NUTRACEUTICAL ASPECTS AND PHYSIOLOGICAL PARAMETERS OF CYANOBACTERIAL STRAIN *SPIRULINA*

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ABSTRACT

Cyanobacteria have a long evolutionary history and documented fossil records date back to about 3500 million years ago. One of the most important cyanobacteria is *Spirulina* which is also called super food because it has many nutraceuticals values. It is commercially produced in large outdoor ponds under controlled conditions. Potential health benefits of *Spirulina* are mainly due to its chemical composition, which includes proteins (the highest protein content of any natural food, 55–70 %), carbohydrates, essential amino acids, minerals (especially iron), essential fatty acids, vitamins and pigments. In this respect, three major bioactive components of *Spirulina*, the protein phycocyanin (a biliprotein pigment), sulfated polysaccharides and gamma linolenic acid seem to play significant roles in imparting improved human body functions. In the present study *Spirulina* strain shows maximum value of physiological parameters at 14th days of incubation. And also show high antioxidant activity (13.29 μmol Trolox g^{-1}).

Key words: *Spirulina* , β-carotene, Physiological parameters and Antioxidant activity.

INTRODUCTION

Blue-green algae (Cyanobacteria) are among the most primitive life forms on Earth. They share several characteristics with both, prokaryotes and eukaryotes. The cellular structure of cyanobacteria belongs to simple prokaryote. They share features with plants, as they have the ability to perform photosynthesis but they lack a plant cell wall. Interestingly, they also share characteristics of the animal kingdom as they contain complex sugars similar to glycogen on their cellular membrane (Vonshak A. 1997).

One of the trends in biotechnology is associated with Blue green microalgae *Spirulina platensis* which have been widely employed as food and feed additives in agriculture, food industry, pharmaceuticals, perfume making, medicine and science (Vonshak A. 1997).The protein is an essential component of diet (Usharani G 2012). *Spirulina* has been used as a complementary dietary ingredient of feed for fish, shrimp and poultry and increasingly as a protein and vitamin supplement to aqua feeds (Campanella L 1999).*Spirulina platensis* has been used as food for centuries by different populations and only rediscovered in recent years. Once classified as the "blue-green algae'', it does not strictly speaking belong to the algae, even though for convenience it continues to be referred to in that way. It grows naturally in the alkaline waters of lakes in warm regions (Pelizer et al. 2002). China is using this micro alga as a partial substitute of imported forage to promote the growth, immunity and viability of shrimp (Usharani G 2012).

In terms of nutrition, *Spirulina* is a rich food source of macro- and micronutrients including high quality protein, iron, gama-linolenic acid, vitamins, minerals, sulfated polysaccharides and phycocanin (Usharani G 2012).Hence Spirulina is of great interest as it offers the possibility of being used as a functional food. This term refers to those foods that have proven to aid specific body functions, yielding health-promoting properties and/or reduce the risk of disease beyond its nutritional functions. Moreover, *Spirulina* has also proven to have good acceptance as of its organoleptic properties (thus making it a possible prospect for food or a nutrition supplement) and it has not exhibited neither acute nor chronic toxicities, making it safe for human consumption. Various nutrients present in this alga have some medical significance as well including prostaglandin synthesis and induction of the regulation of blood pressure, cholesterol synthesis, inflammation and cell proliferation, no excess calories and fats thereby controlling obesity and premenstrual stress.

An organelle called phycobillisome is present in the *Spirulina*, which is a macromolecular complex responsible for harvesting light required for the accomplishment of the process of photosynthesis. This complex contains certain chromoproteins called phycobilliproteins. These phycobilliproteins are majorly classified into phycocyanin, allophycocyanin and phycoerythrin. All of these are composed of a and b protein subunits carrying different isomeric linear tetrapyrrole prosthetic groups (bilin chromophore) which differ in the arrangement of their double bonds.

