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Comparative pigment profiles of different *Spirulina* strains

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***Spirulina platensis* (SP) has diverse biological activity. Due to the high content of highly valuable proteins, indispensable amino acids, β-carotene, vitamins, and other pigments, mineral substances, indispensable fatty acids and polysaccharides, it has been found suitable for use as bioactive additive. In the current study seven strains of S*pirulina* were tested for its pigment profile. Phycobiliproteins are important accessory pigments in *Spirulina*. These consist of phycocyanin, (PC) allophycocyanin, (APC) and phycoerythrin, (PE). Highest PC and APC were recorded in Spirulina platensis (Sp_7, mutant) followed by PC and APC contents recorded in its wild strain (Sp_4). PC content in S. lonar (Sp_6) and APC in Sp_5 and Sp_6 recorded third ranking. Among the strains tested Sp $_4$ showed significantly highest amount of pigments when compared to others. Interaction studies indicated that Sp_7 showed highest PC content followed by its wild strain Sp_4. The PC content in Sp_2, Sp_3 and Sp_6 assumed third ranking at 15th day of incubation. Interaction analysis based upon LSD ranking showed the highest content was observed by the contents exhibited by Sp_4 followed by Sp_2, Sp_3, Sp_7, which was further followed by Sp_5 at similar incubation time i.e. 25th day of incubation. In β-carotene analysis Strains x days of incubation interaction studies were significant and the top ranked combinations were Sp_4 at 15th day (295.6μg g^{-1} dry weight), 20th day (289.5μg g^{-1} dry weight) and at 25th day of incubation (282.4μg g^{-1} dry weight).**

Key Words: *Spirulina*, Phycobiliproteins, *β*- carotene

The cyanobacterium, *Spirulina*, has been already commercially exploited in several countries in health foods and therapeutic preparations because of its valuable constituents, particularly proteins and vitamins. (Benneman, 1990; Venkararaman and Becker, 1988). Carotenoids form an important medicinal and biotechnological class of natural pigments and range from yellow to red. According to Borowitzka (1988), there are over 400 known carotenoids and very few of these are used commercially which include β-carotene (licophin), zeaxanthin, astaxathin and lutein. These structurally diverse pigments have many different biological functions, such as species-specific coloration, photoprotection, light harvesting, and they also serve as precursors for many hormones (Vershinin, 1999). Small-scale production of phycocyanin and phycoerythrin using a range of microalgal species has been reported in literature

(Borowitzka and Borowitzka, 1988). In addition to chlorophyll *a* and carotenoids, cyanobacterial strains are also known to accumulate phycobiliproteins (phycocyanin, phycoerythrin and allophycocyanin). Cyanobacterial pigments comprise the most colourful and attractive components in these microorganisms. Screening programs all over the world have further confirmed the diversity and rich repertoire of pigments, which can revolutionize the industrial uses of "colours" with their nutraceutical and pharmaceutical value (Prasanna *et al*., 2007).

Blinkova,*et al*., 2001 provided interesting information on *Spirulina platensis* (SP) having diverse biological activity. Due to the high content of highly valuable proteins, indispensable amino acids, vitamins, beta-carotene and other pigments, mineral substances, indispensable fatty acids and polysaccharides, it has been found suitable for use as bioactive additive.

The phycobiliproteins represent the major photosynthetic accessory pigments in cyanobacteria, along with chlorophyll *a*. Phycobiliproteins are a family of highly soluble and reasonably stable fluorescent proteins derived from cyanobacteria. There are three basic types of biliproteins phycoerythrin (PE, λmax 560 nm), phycocyanin (PC, λmax 615 nm, blue pigment) and allophycocyanin (APC, λmax 652 nm, bluish green pigment). Cyanobacteria have all three types of phycobilins APC and PC are always present and PE is found in some organisms and not in others (Prasanna *et al*., 2010).

