Development of fly ash based blue green algal inoculants for rice crop production

A thesis submitted in fulfillment of the requirement

for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

Rajinder Kaur

(Registration No.: 901000001)

Department of Biotechnology

Thapar University,

Patiala, Punjab

July, 2014

Certificate

It is hereby certified that **"Development of fly ash based blue green algal inoculants for rice crop production"** which is **submitted by Miss Rajinder Kaur, (Regd. No. 901000001),** in fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology, Thapar University, Patiala, Punjab, India, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

Dr. Dinesh Goyal

Supervisor
Professor and Head
Department of Biotechnology
Thapar University
Patiala – 147004
India

Declaration

I, hereby declare that the work which is being presented in this thesis "**Development of fly ash based blue green algal inoculants for rice crop production"** submitted by me for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the kind supervision of Prof. Dinesh Goyal, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

Date: 18 July '2014
Place: PATIALA

R. Kaur

Rajinder Kaur

Acknowledgement

I would like to take this opportunity to thank many people who have helped and encouraged me throughout this study. Without their help, support and encouragement, I would never have been able to accomplish this task. It is a pleasant aspect that I have now the opportunity to express my gratitude to all of them. First and foremost, I must acknowledge and thanks to the ***GOD****, the almighty (****Waheguru ji****), for the showers of blessings throughout my research work to complete the research successfully. I always feel his presence and support in my hard and happy times.*

Firstly, from the core of my heart, I am thankful to my supervisor ***Dr. Dinesh Goyal,*** *Professor and Head of Department of Biotechnology) and Executive Director, Science & Technology Entrepreneur's Park, Thapar University, Patiala, for providing me an opportunity to work under his guidance and supervision, assisting with all kinds of support and inspiration, wide counsel, constant encouragement, sincere criticism, valuable suggestions and comments, expertise and fruitful advice throughout this investigation and preparation of the thesis.*

I wish to express my thanks to ***Dr. Prakash Gopalan****, Director, Thapar University, Patiala, for providing infrastructure and helping me towards the smooth and timely completion of my research work.*

I extend my sincere words of thanks to ***Dr. Pramod K. Bajpai****, Dean (Research and Sponsored Projects) Thapar University, Patiala, for all the facilities which have been immensely helpful for the completion of my work.*

My special thanks to *the rest of my doctoral committee:* ***Dr. Sanjai Saxena; Dr Ganguly*** *DBT, TU and* ***Dr. Raj Kumar Gupta*** *(CHED, TU) for their constant encouragement, insightful comments, relevant suggestions and needful help during various stages of the work.*

I am highly thankful to ***Dr. Kulvir Singh*** *(Head) and* ***Dr. B.N Chudasama,*** *School of Physics and Material Science for their timely help, invaluable suggestion, encouragement and motivation during my PhD work.*

I am thankful to of ***STEP (Science & Technology Entrepreneurs Park)*** *for their instrument assistance and co-operation.*

I extend my thanks to all the faculty members of Department of Biotechnology, Thapar University, Patiala for providing necessary guidance during my research work. I am thankful to the office and laboratory staff of the Department of Biotechnology & Environmental Sciences for all the help and cooperation.

This research has been supported and funded by ***National Bank for Agriculture and Rural Development (NABARD),*** *Regional office, Punjab for organizing various field trials.*

Thanks also to all my colleagues especially, ***Ms. Gurdeep Kaur, Ms Mahiti Gupta, Ms Divya, Mr. Nadeem Akhtar, Prerna*** *at the Department of Biotechnology & Environmental Sciences, for providing constant support, encouragement and a good working atmosphere that has given me necessary time to relax from my work.*

I feel lacunae of words to acknowledge ***Late Ms Shipra Misra*** *who left us untimely. Her absence was felt deeply.*

Taking this opportunity I wish to heartily thank my friend ***Ms. Samita Thakur, Ms.Chandni****,* ***Ms Noor Humam Sulaiman, Ms Amita, Ms Sandhya Mehra, Ms Tamara Humam Sulaiman*** *for their love, affection, prayers, help and the constant encouragement they provided at all time.*

I want to mention the names of my M.Sc and M.Tech students ***Ms. Shilpi Grover*** *and* ***Ms. Jyotika Chugh*** *in our Industrial Biotechnology lab for their cheerful company.*

I feel a deep sense of gratitude for my parents and in-laws family who have always showered unconditional love on me, encouraged and supported me in every aspect. They formed a part of my vision and taught me the good things that really matter in life. My dearest sisters ***Ms. Virender Kaur, Jatinder Kaur, Parmjeet Kaur*** *and brother* ***Jeevanjot Singh*** *for their unconditional love and affection and all the support they have provided without which I would not have been able to achieve my goal.*

Finally, I wish to extend a very special thanks to my husband ***Mr. Pargat Singh*** *for his unrelenting support, optimism and giving me immense time to complete this research work*

Last but not the least, I wish to acknowledge all those, whose names have not figured here, but who helped me in any form, prayed and showered their blessing during the period of my research work.

Place:

Date: Rajinder Kaur

This Thesis

Is

Dedicated to my beloved

Parents

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LIST OF ABBREVATIONS

BGA	Blue green algae
DW	Distilled water
EC	Electrical conductivity
g	Gram
ha	Hectare
kg	Kilogram
min	Minute
TCP	Tri calcium phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
L	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
ppm	Parts per million
w/v	Weight by volume
v/v	Volume by volume
MMT	Montmorillonite
FA	Fly ash
U	Urea
TN	Total Nitrogen
ARA	Acetylene reductase assay

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ABSTRACT

Seven filamentous heterocystous region specific blue green algae were isolated from rhizospheric zone of paddy field soil and pond flyash and were identified as *Calothrix* sp., *Anabaena flos-aquae, Desmonostoc* sp DGRKC, *Nostoc commune*, *Nostoc* sp. PS1, *Nostoc* sp. DGRKF and *Anabaena* sp. *Anabaena* sp. (Isolate G) showed maximum dry biomass production by 3.45 mg/mL and *Desmonostoc* sp DGRKC (Isolate C) showed highest nitrogenase activity (32.2 ηmole C_2H_4/mg dry wt/hr), total nitrogen content (0.127 %), heterocyst frequency (18.2%) and nitrate reductase activity (31.0162 µmole NO^-_2). *Anabaena flos-aquae* (Isolate B) showed maximum chlorophyll content (3.79 µg/ml), nitrate reductase activity (27.62 µmole NO^-_2) and nitrogenase activity by 20.31 ηmole C_2H_4/mg dry wt/hr. Fly ash showed high thermal stability with least weight loss as observed in TGA and SEM graph indicated that flyash is composed of spherical structures with more surface area for interaction. XRD and EDS studies showed that amorphous content of ash consists of calcium oxide, potassium and major crystalline phases observed were quartz (SiO_2) and aluminium silicon oxide ($Al_{4.52}Si_{1.48}$) and haematite (Fe_2O_3). Charcoal, was amorphous in nature consisting of carbon and graphite. Soil and montmorillonite showed similar results in XRD, FTIR and thermal analysis revealed porous nature with silica as major constituent. The impact of consortium of filamentous nitrogen fixing cyanobacteria comprising *Anabaena variabilis, Aulosira fertilissima, Nostoc muscorum* and *Tolypothrix tenuis* inoculants on different rice cultivars (PUSA 1121, PR 118, PAU 201, SHABNAM and BASMATI 1401improved the grain yield of rice over a period of three years by 8qtl/ha for Basmati 1407 and 5qtl/ha for SHABNAM and PUSA 1121 as compared to urea alone and control, whereas flyash based cyanobacterial biofertilizer (BGA) application alone showed increase by 6 qtl/ha for rice variety BASMATI 1401 followed by 4 qtl/ha for PUSA1121 and by 3 qtl/ha for

PR118 and SHABNAM. Increase in crop yield by 2-6 qtl/ha over control among all rice cultivars supports a positive role of fly ash based algal biofertilizers in soil is consistent with diazotrophic potential of the cyanobacterial strains used. There was an increase in soil organic carbon from 0.32 to 0.53 %, total nitrogen from 0.042 to 0.067 % and phosphorus content from 7.9-27.70 mg/kg as a result of application of cyanobacterial biofertilizers. Fly ash based algal biofertilizers makes rice production system more viable and reduces the ecological hazards caused due to synthetic fertilizers which can serve as an important component of the integrated plant nutrient supply system with net savings in urea by 15-35 %.

SYNOPSIS

Rice is a staple food for about 50 per cent of the world's population that resides in Asia, where 90 per cent of the world's rice is grown and consumed. In India, rice occupies about 44mha of the cultivated area, out of which 20.5 mha are irrigated lands and would need to produce 143 million tons of rice to meet the growing demand by 2030 (Subbaiah *et al.*, 2001). Cyanobacteria (Blue Green Algae) are the major component for nitrogen fixation in paddy cultivation and also for high yield production (Saadantia and Riahi, 2009). It has been reported that excessive utilization of chemical fertilizers has made nitrogen as the second limiting factor after water, for which the use of fertilizers becomes necessary to prevent soil deterioration and maintain soil fertility (Kalra and Jain, 2003). Species of *Nostoc, Anabaena, Tolypothrix, Aulosira, Cylindrospermum, Scytonema, Westellopsis* 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. Indian agriculture was mostly organic before the advent of the Green Revolution however, widespread adoption of nutrient responsive and high yielding varieties and use of inorganic fertilizers, weedicides and insecticides resulted in high crop yield (Pabbi, 2008; Katiyar *et al*., 2012). Amongst the array of biofertilizers developed for different crops, cyanobacteria popularly known as blue green algae, constitute the most important inputs in rice cultivation and application of such algal biofertilizers has proved to be sustainable, ecofreindly, cheap, easily manageable and improves the nutrient status as well as soil health (Dhar *et al*., 2007; Pereira *et al*., 2009).

Punjab, the 'food bowl' of India, where high yield has been the main pursuit of farmers, organic farming has never been a preferred option because of low output and yield loving farmers are inclined to intensive farming or 'chemical farming' (Benbi *et al*., 2009). Inherent nutrient status of Punjab soils has been depleted at a faster rate since green revolution, resulting in macro and micro nutrient deficiencies in crop plants (Verma. *et al*., 2005). With

growing realization that chemical based agriculture is unsustainable and is slowly leading to ecological imbalance, the emerging ecological problems can be tackled with the emergence of the concept of “organic farming” minimizing use of chemical fertilizer and increasing dependence on biological inputs like compost, green manures and biofertilizers (Rai, 2000; Choudhary and Kennedy, 2005).

Fly ash is the major solid by product produced in thermal power stations generated in large quantity (Mittra, 2005; Jawahar and Vaiyapuri, 2010). It acts as a useful ameliorant that improves the physical, chemical and biological properties of problem soils and is a source of readily available plant macro and micronutrients. In conjunction with organic manure and microbial inoculants, fly ash can enhance plant biomass production from degraded soils (Jala and Goyal, 2006).Fly ash fulfils the requirement of a suitable carrier material in being cheap, easily available in bulk, favourable pH and high water holding capacity (WHC) which makes it compatible with native soil and improves K, Ca, Mg and S status of deficient soil (Gaind and Gaur, 2002; Sharma and Kalra, 2006) little contribution towards nitrogen and phosphorus (Gaind and Gaur, 2002; Gaind & Gaur, 2004). Use of fly ash as carrier in the algal biofertilizers could prove to be an effective way of its utilization in a useful manner and restoring soil nutrients. Hence, attempts should be made to partially or completely replace chemical fertilizers by biofertilizers to restore the nutrients and to protect soil fertility to enhance crop productivity.

Present studies was proposed to screen, isolate and study cyanobacterial diversity from local rice fields of Patiala Distt., Punjab and develop fly ash based algal biofertilizers for field application with following objectives:

OBJECTIVES:

1. Isolation and characterization of efficient nitrogen fixing blue green algae from paddy field soils and ash pond samples.
2. Impact of fly ash on growth and nitrogen fixation by selected blue green algal isolates.
3. Fly ash based blue green algal inoculants formulation and their evaluation on growth and yield of rice.

1. Isolation and characterization of efficient nitrogen fixing blue green algae from paddy field soils and ash pond samples.

Seven filamentous heterocystous region specific blue green algae were isolated from paddy field soils of Nabha and Patiala, Punjab, India and pond fly ash from National Thermal Power Station (NTPC), Rihandnagar, U.P, India and were identified morphologically using the keys given by Fritsch (1949); Komarak (2006) and Hrouzek *et al*., 2013 as *Calothrix* sp. (Isolate A), *Anabaena flos-aquae* (Isolate B), *Desmonostoc* sp. (Isolate C), *Nostoc commune* (Isolate D), *Nostoc sp.*PS1(Isolate E), *Nostoc sp.* (Isolate F) and *Anabaena* sp (Isolate G). Comparative study of growth, nitrogen fixation and biochemical attributes in terms of dry biomass (Richmond and Gobbelaar, 1986), Chlorophyll estimation (Mckiney, 1941), total nitrogen content by Kjeldahl method (Piper, 1960), nitrogenase activity (Hardy *et al*., 1973), nitrate reductase activity (Lowe and Evans, 1964), indole acetic acid production (Glickmann and Dessaux, 1995) of seven blue green algal isolates with ARM cultures viz., *Anabaena variabilis* (ARM 441); *Nostoc muscorum* (ARM 442); *Tolypothrix tenius* (ARM 443) and *Aulosira fertilissimia* (ARM 444) procured from Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), Indian Agricultural Research Institute (IARI), New Delhi

which are predominantly used as a multani mitti based biofertilizers for N increment in rice cultivation (Mishra and Pabbi, 2004) was done. Growth study revealed that among all the seven blue green algal isolates, Isolate G showed maximum dry biomass (3.45 mg/mL), Isolate C showed highest nitrogenase activity by 32.2 ηmole C_2H_4/mg dry wt/hr, total nitrogen content (0.127 %) heterocyst frequency (18.2 %) and nitrate reductase activity (31.0162 µ mole NO^-_2); Isolate B showed maximum chlorophyll content (3.79 µg/mL), nitrate reductase activity (27.62 µ mole NO^-_2) and nitrogenase activity by 20.31 mole C_2H_4/ mg dry wt/hr. Isolate C, B, and F were selected based on highest nitrogen fixation, total nitrogen content and heterocyst frequency and were further characterised using molecular identification techniques such as 16S rRNA molecular identification techniques which involved DNA isolation, PCR amplification, DNA sequencing and sequence submission in NCBI database Identification of three isolates *Desmonostoc* sp DGRKC, *Nostoc sp.*DGRKF and *Anabaena flos-aquae* were confirmed by molecular studies and their nucleotide sequence were submitted in NCBI database under the nucleotide accession numbers KM083062 and KM083063.

2. Impact of fly ash on growth and nitrogen fixation by selected blue green algal isolates.

Fly ash in dried form at different concentration 0, 5, 10 and 20% (w/v) was added to BG 11 (-N) growth medium and distilled water (DW) to examine its impact on growth and nitrogen fixation by heterocystous filamentous blue green algal strains. Metal uptake by algal biomass of 30 days grown culture was studied by nitric acid digestion method (Page *et al.*, 1982) using atomic absorption spectrophotometer (BGC 932 AA) in order to accesses the metal removal potential of strains. *Nostoc* sp., *Anabaena flos-aquae, Desmonostoc* sp. DGRKC and *Anabaena variabilis* (ARM 441) showed prolific growth in BG 11 (-N) medium

amended with 5 and10% fly ash with almost same range dry biomass (2.25-3.5 mg/mL). Nitrogen fixation and total nitrogen content of 37.2 - 40.4 mole C_2H_4/mg dry wt/hr and 0.784-0.883% was observed in *Desmonostoc* sp. grown in BG 11amended with 5 % and 10 % fly ash as compared to control (BG 11-0 % FA). However all algal isolates showed decreased nitrogen fixation when grown at higher concentration of fly ash (20 %) both in distilled water (DW) and BG 11 medium. *Anabaena variabilis* (ARM 441) showed highest range of nitrogen fixation of 32.5 mole C_2H_4/mg dry wt/hr and total nitrogen content by 0.509 % when grown in BG 11 (-N) medium amended with 5 % fly ash whereas *Nostoc muscorum* (ARM 442) showed maximum nitrogenase activity (23.14 ηmole C_2H_4/mg dry wt/hr) and total nitrogen content (0.398 %) in BG11 medium amended with 5 % fly ash.

Chromium uptake (Cr) was observed maximum in *Anabaena flos-aquae* (3.73 mg/g), *Nostoc commune* (4.85 mg/g) at BG 11 medium amended with 5 % flyash whereas *Nostoc* sp DGRKF(3.98 mg/g) showed at BG 11 (-N) medium amended with 10 % flyash respectively. Howerever, *Nostoc muscorum* (ARM 442 mg/g) showed maximum uptake of Cr (3.65 mg/g), Pb (2.12 mg/g) at BG 11(-N) medium amended with 10% flyash respectively. *Calothrix* sp. showed maximum uptake of Zn (4.52 mg/g) and Pb (2.52 mg/g) in BG 11(-N) medium amended with 20% fly ash as compared to control, however Cu (0.825 mg/g) and Cr (0.950 mg/g) maximum uptake was showed in BG 11 (-N) amended with 10% flyash. Increased accumulation of metals in blue green algal isolate grown in BG 11(-N) medium amended with 5, 10 and 20% fly ash which confirms that metal concentrations was within the range in all algal strains.

3. Fly ash based blue green algal inoculant formulation and their evaluation on growth and yield of rice.

For development of fly ash based blue green algal inoculants, mineralogical and physico chemical properties of fly ash were studied and compared with that of soil, charcoal and montmorillonite. Electrostatic precipitator (ESP) coal fly ash (FA) was collected from Guru Gobind Singh Super Thermal Power Plant, Ropar, Punjab and was directly analyzed for its physiochemical properties after air-drying. Wood charcoal and montmorillonite was purchased from local market located in Patiala, Punjab as per required quantity. Garden soil was collected from experimental plot Science & Technology Entrepreneur's Park (STEP), Thapar University, Patiala. Soil, charcoal and montmorillonite were crushed into fine powder and air dried for their physiochemical properties.

Coal fly ash, montmorillonite, charcoal and soil was analysed for its mineralogical content and thermal stability by X-ray diffraction (XRD), thermal gravimetric analysis (TGA), fourier-transform infrared spectroscopy (FTIR) and physicochemical properties for pH, EC (µS/cm) by Jackson's method (1967), water holding capacity (WHC) and bulk density were determined using the protocol given by Black et al. (1965), BET (Brunauer – Emmett-Teller) and coarse grain accumulation was done by sieve method of Sugita (2001). Fly ash showed high thermal stability with least weight loss as observed in TGA and SEM graph indicated that flyash is composed of spherical structures with more surface area for interaction, XRD and EDS studies showed that amorphous content of ash consists of calcium oxide, potassium and major crystalline phases observed were quartz (SiO_2) and aluminium silicon oxide ($Al_{4.52}Si_{1.48}$) and haematite (Fe_2O_3). Charcoal, was amorphous in nature consisting of carbon and graphite. Soil and montmorillonite showed similar results in XRD, FTIR and thermal analysis having porous nature with silica as major constituent. Fly ash was found to be alkaline in nature having pH 7.85 and electrical conductivity 0.14 µS/m, good water holding

capacity (62%) and various macro and micronutrients as compared to other material viz. soil, charcoal, montmorillonite.

Effect of consortium of seven isolated blue green isolates (*Calothrix* sp, *Anabaena flos-aquae*, *Desmonostoc* sp. DGRKC, *Nostoc commune*, *Nostoc* sp.PS1, *Nostoc* sp.DGRKF, and *Anabaena* sp.) and consortium of four ARM blue green algal strains (*Anabaena variabilis* (ARM 441); *Nostoc muscorum* (ARM 442); *Tolypothrix tenius* (ARM 443) and *Aulosira fertilissimia* (ARM 444) with different treatments of fly ash (100 %), soil (100 %), montmorillonite (100 %), fly ash + soil (50:50 %) and fly ash + montmorillonite (50:50) on rice cultivar PUSA 1121 were examined by pot experiment. Fly ash + soil (50:50) with of seven blue green algal isolates showed maximum grain yield (14.3 g per pot), organic carbon (0.43%), phosphorus content (17mg/kg) and total nitrogen content (0.168 %) respectively.

Field trial on impact of consortium of fly ash based BGA (filamentous nitrogen fixing blue green algae) inoculants on different rice cultivars (PUSA 1121, PR 118, PAU 201, SHABNAM and BASMATI 1401 were studied for three consecutive years (2010-2013) on 20 acres of trials in different villages along with control. From an average production of 113kg of wet algal biomass in algal ponds, 381 packets of fly ash based cyanobacterial biofertilizers (500gm per packet) were made which were applied for field trials of rice cultivation.

Co application of cyanobacterial biofertilizers @ 500 g/ha with recommended dose of urea @ 60 kg/ha improved the grain yield of rice over a period of three years by 8qtl/ha for Basmati 1407 and 5-6qtl/ha for SHABNAM and PUSA 1121as compared to urea alone and control whereas BGA application alone showed increase by 6 qtl/ha for rice variety BASMATI 1401 followed by 4 qtls/ha for PUSA1121 and by 3 qtl/ha for PR118 and SHABNAM. Urea alone application @60 kg/ha showed increase only by 2-3qtl/ha for PUSA1121 and BASMATI 1401 whereas decrease in yield was observed by 2

qtl/ha by third year for PAU 201 and PR 118. Control plots also showed decrease in grain yield from 3 qtl/ha for BASMATI 1407 (10-07 qtl/ha) to 6 qtl/ha for PR118 (20-14 qtl/ ha).There was increase in soil organic carbon from 0.32 to 0.53%, total nitrogen from 0.042 to 0.067% and phosphorus content from 7.9-27.70 mg/kg as a result of application of cyanobacterial biofertilizers. In RBD block experiment yield of rice increased in BGA + Urea inoculated blocks at three different agricultural sites over control. Flyash based cyanobacterial biofertilizers (BGA @ 500 g/ha) in combination with nitrogenous fertilizers (Urea @60 kg/ha) was effective in increasing yield of rice crop and soil nutrients in three years (2010-2013 for all five rice cultivar viz. PUSA 1121, PAU201, SHABNAM, PR118 and Basmati 1407.

Chapter 1 INTRODUCTION

Indian agriculture was mostly organic before the advent of the Green Revolution. However, widespread adoption of nutrient responsive and high yielding varieties and use of inorganic fertilizers, weedicides and insecticides resulted in high crop yield (Pabbi, 2008; Katiyar *et al*., 2012). Cyanobacteria (Blue Green Algae) are the major component for nitrogen fixation in paddy cultivation and also for high yield production (Saadantia and Riahi, 2009). It has been reported that excessive utilization of chemical fertilizers has made nitrogen as the second limiting factor after water, for which the use of bio-fertilizers becomes necessary to prevent soil deterioration and maintain soil fertility (Malik and Thapiyal, 2009). Use of bio-fertilizers can prevent the depletion of soil organic matter content (Jeyabal and Kuppuswamy, 2001; Choudhury *et al*., 2014). Compulsion to grow more for food security has led farmers to overlook food quality norms and indiscriminate use of natural resources. Punjab, the 'food bowl' of India, where high yield has been the main pursuit of farmers, organic farming has never been a preferred option because of low output and yield loving farmers are inclined to intensive farming or 'chemical farming' (Benbi and Brar, 2008). Inherent nutrient status of Punjab soils has been depleted at a faster rate since unshering of the green revolution, resulting in macro and micro nutrient deficiencies in crop plants (Verma *et al*., 2005). With growing realization that chemical based agriculture is unsustainable and is slowly leading to ecological imbalance, the emerging ecological problems can be tackled with the emergence of the concept of "organic farming" minimizing use of chemical fertilizer and increasing dependence on biological inputs like compost, green manures and biofertilizers (Rai, 2000; Choudhary and Kennedy, 2005).

Rice is a staple food for about 50 per cent of the world's population that resides in Asia, where 90 per cent of the world's rice is grown and consumed. In India, rice occupies about

44mha of the cultivated area, out of which 20.5 mHa are irrigated lands and would need to produce 143 million tons of rice to meet the growing demand by 2030 (Subbaiah *et al.*, 2001). Paddy soil in Punjab requires cheap synthetic nitrogen source irrespective of chemical fertilizers to enhance more productivity in eco-friendly manner. Organic farming has been proved to have the potential in providing benefits in terms of environmental protection, conservation of non-renewable resources and improved food quality (Worthington, 2001; Haas *et al*., 2005).

Biofertilizers are best developed substitute for chemical fertilizers such as cyanobacteria which are capable of fixing nitrogen (Elanwar *et al*., 2010). Amongst the array of biofertilizers developed for different crops, cyanobacteria popularly known as blue green algae, constitute the most important inputs in rice cultivation (Dhar *et al*., 2007). In addition, the use of cyanobacteria as biofertilizers can improve plant growth and crop yield as they add organic matter to soil, thus improving soil texture (Maqubela *et al*., 2009). Commercially algal biofertilizers could be produced comprising of filamentous nitrogen fixing cyanobacteria selected from rice fields to generate a technological package compatible with its use for the rice crop. Application of such algal biofertilizers has proved to be sustainable, ecofreindly, cheap, easily manageable and improves the nutrient status as well as soil health (Pereira *et al*., 2009). Growth of nitrogen fixing cyanobacterial species in rice fields plays a critical role in sustenance of soil fertility (Roger, 1982) and exhibits a great ecological significance in the rhizosphere and endophytic colonization (Prasanna *et al*., 2009). Field trials carried out over the last two decades under the All India Coordinated Research trials, using rural oriented blue green algal (BGA) biofertilizers developed at Indian Agricultural Research Institute (IARI), New Delhi revealed that BGA can provide 25-30 kg N/ha/season (Venkataraman, 1981) and an increase of upto 30% of the paddy crop yield (Venkataraman, 1981; Goyal *et al*., 1997). BGA plays a vital role in reducing oxidizable matter content of the

soil, provides oxygen to the submerged rhizosphere, amerliorate salinity and buffer the pH, solubilize phosphates and increase the fertilizer use efficiency of crop plants (Mandal *et al.*, 1999). The water logged conditions, high humidity, temperature, and shade provided by the paddy crop canopy afford optimal conditions of rapid multiplication for cyanobacteria (Whitton, 2000). 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; Dhar *et al*., 2007). It improves water holding capacity of soil and increases soil aggregation and leads to population build up with enhanced microbial activity.

Coal fly ash is the major solid by product produced as a result of coal burning in Thermal power stations and is generated in large quantity (Rizvi and Khan, 2009; Jawahar and Vaiyapuri, 2010). It acts as a useful ameliorant that improves the physical, chemical and biological properties of problem soils and is a source of readily available plant macro and micronutrients and in conjunction with organic manure and microbial inoculants, fly ash can enhance plant biomass production from degraded soils (Jala and Goyal, 2006). Fly ash act as soil amending agent improving physical properties of the soil but also contribute to better growth and yield of rice (Mishra *et al*., 2007). The large scale use of fly ash in agriculture and wasteland development holds a potential to increase on an average 15% yield of grains, oil seeds, sugarcane, cotton and about 25–30% of vegetables resulting in another green revolution (Kumar *et al*, 2014). It is evident from the results that the addition of fly ash (10 - 200 tons per ha) increased the yield of different crops from 10-40%. Fly ash has been advocated as a promising material (or amendment) for reclaiming wastelands or mine spoils (Adriano and Weber, 2001). Millions of hectares of land rendered wasteland due to strip

mining of coal have been effectively reclaimed and stabilized using fly ash. Fly ash has also been used for the neutralization of acidic mine spoils and restoration of nutrient balance in alkali wastelands and as a safe barnyard paving resource (Singh and Rawat, 1993; Stout *et al.*, 1999).

Carrier based algal biofertilizers can make the rice production system more viable and reduce the ecological hazards caused due to synthetic fertilizers and can serve as one of the component of integrated plant nutrient supply system (Mittra *et al*., 2005). Fly ash fulfils the requirement of a suitable carrier material in being cheap, easily available in bulk, favourable pH and high water holding capacity (WHC) which makes it compatible with native soil and improves K, Ca, Mg and S status of deficient soil (Srivastva and Chhonkar, 2000; Deshmukh *et al*., 2000; Gaind and Gaur, 2002; Sharma and Kalra, 2006) with little contribution towards nitrogen and phosphorus (Gaind and Gaur, 2002; Gaind and Gaur, 2004). Use of fly-ash as carrier in the algal biofertilizers could prove to be an effective way of its utilization in a useful manner and restoring soil nutrients. Hence, attempts should be made to partially or completely replace chemical fertilizers by biofertilizers to restore the nutrients and to protect soil fertility to enhance crop productivity. Present study has been proposed to screen, Isolate and study cyanobacterial diversity from local rice fields of Patiala Distt., Punjab and develops fly ash based algal biofertilizers for field application with following objectives:

Objectives:

1. Isolation and characterization of efficient nitrogen fixing blue green algae from paddy field soils and ash pond samples.
2. Impact of fly ash on growth and nitrogen fixation by selected blue green algal Isolates.
3. Fly ash based blue green algal inoculant formulation and their evaluation on growth and yield of rice.

Filamentous and heterocystous blue green algae were Isolated from rhizospheric soil samples collected from different villages of Patiala, Punjab and pond fly ash from NTPC, Rihandnagar, U.P, India by enrichment culture technique and were screened for growth, nitrogen fixation activity, heterocyst frequency, total nitrogen content and biochemical attributes such as nitrate reductase activity and indole acetic acid production. Isolates were identified morphologically and then best strains with high nitrogen fixation potential were identified by 16S rRNA molecular identification technique and the DNA sequence was submitted in NCBI database. Coal fly ash (FA) was collected from Guru Gobind Singh Super Thermal Power Plant, Ropar, Punjab and was analyzed for its physio-chemical properties. Fly ash in dried form at different concentrations 0, 5, 10 and 20% (w/v) was added to BG 11(-N) growth medium and distilled water (DW) to examine its impact on growth and nitrogen fixation by heterocystous filamentous blue green algae. Crop yield of different rice cultivars (*Oryzae sativa*) PUSA 1121, PR 118, PAU 201 were observed to see the effect of fly ash based BGA biofertilizers as well as on soil physico-chemical properties on agricultural land and compared with urea application.

Chapter 2 REVIEW OF LITERATURE

2.1 Blue green algae

Cyanobacteria, phototrophic prokaryotes is the scientific name for "Blue Green Algae" or “Pond scum” (Stanier, 1971) that are a group of gram negative (Eubacteria) which possesses oxygen evolving photosynthetic apparatus similar to that of higher plant chloroplast. Many of these forms are able to convert molecular nitrogen to ammonia with the help of nitrogen fixing enzyme *nitrogenase,* which is present in highly specialized cells known as heterocyst (Wolk *et al*., 1994). In comparison with eukaryotic algae, cyanobacteria have a high ability to compete for ammonium than nitrate under nitrogen limiting condition. Judicious use of these blue green algae could provide entire rice acreage of India as much nitrogen as obtained from 15 – 17 lakh tonnes of urea (Sharma *et al*., 2000). Supplementation of chemical fertilizer with blue green algae could conserve upto 30% of commercial fertilizers and it is generally believed that the nitrogen fixed by these organisms is made available to the rice plants through exudation or autolysis and microbial decomposition. In addition to contributing biologically fixed nitrogen and adding organic matter to soil, blue green algae are also known to excrete growth promoting substances, solubilize insoluble phosphates, improve fertilizer use efficiency of crop plants and amend the physical and chemical properties of soil. They have also been shown to have ameliorating effects on saline and saline alkali soils, increasing soil aggregate size, thereby, correcting soil compaction, reduce oxidizable matter of the soil and narrowing down the C:N ratio (Swaranlakshmi *et al*., 2007). Nitrogen fixing filamentous cyanobacteria occurs in wide range of habitats, mainly rice-field ecosystem and agricultural fields (Whitton 1970; Chunleuchanon, 2003; Kaushik, 2004; Dhar *et al*., 2007). In rice field among photosynthetic aquatic organisms, investigations have been emphasized more on

isolation and identification of nitrogen fixing cyanobacterial populations in agro-ecosystems for sustainable agriculture.

2.2 Classification of blue green algae

Cyanobacteria are classified as oxygenic photosynthetic bacteria lacking a nucleus were first classified as plant constituting the class *Schizomycetes* and phylum *Schizophyta* (Nageli, 1857), then it was placed in the phylum Monera, kingdom Protista (Haeckel, 1866) and finally was classified with algae under plant kingdom that was named as blue green algae due to presence of different photosynthetic pigents such as chlorophyll a, phycocyanin, phycoerythryn (Stanier and Cohen, 1977) and latter reclassified as Prokaryotes by Chatton in 1925. Morphologically cyanobacteria are classified into five orders (Figure 3) viz., Pleurocapsales (Rippka et al., 1979) Chroococcales, Oscillatoriales, Nostocales and Stigonematales (Fritsch 1949, Castenholz and Waterbury, 1989; Komarek, 2006).

2.2.1Chroococcales: This order is characterized by unicellsular colonies, aggregated in colonies, reproduces by binary fission, lack differentiation between apical and basal structures. This order includes colonial cells type species of *Cyanothece*, *Chroococcus*, *Aphanothece*, *Merismopodia* and *Eucapsis*.

2.2.2 Pleurocapsales: Unicellsular algae having multiple fission type of reproduction and are found in marine, fresh water and terrestrial habitat (Caudales *et al*., 2000). Cyanobacteria such as *Pleurocapsa*, *Cyanocystis* and *Chamaesiphon* are included in this order.

2.2.3 Oscillatoriales: These are freshwater phytoplanktons filamentous in nature, reproduction is by fragentation, consists of hormogonia (Whitton and Peat, 1969). Cyanobacteria included in this order are *Spirulina*, *Oscillatoria*, *Phormidium*, *Arthrospira*, *Lyngbya* and *Microcoleus*.

2.2.4 Nostocales and Stigonematales: Both are monophyletic filamentous cyanobacteria, simple or branched, consist of mucilaginous sheath and are considered as heterocystous. Stigonematales consists of hormogonia, truly branched trichomes and develops heterocysts in certain conditions (Ripkka *et al*., 1979). Nostocales includes *Nostoc*, *Anabaena*, *Cylindrospermum*, *Rivularia*, *Gleotrichia*, *Aphanizomenon*, *Tolypothrix* and *Aulosira* whereas Stigonematales includes *Stigonema*, *Fisherella* and *Hapalosiphon* species.

2.3 Morphology and cellsular organization of cyanobacteria

2.3.1 *Vegetative cells***:** The blue green algal vegetative cells is of prokaryotic type, lacking membrane bound DNA, chloroplasts, plastids, mitochondria and golgi apparatus (Roger, 1982). Nuclear material is comprised of granules in centroplasm and chloroplasm contains pigents concentrated ín phycobilisomes located on the surface of thylakoids (photosynthetic lamella). Vegetative cells of *Anabaena* sp are larger and spherical in shape than other heterocystous cyanobacteria such as *Nostoc* and *Tolypothrix* species (Komarek, 2006).

2.3.2 *Spores :* Heterocystous blue green algae (Nostocaceae and Rivulariaceae) produce large sized spores or akinetes which are resistant to adverse conditions (Figure 1). Akinetes are resting state cells of cyanobacteria viable for long time periods (Olsson *et al*., 2009). Akinetes present in heterocystous blue green algae germinates when conditions are favourable for growth and are common among freshwater forms but uncommon in marine habitat (Thajuddin and Subramanian, 2005). Akinetes formation and differentiation in Anabaena oscillarioides characteristically begins in two vegetative cells adjacent to heterocyst when observed in late exponential phase of growth in batch culture (Ahluwalia *et al*., 1989).

2.3.3 *Hormogonia:* Motile filaments of cells observed in the order of Nostocales and Stigonematales formed during asexual reproduction (Figure 1). Hormogonia in the order

Nostocales and genera *Hapalosiphon* and *Fischerella* are produced terminally in lateral branches and are separated from vegetative cells without forming necrotic cells (Hindak, 2012). *Anabaena* shows gliding movement by the presence of hormogonia and these characteristics differs *Anabaena* from *Nostoc* sp. (Phillipis *et al*., 2000).

2.3.4 *Heterocysts:* Round, squared or rectangular shaped cells are produced by vegetative cells intercalary or basally under nitrogen deficient conditions observed in Nostocales (*Anabaena*, *Nostoc*, *Tolypothrix* and *Aulosira*), Stigonematales (*Hapalosiphon*, *Stigonema*) and Rivulariaceae (*Calothrix*). Heterocysts are specialized cells for nitrogen fixation and are enlarged than vegetative cells (Stewart, 1967; Fay *et al*., 1968). Cells wall of heterocysts consists of three layers comprising fibrous outer layer, non cellsulose polysaccharide middle homogeneous layer and inner layer made up of glycolipid (Lang, 1968).

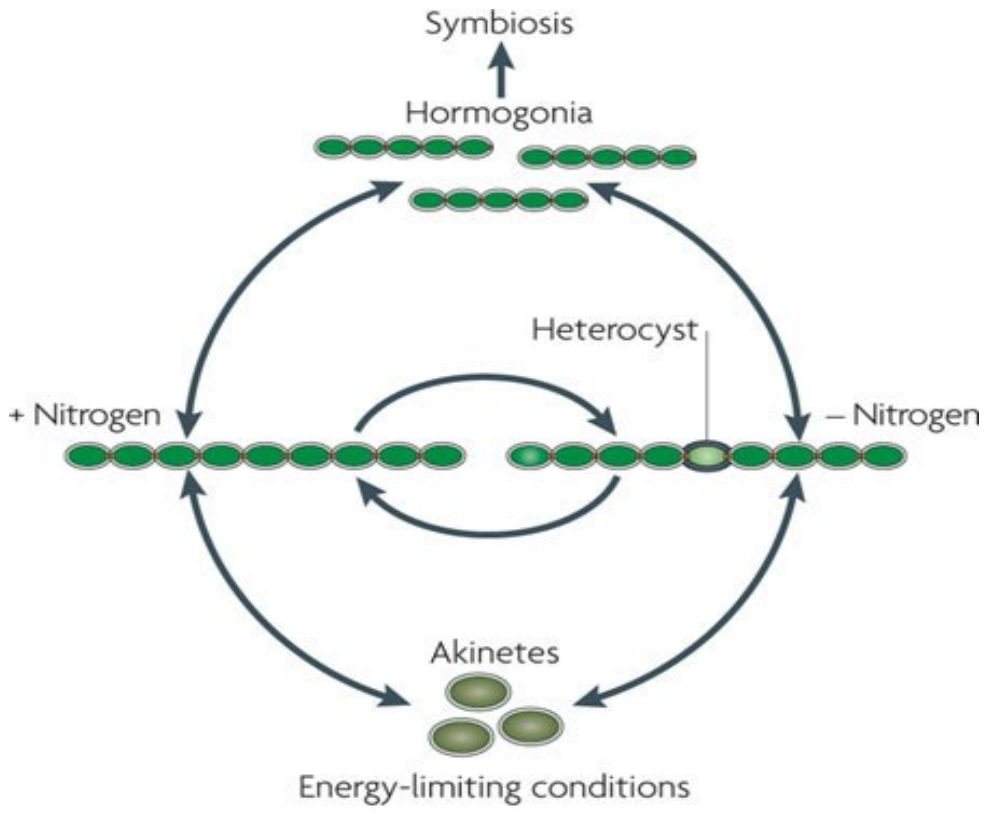


Figure 1. Life cycle of heterocyst forming blue green algae (Flores and Herrero, 2010).

These specialized heterocysts convert atmospheric nitrogen into combined form of ammonium (NH_4) or nitrate ions (NO_3) (Haselkorn, 2007). Free elemental nitrogen (N_2) bound with nitrogenase enzyme complex and ferrodoxin reduces to Fe protein that bind to ATP molecule and reduces molybdenum-iron protein (Mo-Fe protein) producing HN=NH complex by donating electrons to N_2. HN=NH complex is reduced to $H_2N{=}NH_2$ which in turn reduces to $2NH_3$ (**Figure 2)** (Howath and Codd, 1985; Margheri *et al*., 1990; Haselkorn, 2007).

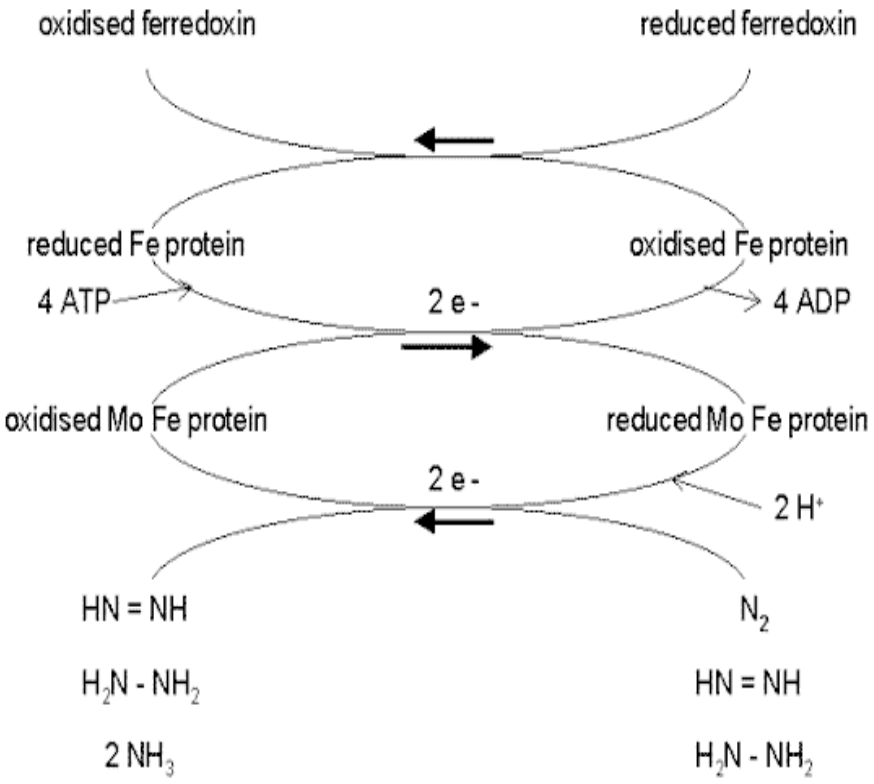


Figure 2. Nitrogen fixation in heterocystous blue green algae (Howath and Codd, 1985; Margheri *et al*., 1990; Haselkorn, 2007).

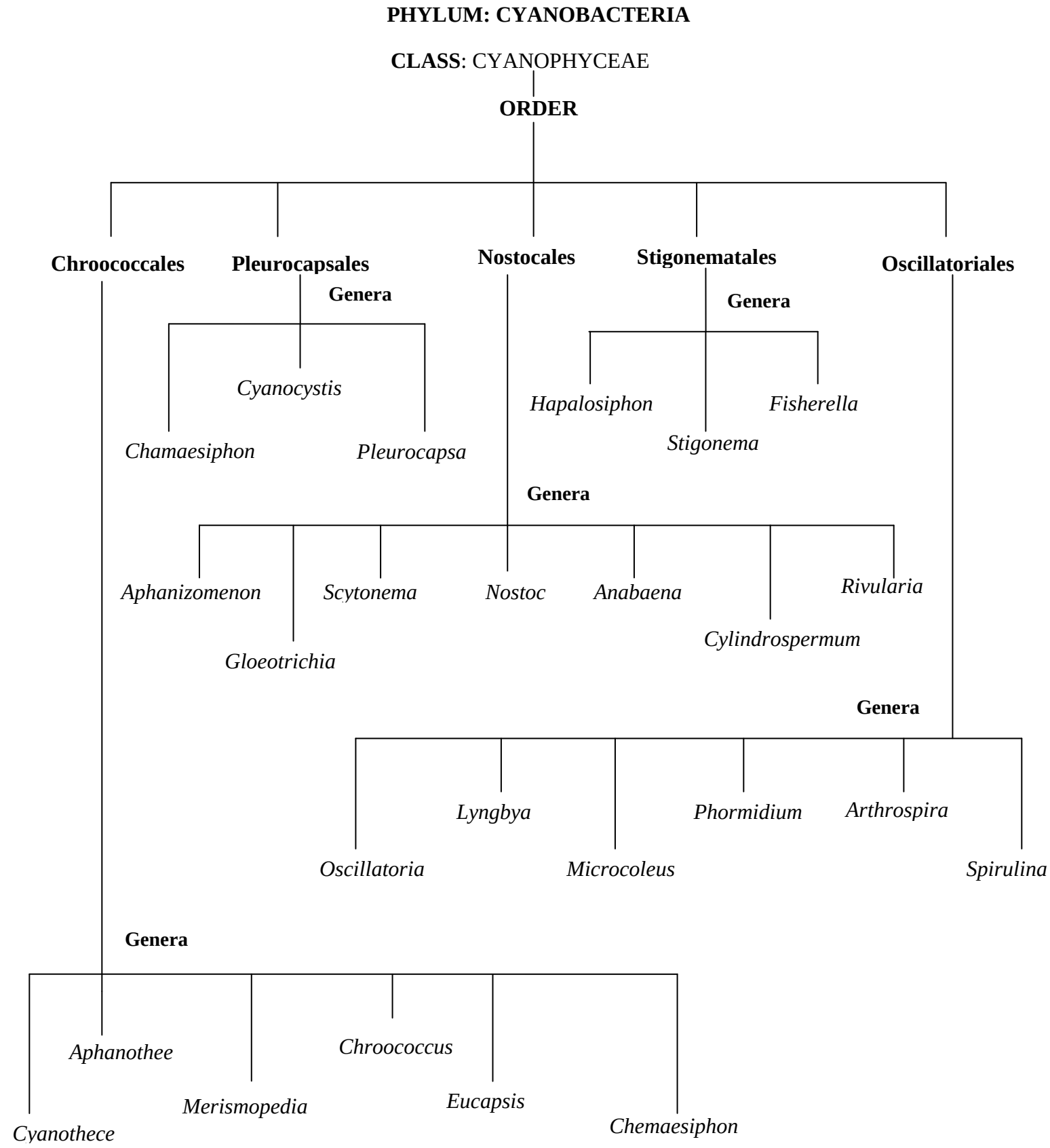


Figure 3. Classification of the Phylum: Cyanobacteria (Rippka *et al*., 1979; Roger, 1982)

2.4 Cyanobacterial diversity in rice fields

Cyanobacteria comprise a large and heterogeneous assemblage of microorganisms which exhibit a wide range of morphological diversity, ranging from unicellsular to branched filamentous organization. Flooded rice fields are known to harbour several biological nitrogen fixations, among which are blue green algae inhabit flooded waters and soil surfaces of rice (Reddy and Roger, 1988; Ladha and Reddy, 2003; Bei *et al*, 2013). Many of them can differentiate heterocysts for nitrogen fixation, akinetes for surviving and hormogonia for gliding movements (Komarek, 2006). Species of *Nostoc, Anabaena, Tolypothrix, Aulosira, Cylindrospermum, Scytonema, Westellopsis* and several other genera are widespread in Indian rice field soils and are known to contribute significantly to soil fertility (Venkataraman, 1981; Kaushik, 1991; Nayak *et al*., 2001; Nayak *et al.*, 2004) (Table 2.1)**.** Katoh et al. (2012) reports that the *Nostoc* species are very useful in agricultural applications because of their nitrogen fixation activity, extracellsular polysaccharide, photosynthetic system, and particularly desiccation tolerance ability and these properties helps to improve the quality of nutrient-poor soils. Cyanobacterial population increased from 14.6×10^4 to 141.0×10^4 cfu/g soil and nitrogen fixation ranged from 1.84 to 8.13 mg N 50/mL as observed in 18 heterocystous cyanobacteria Isolated from rice fields of 11 district of Bangladesh (Begum *et al*., 2008). A detailed comparative study from five different states of North Eastern region of India revealed that 450 unialgal cyanobacteria was Isolated in pure form the selected Isolates were identified by Desikacharya (1959) and Anagnostidis and Komarek (1988). Isolated cultures were examined for their cultural behaviour and released ammonia in nutrient media to detect nitrogen contribution in soil and water bodies in the form of released extracellsular ammonia (Tiwari *et al*., 1972). 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 *et al*., 2010).

Table 2.1 lists blue green algae of different habitats of rice field.

S.No	Habitat	Cyanobacterial species	References
1.	Bottom dwellers	*Aphanothece, Microcoleus, Oscillatoria*	Venkataraman and Neelakantan, 1967 Nayak *et al*., 2009
2.	Phytoplankton	*Merismopedia, Aphanothece, Oscillatoria, Lyngbya, Anabaena*	Roger, 1982 Prasanna and Nayak, 2007
3.	Free floating	*Aphanothece, Anabaena, Aulosira, Gleotrichia*	Roger, 1982 Rippka and Waterbury, 1977
4.	Wet soil inhabitants	*Aphanothece, Microcoleus, Oscillatoria*	Venkataraman, 1976 Kaushik *et al*., 1994 Prasanna and Nayak, 2007
5.	Moist soil forms	*Aphanothece, Oscillatoria, Lyngbya, Porphyrosiphon, Nostoc, Anabaena, Scytonema, Chlorogloeopsis, Westiellopsis*	Rippka *et al*, 1979 Roger, 1982 Pabbi, 2008 Prasanna and Nayak, 2007
6.	Epiphytes	*Calothrix, Microchaete, Gloetrichia, Hapalosiphon*	Irisarri *et al*., 2001
7.	Nitrogen deficient	*Nostoc, Anabaena, Aulosira, Calothrix, Westeillopsis*, and *Scytonema*.	Venkataraman, 1975 Prasanna and Nayak, *2007*
8.	Rhizosphere of rice and wheat	*Anabaena, Nostoc, Weistellopsis, Calothrix*	Venkataraman, 1975 Goyal, 1993
9.	Intracellsular colonization of roots	*Nostoc, Anabaena, Calothrix*	Roger, 1982 Prasanna and Nayak, 2007

Paddy field soil provides environmental conditions favourable for the growth of blue green algae (Watanabe and Yamamoto, 1971). Different heterocystous and filamentous cyanobacterial species reported in other countries in rice field soil also comprises the same group as found in India (Table 2.2). Irisarri et al. (2001) reported the presence of *Nostoc* and *Anabaena sp*. in the rice soil of Uruguay. *Nostoc, Anabaena, Gloeotrichia, Cylindrospermum*

was reported in soil of Chile (Pereira *et al*., 2005). *Oscillatoria, Anabaena, Nostoc, Calothrix, Nodularia, Cylindrospermum, Aphanocapsa* and *Microcystis* were reported in soils of Korea and St. Francis County, Arkansas (Kim, 2006; Smith, 2008).

Table 2.2 Blue-Green algae from rice soil found in India and other countries

S.No	Countries	Cyanobacterial genera	Reference
1.	India	*Nostoc, Anabaena, Aulosira, Tolypothrix, Cylindrospermum, Rivularia, Gloeotrichia, Hapalosiphon, Lyngbya, Calothrix, Phormidium, Spirulina, Aphanothece, Fischerella*	Venkataraman, 1975; Prasanna and Nayak, 2007; Srivastava *et al*., 2009
2.	Uruguay	*Nostoc* and *Anabaena*	Irisarri *et al*., 2001
3.	Chile	*Nostoc , Anabaena, Gloeotrichia, Cylindrospermum*	Pereira *et al*., 2005
4.	China	*Nostoc, Oscillatoria, Anabaena, Gloeocapsa, Scytonema, Leptolyngbya, Phormidium, Microcoleus, Spirulina, Chroococcidiopsis, Synechococcus, Cyanothece, Chamaesiphon, Synechosystis*	Song *et al*., 2005 Lin *et al*., 2013
5.	Bangladesh	*Nostoc, Phormidium, Synechosystis*	Begum *et al*., 2008
6.	Spain	*Nostoc, Phormidium, Synechosystis*	Quesada *et al*., 1996
7.	Czech Republic	*Aulosira* sp. and *Nostoc*	Lukesova *et al*., 2009
8.	Thailand	*Nostoc, Calothrix, Anabaena*	Chunleuchanon *et al*., 2003
9.	Korea	*Oscillatoria, Anabaena, Nostoc, Calothrix, Nodularia, Cylindrospermum, Aphanocapsa*	Kim, 2006
10.	St. Francis County, Arkansas	*Anabaena, Aphanocapsa, Cylindrospermum, Nostoc, Oscillatoria, Microcystis*	Smith, 2008

Literature survey reports different cyanobacterial flora of rice fields of India (Ahmed *et al*., 1999; Tiwari *et al*., 2000; Nayak *et al*., 2001; Kaushik and Prasanna, 2002; Mishra and Pabbi, 2004; Choudhury and Kennedy, 2005; Rai, 2006; Nayak and Prasanna 2007; Dey *et al*., 2010).

Overall in India (Table 2.3), it was reported that on an average, blue green algae accounted for 33% of the 2213 soil samples and up to 50% of the total algae were BGA in southern and eastern states (Venkataraman, 1975; Kaushik, 2005; Pabbi 2008). High density ($2\times10^3 – 10^6$ CFU g^{-1} dry soil) of N_2 fixing BGA was reported in the Indian paddy soils (Pabbi, 2008). However, their occurrence varies depending upon the climatic factors and soil conditions. Most predominant nitrogen fixing genera in Indian paddy soils are *Anabaena, Nostoc, Aulosira, Calothrix, Tolypothrix, Aphanothece* and *Gleotrichia*. In Southwest India, both heterocystous and non heterocystous forms of cyanobacteria was reported which included *Hapalosiphon, Anabaena, Nostoc, Cylindrospermum, Calothrix, Phormidium, Oscillatoria* and *Lyngbya* whereas soil samples from rice field soils of Baraich district of Uttar Pradesh showed abundance of heterocystous forms represented by *Nostoc, Anabaena, Calothrix, Scytonema, Tolypothrix, Hapalosiphon and Westiellopsis* (Misra *et al*., 2001; Pabbi, 2008). In Jammu & Kashmir, isolation from soil samples collected from dry patches showed the presence of heterocystous forms belonging to *Anabaena, Calothrix, Nostoc, Tolypothrix* and *Westiellopsis* (Misra *et al*., 2004; Pabbi, 2008). The abundance of cyanobacteria in the rice fields may be attributable to favourable environment with respect to their requirement for light, water, high temperature and nutrient availability. Nayak and Prasanna (2007) reported more heterocystous forms while studying cyanobacterial abundance and diversity in rice field soils of India.

Table 2.3 Blue-Green algae obtained from rice soil in India

S.No	Locations	Genus	References
1.	Uttar Pradesh	*Tolypothrix, Anabaena, Nostoc, Gloeocapsa, Oscillatoria, Hapalosiphon, Lyngbya, Phormidium, Calothrix, Westiellopsis.*	Venkataraman, 1975 Nayak *et al*., 2001 Prasanna and Nayak, 2007 Srivastava *et al*., 2009
2.	Kerala	*Cylindrospermum, Phormidium, Calothrix, Anabaena, Nostoc*	Venkataraman, 1975 Subramanian,1992 Srivastava *et al*., 2009
3.	Meghalaya	*Cylindrospermum, Anabaena, Nostoc*	Tiwari and Singh, 2010 Nongbri and Syiem, 2012
4.	Assam	*Gloeocapsa, Anabaena, Cylindrospermum, Nostoc, Aulosira, Lyngbya, Phormidium, Calothrix, Westiellopsis.*	Venkataraman, 1975 Yashmin, 2003 Rout and Borah, 2009 Bharadwaj and Baruah, 2013.
5.	Tamil Nadu	*Anabaena, Gloeocapsa, Oscillatoria, Hapalosiphon, Lyngbya, Phormidium, Calothrix, Westiellopsis, Plectonema, Scytonema*	Venkataraman, 1975 Thajuddin and Subramanian,1992 Thajuddin *et al*., 2002 Muthukumar *et al*., 2007
6.	Orissa	*Phormidium, Hapalosiphon, Anabaena, Nostoc, Calothrix*	Sahu *et al*., 1996 Dey *et al*., 2010
7.	Bihar	*Phormidium, Oscillatoria, Hapalosiphon, Anabaena, Calothrix*	Prasanna and Nayak, 2007
8.	Jammu & Kahmir	*Westiellopsis, Phormidium, Gloeothece Calothrix, Nostoc.*	Venkataraman, 1975 Bhushan and Kumar, 2013
9.	Punjab	*Calothrix, Anabaena, Nostoc, Cylinderospermum*	Venkataraman, 1975 Nayak, 2009
10.	Karnataka	*Cylinderospermum, Tolypothrix, Nostoc, Phormidium, Lyngbya*	Venkataraman, 1975 Subramanian, 1992

2.5 Beneficial uses of blue green algae

2.5.1 Blue Green Algae as Biofertilizers (Algalization)

The agricultural uses of nitrogen fixing blue green algae are well known and their contribution in maintenance of the fertility of rice field soils has been well documented all over the world. In India alone, the beneficial effects of cyanobacteria on yield of many rice varieties have been demonstrated in a number of field locations. Literature survey reports that blue green algae were recognized as the first nitrogen fixing agent in the soil of paddy field attributing soil fertility ecologically (De, 1939; Stewart, 1967; Venkataraman 1981; Roger, 1982) and application of BGA during rice cultivation could substitute nitrogen fertilizer (Goyal, 1993; Mishra and Pabbi, 2004). Wetland rice fields can provide an ideal condition for the growth of cyanobacteria, fixing 25–30 kg N ha^{-1} $crop^{-1}$, and reducing the use of urea fertilizer in rice culture by 30% (Hashem, 2001; Choudhury *et al*., 2014). Blue green algae consists of enzyme Nitrogenase that convert atmospheric nitrogen into ammonia (NH_3), or nitrates (NO_3) which are readily absorbed by plants and is converted into nucleic acid and proteins (Roger and Ladha, 1992). Algalization of BGA in rice cultivation promotes organic farming without usage of chemical fertilizers and production of organic basmati rice has been reported to develop a potential export market in the country (Mulbry *et al*., 2008).

Biological nitrogen fixation (BNF), a microbiological process which convert's atmospheric nitrogen into a plant-usable form, offers this alternative. It has been found that BNF technologies are economically viable, ecologically sound and socially accepted (Ladha and Reddy, 2003). The technology has been easily adopted by farmers for multiplication at their own level. 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 (Ariosa *et al*., 2004). The fertility of rice soils is known to be sustained by the process of biological nitrogen

fixation and much interested has been generated in improving the nutrient status of rice soils by enrichment with diazotrophic cyanobacteria and symbiotic systems such as *Azolla* (a water fern which harbors cyanobacteria in its leaf cavities) as biological inputs (Nayak *et al*., 2004).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). The nutrient balance for total N, available N, total P and available P was observed positive in biofertilizers and chemical fertilizer-treated plots. The total and available K was observed negative balance in all the treatments. Blue green cyanobacteria 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). Cyanobacteria also improve soil characteristics by, modifying texture size and subsequent aeration (Ibraheem, 2007), increasing phosphorus content (Fuller and Rogers, 1952) and enhancing carbon content and water holding capacity (Richert *et al*., 2005).

2.5.2 Other potential uses of blue green algae

Blue green algae or cyanobacteria hold immense potential in food production providing good source of proteins, vitamins and minerals, pharmaceutics as well as therapeutics for example S*pirullina*, in hydrogen production for example *Oscillatoria*, *Synechococcus*, *Microcystis* (Prescott, 1969; Khan *et al*., 2005). Cyanobacteria are reported for showing immense potential in industrial effluent, chemical industries, waste water treatment, solar energy conversion and bioremediation of aquatic and terrestrial habitats (Cairns and Dickson, 1971; Dubey et al., 2011). Table 2.4 summarizes other beneficial uses of cyanobacteria.

Table 2.4 Beneficial uses of cyanobacteria

a**)** *Pharmaceutics (Drug discovery) and Therapeutics*

S.No	Potential Use	Reference
1.	Natural products with antitumor and antimicrobial activity (*Cylindrospermopsis raciborskii* CYP011K and *Nostoc sp*. CENA69)	Silva *et al*., 2014
2.	Acetylcholinesterase inhibitory activity (*Nostoc* sp. str. *Lukesova* 27/97 and *Nostoc ellipsosporum Rabenh.* str. *Lukesova* 51/91)	Zelik *et al*., 2009
3.	Effective for the treatment of gout, fistula and anti cancer activity (*Nostoc* species*; Lyngbya majuscule; Oscillatoria* sp.)	Rezanka and Dembitsky, 2006; Nair and Bhimba, 2013
4.	Source of vitamins (biotin, thiamine, riboflavin, folic, ascorbic and nicotinamide (*Aphanizomenon flos-aquae*)	Rezanka and Dembitsky, 2006
5.	Protein –synthesis and effective against Diabetes and Arthritis (*Spirulina*)	Rezanka and Dembitsky, 2006
6.	Antiviral activity (biologically active against Human Immunodeficiency virus - HIV) (*Spirulina* sp., *Nostoc* sp., *Scytonema* sp)	Singh *et al*., 2011
7.	Anti bacterial activity (active against nosocomial infections like methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*)	Reinert *et al*., 2007; Singh *et al*., 2011
8.	Immunomodulatory activity (cyanobacteria such as *Spirulina* increases phagocytic activity, increased antigen production and increased natural killer cells-mediated antitumor activity in chicken)	Qureshi and Ali, 1996 Singh *et al*., 2011
9.	Antiprotozoal activity (Compounds obtained from *Oscillatoria*, *Lyngbya* and *Fischerellambigua* was effective against *Trypanosoma cruzi, Leishmania Mexicana, Plasmodium falciparum*)	Wright *et al*.,2005; Mcphail , 2007;
10.	Protease inhibitor activity (compound obtained *Microcystis* was effective against Chymotrypsin)	Bister, 2004

2.4b. *Microalgae reported for Biodiesel production*

S.No	Taxa	Reference
11.	*Synechococcus elongatus*	Nicole *et al*.,2013
12.	*Cylindrotheca* sp., *Dunaliella primolecta, Nannochloropsis* sp. *Botryococcus braun , Chlorella* sp.	Chisti, 2007
13.	*Scenedesmus obliquus* and *Chlorella vulgaris*	Singh *et al*., 2011
14.	*Synechocystis* sp., *Oscillatoria* sp, *Lyngbya limnetica and Scytonema bohneri*	Rajeshwari and Rajashekhar, 2011
15.	*Phormidium laminosum* and *Gloeobacter violaceus*	Maslova *et al*., 2004

2.4c. Blue green algae reported for Hydrogen production

S.No	Taxa	Reference
16.	*Anabaena cylindrical, Oscillatoria brevis, Calothrix scopulorum; Synechococcus* sp.	Hosmani, 1998
17.	*Anabaena flos-aquae, Nostoc muscorum, Nostoc linckia*	Masukawa *et al*.,2001
18.	*Microcystis* sp.*and Gloebacter* sp.	Hosmani, 1998
19.	*Synechocystis* sp. *and Aphanocapsa montana*	Hernandez and Olguin, 2002
20.	*Spirulina platensis*	Chisti, 2007

2.6 Rice

Rice (*Oryza sativa*) is monocot plant, of the grass family (Poaceae). As a cereal grain, it is the most popular cereal worldwide, serving as a stable food for 39 countries and nearly half of the world's population (Juliano, 1993). Globally rice is considered as dietary energy source providing 22% of total energy intake (Kainuma, 2004). Rice is the second highest worldwide produced and consumed staple food and increasing ratio of population demands more production of rice to meet its consumption (Kubo and Purevdoj, 2004). Rice is cultivated under different climatic conditions (temperature, arid, semi-arid, humid etc). Based on soil-water conditions rice production ecosystems include rainfed lowland, irrigated lowland, rainfed upland and irrigated upland. Food and Agriculture Organization (FAO) of the United Nations revealed that global rice production may no longer be stable in the future by 21st century. Global rice production in 2013 reports 744.9 million tons with relatively 1.1 % increase as compared to previous season. Production rate was low in several important producers in Asia, including China, Malaysia and Loas due to climatic conditions whereas India, Indonesia and Pakistan produced higher grain yield of rice (www.fao.org). India is the largest producer of white and brown rice and accounts for 20% of the world's production and Indian Agriculture Ministry reports that in India total area under rice cultivation is around 36-37 million hectares and over 80% of planting is done during July to August. Indian government reports that rice production in 2013-14 (October - September) is predicted to reach a highest record of 106.19 million tons, about 1% increase from the previous year (Figure 4) (www.oryzae.com).

