STUDIES ON NITROGEN FIXING BLUE GREEN ALGAE AND MASS CULTIVATION

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Studies on nitrogen fixing blue green algae and mass cultivation"** in partial fulfilment of the requirement for the award of the degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab, is an authentic record of my own work during the period of six months from Jan 2011 to June 2011, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology and Environmental Science, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other University.

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on nitrogen fixing blue green algae and mass cultivation"** submitted by Kamal Malhotra in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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DEDICATED

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LIST OF ABBREVATIONS

BGA	-	Blue green algae
DW	-	Distilled water
EC	-	Electrical Conductivity
gm	-	gram
ha	-	hectare
kg	-	kilogram
K_2HPO_4	-	Dipotassium hydrogen phosphate
L / Ltr	-	Litre
mg	-	milligram
$MgSO_4$	-	Magnesium sulphate
ml	-	millilitre
µg	-	microgram
(–N)	-	without nitrogen
(+N)	-	with nitrogen
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
ppm	-	parts per millions
v/v	-	volume by volume
w/w	-	weight by weight

ABSTRACT

Cyanobacteria or blue green algae (BGA) are ecologically important group of organisms since some forms are capable of fixing atmospheric nitrogen. In the present investigation region specific cyanobacteria were isolated from soil samples of paddy field collected from 20 different sites of Patiala district, Punjab and physico-chemical properties were analysed. The soils were mainly alkaline with electrical conductivity (EC) ranging from 100 to 282 µS, organic carbon ranging from 0.37 to 0.85% whereas total nitrogen ranged from 0.035 to 0.087%, available nitrogen ranged from 1.8 to 7.4 mg/kg and available phosphorus from 2.8 to 19.5 mg/kg. Growth of heterocystous cyanobacteria in terms of dry biomass production (mg/ml) and total nitrogen was studied at different intervals in order to assess the nitrogen fixing potential of cyanobacteria.

Total nitrogen was highest in *Anabaena variabilis* (ARM 441) (0.011%) and dry biomass production was highest in *Tolypothrix tenuis* (ARM 443) (1.70 mg/ml). Heterocyst frequency also highest in *Anabaena variabilis* (ARM 441) by 14.9% which was also the case with region specific isolate 6. End point growth yielded maximum chlorophyll content (9.3 µg/ml) in *Aulosira fertilissima* (ARM 444). Wet biomass production in outdoor algal ponds resulted in an average yield of 1.68 kg/m^2 after 7 days, 2.9 kg/m^2 after 14 days and 4.8 kg/m^2 after 21 days whereas dry biomass production was 0.21 kg/m^2 after 7days, 0.59 kg/m^2 after 14 days and 0.65 kg/m^2 after 21 days during the month of March, 2011.

CHAPTER 1 INTRODUCTION

Blue green algae (BGA) also known as cyanobacteria exhibits great morphological and metabolic diversity and have great biological significance with novel characteristic for large scale application to overcome many of the problems related to food, energy, medicine and environmental degradation. BGA plays an important role in industry, agriculture and environment.

Cyanobacteria are heterogeneous group of prokaryotic, principally photosynthetic organisms and resemble the eukaryotic algae in many ways, including morphological characteristics and ecological niches, and were at one time treated as algae; hence they are well known as blue-green algae. Cyanobacteria are the only group of organisms that are able to reduce nitrogen and carbon in aerobic conditions, a fact that may be responsible for their evolutionary and ecological success. Unlike most photosynthetic organisms, cyanobacteria are blue-green or greyish-brown in colour rather than plain green. The blue-green colour of cells (cyan means blue-green) is due to the combination of green chlorophyll pigment and a unique blue pigment (phycocyanin). In cyanobacteria, there is naked circular double stranded DNA which is in the centre of cytoplasm equivalent to single large chromosome known as nucleoid and some cyanobacteria also contains one or more than one plasmids.

Cyanobacteria are genetically diverse and occupy a broad range of habitats across all latitudes, widespread in freshwater, marine and terrestrial ecosystems, and they are found in the most extreme niches such as hot springs, salt works, and hyper saline bays. Cyanobacteria naturally occur in water logged conditions and have ability to attach to the surface and form biofilms. Their occurrence in water is controlled by environmental factors such as temperature, light intensity, nutrients (phosphate and nitrate) availability and water stability. pH is a very important factor in growth, establishment and diversity of cyanobacteria, which have generally been reported to prefer neutral to slightly alkaline pH for optimum growth (Kaushik, 1994 and Singh, 1961). Soil pH is also known to have a selective effect on the indigenous algal flora, especially cyanobacteria and their succession and abundance in soil. The water must be in a temperature range that will support the specific algal species being grown.

Cyanobacteria are diazotrophic nitrogen fixers since many species of cyanobacteria can also “fix” atmospheric nitrogen—that is, they can transform the gaseous nitrogen of the air into compounds that can be used by living cells. Some filamentous forms have the ability to differentiate into several different cell types: vegetative cells, the normal, photosynthetic cells that are formed under favourable growing conditions; akinetes, the climate-resistant spores that may form when environmental conditions become harsh; and thick-walled heterocysts, which contain the enzyme nitrogenase, vital for nitrogen fixation. Particularly efficient nitrogen fixers are found among the filamentous species that have specialized cells called heterocysts. Heterocysts may also form under the appropriate environmental conditions (anoxic) when fixed nitrogen is scarce**.** Heterocyst-forming species are specialized for nitrogen fixation and are able to fix nitrogen gas into ammonia (NH_3), nitrites (NO^{-2}) or nitrates (NO^{-3}) which can be absorbed by plants and converted to protein and nucleic acids (atmospheric nitrogen is not bioavailable to plants).

Rice plantations utilize healthy populations of nitrogen-fixing cyanobacteria (*Azolla*) for use as rice paddy fertilizer. The paddy-field ecosystem represents a unique aquatic-terrestrial habitat, which provides a favourable environment for growth and nitrogen fixation by cyanobacteria, meeting their requirements for light, water, elevated temperature and nutrient availability. This, in turn, has been considered as one of the major reasons for the relatively stable yield of rice under flooded conditions and maintenance of the productivity of rice fields (Roger *et al*., 1993). BGA improve soil health and maintain a continuous supply of crop nutrients. It improves water holding capacity of soil and increases soil aggregation. BGA leads to population build up and enhances the microbial activity. BGA biofertilizer is eco-friendly and non-polluting to the environment due to which cyanobacteria have been widely employed as inoculants for enhancing soil fertility and improving soil structure, besides enhancing crop yields, especially in rice (Venkataraman, 1972; Kaushik, 2004; Nayak *et al*., 2004; Dhar *et al*., 2007).

Extensive research on different fundamental and applied aspects of algae has demonstrated that algal biomass can be used for diverse application. In many cases there is utilization of whole algal biomass or certain valuable constituents are extracted including metabolites and enzymes. A number of processes have been developed for algal mass cultivation and isolation of valuable constituents from these organisms. The ponds in which the blue green algae are cultivated are usually called as “raceway ponds”. One of the major advantages of open ponds is that they are easier to construct and operate than most closed systems. However, major limitations in open ponds include poor light utilization by the cells, evaporative losses, diffusion of CO_2 to the atmosphere, and requirement of large areas of land, contamination by predators and other fast growing heterotrophs have restricted the commercial production of algae in open culture systems to only those organisms that can grow under extreme conditions. Also, due to inefficient stirring mechanisms in open cultivation systems, their mass transfer rates are very poor resulting to low biomass productivity due to which closed bioreactors are used. In closed ponds, the control over the environment is much better than that for the open ponds but the cost of closed pond system is more than the open ponds.

Cyanobacteria are very important for the health and growth of many organisms, and also environment. They are one of the very few groups of organisms that can convert inert atmosphere nitrogen to an organic form, such as nitrate or ammonia. Ever since the importance of these organisms was recognized, considerable amount of research has been carried out to evolve methods and means to utilize these organisms effectively. Improvements in the fertility status of rice fields in tropics by utilizing these as nitrogen input have led to their agronomic potential.

The aim of the present work was to isolate and characterize the heterocystous BGA from paddy fields of Punjab, study growth and mass cultivation of different heterocystous cyanobacteria which are commonly used for algalization in paddy cultivation.

CHAPTER 2 REVIEW OF LITERATURE

Cyanobacteria also known as blue-green algae or blue-green bacteria belong to phylum Cyanophyta since they obtain their energy through photosynthesis. The term "cyanobacteria" comes from the colour of the bacteria and their name derived from bluish pigment phycocyanin, which they use to capture light for photosynthesis. They possess chlorophyll a, the same light harvesting pigment which is present in photosynthetic eukaryotes and are morphologically diverse group of gram negative bacteria (Giavannoni *et al*., 1988), which comprises of unicellular forms- with only vegetative cells and filamentous forms- that differentiates into various forms as specialized cells (Castenholz and Waterbury, 1989). They have oxygen evolving photosynthetic mechanism which is similar to chloroplast of algae and higher plants i.e. they are photoautotrophs and the recent ancestor in cyanobacteria evolutionary line gave rise to chloroplasts (Douglas, 1994). Cyanobacteria have an elaborate and highly organized system called as thylakoids which are involved in photosynthesis. During photosynthesis, water is used as electron donor for carbon dioxide fixation, evolving oxygen as a by-product though some may use hydrogen sulphide as occur among all photosynthetic bacteria (Haselkorn, 1978).

2.1 Nitrogen fixation by cyanobacteria

Nitrogen is an essential plant nutrient which is required for better agricultural yield and their deficiency contribute to reduced productivity throughout the world. Four-fifths of the atmosphere consist of molecular or dinitrogen, but is metabolically unavailable directly to higher plants or animals. It is available to some microorganisms through biological nitrogen fixation (BNF) in which atmospheric nitrogen is converted to ammonia by the enzyme *nitrogenase.* Special nitrogen fixing cells known as heterocysts have been found in cyanobacteria by Adams (2003). The heterocysts are thick-walled cell inclusions that are impermeable to oxygen; they provide the anaerobic (oxygen-free) environment necessary for the operation of the nitrogen-fixing enzymes. These are the nitrogen fixing stations of heterocystous algae and the various non – heterocysts form fixed nitrogen anaerobically (Prasanna and Kaushik, 1994). Nayak and Prasanna (2007) observed more heterocystous forms while studying cyanobacterial abundance and diversity in rice field soils of India.

Cyanobacteria are wide spread in marine, fresh water and terrestrial environments and are capable of utilizing atmospheric free nitrogen in the synthesis of cell material (De, 1939). In micro toxic condition, they have strategy to fix nitrogen in well oxygenated conditions even without heterocyst (Stal, 2008).

BNF offers an economically attractive and ecologically sound alternative of reducing external nitrogen and help in improving the quality and quantity of internal source. Some non-leguminous plants have ability to fix nitrogen either through exogenous or endogenous symbiosis with nitrogen fixing microorganisms like some cereal crops of commercial importance like rice, wheat, maize and millets are found to have association with microorganisms that are capable of assimilating atmospheric nitrogen and this is a potential source of nitrogen for agriculture and are of great economic importance.

Cyanobacteria are free living and have independent existence though many of them have capability to form associations with protista, fungi and plants, ranging from unicellular algae to angiosperms (Rai *et al*., 2000; Gusev *et al*., 2002). Cyanobacteria are intensively used in biotechnology (Singh *et al*., 2005). They add organic matter, synthesize and liberate amino acids, vitamins and auxins, reduce oxidizable matter content of the soil, provide oxygen to the submerged rhizosphere, ameliorate salinity, buffer the pH, solubilize phosphates and increase the efficiency of fertilizer use in crop plants (Mandal *et al.,*1999; Kaushik, 2004). From one of the studies, it was found that cyanobacteria can be used as phytoremediation tool for the removal of Ni from the moderately contaminated waste water (Shukla and Tripathi, 2009).

2.2 Determination of nitrogen in cyanobacteria

Determination of nitrogen fixation of blue green algae and *Azolla sp.* made use of moist soil cores, which was used in field studies in which chemical fertilizers, blue green algae and *Azolla* biofertilizers had been used in association with rice crop which involve the collection of fresh and moist soil cores (0-30mm) using soil auger, incubated with 10% acetylene in air tight glass vials under field conditions for three hours and ethylene was measured using gas chromatography (Prasanna *et al*., 2003). Amount of nitrogen in cyanobacteria can also be determined by Kjeldahl method (Xueyan, 2010). In this method samples are heated with acid and catalyst, to dissolve proteins and let the dissolved ammonia combine with the acid to produce ammonium sulphate. Then with series of processes like digestion process, distillation process and titration process nitrogen amount can be determined.

