

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 K_m 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 Planteavlslaboratorium, 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 2 ml 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.

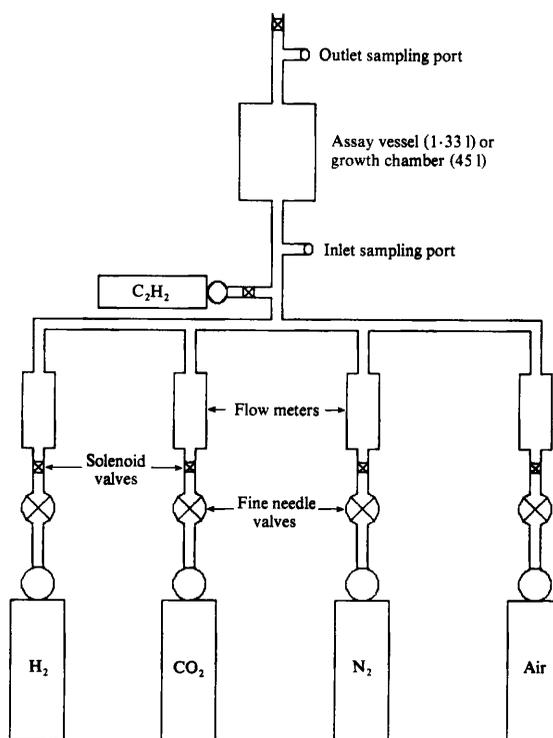


Fig. 1. Schematic diagram of a flow-through system for culturing or C_2H_2 reduction assay in a constant stream of a defined gas mixture.

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 H_2 and O_2 ($0.3855 \text{ g copper loss} \equiv 1 \text{ ml } H_2 + 0.5 \text{ ml } O_2$) 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 C_2H_2 . Since C_2H_2 was reported to inhibit hydrogenase activity in N_2 -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 C_2H_2 , indicating that no inhibition occurs.

Effect of partial pressure of H_2 (pH_2). The influence of pH_2 on hydrogenase activity of whole cells is presented in Fig. 3. The hydrogenase activity increased linearly from 0.03 atm H_2 , reaching a maximum velocity at 0.21 atm H_2 . The pH_2 for half-maximal velocity was approximately 0.15 atm.

Measurement of nitrogenase activity (C_2H_2 reduction)

Effect of partial pressure of O_2 (pO_2). Table 1 shows the effect of varying pO_2 on C_2H_2 reduction by a suspension of autotrophically growing *D. gummosa*, using the conventional technique. The C_2H_4 concentrations indicated are mean values of triplicate tubes used in one of

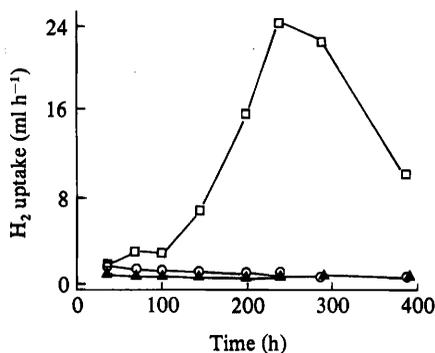


Fig. 2

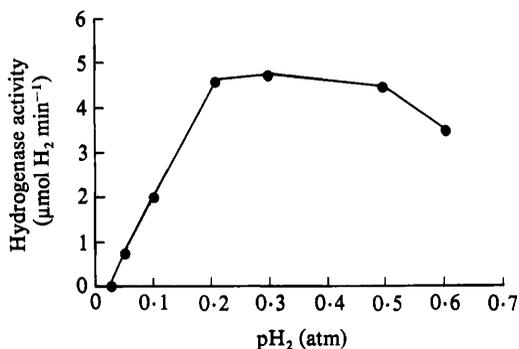


Fig. 3

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 pH₂ 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 = 63000 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

Initial pO ₂ (atm)	C ₂ H ₄ concn (p.p.m.) at various times (h) (means of three replicate tubes)					Final H ₂ concn (%)
	0	1	3	6	22	
0	1.37	1.32	2.13	2.57	5.37	4.0
0.005	1.36	1.21	2.03	3.26	4.5	2.8
0.010	1.18	1.20	2.57	4.05	5.11	2.2
0.020	1.23	1.24	4.53	7.21	10.98	0.4
0.040	1.35	1.12	3.37	6.59	8.93	0.0
0.070	1.45	1.29	1.66	4.86	10.61	0.0
0.100	1.43	1.26	1.62	4.27	13.3	0.0
0.130	1.54	1.4	4.14	9.67	28.03	0.0

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

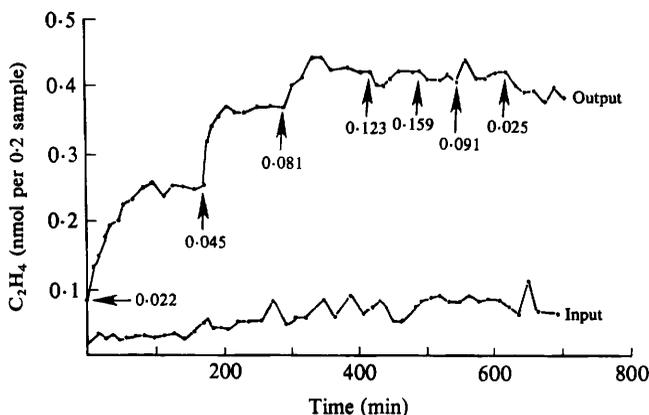


Fig. 4. Effect of pC_2H_2 on C_2H_2 reduction in autotrophically growing *D. gummosa* in a flow-through assay system. Readings of C_2H_4 concentration were taken at 15 min intervals. Arrows indicate the times at which the pC_2H_2 (atm) was altered to the value indicated beneath the arrow. A 17 d old 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 pC_2H_2 of 0.1 atm used in the following experiments was sufficient to saturate the nitrogenase enzyme.

