JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

An Efficient Method for Extraction of Astaxanthin from Green Alga *Haematococcus pluvialis*

R.SARADA, R.VIDHYAVATHI, D. USHA, AND G. A. RAVISHANKAR*

Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore 570 020, India

Haematococcus pluvialis is one of the potent organisms for production of astaxanthin, a high value ketocarotenoid. Astaxanthin is accumulated in thick-walled cyst cells of *Haematococcus*. The thick cell wall is made up of sporopollenin-like material, algaenan, which hinders solvent extraction of astaxanthin. In the present study, an improved method for extraction of astaxanthin without homogenization of cells is reported. Extractability of astaxanthin from cyst cells was evaluated by treating cells with various solvents and pretreating the cells with organic and mineral acids at 70 °C followed by acetone extraction. Hydrochloric acid treatment facilitated 86−94% extractability of astaxanthin. Treatment time, temperature, and concentration of the acid were found to be critical factors for maximum extractability. The treatment did not affect the astaxanthin ester profile and the treated cells can be preserved until further use.

KEYWORDS: ***Haematococcus*; astaxanthin; total carotenoid; extractability**

INTRODUCTION

Haematococcus is a fresh water, motile green alga having commercial importance owing to its ability to accumulate ketocarotenoid, astaxanthin up to 2−3% (w/w) on a dry weight basis. Astaxanthin in *Haematococcus* cells predominantly exists in 3*S*,3′*S* form, which is biologically active (*1*). Astaxanthin is extensively used as a pigmentation source in aquaculture and poultry industries and gained applications in nutraceutical and pharmaceutical industries due to its superior antioxidant activity (*2*, *3*). Astaxanthin was shown to have 500-fold stronger free radical antioxidant activity than vitamin E and 38-fold than β-carotene (*4*, *5*). The antioxidant properties of astaxanthin are believed to have a key role in protection against UV-light photooxidation, inflammation, cancer, *Helicobacter pylori* infection, aging and age-related macular degeneration, or enhancement of the immune response, liver function, and heart, eye, joint, and prostate health (*3*).

Haematococcus has two distinct phases in its life cycle, vegetative growth phase and encysted secondary carotenoid accumulation phase. The *Haematococcus* encysted cell wall consisted of 70% carbohydrates (66% hexoses), 3% cellulose, and 6% proteins and consisted of 3% acetolysis resistant material (*6*). The thick encysted cell wall hinders carotenoid extraction by solvents and bioavailability of astaxanthin (*7*). It was also reported that feeding encysted intact cells to Rainbow trout did not help in pigmentation. Cellular astaxanthin was bioavailable only after feeding of cracked or broken cells (*8*). Therefore, the cells have to be homogenized for efficient astaxanthin extraction or bioavailability. Different methods were reported for extraction of astaxanthin using solvents, treatment with extracellular enzymes followed by solvent extraction (*9*), DMSO (*10*), and cell disruption processes (*11*). All these methods result in loss of pigment to some extent or the other and are difficult to apply on a large scale.

To minimize the loss, homogenization under high pressure at cryogenic temperatures is practiced (*12*). The present study focused on development of an efficient method for extraction of astaxanthin from *Haematococcus* without involving any homogenization procedures.

MATERIALS AND METHODS

Algal Culture. *H. pluvialis* (SAG 19-a) was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany, and grown in an autotrophic medium as detailed in Tripathi et al. (*13*). The cultures were incubated at 25 ± 1 °C under a light:dark (16:8 h) cycle with 1.5 klux intensity for a period of 1 week. Later, the cultures were incubated at a continuous light intensity of 3.0 klux with the addition of 0.2% NaCl and 4.4 mM sodium acetate for a period of 2 weeks. The encysted cells which appeared red due to accumulation of carotenoids were harvested by centrifugation at 5000 rpm for 5 min at 4 °C. Harvested biomass was lyophilized and used for extraction purposes.