2.3 Occurrence and abundance of cyanobacteria in rice fields

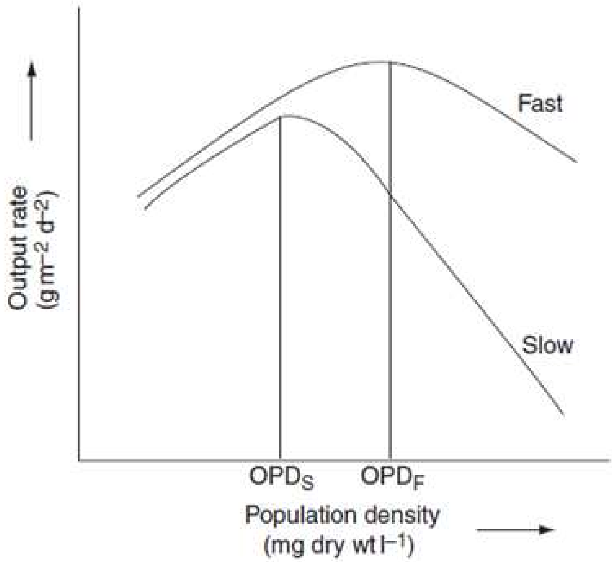
Nitrogen (N) is a most limiting nutrient which is required in the largest quantity for lowland rice production. Development of fertilizer-responsive varieties along with realization of importance of nitrogen by farmers, has led to the use of nitrogen fertilizer in rice crops. But unfortunately a substantial amount of the N fertilizer is lost through different mechanisms causing environmental pollution problems. Crop plants are able to use about 50% of the applied N fertilizers, while 25% is lost from the soil–plant system through leaching, volatilization, denitrification and due to many other factors causing not only an annual economic loss but also cause pollution to the environment. Nitrogen-fixing systems offer an economically attractive and ecologically sound means of reducing external inputs and improving internal resources. Efficient nitrogen fixing strain like *Nostoc linkia, Anabaena variabilis, Aulosira fertilisima, Calothrix sp., Tolypothrix sp.,* and *Scytonema sp.* were identified from various agro - ecological regions and utilized for rice production (Prasad and Prasad, 2001). Blue Green Algae find a favourable abode in the waterlogged conditions of rice fields and provide inexpensive nitrogen to plants besides increasing crop yield by making the soil fertile and productive. Venkataraman (1961) coined the term *'algalization'* to denote the process of application of blue-green algal culture in field as biofertilizer and it helps in creating an environment – friendly agro ecosystem that ensures economic viability in paddy cultivation while saving energy intensive inputs. Rhizobium and legumes are found to be major source of nitrogen in most cropping systems and *Anabaena* and *Azolla* are of particular value to flooded rice crop. They have been exploited as biofertilizers in agriculture, wherein they are known to contribute 20-25 kg N/ha/season and enhance soil fertility (Prasanna and Kaushik, 2006). It was found that nitrogen fixation by some diazotrophic bacteria like *Azotobacter*, *Clostridium*, *Azospirillum*, *Herbaspirillum* and *Burkholderia* can be used in place of N fertilizer and *Rhizobium* can promote the growth physiology or improve the root morphology of the rice plant (Choudhury and Kennedy, 2004). Biological nitrogen fixation (BNF), a microbiological process which convert's atmospheric nitrogen into a plant-usable form, offers this alternative. The technology has been easily adopted by farmers for multiplication at their own level. Species of *Nostoc, Anabaena*, *Tolypothrix, Aulosira, Cylindrospermum, Scytonema, Westiellopsis* and several other genera were widespread in Indian rice field soils and were known to contribute significantly to their fertility (Venkataraman, 1981; Kaushik, 1991; Nayak *et al.*, 2004).

Because of the current concern on environment and soil health, biological nitrogen fixation is gaining importance in rice ecosystem by the continuous use of nitrogenous fertilizer and need for improved sustainable rice productivity. In addition, due to increased cost of non- renewable chemical fertilizers facilitates the use of renewable indigenous biological N_2-fixation system as source of N_2 for the rice. Since the discovery of the cyanobacteria in N gain under flooded conditions, many inoculation experiments have been conducted using cultured cyanobacteria to improve soil fertility and grain yields of rice. Roger and Watanabe (1986) reported that cyanobacterial inoculation increase rice yields only by an average of 337 kg grain ha^{-1} $crop^{-1}$ Heterotrophic bacterial BNF is 7 kg N ha^{-1} (App *et al*.,1986), ranging from 11-16 kg N ha^{-1} which contributes 16-21% of total rice N requirement (Shrestha and Ladha, 1996). It has been recognized for a long time that associative N_2-fixing biological systems in wetlands enrich the soil organic N pool and supply up to 113 kg N/ha to rice crop depending upon the ecosystem, cultural practices and rice variety grown (Watanabe *et al*., 1977; Rao *et al*.,1998; Ariosa *et al*., 2004). In one of the studies it was reported that exogenous supply of N fertilizer counteracts N_2-fixation in the rhizosphere. Several studies have illustrated almost complete and long lasting inhibitory effect of N fertilizers on the N_2-fixing activity of free-living cyanobacteria (Roger and Kulasooriya, 1980). The exogenous supply of nitrogenous fertilizer to lowland rice significantly inhibited N fixation but improved plant growth. Inhibitory effect of exogenous supply of N fertilizer indicates limited potential of associative N_2 fixation to significantly benefit agriculture (Shrestha and Maskey, 2005). Quantification of cyanobacterial population and nitrogen fixation of 18 heterocystous cyanobacteria isolated from rice fields of 11 district of Bangladesh have been studied and it was reported that no. of cyanobacterial population varied from 14.6×10^4 to 141.0×10^4/g soil and nitrogen fixation of *Nostoc, Linkia* isolated from rice fields were ranged from 1.84 to 8.13 mg N 50/ml (Begum and Mandal *et al*., 2008). Cyanobacterial diversity and influence of pH, organic carbon (%) and conductivity has been studied in some local rice fields of Orissa and highly positive correlation was found between cyanobacterial population and soil pH (r≥9) (Dey and Tayung, 2010).

2.4 Biomass estimation of cyanobacteria

Microalgae are very efficient solar energy converters which produce a great variety of metabolites. Man has always tried to take advantage of these properties through algal mass culture. Cyanobacteria are unique photosynthetic diazotrophs that have contributed to the fertility of rice fields for centuries. The mass multiplication of cyanobacteria for field application has gained momentum and is seen as a viable option to cut down fertilizer costs. The soil-based cyanobacterial inoculum is one of the primary modes of algalization of the rice fields. In such a scenario, the actual biomass production by the cyanobacteria needs to be estimated. Microalgae, a broad category encompassing eukaryotic microalgae and cyanobacteria, has been cultivated to produce biomass for a wide range of applications, including animal and human nutrition, the health sector, cosmetics and agriculture (biofertilizers) (Metting, 1996; Spolaore *et al.*, 2006; Thajuddin *et al*., 2005; Tan, 2007; Becker, 2007).

An important application for the cultivation of microalgae is the production of biomass for energy purposes. Microalgae produce biomass, which is converted into energy or an energy carrier through a number of energy conversion processes. Microalgae biomass contains considerable amounts of proteins **(**Becker, 2007) and on the basis of biomass composition the quantity of nitrogen (N) required as fertilizer is estimated to be 8–16 tons N/ha, which means that microalgae production involves enormous amounts of N fertilizers. Maximal culture productivity is obtained only when culture nutritional requirements are satisfied and temperature is about optimal. There exists, indeed, a strong interaction between light and temperature, well-illustrated in the study of Collins and Boylen (1982) who investigated the physiological response of *Anabaena variabilis* to instantaneous exposure to various combinations of light intensity and temperature. An elementary aspect of the interaction of light and temperature thus revealed is that the optimal temperature for photosynthesis increases with increasing light intensities.



A schematic view of the effect of the stirring rate on culture productivity in relation to the population density (after Richmond & Grobbelaar, 1986).

A basic principle thus unfolds: the higher the intensity of the light source, the higher (potentially) becomes the optimal population density and the more significant the degree to which the extent of mixing may exert on the output rate of cell mass. This has been elucidated in the work of Hu *et al*., (1996 a, b).

The effect of nitrogenous fertilizers on alga *Anabaena cylindrica* was studied and concluded that (i) the addition of such fertilizers to the natural population of blue-green algae affects the nitrogen fixation and (ii) among the two types of fertilizers the depressive effect of ammonium nitrogen is likely to be more than the nitrate nitrogen (Stewart *et al*., 1975). Effect of cultural conditions on biomass and nitrate reductase activity in six strains of *Anabaena* isolated from paddy field soils of Ganjam district in presence of pH, temperature, copper & molybdenum, $NaNO_3$, NH_4Cl, and Urea was conducted, NR activity and biomass was affected under varied concentration and maximum NR activity was observed in *Anabaena* sp. at pH 8.5 and 35^{o}C temperature whereas biomass was maximum in *Anabaena variabilis* at pH 8.5 and temperature 35^{o}C (Padhi and Behura *et al*., 2010).

2.5 Plasmid profile of cyanobacteria

The cyanobacteria, traditionally known as the blue-green algae, are photoautotrophic organisms with a number of interesting properties which make them very attractive for molecular biological studies. Some unicellular cyanobacteria contain extra chromosomal (plasmid) DNA (Asato and Ginoza, 1973; Roberts and Koths, 1976). Plasmids have proven extremely useful in the genetic analysis of various bacteria. Because of structural similarity combined with the biochemical complexity of the higher organisms, cyanobacteria serve as attractive model organisms for studying biological problems at molecular level (Doolitle, 1979 and Shestakov *et al*., 1987). It has been reported that most of unicellular and filamentous cyanobacteria contain plasmids. Plasmid distribution among different cyanobacteria was studied using a simplified method for screening and characterization of plasmid DNA in cyanobacteria (Goyal, 1992). Genetically engineered strains of cyanobacteria performing desirable functions have been developed by using cyanobacteria plasmids (Flores and Wolk, 1985; Chauvat *et al*., 1988; Houmard and Tandeau de Marsac, 1988; Goyal, 1990). Large scale isolation and purification of plasmid from filamentous non-heterocystous cyanobacteria and its restriction enzyme analysis was done (Goyal and Venkataraman, 1992).

3.1 Physicochemical analysis of soil samples collected from paddy fields

3.1.1 pH

pH was determined as per the method given by Jackson (1967) in a soil-water suspension of 1:2 ratio. Soil (10 gm) was placed in a 100 ml beaker and 20 ml of distilled water was added and the soil was stirred well for five minutes and kept undisturbed for some time followed by stirring again. pH was measured using a Thermo Orion Model 290 pH meter after calibration with buffers of pH 4.0, 7.0 and 9.2

3.1.2 Electrical conductivity

Electrical conductivity was measured in $\mu S\ cm^{-1}$ as per the method given by Jackson (1967). Soil (10 gm) was placed in a 100 ml beaker and 20 ml distilled water was added. The soil-water mixture was allowed to stand undisturbed until the soil settled completely. The conductivity meter (Orion Model 125) was calibrated with 0.01 M potassium chloride (1413 $\mu S\ cm^{-1}$).

3.1.3 Organic carbon

Total organic carbon was estimated as per the method given by Walkley and Black (1934).

Reagents

a) 1 N $K_2Cr_2O_7$: 49.04 gm of potassium dichromate per litre of solution.
b) 0.5 N ferrous ammonium sulphate: 198 gm salt per litre of solution.
c) Diphenylamine indicator: 0.5 gm of diphenylamine in a mixture of 20 ml water and 100 ml concentrated sulphuric acid.
d) Concentrated sulphuric acid.
e) Orthophosphoric acid (85%)
f) Sodium fluoride (NaF)

Procedure

1. Soil sample (1 gm) was taken in a 500 ml conical flask followed by the addition of 10 ml of 1N $K_2Cr_2O_7$. The flasks were swirled for mixing the soil and reagent.
2. Added 20ml of H_2SO_4 and the flask was allowed to stand undisturbed for 30 minutes after which 200 ml of distilled water was added.
3. To the mixture, 10 ml of orthophosphoric acid, 0.5 gm of NaF and 1 ml diphenylamine indicator was added.
4. The contents were ultimately titrated with freshly prepared 0.5 N ferrous ammonium sulphate till the end-point is observed from blue-violet to green. A blank was also run without soil sample.

Calculations

$$\text{Organic carbon (\%)} = \frac{10\ (B-T) \times 0.003 \times 100}{B \times \text{Wt. of soil (gm)}}$$

Where, B is the volume of ferrous ammonium sulphate solution required for blank titration and T is the volume of ferrous ammonium sulphate solution required for soil sample titration.

3.1.4 Total Nitrogen

Total nitrogen was estimated as per the Kjeldahl method given by Piper (1960).

Reagents

a) Concentrated H_2SO_4.
b) 0.02 N H_2SO_4.
c) Sulphuric-Salicylic acid: 1 gm salicylic acid mixed with 30 ml sulphuric acid.
d) Sodium thiosulphate.
e) 4% boric acid.
f) Mixed indicator. 0.066 gm of methyl red and 0.099 gm of bromocresol green dissolved in 100 ml of ethyl alcohol.
g) 50% NaOH.
h) Digestion mixture: 10 gm HgO, 5 gm $CuSO_4$ and 100 gm K_2SO_4 (2:1:20).

Procedure

1. Soil sample of 5 gm was mixed thoroughly with sulphuric-salicylic acid followed by 5 gm of sodium thiosulphate. Heating was carried out for 5 minutes followed by cooling and addition of 10 gm digestion mixture. The contents were mixed well in a Kjeldahl flask.
2. The flask was kept in the digestion chamber at 100°C for two hours.
3. The colour change was monitored from dark brown to greenish white after which the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15-20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator till the end-point colour changes from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 until the end point colour changed from green to pink.

Calculations

$$\text{Total N \%} = \frac{(T-B) \times \text{Normality of } H_2SO_4 \times 1.4 \times 300}{\text{Weight of sample}}$$

where, T is the titre value for sample and B is for blank.

3.1.5 Available Nitrogen

Available nitrogen was estimated as per the method given by Subbiah and Asija (1956).

Reagents

a) 0.32% Potassium per manganate: 3.2 gm of $KMnO_4$ dissolved in water and final volume made up to 1 L.
b) 2.5% NaOH: 25 gm of sodium hydroxide pellets dissolved in water and volume made up to 1 L.
c) 2% boric acid: 20 gm of boric acid powder dissolved in warm H_2O by stirring and diluted to 1 L.

d) Mixed indicator: 0.066 gm of methyl red and 0.099 gm of bromocresol green dissolved in 100 ml of ethyl alcohol. 20 ml of the mixed indicator added to each litre of 2% boric acid solution and final pH adjusted to 4.5 with dilute HCl or dilute NaOH.

e) 0.1N potassium hydrogen pthalate: Dissolved 20.422 gm of the salt in water and dilute to 1 L.

f) 0.1N NaOH: 4gm of NaOH dissolved in water and diluted to 1 L, standardized against 0.1 N potassium hydrogen pthalate solution.

g) 0.02 N H_2SO_4: 0.1 N H_2SO_4 prepared by adding 2.8 ml of concentrated H_2SO_4 to about 990 ml of distilled water. From this 0.02 N H_2SO_4 made by diluting a suitable volume five times with distilled water and standardized against 0.1 N NaOH solution.

