

ACTIVATION OF BACILLUS STEAROTHERMOPHILUS
SPORES

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ABSTRACT

ACTIVATION OF BACILLUS STEAROTHERMOPHILUS SPORES

By

Yusef Essanusi El-Mabsout

The objective of this study was to investigate the activation of Bacillus stearothermophilus spores after mild heat treatments at low pH. The spores were produced in 32-oz. bottles containing a layer of Nutrient Agar + 0.03% MnSO_4 adjusted to pH 6.8 (NAM). The bottles were surface inoculated and incubated at 55 C for 2 days. The spores were harvested, washed 3 times with sterile distilled water, treated with 0.1 mg/ml lysozyme for 2 hr at 37 C, and washed 3 times with distilled water. The cleaned spore suspension was stored at 4 C.

Initially, >80% of the spores were dormant as determined by comparison of direct microscopic counts and the numbers of spores which would form colonies on NAM at 55 C. Spore suspensions in distilled water were adjusted to pH 1.1-4.0 with HCl and heated at 40-70 C for 30-120 minutes. The effects of various treatments on spore activation were determined by plating on NAM at 55 C. The rate of spore activation was accelerated during treatments at pH 1.1-2.0 and 60-70 C.

Activation of spores was paralleled by transformation of spores to a more heat-sensitive form. Normal or

heat-resistant spores were not significantly affected at pH 7.0 by heating below 100 C ($D_{86} > 1000$ minutes). However, at pH 7.0, the heat-sensitive spores were destroyed during heating at 86 C ($D_{86} \approx 9$ minutes). When activation of spores was $>90\%$ as measured by colony formation on NAM, heating at 86 C and pH 7.0 could be used to measure the fraction of spores which were heat-resistant, and thus, presumably not activated. By following the destruction of spores during heating at 86 C it was apparent from the initial counts that the heat-sensitive forms were viable, activated spores.

Treatment at pH 1.1-2.0 and 60 C for 60 minutes was considered to provide the best conditions for activation in this investigation. While treatment at 70 C resulted in faster activation, results indicate that some spores may be inactivated during treatment at that temperature. Heating the spores for 90 or 120 minutes at 60 C also resulted in less than maximal plate counts. The results of this investigation indicate that activation of B. stearothermophilus spores at low pH may be used under certain conditions as a substitute for a severe heat shock.

ACTIVATION OF BACILLUS STEAROTHERMOPHILUS SPORES

By

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INTRODUCTION

The study of activation of Bacillus stearothermophilus spores is of great importance to food microbiologists due to the unique heat resistance and high degree of dormancy of the mature spores. Because of their extreme heat resistance, they are often used in the food industry as an indicator of the adequacy of the thermal process used for commercial sterilization. Also, large annual economical losses in the canning industry are partially attributed to flat sour spoilage of low acid foods, such as peas, beans, whole kernel corn and cream-style corn, caused by B. stearothermophilus. Accordingly, the food industry is quite concerned with the methodology involved in determining spore counts in raw materials and the survival of spores in finished products. The concern over activation of B. stearothermophilus spores is due to the fact that when a food sample is evaluated for the presence of these organisms, only a small fraction of the spores will form colonies on appropriate media while the majority of the spores remain dormant. Thus resulting in a lower count than the actual number of thermophilic spores present in the sample.

Presently, methods used in determining flat sour organisms utilize severe heat treatments for activation of

spores. This may result in under-estimating the spore content of a given food sample due to a variety of reasons, such as over-heating or killing some of the spores, heat-induced dormancy, or inadequate conditions for activation.

The objective of this study was to investigate the activation of B. stearothermophilus spores by mild heat treatments at low pH.

REVIEW OF LITERATURE

Activation of Bacterial Spores

As stated by Murrell (1961): "Viable spores failing to germinate in apparently favorable conditions are said to be dormant". In most cases fresh spores are dormant and will not germinate, even under favorable conditions unless they are preheated or otherwise treated. The process by which dormant spores are converted into spores which have the ability to germinate is called activation. In some cases, slow germination may take place even without previous treatment, therefore, activation may be defined as: the conditioning treatment or the process by which spores are rendered less dormant and germination of spores is accelerated.

Historically, mold spore activation was discovered before activation of bacterial spores. Shear and Dodge (1927) were the first to apply heat to mold spores for activation and subsequent germination. On the other hand Weizmann⁽¹⁾ (1919) was the first to apply heat activation to bacterial spores. Although he did not realize its direct effect on spores, he used a heat treatment of 90-100 C for one to two minutes to activate Clostridium acetobutylicum spores for use in the fermentation of acetone. In his

(1) Gould and Hurst, 1969

opinion, he heated the spore suspension to eliminate contamination with vegetative cells which might have interfered with normal fermentation by inhibiting spore germination or competition for nutrients. However, Curran and Evans (1945) were the first to note and understand the direct activation effect of heat on bacterial spores. They observed that in a given spore suspension, a greater number of spores germinated if the suspension was given a mild heat treatment before plating (Desrosier and Heiligman, 1956). Curran and Evans (1945) studied both mesophilic, as well as thermophilic strains, including Bacillus stearothermophilus strain NCA 1518.

Although heat treatment is not the only method of activation, the majority of the reports on activation have concerned use of heat. Many workers studied the effects of heat on different spore suspensions and different types and strains of spores. Other methods have also shown results similar to heat activation. In this review, heat activation as well as activation by other methods will be discussed.

Heat Activation

Heat activation is defined by some workers as a sub-lethal heat treatment (Fields, 1970) and it is the simplest method of activation (Keynan and Evanchik, 1969). Spores of different species vary in their temperature requirements for activation. These variations also exist among different strains of the same species and among different batches of spores produced from the same strain. Brachfeld (1955) and

Titus (1957) showed that spores of B. stearothermophilus could be activated at temperatures above 100 C. In another study, Finely and Fields (1962) found that maximal activation of B. stearothermophilus spores occurred only at temperatures above 100 C, namely 100-115 C depending upon the strain and the spore suspension. They also noted heat-induced dormancy when the spores were heated in distilled water at 80, 90 and 100 C. Similar studies on heat activation showed that only a few minutes at 60 C were required for optimal activation of spores of some strains of Bacillus megaterium whereas spores of B. stearothermophilus and other thermophilic and thermotolerant bacteria required from 105-115 C for optimal activation. It has also been shown that Bacillus coagulans required 5 minutes of heating at 85 C (Desrosier and Heiligman, 1956) and Bacillus cereus T required 45 minutes of heating at 65 C (Keynan et al., 1964) for optimal activation.

In addition to species and strain differences in heat requirements for activation, which are endogenous to the spore, other factors also affect heat activation. One factor is the composition of the medium on which growth and sporulation occurs. As an example, Keynan et al. (1961) found that spores grown on media containing different amounts of Ca^{++} and phenyl alanine required different periods at 65 C to obtain maximum activation rates and the times necessary for activation correlated well with the Ca-dipicolonic acid (DPA) content of the spores.

The extent of heat activation is also dependent upon other factors. These include: composition of the heating medium; the presence of salts and heavy metals; and the pH of the medium.

