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Evaluation of *Tolypothrix* Germplasm for Phycobiliprotein Content

R. PRASANNA[a], B.M. PRASANNA[b], S.A. MOHAMMADI[b], P.K. SINGH[a]

[a]*National Center for Conservation and Utilization of Blue-Green Algae,* [b]*Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012, India*

e-mail prasanna@ndf.vsnl.net.in
fax +91 112 576 6420

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ABSTRACT. Twenty *Tolypothrix* strains, including 15 strains of *T. tenuis*, three strains of *T. ceylonica* and one strain each of *T. nodosa* and *T. bouteillei*, were evaluated for their phycobiliprotein content and composition. Significant differences among the *Tolypothrix* strains were found at both inter- and intra-specific levels in the production of phycobiliprotein constituents – phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE). Four specific parameters, *viz*. PC or PE content, total phycobiliprotein and total protein content, and percentage of phycobiliproteins, in a mixture of total proteins were used to select four *T. tenuis* and one *T. ceylonica* strain as useful for phycobiliproteins production.

Microalgae have emerged as important sources of proteins and value-added compounds with pharmaceutical and nutritional importance. Initially considered as 'laboratory curiosities' or 'nuisance agents in water bodies', cyanobacteria or blue-green algae (BGA) now form an important component of integrated nutrient management in agriculture and are exploited in commercial biotechnological ventures (Benemann and Weissman 1984; Kronick 1986) as a source of pigments, vitamins, phycocolloids, immunodiagnostic agents and therapeutics (Borowitzka 1984). The water-soluble fluorescent phycobiliproteins are exclusive to red algae, cyanobacteria and cryptomonads, and are involved as antenna pigments in harvesting of light for photosynthesis (Gantt 1980). These pigments are efficiently exploited as fluorescent tags, immunological diagnostic agents, cosmetics and food coloring agents (Glazer and Stryer 1984).

Phycobilins form supramolecular complexes (phycobilisomes) and can be classified into several groups based on their long-wavelength visible-absorption properties. Two large groups are phycoerythrins (red) and phycocyanins (blue), whose absorption maxima range between 490–570 nm and 610–665 nm, respectively. Further classification of these pigments is based on the constituent proteins, exact location of the maximum absorbance and specific shape of the absorbance spectrum (MacColl and Guard-Friar 1987). Phycobiliproteins participate in an energy transfer chain (phycoerythrin → phycocyanin → allophycocyanin → chlorophyll *a*) within the phycobilisomes (Ley *et al*. 1977). Some spectral properties, such as constant fluorescence over a wide range of pH and ability to conjugate with biologically important molecules (without alterations in spectral characteristics) make the phycobiliproteins particularly useful for fluorescent labeling applications including flow cytometry, fluorescent immunoassays and fluorescence microscopy. Among diverse cyanobacterial genera, the filamentous nitrogen-fixing forms are particularly attractive for commercial production of high-quality biomass (Guerrero *et al.* 1988). Phycobiliproteins in these organisms can constitute up to 50 % soluble protein and about 24 % of the cell dry biomass (Gantt 1980).

Characterization of cyanobacterial strains for various attributes (El-Zahraa and Zaki 1999; Agrawal and Misra 2002; Agrawal and Singh 2002), particularly those related to nitrogen fixation, is useful for their utilization as biofertilizers. The *National Center for Conservation and Utilization of Blue-Green Algae* (NCCUBGA) at New Delhi (India) houses a large collection of heterocystous and non-heterocystous cyanobacterial strains from various agro-ecological habitats of the country. Among the heterocystous forms, the genus *Tolypothrix* has a high level of phycobilins (Glazer and Stryer 1984) and is adaptable to even harsh environments (Roychoudhury and Kaushik 1989). Cyanobacteria such as *Tolypothrix* can potentially be used as sources of important compounds, *e.g.,* phycobiliproteins. The bright-colored phycobilin pigments absorb in a broad spectral range (450–660 nm), and consist generally of three main pigments – phycocyanin (λ_{max} = 615 nm), allophycocyanin (652 nm) and phycoerythrin (562 nm). The ratio of these pigments and amount of phycobilisomes are influenced by growth conditions (*e.g*., incandescent or fluorescent light) and environmental stress-factors, such as heat and cold, high visible and UV-B radiation, *etc*.

