IMPACT OF FLY ASH ON PLANT GROWTH PROMOTING ACTIVITY OF CYANOBACTERIA

A Dissertation report

Submitted in partial fulfillment of the requirements for the award of the degree

Of

MASTER OF SCIENCE

IN

MICROBIOLOGY

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Impact of fly ash on plant growth promoting activities of cyanobacteria."** in partial fulfillment of the requirement for the award of the degree of Master of science in Microbiology, Department of Biotechnology, Thapar University, Patiala, Punjab, is an authentic record of my own work during the period of six months from Jan 2014 to June 2014, under the supervision of Dr. Dinesh Goyal, Professor and Head of Department of Biotechnology, 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 dissertation entitled **"Impact of fly ash on plant growth promoting activity of cyanobacteria"** submitted (Registration No: 301205012) by Shilpi in partial fulfillment of the requirement for the award of degree of Master of Science in Microbiology, to Thapar University, Patiala is a record of student's own work carried out by her. The report has not been submitted for the reward of any other degree or certificate in this or any other university or institute.

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LIST OF ABBREVATIONS

BGA	Blue green algae
DW	Distilled water
EC	Electrical conductivity
Gm	Gram
Ha	Hectare
Kg	Kilogram
Min	Minute
TCP	Tri calcium phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
L/ Ltr	Litre
Mg	Milligram
ml	Milliliter
BNF	Biological nitrogen fixation
IAA	Indole acetic acid
FA	Fly ash
NR	Nitrate reductase
(-N)	Media without nitrogen
NEDD	1-napthyl diamine dichloride
HCN	Hydrogen cyanide
Ppm	Parts per million
w/v	Weight by volume
v/v	Volume by volume

ABSTRACT

*Anabaena variabilis (*ARM441), *Aulosira fertilissima* (ARM444), *Nostoc muscorum* (ARM442), *Tolypothrix tenuis* (ARM443) procured from IARI, New Delhi were studied for plant growth promoting activity such as Indole acetic acid production, Nitrate reductase assay and phosphate solubilization. Among four cyanobacterial strains *Aulosira fertilissima* (ARM 444) showed maximum Indole acetic acid (0.050µg/ml) in the presence of tryptophan (1mgml), Nitrate reductase activity (0.543mg/g), biomass production (2.12mg/ml), solubilisation of tricalcium phosphate (5.86ppm) and chlorophyll content (0.35mg/ml), whereas *Nostoc muscorum* (ARM 442) showed least plant growth promoting activities. Effect of fly ash at different concentration (0, 1, 2, 5, 10 and 20%) on growth and PGP activity was studied on *Anabaena variabilis* (ARM441) and *Aulosira fertilissima* (ARM 444) and it was revealed that 10% fly ash (w/v) in BG-11 medium showed maximum plant growth promoting activity, Nitrate reductase activity, Indole acetic acid production and solubilization of phosphorous and dry biomass production. At higher concentration (20%) of fly ash there was decline plant growth promoting activity.

CHAPTER 1 INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a group of naturally occurring soil bacteria that enhance plant growth and yield by producing various plant growth promoting substances (Jay Shankar., 2013). Soil beneficial microbial formulations or biofertilizers are well known to improve the soil nutritive value in an agricultural system and therefore are important components of organic farming, which help to nourish the crops through required nutrients. These micro organisms are considered as PGPR as they help to fix atmospheric nitrogen, solubilize and mobilize phosphorus, translocate micro elements like zinc, copper, release hormones, vitamins and amino acids and hence improve soil health and increase crop production.

PGPR increases plant growth directly due to biological nitrogen fixation or by increasing the availability of nutrients in the rhizosphere or indirectly by inducing phytohormones production such as auxins, cytokinins, gibberellins.

Various known species of *Rhizobium* for legumes, blue green algae (BGA) or cyanobacteria (*Aulosira*, *Nostoc*, *Anabaena* etc.) and *Azolla* (a fern containing N_2-fixing BGA, *Anabaena azollae*) for wet land rice and *Azotobacter/Azospirillum* for several other crops play a significant role in agriculture due to PGP activity.

Cyanobacteria effectively promote plant growth by fixing nitrogen acting as fertilizer and producing plant growth hormones. Large number of cyanobacteria excretes organic and inorganic acid. The ability to form plant hormones is another property of cyanobacteria that stimulate and facilitate plant growth (PGPR).

These inoculants currently seem to be offering a potentially environmental friendly alternative to chemical fertilizers (Sinha et al., 2002; Rai, 2006). These are photosynthetic prokaryotes that colonize and adapt to a wide array of environmental conditions (Paerl et al., 2000, Kirlwood et al., 2008).

Rice crop utilize healthy population of nitrogen-fixing cyanobacteria for use as biofertilizer. 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. Since it 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).

Several studies have reported that cyanobacteria can improve the plant growth by improving the soil structure and excrete extracellular polysaccharides that help in soil aggregation and water retention (Hill et al., 1994; Maqubela et al., 2009). The aim of the present work was to evaluate the PGP activity of different heterocystous filamentous cyanobacteria used in rice cultivation and study the impact of soil amendment such as fly ash on PGP activity of cyanobacteria.

CHAPTER 2 REVIEW OF LITERATURE

Cyanobacteria also known as blue-green algae belong to phylum Cyanophyta and are photosynthetic gram negative prokaryotes that are found in fresh and salt water which capture sunlight for energy using chlorophyll a and various accessory pigments. Their cell size is usually less than 10µm and thallus range from unicell, small clusters, filaments or large colonies. (Zaccaro, 2000).

Habitat	Cyanobacterial species
Bottom dwellers	*Aphanothece, Microcoleus, Oscillatoria*
Phytoplankton	*Merismopedia, Oscillatoria, Anabaena*
Free Floating	*Anabaena, Aulosira, Gleotrichia*
Wet soil inhabitants	*Aphanothece, Microcoleus, Oscillatoria*
Moist soil forms	*Oscillatoria, Nostoc, Anabaena,*
Epiphytes	*Calothrix, Gloetrichia, Hapalosiphon*
Nitrogen deficient	*Nostoc, Anabaena, Aulosira, Calothrix*
Rhizosphere of rice and wheat	*Anabaena, Nostoc, Weistellopsis, Calothrix*

Table 1: Some cyanobacterial species with their Habitat (Prassana et al., 2007)

Plant growth promoting activities like nitrogen fixation, phosphate solubilisation, nitrate reductase assay and phytohormones production which makes cyanobacteria used as PGPR in crop field. Cyanobacteria use phytohormonal signaling (direct mechanism of phytostimulation) as a tool for plant growth promotion and the biosynthesis of cytokinins and IAA is considered to be crucial for plant growth and development. Cyanobacteria are known (*Anabena, Nostoc, Aulosira, Tolypothrix)* to have the ability to release phytohormones in the rhizosphere from where plant roots may absorb these hormones.

Cyanobacteria as clay based inoculums consisting of *Nostoc, Anabaena, Aulosira and Tolypothrix* is effectively applied in agricultural field, and they are able to fix atmospheric nitrogen and used as inoculants for paddy crop grown under upland and lowland conditions.

Anabaena in association with water fern *Azolla* contributes nitrogen up to 60Kg/ha/Season and also enriches soil with organic matter (Priyadarshani and Rath, 2012).

2.1 Algalization

Cyanobacteria are nitrogen fixing stations represented by heterocystous algae and non-heterocystous forms that nitrogen anaerobically (Prasanna and Kaushik, 1994). Nayak and Prasanna (2007) observed more heterocystous forms while studying cyanobacteria abundance and diversity in rice fields which is known for nitrogen fixation as they have potential to reduce atmospheric dinitrogen to ammonium ion (Zaccaro et al., 2000). **S**ome of common filamentous heterocystous forms are *Anabaena, Aulosira, Calothrix, Nostoc, Tolypothrix, Stigonema* (Stewart et al 1979, Prasanna and Kaushik, 1995). Non-Heterocystous filamentous forms capable of nitrogen fixation are species of *Oscillatoria, Trichodesmium, Pseudanabaena, Lyngbya* (Wyatt and Silvey,1960 ; Stal and Krumbein, 1985). Specialised nitrogen fixing cells known as heterocysts are present in cyanobacteria having enzyme nitrogenase (Adam 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.

"Algalization**"** was coined by Venkataraman in 1961 for biofertilizers of BGA creating an environment friendly agro ecosystem that ensures economic viability in paddy cultivation while saving energy intensive inputs. Cyanobacteria as nitrogen fixers are exploited as biofertilizers in agriculture, with the contribution of 20-25kgN/ha/Season and enhance soil fertility (Prasanna and kaushik, 2009).

Roger and Watanabe (1986) reported that cyanobacterial inoculation increases rice yields by an average of 337Kg 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).

In one of the studies it was reported that exogenous supply of nitrogen fertilizer counteracts nitrogen fixation in the rhizosphere. This exogenous supply of nitrogenous fertilizer to lowland rice significantly inhibited N_2 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).

2.2 Plant Growth Regulators

Plant growth regulators are small organic molecules produced by higher plants, lichens, bacteria, actinomycetes and cyanobacteria (Shrivastava and Banerjee, 2009). Cyanobacteria in addition to contributing nitrogen benefit crop plants by producing various plant growth promoting substances. (Priyadarshani and Rath, 2012).Some growth promoting regulators are given below:

Cyanobacteria species	**Growth promoting substances**	**References**
Cylindrospermum sp	VitaminB_{12}	(Venkataraman and Neelakantan ,1967)
Tolypothrix tenius	Vitamin B_{12}	(Okuda and Yamaguchi, 1960)
Nostoc muscorum	Vitamin B_{12}	(Misra and Kaushik, 1989)
Nostoc, Hapalosiphon	Auxin like Indole-3-acetic acid, Indole-3-propionic acid	(Mishra and Kaushik, 1989)
Calothrix	Cytokinins	(Stirk et al., 1999)
Spirulina	Jasmonic acid	(Stirk et al., 1999)

Table 2 – Growth promoting regulators reported from different cyanobacterial strains

Phytohormones production is wide spread among microorganisms including bacteria and cyanobacteria (Tsavkelova et al., 2006). The ability to secrete hormones a major characteristic of rhizospheric, epiphytic, symbiotic bacteria known as PGPR. Cyanobacteria are capable to synthesis almost all classes of phytohormones such as auxins, cytokinins, gibberellins, abscisic acid and ethylene (Rodriguez et al., 2006; Manickavelu et al., 2006). Despite numerous reports about cytokinins and auxins like activity in cyanobacteria (Stirk et al 2002, Shanab et al 2003) limited amount of literature (Stirk et al., 1999) is available on direct determination of the phytohormones particularly cytokinins.

Cyanobacteria can benefit plants and used as PGPR for crop production by release of hormones such as Auxins (Sergeeva et al., 2002; Parsanna et al., 2010), Gibberellins (Rodriguez et al., 2006), Abscissic acid, Cytokinins (Stirk et al., 2002; Hussain and Hasnain 2009).They are also capable of fixing atmospheric nitrogen (Osman et al., 2010) and solubilisation of mineral phosphates (Banerjee and yasmin, 2009)

Cyanobacteria species	**Characteristics**	**PGP activity**	**PGP activity in crops**
Azolla sp	Free floating water fern	IAA production	Rice and wheat crop
Calothrix sp	Nitrogen fixing blue green algae	Auxin and several amino acids.	Rice and legumes
Anabaena.sp	Nitrogen fixing blue green algae	Auxins, Gibberellin and amino acids.	Wheat, Rice and legumes
Nostoc.sp	Nitrogen fixing blue green algae	Auxins, Gibberellin and amino acids	Legumes and rice
Cylindrospermum sp	Nitrogen fixing fresh water blue green algae	Gibberellin and Auxins.	Wheat and mung beans
Plectonema sp	Nitrogen fixing blue green algae	Auxin, Gibberellin and amino acids.	Wheat and legumes
Oscillatoria.sp	Nitrogen fixing blue green algae	Cytokinins and Auxins	Wheat and Rice crops

Table 3: Some of the cyanobacteria reported for plant- growth promoting activity (Prasanna et al., 2009; 2010)

Phytohormones play an important role as signals and regulators of growth and developments in plants. Auxins, among them in particular Indole-3-acetic acid (IAA), are the most studied plant growth regulators (Sergeeva and Liaimer, 2002) Auxin is one of the plant growth regulator produced from the bacteria, fungi, cyanobacteria and plants. Auxins are classified based on the occurrence by natural source or synthetic (Stirk et al., 1999).

Auxins are the main phytohormones which regulate the growth, morphogenesis, adaptive and repair process. It also helps in root formation and elongation of the plant which promote ethylene production and ripening of fruits. Auxins are present in more than one form such as active compound indole acetic acid which is a common natural auxin. The chemical molecular structure of IAA is shown in Figure 1:

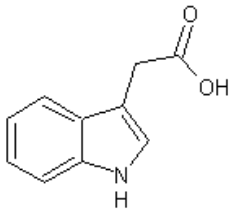

Figure1. Molecular structure of IAA

The amino acid tryptophan is commonly regarded as the precursor for the biosynthesis of auxin in plants (Sergeeva et al, 2002). By one pathway tryptophan is converted to Indole pyruvic acid via transaminase reaction, which requires a keto acid and Pyroxidal phosphate in addition to the enzyme. Indole pyruvic acid is next decarboxylated to indole acetaldehyde in a reaction requiring a decarboxylase thiamine pyrophosphate. Either an oxidase or dehydrogenases then oxidize indole acetaldehyde to IAA (Pattern and Glick, 1996).

Sergeeva et al, (2002) reported that cyanobacteria have the capacity to accumulate IAA endogenously and release. Addition of tryptophan was found to stimulate the accumulation and release of IAA, indicative of a tryptophan dependent production of IAA. In the earlier studies, several strains of *Anabaena* (Prasanna et al., 2008) and cyanobacterial isolates from rhizosphere (Prasanna et al, 2009) exhibited the ability to excrete (mostly in the range of 1-3µg/ml) in the nitrogen free BG-11 medium without added tryptophan.

