Effect of Dissolved Oxygen on Growth Yield and Aldolase Activity in Chemostat Culture of *Azotobacter vinelandii*

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SUMMARY

Glucose-limited and oxygen-limited chemostat cultures of *Azotobacter vinelandii* were agitated at 240, 340, 560 and 780 rev./min. In the oxygen-limited culture the growth yield ranged from 0.2 to 0.25 mg. bacteria/mg. glucose, considerably higher than 0.03 to 0.18 observed when glucose was limiting. This finding suggests that the dissolved oxygen interferes in the metabolism of growing *Azotobacter*. Aldolase activity, but not that of glyceraldehyde-3-phosphate dehydrogenase, isocitrate dehydrogenase or isocitrate lyase, was proportional to dissolved oxygen concentration in each culture. The carbon balance indicated no products other than the bacteria and carbon dioxide.

INTRODUCTION

It is well known that the oxygen uptake rate of *Azotobacter vinelandii* and its nitrogen-fixation capability are affected remarkably by oxygen tension (Parker & Scutt, 1960; Dilworth, 1962). Tschapek & Giambiagi (1955) reported that the multiplication rate and nitrogen-fixing efficiency of *Azotobacter* increased on lowering the partial pressure of oxygen in the culture. The highest growth yield of *Azotobacter* was observed when the aeration rate was decreased to 18.7 mmol O₂/l./h. (Khmél, Gabinskaya & Ierusalimsky, 1965).

The growth yield from glucose of *Azotobacter vinelandii* in a glucose-limited chemostat culture increased from 0.03 to 0.12, with an increase in dilution rate from 0.1 to 0.35 h.⁻¹ (Nagai, Nishizawa & Aiba, 1969). This finding suggested that the decrease of dissolved oxygen concentration in the chemostat culture accompanying the increase in dilution rate might have caused the increase in growth yield.

The principal purpose of the present work was to determine the most probable mode of oxygen interference with the growth yield by investigating oxygen-limited and glucose-limited chemostat cultures. According to the previous observation (Nagai, Nishizawa & Aiba, 1969), most of the glucose consumed (83 to 97%) was converted to carbon dioxide in the glucose-limited chemostat culture. This is an extremely high ratio of carbon dioxide evolved to glucose consumed.

A specific oxidative mechanism for glucose breakdown via the pentose cycle without the participation of phosphate hexokinase has been proposed by Mortenson & Wilson (1954) and Mortenson, Hamilton & Wilson (1955). The tricarboxylic acid cycle has also been observed by Stone & Wilson (1952*a, b, c*). Consequently, it seemed worthwhile to measure the activities of aldolase, glyceraldehyde-3-phosphate dehydrogenase, isocitrate dehydrogenase and isocitrate lyase, all of which are relevant to carbon dioxide evolution.

METHODS

Bacterium and culture conditions. *Azotobacter vinelandii* IAM 1078 (ATCC 9046) was grown at $30^{\circ} \pm 0.05^{\circ}$ in (g./l.): glucose, 8; KH_2PO_4 , 0.2; K_2HPO_4 , 0.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl , 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 was 7.0 to 7.2. Four series of continuous cultures at constant agitation speeds, 240, 340, 560 and 780 rev./min. were carried out. Culture equipment used was described previously (Nagai *et al.* 1969).

Analytical procedures. Bacterial mass, residual glucose and carbon dioxide evolution rate in each chemostat culture were determined as described previously; conditions for the establishment of chemostat cultures were also as before (Nagai *et al.* 1969). Dissolved oxygen concentration was determined using a Beckman-Toshiba type 777 oxygen electrode.

Preparation of cell-free extract. Samples taken from the chemostat overflow (about 1000 ml.) were centrifuged at 8000g for 5 min. at 5° , washed with cold saline (0.8%) and again centrifuged at 8000g for 5 min. The sediment was washed with phosphate buffer (0.015 M, pH 7.0) and homogenized (B. Braun Melsungen 2876) using glass beads of diameter 0.1 mm. The homogenized suspension was centrifuged at 25,000g for 10 min. at 5° . The supernatant solution was used for the determination of enzyme activities.

Aldolase. A kit (C. F. Boehringer Mannheim GmbH, Biochemica, Germany) was used. The rate of decrease in NADH_2 was measured at 340 nm. and the molecular extinction was taken as $6.22 \times 10^6 \text{ cm}^2/\text{mole}$.

Glyceraldehyde-3-phosphate dehydrogenase. Krebs's (1955) method was modified in that NADP was used instead of NAD. The rate of increase in NADPH_2 was measured at 340 nm. and the molecular extinction was also taken as $6.22 \times 10^6 \text{ cm}^2/\text{mole}$.

Isocitrate lyase. Dixon & Kornberg's (1959) method was used. Glyoxylic phenylhydrazone oxidized was measured at 324 nm. and the molecular extinction was taken as $1.7 \times 10^7 \text{ cm}^2/\text{mole}$.

