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A Review on Standard Methods for Identification of BGA, Growth Factors of BGA, Culture of Algal Biofertilizer, N_2 Fixation by BGA, Rural Production Technology of BGA on the Habitat of Sidhi Region (M.P.)

Anil Kumar Singh[1], A. P. Singh[2], Naveen Gaurav[3], Abhishekh Srivastava[4] and Hira Singh Gariya[5]

[1]Research Scholar of A.P.S. University, Rewa, M.P. India.
[2]Department of Botany Govt. P.G. Science College, Rewa, M.P. India
[3]Assistant Professor Department of Biotechnology, S G R R P G College Dehradun, U.K. India
[4]Assistant Professor, Department of Botany Govt. S.V. College Teonthar, M.P. India
[5]B.Sc. Department of Biotechnology, S G R R P G College Dehradun, U.K. India
*Corresponding author email: naveensri17@gmail.com

Abstract

After surveying the one locality of the Sidhi District nearby Rewa division the BGA list was prepared by collecting and identifying the Blue Green Algae (BGA) of specific locality of Village Mawai nearby 10 km from Churhat, District-Sidhi (M.P.). Collection and Identification of the BGA done with the help of various resources and important websites available on the internet. The extensive survey of Sidhi region and surrounding area was done during the primary phase of study to know the distribution pattern of BGA of Sidhi region. **Biometrical:** After detail survey of site in 5 different spot were selected for present investigation. **Climate data:** Month-wise climatic datas were noted such as maximum and minimum temperature, maximum and minimum humidity, rainfall from Kuthulia office, Rewa. Average values were calculated on the basis of data obtained.

Keywords: Sidhi District, BGA, Village Mawai, Biometrical, climate data, climatic datas etc.

Introduction

The four nitrogen fixing cyanobacteria *Aulosira, Tolypothrix, Anabaena* and *Nostoc* which were isolated from the soil experimented. These cyanobacterial species were selected on the basis of their wide occurrence, fast growth and high nitrogen fixing ability. All organisms were used as biofertilizers. Isolation of the organisms in axenic cultures is essential to the study of heterotrophic growth. Different methods have been tried to obtain axenic cultures in the course of the present study, including repeated sub culturing on agar plates, repeatedly washing cells in sterile medium *(Pringsheiem, 1946),* and treatment of the algae with dilute chlorine water *(Fogg, 1942).* The use of antibiotics seemed to offer a possible method for freeing blue-green algae of bacterial contaminants. Therefore various antibiotics were employed, and a bacteria free culture of *Aulosira, Tolypothrix, Anabaena, Nostoc* was found after treatment with neomycin and the alga. Millipore filters (pore diameter 0.45 µm) were used for sterilising neomycin. Experiments were carried out in boiling tubes containing 5 ml of mineral medium plus a range of concentrations of neomycin (5-20 µg/ml). A small amount of inoculums was transferred into each tube and incubated in the dark at 35^0C for 24 h. After the incubation period in the dark, 0-5 ml was transferred from each tube to neomycin-free medium in the light. After 10 days of incubation, the algal growth was obviously inhibited (the cells became colorless) in the high concentrations (14-20 µg/ml). Growth occurred in concentrations of 5-12 µg/ml (somewhat inhibited in 12 µg/ml concentration). 1 ml quantities of the cultures were tested for bacterial growth by incubation into bacterial tests. The algal cells grown in the presence of 12 µg/ml neomycin showed no bacterial growth after incubation into the bacterial testing media. The alga has been maintained for several months by serial subculture in sterile medium. Regular sterility tests and direct microscopical examinations showed it to be still bacteria-free.

Procedure

1) One site of paddy fields with 5 spots was selected depending on their different environmental influences *viz.,* Village Mawai nearby 10 km from Churhat, District-Sidhi (M.P.).

2) Water and soil samples from the selected 5 spots of paddy field in the site during the growing seasons of Kharif was analyzed for their physico-chemical parameters. Analysis

was carried out from year 2011 to 2012 with the using of standard methods described in "Standard Methods" (APHA, 1995).

