Growth Yields and "Maintenance Energy Requirement" in Thiobacillus Species Under Energy Limitation

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Abstract. Molar growth yield studies on chemostat cultures of Thiobacillus neapolitanus grown in thiosulfate-minerals medium have confirmed earlier observations that the dry weight increased linearly with the dilution rate. The observed increase can be explained neither by a change in cell composition nor by the observed excretion of organic compounds. The increase of the molar growth yield over the full range of growth rates, that is also observed in other obligate chemolithotrophs, was not found in the facultatively chemolithotrophic Thiobacillus A2, grown on thiosulfate or formate. The interpretation of the results in terms of "maintenance energy requirement" is discussed. It is concluded that these results do not allow a mathematical treatment according to the empirical formula of Pirt.

Key words: Growth yield — Thiobacillus — Maintenance — Chemolithotroph — Thiosulfate — Formate — Energy limitation — Continuous culture.

The thiobacilli are known for their ability to grow in inorganic media using a reduced inorganic sulfur compound as energy source and carbon dioxide as their sole carbon source (Vishniac and Santer, 1957; Kelly, 1972; Rittenberg, 1969). Within this group the obligately chemolithotrophic thiobacilli are virtually restricted to an autotrophic mode of growth since they cannot obtain energy from the oxidation of organic compounds and can utilize organic compounds only to a limited extent (Rittenberg, 1969, 1972; Kelly, 1972). In contrast the facultatively chemolithotrophic (also called mixotrophic) thiobacilli comprise a group of organisms with a large metabolic flexibility which enables them to grow autotrophically, mixotrophically and heterotrophically on a variety of single, or mixtures of, inorganic and organic substrates (Rittenberg, 1969, 1972; Kelly, 1972).

In our studies on the effect of organic compounds on the growth yields of chemostat cultures of several obligately chemolithotrophic thiobacilli (Kuenen and Veldkamp, 1973) and in more recent work on the effect of varying growth rates on the physiology of thiobacilli it was observed that the growth yield of these organisms increased with increasing growth rate up to near its maximum growth rate. This phenomenon had already been known for Thiobacillus neapolitanus since it was described some years ago by Hempfling and Vishniac (1967). This is in contrast to the commonly observed pattern in most heterotrophic bacteria where yields of cultures, grown under limitation by the carbon- and energy source, are nearly constant over a wide range of growth rates (Herbert et al., 1963; Tempest, 1970). Thus far no explanation is available for the different behaviour of cultures of these chemolithotrophs.

It was feasible that the observed increase of yields with increasing dilution rate was related to the chemolithotrophic nature of the organisms studied. In the underlying study it was attempted to find a physiological explanation for this phenomenon. It was decided to include the mixotrophic Thiobacillus A2 in our studies for comparison. T. A2 cannot only grow autotrophically on thiosulfate but also on formate, and therefore can be grown autotrophically either on a true inorganic or on a true organic compound as energy substrate.

Materials and Methods

Organisms. Thiobacillus neapolitanus (strain X) and Thiobacillus A2 (Taylor and Hoare, 1969), kindly provided by Dr. D. W. Smith, were maintained by monthly subculture on agar medium described below.

Media. The medium for cultivation of T. neapolitanus contained (% w/v): NH₄Cl, 0.1; MgSO₄ · 7 H₂O, 0.15; KH₂PO₄, 0.05; K₂HPO₄, 0.05; Na₂S₂O₃ · 5 H₂O, 0.1; in deionized water, plus 2 ml per liter of a trace element mixture (Vishniac and Santer, 1957). The final pH was adjusted to 6.8. In one experiment 0.2 mM of methionine was added to the medium. The medium was sterilized by autoclaving for 20—
40 min depending on the volume. Phosphates and thiosulfate were sterilized separately in 10% of the volume. The methionine had been sterilized separately by filtration through a membrane filter of 0.2 μm pore size (Sartorius). The basal medium for cultivation of T. A2 contained (g/1): NH₄Cl, 0.3; MgSO₄·7H₂O, 0.1; Na₃H₂P₂O₇·2H₂O, 0.525; KH₂PO₄, 0.15; Na₂S₂O₃·10H₂O, 1.0 or sodium formate, 0.68; in deionized water plus 5 ml per liter of the trace element solution. The final pH of the media was adjusted to 8.0. The media were sterilized as described above. Magnesium sulfate plus trace element mixture, and thiosulfate or formate were sterilized separately in 10% of the volume.

