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Ammonium translocation in Cyanobacteria and their possible use as Biofertilizers.

Interim Report No-5

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ISOLATION OF MUTANTS STRAINS WHICH CONTINUOUSLY RELEASE AMMONIA.

Nitrogen fixing cyanobacteria which reduce dinitrogen to ammonia release the ammonia during growth in very small quantities and made available the fixed products to the rice plants mainly through exudation, autolysis and microbial decomposition. However if the ammonium assimilation is blocked by the L-glutamate analogue, MSX the fixed ammonia comes out in larger quantities (Zimmerman and Boussiba,1987). Mutants resistant to MSX are able to release significant amount of ammonia and may contribute to the growth and nitrogen nutrition of rice plants (Lettore et al., 1986).

Anabaena siamensis [1], a rice field isolate possesses higher growth rate (dt 3-4h) and adaptation to varied temperatures (25 to 40°C) and salinity ranges (1-2%) (Antarikanonda,1982) and the physiological parameters of this cyanobacterium is compared with another nitrogen fixer, Anabaena azollae in Table 1. Since this strain harbours many favorable features to acclimatize the rice field environment it was thus selected as a suitable candidate for strain improvement.

Material and methods :

Cyanobacterium

Anabaena siamensis, a rice field isolate from Thailand was obtained from the Sammlung von Algenkulturen, University of Gottingen, F.R.G.

Growth conditions

A.siamensis was cultivated in 500ml sterilized glass columns inside a transparent plexiglass circulating water bath. Water temperature was maintained at 30°C. A constant photon flux of 175 $\mu E\ m^{-2}s^{-1}$ at the surface of the growth vessel was supplied laterally by a battery of 8 cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1.5% CO_2. Under this condition the pH was maintained at 7.0-7.2. The standard growth medium was AS medium (Antarikanonda, 1982). Unless otherwise stated, culture was sampled during the logarithmic growth phase for use in the different experiments.

Determination of ammonium concentration and growth :

Chlorophyll-a was determined as described by Boussiba et al.,(1987). Protein was determined accordingto Lowry et al., (1951). Ammonium was quantified using Solorzano's phenolhypochlorite method (1969).

Measurement of Oxygen consumption and evolution :

The rates of oxygen evolution in the light (photosyn- thesis) and of oxygen consumption in the dark (respira-tion) were measured in filament suspensions a concen-tration of 10.0µg chlorophyll ml^{-1} at 30°C with a Clark type electrode (Yellow Springs,Ohio Instruments Co.) Connected to a recorder (Vogor 310). The light intensi- ty at the surface of the electrode cell was 700 $\mu Em^{-2}s^{-1}$.

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Enzyme assays

Nitrogenase activity was estimated by the acetylene reduction assay (Stewart, 1967). Samples of 4.6 ml of cyanobacterial culture, washed in fresh BG-11 medium were placed in a 25ml Wheaton bottle sealed with a flanged rubber septum. The Wheaton bottles were subjected to rotary shaking and illuminated with a quantum flux of 75µE $m^{-2}s^{-1}$, during the assay. Filaments were allowed 10 min. of acclimatization before injection of acetylene (C_2H_2). Ethylene (C_2H_4) was analysed on an HP 5890 gas chromatograph using a stainless steel column packed with a Porapack-N (0.2 cm i.d., 265 cm length). Nitrogenase activity was expressed as µmol C_2H_4 produced /mg Chl/h.

Glutamine synthetase assay was performed in concen- trated suspensions (1mg protein ml^{-1}). Cells were then permeabilised with 2% toluene for one minute and kept in ice for 15min. Activity was measured as transferase (Sampio, 1969).

Mutagenesis

Mutagenesis with ethylmethanesulfonate was carried out as reported by Spiller et al., (1986). Filaments (3-6 cells/filament) from 50ml log phase grown culture were taken and centrifuged at 3500rpm at room temperature. Then washed with AS medium and suspended in medium containing 5mM NH_4^+. Ethylmethanesulfonate was added to the suspension at a final concentration of 1% and incubated in a shaker at room temperature for 90 min. with continuous illumination.

