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THE FERMENTATION PRODUCTION OF SPORES OF *BACILLUS STEAROTHERMOPHILUS* VAR. *CALIDOLACTIS*

by

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ABSTRACT

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The Fermentation Production of Spores of Bacillus stearothermophilus var. calidolactis.

Major Professor: Dr. Gregory Blank.

Rough (Rh) and smooth (Sm) spore variants of Bacillus stearothermophilus var. calidolactis were produced via submerged aerobic fermentation at 45 and/or 55°C in a nutrient broth, phosphate-based liquid medium. The growth of the Sm variant at 55°C was shown to increase with aeration. The growth of the Rh variant at similar temperatures indicated only slight variation in growth with increased aeration. Peak lysozyme spore counts were observed to occur mainly at 18 hours of fermentation coinciding with peak vegetative growth.

Thermocin, produced by the Rh variant, at 0.55-0.60 A.u./ml, showed partial to complete inhibition of cellular growth and sporogenesis of the Sm variant. Fermentation cultures containing mixed variants, Rh:Sm(%); 60:40; 40:60;

20:80; were shown to progressively decrease in Rh variant population during the course of growth with a concomitant increase in the Sm variant population. Inocula containing initial Rh:Sm(%), 80:20, were shown to completely revert to the Rh variant form within 30 minutes. Inocula containing Rh:Sm(%), 20:80, showed the obverse effects, all recoverable counts within 30 minutes were of the Sm variant form. Aeration showed little effect in the maintenance of dominance. Peak spore production in cultures containing mixed variants was shown to coincide with peak total viable cell counts regardless of aeration. Thermocin production was noted in those fermentations initially containing 80, 60 and 40% Rh. The thermocin titer was shown to occur as early as 30 minutes and it persisted for 6 hours without any increase.

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INTRODUCTION

Differences in antibiotic sensitivity between rough and smooth variants of Bacillus stearothermophilus var. calodolactis have created some potential concerns regarding the validity and sensitivity of the Delvotest P (Lee Wing and Blank, 1981). In addition, recent investigations by Hii and Blank (1981), have indicated the presence of a bacteriocin-like agent, thermocin, produced by the rough variant, being highly antagonistic towards the smooth variant.

Since the commercial production of Bacillus stearothermophilus var. calidolactis spores is produced commercially by an aerobic submerged fermentation process, conditions governing sporogenesis in these variants were investigated aerobically. In particular, fermentation parameters such as aeration and temperature, were investigated as critical factors governing the growth and/or dominance of these variants as well as the effect of thermocin on pure and mixed smooth variant cultures. Both temperature and aeration have been reported previously (Hill and Fields, 1967; Kauppinen, 1969), to have a profound influence on the growth and/or dominance of these variants.

REVIEW OF LITERATURE

(1) Sporulation

It has been fairly well established that sporulation is a normal metabolic phenomenon among sporogenic bacteria and that the processes involved are quite different from those associated with vegetative growth (Halvorson, 1961). Although a rather large amount of work has been reported concerning this phenomenon in recent years, knowledge regarding the nature of sporogenesis, particularly the physiological and biochemical changes occurring during the period when multiplication ceases and sporulation begins, is still somewhat limited. This may be partially attributed to the use of bacterial cultures containing heterogeneous populations in which the processes of a particular phase of growth or sporulation are complicated by the presence of cells in other various phases of growth. Further complications have arisen from the many reported differences such as morphology, metabolism and chemical composition which exist among species and strains (Halvorson, 1961).

(a) Induction of sporogenesis

Bacterial sporogenesis is basically a specialized form of adaptation of a cell to accessible nutrients in an

environment. Following the logarithmic growth phase, spore formation normally occurs; the generation time increases because of a limited amount of nutrients. Spore formation may also be induced in the primary cell which forms after germination and spore outgrowth (Gould, 1969). After a decrease in nutrient level and ceasation of growth, several changes occur in the bacterium which has undergone sporogenesis. Upon rearrangement, a cell division requiring a long generation time occurs initiated by the forespore septum. These sequential processes will eventually lead to the production of mature spores. During the period of forespore septum formation, the cell (sporangium) achieves the ability to develop into either a vegetative cell or a spore. Lack of nutrients will normally allow for the development of a spore while restoration of depleated nutrients allows for what is known as rejuvenation, resulting in the development of a vegetative cell.

Soon after the critical period of forespore formation, a stage known as irreversible commitment to sporogenesis is reached. This stage is basically characterized by a series of processes, morphogenetic in nature, which eventually lead to irreversible changes in the forespore, resulting in its development into a spore.

If the situation existed where all the nutrients had become exhausted in a growing culture then it would be relatively easy to distinguish which conditions would lead to the induction of sporogenesis. However, sporogenesis can occur in both continuous and batch cultures. Whether or not a growing vegetative cell will reach the stage of irreversible commitment to sporogenesis will depend greatly on the growth rate since slower growth rates will favor sporogenesis.

There are two very definite external inducing factors of sporulation: the production of certain catabolites and minimized levels of available nutrients. Studies by Manteifel (1948) and Grelet (1957) have revealed several such factors whose limitation, induced sporogenesis; the most significant factors found were nitrogen and carbon sources, certain inorganic compounds and growth factors.

Sporulation may also be induced through the presence of certain external factors in the sporulation medium and/or the release of such factors from growing cells. Wooley and Collier (1966) found the Clostridium roseum required two peptide components found in trypticase media for sporulation. Srinivasan (1966) showed that Bacillus cereus T and Bacillus

subtilis underwent sporulation if a ninhydrin sporulation factor was added to vegetative cell cultures in the pre-sporulation stage. Studies by Bergere and Hermier (1965) revealed the presence of an unknown factor which when supplied to a growing culture of Clostridium butyricum reduced the growth rate and induced sporulation. Kerravala et al (1964) and Schaeffer et al (1963) discovered that Bacillus subtilis and Bacillus megaterium sporulated and extent and speed of sporulation depended on the level of carbon and nitrogen sources.

(b) Environmental factors affecting spore yield and quality

The most significant environmental factors which affect sporulation, spore yield and quality include: the level and type of nutrients available, the presence of minerals, temperature, pH and aeration.

(i) The level of available nutrients

Available nutrients in high levels may promote growth but slow the induction of sporogenesis thereby decreasing spore yield. Whether or not this will occur is dependent upon available oxygen and pH changes.

Bernlohr and Novelli (1960) found that pH changes, aeration rate and concentration of glucose were all interrelated factors in the sporulation of Baccillus licheniformis. Nasuno and Asai (1960) studied the effects of glucose and nitrogen sources on strains of Clostridium butyricum and found that sporulation occurred quite easily regardless of the levels of glucose or nitrogen supplied. Studies performed by Jerusalimskij and Rukina (1959) on a continuous culture of Clostridium butyricum revealed that sporogenesis was dependent upon the depletion of nitrogen sources and vitamins. Hardwick and Foster (1952) demonstrated that Bacillus mycoides grown in a nitrogen-rich medium sporulated readily whereas if grown in a medium low in nitrogen, sporulation did not occur.

Studies by Williams and Harper (1951) found that sporulation of Bacillus cereus was reduced markedly if leucine was deleted from their medium. Krask (1953) observed that much more glutamic acid was required in their medium for sporulation to be achieved than was required for growth. Ordal (1957) demonstrated a sulfur requirement for the sporulation of Bacillus coagulans. The organism was first grown without sulfur-containing amino acids (methionine and cystine) and sporulation was reduced

drastically from 75-90% to 5-10%. When the medium was fortified with inorganic sulfate and methionine and cystine left out the organism sporulated 70-90%.

Grelet (1955) working with Bacillus cereus strains found that if the organism was cultured in a glucose-amino acid-salt medium, good sporulation occurred when alanine, leucine, isoleucine and valine became limiting. The effect of carbon sources on sporulation has been studied by several researchers. Foster and Heiligman (1949) found that addition of 2 mg/ml of glucose greatly increased the sporulation (2500%) of Bacillus cereus. Other, however, have found that sporulation was enhanced in some Bacilli species only if glucose became exhausted or was absent from the culture medium. Ordal (1957) observed that Putrefactive Anaerobe 3659 sporulated in the presence of glucose but the spore yields were quite low. This seemingly adverse effect of glucose on the sporulation of certain Bacilli species was further supported by Hardwick and Foster (1952) and Halvorson et al (1956).

A study by Ordal (1957) revealed that Bacillus coagulans exhibited a sporulation requirement for folic acid or p-aminobenzoic acid (PABA). In the medium approximately 90%

of the culture sporulated but when folic acid was removed it decreased to 10%. This effect was remedied when PABA was added and sporulation increased to 75-90%. Addition of adenine, adenosine, guanine, uracil, inosine and thymine at levels of 10 µg/ml had little effect on sporulation. Higher concentrations of 100 µg/ml of these nutrilites almost entirely suppressed growth of Bacillus coagulans. Muhammed et al (1975) studied the nutritional requirements for sporulation of several strains of Clostridium perfringens. In a chemically defined medium it was found that alanine, aspartic acid and methionine highly stimulated sporulation and that some strains required riboflavin, isoleucine, serine, lysine and butanol for increased sporulation.

(ii) Mineral composition of the growth medium

Different anions and cations have been reported to affect sporogenesis as well as spore yield and quality in Bacilli species. Salt effects on sporogenesis were reported as early as 1889 by Behring and Schreiber (1896). Cook (1931) and Tarr (1932) showed that good sporulation was possible in several aerobic bacteria in a mineral salts medium. Roberts (1934) achieved 60-70% sporulation of Bacillus subtilis in a mineral supplemented medium.

Fabian and Bryant (1933) observed increased sporulation among four mesophilic aerobes: Bacillus cereus, Bacillus subtilis, Bacillus mesentericus and Bacillus megaterium if a peptone medium was supplemented with cations of univalent chloride salts such as NaCl, KCl, NH_4Cl and LiCl. Divalent or trivalent chloride salt cations had not influence on sporulation. Foster and Heiligman (1949) studied the effect of potassium on the sporulation of Bacillus cereus. Potassium along with several other cations has been shown to influence sporulation of Bacilli species (Fabian et al, 1933, Perdue, 1933) and its presence in aerobic spores has been revealed by spectrochemical analysis (Curran et al 1943). It was found that the addition of potassium led to a 1000% increase in spore yield, suggesting a definite role for potassium in the sporulation process.

Manganese is specifically required during presporulation and sporulation of several Bacilli species, (Amaha et al, 1956, Donnellan et al, 1964; Charney et al, 1951; Grelet, 1952 and Weinberg, 1955) and possibly plays a role in the activation of some enzymes involved in spore formation. The specificity of manganese for the sporulation of Bacillus subtilis was observed by Charney et al (1951). Sporulation

was negligible without added manganese in both a chemically defined and complex organic media. These findings were further supported by Curran and Evans (1954) who found that iron, used in large amounts, replaced manganese in manganese-deficient media. Weinberg (1955, 1964) also found a manganese requirement for sporulation in Bacillus megaterium strains. Amaha et al (1956) observed the sporulation requirements of three strains of Bacillus coagulans var. thermoacidurans on agar slants and in shake cultures. On peptone-containing agar media, sporulation was markedly stimulated by the addition of manganous sulfate, nickel sulfate or cobalt sulfate at a concentration of 1 ppm. Addition of manganese alone was sufficient to stimulate a high degree of sporulation.

Studies carried out by Grelet (1951, 1952) revealed sporulation requirements of Bacillus megaterium concerning various minerals. Shake cultures in a glucose-mineral-salts based medium were used and the effect of various mineral constituents on sporulation was observed. Depletion of Mn^{++}, Mg^{++} and K^{+} prevented sporulation indicating the necessity of these minerals for sporulation. Brewer et al (1946) using Bacillus anthracis studied the effects of Ca^{++}, Fe^{+++},

Mn^{++} and Mg^{++} on sporulation. Manganese and magnesium were required for sporulation, calcium and iron were not, but addition of excess Ca^{++} and Fe^{+++} increased spore yields. A few other minerals have been observed to be essential for sporulation; zinc for Bacillus coagulans var. thermoacidurans Ward (1947) and for Bacillus cereus Lundgren et al (1960, 1962), trace amounts of copper for strains of Bacillus cereus and Bacillus megaterium, Kolodziej et al (1964) and molybdenum for Bacillus megaterium, Kolodziej et al (1964).

Studies have also indicated that the presence of calcium above certain minimum levels appears to be a factor in the production of thermostable spores; Donnellan et al (1964); Curran (1957); Grelet (1952); Levinson et al (1964); Young et al (1962). It appears that calcium contributes to spore stability through the formation of internal bonding with the peptides found in much of the spore contents.

A plethora of published reports seems to indicate that mineral salts are essential for bacterial spore formation. The fact that spores are not produced in synthetic media without added salts, and, the fact that in complex organic media the inadequacy of spore formation can be remedied

through the addition of suitable minerals support this contention. The exact level of minerals required for sporulation will vary with the organism, and with cultural environmental conditions such as type and concentration of nutrients, amount of available oxygen, pH and temperature.

(iii) Effects of pH

Data of pH optima and pH changes for sporulation have been reviewed; Knaysi, (1948); Leifson, (1930); Murrell, (1961); Halvorson, (1962). Leifson (1930) working with various Bacilli and Clostridia species, found that the pH optima for sporulation were close to neutral for most species studied. Knaysi (1948), also working with various Bacilli, observed a similar requirement for a pH optimum near neutral for maximal sporulation. Recent studies have shown that although the pH optima for sporulation in Bacilli species is near neutrality, once cultures undergo logarithmic growth, a drop in pH results. This has been reported to be due to the production of several organic acids such as pyruvic and acetic. Once these acids are utilized this results in a subsequent rise in pH; Halvorson (1957); Bernlohr (1960); Lundgren and Beskid (1960); Nakata and Halvorson (1960).

(iv) Effects of temperature

Growth temperatures have been shown to affect growth, spore yield and spore properties. Lechowich and Ordal (1962) observed that an increased temperature during both growth and sporulation resulted in increased heat resistance of spores of Bacillus coagulans and Bacillus subtilis. Spores of Bacillus subtilis exhibited an increased heat resistance as well as an increased dipicolinic acid (DPA) content and cation content (Ca^{++}, Mg^{++}, Mn^{++}) as the sporulation temperature was increased from 30°C to 45°C. Bacillus coagulans spores exhibited increased heat resistance as the sporulation temperature was gradually increased from 30°C to 45°C to 52°C. The DPA content decreased and the cation content remained relatively constant.

In a study by Murrell and Warth (1965) the optimum temperature for maximum heat resistance of Bacillus cereus spores was found to be 30°C. Shifts in temperature, either lower or higher than 30°C, resulted in a decrease in the number of spores produced and in their heat resistance.

Lundgren and Beskid (1960) developed a chemically defined medium which supported good growth and sporulation

of Bacillus cereus. Ultraviolet light induced mutants were produced from the normal culture when grown at 37°C. The mutants isolated appeared to be temperature sensitive since they sporulated when grown at 28°C but could not sporulate when grown at 37°C. Similar mutants were prepared by Cooney and Lundgren (1962) and it was found that temperature affected sodium,calcium,potassium and zinc uptake.

(v) Aeration effects

Increased aeration was reported as a requirement for optimal sporulation of several aerobic Bacilli (Halvorson,1957; Roth et al,1955). Two metabolic systems were shown to become functional in aerobic sporeformers during the pre-sporulation stage: the tricarboxylic acid cycle (TCA) and the formation and subsequent breakdown of poly-β-hydroxybutyrate (PHB). A higher aeration rate was required for the degradation of PHB which accumulated before sporulation. Slepecky and Law (1961) found that the utilization of PHB was directly related to the ability of Bacillus megaterium to sporulate.

Studies on the effects of oxygen on the growth and sporulation of anaerobic sporeformers revealed that it

inhibits both growth and sporulation; however, near the end of sporogenesis it was shown to promote spore liberation.

(c) Development of heat resistance of spores

During sporulation there is an accumulation of calcium and DPA in spores. DPA in bacterial spores is of particular interest because of its possible role in developing heat resistance in spores. DPA has been reported to act as a strong chelating agent occurring only in bacterial endospores. It is usually present in high concentrations, (4-15% dry weight). It is synthesized just prior to the formation of resistant spores, and is completely lost during germination (Halvorson 1961). Several functions have been assigned for the role of DPA: overall heat resistance in spores via protein stabilization and removal of water; calcium accumulation in sporulating cells by chelation; stimulation of electron transport system; enzyme stability and stimulation of germination.

Heat resistance has been shown to be related to the presence of calcium (Halvorson, 1961; Abdel-Gadir and Scholefield, 1975). Spores low in calcium are more sensitive to heat inactivation and a correlation between

Ca^{++} content and heat resistance has been observed in several organisms, (Halvorson, 1961). It has also been reported that decreasing amounts of DPA in bacterial spores will result in reduced heat resistance in such spores.

The accumulation of calcium has long been observed to correlate with the development of heat resistance. DPA precedes heat stable spore formation and follows the appearance of spore-like bodies, therefore, DPA synthesis probably occurs either prior to or together with calcium accumulation, (Halvorson, 1961).

The possible interrelationship of calcium and DPA in heat resistance may be considered as follows:

(i) Calcium would act as a catalyst in synthesizing DPA, the DPA being required for heat resistance.

(ii) DPA may be required for calcium accumulation, the calcium being required for heat resistance.

(iii) DPA and calcium might both be required in heat resistance, the mechanism being the type of binding between calcium, DPA and spore protein which makes them most heat resistant, (Halvorson, 1961).

The nature of heat resistance in bacterial endospores has been the subject of considerable investigation. Curran et al (1943) studied the mineral content of both vegetative cells and spores of twelve Bacilli species and found that spores contained much more mineral content than the vegetative cells. A strong correlation was also shown to exist between higher cation concentration and increased heat resistance in these spores.

Cohen and Wiener (1954) examined spores of Bacillus megaterium spectrochemically and found that these spores contained high levels of mangnesium and calcium. Vinter (1956) using spectrochemical analysis also found that Bacillus megaterium spores possessed over five times as much calcium as did the vegetative cells.

Sugiyama (1951) observed that Clostridium botulinum spores contained high levels of iron and calcium and that their thermal resistance was related to the high level of these cations. In a study by Amaha and Ordal (1957) it was found that the heat resistance of Bacillus coagulans var. thermoacidurans spores increased through the addition of calcium and manganese to the sporulation medium. Slepecky and Foster (1959) observed that Bacillus megaterium

spores, produced in a high calcium medium, possessed a high thermal resistance whereas spores produced in a medium high in zinc and manganese but low in calcium showed less heat resistance.

The first studies correlating DPA synthesis and development of heat resistance were carried out by Halvorson, (1957) on Clostridium roseum and Hashimoto et al, (1960) on Bacillus cereus.

Day and Costilow (1964) observed that the heat resistance among Clostridium botulinum spores and the synthesis of DPA were closely associated. This resistance to higher temperatures is not acquired with simultaneous production of maximum DPA levels in the spores. A delay or maturation period was reported to allow for the development of the cortex. Studies have shown an interrelationship between cortical mucopeptides, calcium and DPA in the formation of thermoresistant spores, (Warth et al, 1963; Lewis et al, 1960). The final conclusion drawn in these studies was that the cortex contributes to the retention of calcium and DPA in spores.

The complex interaction of DPA and calcium with cortical mucopeptides and other spore contents can be influenced by many factors during sporogenesis. Each species varies in

how these components interact with one another within their own spore structures. Other factors may show interplay with these components, such as; protein stabilization by calcium alone, changes in the physicochemical nature of nucleic acids and proteins, changes in the tertiary structure of cystine-rich proteins along the spore periphery so that no common mechanism can be established for the optimal interaction of these components resulting in maximum resistance valid for all Bacilli and Clostridia species.