Continuous cultivation was carried out in the equipment described by Harder et al. (1974). Further conditions were as described previously (Kuenen and Veldkamp, 1973). The oxygen concentration in the culture was automatically controlled at 50% air saturation.

Thiosulfate limited cultures were maintained at the desired pH by automatic addition of 1 M Na₂CO₃.

Formate limited cultures were automatically titrated to pH 8.0 with 1 N H₂SO₄.

Contamination by Other Bacteria. All cultures were frequently checked for contaminants on plates and in liquid medium of the appropriate thiosulfate medium and of test medium containing (% v/v) sodium lactate 0.1; sodium acetate 0.1; glucose 0.2; yeast extract 0.3; tap water, pH 7.0–8.0.

Viability. Viability was always more than 95% as measured on appropriate agar media by the method of Postgate (1969). Moreover the E₀₂₅₀ and E₀₉₀ of the supernatant was always below 0.1 indicating negligible numbers of dead cells in the cultures.

Dry Weight, Protein and Yield Calculations. Dry weight and protein were determined as described by Kuenen and Veldkamp (1972). Reproducibility of the assay was better than ± 5% for both assays. The standard deviation was better than ± 2.5% (Kuenen and Veldkamp, 1973).

Yields were calculated from dry weight data using the concentration of thiosulfate or formate corrected for the dilution of the medium by titration fluids. Direct assay of thiosulfate (Sörbo, 1957) and formate (Lang and Lang, 1972) were used to measure the concentration of the respective compound in the medium reservoir.

Excreted organic carbon in the supernatant was measured directly with a Total organic carbon analyzer (Beckmann). Prior to the assay the supernatants of cultures were acidified to pH 4.3 with sulfuric acid and bubbled with nitrogen gas for 5 min, to reduce the concentration of inorganic carbon.

Alternatively, total excreted organic material was estimated by addition of ¹⁴C-carbonate to a steady state culture and measurement of incorporation of acid stable ¹⁴C-carbon into cell material and the supernatant. Further details of this method are described by Cohen et al. (1979).

Miscellaneous Methods. RNA was measured by the orcinol method (Harder and Veldkamp, 1967). For the phosphate assay cells were destructed according to Amen and Dubin (1960). Phosphate was determined with the method described by Chen et al. (1956). Elementary analysis of dry cells was carried out by the Analytical Department of the Chemical Laboratories of the University of Groningen.

Chemicals. All chemicals used were of analytical grade (Merck or BDH).

Results

To study the effect of growth rate on the amount of cell material formed during oxidation of thiosulfate, *Thiobacillus neapolitanus* was grown in a chemostat using a thiosulfate-limited minerals medium. Figure 1 shows that the dry weight of the culture clearly increased with increasing dilution rate. A similar increase had been observed earlier in cultures of *T. neapolitanus* (Hempfling and Vishniac, 1967), and in *Thiomicrospira pelophila* and *Thiobacillus thioparus* (Kuenen and Veldkamp, 1973).

The observed increase could be due to a gross change in cell composition and therefore different cell parameters such as protein content, RNA- and phosphorus content were measured in chemostat grown cultures of *T. neapolitanus*.

As can be seen from Fig. 1 the protein content of the cells slowly decreased with increasing dilution rate whereas the RNA content of the cells clearly increased with increasing growth rate. This is a general phenomenon commonly found in heterotrophic bacteria and due to the increased concentration in the cell of ribosomal RNA needed for the increased rate of protein synthesis. As roughly 10% of RNA consists of phosphorus the concomitant increase in phosphorus content should be expected.

It was concluded from these results that the apparent increase in dry weight could not be due to a major change in cell composition.

In *T. neapolitanus* thiosulfate does not only serve as energy source and electron donor for the reversed electron transport, but also as sulfur source since *T. neapolitanus* is unable to assimilate sulfate (Kelly, 1972). Therefore in *T. neapolitanus* the relatively low yields at low dilution rates might be caused by actual sulfur limitation.

