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Research article

Phylogenetical characterization of cyanobacteria from paddy field of Chhattisgarh (Part ii)

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ABSTRACT

The above article consist the rest part of Phylogenetical Characterization of Cyanobacteria from Paddy Field of Chhattisgarh. Previous article consist of more physical study data. The above article is presented with measurement of pigments, measurement of the growth of the phylotype, estimation of calcium, phosphorus and water holding capacity. With respect to Chhattisgarh, rice is the principal crop of the state. India Covers 66% of cultivable land and mostly grown under kharif cropping season. To increase sustained productivity without decreasing soil quality, algal bio fertilizers are used widely now days in the state. Use of local isolates as algal inoculants is being stressed due to their competitiveness in the field for establishment of better ecological adaptability for developing composite starter. Culture of algal bio fertilizer programmer on a regional basis. Survey, isolation and screening of stress-tolerant cyanobacteria have been started at various parts of India. The soils of Chhattisgarh state comprise mostly of iron- rich red soil, laterite soil, and red and yellow soil and brown forest soil. So the above study comprises the characterization of physical and chemical properties collected from 4 districts of Chhattisgarh state.

Keywords: Cyanobacteria, Paddy field, Soil pH, Soil organic content.

INTRODUCTION

Cyanobacteria also referred as blue-green algae are the pioneer oxygenic phototrophs on earth whose distribution around the world is suppressed only by bacteria. Fossil evidence points to their presence in geographically diverse regions during the pre-cambrian (2 to more than 3.5 billion years ago). They are the large morphologically diverse group of phototrophic prokaryote, which occur in almost every habitat on earth. This versatility may explain the remarkable lack of morphological (and presumably physiological) change seen in 3.5 billion years old fossilized cyanobacteria and their modern day counterparts. Their long evolution history has been marked by key geographical and biotic transitions including the creation of oxygenic photosynthesis, a prerequisite for the development of proliferation of metabolically complex microbial and higher eukaryotic life forms [1].

They are very close to bacteria than higher plants. Cells are generally blue-green to violet but sometimes red or green. The green of the chlorophyll is masked by the blue accessory pigment phycocyanin. These pigments lie in phycobilisoms, which lie on thyo-alkaloid. Their cell walls are made of amino sugars and amino acids. Flagella are completely absent and the moment in the members is accomplished by gliding motion [2]. Their evolutionary antiquity and ability to fix CO_2 using water as an electron donor provided them with a decisive edge over other microbes to dominate the biosphere in the past and inhabit extreme environmental niches. Majority of the extinct species of cyanobacteria are obligate photoautotrophs, though a small fraction is mixotroph. The light dark cycle is perhaps the most significant and persistent selection agent that has shaped the physiology of photoautotrophs all through their origin and evolution. This clearly points to a dominant role of diuranal rhythm on regulation of the mode of carbon utilization in cyanobacteria [3]. The role of cyanobacteria as biological inputs in agriculture has been well documented and substantiated has reported that algal inoculation was

equally effective for the high yielding dwarf rice (*Oriza sativa* L.) varieties under high fertility. Cyanobacteria also occupy a variety of terrestrial environments. Soil is one of the most potential habitats for algal growth particularly in moist or waterlogged conditions. They play a significant role in maintaining soil fertility and in soil reclamation [4].

MATERIAL AND METHOD

Characterization of cyanobacterial strains can be done using morphological, biochemical or physiological attributes in recent times Macromolecules such as Proteins and DNA have been used for profiling the strains these are more reliable and discriminatory besides economical in terms of time and costs [5].This section embodies the common procedures applied frequently throughout the course of present investigation. All investigations in the soils of paddy fields were performed in the four divided Blocks of Chhattisgarh regions. Soil samples of paddy fields were collected randomly from Bilaspur, Champa, Raipur, and Durg, districts of Chhattisgarh. Soil samples from paddy fields were collected in the month of August and September. During this period the age of paddy crops was 2-3 months. The soils were contained fertilizers which were used by the farmers.

