

Phycocyanin from *Spirulina* sp: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin[☆]

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Abstract

A number of drying methods studied for the processing of *Spirulina* (crossflow dried, spray dried and oven dried) resulted in approximately 50% loss of phycocyanin. Therefore fresh biomass was suitable for phycocyanin extraction. Of the extraction methods tested, freezing and thawing of cells, homogenisation using a mortar and pestle in the presence of abrasive material and homogenisation using a blender at 10 000 rpm yielded 19.4 ± 0.4 mg phycocyanin per 100 mg dry weight of *Spirulina* while water extraction was a slow process. Acid treatment also resulted in phycocyanin leaching. Phycocyanin was stable over a pH range of 5–7.5 at $9 \pm 1^\circ\text{C}$, whereas temperature beyond 40°C led to instability. The pigment phycocyanobilin was separated from the phycocyanin. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Spirulina*; Phycocyanin; Stability; Drying methods; Extraction procedures

1. Introduction

The Cyanobacterium *Spirulina* has been commercialised in several countries for its use in health foods and for therapeutic purposes due to its valuable constituents particularly proteins and vitamins [1,2]. The growing awareness of importance of natural colours especially food and cosmetic colourants has placed great demand on biological sources of natural colours. Cyanobacteria and algae possess a wide range of coloured components including carotenoids, chlorophyll and phycobiliproteins [3]. The principal phycobiliproteins are phycocyanin, allo-phycocyanin and phycoerythrin which are made up of dissimilar α and β polypeptide subunits [3]. Phycobiliproteins are assem-

bled into particles named phycobilisomes which are attached in regular arrays to the external surface of the thylakoid membrane and act as major light harvesting pigments in cyanobacteria and red algae. Phycobilisomes consist of allophycocyanin cores surrounded by phycocyanin on the periphery. Phycocyanin is the major constituent while allophycocyanin functions as the bridging pigment between phycobilisomes and the photosynthetic lamella [4]. Phycocyanin is used as colourant in food (chewing gums, dairy products, ice sherbaths, gellies etc) and cosmetic such as lipstick and eye liners in Japan, Thailand and China [5]. It was also shown to have therapeutic value (immunomodulating activity and anti cancer activity) [6]. Owing to its fluorescence properties it has gained importance in the development of phycofluor probes for immunodiagnos- tics [7].

In view of the multiple uses of phycocyanin we have investigated different extraction procedures and the effect of processing methods on phycocyanin quality and yields.

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Table 1
Phycocyanin content in *Spirulina* dried by different drying methods

Drying method	Temperature (°C)	Drying time (h)	% Phycocyanin recovery
Wet biomass (control)	–	–	100
Cross flow dried	60	7	55
Spray dried	150	–	55.5
Oven dried	60	7	54

2. Materials and methods

2.1. Cyanobacterium strains

Spirulina platensis (CFTRI) is a local isolate, maintained under standard conditions for the last 18 years and used in pilot plant cultivation.

2.2. Medium

A modified Zarrouks medium [8] containing the following constituents (all in g litre⁻¹ NaHCO₃ 16.8 g; K₂HPO₄ 0.5 g; NaNO₃ 2.5 g; NaCl 1.0 g; MgSO₄·7H₂O 0.2 g; FeSO₄·7H₂O 0.01 g; K₂SO₄ 1.0 g; CaCl₂·2H₂O 0.04 g; EDTA 0.08 g was used to grow the *Spirulina* culture.

2.3. Culture conditions and growth

The *Spirulina* culture was grown indoors in a thermostat at 30 ± 2°C with an illumination of 4 K.lux light intensity. Ten culture tubes of 250 ml volume were used and were supplied with 300–400 ml of air/min/tube. The cultures were grown for a period of 9 days and growth was monitored spectrophotometrically at 560 nm.

2.4. Drying method

The harvested biomass of *Spirulina* was subjected to cross flow drying, spray drying, sundrying and oven drying methods (Table 1). Phycocyanin was estimated from these samples and was compared with the content of phycocyanin extracted from wet biomass.

2.5. Extraction procedures

Phycocyanin was extracted from the wet biomass of *Spirulina* using the following methods.

