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A perspective towards development and commercialization of potential BGA biofertilizers of Assam, North East India and carrier materials for BGA mass production and inoculum development

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ABSTRACT

Blue green algae are a group of gram negative photosynthetic prokaryotes mostly known as cyanobacteria which has drawn worldwide attention for the nitrogen fixing ability and their use in agriculture. BGA are very common in Indian in rice fields .The present study is focused towards developing ecofriendly technique for mass production and suitable bio-inoculum development for field application of BGA strains. For that purpose three BGA strain namely Anabaena torulosa, Anabaena doliolum and Calothrix marchica were isolated from selected rice field of Assam, North East India. Growth behaviour and potentiality of all the three strains were explored in terms of their biomass production, chlorophyll –a and total chlorophyll content, packed cell volume (PCV) and their production of IAA like substances. The Intrinsic antibiotic resistance profile (IARP) test and compatibility study among the three aforementioned BGA isolates were also carried out for their efficient growth and biomass production for application and development of biofertilizer technology, three biowastes namely paddy straw, sugarcane trash and water hyacinth were analyzed for substrate preparation. It was Anabaena torulosa which showed maximum biomass yield of 18.33 mg/100ml and N-content of 10.16% obtained when paddy straw was taken as substrate. Again paddy straw showed best result of 28.16 mg/100ml and N-content of 20.33%, when composite inoculation of all the three stains was considered. Based on these findings an integrated BGA immobilized inoculum was also formulated with Luffa cylindrica which is locally known as bhol and with sugarcane trash. The experiment conducted was aimed to achieve suitable biofertilizer production at a very low cost using cheapest substrate which is far superior to harmful chemical fertilizers available in market.

Keywords: Biofertilizer, Indole acetic acid, biomass, bioinoculum.

INTRODUCTION

Cyanobacteria (or cyanophyceae) are non-motile, planktonic, occasionally forming blooms and belong to the kingdom of eubacteria and to the division of cyanophyta. They are gram negative and are common in some extreme environments. Cyanobacteria are a large and morphologically diverse group ([1],[2]) which can thrive in all kinds of waters with some species thriving in freshwater while others thrive in brackish water or the marine environment.

Chemical fertilizers are needed to get good crop yields, but their use can be harmful for the environment and their cost cannot seems to make economic agricultural products [3]. Thus, attempts have been undertaken to substitute chemical fertilizers with biofertilizers, such as cyanobacteria (blue green algae), which are capable of fixing atmospheric N ([4],[5]). In addition, the use of cyanobacteria as biofertilizers can improve plant growth and crop yield since they add organic matter to soil[6], thus improving soil structure ([6], [7]). The positive effect on crop yield was due to their release of various biologically active substances such as gibberellin, auxin, cytokinins ([8],[9]), vitamins, amino acids, polypeptides, antibacterial and antifungal substances and polymers, especially exopolysaccharides [10]. Cyanobacteria can also be used for bioremediation since they can take up heavy metals [11]. However, cyanobacteria (some species of the genera Microcystis, Nostoc, Oscillatoria, and Anabaena) might be harmful as they can synthesize toxic secondary metabolites such as microcystins ([12],[13]) which can be accumulated in plant tissues and be carried through the food chain [14].

Most studies on the use of cyanobacteria as biofertilizers have concerned rice and few wheat, maize, and cotton crops, generally with enhancement of yield of rice ([15],[16]), and contents of N and other nutrients, sugar, amino acids, growth regulators, and protein in wheat ([17],[18],[19]). Studies have also been carried out on the effect of cyanobacteria as partial substitute for chemical fertilizers. Soil fertilization with urea and soil inoculation with *Nostoc muscorum* and *T. tenuis* increased C content, dry weight, and shoot length of rice compared to control[20]. Moreover, the biofertilization with a mixture of N fixing cyanobacteria (*Nostoc commune, Nostoc linckia, Nostoc sp.,* and *Anabaena iyengarii var. tenuis*) decreased the use of N fertilizer by 50%, to get the same grain yield and quality of rice compared with the full dose of chemical fertilizer [21]. Therefore, the beneficial influence of N fixing cyanobacteria on rice has been well documented. While a serious drawback to unicellular micro-algal cultivation is the harvesting of the biomass due to the microscopic dimensions of Microalgae (0.5–30μ) ([22],[23]). In essence, harvesting means that the algal biomass is separated from the liquid cultivation medium. As a result algal biomass is concentrated or dewatered, forming a slurry that consists of 5–15% dry solids [24]. Harvesting of biomass from the broth is thought to contribute 20–30% of the total cost of biomass production [22]. Filamentous cyanobacteria, with dimensions of around 200 nm can help reduce the harvesting problem because they may be harvested relatively easily by filtration. In addition, some filamentous cyanobacteria form aggregates and can be harvested by sedimentation or by flotation ([25],[26]). Cyanobacteria have relatively high biomass productivity Moreover, the composition of algal biomass in lipids, proteins and carbohydrates can be affected and consequently manipulated by various cultivation factors .The habitats and the ecological requirements of cyanobacteria are diverse and depend on the genera and even on the strain. For example, *Spirulina platensis* shows growth at pH 9.0– 10.0 and grows well at 11.5 but not at 7.0, while Anabaena sp. displays optimal growth at pH 7.4–8.4 and its productivity decreases significantly at pH values higher than 9 [27].

