Autotrophic Growth and Nitrogen Fixation in Derxia gummosa

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Initial attempts to grow N₂-fixing Derxia gummosa autotrophically on H₂, CO₂, O₂ and N₂ in a closed system yielded variable results. Poor growth was found to be due to rapid O₂ depletion and the requirement for an agar surface. In a closed system, C₂H₂ reduction assays could not be carried out due to complete consumption of H₂. Hence a flow-through culturing technique was developed to supply gases at a constant partial pressure and to perform C₂H₂ reduction assays in a continuous flow system. Hydrogenase of autotrophic D. gummosa was not inhibited by C₂H₂, even at 0.5 atm, and the Kₘ of hydrogenase for H₂ was approximately 0.15 atm. The effects of O₂ and H₂ on C₂H₂ reduction were examined, using the flow-through assay system. The rate of C₂H₂ reduction decreased below 0.074 atm H₂, suggesting that ATP and reductant supply were limiting the nitrogenase activity.

INTRODUCTION

Derxia gummosa, an obligate aerobe of the family Azotobacteraceae, is described as a 'typical awkward nitrogen-fixer' (Hill & Postgate, 1969) because its growth under N₂-fixing conditions is variable and unreliable (Tchan & Jensen, 1963; Hill & Postgate, 1969). Colony variations (massive and thin types), production of tenaceous gum, an apparent need for microaerophilic conditions during N₂ fixation and some unknown factors have made the study of N₂ fixation by D. gummosa difficult.

Autotrophic growth and N₂ fixation were studied as two independent traits until the discovery of a N₂-fixing hydrogen bacterium (Ooyama, 1971). Later, many H₂-oxidizers were proved to fix N₂ and many N₂-fixers were shown to be capable of H₂ oxidation. Some of the latter were also capable of autotrophic growth (Hanus et al., 1979). Pedrosa et al. (1980) were the first to report that D. gummosa can grow autotrophically by aerobic H₂-dependent CO₂ incorporation. In an autotrophically growing, N₂-fixing D. gummosa, electrons donated by H₂ will be channelled to N₂ for N₂ fixation, to CO₂ for carbon fixation and to O₂ as terminal electron acceptor for bioenergetic purposes. Hence the study of autotrophic N₂ fixation should prove rewarding in providing information on the regulation of electron flow.

We have attempted to establish the optimum gas proportions and other cultural conditions for autotrophic growth and N₂ fixation by D. gummosa. Initial attempts to grow the organism autotrophically were not reproducible and growth was very scanty whenever it occurred. This was shown to result from fast O₂ depletion in the sealed growth chamber. Here we describe a continuous gas flow system giving consistent autotrophic growth of D. gummosa on agar surfaces, with provision for a continuous flow-through C₂H₂ reduction assay. The results obtained with this system provide information on reasons for the difficulty experienced in growing D. gummosa.

METHODS

Organism. Derxia gummosa strain 1-15 was originally obtained from H. L. Jensen, Statens Planteavls Laboratorium, Lyngby, Denmark.

Media and growth conditions. The medium of Tchan & Jensen (1963) for D. gummosa consisting of (g per litre of distilled water): K₂HPO₄, 0.5; MgSO₄.7H₂O, 0.25; NaCl, 0.1; FeSO₄.7H₂O, 0.1; CaCl₂, 0.1; Na₂MoO₄.2H₂O,
0.005 (final pH 7.0) was used. Davis agar (Davis Gelatine NZ Ltd) was used as the solidifying agent, at 1.5% (w/v) concentration unless mentioned otherwise.

(a) **Solid culture.** Twelve Petri dishes containing agar medium were inoculated with a suspension of autotrophically grown *D. gummosa* and placed in an anaerobic jar of 3-5 l capacity (Oxoid; code HP11). The jar was evacuated and filled with the desired gas mixture and incubated at 30 °C.

