

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Evaluation of Efficient Extraction Methods for Recovery of Photosynthetic Pigments from Microalgae

¹A. Ilavarasi, ²D. Pandiaraj, ³D. MubarakAli,

²M.H. Mohammed Ilyas and ¹N. Thajuddin

¹Department of Microbiology, School of Life Sciences,
Bharathidasan University, Tiruchirappalli, India

²Department of Botany, Jamal Mohammed College, Tiruchirappalli, India

³Central Inter-Disciplinary Research Facility, Mahatma Gandhi Medical College and
Research Institute Campus, Puducherry, India

Abstract: Microalgal species are known to have pigments in their cellular constitute at the maximum and are valuable bioactive products. In the present study focused was on the evaluation of efficient extraction methods for photosynthetic pigments from microalgal species. They are, *Chlorella* sp., *Acrochaete* sp., *Phormidium chlorinum*, *Jaaginema pseudogeminatum* and *Chroococcus* sp. There are four different extraction methods were adopted for active recovery and are economically feasible such as direct extraction, mechanical grinding, heating and preheated solvent method. It was found that mechanical grinding method has extract two fold increased amount than the other methods. Additionally, this methods is inexpensive, less laborious and active extraction. It is suggested that this method could be used for the extraction of photosynthetic pigments from microalgae for pharmaceutical to biotechnological purpose.

Key words: Microalgae, pigment extraction, carotenoids, chlorophyll

INTRODUCTION

Microalgae are oxygenic photosynthetic organism comprises of both prokaryotic and eukaryotic forms. They are responsible for approximately half of the atmospheric oxygen and simultaneously use the greenhouse gas carbon dioxide. These microalgae possess pigments such as chlorophyll and carotenoids. Pigments like chlorophyll and carotenoids are used to assess algal biomass and the phylogenetic composition of an aquatic ecosystem (Millie *et al.*, 1993; Jeffrey *et al.*, 1997; Al-Kahtani *et al.*, 2007; Adesalu and Nwankwo, 2008). Various extraction techniques are commonly employed to extract these photosynthetic pigments (MacKinney, 1941; Porra *et al.*, 1989; Karsten *et al.*, 1998; Pasquet *et al.*, 2011). Apart from the physical extraction techniques, some enzymes have also been reported to be used in pigment extraction (Deniaud *et al.*, 2003; Kim *et al.*, 2005; Choudhari and Ananthanarayan, 2007).

Precise analysis of algal photosynthetic pigments depends on the efficiency of the extraction technique. Each technique has its own merits and demerits. While evaluating any pigment extraction technique, the parameters like extractability, compatibility, precision, simplicity and safety (Wright *et al.*, 1997) must be

considered. For complete extraction of pigments, the chloroplast membrane should be solubilized in a solvent. In the present study, acetone was chosen as an extraction solvent as it extracts most of the photosynthetic pigments, in a wide range of polarity and furthermore, acetone 90% is recommended for phytoplankton pigment analysis (Strickland and Parsons, 1972; Jeffrey and Hallegraeff, 1987; Parsons *et al.*, 1984). Yet, pigment extraction has also been performed in 100% acetone to limit chl α hydrolysis by the thylakoid-bound enzyme chlorophyllase, present in most diatom species and chlorophyceae. The next thing is that the protocol which favours complete pigment recovery in one organism does not suit for the other. So, it is essential to use an extraction method which is suitable for most of the microalgae and at the same time that method should be easy to follow in any laboratories. Apart from the extraction techniques, these pigments were quantified and identified by spectrophotometric and chromatographic techniques (Jeffrey *et al.*, 1997).

With these considerations, the present work was aimed to compare the efficiency of four different conventional pigment extraction techniques via.: (1) Direct extraction method, (b) Preheated solvent method, (3) Mechanical grinding method and (4) Heating method among morphologically different forms of algae.

MATERIALS AND METHODS

Culture: *Phormidium chlorinum*, *Jaaginema pseudogeminata*, *Chroococcus* sp., *Chlorella* sp. and *Achrochaete* sp. were obtained from Microalgal Culture Collection Centre, Department of Microbiology, Bharathidasan University, Tiruchirappalli- 24, Tamil Nadu, India.