Beers (1958) observed that it was impossible to activate dry lyophilized spores and spores suspended in a high concentration of glycerol. Earlier Powell and Hunger (1966) and Maeda et al. (1975) reported that heat activation must be carried out in the presence of water. Finely and Fields (1962) and Fields and Finely (1963) studied the effects of phosphates and carbohydrates in phosphate buffer. They found that regardless of strain and suspension source, phosphate buffer (0.0083 M) influenced the germination of B. stearoothermophilus. In another experiment, Fields and Finely (1963) found that different spore germination responses occurred when spores were heated in the presence of monosaccharides, disaccharides and polysaccharides in 0.0083 M phosphate buffer (pH 7.1). These differences were observed among various strains, and among spore suspensions from the same strain. In decreasing order of efficiency glucose, lactose, peptone, skim milk, glucose-nutrient agar, beef extract, glucose-nutrient broth, distilled water and sodium chloride were found to affect heat activation (Curran and Evans, 1945). On the contrary, Busta and Ordal (1964) noted no effect on heat activation for various suspending media containing glucose, xylose, robose, NaCl, or sodium phosphate, nor was there a marked effect due to change in

pH from 5.0 to 8.0 when heat activation of Bacillus subtilis strain 5230 spores was carried out at 75 C. Other investigators (Halmann and Keynan, 1962; Heynan et al., 1965) showed that salts interfere with germination if present during heat activation. However, if the spores were washed after the heat treatment, germination was not affected. This suggested that salts did not affect activation, but interfered only with germination.

Various reports also show that heat activation of bacterial endospores is pH dependent. Keynan et al. (1964) showed that B. cereus T activation was inhibited at pH values above 8.5. At pH 7.0, activation was optimal when spores were heated at 60 C for one hour. In comparison, 10 minutes at 60 C gave similar activation when the pH was below 2.0. It has also been demonstrated that some spores respond differently to pH variation during heat activation. Gibbs (1966) found that activation of Clostridium bifermentans spores was optimal at high pH values and inhibited at low pH.

The effect of carbohydrates on heat activation of B. stearothermophilus was studied by Fields and Finely (1964). Their results show that with increased carbohydrate concentrations (using 0.001, 0.01 and 0.1 M glucose, fructose, sucrose, maltose, dextrans and starch), the dominant trend was reduced activation. Fields (1964) found that heat activation of B. stearothermophilus spores at 110 C in 20% sucrose solution caused the rough variant to decrease in a

mixed population of rough and smooth variants of strain M. The smooth variant, however, increased. Similarly, the rough variant of the strain NCA 1518 of B. stearothersophilus decreased when heated in a mixed population at 110 C in the presence of 20% sucrose solution. Other studies have also shown that age of the spores and the presence of lysozyme have a marked effect on heat activation (Fields and Jenne, 1962).

Methods of Activation Other than Heat

Although heat application is the predominant method for activation of bacterial spores, other methods have also been described. These methods involve the use of low pH, Ca-DPA, reducing and surface active agents and other chemicals and ionizing radiation.

Low pH

Normally, spores are heat activated and germinated at normal pH. However, heat activation as described earlier is pH dependent and the lower the pH, the lower the temperature required for activation. Moreover, low pH replaces heat activation under certain conditions. In a study of dormancy and activation of bacterial spores, Lewis et al. (1965) showed that the rate of germination of B. stearothersophilus spores increased from 18 to 80% when they were subjected to pH 1.5 for 80 minutes at 25 C. They also showed that dormancy was restored if the spores were exposed to 0.02 M Ca^{++} ions (pH 9.7). Keynan et al. (1964) reported that spores

of B. cereus T germinated spontaneously (without heat activation) when incubated for a long time in a buffer solution at low pH. Brown et al. (1968) in studying activation of B. stearrowthermophilus spores found that spores subjected to 0.5 N HCl at 25 C gave increased colony counts which were equal to the total counts of spores.

Ca-DPA

"Since the demonstration by Powell (1953) that bacterial spores contained dipicolinic acid (DPA), there has been much interest in its biological role", (Lee and Ordal, 1963). Riemann and Ordal (1961) showed that spores of B. subtilis and B. cereus T. germinated in solutions of equimolar concentrations of Ca^{++} and DPA without heat activation. Later, Lee and Ordal (1963), showed that B. megaterium spores were activated and germinated spontaneously in distilled water when dormant spores were placed in a solution of 40 mM Ca-DPA (pH 7.0) for 70 minutes at 7 and 10 C. However, germination of activated spores was inhibited by a chelating agent, o-phenanthroline, and by various cations such as Cu^{++} , Fe^{++} , Ag^{+} and Hg^{++} . The activation effect was reversed by treatment with acid. In another study, Ca-DPA incorporated into tryptone-glucose yeast extract agar was used to germinate spores of B. subtilis, B. stearrowthermophilus, B. megaterium and B. coagulans for their enumeration on media without heat activation (Busta and Ordal, 1964). However, Freese and Cashel (1965), reported that

spores of B. subtilis 168 did not germinate when inoculated in Ca-DPA for 160 minutes but were only activated for L-alanine germination and 98% of the spores remained refractile after the Ca-DPA treatment.

Reducing agents and other chemical compounds

Reducing agents which reduce disulphide bonds such as thioglycollate and mercaptoethanol were found to replace heat treatment as a means of activation. Keynan et al. (1964) found that when spores of Bacillus cereus strain T were incubated in 0.2 M thioglycollate or 0.2 M mercaptoethanol at 28 C, partial germination occurred after washing and addition of L-alanine and adenosine. However, a long time was required (12 hours) to observe the activation effect of these reducing agents. In another study, (Gibbs, 1966) thioglycollate failed to activate C. bifermentans spores which suggests that spores of different species respond differently to treatment with reducing agents. Antibiotics such as D-cycloserine and O-carbamyl-D-serine, and polar solvents like dimethylformamide also were used as replacements of heat for spore activation (Gould, 1966). Recently, ionizing radiation has been used to activate spores of B. cereus PX (Gould and Ordal, 1968) and B. megaterium (Levinson and Hyatt, 1960).

Generally speaking, activation of bacterial endospores is reversivle. However, there are a few exceptions where activation has been shown to be irreversible (Busta and

Ordal, 1964; Keynan et al., 1965 and Gibbs, 1966). Refractibility and heat resistance of spores are not affected by the activation process.

The mechanism through which activation occurs is still controversial, and no single hypothesis has yet been suggested that could account for all of the known activation treatments and conditions. However, many of these hypotheses deal with a particular factor which may trigger activation such as a change in spore permeability (Lee and Ordal, 1963), deactivation of an inhibitor or toxic factor (Mefferd and Campbell, 1961), reorientation of spore components and denaturation of a spore protein (Keynan et al., 1964). However, more information is required before the real nature of activation is known.

Chemical Manipulation
of the Heat Resistance of
Bacillus stearothermophilus Spores

The refractile dormant spores of B. stearothermophilus are known to be extremely heat resistant. Among the factors that are known to influence their heat resistance are age of the spores, the chemical composition of the medium on which spores were produced, the growth and sporulation temperature, pH and composition of the heating medium (Stumbo, 1973), and the chemical state of the spore (Alderton and Snell, 1969). However, only those pertaining to chemical manipulation of the heat resistance of spores will be discussed below.

Certain divalent cations such as Ca^{++} and Mn^{++} play an important role in determining the thermal stability of mature spores. Amaha and Ordal (1957) showed that heat resistance of bacterial spores was reduced when the concentration of Ca^{++} and Mn^{++} was reduced below certain levels in the sporulation medium. On the contrary, a high phosphate level in the medium markedly decreased the heat resistance (El-Bisi and Ordal, 1956). The chemical composition of mature spores also affects their heat resistance. The results obtained by Murrell and Warth (1965) indicated a significant relationship between heat resistance and Ca^{++} content of the spores. Their results also showed that the lowest heat resistance occurred in spores having the highest Mg:Ca ratio; in heat resistant spores, Ca content was high, Mg:Ca ratio was fairly low, and Ca:DPA ratio was high. However, in mature heat resistant spores, Ca^{++} and DPA occurred in nearly equimolar amounts (El-Bisi et al., 1962) and these compounds were released together with spore peptides during germination (Powell and Strange, 1953). Further studies on the physico-chemical properties of spores have shown that mature spores under pH-controlled conditions were capable of undergoing cation exchange (Alderton and Snell, 1963). Divalent cations such as Ca^{++} and Mg^{++} (presumably responsible for the increased heat resistance of mature spores) are replaced by hydrogen ions or vice versa, thus allowing formation of hydrogen-form (H-form) and calcium-form (Ca-form) spores. As stated by

Alderton and Snell (1963), the spore cation exchange system resembles that of a resinous, weak cation exchange system, such as methacrylic acid polymers, rather than the strong sulfonic acid type of cation exchange system. They also suggested that carboxyl, phosphorus-containing and chelating groups which are pH controlled are possible functional groups for base binding in the spore cation exchange system.