The paddy fields of Chhattisgarh were divided in to 4 Blocks:-

Block 1 : Bilaspur, Bilha

Block 2 : Champa, Baradwar

Block 3 : Raipur, Bhatapara

Block 4 :Durg, Rajnandgaon

Physico-chemical Analysis of Soil

The soil is a porous mixture of inorganic particles, organic matter, air and water. This mixture also contains a large variety of living organisms. The inorganic particles are organic matter make up the soil solids, white and soil pore space is occupied by air and water. The soil environment is influenced by several factors including geological, physico-chemical and biological properties of the soil, climate and human activities prevailing in that area. Soil analysis includes soil sampling and analysis for different physico-chemical properties of the soil. Soil texture and structure are two important physical properties of the soil that influence soil aeration, water retention and water movement. Sandy soils are well aerated but have low water holding capacity. Granulated clayey soils are not only well aerated and well drained but they can retain sufficient water and nutrients for plant use. A number of physicochemical properties such as pH, electrical conductivity and organic carbon were analyzed from different Blocks of Paddy fields.

Determination of water holding capacity

Weight was measured of empty moisture. Saturated soil sample was taken in moisture can and weight was measured. Moisture can with moist soil was kept inside the oven. It was repeated upto a constant value was reached. The reading was measured in triplicate. Calculation involved in the estimation of water holding capacity is as follows:

Weight of empty moisture can = (x)

Moisture content in soil = (y–z)

Weight of oven dry soil = (z–x)

Weight of moisture can + Moist Soil = (Y)

Weight of moisture can + Oven dry Soil = (z)

$$\text{Percentage moisture is soil} = \frac{(y-z)}{(z-x)} \times 100$$

Determination of available phosphorus (14)

2.5 g air-dried soil sample was taken in a 125 ml Erlenmeyer flask. A little amount of activated charcoal was added. 50 ml $NaHCO_3$ solution was added in each flask. Solution was shaked for 30 minutes. Blank experiment was carried out without using soil. Extract was filtered by using what man No. 40 filter paper. 10 ml of extract was taken in 50 ml volumetric flask and 10 ml distilled water and one drop of p-nitrophenol indicator was added. New the content was acidify to pH 5.0 by adding 2.5 m H_2SO_4 dropwise till colour was disappeared. 8 ml of the Murphy-riley solution was added and volume was made upto 50 ml. with distilled water. After 15 minutes, the intensity blue colour on spectro photometer at 730 nm was measured.

$$\text{Available p}\ (\text{kg ha}-1) = \frac{\text{C x Volume of extract}}{\text{Volume of aliquot}} \times \frac{2.24}{\text{Weight of soil}}$$

Where C = mg phosphorus in aliquot (obtained from standard curve)

Determination of Calcium

50 ml of test sample was taken in a conical flask 50 ml of distilled water was added to it. Raised the pH to 12-13 by adding 2 ml N/20 NaOH 1-2 drops of the indicator was added and titrated with EDTA to the end point. Calculation done to determine calcium is as follows:

When the EDTA titrant is 0.02 N then

$$\text{mg/}l\ calcium\ as\ Ca = \frac{ml\ EDTA\ titrant\ x\ 1000}{ml\ sample\ taken\ for\ titration} \times 0.40$$

$$\text{mg/l calcium as CaCO3} = \frac{\text{ml EDTA titrant x 1000}}{\text{ml sample taken for titration}}$$

Numerical evaluation of distributional pattern of cyanobactria

The proportionate occurrence of different cyanobactria in a soil sample is converted to numerical values as dominant, very common, rare and very rare. The total number of soil samples from an area harboring a particular alga, irrespective of its proportionate distribution is taken as "numerical total". The numerical total was multiplied with the corresponding numerical value 1-5, depending upon the comparative preponderance to obtain "rated total". The rated

total was divided by the total number of soil samples from the area and converted to percentage to give rated score for those particular cyanobacteria in the area. This rated score was used to compare the proportionate distributional pattern of algae in the area.

$$Rate\ Score\ (\%) = \frac{Rated\ total\ x\ 100}{Total\ no.of\ Soil\ samples\ collected}$$

Collection and Isolation of Cyanobacteria from soils of paddy fields

Cyanobacteria were isolated from soils of paddy fields of different blocks of Chhattisgarh state. 8-10 representative randomized surface soil samples were collected from each block. These soil samples were inoculated separately in separate petriplates. After proper incubation 4-5 isolates were observed in each petriplate. Approximately 40-50 isolates were collected from each block. These were containing species of cyanobactria and green algae. These were purified by sub-culturing methods, and after purification 10 isolates were selected for physiological and molecular studies. Approximate 8-10 soil sample were collected from each block these soil samples were inoculated separately in petriplates. After proper incubation 4-5 isolates were observed in each plate. Approximate 40 to 50 isolates were collected from each block these were containing species of cyanobacteria and green algae. These were purified later by sub culturing method for cyanobactria only and after appropriate purification only 11 cyanobacterial isolates out of 169 isolates were selected for preliminary morphological identification using trinocular laboratory microscope (Carlzeiss model Axiostar plus A - 80 with Canon digital camera). Shrivastav (2000). Latter on the molecular characterization of only 10 isolates from the 11 morphotypes were carried out based upon phylogenetic analysis [6].