Table 2.5 Rice production (%) of top ten states of India

S.No	State	Production (%)
1.	West Bengal	15.80
	Andhra Pradesh	12.71
2.	Uttar Pradesh	11.91
3.	Punjab	10.86
4.	Orissa	7.31
5.	Tamil Naidu	7.06
6.	Chattisgarh	5.40
7.	Bihar	5.34
8.	Karnataka	3.70
9.	Haryana	3.61

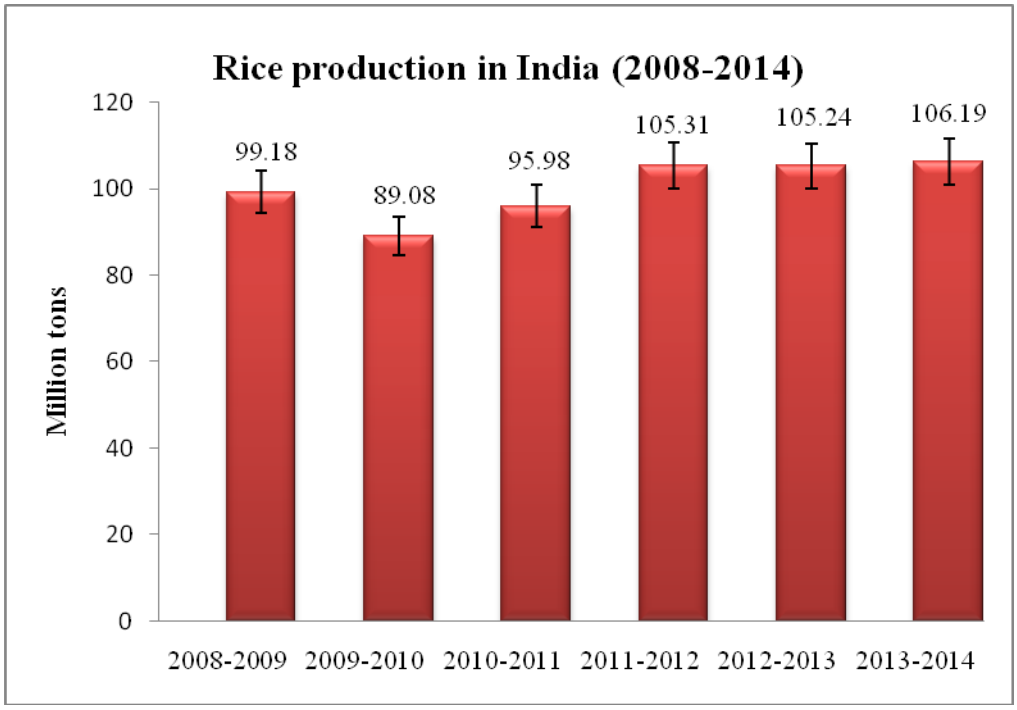


Figure 4 Rice productions in India

Source: Ministry of Agriculture; http://oryza.com/news/rice-news/india-government-projects-2013-14-rice-production-record-10619-million-tons

Table 2.6 Worldwide production and consumption of rice (kg/Ha)

a. Worldwide Rice Production (Kg/Hectare) (2008/09-2013/14)
http://www.airea.net/page/65/world-rice-production-consumption-and-stocks

Countries	**2008/09**	**2009/10**	**2010/11**	**2011/12**	**2012/13**	**2013/14**
Bangladesh	31200	31000	31700	33700	34000	34200
Brazil	8570	7929	9300	7888	8160	8500
Burma	11200	11642	10528	10816	10666	11000
Cambodia	3992	4056	4233	4268	4600	4900
China	134330	136570	137000	140700	143000	144000
Egypt	4673	4564	3100	4250	4675	4850
India	**99180**	**89090**	**95980**	**105310**	**104000**	**108000**
Indonesia	38310	36370	35500	36500	37500	37700
Japan	8029	7711	7720	7646	7756	7720
Korea South	4843	4916	4295	4224	4006	4220
Nigeria	2632	2234	2818	2877	2370	3100
Pakistan	6900	6800	5000	6200	6000	6200
Philippines	10755	9772	10539	10700	11350	11700
Thailand	19850	20260	20262	20460	20200	21100
Vietnam	24393	24993	26371	27152	27650	27850
Others	33298	35598	37360	37251	37952	38183
Subtotal	442155	433505	441706	459942	463885	473223
United States	6546	7133	7593	5866	6334	6038
World Total	**448701**	**440638**	**449299**	**465808**	**470219**	**479261**

b) Worldwide Rice Consumption (Kg/Hectare) (2008/09-2013/14)
http://www.airea.net/page/65/world-rice-production-consumption-and-stocks

Countries	**2008/09**	**2009/10**	**2010/11**	**2011/12**	**2012/13**	**2013/14May**
Bangladesh	31200	31600	32400	34300	34500	34700
Brazil	8400	8477	8200	7928	7900	7800
Burma	10800	10890	10100	10200	10200	10250
Cambodia	3220	3270	3370	3450	3615	3800
China	133000	134320	135000	139600	144000	147000
Egypt	4270	3940	3300	3620	3900	4000
India	**91090**	**85508**	**90206**	**93334**	**96100**	**98500**
Indonesia	37100	38000	39000	39550	40000	40300
Japan	8326	8200	8200	8050	8250	8150
Korea South	4789	4701	5175	4905	4612	4497
Nepal	2880	3060	2713	3224	3320	3350
Nigeria	4220	4350	5000	5400	5700	5900
Philippines	13100	13125	12900	12850	12925	12925
Thailand	9500	10200	10300	10400	10500	10600
Vietnam	19000	19150	19400	19650	20100	20600
Others	50568	52370	53923	56188	57500	58212
Subtotal	433097	434092	441190	456007	466040	473187
United States	4082	4016	4317	3470	3810	3657
World Total	437179	438108	445507	459477	469850	476844

2.7 Carrier for algal biofertilizers

Conventionally, soil has been used as a carrier for cyanobacterial biofertilizers whereas in one study it was reported that soil based inoculum have proved to be disadvantageous due to poor inoculum loading, heavy contamination and its bulky nature (Bisoyi & Singh 1988; Reynand and Meeting 1988; Jha *et al*., 2004). Paddy straw (Kaushik and Prasanna 1998), multanimitti (Goyal *et al*., 1997), sugarcane waste, rice husk (Kannaiyan 2000) and coconut coir (Malliga *et al*., 1996) was developed as new carrier material (Jha and Prasad, 2005). Best approach suggested for useful carrier material for cyanobacteria was to utilize plant biomass with insecticidal properties as a carrier material to control cyanobacterial grazers (Jha and Prasad, 2005). Shelf-life of cyanobacterial biofertilizer can be augented by selecting translucent packing material, dry mixing and paddy straw as a carrier (Jha *et al*., 2005). Dry mixing with a ratio of 50:50 (carrier:cyanobacteria) gave better inoculum loading and shelf-life and decrease in cyanobacterial population was reported least in dried cyanobacterial flakes, indicating its possibility to develop without any carrier material mixing (Jha *et al*., 2005). Field experiment was conducted to compare the effect and efficiency of two carrier based blue green algal (BGA) biofertilizers developed from wheat straw and multanimitti with the traditional soil based BGA biofertilizer, on the grain yield of rice cultivar 'PNR 381'for a period of three years at IARI farms, located at New Delhi, India. Treatments included five levels of nitrogenous fertilizer urea and their interaction with the three types of BGA biofertilizers. Highest grain yield was obtained with the application of multani mitti based biofertilizer along with 90kg urea/ha, whereas straw based and soil based biofertilizers treatments showed highest yield when supplemented with 90 and 120 kg N/ha (Dhar *et al*., 2007) respectively. The coir waste BGA application resulted in increase in root and shoot length, colour of leaves and number of roots (Malliga *et al*., 1996; Pabbi, 2008). Field trials conducted using straw based, soil based and multani mitti based BGA biofertilizers and it

was reported that multani mitti based biofertilizer gave highest yield followed by straw based and soil based BGA inoculants (Pabbi, 2008).

2.8 Coal fly ash production and utilization

Coal fly ash is the solid by product of coal combustion obtained from Thermal power plant all over the world. With increasing demand of energy, coal based Thermal power plants are generating huge amount of fly ash for which safe disposable and effective utilization is of major concern (Gupta *et al*., 2013). During the last decade, a dramatic increase in coal ash production has been reported and its utilization rate is lower than the production rate (Table 2.7). USA, Germany, France and Netherland utilize 70% of the total coal fly ash produced in different aspect whereas India has only 15% utilization rate. Largest fly ash producing countries reported were China (395.5 Mt), India (200 Mt) and USA (118.1 Mt) for which effective utilization rate was reported 265Mt for China, 14.5 Mt for India and 49.7Mt for USA (Heidrich *et al*., 2013). In India coal fly ash production rate will reach up to 300-400 MT/year by 2016-17 (http://fly ash2012.missionenergy.org) and has been regarded as useful accomodity for utilization in different sectors such as in manufacturing cement, concrete, bricks (Singh 1998; Asokan *et al*. 2005), wood substitute products (Saxena and Prabhakar 2000; Haynes, 2009) for soil stabilization, in road base/embankments and consolidation of ground, land reclamation and as a soil amending agent in agriculture (Jala and Goyal 2006; Alam and Akhtar, 2011). Fly ash has been recognized as a potential soil amendment for increasing the availability of mineral nutrients to improve soil fertility and plant growth (Mittra *et al*., 2005; Lee *et al*., 2006; Pandey and Singh, 2010).

Table 2.7: Worldwide production and utilization rate of coal fly ash (Heidrich et al., 2013)

Countries	Production in million of tons (Mt)	Utilization in million of tons (Mt)
USA	118.1	49.7
Europe and Eurasia	52.6	47.8
Russian Federation	26.6	5.0
Middle east and Africa	32.2	3.4
Australia	13.1	6.0
India	200	14.5
China	395.5	265
Other Asia Pacific	16.7	11.1

2.8 Physicochemical characteristics of soil, charcoal, montmorillonite and coal fly ash

Coal fly ash is composed mainly of the inorganic constituents of the coal: oxides of silicon, aluminium, iron and calcium which acts as a useful ameliorant that improve the physical, chemical and biological properties of problem soils having deficiency of macro (Nitrogen, Phosphorus, Potassium) and micronutrient nutrients (Cu, Zn, Fe,) (Singh, 2009; Jala and Goyal, 2006). The physiochemical similarity of fly ashes to alumina silicate enables fly ash to be transformed into materials with a zeolite crystalline structure under appropriate hydroThermal treatment (Lee *et al*., 2006). Addition of coal fly ash to soil increases the availability of silica (Si), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S) and other nutrients (Elseewi *et al*., 1980). Wood charcoal, a rich carbon source, is used as adsorbents for environmental purification and humidity regulation (Pulido *et al*., 1996; Hata *et al*., 1998). Soil is a complex mixture of chemicals and organisms some of which are usually organized at the nanolevel (Nenadovic *et al*., 2010). Montmorillonite particles are considered as nano-filler composites as it consists of hydrated form of minerals (Paluszkiewicz *et al*., 2011). Montmorillonite is a kind of 2:1 type layered silicates and

further investigations on montmorillonite for synthesis of inorganic-organic clay complexes might be of great potential applications in remediation of polluted environment (Xue *et al*., 2007).

2.9 Use of fly ash as carrier for microbial inoculants

Development of bio-fertilizer strains of nitrogen fixers and phosphate solubilizers using fly-ash as carrier material showed encouraging results with respect to other commercially available carrier materials and is useful because it increases pH of soil and converts nutrients in available form (Kumar, 2014)**.** Use of fly-ash as bio-formulation is useful because it increases pH of soil and converts the nutrients in available form (Dwivedi, 2007). Use of fly ash (40 t/ha) in combination with bacterial inoculum (*Pseudomonas striata*) showed positive effect on yield of soyabean with higher uptake of phosphorus by grains without any detrimental effect on bacterial population (Gaind and Guar, 2002). Microbial formulation with carrier materials enabled easy-handling, long term storage and effectiveness of biofertilizers. Since beginning for large scale production and usage of biofertilizers, several carrier materials have been tried like farm yard manure (FYM), compost, peat soil, coal, charcoal, cellsulose powder, lignite, talc, bagasse, sedge peat, press mud, teak leaf meal, coconut shell powder, montmorillonite (Goyal *et al*., 1997; Kannaiyan, 2000; Pabbi, 2008). Significant increase in the population of phosphate-solubilizing bacteria (PSB) and bioavailability of the nutrients was observed when fly ash was mixed with organic matter such as cowdung and inoculated with earthworms (*Eisenia foetida*) for 50 days (Bhattacharya *et al*., 2002). Different microbes such as *Pseudomonas* sp., *Fusarium* sp., and *Aspergillus* sp., have been Isolated from fly ash amended soil (Malik and Thapliyal, 2009). Cyanobacterium, *Anabaena doliolum* growth in BG 11 agar plates with different concentration of fly ash (0, 10, 20, 30, 40 and 50%) accumulated different metals like Fe, Ni, Zn and Cu reducing toxicity on plants (Rai *et al*., 2000). Moreover, fly ash used as a carrier for for diazotrophs

and phosphobacteria like *Azsospirillum barsilense*, *Bacillus circulans* and *Azotobacter chroococcum* showed maximum viability in 10% fly ash whereas *Pseudomonas straita* could proliferate in soil and fly ash mixture (Gaind and Gaur, 2004; Malik and Thapliyal, 2009).

2.9.1 Use of fly ash in Agriculture

Study has revealed that fly ash act as a soil conditioner and fertilizer for which field experiment was conducted in villages around the National Capital Power Project, Ghaziabad, Uttar Pradesh and IARI farm, New Delhi to evaluate the effect of fly ash incorporation on soil properties and growth and yield of wheat (*Triticum aestivum* L.), mustard (*Brassica juncea* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) in Table 2.8 a. Fly ash could be regarded as a pesticides as more than 50 species of insect pests were found to be susceptible to fly ash treatment in many crops (Narayanasamy, 2003; Basu *et al*., 2009).It was found that grain yield of maize increased in fly ash – treated plots with the addition of ash up to a maximum addition of 10 t/ha, whereas paddy yield was similar to that with no fly ash treatment. Fly ash addition to soil results in lower bulk density, hydraulic conductivity and improved moisture retention at field capacity and wilting point with no changes in available water (Kalra *et al*., 1997). An attempt was made to develop an integrated plant nutrition system (IPNS) utilizing fly ash (FA), and paper factory sludge (PFS), along with farm yard manure (FYM), crop residue (CR) and chemical fertilizers (CF) for rice-peanut cropping system (Mittra *et al*., 2005). The results indicated that prospect of safe disposal and utilization of fly ash and organic wastes in agriculture for retaining productivity of problem soils, reduced the usage of costly chemical fertilizer, bring greater economy in cultivation and minimizing environmental problems. Fly ash up to 50t/ha was used to study the effect on the soil properties and yield of wheat (*Triticum aestivum* L.), mustard (*Brassica juncea* L.), rice (*Oryza sativa* L.) and maize (*Zea mays*, L.). The yield of wheat was observed to increase for 20t/ha fly ash while paddy and mustard were observed to survive well in soil amended with

10t/ha of fly ash, all three crop plants showed improved growth over control (Kalra *et al.*, 2003). Similar studies was undertaken in Orissa where fly ash was used for amending soil at levels equivalent to 0, 1, 2.5, 5, 10 and 15 metric tons per ha in which rice was grown and elemental residues of amended soil and plant parts were enumerated. Fly ash amendments caused significant improvement in soil property with enhancement of K, P, Mn, Ni, Co, Pb, Zn, Cu, Cr, and Cd followed by increased germination percentage of rice seeds, plant growth (shoot length, leaf area and pigment composition) and yield (panicle length, seeds per panicle, seed weight and yield per plant) of rice (Dwivedi, 2007), also accumulation of Cu, Pb, Cr, Cd in seeds of plants grown in FA amended @ 10 metric tons per hectare soil was observed to be within permissible range (Mishra *et al*., 2005) (Table 2.8a). It has been reported that a mixture of fly ash and phosphorus – gypsum should reduce P loss from rice paddy soils and increase soil fertility. In a study, mixture of fly ash and phosphorus-gypsum (50:50, wt/wt) was used in rice cultivation to supply Ca and Si to rice while reducing B toxicity. High Ca content in this mixture converts water – soluble P to less soluble forms and thereby reduces the loss of soil P to surface runoff (Lee *et al*., 2007).

Dwivedi et al. (2007) experimented to observe growth performance and biochemical responses of three rice (*Oryzae Sativa* L.) cultivars viz., Saryu – 52, Sabha – 5204, and Plant - 4 grown in garden soil (GS, control) and various amendments of fly ash for a period of 90 days and its effect on growth and productivity of plant was evaluated for metal accumulation in the plants. It was observed that the toxicity of FA at higher concentration (≥ 50%) was reflected by the reduction in photosynthetic pigents, protein and growth parameters viz., plant height, root biomass, number of tiller, grains and straw weight. However, at lower concentrations (10-25 %), FA enhanced growth of the plants. Rice varieties Saryu – 52 and Sabha – 5204 were more tolerant and showed improved growth and yield at lower FA

application doses as compared to Plant-4 and were found suitable for cultivation in FA amended agricultural soils for better crop yields.

Blue green algal biofertilizers plays a vital role in ameliorating the nitrogen demand and fly ash to the growth and yield of rice. Field experiment was conducted to analyze growth performance, elemental composition (Fe, Si, Zn, Mn, Cu, Ni, Cd and As) and yield of rice (*Oryzae sativa* L. cv. Saryu-52) grown under different doses of fly-ash (FA applied @ 10 and 100 t ha^{-1} denoted as FA10 and FA100, respectively) mixed with garden soil (GS) in combination with nitrogen fertilizer (NF; applied @ 90 and 120 kg/ha denoted as NF 90 and NF120, respectively) and blue green algae biofertilizer (BGA; applied @ 12.5 kg/ha denoted as BGA12.5). Significant enhancement in growth was observed in plants grown on amended soils as compared to GS and best response was obtained in amendment of FA@ 10 kg + NF@ 90 kg+ BGA@ 12.5 kg per hectare. Accumulation of Si, Fe, Zn and Mn was higher than Cu, Cd, Ni and As. Arsenic accumulation was detected only in FA100. Inoculation of BGA12.5 caused slight reduction in Cd, Ni and As content of plants as compared to NF120 amendment. The high levels of stress inducible non-protein thiols (NP-SH) and cysteine in FA100 were decreased by application of NF and BGA indicating stress amelioration. Study suggested integrated use of FA, BGA and NF for improved growth, yield and mineral composition of the rice plants besides reducing the high demand of nitrogen fertilizers (Tripathi *et al.*, 2008). Arivazhagan et al. (2011) conducted several field trials using fly ash as a soil amendment and found that application rate of 40–50 MT $ha^{-1}$was more effective with 15-20% increase of crop yield for cereals, 20-30% for sugarcane (*Saccharum* spp.), 40% for corn (*Zea mays* L.), 25% for potato (*Solanum tuberosum* L.), 30% for mustard (Brassica napus L.), and 10% for vegetables. Singh et al. (2011) reported high application rate of fly ash (60-240MT ha^{-1}) for increased root and shoot length, pigment concentration and yield of forage legumes and vegetables (Table 2.8 b and c).

Several researchers have reported that fly ash could be used as soil amendment and liming material for stabilizing pH of acidic soil (Singh *et al*., 2011; Skousen *et al*., 2013) improving soil physico-chemical properties such as porosity, water holding capacity, root penetration and fertility leading to increased biomass production (Khan and Khan, 1996; Singh *et al*., 1997; Singh *et al*., 2011) and enhances plant micro (Ca, Mg, K, and S) and macronutrients (B, Cu, Fe, Mn, Zn, etc.) (Stewart and Daniels, 1992; Skousen *et al*., 2013). Field and nursery trial on pulses (soyabean) and leguminous crops (pea) reported that application of fly ash at 20 to 40% (w/w) was more effective in promoting the plant growth and increasing yield crop (Gaind and Gaur, 2002; Siddiqui and Singh, 2005; Singh *et al*., 2010). Fly ash was found to be effective in post-harvest preservation of five commonly used pulses; viz. soybean (*Glycin max*), bengal gram (*Cicer arietinum*), green gram (*Vigna radiata*), black gram (*V. mungo*) and red gram (*V. unguiculata*) against the attack of pulse beetle, *Callosobruchus chinensis* till one year (Mendki *et al*., 2001). Different forestry species such as *Leucaena leucocephala, Cassia siamea, Albizia lebbek, Dalbergia sissoo, Eucalyptus* sp., *Acacia* sp., *Casuarina* sp., *Toona ciliate* and *Populus deltoides* have been grown at different fly ash rates (0, 5, 10, 20, 50, 100 %) in combination with farmyard manure and microbial inoculants showed increased plant height and collar diameter (Jala and Goyal, 2006) (Table 2.8d).

Table 2.8 Fly ash application rates used in Agriculture

a) Cereals and Oil seed

Cereal	FA doses and treatment	Remarks	References
Rice	FA application @ 0, 10, 12.5, 15, 17.5 and 20 tha^{-1} with chemical fertilizers @ NPK 80:40:40 kg/ha	A gradual increase in grain and straw yield was observed with increase in ash application up to 17.5t ha^{-1} over control.	Sarangi *et al*., 2001
Wheat ***Mustard***	*Maize*: fly ash application was 0 (NFA), 5, 10, 15, 20 t ha^{-1} at the time of sowing at one location. *Rice*: 10 and 20 t ha^{-1}	*Maize*: Increased crop yield over the Control (NFA) was for flyash application @10 t ha^{-1} *Rice*: 20 t ha^{-1} of FA application showed increased grain yield.	Kalra and Jain, 2003
Rice ***Maize***	*Wheat:* 0, 2.5, 5, 7.5, 10 15, 20, 25, 30, 35, 40 and 50 t ha^{-1}. *Mustard* : 5, 10, 15, 20, 25, 30, 35, 40 and 50 t ha^{-1}	*Wheat*: 20 t ha−1 of FA application showed higher yield. *Mustard*: Increased yield was observed for FA application up to 20 t ha^{-1} over NFA.	
Rice ***Mustard***	FA alone @ 0t ha^{-1} & 10t ha^{-1} and with the combination of organic wastes such as farmyard manure (FYM @ 5t ha^{-1}), fresh paddy straw (PS@ 5t ha^{-1}) and green manure (G@ 2.5t ha^{-1})	Integrated use of fly ash, organic wastes and chemical fertilizers was effective in increasing yield of a rice-mustard cropping sequence as compared to continuous use of chemical fertilizers only. Application of fly ash alone was effective in rising soil available P.	Rautaray *et al*., 2003
Wheat	4.5 Mg ha^{-1} biogas slurry (SL); 15 Mg ha^{-1} biogas slurry (SH);4 Mg ha^{-1} fly ash (FL); 8 Mg ha^{-1} fly ash (FM) and 12 Mg ha^{-1} fly ash (FH) with unamended control (C); Biogas slurry contained 15.0, 5.0, and 10.0 g kg^{-1} N, P, and K N_2 fertilizer : Urea @120 kg N ha^{-1} P fertilizer :SSP @ 26.2 kg ha^{-1} K fertilizers : KCl @50 kg ha^{-1}	Highest grain yield of 6.21 Mg ha^{-1} was observed in 15 Mg ha−1 biogas slurry treatment (SH). Fly ash and biogas slurry should be used as soil amendments for obtaining short-term and long-term benefits in terms of production increments and soil amelioration.	Garg *et al*., 1997

Groundnut ***Mustard*** ***Wheat*** ***Rice***	Application rate of FA: Rice and groundnut: 40 t ha^{-1} Mustard , Wheat: 10-15 t ha^{-1} Control: without amendment	Percentage yield increase over control for rice and groundnut was 14-25% and 10-46% for wheat and mustard.	Kumar *et al*., 2010
Rice	Three rice cultivars viz., Saryu-52, Sabha-5204, and Pant-4 grown in garden soil (GS, control) and various amendments (10%, 25%, 50%, 75% and 100%) of FA for a period of 90 days	Lower concentrations of FA (10-25%) enhanced growth of the plants whereas at higher concentration (50%) the toxicity of FA was reflected by the reduction in photosynthetic pigents, protein and growth parameters viz., plant height, root biomass, number of tillers, grain and straw weight.	Dwivedi *et al*., 2007
Rice	FA @ 0, 40, 80, 90 and 120 Mg ha^{-1} N-P_2O_5-K_2O @ 120-48-80 kg/ha	Increased grain yield was observed around 90 kg ha^{-1} with increased available phosphorus (786 mg/kg) and silicon.	Lee *et al*., 2007
Rice	Six FA treatment (0, 1, 2.5, 5, 10 and 15 Mg/ha) were done on RBD plot for five consecutive seasons, 1999 *rabi* to 2001 *rabi*.	Data indicated that flooded – rice soil amended at 10 $MTha^{-1}$ FA improved physical properties of the soil and yield of rice.	Mishra *et al*., 2007
Rice	10 and 100 t ha^{-1} of FA was mixed with garden soil (GS) in combination with nitrogen fertilizer (NF; applied @ 90 and 120 kg ha^{-1} and blue green algae biofertilizer (BGA; applied @ 12.5 kg ha^{-1})	FA_{10} + NF_{90} + $BGA_{12.5}$ enhanced more significant growth as compared to garden soil with accumulation of Si, Fe, Zn and Mn.	Shukla *et al*., 2008
Wheat	Fly ash based *Azotobacter* and *Azospirillum* formulations alone (10 g formulation + 10 g farm yard manure + 0.5 g gum Arabic + 50 mL water per 1 kg of seed) and in combination with chemical fertilizer (Urea @ 300 kg/ha)	Bio-fertilizers treated soil significantly increased microbial population in soil where as chemical fertilizers treated soil reduced microbial population. Safe and effective utilization of fly ash.	Kumar *et al*., 2010

Rice* and *Peanut	Fly ash application @ 0, 5 and 10 t ha^{-1} with organic materials such as FYM (Farm Yard Manure) or PFS (paper factory sludge) or CR (crop residue). Organic materials were applied in quantity to supply 30 kg N ha^{-1} and lime at 2 t ha^{-1}. N: P_2O_5: K_2O @ 90: 60: 40 kg/ha	10 tha^{-1} of fly ash application with organic sources and chemical fertilizer increased the grain yield and nutrient uptake of rice, and pod yield of peanut compared to chemical fertilizers alone. Fly ash reported as a soil ameliorates and good source of micronutrients and Ca, Mg in acidic soil.	Mittra *et al*., 2005
Rice	Field experiments were carried out to evaluate rice (Oryza sativa) productivity in silt loam and loamy sand soils to which 0, 40, 80, and 120 Mg/ha of fly ash were added with 2 Mg/ha and Si as a control The rates of chemical fertilizer : 60 kg N ha^{-1} as urea 48 kg P_2O_5 ha^{-1}as fused phosphate 56 kg K_2O ha^{-1} as potassium chloride	The highest rice yield was observed at 90 Mg/ha FA with increased uptake of Si, P and K by the rice plants without any excessive uptake of heavy metals in the submerged paddy soil. Fly ash could be a good supplement to other inorganic soil amendments to improve the nutrient balance in paddy soils.	Lee *et al*., 2006
Rice	Control (without amendment) Fly ash (FA @ 50 t ha^{-1}) Farmyard Manure (FYM @ 50 t ha^{-1}) FA+FYM (each @50 t ha^{-1})	Effective treatment was FA+FYM (each @50 t ha^{-1}) with increased grain yield by 92% over control	Singh *et al*., 2011
Corn	58% FA+ 42% soil 79% FA+ 21% soil with mixture of cow manure at 0, 60 or 120 tonnes per hectare, sewage sludge at 0, 60, or 120 tonnes per hectare or chicken manure 0, 25, or 50 tonnes per hectare	Increased yield over control was observed for 58% fly ash application with 42% soil and boron accumulation in corn leaves was at 79% FA addition.	Sajwan *et al*., 1996

b) Pulses and Leguminous plants

Crop	FA doses and treatment	Remarks	References
Soybean *Bengal gram* *Green gram* *Black gram* *Red gram*	Pulses were deliberately infested with stored grain pest, commonly known as pulse beetle, *Callosobruchus chinensis* and treated with 1 g fly ash per 5 kg of pulses under ambient storage conditions for 18 months.	No adult C. chinensis were found in pulses treated with fly ash even after 12 months of treatment. After 18 months, bengal gram was most infested whereas soybean and black gram were least infested. There was no effect of fly ash on the nutritional quality and percent germination of pulses.	Mendki *et al*., 2001
Soyabean	FA: 20, 40, 60 and 80 t/ha with N and P fertilizer. Phosphate solubilizer: *Pseudomonas striata.*	40 t/ha of FA with P. striata inoculation improved the bean yield and P uptake by grain	Gaind and Gaur, 2002
Pea (Pisum sativum)	FA rates @ 0, 25, 50, 70 and 100% fly ash in soil	Accumulation of Zn, Cu, Ni and Fe was observed in all treatment	Tripathi *et al*., 2008
Pea (Pisum sativum)	FA application @ 0, 20, and 40% with soil Microbial Inoculant: Phosphate solubilizing microorganism *Pseudomonas striata* and root-nodule bacterium *Rhizobium* sp with inoculated root-knot nematode *Meloidogyne incognita*	FA application rates of 20 and 40% fly ash with soil was effective for plant growth both in nematode inoculated and uninoculated plants	Siddiqui and Singh, 2005
Cow pea (Vigna sinensis)	FA : soil (w/w) @ 20, 40, 60, 80 and 100% levels	Lower rates of FA application (20 and 40%) increased plant growth, yield, pigent and protein content of pea.	Singh *et al*., 2010

c) Vegetables

Vegetables	FA doses and treatment	Remarks	References
Tomato	Pot trials were conducted with fly ash and normal field soil were mixture (vol'vol) in 11 proportions, i.e. 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and I00%.	Plant growth, yield, (flowering, fruiting, frruit weight/plant, mean fruit weight), carotenoids and chlorophylls were mostly enhanced in the treatments with 40-80% fly ash, being optimal at 50 or 60%.	Khan and Khan, 1996
Tomato	Fly ash application to soil by broadcasting or in rows @ 1, 2, 3 and 4 kg ash m^{-2} in place of inorganic fertilizers. Control plots with NPK (40:20:20 kg $acre^{-1}$) and compost without fly ash. Tomato cultivars: Pusa Ruby, Pusa Early Dwarf and New Uday, and on wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici*	Increased yield was observed in tomato cv. Pusa Ruby (39 ± 61 and 9 ± 24%) for fungus inoculated and uninoculated plants at 3 or 4 kg ash m^{-2} followed by Pusa Early Dwarf (31 ± 61 and 17 ± 34%) and New Uday (21 ± 35 and 4 ± 22%).	Khan *et al*., 2001
Palak (Beta vulgaris)	FA (0%, 5%, 10%, 15% and 20%)	The study remarks that *B. vulgaris* plant was sensitive to FA and lower concentration of FA could be regarded as soil amendment for better management of few crops.	Singh *et al*., 2008
Cabbage	Different amendments of tannery sludge/ fly ash mixture 5, 10, 15, 20 and 100% @ 15kg along with one set of control (garden soil). A = 4:1 (4 part tannery sludge + 1 part FA) B = 4:2 (4 part tannery sludge + 2 part FA)	OC and OM increased with increase in lower amendments with both the mixtures (A and B), while, in higher amendments, values decreased in comparison to their respective controls. The analysis of the results suggested that mixture A (>10%) was found suitable to grow the plants of *B. campestris* due to better yield and less accumulation of metals in the seeds	Gupta *et al*., 2002

Potato	Composting of FA was carried out with and without epigeic earthworm (E. fetida) using five combinations of fly ash (FA) and organic matter (OM) in the form of cowdung viz. FA alone, OM alone, FA + OM (1:1), FA + OM (1:3) and FA + OM (3:1)	Advantage of vermicomposted FA and organic matter (1:1) @ 10 t ha^{-1} in potato cultivation was evidenced by significant increase in crop yield due to enhanced soil fertility and reduced risk of heavy metal toxicity.	Bhattacharya *et al.*, 2012.
Palak ***Mung bean*** ***Chilli***	FA application: 0, 5, 10, 15, 20, 25 and 30%	Increased yield was recorded at 25% FA application with increased number of leaves, plant height and biomass whereas above 25% FA growth and yield declined.	Katiyar *et al.*, 2012

d) Forestry and tree plantation

Species	FA doses and treatment	Remarks	References
Leucaena leucocephala *Cassia siamea* *Albizia lebbek* *Dalbergia sissoo* *Gelina arborea*	Control (No fly ash, only 20 kg soil) Soil +5% fly ash Soil + 10% fly ash Soil + 20% fly ash, and Soil + 50% fly ash	All five tree species showed increased collar diameter, height, number of leaves and leaf at 20% (w/w) FA addition	Pandey *et al*., 1996
Eucalyptus sp. *Acacia sp.* *Casuarina sp.*	FA @ 0-24% (0, 6, 12, 18, 24%)	20% increase in the growth of all three trees were noted around 12-18%	Goyal *et al*., 2002
Acacia auriculiformis *Leucaena leucocephala* *Toona ciliate* *Populus deltoides*	Lagoon ash (210g) + 30% vermiculite (90g) + 30% (w/w) sewage sludge compost Rhizobium Soil alone and with combination 10% FA 5% distillery waste 20%farmyard manure (FYM) Microbial inoulant: *Pseudomomas* .sp +*Azotobacter* sp.	Lagoon ash amended with 30% sewage sludge compost showed better growth for Acacia auriculiformis than that amended with 30% vermiculite whereas both the treatment were ineffective for Leucaena leucocephala Combined application of FA (10%) + FYM (20%) +Microbial inoculants showed increased plant height, collar diameter and total dry biomass.	Chueng *et al*., 2000
Leucaena Leucocephala	Soil:100% Mine waste (MW):100% Coal fly ash:100% Soil : Mine waste: (50:50)%, Soil: Coal fly ash =(50 :50)%	Coal fly ash: Soil (50:50) % and Mine Waste: Soil (50:50) % showed increased biomass yield	Bisht *et al*., 2011

2.9.2 **Impact of fly ash on nitrogen fixing blue green algae**

Cyanobacteria reduces toxicity of fly ash by accumulating metals in their cells with consequent detoxification responses (Kramer *et al*., 1996) as the fly ash contains many essential elements for plant growth like Na, K, Ca, Cu, Mg, Fe, Zn, B and Mo along with some of the constituent metals e.g., zinc, copper and molybdenum, which are required in traces for algal growth for various physiological and biochemical processes. Fly ash has been tested for cyanobacterial growth (Banerjee, 1992; Banerjee and Deb, 1993), where several cyanophycean algae have been reported to accumulate significant amounts of toxic metals from fly ash contaminated surface water (Rai *et al*., 2005). Application of nitrogen fixing cyanobacteria inoculants to enhance N and P status and reducing metal toxicity of fly ash has also been reported by Rai and his coworkers in 2000. Fly ash disposal in aquatic ecosystem increases the salinity (Carlson and Adriano, 1993) that correlates with increase in population of BGA (Sellner *et al*., 1988) which is in accordance with the earlier reports that nitrogen fixing taxa have tolerance to high salinity and boron. This reveals that blue green algae are the most successful early colonizers in fly ash contaminated areas (Carlson and Adriano, 1993; Gupta *et al*., 2002), where boron and other minerals present in fly ash are essential for growth and nitrogen fixation by heterocystous BGA, (Bonilla *et al*., 1990).

2.9.3 **Future prospects of coal fly ash and blue green algal inoculants in agriculture**

Huge generation of coal fly ash from Thermal power plants (TPP's) is of great concern due to its disposal problems and its effective utilization based on its typical characteristics as soil ameliorant in agriculture and forestry is one of the potential area (Ram *et al*., 2011; Goyal and Jala, 2006). More field trials and demonstration of controlled application of fly ash in different soil types and agro-climatic conditions for longer durations are essential to establish its usage as soil amendment agent (Pathan 2003; Ram *et al*., 2011; Skousen *et al*., 2013). Attempts have to be made to study the effect of trace elements and radioactive elements

present in fly ash on soil properties for specific crop (Skousen *et al*., 2013). Intergovernmental Panel on Climate Change (IPCC) assumes that lime application contributes to global warming assuming that carbon dioxide (CO_2) is finally released to the atmosphere for which coal fly ash application could replace lime in agriculture without any environmental threat (Basu *et al*., 2009). Several studies have reported that application of fly ash as bioformulations is more effective and useful in crop production with increased grain yield, improved soil properties with reduction in use of chemical fertilizers and lowering input cost (Gaind and Gaur, 2004; Kumar, 2014). Indiscriminate use of chemical fertilizers has led to soil deterioration which has resulted in low crop productivity. Appropriate combination of coal fly ash with organic manure, microbial inoculants and reduced dosage of chemical fertilizers could be regarded as the best strategy for gainfully using fly ash in agriculture (Mittra *et al*., 2003; Ashokan, 2006; Jala and Goyal, 2006; Ahmaruzzaman, 2010; Masto, 2014).

Chapter 3 MATERIALS AND METHODS

3.1 Isolation and characterization of efficient nitrogen fixing blue green algae from paddy field soils and ash pond samples

3.1.1 Collection of paddy field soils and ash pond samples

a) Paddy field soils

For isolation of efficient nitrogen fixing BGA from organic paddy field soils, samples were collected from rhizospheric zone of rice fields of villages Shauli, Palia Khurd, Dittupur, Gunike, Rajgarh and Sibro, District Patiala (30.7900° N, 76.7800° E), Punjab, India. Soil samples to the depth of 6-10cm were drawn carefully and were collected in sterile plastic bags and stored at 4 ºC in laboratory and further processed for physicochemical analysis isolation.

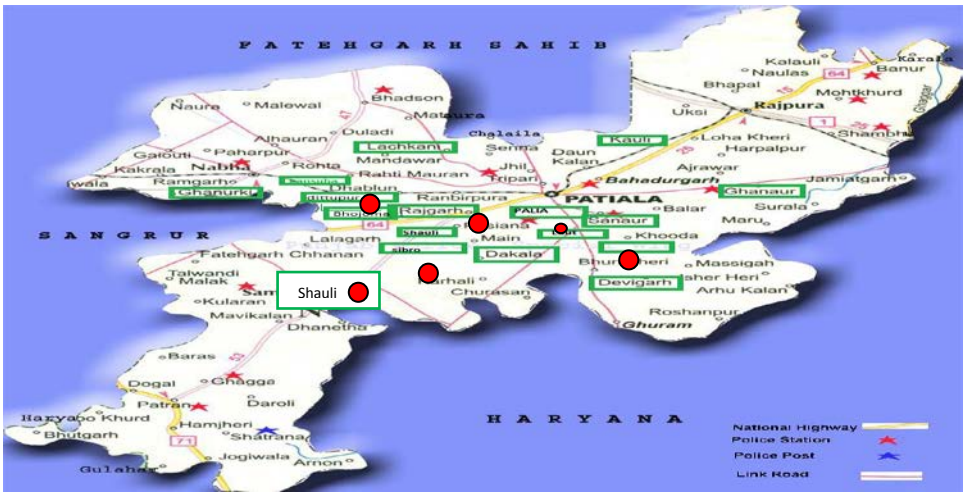


Figure 3.1 Villages with green blocks viz., Devigarh, Dakala, Sibro, Ajnoida, Pedhna, Palia Khurd, Ghanaur, Gunike, Rajgarh, Bhojomajri, Dittupur, Shauli, Lachkani, Kauli, Laut, Sanaur, Gaushala, Pedhna, Bhawanigarh were selected for various field and RBD trials.

- Indicates villages (Shauli, Palia Khurd, Dittupur, Gunike, Rajgarh and Sibro) were soil sample collection of isolation of region specific blue green algae

b) Collection of ash pond samples

Ash pond samples were collected from Rihand Dam, National Thermal Power Cooperation (NTPC), Rihandnagar, Uttar Pradesh (24°12′9″N 83°0′29″E 24.2025°N 83.00806°E), India Dried fly ash was directly analyzed for its physicochemical properties.

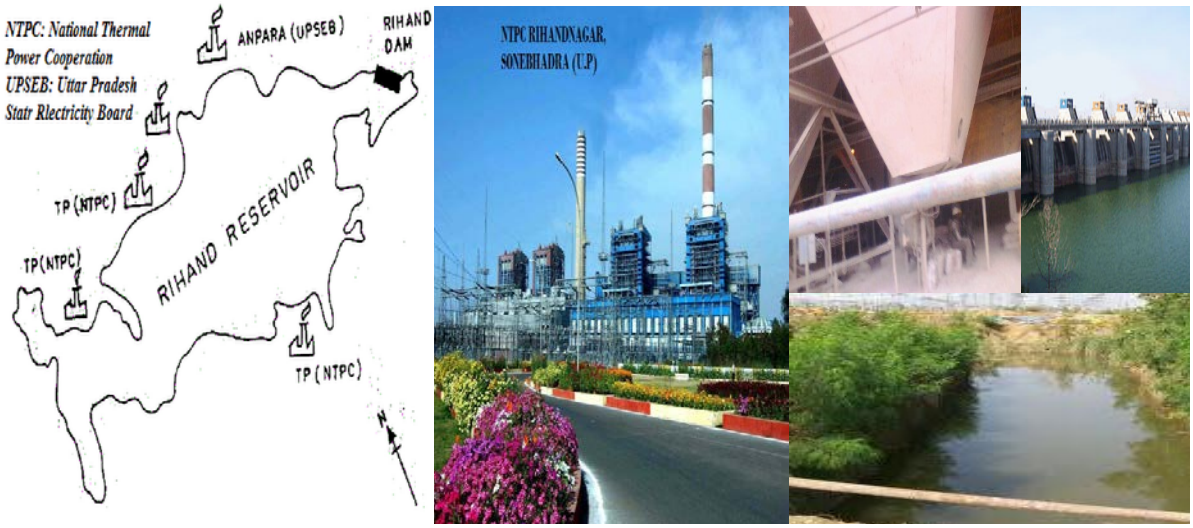


Figure 3.2 Map of area from where pond ash was collected.

3.1.2 Isolation and screening of heterocystous blue green algae (cyanobacteria)

Soil samples were collected from organic paddy fields from nearby region of Patiala district for the isolation of nitrogen fixing blue green algae. One gram of soil was inoculated in 25 mL sterilized BG-11 medium (-N) in 100 mL conical flasks and incubated under 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 hours 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. These unialgal cultures were picked up from the plate and transferred to agar slant.

Composition of BG-11 medium (Stanier *et.al*, 1971)

Constituent's	g/L
Sodium nitrate	1.5
K_2HPO_4	0.040
$MgSO_4.7H_2O$	0.075
$CaCl_2.H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA	0.001
Sodium carbonate	0.020
Trace metal mix	1 mL
Distilled water	1000 mL

Trace metal mix composition

The trace metal A5 solution contained the following constituents in g/L

H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494
Distilled water	1000 mL

3.1.3 Growth and physiological studies

3.1.3.1 Microscopic observations

Microscopic examination of the growth observed in the flasks was undertaken periodically in order to identify the forms present. Identification was done following the keys given by Fritsch (1949); Komark (2008); Ramirez (2011); Uher (2007) and Hrouzek *et al*., 2013). Pure culture was observed under microscope. The cell shape and size were observed, measured by micrometry and documented as microphotograph. Photographs were taken using the light microscope attached with Olympus trinocular microscope. The specimens were observed at 40X magnification.

3.1.3.2 *Nitrogen fixation ability*

Acetylene reduction assay (ARA). Gas chromatographic quantification of ethylene formed was utilized as an index of nitrogen fixation. Commercially available ethylene was utilized for quantification and vials with an equivalent volume of water served as controls (Hardy *et al*., 1973). Measurement of acetylene reduction activity (ARA) was done after 7, 14 and 21 days of incubation. Acetylene (10% v/v) was injected after removal of equivalent amount of air and incubation was done in light for 90 min. The ARA values were expressed as mole ethylene produced per mL culture. All the measurements were taken in triplicate.

3.1.3.3 Total nitrogen estimation **(Piper, 1960)**

Reagents

a) Catalyst mixture: For algae- 10 g anhydrous sodium sulphate + 1g $CuSO_4.5H_2O$ mixed in the ratio 10:1
b) Mixed indicator: Dissolve 0.5 g of Bromophenol green and 0.1g of methyl red indicator in 100mL of ethanol.

c) Boric acid solution: Dissolve 40 g H_3BO_3 in 1 L of distilled water; add 5mL 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_2CO_3 impurities.

e) Conc. H_2SO_4- Specific gravity 1.84

f) Sodium thiosulphate

g) 0.01N H_2SO_4

Procedure

1. Algal sample (50 mL) was placed in 500mL Kjeldahl digestion tube.
2. Added 25mL conc. H_2SO_4 (allowed to stand for 30min).
3. To the above mixture 5 g of sodium thiosulphate was added and allowed to stand for 30min. After that 2-3 g catalyst mixture was added into the flask and glass beads were added to prevent bumping. 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 0.01N 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 30min).
9. To the above mixture 5 g of sodium thiosulphate was added and allowed to stand for 30min. After that 2-3 g catalyst mixture was added into the flask and glass beads were

added to prevent bumping. First heated slowly till the frothing continues 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.
12. 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.
13. This was followed by titration against 0.01N sulphuric acid.

Calculations

Amount (g.) 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.

3.1.3.3 Microscopic determination of heterocyst frequency of cyanobacteria

Procedure

1. Fixed the material in Lugol's iodine solution (dissolved 5g iodine in 100mL 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

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

3.1.3.4 Biomass estimation

Dry biomass estimation (Richmond and Gobbelaar, 1986)

1. The Whatman filter was soaked in water to saturate and dried overnight.
2. The weight of the dried filter paper was noted as the initial reading.
3. Took some beads (4-5mm) in beaker and dried them in oven.
4. 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.
5. This was kept for drying and transferred to hot air oven maintained at about 60ºC, till constant weight was recorded at room temperature.
6. The difference between initial and final reading of the weight gave the dry biomass in mg/mL.

3.1.3.4 Chlorophyll estimation **(Mckiney, 1941)**

1. Algal suspension of 10mL was filtered through Whatman filter paper no.42 and washed with sterile double distilled water.
2. 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.
3. The oak ridge centrifuge tubes were tightly packed, vigorously shaken and kept in water bath at 60^0C for 30min, which led to extraction of chlorophyll into the solution.
4. Samples were removed from the water bath and allowed to cool at room temperature, made the volume again to 10mL by adding 96 % methanol and centrifuged at 8000 rpm for 10 minute.

5. 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 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 650nm wavelength

E_{665}= Absorbance at 665nm wavelength

3.1.3.5 Nitrate reductase (NR) activity **(EC 1.6.6.1., 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.

(i) Reagents:

1% sulphanilamide in 100mL of 1N HCl.

0.2% NEDD (N-1-naphthyl ethylene diamine dichloride)

(ii) Procedure: For the assay of the enzyme, a known volume (10 mL) of cyanobacterial suspension was taken. It was centrifuged (4000g, 10 mins) and pellet was washed with sterile water. The pellet was then incubated overnight in basal medium 47containing 10mM $NaNO_3$ to induce NR. After incubation, one mL of sample was taken and to this added 2 mL of reagent A. Mixed them well and after 15 minutes, added 2mL of reagent B. The pink coloured was allowed to develop for 15 mins. Absorbance was recorded at 540 nm and the values were calibrated against the standard curve using $NaNO_2$. The NR activity was expressed in terms of μ mole NO_2/mL.

3.1.3.6 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 Fe Cl_3 and 49 mL of water and 49 mL of 70% perchloric 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 4000 rpm 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.

3.1.4 Molecular Studies

3.1.4.1 DNA extraction

Total genomic DNA was extracted by a modification of a method by Smoker and Barnum (1998). A 1 mL aliquot of mid-to late log phase culture was pelleted by centrifugation, the medium was decanted, and the pellet was resuspended in 500 μL of 50 mM. Tris- HCl (pH 8.0)-5 mM EDTA (pH 8.0)- 50 mM NaCl. Lysozyme was added to obtain a final concentration of 1 mg/mL, and solution was incubated at 55°C for 30 mins. After the addition of 10 μL of proteinase K (10 mg/mL) and 20 μL of 10% sodium dodecyl sulfate, the mixture was incubated at 55°C for 10 min or until the solution cleared (complete cells lysis). The solution was chilled on ice and extracted with an equal volume phenol-chloroform isoamyl alcohol (25:24:1 v/v, Sigma).The organic extraction was repeated, and the supernatant was added to an equal volume of 4M ammonium acetate. Total genomic DNA was precipitated by the addition of 2 volumes of isopropanol followed by centrifugation for 10 min at room temperature. The pellet was washed with 70% ethanol, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C and further diluted in TE buffer prior to use in PCR. DNA concentration were estimated directly from ethidium bromide florescence in

agarose gel (0.8 % in 1X TAE buffer) against standard quantities of 1 Kb λ bacteriophage DNA, by using a gel documentation system and associated software.

3.1.4.2 16S rRNA gene amplification

Fragents of the 16S rRNA gene were amplified by the method of Wilmotte *et al.,* 1993; Nelissen *et al.,*1994. The amplifications were performed with DNA Thermal cycler (Verti® – 96 well Thermal cycler, Applied Biosystems, USA). The PCR conditions for the arbitrary primer were as specified by Nubel *et al.,* (1997). The primers were synthesized by XDT Technologies (Germany). The PCR cycle for primer was: Initial denaturation at 94°C for 6 minutes, 35 cycles of cyclic denaturation at 94°C for 1 minute, cyclic annealing at 56°C for 1 minute, cyclic extension at 72°C for 1 minutes and finally the final extension at 72°C for 7 minutes. After the reaction was completed, 10 μL of amplified DNA was separated on 1.2% low melting agarose (Siga, USA), stained with ethidium bromide and recorded by Gel Doc System.

3.1.4.3 Sequencing and phylogenetic analysis of 16S rRNA gene

The result of 16S rRNA gene amplified product in Fig. 1 shows the product obtained after running PCR in suitable condition with appropriate primer, were subjected to detection for its presence on agarose gel electrophoresis. The amplified product was further send for sequencing at Chromous Biotech, Bangalore. BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) was carried out against GenBank databasebefore submission. The 16S rDNA sequences reported in this study was multiple-alignedusing CLUSTAL W, Version 1.7 (Thompson *et al.,* 1994) with a selection of cyanobacterial reference sequences obtained from GenBank (NCBI). Phylogenetic tree was constructed using the Neighbour-joining (NJ) algorithm (Saitou and Nei, 1987) of MEGA 5.05; Tamura et al. (2011), with multiple substitutions corrected and positions with gaps excluded.

3.2 Impact of fly ash on growth and nitrogen fixation by selected blue green algal isolates.

Electrostatic precipitator (ESP) coal fly ash (FA) was collected from Sturdy Industries Ltd. village Saidpura, Derabassi (Punjab). The main source of fly ash for Sturdy Industries Ltd. is Guru Gobind Singh Super Thermal Power Plant, Ropar, Punjab and was directly analyzed for its physiochemical properties after air-drying. Fly ash in dried form at different concentration 0, 5, 10 and 20% (w/v) was added to BG 11 growth medium and distilled water (DW) (w/v) to examine its impact on growth and nitrogen fixation by heterocystous filamentous blue green algal strains. Different blue green algal isolates was grown in BG 11 (-N) media and distilled water (DW) amended with different concentrations of Fly ash (FA) and growth performance of individual Isolate has been evaluated. BG11-N media was prepared as per composition given by Stainer et al., 1971. Fly ash was weighed according to different concentrations i.e. 0%, 5%, 10%, 20% and added to 250 mL flasks containing 100 mL of BG11 (-N) media and distilled water (DW). Flasks were inoculated by equal amount (4 mL) of exponentially growing cultures of seven different blue green algal Isolates along with four ARM cultures were maintained at 22-24°C for 30 days to grow. Observations for different parameters i.e., Biomass estimation, and heavy metal uptake by algae were recorded after 30 days. Biomass estimation was done by filtering the algal mass grown in fly ash by filtration assembly with whatman filter paper no 1. Dried biomass along with Fly ash was stored in packets and labeled accordingly. All dried biomass samples were analyzed for the presence of heavy metal content in them. For metal analysis 1 g of each sample was wet digested in HNO_3:$HClO_4$ (3:1, v/v) mixture at 80°C. The digested samples were dissolved in 50 mL HNO_3:$HClO_4$ (3:1, v/v) mixture and filtered with what man filter paper no 1. The filtrate for each sample was collected and analyzed for metal content of different trace metals. (Cr, Cu,

Pb, Zn) (Rai *et al*., 2000). The heavy metal content was estimated by Atomic Absorption Spectrophotometer. Fly ash which was not inoculated by any algal isolate was taken as blank.

3.3 Fly ash based blue green algal inoculant formulation and their evaluation on growth and yield of rice.

3.3.1 Physical and mineralogical characterization of coal fly ash, montmorillonite, charcoal, and soil

3.3.1.1 Collection and processing of different carrier materials

Electrostatic precipitator (ESP) coal fly ash (FA) was collected from Sturdy Industries Ltd. village Saidpura, Derabassi (Punjab). Wood charcoal and montmorillonite was purchased from local market located in Patiala, Punjab as per required quantity. Garden soil was collected from experimental plot Science & Technology Entrepreneur's Park (STEP), Thapar University, Patiala. Soil, charcoal and montmorillonite were crushed into fine powder and air dried for their physiochemical properties.

3.3.1.2 Microstructural Characterisation

3.3.1.2.1 X- ray diffraction: Samples were characterized using X- ray diffraction to identify the formed phases. The powdered sample were pressed into the aluminium holder and subjected to Cu-K radiataion between 10-80ºC. X-ray powder diffractions study was performed at room temperature using PANalytical X'Pert PRO system with Ni-filter. During experiment the step size was 0.013°/min and X-ray diffractogram of different materials were obtained and identified. The elemental compositions of the present studied samples were determined by using High Score Plus software associated with PANalytical X'Pert PRO system. This software uses the area under the curve of different peak of different phases to calculate the quantitative percentage of different phases. Total element concentration of Cr,

Pb, Mn, Zn, Cu, Ni, Co, Mo, Cd, Se, As, Fe and S in coal fly ash was analysed with strong nitric acid digestion method followed by analysis with Atomic absorption spectrophotometer (GBC 932AA) (Page *et al*., 1982).

3.3.1.2.2 Differential Thermal analysis DTA /TGA: was performed in nitrogen atmosphere using Perkin Elmer (Model: Diamond Pyris TG/DTA analyzer) to check thermal stability and any phase transition. The Differential Thermal analysis/ Thermal gravimetric measurements of the samples were performed using Al_2O_3 powder as reference material in nitrogen atmosphere at 10 °C/min heating rate from 50 °C to 900 °C. The temperature and weight loss detection limit of the instrument are ± 1ºC and 0.001mg respectively.

3.3.1.2.3 Fourier transform infrared spectra (FTIR): FTIR spectra were obtained at room temperature by using Perkin Elmer model RZX spectrometer in the region 400-2000 cm^{-1}. The spectrum of each sample was normalized to the spectrum of blank KBr.

***3.3.1.2.4 Scanning electron micrograph (SEM)*:** The microstructural study was carried out by a scanning electron microscope (JSM-6510LV, JEOL). For microstructural study, all the powdered samples of fly ash, charcoal, montmorillonite and soil were sputtered before the measurement to study the morphology and particle size respectively.

***3.3.1.2.5 Surface area*:** "Fineness" is quantified by the specific surface of a material. The specific surface is defined as the "number of units of surface area "contained in a "unit weight" of a material. BET (Brunauer -Emmett-Teller) method was used to measure the specific surface area of the samples using area-meter Π by nitrogen gas absorption in a flow rate 4.5 L/h.

3.3.1.2.6 Coarse Grain Accumulation*: Coarse grain accumulation* of coal fly ash, charcoal, montmorillonite and soil was done by dry sieve analysis followed by hydrometer. Each sample was carefully sieved with round test sieves with a hole diameter of 4mm, 2mm, 1mm, 0.500mm,0.300 mm, 0.150 mm, 0.075 mm, 0.025 mm and 0.01 mm, in accordance with ISO 1953 (Sugita and Marumo, 2001).

3.3.2 Process development for use of fly ash as a carrier for BGA biofertilizers

3.3.2.1 Mass cultivation of blue green algae

3.3.2.1.1 Small Scale Production (lab scale)

100 mL of BG-11 medium in 2L flasks was inoculated with different cultures and incubated at 28±2˚C under discontinuous illumination at 16:8 hours light-dark cycle at 2500-3000 lux light intensity. After observing adequate growth, the flasks were transferred to the glasshouse (Figure 3.3).

3.3.2.1.2 Pilot scale culture production in algal ponds

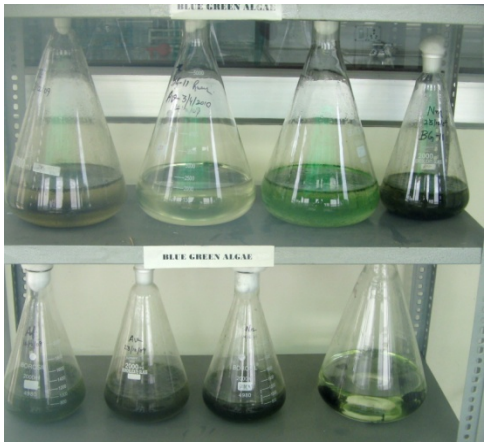
For the large scale production of algal biomass, algal cultures grown in small scale were transferred from flasks and tubs to the algal ponds for mass production (Figure 3.4).

1. The production is carried out in algal ponds in poly house 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.
2. Each algal pond was filled with tap water upto a height of 18 – 20 cm and dipotassium hydrogen phosphate (K_2HPO_4) and magnesium sulphate hepta hydrate ($MgSO_4.7H_2O$) was added (10 g/10 L of water).
3. Starter culture, a mixture of *Tolypothrix tenius* (ARM444), *Nostoc muscorum* (ARM442), *Anabaena variabilis* (ARM 441), *Aulosira fertilissima* (ARM444) was inoculated in each multiplication unit.

4. Malathion (5-10 mL per pond) or Carbofuran (3% granules, 20 g per pond), was added to prevent insect breeding.

3.3.2.2 Harvesting of algal inoculants after their 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. Thick mat of blue green algae was collected, dried and then was mixed with the sterile fly ash and packed in the packets. Quality check was done of the manufactured algal biofertilizer packets by serial dilution and plating technique. The resultant material was checked for the number of propagules (cfu/g) of the material. The result showed that there were 10^3 – 10^4 cfu/g of the carrier material (Figure 3.4)



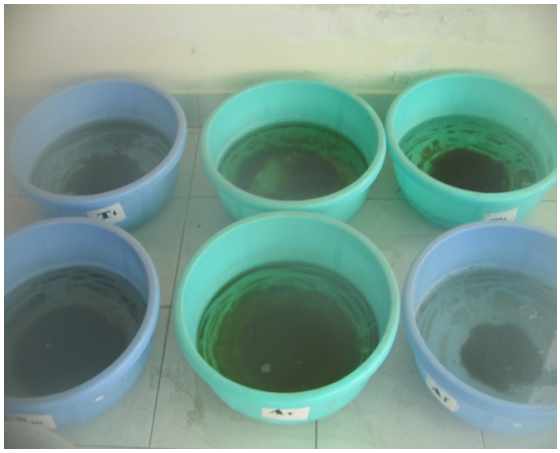
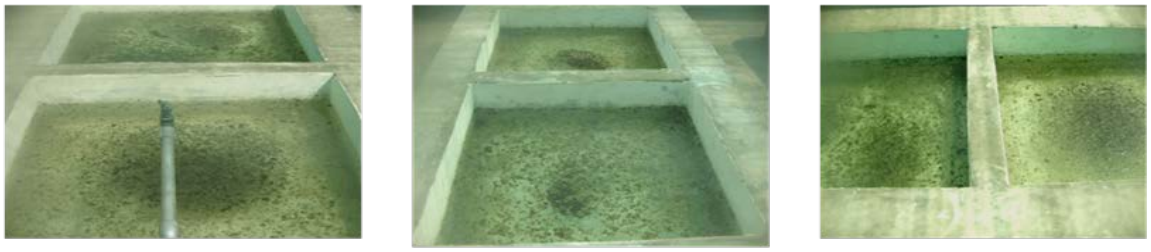
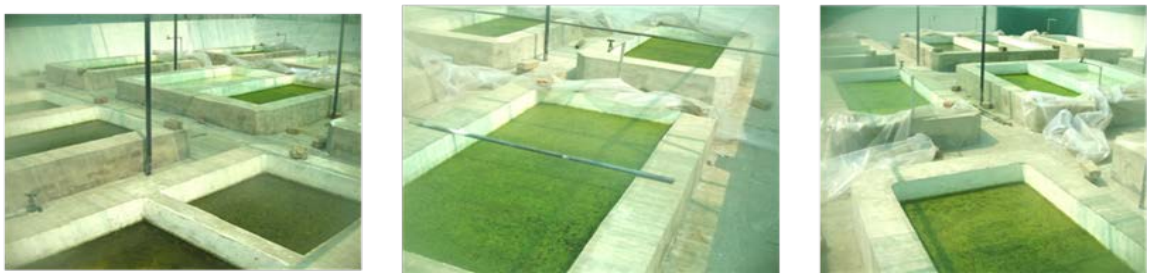


Figure 3.3 Small scale production of BGA inoculants



Inoculation done in the algal ponds



⇩

⇩

Algal slurry was mixed with the carrier material (fly ash)

⇩

Fly ash based blue green algal biofertilizers (500g per packet)

Figure 3.4: Large scale production of BGA inoculants, harvesting of algal biomass and manufacturing fly ash based BGA biofertilizers

3.3.3 Pot experiment and field trial

3.3.3.1Pot experiment

A pot culture experiment was designed to evaluate the effect of consortium of selected blue green algal Isolates and consortium of ARM cultures on the yield of rice (variety Pusa 1401) with different combination of carrier materials as given below under glasshouse conditions, using unsterile soil (12 kg soil per pot) taken from experimental garden STEP, Thapar University. Rice seedlings 3-weeks old were transplanted into each pot (7 seedlings per pot) with equal spacing and irrigation was given according to routine agronomic practices. Different carrier based consortium of seven blue green algal isolates and four ARM cultures were prepared and applied at the rate 5 µg (dry weight) per pot. Physico chemical properties i.e pH, organic carbon, total nitrogen and available phosphorus were recorded before and after the inoculation of the blue green algal consortium at the time of harvest (90 days after transplanting). Grain yield (in g per pot) of the rice were recorded at the time of harvest.

