THE GROWTH OF AEROBIC THERMOPHILIC BACTERIA

A. IMŠENECKI AND L. SOLNZEVA

Institute of Microbiology of the U. S. S. R. Academy of Sciences

Received for publication November 20, 1944

The thermophilic microbes are usually defined as organisms capable of growing at high temperatures. Such a definition does not imply that life at high temperature is actually linked with a modified physiology of nutrition, reproduction, etc. The microbes adapt themselves not only to high temperatures, but also to the physico-chemical peculiarities of their habitat at these temperatures. Such an ecological approach suggests that the thermophiles represent a rather isolated group of microbes differing somewhat in their biology from mesophilic bacteria.

It was pointed out in previous publications that the biochemical activity of thermophilic bacteria is extremely high (Imšenecki, 1941a, 1941b). The leading role among the causes underlying this increased activity belongs to rapid growth of thermophiles, and for this reason the present study deals with the peculiarities of reproduction of the sporulating aerobic thermophilic bacteria. Some parallel experiments were also conducted with mesophilic bacteria.

The Behavior of Thermophilic Bacteria at Relatively Low Temperatures

Thermophilic bacteria very often occur at temperatures lower than those necessary for their growth. Hence it is commonly held that under such conditions the thermophiles are in a resting state (mostly in the spore state) and do not participate in the mineralization of organic matter. Revival of the thermophiles and the beginning of their active life is caused by a rise of temperature as linked, e.g., with heating of the superficial layers of the soil by sunlight or with self-heating of the disintegrating organic matter.

Yet, inactivity of thermophiles at relatively low temperatures is sometimes denied. It was shown in a number of studies that the thermophiles are able to propagate not only at high but also at low temperatures, such as 20 to 30 C (Koch and Hoffmann, 1911; Wallace, 1924; Tanner and Wallace, 1925; Hansen, 1933). It will be noted that growth of the thermophilic bacteria at these low temperatures proceeds so slowly that the division of one cell takes 6 hours (Hansen, 1933). Hence the development of the thermophiles at low temperatures has been overlooked. Thus, the thermophiles live and reproduce at relatively low temperatures and participate in the circulation of matter.

Attention was therefore paid, in the present study, to the capacity of thermophilic bacteria to grow at different temperatures. By inoculating meat peptone agar plates with the contents of the intestine of various animals, soil, or slime, it is easy to cultivate colonies at 60 C. However, the overwhelming part of the colonies belongs to species which in pure cultures grow rapidly not only at 60 C but also at such low temperatures as 25 to 30 C. The optimum temperature

range for growth of these species is 40 to 50 C. Hence the great majority of the forms cultivated at 60 C belong not to the truly thermophilic but to thermotolerant species.

As to true thermophiles, i.e., species whose optimal range of growth lies between 55 to 65 C, one may distinguish two groups of organisms. One of them develops at 60 C but does not show any growth during several days at 28 to 30 C. Following the general biological nomenclature one may call them stenothermal thermophiles.

The representatives of the other group of thermophilic bacteria also show a growth optimum at about 60 C, but a slight growth may also be seen at such low temperatures as 28 to 30 C, that is to say, these are eurithermal thermophiles. It is important in determining the temperature range of growth that tests also be made for visible growth on the surface of agar at relatively low temperatures. However, in liquid media or in the condensation water in agar test tubes such apparently negative tube cultures may show slow growth, which brings about an

TABLE 1

Cultivation of thermophilic bacteria at 28 to 30 C

(The figures correspond to the number of cells in thousands per 1 ml as found by direct count)

