FACTORS AFFECTING GROWTH AND SPORE FORMATION OF *BACILLUS STEAROTHERMOPHILUS*[1, 2]

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The effect of aeration on growth and spore formation of mesophilic bacilli has been the subject of a number of studies, but the importance of this factor in thermophiles has attracted little interest. The literature emphasizes decreased solubility of oxygen in liquid media at higher temperatures as a major limiting factor in growth and spore formation of thermophiles. Thus, Imsenecki and Solnzeva (1945) reported the necessity of aeration for maximum vegetative growth of stenothermophiles in liquid cultures. An increased oxygen demand due to decreased solubility of this gas at elevated temperatures was noted by Allen (1953), but Baker *et al.* (1955) observed that the solubility of oxygen in liquid media was only one-half as great at 60 C as at 30 C. Gaughran (1946) found that as the surface to volume ratio of a liquid medium was decreased the viable vegetative count of thermophilic bacteria also decreased. The absence of spores in his cultures "may have been the result of a very low oxygen tension in the liquid medium."

This study was undertaken to determine the effects of type of inoculum and aeration upon the production of vegetative cells and spores of *Bacillus stearothermophilus* at 37 C and 55 C.

EXPERIMENTAL METHODS

Culture. The organism used in this study was *B. stearothermophilus*, National Canners Association number 1518. The morphological and physiological characteristics of this organism were in general agreement with the descriptions of Smith *et al.* (1952).

Media. Complex media were screened for growth and spore formation of the test culture. Most were unsatisfactory for spore formation or were highly colored and therefore unsuitable for turbidimetric determination of growth. Tryptose Basamin glucose broth was satisfactorily low in color and consistently yielded good quantities of cells and spores at 37 C and 55 C. This medium consisted of: Basamin, 0.3 per cent; tryptose, 0.2 per cent; glucose, 0.2 per cent; K_2HPO_4, 0.3 per cent; and KH_2PO_4, 0.1 per cent. An average analysis of Basamin is indicated in table 1. Attempts to improve cell and spore production by addition of mineral salts were uniformly unsuccessful.

Inoculum. Preliminary determinations of growth showed considerable variation in rapidity and quantity of growth and spore formation depending upon the type of inoculum. Twelve-hr washed suspensions of tryptose Basamin glucose broth cultures of *B. stearothermophilus* were used as vegetative inocula after dilution to an arbitrary reading of 49 on a Klett-Summerson photoelectric colorimeter. Initial spore inocula were obtained by culturing at 37 C and 55 C in large flasks containing small amounts of tryptose Basamin glucose broth. Cross-inoculation of the spores obtained at 55 C, to the growth medium at 37 C, and conversely, showed no significant difference in growth or sporulation due to temperature of production of the inoculum. Spores produced at 37 C were used in further experiments since greater percentage sporulation was consistently obtained at this temperature. Spore inocula were prepared according to the method of Long and Williams (1958), diluted to a reading of 49 on the colorimeter, and stored frozen until used. Unused suspensions were discarded after 10 days. Vegetative cells of this organism showed complete killing upon pasteurization at 95 C for 20 min. Viable vegetative cells in spore inocula were killed by this treatment prior to use.

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[2] This work was taken from a dissertation submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of Texas, Austin.

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TABLE 1

*Average analysis of Basamin**

Chemical analysis	%
Solids	96.0
Total nitrogen	11.5
Amino nitrogen (Van Slyke)	5.0
Amino of total nitrogen	57.0
Peptone nitrogen	2.5
Proteose nitrogen	1.0
Polypeptides, purines, pyrimidines and amide nitrogen; by difference	3.0
Carbohydrates; by difference	16.6
Reducing substances	Trace
Ether extractibles	1.5
Total ash	11.0
Sodium chloride	0.5
Amino acid composition	
Arginine	3.5
Histidine	1.5
Lysine	6.5
Tyrosine	4.0
Tryptophan	1.0
Phenylalanine	3.5
Cystine	1.6
Methionine	2.0
Threonine	3.3
Leucine	6.4
Isoleucine	4.7
Valine	4.8
Vitamin content	*mg/100g*
Thiamine	5.0
Riboflavin	5.0
Niacin	50.0
Pantothenic acid	10.0
Pyridoxine	2.5
Biotin	0.3
Folic acid	2.0
Choline	200.0

* Anheuser-Busch Company, St. Louis, Missouri.

Aeration. One-L quantities of tryptose Basamin glucose broth in 2-L Erlenmeyer flasks were aerated at 0, 250, 500, 750, and 1000 ml per min with filtered compressed air by means of coarse porosity sintered glass spargers. In comparative studies on the effects of aeration at given rates, duplicate control flasks were run at 37 C and 55 C at 0 aeration and duplicates at the designated aeration rates.