MATERIALS AND METHODS

Test organism

Cyanobacterial strain from genus *Spirulina* was procured from algal biotechnology laboratory, Department of Microbiology, Chaudhary Charan Singh University, Meerut. **(Fig.1)**

Growth and Maintenance

Cyanobacterial strain from *Spirulina* was grown and maintained in chemically defined modified Z-Medium (Zarrouk, 1966) at 28 ± 2ºC under a light intensity of 52-55 µmol photon m-2 s -1 and L:D cycles of 16:8 hours.

pH of the medium was maintained in the range of 9.5-10.5 for optimal growth of cultures. Solid agar based medium was prepared by dissolving 12-15 g of purified agar in 1 liter of medium (1.2-1.5%) and autoclaved before use for maintenance of the cultures. Mass production of strains on large scale was done in photo bioreactor (Applikon) and raceways system. After fourteen days of incubation, the cultures were subjected to streaking on agar based Z-Medium for obtaining discrete colonies under similar cultural conditions. These colonies were picked up and inoculated in 50 mL flasks containing 20 mL Z-Medium and incubated till exponential phase, 14th day. The strains used in the present study varied in their morphological characters and the identification was authenticated based upon the keys given by Desikachary (1959) and Geitler (1932). The purity of the cultures was examined by microscopic observations at different stages of growth and the purity was maintained by streaking the cultures regularly on agar plates having suitable media. **(Fig.2)**

Physiological parameters

Estimation of Chlorophyll (McKinney, 1941)

A known volume (10 mL) of homogenized cyanobacterial suspension was taken and subjected to centrifugation (4000g, 10 min). The chlorophyll was extracted from pellet with equal volume of methanol (95%) in a water bath (60˚C, 30 min). The suspension was centrifuged and the absorbance of the supernatant was measured at 650 and 665 nm against 95% methanol as blank.

Dry weight (mg /mL)

A known volume (50 ml) of Cyanobacterial suspension was homogenized and filtered through a sintered glass apparatus on a preweighed Whatman No. 42 filter paper. This was then oven dried at 60˚C and cooled in a desiccator until constant weight was achieved. The difference in two weights was recorded as dry weight (Sorokin, 1973).

Carotenoids (Jensen, 1978)

(i) Reagent: Acetone (85%)

(ii) Procedure

A known volume (10 mL) of homogenized cyanobacterial suspension was subjected to centrifugation (4000g, 10 min.). Discarded the supernatant and washed the pellet 2- 3 times with distilled water to remove traces of adhering salts. To the pellet, added 2- 3 mL of acetone (85%) and stored at 40C for a week. Centrifuged and collected the supernatant containing pigment. Repeated the extraction from pellet with acetone till the supernatant became colorless. Pooled all the fractions of supernatants and made up to a final known volume. Took the OD at 450nm using 85% acetone as blank and calculated the total amount of carotenoids as follows:

Carotenoids (mg/mL) = [D x V x f x10] / 2500

Estimation of Total soluble proteins (Lowry *et al*. 1951; Herbert *et al*. 1971)

(i) Reagents:

(a)1N sodium hydroxide solution

(b) (i) 5% sodium carbonate

(ii) 0.5% copper sulphate ($CuSO_4.5H_2O$) solution in 1% sodium potassium tartarate

2 mL of reagent B (ii) was mixed with 50 ml of freshly prepared reagent B (i)

(c) 1N Folin-ciocalteau reagent

A known volume (0.5 mL) of homogenized cyanobacterial suspension was taken in test tubes. To this, 0.5 mL of reagent (a) was added. The tubes were then heated in a boiling water bath for 10 mins. and cooled in running tap water. Subsequently, 2.5 mL of reagent (b) was added in each and the tubes were incubated at room temperature for 10 mins. After this, 0.5 mL of reagent (c) was added and the tubes were kept at room temperature for 15 mins. The intensity of blue colour was read as absorbance at 650 nm against appropriate blank. The protein content was estimated using a standard calibration curve prepared from bovine serum albumin and expressed in terms of mg/mL.

Estimation of β-carotene

(i) Extraction

5 gm of fresh wet biomass of *Spirulina* strains were weighed into 200 mL amber colored flask wrapped with aluminum foil and was homogenized in a blender. A mixture of hexane-ethanol, 1:1 (vol/vol), was then added to the flask and sonicated continuously for 10 min on an ultrasonicator (Misonix Ultrasonic Liquid Processor, NY, U.S.A). The extraction was repeated until sample became colorless. The combined extract was transferred to a separating funnel and 5 mL of distilled water was added to separate into two distinct polar and non polar layers. The non polar hexane layer containing β-carotene was collected and concentrated in a rotary evaporator (Heidolph, Germany) till dryness. Residue was dissolved in 25 mL of methyltert-butyl ether (MTBE) and sample was analyzed by HPLC.