The mutgenised filaments were washed with medium without NH_4^+ and suspended in the same medium and incubated at 48°C for 40 min. to aid depurination of alkylated guanine residues. The cells were then collected by centrifugation and suspended in 3mM NH_4^+ containing medium and incubated in light at room temperature with gentle shaking (100 rpm) for 10h to allow the mutagenised chromosome for segregation.

Selection of mutants

The mutagenised filaments were placed on MSX (AS) agar medium and incubated at 30°C with continuous illumination. Colonies appearing on 500µM MSX con- taining agar medium were streaked in pH indicator medium and those who alter the pH of the medium to the alkaline range were numbered and designated as ammonium excreting mutants. Among them one named A.siamensis SS1 was taken for further studies in liquid medium. The pH of the pH indicator medium was 6.7. An increase in pH of 0.2 altered the colour of the medium from orange to red if phenol red indicator was present in the medium.

Results :

Concentrations from 5 to 100µM/ml of MSX reduce the growth significantly when compared to the control (Fig 1) and 500µM/ml exerts complete inhibition of growth of *A.siamensis* (Fig 2). The release of ammonia in the absence of MSX is insignificant. An MSX resistant mutant *A.siamensis* **SS1** was isolated by EMS treatment for 90 min. as reported by Spiller et al., (1986). Growth of the mutant in liquid medium with and without MSX is equal to that of the parent strain (Fig 3). Ammonia release in the absence of MSX is observed up to 4 days and as the culture is dense by the population ammonium depletion occurs probably taken up by the cells as a consequence of reduction in nitrogenase activity (Table 2). In the presence of MSX ammonia release is observed only during the lag period and when the growth is progressing there is no significant release of ammonia (Fig 4). The rate of ammonium production of the mutant is 0.8 mM /mg Chl/hr. during the early log period (Fig 5).

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CONCLUSIONS

- The MSX resistant mutant, A.siamensis SS1 exhibits the same growth rate of the wild type in the presence and absence of 500 μM MSX .
- GS activity of the mutant in the presence and absence of MSX is reduced more than 50% of that of the parent.
- Ammonium release is observed only in the early log phase.
- Since the main ammonium assimilation pathway is not inhibited the released ammonium, is being taken up probably as the culture becomes dense.
- Experiments to optimise the conditions for the sustained photoproduction of ammonium in continuous cultures are under progress.

