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Effect of various solvents on chlorophyll and carotenoid extraction in green algae:*Chlamydomonas reinhardtii* and *Chlorella vulgaris*

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ABSTRACT

Content of plant pigments such as chlorophylls [chlorophyll a (Chl. a) and chlorophyll b (Chl. b)] and carotenoids (xanthophylls and carotenes) may vary depending on species or sometime within the species, but less efforts have been made with primitive plants such as algae. Moreover, estimation of pigments is one of the regular practices to estimate the biomass of particular plant or algae. In the present investigation, both Chlamydomonas reinhardtii (C. reinhardtii) and Chlorella vulgaris (C. vulgaris) belongs to class Chlorophyceae of green algae were selected to check their pigment contents using different solvents such as acetone, ethanol, methanol and diethyl ether (DEE). Prior to biomass estimation, both the cultures were grown in tris-acetate phosphate (TAP) medium under in vitro cultures and experiment was carried out with log phase cells. Results revealed that Chl. a and Chl. b were higher with ethanol and acetone extracts, respectively in C. reinhardtii. Moreover, Chl. a and Chl. b were higher with methanol and ethanol extracts in C. vulgaris. In addition, total chlorophyll content was high with ethanol extracts in both C. reinhardtii (25.8 µg/mL) and C. vulgaris (23.0 µg/mL) when compared to extracts of other three solvents. In contrast, total carotenoids were more with ethanol extract of C. reinhardtii (8.0 µg/mL) and acetone proved as best solvent for total carotenoids extraction in C. vulgaris (7.4 µg/mL). Depending on the solvent, variation in contents of chlorophylls and carotenoids were found in both the species. The data also indicated that contents of pigments and their composition were different in each alga though both belong to same family.

Keywords: Chlorophylls, carotenoids, acetone, ethanol, methanol, DEE

INTRODUCTION

Plant biomass is nothing but a form of energy which is located in different plant parts such as leaf, stem including root. In extent, research on enhancement of biomass production is a continuous process in plant science or agricultural sector to provide food, medicine, nutraceuticals etc., for the future generations (Khoo *et al.*, 2011 and Welfle *et al.*, 2014). Moreover, estimation of yield through biomass content is very common process and most of the researchers will use this method to get appropriated data all over the world. Evaluation of content of chlorophylls such as chlorophyll *a* and chlorophyll *b* in turn total chlorophylls and carotenoids namely xanthophylls and carotenes is one of the best ways to estimate the biomass of particular organism (Cha *et al.*, 2010). Though we have advanced facilities or instruments, but still facing minor error in the process of evaluation of plant pigments. Selection of solvent is also an important factor to know the amount of pigment in respective plant or algal cultures. Acetone is the most often used solvent to estimate the chlorophylls which is universally accepted, but each plant or alga has its own pigments in different composition (Alam *et al.*, 2018). Specifically algae, belong to primitive group of plants which classified again depending on habitat, size (macro or micro) and pigment composition including metabolites. Chlorophylls (Chl. *a* and Chl. *b*) are abundant in nature which gives green color to plants and have light harvesting role in the process of photosynthesis (Costache *et al.*, 2012). In addition these are also used as antioxidant and antimutagenic agents and helpful in pharamaceutical and cosmetic industries (Jayashree *et al.*, 2016). Carotenoids contain yellow, orange and red colored xanthophylls (x) and carotenes (c) which are distributed widely in green, red, blue-green, red and brown algae (Khoo *et al.*, 2011). Carotenoids also absorb the light in photosynthesis and protect the chlorophylls from photodamage (Osterrothova *et al.*, 2019). Similarly like chlorophylls, carotenoids also possess antioxidant capacity and prevent certain diseases. Moreover based on pigment and metabolite composition, algae are divided in to green, red and brown groups (Milledge *et al.*, 2014). But choosing the same group of algae is

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worthy to estimate pigments accurately using various solvents. In the present investigation, *Chlamydomonas reinhardtii* and *Chlorella vulgaris* were selected as experimental cultures to estimate the pigments. Both the species belongs to green algae and lives in fresh water conditions (Jayashree *et al.*, 2016). Most important similarity is both are unicellular organisms and compatible to estimate the pigments. With this background, experiments were carried out by choosing various solvents such as ethanol, methanol and diethyl ether (DEE) apart from regular acetone. All the four solvents have different flammable, volatile and toxic nature and extraction of each pigment in both the species is completely depends on the chemical nature of solvent. There were number of reports suggested that these solvents are helpful for extraction of algal pigments (Vimala and Poonghuzhali, 2014; Alam *et al.*, 2018). So, in the present work we choose two green algal members and initially cultured under *in vitro* conditions. Further extraction and estimation of chlorophylls and carotenoids from *in vitro* grown cultures were carried out using four different solvents to know their effect on pigment extraction.