(ii) HPLC analyses

β -carotene content was analyzed using HPLC method (Dewanto, 2002). HPLC apparatus consisted of a water e2695 quaternary pump with auto injector (20 µLloop) and a 2998 photodiode array detector (Waters Corp., Milford, Mass., U.S.A.) and a 25 cm x 4.6 mm dia and 5µm C30 YMC column (Waters Corp., Ireland). Two milliliters of each sample solution was filtered through a 0.4-µm nylon filter before injection into HPLC. The mobile phase comprised of isocratic mixture of MTBE: methanol, 70:30 (v/v) at a flow rate of 1 mL/min. Concentration of standard solutions was calculated using the molar extinction coefficient of 13.9 $x10^4$ for β-carotene in hexane and peaks were detected at 453 nm. Results were expressed as mg/100 g fresh weight. A computer using an "Empower" software programmed integrated the peak areas automatically.

Antioxidant activity

Free radical scavenging activity using DPPH assay

DPPH assay is based on the measurement of the scavenging ability of antioxidants toward the stable radical DPPH (Brand-Williams, Cuvelier, & Berset, 1995). A 3.9 mL aliquot of a 0.0634 mM of DPPH solution in methanol (95%) was added to 0.1 mL of each extract and shaken vigorously. Change in the absorbance of the sample extract was measured at 515 nm for 30 min. The percentage inhibition of DPPH of the test sample and known solutions of Trolox were calculated by the following formula:

$$\% \text{ Inhibition} = 100 \times (A_0 - A) / A_0$$

Where A_0 was the beginning absorbance at 515 nm, obtained by measuring the same volume of solvent, and A was the final absorbance of the sample extract at 515 nm. Methanol (95%) was used as blank. Results were expressed as µmol TE/g fw.

Determination of ferric reducing antioxidant power (FRAP) assay

FRAP was performed according to the procedure described by Benzie and Strain (1996). The FRAP reagent included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM FeCl3in the ratio 10:1:1 (v:v:v). Three mL of the FRAP reagent was mixed with 100 µL of sample extract in a test tube, vortexed and incubated at 37 °C for 30 min in a water bath. Reduction of the ferric-tripyridyltriazine to the ferrous complex formed an intense blue color which was measured in UV vis spectrophotometer (Varian Cary 50) at 593 nm at the end of 4 min. Results were expressed in terms of µmol TE/g fw.

ABTS assay

Two gram of cyanobacteria suspension was weighed and filled up with 25 ml of 80% (vol.) ethanol. The samples were then extracted using a high-speed homogeniser (19000 r/min, 2 min, UltraTurrax T-25 basic; IKA Werke GmbH, Staufen, Germany), and the homogenate obtained was centrifuged (1000 × *g*, 20 min). The obtained supernatant was transferred into a measuring flask and topped up with 80% ethanol to 25 ml. In the extract prepared in this way, the antioxidant activity was determined in accordance with the previously described protocol (Tarko *et al.* 2009). The ABTS stock solution (7 mM)was prepared through reaction of 7 mM ABTS and 2.45 mM of potassium persulphate as the oxidant agent. The working solution of ABTS was obtained by diluting the stock solution in ethanol to give an absorption of 0.70 ± 0.02 at 734 nm. Sample extract 10 µL was added to 90 µL of ABTS solution and absorbance readings at 734 nm were taken at 30 °C for exactly 10 min after initial mixing. The percentage inhibition of ABTS of the test sample and known solutions of Trolox were calculated by the following formula: %Inhibition = 100 x (A_0 _ A)/A_0 where A_0 was the beginning absorbance at 734 nm, obtained by measuring the same volume of solvent, and A was the final absorbance of the test sample at 734 nm. Antioxidant capacity was calculating using a standard curve obtained by measuring the absorbance of synthetic vitamin E solutions (Trolox) and expressed in µmol Trolo/g of dry mass.

Results

The final results for different physiological parameters at different days of incubation (7, 14, 21 and 28 Days) are:

Chlorophyll

Cyanobacterial strain shows maximum concentration at 14^{th} days of incubation. **(Fig.4)**

Carotenoids

Cyanobacterial strain shows maximum concentration at 14^{th} days of incubation. **(Fig.5)**

Dry Weight

Cyanobacterial strain shows maximum dry weight at 28^{th} days of incubation. **(Fig.3)**

Total Soluble Proteins

Cyanobacterial strain shows maximum concentration at 14^{th} days of incubation. **(Fig.6)**

Extraction and estimation of β-carotene

β-carotene concentration in cyanobacterial strain was evaluated (260.39 mg/100 gdwt.). **(Fig.7)**

Antioxidant activity

The levels of antioxidants in hexane extract was 3.64µmol Trolox g^{-1} in tested strain based upon FRAP assay in hexane extract. The obtained data also showed a diverse antioxidant capacity in the various fractions of the extracellular substances of the studied strain.

