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SUSTAINED AMMONIA PRODUCTION BY IMMOBILIZED FILAMENTS OF THE NITROGEN-FIXING CYANOBACTERIUM *ANABAENA* 27893

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SUMMARY

Whole filaments of the N_2-fixing cyanobacterium *Anabaena* ATCC 27893 have been immobilized by entrapment in calcium alginate gel beads. In a continuous flow fluidized bed reactor sustained photosynthesis, N_2-fixation, and ammonia production have been achieved over a 130 hour period, the longest tested.

INTRODUCTION

Cyanobacteria are O_2-evolving photosynthetic prokaryotes, certain species of which fix N_2 (Stewart, 1980). Such organisms use sunlight as an energy source and reductant is provided by the photolysis of water. Thus, unlike the Haber-Bosch process of chemical N_2-fixation which requires high temperatures and pressures (see Chatt, 1980), cyanobacteria fix N_2 independently of a fossil fuel supply. Most aerobic N_2-fixing cyanobacteria are heterocystous forms in which the heterocysts act as a N_2-fixing factory (Stewart *et al.*, 1969) with the fixed carbon necessary to sustain nitrogenase activity being provided by the adjacent vegetative cells (Wolk, 1968).

Such N_2-fixing cyanobacteria normally use the nitrogen which they fix for further growth. What we have done in this study is to devise a mechanism whereby the fixed nitrogen is released extracellularly as ammonia rather than being assimilated. We have made use of a previous

finding from this laboratory that N_2-fixing cyanobacteria can liberate extracellularly, as ammonia, over 90% of the N_2 fixed if the primary enzyme in ammonia assimilation, glutamine synthetase is inhibited (Stewart and Rowell, 1975). In order to achieve sustained high rates of NH_4^+ production, it is important to be able to immobilize the cyanobacterium in such a way that it can be used in a continuous flow reactor at high dilution rates, to produce ammonia, without washout of the organism. To date, no information is available on the immobilization of cyanobacteria for biotechnological use, apart from reports on H_2 production by *Anabaena cylindrica* adsorbed to glass beads (Lambert *et al.*, 1979; Smith and Lambert, 1981). We report here on the successful immobilization of *Anabaena* ATCC 27893, on sustained metabolic activity by the immobilized cyanobacteria and on sustained NH_4^+ production from sunlight, air and water.

MATERIALS AND METHODS

Organism. *Anabaena* 27893, obtained from the American Type Culture Collection, was grown in pure culture in BG-11_o medium (Rippka *et al.*, 1979) in a 5 l chemostat at a dilution rate of 0.004 h^{-1} at 22°C. The culture was stirred magnetically and sparged with sterile air at a rate of 24 l h^{-1}. Illumination was provided by white fluorescent tubes which provided a photon flux density of 100 µE m^{-2} s^{-1} at the surface of the vessel.

Immobilization. Cells were harvested by centrifuging 5 l of culture at 2000 x g for 15 minutes and resuspending the pelleted cells in 150 ml of fresh BG-11_o medium. Alginic acid (Type IV, Sigma) (9 g) was dissolved in 150 ml of distilled water at 45°C and allowed to cool to room temperature. The *Anabaena* suspension and the alginic acid solution were then thoroughly mixed and calcium alginate gel beads were prepared by dropping the mixture, drop by drop via a syringe canula, into a solution of 0.1 M calcium chloride. The beads, each about 2 mm in diameter, so produced, were then hardened by leaving them for 30 min in the calcium chloride solution at 5°C. They were then rinsed in BG-11_o medium. About 300 ml of cell suspension and alginic acid solution produced 150 ml of beads.

The Bioreactor. A three-phase fluidized bed column, 21 mm in internal diameter and 300 ml total volume containing 130 ml of beads was used. Air (12 l h^{-1}) and BG-11_o medium (140 ml h^{-1}) were pumped into the column via the bottom. The outlet, at the top, was pumped into a fraction collector. Complete mixing of the void volume (170 ml) occurred within 5 min. The photon flux density, provided by white fluorescent tubes, was 65 µE m^{-2} s^{-1} at the surface of the column and the operating temperature was 22°C.