Treatment of Cells and Extraction of Carotenoids. Ten milligrams of biomass was homogenized in a mortar and pestle in the presence of neutral glass powder (Borosil Glass Works Ltd., India) extracted with acetone and centrifuged at 5000 rpm for 10 min. The supernatant was used for estimation of total carotenoid and total astaxanthin. Ten milligram quantities of biomass were individually treated with acetone (1 h), acetone (24 h), methanol (24 h), and dimethyl sulfoxide (DMSO) with 5 drops of glacial acetic acid (*10*), centrifuged at 5000 rpm for 10 min at 4 °C, and supernatants were used for estimation of extractable astaxanthin. Another set of 10 mg quantities of biomass was separately

* To whom correspondence should be addressed. Tel.: +91-821-2516501. Fax: +91-821-2517233. E-mail: pcbt@cftri.res.in.

10.1021/jf060737t CCC: $33.50
Published on Web 09/12/2006

treated with different concentrations (1−10 N) of hydrochloric acid (HCl) and 2 N formic acid, citric acid, acetic acid, and tartaric acid at 70 °C, cooled, centrifuged, washed with 2 mL of distilled water, and treated with acetone for 1 h and the supernatants were used for estimation of extractable astaxanthin. All the steps were carried out in dim light.

Removal of Chlorophyll. Encysted cells of *Haematococcus* were treated with 5% methanolic KOH at 70 °C for 5 min. The cells were separated by centrifugation at 5000 rpm for 10 min at 4 °C, washed with 2 mL of distilled water, and processed for astaxanthin extraction and estimation.

Estimation of Carotenoids and Chlorophylls. The absorbance of the extracts were read at 470, 480, 645, and 661.5 nm using a Shimadzu UV-160A Spectrophotometer. Chlorophyll and total carotenoid contents were calculated using Lichtenthaler equations (*14*). Astaxanthin was analyzed by the method of Davies (*15*). Extractability of astaxanthin was calculated for all samples as per the procedure of Kobayashi et al. (*9*) by the formula

$$\text{extractability (\%)} = \text{free astaxanthin } (\mu\text{g mL}^{-1})/\text{total astaxanthin } (\mu\text{g mL}^{-1}) \times 100$$

Analysis by TLC. Extracted carotenoids from control and treated *Haematococcus* cells were concentrated and separated on silica gel 60 F_{254} TLC plates (10 × 10 cm, Merck) and developed using acetone: *n*-hexane (30:70) solvent for 20 min.

Analysis of Carotenoids by HPLC. Carotenoid extracts were subjected to HPLC (Shimadzu LC 10A) analysis using reversed phase C18 (Supelco, 25 cm × 4.6 mm) column. The following solvents were used at a flow rate of 1.25 mL min^{-1}: (A) acetone and (B) methanol: H_2O (9:1 v/v). The separation of carotenoids was achieved by a gradient between solvents A and B for 40 min as follows: B was run at 80 to 20% for 25 min, 20% for 10 min, and 20 to 80% for 5 min. The separated carotenoids and astaxanthin esters were identified using a photodiode array detector (*16*). The peaks were integrated at 476 nm to quantify free astaxanthin and astaxanthin esters. β-Carotene and astaxanthin (Sigma) were used as standards.

Estimation of Chemical Constituents. Fat, total protein, and ash content of *Haematococcus* encysted cells were analyzed by AOAC methods (*17*). The carbohydrate content was estimated by a phenol sulfuric acid method (*18*).

Experimental Design and Data Analysis. Each experiment was repeated twice with two replications. All the observations and calculations were made separately for each set of experiments and were expressed as mean ± standard deviation. The significance ($p < 0.05$) of the variables studied was assessed by simple student 't' test using Microsoft Excel Xp. The mean separations were performed by Duncan's multiple range test for segregating means where the level of significance was set at $p \leq 0.05$ (*19*).

RESULTS AND DISCUSSION

Extractability of Carotenoids from *Haematococcus* Cells Treated with Different Solvents and Acids. *Haematococcus* cells that were selected for this study contained 2% total carotenoid (w/w) and astaxanthin constituted 88% of total carotenoids and 1.76% w/w of dry biomass. Astaxanthin exists as monoester, diester, and free astaxanthin at 72.0, 27.5, and 0.5%, respectively. The extractability of astaxanthin from this alga was evaluated using different solvents and different acids for pretreatment followed by acetone extraction. It is evident from **Table 1** that extractability of astaxanthin was considerably less when cyst cells were extracted with solvents. However, with DMSO in the presence of a few drops of glacial acetic acid at 70 °C, the extractability increased significantly. Among the acids tested, HCl treatment followed by acetone extraction resulted in 86.40 ± 1.32% (w/w) of astaxanthin extractability (**Table 1**). The extractability of astaxanthin increased with increasing concentration of HCl (**Figure 1**). However, at higher concentrations (>4 N HCl) the cells were observed to be floating and the astaxanthin content was found to be decreased significantly. Low concentration (1−2 N) with 5−10 min of heating at 70 °C resulted in 96 ± 3% extractability. The cells after extraction when observed under microscope appeared colorless with intact cell wall (**Figure 2**). Thus, the results indicated that the treatment facilitated extraction of astaxanthin without homogenization of cells.