1. Water extraction: *Spirulina* biomass was suspended in distilled water and the phycocyanin leached out was estimated spectrophotometrically [9].
2. Homogenisation of cells in a mortar and pestle: Biomass was homogenised in a mortar and pestle in the presence of acid washed neutral sand using 50 mM sodium phosphate buffer pH 6.8. The extract

was centrifuged and the supernatant contained phycocyanin. The pellet was re-extracted with buffer to ensure complete recovery of phycocyanin.

3. Freezing and thawing: Phycocyanin was extracted by repeated freezing and thawing of cells in 50 mM phosphate buffer pH 6.8 and estimated by the method of Sigelman and Kycia [9] and the amount of phycocyanin was calculated as mg phycocyanin per ml using the equation $O.D \text{ at } 615 \text{ nm} - 0.474 (O.D \text{ at } 652 \text{ nm})/5.34$ [9]. The same method was followed for estimation of phycocyanin extracted by other procedures.
4. Homogenisation in Virtimixer (The Virtis Company, Gardiner NY 12525) at different speeds: The harvested wet biomass suspended in 50 mM phosphate buffer pH 6.8 was subjected to 5, 10, 20 × 1000 rpm for 10 min duration intermittently.
5. Acid extraction: The wet biomass was treated with different concentrations of hydrochloric acid (2, 4, 6, 8 and 10 N) at room temperature. At different time intervals (2, 4, 24 h) samples were centrifuged and supernatants taken for phycocyanin estimation.

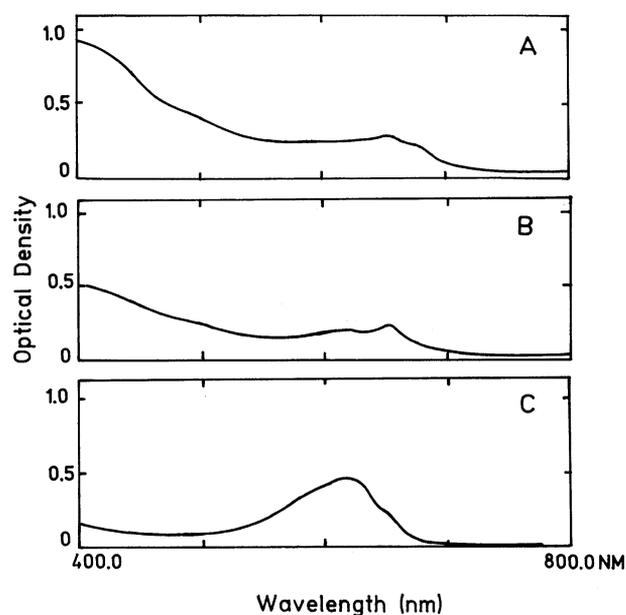


Fig. 1. Spectra of phycocyanin from *Spirulina* dried by: A, Spray dried; B, Cross flow dried; and C, Fresh biomass.

Table 2
Comparison of different methods for phycocyanin extraction from wet cells of *Spirulina*

Method	Amount of phycocyanin extracted expressed in mg phycocyanin from 100 mg dry biomass ^a							
	1st extraction		2nd extraction		3rd extraction		Total	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Freezing and thawing	17.66	14.16	1.3	4.1	0.51	0.8	19.47	19.06
Homogenisation (mortar and pestle)	17.58	11.72	2.07	8.12	–	–	19.66	19.84
Water extraction	14.68	9.64	2.57	3.95	2.01	5.54	19.26	19.12

^a Treated refers to cells treated with 0.02 N HCl for 30 s. Control refers to cells not treated with 0.02 N HCl.

2.6. Stability studies of phycocyanin

The stability of phycocyanin was studied at pHs ranging from 2.5 to 13 using different buffers at both room temperature ($25 \pm 2^\circ\text{C}$) and low temperature ($9 \pm 1^\circ\text{C}$). Citrate phosphate buffer in the range of 2.5–7.0 pH, phosphate buffer 7.5–8.0, Tris–HCl buffer 8.5–11, glycine buffer 12.5–13 were used. Temperature stability was studied at 10, 30, 45 and 55°C by incubating samples in water baths maintained at the specified temperature. The stability of phycocyanin was also studied at -4°C .

