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ABSTRACT

EFFECTS OF CHEMICAL TREATMENTS ON THE HEAT RESISTANCE OF BACILLUS COAGULANS 43P SPORES

By

Apinya Assavanig

The effects of chemical treatments on the heat resistance of Bacillus coagulans 43P spores were studied. The spores were produced using both TAM-sporulation (TAM) agar and thermoacidurans (TA) agar, pH 6.8. A culture of B. coagulans 43P was inoculated and incubated at 45 C for 4 to 7 days to obtain > 95% sporulation. The spores were harvested, washed 3 times with sterile cold distilled water, treated with 0.75 mg lysozyme/ml for 3 to 4 hours at room temperature, and washed 3 times with sterile cold distilled water. The cleaned spore suspension was stored at 4 C.

Determination of heat resistance was carried out using B. coagulans spore suspensions prepared as follows: (1) untreated B. coagulans spores; (2) spores treated with thioglycollic acid solutions (0.5% and 12.5% w/v at pH 5.4 and 5.2, respectively); (3) lyophilized spores; (4) acid-treated spores prepared from (1), (2), and (3); and

(5) Calcium-treated spores prepared from (1), (2), and (4). The spore suspensions were heated at various temperatures and survivor curves were prepared by plotting the log survivors versus the heating times in minutes. The D-value was calculated for each heating trial.

There was no substantial difference in heat resistance among the natural and treated spores of B. coagulans; D_{100} -values ranged from 6.5 to 10 minutes. In contrast, previous investigations have shown that Ca-form spores have a 24-fold and 1000-fold higher heat resistance than H-form spores for C. botulinum and B. stearothermophilus, respectively.

The survivor curves of untreated spores contained a pronounced shoulder due to heat activation during the initial 15-25 minutes of heating. After treatment with thio-glycollic acid, the shape of the survivor curve changed and a portion of the spores appeared to be more sensitive to heat.

The results of this investigation indicate that the heat resistance of B. coagulans spores was not readily manipulated by chemical treatments. There was no substantial difference in the heat resistance of spore preparations produced using conventional methods for the preparation of

H-form and Ca-form spores. This resistance to chemical manipulation may be important in the survival of B. coagulans spores in thermally processed tomato products.

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INTRODUCTION

During the first few years of manufacture of tomato juice there were several outbreaks of what came to be known as "flat-sour" spoilage, characterized by the development in varying degrees of intensity of a medicinal or "phenolic" taste, and accompanied by a drop of 0.3 to 0.5 in pH. Cans of spoiled juice remained flat, but there was always a loss of vacuum. This type of spoilage is now rare because empirical methods for control have been developed (Stern et al., 1942).

However, microbial spoilage of heat-processed canned foods may be caused by microorganisms that survive thermal processes or that gain entrance through container leakage subsequent to processing. From the standpoint of establishing sterilization processes, spore-bearing bacteria are the organisms of chief concern except in high acid products (Stumbo, 1973).

Berry (1933) microscopically examined spoiled tomato juice and revealed the presence of an abundance of large, rod-shaped, vegetative cells. He successfully isolated

a spore-forming organism which he described under the name Bacillus thermoacidurans. Later, this organism was named B. coagulans or B. coagulans var. thermoacidurans.

Bacillus coagulans is an important food spoilage organism and has caused considerable economic loss in the tomato juice industry.

The heat resistance of spores is a variable property, and mature, dormant Bacillus spores can be manipulated between heat-sensitive and heat-resistant forms by chemical treatment (Alderton and Snell, 1963; Alderton et al., 1964; Alderton and Snell, 1969a, b; Alderton et al., 1976). The spores of B. coagulans, in nature, exhibit a variable resistance to the conventional thermal processes recommend for such acid foods (El-Bisi and Ordal, 1956a). The purpose of this investigation was to determine the effects of chemical treatment on heat resistance of B. coagulans spores.

REVIEW OF LITERATURE

Bacillus coagulans is a noncapsulated rod-shaped organism with truncate to round ends. It occurs singly, in pairs, and occasionally in short chains. It is motile by means of peritrichous flagella. Twenty-four-hour cultures are gram-positive while older cultures are both gram-positive and -negative. This organism is a facultative anaerobe which is normally classified as a thermophile. Spores are produced without bulging the sporangia (Sarles and Hammer, 1932; Berry, 1933).

The sporulation process is an important characteristic of the genus Bacillus. This process results in the formation of a single spore within a sporangium derived from a single vegetative cell. The spore is highly refractile to light, due to the high refractive index of the spore components, as evidenced by its bright appearance under phase-contrast microscopy. The spore also has an extremely low metabolic activity and a high density, and contains dipicolinic acid (DPA) and large amounts of calcium. It is resistant to usual staining techniques, heat, ultraviolet and

gamma-ray irradiation and most chemicals.

According to Gordon et al. (1973), the synonyms of B. coagulans used by earlier investigators include B. thermoacidurans (Berry, 1933), B. dextrolacticus (Anderson and Werkman, 1940), B. thermoacidificans (Renco, 1942), Lactobacillus cereale (Olsen, 1944). Smith et al. (1952) named the organism B. coagulans.

Incidence of the Organism

Bacillus coagulans is indigenous in soil. It is not pathogenic to humans or guinea pigs when taken orally; nor to tomato plants or green or ripe tomatoes (Berry, 1933).

In 1915, B. coagulans was described as the cause of an outbreak of coagulation in evaporated milk. The organism was present in the spoiled milk even though some of the milk had been processed at 113.3 C (236 F) for thirty-six minutes. The spoiled milk had been held at the condensery for at least ten days prior to shipment and was apparently normal. Therefore coagulation of the milk did not occur rapidly. Most of the abnormal cans were firmly curdled, but a few had a soft, flaky curd with considerable whey. The spoiled milk had a sweetish, cheesy odor, somewhat resembling the odor of Swiss cheese, and a sour cheesy flavor. The odor

and flavor were not unpleasant and there was no putrefaction. Acid determinations were measured on the milk that coagulated during the outbreak and on the milk that coagulated after being inoculated with a pure culture of the organism. A considerable increase in acidity occurred, i.e., the acidity of normal milk was 0.48% while that of the spoiled milk was 1.05%, calculated as lactic acid (Sarles and Hammer, 1932).

Cordes (1928) found B. coagulans responsible for an outbreak of "flat-sours" in evaporated milk. Moreover, B. coagulans caused flat-sour spoilage of commercially canned tomato juice. The organism was isolated from off-flavored canned tomato juice. While the spoilage was eventually of the "flat-sour" type, the off-flavor was observed some time before any change in pH occurred (Berry, 1933).

Bacillus coagulans is important to the canning industry since it is one of the few sporeforming organisms capable of growing in an acid food product, such as tomato juice, and producing flat-sour spoilage. Spoilage of foods is ordinarily accompanied by marked physical changes in the food as well as by the presence of large numbers of the causative microorganisms. However, flat-sour spoilage is often difficult to detect by appearance and the plate counts of spoiled foods are always low and often zero. Little or no

gas is produced to reduce the vacuum in the container; therefore, detection of flat-sour spoilage is impossible to determine without opening the container. Acid produced by the organisms during growth eventually will limit growth and cause death of the organism. Thus, the organism is difficult to isolate from the spoiled product (Becker and Pederson, 1950; Stumbo, 1973).

The sporadic nature of flat-sour spoilage in tomato products is based on a combination of factors: low-acid tomatoes, the presence of B. coagulans, and an opportunity and sufficient time for the organism to grow and acclimate to an abnormally low pH and then produce resistant spores. The occurrence of these factors is found in the mixture of soil and tomato juice often present on the surfaces of tomatoes in crates, or in decaying tomatoes in which a miscellaneous flora of yeasts and molds may raise the pH to a point permitting growth and spore production by B. coagulans (Becker and Pederson, 1950).

Factors Affecting Growth and Sporulation

Temperature. Becker and Pederson (1950) showed that B. coagulans grew at 45 and 55 C, and most strains grew at 63 to 65 C (water bath temperature). However, this

organism is not an obligatory thermophile since growth occurred at temperatures as low as 18 C. Optimum growth occurred between 37 and 45 C. Berry (1933) stated that the optimum growth temperature of B. coagulans was between 40 and 60 C and that 37 C was the optimum for the development of flat-sour spoilage.

In general, the temperature of incubation also affects the rate and the amount of spore formation. The optimum temperature for sporulation is close to that for growth, but the range is narrower. The amount of sporulation is reduced by unfavorable temperatures (Ordal, 1957; Stumbo, 1973).

Hydrogen ion concentration or pH. With respect to acidity, Cameron and Esty (1940) classified food into four groups:

- 1) Low-acid foods: pH 5.0 and higher.
- 2) Medium- (or semi-) acid foods: pH 5.0 to 4.5.
- 3) Acid-foods: pH 4.5 to 3.7.
- 4) High-acid foods: pH 3.7 and lower.

Bacillus coagulans is acid-tolerant to a certain degree. It is capable of causing the spoilage of acid foods, particularly tomatoes and tomato products. It can grow at

pH 4.0 or slightly lower and grows well in tomato products and some other semi-acid foods at pH 4.0 to 4.6 (Stumbo, 1973).

Rice and Pederson (1954b) suggested that various acidic parameters, e.g., pH, titratable acidity, buffer capacity, total acidity, and individual organic acids were involved in the natural inhibitory activity of tomato juice toward the growth of B. coagulans. However, pH was the most significant factor.