EXP. NO.	BACILLUS SP. (STRAIN 1) EURITHERMAL THERMOPHILE		B. DIASTATICUS STENOTHERMAL THERMOPHILE	
	Initial cell number	After 5 days	Initial cell number	After 5 days
1	510	50,064	6,115	2,675
2	2,675	35,287	1,656	2,038
3			4,713	5,350
Avg.....	1,598	42,676	4,161	3,354

opacity of the fluid. This may be illustrated by the protocols of two experiments. In the first of these the culture of the thermophile, *Bacillus* sp. (strain 1), was inoculated on meat peptone broth of 1-cm depth. No perceptible growth was noted at 28 to 30 C on meat peptone agar by the naked eye during 3 to 4 days. In the second experiment the development of the thermophile *Bacillus diastaticus* nov. sp., which intensely hydrolyzes starch, was studied. The description of this species has been given elsewhere (Imšenecki *et al.*, 1942). *Bacillus diastaticus* was cultivated on 5 per cent potato decoction with 0.1 per cent chalk, also in a thin layer of liquid. In both experiments the number of cells was determined in the contents of the flasks immediately after inoculation and after 5 days' incubation in a thermostat at 28 to 30 C. The method of direct count of stained bacteria was used.

The figures of table 1 show that in 5 days the number of cells of *Bacillus* sp. increased by approximately 27 times. Hence, although slowly, the bacteria do reproduce, and the species in question may be classified as an eurithermal thermophile.

As to the amylolytic bacterium, *Bacillus diastaticus*, which at 60 C develops vigorously on the potato decoction, no growth whatever was noted in the same medium during 5 days' cultivation at 28 to 30 C. Such a behavior is characteristic of stenothermal thermophiles, which occur in nature much less frequently than the representatives of the first group.

The species not developing at low temperatures, i.e., stenothermal thermophiles, include also *Bacillus cellulosae-dissolvens*. A pure culture of this species rapidly fermented cellulose at high temperatures but not at relatively low temperatures. This is shown by the following experiment. In the midst of fermentation the flask with the decomposing cellulose was removed from the thermostat and the fermentation products were determined in the culture fluid. Then the flask was kept at 20 C during 10 days and the analyses repeated. In both experiments the same amount of hydrolytic products of cellulose and of volatile acids was found in the medium. Hence at relatively low temperatures cellulose is not fermented. Some investigators who dealt only with stenothermal species concluded that at a relatively low temperature no growth occurs. In studying eurithermal species one could make the mistake of attributing to all thermophiles the capacity of growth at low temperatures. In fact, however, there exist in nature both groups of thermophiles.

Growth of Thermophilic Bacteria at High Temperatures

Experiments were conducted on *Bacillus diastaticus* and *Bacillus* sp. (strain 1) to get an idea as to the rate of growth of the thermophiles at 60 C. Erlenmeyer flasks of 250-ml capacity were filled with thin layers (1 cm) of the liquid medium previously mentioned. The flasks were inoculated with 17-hour cultures on liquid media. Cell counts were made immediately after inoculation and in cultures at different ages. Prior to inoculation, the cultures were diluted in a liquid nutrient medium brought to 60 C. In the experiments with *Bacillus* sp. the number of cells was determined both by direct cell count and by plating on meat peptone agar. To secure cell counts in young cultures the flasks were inoculated with relatively large doses of microbes. In *Bacillus diastaticus* the cell count was made by plating in petri dishes on solid nutrient medium of the following composition: 20 per cent potato decoction, 100 ml; peptone, 0.5 g; chalk, 0.1 g; agar, 2.0 g. The results of these experiments are summarized in tables 2 and 3.

It will be seen that the number of cells of *Bacillus* sp. and *Bacillus diastaticus* rapidly increases and very soon reaches its maximum. Reproduction of thermophilic bacteria begins almost at once after inoculation of the culture. The initial stationary phase is either absent or is so short that our methods did not reveal it. Growth is more rapid in the thermophiles than in the case of mesophiles, that is, the generation time is shorter. In the thermophilic bacteria there occurs a more rapid diminution of the number of cells in the culture, and hence the stationary phase is followed by a rapid decline. Mesophiles are characterized by a slower diminution of the number of bacteria.

