THE UNIVERSITY OF MANITOBA

THE ANTIBIOTIC SENSITIVITIES OF ROUGH AND SMOOTH VARIANTS OF *BACILLUS STEAROTHERMOPHILUS* VAR. *CALIDOLACTIS*

by

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TABLE OF CONTENTS

TABLE OF CONTENTS Continued

TABLE OF CONTENTS Continued

LIST OF TABLES

LIST OF TABLES Continued

LIST OF TABLES Continued

LIST OF TABLES Continued

LIST OF FIGURES

LIST OF FIGURES Continued

LIST OF FIGURES Continued

ABSTRACT

Lee Wing, Phillip. M.Sc., The University of Manitoba. October, 1980. The Antibiotic Sensitivities of Rough and Smooth Variants of Bacillus stearothermophilus var. calidolactis. Major Professor: Dr. Gregory Blank.

Rough and smooth spore variants of Bacillus stearothermophilus var. calidolactis, subcultured from commercially available Delvotest® P ampoules, were evaluated in terms of their antibiotic sensitivities using both a simulated Delvotest® P and disc assay procedure. Differences in antibiotic sensitivity of the rough and smooth variants were observed with penicillin and tetracycline using both types of test procedures. The greatest difference in antibiotic sensitivities between the rough and the smooth variants was observed with tetracycline when tested using the disc assay method. The pure rough and pure smooth variants showed minimum detectable levels of 40.0 and 2.0 μg/ml, respectively. These differences in sensitivities by the pure rough and the pure smooth variants towards tetracycline were further demonstrated when varying proportions of mixed spore populations were evaluated using a disc assay method.

The minimum detectable concentration of penicillin by the pure rough and pure smooth variants using the simulated Delvotest® P method was 0.01 and 0.10 Iu/ml, respectively. The sensitivity of the pure rough and pure smooth variants to penicillin was increased when the disc assay procedure was employed viz, 0.004 and 0.005 Iu/ml, respectively.

The varying surface:volume ratios on the performance of the simulated Delvotest® P method showed no effect; the nature of the antibiotic diluent, however, influenced the pH response in Tryptone Glucose Skim Milk base medium.

INTRODUCTION

Several microbiological methods have been developed for the detection of penicillin and other antibiotics in milk. In most of these assay procedures, a time period of 16 to 18 hours is required in order to give test results. Such methods are generally too time consuming to be of use for commercial dairies and/or regulatory agencies. A rapid method for the detection of antibiotics in milk which is both sensitive and accurate would be of great benefit in dealing with this serious problem.

One of the methods presently used for the detection of antibiotic residues in milk and dairy products is based on the acid-production of Bacillus stearothermophilus var. calidolactis. This test is commercially available under the trade name Delvotest® P, and is described as being simple, sensitive, reliable and quick. The Delvotest® P method is an agar diffusion test employing a fixed level of germinative spores/ml incubated at a temperature of 63°C - 66°C for 2½ hours. This test microorganism was chosen because of its thermophilic nature which, when incubated at 63°C - 66°C, precludes the growth of many interfering microorganisms naturally occuring in milk; Bacillus stearothermophilus var. calidolactis is also highly penicillin sensitive.

Previous studies performed in this department have revealed the presence of rough and smooth variants of Bacillus stearothermophilus var. calidolactis isolated from commercially available Delvotest® P ampoules when subcultured in a tryptone yeast extract base medium. Although the

existence of rough and smooth variants of Bacillus stearothermophilus have been documented, their antibiotic sensitivities have so far not been evaluated.

In this investigation, pure rough and pure smooth variants of Bacillus stearothermophilus var. calidolactis were subcultured from commercially available Delvotest® P ampoules. The antibiotic sensitivities of these variants were tested separately against penicillin, chloramphenicol, tetracycline and bacitracin, using a simulated Delvotest® P and disc assay method. These antibiotic sensitivity tests were performed in order to evaluate and delineate the possible effect of rough and smooth variants on the performance and sensitivity of the Delvotest® P method.

REVIEW OF LITERATURE

THE USE OF ANTIBIOTICS IN THE DAIRY INDUSTRY

Antibiotic therapy is presently used as one of the principal methods for the treatment of infectious diseases such as mastitis in dairy cattle. Among the various types of antibiotics available, penicillin has been one of the most widely used in the treatment of these diseases. Antibiotics can be administered to the diseased animal by infusion, injection or orally, depending on the type of infection or personal preference (Yusuf et al., 1960). Unfortunately, all three routes of antibiotic administrations can permit the antibiotic to ultimately reach the milk supply, thus causing problems to the milk producer, processor and consumer.

Some of the problems created by the presence of antibiotics in milk include slow or complete absence of acid production by bacterial starter cultures employed in the manufacture of various cultured dairy products and the onset of hypersensitive reactions in antibiotic sensitized individuals (Krinke, 1950).

Welch et al. (1948) were the first to report the presence of residual levels of penicillin in milk following intramuscular injections of cows treated for mastitis. Although the need of intramammary antibiotic preparation for the control of mastitis in dairy cattle is universally accepted, the attendant risk of milk contamination has always been a cause for concern to dairy technologists and health authorities (Vilim et al., 1979). The problem of antibiotic residues in milk and

dairy products require continuous monitoring on the part of the dairy industry and regulatory agencies in order to reduce and/or avoid a public health crises. In Canada, the Food and Drugs Act and Regulations states that "when an antibiotic is used for lactating cattle, the period of time that must elapse after the last antibiotic treatment in order that the milk from treated lactating animals will contain no antibiotic residue, should not exceed 96 hours" (F.D.A.R., 1975).

METHODS USED FOR THE DETECTION OF ANTIBIOTICS IN MILK

The methods involved in the detection of residual levels of antibiotics in milk are diverse and are based on several principles:

(a) The Reduction of Dyes

These tests utilize a special group of dyes which are incorporated into appropriate microbial growth media, seeded with susceptible, sensitive test microorganisms. The method depends on the ability of the microorganism upon incubation, to grow and utilize the oxygen dissolved in the growth media, which in turn lowers the oxidation-reduction potential of the growth media. To measure the microbial activity and to demonstrate visibly the rate of oxygen depletion, an oxidation-reduction indicator is added. Various dyes have been used in this type of test and include rezazurin, (Hietaranta and Timroth, 1953); methylene blue, (Schipper and Peterson, 1951); triphenyl tetrazolium chloride,(Neal and Calbert, 1956).

(b) Agar Diffusion

The agar diffusion test is based on the formation of zones of inhibition which appear on test plates seeded with susceptible, sensitive test microorganisms, specifically selected for the antibiotic being

tested. The methods of interpretation have varied from susceptibility based on zone-on-zone readings to susceptibility based on measurements of the zone diameter (Balows, 1974). Two main types of assays which utilize an agar diffusion method for the detection of antibiotics in milk are:

(i) Cylinder-plate Method

This method was first outlined by Carter, (1974) and is currently cited in Standard Methods for the Examination of Dairy Products (A.P.H.A., 1978). The method involves the use of the microorganism Sarcina lutea and was developed to detect penicillin residues in milk and dairy products (Ledford and Brown, 1977). Prior to the development of the Delvotest® P method, the Sarcina lutea cylinder plate method was adapted by government agencies to monitor antibiotics in non-fat dairy milk. This method, although more sensitive than the disc plate method, is more complex and requires 16 - 18 hours of incubation.

A variation of the cylinder plate method, as described by Kabay, (1971), utilized a cylinder cup assay, employing Sarcina lutea as the test organism. This method was shown to be sensitive to 14 different antibiotics; the minimum concentration of penicillin detected was 0.01 Iu/ml.

(ii) Disc-plate Method

A detailed procedure outlining this test method is described in Standard Methods for the Examination of Dairy Products (A.P.H.A., 1978). The standard method presently used utilizes agar plates seeded with spores of Bacillus subtilis (Arret and Kirshbaum, 1959). Spores of Bacillus megaterium have also been used in the disc-plate method (Read et al., 1971). Studies performed by Forschner (1973), using spores of Bacillus

stearothermophilus var. calidolactis as the test organism in a disc-assay, have shown that the organism was sensitive to 0.004 Iu penicillin/ml, when incubated at 70°C for 3 hours. On the other hand, a sensitivity of 0.05 Iu penicillin/ml was obtained when Bacillus subtilis was used, with an incubation temperature of 32°C for 14 - 24 hours. Kaufmann (1977), using Bacillus stearothermophilus var. calidolactis in a disc agar method, showed that their test method was more sensitive than either the official disc or the cylinder assay method used for antibiotic assays.

(c) Radioactive Tracing(Charm Test)

This test is specifically used for the detection of penicillin in milk. The general principle involves the affinity of a binder for a ^{14}carbon agent. Penicillin or penicillin derivatives interfere with the binding of a carbon 14. A penicillin analyzer then measures the amount of carbon 14 that is bound and the results are displayed in a digital readout on the analyzer. Thus, the higher the number displayed, the lower the penicillin concentration.

This method, used for the detection of penicillin in milk, is able to give results in less than 15 mins with a sensitivity for penicillin as low as 0.001 Iu/ml. Although the method is simple, fast and easily reproducible, its disadvantage lies in its specificity for only one antibiotic, i.e. penicillin. The high initial cost for the equipment also makes it unaccessible to many dairy industries. This test is commercially available and supplied by Penicillin Assays Inc., 33 Harrison Avenue, Boston, Ma. (Anon 1979).

(d) Acid Production

These tests are based on the inhibition of acid production by the

test organism when antibiotics are present. The method depends on the ability of the microorganism, upon incubation, to produce an acidic end product, mainly lactic acid. The inhibition of microbial growth due to the presence of antibiotics is determined by the reduction in acid production by the test organism. Various test organisms which have been used include: Streptococcus thermophilus (Collins, 1957), Streptococcus agalactiae (Watts et al., 1945), Lactobacillus bulgaricus (Ullberg, 1952), commercial dairy starter cultures (Reuhe, 1950) and Bacillus stearothermophilus var. calidolactis (Van Os et al., 1975). Another test assay using Bacillus stearothermophilus var. calidolactis C953 is commercially available as the BR Foss test. This test procedure is simple and is based on the reduction of the dye brilliant black by the test organism, after an incubation period of 3 hours. A concentration of 0.004 Iu penicillin/ml can be detected using this method. Van Os et al. (1975), described a variation of the Bacillus stearothermophilus var. calidolactis method, which has been developed by Gist-Brocades, Delft, Holland, as Delvotest® P.

The commercially available Delvotest® P is one of the methods presently used for the detection of various antibiotic residues in milk and other diary products. The test kit consists of two main important components:

(i) sealed ampoules, each containing approximately 2.0×10^6/ml of highly purified, germinative spores of Bacillus stearothermophilus var. calidolactis, and

(ii) nutrient tablets, each containing 0.5 mg tryptone, 5.0 mg glucose, 2.0 mg non-fat dry milk, and 0.25 mg bromocresol purple.

The tablets containing the nutrients and bromocresol purple are

added to ampoules containing plain agar with spores of Bacillus stearothermophilus var. calidolactis. After the addition of 0.1 ml of milk sample, the ampoule is incubated for 2½ hours in a water-bath at 63° - 66°C. In the absence of antimicrobial substances, the whole of the solid medium turns yellow, while it remains purple in the presence of sufficiently high concentrations of antibiotics. The test method has a very high sensitivity for penicillin. Other advantages include a rapid completion time of the test, simple equipment required, less interference from natural inhibitors in the milk, and greater stability of the activated spores. Reproducible results were obtained with test materials produced in different batches on a large scale, and the test ampoules were shown to be stable for at least 12 months; the nutrient tablets, when stored in dry conditions, were stable for at least 6 months.

Research has shown that the Delvotest® P method can detect as low as 0.004 Iu penicillin/ml of milk sample and is not affected by detergent residues in milk which may occur occasionally. Natural antimicrobial substances in raw milk hardly interfere with the test. This was proven in experiments with untreated churn milk samples (Van Os et al., 1975). Many of the antimicrobial substances which interfere with the test can be prevented by special treatments e.g. boiling. As indicated by Standard Method for the examination of dairy products, this boiling consists of a heat treatment of 1 hour at 100°C.

The possibility of reducing the duration of the test to 1½ hours by preincubating the medium and nutrients can also be achieved.

Work performed by Stahouders and Hassing (1976), investigated the reproducibility of the Delvotest® P method and the extent to which these

tests correlated with each other using various batch lots.

STAGES IN SPORE DEVELOPMENT

(a) Dormancy

Dormancy is a stage or condition of a living organism that is characterized by a lack of metabolism and developmental processes. The extreme case of dormancy is characterized by no metabolic activity and has been designated as cryptobiosis. The retention of viability for extended periods of time by spores implies the cryptobiotic state. The degree of dormancy of individual spores is quite variable. Factors that maintain, augment or induce dormancy include temperature, ionic environment, pH, exchangeable cation load, water activity, oxygen, oxidation-reduction as well as the presence of protein agents, metabolic inhibitors, analogs and antibiotics (Gould, 1969). With aging, heating, reductants, low or high pH, or altered exchangeable cation load the optimally dormant state may be transformed into an activated state. The process of activation terminates the state of dormancy temporarily without terminating cryptobiosis in the spore. Dormancy is considered to be broken completely when the germination rate of the spore suspension is maximal.

Although the exact function of dormancy is not known three roles have been ascribed to spores, including enhancement of survivability, disseminability and coordination of development with favorable environmental conditions (Sussmanona and Halvorson, 1966).

Several distinct and physiologically independent stages of maturation have been shown to be involved in the

transformation of a dormant spore into a vegetative cell. These processess have been termed activation, germination and finally, outgrowth.

(b) Activation

Activation is the initial process which conditions the dormant spore to germinate under appropriate conditions. The activated spore retains most of the important properties found in the dormant spore state such as resistance to heat and radiation, nonstainability and refractility.Activation, however, causes changes in the germination requirements for the spore and effects the ultimate germination rate which otherwise is slow and often incomplete. Other effects include changes in morphology, permeability and spore composition as well as changes in the metabolic activity of the spore (Keynan and Evenchik, 1969). The process of activation is in most cases reversible and, as noted by Keynan and Halvorson (1965), does not occur in all spores. The need for this process depends on the nature of the spore type, the previous history of the spore and the type of environmental conditions such as temperature and pH, which can induce the transformation of the dormant spore into a vegetative cell.

The simplest method of spore activation is exposure to sub-lethal heat, but other treatments have been shown to replace this heat effect. Work done by Finley and Fields (1962), has shown that at temperatures above 100^{o}C, activation of Bacillus stearothermophilus strain 1518 occured, while at temperatures below 100^{o}C, heat induced dormancy was observed. Reports verifying the above finding were earlier documented by Brachfeld (1955). Curran and Evans (1945), however, demonstrated that sub-lethal heat (62^{o} to 65^{o}C) could also induce dormant spores to

germinate.

A number of reports exist, indicating that the composition of the medium in which the spores are suspended during heating (the menstruum) influences the degree of activation. This was first reported by Curran and Evans (1945), who showed that the nature of the menstruum determined the degree of activation in their strains. Powell and Hunter (1955), later reported that heat activation must be carried out in the presence of water. It was further observed that it was impossible to activate lyophilized spores, or spores resuspended in a high concentration of glycerol (Beers, 1958). These results indicate the importance of the role of water for activation.

A study of the kinetics of heat activation showed that the activation energy attained is high and is similar to that observed in the heat denaturation of macromolecules. The utilization of heat for spore activation can also give rise to the following conditions (Busta and Ordal, 1964):

i) heat killing of the spores;

ii) heat damage of some specific enzymes involved in germination;

iii) quantitative population changes in heterogeneous spore suspensions;

iv) permanent changes in germination requirements; and

v) heat induced dormancy.

The process of heat induced dormancy was observed with spores of two strains of Bacillus stearothermophilus when heated in distilled water at 80°, 90° and $100^{\circ}C$ (Finley and Fields, 1962). It was found that heat treatment at these temperatures gave a reduced metabolic activity effect. Other methods which have been used for effective activation of spores are summarized by Keynan and Evanchik, (1969):

i) low pH and reducing agents;

ii) calcium-dipicolinic acid; (Ca-DPA);

iii) ionizing radiation;

iv) activation by various chemicals; and

v) ageing

The activation mechanism has been postulated by several workers (Gould and Hitchens, 1963; Lee and Ordal, 1963), as a change involved in spore permeability. The evidence that spore permeability is altered during activation is based on the following observation: increased uptake of oxygen by activated spores when glucose is added; a need for less L-alanine for the saturation of germination rates; excretion of Ca-DPA and some amino acids during activation (Keynan and Evenchik, 1969); and finally, more direct proof by Gould and Hitchens, (1963), who observed that exposure of spores to mercaptoethanol, a treatment which induces activation, enables a molecule as large as lysozyme, to penetrate the spore coat and reach its substrate located in the cortex of the spore.

Other explanations for the activation effect have been given by Keynan et al. (1964), who reported that the activation mechanism could be considered as a process in which molecular changes occured in proteins located in the various spore structures. Freeze and Cashel (1965), hypothesized on the mechanism by which activation results in the release of a factor stimulating germination. According to this hypothesis, when the spore is heated, a stimulating factor becomes available, which leads to the onset of germination. This substance, however, is unstable and disappears if germination is prevented. Studies performed by these researchers suggested that the hypothetical stimulating substance might

be either Ca^{2+} or DPA, or both.

Halvorson et al. (1958), however, regarded the mechanism of activation as one possibly involving the inactivation of an inhibitor. They showed that dipicolinic acid, which is a potent chelating agent, stimulated the activity of the endogenous electron transport system in spores. They assumed therefore, that activation might lead to the release of DPA from its bound form. The liberated DPA then removes an inhibitory metal by chelation, promoting the flow of electrons from substrate to molecular oxygen. This hypothesis, however, assumes the existence of a metal which maintains the dormant state of the spore.

(c) Germination

Activation results in a spore which is poised for germination but still retains most of the original dormant spore properties. The activation process results in the conversion of a resistant and dormant spore into a sensitive and metabolically-active spore form. The changes which occur during germination (Gould, 1969), include:

i) changes in resistance

ii) breaking of dormancy

iii) depolymerization and excretion of spore constituents

iv) cytological changes

v) optical density changes

vi) phase darkening

viii) increase in permeability, and

ix) increase in spore volume.

Various physical and chemical factors have been found which are able to initiate germination. Some of these factors include the use of specific amino acids (Wolf and Mahmoud, 1957); ribosides (Hills, 1949);

sugars (Wolf and Thorley, 1957); non-metabolizable germinants such as ions (Levinson and Sevag, 1953); surfactants (Rode and Foster, 1960); chelates (Riemann and Ordal, 1961); environmental factors such as pH (Church et al., 1954); temperature (Knaysi, 1964); water activity (Hagen et al., 1967) and ionic strength (Rode and Foster, 1962).

(d) Pre-emergence and Outgrowth

After the completion of germination, a process of synthesis of new macromolecules and a highly ordered sequence of biosynthetic events occur resulting in the differentiation of structures characteristic of the vegetative cell (Kobayashi et al., 1965). Both physical and chemical requirements were found to give optimum spore outgrowth resulting in the emergence of a new vegetative cell. These include temperature, inorganic material ie. sulphur, phosphorus; amino acids and pH (Strange and Hunter, 1968). During the conversion of a spore to a vegetative cell, a highly ordered sequence of biosynthetic events occurs leading to a culture which divides synchronously for at least several generations. During outgrowth, progressive changes in structure, metabolic activity, classes of ribosomal particles, and patterns of RNA synthesis have been observed (Kobayashi et al., 1965). Germination and outgrowth can, therefore, be considered as a process of intracellular differentiation involving a series of distinct structural and functional alterations.

BACILLUS STEAROTHERMOPHILUS

The first named and described variety of Bacillus stearothermophilus was isolated from cans of spoiled corn and string beans (Smith et al., 1952). This species has long been of great importance and concern in the canned food industry. The spores of this organism are extremely heat-resistant,

surviving heat treatments at 120°C for 35 mins in pH 6.95 phosphate buffer (William et al., 1937). The vegetative cell on the other hand is capable of growth at temperatures of 70°C or slightly higher, and is responsible for flat sour spoilage of low acid canned foods (Esty and Stevenson, 1925). These spoilage organisms are usually facultative anaerobes and obligate thermophiles.

The following characterization of Bacillus stearothermophilus was based on a study of 98 cultures, all incubated at 45° to 50°C during the observation period (Smith et al., 1952):

Vegetative Rods

0.9 μ to 1.0 μ by 2.5 μ to 3.5 μ; motile; gram variable.

Spores

1.0 μ to 1.2 μ by 1.5 μ to 2.2 μ; characteristically variable in size; oval; located terminally to subterminally within the sporangia.

Colonies

Non-distinctive; pinpoint to small; round to irregular; translucent to opaque; rough to smooth.

Nutrient Agar Slant

Growth variable; ranging from thin, scant, rough and non-spreading to good, opaque, smooth and spreading.

Growth Temperature

Maximum temperature - 65° to 75°C; minimum temperature - 30° to 45°C.

Hill and Fields (1967a), studied the influence of temperature upon the growth and interaction of rough and smooth variants of Bacillus stearothermophilus NCA 1518. They found that a low growth temperature (45°C) favoured the rough variant type. The smooth variant however, did

not grow well at this lower temperature. It was then concluded that the ultimate effects of these temperatures were due to enzymatic responses rather than changes in the nutritional requirements of the variants.