An important factor in the production of algal biomass is the selection of the strain that is best suited to the environmental and cultivation conditions. Some of the filamentous cyanobacteria genera are: Anabaena, Anabaenopsis, Aphanizomenon, Nadularia, Oscilatoria, Spirulina, Phormidium, Nostoc, Nostochpsis, and Scytonema. In Table 1 the biomass concentration, the productivity and the biomass composition of selected cyanobacteria are listed. In order for a culture to be successful, various environmental and operational factors, which affect the biology and habitats of the organisms, must be taken into account. These factors also affect cyanobacterial biomass productivity as well as biomass composition. The most important factors are: nutrients, pH and alkalinity, light and cultural cell density, temperature, and contamination by other microorganisms. The composition of cyanobacterial biomass composition is affected by

nitrogen nutrition. Besides the essential nutrients mentioned above cyanobacteria also require for their growth a number of other macro-nutrients in considerable amounts, including sulphur (S), calcium (Ca), magnesium (Mg) and potassium (K) . Micronutrients required include molybdenum (Mo), iron (Fe) nickel (Ni) , copper (Cu), zinc (Zn), cobalt (Co), boron (B), manganese (Mn) and chloride (Cl). Temperature is an important physical factor, which strongly influences the oxygen evolving activity of the photosystem II (PSII), has a number of effects on the cyanobacterial membranes and influences nutrient availability and its uptake ([28],[29]). There is a connection between temperature, light and photoinhibition. At low temperatures cyanobacteria are photoinhibited by high light intensities and thus temperature can be considered as the most important limiting factor in outdoor cultivation during the winter. However, photoinhibition can be considerably reduced by an increase in temperature ([29],[30]). Optimum temperature for biomass production is genera and strain dependent ([29],[31]). In Table: 2 the minimum and maximum temperature and the growth rate of some cyanobacteria are listed. Temperature is a factor that also affects cyanobacterial biomass composition. ([32],[33], reported a variation in biomass composition as the temperature rises. Cyanobacteria absorb light mainly in the 400–500 nm and 600–700 nm light wavelength range. Cyanobacterial growth rates are enhanced by increasing light density up to the point of light saturation, at which point photosynthetic activity reaches its maximum ([32] ,[34]). At high light densities, the photosynthetic capacity decreases and cyanobacterial growth is inhibited.

Table-1: The biomass concentration, the productivity and the biomass composition of some of the selected cyanobacteria

Species	Biomass concentration	Productivity	Protein (%)	Lipid (%)	Carbohydrates (%)	References
Anabaena cylindrica	-	-	43–56	4–7	25–30	Becker,1994
Anabaena cylindrica	-	-	-	10.7–12.3	-	Sallal et al.,1990
Anabaena sp.	-	9–35 g/m2 d	-	-	-	Moreno et al.,2003
Anabaena variabilis	-	6–13 g/m2 d	51.9	22.7	9.2	Fontes et al.,1987
Nostoc sp.	0.2–0.3 g/L	-	-	-	30.66-32.85	Rodjaroen et al.,2007
Oscillatoria rubescens	0.05–0.327 g/L	-	28-53.8	1.3-12.8	-	Piorreck et al.,1984
Oscillatoria sp.	-		49.3-79.4	-	-	Hashimoto et al.,1989
Oscillatoria sp	0.26g/L	-	-	-	19.32	Rodjaroen et al .,2007
Phormidium angustissimum	-	-	-	-	28.48	Rodjaroen et al .,2007
Spirulina maxima	-	0.21-0.25g/Ld	60-71	6-7	13-16	Becker,1994
Spirulina platensis	-	-	46-63	4-9	8-14	Rodjaroen et al .,2007
Spirulina platensis	-	0.06-0.42g/Ld	-	-	-	Reichert et al.,2006

Contamination and competition is another factor of outdoor cultures of cyanobacteria which suffer from contamination by other microorganisms such as bacteria, fungi, yeasts and other microalgae genera, the metabolites or growth rates of which may inhibit the growth of the cultivated algae [35].

Table-2: The minimum and maximum temperature and the growth rate of some cyanobacteria.

Species	T min (°C)	T opt (°C)	Growth rate μ max (1/d)	References
Anabaena variabilis	–	35	–	Vonshak et al.,2000
Anabaena variabilis	<10	35	1.1	Robarts RD Zohary,1987
Oscillatoria	<15	27	–	Talbot et al.,1991
Spirulina maxima	15	30–35	0.26–0.45	Vonshak et al.,2003
Spirulina platensis	15	25–30	0.46–0.58	Vonshak et al.,2003

Considering the potentiality of blue green algae as a most important natural resource of biological nitrogen fixing agent it becomes an urgent need to developing low cost technique for mass cultivation of BGA biomass [36]. This piece of work focuses on the cultivation of cyanobacteria by the production of biomass besides using a low cost substrate and considering many other biological compost and waste materials . Moreover in recent times solid matrices

such as polyurethane foam, sugarcane waste and paper waste have been used for the mass production of carrier based immobilized cyanobacterial inoculum ([37],[38],[39]).