Of considerable interest is the comparison of the results obtained with *Bacillus*

sp. (strain 1) by determining the number of cells by cell count and inoculation (table 2). By comparing the direct count and the plate count it is possible to elucidate the correlation between viable and dead cells and those which do not germinate on the surface of solid media. It will be seen that the number of cells obtained by direct count increases more rapidly than that obtained by

TABLE 2

Growth of the thermophilic bacteria (Bacillus sp., strain 1)

(The figures correspond to the number of cells in thousands per 1 ml as found by direct count)

EXP. NO.	IMMEDIATELY AFTER INOCULATION		AGE OF CULTURE									
			4 hours		6 hours		17 hours		24 hours		36 hours	
	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count
1	8,056	3,185	11,176	43,567	27,296	74,396						
2	5,160	1,401	17,720	7,134	81,760	38,090						
3	8,240	764	29,340	20,255	45,260	35,160						
4	2,233	11,720					258,200	287,137	101,500	210,703		
5	470	6,115					289,100	332,233	61,300	339,622		
6	5,760	5,605					145,900	446,629	111,800	273,634		
7	7,470	8,663					195,000	500,897	204,800	329,431		
8	1,031	4,968									12,040	19,873
9	484	2,675									10,160	42,803
10	709	4,104									9,840	36,434
Avg..	1,895	4,274	19,412	23,652	51,439	49,215	222,050	391,724	119,850	288,349	10,680	33,037

TABLE 3

Growth of the thermophilic amylolytic bacteria (Bacillus diastaticus)

(The figures correspond to the number of cells in thousands per 1 ml)

EXP. NO.	IMMEDIATELY AFTER INOCULATION (CONTROL)	AGE OF CULTURE		
		24 hours	48 hours	72 hours
1	2.8	20,400	1,490	370
2	13.6	24,300	830	1,120
3	15.2	7,000	2,900	59
4	7.4	3,800	600	240
Avg.....	9.75	13,875	1,455	447

plating. This discrepancy reaches a maximum at the apex of the curve (maximum stationary phase), which suggests that the number of dying cells rises continuously. It will also be noted that if the dead bacteria did not rapidly disintegrate, they would have been counted, even though reproduction of the cells had stopped. Microscopic examination of cultures of different ages completely confirms this view. In relatively young thermophile cultures there always occur

poorly staining ghosts of cells, as well as cells with granular contents. The morphological analysis reveals extremely rapid aging of the culture and early disintegration of the cells. Dead cells are usually numerous in 24-hour cultures, in which amorphous granular debris accumulates.

Thus, not only are thermophilic bacteria characterized by extremely rapid reproduction, but also no less characteristic is the rapid aging and dying of the cells. The whole rate of life of thermophiles is increased; the faster the division of the cells, the sooner their death. In spite of the fast propagation of the thermophiles, the total number of cells in the cultures without aeration is relatively low. For *Bacillus* sp. the maximum number of cells per 1 ml of culture amounts (direct count) to about 392 million; for *Bacillus diastaticus* to only 14 million. It seemed therefore worth while to determine the maximum number of cells in the cultures of mesophilic sporulating bacteria. For this purpose cultures of *Bacillus malabarensis* were taken, as well as *Bacillus megatherium* and *Bacillus pseudoanthracis*, because our strains of these species do not form a film which impedes cell count. The mesophiles were cultivated in flasks with meat

TABLE 4

Number of cells in the cultures of mesophilic bacteria

(The figures correspond to the number of bacteria in thousands in 1 ml)

B. MALABARENSIS		B. MEGATHERIUM		B. PSEUDOANTHRACIS	
Initial number	After 48 hr	Initial number	After 48 hr	Initial number	After 48 hr
17,325	2,430,601	19,109	1,959,258	21,147	2,140,152
8,663	830,583	21,911	1,643,331	10,446	1,457,342
15,542	1,070,076	23,185	1,757,982		
Avg 13,843	1,443,753	20,510	1,801,295	15,797	1,798,747

peptone broth; in other respects the methods of study were similar to those previously described. It was shown by preliminary experiments that the number of cells in the cultures of all three mesophile species gradually increases, attaining a maximum in 48-hour cultures.