MATERIALS AND METHODS

In the present experiments, two important green algal species i.e*. C. reinhardtii* and *C. vulgaris* were collected from Acharya Nagarjuna University and University of Madras, India. After collection they were stored as glycerol stocks in -80ºC and some of the cultures were maintained in agar plates for regular experiments. These plates were subcultured every two weeks to get fresh algal samples for inoculation. Further algal medium preparation, inoculation and maintenance of algal cultures including extraction and estimation of pigments are the various stages in the present work.

Prior to preparation of algal medium, glassware were washed properly using appropriate detergent solution. Further clean under running tap water and later with distilled water and finally dried using hot air oven. Tris-acetate phosphate (TAP) medium was used for initial algal growth and development. All the experiments were carried out under *in vitro* conditions within the laboratory. The pH of the algal medium i.e. TAP was adjusted to 7.0 using 0.1 N HCl or 0.1 N NaOH solutions. Generally 250 ml conical flasks (with 50 ml TAP) were used for algal growth, but for certain period algal culture initiation was also carried out using 30 ml serum vials (with 5.0 ml TAP). All the media prepared were autoclaved at 121 ºC and 15 lbs/in^2 for 20 min. At least ¾ empty space is must in the flask or vial for better algal growth. Normally growth of each alga will take 2-3 days depends on the species in TAP medium, so it is must to keep plenty of gaseous phase. After the completion of sterilization, inoculation was performed in laminar air flow (LAF) chamber. Before to inoculation, all the requirements such as sterilized inoculation needles, small wood sticks, algal medium, algal samples etc., were transferred to inside of the LAF chamber. Inoculation was carried out using sterilized loops and small wood sticks and all the cultures were incubated in an orbital shaker with 120 rpm at 25ºC. Inoculated algal species were grown under normal light condition i.e. at a photo flux density of 40-50 μEm^2S^{-1} of white fluorescent tubes. Extraction and estimation of algal pigments i.e. chlorophylls and carotenoids were carried out using Lichtenthaler (1987) and Arnon's (1949) methods. After reaching the log phase both the algal cultures were removed and used for pigment estimation. Statistical work i.e. one-way ANOVA Tukey multiple analysis was carried out in the present work to know the significant differences between the obtained data.

RESULTS AND DISCUSSION

C**hlorophyll and carotenoid contents in C. reinhardtii extracts**

Among all the *C. reinhardtii* extracts, high content of Chl. *a* was found in ethanol extract when compared to diethyl ether or methanol or acetone extracts (Table-1). Though ethanol is flammable, it is safer solvent than methanol or diethyl ether and less toxic for pigment (Amin *et al.*, 2018). In fact Saini and Keum (2018) emphasized that both ethanol and acetone are safer solvents for chlorophyll extraction than diethyl ether and methanol. Moreover in spectrophotometric analysis, polystyrene plastic cuvettes may be helpful with ethanol extract extraction. The extracts of diethyl ether is also contain considerable amount of Chl. *a* and did not shown significant difference with ethanol extract (Table 1). Recently Etemadian *et al.* (2017) also

proved that diethyl ether is one of the best extractant for chlorophylls in two algal species namely *Polycladia myrica* and *Colpomenia sinuosa*.Though acetone is a regular solvent for chlorophyll estimation, surprisingly acetone extract of *C. reinhardtii* exhibits less Chl. *a* content in this species. In contrast, content of Chl. *b* was more in acetone extract when compared to other three solvent extracts of C. *reinhardtii* (Table1). In conclusion, with respect to Chl. *b*, there were no significant difference was observed with ethanol or DEE extracts when compared to content from acetone extract. Similarly Vimala and Poonghuzhali (2015) observed that acetone is best solvent for Chl. *b* isolation in *Ulva reticulata*, *Sargassum ilicifolium* and *Hydroclathrus clathratus.*

Table1: Quantification of Chlorophyll *a* and Chlorophyll *b* (µg/ ml) in various chemical solvents in *C. reinhardtii*(numbers in the brackets indicates CD values)

C. reinhardtii	Acetone	Ethanol	DEE	Methanol
Chlorophyll *a*	13.7±0.38 (3.0)	18.0±0.17 (3.0)	16.8±1.00 NS	16.3±1.60 NS
Chlorophyll *b*	9.0±0.73 (2.4)	7.7±0.73 NS	7.1±1.15 NS	2.7±1.10 (2.4)

Estimation of total chlorophyll revealed that ethanol extract contain more total chlorophyll (25.8 µg/mL) when compared to other three solvent extracts (Fig. 1). Hosikian *et al.* (2010) emphasized the role of ethanol in chlorophyll isolation and its advantages in various microalgal species. Similarly total carotenoids were also high in ethanol extract (8.0 µg/mL) when compared to acetone or methanol or DEE solvent extracts (Fig. 2). Overall ethanol proved as best solvent for both total chlorophyll and carotenoid extraction in *C. reinhardtii*. Poojari *et al.* (2016) also highlighted the ethanol importance in carotenoid isolation and estimations specifically for algal species.