MATERIALS AND METHODS

Organism

The different strains of *Spirulina* employed in this investigation were procured from the germ plasm collection of CCUBGA, IARI, New Delhi. The identification of the *Spirulina* cultures was authenticated based upon the keys given by Desikachary (1959). The details regarding sources of these strains and their accession numbers are as under:

Strain No. CCC No.	**Cultures, Source**
Sp_1	*Spirulina platensis* (West Germany)
Sp_2	*Spirulina platensis (*Israel)
Sp_3	*Spirulina platensis* (Vietnam)
Sp_4	*Spirulina platensis* (CFTRI, Mysore, wild)
Sp_5	*Spirulina maxima* (CFTRI, Mysore)
Sp_6	*Spirulina lonar* (China)
Sp_7	*Spirulina platensis* (Mutant of Sp_4)

The *Spirulina* cultures were maintained and grown routinely in batch cultures in modified Zarrouk's medium (ZM).

Phycobiliproteins

A known volume of homogenized suspension was taken and centrifuged (3000 x g, 5 minutes). Phycobiliprotins were extracted completely from the pellet using equivalent volume of 0.05M phosphate buffer by repeated freezing and thawing. The absorbance of the supernatant was read at 615, 652 and 562 nm (Bennet and Bogorad, 1973)

The phycobilins were calculated (µg mL^{-1}) using the formula:

$$\text{Phycocyanin (PC)} = \frac{(A_{615})-(0.475 \times A_{652})}{5.34}$$

$$\text{Allophycocyanin (APC)} = \frac{(A_{652}) - (0.208 \times A_{615})}{5.09}$$

$$\text{Phycoerythrin (PE)} = \frac{(A_{562}) - (2.41 \times PC) - (0.849 \times APC)}{9.62}$$

Total Carotenoids

A known volume of homogenized algal suspension was centrifuged at 3000 rpm for 5 minutes. The pellet was washed with distilled water 2-3 times to remove traces of adhering salts. To the pellet, added 2-3 mL of acetone (85%) which was then subjected to repeated freezing and thawing. The suspension was centrifuged and the supernatant containing pigment was collected. The extraction was repeated till the supernatant became colorless, for complete recovery of carotenoids. The pooled fractions of supernatants were made-up to a final known volume. The absorbance was taken at 450nm using 85% acetone as blank and the total amount of carotenoids was calculated in $mgmL^{-1}$ as follows:

$$C = \frac{D \times V F}{2500X100}$$

D = OD at 450nm
V = Volume of the extract, and
F = Dilution factor
(Assuming that average extinction coefficient of pigments is 2500)

β - carotene content

β-carotene was estimated using HPLC system (Waters, 501) based upon reversed phase liquid chromatography.

Stationary phase	:	Octadecylsilane (ODS)
Mobile phase	:	Degassed carbinol and acetonitrile in 9:1 ratio.
HPLC system	:	Isocratic system
Isocratic HPLC system		
High-pressure pump	:	Waters 501 HPLC pump Millipore
Column	:	Lichrosorb RP-8 (5µm) column of length 25 cm
Detector	:	Water™ 486 Turnable Absorbance Detector.
Recorder	:	Computer controlled data system (Waters 746 Data Module Millipore)
Flow rate	:	1.5 ml min^{-1}

A known volume of homogenized suspension was taken and centrifuged at 3000 g for 5min. To the pellet, HPLC grade carbinol (methanol) was added and repeatedly vortexed at regular intervals for the complete release of pigments. The supernatant was filtered through Whatman No. 42 filter paper. Care had been taken to avoid exposure to light by wrapping the tubes with aluminum foil at all stages of extraction. 300µl sample was injected into the HPLC system with the help of a micro syringe.100-ppm of standard β-carotene was dissolved in carbinol and the β-carotene was quantified following the method of Braumann and Griemme (1981).

