Reassessment of Maintenance and Energy Uncoupling in the Growth of *Azotobacter vinelandii*

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SUMMARY

Growth yields of *Azotobacter vinelandii* from carbon source and oxygen were reassessed to define more clearly the terms, maintenance and true growth yield.

In oxygen-limited chemostat cultures, the maintenance coefficients, m (mmol glucose/mg bacteria/h) and m_o (mmol O_2 /mg bacteria/h) were 8×10^{-4} and 5.5×10^{-3} ; i.e. $6m \approx m_o$. This relationship between m and m_o explains the perfect oxidation of carbohydrate (glucose) by maintenance metabolism. Values of the true growth yield, Y_G (mg bacteria/mmol glucose) and Y_{G_0} (mg bacteria/mmol O_2) were 46 and 13; these values were lower than those recorded for other micro-organisms.

In glucose-limited chemostat cultures, fairly high values of m and m_o were observed and in addition, negative values of Y_G and Y_{G_0} were obtained. Energy-uncoupled growth could account for these negative values.

INTRODUCTION

From a mass balance of energy substrate, Pirt (1965) subdivided the total rate of substrate utilization into the rate for cellular maintenance and that for cell growth, i.e.

$$\frac{1}{Y_{x/s}} = \frac{m}{\mu_x} + \frac{1}{Y_G}, \quad (1)$$

where $Y_{x/s} = dX/-dS$, total growth yield (mg bacteria/mmol glucose); $\mu_x = (1/X)(dX/dt)$ specific growth rate (h^{-1}); m = maintenance coefficient (mmol glucose/mg bacteria/h); Y_G = true growth yield (g bacteria/mol glucose). Marr, Nilson & Clark (1963) introduced originally the specific maintenance rate, a (h^{-1}), and derived the following equation:

$$\frac{1}{Y_{x/s}} = \frac{a}{Y_{\max} \mu_x} + \frac{1}{Y_{\max}}. \quad (1')$$

If the values of m and Y_G in equation (1) or those of a and $Y_{\max}$ in equation (1)' are constant, a linear correlation should be obtained between the reciprocals of $Y_{x/s}$ and μ_x . Indeed, using chemostat cultures, Marr *et al.* (1963), Pirt (1965) and other workers verified experimentally equation (1) or (1)' with respect to *Escherichia coli* (Marr *et al.* 1963; Schulze & Lipe, 1964), *Aerobacter aerogenes*, *A. cloacae* (Pirt, 1965) and *Penicillium chrysogenum* (Righelato, Trinci, Pirt & Peat, 1968).

However, some exceptions, apparently contradictory to equation (1) or equation (1)', must be mentioned. First, Pirt (1965) pointed out that the relationship between the reciprocals of $Y_{x/s}$ and μ_x for rumen bacteria (Hobson, 1965) deviated from the linear relationship. Secondly, when a glucose-limited chemostat culture of *Azotobacter vinelandii* was disrupted by suddenly changing the glucose concentration in order to check whether or not equation (1) holds true even in the unsteady state accompanying the disruption, the reciprocal plot resulted in the negative value of Y_G ; a preliminary assumption that Y_G was a hyperbolic

function of μ_x resolved this inconsistency (Nagai, Nishizawa, Endo & Aiba, 1968), but there are other possible explanations.

The purpose of this work is to elaborate the true growth yield, Y_G , and the maintenance coefficient, m , from mass balance relationships for carbon and oxygen such that a clearer definition of the terms (Y_G and m) applicable to organisms other than *Azotobacter vinelandii* can be obtained.

METHODS

Bacterial strain and culture conditions. *Azotobacter vinelandii* IAM 1078 (ATCC 9046) was grown at $30 \pm 0.5^\circ\text{C}$ in (g/l): glucose, 5 or 8; KH_2PO_4 , 0.2; K_2HPO_4 , 0.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; sodium citrate, 0.05; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005; $\text{Fe}_2(\text{SO}_4)_3 \cdot 3\text{H}_2\text{O}$, 0.005; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; pH 7.0 to 7.2. The fermenter vessel used was 30 l (working volume 15 l) and aeration rate was fixed at 9 l/min throughout. Glucose-limited and oxygen-limited chemostat cultures could be established by controlling the agitation speed in each run. The bacterial mass, residual glucose and respiration rate in each chemostat culture were measured by standard methods described elsewhere (Nagai, Nishizawa & Aiba, 1969).

