spore counts were made, using duplicate tubes and triplicate plates for each tube. Two recovery media were used in this study. Thermoacidurans agar, as described in the Difco manual (except that pH was adjusted to 7.0), was used in the buffer studies and in the method of spore preparation studies, while dextrose tryptone agar, the medium generally utilized for the detection of "flat sour bacteria" in canned foods, was used in the tomato and O.T.J. studies. All plants were incubated at 52°C for 48 hours.

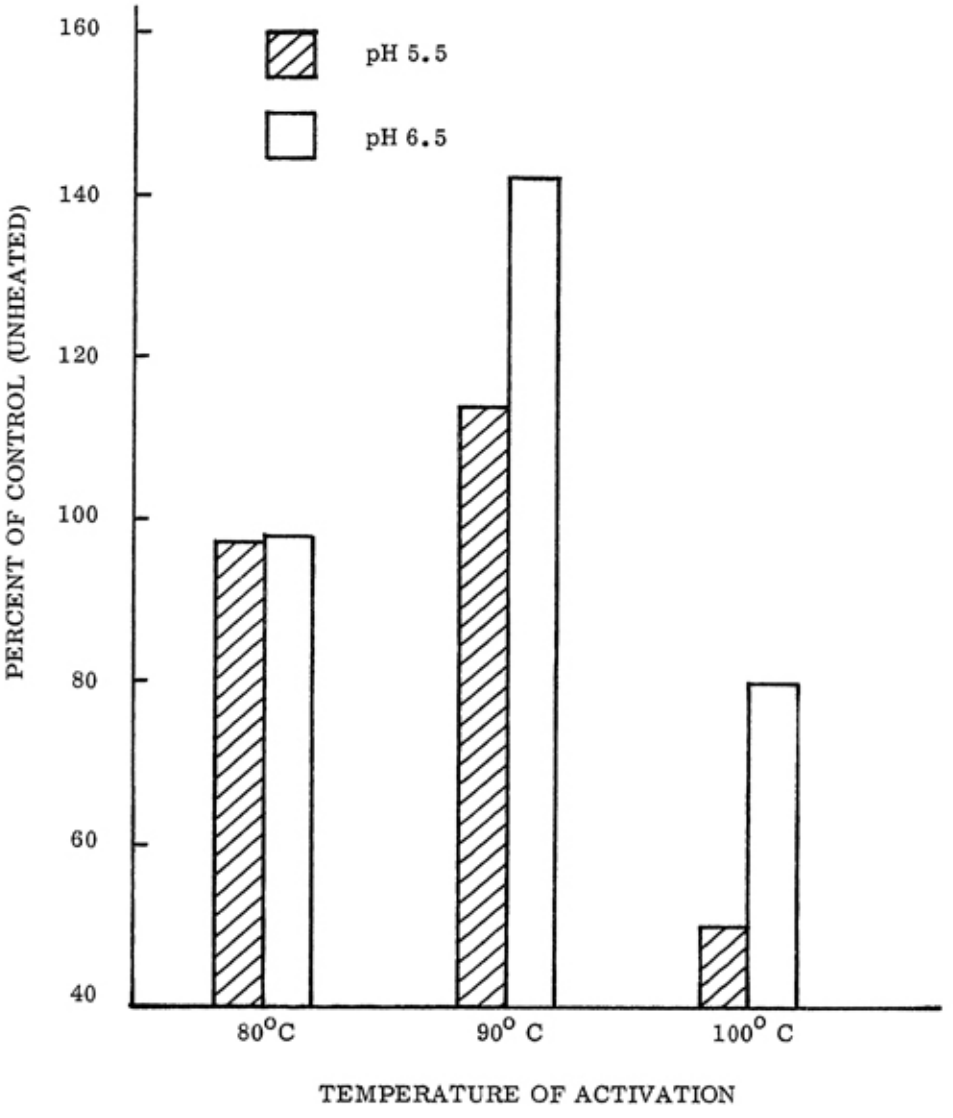
RESULTS

Effect of Temperature, pH and Molar Concentration

In Figures 1 and 2, the effect of temperature, pH of phosphate buffer and bacterial strains on heat activation of spores are shown. In Figure 1, the results of M/120 phosphate buffer are graphed. Similar results were obtained with M/500 and M/1000 for strains 27 and 25.