Water extract fractions depicted better antioxidant levels in comparison to hexane and ethyl acetate fractions based upon DPPH assay. Aliquot volume used for water extract was 10µL which was 50 times less than hexane or ethyl acetate extract and water was shown to be very strong solvent for antioxidant extraction. The highest radical-scavenging power with DPPH assay was 11.29 µmol Trolox g^{-1} .

Antioxidant activity shown by ABTS assay is higher than FRAP and DPPH assay. The scavenging capacity was highest (13.29 µmol Trolox g^{-1}).The trend observed in DPPH assay was seen to be similar to that of ABTS assay and interestingly, the overall value of total antioxidant activity in DPPH were lower than those obtained in ABTS assay.

Discussion

Cyanobacteria are morphologically diverse group of photoautotrophic bacteria showing oxygenic photosynthesis whose classification in accordance with the Botanical (Anagnostidis and Komarek 1985) and Bacterial (Castenholz 1989; Rippka *et al*., 1979) codes is almost entirely based on phenotypic traits. The classification of these organisms is complicated owing to the misidentification of the strains, the lack of isolates and genetic information for many morphopophytes as well as inadequate morphological data on many genetically characterized strains (Wilmotte and Herdman, 2001). The phenotype of cyanobacteria is known to change occasionally during prolonged laboratory cultivation (Gugger *et al.*, 2002a; Lehtimaki *et al,*. 2000), which makes their identification sometimes difficult.

In addition to morphological diversity and widespread distribution, cyanobacteria reflect a broad spectrum of physiological properties and tolerance to environmental stress (Tandeau de Marsac and Houmard 1993). Cyanobacteria are good source of pigments, vitamins, polysaccharides, proteins, pharmaceuticals and other biologically active compounds of high commercial value (Thajuddin and Subramanian 2005). Little attention has been given to the general features of physiology of cyanobacteria with the specific attention given to nitrogen fixation and some aspects of photosynthesis (Brown and Webster 1953; Fog 1947).

Total soluble proteins also differed and it was highest (10.24 mg g^{-1}dry wt.) in *Spirulina* at 14th day of incubation in *Spirulina* strain

Statistically significant differences in the contents of β-carotene depending on the studied strain of cyanobacteria were observed. The highest concentration of β-carotene was found in strain (260.39 mg/100 g DM).

Conclusion

Finally, from the present study, conclusion is that the *Spirulina* strain can be considered as the best strain in terms of total soluble proteins, lipid, antioxidant activity and fatty acid profile.

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References

Kumar D, Kumar N, Pabbi S, Walia S, and Dhar DW Protocol optimization for enhanced production of natural colors in *Spirulina*. Indian J Plant Physiol, 18 (2013) 308.

Kumar D, Dhar DW ,Nehra P & Kumar N, Similarity analysis of Spirulina/arthrospira strains on the basis of phycocyanin operon locus (cpc-IGS-cpcA) and 16 S r RNA gene sequences. Indian J Biotechnol,16 (2017) 84.

Kumar D, Nehra P, Saxena S & Dhar DW, Quantification and Purification of C-Phycocyanin from cyanobacterial strains *Anabaena* and *Phormidium*

Zarrouk C (1966). Contribution a l'etud d'une facteure physiques et la. Photosynthesis de *Spirulina platensis* (Setch et Gardner) Geitler. Ph.D. Thesis, University of paris, France.

Zeigler DR (2003). Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int J Syst Evol Microbiol* **53:** 1893- 1900.

Zhang GW, Koti WM and Daggard G (2002). *Lett Appl Microbiol* **35:** 247–250.

Zhang T and Fang HHP (2006). Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl Microbiol Biotechnol* **70:** 281-289.

Mackinney G (1941). Absorption of light by chlorophyll solutions. *Journal of Biology and Chemistry* **140**:315-322.

Lowry OH, Farr AL, Rosebrough NJ and Randall RJ (1951). Protein measurement with the folin phenol reagent. *Journl of Biology and Chemistry* **19**3:265-275.

Garcia-Dominguez M, Reyes JC and Florencio FJ (1999). Glutamine synthetase inactivation by protein-protein interaction. *Proc Natl Acad Sci USA* **96:** 7161-7166.

Garcia-Pichel F (2001). Phylogenetic and morphological diversity of cyanobacteria in soil desert crusts from the Colorado Plateau. *Appl Environ Microbiol* **67:** 1902–1910.

