The Effect of Growth Conditions on Respiratory Activity and Growth Efficiency in Facultative Anaerobes Grown in Chemostat Culture

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SUMMARY

The effects of temperature, pH, dilution rate and oxygen tension on the yield coefficient and respiration rate of *Klebsiella aerogenes* and *Escherichia coli* grown in continuous culture were studied. Reduced cell yields and increased oxygen uptake rates were obtained at low oxygen tension (2 to 5 mmHg) without any increase in the concentration of extracellular organic carbon. These effects and changes in the respiration rate with changes in the dilution rate are discussed in relation to the 'energy efficiency' of the cells.

INTRODUCTION

In the majority of microbial processes in industry the aim is to convert a maximum amount of substrate to cells or useful product. For waste treatment, however, it is desirable to oxidize substrate to carbon dioxide as fast as possible with minimum production of cell mass. At present most sewage and industrial wastes are treated in completely aerobic systems chiefly because this is thought necessary to maintain the physical characteristics of a fast settling sludge. Assuming that other methods for removing solids from suspensions will be found, it is important to know what conditions would be expected to produce least cells for maximum oxidation rates.

Klebsiella aerogenes respired faster at low oxygen tensions below a 'critical value' than under fully aerobic conditions and in this state the cell yield was decreased (Harrison & Pirt, 1967).

The 'potential' respiration rate (i.e. respiration rate of a sample when exposed to excess oxygen and excess substrate) of cells grown in continuous culture was higher in anaerobically grown cultures than in fully aerobic cultures (Harrison, MacLennan & Pirt, 1969). In the present work an attempt is made to determine the way in which the physiology of the cell changes to adapt to different growth conditions.

METHODS

The organisms used in this work were *Escherichia coli* I (NCIB 86) and *Klebsiella aerogenes* 8017 (NCIB).

Culture technique. The bacteria were grown in a 1.5 l. Porton-type chemostat vessel (Elsworth, Meakin, Pirt & Capell, 1956) equipped with automatic control and recording of pH and temperature. The dissolved oxygen tension in the culture was recorded using either a Mackereth probe (Mackereth, 1964) or a steam sterilizable version of this probe

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(Harrison & Melbourne, 1970). A synthetic medium was used containing glucose (5.0 g./l.) as the sole, limiting carbon source (Harrison & Pirt, 1967). The oxygen tension in the culture was regulated via the proportions of air and nitrogen in the feed gas. Anaerobic conditions were obtained by feeding 'white spot' nitrogen gas (BOC, Wembley, Middlesex). No steps were taken to eliminate the small amount of oxygen dissolved in the medium in the reservoir or to prevent diffusion through silicone bungs, etc.

Analytical techniques. Dry weights were determined on 30 ml. centrifuged, washed samples. Glucose was determined by the hexokinase method (Slein, 1965) and by the anthrone method (Trevelyan & Harrison, 1952). Cell and supernatant carbon determinations were made using an infrared carbon analyser (Beckman Instruments), and cell nitrogen was determined by the Kjeldahl technique (Magee, 1955); these determinations were carried out by the Analytical Section of the Laboratory.

Carbon dioxide production was monitored by passing the effluent gas from the culture through a 'Lira 300' infrared CO₂ analyser (Mine Safety Appliances Co. Ltd, Glasgow). The oxygen in the gas phase was measured using a Mackereth oxygen probe (Briggs, Melbourne & Eden, 1966). The gas was moistened and temperature equilibrated before passing through the probe assembly.

Measurement of Q_{O_2} . The 'potential' respiration rate of cells grown under glucose-limited conditions was measured by diluting samples from the culture in air-saturated buffer, adding excess glucose (10 mg./ml.) and following the oxygen uptake in an oxygen electrode respirometer (Rank Bros., Bottisham, Cambridgeshire).

The 'potential' Q_{O_2} was calculated from

$$Q_{O_2} \text{ (mmole/g. h.)} = \frac{r}{xd} \quad (1)$$

where r = rate of depletion of oxygen concentration as measured in the oxygen electrode cell (mmole/l. h.), d = dilution factor, x = concentration of organism (g./l.).

The '*in situ*' Q_{O_2} of the culture, used for the respiration rate of cells as they grow in the culture vessel, was calculated from

$$Q_{O_2} \text{ (mmole/g. h.)} = (C_1 - C_2) \frac{P}{760} \frac{273}{T + 273} \frac{f}{100} \frac{60}{22.4} \frac{1}{xV} \quad (2)$$

where C_1 = oxygen in gas supply (%), C_2 = oxygen in effluent gas (%) corrected for volume change if necessary, P = atmospheric pressure (mmHg), T = temperature of culture (°C), f = gas flow-rate (ml./min.), x = concentration (dry weight) of bacteria in a culture (g./l.), V = volume of culture.