Results and Discussion

Phycobiliproteins are important accessory pigments in *Spirulina*. These consist of phycocyanin, (PC) allophycocyanin, (APC) and phycoerythrin, (PE). Highest PC and APC were recorded in *Spirulina platensis* (Sp_7, mutant) followed by PC and APC contents recorded in its wild strain (Sp_4). PC content in *S. lonar* (Sp_6) and APC in Sp_5 and Sp_6 recorded third ranking. There was an enhancement in PC content with incubation time and the peak was recorded on 15th day of incubation. However, APC content recorded peak on 25th day of incubation. Interaction studies indicated that Sp_7 showed highest PC content followed by its wild strain Sp_4. The PC content in Sp_2, Sp_3 and Sp_6 assumed third ranking at 15th day of incubation. Highest APC content was observed in Sp_7 at 25th day of incubation followed by its content on 20th day of incubation which was *at par* with the APC content in Sp_4 at 15th day of incubation followed by the content at 25th day of incubation. The APC content in Sp_4 and Sp_6 on 20th day of incubation assumed third ranking. (Table 1)

Phycobiliproteins are organized in supra molecular aggregates called phycobilisomes in order to maximize energy transfer to the chlorophyll- protein complexes located at thylakoid membrane. Highest phycocyanin (PC) and Allophycocyanin (APC) were recorded in *Spirulina platensis* (Sp_7); however, highest phycoerythrin (PE) was recorded in its wild strain (SP_4). Peak in the PC and APC contents were recorded on 15th day of incubation while peak for PE was recorded on 25th day of incubation. Phycocyanins have been extensively studied due to their involvement in photosynthesis as major accessory pigments (Prasanna *et al*., 2003). High content of phycobiliproteins has been reported from *Spirulina* (Sarada *et al*., 1999) and these pigments from *Spirulina platensis* were investigated in relation to their possible role as a reserve source of nitrogen. This is of particular interest because biliproteins may comprise a large part of the total algal protein, which could be used as a source of nutrition for humans and animals (Clement *et al*., 1967).

β-carotene exists in 6 isomeric forms, *viz*. 15-cis (10%), 9-cis (41%), all trans (42%) and two unidentified isomers (16%) (Ben Amotz and Avron, 1982). This is commercially produced from *Dunaliella salina* and *D. bardawil* and contains high retinol conversion biopotency (Amotz *et al*., 1988). The β-carotene is now being sold at very high rate. The work on the commercial production in Israel and Australia are significant (Amotz and Avron 1982; Borowitzka and Borowitzka 1986; Benemann 1988). *Spirulina* was also identified for its anticancer property due to β-carotene (Peto *et a*l., 1981).

Table 1. Comparative phycobiliproteins (µg mL^{-1}) in *Spirulina* strains at different days of incubation

a) Phycocyanin

	Days					
Strains	**5**	**10**	**15**	**20**	**25**	**Mean**
Sp$_1$	5.18	35.98	64.65	43.2	40.36	37.88
Sp$_2$	6.08	42	74.13^{c}	54.8	49.34	45.27
Sp$_3$	7.34	44.24	75.27^{c}	53.63	47.94	45.69
Sp$_4$	9.52	55.33	79.35^{b}	57.95	57.3	51.89^{b}
Sp$_5$	7.37	47.29	71.36	52.05	49.34	45.48
Sp$_6$	7.95	52.28	73.46^{c}	55.28	51.31	48.06^{c}
Sp$_7$	9.24	55.29	82.89^{a}	57.17	57.2	57.36^{a}
Mean	7.53	47.49	74.44	53.44	50.4	
		SE (m)		**CD (P= 0.05)**		
Strains (S)		0.497		0.991		
Days (D)		0.42		0.838		
S x D		1.111		2.216		