Analytical methods. Beads (0.1 - 0.5 g fresh weight) were removed from the top of the column to measure photosynthetic oxygen evolution, acetylene reduction and chlorophyll contents. Photosynthetic oxygen evolution was measured using a Rank Pt-Ag O_2 electrode at 22°C at saturating light provided by a tungsten filament lamp. Acetylene reduction was determined as before (Stewart *et al.*, 1967) over a 60 min incubation with 0.8 ml $BG\text{-}11_o$ plus 0.6 ml acetylene at 80% of the saturating light intensity for acetylene reduction. The chlorophyll was extracted overnight in methanol in the dark at -20°C. Absorbance of the filtrate (Whatman No. 1 paper) was measured at 663 nm and the chlorophyll *a* concentration calculated according to Mackinney (1941). Ammonia levels in the effluent fractions were determined by the method of Solorzano (1969).

RESULTS AND DISCUSSION

Table 1 shows the initial effects of entrapment in calcium alginate gel on the ability of *Anabaena* 27893 to evolve O_2 photosynthetically and to reduce C_2H_2.

Table 1. The effects of immobilization on photosynthetic oxygen evolution and acetylene reduction by the N_2-fixing cyanobacterium *Anabaena* 27893

	Relative rates of photosynthetic oxygen evolution	Relative rates of nitrogenase activity (acetylene reduction)
Liquid cell suspension	100	100
Newly immobilized cells	73	7
Immobilized cells after 3 hours	n.d.	43
Immobilized cells after 24 hours	75	67
Immobilized cells after 530 hours	98	75
Immobilized cells after 1157 hours	100	n.d.

For photosynthetic O_2 evolution, the 100% rate was 149 nmoles O_2 evolved.μg chl$a^{-1}h^{-1}$; for acetylene reduction the 100% rate was 3.8 nmoles C_2H_2 reduced.μg chl$a^{-1}h^{-1}$. n.d. = not determined.

The rate of photosynthetic O_2 evolution of the newly entrapped filaments was 73% of the rate of free-living cells taken from the chemostat, whereas the rate of acetylene reduction by freshly prepared beads was only 7% of that of the free-living cells prior to entrapment. Nitrogenase activity did recover, however, to 43% of the rate of free-living cells within 3 h and to 67% of the free-living rate after 24 h (Table 1). Relative acetylene reduction rates did not decline within 530 h of immobilization and photosynthetic oxygen evolution rates remained approximately constant beyond 1100 h (Table 1).

Fig. 1 provides detailed data on the trends in photosynthetic O_2 evolution and acetylene reduction which were measured over a 200 h period. During the first 48 h the medium used was BG-11_o. After 48 h, this was supplemented with 10 µM L-methionine-DL-sulphoximine (MSX) to inhibit glutamine synthetase activity (Stewart and Rowell, 1975) and to promote ammonia release. This treatment inhibited the glutamine synthetase activity of the entrapped organism (unpublished data).

Fig. 1a shows that photosynthetic O_2 evolution rates were unaffected by the addition of MSX for up to 50 h but then declined over the subsequent 50 h to a rate which at 192 h was approximately 50% of the initial rate.

Fig. 1b shows that after an initial loss of activity immediately on entrapment, nitrogenase activity recovered rapidly and remained steady for 150 h. The addition of MSX had no effect for approximately 100 h but then activity declined probably as a result of reduced photosynthetic activity (see Fig. 1a). Chlorophyll concentrations increased by less than 10% during the 200 h duration of the experiment indicating that there was no significant increase in cyanobacterial biomass in the beads (data not shown).

NH_4^+ was detectable in the effluent within 10 h of adding MSX. The highest concentrations of NH_4^+ recorded so far were 290 µmoles l^{-1} of effluent. This value yields a reactor productivity of 130 µmoles l^{-1} h^{-1} of NH_4^+, and a specific rate of NH_4^+ production of 8.5 µmoles. mg chlorophyll a^{-1} h^{-1}. Fig. 1c shows a profile of NH_4^+ production which declined after 100 h then oscillated throughout the remainder of the run. However, after 200 h the NH_4^+ production rate still amounted to 70% of the maximum achieved in this time course.