*T. neapolitanus* is able to incorporate methionine (Kelly, 1969) which should at least be able to partially
Table 1. Dry weight and protein content of thiosulfate (10%)-limited chemostat cultures of *Thiobacillus neapolitanus* grown at \( D = 0.05 \text{ h}^{-1} \) and \( D = 0.2 \text{ h}^{-1} \) in the presence or absence of 0.2 mM methionine

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dilution rate h(^{-1})</th>
<th>Dry weight mg/l</th>
<th>Protein mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiosulfate minerals</td>
<td>0.05</td>
<td>179</td>
<td>120</td>
</tr>
<tr>
<td>Thiosulfate minerals +0.2 mM methionine</td>
<td>0.05</td>
<td>178</td>
<td>123</td>
</tr>
<tr>
<td>Thiosulfate minerals</td>
<td>0.20</td>
<td>202</td>
<td>123</td>
</tr>
<tr>
<td>Thiosulfate minerals +0.2 mM methionine</td>
<td>0.20</td>
<td>206</td>
<td>123</td>
</tr>
</tbody>
</table>

Fig. 2. Dry weight, RNA-, and protein-content of thiosulfate-limited chemostat cultures of *Thiobacillus A2* as a function of the dilution rate. Symbols as in Fig. 1

release a possible sulfur-limitation. This in turn should result in a clear increase in yield, if indeed the culture had been sulfur-limited. To test this possibility *T. neapolitanus* was grown in a thiosulfate-limited chemostat at a low and high dilution rate (0.05 and 0.2 h\(^{-1}\) respectively) in the presence or absence of 0.2 mM methionine (Table 1). No significant difference (<2%) in steady state values of dry weight and protein-content of the cultures were observed indicating that at the dilution rates tested sulfur-limitation during thiosulfate-limitation does not occur.

For comparison, the facultatively autotrophic *Thiobacillus A2* was grown in a thiosulfate-limited chemostat and again dry weight and several other cell parameters were measured. Figure 2 shows that the dry weight increased with the dilution rate from \( D = 0.015 \) to \( D = 0.05 \text{ h}^{-1} \) and, unexpectedly decreased above \( D = 0.05 \text{ h}^{-1} \). Several tests were carried out to ensure that thiosulfate was the growth limiting substrate at all dilution tested. At a dilution rate of 0.08 h\(^{-1}\), where a clear drop of total dry weight could be observed, an increase in the thiosulfate concentration of the inflowing medium resulted in the expected increase in the dry weight. No thiosulfate, polythionates, or sulfite could be detected, and the rate of sulfuric acid production was as to be expected from complete conversion of thiosulfate to sulfate. This finding refutes the possibility that a substrate other than thiosulfate was growth limiting. Also, it should be mentioned here that *T. A2* is able to reductively assimilate sulfate since it can grow in media without any added reduced sulfur source (Taylor and Hoare, 1969). Thus, as sulfate is in excess, limitation by the available sulfur-source will not occur in these cultures.

*T. A2* is able to grow autotrophically not only on thiosulfate-minerals medium but also on formate-minerals medium, whereby formate is used as energy source and electron donor (Kelly et al., 1979, in press). As can be seen from Fig. 3 no obvious increase in the dry weight with increasing dilution rates was observed in formate-limited cultures of *T. A2*. Changes in RNA content in both thiosulfate- and formate grown cells were similar to that observed in *T. neapolitanus* (compare Fig. 2 and 3 with Fig. 1).

The cell composition of *T. A2*, taken from thiosulfate or formate grown chemostat cultures, at a dilution rate of 0.05 h\(^{-1}\), was essentially the same, namely \( \text{C}_8\text{H}_{19}\text{O}_8\text{N}_2 \).

Thus the increase of dry weight with increasing dilution rate which is apparent over the full range of growth rates seems to be limited to cultures of the obligatory chemolithotrophic *T. neapolitanus, T. thioparus* and *Thiomicropsira pelophila*. In an attempt to find an explanation for this phenomenon, the organic carbon content of the supernatant liquid of *T. neapolitanus* was measured using radioactively labeled carbon dioxide (see Materials and Methods). For comparison
the same was done in cultures of T. A2, using an organic carbon analyzer. In supernatant of T. A2 cultures no organic carbon could be detected. In thiosulfate limited \textit{T. neapolitanus} cultures, however, between 8 - 15\% of the total radioactively \textsuperscript{14}C-labeled carbon assimilated by the culture was present as soluble organic material in the supernatant. The excretion was 15\% at a dilution rate of 0.03 h\textsuperscript{-1}, decreased to 8\% at \( D = 0.2 \) h\textsuperscript{-1} and then remained 8\% at higher growth rates. Details of these experiments and further analysis of these excretion products will be described in the following paper (Cohen et al., 1979). The dry weights of \textit{T. neapolitanus} were corrected for the excretion products assuming, in a first approximation, a carbon content of the excreted products of 50\% of the dry weight. Although excretion was lower at higher growth rates the corrected yield still increased with increasing dilution rate. The corrected yields are presented in the reciprocal plot of Fig. 4, which will be discussed below.