Taxonomic studies on two strains of Bacillus stearothermophilus performed by Finley and Fields (1962), noted that one strain consistently grew at 70°C, whereas the other grew at 65°C and not 70°C. This inability of the particular strain to grow at 70°C may have indicated that there may be a comparable intrinsic difference in the heat activability of the spores of these two strains. Research by Long and Williams (1959), further showed that some strains of Bacillus stearothermophilus were dependent upon the surface:volume ratio of the media for growth at low temperatures.

Species Variation

Apart from differences in colony structure, due to species differences, the bacteria of a single species may form several distinct types of colonies. Two colonial types which have been isolated from various bacterial species include smooth colonies of regular outline and form, and rough colonies of a more granular and irregular character. These differences in colony form are usually outward expressions of significant morphological and physiological differences in the particular organisms concerned (Hanshelwood, 1947).

The factors which favour the establishment of a smooth population over a rough should be recognized and investigated. Oxygen tension has been shown to affect differentially the growth of strains of many organisms. Wessman (1964) found that oxygen limitation favoured the growth

of non-smooth forms of Pasteurella haemolytica and that this selectivity did not occur in aerated cultures. Similar results were obtained by Altenbern et al. (1957), who stated that the non-smooth type of Brucella abortus grew at a faster rate than the smooth at low air pressure and that the rough had a greater affinity for oxygen in the terminal hydrogen transport system. Other bacterial species which have shown these colonial variations include Diplococcus pneumoniae (Carta and Firshein, 1962) and many species belonging to the genus Bacillus (Smith et al., 1952). Carta and Firshein (1962), showed that two factors produced by one variant type inhibited the multiplication of the other variant. One of the factors was the presence of an endotoxic substance which was part of the cell itself. The inhibitory factors, however, have not been characterized or identified.

VARIANTS OF BACILLUS STEAROTHERMOPHILUS

(a) Morphological and Colonial Characteristics

Cultures of Bacillus stearothermophilus have long been recognized (Michner, 1953; Fields, 1963) to contain both rough and smooth variants according to their colonial and morphological characteristics. Variations in size of the rough and smooth variants of the vegetative cells of Bacillus stearothermophilus NCA 1518 were first reported by Fields (1963), who showed that the surface colony of the smooth variants was round with a central opaque spot. The size of these smooth vegetative cells had a mean of 1.5 μ x 0.5 μ with a maximum of 2.0 μ x 0.5 μ and a minimum of 1.0 μ x 0.5 μ. The rough variants, however, showed colonies with an irregular margin; the surface colonies of the rough variants also tended to be larger than the smooth variants when grown on the same medium

and under the same experimental conditions. The vegetative cells of the rough variant were considerably longer having a mean of 3.6μ x 0.5μ with maximum length of 5.0μ x 0.5μ and a minimum of 2.0μ x 0.5μ. A report by Scholefield and Abdelgadir (1974) also indicated that the rough variant produced a larger vegetative cell and spore. Intermediate forms of Bacillus stearothermophilus NCA 1518 have also been reported by Humbert et al. (1972). They reported three types of colonies which arose when the stock spore suspension of Bacillus stearothermophilus NCA 1518 was inoculated onto trypticase-soy agar. One type of colony was smooth, while the other two were of the rough type, giving an acid and an alkaline reaction on dextrose tryptone agar. The rough (alkaline) and the rough (acidic) strain had a strong tendency to autolyse after they had reached their maximum cell concentration or, if they were held at room temperature. The smooth strain cells were quite stable under similar conditions.

Work performed by Hill and Fields (1967b) suggested that at least two possible interactions between rough and smooth variants of Bacillus stearothermophilus NCA 1518 could occur:

(1) A rough population could be established in an acid medium and by production of basic substances, raise the pH sufficiently to stimulate growth of the smooth variant.

(2) In a medium which permits growth of the rough variant but is nutritionally inadequate for the smooth variant, the rough may produce metabolic by-products which enable the smooth variant to grow.

Previous work performed by Hill and Fields (1967a) showed that a population of Bacillus stearothermophilus NCA 1518 initially containing

a mixed proportion of rough and smooth variants, quickly reverted to an all smooth variant population, except when grown at 45°C.

Studies performed by Humbert et al. (1972) further showed that there was reason to believe that mutations in colonial morphology occurred in their population of Bacillus stearothermophilus NCA 1518. Hill and Fields (1967a) postulated that when the mutation from smooth to the rough colonial type occurred, there was a concurrent drop in the heat resistance and a change in a number of nutritional and biochemical characteristics. Humbert et al. (1972), however, showed that this was not necessarily true, and concluded that when there was a mutation in the Bacillus stearothermophilus strain from smooth to rough, the nutritional and biochemical capabilities of the mutant strain usually remained essentially unchanged from those of the parent. As noted by these researchers, it was possible for mutation to occur in both the rough and smooth variants of Bacillus stearothermophilus NCA 1518.

(b) Ultrastructure of the Spores

Studies performed on the ultrastructure of spores of Bacillus stearothermophilus NCA 1518 by Rothman and Fields (1966), indicated that the spore wall of both the rough and the smooth variant was composed of three layers. In the smooth variant, however, these layers appear to adhere together, whereas in the rough, the layers were distinctly separated. The ultrastructure studies performed also illustrated that the smooth variant had a thicker spore wall as compared to the rough variant and that a two-layer area of less electron density was immediately adjacent to the spore wall of the smooth variant. The rough variant, on the other hand, contained a thicker cortex as compared to the smooth

variant and also contained a core wall, or a layer surrounding the core of the spore, which was not observed with the smooth variant.

(c) Heat Resistance of the Spores

Fields (1963) reported that the temperatures needed to activate smooth variant spores, inactivated the rough variant spores. Rothman and Fields (1966) later reported D-values (that time of heating at a temperature to cause a 90 percent reduction in the count of viable spores) at 121°C of 2.32 and 1.42 minutes for smooth and rough variant spores of Bacillus stearothermophilus NCA 1518, respectively. Bigelow (1921) found that the F-value of Bacillus stearothermophilus NCA 1518 was 5.5 minutes at 121°C. Reed et al. (1951) however, published F-values (that time in minutes required to destroy the organism in a specified medium at 250°F or 121°C) for this organism of 25.3 minutes in phosphate buffer, pH 7.0. Fields and Finley (1962) found the F-value at 121°C to be 7.0 minutes for a suspension of NCA 1518. Rothman and Fields (1966) later explained these differences in F-value as reflections of traditionally recognized factors such as: nutritional status of the organism, the suspending mestruum, the age and concentration of the spore crop. The differences in F-values obtained between spore suspensions of the same organism, however, may have been due to the presence of variant forms in the spore suspension.

(d) Growth Conditions and Pnysiological Characteristics

The nature of the growth medium and the pH were found to have an influence on the growth and interaction of rough and smooth variants of Bacillus stearothermophilus. Studies performed by Hill and Fields (1967a) showed that oxygen tension affected the amount of acid produced by the rough and smooth variants of Bacillus stearothermophilus NCA 1518. The smooth variants produced more acid than the rough, but the amount of acid was independant of the oxygen availibility. The rough variant, however,

showed more acid accumulation as the oxygen tension was lowered. Further studies have shown that the rough variant not only had a lower nutritional requirement than the smooth variant, but that the generation time included temperature and oxygen tension.

Fields (1966) studied the growth of the rough and the smooth variants of Bacillus stearothermophilus NCA 1518 in pure and mixed cultures in pea extract. He found that in mixed cultures the smooth variant produced acids and/or other metabolites which inhibited the growth of the rough variant. His results also indicated that the rough variant produced basic substances during growth in pea broth.

The pathway of glucose metabolism in the rough and smooth variants of Bacillus stearothermophilus NCA 1518 has been studied by Hill et al. (1967). They found that the Embden-Meyerhof pathway was more active in the smooth variant as compared to the rough variant type. The rough variant, however, utilized glucose via the EM pathway exclusively or in combination with a pathway other than the hexose monophosphate shunt.

(e) Heat Activation

The use of sublethal heat (62^{o} to $65^{o}C$) to induce dormant bacterial spores to germinate was performed initially by Curran and Evans (1945). Later, both Brachfeld (1955) and Titus (1957) showed that the spores of Bacillus stearothermophilus could be activated at temperatures above $100^{o}C$. Research performed by Finley and Fields (1962) showed that heat-induced dormancy occurred when spores of Bacillus stearothermophilus were heated in distilled water at 80^{o}, 90^{o} and $100^{o}C$; temperatures greater than $100^{o}C$, however, resulted in activation. Maximal activation occurred at temperatures between 100^{o} and $115^{o}C$, depending on the strain

and nature of the variants in the spore suspension i.e. consisting of one pure variant type or mixed. The optimum temperature for heat activation for both the rough and the smooth variants of Bacillus stearothermophilus strain NCA 1518 was shown to be 110°C. The heating times for both variants at 110°C were found to differ. The smooth variant was shown to require approximately 15 minutes more than the rough variant in order to achieve optimum activation (Fields, 1963).

(f) Germination and Outgrowth

Finley and Fields, (1962) working with Bacillus stearothermophilus NCA 1518, reported that phosphate buffer lowered the heat resistance of the spore and also influenced the rate and extent of germination of the spore. Brachfeld (1955), however, previously reported that phosphate buffer significantly decreased the average plate count. Similarly, Williams and Hennessee (1956), noted that the apparent heat resistance of Bacillus stearothermophilus spores was affected by the concentration of the phosphate buffer contained in the heating mestruum.

Comparative plate count data obtained from spores suspended and heated in distilled water and in M/120 phosphate buffer at both maximal and submaximal activation temperatures showed that the phosphate menstruum had a definite, inhibitory effect on spore germination and outgrowth (Finley and Fields, 1962). The comparative data also indicated that there is a direct relationship between the magnitude of heat activation and the magnitude of phosphate inhibition.

Other factors affecting the germination process of Bacillus stearothermophilus have been reported by various researchers and include:

i) the concentration of spore suspension (Curran and Pallansch, 1963);

ii) inhibitory compounds (eg. nisin) which may be produced by the natural flora of milk (McGillivray et al., 1966);

iii) osmotic pressure (Fields, 1964);

iv) calcium dipicolinate (Busta and Ordal, 1964);

v) carbon dioxide (Cook et al., 1964).

ANTIBIOTICS AND ANTIMICROBIAL ACTION

Antibiotics have been divided into two groups: bactericidal antibiotics (ie. penicillin) which kill or cause the lysis (dissolution) of invading bacteria, and bacterostatic antibiotics (ie. chloramphenicol) which merely inhibit bacterial growth and replication.

(a) Penicillin

Potassium penicillin G is a highly soluble salt. When susceptible bacteria are grown in the presence of lethal concentrations of penicillin, lysis occurs. If sublethal concentrations are used, large, swollen filamentous forms are produced. Such effects are observed only with growing bacteria. This antibiotic is effective against both gram positive and gram negative bacteria. It has been known for may years that penicillin is a selective inhibitor of bacterial cell wall synthesis in multiplying bacteria. This inhibitory action is, however, incomplete because cell wall construction and cell division continue in the presence of penicillin (Huckers and Bennett, 1975). Penicillin specifically inhibits the last step in peptidoglycan synthesis, the cross linking of the linear peptidoglycan strands. When bacteria are grown in the presence of penicillin, uncross-linked uridine nucleotide intermediates of peptidoglycan synthesis accumulate. Studies performed by Vinter (1965) showed that when lethal concentrations of penicillin were present at the beginning of the germination stage of Bacillus stearothermophilus spores, the incorporation

of [^{14}C] DAP was inhibited until the antibiotic was degraded. The penicillin inhibited the cortex formation and retention of calcium and DPA in the sporulating variants.

(b) Tetracycline

Tetracycline is the parent member of a group of broad spectrum antibiotics that are equally effective against gram-positive and gram-negative bacteria. They inhibit protein synthesis due to the high intracellular concentration of the antibiotic. Tetracycline molecules form a complex with the magnesium ions that are present in the bacterial cell wall and on the surface of the cytoplasmic membrane. The complex is then transported across the membrane and the tetracycline molecules are released into the cytoplasm and act upon the ribosomes. The process results in an intracellular concentration of antibiotics up to 30 times the concentration outside the cell (Hammond and Lambert, 1978).

(c) Bacitracin

The mode of action of this antibiotic resembles that of penicillin, in that it causes spheroplast formation and the accumulation of nucleotide precursors of peptidoglycan. The specific site of action is concerned with the lipid-soluble carrier molecule which transports the disaccharide-pentapeptide presursor across the cell membrane for assembly outside the membrane. In the last step of this stage of peptidoglycan biosynthesis, the pyrophosphate form of the lipid-soluble carrier is dephosphorylated to yield inorganic phosphate and the carrier lipid. It is this last step that is specifically inhibited by bacitracin and the carrier lipid is thus prevented from re-entering the reaction cycle of peptidoglycan synthesis (Hammond and Lambert, 1978). Bacitracin is

highly active against many gram-positive bacteria.

(d) Chloramphenicol

This broad spectrum antibiotic acts only upon bacterial ribosomes, leading to inhibition of peptide bond formation. Studies performed by Ryter and Szulmajster (1965) showed that the development of forespores of Bacillus subtilis which have not yet reached the stage of coat formation, was inhibited by chlormaphenicol. The antibiotic blocked the stage involving coat formation, however, it did not impair cortex formation.

MATERIAL AND METHODS

MATERIALS

The bacterial culture used throughout this investigation was Bacillus stearothermophilus var. calidolactis, rough and smooth variants, obtained directly by subculturing commercially available Delvotest® P ampoules supplied by the Laboratory of Technical Microbiology of the Technical University, Delft, The Netherlands.

The antibiotics used were:

(a) Penicillin-G (1595 International Units/mg)

(b) Bacitracin (56300 International Units/gram)

(c) Tetracycline (crystalline)

(d) Chloramphenicol (crystalline)

All antibiotics were obtained from the Sigma Chemical Company, St. Louis, Missouri, U.S.A.

METHODS

1. Preparation of Media

(a) Tryptone Yeast Extract Glucose (TYG) Medium

The TYG medium described by Kim and Naylor, (1966) was used initially for the preparation of the vegetative cells of Bacillus stearothermophilus var. calidolactis. The medium contained the following:

Tryptone	10.0 g
Glucose	5.0 g

Yeast Extract	5.0 g
K_2HPO_4	5.0 g
Distilled Water	1000 ml

and was adjusted to pH 7.2 with 0.1N NaOH prior to sterilization.

(b) Sporulating Agar

The sporulation agar used for spore production was described by Kim and Naylor (1966). This medium was used for the sporulation of the variant spore types after initial vegetative growth in TYG media was achieved. The sporulation agar consisted of the following:

Nutrient broth	8.0 g
Yeast Extract	4.0 g
$MnCl_2 \cdot 4H_2O$	10.0 ppm
Agar	20.0 g
Distilled Water	1000 ml

and was adjusted to pH 7.2 with 0.1N NaOH prior to sterilization. Two hundred milliliter volumes of the sporulation agar were used for each 1-litre Roux Bottle, sterilized and allowed to solidify in a horizontal position.

(c) Tryptone Glucose Skim Milk (TGS) Medium

This medium consisted of a simulated Delvotest® P test nutrient tablet as described by Van Os et al. (1975). The medium contained the following:

Tryptone	0.0833%
Glucose	0.833 %
Skim Milk Powder	0.333 %
Bromocresol Purple	0.004 %

and was adjusted to pH 7 with 0.1N HCl prior to sterilization.

2. Preparation of Vegetative Cell Inoculum

The contents of the Delvotest® P ampoules were initially tempered in a water bath at 100°C for 1 minute in order to melt the agar suspending the bacterial spores. The spores were then directly inoculated into sterile TYG medium and incubated at 53°-55°C in a New Brunswick Scientific gyratory shaker, G-25, 1" circular orbit, operating at 200 r.p.m. for 14 - 18 hours.

The resultant vegetative growth (stock) was then used as the inoculum for the preparation of the rough and smooth variants of Bacillus stearothermophilus var. calidolactis. The stock was initially serially streaked on TYG agar plates, followed by incubation at 55°C for 48 hours. The morphological rough and smooth variants were selected and picked from the agar surface on the basis of their colony variation, using a method of serial, single-colony selections on TYG agar. All plates were incubated at 55°C for 18 hours. The rough and smooth variants were both isolated and successively streaked separately five times on TYG agar in order to validate the purity of the inocula before they were used for spore crop production.

Vegetative cell growth was obtained by separate inoculation of pure colonies of the pure rough and the pure smooth variants in TYG medium, followed by incubation at 55°C for 18 hours. Pure lines of both rough and smooth variants, as well as the stock culture, were maintained in the spore state, suspended in sterile distilled water and stored at approximately 4°C. The rough and smooth variants were periodically streaked out on TYG media in order to check their purity.

3. Spore Production

Five milliliter aliquots of the respective vegetative cells were distributed evenly over the surface of the sterile sporlating agar with the aid of sterile glass beads, and incubated at 55°C. At 24 hour intervals, a spore stain was performed on a sample obtained from the Roux bottles in order to determine the extent of sporulation. An incubation period of seven days was shown to give maximum spore yield for the rough and smooth mixed (stock), pure smooth and pure rough variants of Bacillus stearothermophilus var. calidolactis.

(a) Spore Harvest

After incubation, the resultant spore crop was harvested by washing the surface of the agar with 10 ml of sterilized, cold (4°C) distilled water. The spore crop from each Roux bottle was then quickly pooled into a sterile flask and centrifuged at 1°C for one hour at 400 x g using a Sorval superspeed RC2-B automatic refrigerated centrifuge. Centrifugation was carried out asceptically.

(b) Treatment of Spores with Lysozyme

After centrifugation, the supernatant was discarded and an equal volume of cold (4°C) distilled water was added to resuspend the spore pellet. A 1% solution of lysozyme was added to the resuspended spore pellets at a rate of 1% v/v. The spore suspension, together with the lysozyme, was incubated overnight at 1° - 3°C to ensure liberation of all endospores from the sporangia, and lysis of any unsporulated cells contained in the preparation (Kim and Naylor, 1966).

(c) Separation of Spores from Lysed Sporangia

After lysozyme treatment, the spores were washed repeatedly with

cold, sterile distilled water, then centrifuged (400 x g) at 1°C. The spore pellet was finally resuspended to its original volume using sterile cold (4°C) distilled water. The purity of the spore suspension was checked by microscopic examination using Fleming's Method of spore staining (Cowan and Steel, 1970).

The above procedures were used for the preparation of the stock (rough and smooth mixed), pure rough and pure smooth spores of Bacillus stearothermophilus var. calidolactis.

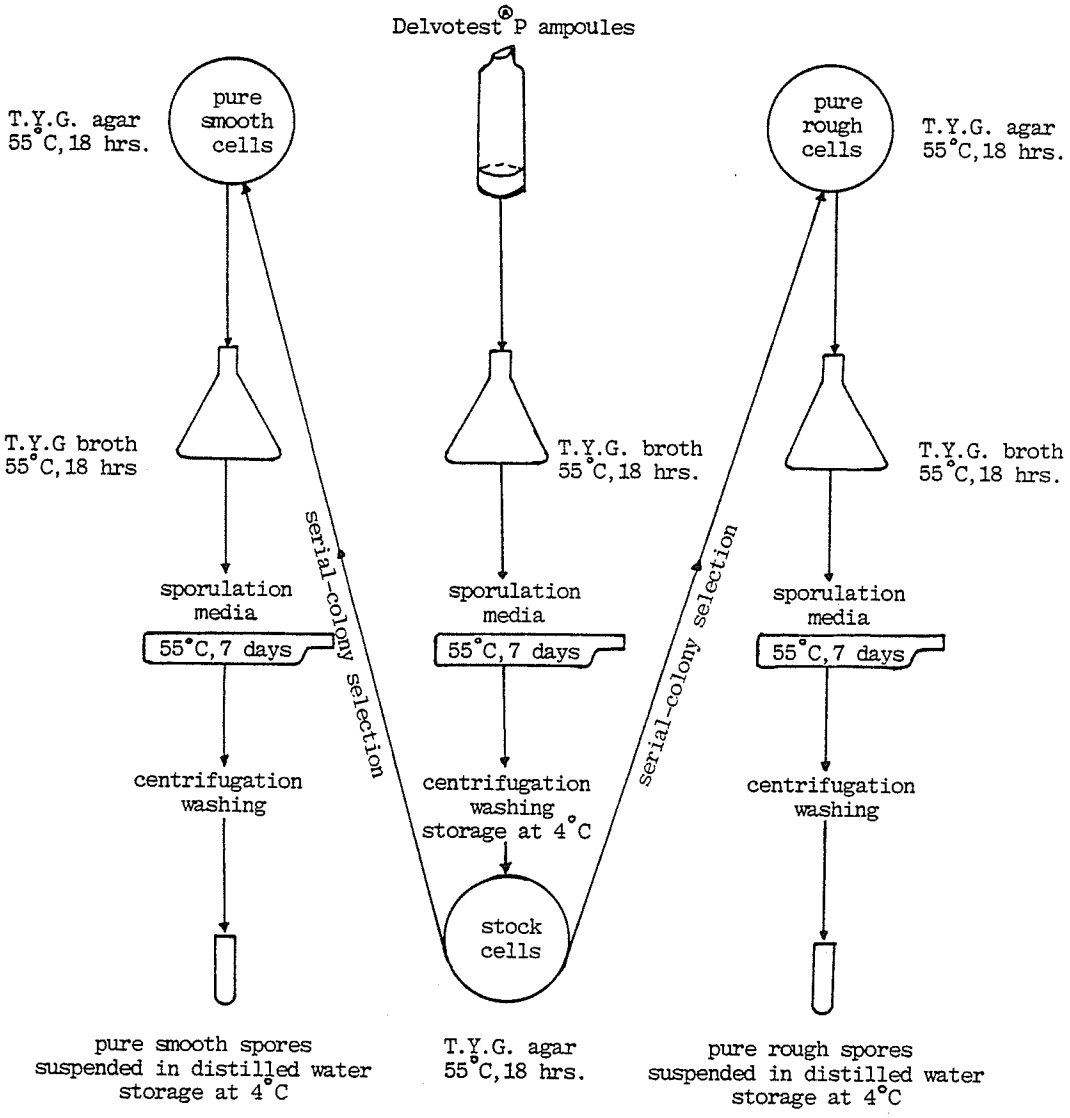
(d) Spore Inoculum Standardization

The spore concentrations were standardized using a Perkin Elmer Hitachi model III spectrophotometer set at a wavelength of 340 nm. Sterile distilled water was used as the blank. Viable spore counts were determined by dilution plate counts on TYG agar incubated at 55°C for 48 hours. The standardized spore suspensions used throughout the study ranged from 1×10^6 to 4×10^6 spores/ml. A diagramatic scheme used for spore and vegetative cell production is shown in figure 1.