Tomato juice often appears to be an unfavorable growth medium for B. coagulans. Many B. coagulans strains grew very slowly and required a period of acclimatization, i.e., repeated transfers from juice to juice, before producing the typical flat-sour spoilage (Stern et al., 1942). Growth in tomato juice of pH 4.2 to 4.3 was very slow even at 37 C. Using a proteose peptone acid medium, Stern et al (1942) found the optimum pH for growth of B. coagulans was about 5. At various pH levels in tomato juice, the size of inoculum also affected the growth of B. coagulans. As the concentration of spores in the inoculum was lowered, the culture required a more favorable pH for growth. It was impossible to indicate the absolute minimum pH at which a given concentration of spores would show growth since the

acid tolerance varied for each strain of B. coagulans employed (Rice and Pederson, 1954a).

Sporulation usually occurred between pH 5.15 and 6.95. A few spores were produced in media with a pH as low as pH 4.5, or as high as 7.8, but sporulation was not observed below or above these limits (Becker and Pederson, 1950). With some strains, pH values not affecting vegetative growth may partially or completely prevent spore formation. Bacillus coagulans requires relatively low pH levels, about 5.4 to 5.7, for optimum sporulation (Stumbo, 1973). Nevertheless, Ordal (1957) reported that a six-day old slant culture of B. coagulans (NCA strain 43P) incubated at 45 C had maximum sporulation at pH values close to 6.5, although the organism appeared to grow equally well over the pH range 5.0 to 7.5.

Nutritional requirements. The nutritional needs of B. coagulans varied depending upon the temperature of incubation (Campbell and William, 1953). In a study of the vitamin requirements of B. coagulans strains at 37 and 55 C, Cleverdon et al. (1949) found that niacin, thiamine, and biotin were required for growth at both temperatures. Growth and sporulation were most abundant at the lower temperature

of incubation.

Ordal (1957) showed that the omission of the sulfur-containing amino acids (methionine and cystine) from media markedly reduced the sporulation of B. coagulans. However, upon fortification of the medium with 10 ppm MnSO_4 , sporulation equalled that in the complete medium. He suggested that instead of a methionine requirement the organism actually had a sulfur requirement which was greater for sporulation than for growth. Furthermore, he found that L-alanine exhibited some stimulatory effect toward the sporulation of B. coagulans even though this compound is an active stimulant of spore germination.

Bacillus coagulans requires more folic acid or para-aminobenzoic acid (PABA) for sporulation than it does for growth. The omission of folic acid markedly reduced the percentage of sporulation, although this requirement could be fulfilled by PABA. Addition of various purine and pyrimidine bases in place of folic acid or PABA showed little or no effect on sporulation (Ordal, 1957).

The addition of malate, succinate, or α -ketoglutarate to shake cultures of B. coagulans in thermoacidurans broth markedly stimulated sporogenesis (Amaha et al., 1956; Ordal, 1957).

The role of minerals also has a great effect on sporogenesis. Sporulation of B. coagulans in three peptone-containing agar media was greatly enhanced by the addition of manganese (Mn^{++}), cobalt (Co^{++}), or nickel (Ni^{++}) (Amaha et al., 1956).

Oxygen. Bacillus species require oxygen for the production of spores. However, Sarles and Hammer (1932) reported that B. coagulans grew in canned milk, although an analysis of the headspace gas after an incubation period of 75 days revealed no oxygen was present. The organism was therefore regarded as a facultative anaerobe since it was capable of spore germination and vegetative growth in canned tomato juice (Berry, 1933). Spore germination, vegetative growth and spore formation of strains of B. coagulans occurred in oxygen concentrations as low as 0.1 mm (mercury) on agar media. In the absence of oxygen, however, some of these cellular functions occurred (Rice and Pederson, 1954).

Thermal Resistance of Spores

Among biological systems, bacterial endospores exhibit the greatest degree of thermoresistance. The heat resistance of bacterial spores may exceed that of the vegetative form by factors of 10^5 or more (Gould and Dring, 1974).

The resistance of bacteria to heat can be affected by many factors, e.g., inherent resistance, environmental influences active during the growth and formation of cells or spores, and environmental influences active during the time of heating of the cells or spores (Schmidt, 1957).

Inherent resistance. Variation in inherent resistance occurs not only within species but also within different strains of the same species. Different strains of the same species grown in the same medium and heated in the same menstruum may have widely different resistances (Stumbo, 1973).

Age. The age of spores may have some effect on their heat resistance. Esty and Meyer (1922) found that young, moist spores are more resistant than old spores. However, Williams (1936) found no correlation between the age and resistance of spores of some species.

Growth and sporulation temperature. El-Bisi and Ordal (1956a, b) and Lechowich and Ordal (1962) reported that the growth temperature of B. coagulans markedly affected the thermal resistance of the spores produced. The thermal resistance of spores was increased by increases in the growth temperature.

Medium and pH. The phosphate concentration in the growth medium exhibited a highly significant effect on the thermal death rate of B. coagulans spores. El-Bisi and Ordal (1956a) found a decrease in heat resistance as well as in the calcium content of spores of B. coagulans, with increasing phosphate concentrations in the sporulation medium. They postulated that the phosphate anion in the sporulation medium interfered with the availability of divalent cations to the sporulating cells.

The addition of extra calcium or manganese to the sporulation medium caused a significant increase in thermal resistance of B. coagulans spores, whereas the addition of magnesium had no effect (Amaha and Ordal, 1957). Lechowich and Ordal (1962) found that when heat resistance of B. subtilis spores increased, amounts of calcium, magnesium, manganese, and dipicolinic acid (DPA) in the spores also increased. This relationship did not occur in the spores of B. coagulans. In this case the DPA content was lower in spores which exhibited a higher degree of heat resistance.

Heating medium. The nature of the suspending medium during heat treatment has a marked effect on resistance of the spore (Anderson et al., 1949). Increased

calcium or magnesium ion concentrations in the suspending medium enhanced thermal resistance of some species (Sugiyama, 1951), and low concentrations of NaCl also tended to increase the resistance of many organisms (Esty and Meyer, 1922; Stumbo, 1973). Ordal and Lechowich (1958) showed that spores of B. coagulans exhibited the highest heat resistance when heated in M/40 phosphate buffer as compared to heating in phosphate buffers of higher or lower concentrations, M/100 glycylglycine, M/100 trihydroxymethylaminemethane buffer, M/100 ethylenediaminetetraacetic acid solution or distilled water.

When organisms were heated in different foods, they had different degrees of thermal resistance. Anderson et al. (1949) found that with increases in salt concentration of tomato juice, as well as increases in citric, acetic, and lactic acids, a progressive decrease in the thermal destruction time of B. coagulans was observed. On the other hand, sucrose and dextrose enhanced the heat resistance of the organism.

Chemical Manipulation of the Heat Resistance of Spores

Thermal resistance of spores can be manipulated by chemical means from ordinary heat resistance into

heat-sensitive and heat-resistant states (Alderton and Snell, 1969a, b; Alderton et al., 1976). Due to the chemical methods used, Alderton and his coworkers called the heat-sensitive and heat-resistant states of the spores hydrogen (H)-form and calcium (Ca)-form spores, respectively. The differences in heat resistance between the two states, based on the rate of destruction at a given temperature, differed up to 1000-fold. These changes in heat resistance were reversible and were controlled by chemical treatment of a cation exchange system contained within the spore. The change in heat resistance was a property of the spore and persisted in a new environment after removal of the reagents used to effect the change in heat resistance (Alderton and Snell, 1969a). I am unaware of any literature concerning chemical manipulation of the heat resistance of B. coagulans spores. However, many studies had been done on other spores of Bacillus and Clostridium species (Alderton and Snell, 1963; Alderton et al., 1964; Alderton and Snell, 1969a, b; Alderton et al., 1976).

Hydrogen (H)-form spore. Preparation of H-form spores was carried out by placing spores in nitric or hydrochloric acid solutions (Alderton et al., 1964; Alderton et al., 1976). Alderton and Snell (1963) found that

B. megaterium spores were more easily heat killed at low pH. Under those conditions, hydrogen ions, having the highest exchange potential, would displace other cations in the spore cation exchange system. The H-form spores of B. stearothermophilus (NCA 1518) had a heat resistance approximately 1000-fold less than that of Ca-form spores at the same temperature (Alderton and Snell, 1969b). Likewise, sensitive (H)-form spores of Clostridium botulinum 52A were 24-fold less heat resistant than Ca-form spores at 235 F (Alderton et al., 1976).

Calcium (Ca)-form spore. The heat-resistant (Ca)-state of spores can be obtained from either the H-form spore or the native spore by treatment in calcium buffer solution at high pH. Alderton et al. (1964) found that the cations of the phosphate buffer at pH 7.9 partially convert the spores to heat resistant form during the heat resistance test itself. At a lower pH (5.7), monovalent cations could not compete effectively with hydrogen ions for the ion exchange sites and the spores remained in the heat sensitive state. Recently, Alderton et al. (1976) reported the heat resistance of Ca-form spores, either in food products or in laboratory media, was much greater than that of untreated

spores. However, the higher resistance obtained by chemical treatment to the Ca-form was reduced when the spores were held at elevated but sublethal temperatures before terminal heating. Furthermore, conversion of Ca-form spores to their heat-sensitive (H)-form could be done by acidification of products containing the spores (Alderton et al., 1976).

Effect of Thioglycollate on Spores

Bacterial endospores germinate after heat activation. However, reducing agents such as thioglycollic acid and mercaptoethanol were found to imitate heat activation of bacterial endospores. Activation caused by reducing agents is reversible, but their effectiveness in activating spores is incomplete. Keynan et al., (1964) illustrated partial activation of B. cereus strain T which occurred after 12 hours exposure to either 0.2 M mercaptoethanol or 0.2 M thioglycollic acid at 28 C. The optimum pH for activation was about 6. On the other hand, Gibbs (1966) found that thioglycollate failed to activate C. bifermentans spores which indicated that the response to treatment with reducing agents varied among spores of different species. The activation process does not affect the refractility and heat resistance of bacterial spores (Gould and Hitchin,

1963a, b; Rowley and Levinson, 1967).