Medium and culture conditions: *Phormidium chlorinum* and *Jaaginema pseudogeminata*, *Chroococcus* sp. and *Achrochaete* sp. were subcultured in MN⁺ medium (Rippka, 1988) whereas *Chlorella* sp. was sub cultured in Half Strength Chu 10 medium (Ilavarasi *et al.*, 2011). The cultures were maintained at 25±2°C with 1.5 klux in 12:12 h photoperiod.

Extraction methods: All the cultures were harvested at their respective stationary phases and lyophilized. Lyophilized algal cells were used for each conventional extraction method and all the extractions were performed in triplicates. After extraction, the absorbance of the supernatants was measured at 663 and 470 nm for chlorophyll a and carotenoids, respectively. The pigment content was estimated based on the equations proposed by Lichtenthaler (1987).

Direct extraction: For 30 mg dried cells, 10 mL acetone was added and kept in a rotary shaker for 24 h followed by centrifugation at 8,000 rpm for 10 min. The supernatant was collected and the absorbance was measured at corresponding wavelengths.

Preheated solvent method: For 30 mg of lyophilized cells, 10 mL of preheated acetone was added and it was kept in rotary shaker for 15 min followed by centrifugation at 8,000 rpm for 10 min. The supernatant was collected and their absorbance was measured.

Mechanical grinding method: Thirty milligram of lyophilized cells were taken in a mortar and pestle with a pinch of glass powder. Initially, 2 mL of acetone was added followed by grinding for 2-3 min. The solvent- ground cell mixture was centrifuged and the supernatant was collected in a separate tube. The procedure was repeated until the pellet become colourless and the collected supernatants were pooled. The absorbance of the supernatant was measured.

Heating method: Thirty miligram of lyophilized cells were taken in a boiling tube along with 10 mL of acetone. The solvent-cell mixture was kept at 50°C in a water bath for

3 h which has been shaken manually once in 30 min. The contents were cooled, centrifuged and the supernatant was collected. The absorbance of the supernatant was measured at their corresponding wavelengths.

RESULTS AND DISCUSSION

The present study was initiated in order to evaluate the efficiency of conventional pigment extraction methods. In this study four different extraction methods viz., (a) direct extraction, (b) mechanical grinding method, (c) preheated solvent method and (d) heating method were employed to evaluate the pigment extraction efficiency. At the same time, morphologically different forms of microalgae (Fig. 1) were taken in this study, in order to know the versatility of the pigment extraction method. Pigments like chlorophyll and carotenoids are used to assess algal biomass from a particular environment and at the same time, they are also used to assess the algal growth under controlled environmental conditions.

Figure 2 shows the overall pigment yield of the mechanical grinding method. Error bars indicate standard errors (n = 5). *Chlorella* sp. showed maximum chlorophyll a and carotenoid content of about 2.45 and 0.27 mg g⁻¹ of lyophilized cells whereas the *Acrochaete* sp. found to have 1.75 and 0.25 mg g⁻¹ of chlorophyll a and carotenoid, respectively. In case of *Phormidium chlorinum*, the chlorophyll a and carotenoid content were found to be 2.97 and 0.71 mg g⁻¹ whereas, in *Jaaginema pseudogeminatum* it was about 1.14 and 0.33 mg g⁻¹, respectively. Regarding the pigment content of *Chroococcus* sp. the chlorophyll a and carotenoid content of about 0.49 and 0.13 mg g⁻¹ were recorded. The extraction protocols in the literature are diverse and represent the simple fact that no one protocol is sufficient for use on all varieties of algae.