MATERIALS AND METHODS

The strains *Anabaena torulosa, Anabaena doliolum* and *Calothrix marchica* were isolated from the rice fields of Assam, North East of India. All the samples were pure cultured by agar plating method and transferring each colonies to BG11 liquid culture media. For our experiment fluorescent lamps were used as a source of light and intensity was adjusted to 2500~3500 lux for all the culture flasks and 16 hrs of light and 8 hrs of dark cycles were repeated for growth of all the cultures. The temperature was adjusted to 25.C for all the flasks.

2.1 Determination of Biomass, Chlorophyll-a and total Chlorophyll Content

Each cyanobacterial culture was harvested at 15 days , 30 days and 45 days. Biomass were collected by filtering the media through Whatman No 41 filter paper and finally weighed to record the biomass. In this investigation the final biomasses were recorded at specific time intervals of incubation at their stationary phases which showed maximum growth. Total biomass was calculated on the basis of weight showed maximum biomass production and hence taken for further evaluation. The curve of chlorophyll-a and total chlorophyll content was estimated as an index of algal growth. The pigment was extracted and estimated by using cold extraction method [40] and expressed in mg/ml of fresh culture.

2.2 Determination of total N-content

After biomass determination of each BGA isolates, 0.1 gm of dry algal flakes was collected and determination of total N-content by Micro Kjeldahl method [41]. The biomass production and total N content were found varied among the BGA isolates.

2.3 Determination of packed cell volume (PCV) as an index of growth

For determination of packed cell volume, a small amount of the sample (10 ml) was removed from the uniformly disposed suspension culture aseptically and centrifuged at 500 rpm for 15 minutes in 15 ml graduates centrifuged tubes. The packed cell volume is expressed as ml pellet ml^{-1}culture. The packed cell volume was taken at 3 consecutive interval of time in 15 days, 30 days and at 45 days.

2.4 Determination of intrinsic antibiotic resistant profile (IARP)

IARP of the isolates was determined against five standard antibiotics viz. ampicillin, chloremphenicol, gentamycin, kanamycin and streptomycin at concentrations ranging (5-100ppm) in liquid BG_{11} media. Stock solution of each antibiotic and BG_{11} liquid media were prepared by dissolving 1 gm of each antibiotic in 100ml and centrifuged at 10,000 ppm. The pH of the medium was adjusted with 0.1 N of KOH or NaOH and maintained at 7-7.5 and analyzed for its growth behaviour for one month.

2.5 Determination of Indole-acetic acid (IAA) like substance production:

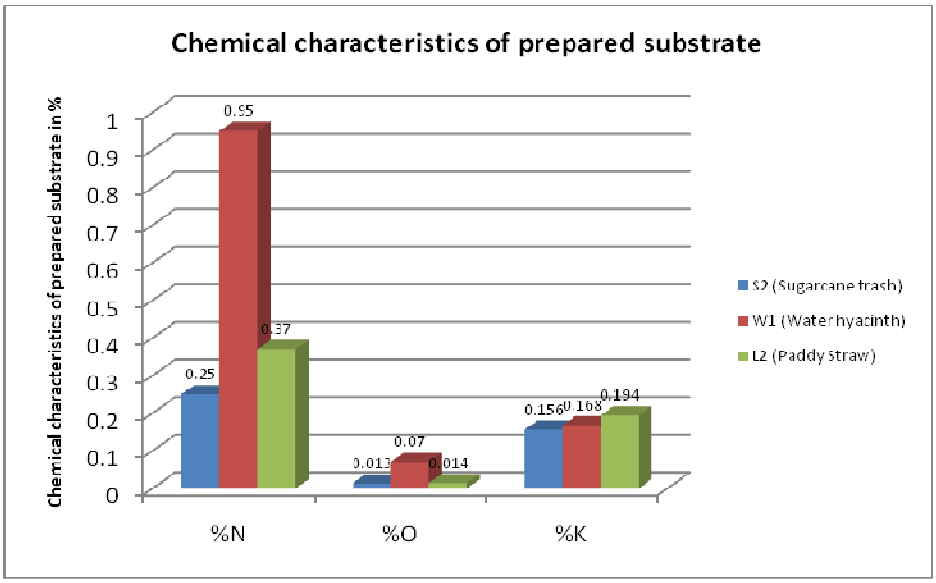
To check for IAA like substance production conical flasks in triplicates containing 50 ml of BG_{11} media for each culture were inoculated with 0.1 mg/ml isolates for 15days. Each broth culture was centrifuged at 10,000 rpm at 4^oC for 15 minutes and supernatant was acidified to pH 3 with 1 N HCL. The suspension were divided in two equal parts for determination of IAA like substances.One part of supernatant was extracted sequentially thrice with 10 ml of ethyl acetate at 30 minutes interval. The ethyl acetate fraction was allowed to evaporated to dryness. The residue was dissolved in 3 ml of absolute method and mixed with 4 ml of Fe–$HClO_4$ reagent (i.e.