Calculation of yield coefficients. The steady-state yield coefficient from glucose was calculated from

$$Y_{\text{glucose}} = \frac{x}{s_i - s_0} \quad (3)$$

where x = concentration of organisms (g./l.), s_i = glucose concentration in the feed medium (g./l.), s_0 = steady-state glucose concentration in the culture (g./l.).

Usually, s_0 was negligible compared with s_i , so that $Y_{\text{glucose}} = x/s_i$. The steady-state yield coefficient from oxygen (Y_{O_2}) was calculated from

$$Y_{O_2} = \frac{\mu}{Q_{O_2}} \quad (4)$$

where μ = specific growth rate (h.⁻¹) = dilution rate Q_{O_2} = specific respiration rate *in situ* (mmole/g. h.).

RESULTS

Effect of growth conditions on growth yield

Temperature affects the maximum growth rate (Ingraham, 1962) and growth yield (Senez, 1962) of bacteria. Table 1 shows the growth yields obtained for a glucose-limited culture of *Escherichia coli* at different temperatures. The growth yield constant was unaffected by the temperature, between 15° and 36°, although the maximum growth rate was much lower at 15° than at 30° or 36°. To obtain a steady state at 15° the dilution rate had to be lowered to 0.05 h.⁻¹.

Table 1. *The effect of temperature on growth yield of a glucose-limited chemostat culture of Escherichia coli*

Cultural conditions: pH = 6.5

Temperature (°C)	Growth rate (h. ⁻¹)	Yield constant (g. cells/g. glucose)
15	0.05*	0.37 ± 0.01
30	0.15	0.38 ± 0.02
36	0.15	0.37 ± 0.02
42	No growth	—

* This was the maximum growth rate that could be achieved under these conditions.

Table 2. *The effect of pH on the growth yield and Q_{O_2} of a glucose-limited chemostat culture of Escherichia coli*

Cultural conditions: temperature 35°, growth rate 0.15 h.⁻¹

pH value	Yield constant (g. cells/g. glucose)	Q_{O_2} * (in situ) (mmole O ₂ /g. h.)
8.2	0.35 ± 0.02†	5.5 ± 0.3
7.5	0.39 ± 0.02†	5.5 ± 0.3
7.0	0.37 ± 0.02†	n.d.
6.6	0.37 ± 0.02†	4.9 ± 0.4
5.4	0.32 ± 0.02	8.3 ± 0.3

n.d. = Not determined.

* Each value is the mean of duplicate estimations.

† Not significantly different at the 95% confidence level.

The effect of pH on a glucose-limited culture is shown in Table 2. The organism grew at a rate of 0.15 h.⁻¹ between pH 5.4 to 8.2. There was no significant effect of pH on growth yield between pH 6.6 to 8.2. However, at pH 5.4 a lower yield of cells was obtained; the difference being significant at the 95% confidence level. (Significance was judged by the *t*-test applied to the technical error which was calculated from the results of more than 30 samples.) This lower yield was accompanied by a greater Q_{O_2} than that observed at higher pH values. At pH 5.4 more substrate was expended in energy production and less was converted into cell material. Although a lower Q_{O_2} value was obtained at pH 6.6 than at 7.5 this difference was within the limit of technical error.

Effect of oxygen supply on growth yield and Q_{O_2}

Harrison & Pirt (1967) showed that the 'in situ' Q_{O_2} of a culture of *Klebsiella aerogenes* was highest when the oxygen supply limited growth. In *Escherichia coli* as well as in

K. aerogenes the 'in situ' Q_{O_2} was higher under limited oxygen conditions than in the presence of excess oxygen (Tables 3 and 4). The 'potential' Q_{O_2} was higher than the 'in situ' Q_{O_2} under all conditions of oxygen supply. With *E. coli* the maximum 'potential' Q_{O_2} was observed under limited oxygen conditions whereas with *K. aerogenes* the maximum value was attained after the organisms had been grown anaerobically for 3 days, when it was nearly double that found after growth with a limited supply of oxygen.