Procedure

1. Weighed 5 gm of soil sample and placed in 800 ml Kjeldahl flask.
2. The soil was moistened with 10 ml distilled water and any adhering soil on the neck was washed down followed by addition of 100 ml of 0.32% $KMnO_4$. Glass beads measuring 0.4 mm were added to prevent bumping.
3. 20 ml of 2% boric acid containing mixed indicator was measured in a 250 ml conical flask and placed under the receiver tube. The receiver tube end was dipped in the boric acid.
4. Tap water was allowed to run into the condenser for cooling.
5. 100 ml of 2.5 % NaOH solution was added and the rubber stopper was quickly fitted in the alkali trap.
6. The heaters were switched on to continue distillation until about 100 ml of distillate was collected.
7. The conical flask containing distillate was removed before switching off the heater to avoid back suction.
8. The distillate was titrated against 0.02 N H_2SO_4 in a burette until a pink colour started appearing. A blank was run without soil.

Calculations

Available N in sample in ppm = (X) x 0.00028 x 100/5 where X stands for the titre value of 0.02 N H_2SO_4 consumed

3.1.6 Available phosphorus

Available phosphorus in the alkaline soil was estimated as per the method given by Olsen *et al.*, (1954).

Reagents

a) 0.5 M $NaHCO_3$ extracting solution: 84 gm of sodium bicarbonate was added in distilled water and the volume was made up to 2 L. The pH was adjusted to 8.5 with 1 M or 1 N NaOH.
b) Reagent A: 12.0 gm of ammonium molybdate in 250 ml distilled water and 0.2908 gm of antimony potassium tartrate in 100 ml distilled water was added to 1000 ml of 2.5 M H_2SO_4, mixed thoroughly and volume made upto 2 L. with distilled water.
c) Reagent B (freshly prepared): 1.058 gm of ascorbic acid in 200 ml of reagent A and mixed.
d) Stock Standard P solution (50 ppm P): 0.2917 gm KH_2PO_4 dissolved in water to a final volume of 1 L.
e) Working Standard P solution (1 ppm): 20 ml of (50 ppm P) solution diluted to 1 L.

Procedure

1. Soil sample (2.5 gm) was placed in a 100 ml Erlenmeyer flask followed by the addition of 50 ml extracting solution.
2. The solution was kept on a shaker for 30 minutes and filtered through Whatman No. 42 filter paper.
3. 10 ml aliquot of the filtrate was transferred to a 100 ml beaker followed by addition of 1 ml of 2.5 M H_2SO_4, 15.5 ml of distilled water, 8 ml of Reagent B and another 15.5 ml of distilled water.
4. A blank was prepared as above. For the standard curve: 0, 2, 5, 10, 15 and 20 ml of standard solution was placed in 50 ml volumetric flasks separately. 10 ml of extracting solution , 1.0 ml of 2.5 M H_2SO_4, 8 ml Reagent B was added and the final volume was made upto 50 ml. The P concentrations of these solutions were 0.04, 0.1, 0.2, 0.3 and 0.4 ppm respectively. After 10 minutes, the P concentration was read at 882 nm.

Calculations

P in sample (mg kg^{-1}) = P in extract (mg l^{-1}) x 20 (the standard sample to solution ratio)

3.2 Isolation and screening of heterocystous cyanobacteria from paddy fields

Soil samples were collected from paddy fields from nearby region of Patiala district for the isolation of nitrogen fixing blue green algae. Soil of 1 gm was inoculated in 25 ml sterilized BG-11 medium (-N) in 100 ml conical flasks and incubated under optimal growth conditions at 28±2ºC temperature and 2500-3000 lux light intensity provided by cool day light fluorescent lamp for 21 days with 16:8 light/dark cycle. Flasks were observed daily for algal growth, after 12-15 days visible growth were observed then 6-7 wet mounts from each flasks were prepared by lifting the algal growth from surface of the soil, water and wall of the soil was lifted and suspended in 5 ml sterilized distilled water in test tube, shaken vigorously to make a homogenous suspension. 0.5 ml of this suspension was seeded on an agar plate with help of a sterilized pipette. The plate was observed regularly and isolated colonies were picked up and examined under microscope for morphological studies. These unialgal cultures were picked up from the plate and transferred to agar slant.

3.2.1 Culture Methods

Four cyanobacterial strains viz. *Anabaena variabilis* (ARM 441), *Aulosira fertilissima* (ARM 444), *Nostoc muscorum* (ARM 442) and *Tolypothrix tenuis* (ARM 443) were procured from National Centre For Conservation and Utilisation of Blue Green Algae, Division of Microbiology, IARI, New Delhi. The cyanobacterial cultures were maintained and grown routinely in batch culture in BG-11 medium (Stanier *et.al*, 1971). BG-11medium was prepared using the double distilled water, pH was kept in range of 7.0 – 7.27 for the optimal growth of cultures. Growth media were sterilized in autoclave at 121˚C at 15 psi (1.06 kg/m^2 pressure) for 20 minutes. The glasswares were sterilized in hot air oven at 180˚C for 1-2 hours.

Composition of BG-11 medium (-N)

CONSTITUENTS	gm/L
1. K_2HPO_4	0.04
2. $CaCl_2.H_2O$	0.036
3. Citric acid	0.006
4. Ferric ammonium citrate	0.006
5. EDTA (di sodium magnesium salt)	0.001
6. Sodium carbonate	0.02
7. Trace metal mix	1 ml

Trace metal mix composition

The trace metal A5 solution (Arnon, 1938) contained the following constituents in gm/L

CONSTITUENTS	gm/L
1. H_3BO_3	2.86
2. $MnCl_2.4H_2O$	1.18
3. $ZnSO_4.7H_2O$	0.222
4. $Na_2MoO_4.2H_2O$	0.39
5. $CuSO_4.5H_2O$	0.079
6. $Co(NO_3)_2.6H_2O$	0.0494

This composition was for BG-11 media for heterocyst that grow in nitrogen free media whereas for non-heterocyst algal inoculants nitrogen supplement was added by Sodium nitrate ($NaNO_3$).

3.3 GROWTH STUDIES

Growth studies of different heterocystous cyanobacterial strains in BG-11 medium (-N) were done by estimation of biomass

3.3.1 Dry biomass estimation (Richmond and Gobbelaar, 1986)

a) The Whatman filter was soaked in water to saturate and dried overnight.
b) The weight of the dried filter paper was noted as the initial reading.
c) Took some beads (4-5 mm) in beaker and dried them in oven.
d) The cultures were homogenized by vigorous shaking by adding dried beads to it and 100 ml of culture was taken and filtered through previously dried paper using vacuum filtration assembly. Noted the wet weight.

e) This was kept for drying and transferred to hot air oven maintained at about 60ºC, till constant weight was recorded at room temperature.

f) The difference between initial and final reading of the weight gave the dry biomass in mg/ml.

3.3.2 Chlorophyll estimation (Mckiney, 1941)

a) Algal suspension of 10 ml was filtered through Whatman filter paper no.42 and washed with sterile double distilled water.

b) Algal biomass along with filter paper was transferred to oak ridge centrifuge tubes and the level of methanol was marked on the oak ridge centrifuge tubes.

c) The oak ridge centrifuge tubes were tightly packed, vigorously shaken and kept in water bath at 60^{0}C for 30 min, which led to extraction of chlorophyll into the solution.

d) Samples were removed from the water bath and allowed to cool at room temperature, made the volume again to 10 ml by adding 96% methanol and centrifuged at 8000 rpm for 10 minute.

e) Pigment of solution was analysed using spectrophotometer (in terms of O.D) by comparing a sample of unknown transmission against a blank (96% methanol alone) of 100% transmission at 650 nm and 665 nm.

Calculations

Total chlorophyll= $2.55\times10^{-2}.E_{650} + 0.4 \times10^{-2}. E_{665}$ mg/ml

Where, E_{650} = Absorbance at 650 nm wavelength

E_{665}= Absorbance at 665 nm wavelength

3.3.3 Total nitrogen estimation (Kjeldahl, 1960)

Reagents

a) Catalyst mixture: For algae- 10 gm anhydrous sodium sulphate + 1 gm $CuSO_4.5H_2O$ mixed in the ratio 10:1
b) Mixed indicator: Dissolve 0.5 gm of Bromophenol green and 0.1 gm of methyl red indicator in 100 ml of ethanol.
c) Boric acid solution: Dissolve 40 gm H_3BO_3 in 1 L. of distilled water; add 5 ml of indicator per litre of Boric acid solution.
d) NaOH solution- 40% of NaOH solution. This solution is allowed to stand for 24-48 hrs as to precipitate out Na $(CO_3)_2$ impurities.
e) Conc. H_2SO_4- Specific gravity 1.84
f) Sodium thiosulphate
g) 0.01 N H_2SO_4

Procedure

1. Algal sample (50 ml) was placed in 500 ml Kjeldahl digestion tube.
2. Added 25 ml conc. H_2SO_4 (allowed to stand for 30 min).
3. To the above mixture 5 gm of sodium thiosulphate was added and allowed to stand for 30 min. After that 2-3 gm catalyst mixture was added into the flask and glass beads were added to prevent bumping. First heated slowly till the frothing continue and after that heat it briskly. Digestion was continued for one hour until the digest get clear.
4. Placed this flask with material for automatic distillation by Kjeldahl unit.
5. To this solution or digest 150 ml distilled H_2O was added, cooled and again added 120 ml of 40% NaOH along the sides of Kjeldahl flask and connected the distillation system. Collected the ammonia evolved in 25 ml boric acid in 250 ml flask (to which mixed indicator has already been added) continue till 150 ml distillate was collected.
6. This was followed by titration against 0.01 N sulphuric acid.
7. Algal sample (50 ml) was placed in 500 ml Kjeldahl digestion tube.
8. Added 25 ml conc. H_2SO_4 (allowed to stand for 30 min).
9. To the above mixture 5 gm of sodium thiosulphate was added and allowed to stand for 30 min. After that 2-3 gm catalyst mixture was added into the flask and glass beads were added to prevent bumping. First heated slowly till the

frothing continue and after that heat it briskly. Digestion was continued for one hour until the digest get clear.

10. Placed this flask with material for automatic distillation by Kjeldahl unit.
11. To this solution or digest 150 ml distilled H_2O was added, cooled and again added 120 ml of 40% NaOH along the sides of Kjeldahl flask and connected the distillation system. Collected the ammonia evolved in 25 ml boric acid in 250 ml flask (to which mixed indicator has already been added) continue till 150 ml distillate was collected.
12. This was followed by titration against 0.01 N sulphuric acid.

Calculations

Amount (gm.) of N_2 in samples = (ml of acid for sample – ml of acid for black) × Normality of acid × 14×10^{-3}

% of N_2 in samples = Amount of N_2 in samples ×100/ sample weight in grams.

3.3.4 Microscopic determination of heterocyst frequency of cyanobacteria

Procedure

1. Fixed the material in Lugol's iodine solution (dissolved 5 gm iodine in 100 ml of 10% potassium iodide solution)
2. Observed it at 100X magnification using oil emersion under the microscope.
3. Counted the heterocyst as well as vegetative cells in triplicate.
4. Expressed the heterocyst abundance as percentage over total population of the cells.

Calculation

$$\text{Heterocyst frequency (\%)} = \frac{\text{Total no. of heterocyst}}{\text{Total no. of cells}} \times 100$$

3.3.5 Mass Cultivation

i. Small Scale Production (lab scale)

BG-11 medium (100 ml) was inoculated in 2 L flasks with different cultures and incubated at 28±2˚C under discontinuous illumination at 16h: 8h light/dark cycle at 2500-3000 lux light intensity. After observing adequate growth, the flasks were transferred to the glasshouse. For the large scale production of algal biomass, algal cultures grown in small scale were transferred from flasks and tubs to the algal ponds for mass production.

ii. **Mass cultivation of blue green algae**

a) The production was carried out in algal ponds in polyhouse at Building Centre (Multiplication unit). Each algal pond before inoculation was cleaned and dried properly and then inoculated with the mixture of different algal cultures
b) Each algal pond was filled with tap water upto a height of 18 – 20 cm and 10 gm dipotassium hydrogen phosphate (K_2HPO_4) and 10 gm magnesium sulphate hepta hydrate ($MgSO_4.7H_2O$) was added.
c) Starter culture, a mixture of *Tolypothrix tenuis* (ARM 444), *Nostoc muscorum* (ARM 442), *Anabaena variabilis* (ARM 441), *Aulosira fertilissima* (ARM 444) was inoculated in each multiplication unit.
d) Malathion (5-10 ml per pond) or Carbofuran (3% granules, 20 gm. per pond), was added to prevent insect breeding.

3.3.6 Harvesting of algal biomass after mass cultivation

Under favourable conditions (temperature 32 - 38º C), the growth of blue-green algae was rapid and a thick algal mat was formed on the surface of the water in about 10-15 days. During this period, water was added periodically to maintain the water level around 10 cm. The biomass harvested was mixed with equal amount of sterilized soil (w/w), mixed well and dried in sun.

3.4 Determination of end point growth of nitrogen fixing heterocystous cyanobacteria

Procedure

1. Inoculation of different algal cultures was done in 50 ml BG11 (-N) media in 150 ml flasks in triplicate.
2. Incubated the cultures for 21 days at 28±2˚C under discontinuous illumination at 16h: 8h light/dark cycle at 2500-3000 lux light intensity for adequate growth of cultures.
3. After the completion of incubation period, homogenized the cultures by shaking with dried glass beads.