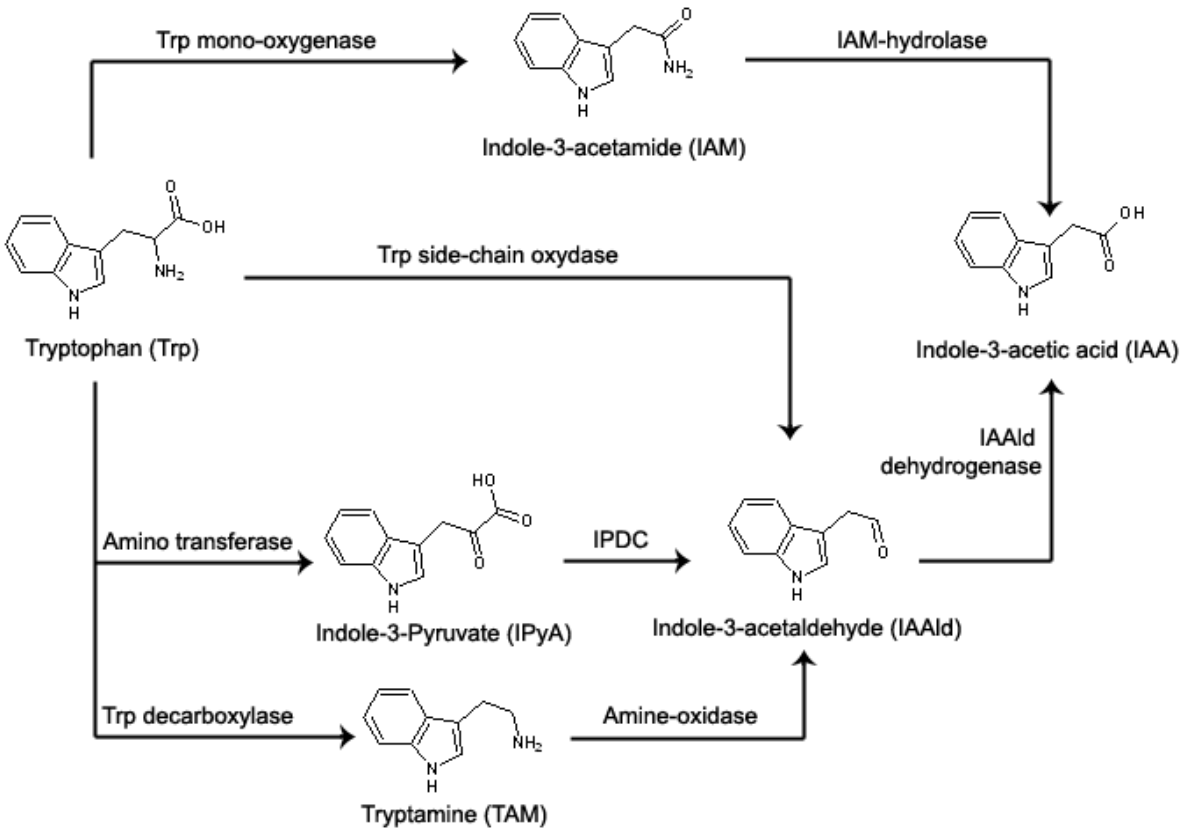

Figure2: Tryptophan dependent IAA biosynthesis pathways Spaepen et al, 2007.

Cyanobacteria including *Anabaena, Nostoc, Calothrix, Cylindrospermum, Gloeothece, Plectonema, Synechocystis* have been reported to produce IAA. IAA is determined in cyanobacteria by colorimetric as well as chromatographic methods (Hussain et al., 2010). Mishra and kaushik (1989) reported that external medium used for growth of cyanobacteria was found to be rich in some amino acids like histidine, serine, glycine. In addition to amino acid some polysaccharides were also found i.e. galatose, fructose, xylose.

PGPR	PGP activity	PGP activity in crops	References
Rhizobium.sp	IAA, Siderophores, HCN	Rice	Ahmed and khan,2009
Azotobacter.sp	Indole-3 acetic acid	Maize	Zahir et al,2000
Pseudomonas.sp	IAA, Siderophores, HCN	Wheat, Maize	Dey et al, 2004
Bacillus.sp	Antifungal activity, IAA	Wheat, spinach	Cazorla et al.,2007
Bradyrhizobium.sp	Siderophore, HCN	Rice, Brassica	Wani et al., 2007
Azospirillum.sp	IAA, Nitrogenase activity	Wheat	Elisete et al.,2008

Table 4: Some other Bacterial species reported as PGPR for the plant growth promoting activity in different crop yield.

2.3 Nitrate reductase (NR) Activity by different cyanobacteria

The Nitrate reductase is a substrate inducible enzyme and its activity is based upon its ability to reduce nitrate to nitrite that is measured by diazocoupling method (Lowe and Evans., 1964).

This test determines whether the microbe produces nitrate reductase and nitrite reductase. The two enzymes catalyze two reactions involved in converting starting compound nitrate into end product nitrogen gas. The reduction reaction is given below:

Nitrate reductase Nitrite reductase

NO_3 ----------> NO_2----------> N_2

Nitrate nitrite nitrogen gas

The reduction of nitrate to nitrite to form nitrous acid which reacts with diazotized sulfanilic acid. This diazotized sulfanilic acid that is produced reacts with the α-naphthylamine to form the red complex.

The assimilation of nitrate takes place through three successive steps (Frias et al 1997)

a) Nitrate enters the cyanobacterial cell mainly by means of an active transport system.
b) Then, intracellular nitrate is reduced to ammonium through two sequential reactions catalyzed by nitrate reductase and nitrite reductase respectively.

c) Ammonium resulting either from nitrate assimilation or resulting from nitrogen fixation.

Nitrate represents the immediate form of nitrogen for most plants in their natural habitat. After being taken up, nitrate must be reduced to ammonium to form the different organic nitrogenous compounds. Ammonium, the end product of assimilatory nitrate reduction, behaves as an antagonist of nitrate assimilation for all types of organisms (Flores et al 1980).

2.4 Phosphate Solubilization

Phosphorous is one of the major essential macronutrient for plants, which applied to the soil in the form of phosphatic manure. It is the second important key element after nitrogen in terms of quantitative plant nutrients (Sharma et al 2013).

Diverse group of microorganisms such as bacteria, fungi, actinomycetes, yeast are known to solubilize insoluble form of inorganic phosphate (Jenzen et al 1989).On an average of uptake of added phosphatic fertilizer to plant ranges from 15-20% due to chemical fixation of phosphorous as either iron or aluminium phosphates in acid soil and calcium phosphate under alkaline soil (Yandigeri et al, 2010). For cyanobacteria, phosphorous is necessary for growth and nitrogen fixation. Blue green algae like phosphorous solubilsing bacteria are known to have ability to mobilize bound phosphatases. Among various mechanisms reported by which phosphate may be liberated from insoluble minerals by BGA includes production of organic acid, synthesis of chelators, enzyme solubilisation, Dissimilation reduction of ferric ions (Whitton et al.,1991). Morever ability of blue green algae to release soluble orthophosphate (P_i) from rock phosphate ore make this phenotype of great potential importance for the development of eco-rational phosphate fertilizer technologies for agriculture.

Phosphate solubilizing microbes	**Examples**
Bacteria	*Pseudomonas, Xanthomonas, Enterobacter*
Fungi	*Penicillum, Aspergillus, Trichoderma*
Yeast	*Yarrowia lipolytica,Schizosaccromyces pombe*
Blue green algae	*Tolypothrix, Scytonema, Hapalosiphon*

Table 5: Phosphate solubilizing microorganisms (Sharma and Sayyed et al., 2013)

2.5 Hydrogen Cyanide (HCN) production

Hydrogen Cyanide (HCN) production is an inorganic compound. It is colorless, extremely poisonous liquid that boils slightly above the room temperature at 26°C. Hydrogen cyanide is a linear molecule with a triple bond between carbon and nitrogen.

HCN inhibits the electron transport thereby the energy supply to the cell is disrupted leading to the death of organism. It inhibits the proper functioning of enzymes and natural receptors by the reversible mechanism of inhibition. It is also known to inhibit the action of cytochrome oxidase (Gebring et al., 1993).HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Defago et al., 1990).

2.6 Cyanobacteria as biofertilizer for crop productivity.

The agricultural importance of cyanobacteria in rice cultivation is directly related with their ability to fix nitrogen and other positive effects for plants and soil. The paddy-field ecosystem represents a unique aquatic-terrestrial habitat, which provides a favorable environment for growth of and nitrogen fixation by cyanobacteria, meeting their requirements for light, water, elevated temperature and nutrient availability. This, in turn, is considered to be one of the major reasons for the relatively stable yield of rice under water logged conditions and maintenance of the productivity of rice fields (Roger et al., 1993).

Cyanobacteria also 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., 1998, Kaushik 2004).

Cyanobacteria species were recommended to be used as biofertilizers instead of utilizing the expensive industrial chemical fertilizers. This was because of the increased cost of chemical fertilizers that cause soil and water pollution. In comparison, these microbes are a cheap source of N, which does not cause pollution. Thus, current study aimed to investigate the influence of different application methods of these inoculants on yield and yield quality of common bean under two levels of mineral nitrogen fertilization.

Field Study: Studies performed by Sergeeva (2002) on the production of phytohormones reported tryptophan is considered as main precursor for IAA accumulation in cell extracts and supernatant of the algal strains**.** Similar studies were performed by Meudt et al., (1967**)** reported that the use of DMCA reagent gives the relative colorimetric analysis than salkowski or Ehrlich's reagent. DMCA reagent yields a wine red color with IAA product during oxidation process. Prasanna et al, (2010) found that tryptophan is not essential as precursor for IAA biosynthesis. They performed the further study to identify genes involved in the tryptophan independent pathway for IAA biosynthesis.

Natesan et al., (1989) reported that the *Anabaena* strain solubilized extracellular tricalcium phosphate through increased phosphatase activity. Mishra and Kaushik., (1989) reported the presence of auxin in cyanobacterial strains like *Nostoc muscorum* and *Hapalosiphon fontinalis* and their quantities were 3.76 and 4.48µg/g respectively. Avena growth test was performed for auxins has indicated the growth of auxins in both strains and the quantity was <0.001mg/l. Ragini and Pankaj, (2012) studied the comprehensive view of cyanobacteria which reported that cyanobacteria are bacteria carried photosynthesis and unicellular and grow in colonies large enough to see and their distinction of being oldest known fossils.

Study performed by Yandigeri et al., (2010) on Phosphate solubilisation by Anabaena reported that these strain used phthalic acid as possible mode of phosphorous solubilization. Chouhan; Yadav et al., (2013) studied on soil cyanobacteria to evaluate metabolite production during various incubation in their culture filtrate and it was found that amount of biomass, IAA, protein was increased with incubation time and show maximum concentration after 30 days respectively.

Zaccaro, (2000) studied on plant growth promoting activity of cyanobacteria which revealed that cyanobacteria contribute to soil fertility , produce bioactive substances like amino acids, exopolysaccrides and act as bioremediating agent to improve the yield of fertile soil. Prasanna et al., (2009) studied on rhizospheric dynamics of inoculated cyanobacteria and their growth promoting role in rice crop which revealed that it help to select promising strain for developing carrier based inoculants to promote the growth of crop and soil micro flora.

2.7 Organic Farming

Organic Farming is recognized as the best known alternative to the conventional agriculture. It is a crop production system that avoids the use of synthetic and chemical inputs like pesticides, growth regulators, fertilizers, and livestock feed additives. Organic manure such as farmyard manure, manure, compost, vermicompost, biofertilizers, biopesticides etc. Among the entire component biofertilizers are very important as they are ready to use live formulates of beneficial microorganisms which applies on seed, root or soil (Das et al.2014). Parr et al., (1994) found that the use of microbial inoculants has obtained much more prominence of enhancing productivity of organic farming system due to ability of these organisms to release the bound nutrients in most organic matter at required times for crop utilization.

2.7.1 Benefit of Biofertilizers over Chemical fertilizers

Fertilizers increases efficiency and obtain better quality of product recovery in agricultural practices. It is one of the most important ways. Non-organic fertilizers mainly contain phosphate, nitrate ammonium and potassium salts. However in recent years, fertilizer consumption increased exponentially throughout the world cause serious environmental problems. Fertilizers may affect the accumulation of heavy metals in soil and plant system. Plant absorbs the fertilizers through soil, they enter the food chain. Thus the fertilization leads to water, soil, air and water pollution (Serpil et al., 2012).

Due to harmful effects of chemical fertilizers on crop, researchers have started showing their interest in biofertilizers. Biofertilizers, more commonly known as microbial inoculants, are artificially multiplied cultures of certain soil organisms that can improve soil fertility and crop productivity. Besides accessing nutrients, for current intake as well as residual, different biofertilizers also provide growth-promoting factors to plants and some have been successfully facilitating composting and effective recycling of solid wastes. By controlling soil borne diseases and improving soil health and soil properties these organisms help not only in saving, but also in effectively utilizing chemical fertilizers and results in higher yields rates.

Function	Organisms	Crops
Nitrogen fixation	*Rhizobium,* *Acetobacter,* *Azospirillum,* Blue green algae such as *Anabaena, Nostoc, Aulosira*	Pea , gram, Arhar Maize, cottony, paddy Sugarcane Paddy
Phosphate solubilisation	*Pseudomonas, Aspergillus,* *Penicillum, Bacillus*	All crops
Organic matter decompostion	*Trichoderma, Bacillus,*	Forest tree species
Growth accelerators	*PGPR, Xanthomonas*	All crops

Table 6: Microorganisms being used as biofertilizers for various crops (Dixit, 2002)

2.8 Fly ash as ameliorating agent

The global energy need has increased with rapid industrialization, which to a large extent has been met by fossil fuels. Coal is an exhaustible energy source, which play a crucial role in meeting the ever-increasing energy demands of countries around the world. Combustion of coal in thermal power stations produces a variety of residues-fly ash, bottom ash, coal gasification ash. Fly ash is an amorphous mixture of ferroaluminosilicate minerals generated from combustion of ground or powdered coal at temperature ranging from 400-1500°C with 2%excess air (Mattigod et al.1990).It is chemically heterogenous in nature on account of being composed of large number of trace and heavy metals in variable proportions.

Fly ash alters the physical properties of soil such as texture, bulk density, water holding capacity and particle size distribution. Fly ash is considered as useful ameliorant which improve physical, biological and chemical properties of problematic soils and enhance the macro and micro-nutrients for plants (Jala and Goyal, 2006). It also increases the nutrient content of the soil which is beneficial for the plant growth.

CHAPTER 3 MATERIALS AND METHODS

3.1 Growth of heterocystous cyanobacteria

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.

Figure 3: Algal cultures grown in racks

3.2 Culture methods

Four cyanobacterial strains i.e *Anabaena variabilis* (ARM441), *Aulosira fertilissima* (ARM444), *Nostoc muscorum* (ARM442) and *Tolypothrix tenuis* (ARM443) were procured from National Centre for Conservation and Utilization 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-11 medium was prepared using double distilled water; pH was kept in range of 7.0-7.3 for optimal growth of cultures. Growth media were sterilized in autoclave at 121°C at 15 psi 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	1ml

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 grows 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 hetrocystous cyanobacterial strains in BG-11 medium (-N) were done by estimation of biomass.