Although several hypotheses have been proposed for the mechanism of activation, most workers have suggested that reducing agents act by denaturing spore coat protein via reduction of disulfide linkages. This change in structure of protein could be responsible either for the exposure of the active enzymatic sites necessary for germination or for the increase in the accessibility of the substrate to those sites (Gould and Hitchins, 1963a; Keynan et al., 1964; Rowley and Levinson, 1967).

Other investigators have reported that rupture of disulfide bonds by reducing agents allowed the spores of Bacillus and Clostridium species to become sensitive to lysozyme and hydrogen peroxide, resulting in a phase-darkening of the treated spores, loss of dipicolinic acid and almost complete lysis of the spores (Gould and Hitchins, 1963a, b). Furthermore, at pH 2.6 thioglycollate enhanced an exchange of H^+ and spore cations, thus allowing the spores to become heat sensitive (Rowley and Levinson, 1967).

MATERIALS AND METHODS

Test Organism

Bacillus coagulans 43P strain was obtained from the culture collection of the Department of Food Science and Human Nutrition, Michigan State University. The frozen APT broth-culture was thawed and incubated at 55 C for 1 day. An active culture was produced by streaking 1 loopful of the original culture onto Bacto-TAM Sporulation (TAM) agar (Difco). After incubation for 24 hr at 55 C, a single colony was picked and transferred to a TAM agar slant for culture maintenance. The identity of the organism was confirmed according to the methods in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Media

Bacto-Thermoacidurans (TA) agar (Difco) and TAM agar were used in this investigation. Both media contained 0.5% proteose peptone (Difco), 0.5% yeast extract (Difco), 0.5% dextrose (Difco), and dibasic potassium phosphate, 0.05% in TAM agar and 0.4% in TA agar. The primary

difference in ingredients was the incorporation of 0.02% manganese sulfate in TAM agar but not in TA agar. The pH of both media was adjusted to 6.8.

Preparation of Spore Suspension

Bacillus coagulans spores were produced using procedures described by Lechowich and Ordal (1962) and Stumbo (1973). The culture was inoculated on a TAM agar slant and incubated at 45 C for 2 days. After microscopic examination showed > 95% sporulation, the surface growth was suspended in a small amount of sterile distilled water by scraping the surface with a sterile glass rod. The suspension was collected in a sterile screw-cap test tube and heated in a water bath at 80 C for 20 minutes to destroy vegetative cells and to activate the spores for germination. This heat-shocked suspension was used to inoculate large bottles containing layers of TAM agar for mass spore production.

Each bottle of TAM agar was inoculated with 2 ml of heated spore suspension. The bottle was tilted back and forth until the inoculum covered the entire surface. After inoculation, the bottles were incubated at 45 C for 4 to 7 days until growth and sporulation were virtually complete.

The spores were harvested separately from each bottle with 100 ml cold sterile distilled water by scraping the growth from the agar surface with a sterile glass rod. The crude spore suspension was then centrifuged at 5000 x g for 30 min in a Sorvall RC II refrigerated centrifuge, washed 3 times by centrifugation in cold sterile distilled water, and suspended in 50 ml cold sterile distilled water.

Later, the spores were suspended in 0.75 mg lysozyme/ml in 0.1 N potassium chloride and shaken for 3 to 4 hours at room temperature to facilitate the degradation and removal of sporangial fragments and cellular debris. After 3 additional washings in cold sterile distilled water, the spore suspension was considered to be free of foreign material and appeared clean when examined under dark phase-contrast microscopy (1000 x). The spores were counted with the aid of a Petroff-Hausser Bacteria Counter (C.A. Hausser & Son, Philadelphia, Pa.). The spores were stored at 4 C in screw-cap bottles containing small glass beads in suspensions containing approximately 6.0×10^8 to 9.0×10^8 spores/ml.

Treatment of Spores

Thioglycollate-treated spores. Thioglycollic acid, 1% and 25% (w/v), were prepared by adding 1 g and 25 g,

respectively, of sodium thioglycollate to 48 g 8 M urea with equivalent amounts of concentrated hydrochloric acid (HCl), followed by diluting to 100 ml with distilled water (Gould and Hitchins, 1963a, b). The spore suspensions were treated with the thioglycollic acid solutions at a ratio of 1:1 and incubated at 37 C for 2.5 hours. After incubation the treated spores were centrifuged, washed 3 times with cold sterile distilled water and suspended in sterile distilled water at a concentration of approximately 10^8 spores/ml.

Lyophilized spores. Twenty-five milliliters of spore suspension were freeze-dried using a VirTis Freeze-Mobile lyophilizer. The lyophilized spores (12.8 g) were suspended in 8 ml of cold sterile distilled water, and counted prior to heat treatment.

Acid-treated spores.

1) Untreated spore suspension was mixed with 0.2 N HCl in a 1:1 ratio giving a final concentration of 0.1 N HCl. After 16 hours of incubation at 25 C, the spores were centrifuged and washed 3 times (Alderton et al., 1976).

2) Thioglycollate-treated spores were mixed with 0.2 N HCl at a 1:1 ratio, and incubated at 50 C for 2 hours, followed by centrifugation and washing with cold sterile

distilled water.

3) Lyophilized spores were also treated with both methods described above (1 and 2).

Calcium-treated spores.

1) An untreated spore suspension was mixed with an equal volume of 0.4 M calcium acetate (CaAc) adjusted to pH 8.5 and incubated at room temperature for 140 hours. The spores were centrifuged and washed 3 times as described above (Alderton et al., 1976)

2) Hydrogen-treated spores were treated with calcium by adding 0.4 M CaAc at a 1:1 ratio and incubating the mixture at 50 C for 140 hours; or alternatively, H-treated spores were allowed to stand overnight in an equal volume of 0.04 M CaAc, pH 9.7, at 21 C, followed by holding this suspension in a water bath at 80 C for 3 hours (Alderton and Snell, 1969b). After treatment the spores were centrifuged and washed 3 times.

3) Thioglycollate-treated spores were then mixed as described above (2) with 0.04 M CaAc.

After washing, the spores from each treatment were suspended in cold sterile distilled water at a concentration of approximately 10^8 to 10^9 spores/ml.

Determination of Heat Resistance of Spores

The following is a modification of methods described by El-Bisi and Ordal (1956a, b) and Lechowich and Ordal (1962). One milliliter of spore suspension was added at zero time into 0.025 M potassium phosphate buffer which had been adjusted to pH 7.0 with 0.1 N NaOH, to make approximately 10^7 spores/ml. The heating bath was equilibrated at the desired temperature, i.e. 75, 85, 96, or 100 C before the spores were added. Samples were withdrawn at various intervals, discharged into previously chilled phosphate buffered dilution blanks, and cooled in an ice bath. The samples were diluted and plated in duplicate on either TAM or TA agar plates. All plates were incubated at 45 C and colonies were counted after incubation for 24 to 48 hours. Logarithms of the mean plate counts for each sample, i.e. log survivors, were plotted versus the heating times in minutes. The D-value, time required to destroy 90% of the spores, was calculated for each heating trial from the slope of the survivor curve ($D = -1/\text{slope}$).

RESULTS AND DISCUSSION

Growth and Sporulation

Many studies indicated that B. coagulans grew well and sporulated at 50 to 55 C on a proteose peptone acid medium, namely thermoacidurans agar. Sporulation usually occurs after 2 to 4 days incubation (Stern et al., 1942; Stumbo, 1973; Townsend et al., 1956). Using TAM agar (TA agar fortified with 0.02% MnSO_4) adjusted to pH 6.8, 90% sporulation was obtained when the organisms were incubated at 30, 45, and 52 C for 12, 6, and 4 days, respectively (Lechowich and Ordal, 1962). In the present investigation, preliminary experiments showed that B. coagulans 43P grew well but sporulated poorly on TAM agar at 55 C. When the incubation was continued for 5 days, the organism died. However, sporulation occurred at 45 C. In order to obtain >95% sporulation, 2 days of incubation on TAM agar slants was required whereas 4 to 6 days of incubation was needed on the same agar in bottles. Thus, TAM agar incubated at 45 C was used in subsequent experiments for the recovery of surviving spores after various heat treatments.

Later, TA agar, pH 5, was used instead of TAM agar. The primary differences between the two media were their pH and the incorporation of 0.02% MnSO_4 in TAM agar but not in TA agar. However, there was no substantial difference in the numbers of spores obtained when using TA agar adjusted to pH 6.8 with 0.1 N NaOH, or TA agar plus 0.02% MnSO_4 adjusted to pH 6.8, or TAM agar pH 6.8. Thus, TA agar adjusted to pH 6.8 was used in subsequent work, including spore production and recovery of viable spores after heat treatment. Most workers indicated that addition of MnSO_4 to the medium enhanced the sporulation of various Bacillus species and stimulated the development of colonies from viable spores (Amaha et al., 1956; Amaha and Ordal, 1957; Ordal, 1957; Curran, 1957; Alderton and Snell, 1969). However, deleting MnSO_4 from the medium in this study had no effect on either sporulation or the total plate counts of the spores.

Heat Resistance of Untreated Spores

Figure 1 shows the survivor curve for untreated B. coagulans spores heated at 100 C in 0.025 M potassium phosphate buffer, adjusted to pH 7.0. A plateau or shoulder was initially present after heating for 0 to 35 minutes.

