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ORIGINAL ARTICLE

Responses of Wild type and Mutant strains of Cyanobacterium *Anabaena variabilis* under Immobilized Condition

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ABSTRACT
Parent wild type [Av (P)], its multiple herbicide resistant [Av (MHR)^r] and ammonia excretory multiple herbicide resistant mutant [Av (MHR) Eda^r] cells of Anabaena variabilis were immobilized on sodium alginate, polyurethane foam, sugarcane waste, paper waste and growth, nitrogenase activity and ammonia excretion were investigated. The growth measured by chlorophyll a determination showed that the growth of immobilized mutant cells were more rapid than immobilized wild type cells and for both two types of cells the immobilized cells grew better than the free living cells. The nitrogenase activity was 24%-66% higher in immobilized state as compared to free living condition. Free living cells of wild type and multiple herbicide resistant mutant almost did not excrete any ammonia but their immobilized counter parts had the ammonium ion excretion activity. Moreover immobilization stimulated the activity of ammonium ion excretion 22%-74% greater than the free living cells. In general, immobilization of cyanobacterial strains on solid matrices stimulated growth, nitrogenase activity and ammonia excretion when compared to free- living condition. Among the substrates used, sodium alginate proved to be the best facilitating with highest growth and metabolic activities as compared to other matrices. Immobilized ammonia excretory multiple herbicide resistant strain [Av (MHR) Eda^r] showed maximum growth, nitrogenase and ammonia excretion. The excretion of higher quantities of ammonia of such biofertilizer strains has important significance in rice crop productivity.
Keywords: *cyanobacteria, immobilization, growth, ammonia excretion, nitrogen fixation*

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INTRODUCTION

The nitrogen-fixing cyanobacteria form a prominent component of microbial population in rice paddy fields, since they significantly contribute to fertility as natural biofertilizers [17]. Rice crop has significantly responded to cyanobacterial inoculants, because the rice field conditions provide an ideal environment for the luxurious growth of blue-green algae [11, 22]. A series of pot culture trials and field studies conducted have shown that N_2-fixing cyanobacteria are beneficial in increasing the rice yield [11, 20, 21,12]. Although rice fields provide favourable environment for the development of cyanobacteria, it is generally assumed that nutrients fixed by cyanobacteria are made available to the rice plants through exudation, autolysis and microbial decomposition. However, under field conditions, only a part of this fixed nitrogen is available to the rice plants, some being either reincorporated by the microflora or volatilised. Under these circumstances, it is difficult to control the flow of nitrogen compounds needed for the development of the rice plants. The breakthrough for this problem will be the development of cyanobacterial strains, which release ammonium continuously into the field. Ammonia excreting mutants of cyanobacteria have been isolated for cyanobacteria like *Nostoc muscorum*, *Anabaena variabilis* [19, 10, 16, 4]. However, whether such mutants can compete with natural populations in rice fields is still an open question.

The process can be successfully enhanced by immobilizing these diazotrophic cyanobacteria on suitable matrices [2, 6]. It has been reported that immobilization of N_2- fixing cyanobacteria on solid substrates stimulated growth and ammonia excretion than in free living condition [6]. A field experiment with paddy rice and polyurethane foam immobilized *A. azollae* strains indicated that the cyanobacteria excreted significant amounts of ammonia into the flood water in the rice fields resulting in increased chlorophyll content of plants and increased the rice grain and straw yields [5].

Thus the present investigation was aimed to study the effect of immobilization using different substrates (Polyurethane foam, sugarcane waste bagasse, unused paper waste and sodium alginate) on the growth, nitrogenase activity and ammonia excretion of wild type and mutant strains of *Anabaena variabilis*.

MATERIALS AND METHODS

Organisms and growth conditions: The axenic clonal culture of wild type N_2-fixing cyanobacterium *Anabaena variabilis* [*Av* (P)], a rice field isolate, its spontaneous mutant [*Av* (MHR)^r] exhibiting resistance to the lethal dosages of herbicides: Arozin, Alachlor, Butachlor, 2,4-D, Atrazine, DCMU [13, 14] and its multiple herbicide ammonia excretory resistant strain [Av (MHR) Eda^r] was cultivated in BG_{11} medium [8], devoid of any combined nitrogen source (N_2-medium). Cultures were incubated in an air-conditioned culture room maintained at 25° ± 1°C fitted with cool day fluorescent light. Photon flux density of light on the surface of the vessel was 45 $\mu Em^{-2} s^{-1}$ for 18 h d^{-1}.

Immobilization: The cells were immobilized by entrapment in Polyurethane foam, sugarcane waste bagasse and paper waste by the method of Balachander and Kannaiyan [1] and sodium alginate gel following the method of [3]. Cyanobacteria were prepared for analysis by gently releasing them from the immobilized state using a sterile pestle and mortar.

Measurement of Growth: Growth was estimated at regular intervals by measuring Chlorophyll *a* by the method of Mackinney [7].

Measurement of Nitrogenase activity: Nitrogenase activity of cyanobacterial strains was measured by acetylene reduction technique using Gas Chromatograph as described by Stewart *et. al.*[17].