The H-form spores have a markedly lower heat resistance than Ca-form spores. This difference in heat resistance is of a large magnitude. Alderton and Snell (1969b) stated that the temperature at which equal survivor rates of B. stearothermophilus spores are obtained changes about 28 C between the heat-sensitive (H-form) and the heat-resistant (Ca-form). The chemical states of spores can be prepared by treating mature spores with an acid (e.g. HCl) or buffered metal cations (e.g. Ca(OH)_2 in calcium acetate buffer). The change in heat resistance of spores which is introduced by such treatments persists even after the spores are thoroughly washed and the rate of change can be manipulated by changing the pH and the temperature as well as the duration of the treatment (Alderton and Snell, 1969b).

MATERIALS AND METHODS

Organism

The organism used in this study was B. stearoothermo-
philus E-2 obtained from the culture collection of the
Department of Food Science and Human Nutrition at Michigan
State University. It was maintained in a frozen form in
nutrient broth (Difco) and stored at -20 C.

The frozen culture was thawed and incubated at 55 C
for 48 hr., and nutrient agar (Difco) slants (NA) were ino-
culated and incubated at 55 C for 48 hours. One-ml portions
of the 48-hr NB were used to inoculate double strength
nutrient agar + 0.03% MnSO_4 (NAM) plates, and the plates
were incubated for 3 days at 55 C. The cultures were har-
vested by adding 4 ml of sterile deionized water to each
plate and rubbing the growth off with a sterile glass rod.
The harvested cells were centrifuged at 4080 x g for 20
minutes in a SorVal Superspeed RC-2 automatic refrigerated
centrifuge, suspended in sterile water and cleaned 3 times
by centrifugation. The spores and cells were suspended in
water and stored at 5 C. This spore suspension was used in
preliminary investigations.

For preparing larger amounts of spores, 32-oz. pre-
scription bottles containing 200 ml of NAM were sterilized

at 121 C for 15 minutes. After solidification of the agar, bottles were left on their wide side for 2 days over the surface of the agar. The bottles were incubated for 2 days at 55 C. Spores were harvested in 10 ml sterile, distilled water, centrifuged, cleaned 3 times with sterile, distilled water, treated with 0.1 mg/ml lysozyme, 3 x crystalline (Nutritional Biochem. Corp., Cleveland, Ohio), at 37 C for 2 hr, and cleaned 3 times with deionized sterile water by centrifugation. The cleaned spore slurry was suspended in water and stored at 4 C.

Preparation of Heat Sensitive Spores

Heat sensitive spores (H-form) were prepared by mixing normal spores in a solution described by Alderton and Snell (1969a) containing 1% tryptone, 0.5% glucose and 0.1% soluble starch. The pH of the solution was adjusted to 1.1 with 3N HCl. The spore suspension was incubated overnight at 20 C, centrifuged, washed 3 times and suspended in sterile, deionized water. Heat sensitive spores were also formed by treatment with some of the methods described below in the section on Activation of Spores.

Preparation of Heat Resistant Spores

Heat resistant spores (Ca-form) were prepared by mixing normal spores in a solution described by Alderton and Snell (1969a) containing 0.02 M calcium acetate, adjusted to pH 9.7 with aqueous $\text{Ca}(\text{OH})_2$. The suspension was incubated overnight at 20 C and heated in a steam cabinet for 15

minutes. The suspension was centrifuged, washed 3 times and suspended in deionized sterile water.

Activation of Spores

Temperature, time and pH were the main factors considered in this investigation. The general approach was to change one of these factors at a time, thus allowing for the evaluation of its effects on activation of spores.

In one set of experiments, 5-10 ml samples of spore suspension were pipetted into screw-capped test tubes (Pyrex no. 9825) each containing 10 ml of 0.2N HCl giving a final pH of 1.1. These sample suspensions were stored at 50 C for 0, 30, 60, 90 or 120 minutes.

For determining the effect of temperature, 4-10 ml samples of normal spore suspensions were added to screw-capped test tubes containing 10 ml of 0.2N HCl. These sample suspensions were incubated at 40, 50, 60 or 70 C for one hour.

For determining the effect of pH, 4-10 ml aliquots of acid-buffer solutions (pH adjusted to 1.1, 2.0, 3.0 or 4.0) were added to screw-capped test tubes. Each contained 10 ml of cleaned normal spore suspension. These sample suspensions were incubated for 1 hr in a water bath adjusted to 60 C.

Other methods used in this study included the standard method for the determination of thermophilic flat sour spores described by the American Public Health Association

(APHA, 1966) and the method described by Dr. J.C. Canada of Gerber Products Company (Personal communication) which is used for examining raw materials for heat resistant aerobic thermophiles and flat-sour spores. A description of this method is given below.

A sample was weighed and dispersed in H_2O by blending or shaking at 1:10. Twenty milliliters of the suspension were pipetted into a 250-ml Erlenmeyer flask containing 100 ml sterile dextrose-tryptone agar with brom cresol purple. The flask was cotton plugged and swirled to mix contents and autoclaved at 108 C (5 psi) for 10 minutes. The autoclave was previously standardized to give a heat treatment equivalent to $F_0^{18} = 0.74$ when heating was completed with the autoclave set for slow exhaust. The flask was immediately removed and the contents were cooled to 43-45 C in running water. The contents were poured into 4-5 petri dishes and the plates were inverted and incubated at 50-55 C for 48 hrs.

Heat Treatment at 86 C

One milliliter of each treated sample was added to 9 ml of phosphate buffer, pH 7.6, in a screw-capped test tube. The sample suspensions were heated in a water bath at 86 C and consecutive dilutions were made from suspensions which had been heated for various time intervals. Appropriate dilutions were surface-plated on NAM plates, and the plates were inverted and incubated at 55 C for 2 days.

EXPERIMENTAL RESULTS

Growth and Sporulation

Most workers indicate that B. stearothermophilus sporulates readily on nutrient agar, a nutritious medium which contains 0.3% beef extract, 0.5% peptone and 1.5% agar. However, preliminary investigation showed that B. stero-thermophilus strain E-2 grew poorly and failed to sporulate on several media such as dextrose tryptone brom cresol purple agar (DTBA), plate count agar (PCA), and nutrient agar (NA).

It is mentioned in the literature that $MnSO_4$ can be used to supplement nutrient agar in order to enhance the production of B. stearothermophilus spores (Alderton and Snell, 1969; Titus, 1957). When 0.03% $MnSO_4$ was incorporated into NA in this investigation, better growth and sporulation was observed. Luxuriant growth and approximately 80% sporulation was observed when B. stearothermophilus strain E-2 was cultured on NAM for 2 days at 55 C. Thus, NAM was used in subsequent experiments.

Measurement of Activation by Plating

Table 1 shows average plate counts of spores which have been activated at pH 2.0 and 50 C for different periods vs. nonactivated spores. The total count of spores, as

determined by direct microscopic count, was $\sim 9 \times 10^7$ spores/ml. Colony counts were obtained from plates containing a 10^{-6} dilution of the original spore suspension.