(Grossman *et al*. 1993). Phycocyanin and allophycocyanin are universally present in cyanobacteria; phycoerythrins are widely distributed among all taxonomic groups and form the spectroscopically variable class of phycobiliproteins (Glazer and Bryant 1975; Stanier and Cohen-Bazire 1977).

T. tenuis has been intensively studied with respect to phycobilin formation under various environmental conditions (Fujita and Hattori 1961). Scheibe (1972) isolated a phytochrome-like photoreversible pigment from *T. tenuis* that exhibited several plant phytochrome-like characters (Glazer 1988). We studied a set of *Tolypothrix* strains belonging to four different species for phycobiliprotein production in order to assess the intra- and interspecific strain variation in phycobiliprotein content and composition and to identify strains useful for further exploitation.

MATERIALS AND METHODS

Strains and growth conditions. The twenty selected *Tolypothrix* strains used, including their geographical sources, are given in Table I. Unialgal isolates designated as 'ARM' (Agricultural Research *Myxophyceae*) strains were maintained at NCCUBGA. The strains were cultured in BG-II medium (Stanier *et al*. 1979) at 29 °C and light intensity of 50–55 μmol phyton $m^{-2}\,s^{-1}$ under light–dark (14/10 h) cycles. Stationary cultures were utilized for extraction of the pigments and/or proteins during the light period. Growth conditions for the cultures were standardized by measuring total proteins in the culture after 5, 10 and 15 d of growth.

Table I. List of *Tolypothrix* strains used

Species	Strain	Origin of isolate (India)	Species	Strain	Origin of isolate (India)
T. tenuis	ARM74	Allahabad, Uttar Pradesh	*T. tenuis*	ARM426	Chandrapur, Maharashtra
	75	Allahabad, Uttar Pradesh		460	Rajasthan
	76	Allahabad, Uttar Pradesh	*T. ceylonica*	485	Kalyani, West Bengal
T. nodosa	91	Rajasthan	*T. tenuis*	520	Kota, Rajasthan
T. tenuis	113	Rajasthan		528	Kota, Rajasthan
	172	Kanyakumari, Tamil Nadu		531	Kota, Rajasthan
	173	Kanyakumari, Tamil Nadu	*T. ceylonica*	543	Kota, Rajasthan
T. ceylonica	397	*unknown*	*T. tenuis*	586	*unknown*
T. tenuis	424	Rampur, Maharashtra	*T. bouteillei*	592	*unknown*
	425	Gadichirdi, Maharashtra	*T. tenuis*	617	*unknown*

Estimation of phycobiliprotein composition and total protein content. The concentration of c-phycocyanin (PC), allophycocyanin (APC) and c-phycobiliprotein (PE) was estimated according to Bennett and Bogorad (1971). A homogenized culture of each strain (10 mL) was centrifuged at 83 Hz; the pellet was mixed with 10 mL phosphate solution (in mmol/L, trisodium phosphate 50, disodium hydrogen phosphate 100, sodium dihydrogen phosphate 100). The sample was subjected to repeated freezing and thawing cycles until the pellet became colorless. The suspension was then centrifuged at 83 Hz and clear cell-free supernatant was used for estimation of absorbance at 562, 615 and 652 nm (*Beckman*, model DU64). Phycocyanin–phycoerythrin ratio and percentage of phycobiliproteins to total proteins were estimated for each culture.