(2) Bacillus Stearothermophilus

Bacillus stearothermophilus is the typical organism causing flat sour spoilage in low acid foods (foods with a pH above 5.3; including such foods as peas, corn, lima beans, meats, fish, poultry and milk). This kind of spoilage derives its name from the fact that the ends of the can of food remain flat, that is, they have a normal concavity, during souring, or the development of lactic acid in the food by the flat sour bacteria. The existence of the organism was known early in the food canning industry; the organism was first isolated by Donk, (1920).

The following are some selected characteristics of Bacillus stearothermophilus: (Bergey's Manual, 1975)

Vegetative rods

0.6 to 1.0 μ by 2.0 to 3.4 μ; motile.

Sporangia

Swollen; racket-shaped.

Spores

1.0 to 2.0 μ by 1.5 to 2.2 μ; oval and subterminal to terminal.

Colonies

Not distinctive; pinpoint to small; round to irregular; translucent to opague; smooth to rough.

Growth temperature

Optimum growth 50-65°C; maximum temperature 65-75°C; minimum temperature 30-45°C.

(a) The existence of bacterial variants

The term variation refers to the fact that within bacterial strains, variant forms exist which differ from each other morphologically, physiologically and biochemically. Bacterial variations have been shown to arise either spontaneously or by induction, using mutagenic agents

or by recombination, following some method of transfer of genetic material, (Krueger, 1973).

The existence of both rough (Rh) and smooth (Sm) variants in B. stearothermophilus was first observed by Michner (1953). However, this species variation, that is, existence of both Sm and Rh variant forms of an organism is also known to exist in other genera as well.

Altenbern et al (1957) observed metabolic and population changes in Brucella abortus. It was found that differences in the terminal respiratory systems enabled Rh variants of B. abortus to grow after oxygen deprivation inhibited the virulent, Sm cells. Carta and Firshein (1962) studied population changes in Diplococcus pneumoniae. A shift from virulent (Sm) populations to avirulent (Rh) strains was observed. This selective population change was due to a toxic factor produced by large numbers of avirulent (Rh) cells inhibiting the multiplication of virulent (Sm) cells. Wessman (1964) studied the inter-relationships of smooth and nonsmooth variants of Past eurella haemolytica.

Smooth and nonsmooth variants were found to be similar biochemically but differed in their virulence. The presence

of nonsmooth cells in mixed cultures severely limited the growth of smooth cells. The critical factor appeared to be the amount of oxygen in the culture where selective inhibition of smooth variants occurred under reduced air pressure. Rh and Sm variants have also been found in Pseudomonas aeruginosa and Bacillus coagulans (Bisset, 1955).

(b) Morphology and spore structure of variants

The morphological differences between the Rh and Sm variants of B. stearothermophilus have been reported by Fields (1963). Surface colonies of the Sm variant were observed to be round with a central opaque spot. The vegetative cells consisted of short rods with a maximum length of 2.0 x 0.5 μ and a minimum of 1.0 x 0.5 μ. The Rh variants on the other hand, showed colonies with an irregular margin. When grown on the same medium under the same environmental conditions, the surface colonies of the Rh variants tended to be larger than the Sm variant. The vegetative cells of the Rh variant were considerably longer with a maximum length of 5.0 x 0.5 μ and a minimum of 2.0 x 0.5 μ.

Ultrastructure studies of these spores were performed by Bradley and Franklin (1958) and Rotman and Fields (1966).

Bradley and Franklin (1958) observed that spores of B. calidolactis were very similar to B. stearothermophilus but some ribbing was usually present in B. calidolactis. B. stearothermophilus spores were reported to be rough in appearance because of surface contamination. B. calidolactis spores were 1.2 to 2.2 μ by 0.0 μ; the surface is usually patterned with irregularly spaced, parallel, longitudinal ribs, often indistinct.

Rotman and Fields (1966) carried out electron microscopy studies and found the presence of three layers in the Rh spore wall. The Sm variant spore wall was thicker than the Rh variant spore wall by ca. 175.3°A. A significant difference between the variants appeared to be the observation that the Rh variant spore well layers were separated to a greater extent than in the Sm variant.

Inside the Sm variant spore wall, two layers of low electron density were observed which could not be seen in Rh variant spore sections. This structure, along with the thick spore wall, has been suggested to act as a mechanical barrier to heat in Sm variant spores. In addition, it was also observed that Rh variant spores showed a thicker cortex than did the Sm variant spores, although

the exact significance of the spore cortex is still unknown. Some researchers consider the cortex to be the site of DPA and calcium storage which in turn is known to affect the heat resistance of the spores. Other researchers have suggested that DPA is found in the core and that the core contains a high concentration of mineral matter, (Halvorson, 1961). The fact that the Rh variant spore was shown to possess more cortex than the Sm variant spore did not seem to be related to heat resistance since the Rh variant was shown to be the less heat resistant of the two variants, (Hill and Fields, 1967).

Much of the literature reported on B. stearothermophilus indicates that the spores of this organism are ubiquitous in nature, non-pathogenic, difficult to destroy with heat or chemicals and are hard to characterize with regard to growth and sporulation requirements.

(c) Growth of rough and smooth variants

(i) Mineral and media composition

Dahl (1955) revealed that nitrate, after reduction to nitrite, stimulated the sporulation of a strain of B. stearothermophilus. The greatest percentage of spores was

obtained at 0.1 per cent nitrate and 0.01-0.05 per cent nitrite. Yao and Walker (1967), described a liquid medium for the production of spores of B. stearothermophilus. It was observed that supplementation with Mn^{++}, Ca^{++} and NO_3^- was required for maximal sporulation, which occurred after 18 to 24 hours of growth. A study by Kim and Naylor (1966) showed that 10 ppm of manganese was necessary in a sporulation medium to facilitate maximum spore yield by B. stearothermophilus. Guzman et al (1971) further supported this contention for a manganese requirement when it was observed that 10 ppm of MnSO4 added to the medium enhanced sporulation of B. stearothermophilus spores.

The effects of calcium, cobalt and manganese in various media on the sporulation of the Rh and Sm variants of B. stearothermophlus was investigated by Rotman and Fields (1969). In nutrient agar, calcium was found to have a partial inhibitory effect on the sporulation of the Rh variant. Cobalt in nutrient agar reduced sporulation of the Sm variant. Concentrations of 6 ppm and higher of cobalt, moderately stimulated sporulation of the Rh variant. Manganese (1 ppm) stimulated sporulation of the Rh variant in nutrient agar. In nutrient broth, fortification of the aerated broth with manganese (1 ppm) resulted in an

85 per cent increase in sporulation of the Sm variant. Cobalt also stimulated the sporulation of Sm variant in nutrient broth.

Tandon and Gollakota(1971) investigated the growth and sporulation of the Rh variant of B. stearothermophilus in a chemically defined medium. The medium (V-medium) consisted of various amino acids, vitamins and mineral salts; sporulation was greatly enhanced through the addition of glutamate and lysine. Higher incubation temperatures were adopted and it was found that 60°C greatly speeded up sporulation. The pH decreased steadily after initial growth (5.2) in V-medium but after sporulation was complete in 24 hours it rose to 8.4, acid free spores were visible after 20 hours.

A study of the Sm variant of B. stearothermophilus by Thompson and Thames (1967) revealed that maximal spore yields were obtained after 20 hours. A requirement of oxygen for rapid vegetative growth and sporulation was demonstrated. It was also found that manganese (15 to 30 ppm) stimulated sporulation.

The effects of media composition and pH on the growth and interaction of Rh and Sm variants of B. stearothermophilus

were examined by Hill and Fields (1967). Nutritional studies revealed that the Sm variant required arginine, histidine, methionine, valine, isoleucine, biotin and thiamine for growth. The Rh variant was found to have a less demanding nutritive requirement as compared to the Sm type, requiring only methionine and perhaps biotin for growth.

(ii) pH

Studies investigating the effect of pH on the growth of Rh and Sm variants in pure and mixed cultures in nutrient broth at pH 6.0, 7.0 and 8.0 were carried out by Hill and Fields (1967). The Rh variant which was shown to be much less sensitive to acid and alkaline environments and showed an increase of 3 hours in lag time at pH 6.0 and 8.0 as compared to pH 7.0; the Sm variant had an increase in lag time of 37 hours at pH 6.0 and 47 hours at pH 8.0 as compared to pH 7.0.

In trypticase soy agar, the Sm variant produced very little basic substances while the Rh variant produced large amounts of these substances. Two possible interactions were given for this occurrence:

(a) A Rh population could be produced in an acid medium and by producing basic substances raise the pH enough to stimulate Sm variant growth; OR

(b) In a medium permitting Rh variant growth but nutritionally inadequate for Sm variant growth, the Rh variant may produce metabolites allowing the Sm variant to grow.

(iii) Oxygen tension

The influence of oxygen tension on the growth and interaction of Rh and Sm variants of B. stearothermophilus in pure and mixed cultures was investigated by Hill and Fields (1967). They observed that oxygen did have an influence on the growth of these organisms by affecting the generation time of the two variants. At 55°C, the pure Rh population showed a lower generation time when grown under reduced O_2 tension while the generation time of the Sm variant was increased by low oxygen tension. Oxygen tension was also shown to affect the amounts of acid produced by the variants. The Sm variant produced more acid than the Rh variant, but the amount of acid was independent of oxygen availability. As the oxygen tension was lowered, however, the Rh variant showed more acid accumulation.

In a previous study by Hill and Fields (1967), it was found that the Rh variant had an active tricarboxylic acid (TCA) cycle while the Sm variant exhibited very little TCA activity. The Rh would be expected to accumulate more acids under anaerobic conditions where its TCA activity would be reduced while the Sm would show little dependence upon oxygen for its acid production.

Kauppinen (1969) examined the effects of aeration on the growth and sporulation of Sm and Rh variants of B. stearothermophilus. The Sm variant was found to have a higher oxygen demand than the Rh variant during exponential growth. Increased aeration of Sm variant cultures resulted in a higher growth rate and increased sporogenesis, whereas the Rh variant grew at a constant rate and sporulated at a higher rate irrespective of the aeration rate.

(iv) Temperature of growth

The growth temperature has also been shown to affect Rh and Sm variants of B. stearothermophilus (Hill and Fields, 1967). The Sm variant was shown to be more sensitive to a low temperature (45°C); the generation time of the Sm variant in a pure population decreased more than threefold when the temperature was raised to 55°C. A

further temperature increase to 65°C resulted in a twofold decrease in the generation time of the Sm variant. The Rh variant, on the other hand, exhibited a twofold decrease in generation time for each 10°C increase in temperature.

(v) Metabolism

Hill et al (1966) studied the pathways of glucose metabolism in the Rh and Sm variants of B. stearothermophilus. The Rh variant is generally less active biochemically and nutritionally less demanding than the Sm variant. Since the Rh and Sm variant transitions are thought to be the result of a biochemical mutation, differences in metabolism should exist between the two variants, (Braun, 1965).

Hill and Fields showed that the Embden-Meyerhof (EM) pathway was more active in the Sm variant than the Rh variant whereas the TCA cycle was more active in the Rh variant than in the Sm variant.

Jung et al (1973) observed metabolic differences between B. stearothermophilus grown at 37°C and 55°C. Thermophilic growth cultures (55°C) showed high activities of glyceraldehyde - 3 - phosphate dehydrogenase and alcohol dehydrogenase enzyme activity whereas thermophilic cultures grown at 37°C showed little activity of either enzyme.

(vi) Antibiotics

A review of the literature has indicated that very little information is available concerning the antibiotic sensitivities of Rh and Sm variants of B. stearothermophilus. Recent studies, however, by Lee Wing and Blank (1980) performed on the antibiotic sensitivities of Rh and Sm variants of B. stearothermophilus var. calidolactis with penicillin, chloramphemicol, tetracycline and bacitracin have revealed that these variants showed different antibiotic responses particularly towards tetracycline and penicillin.

The inhibition of bacteria by closely related species which produce a bacteriocin has long been observed (Johnson et al, 1966; Hill and Fields, 1967; Shafia, 1966; Cole, 1973; Yule and Barridge, 1976; Sharp et al, 1979). Bacteriocins which are produced by thermophiles are designated as thermocins. Thermocins are unique bacteriocins because they are found extracellularly in an environment destructive to most other bacteriocins.

Yule and Barridge (1976) reported the existence of a thermocin produced by B. stearothermophilus. The thermocin was highly thermostable, of low molecular weight and composed mainly of protein and carbohydrate. Shafia

(1966) examined 22 strains of B. stearothermophilus and found 12 to be bacteriocinogenic. Some species produced thermocins which were active on many host strains while others were inhibitory to only one host strain. Hill and Fields (1967) showed that a heat-labile, bacteriostatic agent capable of slowing the growth of the Rh variant of B. stearothermophilus was produced by the Sm variant.

Recent work by Hii and Blank (1981) revealed the production of a thermocin by the Rh variant of B. stearothermophilus var. calidolactis which showed antagonistic activity against the Sm variant. Maximum thermocin activity occurred during the exponential phase of growth.

The thermocin was found to be stable against both heat and pH treatment.

(vii) Effect of heat and heat activation

Curran and Evans (1944) were the first workers to demonstrate that sublethal heat (62 to 95°C) could induce dormant spores to germinate. The first reference to activation of spores at temperatures greater than 100°C was made by Brachfeld (1955). In this study with B. stearothermophilus NCA 1518, it was found that spores

suspended in distilled water and heated to 105°C for five minutes gave maximal plate counts. Titus (1957) working with a isolate of B. stearothermophilus reported that heating spores of this strain at 110°C for 6 to 10 minutes resulted in maximal activation.

A study by Finley and Fields (1961) showed that when spores of two strains of B. stearothermophilus 1518 were heated at temperatures above 100°C activation occurred. A heat treatment of 115°C for three minutes was required to induce maximal activation in one suspension of strain 1518 spores whereas a heat treatment of 110°C for 7 to 10 minutes was adequate for the other suspension of strain 1518 spores. The degree to which the spores could be activated was strain dependent and variable among spore suspensions of the same strain.

A further extension of this study by Fields (1962) reported on the effects of heat on Rh and Sm variants of B. stearothermophilus. The effect of heat (110°C) on spore suspensions of mixed variants (Rh and Sm) was difficult to measure as the Rh variant was not as heat-resistant. While the Rh variant was activated in a shorter time, the Sm variant was not activated; when the Sm variant was

activated, the Rh was killed. When mixed cultures were subjected to lethal temperatures (120°C), only the Sm variant survived. In a mixed population, part of the spore population was being activated, while the other part was being inactivated.

(viii) Heat resistance

A number of investigations have shown that the heat resistance of B. stearothermophilus spores varies widely. The heat resistance of these spores was shown to vary with the composition of the growth medium (Williams and Robertson, 1954; Cook and Gilbert, 1968; Abdel-Gadir and Scholefield, 1975; Mayou and Jezeski, 1977); the temperature of growth (Amaha et al, 1957; Woese, 1960; Wang et al, 1964, Anderson and Friesen, 1974); the nature of the heating menstruum (Williams and Hennessee, 1956; Gautheir et al, 1978; Reed et al, 1951; Murrell and Scott, 1966; Collier and Townsend, 1956; Alderton and Snell, 1970; Harnulv et al, 1977); the particular strain involved and the variant type within the strain.

Cook and Gilbert (1968) found increasing spore heat resistance with increasing sporulation temperature. Heat resistance was correlated with manganese sulfate in the

sporulation medium at high concentrations (1000 ppm). Mayou and Jezeski (1977) found that heat resistance of B. stearothermophilus spores was greater with spores grown on nutrient agar fortified with milk than those grown on nutrient agar with 40 ppm of manganese sulfate. Reed et al (1951) determined the heat resistance of B. stearothermophilus in various vegetables and found z values ranging from 15.6 to 23.2 F and F_{250} values ranging from 21 to 44 min. Mayou and Jezeski (1977) found that B. stearothermophilus spores had a D_{250} = 2.4 minutes in reconstituted nonfat dry milk (10%) and D_{250} = 3.5 min in skim milk, pH 6.5. Abdel-Gadir and Scholefield (1975) observed significant differences in heat resistance of spores of B. stearothermophilus produced in solid and liquid media. Spores harvested in solid media exhibited greater heat tolerance.

The heat resistance data of Humbert et al (1972) indicated that spores of the Sm variant of B. stearothermophilus were more heat-resistant than the spores of the Rh variety. This further supported the results obtained by Fields (1963), Rotman and Fields (1966) and Scholefield and Abdel-Gadir (1974).

Humbert *et al* (1972), showed that when a mutation occurs from Sm to Rh, the heat resistance of the mutant does not appear to be affected. The variability in the heat resistance of duplicate spore suspensions of the same strain indicates that this variation might be one major reason for the variety of thermal resistance values published in the literature for *B. stearothermophilus.*

MATERIALS AND METHODS

(1) Source of microorganisms

The organism used during the course of this investigation was Bacillus stearothermophilus var. calidolactis. The Rh and Sm variants of this organism were isolated from Delvotest P ampules purchased from the Technical Microbiology Laboratory of the Technical University, Delft, Netherlands. The variants, isolated by their colonial growth appearance, were repeatedly sub-cultured on Tryptic Soy Agar (TSA) plates incubated at 45°C and/or 55°C for 18-24 hours in order to maintain viability and purity. The plates were stored at 4°C.

(2) Growth media

(a) Tryptic yeast extract glucose broth (TYG)

The TYG medium described by Kim and Naylor (1966) was used for the initial preparation of Rh and Sm vegetative cells (pre-inocula, stage 1) and consisted of (g/L):

Tryptone (Difco)	10.0
Yeast Extract (Difco)	5.0
Glucose	5.0
K_2HPO4	2.0
Distilled water	1000 mL

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

(b) NP medium

NP medium was used for pre-inocula growth, (stage 2; Yao and Walker, 1967) and consisted of 0.8% nutrient broth (Difco) dissolved in 0.1 N phosphate buffer adjusted to pH 7.2 with 0.1 N NaOH prior to sterilization.

(c) NPM medium

The fermentation broth used for spore production was based on the medium described by Yao and Walker (1967). The medium consisted of NP broth supplemented with the following:

Mn^{2+}	2 µg/mL
Ca^{2+}	2 µg/mL
NO_3^-	2 µg/mL

and was adjusted to a pH of 7.2 with 0.1 N NaOH prior to sterilization. Unless otherwise specified, the final volume of the fermentation broth was 2.5 L.

(3) Spore production

(a) pre-inocula

The pre-inocula used for subsequent spore production was grown in two successive stages. Stage 1 consisted of inoculating discrete, purified colonies of Rh or Sm variants into 250-mL Erlenmeyer flasks containing 120 mL of TYG broth. The inoculated flasks were then incubated at 55°C (Sm variant) and 45°C or 55°C (Rh variant) for 18-24 hours on a New Brunswick gyratory shaker incubator operating at 150 r.p.m. After incubation, 1 mL aliquots of the broth cultures (stage 2) were transferred into 250 mL-Erlenmeyer flasks each containing 125 mL of NP broth. The variants were again incubated at their respective temperature(s) for 18-24 hours.

(b) fermentation inocula

The fermentation production of spores was carried out by inoculating the fermentation vessel with 125 mL of pre-inocula obtained from stage 2. This inocula corresponded to a 5% V/V level. The final volume of the inoculated fermentation broth was 2.5 L.

4) Fermentation production of spores

(a) fermentor design

A Virtis, 4-L, bench-top fermentor equipped with a magnetic drive impellor was used for the fermentation production of spores.

(b) temperature

A temperature of 55°C was used for the production of Sm variant spores while temperatures of 45°C and/or 55°C were used for the Rh variant. The fermentation temperature was controlled by immersing the fermentation vessel in a thermostatically controlled water bath.