4. Spore Heat Activation

Standardized spore suspensions contained in distilled water, such that the final volume contained 10 ml of spore suspension and heating mestruum, were heat treated at various temperatures, viz, 70°, 80°, 90°, 100° and 110°C. Several series of tubes containing standardized spores were used for each activation temperature studied. Nine tubes constituted a series; one tube served as an unheated control and the others were subjected to heat for varying lengths of time up to 60 minues. The first tube was removed from the heating bath after the heating menstruum reached the designated temperature. This was considered "0" time. There-

FIG. 1 Flow diagram outlining the stepwise procedures for the separation of rough and smooth variants of Bacillus stearothermophilus var. calidolactis from Delvotest® P ampoules.



after, the tubes were removed at varying time intervals, viz, 1, 5, 15, 20, 30, 45 and 60 minutes. Heat activation studies at or below 100°C were performed in a Blue M magni whirl thermostatically controlled water-bath using standard 8 x 150 mm pyrex glass tubes. Studies performed at temperatures greater than 100°C utilized a Blue M magni whirl thermostatically controlled oil-bath, using 6 x 150 mm standard screw top pyrex tubes. After appropriate heating, the designated tubes were immediately removed and rapidly cooled to room temperature by use of a cold water bath. The time-temperature criteria required for optimum activation of the various spore variant types were correlated with the minimum time required to change the pH of TGS medium from 6.8 to 5.4. All pH measurements were performed using a Fisher Accumet Model 520 digital pH/ion meter; visual changes in the pH of the medium were followed by observing the colour change of the bromocresol purple indicator.

The optimum activation temperature and time were determined for the stock spores as well as the pure rough and the pure smooth variants.

5. Determination of Antibiotic Sensitivities

(a) Simulated Delvotest® P Method

Simulated Delvotest® P, performed in standard 8 x 150 mm pyrex tubes, containing 10 ml of TGS media, were inoculated with respective standardized heat activated spore suspensions containing the various concentrations of antibiotics under study. Simulated Delvotest® P media, inoculated only with respective standardized heat activated spore suspensions served as controls. The time taken for the simulated control Delvotest® P medium to achieve a pH of 5.4 was used as the maximum incubation time

for the test and was determined for each variant studied. A visual reference point, corresponding to a pH of 5.9 was used to determine the minimum inhibitory concentration of the antibiotic under study. This visual reference point corresponded to that pH at which the indicator, bromocresol purple, initially started to change colour from purple to a faint purple-yellow. (Figure 2).

(b) Disc Assay Method

Petri plates containing 10 ml of TYG agar, seeded with heat activated spores (2×10^6 spores/ml) were used to determine the various antibiotic sensitivities of the rough and the smooth variants. Sterile blank discs, $\frac{1}{2}$" (1.27 cm) in diameter (Difco) were capillary wetted with the various antibiotics and gently layered on the surface of the solidified agar bed. Petri plates having an inside diameter of 85 mm were used throughout this study. All plates were inverted and incubated at 55°C for 6 hours. Perceptible, clear zone diameters of inhibition were measured to the nearest millimeter, perpendicularly to both axis, and were recorded as the average of triplicate discs on duplicate plates. All antibiotics were freshly prepared on the same day of assay and suitable diluted in distilled water or milk, using appropriate controls.

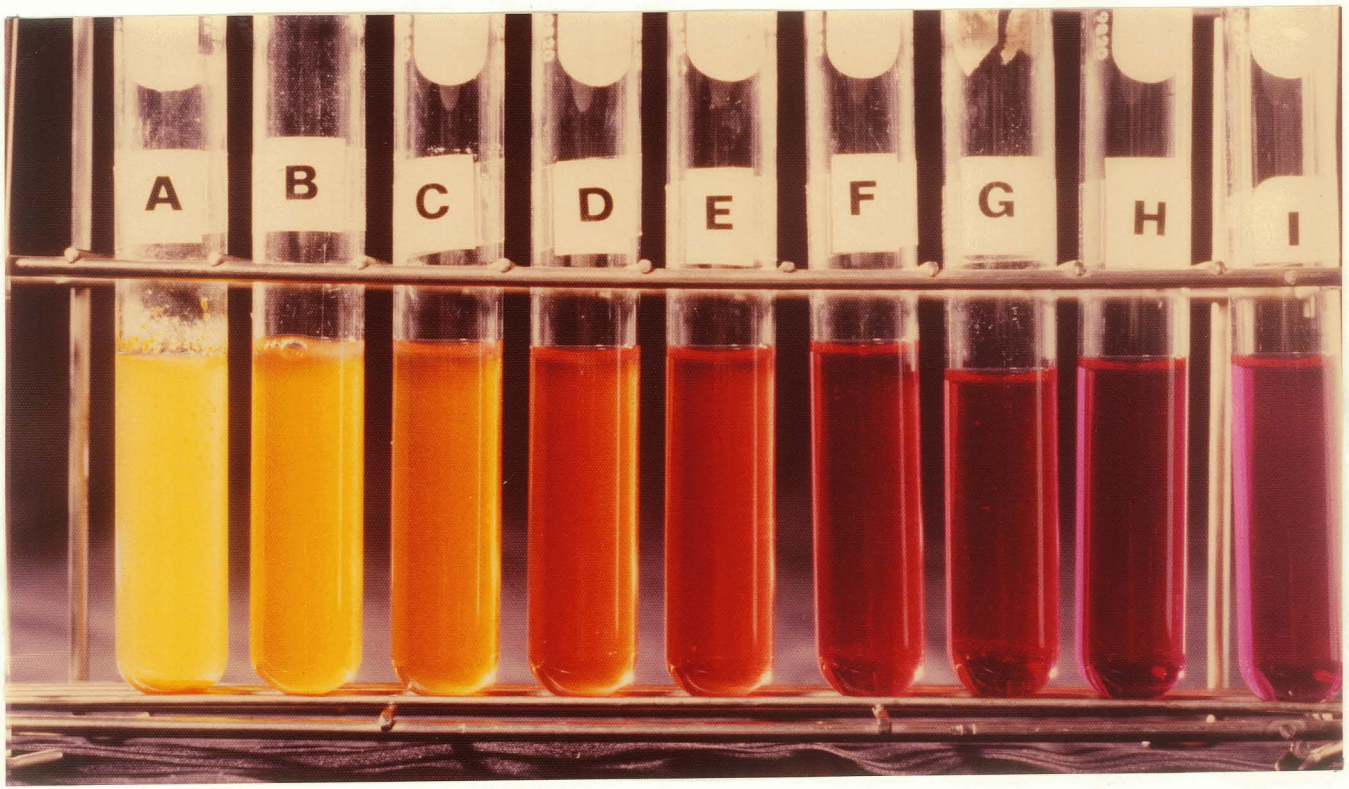
6. Determination of Antibiotic Sensitivities of Various Mixed Proportions of Rough and Smooth Variants of Bacillus stearothermophilus var. calidolactis

(a) Simulated Delvotest® P Method

Spore suspensions of pure rough and pure smooth variants of Bacillus stearothermophilus var. calidolactis, each standardized to contain approximately 2×10^6 heat activated spores/ml were inoculated into a

FIGURE 2. pH changes in TGS media containing 0.004% bromocresol purple.

(A) pH 5.26 (B) pH 5.45 (C) pH 5.61
(D) pH 5.81 (E) pH 5.91* (F) pH 6.04
(G) pH 6.23 (H) pH 6.56 (I) pH 6.88



* Reference pH

sequence of test tubes containing TYG medium as follows:

Pyrex Test Tubes (8 x 10mm)	% Rough	% Smooth
1	0	100
2	20	80
3	40	60
4	60	40
5	80	20
6	100	0

The final volume in each test tube was 10 ml with each variant type being heat activated separately at 70°C for 15 mins. prior to inoculation. The inoculated test tubes, set up to contain a fixed proportion of rough and smooth heat activated spores, were then each tested for antibiotic sensitivity using a selected range of antibiotic concentrations.

A second sequence of identical test tubes containing no antibiotics were used as the control. All test tubes were incubated at 45°C in a water bath. The maximum incubation time employed for each spore proportion tested was determined by monitoring the time required to change the pH of the corresponding control from 6.8 to 5.4 (colour change from purple to yellow).

In another study, 50% pure rough variant spores were mixed with an equal amount of pure smooth variant spores and heat activated at 110°C for 15 mins. These spores were inoculated into TGS medium, giving a final volume of 10 ml and incubated at 66°C, until the pH of the medium was observed to change from 6.9 to 5.4. The time required for this colour change was then recorded.

(b) Disc Assay Method

A sequence of six test tubes containing varying proportions of the same fixed population of pure rough and pure smooth variant spores (as described previously) were seeded into TGS agar and poured into petri plates, having an inside diameter of 85 mm. A final volume of 10 ml of the agar-spore suspension was used throughout the study. The method previously described for the determination of the minimum inhibitory concentration of antibiotics on the pure variants using the disc assay method was utilized. All plates were inverted and incubated at 55°C for 6 hours, after which zone diameters of inhibition were measured and recorded.

7. The Effect of Varying the Surface to Volume Ratio on the Performance of the Simulated Delvotest®P Method Using Bacillus stearothermophilus var. calidolactis

The effect of varying the surface to volume ratio (ie. the relative surface exposures of the media) on the performance of the simulated Delvotest®P method using the rough and smooth variants of Bacillus stearothermophilus var. calidolactis was determined using varying sized test tubes. The surface to volume ratios studied in this investigation are outlined as follows:

Test Tube Dimensions (mm)	Diameter at Media Surface (mm)	Media Depth (mm)	Media Volume (ml)	Surface to Volume Ratio
16 x 150	16.0	30.5	5.0	40:1
16 x 150	16.0	55.0	10.0	20:1
18 x 150	18.0	65.0	15.0	17:1
18 x 150	18.0	83.0	20.0	13:1

RESULTS

Spore suspensions of Bacillus stearothermophilus var. calidolactis exhibit the phenomenon of biological dormancy, such that the process of germination progresses very slowly and/or incompletely unless some method of activation is applied to the spores.

In this study, preliminary investigations were initiated in order to determine the optimal conditions necessary for the heat activation of the spore variants. The various antibiotic sensitivities for the spore variants were then subsequently evaluated using a simulated Delvotest® P and disc assay method.

Heat Activation

Heat activation studies using rough and smooth mixed (stock) variants of Bacillus stearothermophilus var. calidolactis were performed at temperatures of 90°, 100° and 110°C for 1 to 60 minutes, holding time. The results (Tables 1 and 2) indicated that 90° and 100° were sub-optimal for the activation of the spore stock. A temperature of 110°C (Table 3) applied for approximately 15 minutes showed maximum heat activation. This time-temperature combination, when applied to either a pure smooth or a pure rough spore population, however, showed minimal spore activation effects (Figure 3).

The pure smooth variants were optimally heat activated at 70°C for approximately 15 minutes (Table 4). A temperature of 45°C was also established as the optimal incubation temperature, regardless of the time and temperature used for the heat activation. Incubation temperatures greater than 45°C progressively decreased the rate of pH change

TABLE 1. Changes in the pH of TGS medium inoculated with rough and smooth mixed variants of Bacillus stearothermophilus var. calidolactis, heat activated at 90°C for varying lengths of time. Incubation time and temperature was 180 minutes and 66°C, respectively.

Heat[@] Activation Time (mins)	pH change						
	Incubation Times (mins)						
	0	30	60	90	120	150	180
1	6.41	6.37	6.41	6.41	6.27	6.14	5.64
5	6.43	6.41	6.41	6.33	6.28	6.01	5.62
15	6.42	6.42	6.41	6.38	6.23	5.90	5.48
20	6.41	6.41	6.27	6.27	6.26	5.86	5.43
30	6.52	6.50	6.50	6.50	6.32	5.86	5.43
45	6.50	6.50	6.50	6.43	6.27	5.76	5.36
60	6.50	6.50	6.50	6.44	6.21	5.74	5.29

@ come up time was 6 minutes

TABLE 2. Changes in the pH of TGS medium inoculated with rough and smooth mixed variants of Bacillus stearothermophilus var. calidolactis, heat activated at 100°C for varying lengths of time. Incubation time and temperature was 180 minutes and 66°C, respectively.

Heat Activation Time (mins)@	pH change						
	Incubation Times (mins)						
	0	30	60	90	120	150	180
1	6.41	6.41	6.38	6.34	6.21	5.88	5.48
5	6.41	6.40	6.39	6.28	6.20	5.87	5.26
15	6.43	6.42	6.39	6.27	6.16	5.87	5.16
20	6.42	6.41	6.38	6.26	6.14	5.77	5.11
30	6.41	6.40	6.37	6.23	6.10	5.65	5.10
45	6.34	6.34	6.32	6.20	6.01	5.66	5.09
60	6.42	6.42	6.40	6.38	6.22	5.64	5.04

@ come up time was 8 minutes

TABLE 3. Changes in the pH of TGS medium inoculated with rough and smooth mixed variants of Bacillus stearothermophilus var. calidolactis, heat activated at 110°C for varying lengths of time. Incubation time and temperature was 180 minutes and 66°C, respectively.

Heat@ Activation Time (mins)	pH change						
	Incubation Times (mins)						
	0	30	60	90	120	150	180
1	6.54	6.54	6.45	6.33	5.89	5.41	4.87
5	6.54	6.54	6.48	6.32	5.80	5.28	4.81
15	6.60	6.54	6.47	6.25	5.77	5.25	4.81
20	6.56	6.53	6.47	6.28	5.96	5.33	4.85
30	6.52	6.53	6.50	6.31	5.86	5.34	4.86
45	6.53	6.53	6.48	6.30	5.87	5.29	4.85
60	6.54	6.54	6.48	6.28	5.80	5.28	4.86

@ come up time was 9 minutes

FIGURE 3. Changes in the pH of T.G.S. medium inoculated with pure rough, pure smooth, and rough and smooth mixed variants of Bacillus stearothermophilus var. calidolactis, heat activated at 110°C for 15 mins. Incubation temperature was 65°C.

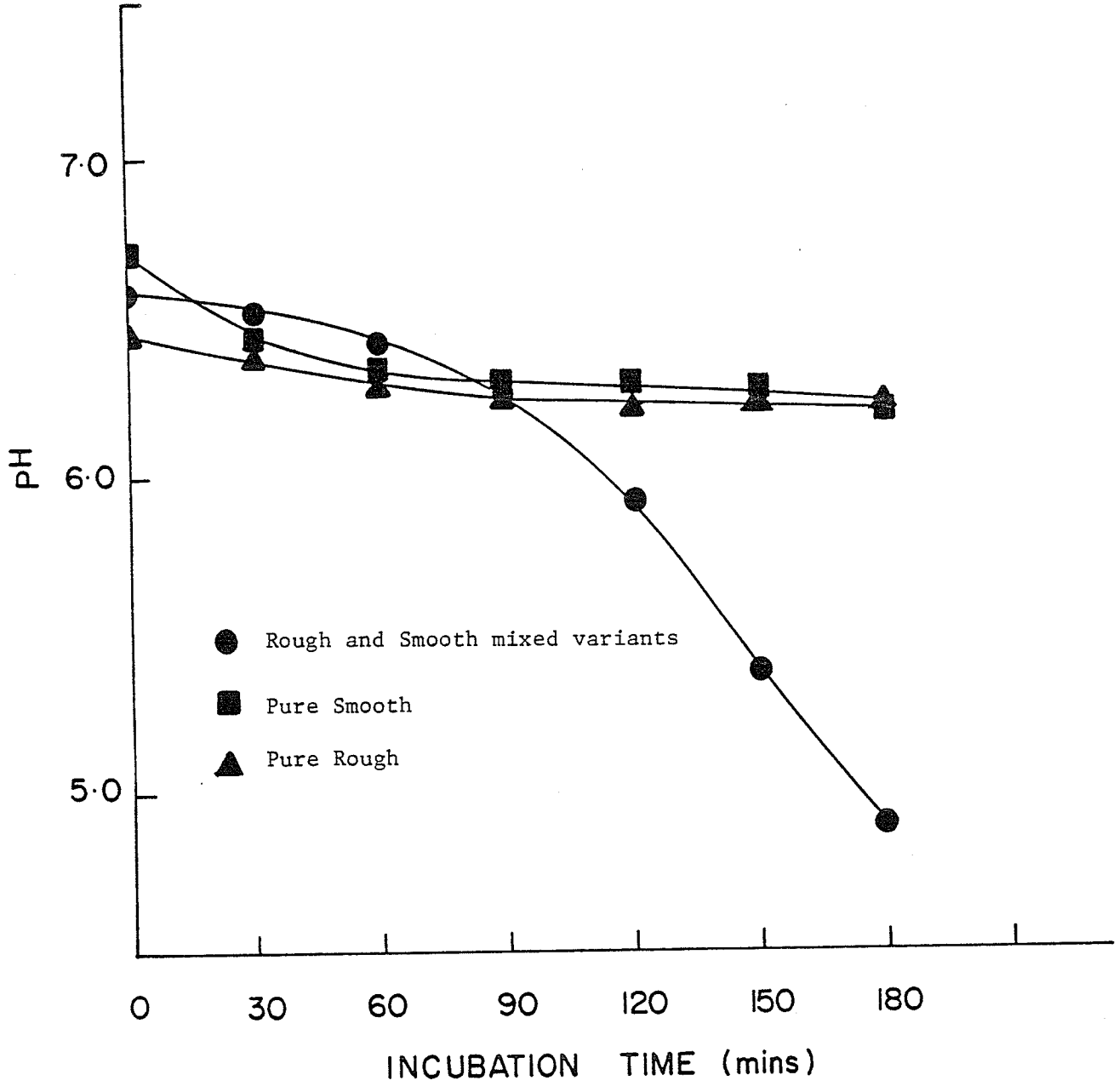


TABLE 4. Changes in the pH of TGS medium inoculated with pure smooth variants of Bacillus stearothermophilus var. calidolactis, heat activated at 70°C for varying lengths of time. Incubation time and temperature was 180 minutes and 45°C, respectively.

Heat@ Activation Time (mins)	pH change						
	Incubation Times (mins)						
	0	30	60	90	120	150	180
1	6.76	6.74	6.71	6.63	6.43	6.17	5.86
5	6.79	6.75	6.71	6.62	6.42	6.20	5.87
15	6.78	6.71	6.71	6.59	6.35	6.09	5.83
20	6.77	6.72	6.71	6.62	6.42	6.17	5.88
30	6.77	6.73	6.71	6.62	6.42	6.16	5.88
45	6.77	6.74	6.70	6.62	6.42	6.15	5.88
60	6.78	6.74	6.71	6.61	6.40	6.14	5.87

@ come up time was 1.5 minutes

in the simulated Delvotest® P method (Tables 5, 6, 7, 8 and 9).

Optimal heat activation conditions for the rough variants were not established in this invesigation. Initially, temperatures of 60°, 70°, 80°, 90°, 100° and 100°C were used to induce activation of the rough variant. These temperatures, applied from 1 to 6 minutes, failed to give optimum activation as indicated by the pH changes in TGS medium (Tables 10, 11, 12, 13, 14 and 15).

Incubation temperatures of 45°, 55° and 66°C were used in conjunction with the various heat activation combinations. A temperature of 70°C for 15 minutes was arbitrarily chosen for the heat activation of the rough variant. An incubation temperature of 45°C was chosen and the duration of the test was extended from 3 hours to 6-6¼ hours (Figure 4).

Antibiotic Sensitivities of Rough and Smooth Variants of Bacillus Stearothermophilus var. calidolactis

(a) Simulated Delvotest® P Method

The minimum inhibitory concentration of penicillin, chloramphenicol tetracycline and bacitracin dissolved in water as the diluent, was determined for the pure rough and pure smooth variants of Bacillus stearothermophilus var. calidolactis using a pH of 5.9 as a reference end point. In addition, the minimum inhibitory concentration of penicillin was determined for the rough and smooth mixed (stock) variants. Figures 5, 6, 7 and 8 illustrate the pH changes in TGS medium (simulated Delvotest® P medium) containing pure rough and pure smooth spores, incubated with the various antibiotics concentrations. The minimum inhibitory concentration of penicillin, chloramphenicol, tetracycline and bacitracin determined for the pure rough variant was 0.01 Iu/ml, 1.0 μg/ml, 0.05 μg/ml and 0.4 Iu/ml, respectively.