Treatment details of different carrier material are as follows:

T1 Soil 100%

T2 Montmorillonite 100%

T3 Fly ash + soil (50:50)

T4 Fly ash + Montmorillonite (50:50)

T5 Fly ash + Montmorillonite (10:50)

T6 Fly ash 100%

Control soil without the inoculation of blue green algal consortium was also studied.

3.3.3.2 Field trial sites and Random Block designs

A three year field trial was conducted during kharif season (June-October) at different villages of Patiala, Punjab for different rice cultivars viz: PUSA 1121, PR 118, PAU 201 at one hectare of agricultural land. Field trials and pot experiments will be carried out to see the effect of carrier based blue green algal biofertilizers and urea on the growth and yield of paddy crop over control in. Soil samples will be taken at regular intervals to study the cyanobacterial population and for physicochemical characterization. Details of field and nursery trial treatment are given below:

Experimental design	Randomized Block design
Treatment groups	4
Replications	3

Treatment details:

I. Control (without BGA / Urea)

II. Urea @ 30 kg per hectare

III. Urea (20 kg) + BGA(500 g)

IV. BGA @ 500 g per hectare

Soil sampling

For field trials soil samples were collected from 20 different agricultural fields of Patiala and Nabha (villages highlighted in green blocks in the map given in Figure 3.1). Soil from depth of 30 inches from five different sites on the same field was collected before transplantation and after application of algal biofertilizers. Soil collection from 25 different agricultural lands was done at different intervals of time during the cropping period. Soil samples collected from different agricultural fields were air dried in the shade and sieved through 2mm sieve

for physical analysis and through 0.2mm sieve for chemical analysis. pH, electrical conductivity, organic carbon content, was estimated using standard protocols

3.3.4 Physiochemical analysis

3.3.4.1 Chemical Analysis

pH

pH was determined as per the method given by Jackson (1967) in a soil-water/fly ash water suspension of 1:2 ratio. Ten g of soil/fly ash was placed in a 100 mL beaker and 20mL 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.

Electrical conductivity

Electrical conductivity was measured in $\mu S\ cm^{-1}$ as per the method given by Jackson (1967). Ten g of soil /fly ash 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 (Cyberscan con 510) was calibrated with 0.01 M potassium chloride (1413 $\mu S\ cm^{-1}$).

Organic carbon

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

Reagents

1. 1 N $K_2Cr_2O_7$: 49.04 g of potassium dichromate per litre of solution.
2. 0.5 N ferrous ammonium sulphate: 198 g salt per litre of solution.

3. Diphenylamine indicator: 0.5 g of diphenylamine in a mixture of 20 mL water and 100 mL concentrated sulphuric acid.
4. Concentrated sulphuric acid.
5. Orthophosphoric acid (85%)
6. Sodium fluoride (NaF).

Procedure

1. 1 g of soil / fly ash 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. 20 mL of H_2SO_4 was added 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 g 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.

Calculation

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

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.

Available phosphorus

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

Reagents

1. 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 1M or 1N NaOH.
2. Reagent A: 12.0 g of ammonium molybdate in 250 mL distilled water and 0.2908g of 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 2 L with distilled water.
3. Reagent B (freshly prepared): 1.058g of ascorbic acid in 200 mL of reagent A and mixed.
4. Sulphuric acid (2.5 M): 140 mL of concentrated H_2SO_4 diluted to 1 L.
5. Stock standard P solution (50 ppm P): 0.2917 g KH_2PO_4 dissolved in water to a final volume of 1 L.
6. Working standard P solution (1 ppm): 20 mL of (50 ppm P) solution diluted to 1 L.

Procedure

1. 2.5 g soil/fly ash 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. Ten 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.

Calculation

P in sample (mg / kg) = P in extract (mg / L) x 20 (the standard sample to solution ratio)

Available phosphorus in acidic soil/fly ash was estimated as per the method given by Bray and Kurtz (1945).

Reagents

1. Ammonium fluoride (extracting solution): 22.2 g of NH_4F dissolved in 41.6 mL HCl and volume made upto 2 L.
2. Reagent A: 12.0g of ammonium molybdate in 250 mL distilled water and 0.2908 g antimony potassium tartarate in 100 mL distilled water. These two solutions were mixed, 1000 mL of 2.5 M H_2SO_4 was added and volume made upto 2 L with distilled water.
3. Reagent B (freshly prepared): 1.058g of ascorbic acid dissolved in 200 mL of reagent A and mixed.
4. Sulphuric acid (2.5 M): 140 mL of concentrated H_2SO_4 diluted to 1 L.
5. Stock Standard P solution (50 ppm P): Dissolved 0.2917g of KH_2PO_4 in water to a final volume of 1 L.
6. Working Standard P solution (1 ppm): Diluted 20 mL of (50 ppm P) solution to 1 L.
7. 40% NaOH.

Procedure

1. 2.5 g soil/fly ash was taken in a 100 mL flask and 25 mL extracting solution was added.
2. The solution was kept on a shaker for 30 minutes and filtered through Whatman filter paper No. 42.
3. The 2 mL aliquot of the filtrate was transferred to a 100 mL beaker followed by addition of 20 mL of distilled water, 8 mL of reagent B and 20 mL of distilled water.
4. Blank prepared as above. For the standard curve: 0, 2, 5, 10, 15 and 20 mL of standard solution were measured in 50 mL volumetric flasks. Two mL of extracting solution and 8 mL Reagent B was added and the final volume was made upto 50 mL. The P concentrations of these solutions were 0.0, 0.04, 0.1, 0.2, 0.3 and 0.4 ppm respectively. After 10 minutes, the P concentration was read at 882 nm.
5. For total phosphorus 1 g soil was digested with HNO_3 and $HClO_4$ in the ratio 3:1 on hot pLate at 100°C untiL a whitish brown mass was obtained.
6. The sample was treated with a HCl and water mixture and filtered through Whatman No. 42 filter paper and the filtrate was stored in bottles.
7. 10 mL of filtrate was taken and pH adjusted to 5.0 using 40% NaOH and volume was made up to 50 mL. 2 mL aliquot was taken and analysis carried out as in step 3 and 4.

Calculation

P in sample (mg/ kg) = P in extract (mg / L) x 10 (the standard sample to solution ratio)

Available Nitrogen

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

Reagents

1. 0.32 % Potassium per manganate: 3.2 g of $KMnO_4$ dissolved in water and final volume made up to 1 L.
2. 2.5 % NaOH: 5 g of sodium hydroxide pellets dissolved in water and volume made up to 1 L.
3. 2% boric acid: 20g of boric acid powder dissolved in warm water by stirring and diluted to 1 L.
4. Mixed indicator: 0.066 g of methyl red and 0.099 g 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 dilutes NaOH.
5. 0.1N potassium hydrogen pthalate: Dissolve 20.422 g of the salt in water and dilute to 1 L.
6. 0.1N NaOH: 4g of NaOH dissolved in water and diluted to 1L, standardized against 0.1 N potassium hydrogen pthalate solution.
7. 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. 5 g of soil/fly ash was weighed 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 burrette until a pink colour started appearing. A blank was run without soil.

Calculation

Available N (ppm) = (X) x 0.00028 x 100/5

where X stands for the titre value of 0.02 N H_2SO_4 consumed

Total Nitrogen

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

Reagents

1. Concentrated H_2SO_4.
2. 0.02 N H_2SO_4.
3. Sulphuric-Salicylic acid: 1 g salicylic acid mixed with 30 mL sulphuric acid.
4. Sodium thiosulphate.

5. 4% boric acid.

6. Mixed indicator. 0.066 g of methyl red and 0.099 g of bromocresol green dissolved in 100 mL of ethyl alcohol.

7. 50% NaOH.

8. Digestion mixture: 10 g HgO, 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20).

Procedure

1. 5 g soil/fly ash was mixed thoroughly with sulphuric-salicylic acid followed by 5g of sodium thiosulphate. Heating was carried out for 5 minutes followed by cooling and addition of 10g 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 color 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 condensor 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 color changes from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 until the endpoint colour changed from green to pink.

Calculation

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

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

Heavy Metal analysis **(Total Fe, Mn, Ni, Cr, Pb, Zn, Ca, Mg, Na)**

Estimation of total metals was done as per the method given by Page *et al*. (1982).

Reagents

1. Concentrated perchloric acid ($HClO_4$) and nitric acid (HNO_3).
2. Acid water solution containing HCl and water in a 1:1 ratio.

Procedure

1. 1g of soil/fly ash/plant sample was placed in a 150 mL beaker.
2. HNO_3 and $HClO_4$ in a 3:1 ratio were added to the sample.
3. The sample was digested on a hot plate at 100°C for 3-4 hours until a whitish brown dry mass was obtained.
4. The samples after digestion were treated with acid water mixture and filtered through Whatman No.42 filter paper.
5. The filtrate was analyzed for total Cr, Pb and Cd in both soil samples using an atomic absorption spectrophotometer (GBC 932AA). Metals (Pb, Cr and Cd) along with their sensitivity Limits are as follows.

Element Sensitivity (µg/ mL)

Element	Sensitivity (mg/mL)
Cr	0.00005
Fe	0.00005
Ni	0.00004
Pb	0.00006
Zn	0.000008
Mn	0.00002
Ca	0.00002

Mg	0.000003
Na	0.000004

Calcultion:

Heavy metals in sample (mg / kg) = heavy metal in extract (mg / L) x dilution factor

Wt. of sample taken

3.3.4.2 Physical analysis

Water holding capacity: Black *et al.* (1965).

Apparatus

Circular brass boxes (keen boxes) of 5.6 cm internal diameter and 1.6 cm depth were taken which had 0.75 mm holes spaced 4 mm apart at the bottom. Each box is fitted with a brass Lid.

Procedure

1. A filter paper strip of the size of the base of the keen box was cut.
2. The filter disc was weighed and placed in a petridish containing water for measuring the moisture absorbed by the filter paper.
3. The disc was placed at the bottom of the keen box and weighed followed by filling of the box with soil/fly ash. Each time the box was tapped to make a uniform soil/fly ash column.
4. The box containing soil/fly ash was weighed and kept in a petridish containing water for overnight saturation.
5. The box was removed the next day, wiped and weighed followed by overnight drying at 80^{o} C in the oven in order to obtain constant weight.
6. The box containing oven-dry soil/fly ash was weighed finally at room temperature.

Calculation

Weight of box+ filter paper = W1

Weight of the box +oven dry soil = W2

Weight of the box+ soil after moistening = W3

Weight of dry soil = W2-W1

Weight of moisture absorbed = W3-W2

Moisture absorbed by filter paper = W4

Moisture held by soil alone = W3-W2-W4

Water holding capacity of the soil = W3-W2-W4/W2-W1x100

Bulk density

Bulk density was measured as per method given by Black *et al*. (1965).

Apparatus

Specific gravity bottle.

Procedure

1. The specific gravity bottle was weighed and the volume of water, which could fill it up to the brim, was measured.
2. The bottle was filled with soil/fly ash and weighed.

Calculation

Weight of empty bottle = W1

Weight of bottle and soil = W2

Weight of soil = W2-W1

Volume of the soil or volume of water needed to fill the bottle = V mL

Bulk density of the soil/fly ash = $\frac{W2- W1}{V}$ g / m^3

Statistical Analysis

The various statistical parameters were analysed as per the methods given by Rao, 1996 using Graph pad Prism Software 2.01 and Microsoft Excel.

Coefficient of dispersion or variation

To compare the variability of two series, which differ widely in their averages, a reLative measure of dispersion is used which is known as coefficient of variation or dispersion. The ratio of a measure of dispersion to an average will give the coefficient of dispersion.

Coefficient of dispersion is defined as:

i. $\frac{\text{Mean deviation}}{\text{Median}} \times 100$ ii. $\frac{\text{Mean deviation}}{\text{Mean}} \times 100$

iii. $\frac{\text{Quartile deviation}}{\text{Median}} \times 100$ iv. $\frac{\text{Standard deviation}}{\text{Mean}} \times 100$

where the fourth definition is the well-known coefficient of variation (CV).When the variability of two series is compared, the series having greater CV is said to have greater variation than the other and the series having lower cv is said to be more homogenous than the other.

The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $p<0.05$.

4.1 Isolation and characterization of efficient strains of blue green algae from paddy fields

Paddy field soil from seven different villages of Nabha and Patiala, Punjab and pond fly ash from National Thermal Power Station (NTPC), Rihandnagar, U.P was collected and through enrichment culture technique seven blue green algae were isolated. These isolates were identified morphologically using keys of Fritsch (1949); Komarak, 2006 and Hrouzek (2013) as *Calothrix* sp. (Isolate A), *Anabaena flos-aquae* (Isolate B), *Desmonostoc* sp. (Isolate C), *Nostoc commune* (Isolate D), *Nostoc* sp. PS1 (Isolate E), *Nostoc sp.* (Isolate F) and *Anabaena* sp. (Isolate G). These Isolates were screened for filamentous, heterocystous, nitrogen fixing blue green algae in order to isolate region specific strains. Comparative study of growth, nitrogen fixation and biochemical attributes in terms of dry biomass (Richmond and GobbeLaar, 1986), Chlorophyll estimation (Mckiney, 1941), total nitrogen content by Kjeldahl method (Piper, 1960), nitrogenase activity (Hardy *et al*., 1973), nitrate reductase activity (Lowe and Evans, 1964), indole acetic acid production of seven blue green algal Isolates with ARM cultures viz., *Anabaena variabilis* (ARM 441); *Nostoc muscorum* (ARM 442); *Tolypothrix tenius* (ARM 443) and *Aulosira fertilissima* (ARM 444) procured from Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), Indian Agricultural Research Institute (IARI), New Delhi. Algal isolates screened for highest nitrogen fixation, total nitrogen content and heterocyst frequency, were further characterised using molecular identification techniques such as 16S rRNA molecular identification techniques which involved DNA isolation, PCR amplification, DNA sequencing and sequence submission in NCBI database

4.1.1 Physico-chemical characterization of paddy field soil

Physico chemical analysis of paddy field soil collected from nearby villages of Patiala (30.79° N, 76.78° E) reveals that soil was alkaline in nature with pH ranging from 7.81 -8.54 and EC (µS/cm) ranged from 113-222.5 (Table 4.1.1). Organic carbon content (%) ranged from 0.22 -0.35, available phosphorus (mg/kg) ranged from 9-19.03 and total nitrogen (%) ranged from 0.019 -0.027 respectively. Water holding capacity of Patiala soils varied from 25-38 % and bulk density (g/m^3) from 0.32-0.58. Punjab soils are alkaline in nature with low to moderate carbon content and nitrogen content (Benbi, 2009 and Saxena *et al*., 1999). Pond fly ash collected from National Thermal Power Cooperation (NTPC), Rihandnagar, Uttar Pradesh (25.15° N, 82.60° E) was acidic in nature with pH 3.5 and EC (µS/cm) 460. Organic carbon and nitrogen content (%) was 0.43 and 0.011 while phosphorus content observed was 14 mg/kg. Water holding capacity and bulk density of pond fly ash was noted to be 44% and 0.98 g /m^3 respectively.

4.1.2 Isolation of efficient strains of blue green algae

Seven filamentous, heterocystous blue green algae were Isolated from paddy field soils of Nabha and Patiala, Punjab (Isolate A, Isolate C, Isolate D, Isolate E, Isolate F, Isolate G) and one (Isolate B) from pond fly ash from National Thermal Power Station (NTPC), Rihandnagar, U.P (Table 4.1.2). Blue green algal isolates were identified morphologically through microscope and to screen the most efficient strain among the seven algal Isolates their growth and biochemical studies in terms of biomass, chlorophyll content, nitrate reductase activity (NR), indole acid production (IAA), heterocyst frequency, total nitrogen and nitrogenase activity (ARA) were studied and compared with the studies of four efficient heterocyst nitrogen fixing blue green algae, *Anabaena variabilis* (ARM 441); *Nostoc muscorum* (ARM 442); *Tolypothrix tenius* (ARM 443) and *Aulosira fertilissima* (ARM 444) were procured from Centre for Conservation and Utilisation of Blue Green Algae

(CCUBGA), Indian Agricultural Research Institute (IARI), New Delhi which were predominantly used as a multani mitti based biofertilizers for N increment in rice cultivation (Mishra and Pabbi, 2004).

Table 4.1.1 Physicochemical characteristics of soil collected from nearby villages of Nabha and Patiala

Parameters	Villages of Nabha and Patiala						Pond fly ash
	Shauli	Palia Khurd	Dittupur	Gunike	Rajgarh	Sibro	NTPC, U.P
pH	8.54±0.05	7.81 ± 0.65	8.53 ± 0.59	8.09±0.31	8.02 ± 0.20	8.15 ± 0.27	3.5 ± 0.01
EC (µS/cm)	113±0.09	171.65±0.11	182 ±0.96	168.90±0.01	135.3± 0.25	222.5 ±1.2	460 ± 1.51
Organic Carbon (%)	0.28±0.07	0.35±0.14	0.35±0.05	0.30±0.01	0.34±0.01	0.22±0.05	0.43±0.05
Available Phosphorus (mg /kg)	9.00±0.32	24.00±0.69	11.47±0.02	19.03±0.53	15.06±0.29	10.11±0.64	14±0.01
Total Nitrogen (%)	0.026±0.01	0.021±0.01	0.027±0.008	0.019±0.001	0.024±0.001	0.013±0.002	0.011±0.002
Bulk density (g /m^3)	0.58±0.06	0.47±0.01	0.44±0.02	0.32±0.01	0.51±0.09	0.54±0.23	0.98±0.16
Water holding capacity (%)	31.00±0.06	29.00±0.1	33.00±0.05	25.00±1.2	36.00±0.7	38.00±0.55	44.00±0.95

Values are average of Mean±SE (n=3)

4.1.2 Microscopic studies

Region specific blue green algal varied in their morphological characters and were identified microscopically based upon the keys given by Fritsch (1949); Komarek, (2006); Ramirez, (2011); Uher, (2007) and Hrouzek et al. (2013). Pure culture was observed under the microscope. The cell shape and size (length and width of the vegetative cellss, heterocysts and akinetes) of different filaments of each strain from late exponential growing fresh cultures were observed, measured by micrometry and documented as microphotograph (Plate 1). The most predominant genus in the study was *Nostoc* (3 strains) followed by *Anabaena* (2 strains) and one species each of *Desmonostoc* and *Calothrix* respectively. Morphological characteristic of the Isolates are given in the Table 4.1.3. The common features based on which the Isolates were identified using different keys are described below:

Isolate A was identified as *Calothrix* sp. since heterocysts were observed at one end of the trichome and vegetative cellss were cylindrical in shape (Anagnostids and Komarek, 1985; Uher, 2007). Isolate B and Isolate G showed resemblance to *Anabaena* sp. showing spirally arranged spherical to cylindrical cells in multiple chains and akinetes were observed cylindrical in shape present at a distance from heterocysts (Fritsch 1949; Komarek and Zapomelova, 2007). Morphological characterisation done by Komarek and Komarkova in 2006 on various strains of *Anabaena* species indicates that *Anabaena flos-aquae* showed spherical vegetative cells of size 4.3-11.4×4.3-5.7µm, heterocysts of size 5-6.4 ×10-17.8µm and akinetes of size 30-54.3× 5.4-9.3 µm and our strain (Isolate B) showed cellular size of similar range, vegetative cells of size 5.5×4.7 µm, heterocysts of 6.6×4.1µm and akinetes of 23×10.2µm. Isolate C showed mixed resemblance to *Desmonostoc* sp. and *Nostoc* sp. showing long vegetative filaments with mucilaginous sheath. *Desmonostoc* is the newLy reported genus which is a mixture of *Nostoc muscorum* and several other strains of *Nostoc* showing similar morphology and phylogenetic resemblance (Hrouzek *et al*., 2013). Isolate D

was identified as *Nostoc commune* using the keys of Fritsch 1943, Ramirez *et al.*, 2011 showing uncoiled filaments with granulated akinetes and terminal heterocysts. Also, Novis and Smissen in 2006 described that *Nostoc commune* have spherical to ellipsoidal vegetative cells of size 6.4 ×3.6 µm and our strain showed similar range of size in vegetative cells (6.17 µm Long and 3.18 µm wide) respectively. Isolate E and F showed close resemblance to *Nostoc* sp. having thick mucilaginous sheath of barrel or spherical to shaped cells and akinetes present near or far away from heterocysts. Motility is restricted in *Nostoc* but are observed in *Anabaena* (Rippka *et al*., 1979), that is considered as unreliable characteristics for identification of strains from culture collection (De Phillips *et al*., 2000; Orietta, 2008).

Table 4.1.2 Classification of isolated filamentous blue green algae from selected location.

S.No	Location	Isolate Designation	Class	Order	FamiLy	Genus	Species	Reference
				Paddy field soil				
1.	Shauli	A	Cyanophyceae	Nostocales	Rivularia	*Calothrix*	---	Uher, 2007
2.	Dittupur	C	Cyanophyceae	Nostocales	Nostocaceae	*Desmonostoc*	---	Hrouzek *et al*., 2013
3.	Gunike	D	Cyanophyceae	Nostocales	Nostocaceae	*Nostoc*	*commune*	Ramirez, 2011
4.	Rajgarh	E	Cyanophyceae	Nostocales	Nostocaceae	*Nostoc*	---	Ramirez, 2011
5.	Sibro	F	Cyanophyceae	Nostocales	Nostocaceae	*Nostoc*	---	Ramirez, 2011
6.	Palia Khurd	G	Cyanophyceae	Nostocales	Nostocaceae	*Anabaena*	---	Rajaniemi et al., 2005 Komarek and Zapomelova, (2007)
				Pond fly ash				
7.	NTPC Rihandnagar, U.P	B	Cyanophyceae	Nostocales	Nostocaceae	*Anabaena*	*flos-aquae*	Rajaniemi et al., 2005 Komarek and Zapomelova, (2007)

Table 4.1.3 Morphological characters of the BGA genera isolated using keys of Fritsch (1949); Komark, (2008); Ramirez, (2011); Uher, (2007) and Hrouzek et al. (2013).

Isolate No.	Genera	Morphological characters
		Heterocystous forms
A	***Calothrix* sp.**	Filaments were straight and gelatinous with yellowish brown sheath, single spherical heterocysts smaller in size having width 6.87µm than vegetative cells, tapered trichomes, hormogonia were identified. Cells size observed was 3.1 µm in length and 4.98 µm in width.
B	***Anabaena flos-aquae***	Filamentous with thallus, trichomes were untapered, Hormogonia were absent, vegetative cells were ellipsoidal to cylindrical in shape of 5.5 µm in length and 4.7 µm in width. Heterocysts were spherical to cylindrical eLongated of 6.6 µm long and 4.1 µm braod. Akinetes were colourless and cylindrical in shape of 23 µm long and 10.2 µm, present at a distant from heterocysts and trichome width was 3.8 - 6.59 µm.
C	***Desmonostoc* sp.**	Filaments were long chained with thick mucilaginous sheath. Heterocyst was terminal and intercalary of 5.2 µm in length and 3.8 µm in width, akinetes were elongated or oval shaped of 8.1µm Long and 5.3 µm broad, present at the end of heterocysts. Hormogonia were present.
D	***Nostoc commune***	Filaments were unbranched with straight trichomes, vegetative cells were elliptical to spherical of 6.17 µm long and 3.18 µm wide, apical cells were oval in shape, heterocysts are spherical and intercalary of 6.25 µm long and 4.3µm in width. Colourless akinetes with oval shaped, adjacent to heterocysts or to a distance of 2-4 cells having 21.6 µm length and 7.1µm width and trichome of size 5.01µm was observed.
E	***Nostoc* sp. *(PS1)***	Round or barrel shaped vegetative cells of size (2.5 µm ×3.7 µm) with mucilaginous sheath were observed. Heterocyst cells were spherical in shape of size 3.3 µm×4.5 µm) and hormogonia were

		observed. Akinetes were spherical in shape of 4.75 µm in length and 6.2 µm in width and trichomes were colourLess.
F	***Nostoc* sp.**	Filamentous with thick mucilaginous sheath, trichomes were straight, vegetative cells were short and cylindrical of 5.92 µm in length and 3.6 µm in width, heterocysts were single and intercalary of 6.4 µm in length and 4.1µm width, akinetes were colourless and spherical having 22.6 µm length and 5.7 µm width, present at the end of heterocysts.
G	***Anabaena isolate***	Coiled filaments without mucilaginous sheath having spherical to cylindrical vegetative cells (5.1 µm ×4.5 µm) with trichomes of 5.63 µm width and oval shaped akinetes present at a distant from heterocyst cells of size 7.56 µm ×18.7 µm. Heterocyst were cylindrical in shape of 5.1 µm in length and 7.6 µm in width.

4.1.1.1 Filamentous, heterocystous cyanobacterial strain

Filamentous, heterocystous nitrogen fixing blue green algal isolates from paddy field soil and pond fly ash procured were examined for heterocyst frequency, total nitrogen and nitrogenase activity and compared with four filamentous heterocystous ARM cyanobacteria procured from Indian Agricultural Research Institute (IARI), New Delhi (Table 4.1.4). Nitrogen fixing potential of blue green algae is important to determine as it provides an estimate of nitrogen increment in soils of rice cuLtivated fields (Roger, 1996; Prasanna *et al*., 2003). Heterocyst frequency (%) was determined as number of heterocysts present in the Log phase culture (15 days old) when grown in nitrogen free medium (BG11 –N). Heterocysts are morphologically distinct from vegetative cells being enlarged in size than vegetative cells and are specialised for in nitrogen free medium. The present study reveals that among the seven isolates (Isolate A, B, C, D E, F, G), three algal isolate (Isolate C, B and F) had good heterocyst frequency

(%), total nitrogen content (%) and nitrogenase activity (mole C_2H_4/mg dry wt/hr). Isolate C (*Desmonostoc* sp.) showed the maximum heterocyst frequency of 18.2%, total nitrogen content of 0.127% and nitrogenase activity (ARA) of 32.2 mole C_2H_4/mg dry wt/hr, followed by Isolate B (*Anabaena flos-aquae*) showing 13% heterocyst frequency, total nitrogen content of 0.069% and nitrogenase activity of 20.31 mole C_2H_4/mg dry wt/hr, followed by Isolate F (*Nostoc* sp.) showing 12% heterocyst frequency, total nitrogen content of 0.051% and nitrogenase activity of 18.08 mole C_2H_4/mg dry wt/hr respectively. Among four ARM cultures procured from from Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), Indian Agricultural Research Institute (IARI), New Delhi, *Anabaena variabilis* (ARM 441) showed highest heterocyst frequency (15%) , total nitrogen content (0.087%) and nitrogenase activity (25 mole C_2H_4/mg dry wt/hr), followed by *Nostoc muscorum* (ARM 442) which showed heterocyst frequency by 12.24% and nitrogenase activity by 19 mole C_2H_4/mg dry wt/hr which were almost similar to that observed in Isolate B (*Anabaena flos-aquae*) and Isolate F (*Nostoc* sp.). Literature supports our findings for ARM cultures that log phase (12-15 days old) grown cultures of *Anabaena sp.* showed heterocyst frequency of 2.2-25% (Thiel, 1993; Prasanna *et al.*, 2006), total nitrogen content from 0.050-2.45% (Vyas and Kumar, 1994) and nitrogenase activity of 13-27 mole C_2H_4/mg dry wt/hr (Kangatharalin *et al*., 1992), whereas *Nostoc muscorum* showed heterocyst frequency of 7-15% total nitrogen of 0.036-0.061% (Sekina and Atef, 2010) and nitrogenase activity of 10-22.2 mole C_2H_4/mg dry wt/hr (Sholkamy *et al*., 2012). Isolate C (*Desmonostoc* sp.) showed maximum total nitrogen content among all the blue green algal strains examined.

Table 4.1.4 Nitrogen fixation by blue green algal strains (15 days old) grown in BG11 (-N) medium

Strain	Genera	Heterocyst frequency (%)	Total Nitrogen (%)	Nitrogenase activity (mole C_2H_4/mg dry wt/hr)
A.	***Calothrix sp***	6.1 ± 0.10^{h}	0.025 ± 0.001^{g}	5.61 ± 1.20^{h}
B.	***Anabaena flos-aquae***	13.0 ± 0.50^{bc}	0.069 ± 0.004^{c}	20.31 ± 0.64^{c}
C.	***Desmonostoc sp.***	**18.2 ± 0.62^{a}**	**0.127 ± 0.001^{a}**	**32.2 ± 0.97^{a}**
D.	***Nostoc commune***	9.6 ± 0.53^{fg}	0.038 ± 0.003^{f}	10.9 ± 0.62^{f}
E.	***Nostoc sp. PS1***	9.1 ± 0.10^{fg}	0.038 ± 0.017^{f}	8.58 ± 0.8^{g}
F.	***Nostoc sp.***	12 ± 0.80^{cd}	0.051 ± 0.002^{d}	18.08 ± 1.0^{d}
G.	***Anabaena sp***	10.72 ± 0.04^{de}	0.047 ± 0.014^{de}	17.13 ± 1.4^{d}
ARM 441	***Anabaena variabilis***	**15 ± 0.70^{b}**	**0.087 ± 0.001^{b}**	**25.02 ± 0.25^{b}**
ARM 442	***Nostoc muscorum***	12.24 ± 0.32^{c}	0.049 ± 0.012^{de}	19 ± 0.47^{cd}
ARM 443	***Tolypothrix tenuis***	8.74 ± 0.14^{g}	0.037 ± 0.007^{f}	10 ± 0.81^{f}
ARM 444	***Aulosira Fertilissima***	10.3 ± 0.09^{ef}	0.042 ± 0.001^{ef}	15 ± 0.24^{e}

(Mean±SE, n=3); Mean values with the same Letter were not significantly different, based on ANOVA followed by Tukey's test at P≤0.05

4.1.1.2 Growth and physiological studies

Overall, growth studies at time interval of seven days indicates that all the blue green algal isolates and ARM procured cultures showed progressive increase in biomass, chlorophyll content, NR activity and IAA production **(**Table 4.1.5) (Figure 4.1a and b).

Growth and physiological studies in terms of dry biomass, chlorophyll, nitrate reductase activity (NR) and phytohormone indole acetic acid (IAA) production were studied for 30 days at interval time of seven days. Cyanobacterial strain produces biologically active growth

promoting substance (Prasanna *et al*., 2013). Among seven Isolates (Isolate A, B, C, D, E, F and G), Isolate G (*Anabaena* sp.) showed maximum dry biomass of 3.45 mg/mL which was even more than ARM procured cultures as observed in *Tolypothrix tenius* (ARM 443) by 3.01 mg/mL and minimum was observed in Isolate D (*Nostoc commune*) by 1.70 mg/mL. Chlorophyll content (µg/mL) was observed maximum in Isolate B (*Anabaena flos-aquae*) by 3.79 and minimum was observed in Isolate C (*Desmonostoc* sp.) by1.58 and Isolate F (*Nostoc* sp.) by 1.56 respectively. Nitrate reductase activity and indole acetic acid production was observed maximum in Isolate C (*Desmonostoc* sp.) by 31.01 µ mole NO^-_2 and 0.29 µg/mL and minimum was observed in Isolate A (*Calothrix* sp.) by 7.25 µ mole NO^-_2 and 0.06 µg/ mL. ARM procured cultures showed maximum chlorophyll content in *Anabaena variabilis* (ARM 441) by 4.58 µg/mL followed by maximum nitrate activity (28.36 µ mole NO^-_2 and IAA production (0.27 µg/mL) whereas least biomass was observed *Aulosira fertilissima* (ARM 444) by 1.96 mg/mLand least chlorophyll content and IAA production was observed in *Tolypothrix tenius* (ARM 443) by 1.20 µg/mL, 0.06 µg/mL respectively. According to earlier reports various strains of *Anabaena* had chlorophyll content of 0.11-3.40 µg/mL, nitrate reductase activity of 2.83-31.83 µmole NO_2^- (Prasanna *et al*., 2006; Nayak *et al*., 2004; Pereira *et al*., 2009), indole acetic acid production from 0.001 - 2 µg/mL (Sergeeva *et al*, 2002; Prasanna *et al*., 2013) and nitrogenase activity by 0.5-7.05 n mole/mL/C_2H_2 /h (Nayak *et al*., 2004). Similarly *Nostoc muscorum* showed dry biomass from 0.05-3.0 mg/mL, indole acid production (IAA) from 0.005-0.43 µg/L (Sekina and Atef, 2010), chlorophyll content from 1.70-4.10 µg/mL (Oinam *et al*., 2010), nitrate reductase activity ranges from 3.6-29.26 µmole NO_2^- (Syiem, 2007) and nitrogen fixation rates as reported for *Nostoc commune*, *Nostoc ellipsosporum*, *Nostoc linckia* and *Nostoc* species was by 0.55-4.54 mole C_2H_2 h^{-1} (Pereira *et al*., 2009). Comparative growth and biochemical studies of seven blue

green algal isolates and four ARM cultures reveals maximum dry biomass by Isolate G, nitrate reductase activity and indole acetic acid production by Isolate B and C respectively.

Table 4.1.5 Growth and physiological studies of BGA strains grown in BG 11 (-N) medium for 30 days.

Strain	Isolated Strain	Dry biomass (mg/mL)	Chlorophyll (µg mL^{-1})	NR (µ mole NO^{-}_{2})	IAA (µg/mL)
A.	***Calothrix* sp.**	2.14 ± 0.5^{def}	1.8 ± 0.03^{h}	7.25 ± 0.06^{h}	$0.\ 06\pm0.003^{i}$
B.	***Anabaena flos-aquae***	3.37 ± 1.2^{b}	**3.79 ± 1.04^{b}**	**27.62 ± 0.01^{b}**	**$0.\ 21\pm0.08^{b}$**
C.	***Desmonostoc* sp.**	2.75 ± 0.7^{bc}	1.58 ± 0.36^{i}	**31.01 ± 1.9^{a}**	**$0.\ 29\pm0.11^{a}$**
D.	***Nostoc commune***	1.70 ± 0.4^{fg}	2.14 ± 0.5^{f}	16.52 ± 0.29^{f}	0.05 ± 0.02^{i}
E.	***Nostoc* sp.PS1**	2.01 ± 0.04^{defg}	2.95 ± 0.41^{e}	12.02 ± 1.5^{g}	0.10 ± 0.04^{c}
F.	***Nostoc* sp.**	1.64 ± 0.004^{g}	1.56 ± 0.09^{i}	20.95 ± 0.33^{d}	0.14 ± 0.001^{f}
G.	***Anabaena* sp.**	3.45 ± 0.21^{a}	2.01 ± 0.05^{g}	18.31 ± 0.74^{e}	0.19 ± 0.002^{e}
ARM 441	***Anabaena variabilis***	2.50 ± 0.7^{cd}	4.58 ± 1.25^{a}	28.36 ± 1.3^{b}	0.27 ± 0.001^{d}
ARM 442	***Nostoc muscorum***	2.35 ± 0.10^{cde}	3.60 ± 1.7^{c}	23.5 ± 0.37^{c}	0.10 ± 0.05^{h}
ARM 443	***Tolypothrix tenuis***	3.01 ± 1.1^{ab}	1.20 ± 0.65^{j}	14.65 ± 1.14^{fg}	0.07 ± 0.002^{i}
ARM 444	***Aulosira fertilissima***	1.96 ± 0.26^{efg}	3.08 ± 0.08^{d}	12.03 ± 0.38^{g}	0.12 ± 0.004^{j}

(Mean±SE, n=3); Mean values with the same letter were not significantly different, based on ANOVA followed by Tukey's test at P≤0.05

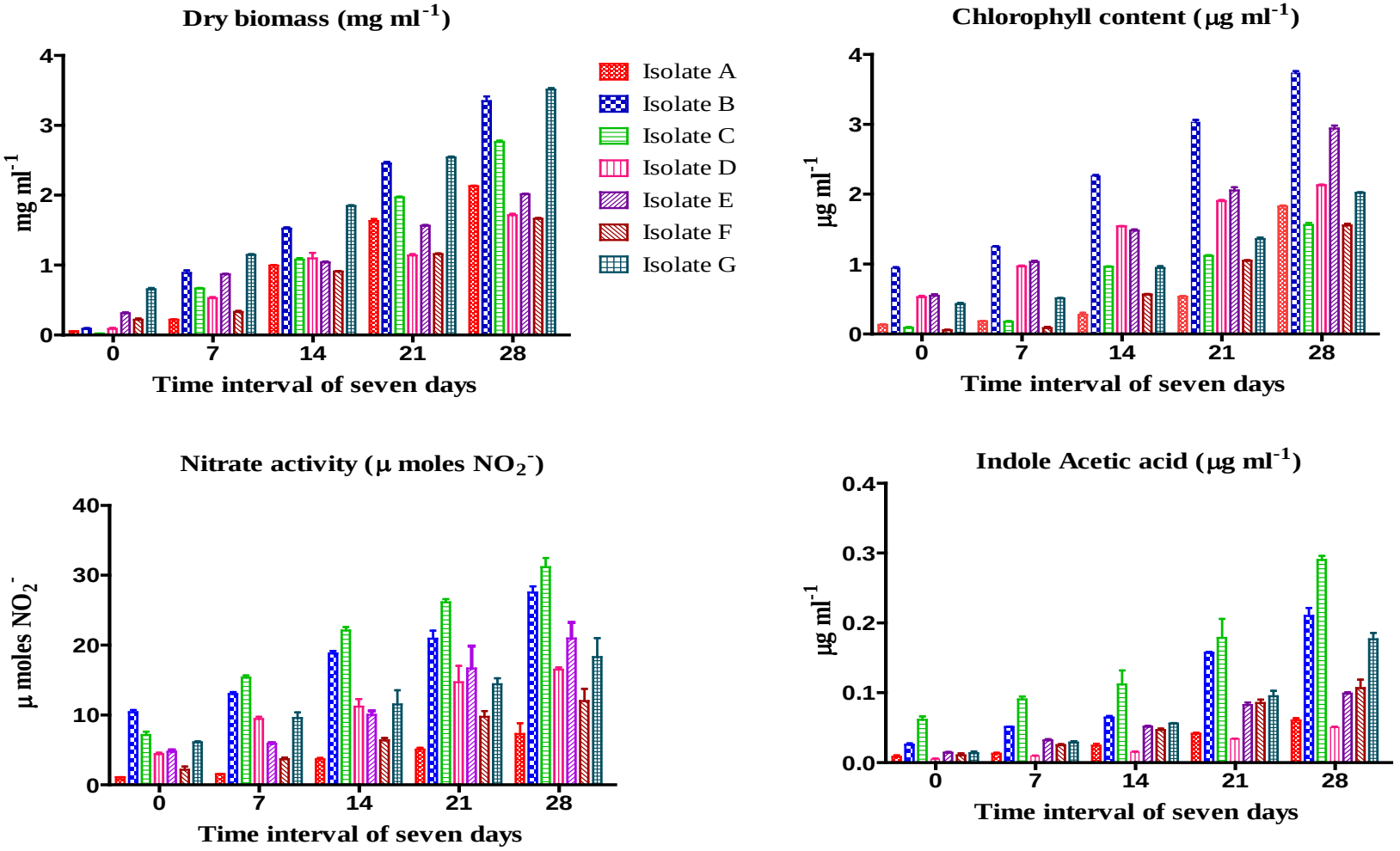


Figure 4.1 a: Growth and biochemical studies of blue green algal isolates at time interval of seven days.

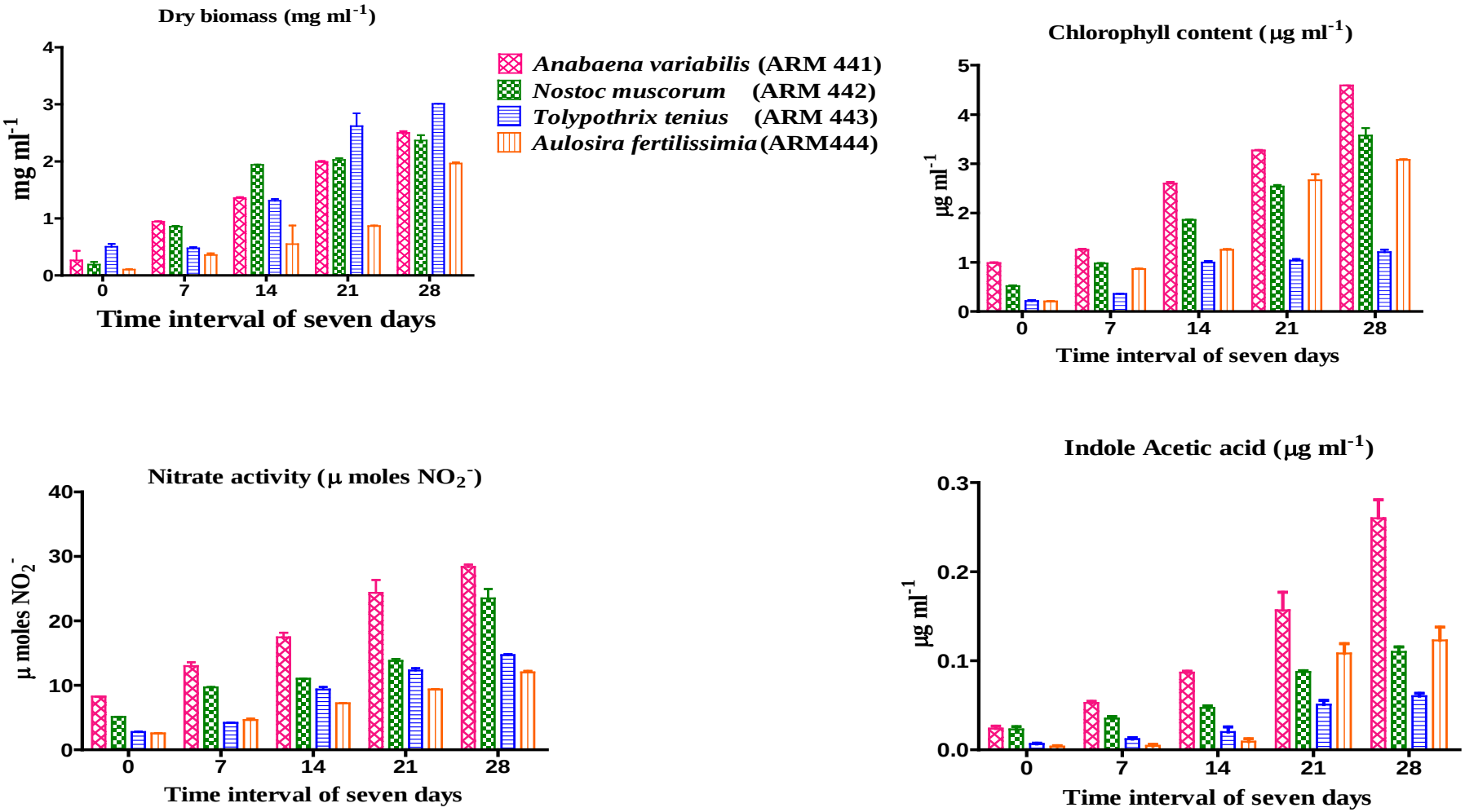


Figure 4.1b: Growth and biochemical studies of ARM cyanobacterial strains at time interval of seven days for one month.

4.2 Molecular studies

Three blue green algal isolates (Isolate B, C and F) were selected out of seven, based on highest nitrogen fixation and heterocyst frequency and were further characterised using 16S rRNA molecular identification technique. Nucleotide sequence data was submitted in NCBI database.

4.2.1 DNA extraction and 16S rRNA gene amplification

Total genomic DNA was extracted from the screened isolates (Isolate B, C and F) were subjected to 16S rRNA amplification using universal primers 27-F-5'AGAGTTTGATCCTGGCTCAG-3' and 1492-R-5'TACCTTGTTACGACTT-3'. The DNA bands formed for all the species were above 1500bps on comparing to the marker DNA (Figure 4.2). Similar type of banding patter was observed in Isolate B (Lane 2 &3), Isolate C (Lane 4 & 5) and Isolate F (Lane 6 & 7).

4.2.2 Phylogenetic analysis of blue green algal Isolates (Isolate B, C and F)

The 16S rRNA gene sequences of the taxa examined and the sequences of reference organisms obtained from databases were multiple aligned using CLUSTAL W. Jukes-Cantor distances, generated by pairwise comparisons of the Isolates, were used to create a phylogenetic tree by Neighbor-joining analysis. Constructed phylogenetic tree (Figure 4.3) revealed that, the sequences of taxa examined matches to the genus already existing sequences in NCBI. The 16S rRNA gene sequences formed three groups in the phylogenetic tree as heterocystous blue green algae. Taton *et al.*, (2006) amplified seventeen morphospecies and twenty eight 16S rRNA taxonomic units belonging to the Oscillatoriales, Nostocales and Chroococcales were identified.

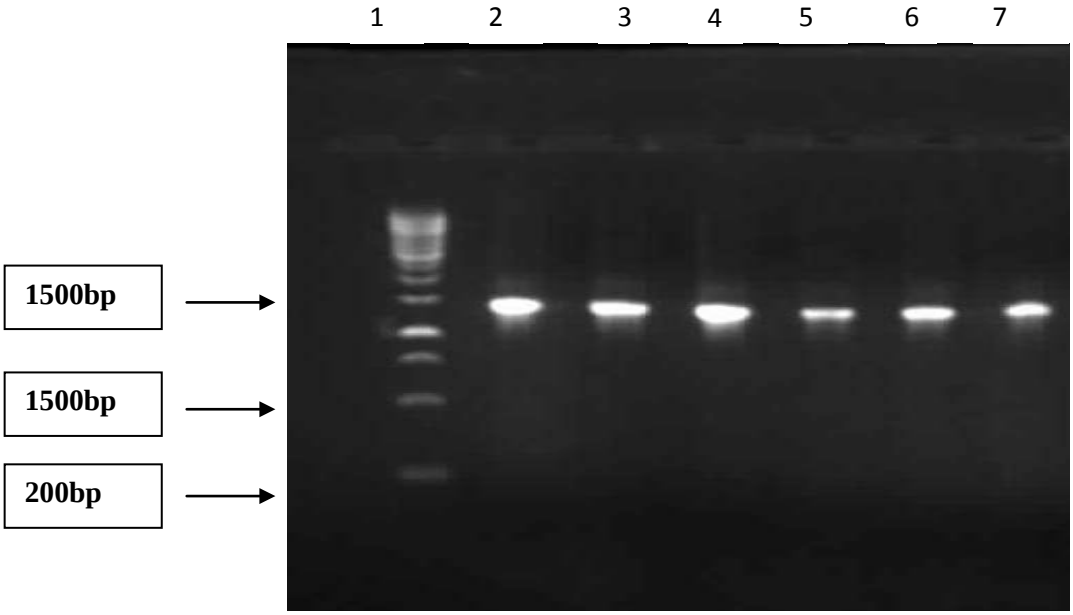


Figure 4.2: 16SrRNA ampLified PCR products of Isolate B, C and F.

Lane 1:1KB Ladder, Lane 2 and 3: Isolate C; Lane 4 and 5: Isolate D; Lane 6 and 7: Isolate F

4.2.2 BLAST analysis of Isolate B, C and F

The partial sequences of 16S rRNA of Isolate A and Isolate F were BLAST and compared with already existing sequences in the database to identify the most probable similarity with high expect value. BLAST analysis of Isolate B showed mixed similarity with *Anabaena flos-aquae*, *Anabaena* sp. and *Nostoc* sp. whereas sequence of Isolate C showed closed resemblance to Desmonostoc sp. PCC 8306 (100% identity & query coverage), *Nostoc* sp. and *Anabaena* sp. by 99% identity. 58% heterocystous cyanobacteria belonging to genera Nostocales shows highest similarity with species of *Anabaena* and *Nostoc* (Srivastava, 2009; Nayak and Prasanna, 2007). BLAST analysis of Isolate F revealed that 9 species of *Nostoc* and 2 species of *Anabaena* showed closed similarity (query coverage of 94% and identity of 75%) respectively. Through Nucleotide BLAST analysis, 12-20 relevant reference sequences showing close similarity to the sequence of B, C and F were downloaded from genbank database and were aligned using multalin for phylogenetic tree construction.

4.2.3 Phylogenetic analysis of Isolate B, C and F

Isolate B

Phylogenetic analysis (NJ tree) based on 16S rRNA gene sequence of Isolate B reveals that cluster 1 showed *Anabaena flos-aquae* DQ234823 and *Anabaena flos-aquae* DQ234825 supported by highest bootstrap value of 82 followed by *Nostoc* sp. FJ70580 and *Anabaena dolilum* GU396094 (Figure 4.3a). Proper discrimination between *Anabaena* and *Nostoc* strain was difficuLt to find out based on morphological and molecular characterisation as a close genetic relationship occurs among these species (Tamas *et al*., 2000; Rajaniemi *et al*., 2005). The 16S rRNA tree of strain *Anabaena flos-aquae* contains mixed genera of *Anabaena* and *Aphanizomenon* (Gugger *et al*, 2002). Cluster 2 showed paraphyletic *Anabaena* strains

containing *Anabaena doliolum* GU396094, *Anabaena* sp KJ652540, and *Anabaena flos-aquae* AB042858 whereas Cluster 3 and 4 depicted the mixed resemblance with *Anabaena* and *Nostoc* strains respectively.

Isolate C

The sequence similarity of Isolate C was observed within Cluster 1 containing strains of *Desmonostoc* sp HG004584, *Desmonostoc* sp. HG004579 and *Desmonostoc* sp HG004586 with highest bootstrap value of 99-100% (Figure 4.3b). Cluster 2 also depicts closed resemblance to *Desmonostoc* sp KF417429, two strains of *Nostoc muscorum*, *Nostoocaceae* GQ389643 and *Nostoc* sp AY742454 with highest bootstrap value of 89%. Cluster 3 contained two species of *Desmonostoc* sp. and one of *Nostoc* sp having bootstrap value of 73%. Previous studies indicate that species of *Nostoc* strain shows closely related monophyletic taxon with *Desmonostoc* sp. (Hrouzek *et al*., 2005; Hrouzek *et al*., 2013).

Isolate F

The Neighbour joining analysis of Isolate F showed 4 clusters (Figure 4.3 c), among which the most sequence similarity was shown in cluster 1 by 99% consisting of *Nostoc* sp. KF953512, *Nostoc* sp. KF953506, Nostoc sp. HE974997 and *Nostoc* sp. KF953509 whereas cluster 2 and cluster 3 showed sequence similarity by 64- 91% with *Nostoc cranium*, *Nostoc entophytum*, *Nostoc* sp KC699844 and *Nostoc linckia* GQ1657550. Cluster 4 showed mixed resemblance containing two strains of *Anabaena* sp. and one of *Nostoc calcicola* supported by 60% bootstrap value.

4.2.4 Nucleotide accession numbers

16S rRNA gene sequences of isolate C and F determined in this study were deposited in Gen-Bank database of the NCBI under the accession numbers KM083062 and KM083063.

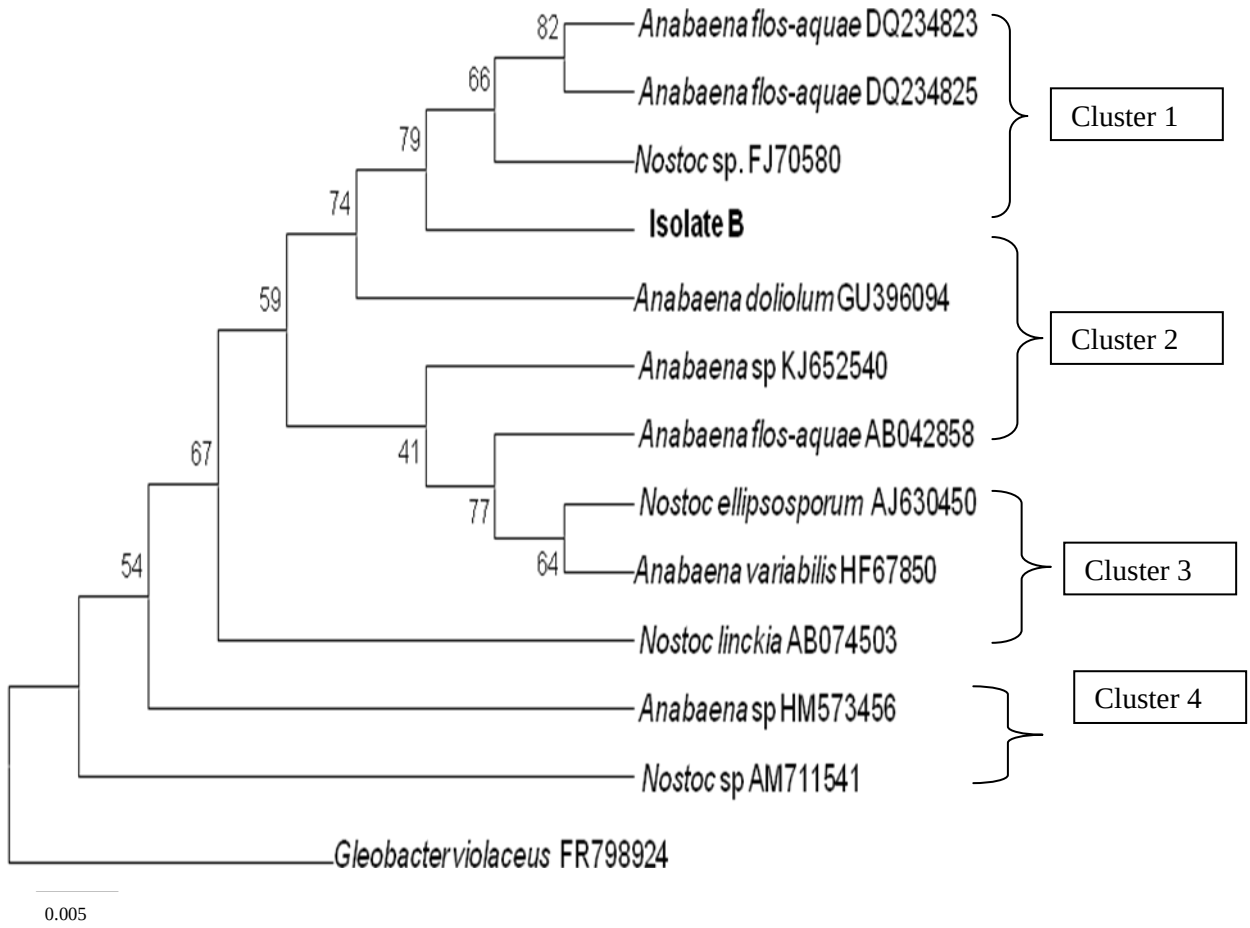


Figure 4.3a Phylogenetic (Neigbhour-Joining) tree based on 16S rRNA gene sequence of Isolate B, showing its relationship with representatives of other related taxa. *Gleobacter violaceus* (FR798927) was used as out group.

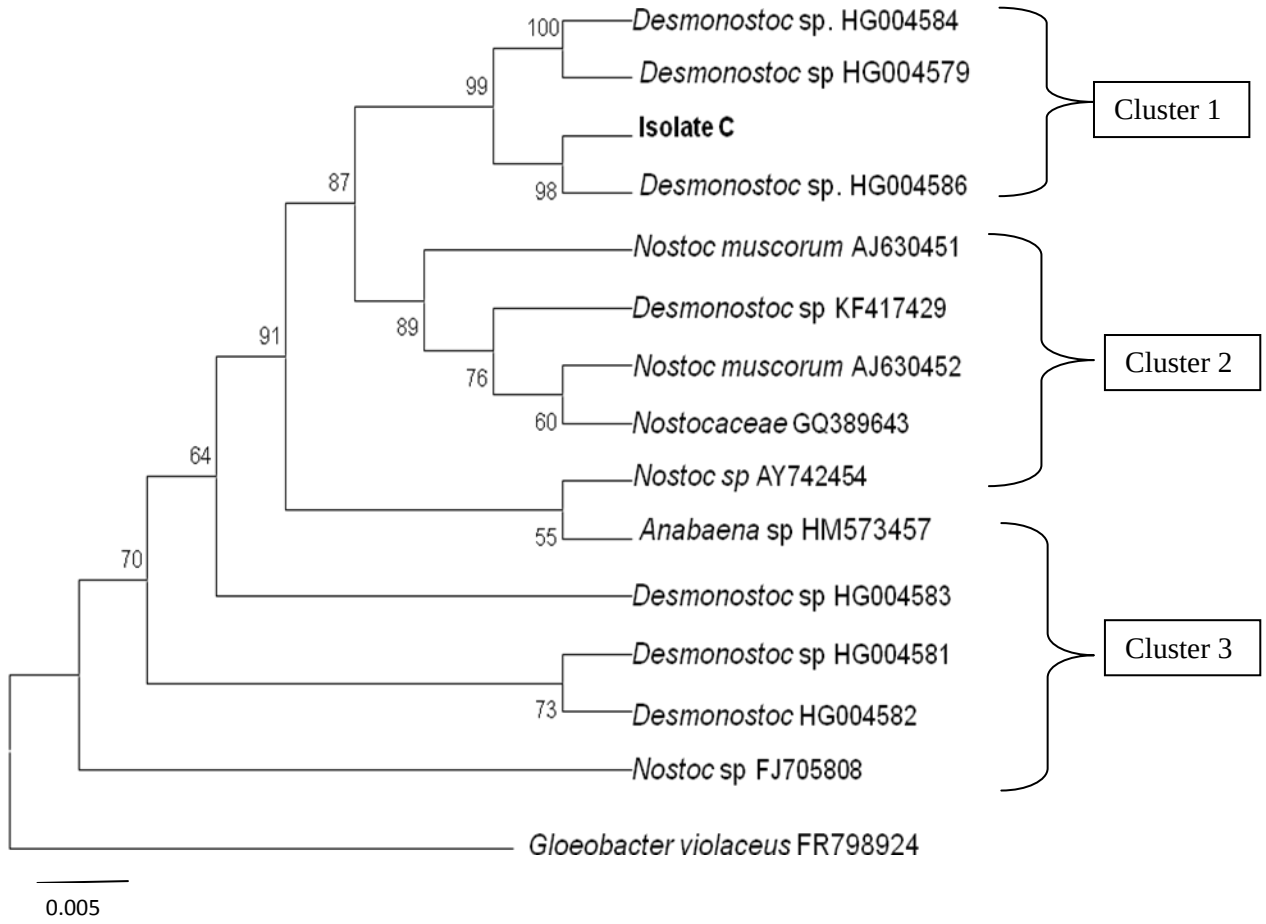


Figure 4.3b Phylogenetic (Neigbhour-Joining) tree based on 16S rRNA gene sequence of Isolate C, showing its relationship with representatives of other related taxa. *Gleobacter violaceus* (FR798927) was used as out group.