Halden C, Hansen M, Nilsson NO, Hjerdin A and Sall T (1996). Competition as a source of errors in RAPD analysis. *Theor Appl Gen* **93:** 1185-1192.

Hall GC, Flick MB Gherne RL and Jensen RA (1982). Biochemical diversity for biosynthesis of aromatic amino acids among the cyanobacteria. *J Bacteriol* **149:** 65-78.

Santos SR and Ochman H (2004). Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environ Microbiol* **6:** 754-759.

Vonshak A (1997) *Spirulina*: growth, physiology and biochemistry. In *Spirulina platensis (Arthrospira)*: physiology, cell-biology and biotechnology. Vonshak, A. (Ed.), Taylor & Francis, London, 43-65.

Vonshak A and Tomaselli L (2000). *Arthrospira* (*Spirulina)*: Systematics and ecophysiology. In: A. Whitton and E Potts (eds.), The Ecology of Cyanobacteria, pp505-522, Kluwer Academic Publishers. The Netherlands.

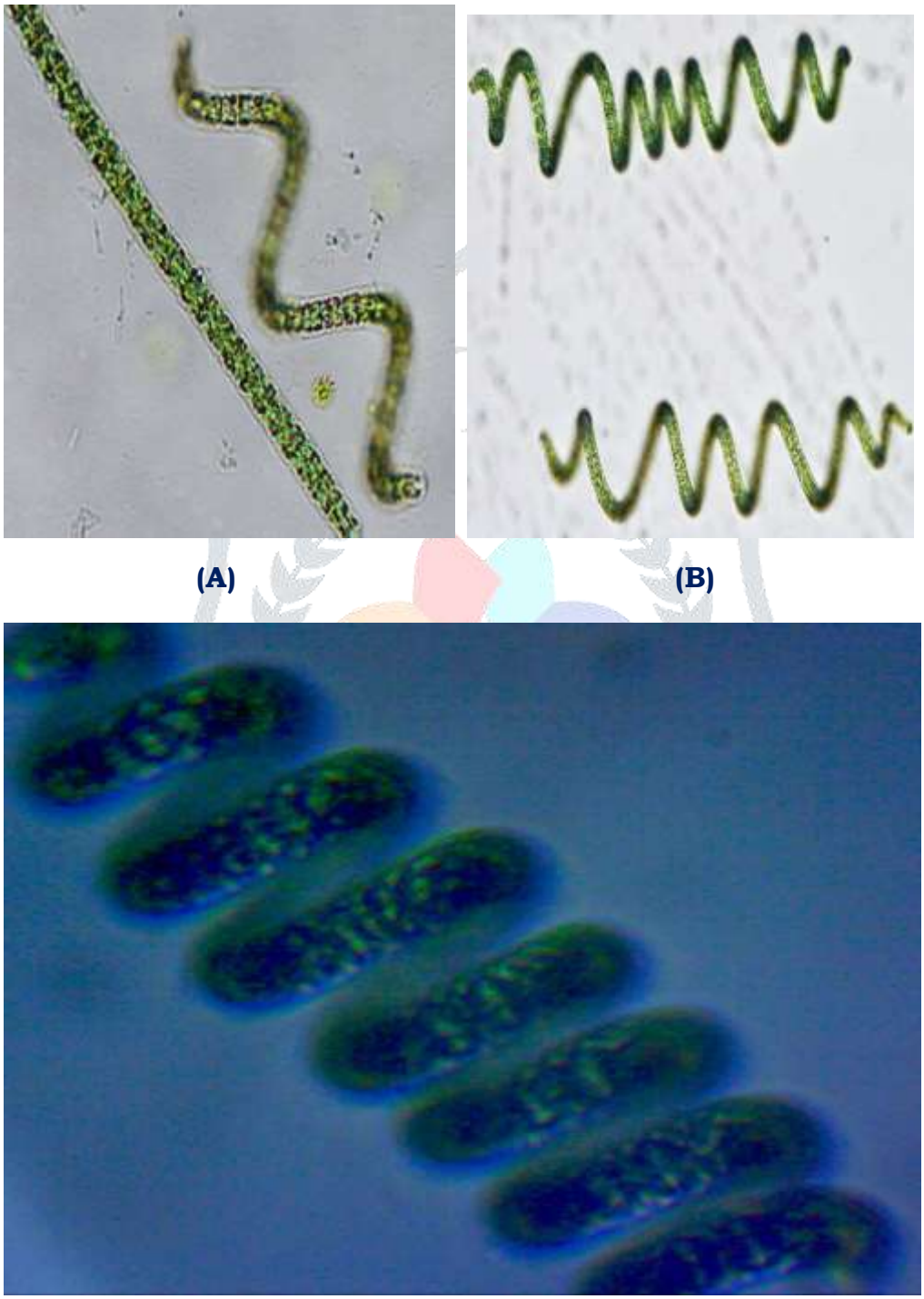

Fig.1: (A) and **(B)** Photomicrograph **(C)** Electron micrograph of *Spirulina*