Selected *Tolypothrix* strains were also analyzed for their protein content according to Herbert *et al.* (1971). Culture suspensions (0.5 mL) were denatured using 0.5 mL of 1 mol/L sodium hydroxide. The samples were heated in a boiling water bath for 10 min, followed by color development through sequential addition of disodium carbonate, sodium potassium tartrate, copper sulfate and Folin–Ciocalteau reagent. A_{650} of cell-free filtrates was measured and compared with protein standard solutions of bovine serum albumin and lysozyme.

Statistical analysis. The data recorded for phycobiliprotein content in various strains was subjected to ANOVA (analysis of variance) in accordance with the experimental design (completely randomized block design) using MSTAT-C statistical package, to quantify and evaluate the sources of variation. The linear model used for ANOVA was $Y_{ij} = \mu + S_i + \varepsilon_{ij}$, where Y_{ij} denotes an observation for the *i*-th strain for *j*-th variable, μ overall mean, S_i refers to effect of *i*-th strain and ε_{ij} denotes random error associated with *i*-th strain and *j*-th variable. Orthogonal contrast was performed through pair-wise comparison of species

(Pearce 1992) to further analyze the inter-specific differences in terms of phycobiliprotein and total protein content.

Duncan's multiple-range test (DMRT) was carried out using MSTAT-C to compare the mean phycobiliprotein and total protein contents of the strains. DMRT uses several critical values; the critical values for each sub-set of mean pairs are associated with the same number of steps, R = 2, 3, …, k. Duncan's procedure also uses a different error rate $a_R = (1–a)^R$ for each subset associated with R steps, R = 2, 3, …, k, in calculating all critical values. This procedure facilitated the identification of a subset of *Tolypothrix* strains with the highest content of specific constituents of phycobiliproteins and total phycobiliprotein. Alphabetical ranking of the strains is based on the mean values of specific traits. For instance, a strain with rank 'A' is the best among the selected strains for a given trait.

To analyze the interrelationships among PC, APC and PE, and to determine their direct and indirect effects on total phycobiliprotein content path coefficient analysis was carried out using standard procedures (Wright 1921; Dewey and Lu 1959).

RESULTS AND DISCUSSION

Tolypothrix germplasm including fifteen *T. tenuis* strains, three *T. ceylonica* strains and one each of *T. nodosa* and *T. bouteillei*, was analyzed with respect to phycobiliprotein constituents. ANOVA indicated significant differences among the strains in PC, APC and PE content (Table II). Orthogonal comparison of strains belonging to four different species for their ability to produce phycobiliproteins also revealed significant differences, except in the case of *T. ceylonica vs*. *T. nodosa* (Table III). Comparison of the mean contents of PC, APC and PE revealed significant intra- and interspecific variation among the strains (Table IV). ARM172, ARM173 and ARM74 had the highest PC content, while ARM113 and ARM528 were rich in PE. APC content was found to be highest in ARM460. The high PC/PE ratio in ARM172 attests to high PC production while the low PC/PE ratio in ARM113 and ARM528 (in combination with their high PE content) points to a high PE production. None of the non-*T. tenuis* strains displayed a high PC or PE production, except ARM485 (*T. ceylonica* strain with a higher PE and PC content). In general, *T. ceylonica* strains exhibited moderate PC and PE content.

Table II. ANOVA for phycobiliprotein constituents in *Tolypothrix* strains

Sources of variation	Degrees of freedom	Mean sum of squares[a]		
		PC	APC	PE
Strains	19	4.99	1.20	5.40
Error	40	0.07	0.02	0.02

[a]All F values significant at p = 0.05.