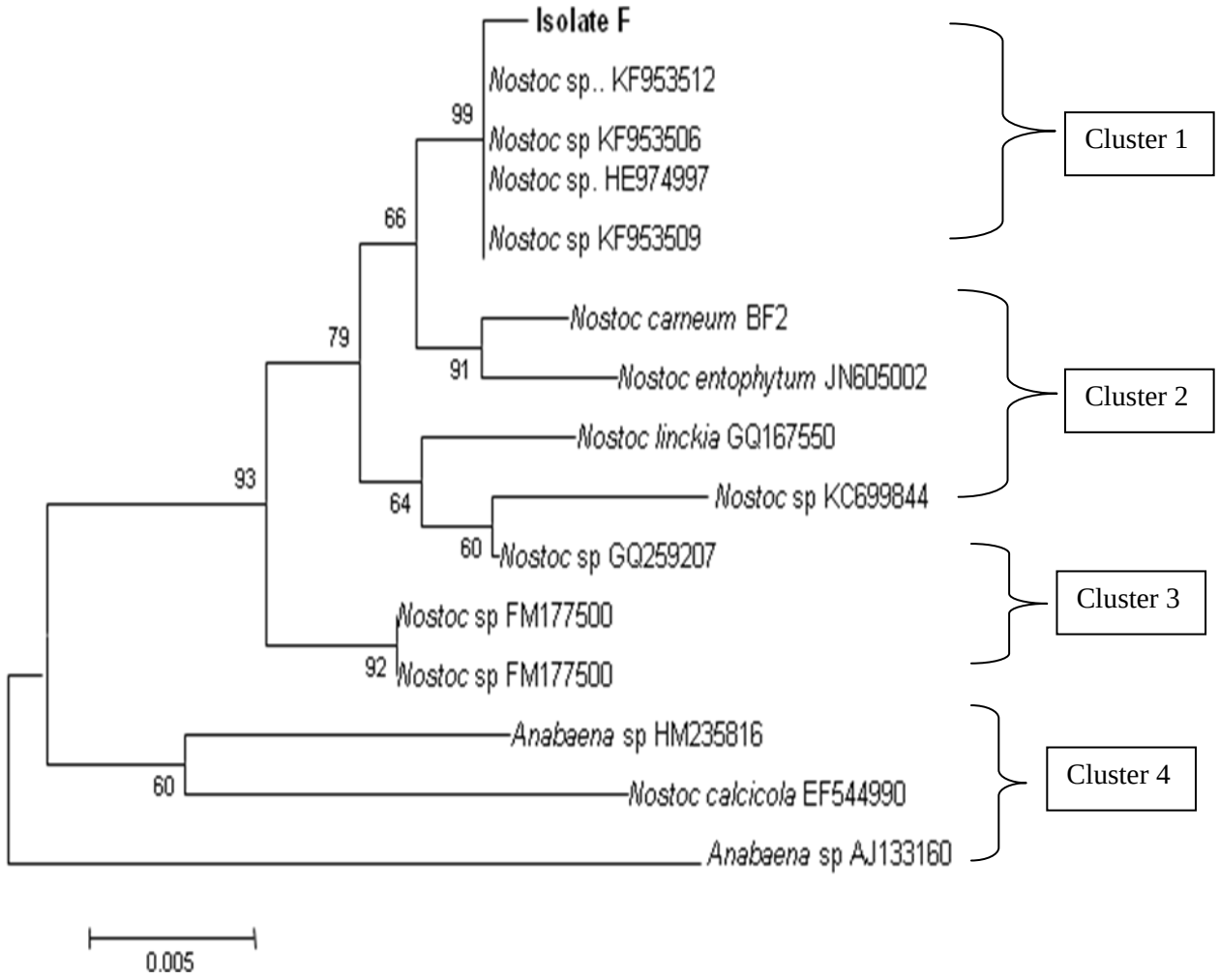
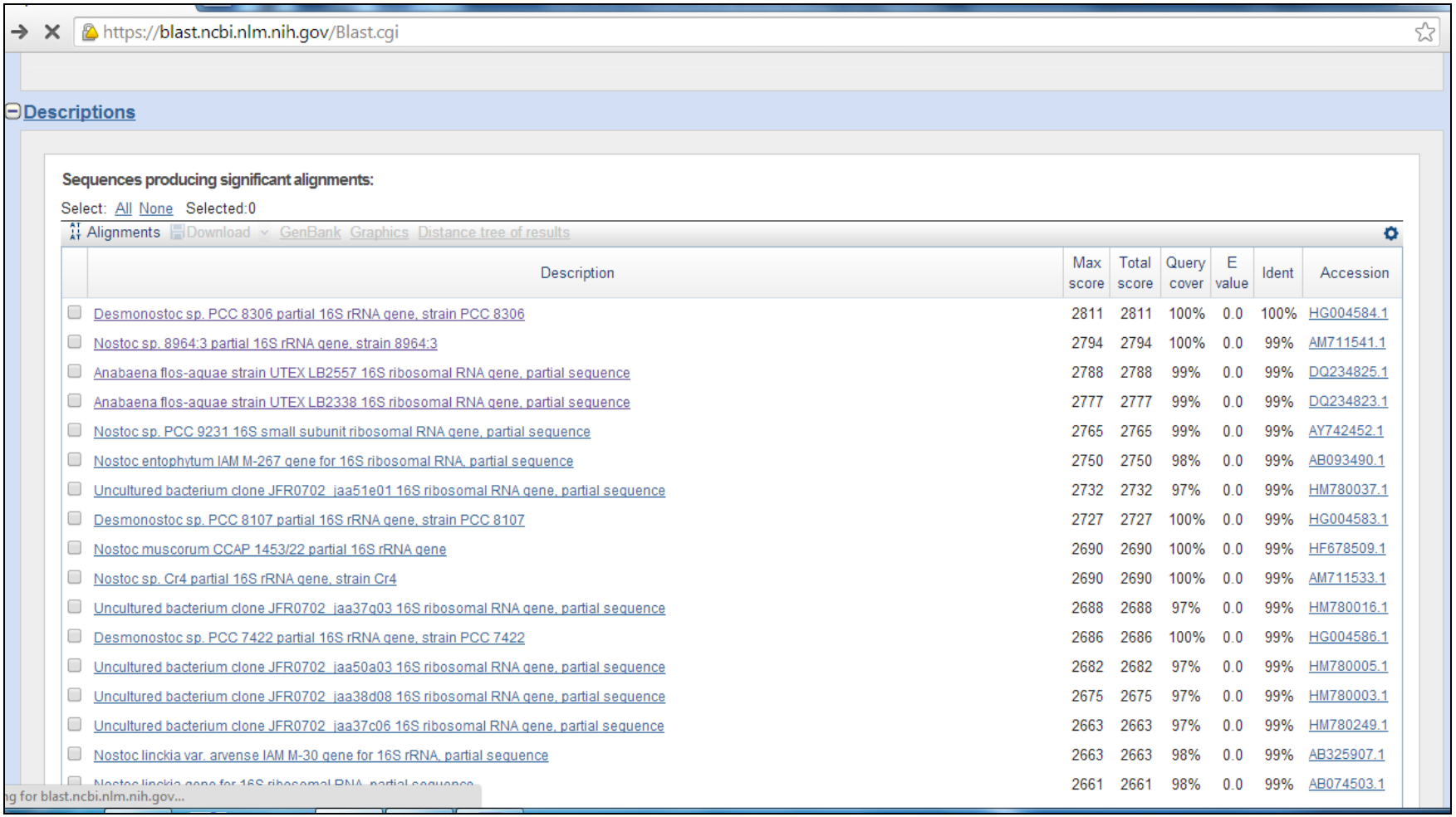
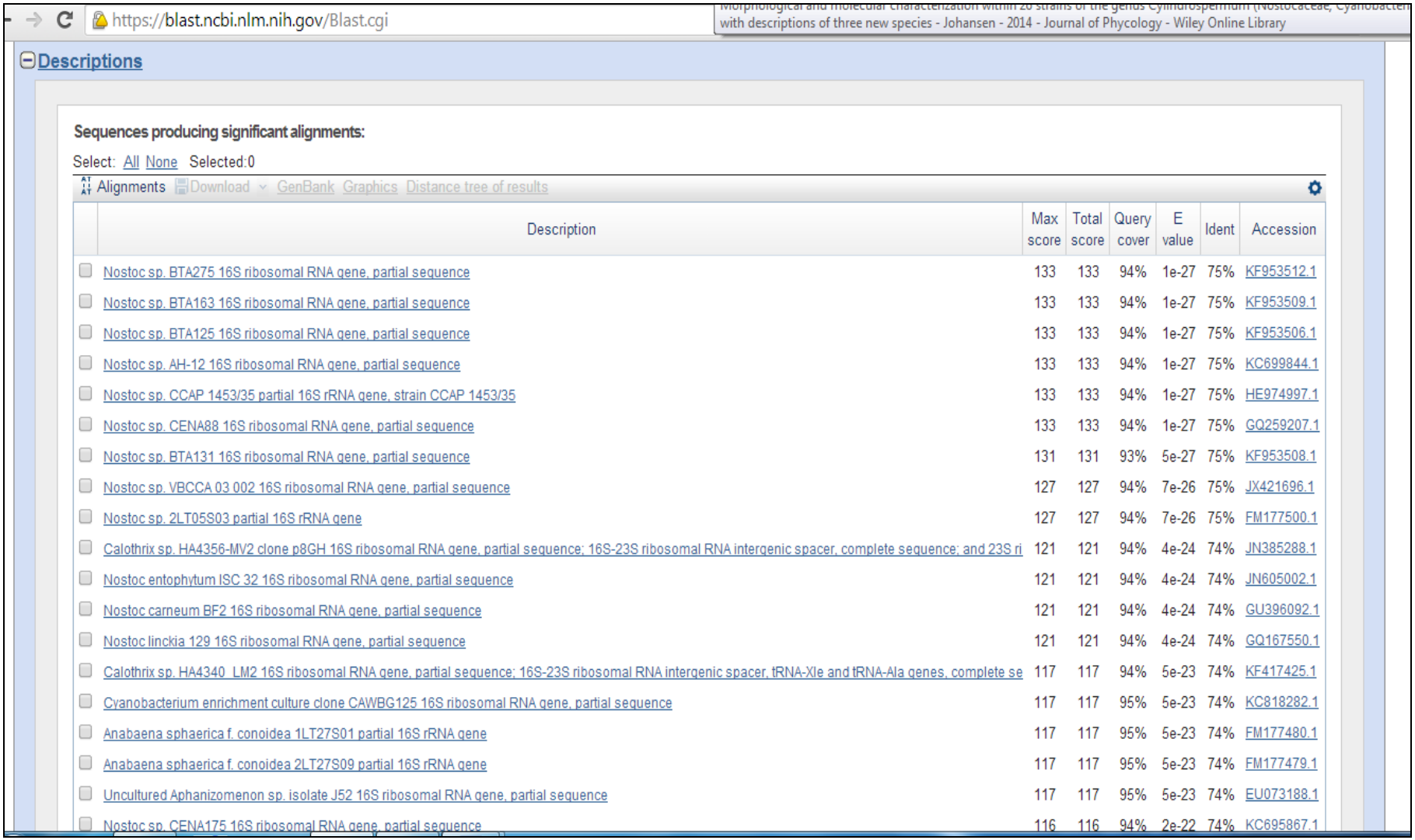


Figure 4.3c Phylogenetic (Neigbhour-Joining) tree based on 16S rRNA gene sequence of Isolate F, showing its relationship with representatives of other related taxa.



BLAST analysis of Isolate C (*Desmonostoc* sp. DGRKC)



BLAST analysis Isolate F (*Nostoc* sp.DGRKF)

4.2 Impact of fly ash on growth and nitrogen fixation by selected blue green algal Isolates

Coal fly ash procured from Guru Gobind Singh Super Thermal Power Plant, Ropar, Punjab was characterized for its physiochemical properties and its impact on growth and nitrogen fixation by selected blue green algal isolates viz., *Calothrix* sp, *Anabaena flos-aquae, Desmonostoc* sp. DGRKC., *Nostoc commune, Nostoc sp.*PS1, *Nostoc* sp.DGRKF, and *Anabaena* sp. was studied and compared with *Anabaena variabilis* (ARM 441); *Nostoc muscorum* (ARM 442); *Tolypothrix tenius* (ARM 443) and *Aulosira fertilissima* (ARM 444) procured from IARI, New Delhi.

Coal fly ash in dried form at different concentration 0, 5, 10 and 20% (w/v) was added to BG 11(-N) growth medium and distilled water (DW) separately to examine its impact on growth and nitrogen fixation by heterocystous filamentous blue green algal strains and to find out optimum concentration of fly ash that supports growth and nitrogen fixation. Metal uptake by algal biomass of 30 days grown culture was studied by nitric acid digestion method (Page *et al*, 1982) in order to accesses the metal removal potential of blue green algal strains.

4.2.1 Growth of blue green algal strain with different concentration of fly ash

The growth performance of different blue green algal strains amended with different concentrations of fly ash (0%, 5%, 10%, and 20%) for 30 days was examined and prolific growth in all the algal strains were observed in BG 11(-N) medium amended with 5 and 10% fly ash as compared to control (0% FA). All the algal strains showed similar trend (Table 4.2.1; Figure 5.1). At 20% fly ash growth of algal strains was negligible which could be due to phytotoxicity caused by fly ash (Rai *et al.,* 2000). *Nostoc* sp. showed good growth in BG 11 amended with 5- 10% fly ash whereas *Anabaena variabilis*, *Anabaena flos-aquae* and *Desmonostoc* sp.DGRKC showed growth in BG 11 amended with 5,10 and 20% fly ash and showed almost same dry biomass (mg/L) as observed after 30 days of growth at 5 and 10% fly ash (Figure 4.2.2). Similar difference was reported in previous studies done by Zhou *et al*. (1998) and Rai et al. (2000) that *Nostoc commune* and *Nostoc calcicola* showed less growth on fly ash bed as compared to *Anabaena doliolum*.

Table 4.2.1 Growth of BGA strains growth observed after 30 days in BG 11(-N) medium and distilled water (DW) amended with different concentration of fly ash (0, 5, 10 and 20%).

+++ProLific growth

Isolate Designation	**Blue green algal strain**	***Growth (after 30 days)***							
		BG11- N				Distilled water			
		0%	5%	10%	20%	0%	5%	10%	20%
A.	*Calothrix* sp	+++	+++	+	+	++	+	+	+
B.	*Anabaena flos-aquae*	+++	+++	+++	++	++	++	++	+
C.	*Desmonostoc* sp.	+++	+++	++	++	++	++	++	+
D.	*Nostoc commune*	++	++	+	+	++	++	+	+
E.	*Nostoc* sp. PS1	+++	+++	++	++	++	++	+	+
F.	*Nostoc* sp.BTA 275	+++	+++	++	+	++	++	+	+
G.	*Anabaena* sp.	+++	+++	++	++	++	++	+	+
ARM 441	*Anabaena variabilis*	+++	+++	++	++	++	++	++	+
ARM 442	*Nostoc muscorum*	++	+++	+	+	++	++	++	+
ARM 443	*Tolypothrix tenuis*	+++	+++	++	+	++	++	+	+
ARM444	*Aulosira fertilissima*	+++	+++	++	+	++	+	+	+

++Average growth

+NegligibLe growth

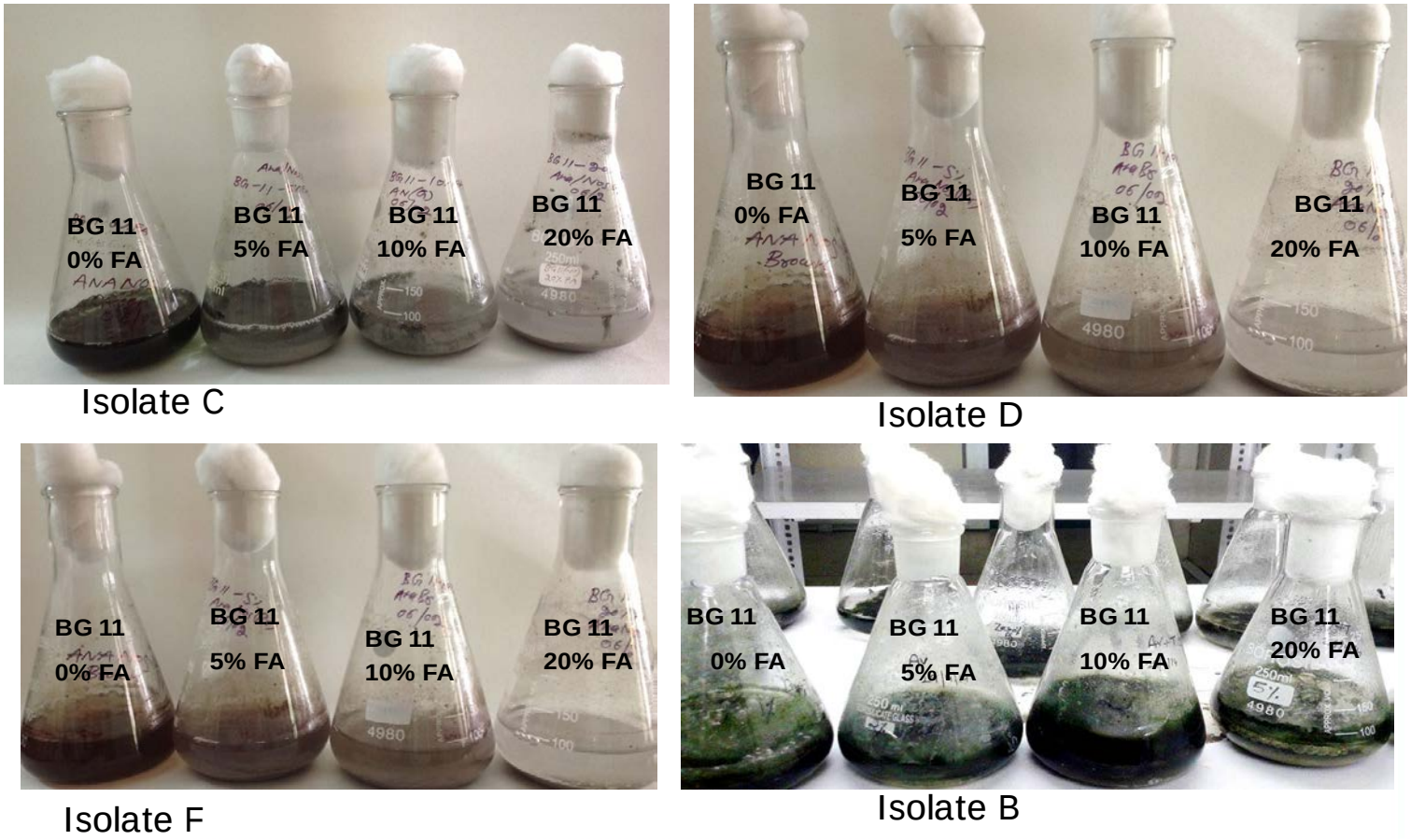


Figure 5.1 Growth of blue green algal isolates observed on 30th day in BG 11(-N) medium amended with different concentration of fly ash (0, 5, 10 and 20%)

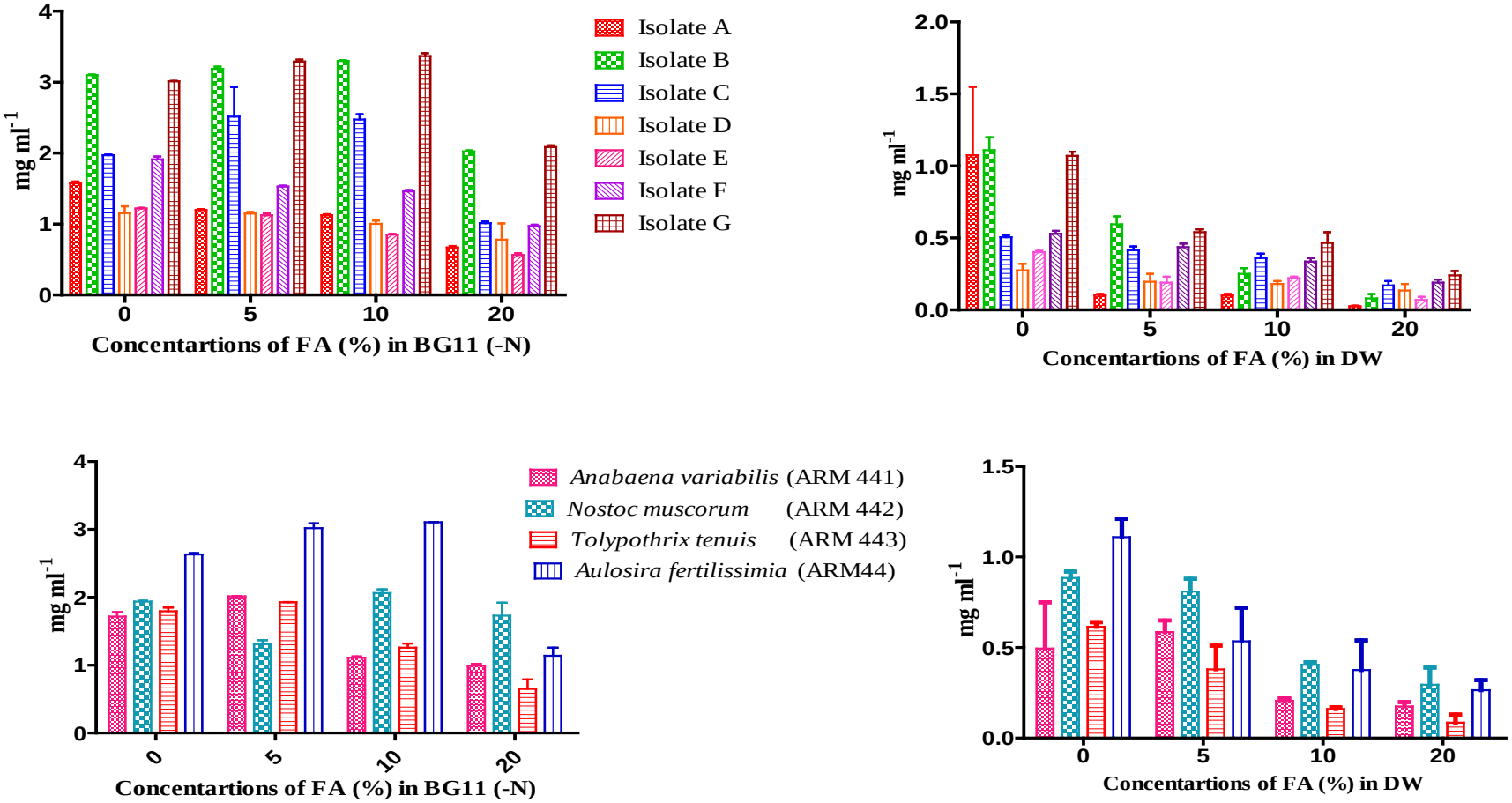


Figure 5.2 Dry biomass (mg/mL) of blue green algal strains grown for 30 days in BG 11(-N) medium and distilled water (DW) amended with different concentration of fly ash (0, 5, 10 and 20%).

4.2.2 Impact of fly ash on nitrogen fixation of blue green algae

Impact of fly ash on the nitrogen fixation of 30 days old blue green algal cultures grown in BG 11 (-N) medium and distilled water (DW) at different concentration of fly ash @ 0, 5, 10 and 20 % reveals that BG 11(-N) medium amended with 5 and 10% fly ash (FA) showed increased nitrogenase activity (ARA) and total nitrogen content (%) in all the seven algal isolates (Table 4.2.2a). Maximum nitrogen fixation and total nitrogen content of 37.2 – 40.4 mole C_2H_4/mg dry wt/hr and 0.784-0.883% was observed in *Desmonostoc* sp.DGRKC grown in BG 11amended with 5% and 10% fly ash as compared to control (BG 11-0% FA). However these algal isolates showed decreased nitrogen fixation when grown in higher concentration of fly ash (20%) both in distilled water (DW) and BG 11 medium. Alkaline fly ash increases the growth of blue green algae as blue green algae are alkali tolerant and fly ash inhibits essential elements for growth and inherent tolerance of high alkalinity (Rai et al., 2000). *Anabaena variabilis* (ARM 441) showed highest range of nitrogen fixation of 32.5 mole C_2H_4/mg dry wt/hr and total nitrogen content by 0.509 % when grown in BG 11 (-N) medium amended with 5% fly ash whereas *Nostoc muscorum* (ARM 442) showed maximum nitrogenase activity (23.14 mole C_2H_4/mg dry wt/hr) and total nitrogen content (0.398 %) in BG11 medium amended with 5% fly ash (Table 4.2.2b). Fly ash is the reservoir of all the essential elements for plant growth like Na, K, Ca, Cu, Mg, Fe, Zn, B and Mo, however some constituent metals like zinc, molybdenum and zinc present are used by blue green algae in small quantities for various physiological and biochemical process (Round, 1973; Rai *et al.*, 2000). The present study reveals that the 5 and 10% fly ash showed increased nitrogen fixation in blue green algal strains when grown in BG11 (-N) medium. It was reported that fly ash amendment alone at low concentration (5-20%) with organic manures organic manures significantly increased concentration of phosphate-solubilizing bacteria (PSB) (Bhattacharya, 2002; Malik and Thapliyal, 2009).

Table 4.2.2a : Nitrogen fixation in terms of nitrogenase activity (ARA) in mole C_2H_4/mg dry wt/hr and total nitrogen content (TN) in % observed in seven blue green algal Isolates grown in BG 11(-N) medium and distilled water (DW) amended with different concentrations of fly ash (0, 5, 10 and 20%).

Treatments	*Calothrix* sp.		*Anabaena flos-aquae*		*Desmonostoc sp.*		*Nostoc commune*		*Nostoc sp PS1*		*Nostoc sp BTA 275*		*Anabaena sp.*	
	ARA	**T N**	**ARA**	**T N**	**ARA**	**T N**	**ARA**	**T N**	**ARA**	**T N**	**ARA**	**T N**	**ARA**	**T N**
BG11-0% FA	5.50±0.01	0.169±0.010	17.9±0.30	0.225±0.040	30.5±1.10	0.457±0.020	9.58±0.60	0.192±0.010	9.01±0.50	0.106±0.050	15.2±0.50	0.168±0.050	13.98±0.1	0.135±0.003
BG11-5% FA	6.01±0.20	0.226±0.014	**19.26±0.05**	0.599±0.020	37.2±0.47	0.784±0.050	10.2±0.10	0.268±0.700	13.1±1.20	0.362±0.020	**17.3±0.90**	0.323±0.010	15.26±1.0	0.207±0.051
BG11-10%FA	6.59±0.01	0.219±0.500	**27.58±0.60**	**0.635±0.110**	**40.4±0.50**	**0.883±0.090**	**12.56±0.70**	**0.295±0.500**	**13.6±0.96**	**0.291±0.080**	**22.4±0.10**	**0.349±0.070**	17.9±1.3	0.195±0.020
BG11-20%FA	4.21±0.40	0.159±0.010	16.2±0.20	0.301±0.050	29.7±0.10	0.541±0.010	10.20±0.40	0.124±0.040	10.02±0.20	0.201±0.050	15.20±0.60	0.253±0.040	15.61±2.1	0.101±0.001
DW-0% FA	3.21±0.01	0.039±0.001	8.1±0.33	0.026±0.001	**16.01±0.50**	**0.034±0.002**	5.6±0.09	0.026±0.001	3.15±0.10	0.019±0.001	7.59±0.96	0.013±0.004	05.2±0.4	0.021±0.004
DW-5% FA	3.09±0.50	0.021±0.003	6.9±0.14	0.017±0.001	**15.8±0.40**	**0.031±0.001**	4.1±0.52	0.022±0.005	2.27±0.50	0.012±0.001	7.52±0.70	0.010±0.001	04.96±0.9	0.015±0.001
DW-10% FA	2.51±0.02	0.014±0.003	6.6±0.17	0.011±0.003	11.9±0.50	0.025±0.002	3.5±0.13	0.021±0.010	1.99±0.10	0.007±0.001	6.01±0.14	0.009±0.001	03.54±0.7	0.011±0.003
DW-20% FA	1.04±0.08	0.008±0.001	5.3±0.04	0.009±0.001	9.6±0.80	0.019±0.001	2.5±0.51	0.015±0.004	0.69±0.10	0.005±0.001	3.71±0.10	0.006±0.001	02.6±0.5	0.004±0.001

Values are average of Mean±SE (n=3) ; Acetylene Reductase Assay (ARA) (mole C_2H_4/mg dry wt/hr); Total nitrogen content (TN)

Table 4.2.2b : Nitrogen fixation in terms of nitrogenase activity (ARA) in mole C_2H_4/mg dry wt/hr and total nitrogen content (TN) in % observed in four ARM procured blue green algal strains grown in BG 11(-N) medium and distilled water (DW) amended with different concentrations of fly ash (0, 5, 10 and 20%).

Treatments	***Anabaena variabilis* (ARM 441)**		***Nostoc muscorum* (ARM 442)**		***Tolypothrix tenuis* (ARM 443)**		***Aulosira fertilissima* (ARM 444)**	
	ARA (mole C_2H_4/mg dry wt/hr)	T N(%)	ARA (mole C_2H_4/mg dry wt/hr)	T N (%)	ARA (mole C_2H_4/mg dry wt/hr)	T N (%)	ARA (mole C_2H_4/mg dry wt/hr)	T N (%)
BG11-0% FA	28.1±0.96	0.292±0.001	17.00±0.54	0.103±0.001	12.00±1.50	0.133±0.012	13.80±0.25	0.140±0.020
BG11-5% FA	**32.5±0.52**	**0.509±0.001**	21.25±0.86	0.223±0.06	14.00±0.59	0.217±0.001	14.63±0.48	0.201±0.005
BG11-10%FA	30.0±0.14	0.501±0.002	**23.14±0.47**	**0.398±0.02**	13.78±0.25	0.231±0.050	15.01±0.99	0.295±0.010
BG11-20%FA	21.0±0.33	0.109±0.005	20.01±0.14	0.089±0.01	08.50±0.01	0.124±0.03	06.21±0.13	0.171±0.031
DW-0% FA	15.0±1.20	0.121±0.04	10.96±0.69	0.056±0.001	06.90±0.54	0.104±0.001	05.70±0.09	0.125±0.050
DW-5% FA	13.3±0.58	0.118±0.001	11.02±0.54	0.025±0.001	06.20±0.09	0.072±0.002	04.58±0.51	0.110±0.002
DW-10% FA	11.0±1.60	0.113±0.04	10.58±0.29	0.010±0.002	05.58±0.70	0.043±0.001	03.61±0.03	0.013±0.005
DW-20% FA	07.1±0.02	0.009±0.001	05.01±0.02	0.006±0.001	03.02±0.50	0.022±0.005	02.05±0.01	0.006±0.001

Values are average of Mean±SE (n=3) ; Acetylene Reductase Assay (ARA) (mole C_2H_4/mg dry wt/hr); Total nitrogen content (TN)

4.2.3 Uptake of heavy metals from fly ash by selected blue green algal strains

Among all the blue green algal isolates, *Calothrix* sp. showed maximum uptake of Zn (4.52 mg/g) and Pb (2.52 mg/g) in BG 11(-N) medium amended with 20% fly ash as compared to control, however Cu (0.825 mg/g)and Cr (0.950 mg/g) showed maximum uptake in BG 11 (-N) amended with 10% fly ash (Table 4.2.3a). Previous studies reported that fourteen day old culture of *Calothrix marchia* showed higher adsorption of Lead (Pb^{2+}) from 0.253 -1.16 mg/g with low dry biomass (mg/g) (Ruangsomboon *et al*., 2006). Algae are also well known for their capacity to accumulate metals from wastewater since many heavy metals e.g Cu, Fe, Mn, Zn, Co and Mo are required as essential micronutrients (Harish *et al*., 2008). Chromium uptake (Cr) was observed maximum in *Anabaena flos-aquae* (3.73 mg/g), *Nostoc commune* (4.85 mg/g) at BG 11 medium amended with 5% fly ash whereas *Nostoc* sp DGRKF (3.98 mg/g) showed at BG 11 (-N) medium amended with 10% fly ash respectively. Howerever, *Nostoc muscorum* (ARM 442 mg/g) showed maximum uptake of Cr (3.65 mg/g), Pb (2.12 mg/g) at BG 11(-N) medium amended with 10% fly ash respectively (Table 4.2.3b). *Anabaena variabilis* (ARM 441) showed maximum uptake of Cu (0.313 mg/g)and Pb (2.01 mg/g) in BG 11 (-N) medium amended with 5% fly ash whereas Cr uptake (1.21 mg/g) at 10% fly ash and Zn uptake (0.697 mg/g) at 20% fly ash grown in BG 11(-N) medium. Blue green algal strains are known for eliminating heavy metal from fresh water (Inthorn *et al*, 2002) *Nostoc Linckia, Nostoc calcicola* were reported for chromium and zinc uptake (Anjana *et al*., 2007) whereas *Nostoc muscorum* and *Anabaena s*p. for Cu and Pb uptake (Sheek *et al*., 2005). Khattar *et al.*, (2001) reports that *Anabaena variabilis* is tolerant to chromium and has a good potential for the removal of Cr ions from industrial effluents.The availability, uptake, and subsequent toxicity of a particular metal depend upon several factors including pH, amount of biomass present in the system, and the metal species and its concentration (Rai and Dubey, 1988; Rai *et al*., 1999; Yetis, 1999; Khattar, 2001).

Increased accumulation of metals in blue green algal isolate grown in BG 11(-N) medium amended with 5, 10 and 20% fly ash which confirms that metal concentration was balanced between the algal strains. Mass balance of metals determined a balance metal concentration between fly ash and alga (*A.doliolum*) confirms no loss of metals (Rai *et al*., 2000).

Table 4.2.3 a. Metal (Cu, Zn, Cr and Pb) uptake (mg/g) by BGA isolates grown in BG 11(-N) medium and distilled water (DW) amended with different concentrations of fly ash (0, 5, 10 and 20%).

Metal concentration (mg/g) in 30 days grown blue green algae				
	Cu	**Zn**	**Cr**	**Pb**
		***Calothrix* sp.**		
BG11 -0%FA	0.402±0.010	1.12±0.030	0.291±0.020	0.863±0.004
BG11-5% FA	0.639±0.080	2.06±0.005	0.832±0.040	1.520±0.001
BG11-10%FA	**0.825**±0.005	3.95±0.010	**0.950**±0.050	2.010±0.063
BG11-20%FA	0.543±0.030	**4.52**±0.050	0.617±0.010	**2.520**±0.012
DW -0% FA	0.119±0.020	1.03±0.020	0.523±0.030	0.357±0.010
DW -5% FA	0.213±0.001	1.13±0.010	0.890±0.005	0.512±0.010
DW-10% FA	0.227±0.004	2.01±0.060	0.713±0.010	0.320±0.050
DW-20% FA	0.014±0.001	1.96±0.001	0.396±0.040	0.291±0.010
		Anabaena flos-aquae		
BG11 -0%FA	0.011±0.001	0.201±0.010	1.70±0.011	0.011±0.003
BG11-5% FA	**0.077**±0.001	**0.495**±0.005	**3.73±0.002**	0.037±0.001
BG11-10%FA	0.075±0.001	0.421±0.001	2.95±0.018	**0.054**±0.006
BG11-20%FA	0.046±0.003	0.252±0.008	2.71±0.100	0.049±0.001
DW -0% FA	0.013±0.001	0.101±0.002	1.05±0.030	0.002±0.001
DW -5% FA	0.056±0.001	0.213±0.001	1.24±0.010	0.034±0.001
DW-10% FA	0.067±0.001	0.185±0.001	1.05±0.001	0.019±0.001
DW-20% FA	0.010±0.003	0.123±0.005	0.96±0.007	**0.011±0.001**
		Desmonostoc* sp. *DGRKC		
BG11 -0%FA	0.053±0.001	0.193±0.030	0.631±0.10	0.083±0.005
BG11-5% FA	**0.061±0.001**	0.462±0.100	**0.962±0.10**	**0.174**±0.002
BG11-10%FA	0.054±0.001	**0.87±0.010**	1.100±0.30	0.235±0.001
BG11-20%FA	0.031±0.001	0.317±0.020	**1.410±0.12**	**0.428±0.004**
DW -0% FA	0.022±0.001	0.119±0.003	0.547±0.01	0.021±0.003
DW -5% FA	0.021±0.001	0.095±0.001	0.981±0.08	0.038±0.001
DW-10% FA	0.016±0.003	0.053±0.001	0.872±0.10	0.047±0.001
DW-20% FA	0.001±0.002	0.014±0.001	0.533±0.01	0.032±0.001
		Nostoc commune		
BG11 -0%FA	0.021±0.001	0.114±0.005	1.75±0.005	0.302±0.001
BG11-5% FA	0.066±0.001	**0.393±0.021**	**4.89±0.50**	**0.491±0.001**
BG11-10%FA	**0.078±0.003**	0.538±0.005	4.32±0.17	0.350±0.005
BG11-20%FA	0.059±0.010	**0.831±0.001**	4.19±0.03	0.068±0.002
DW -0% FA	0.014±0.001	0.140±0.003	2.29±0.01	0.448±0.001
DW -5% FA	0.011±0.001	0.102±0.009	1.04±0.07	**0.535±0.080**
DW-10% FA	0.009±0.001	0.093±0.002	0.94±0.03	0.113±0.007
DW-20% FA	0.002±0.001	0.041±0.001	0.41±0.01	0.171±0.005

Values are average of Mean±SE (n=3)

	Cu	Zn	Cr	Pb
		***Nostoc* sp. PS1**		
BG11 -0%FA	0.113±0.001	0.203±0.028	0.157±0.001	0.120±0.001
BG11-5% FA	0.363±0.001	0.395±0.031	0.703±0.001	**0.337±0.009**
BG11-10%FA	0.598±0.001	**0.431**±0.006	**1.730±0.020**	**0.586±0.003**
BG11-20%FA	0.521±0.100	0.117±0.010	1.500±0.018	0.124±0.001
DW -0% FA	0.169±0.003	0.129±0.010	0.101±0.001	0.023±0.001
DW -5% FA	0.267±0.001	0.217±0.005	0.133±0.010	0.084±0.007
DW-10% FA	0.154±0.005	0.212±0.003	0.437±0.100	0.211±0.001
DW-20% FA	0.176±0.100	0.108±0.012	0.491±0.010	0.094±0.006
		Nostoc* sp. *DGRKF		
BG11 -0%FA	0.142±0.011	0.113±0.018	0.217±0.002	0.062±0.006
BG11-5% FA	0.430±0.036	0.357±0.015	1.560±0.008	**0.783±0.026**
BG11-10%FA	**0.733±0.004**	**0.499±0.001**	**3.980±0.001**	**0.612±0.011**
BG11-20%FA	0.714±0.031	**0.683±0.030**	1.720±0.031	0.291±0.007
DW -0% FA	0.103±0.019	0.191±0.034	0.270±0.001	0.072±0.001
DW -5% FA	0.111±0.010	0.146±0.003	0.140±0.001	0.116±0.001
DW-10% FA	0.116±0.027	0.090±0.005	0.100±0.022	0.133±0.005
DW-20% FA	0.098±0.008	0.017±0.001	0.130±0.025	0.181±0.002
		***Anabaena* sp.**		
BG11 -0%FA	0.012±0.06	0.047±0.005	0.101±0.009	0.132±0.011
BG11-5% FA	0.069±0.01	0.443±0.011	0.285±0.001	0.524±0.031
BG11-10%FA	0.113±0.02	**0.656±0.018**	**0.339±0.005**	**0.685±0.016**
BG11-20%FA	**0.145±0.01**	0.519±0.007	0.121±0.005	0.677±0.014
DW -0% FA	0.037±0.01	0.039±0.001	0.024±0.001	0.107±0.005
DW -5% FA	0.059±0.03	0.041±0.002	0.083±0.011	0.266±0.007
DW-10% FA	0.065±0.01	0.042±0.001	0.051±0.001	0.217±0.001
DW-20% FA	0.041±0.02	0.038±0.001	0.039±0.005	0.106±0.003

Values are average of Mean±SE (n=3)

Table 4.2.3b. Metal (Cu, Zn, Cr and Pb) uptake (mg/g) by ARM procured blue green algal strains grown in BG 11(-N) medium and distilled water (DW) amended with different concentrations of fly ash (0, 5, 10 and 20%).

	Cu	**Zn**	**Cr**	**Pb**
		***Anabaena variabilis* (ARM 441)**		
BG11 -0%FA	0.156±0.01	0.117±0.010	0.524±0.030	0.78±0.010
BG11-5% FA	**0.313**±0.001	0.229±0.001	0.891±0.050	**2.01±0.011**
BG11-10%FA	0.280±0.005	0.547±0.001	**1.210**±0.005	1.13±0.040
BG11-20%FA	0.210±0.030	**0.697±0.050**	0.964±0.001	1.02±0.001
DW -0% FA	0.170±0.001	0.212±0.020	0.130±0.001	0.35±0.050
DW -5% FA	0.090±0.001	0.191±0.001	0.150±0.001	0.89±0.001
DW-10% FA	0.150±0.003	0.114±0.005	0.110±0.003	0.81±0.002
DW-20% FA	0.080±0.001	0.101±0.010	0.060±0.001	0.65±0.005
		***Nostoc muscorum* (ARM 442)**		
BG11 -0%FA	0.122±0.010	0.019±0.001	0.542±0.050	0.920±0.005
BG11-5% FA	0.362±0.004	0.167±0.050	1.330±0.001	1.370±0.001
BG11-10%FA	**0.729±0.010**	0.231±0.010	**3.65**±0.001	**2.120±0.050**
BG11-20%FA	0.625±0.005	**0.254±0.050**	2.41±0.050	1.920±0.010
DW -0% FA	0.119±0.030	0.013±0.040	0.81±0.010	0.212±0.020
DW -5% FA	0.271±0.010	0.127±0.010	0.56±0.110	0.988±0.050
DW-10% FA	0.219±0.040	0.114±0.001	0.21±0.050	0.885±0.090
DW-20% FA	0.138±0.010	0.102±0.004	0.07±0.001	0.571±0.001
		***Tolypothrix tenuis* (ARM 443)**		
BG11 -0%FA	0.117±0.040	0.016±0.001	0.113±0.001	0.85±0.050
BG11-5% FA	0.215±0.050	0.113±0.001	0.297±0.050	**1.93±0.120**
BG11-10%FA	**0.367±0.004**	**0.229±0.004**	**0.424±0.005**	1.32±0.050
BG11-20%FA	0.304±0.010	0.212±0.001	0.415±0.001	0.79±0.010
DW -0% FA	0.103±0.010	0.010±0.004	0.102±0.020	0.24±0.001
DW -5% FA	0.199±0.010	0.017±0.010	0.116±0.004	0.51±0.013
DW-10% FA	0.211±0.003	0.014±0.005	0.102±0.001	0.57±0.005
DW-20% FA	0.280±0.005	0.013±0.001	0.08±0.050	0.43±0.060
		***Aulosira fertilissima* (ARM 444)**		
BG11 -0%FA	0.183±0.003	0.101±0.001	0.150±0.020	1.05±0.052
BG11-5% FA	0.299±0.004	0.294±0.010	**0.410**±0.050	**3.09**±0.047
BG11-10%FA	**0.373±0.010**	0.319±0.020	0.381±0.050	3.11±0.078
BG11-20%FA	0.225±0.005	**0.345±0.004**	0.279±0.080	2.26±0.051
DW -0% FA	0.123±0.001	0.085±0.001	0.187±0.010	1.21±0.053
DW -5% FA	0.192±0.001	0.113±0.005	0.203±0.020	1.72±0.014
DW-10% FA	0.215±0.001	0.107±0.001	0.123±0.001	1.05±0.011
DW-20% FA	0.117±0.050	0.095±0.010	0.119±0.010	0.93±0.058

Values are average of Mean±SE (n=3)

4.3 Fly ash based blue green algal inoculant formulation and their evaluation on growth and yield of rice

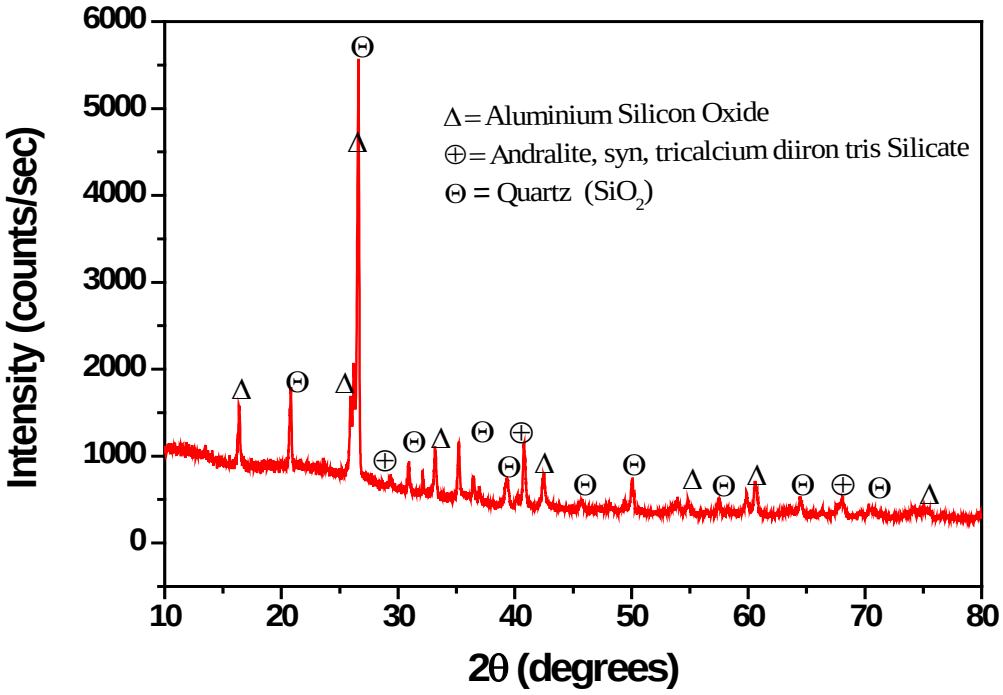
For development of fly ash based blue green algal inoculants using fly ash as carrier material, mineralogical and physico-chemical properties of fly ash were studied and compared with that of soil, charcoal and montmorillonite. Coal fly ash, montmorillonite, charcoal and soil was analysed for its mineralogical content and Thermal stability by X-ray diffraction (XRD), Thermal gravimetric analysis (TGA), fourier-transform infrared spectroscopy (FTIR) and physicochemical properties. Further, nursery trial was conducted for paddy crop with different treatment of isolated nitrogen fixing filamentous cyanobacterial and carrier materials viz. soil, montmorillonite and fly ash to observe their effect on growth and nutrient status of soil.

Selected blue green algae were grown on mass scale and the process for fly ash based blue green inoculants was developed. Fly ash based BGA inoculants were put on 20 acres of field trials in different villages along with control and compared with urea treatment, to develop a strategy for large scale usage of fly ash based blue green algal inoculants in farmer's paddy field and to see its impact on crop yield.

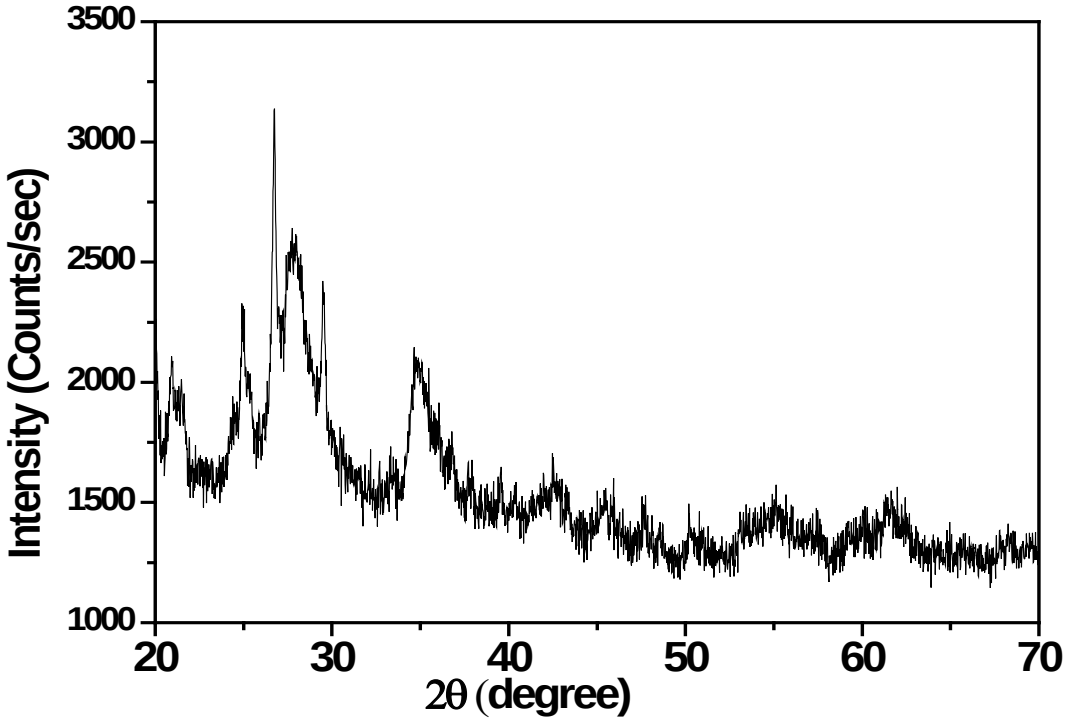
4.3.1 Physical and mineralogical characterization of coal fly ash, montmorillonite, charcoal, and soil

4.3.1.1 ***Mineralogical composition:*** X- ray diffraction pattern showed that major crystalline phases in coal fly ash were of quartz (SiO_2), mullite ($Al_{4.56}Si_{1.44}O_{9.72}$) and hematite ($Fe_2O_3$) and minor constituents comprised of oxides of magnesium, potassium and calcium (Figure 6.1a; Table 5.1.1a). Mineralogical study confirmed that coal fly ash contains basic mineral eLement (N, P, K, Fe, S, Ca) (Table 5.1.1b) in readily available ionic form, which can Lead to their increased uptake by plants. Major matrix elements of coal fly ash were found to be AL and Si, together with significant percentage of K, Fe, S, Ca, and Mg. Lee et al. (1999) reported that major crystalline phases in XRD pattern of fly ash are of quartz and mullite. Agricultural utilization of coal fly ash has been proposed because of its considerable mineral content of macronutrient (K, Ca, Mg, S, P) and secondary source of micronutrients (Cu, Fe, Mn, Zn etc) for soil deficient in nutrients (Sikka and Kansal, 1995; Kalra *et al*., 1997; Adriano and Weiber, 2001; Jala and Goyal, 2006; Skousen *et al*., 2013). XRD pattern of wood charcoal showed the crystalline structure of cellulose and graphite with different forms of amorphous carbon and no clear diffraction peaks were detected due to the decomposition of cellulose structure at high temperature (Figure 6.1b). XRD pattern of wood charcoal shows the crystalline phase of graphite, diamond and fullerene along with some amorphous materials (Zhao, 1997). XRD pattern of soil showed the presence of kaolin, quartz and feldspate (Figure 6.1c). Quartz in the soil has primary (volcanic) origin and is most frequent in acidic soil (Nenadovic *et al*., 2010), whereas XRD of montmorillonite showed the presence of three crystallographic phases of calcium–magnesium montmorillonite $Ca_{0.2}(Al.Mg)_2Si_4O_{10}(OH)_2.4H_2O$, magnesium montmorillonite ($MgO.Al_2O_3.5SiO_2.xH_2O$) and quartz (SiO_2) (Figure 6.1d). Concentration of heavy metals in coal fly ash was found to be low (As, Cd, Se, Pb, Ni and Co), which is supported by the earlier findings that the heavy metal (As, Cd, Se etc.) present in coal fly ash do not leach into groundwater, when applied to

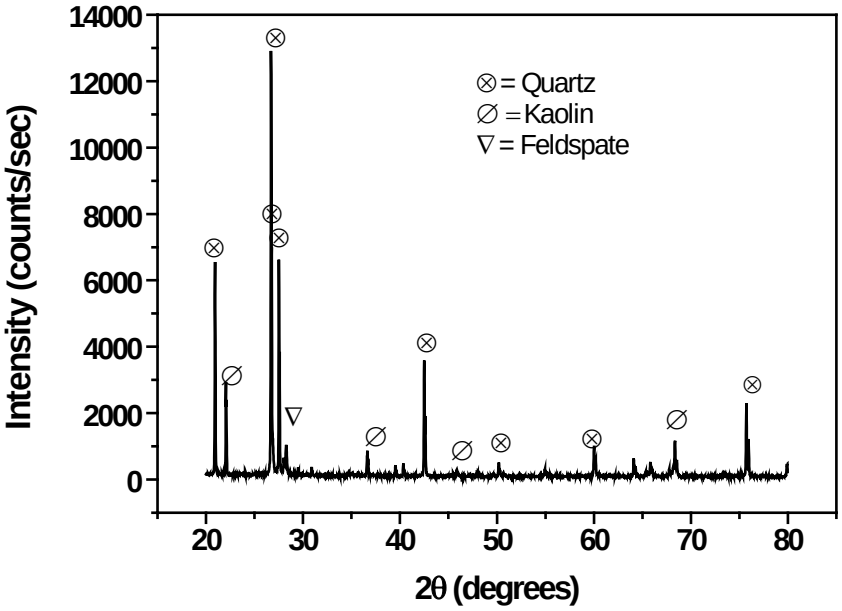
soils and do not leach from products that contain coal fly ash (Sushil and Batra, 2006; Seshadri *et al*., 2011; Skousen *et al*., 2013). The heavy metals can limit the survival and growth of plants and microbial population but in general the heavy metal concentrations of Indian coal ash are reported low as compared to ash from other parts of world, unlikely to affect ground water quality (Sushil and Batra, 2006). Coal fly ash used in fixed quantities represents a good carrier material and will not cause leaching problem.



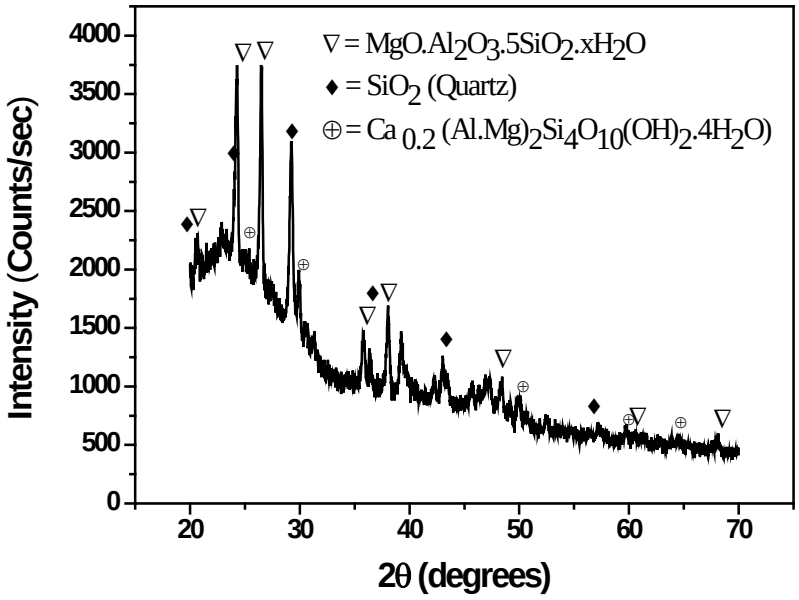
a) Fly ash



b) Charcoal



c) Soil



d) Montmorillonite (MMT)

Figure 6.1 X-Ray Diffraction of fly ash, charcoal, soil and montmorillonite

XRD of fly ash (a) shows the presence of quartz (SiO_2), mullite ($Al_{4.56}Si_{1.44}O_{9.72}$), haematite and small amount of calcite and no magnetite. Soil (c) and montmorillonite (d) mainly consists of kaolin, quartz and feldspar, whereas charcoal (b) shows crystalline phase of amorphous carbon and graphite.

Table 5.1.1 (a) X-Ray Diffraction (XRD)

Chemical composition of fly ash, charcoal, soil and montmorillonite as determined by XRD analysis. All values are reported as weight percentage.

Compound & Concentration (%)	**Fly ash**	**Charcoal**	**Soil**	**Montmorillonite**
SiO_2	54	28	65	68
AL_2O_3	40	0.35	20	21
MgO	0.95	0.67	13	6
CaO	1.2	0.81	0.97	---
FeO	3.8	0.19	1	5
CO_2	0.30	70	---	---

Table 5.1.1b: Total elemental concentration of coal fly ash (ppm)

Element	**Concentration (ppm)**
Fe	1581±0.01
Zn	59.54±0.05
Mn	28.00±0.01
Pb	18.00±0.02
Co	03.96±0.50
Cr	05.32±0.10
Ni	21.4±0.01
Cu	17.09±0.01
S	1901±0.06
Mo	41±0.15
Se	03.8±0.25
As	06.1±0.10
Cd	BDL

***BDL: below detection Limit**

4.3.1.2 ***Differential Thermal and Thermal Gravimetric Analysis:*** Thermal study was carried out to observe thermal decomposition of humic substances and organic matter in fly ash, charcoal, montmorillonite and soil. Among all material fly ash showed stable thermal effect from 100-500 ºC. Weight loss observed upto 200°C was primarily due to loss of water and carbon that was formed during the hydration process as confirmed by XRD analysis (Figure 6.2a). The endothermic peak at 448°C associated with the weight loss between 400 and 500°C is caused by water and carbon decarboxylation. Finally the third endothermic peak at 647°C is due to the loss of CO_2 from calcium carbonate. When fly ash is in contact with water, limited amount of calcium and aluminium ions are released in the solution. For this reason, the hydration of mixtures with fly ash is severely retarded at early ages until additional activators such as alkali, calcium hydroxides, or sulphates are present in the medium (Kuceba, 2004).The TGA plot of charcoal showed total weight loss during pyrolysis is more than 95%. Our results are supported by the findings of Cohen and his co-workers (2006) that the TGA plot showed total weight loss during pyrolysis of charcoal above 95%. The DTA plot showed that the major phase change occurs at 500 °C (Figure 6.2b). The TGA curve of charcoal goes back to low temperature side at about 480 °C and this decrease in the temperature can be due to endothermic reactions. Soil and montmorillonite showed similar decomposition. TG profile of soil and montmorillonite indicates a weight loss between 300 and 550ºC which is due to water, an exothermic energy released was observed at 350ºC followed by endothermic peak, showing transition with gain of energy at 550 ºC (Figure 6.2 c and d). The results led us to conclude that coal fly ash has more thermal stability as compared to other materials followed by well stabilised organic matter with high degree of humification. Presumably, during the organic matter transformation process, the formation of humic-like substances enabled the formation of complexes characterised by a strong thermal stability (http://natres.psu.ac.th/Link/SoilCongress/bdd/symp40/681-t.pdf).

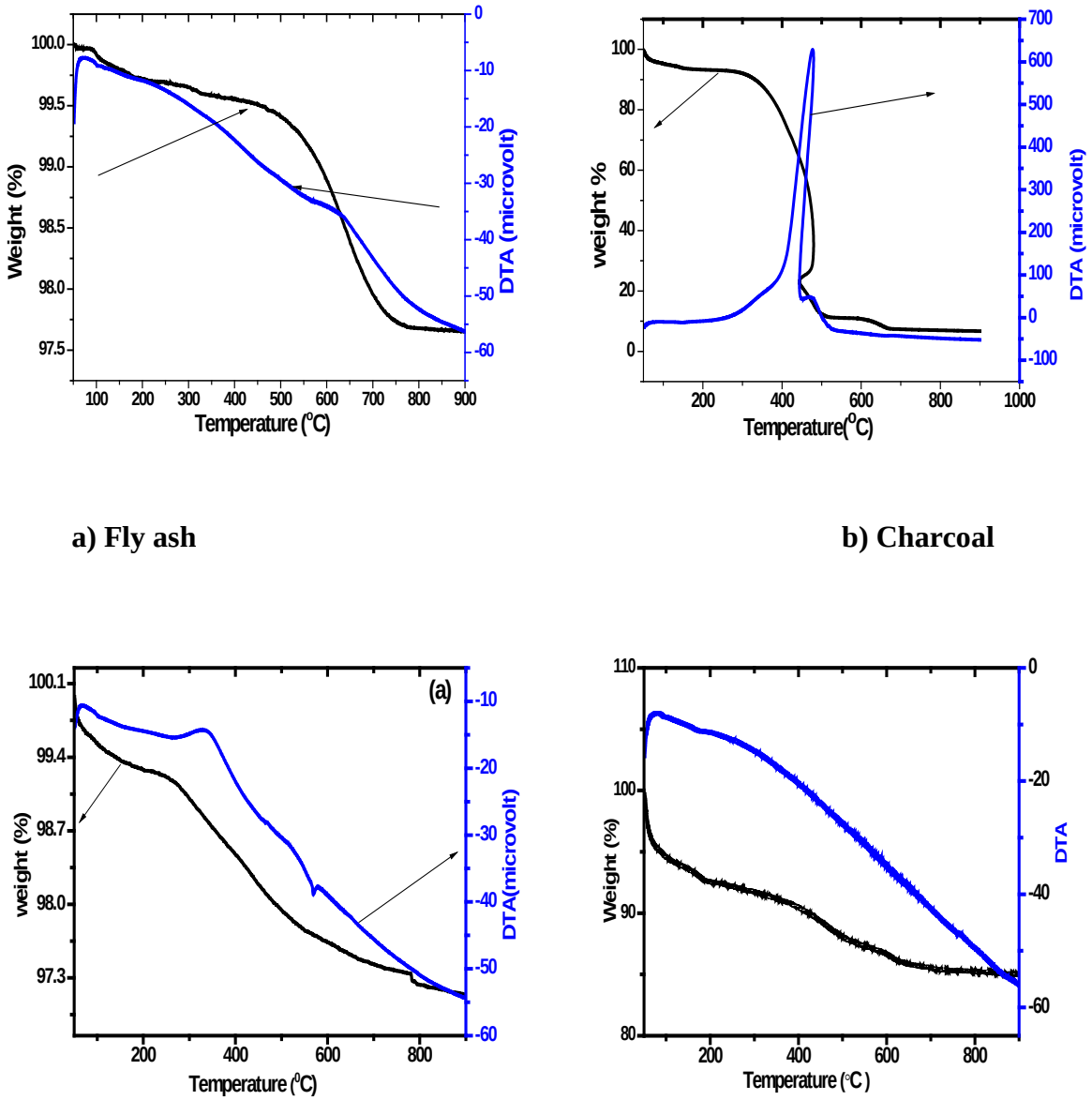
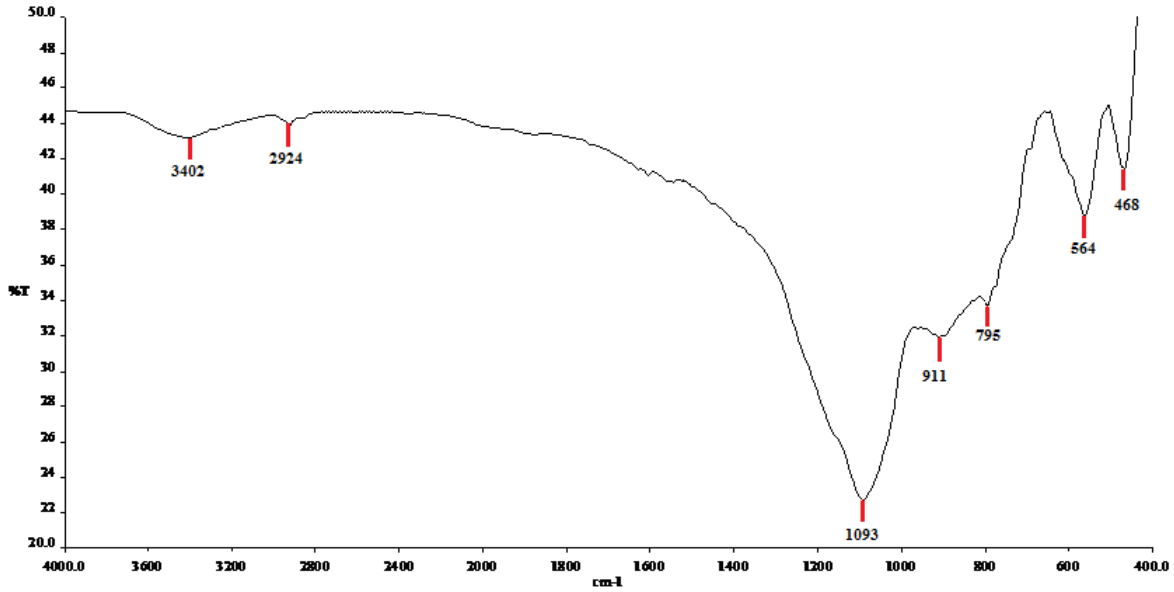


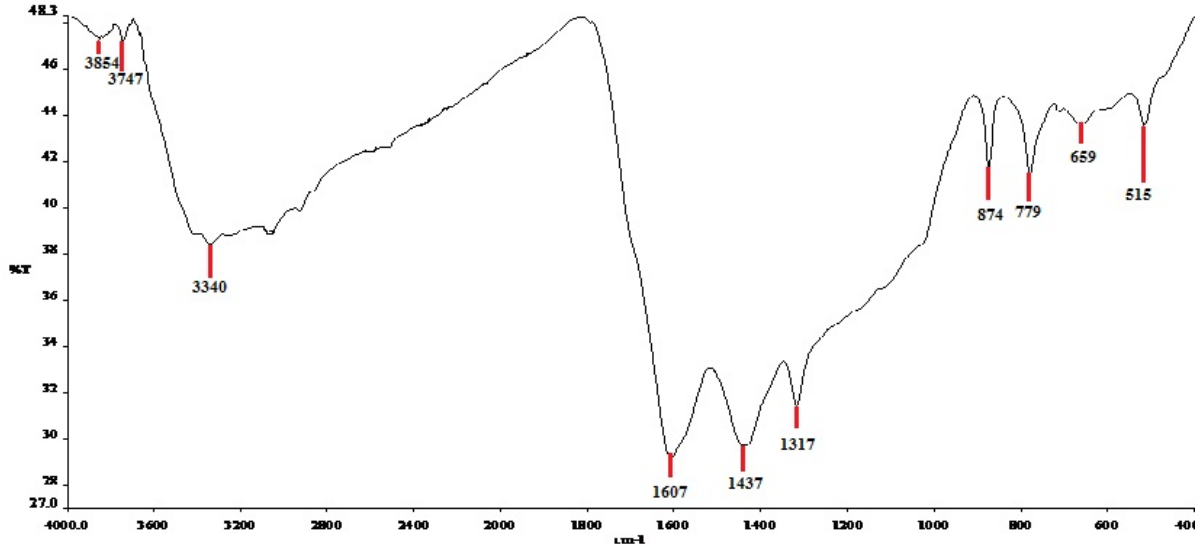
Figure 6.2 ***Differential Thermal and Thermal Gravimetric Analysis*** **TGA/DTA** curves of (a) Fly ash (a) shows stable thermal effect from 100-500ºC whereas in charcoal (b) total weight loss observed during pyrolysis was more than 95%. A similar trend of decomposition of weight loss was observed in soil(c) and montmorillonite (d) between 300 and 550 ºC.