3.3.1 Dry biomass production (Richmond and Gobbelaar, 1986)

a) The weight of dried Whatmann filter paper 42 was noted as initial reading.
b) The algal cultures were homogenized by vigrous shaking by adding glass beads to it and 10 ml of culture was taken and filtered through previously dried filter paper and noted its wet weight.

c) This was kept for drying and transferred to hot air oven maintained at about 60°C,till constant weight was recorded at room temperature.
d) The difference between initial and final reading of the weight gave the dry biomass in mg/ml.
e) Dry biomass production was studied after 7 days and studied the growth upto 30 days of inoculation.

3.3.2 Chlorophyll production (McKinney, 1941)

Reagent: Methanol (95%)

Procedure:

a) Algal suspension of 10ml was filtered through Whatmann 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 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°C for 30min, which lead 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 8000rpm for 10 minute.
e) Pigment of the solution was analyzed by spectrophotometer by comparing a sample of unknown transmission against a blank (96% methanol) of 100% transmission at 650 nm and 665nm.
f) Chlorophyll production was observed after every 7 days of inoculation and growth was studied up to 30 days of incubation.

Calculations

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

Where,

E_{650}=Absorbance at 650nm wavelength

E_{665}= Absorbance at 665nm wavelength

3.4 Plant growth promoting traits of different filamentous heterocystous cyanobacteria

Some plant growth promoting activities which produced growth stimulating hormone i.e. Indole acetic acid (IAA), Nitrate reductase activity, Solubilization of TCP.

3.4.1 Indole acetic acid estimation (Glickmann and Dessaux, 1995)

Stock solution for standard: Dissolve the 20mg IAA in 20ml of Acetone i.e. concentration of stock solution was 1mg/ml.

Reagent : Salkowski Reagent =Mix 2ml of 0.5M $Fecl_3$ and 49ml of water and 49ml of 70% percloric acid.

Procedure

a) The algal cultures were grown on BG-11 media, pH 7 at 25°C for 15 days supplemented with filter sterilized tryptophan of concentration 1mg/ml.
b) After 15 day, cyanobacterial cells were removed by centrifugation at 4000rpm for 20 minutes at 4°C.
c) Then, Salkowski reagent was added to the supernatant in a ratio of 1:2(v/v) and incubated for 30 minutes in dark conditions at room temperature.
d) Concentration of IAA estimated by taking absorbance at 535nm against a control of 1 ml culture medium and 2 ml of Salkowski reagent.
e) IAA production was studied with 30 days of inoculation.

3.4.2 Solubilisation of TCP by different filamentous heterocystous cyanobacteria.

Phosphate solubilisation was done by Available phosphorous of different heterocystous cyanobacterial cultures.

Available phosphorous in Cultures was estimated as per the method given by **Olsen et al., 1954.**

Reagents

a) 0.5 M $NaHCO_3$ extracting solution: 84 g 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 IM or 1N NaOH.
b) Reagent A: 12.0 g Ammonium molybdate in 250 ml distilled water and 0.2908 g antimony potassium tartarate in 100 ml distilled water was added to 1000 ml of 2.5 M H_2SO_4, mixed thoroughly and volume made upto 2L with distilled water.

c) Reagent B (freshly prepared): 1.058 g ascorbic acid in 200 ml of reagent A and mixed.
d) Sulphuric acid (2.5 M): 140 ml of concentrated H_2SO_4 diluted to 1 L.
e) Stock Standard P solution (50 ppm P): 0.2917 g KH_2PO_4 dissolved in water to a final volume of 1 L.
f) Working Standard P solution (1 ppm): 20 ml of (50 ppm P) solution diluted to 1L.

Procedure:

a) Filtered the 10ml of homogenized culture treated with TCP (1mg/ml) through Whatmann Filter paper No.42.
b) Transferred 10ml aliquot of the culture filtrate 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 again 15.5 ml of distilled water.
c) Prepared a blank as above. For standard curve: Took 0, 2, 5, 10, 15 and 20 ml of standard solution in 50 ml volumetric flasks separately. Added 10 ml of extracting solution, 1.0 ml of 2.5 M H_2SO_4, 8 ml Reagent B and make the final volume up to 50 ml.
d) The P concentrations of these solutions were 0.04, 0.1, 0.2, 0.3 and 0.4 ppm respectively. After 10 minutes, read the P concentration at 882 nm.

Calculations:

P in culture (ppm) = P in extract (ppm) x 20 (the standard soil to solution ratio)

3.4.3 Nitrate reductase(NR) Activity (Lowe and Evans,1964)

The nitrate reductase is a substrate inducible enzyme and its activity is based upon its ability to reduce nitrate to nitrite that is measured by diazocoupling method.

Composition of Basal medium.

Constituents	gm/L
1.$NaNO_3$	25
2.$CaCl_2.2H_2O$	2.5
3.$MgSO_4.7H_2O$	7.5
4.K_2HPO_4	7.5
5.KH_2PO_4	17.5
6.NaCl	2.5

7.EDTA KOH	50.0 31.0
8.$FeSO_4.7H_2O$ H_2SO_4	4.98 1ml
9.H_3BO_3	11.42
10.Trace element solution	1ml

Composition of Trace element solution:

CONSTITUENTS	gm/L
1.$MnCl_2.4H_2O$	1.44
2.$ZnSO_4.7H_2O$	8.82
3.MoO_3	0.71
4.$CuSO_4.5H_2O$	1.57
5.$Co(NO_3)_2.6H_2O$	0.49

Reagents:

a) 1% sulphanilamide in 100ml of 1N HCL.
b) 0.2% NEDD(N-1-naphthyl ethylene diamine dichloride)

Procedure

Stock solution: Dissolve 0.075g $NaNO_2$ in 100 ml of water i.e concentration of stock solution was 500µg NO_2/ml.

Standared Curve

Pipetted into 100 ml volumetric flasks 0, 5, 10, 20 and 50 ml of nitrite standard (corresponding to 0, 2.5, 5, 10 and 25 µg of nitrite) and diluted to about 50 ml with water. Add to each of the flasks 5ml of sulfanilamide solution and mix. After 3 min add 1 ml of coupling reagent, dilute to mark with water, mix and let stand for 15 min. Measure the absorbance of the solutions against water at 540 nm.

Sample:

a) 10ml of cyanobacterial suspension was taken and centrifuged (4000g, 10min) and pellet was washed with sterile water.
c) The pellet was incubated overnight in basal medium containing $NaNO_3$ to induce NR.

d) After incubation, one ml of sample was taken and to this added 2ml of reagent A.
e) Mixed them well and after 15 minutes, added 2 ml of reagent B.
f) The pink colored was allowed to develop for 15 minutes.
g) Absorbance was recorded at 540nm and the values were calibrated against the standard curve using $NaNO_2$.

Calculations:

Content of nitrite=A×5÷W

Where,

A = Amount of nitrite read from the standard curve (μg)

W = Weight of sample (g)

3.4.4 Hydrogen cyanide (HCN) production (Bakker and Scippers, 1987)

Reagents:

a) 2% Sodium carbonate
b) 0.5% picric acid

Procedure

a) 5ml of cyanobacterial suspension were spreaded on Petri dishes containing BG-11 media supplemented with Agar (1.6%).
b) A Whatmann No.1 filter paper soaked in 2% (w/v) sodium carbonate in 0.5 % (w/v) picric acid solution was placed inside the lid of a Petri dish.
c) The plates was then sealed with parafilm and incubated at 25°C for 3-4 days.

3.5 Characterization of fly ash

Coal fly ash procured from Guru Gobind Singh Thermal Power Plant (GGSTPP), Ropar showed alkaline pH of 7.85, electrical conductivity of 0.14μS/m, bulk density of 0.99 g/cm3 and water holding capacity of 62%.

3.6 Effect of coal fly ash on Plant growth promoting activities of different heterocystous cyanobacteria.

Effect of coal fly ash on plant growth promoting activities like Indole acetic acid, Nitrate Reductase Activity, Dry Biomass Production, Solubilisation of TCP (1mg/ml) was studied.

3.6.1 Effect of coal fly ash on Indole acetic acid estimation by different cyanobacteria.

Reagents: Salkowski Reagent =Mix 2ml of 0.5M $Fecl_3$ and 49ml of water and 49ml of 70% percloric acid.

Procedure:

a) The algal cultures were grown on BG-11 media, pH 7 at 25°C for 15 days. After 15 days these cultures were supplemented with filter sterilized tryptophan of concentration 1mg/ml.
b) Algal cultures were treated with different concentrations (%) of fly ash i.e. 0, 1,2,5,10,20.
c) Then cyanobacterial cells amended in fly ash were removed by centrifugation at 4000rpm for 20 minutes at 4°C.
d) Then, Salkowski reagent was added to the supernatant in a ratio of 1:2(v/v) and incubated for 30 minutes in dark conditions at room temperature.
e) Concentration of IAA estimated by taking absorbance at 535nm against a control of 1 ml culture medium and 2 ml of Salkwoski reagent.

3.6.2 Effect of coal fly ash on nitrate reductase activity by different cyanobacteria.

Reagents:

a) 1% sulphanilamide in 100ml of 1N HCL.
b) 0.2% NEDD (N-1-naphthyl ethylene diamine dichloride.

Procedure:

a) 10ml of cyanobacterial suspension amended in different concentration (0%, 1%, 2%,5%,10%,20%) of fly ash was taken and centrifuged (4000g,10min) and pellet was washed with sterile water.
b) The pellet was incubated overnight in basal medium containing $NaNO_3$ to induce NR.
c) After incubation, one ml of sample was taken and to this added 2ml of reagent A.
d) Mixed them well and after 15 minutes, added 2 ml of reagent B.
e) The pink colored was allowed to develop for 15 minutes.
f) Absorbance was recorded at 540nm and the values were calibrated against the standard curve using $NaNO_2$.

Calculations:

Content of nitrite=A×5÷W

Where,

A = Amount of nitrite read from the standard curve (μg)

W = Weight of sample (g)

3.6.3 Effect of coal fly ash on dry biomass production by different heterocystous cyanobacteria.

a) The weight of dried Whatmann filter paper 42 was noted as initial reading.
b) The algal cultures were homogenized by vigorous shaking by adding glass beads to it and 10 ml of culture amended with different concentrations of Fly ash was taken and filtered through previously dried filter paper and noted wet weight of algal cells with fly ash.
c) This was kept for drying and transferred to hot air oven maintained at about 60°C, till constant weight was recorded at room temperature.
d) The difference between initial and final reading of the weight gave the dry biomass in mg/ml

3.5.4 Effect of coal fly ash on phosphate solubilisation by different heterocystous cyanobacteria.

Reagents:

a) 0.5 M $NaHCO_3$ extracting solution: 84 g 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 IM or 1N NaOH.
b) Reagent A: 12.0 g Ammonium molybdate in 250 ml distilled water and 0.2908 g antimony potassium tartarate in 100 ml distilled water was added to 1000 ml of 2.5 M H_2SO_4, mixed thoroughly and volume made up to 2L with distilled water.
c) Reagent B (freshly prepared): 1.058 g ascorbic acid in 200 ml of reagent A and mixed.
d) Sulphuric acid (2.5 M): 140 ml of concentrated H_2SO_4 diluted to 1 L.
e) Stock Standard P solution (50 ppm P): 0.2917 g KH_2PO_4 dissolved in water to a final volume of 1 L.
f) Working Standard P solution (1 ppm): 20 ml of (50 ppm P) solution diluted to 1L.

Procedure:

a) Filtered the 10ml of homogenized culture amended with different concentrations of Fly ash (0%, 5%, 10%, 20%) through Whatmann Filter paper No.42.

e) Transferred 10ml aliquot of the culture filtrate 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 again 15.5 ml of distilled water.

f) Prepared a blank as above. For standard curve: Took 0, 2, 5, 10, 15 and 20 ml of standard solution in 50 ml volumetric flasks separately. Added 10 ml of extracting solution, 1.0 ml of 2.5 M H_2SO_4, 8 ml Reagent B and make the final volume up to 50 ml.

g) The P concentrations of these solutions were 0.04, 0.1, 0.2, 0.3 and 0.4 ppm respectively. After 10 minutes, read the P concentration at 882 nm.

Calculations:

P in culture (ppm) = P in extract (ppm) x 20 (the standard soil to solution ratio)

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Growth Studies

Growth of different heterocystous cyanobacterial strains was studied by estimation of dry biomass production (mg/ml) and chlorophyll production (mg/ml) after 30 days of incubation.

4.1.1 Dry Biomass (mg/ml) of different heterocystous cyanobacteria.

Dry Biomass was estimated by Richmond and Gobbelaar, 1986 on dry weight basis (mg/ml)

S.No	Cyanobacterial strains	0^{th} day	7^{th} day	14^{th} day	21^{st} day	28^{th} day
1	*Anabaena variabilis* (ARM 441)	0.009±0.01	0.21±0.19	0.58±0.01	1.25±0.20	1.75±0.31
2	*Nostoc muscorum (*ARM 442)	0.05±0.02	0.16±0.09	0.42±0.14	1.15±0.16	1.52±0.29
3	***Aulosira fertilissima* (ARM 444)**	**0.1±0.05**	**0.25±0.16**	**0.65±0.18**	**1.35±0.15**	**2.12±0.36**
4	*Tolypothrix tenuis* (ARM 443)	0.005±0.04	0.19±0.10	0.50±0.18	1.16±25	1.68±0.31

Table 7: Dry biomass production (mg/ml) of heterocystous cyanobacteria strains at different intervals of time.

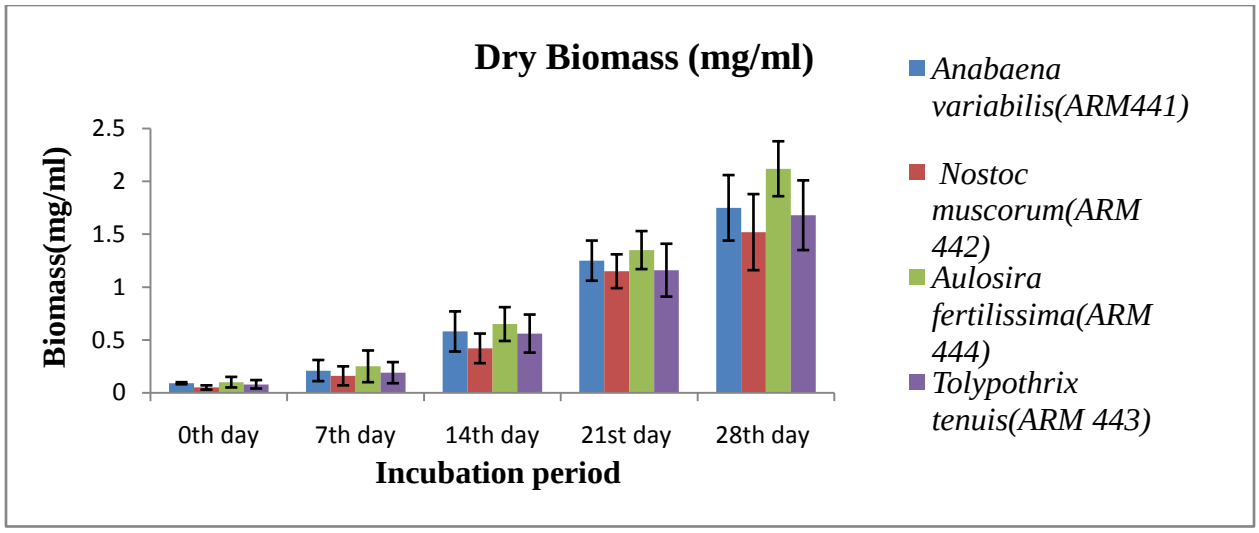

Figure 4: Dry biomass (mg/ml) of heterocystous cyanobacterial strains after 30 days incubation.