(b) **Liquid culture.** *D. gummosa* was inoculated into 100 ml liquid medium in a 1-litre flask, which was incubated at 30 °C on a gyrotary shaker at 250 r.p.m. The mouth of the flask was sealed with a rubber bung fitted with a three-way stopcock, one free end of which was connected to an electrolytic generator. The other free end was used for evacuation, filling and sampling of gases. The decrease in pressure due to consumption of gases in the culture flask brought electrolyte (dilute H2SO4) into contact with the electrodes in an electrolytic generator which then produced H2 and O2 in the molar ratio of 2:1 until the gas space was at atmospheric pressure. According to Faraday's law of electrolysis, the products of electrolysis are proportional to the current consumed, which was measured with a copper coulometer connected in series in the circuit. Hence the loss of copper is proportional to the gas consumption in the flask. The partial pressure of CO2 (pCO2) within the flask was maintained at 0.1 atm by including in the flask a test tube containing 2ml NaHCO3 solution (2.5%, w/v) buffered with 1 M-Tris at pH 8.5.

(c) **Biphasic culture.** This was similar to liquid culture except that the flask contained 200 ml agar medium (3%, w/v, agar) overlaid with 100 ml liquid medium.

**Acetylene reduction assays.** (a) **Conventional technique.** Vacutainer tubes (10 ml) (Terumo Corp.) were evacuated and filled with 0-1 atm C2H2, 0.05 atm CO2, 0.05 atm H2 and complementary proportions of O2 and N2. A 1 ml sample of a suspension of autotrophically growing *D. gummosa* was injected through the septum. Gas samples of 0.2 ml were analysed by gas chromatography (Varian 3700) using a flame ionization detector to follow C2H4 formation.

(b) **Flow-through assay.** This was done in a plastic lunch box (18.5 × 12 × 6 cm) with a snaptight lid. Two brass T-pieces having a septum and placed diagonally on either end of the box served as inlet and outlet ports. The gas mixture was supplied by a gas-mixing manifold attached to cylinders of H2, air, CO2, N2 and C2H2. Pressures were controlled by two-stage pressure regulators. The gas flow rates were controlled by fine needle valves and monitored with flow meters. A solenoid valve was used to cut off the gas supply (Fig. 1). Eight Petri dishes with growing culture were placed in the box and gas mixture was passed through it at a constant flow rate of between 300 and 500 ml min⁻¹.

**Gas analysis.** H2, O2, CO2 and N2 were separated on a CTR-1 concentric column (1-83 m × 3 mm; Alltech Associates) using Ar as carrier gas (30 ml min⁻¹) and measured with a thermal conductivity detector (Varian 3700) at 101 mA current. The column oven temperature was 40 °C.

CH4, C2H4 and C2H2 were separated on a Porapak T column (1-83 m × 3 mm) using N2 as carrier gas. The column oven temperature was 80 °C. A flame ionization detector (Varian 3700) was used to quantify the gases. All C2H4 concentrations were corrected with reference to the CH4 peak, to account for slight variations in flow rate of the assay system and for injection errors. The areas of the peaks were measured with an Autolab Minigrator (Spectra-Physics).

**Hydrogenase activity.** This was estimated by H2-dependent methylene blue reduction, measured photometrically. The reaction mixture was 5 ml phosphate-buffered saline (0.02 M-phosphate, pH 7.0) containing 20 p.p.m. methylene blue. The methylene blue solution was deoxygenated by boiling, flushed with H2 and equilibrated at 30 °C for 30 min. Ar was used as the filler gas when variable H2 concentrations were required. The reaction was started by adding 100 μl *D. gummosa* suspension and the absorbance was measured at 660 nm. The rate of methylene blue reduction was determined from the slope of the curve, which indicates the hydrogenase activity.