4.3.1.3 ***Fourier-transform infrared spectroscopy (FTIR):*** FTIR is an alternate approach to determine organic matter and inorganic portion of soil (Cox, 2000). FTIR spectra were helpful in detecting fingerprints of minerals attributed to Si-O-Si stretching and C-O stretching vibrations in coal flyash and other materials. FTIR of fly ash showed that the most prominent peaks in the spectra originate from Si-O and Al-O stretch vibrations. Band near 1090-1200 cm^{-1} is due to Si-O stretch vibrations. Zone corresponding to Si–O and Al–O vibrations in original fly ash is 1080-1090 cm^{-1} (Figure 6.3a and Table 5.2.2). This shift was due to penetration into original structure Si–O–Si as it was observed in zeolites of fly ash by Skvara et al. (2006). Generally, the main component of coal fly ash is amorphous aluminosilicate, quartz (SiO2) and mullite ($3Al_2O_3.2SiO_2$) existing as a crystalline substance in coal fly ash and this amorphous materials of Si and Al can be changed into zeolite crystals by hydrothermal treatment. XRD data did not show zeolites peak as zeolite concentration was low (less than 1 % by wt). Fly ash amended soil show its natural zeolitic characteristics that helps in soil improvement by maintaining soil moisture and fertilizer component as NH_4^+ and K^+ (Querol *et al*., 2002; Murayama *et al*., 2003). The usage of the synthesized zeolites as soil improvement makes a contribution to return burnt coal residue to soil in an environment friendly manner (Murayama *et al*., 2003). Infrared spectra of charcoal showed that the carbon double bonds and aromatic rings were formed at carbonization temperature of 600°C. Wood charcoal carbonized at 1600°C was partly graphitized, a finding supported by the results of X-ray diffraction (Figure 6.3b and Table 5.2.2). The band at 3340 cm^{-1} was due to the absorption of water molecules as result of an O-H stretching mode. The O-H mode at 3300 cm^{-1} decreased with an increasing carbonization temperature while the band at 2890 is attributed to C-H interaction with the surface of the carbon. It is reported that in case of the original wood aromatic absorption originating from lignin was detected at about 1600 -1510 cm^{-1} (Hata *et al*., 1998). These aromatic modes are found in charcoal but moved to a lower

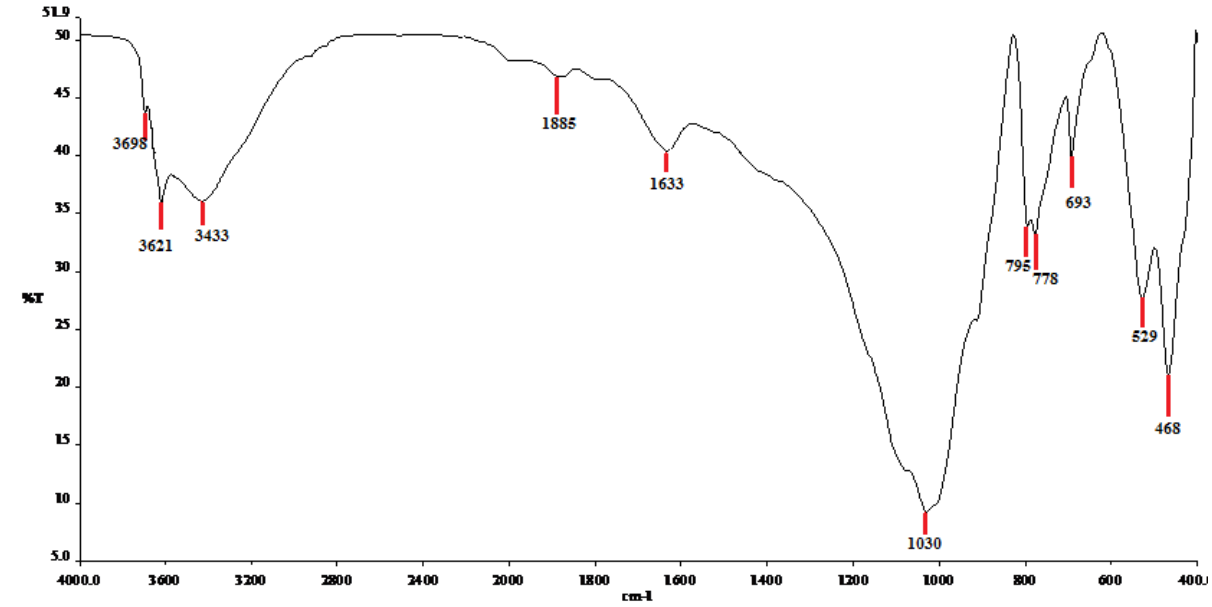
wave number with the increase in carbonization temperature. FTIR analysis of soil showed absorption bands at 3695, 3545, 1700 cm^{-1} (Figure 6.3c and Table 5.2.2) which were characteristic to soil humic compounds followed by the sharp band at 1031 cm^{-1} which can be assigned to the Si–O stretch (Cox *et al*., 2000; Choe *et al*., 2010). In the range of 900–1180cm^{-1}, spectra of soil include fingerprints of minerals, attributed to Si–O–Si stretching and C–O stretching vibrations, as interpreted by a FTIR spectral library (Cohen et al., 2006). FTIR spectra of montmorillonite was characterised by typical bands responsible for stretching vibrations of O–H near 3440–3620 cm^{-1} and Si–O (1100–1035 cm^{-1}) bonds (Figure 6.3d and Table 5.2.2). The bands between170-710 cm^{-1} was attributed to the SiO_2 andAl_2O_3 lattice and bands near 840 and 1030 were due to bending vibrations of the structural OH bound to octahedral cations (Bishop, 2004). Analysis of FTIR –spectra of montmorillonite indicates typical bands responsible for stretching vibrations of O-H (υ = 3440 -3620 cm^{-1}) and Si–O (υ=1113-1035cm^{-1}) bonds and bending vibrations of Al Mg OH (υ =830–840 cm^{-1}) and Al Al OH (υ = 915–925 cm^{-1}) bonds (Paluszkiewicz *et al*., 2008; Paluszkiewicz *et al*., 2011). Characteristic bending vibrations of Si–O bonds at $\upsilon 1$ = 692 cm^{-1} and $\upsilon 2$ = 529 cm^{-1} (Tyagi *et al*., 2006; Patel *et al.*, 2007; Paluszkiewicz *et al*., 2011) were also observed.



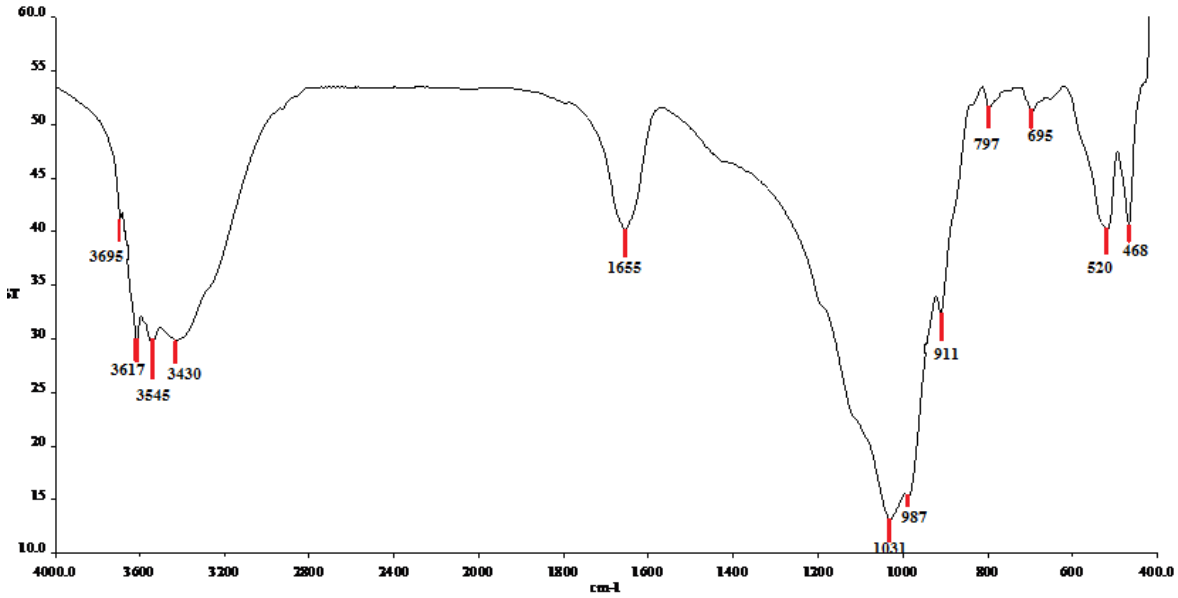
a) Flyash



b) Charcoal



c) Soil



d) Montmorillonite

Figure 6.3: Fourier-transform infrared spectroscopy (FTIR) - Si–O and Al–O vibrations in original fly ash (a) is 1080-1090 cm^{-1} and in charcoal (b), bands stretching at 3340 cm^{-1} was observed to be O-H stretching mode and charcoal carbonized at 1600°C was partly graphitized. Soil (c) exhibits absorption bands at 3695, 3545, 1700 cm^{-1} whereas montmorillonite(d) shows typical bands responsible for stretching vibrations of O–H near 3440–3620 cm−1 and Si–O (1100–1035 cm−1) band.

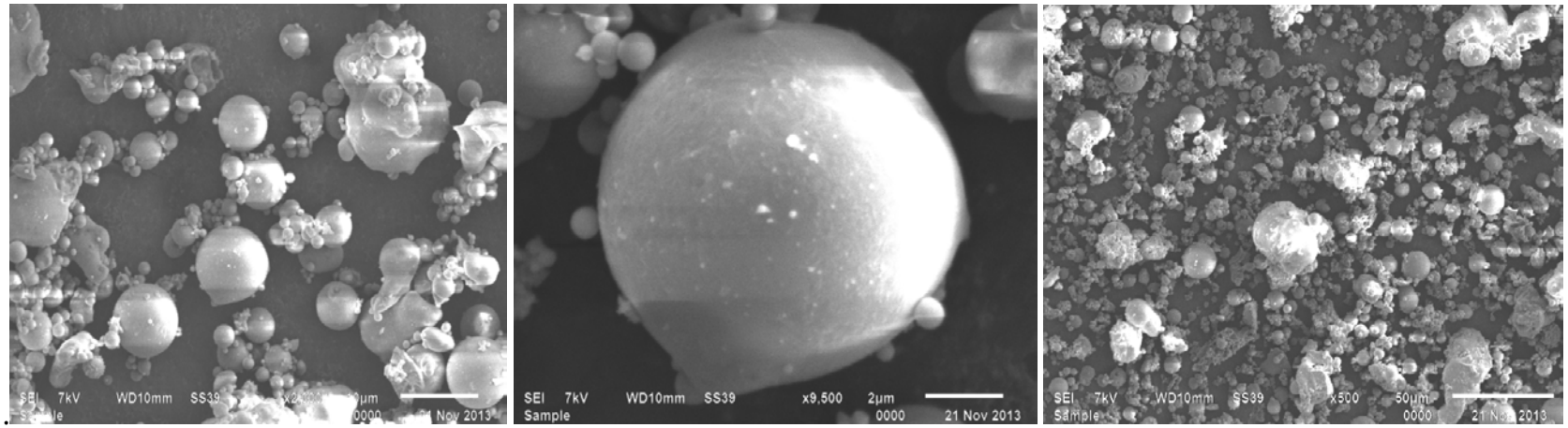
Table 5.2.2 Fourier-transform infrared spectroscopy (FTIR)

Band assignments of peak components as observed in flyash, charcoal, soil and montmorillonite**.**

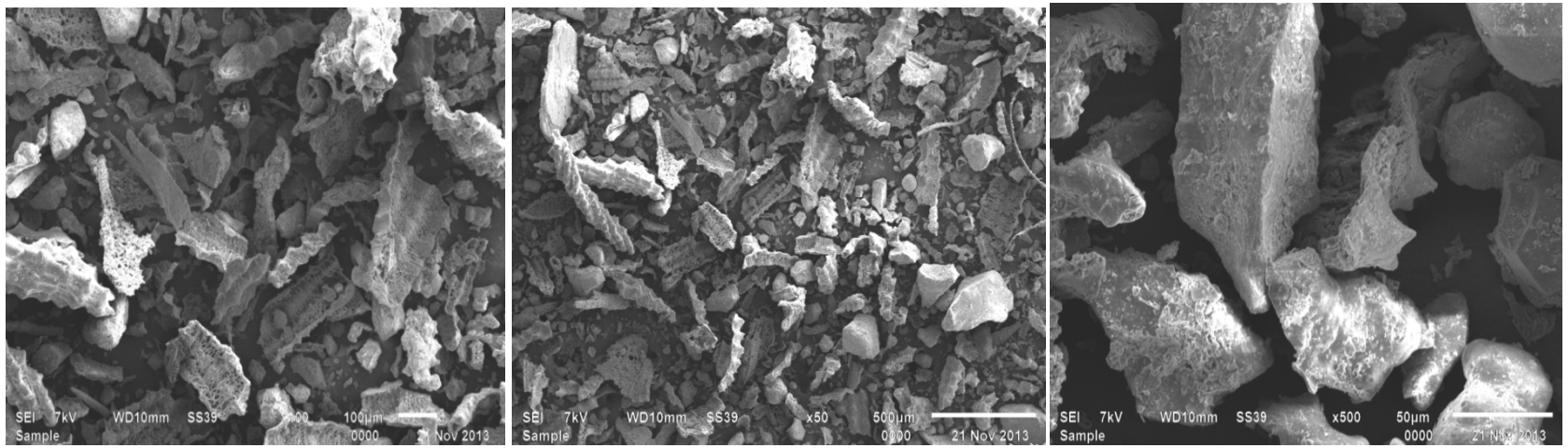
Wavenumber range (cm^{-1})	**Band assignment**			
	Flyash	**Charcoal**	**Soil**	**Montmorillonite**
450-520	Si-O	Graphite, C-OH, C=C	Si-O-Si	Quartz /Structural Lattice modes
550-1100	Si-O, Al-O	Aromatic mode	Si-O-Si	Si-O, AlMgOH, $(Al)_2OH$
1120-1600	---	Aromatic mode	C-O, O-H	Si-O, H-O-H bending of structural water of Montmorillonite
1600-1700	---	C=C, C=O	O-H	---
2900–3400	C=O, Si-O-Si, O-H	O=H stretching	O-H	---
3440–3620	---	O=H stretching	O-H	O-H

4.3.1.4 ***Scanning electron micrograph (SEM) & Energy dispersive spectrometry (EDS):*** Morphology study using SEM shows that fly ash has a smooth spherical surfaces i.e cenospheres and plenospheres having more surface area for interaction (Figure 6.4a). Flyash particles are hollow, empty spheres (cenospheres) filled with smaller amorphous particles and crystals (plerospheres) (Jala and Goyal, 2006). SEM micrograph showed dense microstructure having particle size with equivalent diameter ranging from 2 to 50μm. EDS of surface of coal fly ash supported the XRD data with high mineralogy content of fly ash rich in Fe, C, Ca, SiO_2, Al, K with few traces of Zn and Cu (Figure 7.1a; Table 5.3.1). Coal fly ash mainly comprised of amorphous alumino-silicate spheres, a smaller amount of iron-rich spheres and the majority of the iron-rich spheres had two components: iron oxide and amorphous alumino-silicate, calcium, the fourth most abundant element found in the coal fly ash, was associated with oxygen, sulfur or phosphorous (Barbara *et al*., 2006). SEM micrographs of charcoal showed a packed fibrous structure having multiple pores showing uniform wood parenchyma in the charcoal and particle size ranging from 50-100μm (Figure

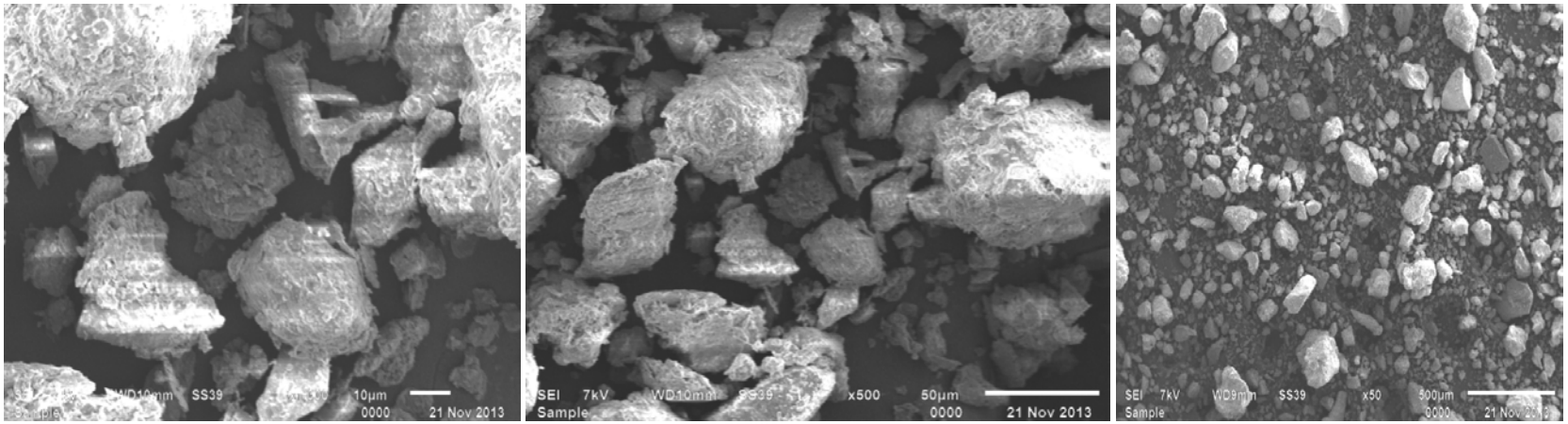
6.4b). Microstructure of soil confirms the broad distribution of particles ranging from few micrometers to hundreds of micrometer consisting of Kaolin and quartz particles. SEM micrograph with higher magnification confirmed that soil particles were porous in structure and cracked at various stages which are responsible for many soil properties. Aggregate structure observed comprised of iron, magnesium and potassium in small quantities was clearly visible both in case of soil and montmorillonite as observed in EDS graphs (Table 5.3.1; Figure 7.1 c and d). SEM micrographs of soil and montmorillonite supported the particles have size in the range of 10 to 500 μm and clearly defined porosity similar to clay (Nenadovic *et al*., 2010). In case of montmorillonite also, SEM studies indicated that agglomerates of the MMT particles showed a plate like morphology characteristic of layered silicates and EDS analysis also supports the 60% presence of silica (Figure 7.1 c and d; Table 5.3.1). SEM and EDS results concluded that in comparison to charcoal, montmorillonite and soil, coal fly ash samples contains essential macronutrients including P, K, Ca, Mg and S and micronutrients like Fe, Mn, Zn, Cu, Co that are beneficial in crop yield of many agricultural crops (Basu *et al*., 2009) .



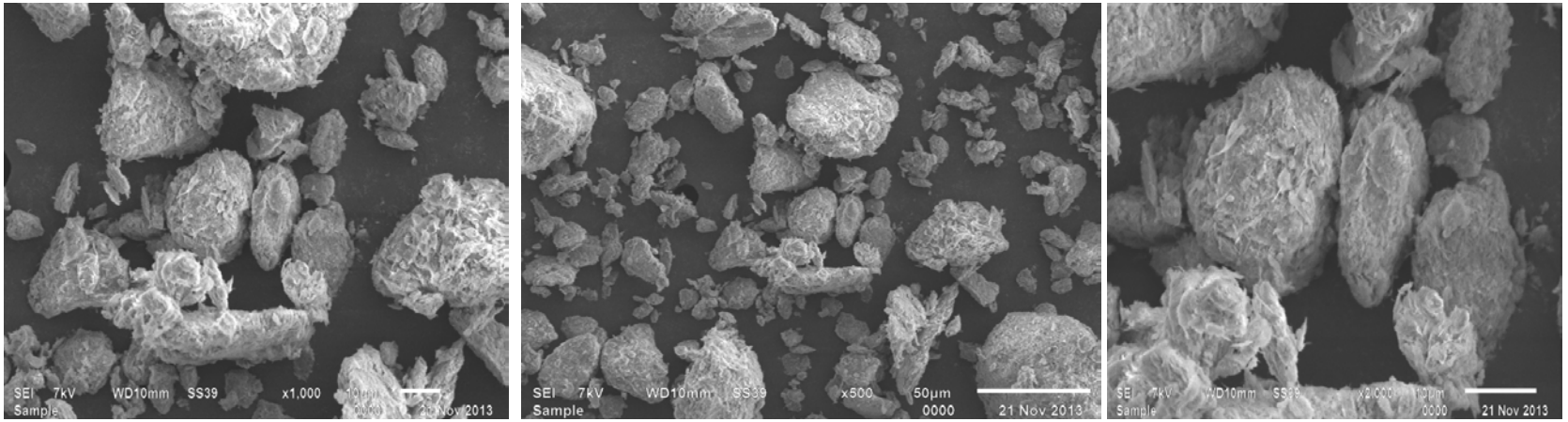
a) Fly ash



b) Charcoal

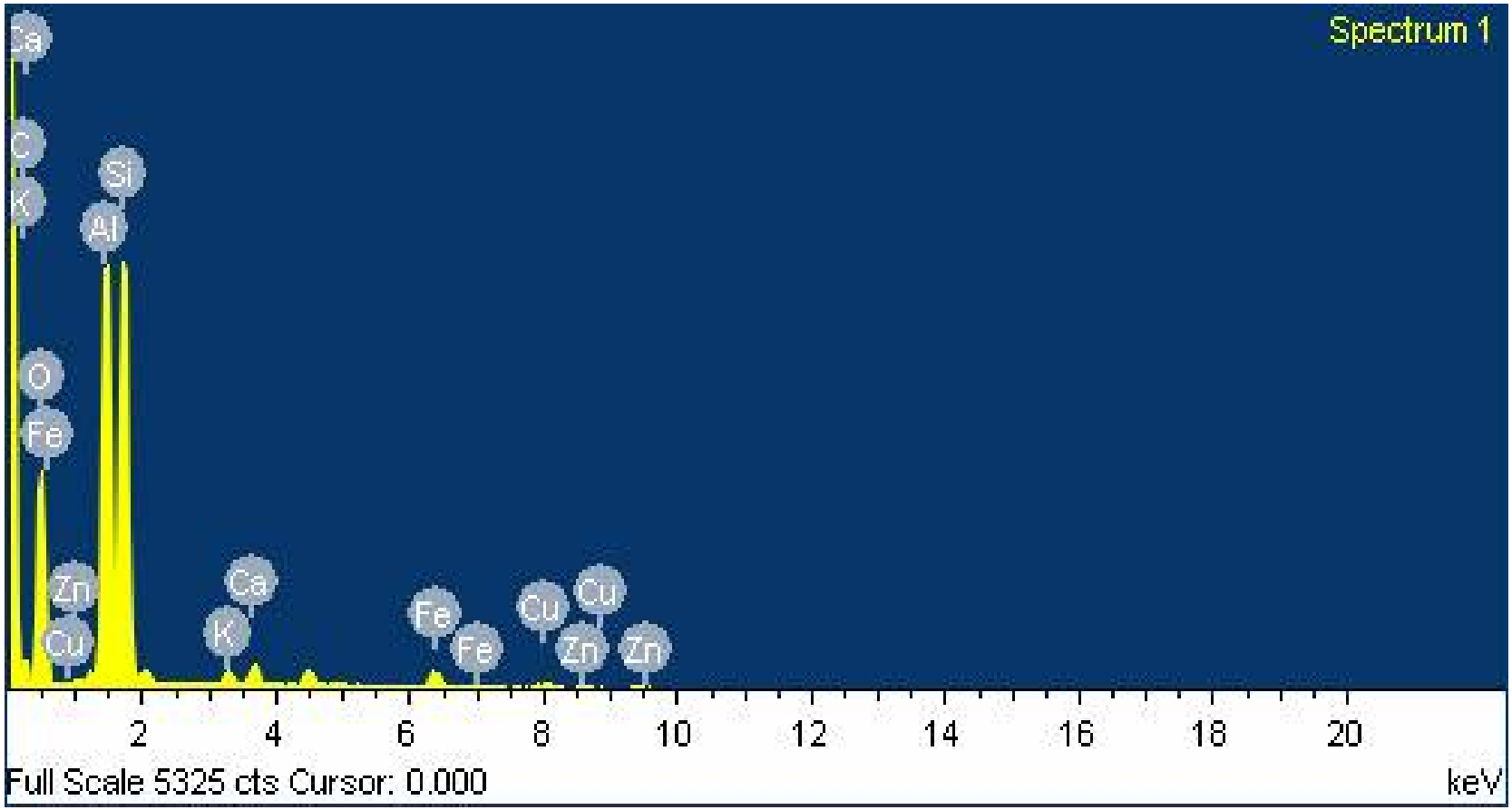


c) Soil

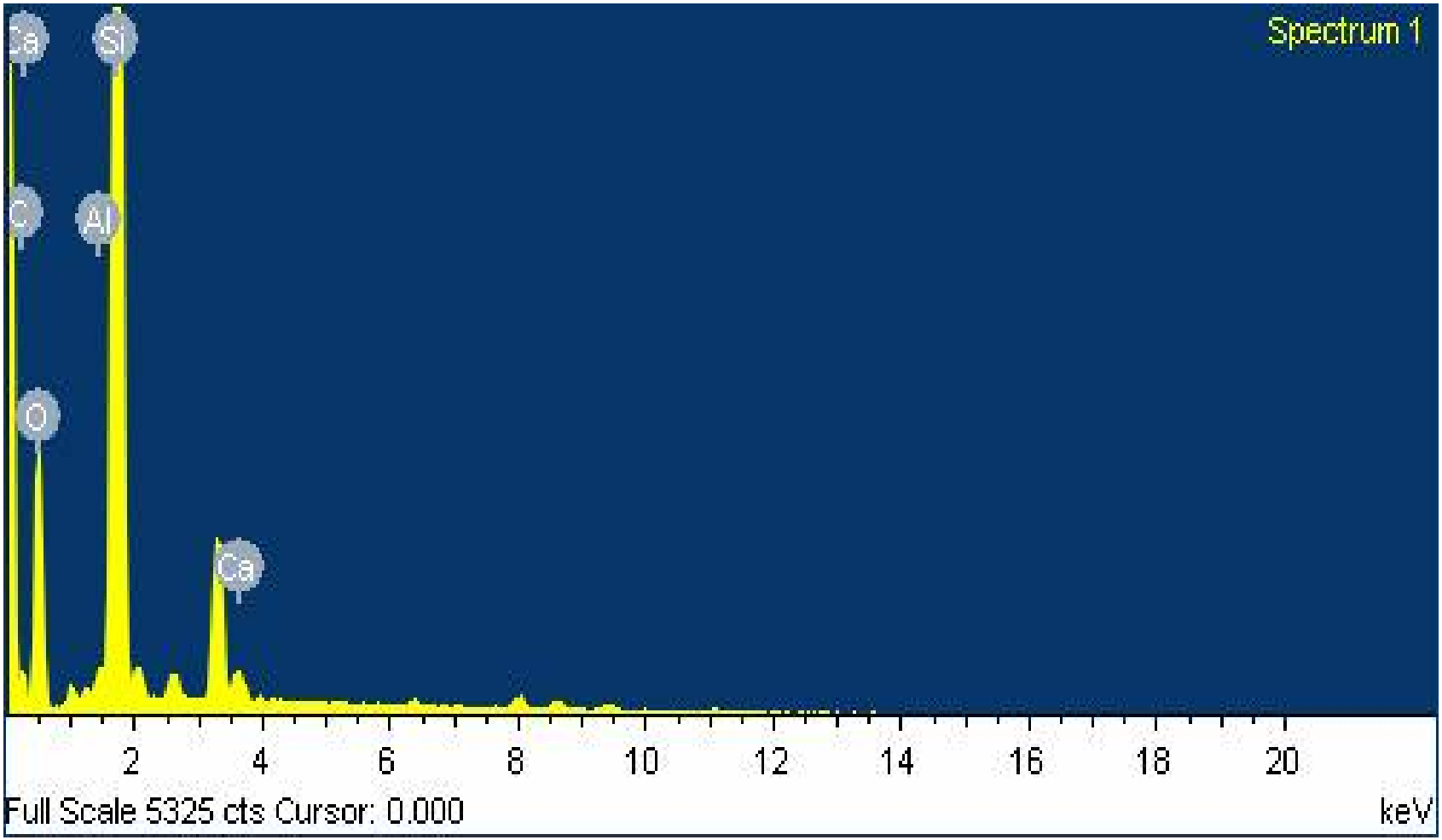


d) Montmorillonite

Figure 6.4 Scanning electron micrograghs (SEM): Fly ash (a) showed smooth spheres of cenospheres and plenospheres having more surface area for interaction having particle size from 2µm-50 µm. Charcoal(b) showed a fibrous structure of parenchyma cells of wood and particle size ranging from 50-100 µm. Soil(c) and montmorillonite(d) showed agglometric structures and particle size ranging from 10-500µm.


Spectrum 1
Ca
C
K
Si
Al
O
Fe
Zn
Cu
Ca
K
Fe
Fe
Cu
Cu
Zn
Zn
2
4
6
8
10
12
14
16
18
20
Full Scale 5325 cts Cursor: 0.000
keV

a) Flyash


Spectrum 1
Si
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Full Scale 5325 cts Cursor: 0.000
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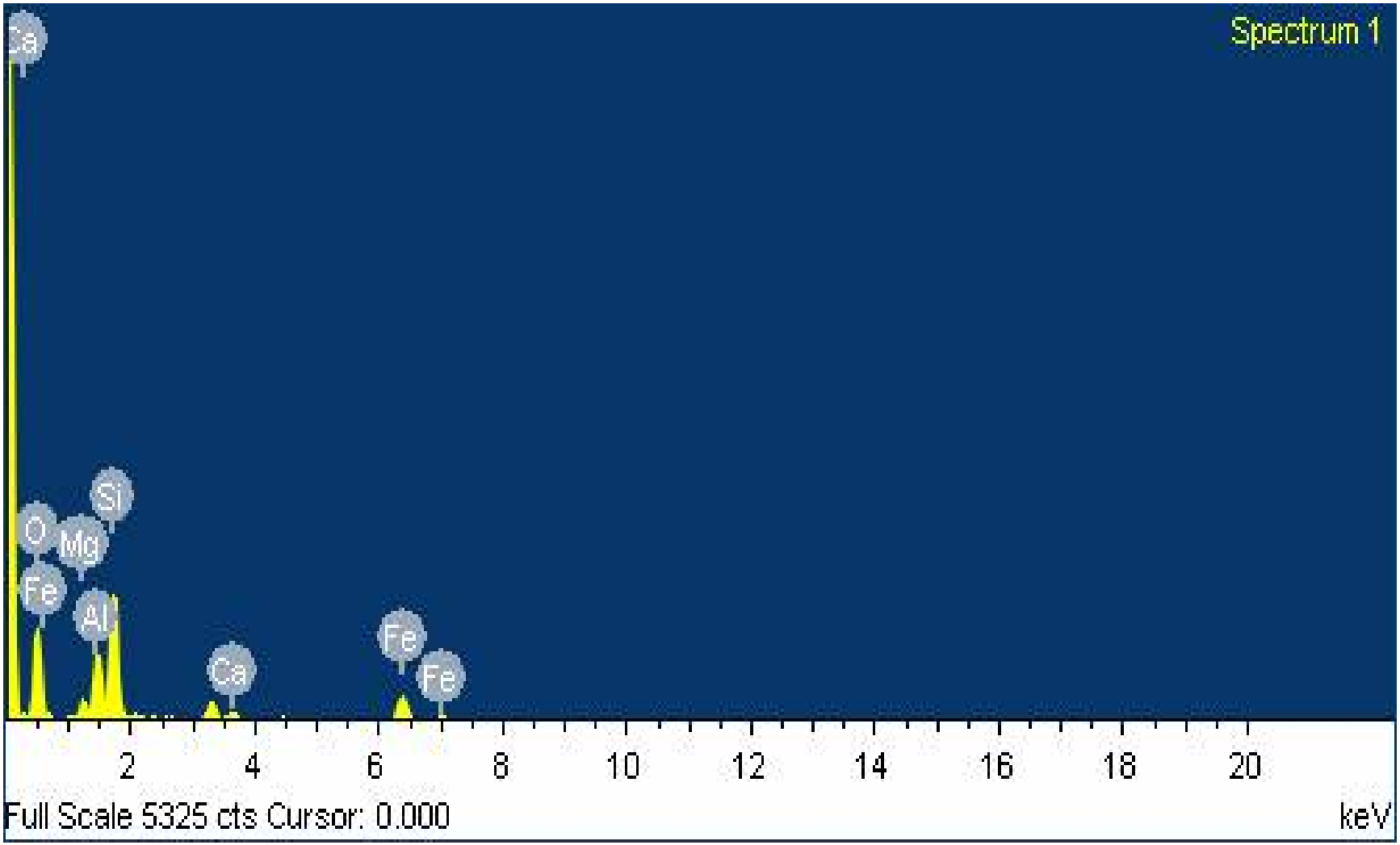
b) Charcoal


Spectrum 1
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Full Scale 5325 cts Cursor: 0.000
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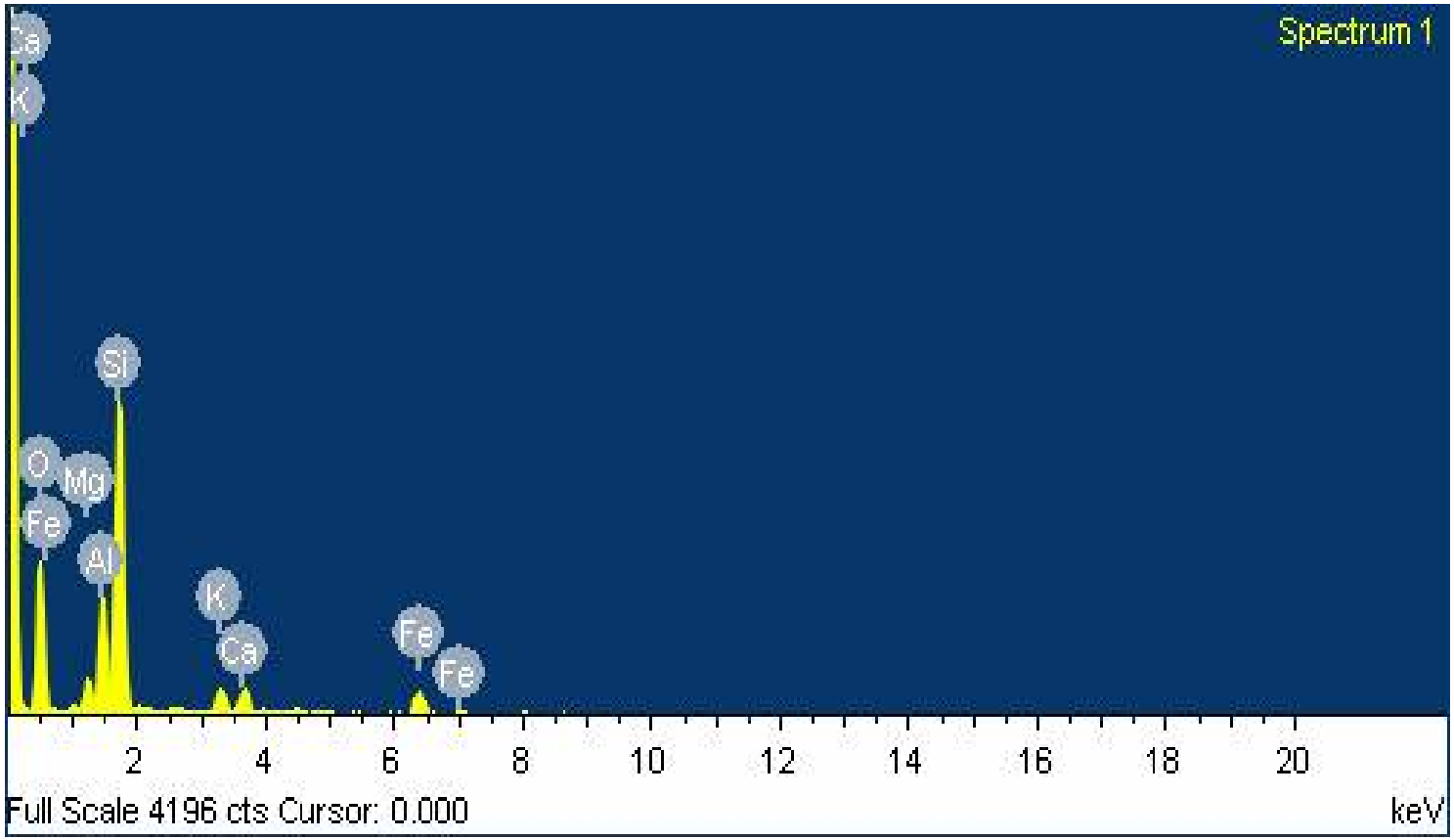
c) Soil



d) Montmorillonite

Figure 7.1 Energy dispersive spectrometry (EDS): Fly ash (a) showed the presence of aluminosilicate, calcium, iron, potassium, zinc and copper. Charcoal (b) showed the presence of carbon with some traces of calcium and aluminium. Soil (c) and MMT (d) showed the maximum presence of silica followed by aluminium, magnesium and calcium.

Table 5.3.1 Energy dispersive spectrometry (EDS**)** observed in flyash, charcoal, soil and montmorillonite

Element	**Weight (%)**	**Atomic wt (%)**	**Compd (%)**	**Formula**
Flyash				
C K	11.20	16.2	40.9	CO_2
Al K	00.40	00.2	00.7	Al_2O_3
Si K	26.90	16.8	37.7	SiO_2
Ca K	00.60	00.3	00.8	CaO
O	60.90	66.5	---	---
Total	100.00			
Charcoal				
C K	11.2	16.2	40.9	CO_2
Al K	00.4	00.2	00.7	Al_2O_3
Si K	26.9	16.8	37.7	SiO_2
Ca K	00.6	00.3	00.8	CaO
O	60.9	66.5	---	---
Total	**100.00**			
Soil				
Mg K	03.7	03.4	06.2	MgO
Al K	11.3	09.3	21.4	Al_2O_3
Si K	24.2	19.0	51.8	SiO_2
Ca K	01.5	00.8	02.1	CaO
Fe K	14.5	05.7	18.6	FeO
O	44.8	61.8	---	---
Total	**100.00**			
Montmorillonite				
Mg K	03.0	02.7	05.0	MgO
Al K	09.5	07.6	18.0	Al_2O_3
Si K	28.3	21.7	60.6	SiO_2
K K	02.9	01.6	03.5	K_2O
Ca K	02.7	01.4	03.7	CaO
Fe K	07.1	02.7	09.1	FeO
O	46.5	62.3	---	---
Totals	**100.00**			

4.3.1.5 ***Coarse grain accumulation:*** Particle size distribution (Table 5.3.2) of fly ash indicates presence of fine grained particles with more than about 70% of the particles finer than 0.075mm. Silt size particles in coal fly ash may often be substituted for topsoil in surface mine lands, thereby enhancing physical conditions of soil especially water holding capacity (Basu *et al*., 2009; Jala and Goyal, 2006; Skousen *et al*., 2013). Soil has substantial quantity of fines which is 51% followed by montmorillonite (47%) and charcoal (27%). 40-50% of the particles are finer than 0.025 mm in fly ash, whereas in soil it is 45%. Charcoal and montmorillonite has 16% and 28% of 0.025 mm fines. It is reported that the number of particle size classes measured by sieving in soil could be reduced to three, <0.05, 0.05–0.2, and 0.2–2 mm, which enabled 87.9% of the soil samples to be discriminated (Sugita and Maumo, 2001) and fly ash particles which are typically spherical and glassy with particle diameter ranging from 1 to 150μm (Yilamz, 2012). The extensive investigation carried out on Indian coal ashes demonstrates that the fly ashes consist predominantly of silt-size fraction with some clay-size fraction and based on the grain-size distribution, the coal ashes can be classified as sandy silt to silty sand (Jala and Goyal, 2006).

Table 5.3.2 Coarse grain accumulation

S.No	Particle size (mm)	Fly ash	Charcoal	Montmorillonite	Soil
1.	4	100	96±0.12	94±0.18	100
2.	2	100	90±0.09	88±0.14	100
3.	1	96±0.01	83±0.01	82±0.05	98±0.01
4.	0.500	87±0.03	70±0.06	77±0.07	88±0.02
5.	0.300	81±0.01	61±0.01	60±0.11	82±0.01
6.	0.150	76±0.05	43±0.01	52±0.07	68±0.01
7.	0.075	70±0.01	27±0.02	47±0.04	51±0.02
8.	0.025	51±0.41	16±0.04	28±0.01	47±0.01
9.	0.010	42±0.01	10±0.02	18±0.01	38±0.05

(n=3,Mean± SE)

4.3.1.*6 Physical characterisation*: Coal fly ash to be used in agriculture and for microbial formulation depends upon its acidic or alkaline nature (Table 5.4.1). pH of all powdered material was found to be alkaline ranging from 7.85±0.12 in coal fly ash to 8.22±0.05 as observed in soil. Montmorillonite and charcoal was alkaline having pH 8.27±0.1 and 8.19±0.02 respectively. The pH of fly ash generally varies from 4.5-12, however worldwide coal flyash are alkaline including that from India (Ram and Masto, 2009; Ram *et al*., 2011). Electrical Conductivity (EC) (µS/cm) of fly ash was 0.14±0.02 whereas of charcoal it was 2.87±0.01. Addition of coal fly ash to soil improves physico-chemical properties of soil such as pH, EC, porosity, water holding capacity, root penetration and fertility leading to increased biomass production (Khan and Khan, 1996; Singh *et al*., 1997; Singh *et al*., 2011). Coal flyash was effectively used for reclamation stabilization and was helpful in neutralization of acidic mine spoils and restoration of nutrient balance in alkaline wastelands (Jala and Goyal, 2006). Soil and montmorillonite showed EC ranging from 2.45±3.75 to 15.5±25.8 respectively (Table 5.4.1). Bulk density (g/cm^3) of fly ash and soil was 0.99 and 0.43 whereas of charcoal and soil it was found to be 2.05 and 1.36 respectively. Water holding capacity was maximum in charcoal (198%) followed by montmorillonite (84.3%), fly ash (62%) and soil (39.9%). Indian fly ash has a vast potential for use in agriculture as an amendment especially due to its alkaline pH, low bulk density and high water holding capacity, which are conducive for plant growth (Ram and Masto, 2009; Ram *et al*., 2011). Surface area (m^2/g) was observed maximum in montmorillonite (79.3) followed by soil (5.45), charcoal (4.90) and fly ash (0.96). Fly ash addition generally decreased the bulk density of soils, which in turn improved soil porosity and workability and enhanced water retention capacity (Page *et al*., 1979; Jala and Goyal, 2006). Montmorillonite showed high specific surface area of 79.3 m^2/g by BET method that is due to its two layered particles with a thickness of 4 to 10 unit layers (Macht *et al*., 2010) and the bulk density for montmorillonite ranges between 2 to 2.7

g/cm^3 (Chitale and Sigal, 2000). Coal fly ash is abundant in India as compared to charcoal and montmorillonite but the percentage of utilization of coal fly ash in India is only 38% and rest of this were dumped into basin or landfill near power plants which is not environmentally safe and hence the utilization percentage of coal fly ash can be increased in agriculture sector demonstrating its positive benefits for improving soil properties by buffering soil, increasing moisture content, improved soil porosity and increasing crop yield (Ram and Masto, 2010; Ram *et al*., 2011; Jala and Goyal, 2006).

Table 5.4.1. Physiochemical characterisation of flyash, charcoal, soil and montmorillonite.

S.No	Parameters	Fly ash	Charcoal	Soil	Montmorillonite
1.	pH	7.85±0.12	8.19±0.02	8.22±0.10	8.27±0.10
2.	EC (µS/m)	0.14±0.01	2.87±0.01	2.45±0.05	15.5±0.02
3.	Bulk density(g/cm^3)	0.99±0.01	0.43±0.01	1.36±0.01	2.05±0.06
4.	Water holding capacity (%)	62.0±2.70	198±0.30	39.6±2.03	84.3±1.30
5.	Surface area (m^2/g)	0.96	4.90	5.45	79.27

4.3.2 Process development for use of fly ash as a carrier for BGA biofertilizers

4.3.2.1 Growth (Dry Biomass) in algal ponds

Total biomass production in one month from nine algal ponds was 12.47 kg on dry weight basis and 42.4 kg on wet weight basis respectively. With increase in time, the biomass of algal inoculants in all the ponds increased from the date of inoculation under favorable conditions. It was reported that on an average 75 g/m^2/day of algal biomass would be possible in the Indian condition under properly designed outdoor algal pond and operated efficientLy to maximise algal product yield (Sudhakar, 2014). Average one year production from algal ponds was 113 kg of wet algal biomass was produced from which 381 packets of fly ash based cyanobacterial biofertilizers (500 g per packet) were prepared and applied for field trials of rice cultivation. Successful algal biomass cultivation at large scale is a key limiting step for the production of high-value products and algal biofuels, and crop protection against undesirable biomass losses will be a critically important component of commercialization efforts (Smith and Crews, 2014).

Pot trial

Blue green algal biofertilizer comprising of consortium of seven blue green algal isolates (*Calothrix* sp, *Anabaena flos-aquae, Desmonostoc* sp., *Nostoc commune, Nostoc* sp.PS1, *Nostoc* sp. DGRKF, and *Anabaena* sp.) and consortium of four ARM blue green algal strains (*Anabaena variabilis* (ARM 441); *Nostoc muscorum* (ARM 442); *Tolypothrix tenius* (ARM 443) and *Aulosira fertilissima* (ARM 444) were prepared using different carrier materials (fly ash (100%), soil (100%), montmorillonite (100%), fly ash + soil (1:1) and fly ash + montmorillonite (1:1). Their effect on rice cultivar PUSA 1121 was examined in pot experiment under glasshouse conditions, using nonsterile soil with a control without inoculation of blue green algal consortium. Effect of blue green algal consortium of region

specific isolates was compared with the consortium of ARM cultures on soil physicochemical properties and grain yield (g per pot) after 90 days at the time of harvest. Table 5.4.1 gives the physicochemical properties of soil before the inoculation of BGA consortium. At the time of harvest (after 90 days of inoculation) treatment T3 involving fly ash + soil (1:1) inoculated with consortium of region specific blue green algal isolates showed highest organic carbon content (0.43 %) , total nitrogen (0.168 %) followed by available phosphorus (17.4 mg/kg) as compared to control (Table 5.4.2). Treatment T4, combination of FA+MMT (1:1) also showed enhancement of organic carbon and total nitrogen by 0.37 % and 0.160 % in soil inoculated with consortium of region specific blue green algae as compared to control.

However, similar results were observed in soil inoculated with consortium of ARM procured cultures for combination of carrier material fly ash and soil (1:1) treatment T3 with nitrogen content of 0.149 % and carbon of 0.39 % respectively (Table 5.4.2). Significant enhancement of organic carbon, total nitrogen and phosphorus content observed for combination of carrier material fly ash+ soil (1:1) over control could be contributed by growth promoting role of cyanobacterial inoculants in the rhizospheric zone of rice fields (Prasanna *et al*., 2009) and fly ash which itself contained some available P (Gaind and Gaur, 2002). Past studies have also shown that single strain inoculations are ineffective (Egamberdiyeva and Hoflich 2004) while mixtures provide more consistency (Belimov *et al*.1995; Prasanna *et al*., 2009).

Highest grain yield (g per pot) of 14.3 and 12.75 was recorded in treatment T3, fly ash +soil (50:50) for both inoculated by consortium of seven isolated blue green algal strains and with consortium of four ARM cultures as compared to control (Figure 8.1). Valiente *et al*. (2000) reported no vast differences in grain yield and nitrogen uptake by pLant when applied with cyanobacteria and chemical fertilizers (ammonium sulphate) in a field experiment for rice production. Soil based algal inoculum, was developed for the benefit of small and marginal farmers as a cheap and easily adaptable method for the production of rice crop with little

capital investment (Venkataraman, 1981; Dhar *et al*., 2007). For increased crop yield, additional nutrients in the form of fertilizers are applied to the soil which mainLy compensate for the deficiency of major nutrients like nitrogen, phosphorus, and potassium in the soil, however fly ash being the reservoir of a number of macro and micronutrients, could be used to treat the soils deficiencies of these minerals (Malik and Thapliyal, 2009). A number of carriers have been used for cyanobacterial inoculants, such as soil, wheat straw montmorillonite (Venkataraman 1972; Kaushik 2004; Jha and Prasad, 2004; Dhar *et al*. 2007) and vermicuLite (Prasanna *et al*., 2013), however in the present study fly ash with combination of soil (1:1) was observed as a good carrier material in pLace of soil or MMT alone for showing highest nitrogen, carbon and phosphorus content. Local strains can be used as biofertilizer for the improvement of growth of many important crops, exorbitant cost of fertilizers and greater consciousness on environmental protection (Tantawy and Atef, 2010).

Table 5.4.1 Physicochemical analysis of soil used in pot culture experiment before the transpLantation of of rice and inoculation of BGA consortium.

Parameters	**Mean±SE**
pH	8.45±0.070
Organic Carbon (%)	0.20±0.010
Total Nitrogen (%)	0.011±0.004
Ava. Phosphorus ($mgkg^{-1}$)	5.410±0.600
Water holding capacity (%)	33.00±0.100

Table 5.4.2 Influence of consortium of BGA inoculants on soil physico chemical properties in pot experiment with rice crop after 90 DAT (Days after transplanting).

Treatment		*Region specific blue green algal isolates*				*BGA consortium of ARM cultures*			
		pH	Organic Carbon (%)	Total Nitrogen (%)	Ava. P ($mgkg^{-1}$)	pH	Organic Carbon (%)	Total Nitrogen (%)	Ava. P ($mgkg^{-1}$)
T1	Soil 100%	8.47 ± 0.25^{a}	0.30 ± 0.1^{cd}	0.057 ± 0.005^{c}	11.6 ± 0.52^{d}	8.44 ± 0.5^{b}	0.29 ± 0.04^{bc}	0.075 ± 0.005^{d}	12.95 ± 1.3^{c}
T2	MMT 100%	8.30 ± 0.07^{a}	0.35 ± 0.04^{bc}	0.102 ± 0.002^{c}	10.25 ± 0.98^{e}	8.33 ± 0.24^{c}	0.31 ± 0.01^{b}	0.095 ± 0.012^{c}	11.69 ± 1.10^{d}
T3	Fly ash +soil (50:50)	8.38 ± 0.69^{ab}	**0.43 ± 0.01^{a}**	**0.168 ± 0.010^{a}**	17.4 ± 0.62^{a}	8.21 ± 0.58^{e}	**0.39 ± 0.01^{a}**	**0.149 ± 0.034^{a}**	14.98 ± 0.96^{b}
T4	Fly ash + MMT (50:50)	8.15 ± 0.14^{ab}	0.37 ± 0.03^{ab}	0.160 ± 0.023^{a}	14.65 ± 0.13^{b}	8.29 ± 0.17^{d}	0.33 ± 0.02^{b}	0.137 ± 0.001^{b}	15.74 ± 0.52^{a}
T5	Fly ash + MMT (10:50)	8.11 ± 0.05^{ab}	0.31 ± 0.07^{cd}	0.127 ± 0.001^{b}	12.69 ± 1.3^{c}	8.13 ± 0.22^{f}	0.30 ± 0.001^{bc}	0.120 ± 0.011^{b}	11.25 ± 0.95^{d}
T6	Fly ash 100%	7.82 ± 0.58^{b}	0.29 ± 0.04^{c}	0.099 ± 0.001^{d}	12.04 ± 0.96^{cd}	7.68 ± 0.51^{g}	0.24 ± 0.007^{ab}	0.092 ± 0.004^{b}	13.01 ± 0.62^{c}
Control	Soil without inoculum	8.45 ± 0.22^{a}	0.19 ± 0.01^{d}	0.010 ± 0.002^{e}	5.1 ± 1.1^{f}	8.44 ± 0.6^{a}	0.21 ± 0.001^{c}	0.007 ± 0.001^{e}	4.54 ± 0.32^{e}
L.S.D (P<0.05)		**0.198**	**0.038**	**0.002**	**0.57**	**0.036**	**0.054**	**0.003**	**0.379**

(Mean±SE, n=3); Mean values with the same letter were not significantly different, based on ANOVA followed by Tukey's test at P≤0.05

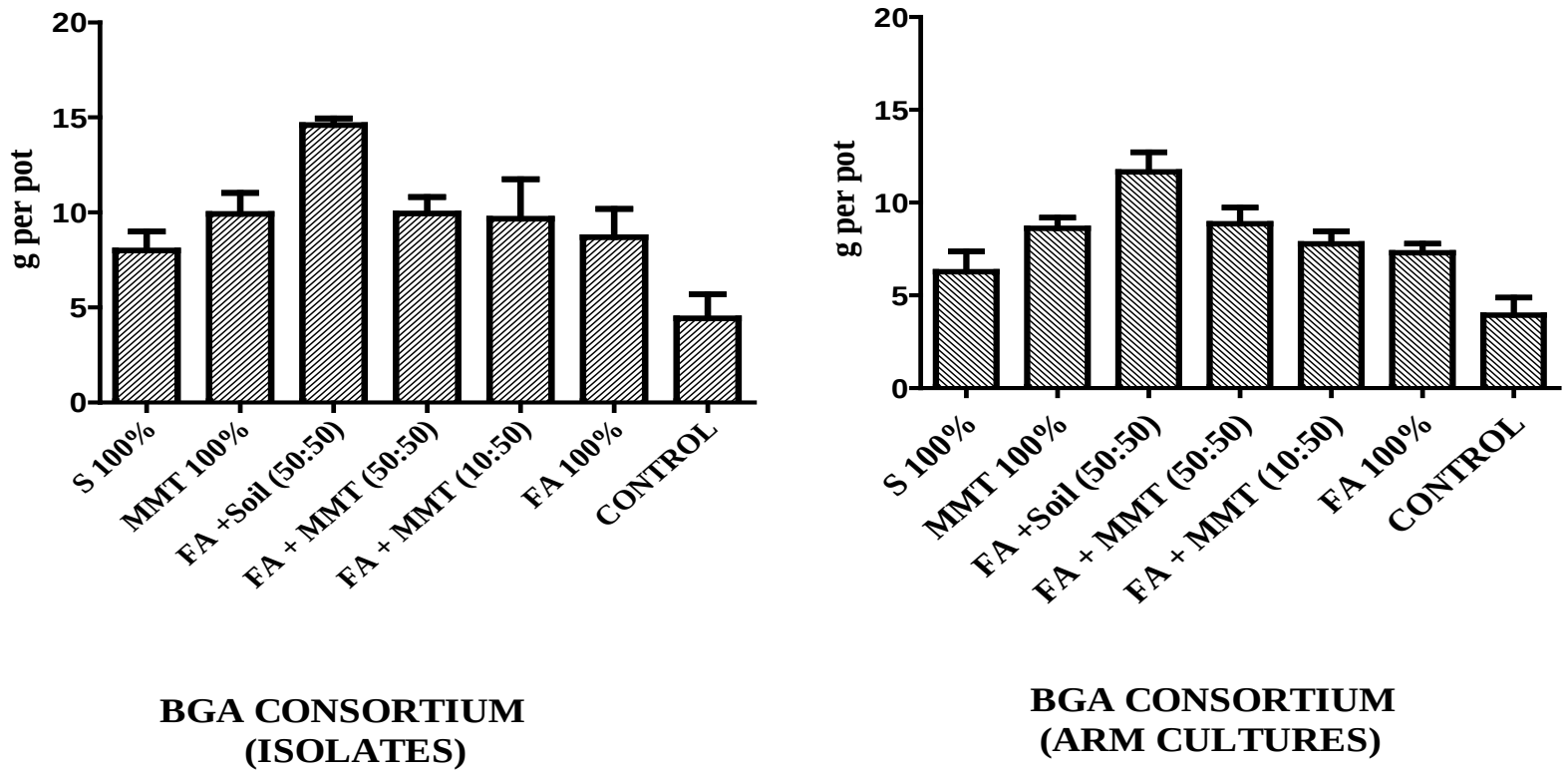


Figure 8.1 Effect of inoculation of consortium of region specific BGA isolate and of BGA (ARM cultures) on grain yield of rice crop (g per pot).

Field trial

Characterisation of soil before and after application of biofertilizers

Physiochemical properties of field soil such as pH, organic carbon (%), Total Nitrogen (%) and available Phosphorus (mg/kg) were estimated before and after application of biofertilizers in different villages of Punjab to study the effect of biofertilizers on nutrient profiLe in soils (Table 5.4.3). Soil micro and macro nutrients were increased as a result of application of biofertilizers. Overall mean value of pH of soils in twenty sites of Patiala was observed to be 7.54±0.5 before sowing of crop and 7.59 ± 0.05 at the time of harvesting of crop over control (Table 3). Organic carbon content (%) on application of algal biofertilizers during rice cultivation in 2011 showed significant increase in soils of some villages including village Dittupur from 0.36 % before sowing to 0.51 % at the harvesting over control whereas in village Khanoure the increase in carbon content was observed from 0.21% before sowing to 0.32% at the time of harvesting. Organic carbon content before sowing ranged from 0.18% in village Khanoure and Dittupur to 0.34- 0.36% in village Dakala and Dittupur respectively whereas at the time of harvesting it ranged from 0.24% in agricultural land of village Sibro to 0.51% in another land of village Dittupur (Table 3). Significant increase in the overall mean value of total nitrogen content from 0.028 % before sowing to 0.049% at the time of harvesting of crop over control in the soils of twenty villages of Patiala, Punjab was observed. Application of algal biofertilizers showed increase in mean value of phosphorus from 13 mg/kg before sowing to 18 mg/kg at the time of harvesting of crop over control (Table 3). Phosphorus content (mg/kg) ranged from 8.6 as observed in soils of village Bhojomajri to 23 in village Palia Khurd before sowing of crop whereas at the time of harvesting of 144 days the P content (mg/kg) ranged from 13 in village Bhojomajri to 26 in village Gunika respectively.

Table 5.4.3 Characterisation of soil before and after application of algal biofertilizers

	Before sowing of crop				After harvesting of crop			
Farmer's Name / Village	**pH**	**Organic Carbon (%)**	**Total Nitrogen (%)**	**Available Phosphorus (mg/kg)**	**pH**	**Organic Carbon (%)**	**Total Nitrogen (%)**	**Available Phosphorus (mg/kg)**
Acchhar singh, Bhojomajri	7.86±0.04 c	0.26±0.03 cdefgh	0.023±0.002 ef	11.03±1.04 defghi	7.70±0.02 fg	0.33±0.02 d	0.043±0.002 fg	15.3±2.1 cde
Rajinder singh, Bhojomajri	7.53 ± 0.02 d	0.32±0.01 ab	0.021±0.002 f	8.6±1.18 i	7.35± 0.04 hi	0.42±0.02 bc	0.041±0.001 gh	13.3±1.5 e
Darshan singh, Sibro	7.13 ± 0.02 h	0.19±0.03 ij	0.027±0.002 cdef	12.2±2.27 cdef	7.05±0.03 j	0.24±0.02 e	0.032±0.002 i	13.5±1.3 e
Malkit singh, Khanoure	7.29 ± 0.02 fg	0.18±0.02 j	0.030±0.001 cdef	12±0.60 cdefg	7.30±0.02 i	0.31±0.03 de	0.045±0.002 fg	14.4±0.6 cde
Kamalvir singh, Narainganj	7.10±0.02 hi	0.23±0.02 efghij	0.018±0.002 f	13.7±1.07 cde	7.14±0.06 j	0.36±0.04 bcd	0.036±0.003 hi	15.1±0.3 cde
Chamkaur singh, Kansuha	6.64 ± 0.03 k	0.28±0.01 bcdef	0.023±0.001 def	9.78±0.7 fghi	7.04±0.03 j	0.35±0.03 bcd	0.044±0.001 fg	16.3±0.6 cde
Guashala, Nabha	8.52 ± 0.01 a	0.33±0.02 ab	0.037±0.003 b	10.7±0.52 efghi	8.39±0.02 a	0.43±0.02 b	0.046±0.003 efg	20.7±0.5 b
Satguru singh, Gunika	7.44 ± 0.03 e	0.25±0.02 defghi	0.035±0.002 b	17.7±1.5 b	7.96±0.04 cd	0.37±0.02 bcd	0.081±0.002 a	26±1 a
Balwant singh, Khanoure	7.03 ± 0.02 hi	0.21±0.01 ghij	0.022±0.001 f	10.4±0.6 fghi	7.16±0.07 j	0.32±0.01 de	0.043±0.003 fg	14.7±0.5 cde
Paramjit singh, Dittupur	8.03 ± 0.02 b	0.36±0.02 a	0.020±0.004 f	12.2±0.8 cdefg	8.08±0.05 b	0.51±0.02 a	0.049±0.001 def	16.4±0.5 cde
Harisingh, Laut	7.15 ± 0.03 h	0.27±0.02 bcdefg	0.044±0.003 a	14.3±0.61 c	7.44±0.02 h	0.31±0.02 de	0.060±0.002 b	17±0.9 cd
Kripalsingh, Paliakhurd	6.86±0.03 j	0.24±0.03 efghij	0.03±0.001 bcd	8.8±0.5 hi	6.68±0.04 k	0.42±0.01 bc	0.052±0.001 cde	19±0.2 bc
GurmaiL singh, Gunika	8.01±0.03 b	0.29±0.02 abcde	0.035±0.002 b	14±1 cd	8.03±0.02 bc	0.34±0.03 d	0.041±0.002 gh	17.23±0.4 c
Sukhwinder singh, Dittupur	7.92±0.02 c	0.21±0.02 hij	0.023±0.002 def	9.6±0.6 fghi	8.04±0.04 bc	0.35±0.04 bcd	0.045±0.002 fg	13.7±0.5 de
Swaran singh, ShauLi	8.07 ± 0.02 b	0.31±0.01 abcd	0.024±0.001 def	11.9±1 cdefgh	8.05±0.03 bc	0.33±0.01 d	0.052±0.001 cde	16.6±0.6 cde
Chand singh, ShauLi	7.69 ± 0.04 d	0.23±0.03 efghij	0.030±0.002 bcde	18±0.1 b	7.59±0.07 g	0.30±0.02 de	0.054±0.003 cd	25.3±1.1 a
Avatar singh, Dittupur	7.92± 0.02 c	0.18±0.02 j	0.021±0.001 f	9.3±0.6 ghi	7.95±0.03 cd	0.25±0.03 e	0.047±0.003 efg	17.3±0.5 bc
Charan singh, PaliaKhurd	7.31 ± 0.01 f	0.24±0.04 efghij	0.036±0.003 b	22.7±1.5 a	7.86±0.03 de	0.32±0.04 de	0.057±0.002 bc	27±1.5 a
Brijender singh, Dakala	7.85±0.04 c	0.34±0.02 ab	0.019±0.005 f	14.3±1.5 c	7.76±0.04 ef	0.43±0.41 b	0.048±0.002 def	16.3±1.1 cde
Pardeep Singh Rajgarh	7.23±0.03 g	0.22±0.03 fghij	0.032±0.004 bc	20±1 ab	7.29±0.02 i	0.34±0.03 d	0.61±0.003 b	25±2 a
Overall Mean (*n*=20)	**7.54±0.5**	**0.26±0.05**	**0.028±0.01**	**13.10±3.9**	**7.59±0.5**	**0.35±0.06**	**0.049±0.01**	**17.84±4.3**

Values sharing a common letter within a column are not significant at $p<0.05$; values are Mean ± SD (*n*=3)

6.3.1 Impact of field application of cyanobacterial biofertilizers on different rice cultivars

Soil pH (Table 5.4.4)

Three years (2010-2013) comparative study of Urea, BGA and BGA+Urea applied fields over control fields without any treatment revealed that urea applied plots showed increased pH in soils as observed in each rice cultivars whereas BGA and BGA +Urea applied plots showed decrease in pH in as compared to control and urea applied plots. Continuous application of fly ash based BGA biofertilizer (500g per ha) alone showed gradual decrease in pH in soil of rice varieties as compared to control and urea applied plots. Increase in pH of soil recorded in Urea treated plots was 8.27 to 8.47 for PAU 201, 8.48 to 8.71 for PUSA 1121, 8.48 to 8.77 for SHABNAM, 8.35 to 8.56 for Basmati 1401 and 8.43 to 8.67 for PR 118 respectively. It is reported that adding nitrate or chemical fertilizers will decrease acidity and increases the pH of soil (Smith, 2008). BGA and BGA+ Urea plots showed gradual decrease in pH from 8.23 and 8.54 to 8.08 and 8.34 for rice variety PAU 201, 8.06 and 8.71 to 7.82 and 8.45 for PUSA 1121, 8.42 and 8.62 to 8.20 and 8.21 for SHABNAM, 8.32 and 8.35 to 8.13 and 8.25 for Basmati 1401 and 8.33 and 8.43 to 8.06 and 8.17 for PR 118 respectively. Coal fly ash is mostly utilized as a soil amendment in agriculture, used to buffer the soil pH (Phung *et al*., 1978), improving soil texture (Chang *et al*., 1977), improving the nutrient status of the soil (Rautaray *et al*., 2003; Singh *et al*., 2010). Integrated application of BGA biofertilizers and chemical fertilizers helped to maintain the pH and eLectrical conductivity (EC) of the soil (Dhar *et al*., 2007).

