

THE DYNAMIC NATURE OF THERMOPHILY*

By MARY BELLE ALLEN

(From *Hopkins Marine Station, Pacific Grove, California, and the Department of Chemistry, Laboratories of The Mount Sinai Hospital, New York*)

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Ever since the importance of proteins for the metabolic processes of living organisms has been recognized, there have from time to time been speculations as to what sort of protein could be present in those organisms which are known to grow and metabolize at temperatures above the coagulation point of typical proteins. Since these thermophilic organisms engage in enzymatic activities at temperatures at which most enzymes from ordinary mesophilic forms are inactivated, it has been accepted as obvious by most of those who have considered the problem that they must contain heat-resistant enzyme proteins. The present investigation, in fact, was started with the ultimate aim of finding out in what ways such heat-resistant proteins differ from the more usual types.

Although we do not know the nature of the difference between thermophilic and mesophilic microbes, it is possible, from what is known about thermophils, to describe certain properties of this difference. In the first place, all the evidence which is available indicates that the metabolic processes of thermophils are essentially similar to those of mesophils. As with mesophils, a wide variety of materials can be used as sources of energy and for the production of cell materials by these organisms. In the few cases which have been investigated from this point of view, the products of the breakdown of these nutrients are the same as those found for analogous mesophilic forms. Enzymes of thermophilic organisms must therefore carry out the same functions as those of ordinary bacteria.

Second, there is no natural dividing line between thermophils and mesophils. Within the group of aerobic spore-forming bacteria, for example, a continuous spectrum of organisms from those which will not tolerate temperatures above 35° to those which will not grow below 40–45°C. can easily be assembled. Any division into thermophils and mesophils on the basis of a set temperature is thus purely arbitrary and a matter of convenience, rather than the expression of any discontinuous natural separation. The difference between the two types, therefore, must be one which is susceptible to continuous variation.

And third, the property of thermophily is one which can be gained and lost, at least in some bacteria, with a frequency comparable to that of other variable

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characteristics. The classical example of adaptation of a microorganism to a higher or lower temperature range than that within which it initially could grow can be found in the work of Kluver and Baars (1932) and of Starkey (1938) on *Sporovibrio desulfuricans*. In this case, cultures isolated at 55°, which would not grow below 40° when isolated, could be transformed to cultures which would grow well at low temperatures, and the reverse process could be demonstrated with cultures isolated at 25°. In both cases, the change was accomplished by a series of transfers at successively lower or higher temperatures, with a sort of crisis occurring at 40°. On the other hand, Casman and Rettger (1933) were unable to adapt *B. subtilis* to higher temperatures by a series of transfers at gradually increasing temperatures. Experience in this laboratory has shown that the ease of adaptation of thermophiles to low temperatures varies with the culture used, some strains being able to grow below their original minimum temperature after a few transfers at the minimum, while others are refractory toward such adaptation. Gain of the ability to grow at high temperatures may be the result of a discontinuous change rather than a gradual adaptation. By using techniques of mass inoculation, such as are used for the selection of mutants, it has been possible to isolate thermophilic variants from several pure cultures of ordinary aerobic spore formers, including such typical mesophiles as *B. polymyxa* and *B. megatherium*. These observations suggest that the discrepancy in the results obtained by Kluver and Baars on the one hand, and by Casman and Rettger on the other, might be explained by the differences in methods used; Kluver and Baars transferred large inocula, Casman and Rettger, small ones. Although much needs to be done on the nature of this type of variation and the conditions for obtaining such variants, it seems established that it is possible to obtain thermophilic bacteria from mesophilic ones under laboratory conditions. It seems reasonable to conclude from this that a relatively small change must be involved in the transformation from mesophily to thermophily.

Experimental studies on several aerobic and facultatively anaerobic thermophilic spore-forming bacteria have shown that the ability of these organisms to grow and metabolize at high temperatures is not, or at least is not primarily, due to an intrinsic structural stability of their proteins which enables them to resist these extreme conditions, but is predicated upon the active metabolism of the bacteria. When active metabolism is impossible, the bacteria are no more heat-resistant than mesophilic forms.