TABLE 5. Changes in the pH of TGS medium inoculated with pure smooth variants of Bacillus stearothermophilus var. calidolactis, heat activated at 70°C for varying lengths of time. Incubation time and temperature was 180 minutes and 55°C, respectively.

Heat Activation Time (mins)@	pH change						
	Incubation Times (mins)						
	0	30	60	90	120	150	180
1	6.76	6.73	6.72	6.72	6.71	6.71	6.69
5	6.77	6.73	6.73	6.72	6.71	6.71	6.69
15	6.77	6.74	6.72	6.72	6.71	6.71	6.70
20	6.75	6.74	6.73	6.71	6.71	6.71	6.71
30	6.77	6.73	6.73	6.72	6.72	6.72	6.71
45	6.77	6.74	6.73	6.72	6.72	6.71	6.71
60	6.76	6.73	6.73	6.73	6.73	6.72	6.72

@ come up time was 1.5 minutes

TABLE 6. Changes in the pH of TGS medium inoculated with pure smooth variants of Bacillus stearothermophilus var. calidolactis, heat activated at 80°C for varying lengths of time. Incubation time and temperatures were 180 minutes and 45°C, 55°C and 66°C, respectively.

Incubation temperature	Activation@ time (mins)	Initial pH	Final pH
45°C	1	6.89	6.10
	5	6.95	6.14
	15	6.91	6.08
	20	6.94	6.14
	30	6.92	6.18
	45	6.92	6.24
	60	6.91	6.20
55°C	1	7.00	6.96
	5	7.02	6.96
	15	7.03	6.95
	20	7.00	6.97
	30	7.03	6.98
	45	7.02	6.98
	60	7.00	6.99
66°C	1	7.26	7.22
	5	7.26	7.21
	15	7.24	7.22
	20	7.25	7.22
	30	7.27	7.22
	45	7.29	7.23
	60	7.29	7.23

@ come up time was 3 minutes

TABLE 7. Changes in the pH of TGS medium inoculated with pure smooth variants of Bacillus stearothermophilus var. calidolactis, heat activated at 90°C for varying lengths of time. Incubation time and temperatures were 180 minutes and 55°C and 66°C, respectively.

Incubation temperature	Activation@ time (mins)	Initial pH	Final pH
55°C	1	6.91	6.87
	5	6.92	6.88
	15	6.92	6.88
	20	6.92	6.88
	30	6.93	6.87
	45	6.91	6.88
	60	6.93	6.88
66°C	1	6.65	6.61
	5	6.65	6.67
	15	6.66	6.61
	20	6.62	6.61
	30	6.63	6.61
	45	6.66	6.62
	60	6.69	6.61

@ come up time was 6 minutes

TABLE 8. Changes in the pH of TGS medium inoculated with pure smooth variants of Bacillus stearothermophilus var. calidolactis, heat activated at 100°C for varying lengths of time. Incubation time and temperature was 180 minutes and 66°C, respectively.

Activation@ time (mins)	Initial pH	Final pH
1	6.82	6.75
5	6.79	6.71
15	6.78	6.73
20	6.75	6.75
30	6.75	6.71
45	6.75	6.73
60	6.92	6.75

@ come up time was 8 minutes

TABLE 9. Changes in the pH of TGS medium inoculated with pure smooth variants of Bacillus stearothermophilus var. calidolactis, heat activated at 110°C for varying lengths of time. Incubation time and temperatures were 180 minutes and 45°C and 66°C, respectively.

Incubation temperature	Activation time (mins)	Initial pH	Final pH
45°C	1	6.96	6.94
	5	6.94	6.95
	15	6.98	6.95
	20	6.94	6.95
	30	6.97	6.97
	45	6.95	6.95
	60	6.93	6.93
66°C	1	6.84	6.38
	5	6.82	6.43
	15	6.75	6.40
	20	6.72	6.49
	30	6.77	6.43
	45	6.77	6.41
	60	6.75	6.41

@ come up time was 9 minutes

TABLE 10. Changes in the pH of TGS medium inoculated with pure rough variants of Bacillus stearothermophilus var. calidolactis, heat activated at 60°C for varying lengths of time. Incubation time and temperature was 180 minutes and 45°C respectively.

Activation@ time (mins)	Initial pH	Final pH
1	6.95	6.90
5	6.95	6.90
15	6.95	6.91
20	6.95	6.90
30	6.96	6.89
45	6.93	6.90
60	6.95	6.90

@ come up time was 1 minute

TABLE 11. Changes in the pH of TGS medium inoculated with pure rough variants of Bacillus stearothermophilus var. calidolactis, heat activated at 70°C for varying lengths of time. Incubation time and temperatures were 180 minutes and 45°C and 55°C, respectively.

Incubation temperature	Activation time (mins)[@]	Initial pH	Final ph
45°C	1	6.75	6.67
	5	6.75	6.67
	15	6.74	6.66
	20	6.75	6.66
	30	6.74	6.64
	45	6.74	6.64
	60	6.74	6.64
55°C	1	6.76	6.70
	5	6.77	6.70
	15	6.77	6.70
	20	6.75	6.71
	30	6.76	6.72
	45	6.76	6.72
	60	6.75	6.72

@ come up time was 1.5 minutes

TABLE 12. Changes in the pH of TGS medium inoculated with pure rough variants of Bacillus stearothermophilus var. calidolactis, heat activated at 80°C for varying lengths of time. Incubation time and temperatures were 180 minutes and 45°C, 55°C, and 66°C, respectively.

Incubation temperature	Activation@ time (mins)	Initial pH	Final pH
45°C	1	6.91	6.91
	5	6.92	6.92
	15	6.92	6.91
	20	6.94	6.91
	30	6.93	6.93
	45	6.92	6.91
	60	6.91	6.91
55°C	1	7.02	6.96
	5	6.97	6.97
	15	6.99	6.95
	20	6.99	6.96
	30	7.00	6.97
	45	6.98	6.96
	60	6.99	6.96
66°C	1	7.19	7.17
	5	7.22	7.16
	15	7.19	7.18
	20	7.21	7.17
	30	7.21	7.17
	45	7.22	7.17
	60	7.20	7.18

@ come up time was 3 minutes

TABLE 13. Changes in the pH of TGS medium inoculated with pure rough variants of Bacillus stearothermophilus var. calidolactis, heat activated at 90°C for varying lengths of time. Incubation time and temperatures were 180 minutes and 55°C and 66°C respectively.

Incubation temperature	Activation[@] time (mins)	Initial pH	Final pH
55°C	1	6.88	6.84
	5	6.84	6.81
	15	6.87	6.81
	20	6.87	6.82
	30	6.85	6.82
	45	6.86	6.83
	60	6.86	6.81
66°C	1	6.67	6.62
	5	6.68	6.62
	15	6.66	6.63
	20	6.67	6.61
	30	6.66	6.66
	45	6.74	6.65
	60	6.68	6.66

@ come up time was 6 minutes

TABLE 14. Changes in the pH of TGS medium inoculated with pure rough variants of Bacillus stearothermophilus var. calidolactis, heat activated at 100°C for varying lengths of time. Incubation time and temperature was 180 minutes and 66°C respectively.

Activation@ time (mins)	Initial pH	Final pH
1	6.72	6.69
5	6.73	6.64
15	6.73	6.65
20	6.72	6.64
30	6.71	6.62
45	6.72	6.63
60	6.70	6.62

@ come up time was 8 minutes

TABLE 15. Changes in the pH of TGS medium inoculated with pure rough variants of Bacillus stearothermophilus var. calidolactis, heat activated at 110°C for varying lengths of time. Incubation time and temperatures were 180 minutes and 45°C and 66°C, respectively.

Incubation temperature	Activation[@] time (mins)	Initial pH	Final pH
45°C	1	6.97	6.97
	5	6.94	6.94
	15	6.97	6.96
	20	6.97	6.96
	30	6.96	6.96
	45	6.97	6.97
	60	6.95	6.95
66°C	1	6.43	6.41
	5	6.31	6.31
	15	6.40	6.38
	20	6.43	6.40
	30	6.40	6.40
	45	6.42	6.42
	60	6.42	6.42

@ come up time was 9 minutes

Figure 4. Changes in the pH of T.G.S. medium inoculated with pure rough and pure smooth variants of Bacillus stearothermophilus var. calidolactis, heat activated at 70°C for 15 mins. Incubation temperature was 45°C.

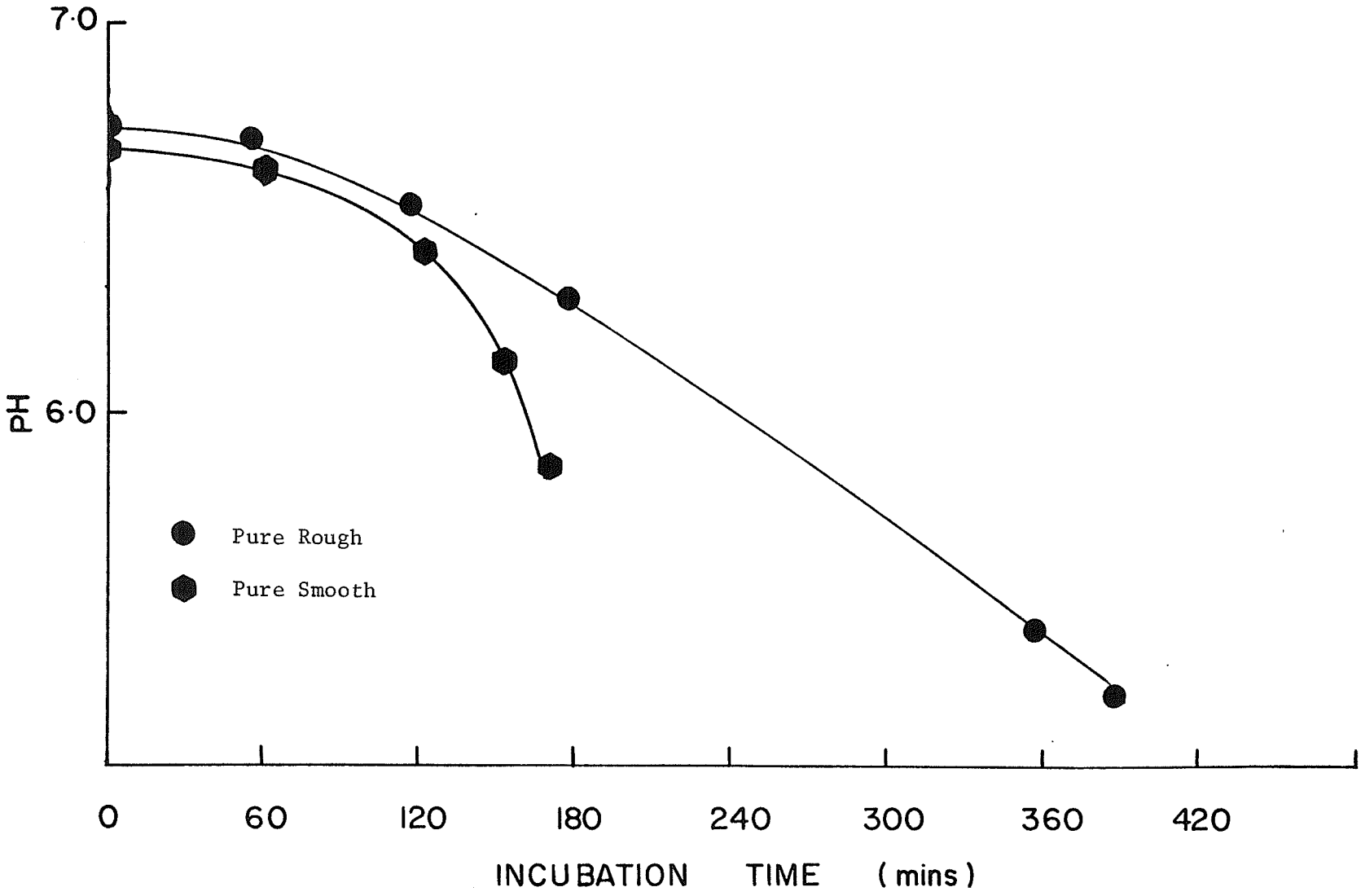


FIGURE 5. Changes in the pH of T.G.S. medium containing varying concentrations of penicillin inoculated with pure smooth and pure rough variants of Bacillus stearothermophilus var. calidolactis. Incubation temperature was 45°C.

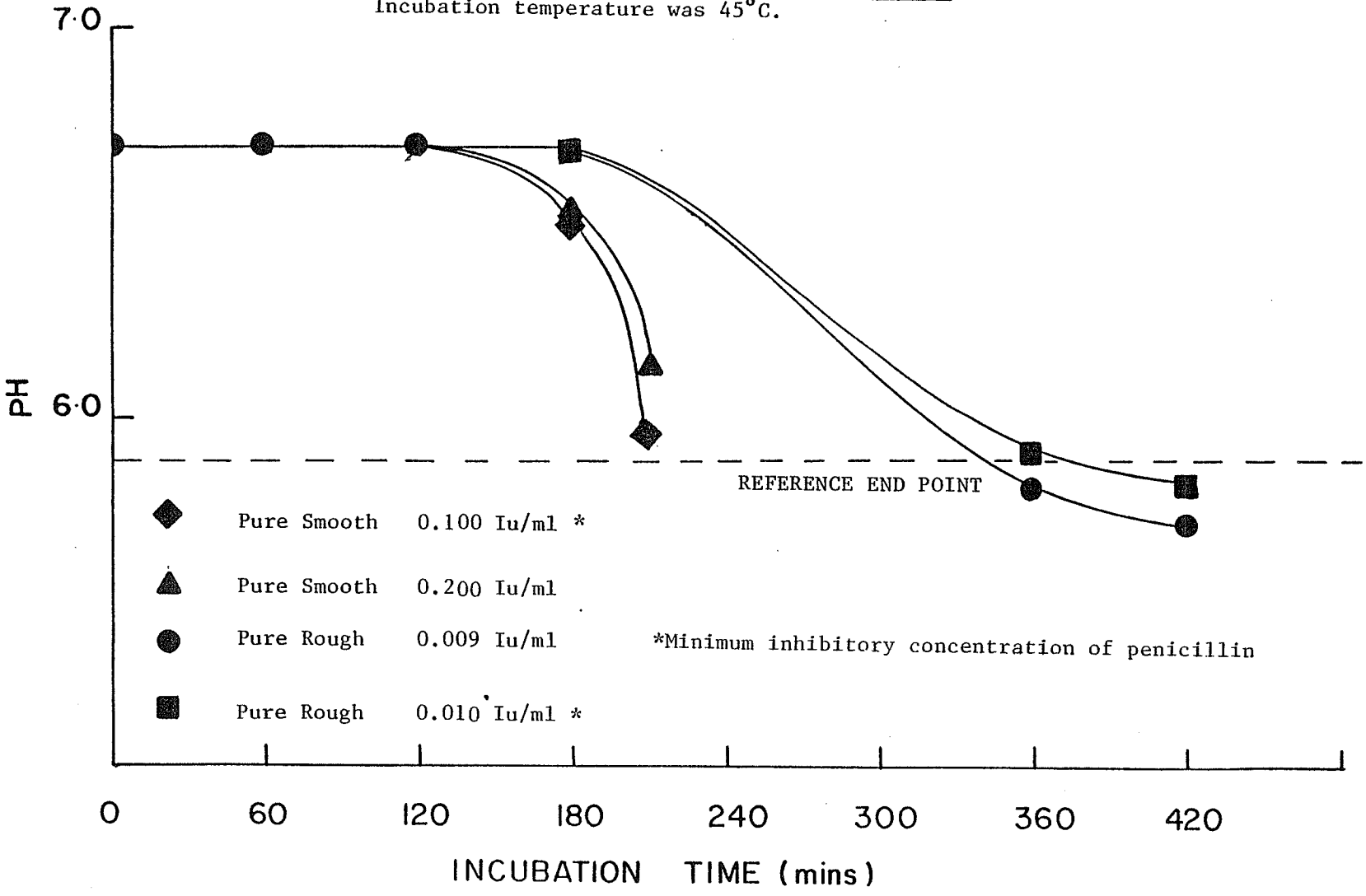


FIGURE 6. Changes in the pH of T.G.S. medium containing 1.0 μg/ml chloramphenicol, inoculated with pure smooth and pure rough variants of Bacillus stearotheromphilus var. calidolactis. Incubation temperature was 45°C.

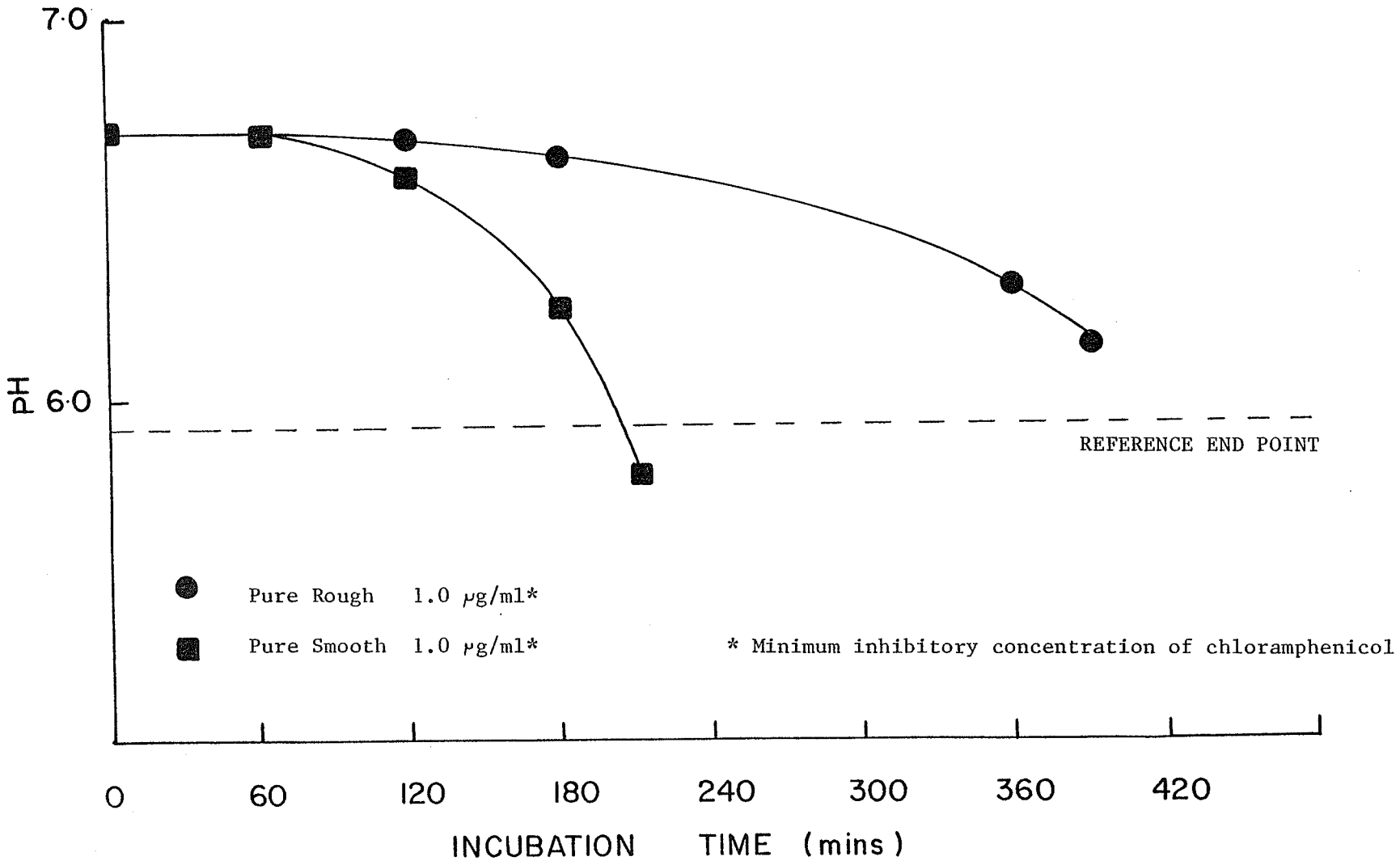


FIGURE 7. Changes in the pH of T.G.S. medium containing varying concentrations of tetracycline, inoculated with pure smooth and pure rough variants of Bacillus stearothermophilus var. calidolactis. Incubation temperature was 45°C.