Soil organic matter status (Table 5.4.5)

The combined application of BGA + Urea plots and BGA biofertilizers applied plots showed significant changes in organic carbon content in three years as compared to urea applied

plotand control plots. Over the period of three years among all five rice varieties maximum carbon content increased was observed in PUSA 1121 showed increased from 0.32 to 0.53 by third year (2012-2013) in BGA+ Urea applied plots and from 0.30% to 0.49% in BGA applied plots whereas urea applied plots increase from 0.28% to 0.37% respectively. Studies reported that the balanced use of fertilizers alone or conjoint use of inorganic with organics resulted in a significant build – up of organic carbon and available N, P and K over three long decades leading to sustained soil fertility and productivity (Verma *et al*., 2005)

Soil available Phosphorus (mg/kg) (Table 5.4.6)

Phosphorus constitutes second most important macronutrient for crop development as recorded during the field trials conducted showed increase in each rice cultivar during three years of cultivation from 9.5 -21mg per kg in rice cultivar PAU 201& SHABNAM in the first year (2010-2011) to 12.9- 27 by third year (2012-2013) respectively. Overall increase in P content was observed in BGA + Urea and BGA applied plots in three years. For PAU201 this increase ranged from 8.63 mg/kg and 17.6 mg/kg as recorded before sowing in 2010-2011 to 12.9 mg/kg and 18.77 mg/kg. Similar results were recorded Basmati 1407 and PR118 from 9.8– 13.2 mg/kg in previous year (2010-2011) to 14.78 -21.09 mg/kg in third year (2012-2013) respectively as compared to control plots. Long-term cropping systems and fertiLity practices significantly alter rice productivity and soil properties for which fertilizer rate shouLd be readjusted. Therefore biological nitrogen fixation with P accumulation, NPK value of soil in rice cultivation could be improved (Lee *et al.*, 2008). Cyanobacteria are agents of carbon sequestration enhancing N and P in soils that promotes plant growth and are significant in sustainable management of the rice ecosystem (Prasanna *et al*., 2009). Urea applied plots showed marginal change in P content for rice variety SHABNAM and Basmati 1407 from 14 mg/kg and 10 mg/kg in 2010 to 15.89 mg/kg and 10.09 mg/kg in 2013 whereas other three rice cultivar viz. PAU201, PUSA 1121 and PR118 showed decrease in P

content (Fig). Long term fertilization affects the soil properties and exhibits a detrimental effect on soil health (Kanwar and Katyal, 1997; Verma *et al*., 2012).

Soil total nitrogen (%) (Table 5.4.7)

In BGA +Urea applied plots total nitrogen was found to increase significantly followed by BGA biofertilizers applied plots in three years regular application as compared to urea treated plots. Rice cultivar PUSA 1121 & SHABNAM showed increase in total nitrogen content in BGA +Urea treated plots upto 0.067% and 0.63% by third year (2012-2013) from its initial status as recorded before sowing in first trial (2010-2011) of 0.021% and 0.024% . Previous studies support the result that fly ash used at lower concentration (10-25%) enhances more growth and blue green algae plays a vital role in reducing nitrogen demand and enhance growth in plants grown in fly ash amended soils (FA 10% +NF90%+BGA 12.5%) (Dwivedi *et. al*., 2007; Tripathi *et al*., 2008). Overall increase in nitrogen content in urea applied plots @60 kg per hectare for all five rice cultivars was 0.042 -0.045% in third year (2012-2013) from 0.022 -0.025% before sowing in first year (2010-2011) respectively. Blue green algae fixes nitrogen in soil is supported by the observation in three year study that bga applied plots showed increase in total nitrogen for all rice cultivars in an ecological manner whereas increase in total nitrogen content as observed in urea applied plots was not much significant and valuable as it showed less increment of nitrogen content as compared to BGA+Urea and BGA applied plots. BGA biofertilizers showed a tremendous input of nitrogen content as observed before sowing in first year (2010-2011) to after harvesting in third year (2012-2013) from 0.019% and 0.021% to 0.055% and 0.049% in case of rice cultivar PR118 and Basmati 1404 whereas increase for rice cultivar PAU 201 was from 0.023% to 0.055%, 0.026% to 0.061% for PUSA 1121and 0.030% to 0.060% for SHABNAM which almost respectively doubles the content of nitrogen in three years wheras decline in nitrogen content in control

fields as recorded over three years with no treatment as observed for all rice cultivars. This increase in total nitrogen is confirmed by those reported by Adam (1999) which can be due to nitrogen fixation and nitrate reductase activities of cyanobacteria, or to uptake of NH_4^+ and uptake of amino acids and peptides produced by cyanobacteria.

6.3.2 Impact of cyanobacterial biofertilizers on crop yield in comparison to urea (Table 5.4.8)

Significant differences in grain yield were observed on studying the independent influence of Urea, BGA, BGA+Urea applied plots over control plots during three years (2010-2013). Overall three years study revealed significant enhancement in grain yield in combined application of BGA +Urea over control for all rice variety For BGA +Urea applied plots maximum increase in grain yield was observed in BASMATI 1401 by 8 q/ha from 15 q/ha in first year to 23 q/ha by third year followed by PUSA 1121 by 6 q/ha from 19 q/ha in first year to 25 q/ha in third year. SHABNAM showed increase by 5q/ha followed by PR118 (4 q/ha) and PAU 201(3 q/ha) respectively. Results are supported by the findings that algalization in the presence of recommended levels of nitrogenous fertilizers results in significantly higher yields in comparison to uninoculated treatments (Dhar *et al*., 2007). Integrated nutrient supply which involves the conjunctive use of fertilizers and organic sources assumes great importance in recent years due to consistently increasing trend in the cost of fertilizers. First year field trial (2010-2011) showed marginal increase in grain yield in urea applied plots by 24 q/ha for rice variety PR 118 & 23 q/ha for PAU 201 and BGA applied plot showed grain yield of 23 q/ha for PR118 & 22 q/ha for PAU 201 respectively whereas by third year (2012-2013) field trial the urea applied plot showed decrease in grain yield as compared to BGA applied plots. The application of BGA biofertilizers led to a definite increase in grain yield over three years in all rice varieties as compared to urea treated plots and control plots. Unfortunately a substantial amount of nitrogenous fertilization (Urea) causes environmental

pollution problems with depletion of soil nutrient, however, utilization of biological nitrogen fixation technology can supplement the use of urea-n, reducing the environmental problems to a considerable extent by improving nitrogen use efficiency (Choudhury *et al*., 2014). There is conclusive evidence that nitrogen fixed by cyanobacteria is made available to rice as well as other plant or microbial life (Mandal *et al*., 1998; Nayak 2004., Prasanna *et al*., 2009). Maximum grain yield for urea applied plotby third year (2012-2013) was 22q/ha for PR118 and 21q/ha for PAU 201 whereas BGA showed gradual increase by 26 q/ha for PR118 and 24q/ha for PAU201 respectively. It was reported that increased level of urea application decreased nitrogenase activity whereas Azolla or Azolla +BGA application exhibits higher nitrogenase activity (Prasanna *et al*., 2003; Venkataraman, 1981). Earlier study by Pereira et al (2009) that the use of biofertilizer allowed a 50% decrease in the use of synthetic nitrogen fertilizer with similar results with respect to grain yield. Long time studies being carried out at several locations in India indicated that application of all the needy nutrients through chemical fertilizers have drastic effect on soil properties leading to unsustainable yields (Jaga and Upadhayay, 2013; Dhar *et al*., 2007). Control fields with no nutrient supplement showed decrease in grain yield by third year (2012-2013) as compared to first year (2010-2011)for all rice cuLtivars which couLd be due to stressful soil conditions (low soil moisture and nutrient availability) (Singh *et al*., 2011). Algal fertilizers possess a number of distinct environmental advantages compared to conventional fertilizers and manure. In addition to the potential predictability of algal fertilizers in different soils, algal biomass constitutes a stable, transportable and highly concentrated form of transformed manure nutrients and the economic balance becomes more favorable if values from algae as a byproduct (e.g., fertilizer sale) and/or the value of nutrient recovery from the watershed can be realized (Mulbury *et al*., 2008).

6.4 Reduction in rate of application of chemical fertilizers based on farmer's response.

Currently about 60 % of the farmer are practising intensive farming in order to increase the yield and 40% have opted for organic farming in the villages of Patiala and Nabha. It is evident that increased application of chemical fertilizers at high rates has boosted agricultural production in the country but it has also caused adverse impact on soil and water as well as on environment (Narayanan, 2005). Several studies on the effects of use and application of biofertilizers for crop cultivation supports our findings that application of fly ash based biofertilizers reduce the usage of chemical fertilizers. It is reported that the use of algal biofertilizers allowed a 50% decrease in the use of synthetic nitrogen fertilizer (from 100 kg N ha^{-1} to 50 kg N ha^{-1}) with increased grain yield over control for rice crops in Chile (Pereira *et al*., 2009). Also, Blue green algal (BGA) application was observed to reduce the chemical nitrogen fertilizer requirement by 30 kg/ha and their application is an economical strategy in paddy cultivation (Tripathi *et al*., 2008) and cyanobacterial formulation increases the grain yield of rice crop (Innok *et al*., 2009). Fly ash as carrier material for microbial inoculants had no negative impact on cells viability and the shelf life of fly ash based biofertilizers was comparable with that of charcoal or montmorillonite. Efficacy of algal biofertilizers has resulted in reduction in application of urea and SSP in an ecofreindly and safe manner. In addition to the increase in the crop yield, farmers also reported improvement in size of grains and luster for better quality and marketability.

Table 5.4.4. Influence of fly ash based BGA biofertilizers on soil pH during three years field trial (2010-2013)

Rice Varieties	Treatment	2010-2011			2011-2012			2012-2013		
		Control		Applied	Control		Applied	Control		Applied
		BS	AH	AH	BS	AH	AH	BS	AH	AH
PAU 201	**B**	8.23±0.17^{ab}	8.28±0.30^{a}	8.15±0.02^{h}	8.29±0.01^{f}	8.33±0.04^{fg}	8.12±0.01^{h}	8.35±0.02^{ef}	8.41±0.01^{efg}	8.08±0.07^{hi}
	B+U	8.54±0.03^{ab}	8.56±0.40^{a}	8.45±0.04^{bc}	8.57±0.02^{abc}	8.59±0.01^{bc}	8.40±0.03^{d}	8.61±0.01^{abcd}	8.67±0.02^{abcd}	8.34±0.03^{e}
	U	8.27±0.02^{ab}	8.31±0.60^{a}	8.35±0.03^{de}	8.30±0.01^{f}	8.32±0.02^{g}	8.41±0.01^{d}	8.42±0.02^{def}	8.31±0.02^{fg}	**8.47±0.01^{d}**
PUSA	**B**	8.06±0.03^{b}	8.12±0.10^{a}	8.07±0.02^{i}	8.12±0.04^{g}	8.18±0.04^{h}	8.03±0.02^{i}	8.21±0.20^{f}	8.24±0.03^{g}	**7.82±0.01^{j}**
1121	**B+U**	8.71±0.2^{a}	8.66±0.10^{a}	**8.61±0.02^{a}**	8.63±0.1^{a}	8.68±0.02^{ab}	**8.52±0.01^{c}**	8.69±0.01^{abc}	8.72±0.03^{abc}	8.45±0.03^{d}
	U	8.40±0.03^{ab}	8.43±0.02^{a}	8.43±0.01^{cd}	8.44±0.01^{de}	8.49±0.02^{cde}	8.60±0.02^{b}	8.43±0.09^{def}	8.46± 0.03^{def}	8.71±0.02^{ab}
Shabnam	**B**	8.42±0.4^{ab}	8.63±0.30^{a}	8.39±0.06^{cde}	8.64±0.04^{a}	8.67±0.02^{ab}	8.30±0.04^{e}	8.72±0.24^{ab}	8.76±0.01^{ab}	8.20±0.5^{fg}
	B+U	8.62±0.02^{ab}	8.66±0.30^{a}	8.31±0.02^{ef}	8.67±0.01^{a}	8.75±0.02^{a}	8.25±0.03^{ef}	8.76±0.04^{a}	8.80±0.02^{a}	8.21±0.01^{fg}
	U	8.48±0.04^{ab}	8.50±0.01^{a}	**8.56±0.01^{a}**	8.58±0.02^{ab}	8.65±0.02^{b}	**8.71±0.03^{a}**	8.60±0.11^{abcd}	8.72±0.02^{abc}	8.77±0.01^{a}
Basmati	**B**	8.32±0.27^{ab}	8.42±0.30^{a}	8.22±0.03^{gh}	8.43±0.02^{de}	8.48±0.1^{de}	8.20±0.02^{fg}	8.47±0.02^{bcde}	8.53±0.03^{cde}	8.13±0.02^{ghi}
1401	**B+U**	8.48±0.20^{ab}	8.46±0.90^{a}	8.33±0.03^{e}	8.47±0.03^{d}	8.53±0.03^{cd}	8.31±0.02^{e}	8.53±0.02^{abcde}	8.72±0.25^{abc}	8.25±0.02^{ef}
	U	8.35±0.02^{ab}	8.39± 0.01^{a}	8.43±0.01^{c}	8.37±0.01^{ef}	8.40±0.06^{efg}	8.48±0.01^{c}	8.40±0.01^{def}	8.49±0.02^{def}	**8.56±0.04^{c}**
PR 118	**B**	8.33±0.02^{ab}	8.35±0.60^{a}	8.25±0.03^{fg}	8.37±0.02^{ef}	8.42±0.02^{ef}	8.14±0.03^{gh}	8.45±0.03^{cdef}	8.49±0.1^{def}	8.06±0.03^{i}
	B+U	8.45±0.39^{ab}	8.48±0.40^{a}	8.31±0.02^{ef}	8.47±0.02^{cd}	8.47±0.03^{de}	8.26±0.02^{ef}	8.49±0.1^{bcde}	8.53±0.05^{cde}	8.17±0.05^{fgh}
	U	8.43±0.02^{ab}	8.47±0.01^{a}	8.53±0.01^{ab}	8.48±0.01^{bcd}	8.52±0.03^{cd}	**8.59±0.01^{b}**	8.51±0.03^{abcde}	8.56±0.01^{bcde}	**8.67±0.01^{b}**
L.S.D (P<0.05)		**0.31**	**0.52**	**0.04**	**0.05**	**0.05**	**0.04**	**0.14**	**0.12**	**0.05**

(Mean±SE, n=3); Mean values with the same Letter were not significantly different, based on ANOVA followed by Turkey's test at P≤0.05

BS: before sowing; AH: after harvesting B= cyanobacterial biofertilizers alone (500 g/ha); B+U = cyanobacterial biofertilizers (500 g/ha) + Urea (60kg ha^{1}); U= urea alone (60kg/ha); C= control fields with no nutrient supplement.

Table 5.4.5 Influence of fly ash based BGA biofertilizers on soil organic carbon content (%) during three years field trial (2010-2013)

Rice Varieties	Treatment	2010-2011			2011-2012			2012-2013		
		Control		Applied	Control		Applied	Control		Applied
		BS	AH	AH	BS	AH	AH	BS	AH	AH
PAU 201	**B**	0.24±0.04^{a}	0.20±0.08 ^{a}	0.31±0.02^{ab}	0.19±0.03^{ab}	0.15±0.04^{a}	0.36±0.02^{abc}	0.13±0.04^{bc}	0.09±0.02 ^{b}	0.42±0.03^{abc}
	B+U	0.29±0.01 ^{a}	0.22±0.05 ^{a}	0.36±0.04 ^{ab}	0.22±0.05 ^{ab}	0.18±0.05 ^{a}	0.40±0.03^{ab}	0.19±0.05^{b}	0.11±0.03 ^{ab}	0.47±0.20^{abc}
	U	0.25 ±0.01 ^{a}	0.21 ±0.02 ^{a}	0.28 ±0.03^{ab}	0.21 ±0.02 ^{ab}	0.16 ±0.04 ^{a}	0.29 ±0.02^{bc}	0.13 ±0.02^{bc}	0.10 ±0.04^{ab}	**0.32± 0.03^{c}**
PUSA 1121	**B**	0.30±0.04 ^{a}	**0.25±0.05** ^{a}	0.31±0.02^{ab}	0.21±0.03 ^{ab}	0.16±0.03 ^{a}	0.37±0.05^{abc}	0.17±0.04^{b}	0.12±0.03 ^{a}	0.49±0.02^{abc}
	B+U	**0.32±0.05** ^{a}	0.30±0.02 ^{a}	0.35±0.02 ^{ab}	0.23±0.03 ^{ab}	0.18±0.01 ^{a}	**0.43±0.01^{a}**	0.15±0.01^{b}	0.08±0.02 ^{b}	**0.53±0.02^{a}**
	U	0.28 ±0.01 ^{a}	0.25 ±0.01 ^{a}	0.27± 0.01^{b}	0.23± 0.01 ^{ab}	0.21 ±0.02 ^{a}	0.32 ±0.02^{abc}	0.22 ±0.03^{a}	0.18± 0.03 ^{a}	0.37 ±0.02^{abc}
Shabnam	**B**	0.23±0.05 ^{a}	0.19±0.02 ^{a}	0.28±0.03^{ab}	0.21±0.02 ^{ab}	0.14±0.03 ^{a}	0.33±0.04^{abc}	0.12±0.01^{b}	0.11±0.04 ^{b}	0.45±0.10^{abc}
	B+U	0.31±0.02 ^{a}	**0.27±0.07** ^{a}	0.37±0.06^{ab}	0.28±0.06^{a}	0.22±0.02 ^{a}	0.42±0.02^{a}	0.23±0.06 ^{a}	0.17±0.04 ^{a}	**0.51±0.03^{ab}**
	U	0.27 ±0.03 ^{a}	0.23± 0.03 ^{a}	0.29 ±0.02^{ab}	0.22 ±0.04 ^{ab}	0.20 ±0.02 ^{a}	0.34 ±0.02^{abc}	0.18 ±0.01 ^{b}	0.13 ±0.03 ^{a}	0.38 ±0.02^{bc}
Basmati 1401	**B**	0.23±0.02 ^{a}	0.20±0.02 ^{a}	0.31±0.02^{ab}	0.20±0.03 ^{ab}	0.14±0.02 ^{a}	0.35±0.04^{abc}	0.11±0.02^{c}	0.06±0.04 ^{c}	0.41±0.03^{abc}
	B+U	**0.29±0.03** ^{a}	0.24±0.08 ^{a}	0.39±0. 01^{a}	0.23±0.02 ^{ab}	**0.18±0.03** ^{a}	0.43±0.02^{a}	0.19±0.05^{bc}	0.13±0.02 ^{ab}	0.46±0.03^{abc}
	U	0.23± 0.03 ^{a}	0.20 ±0.01^{a}	0.28 ±0.01^{ab}	0.21± 0.06 ^{ab}	0.15 ±0.03 ^{a}	0.29 ±0.02^{bc}	0.30± 0.06^{a}	0.11± 0.05^{b}	**0.33 ±0.01^{c}**
PR 118	**B**	0.22±0.03 ^{a}	0.19±0.03 ^{a}	0.29±0.10^{ab}	0.17±0.04^{b}	0.16±0.11 ^{a}	0.37±0.04^{abc}	0.12±0.02 ^{a}	0.10±0.03^{bc}	0.43±0.02^{abc}
	B+U	0.27±0.05 ^{a}	0.21±0.06 ^{a}	0.34±0.04^{ab}	0.22±0.04 ^{ab}	**0.19±0.02** ^{a}	**0.43±0.03^{a}**	0.17±0.06 ^{a}	0.11±0.02 ^{a}	0.50±0.03^{ab}
	U	0.23 ±0.02 ^{a}	0.20 ±0.01 ^{a}	0.26 ±0.02^{b}	0.21 ±0.06 ^{ab}	0.15± 0.01^{a}	0.29± 0.01^{bc}	0.16± 0.04 ^{a}	0.10± 0.13 ^{a}	0.35± 0.01^{bc}
L.S.D (P<0.05%)		**0.07**	**0.10**	**0.07**	**0.05**	**0.05**	**0.06**	**0.34**	**0.52**	**0.10**

(Mean±SE, n=3); Mean values with the same Letter were not significantly different, based on ANOVA followed by Turkey's test at P≤0.05

BS: initial Level of SOC before sowing; AH: Level of SOC after harvesting ; B= cyanobacterial biofertilizers alone (500 g/ha); B+U = cyanobacterial biofertilizers (500 g/ha) + Urea (60kg/ha); U= urea alone (60kg/ha); C= control fields with no nutrient supplement.

Table 5.4.6 Influence of fly ash based BGA biofertilizers on available phosphorus (mg/kg) of soil during three years field trial (2010-2013)

Rice Varieties	Treatment	2010-2011			2011-2012			2012-2013		
		Control		Applied	Control		Applied	Control		Applied
		BS	AH	AH	BS	AH	AH	BS	AH	AH
PAU 201	**B**	08.63±0.3^{e}	08.1±0.9^{d}	09.5±0.9^{hi}	08.20±0.7^{fg}	07.4±0.3^{e}	10.5±0.50^{fg}	07.90±0.10^{cd}	07.32±0.3^{bcde}	12.9±0.6^{g}
	B+U	**17.6±1.20^{a}**	**14.7±0.6^{a}**	16.6±0.6^{b}	12.0±1^{abc}	9.4±0.6^{bcd}	17.7±1.30^{bc}	8.92±0.2^{bc}	7.80±0.3^{bcd}	18.77±1.1^{cd}
	U	8.76±0.05^{e}	8.63±0.7^{cd}	8.23±0.03^{i}	8.22±0.03^{fg}	8.0±0.02^{de}	8.23±0.01^{g}	7.70±0.2^{cd}	7.0±0.02^{de}	**8.10±0.2^{i}**
PUSA 1121	**B**	15.3±2.5^{ab}	11.4±0.8^{b}	14.8±0.5^{bcd}	11.3±0.8^{bc}	9.5±0.3^{bc}	16.3±2.50^{bcd}	8.81±0.2^{bc}	7.99±0.2^{bc}	18.36±0.3^{cd}
	B+U	10.5±0.7^{de}	8.9±1.7^{bcd}	13.7±1.5^{cde}	8.02±1.4^{fg}	7.85±0.7^{e}	15.4±0.70^{cd}	7.47±0.6^{d}	**6.80±0.70^{e}**	**19.14±0.1^{c}**
	U	13±0.5^{bcd}	11.8±0.1^{b}	12.3±0.02^{efg}	10.7±0.1^{cde}	8.4±0.01^{cde}	11.0±0.10^{f}	7.3±0.10^{d}	7.10±0.10^{cde}	10.00±0.10^{h}
Shabnam	**B**	17.4±1.3^{a}	14±0.90^{a}	**21.1±0.6^{a}**	13.24±1^{ab}	**11.96±0.9^{a}**	25.3±0.60^{a}	**11.2±0.2^{a}**	8.20±0.20^{b}	**27.54±0.8^{a}**
	B+U	8.8±1.30^{e}	7.9±0.1^{e}	10.9±1^{gh}	7.84±0.9^{g}	7.27±0.4^{e}	14.5±0.40^{d}	7.22±0.1^{d}	**6.59±0.20^{e}**	17.30±0.9^{de}
	U	14.3±0.2^{abc}	12±0.03^{b}	13±0.1^{defg}	13.63±0.3^{a}	11.76±0.01^{a}	15.2±0.30^{cd}	9.67±0.02^{b}	7.78±0.10^{bcd}	15.85±0.1^{ef}
Basmati 1401	**B**	11.3±1.5^{cde}	9.4±0.5^{bcd}	12.4±0.7^{efg}	9±0.6^{defg}	9.96±0.01^{b}	13.7±0.60^{de}	8.47±0.8^{bcd}	6.99±0.01^{de}	14.70±0.9^{fg}
	B+U	9.8±1.20^{de}	8.6±0.5^{cd}	11.9±0.2^{efg}	8.4±0.3^{fg}	7.96±0.06^{de}	14.6±0.60^{d}	**7.45±0.03^{d}**	7.03±0.01^{de}	15.19±0.6^{f}
	U	10.0±0.11^{de}	9.21±0.2^{b}	11.3±0.3^{fgh}	8.56±0.01^{efg}	8.34±0.01^{cde}	11.6±0.40^{ef}	8.12±0.01^{cd}	7.12±0.1^{cde}	10.04±0.03^{h}
PR 118	**B**	13.2±1.1^{bcd}	11.2±1^{bc}	15.4±0.6^{bc}	10.9±0.8^{cd}	8.36±0.03^{cde}	19.9±0.10^{b}	9.65±0.01^{b}	7.78±0.05^{bcd}	**21.7±0.02^{b}**
	B+U	11.51±1.4^{cde}	8.3±0.5^{d}	13.6±1.5^{cdef}	9.9±0.1^{cdefg}	8.28±0.1^{cde}	16.2±0.80^{cd}	7.96±0.03^{cd}	6.84±0.10^{e}	18.45±0.9^{cd}
	U	12±0.8^{bcde}	11.4±1^{bc}	12.31±0.2^{efg}	10.1±0.2^{cdef}	8.34±0.04^{cde}	10.9±0.01^{f}	8.21±0.03^{cd}	10.8±0.30^{a}	**7.82±0.02^{i}**
L.S.D (P<0.05%)		**1.92**	**1.58**	**1.27**	**1.17**	**0.79**	**1.42**	**0.72**	**0.51**	**0.99**

(Mean±SE, n=3); Mean values with the same Letter were not significantly different, based on ANOVA followed by Turkey's test at P≤0.05

BS: initial Level of SOC before sowing; AH: Level of SOC after harvesting ; B= cyanobacterial biofertilizers alone (500 g/ha); B+U = cyanobacterial biofertilizers (500 g/ha) + Urea (60 kg/ha); U= urea alone (60 kg/ha); C= control fields with no nutrient supplement.

Table 5.4.7 Influence of fly ash based BGA biofertilizers on soil total nitrogen content (%) during three years field trial (2010-2013)

Rice Variety	Treatment	2010-2011			2011-2012			2012-2013		
		Control		Applied	Control		Applied	Control		Applied
		BS	AH	AH	BS	AH	AH	BS	AH	AH
PAU 201	**B**	0.023±0.002 [a]	0.020±0.005 [a]	0.040±0.001 [a]	0.019±0.001 [a]	0.015±0.01 [a]	0.049±0.001 [a]	0.014±0.003	0.010±0.002 [a]	0.055±0.004 [a]
	B+U	0.027±0.003 [a]	0.021±0.001 [a]	0.037±0.004 [a]	0.025±0.001 [a]	**0.019±0.002** [a]	0.047±0.001 [a]	**0.017±0.004** [a]	0.013±0.003 [a]	**0.057±0.003** [a]
	U	0.024±0.005 [a]	0.022±0.001 [a]	0.031±0.001 [b]	0.019±0.001 [a]	0.014±0.003 [a]	0.037±0.005 [b]	0.013±0.002 [a]	0.010±0.001 [a]	**0.045±0.001** [a]
PUSA 1121	**B**	0.026±0.002 [a]	0.022±0.002 [a]	0.038±0.002 [a]	0.019±0.002 [a]	**0.016±0.01** [a]	0.050±0.001 [a]	0.015±0.001 [a]	0.012±0.002 [a]	**0.061±0.002** [a]
	B+U	0.021±0.003 [a]	0.015±0.003 [b]	0.037±0.001 [ab]	0.016±0.003 [a]	0.013±0.003 [a]	0.042±0.001 [a]	0.011±0.001 [ab]	0.009±0.001 [a]	**0.067±0.003** [a]
	U	0.024±0.001 [a]	0.018±0.004 [a]	0.031±0.005 [b]	0.015±0.003 [a]	0.012±0.001 [a]	0.035±0.002 [b]	**0.010±0.001** [ab]	0.007±0.001 [b]	0.042±0.003 [b]
Shabnam	**B**	**0.030±0.002** [a]	0.025±0.001 [a]	0.041±0.001 [a]	0.023±0.002 [a]	0.017±0.001 [a]	0.051±0.003 [a]	0.015±0.004 [a]	0.011±0.002 [a]	0.060±0.005 [a]
	B+U	0.024±0.003 [a]	0.019±0.002 [a]	**0.046±0.001** [a]	0.018±0.002 [a]	0.012±0.002 [a]	**0.053±0.002** [a]	0.012±0.003 [a]	0.008±0.003 [a]	**0.063±0.002** [a]
	U	0.025±0.001 [a]	0.021±0.004 [a]	0.029±0.001 [ab]	0.019±0.001 [a]	0.015±0.001 [a]	0.037±0.001 [ab]	0.011±0.003 [ab]	0.008±0.001 [a]	0.048±0.001 [ab]
Basmati 1401	**B**	0.021±0.003 [a]	0.017±0.004 [a]	0.031±0.002 [ab]	0.016±0.001 [a]	0.011±0.002 [a]	0.043±0.003 [ab]	0.012±0.002 [ab]	0.008±0.001 [a]	**0.049±0.004** [ab]
	B+U	0.023±0.001 [a]	0.018±0.001 [a]	0.043±0.004 [a]	0.018±0.001 [a]	0.013±0.001 [a]	0.047±0.001 [a]	0.011±0.002 [ab]	0.007±0.001 [b]	0.054±0.001 [a]
	U	0.020±0.001	0.015±0.001 [b]	0.030±0.003 [ab]	0.013±0.002 [ab]	0.011±0.003 [a]	0.041±0.005	0.010±0.001	**0.006±0.002**	0.045±0.001
PR 118	**B**	**0.019±0.001** [b]	0.016±0.004 [ab]	0.038±0.002 [a]	0.014±0.004 [ab]	0.011±0.004 [a]	0.048±0.001	0.011±0.002	0.006±0.001	0.055±0.010
	B+U	0.023±0.003 [a]	0.019±0.001 [a]	0.045±0.001 [a]	0.018±0.003 [a]	0.011±0.003 [a]	**0.054±0.001**	0.010±0.003	0.007±0.001	**0.058±0.003**
	U	0.022±0.002 [a]	0.015±0.001 [b]	0.036±0.007 [a]	0.013±0.002 [ab]	0.010±0.001 [a]	0.047±0.003	0.008±0.001	**0.005±0.001**	0.053±0.010
L.S.D (P<0.05%)		**0.001**	**0.006**	**0.002**	**0.001**	**0.003**	**0.005**	**0.002**	**0.001**	**0.005**

(Mean±SE, n=3); Mean values with the same letter were not significantly different, based on ANOVA followed by Turkey's test at P≤0.05

BS: initial Level of N before sowing; AH: Level of N after harvesting; B= cyanobacterial biofertilizers alone (500 g/ha); B+U = cyanobacterial biofertilizers (500 g/ha) + Urea (60 kg/ha); U= urea alone (60 kg/ha); C= control fields with no nutrient supplement.

Table 5.4.8 Influence of fly ahs based BGA biofertilizers over nitrogenous fertilizers on average grain yield of rice.

Rice Varieties	2010-2011 (q ha^{-1})				2011-2012 (q ha^{-1})				2012-2013 (q ha^{-1})			
	C	**U**	**B**	**B+U**	**C**	**U**	**B**	**B +U**	**C**	**U**	**B**	**B+U**
PAU 201	20^{a}	**23^{a}**	22^{a}	**24^{ab}**	18^{a}	22^{ab}	23^{ab}	**25^{ab}**	16^{a}	21^{a}	24^{ab}	**27^{a}**
PUSA 1121	13^{b}	16^{b}	15^{b}	19^{bc}	11^{b}	17^{bc}	18^{bc}	21^{abc}	09^{bc}	18^{ab}	19^{abc}	25^{a}
SHABNAM	11^{b}	14^{b}	14^{b}	16^{c}	10^{b}	15^{bc}	16^{c}	19^{bc}	08^{bc}	13^{b}	17^{c}	21^{a}
BASMATI 1401	10^{b}	13^{b}	12^{b}	15^{c}	09^{b}	14^{c}	15^{c}	18^{c}	**07^{c}**	16^{b}	18^{bc}	23^{a}
PR 118	20^{a}	**24^{a}**	23^{a}	**26^{a}**	17^{a}	24^{a}	25^{a}	**28^{a}**	14^{ab}	**22^{a}**	**26^{a}**	**30^{a}**
L.S.D (P<0.05)	**3.08**	**3.99**	**4.36**	**4.38**	**3.61**	**4.86**	**3.90**	**5.02**	**3.96**	**3.67**	**4.86**	**4.84**

(Mean±SE, n=3); Mean values with the same letter were not significantly different, based on ANOVA followed by Turkey's test at P≤0.05
B= cyanobacterial biofertilizers alone (500 g/ha); B+U = cyanobacterial biofertilizers (500 g/ha) + Urea (60 kg/ha); U= urea alone (60 kg/ha); C= control fields with no nutrient supplement.

6.5 Random block designs (RBD) for flyash based BGA biofertilizers

RBD plots were constructed on one hectare of agricultural land in village Ajnoida, Pedhna and Rajgarh during kharif season (May-October, 2011) to observe the effect of fly ash based BGA biofertilizers on yield of rice cultivar PR118 with the treatment of Urea, BGA, BGA+UREA and control. In each village one hectare of land (190 ft×220 ft) (41800 sq ft) was divided into four bighas, each of 837.60 sq m for four different treatments. Urea was applied @ 10kg/bigha; BGA@ 500 g/bigha and BGA+UREA @ 5kg urea per bigha and BGA @ 500 g/bigha. Control plots with no inoculation.Physicochemical properties observed in the RBD plots showed that total nitrogen content increased in BGA+UREA applied plots in village Ajnoida by 0.069%, in village Pedhna by 0.067% and in village Rajgarh by 0.045%. However, increase in organic carbon content was noticed in BGA inoculated plots (500 g per bigha) by 0.37 in village Ajnoida), 0.54 % in village pedhna and 0.48% in village Rajgarh respectively. Nitrogen fixing cyanobacteria or blue green algae are ecologically significant inputs in rice cultivation in the tropics and continued application of chemical fertilizers, over long periods is known to bring about drastic changes in soil properties, however, integrated application of BGA biofertilizers and chemical fertilizers helped to maintain the pH, nitrogen and carbon content of the soils (Rojer and Ladha, 1992; Dhar *et al*., 2007).

Similarly grain yield (q/bigha) was noticed in BGA+ UREA treated plots by 7 q per bigha in village Pedhna followed by village Ajnoida by 6 q per bigha and by 5.3 q per bigha in village Rajgarh respectively (Table 5.5.1). Grain yield of rice crop increases with the controlled application of N-fertilizers, however the nitrogenase activity of blue green algae decreases with increase dose on N-fertilizers (Valiente *et al*., 2000). Control plots showed less grain yield in all three villages ranging from 2-3 q per bigha. Overall increase in grain yield in

inoculated plots as compared to control plot was by 2 -3q per bigha in BGA inoculated plots and by 2-4 q per bigha in BGA+Urea inoculated plots.

Table 5.5.1 Physico chemical analysis of soil in RBD Plots

Parameters	Control	Urea	BGA	BGA+ UREA
		Village Ajnoida		
pH	07.37 ± 0.010[a]	07.38±0.18[a]	07.240 ± 0.02[b]	07.29 ± 0.11[b]
EC (µS/cm)	69.54 ± 0.012[ab]	71.00 ± 0.16[ab]	67.000 ± 0.32[b]	73.00 ± 0.02[a]
Organic Carbon (%)	00.22±0.001[c]	00.27±0.001[b]	**00.370±0.01[a]**	0.35±0.01[a]
Available P (mg/kg)	10.01±0.070[b]	11.12±0.02[a]	10.560±0.12[b]	11.50±0.35[a]
Total N (%)	0.023±0.001[d]	0.041±0.007[c]	**0.058±0.003[b]**	**0.069±0.002[a]**
		Village Pedhna		
pH	8.11±0.36[a]	8.13±0.24 [a]	8.10±0.35[a]	8.12±0.13[a]
EC (µS/cm)	69±0.12[b]	77±0.14 [a]	70±0.78[b]	75±0.38[a]
Organic Carbon (%)	0.25±0.01[d]	0.30±0.13[c]	**0.54±0.01[a]**	0.50±0.03[b]
Available P (mg/kg)	11.92±0.56	10±0.98	12.98±0.34[b]	14.23±0.87[a]
Total N (%)	0.011±0.001[d]	0.044±0.002[c]	0.058±0.007[b]	**0.067±0.001[a]**
		Village Rajgarh		
pH	7.67±0.10[a]	7.69±0.92[a]	7.48±0.51[a]	7.52±0.006[a]
EC (µS/cm)	133±0.30[c]	144±0.35[a]	138±0.49[b]	140±0.93[b]
Organic Carbon (%)	0.34±0.02[d]	0.41±0.06[c]	0.48±0.02[a]	0.44±0.08[b]
Available P (mg/kg)	15.06±0.29[d]	16.12±1.83[c]	16.18±0.24[b]	17.01±0.46[a]
Total N (%)	0.022±0.06 [d]	0.038±0.009[c]	0.040± 0.02[b]	**0.045±0.006[a]**

(Mean±SE, n=3); Mean values with the same letter were not significantly different, based on ANOVA followed by Turkey's test at P≤0.05B= cyanobacterial biofertilizers alone (500 g/ha);
B+U =cyanobacterial biofertilizers (500 g/ha) + Urea (60 kg/ha); U= urea alone (60kg/ha);
C= control fields with no nutrient supplement

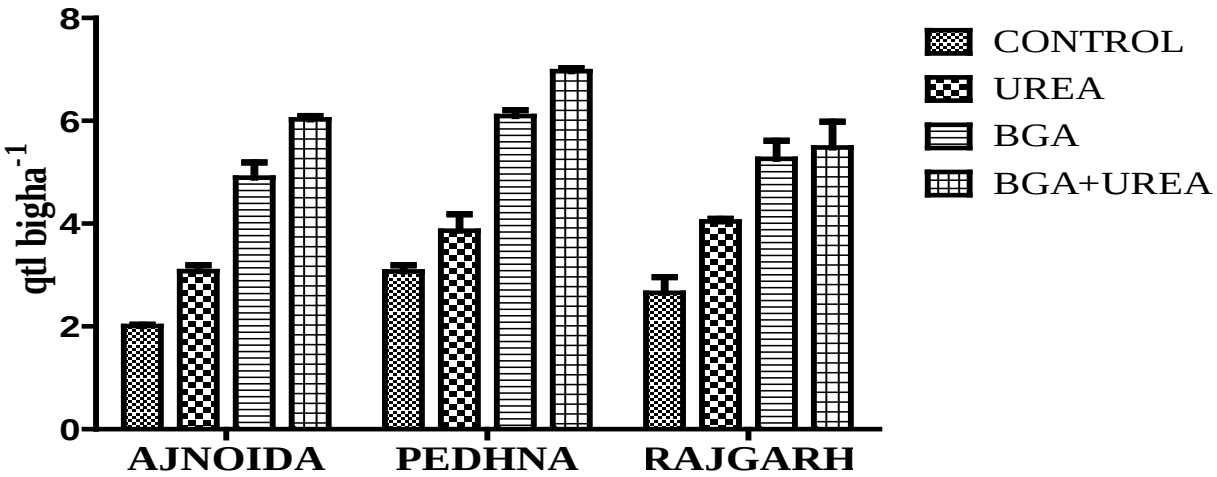


Figure 8.2 Yield Productivity Data (q per ha) RBD Plots

Urea	20 -30 kg per bigha
BGA	500 g per bigha
BGA + UREA	500 g + 30 kg Urea

CHAPTER 5 MAJOR CONTRIBUTION AND NEW FINDINGS

1. Seven filamentous heterocystous region specific blue green algae were Isolated from paddy field soil and pond fly ash and identified as *Calothrix* sp., *Anabaena flos-aquae*, *Desmonostoc* sp. DGRKC, *Nostoc commune*, *Nostoc* sp.PS1, *Nostoc* sp. DGRKF and *Anabaena* sp.

2. *Anabaena flos-aquae*, *Desmonostoc* sp. DGRKC and *Nostoc* sp. DGRKF showed highest nitrogen fixation, heterocyst frequency, total nitrogen content and nitrate reductase activity.

3. *Calothrix* sp. showed maximum uptake of Zn (4.52 mg/g) and Pb (2.52 mg/g), *Nostoc commune* and *Anabaena flos-aquae* showed maximum uptake of chromium by 4.85 mg/g and 3.73 mg/g respectively and copper uptake was maximum in *Calothrix* sp.(0.825 mg/g) and *Nostoc* sp DGRKF (0.733 mg/g).

4. The present study revealed that coal fly ash at 5 and 10% (w/v) addition in BG 11(-N) medium increased in biomass and nitrogen fixation with no negative impact on nitrogen fixation by blue green algal strains.

5. Mineralogical composition of coal fly ash ascertains its applicability as a carrier for different microbial inoculants for soil application in agriculture which can act as an economic source of nutrient supplement for crop plants.

6. Use of fly ash as a carrier has an advantage over other carrier material as it has high water holding capacity (WHC) and is inert material and provides other trace elements for growth of BGA and plants.

7. Process developed pertains to new use of fly ash as carrier for blue green algae. Use of fly ash as carrier in the algal biofertilizers proves to be an effective way of utilization of fly ash in a useful manner in restoring soil nutrients.

8. Increase in crop yield by 2-6 q/ha over control among all rice cultivars supports a positive role of fly ash based algal biofertilizers.

9. With the application of BGA biofertilizers soil nitrogen and organic carbon was increased by 0.042 to 0.067 % and 0.32 to 0.53 % respectively.

10. Field survey and trials conducted at different villages of Patiala confirms positive response of farmers and value addition of fly ash based algal biofertilizers to the soil in enhancing crop productivity with reduction in usage of urea.

SUMMARY

1. Isolation and characterization of efficient nitrogen fixing blue green algae from paddy field soils and ash pond samples.

Seven filamentous heterocystous region specific blue green algae were Isolated from paddy field soils of Nabha and Patiala, Punjab, India and pond fly ash from National Thermal Power Station (NTPC), Rihandnagar, U.P, India and were identified as Calothrix sp. (Isolate A), *Anabaena flos-aquae* (Isolate B), *Desmonostoc* sp. (Isolate C), *Nostoc commune* (Isolate D), *Nostoc* sp. PS1 (Isolate E), *Nostoc* sp. (Isolate F) and *Anabaena* sp. (Isolate G). Growth study revealed that among all the seven BGA isolates, Isolate G showed maximum dry biomass (3.45 mg/mL), Isolate C showed highest nitrogenase activity by 32.2 mole C_2H_4/mg dry wt/hr , total nitrogen content (0.127 %) heterocyst frequency (18.2%) and nitrate reductase activity (31.0162 µ mole NO^-_2); Isolate B showed maximum chlorophyll content (3.79 µg/mL), nitrate reductase activity (27.62 µ mole NO^-_2) and nitrogenase activity by 20.31 mole C_2H_4/mg dry wt/hr.

Isolate C, B, and F were selected based on highest nitrogen fixation, total nitrogen content and heterocyst frequency and were further characterised 16S rRNA molecular identification technique. Identification of three Isolates *Desmonostoc* sp DGRKC (Isolate C), *Nostoc sp.*DGRKF (Isolate F) and *Anabaena flos-aquae* (Isolate B) were confirmed by molecular studies and their nucleotide sequences were submitted in NCBI database under the nucleotide accession numbers KM083062 and KM083063.

2. Impact of fly ash on growth and nitrogen fixation by selected blue green algal Isolates.

Fly ash in dried form at different concentration 0, 5, 10 and 20% (w/v) was added to BG 11(-N) growth medium and distilled water (DW) to examine its impact on growth and nitrogen fixation by heterocystous filamentous blue green algal strains. *Nostoc* sp., *Anabaena flos-aquae*, *Desmonostoc* sp. DGRKC and *Anabaena variabilis* (ARM 441) showed proLific growth in BG 11 (-N) medium amended with 5 and 10% fly ash with almost same range dry biomass (2.25-3.5 mg/mL). Maximum nitrogen fixation and total nitrogen content of 37.2 – 40.4 mole C_2H_4/mg dry wt/hr and 0.784-0.883% was observed in *Desmonostoc* sp. grown in BG 11amended with 5% and 10% fly ash as compared to control (BG 11-0% FA). However all algal isolates showed decreased nitrogen fixation when grown at higher concentration of fly ash (20%) both in distilled water (DW) and BG 11 medium. *Anabaena variabilis* (ARM 441) showed highest range of nitrogen fixation of 32.5 mole C_2H_4/mg dry wt/hr and total nitrogen content by 0.509 % when grown in BG 11 (-N) medium amended with 5% fly ash whereas *Nostoc muscorum* (ARM 442) showed maximum nitrogenase activity (23.14 mole C_2H_4/mg dry wt/hr) and total nitrogen content (0.398 %) in BG11 medium amended with 5% fly ash.

Chromium uptake (Cr) was observed maximum in *Anabaena flos-aquae* (3.73 mg/g), *Nostoc commune* (4.85 mg/g) at BG 11 medium amended with 5% fly ash whereas *Nostoc* sp DGRKF(3.98 mg/g) showed at BG 11 (-N) medium amended with 10% fly ash respectively. Howerever, *Nostoc muscorum* (ARM 442 mg/g) showed maximum uptake of Cr (3.65 mg/g), Pb (2.12 mg/g) in BG 11(-N) medium amended with 10% fly ash respectively. *Calothrix* sp. showed maximum uptake of Zn (4.52 mg/g) and Pb (2.52 mg/g) in BG 11(-N) medium amended with 20% fly ash as compared to control, however Cu and Cr uptake was maximum by 0.825 mg/g and 0.950 mg/g in BG 11 (-N) amended with 10% fly ash. Increased accumulation of metals by blue green algal isolates grown in BG 11(-N) medium amended with 5, 10 and 20% fly ash was observed.

3. Fly ash based blue green algal inoculant formulation and their evaluation on growth and yield of rice.

For development of fly ash based blue green algal inoculants, mineralogical and physico chemical properties of fly ash were studied and compared with that of soil, charcoal and montmorillonite.

Fly ash showed high thermal stability with least weight loss as observed in TGA and SEM graph indicated that fly ash is composed of spherical structures with more surface area for interaction, XRD and EDS studies showed that amorphous content of ash consists of calcium oxide, potassium and major crystalline phases observed were quartz (SiO_2) and aluminium siLicon oxide ($Al_{4.52}Si_{1.48}$) and haematite (Fe_2O_3). Charcoal, was amorphous in nature consisting of carbon and graphite. Soil and montmorillonite showed similar results in XRD, FTIR and thermal analysis having porous nature with silica as major constituent.

Field trial on impact of consortium of fly ash based BGA inoculants on different rice cuLtivars (PUSA 1121, PR 118, PAU 201, SHABNAM and BASMATI 1401 were studied for three consecutive years (2010-2013) on 20 acres of trials in different villages along with control. From an average production of 113 kg of wet algal biomass in algal ponds, 381 packets of fly ash based cyanobacterial biofertilizers (500 g per packet) were made which were applied for field trials of rice cultivation. Co application of cyanobacterial biofertilizers @ 500g/ha with recommended dose of urea @ 60 kg/ha improved the grain yield of rice over a period of three years by 8 q/ha for Basmati 1407 and 5-6 q/ha for SHABNAM and PUSA 1121 as compared to urea alone and control whereas BGA application alone showed increase by 6 q/ha for rice variety BASMATI 1401 followed by 4 q/hafor PUSA1121 and by 3 q/ha for PR118 and SHABNAM. Urea alone application @ 60 kg/ha showed increase only by 2-3q/ha for PUSA1121 and BASMATI 1401 whereas decrease in yield was observed by 2 q/ha by third year for PAU 201 and PR 118. Control plots also showed decrease in grain yield

from 3 q/ha for BASMATI 1407 (10-07 q/ha) to 6 q/ha for PR118 (20-14 q/ha).There was increase in soil organic carbon from 0.32 to 0.53%, total nitrogen from 0.042 to 0.067% and phosphorus content from 7.9-27.70 mg/kg as a result of application of cyanobacterial biofertilizers. In RBD block experiment yield of rice increased in BGA + Urea inoculated blocks at three different agricultural sites over control. Fly ash based cyanobacterial biofertilizers (BGA @ 500 g/ha) in combination with nitrogenous fertilizers (Urea @ 60 kg/ha) was effective in increasing yield of rice crop and soil nutrients in three years (2010-2013 for all five rice cultivar viz. PUSA 1121, PAU 201, SHABNAM, PR 118 and Basmati 1407.

Fly ash based algal biofertilizers makes rice production system more viable and reduce the ecological hazards caused due to synthetic fertilizers and serves as one of the component of integrated plant nutrient supply system through net saving's in urea by 15-35% which is an indirect income for farmers.The process development and standardised is ecofreindly and does not include costly plant and machinery and can be adopted easily by farmers.

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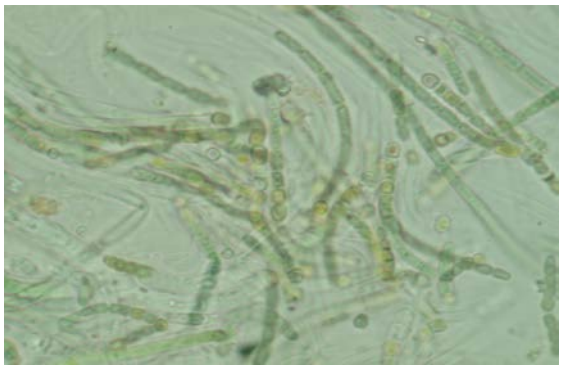
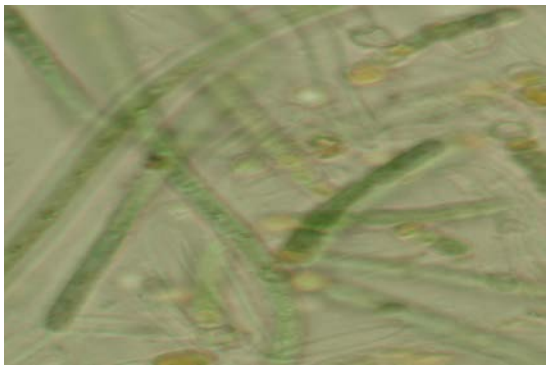

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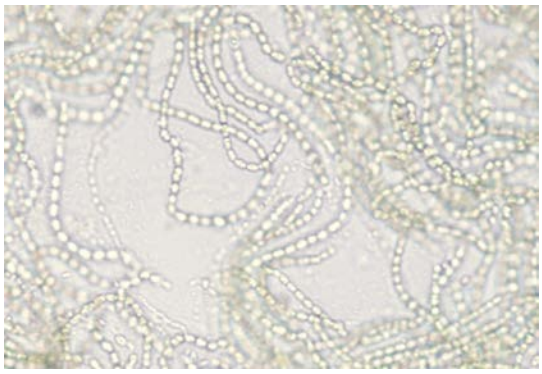

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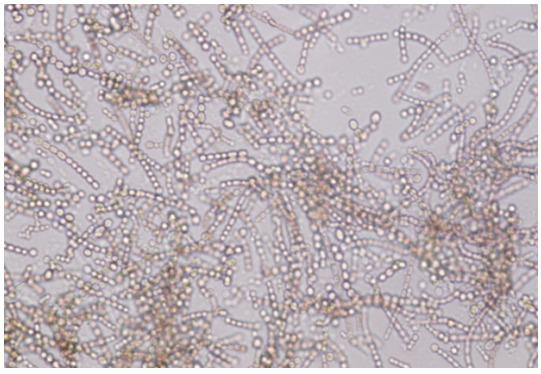
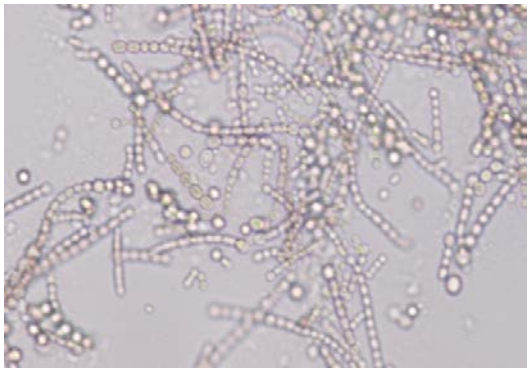

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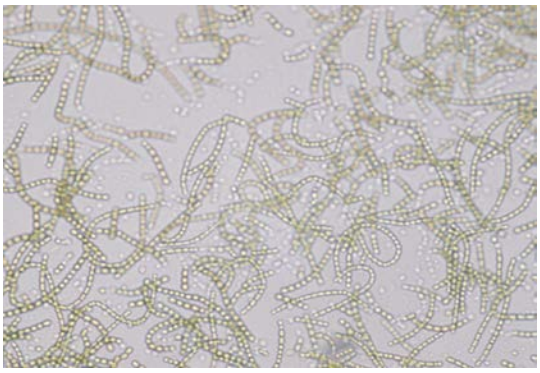


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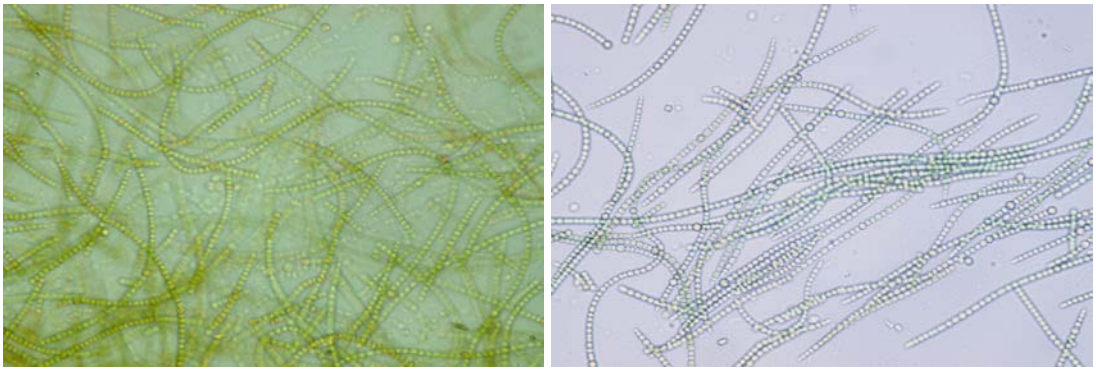

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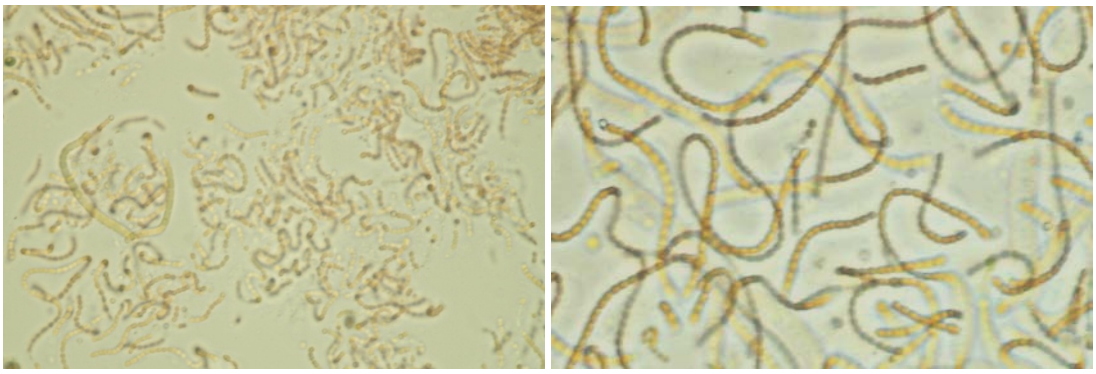

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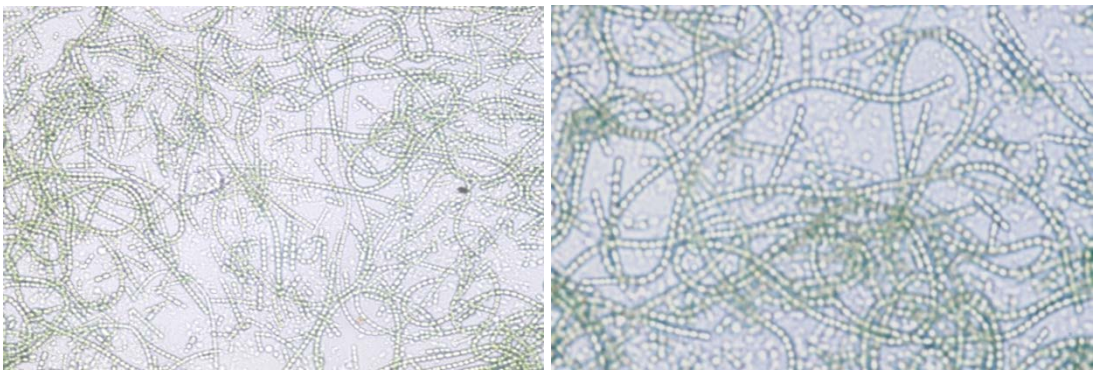

Isolate G: *Anabaena sp.*

PLATE1. Efficient strains of BGA isolated from paddy field soil and as pond sample.

Isolate A: *Calothrix* sp.; Isolate B: *Anabaena flos-aqaue* (ash pond BGA); Isolate C: *Desmonostoc* sp. DGRKC; Isolate D: *Nostoc commune*; Isolate E: *Nostoc* sp.PS1; Isolate F: *Nostoc* sp.*DGRKF 275*; Isolate G: *Anabaena* sp.

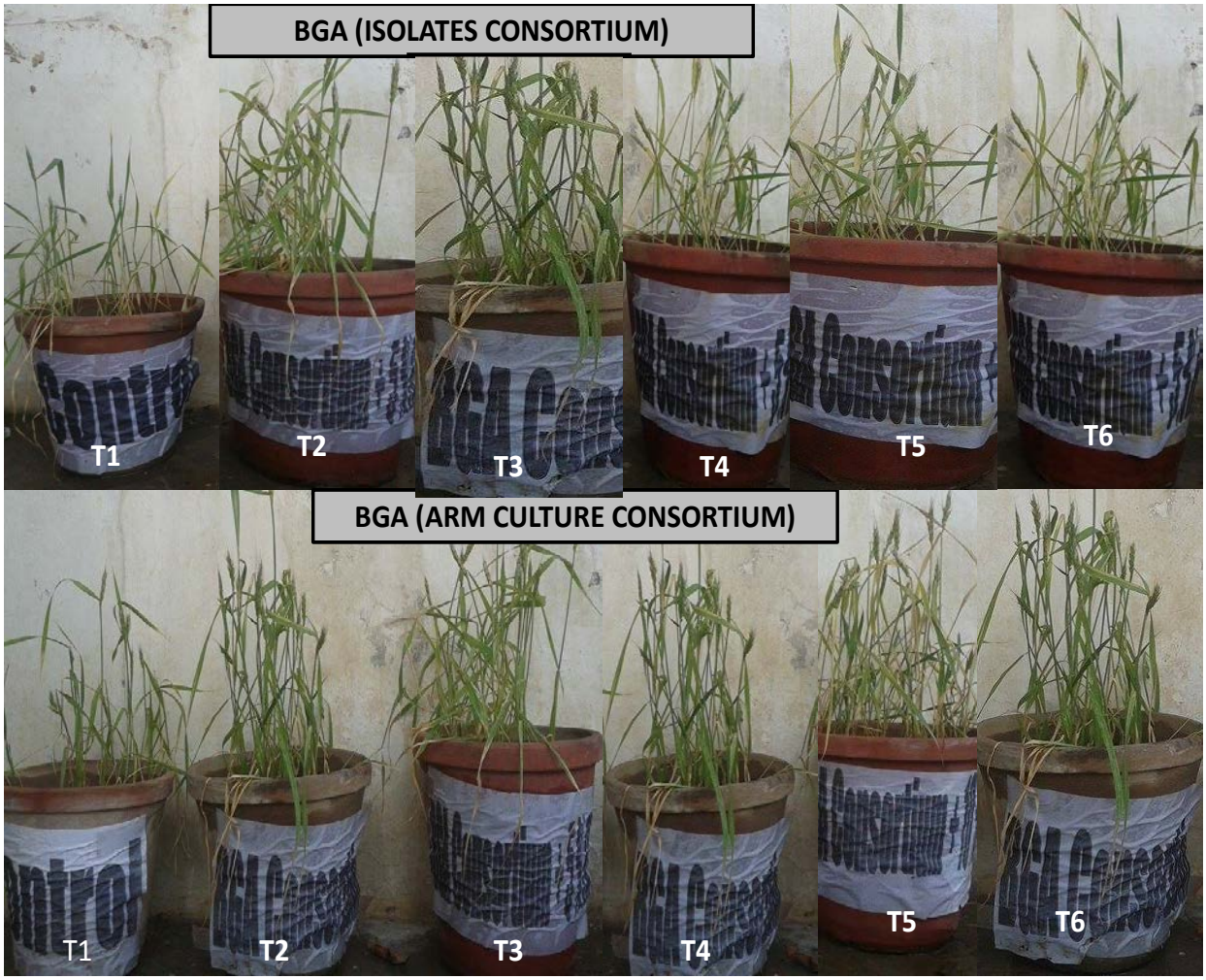

Plate 2: Influence of consortium of BGA inoculants on soil properties during pot experiment of rice crop after 90 DAT

Treatment details of different carrier material are as follows:

T1 Control (Only soil)

T2 Montmorillonite 100%

T3 Fly ash + soil (50:50)

T4 Fly ash + Montmorillonite (50:50)

T5 Fly ash + Montmorillonite (10:50)

T6 Fly ash 100%

Village Dittupur **Village Laut** **Village Shauli**

Village Bhojomajri **Village Gunike** **Village Khanoure**

Village Bhojomajri **Village Laut** **Village Palia Khurd** **Village Shauli**

PLATE 2: Different agricultural sites where field trials were conducted for three consecutive years (2010-2013) for rice crop.

a) Village Pedhna

b) Village Ajnoida

PLATE 3 RBD plots conducted at different villages of Nabha and Patiala, Punjab.

