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Growth Requirements of some Thermophilic and Mesophilic Bacilli

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SUMMARY: To approach reproducibility of mass cultures as sources of enzymes, the growth requirements of two mesophils belonging to *Bacillus licheniformis* and *B. circulans*, and three thermophils belonging to *B. licheniformis*, *B. circulans* and *B. stearothermophilus*, were determined in metal-buffered media.

The mesophilic *B. licheniformis* required glycerol or glucose, also alanine, aspartate, glycine, glutamate, arginine, histidine and lysine. Cytidylic acid or guanylic acid further increased growth; adenosine was inactive. The thermophilic *B. licheniformis* required for good growth either glycerol, gluconate, or glucose, also succinate or glutamate; glutamate was replaceable by the combination of arginine + histidine + proline. In glycerol media, either DL-malate alone, or citrate plus either acetate or fumarate supported growth; neither acetate nor fumarate were effective by themselves. In the absence of intermediates of the citric acid cycle, sodium bicarbonate or a combination of protogen + acetate allowed growth. With acetate present, protogen was replaceable by thiamine.

The *B. stearothermophilus* strain utilized glucose or sucrose; thiamine, biotin and nicotinic acid were required. A combination of arginine + histidine + isoleucine increased growth. The carbohydrate requirement appeared absolute.

The thermophilic *B. circulans* required glutamate or a combination of succinate + arginine + histidine; these sufficed for moderate growth. Supplementation with glycerol, sucrose, gluconate or glucose increased growth. Further addition of acetate decreased the lag phase. For the mesophilic *B. circulans*, either glucose or gluconate were suitable substrates; thiamine, biotin, riboflavin, and 'reduced sulphur' were required.

The advantages of thermophilic bacteria for studies of enzyme turnover are defined in recent reviews (Clegg & Jacobs, 1953; Allen, 1953). Before testing the thermostability of enzymes in thermophilic bacteria, chemically-defined culture media suitable for mass cultures were needed to ensure good yields of organisms and enzymes, and reproducibility of enzymic activities. Such media were especially desirable for work with proteolytic enzymes whose activities are conspicuously dependent upon Mn, Mg, Co, Ca or Zn ions (Smith, 1951; Crook, 1951). The concentration of these ions in natural materials varies widely.

Knight & Proom (1950) noted that many strains of *Bacillus circulans* grew poorly in defined media suitable for strains of other species. One of our mesophils and one of our facultative thermophils belonged to the *B. circulans* group, and it was therefore of interest to extend the study of Knight & Proom. The basal medium devised for this purpose appears suitable for mass cultures of our *B. circulans* strain and also for mesophilic and thermophilic variants

belonging to several species and for a facultatively thermophilic strain of *B. stearotherophilus*—the species which includes the obligately thermophilic bacilli—since the technique devised for this purpose was simple, and the information so obtained was readily applicable to the mass cultivations needed for isolating enzymes in quantity. The definitions employed here for degrees of thermophily are those of Clegg & Jacobs (1953).

MATERIALS AND METHODS

The culture methods were essentially those described elsewhere for aerobic protists (Hutner, 1950; Hutner, Provasoli & Filfus, 1953). Experimental media in 5 ml. amounts were distributed in 25 ml. Pyrex Erlenmeyer flasks provided with glass or aluminium caps. In later work, to conserve space, media were distributed in 5 ml. amounts in 10 ml. borosilicate flasks having a broad base, thus resembling Fernbach flasks in shape; these flasks (Kimble Glass Company, Toledo, Ohio) allowed a greater surface: volume ratio (i.e. better oxygenation), and the flasks were less top-heavy. The flasks were incubated within chambers formed by inverting one Pyrex tray over another and sealing the joint with cellulose tape. Each chamber held up to thirty-eight 10 ml. flasks. Water was added to each tray to minimize the hazards of evaporation and uneven temperatures which are intensified at the incubation temperature for the thermophils. The temperatures of incubation, 37° for the mesophils and 55° for the thermophils, were read from thermometers kept in uninoculated flasks of culture medium; the difference between the air temperature in the incubator and that of the cultures was less than 0.5°. Maximum growth for all cultures usually required 5–7 days.

