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Measurements of Nitrogenase Activity of *Gluconacetobacter diazotrophicus* Colonies on Solid Medium

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Abstract

The nitrogenase activity of *Gluconacetobacter diazotrophicus* colonies on solid LGI-P medium was examined by H_2 evolution and acetylene reduction assays under 2 kPa and 20 kPa O_2 partial pressures (pO_2). Colonies incubated at 20 kPa of pO_2 produced large quantities of extracellular polysaccharides and formed thick pellicles, while colonies at 2 kPa of pO_2 formed very thin pellicles. H_2 evolution rate was reduced for both cultures in the presence of acetylene, but not abolished even at 30% acetylene. Because of this incomplete saturation of nitrogenase by acetylene, the acetylene reduction assay underestimated at least 13 to 17% of total nitrogenase activity of *G. diazotrophicus* colonies. The percentage of remaining H_2 produced in acetylene reduction assay showed no significant difference between colonies incubated at 2 kPa and 20 kPa of pO_2, suggesting that the incomplete saturation of nitrogenase by acetylene was not caused by the pellicle as diffusion barrier. Our study shows that to measure total nitrogenase activity (TNA) of *G. diazotrophicus* by acetylene reduction assay, the H_2 evolution rate should be monitored.

Keywords: *Gluconacetobacter diazotrophicus*, nitrogenase activity, acetylene reduction assay, H_2 evolution

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1. Introduction

Gluconacetobacter diazotrophicus (Yamada et al., 1997), formerly *Acetobacter diazotrophicus*, is a N_2-fixing bacterium originally isolated from sugarcane stems, roots, leaves and rhizosphere (Cavalcante and Döbereiner 1988; Gillis et al., 1989; Li and MacRae, 1991). It was subsequently isolated from sugarcane mealybug (Ashbolt and Inkerman, 1990), coffee, millets, and pineapples (Jimenez-Salgado et al., 1997; Loganathan et al., 1999; Tapia-Hernandez et al., 2000). Inoculation of sweet potato, sweet sorghum, maize, wheat and rice with *G. diazotrophicus* has also been reported (Paula et al., 1991; Sevilla and Kennedy, 2000). The potential to fix and transfer N to the associated plant partner (Cojho et al., 1993) has stimulated active research on this bacterium.

Measuring N_2-fixation rate is an essential part of research on N_2-fixing bacteria. Although the measurements of whole-organism N-increment and isotope techniques are valuable for seasonal measurements and field studies, they are not appropriate for physiological studies that require dynamic measurements of nitrogenase activity (Hunt and Layzell, 1993). The only two methods that allow short-term measurement of nitrogenase activity in undisturbed conditions are the acetylene reduction assay and H_2 evolution method. Acetylene reduction assay has been used to assess the nitrogenase activity in most studies of *G. diazotrophicus* (Fu et al., 1988; Fuentes-Ramirez et al., 1993; James et al., 1994; Dong et al., 1994; Reis and Döbereiner, 1998). It is generally believed that in an atmosphere of 10% acetylene, all electron flow through nitrogenase is used to reduce acetylene to ethylene (Hardy et al., 1973; Hunt and Layzell, 1993). The production of ethylene from acetylene is presumptive evidence for the presence of nitrogenase and provides an indirect measure of total nitrogenase activity (Hardy et al., 1973; Hunt and Layzell, 1993). However, Dong et al. (1995) showed that nitrogenase of *G. diazotrophicus* incubated in air was not saturated by acetylene even at a high partial pressure of acetylene (30%). The objective of this study is to assess the effect of O_2 concentration on the bacterial colonies and to further study the validity of acetylene reduction assay in determining total nitrogenase activity in these colonies.

2. Materials and Methods

Bacterial strain and culture conditions

G. diazotrophicus strain PAL-5 was cultured on solid N-free LGI-P medium with the pH adjusted to 5.5 (Cavalcante and Döbereiner, 1988). Some bacteria

were cultured on agar plates for the observation of colony structures at different atmospheric partial pressures of O_2. Bacteria were also inoculated on to agar slopes (7 ml) in 17 ml test tubes for the measurements of nitrogenase activity. The cultures were incubated at 30°C in a N_2-O_2 gas mixture (20 kPa or 2 kPa of pO_2) for 5 days before assays for nitrogenase activity. The pO_2 was maintained by replacing the gas with freshly made N_2-O_2 gas mixture at 36, 72, and 96 hours of incubation, and immediately before the assays. The culture tubes were incubated in a water bath of 30°C and sealed by gas-tight serum stoppers during the analyses.