Chlorophyll and carotenoid contents in C. vulgaris extracts

Methanol extract of *C. vulgaris* resulted high amount of Chl. *a* when compared to all other three solvent extracts (Table2). In consistent with present results, Molnar *et al.* (2013) reported that methanol solvent is best for Chl. *a* extraction from *Chlorella vulgaris*. Less amount of Chl. *a* was noticed with diethyl ether extract which showed significant difference with methanol extract. In contrast, Chl. *b* was found more in ethanol extract, which is notable observation in *C. vulgaris* (Table2).

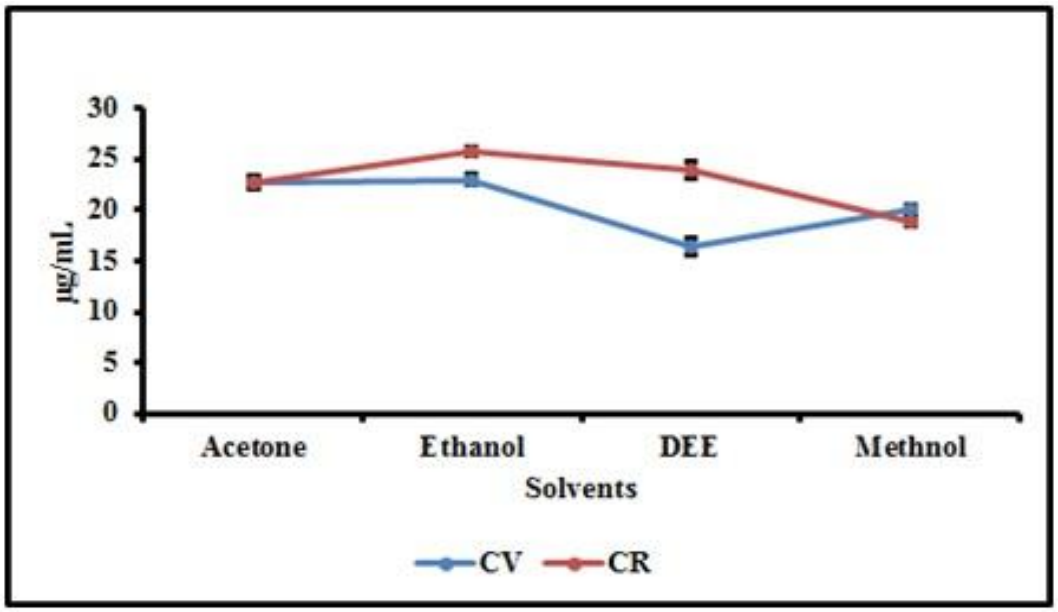

Figure1: Total chlorophyll content of both *C. vulgaris* and *C. reinhardtii* with different solvents

Here again methanol extract resulted less Chl. *b* as observed in *C. reinhardtii*. Saini and Keum (2018) and Hosikian *et al.* (2010) were also reported that ethanol is best solvent for chlorophyll extraction in algal species.

Table2: Quantification of Chlorophyll *a* and Chlorophyll *b* (µg/ ml) in various chemical solvents in *C. vulgaris*(numbers in the brackets indicates CD values)

C. vulgaris	Acetone	Ethanol	DEE	Methanol
Chlorophyll *a*	14.9±0.52 NS	13.7±0.33 (2.2)	10.2±0.77 (2.2)	16.6±0.89 (2.2)
Chlorophyll *b*	8.1±1.39 NS	9.2±1.53 (4.5)	6.2±0.70 NS	3.7±1.40 (4.5)