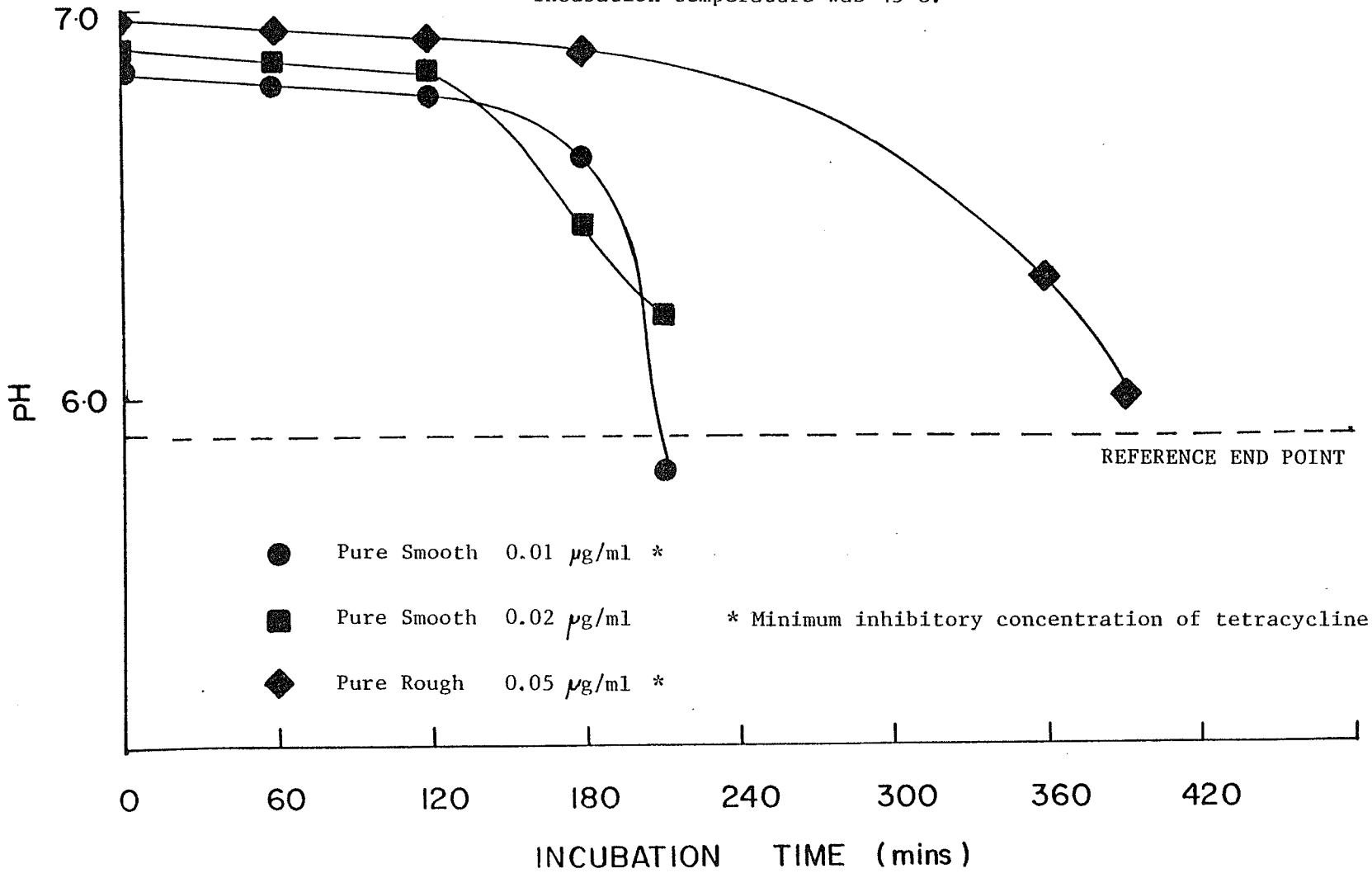
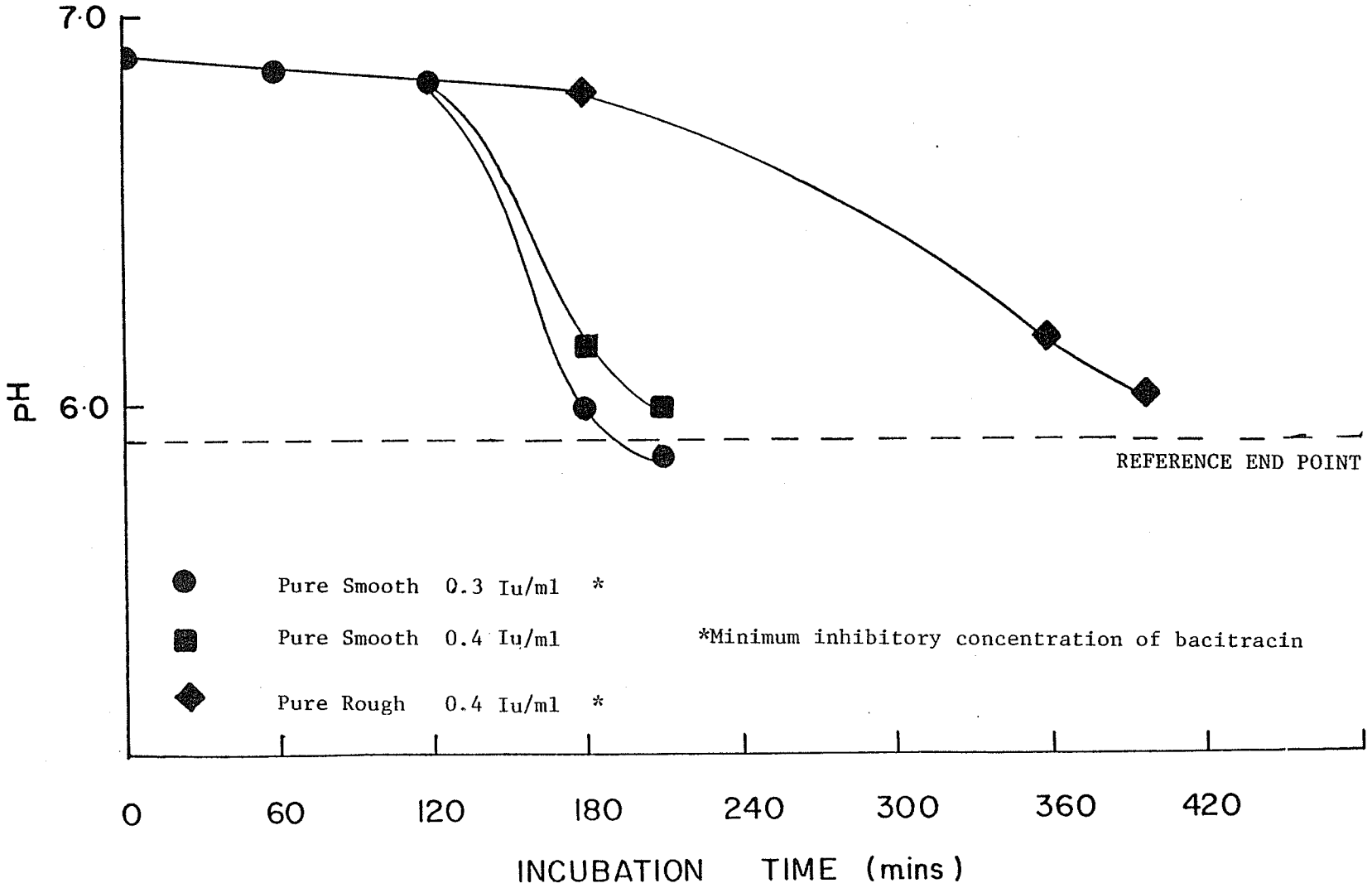


FIGURE 8. Changes in the pH of T.G.S. medium containing varying concentrations of bacitracin, inoculated with pure smooth and pure rough variants of Bacillus stearothermophilus var. calidolactis. Incubation temperature was 45°C.



The pure smooth variants on the other hand showed minimum inhibitory concentrations of 0.10 Iu/ml penicillin, 1.0 μg/ml chloramphenicol, 0.02 μg/ml tetracycline, and 0.4 Iu/ml bacitracin. The minimum inhibitory concentrations of bacitracin and chloramphenicol were identical for both the pure smooth and pure rough variants.

Although the pure smooth variant was somewhat more sensitive to tetracycline as compared to the pure rough variant, the greatest difference in antibiotic sensitivities between the spore variants occured with penicillin; the rough variant was approximately ten times more sensitive as compared to the smooth when the simulated Delvotest® P method was utilized (Table 16).

Figure 9 illustrates the pH changes in TGS medium inoculated with mixed smooth and rough spores (stock) containing 0.004 Iu/ml penicillin. This level of penicillin was determined to be the minimum inhibitory concentration for the stock spores. The pH changes of actual Delvotest® P ampoules inoculated with 0.004 Iu penicillin/ml are also illustrated in Figure 9.

The sensitivities of pencillin, chloramphenicol, bacitracin, tetracycline, dissolved in 2% pasteurized milk as the diluent, were not evaluated against the pure rough and the pure smooth variants because of the buffering capacity exhibited by the diluent. This buffering capacity retarded pH changes, and as such, the reference end point could not be attained within the time specification of the test.

Figure 10 illustrates the pH change in TGS medium inoculated with pure rough and pure smooth variants of Bacillus stearothermophilus devoid of any dissolved antibiotics. These controls were made up to appropriate volumes using either 2% pasteurized milk or distilled water.

TABLE 16. Minimum inhibitory concentrations of various antibiotics on pure rough and pure smooth variants of Bacillus stearothermophilus var. calidolactis, using a simulated Delvotest®P method.

Antibiotic	Minimum Inhibitory Concentration		
Penicillin	(stock)	0.004	Iu/ml
	(smooth)	0.100	Iu/ml
	(rough)	0.010	Iu/ml
Chloramphenicol	(smooth)	1.0	μg/ml
	(rough)	1.0	μg/ml
Tetracycline	(smooth)	0.02	μg/ml
	(rough)	0.05	μg/ml
Bacitracin	(smooth)	0.40	Iu/ml
	(rough)	0.40	Iu/ml

FIGURE 9. Changes in the pH of (a) TGS medium inoculated with stock spores (mixed rough and smooth) variants of Bacillus stearothermophilus var. calidolactis and (b) actual Delvotest®P ampoules, both containing 0.004 Iu/ml penicillin.

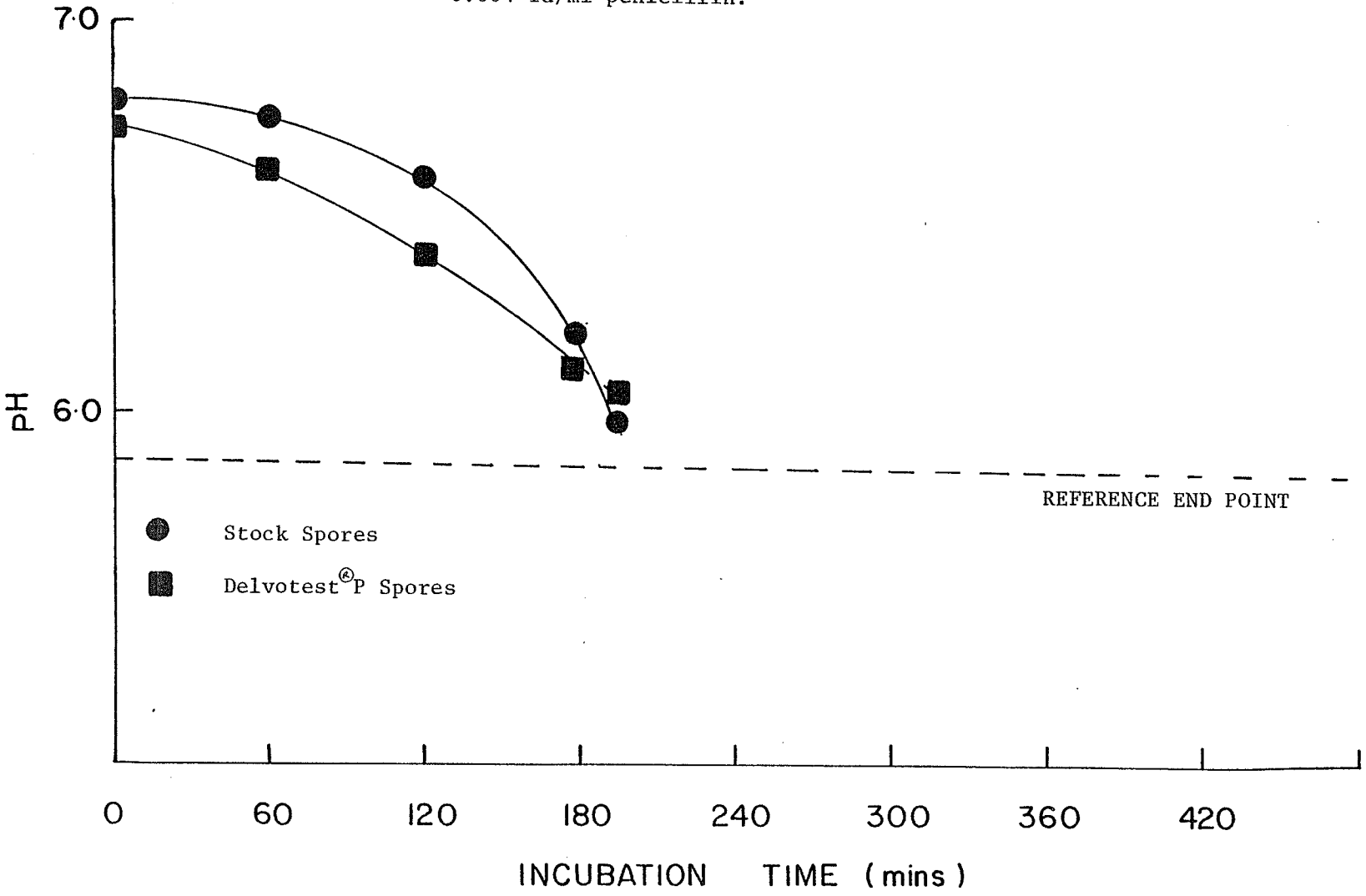
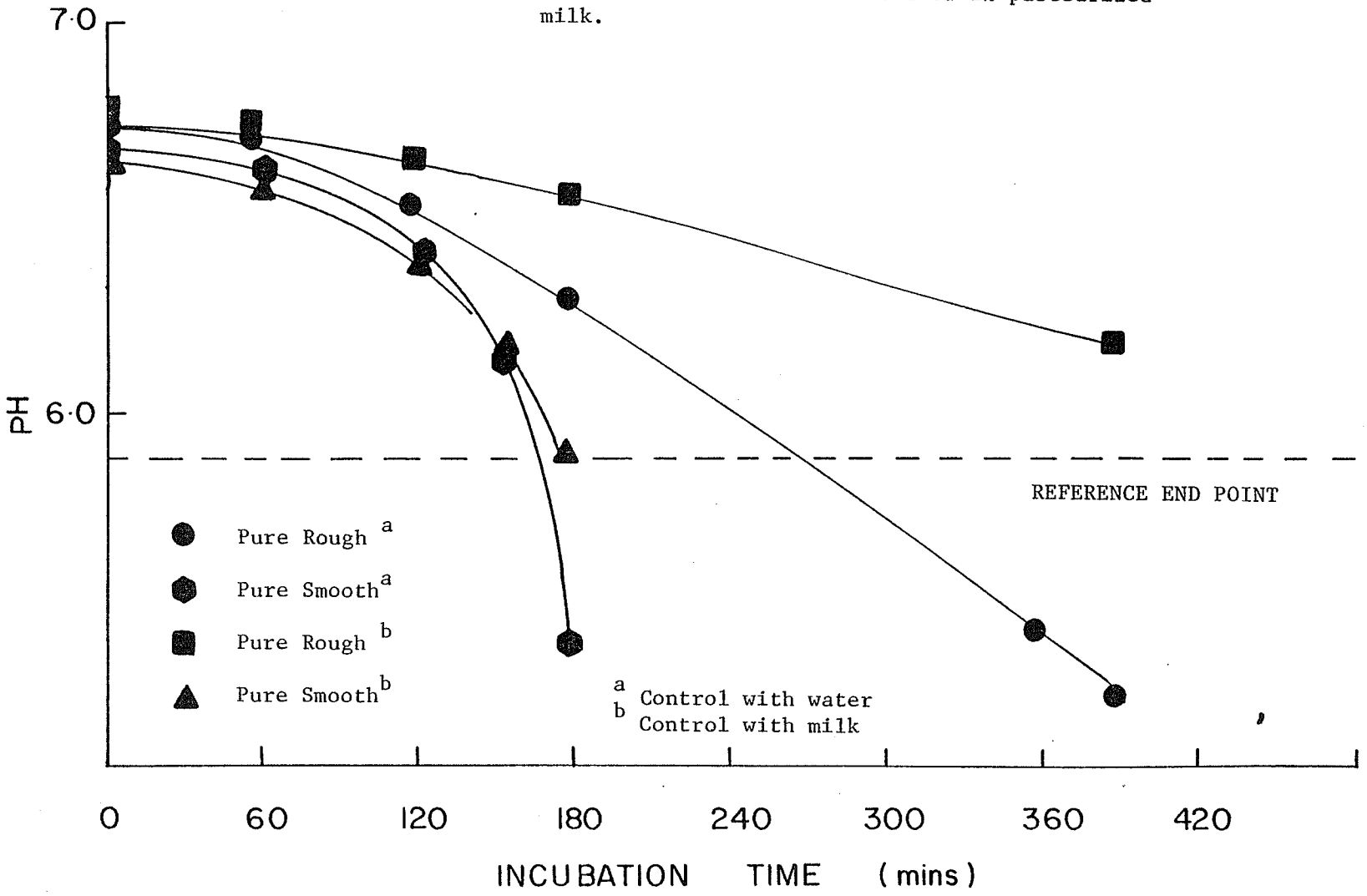


FIGURE 10. Changes in the pH of TGS medium inoculated with pure rough and pure smooth variants of Bacillus stearotheromphilus var. calidolactis devoid of any dissolved antibiotics. These controls contain either water or 2% pasteurized milk.



Simulated Delvotest® P tests containing water as the diluent showed a final pH of 5.4 and 5.5 for the rough and the smooth variants, respectively, while those simulated Delvotest® P tests containing milk as the diluent showed a final pH of 6.3 and 5.9 for the rough and the smooth variants, respectively.

(b) Disc Assay Method

The minimum inhibitory concentrations of penicillin, chloramphenicol, tetracycline and bacitracin for the pure rough variant, examined by the disc assay procedure were 0.004 Iu/ml, 1.0 μg/ml, 40.0 μg/ml and 35 Iu/ml, respectively. These antibiotics were freshly prepared using distilled water as the diluent. When the antibiotic concentrations of penicillin, chloramphenicol, tetracycline and bacitracin were prepared using 2% pasteurized milk, the minimum inhibitory concentration detected for the pure rough variant was 0.008 Iu/ml, 1.0 μg/ml, 30 μg/ml and 35 Iu/ml, respectively (Table 17).

Figure 11 illustrates the zone of inhibition produced by the pure rough variants of Bacillus stearothermophilus var. calidolactis seeded into TYG agar, using a disc assay method. The sterile disc was impregnated with 0.004 Iu/ml penicillin.

The pure smooth variants of Bacillus stearothermophilus var. calidolactis showed a minimum inhibitory concentration of 0.005 Iu/ml penicillin, 1.0 μg/ml chloramphenicol, 2.0 μg/ml tetracycline and 40.0 Iu/ml bacitracin. These antibiotics were freshly prepared and suitably diluted using distilled water. The minimum inhibitory concentrations detected for antibiotics dissolved in pasteurized 2% milk were 0.01 Iu/ml penicillin, 1.0 μg/ml chloramphenicol, 2.0 μg/ml tetracycline and 40 Iu/ml bacitracin (Table 17).

Figure 12 illustrates the zone of inhibition produced by the pure smooth variants of Bacillus stearothermophilus var. calidolactis seeded into TYG agar using a disc assay method. The sterile disc was impregnated with 0.005 Iu/ml penicillin.

TABLE 17. Minimum inhibitory concentrations of various antibiotics on rough and smooth variants of Bacillus stearothermophilus var. calidolactis, using a disc assay method.

Antibiotic / diluent		Distilled Water[a]		2% Milk[b]	
Antibiotic		MIC	Zone Diameter[c] (mm)	MIC	Zone Diameter[c] (mm)
Penicillin	(smooth)	0.005 Iu/ml	14.5	0.01 Iu/ml	15.0
	(rough)	0.004 Iu/ml	14.5	0.008 Iu/ml	14.0
Chloramphenicol	(smooth)	1.0 µg/ml	14.5	1.0 µg/ml	14.5
	(rough)	1.0 µg/ml	14.5	1.0 µg/ml	15.0
Tetracycline	(smooth)	2.0 µg/ml	14.0	2.0 µg/ml	15.0
	(rough)	40.0 µg/ml	14.5	30 µg/ml	14.5
Bacitracin	(smooth)	40 Iu/ml	14.0	40 Iu/ml	14.5
	(rough)	35 Iu/ml	14.0	35 Iu/ml	14.0

[a] All antibiotic stock solutions and appropriate dilutions were freshly prepared using distilled water.

[b] All antibiotic stock solutions were initially prepared using distilled water. The final appropriate dilutions were, however, prepared using 2% freshly pasteurized milk.

[c] All zone diameters were measured both vertically and horizontally to the nearest millimeter and represent the average of duplicate discs on triplicate plates.