**RESULTS AND DISCUSSION**

**Chemoautotrophic growth**

Initial attempts to grow *D. gummosa* in Petri plates on agar containing mineral medium supplied with H2, O2, N2 and CO2 (5:1:89:5, by vol.) yielded variable results. An analysis of the change in gas composition over the growing culture showed that O2 was rapidly depleted and frequent replenishments were necessary. Growth was much improved when the culture was grown in a perspex box (50 × 30 × 31 cm) under a stream of gas mixture. The box contained 60–80 agar plates seeded with *D. gummosa*. Fig. 1 is a schematic diagram of the flow-through apparatus used.

Since agar culture was not convenient for experimentation on cells, we also resorted to liquid culture, but this gave very poor growth. Since we had maintained similar cultural conditions in both solid and liquid cultures, it was apparent that agar in some way produced superior conditions for growth.
Effect of agar on autotrophic growth of *D. gummosa*

We compared the growth of autotrophic *D. gummosa* in biphasic culture and liquid culture, with and without agar extract. Single flasks were used and the experiment was repeated three times, the same results being obtained each time. The results obtained from one of the experiments are presented in Fig. 2. Here, the loss of copper from the anode of the coulometer was taken as equivalent to the amount of electrolytically produced H₂ and O₂ (0.3855 g copper loss = 1 ml H₂ + 0.5 ml O₂) and also as an indication of the hydrogenase activity of *D. gummosa*. The results indicated that a solid agar surface promoted growth and that an agar extract containing soluble material could not be substituted for it.

Hydrogenase activity

**Effect of C₂H₂.** Since C₂H₂ was reported to inhibit hydrogenase activity in N₂-fixing bacteria (Smith *et al.*, 1976), it was necessary to establish the extent of inhibition, if any, with *D. gummosa*. No significant differences were found in the rate of methylene blue reduction even at 0.5 atm C₂H₂, indicating that no inhibition occurs.

**Effect of partial pressure of H₂ (pH₂).** The influence of pH₂ on hydrogenase activity of whole cells is presented in Fig. 3. The hydrogenase activity increased linearly from 0.03 atm H₂, reaching a maximum velocity at 0.21 atm H₂. The pH₂ for half-maximal velocity was approximately 0.15 atm.

Measurement of nitrogenase activity (C₂H₂ reduction)

**Effect of partial pressure of O₂ (pO₂).** Table 1 shows the effect of varying pO₂ on C₂H₂ reduction by a suspension of autotrophically growing *D. gummosa*, using the conventional technique. The C₂H₄ concentrations indicated are mean values of triplicate tubes used in one of
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Fig. 2. H₂ uptake by autotrophically growing D. gummosa in liquid medium (▲), liquid medium + agar extract (○) or biphasic culture (□) as measured by loss of copper in a copper coulometer. The flask contained 0.8 atm H₂, 0.02 atm O₂, 0.1 atm CO₂ and 0.08 atm N₂.

Fig. 3. Effect of pH₃ on hydrogenase activity by autotrophically growing D. gummosa. Hydrogenase activity was calculated from the rate of change of A₆₆₀ using the molar absorption coefficient value of ε₆₆₀ = 63 000 l mol⁻¹ cm⁻¹. A cell concentration equivalent to 1-2 mg protein was used per assay. A typical value for hydrogenase activity would be 3-5 μmol H₂ (mg protein)⁻¹ min⁻¹.

Table 1. Effect of pO₂ on C₂H₂ reduction by autotrophically growing D. gummosa, measured by the conventional technique

<table>
<thead>
<tr>
<th>Initial pO₂ (atm)</th>
<th>C₂H₄ concn (p.p.m.) at various times (h) (means of three replicate tubes)</th>
<th>Final H₂ concn (%)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>1.37 1.32 2.13 2.57 5.37</td>
<td>4.0</td>
</tr>
<tr>
<td>0.005</td>
<td>1.36 1.21 2.03 3.26 4.5</td>
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<td>0.0</td>
</tr>
<tr>
<td>0.100</td>
<td>1.43 1.26 1.62 4.27 13.3</td>
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</tr>
<tr>
<td>0.130</td>
<td>1.54 1.4 4.14 9.67 28.03</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The experiments. C₂H₂ reduction was negligible in the initial stages, but increased with time after about 3 h. The total amount of C₂H₄ increased with increase in initial pO₂ up to the highest value tested, but concurrent analysis of the samples at 22 h showed that H₂ was completely consumed in all the treatments above 0.04 atm O₂. Decreasing the cell density and/or increasing the surface area did not alleviate the problem of rapid H₂ removal. Hence a flow-through C₂H₂ reduction assay system, where the gas mixture was continuously replenished, was designed (see Methods). This system was used in the experiments described hereafter. In this system each point of analysis (output - input value) represents the rate of C₂H₂ reduction, unlike the situation in a closed vessel (conventional technique) where C₂H₄ concentration is cumulative.