1 ml of 0.5 M $FeCl_3$ and 50 ml of 35% $HClO_4$) 2-3 drops of orthophosphoric acid were added and incubated for 25 minute at room temperature. Lastly a standard curve was prepared using absorbance readings of standard IAA solution and used to determine concentration of the IAA like substance in the culture broths [42].

2.6 Assessment of compatibility among the isolates of Cyanobacteria for mass scale multiplication (*in vitro*) and use as biofertilizer agents

Compatibility among the *Anabaena torulosa*, *Anabaena doliolum* and *Calothrix marchica* were tested by quantifying their growth promoting mechanisms in mixed culture in comparison to their pure culture and performance evaluation as mixed inocula in different combinations on field condition in enhancing mass production.

Figure-1: Chemical characteristics of prepared substrate



2.7 *In vivo* studies for mass multiplication of BGA based on different locally available biological composts and waste materials

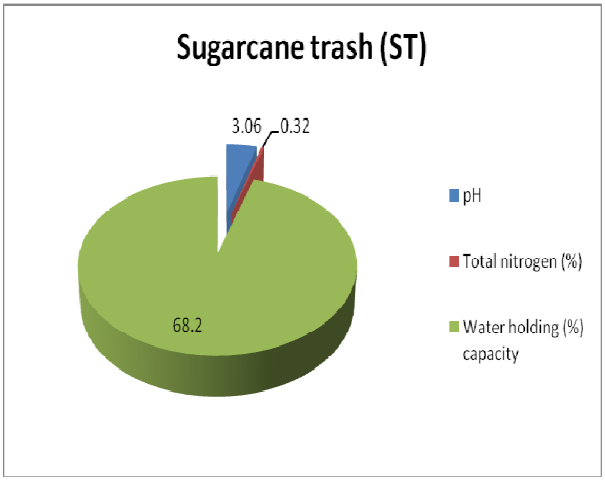
Three types of different biodegradable wastes and composts were considered and tested in the experiment which were paddy straw, sugarcane trash and water hyacinth. To get a good quality of compost, above mentioned locally available biodegradable wastes were collected for the experiment: Shallow pits were dug into the ground measuring (1m×1m×0.2m). and it was leveled uniformly and layered with a polythene sheet (0.5 mm thickness) to hold water and to prevent leakage .The collected wastes were chopped, shredded split or bruised to increase their surface area. The prepared wastes was kept into the pit separately i.e. in a single pit there should be a single waste product. each pit was covered with a thin polythene sheet to prevent rain water and requisite amount for maitaining moisture level at 80% The temperature inside should range from 32^o-60^o. The pit was agitated regularly to enhance aeration and regulate temperature. Finally all the pit cemented with soil and kept them for a reasonable time to get a mature and good quality of compost. Table 9 describes the chemical characteristics of prepared compost and soil sample.

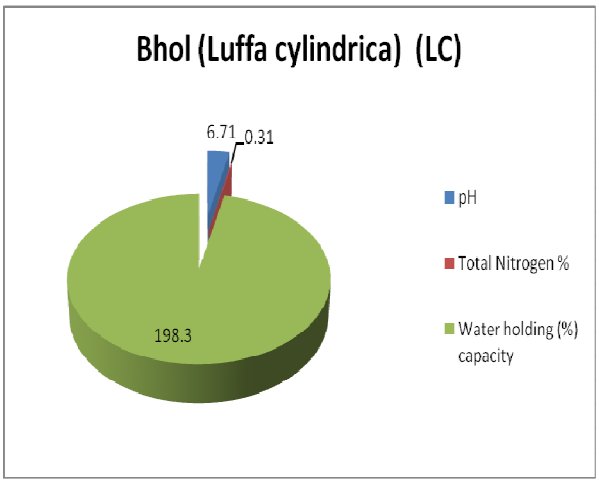
2.8 BGA compatibility and mass culture for use as biofertilizer and selection and preparation of bio carrier materials for optimization of immobilized BGA inoculum production :

The mass multiplications of BGA inocula was performed in earthen pots having a diameter of 10 inch and a depth of 7 inch with volume of 5 litres each. to assess the compatibility among the three BGA isolates (*in vivo)* . The growth study of BGA strain in normal BG_{11} media was considered as control . 500 gm of powdered compost was added to each of the pots containing 5 lit of water and the pH adjust in between 6.5 –7 and the cultures were allowed to grow for a time period of six months alone and in combinations after inoculating with 10 mg inoculum each. The cultures were allowed to multiply until thick mat was formed on the surface.

Ridge guard (*Luffa cylindrica*) which locally known as 'bhol' and sugarcane trash were collected for bio carrier based BGA inoculum production.The materials were washed and properly dried . A homogenize mixture of the three isolates was prepared and finally cultured in a metallic tray. The collected algal mat was mixed with the carrier materials and sun dried. The BGA mixed carrier materials were packed in different polythene bags separately. The quality assessment of prepared immobilized inoculum was recorded after a period of 7 and 15 months .

Figure-2: Characteristics in terms of pH, total N and water holding capacity of collected carrier materials in Pie diagram.