2.6.1. Delinking of pigment from the protein

Various reagents viz 10% sodium dodecyl sulphate, 8 M urea, beta-mercaptoethanol were tested at room temperature to denature protein and release the pig-

ment. Treatment with 10 N hydrochloric acid at 80°C was also tested for breaking the covalent bonds between protein and chromophore of phycocyanobilin.

3. Results and discussion

3.1. Effect of drying methods on phycocyanin quality and quantity

The harvested biomass of *Spirulina* was subjected to various drying methods (Table 1). The phycocyanin content extracted from fresh biomass using the freezing and thawing method was considered as 100%. There was considerable loss of phycocyanin in dried samples. The phycocyanin extracted from dried samples also showed variations in spectra when compared to phycocyanin

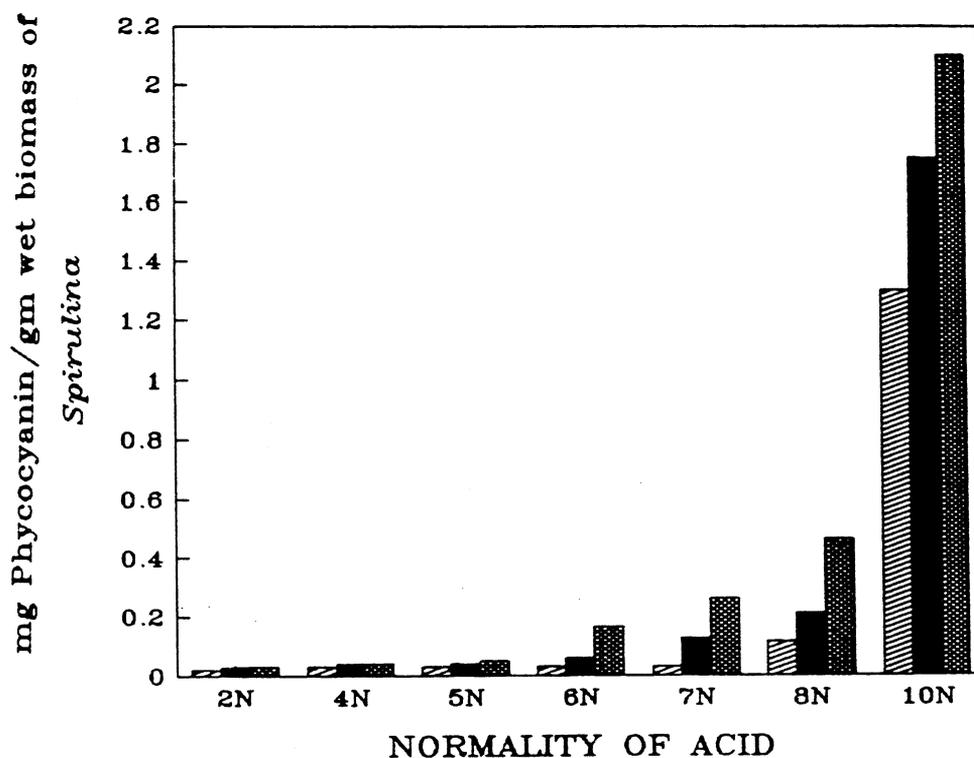


Fig. 2. Phycocyanin extraction from *Spirulina* wet biomass with different concentrations of hydrochloric acid: 2 h (▨); 4 h (■); and 24 h (▩).

cyanin from fresh biomass (Fig. 1). The phycocyanin sample extracted from fresh biomass showed a major peak at 615 nm while that extracted from dried samples showed an additional peak at 652 nm. The significant loss of phycocyanin in dried samples (Table 1) could be due to its peripheral position in phycobilisomes on the thylakoid membrane [4] and attributable to its sensitivity to temperature.