(c) aeration

Filtered air was fed into the fermentation vessel by means of a single probe sparger. Air was filter sterilized by means of glasswool, activated carbon, air-line filters. Aeration was controlled by the use of a flow-meter connected to an air/line pressure guage. Aeration rates of 0.2, 0.4 and 0.6 ml/min/ml medium were used in this investigation.

(d) foam control

Foam formation in the fermentor was controlled by periodic addition of sterile antifoam (Dow-Corning Antifoam A and Antifoam B emulsion).

(e) sterilization

The fermentor vessel, fermentation broth, conduit and filters were sterilized routinely at 121°C for 20 minutes at 15 p.s.i.

(f) sample withdrawal

Time course spore production was monitored via automatic withdrawal of samples through the use of Masterflex peristolic pumps controlled by automatic trip timers. Samples (ca. 50 mL) were fed via sterile conduit into flasks maintained at 4°C.

(g) total colony enumeration

(i) total viable cell count

Fermentation broth samples were plated using standard plate count medium (SPC, Difco) for total colony counts, comprised mainly of sporangia and vegetative cells. Enumeration was facilitated by serial dilution using

physiological saline as the diluent. Plates were incubated at 55°C and/or 45°C for 18-24 hours.

(ii) lysozyme treatment

Collected fermentation broth samples were subjected to centrifugation at 10,000 xg at 4°C for 20 minutes using a Sorvall superspeed RC2B automatic refrigerated centrifuge. The resultant pellets were resuspended in sterile physiological saline by Vortex mixing and similarly centrifuged. This procedure was continued for at least three times or until the supernatant fluid was rendered clear. The spore pellets were resuspended in a final volume of 10 mL of sterile physiological saline. The pellets were then treated with a 1% V/V lysozyme solution (Sigma Chemical Co.) overnight at 4°C. The efficiency of the lysozyme treatment was periodically checked by the Fleming method of direct spore staining (Cowan and Steel, 1970). Following lysozyme treatment, the lysozyme treated pellets were subjected to centrifugation of 50 xg for 5 minutes at 4°C. The supernatant containing cell debris was discarded and the spore pellets were resuspended in sterile physiological saline to their original volume (ca. 10 mL).

(iii) heat activation treatment

Approximately 5 mL from each collected fermentation broth sample was withdrawn via a sterile pipette and placed into 6 x 150 mm pyrex screw-cap test tubes. The test tubes were then placed in a thermostatically controlled oil-bath (Blue M, Magniwhirl) and heat activated for 15 minutes at 110°C. The heat activated spore suspensions were enumerated via pour plate technique using SPC medium with sterile physiological saline as the diluent. Incubation was carried out at 45°C and/or 55°C for 18-24 hours. Come-up-time was monitored.

(iv) thermocin treatment

(A) thermocin production

Thermocin production was accomplished with Rh isolates inoculated into NPM medium as outlined in Section III(3)(b). Temperatures of 45°C and/or 55°C were used. The fermentation was carried out for 6 hours after which the vessel contents were centrifuged at 10,000 xg for 20 minutes at 4°C. The resulting supernatant was then concentrated by rotary film evaporation at 40°C using a Buchler Instruments flash evaporator. The supernatant was concentrated to a final volume of approximately one-third of its original volume.

(B) thermocin assay

(1) indicator organism

B. stearothermophilus var. colidolactis, Sm variant, was grown in 1-L Roux bottles each containing 200 mL of TSA. Growth was carried out at 55°C for 18-24 hours after which the resultant cells were gently washed off the surface of the slant with 10 mL of sterile physiological saline. Sterile glass beads (5 mm diameter) were added to each Roux bottle slant after agar solidification. This allowed for uniform distribution of the inocula and aided in dislodging the cellular growth from the surface of the slants.

The harvested cells were pooled into a sterile flask and then centrifuged at 10,000 xg for 20 minutes at 4°C. The centrifuge tubes were sterilized prior to use. The resultant pellets were resuspended in approximately 10 mL of sterile physiological saline by Vortex mixing. The stock suspension of cells was then standardized with additional sterile saline at absorbance = 0.6, 410 nm. by means of a Hitachi Perkin-Elmar Coleman 111 Spectrophotometer.

(2) assay plates

Assay plates consisted of disposable (85 mm I.D.) petri dishes each containing 7.0 mL sterile TSA and 1.4 mL standardized Sm variant inoculum. The prepared plates contained a final concentration of approximately 1×10^6 cells/mL assay medium. Assay plates were stored at 4°C, for a maximum of three days until needed, in plastic sleeve containers.

(3) thermocin activity

The thermocin activity was determined by spot-plating serial dilutions of the Rh variant onto previously prepared assay plates. Varying concentrations of thermocin were prepared by the addition of sterile physiological saline. All assay plates were incubated at 55°C for 6 hours.

Thermocin activity is expressed in arbitrary units (Au). The thermocin titer was determined by taking the reciprocal of the highest dilution of thermocin that produces a visible zone on a lawn of B. stearothermophilus var. calidolactis, Sm variant cells.

(v) mixed culture growth studies

Both Rh and Sm variants (stage 1, pre-inocula) were prepared as outlined in Section III(3)(a) the only

modification being that both variants were grown at 55°C. The fermentation production of both Rh and Sm spores was carried out by simultaneously inoculating the fermentation vessel with both Rh and Sm pre-inocula up to a total volume of 125 mL in order to maintain a 5% V/V inoculum level. The final volume of the inoculated fermentation broth was still 2.5 L. The mixed cultures chosen for this investigation were as follows Rh: Sm: %; 80:20; 60:40; 40:60; 20:80. Each mixed culture study was subjected to aeration rates of 0.2 and 0.6 ml/min/ml. Total colony counts and lysozyme treated counts were carried out for each resultant spore suspension via pour plate as outlined in Section III(4)(g) (i) and (ii); the only modification being an incubation temperature of 55°C was used for all the prepared plates. Rh and Sm spores were enumerated based solely on colonial morphology.

RESULTS AND DISCUSSION

(1) Time Course Growth Studies, Sm Variant at 55°C Under Varying Aeration Rates

Time course growth sutides for the smooth variant at 55°C, under varying aeration rates are illustrated in Figures 1-3. The total viable cell count (TVC), including sporangia, regardless of aeration rate was maximal at 18 hours of fermentation, thereafter decreasing. This decrease in TVC has been similarly noted towards the end of the cultivation period by Thompson and Thames (1967) and Kauppinen (1969). It would appear that this decrease in TVC is due to substrate exhaustion, although residual carbohydrate was not monitored, and that ensuing lysis resulted as indicated by an increase in pH during this time period. Aeration rates of 0.2-0.6 ml/air/min/ml fermentation medium were shown to progressively increase the peak TVC counts at 18 hours. The exponential specific growth rates of the Sm variant were shown to vary with the aeration rate. Aeration rates of 0.2, 0.4 and 0.6 ml/min/ml yielded exponential specific growth rates (μ) of 0.56, 0.64 and 0.80 h^{-1}, respectively (Appendix Table 16).

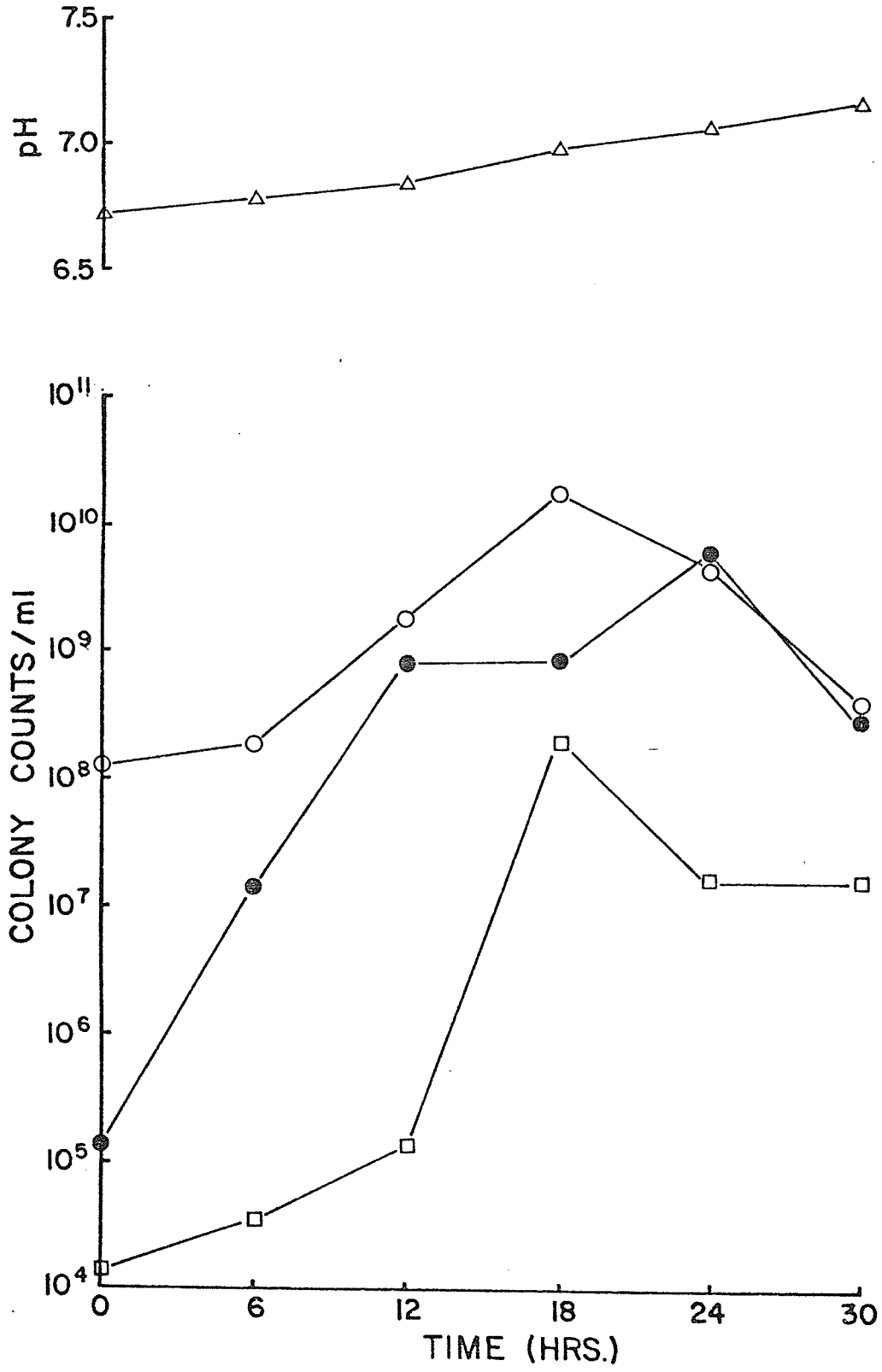
Exponential growth usually began after initial inoculation into the growth medium. Aeration at 0.2 ml/min/ml did, however, show a slight lag phase lasting approximately 6 hours.

The absence of a lag phase at the higher aeration rates may be attributed to the concerted effect of aeration and media composition. According to Kauppinen (1969), higher aeration rates increase the growth rate of the Sm variant because the adaptive TCA cycle enzymes are formed more readily or in greater amounts. Growth of the Sm variant in highly aerated medium would therefore preclude a lag phase. In addition, the growth of the pre-inocula in a similar fermentation medium, would also decrease the time necessary for adaptive enzyme synthesis.

The slight lag phase observed with the lower aeration rate may be partially due to the aeration, per se. Long and Williams (1959) reported that decreased oxygen tension in liquid media at higher temperatures was a major limiting factor in growth and sporulation of thermophiles. An increased oxygen demand due to decreased solubility of this gas was noted by Allen (1953) and is particularly evident at these high temperatures. Gaughren (1946) found that as

Figure 1. Time course growth studies of B. stearothermophilus var. calidolactis, Sm variant at 55°C. Aeration: 0.2 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, ●–●; heat activated count/ml, □–□.

(Appendix Table 1)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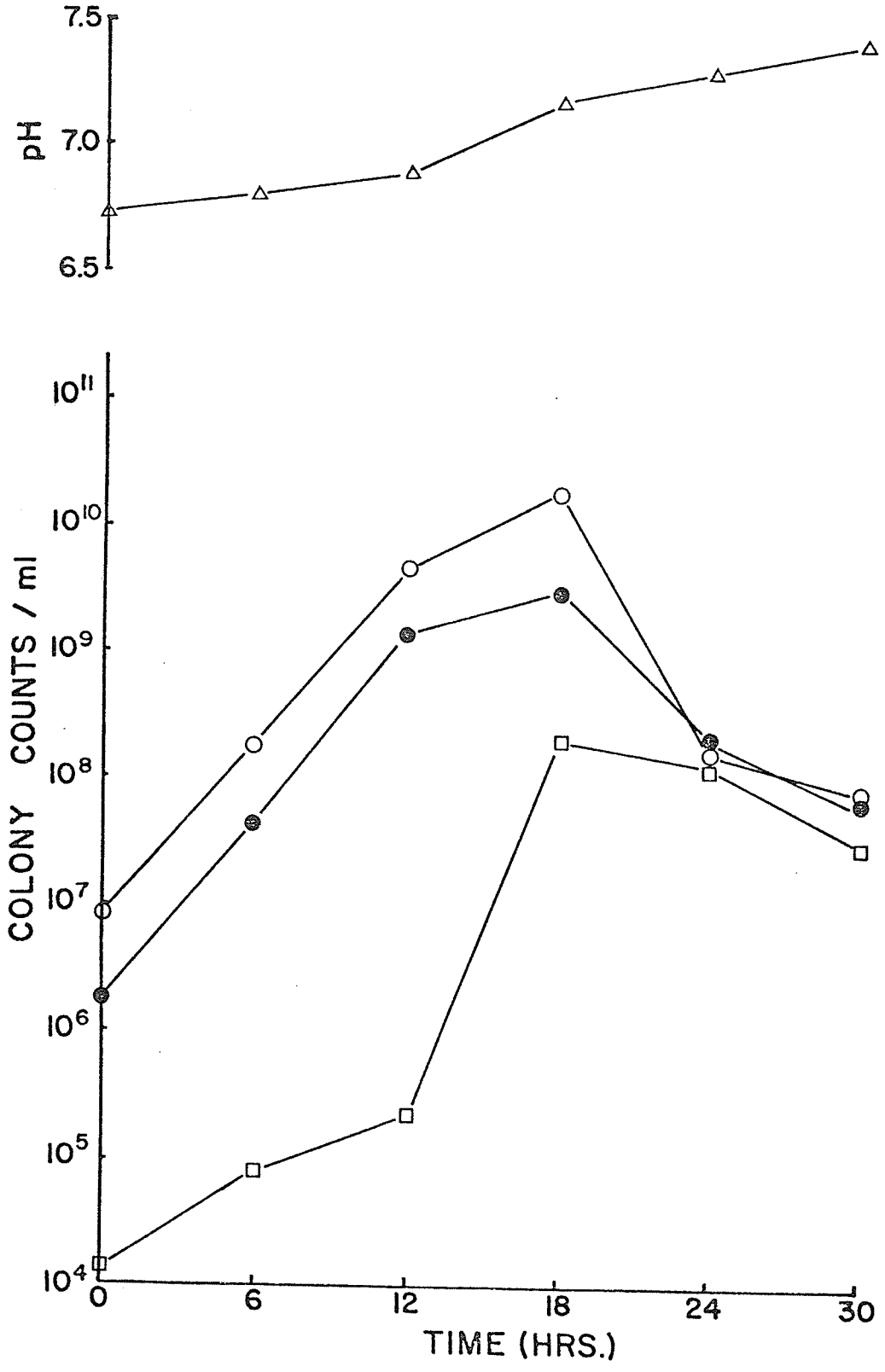
pH
7.0
6.5
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
COLONY COUNTS/ml
0
6
12
18
24
30
TIME (HRS.)

Figure 2. Time course growth studies of B. stearothermophilus var. calidolactis, Sm variant at 55°C. Aeration: 0.4 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, ●–●; heat activated count/ml, □–□.

(Appendix Table 2)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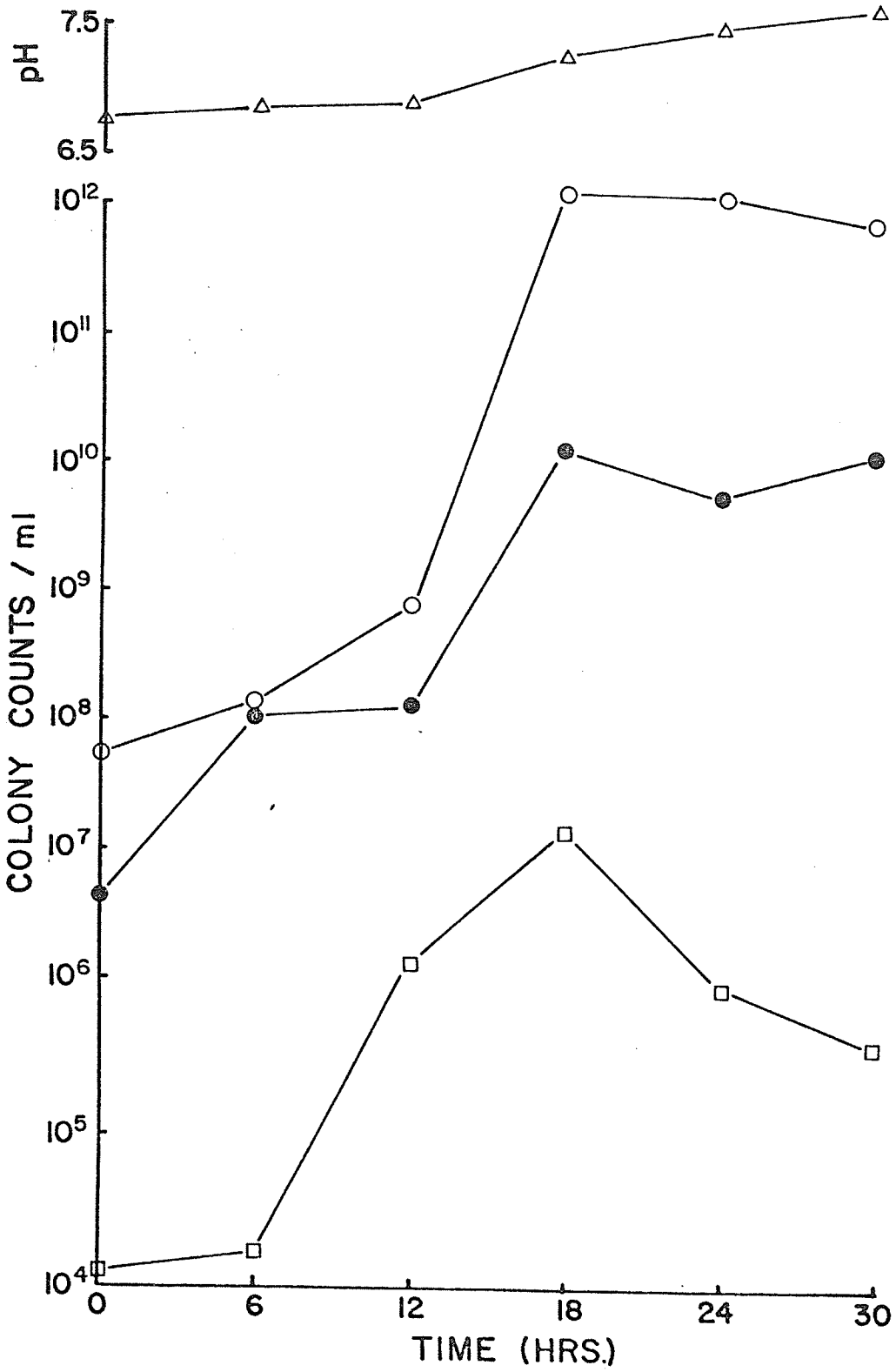
6.5
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 3. Time course growth studies of B. stearothermophilus var. calidolactis, Sm variant at 55°C. Aeration: 0.6 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, ●–●; heat activated count/ml, □–□.