Effect of pO_2 . Plate cultures of *D. gummosa* equilibrated for 24 h in an atmosphere of 0.048 atm O_2 were exposed to various pO_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 pO_2 increased the rate of C_2H_2 reduction, which stabilized at a new higher value, and lowering the pO_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 pO_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' pO_2 , it was possible to use a greater number of different pO_2 values (Fig. 6). The steady-state rates of C_2H_2 reduction again appeared to increase with increase in pO_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 pO_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 pO_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 pO_2 .

Effect of pH_2 . Cells were starved of H_2 for 2 h and C_2H_2 reduction was followed at various pH_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 pH_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 pH_2 to 0.147 atm no marked effect was observed. However, when the pH_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 .

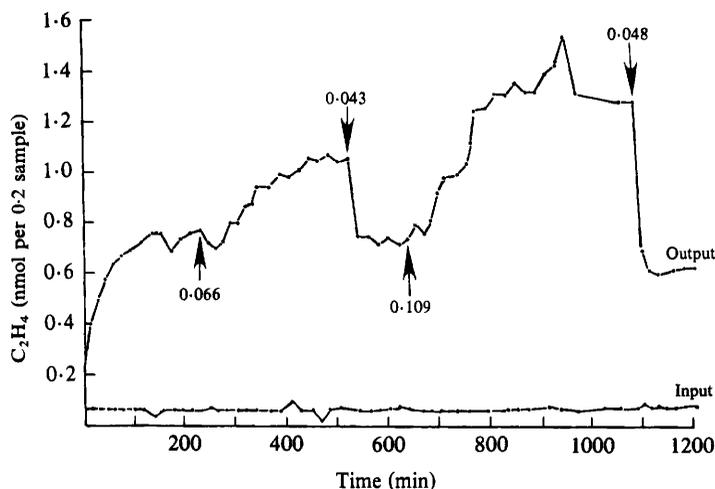


Fig. 5. Effect of pO_2 on C_2H_2 reduction by autotrophically growing *D. gummosa* in a flow-through assay system. Readings of C_2H_4 concentration were taken at 15–20 min intervals except at 1015 min, where a 90 min interval was used. Arrows indicate the time at which the pO_2 (atm) was changed to the 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.

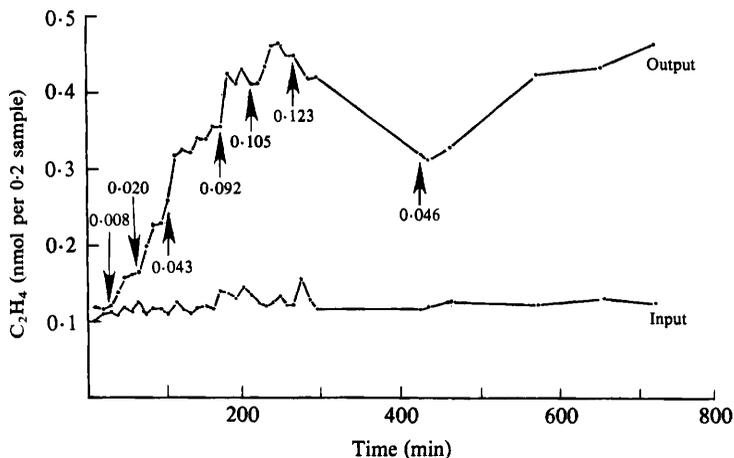


Fig. 6. Effect of pO_2 on C_2H_2 reduction by autotrophically growing *D. gummosa* in a flow-through assay system. Readings of C_2H_4 concentration were taken at 10 min intervals up to 315 min, after which the interval was variable. Arrows indicate the time at which pO_2 (atm) was changed to the value 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 & Wölflé (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 ,

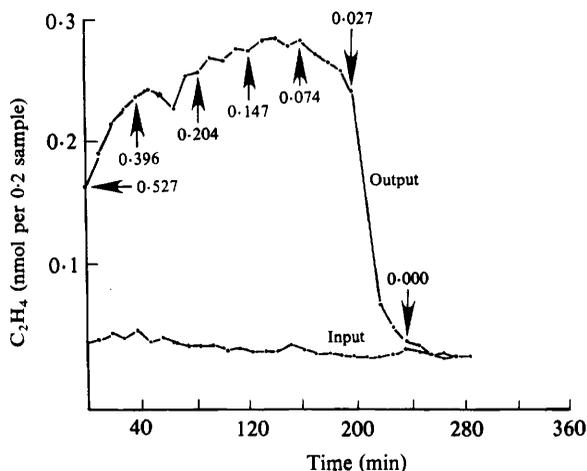


Fig. 7. Effect of pH_2 on C_2H_4 reduction by autotrophically growing *D. gummosa* in a flow-through assay system. Readings of C_2H_4 concentration were taken at 10 min intervals. Arrows indicate the time at which the pH_2 (atm) was changed to the value beneath the arrow. A 13 d old culture grown on 0.231 atm H_2 , 0.049 atm O_2 , 0.05 atm CO_2 and 0.67 atm N_2 was used.

no C_2H_4 reduction occurred. This could be due either to O_2 sensitivity of nitrogenase, which is prevented when H_2 is present, or to severe limitations in the supply of ATP and reductant to nitrogenase, in the absence of H_2 . These data now enable us to establish a set of conditions that can yield reasonable autotrophic growth of N_2 -fixing *D. gummosa*. Studies are in progress to define further the O_2 and H_2 requirements for autotrophic growth and N_2 fixation, factors which regulate the allocation of electrons from H_2 to various electron acceptors, and to determine the pathways of electron transfer.

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