A Defined Medium for the Growth of the Thermophilic Actinomycete Micromonospora vulgaris

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SUMMARY: Biotin and methionine are essential growth factors for the aerobic thermophilic actinomycete Micromonospora vulgaris. Biotin can be partially replaced by Tween 80 (sorbitan mono-oleate). No aerial growth is obtained in the absence of methionine. A chemically defined medium containing mineral salts, phosphate buffer (pH 6.8), soluble starch, a mixture of 18 synthetic amino acids (including methionine) and biotin will support growth of the organism.

Throughout the studies of Erikson (1952, 1958), Erikson & Webley (1953) and Webley (1954) on the aerobic thermophilic actinomycete Micromonospora vulgaris it was found necessary to use in the main a complex medium (C.P.S.) for growth. Erikson (1952) did, however, show that the organism would grow in the presence of Czapek's mineral salt solution containing 1% (w/v) soluble starch, 1% (w/v) vitamin-free acid hydrolysate of casein and 0.01% (w/v) autolysed yeast extract. The present work deals with the essential growth factor requirements of the organism and the establishment of a chemically defined medium for growth.

METHODS

Organism. The experiments presented here were performed with strains H, B, M and F of Micromonospora vulgaris as used by Erikson (1953).

Preparation of spore suspensions. For this purpose 2-day growth on cellophane circles over solidified C.P.S. medium was used (Erikson & Webley, 1953). The surface growth from a plate with well-developed aerial mycelium was covered with 4 ml. sterile 0.0133M-phosphate buffer (pH 6.8) and gently detached into the buffer by rubbing with a bent sterile glass rod. The suspension obtained was transferred to a 50 ml. flask containing a few sterile glass beads (3-4 mm. diameter) and shaken gently by hand for 1 min. to detach the spores which were then separated from the aerial mycelium by filtration through sterile glass wool. The spore suspension was washed four times on the centrifuge with sterile buffer and finally suspended to give about 1 x 10^8 spores/ml. (haemocytometer count). This suspension was preheated for 1 min. at 100° and 0.1 ml. used for inoculation.

Growth experiments. These were carried out in 100 ml. filter flasks containing 10 ml. medium. The flasks were sterilized with cotton-wool plugs in side arm and neck. After inoculation the plug in the neck was replaced by a tightly-fitting rubber stopper. In this way little evaporation took place from the shallow liquid layer during the incubation period of 8-10 days at 58-60°. To
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To ensure a water-saturated environment for maximum production of aerial growth (see Erikson, 1953) the side arms of the filter flasks were closed by means of short pieces of pressure tubing clipped with screw clamps. No evidence of limitation of growth by lack of oxygen was observed under these conditions. The basal mineral salt solution used was as follows (%, w/v): NaCl, 0.02; MgSO₄·7H₂O, 0.02; CaCl₂, 0.005; K₂HPO₄, 0.05; FeCl₃ trace; pH 6.8. Soluble starch 1% (w/v) was included in all experiments (unless otherwise stated) and further additions were made as described later. The flasks were set up in duplicate and examined daily for surface and bottom growth. Owing to the complex morphology of *Micromonospora vulgaris* (Erikson, 1953) it was not found possible to record the results of the growth experiments by conventional methods. Also, because of the inherent variability of a thermophil of such complexity of structure, it was found that not all the flasks gave uniform growth. It was therefore necessary to repeat each growth experiment several times before coming to definite conclusions. As the strains behaved similarly in their responses the results recorded are applicable to all of them.

**RESULTS**

*Influence of increasing pH values of the medium on the growth of Micromonospora vulgaris*

It was consistently observed that when the organism was grown in the presence of the basal mineral salts, soluble starch, 2% (w/v) vitamin-free Casamino acids (Difco) and 0.01% (w/v) yeast extract (Difco) the medium became increasingly alkaline (rising to pH 9.0) after 8–10 days. A similar effect was obtained by Baker, Sobotka & Hunter (1953) during the growth of thermophilic bacteria. With *Micromonospora vulgaris* this increasing alkalization of the medium brought about autolysis, particularly of the aerial growth. Attempts to overcome this by the use of the organic buffer recommended by Baker et al. (1953) were unsuccessful. Experiments were next carried out in which varying concentrations of Sørensen’s phosphate mixture (pH 6.8; Clark, 1928) were added to the above medium. It was found that development of aerial growth took place in the presence of the buffer at a final concentration of 0.02M (0.717% (w/v) Na₂HPO₄·12H₂O + 0.272% (w/v) KH₂PO₄). Under these conditions the aerial mycelium did not rapidly autolyse and therefore in all subsequent growth experiments this buffer concentration was included.

*Replacement of the vitamin-free Casamino acids by a mixture of synthetic amino acids*

The first experiments in this part of the work were performed with a mixture of the 18 amino acids in hydrolysed casein according to the proportion of each present (Block, 1945) so as to give a mixture approximately equal to 2% (w/v) vitamin-free Casamino acids. The following were the amounts (g./100 ml. culture medium): L-arginine 0.077, L-histidine 0.046, L-lysine 0.146, L-tyrosine 0.127, L-tryptophan 0.026, L-phenylalanine 0.095, L-cystine 0.0056,
DL-methionine 0.064, DL-serine 0.188, DL-threonine 0.075, L-leucine 0.181, L-isoleucine 0.12, DL-valine 0.128, L-glutamic acid 0.445, DL-aspartic acid 0.068, glycine 0.011, DL-alanine 0.056, proline 0.147. This mixture in the presence of the mineral salts and soluble starch + buffer + yeast extract gave aerial and vegetative growth of all the strains of *Micromonospora vulgaris* within 48 hr. The replacement of starch by inulin, sucrose, glucose or fructose in this medium did not improve the growth. Poor development of aerial mycelium was obtained when the starch was omitted (cf. Erikson, 1958).