RESULTS AND DISCUSSION

The three strains of BGA namely *Anabaena torulosa*, *Anabaena doliolum* and *Calothrix marchica* were isolated by serial dilution method [43] and cultured in BG11 medium [44]. The BGA strain *A.torulosa* was blue green in colour, thallus mucilaginous; trichome 5 µ broad, apical cell acutely conical ; cells barrel shaped ;heterocyst sub spherical , 6 µ broad and 6-8 µ long; akinates presents on both sides of the heterocyst. Similarly *A. doliolum* was pale blue green in colour, thallus mucilaginous, trichome single, filaments straight curved or slightly coiled, 3.6-4-2 µ broad, slightly tappering at the ends with conical apical cells. Cells barred shaped, heterocyst, barred shaped 5.2-6.3 µ broad and 6.3-9.4 µ long, epispore thick, smooth and hyaline or yellow brown in color. However *C. marchica* was observed olive green in colour, filaments straight or slightly bent,single ,at the base 5-6 µ broad with a close thin colourless sheath ;trichome gradually attenuated into a hair; cells nearly as long as broad ,end cell conical; heterocyst single and hemispherical [45].

The results of the biomass study, chlorophyll-a and total chlorophyll, total N content and PCV of above mentioned three strains in three different days of interval are presented in table 3 and 4. It was observed that among the all three stains, *A. torulosa* which showed highest amount of biomass (0.186 mg/100ml) as well as highest amount of chlorophyll a (0.67mg/ml) and total chlorophyll content (0.91mg/ml) and total N content (2.64%) at 45 days of growth in comparison to *A.doliolum* and *C. marchica*.

Table 3: Estimation of Biomass production and determination of Chlorophyll-a and total Chlorophyll of the three BGA Isolates (mg/ml) ± SE, at three consecutive intervals of time

BGA Isolates	Day 15			Day 30			Day 45		
	Biomass Production (mg/100ml) ± SE	Chl-a (mg/ml ± SE)	Total Chl. (mg/ml ± SE)	Biomass Production (mg/100ml) ± SE	Chl-a (mg/ml ± SE)	Total Chl. (mg/ml ± SE)	Biomass Production (mg/100ml) ± SE	Chl-a (mg/ml ± SE)	Total Chl. (mg/ml ± SE)
Anabaena torulosa	0.061 ± 0.001	0.18±0.001	0.26±0.002	0.061 ± 0.001	0.41±0.001	0.60±0.001	0.186 ± 0.00	0.67±0.006	0.91±0.004
Anabaena doliolum	0.054 ± 0.00	0.13±0.00	0.20±0.001	0.054 ± 0.00	0.29±0.001	0.39±0.001	0. 162 ± 0.01	0.45±0.003	0.62±0.01
Calothrix marchica	0.030 ± 0.001	0.096±0.002	0.136±0.01	0.030 ± 0.001	0.193±0.01	0.273±0.002	0.092±0.0001	0.29±0.002	0.41±0.001

(Values are average of three replicates)

The study of packed cell volume (PCV) also indicated that *A.torulosa* was the efficient strains with highest amount of PCV (1.70ml pellet/ml) recorded at same very days, which was followed by *A.doliolum* and *C.marchica*. Table 4 shows the results of PCV and the total N content respectively.

Table 4: Determination of Packed Cell Volume (PCV) of BGA Isolates (ml pellet/10 ml of culture ± SE) and Total N content of BGA Isolates (% ± SE)

BGA Isolates	Day 15		Day 30		Day 45	
	Packed Cell Volume (PCV) (ml pellet ml^{-1} ± SE)	Total N Content (%±SE)	Packed Cell Volume (PCV) (ml pellet ml^{-1} ± SE)	Total N Content (%±SE)	Packed Cell Volume (PCV) (ml pellet ml^{-1} ± SE)	Total N Content (%±SE)
Anabaena torulosa	0.56 ± 0.06	0.88 ± 0.00	1.13 ± 0.02	1.56 ± 0.001	1.70 ± 0.06	2.64 ± 0.001
Anabaena doliolum	0.49 ± 0.08	0.74 ± 0.001	0.99 ± 0.06	1.49 ± 0.0001	1.50 ± 0.08	2.24 ± 0.001
Calothrix marchica	0.26 ± 0.02	0.72 ± 0.01	0.53 ± 0.02	1.60 ± 0.001	0.80 ± 0.06	2.41 ± 0.00

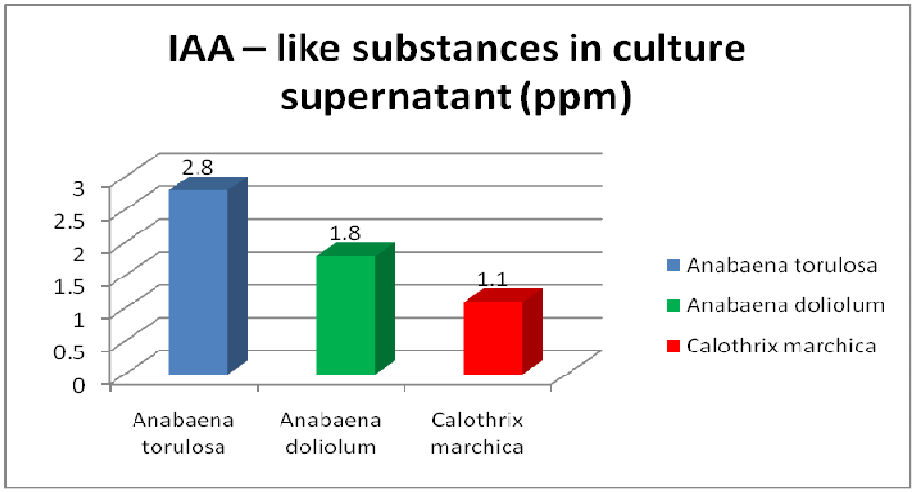
The growth curve towards the efficiency of intrinsic antibiotic resistance profile test (IARP) against five common antibiotics namely streptomycin sulfate, chloremphenicol, ampicillin, gentamycin and kanamycin (Table 5 and 6 depicts the IAR profile of BGA isolates), also