3.2. Comparison of different extraction methods for yield of phycocyanin

Comparison was made among different procedures for phycocyanin extraction with freshly harvested biomass and the data are presented in terms of mg phycocyanin per 100 mg dry weight of *Spirulina* (Table 2). Treatment with 0.02 N HCl for 30 s facilitated extraction of phycocyanin. Hence in the present study comparison was made between acid treated and untreated biomass by different extraction methods (Table 2). Of these methods water extraction method is the slowest and takes 3–4 days for comparable yields of phycocyanin while homogenisation either by mortar and pestle or Virtimixer and freezing and thawing takes 10–12 h. As shown in Table 2, 2–3 cycles of freezing

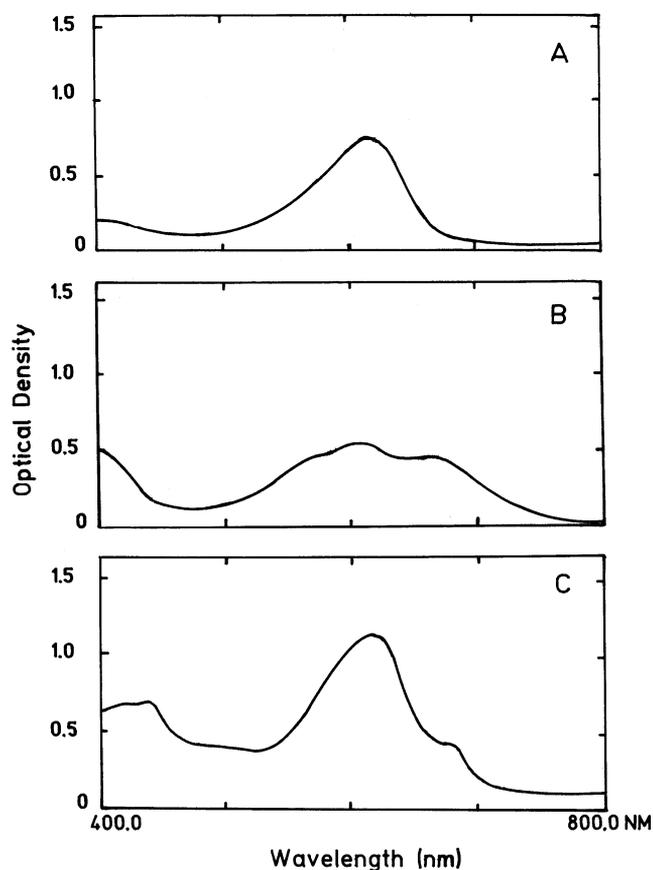


Fig. 3. Spectra of phycocyanin extracted with: A, water; B, Hydrochloric acid; and C, homogenisation in blender.

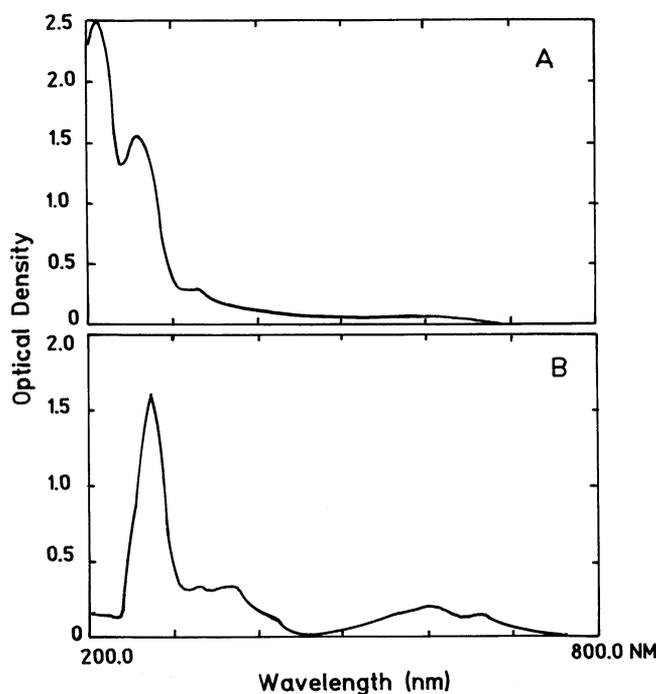


Fig. 4. Spectra of phycocyanin treated with 10 N HCl at 80°C for 15 min followed by chloroform extraction: A, Spectra of sample in acidified aqueous phase; and B, Spectra of sample in chloroform (Indicating separation of protein and pigment).