Measurement of nitrogenase activity

H_2 evolution of *G. diazotrophicus* colonies under the same pO_2 as incubation was measured first without acetylene for every culture. The temperature was maintained at 30°C by placing the tubes in a water bath. Since it is difficult to determine the bacterial number accurately, and the number of bacterial cells may not be direct proportion to the N_2-fixing capacity as the nitrogen-fixing colony needs a minimum size to keep the optimal pO_2 inside the colony, the rate of H_2 evolution was expressed as nmol/hr/culture in this experiment. This is a measure of apparent nitrogenase activity (ANA) of *G. diazotrophicus* culture for each tube.

To study the validity of acetylene reduction assay in estimating total nitrogenase activity (TNA), the H_2 and ethylene production of the cultures were measured at 2.5%, 10%, 20% and 30% (v/v) acetylene with the same pO_2 as incubation in the headspace. H_2 and ethylene production rates by individual cultures have then been normalized to 100% of H_2 production in N_2-O_2 gas mixture (% ANA).

All analyses were done using a portable gas chromatograph fitted with a Porapak N column (Alltech, Guelph, Ont., Canada) and a semiconductor sensor (Model 822, Figaro Engineering Inc., Osaka, Japan) (Holfeld et al., 1979). An airflow of 23 ml min^{-1} was used as carrier gas at room temperature.

Five replicates per treatment were measured in all experiments. Means ± standard error are presented.

3. Results

Morphology of culture colonies

Gluconacetobacter diazotrophicus typically formed orange colonies on LGI-P medium supplemented with bromothymol blue as the bacterial cells accumulated the pH indicator and changed its color in acid production. When