(Appendix Table 3)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7.5
6.5
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

the surface to volume ratio of a liquid medium was decreased the viable vegetative count of thermophilic bacteria also decreased due to poor oxygen diffusion. Since the surface to volume ratio in these fermentations was maintained at a constant level, it is plausible that the rate of oxygenation coupled with decreased solubility at 55°C affected the TVC at this aeration level.

The lysozyme spore count (LSC) showed maximum numbers at 24 hours of fermentation with an aeration rate of 0.2 ml/min/ml. Increasing the aeration rate to 0.4-0.6 ml/min/ml subsequently decreased the time period for maximum LSC which occurred at 18 hours fermentation. It has been reported that spore formation normally occurs after the logarithmic growth phase when generation time increases due to limitation of nutrients (Gould, 1969). In these studies lysozyme treated spores showed maximal numbers which coincided to peak TVC with the exception of the lowest aeration rate. In all cases lysozyme treated spores decreased and/or remained constant following peak formation This decrease in lysozyme treated spores following sporogenesis for the Sm variant has been reported by Kauppinen (1969), who stated that the lysis of the developing spores of the Sm variant can be partially attributed to the poor

efficiency of the TCA cycle (Hill et al, 1967). According to Benger (1962), the TCA cycle is indespensible for the biosynthesis of DPA which is necessary for the maturation of spores.

The heat activated count (HAC) also showed maximum numbers at 18 hours fermentation. The degree of sporulation (HAC) in the Sm cultures remained fairly constant with aeration rates of 0.2-0.4 ml/min/ml. Aeration rate of 0.6 ml/min/ml, however, showed decreased peak counts. Kauppinen and Hjerp (1969), reported that sporogensis of Sm variant of B. stearothermophilus increased with increasing aeration in the cultures although most of the spores disappeared soon after sporogenesis. On the other hand, Thompson and Thames (1967), observed suppression of sporulation at higher aeration rates which they attributed to rapid germination of the newly formed spores of the Sm form.

The pH which initially ranged from 6.72-6.76, progressively increased reaching slightly higher pH values at the higher aeration rates. According to Hill and Fields (1967) and Hill et al (1966), the Sm variant of B. stearothermophilus was found to produce acid during growth. The amount of acid produced was independent of oxygen availability.

These acids were gradually utilized by the Sm variant resulting in a subsequent rise in pH regardless of the aeration rate. The fermentation studies conducted in this investigation, however, revealed no acid accumulation. It would appear that any organic acids produced during the growth of the organism were immediately utilized or neutralized by deamination products resulting from the metabolism of tryptone. In addition, the increase towards an alkaline pH may also be due to deamidization reactions particularly prevelent in growth media containing little or no available carbohydrate. Since many of the organic acids synthesized by the Sm variant during the initial stages of growth are attacked by adaptive enzymes, the lack of accumulated organic acids during these fermentations would be expected, since the inocula exponentially grown in a similar medium would already contain high levels of these adaptive enzymes. The presence of these adaptive enzymes in the inocula could therefore attack the organic acids as they were being synthesized.

(2) Time Course Growth Studies, Rh Variant at 45°C Under Varying Aeration Rates

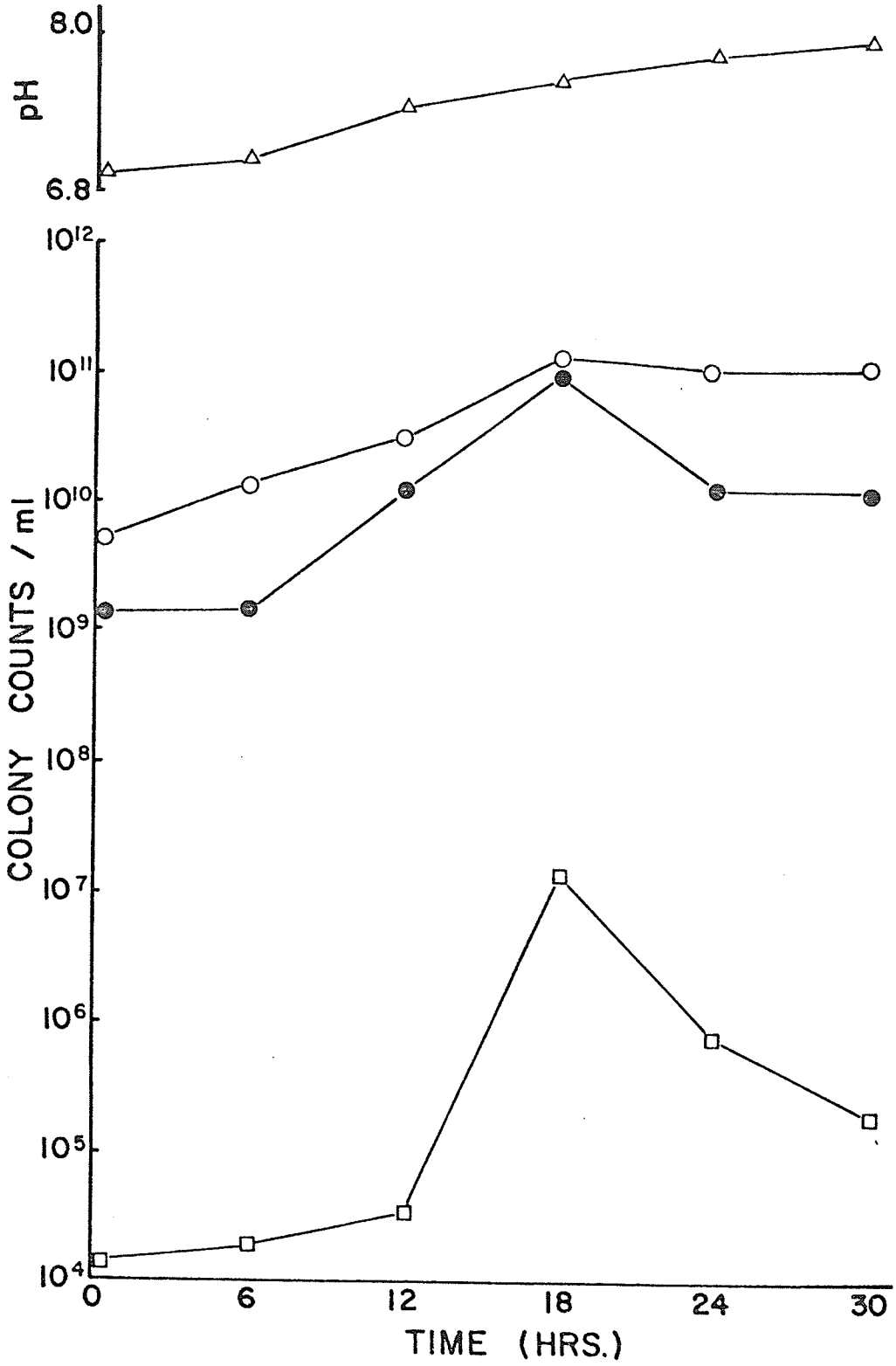
Time course growth studies for the Rh variant at 45°C under varying aeration rates are illustrated in Figures 4-7. The TVC including sporangia regardless of aeration was

maximal at 18 hrs of fermentation, thereafter decreasing, although non-aerated fermentations showed only slight vegetative growth. The exponential specific growth rates for the Rh variant were shown to vary with the aeration rate. Aeration rates of 0.2-0.6 ml/min/ml yielded exponential specific growth rates (μ) of 0.25, 1.60 and 1.06 h^{-1}, respectively (Appendix Table 16). The effect of aeration on the growth of the Rh variant has been reported by several authors although their results are somewhat conflicting.

Thompson and Thames (1967) observed that non-aerated cultures of the Rh variant of B. stearothermophilus exhibited severely decreased vegetative growth. When air was supplied at various rates (0.13-2.7 L/min/L medium), full vegetative growth and sporulation followed. As shown in Figure 7, it appeared that the lack of suitable aeration allowed for sporulation which was maximal at 18 hours but hindered vegetative growth. Hill and Fields (1967) also reported that the Rh variant, grown in a liquid medium devoid of aeration, showed a decreased generation time. Kauppinen (1969), however, reported that the Rh variant was insensitive to aeration, growing at a constant rate under all aeration rates investigated.

Figure 4. Time course growth studies of B. stearothermophilus var. calidolactis, Rh variant at 45°C. Aeration: 0.2 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□; heat activated count/ml, ●–●.

(Appendix Table 4)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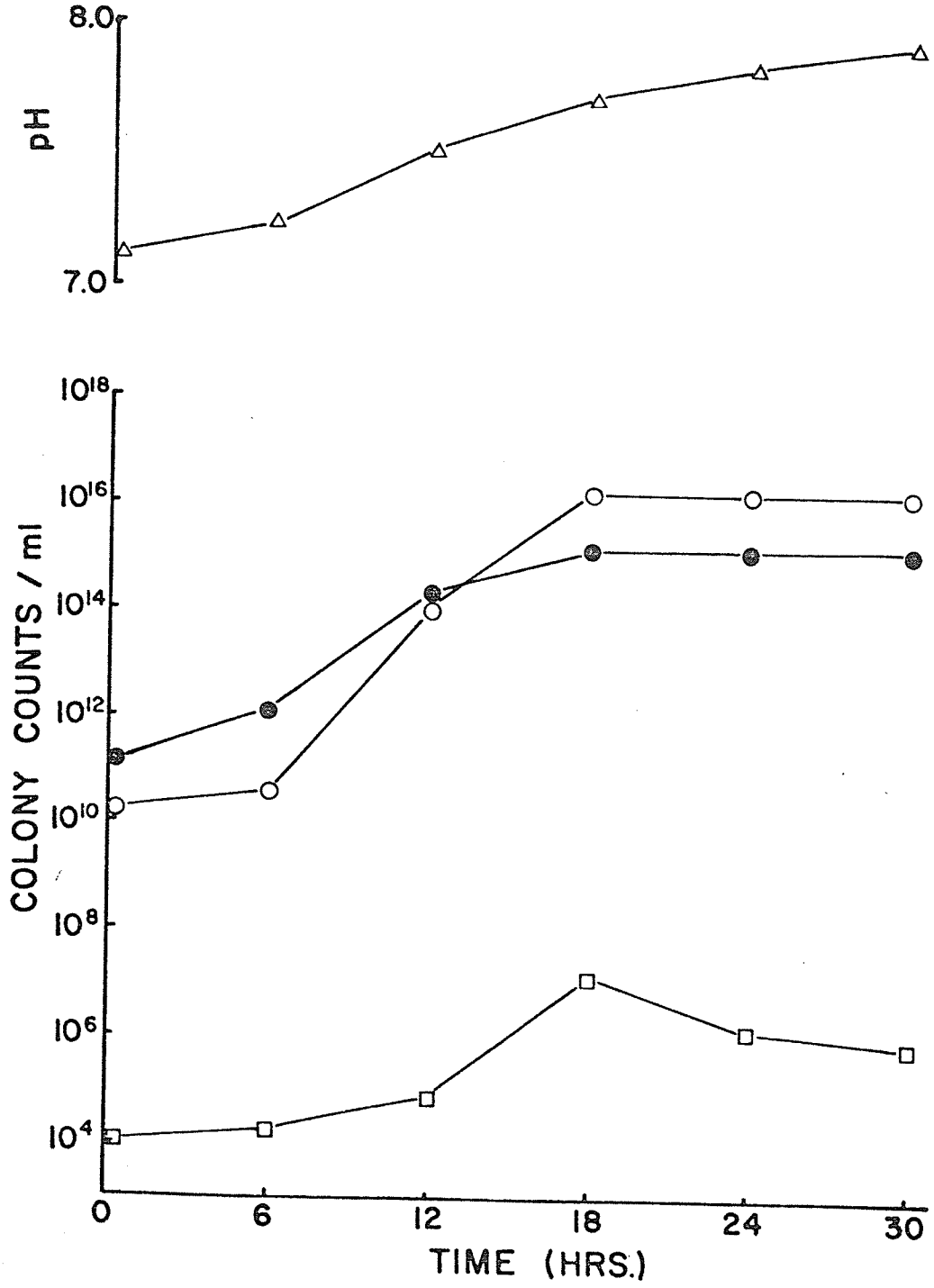
pH
6.8
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 5. Time course growth studies of B. stearothermophilus var. calidolactis, Rh variant at 45°C. Aeration: 0.4 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, ●–●; heat activated count/ml, □–□.

(Appendix Table 5)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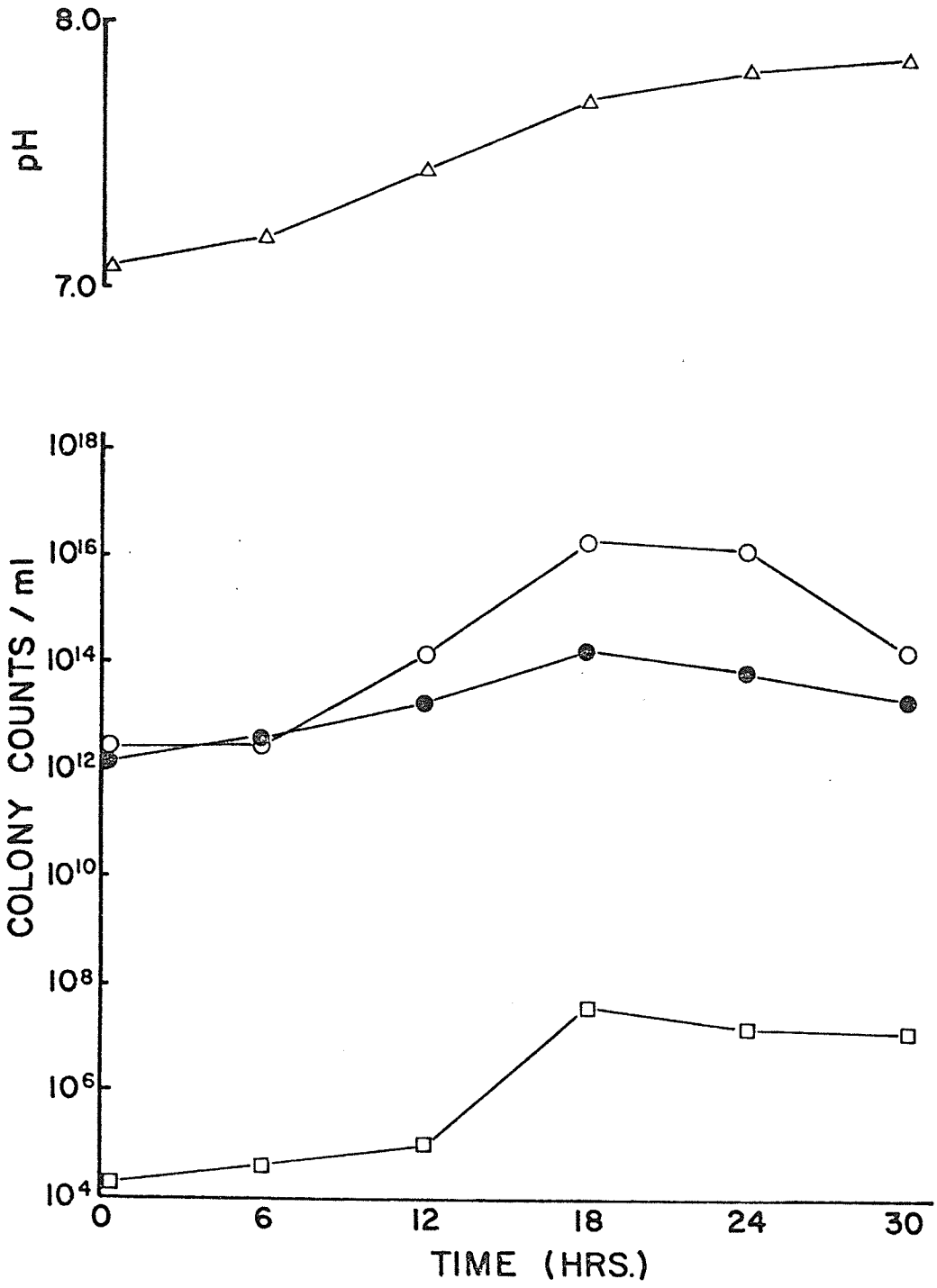
pH
7.0
10^{18}
10^{16}
10^{14}
10^{12}
10^{10}
10^{8}
10^{6}
10^{4}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 6. Time course growth studies of B. stearothermophilus var. calidolactis, Rh variant at 45°C. Aeration: 0.6 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, ●–●; heat activated count/ml, □–□.

(Appendix Table 6)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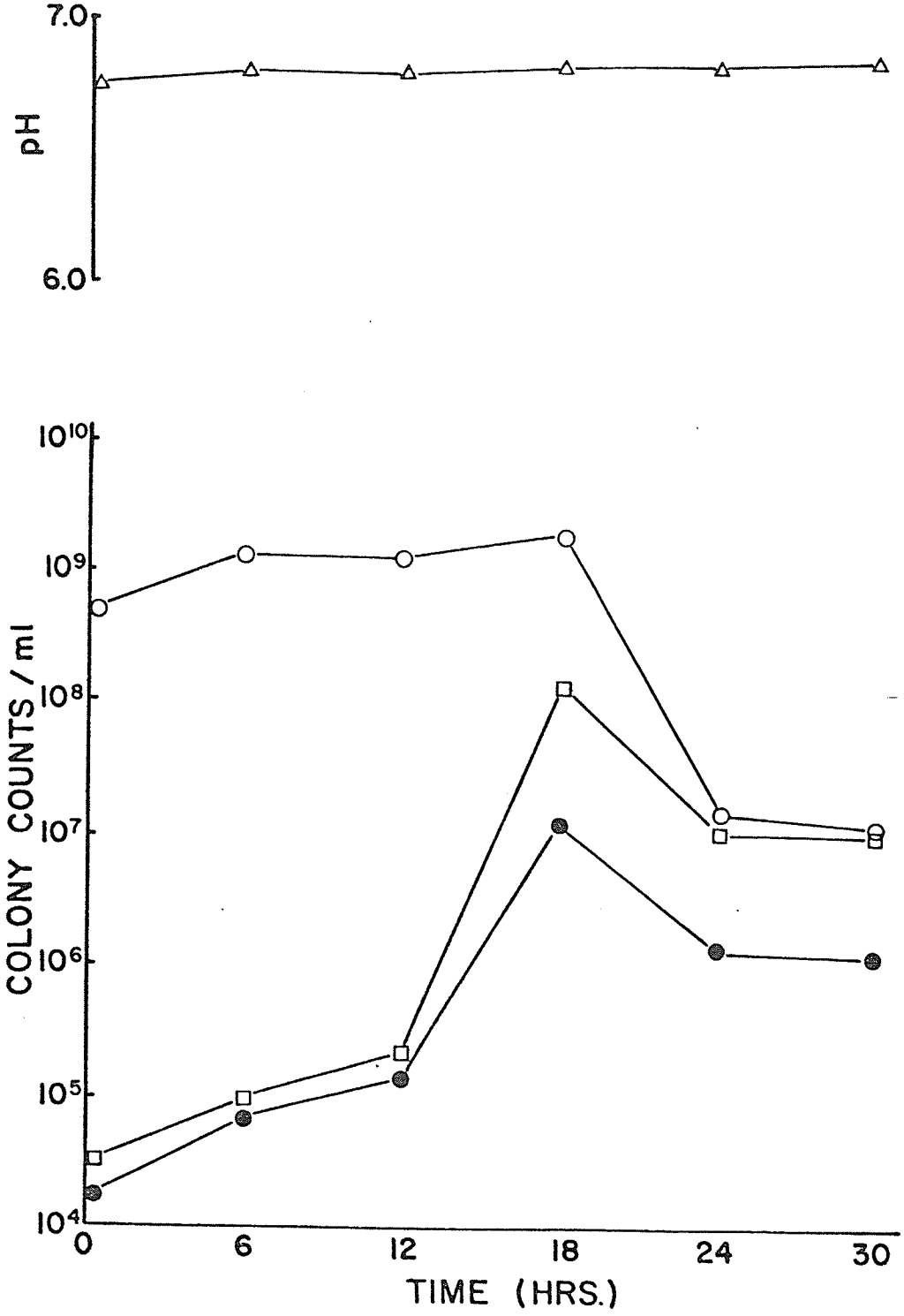
pH
7.0
10^{18}
10^{16}
10^{14}
10^{12}
10^{10}
10^{8}
10^{6}
10^{4}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 7. Time course growth studies of B. stearothermophilus var. calidolactis, Rh variant at 45°C. No aeration. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□; heat activated count/ml, ●–● .