The thermophilic bacteria used in these experiments were isolated from soil and stable manure at 55–65°C. In their general properties, other than temperature range, they resemble members of the *subtilis* group. All have a maximum temperature of ca. 65°C., while the minima vary from 25–45°, depending on the strain. Descriptions are being published elsewhere (Allen, 1949).

Killing rates were determined by washing the cells from a 3 to 6 hour old slant

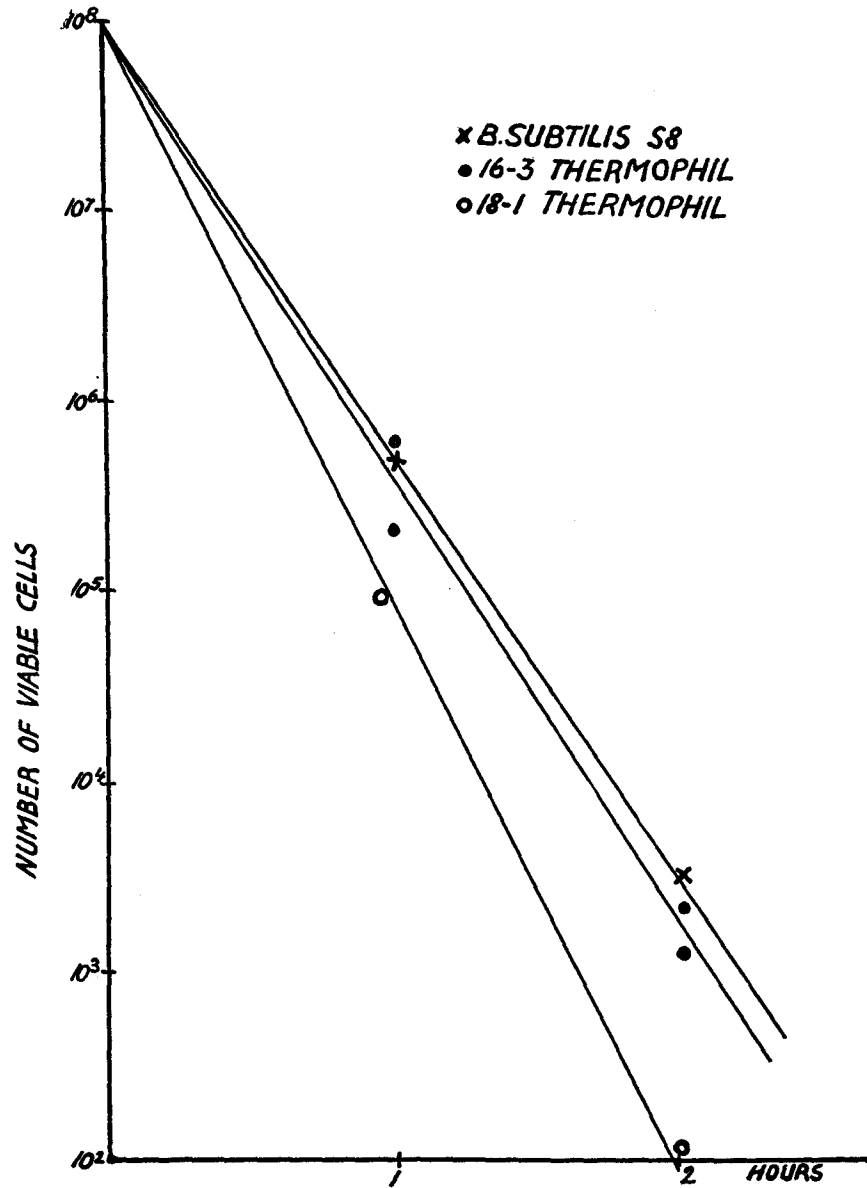


FIG. 1. Killing rates of two strains of thermophilic bacteria compared with that of the mesophilic *B. subtilis* S8.

(maximum growth is reached in 6 to 8 hours), suspending in a bicarbonate buffer of pH ca. 8 (which is optimum for growth when nutrients are present), incubating in

a 55° water bath, removing samples from time to time, and plating appropriate dilutions.

