Microthrix parvicella, a Filamentous Bacterium from Activated Sludge: Growth on Tween 80 as Carbon and Energy Source

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Microthrix parvicella, cultivated in a medium with Tween 80 and Casamino acids, utilized only the oleic acid moiety of Tween 80 as carbon and energy source. The cell yield from Tween 80 was about 0.32 g dry weight of cells per g of Tween 80 consumed. As only the oleic acid moiety of Tween 80 was utilized, the cell yield from oleic acid was 1.3 g dry weight of cells per g oleic acid consumed. The amount of carbon produced as CO₂ was less than 30% of the oleic acid-carbon and this low value was in agreement with the high cell yield. In batch culture M. parvicella stored large amounts of lipid material during the early growth phase. The fatty acids of the lipid globules were similar to the fatty acids supplied as carbon source. The percentage composition of the biomass changed to give C/N percentage ratios of about 15 during the early growth phase due to the high concentration of internal lipids and the low concentration of protein. The growth rate in batch culture was about 0.016 h⁻¹ but was affected by the concentration of Casamino acids in the medium.

INTRODUCTION

Waste water treatment by the activated sludge process does not always operate satisfactorily because of the poor settling properties of the sludge. Abundant growth of filamentous bacteria usually causes this poor settling ('bulking sludge'). As many as 25 types of filamentous bacteria have been observed microscopically in activated sludge (Eikelboom, 1975; Eikelboom & Buijsen, 1981). However, only a limited number of these types are able to grow so abundantly in activated sludge that bulking sludge develops. Microthrix parvicella, a thin non-sheathed filamentous bacterium often causes bulking in oxidation ditches and low-loaded activated sludge plants (Eikelboom, 1982).

The physiological characteristics of an organism determine to a great extent its occurrence in a mixed culture like activated sludge. A detailed study of the physiology of M. parvicella may contribute to the development and application of selective methods for controlling this organism in the activated sludge process.

The nutritional requirements of M. parvicella have been elucidated. The organism utilizes long chain fatty acids, preferably the oleic acid moiety of Tween 80, as carbon and energy source and requires reduced nitrogen and reduced sulphur compounds for growth (Slijkhuis, 1983). Its growth in a complex medium shows remarkable changes in the protein and lipid components as a percentage of cell dry weight during growth (Slijkhuis & Deinema, 1982).

This report deals with the growth characteristics of M. parvicella in batch culture.

METHODS

Organism and growth conditions. Microthrix parvicella strain B was isolated from activated sludge from an oxidation ditch at Bennekom, The Netherlands. Stock cultures were kept on slants of medium A agar. Medium A (Slijkhuis, 1983) contained (g -1): Tween 80 (polyoxyethylene sorbitan mono-oleate; Sigma), 4; bacteriological peptone (Oxoid), 4; (NH₄)₂SO₄, 0.8; K₂HPO₄, 8.2; KH₂PO₄, 0.35; MgSO₄ + 7H₂O, 0.075; CaCl₂, 0.05 and trace

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elements (mg l⁻¹): FeCl₃, 6H₂O, 5; MnSO₄, H₂O, 3; ZnSO₄·7H₂O, 0·1; CuSO₄·5H₂O, 0·1; H₃BO₃, 0·1; Na₂MoO₄·2H₂O, 0·05; CoCl₂·6H₂O, 0·05; vitamin stock solution, 10 ml; pH 8. The vitamin stock solution (final pH adjusted to 3 with HCl) contained (mg l⁻¹): pantothenic acid, 10; nicotinamide, 10; pyridoxine, 10; p-aminobenzoic acid, 10; inositol, 10; thiamin, 10; riboflavin, 10; choline, 10; biotin, 10; cyanocobalamin, 0·5; folic acid, 0·5. Medium C was medium A without bacteriological peptone. Medium D was medium A with bacteriological peptone replaced by Casamino acids (Merck), 2 g l⁻¹. In medium D of, Tween 80 was replaced by 4 g Tween 60 l⁻¹ (polyoxyethylene sorbitan mono-stearate; Lamers & Indemans, 's Hertogenbosch, The Netherlands).

Media were sterilized by autoclaving (15 min, 121 °C); phosphate buffer (sterilized separately) and vitamin stock solution (sterilized by Setz filteration) were added to the cooled media.