Table 1. Astaxanthin Extractability from *Haematococcus* Encysted Cells Using Different Solvents with and without Acid Treatment

treatment[a]	astaxanthin extractability[b] (%)
acetone (1 h)	11.25 ± 0.50 cd
acetone (24 h)	14.13 ± 0.66 cd
methanol	18.62 ± 0.78 cd
DMSO	66.64 ± 0.61 ab
HCl	86.40 ± 1.32 a
tartaric acid	22.00 ± 0.44 c
citric acid	3.44 ± 0.11 d
acetic acid	19.38 ± 1.10 cd
formic acid	8.00 ± 0.23 cd

[a] Note: All acids were tested at 2 N concentration for 10 min. [b] Each value represents the mean of two separate experiments each with two replicates. Means within a column followed by the same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

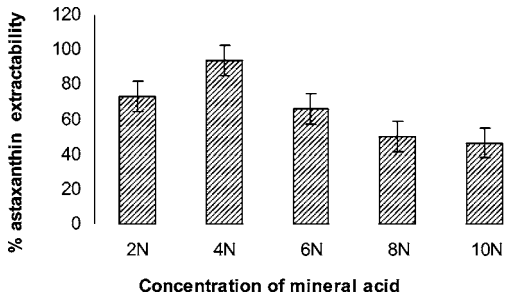

Figure 1. Astaxanthin extractability from *Haematococcus* encysted cells after treatment with HCl at 70 °C for 2 min.

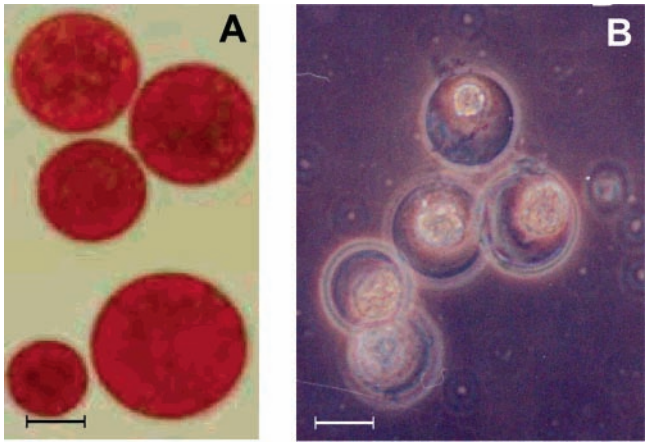

Figure 2. Photomicrographs of *Haematococcus* encysted cells (scale bar 22 μm). (A) before extraction and (B) after extraction of carotenoids.

Carotenoid Profile of *Haematococcus* Extract before and after Treatment with Hydrochloric Acid. The absorption spectra of astaxanthin extract from control and treated cells was observed to be the same, showing characteristic peak in the region of 475 nm (**Figure 3**). It was also evident from the spectra that the extract has not shown a peak in the 645−660 nm region, indicating the absence or traces of chlorophyll. The astaxanthin extracted from *Haematococcus* cells after homogenization and from HCl-treated cells without homogenization were analyzed