Among the five organisms, *Chlorella* sp., *Acrochaete* sp. was found to have rigid fibrillar cell wall composed of polysaccharides whereas the rest of the organisms possess gelatinous cell wall, which has been completely lysed using this method. The important step in each and every extraction method is cell lysis. The contact of cells with solvent usually leads to cell lysis to some extent. In order to obtain efficient cell lysis, here the cells were ground in acetone along with glass powder. The cells were lysed upon solvent and grinding treatment (data not shown). This is one of the simple mechanical disruption methods which is commonly used in many of the phycology laboratories. The mechanical grinding leads to rise in temperature that may leads to pigment

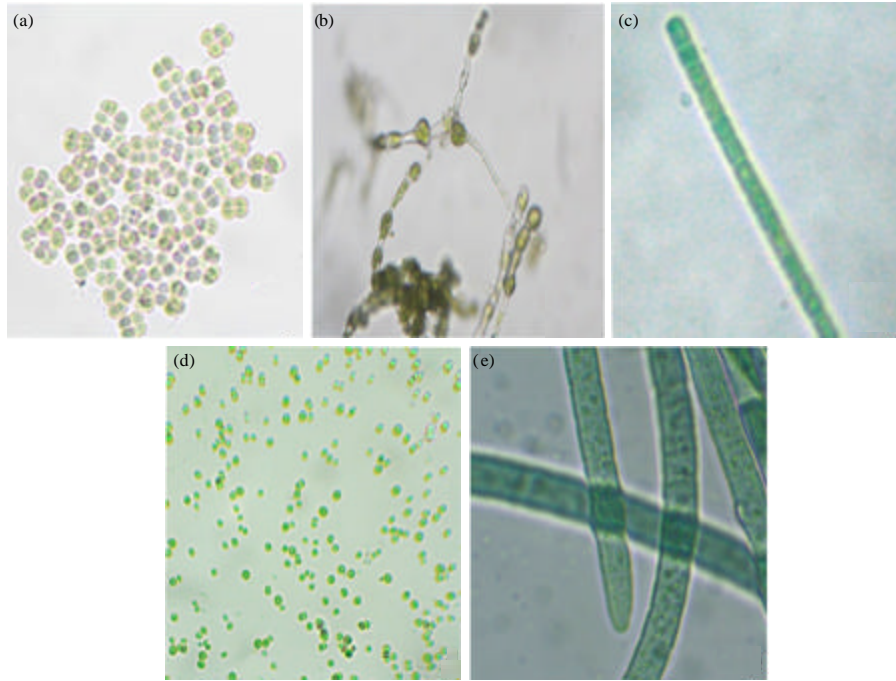


Fig. 1(a-e): Various morphotypes of microalgae (a) *Chroococcus* sp., (b) *Acrochaete* sp., (c) *Phormidium chlorinum*, (d) *Chlorella* sp. and (e) *Jaaginema pseudogeminatum* used in the current study

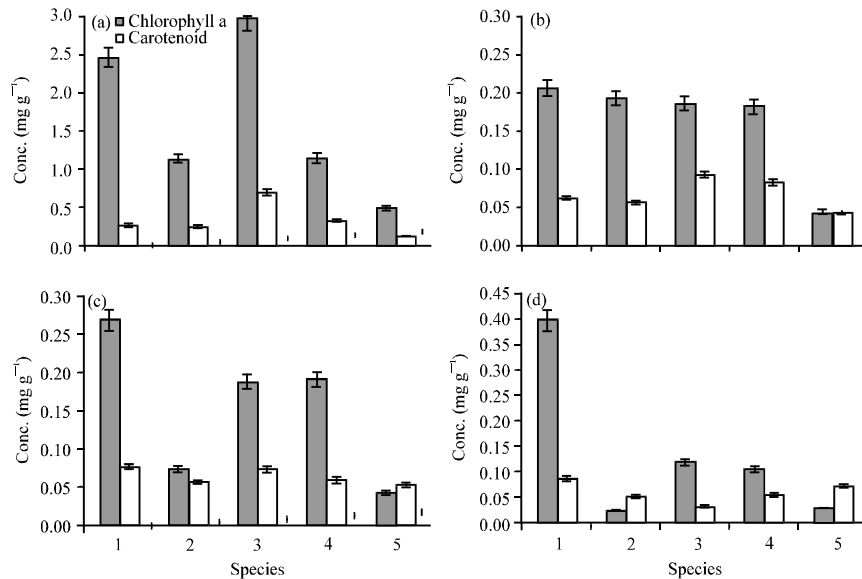


Fig. 2(a-d): Estimated pigment content of algal cells using different pigment extraction techniques, (a) Mechanical grinding method, (b) Direct extraction method, (c) Heating method and (d) Preheated solvent method, 1: *Chlorella* sp., 2: *Acrochaete* sp., 3: *Phormidium chlorinum*, 4: *Jaaginema pseudogeminatum* 5: *Chroococcus* sp.), error bars indicate standard errors

degradation. In order to avoid pigment degradation while extraction the whole extraction procedure was carried out under low temperature.