Estimation of Ammonia: Extra cellular product of ammonium was assayed by employing the method of Solarzano [15].

RESULTS AND DISCUSSION

The cyanobacterial strains colonized well in all the solid matrices. However, the immobilization of the cultures was much better in sodium alginate followed by polyurethane foam, sugarcane waste and paper waste. The immobilization of cyanobacterial cultures in solid matrices showed relatively higher growth when compared to free-living condition. Among the cyanobacterial strains, maximum growth was recorded in *Av* (MHR) Eda^r followed by *Av* (MHR)^r and *Av* (P). Among the substrates sodium alginate proved to be better immobilizing substrate. Maximum growth was recorded in *Av* (MHR) Eda^r immobilized in sodium alginate with 80% increase in Chlorophyll *a* content followed by polyurethane foam (77%), sugarcane waste (72%), paper waste (68%) as compared to free cells by the end of 7th day of growth (table 1).

The immobilized cyanobacterial cells showed higher nitrogenase activity as compared to free cells. The nitrogenase activity of cyanobacterial strains were 24%-66% greater in different solid matrices than free living counterparts. Highest nitrogenase activity (66%) was recorded in *Av* (MHR) Eda^r immobilized in sodium alginate followed by polyurethane foam (64%), sugarcane waste (59%) and paper waste (53%) by the end of 7th day of growth (table 2).

The cyanobacterial cultures when immobilized in solid matrices excreted ammonia into the medium. Cyanobacteria immobilized in Sodium alginate and polyurethane foam showed significantly higher ammonia excretion than the other substrates. Maximum ammonia liberation was observed in *Av* (MHR) Eda^r (74%), followed by *Av* (MHR)^r (62%) and *Av* (P) (53%) immobilized in sodium alginate as compared to free living cells (table 3).

Table 1. Effect of immobilization of cyanobacterial strains in different solid matrices on chlorophyll *a* ($\mu g ml^{-1}$) by the end of 7th day of growth.

Strains	Free Cells	Paper waste	Sugarcane waste	Polyurethane foam	Sodium Alginate
Av (P)	1.72±0.3	2.3±0.4	2.8±0.3	3.0±0.2	3.8±0.4
Av (MHR)^r	2.24±0.5	3.5±0.5	4.8±0.4	5.0±0.6	6.0±0.5
Av(MHR)Eda^r	3.0±0.5	9.4±0.7	10.6±0.9	13.0±1.0	14.7±1.0

The data is an average (± SEM) of three independent experiments

Table 2. The nitrogenase activity (nmol.C_2H_4. μg^{-1}chl *a* h^{-1}) of the mutants and its parent wild type cells of *Anabaena variabilis* in free-living and immobilized states by the end of 7th day of growth.

Strains	Free Cells	Paper waste	Sugarcane Waste	Polyurethane foam	Sodium Alginate
Av (P)	2.5±0.2	3.2±0.3	4.0±0.5	4.2±0.6	5.3±0.7
Av (MHR)^r	2.5±0.3	3.7±0.2	4.6±0.6	5.3±0.6	6.5±0.8
Av(MHR)Eda^r	15.2±2.6	38.6±4.2	41.2±4.6	50.3±4.9	57.6±5.0

The data is an average (± SEM) of three independent experiments

Table 3. The ammonium excretion activity (μmol. ammonia mg^{-1} chl *a* h^{-1}) of the mutant and its parent wild type cells of *Anabaena variabilis* in free-living and immobilized states by the end of 7th day of growth.

Strains	Free Cells	Paper waste	Sugarcane Waste	Polyurethane foam	Sodium Alginate
Av (P)	6.4±0.7	8.4±0.8	9.6±0.9	11.2±1.3	12.0±2.5
Av (MHR)^r	10.2±1.2	15.5±1.6	18.0±1.8	19.3±2.7	20.2±2.3
Av(MHR)Eda^r	18.0±1.4	38.2±2.3	44.1±3.5	50.0±4.6	53.2±4.8

The data is an average (± SEM) of three independent experiments

In the present investigation cyanobacterial strains immobilized in solid matrices retained the same morphology as that of free cells. The growth curves measured by determination of chlorophyll *a* showed that the growth of immobilized mutant cells was more rapid than wild type cells and for both, the immobilized cells grew better than the free cells. Nitrogenase activity was also greater in immobilized condition than in free living state. Free cells of *Av* (P) and *Av* (MHR)^r almost did not excreted any ammonium ion, but their immobilized counterparts had the ammonium excretion activity. Maximum growth, nitrogenase and ammonium excretion activity was recorded in cells immobilized in sodium alginate followed by polyurethane foam, sugarcane waste and paper waste. Highest growth and metabolic activities were recorded in immobilized *Av* (MHR) Eda^r as compared to other immobilized strains.

Thus the solid substrates provided a support for better growth of cyanobacterial strains. Accelerated growth and change in the membrane permeability of cyanobacteria lead to higher ammonia excretion [9]. The ammonia excretion property of these immobilized strains has practical significance under rice field conditions and this may be exploited for continuous ammonia production in rice field for higher productivity.

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