3) The identification of the isolated BGA from the site was carried out by the using of standard keys *(Desikachary, 1959; Anand, 1989; Santra, 1993).*
4) Lucky drop method has been used for Quantitative analysis of the sample as mentioned in *APHA (1995)*
5) The three types of cyanobacteria determined i.e. heterocystous, non-heterosystous and unicellular in Kharif seasons of paddy cultivation in site.
6) To obtain dominant heterosystous form of BGA, algal samples collected from paddy field and were identified by the using of standard keys (Desikachary, 1959).
7) *Aulosira, Tolypothrix, Anabaena, Nostoc* were identified in selected site.
8) Clonal cultures were obtained from a single filament, grown in BG_{11} medium *(Stainer,,et.al.,1971)* and it was maintained by the repeated sub culturing.
9) The effect of various concentrations of commercial fertilizers locally used on biomass content (g/10ml) *(Richmond and Grobbelaar, 1986),* chlorophyll "a" (µg/ml) *(Mckinney, 1941),* total proteins (µg/ml) *(Lowry, et.al.,1951)* and total carbohydrates (µg/ml) *(Dubois, et.al., 1956)* was studied in *Aulosira*, *Tolypothrix*, *Anabaena*, *Nostoc*.
11) The effect of different concentrations of commercial pesticides used locally Rogar 30, Butachlor, Monocrotophosr and Phorate on biomass content (g/10ml), chlorophyll a content (µg/ml), total proteins (µg/ml)) and total carbohydrates (µg/ml) was studied in *Aulosira*, *Tolypothrix*, *Anabaena*, *Nostoc*.

Methodology

The soil used in this study was taken at 0 - 20 cm depth supplied from Village Mawai nearby 10 km from Churhat, District-Sidhi (M.P.) Soil samples were air dried to be used for isolation of microalgae and other samples were autoclaved for undergoing Pot experiment. Some mechanical, physical and chemical properties of soil were analysed before conducting the Pot experiment.

Isolation of the organisms

Aulosira, Tolypothrix, Anabaena, Nostoc was isolated from the soil surface of a rice field in site in 5 different spots. The location of *Aulosira*, *Tolypothrix*, *Anabaena*, *Nostoc* was collected from

soil in site in 5 different spots. The location of site is at latitude of 24^0:47' and at a longitude of 81^0:73'.

Isolation and identification of microalgae

One gram of air dry soil samples was spread over different types of solid media in Petri dishes and incubated under fluorescent illumination at 33^0C. After 2 to 3 weeks the green mat that appeared on the surface of the dishes was picked up and sub-cultured in sterile liquid media (BG_{11}, Bold's Basal medium, Chu's medium, Allen's medium and modified Watanabae medium). Also, ten grams of soil samples were placed in 250 ml.

Erlenmeyer flasks each containing 100 ml of the sterile medium and left under the favourable growth conditions. Any coloured growth was picked up, sub-cultured and streaked several times in a new plate. Successive transfers from liquid to solid media and resub-culturing yield unialgal cultures. Identification of microalgae was done according to *Desikachary (1959).*

The colonies of *Aulosira, Tolypothrix, Anabaena, Nostoc* which appeared to be the dominant species were isolated and purified. Bacteria free cultures were obtained by washing according to *Hoshaw and Rosowski (1973).* Uni-algal cultures were subjected to centrifugation for 10 minutes at 3000 rpm, thereafter the supernatant was decanted. The washing process and centrifugation were repeated many times (not less than 6 times). Finally, the cyanobacterial mat was transferred to flasks containing sterile culture medium and incubated for growth. Repeated transfer of cyanobacterial cells to distilled water or liquid medium and centrifugation may lead to purification of the cyanobacteria.