The rapid decline in numbers of viable cells when vegetative cells of several thermophils are suspended in a buffer solution without added nutrients, at 55°, is shown in Fig. 1, together with comparable results on mesophils. It will be seen that the rate of killing is of the same order of magnitude in both cases. Addition of glucose has little, if any, effect on the death rate, but if nitrogenous

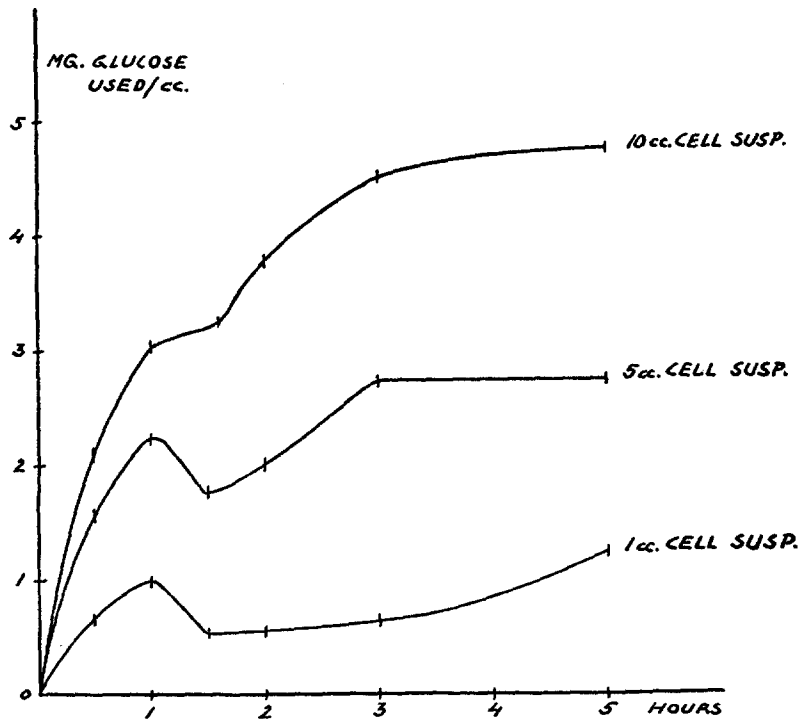


FIG. 2. Utilization of glucose by resting cells of thermophil 16-3 at 55°.

nutrients are added, the thermophils grow, while the mesophils continue to die. Microscopic observation of a suspension of thermophils which has been incubated in buffer at 55° for some time shows very few intact cells, mostly amorphous clumps of precipitated protein and other detritus.

Similar results are obtained if, instead of the viability of cells, the activities of enzyme systems in resting cells at high temperatures are investigated. Among the thermophilic bacteria which have been studied are several which ferment glucose to a complex mixture of products, resembling those obtained from the mesophilic Ford strains of *B. subtilis* (Allen, 1949). The utilization of glucose under anaerobic conditions by resting cells of one of these bacilli is shown in Fig. 2.

Glucose utilization by resting cells was determined with 6 to 8 hour old cultures, grown aerobically on yeast-glucose-calcium carbonate medium (older or younger cells, or those grown on other ordinary media, are not as active). Such cells were suspended in a 1 per cent glucose, 1 per cent sodium bicarbonate medium and incubated anaerobically at 55°. Samples were removed at intervals and the reducing sugar was determined by the Luff-Schoorl method.