(Appendix Table 7)


7.0
pH
6.0
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Cultures grown at 0.4 and 0.6 ml/min/ml both showed an initial lag phase lasting approximately 6 hours. The exact cause of this lag formation within these cultures is not known. The information furnished by Thompson and Thames; 1967, would preclude the occurrence of a lag due to aeration and/or adaptive enzyme synthesis effects. As specific growth rate constants were also shown to vary in these cultures, the presence of a lag during growth and the differences noted in the growth rate under varying aeration may be both partially attributed to the difference in the initial inocula used to seed these fermentations.

The LSC also showed maxima numbers at 18 hours of fermentation which corresponded to peak vegetative growth. The degree of sporulation in the Rh grown cultures varied with aeration. Sporogenesis began immediately in non-aerated cultures, showing a rapid increase at the mid-stationary phase of growth. In aerated cultures, (0.2-0.4 ml/min/ml), rapid sporogenesis began at the early exponential phase. This same effect was noted in the highest aerated culture, although rapid sporogenesis was not noted. A decrease in LSC was observed in non-aerated and 0.2 ml/min/ml aerated cultures. The decrease in LSC counts coincided with the death phase of the organism while the

later decreased during the early stationary phase. LSC in the 0.4 and 0.6 ml/min/ml aerated cultures did not show a decrease.

Kauppinen (1969), reported that the Rh variant sporulated at a high rate irrespective of the aeration rate up to certain levels but that at high aeration rates, (0.8-1.0 ml/min/ml), sporogenesis of the Rh variant was delayed. Thompson and Thames (1967), demonstrated the susceptibility of sporulation of the Rh variant to nutritional influences. A repression of sporulation in the presence of enhanced growth occurred at aeration rates in excess of 0.67 ml/min/ml. This similar effect is observed in Figure 6 where at an aeration rate of 0.6 ml/min/ml sporulation was somewhat suppressed as compared to Figures 4 and 5 where increasing aeration correspondingly increased sporulation.

The HAC also showed maximum numbers at 18 hours fermentation thereafter decreasing for aeration rates of 0.0 and 0.2 ml/min/ml and somewhat plateaued for aeration rates of 0.4 and 0.6 ml/min/ml. This was observed as stated before by Thompson and Thames (1967) and Kauppinen where at high aeration rates the sporulation of the Rh variant was somewhat suppressed and at lower aeration the

Rh variant sporulated at a high rate.

The initial pH of the Rh cultures ranged from 6.74 to 7.11; in the non-aerated cultures, the pH remained fairly constant throughout the 30 hours of fermentation (6.74-6.84). No detectable acid accumulation was evidenced and if produced it was utilized or neutralized upon formation. Under aeration rates of 0.2-0.6 ml/min/ml, the pH progressively increased during the course of these fermentations. The Rh variant has been reported to possess an active TCA cycle and also is capable of producing several basic compounds during the course of its growth and sporulation, (Hill and Fields, 1967; Kauppinen, 1969; Hill et al, 1966; Tandon and Gollakota,1971).

(3) Time Course Growth Studies, Rh Variant at 55°C Under Varying Aeration Rates

Time course growth studies for the Rh variant at 55°C at varying aeration rates are illustrated in Figures 8-10. The TVC including sporangia regardless of aeration rate was maximal at 18 hours of fermentation, thereafter decreasing. The peak in vegetative growth was shown to progressively increase as the aeration rate was increased. The exponential specific growth rates of the Rh variant

were shown to vary only slightly with the aeration rate used. Exponential specific growth rates (μ) of 1.1, 1.5 and 1.1 h^{-1}, (Appendix Table 16), were obtained for aeration rates of 0.2-0.6 ml/min/ml respectively. The differences noted in the peak TVC, when correlated to the relative constancy of the respective specific growth rate constants, can be partially explained by the differences in the inocula used to seed the fermentations and the apparent lag formation at the lower aeration rates.

The LSC also showed maximum numbers at 18 hours of fermentation, thereafter decreasing. These peak LSC coincided to the beginning of the stationary phase of vegetative growth. Increasing the aeration rate from 0.2-0.4 ml/min/ml, showed an approximate 2 log increase in LSC. The aeration rates of 0.4-0.6 ml/min/ml showed somewhat similar counts.

The pH in these Rh cultures rose slightly, showing no signs of acid accumulation. Basic material accumulation was more rapid in the lowest aerated culture.

Thermocin production by these cultures under varying aeration is given in Table 1. Thermocin activity was

Figure 8. Time course growth studies of B. stearothermophilus var. calidolactis, Rh variant at 55°C. Aeration: 0.2 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□.

(Appendix Table 8)

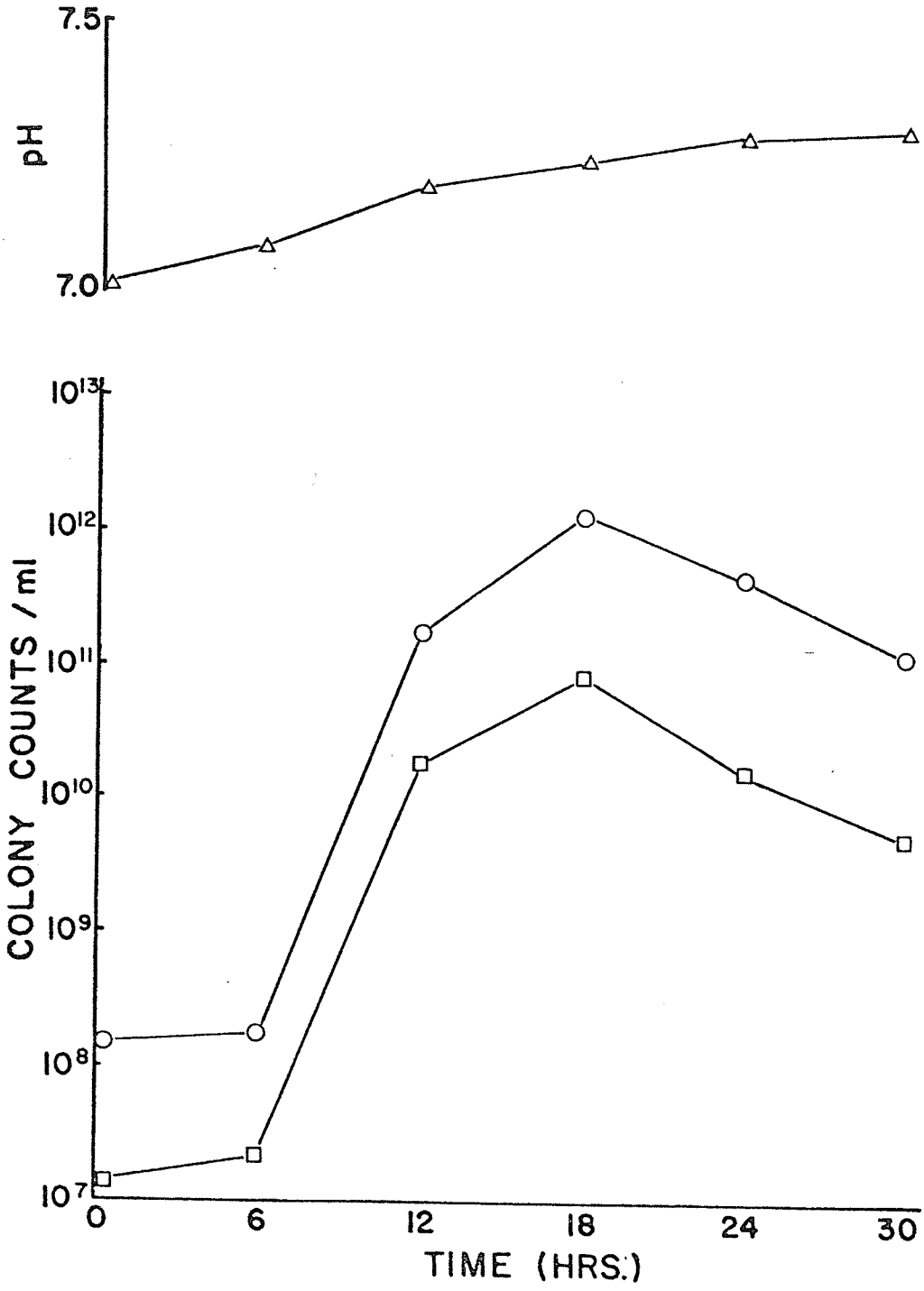

7.5
pH
7.0
10^{13}
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 9. Time course growth studies of B. stearothermophilus var. calidolactis, Rh variant at 55°C. Aeration: 0.4 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□.

(Appendix Table 9)

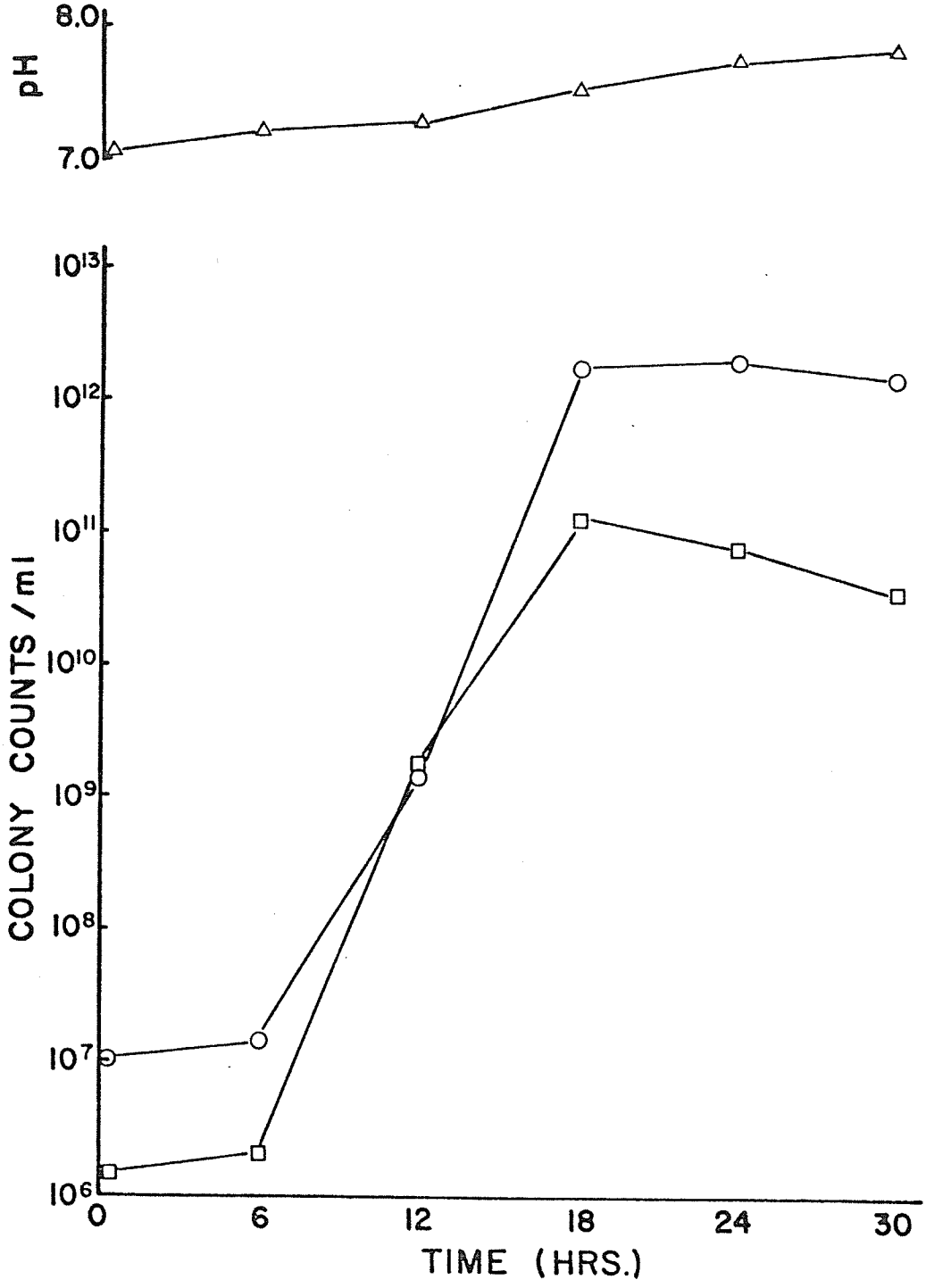
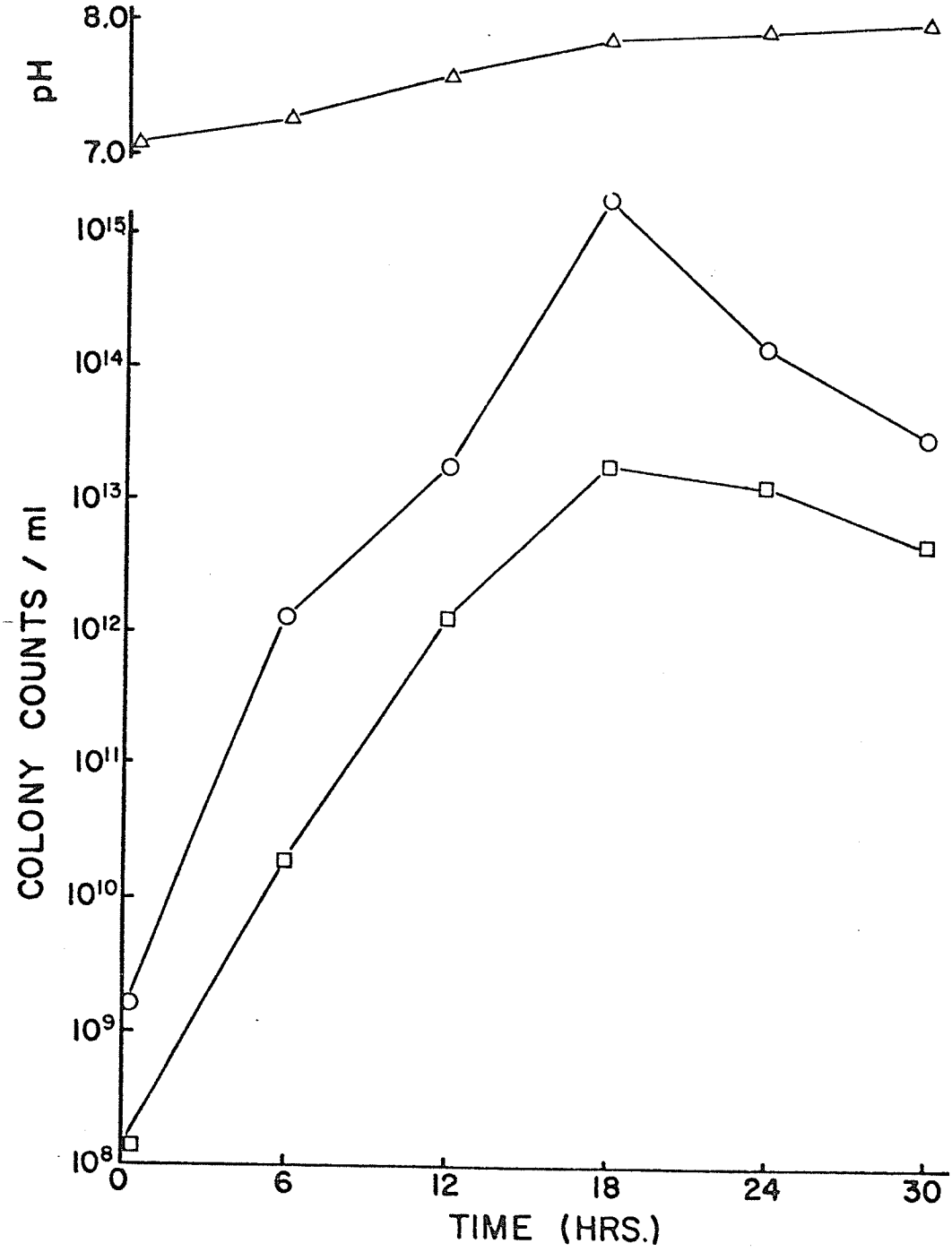

8.0
pH
7.0
10^{13}
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 10. Time course growth studies of B. stearothermophilus var. calidolactis, Rh variant at 55°C. Aeration: 0.6 ml/min/ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□.

(Appendix Table 10)


8.0
7.0
pH
10^{15}
10^{14}
10^{13}
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

shown to increase with increasing aeration rates. Peak thermocin titers were observed at 12 hours at the higher aeration rates and at 24 hours for the lowest aeration rate. The peak thermocin titers were shown to remain constant for the duration of growth. Since the thermocin produced by the Rh variant has been shown to be both heat and pH stable (Yule and Barridge, 1976) it is plausible that the peak levels of thermocin produced were not degraded nor did it cause any isoantagonism. Since isoantagonistic effects have not been reported by the Rh variant (Shafia, 1966), it seems plausible that the thermocin which was produced would not affect the growth of the Rh variant itself, and would have no or minimal effects on LSC. The peak thermocin titers observed were noted to correspond to the late or end stages of exponential growth for the higher aeration rates (0.4-0.6 ml/min/ml). Peak thermocin production at the lower aeration rate occurred during the stationary and/or death phase. It appeared from these studies that in addition to aeration, peak thermocin titers were correlated to the amount of growth (Sharp et al, 1979).

Table 1: Thermocin production, Rh variant at 55°C produced under varying aeration

Thermocin Activity

(A.u./ml)

Time	Aeration 0.2	0.4	0.6
0	1	2	1
6	2	3	3
12	3	4	6
18	3	5	6
24	4	5	6
30	4	5	6

(4) Time Course Growth Studies, Sm Variant at Varying Thermocin Concentration

The effect of thermocin on the growth of B. stearothermophilus var. calidolactis, Sm variant is shown in Figures 11-13. As indicated, thermocin concentrations of 0.55-0.60 A.u./ml showed an antagonistic effect on the TVC and LSC. Sm cultures grown in the presence of 0.3 A.u./ml exhibited maximum TVC and LSC at 18 hours. These peak counts were shifted to 12 hours at the higher thermocin concentrations. TVC in cultures with 0.6 A.u./ml showed no growth and declined rapidly after 12 hours. The LSC increased during the stationary phase of growth. These counts also decreased after 12 hours.

The minimum antagonistic activity for the Sm variant in these studies appears to be 0.55-0.60 A.u./ml. Although thermocin added at 0.55 A.u./ml showed only partial inhibition it is believed that this concentration of thermocin would also show complete inhibition if similar initial inocula were used. It would appear, then, that only partial inhibition of growth was observed in the culture containing 0.55 A.u./ml rather than complete inhibition as shown in Figure 13 because of the relative access of cells to thermocin (Cole, 1973).

Figure 11. Effects of thermocin on the growth of B. stearothermophilus var. calidolactis, Sm variant at 55°C. Thermocin activity 0.3 A.U./ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□.