All stock cultures were maintained on: Nutri-Peptide (Baltimore Biological Laboratory), 0.5 g.; beef extract (Difco), 0.5 g.; agar, 2.0 g.; distilled water to 100 ml.; pH 6.5–7.0. Subcultures were made monthly and stored in the refrigerator after incubation. Bacteria scraped from agar slopes and suspended in the basal medium served as inocula. Growth was measured in optical density (o.d.) units as determined with a Welch Densichron. An o.d. of 1.0 equalled 0.50–0.55 g. dry-wt. washed bacteria/litre.

The basal medium shown in Table 2, when supplemented with appropriate substrates and growth factors, allowed heavy growth of the organisms listed in Table 1; this medium could be made in double strength and refrigerated without precipitation.

The concentrations of metals listed in Table 2 refer to the metal content of the salts used; these were usually sulphates rather than the hygroscopic chlorides. Calcium was prepared as CaCO₃ dissolved in HCl. Molybdenum salts are poorly soluble in acid solutions and were not combined with other metals in one acidic stock solution. To avoid precipitation the molybdenum stock solutions were adjusted to pH 8.0–8.5 with alkali.

Transaconitic acid + triethanolamine served as buffers. Under the conditions of these experiments there was no evidence that this buffer pair had substrate activity. The pronounced alkalization of the media (the pH

may rise above 8.5) resulting from the consumption of organic acids and the liberation of ammonia from the amino-acids limited growth, making the use of concentrated buffer desirable.

Table 1. *Species of Bacillus used*

Strain	Mesophil	Source
<i>B. licheniformis</i> , no. S8 (Ford strain)		I. M. Gunsalus
<i>B. circulans</i>		ATCC, no. 7046
	Thermophil	
<i>B. licheniformis</i> , no. S8		Thermophilic variant of the Ford strain isolated by M. B. Allen
<i>B. circulans</i> , no. 7-2		Isolated from soil by M. B. Allen
<i>B. stearothermophilus</i> , no. B1102		Originally from the Northern Regional Laboratory; sent through the courtesy of W. G. Walter.

Table 2. *Basal medium*

Ethylenediamine tetraacetic acid (EDTA)	0.05 g.	NH ₄ Cl	0.12 g.
KH ₂ PO ₄	0.02 g.	Ca (as Cl)	0.001 g.
MgSO ₄ ·7H ₂ O	0.05 g.	Transaconitic acid	0.5 g.
Mo (as ammonium molybdate)	0.001 g.	Triethanolamine	1.0 g.
'Metals 42'	1.5 ml.	Distilled water to	100 ml.

EDTA was used as the free acid purified by several recrystallizations from water; this preparation was a gift from the Bersworth Chemical Co., Framingham, Mass. The pH value of the medium was adjusted with KOH or H₂SO₄. The initial pH of the medium was 6.0-7.0 depending on the tolerance to the lower pH limit of the organism. One ml. of 'Metals 42' contains (mg./ml.): EDTA, 2.5; Zn, 4.0; Mn, 3.0; Cu, 0.25; Fe, 0.1; B (as H₃BO₃), 0.1; Co, 0.04.

RESULTS

Bacillus circulans—mesophilic strain

Substrates. Initial pH 6.0. Glucose or gluconate (0.5%; % means % w/v, throughout unless otherwise indicated) were equally effective as substrates. Gluconate offered the advantage of being obtainable in pure form from the δ -gluconolactone. The excess of chelating metals in the basal medium helped to compensate for the metal-binding effect of gluconate; the effectiveness of gluconate had been overlooked in previous experiments because of the metal deficiencies induced by its chelating ability.

Growth factors. Thiamine HCl (0.2 mg./100 ml.), biotin (0.5 μ g./100 ml.) and riboflavin (5.0 μ g./100 ml.) appear to be absolute requirements. 'Reduced sulphur', i.e. compounds more reduced than sulphates on the biosynthetic pathway to cystine and methionine, is also an absolute requirement (Table 3).

