The Nutrition of *Zymomonas anaerobia*

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The genus *Zymomonas* includes two species, *Zymomonas mobilis* and *Zymomonas anaerobia*. Millis (1956) has described some of the biochemical characteristics of *Z. anaerobia* and presented evidence for an essentially quantitative conversion of glucose to ethanol and CO₂. McGill, Ribbons & Dawes (1965) and McGill (1966) demonstrated the operation of the Entner-Doudoroff (1952) pathway and studied the metabolism of glucose and fructose by this organism and the molar growth yields.

The first nutritional studies were carried out with *Zymomonas mobilis* by Belaïch & Senez (1965), who showed that either an amino acid mixture or, less effectively, ammonium salts, supported growth with glucose as the energy source if supplemented with calcium pantothenate. The present report concerns the nutritional requirements of *Z. anaerobia* which differ significantly from those of *Z. mobilis*.

METHODS

Organism. *Zymomonas anaerobia*, strain NCIB 8227, was maintained as stab cultures in nutrient agar and by subculture in liquid medium under O₂-free nitrogen in a Fildes-McIntosh jar; growth was at 30°.

Media. The standard liquid medium for maintenance contained: Difco Bacto-peptone, 10 g., Difco yeast extract, 10 g. and D-glucose, 20 g./l. distilled water; solid media were prepared with 15 g. New Zealand agar substitute (Hopkin & Williams Ltd.)/l. This standard medium was modified in various ways to define the nutritional requirements of the organism.

Defined medium. Belaïch & Senez (1965) described a defined medium which, plus calcium pantothenate, supported growth of *Zymomonas mobilis*. It contained, per l. of tris-maleate buffer, pH 6.8: KH₂PO₄, 10 mg.; NH₄Cl, 1 g.; MgSO₄·7H₂O, 238 mg.; CaCl₂, 1.1 mg.; FeSO₄·7H₂O, 50 mg.; ZnSO₄·7H₂O, 7.2 mg.; MnSO₄·H₂O, 4.2 mg.; CuSO₄·5H₂O, 1.4 mg.; CoSO₄·7H₂O, 1.4 mg.; KCl, 50 mg. and NaCl, 50 mg., plus the following amino acids at 60 mg./l.: L-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine, L-hydroxyproline, L-isoleucine, L-leucine, L-lysine, DL-methionine, L-ornithine, DL-phenylalanine, L-proline, DL-serine, L-threonine, DL-tryptophan, L-tyrosine and L-valine. This medium was used in place of the Bacto-peptone of the standard medium, and glucose, together with either yeast extract or the appropriate vitamin solutions, were added to it.

Energy source. The standard medium was varied by substituting 2% (w/v) fructose, sucrose, gluconate, glycerol or pyruvate for the glucose. All energy sources were sterilized separately by Millipore filtration, and added to the otherwise complete medium.

Vitamins. Sterile stock solutions (100 µg./ml.) were prepared of each of the following

eleven vitamins, the pH being adjusted to 7.0 where necessary: *p*-aminobenzoic acid, biotin, cyanocobalamin, folic acid, inositol, lipoic acid, nicotinic acid, calcium pantothenate, pyridoxine hydrochloride, riboflavin and thiamine hydrochloride. A solution of haem was similarly prepared. The vitamins were added aseptically to either Bacto-peptone (1%)–glucose (2%) medium to a final concentration of 1 µg./ml., or were added to the defined amino acids–glucose medium to give a final concentration of 1 or 20 µg./ml.

Amino acid requirements. The amino acid requirements were investigated by the methods described by Seaman (1963) and also by adding individual amino acids to media containing 2% (w/v) glucose, 20 µg./ml. of biotin and lipoic acid, and inorganic salts.

Purines and pyrimidines. Adenine, guanine, hypoxanthine, xanthine, cytosine, thymine and uracil were added singly or in combination to the defined medium to give each a final concentration of 60 µg./ml.

Fatty and keto acids. *n*-Caproic, α -oxobutyric and α -oxoisovaleric acids were added singly or together to give each a final concentration of 17 µg./ml.

Inocula. To avoid the carry-over of nutrients, cells grown in 12.5 ml. of either standard or defined media were harvested under aseptic conditions, washed and resuspended in 12.5 ml. of sterile 0.067 M-Na K-phosphate buffer (pH 7.1); 0.1 ml. of the resulting suspension was used to inoculate 12.5 ml. of medium.

Growth conditions and measurement. Growth was obtained in $6 \times \frac{1}{2}$ in. pyrex tubes, containing 12.5 ml. of the appropriate medium, in a Fildes–McIntosh jar under O₂-free nitrogen. When growth was complete, the cultures were transferred quantitatively to 25 ml. volumetric flasks and made to the mark with 0.067 M-phosphate buffer (pH 7.1). The extinction was measured at 570 nm., and the dry weight read from a calibration curve relating extinction to bacterial dry weight over a range of 0 to 200 µg./ml. Results are recorded as the mean of replicate determinations, the range of which did not exceed 10 µg./ml., carried out in at least three independent experiments.

Chemicals. The chemicals used were of analytical reagent quality wherever possible.

RESULTS

Energy source. Peptone-yeast extract medium cannot support the growth of *Zymomonas anaerobia* in the absence of glucose. While fructose can replace glucose, giving altered fermentation products (McGill *et al.* 1965; McGill, 1966), neither sucrose, gluconate, glycerol nor pyruvate did so.