Fig.2: Growth and Maintenance of Cyanobacterial strain

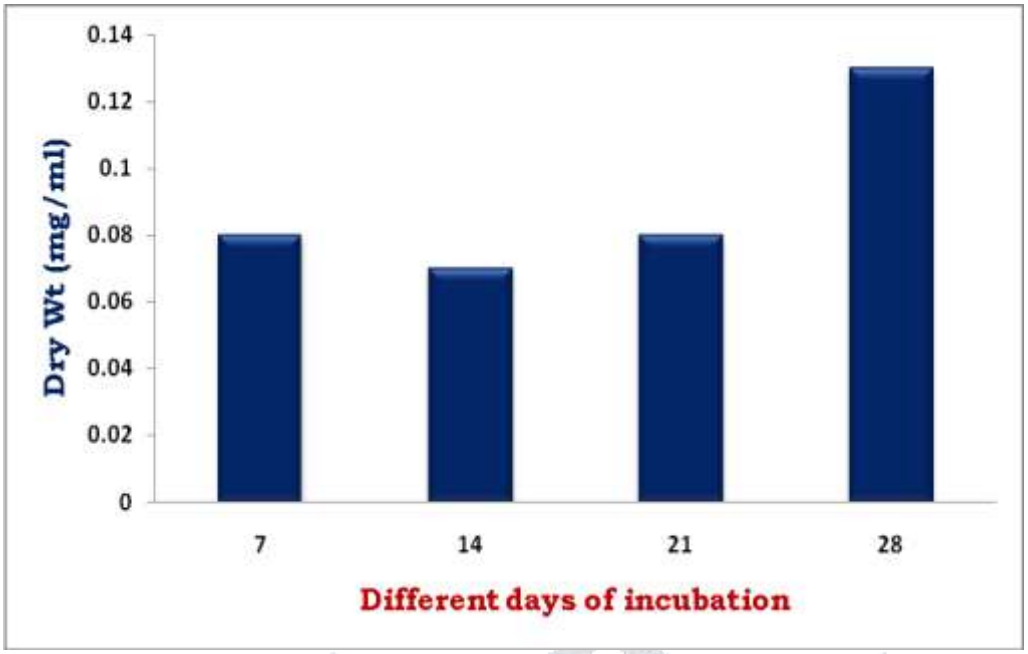

Fig.3: Dry weight (mg/ml) at different days (7, 14, 21 and 28th) of incubation

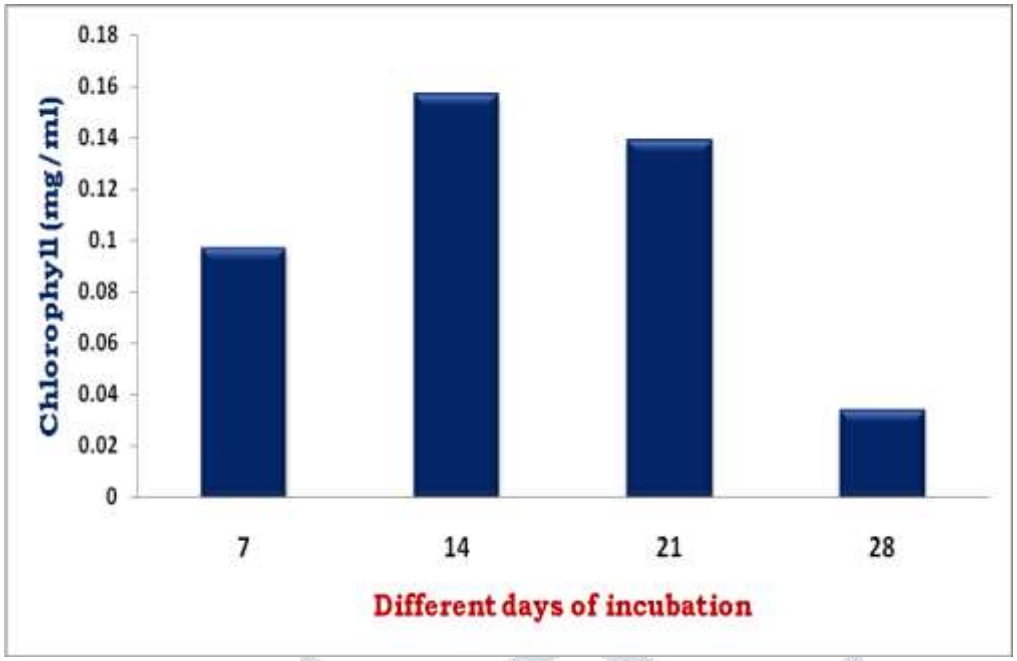