Some years ago Pirt (1965) introduced the concept of maintenance energy requirement to explain the often observed decrease of yield at relatively low growth rates. He presented a linear relationship

\begin{equation}
\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_0}
\end{equation}

where \( Y \) is the molar growth yield coefficient, \( \mu \) the growth rate, \( Y_0 \) the growth yield corrected for maintenance, and \( m \) is the maintenance coefficient. If the reciprocal \( \frac{1}{Y} \) is plotted against the reciprocal \( \frac{1}{\mu} (= D \) in the chemostat) a straight line is obtained if the parameter, \( m \), is a constant. Most yield data obtained from continuous culture studies on heterotrophic bacteria indeed fit a straight line when plotted in this way.

If the yield data of our experiments, and of earlier experiments as mentioned before (Kuenen and Veldkamp, 1973) are used for a plot of the reciprocal of yield versus the reciprocal of the dilution rate in order to obtain figures for the maintenance requirement, no straight lines are obtained. Figure 4 shows such a plot for \textit{T. neapolitanus} grown on thiosulfate, corrected and not corrected for organic excretion products. It can be seen that it is possible to draw 2 lines with different slopes fitting the data (Hempfling and Vishniac, 1967), but another obvious possibility is to draw a continuous line showing a gradual increase of the efficiency with which the available energy is utilized for growth. It should be pointed out that in the latter case the Pirt equation cannot be used to describe this phenomenon since it requires a linear relationship between the reciprocals of yield coefficient and the dilution rate.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig4.png}
\caption{Reciprocal molar growth yield coefficient \((1/Y, \text{mole/gram dry weight})\) of thiosulfate limited chemostat cultures of \textit{T. neapolitanus} as a function of reciprocal dilution rate \((1/D, \text{h})\) (Pirt, 1965), showing a non-linear function both for uncorrected yield values \((\Delta)\) (based on data of Fig. 1), and yield data corrected for excreted organic products. \( (\odot) \) The dotted lines have been drawn assuming a discontinuous linear relationship between \( 1/Y \) and \( 1/D \)}
\end{figure}

\textbf{Discussion}

The experimental maximum yield coefficients observed for growth on thiosulfate in the obligately chemolitho-trophic \textit{Thiobacillus neapolitanus}, \textit{Thiobacillus thio-parus} and \textit{Thiomicrospira pelophila} and the mixotrophic \textit{Thiobacillus A2} fall in a similar range from about 5.0 to 6.0. These figures are 20 - 30\% lower than the maximum experimental values reported for \textit{T. neapolitanus} at similar growth rates (Hempfling and Vishniac) and are only half of the yields reported for \textit{Thiobacillus denitrificans} grown aerobically and / or anaerobically (Timmer-ten Hoor, 1976; Justin and Kelly, 1978). One of the obvious explanations for these discrepancies may be the differences in conditions for growth. However, a comparison of methods employed by the respective authors does not reveal such differences: All experiments were carried out in a thiosulfate minerals medium in thiosulfate (or nitrate-) limited continuous cultures. Another explanation may be found in differences in the amount of ATP obtained from thiosulfate in the various strains or species used. In particular this may be true for \textit{T. denitrificans} which possesses the APS pathway for substrate level phosphorylation (Peck, 1962; Timmer-ten Hoor, 1976; Aleem, 1975) whereas in our strain of \textit{T. neapolitanus} and in \textit{T. A2} APS-reductase could not be detected (unpublished results; Aleem, 1975; Silver and Kelly, 1976). Hempfling and Vishniac (1967) assumed that their strain of \textit{T. neapolitanus} did possess a pathway for substrate phosphorylation which would contribute 30\% of the total energy generated.

Thiosulfate grown cultures of \textit{T. A2} show a rather unusual yield pattern. The increase of yields up to half of the maximum growth rate might be analogous with
the increase observed in yields of the obligate chemolithotrophic. The unexplained drop in yields above a dilution rate of 0.05 h\(^{-1}\) is presently under investigation. When T. A2 was grown mixotrophically on growth limiting mixtures of thiosulfate and formate or acetate the contribution of thiosulfate to the total yield did not decrease above 0.05 h\(^{-1}\) (J. C. Gottschal and J. G. Kuenen, in preparation).

