EFFECT OF TEMPERATURE ON GROWTH CHARACTERISTICS OF *BACILLUS STEAROTHERMOPHILUS*

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Summary

The maximum temperature at which *B. stearothermophilus* will grow is considerably decreased if a simple medium free of cofactors is used.

Such an increase of thermosensitivity could be caused by an imbalance of the cellular system or a shortage of essential components which the organisms cannot produce from the simple medium at all or at the rate required, or by both of these factors.

The length of the lag period is determined probably by both the adaptation mechanism to a different temperature and the recovery period from a thermal shock during the harvesting procedure.

The number of generations which will grow in a simple, defined medium ($MgSO_4.7H_2O$ 0·08%, $CaCl_2.2H_2O$ 0·018%, KNO_3 0·05%, NH_4Cl 0·28%, glycerol 4%, sodium glycerophosphate 0·6%, and glucose monohydrate 1% in distilled water) at a given temperature is probably determined by the presence of an inhibitory material produced and sustained inside the cell as well as other factors. If the rate of formation of such a material is temperature-dependent, growth would be limited to fewer generations at higher temperatures. Experimental evidence supports such a suggestion.

I. Introduction

As part of an investigation of environmental effects on thermophilic bacteria the influence of temperature on a number of growth characteristics of *Bacillus stearothermophilus* was studied. The growth characteristics studied included the duration of the lag and log periods, the mean generation time, and the yield of biomass per experiment and per generation. Evidence was found also for a possible factor involved in determining the number of generations which will grow in a specified medium under a given set of conditions. The importance of standardizing experimental conditions during bacterial growth has been discussed elsewhere (Bubela and Oberhauser 1966; Scrimshaw, Bubela, and Oberhauser 1966; Bubela, Scrimshaw, and Oberhauser 1967). To obtain meaningful results an apparatus for controlled growth of the microorganisms and a standardized experimental method had to be devised.

Several defined media for thermophilic microorganisms have been described in the literature (Campbell and Williams 1953; Baker *et al.* 1960) all of them containing vitamins, cofactors, and other relatively complex organic compounds. A medium containing organic compounds no more complex than glycerophosphate is described in this paper.

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II. Materials and Methods

All percentages are expressed as w/v, and all reagents are A.R. grade unless otherwise defined. Sodium hydroxide or phosphoric acid was used for pH adjustment.

Microorganisms

Bacillus stearothermophilus type 1503–4 was kept and propagated as described previously (Bubela and Holdsworth 1966*a*). The stock cultures, inocula, and growth media varied according to the experimental requirements.

Medium A

K_2HPO_4 3%, KH_2PO_4 0·1%, Polypeptone 0·5% (Baltimore Biological Lab.), Basamine 0·3% (Basamine–Bush, Anhauser Bush Inc.), glucose monohydrate 1% in distilled water. The medium was sterilized for 30 min at 2 atm. The pH of the medium was adjusted to 6·8.

Medium B

$MgSO_4.7H_2O$ 0·08%, $CaCl_2.2H_2O$ 0·018%, KNO_3 0·05%, NH_4Cl 0·28%, glycerol 4%, sodium glycerophosphate 0·6%, and glucose monohydrate 1% in distilled water. The medium was adjusted to pH 6·8 and sterilized by filtration through Millipore membrane HAWP 04700–HA (0·45 μ).

Apparatus for Control of Bacterial Growth

This apparatus was similar to that described previously (Bubela and Oberhauser 1966) with the difference that the medium was circulated by a peristaltic pump during the growth period through a densitometer and the change of density of the medium due to the bacterial growth was recorded automatically.