Table 3. *The effect of oxygen supply on the respiration rate, yield constant and supernatant carbon of a glucose-limited chemostat culture of Escherichia coli*

Culture conditions: pH 6.5, temperature 35°, growth rate 0.15 h.⁻¹

Growth conditions	Q_{O_2} (mmole/g. h.)		Yield constant (g. cells/g. glucose)	Supernatant carbon (mg./l.)
	<i>In situ</i>	Potential		
Oxygen in excess	5.0	5.4	0.36 ± 0.02	131
	5.0	6.2		150
Limited oxygen	8.8	16.0	0.16 ± 0.02	830
Anaerobic	—	8.6	0.08 ± 0.01	1300
		9.0		1400
		8.8		

Table 4. *Effect of oxygen supply on the respiration rate, yield constant and supernatant carbon of a glucose-limited chemostat culture of Klebsiella aerogenes*

Culture conditions: pH 6.5, temperature 30°, growth rate 0.21 h.⁻¹

Growth conditions	Q_{O_2} (mmole/g. h.)		Yield constant (g. cells/g. glucose)	Supernatant carbon (mg./l.)	Supernatant glucose (mg./l.)
	<i>In situ</i>	Potential			
Oxygen in excess (O ₂ tension 5–150 mmHg)	5.6 ± 0.3	9.1 ± 0.3	0.43 ± 0.02	255 ± 50	< 50
Limited O ₂ (O ₂ tension < 5 mmHg)					
Oxygen supply < demand	9.7 ± 0.3	10.9 ± 0.4	0.26 ± 0.06	233 ± 90	< 50
Oxygen supply ≤ demand	7.2 ± 0.6	12.7 ± 0.8	0.14 ± 0.2	900 ± 170	< 50
Anaerobic					
For 1 day	—	10.6 ± 0.7	0.13 ± 0.03	860 ± 170	< 50
For 3 days	—	22.4 ± 0.3	0.08 ± 0.01	1020 ± 90	< 50

The yield constants were highest under fully aerobic conditions, lower with limited oxygen and least under anaerobic conditions. The supernatant carbon increased as the oxygen supply was diminished, because products were formed by glucose fermentation. When a culture of *Klebsiella aerogenes* was only just oxygen-limited (Table 4) the dissolved carbon concentration was about the same as the fully aerobic value, although the cell yield was decreased by nearly 40%. This is the phase of 'uncoupled growth' which may give rise to stable oscillations (Degn & Harrison, 1969) and, in fact, oscillations were observed at one stage of this experiment.

Effect of growth rate on cell yield and Q_{O_2} of Klebsiella aerogenes

Fig. 1*a* shows the effect of changing growth rate on the 'in situ' and 'potential' Q_{O_2} . At growth rates from 0.05 to 0.6 h.⁻¹ the 'in situ' Q_{O_2} rose linearly. This would be expected if growth were energy-limited according to the relation

$$Q_{O_2} = \frac{\mu}{Y^{ATP}N} + \frac{M}{N} \quad (5)$$

where Q_{O_2} = specific respiration rate (mole/g. h.), μ = specific growth rate (h.⁻¹) = dilution rate, Y^{ATP} = cell yield coefficient for ATP (g./mole) as proposed by Bauchop & Elsdon (1960), N = number of moles ATP produced/mole oxygen taken up, M = maintenance ATP requirement (moles/g. h.).

Fig. 1. The effect of growth rate on cell dry wt concentration and Q_{O_2} . ○, potential Q_{O_2} ; ●, in situ Q_{O_2} ; □, cell dry wt; ×, glucose concentration. Culture conditions: pH 6.5, temperature 30°.

The point where the extrapolated line cuts the Q_{O_2} axis gives the maintenance respiration rate (1.4 mmole/g. h.) which is equal to M/N . Assuming three sites of oxidative phosphorylation and two substrate-level phosphorylations/glucose molecule, $N = 6.3$, whence $M = 8.8$ mmole ATP/g. h.

The 'potential' Q_{O_2} increased more slowly with growth rate than the 'in situ' Q_{O_2} . At a growth rate of 0.1 h.⁻¹ the 'potential' Q_{O_2} was about three times greater than the 'in situ' Q_{O_2} , but at a growth rate of 0.06 h.⁻¹ there was little difference between the 'potential' and 'in situ' Q_{O_2} . At a very low growth rate (0.025 h.⁻¹) there was a sharp fall in 'potential' Q_{O_2} . This might be due to the high proportion of non-viable cells present under these conditions (Postgate & Hunter, 1962).