Isolation of cyanobacteria

Water and soil dilution were prepared 10^{-2} and 10^{-3} and nitrogen free media, Tretykova (A Medium) and modified Benecke's solutions (B Medium) were solidified with 15 gram agar. A dilution was added to plates 1 ml from 10^{-2} and 10^{-3} and after that incubation of plates for photoperiod of 30-35 days in incubator set was done at 26-30^0C and 12/12 h *(Kaushik 1987).* A part of colonies was removed for the identification purification and multiplication of colonies by a loop and transferred to a liquid media shaken in a shaker at 26-30^0C and 12/12 h photoperiod.

Algal Isolation

This BGA was isolated and carried out the alga *Tolypothrix*, *Anabaena*, *Nostoc* and *Aulosira* in the site of Sidhi district and, a suitable culture medium found as De's solution *(De, 1939).* These alga Tolipothrix, *Nostoc*, *Anabaena* and *Aulosira* have been identified *(Geitler, 1932)*. The curious mucilaginous envelop differs it from the original, surrounding the heterocyst was not observed by Lemmermann.

Pure culture Isolation of Alga

For the study of nitrogen fixation isolation in pure culture is necessary preliminary. Although, it is necessary that the origin of cultures used should have from the one individual and the possibility of various physiological races of a algal species may exist. Such type of unialgal cultures of *Anabaena cylindrica* were isolated a specific single filament with a sterile capillary pipette, under low powered microscope inculcating into a sterile culture medium. The culture was still contaminated with bacteria obtained by this method. A little part of algal material was washed in sterile water and plated out but before that the material was immersed in the chlorine water for two minute containing a concentration of 25 ppm.

The Growth of BGA species *Tolipothrix, Nostoc, Anabaena* and *Aulosira* was maintained in BG_{11} medium (without nitrate salts, *Rippka and Stanier, 1978)* in 250 ml shaker flasks (50 ml media), at room temperature, under constant lighting from a 25 cm fluorescent light bulb. Cultures were sub-cultured to fresh media in 250 ml shaker flasks (2% v/v inoculums) approximately every 2 months.

For larger scale growth, a one liter airlift fermentor containing 250 ml of BG11 was inoculated with an entire shaker flask (~50 ml) of *Tolipothrix, Nostoc, Anabaena* and *Aulosira* and allowed to incubate under a bank of four 25 cm fluorescent light bulbs (incident light ~ 80 mol photons $m^{-2}s^{-1}$), at 27^0C, with continual sparging of sterile air for about 5-7 days. The culture would typically be near saturation (determined qualitatively by the amount of green color) in about 5-7 days under the conditions described. This culture was then used as an inoculum for a 2-l airlift fermentor.

Standard growth conditions

The growth in light was carried out at 35^0C in flasks incubated in the tank at 2000 or 5000 lux and shaken 64 times per min through a horizontal movement of 30 mm. The growth in dark was carried out under the same conditions in flasks painted with black paint, and then wrapped in aluminium foil, and black polythene.

Growth measurements

During growth of the *Tolipothrix, Nostoc, Anabaena* and *Aulosira*, estimations were made in various experiments, of the number of total cells, and dry weight of the algae. The following Standard procedure was used in harvesting. The culture was removed from the growth flask to a volumetric cylinder, and the volume was measured. It was then centrifuged at 5000 x g for 5 min. The algal material was washed with glass distilled water and re-centrifuged at 5000 x g for 5 min. This procedure was repeated twice. The material was then transferred to vitreosil crucibles (previously dried at 105^0C) and dried for 24 hours at 105^0C Growth rates were expressed in terms of the relative growth constant, k, in $\log_{10}$ day units *(Fogg, 1965).*

$$k = \frac{\log 10\ N - \log 10\ N0}{T}$$

where t= Days after inoculation, N= Yield after t days N_0= The total amount of cell material in the culture at zero time.

The mean doubling time, G, can be derived from k:

$$G = \frac{0.301}{K}$$

For certain experiments 0.2 mm haemocytometer was used for counting the number of cells in filament/ml and the total number of cells/ml. Before counting of the cells, the culture was treated with an ultrasonic probe for 1 min. to break the clumps. Three replicate counts were always made.