Preparation of culture media

Chu-10, BG-11 and modified Hughes medium were tested for isolation of cyanobacterial populations but the medium BG-11 supported the luxuriant growth of majority of the cyanobacterial isolates[9, 10]. Stock solutions of all the ingredients were prepared and kept in screw capped bottles. Volume of each ingredient was taken accordingly for 1 litre of the medium. pH of the solution was maintained between 7.5-8.0. For adjusting the pH, N/10 solution of NaOH and HCl were used. BG-11 medium was used for cultivation of isolates. 10 ml of each ingredient was taken and 1 ml of trace element mix was taken for the preparation of 1000 ml of medium.

Composition of BG-11 medium

Component's	**g/l**
Sodium nitrate	1.5
Di-potassium hydrogen phosphate	0.04
Magnesium sulphate	0.075
Calcium sulphate	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (Disodium magnesium salt	0.001
Sodium carbonate	0.02
Trace elements mix	1 ml

Trace elements mix

Component's	**g/l**
Boric acid	2.86
Manganese chloride	1.81
Zinc sulphate	0.222
Sodium molybdate	0.039
Copper sulphate	0.079
Cobalt nitrate	0.0494

Sterilization

The BG-11 growth medium used for the routine culturing of cyanobacterial isolates was steam sterilized in an autoclave at a steam pressure of 15 lb per square inch at a temperature of 121°C. All the glasswares were sterilized in a hot air oven at 160°C for 2 hrs before being used

Isolation and Growth condition

BG-11 medium having solidifying agent (Agar-Agar) was taken in petriplates and green soil scrape from paddy fields were inoculated on the surface of solid medium. Plates were incubated under light intensity of 3000 lux, 16 hr light and dark period at 25°C ± 1°C for 15 days.After incubation greenish colonies were observed. These were transferred into another plate. Now through sub-culturing method, these colonies were again transferred into the medium by sterilized tooth pricks.

Purification of cultures

Contaminated cultures were made unicyanobacterial by streak plate method. Streptomycin antibiotic was used to inhibit the growth of contaminants like other bacteria and fungi.

Procedure

After bright field microscopic observation different colonies were transferred into broth medium separately.

Flask cultures were incubated.

After proper incubation colonies were again observed through light microscope.

The purity of cultures was maintained by sub-culturing.

Finally the pure cultures of cyanobacteria were obtained.

Green soil scrape from paddy fields were inoculated in N^+ and N^- BG-11 medium separately but growth were observed only in N^+.

Maintenance of culture

To reduce frequent sub-culturing without losing viability, the unicyanobacterial cultures were grown on Agar slant with appropriate medium contained in a screw capped culture tubes as well as in conical flask containing liquid medium. The cotton plugs were then replaced by pre-sterilized, bakelite screw caps. The experiments were run in

duplicates. Cultures were then transferred to stock culture room under conditions (at temperature of 25°C ± 1°C under light intensity of 3000 lux for 16 hr light and dark) which were just sufficient to keep them in viable state. All manipulation involving the transfer of cultures in the liquid media or on agar plates and agar slants were carried out under aseptic conditions on a laminar flow.

Measurement of Growth

Growth experiments were performed in 250 ml flask containing 100 ml media, exponentially growing liquid culture was harvested and inoculated into fresh medium. The culture was maintained in culture room as mentioned above. Growth was recorded by following change in optical density at 760 nm using UV/VIS spectrophotometer (Shimatzu) alternatively growth was also monitored by following changes in protein concentration per unit volume of culture [10].

Estimation of chlorophyll-a

Chlorophyll a was estimated by the following method, 4 ml aliquot of culture was centrifuged (5.000 xg, 10 min.) and the pellets were suspended and thoroughly mixed with 4 ml methanol. After 30 min. of incubation, cell debris was removed by centrifugation. Chlorophyll a content in methanol extracted supernatant was measured at 665 nm employing standard extinction coefficient [11]. Chl a µg/ml = 12.7 x $O.D._{665}$

Phycocyanins

20 ml of exponentially growing cells ware pelleted and suspended in 85% acetone and incubated in dark for 12 hrs at 4ºC. The supernatant was separated by centrifugation. The acetone insoluble fraction was mixed with 10 ml distilled water and exposed to repeated freezing and thawing (3-4 times). Finally the suspension was sonicated in Soniprep-150 (MSE. England) at 150- 200 miliampieres for 2 min at 4ºC and centrifuged. Clear suspended and acetone soluble fraction were examined spectrophotometerically with systronics spectrophotometer at 620 nm [12].