4. Different set of cultures were then used for estimation of dry biomass, chlorophyll and total nitrogen as described in previous section 3.3.1, 3.3.2 and 3.3.3

3.5 Plasmid Isolation (Goyal, 1992)

Reagents

a) GTE buffer: 50 mM Glucose, 25 mM Tris HCl, 10 mM EDTA
b) Lysozyme: stock concentration 100 mg/ml, working concentration 5 mg/ml
c) NaCl: Stock concentration 5 M, working concentration 1 M
d) Tris-saturated phenol
e) Chloroform: isoamyl- alcohol (24:1,v/v)
f) Ethanol 95% and 70%
g) Agarose 0.8%
h) 5X TBE buffer (500 ml): Tris-HCl 27 gm, Boric acid 13.7 gm, EDTA (0.5 M) 10 ml having pH 8.3
i) EtBr 10 µl
j) TE buffer: 10 mM Tris, 1 mM EDTA, pH 8
k) 6× gel loading buffer i.e. bromophenol blue (3 µl)

Procedure

1. Algal cells from 100-200 ml of exponentially growing cultures (14 days old) cultures were harvested by centrifugation at 8000 rpm, 10 min at 4°C.
2. Discard the supernatant and washed the pellet with 200 µl GTE buffer (glucose, 50 mM; Tris-HCl, 25 mM; EDTA, 10 mM), pH 8 and vortex and centrifuged it at 8000 rpm, 10 min at 4°C.
3. Again suspended the pellet in the same buffer (500 µl) containing lysozyme (5 mg/ml) and kept at 37°C for 1 hour.
4. After this ¼ of the initial volume (200 µl) of freshly prepared solution II (10% SDS in 0.2N NaOH) was added and kept at 37°C for 15-30 min in water bath.
5. Then, 5 M NaCl was added to a final concentration of 1 M (i.e. 140 µl of 5 M) and kept overnight at 4°C.

6. The cell debris was removed by centrifugation at 14000 rpm, 30 min at 4^{o}C and the supernatant was extracted twice with tris saturated phenol (i.e. added equal vol. of tris saturated phenol in supernatant) and centrifuged at 8000 rpm, 10 min and 4^{o}C.
7. Supernatant was extracted twice with chloroform: isoamyl alcohol (24:1, v/v) and centrifuged at 8000 rpm, 10 min and 4^{o}C.
8. To the clear aqueous phase, 2 vols. of cold ethanol (95%) were added and kept overnight at -20^{o}C.
9. The precipitated DNA was recovered by centrifugation at 10000 rpm at 4^{o}C for 10 min.
10. Ethanol was then drained off completely from the tube. The pellet of DNA was washed with 70% ethanol, vortexed it and centrifuged at 8000 rpm at 4^{o}C for 10 min, discard supernatant and dried at room temperature for 2 hours.
11. Suspend it in 150 μl of TE buffer, pH 7.5 and store at 4^{o}C.
12. The DNA samples were analysed by agarose gel electrophoresis.
13. 0.8% of agarose gel was made in 0.5 X TBE buffer having 10 μl EDTA.
14. The samples (5 μl) were mixed with 6× gel loading buffer i.e. bromophenol blue (3 μl) and autoclaved distilled water (3 μl) and run the ladder (5 μl)
15. Electrophoresis was carried out at room temperature for 2-3 hours or until the dye reached the bottom of the gel.
16. Fluorescent DNA-EtBr complexes were then observed in transmitted UV light and photographed through gel doc.

4.1 Physico-chemical analysis of soil samples from paddy field of different farmers in Patiala District

Physico-chemical properties like pH, EC, total organic carbon, total nitrogen, available nitrogen and phosphorus are indicators of soil quality in understanding the nutrient status of soil and therefore were examined to study the soil fertility status.

1) **pH of soil samples collected from different paddy fields**

Soil Sample	**pH**			
	R1	**R2**	**R3**	**Mean ±SE**
1	7.95	7.50	7.56	7.7±0.14
2	8.02	7.98	7.99	8.0±0.01
3	7.38	8.10	8.40	8.0±0.30
4	7.56	7.26	7.45	7.4±0.009
5	8.49	7.89	8.00	8.1±0.18
6	7.50	7.00	7.20	7.2±0.15
7	7.79	7.90	7.60	7.8±0.09
8	7.21	8.00	8.20	7.8±0.30
9	8.64	8.20	8.00	8.3±0.19
10	7.87	7.10	7.50	7.5±0.22
11	7.02	7.26	7.10	7.1±0.07
12	7.81	7.36	7.50	7.6±0.13
13	8.39	8.00	8.10	8.2±0.11
14	8.51	7.80	8.30	8.2±0.21
15	7.48	7.50	7.30	7.4±0.06
16	7.90	8.25	8.15	8.1±0.10
17	8.48	7.90	8.20	8.2±0.17

Table 1: pH of soil samples collected from different paddy fields

The soil was alkaline in nature and its pH varies from 7.12±0.07 to 8.28±0.19 (Table1)

2) EC (µS) of soil samples collected from paddy field

Soil Sample	**EC (µS)**			
	R1	**R2**	**R3**	**Mean ±SE**
1	106.0	108.0	116.0	110±3.1
2	127.9	133.5	137.0	133±2.7
3	101.0	106.0	105.8	104±1.6
4	120.4	124.3	126.0	124±1.7
5	124.2	125.6	128.5	126±1.3
6	164.0	161.0	158.0	161±1.7
7	100.0	100.0	100.1	100±0.03
8	100.1	105.1	100.6	102±1.6
9	288.0	281.0	278.0	282±3.0
10	119.0	109.8	117.0	115±2.8
11	150.0	153.0	149.0	151±1.2
12	119.1	117.6	125.6	121±2.5
13	105.0	101.0	107.0	104±1.8
14	117.0	127.0	122.6	122±2.9
15	187.0	190.2	198.0	192±3.3
16	121.1	113.0	109.0	114±3.6
17	242.0	253.0	243.0	246±3.5

Table 2: Electrical conductivity (µS) of different soil samples of paddy field

Increase in electrical conductivity of soil, increases the availability of soluble salts (Pitchel and Hayes, 1990). The total soluble salt content of these soils expressed as electrical conductivity (EC) varied from 100±0.03 to 282±2.96 (µS) (Table 2). The mean EC of soil sample was found to be 141.58 (µS).

3) Organic carbon (%) of soil samples collected from paddy field

Soil Sample	Organic carbon (%)			
	R1	**R2**	**R3**	**Mean ±SE**
1	0.75	0.73	0.71	0.73±0.01
2	0.64	0.60	0.59	0.61±0.015
3	0.63	0.61	0.65	0.63±0.01
4	0.89	0.83	0.85	0.85±0.02
5	0.73	0.69	0.65	0.69±0.02
6	0.71	0.73	0.69	0.71±0.01
7	0.64	0.68	0.60	0.64±0.02
8	0.48	0.49	0.49	0.49±0.003
9	0.41	0.43	0.41	0.42±0.01
10	0.64	0.63	0.66	0.64±.008
11	0.61	0.65	0.63	0.63±0.012
12	0.55	0.57	0.58	0.56±0.008
13	0.695	0.71	0.70	0.70±0.004
14	0.45	0.48	0.46	0.46±.009
15	0.57	0.58	0.56	0.57±0.006
16	0.45	0.47	0.48	0.47±0.009
17	0.36	0.37	0.38	0.37±0.006

Table 3: Organic carbon (%) of different soil samples of paddy field

The organic carbon content (%) of soil sample varied from 0.37±0.006 to 0.85±0.02 with the mean value of 0.6% (Table 3). The distribution of soil samples with respect to organic carbon content indicates that 17% of soil sample fall in organic carbon range of 0.3 to 0.5%, 53% of soil sample having 0.5 to 0.7% organic carbon content whereas only 17% soil sample has more than 0.7% content of organic carbon. Soil from paddy field had high range of organic carbon content which is also an indicator of available nitrogen status of soil.

4) **Total nitrogen (%) of soil samples collected from paddy field**

Soil Sample	Total nitrogen (%)			
	R1	**R2**	**R3**	**Mean ±SE**
1	0.089	0.086	0.085	0.087±0.001
2	0.067	0.072	0.063	0.067±0.003
3	0.064	0.059	0.060	0.061±0.002
4	0.074	0.078	0.075	0.076±0.001
5	0.062	0.069	0.059	0.063±0.003
6	0.068	0.061	0.069	0.066±0.003
7	0.035	0.030	0.039	0.035±0.003
8	0.074	0.079	0.075	0.076±0.002
9	0.084	0.086	0.082	0.084±0.001
10	0.052	0.049	0.059	0.053±0.003
11	0.066	0.069	0.062	0.066±0.002
12	0.030	0.035	0.039	0.035±0.003
13	0.080	0.085	0.083	0.083±0.001
14	0.064	0.070	0.060	0.065±0.003
15	0.072	0.078	0.070	0.073±0.002
16	0.084	0.079	0.084	0.082±0.002
17	0.045	0.050	0.049	0.048±0.002

Table 4: Total nitrogen (%) of different soil samples of paddy field

Total nitrogen content (%) of soil samples collected from paddy fields varied from 0.035±0.003 to 0.087±0.001 (Table 4). Distribution of soil samples with respect to total nitrogen content showed that only 5% of soil sample fall in 0.005 to 0.01% of total nitrogen range, 11% soil sample nitrogen in range of 0.01-0.05% whereas most of the soil sample i.e. 76% had nitrogen in range of 0.05-0.1%. Overall soil sample of paddy field were rich in total nitrogen content.

5) Available nitrogen (ppm) of soil samples collected from paddy field

Soil Sample	Available nitrogen (ppm*)			
	R1	**R2**	**R3**	**Mean ±SE**
1	5.6	4.2	2.7	4.2±0.84
2	7.7	5.5	2.3	5.2±1.6
3	6.5	6.8	6.0	6.4±0.23
4	6.1	5.9	3.2	5.1±0.94
5	5.1	3.9	2.2	3.7±0.84
6	6.0	4.9	3.0	4.6±0.88
7	1.9	1.8	1.7	1.8±0.06
8	3.5	3.0	2.2	2.9±0.38
9	2.7	2.5	2.3	2.5±0.12
10	5.0	4.9	4.1	4.7±0.28
11	4.4	4.3	4.0	4.2±0.12
12	6.0	4.5	2.3	4.3±1.07
13	3.5	2.8	2.2	2.8±0.38
14	7.8	7.5	7.0	7.4±0.23
15	4.3	4.8	4.5	4.5±0.15
16	5.9	5.1	5.5	5.5±0.23
17	5.0	4.9	5.52	5.1±0.19

ppm* = mg/kg

Table 5: Available nitrogen (in ppm) of different soil samples of paddy field

Available nitrogen content of soil samples of paddy field of Patiala district ranged from 1.8±0.06 to 7.4±0.23 which showed that soil is rich in available nitrogen content (Table 5). 24% of soil sample had available nitrogen from 1.5 to 3 ppm, 29% had 3-4.5 ppm, 41% had 4.5 to 6 ppm and only 12% of soil sample having available nitrogen content above 6 ppm.

6) Available phosphorus (ppm) of soil samples collected from paddy field

Soil Sample	Available phosphorus (ppm*)			
	R1	**R2**	**R3**	**Mean ±SE**
1	7.8	13.2	5.2	8.7±2.4
2	4.0	3.5	2.0	3.2±0.6
3	2.1	4.0	2.2	2.8±0.62
4	19.5	20.0	18.9	19.5±0.32
5	5.0	5.6	2.9	4.5±0.82
6	7.1	11.2	6.3	8.2±1.52
7	4.5	7.8	4.1	5.5±1.17
8	3.8	7.0	3.7	4.8±1.1
9	6.5	10.6	6.0	7.7±1.46
10	4.2	7.6	4.0	5.3±1.17
11	5.5	9.1	4.9	6.5±1.3
12	2.0	4.2	2.1	2.8±0.72
13	8.5	14.0	7.0	9.8±2.12
14	6.2	10.8	6.0	7.7±1.57
15	7.8	13.2	5.3	8.8±2.3
16	5.0	9.0	4.3	6.1±1.5
17	2.1	4.5	2.2	2.9±0.79

ppm*= mg/kg

Table 6: Available phosphorus (ppm) of different soil samples of paddy field

Available phosphorus content of soil samples of paddy field of Patiala district ranged from 2.8±0.72 to 19.5±0.32. 35% of soil samples fall in range of 2.5 to 5 ppm, 24% fall in range of 5-7.5 ppm, 35% fall in range of 7.5 to 10 ppm whereas highest phosphorus content was found to be 19.5 ppm in sample 4 (Table 6).

Soil Sample	Comparison of physico-chemical analysis of soil samples collected from paddy fields					
Chemical analysis	pH	EC (µS)	Organic Carbon (%)	Total Nitrogen (%)	Available Nitrogen (ppm)	Available Phosphorus (ppm)
	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE
1	7.7±0.14	110±3.05	0.73±0.01	0.087±0.001	4.2±0.84	8.7±2.4
2	8.0±0.01	133±2.65	0.61±0.015	0.067±0.003	5.2±1.6	3.2±o.6
3	8.0±0.30	104±1.6	0.63±0.01	0.061±0.002	6.4±0.23	2.8±.62
4	7.4±0.09	124±1.7	0.85±0.02	0.076±0.001	5.1±0.94	19.5±0.32
5	8.1±0.18	126±1.3	0.69±0.02	0.063±0.003	3.7±0.84	4.5±0.82
6	7.2±0.15	161±1.7	0.71±0.01	0.066±0.003	4.6±0.88	8.2±1.52
7	7.8±0.09	100±0.03	0.64±0.02	0.035±0.003	1.8±0.06	5.5±1.17
8	7.8±0.30	102±1.6	0.49±0.003	0.076±0.002	2.9±0.38	4.8±1.1
9	8.3±0.19	282±2.96	0.42±0.01	0.084±0.001	2.5±0.12	7.7±1.46
10	7.5±0.22	115±2.8	0.64±.008	0.053±0.003	4.6±0.29	5.3±1.17
11	7.1±0.07	151±1.2	0.63±0.012	0.066±0.002	4.2±0.12	6.5±1.3
12	7.6±0.13	121±2.5	0.56±0.008	0.007±0.002	4.3±1.07	2.8±0.72
13	8.2±0.11	104±1.8	0.70±0.004	0.083±0.001	2.83±0.38	9.8±2.12
14	8.2±0.21	122±2.9	0.46±.009	0.065±0.003	7.4±0.23	7.7±1.57
15	7.4±0.06	192±3.3	0.57±0.006	0.073±0.002	4.5±0.15	8.8±2.3
16	8.1±0.10	114±3.6	0.47±0.009	0.082±0.002	5.5±0.23	6.1±1.5
17	8.2±0.17	246±3.5	0.37±0.006	0.048±0.002	5.1±0.19	2.9±0.79

Table 7: Comparison of physico-chemical analysis of soil samples collected from paddy fields

The physico-chemical properties of soil samples collected from paddy fields showed that soil pH was alkaline and ranging from 7.1±0.07 to 8.3±0.19, electrical conductivity varied from 100±0.03 to 282±2.96 (µS), organic carbon content (%) varied from 0.37±0.006 to 0.85±0.02, total nitrogen content varied from 0.007±0.002 to 0.087±0.001. Similarly, available nitrogen and phosphorus varied from 1.8±0.06 to 6.4±0.23 and 2.8±0.62 to 19.5±0.32 respectively (Table 7).