Growth conditions and preparation of the cyanobacterial inoculums

Replicates of each cyanobacterial species were cultured in Erlenmeyer flasks (1 L capacity), each containing 300 ml of BG11 free-nitrogen nutrient medium which was the most suitable medium for growth of both organisms. All flasks were incubated in an illuminated incubator at 33^0C±2 and light intensity of 2000 lux ($39\mu EM^{-2}S^{-1}$) using white fluorescent lamps. Subculturing was repeated periodically to obtain stock cyanobacterial biomass, to be used in further experiments in this study. Certain inocula of 7 days old cyanobacterial cultures were incubated in optimum growth conditions for 21 days to ensure the formation of secondary metabolites. Cyanobacterial inoculum was adjusted to be 10 ml of the cyanobacterial suspension (cells in the exponential phase) that contain approximately 10µg/ml chlorophyll "a". Separation of the cyanobacterial culture filtrate was carried out by centrifugation. Cyanobacterial cell suspensions were prepared by mixing the cyanobacterial mats with distilled water to the original volume. Initial chlorophyll a, total carbohydrates, proteins, and total nitrogen of both cyanobacteria were determined. Nitrogenase enzyme activity was also determined.

Tests of purity of the cultures

Tests were made with the following media:

(1) Beef peptone agar, (2) Malt extract agar, (3) Yeast extract agar

The compositions of the three media are shown in table. The media were sterilized by autoclaving for 15 min at 15 lb per sq inch. Incubation was in the dark at 35^0C for 48 h. Direct microscopical examinations of the cultures were also made, and in fact absence of visible bacteria in old algal cultures is perhaps the single most reliable method of confirming that a culture is axenic.

Chemicals

Except for the items listed below, all chemicals were obtained from the agencies and were of Analar grade. The exceptions were:

The composition of beef peptone, malt extract, and yeast extract agar media.

Chemical	Beef Peptone Agar	Malt Extract agar	Yeast extract agar
Bovril	3	–	–
Peptone	10	–	–
Malt-Difco	–	20	–
Yeast extract	–	–	30
Agar	18	18	18

Culture methods

The cultures were obtained from various sources. In addition to those obtained from Cambridge and Indiana culture collections, one other was isolated from site and obtained in an axenic state.

Again the cultures were obtained from various sources and included one isolate made by the author from a sample from site.

Stock cultures were maintained and experiments were carried out in 100 ml conical were soaked overnight in a hot 2% 'Quadralenel laboratory detergent scrubbed, and rinsed thoroughly in were finally rinsed three times in and dried in an oven at 100^0C. Pyrex flasks. These (w/v) solution of after soaking they were hot water. The flasks glass distilled water Pipettes were cleaned by soaking overnight in a mixture of one volume of saturated sodium nitrate and six volumes of concentrated sulphuric acid. They were rinsed thoroughly in tap water and were given a final rinse in glass distilled water.

Apparatus for growth of cultures

Growth was carried out in flasks incubated in tanks (warm white) illuminated from by a bank of fluorescent tubesh light intensities were adjusted by reducing the number of tubes and by wrapping layers of black fine-meshed cloth round the flasks. The apparatus was designed to make easy and rapid modifications of the incubation temperature and shaking rate. An orbital incubator was modified to provide complete darkness conditions and was used for re-checking the growth of the cultures in the dark.

Inoculum material

In general, the inoculum material was composed of 1 ml of a culture between 23 to 40 days old, which had been grown in the light or in the dark under the standard growth conditions.

While sub-culturing material from dark to dark, special care was taken to avoid picking up any trace of light which might possibly have a photomorphogenetic effect on the algae. Before autoclaving, pipettes were wrapped in aluminium foil, and inoculation was carried out in the dark near the bunsen flame, in a sterilised room. The modified flasks for growth in dark were immediately wrapped in aluminium foil and black polythene.