Table 1. Activation of spores at pH 2.0 and 50 C as determined on NAM agar after incubation for 48 hr at 55 C.

	Time of activation treatment in minutes				
	0	30	60	90	120
Colonies/plate ^a	9	19	74	55	51

^aNumbers represent the average count on duplicate plates at a 10^{-6} dilution.

These results indicate that activation has taken place since the plate counts increased from 9×10^6 at 0 Time (untreated control) to 7.4×10^7 at 60 minutes, which represents the optimal activation time under these conditions. The counts decreased slightly during extended treatment.

When activation was carried out at a higher temperature (60 C), the same trend was observed. However, higher activation was obtained with the 60 C treatment (Table 2). After 60 minutes, plate counts increased more than 8-fold,

Table 2. Activation of spores at pH 2.0 and 60 C as determined on NAM agar after incubation for 48 hr at 55 C.

	Time of activation treatment in minutes				
	0	30	60	90	120
Colonies/plate ^a	10	31	83	67	59

^aNumbers represent the average count on duplicate plates at a 10^{-6} dilution.

from 1.0×10^7 to 8.3×10^7 spores/ml. The data also show that the numbers of activated spores increased to at least 90% of the total count of spores in the original spore suspension (9.0×10^7 spores/ml).

Heat Resistance of H-form and Ca-form Spores

Data presented in Figure 1 show survivor curves for heat sensitive (H-form) and heat resistant (Ca-form) spores when heated at 86 and 100 C. These data indicate that the Ca-form spores were extremely heat resistant and the total number of spores remained virtually unchanged when heated for 30 minutes at 86 or 100 C. On the other hand, 99.95% of the H-form spores were killed after the first 5 minutes of heating at 100 C. In addition, when the H-form spores were heated at 86 C, over 99.99% of the total count of spores were destroyed after 30 minutes.

Heat Resistance of Activated Spores

Spores subjected to an activation treatment of 60 minutes at pH 2.0 and 60 C show a marked decrease in their heat resistance. Table 3 shows the number of treated spores which survived heating at 86 C.

Table 3. Survival of activated (treated at pH 2.0 and 60 C for 60 minutes) spores at 86 C as determined on NAM agar after incubation for 48 hr at 55 C.

	Heating time in minutes at 86 C					
	0	5	10	15	20	30
Colonies/plate ^a	300	249	183	63	38	5

^aNumbers represent the average count on duplicate plates at a 10^{-5} dilution.

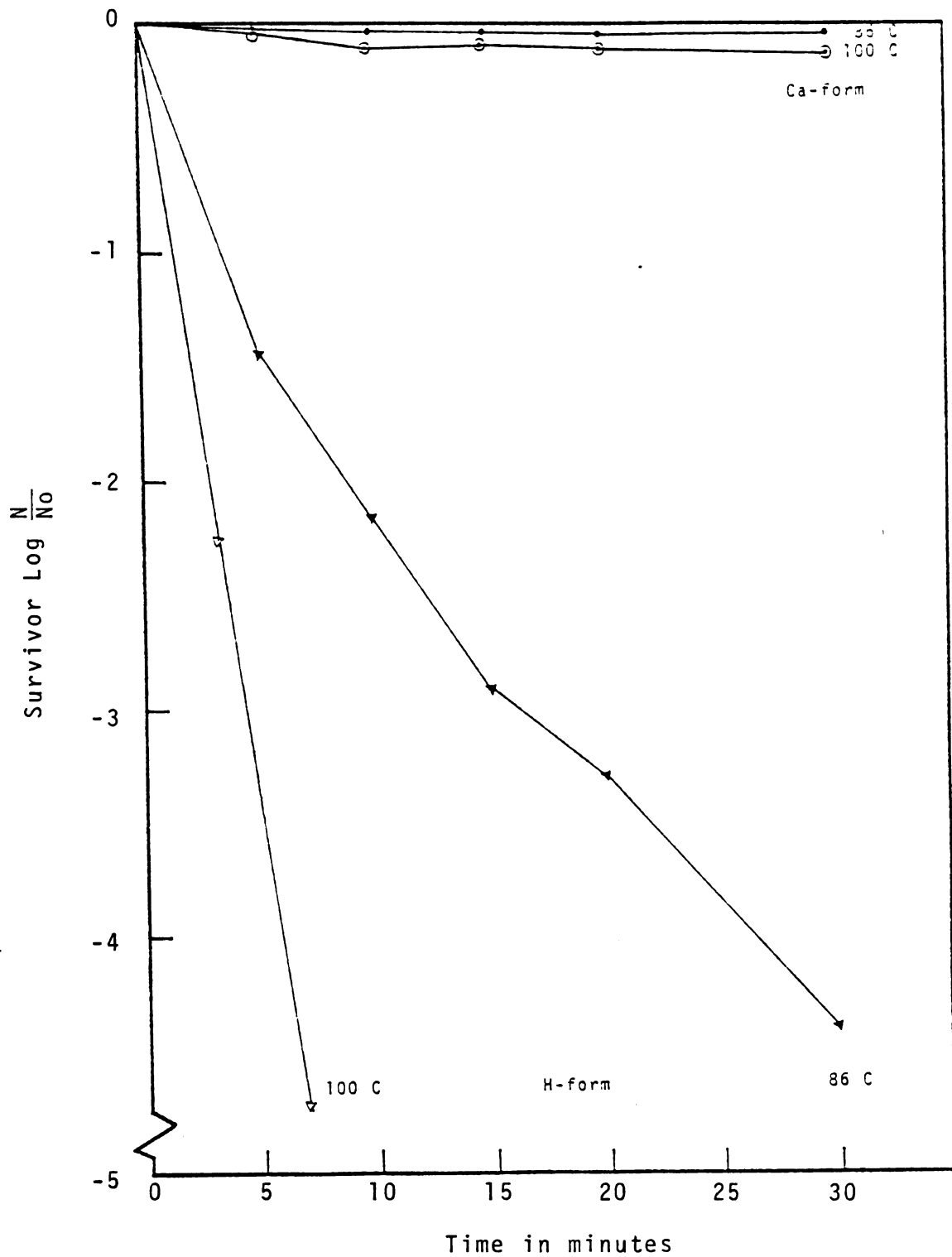


Figure 1. Survivor curves of H-form and Ca-form spores heated at 86 and 100 C.

These data indicated that over 90% of the spores were heat sensitive. This is comparable to the percentage of spores activated during treatment at pH 2.0 and 60 C for 60 minutes (Table 2). Thus, during activation under these conditions (60 C and pH 2.0) spores were transformed to a heat-sensitive state. Data obtained on the heat resistance of untreated spores at 86 C indicate that B. stearothermophilus spores are very heat resistant. This confirms the previous conclusion that activation of spores was accompanied by their sensitization to heat.

In measuring activation, the use of plate counts (before and after activation) is limited to ~80-90% of the total count. However, at higher levels, activation can be approximated by measuring the proportion of heat sensitive spores. Thus, heating at 86 C was used in the following experiments to monitor the proportion of spores which were activated, i.e. heat-sensitive.

Activation of Spores at pH 1.1

Effect of temperature

Figure 2 shows survivor curves for spores which were treated at pH 1.1 for 60 minutes at temperatures of 40, 50, 60 or 70 C, and then heated at 86 C. Results indicate that at least 90% of the spores were activated (heat sensitive) during treatment at 40 and 50 C. Activation increased to approximately 99.9% and 99.99% for treatment at 60 and 70 C, respectively.