For strain 27, the percent recovery at 80°C and at pH 5.5 and 6.5 was 97 and 98 respectively. Maximum recovery was obtained by heating at pH 6.5 and 90°C. Differences in pH had a major effect when the spores were heated at 90°C

Figure 1. Effect of pH and Temperature on Spores of Bacillus coagulans Strain 27 in M/120 Phosphate Buffer. Heat Shocked for 13 Minutes.

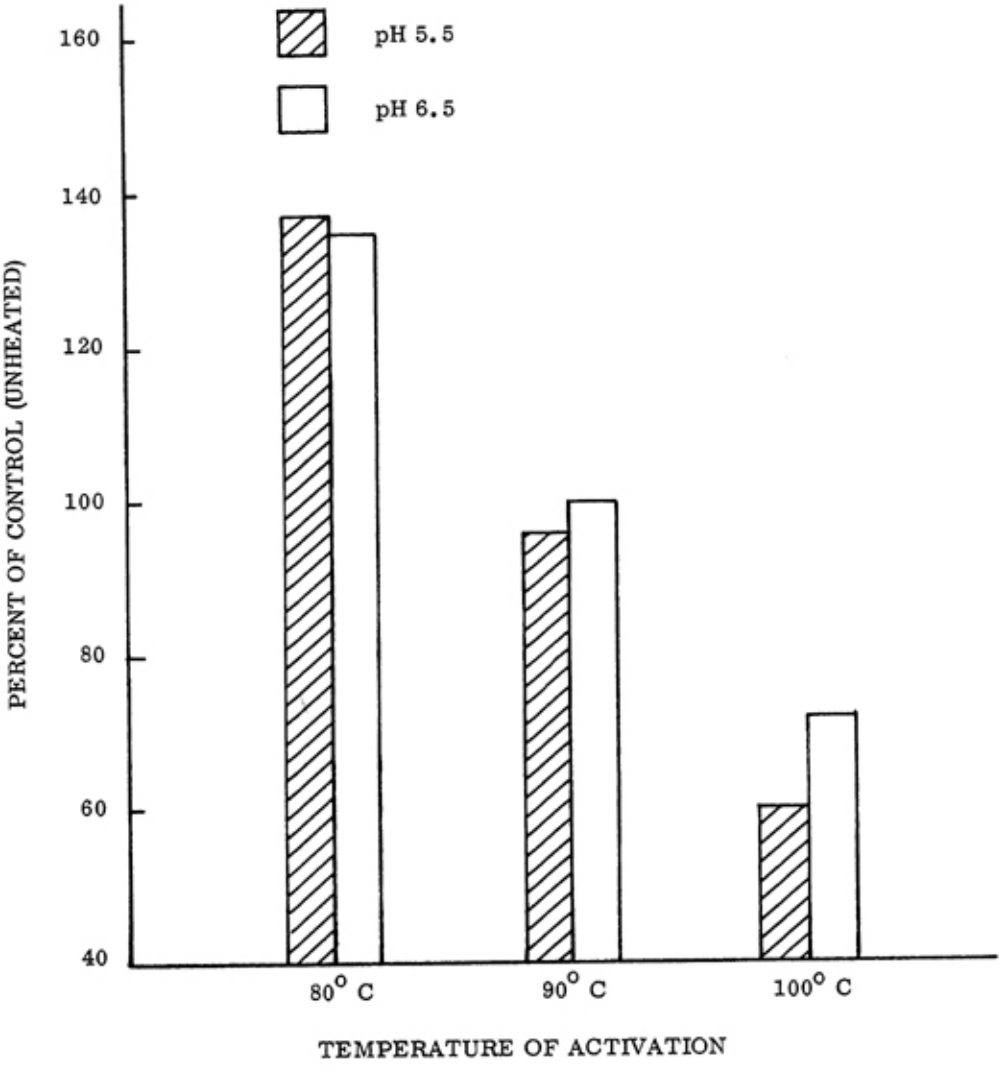


and 100°C. There was a difference of 28 percent at 90°C and 30 percent at 100°C, while there was a difference of only 1 percent at 80°C. The percent decline in counts at pH 5.5 for 90°C to 100°C was 64 percent and at pH 6.5 was 62 percent.