Types of media

Macro-nutrient solutions

Macro nutrient solutions were prepared as follow: 1500 mg NaNO3; 40 mg K2HPO4; 75 mg MgSO4.7H2O; 36 mg CaCl2.2H2O; 6 mg Citric acid; 20 mg Na2CO3; 1 mg Na2EDTA; 6mg Ferric ammonium citrate. NaNO3 was omitted to obtain a medium free of combined nitrogen. The above salts were dissolved in one liter distilled water, and then one ml of the micro-element solution was added.

Micro-nutrient solutions

Micro nutrient solutions were prepared as follows:

2.86g H3BO3; 1.81g MnCl2.4H2O; 0.222g ZnSO4.7H2O; 0.39g

Na2MoO4.2H2O; 0.079g CuSO4.5H2O; 0.0494g Co(NO3)2.6H2O.

These salts were dissolved in one liter distilled water.

2- Bold's Basal medium *(Bischoff and Bold, 1963)*

Six stock solutions (in distilled water) 400 ml in volume, each containing one of the following salts in the concentrations listed were prepared:

l0g NaNo3; 1g CaCl2.2H2O; 3g MgSO4.7H2O; 3g K2HPO4; 7g

KH2PO4; 1g NaCl.

Ten ml of each stock solution and one ml of each of the following stock trace element solutions were added to 940 ml distilled water. NaNO3 was omitted to obtain a medium free of combined nitrogen.

Micro-nutrient solutions

50g Na2-EDTA in 1litre distilled water; 4.98g FeSO4.7H2O in one liter of acidified water (1 ml conc.H2SO4); 11.42g H3BO3 in one liter distilled water; the following amounts were added together to one liter distilled water 0.71g MoO3; 1.44g MnCl2.4H2O; 8.82g ZnSO4.7H2O; 1.57g CuSO4.5H2O and 0.049g Co(NO3)2 6H2O.

Modified Chu's medium *(Gerloff,et.al.,1950)*

It has the following composition: 7.08g Ca(No3)2. 4H2O; 0.87g K2HPO4; 2.43g MgSO4.7H2O; 27.8mg FeSO4.7H2O; 1.9mg MnCl2.4H2O; 37.0mg EDTA (disodium salt).

All were dissolved separately each in 100 ml of distilled water. Ca (NO3)2.4H2O and KNO3 were not added.

Soil extract

200g of soil were added to 300 ml distilled water, autoclaved and then filtered after cooling. The following volumes were taken from the above solutions:

1 ml of K2HPO4; 2.46 ml of MgSO4.7H2O; 1 ml of FeSO4.7H2O;

1 ml of MnCl2.4H2O; 10 ml EDTA; 25 ml of soil extract, then completed to one litre with distilled water and autoclaved.

Allen's medium *(Allen, 1968)*

The following weights of salts were dissolved in one litre distilled water:

1.5g NaNo3; 0.075g MgSO4.7H2O; 0.027g CaCl2.2H2O; 0.039g K2HPO4; 0.02g Na2CO3; 0.001g EDTA; 0.006g citric acid; 0.006g ferric citrate and 0.058g Na2SiO3.9H2O. NaNO3 was not added to the medium, one ml of trace elements solution was added then autoclaved.

Micro-nutrient solutions

2.86g H3BO3; 1.81g MnCl2.4H2O; 0.222g ZnSO4.7H2O; 0.391g Na2MoO3.2H2O; 0.079g CuSO4.5H2O; 0.049g Co(NO3)2.6H2O, all were dissolved in 1litre distilled water.

Modified Watanabae medium *(EL-Nawawy et al, 1958)*

Macro-nutrient solutions

To 0.3g K2HPO4; 0.2g MgSO4.7H2O; 0.2g K2SO4; 0.1g CaCO3; 2.0g glucose; 0.2 ml of FeCl3 (1% freshly prepared); one ml of the micro-nutrient solution, 800 ml of distilled water were added and the media was autoclaved. Glucose was dissolved in the least amount of distilled water (200 ml) and autoclaved separately. After autoclaving it was added to the media under aseptic conditions.