Replication

The microorganisms were collected in the middle of the log phase, washed with corresponding medium, and diluted to give a bacterial concentration of 1000/ml. An aliquot of 0·1 ml was then transferred to a Petri dish containing the required agar medium and incubated at the temperature under investigation. When bacterial colonies became visible the plate was "replicated" by the method of Lederberg and Lederberg (1952). Where "microreplication" was desired the method was altered as follows: the inoculated plates were removed from the incubator before the colonies could be detected by the naked eye, and several small rectangles about 4 mm square were removed from the agar with a sterile scalpel and observed directly under phase contrast with ×980 magnification. If clusters of 5–50 bacteria could be detected under the microscope, such a plate was used for replication and both the replica plate and the original plate were incubated until colonies appeared. Then the relative position and number of the colonies were compared

Incubation of Plates

To prevent water losses from the plates during the incubation at temperatures over 50°C the plates were placed upside down in tins containing a small beaker with distilled water and the tins were closed with well-fitting lids. Under such conditions the plates could be incubated for several weeks without any deterioration due to water losses.

Dry Weight Estimation of the Biomass

Two methods were used for the estimation of the dry weight of the biomass:

(1) Aliquots were dried to constant weight at 105°C.

(2) The bacterial suspension (10 ml) was passed through Millipore Solvinert UG filter (0·25 μ). The filters were washed with 5 ml distilled water and then dried at 106°C to constant weight. A filter treated with distilled water and dried was used as a "blank".

Estimation of Mean Generation Time

Mean generation time was calculated from the formula

$$(\log n - \log n_0)/t = \log 2/t_m,$$

where n is the dry weight at time t_n, n_0 is the dry weight at time t_{n_0}, t is the time interval $t_n - t_{n_0}$ in minutes, and t_m the mean generation time which was estimated for the middle region of the log phase.

Inhibition Test

To test whether a growth-inhibitory substance appeared in the medium, the following procedure was used. An inoculum in medium B was prepared from a slope of medium B and grown at 53°C for 16 hr. The microorganisms were harvested and resuspended in 500 ml of medium B to give a reading of 20 on the Klett colorimeter (filter No. 54) using medium free of organisms as a blank. The organisms were then grown (cf. preparative medium) for 24 hr at 59°C. The preparation was freed of the organisms by centrifuging for 30 min at 10,000 *g* and filtering twice through a sterile Millipore HAWP 04700–HA filter (0·45 μ). The sterile medium was then divided into five lots to which the following sterile materials were then added:

1, sodium glycerophosphate 0·6% (w/v);
2, glucose 12% (w/v);
3, sodium glycerophosphate and glucose;
4, no addition;
5, no addition.

Freshly harvested organisms grown at 53°C in medium B were added to lots 1, 2, 3, and 4 to obtain a reading of 20 on the Klett colorimeter (filter No. 54), using sterile medium as a blank. No organisms were added to lot 5 which served as a blank for sterility. Lot 6 was prepared from 100 ml of medium B incubated at 59°C for 24 hr and then inoculated with freshly harvested organisms grown at 53°C. All samples were then incubated for 24 hr at 53°C and checked for bacterial growth.