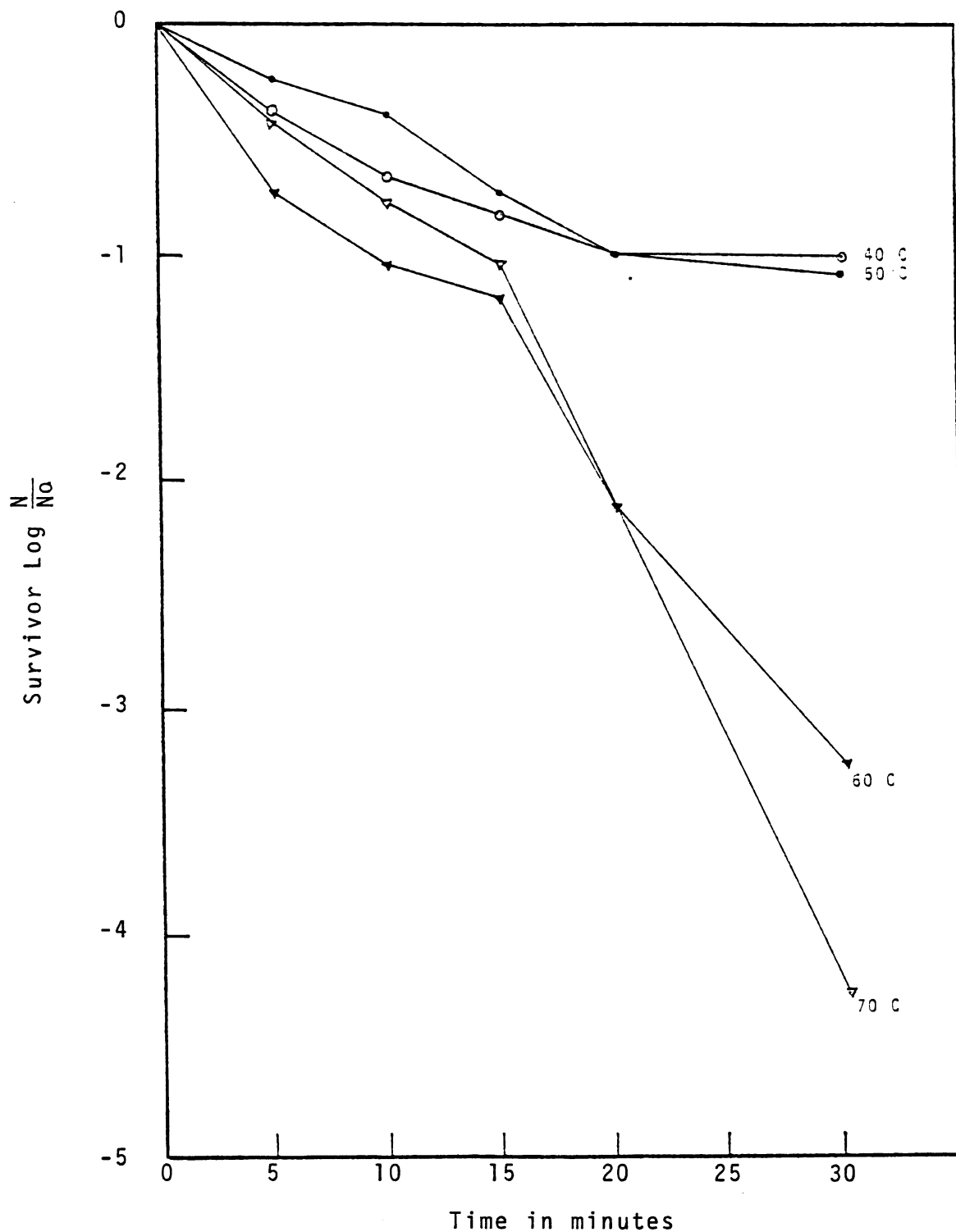


Figure 2. Survivor curves of spores heated at 86 C. Spore were incubated at different temperatures for 60 minutes at pH 1.1 before heating at 86 C.

Rate of activation at 50 C

Figure 3 shows a survivor curve during heating at 86 C for spores treated at 50 C and pH 1.1 for various periods of time. The data show that 55% of the total spores were heat sensitive (activated) when treated for 30 minutes under the aforementioned conditions. Similarly over 90% of the spores were activated by treatment for 60 and 90 minutes. The 120-minute treatment gave approximately 99.9% activation.

Effect of pH on Activation of Spores

For spores activated for 60 minutes at 60 C, maximum activation occurred at pH 1.1-2.0. At higher pH values (3.0 and 4.0), no significant activation was observed. Figure 4 shows survivor curves for spores heated at 86 C after treatment for 60 minutes at 60 C and pH values of 1.1, 2.0, 3.0 or 4.0. These data indicate that over 99.9% of the spores were activated at pH 1.1 and 98% at pH 2.0. At pH values of 3.0 and 4.0 activation was minimal.

The effect of pH was also shown by the fact that over 99.99% activation was obtained when the spores were treated at pH 1.1 and 20 C overnight (Figure 1). On the contrary, an overnight treatment of spores at pH 9.7 and 20 C resulted in essentially no activation.

Activation of Thermophilic Flat Sour Spores in Flour Samples

Data obtained (Table 4) indicated that few thermophilic flat sour spores were activated by the low pH-mild heat (pH 1.1 and 60 C) treatment. Higher plate counts were