Table 5. *A comparison of growth parameters between the logarithmic phase of batch cultures and high-rate continuous culture of Klebsiella aerogenes*

	Growth rate (h. ⁻¹)	Q_{O_2} (mmole/g. h.)		Y_{glucose} (g./g.)	Y_{O_2} (g./mole)
		<i>In situ</i>	Potential		
Batch culture	0.87	17.5	14.2*	0.39	39
Continuous culture	0.6	14.0	15.0	0.43	43
	0.75	25.0	24.0	0.23	30

* Value obtained for washed, resuspended cells.

When the dilution rate was increased to 0.65 h.⁻¹ the cell concentration began to fall, but complete washout of culture did not occur; instead, the cell dry weight concentration settled at a new low level and a steady state was maintained for over 5 days. This steady state was accompanied by a high glucose concentration and both the 'potential' and 'in situ' Q_{O_2} values were much higher than at a dilution rate of 0.6 h.⁻¹ (Fig. 1*a*). Samples of a culture in this state were analysed for glucose by both the enzyme and anthrone methods. The results of the enzyme assay, which is specific for D-(+)-glucose, is shown in Fig. 1*b*. The anthrone method, which is non-specific and detects many types of reducing sugar including their polymers, gave values many times higher than the enzyme method. This discrepancy may have been caused by the production of extracellular polysaccharide in the culture.

The physiological state of the cells would appear to have been changed by increasing the growth rate from 0.60 h.⁻¹ to 0.65 h.⁻¹. In order to see whether the same physiological characteristics prevailed during the logarithmic phase of growth in batch culture, a batch culture of *Klebsiella aerogenes* was grown from a small inoculum in the continuous culture vessel under the same conditions of pH, temperature and stirring as the continuous culture. In batch culture the cells grew at 0.87 h.⁻¹, but did not exhibit the high Q_{O_2} and low growth yield which had been observed with the continuous culture grown at a growth rate of 0.75 h.⁻¹ (Table 5).

According to conventional continuous culture theory (Herbert, Elsworth & Telling, 1956) the maximum attainable dilution rate in chemostat culture should be equal to the logarithmic growth rate of a batch culture under identical conditions. However, on increasing the dilution rate of the chemostat culture to 0.85 h.⁻¹ the culture diluted out completely.

Herbert *et al.* (1956) also reported reduced cell concentrations at high growth rates in continuous culture and attributed this to imperfect mixing. However, in our experiments, the culture vessel was vigorously stirred at a rate of 900 rev./min. Possibly a medium constituent other than glucose became growth-limiting at high dilution rates. This could

occur if the requirement for this substance increased with growth rate, as in the case of magnesium (Tempest, Hunter & Sykes, 1965). However, the concentrations of all medium constituents apart from glucose were doubled without having any effect on cell concentration at a dilution rate of 0.75 h^{-1} . Addition of casamino acid and yeast extract (0.1 %, w/v) did not have any effect, neither did increasing the CO_2 concentration in the gas phase up to 4 %. The nature of the limiting substance, if it exists, was not determined. Possibly the cells themselves produce a growth-regulating substance which can be maintained at an optimum level in batch culture or continuous culture at low dilution rates but which at high dilution rates washes out faster than it is formed.

DISCUSSION

The efficiency of growth of micro-organisms supplied with a single, limited source of both carbon and energy may be reduced if either the yield of utilizable energy from the substrate diminishes or if the energy requirement of the cells increases. In either case an inverse correlation would exist between respiration rate and cell yield. In this work little change was observed in the growth efficiency of *Escherichia coli* over a 20° range of temperature which would indicate that the energy requirement for growth is little affected by temperature. However, the growth rate at 15° was only 0.05 h^{-1} compared with 0.15 h^{-1} at 30° and 36° . From Fig. 1 it can be seen that the growth yield of *Klebsiella aerogenes* grown at 30° is considerably lower at 0.05 h^{-1} than 0.15 h^{-1} . This is accounted for by the comparatively greater proportion of energy that must be used for cell maintenance (equation (5)). If a similar relationship between cell yield and growth rate at 30° applies to cultures of *E. coli* then it might be predicted that raising the temperature from 15° to 30° at a growth rate of 0.05 h^{-1} would cause a decrease in the growth yield. Such a result would indicate a dependence of energy of maintenance on temperature, the energy required for this purpose being decreased at low temperatures. The decreased efficiency of growth on lowering the pH of an *E. coli* culture to 5.4 may be caused by an increased energy requirement to pump ions in order to maintain a constant intracellular pH.

The concept of growth yield coefficient from ATP (Y^{ATP}) introduced by Bauchop & Elsdén (1960) is very useful for interpreting the energetics of cell growth. ATP is the 'coinage' for energy within a cell so that Y^{ATP} represents the efficiency of energy utilization by the cell.