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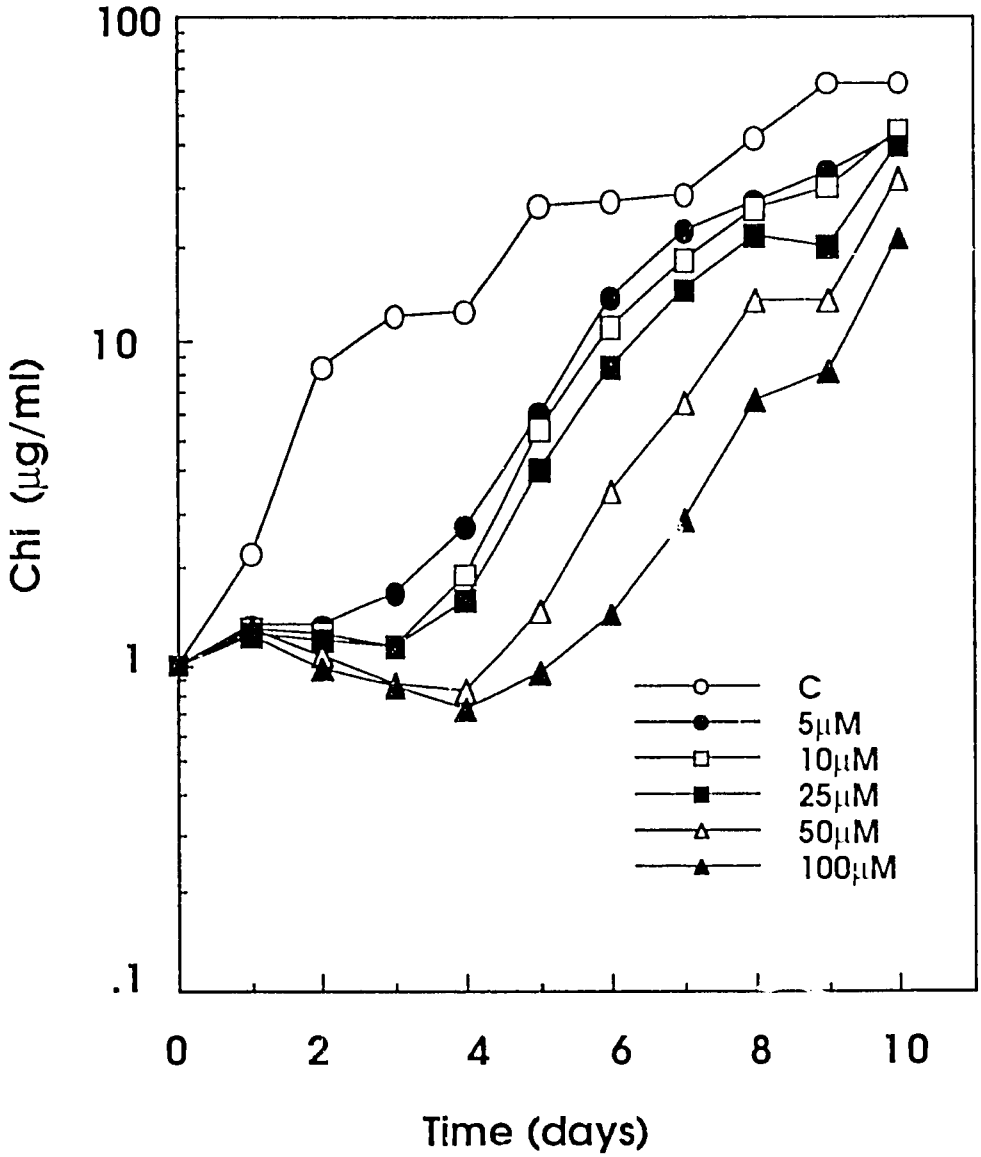

Fig 1 : Influence of MSX on the growth of Anabaena siamensis in liquid medium.