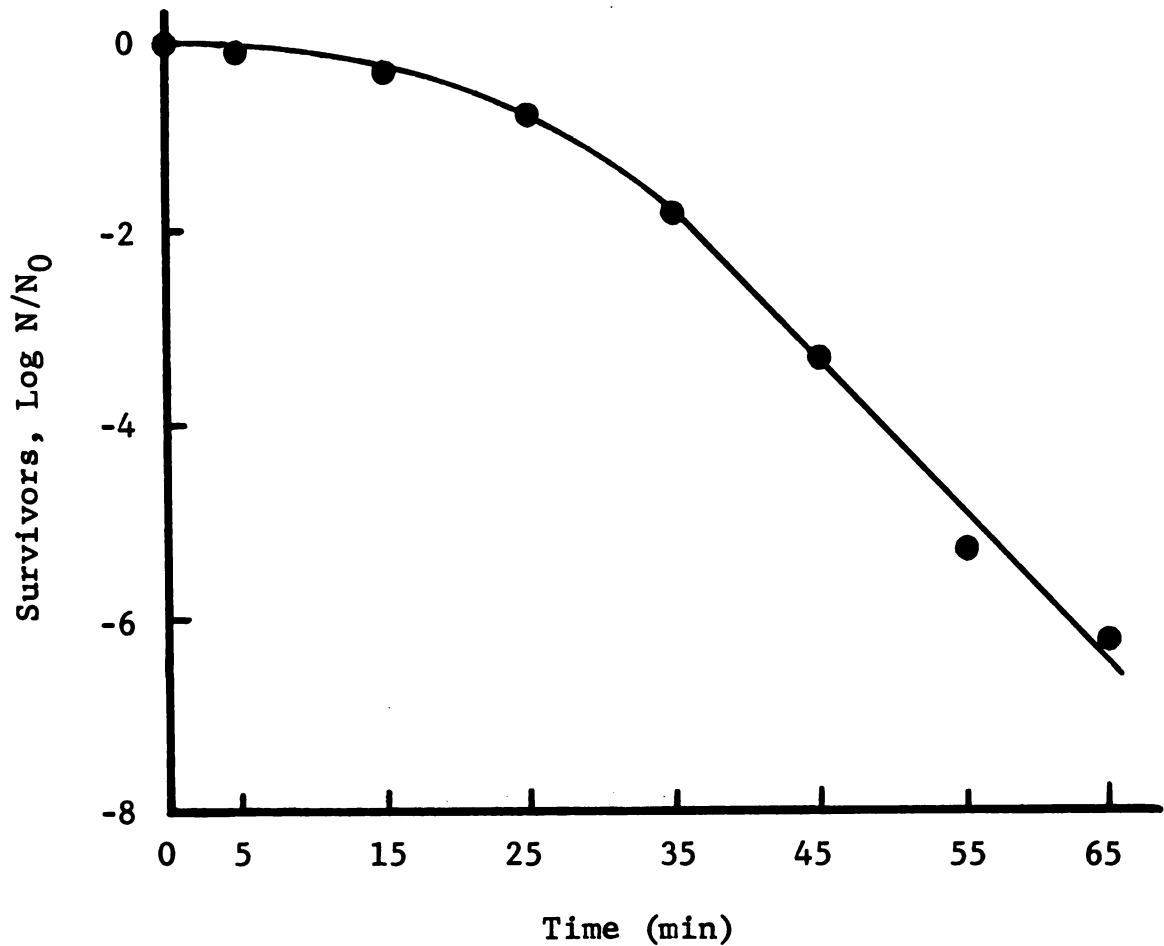


Figure 1. Survivor curve of untreated *B. coagulans* spores, heated at 100 C in 0.025 M potassium phosphate buffer, pH 7.0. The survivor curve was plotted from the logarithmic mean obtained from four separate experiments.

The shoulder was apparently due to heat activation of the spores. The temperature and duration of optimal heating for the activation of spores varied widely among different species and even among different spore preparations of the same strain (Keynan et al., 1964). Activation of some spores would compensate for destruction of some spores, resulting in a false decrease in the inherent rate of death of the organisms. The exact character of the shoulder depends upon the degree of predominance of one rate over the other. After 35 minutes the heat activation was essentially complete and the destruction rate remained constant. The destruction of the untreated spores was logarithmic. Youland and Stumbo (1953) found that the order of death of B. coagulans spores subjected to moist heat was logarithmic. In contrast, Frank and Campbell (1957) indicated that the survivor curve for B. coagulans spores heated at 107.2 C was non-logarithmic. However, different shapes of survivor curves have been observed and they have been discussed by many investigators (El-Bisi and Ordal, 1956b; Stumbo, 1973).

The thermal death rate of untreated spores at 100 C was determined as the time required to destroy 90% of the spores at 100 C (decimal reduction time, or D_{100} -value). The D_{100} -value was 6.5 minutes. This result was in

reasonable agreement with values reported by other investigators. El-Bisi and Ordal (1956b) determined the D-value of B. coagulans spores was 8.31 minutes at 96 C whereas Lechowich and Ordal (1962) found the $D_{98.5}$ -value was 11 minutes.

Heat Resistance of HCl-treated and Ca-treated Spores

Spores of many Bacillus and Clostridium species can be manipulated between the heat-sensitive or H-form and heat-resistant or Ca-form by chemical treatments (Alderton and Snell, 1963; Alderton and Snell, 1969a, b; Alderton et al., 1976). Conventional procedures for chemical manipulation of spores were also expected to affect B. coagulans spores.

HCl-treated spores. Conventional treatment with hydrochloric acid (HCl) was used in an attempt to convert spores of B. coagulans to the H-form. The HCl-treated spores were heated at 100 and 96 C, and the resulting survivor curves are presented in Figure 2. The D_{100} -value of the HCl-treated spores was 7 minutes and the D_{96} -value was 30 minutes. The curves were quite similar to that obtained for untreated spores. The heat activation period ranged from 0 to 25 minutes at 100 C and from 0 to 35 minutes at 96 C. Several

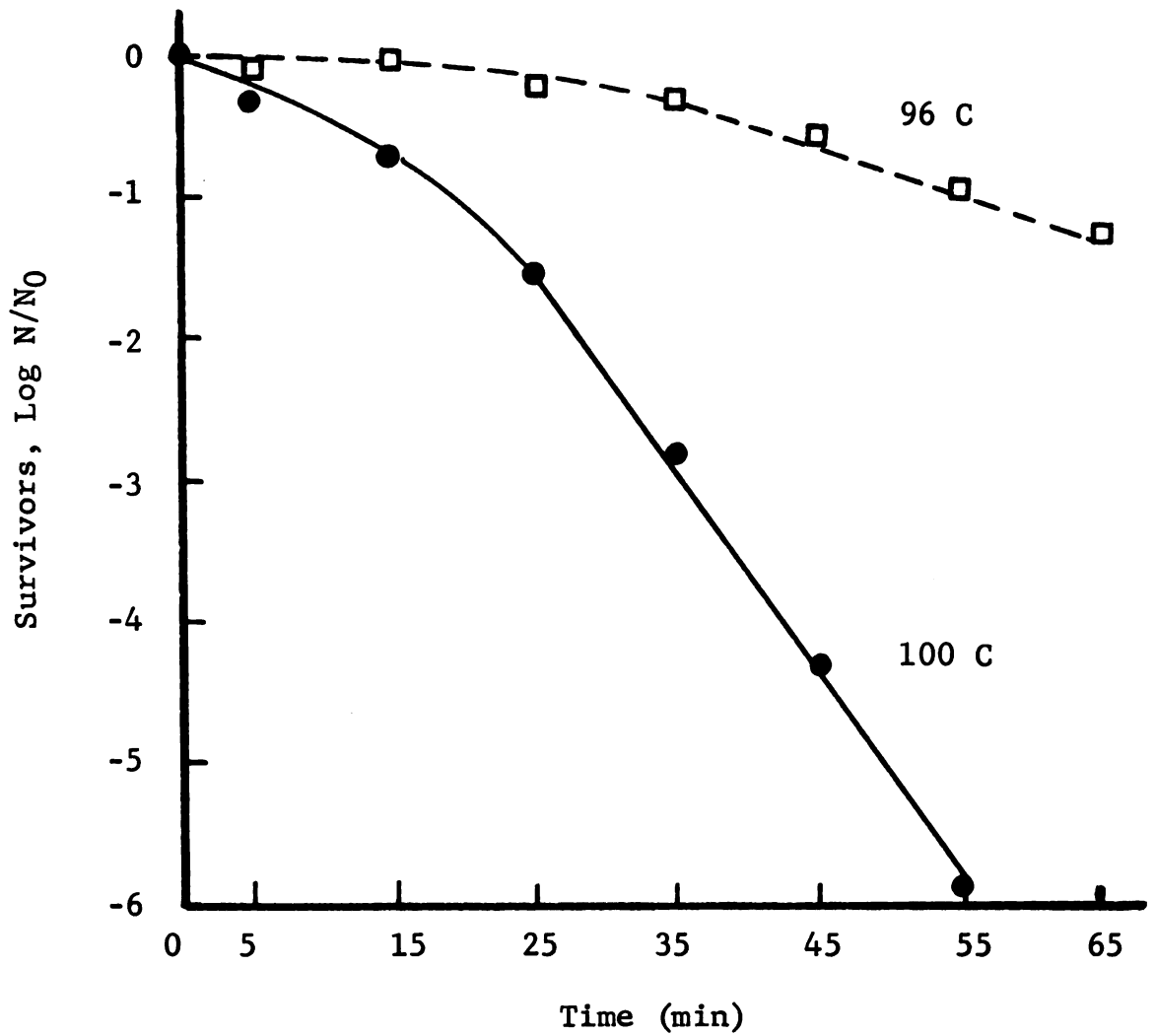


Figure 2. Survivor curves of HCl-treated spores, heated at 96 and 100 C. The survivor curve at 100 C was plotted from the logarithmic mean obtained from duplicated experiments.

reports have indicated that the time required for heat activation decreased as the temperature increased. Thus, at higher temperatures, the activation may occur so quickly that it would not be detectable (Stumbo, 1973).