Isocitrate dehydrogenase. Kornberg's (1955) method was employed. The rate of increase in NADPH_2 was measured at 340 nm., taking the molecular extinction as $6.22 \times 10^6 \text{ cm}^2/\text{mole}$.

Soluble protein. The method of Gornall, Bardawill & David (1949) was used.

RESULTS AND DISCUSSION

Four series of chemostat cultures of *Azotobacter vinelandii* were carried out at constant agitation speeds of 240, 340, 560 and 780 rev./min. Data for those at 340 and 560 rev./min. are shown in Fig. 1 and 2. When agitation was maintained at 340 rev./min. typical oxygen-limited chemostat cultures were obtained as shown in Fig. 1. In this run, dissolved oxygen concentrations were controlled at low levels ranging from 0.1 to 0.4 p.p.m., but glucose concentrations were fairly high; the lowest level observed was 0.44 mg./ml. (440 p.p.m.) at a dilution rate of 0.1 h^{-1} (see Fig. 1). In the glucose-limited chemostat culture of Nagai *et al.* (1969) glucose concentration was around 10^{-3} mg./ml. (1 p.p.m.). Experimental data for 240 rev./min. were similar to Fig. 1 (oxygen-limited), but the cell concentration was lower because of the lower supply rate of oxygen.

Fig. 2 shows the experimental results when the agitation speed was moderate at 560 rev./min. In contrast to Fig. 1, glucose and dissolved oxygen concentrations exhibited peculiar patterns with respect to the dilution rate. Glucose concentration was nearly zero when the dilution rate was less than 0.2 h^{-1} (Fig. 2), but the concentration became higher when the

dilution rate was greater than 0.2 h^{-1} . Apparently, the chemostat culture obtained at an agitation speed of 560 rev./min. was characterized by two limiting factors. It was oxygen-limited at dilution rates greater than 0.2 h^{-1} , and glucose-limited when the dilution rate was lower than 0.2 h^{-1} .

Growth yield from glucose. The growth yield from glucose of *Azotobacter vinelandii* in glucose-limited chemostat culture increased from 0.03 to 0.12 mg. bacteria/mg. glucose, following an increase in dilution rate from 0.1 to 0.35 h^{-1} when the glucose concentration

Fig. 1

Fig. 2

Fig. 1. Bacterial mass, glucose, and dissolved oxygen concentrations, specific rate of CO_2 evolution and yield from glucose in oxygen-limited chemostat culture of *Azotobacter vinelandii*. Agitation speed, 340 rev./min. ○—○, Glucose concentration in chemostat culture; ●—●, glucose concentration in fresh medium; △—△, bacterial concentration; □—□, dissolved oxygen concentration; ▲—▲, CO_2 evolution; ◇—◇, growth yield from glucose.

Fig. 2. Bacterial mass, glucose and dissolved oxygen concentrations, specific rate of CO_2 evolution and yield from glucose in glucose-limited (dilution rate $< 0.2 \text{ h}^{-1}$) and in oxygen-limited (dilution rate $> 0.2 \text{ h}^{-1}$) chemostat culture of *Azotobacter vinelandii*. Agitation speed, 560 rev./min. ○—○, Glucose concentration in chemostat culture; ●—●, glucose concentration in fresh medium; △—△, bacterial concentration; □—□, dissolved oxygen concentration; ▲—▲, CO_2 evolution; ◇—◇, growth yield from glucose.

in the fresh medium was 5 mg./ml. (Nagai *et al.* 1969). In the oxygen-limited chemostat culture in the present work (Fig. 1) the yield was considerably higher, ranging from 0.2 to 0.23. In the glucose-limited culture (glucose concentration in fresh medium 8 mg./ml.) the yield agreed with the previous finding (Nagai *et al.* 1969).

These results suggested that the growth yield depended on the dissolved oxygen concentration. The relationship between the growth yield and dissolved oxygen concentration is summarized in Fig. 3, extracting relevant data from the experiments at 240, 340, 560 and 780 rev./min.

A similar observation was presented by Dalton & Postgate (1969) for chemostat cultures

of *Azotobacter chroococcum*. They suggested that the physiological meaning of the term 'carbon-limited' becomes equivocal, because nitrogen-fixation efficiency which ought to have been limited by carbohydrate was decreased by oxygen.

Fig. 3. Effects of dissolved oxygen concentration on yield from glucose and specific evolution rate of carbon dioxide in chemostat culture of *Azotobacter vinelandii*. Data points were reproduced from Fig. 1 and 2 and additional data from experiments at 240 rev./min. (oxygen-limited) and 780 rev./min. (glucose-limited) were cited. Open and solid symbols indicate growth yield from glucose and specific evolution rate of carbon dioxide, respectively. ○, ●, 780 rev./min.; △, ▲, 560 rev./min.; □, ■, 340 rev./min.; ◇, ◆, 240 rev./min.