RESULTS AND DISCUSSION

Energy-balance equations for cell growth. Equation (1) can be rearranged as follows:

$$\nu = m + \mu_x/Y_G, \quad (2)$$

where $\nu = (1/X) (-dS/dt)$, specific rate of glucose utilization (mmol glucose/mg bacteria/h). Assuming that carbon dioxide and water are the only metabolites other than the bacteria, Johnson (1964), presented the following balance equation which deals with oxygen requirement:

$$A\nu = B\mu_x + Q_{O_2}, \quad (3)$$

where A is the amount of oxygen required for combustion of glucose (or glycerol) (mmol O_2 /mmol glucose), B is the amount of oxygen required for combustion of cells (mmol O_2 /mg bacteria) = 0.042 (Johnson, 1964), and Q_{O_2} is the specific rate of respiration (mmol O_2 /mg bacteria/h). In the light of mass balance, equation (3) is essentially along the same line of equation (1) or (1)'. $A\nu$ on the left-hand side of equation (3) is the amount of oxygen required for a complete combustion of substrate consumed, while the first and second terms on the right-hand side of the equation represent the amount of oxygen needed for a complete combustion of bacteria synthesized and that amount required for the bacterial synthesis, respectively.

Payne (1970) employed total energy in place of oxygen requirement mentioned previously in equation (3), and from the concept of energy conservation he subdivided the total energy derivable from the medium ($\propto A\nu$) into the energy responsible for the bacterial growth ($\propto B\mu_x$) and that expended in catabolism ($\propto Q_{O_2}$).

Analogous to equation (1) or (2), the specific respiration rate can be described in terms of maintenance and growth (Herbert, 1958; Schulze & Lipe, 1964; Pirt, 1965; Righelato *et al.* 1968; van Uden, 1968; Harrison & Loveless, 1971):

$$Q_{O_2} = (Q_{O_2})_m + (Q_{O_2})_G \quad (4)$$

or

$$Q_{O_2} = m_o + \frac{1}{Y_{GO}} \mu_x, \quad (5)$$

Fig. 1

Fig. 2

Fig. 1. Specific rate of glucose consumption against dilution rate in glucose-limited and oxygen-limited chemostat cultures of *Azotobacter vinelandii*. ●, Oxygen-limited at 240 rev./min, $S_0 = 45$; □, oxygen-limited at 340 rev./min, $S_0 = 44$; △, glucose-limited at 540 rev./min, $S_0 = 43$; ○, glucose-limited at 780 rev./min, $S_0 = 44$; ×, glucose-limited at 780 rev./min, $S_0 = 27$; S_0 , glucose concentration in fresh medium (mmol/l); rev./min, agitation speed.

Fig. 2. Specific rate of respiration against dilution rate in glucose-limited and oxygen-limited chemostat cultures of *Azotobacter vinelandii*. ●, □, Oxygen-limited; △, ○, ×, glucose-limited (for details see in Fig. 1).

where m_o is the maintenance coefficient for respiration (mmol O_2 /mg bacteria/h) and Y_{GO} is the $dX/(dO_2)_G$, true growth yield for oxygen (mg bacteria/mmol O_2). From equations (3) and (5),

$$\nu = \frac{m_o}{A} + \frac{1}{A} \left(B + \frac{1}{Y_{GO}} \right) \mu_x. \quad (6)$$

From equations (2) and (6),

$$m = \frac{m_o}{A}, \quad (7)$$

$$\frac{1}{Y_G} = \frac{1}{A} \left(B + \frac{1}{Y_{GO}} \right). \quad (8)$$

Now, the maintenance coefficient, m_o , appearing in equation (5) is interpreted as that corresponding to the complete oxidation of glucose, while the specific rate of glucose metabolized for growth, μ_x/Y_G , in equation (2) is re-interpreted as a sum of that rate incorporated into the cellular material, $B\mu_x/A$, and another expended in catabolism, μ_x/AY_{GO} .