Study was performed to observe the growth of *Aulosira fertilissimia* (ARM444), *Anabaena variabilis* (ARM441), *Nostoc muscorum* (ARM442), *Tolypothrix tenuis* (ARM443).

Table 7 and Fig.4 showed growth of cyanobacterial strain observed as dry biomass (mg ml^{-1}). Overall, biomass increased with increase in time interval of seven days. *Aulosira fertilissima* showed maximum increased biomass (0.1mg/ml) as zero day to (2.12 mg/ml) observed at 28^{th} day followed by *Anabaena variabilis* (1.52 mg/ml), *Tolypothrix tenuis* (1.68 mg/ml), *Nostoc muscorum*(1.52 mg/ml) at 28^{th} day. Chouhan et al., 2013 reported that total biomass produced by cyanobacterial strain increased with increase in time intervals as observed in *Nostoc s*pp.

4.1.2 Chlorophyll production (mg/ml) of heterocystous cyanobacterial strains

S .No	Algal strains	0th day	7th day	14th day	21st day	28th day
1	*Anabaena variabilis* (ARM 441)	0.09 ±0.01	0.11±0.01	0.18±0.04	0.27±0.02	0.29 ±0.14
2	*Nostoc muscorum (*ARM 442)	0.08±0.01	0.10±0.01	0.14±0.03	0.24 ±0.05	0.27±0.005
3	***Aulosira fertilissima* (ARM 444)**	**0.07±0.01**	**0.13±0.20**	**0.20±0.04**	**0.23±0.05**	**0.35±0.01**
4	*Tolypothrix tenuis* (ARM 443)	0.06±0.02	0.14±0.01	0.22±0.12	0.24±0.02	0.28±0.015

Table 8: Chlorophyll production (mg/ml) of heterocystous cyanobacterial strains.

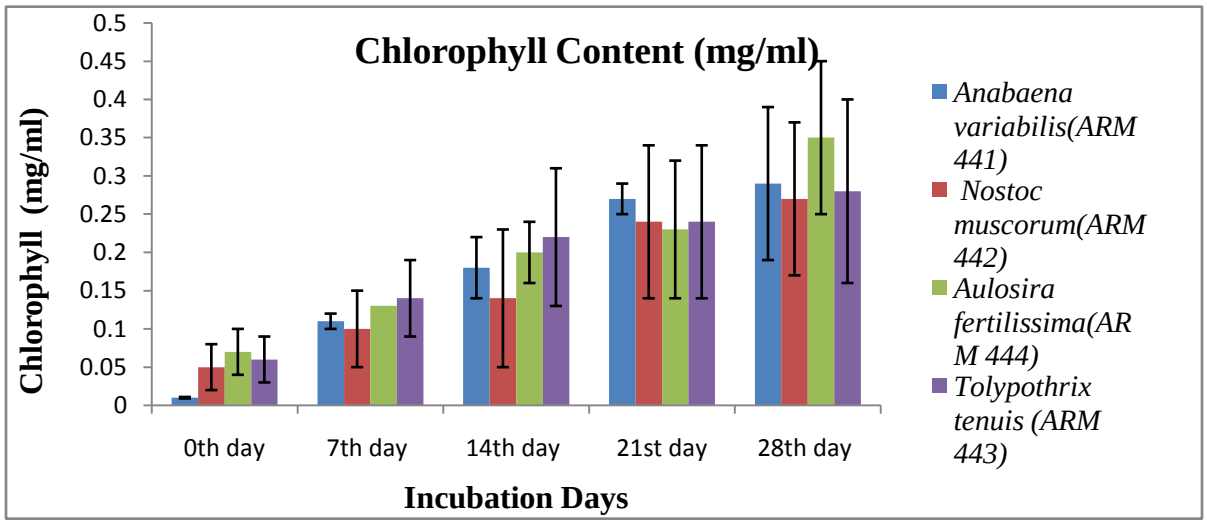

Figure. 5: Chlorophyll (mg/ml) of different cyanobacterial strains at different time intervals.

Table 8 and Fig.5 showed maximum chlorophyll content in *Aulosira fertilissima* (ARM444) from 0.07 mg/ml at zero day to 0.35 mg/ml at 28th day followed by *Anabaena variabilis* (ARM 441) 0.29±0.14 mg/ml, *Tolypothrix tenuis* (ARM443) 0.28±0.01 mg/ml, *Nostoc muscorum* (ARM442) 0.27±0.14 mg/ml when observed at 28th day. Increase in chlorophyll content could be

due to increase in biomass production. Sayed et al., 2010 studied growth of two cyanobacterial strain, *Anabaena* sp and *Calothrix* sp and reported that chlorophyll-a content increases with the amendment of nutrient supplementation of inorganic carbon source.

4.2 Plant growth promoting activity (PGP) of cyanobacterial strains

4.2.1 Indole acetic acid (IAA)

S.No	Concentration of IAA (µg/ml)	Volume of IAA(µl)	Volume of media(µl)	Volume of Salkowski Reagent (ml)	Incubation	Optical density at 535nm
1	0	0	1000	2	Incubated at room temperature in dark conditions	0.00
2	5	5	995	2		0.006
3	10	10	990	2		0.233
4	20	20	980	2		0.254
5	30	30	970	2		0.308
6	40	40	960	2		0.437
7	50	50	950	2		0.509
8	60	60	940	2		0.603
9	70	70	930	2		0.699
10	80	80	920	2		0.785
11	90	90	910	2		0.845
12	100	100	900	2		0.935

Table 9a: OD at 535nm for standard Curve of IAA production

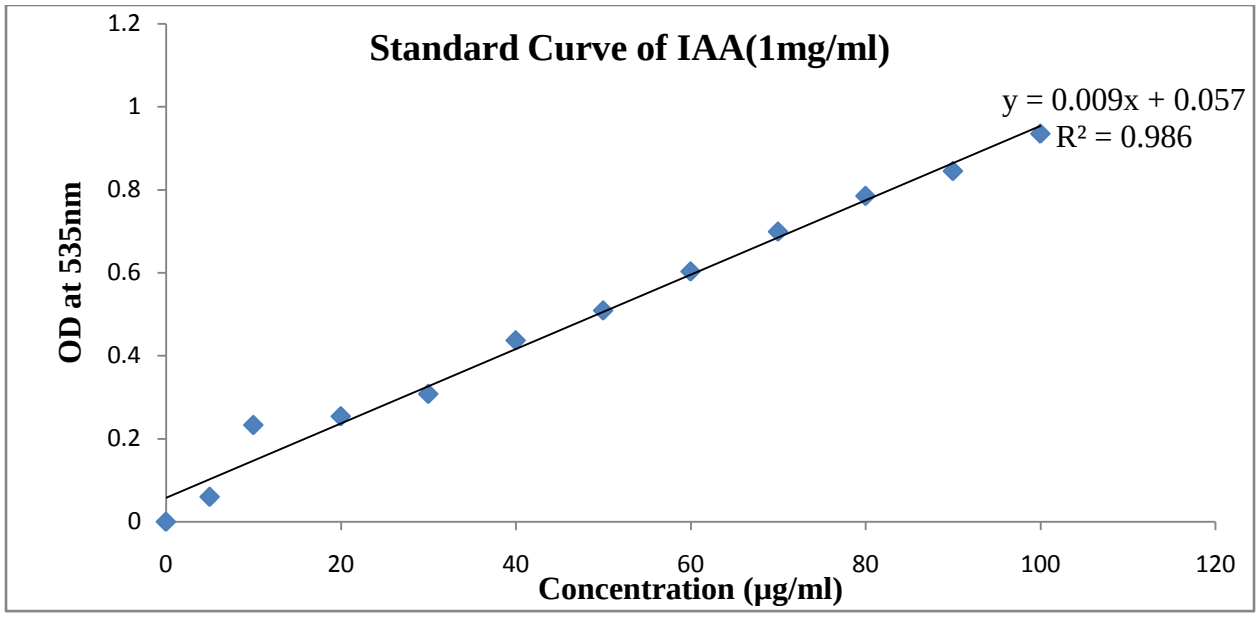

Figure 6a: Standard Curve of IAA production

S.No	Cyanobacterial Strains	0th Day	7th Day	14th Day	21st Day	28th Day
1	Nm	0.007±0.003	0.013±0.009	0.019±0.000	0.025±0.002	0.030±0.013
2	Nm+T	0.014±0.001	0.019±0.057	0.020±0.002	0.036±0.001	0.042±0.028
3	**Af**	**0.003±0.001**	**0.013±0.005**	**0.025±0.001**	**0.037±0.001**	**0.042±0.014**
4	**Af+T**	**0.010±0.003**	**0.027±0.011**	**0.039±0.002**	**0.045±0.002**	**0.050±0.011**
5	Av	0.004±0.002	0.018±0.131	0.027±0.002	0.030±0.001	0.041±0.009
6	Av+T	0.017±0.002	0.023±0.005	0.035±0.001	0.043±0.001	0.048±0.005
7	Tt	0.008±0.002	0.015±0.001	0.026±0.001	0.032±0.002	0.035±0.005
8	Tt+T	0.013±0.002	0.027±0.459	0.032±0.007	0.035±0.001	0.045±0.003

Table 9b: IAA estimation of cyanobacterial strains at different time intervals

Mean±SE(n=3)

Nostoc muscorum	*= Nm;*	*Nostoc muscorum* + Tryptophan (1mg/ml)	=Nm+ T
Aulosira fertilissimia	*=Af:*	*Aulosira fertilissimia* + Tryptophan (1mg/ml)	=Af+T
Anabaena variabilis	*=Av;*	*Anabaena variabilis*+ Tryptophan (1mg/ml)	=Av+T
Tolypothrix tenuis	*=Tt;*	*Aulosira fertilissimia* + Tryptophan (1mg/ml)	=Tt+T

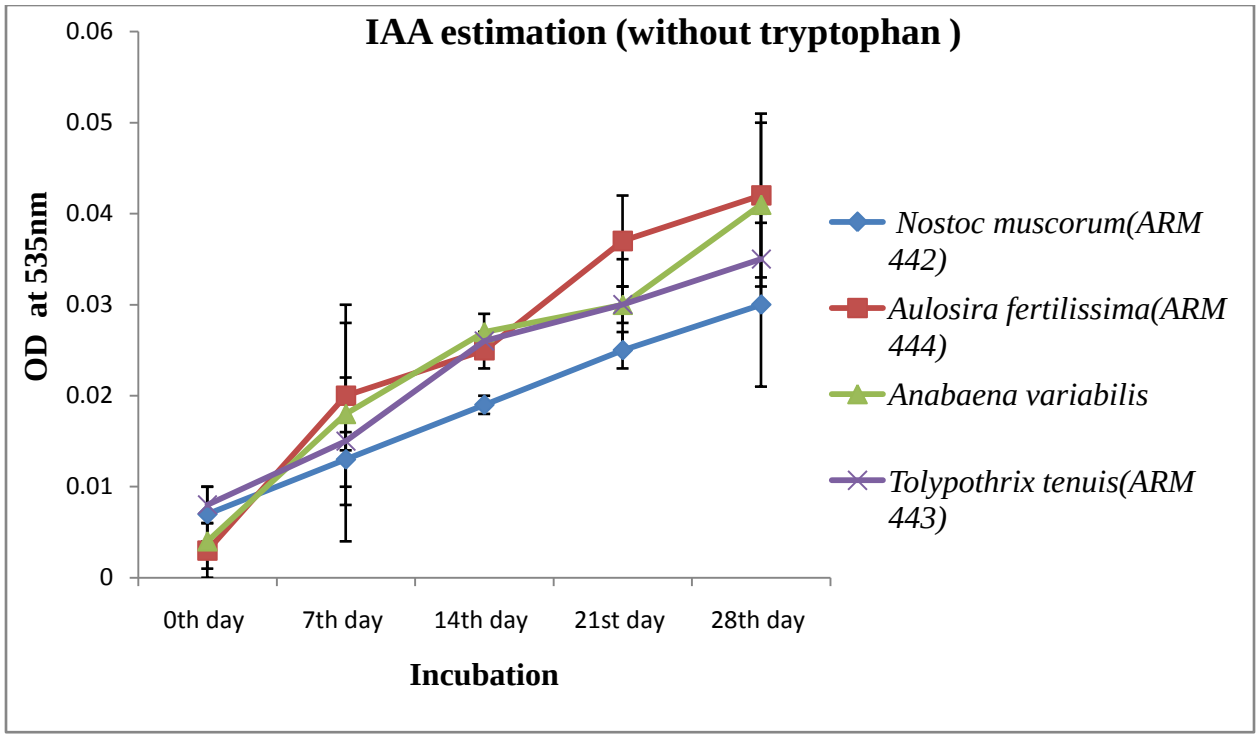

Figure 6b: IAA estimation of different cyanobacterial strains without tryptophan.