Media were inoculated (10 ml into 100 ml fresh medium) from a culture grown for 10 d in a medium of identical composition. Cultures of M. parvicella, M. panicella, and H. SLIJKHUIS, J. W. VAN GROENESTIJN and D. J. KYLSTRA

Cultures treatment. Cultures (75 ml) were centrifuged (20 min, 4000 g in a swing-out rotor) and the supernatant was partly removed by decanting (used medium). The loosely packed cell was filtered by membrane filtration (cellulose acetate membrane filters, pore size 0·45 μm) and washed twice with NaCl solution (2·5 g l⁻¹). The residue was taken up in 75 ml of this solution. Washed cells were disintegrated by ultrasonic treatment (Branson sonifier B-12). All filaments were broken after 2 × 1 min treatment at 35 W as determined by microscopic observation.

Analyses. Dry weight was determined in duplicate using 20 ml washed cell suspension. These suspensions and blanks containing NaCl (2·5 g l⁻¹) were dried for 20 h at 103 °C.

Protein content of disintegrated cell suspensions was determined by the Lowry method as modified by Herbert et al. (1971). Blanks with the disintegrated cell suspension but without reagents had to be included because of light scattering due to the lipids present in cells. Bovine serum albumin was used as standard.

Lipids in washed cell suspensions were determined as oleic acid. Suspensions were digested with 1 M HCl at 100 °C for 2 h and extracted with chloroform. The oleic acid concentration was determined spectrophotometrically after reaction with concentrated H₂SO₄ (Zevenhuizen, 1974; Zevenhuizen & Ebbink, 1974).

The total amount of carbohydrates in washed cell suspensions was determined as glucose with the anthrone reagent in H₂SO₄ (Herbert et al., 1971).

RNA in washed cell suspensions was determined as ribose by the orcinol method (Herbert et al., 1971). RNA from yeast (Koch-Light) was used as standard.

Total organic carbon content of disintegrated cell suspensions and of used medium was determined in a Beckman model 915 A Total Organic Carbon Analyzer by subtracting the amount of inorganic carbon from the amount of total carbon. A solution of NaHCO₃ and an acidified solution (0·1 M-H₂SO₄) of phthalic acid were used as standards.

To determine total nitrogen, samples of washed cell suspensions and of used medium were digested (Kjeldahl). Ammonia from these samples and from non-digested samples of used medium was distilled off and determined with Nessler's reagent (American Public Health Association, 1975; no. 421).

Total phosphate was determined by digesting a suitable volume of a washed cell suspension by heating with persulphate. The liberated orthophosphate was determined by the ascorbic acid method (American Public Health Association, 1975; no. 425 C and no. 425 F).

A new rapid method to determine Tween 80-oleic acid concentration in the medium was used. Tween 80 in samples of used medium (5 ml) was saponified by adding 0·5 ml 10 M-NaOH and heating the samples in loosely glass-stoppered tubes for 10 min in a boiling water bath. After cooling in tap water, the samples were acidified by adding 1 ml 5 M-H₂SO₄ and then thoroughly mixed for 1 s. The scattering due to the colloidal suspension of oleic acid was measured after 10 min at 620 nm. The sample cell of the spectrophotometer had to be cleaned frequently with ethanol because of adsorption of oleic acid to the glass wall. Tween 80 in non-inoculated medium was used as a standard. It gave a linear response from 0·0 to 0·6 g l⁻¹.

Chromatography. Chloroform extracts of digested washed cell suspensions were saponified and methylated (Brian & Gardner, 1967). Methyl esters of fatty acids were separated in a gas chromatograph equipped with a flame ionization detector at a gas flow rate of 20 ml nitrogen min⁻¹. The column was 2000 x 4 mm stainless steel containing 10% (w/w) diethylene glycol succinate on Chromosorb G·HP (80–100 mesh) and operating at 180 °C. Methyl esters of various pure fatty acids (Sigma) were used as standards.

Carbon dioxide production. Inoculated medium (165 ml) was placed into a sterilized vial (inner diameter 6 cm, total volume 900 ml), the cotton plug pushed down and the vial closed by a rubber stopper. The rubber stopper was perforated for the air inlet and outlet and cultures were stirred by a magnetic stirrer, separated from the vial by an isolating plate (Tempex, 2 cm). Experiments were carried out at 25 °C. The air inlet and outlet were connected to a gas distributor and an infra-red gas analyser (both purchased from the Analytical Development Co., UK). Differences between the CO₂ concentrations of the air stream through samples and that through fresh medium (reference) were intermittently recorded.
Growth of *M. parvicella* on Tween 80

**RESULTS**

The results in Fig. 1(a) show cell biomass, cell protein and Tween 80-oleic acid concentration in used medium during growth of *M. parvicella* in medium D, in which Tween 80, the carbon and energy source, was the growth-limiting substrate. The pH of the strongly buffered culture decreased from 7.8 (inoculated medium) to 7.5 (after 20 d incubation). The amounts of total...
organic carbon and nitrogen, the C/N percentage ratio of the biomass (Fig. 1 b) and the carbon dioxide production of a growing culture were also determined (Fig. 2). The carbon and nitrogen values, as shown in Figs 1 (b) and 2, supplemented with the organic carbon and nitrogen values of used medium, enabled the calculation of C- and N-balances of growing cultures (Fig. 34 b).