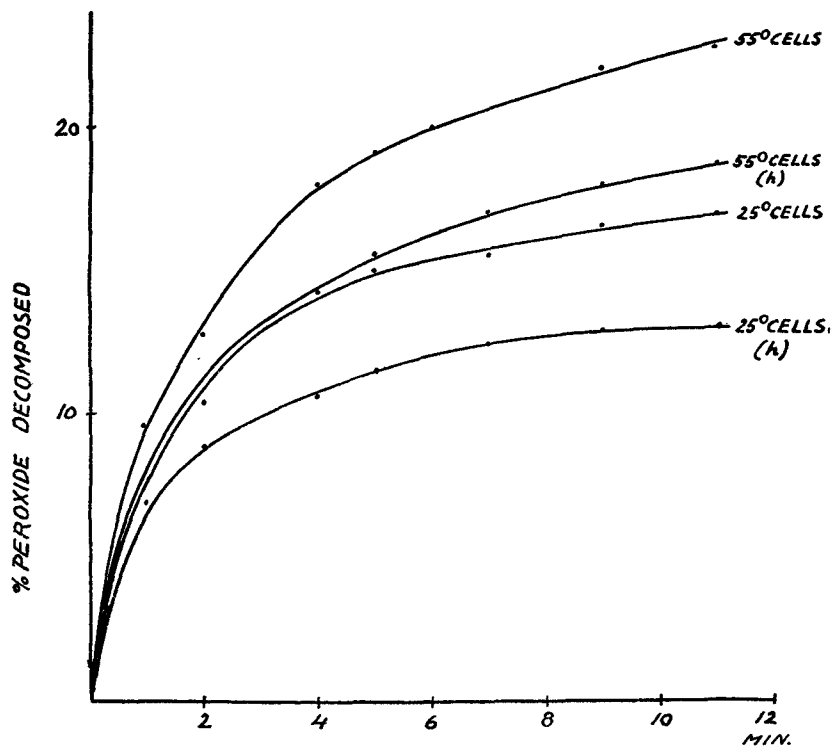


FIG. 3. Breakdown of H_2O_2 by cell-free extracts of thermophil strain 16-2 at 55°. Cultures grown at 25 and 55°C. Curves marked (h) were obtained with preparations heated 10 minutes at 55° in absence of substrate.

Glucose disappears rapidly at first, then the reaction stops, and from then on, often after a period of regression, follows a halting and irregular course, which can be accounted for on the basis of growth of a few bacteria on products released by lysis of the majority of the organisms. Likewise, when cell-free extracts of a thermophil were prepared and the decomposition of hydrogen peroxide by this preparation was observed, the enzymes involved were rapidly inactivated, as shown in Fig. 3.

Cell-free extracts for catalase plus peroxidase determinations were prepared from cells grown on yeast agar, frozen, dried *in vacuo*, and the dry cells ground to a powder. This powder was extracted with 0.01 M phosphate buffer, pH 7; 60-70 per cent of the

peroxide-decomposing systems were obtained in the extract. Since manometric determinations did not seem feasible at high temperatures, the peroxide remaining was determined by iodimetric titration of samples at frequent intervals.

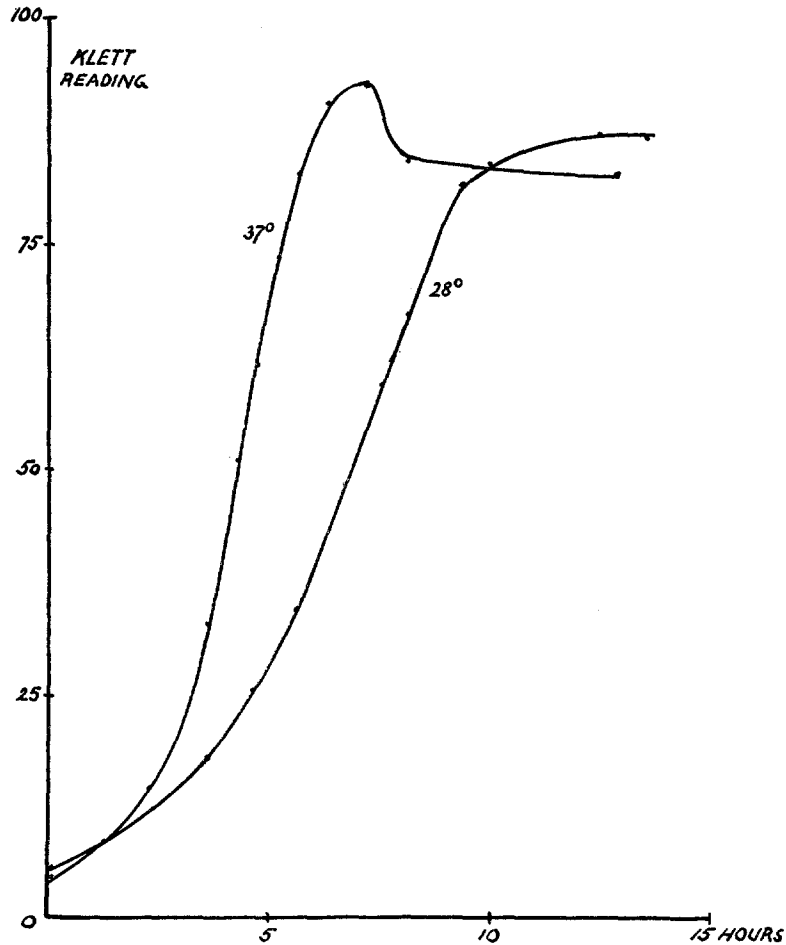


FIG. 4. Turbidometric growth curves of *Bacillus subtilis* at 37 and 28°C.