Table III. ANOVA and orthogonal contrast for total phycobiliprotein content in *Tolypothrix* strains analyzed

Sources of variation	Degrees of freedom	Mean sum of squares[a]	
		phycobiliprotein	total protein
Strains	19	18.9	356117
T. tenuis vs. T. ceylonica	1	8.7	110009
T. tenuis vs. T. bouteillei	1	16.5	427781
T. tenuis vs. T. nodosa	1	6.3	701251
T. ceylonica vs. T. bouteillei	1	4.1	162678
T. ceylonica vs. T. nodosa	1	0.4 ns	321867
T. bouteilli vs. T. nodosa	1	1.3	17931
Residual	13	24.8	386516
Error	40	0.1	282

[a]All F values significant at p = 0.05; ns – non-significant.

The total phycobiliprotein content after a 10-d incubation (production optimum) ranged from 42 mg/L in ARM76 to 850 mg/L in ARM528 (both belonging to *T. tenuis*). *T. tenuis* strains ARM113, ARM172 and ARM528 had a high phycobiliprotein content, while strains ARM586, ARM173 and ARM75 had the highest total protein content (Table V). Strain ARM172 had a high content of phycobiliproteins as well as a high total protein content. *T. nodosa* and *T. bouteillei* strains had low total protein content while the *T. ceylonica* strains were comparable to the majority of *T. tenuis* strains. The percentage of phycobiliproteins in the total proteins was the highest in strain ARM113, followed by ARM485 (*T. ceylonica* strain) and ARM74 (*T. tenuis* strain).

Correlation coefficients showed significant positive associations between all characters except PC and PE, and APC and total phycobiliprotein content. Path coefficient analysis showed that PC and PE directly affect total phycobiliprotein content while the effect of APC is mostly indirect (Fig. 1).

Table IV. Concentration of phycobiliprotein constituents[a] in *Tolypothrix* strains

Strain ARM	PC		APC		PE		PC/PE
	mean	rank[b]	mean	rank[b]	mean	rank[b]	
T. tenuis							
74	29	B	12	C	22	C	1.3
75	1	G	1	I	2	H	0.5
76	6	EFG	3	HI	6	H	1.0
113	16	D	17	B	52	A	0.3
172	53	A	16	B	3	H	7.6
173	31	B	10	CD	7	G	4.4
424	15	D	9	DE	14	E	1.1
425	8	EF	6	FG	14	E	0.6
426	4	FG	4	GH	1	H	4.0
460	5	EFG	24	A	14	E	0.4
520	3	G	1	I	1	H	3.0
528	19	D	12	C	36	B	0.5
531	14	D	9	DE	21	C	0.6
586	2	G	1	I	2	H	1.0
617	24	C	8	EF	18	D	1.3
T. nodosa							
91	6	EFG	15	B	2	H	3.0
T. ceylonica							
397	9	E	3	HI	11	F	0.8
485	18	D	11	CD	23	C	0.8
543	3	G	1	I	3	H	1.0
T. bouteillei							
592	6	EFG	6	FG	2	H	3.0
Mean ± SE	14 ± 1.5		8 ± 0.8		11 ± 0.8		
Range	1–53		1–24		1–52		

[a]Mean values, g/L; PC – phycocyanin, APC – allophycocyanin, PE – phycoerythrin.
[b]Based on DMRT of mean values.

Based on PC or PE content, total phycobiliprotein content, total protein content and ratio of phycobiliproteins to total proteins, four *T. tenuis* strains (ARM113, ARM172, AM528 and ARM74) were considered as prospective sources of phycobiliproteins. No clear association was found between the geographical origin (in India) of *Tolypothrix* isolates and their phycobiliprotein yield (the above strains originated from three states in India – Rajasthan, Tamil Nadu and Uttar Pradesh, with widely diverse agro-ecological features).