Micro-nutrient solutions

2.8g H3BO3; 0.22g ZnSO4.7H2O; 0.08g CuSO4.5H2O; 0.02g MoO3 and 1.8g MnCl2.4H2O, all were dissolved in 1 liter distilled water.

Media

The media were prepared with glass distilled water and chemicals of Analar grade. Two basic inorganic media were used, one containing a source of combined nitrogen, which is a modification of the ICI medium of *Kratz* And *Myers (1955),* and here termed 1AC1 medium, and the other lacking combined nitrogen which is a modification of the medium of *Fogg (1949),* and here termed 'AD' medium.

Sterilisation

Flasks were stoppered with non-absorbent cotton wool plugs. Basal media, and media containing sucrose or sodium acetate, were sterilised by autoclaving at 15 lb per sq inch for 15 min, whilst those containing fructose galactose, glucose or maltose were sterilised by autoclaving at 10 lb per sq inch for 10 minutes. Pipettes were sterilised in a similar manner to the basal media.

Inoculation was carried out in a room partially sterilised by ultra-violet irradiation, with other precautions being taken to minimise contamination. Before inoculation the bench was cleaned

with absolute ethanol and the room sprayed with absolute ethanol to remove suspended material from the atmosphere.

Soil conditioners

The synthetic conditioner used in the present investigation was a cross-linked sodium polyacrylamide commercially known as broadleaf-P4. It is granular, insoluble hydrophilic and absorbs water 400 times its weight. Broadleaf-P4 was homogeneously mixed with sterile air dry sandy soil at 0.2 % (W/W).

Determination of Biomass, Total chlorophyll content and chlorophyll –a

Each culture of cyanobacteria was harvested on 7, 14, 21 and 28 days, in between the time of interval the biomass were get collected by filtering of the media through 41 no filter paper of Whatman and after that in the finally weighted to record the biomass. By the way of this research method or investigation the record of last biomass noted at specific time intervals for incubation, which showed there maximum growth at their stationary phases. The total biomass was taken for further evaluation as the biomass calculated on the basis of weight of maximum biomass production showed. The total chlorophyll content and the curve of chlorophyll–a has been estimated like a index for growth of alga. The estimation of extracted pigment was done by the using cold extraction method and, also expressed of fresh culture in ml/mg *(Arnon, 1949).*

Determination of total N-content

By the using of Micro Kjeldahl method total N- content was determined and 0.1 gm dry flakes of alga were collected after the biomass determination of isolates of BGA. The total N content and biomass production were analysed *(Jackson, 1973).*

In vivo studies on the production of BGA isolates in respect to the seasonal growth

To observe the growth of BGA culture a separate field experiment has been carried out in different seasons throughout the year. A temporary bed of bamboo culture was prepared for this experiment and they were cultured in poly bag measuring (12cm×12cm×18cm) in BG_{11} medium to assess the growth among the BGA isolates. In each of poly bag 500 ml of BG_{11} was poured in three replicates and in the interval of 07, 14, 21 and 28days the growth of culture was observed.

Considering three replicates also observed the multiple culture of BGA and during the time of field and pot experiments meterological parameters were also recorded.

In vivo studies for mass multiplication of BGA based on sterile and non sterile paddy soils as substrate

Paddy soils, both sterile and non–sterile were evaluated and considered for the experiment and chemical characteristics of soil samples were analysed as per methods.

Measurement of physical features

The light intensities were measured with an EEL Light Master Photometer at the surface of the water, and oriented at right angles to the source of light. Light intensity is expressed in lux. 20–1000 lux is referred to as low light and 6000 lux onward as high light. Temperature is expressed on the centigrade scale (^{0}C).