The experimental results of glucose- and oxygen-limited chemostat cultures are shown in Fig. 1 and 2 (cf. equations (2) and (5)). If the values of m , m_o , Y_G and Y_{GO} are constant respectively, linear relationships are expected between ν and $\mu_x (= D)$, or Q_{O_2} and $\mu_x (= D)$. For oxygen-limited chemostat cultures, positive values of (m , Y_G ; m_o , Y_{GO}) were obtained. Several workers also presented this kind of correlation (Herbert, 1958; Schulze & Lipe, 1964; Pirt, 1965; Righelato *et al.* 1968; van Uden, 1968). However, an apparently unacceptable result is noted from Fig. 1 and 2 for the glucose-limited chemostat culture; large values

Table 1. *True growth yields and maintenance coefficients for various organisms*

Organism	Limiting factor	Substrate	m	m_o	m_o^*	Y_G	Y_G^*	Y_{GO}	Reference
<i>Aerobacter aerogenes</i>	Glycerol	Glycerol	0.83	3.4	2.9	51	47	30	Herbert (1958)
<i>A. clocae</i>	Glucose	Glucose	0.5	—	3.0	79	—	—	Pirt (1965)
<i>Azotobacter vinelandii</i>	Oxygen	Glucose	0.8	5.5	4.8	46	50	13	This work
<i>Escherichia coli</i>	Glucose	Glucose	0.3	0.5	1.8	95	90	41.5	Schulze & Lipe (1964)
<i>Penicillium chrysogenum</i>	Glucose	Glucose	0.12	0.74	0.72	81	97	50	Righelato <i>et al.</i> (1968)
<i>Saccharomyces cerevisiae</i>	Glucose	Glucose	0.08	0.60	0.48	91	85	34.5	von Meyenburg (1969)

Unit: m (mmol substrate/g bacteria/h); m_o (mmol O_2 /g bacteria/h); Y_G (g bacteria/mol substrate); Y_{GO} (g bacteria/mol O_2).

* Value calculated by equation (7) or (8), $A = 6$ (mol O_2 /mol glucose) or 3.5 for glycerol, $B = 41.7$ (mmol O_2 /g bacteria).

of m , m_o and negative values of Y_G , Y_{GO} . A concept of energy-uncoupled growth could resolve the unacceptable observation.

Values of m , m_o , Y_G and Y_{GO} which are assessed from Fig. 1 and 2 and values cited from the data published by other workers on some other organisms are summarized in Table 1. Values of m and m_o depended on the species of organisms; fairly low values were observed with *Penicillium chrysogenum* and *Saccharomyces cerevisiae*. Except for *Escherichia coli*, m_o^* values calculated from equation (7) are in good agreement with those observed. This agreement supports the hypothesis that maintenance metabolism results in the complete oxidation of glucose (glycerol). The fairly large difference between m_o and m_o^* for *E. coli* in the table might have originated from the usual procedure of Q_{O_2} measurement with a Warburg manometer, i.e. the cells were transferred from the glucose-limited chemostat culture to the manometer, thus disrupting the glucose-limited environment. On the other hand, the values for m_o for other organisms in Table 1 were assessed from Q_{O_2} measurements with chemostat cultures by observing the difference in oxygen concentrations between inlet and outlet air.

Values of Y_G and Y_{GO} for *Azotobacter vinelandii* in Table 1 are lower than those for other organisms. This fact is ascribed to the higher energy requirement for nitrogen fixation from air in this organism. In addition to the previous agreement of m_o between observation and calculation, another fact that Y_G values estimated directly from the measurement of v with the use of equation (2) agreed favourably with Y_G^* values assessed from Q_{O_2} measurements, utilizing equation (8) justifies equation (3).