The extraction period is a significant aspect in the recovery and interpretation of pigment yields. In this method, the cell to solvent contact time was about one

hour, this facilitates the complete recovery of pigments from the cell. In contrast to the present study, it was reported that short extraction time may fail to recover pigments completely (Buffan-Dubau and Carman, 2000; Cartaxana and Brotas, 2003). In this study upon repeated extraction, the color of the cell has been changed from green to pale white revealed that the pigments were completely recovered from the cell. It was also reported that mechanical disruption can improve the extraction efficiency of the solvent depending on the solvent used and if the sample was freeze-dried. Pigment extraction methods from algae were evaluated by many of the researchers. Hagerthey *et al.* (2006) evaluated various methods of pigment extraction for periphyton group of algae. From the estimation, it was found that the mechanical grinding method is efficient than the rest of the pigment extraction methods (Table 1).

Figure 3a shows the yield of pigment using direct extraction method. The error bars indicate the standard errors. In *Chlorella* sp. the chlorophyll a and carotenoid content of about 0.20 and 0.06 mg g⁻¹ were estimated using the direct extraction method. *Acrochaete* sp. showed 0.19 and 0.05 mg g⁻¹ of chlorophylla and carotenoid respectively, whereas, in *Phormidium chlorinum* showed 0.18 and 0.09 mg g⁻¹ correspondingly. *Jaaginema pseudogeminatum* showed chlorophyll a and carotenoid content of about 0.18 and 0.08 mg g⁻¹ whereas, in *Chroococcus* sp. it was 0.04 and 0.04 mg g⁻¹ chlorophyll a and carotenoid content, respectively. The direct extraction method yielded less amount of pigment when compared to the mortar and pestle method.

In the direct extraction method, the cell to solvent contact time was about 24 h which is high when compared

Table 1: Various pigment extraction methods used in the present study

Criteria	Extraction methods			
	Direct	Preheated solvent	Mechanical grinding	Heating
Solvent	Acetone	Acetone	Acetone	Acetone
Solvent-cells contact time	24 h	30 min	1 h	3 h
Time taken for extraction	24 h+10 min	40 min	1 h+10 min	3 h+10 min
Extraction efficiency	Minimum	Below minimum	Maximum	Minimum