4.2 Isolation of heterocystous cyanobacteria from paddy fields

In the present work, five different types of blue green algae (Isolate 5, Isolate 6, Isolate 7, Isolate 8 and Isolate 9) were isolated from the soil samples collected from organic paddy fields of Patiala District, Punjab and were studied for their morphological characteristic under the microscope. The isolates were purified and made uni-algal by plating on BG-11 (-N) media and subjected to microscopic examination (Plate 3: Isolate 5, Isolate 6, Isolate 7, Isolate 8, Isolate 9) for identification and determination of heterocyst frequency. Isolate 2, 3 and 4 were taken from previous experimental studies done on "Growth of heterocystous blue green algae used for algalization in paddy cultivation".

4.3 Growth Studies of different heterocystous cyanobacterial strains

Growth studies of different strains of cyanobacteria were studied in BG-11 medium (-N) which are as follows

4.3.1 Dry Biomass

Dry biomass estimation (mg/ml) at different intervals of time				
S. No.	**Cyanobacterial strains**	**Mean ±SE (7 days incubation period)**	**Mean ±SE (14 days incubation period)**	**Mean ±SE (21 days incubation period)**
1	*Anabaena variabilis* (ARM 441)	0.05±0.01	0.11±0.02	0.32±0.06
2	*Aulosira fertilissima* (ARM 444)	0.08±0.02	0.17±0.02	0.67±0.09
3	*Nostoc muscorum* (ARM 442)	0.10±0.02	0.18±0.01	0.81±0.11
4	*Tolypothrix tenuis* (ARM 443)	0.14±0.06	0.27±0.06	1.70±0.11
5	Isolate 2	0.14±0.05	0.23±0.06	0.30±0.09
6	Isolate 3	0.20±0.02	0.32±0.05	0.99±0.03
7	Isolate 4	0.11±0.04	0.16±0.05	0.37±0.10

Table 8: Dry biomass estimation of heterocystous cyanobacterial strains at different intervals of time

Biomass was expressed on dry weight (mg/ml) basis (Table 8, Fig.1) which showed that the growth of all organisms followed an increasing trend with increase in time of incubation. Maximum dry biomass was observed in *Tolypothrix tenuis* (ARM 443) followed by Isolate 3 and *Nostoc muscorum* (ARM 442) showed comparatively better growth after *Tolypothrix tenuis* (ARM 443) among procured cultures.

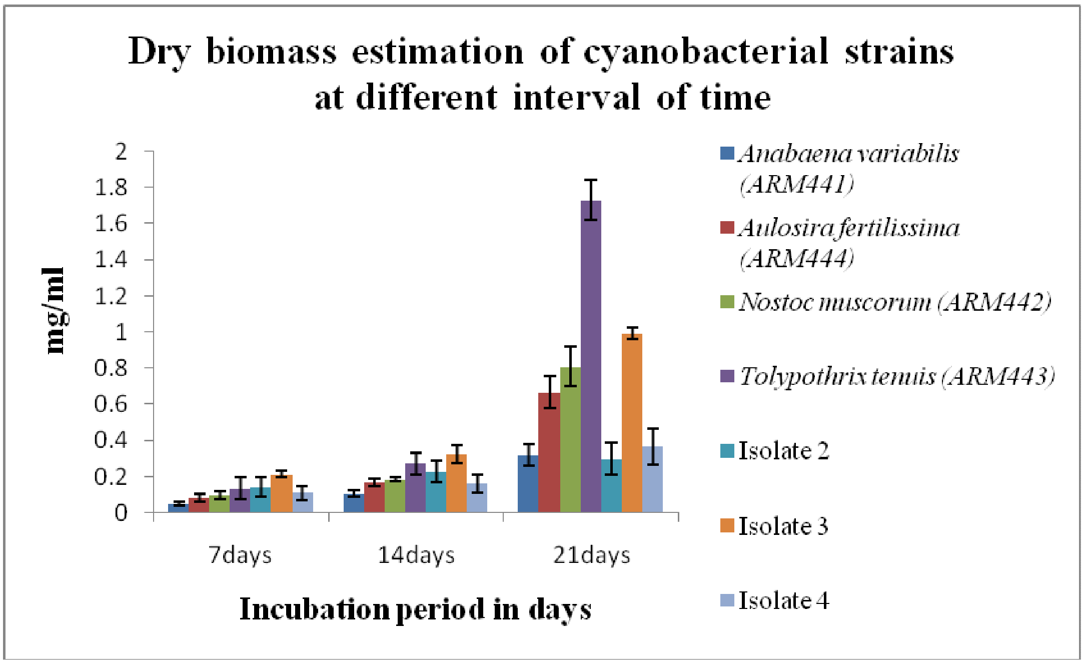


Fig 1: Dry biomass estimation of cyanobacterial strains at different interval of time

4.3.2 Total Nitrogen

Total nitrogen (%) estimation at different intervals of time				
S.no.	**Cyanobacterial Strains**	**Mean ±SE (7 days incubation period)**	**Mean ±SE (14 days incubation period)**	**Mean ±SE (21 days incubation period)**
1	*Anabaena variabilis* (ARM 441)	0.0043±0.0007	0.0075±0.0008	0.011±0.0008
2	*Aulosira fertilissima* (ARM 444)	0.0019±0.00037	0.004±0.00027	0.0073±0.0014
3	*Nostoc muscorum* (ARM 442)	0.0027±0.0012	0.0077±0.0007	0.0056±0.00083
4	*Tolypothrix tenuis* (ARM 443)	0.0012±0.0002	0.0013±0.0001	0.0051±0.00064
5	Isolate 2	0.002±0.0003	0.0031±0.0002	0.0045±0.0003
6	Isolate 3	0.0023±0.0003	0.0025±0.0003	0.004±0.00023
7	Isolate 4	0.0015±0.00024	0.0022±0.0003	0.0035±0.0005

Table 9: Total nitrogen (%) estimation of heterocystous cyanobacterial strains at different intervals of time

Total nitrogen was estimated in different cyanobacterial cultures which showed that the total nitrogen content (%) increased with increase in time (Table 9, Fig. 2). The total nitrogen content (%) was highest in *Anabaena variabilis* (0.011±0.0008) followed by *Aulosira fertilissima* (0.0073±0.0014), *Nostoc muscorum* (0.0056 ± 0.00083) and least in Isolate 4 (0.0035±0.0005).

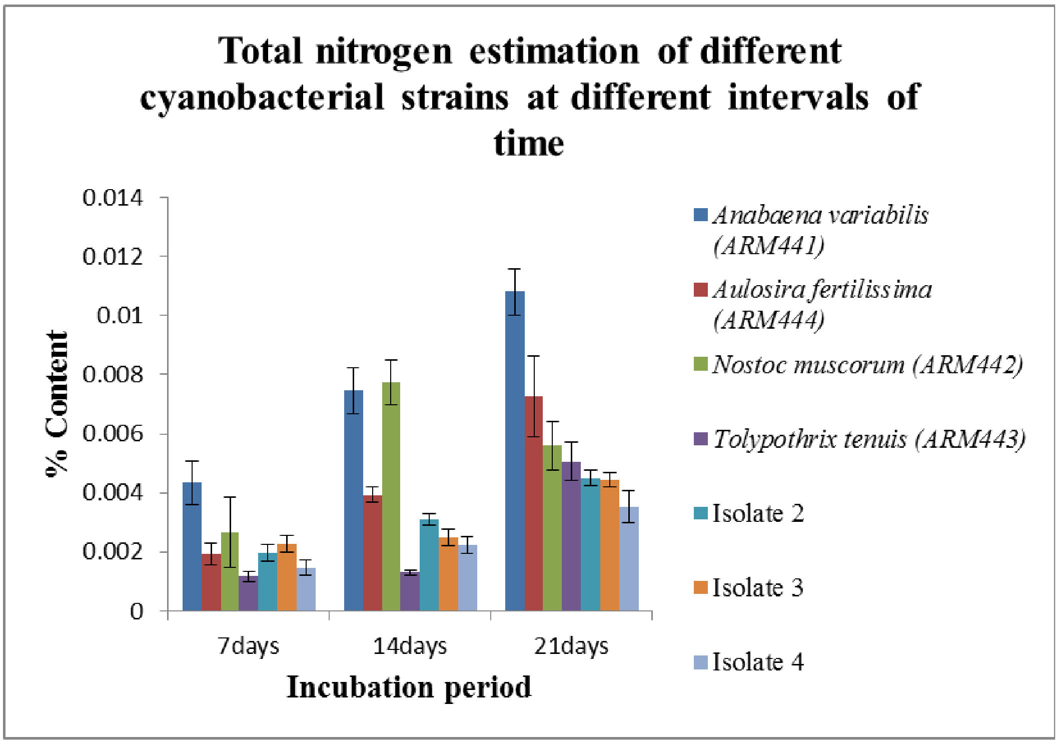


Fig 2: Total nitrogen (%) estimation of heterocystous cyanobacterial strains at different intervals of time

4.3.3 Mass cultivation

i. Small Scale Production (lab scale)

BG-11 (100ml) medium was inoculated with different cultures and incubated at 28±2˚C under discontinuous illumination at 16h: 8h light/dark cycle at 2500-3000 lux light intensity. After observing adequate growth, the flasks were transferred to the glass house.

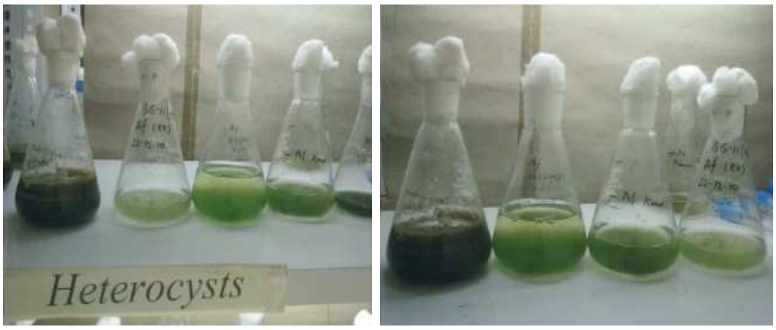


Fig 3: Algal cultures growing in racks

ii. Mass cultivation of BGA

For the large scale production of algal biomass, algal cultures grown in small scale were transferred from flasks and tubs to the algal ponds for mass production. The cultures isolated were mass produced in algal ponds in a polyhouse. Before inoculation 10 gm dipotassium hydrogen phosphate (K_2HPO_4) and 10 gm magnesium sulphate hepta hydrate ($MgSO_4.7H_2O$) was added.

Inoculation was done in the algal ponds for mass multiplication

Fig 4 a: Algal ponds before inoculation; Fig 4 b, c: Inoculation of algal ponds

Fig 5 : Mass multiplication of blue green algal inoculants in various ponds

Dimensions of Algal pond

Length of each pond = 7 feet 6 inches i.e.228.6 cm

Width of each pond = 3 feet 6 inches i.e. 106.68 cm

Depth of pond = 6 inches i.e. 15.24 cm

Area of each pond = 2.44 m^2

Volume of each pond ≈ 372 litres

4.3.4 Harvesting of algal biomass after mass cultivation

Thick mat of blue green algae was observed and harvesting was done after 21 days of inoculation by using nylon net having pore size of 1mm. Thick biomass was collected in the plastic tub and were kept for sun drying.

During harvesting, polyhouse temperature ranges from 38ºC to 35ºC, water temperature ranges from 20ºC to 22ºC and pH of water ranges from 7.5 to 8.8

Fig 6 : Harvesting of algal biomass after their mass cultivation i.e. after 21 days of inoculation

After 4-5 days of air drying with moisture content of 40-45% of the dry biomass was mixed with suitable carrier material and then packed in the packets @ 500 gm per packet that is used for 1 acre of paddy field.

4.3.5 Heterocyst frequency (%) of different cyanobacterial strains

Determination of heterocyst frequency is an important parameter for identification of nitrogen fixing cyanobacterial strains.

Heterocyst frequency (%)					
S.no.	**Cyanobacterial strains**	**R1**	**R2**	**R3**	**Mean ±SE**
1	*Anabaena variabilis* (ARM 441)	15.40	14.04	15.30	14.9±0.44
2	*Aulosira fertilissima* (ARM 444)	8.05	14.42	10.32	10.9±1.86
3	*Nostoc muscorum* (ARM 442)	10.14	8.87	11.78	10.3±0.84
4	*Tolypothrix tenuis* (ARM 443)	18.54	14.73	20.32	17.9±1.65
5	Isolate 5	13.33	16.95	12.28	14.2±1.4
6	Isolate 6	15.79	14.29	14.49	14.9±0.47
7	Isolate 7	10.98	11.25	10.25	10.8±0.3
8	Isolate 8	15.00	13.20	14.54	14.3±0.54
9	Isolate 9	9.84	12.73	12.30	11.6±0.9

Table 10: Heterocyst frequency (%) of different cyanobacterial strains

From the Table 10, Fig. 7 it was concluded that maximum heterocyst frequency was observed in *Tolypothrix tenuis* (ARM 443) i.e. 17.9±1.65 and minimum heterocyst frequency was observed in *Nostoc muscorum* (ARM 442) i.e. 10.3±0.84 and after *Tolypothrix tenuis* (ARM 442) heterocyst frequency of *Anabaena variabilis* (ARM 441) was found to be 14.9±0.44 and average heterocystous frequency in all 4 strains was 13.5%.