The results of the experiments are summarized in table 4, which shows that in mesophiles the number of bacteria in the culture is very great, viz., from 1,443 to 1,801 million, on the average. Thus, in thermophile cultures the absolute number of cells is considerably less than in the mesophiles. This difference is probably less than appears to be the case since in the thermophile cultures autolysis and decomposition of the cells may have already begun, whereas in mesophiles autolysis begins much later. However, this discrepancy cannot be great as fewer cells are seen in very young thermophile cultures in which disintegration of the cells has not as yet begun.

A suggestion was made, on ecological grounds, to account for the difference in the number of cells in the cultures of thermophilic and mesophilic bacteria. The amount of dissolved oxygen in water is known to decrease with an increase in temperature, and it is logical to expect that one restricting factor in the growth

of thermophiles is insufficient aeration of the medium. This seemed quite plausible as observations on thermophilic bacteria made during many years show that aerobic thermophiles reproduce well only in a thin layer of liquid medium. In flasks with a high column of fluid, development of the thermophile cultures is greatly impeded. The following experiments were aimed at an experimental verification of this suggestion.

Effect of Aeration on the Rate of Growth

The following methods were used. Glass cylinders of 50-ml capacity were filled with 30 ml of the liquid medium previously mentioned. A rubber tube with minute orifices in its lower end was immersed in the medium. The tube was passed through the cotton stopper of the cylinder. The lower end of the tube was hermetically closed with a glass rod, whereas the upper end was connected with a glass tube through which sterile air heated to 60 C was passed. The medium within the cylinders was inoculated with 2 ml of an 18-hour culture grown in a liquid medium at 60 C. Immediately after inoculation the number

TABLE 5

Effect of aeration of culture on the growth of thermophilic bacteria

(The figures correspond to the number of bacteria in thousands in 1 ml)

EXP. NO.	BACILLUS DIASTATICUS			EXP. NO.	BACILLUS SP. (STRAIN 1)		
	Initial number	After 6 hr	After 8 hr		Initial number	After 6 hr	After 8 hr
1	16,306	324,699	737,079	1	10,446	1,283,072	2,225,758
2	8,408	236,436	438,731	2	25,478	1,494,794	3,020,672
				3	6,879	1,291,735	1,635,688
Avg...	12,357	330,068	587,905		14,268	1,359,200	2,294,039

of cells in the cylinders was determined by direct count, and the cylinders were placed in a thermostat at 60 C. Air was passed through the medium throughout the experiment. Cell counts were made of the culture after 6 and 8 hours of aeration.

It is seen from the data of table 5 that aeration appreciably accelerates the reproduction of thermophilic bacteria. Under these conditions the number of cells per 1 ml of culture of *Bacillus diastaticus* attains 587 million after 8 hours, and 2,294 million in the case of *Bacillus* sp. A comparison of these figures with those obtained in the nonaerated cultures (thin layer) shows that aeration greatly increases the number of cells.

Thus, by means of aeration it is possible to shift appreciably the growth curve of thermophilic bacteria and to obtain the maximal number of cells in less time. In cultivating *Bacillus diastaticus* in large volumes of liquid for the production of amylase preparations, the cultivation time of the thermophiles can be reduced to a minimum. Thus, upon inoculation of the medium with 5 to 10 per cent of culture, and with more perfect aeration than in the laboratory experiments,

reproduction of the bacillus and the accumulation of amylase in the medium attain a maximum after 4 to 5 hours.

DISCUSSION

As a result of adaptation to high temperatures there have arisen in nature thermophilic microbes among which occur species the temperature optimum of which surpasses the temperature causing death of the mesophiles. Such an adaptation can be simulated under laboratory conditions; by means of a gradual increase of temperature it is possible to obtain forms adapted to higher temperatures (Dallinger, 1887; Dieudonné, 1894; Tsiklinsky, 1899). However, an appreciable change of the temperature maximum requires a considerable span of time. Thus, the sudden formation of thermophiles, which is considered possible (Lieske, 1921; Kluyver and Baars, 1932), seems very doubtful.