b) Phycoerythrin

	Days					
Strains	**5**	**10**	**15**	**20**	**25**	**Mean**
Sp$_1$	2.45	13.35	31.46	26.24	31.27	20.95
Sp$_2$	3.02	13.02	32.65	27.42	33.22	21.86
Sp$_3$	2.34	14.38	32.6	29.27	32.28	22.17^{c}
Sp$_4$	3.86	16.3	44.95^{b}	37.21	46.32^{a}	29.72^{a}
Sp$_5$	2.34	10.95	35.55	28.12	36.23	22.64^{c}
Sp$_6$	2.34	11.28	33.32	29.43	34.45	22.16
Sp$_7$	3.3	13.57	38.2	32.17	39.28^{c}	25.31^{b}
Mean	2.81	13.26	35.53	29.98	36.15	
		SE (m)		**CD (P= 0.05)**		
Strains (S)		0.234		0.468		
Days (D)		0.198		0.395		
S x D		0.524		1.046		

c) Allophycocyanin

	Days					
Strains	**5**	**10**	**15**	**20**	**25**	**Mean**
Sp$_1$	3.49	16.01	36.03	30.44	33.61	23.92
Sp$_2$	3.62	16.32	38.38	32.8	35.31	25.29
Sp$_3$	3.32	18.27	38.21	32.51	40.21	26.51
Sp$_4$	4.34	26.4	51.35^{b}	45.95^{c}	50.27^{bc}	35.66^{b}
Sp$_5$	3.42	21.33	42.21	39.35	41.29	29.52^{c}
Sp$_6$	3.43	19.34	40.64	45.56^{c}	41.75	30.14^{c}
Sp$_7$	7.44	29.21	46.25^{c}	51.45^{b}	62.32^{a}	39.33^{a}
Mean	4.15	20.98	41.87	39.72	43.54	
		SE (m)		**CD (P= 0.05)**		
Strains (S)		1.101		2.196		
Days (D)		0.93		1.856		
S x D		2.461		4.911		

a, b, c, ranking in ascending order based on LSD grouping

Sp$_1$, Sp$_2$, Sp$_3$ (***Spirulina platensis***) **; Sp$_4$** (***Spirulina platensis,*** wild); **Sp$_5$** (***Spirulina maxima***) **Sp$_6$** (***Spirulina lonar***) ; **Sp$_7$** (***Spirulina platensis,*** mutant)

Table 2. Comparative total carotenoids (mg mL^{-1}) in *Spirulina* strains at different days of incubation

Strains	Days					Mean
	5	10	15	20	25	
Sp_1	2.45	2.45	21.83	21.69	46.41^c	18.97
Sp_2	2.66	3.78	22.11	23.28	51.38^b	20.64^c
Sp_3	2.22	3.53	21.73	23.30	51.44^b	20.46^c
Sp_4	3.53	4.64	26.67	27.50	55.91^a	23.65^a
Sp_5	3.23	4.27	21.45	22.10	46.25^c	19.45
Sp_6	3.33	4.32	22.21	21.85	43.32	19.01
Sp_7	3.55	4.56	25.49	24.45	51.54^b	21.92^b
Mean	3	4.12	23.17	23.44	49.46	
		SE (m)		**CD (P= 0.05)**		
Strains (S)		0.229		0.456		
Days (D)		0.194		0.386		
S x D		0.513		1.023		
a, b, c, ranking in ascending order based on LSD grouping						
Sp_1, Sp_2, Sp_3 (***Spirulina platensis***) **;** **Sp_4** (***Spirulina platensis,*** wild); **Sp_5** (***Spirulina maxima***) **Sp_6** (***Spirulina lonar***) ; **Sp_7** (***Spirulina platensis,*** mutant)						

Table 3. Comparative β - carotene content (μg mL^{-1}) of *Spirulina* strains at different days of incubation