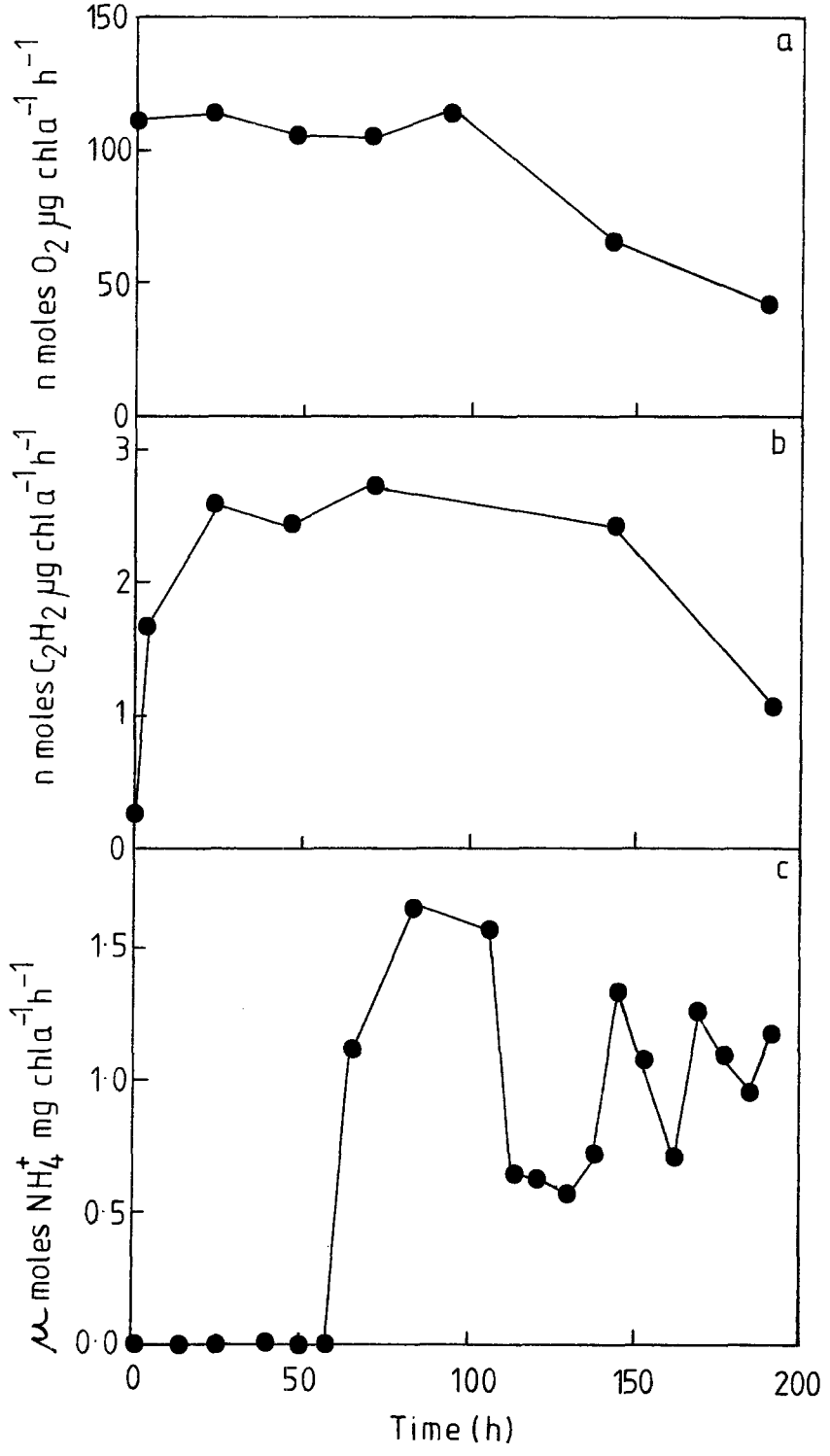

Fig. 1. Rates of photosynthetic oxygen evolution (a), acetylene reduction (b) and NH_4^+ production (c) in a fluidized bed reactor over 200 h. Filaments were immobilized at t = 0, the medium was supplemented with 10 µM MSX at t = 48.

This is the first report of extracellular ammonia production from light, air and water by immobilized N_2-fixing photosynthetic microorganisms. It is clear from such data that N_2-fixing cyanobacteria readily survive entrapment and that by adding compounds which inhibit glutamine synthetase it is possible to produce NH_4^+ extracellularly at room temperature from light, air and water. We have made no attempt to optimise the system so far, but simply to show that ammonia can be produced in this way. It is our aim now to optimise such systems both in terms of achievable rates and times over which the NH_4^+ is produced.

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