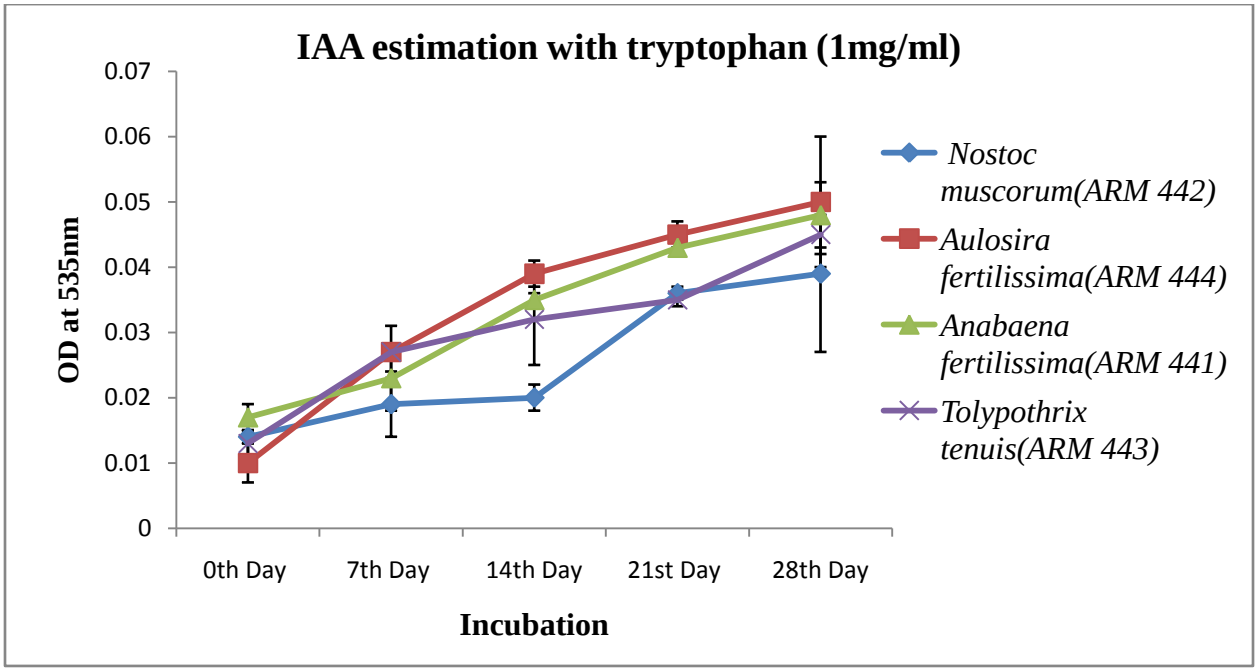

Figure 6c: IAA estimation of different cyanobacteria with tryptophan.

Indole-3 –acetic acid, a plant growth regulator and when a cyanobacterial strain multiplies in soil, they secrete extracellular IAA during stationary phase enhancing plant growth. Four cyanobacterial strains such as *Anabaena variabilis, Aulosira fertilissima, Tolypothrix tenuis*, *Nostoc muscorum* were screened for IAA production with or without L-tryptophan and it was revealed that maximum IAA production was observed in the presence of L -tryptophan (1mg/ml). Since L-tryptophan is the main precursor for IAA production (Perumal Varalakshmi et al., 2012). Maximum IAA production was observed in *Aulosira fertilissima* (ARM444) 0.050µg/ml followed by *Anabaena variabilis* (ARM441) 0.048µg/ml, *Tolypothrix tenuis* (ARM443) 0.045 µg/ml and *Nostoc muscorum* (ARM442) 0.042 µg/ml at 28th days of inoculation. Sergeeva, 2002 reported that tryptophan was considered as a main precursor for IAA accumulation in cell extracts and supernatant of algal strains. Similar results were reported by Chouhan et al., 2013 that IAA production increased with incubation time and showed maximum concentration after 30 days as observed in *Nostoc* spp.

4.2.2 Nitrate reductase (NR) activity (mg/g) of different cyanobacterial strain.

S.NO	Concentration of $NaNO_2$ (µg/ml)	Volume of $NaNO_2$(ml)	Volume of water (ml)	Volume of Reagent A(ml)	Volume of Reagent B (ml)	Incubation	Optical density at 540nm
1	0	0	50	5	0.5	Incubated at room temperature for 2 min	0
2	0.125	0.25	49.8	5	0.5		0.016
3	0.25	0.5	49.5	5	0.5		0.075
4	0.5	1.0	49.0	5	0.5		0.096
5	0.75	1.5	48.5	5	0.5		0.145
6	1.0	2.0	48.0	5	0.5		0.187
7	1.25	2.5	47.5	5	0.5		0.245
8	1.5	3.0	47.05	5	0.5		0.278

Table 10a: OD at 540nm for standard curve of sodium nitrite ($NaNO_2$)

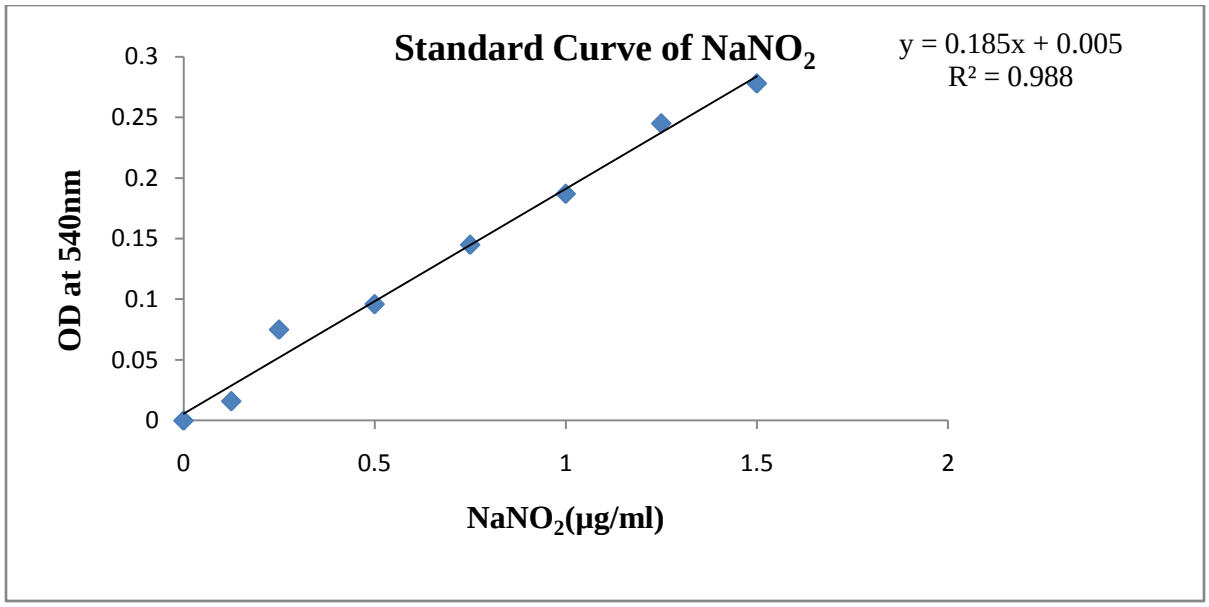

Figure 7a: Standard Curve of Sodium nitrite ($NaNO_2$)

S.No	Cyanobacterial strains	0th day	7th day	14th day	21st day
1	*Anabaena variabilis (ARM 441)*	0.187±0.01	0.395±0.04	0.408±0.02	0.523±0.02
2	*Nostoc muscorum (ARM 442)*	0.201±0.02	0.312±0.03	0.445±0.03	0.474±0.03
3	*Aulosira fertilissima (ARM 444)*	0.262±0.03	0.326±0.03	0.411±0.04	0.543±0.04
4	*Tolypothrix tenuis (ARM 443)*	0.145±0.05	0.203±0.04	0.294±0.03	0.318±0.03

Table 10b: Nitrate reductase (NR) activity of different cyanobacteria at different time intervals

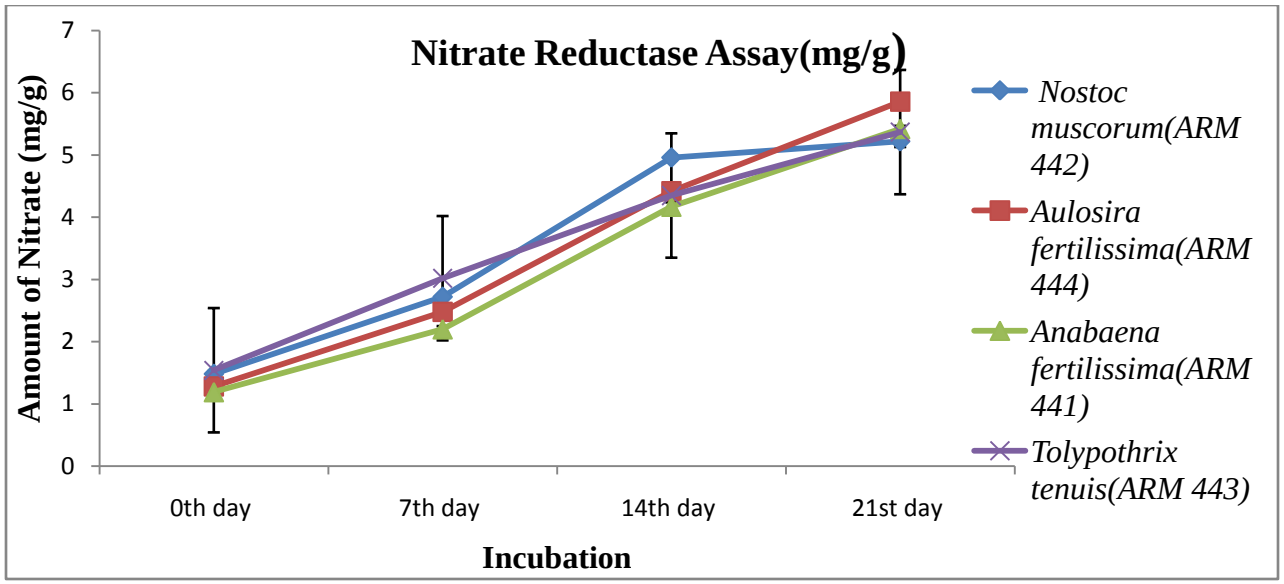

Figure 7b: Nitrate reductase (NR) activity of different heterocystous cyanobacteria strain.

In Table 10b and Fig.8b *Aulosira fertilissima* (ARM444) showed highest NR activity 0.262±0.03mg/g at zero day to 0.543±0.04 mg/g at 28th day followed by *Anabaena variabilis* (ARM 441) 0.523mg/g, *Nostoc muscorum (*ARM442) 0.474mg/g, *Tolypothrix tenuis* (ARM443) (0.318mg/g) after 30 days of incubation. Nitrate reductase activity was shown by cyanobacterial strains in the presence of nitrate at the concentration of 10mM as the nitrogen source that induces NR in the basal media (Lowe and Evans, 1964). NR activity increases with increase in biomass content as increased with incubation time. Flores et al., (1981) studies on cellular activity of nitrate reductase in different cyanobacteria which revealed that nitrate play an active role in nitrate reductase synthesis in filamentous nitrogen fixing strains of *Anabaena* sp and *Nostoc* sp with ammonium acting as an antagonist with regard to nitrate.

4.2.3 Solubilisation of TCP by different heterocystous cyanobacteria.

S.No	Concentration of PO_4^- (ppm)	Volume of KH_2PO_4(ml)	Volume of Extracting solution(ml)	Volume of H_2SO_4 added(ml)	Volume of Reagent B (ml)		Optical density at 882nm
1	0	0	10	1	8	Make up the volume up to 50ml with dd.H_2O	0.00
2	0.04	2	10	1	8		0.08
3	0.1	5	10	1	8		0.018
4	0.2	10	10	1	8		0.357
5	0.3	15	10	1	8		0.520
6	0.4	20	10	1	8		0.70

Table 11a: OD at 882nm for standard curve of available phosphorous.

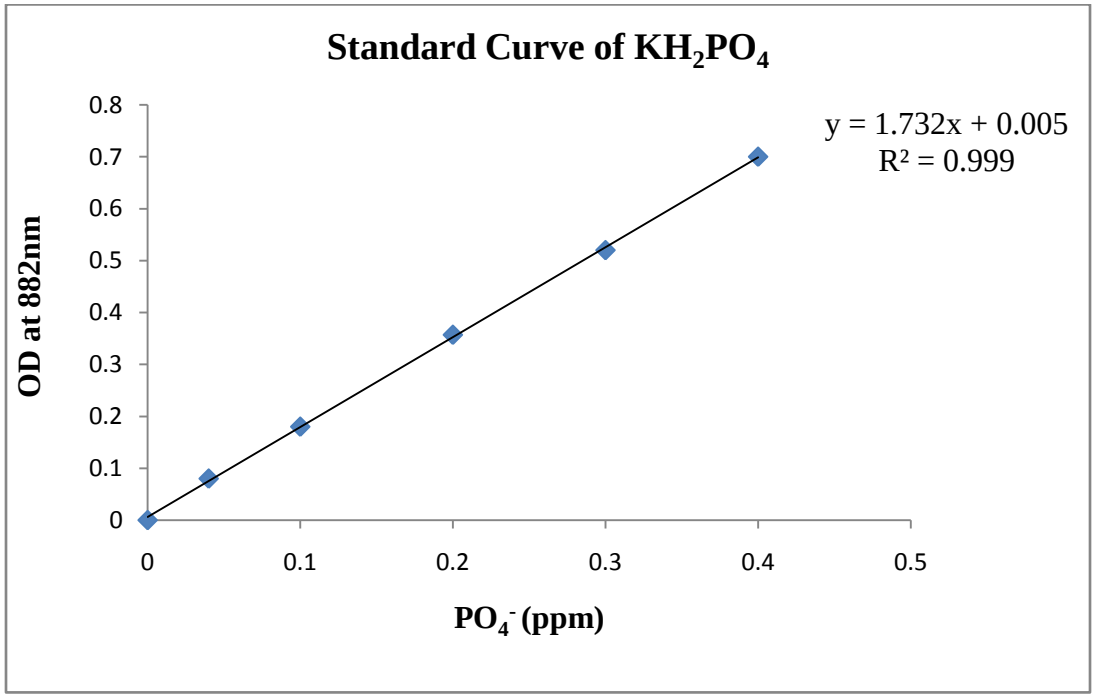

Figure 8a: Standard curve of available phosphorous

		(Concentration in ppm)			
S.No	**Cyanobacterial strains**	**0th day**	**7th day**	**14th day**	**21st day**
1	*Nostoc muscorum(ARM 442)*	1.48±0.5	2.72±0.8	4.96±0.9	5.22±1.1
2	***Aulosira fertilissima(ARM 444)***	**1.28±0.5**	**2.48±0.9**	**4.42±0.6**	**5.86±1.1**
3	*Anabaena fertilissima(ARM 441)*	1.19±0.6	2.2±0.7	4.17±0.5	5.42±1.2
4	*Tolypothrix tenuis(ARM 443)*	1.54±0.7	3.02±0.5	4.35±0.9	5.37±1.1

Table 11b: Solubilization of TCP (1mg/ml) by different heterocystous cyanobacteria.