Fig.4: Chlorophyll (mg/ml) at different days (7, 14, 21 and 28th) of incubation

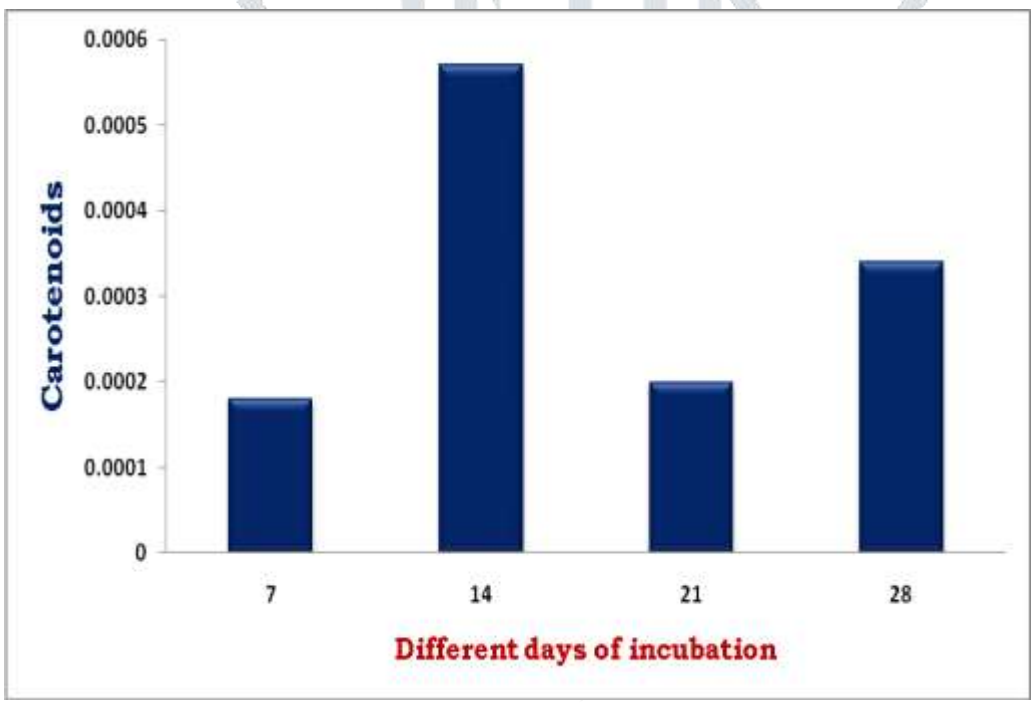

Fig.5: Carotenoids (mg/ml) at different days (7, 14, 21 and 28th) of incubation