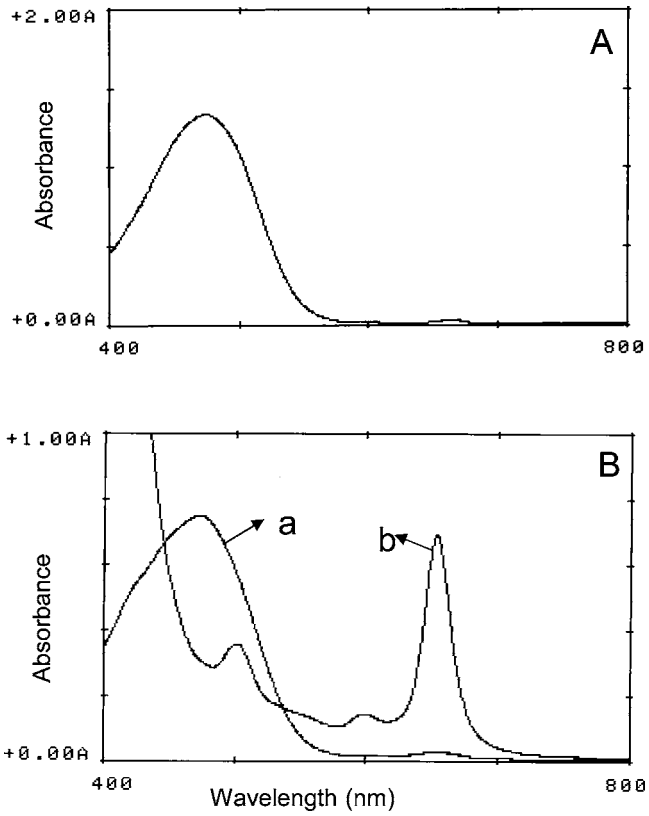

Figure 3. Absorption spectra of carotenoid extract from encysted *Haematococcus* cells: (A) Control cells and (B) carotenoid extract (a) and chlorophyll extract (b) from methanolic KOH-treated cells.

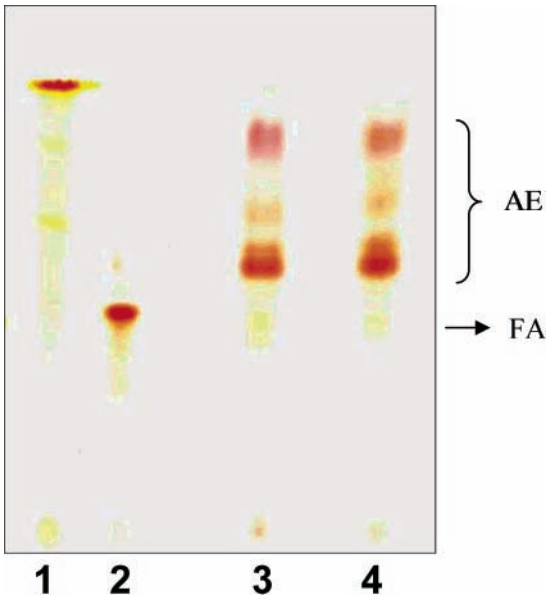

Figure 4. TLC separation of carotenoids extracted from *Haematococcus* cells: (1) Standard β-carotene; (2) standard free astaxanthin; (3) control cells; (4) 2 N HCl treated cells. AE: astaxanthin esters; FA: free astaxanthin.

by both TLC and HPLC and compared. The TLC analysis showed no difference in the band pattern (**Figure 4**). HPLC profiles of both samples (**Figure 5**) were identical, indicating that treatment of cells with HCl did not affect the astaxanthin esters.

Extraction of astaxanthin from encysted cells is a critical step in downstream processing of *Haematococcus* as the cells consisted of thick algaenan-type cell wall which hinders solvent