The thermophilic bacteria present an isolated group of microbes of secondary origin which have adapted themselves to life in surroundings with a high temperature. This group may include thermophilic strains of mesophiles, as is the case with bacteria fermenting cellulose, as well as species which do not appear to have parallel forms among the mesophiles. Some thermophiles have lost the capacity to grow, even slowly, at relatively low temperatures, whereas others have retained this capacity.

Life at high temperatures calls forth a change in the whole biology of the microbes. Thus, characteristic of the thermophiles is rapid growth at high temperatures. Even in the slowly multiplying actinomycetes, thermophilic strains show rapid growth (Imšenecki and Avdievich, 1944). Vigorous reproduction of the thermophiles is followed by rapid aging, death, and disintegration of the cells. These bacteria obey the general law that the more rapid the reproduction, the shorter the survival time. Both phenomena, intense growth and rapid death, occur at the optimal temperature (for reproduction) of 55 to 65 C, but this temperature is not optimal for the preservation of the species.

For practical purposes, rapid reproduction of bacteria is essential, whether or not there occur degenerative changes and mass death of the cells. In those cases in which rapid reproduction of the bacteria and accumulation of enzymes in the medium are required, the thermophilic microbes have some advantages. It is probably the early disintegration and autolysis of the thermophilic microbes, with the release of the intracellular enzymes, that account for the more rapid accumulation of the enzymes in the medium with the thermophiles than occurs with the mesophiles.

SUMMARY

Two groups may be distinguished among the thermophilic bacteria: stenothermal thermophiles not developing at relatively low temperatures, and eurithermal thermophiles growing at these temperatures.

Rapid reproduction at high temperatures is one of the most characteristic peculiarities of thermophiles. Their growth curve differs from that of mesophiles.

Following rapid reproduction the cells of thermophiles soon die away, undergoing autolysis and disintegration. Accelerated aging of the culture is also one of the thermophile peculiarities.

Aeration of the culture appreciably accelerates reproduction of the aerobic thermophilic bacteria.

REFERENCES

DALLINGER, W. 1887 The president's address. J. Roy. Microscop. Soc., 185.

DIEUDONNÉ, A. 1894 Beiträge zur Kenntnis der Anpassungsfähigkeit der Bakterien an ursprünglich ungünstige Temperaturverhältnisse. Zentr. Bakt. Parasitenk., **16,** 965.

HANSEN, A. 1933 The growth of thermophilic bacteria. Arch. Mikrobiol., **4,** 23.

IMŠENECKI, A. 1941a Biochemical activity of thermophile bacteria. (In Russian.) Compt. rend. U. S. S. R., **10,** 671.

IMŠENECKI, A. 1941b O scorosti processov, visivaemich mesophilnimi i termophilnimi bacterijami. (Rate of the processes called forth by mesophilic and thermophilic bacteria.) Microbiology U. S. S. R., **10,** 385.

IMŠENECKI, A., AND AVDIEVICH, N. 1944 Rost termophilnich actinomicetow. (The growth of thermophilic actinomyces.) Microbiology U. S. S. R. In press.

IMŠENECKI, A., SOLNZEVA, L., AND KUSIURINA, L. 1942 Termophilnie amilolitichеskije bacterii. (Thermophilic amylolitic bacteria.) Microbiology U. S. S. R., **11,** 29.

KLUYVER, A., AND BAARS, J. 1932 On some physiological artifacts. Proc. Acad. Sci. Amsterdam, **35,** 270.

KOCH, A., AND HOFFMANN, C. 1911 Über die Verschiedenheit der Temperaturansprüche thermophiler Bakterien in Boden und in künstlichen Nährsubstraten. Zentr. Bakt. Parasitenk., **II, 31,** 432.

LIESKE, R. 1921 Morphologie und Biologie der Strahlenpilze. Berlin.

TANNER, F., AND WALLACE, G. 1925 Relation of temperature to the growth of thermophilic bacteria. J. Bact., **10,** 421.

TSIKLINSKY. 1899 Sur les microbes thermophiles des sources thermales. Ann. inst. Pasteur, **10,** 788.

WALLACE, G. 1924 Thesis. Univ. Illinois. (Cited by Hansen, 1933.)