Stimulatory factors. In the presence of either glucose or gluconate and the other required nutrients, acetate (0.04% Na acetate trihydrate) was stimulatory. Cyanocobalamin (vitamin B₁₂) was slightly stimulatory in the presence of L-cystine (4.0 mg./100 ml.). This stimulation was also evident when the cystine was replaced by thiosulphate; DL-methionine (8.0 mg./100 ml.) as the sole source of reduced sulphur yielded the same amount of growth as that

permitted by cyanocobalamin *plus* either cystine or thiosulphate. It increased the density of growth from about 0.75 to 0.95. Table 3 combines several experiments indicating the need for reduced sulphur carried out under similar conditions.

Table 3. *Reduced sulphur requirement for Bacillus circulans mesophil in gluconate medium*

	Sulphur com- pounds (mg./ 100 ml.)	Optical density		Sulphur com- pounds (mg./ 100 ml.)	Optical density
None	0	0	DL-Homocystine	1.0	0.28
L-Cystine	1.0	0.44		4.0	0.56
	4.0	0.66		8.0	0.72
	8.0	0.78	Glutathione	5.0	0.11
DL-Methionine	3.0	0.36		10.0	0.32
	5.0	0.56		50.0	0.58
	10.0	0.62	Na ₂ thiosulphate 5H ₂ O	10.0	0.38
L-Cysteine hydro- chloride.H ₂ O	1.0	0.52		50.0	0.52
	4.0	0.7		100.0	0.78
	8.0	0.82	Na ₂ SO ₄ (anhydrous)	10.0	0
Methionine sulfoxide	3.0	0.22		50.0	0
	5.0	0.46		100.0	0
	10.0	0.56			

Citric acid cycle components (DL-malate, fumarate, succinate, citrate) tested through a wide concentration range (0.01–0.5), and related compounds (L-glutamate, DL-alanine, and DL-aspartate) neither supported growth in the absence of glucose or gluconate nor were they stimulatory in the presence of glucose or gluconate.

Bacillus circulans—thermophilic strain

Substrates. Initial pH 6.0. Glucose, sucrose, gluconate, or glycerol in concentrations of 1.0%, in combination with (a) L-glutamate (0.5%), or (b) succinate (0.3%) + L-arginine hydrochloride (0.05%) + histidine hydrochloride (0.02%) supported dense growth (O.D. 1.7). Glutamate or the succinate + arginine + histidine combination supported moderate growth (O.D. 0.7). The addition of acetate (0.04% Na acetate trihydrate) to these media decreased the lag time. The requirement for glutamate or for succinate + arginine + histidine is absolute.

Bacillus licheniformis—mesophilic strain

Substrates. Initial pH 6.5. Glycerol (1.0%) or glucose (1.0%). Sucrose or gluconate did not support growth.

Growth factors. The following amino-acids represent absolute requirements: DL-alanine (0.05%), DL-aspartate (0.05%), L-glutamate (0.5%), glycine (0.05%), L-arginine hydrochloride (0.05%), L-histidine hydrochloride (0.02%), and DL-lysine hydrochloride (0.05%).

Stimulatory factors. The addition of cytidylic acid or guanylic acid (up to 0.01 %) increased growth from O.D. 0.45 to 0.9; the corresponding nucleosides and free purine and pyrimidine bases were inactive. Alkali-hydrolysed yeast nucleic acid was stimulatory to the expected extent. The nucleic acid was hydrolysed by suspending yeast nucleic acid in water, bringing the preparation to pH 9.5, autoclaving for 5 min. at 115–120°, then neutralizing.

This organism grew in flakes; the others grew diffusely.

Bacillus licheniformis—thermophilic strain

Substrates. Initial pH 6.5. Glycerol (0.5 %), glucose (0.5 %) or gluconate (0.05 %) allowed growth in the presence of glutamate or succinate. Glucose or gluconate was not as effective as glycerol. A combination of L-arginine hydrochloride (0.05 %) + L-histidine hydrochloride (0.02 %) + L-proline (0.005 %) also supported growth in the glycerol medium. It was advantageous to add 1.0 mg./100 ml. additional Mn for this strain.

The utilization of intermediates of the citric acid cycle in glycerol media was surveyed. DL-Malate (up to 0.1 %) supported growth. Citrate (as tripotassium citrate monohydrate, 0.05 %) *plus* either Na acetate trihydrate (0.01 %) or Na fumarate (0.1 %) also supported growth. Acetate or fumarate alone, tested up to the toxic limit, did not support growth. Potassium citrate monohydrate was not employed in concentrations above 0.05 % because of the induction of metal deficiencies through its chelating ability.