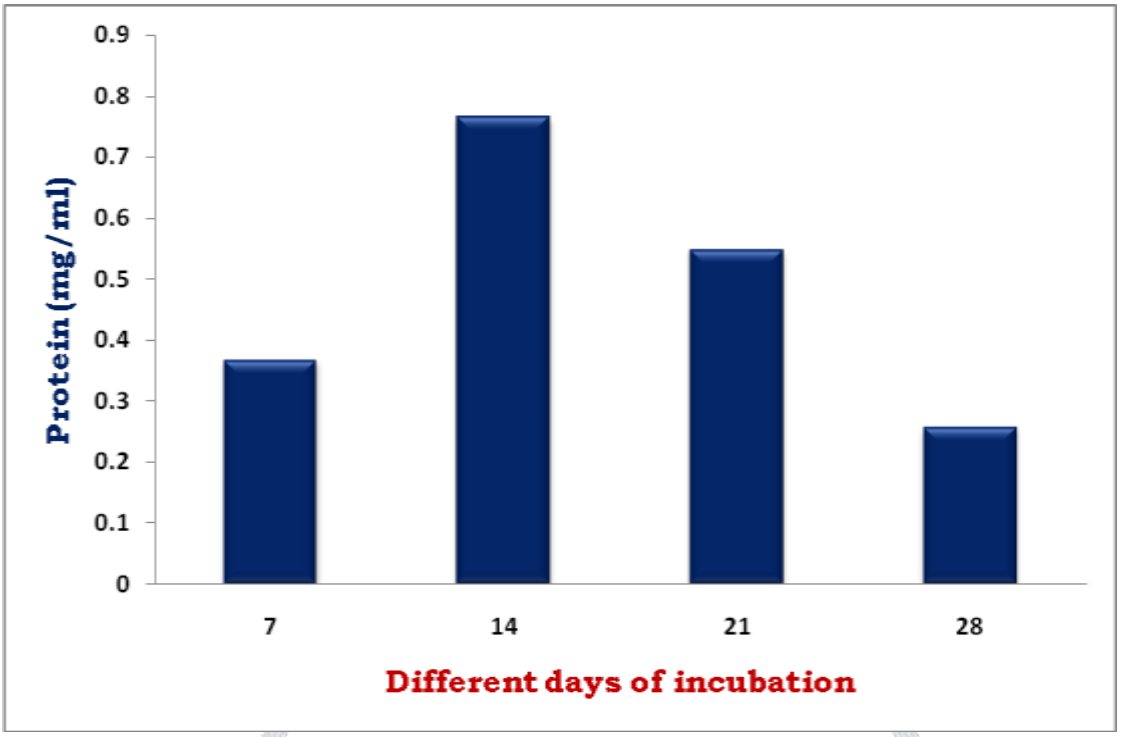

Fig.6: Protein (mg/ml) at different days (7,14,21 and 28th) of incubation

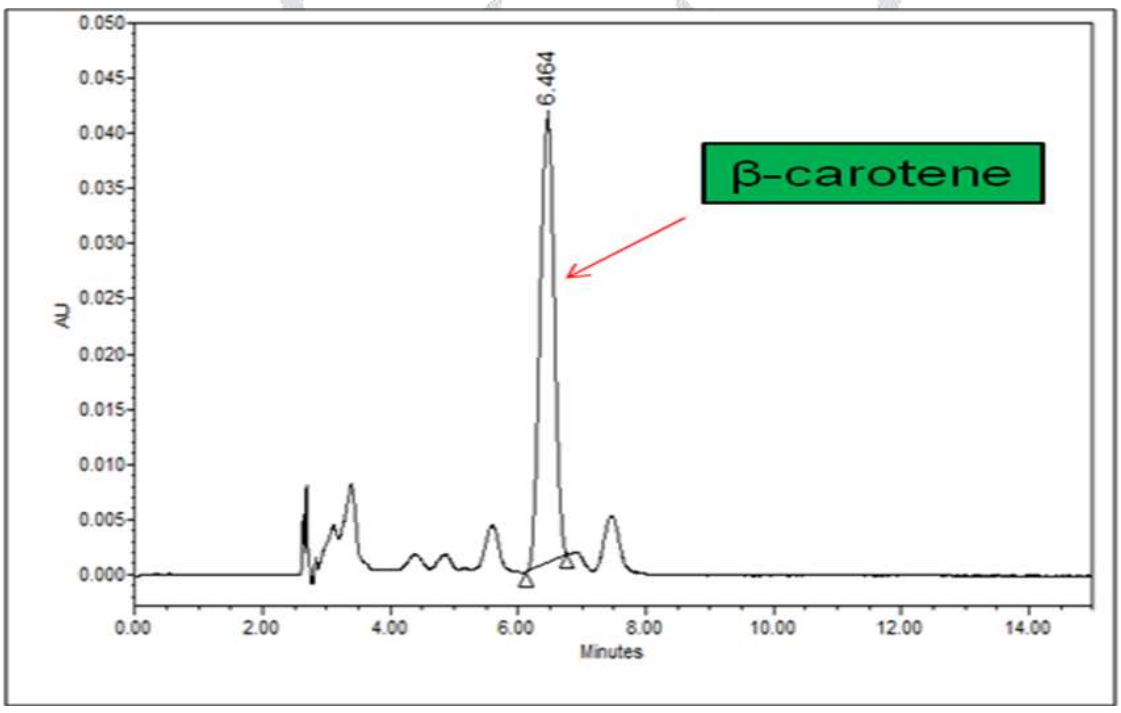

Fig.7: HPLC Chromatogram of β-carotene