Energy-uncoupled growth. Results from anaerobic cultures suggest that Y_{ATP} is 10.5 g bacteria/mol ATP (Bauchop & Elsdén, 1960; Hadjipetrou, Gerrits, Teulings & Stouthamer, 1964). The paucity of Y_{ATP} values for aerobic cultures, on the other hand, originates from the lack of information on energy yield and expenditure. However, according to Stouthamer (1962) the values of Y_{ATP} for aerobically grown cells of *Gluconobacter liquefaciens* using various carbohydrates were also 10 g bacteria/mol ATP. Hernandez & Johnson (1967b) proposed that some factor other than ATP might have become the growth-limiting factor in an aerobic culture of *Candida utilis* and *Saccharomyces carlsbergensis*, because the value of Y_{ATP} estimated from the value of Q_{O_2} measured and P/O ratio assumed was about 7;

Fig. 3. Total growth yield for oxygen uptake against dissolved oxygen concentration in glucose-limited and oxygen-limited chemostat cultures of *Azotobacter vinelandii*. ●, □, Oxygen-limited; △, ○, ×, glucose-limited (for details, see in Fig. 1).

they suggested the possibility that some fraction of ATP synthesized might have been wasted as heat. Similar phenomena designated as energy-uncoupled growth have been observed in anaerobic cultures of *Escherichia coli* (Hernandez & Johnson, 1967a) and in aerobic cultures of *Desulfovibrio desulfuricans* and *Aerobacter aerogenes* (Senez, 1962) and *Klebsiella aerogenes* (Harrison & Loveless, 1971).

Before proceeding to discuss the energy-uncoupled growth of *Azotobacter vinelandii* it is necessary to refer briefly to the inhibitory effect of dissolved oxygen on the microbial growth of this organism, because energy-uncoupled growth is closely related to this inhibitory phenomenon (Phillips & Johnson, 1961; Ackrell & Jones, 1971; Nagai, Nishizawa, Onodera & Aiba, 1971). Phillips & Johnson (1961) and Nagai *et al.* (1971) pointed out that an oxygen-wasting system functions when dissolved oxygen exceeds a critical level. From the analysis of enzymes responsible for growth in glucose-limited chemostat culture, Nagai *et al.* (1971) showed that the oxygen-wasting system became predominant at high oxygen concentration.

Total growth yields for oxygen, $Y_{x/o}$ (mg bacteria/mmol O_2) obtained from glucose- and oxygen-limited chemostat cultures are correlated with dissolved oxygen concentration (Fig. 3), regardless of the limiting conditions.

We now develop a hypothesis which explains the negative yield constants illustrated in Fig. 1 and 2. Let Q_{o_2} be subdivided into the following terms:

$$Q_{o_2} = (Q_{o_2})_g + (Q_{o_2})_v, \quad (9)$$

where $(Q_{o_2})_v$ is the specific rate of respiration relevant to energy-uncoupling (mmol O_2 /mg bacteria/h). The second term, $(Q_{o_2})_v$, on the right-hand side of equation (9), incorporates the term, $(Q_{o_2})_m$, attributable to maintenance, and another term due to energy loss. By replacing $(Q_{o_2})_m$ with $(Q_{o_2})_v$ the abnormally high values for m_o obtained by plotting the data from glucose-limited chemostat cultures of *Azotobacter vinelandii* according to equation (5) can be explained. When energy loss can be disregarded, the use of $(Q_{o_2})_v$ in exchange for $(Q_{o_2})_m$ is not always required.

Fig. 4. Oxygen waste in *Azotobacter vinelandii* affected by dissolved oxygen concentration in glucose-limited chemostat cultures (for symbols, see in Fig. 1).