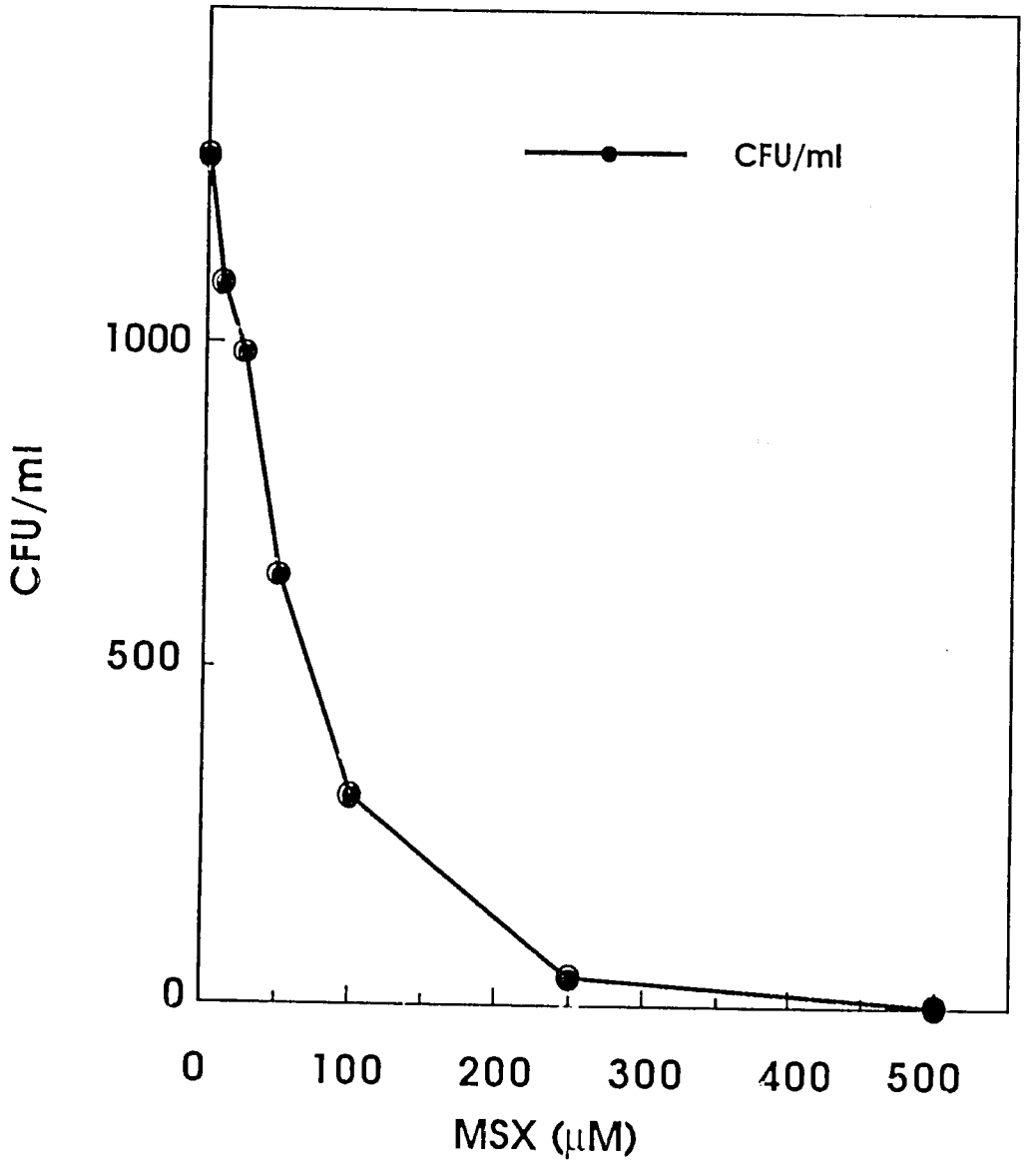

Fig 2 : Influence of MSX on the growth of A.siamensis in solid medium.