Carotenoid

40 ml thick culture (O.D. 0.6-1.0) was pelleted by centrifugation at 10,000 xg for 10 min and suspended in 60 ml of petroleum ether. The mixture was well vortexed and washed several times by water (75ml). Thereafter, the upper layer of the petroleum ether was taken for further extraction. Whereas remaining water layer was discarded. To upper layer, 40 ml of 90% methyl alcohol was added by constant stirring. The two layers were allowed to separate from each other. In the upper petroleum layers (30 ml), 15 ml of methyl alcohol (30 %) KOH solution was gently added, 30 ml of water was also added and the whole mixture was vortexed. The two layers were allowed to separate. The lower orange petroleum ether fraction was of carotenoids (24).

Measurement of pigments

The following calculation was used for quantitative estimation of different pigments found in cyanobacterial isolates CB_1 to CB_{15} (25).

Phycocyanin= (0.19 A_{622} - 0.06 a_{678}) x 10^{-3} µg/ml

Chlorophyll a = (14.5 a_{678} - 0.56 A_{622}) x 10^{-3} µg/ml

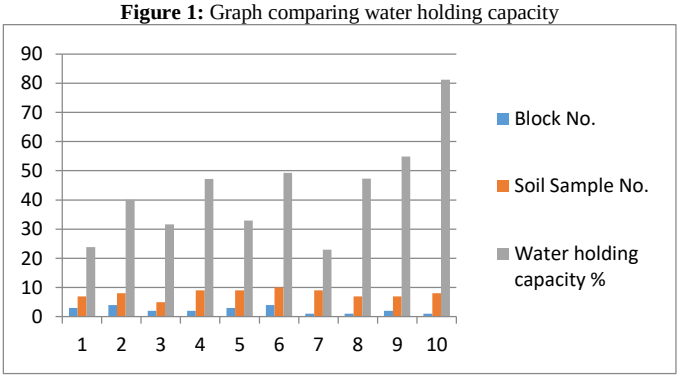
Carotenoid = (7.6 A_{480} - 3.6 A_{510}) x 10^{-3} µg/ml.

RESULT

Determination of water holding capacity

Water plays a very significant role in soil plant growth relationship. In fact water in a regulator of physical, chemical and biological activities in the soil. Water holding capacity of the soil is the amount of water in a soil when its total pore space both macro and micro is completely filled with water. Fig. 14 showed that 81.21% is water holding capacity of block 01 which ranges from 22.99 to 81.21% the lowest capacity is shown by other block no. 3 (23.87%). Soil texture and structure are two important physical properties of the soil that influence soil aeration, water retention and water movement. Sandy soils are well aerated but have low water holding capacity. Granulated clayey soils are not only well aerated and well drained but they can retain sufficient water and nutrients for plant use. Heavy clayey soils are not well aerated and drained. The soil of block no. 1 granulated clayey and block 03 was sandy clay, 02 - clay and block no. 4 was clay loam. On the basis of analysis of soil texture it can be said that maximum no. 7 cyanobacteria were isolated from block no. 01 and 02 which has granulated clayey and clayey soil, known to have high water holding capacity. Result is shown in figure no.1.

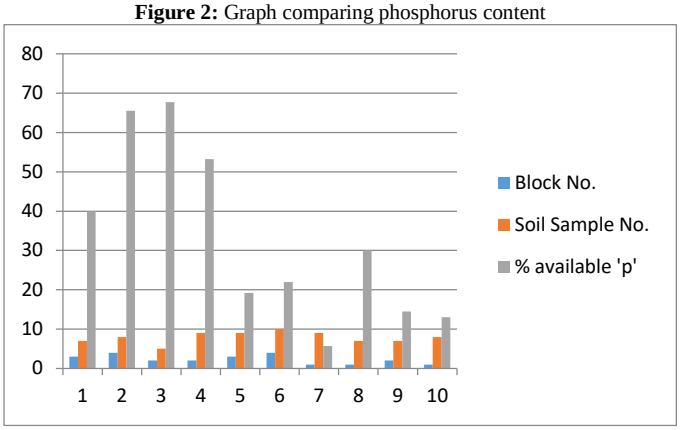
Figure 1: Graph comparing water holding capacity