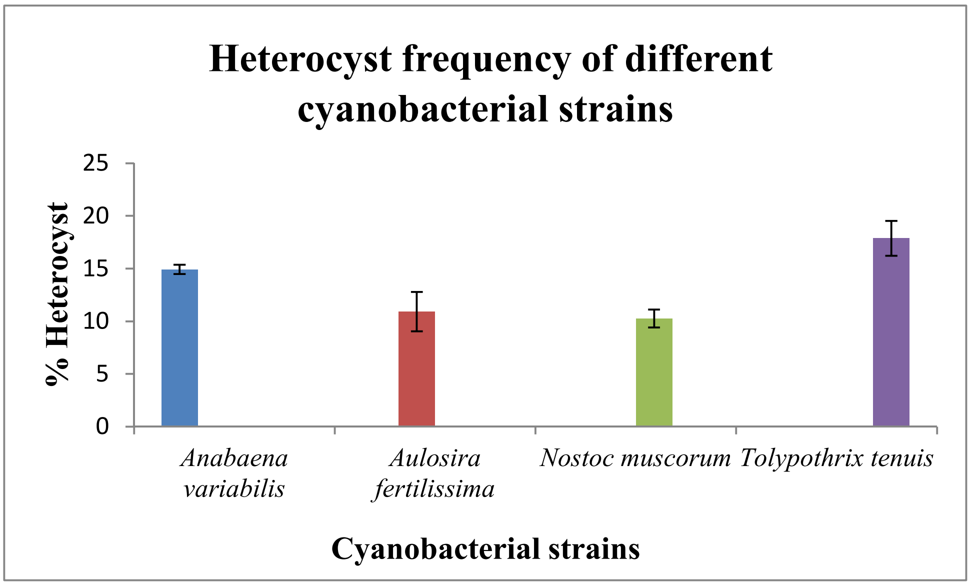


Fig 7: Heterocyst frequency (%) of different cyanobacterial strains

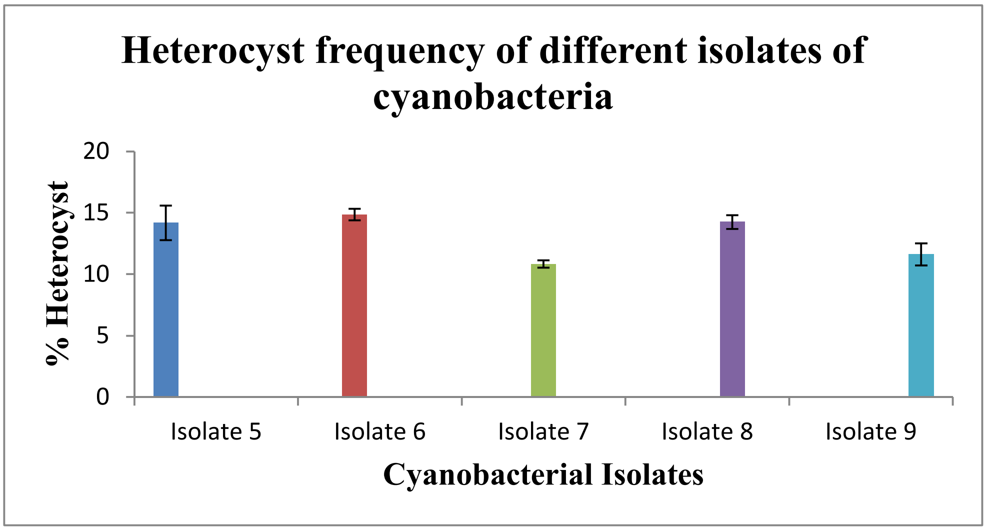


Fig 8: Heterocyst frequency of different isolates of cyanobacteria

Out of 5 isolates of heterocystous cyanobacteria, maximum heterocyst frequency was observed in Isolate 6 i.e. 14.9±0.47 followed by Isolate 8 i.e. 14.3±0.54 whereas minimum heterocyst frequency was observed in Isolate 7 i.e. 10.8±0.3 and average heterocystous frequency in all these 5 isolates was 13.2% (Table 10, Fig. 8)

4.4 End point growth of cyanobacteria

4.4.1 Dry biomass estimation (mg/ml) of heterocystous cyanobacterial strains after 21 days of incubation

Dry biomass estimation (mg/ml)					
S.no.	**Cyanobacterial strains**	**R1**	**R2**	**R3**	**Mean ±SE**
1	*Anabaena variabilis* (ARM 441)	0.36	0.20	0.40	0.32±0.06
2	*Aulosira fertilissima* (ARM 444)	0.50	0.80	0.70	0.67±0.09
3	*Nostoc muscorum* (ARM 442)	0.40	0.84	0.98	0.74±0.17
4	*Tolypothrix tenuis* (ARM 443)	0.83	1.00	0.95	0.92±0.11
5	Isolate 2	1.02	0.85	0.93	0.93±0.09
6	Isolate 3	0.30	0.22	0.25	0.25±0.03
7	Isolate 4	0.56	0.24	0.30	0.37±0.1

Table 11: Dry biomass estimation (mg/ml) of heterocystous cyanobacterial strains after 21 days of incubation

Biomass was expressed on dry weight (mg/ml) basis. The end point biomass i.e. maximum growth in different heterocystous strains after 21 days of inoculation was observed (Table 11, Fig 9) and it was concluded that maximum biomass was obtained in *Tolypothrix tenuis* (ARM 443) i.e. 0.92±0.11 and minimum was observed in Isolate 3 i.e. 0.05±0.03 and the average biomass production in all cyanobacterial strains were found to be 0.55 mg/ml.

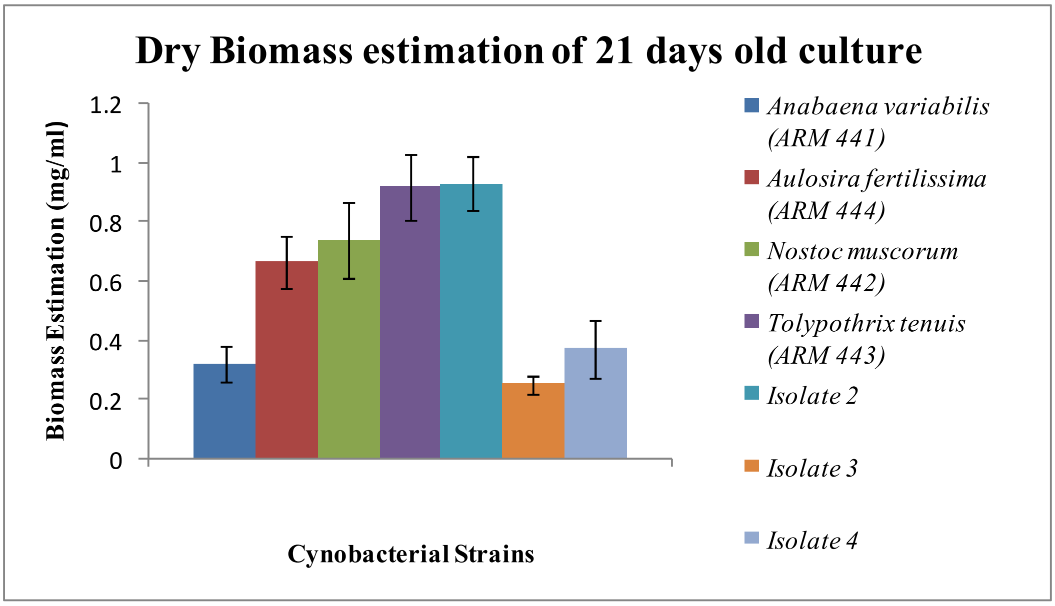


Fig 9: Dry biomass estimation (mg/ml) of heterocystous cyanobacterial strains after 21 days of incubation

4.4.2 Chlorophyll estimation (µg/ml) of heterocystous cyanobacterial strains after 21 days of incubation

Chlorophyll estimation (µg/ml)					
S.no.	**Cyanobacterial strains**	**R1**	**R2**	**R3**	**Mean ±SE**
1	*Anabaena variabilis* (ARM 441)	2.8	3.5	3.0	3.1±0.21
2	*Aulosira fertilissima* (ARM 444)	9.26	8.78	9.88	9.3±0.32
3	*Nostoc muscorum* (ARM 442)	2.7	3.8	3.0	3.2±0.33
4	*Tolypothrix tenuis* (ARM 443)	1.45	1.50	1.20	1.4 ±0.09
5	Isolate 2	1.90	2.00	1.85	1.9±0.04
6	Isolate3	3.2	3.5	4.0	3.6±0.23
7	Isolate 4	2.8	2.5	3.3	2.9±0.23

Table 12: Chlorophyll estimation (µg/ml) of heterocystous cyanobacterial strains after 21 days of incubation

Chlorophyll expressed in µg/ml. (Table 12, Fig 10) at the end point growth in different heterocystous strains after 21 days of inoculation was observed. Maximum was in *Aulosira fertilissima* (ARM 444) i.e. 9.3±0.32 whereas minimum was observed in *Tolypothrix tenuis* (ARM 443) i.e. 1.4 ±0.09 and the average chlorophyll content in all cyanobacterial strains was 3.36 µg/ml.

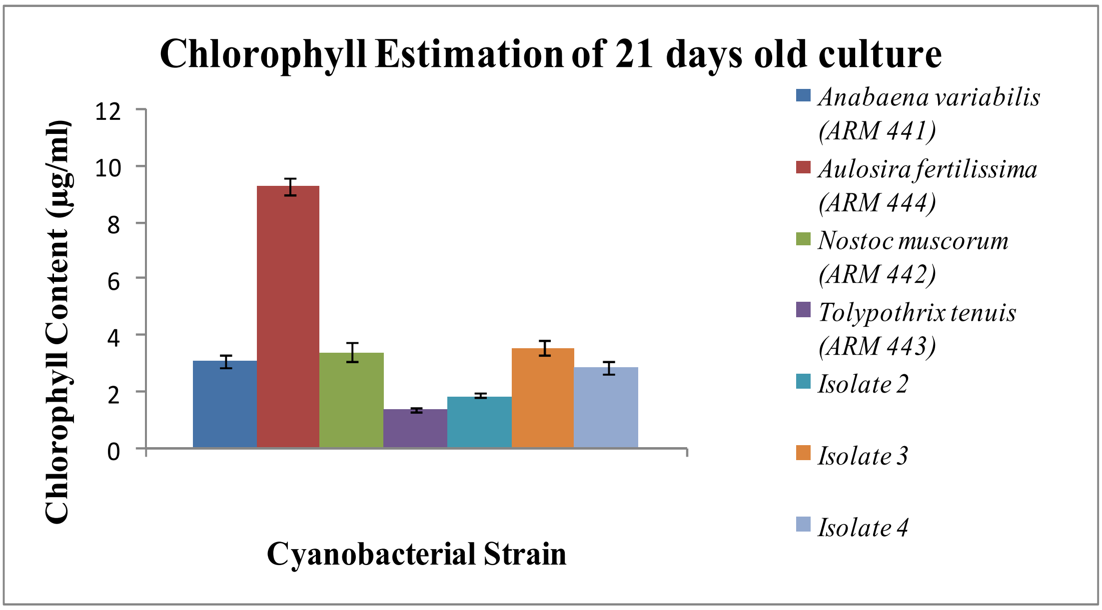


Fig 10: Chlorophyll estimation (µg/ml) of heterocystous cyanobacterial strains after 21 of days incubation

4.4.3 Total nitrogen estimation (%) of heterocystous cyanobacterial strains after 21 days of incubation

Total nitrogen estimation (%)					
S.no.	**Cyanobacterial strains**	**R1**	**R2**	**R3**	**Mean ±SE**
1	*Anabaena variabilis* (ARM 441)	0.0105	0.0096	0.0123	0.011±0.0008
2	*Aulosira fertilissima* (ARM 444)	0.0056	0.0062	0.0100	0.0073±0.0014
3	*Nostoc muscorum* (ARM 442)	0.0068	0.0040	0.0060	0.0056±0.00083
4	*Tolypothrix tenuis* (ARM 443)	0.0050	0.0062	0.0040	0.0051±0.00064
5	Isolate 2	0.0049	0.0040	0.0046	0.0045±0.00026
6	Isolate 3	0.0048	0.0040	0.0045	0.004±0.0002
7	Isolate 4	0.0046	0.0030	0.0030	0.0035±0.0005

Table 13: Total nitrogen estimation (%) of heterocystous cyanobacterial strains after 21 days of incubation

Total nitrogen content (%) of all cyanobacterial strains after 21 days of inoculation was determined and *Anabaena variabilis* (ARM 441) was found to have maximum total nitrogen that is 0.011±0.0008 followed by *Tolypothrix tenuis* (ARM 443) that is 0.0051±0.00064 and Isolate 4 was found to contain minimum nitrogen content that is 0.0035±0.0005 with an average total nitrogen content of 0.0054% (Table 13, Fig.11).