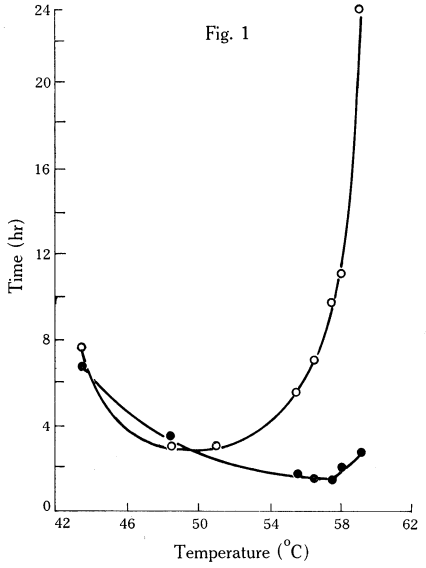
III. Results

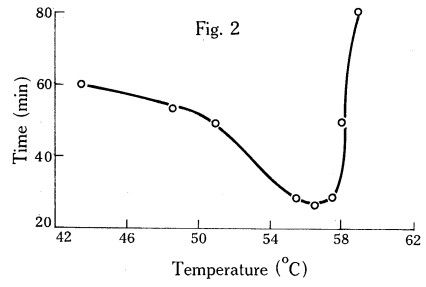
When *B. stearothermophilus* was grown in medium B, the temperature of the growth medium, the duration of lag and log phases, and the biomass expressed in milligrams dry weight at the end of the log phase were measured. Mean generation time, number of generations per log phase, and biomass per generation per millilitre medium at the end of the log phase (expressed in milligrams dry weight) were calculated from such measurements. The relationship between the temperature and the duration of the lag and log phases respectively is presented in Figure 1. The minimum lag value corresponds to a temperature of about 50°C and the minimum log value was obtained at about 56°C. The calculated values for mean generation times as plotted against the temperature are presented in Figure 2. The number of generations was plotted against the temperature, against dry weight per millilitre of medium at the end of the log phase, as well as against the apparent dry weight per generation per millilitre of medium. The graphs are presented in Figure 3. The number of generations and the dry weight decrease with the experimental temperature but the weight per millilitre per generation is constant.

The results of the inhibition test (see Section II) are presented below:

Lot No.	Inoculation	Growth	Lot No.	Inoculation	Growth
1	+	+	4	+	+
2	+	+	5	−	−
3	+	+	6	+	+

They indicate that the microorganisms do not liberate a component into the medium which limits the number of generations during the log phase, that the medium is sufficiently sterile not to produce a visible growth from any contaminating microorganisms within 24 hr when incubated at 53°C, that the energy source of the medium is not exhausted at the end of the log phase, and finally that the medium itself is not altered sufficiently after 24 hr at 59°C to prevent bacterial growth after a fresh inoculation. The pH of the medium did not change appreciably during the growth of the organisms. There was no appreciable difference in the intensity of the bacterial growth in lots 1–4 and lot 6.





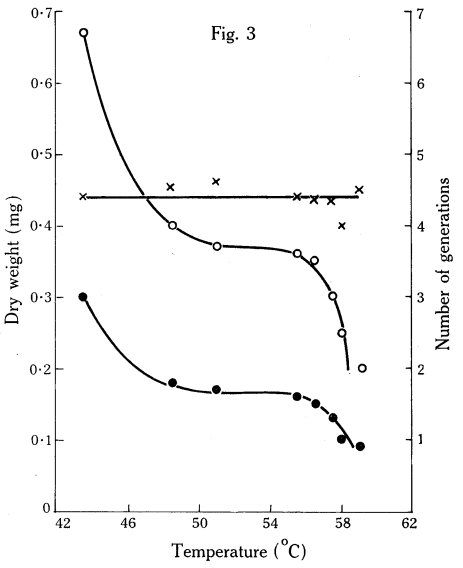


Fig. 1.—Duration of the lag (○) and log (●) phases plotted against the experimental temperature.

Fig. 2.—Calculated mean generation times plotted against the experimental temperature.

Fig. 3.—Number of generations (○), the maximum dry weight at the end of the log period in milligrams per millilitre (●), and the dry weight per generation per millilitre (×), plotted against the experimental temperature.

When the bacteria were plated on a medium B agar plate at 53°C and replicas then made onto a medium A agar plate and incubated at 63 or 53°C, the same number of colonies located at corresponding areas was obtained. Similar results were obtained when the "micro-replica" technique was used.

The organisms grew equally well on complex medium A agar plates, simple medium B agar plates, and simple medium B silica plates, if incubated at 53°C. The microorganisms did not grow on medium B above 59°C but colonies were observed

up to 69°C on medium A. The results indicate that the microorganisms can grow in the absence of relatively complex organic molecules. The possibility that the organisms assimilate some components present in agar was eliminated by the observed growth on silica gel plates.

IV. Discussion

Before any responses of *B. stearothermophilus* to the temperature in medium B could be considered, it was necessary to establish that the differences in measurements were not due to bacterial contamination, selection, or mutation. When replica plates of the microorganisms were made from simple medium B agar or silica gel plates grown at 53°C on to complex medium A agar plates a growth resulting in the same number of colonies at corresponding locations was observed at temperatures above 60°C. Such an observation was interpreted as evidence that it was the same genotype that was being examined at the various temperatures and on the two different media.