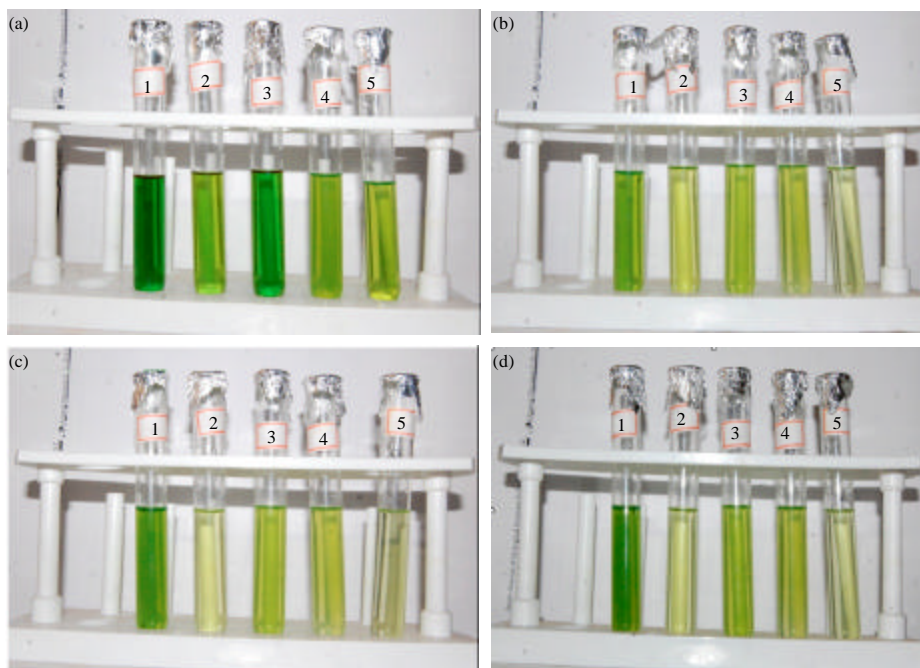


Fig. 3(a-d): Pigment recovered from algal cells using different pigment extraction techniques, (a) Mortar and pestle method, (b) Direct extraction method, (c) Heating method and (d) Preheated solvent method, 1: *Chlorella* species 2: *Acrochaete* species 3: *Phormidium chlorinum*, 4: *Jaaginema pseudogeminatum* 5. *Chroococcus* species

to the contact time of the rest of the methods. Under good extraction conditions, solid substances i.e., the cell substances should dissolve in respective solvent rapidly and totally in a short period. However, this is not always the case when dealing with cells having complex cell wall. Moreover without any pretreatment, cells with complex cell wall require long extraction period. Former studies showed that long duration of extraction procedure can increase the formation of degradation products like chlorophyllide a and chl a allomers and epimers (Furuya *et al.*, 1998; Cartaxana and Brotas, 2003). Although, we did not quantify degradation products, Furuya *et al.*, 1998 found that chlorophyll degradation products for a 5 h extraction using DMF were more than 2-fold greater than a 2 h extraction; thus, it is likely that 2 h extraction minimizes pigment degradation. In this regard, the present study showed that direct extraction procedure does not suit much for photosynthetic pigment extraction from algae.

Figure 3b shows the yield of pigment using heating method. In *Chlorella* sp. the yield of chlorophyll a was higher in heating method followed by mortar and pestle method. But the pigment yield was very low when compared to the mortar and pestle method. The pigment yield may be affected by increase in temperature of this extraction method. Figure 3c shows the overall yield of pigment using preheated solvent method. This type of extraction does not suit much for microalgae used in the present study. The pigment yield was very low in this extraction method when compared to the rest of the extraction methods. Schumann *et al.* (2005) reported a suitable pigment extraction method for the extraction of chlorophyll from green microalgae colonizing building facades. Similarly, many pigment extraction protocols for photosynthetic pigments were evaluated by many researchers (Henriques *et al.*, 2007). From the overall results (Fig. 3d), it was found that mechanical grinding method yielded maximum amount of pigment than the rest of the methods employed this study.

CONCLUSION

The present study states that the mechanical grinding proved to be efficient among the conventional pigment extraction methods and it will be used for the recovery of pigments from microalgae. Moreover, this pigment extraction method can be further used for exploitation of various photosynthetic pigments from microalgae.

ACKNOWLEDGMENT

The first author acknowledges Department of Science and Technology (DST), India for providing INSPIRE-JRF fellowship (DST/INSPIRE Fellowship/2011/IF110368).