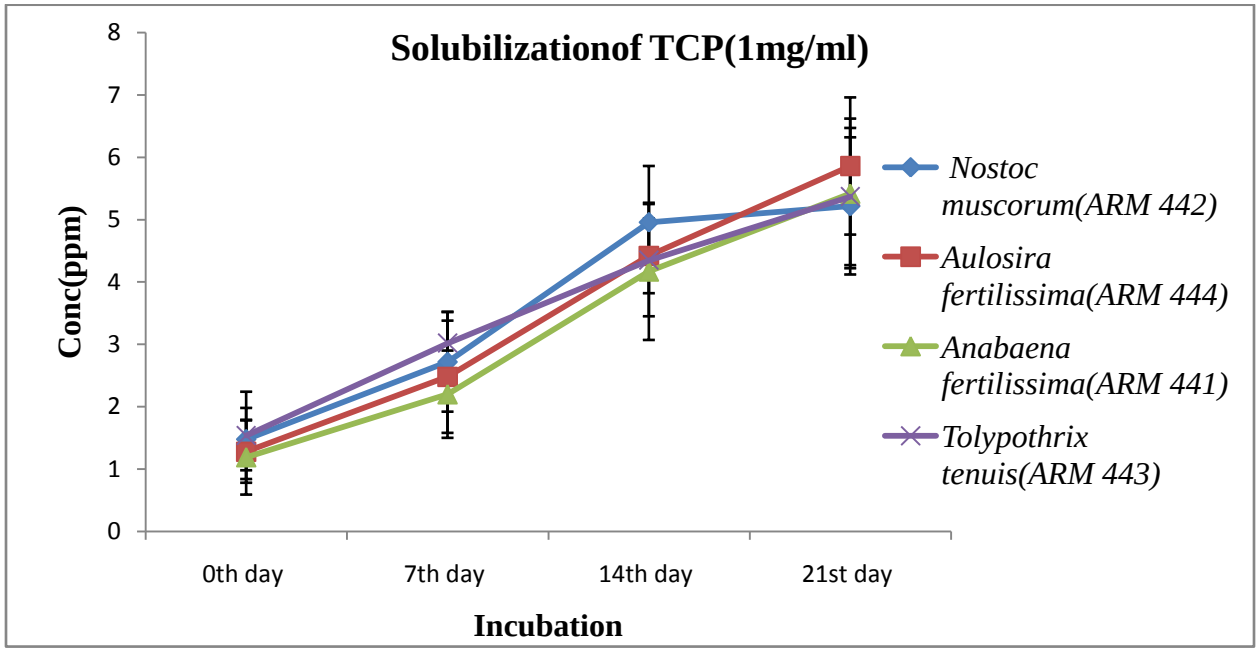

Figure8b: Solubilization of TCP (1mg/ml) by different heterocystous cyanobacterial strains.

All cyanobacterial strains should solubilization of insoluble tricalcium phosphate (TCP) with gradual increase in available phosphorous at different time intervals of seven days. Yandigeri et al., (2010) reported that microorganism solubilizes insoluble phosphates and only a part of it is used for their own growth and metabolism. *Aulosira fertilissima* showed maximum solubilization of TCP (1mg/ml) from 1.28 ppm as zero day to 5.86 ppm at 28th day followed by *Anabaena variabilis* (5.42 ppm), *Tolypothrix tenuis* (5.37ppm), *Nostoc muscorum* (5.22 ppm) as observed on 28th day after the inoculation. Natesan et al., 1989 reported that the *Anabaena* strain solubilized extracellular tricalcium phosphate through increased phosphatase activity.

4.2.4 HCN production

In Blue Green algae there was no HCN production reported

Figure 9: No Results were observed on BG-11 agar plates for HCN production in different cyanobacterial strains.

4.3 Effect of Coal Fly ash on Plant growth promoting activity of different heterocystous cyanobacteria.

4.3.1a: Effect of coal fly ash on Indole acetic acid (IAA) production by *Anabaena variabilis*

Sr.No	% of fly ash	0th day	7th day	14th day	21st Day	28th day
1	0	0.002±0.001	0.006±0.001	0.010±0.001	0.015±0.001	0.019±0.001
2	0+T	0.004±0.001	0.010±0.001	0.013±0.001	0.020±0.001	0.021±0.001
3	1	0.005±0.002	0.009±0.001	0.014±0.001	0.016±0.001	0.023±0.001
4	1+T	0.008±0.001	0.013±0.001	0.017±0.001	0.025±0.001	0.027±0.001
5	2	0.006±0.001	0.012±0.001	0.019±0.001	0.021±0.001	0.029±0.001
6	2+t	0.009±0.001	0.018±0.001	0.020±0.001	0.027±0.001	0.032±0.001
7	5	0.010±0.001	0.015±0.001	0.021±0.001	0.029±0.001	0.035±0.001
8	5+T	0.011±0.001	0.020±0.001	0.029±0.001	0.034±0.01	0.037±0.001
9	**10**	**0.012±0.001**	**0.019±0.001**	**0.032±0.002**	**0.038±0.001**	**0.040±0.001**
10	**10+T**	**0.015±0.001**	**0.025±0.001**	**0.036±0.001**	**0.043±0.001**	**0.045±0.001**
11	20	0.007±0.001	0.010±0.001	0.016±0.001	0.019±0.001	0.020±0.001
12	20+T	0.009±0.001	0.013±0.001	0.020±0.001	0.021±0.001	0.023±0.001

Mean±SE (n=3) T=tryptophan (1mg/ml) (maximum yield in bold)

Table 12a: IAA production by *Anabaena variabilis* at different concentration of fly ash

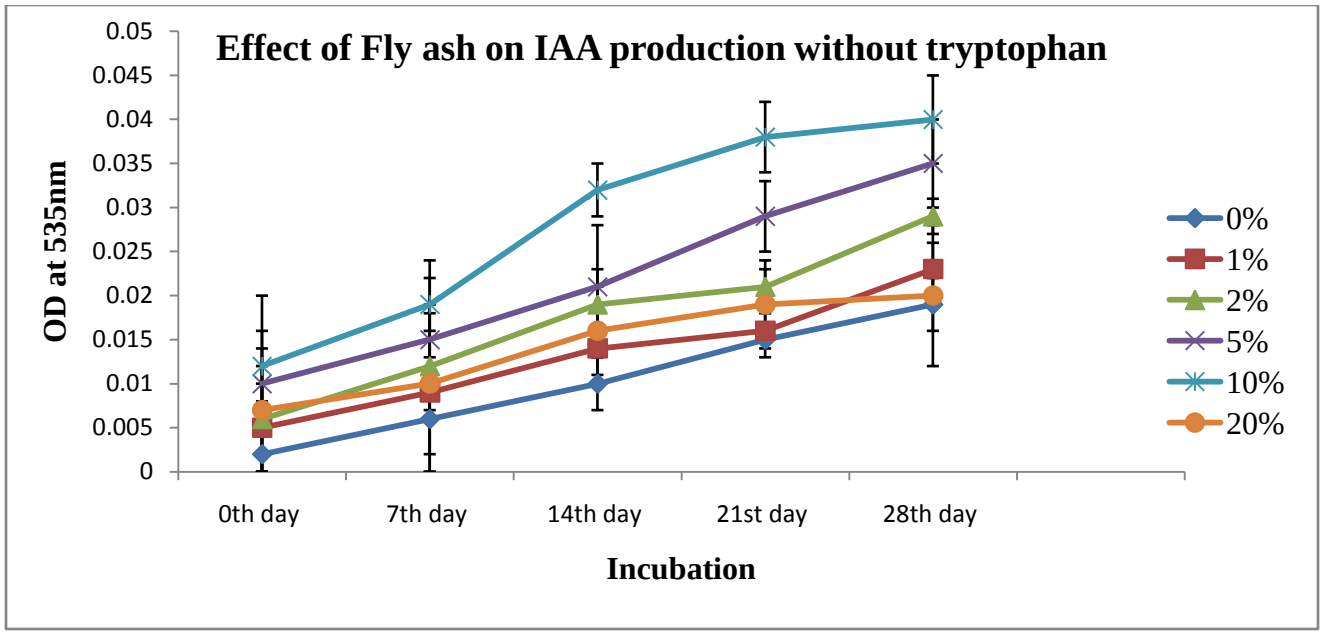

Figure 10a: Effect of fly ash on IAA production by *Anabaena variabilis* without tryptophan.

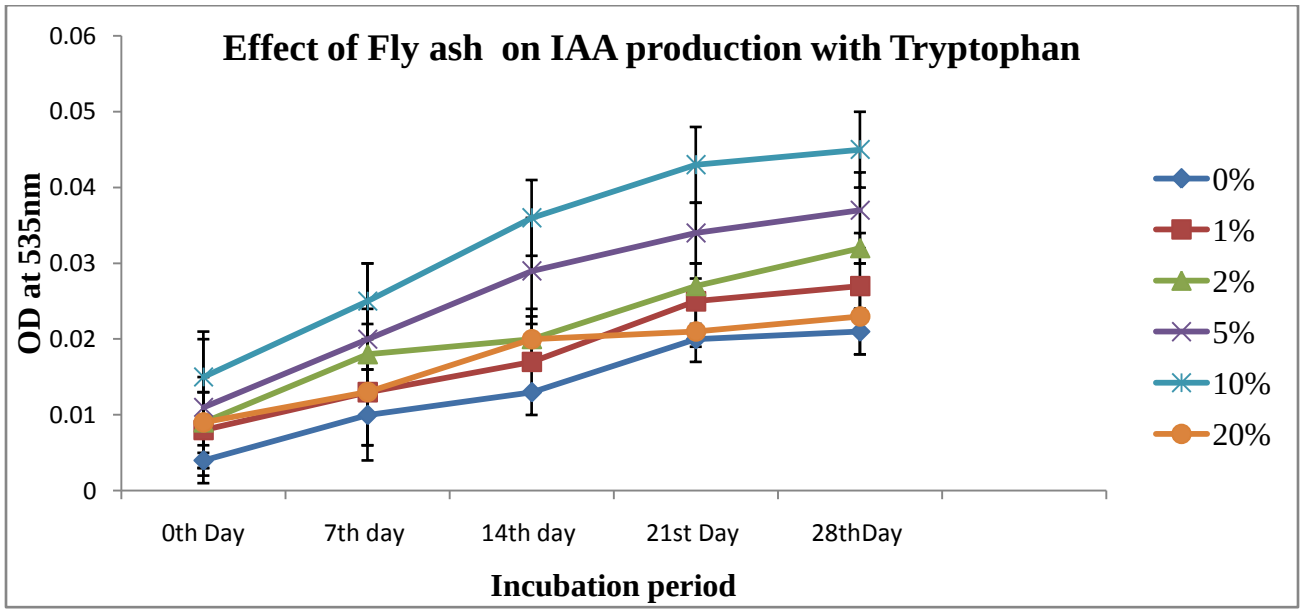

Figure 10b: IAA production of *Anabaena variabilis* with tryptophan (1mg/ml) at different concentration of fly ash.

4.3.1b: Effect of coal fly ash on IAA production by *Aulosira fertilissima*

S .no	%Fly ash	0th day	7th day	14th day	21st Day	28th day
1	0	0.002±0.010	0.006±0.001	0.010±0.001	0.015±0.001	0.017±0.001
2	0+t	0.004±0.001	0.007±0.001	0.015±0.001	0.021±0.001	0.022±0.001
3	1	0.003±0.001	0.008±0.001	0.013±0.001	0.018±0.001	0.021±0.002
4	1+t	0.005±0.002	0.010±0.001	0.020±0.001	0.026±0.002	0.029±0.001
5	2	0.007±0.001	0.012±0.001	0.019±0.001	0.023±0.001	0.025±0.001
6	2+t	0.009±0.001	0.014±0.001	0.023±0.001	0.027±0.001	0.033±0.001
7	5	0.010±0.001	0.016±0.001	0.021±0.002	0.029±0.001	0.034±0.001
8	5+t	0.013±0.001	0.021±0.001	0.030±0.001	0.037±0.001	0.045±0.001
9	**10**	**0.011±0.001**	**0.023±0.001**	**0.029±0.001**	**0.036±0.001**	**0.049±0.001**
10	**10+t**	**0.019±0.001**	**0.027±0.001**	**0.042±0.001**	**0.049±0.001**	**0.052±0.001**
11	20	0.008±0.001	0.012±0.001	0.019±0.001	0.020±0.001	0.024±0.001
12	20+t	0.010±0.002	0.015±0.001	0.024±0.001	0.026±0.001	0.027±0.001

Mean±SE (n=3) T=Tryptophan (1mg/ml) (maximum yield in bold)

Table 12b: Effect of fly ash on IAA production by *Aulosira fertilissima*

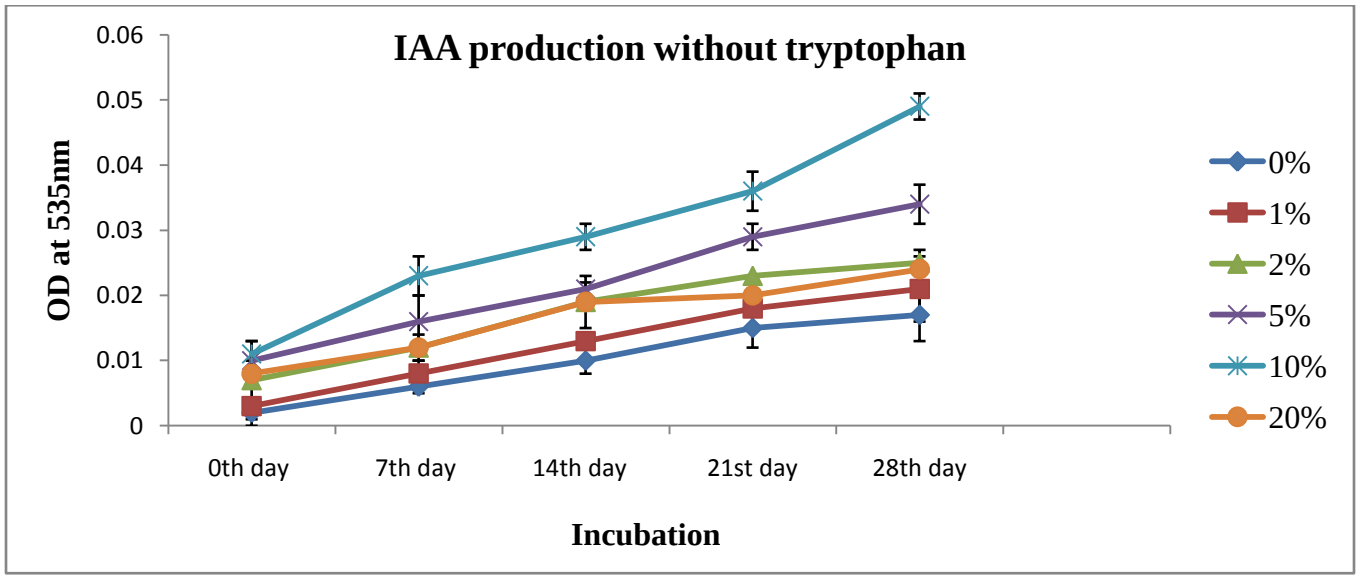

Figure 10c: IAA production of *Aulosira fertilissima* without tryptophan with different concentration of Fly ash.