and thawing followed by centrifugation resulted in 90% (17.66 mg phycocyanin) of the total extracted (19.47 mg) phycocyanin from acid treated samples while in untreated samples, 62% of the phycocyanin (14.16 mg) was extracted. The remaining phycocyanin was extracted in a subsequent two steps (Table 2). In the water extraction process, phycocyanin leaching was very slow and observed only in cells harvested in late exponential phase. Although 19.12–19.28 mg of phycocyanin was obtained in three extraction steps, each step took 1 day. To prevent contamination sodium azide (0.02% w/v) was added. Homogenisation using mortar and pestle in the presence of acid washed neutral sand resulted in 90% (17.58 mg) of the total (19.84 mg) phycocyanin extracted. Repetition of this step 2–3 times resulted in complete extraction. Homogenisation using a 'virtimixer' at 10 000 rpm for 10 min intermittently at 9°C yielded 19.02 mg of phycocyanin.

The results obtained for the effect of increasing concentration of hydrochloric acid on extraction of phycocyanin with time are shown in Fig. 2. Phycocyanin extraction was achieved only with 8–10 N hydrochloric acid. The microscopic observation of cells indicated complete disintegration of cells at these concentrations and also resulted in separation of the pigment phycocyanobilin from phycobiliprotein, phycocyanin as also reported by O'hEocha [10].

The extraction method employed also showed differences in spectral properties of the phycocyanin (Fig. 3).

The sample extracted by freezing and thawing showed only one absorption peak of phycocyanin, while the sample extracted by homogenisation showed a minor second peak at 678 nm indicating the contamination of chlorophyll which is due to disintegration of cells. The quality of phycocyanin required would influence the selection of suitable extraction method. However for extraction of phycocyanin only fresh biomass was suitable. For purification of phycocyanin-freezing and thawing method and for routine estimations of phycocyanin in small samples, homogenisation in mortar and pestle would be ideal procedures to follow.

3.3. Separation of protein and pigment

Treatment of phycobiliprotein with 10% sodium dodecyl sulphate, 8 M urea and, β -mercaptoethanol which are generally used for denaturing proteins did not separate the pigment from the protein. Phycocyanin lost its colour in 8 M urea. To elucidate the nature of the covalent linkage between chromophore and protein, reagents which cleave ester, ether and polypeptide bonds were used [10,11]. Brown et al. [12] established the presence of a thioether linkage between phycocyanobilin and cysteine at each chromophore attachment site on α and β subunits of phycocyanin in the

