Immobilization of *Anabaena azollae* from *Azolla filiculoides* in polyvinyl foam for ammonia production in a photobioreactor system

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*Anabaena azollae* (AS-DS), isolated from *Azolla filiculoides* and grown in nitrogen-free medium, was immobilized in 5-mm-cube polyvinyl foam pieces and incorporated into a photobioreactor system for the production of NH$_3$. NH$_3$ was produced continuously and in significant amounts. Benlate (methyl-1-butyl-carbamoyl)-2-benzimidazole carbamate) at 5 ppm and L-methionine-D,L-sulphoximine at 50 μM stimulated NH$_3$ production continuously for a period of 1 week.

Key words: Ammonia excretion, *Anabaena azollae* (AS-DS), Benlate, immobilization, MSX, photobioreactor, polyvinyl foam.

*Azolla* is an aquatic fern which fixes N$_2$ from the atmosphere in association with a cyanobacterium, *Anabaena azollae*. The *Azolla*-*Anabaena* symbiosis is known to fix significant amounts of N$_2$ and produce large amounts of biomass (Moore 1969). *Azolla* can be effectively utilized as a biofertilizer in rice production and can contribute 40 to 60 kg N ha$^{-1}$ per crop cycle (Watanabe et al. 1977; Lumpkin & Plucknett 1980; Kannaiyan 1986). The cyanobacterial symbiont *An. azollae* is associated with the upper leaf lobe of *Azolla*, where it fixes considerable amounts of N$_2$ (Shi & Hall 1988). The N$_2$-fixing, NH$_3$-secretion activity of *Anabaena azollae* strains (isolated from *Azolla filiculoides* and *A. microphylla*) under free-living conditions in N-free medium was demonstrated by Samal & Kannaiyan (1992).

*Anabaena azollae* and *A. variabilis*, when immobilized in polyurethane and polyvinyl foams and calcium alginate beads, have been shown to release NH$_3$ extracellularly (Kerby et al. 1986; Brouers et al. 1988). The present paper deals with the immobilization of *An. azollae* (isolated from rice paddy-grown *Azolla filiculoides*) in polyvinyl foam and its incorporation in a photobioreactor system for the continuous production of ammonia.

**Materials and Methods**

*Culture of Anabaena azollae*

*Anabaena azollae* (AS-DS) was isolated in Coimbatore, India, from rice paddy-grown *Azolla filiculoides* and cultured in N-free BG-11 (Stanier et al. 1971). The culture was maintained in a growth cabinet at 26 ± 1°C and an irradiance of 65 μmol photons m$^{-2}$ s$^{-1}$ (cool white fluorescent tubes). Actively growing young cultures (1 week old) were used for inoculation.

*Immobilization of Anabaena azollae*

White polyvinyl foam (Extrasorb Ltd, Preston, UK) was cut into 5-mm-side cubes and added to 250-ml conical flasks, at 2 g per flask, each containing 100 ml N-free BG-11 medium which had been autoclaved at 125 kPa for 30 min. The *An. azollae* was inoculated into the flask and incubated for 15 days under the same conditions as the inocula.

*Photobioreactor System*

A water-jacketed (27°C) photobioreactor glass column, (30 cm long x 2 cm diam.) was filled with the polyvinyl foam containing immobilized *An. azollae*. The column was connected to a flask containing BG-11 medium (continuously stirred) via a peristaltic pump (Figure 1). The medium was trickled at 2 ml h$^{-1}$ through the foam from the top of the column. Illumination of the photobioreactor was provided by fluorescent lamps, with an irradiance of 60 μmol photons m$^{-2}$ s$^{-1}$ at the column surface. The
Fluorescent lamps

Air input

BG-11 medium

Stirrer

Water bath

Sample collector

White polyvinyl foam with immobilized

Anabaena azollae

Figure 1. Schematic diagram of the photobioreactor system used for the production of NH$_3$ by foam-immobilized Anabaena azollae.

effluent was analysed daily for NH$_3$. After one week, the BG-11 medium was mixed with 50 μM MSX (L-methionine-D,L-sulphoximine) (Sigma, Poole, UK) and the column effluent was analysed for NH$_3$ for a further week. Thereafter, the column was packed with a new batch of foam with immobilized An. azollae and BG-11 was passed through the column for 1 week. Then the systemic fungicide Benlate, (methyl-l-butyl-carbamoyl)-2-benzimidazol carbamate) (Dupont, Wilmington, USA), was added at 5 ppm to the BG-11 medium. The NH$_3$ content of the effluent was measured for a further week. The foam was then removed and its dry weight determined.

Determination of NH$_3$

The photobioreactor effluent medium was sampled daily and analyzed for NH$_3$ by the method of Solorzano (1969).

Scanning Electron Microscopy

Sections of foam with immobilized cells of An. azollae were examined using freeze-drying methods after fixing for 12 h at 20°C in 2.5% (w/v) glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) containing 0% (w/v) sucrose and then washing well in the same buffer containing 12% (w/v) sucrose (Markov et al. 1993). The specimens were dried in a FD 500 (Emscope, UK). Dried specimens were gold-coated in a Polaron E5100 coater and examined in a Jeol JSM 255 scanning microscope at an accelerating voltage of 15 or 25 kV.

Statistical Calculations

Analysis of variance was performed using the ANOVA program of an SPSS-X package installed on a VAX computer.

Results and Discussion

Anabaena azollae immobilized very well in the foam cubes, the filaments penetrating readily into the pores of the foam (Figures 2 and 3). There were no significant differences in the rates of NH$_3$ secretion by the immobilized An. azollae cells over the 7 days of measurement (Table 1). However, MSX treatment increased NH$_3$ production 5- to 10-fold.

Similarly the addition of Benlate to BG-11 medium increased NH$_3$ production 5- to 10-fold, but the rate of production started to decrease after day 2, albeit not statistically significantly (Table 1). The results show that treatment with the systemic fungicide, Benlate, induced NH$_3$ production (comparable with MSX treatment) at rates significantly higher than control rates (Table 2). It is also important to note that An. azollae (AS-DS) secreted significantly higher amounts of NH$_3$ in the foam-immobilized state than when free-living, even without the addition of MSX or Benlate (data not shown).

N$_2$-fixing cyanobacteria, both symbiotic and free-living species, are known to provide NH$_3$ to rice plants under natural ecological conditions (Kannaiyan 1992). It has been reported that certain mutant strains of cyanobacteria, such as An. variabilis, are capable of secreting the NH$_3$ into the natural environment (Spiller et al. 1986).