Table V. DMRT of the mean phycobiliprotein and total protein content of *Tolypothrix* strains

Strain ARM	Total phycobiliprotein, mg/L		Total protein, g/L		%[a]
	mean	rank[b]	mean	rank[b]	
T. tenuis					
74	630	C	1.18	F	53.2
75	42	I	1.49	C	2.8
76	120	HI	1.21	F	9.9
113	850	A	1.03	G	82.7
172	720	B	1.41	D	50.9
173	483	DE	1.58	B	30.6
424	380	F	0.97	I	30.6
425	281	G	0.70	L	40.1
426	90	HIJ	0.35	N	25.4
460	430	EF	1.03	G	41.7
520	50	J	0.76	J	6.5
528	667	BC	1.31	E	50.8
531	440	E	1.02	G	43.2
586	53	J	1.79	A	3.0
617	500	D	0.99	HI	50.7
T. nodosa					
91	233	G	0.62	M	37.5
T. ceylonica					
397	234	G	1.02	G	23.0
485	520	D	0.97	I	53.5
543	70	IJ	1.01	GH	6.9
T. bouteillei					
592	140	H	0.73	K	19.1
Mean ± SE	346± 20		106 ± 8		
Range	42–850		35–179		

[a]Relative content of phycobiliprotein to total protein. [b]Based on DMRT of mean values.

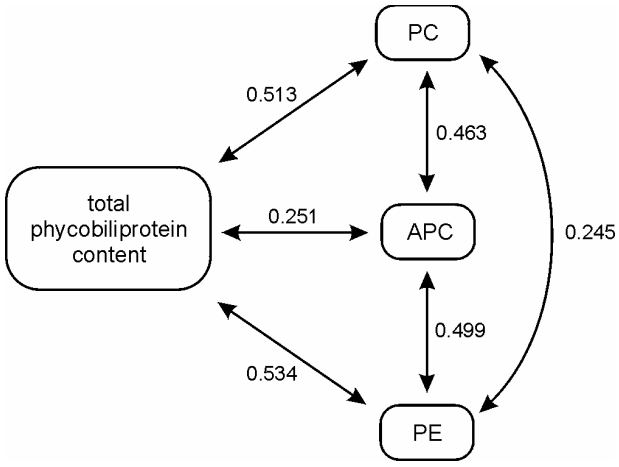

Fig. 1. Path analysis illustrating direct and indirect effects of individual constituents (PC – phycocyanin, APC – allophycocyanin, PE – phycoerythrin) on total phycobiliprotein content in the *Tolypothrix* germplasm; *numbers at arrows* – path coefficients, for further details *see Materials and Methods*).

Considerable inter- and intraspecific diversity or phycobiliprotein composition and content was also described by Gantt (1980) who observed that the levels of PC, APC and PE in total phycobiliprotein content vary not only with species but are also influenced by environmental parameters. Many cyanobacterial species vary in PC/PE ratio, which may regulate the wavelength range of the light absorbed for photosynthesis; this phenomenon is referred to as 'chromatic adaptation' (Tandeau de Marsac 1977). Such changes in the light-harvesting complex offer an adaptive advantage, since PC can efficiently absorb red light while PE absorbs green light. Our strains showed a wide range of PC/PE ratio.

Strains ARM172, ARM460 and ARM173 produced high levels of phycocyanin, ARM113, ARM528 and ARM75 were good phycoerythrin producers, and ARM460 had high levels of allophycocyanin; the best total phycobiliprotein producers were *T. tenuis* ARM113, ARM172 and ARM528. These strains could be used for optimizing phycobiliprotein production for commercial purposes. The majority of *T. tenuis* strains had a high total protein content. *T. tenuis* ARM172 and ARM113 showed a high phycobiliprotein production, reflected in a high ratio of phycobiliprotiens to total proteins. Strain *T. ceylonica* ARM485 exhibited also high PC production and a fairly high relative phycobiliprotein content. Strains ARM113, ARM172, ARM528 and ARM74 could ensure a considerable production of pigment-extracted biomass and can serve as protein-rich feed supplements. Path coefficient showed that PC and PE directly affect phycobiliprotein content while APC does not.

Tolypothrix, a cyanobacterial genus of wide distribution, can be used as a biofertilizer and also as a source of a number of products, particularly phycobiliproteins.

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