provided support that they were different strains as they showed different sensitivity towards different antibiotics. It was observed that isolates *A.torulosa* showed a good resistance capability towards the two antibiotic namely streptomycin sulfate and ampicillin. Similarly *A.doliolum* also showed resistance towards chloramphenicol and ampicillin.But the isolate *C.marchica* showed exceptionally higher sensitivity towards all the five antibiotics.

Table –5: *In vitro* culture of BGA isolates in BG_{11} media taking as control for intrinsic antibiotic resistance profile (IARP) test (culture period 30 days)

BGA Isolates	Growth parameters when BG11 media taken as control		
	Biomass (mg/100ml± SE)	Chl-a (mg/ml ± SE)	Total N content (%± SE)
Anabaena torulosa	0.168 ± 0.002	0.42 ± 0.0003	2.38 ± 0.001
Anabaena doliolum	0.154 ± 0.004	0.37 ± 0.001	2.27 ± 0.001
Calothrix marchica	0.089 ± 0.001	0.21 ± 0.002	1.51 ± 0.002

Figure 3: Content of IAA like substances in the culture supernatant of BGA isolates



There was no detectable amount of IAA like substances was determined in the uninoculated control medium. Among the three isolates, more quantity of IAA-like substances (2.8ppm)was detected in culture supernatant of *A.torulosa* which was followed by *A.doliolum* (1.8 ppm) and *C.marchica* (1.1ppm). Figure 3 describes the Indole-Acetic Acid (IAA) like substance production.

In vitro compatibility study of efficient BGA isolates were carried out in pure culure form or in mixed culture (table 7). The results indicated that *A.torulosa* was the most efficient isolates in pure culture state in all considering growth parameters. Whereas when the all three isolates mixed together and cultured for the same period of time, it gives much more better results in comparison to their pure culture form in all considering growth parameters. It was observed that when mixed culture of *A.torulosa, A.doliolum* and *C.marchica* performed, the quantity of biomass (0.192mg/100ml), total chlorophyll content (0.94mg/ml) and total N-content (4.51%) increased as well as the the IAA like substance (6.4ppm) also found to be higher in composite culture broth.

Table – 6: Intrinsic antibiotic resistance profile (IARP) test of thr three BGA isolates (Culture period 30 days)

BGA Isolates	Streptomycin Sulfate														
	10 (ppm)			25 (ppm)			50 (ppm)			75 (ppm)			100 (ppm)		
	Biomass (mg/100ml ± SE)	Chl-a (mg/ml ± SE)	N Content (%± SE)	Biomass (mg/100ml ± SE)	Chl-a (mg/ml ± SE)	N Content (%± SE)	Biomass (mg/100ml ± SE)	Chl-a (mg/ml ± SE)	N Content (%± SE)	Biomass (mg/100ml ± SE)	Chl-a (mg/ml ± SE)	N Content (%± SE)	Biomass (mg/100ml ± SE)	Chl-a (mg/ml ± SE)	N Content (%± SE)
AT_X	0.063 ± 0.001	0.032 ± 0.00	0.124 ± 0.001	0.061 ± 0.00	0.041 ± 0.001	0.88 ± 0.001	Trace	-	-	-	-	-	-	-	-
AD_Y	Trace	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CM_O	Trace	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorem phenicol															
AT_X	-	-	-	-	-										
AD_Y	0.043 ± 0.001	0.09 ± 0.00	0.121 ± 0.001	0.024 ± 0.00	0.03 ± 0.001	0.130 ± 0.001	0.026 ± 0.001	0.06 ± 0.00	0.101 ± 0.002	Trace	-	-	-	-	-
CM_O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ampicillin															
AT_X	0.042± 0.001	0.021± 0.00	0.94± 0.001	0.038± 0.001	0.024± 0.001	0.98± 0.001	0.014± 0.002	0.021± 0.001	0.64± 0.002	0.011± 0.001	0.018± 0.001	0.62± 0.002	-	-	-
AD_Y	0.062± 0.002	0.042± 0.001	0.112± 0.001	0.031± 0.001	0.041± 0.001	0.108± 0.001	Trace	-	-	-	-	-	-	-	-
CM_O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentamycin															
AT_X	Trace	-	-	-	-	-	-	-	-	Trace	-	-	-	-	-
AD_Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CM_O	Trace	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kanamycin															
AT_X	Trace	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AD_Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CM_O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 7- *In-vitro* Biomass production, total nitrogen content, total chlorophyll content and production of IAA like substance in pure and mixed culture of inoculants strains of *Anabaena torulosa, Anabaena doliolum* and *Calothrix marchica*