ANNEXURE

NCBI GenBank detaiLs of blue green algal Isolates

BankIt1737815 Seq1 KM083062 Desmonostoc sp. DGRKC (Isolate C)
BankIt1737815 Seq2 KM083063 Nostoc sp. DGRKF (Isolate F)

```
LOCUS       Seq1                 213 bp    DNA     Linear       02-JUL-2014
DEFINITION  Desmonostoc sp. DGRKC 16S ribosomal RNA gene, partial sequence.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      Desmonostoc sp. DGRKC
  ORGANISM  Desmonostoc sp. DGRKC
            UncLassified.
REFERENCE   1  (bases 1 to 213)
  AUTHORS   Kaur,R. and Goyal,D.
  TITLE     Sequence of nitrogen fixing blue green algae Isolated from paddy
            field soils
  JOURNAL   UnpubLished
REFERENCE   2  (bases 1 to 213)
  AUTHORS   Kaur,R. and Goyal,D.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2014) Department of BiotechnoLogy, Thapar
            University Patiala, Bhadson road, Patiala, Punjab 147 004, India
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Particulate Science and Technology: An International Journal

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/upst20

Mineralogical Studies of Coal Fly Ash for Soil Application in Agriculture

Rajinder Kaur [a] & Dinesh Goyal [a]

[a] Department of Biotechnology , Thapar University , Punjab , India

Accepted author version posted online: 29 Jul 2014.

To cite this article: Rajinder Kaur & Dinesh Goyal (2014): Mineralogical Studies of Coal Fly Ash for Soil Application in Agriculture, Particulate Science and Technology: An International Journal, DOI: 10.1080/02726351.2014.938378

To link to this article: http://dx.doi.org/10.1080/02726351.2014.938378

Mineralogical Studies of Coal Fly Ash for Soil Application in Agriculture

Rajinder Kaur[1], Dinesh Goyal[1]

[1]Department of Biotechnology, Thapar University, Punjab, India

Address correspondence to Dinesh Goyal, Department of Biotechnology & Environmental Sciences (DBTES), Thapar University, Bhadson road, Patiala, 147004, Punjab, India.E-mail: dgoyal@thapar.edu

Abstract

Coal fly ash procured from Guru Gobind Singh Super Thermal Power Plant, Ropar, Punjab, India was analysed for its mineralogical content and thermal stability by X-ray diffraction (XRD), thermal gravimetric analysis (TGA), fourier-transform infrared spectroscopy (FTIR) and physicochemical properties. XRD studies showed that major crystalline phases observed were quartz (SiO_2) and aluminium silicon oxide ($Al_{4.52}Si_{1.48}$) with macro and micro element (N, P, K,Mg, Zn, S and Fe). Fly ash showed thermal stability upto 500 ºC and reduction in weight was upto 200°C, primarily due to loss of water and decarboxylation asrevealed by TGA plots. FTIR of fly ash showed that the most prominent peaks in the spectra correspondedto Si-Oand Al-Ostretch vibrations. Coarse grain accumulation of fly ash indicated presence of 70% of fine grained particles of 0.075mm. Coal fly ash was alkaline in nature (pH 7.85±0.03) with electrical conductivity (0.14±0.02µS/m), water holding capacity (62%) andlow bulk density (0.99g /cm^3) having surface area of 0.96 m^2 /g. With properties similar to that of soil coal flyash represents a suitable material for use in specific quantities as a soil amending agent in agriculture.

KEYWORDS: fly ash, thermal stability, XRD, FTIR, physicochemical analysis, coarse grain accumulation.

1. INTRODUCTION

Fly ash, a by-product of the coal-burning industry, has been recognized as a potential soil amendment for increasing the availability of mineral nutrients to improve soil fertility and plant growth (Mittra et al. 2005; Lee et al. 2006; Pandey andSingh 2010). Flyash is utilised in manufacturing cement, concrete, bricks (Singh 1998; Asokan et al. 2005), wood substitute products (Saxena and Prabhakar2000; Haynes 2009) for soil stabilization, in road base/embankments and consolidation of ground, land reclamation and as a soil amending agent in agriculture (Jala and Goyal 2006; Haynes 2009; Alam and Akhtar 2011). Presence of almost all essential plant nutrients in ionic form and positive impact on physico-chemical properties of soil makes fly ash a useful adjunct for crop production especially on variously problem soils and waste lands (Mittra et al. 2005; Pandey and Singh 2010; Singh et al. 2013;Masto et al.2012). Problem soil refers to deficiencies of macronutrients (N, Pand K) and micronutrients (Zn,Fe,Cu and Mn) which are essential for plant growth (Singh 2013; Srinivasan 2013). Presence of K, Zn, Mg, Na, Ca and Fe in flyash makes it suitable material for reclaiming problem soil by increasing available nutrients for plant growth (Sharma and Kalra2006). Indian fly ashes have low bulk density, high water holding capacity and porosity, rich silt-sized particles, alkaline nature, negligible solubility, and reasonable plant nutrients (Ram et al.2011).A careful assessment on physicochemical propertiesof both fly ash and soil is required before its application as a soil-ameliorating agent. In the present study coalflyash collected from

Guru GobindSingh Super Thermal Power Plant, Ropar, Punjab, India was studied for its mineralogical composition and physicochemical parameters for its safe and effective application in agriculture as a soil ameliorating agent.

2. MATERIALS AND METHODS

2.1.1 C*ollection, Processingof Fly Ash And Soil*

Coal fly ash was collected from Guru Gobind Singh Super Thermal Power Plant, Ropar, Punjab, India. Field soil was collected from experimental plot of Science & Technology Entrepreneur's Park (STEP), Thapar University, Patiala, India. Prior to analysis fly ash and soil samples were air dried in shadeand passed through 2mm sieve for physical analysis and 0.2mm sieve for chemical analysis.

2.1.2 *Physicochemical Analysis Of Fly Ash And Soil*

The physicochemical properties of fly ash and soil werecarried out following standard protocols. pH and electrical conductivity (EC) were measured as per the protocol given by Jackson (1967), water holding capacity (WHC) and bulk density were determined using the protocol given by Black et al.(1965).Surface area in terms of "number of units of surface area "contained in a "unit weight" of a material was done by BET (Brunauer – Emmett-Teller) and coarse grain accumulation was done by sieve method of Sugita (2001), using round sieves of different set of different pore size (2mm, 0.500mm, 0.075 mm, 0.025 mm, 0.010 mm). Total element concentration (mg kg^{-1}) of nitrogen (N), phosphorus (P), potassium (K), zinc (Zn), sulphur (S), magnesium (Mg), chromium (Cr, cadmium (Cd), iron (Fe) and lead (Pb) in coal fly ash and soil was analysed after

concentrated nitric acid digestion (Page et al.1982) by Atomic absorption spectrophotometer (GBC 932AA).

2.2. Microstructural Characterisation

2.2.1 X- Ray Diffraction

Samples were characterized using X- ray diffraction to identify the formed phases (Singh et al. 2012). The powdered sample of fly ash was pressed into the aluminium holder and subjected to Cu-K radiation between 10-80ºC. X-ray powder diffraction study was performed at room temperature using PANanalyticalX'Pert PRO system with Ni- filter. During the experimentstep size was 0.013°/min and X-ray diffractogram of coal fly ash was obtained and identified.

2.2.2 Differential Thermal And Thermal Gravimetric Analysis (DTA /TGA)

Differential thermal analysis (DTA) was performed in nitrogen atmosphere using Perkin Elmer (Model: Diamond Pyris TG/DTAanalyzer) to check thermal stability and phase transition (Singh et al. 2012). Thermal gravimetric measurements of the samples were performed using Al_2O_3 powder as reference material in nitrogen atmosphere at heating rate of 10 °C/min from 50 °C to 900 °C. The temperature and weight loss detection limit of the instrument are ± 1ºC and 0.001mg respectively.

2.2.3 Fourier Transform Infrared Spectra (Ftir)

FTIR spectra were obtained at room temperature by using Perkin Elmer model RZX spectrometer in the region 400-2000 cm^{-1}. The spectrum of each sample was normalized to the spectrum of blank potassium bromide (KBr)(Singh et al. 2012).

3. RESULTS AND DISCUSSION

3.1 Physico-Chemical Properties Offly Ash And Soil

Fly ash collected from Guru GobindSinghThermal Power Plant, Ropar, India had pH 7.85, electrical conductivity (EC) 0.14 μS/m, bulk density 0.99 g/cm^3 and surface area 0.96 m^2/gm,which were lower than soil except high water holding capacity which was 62 %, higher than soil (Table1).Alkaline coal fly ashes are favourable for soil application in large quantities as it neutralizes acidity, raises pH associated with acid soil, reduces hydraulic conductivity of the soil and reduces acid mine drainage problem (Chang et al.1977; Gitari et al. 2006; Skousen et al. 2013). High water holding capacity with low bulk density of coal fly ash makes it a suitable soil amending agent,since soils amended with fly ash soils have tendency to reduce bulk density, increase soil porosityas well as water holding capacity with improvement in soil texture (Chang 1977; Page 1979; Sharma and Kalra 2006;Mahale et al. 2012).Flyash also increases essential plant nutrient availability which further lead to increase in plant growth and crop yield (Jala and Goyal 2006;Mishra et al. 2012; Kishore et al. 2009).Coarse grain accumulation (Table 2) of fly ash indicates presence of fine grained particles with more than about 70% of the particles finer than 0.075mm as compared to soil. Indian fly ashes are predominantly silt sized fine material with 60-90% of fly ash particles finer than 0.075 mm sieve (Rai et al.2010). The silt-sized particles, low bulk density (BD), higher water holding capacity (WHC),

favorable pH, and plant nutrients in fly ash has also been reported by Ram and Masto (2014) as a potential soil amendment agent .Coal fly ash showed the presence of major elements of phosphorus (19.02 mg kg^{-1}); potassium (52 mg kg^{-1}) and nitrogen (0.009%); microelements were iron (1581 mg kg^{-1}); zinc (59.54 mg kg^{-1}); sulphur (1901 mg kg^{-1}); magnesium (101.5 mg kg^{-1}) and heavy elements were chromium (5.32 mg kg^{-1}), lead (18 mg kg^{-1}) and cadmium was below detection limit. Concentration of major and micronutrients in coal flyash is compared with soil in Table 3. Fly ash contains significant quantities of Si, Al, Fe, Ca, K, S, Na, Mo and trace elements of As, Pb, Se and Cd (Chang et al. 1977; Ram and Masto 2009; Adriano et al. 2001; Skousen 2013). Presence of heavy metals and trace metals restricts the direct application of coal fly ash in soil at high concentration, although these elements are low and in permissible range as reported in coal Indian fly ashes by Rautray et al. (2009). Previous studies reports that fly ash application at 1, 5, 10, 18, 20, 30 and 40% (w/w) in soil has been reported to increase its pH, electrical conductivity, modify water retention capacity and improves plant growth condition on mine soils (Kalra et al. 2000, Khan et al. 1997,Goyal et al. 2002, Hu et al. 2004).

3.2 Microstructural Characterisation

3.2.1 X –Ray Diffraction (XRD)

Major crystalline phases in fly ash detected were of quartz (SiO_2), mullite ($Al_{4.56}Si_{1.44}O_{9.72}$) with small amount of calcium oxide (CaO), iron oxide (Fe_2O_3), magnesium oxide (MgO), sodium oxide (Na_2O) and potassium oxide (K_2O) respectively. (Table 3) (Figure1).Literature reports that Indian coal fly ash is an amorphous,

ferroaluminosilicate material predominantlyenriched in oxides of Si, Al, Fe and Ca (95–99%) and Na, P, K and S (0.5–3.5%) and the remainder of the ash is composed of trace elements (Kumar et al. 2000,Basu 2009,Mishra and Das 2010).These available nutrients could satisfy the growth demand of plants and neutralizes the pH of soil. Coal fly ash comprised of quartz, mullite and iron oxide is considered as chemical conditioner of degraded soil for nutrient restoration managing soil fertility and is used in synthesis of artificial zeolite for water treatment because of high content of aluminosilicate glass(Querol et al.1997., Hwa et al. 1997; Pathan et al. 2003; Gatima et al. 2005).

3.2.2 Differential Thermal And Thermal Gravimetric Analysis (DTA/TGA)

To study the thermal chemical behaviour of coal fly ash in terms of degree of decomposition of unburnt carbon present in coal fly ash DTA/TGA was conducted (Miyazawa et al. 2000).TGAplot showed stable thermal effect upto500°C with weight loss of nearly 0.5% was observed at 200°C, primarily due to loss of water and decarboxylation(Figure 2).Terzic et al. (2013) also reported reduction in weight of coal flyashat 200 °C, which corresponded to the volatilization of the water. Coal fly ash showed stable thermal effect with less weight loss that supports its application in soil would enhance soil organic carbon portion maintaining humus content in the soil (Leifeld et al. 2006).

3.2.3 Fourier-Transform Infrared Spectroscopy (Ftir)

To understand the surface chemistry in terms of structure and bonding of raw fly ashand to determine silicate backbone of coal flyashFTIR was carried out. The FTIR

spectroscopic studies reveal that band near 564- 796 cm^{-1} and 911-1090 $cm^{-1}$was due to Al–O and Si–O stretch vibrations, whereas band near to 2890 cm^{-1} was due to C-Hvibrations in original fly ash followed by O-H stretch vibration at 3402 respectively (Figure 3). Results are supported by the findings of Sarkar and his co-workers in 2006 that strong mineral bands (Si –O –Si/Si –O stretching) are observed at 1031-1095 cm^{-1} in all the fractions of Indian fly ash (magnetic/non-magnetic) that marks the presence of kaolinite, quartz and mullite. Peak 2887 cm^{-1} and above could be assigned to C-H stretching vibration of organic contaminants or some hydrocarbonpresent in fly ash, whereas peak observed at 3096-3553 corresponds to O-H bonding (Katara et al. 2013). Previous FTIR studies reports that coal fly ash modified with humic substances could be evaluated for agricultural uses and as soil conditioner (Chassapis et al. 2010).

4. CONCLUSION

Coal fly ash from Guru Gobind Singh Thermal Power Plant, Ropar India was alkaline with high water holding capacity and porosity, low bulk density, rich silt and sand sized particles and thermally stable. Microstructural and physico-chemical characterization of coal fly ash reveals that flyash consists of major crystalline phase of mullite and quartz and contains various nutrients such as N (0.009%), P (19.02 mg kg^{-1}) K (52 mg kg^{-1}), S (1901 mg kg^{-1}), Fe (1581 mg kg^{-1}), Zn (59.54 mg kg^{-1}) and Mg (101.5 mg kg^{-1}),which makes it as a potential inorganic soil ameliorant to restore soil nutrient deficiency and improving crop yield. Fly ash amendments in soil at controlled rates are recommended for its effective and gainful utilization in agriculture.

ACKNOWLEDGMENTS

The authors are thankful to Director, Thapar University, Patiala and Science & Technology Entrepreneur's Park (STEP), TU, for providing infrastructural and support and to Fly ash unit (FAU), Department of Science &Technology (DST) and National Bank for Agriculture and Rural Development (NABARD) for financial support.

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Table 1. Physico -chemical characterisation of Fly ash and soil

S. No	Parameters	Fly ash	Soil
1.	pH	7.85±0.12	8.22±0.1
2.	EC (μS/m)	0.14±0.02	2.45±3.75
3.	Bulk density(g/cm^3)	0.99±0.01	1.36±0.01
4.	Water holding capacity (%)	62.0±2.7	39.6±2.03
5.	Surface area (m^2/gm)	0.96±0.1	5.45±0.01

(n =3, Mean ± SE)

Table 2. Coarse Grain accumulation (Comparison of Percentage (%) finer for Fly ash and soil sample)

S. No	Particle size (mm)	Fly ash	Soil
1.	2	100	100
2.	0.500	87±0.03	88±0.02
3.	0.075	70±0.01	51±0.02
4.	0.025	51±0.41	47±0.01
5.	0.010	42±0.01	38±0.05

(n =3, Mean ± SE)

Table 3. Chemical composition of fly ash and soil

Element	Fly ash (mg kg^{-1})	Soil (mg kg^{-1})
P	19.02±0.1	7.43±0.2
K	52±0.5	11.5±0.1
N (%)	0.009±0.3	0.007±0.05
Fe	1581±0.01	116.28±0.1
Zn	59.54±0.05	BDL
Pb	18±0.02	12.95±0.5
Cr	5.32±0.1	0.95±0.08
S	1901±0.06	59.2±0.02
Mg	101.5±0.15	95±0.04
Cd	BDL	BDL

Table 4. Spectral bands observed at different wavelength in the peaks of Fourier transform infrared spectra (FTIR) of fly ash

S. No	Wavenumber (cm^{-1})	Bonds
1.	468	Si-O
2.	564- 796	Al-O
3.	911-1090	Si-O
4.	2890	C-H
5.	3402	O-H

Figure 1. X –Ray diffraction (XRD) of fly ash.

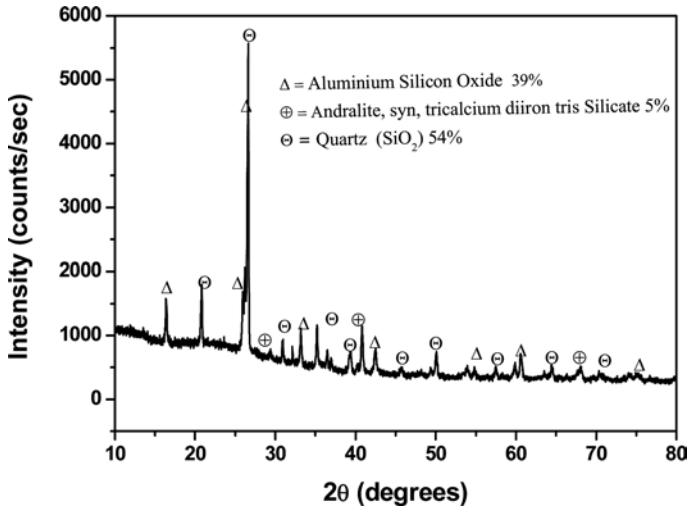


Figure 2. Differentialthermal and thermal gravimetric analysis of flyash.

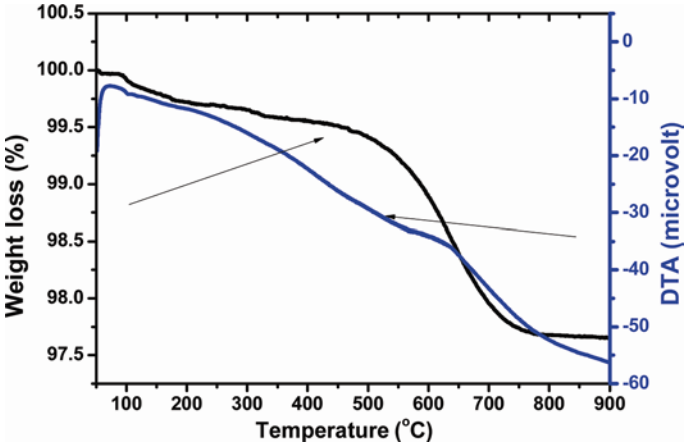
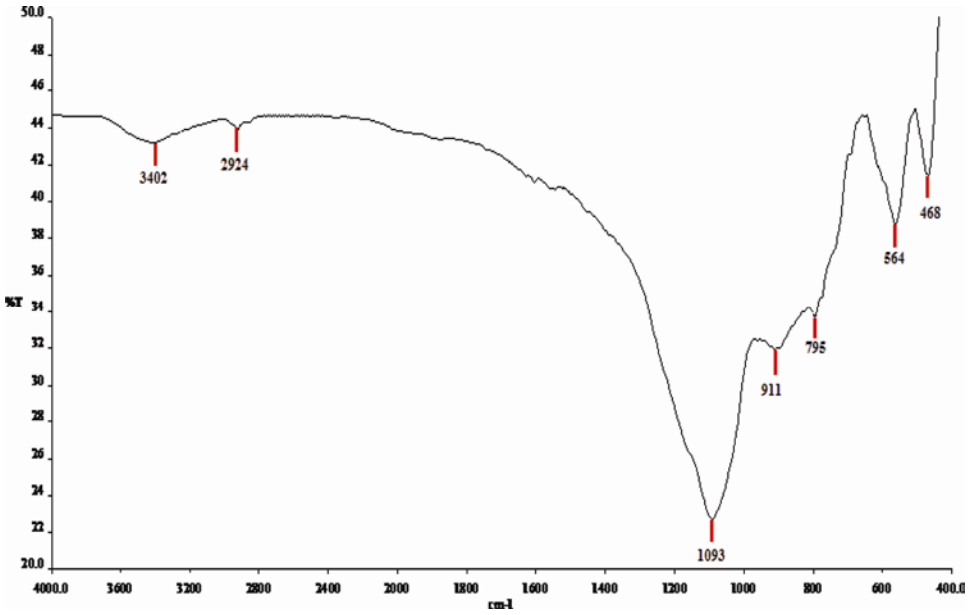


Figure 3. Fourier-transform infrared spectroscopy (FTIR) of fly ash.



VEGETOS

Vol. 27 (2) : 291-300 (2014)

DOI: 10.5958/2229-4473.2014.00047.0

Soil Application of Fly Ash Based Biofertilizers for Increased Crop Production

Rajinder Kaur and Dinesh Goyal*

Received: 6.12.13/ Revised: 29.5.14/ Accepted: 20.7.14/ Published online: 30 September, 2014
This article is published in open access at www.vegetosindia.org

Abstract

Field trials were conducted in five consecutive years (2006-2011) in 20 different villages of Nabha, District Patiala, Punjab to observe the effect of fly ash based bacterial bio fertilizers viz., nitrofix and phosul on wheat (HD-2967and PBW-343) and blue green algal biofertilizers on rice (PUSA 1121and Basmati 1401) cultivars to reduce the application of chemical fertilizers, improve soil nutrient status and crop yield. Positive response and upward trend in wheat and paddy crop was observed with significant increase in paddy yield ranging from 5-23 quintals per acre in five year (2006-2011). Increase in yield of wheat was observed during field demonstration from 2 quintals per acre in 2006 to 20 quintals per acre in 2011 with reduction in application of urea and SSP from 6-30%. An overall increase in soil organic carbon from 0.10-0.51%, total nitrogen from 0.001-0.08% and available phosphorus from 5-26 mg/kg of soil was observed during field trials conducted during 2006-2011 as a result of application of microbial inoculants.

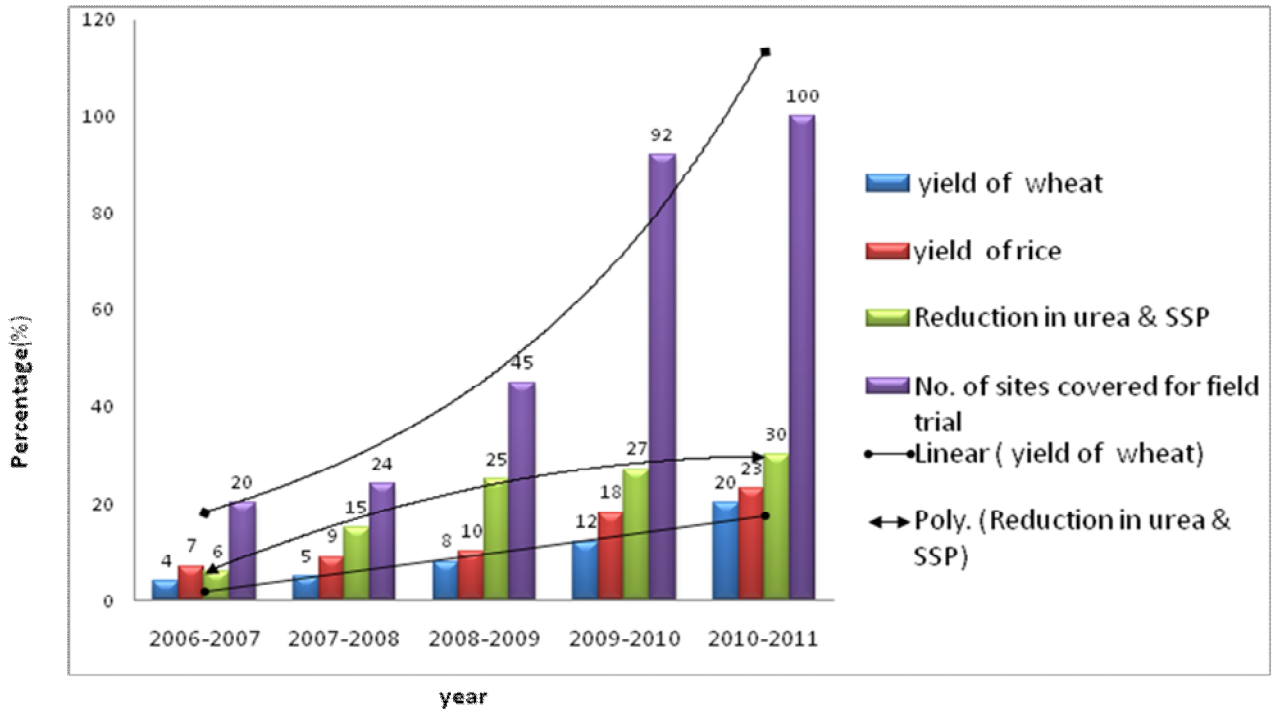
Key words: Bio-fertilizers, flyash, wheat, paddy, urea & SSP reduction, soil nutrients

Introduction

Utilization of fly ash in agriculture in an effective manner has become essential to prevent soil deterioration and replenishing other soil nutrients and is an alternative approach for fly ash management (Cheung *et al.* 2000, Jala and Goyal 2006, Pandey 2010). Fly ash is produced as a by-product of burning coal in thermal power plants and its production in India will exceed 200 million tons by 2020 (Kalra *et al.* 1998). Fly ash amendment in soil improves K, Ca, Mg and S status of deficient soil (Srivastva and Chhonkar 2000, Deshmukh *et al.* 2000, Gaind and Gaur 2002), however N and P deficient soil could be restored by the co application of fly ash and microbial inoculants. In present era, soil deterioration due to excessive usage of chemical fertilizers has been already reported for which application of organic materials are strongly recommended as an alternative supplement to chemical fertilizers so as to retain soil productivity (Kanu *et al.* 2013, Laudicia *et al.* 2011). Fly ash has been evaluated for possible use as carrier for *Azotobacter* and *Azospirillum* formulation in wheat crop to reduce the application of chemical fertilizers (Kumar and Singh 2010). Fly ash has also been used for neutralization of acidic mine spoils and restoration of nutrient balance in alkaline wastelands (Adriano *et al.* 1980, Jala and Goyal 2006). Studies showed that fly ash amended at 1% and 5% application rates improved soil and plant growth conditions on mine soil in China (Skousen *et al.* 2013). A number of forestry species like *Dalbergia sisso*, *Albizzia lebbek, Eucalyptus tereticornis Acacia auriculiformis, Populus deltoides, Acacia nilotica* and flowering and aromatic plants like marigold, lemongrass, gladioli and lily have been successfully grown on fly ash amended wastelands resulting in biomass production without any adverse effects (Adholeya *et al.* 1997, Goyal *et al.* 2002, Aggarwal and Goyal 2007). Flyash and its different combinations with soil (w/w) were tested for use as a carrier for diazotrophs (*Azotobacter chroococcum, Azospirillum brasilense*) and phosphorous bacteria *Bacillus circulans, Pseudomonas striata*), which showed their maximum viability in flyash alone or soil:flyash (1:1) combination (Gaind and Gaur 2004). In conjuction with organic manure and microbial inoculants, flyash enhances plant biomass production from degraded soils. Chemical fertilizers are one of the major nutrient suppliers besides organic and green manures, despite excessive use of chemical fertilizers, the gap between the nutrient removal and replenishment is significantly high. The microbial inoculation in the form of seed bacterization has proved beneficial for the maintenance of soil health and the use of suitable carrier, capable of supporting high viable microbial population for a prolonged duration

Department of Biotechnology and Environmental Sciences, Thapar University, Patiala 147004, Punjab, India
*Corresponding author E-mail: dgoyal@thapar.edu

Fig 1. Crop productivity, reduction in application of urea and SSP and number of sites put under field trial over five year period.



(Gaind and Gaur 2004). Application of biofertilizers along with organic manure significantly improved the quality parameters, nutrient uptake and fertility status of soil over control. Organic treatments i.e FYM (Farmyard manure), PM (Poutry manure) and vermicompost with the application of biofertilizers (*Azotobacter.* sp and Phosphorus solubilising bacteria) significantly improves NPK status of the soil over control along with some secondary nutrients. (Yadav *et al.* 2013). Number of carrier material including charcoal soil mixture, wheat bran (Gaind and Gaur 1990) peat, press mud (Jauhri and Philip 1984), calcium alginate, rock phosphate based soil implant formulation (Viveganandan and Jauhri 2000, 2002) have been found successful in maintaining high shelf life of phosphobacteria and diazotrophs, still there is wide scope of exploring cheap and easily available waste material as a carrier for bio inoculants (Gaind and Gaur 2004). The present investigation was carried out with the objective to assess the effect of fly ash based biofertilizers in cultivation of wheat and rice to reduce the utilization of chemical fertilizers in different villages of Nabha, District Patiala, Punjab. Fly ash based biofertilizers were developed and used for field trial to examine the combined effect of fly ash with microbial inoculants on yield of wheat and rice.

Materials and Methods

The ESP fly ash was collected at yearly intervals from Sturdy industries, Guru Gobind Singh Thermal power plant, Ropar, Punjab and analyzed for pH and electrical conductivity (Jackson 1973), organic carbon (Walkley and Black 1934), available phosphorous (Olsen 1945), and total nitrogen (Piper 1960).

Development of carrier based microbial inoculants:

Bacterial inoculants such as nitrogen fixing bacterium *Azotobacter* and phosphate solubilising bacterium *Pseudomonas* strains were grown on Jensen medium and Pikovskaya medium for 6-7 days as per Bureau of Indian Standards (BIS) viz. BIS: 9138-2002 and BIS: 14807-2000 respectively (Agarwal 2005). After checking the culture for purity and proper growth, the culture was transferred into fermentors for large scale production containing sterilized medium grown for 3-4 days at 30°C with constant stirring. The well grown culture was directly mixed manually aseptically in sterilised fly ash in 1:1 ratio and 250 gm was packed in polythene bags and stored at 4°C under dark conditions till further use. BGA biofertilizers containing a mixture of heterocystous nitrogen fixing BGA–*Anabaena variabilis, Tolypothrix tenuis, Nostoc muscorum, Aulosira fertilissimia* procured from National Centre for Conservation and Utilisation of Blue Green Algae, Division of Microbiology, IARI, New Delhi were grown separately in open algal ponds, harvested by filtration using nylon nets and wet algal biomass slurry was mixed thoroughly, dried and 500 gm of powdered fly ash based BGA inoculum was packed in polythene bags and stored in cool, dry and dark conditions till further use. The algal biofertilizers produced

Table 1. Physicochemical analysis of fly ash

Parameters	Mean ± SE
pH	7.85±0.12
EC (µS)	0.14±0.02
Organic Carbon (%)	0.06±0.03
Total Nitrogen (%)	0.15±0.01
Available Nitrogen (%)	0.004±0.5
Available Phosphorus (ppm)	20.4±1.0
Available Sulphur (ppm)	254.7±17
Water Holding capacity (%)	62.0±2.7
Fe (ppm)	1581±0.01
Zn (ppm)	59.54±0.05
Mn (ppm)	28±0.01
Pb (ppm)	18±0.02
Co (ppm)	3.96±0.5
Cr (ppm)	5.32±0.1
Ni (ppm)	8.4±0.01
Cd (ppm)	BDL*

*BDL = Below Detection Limit

contained 10^5-10^6 propagules per gram of carrier material.

Soil characteristics and field trials

Field trials were conducted at different agricultural villages of Nabha district Patiala, Punjab viz., Bhojomajri, Sibro, Khanoure, Narainganj, Kansuha, Gaushala, Gunika, Dittupur, Laut, Palia Khurd, Shauli and Dakala for five consecutive year's viz. 2006 – 2011 to study the effect of application of flyash based bacterial and algal biofertilizers on yield of wheat (HD-2967and PBW-343) and rice (PUSA 1121 and Basmati 1401) varieties respectively and to reduce the application of chemical fertilizer. Punjab, located between 30.79° N latitude and 76.78° E longitudes, one of the north Indian states has a subtropical, semiarid and monsoonal climate with topography varying from sand dunes to piedmont plains. The average monthly air temperature ranges between 13 ◦C in January and 34 ◦C in June. Annual rainfall ranges from 1250 mm in the North to 350 mm in the Southwest. More than 70 percent of the annual rainfall occurs during the monsoon season from July to September (Benbi *et al.* 2009). Soil treatment of flyash based microbial inoculants was done by mixing 250gm of flyash based bacterial inoculum with 4 kg of dry sieved soil and broadcasted in one acre of land under wheat cultivation at seedling stage. Similarly, 500gm of flyash based BGA inoculum was mixed with 4 kg of dry sieved soil and was broadcasted over the standing water in the rice field in one acre area. Application was done immediately after the transplantation and farmers were informed to apply urea or pesticides only 7 -8 days either before or after algalization. Overall 2032 acres of agricultural land was put under demonstration for wheat and rice cultivation

Table 2. Production of fly ash based bacterial (nitrofix and phosul) and blue green algal (BGA) inoculants during 2006 -2011 for field application

Microbial Inoculants	Production of fly ash based bacterial and blue green algal bio fertilizers (Kgs)	Area covered (acre)	Number of sites put under trial
2006 -2007			
Nitrofix	65	48	12
Phosul	67	56	12
BGA	29	42	08
2007 -2008			
Nitrofix	157	91	14
Phosul	151	94	14
BGA	25	43	10
2008 - 2009			
Nitrofix	250	224	20
Phosul	250	200	20
BGA	30	50	25
2009 – 2010			
Nitrofix	220	200	53
Phosul	234	200	57
BGA	70	77	39
2010 – 2011			
Nitrofix	360	320	61
Phosul	321	301	59
BGA	74	86	40
Total	**2303**	**2032**	**444**

Table 3. Physicochemical analysis of soil of different fields put under trial in Patiala district

	Before sowing of crop				After harvesting of crop			
Farmer's Name / Village	**pH**	**Organic Carbon (%)**	**Total Nitrogen (%)**	**Available Phosphorus (mg kg^{-1})**	**pH**	**Organic Carbon (%)**	**Total Nitrogen (%)**	**Available Phosphorus (mg kg^{-1})**
Acchhar singh, Bhojomajri	7.86±0.04 c	0.26±0.03 cdefgh	0.023±0.002 ef	11.03±1.04 defghi	7.70±0.02 fg	0.33±0.02 d	0.043±0.002 fg	15.3±2.1 cde
Rajinder singh, Bhojomajri	7.53 ± 0.02 d	0.32±0.01 ab	0.021±0.002 f	8.6±1.18 i	7.35± 0.04 hi	0.42±0.02 bc	0.041±0.001 gh	13.3±1.5e
Darshan singh, Sibro	7.13 ± 0.02 h	0.19±0.03 ij	0.027±0.002 cdef	12.2±2.27 cdef	7.05±0.03 j	0.24±0.02 e	0.032±0.002 i	13.5±1.3 e
Malkit singh, Khanoure	7.29 ± 0.02 fg	0.18±0.02 j	0.030±0.001 cdef	12±0.60 cdefg	7.30±0.02 i	0.31±0.03 de	0.045±0.002 fg	14.4±0.6 cde
Kamalvir singh, Narainganj	7.10±0.02 hi	0.23±0.02 efghij	0.018±0.002 f	13.7±1.07 cde	7.14±0.06 j	0.36±0.04 bcd	0.036±0.003 hi	15.1±0.3 cde
Chamkaur singh, Kansuha	6.64 ± 0.03 k	0.28±0.01 bcdef	0.023±0.001 def	9.78±0.7 fghi	7.04±0.03 j	0.35±0.03 bcd	0.044±0.001 fg	16.3±0.6 cde
Guashala, Nabha	8.52 ± 0.01 a	0.33±0.02 ab	0.037±0.003 b	10.7±0.52 efghi	8.39±0.02 a	0.43±0.02 b	0.046±0.003 efg	20.7±0.5 b
Satguru singh, Gunika	7.44 ± 0.03 e	0.25±0.02 defghi	0.035±0.002 b	17.7±1.5 b	7.96±0.04 cd	0.37±0.02 bcd	0.081±0.002 a	26±1 a
Balwant singh, Khanoure	7.03 ± 0.02 hi	0.21±0.01 ghij	0.022±0.001 f	10.4±0.6 fghi	7.16±0.07 j	0.32±0.01 de	0.043±0.003 fg	14.7±0.5 cde
Paramjit singh, Dittupur	8.03 ± 0.02 b	0.36±0.02 a	0.020±0.004 f	12.2±0.8 cdefg	8.08±0.05 b	0.51±0.02 a	0.049±0.001 def	16.4±0.5 cde
Harisingh, Laut	7.15 ± 0.03 h	0.27±0.02 bcdefg	0.044±0.003 a	14.3±0.61 c	7.44±0.02 h	0.31±0.02 de	0.060±0.002 b	17±0.9 cd
Kripalsingh, Paliakhurd	6.86±0.03 j	0.24±0.03 efghij	0.03±0.001 bcd	8.8±0.5 hi	6.68±0.04 k	0.42±0.01 bc	0.052±0.001 cde	19±0.2 bc
Gurmail singh, Gunika	8.01±0.03 b	0.29±0.02 abcde	0.035±0.002 b	14±1 cd	8.03±0.02 bc	0.34±0.03 d	0.041±0.002 gh	17.23±0.4 c
Sukhwinder singh, Dittupur	7.92±0.02 c	0.21±0.02 hij	0.023±0.002 def	9.6±0.6 fghi	8.04±0.04 bc	0.35±0.04 bcd	0.045±0.002 fg	13.7±0.5 de
Swaran singh, Shauli	8.07 ± 0.02 b	0.31±0.01 abcd	0.024±0.001 def	11.9±1 cdefgh	8.05±0.03 bc	0.33±0.01 d	0.052±0.001 cde	16.6±0.6 cde
Chand singh, Shauli	7.69 ± 0.04 d	0.23±0.03 efghij	0.030±0.002 bcde	18±0.1 b	7.59±0.07 g	0.30±0.02 de	0.054±0.003 cd	25.3±1.1 a
Avatar singh, Dittupur	7.92± 0.02 c	0.18±0.02 j	0.021±0.001 f	9.3±0.6 ghi	7.95±0.03 cd	0.25±0.03 e	0.047±0.003 efg	17.3±0.5 bc
Charan singh, PaliaKhurd	7.31 ± 0.01 f	0.24±0.04 efghij	0.036±0.003 b	22.7±1.5 a	7.86±0.03 de	0.32±0.04 de	0.057±0.002 bc	27±1.5 a
Brijender singh, Dakala	7.85±0.04 c	0.34±0.02 ab	0.019±0.005 f	14.3±1.5 c	7.76±0.04 ef	0.43±0.41 b	0.048±0.002 def	16.3±1.1 cde
Pardeep Singh Rajgarh	7.23±0.03 g	0.22±0.03 fghij	0.032±0.004 bc	20±1 ab	7.29±0.02 i	0.34±0.03 d	0.61±0.003 b	25±2 a
Overall Mean (*n*=20)	**7.54±0.5**	**0.26±0.05**	**0.028±0.01**	**13.10±3.9**	**7.59±0.5**	**0.35±0.06**	**0.049±0.01**	**17.84±4.3**

Values are Mean ± SD (*n*=3)Mean values with the same letter were not significantly different, based on ANOVA followed by Turkey's t est at P≤0.05

Table 4. Increase in yield (qtls per acre) over control in rice and wheat as a result of application of flyash based bacterial and blue green algal inoculants

Year	Wheat (qtls/acre)	Rice (qtls/acre)	Reduction in application of Urea & SSP (%) per acre of land (Farmer's Feedback)
2006-2007	02-04	05-09	06
2007-2008	02-05	06-11	15
2008-2009	07-08	09-10	25
2009-2010	03-12	06-18	27
2010-2011	06-20	06-23	30

*SSP: Single super phosphate

during 2006-2011 respectively covering 444 different sites. Field visits were conducted at different time intervals of crop development to take the feedback from farmers before and after harvesting of the crop to assess crop yield. Soil samples were collected from farmer's fields before and after trials for analysis different parameters such as pH, organic carbon, total nitrogen and phosphorous to ascertain the role of fly ash based microbial inoculants on soil nutrient status. Also, soil application of fly ash based microbial inoculants proves to be easiest, non laborious and effective method. The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $p<0.05$.

Results and Discussion

Characterization of electrostatic precipitator (ESP) fly ash

Fly ash from electrostatic precipitator (ESP) was alkaline having pH 7.85 with electrical conductivity of 0.14 (µS) (Table 1). Available sulphur was 254 ppm and phosphorus was 20.4 ppm whereas available nitrogen and organic carbon content was found to be 0.004 and 0.06 % respectively. Heavy metals in fly ash were in the order Fe (1581ppm) > Zn (59.54ppm) > Mn (28ppm) > Pb (18 ppm) > Ni (8.4ppm) > Co (3.96 ppm) > Cr (5.32 ppm) > Cu (1.51ppm) > Cd (BDL). Water holding capacity was higher in fly ash which was 62%. Alkaline pH of fly ash enhances mineralization of organic matter and promotes nutrient supply to the plants (Mittra *et al.* 2005). Addition of fly ash in soil affects its chemical composition due to increased concentration of various elements, which is beneficial for plant growth when applied at lower concentrations but becomes toxic at higher doses (Gupta *et al.* 2002). Fly ash is often used as soil amending agent (Sikka and Kansal 1995, Tripathi *et al.* 2004, Mittra *et al.* 2003, Jala and Goyal 2006) and in combination with organic and chemical fertilizers, increased the grain yield and nutrient uptake of rice and peanut (Mittra *et al.* 2003, Dwivedi *et al.* 2007).

The heavy metal content in fly ash is within the range as found in common cropland soils (Jala and Goyal 2006). Therefore 250-500gm of fly ash based microbial inoculants per acre of land will not lead to increase of heavy metal rather would supplement certain micronutrient deficiencies in soil. An advantage of fly ash as a carrier is that it is a source of micronutrients and trace elements such as Zn, Cu, Fe, etc which affects crop plants in a positive way.

It is already reported that Indian fly ash provides the uptake of vital nutrients and minerals (Ca, Mg, Fe, Zn, Mo, S and Se) by crops and vegetation and can be considered as a potential growth improver, hence it serves as a good fertilizers (Senapati 2011).

Production and field demonstration of application of biofertilizers

During last five years (2006-2011) overall production of fly ash based algal and bacterial biofertilizers was 2303 kgs which was applied on 2032 acres of agricultural land for field demon-

Table 5. Overall range of physicochemical characteristics of soil from different field during five years.

Soil Parameters	pH	EC (µS/cm)	Organic Carbon (%)	Available Nitrogen (mg per kg)	Total Nitrogen (%)	Available Phosphorus (mg kg^{-1})
2006-2007	6.00-9.53	142-251	0.10-0.27	08-43	0.001-0.021	05-14
2007-2008	6.01-9.47	141-223	0.11-0.31	09-47	0.003-0.035	06-18
2008-2009	6.00-8.98	140-247	0.13-0.45	11-51	0.005-0.058	09-21
2009-2010	6.28-8.96	143-212	0.23-0.49	11-58	0.011-0.077	11-23
2010-2011	6.64-8.52	149-254	0.18-0.51	13-64	0.018-0.081	9-26

stration covering 444 sites of Punjab (Table 2). We observed a gradual increase in the demand for these biofertilizers. Production of fly ash based nitrogen fixing bacterial biofertilizers (Nitrofix) increased from 65kgs in 2006 to 360kgs in 2011 and area under trial increased from 48 to 86 simultaneously, whereas production of phosphate solubilising bacterial biofertilizers (Phosul) increased from 67 in 2006 to 321 in 2011 and area covered increased from 56 to 301 in 2011. Production of algal biofertilizers increased from 29 kgs in 2006 -2007 to 74 kgs in 2010-2011 depending upon the farmer's demand. Area under trial increased gradually from 42 acres in 2006-2007 to 86 acres in 2010-2011in different villages. A positive response regarding the effect of algal biofertilizers on rice cultivation was observed by the farmers with reduced application of urea.

Characterisation of soil before and after application of biofertilizers

Physicochemical properties of field soil such as pH, organic carbon (%), Total Nitrogen (%) and available Phosphorus (mg per kg) were estimated before and after application of biofertilizers in different villages of Punjab to study the effect of biofertilizers on nutrient profile in soils. Soil micro and macro nutrients were increased as a result of application of biofertilizers (Table 2).

pH

Overall mean value of pH of soils in twenty sites of Patiala was observed to be 7.54±0.5 before sowing of crop and 7.59 ± 0.05 at the time of harvesting of crop over control (Table 3). The result indicates that there was no such variation in pH of soils after application of biofertilizer. One year data (2010-2011) for rice cultivation with the application of algal biofertilizers showed pH of soil ranged from 6.64 as observed in village Kansuha to 8.52 in soils of village Gaushala, whereas after 144 days of harvesting pH ranged from 6.68 in village Palia khurd to 8.39 in soils of village Gaushala, Nabha. However, five years (2006 -2011) study on effect of biofertilizers on the soil pH in different villages of Patiala was observed to be in the range from 6 -9.53 in 2006-2007 to 6.64-8.52 in 2010-2011 (Table 5). The decline in soil pH with years of cropping may be attributed to soil submergence during the rice-growing period with concomitant application of urea over the years (Benbi *et al*. 2009). Significant number of soil samples from village Laut, Rajgarh, Bhojomajri, Sibro, Khanaure, Dittupur had pH values ranging from 7.06 – 8.08 respectively. Soils of Mansa district, an area which is a part of Indo–Gangetic alluvial plain located in the arid tract of Punjab, India had alkaline pH ranging from 8.00 to 9.7 (Verma *et al*. 2005). Physiochemical properties of soils of Fatehgarh Sahib, Punjab also showed that soils were alkaline in reaction (pH varied from 7.42 to 9.47) and usually increased with increasing soil depth (Verma et al., 2005). Algal biofertilizers ameliorate salinity, buffer the pH, solubilise phosphates and increases the efficiency of fertilizer use in crop plants (Mandal *et al.* 1998, Kaushik 2004, Prasanna *et al*. 2009). pH of soil amended with fly ash ranged from 8.12-9.09 during cultivation of wheat, mustard, rice, maize and lentil (Kalra *et al*. 2002).

Organic Carbon

Organic carbon content (%) on application of algal biofertilizers during rice cultivation in 2011 showed significant increase in soils of some villages including village Dittupur from 0.36% before sowing to 0.51% at the harvesting over control whereas in village Khanoure the increase in carbon content was observed from 0.21% before sowing to 0.32% at the time of harvesting. Organic carbon content before sowing ranged from 0.18% in village Khanoure and Dittupur to 0.34- 0.36% in village Dakala and Dittupur respectively whereas at the time of harvesting it ranged from 0.24% in agricultural land of village Sibro to 0.51% in another land of village Dittupur (Table 3). In almost all the field trials soil organic carbon was increased from time of sowing to the time of harvesting as a result of application of microbial inoculants. Five years study showed increase in carbon content with the application of biofertilizers in soils of different agricultural land of Punjab from 0.10-0.27 % as observed in 2006-2007 to 0.18-0.51% in 2010-2011 respectively (Table 5). Organic carbon content in soils of Mansa district was found to be low ranging from 0.02 to 0.40% (Verma *et al.* 2005). Fly ash acts as a soil conditioner increasing soil carbon and nitrogen content (Kalra *et al.* 1998) and application of blue green algae to the soil increases the organic carbon content (Mandal *et al*. 1998, Kaushik 2004, Nayak *et al.* 2004, Prasanna *et al*. 2009).

Total Nitrogen (%)

Blue green algal biofertilizers were used in paddy cultivation so as to increase the nitrogen content in soil through biological nitrogen fixation and reduce the application of urea. Significant increase in the overall mean value of total nitrogen content from 0.028 % before sowing to 0.049% at the time of harvesting of crop over control in the soils of twenty villages of Patiala, Punjab was observed. Application of BGA biofertilizers improves nitrogen content of paddy soils through fixation of atmospheric nitrogen growth and productivity of plant (Kaushik

2004, Tripathi *et al*. 2008, Pabbi 2008, Prasanna *et al*. 2009). Total nitrogen content (%) ranged from 0.018% -0.44% in soils of villages of Narainganj, Kansuha, Laut Palia Khurd and Gunika before sowing of crop whereas the percentage ranged from 0.032% in village Sibro to 0.081% in village Gunika at the time of harvesting of crop (Table 3). However, total nitrogen (%) in soil samples observed during 2006-2011 showed significant increase from 0.001in 2006 to 0.081 % in 2011 that proves nitrogen content in soil has been improved by the application of nitrofix and algal biofertilizers (Table5). Fly ash as a carrier for algal biofertilizers had a great impact on soil Nitrogen, N-transformation process and paddy yields (Singh *et al*. 2011). Recent investigations also suggests that fly ash can find better application if combined with organic amendments, nitrogen fertilizers (NF) and blue green algae (BGA) biofertilizers (Rautaray *et al*. 2003, Tripathi *et al*. 2004, Rai *et al*. 2004, Tripathi *et al*. 2008).

Available Phosphorus (mg/kg)

During rice cultivation in 2010-2011 soils of twenty villages with application of algal biofertilizers showed increase in mean value of phosphorus from 13mg kg^{-1} before sowing to 18 mg kg^{-1} at the time of harvesting of crop over control (Table 3). Phosphorus content (mg kg^{-1}) ranged from 8.6 as observed in soils of village Bhojomajri to 23 in village Palia Khurd before sowing of crop whereas at the time of harvesting of 144 days the P content (mg kg^{-1}) ranged from 13 in village Bhojomajri to 26 in village Gunika respectively. Five years (2006-2011) study on soil nutrient with the application of biofertilizers in different villages of Punjab showed significant increase in phosphorus content from 5-14 mg kg^{-1} as observed in year 2006 -2007 to 9-26 mg kg^{-1} in 2010-2011 over control (Table5).