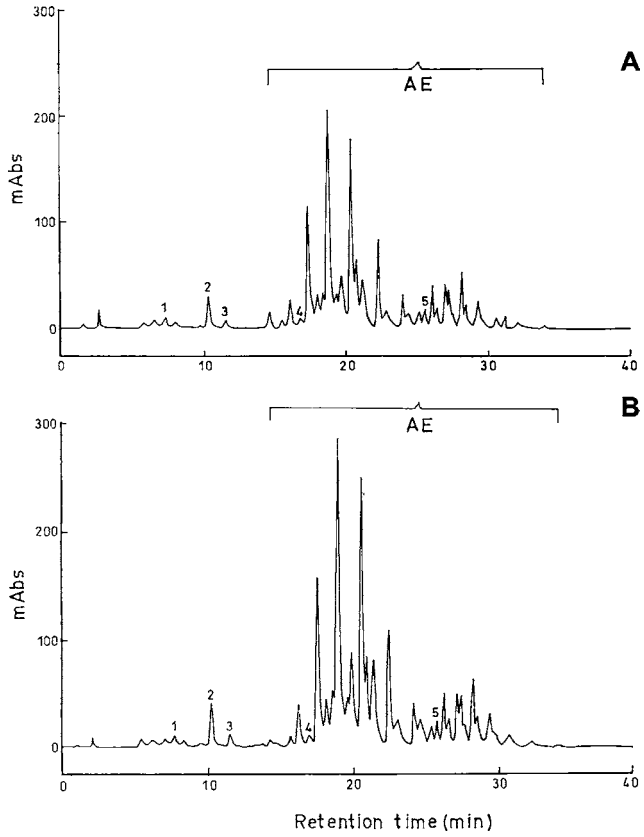

Figure 5. HPLC analysis of carotenoids extracted from *Haematococcus* cells: (A) control cells; (B) 2 N HCl treated cells. Free astaxanthin (1), lutein (2), canthaxanthin (3), echinenone (4), β-carotene (5), and astaxanthin esters (AE).

extraction of astaxanthin from intact encysted cells (*20*). There are several reports on the treatment of cells to enhance the extractability and bioavailability of astaxanthin. Kobayashi et al. (*9*) evaluated different conditions for selective removal of chlorophyll and extraction of astaxanthin from *Haematococcus* cells. They recovered 70% astaxanthin when red cysts were treated with 40% acetone at 80 °C for 2 min followed by lyophilization or enzymatic treatment with a combination of three enzymes: kitalase, cellulose, and Abalone acetone powder (mainly β-glucuronidase).

Mendes-Pinto et al. (*11*) compared the effect of different cell disruption processes like acid, alkali, and enzyme treatment, autoclaving for 30 min, 121 °C, spray drying inlet 180 °C, outlet 115 °C, and mechanical disruption methods for extraction of astaxanthin. They found that autoclaving and spray drying treatments facilitated cell disruption by ultrasonication, thereby resulting in better astaxanthin recovery (85%) which is otherwise resistant to disruption by ultrasonication alone. They also reported that enzymatic treatment of *Haematococcus* cells or exposure to alkali or acid resulted in a significant loss (20−35%) of total carotenoids as a direct result of processing. Bubrick (*12*) described grinding of dried *Haematococcus* biomass at cryogenic temperature (−170 °C) in the presence of butylated hydroxytoluene. Boussiba et al. (*10*) described treatment of cyst cells with 5% KOH in 30% methanol to destroy the chlorophyll, and extraction with DMSO with a few drops of glacial acetic acid by homogenization and heating at 70 °C for 10 min to recover astaxanthin.

In the present study, an improved method was developed for efficient extraction of astaxanthin without homogenization of cells either by mechanical means or by sonication. Treatment

of cells with HCl up to 4 N at 70 °C facilitated solvent extraction of astaxanthin and higher than 4 N was not required as the astaxanthin content was affected. Thus, the results indicated that the concentration and contact time of acid and incubation temperature are the critical factors for efficient extraction. Although Mendes-Pinto et al. (*11*) reported treatment with HCl for 15 and 30 min at room temperature, they could achieve efficient extraction of astaxanthin only after sonication. The present results showed 90% extractability (**Figures 1** and **2**) of astaxanthin when cells were treated with HCl at 70 °C alone without homogenization. This probably may be due to cleavage of some vital bonds in the cell wall during hydrochloric acid treatment with heat which facilitated solvent extraction of astaxanthin. The astaxanthin esters were not affected by this treatment. The treatment for removal of chlorophyll with methanolic KOH has resulted in significant loss of (30−40%) astaxanthin content (**Figure 3B**). This step can be included only when pure astaxanthin is required. The chlorophyll content in the normal process of extraction from cyst cells constitutes less than 0.2% (w/w). The present method of extraction avoided use of enzymes, autoclaving, sonication, or homogenization of cells. The treated cells can be stored at low temperature or freeze-dried and stored until further use. Since the encysted cells of *Haematococcus* consist of 28 ± 3% fat, 31.5 ± 2.5% carbohydrate, 26.0 ± 2% protein, and 4.6 ± 0.3% ash, the colorless residual biomass after astaxanthin extraction can be used as a supplement in feeds.