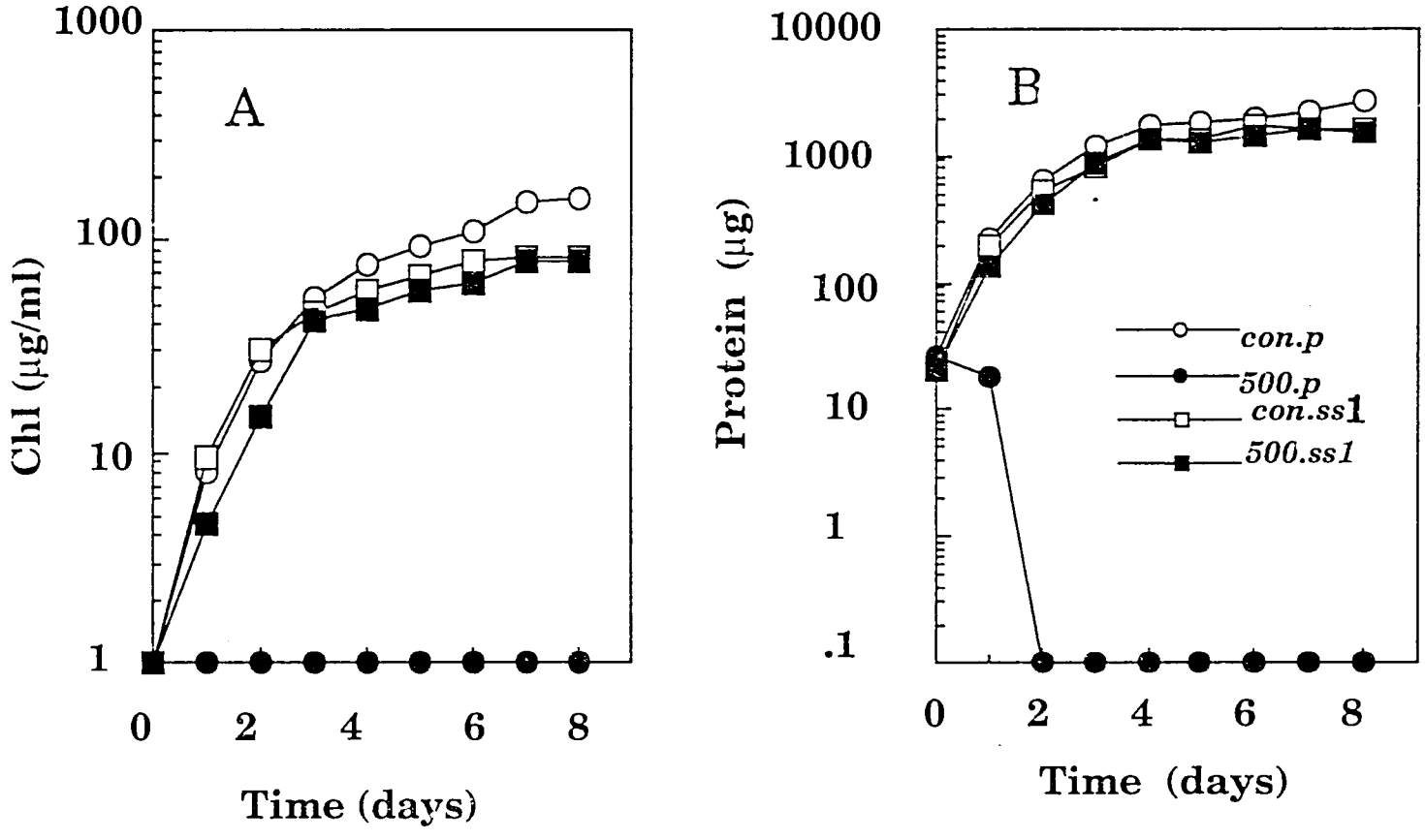

Fig 3 : Comparison of growth of the wild, A.siamensis and the mutant, SS1 in terms of Chlorophyll (A) and Protein (B) .