The inactivation of enzymes of thermophilic bacteria in resting cell suspensions at high temperatures is also apparent in the recently published results of Gaughran (1949) on the temperature coefficients of several enzymes from thermophilic bacilli. His curves for the activity at different temperatures of several dehydrogenases, of the cytochrome-*p*-phenylenediamine system, of catalase, and of aerobic respiration all show an inactivation of the respective enzyme systems at temperatures from 45° upward.

It may also be noted that phenomena of enzyme inactivation and cell destruction can be observed in mesophilic bacteria at temperatures commonly used in bacteriological studies. The results of Monod (1942) showed that *B. subtilis* at 37° lysed rapidly at the end of growth, when nutrients were exhausted. Since this behavior of *B. subtilis* was similar to that observed with thermophilic cultures, it was of importance to know whether such lysis is a property of members of the genus *Bacillus* regardless of temperature, or whether it is due to destruction of cellular constituents as a result of relatively high temperatures. Growth curves of *B. subtilis* were therefore run at 37° and at 28°, with results as shown in Fig. 4.

The Ford strain S8, obtained from Dr. I. C. Gunsalus, was used. Cultures were grown anaerobically in a medium consisting of glucose 0.2 per cent, yeast autolysate 5 per cent, buffered with M/15 potassium phosphate, pH 7.5. Growth was determined by measuring the optical density in a Klett colorimeter.

These results show clearly that lysis of cells at the end of growth is a temperature-dependent phenomenon.

The oxidation of glucose by resting cells of *B. subtilis* at 37° is compared with the same process by thermophil cells at 55° in Fig. 5. At 37°, the cells become inactive only after 2 hours, while at 55° 10 to 15 minutes suffices for destruction of the enzymes involved.

It is therefore apparent that 37° is a sufficiently high temperature for enzyme destruction to be measurable, as would be expected from other results of Monod on *E. coli*, in which he shows that at temperatures above 30° the logarithm of rate of growth is no longer proportional to the reciprocal of the absolute temperature and the yield of cells per unit of nutrient falls off. It is only at higher temperatures, however, that these effects become so large as to dominate the whole metabolic pattern of the organism.

We thus have evidence that essential catalytic systems of thermophils are rapidly destroyed at temperatures at which these organisms grow readily. Their ability to grow under such conditions must therefore be at least partly due to a capacity for replacing enzymes faster than they are destroyed by heat, although other factors may be involved. Heat-resistant hydrolytic enzymes are well known, and it is not necessary to assume that all constituents of the thermophil cell require rapid repair; but the ability to rapidly resynthesize inactivated cell materials is certainly of prime importance for thermophils.

In order to account for this great synthetic ability at high temperatures, as well as for the observed fact that many thermophils grow poorly, if at all, at low temperatures, it seems necessary to assume a higher temperature coefficient of enzyme synthesis in these organisms than in the mesophils. Unfortunately, nothing is known about the temperature coefficients for synthesis of enzymes, and an experimental approach to this problem, especially at high temperatures,

may be difficult to achieve. Temperature coefficients for the reactions catalyzed by a variety of enzymes have been measured, as well as those for destruction of a number of enzymes. Activation energies for enzyme action lie in the range 5000 to 25,000 cal./mol, while those for enzyme destruction are much higher—from 40,000 to 100,000 cal./mol (an activation energy of 13,000 cal./mol corresponds roughly to a Q_{10} of 2 between 20 and 30°C.). The only available data