In order to explain the unexpected arithmetic growth and the unusual change in the C/N ratio of the cell material, the bacteria were analysed for various polymers (Fig. 4). The initial internal lipid content of the biomass was extremely high (about 25% of the dry weight after an incubation period of 1 d and more than 35% after 4 d; Fig. 4). These lipids appeared as large globules of low electron density in the cells (Fig. 5). The globules also became clearly visible by staining with
Fig. 5. Inclusions of *M. parvicella*. Electron micrographs of a section of cells, grown for 30 h in medium D, showing large lipid globules (a) and of cells, grown for 12 d in medium D (b). The bar markers represent 100 nm.
Table 1. Distribution of palmitic, stearic and oleic acids in culture medium D (containing Tween 80) and D_{60} (containing Tween 60) and in M. parvicella cells grown in these media

Saturated and unsaturated fatty acids with a chain length of 12 to 18 carbon atoms were determined by GC of their methyl esters as described in Methods.

<table>
<thead>
<tr>
<th>Percentage of total fatty acids</th>
<th>Palmitic acid</th>
<th>Stearic acid</th>
<th>Oleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium D (Tween 80)</td>
<td>7</td>
<td>-</td>
<td>86</td>
</tr>
<tr>
<td>Biomass grown in medium D*</td>
<td>6</td>
<td>-</td>
<td>72</td>
</tr>
<tr>
<td>Medium D_{60} (Tween 60)</td>
<td>41</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td>Biomass grown in medium D_{60}*</td>
<td>29</td>
<td>55</td>
<td>8</td>
</tr>
</tbody>
</table>

-, Not detectable.
* Means of samples taken from M. parvicella cultures grown for 1 and 2 d (four analyses).

Table 2. C/N and C/P percentage ratios of biomass of M. parvicella during growth in medium A

<table>
<thead>
<tr>
<th>Time of growth (d)</th>
<th>C/N ratio of biomass</th>
<th>C/P ratio of biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4-7</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>8-8</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>5-0</td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>4-2</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 3. Effect of Casamino acids on dry weight and percentages of protein and oleic acid in M. parvicella during growth in medium C

The inoculum (10%, v/v) was derived from a culture grown for 10 d in medium D.

<table>
<thead>
<tr>
<th>Concen of Casamino acids in medium (g l^{-1})</th>
<th>Dry wt of biomass (g l^{-1})</th>
<th>Protein (% of dry wt)</th>
<th>Oleic acid (% of dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after inoculation (d)</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>0</td>
<td>0.11</td>
<td>0.32</td>
<td>0.41</td>
</tr>
<tr>
<td>0.5</td>
<td>0.11</td>
<td>0.41</td>
<td>0.80</td>
</tr>
<tr>
<td>1.0</td>
<td>0.11</td>
<td>0.47</td>
<td>0.98</td>
</tr>
<tr>
<td>2.0</td>
<td>0.11</td>
<td>0.68</td>
<td>1.10</td>
</tr>
<tr>
<td>5.0</td>
<td>0.11</td>
<td>0.91</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Sudan Black. To obtain some information concerning the chemical composition of the lipid granules, a qualitative analysis of the fatty acids of washed cells of M. parvicella during the early growth phase (1 and 2 d after inoculation) was made. Oleic acid was the main fatty acid when the organism was grown in medium D, whereas stearic and palmitic acids were present in large amounts in cells grown in medium D_{60} (Table 1). The nature of the lipids in cells grown for 30 h in medium D and cells grown for 12 d in medium D was investigated by infra-red spectroscopy, which suggested that the fatty acids in the lipids were present mainly in esterified form (results not shown).