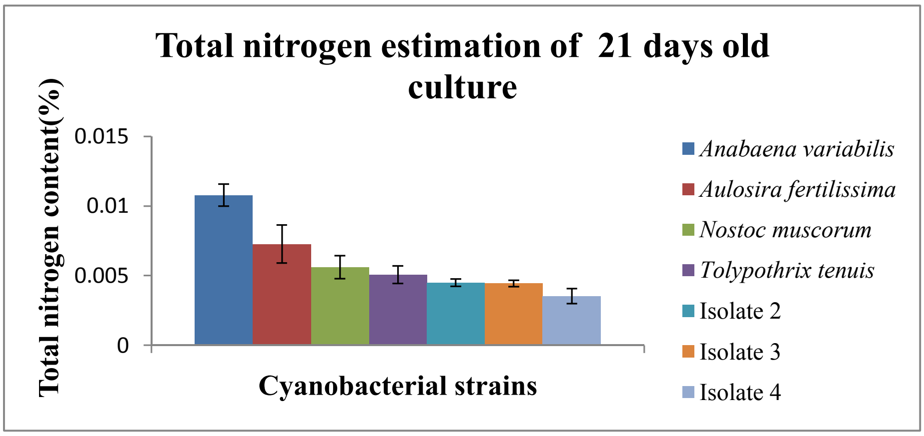


Fig 11: Total Nitrogen estimation of heterocystous cyanobacterial strains after 21 days of incubation

Determination of Biomass, Chlorophyll and Total nitrogen				
S.No.	**Cyanobacterial strains**	**Biomass (mg/ml)**	**Chlorophyll (µg/ml)**	**Total Nitrogen (%)**
		Mean ±SE	Mean ±SE	Mean ±SE
1	*Anabaena variabilis* (ARM 441)	0.32±0.06	3.10±1.2	0.0110±0.0008
2	*Aulosira fertilissima* (ARM 444)	0.67±0.09	9.30±0.32	0.0073±0.0014
3	*Nostoc muscorum* (ARM 442)	0.74±0.17	3.18±0.63	0.0056±0.00083
4	*Tolypothrix tenuis* (ARM 443)	0.92±0.53	1.38±0.09	0.0051±0.00064
5	Isolate 2	0.93±0.29	1.92±0.04	0.0045±0.00026
6	Isolate 3	0.05±0.03	3.60±0.5	0.004±0.0002
7	Isolate 4	0.37±0.1	2.90±0.4	0.0035±0.0005

Table 14: Determination of end point growth of nitrogen fixing heterocystous cyanobacteria

End point growth of cyanobacterial cultures after 21 days of inoculation was determined in terms of chlorophyll content, total nitrogen and biomass among different cyanobacterial strains and it showed the variation i.e. maximum biomass was observed in Isolate 2 (0.93±0.29) followed by *Tolypothrix tenuis* (0.92±0.35), chlorophyll content were maximum in *Aulosira fertilissima* and *Anabaena variabilis* were estimated to be contain maximum total nitrogen (%) i.e. 0.01±0.008 (Table 14).

4.5 Biomass (kg per m^2) in different algal ponds

Incubation	Wet Biomass per unit area (kg per m^2) in each pond									
(Days)	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Pond 6	Pond 7	Pond 8	Pond 9	Pond 10
07	2.0 ±0.29	1.8 ±0.23	1.5 ±0.11	1.8 ±0.10	1.5 ±0.26	1.2 ±0.20	1.4 ±0.16	1.8 ±0.25	2.0 ±0.18	1.76 ±0.2
14	3.0 ±0.21	3.0 ±0.15	2.4 ±0.3	3.1 ±0.19	3.4 ±0.22	2.5 ±0.26	2.1 ±0.30	3.5 ±0.25	3.65 ±0.19	2.3 ±0.30
21	5.2 ±0.23	4.8 ±0.16	4.2 ±0.25	5.0 ±0.30	4.0 ±0.26	4.1 ±0.27	4.6 ±0.33	5.7 ±0.17	6.2 ±0.15	4.5 ±0.27

Table 15: Wet Biomass (kg per m^2) in different algal ponds

Growth (Biomass kg per m^2) of mixed culture of heterocystous cyanobacteria (*Anabaena variabilis* ARM 441, *Aulosira fertilissima* ARM 444, *Nostoc muscorum* ARM 442, *Tolypothrix tenuis* ARM 443) in each pond containing tap water, $MgSO_4$ (0.075 gm/L) and K_2HPO_4 (0.04 gm/L) was estimated. The peak growth upto 21 days of incubation was observed at weekly intervals in each pond. With increase in time, the biomass of algal inoculants in all the ponds increased from the date of inoculation. Average biomass production per unit area on wet weight basis was highest in pond no. 9 with 3.95 kg/m^2 harvested after 21 days of inoculation whereas in pond 10 it was 2.85 kg/m^2 (Table 15, Fig 12).

Therefore, on an average per unit area biomass productivity in all algal ponds on wet weight basis after 7 day was 1.68 kg/m^2, 2.9 kg/m^2 after 14 days and 4.8 kg/m^2 after 21 days. Total biomass production on wet weight basis after 21 days in all algal ponds was 48.3 kg.

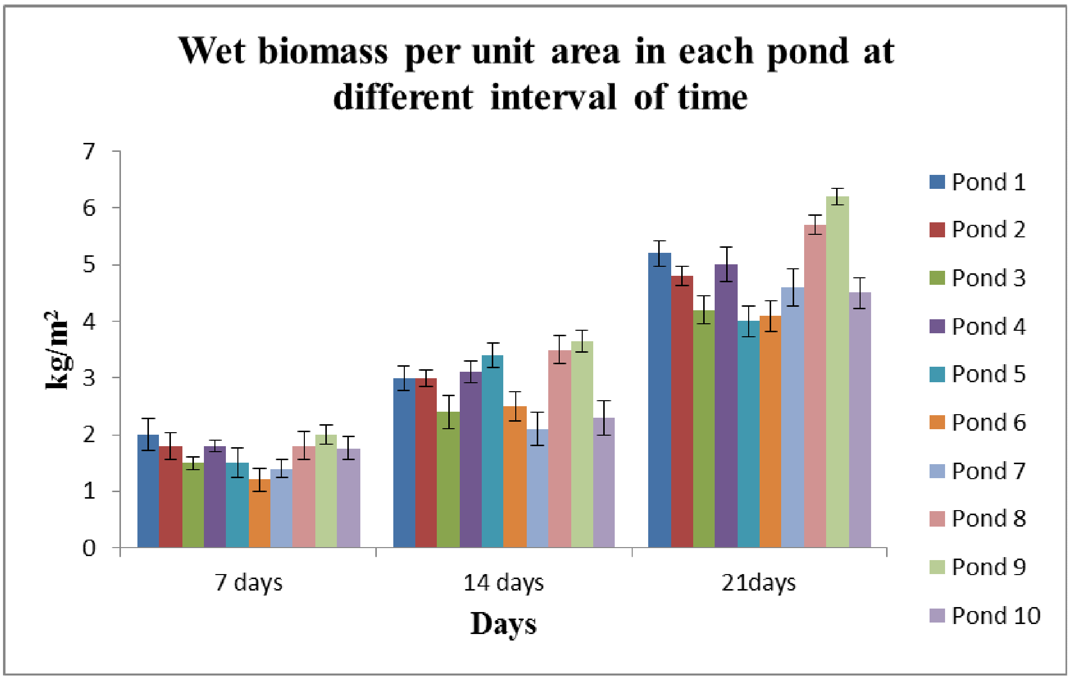


Fig 12: Wet Biomass (kg per m^2) in different algal ponds

Incubation	Dry Biomass per unit area (kg per m^2) in each pond									
(Days)	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Pond 6	Pond 7	Pond 8	Pond 9	Pond 10
07	0.43± 0.014	0.33± 0.023	0.14± 0.02	0.11± 0.019	0.05± 0.014	0.057± 0.01	0.26± 0.017	0.23± 0.018	0.35± 0.024	0.16± 0.025
14	0.63± 0.03	0.40± 40.02	0.41± 0.03	0.74± 0.023	0.63± 0.026	0.49± 0.009	0.76± 0.034	0.83± 0.018	0.82± 0.027	0.2± 0.01
21	0.82± 0.012	0.52± 0.012	0.45± 0.01	0.73± 0.03	0.66± 0.03	0.52± 0.02	0.72± 0.05	0.83± 0.03	0.88± 0.02	0.34± 0.02

Table 16: Dry Biomass (kg per m^2) in different algal ponds

Average biomass production per unit area was highest in pond no. 9 with 0.68± 0.17 kg/m^2 harvested after 21 days of inoculation whereas in the pond no.10 it was 0.23±0.05 kg /m^2 (Table 16, Fig 13). Therefore, on an average per unit area biomass productivity in all algal ponds on dry weight basis after 7 day was 0.21kg/m^2, 0.59 kg/m^2 after 14 days and 0.65 kg/m^2 after 21 days. Total biomass production on dry weight basis after 21 days in all algal ponds was 6.47 kg.

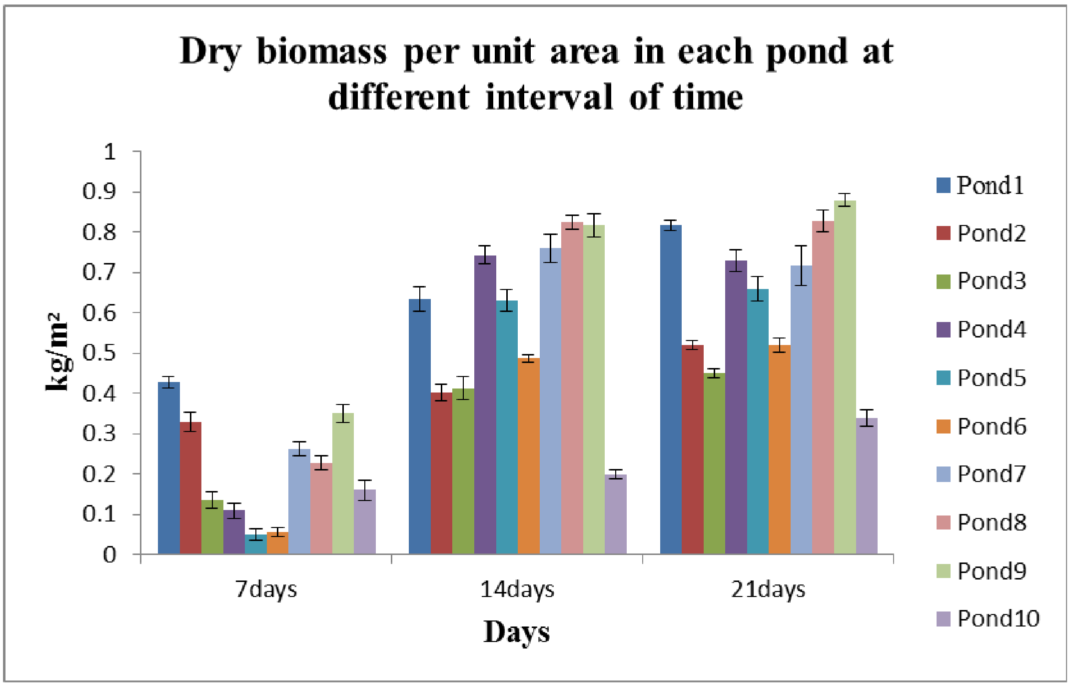


Fig 13: Dry biomass (kg per m^2) in different algal ponds

4.5.1 Observation of temperature, pH and water level at different intervals of time in each pond

	Variation of pH in each pond									
Incubation (Days)	**Pond 1**	**Pond 2**	**Pond 3**	**Pond 4**	**Pond 5**	**Pond 6**	**Pond 7**	**Pond 8**	**Pond 9**	**Pond 10**
0	7.3	7.4	7.5	7.5	7.3	7.5	7.3	7.5	7.3	7.5
07	7.5	7.6	7.6	7.8	7.6	7.8	7.4	7.6	7.5	7.7
14	8.0	7.9	8.0	8.2	8.3	8.1	7.6	7.9	7.8	8.2
21	8.9	8.2	8.3	8.9	8.6	8.6	7.7	8.4	8.0	8.5
	Variation of Temperature (°C) in each pond									
Incubation (Days)	**Pond 1**	**Pond 2**	**Pond 3**	**Pond 4**	**Pond 5**	**Pond 6**	**Pond 7**	**Pond 8**	**Pond 9**	**Pond 10**
0	24	23	23	25	25	23	25	25	25	24
07	23	23	22	24	23	23	23	24	23	23
14	23	22	23	22	22	22	23	23	23	23
21	22	21	21	22	22	22	21	22	22	22
	Variation of Water level (inch) in each pond									
Incubation (Days)	**Pond 1**	**Pond 2**	**Pond 3**	**Pond 4**	**Pond 5**	**Pond 6**	**Pond 7**	**Pond 8**	**Pond 9**	**Pond 10**
0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
07	5.7	5.8	5.6	5.7	5.2	5.5	5.5	5.7	5.5	5.8
14	5.5	5.0	5.0	5.5	5.0	5.3	5.7	5.2	5.5	5.0
21	5.0	4.9	4.7	5.3	4.9	5.0	5.4	4.9	5.0	5.0

Table 17: Temperature, pH and water level in each algal pond

Temperature, pH and water level shows that pH of all algal ponds ranges between 7.3-8.6, water level varies from 5-6 inches and water temperature ranges between 21ºC-25ºC (Table 17).