Ca-treated spores. The Ca-treated spores of B. coagulans were prepared from both untreated and HCl-treated spores. The spores were heated at 100 C. The survivor curves, as shown in Figure 3, were similar to those of both untreated and HCl-treated spores. The heat activation period ranged from 0 to 30 minutes. The D_{100} -values were 7 and 9.5 minutes for Ca-treated spores prepared from untreated and HCl-treated spores, respectively.

These preliminary studies indicated that the thermal resistance of B. coagulans 43P spores was not substantially different among untreated, HCl-treated, and Ca-treated spores. The D_{100} -values ranged from 6.5 to 9.5 minutes. In contrast, in previous studies, Ca-form spores had a 24-fold and 1000-fold higher heat resistance than H-form spores for C. botulinum and B. stearothermophilus, respectively (Alderton and Snell, 1969b; Alderton et al., 1976). Also, Ca-form spores of C. botulinum were 5 to 10 times more heat resistant than untreated spores. Moreover, the heating

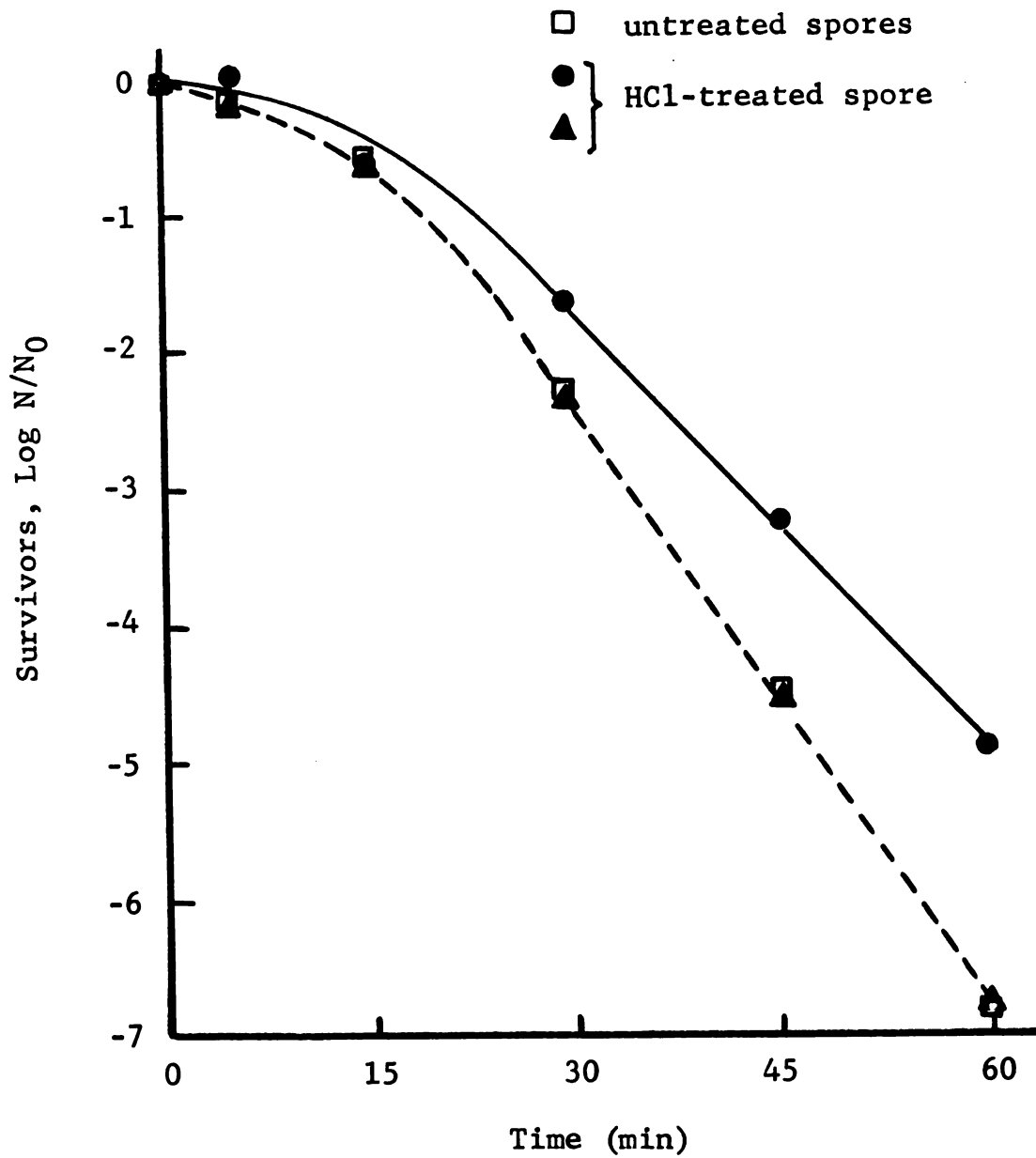


Figure 3. Survivor curves of Ca-treated spores, heated at 100 C. The spores were prepared from untreated and HCl-treated spores.

temperature had to be changed about 28 C in order to get equal survivor rates between H-form and Ca-form spores of B. stearothermophilus (Alderton and Snell, 1969a). Thomas and Russell (1975) reported that $>99.9\%$ of H-form spores of B. pumilus were killed after 60 minutes of heating at 77 C whereas only 50 and 0% killing were found in Ca-form and natural form spores. Most workers indicated that the difference in heat resistance was of a large magnitude. The chemical states of spores can be prepared by treating mature dormant spores with an acid (e.g., HCl, and HNO₃) to form H-form spores or buffered metal cations (e.g., calcium acetate at pH 9.7) to form Ca-form spores. The change in heat resistance of spores which is introduced by such treatments persists even after the spores are thoroughly washed. The rate of change is affected by the pH and the temperature as well as the duration of the treatment (Alderton and Snell, 1963; Alderton et al., 1964; Alderton and Snell, 1969a; El-Mabsout, 1977).

Heat Resistance of Lyophilized Spores and Lyophilized Acid-treated Spores

The primary difference between the experiments described herein and those of Alderton and Snell (1969b) and Alderton et al. (1976), was that Alderton and coworkers

used lyophilized spores. Thus, the heat resistances of untreated and acid-treated spores were determined for lyophilized spores.

Lyophilized spores. Lyophilized spores were heated at 100 C. The survivor curve, as shown in Figure 4, indicated that the lyophilization process had some influence on the thermal resistance of the spores. Apparently some spores were either germinated or converted into heat-sensitive forms since they were readily destroyed during the first 5 minutes of heating. The middle portion of the curve (from 5 to 25 minutes) was influenced by the heat activation of some spores that were not heat-sensitive. The destruction rate increased after 25 minutes of heating, and the D_{100} -value was 8.5 minutes, which is similar to that of the natural spores.

Acid-treated spores. Lyophilized spores were acid-treated and then heated at 85 C. The survivor curve (Figure 4) also showed the destruction of heat-sensitive forms during the first period of heat treatment. However, the D_{85} -value could not be determined accurately from the experimental data. Since the upper portion of the curve was similar to that of the lyophilized spores, the acid