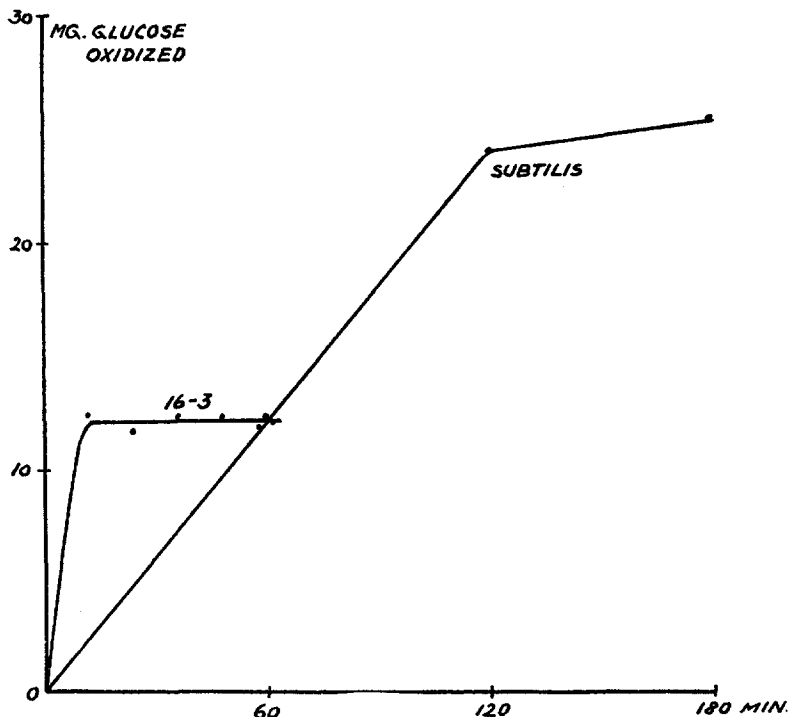


FIG. 5. Comparison of glucose oxidation by resting cells of *Bacillus subtilis* at 37° with that by thermophil 16-3 at 55°. 100 mg. were added in both cases.

on temperature coefficients of thermophil enzymes are those of Gaughran, which indicate activation energies of the same order of magnitude—perhaps at times somewhat higher—than those previously known for the same systems in mesophils. Until a means for measuring it has been devised, the higher temperature coefficient for enzyme synthesis in thermophils must remain a useful assumption, which makes possible a simple interpretation of otherwise puzzling data.

It is a consequence of the rapid inactivation and resynthesis of enzymes at high temperatures that an appreciable amount of nutrient will be used up in maintaining a stable population of thermophils under such conditions. Since it has been shown that enzymes wear out, even at ordinary temperatures (*cf.*

Morel (1933), for example), some nutrient will be used for this purpose under any conditions; but because of the large temperature coefficients for enzyme inactivation, the amount required should increase rapidly with temperature. That it does so is shown by the generally observed decrease in yield per unit amount of nutrient added as the temperature increases. In the case of the thermophilic bacilli it is possible to obtain an estimate of the quantity of nutrient required for repair, since these bacteria possess the property of lysing rapidly when their supply of nutrients is exhausted. Only preliminary results on the amounts of nutrients required to maintain a stable population are so far available, and quantitative figures cannot as yet be given, but the data obtained are in accordance with greatly increased nutrient requirements for maintenance of high temperature forms.

Combining the assumption of a higher temperature coefficient for enzyme synthesis with the demonstrated requirement of a minimum amount of nutrition to replace cell materials which have been destroyed during metabolism, it becomes possible to explain, not only the high maximum temperatures for growth of thermophils, but also the high minimum temperatures observed in some cases. The maximum temperature is reached when thermal enzyme destruction overtakes enzyme synthesis; and the minimum is reached when the rate of enzyme synthesis has dropped to a point where it cannot compensate for the destruction of enzymes as a consequence of metabolism. In the case of the mesophil, or even the "facultative" thermophil, the second factor may never become important, but for the obligate thermophil, with its more sharply temperature-dependent enzyme synthesis, it may well become a limiting factor at relatively elevated temperatures.

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SUMMARY

1. Evidence for a close relation between thermophilic and mesophilic bacteria is discussed.
2. It is shown that in the absence of nutrients thermophilic bacteria at 55°C. die as rapidly as mesophilic bacteria, and that enzyme systems of the thermophils are rapidly inactivated at this temperature.
3. It is concluded that the thermophils can live at high temperatures because they can synthesize enzymes and other cellular constituents faster than these are destroyed by heat.
4. In order to account for this great synthetic capacity at high temperatures, and for the high minimum temperatures observed for many thermophils, it is postulated that these organisms have a higher temperature coefficient of enzyme synthesis than mesophils.

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