4.6 Plasmid profile of cyanobacteria

Lane 1: Isolate 5
Lane 2: Isolate 6
Lane 3: Isolate 7
Lane 4: Isolate 8
Lane 5: Isolate 9
Lane 6: Isolate 10*
Lane 7: Hind III
Lane 8: Isolate 11*
Lane 9: Isolate 12*
Lane 10: Isolate 13*
Lane 11: Isolate 14*
Lane 12: pUC 19
Lane 13: pUC 19
Lane 14: Isolate 15*

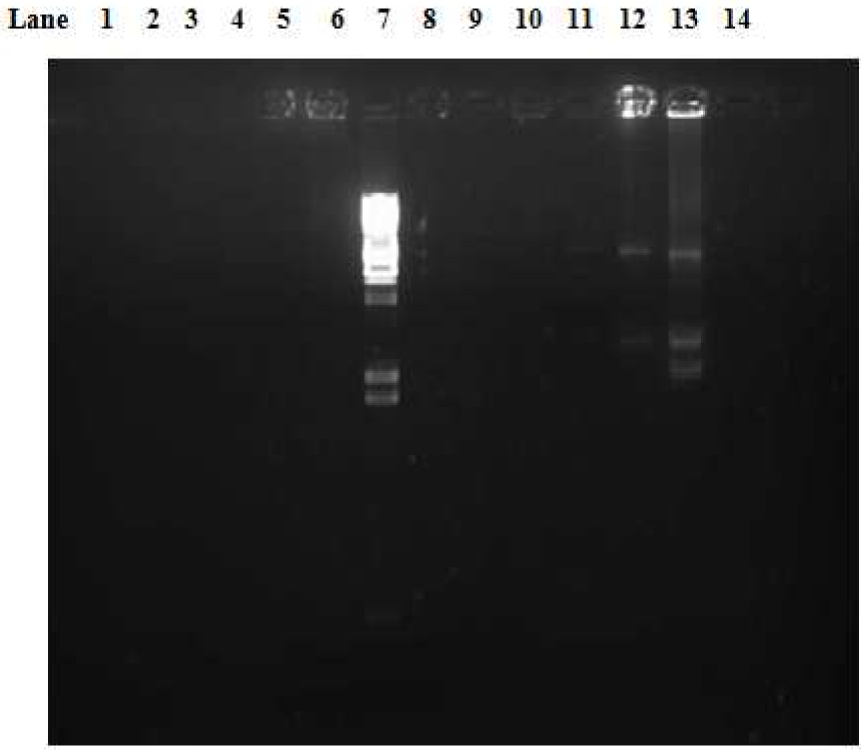
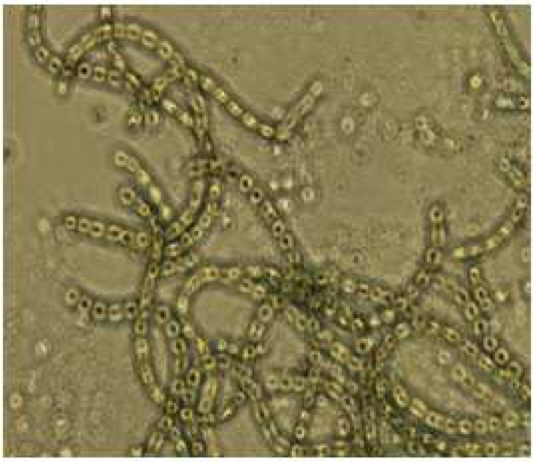


Plate 1: Plasmid profile of cyanobacteria

Isolate 5,6,7,8 and 9 were heterocyst cyanobacteria

*Isolate 10, 11, 12, 13, 14 and 15 were non-heterocyst cyanobacteria

No plasmid was found in all the cyanobacteria studied using the method of isolation as per the method given by Goyal, 1992.



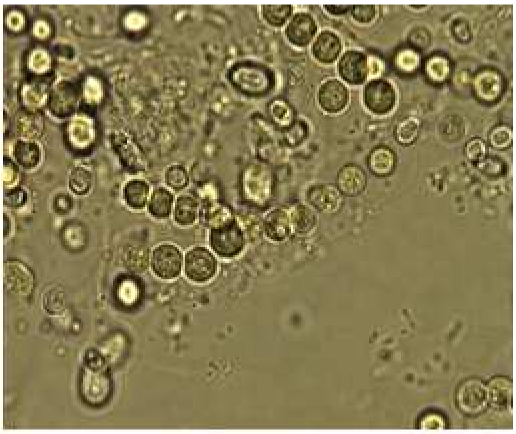
A: Anabaena variabilis ARM 441

B: *Nostoc muscorum* ARM 442

C: *Tolypothrix tenuis* ARM 443

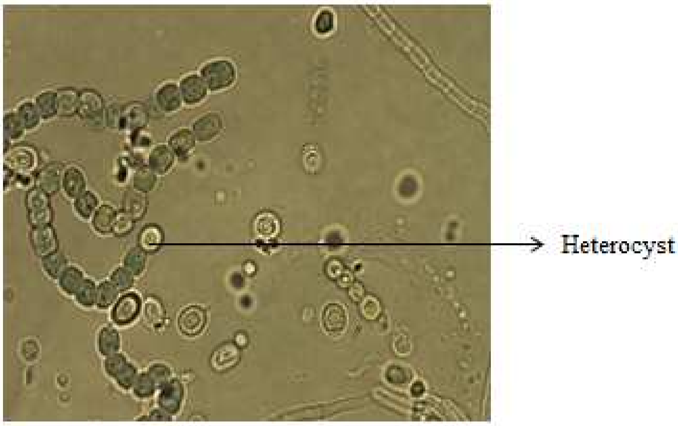
D: *Aulosira fertilissima* ARM 444

Plate 2

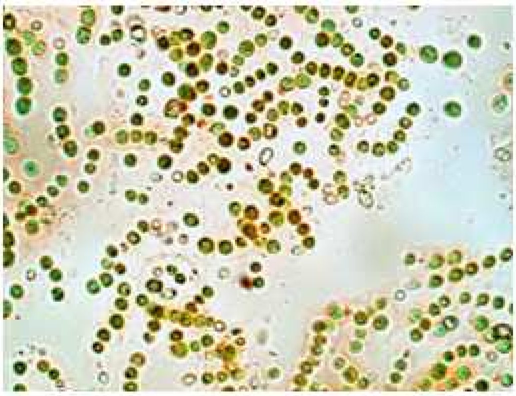


A: *Isolate 5*

B: *Isolate 6*



C: *Isolate 7*



D: *Isolate 8*

E: *Isolate 9*

Plate 3

CHAPTER 5 DISCUSSION

5.1 Physico-chemical analysis of soil samples from paddy field of different farmers in Patiala District

Physicochemical analysis of soil samples collected from different paddy fields of Patiala district for parameters like pH, EC, organic carbon, total nitrogen, available nitrogen and available phosphorus was done. pH being an important parameter to determine acidic or basic nature of soil. In the present study soil pH was found to be alkaline in the range of 7.12 to 8.28 which is in confirmation with earlier finding which showed that among different physico-chemical properties pH is important in determining growth, establishment and diversity of cyanobacterial flora, which is generally been reported to prefer neutral to slightly alkaline (Roger and Kulasooriya 1980, Kaushik 1994). Our result supports the fact that most diverse cyanobacteria *Anabaena* and *Nostoc* prefer neutral to slightly alkaline soil (Nayak and Prasanna, 2007).

EC is an important factor in determining the salinity of soil. Increase in electrical conductivity of soil, increases the availability of soluble salts (Pitchel and Hayes, 1990).

Soil sample of paddy field showed high range of organic carbon content which is an indicator of available nitrogen status of soil thus soil is also rich in available nitrogen content. The quantification of organic carbon content helps in determining the soil quality and productivity (Krishnan *et al.,* 2009). Total nitrogen content was in the range of 0.035% to 0.087%, which showed that soil of paddy field of Patiala district is also rich in total nitrogen content. Available nitrogen and phosphorus determines the amount of nitrogen and phosphorus which is easily available to the plants. Chemical analysis showed that available nitrogen and phosphorus content of soil samples varied from 1.8 ppm to 7.4 ppm and 2.8 ppm to 19.5 ppm respectively which showed that soil of paddy filed is rich in nitrogen and phosphorus content.

5.2 Isolation of heterocystous cyanobacteria from paddy fields

Five different types of heterocystous cyanobacteria were isolated from the soil sample of different paddy fields of Patiala district. The results of the present studies have demonstrated that heterocystous BGA was recorded from rice field soils. Cyanobacteria were the first reported to be the main components of the microbiota in rice fields (Ladha and Reddy, 2003) and play an important role in the maintenance and build-up of soil fertility, consequently increasing rice growth and yield (Roger and Ladha, 1992, Kaushik and Prasanna, 2002). Tropical soils of India and particularly that of flooded rice field often have a diverse flora of morphologically distinct form (Tiwari 1972). In general, the BGA show dominance over other classes of algae and principal genera having the maximum no. of nitrogen fixing species are *Aulosira* species, *Nostoc* species, *Anabaena* species and *Cylindrospermum* species. Efficient nitrogen fixing strain like *Nostoc linkia, Anabaena variabilis, Aulosira fertilissima, Calothrix sp., Tolypothrix sp.,* and *Scytonema sp.* were identified from various agro - ecological regions and utilized for rice production (Prasad and Prasad, 2001). These five isolates were found to contain heterocyst. These are the nitrogen fixing stations of heterocystous algae and the various non – heterocysts form fixed nitrogen anaerobically (Prasanna and Kaushik, 1994). Heterocyst frequency of these isolates was estimated to determine their nitrogen fixing potential and maximum heterocyst frequency was found in Isolate 6. Prasanna (2007) recorded more heterocystous forms while studying cyanobacterial abundance and diversity in rice field soils of India.

5.3 Growth studies of heterocystous cyanobacteria

Blue-green algae are considered as the ideal biological fertilizer for the improvement of soil fertility and its long term sustainability under wet land rice ecosystem. Role of the blue-green algae *(e.g. Aulosira, Anabaena, Cylindrospermum, Nostoc, Tolypothrix)* in the paddy fields was realized much earlier (Singh, 1961) and their practical utility as a source of organic nitrogen fertilizer for rice has been well recognized. De (1939) was the first to suggest a positive role of blue green algae (BGA) in the sustenance of the nitrogen status of rice fields. Species of *Nostoc, Anabaena*, *Tolypothrix, Aulosira, Cylindrospermum, Scytonema, Westiellopsis* and several other genera were widespread in Indian rice field soils and were known to contribute significantly to their fertility (Venkataraman, 1981; Kaushik, 1991; Nayak *et al.*, 2004). These heterocystous cyanobacteria are present in abundance in rice fields but their occurrence varies depending upon soil conditions and other climatic factors. The present study has been carried out on four heterocystous cyanobacteria and some isolates in terms of growth studies which involve dry biomass estimation and total nitrogen estimation. Biomass and total nitrogen content of heterocystous cyanobacterial strains were estimated at different intervals of time that is after 7, 14 and 21 days of inoculation and it was observed that both biomass and total nitrogen content increases with increase in time. *Tolypothrix tenuis* (ARM 443) showed the maximum growth in terms of dry biomass that is 1.70 mg/ml followed by Isolate 3 (0.99 mg/ml). Similarly maximum Total nitrogen content was observed in *Anabaena variabilis* (0.011%) followed by *Aulosira fertilissima* (0.0073%). A wide variation was observed amongst and within the different strains of cyanobacteria with respect to biomass production and nitrogen fixing ability. In the growth studies, heterocyst frequency of different cyanobacterial strains were also determined and maximum heterocysts were found to be in *Tolypothrix tenuis* (ARM 443) that is 17.9%.

The present study highlighted the variability existing among different cyanobacterial strains with respect to growth parameter. In one of the study distinct variations in growth and other physiological parameters (like inter/intra generic differences/ similarities) were observed in cyanobacterial strains of *Nostoc, Anabaena*, and *Calothrix* (Narayan *et al.,* 2006).

5.4 Mass cultivation of blue green algae

Mass cultivation of heterocystous cyanobacteria has been carried out in algal ponds for use as biofertilizer. Japanese workers (Watanabe, 1965) developed techniques for mass cultivation of blue-green algae to be used as biofertilizer in paddy fields. Maximal culture productivity is obtained only when culture nutritional requirements are satisfied and temperature is about optimal. Biomass estimation of all algal ponds at different interval of time on wet weight basis showed that the average biomass productivity per unit area in all algal ponds after 7 days,14 days and 21 days were found to be 1.68 kg/m^2, 2.9 kg/m^2 and 4.8 kg/m^2 . Similarly, dry weight basis showed that the average biomass productivity per unit area in all algal ponds after 7 days, 14 days and 21 days were found to be 0.21 kg/m^2, 0.59 kg/m^2 and 0.65 kg/m^2 respectively and the total biomass production on dry weight basis after 21 days in all algal ponds was found to be 6.47 kg.

5.5 End point growth of cyanobacteria

End point growth estimation of cyanobacterial cultures was done to determine the peak growth after 21 days of inoculation to compare the chlorophyll content, total nitrogen and biomass among different cyanobacterial strains and it showed the variation i.e. maximum biomass was observed in Isolate 2 (0.93 mg/ml) followed by *Tolypothrix tenuis* (0.92 mg/ml), chlorophyll content was maximum in *Aulosira fertilissima* (9.3 µg/ml) and *Anabaena* variabilis was estimated to be contain maximum total nitrogen i.e. 0.01%.

5.6 Plasmid profile of cyanobacteria

Some unicellular cyanobacteria contain extra chromosomal (plasmid) DNA (Asato and Ginoza, 1973; Restaino and Frampton, 1975; Roberts and Koths, 1976). Plasmids have proven extremely useful in the genetic analysis of various bacteria. But some species of cyanobacteria donot contain plasmid, similarly in our experiment no plasmid was reported (Goyal, 1992).

CHAPTER 6 CONCLUSION

1. Five different heterocystous cyanobacteria have been isolated from paddy fields of Patiala district and the soil sample of paddy field was found to be alkaline with electrical conductivity (EC) ranging from 100 to 282 µS, organic carbon ranging from 0.37 to 0.85% whereas total nitrogen ranged from 0.035 to 0.087%, available nitrogen ranged from 1.8 to 7.4 mg/kg and available phosphorus from 2.8 to 19.5 mg/kg.
2. The maximum growth in terms of dry biomass was seen in *Tolypothrix tenuis* (ARM 443) ranging from 0.14 to 1.70 mg/ml. Total nitrogen content was maximum in *Anabaena variabilis* **(**ARM 441) ranging from 0.0043 to 0.011% whereas heterocyst frequency was found maximum in *Anabaena variabilis* (ARM 441) by 14.9% which was also the case with region specific Isolate 6.
3. End point growth of cyanobacterial cultures was done to determine the peak growth after 21 days of inoculation and maximum biomass was observed in Isolate 2 (0.93 mg/ml) followed by *Tolypothrix tenuis* (ARM 443) (0.92 mg/ml), chlorophyll content was maximum in *Aulosira fertilissima* (ARM 444) (9.30 µg/ml) and *Anabaena variabilis* (ARM 441) was estimated to be contain maximum total nitrogen i.e. 0.01%.
4. Mass cultivation of heterocystous cyanobacterial culture was done and on an average biomass production in 10 algal ponds after 21 days was estimated to be 0.65 kg/m^2 and total biomass production after 21 days was 6.47 kg on dry weight basis and on wet weight basis the average biomass production after 21 days was 4.8 kg/m^2 and the total biomass production was 48 kg.
5. From the present investigation it was concluded that heterocystous nitrogen fixing cyanobacteria i.e. *Tolypothrix tenuis* (ARM 443) and *Anabaena variabilis* **(**ARM 441) are efficient nitrogen fixers for application in paddy cultivation.

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