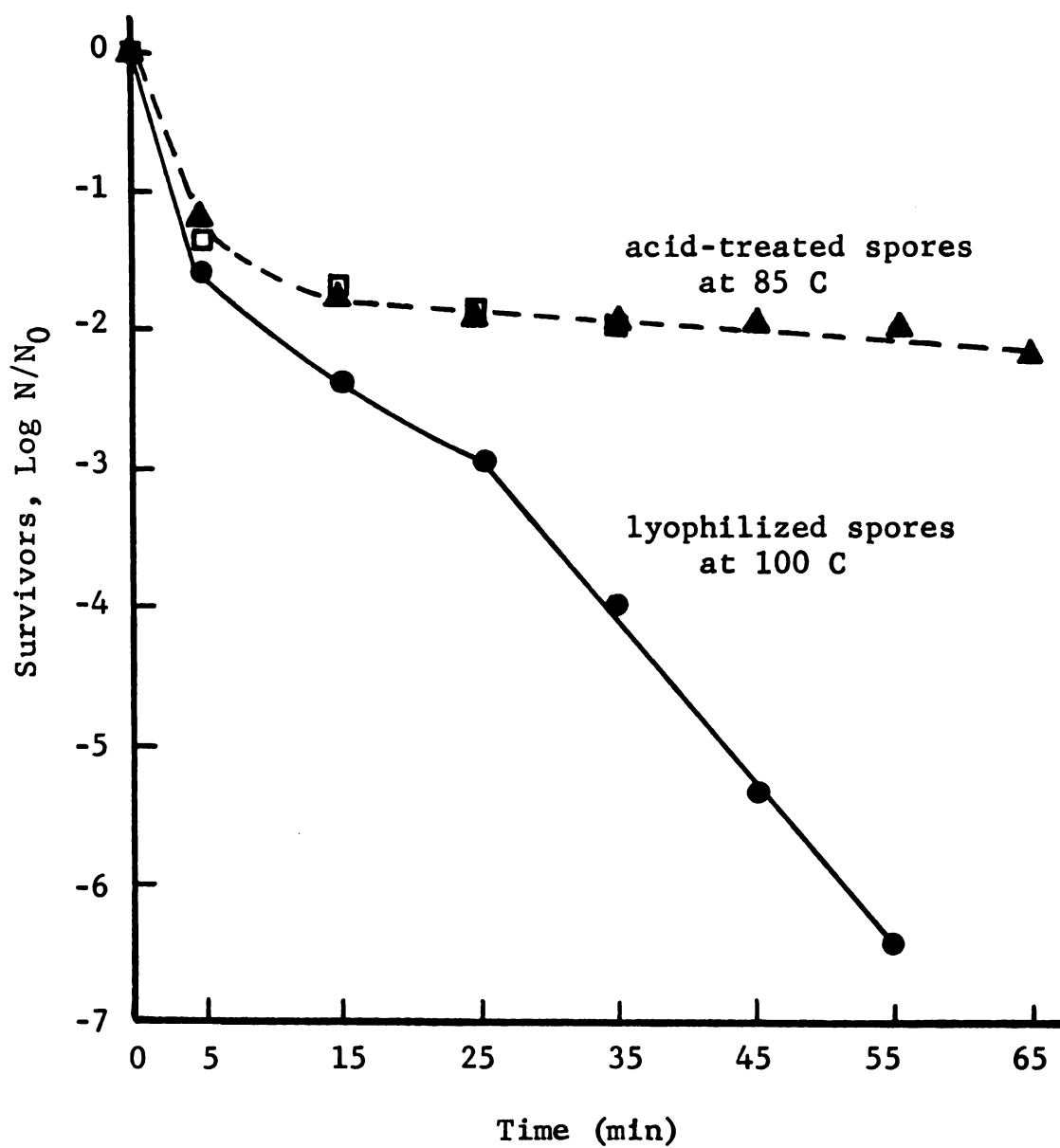


Figure 4. Survivor curves of lyophilized spores and lyophilized, acid-treated spores, heated at 100 and 85 C, respectively.

treatment did not appear to affect the portion of lyophilized spores which were heat-resistant.

Heat Resistance of Thioglycollate-treated Spores

Bacillus coagulans spores were exposed to 1 and 25% (w/v) thioglycollic acid in 8 M urea at pH 5.4 and 5.2, respectively. Figure 5 shows the survivor curve for 0.5% and 12.5% thioglycollate-treated spores heated at 100 C in 0.025 M potassium phosphate buffer, pH 7.0. The shape of the curve was different from that of the untreated spores since there was no plateau or shoulder on the survivor curve for the thioglycollate-treated spores when heated at 100 C. However, the heat resistance of the thioglycollate-treated spores ($D_{100} = 6.7$ min) was similar to that of the untreated spores.

The survivor curve for 0.5% thioglycollate-treated spores heated at 75 C is also shown in Figure 5. There was little destruction and a D_{75} -value could not be accurately determined from the curve. As expected, the data indicated that when the heating temperature was lowered, the thermal destruction rate of the thioglycollate-treated spores decreased.

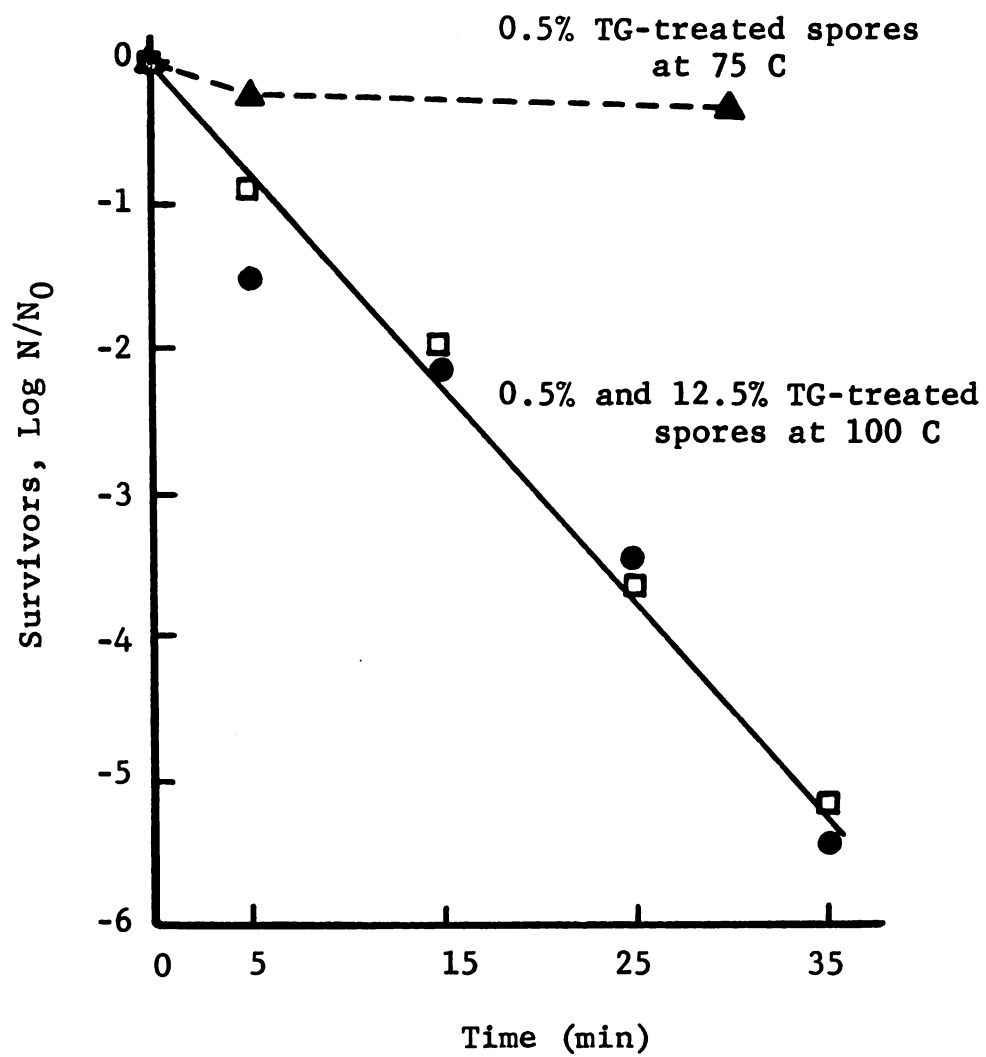


Figure 5. Survivor curves of 0.5% and 12.5% thioglycollate (TG)-treated spores, heated at 75 and 100 C.

Reducing agents such as thioglycollic acid and mercaptoethanol were found to partially replace heat treatment as a mean of spore activation. Incomplete activation could result if the disulfide bonds in the spores distributed so that only a portion was accessible to added reducing agents (Keynan et al., 1964). Gould and Hitchins (1963a) found that when B. cereus spores were incubated in thioglycollic acid (1% v/v; 8 M urea) at 37 C for 24 hours, no decrease in viability occurred nor was there a loss of spore refractility under phase-contrast microscopy. However, when thioglycollate-treated spores were subsequently transferred to lysozyme, loss of viability occurred at a much slower rate than loss of phase brightness. In another study (Rowley and Levinson, 1967), spores of B. megaterium QM B 1551 treated with thioglycollate (0.4 M, pH 2.6) at 30 C for 5 minutes retained the characteristics of dormant spores but they became heat-sensitive. The percentage of germination of thioglycollate-treated spores over a 5-hour period in glucose was markedly reduced. However, germinability and heat-resistance were restored by exogenous cations which suggested that the thioglycollate treatment resulted in the loss of spore ions essential for normal germination in glucose and for heat resistance (Rowley and Levinson, 1967).

Heat Resistance of Acid-treated and Ca-treated Spores
Prepared From Thioglycollate-treated Spores

Acid-treated spores. Figure 6 shows the survivor curves for acid-treated spores prepared from 0.5% and 12.5% thioglycollate-treated spores. The data indicated that $< 90\%$ of the acid-treated spores prepared from 0.5% thioglycollate-treated spores were killed after 5 minutes heating at 85 C in 0.025 M potassium phosphate buffer, pH 7.0. In addition, $> 99\%$ of the acid-treated spores prepared from 12.5% thioglycollate-treated spores were destroyed after heating for 15 minutes. The results indicated that some heat-sensitive spores were formed, or, less likely, that some spores germinated during acid treatment of spores previously exposed to 0.5% or 12.5% thioglycollic acid. Treatment with the higher concentration of thioglycollic acid resulted in a greater conversion of spores to heat-sensitive forms during subsequent treatment with acid. Most workers have indicated that exposure of spores to a low pH results in the loss of spore cations (Levinson and Hyatt, 1964; Rode and Foster, 1966b), a change to a heat-sensitive form (Alderton and Snell, 1963) and in greater germinability (Rode and Foster, 1966a), depending upon calcium and other cations. Therefore, when the disulfide bonds in the spore coat were unmasked by