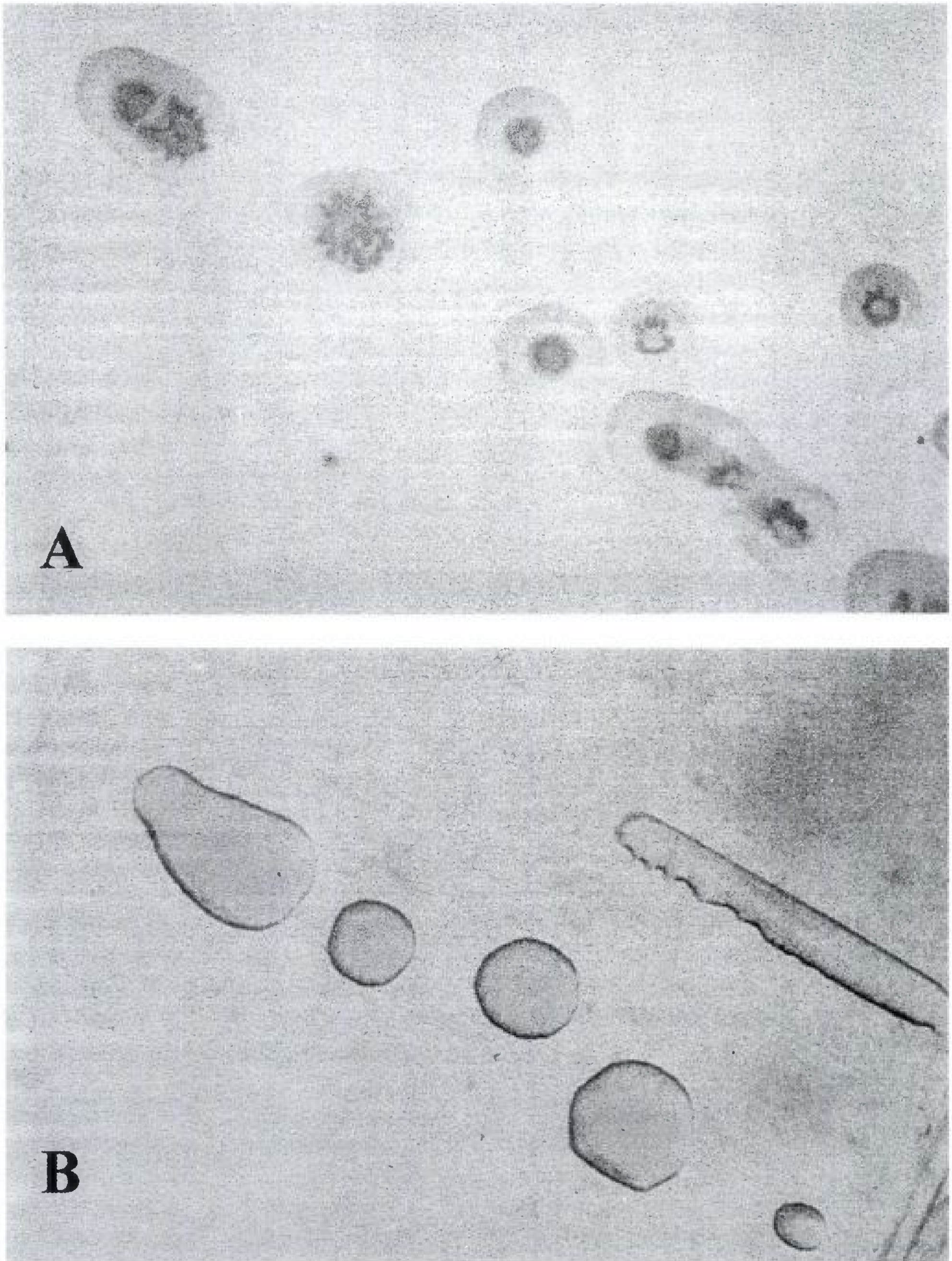


Figure 1. *Gluconacetobacter diazotrophicus* colonies on solid LGI-P medium. (a). Colonies incubated at 20 kPa of pO_2. (b). Colonies incubated at 2 kPa of pO_2. Magnification: 5×.

cultured at 20 kPa of pO_2, the bacterium formed thicker mucilaginous pellicles (Fig. 1a) than those cultured at 2 kPa of pO_2 (Fig. 1b). The colonies cultured at 20 kPa of pO_2 had orange mass in the center indicating the presence of a large number of bacteria in the colony interior (Fig. 1a). In contrast, the colonies incubated at 2 kPa of pO_2 were uniformly orange in color indicating the growth of bacterial cells extended to the edge of the colonies (Fig. 1b). Nitrogenase activity was very sensitive to increased pO_2. H_2 evolution and acetylene reduction rate declined significantly a few minutes after the bacteria cultured at 2 kPa pO_2 was exposed to 20 kPa of pO_2 (data not shown).

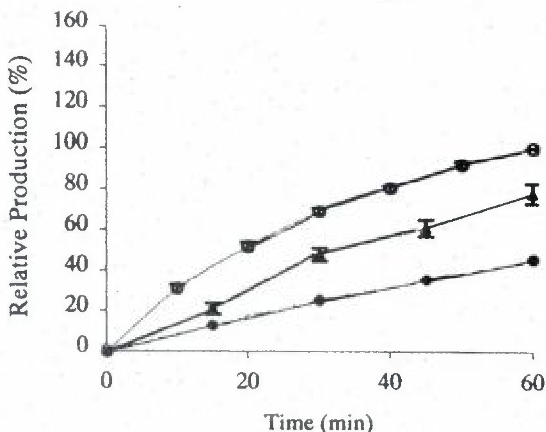
H_2 evolution in the presence of acetylene

In N_2-O_2 without acetylene, the H_2 production of *G. diazotrophicus* continued at a steady rate for over a hour (Fig. 2). When acetylene was added to the culture tubes, ethylene production was detected with a reduction in H_2 evolution. At 10% acetylene and 20 kPa of pO_2, the standard conditions for acetylene reduction assays, H_2 evolution continued. After 1 hr of exposure to acetylene, the quantity of H_2 evolved was 45% of that in N_2-O_2 (ANA) treatment (Fig. 2a) and the quantity of ethylene produced was only 78% of H_2 evolution in N_2-O_2. A similar pattern was observed at 2 kPa of pO_2 (Fig. 2b): H_2 evolution and ethylene production occurred concurrently in 10% acetylene and the relative production of H_2 and ethylene in one hour were 80% and 141% respectively of H_2 production in N_2-O_2.

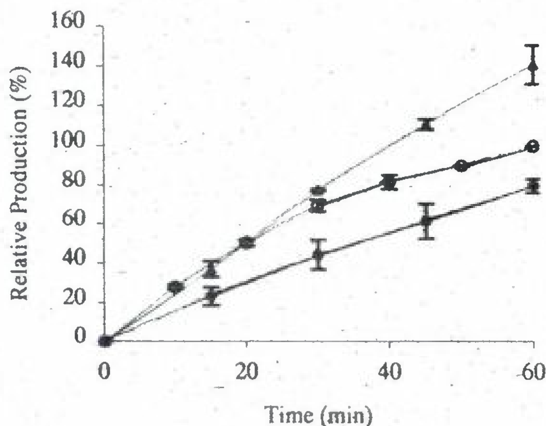
The relative production rates of H_2 and ethylene in various concentrations of acetylene are shown in Tables 1 and 2. Apparent nitrogenase activity (ANA) was defined as the rate of H_2 evolution in N_2-O_2. When the concentration of acetylene increased, the relative production rate of H_2 decreased and that of ethylene increased. In 30% acetylene, ethylene production rate increased substantially to 285% of ANA at 20 kPa of pO_2 and 237% of ANA at 2 kPa of pO_2. However, H_2 evolution never ceased and remained to be 13 to 17% of ANA under 30% acetylene.