Replacement of yeast extract

No growth of the organism took place in the presence of either vitamin-free Casamino acids or of the amino acid mixture used above unless (in addition to the mineral salts, buffer, and soluble starch) 0.01% yeast extract was added. Attempts were made to replace the yeast extract by known growth factors. It was found that of those tried biotin (0.00625 μg./ml.), and to a lesser extent 1/1000 (w/v) Tween 80, could replace the yeast extract. The following well-known accessory factors singly or in combination gave negative results—vitamin B₁, riboflavin, pyridoxal, folic acid, nicotinic acid, pantothenic acid, ascorbic acid, p-aminobenzoic acid, inositol, β-alanine, adenine, uracil, vitamin B₁₂, cytochrome c and thioctic acid.

Tween 80 (polyoxyethylene sorbitan mono-oleate), which could partially replace biotin, could not be replaced by Tween 40 (polyoxyethylene sorbitan monopalmitate) or oleic acid. Williams, Broquist & Snell (1947) similarly found that, for growth, certain lactic acid bacteria responded to the addition of Tween 80 but not to the other two substances. These workers have shown that the growth response of lactic acid bacteria to Tween 80 is due to oleic acid provided in a non-toxic form by this compound.

It has been shown (Schaefer, Cohen & Middlebrook, 1955) that the biotin requirement of strains of *Mycobacterium tuberculosis* can be abolished when the cultures are incubated in an atmosphere containing 1–5% (v/v) carbon dioxide. Similar attempts to replace biotin by CO₂ for the growth of *Micromonospora vulgaris* were unsuccessful.

Need for methionine for development of aerial growth

Campbell & Williams (1958) showed that certain strains of thermophilic spore-forming bacteria have absolute requirements for certain amino acids and vitamins for growth at 55°. It was therefore considered of interest to see if *Micromonospora vulgaris* also required any of the amino acids in the synthetic mixture used above as accessory factors. For this purpose growth experiments were set up, using the procedure of Campbell & Williams (1958), namely, the omission of individual amino acids from the medium containing the synthetic amino acid mixture + mineral salts + buffer + starch + biotin (0.00625 μg./ml.). It was found from these experiments that the presence of methionine was essential for the production of aerial growth; in its absence vegetative growth was obtained. Further experiments showed that methio-
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nine was effective at a final concentration of 0.008% (w/v). D-, L-, or DL-

methionine were all equally effective; they could not be replaced by cysteine,
cystine or thiosulphate.

Further attempts to simplify the medium.

Since, with the exception of methionine, the remaining amino acids did not
appear to be essential for growth, attempts were made to decrease their
number in the medium. Chromatographic analysis (kindly performed by
Dr R. I. Morrison of this Institute) of the culture medium after growth of
Micromonospora vulgaris for 9 days on 2% (w/v) vitamin-free Casamino acids +
mineral salts + buffer + starch + biotin (0.00625 μg./ml.) showed that there had
been a decrease in the amounts of the following amino acids: arginine, serine,
threonine, alanine, valine, isoleucine, leucine, glutamic acid, glycine, aspartic
acid. Growth experiments were set up with these 10 amino acids in the usual
way and methionine (0.008% (w/v)) and biotin (0.00625 μg./ml.) were added.
After a lag period of 8–5 days, growth with production of aerial mycelium
took place. It was generally inferior to that obtained with the complete
amino acid mixture.

DISCUSSION

Considering its morphological complexity, the absolute growth factor require-
ments (biotin and methionine) of Micromonospora vulgaris are relatively
simple when compared with certain strains of aerobic thermophilic bacteria
studied by Campbell & Williams (1953). The fact that Tween 80 can partially
replace biotin for growth of M. vulgaris indicates that oleic acid is also required
by the organism. It has been suggested by Williams et al. (1947) that one
function of biotin is to catalyse the synthesis of oleic acid. The necessity for
the SH- containing amino acid, methionine, for the production of aerial growth,
is of particular interest in the light of previous work (Webley, 1954), in which it
was suggested that the specific inhibitory effect of oxygen on the production
of aerial mycelium by M. vulgaris is connected with the inactivation of thiol-
containing enzymes. Another feature is the poorer growth obtained in the
presence of biotin and methionine when the amino acids in the defined mixture,
based on vitamin-free Casamino acids, are limited to those shown to be
utilized (by chromatography) during the growth of the organism. Snell (1951)
pointed out that, although the explanation for this phenomenon is in most
cases not known, it often lies in one of two directions, viz. an imbalance of
amino acids may be present in the limited mixture, or amino acids which are
not essential when omitted from a complete medium may be essential precursors
in a more restricted medium. The fact that a lag period was always noted for
growth in the simplified medium in this work lends support to the latter
possibility operating with M. vulgaris.
REFERENCES


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