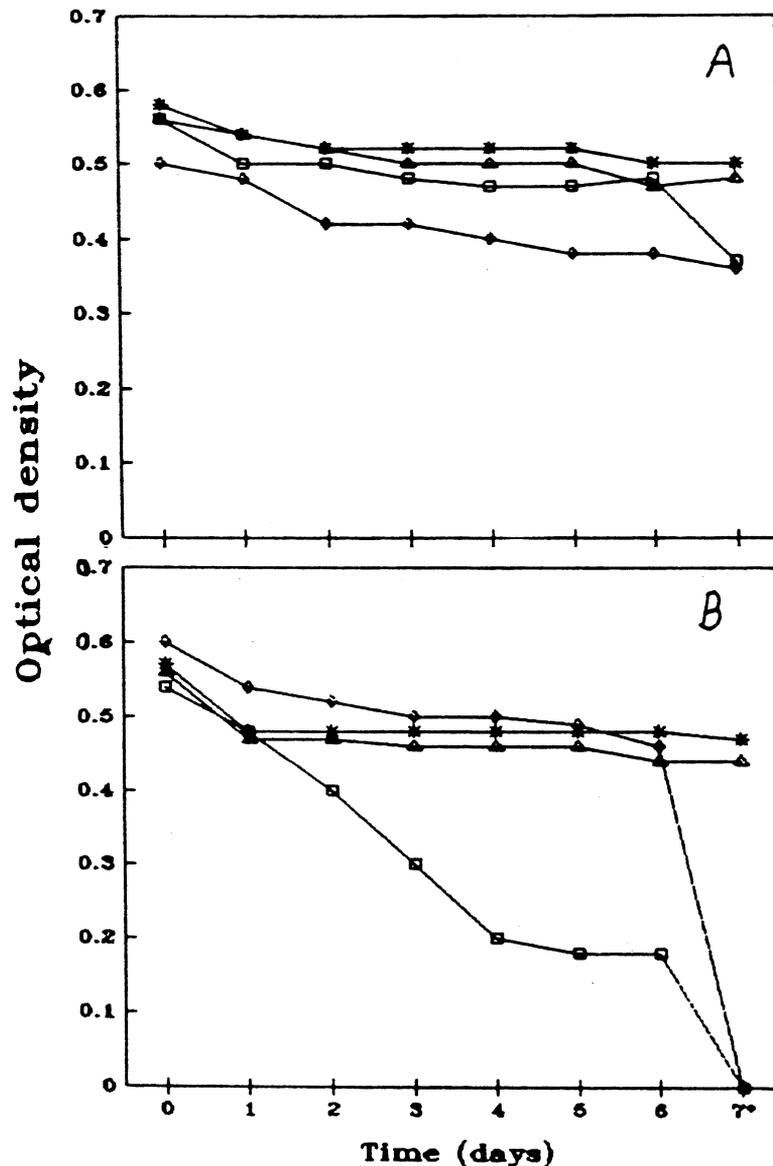


Fig. 5. Stability of phycocyanin at different pH values: A, Stability of phycocyanin at $9 \pm 1^\circ\text{C}$; and B, Stability of phycocyanin at $25 \pm 2^\circ\text{C}$, 7* indicates 30th day. \diamond - pH 5.0; \triangle - pH 6.0; * - pH 7.0; and \square - pH 8.0.

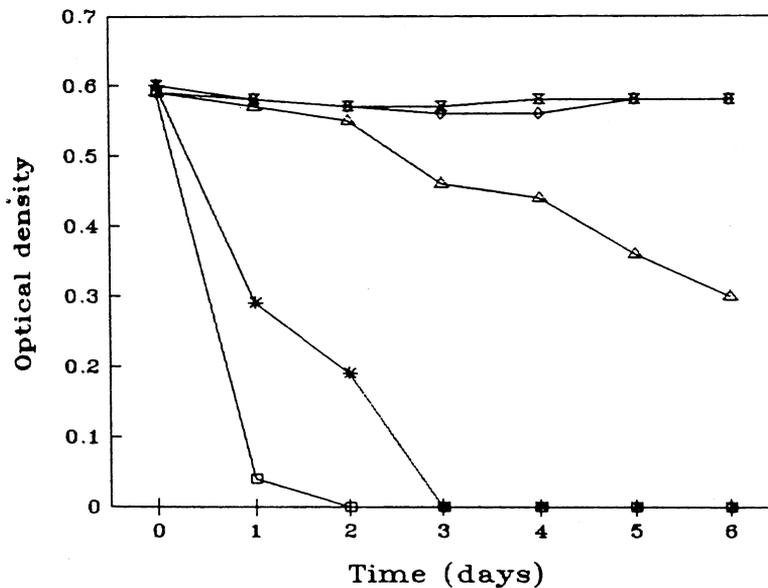


Fig. 6. Stability of phycocyanin at different temperatures: ▽—4°C; ◇—9 ± 1°C; △—30°C; *—45°C; and □—55°C.

unicellular rhodophyte *Cyanidium caldarium*. Treatment of phycocyanin with 10 N HCl for 15 min at 80°C followed by extraction with chloroform resulted in pigment (phycocyanobilin) separation. The absence of absorption peaks in the visible region in acid phase after chloroform extraction (Fig. 4) confirms complete hydrolysis and extraction of pigment. The chloroform extract showed two absorption peaks (662 and 607 nm) and the spectral properties were similar to the reported values for phycocyanobilin [10].

3.4. Stability studies of phycocyanin

The pH stability studies for a period of 4 weeks indicated that the phycocyanin was stable over a pH range of 5–7.5 at ambient temperature (25 ± 2°C) and 9 ± 1°C (Fig. 5A and Fig. 5B). At lower temperature, phycocyanin was stable for longer periods than at room temperature. Below and above 5–7.5 pH range the pigment lost its colour gradually.

The effect of temperature on phycocyanin stability indicated that it was highly unstable at 45°C and above (Fig. 6). Phycocyanin lost its colour and the spectra taken before and after treatment at 50°C showed the disappearance of an absorption peak at 615 nm (Fig. 7) Above 30°C it gradually lost its colour. It is quite stable at 10 and 4°C for a long time.