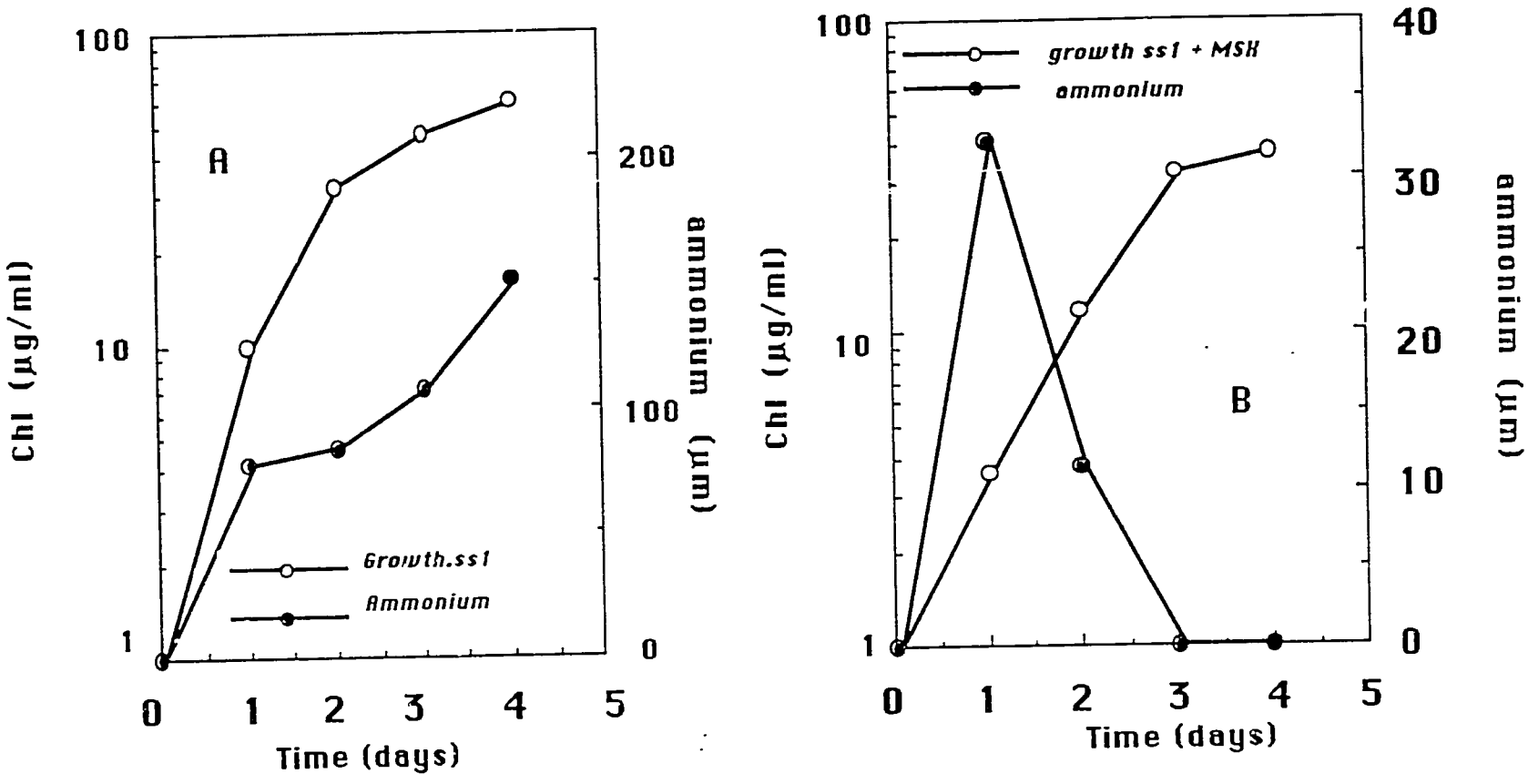

Fig 4: Comparison of ammonium release in relation to the growth by A.siamensis SS1 in the presence and absence of MSX.