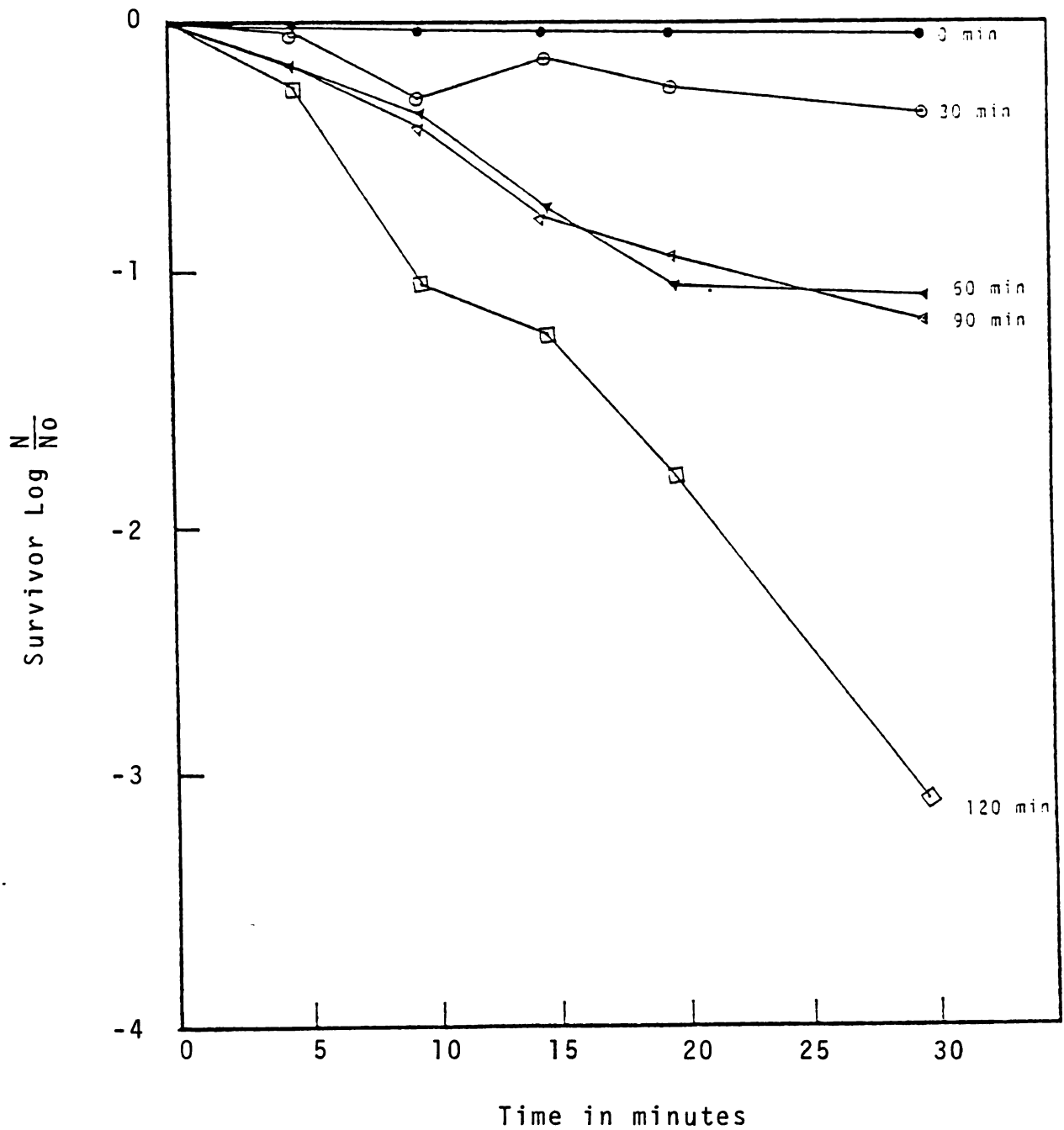


Figure 3. Survivor curves of spores heated at 86 C. Spores were incubated for different periods at pH 1.1 and 50 C before heating at 86 C.

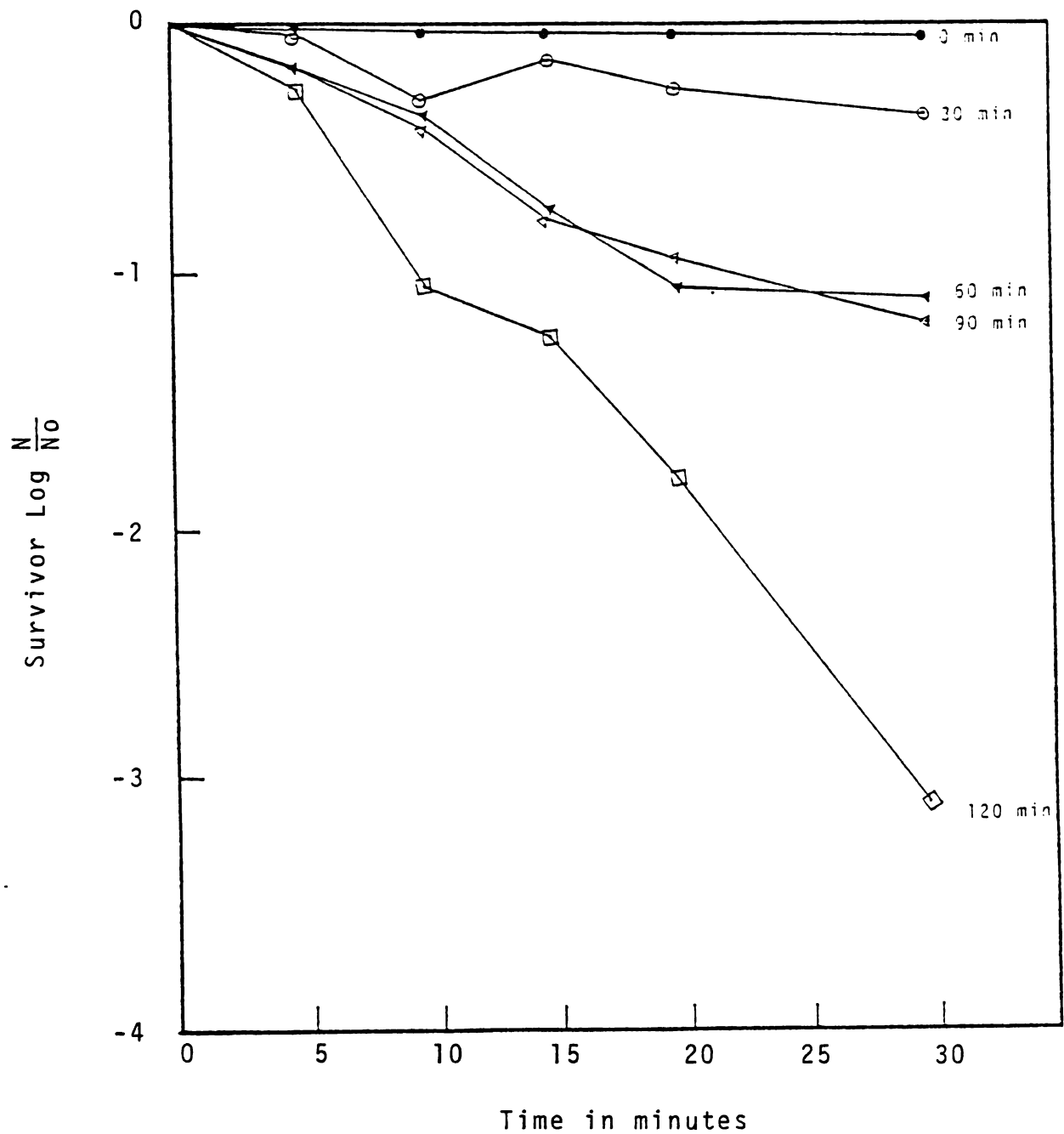


Figure 3. Survivor curves of spores heated at 86 C. Spores were incubated for different periods at pH 1.1 and 50 C before heating at 86 C.