Sodium bicarbonate added aseptically to the glycerol medium in the absence of citric acid cycle intermediates allowed dense growth (O.D. up to 1.2). When the pH of the medium was raised to 7.0, protogen (supplied as DL-thioctic acid, 6 : 8-dithiooctanoic acid, for which we are indebted to Dr E. L. R. Stokstad of the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N.Y.) at 40 µg./100 ml. supported growth in the presence of 0.03 % Na acetate trihydrate; higher concentrations of acetate were toxic. Thiamine hydrochloride (0.1 mg./100 ml.) replaced thioctic acid in the presence of acetate under these conditions.

Bacillus stearothermophilus—facultative thermophilic strain

Substrates. Initial pH 6.0. Glucose (1.0 %) or sucrose (1.0 %). The addition of 0.04 % acetate as Na acetate trihydrate was stimulatory.

Growth factors. Thiamine hydrochloride (15 µg./100 ml.), biotin (0.9 µg./100 ml.), and nicotinic acid (0.15 mg./100 ml.) represent absolute requirements.

Stimulatory factors. The addition to the glucose medium of a combination L-arginine hydrochloride (0.01 %) + L-histidine hydrochloride (0.0045 %) + DL-isoleucine (0.04 %) doubled growth (final O.D. 1.0).

DISCUSSION

Most recent attempts to explain thermophily ascribe such growth to an exceptionally rapid synthesis of enzymes which outstrips the thermally accelerated destruction of enzymes. That is to say, thermophily is viewed as

an active process and not solely as a result of the possession of thermostable key enzymes (Allen, 1958; Clegg & Jacobs, 1958). This postulated rapidity of synthesis and destruction of cell substance enhances the need for culture media which allow the organism to carry out essential syntheses at the highest possible rates. Such nutritionally adequate media should also, by simple modification, lend themselves to the manipulation of inorganic and organic components. For instance, in studying proteolytic enzymes it might be desirable to measure their production and activity as a function of concentration of the divalent ions active as co-factors. The relatively inexpensive defined media devised appear to be adequate for mass cultures, despite their simplicity; supplementation with complex materials (yeast autolysate, liver extracts, peptones, etc.) seldom increased growth more than 0.2–0.4 o.d. units.

Since this investigation was a survey of growth requirements in so far as this knowledge was needed for mass cultures, neither analysis of the kinetics of any isolated metabolic reaction nor an exhaustive analysis of any of the various nutritional patterns encountered was attempted; at this stage final growth was the index of adequacy of culture media. It proved necessary to inspect cultures at frequent intervals to avoid missing peak growth because rapid autolysis was likely to follow the attainment of full growth (Allen, 1950). These visual inspections of growth gave the impression that growth under these conditions was more rapid in the media which permitted the denser final growths.

Oxygen supply probably most commonly limits growth of these cultures in complete media. At elevated temperatures oxygen consumption, as indicated in the reviews previously cited, is accelerated; concurrently the solubility of oxygen in water is diminished. The use of media in shallow layers was mandatory in the technique employed here.

The diversity of nutritional requirements encountered with aerobic spore-forming bacteria suggests that they are biochemically more heterogeneous than is suggested by their shared morphological features. Even within a single species, e.g. *B. licheniformis*, the heterogeneity is striking. The findings of Campbell & Williams (1958) indicate that a comparable heterogeneity holds for the obligate thermophiles. They showed that strains of *B. stearothermophilus* exhibited a variety of growth requirements; in many strains, moreover, these requirements depended on the temperature. In most instances, as in 'temperature' mutants, cultures incubated at the higher temperatures had additional exogenous requirements; less frequently the opposite was true. Consequently, the 'absolute' requirements listed in the present paper may not hold for other than the stated temperatures. The nutrition of the aerobic spore-formers as outlined to date gives the impression of a sampling of a diversity of nutritional patterns.