(Appendix Table 11)

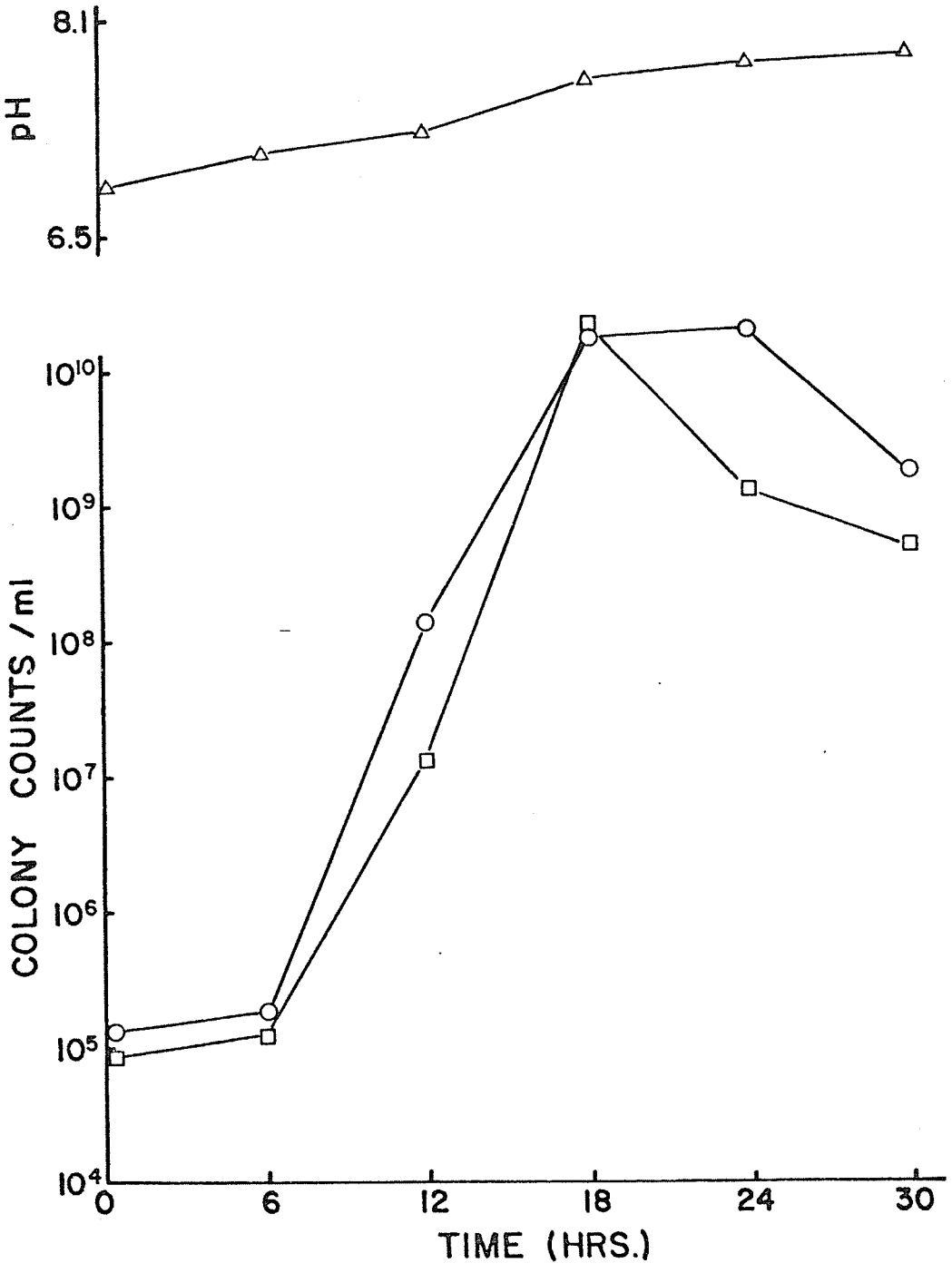

8.1
pH
6.5
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 12. Effects of thermocin on the growth of B. stearothermophilus var. calidolactis, Sm variant at 55°C. Thermocin activity 0.55 A.U./ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□.

(Appendix Table 12)

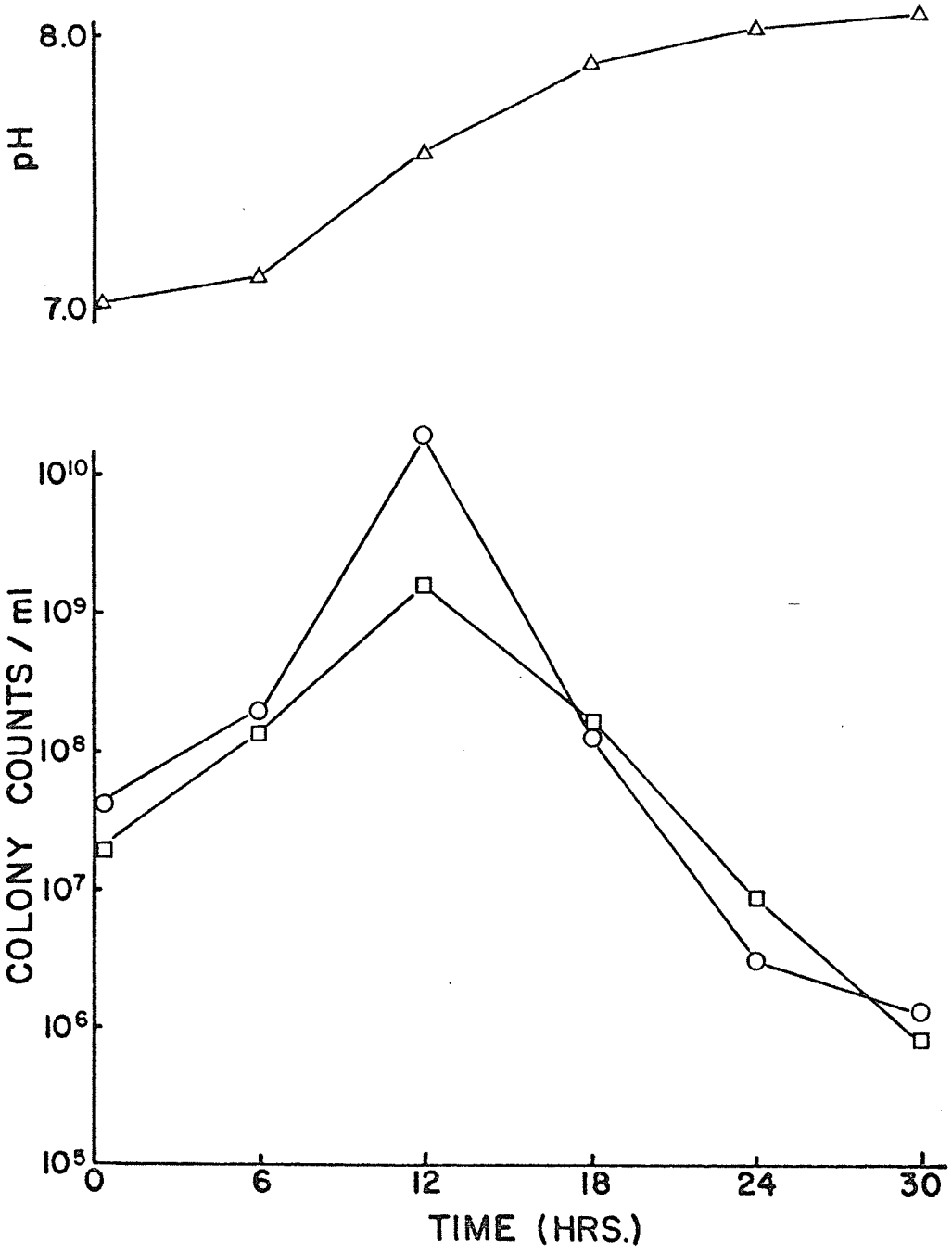
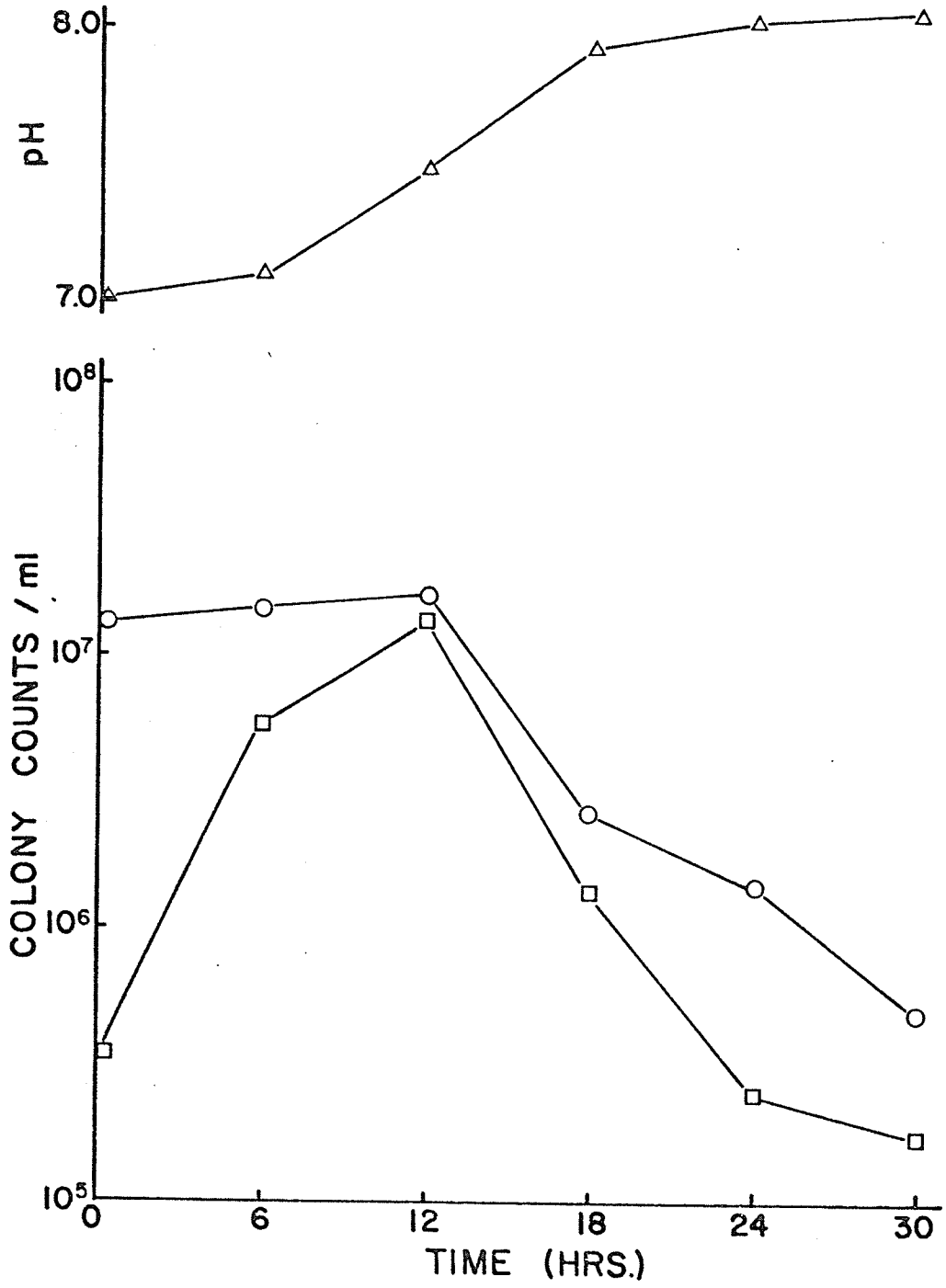

8.0
pH
7.0
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

Figure 13. Effects of thermocin on the growth of B. stearothermophilus var. calidolactis, Sm variant at 55°C. Thermocin activity 0.6 A.U./ml. pH, △–△; total viable cell count/ml, ○–○; spore count/ml, □–□.

(Appendix Table 13)


8.0
7.0
pH
10^8
10^7
10^6
10^5
COLONY COUNTS / ml
0
6
12
18
24
30
TIME (HRS.)

The results obtained in this investigation are in agreement with studies performed by Hii and Blank, 1981. These researchers also exhibited an antagonistic effect on the Sm variant via thermocin produced by the Rh variant.

The pH profiles in these cultures showed an increase towards the alkaline values which is particularly noticeable with the higher thermocin containing cultures. This increase in pH towards alkalinity can be partially attributed to the production of basic substances produced by the growing cultures and/or the liberation of such material upon cellular lysis.

(5) Spore Production Using Mixed Variants at 55°C Under Varying Aeration Rates

Spore production using mixed variants is illustrated in Figures 14-19. Growth cultures initially containing 80% Rh and 20% Sm were shown to completely revert to the Rh variant form as early as 30 minutes after the addition of the inocula. Mixed variant populations initially containing, Rh:Sm(%); 60:40; 40:60; 20:80 were shown to progressively decrease in Rh variant population during the course of growth with a concomitant increase in the Sm variant population.

Results from this investigation appeared to indicate that fermentations containing a Rh population less than 80%, progressively decreased. The exact lower limit of a Rh population required to maintain Rh dominance was not determined although it appears to be between 60-80%. It is interesting to note that fermentations containing initial 20% Rh and 80% Sm were very quickly converted to the Sm variant form, the converse of the 80% Rh and 20% Sm fermentations.

According to Fields (1963), the occurrence of Rh variants in spore populations is probably linked with population pressure. Population pressure can act as a selection pressure, permitting the establishment of any variant that can grow and remain under environmental conditions that exist. This same explanation can be applied to the results obtained in this investigation, in that populations of mixed variants will achieve a dominance dependent upon the occurrence or numbers of that variant initially present in a higher proportion. In addition, differences in nutritional or growth requirements between the variants and their generation time will also affect variant dominance (Fields, 1963).

The specific growth rate constants (μ, h^{-1}) for the mixed variants, Rh: Sm:% ; 80:20; 60:40; 40:60 and 20:80, at an aeration rate of 0.2 ml/min/ml were: 1.7, 1.4, 0.6 and 0.74, respectively.

Using an aeration rate of 0.6 ml/min/ml the specific growth rate constants were: 1.3, 2.4, 1.4, 1.7 h^{-1}, respectively.

In all mixed fermentation cultures aside from Rh: Sm: 80:20% the mean generation time was shown to substantially decrease. This decrease in mean generation time can be partially attributed to the growth of the Sm variant and its increasing dominance during the fermentation which has been reported by Hill and Fields (1967) to have a shorter generation time. Mixed cultures containing Rh: Sm: 80:20% did not show this same trend apparently due to the dominance of the Rh variant throughout the cultural period. As previously shown in this investigation, pure Rh variants grown at 55°C showed only slight growth promoting effects as aeration was increased. The mixed cultures containing Rh: Sm: 80:20% were again noted to show only minimal effects under increased aeration.

Earlier studies performed with the pure Sm variant at 55°C indicated a higher generation time as compared to the

Rh variant. It would appear from these studies that the generation time of the mixed variants is not a function of the individual mean generation time of the variants involved. This disparity may be due to factors occurring during the fermentation period particularly during those periods of time when Rh to Sm variations are occurring.

It is interesting to note that the mixed fermentation culture containing Rh: Sm: 80:20% which maintained Rh dominance, displayed specific growth rates which were similar to those obtained with the pure Rh variant culture at 55°C (μ = 1.1, aeration 0.2 ml/min/ml; μ = 1.1, aeration 0.6 ml/min/ml). The mixed fermentation cultures with Rh: Sm: %; 40:60 and 20:80, which showed increasing Sm dominance, also exhibited specific growth rates, at 0.2 ml/min/ml aeration, similar to the pure Sm variant cultures grown at 55°C (μ = 0.56, aeration 0.2 ml/min/ml; μ = 0.8, aeration 0.6 ml/min/ml).

Fields (1966) and Hill and Fields (1967), working with B. stearothermophilus NCA 1518 Rh and Sm variants, demonstrated a difference in the lag and generation time of the two variants which was partially attributed to the type of cell division exhibited by these variants. According to Bisset (1955), the Sm variant types of bacteria

divide by the constriction of the cell wall while the Rh variant divides by the formation of a complete cross-wall which subsequently splits. It has also been demonstrated that differences in nutritional requirements and aeration exist between these variants (Humbert et al, 1972). Ordal (1957), showed that Mn^{++} may have an influence on metabolite accumulation. Studies with Brucella have shown that Mn^{++} affects the metabolism of the Sm variant and favors the establishment of the Rh variant in initially Sm cultures (Braun, 1953). The results obtained at a higher aeration rate, 0.6 ml/min/ml are presented in Figures 14-19.

Work performed by Hill and Fields (1967), investigating mixed population growth also observed that an initial Rh dominant population quickly became predominantly Sm at 55°C and that the dominance of the Rh was maintained only at 45°C. The decrease in the Rh population was shown to occur regardless of aeration, albeit, the decrease in the Rh population was delayed by approximately 1 hour under limited aeration.

(6) Time Course Growth Studies and Thermocin Production Using Mixed Culture Variants at 55°C Under Varying Aeration Rates

The time course growth studies and thermocin production of B. stearothermophilus var. calidolactis using mixed

Figure 14. Spore production using mixed variants of Bacillus stearothermophilus var. calidolactis. Rh : Sm: 60: 40%. Aeration at 0.2 ml/min/ml. ○—○ , Rh; □—□ , Sm.

(Appendix Table 14).

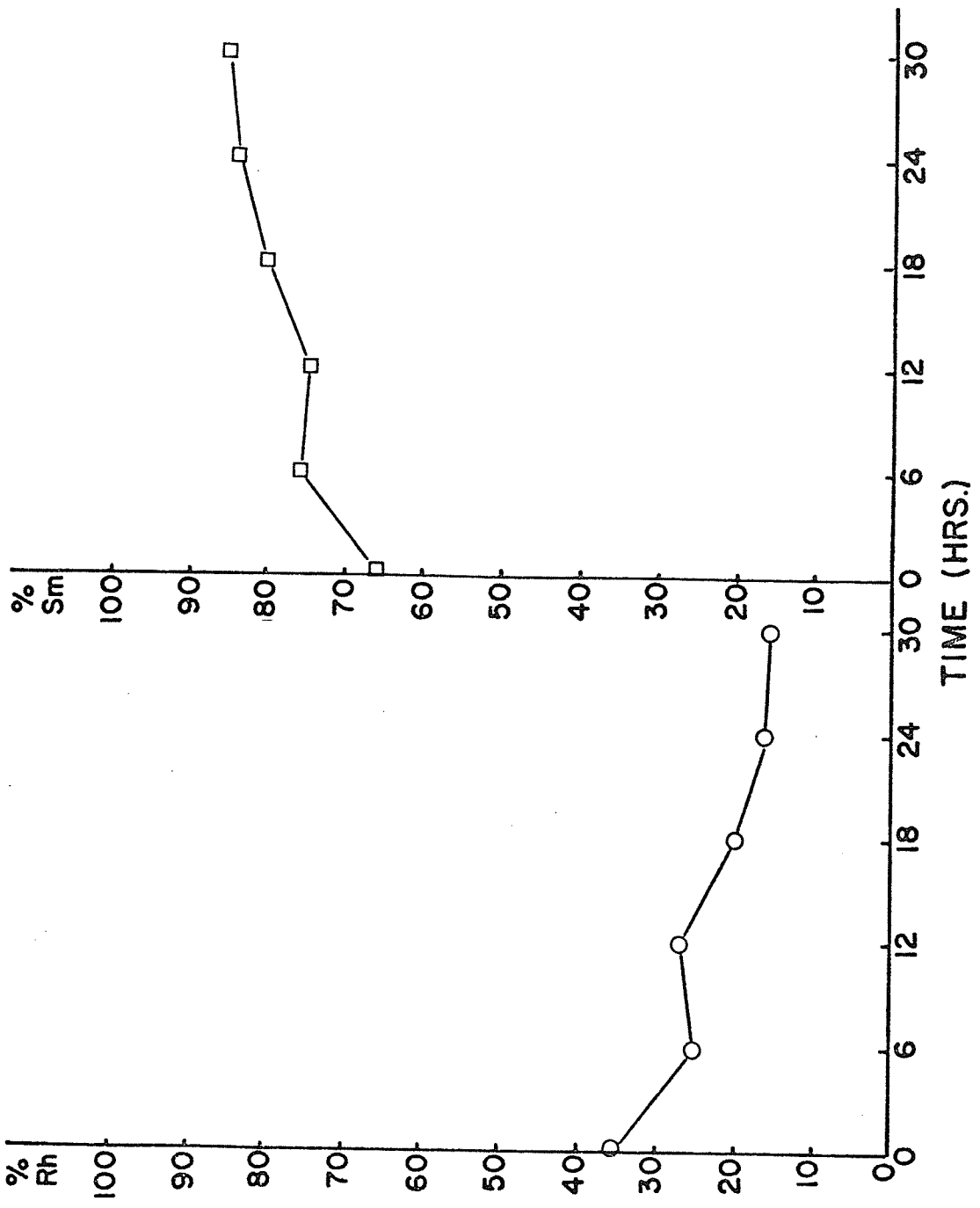

% Rh
% Sm
100
90
80
70
60
50
40
30
20
10
0
6
12
18
24
30
TIME (HRS.)