REFERENCES

- Adesalu, T.A. and D.I. Nwankwo, 2008. Effect of water quality indices on phytoplankton of a sluggish tidal creek in Lagos, Nigeria. *Pak. J. Biol. Sci.*, 11: 836-844.
- Al-Kahtani, M.A., A.M. Youssef and A.A. Fathi, 2007. Ecological studies on Al-khadoud spring, Al-Hassa, Saudi Arabia. *Pak. J. Biol. Sci.*, 10: 4063-4068.
- Buffan-Dubau, E. and K.R. Carman, 2000. Extraction of benthic microalgal pigments for HPLC analyses. *Marine Ecol. Progress Ser.*, 204: 293-297.
- Cartaxana, P. and V. Brotas, 2003. Effects of extraction on HPLC quantification of major pigments from benthic microalgae. *Archiv Hydrobiologie*, 157: 339-349.
- Choudhari, S.M. and L. Ananthanarayan, 2007. Enzyme aided extraction of lycopene from tomato tissues. *Food Chem.*, 102: 77-81.
- Deniaud, E., J. Fleurence and M. Lahaye, 2003. Preparation and chemical characterization of cell wall fractions enriched in structural proteins from *Palmaria palmata* (Rhodophyta). *Botanica Marina*, 46: 366-377.
- Furuya, K., M. Hayashi and Y. Yabushita, 1998. HPLC determination of phytoplankton pigments using N, N-dimethylformamide. *J. Oceanogr.*, 54: 199-203.
- Hagerthey, S.E., J.W. Louda and P. Mongkronsri, 2006. Evaluation of pigment extraction methods and a recommended protocol for periphyton chlorophyll a determination and chemotaxonomic assessment. *J. Phycol.*, 42: 1125-1136.
- Henriques, M., A. Silva and J. Rocha, 2007. Extraction and Quantification of Pigments from a Marine Microalga: A Simple and Reproducible Method. In: *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, Mendez-Vilas, A. (Ed.) Formatex, Spain, pp: 586-593.
- Ilavarasi, A., D. Mubarakali, R. Praveenkumar, E. Baldev and N. Thajuddin, 2011. Optimization of various growth media to freshwater microalgae for biomass production. *Biotechnology*, 10: 540-545.
- Jeffrey, S.W. and G.M. Hallegraeff, 1987. Chlase distribution in ten classes of phytoplankton: A problem for CHL analysis. *Marine Ecol. Progress Ser.*, 35: 293-304.

- Jeffrey, S.W., R.F.C. Mantoura and S.W. Wright, 1997. Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods. UNESCO, Paris, Pages: 261.
- Karsten, U., J. Maier and F. Garcia-Pichel, 1998. Seasonality in UV-absorbing compounds of cyanobacterial mat communities from an intertidal mangrove flat. *Aquat. Microb. Ecol.*, 16: 37-44.
- Kim, D.H., J.H. Kim, S.E. Bae, J.H. Seo, T.K. Oh and C.H. Lee, 2005. Enhancement of natural pigment extraction using *Bacillus* species xylanase. *J. Agric. Food Chem.*, 53: 2541-2545.
- Lichtenthaler, H.K., 1987. Chlorophyll and carotenoids pigments of photosynthetic biomembranes. *Methods Enzymol.*, 148: 350-382.
- MacKinney, G., 1941. Absorption of light by chlorophyll solutions. *J. Biol. Chem.*, 104: 315-322.
- Millie, D.F., H.W. Paerl and J.P. Hurley, 1993. Microalgal pigment assessments using high-performance liquid chromatography: A synopsis of organismal and ecological applications. *Can. J. Fish. Aquat. Sci.*, 50: 2513-2527.
- Parsons, T.R., Y. Maita and M. Lalli, 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford, ISBN: 9780080302874, Pages: 173.
- Pasquet, V., J.R. Cherouvrier, F. Farhat, V. Thiery and J.M. Piot *et al.*, 2011. Study on the microalgal pigments extraction process: Performance of microwave assisted extraction. *Process Biochem.*, 46: 59-67.
- Porra, R.J., W.A. Thompson and P.E. Kriedemann, 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochem. Biophys. Acta*, 975: 384-394.
- Rippka, R., 1988. Isolation and purification of cyanobacteria. *Methods Enzymol.*, 167: 3-27.
- Schumann, R., N. Haubner, S. Klausch and U. Karsten, 2005. Chlorophyll extraction methods for the quantification of green microalgae colonizing building facades. *Int. Biodeterior. Biodegrad.*, 55: 213-222.
- Strickland, J.D.H. and T.R. Parsons, 1972. A practical handbook of seawater analysis. *Bull. Fish. Res. Bd., Canada*, 167: 311-311.
- Wright, S.W., S.W. Jeffrey and R.F.C. Mantoura, 1997. Evaluation of Methods and Solvents for Pigment Extraction. In: *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*, Jeffrey, S.W., R.F.C. Mantoura and S.W. Wright (Eds.). UNESCO Publishing, Paris, pp: 261-282.