Growth Characteristics of an Oxygen-resistant Hydrogen Bacterium Strain N34

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The growth characteristics of the oxygen-resistant strain of hydrogen bacterium N34 in a 40% O₂-culture were compared with those in a 4% O₂-culture in order to clarify the mechanism of oxygen-resistance. Growth inhibition was observed with decreased CO₂ tension indicating the inhibition of CO₂-fixation. Cell growth in 40% O₂-culture was associated with stimulated oxyhydrogen reaction. There was no significant difference in cell composition. Each of the efficiency of energy conversion and the yield coefficients, Y_{H_2} and Y_{O_2} , in the 40% O₂-culture was about half of that in the 4% O₂-culture. The yield coefficient, Y_{CO_2} , or the carbon recovery, however, was almost equal between the two kinds of cultures. The mechanism of oxygen-resistance was discussed.

It seems to be a general phenomenon that high oxygen tension inhibits the cell growth of hydrogen bacteria; the majority of hydrogen bacteria are unable to grow under more than 40% O₂.¹⁾ Some hydrogen bacteria, as exceptions, were reported to be able to grow under high oxygen tension,^{2,3)} but the mechanisms of the oxygen-resistance in these organisms have not been mentioned yet. The growth inhibition by high oxygen tension has been considered to be the result of the high oxygen-sensitivity of hydrogenase⁴⁾ and many attempts to stabilize or protect hydrogenase against oxygen inactivation have been reported.^{5~9)} It has been reported that high oxygen tension also represses hydrogenase formation.^{10~13)}

Oxygen-resistant hydrogen bacterium strain N34,¹⁴⁾ capable of cell growth under 40% O₂ without a growth lag phase, was obtained by the adaptation of oxygen-sensitive strain N34¹⁵⁾ to 40% O₂. Recently, the adaptation of growing cultures of *Chlorella*¹⁶⁾ to 80% O₂ was reported and it was assumed to be a phenotypic change.

We have previously described¹⁷⁾ that the hydrogen-oxidizing system was not protected from the influence of 40% O₂ in oxygen-resistant resting cells and that the hydrogenase formation system seemed to be protected from

oxygen inhibition in the oxygen-resistant proliferating cells.

The present paper deals with comparisons of growth characteristics between 4% O₂- and 40% O₂-cultures of oxygen-resistant hydrogen bacterium strain N34¹⁴⁾ to clarify the mechanisms of the oxygen-resistance.

MATERIALS AND METHODS

Organism. Oxygen-resistant hydrogen bacterium strain N34¹⁴⁾ obtained by the adaptation of oxygen-sensitive strain N34 to high oxygen tension was used for the investigations.

Culture medium and growth conditions. The medium used for the autotrophic culture was composed of KH₂PO₄ 300 mg; K₂HPO₄ 400 mg; urea 1 g; MgSO₄·7H₂O 200 mg; FeSO₄·7H₂O 50 mg; ZnSO₄·7H₂O 0.05 mg; Na₂MoO₄·2H₂O 0.1 gm, made up to 1 liter with distilled water. The solution of urea was filter-sterilized (membrane filter, 0.2 μm pore size) and added aseptically. The seed culture was carried out on agar plate medium under an atmosphere of 80% H₂+10% O₂+10% CO₂. Cultivation was performed in 1 liter-suction flasks containing 10 ml of the medium. After inoculation, flasks were evacuated, refilled with gas-mixtures of various compositions and shaken reciprocally (120 rpm, stroke length 7 cm) at 35°C. The gas phase was not replaced during cultivation.

Measurement of growth. Cell growth was determined by

measuring the optical density with a photometer (Shimadzu made Baush & Lomb Spectronic 20 model) at 450 nm. The specific growth rate, $\mu_{\max}$, was estimated during the exponential growth phase.

Measurement of dry cell weight. The dry cell weight of washed cells was measured after the weight had reached a constant value on drying at 105°C.