Determination of available phosphorus

Phosphorus in soils ranges from 0.01 to 0.3% and occur in several forms and combinations. The apatite group of primary minerals is the original source of about 95% or more of the soil phosphorus. The total amount of phosphorus present in soils is not available to the soil microflora and plants, only small fraction of it may be available which is of direct relevance in assessing the phosphorus fertility levels. The maximum percentage of available 'p' was shown by block no. 02 (67.75%) as compare to other blocks, which exhibited low content of available 'p' to the soil microflora. Cyanobacterial growth in flooded rice soils have also been reported to influence the availability of P, Zn, Mn, Fe. Result is shown in figure no.2.

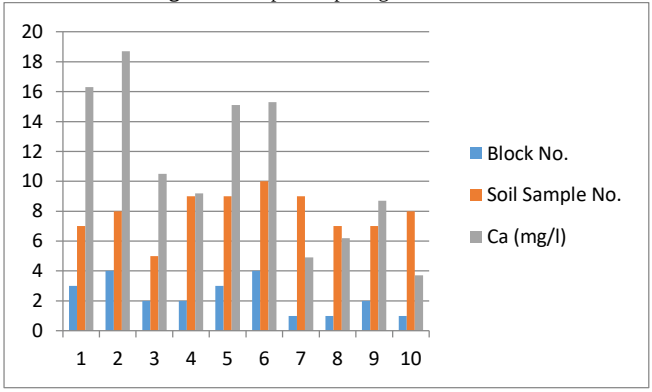
Figure 2: Graph comparing phosphorus content



Determination of Ca^{++}

The availability of Calcium ions influences not only crop growth but also the occurrence, abundance and distribution of the different types of soil microflora. Figure exhibited the available calcium ion in the soil which included maximum amount (18.7 mg/l) of Ca available was in block no. 04 soil sample and least in block no. 01 (3.7 mg/l). The soil of block 04 was suitable in raising growth conditions of soil microflora. This is due to the outstanding contribution of Ca to the cell structure stability and in behaviour or nuclei. Calcium is important in maintaining the stability of cell structure. As exhibited in figure the rated score of CB-4, CB-10 was higher as compare to other isolates, it indicated the proportionate distribution pattern of cyanobacteria in all the four blocks selected for the present study. Result is shown in figure no.3

Figure 3: Graph comparing calcium content



Measurement of pigments

Cellular components the analysis of pigments is most widely developed. Cyanobacteria have chlorophyll a as the major light harvesting pigment alongwith carotenoids and phycobilins as accessory pigments.

It was evident from figure that increased chlorophyll content (0.0085726 g/ml) was show by CB-10 as compared to other phylotypes, lowest (0.0001014 g/ml) was observed in CB-07. However reduced phycocyanin content (0.000001 g/ml) was observed in CB-04 phylotype. CB-13 showed inhanced content of caroteenied (0.004656 g/ml) as compared to other phylotypes. The concentration of various pigments in different phylotypes was in order of chlorophyll < carotenoides < phycocyanin. No single phylotypes have shown the increased content of all the 03 pigments i.e. chlorophyll, carotenoids and phycocyanim. Result is shown in figure no.4-6.