The changes in protein, lipid, RNA and carbohydrate concentration during growth in medium D were similar to the results previously shown for growth in medium A (Slijkhuis & Deinema, 1982). However, the maximum value of the C/N percentage ratio of the biomass grown in medium A was lower (Table 2), owing to the more rapid protein synthesis in the peptone-containing medium A than in the Casamino-acids-containing medium D. The phosphorus content of the biomass grown in medium A did not vary during growth; it was about 3.3% of the dry weight and the C/P ratio varied between 32 and 38 (Table 2).
M. parvicella does not utilize sulphates but its sulphur requirement can be satisfied by the addition of 0.5 g Casamino acids l⁻¹ to medium C (Slijkhuis, 1983). Increasing amounts of Casamino acids added to medium C stimulated the rate of protein synthesis and lowered the maximum value of the internal oleic acid as a percentage of biomass (Table 3). The amount of cell protein in medium C (without Casamino acids) doubled only once during an incubation period of 7 d and cells were still completely filled with lipid globules after that period.

DISCUSSION

The results of this work show that the ready growth of Microthrix parvicella in basal medium supplied with Tween 80 and Casamino acids depends on the utilization of the oleic acid moiety of Tween 80 as carbon and energy source. The amount of biomass obtained with this substrate was high (approximately 1.35 g dry weight per g oleic acid consumed) as compared with that reported for various hydrocarbons consumed by Pseudomonas, Nocardia and Mycobacterium strains, viz. from 0.85 to 1.23 g biomass per g of substrate utilized (Raymond & Davis, 1960; Wodzinski & Johnson 1968; Blevins & Perry, 1971). When the biomass reached its maximum value (after an incubation period of 10 d, Fig. 1a), about 70% of the substrate oleic acid-carbon had been converted into biomass and only 28% had been oxidized to carbon dioxide. The high ratio of biomass-C/CO₂-C (2.6) implies a high efficiency of M. parvicella in utilizing the substrate and consequently a high yield from this substrate.

One of the remarkable characteristics of M. parvicella is its ability to store the carbon source supplied in large globules, presumably consisting of lipids, in the early growth phase (Figs 4 and 5). Because protein synthesis in the early growth phase lagged far behind the storage of oleic acid, the C/N percentage ratios were extremely high. When growth proceeded, this ratio markedly decreased because the stored fatty acids were utilized for the synthesis of cell components (Fig. 1b). Owing to this abnormal behaviour, the growth of M. parvicella in a medium with Tween 80 as carbon source was not exponential when expressed as biomass. However, plotting growth as cellular protein gave a normal exponential growth curve, showing a specific growth rate in batch culture of 0.016 h⁻¹ (Fig. 6).

The early storage of long chain fatty acids differentiates M. parvicella from most other micro-organisms that in the presence of an excess of carbon source produce and accumulate reserve material such as glycogen, poly-β-hydroxybutyrates and lipids during the final phase of growth. A Mycobacterium sp. cultivated with Tween 80 as carbon source showed a similar behaviour to that of M. parvicella (Schaefer & Lewis, 1965). The form of the stored long chain fatty acids was similar to that in the supplied substrate, as was shown with Tween 60 and Tween 80 as substrate (Table 1).
The ability to accumulate long chain fatty acids intracellularly as soon as they are available would favour *M. parvicella* in the competition for substrates when living in the activated sludge of waste water treatment plants supplied with domestic sewage. This type of waste water contains considerable amounts of long chain fatty acids (Painter & Viney, 1959) and is therefore an excellent nutrient medium for *M. parvicella*. An additional factor enabling this organism to compete successfully with bacteria living within the sludge flocs may be its occurrence as filaments protruding from the flocs. The long chain fatty acids, probably present in the waste water in an insoluble esterified form, are thought to be adsorbed by the sludge flocs, where they would favour compounds like elementary sulphur, thiosulphate, sulphides or sulphur-containing amino acids. The beneficial effect of Casamino acids in the nutrient medium on growth depends partly on the sulphur-containing amino acids. As the sulphur requirement is fulfilled by 0.5 g Casamino acids l\(^{-1}\) (Slijkhuis, 1983), the response to considerable amounts of these compounds (Table 3) is ascribed to a different effect, possibly protection against oleic acid, as was suggested for the beneficial effect of bacteriological peptone (Slijkhuis, 1983).

A further unusual characteristic of *M. parvicella* concerns its requirement for reduced sulphur compounds like elementary sulphur, thiosulphate, sulphides or sulphur-containing amino acids. The beneficial effect of Casamino acids in the nutrient medium on growth depends partly on the sulphur-containing amino acids. As the sulphur requirement is fulfilled by 0.5 g Casamino acids l\(^{-1}\) (Slijkhuis, 1983), the response to considerable amounts of these compounds (Table 3) is ascribed to a different effect, possibly protection against oleic acid, as was suggested for the beneficial effect of bacteriological peptone (Slijkhuis, 1983).

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### REFERENCES