Analysis of cell composition. Elemental analysis was done with a CHN analyzer (Yanagimoto, Type M-2). Protein content was determined by Lowry's method.¹⁸⁾ Analysis of poly- β -hydroxy butyrate (PHB) was carried out by the method described by Law *et al.*¹⁹⁾

Analysis of gases. The composition of the gas phase in the flask before and after the cultivation was determined with a gas chromatograph (Shimadzu Type GC-4B) as described in the previous paper.¹⁾ The consumption of H₂, O₂ and CO₂ was estimated as the difference between the initial and the final composition of the gas phase.

RESULTS

Effects of oxygen tension on growth rate and cell yield

Table I shows the effects of oxygen tension on growth rate and cell yield of oxygen-resistant strain N34. Cell growth occurred without a lag period under 10%~60% O₂, but both the growth rate and the cell yield were depressed markedly with increased oxygen tension. Gaseous substrates had not been exhausted at the stationary growth phase in each flask. Moreover, the resumption of cell growth occurred when the gas phase was replaced with a gas-mixture of 92% H₂ + 4% O₂ + 4% CO₂ at the stationary growth phase in each flask. These results indicate that the cell growth ceased not due to the deficiency of the substrates or inorganic nutrient sources but to the inhibition effects of oxygen.

Effect of hydrogen tension on growth rate

The effect of hydrogen tension on the growth rate of oxygen-resistant strain N34 was tested both under 4% and 40% O₂. The growth rate was not affected by the increased hydrogen tension.

Effects of carbon dioxide tension on growth rate

As shown in Table II, a positive correlation

TABLE I. EFFECT OF OXYGEN TENSION ON GROWTH RATE AND CELL YIELD OF OXYGEN-RESISTANT STRAIN N34

Gas composition (%)			Growth rate μ (hr ⁻¹)	Dry cell weight ^a (mg/ml)
H ₂	O ₂	CO ₂		
86	10	4	0.226	5.13
76	20	4	0.168	4.06
66	30	4	0.153	2.37
56	40	4	0.116	1.53
46	50	4	0.097	0.94
36	60	4	0.035	0.35

^a Cells were harvested at the end of the culture.

TABLE II. EFFECT OF CARBON DIOXIDE TENSION ON GROWTH RATE OF OXYGEN-RESISTANT STRAIN N34

H₂ tension was 86% and 50% in the 4% O₂- and 40% O₂-cultures, respectively.

CO ₂ tension (%)	Growth rate, μ (hr ⁻¹) under	
	4% O ₂	40% O ₂
0.5	0.108	0.068
1	0.139	0.088
2	0.164	0.103
10	0.187	0.120

exists between the initial CO₂ tension in the gas phase and the specific growth rate; growth inhibition with decreased CO₂ tension was observed.

Comparison of cell compositions

The elemental composition of the cell mass was analyzed as shown in Table III. There were no significant differences in elemental composition between the two kinds of cells. The protein content was higher in the 40% O₂-grown cells, while the PHB content was higher in the 4% O₂-grown cells.

Comparison of material balance

Material balances in 4% O₂- and 40% O₂-cultures of oxygen-resistant strain N34 are summarized in Table IV.

The theoretical stoichiometry of gas consumption is as follows;

from (3),

$$n = \frac{\text{consumed H}_2 \text{ (ratio to CO}_2\text{)}}{2} - 1 \quad (4)$$

TABLE III. CELL COMPOSITION OF OXYGEN-RESISTANT N34 GROWN UNDER 4% O₂ AND 40% O₂

	Oxygen tension	
	4%	40%
Element (%) C	48.11	46.64
H	7.31	6.88
O ^a	28.09	29.30
N	9.65	10.93
Ash	6.84	6.25
Protein (%)	57.9	65.5
PHB (%)	1.8	1.3

^a Oxygen content was calculated as residue.

TABLE IV. GROWTH CHARACTERISTICS OF OXYGEN-RESISTANT STRAIN N34 IN 4% O₂- AND 40% O₂-CULTURES

	Oxygen tension	
	4%	40%
Gas consumption (ml) H ₂	145.4	91.4
O ₂	58.2	40.9
CO ₂	26.4	8.7
Ratio (H ₂ :O ₂ :CO ₂)	5.5:2.2:1	10.5:4.7:1
Cell yield (dry wt. mg)	21.2	6.4
Yield coefficient, ^a Y _{H₂}	1.63	0.78
(g/g) Y _{O₂}	0.26	0.11
Y _{CO₂}	0.41	0.37
Carbon recovery ^b (%)	72.0	63.9
Efficiency of energy conversion, Y _E ^c (%)	28.0	13.4

^a Yield coefficients were calculated by dividing the dry cell weight produced by the weight of gaseous substrate consumed.