Culture plates briefly exposed to air during transfer from the culture chamber to the assay chamber initially did not reduce C₂H₂, but they regained their C₂H₂-reducing activity within 24 h. Therefore plates were always incubated in the assay chamber for 24 h, in an atmosphere containing H₂, O₂, N₂ and CO₂, before C₂H₂ reduction assays were done.

Effect of partial pressure of C₂H₂ (pC₂H₂). The C₂H₂ reduction rate increased with increase in pC₂H₂ from 0.022 atm to 0.081 atm, above which value there was no significant change (Fig. 4). Decrease in pC₂H₂ did not decrease the rate of C₂H₂ reduction within the time tested. This
Autotrophic \( N_2 \) fixation in Derxia gummosa

![Graph](image)

Fig. 4. Effect of p\( C_2H_2 \) on \( C_2H_2 \) reduction in autotrophically growing \( D. \) gummosa in a flow-through assay system. Readings of \( C_2H_4 \) concentration were taken at 15 min intervals. Arrows indicate the times at which the p\( C_2H_2 \) (atm) was altered to the value indicated beneath the arrow. A 17 d old culture grown on 0-364 atm \( H_2 \), 0.03 atm \( O_2 \), 0.05 atm \( CO_2 \) and 0.556 atm \( N_2 \) was used. \( C_2H_2 \) and \( N_2 \) were in complementary proportions during the experiment.

could be due to the ‘memory effect’ caused by high solubility of \( C_2H_2 \) in water. The p\( C_2H_2 \) of 0-1 atm used in the following experiments was sufficient to saturate the nitrogenase enzyme.

**Effect of p\( O_2 \).** Plate cultures of \( D. \) gummosa equilibrated for 24 h in an atmosphere of 0-048 atm \( O_2 \) were exposed to various p\( O_2 \) values by changing the flow rates of air and \( N_2 \) complementary to each other. The results of \( C_2H_4 \) analyses at 10–20 min intervals at the inlet and outlet ports are shown in Fig. 5. At 0-048 atm \( O_2 \), \( C_2H_2 \) reduction activity increased initially and stabilized after 2 to 3 h. An increase in p\( O_2 \) increased the rate of \( C_2H_2 \) reduction, which stabilized at a new higher value, and lowering the p\( O_2 \) to 0-043–0-048 atm caused a rapid decline in the activity, until the original steady-state value was attained.

In a further experiment, with a different culture grown at 0-043 atm \( O_2 \), the cells were starved of \( O_2 \) for 2 h before being exposed to increasing p\( O_2 \) values. With this culture, steady rates of \( C_2H_2 \) reduction were often achieved within 40 min and, by not returning to a constant ‘baseline’ p\( O_2 \), it was possible to use a greater number of different p\( O_2 \) values (Fig. 6). The steady-state rates of \( C_2H_2 \) reduction again appeared to increase with increase in p\( O_2 \) up to 0-105 atm, but activity decreased at 0-123 atm \( O_2 \). This is probably due to the sensitivity of hydrogenase towards \( O_2 \) in this range rather than to denaturation of nitrogenase, since lowering the p\( O_2 \) to 0-046 atm restored the \( C_2H_2 \) reduction activity. The reason for the rise of activity at 0-046 atm \( O_2 \), above the value observed previously at a similar p\( O_2 \), is not apparent and is at variance with the results shown in Fig. 5. However, the results confirm that \( C_2H_2 \) reduction increases with increasing p\( O_2 \).