With strain 25, as shown in Figure 2, there was a linear decline in the percent recovery when the spores were heated at 80°C, 90°C and 100°C. Only at 80°C were the percent recoveries greater than 100 percent, indicating activation. The pH of the suspending menstrua had very little effect on strain 25 since the differences in recoveries at 80°C, 90°C, and 100°C were 2, 4 and 12 percent respectively.

As shown in Figure 3, the effect of temperature on the percent recovery was demonstrated again when the spores of strain 27 were heated at 80°C and

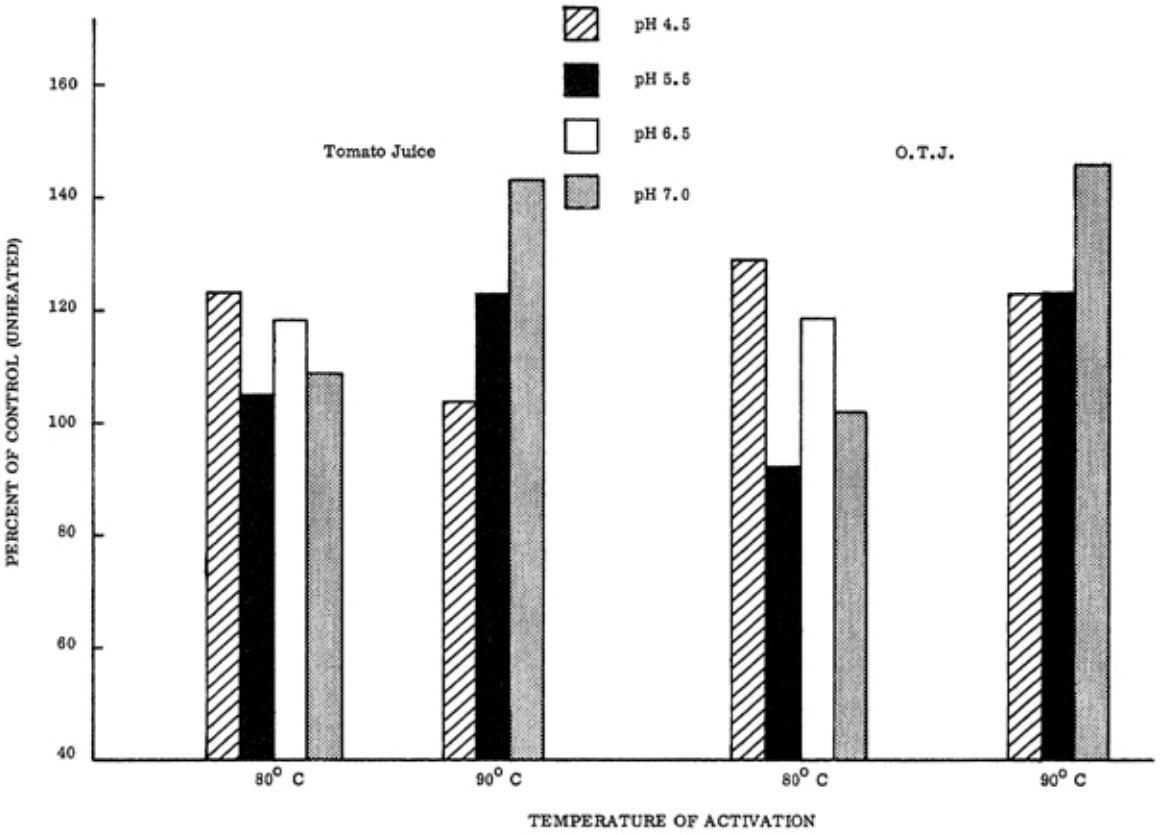
Figure 2. Effect of pH and Temperature on Spores of Bacillus coagulans Strain 25 in M/120 Phosphate Buffer. Heat Shocked for 13 Minutes.