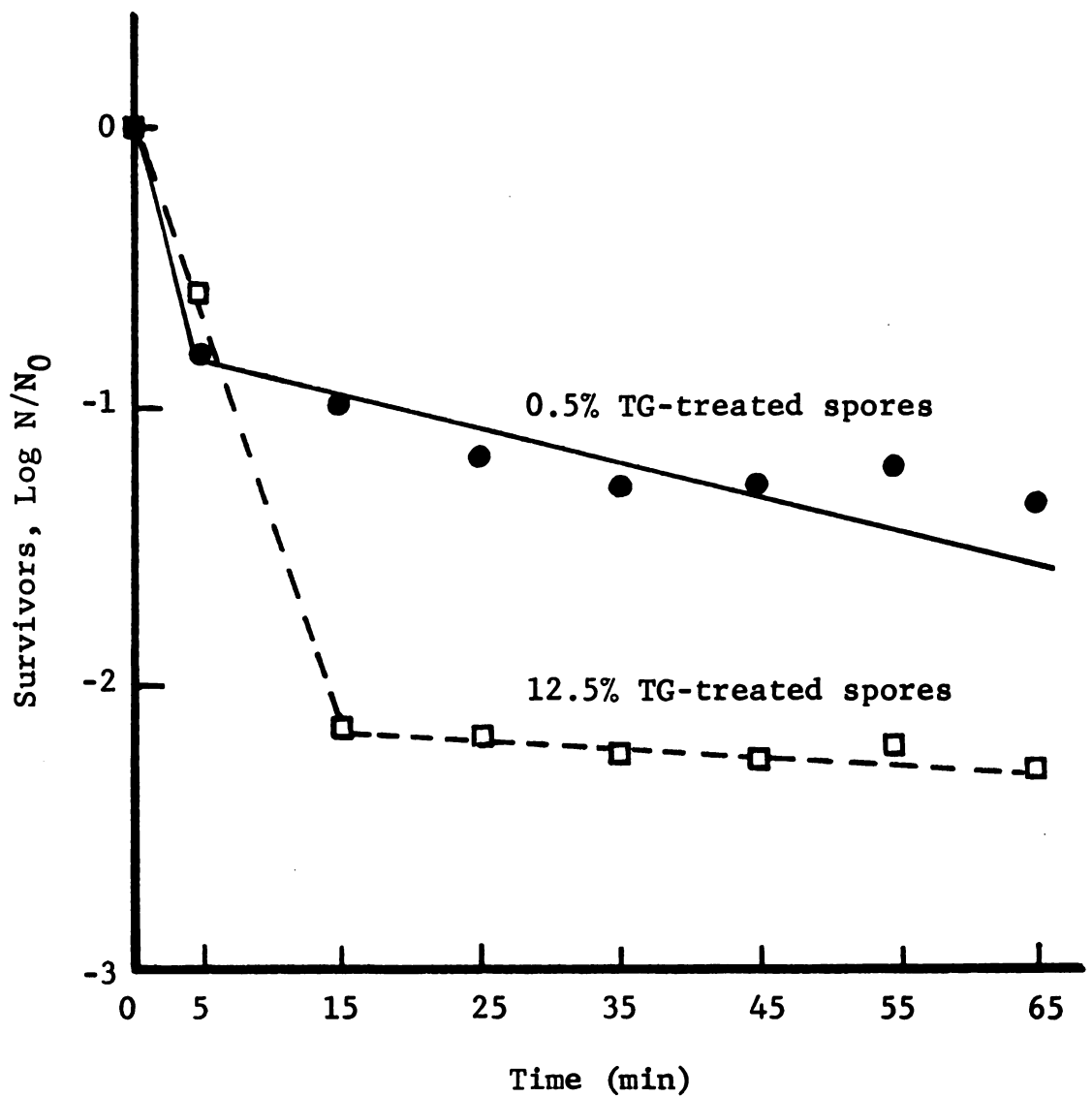


Figure 6. Survivor curves of acid-treated spores, heated at 85 C. The spores were prepared from 0.5% and 12.5% thioglycollate (TG)-treated spores.

thioglycollate and/or the reaction of H^+ ions was increased, at low pH, an exchange of spore cations might be enhanced. The loss of calcium and other ions from the spores treated with thioglycollate at low pH resulted in a decreased heat resistance of the spores (Rowley and Levinson, 1967).

Ca-treated spores. Figure 7 shows the survivor curves of Ca-treated spores previously exposed to 0.5% or 12.5% thioglycollic acid. The shape of the curves was similar to that of the natural spores and did include the heat activation period from 0 to 30 minutes. Although there was a slight difference between the Ca-treated spores previously exposed to 0.5% and 12.5% thioglycollic acid ($D_{100} = 10$ and 7.2 min), the values were not substantially different from that obtained for the natural spores.

Table 1 presents the D_{100} -values of B. coagulans spores obtained after various treatments. The data indicate that chemical treatments did not cause a substantial change in the heat resistance of B. coagulans 43P spores. The D_{100} -values of untreated and chemically treated spore suspensions ranged from 6.5 to 10 minutes. Recently, it was reported that C. botulinum type E spores could not be manipulated to the calcium form since the spores germinated when they were

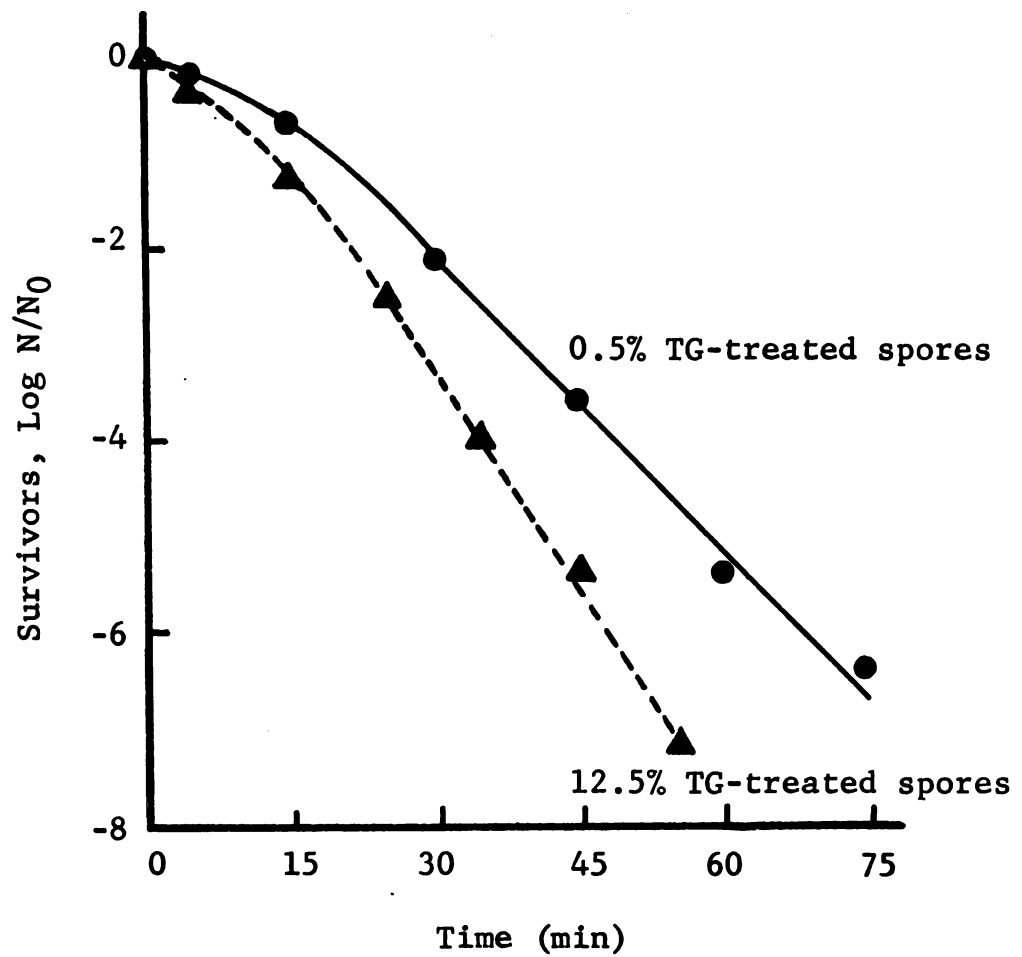


Figure 7. Survivor curves of Ca-treated spores, heated at 100 C. The spores were prepared from 0.5% and 12.5% thioglycollate (TG)-treated spores.

Table 1. D_{100} -values of Bacillus coagulans spores after various treatments

Type of Spore	D_{100} value (min)
Untreated spores	6.5
HCl-treated spores	7.0
Ca-treated spores prepared from untreated and HCl-treated spores	7.0 to 9.5
Lyophilized spores	8.5
0.5% and 12.5% Thioglycollate (TG)-treated spores	6.7
Ca-treated spores prepared from thioglycollate- treated spores: 0.5% TG	10.0
12.5% TG	7.2

incubated in a solution containing calcium (Alderton et al., 1974). I am unaware of any other studies reporting difficulties in chemically manipulating spores to the heat-sensitive and heat-resistant forms.

The existence of separate spore heat resistance states required a change in the constitutive assumption of the nature of spore heat resistance, as well as a change in the common experimental designs and practices based on that assumption. Spores subjected to such heating may or may not, depending on their prior treatment, actually express a significant part of their potential heat resistance (Alderton and Snell, 1969a). Generally, tests of heat resistance of the acid-treated and calcium-treated spores were carried out in water after thorough removal of the reagents (H^+ and buffered metal cations) used in the preparation of the spore suspensions. Also, the tests were carried out in a medium relatively inert with respect to capacity to induce change of state, otherwise, the heating-medium effects exerted by a noninert medium would be confounded with the effect of change of state (Alderton and Snell, 1969a). Alderton et al. (1964) reported that increased heat resistance of the spores could be induced during the process of heating. They found that the death rate of the H-form of B. megaterium spores

was decelerated when heated in 0.02 M calcium acetate buffer at pH 5.7. However, pH of the buffer, temperature and duration of suspension in that buffer before heat treatment influenced the rate of heat adaptation of the spores. The higher the buffer pH, the higher the rate of heat adaptation of the spores. As mentioned previously, at pH 7.9 both mono- and divalent metallic ions (e.g., sodium and calcium buffers) were effective in conferring some degree of heat resistance. On the other hand, at pH 5.7, sodium buffer gave only minor enhancement of heat resistance compared with that shown by calcium ion, since the monovalent sodium ion apparently was unable to compete with protons for the exchange sites in this pH region. Therefore, any arrangement of the chemical environment in such a way that there was an ion exchange between the spores and the external medium, would affect the thermal resistance of the spores (Alderton et al., 1964). Since in this study B. coagulans spores were heated in 0.025 M potassium phosphate buffer at pH 7.0, there is a slight possibility that ion exchange between the spores and buffer medium could occur. Thus, ion exchange between the H-form spores and the medium may increase the thermal resistance of the spores to the same range as those of the untreated and calcium-form spores. However, acid-treated spores were

stored in distilled water and only placed in buffer during heating. Since no increase in heat resistance was observed during heating (Fig. 2), it is unlikely that the buffer is responsible for the absence of heat-sensitive spores in the acid-treated spore suspensions. On the other hand, the buffer may be responsible for the apparent differences in heat resistance of portions of the population in the lyophilized and acid-treated (Fig. 4) and thioglycollate-treated (Fig. 6) spores.