^b Carbon recovery was calculated as the percentage of cell-C to consumed CO₂-C.

^c $Y_E = \frac{\text{produced cells (mg)} \times 4.85}{\text{consumed H}_2 \text{ (mmol)} \times 56.5} \times 100$

When observed values of consumed H₂ (ratio to consumed CO₂) shown in Table IV are put into eq. (4), *n* is calculated to be 1.75 and 4.25 in the 4% O₂- and 40% O₂-cultures, respectively. The other values of *n*, based on consumed O₂ (ratio to consumed CO₂), 2.2 and 4.7 (Table IV), are larger than the calculated ones, respectively, showing that cell growth of oxygen-resistant strain N34 is associated with the stimulated oxygen uptake. Furthermore, the ratio of consumed H₂ + O₂ in the 40% O₂-culture was twice as much than that in the 4% O₂-culture and the difference between the two cultures was 5H₂ + 2.5O₂.

Yield coefficients, Y_{H₂} and Y_{O₂}, in the 40% O₂-culture are approximately half of those in the 4% O₂-culture, while Y_{CO₂} are almost equal to each other.

The carbon recoveries with respect to the percentage of the cell-C to the consumed CO₂-C are also almost equal to each other as for Y_{CO₂} above.

The efficiency of energy conversion, Y_E, was estimated as the percentage of the combustion heat of produced cells (4.85 kcal/g) to that of consumed H₂ (56.5 kcal/mol).²⁰⁾ As shown in Table IV, Y_E in the 40% O₂-culture is about half of that in the 4% O₂-culture.

DISCUSSION

Growth inhibition was observed with decreased CO₂ tension (Table II). There are few data on the effect of CO₂ tension on the cell growth of hydrogen bacteria.^{21~25)} A positive correlation between the initial partial pressure of CO₂ and the specific growth rate was reported for thermophilic hydrogen bacterium strain TH-1, and the dependence of the physiology of strain TH-1 on the partial pressure of CO₂ was speculated.²³⁾ Recently, the inhibition of CO₂-fixation was observed under low CO₂/high O₂ conditions with *Alcaligenes eutrophus*²⁵⁾ as it was assumed to be due to the oxygenase function of ribulose 1,5-bisphosphate carboxylase. The growth inhibition observed with decreased CO₂ tension in strain N34 seems likely to be accounted for by the

inhibition of CO₂-fixation. That Y_{CO_2} or the carbon recovery was almost equal in both 4% O₂- and 40% O₂-cultures (Table IV) shows that the process of cell yield next to the incorporation of CO₂ was not affected by oxygen tension. Thus, the inhibition of CO₂-fixation is presumed to be one possible factor of the depression of the cell yield under high oxygen tension shown in Table I.

The elemental composition of C, H, O and N shown in Table III is similar to that of other hydrogen bacteria.^{20,26)} That the PHB content was higher in the 4% O₂-grown cells (Table III) is due to the oxygen deficiency²⁷⁾ at the end of the 4% O₂-culture.

With respect to the gas consumption, the observed value of consumed O₂ was higher than the calculated one (Table IV). But it is uncertain whether or not this observation supports the existence of the respiratory protection system (if any) as described for *Azotobacter*.²⁸⁾ It seems a rather general phenomenon in the growth of hydrogen bacteria that the observed O₂ consumption is greater than the calculated one, because the ratio of observed gas composition in *Pseudomonas hydrogenovora*²⁰⁾ and *Alcaligenes hydrogenophilus*²⁶⁾ also shows higher consumption of O₂ than calculated from eq. (3).

As already mentioned in the introduction, the hydrogenase synthesizing system was presumed to be protected from the influence of high oxygen tension in proliferating cells of oxygen-resistant strain N34.¹⁷⁾ In the 40% O₂-culture, the augmented oxyhydrogen reaction presumably scavenges the excess O₂ resulting in the protection of the hydrogenase synthesizing system.

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