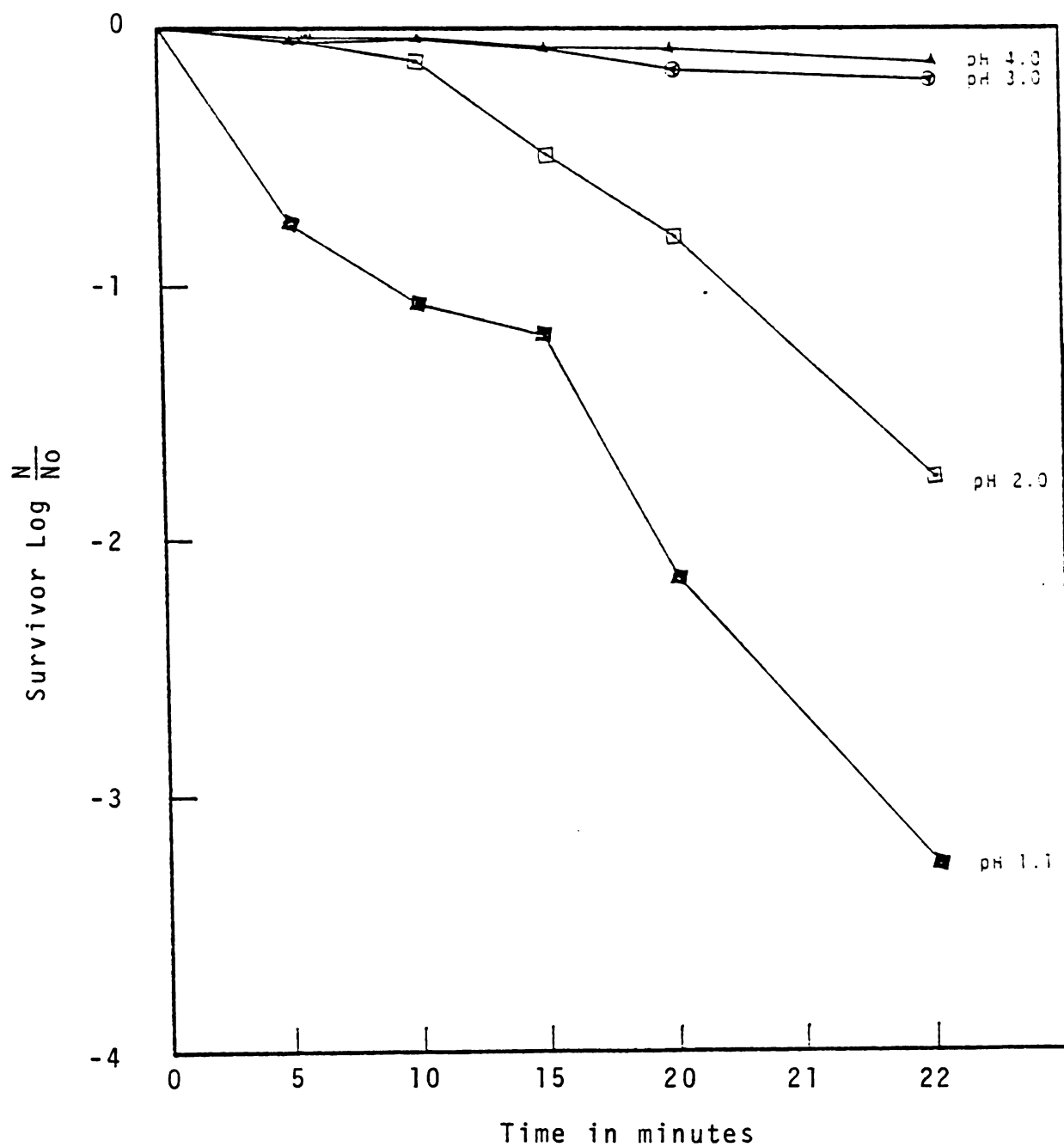


Figure 4. Survivor curves of spores heated at 86 C. Spores were incubated for 60 minutes at 60 C at different pH values before heating at 86 C.

were obtained when the recommended method for enumeration of thermophilic flat sour spores (APHA, 1966) was followed.

Table 4. Enumeration of thermophilic flat sour spores in oat flour.

Method	Spore count per 10g sample
Recommended method	170
Low pH-mild temperature	35
Gerber method	30

DISCUSSION

Preliminary investigations suggested that Mn^{++} was necessary at a certain level in the plating medium for improved growth and sporulation of B. stearothermophilus strain E-2. Similar situations were encountered by other workers. Rowe et al. (1975) found that manganese was required at relatively high concentrations (1.0 μM) for the growth of B. stearothermophilus 1503 in a defined liquid medium. The addition of 15 to 30 ppm of manganese (Mn^{++}) to a defined liquid medium containing tryptone was found to stimulate sporulation of B. stearothermophilus (Thompson and Thames, 1967). Addition of Mn^{++} to nutrient agar for increased sporulation of B. stearothermophilus has been utilized by various investigators (Schmidt, 1950; Kim and Naylor, 1966; Titus, 1957).

The D_{86} -value (decimal reduction time, or the time required to destroy 90% of the spores at 86 C) obtained when B. stearothermophilus strain E-2 spores were heated at pH 7.0 at 86 C indicated significant differences between activated and normal spores. These differences were dependent on the conditions (pH, time and temperature) under which the spores were activated. The minimum D_{86} -value ($D_{86} = \sim 7$ minutes) was observed when the spores were activated at

pH 1.1 for 60 minutes at 70 C. When the temperature of activation was decreased to 60 C and 50 C, the D_{86} value increased to ~9 and 20 minutes, respectively. However, similar D-values were shown for the spores which have been treated overnight at pH 1.1 and 20 C and the spores which have been treated at pH 1.1 at 60 C for 1 hr. Thus, the higher D_{86} -value for spores treated at 50 C probably represent partial activation or a partial change to the heat-sensitive state.

On the other hand, the Ca-form spores have shown extreme heat resistance when heated below 100 C at pH 7.0, indicating a D_{86} -value of over 1000 minutes, which is similar to the D-value obtained with natural (untreated) spores heated under the same conditions.

Difficulties were encountered when attempts were made to compare these findings to those of other workers. That is, the conditions under which their experiments were carried out were different from those which were employed in this study. However, the D-values obtained with natural spores are comparable to those obtained by other workers. When B. stearothermophilus NCIB 8919 spores were heated in water at 100 C, a D_{100} -value of 3000 minutes was obtained (Briggs, 1966). At a higher heating temperature (115 C) a lower D-value ($D_{115} = 22.6$ minutes) was reported by the same worker. For spores of B. stearothermophilus NCIB 8919 a D_{115} value of 18.3 minutes was obtained (Cook and Gilbert, 1968). For spores of B. stearothermophilus ATCC 7953 two D-values

(D_{100} = 459 and 714 minutes) were reported (Murrell and Warth, 1965). The difference between the two values was attributed to differences in Ca:DPA ratios of the spores. A comparison between these findings and the data obtained with B. stearothermophilus E-2 native spores would indicate a fairly good agreement, and the discrepancies are quite explainable in light of the factors which affect heat resistance described earlier in the review of literature.

The effect of acid wash (Slepecky and Foster, 1959) on spore heat resistance was investigated by Murrell and Warth (1965). They reported little effect for such treatment (at 5 C) on the heat resistance of four Bacillus species. The D_{100} -values for these species as measured before and after the treatment were 0.99 and 1.0 minute for Bacillus strain 668, 35.2 and 34.6 minutes for Bacillus strain 636, 270 and 232 minutes for B. coagulans; and 2.38 and 2.17 minutes for Bacillus strain 645. However, when spores of B. stearothermophilus strain NCA 1518 were treated in a liquid culture medium, previously adjusted to pH 3.0 with HCl, for 60 minutes at 70 C, the heat resistance was reduced greatly as compared to the untreated spores.

The time needed to reach a 100,000-fold reduction in the untreated spores was 11 times greater than for heat sensitive spores when both spore suspensions were heated at 115.6 C and pH 5.95 (Alderton and Snell, 1969b). Alderton and Snell (1969b) stated that the change in heat resistance amounted to several hundred-fold between the heat sensitive

and heat resistant forms. Thus the data obtained with H-form and natural spores of B. stearothermophilus E-2 are comparable and the general trends in heat resistance of treated and untreated spores are quite similar to those obtained by other workers.

Various studies have shown that activation of Bacillus stearothermophilus spores will take place primarily at temperatures above 100 C. With spores of B. stearothermophilus NCA 1518, Brachfeld (1955) found that maximum activation was obtained when the spores were heated for 5 minutes at 105 C. He also found that heating at lower temperature (55-85 C) resulted in heat-induced dormancy and lower plate counts. Furthermore, his data have shown that even after heating the spores at 105 C for 5 minutes, complete activation was not observed and a portion of the spores were unable to form colonies.

Lewis et al. (1965) have shown that activation of B. stearothermophilus spores is possible at lower temperatures (25 C) and under conditions of low pH (pH = 1.5). Thus the effect of pH in conjunction with temperature and length of duration on activation of B. stearothermophilus spore was investigated.

The results of this study indicated that B. stearothermophilus E-2 spores were activated by a relatively mild heat treatment (50-70 C) under controlled pH conditions (1.1-2.0). At higher pH value (pH $\geq$ 3.0) and lower temperature (40 C) little activation was observed. The optimal

time for activation under these conditions (pH 1.1-2.0 and 50-70 C) was found to be approximately 60 minutes. However, other treatment times (30, 90 or 120 minutes) at pH 2.0 and 50 or 60 C resulted in less activation.