90°C in tomato juice and O.T.J. at pH of 4.5, 5.5, and 7.0. There was not a definite pattern of recovery, as far as pH, considering both temperatures; but there was at 80°C. The reason why the recoveries were higher at pH 4.5 is not apparent. Regardless of the pH, type of suspending menstrua or temperature of activation, the percent recovery of all treatments was greater than 100 percent except the treatment in which the spores were placed in O.T.J. (pH 5.5, 80°C).

The effects of pH, spore preparation and spore age are shown in Figure 4. The percent recoveries of the old spores were dependent upon pH, with the highest recoveries being obtained at pH 6.5. The new, non-lysozyme-treated spores were activated the most at pH 5.5; however, the difference between pH 5.5 and pH 6.5 was 3 percent, which is a non-significant difference. The old,

Figure 3. Effect of Heating Menstrua and pH on Activation of New, Lysozyme-treated Spores of Bacillus coagulans Strain 27. Heat Shocked for 15 Minutes at Indicated Temperatures.



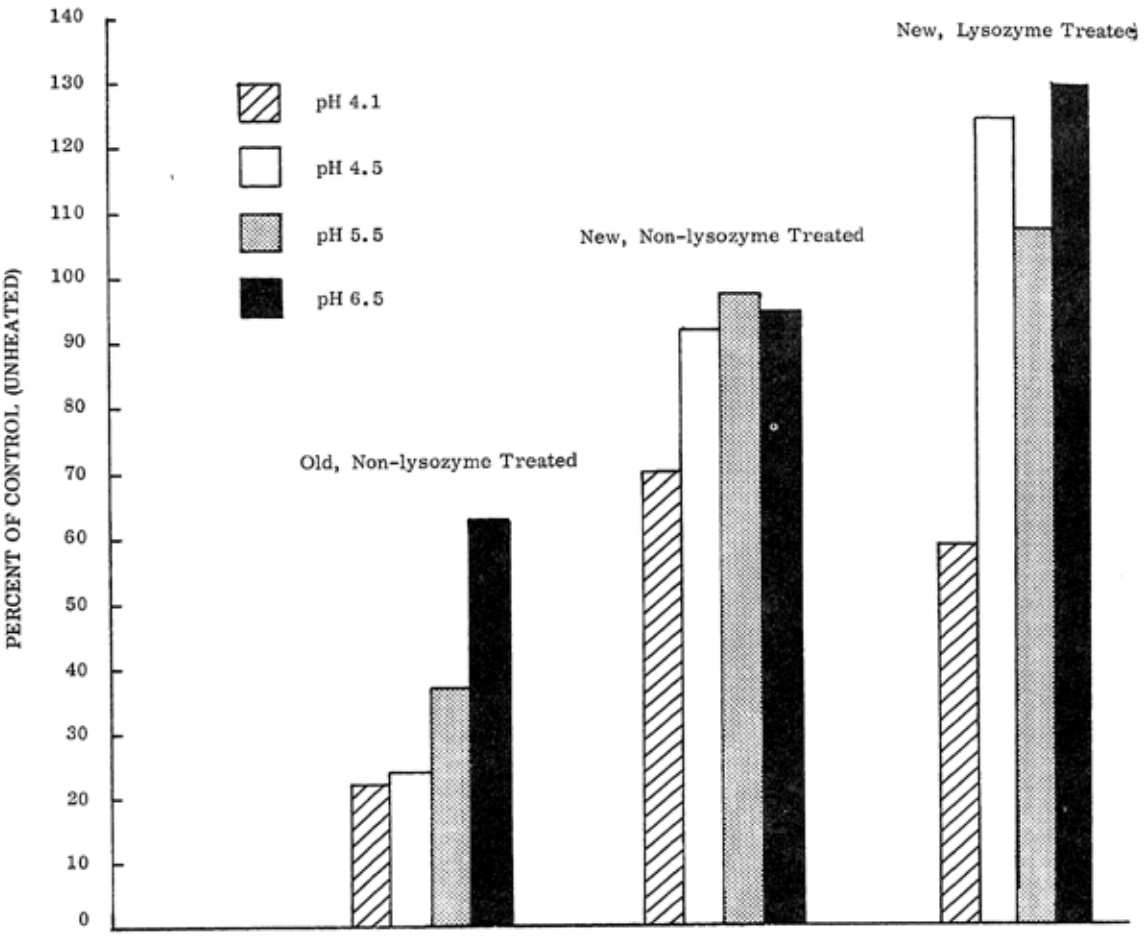
non-lysozyme-treated spores were significantly lower than the new, non-lysozyme-treated spores at all pH units tested.

