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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.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

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).