4. Discussion

Gluconacetobacter diazotrophicus colonies incubated at 20 kPa of pO_2 produced more extracellular polysaccharides and formed thick pellicles compared to those incubated at 2 kPa of pO_2. In addition, bacterial cells were confined to the colony interior at 20 kPa of pO_2 whereas the bacteria grew to the edge of the colonies at reduced O_2 concentration. The pellicles appeared to function as a barrier to O_2 diffusion to avoid the inhibition of nitrogenase activity by high O_2 concentrations. The production of extracellular



a) 20 kPa of pO_2.



b) 2 kPa of pO_2.

Figure 2. Relative production of H_2 and ethylene with or without 10% acetylene in 1 hr. (a) *G. diazotrophicus* colonies incubated at 20 kPa of pO_2. (b) *G. diazotrophicus* colonies incubated at 2 kPa of pO_2. Data are means ± standard errors (n=5). H_2 production in N_2-O_2 (○), H_2 production in 10% acetylene (●) and ethylene production in 10% acetylene (▲).

polysaccharides and mucilaginous colonies has also been reported in other N_2-fixing bacteria such as *Beijerinckia derxii*, *Derxia gummosa* and *Azotobacter chroococcum* (Barbosa and Alterthum, 1992; Hill, 1971; Hill et al., 1972). In *G. diazotrophicus* colonies incubated at 2 kPa of pO_2 H_2 production declined dramatically when they were exposed to 20 kPa of pO_2 (Dong et al., 1995), indicating that the thin pellicles did not provide adequate protection from high pO_2.

Table 1. Relative production rates of H_2 and ethylene by *G. diazotrophicus* in various concentrations of acetylene at 20 kPa of pO_2. Data are means ± standard error (n=5).

Acetylene concentration	Relative production rate (% ANA)	
	H_2	C_2H_4
0	100	0
2.5	87.94 ± 7.22	33.66 ± 5.13
10	45.49 ± 1.70	77.83 ± 4.98
20	33.95 ± 6.65	172.19 ± 23.64
30	16.57 ± 2.11	284.59 ± 38.93

Table 2. Relative production rates of H_2 and ethylene by *G. diazotrophicus* in various concentrations of acetylene at 2 kPa of pO_2. Data are means ± standard error (n=5).

Acetylene concentration	Relative production rate (% ANA)	
	H_2	C_2H_4
0	100	0
2.5	91.14 ± 15.33	83.34 ± 16.16
10	79.92 ± 3.70	140.86 ± 9.90
20	23.15 ± 0.50	170.65 ± 16.82
30	12.94 ± 1.71	236.86 ± 16.07

Nitrogenase simultaneously reduces N_2 to ammonia and protons to H_2 gas. Only a proportion of electron is used for H_2 evolution in N_2-O_2. Total nitrogenase activity (TNA) can be measured by H_2 evolution rate in Ar-O_2 or by acetylene reduction in air. Nitrogenase in legume nodules can be saturated with 10% acetylene (Hunt and Layzell, 1993). At this condition, there is no or little H_2 production and N_2 reduction, almost all the electrons passing through nitrogenase go to acetylene and ethylene production rate gives the measure of the total nitrogenase activity. However, the results of acetylene reduction assays in *G. diazotrophicus* showed that H_2 evolution decreased with increasing of acetylene concentration, but did not cease.

Spiff and Odu (1973), MacRae (1977), and Dong et al. (1995) found that H_2 was still produced by some N_2-fixing bacteria at high partial pressure of acetylene. In this study, H_2 production in *G. diazotrophicus* was detected in

30% acetylene on solid medium at both 20 kPa and 2 kPa of pO_2. Since colonies grown at both 2 kPa and 20 kPa of pO_2 showed similar relative production rates in the presence of acetylene, the physical diffusion barrier presented by the pellicle on colonies are unlikely to be the reason for the incomplete saturation of nitrogenase by acetylene.

To measure total nitrogenase activity by acetylene reduction assay, nitrogenase should be saturated by acetylene. The results of this study showed that at both 2 kPa and 20 kPa of pO_2, H_2 production rate in 30% acetylene was 13–17% of ANA. Dong et al. (1995) demonstrated a similar result for cultures grown at 20 kPa of pO_2. H_2 production in 30% acetylene was 15% of ANA in their study. Therefore, the acetylene reduction assay does not give an accurate measurement of total nitrogenase activity of *G. diazotrophicus* colonies. In 30% acetylene, acetylene reduction assay underestimated total nitrogenase activity of *G. diazotrophicus* by at least 10–20%. The underestimation was even larger at 10% acetylene, the standard condition for the assay. It is likely that this incomplete saturation of nitrogenase by high concentration of acetylene may happen in other N_2-fixing bacteria as well. Our study showed that although the acetylene reduction assay can still be useful for the rapid detection of active nitrogenase, for accurate measure of nitrogenase activity, H_2 production should be monitored during the assay.

Acknowledgments

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