It would appear that the lysozyme treatment and/or the initial heat treatment of 80°C for 10 minutes had an effect upon the new spores since the lysozyme-treated spores were the only spores that could be activated.

DISCUSSION

As Desrosier and Heiligmen (1956) have pointed out, there is a close relation between heat activation and thermal destruction of spores. The data in this report support this point of view, since activation was demonstrated at 80°C and 90°C but not at 100°C at these time-temperature relationships. The 100°C treatment was considered lethal at the time tested. These responses are similar to enzyme reactions in which increasing amounts of heat accelerate the reaction until temperatures are reached in which denaturation occurs. Some reasons which have been advanced for heat activation are: 1, preheating activates certain enzymes associated with germination and respiration (Church and Halvorson, 1956, Cook, 1932, and Tarr, 1933); 2, heat shock supplies the needed energy for germination (Desrosier and Heiligman, 1956); 3, stimulation due to heat was associ-

Figure 4. Effect of Lysozyme Treatment and Age of Spores of Bacillus coagulans Strain 27 in Tomato Juice at Indicated pH. Heat Shocked at 80°C for 15 Minutes.



ated with inactivation of toxic substances present in the spore (Mefferd and Campbell, 1951).

The percent activation in this study is in the same magnitude as that of Desrosier and Heiligman (1956) for strain F.S. 787. This is in sharp contrast to the degree of activation achieved with M strain of *B. stearothermophilus* which was activated 235 percent (Finley and Fields, 1962). The same magnitude of activation occurred in this study with strains 27 and 25 but at different temperatures. There were not any large differences in the magnitude of activation of lysozyme-treated spores when heated in tomato juice or O.T.J.

The data in this study indicate that lysozyme tends to increase heat activation. Similar results have been noted by Guse and Hartsell (1959) for *Bacillus cerens* spores. They also showed that the spores of *Bacillus subtilis var. niger* were repressed. Previous work with two strains of *B. stearothermophilus,* which were lysozyme-treated (Finley and Fields, 1962), showed that both could be activated but one to a higher degree. Whether such differences are intrinsic or extrinsic needs further study.

SUMMARY

The degree of activation in phosphate buffer was affected by pH and temperature. When spores of strain 27 were heated in buffer, maximum activation occurred at 90°C and at pH 6.5 while the maximum activation of strain 25 occurred at 80°C, without any significant difference between pH 5.5 and 6.5. Temperatures of 90°C and 100°C were lethal to the spores of strain 25.

Activation was observed when the spores of strain 27 were heated at 80°C and 90°C, both in O.T.J. and tomato juice except the treatment in which the spores were placed in O.T.J. (pH 5.5, 80°C). At 80°C both in tomato juice and O.T.J., the number of spores germinating did not decrease with a decline in pH. More spores germinated at pH 4.5 than at 5.5. At 90°C, however, the number of germinating spores in tomato juice was greatest at pH 7.0 with the least at pH 4.5, while the greatest number germinating in O.T.J. was at pH 7.0 with no difference between pH 4.5 and 5.5.

Two-year-old, non-lysozyme-treated spores of strain 27, when heated in tomato juices of pH 4.1, 4.5, 5.5 and 6.5, gave increasing recoveries with decreasing pH (highest recoveries at pH 6.5). There was no true activation since at pH 6.5 the recovery, as computed as percent of control, was only 64 percent. New, non-lysozyme-treated spores, when heated in the same menstruum, gave higher recoveries than the two-year-old spores, but still there were no true activation responses when heated at the same temperature. New, lysozyme-treated spores were activated at pH 4.5, 5.5, and 6.5, while only recoveries of 58 percent occurred at pH 4.1.