Figure 4: Growth characteristic of phylotypes of paddy field

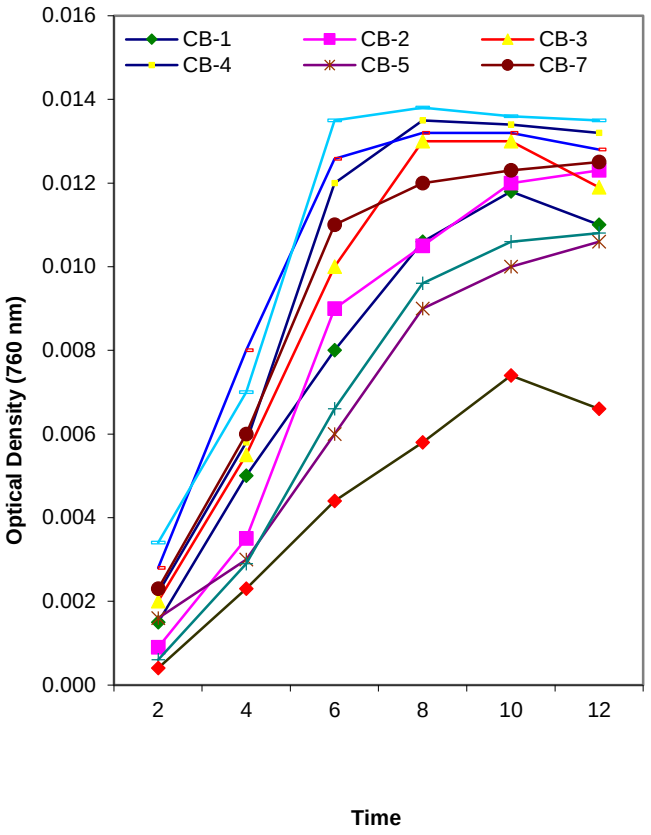


Figure 5: Phycocyanin estimation of Isolates (CB-1 to CB15)

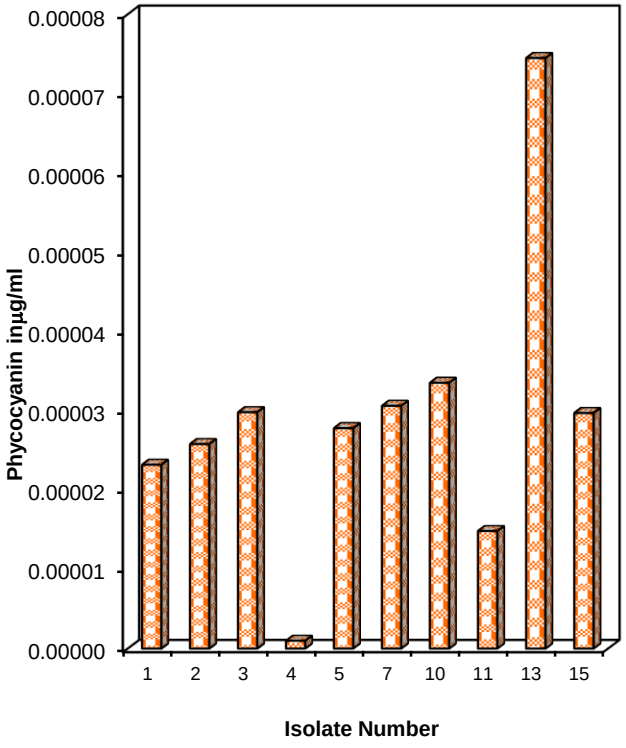
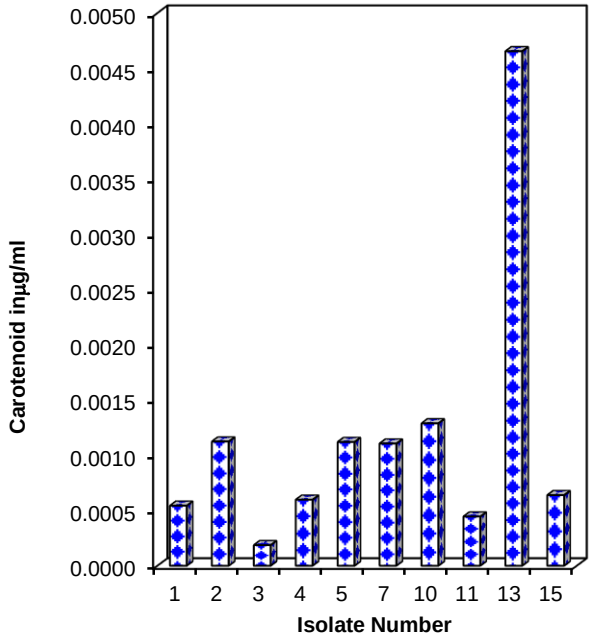


Figure 6: Carotenoid estimation of Isolates (CB-1 to CB15*)*



CONCLUSION

The physico chemical analysis of soils from 04 different selected blocks of Chhattisgarh state has shown that 81.21% was the water holding capacity of block 01. Soil texture and structure are two important physical properties of the soil that influence soil aeration, water retention and water movement. The soil of block 01 was granulated clayey. The available phosphorus was shown to be maximum in block 02. As Ca ions influence occurrence, abundance and distribution of the different types of soil microflora. The maximum content of Ca was found in block 04, which was suitable in raising growth conditions of soil microflora. The pigment synthesis in phylotypes followed the pattern as chlorophyll < carotenoids < Phycocyanin. The nutritional status of cyanobacteria can affect photosynthetic pigment synthesis and utilization of reserve products like cyanophycin during nitrogen starvation or excess.

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