Nitrogenation and oxygenation. Commercial grade nitrogen gas substituted for air served as a control on possible stimulatory effects of agitation. Nitrogen was introduced into cultures by the method used for air. Oxygen was used in the same manner.

Measurement of vegetative growth and sporulation. Vegetative growth was determined turbidimetrically on washed samples, and percentage sporulation was estimated microscopically on a minimum of 10 fields per stained smear.

RESULTS AND DISCUSSION

Aeration at all rates resulted in increased growth in tryptose Basamin glucose broth at 37 C over that obtained at 0 aeration rate (figure 1). The differences obtained were more marked at the higher rates (500 to 1000 ml/min/L). It would seem, upon first consideration, that all of the rates employed would provide oxygen in excess of the amount required to maintain saturation of the medium with oxygen at 37 C. However, comparison of relative turbidities suggested that this was not the case. A possible solution to this apparent discrepancy lies in the low efficiency of oxygen absorption due to large air bubble size and decreased gas-liquid interface at low aeration rates. At higher rates, smaller bubbles and increased interfacial area improved absorption. Therefore, at lower aeration rates there probably existed relatively low oxygen tensions due to inefficiency of aeration and demand of the culture. At rates of

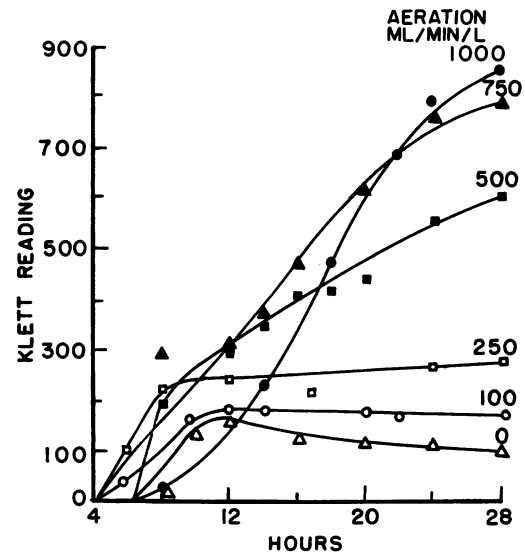

Figure 1. Effect of various aeration rates upon growth of *Bacillus stearothermophilus* NCA 1518 in broth at 37 C.

500 ml/min/L and above, improved absorption of oxygen permitted an approach toward satisfaction of demand which resulted in greater cell yields.

Limitation of growth at lower aeration rates may have been due also to pH changes in the culture. At rates of 500 ml/min/L and above, the pH of the cultures remained within a range of 7.2 to 7.8 throughout the growth period, whereas at the lower aeration rates pH decreased rapidly to about 6.0 for the 100-and 250-ml/min cultures and pH 5.5 to 5.6 for the 0 cultures. These pH changes are characteristic of growth under low oxygen tension.

The results obtained by aerating 55 C cultures containing spore inocula were strikingly different from those observed at 37 C. Aeration at this temperature at all rates prevented germination of the inocula. Without aeration, growth approximated that obtained at 37 C under corresponding conditions (figure 2). The only apparent difference between 0 rate cultures at the two temperatures was a slightly more rapid increase to maximum turbidity at the higher temperature. Pasteurization and subsequent plating of samples from the 55 C aerated cultures indicated that the spore inoculum remained viable during aeration although germination and growth were suppressed. The results obtained at 37 C and 55 C, aerated and unaerated, are summarized in table 2.

Since spore inocula failed to germinate at 55 C with aeration, it was necessary to use vegetative inocula to determine the effect of aeration upon growth at this temperature. Inocula consisted of 1 ml of a 12-hr broth culture per L of growth medium. With this type of

TABLE 2

Effect of aeration on sporulation

Incubation Temp	Aeration Rate	Sporulation	Typical Turbidity Reading*
C	*ml/min/L*	%	
37	0	0–1	140
	100	0–1	180
	250	90–95	285
	500	90–95	600
	750	90–95	770
	1000	90–95	850
55	0	10	145
	100	—	NG†
	250	—	NG
	500	—	NG
	750	—	NG
	1000	—	NG

* Klett-Summerson colorimeter.

† NG = no growth.

Medium: tryptose Basamin glucose broth.

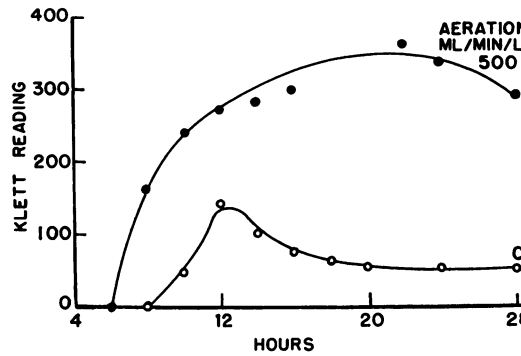

Figure 3. Effect of aeration upon growth of vegetative inocula of *Bacillus stearothermophilus* NCA 1518 in broth at 55 C.