Figure 15. Spore production using mixed variants of Bacillus stearothermophilus var. calidolactis. Rh: Sm: 60: 40%. Aeration at 0.6 ml/min/ml. ○—○, Rh; □—□, Sm.

(Appendix Table 15)

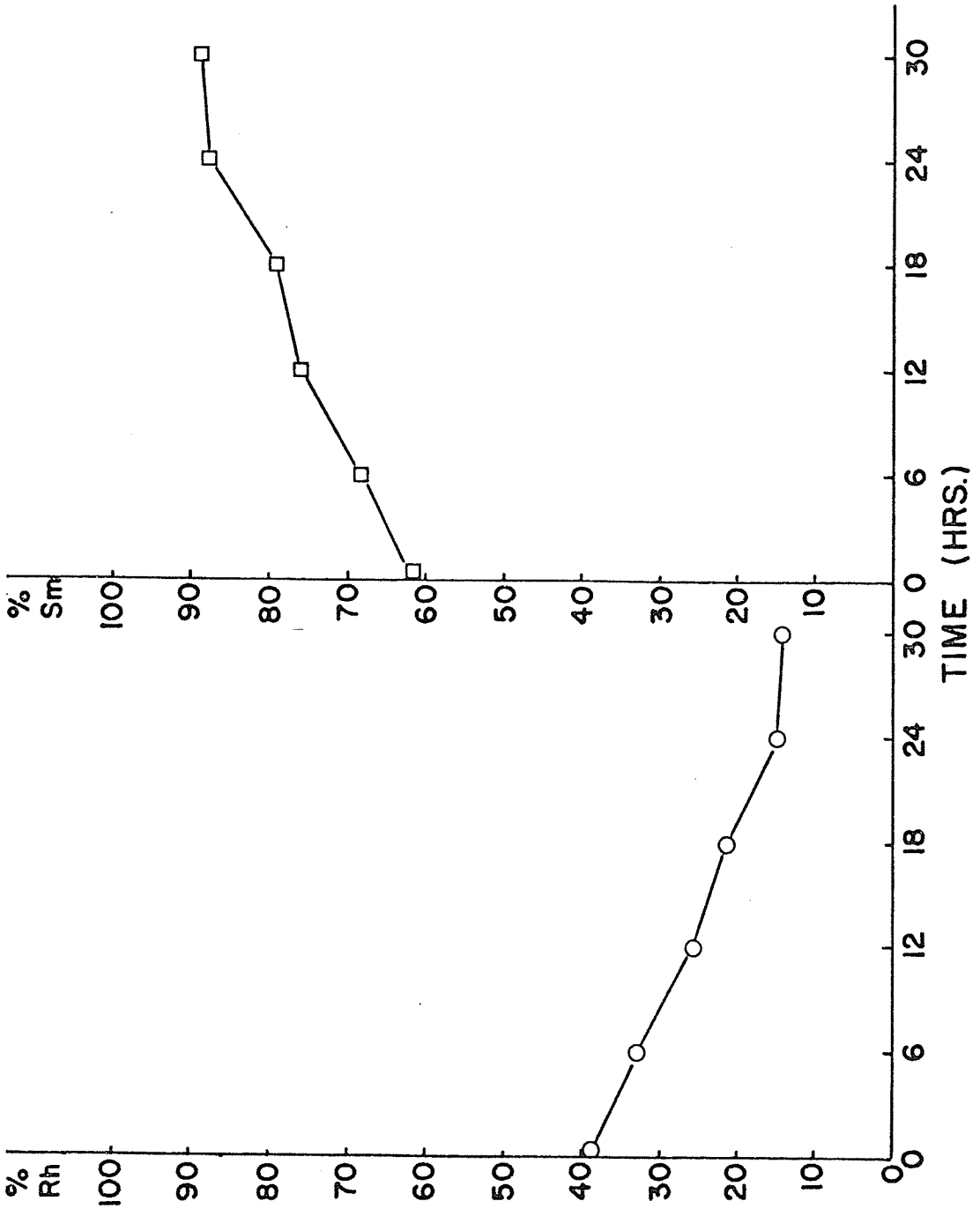

% Rh
% Sm
100
90
80
70
60
50
40
30
20
10
0
6
12
18
24
30
TIME (HRS.)

Figure 16. Spore production using mixed variants of Bacillus stearothermophilus var. calidolactis. Rh : Sm : 40 : 60 %. Aeration at 0.2 ml/min/ml. ○—○ , Rh; □—□ , Sm.

(Appendix Table 14)

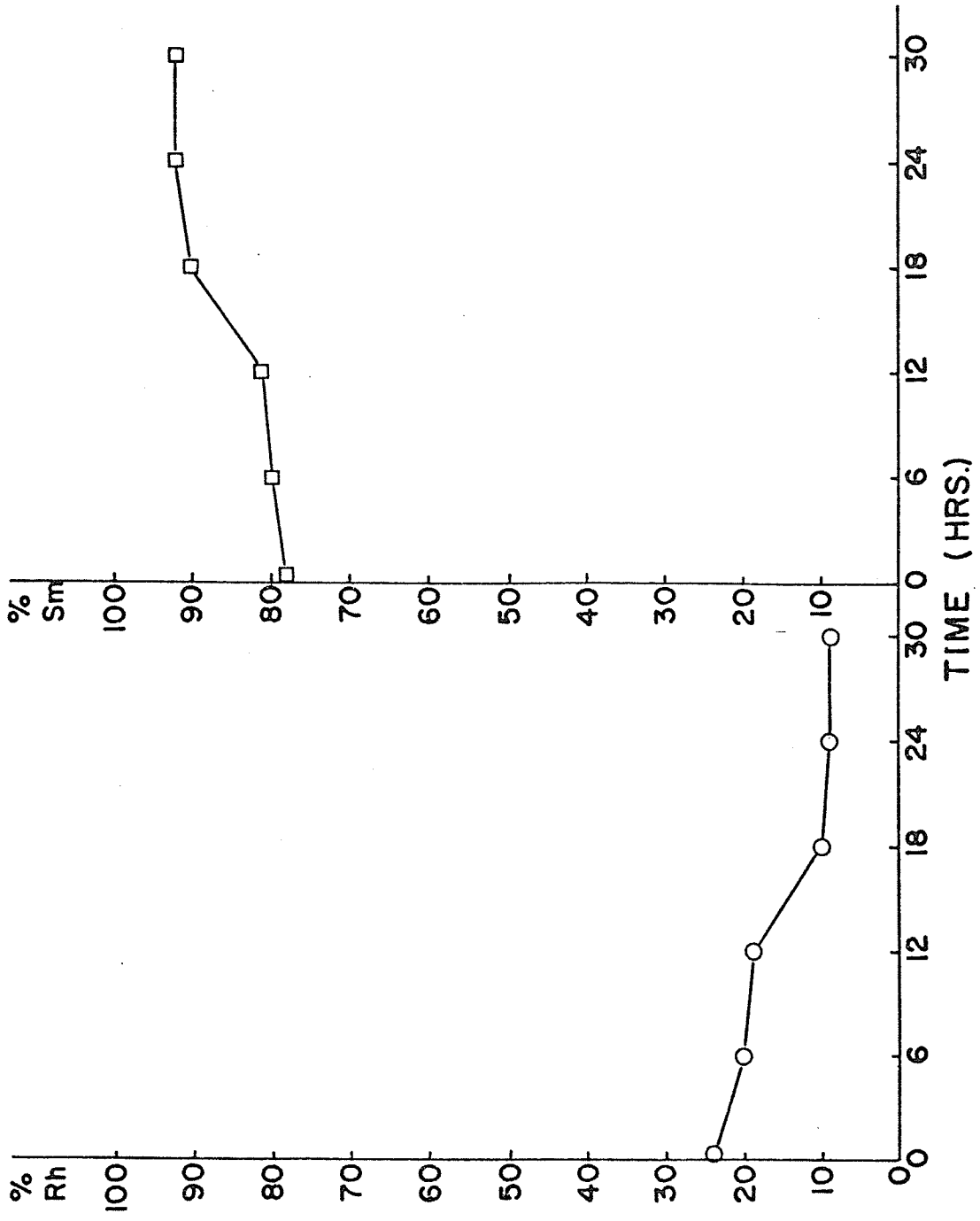

% Rh
% Sm
100
90
80
70
60
50
40
30
20
10
0
6
12
18
24
30
TIME (HRS.)

Figure 17. Spore production using mixed variants of Bacillus stearothermophilus var. calidolactis. Rh : Sm : 40 : 60%. Aeration at 0.6 ml/min/ml. ○–○ , Rh; □–□ , Sm.

(Appendix Table 15)

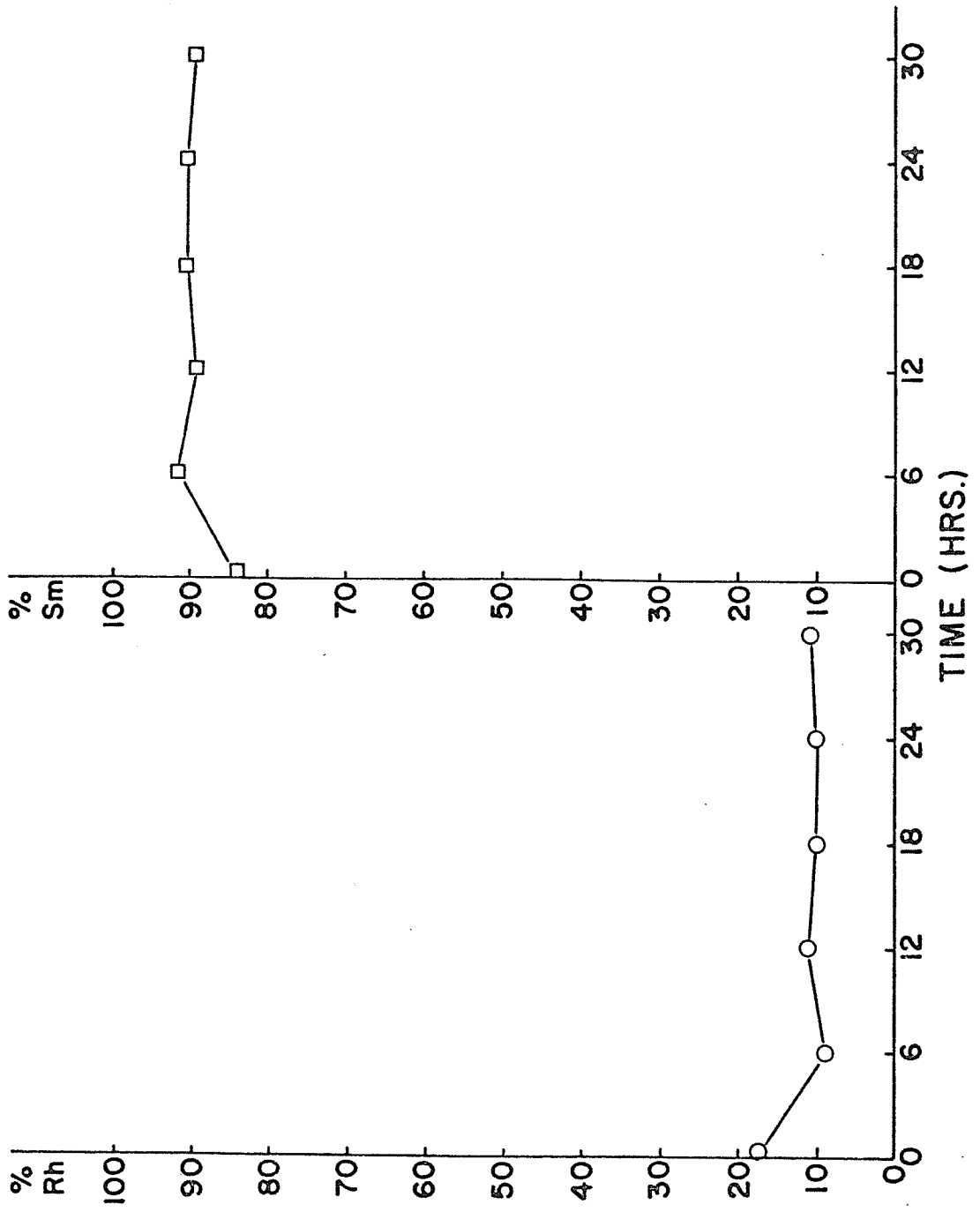

% Rh
% Sm
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
0
6
12
18
24
30
TIME (HRS.)

Figure 18. Spore production using mixed variants of Bacillus stearothermophilus var. calidolactis. Rh: Sm: 20: 80%. Aeration at 0.2 ml/min/ml. O—O , Rh; □—□ , Sm.

(Appendix Table 14)

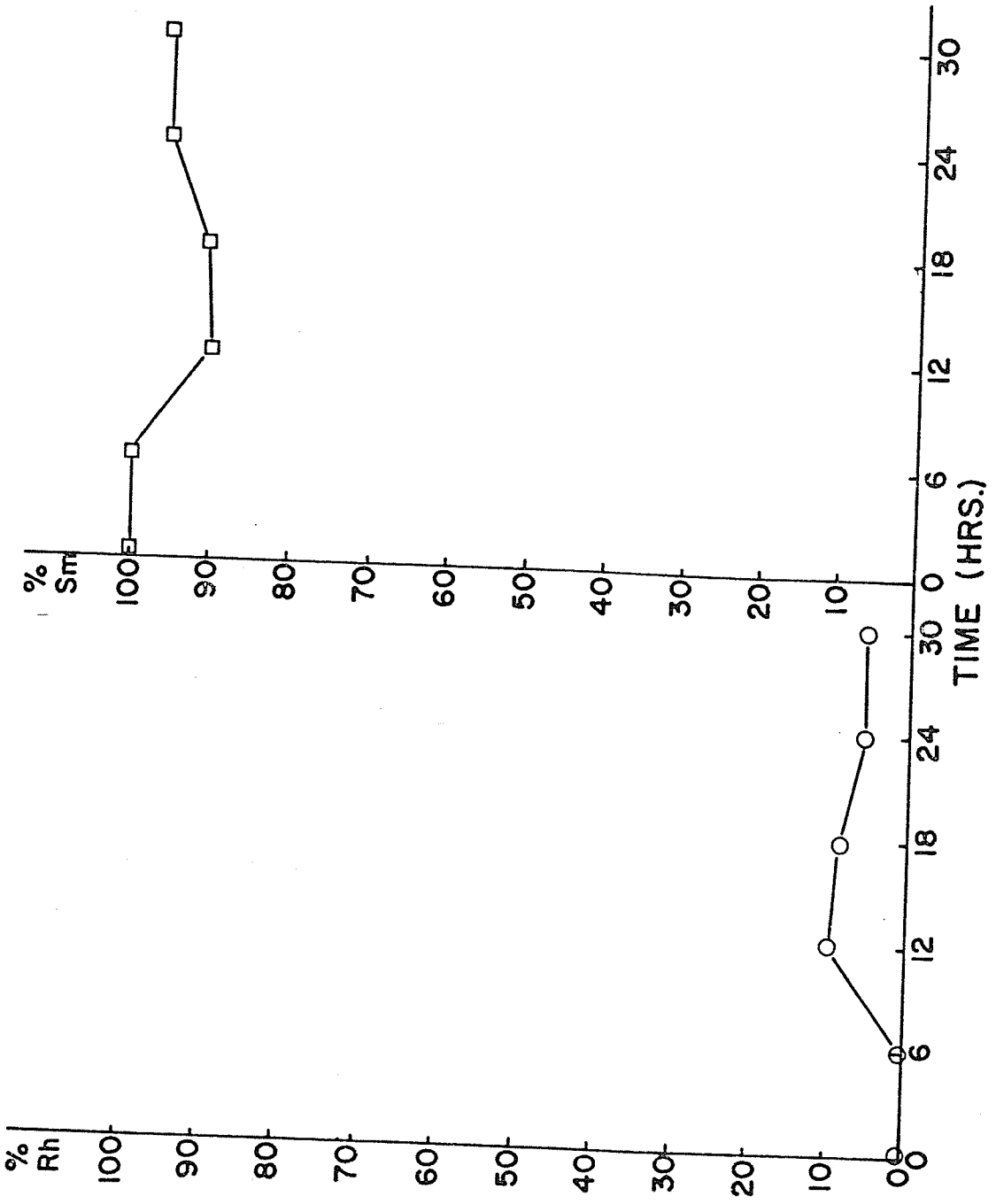
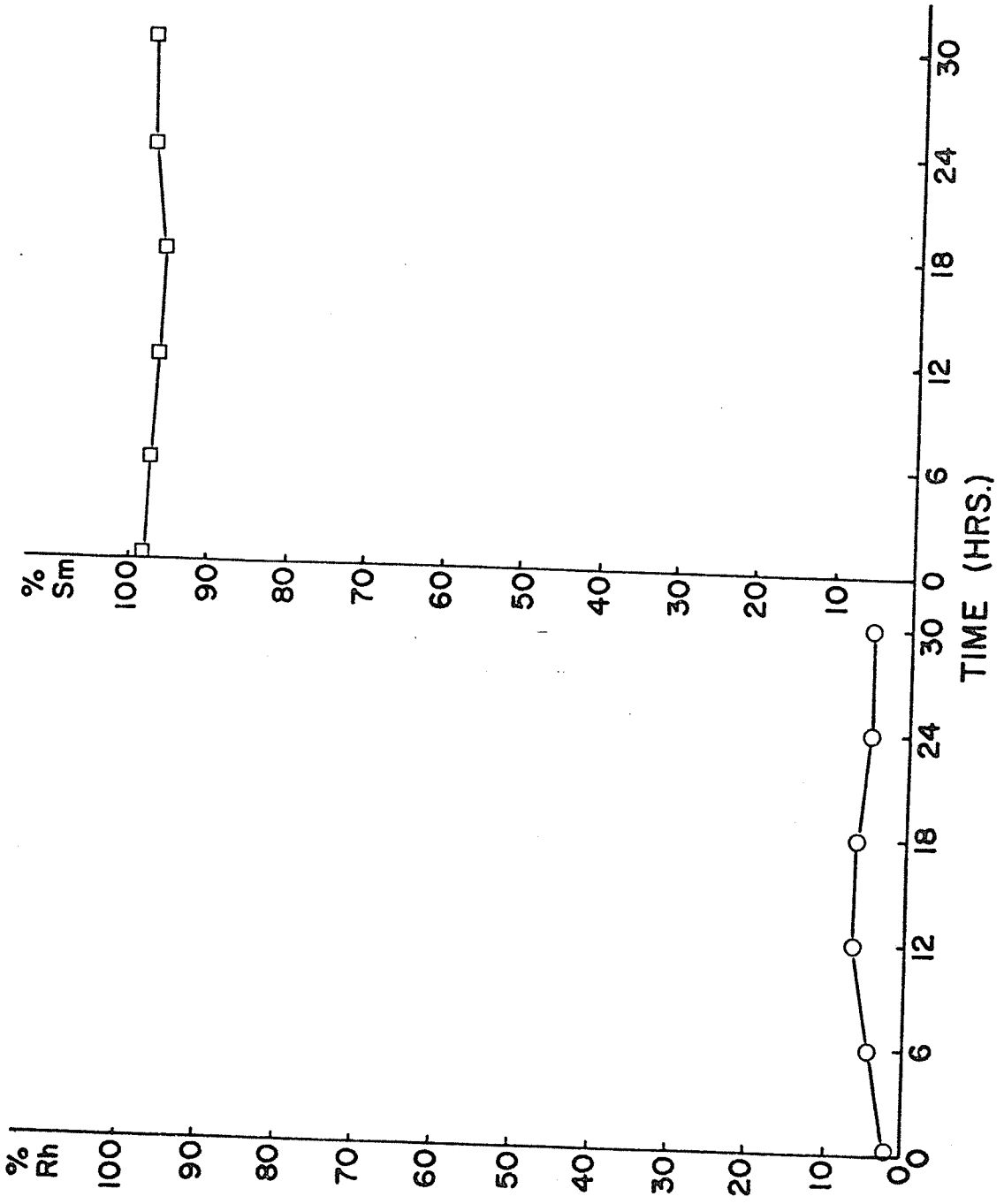

% Rh
% Sm
TIME (HRS.)
0
6
12
18
24
30
10
20
30
40
50
60
70
80
90
100

Figure 19. Spore production using mixed variants of Bacillus stearothermophilus var. calidolactis. Rh : Sm : 20 : 80%. Aeration at 0.6 ml/min/ml. O–O , Rh; □–□ , Sm.