BGA isolates	Biomass Production (mg /100ml ± SE)	Total N Content (% ± SE)	Total Chlorophyll Content (mg/ml ± SE)	IAA like, substances (ppm)
Control (without inoculation)	0	0	0	0
Anabaena torulosa(AT)	0.168 ± 0.002	2.38 ± 0.001	0.62 ± 0.001	2.6 (± 0.16)
Anabaena doliolum(AD)	0.154 ± 0.004	2.27 ± 0.001	0.39 ± 0.001	1.8 (± 0.29)
Calothrix marchica(CM)	0.089 ± 0.001	1.51 ± 0.002	0.39 ± 0.001	1.1 (± 0.22)
AT+AD+CM	0.192 ± 0.002	4.51 ± 0.003	0.94 ± 0.002	6.4 (± 0.12)

(Values are average of three replicates)

Table-8: Mono and multiple BGA isolates culture (*in vivo)* considering normal BG_{11} media as control.

BGA Isolates	Biomass mg/100ml (Mean)	Total N Content (%) (Mean)
AT_x	16.88	6.08
AD_y	12.02	3.23
CM_0	8.16	1.04
$AT_x+AD_y+CM_0$	22.73	9.64
F-test	*	*
SE(d)	0.362	0.277
CD(1%)	1.154	1.011
CD(5%)	1.341	0.399

Thereafter, the growth study and mass multiplication of all the three strains were carried out in field condition considering paddy straw, sugarcane trash and water hyacinth as their substrates.(Figure-2 shows the chemical characteristics of prepared substrates). The mass multiplication of *A.torulosa* in earthen pot considering paddy straw and sugarcane trash as a substrates found higher among all the three strains (table 9) whereas the performance of same strain in water hyacinth substrates was found declined progressively. All the three isolates were not able to grow in field condition when the water hyacinth was taken as substrates for mass multiplication. (table 9). In comparison to control (table 8), the biomass and total N-content of three BGA isolates grown in pure form, recorded higher growth when paddy straw taken as substrate . However composite culture of *A.torulosa, A.doliolum* and *C.marchica* on paddy straw substrates showed significantly increased biomass (28.16mg/100ml) as well as total N-content (20.33%) than that of their respective single culture in earthen pot. Similar effect in case of combine inoculation of all the three strains on sugarcane trash was also observed. There was significant decrease in growth of composite strains when water hyacinth substrate was utilized for their growth. Therefore, to develop multiple BGA inocula with an aim to improve N nutrition in different substrates systems, the composite culture of three BGA isolate with paddy straw substrate were identified as most suitable combination. All the isolates showed synergism with each other in terms of improvement of biomass yield and nitrogen activity. Based on these findings an integrated BGA immobilized inoculum was also formulated with two locally available carrier materials.

Table 9: Mono and multiple BGA isolates culture (*in vivo*) considering sugarcane trash water hyacinth compost and paddy straw as a substrate

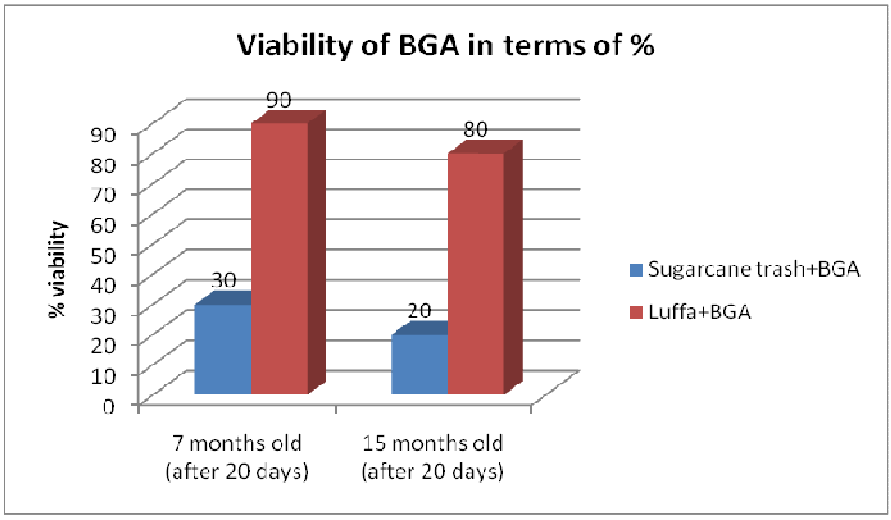
BGA Isolates	Substrate used sugarcane trash (pH 7.63)		Substrate used water hyacinth (pH4.16)		Substrate used Paddy straw (pH7.02)	
	Biomass (mg/100ml) (Mean)	Total N content (%) (Mean)	Biomass (mg/100ml) (Mean)	Total N content (%) (Mean)	Biomass (mg/100ml) (Mean)	Total N content (%) (Mean)
AT_x	12.83	9.17	0	0	18.33	10.16
AD_y	11.83	9.91	0	0	12.41	9.17
CM_0	8.5	5.80	0	0	9.75	4.83
$AT_x+AD_y+CM_0$	16.17	13.92	8.92	2.17	28.16	20.33
F-test	*	*	*	*	*	*
SE (d)	1.01	0.6	0.678	0.377	0.964	0.778
CD (1%)	2.95	1.75	1.980	1.101	2.816	2.272
CD (5%)	2.14	1.27	1.437	0.799	2.043	1.649