CONCLUSIONS

The effects of chemical treatments on the heat resistance of Bacillus coagulans spores were studied. Results showed that there was no substantial difference in the heat resistance of spore preparations produced using conventional methods for the preparation of H-form and Ca-form spores. This is in contrast to numerous reports which indicate that spores of Bacillus and Clostridium species are readily manipulated between the H-form and Ca-form.

When the spores were treated with thioglycollic acid, the shape of the survivor curve changed because the heat activation period disappeared. Acid treatment of thioglycollate-treated spores resulted in a decrease in the heat resistance of a portion of the spores.

These results indicated that the heat resistance of B. coagulans spores was not readily manipulated by chemical treatments. This resistance to chemical manipulation may be important in the survival of B. coagulans spores in thermally processed semi-acid food such as tomato products. Therefore, more research is needed to determine the spore

structures responsible for the ion exchange system involved in heat resistance of the spores.

BIBLIOGRAPHY

BIBLIOGRAPHY

- Alderton, G., Chen, J. K., and Ito, K. A. 1974. Effect of lysozyme on the recovery of heated Clostridium botulinum spores. Appl. Microbiol. 27: 613-615.
- Alderton, G., Ito, K. A., and Chen, J. K. 1976. Chemical manipulation of the heat resistance of Clostridium botulinum spores. Appl. Environ. Microbiol. 31: 492-498.
- Alderton, G. and Snell, N. 1963. Base exchange and heat resistance in bacterial spores. Biochem. Biophys. Res. Commun. 10: 139-143.
- Alderton, G. and Snell, N. 1969a. Bacterial spores: chemical sensitization to heat. Science 163: 1212-1213.
- Alderton, G. and Snell, N. 1969b. Chemical states of bacterial spores: dry heat resistance. Appl. Microbiol. 17: 745-749.
- Alderton, G., Thompson, P. A., and Snell, N. 1964. Heat adaptation and ion exchange in Bacillus megaterium spores. Science 143: 141-143.
- 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: 596-604.
- Amaha, M., Ordal, Z. J., and Touba, A. 1956. Sporulation requirements of Bacillus coagulans var. thermoacidurans in complex media. J. Bacteriol. 72: 34-41.
- Anderson, E. E., Esselen, W. B., Jr., and Fellers, C. R. 1949. Effect of acids, salt, sugar, and other food ingredients on thermal resistance of Bacillus thermoacidurans. Food Res. 14: 499-510.

- Becker, M. E. and Pederson, C. S. 1950. The physiological characters of Bacillus coagulans (Bacillus thermoacidurans). J. Bacteriol. 59: 717-725.
- Berry, R. N. 1933. Some new heat resistant, acid tolerant organisms causing spoilage in tomato juice. J. Bacteriol. 25: 72-73.
- Buchanan, R. E. and Gibbons, N. E. 1974. Bergey's Manual of Determinative Bacteriology. 8th ed. The Williams & Wilkins Co. Baltimore.
- Cameron, E. J. and Esty, J. R. 1940. Comments on the microbiology of spoilage in canned foods. Food Res. 5: 549-557.
- Campbell, L. L., Jr. and Williams, O. B. 1953. The effect of temperature on the nutritional requirements of facultative and obligate thermophilic bacteria. J. Bacteriol. 65: 141-145.
- Cleverdon, R. C., Pelczar, M. J., and Doetsch, R. N. 1949. Vitamin requirements of Bacillus coagulans. J. Bacteriol. 58: 113-114.
- Cordes, W. A. 1928. Bacterial action in the coagulation of evaporated milk. J. Dairy Sci. 11: 46-51.
- Curran, H. R. 1957. The mineral requirements for sporulation. In "Spores" (H. O. Halvorson, ed.), pp. 1-9. American Institute of Biological Science. Washington, D. C.
- El-Bisi, H. M. and Ordal, Z. J. 1956a. The effect of certain sporulation conditions on the thermal death rate of Bacillus coagulans var. thermoacidurans. J. Bacteriol. 71: 1-9.
- El-Bisi, H. M. and Ordal, Z. J. 1956b. The effect of sporulation temperature on the thermal resistance of Bacillus coagulans var. thermoacidurans. J. Bacteriol. 71: 10-16.
- El-Mabsout, Y. E. 1977. Activation of Bacillus stearothermophilus spores. M. S. Thesis. Michigan State University.

- Esty, J. R. and Meyer, K. F. 1922. The heat resistance of the spores of Bacillus botulinus and allied anaerobes. J. Infect. Diseases 31: 650-663.
- Frank, H. A. and Campbell, L. L., Jr. 1957. The nonlogarithmic rate of thermal destruction of spores of Bacillus coagulans. Appl. Microbiol. 5: 243-248.
- Gibbs, P. A. 1967. The activation of spores of Clostridium bifermentans. J. Gen. Microbiol. 46: 285-291.
- Gordon, R. E., Haynes, W. C., and Pang, C. H. 1973. The Genus Bacillus. Agriculture Handbook No. 427. Washington, D. C.
- Gould, G. W. and Dring, G. J. 1974. Mechanisms of spore heat resistance. In "Advances in Microbial Physiology" (A. H. Rose and D. W. Tempest, eds.), pp. 137-164. Academic Press, New York.
- Gould, G. W. and Hitchins, A. D. 1963a. Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulphide bonds. J. Gen. Microbiol. 33: 413-423.
- Gould, G. W. and Hitchins, A. D. 1963b. Sensitization of bacterial spores to lysozyme and hydrogen peroxide with reagents which rupture disulphide bonds. Nature 197: 622.
- Keynan, A., Evenchik, Z., Halvorson, H. O., and Hastings, J. W. 1964. Activation of bacterial endospores. J. Bacteriol. 88: 313-318.
- Lechowich, R. V. and Ordal, Z. J. 1962. The influence of the sporulation temperature on the heat resistance and chemical composition of bacterial spores. Can. J. Microbiol. 8: 287-295.
- Levinson, H. S. and Hyatt, M. T. 1964. Effect of sporulation medium on heat resistance, chemical composition, and germination of Bacillus megaterium spores. J. Bacteriol. 87: 876-886.

- Ordal, Z. J. 1957. The effect of nutritional and environmental conditions of sporulation. In "Spores" (H. O. Halvorson, ed.), pp. 18-32. American Institute of Biological Science. Washington, D. C.
- Ordal, Z. J. and Lechowich, R. V. 1958. Some factors influencing thermal resistance of bacterial spores. Proc. 10th Res. Conf. Amer. Meat Inst. Found. p. 91-99.
- Rice, A. C. and Pederson, C. S. 1954a. Factors influencing growth of Bacillus coagulans in canned tomato juice. I. size of inoculum and oxygen concentration. Food Res. 19: 115-123.
- Rice, A. C. and Pederson, C. S. 1954b. Factors influencing growth of Bacillus coagulans in canned tomato juice. II. acidic constituents of tomato juice and specific organic acids. Food Res. 19: 124-133.
- Rode, L. J. and Foster, J. W. 1966a. Influence of exchangeable ions on germinability of bacterial spores. J. Bacteriol. 91: 1582-1588.
- Rode, L. J. and Foster, J. W. 1966b. Quantitative aspects of exchangeable calcium in spores of Bacillus megaterium. J. Bacteriol. 91: 1589-1593.
- Rowley, D. B. and Levinson, H. S. 1967. Changes in spores of Bacillus megaterium treated with thioglycolate at a low pH and restoration of germinability and heat resistance by cations. J. Bacteriol. 93: 1017-1022.
- Sarles, W. B. and Hammer, B. W. 1932. Observations on Bacillus coagulans. J. Bacteriol. 23: 301-314.
- Schmidt, C. F. 1957. Thermal resistance of microorganisms. In "Antiseptics, Disinfectants, Fungicides and Sterilization" (G. F. Reddish, ed.), 2nd ed., pp. 831-884. Lea & Febiger, Philadelphia.
- Stern, R. M., Hegarty, C. P., and Williams, O. B. 1942. Detection of Bacillus thermoacidurans (Berry) in tomato juice, and successful cultivation of the organism in the laboratory. Food Res. 7: 186-191.

- Stumbo, C. R. 1973. Thermobacteriology in Food Processing. 2nd ed. Academic Press, Inc., New York.
- Sugiyama, H. 1951. Studies on factors affecting the heat resistance of spores of Clostridium botulinum. J. Bacteriol. 62: 81-96.
- Thomas, S. and Russell, A. D. 1975. Sensitivity and resistance to glutaraldehyde of the hydrogen and calcium forms of Bacillus pumilus spores. J. Appl. Bacteriol. 38: 315-317.
- Townsend, C. T., Somers, I. I., Lamb, F. C., and Olson, N. A. 1956. A Laboratory Manual for the Canning Industry. 2nd ed. National Canners Association Research Laboratories.
- Williams, F. T. 1936. Attempts to increase the heat resistance of bacterial spores. J. Bacteriol. 32: 589-597.
- Youland, G. C. and Stumbo, C. R. 1953. Resistance values reflecting the order of death of spores of Bacillus coagulans subjected to moist heat. Food Tech. 7: 286-288.

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