LITERATURE CITED

Amaha, M. and Ordal, Z. J. 1957. Effect of Divalent Cations in the Sporulation Medium on the Thermal Death Rate of *Bacillus coagulans var. thermoacidurans.* J. Bacteriol. *74,* 569-604.

Amaha, M. and Sakaguchi, K. 1952. Nutritional Requirements of Vegetative Cells and Spores of Aerobic Spore Forming Bacilli. J. Agr. Chem. Soc. Japan *26,* 353-359.

Bohrer, C. W. and Reed, J. M. 1949. Tomato Juice Spoilage Studies in 1948. Convention Issue Information Letter No. 1219, Nat'l. Canners' Association, Washington, D. C. pp. 80-83.

Brachfeld, B. A. 1955. Studies on Media Composition and Heat Activation for the Demonstration of Viability of Spores of *Bacillus stearothermophilus.* Ph.D. Thesis. University of Illinois.

Brown, W. L., Ordal, Z. J. and Halvorson, H. C. 1957. Production and Cleaning of Putrefactive Anaerobe 3679. Applied Microbiology *5.* 156-159.

Church, B. D. and Halvorson, H. C. 1956. Effect of Heating and Aging on Germination and Glucose Oxidation of Spores of Aerobic Bacilli. Bact. Proc. pp. 45-46.

Cook, R. P. 1932. Bacterial Spores. Biol .Rer. Cambridge Phil. Soc. 7, 1-23.

Desrosier, N. W. and Heiligman, F. 1956. Heat-Activation of Bacterial Spores. Food Research *21,* 54-62.

Fields, M. L. 1962. The Effect of Oidium lactis on the Sporulation of *Bacillus coagulans* in Tomato Juice. Applied Microbiology *10,* 70-73.

Finley, N. and Fields, M. L. 1962. Heat Activation and Heat-Induced Dormancy of *Bacillus stearothermophilus* Spores. Applied Microbiology *10,* 231-236.

Guse, D. G. and Hartsell, S. E. 1959. Some Effects of Lysozyme Bacterial Spores. Food Technology *13,* 474-475.

Knock, G. G., Lambrechts, M. S. J., Hunter, R. C. and Riley, F. R. 1959. Souring of South African Tomato Juice by *Bacillus coagulans.* J. Sci. Food Agric. *10,* 337-342.

Levenson, H. S. and Sevag, M. G. 1953. The Stimulation of Germination and Respiration of the Spores of *Bacillus megatherium* by Manganese and Monovalent Ion. J. Gen. Phys., *36,* 617-29.

Mefferd, R. B. and Campbell, L. L. 1951. The Activation of Thermophilic Spores by Furfural. J. Bacteriol. *62,* 130-132.

Ordal, Z. J. and Lechowich, V. 1958. Some Factors Influencing Thermal Resistance of Bacterial Spores. Proceedings of the Tenth Research Conference Sponsored by the Research Advisory Council of the American Meat Institute Foundation at the University of Chicago. pp. 91-99.

Pearce, W. E. 1940. Sources of Contamination in the Manufacture of Tomato Juice. Fruit Products Journal *19,* 206-208.

Pederson, C. S. and Becker, M. E. 1949. Flat Sour Spoilage of Tomato Juice. N. Y. State Agr. Expt. Tech. Bull. No. *287,* 1-22.

Tarr, H. L. 1933. Some Observations of the Respiratory Catalysts Present in the Spores and Vegetative Cells of Certain Aerobic Bacilli. Biochem. Jour. *27,* 136-45.

Wessel, D. J. and Benjamin, H. A. 1941. Process Control of Heat Resistant Spoilage in Tomato Juice. Fruit Products Journal *20.* 178-180, 189.

Wildman, J. D. and Clark, P. B. 1947. Some Examples of the Occurrence of Machinery Slime in Canning Factories. J. Assoc. Offici. Agr. Chemists *30,* 582-585.

Williams, O. B. 1940. Sources of Contamination in the Manufacture of Tomato Juice. Fruit Products Journal *19,* 209, 215.

Williams, O. B. and Hennessee, A. D. 1956. Studies on Heat Resistance I. The Effect of Phosphate on Apparent Heat Resistance of Spores of *Bacillus stearothermophilus.* Food Research *21,* 112-116.