(Appendix Table 15)


% Rh
% Sm
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
TIME (HRS.)

culture variants at 55°C are given in Tables 2-9. The TVC counts were shown to progressively increase in each culture aerated at 0.2 ml/min/ml. A clear relationship between TVC and/or LSC and the variant population used could not be discerned. Thermocin activity was noted to be highest in cultures containing a dominant Rh population. Cultures containing 20% Rh showed no thermocin activity. Thermocin when produced by the cultures was detected only between 0-6 hours. The highest thermocin titer obtained was 1 A.u./ml. Thermocin was noted to disappear after 6 hours of culture. The disappearance of thermocin in the culture medium could be partially attributed to its antagonistic effect on the Sm variant. Since thermocin activity was shown to completely disappear after 6 hours, and in most cases TVC still increased, it is plausible that the transition of Rh to Sm was occurring at a faster rate than thermocin production. Although a reduction in the Sm cell population would be expected, the TVC did not show this. This same effect was noted at the higher aeration rate, 0.6 ml/min/ml. Although increased aeration has been shown to favor thermocin production, the increased growth of the cultures would far supercede the antagonistic activity of any thermocin produced.

Table 2: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.2 ml/min/ml. Rh: Sm: 80:20%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	7.01	$1.6 \times 10^{9*}$	1.2×10^{8}	1
6	6.94	2.1×10^{9}	2.0×10^{8}	1
12	7.17	$1.5 \times 10^{15*}$	2.8×10^{14}	0
18	7.30	2.5×10^{15}	2.8×10^{14}	0
24	7.39	1.6×10^{15}	1.2×10^{14}	0
30	7.42	2.0×10^{14}	1.4×10^{13}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Table 3: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.6 ml/min/ml. Rh: Sm: 80:20%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	7.08	2.2×10^{11}	1.1×10^{10}	1
6	7.26	2.3×10^{11}*	2.2×10^{10}	1
12	7.54	1.5×10^{14}	1.9×10^{13}	0
18	7.71	1.2×10^{16}*	8.1×10^{14}	0
24	7.76	8.4×10^{15}	3.4×10^{13}	0
30	7.81	5.0×10^{15}	3.0×10^{13}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Table 4: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.2 ml/min/ml. Rh: Sm: 60:40%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	6.84	3.0×10^{7}*	1.6×10^{7}	1
6	6.92	1.6×10^{7}	1.0×10^{7}	1
12	7.19	2.7×10^{12}*	1.2×10^{11}	0
18	7.29	1.2×10^{12}	9.0×10^{11}	0
24	7.36	1.2×10^{10}	7.9×10^{9}	0
30	7.42	9.2×10^{10}	5.2×10^{9}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Table 5: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.6 ml/min/ml. Rh: Sm: 60:40%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	6.89	3.3×10^{7}	2.3×10^{7}	1
6	7.05	3.8×10^{7}*	3.1×10^{7}	1
12	7.48	1.1×10^{14}	2.0×10^{13}	0
18	7.69	2.5×10^{16}*	2.8×10^{15}	0
24	7.81	1.2×10^{15}	6.9×10^{14}	0
30	7.87	8.7×10^{14}	3.6×10^{13}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Table 6: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.2 ml/min/ml. Rh: Sm: 40:60%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	6.92	$2.3 \times 10^{7*}$	1.8×10^{7}	1
6	6.87	2.8×10^{10}	1.4×10^{9}	0
12	7.11	6.9×10^{10}	8.9×10^{9}	0
18	7.35	$7.1 \times 10^{10*}$	1.2×10^{10}	0
24	7.46	5.7×10^{9}	1.5×10^{9}	0
30	7.51	7.9×10^{9}	1.0×10^{9}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Table 7: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.6 ml/min/ml. Rh: Sm: 40:60%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	6.79	9.5×10^{8}	3.3×10^{7}	1
6	6.84	2.1×10^{10}*	1.9×10^{9}	0
12	7.34	9.2×10^{10}	3.6×10^{10}	0
18	7.51	2.6×10^{15}*	2.0×10^{14}	0
24	7.72	2.2×10^{15}	1.6×10^{14}	0
30	7.81	1.4×10^{14}	6.7×10^{13}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Table 8: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.2 ml/min/ml. Rh: Sm: 20:80%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	7.06	3.3×10^{8}	2.3×10^{7}	0
6	7.01	$3.8 \times 10^{8*}$	2.6×10^{8}	0
12	7.03	3.4×10^{10}	2.0×10^{9}	0
18	7.15	$1.9 \times 10^{11*}$	1.1×10^{10}	0
24	7.25	2.8×10^{10}	8.1×10^{9}	0
30	7.33	2.0×10^{10}	4.3×10^{9}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Table 9: Time course growth study and thermocin production using mixed culture variants at 55°C. Aeration at 0.6 ml/min/ml. Rh: Sm: 20:80%

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	Thermocin activity (A.u./ml)
0	6.83	1.1×10^{10}	8.9×10^{8}	0
6	7.01	$2.0 \times 10^{10*}$	2.0×10^{9}	0
12	7.36	1.3×10^{12}	2.2×10^{11}	0
18	7.65	$2.3 \times 10^{16*}$	1.6×10^{16}	0
24	7.75	1.5×10^{15}	7.9×10^{14}	0
30	7.82	9.7×10^{14}	3.8×10^{13}	0

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

CONCLUSION

Rh and Sm spores of *B. stearothermophilus* var. *calidolactis* were produced in a nutrient broth, phosphate-based liquid medium. Aeration was more important for the growth and sporulation of the Sm variant although thermocin production by the pure Rh variant at 55°C was dependent upon aeration. Fermentations employing mixed variants will normally revert to the all-Sm variant form unless the Rh variant is present in the excess of 80% indicating some population pressure dynamics. The unintentional use of mixed variants as inocula for the large scale production of spores can lead to various problems. The fact that it has been shown that Rh and Sm variants of *B. stearothermophilus* var. *calidolactis* exhibit different antibiotic sensitivities; that the Rh variant produces thermocin which will affect the growth and sporulation of the Sm variant and that the two variants exhibit different growth rates may lead one to question the Delvotest P if uncertainty exists as to whether or not only pure Sm variant spores are contained in the Delvotest P ampules. The effect of thermocin produced by the Rh variant was only of minor importance so long as the Sm population was in excess of the Rh variant and increasing Sm dominance

was occurring faster than thermocin production. Owing to the pH and thermostable nature of thermocin, any residual levels of this antagonistic agent will affect the growth and sporulation of the Sm variant if present in levels in the excess of 0.55-0.60 A.u./ml. Populations containing either pure Sm and/or pure Rh variants remained stable throughout the fermentation period (30 hrs). Long-term stability of these spores, however, is uncertain at the present time.

RECOMMENDATIONS FOR FUTURE INVESTIGATIONS

Although pure spore suspensions were shown to remain stable throughout the cultural time period (30 hrs), the long-range effects on spore stability in the Delvotest P ampules per se, should be investigated. In addition, the growth and interaction of these variants should be investigated using a simulated Delvotest P.

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Appendix Table 1: Time course growth study, Sm variant at 55°C. Aeration at 0.2 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Heat activated spore count (ml)
0	6.72	1.0×10^8	1.4×10^5	1.9×10^4
6	6.77	$2.6 \times 10^{8*}$	1.8×10^7	5.2×10^4
12	6.85	2.4×10^9	1.1×10^9	1.3×10^5
18	6.99	$2.7 \times 10^{10*}$	1.3×10^9	3.3×10^8
24	7.08	6.8×10^9	7.7×10^9	2.1×10^7
30	7.16	6.0×10^8	5.5×10^8	2.0×10^7

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]heat activated at 110°C for 15 minutes

*used in calculating mean generation time

Appendix Table 2: Time course growth study, Sm variant at 55°C. Aeration at 0.4 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Heat activated spore count (ml)
0	6.73	$9.5 \times 10^{6*}$	2.4×10^{6}	1.6×10^{4}
6	6.80	2.4×10^{8}	6.0×10^{7}	8.9×10^{4}
12	6.87	6.4×10^{9}	1.4×10^{9}	3.7×10^{5}
18	7.18	$2.7 \times 10^{10*}$	5.4×10^{9}	3.3×10^{8}
24	7.30	2.0×10^{8}	2.2×10^{8}	1.9×10^{8}
30	7.42	8.8×10^{7}	8.6×10^{7}	4.9×10^{7}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]heat activated at 110°C for 15 minutes

*used in calculating mean generation time

Appendix Table 3: Time course growth study, Sm variant at 55°C. Aeration at 0.6 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Heat activated spore count (ml)
0	6.76	$7.2 \times 10^{7*}$	6.2×10^{6}	1.4×10^{4}
6	6.81	1.6×10^{8}	1.1×10^{8}	2.4×10^{4}
12	6.88	8.7×10^{8}	1.3×10^{8}	1.5×10^{6}
18	7.27	$1.5 \times 10^{12*}$	1.1×10^{6}	1.6×10^{7}
24	7.48	1.1×10^{12}	7.7×10^{9}	9.8×10^{5}
30	7.63	8.5×10^{11}	1.0×10^{10}	7.1×10^{5}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]heat activated at 110°C for 15 minutes

*used in calculating mean generation time

Appendix Table 4: Time course growth study, Rh variant at 45°C. Aeration at 0.2 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Heat Activated spore count (ml)
0	6.88	6.8×10^{9}	1.4×10^{9}	1.3×10^{4}
6	7.04	1.4×10^{10}	1.7×10^{9}	2.6×10^{4}
12	7.44	5.5×10^{10}	1.1×10^{10}	5.5×10^{4}
18	7.67	1.5×10^{11}*	1.1×10^{11}	2.0×10^{7}
24	7.83	1.2×10^{11}	1.9×10^{10}	8.5×10^{5}
30	7.91	1.1×10^{11}	1.4×10^{10}	3.4×10^{5}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]heat activated at 110°C for 15 minutes

*used in calculating mean generation time.

Appendix Table 5: Time course growth study Rh variant at 45°C. Aeration at 0.4 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Heat Activated spore count (ml)
0	7.11	3.2×10^{10}	1.3×10^{11}	1.1×10^{4}
6	7.24	$5.9 \times 10^{10*}$	1.5×10^{12}	2.8×10^{4}
12	7.53	1.2×10^{14}	1.2×10^{14}	8.3×10^{4}
18	7.72	$2.7 \times 10^{16*}$	1.2×10^{15}	1.9×10^{7}
24	7.84	2.0×10^{16}	1.0×10^{15}	1.7×10^{6}
30	7.92	1.9×10^{16}	1.0×10^{15}	8.8×10^{5}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]heat activated at 110°C for 15 minutes

*used in calculating mean generation time

Appendix Table 6: Time course growth study, Rh variant at 45°C. Aeration at 0.6 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Heat Activated spore count (ml)
0	7.09	$3.8 \times 10^{12*}$	3.4×10^{12}	2.7×10^{4}
6	7.18	4.1×10^{12}	4.2×10^{12}	5.4×10^{4}
12	7.43	1.9×10^{14}	2.6×10^{13}	9.3×10^{4}
18	7.70	$2.7 \times 10^{16*}$	2.5×10^{14}	5.5×10^{7}
24	7.81	1.7×10^{16}	8.9×10^{13}	2.0×10^{7}
30	7.85	2.1×10^{14}	3.6×10^{13}	1.5×10^{7}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]heat activated at 110°C for 15 minutes

*used in calculating mean generation time

Appendix Table 7: Time course growth study, Rh variant at 45°C. No aeration

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Heat Activated spore count (ml)
0	6.74	$7.0 \times 10^{8*}$	5.1×10^{4}	2.1×10^{4}
6	6.79	1.5×10^{9}	9.5×10^{4}	8.2×10^{4}
12	6.79	1.0×10^{9}	1.7×10^{5}	1.6×10^{5}
18	6.81	$2.8 \times 10^{9*}$	1.5×10^{8}	1.3×10^{7}
24	6.83	1.9×10^{7}	1.7×10^{7}	1.9×10^{6}
30	6.84	1.1×10^{7}	1.2×10^{7}	1.2×10^{6}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]heat activated at 110°C for 15 minutes

*used in calculating mean generation time

Appendix Table 8: Time course growth study, Rh variant at 55°C. Aeration at 0.2 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Thermocin activity (A.u./ml)
0	7.00	1.8×10^{8}	1.4×10^{7}	0.05
6	7.07	$2.0 \times 10^{8*}$	3.4×10^{7}	0.10
12	7.19	2.4×10^{11}	2.5×10^{10}	0.15
18	7.24	$1.5 \times 10^{12*}$	9.4×10^{10}	0.15
24	7.28	6.2×10^{11}	2.0×10^{10}	0.20
30	7.30	1.0×10^{11}	7.6×10^{9}	0.20

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]expressed in arbitrary units/ml

*used in calculating mean generation time

Appendix Table 9: Time course growth study, Rh variant at 55°C. Aeration at 0.4 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Thermocin activity (A.u./ml)
0	7.05	1.3×10^{7}	1.8×10^{6}	0.10
6	7.25	$1.4 \times 10^{7*}$	2.9×10^{6}	0.15
12	7.30	1.8×10^{9}	2.0×10^{9}	0.20
18	7.58	$2.3 \times 10^{12*}$	1.4×10^{11}	0.25
24	7.74	2.9×10^{12}	8.8×10^{10}	0.25
30	7.83	1.9×10^{12}	5.8×10^{10}	0.25

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]expressed in arbitrary units/ml

*used in calculating mean generation time

Appendix Table 10: Time course growth study, Rh variant at 45°C. Aeration at 0.6 ml/min/ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)	[c]Thermocin activity (A.u./ml)
0	7.04	$2.0 \times 10^{9*}$	1.5×10^{8}	0.05
6	7.22	1.2×10^{12}	2.8×10^{10}	0.15
12	7.59	2.3×10^{13}	1.2×10^{12}	0.30
18	7.83	$2.8 \times 10^{15*}$	2.6×10^{13}	0.30
24	7.89	1.8×10^{14}	1.7×10^{13}	0.30
30	7.94	5.0×10^{13}	6.7×10^{12}	0.30

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

[c]expressed in arbitrary units/ml

*used in calculating mean generation time

Appendix Table 11: Time course growth study of Sm variant at 55°C at varying thermocin concentrations. Aeration at 0.2 ml/min/ml. Thermocin activity at 0.3 A.u./ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)
0	6.89	1.1×10^5	9.5×10^4
6	7.11	$2.2 \times 10^{5*}$	1.3×10^5
12	7.29	1.7×10^8	1.5×10^7
18	7.69	$2.5 \times 10^{10*}$	2.7×10^{10}
24	7.81	2.8×10^{10}	1.3×10^9
30	7.86	2.4×10^9	7.4×10^8

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Appendix Table 12: Time course growth study of Sm variant at 55°C at varying thermocin concentrations. Aeration at 0.2 ml/min/ml. Thermocin activity at 0.55 A.u./ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)
0	7.01	$6.0 \times 10^{7*}$	2.6×10^{7}
6	7.12	2.6×10^{8}	1.3×10^{8}
12	7.57	$2.9 \times 10^{10*}$	2.1×10^{9}
18	7.90	1.4×10^{8}	2.0×10^{8}
24	8.04	5.1×10^{6}	9.8×10^{6}
30	8.09	1.5×10^{6}	9.0×10^{5}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Appendix Table 13: Time course growth study of Sm variant at 55°C at varying thermocin concentrations. Aeration at 0.2 ml/min/ml. Thermocin activity at 0.6 A.u./ml

Fermentation time (hrs)	pH	[a]Total viable cell count (ml)	[b]Lysozyme spore count (ml)
0	6.98	$1.0 \times 10^{7*}$	5.3×10^{5}
6	7.09	1.8×10^{7}	7.6×10^{6}
12	7.48	$2.0 \times 10^{7*}$	1.2×10^{7}
18	7.93	4.1×10^{6}	1.3×10^{6}
24	8.02	1.8×10^{6}	4.0×10^{5}
30	8.06	7.0×10^{5}	2.6×10^{5}

[a]includes sporangia and endospores

[b]treated with 1% V/V lysozyme solution (endospores)

*used in calculating mean generation time

Appendix Table 14: Spore production using mixed variants at 55°C. Aeration 0.2 ml/min/ml

Inocula Rh: Sm (%)	Fermentation Time: 30 minutes Rh: Sm (%)[a]	Fermentation Time: 30 hours Rh: Sm (%)[b]
80:20	100:0	100:0
60:40	35:65	15:85
40:60	23:77	8:92
20:80	0:100	5:95

[a,b]The Rh: Sm (%) obtained at these time intervals were enumerated from LSC counts (Tables 2, 4, 6, 8). Enumeration of the variants was performed solely by colonial morphology.

Appendix Table 15: Spore production using mixed variants at 55°C. Aeration 0.6 ml/min/ml

Inocula Rh: Sm (%)	Fermentation Time: 30 minutes Rh: Sm (%)[a]	Fermentation Time: 30 hours Rh: Sm (%)[b]
80:20	100:0	100:0
60:40	39:61	13:87
40:60	17:83	11:89
20:80	2:98	4:96

[a,b]The Rh: Sm (%) obtained at these time intervals were enumerated from LSC counts (Tables 3, 5, 7, 9). Enumeration of the variants was performed solely by colonial morphology.

Appendix Table 16: Mean generation times for rough and smooth variants at 55°C and/or 45°C under varying aeration rates

All calculations were based on the following formula:

$$\frac{\log n - \log n_0}{t} = \frac{\log 2}{T}$$

where:

T = mean generation time

t = generation time for individual cell populations (in minutes)

n = number of cells/ml at the end of generation

n_0 = initial number of cells or initial inoculum.