(Values are average of three replicates)

Among the two bio-carrier materials viz. Ridge guard (*Luffa cylindrica*) which is locally known as 'bhol' and sugarcane trash used in formulation of immobilized bio inoculum. Figure-3 depicts the characteristics of collected carrier materials in terms of pH, total N and water holding capacity**.** It was bhol which performed significantly higher viability after a period of 7 months and 15 months (figure-4). The formation of profuse scum after 20 days of culture indicating

viability of immobilized BGA inoculum. So from the above study utilization of bhol promises to be a better alternative immobilized bioinoculum in BGA biofertlizer technology.

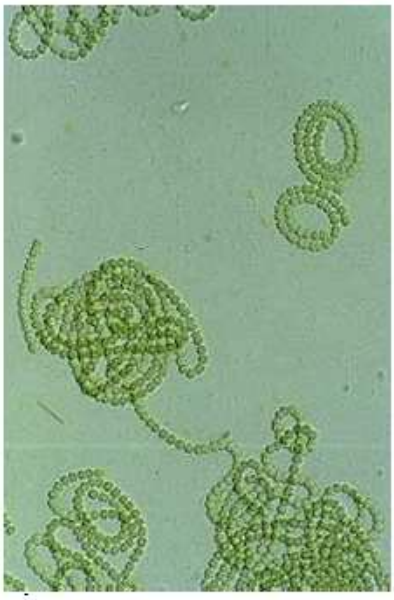
Figure-4: Viability (in terms of %) study of 7 and 15 months old immobilized carrier based BGA inoculum after 20 days of culture



CONCLUSION

In the present investigation, emphasis was given to develop a composite inoculum which comprises with potent BGA species which might be used as algal biofertilizer. The study showed efficiency of three BGA isolates namely *A.torulosa, A.doliolum* and *C.marchica* towards growth curve considering different parameters in laboratory condition as well as in field condition. Presently the soil based BGA inoculum is being produced under field condition and utilized as algal biofertilizers for rice. However , to provide plant nutrients directly to the crops and maintenance of soil fertility status as the non renewable sources of energy are fast depleting. In this respect intensive research work has been made for developing biological process in which non conventional substrates like agricultural, natural, industrial, waste & by production could be used as substrate in the cultivation of cyanobacteria species ([46],[47]). In the later part of evaluation mass cultivation of three species of BGA i.e *A. torulosa, A. doliolum* and *C. marchica* were carried out using three non conventional substrates (paddy straw, sugarcane trash and water hyacinth) were recorded. Similar results have been reported by ([48],[49],[50]), as a single paddy field harbours a number of different nitrogen fixing species those act in a composite manner. Moreover [51] reported that inoculation with composite cultures is more effective than single culture inoculation. During the study it was revealed that growth of composite BGA culture on paddy straw substrate and sugarcane trash substrate was found satisfactory. While the findings recorded in the water hyacinth substrate was not satisfactory. The experimental results show variation among the culture on three substrates due to a number of biotic and abiotic factors. The most common biotic factors may be the parasitic and antagonistic fungi, bacteria, viruses and insects pests. There was little doubt of heavy metal content of water hyacinth as they have the ability to absorb heavy metals like cadmium, lead, copper and zinc which may effect in growth of BGA [52]. Out of the three substrates i.e paddy straw, sugarcane trash and water hyacinth ,paddy straw harboured maximum quantity of biomass (28.16mg/100ml) and as well as total N –content (20.33%). Again satisfactory amount of biomass (19.17mg/100ml) and N-content (13.92%) on sugarcane trash was found. The increased amount of biomass and total N-content of three mixed composite cultures of BGA strains on different substrates was satistically

significant. These results indicated the definite benefits and potentiality of biomass yield and N nutrition from, preparation of composite immobilized BGA inoculum in future.



Photograph-1: The microscopic photograph of *Anabaena torulosa* on BG_{11} media after one month of culture. (Under 40X magnification)

Photograph-2: *Calothrix marchica.* (Under 40X magnification)

Photograph-3: *Anabaena doliolum.* (under 40X magnification)

Photograph-4: Luffa biofertilizer packet for field application

Photograph-5: Sugarcane trash biofertilizer packet for field application

Photograph-6: Germination of 15 month old *Luffa cylindrica* based immobilized BGA.

Photograph-7: BGA cultures inside the laboratory.

Photograph-8: Earthen pot culture for mass multiplication of BGA taking paddy straw as substrate.

All the photographs were taken at Environmental Biotech Lab. Department of Biotechnology, Gauhati University ,Guwahti Assam

ABBREVIATIONS: PCV, Packed cell volume; IAA, Indole acetic acid; IARP, Intrinsic antibiotic resistance profile.

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