FIGURE 11. Zone of inhibition on TYG agar media using a disc assay procedure seeded with pure rough variants of Bacillus stearothermophilus var. calidolactis. Disc impregnation - 0.004 Iu/ml penicillin. (Disc diameter - 12.70 mm)

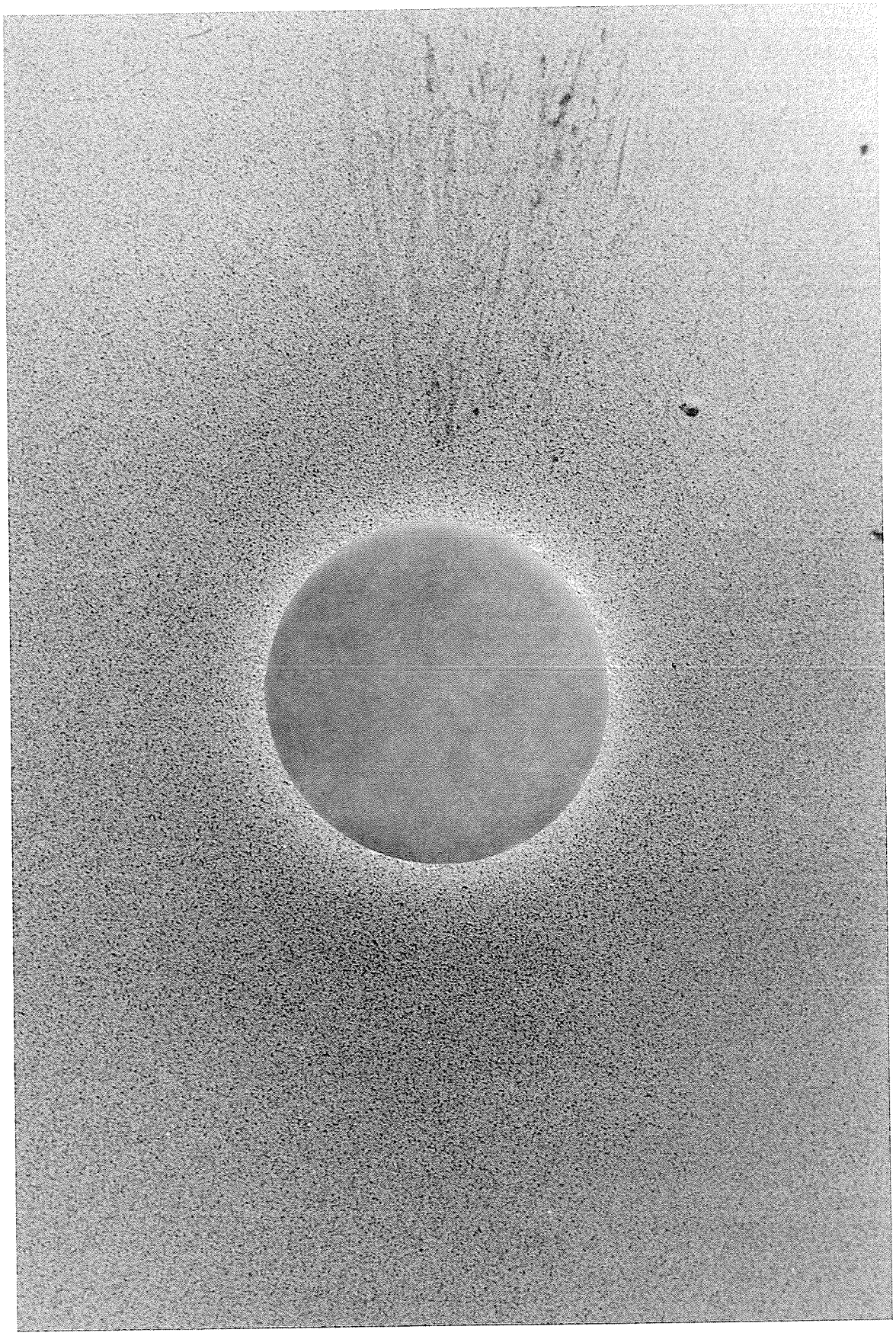
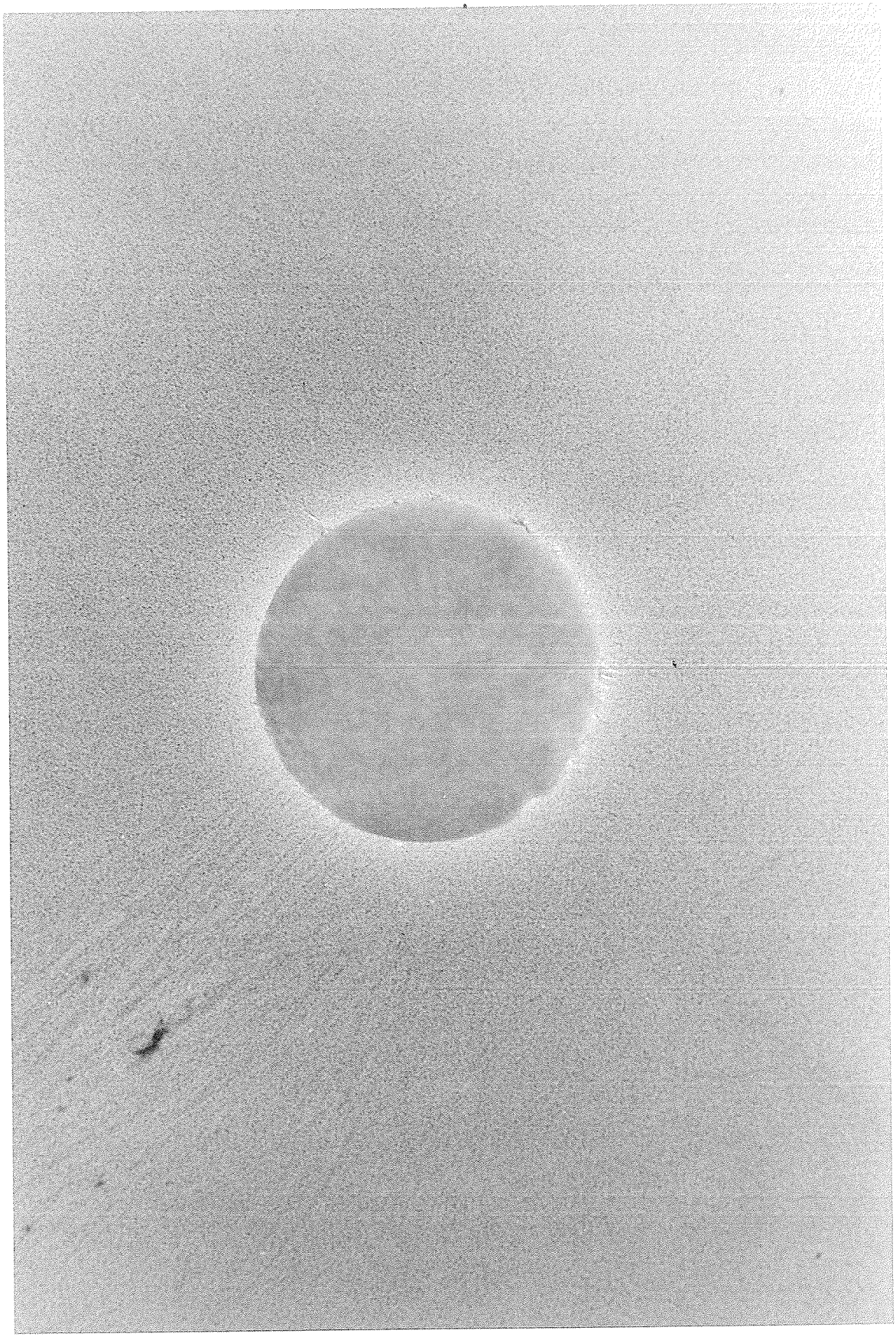


FIGURE 12. Zone of inhibition on TYG agar media using a disc assay procedure seeded with pure smooth variants of Bacillus stearothermophilus var. calidolactis. Disc impregnation - 0.005 Iu/ml penicillin. (Disc diameter - 12.70 mm)



Antibiotic Sensitivities of Mixed Variants of Bacillus stearothermophilus var. calidolactis

(a) Simulated Delvotest® P Method

TGS media inoculated with the following proportions of pure rough and pure smooth spores, 100%R:0%S, 80%R:20%S, 60%R:40%S and 40%R:60%S, all showed varying pH changes during the incubation period. The final pH attained after approximately 6¼ hours was $5.4^{+}_{-}1$. TGS media inoculated with 20%R:80%S gave pH changes characteristic of a pure smooth population (Figure 13).

(b) Disc Assay Method

Petri plates seeded with varying proportions of the mixed variants of Bacillus stearothermophilus var. calidolactis all showed a minimum inhibitory concentration of 0.005 Iu/ml penicillin, 1.0 μg/ml chloramphenicol, and 4 Iu/ml bacitracin (Tables 18, 19 and 20). Double zones of inhibitions were observed when 40 μg/ml tetracycline was used (Table 21).

Plates seeded with 100% of the pure rough variant gave a zone of inhibition which indicated the level of minimum inhibitory concentration of tetracycline (Figure 14 Zone A) while plates seeded with 100% pure smooth variant spores gave an average zone diameter of 27.0 mm (Figure 19 Zone B). Plates seeded with mixed spore variants gave two zones of inhibitions, each characteristic of the mixed proportion of the variants used (Figures 15,16,17 and 18).

Effects of Surface/Volume Ratio on the pH changes of TGS Medium Inoculated with Bacillus stearothermophilus var. calidolactis

Pyrex test tubes varying in their surface to volume ratio were used

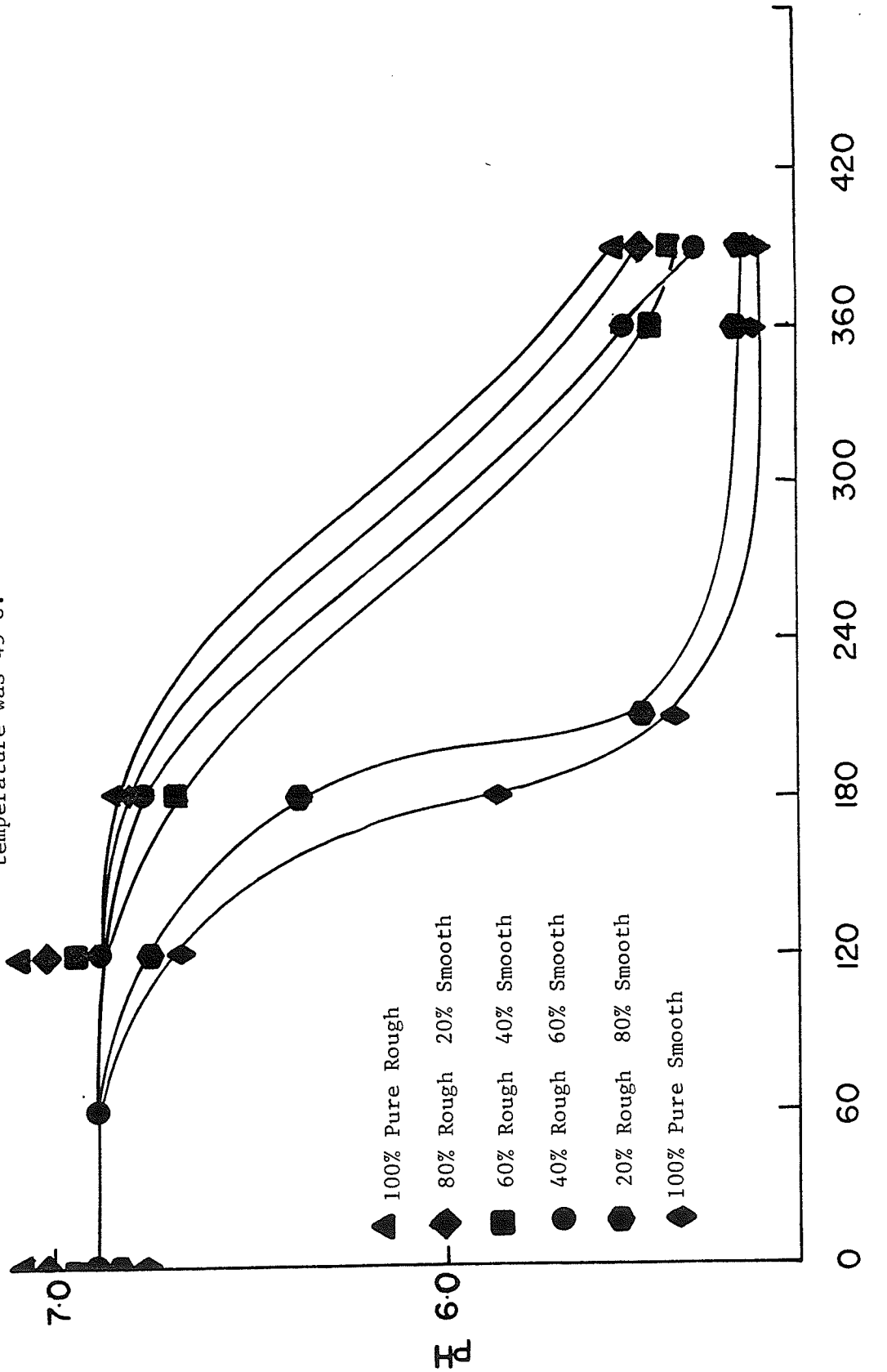


Figure 13. Changes in the pH of TGS medium inoculated with varying proportions of Bacillus stearothermophilus var. calidolactis, heat activated at 70°C for 15 minutes. Incubation temperature was 45°C.

TABLE 18. Effect of 0.005 Iu/ml penicillin on various mixed variant proportions of Bacillus stearothermophilus var. calidolactis, using a disc agar diffusion method.

Mixed Variant Proportions[b]		Zone Diameter (mm)[a]
0% R	100% S	14.0
20% R	80% S	14.5
40% R	60% S	14.5
60% R	40% S	14.0
80% R	20% S	14.0
100% R	0% S	14.0

[a] All zone diameters were measured both vertically and horizontally to the nearest millimeter and represent the average of duplicate discs on triplicate plates.

[b] All mixed proportions of the rough and smooth variants contain a final concentration of 2×10^6 activated spores/ml.

TABLE 19. Effect of 1.0 μg/ml chloramphenicol on various mixed variant proportions of Bacillus stearothermophilus var. calidolactis, using a disc agar diffusion method.

Mixed Variant Proportions[b]		Zone Diameter (mm)[a]
0% R	100% S	14.0
20% R	80% S	14.0
40% R	60% S	14.0
60% R	40% S	14.0
80% R	20% S	14.0
100% R	0% S	14.0

[a] All zone diameters were measured both vertically and horizontally to the nearest millimeter and represent the average of duplicate discs on triplicate plates.

[b] All mixed proportions of the rough and smooth variants contain a final concentration of 2×10^6 activated spores/ml.

TABLE 20. Effect of 4.0 Iu/ml bacitracin on various mixed variant proportions of Bacillus stearothermophilus var. calidolactis, using a disc agar diffusion method.

Mixed Variant Proportions[b]		Zone Diameter (mm)[a]
0% R	100% S	14.5
20% R	80% S	14.0
40% R	60% S	14.0
60% R	40% S	14.0
80% R	20% S	14.0
100% R	0% S	14.0

[a] All zone diameters were measured both vertically and horizontally to the nearest millimeter and represent the average of duplicate discs on triplicate plates.

[b] All mixed proportions of the rough and smooth variants contain a final concentration of 2×10^6 activated spores/ml.

TABLE 21. Effect of 40 μg/ml tetracycline on various mixed variant proportions of Bacillus stearothermophilus var. calidolactis, using a disc agar diffusion method.

Mixed Variant Proportions[b]		Zone Diameter (mm)[a]	
0% R	100% S	---	27.0
20% R	80% S	15.0	26.5[c]
40% R	60% S	16.0	27.0[c]
60% R	40% S	16.0	27.0[c]
80% R	20% S	16.5	27.0[c]
100% R	0% S	16.0	---

[a] All zone diameters were measured both vertically and horizontally to the nearest millimeter and represent the average of duplicate discs on triplicate plates.

[b] All mixed proportions of the rough and smooth variants contain a final concentration of 2×10^6 activated spores/ml.

[c] Two zones observed.

FIGURE 14. Zone of inhibition on TYG agar media using a disc assay procedure seeded with 100% rough variants of Bacillus stearothermophilus var. calidolactis (Zone A). Disc impregnation - 40 µg/ml tetracycline. (Disc diameter - 12.70 mm)

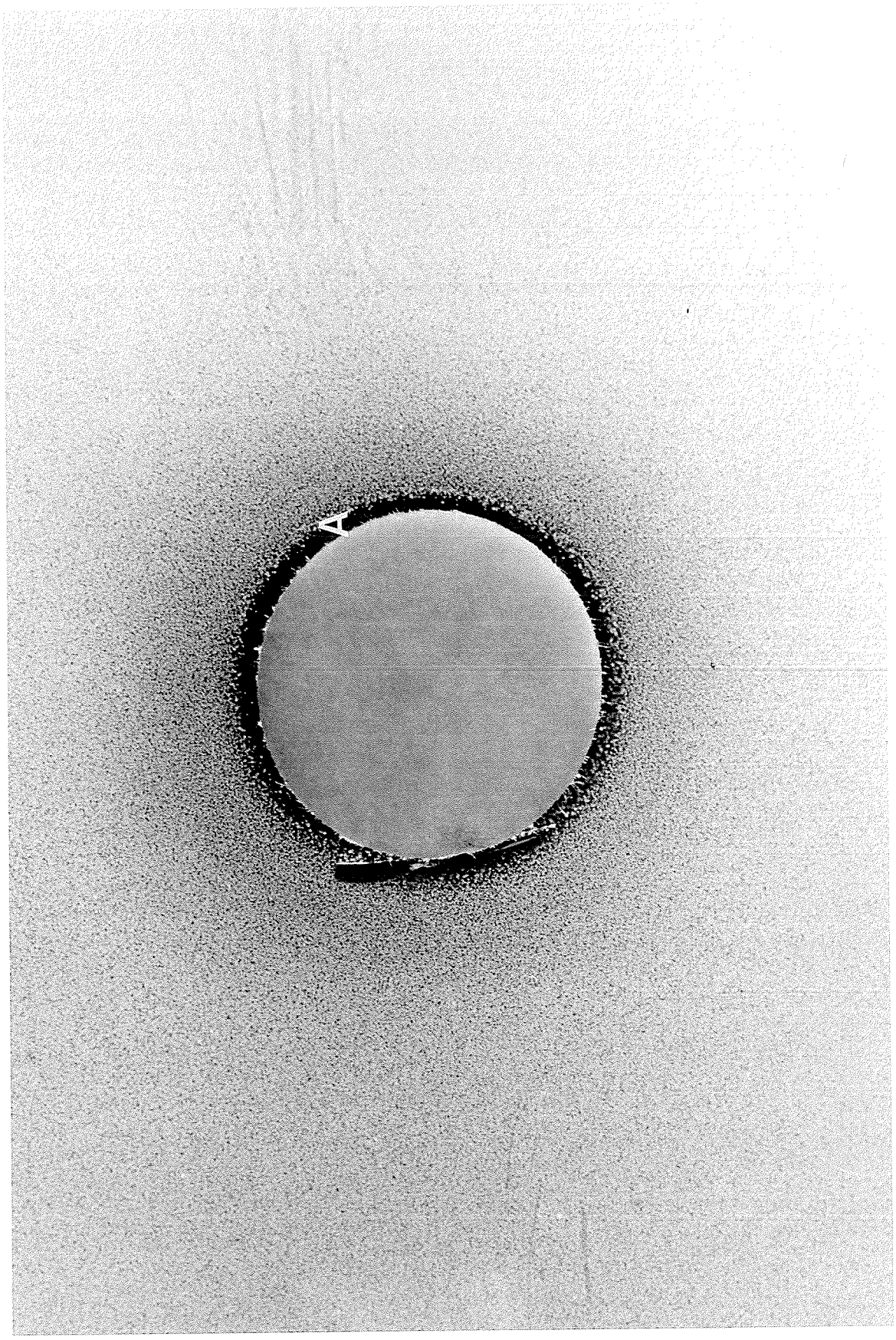

A

FIGURE 15. Zone of inhibition on TYG agar media using a disc assay procedure seeded with 80% rough (Zone A) and 20% smooth (Zone B) variants of Bacillus stearothermophilus var. calidolactis. Disc impregnation - 40 μg/ml tetracycline. (Disc diameter - 12.70 mm)