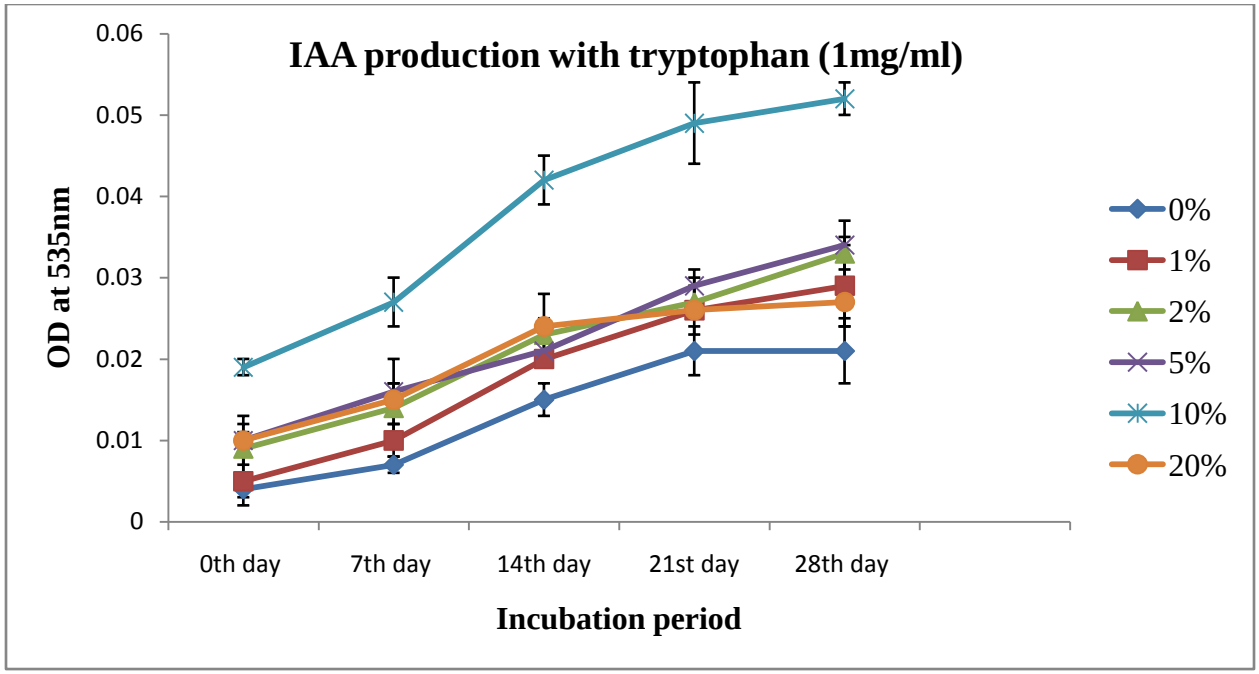

Figure 10d: IAA production of *Aulosira fertilissima* with tryptophan (1mg/ml) at different concentrations of fly ash.

Study on growth performance of *Anabaena variabilis* (ARM441) and *Aulosira fertilissima* (ARM444) at different concentration of fly ash (0, 1, 2, 5, 10, and 20 %) reveals that both cyanobacterial strains showed maximum IAA production with tryptophan (1mg/ml) by (0.045µg/ml) in *Anabaena variabilis* and (0.052 µg/ml) in *Aulosira fertilissima* as observed on 28th day after the inoculation days of incubation time. Tryptophan considered as main precursor of IAA production as it showed maximum IAA production at 10% fly ash. It was concluded that within increase the concentration of fly ash the IAA production was decreased in the presence of tryptophan. Fly ash added at optimum concentration of 10% (w/v) to soil showed no adverse effect on microbial activity. Baath et al., 1995 reported that beyond 10% of fly ash some decrease in microbial activity due to decrease in soil-microbe ratio and increased mobility of metals.

4.3.2a Effect of coal fly ash on Dry Biomass production (mg/ml) by *Anabaena variabilis*

S.No	%of FA	0th day	7th day	14th day	21st day	28th day
1	0	0.20±0.15	0.43±0.04	0.89±0.46	1.01±0.24	1.37±0.19
2	1	0.25±0.01	0.46±0.02	0.91±0.04	1.11±0.04	1.45±0.04
3	2	0.28±0.02	0.51±0.02	1.06±0.03	1.23±0.04	1.54±0.04
4	5	0.32±0.01	0.65±0.01	1.12±0.02	1.49±0.02	1.79±0.03
5	**10**	**0.38±0.01**	**0.78±0.02**	**1.26±0.03**	**1.65±0.02**	**1.93±0.03**
6	20	0.26±0.01	0.32±0.01	0.95±0.02	1.05±0.03	1.10±0.04

Mean ±SE (n=3)

Table13a: Dry biomass production (mg/ml) by *Anabaena variabilis* at different concentration of fly ash.

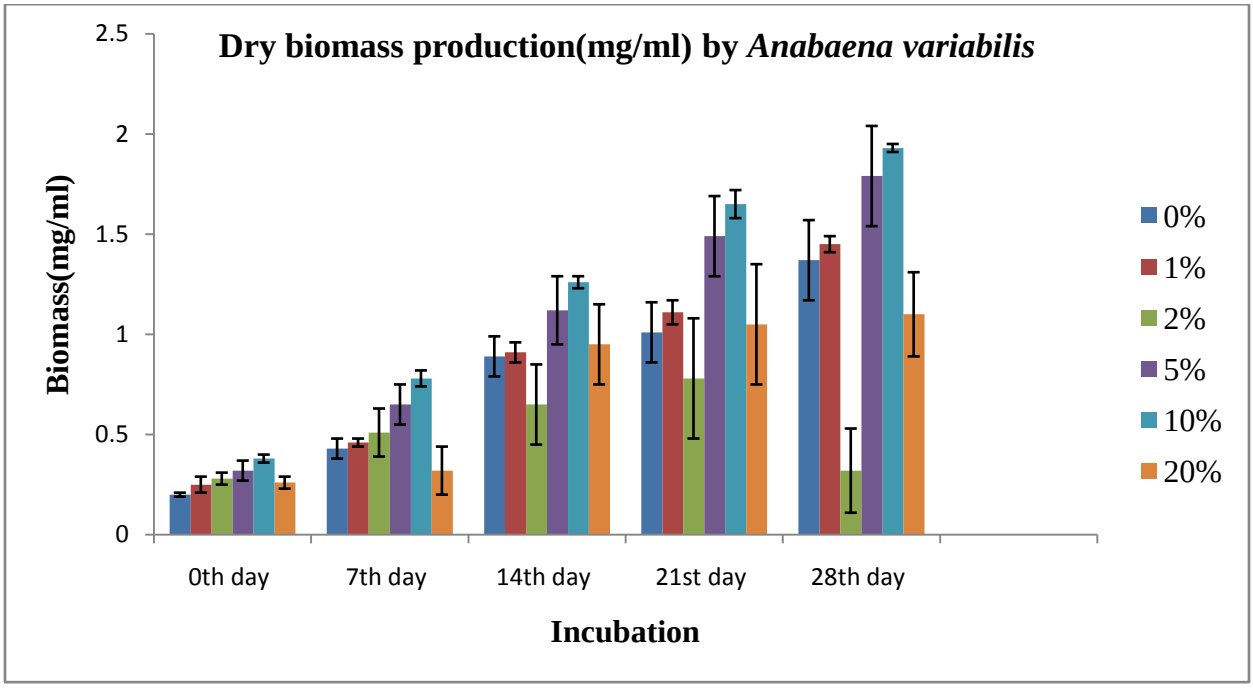

Figure 11a: Dry biomass estimation (mg/ml) of *Anabaena variabilis* at different concentration of fly ash.

4.3.2b Effect of coal fly ash on dry biomass production (mg/ml) by *Aulosira fertilissima*

S.No	%of FA	0th day	7th day	14th day	21st day	28th day
1	0	0.31±0.01	0.46±0.04	0.85±0.46	1.11±0.24	1.83±0.19
2	1	0.42±0.01	0.49±0.02	0.95±0.04	1.21±0.04	1.87±0.04
3	2	0.56±0.02	0.54±0.02	1.06±0.03	1.33±0.04	1.98±0.04
4	5	0.65±0.01	0.69±0.01	1.19±0.02	1.59±0.02	2.29±0.03
5	**10**	**0.84±0.01**	**0.75±0.02**	**1.58±0.03**	**1.98±0.02**	**2.57±0.03**
6	20	0.38±0.01	0.49±0.01	0.89±0.02	1.14±0.03	1.20±0.04

Mean ±SE (n=3)

Table13b: Dry biomass production (mg/ml) of *Aulosira fertilissima* with different concentrations of fly ash

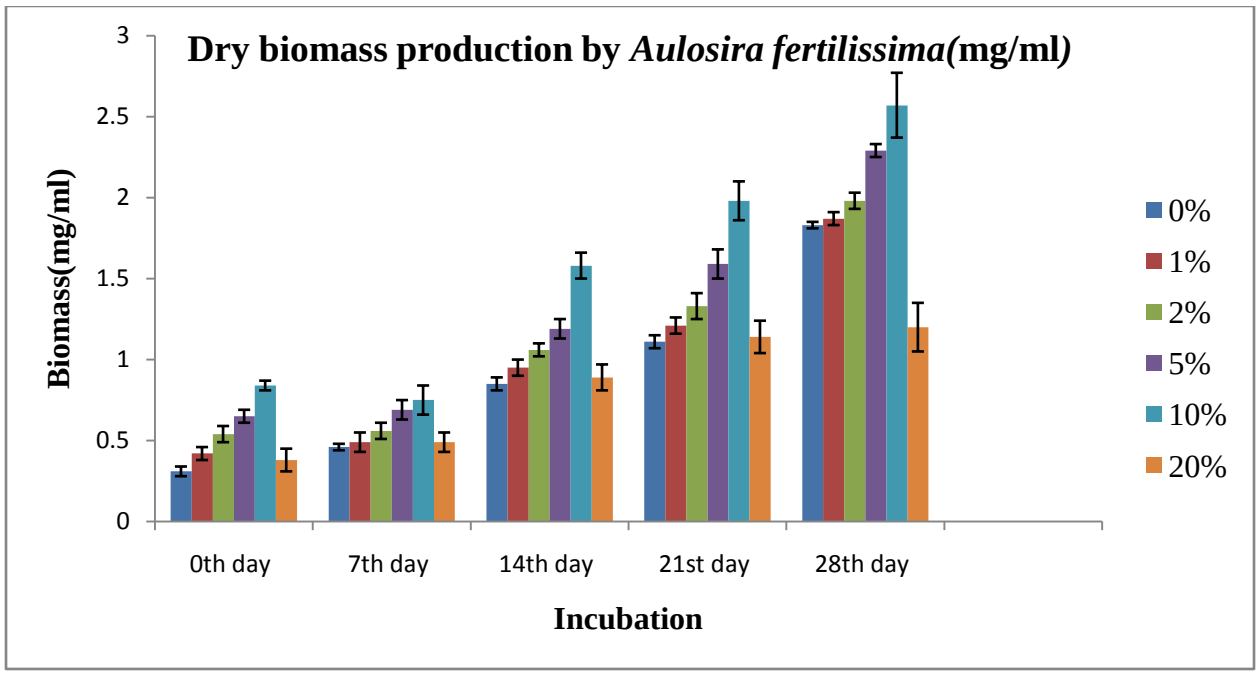

Figure 11b: Dry biomass production (mg/ml) of *Aulosira fertilissima* at different concentrations of fly ash.

Dry biomass production (mg/ml) of *Aulosira fertilissima (*ARM444) was 0.84±0.01 mg/ml at zero day to 2.57±0.03 mg/ml at 28th day of incubation at 10% of fly ash and it was decreased to 1.20±0.04 mg/ml on 28th day at 20% of fly ash, Whereas in *Anabaena variabilis*

(ARM 441) 0.38±0.01mg/ml at zero day to 1.93 mg/ml at 28th day of inoculation. At 20% of fly ash it was decreased to 1.10± 0.04 mg/ml at 28th day. It was concluded that at high concentration of fly ash of blue green algae (*Anabaena variabilis and Aulosira fertilissima*) was decreased due to inhibition of metabolic microbial activity (Rai et al., 2000).

4.3.3 Effect of coal fly ash on nitrate reductase (NR) activity (mg/g) by *Anabaena variabilis.*

S .No	% of Fly ash	0th day	7th day	14th day	21st day
1	0	0.125±0.005	0.206±0.01	0.312±0.015	0.426±0.02
2	1	0.156±0.004	0.256±0.01	0.356±0.012	0.445±0.021
3	2	0.168±0.002	0.324±0.016	0.402±0.02	0.495±0.026
4	5	0.175±0.006	0.395±0.015	0.452±0.02	0.554±0.025
5	**10**	**0.182±0.01**	**0.412±0.02**	**0.592±0.025**	**0.602±0.03**
6	20	0.152±0.005	0.210±0.01	0.356±0.013	0.446±0.021

Mean ±SE (n=3) (maximum yield in bold)

Table 14a: Nitrate reductase (NR) activity (mg/g) of *Anabaena variabilis* at different concentration of fly ash.

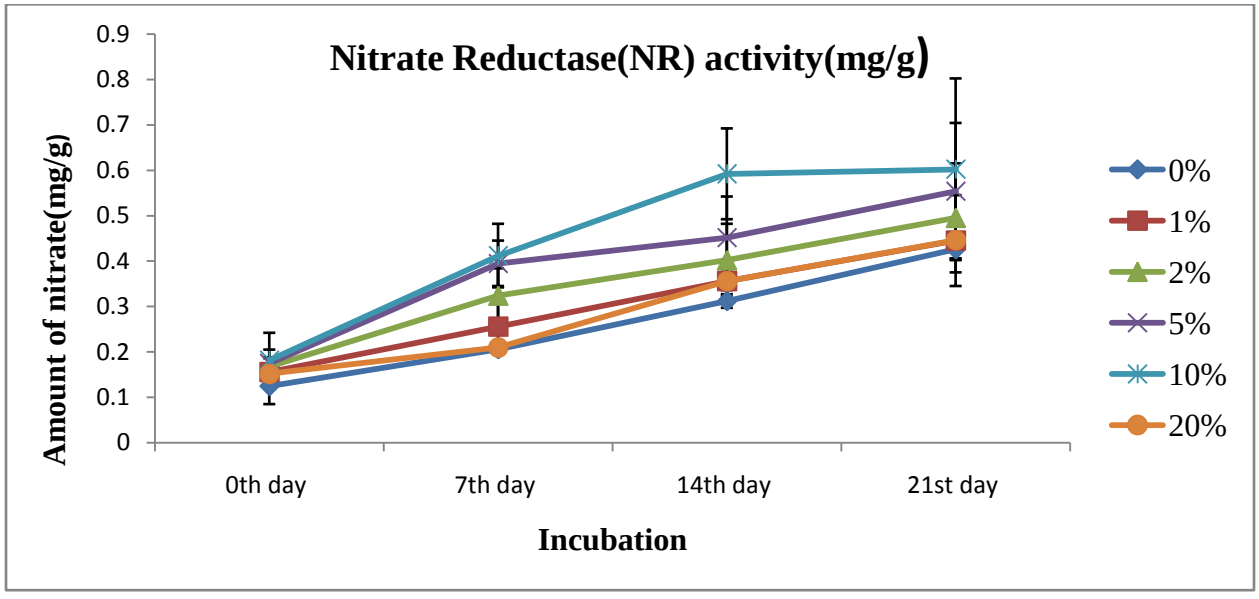

Figure 12a: Nitrate reductase activity (mg/gg) of *Anabaena variabilis* at different concentration of fly ash.