Biochemical analysis

Extraction and determination of photosynthetic pigments

One gram of fresh BGA was extracted using 80 % acetone (v/v) and the extract was filtered. The optical density (O.D) of the extract was measured at 440.5, 645 and 663 nm using a spectrophotometer (pharmacia Biotech-Ultra Spec 2000). The equations given by *Steward (1964)* and *Witham, et.al.,(1971)* were used to calculate chlorophyll a, chlorophyll b, and carotenoids as follows:

1.	mg chl a/g tissue =	$[12.7(O.D_{663})–2.69\ (O.D._{645})\times \frac{V}{1000\times W}]$
2.	mg chl b/g tissue =	$[20.2(O.D_{645})–8.02\ (O.D._{663})\times \frac{V}{1000\times W}]$
3.	mg Total Chl/g tissue =	$[22.9(O.D_{645})–4.68\ (O.D._{663})\times \frac{V}{1000\times W}]$
4.	mg caroteniods / g tissue =	$4.695(O.D_{440})–0.268\times(chla+chlb)$

Where is V = Acetone volume, W = fresh weight of leaves.

Chlorophyll a, chlorophyll b and carotenoids are expressed as mg per g fresh tissue. Chlorophyll (a) determination in the cyanobacteria was carried out according to: APHA, AWWA and WEF (1995).

A known volume of the cyanobacterial suspension was centrifuged and the clear solution was decanted. The cyanobacterial pellets were extracted using 100% hot methanol then centrifuged for 10 minutes at 2000 rpm if needed. The extract was measured against 100 % methanol at 664, 647, and 630 nm. Chlorophyll a content was calculated as follows:

$$\text{Chl a (A)} = 11.85_{\text{O-D664}} - 1.54_{\text{O-D647}} - 0.008_{\text{O-D630}}$$

$$\text{Chl a } \mu\text{g/L} = \frac{A \times v \times 1000}{V}$$

Where :-

v = Methanol volume

V = Sample volume

Extraction and determination of soluble nitrogen

Soluble nitrogen content was determined by dividing the protein content by 6.25. Concerning protein extraction of cyanobacterial cells, it was done with 4 % sodium hydroxide according to *Quian* and *Wang (1989),* and determination according to *Lowry,et.al.,(1951)* as mentioned before.

Total nitrogen

The total nitrogen content of root and shoot tissues was determined according to *Naguib (1969).* A known weight of tissue was digested using 50 % H2SO4 and 30 % HClO3, then ammonia content was estimated in the neutral samples using the Berthelot reaction (*Chaney* and *Marbach, 1962)* methods as follows:

Reagents

I- Phenol sodium nitroprusside: 50gm phenol + 0.25gm sodium nitroprusside / litre distilled H2O (in a dark bottle).

II- Sodium hydroxide, sodium hypochlorite: 25gm NaOH + 30 ml NaO Cl / 1 litre distilled H2O.

1 ml of sample + 1 ml of reagent I + 1 ml of reagent II were placed in a water bath at 37^0C for 15 minutes, after shaking and the volume was completed with distilled H2O according to colour intensity. The absorbance was measured at 630 nm, and calculations were obtained from the Standard curve. Other insoluble nitrogen content was determined by subtracting the values of proteins and soluble nitrogen from that of total nitrogen. Both the total nitrogen content in the cyanobacterial tissues and the total soluble nitrogen secretion were determined by the above mentioned method. The total nitrogen fixed by the cyanobacteria was calculated by summation of total insoluble fixed nitrogen (cyanobacterial masses) and soluble fixed nitrogen (in the growth medium).

Nitrogenase activity

The activity of nitrogenase enzyme was determined by the acetylene reduction technique in both nitrogen fixing cyanobacteria according to *Hardy et al (1973).*

Statistical analysis

In this study data were subjected to statistical analysis according to *Steel* and *Torrie (1980).* The least significant difference between readings and control (LSD) in all treatments was conducted in addition to standard deviation (SD).

Statistical analysis– Significant differences were surveyed at the level of 0.05 in an analysis performed with independent samples

The BGA species were collected from the study site in various spots and the morphological parameters were taken.

The BGA species were grown in earthen pots. The BGA species were grown on the different types of soil. The observation were made and noted in the table. The analysis of different parameters determined as per Standard methods.

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