The yields of T. A2 on formate are very similar to those observed in *Pseudomonas oxalaticus* (Dijkhuizen et al., 1977), but about 20% lower than the yields found for T. A2 grown on formate in "extended" culture (Kelly et al., 1979, in press). In chemostat cultures of T. A2 grown at a dilution rate of 0.14 h\(^{-1}\) no formate could be detected (see Materials and Methods). This was expected since formate was the growth limiting nutrient. Surprisingly, in order to grow T. A2 at the same growth rate in "extended" culture the formate concentration had to be increased to about 20 mM (Kelly et al., 1979, in press). At present there is no explanation for this difference, which certainly needs further investigation.

Our studies on the relation between growth rates and yields of several obligately chemolithotrophic thiothricilli and related organisms have confirmed and further substantiated the observation originally made by Hempfling and Vishniac (1967) showing that in *T. neapolitanus* the cell yields increase with increasing growth rates. We have been unable to find an explanation for this phenomenon. Changes in the cell composition of *T. neapolitanus* at different growth rates were minor and the observed increase in RNA content with growth rate followed a pattern also observed in heterotrophic bacteria (Tempest and Hunter, 1965; Harder and Veldkamp, 1967). In thiosulfate-limited cultures of *T. neapolitanus*, which cannot assimilate sulfite for biosynthesis, the thiosulfate-limitation is not likely to result in limitation of growth by the sulfur source since addition of methionine to these cultures did not result in any increase in cellular dry weight or protein. The excretion of organic products which has been observed in *T. neapolitanus* could not account for the increase of yield with increasing growth rate although excretion was clearly higher at the lower dilution rates.

The increase of yield with increasing dilution rate in thiosulfate-limited cultures has now been observed for three different chemolithotrophic organisms, *T. neapolitanus* (Hempfling and Vishniac, this report), *T. pelophila* (Kuenen and Veldkamp, 1973) and a strain of *T. thioparus* (A. Timmer-ten Hoor, J. G. Kuenen and H. Veldkamp, unpublished). The phenomenon has been related to a sudden increase at elevated growth rates in the maintenance energy requirement of the organism (Hempfling and Vishniac) (cf. Fig. 4). As the yield is higher at the higher dilution rates it should be realized that, if this is the case, the percentage of energy available for biosynthesis has increased with increasing growth rate, in spite of the supposedly higher maintenance energy requirement. In other words, the efficiency of growth has increased with increasing growth rate, and not decreased as may be suggested by assuming a higher maintenance energy requirement. As has been pointed out in the results section it is possible to fit a continuous line to the points obtained in a plot of reciprocal yield versus reciprocal dilution rate. It is obvious that the slope of this line has no meaning in terms of "maintenance energy requirement", since the concept of "maintenance requirement" as postulated by Pirt requires a growth rate independent process.

It is not possible here to discriminate between the "Pirt"- (that is growth rate independent) and growth rate dependent-maintenance energy requirement. It must be stated that if the term "maintenance energy" is used for any energy needed for growth, growth dependent or not, which consumes energy in such a way that it does not become available for biosynthesis, the increase in yield with increasing growth would imply that the percentage of the total available energy which is needed for "maintenance" is going down and not up with increasing growth rate. This conclusion is somewhat analogous to the one recently made by Tempest (1978). This author clearly showed that plots of yield data of cultures in which the maintenance energy requirement had been increased artificially have no physiological meaning and cannot be used to calculate $Y_{\text{max}}$ values.

In their recent studies on *Klebsiella aerogenes* Neijssel and Tempest (1976a and b) have obtained evidence that the maintenance energy requirement is not necessarily a constant but may vary linearly with the growth rate. Such a linear change will still result in a linear relationship when the reciprocal yield is plotted against the reciprocal dilution rate, however, with a different intercept with the ordinate. Neijssel and Tempest explained such a change in maintenance as a reflection of a growth dependent "slip" between energy-generating and -consuming processes. The slip would be caused by the necessity of a cell to "idle" during limitation by any growth-limiting substrate other than the energy source.

In most heterotrophic bacteria the energy source is usually carbon source at the same time. It seems obvious that under growth limitation by such a substrate generally either energy or carbon will be growth limiting. Neijssel and Tempest (1976a and b) have obtained evidence that chemostat cultures of *K. aerogenes* grown under such conditions are, in fact, often carbon and not energy-limited, which would lead to loss of energy by "slip" mechanisms.
When thiobacilli are grown on thiosulfate the substrate serves both as energy source and electron donor for the generation of reducing power. And here again, generally either the process of energy generation or the reversed electron transport (production of reducing power, NADH) will eventually be growth-limiting. It is feasible that the unusual change in growth yield must be interpreted as a reflection of a growth-dependent “slip” due to intracellular limitation by the rate of generation of reducing power.

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References


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