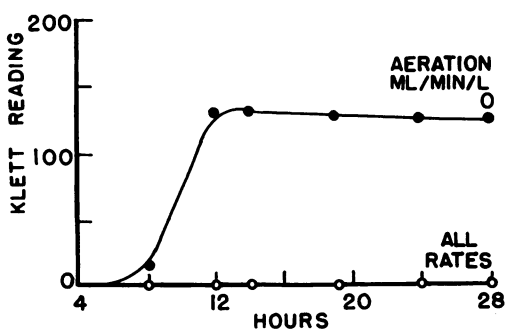

Figure 2. Effect of aeration upon germination of spore inocula of *Bacillus stearothermophilus* NCA 1518 in broth at 55 C.

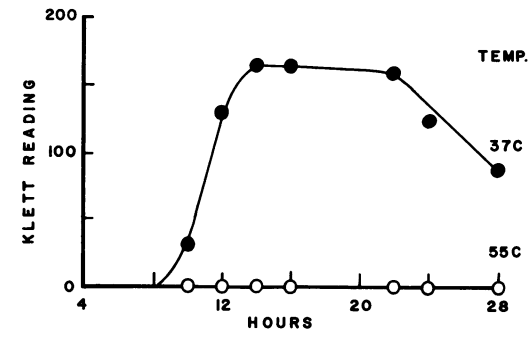

Figure 4. Effect of nitrogen sparging upon growth of *Bacillus stearothermophilus* NCA 1518 from spore inocula in broth at 37 C and 55 C.

inoculum, aeration produced a marked increase in total growth over that found in 0 rate cultures. The results shown in figure 3 represent typical growth at the indicated aeration rates at 55 C. There was only a slight increase in growth with increasing aeration rate regardless of rate employed, indicating optimum absorption of oxygen at all rates.

At the two lower rates of aeration at 37 C, spore formation was extremely limited, generally approximating 1 per cent. At rates higher than 100 ml/min, sporulation in excess of 90 per cent was uniformly obtained (table 2). Using spore inocula, incubation at 55 C yielded a low order of sporulation (10 per cent) at 0 rate, whereas no growth was obtained at higher rates. Although aeration of cultures containing vegetative inocula at 55 C produced good growth, the cultures lysed without sporulation after 5 days.

A marked decrease in absorption of oxygen in the medium at 55 C compared with that at 37 C seems to present an obvious explanation for the observed differences in growth and sporulation (Allen, 1953; Baker *et al.*, 1955; Gaughran, 1946). However, calculation of the absorption coefficients for the two temperatures indicates only a slight loss (14 per cent) in solubility of oxygen in water at 55 C compared with 37 C. This fact might account for decreased growth at the higher temperature but hardly to the extent observed. It therefore seems likely that aeration alone, under the rather rigorous growth conditions imposed by the elevated incubation temperature, exerted a toxicity which inhibited germination and spore formation. This further suggests that the cultural requirements for germination of spores of this strain of *B. stearothermophilus* at 55 C are readily satisfied by essentially anaerobic or microaerophilic conditions.

Sparging spore-inoculated cultures with nitrogen instead of air at 37 C permitted growth comparable to that obtained at 0 rate with the exception that no spore formation occurred (figure 4). The limited growth obtained here was likely due to the presence of small amounts of air in the nitrogen gas. Nitrogen sparging at 55 C completely inhibited germination of the spore inoculum, possibly for the same reason.

Sparging cultures containing spore inocula with oxygen at 37 C permitted only slight growth at even the lowest rate of sparging. It is apparent that an excessive concentration of dissolved oxygen was rapidly attained under these conditions. Oxygenation of spore inoculated cultures at 55 C completely inhibited spore germination and vegetative growth.

SUMMARY

Environmental conditions for production of vegetative cells and spores of a strain of *Bacillus stearothermophilus* at 37 C and 55 C have been described. Aeration of broth cultures at all aeration rates promoted growth and spore formation at 37 C, but inhibited germination of spore inocula and growth at 55 C. Aeration of cultures with vegetative inocula at the higher temperature allowed vigorous vegetative growth, but inhibited spore formation. Vegetative cells produced under these conditions lysed completely during 5 days of incubation. Germination of spore inocula at 55 C was inhibited by sparging with nitrogen gas, whereas at 37 C germination and growth occurred readily although without subsequent spore formation. Sparging of 37 C spore inoculated cultures with oxygen at the lowest rate permitted limited vegetative growth. The same conditions at 55 C completely inhibited growth. No evidence was obtained to show that increased aeration was required for growth and sporulation of thermophiles at elevated temperatures, instead, the converse of this was indicated.

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