ACKNOWLEDGMENT

The authors thank Dr. V. Prakash, Director, CFTRI, for his support to carry out this study.

LITERATURE CITED

(1) Johnson, E. A.; An, G. H. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.* **1991**, *11*, 297−326.

(2) Lorenz, R. T.; Cysewski, G. R. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* **2000**, *18*, 160−167.

(3) Guerin, M.; Huntley, M. E.; Olaizola, M. *Haematococcus* astaxanthin: applications for human health and nutrition. *Trends Biotechnol*. **2003**, *21*, 210−216.

(4) Kurashige, M.; Okimasu, E.; Inoue, M.; Utsumi, K. Inhibition of oxidative injury of biological membranes by astaxanthin. *Physiol. Chem. Phys. Med. NMR* **1990**, *22*, 27−38.

(5) Shimidzu, N.; Goto, M.; Miki, W. Carotenoids as singlet oxygen quenchers in marine organisms. *Fish. Sci.* **1996**, *62*, 134−137.

(6) Hagen, C.; Siegmund, S.; Braune, W. Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation. *Eur. J. Phycol.* **2002**, *37*, 217−226.

(7) Good, B. H.; Chapman, R. L. A comparison between the walls of *Haematococcus* cysts and the loricas of *Dysmorphococcus* and the possible presence of sporopollenin. *J. Phycol.* **1979**, *15*, 17.

(8) Sommer, T. R.; Potts, W. T.; Morrisey, N. M. Utilization of microalgae astaxanthin by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **1991**, *94*, 79−88.

(9) Kobayashi, M.; Kurimura, Y.; Sakamoto, Y.; Tsuji, Y. Selective extraction of astaxanthin and chlorophyll from the green alga *Haematococcus pluvialis. Biotechnol. Tech.* **1997**, *11*, 657−660.

(10) Boussiba, S.; Fan, L.; Vonshak, A. Enhancement and determination of astaxanthin accumulation in green alga *Haematococcus pluvialis. Methods Enzymol.* **1992**, *213*, 386−391.

(11) Mendes-Pinto, M. M.; Raposo, M. F. J.; Bowen, J.; Young, A. J.; Morais, R. Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability. *J. Appl. Phycol.* **2001**, *13*, 19−24.

(12) Bubrick, P. Production of astaxanthin from *Haematococcus. Biores. Technol.* **1991**, *38*, 237−239.

(13) Tripathi, U.; Sarada, R.; Rao, S. R.; Ravishankar, G. A. Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. *Biores. Technol.* **1999**, *68*, 197−199.

(14) Lichtenthaler, H. K. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* **1987**, *148*, 350−382.

(15) Davies, B. H. Carotenoids. In *Chemistry and biochemistry of plant pigments*; Goodwin, T. W., Ed.; Academic Press: London, 1976; pp 38−166.

(16) Brinda, B. R.; Sarada, R.; Kamath, B. S.; Ravishankar, G. A. Accumulation of astaxanthin in flagellated cells of *Haematococcus pluvialis* - cultural and regulatory aspects. *Curr. Sci.* **2004**, *87*, 1290−1295.

(17) Association of Official Analytical Chemists. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 16th ed.; Cunniff, P., Ed.; AOAC International Suite, Maryland, 1997; Volume I.

(18) Sadasivam, S.; Manickam, A. Phenol-sulphuric acid method for total carbohydrate. In *Biochemical methods*, 2nd ed. New Age International Publishers: New Delhi, 1996.

(19) Harter, L. N. Critical values for Duncan's new multiple range test. *Biometrics* **1960**, *16*, 671−685.

(20) Montsant, A.; Zarka, A.; Boussiba, S. Presence of a nonhydrolyzable biopolymer in the cell wall of vegetative cells and astaxanthin-rich cysts of *Haematococcus pluvialis* (Chlorophyceae). *Mar. Biotechnol.* **2001**, *3*, 515−521.

Received for review March 16, 2006. Revised manuscript received August 1, 2006. Accepted August 3, 2006. The award of Senior Research Fellowship to V.R. and U.T. by the Council of Scientific and Industrial Research (CSIR), New Delhi, is gratefully acknowledged.

JF060737T