The only novel phenomenon noted in the present work was with an aberrant agar-liquefying strain of *B. stearothermophilus*—a mutual sparing between riboflavin and calcium. Allen (unpublished) has found this phenomenon to be common among bacilli. The complexity of this calcium-riboflavin relation precludes a detailed treatment at this time. In any event, the media devised

to date, on the basis of preliminary experiments with additional strains, appear to be generally suitable for mass cultures. Several strains of facultatively thermophilic *B. stearothermophilus* obtained from the National Canners Association, Washington, D.C., grew to o.d. 2.4 when the basal medium was supplemented with glucose, acetate, and the vitamins and amino-acids found necessary by Campbell & Williams (1953). A thermophilic strain of *B. coagulans*, no. 139, obtained from Dr Allen, grew to o.d. 2.8 in the basal medium supplemented with glutamate plus either glycerol or carbohydrate. A mesophilic *B. sphaericus* (no. RA91, obtained from Dr Allen) reached o.d. 2.2 in the basal medium supplemented with glucose, acetate, and amino-acids.

The nutritional patterns of the five strains discussed here may have their counterparts elsewhere in microbial nutrition. A requirement similar to that for 'reduced sulphur' in the *B. circulans* mesophil was described for certain mutants of *Bacterium coli* (Lampen, Roepke & Jones, 1947), in Saprolegniaceae (Reischer, 1951), and in *Proteus morganii* (Meyers & Porter, 1944), to list only non-parasitic bacteria; it has not hitherto been reported in bacilli. The growth stimulation of the *B. circulans* mesophil by cyanocobalamin or methionine accords with evidence which implicates cyanocobalamin in methylations. This raises the question of whether the methionine requirements reported by Campbell & Williams for several strains of *B. stearothermophilus* represent either (a) a requirement for 'reduced sulphur', (b) a specific requirement for methionine, or (c) a deficiency in the synthesis of labile methyl groups. The 'glutamate' requirement for the *B. circulans* and *B. licheniformis* thermophils resembles the stimulation described by Wiame & Storck (1953) for a mutant of *B. subtilis*; glutamate here was replaceable, as with the *B. licheniformis* thermophil, by proline. The activity of succinate in replacing glutamate for the *B. circulans* thermophil suggests that the 'glutamate' requirement is essentially a requirement for a citric acid cycle component, and that permeability factors and restrictions in transamination underlie many of the differences among strains. To chart the interrelationships governing the utilization of carbohydrate, glycerol, acetate, citric acid cycle intermediates, and the requirements for thiamins, biotin, and pantothenate, is a task as yet attacked only in piecemeal fashion in metazoan and microbial nutrition. A glutamate requirement for a strain of *B. cereus* was mentioned by Foster & Heiligman (1949). The basis for the requirement for carbohydrate in *B. stearothermophilus*, unexpected in an aerobic organism, is obscure. The non-accumulation of acid suggests that this organism derives energy from oxidation as well as glycolysis. Hendlin (1949) described a strain of *B. subtilis* which required carbohydrates; citric acid cycle intermediates did not support growth.

The stimulation by nucleotides of the *B. licheniformis* mesophil and the inactivity of adenosine contrasts with the repeated observation that adenosine is required for spore germination in *Bacillus* (Pulvertaft & Haynes, 1951; Stewart & Halvorsen, 1953).

Interactions among protogen, thiamine, malate, acetate, and bicarbonate such as that detected in the thermophilic *B. licheniformis* may become familiar now that synthetic protogen is available in the form of DL-thioctic acid.

A similar interaction was described by Lytle, Zulick & O'Kane (1951) for *Streptococcus faecalis*; with gluconate as substrate, acetate was interchangeable with protogen; and malate, succinate, or bicarbonate were in turn interchangeable with protogen or acetate. Sparing of thiamine by protogen + acetate was described for a mutant of *Bacterium coli* (Reed & DeBusk, 1952).

Non-metabolizable EDTA replaced the citrate formerly employed as metal buffer and allowed a clearer demonstration of the relations of substrate to other nutrients. As mentioned, *transaconitic* acid buffer was considered to be unattacked under the present cultural conditions, but there is the possibility that under certain circumstances it may be convertible to the metabolizable *cis* isomer. In future work it is hoped to replace the potentially metabolizable aconitic acid with buffers less likely to be physiologically active.

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