From equations (5) and (9)

$$\begin{aligned}(Q_{O_2})_v &= Q_{O_2} - (Q_{O_2})_g \\ &= Q_{O_2} - \frac{1}{Y_{GO}} \mu_x.\end{aligned}\quad (10)$$

It therefore becomes possible to evaluate $(Q_{O_2})_v$ from the terms on the right-hand side of equation (10), if it is assumed that the value of Y_{GO} from the energy-coupled (oxygen-limited) growth listed in the table for *Azotobacter vinelandii* can also be used for glucose-limited chemostat culture. The values of $(Q_{O_2})_v$ estimated in this way are plotted against dissolved oxygen concentration in Fig. 4. In spite of the difference in experimental details for glucose-limited chemostat cultures, it is noted from Fig. 4 that the specific rate of respiration relevant to energy-uncoupling increased almost linearly with the increase in dissolved oxygen concentration. Thus to a close approximation:

$$(Q_{O_2})_v = U(\text{D.O.}), \quad (11)$$

where U = proportionality constant, D.O. = dissolved oxygen concentration (p.p.m.).

In Fig. 5a dissolved oxygen concentration, D.O., and total rate of O_2 uptake, $Q_{O_2}X$ (mmol O_2 /ml culture/h), are plotted against dilution rate, D (h^{-1}). It is noted from the figure that the increase in dilution rate, D , was accompanied by an appreciable increase in $Q_{O_2}X$, principally emerging from the increase in X (mg bacteria/ml culture). It therefore seems that an increase in $Q_{O_2}X$ resulted in the decrease in dissolved oxygen concentration in each run, provided that the aeration rate and the rotation speed of the impeller were kept constant.

If a straight line is drawn through each set of the data points in Fig. 5(b), then

$$\text{D.O.} = E + F\mu_x, \quad (12)$$

where E = intersection of straight line with ordinate; F = slope of straight line.

Although the values of E and F have no particular significance from a biological viewpoint, the observations on $Q_{O_2}X$ versus D referred to above underlie the negative values of F for the glucose-limited chemostat cultures. In fact, another analysis (Nagai & Aiba, 1972),

Fig. 5 (a) Oxygen requirement of culture (mmol O_2 /ml culture/h), and (b) dissolved oxygen concentration against dilution rate in glucose-limited chemostat cultures of *Azotobacter vinelandii* (for symbols, see in Fig. 1).

utilizing activities of aldolase and glyceraldehyde-3-phosphate dehydrogenase as key parameters in the branched enzymatic kinetics (Waley, 1964), showed the decrease of D.O. as a hyperbolic function of μ_x . The fact that the increase in dilution rate was followed by a sharp decrease of D.O. as shown in Fig. 5(b) was either represented empirically by equation (12) or supported theoretically by the more sophisticated model.

Equations (11) and (12) give:

$$(Q_{O_2})_U = UE + UF\mu_x. \quad (13)$$

Further, equations (10) and (13) yield

$$Q_{O_2} = UE + \left(UF + \frac{1}{Y_{GO}} \right) \mu_x. \quad (14)$$

It is evident from equations (5) and (14) that the terms, m_o and Y_{GO}^{-1} observed with glucose-limited chemostat cultures correspond to UE and $(UF + (1/Y_{GO}))$ respectively. The values $(UF + (1/Y_{GO}))$ or Y_{GO}^{-1} measured for the glucose-limited chemostat cultures (see Fig. 2) are negative because the negative values of UF for the glucose-limited chemostat culture overweighed the value of Y_{GO}^{-1} in equation (14): needless to say, the value of Y_{GO} for the true growth yield should be positive at all times.

Another contradictory phenomenon, the higher values of m and negative values of Y_G for the glucose-limited chemostat cultures (see equation (2) and Fig. 1), could also be re-interpreted by the following equation obtainable from equations (3) and (14).

$$\nu = \frac{UF}{A} + \frac{1}{A} \left(UF + \frac{1}{Y_{GO}} + B \right) \mu_x. \quad (15)$$

The negative values of $Y_e (= (UF + (1/Y_{GO}) + B)^{-1})$ could be explained by the fact that UF values (negative) overweighed positive values of $(1/Y_{GO}) + B$.

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