These results showed that fresh biomass is best for phycocyanin extraction to avoid 50% loss in pigment by various drying methods. The homogenisation method is rapid, while acid extraction would be useful for direct extraction of pigment phycocyanobilin from phycocyanin. Phycocyanin was stable at lower

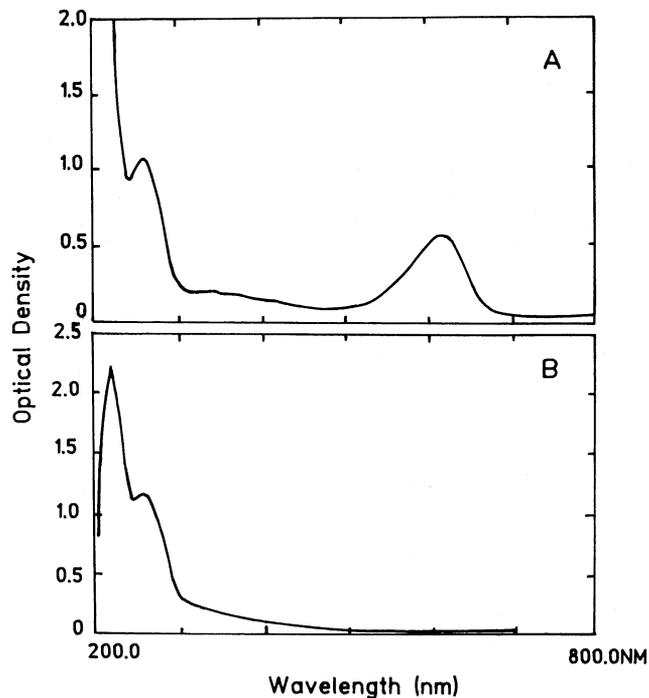


Fig. 7. Spectra of phycocyanin: A, Before incubation; and B, After incubation at 55°C for 15 min.

temperature for longer periods in the pH range of 5.0–7.5.

Acknowledgements

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References

- [1] Benneman JR. Microalgal Biotechnology: Products, Process and Opportunities, vol. 1. Washington: OMEC International Inc, 1988.
- [2] Venkataraman LV, Becker EW. Algal cultivation. In: Venkataraman LV, Becker EW, editors. Biotechnology and utilization of algae. The Indian experience, 1985:12–32.
- [3] Henrikson R. In: Henrikson Robert, editor. Earth food *Spirulina*. Laguna Beach, CA: Ronore Enterprises Inc, 1989.
- [4] Gantt E. Phycobilisomes. *Ann Rev Plant Physiol* 1981;32:327–47.
- [5] Dainippon Ink and Chemicals. Lina blue A (Natural blue colorant of *Spirulina* origin) Technical information. Tokyo, Japan: Dainippon Ink and Chemicals, 1985.
- [6] Iijima N, Shimamatsu H. (inventors; Dainippon Ink and Chemicals assignee). Antitumor agent and method of treatment thereof, US Patent pending. ref p1150-726-A82679, App.15 September, 1982.
- [7] Kronik M, Grossman P. Immunoassay techniques with fluorescent phycobiliprotein conjugates. *Clin Chem* 1983;29:1582–6.
- [8] Zarrouk C. Contribution a letude d'une cyanophycee. Influence de divers factours physiques. et chimiques sur la croissance et la phytosynthese do *Spirulina maxima*, Ph.D Thesis, University of Paris.
- [9] Siegelman HW, Kycia JH. Algal biliproteins. In: Hellebust JA, Craigie JS, editors. Handbook of Phycological Methods. Cambridge: Cambridge University Press, 1978:72–8.
- [10] O'hEocha C. Spectral properties of the phycobilins. 1. Phycocyanobilin. *Biochemistry* 1963;2:375–82.
- [11] O'hEocha C. Biliproteins of Algae. *Ann Rev Plant Physiol* 1965;16:415–34.
- [12] Brown AS, Offner GD, Ehrhardt MM, Troxler RF. Phycobilin-apoprotein linkages in the α and β subunits of phycocyanin from the unicellular rhodophyte, *Cyanidium caldarium* aminoacid sequences of ^{35}S labelled chromopeptides. *J Biol Chem* 1979;254:7803–11.