**Effect of p\( H_2 \).** Cells were starved of \( H_2 \) for 2 h and \( C_2H_2 \) reduction was followed at various p\( H_2 \) values. No \( C_2H_2 \) reduction could be detected, indicating that nitrogenase was inactivated. It was reasoned that, in the absence of \( H_2 \), nitrogenase would be inactivated by \( O_2 \). The experiment was repeated with descending p\( H_2 \) (Fig. 7). The cells had previously been growing at 0-231 atm \( H_2 \) before the atmosphere was changed to 0-527 atm \( H_2 \). The \( N_2 \) flow rate was adjusted to compensate for the increased \( H_2 \) flow rate. Initially, at 0-527 atm \( H_2 \), the \( C_2H_2 \) reduction rate increased during \( C_2H_2 \) equilibration. With decreasing p\( H_2 \) to 0-147 atm no marked effect was observed. However, when the p\( H_2 \) was reduced to 0-074 atm, the rate of \( C_2H_2 \) reduction fell drastically, and essentially no activity was recorded following change to 0-027 atm \( H_2 \).
Fig. 5. Effect of $pO_2$ on $C_2H_2$ reduction by autotrophically growing *D. gummosa* in a flow-through assay system. Readings of $C_2H_4$ concentration were taken at 15–20 min intervals except at 1015 min, where a 90 min interval was used. Arrows indicate the time at which the $pO_2$ (atm) was changed to the value beneath the arrow. A 13 d old culture grown on 0.24 atm $H_2$, 0.048 atm $O_2$, 0.05 atm $CO_2$ and 0.66 atm $N_2$ was used.

Fig. 6. Effect of $pO_2$ on $C_2H_2$ reduction by autotrophically growing *D. gummosa* in a flow-through assay system. Readings of $C_2H_4$ concentration were taken at 10 min intervals up to 315 min, after which the interval was variable. Arrows indicate the time at which $pO_2$ (atm) was changed to the value indicated beneath the arrow. A 14 d old culture grown on 0.233 atm $H_2$, 0.043 atm $O_2$, 0.05 atm $CO_2$ and 0.674 atm $N_2$ was used.

Thus the reduction of $C_2H_2$ was absolutely dependent upon the presence of $H_2$. It is interesting that the $C_2H_2$ reduction rate did not start decreasing until the pH$_2$ was well below the $K_m$ of hydrogenase for $H_2$ as seen from Fig. 3. This is consistent with the theory that $C_2H_2$ reduction at this pH$_2$ is limited by the electrons donated by $H_2$. A similar observation was made by Berndt & Wölfe (1978), who studied the effect of pH$_2$ on $C_2H_2$ reduction by *Xanthobacter autotrophicus* in chemostat culture.

$H_2$ also helps to prevent $O_2$ damage to nitrogenase. In previous experiments the rate of $C_2H_2$ reduction decreased at $O_2$ concentrations above 10–12%. This may have been due to the sensitivity of hydrogenase to $O_2$ in this range (Bowien & Schlegel, 1981). In the absence of $H_2$,
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no C\textsubscript{2}H\textsubscript{2} reduction occurred. This could be due either to O\textsubscript{2} sensitivity of nitrogenase, which is prevented when H\textsubscript{2} is present, or to severe limitations in the supply of ATP and reductant to nitrogenase, in the absence of H\textsubscript{2}. These data now enable us to establish a set of conditions that can yield reasonable autotrophic growth of N\textsubscript{2}-fixing D. gummosa. Studies are in progress to define further the O\textsubscript{2} and H\textsubscript{2} requirements for autotrophic growth and N\textsubscript{2} fixation, factors which regulate the allocation of electrons from H\textsubscript{2} to various electron acceptors, and to determine the pathways of electron transfer.

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REFERENCES