The high available P content is attributed to the regular application of phosphatic fertilizers and immobile nature of phosphate ions in soils, which resulted in accumulation of P in soils (Verma *et al*. 2005). Fly ash had inherent phosphorus which along with soil phosphorous in due course of time might have been converted by microorganisms to available form and in these way microorganisms increases the availability of phosphorus by utilizing the phosphorus present in soil and fly ash (Gaind and Gaur, 1991). Results supports the finding that available phosphorus in soil increases with the application of fly ash, phosphate solubilising bacteria (*Pseudomona. straita*) (Gaind and Gaur 2002) and blue green algae (Prasanna *et al*. 2009).

Crop yield(%)

In 2006-2007 an increase of 5 - 09 qtls per acre in the yield of paddy was observed with the application of BGA biofertilizers whereas increase in yield of wheat ranged from 2-4 qtls per acre (Table 4, Fig 1). Similarly in next year 2007-2008, increase in yield of rice was observed to be 6.25-11qtl per acre over control whereas in wheat yield increased from 2 -5 qtls per acre. In 2008-2009 both rice and wheat showed increase in yield from 7-10 qtls per acre. However in 2009-2010 considerable increase was observed from 6–18 qtls per acre over control in rice and in wheat it ranged from 03-12 qtls per acre. Whereas in 2010-2011 field experiments conducted in different villages of Patiala and Nabha revealed that application of blue green algal and bacterial biofertilizers increases the yield productivity of rice and wheat from 06 qtls per acre to 23 qtls per acre in rice and 20 qtls per acre in wheat respectively. Application of BGA has been demonstrated to increase the productivity of crops like rice (Yanni, 1992), wheat (ABD-All *et al.* 1994) and leguminos plant, *Prosopis juliflora* (Rai *et al*. 2004, Tripathi *et al.* 2008).

Reduction in rate of application of chemical fertilizers based on farmer's response.

During 2006 -2011, there was 6 and 30% reduction in urea and single super phosphate (SSP) application for rice and wheat cultivation respectively (Table 4, Figure 1). Currently about 60 % of the farmer are practising intensive farming in order to increase the yield and 40% have opted for organic farming in the villages of Patiala and Nabha. It is evident that increased application of chemical fertilizers at high rates has boosted agricultural production in the country but it has also caused adverse impact on soil and water as well as on environment (Narayanan 2005). Several studies on the effects of use and application of biofertilizers for crop cultivation supports our findings that application of flyash based biofertilizers reduce the usage of chemical fertilizers. It is reported that the use of algal biofertilizers allowed a 50% decrease in the use of synthetic nitrogen fertilizer (from 100 kg N ha -1 to 50 kg N ha-1) with increased grain yield over control for rice crops in Chile (Pereira *et al*. 2009). Also, Blue green algal (BGA) application was observed to reduce the chemical nitrogen fertilizer requirement by 30 kg ha^{-1} and their application is an economical strategy in paddy cultivation (Tripathi *et al*. 2008). Flyash as carrier material for microbial inoculants had no negative impact on cell viability and the shelf life of flyash based biofertilizers was comparable with that of charcoal or montmorillonite. Efficacy of algal and bacterial biofertilizers has resulted in

reduction in application of urea and SSP in an ecofreindly and safe manner. In addition to the increase in the crop yield, farmers also reported improvement in size of grains and lustre for better quality and marketability.

Conclusion

Five year yield data of wheat and rice from different villages of Punjab showed increase in crop yield from 2 to 20 quintals per acre in wheat and 5 to 23 quintals per acre in rice, which supports the positive effect of application of fly ash based biofertilizers in crop cultivation. Soil nutrients were increased during five year study (2006-2011). Organic carbon content increased (%) from 0.10-0.27 in 2006 to 0.18-0.53 in 2011, total nitrogen (%) increased from 0.001-0.021% in 2006 to 0.021-0.080% in 2011and available phosphorus showed increased from 5-14 mg kg^{-1} in 2006 to 14-27 mg kg^{-1} in 2011 respectively. Overall, increase in soil nutrient resulted in reduction in rate of application of urea and single super phosphate (SSP) per acre by 6 % in 2006 to 30% in 2011. Field trials conducted over five year period showed increase in demand for microbial inoculants and illustrates useful application of fly ash based biofertilizers with increase in crop yield and improvement in soil nutrient status and reduction in rates of application of chemical fertilizers which can be easily adopted as a soil nutrient management strategy on large scale. Use of fly ash as a carrier in biofertilizer formulations (*Azotobacter, Pseudomonas, Blue Green Algae*) is an effective way of utilization of fly ash as a resource material.

Acknowledgements

Authors are thankful to the Director, Thapar University, Patiala and Science & Technology Entrepreneur's Park (STEP), TU, for providing infrastructural support and to Fly ash unit (FAU), Department of Science & Technology (DST), New Delhi and National Bank for Agriculture and Rural Development (NABARD), Chandigarh for financial support.

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Mineralogical comparison of coal flyash with soil for use in agriculture

Rajinder Kaur and Dinesh Goyal*

Department of Biotechnology

Thapar University, Patiala 147004, Punjab, India

Corresponding Author:

Dr. Dinesh Goyal*

Professor

Department of Biotechnology

Thapar University

Bhadson road, Patiala, 147004

Punjab, India.

Phone no:+91 9815601075

Email Id: dgoyal@thapar.edu

Mineralogical comparison of coal flyash with soil for use in agriculture

Rajinder Kaur[a] and Dinesh Goyal[b]

[ab] Department of Biotechnology

Thapar University, Patiala 147004, Punjab, India

Abstract

Mineralogical comparison of coal fly ash with soil and other material such as montmorillonite, charcoal was done by X-ray diffraction (XRD), thermo gravimetric analysis (TGA), Fourier transform infrared spectroscopy (FTIR) and Scanning electron micrograph (SEM) with EDS. Fly ash showed high thermal stability with least weight loss as observed in TGA and SEM graph indicated that flyash is composed of spherical structures with more surface area for interaction, XRD and EDS studies showed that amorphous content of ash consists of calcium oxide, potassium and major crystalline phases observed were quartz (SiO_2) and aluminium silicon oxide ($Al_{4.52}Si_{1.48}$) and haematite (Fe_2O_3). Charcoal, was amorphous in nature consisting of carbon and graphite. Soil and montmorillonite showed similar results in XRD, FTIR and thermal analysis having porous nature with silica as major constituent. Fly ash was found to be alkaline in nature having pH 7.85 and electrical conductivity 0.14 µS/m, good water holding capacity (62%) and various macro and micronutrients as compared to other material viz. soil, charcoal, montmorillonite and hence, its mineralogical composition ascertains its applicability as a carrier for different microbial inoculants for soil application in agriculture which can act as an economic source of nutrient supplement for crop plants.

Keywords: Fly ash, soil, charcoal, montmorillonite, thermal stability, agriculture.

1. Introduction

Coal fly ash is the mineral residue that is obtained as a by product of the combustion of coal for the production of electricity that is generated about 200milion tons of fly ash every year and is expected to increase to about 225 million tons by 2017 in India [1,2]. Effective management of coal fly ash without having any adverse effect on environmental conditions is of great concern. Worldwide sustainable use of coal fly ash is encouraged in cement industry and brick manufacturing, however since coal fly ash contains essential macro and micro nutrients, which are necessary for plant growth; hence it is very useful in order to increase soil fertility and production of crops whereas trace elements and heavy metals have been found in range, which is similar to the range as found in common cropland soils [3,4] and has not effect on soil or food chain. Formulation of microbial inoculation using optimum concentration of flyash does not show any adverse effect on the metabolic activity of microorganisms for specific time period [5,6,7]. Coal flyash has been used in fixed quantities as soil amending agent [8,9,4] without any deleterious effect on soil or soil health or ground water. Establishment of vegetation, flowering and aromatic plants like marigold (*Calendula sp*.), lily (*Lilium sp*.), and raising of forest trees (*Dalbergia sisso, Albizia lebbek, Eucalyptus hybrid, Acacia, Tamarindus, Populus deltoids*) on fly ash basins and landfills serve a variety of functions like stabilizing the ash against wind and water erosion, providing shelter and habitat for wildlife and transforming the area into aesthetically pleasing landscape [3,4,10, 11]. Coal fly ash is composed mainly of the inorganic constituents of the coal: oxides of

silicon, aluminium, iron and calcium which acts as a useful ameliorant that improve the physical, chemical and biological properties of problem soils having deficiency of macro (Nitrogen, Phosphorus, Potassium) and micronutrient nutrients (Cu, Zn, Fe,) [4,12]. Deficiency of zinc, iron, copper, manganese, boron, molybdenum, sulphur has been noticed in soils of India and similar deficiencies are observed in green vegetables and crops [12]. Coal fly ash may be an important source of Si to increase plant cell –wall rigidity that imparts resistance to lodging in rice [13]. The physiochemical similarity of fly ashes to alumina silicate enables fly ash to be transformed into materials with a zeolite crystalline structure under appropriate hydrothermal treatment. The addition of coal fly ash to soil increases the availability of silicon (Si), potassium (K), calcium (Ca), magnesium (Mg), sulphate (S) and other nutrients but not nitrogen (N) [14,15]. Wood charcoal is considered as a very good source of carbon materials and has excellent characteristics and could be replaced and utilized for sustainable forest resource and is used as an adsorbents for environmental purification and as materials regulating humidity [16,17]. Soil is a complex mixture of chemicals and organisms some of which are usually organized at the nanolevel [18]. Montmorillonite particles are considered as nano-filler composites as it consists of hydrated form of minerals [19]. Montmorillonite is a kind of 2:1 type layered silicates and further investigations on montmorillonite for synthesis of inorganic-organic clay complexes might be of great potential applications in remediation of polluted environment [20]. Coal fly ash addition on agricultural land improves wasteland quality and improves agricultural

productivity for which its detailed micro structural characterization and physical studies are important parameters. Risk analysis of coal flyash related to radioactivity and heavy metals reports that majority of Indian coal fly ash are not significantly enriched in radioactive elements compared to common soils or rocks and the activity of levels of gamma emitting radionuclides K, Ra, Ac were within the permissible limits and mixing of fly ash with the soil at 24% v/v was of no consequences [4,21,22] and it is expected that use of fly-ash instead of lime in agriculture can reduce net CO_2 emission, thus reduce global warming also [11]. According to the hazardous waste management and handling rule of 1989, Indian coal fly ash is considered as non hazardous waste and hence, coal fly ash obtained from thermal power plants of India can be treated as a by product rather than waste [23] and its application in agricultural sector can serve as secondary source of nutrients [4,24,25]. The U.S. Environmental Protection Agency (EPA) also recommends coal combustion products such as fly ash as a soil amending agent among municipal biosolids along with variety of composted agricultural byproducts, as well as traditional agricultural fertilizers [26]. Charcoal, montmorillonite are costly for utilisation in pilot scale production of biofertilizers, whereas fly ash is generated in huge quantity as a major solid by product of thermal power plants [27]. To know the elemental composition of coal fly ash and how it is going to affect the soil physicochemical characteristics after amendment and its comparison with other similar materials such as soil, charcoal and montmorillonite becomes important. In the present investigation mineralogical comparison of flyash with soil and other materials such as

montmorillonite and charcoal, which are used as carrier for microbial inoculants for soil application, was carried out in order to ascertain usage of flyash for similar purpose.

2. Materials and Methods

2.1 *Collection and processing of different carrier materials*

Electrostatic precipitator (ESP) coal fly ash (FA) was collected from Sturdy Industries Ltd. village Saidpura, Derabassi (Punjab). The main source of fly ash for Sturdy Industries Ltd. is Guru Gobind Singh Super Thermal Power Plant, Ropar, Punjab and was directly analyzed for its physiochemical properties after air-drying. Wood charcoal and montmorillonite was purchased from local market located in Patiala, Punjab as per required quantity. Garden soil was collected from experimental plot Science & Technology Entrepreneur's Park (STEP), Thapar University, Patiala. Soil, charcoal and montmorillonite were crushed into fine powder and air dried for their physiochemical properties.

2.2 *Microstructural Characterisation*

2.2.1 *X- ray diffraction*: Samples were characterized using X- ray diffraction to identify the formed phases. The powdered sample were pressed into the aluminium holder and subjected to Cu-K radiataion between 10-80ºC. X-ray powder diffractions study was performed at room temperature using PANalytical X'Pert PRO system with Ni-filter. During experiment the step size was 0.013°/min and X-ray diffractogram of different materials were obtained and identified [28]. The elemental compositions of the present studied samples were determined by using High Score Plus software associated with PANalytical X'Pert PRO system. This software uses the area under the curve of different peak of different phases to calculate the

quantitative percentage of different phases [29]. Total element concentration of Cr, Pb, Mn, Zn, Cu, Ni, Co, Mo, Cd, Se, As, Fe and S in coal fly ash was analysed with strong nitric acid digestion method followed by analysis with Atomic absorption spectrophotometer (GBC 932AA) [30].

2.2.2 *DTA /TGA*: Differential thermal analysis (DTA) was performed in nitrogen atmosphere using Perkin Elmer (Model: Diamond Pyris TG/DTA analyzer) to check thermal stability and any phase transition. The Differential thermal analysis/ Thermal gravimetric measurements of the samples were performed using Al_2O_3 powder as reference material in nitrogen atmosphere at 10 °C/min heating rate from 50 °C to 900 °C. The temperature and weight loss detection limit of the instrument are ± 1ºC and 0.001mg, respectively [28].

2.2.3 *Fourier transform infrared spectra (FTIR):* FTIR spectra were obtained at room temperature by using Perkin Elmer model RZX spectrometer in the region 400-2000 cm^{-1}. The spectrum of each sample was normalized to the spectrum of blank KBr [28].

2.2.4 *Scanning electron micrograph (SEM)*: The microstructural study was carried out by a scanning electron microscope (JSM-6510LV, JEOL). For microstructural study, all the powdered samples of flyash, charcoal, montmorillonite and soil were sputtered before the measurement to study the morphology and particle size respectively.

2.2.5 *Surface area*: "Fineness" is quantified by the specific surface of a material. The specific surface is defined as the "number of units of surface area "contained in a "unit weight" of a material. BET (Brunauer – Emmett-Teller) method was used to measure the specific surface area of the samples using area-meter Π by nitrogen gas absorption in a flow rate 4.5 l/h

2.2.8 *Coarse Grain Accumulation: Coarse grain accumulation* of coal fly ash, charcoal, montmorillonite and soil was done by dry sieve analysis followed by hydrometer. Each sample was carefully sieved with round test sieves with a hole diameter of 4mm, 2mm, 1mm, 0.500mm,0.300 mm, 0.150 mm, 0.075 mm, 0.025 mm and 0.01 mm, in accordance with ISO 1953 [31].

2.3 *Physical characterisation:* Air dried coal fly ash, wood charcoal, montmorillonite and garden soil was directly used physical parameters analysis viz. Water holding capacity and bulk density [32], pH and electrical conductivity (EC) [33] respectively. pH and electrical conductivity (EC) was determined in a sample-water suspension of 1:5 ratio. pH was measured using a Thermo Orion Model 290 pH meter after calibration with standard buffers of pH 4.0, 7.0 and 9.2, whereas electrical conductivity was measured by conductivity meter (Orion Model 125) after calibration with 0.01 M potassium chloride (1413 μS cm^{-1}).

3. Results and Discussion

3.1 ***Mineralogical composition:*** X- ray diffraction pattern showed that major crystalline phases in coal fly ash were of quartz (SiO_2), mullite ($Al_{4.56}Si_{1.44}O_{9.72}$) and hematite ($Fe_2O_3$) and minor constituents comprised of oxides of magnesium, potassium and calcium(Fig1a; Table1a). Mineralogical study confirmed that coal fly ash contains basic mineral element (N, P, K, Fe, S, Ca) (Table 1b) in readily available ionic form, which can lead to their increased uptake by plants. Major matrix elements of coal fly ash were found to be Al and Si, together with significant percentage of K, Fe, S, Ca, and Mg. Lee *et al*. [34] reported that major crystalline phases in XRD pattern of fly ash are of quartz and mullite. Agricultural utilization of coal fly ash has been proposed because of its considerable mineral content of

macronutrient (K, Ca, Mg, S, P) and secondary source of micronutrients (Cu, Fe, Mn, Zn etc) for soil deficient in nutrients [3,4,27,35,36,37,38]. XRD pattern of wood charcoal showed the peaks of crystalline structure of cellulose and graphite with different forms of amorphous carbon whereas no clear diffraction peaks were detected due to the decomposition of cellulose structure at higher temperature (Fig 1b). The sharp peaks appeared in charcoal carbonization seemed to originate from the graphite crystalline structure [39-40]. XRD pattern of wood charcoal shows the crystalline phase of graphite, diamond and fullerene along with some amorphous materials [41]. Hata *et al*. [16] also reported that no clear diffraction peaks are obtained in XRD of charcoal below 1000ºC indicating thermal decomposition of cellulose structure and no graphitization in charcoal. XRD pattern of soil showed the presence of kaolin, quartz and feldspate (Fig 1c). Quartz in the soil has primary (volcanic) origin and is most frequent in acidic soil [18], whereas XRD of montmorillonite showed the presence of three crystallographic phases of calcium–magnesium montmorillonite $Ca_{0.2}(Al.Mg)_2Si_4O_{10}(OH)_2.4H_2O$, magnesium montmorillonite ($MgO.Al_2O_3.5SiO_2.xH_2O$) and quartz (SiO_2) (Fig1d). Concentration of heavy metals in coal fly ash was found to be low (As, Cd, Se, Pb, Ni and Co), which is supported by the earlier findings that the heavy metal (As, Cd, Se etc.) present in coal flyash do not leach into groundwater, when applied to soils and do not leach from products that contain coal flyash [3,40,42]. The heavy metals can limit the survival and growth of plants and microbial population but in general the heavy metal concentrations of Indian coal ash are reported low as compared to ash from other parts of world, unlikely to affect ground water quality [42]. Coal fly ash used in fixed quantities represents a good carrier material and will not cause leaching problem.

3.2 ***Differential Thermal and Thermal Gravimetric Analysis:*** Thermal study was carried out to observe thermal decomposition of humic substances and organic matter in fly ash, charcoal, montmorillonite and soil. Among all material fly ash showed stable thermal effect

from 100-500 ºC. Weight loss observed upto 200°C was primarily due to loss of water and carbon that was formed during the hydration process as confirmed by XRD analysis (Fig. 2a). The endothermic peak at 448°C associated with the weight loss between 400°C and 500°C is caused by evaporation of water and loss of carbon as CO_2. Finally the third endothermic peak at 647°C is due to the loss of CO_2 from calcium carbonate. When fly ash is in contact with water, limited amount of calcium and aluminium ions are released in the solution. For this reason, the hydration of mixtures with fly ash is severely retarded at early ages until additional activators such as alkali, calcium hydroxides, or sulphates are present in the medium [43].The TGA plot of charcoal showed total weight loss during pyrolysis is more than 95%. Our results are supported by the findings of Cohen and his co-workers [44] that the TGA plot showed total weight loss during pyrolysis of charcoal above 95%. However the purity of used N_2 was 97% so, it might be possible that air is also present during the reaction. The DTA plot showed that the major phase change occurs at 500 °C (Fig 2b). DTA analysis of pure geological graphite showed that it decomposes around 800–900 °C [44]. The TG curve of charcoal goes back to low temperature side at about 480 degree C and this decrease in the temperature might be due to some analytical errors and endorthermic reactions. Soil and montmorillonite showed similar decomposition. TG profile of soil and montmorillonite indicates a weight loss between 300 and 550ºC, an exothermic energy released was observed at 350ºC followed by endothermic peak, showing transition with gain of energy at 550 ºC (Fig 2c and Fig 2d). The results led us to conclude that coal fly ash has more thermal stability as compared to other materials followed by well stabilised organic matter with high degree of humification. Presumably, during the organic matter transformation process, the formation of humic-like substances enabled the formation of complexes characterised by a strong thermal stability [45].

3.3 Fourier-transform infrared spectroscopy (FTIR): FTIR is an alternate approach to determine organic matter and inorganic portion of soil [46]. FTIR spectra were helpful in detecting fingerprints of minerals attributed to Si-O-Si stretching and C-O stretching vibrations in coal flyash and other materials. FTIR of fly ash showed that the most prominent peaks in the spectra originate from Si-O and Al-O stretch vibrations. Band near 1090-1200 cm^{-1} is due to Si-O stretch vibrations. Zone corresponding to Si–O and Al–O vibrations in original fly ash is 1080-1090 cm^{-1} (Fig 3a; Table 2). This shift was due to penetration into original structure Si–O–Si as it was observed in zeolites of fly ash by Skvara *et al*. [47]. Major component of coal fly ash is amorphous aluminosilicate, quartz (SiO_2) and mullite ($3Al_2O_3.2SiO_2$), which can also be converted into zeolite crystals by hydrothermal treatment [48, 49] having natural zeolitic characteristics that helps in soil improvement by maintaining soil moisture and NH_4^+ and K^+. Infrared spectra of charcoal showed that the carbon double bonds and aromatic rings were formed at carbonization temperature of 600°C. Wood charcoal carbonized at 1600°C was partly graphitized, a finding supported by the results of X-ray diffraction (Fig 3b; Table 2). The band at 3340 cm^{-1} was due to the absorption of water molecules as result of an O-H stretching mode. The O-H mode at 3300cm^{-1} decreased with an increasing carbonization temperature while the band at 2890 is attributed to C-H interaction with the surface of the carbon. It is reported that in case of the original wood aromatic absorption originating from lignin was detected at about 1600 -1510cm^{-1} [16]. These aromatic modes are found in charcoal but moved to a lower wave number with the increase in carbonization temperature. FTIR analysis of soil showed absorption bands at 3695, 3545, 1700 cm^{-1}(Fig 3c; Table 2), which were characteristic to soil humic compounds followed by the sharp band at 1031 cm^{-1} which can be assigned to the Si–O stretch [50, 51] In the range of 900–1180cm^{-1}, spectra of soil include fingerprints of minerals, attributed to Si–O–Si stretching and C–O stretching vibrations, as interpreted by a FTIR spectral library [52]. FTIR

spectra of montmorillonite was characterised by typical bands responsible for stretching vibrations of O–H near 3440–3620 cm^{-1} and Si–O (1100–1035 cm^{-1}) bonds (Fig 3d; Table 2). The bands between 170-710 cm^{-1} were attributed to SiO_2 and Al_2O_3 lattice and bands near 840 and 1030 were due to bending vibrations of the structural OH, bound to octahedral cations [53], however the typical bands responsible for stretching vibrations were due to O-H (υ = 3440 -3620 cm^{-1}) and Si–O (υ=1113-1035cm^{-1}) bonds and bending vibrations were due to Al Mg OH (υ =830–840 cm^{-1}) and Al Al OH (υ = 915–925 cm^{-1}) bonds [19,54]. Analysis of FTIR –spectra of montmorillonite indicates typical bands responsible for stretching vibrations of O-H (and Characteristic bending vibrations of Si–O bonds at υ1 = 692 cm^{-1} and υ2 = 529 cm^{-1} [19,55,56] were also observed.

3.4 Scanning electron micrograph (SEM) & Energy dispersive spectrometry (EDS): Morphology study using SEM shows that fly ash has a smooth spherical surfaces i.e cenospheres and plenospheres having more surface area for interaction (Fig 4a). Flyash particles are hollow, empty spheres (cenospheres) filled with smaller amorphous particles and crystals (plerospheres) [4]. SEM micrograph showed dense microstructure having particle size with equivalent diameter ranging from 2 to 50µm. EDS of surface of coal fly ash supported the XRD data with high mineralogy content of fly ash rich in Fe, C, Ca, SiO_2, Al, K with few traces of Zn and Cu (Table 3a; Fig 5a). Coal fly ash mainly comprised of amorphous alumino-silicate spheres, a smaller amount of iron-rich spheres and the majority of the iron-rich spheres had two components: iron oxide and amorphous alumino-silicate, calcium, the fourth most abundant element found in the coal fly ash, was associated with oxygen, sulfur or phosphorous [57]. SEM micrographs of charcoal showed a packed fibrous structure having multiple pores showing uniform wood parenchyma in the charcoal and particle size ranging from 50-100µm. (Fig 4b). Microstructure of soil confirms the broad distribution of particles ranging from few micrometers to hundreds of micrometer consisting

of Kaolin and quartz particles. SEM micrograph with higher magnification confirmed that soil particles were porous in structure and cracked at various stages which are responsible for many soil properties. Aggregate structure observed comprised of iron, magnesium and potassium in small quantities was clearly visible both in case of soil and montmorillonite as observed in EDS graphs (Table 3c and Table 3d). SEM micrographs of soil and montmorillonite supported the particles have size in the range of 10 to 500 μm and clearly defined porosity similar to clay [18]. In case of montmorillonite also, SEM studies indicated that agglomerates of the montmorillonite particles showed a plate like morphology characteristic of layered silicates and EDS analysis also supports the 60% presence of silica (Fig 4c, Fig 4d &Table 3c, 3d). SEM and EDS results concluded that in comparison to charcoal, montmorillonite and soil, coal fly ash samples contains essential macronutrients including P, K, Ca, Mg and S and micronutrients like Fe, Mn, Zn, Cu, Co that are beneficial in crop yield of many agricultural crops [11,4].

3.5 Coarse grain accumulation: Particle size distribution (Table 4) of fly ash indicates presence of fine grained particles with more than about 70% of the particles finer than 0.075mm. Silt size particles in coal fly ash may often be substituted for topsoil in surface mine lands, thereby enhancing physical conditions of soil especially water holding capacity [3,4,11]. Soil has substantial quantity of fines which is 51% followed by montmorillonite (47%) and charcoal (27%). 40-50% of the particles are finer than 0.025mm in fly ash, whereas in soil it is 45%. Charcoal and montmorillonite has 16% and 28% of 0.025mm fines. It is reported that the number of particle size classes measured by sieving in soil could be reduced to three, <0.05, 0.05–0.2, and 0.2–2 mm, which enabled 87.9% of the soil samples to be discriminated [31] and fly ash particles which are typically spherical and glassy with particle diameter ranging from 1 to 150μm [58]. The extensive investigation carried out on

Indian coal ashes demonstrates that the fly ashes consist predominantly of silt-size fraction with some clay-size fraction and based on the grain-size distribution, the coal ashes can be classified as sandy silt to silty sand [4].

3.6 Physical characterisation: Coal fly ash to be used in agriculture and for microbial formulation depends upon its acidic or alkaline nature. (Table 5) pH of all powdered material was found to be alkaline ranging from 7.85±0.12 in coal fly ash to 8.22±0.05 as observed in soil. Montmorillonite and charcoal was alkaline having pH 8.27±0.1 and 8.19±0.02 respectively. The pH of fly ash generally varies from 4.5-12, however worldwide coal flyash are alkaline including that from India [59,60]. Electrical Conductivity (EC) (µS/cm) of fly ash was 0.14±0.02 whereas of charcoal it was 2.87±0.01. Addition of coal fly ash to soil improves physico-chemical properties of soil such as pH, EC, porosity, water holding capacity, root penetration and fertility leading to increased biomass production [8,38,61]. Coal flyash was effectively used for reclamation stabilization and was helpful in neutralization of acidic mine spoils and restoration of nutrient balance in alkaline wastelands [4]. Soil and montmorillonite showed EC ranging from 2.45±3.75 to 15.5±25.8 respectively (Table 5). Bulk density (g/cm^3) of fly ash and charcoal was 0.99 and 0.43 whereas of montmorillonite and soil it was found to be 2.05 and 1.36 respectively. Water holding capacity was maximum in charcoal (198%) followed by montmorillonite (84.3%), fly ash (62%) and soil (39.9%). Indian fly ash has a vast potential for use in agriculture as an amendment especially due to its alkaline pH, low bulk density and high water holding capacity, which are conducive for plant growth [59,60]. Surface area (m^2/g) was observed maximum in montmorillonite (79.3) followed by soil (5.45), charcoal (4.90) and fly ash (0.96) (Table 5). Fly ash addition generally decreased the bulk density of soils, which in turn improved soil porosity and workability and enhanced water retention capacity [62,4].

Montmorillonite showed high specific surface area of 79.3 m^2/g by BET method that is due to its two layered particles with a thickness of 4 to 10 unit layers [63] and the bulk density for montmorillonite ranges between 2 to 2.7 g/cm^3 [64]. Coal fly ash is abundant in India as compared to charcoal and montmorillonite but the percentage of utilization of coal fly ash in India is only 38% and rest of this were dumped into basin or landfill near power plants which is not environmentally safe and hence the utilization percentage of coal fly ash can be increased in agriculture sector demonstrating its positive benefits for improving soil properties by buffering soil, increasing moisture content, improved soil porosity and increasing crop yield [4,59,60]. Use of coal fly ash at different concentration with other soil amendments such as sewage sludge, lime, farmyard manure, cowdung has shown positive results [3,4,11] in improving physico-chemical properties of problem soils. Flyash has been used as a liming material on acidic or alkaline soil for improving the pH of the problem soil with reduction in use of chemical fertilizers [65].

4. Conclusion: Comparative study for microstructure and physical characterization of four different material fly ash, charcoal, soil and montmorillonite showed that XRD pattern and SEM-EDS graphs confirms the presence of various minerals in fly ash, which may act as potential inorganic soil amendment to restore the soil nutrient balance. Since fly ash is a resultant of coal combustion therefore quartz, mullite and haematite were detected by XRD, whereas a little weight loss through endothermic reaction was detected by TGA/DTA analysis. Among soil, charcoal and montmorillonite, flyash was found to be more thermo stable material with more surface area and inorganic materials comprising aluminosilicate and silt sized particles. FTIR bands in fly ash suggested prominent peaks in the spectra originating from Si-O and Al-O stretch vibration and this shift could be interpreted for fly ash

to be considered as zeolites. Among four different materials fly ash has a good potential and could be directly used in place of soil, montmorillonite and charcoal for the formulation of carrier based microbial inoculants for soil application. Hence, results indicate that coal fly ash is effective for problem soil in an ecofreindly manner and safe strategy.

Acknowledgement:

The authors are thankful to Director, Thapar University, Patiala and Science & Technology Entrepreneur's Park (STEP), TU, for providing infrastructural and support and to Fly ash unit (FAU), Department of Science &Technology (DST) and National Bank for Agriculture and Rural Development (NABARD) for financial support.

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Figure Captions:

Fig 1: XRD pattern of fly ash (a), charcoal (b), soil (c) and montmorillonite (d).

Fig 2: Thermo gravimetric analysis (TGA) TGA/DTA of fly ash (a), charcoal (b), soil(c) and montmorillonite (d).

Fig 3: Fourier-transform infrared spectroscopy (FTIR) of fly ash (a), charcoal (b), soil(c) and montmorillonite (d).

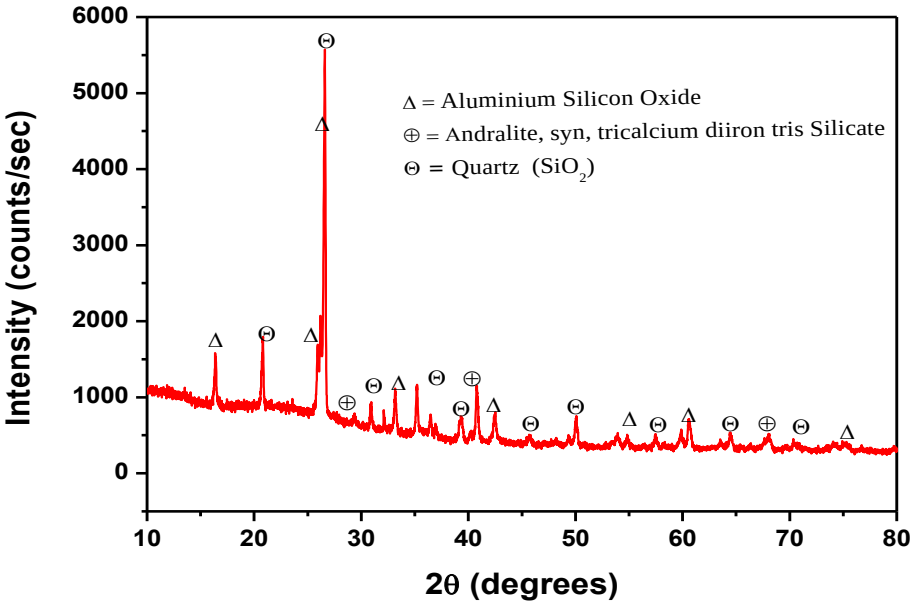
Fig 4: Scanning electron micrograph (SEM) of fly ash (a), charcoal (b), soil(c) and montmorillonite (d).

Fig 5: Energy dispersive spectrometry (EDS) of fly ash (a), charcoal (b), soil(c) and montmorillonite (d).

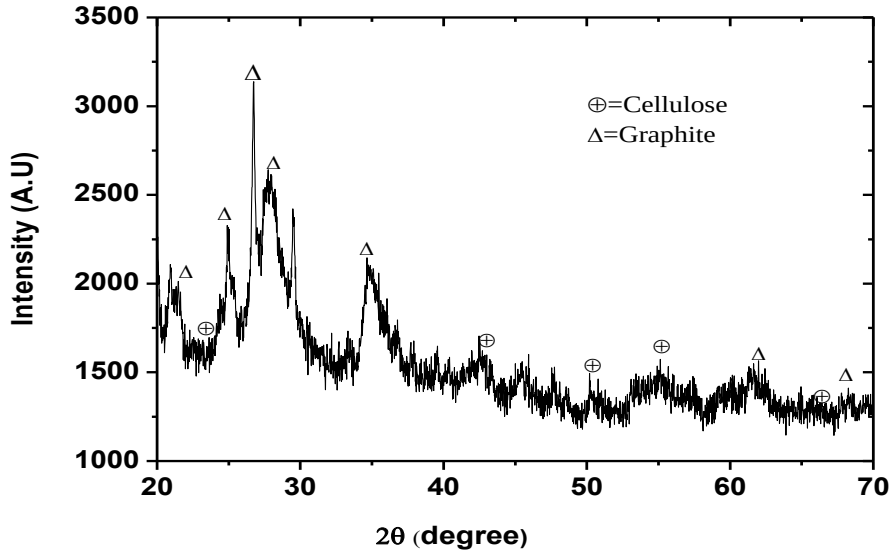
Table Captions:

Fig 1: X-Ray Diffraction of fly ash, charcoal, soil and montmorillonite

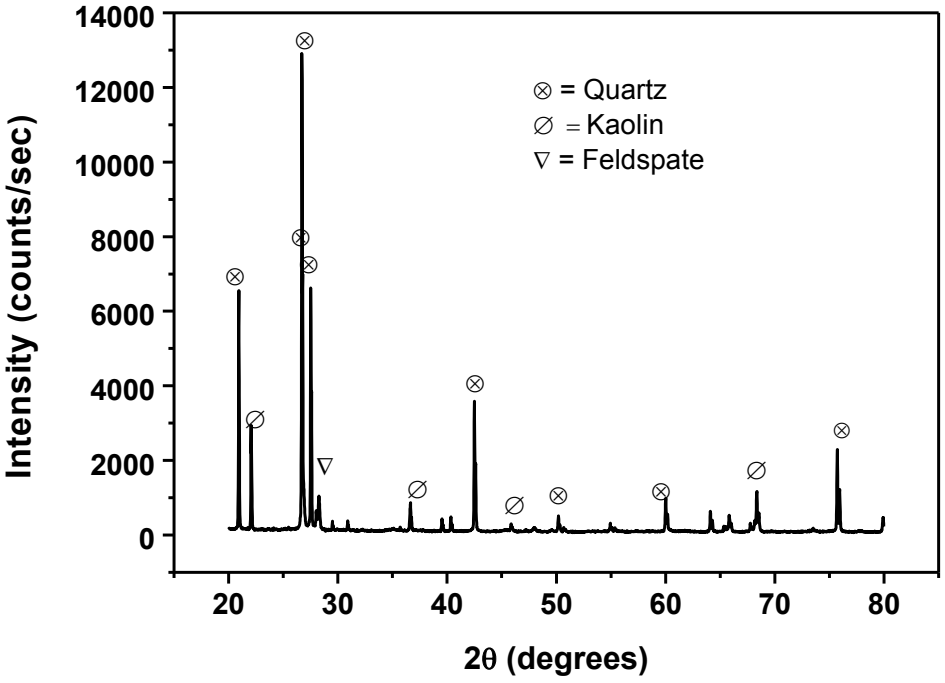
XRD of fly ash (a) shows the presence of quartz (SiO_2), mullite ($Al_{4.56}Si_{1.44}O_{9.72}$), haematite and small amount of calcite and no magnetite. Soil (c) and montmorillonite (d) mainly consists of kaolin, quartz and feldspar, whereas charcoal (b) shows crystalline phase of amorphous carbon and graphite.



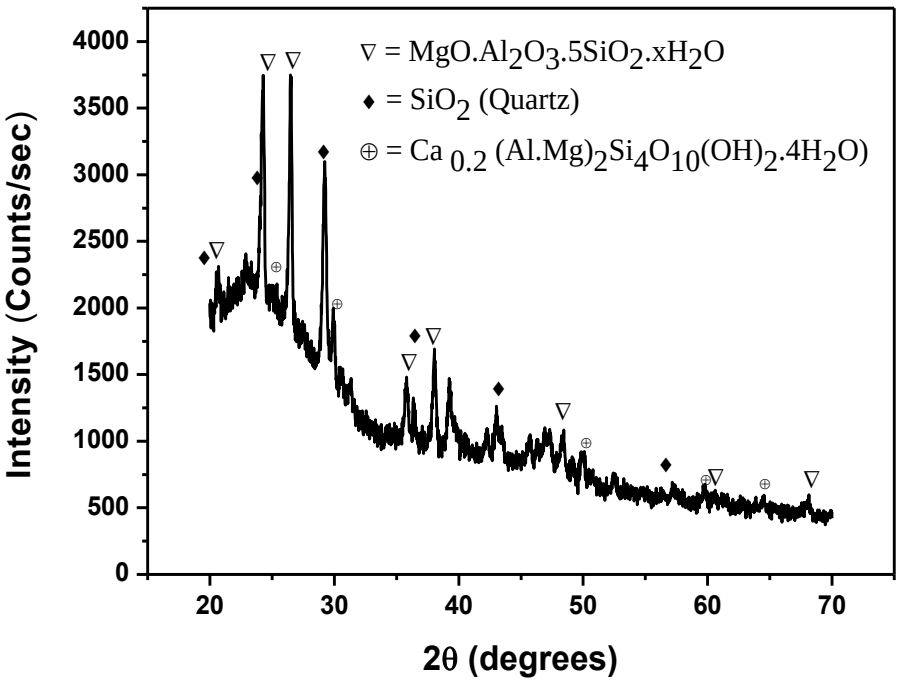
a) XRD of Fly ash



b) XRD of Charcoal

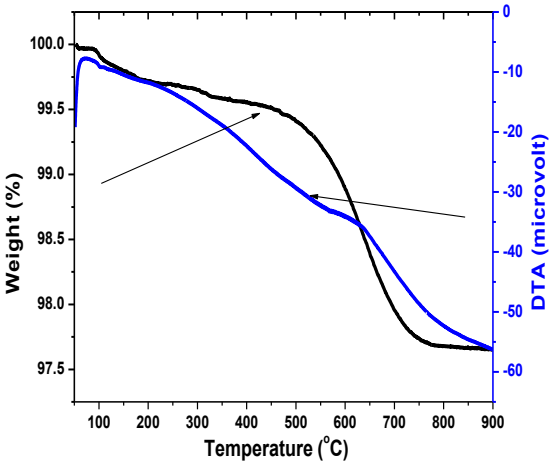


c) XRD of Soil

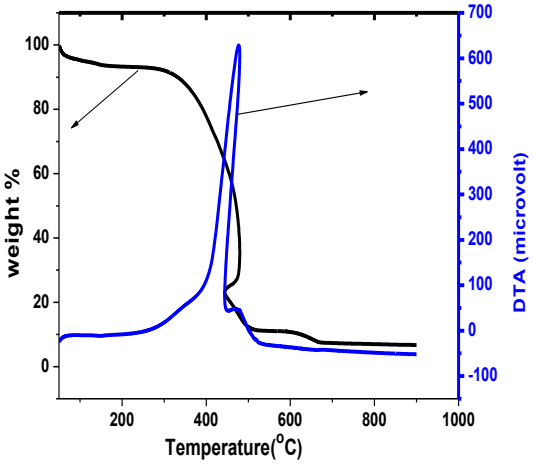


d) XRD of Montmorillonite (MMT)

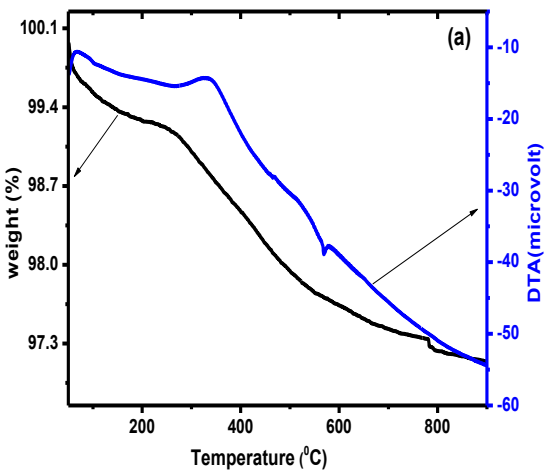
Fig 2: TGA/DTA curves of (a) Fly ash (a) shows stable thermal effect from 100-500ºC whereas in charcoal (b) total weight loss observed during pyrolysis was more than 95%. A similar trend of decomposition of weight loss was observed in soil(c) and montmorillonite (d) between 300 and 550 ºC.



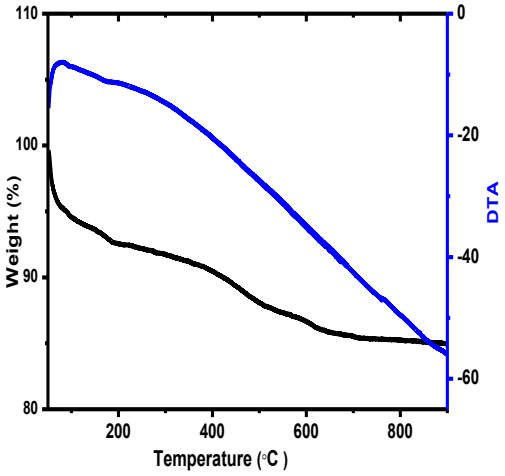
a) Fly ash



b) Charcoal

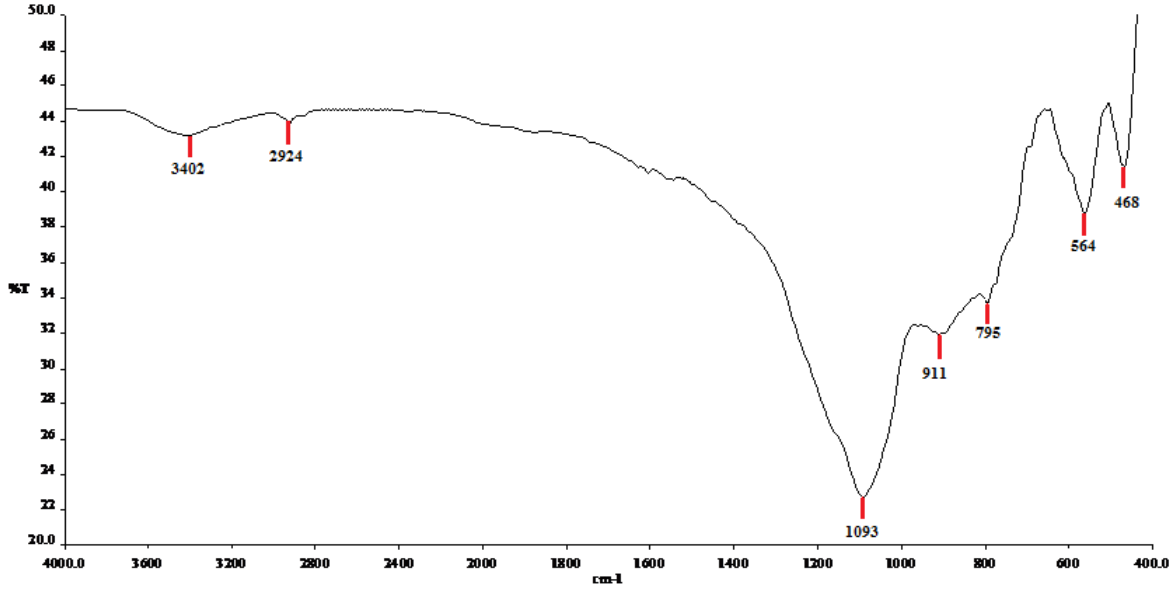


c) Soil

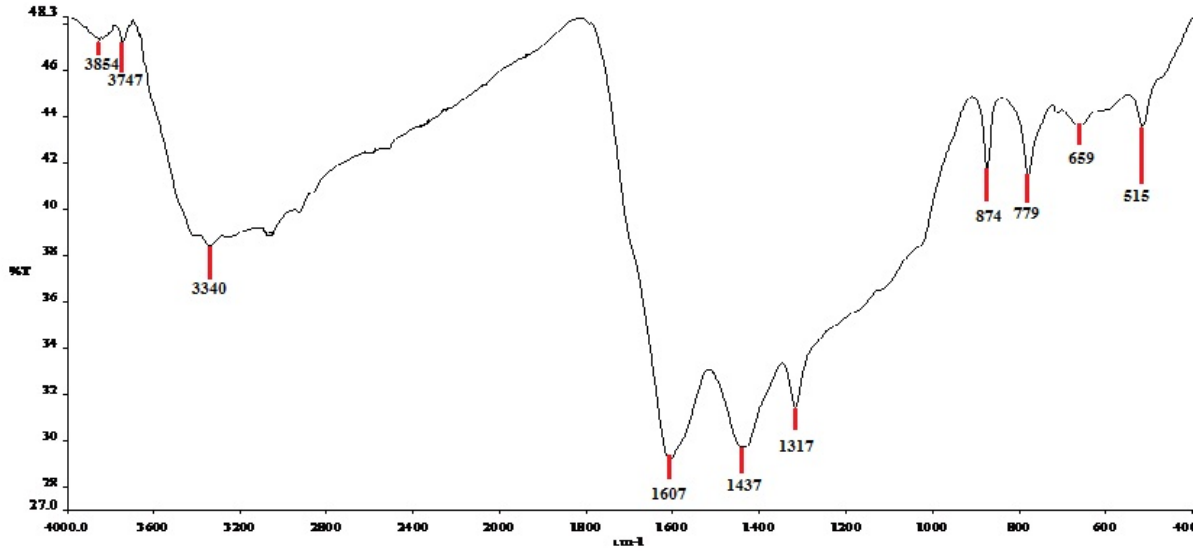


d) Montmorillonite

Fig 3: Fourier-transform infrared spectroscopy (FTIR) - Si–O and Al–O vibrations in original fly ash(a) is 1080-1090 cm^{-1} and in charcoal (b), bands stretching at 3340 cm^{-1} was observed to be O-H stretching mode and charcoal carbonized at 1600°C was partly graphitized. Soil (c) exhibits absorption bands at 3695, 3545, 1700 cm^{-1} whereas montmorillonite(d) shows typical bands responsible for stretching vibrations of O–H near 3440–3620 cm−1 and Si–O (1100–1035 cm−1) band.

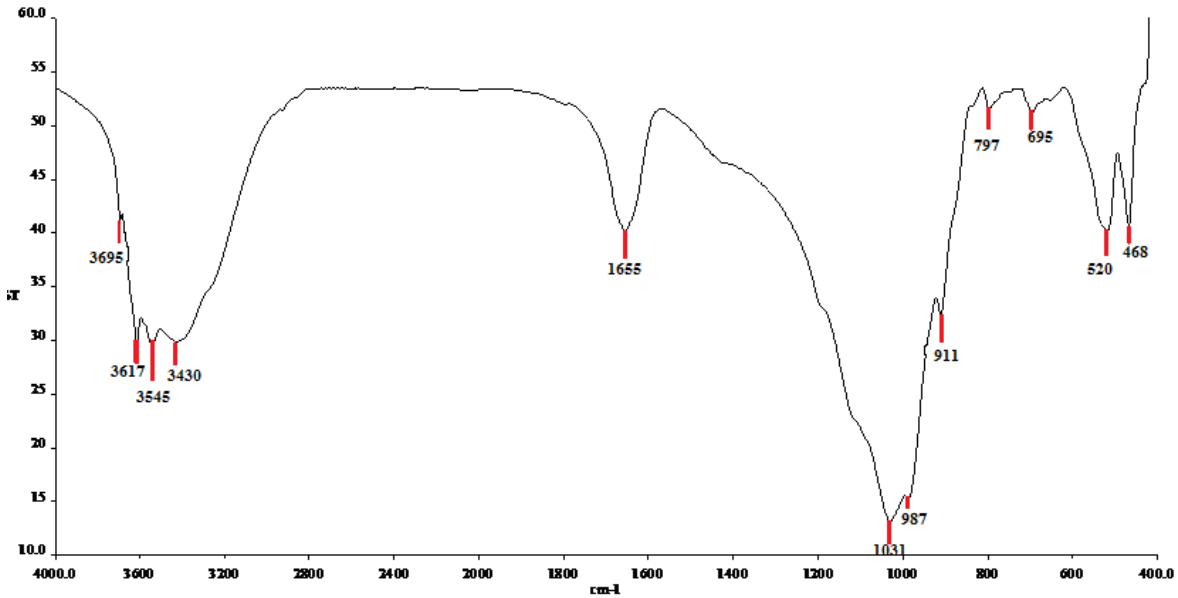


a) Flyash



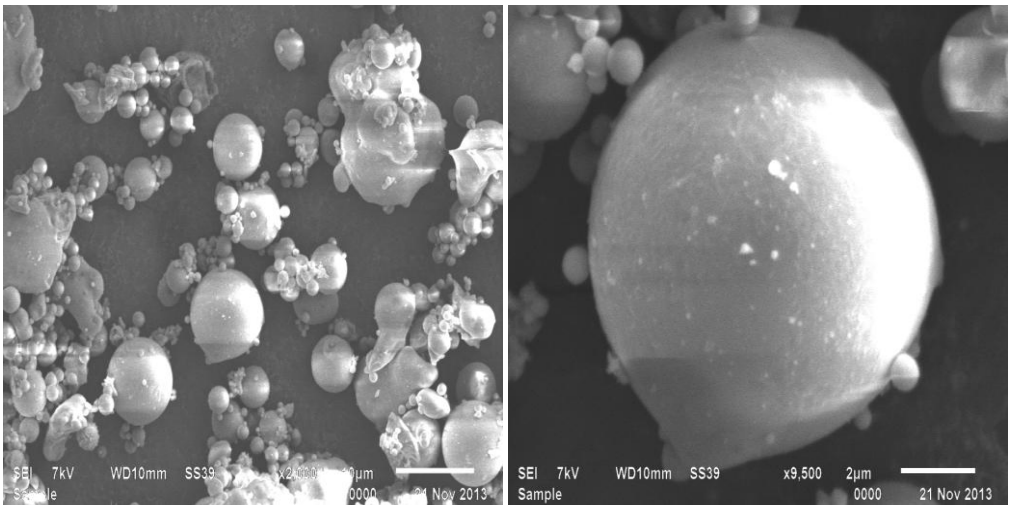
b) Charcoal

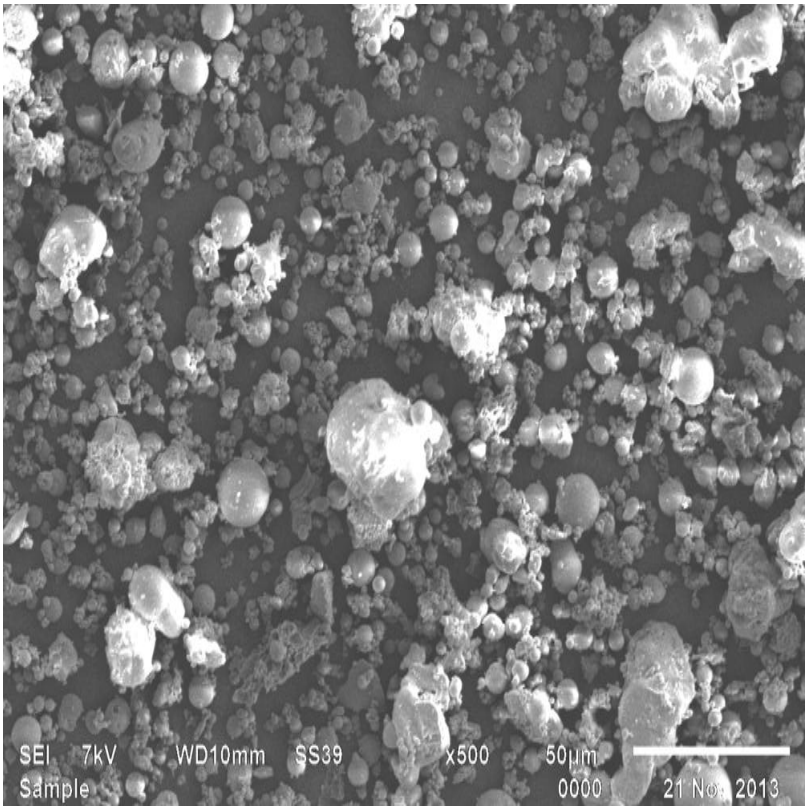
c) Soil



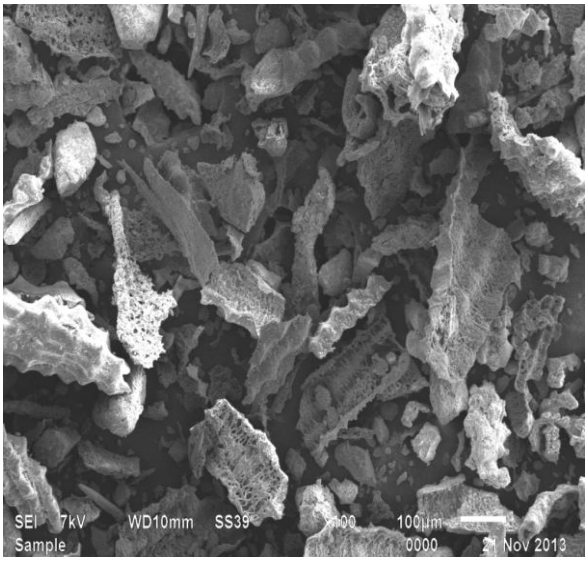
d) Montmorillonite

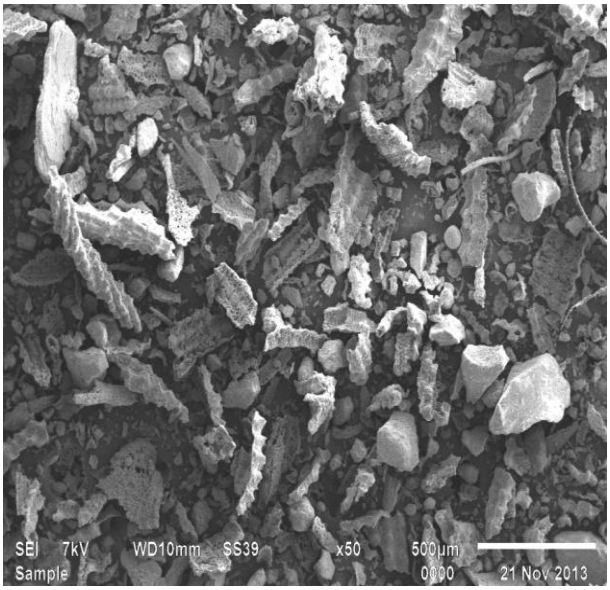
Fig 4: Scanning electron micrograghs (SEM): Fly ash(a) showed smooth spheres of cenospheres and plenospheres having more surface area for interaction having particle size from 2µm-50 µm. Charcoal(b) showed a fibrous structure of parenchyma cells of wood and particle size ranging from 50-100 µm. Soil(c) and montmorillonite(d) showed agglometric structures and particle size ranging from 10-500µm.




SEI 7kV WD10mm SS39 x500 50µm
Sample 0000 21 Nov 2013

a) Fly ash


SEI 7kV WD10mm SS39 100µm
Sample 0000 21 Nov 2013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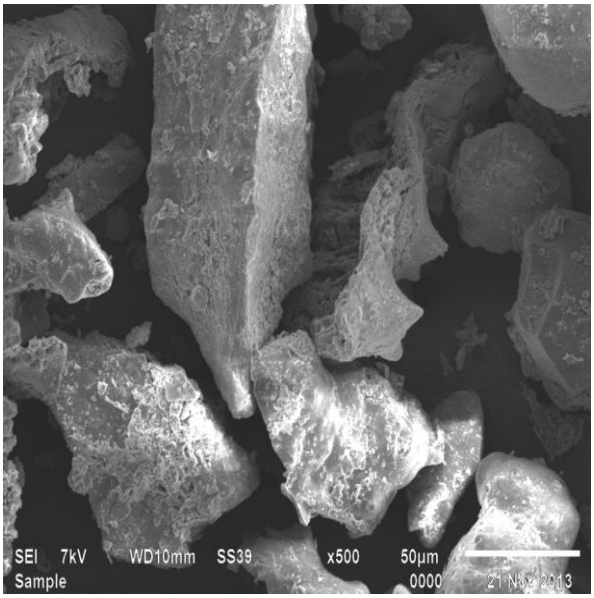
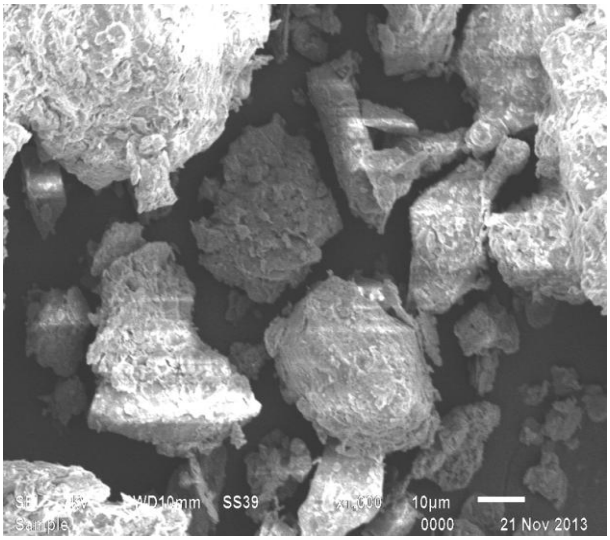
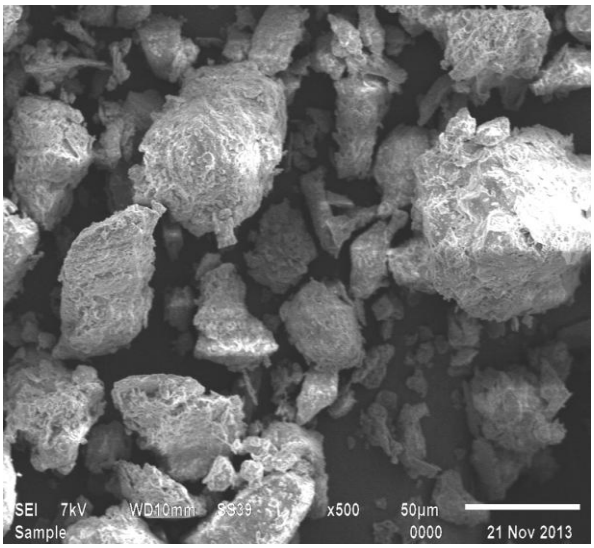
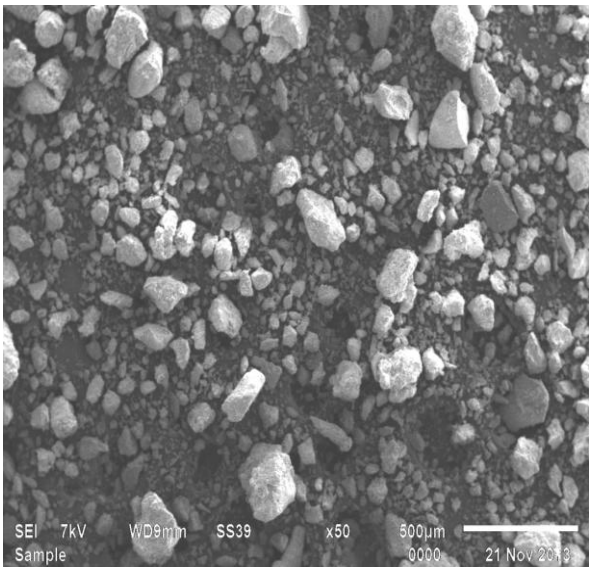
Sample 0000 21 Nov 2013


SEI 7kV WD10mm SS39 x500 50µm
Sample 0000

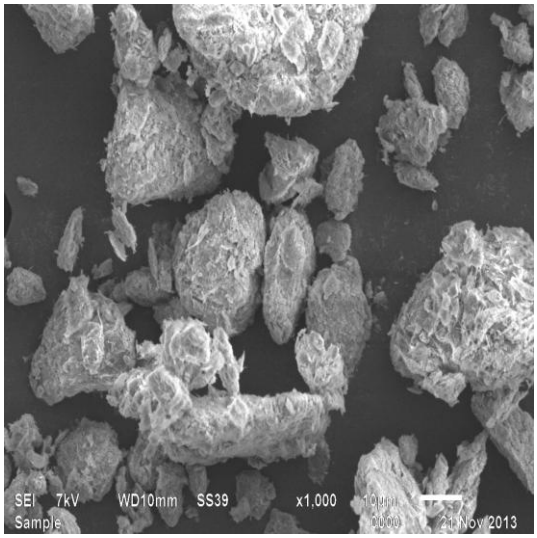
b) Charcoal

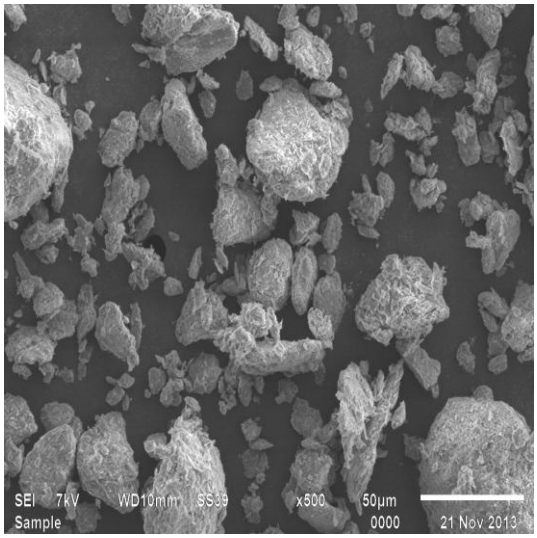

SS39
10µm
0000
21 Nov 2013

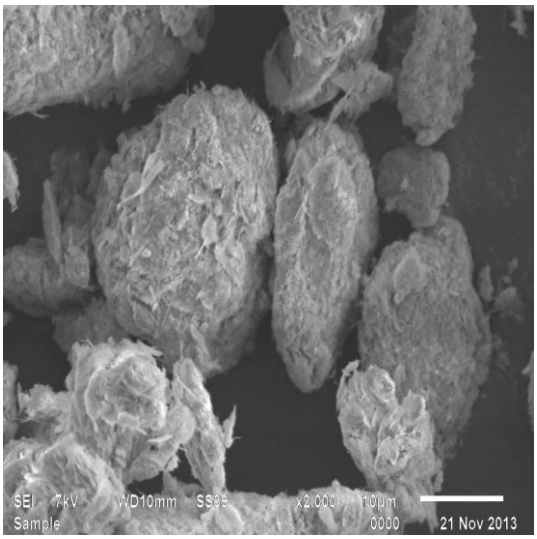

SEI 7kV WD10mm SS39 x500 50µm
Sample
0000 21 Nov 2013


SEI 7kV WD9mm SS39 x50 500µm
Sample
0000 21 Nov 2013

c) Soil

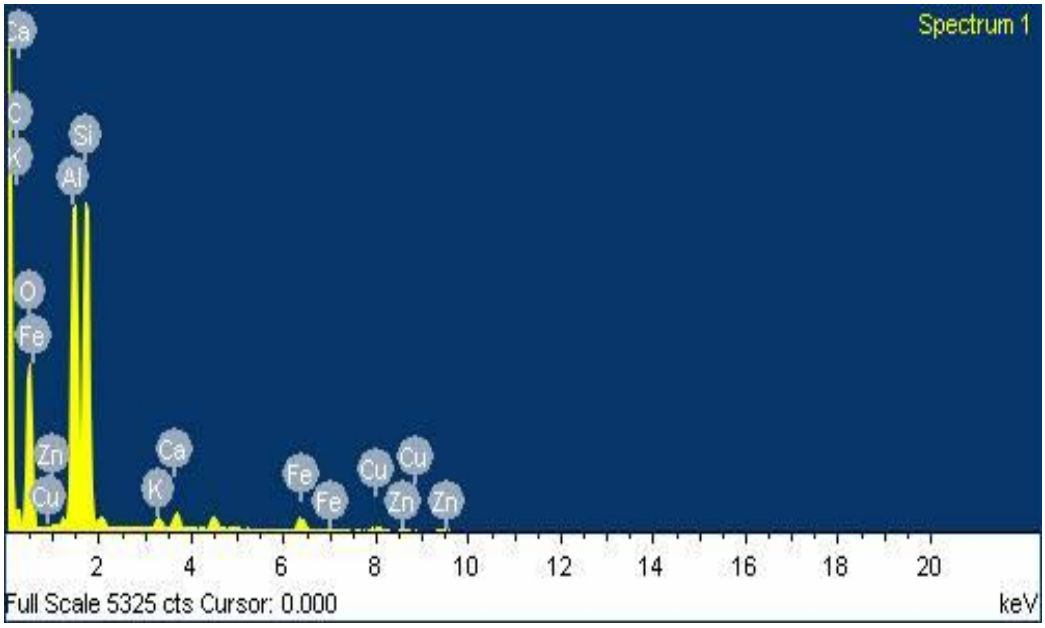




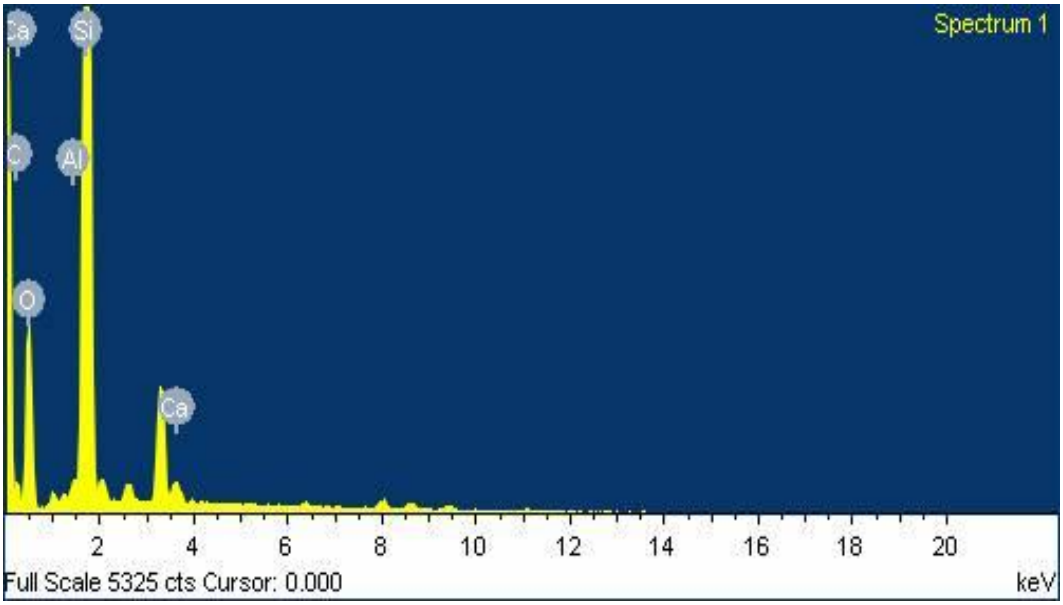


d) Montmorillonite

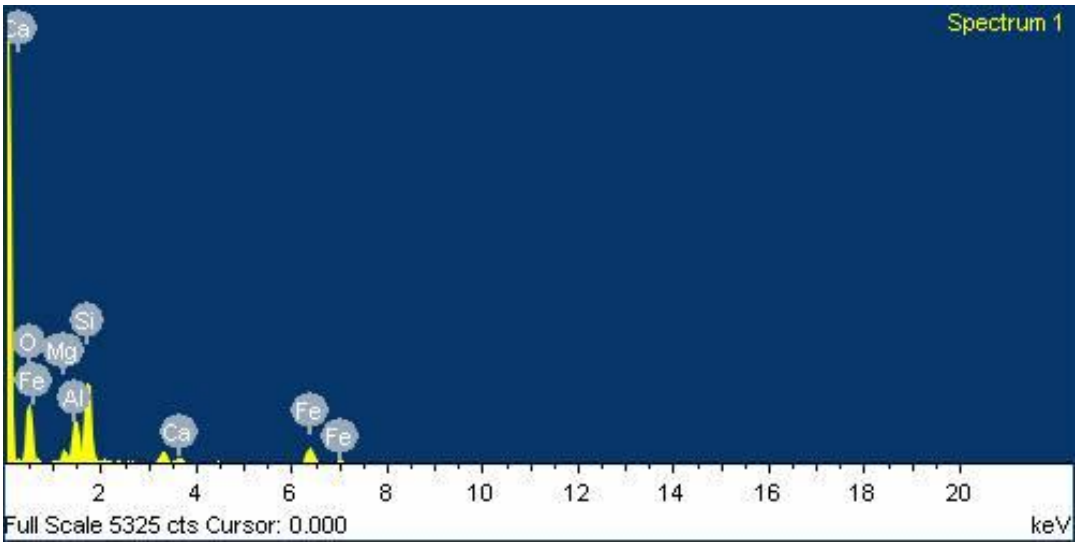
Fig 5: Energy dispersive spectrometry (EDS): Fly ash (a) showed the presence of aluminosilicate, calcium, iron, potassium, zinc and copper. Charcoal (b) showed the presence of carbon with some traces of calcium and aluminium. Soil (c) and MMT (d) showed the maximum presence of silica followed by aluminium, magnesium and calcium.



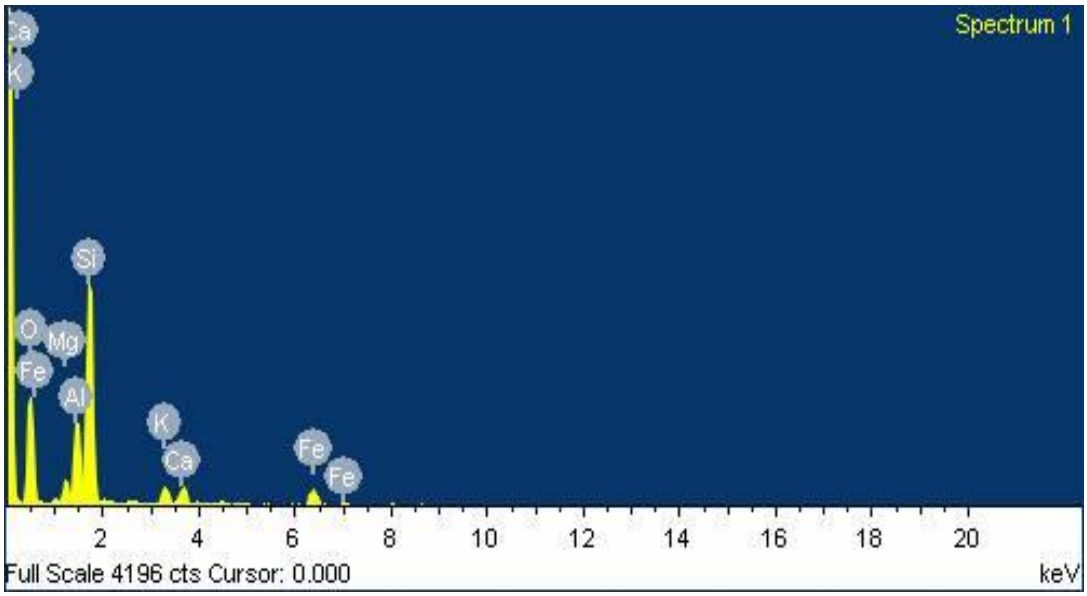
a) Flyash



b) Charcoal



c) Soil



d) Montmorillonite

Table 1 (a) X-Ray Diffraction (XRD)

Chemical composition of fly ash, charcoal, soil and montmorillonite as determined by XRD analysis. All values are reported as weight percentage.

Compound & Concentration (%)	Fly ash	Charcoal	Soil	Montmorillonite
SiO_2	54	28	65	68
Al_2O_3	40	0.35	20	21
MgO	0.95	0.67	13	6
CaO	1.2	0.81	0.97	---
FeO	0.16	0.19	1	5
CO_2	0.30	70	---	---

Table 1 (b): Total elemental Concentration of coal fly ash (ppm)

Element	Concentration (ppm)
Fe	1581±0.01
Zn	59.54±0.05
Mn	28±0.01
Pb	18±0.02
Co	3.96±0.5
Cr	5.32±0.1
Ni	21.4±0.01
Cu	17.09±0.01
S	1901±0.06
Mo	41±0.15
Se	3.8±0.25
As	6.1±0.10
Cd	BDL

***BDL :below detection limit**

Table 2 Fourier-transform infrared spectroscopy (FTIR)

*Band assignments of peak components as observed in flyash, charcoal, soil and montmorillonite***.**

Wavenumber range (cm^{-1})	**Band assignment**			
	Flyash	**Charcoal**	**Soil**	**Montmorrilonite**
450-520	Si-O	Graphite, C-OH, C=C	Si-O-Si	Quartz /Structural Lattice modes
550-1100	Si-O, Al-O	Aromatic mode	Si-O-Si	Si-O, AlMgOH, $(Al)_2OH$
1120-1600	---	Aromatic mode	C-O, O-H	Si-O, H-O-H bending of structural water of MONTMORILLONITE
1600-1700	---	C=C, C=O	O-H	---
2900–3400	C=O, Si-O-Si, O-H	O=H stretching	O-H	---
3440–3620	---	O=H stretching	O-H	O-H

Table 3. Energy Dispersive Spectrometry (EDS) of fly ash, charcoal, soil and montmorillonite.

Element	***Weight%***	***Atomic wt%***	***Compound%***	***Formula***
C K	17.17	23.65	62.93	CO_2
Al K	7.85	4.81	14.84	Al_2O_3
Si K	8.61	5.07	18.42	SiO_2
K K	0.29	0.12	0.35	K_2O
Ca K	0.53	0.22	0.75	CaO
Fe K	1.09	0.32	0.14	FeO
Cu K	0.53	0.14	0.66	CuO
Zn K	0.53	0.13	0.66	ZnO
O	63.40	65.53		
Total	**100.00**			

a) Fly ash

Element	Weight%	Atomic%	Compd%	Formula
C K	11.15	16.21	40.85	CO_2
Al K	0.35	0.23	0.67	Al_2O_3
Si K	26.96	16.77	37.69	SiO_2
Ca K	0.57	0.25	0.80	CaO
O	60.96	66.55		
Total	**100.00**			

b) Charcoal

Element	Weight%	Atomic%	Compd%	Formula
Mg K	3.71	3.37	6.16	MgO
Al K	11.31	9.25	21.37	Al_2O_3
Si K	24.21	19.02	51.79	SiO_2
Ca K	1.46	0.81	2.05	CaO
Fe K	14.49	5.72	18.64	FeO
O	44.82	61.82		
Total	**100.00**			

c) Soil

Element	Weight%	Atomic%	Compd%	Formula
Mg K	3.04	2.69	5.04	MgO
Al K	9.53	7.59	18.01	Al_2O_3
Si K	28.34	21.66	60.62	SiO_2
K K	2.88	1.58	3.46	K_2O
Ca K	2.67	1.43	3.73	CaO
Fe K	7.10	2.73	9.13	FeO
O	46.45	62.33		
Totals	**100.00**			

d) Montmorillonite

Table 4 Coarse grain accumulation

S.No	Particle size (mm)	Fly ash	Charcoal	Montmorrilonite	Soil
1.	4	100	96±0.12	94±0.18	100
2.	2	100	90±0.09	88±0.14	100
3.	1	96±0.01	83±0.01	82±0.05	98±0.01
4.	0.500	87±0.03	70±0.06	77±0.07	88±0.02
5.	0.300	81±0.01	61±0.01	60±0.11	82±0.01
6.	0.150	76±0.05	43±0.01	52±0.07	68±0.01
7.	0.075	70±0.01	27±0.02	47±0.04	51±0.02
8.	0.025	51±0.41	16±0.04	28±0.01	47±0.01
9.	0.010	42±0.01	10±0.02	18±0.01	38±0.05

(n=3,Mean± SE)

Table 5. Physiochemical characterisation of flyash, charcoal, soil and montmorillonite.

S.No	Parameters	Fly ash	Charcoal	Soil	Montmorillonite
1.	pH	7.85±0.12	8.19±0.02	8.22±0.1	8.27±0.1
2.	EC (µS/m)	0.14±0.02	2.87±0.01	2.45±3.75	15.5±25.8
3.	Bulk density(g/cm^3)	0.99±0.01	0.43±0.01	1.36±0.01	2.05±0.06
4.	Water holding capacity (%)	62.0±2.7	198±0.3	39.6±2.03	84.3±1.3
5.	Surface area (m^2/g)	0.96	4.90	5.45	79.27