Nitrogen requirements. Replacement of peptone with ammonium chloride (0.1%) gave a medium capable of supporting some growth (57 cf. 172 µg./ml.). The Seaman (1963) groups of amino acids individually also supported some growth in the range 48 to 60 µg./ml., but the complete mixture of 18 amino acids was necessary to secure the greatest effect (121 µg./ml. increased to 139 in the presence of ammonium chloride). The Seaman (1963) medium, which differs from that of Belaïch & Senez (1965) in having three fewer amino acids and lacking ammonium chloride, supported less growth (121 cf. 146 µg./ml.); the addition of either these amino acids (lysine, ornithine and proline) or ammonium chloride enhanced the total growth to 133 and 139 respectively, the maximum effect (145 µg./ml.) being achieved with supplements of both, when growth equivalent to that in the Belaïch & Senez medium was recorded. Maximum

growth in the defined medium was, however, less than that in peptone (146 cf. 172 $\mu\text{g.}/\text{ml.}$).

The net growth on single amino acids at a much higher concentration than in the Seaman medium (1.35% cf. 0.067%, w/v) revealed that arginine, tryptophan, glutamic acid and cystine were the most effective, giving yields of 87, 87, 83 and 76 $\mu\text{g.}/\text{ml.}$ respectively. Similar results were obtained with an amino acid concentration of 0.27%.

Vitamin requirements. The mixture of 11 vitamins, each at 1 $\mu\text{g.}/\text{ml.}$, could replace 1% yeast extract in peptone-glucose medium, and despite the disparity in concentration give 75% of the growth with the yeast extract (311 cf. 418 $\mu\text{g.}/\text{ml.}$). The addition of haem did not increase the cell yield. Screening experiments indicated that lipoic acid and biotin were the most effective growth factors and this was confirmed by a comparison of the effect of the addition of single vitamins.

Fig. 1. Relationship between total growth of *Zymomonas anaerobia* and biotin and/or lipoic acid concentration in an amino acid-glucose-salts medium. Biotin, ○; lipoic acid, ●; biotin plus lipoic acid, □.

When similar experiments were conducted with defined amino acid media replacing peptone the vitamin mixture was only about 60% as effective as yeast extract (226 cf. 379 $\mu\text{g.}/\text{ml.}$). *p*-Aminobenzoic acid was the only other vitamin to show some effect at 1 $\mu\text{g.}/\text{ml.}$ concentration, but at 20 $\mu\text{g.}/\text{ml.}$ the growth was very much less marked (40 $\mu\text{g.}/\text{ml.}$) than that with lipoic acid (131 $\mu\text{g.}/\text{ml.}$) or biotin (132 $\mu\text{g.}/\text{ml.}$).

The growth response to increasing concentrations of biotin and lipoic acid, singly and combined, is shown in Fig. 1. It will be noted that the combined effect of these two vitamins is not additive.

Purines and pyrimidines. The addition of purines and pyrimidines, singly or combined, to the basal amino acid medium severely depressed the growth yield (e.g. 55 to 67 cf. 148 $\mu\text{g./ml.}$).

Fatty and keto acids. The fatty and oxo acids used did not have any significant effect on the growth of the organism.

DISCUSSION

The two species of the genus *Zymomonas* display many similarities in their metabolism but may be distinguished nutritionally. Both *Z. mobilis* and *Z. anaerobia* require an energy source for growth in complex media although they display a very restricted utilization of carbohydrates. While *Z. anaerobia* can utilize only glucose and fructose, *Z. mobilis* additionally utilizes sucrose (Millis, 1956). Dawes, Ribbons & Rees (1966) found that growth of *Z. mobilis* on sucrose gave appreciably lower molar growth-yield coefficients than those for the equivalent concentrations of glucose plus fructose and observed that growth on sucrose gave rise to a levan, which they characterized. The utilization of sucrose in a peptone-yeast extract medium thus serves readily to distinguish between *Z. mobilis* and *Z. anaerobia*. The molar growth yield of *Z. anaerobia* in peptone-glucose media is 5.89 (McGill, 1966), a value substantially lower than those of 8.3, 7.95 and 8.7 respectively recorded for *Z. mobilis* in comparable medium by Bauchop & Elsdon (1960), Belaïch & Senez (1965) and Dawes *et al.* (1966). It seems, therefore, that *Z. anaerobia* utilizes glucose less efficiently than *Z. mobilis*. Further, unlike *Z. mobilis*, the growth yield on fructose is less than on glucose, a finding which was correlated with a different fermentation pattern when growth occurs on the ketohexose (McGill, 1966). It has also been observed throughout our work with these organisms that *Z. anaerobia* appears more granular in liquid peptone-yeast extract culture than does *Z. mobilis* and there is no difficulty in distinguishing between them visually under these growth conditions.

The organisms also have different vitamin requirements. Calcium pantothenate is the only growth factor required by *Zymomonas mobilis* (Belaïch & Senez, 1965) whereas biotin and lipoic acid are required by *Z. anaerobia*, although their action is not additive and together they give growth equivalent to 64% of that on a mixture containing nine additional vitamins. Presumably biotin is involved in CO_2 -fixation reactions; since lipoic acid is usually associated with α -keto acid dehydrogenases the precise role of such enzymes in this anaerobic organism, which possesses an extremely active pyruvate decarboxylase, requires investigation.

Zymomonas anaerobia is more exacting for amino acids than *Z. mobilis*, which grows reasonably well, although slightly more slowly, on ammonium chloride as the sole nitrogen source, to give a growth yield of 4.09 g. dry weight/mole glucose fermented, compared with a yield of 4.98 in the amino acid medium (Belaïch & Senez, 1965). Maximum growth of *Z. anaerobia* in the defined medium was obtained with the complete mixture of 21 amino acids but arginine, tryptophan, cystine and glutamic acid supported the highest growth when amino acids were presented singly to the organism. *Z. anaerobia*, unlike *Z. mobilis*, did not grow well on ammonium chloride.

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