4.4.2b: Effect of fly ash nitrate reductase (NR) activity (mg/g) by *Aulosira fertilissima*

S .No	%of Fly ash	0th day	7th day	14th day	21st day
1	0	0.128±0.001	0.212±0.010	0.321±0.01	0.415±0.02
2	1	0.165±0.005	0.294±0.01	0.364±0.01	0.435±0.02
3	2	0.178±0.004	0.352±0.01	0.456±0.02	0.496±0.01
4	5	0.184±0.005	0.398±0.015	0.474±0.02	0.525±0.02
5	**10**	**0.195±0.003**	**0.452±0.02**	**0.495±0.01**	**0.585±0.03**
6	20	0.154±0.006	0.256±0.01	0.385±0.01	0.425±0.02

Mean ±SE (n=3)

Table 14b: Nitrate reductase (NR) activity (mg/g) by *Aulosira fertilissima* with different concentrations of fly ash

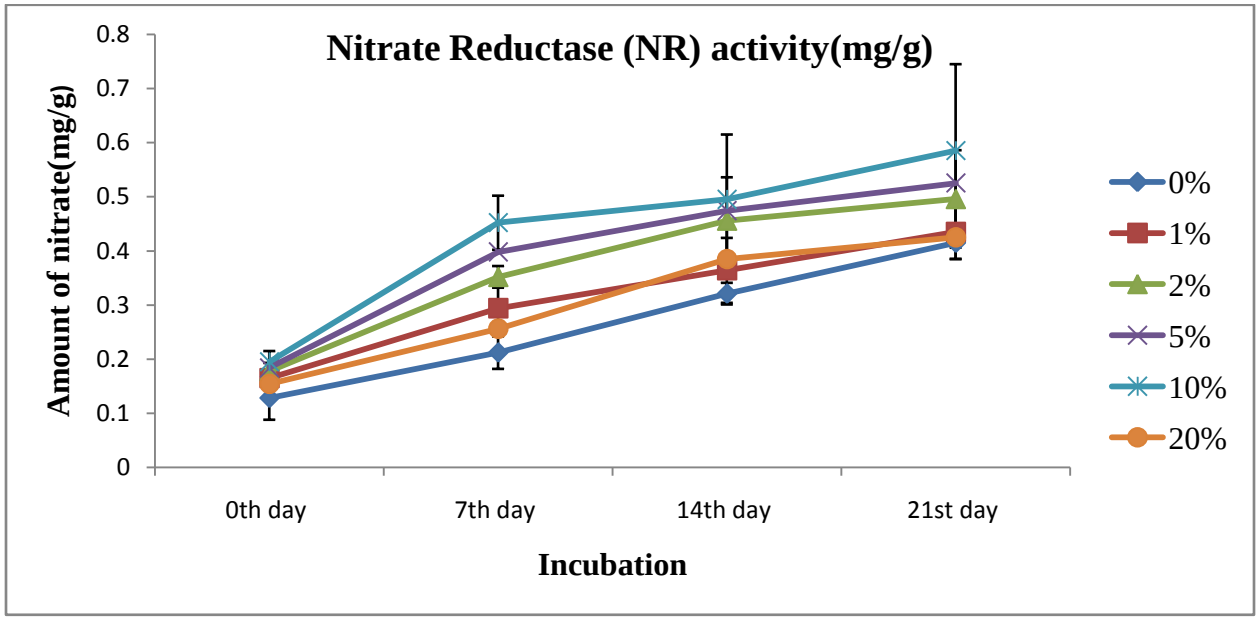

Figure12b: Nitrate Reductase (NR) activity (mg/g) of *Aulosira fertilissima* at different concentration of fly ash.

Maximum NR activity was observed in *Aulosira fertilissima (*ARM444*)* by 0.195± 0.03mg/g at zero day to 0.585±0.03 mg/g on 28th day at 10% of fly ash. However, it decreases from 0.154 mg/g at zero day to 0.425 mg/g at 28th day at 20% of fly ash. Similarly studies were performed to observe the maximum NR activity in *Anabaena variabilis* (ARM441) from 0.182 mg/g at zero

day to 0.602±0.03mg/g on 28th day at 10% of fly ash. 20% fly ash showed decreased NR activity wheras 10% showed maximum NR activity.

4.4.4a Effect of fly ash on solubilization of phosphorous by *Anabaena variabilis*

		Concentration in ppm			
S.No	**% of fly ash**	**0th day**	**7th day**	**14th day**	**21st day**
1	0	0.6±0.3	0.8±0.4	1.2±0.5	1.8±0.7
2	5	0.8±0.2	1.2±0.4	2.3±0.3	3.6±0.5
3	10	0.9±0.1	1.5±0.4	2.7±0.5	4.4±0.3
4	20	1.2±0.3	2.3±0.4	3.4±0.5	4.8±0.4

Table15a: Solubilization of phosphorous by *Anabaena variabilis* at different concentration of fly ash.

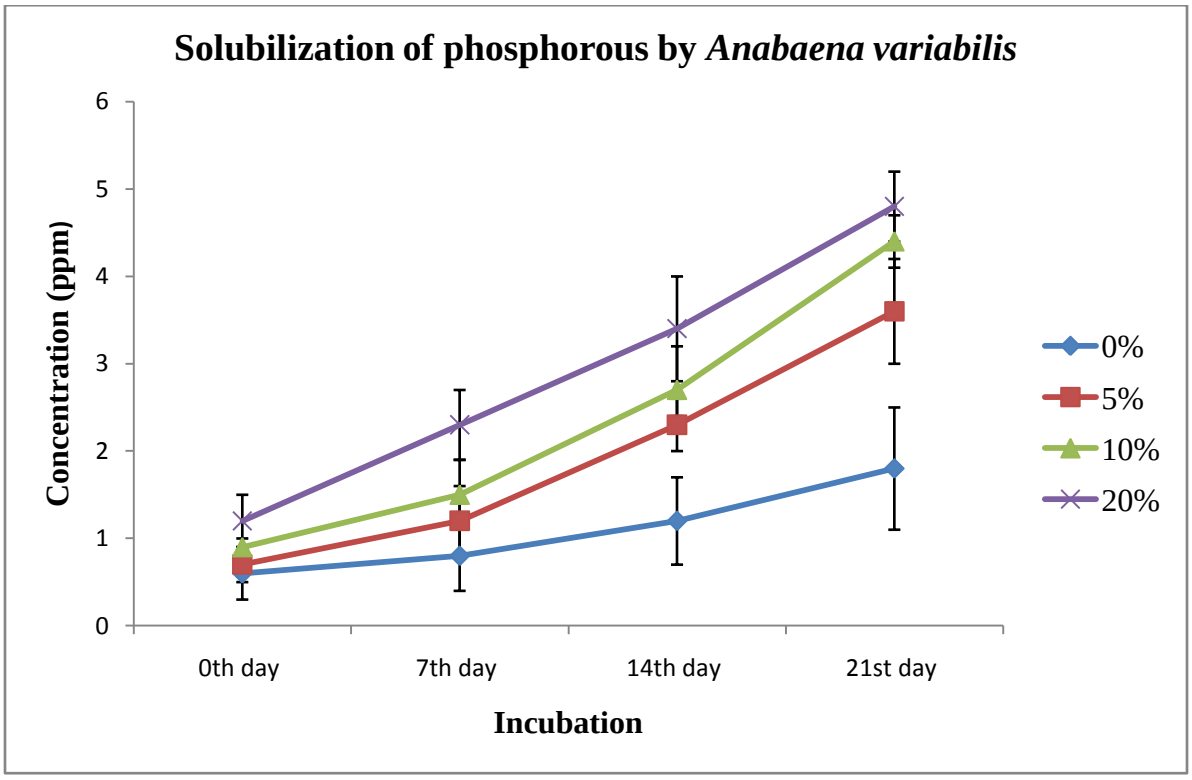

Figure13a: Solubilization of phosphorous by *Anabaena variabilis* at different concentration of fly ash.

4.4.4b: Effect of fly ash on solubilization of phosphorous by *Aulosira fertlissima*

		Concentration in ppm			
S.No	**% of fly ash**	**0th day**	**7th day**	**14th day**	**21st day**
1	0	0.5±0.2	1.4±0.3	1.8±0.3	2.1±0.4
2	5	0.7±0.3	1.5±0.2	2.2±0.2	3.5±0.4
3	10	0.9±0.2	1.7±0.3	2.7±0.4	3.7±0.5
4	20	1.3±0.2	2.1±0.3	2.9±0.3	4.2±0.4

Table15b: Solubilization of phosphorous by *Aulosira fertilissima* at different concentration of flyash.

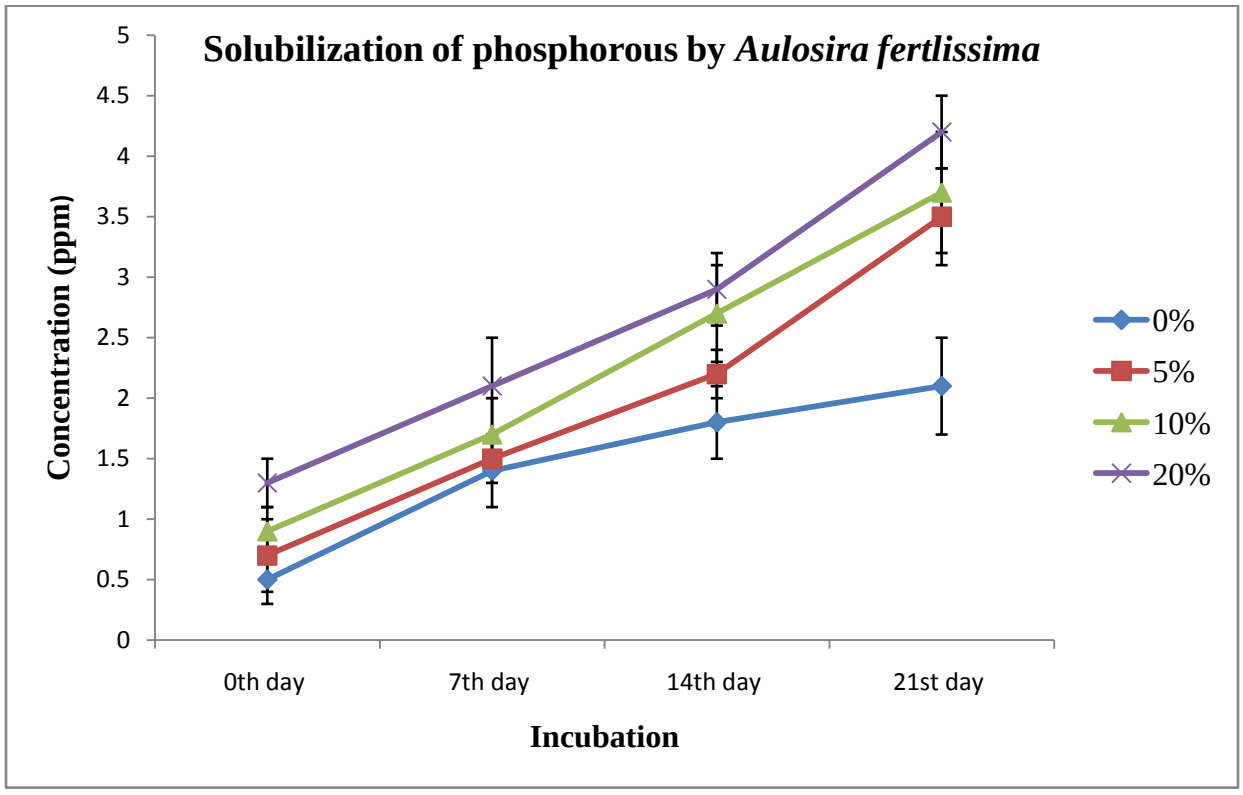

Figure13b: Solubilization of phosphorous by *Aulosira fertilissima* at different concentration of fly ash

Solubilization of phosphorous was maximum in *Aulosira fertilissima* (ARM444) from 1.3 ppm at 20% of fly ash on zero day to 21st day with 4.2 ppm. Maximum solubilization was noticed in 20% of fly ash with 4.2 ppm on 21st day. Similarly growth study was performed in *Anabanena variabilis* (ARM441), solubilization of phosphorous was minimum at 0% of fly ash with 0.6±0.3ppm on zero day and it increased to 21st day. Maximum solubilization was observed in 20% of fly ash at 21st day with 4.8±0.4 ppm after 30 days of incubation time. Fly ash contains phosphorous content, with increase in concentration of fly ash; maximum solubilization of phosphorous also increases at 20% of fly ash.

CONCLUSIONS

1. Plant growth promoting activity of four heterocystous filamentous cyanobacteria strains was studied and it was concluded that among the four cyanobacterial strain *Aulosira fertilissima* (ARM444) showed highest biomass production (2.12±0.36mg/ml), chlorophyll content (0.35±0.01mg/ml), NR activity (0.543±0.04mg/g) and IAA production (0.050±0.01µg/ml) in the presence of tryptophan (1mg/ml) and solubilization of TCP (5.86±1.1ppm). *Nostoc muscorum* (ARM 442) showed minimum PGP activity in terms of IAA production in the presence of tryptophan (0.042±0.02µg/ml), NR activity (0.474±0.03mg/g), Solubilization of TCP (5.22±1.1ppm).
2. IAA production was enhanced in the presence of tryptophan (1mg/ml). Maximum IAA production was observed in *Aulosira fertilissima* (0.050±0.011µg/ml) followed by *Anabaena variabilis*, *Nostoc muscorum* and *Tolypothrix tenuis* in the presence of tryptophan whereas without tryptophan the indole acetic production was (0.042±0.014µg/ml) after 28th day of inoculation.
3. Effect of fly ash at different concentration (0, 1, 2, 5, 10 and 20%) on plant growth promoting activities of heterocystous cyanobacterial strains showed that 10% FA showed maximum NR, IAA and solubilization of phosphorous and this concentration of fly ash was optimum which had no adverse effect on cyanobacterial activity. However, beyond and above 10% decreased growth was observed that could be due to inhibition of metabolic activity.

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