Activation of dormant spores was measured by two methods. These methods are: the regular plating method (plating before and after treatment) and by measuring the heat sensitive spores. The assumption was that if the spores were sensitized to heat by the acid-low heat treatment, even if the acid was removed and heating was carried out at pH 7.0 then the amount of activated spores should be comparable to the amount of heat sensitized ones. The data presented earlier (Tables 2 and 3) indicated that this relationship existed in this investigation. At higher levels of activation (>90% of the total count of spores) the plate count method becomes less sensitive in measuring activation. However, under these circumstances the amount of activation can be more accurately measured by determining the proportion of heat sensitive spores.

When the results of this study were compared to the finding of other workers, a fairly good, but incomplete agreement was obtained. For example, Lewis et al. (1965) found that the treatment of B. stearothermophilus spores at pH 1.5 and 25 C for 80 minutes increased the colony count from 18% of total count of spores to 80%, and the heat resistance of spores was greatly reduced. When these activated spores were exposed to pH 9.7 solution, 0.02 M Ca^{++} ,

dormancy and heat resistance was restored. Results obtained with B. stearothermophilus E-2 revealed over 99.9% activation (of the total count) when the spores were treated at pH 1.1 and 60 °C for 60 minutes. This higher activation effect is probably due to the combined effect of higher temperature (60 °C) and lower pH (1.1) used. However, even at pH higher than 1.5 (pH 2.0), more activation of B. stearothermophilus E-2 spores was obtained (99.9%). These findings indicate that effect of temperature is important in activating spores at low pH.

In another investigation, Brown et al. (1968) reported that the presence of 0.5 N hydrochloric acid at 25 °C increased the colony count of B. stearothermophilus (NCIB 8919) spores to the total microscopic count. Although the time of exposure was not stated in their work, their data show that maximum activation occurred at 28-30 minutes, and that longer exposure to the acid reduced the colony count. However, 60 minutes were required to obtain maximum activation at pH 1.1-2.0 in this study. This difference is probably due to the higher acid concentration, resulting in a lower pH, used by Brown et al. (1968). The reduced colony counts which were observed when the spores of B. stearothermophilus E-2 spores were treated over 60 minutes may be due to inhibition or loss of certain factor(s) which trigger germination or outgrowth. Destruction of the spores is also another possibility. However, microscopic examination of activated spores showed that <1.0% of the total

count of spores were converted to phase dark spores even after 120 minutes at pH 2.0 and 60 C.

These results also indicate that at this specific temperature and pH a critical time is involved, after which the amount of activation as measured by the plate count method was reduced. When the amount of activation was determined by measuring the heat sensitive spores no reduction of activation over time was observed, which suggests that activation has increased but the ability of spores to form colonies on NAM was impaired.

Results on enumeration of thermophilic flat sour spores in flour samples indicated that partial activation occurred when the spores were incubated for 60 minutes at pH 1.1 and 60 C. These results also suggested that some of the spores failed to germinate due to interference of some of the components of flour, such as minerals and salts, with germination (Halmann, 1962 and Keynan et al., 1965) or interference of carbohydrates with activation (Fields and Finely, 1964).

Bacterial spores contain relatively high concentrations (4-15% dry weight) of DPA (Halvorson and Howitt, 1961). Also maturation and development of heat resistance in the bacterial spore parallels the accumulation of calcium in the spore (Vinter, 1956). During germination, both Ca^{++} and DPA were released along with the spore peptides (Powell and Strange, 1953).

Alderton and Snell (1963) reported that divalent cations such as Ca^{++} and Mg^{++} can be replaced by hydrogen ions, in a spore cation exchange system, resulting in a lowered heat resistance for H-form spores. The sites of exchange of these cations, which affect heat activation and heat resistance of mature spores, have not been identified (Alderton and Snell, 1963; Lewis et al., 1965). As stated by Gould and Dring (1975), much circumstantial evidence supports the hypothesis that heat resistance of bacterial spores results partly from the relative dehydration of the central protoplast or core. The relatively "dry" core might be achieved by compressive contraction of the cortex caused by the cation load on the cation exchange system of the spore (Alderton and Snell, 1963; Lewis et al., 1965). However, a more recent hypothesis suggested that heat resistance of bacterial spores results from expansion of the cortex caused by the electrostatic repulsion of negatively charged groups on the cortex peptidoglycan. The results of this study indicate that Ca^{++} plays an important role in determining the heat resistance of the spores and, the treatment at low pH and mild temperature conditions reduces the heat resistance and activates the spores. This might lead to the conclusion that peptidoglycan of the cortex and DPA present in the spore system are acting like an ionic buffer system which maintains a low pool concentration of Ca^{++} within the spore protoplast, thus maintaining dormancy and heat resistance of the spore (Gould and Dring, 1974).

This hypothesis is supported by the fact that when the spores were treated at low pH in this study, dormancy and heat resistance of the spores were lost, and the observation made by Hanson et al. (1972) that DPA-negative mutant spores lost their heat resistance and dormancy rapidly on storage. Further evidence for the role of DPA in maintaining dormancy was reported by Zytkevich and Halvorson (1972) who showed that DPA-less mutant spores of *B. cereus*, *B. megaterium* and *B. subtilis* were unable to germinate with customary germinants. These results suggest that Ca^{++} and DPA were needed for maintaining the heat resistance and the dormant state of bacterial spores.

CONCLUSIONS

Some of the conditions for activation of B. stearo-thermophilus E-2 were studied. A pH of 1.1-2.0 and a temperature of 60 C for 60 minutes were the best conditions for activation, while treatment at 70 C resulted in faster activation.

Activation of spores under these conditions was paralleled by transformation of spores to a more heat sensitive form. Thus, the degree of activation could be measured by determining the amount of heat sensitive spores provided that activation was $\geq 90\%$. Heating at 86 C was used in this investigation to determine the proportion of heat sensitive spores. This provided a more accurate method for determining the degree of activation than the direct plate counting method. Activation of B. stearo-thermophilus E-2 spores at low pH may be used under certain conditions as a substitute for a severe heat shock.

More research is needed to determine the exact time-temperature-pH combination(s) which should be used to obtain optimal activation.

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تَـلْخِصُّ
نَشِيطُ جَرَاثِمِ الْبَاسِيلِ اسْتِيرُوثيرْمَفِلِسْ
إِعْدَادُ
يُوسُفِ السَّنُوسِي الْمُبْسُوطِ

لُحِثَ مِنْ خِلَالِ هَذَا الْبَحْثِ أَنَّهُ يُمْكِنُ تَنْشِيطُ
جَرَاثِمِ الْبَاسِيلِ اسْتِيرُوثيرْمَفِلِسْ لِلْأَنْبَاتِ وَالتَّكَاثُرِ
بِعَامِلَتِهَا تَحْتَ ظُرُوفٍ مُعْتَدَلَةٍ فِي جَوْحِ مُضَيٍّ ، وَقَدْ وَجِدَ
أَنَّ أَكْبَرَ نِسْبَةِ تَنْشِيطٍ قَدْ حَصَلَتْ عِنْدَمَا عُمِلَتْ الْجَرَاثِمُ
لِمُدَّةٍ ٦ دَقِيقَةٍ فِي مُحْلُولٍ أَسْتَهَ الْهَيْدْرُوجِينِ
يَتَرَاوَحُ مَا بَيْنَ ١٠ - ٢٠ ، وَعَلَى دَرَجَةِ حَرَارَةٍ
٦٠ دَرَجَةِ مَثْوِيَةٍ .

وَتَلَبَّتْ أَيْضًا أَنَّ الْجَرَاثِمَ الَّتِي تَنْشِيطُهَا
قَدْ تَحَوَّلَتْ إِلَى جَرَاثِمٍ أَقْلَ مَقَاوِمَةً لِلْحَرَارَةِ .

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