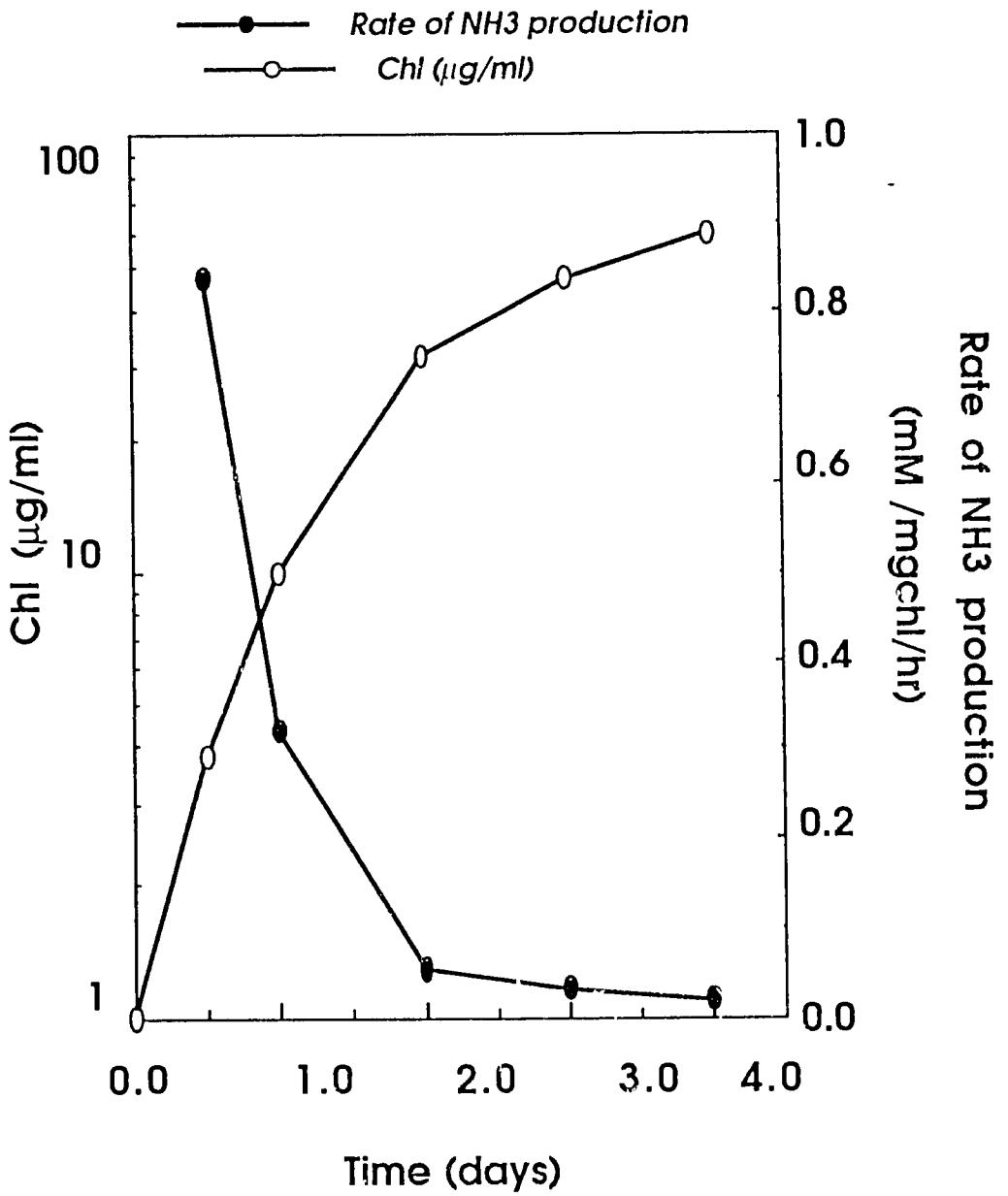

Fig 5 : Rate of ammonium production in relation to the growth of A. siamensis SS1 in the absence of MSX.

RESULTS

TABLE : 1

Comparison of the physiological features of A.siamensis with another nitrogen fixer A. azollae.

Strain	Growth rate dt (h)	Oxygen evolution (µmol/mgchl/h)	Nitrogenase (µmolC_2H_4/mgchl/h)
A. siamensis	5.0	1200	20
A. azollae	9.5	450	30

TABLE : 2

Nitrogenase and GS activity of the parent and the mutant during the 5th day of cultural growth.

Strain	Nitrogenase activity (µmoles C_2H_4/ mgchl/h)		GS activity(%)	
	- MSX	+ MSX	- MSX	+ MSX
A.siamensis	20.0	0..00	100 .00	0.00
A.siamensis SS1	0.00	0.00	37.35	44.91