Strains	Days					Mean
	5	10	15	20	25	
Sp_1	86.5	152.3	211.5	216.5	213.5	176.1
Sp_2	92.2	164.7	222.1	219.5	215.9	182.9
Sp_3	89.4	170.3	226.8	222.9	219.6	185.9^c
Sp_4	97.4	193.5	295.6^a	289.5^b	282.4^c	231.7^a
Sp_5	76.7	144.8	211.7	213	209.5	171.1
Sp_6	77.5	148.5	213.7	211.8	206.4	171.6
Sp_7	81.9	182.7	270.4	265.8	263.1	212.8^b
Mean	86	165.3	236	234.1	230.1	
		SE (m)		**CD (P= 0.05)**		
Strains (S)		0.74		1.48		
Days (D)		0.63		1.25		
S x D		1.67		3.32		
a, b, c, ranking in ascending order based on LSD grouping						
Sp_1, Sp_2, Sp_3 (***Spirulina platensis***) **;** **Sp_4** (***Spirulina platensis,*** wild); **Sp_5** (***Spirulina maxima***) **Sp_6** (***Spirulina lonar***) ; **Sp_7** (***Spirulina platensis,*** mutant)						

The carotenoids in *Spirulina platensis* (Strains Sp_2 and Sp_3) were almost similar and assumed third position in the order of ranking. *Spirulina platensis* (Sp_1) recorded lowest carotenoids (18.97 mg mL^{-1}). Total carotenoids enhanced gradually and linearly with the incubation time with highest content observed at 25th day of incubation (49.46 mg mL^{-1}).

Interaction analysis based upon LSD ranking showed the highest content was observed by the contents exhibited by Sp_4 followed by Sp_2, Sp_3, Sp_7, which was further followed by Sp_5 at similar incubation time i.e. 25th day of incubation.(Table 2)

The β carotene content ranged from the lowest (Sp_5), 171.1 to the highest (Sp_4) 231.7 (µg g^{-1} dry weight) in the *Spirulina* strains examined for this attribute. β carotene content in *Spirulina platensis* (Sp_7, mutant) and in *S. platensis* (Sp_3) recorded second and third ranking. There was a gradual and linear increase in β carotene accumulation with the peak observed at 15th day of incubation followed by a slow decline upto a period of 25th day of incubation. (Table3). The β carotene content showed a typical sigmoid behaviour with highest observed during exponential phase of growth in *Spirulina* strains with incubation time (Saleh *et al*., 2008).

Strains x days of incubation interaction studies were significant and the top ranked combinations were Sp_4 at 15th day (295.6µg g^{-1} dry weight)) 20th day (289.5µg g^{-1} dry weight) and at 25th day of incubation (282.4µg g^{-1} dry weight). Strain Sp_5 (*Spirulina maxima*) at 25th day of incubation showed lowest β carotene content.

Dunaliella β-carotene is a mixture of both *trans* and *cis* isomers unlike synthetic β-carotene which has only trans isomer produced in a 6 step synthesis from β iononone which itself is synthesized in ten steps from acetone. Synthetic β-carotene is a complicated and expensive production system (Benemann 1988). There are large numbers of reports available that sp of *Dunaliella* are able to accumulate large amounts of β-caroten β-carotene (Ben-Amotz *et al*., 1983). β-carotene, especially in cis form is the only natural product which can provide protection against cancer (Shinohara *et al*, 1988), as it can reverse pre cancerous lesions, can provide protection against cataract, enhance the immune system. It is also known to prevent certain photosensitivity disorders, improve antioxidant actions in the tissues and prevent age related muscular degeneration. β-carotene quenches singlet oxygen and provides protection against heart diseases by protecting low-density lipoproteins from oxidation and reduces stress reactions. In animal feeding trials the presence of these pigments especially β-carotene led to the colour enhancement of egg yolks, chicken flesh and fish- scales (Santillan, 1982). The major function of β carotene in the diet is thought to be a source of vitamin A. β carotene has been approved by the FDA as a source as a color additive (exempted from the need for certification) in foods, drugs and cosmetics.

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