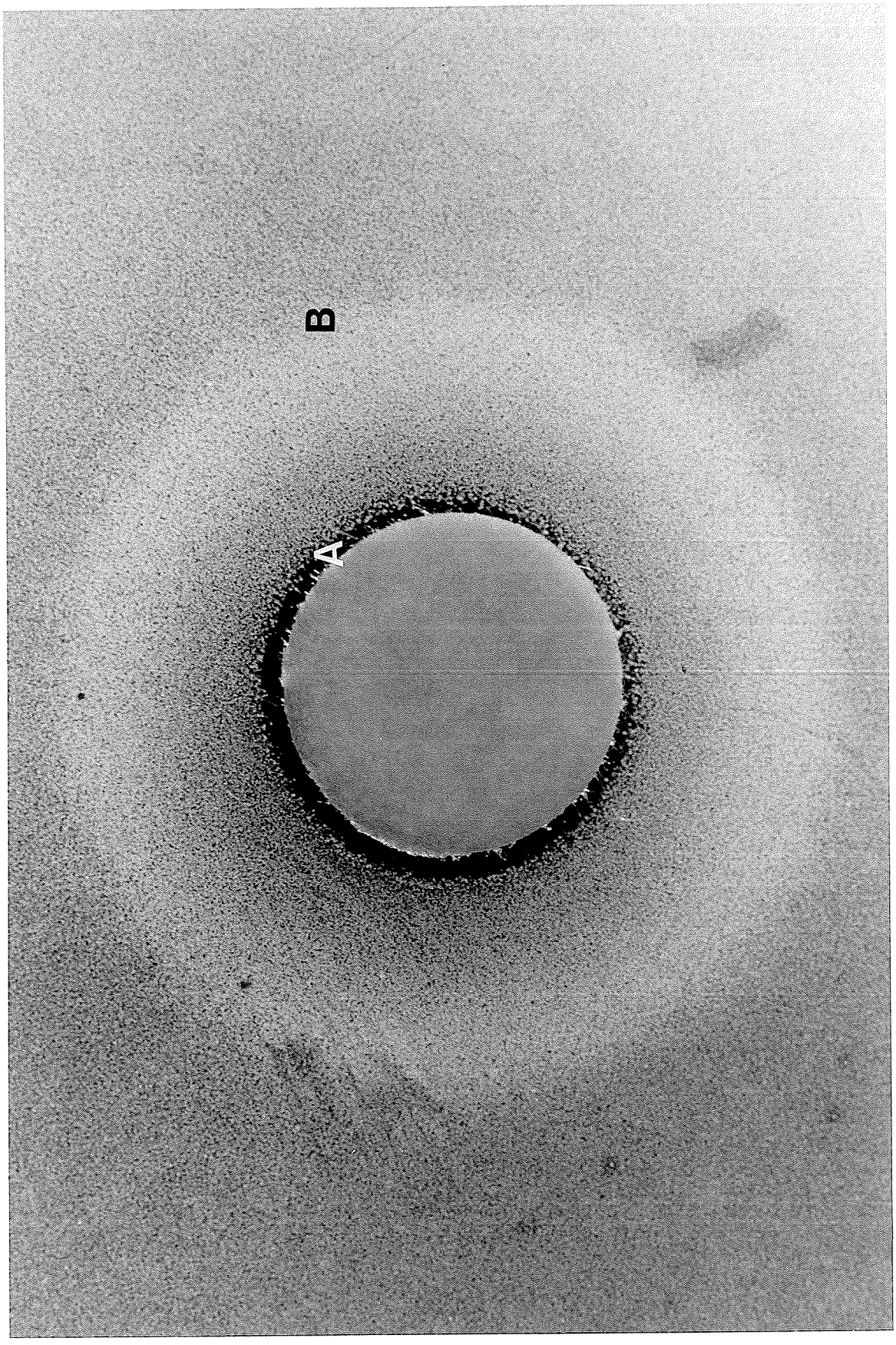

B
A

FIGURE 16. Zone of inhibition on TYG agar media using a disc assay procedure seeded with 60% rough (Zone A) and 40% smooth (Zone B) variants of Bacillus stearothermophilus var. calidolactis. Disc impregnation - 40 μg/ml tetracycline. (Disc diameter - 12.70 mm)

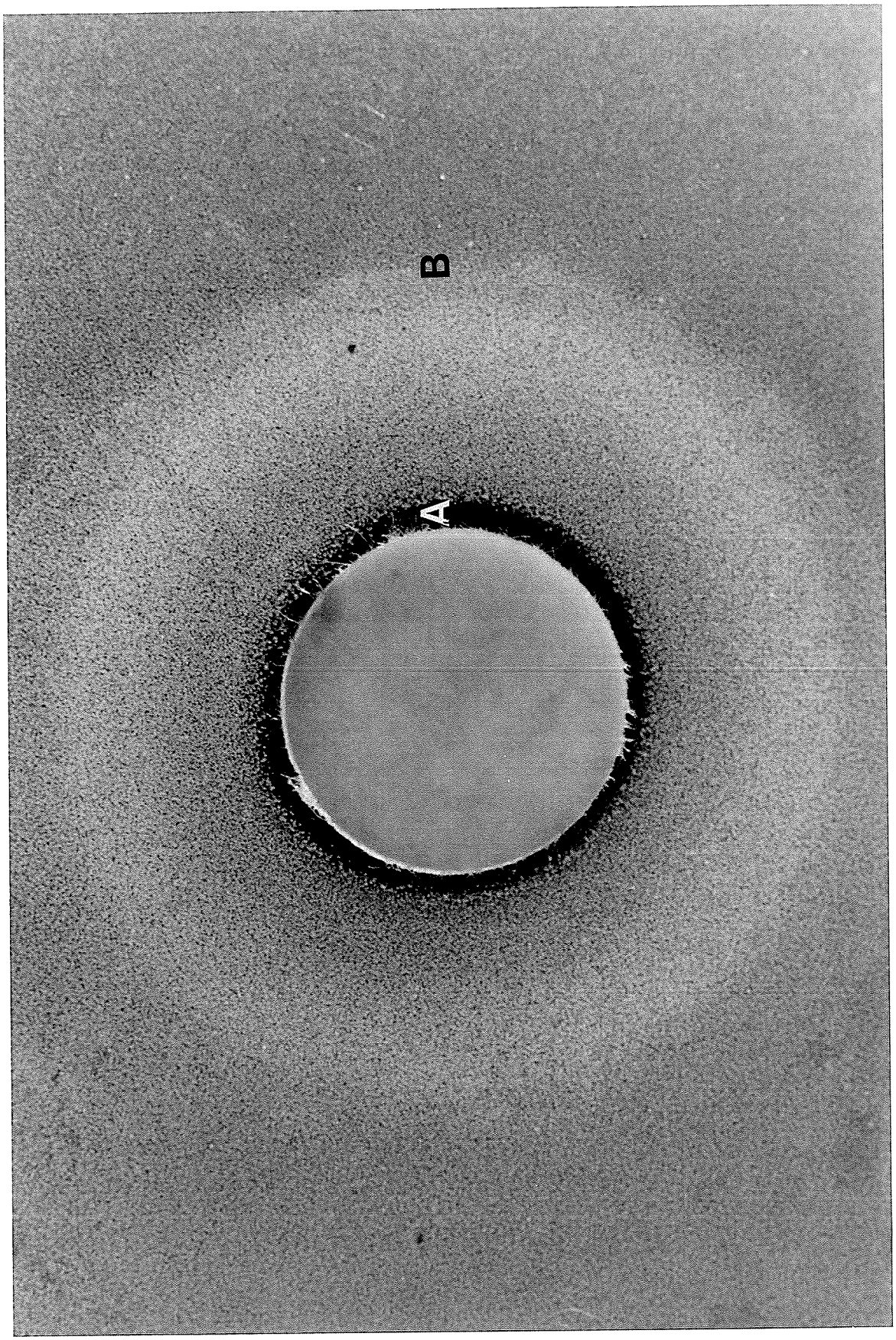

B
A

FIGURE 17. Zone of inhibition on TYG agar media using a disc assay procedure seeded with 40% rough (Zone A) and 60% smooth (Zone B) variants of Bacillus stearothermophilus var. calidolactis. Disc impregnation - 40 μg/ml tetracycline. (Disc diameter - 12.70 mm)

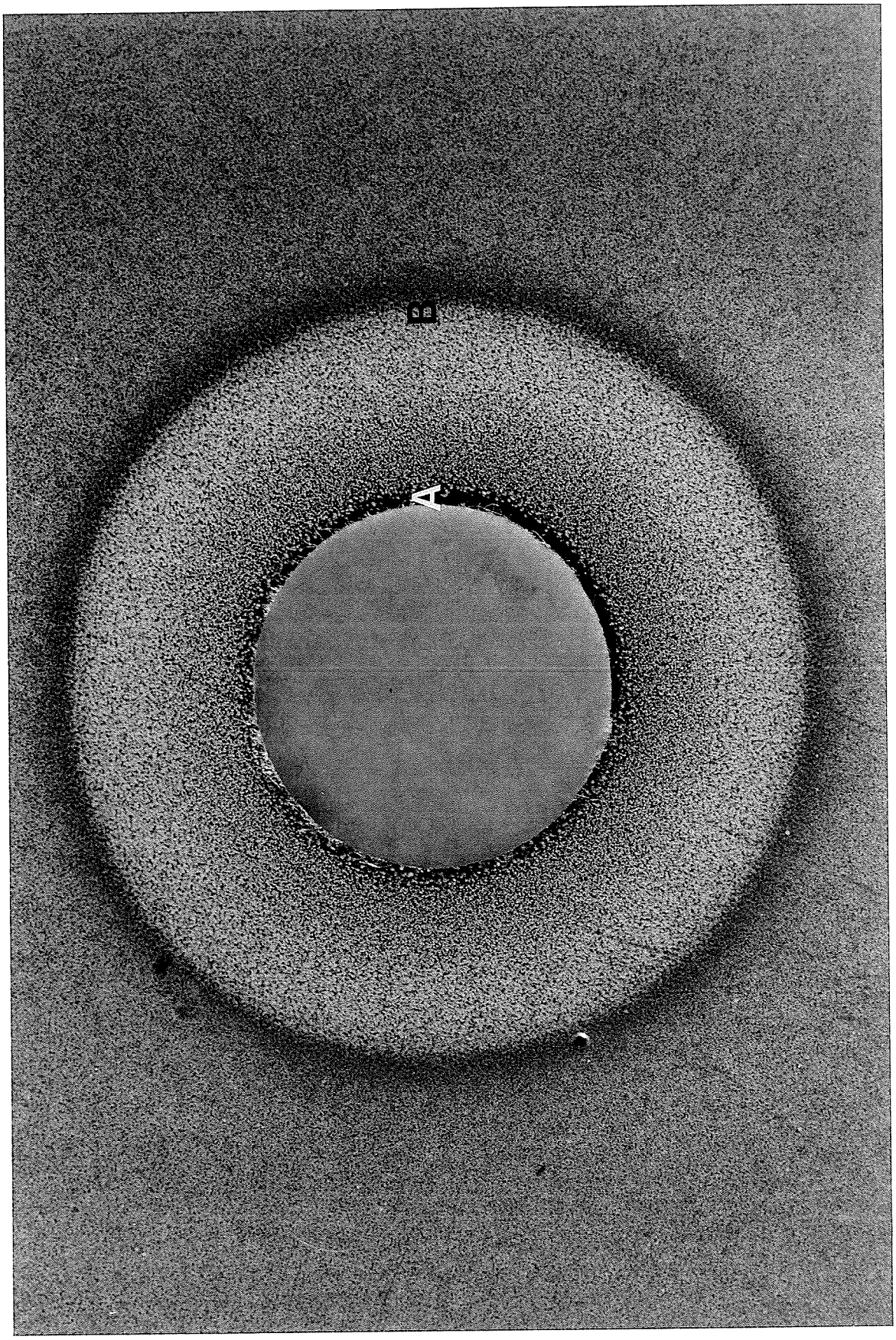

B
A

FIGURE 18. Zone of inhibition on TYG agar media using a disc assay procedure seeded with 20% Rough (Zone A) and 80% Smooth (Zone B) variants of Bacillus stearothermophilus var. calidolactis. Disc impregnation - 40 μg/ml tetracycline. (Disc diameter - 12.70 mm)

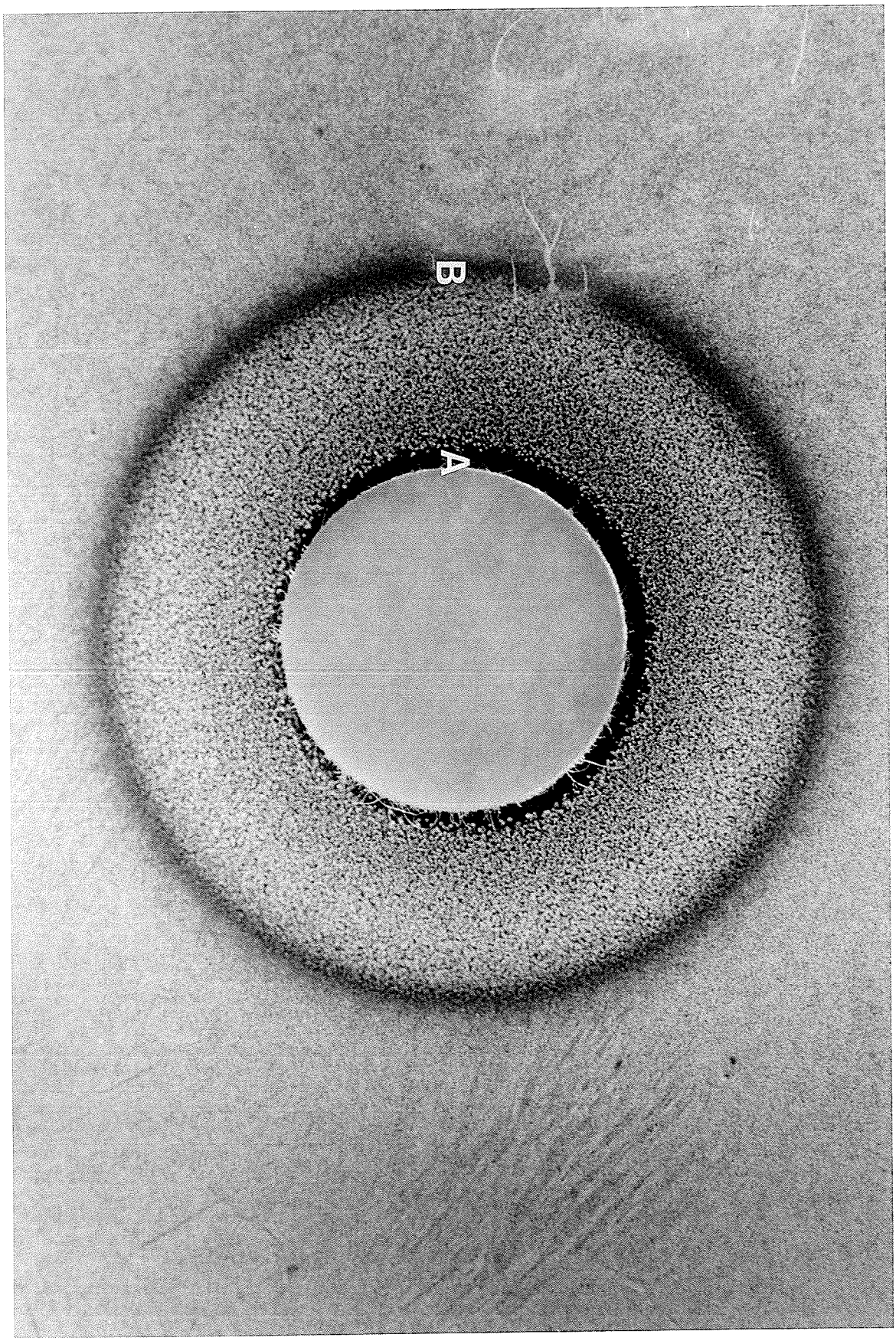
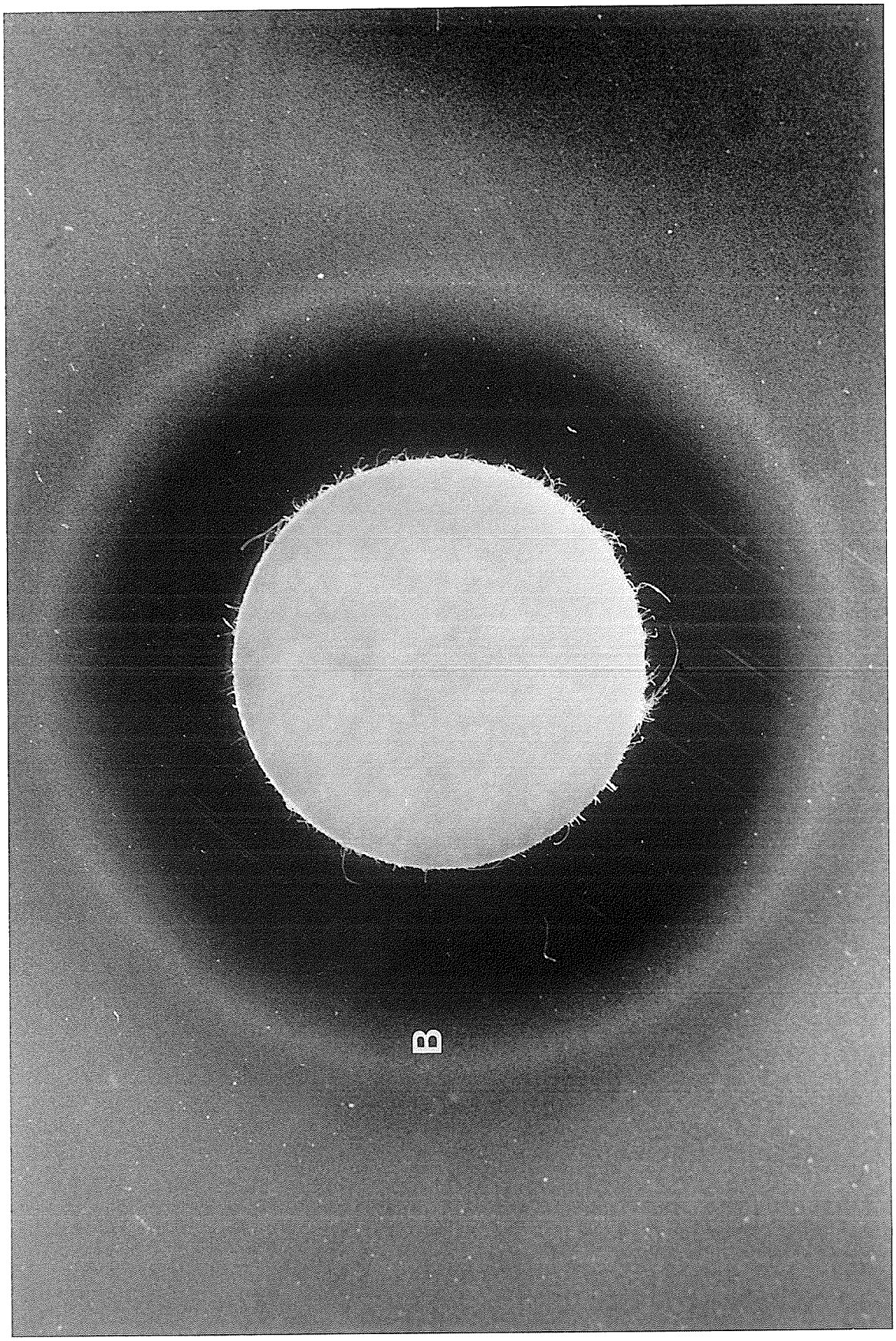

B
A

FIGURE 19. Zone of inhibition on TYG agar media using a disc assay procedure seeded with 100% Smooth (Zone B) variants of Bacillus stearothermophilus var. calidolactis. Disc impregnation - 40 μg/ml tetracycline. (Disc diameter - 12.70 mm)


B

to determine their effect on the pH changes of Bacillus stearothermophilus var. calidolactis inoculated into TGS medium. No variations were observed for the varying surface to volume ratios employed for the pure smooth (Figure 20) and the pure rough variants (Figure 21).

FIGURE 20. Changes in the pH of T.G.S. medium inoculated with pure smooth variants of Bacillus stearothermophilus var. calidolactis, using varying surface to volume ratios.