As the replication method involves transfer of microorganisms from colonies having a relatively high population, the chances for the occurrence of thermoduric or thermophilic mutants is reasonably high; the microreplication method was used in order to minimize this possibility. The concentration of the microorganisms was relatively low at the time of replication (5–50 microorganisms per cluster) and the chance that mutants were selected was therefore considerably decreased. The results obtained were taken as an indication that even if the possibility of mutation or selection cannot be eliminated, the effect of mutants on the results was minimized. The batches of bacteria were checked frequently by the above replication method.

In spite of the fact that *B. stearothermophilus* grows in medium B, the necessity for some specific materials for its survival at higher temperatures is evident from its growth characteristics. Such a requirement could be explained in at least two ways:

(1) There are two main groups of reactions taking place in a cell; the anabolic and degradative reactions. Among degradative reactions we can, for our purpose, include the normal catabolic reactions and such processes as the thermal inactivation or destruction of thermolabile components. With increase in temperature the rate of the degradative processes could be caused to increase disproportionally and lead to death of the cell. It has been shown before (Bubela and Holdsworth 1966*b*) that high turnover of proteins and nucleic acids might be one of the factors enabling *B. stearothermophilus* to survive at high temperatures.

It is possible that certain metabolites required for such a high turnover might be readily available in medium A but are lacking in medium B. Alternatively, it is possible that the cell might be able to produce them in sufficient quantities for growth at temperatures below 60°C but their supply might become critical at higher temperatures.

(2) The enzymes or enzymic systems involved in the formation of certain metabolites might be heat labile and therefore the cell would lack an endogenous supply of such components at extreme temperatures unless they could be obtained from the medium.

It appears possible that mechanisms (1) and (2) may operate concurrently.

It is evident from Figure 1 that both lag and log phases had a temperature-dependent minimum value. The minimum lag was at about 50°C and the minimum log at about 56°C. All the inocula were prepared at 53°C irrespective of the temperatures at which the organisms were later grown during the experimental period. It was surprising therefore that the minimum lag period was not observed at 53°C. During the harvesting of the inoculum, the organisms were centrifuged at 2°C. It could therefore be suggested that the length of the lag period depends not only on the possible changes the organisms have to undergo to become adapted to the new temperature during the experimental period, but also on the recovery from the thermal shock they suffered during the harvesting period.

The minimum log phase was observed at about 56°C. The minimum mean generation time was observed for the same temperature. The corresponding number of generations was calculated to be three.

There could be several causes limiting the number of generations during the growth period. For example, one or more of the essential components of the medium may become exhausted; or the medium may change due to bacterial growth (e.g. changes in pH), or due to the exposure to temperature; or the organisms may produce and liberate into the medium a material which is inhibitory to the organism at a certain critical concentration. The results do not support any of these possibilities.

However, the organisms may produce an inhibitory material which accumulates in the cell and is not necessarily liberated into the medium. Such a component has been recently described in *B. stearothermophilus* (Okamota 1967). This cellular fraction has been partly purified and is present in the *t*-RNA fraction in the log phase. It contains organic phosphate and does not absorb ultraviolet light in the 260–280 mμ region. It does not give a positive reaction for hexoses and pentoses. It inhibits the binding of amino acids to *t*-RNA. If the rate of production of such a component increases with the incubation temperature, then it could correspondingly limit the number of generations. As each daughter cell would contain, at least theoretically, one half of the concentration of such a material from the mother cell, it is evident that with an increased incubation temperature the critical concentration inhibiting the growth would be reached in fewer generations.

If the curve representing the number of generations in Figure 3 is extrapolated to zero, the corresponding temperature would be 60°C; no growth was indeed observed at this temperature in medium B. Similarly the lag period corresponding to 60°C should approach infinity. The value of the lag period for such a temperature as extrapolated from Figure 1 does approach infinity.

In medium A, growth was observed at temperatures higher than 60°C. As the metabolism of the cells of *B. stearothermophilus* probably follows different pathways in different media (Wilson 1966; Wilkes and Holdsworth, unpublished data), further studies are required in this respect.

V. Acknowlegments

The author acknowledges gratefully a gift of *B. stearothermophilus* from Professor W. H. Elliot, Department of Biochemistry, University of Adelaide, South Australia, and the enthusiastic help of Mrs. J. Cottier.

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