Bauchop & Elsdén (1960) obtained a value of 10.5 for the Y^{ATP} of several organisms grown anaerobically under glucose-limited conditions and suggested that this was the value for maximum efficiency. Lower values may be obtained under less favourable growth conditions and, from the data of Harrison & Pirt (1967) for *Klebsiella aerogenes* grown under glucose-limited conditions in a simple synthetic medium, a value of 7.1 may be calculated.

Rearranging equation (5) gives an expression for N , the number of moles of ATP produced/mole of oxygen taken up:

$$N = \frac{\mu}{Y^{\text{ATP}}} \frac{1}{Q_{\text{O}_2} - M/N} \quad (6)$$

M/N , the maintenance respiration rate, has been found to be $1.4 \times 10^{-3} \text{ mole/g. h.}$ If the maximum value of Y^{ATP} of 10.5 is inserted, a value can be obtained for the minimum ATP production/mole of oxygen. For three sites of oxidative phosphorylation and two substrate level phosphorylations, 38 ATP molecules will be produced per mole of glucose and per

6 moles of oxygen, and N will be 6.3. Similarly, for two or one sites of oxidative phosphorylation N will be 4.3 or 2.3 respectively.

Applying equation (6) to the data of Fig. 1, at a growth rate of 0.6 h^{-1} , $N = 4.8$ and at 0.1 h^{-1} $N = 4.3$, whereas at growth rates of 0.65 or 0.75 h^{-1} $N = 3.3$ or 3.1 respectively. The low values at high growth rates represent a partial uncoupling of oxidative phosphorylation. From the data of Table 3 the value of N obtained for a fully aerobic culture of *Escherichia coli* (assuming that the maintenance respiration is similar to that of *Klebsiella aerogenes*) is 4.0 and for oxygen-limited growth is 1.9. The values for *K. aerogenes* calculated from Table 4 are $N = 4.7$ for fully aerobic conditions and $N = 2.4$ for cells which were just oxygen-limited.

This may be interpreted as indicating that there are two sites of oxidative phosphorylation under fully aerobic conditions and that the ATP produced is used efficiently for growth. However, if the Y^{ATP} value for these conditions were less than 10.5, N would be higher, indicating three sites of oxidative phosphorylation, with a high efficiency of energy utilization. The low values of N for limited-oxygen conditions may arise from loss of coupling at a site of oxidative phosphorylation, so that efficiency of ATP production falls, or it may arise from a lower growth efficiency and decreased Y^{ATP} . The former explanation would seem to be the more likely in the light of biochemical studies (Harrison & Maitra, 1969).

Reports that the efficiency of energy metabolism in aerobic cultures is extremely low (Wimpenny, 1969) seem to be based on erroneous calculations which fail to take into account the portion of the substrate converted into cell material. The conclusion from the results reported here is in broad agreement with the views of Chen (1964) and Hadjipetrou, Gerrits, Teulings & Stouthamer (1964) that micro-organisms growing under carbon-limited aerobic conditions may utilize the ATP produced with an efficiency equal to anaerobically grown cells. However, it is clear that under some growth conditions the efficiency can fall to a low value and 'uncoupled' growth occurs (Gunsalus & Shuster, 1961; Senez, 1962).

In processes where cell production is the main aim any degree of 'uncoupling' of growth from energy metabolism clearly is to be avoided, but in waste-treatment systems such uncoupling is desirable as it leads to higher oxidation rates and lower yields of solids. Such studies as those presented here should help to define optimum conditions for oxidation of wastes. For instance, it seems that oxygen-limited conditions and/or high growth rates offer advantages which may be worth further investigation.

Other workers (Hino & Maedo, 1966; Cavari, Avi-dor & Grossowicz, 1968) observed extremely low respiration rates for *Escherichia coli* grown under strictly anaerobic conditions. This discrepancy may arise because although the 'anaerobic' cultures in our experiments contained very little oxygen, no steps were taken to remove the last traces of oxygen. Although the amount of residual oxygen was certainly insignificant as an electron acceptor, there may have been sufficient to act as an inducer of respiratory enzymes. The higher 'potential' respiration rate is one aspect of the diminished cellular efficiency which is a consequence of the loss of the tight control over metabolism which functions in cells in the aerobic steady state of growth (Harrison & Maitra, 1969). There is no correlation between cytochrome content of cells and the 'potential' respiration rate (Harrison *et al.* 1969).

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