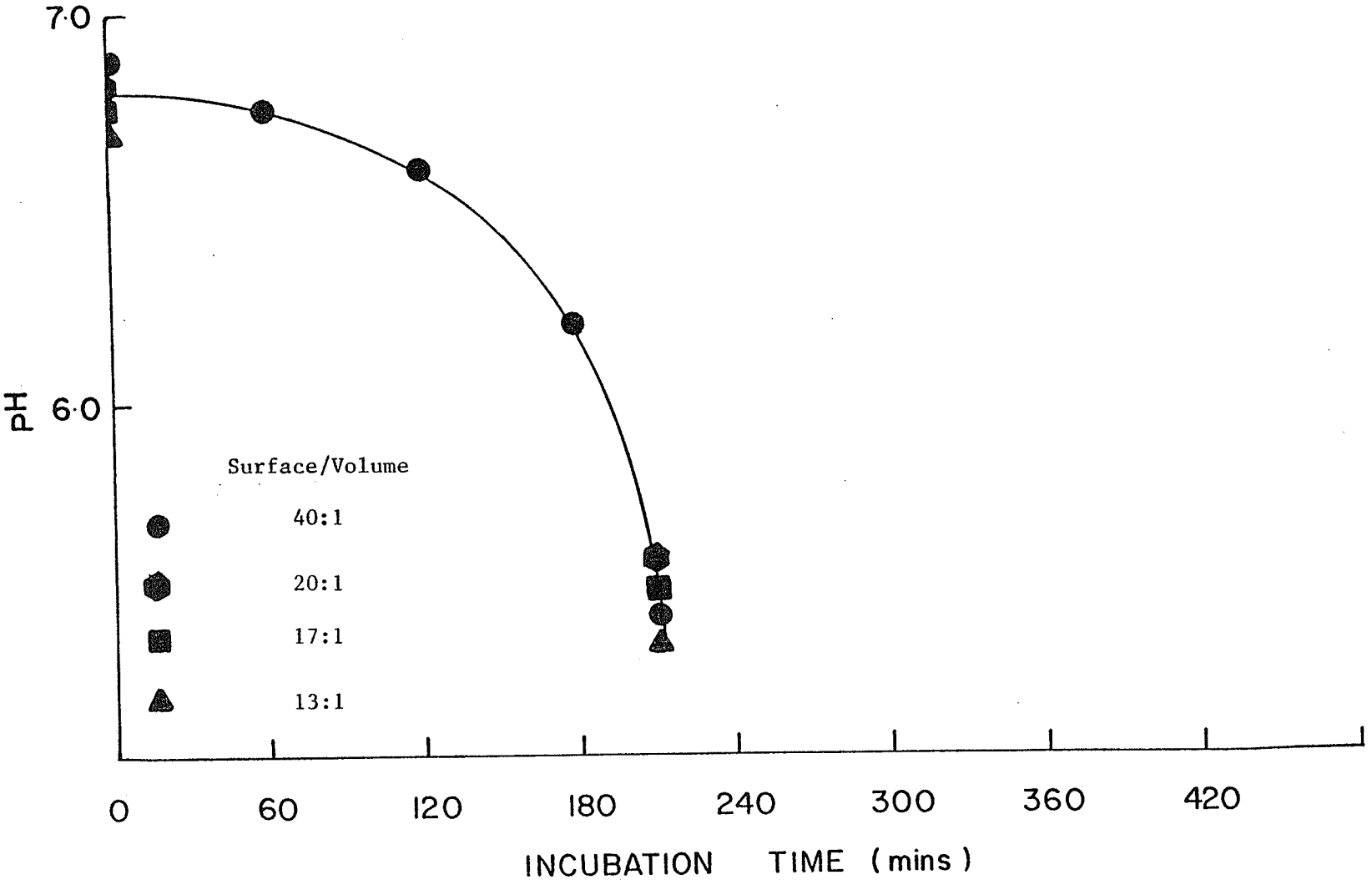
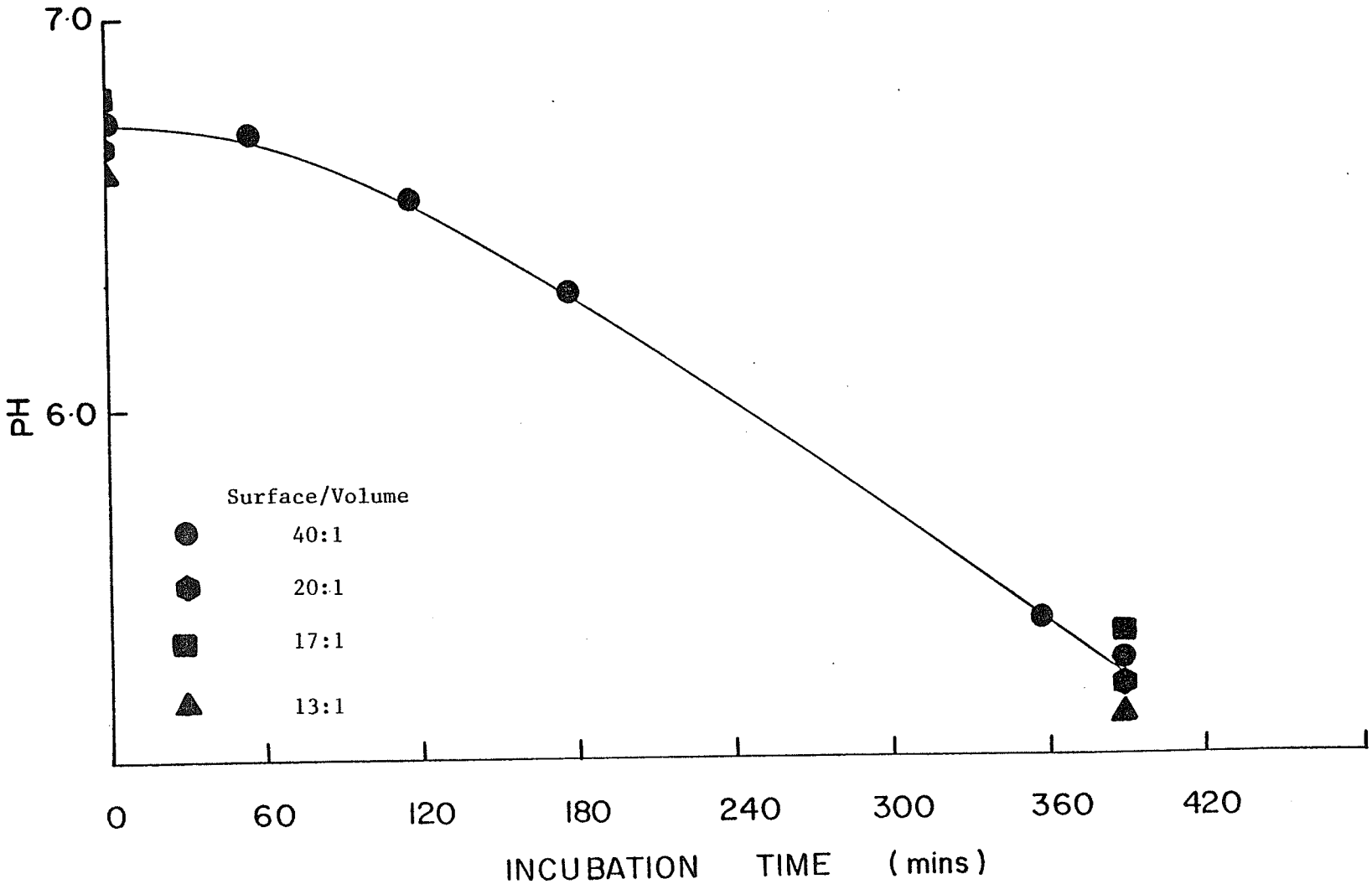


FIGURE 21. Changes in the pH of T.G.S. medium inoculated with pure rough variants of Bacillus stearothermophilus var. calidolactis, using varying surface to volume ratios.



DISCUSSION

The rough and smooth mixed spore variants (stock) of Bacillus stearothermophilus var. calidolactis were optimally heat activated in distilled water at 110°C for 15 minutes. The observed heating time did not include the initial come-up-time of 9 minutes. Although a heating time of 15 minutes was used, only minimal differences in spore stock activation were noted when the time of heat activation was either decreased or increased, as evidenced by the final pH of the TGS media ($4.81 \pm .20$) at 180 minutes of incubation. Finley and Fields (1962) working with two strains of Bacillus stearothermophilus NCA 1518, also showed that their spore suspensions could be heat activated at 110°C for 9 - 15 minutes, based on viable plate count data. These researchers suggested, however, that the degree of spore activation was strain and suspension dependent.

The criterion for optimum heat activation used in this investigation was not, however, based on viable plate count data; optimal heat activation was determined as a function of pH response using simulated Delvotest® P media. The rate of pH change as well as the final pH attained in the TGS medium provided the basis for optimum heat activation.

Time-temperature conditions established for optimal activation of the spore stock were not evaluated against viable plate count data, therefore the optimal heat activation conditions described for the spore stock may not provide optimal conditions for viable plate count evaluations. The assumption that the rate of acid production, within a specified period

of time, could be directly related to the number of viable vegetative cells resulting from spore activation, is not, however, totally correct.

Fields (1966) working with rough and smooth variants of Bacillus stearothermophilus NCA 1518 has already shown distinct differences in the generation times, and acid producing capability of these variants. In addition, Johnson et al. (1966), Shafia (1966), Yule and Barridge (1976), and Sharp et al. (1979), have reported the presence of bacteriocinogenic strains of Bacillus stearothermophilus which were specific for other strains of Bacillus stearothermophilus. The presence of thermocins, possibly variant antagonistic, could influence the growth of one variant when grown in a mixed population.

Hill and Fields (1967a), also showed that a mixed population of the two variants always became predominantly smooth within a few hours of incubation. Although the relative proportions of pure rough and pure smooth variants in the spore stock were not evaluated, repeated isolation of the spore stock from Delvotest® P ampoules in this investigation yielded the same optimum heat activation parameters. These parameters did not change during the course of this investigation when the spore stock was stored at 4°C.

Heat activation at 90°C and 100°C, regardless of the activation time employed, was sub-optimal for spore stock suspensions. Increasing the time of activation from 1 to 60 minutes at these temperatures did, however, increase the heat activation effect as evidenced by a progressive decrease in the final pH. No deactivation was observed when the stock spores were subjected to these temperatures.

The pure smooth variants of Bacillus stearothermophilus var. calidolactis were optimally heat activated at 70°C for 15 minutes. The observed heating

time did not include the initial come-up-time of 1.5 minutes. Minimal differences in the activation of the pure smooth spores were again noted when the time of heat activation was varied from 1 to 60 minutes (pH 5.83$\pm$.02). It was observed in these studies that the temperature of incubation influenced the acid production of the activated pure smooth spore. Incubation at 45^{o}C was found to be optimal for acid production; incubation temperatures of 55^{o}C and 66^{o}C progressively decreased the rate of acid production for the activated pure smooth spores. Incubation at 66^{o}C showed no pH change within the incubation period, despite heat activation. Heat activation at 80^{o}, 90^{o}, 100^{o} and 110^{o}C was sub-optimal for the pure smooth spores.In all cases, however, an incubation temperature of 45^{o}C was found to be optimal.

Optimal heat activation conditions for the pure rough variant were not established in this investigation. Heat activation temperatures of 70^{o}, 80^{o}, 90^{o}, 100^{o} and 110^{o}C using heating times of 1 to 60 minutes, failed to yield significant pH responses in the TGS medium. Incubation temperatures of 45^{o}, 55^{o} and 66^{o}C were used in combination with the various heat activation temperature-time conditions.

Since it is known that at least three main sequential processes are responsible for the changing of a dormant bacterial spore into a vegetative cell, and that these processes are fundamentally different from each other and often exacting in nutritional requirements (Keynan & Evanchik, 1968), there remains the possibility that the heat activation conditions for the pure rough spore could have been established, but that germination and subsequent outgrowth did not occur, or occurred very slowly.

Microscopic examinations were not performed, however, to verify whether activation occured within the heated pure rough populations. An

activation temperature of 70°C for 15 minutes was arbitrarily chosen for the heat activation of the rough variant. The time of incubation was extended to 6¼ hours in order to achieve the desired pH change (5.4).

Extending the incubation time to 6 - 6¼ hours could perhaps reflect a difference in the growth rate of the individual variants. Fields (1966), Hill and Fields (1967a), working with Bacillus stearothermophilus NCA 1518, showed that the pure smooth variant grew faster from the spore inoculum than the rough variant, and also produced more acid than the rough variant type. Oxygen tension was shown to affect acid production of the rough variant.

Radiorespirometric studies performed by Hill et al. (1967), also showed differences in the pathway of glucose metabolism between the pure rough and pure smooth variants of Bacillus stearothermophilus NCA 1518, which could explain the differences in acid production between the spore variants.

Once the heat activation conditions were established, or arbitrarily assigned, the antibiotic sensitivities of the heat activated spore variants were evaluated with penicillin, chloramphenicol, tetracycline and bacitracin, using a simulated Delvotest® P and disc assay method.

Using the simulated Delvotest® P method and water as the antibiotic diluent, the results indicated that the rough variant was approximately ten times more sensitive to penicillin as compared to the smooth variant, as shown by their minimum inhibitory concentrations, 0.01 and 0.1 Iu/ml, respectively. The stock spore suspension (mixed rough and smooth) showed similar minimum inhibitory concentration of penicillin to the commercially available Delvotest® P ampoule (0.004 Iu/ml).

Although a slight difference was observed in the minimum inhibitory concentration of tetracycline between the pure smooth and the pure rough variants, 0.02 and 0.05 μg/ml respectively, these concentrations were $3\frac{1}{2}$ to 4 times more sensitive than the sensitivities reported by Gist Brocades N.V. The minimum inhibitory concentration of chloramphenicol for both the pure rough and pure smooth was the same (1 μg/ml). This minimum inhibitory concentration was none-the-less 8 to 10 times more sensitive than the Delvotest® P method as reported by Gist Brocades N.V.

No differences were observed in the minimum inhibitory concentration of bacitracin when tested against pure rough and pure smooth variants (0.40 Iu/ml). This minimum inhibitory concentration was, however, 4 to 6 times less sensitive than that reported by Gist Brocades N.V.

When 2% pasteurized milk was used as the diluent in a simulated Delvotest® P method, the minimum inhibitory concentration of these antibiotics could not be established using the criteria already established for this test i.e. attainment of a reference end point in ($2\frac{1}{2}$ to 3) and (6 to $6\frac{1}{4}$) hours for the pure smooth and the pure rough variants, respectively, because of a noticeable buffering effect exhibited by the milk. This buffering effect retarded the pH changes exhibited by both the pure rough and the pure smooth variants, when incubated with the minimum inhibitory concentration of those antibiotics established using water as the diluent. Therefore, antibiotics dissolved in milk gave a buffering effect which was apparent when compared to their counterparts i.e. antibiotics dissolved in water.

Using the disc agar diffusion test, the sensitivity of both the pure rough and pure smooth variants tested against bacitracin was 35 Iu/ml and 40 Iu/ml, respectively. The minimum inhibitory concentration in this test procedure was, however, 100 times less sensitive as compared to the

simulated Delvotest® P method.

The minimum inhibitory concentration of the various antibiotics on the pure rough and pure smooth variants of Bacillus stearothermophilus using a disc assay method showed some differences depending upon the nature of the antibiotic diluent. The greatest differences in minimum inhibitory concentration occurred with penicillin. When water was used as the antibiotic diluent, the minimum inhibitory concentration for the smooth and rough variants was 0.005 and 0.004 Iu/ml, respectively. These minimum inhibitory concentrations both showed inhibition zones of 14.5 mm. The minimum inhibitory concentration of the pure smooth and the pure rough variants, when tested against penicillin dissolved in 2% pasteurized milk, showed values of 0.01 and 0.008 Iu/ml, respectively. These minimum inhibitory concentrations showed zones of inhibition corresponding to 15.0 and 14.5 mm, respectively. Differences in sensitivity of the variants due to the nature of the antibiotic diluent could largely reflect the difficulties in discerning the minimum persceptible zones of inhibition, exaggerated by the highly sensitive nature of Bacillus stearothermophilus var. calidolactis to penicillin.

Busta and Speck (1965), Busta (1966), Ashton and Busta (1968) and Mayou and Jezeski (1977) showed that milk contains an inhibitor (possibly the casein fraction in milk) which prevents germinaton and/or outgrowth of Bacillus stearothermophilus spores. It is possible that this explanation may apply to the results obtained with penicillin, but not with tetracycline, because of the highly sensitive nature of the test organism.

The differences in antibiotic sensitivity between pure rough and pure smooth variants, which in turn reflect the minimal detectable concentration levels of antibiotics, could perhaps delineate the various differences already established in the ultrastructure, metabolism and biochemical

properties of the spore variants. Conversely, the lack of differences in antibiotic sensitivities may be due to similarities in ultrastructure, metabolism and biochemical properties of the spore variants. These differences and similarities in the antibiotic sensitivities of the variant populations, could be explained by the mode of action of the various antibiotics and in particular, their selective target sites.

Differences in the antibiotic sensitivities between the pure variant lines and mixed stock variant can be partially explained by non-associative and associative growth, respectively. Hill and Fields (1967a) demonstrated differences and similarities in the generation time between the pure lines and the mixed spore variants. These similarities and differences were dependant upon the dominant variant in the mixture.

In addition, it is quite conceivable that a mixed population of rough and smooth spore variants could undergo transitional mutation. Intermediate spore variants, representing a transition from rough to smooth or vice versa, could exhibit antibiotic sensitivities which are different from either of the pure variant types. This phenomenon was also shown to exist when a mixed population of 50% pure rough and 50% pure smooth variants was heat activated at 110^{o}C for 15 minutes. The resultant heat activated mixed spores failed to give a pH pattern in Delvotest® P medium characteristic of the spore stock (rough and smooth mixed) variants of Bacillus stearothermophilus. Therefore, in order to evaluate mixed spore variants grown associatively, it is imperative that the composition of the initial spore inoculum and growth conditions be the same and/or synchronous growth be maintained. In this way, the number of rough, smooth and transitional intermediate variants will be maintained.

Changes in the pH of simulated Delvotest® P media, inoculated with varying proportions of mixed variants of Bacillus stearothermophilus var. calidolactis containing no antibiotics, exhibited pH trends similar to either the pure rough or the pure smooth variants.

Spore populations of 100% pure rough, 80% rough:20% smooth, 60% rough: 40% smooth and 40% rough:60% smooth exhibited similar pH patterns while mixed spore populations of 20% rough:80% smooth, and 100% pure smooth also exhibited similar pH patterns. Theoretically, the mixed populations containing 40% rough:60% smooth should have exhibited pH changes similar to 20% rough and 80% smooth due to the predominant, faster growing smooth variant. It is assumed that the pH change exhibited is reflective of the dominant variant of the mixed population. It is not known, however, if these pH changes also reflect innate characteristics of transitional variants, if indeed transitional variants were produced during the incubation period.

The simulated Delvotest® P method was not used, however, to evaluate the antibiotic sensitivities of the various proportions of mixed variants, since the results obtained with the controls indicated a pH pattern largely dependant upon the dominant variant in the mixture. The pH trends of these various proportions of mixed variants would be expected to follow the same pH pattern of the least sensitive variant for each antibiotic tested. The differences in antibiotic sensitivities between the varying proportions of the mixed variants were tested against tetracycline using a disc agar diffusion method.

These various proportions of mixed variants when evaluated against 40 μg/ml tetracycline showed two zones of inhibition. These zones of inhibition were characteristic of the more sensitive variant present in

the mixture. Increasing the proportion of smooth variants in the mixture subsequently led to a "phasing in" of a zone of inhibition, characteristic of a pure smooth population.

No differences in pH changes were observed when varying surface to volume ratios of TGS media were inoculated with pure smooth or pure rough variants of Bacillus stearothermophilus. Long and Williams (1959) demonstrated in their studies the importance of varying the surface exposure of broth grown cultures of Bacillus stearothermophilus to atmospheric oxygen. These researchers showed that when strict thermophilic strains of Bacillus stearothermophilus were grown at sub-optimal temperatures, increased surface to volume ratios increased the growth of these cultures. The lack of significant differences in pH change using the various surface to volume ratios would tend to indicate the negligible effects of any real differences in pH observed between simulated Delvotest® and commercially available Delvotest® P ampoules, due to physical constraints.

SUMMARY AND CONCLUSION

Pure lines of rough and smooth variants of Bacillus stearothermophilus var. calidolactis, subcultured from commercially available Delvotest® P ampoules, were heat activated and subsequently evaluated for their antibiotic sensitivity against penicillin, chloramphenicol, tetracycline and bacitracin using a simulated Delvotest® P and disc agar diffusion method. The following results were obtained in this investigation:

1. The stock spore culture (rough and smooth mixed) were optimally heat activated at 110°C for 15 minutes.
2. The pure smooth spore variants were optimally heat activated at 70°C for 15 minutes.
3. The pure rough spore variants were heat activated at 70°C for 15 minutes. This time-temperature relationship was arbitrarily assigned to the variant, since optimum heat activation conditions were not established.
4. The minimum detectable concentration of penicillin detected by the stock spore cultures using a simulated Delvotest® P method was 0.004 Iu/ml.
5. The minimal detectable concentration of penicillin, chloramphenicol, tetracycline and bacitracin dissolved in water, detected by pure smooth variants using a simulated Delvotest® P method, was 0.10 Iu/ml, 1.0 µg/ml, 0.02 µg/ml and 0.40 Iu/ml, respectively.
6. The minimal detectable concentration of penicillin, chloramphenicol, tetracycline and bacitracin dissolved in water, detected by pure rough variants using a simulated Delvotest® P method, was 0.01 Iu/ml,

1.0 μg/ml, 0.05 μg/ml and 0.40 Iu/ml, respectively.

7. The minimal detectable concentration of penicillin, chloramphenicol, tetracycline and bacitracin dissolved in water, detected by pure smooth variants using an agar diffusion method was 0.005 Iu/ml, 1.0 μg/ml, 2.0 μg/ml and 40 Iu/ml, respectively.
8. The minimal detectable concentration of penicillin, chloramphenicol, tetracycline and bacitracin dissolved in water, detected by pure rough variants using an agar diffusion method was 0.004 Iu/ml, 1.0 μg/ml, 40 μg/ml and 35 Iu/ml, respectively.
9. The minimal detectable concentration of penicillin, chloramphenicol, tetracycline and bacitracin dissolved in 2% pasteurized milk, detected by pure smooth variants using an agar diffusion method was 0.01 Iu/ml, 1.0 μg/ml, 2.0 μg/ml and 40 Iu/ml, respectively.
10. The minimal detectable concentration of pencillin, chloramphenicol, tetracycline and bacitracin dissolved in 2% pasteurized milk, detected by pure rough variants using an agar diffusion method was 0.008 Iu/ml, 1.0 μg/ml, 30 μg/ml and 35 Iu/ml, respectively.
11. Varying proportions of mixed variants viz. 100% Smooth, 80% Smooth: 20% Rough, 60% Smooth:40% Rough, 40% Smooth:60% Rough, 20% Smooth: 80% Rough, and 100% Rough, when tested against 40 μg/ml tetracycline gave either one or two zones of inhibition depending on the dominant spore variant present.
12. The surface:volume ratios showed no effects on the performance of the simulated Delvotest® P method.

The differences in antibiotic sensitivities between the pure rough, pure smooth and mixed variants of Bacillus stearothermophilus var.

calidolactis have suggested several possible areas of concern in the performance of the commercially available Delvotest® P method. Rough and smooth spore variants, if indeed present, in commercially available Delvotest® P ampoules could affect the sensitivity of the test in one of two ways:

(a) dependent on the dominant variant present, the time specification for the test may be altered, due to differences in generation time of the variants, and

(b) dependent on the presence and/or proportions of both rough and smooth variants, the minimal detectable concentration could be altered to reflect the more sensitive variant type.

These effects could be further aggravated if stable or transitional mutations occured during the performance of the test and/or during any pre-incubation procedures.

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