Carbon balance for glucose. Fig. 3 shows that the specific rate of carbon dioxide evolution increased almost in proportion to the increase in dissolved oxygen concentration. If bacterial mass and carbon dioxide are the principal products formed from glucose, one can write:

$$\text{Glucose carbon consumed} = \text{Bacterial carbon produced} + \text{CO}_2 \text{ evolved.} \quad (1)$$

Then

$$\alpha_3 \nu = \alpha_1 \mu_x + \alpha_2 Q_{\text{CO}_2} \quad (2)$$

where

$$\nu = -\frac{1}{X} \frac{dS}{dt} = \text{Specific rate of glucose consumption, mg. glucose/mg. bacteria/h.};$$

$$\mu_x = \frac{1}{X} \frac{dX}{dt} = D, \text{ dilution rate, h.}^{-1};$$

$$Q_{\text{CO}_2} = \frac{1}{X} \frac{d\text{CO}_2}{dt} = \text{Specific rate of carbon dioxide evolution, mmoles CO}_2/\text{mg. bacteria/h.};$$

$$\begin{aligned} \alpha_1 &= \text{Carbon content of } Azotobacter \text{ vinelandii} \\ &= 0.45 \text{ mg. carbon/mg.}; \end{aligned}$$

α_2 = Stoichiometric constant for carbon in CO_2

= 12 mg. carbon/mmmole CO_2 ;

α_3 = Stoichiometric constant for carbon in glucose

= 0.40 (= 72/180) mg. carbon/mg. glucose.

Rearranging equation (2),

$$\begin{aligned}\frac{1}{Y_{x/s}} &= \frac{\alpha_1}{\alpha_3} + \frac{\alpha_2}{\alpha_3} \frac{Q_{\text{CO}_2}}{\mu_x} \\ &= 1.12 + 30 \frac{Q_{\text{CO}_2}}{\mu_x}\end{aligned}\quad (3)$$

where

$Y_{x/s}$ = Growth yield = $\frac{\mu_x}{\nu}$ (mg. bacteria/mg. glucose).

Fig. 4. Specific oxidative pathway of glucose degradation in *Azotobacter vinelandii* (Stone *et al.* 1952*a, b, c*; Mortenson *et al.* 1954, 1955). G-6-P = glucose-6-phosphate; 6-PG = 6-phosphogluconate; R-5-P = ribulose-5-phosphate; GA-3-P = glyceraldehyde-3-phosphate; F-6-P = fructose-6-phosphate; F-1, 6-P = fructose-1, 6-diphosphate; DHA-P = dihydroxyacetonephosphate.

When the data from the four experiments at 240, 340, 560 and 780 rev./min. (Fig. 3) were plotted as $1/Y_{x,s}$ against Q_{CO_2}/μ_x , a good straight-line relationship of equation (3) was found. This supported the assumption that no appreciable amount of metabolites other than bacteria and carbon dioxide was produced in both oxygen-limited and glucose-limited chemostat cultures.

The specific oxidative mechanism for glucose breakdown is represented schematically in Fig. 4. The scheme shows that glyceraldehyde-3-phosphate is branched either to fructose-1,6-diphosphate via aldolase or to pyruvate via glyceraldehyde-3-phosphate dehydrogenase. Isocitrate can be converted to succinate and malate via isocitrate dehydrogenase or to the

Fig. 5. Effect of dissolved oxygen concentration on activities of aldolase (●), glyceraldehyde-3-phosphate dehydrogenase (○), isocitrate lyase (△) and isocitrate dehydrogenase (□) in glucose-limited and oxygen-limited chemostat cultures of *Azotobacter vinelandii*.

same acids via isocitrate lyase. Enzyme activities related to these major biochemical steps are obviously concerned in the evolution of carbon dioxide; for example, if aldolase activity is high, recycling of glyceraldehyde-3-phosphate through the pentose cycle may become preponderant culminating in the more active evolution of carbon dioxide.

Activities of the four enzymes in the chemostat cultures at an agitation speed of 560 rev./min. are shown in Fig. 5.

The experimental fact that a fairly large range of dissolved oxygen concentration was observed on changing the dilution rate in this particular chemostat is the reason for using the data obtained at 560 rev./min. (given in Fig. 2). It is evident that activities of isocitrate dehydrogenase, isocitrate lyase and glyceraldehyde-3-phosphate dehydrogenase remained fairly constant, while aldolase activity increased when the cells were subjected to an environment higher in dissolved oxygen. These results are consistent with the observation that the yield from glucose decreased appreciably with the increase in dissolved oxygen concentra-

tion because an increase in aldolase activity would direct more glucose breakdown into the pentose cycle, evolving more carbon dioxide and thus decreasing metabolism via the tri-carboxylic acid cycle leading to biosynthesis.

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