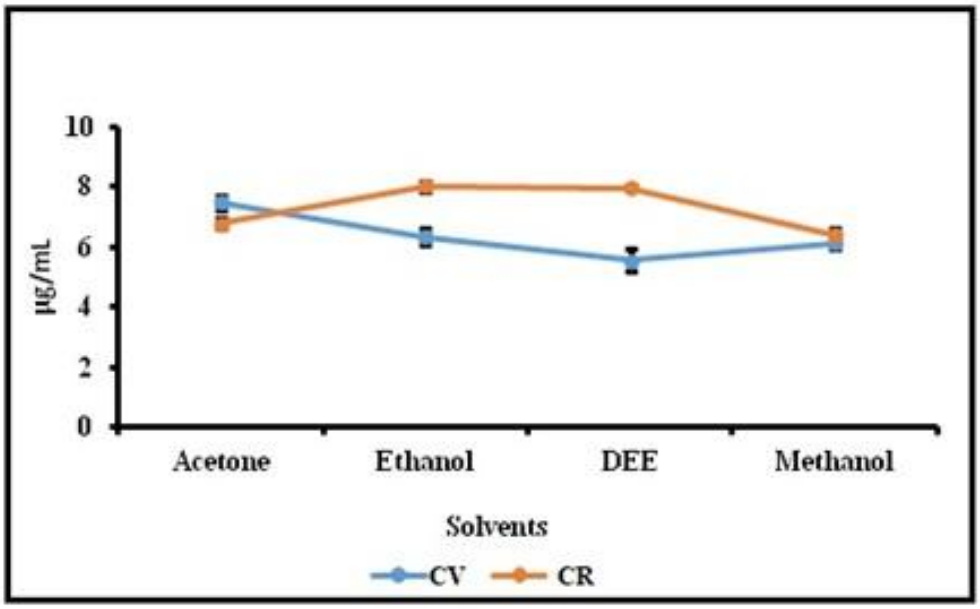

Figure2: Total carotenoids content (x + c) of both *C. vulgaris* and *C. reinhardtii* with different solvents

Overall total chlorophyll was high (23.0 µg/mL) in ethanol extract in this species (Fig. 1). In addition total carotenoids are more (7.4 µg/mL) in acetone extract when compared to other solvent extracts in *C. vulgaris* (Fig. 2). It is a well-known fact that acetone is a regular solvent for pigment extraction. Sumanta *et al.*(2014) proved that acetone is best solvent for carotenoid isolation also in *Adiantum* species. Similarly Grosso *et al.* (2015) found that acetone is universal solvent for carotenoid extraction specifically with marine algal samples. Table 3 explains the order of solvents which are best for pigment extraction in both the species.

Table3: Order of chlorophyll and carotenoid contents in various solvent extracts

Content	*C. reinhardtii*	*C. vulgaris*
Chlorophyll *a*	Ethanol > DEE > Methanol > Acetone	Methanol > Acetone > Ethanol > DEE
Chlorophyll *b*	Acetone > Ethanol > DEE > Methanol	Ethanol > Acetone > DEE > Methanol
Total Chlorophyll	Ethanol > DEE > Acetone > Methanol	Ethanol > Acetone > Methanol > DEE
Carotenoids	Ethanol > DEE> Acetone > Methanol	Acetone > Ethanol > Methanol > DEE

In the present investigation, total chlorophylls were found relatively higher with ethanol extracts in both the species. In contrast, total carotenoids were higher with ethanol extracts in *C. reinhardtii* and the same were more with acetone extracts in *C. vulgaris*. The pigment composition vary depend on the algal species and each solvent has its own capacity extract the pigments.

REFERENCES

Alam, T., Najam, L. and Al Harrasi, A. (2018) Extraction of natural pigments from marine algae. *Journal of Agricultural and Marine Sciences***23**: 81-91.

Amin, A., Chetpattananondh, P., Khan, M.N., Mushtaq, F. and Sami, S.K. (2018) Extraction and quantification of chlorophyll from microalgae *Chlorella* sp. *Materials Science and Engineering* **414**: 1-7.

Arnon, D. (1949) Estimation of total chlorophyll. *Plant Physiology***24**: 1-15.

Cha, K.H., Lee, H.J., Koo, S.Y., Song, D.G., Lee, D.U. and Pan, C.H. (2010) Optimization of pressurized liquid extraction of carotenoids and chlorophylls from *Chlorella vulgaris*. *Journal of Agricultural and Food Chemistry* **58**: 793-797.

Costache, M.A., Campeanu, G. and Neata, G. (2012)Studies concerning the extraction of chlorophyll and total carotenoids from vegetables.*Romanian Biotechnological Letters***17**(5): 7702-7708.

Etemadian, Y., Shabanpour, B., Ghaemi, V. and Kordjazi, M. (2017) Compare the chlorophyll amount in three brown algae species of the persian gulf by using three solvents and applying two formulas. *International Journal of Biochemistry, Biophysics and Molecular Biology***2**(6): 77-79.

Grosso, C., Valentao, P., Ferreres, F. and Andrade, P.B. (2015) Alternative and efficient extraction methods for marine-derived compounds.*Marine Drugs***13**: 3182-3230.

Hosikian, A., Lim, S., Halim, R. and Danquah, M.K. (2010) Chlorophyll extraction from microalgae: A review on the process engineering aspects.*International Journal of Chemical Engineering* **2010**: 1-11.

Jayshree, A., Jayshree, S. and Thangaraju, N. (2016) *Chlorella vulgaris* and *Chlamydomonas reinhardtii*: Effective antioxidant, antibacterial and anticancer mediators.*Indian Journal of Pharmaceutical Sciences***78**(5): 575-581.

Khoo, H.E., Prasad, K.N., Kong, K.W., Jiang, Y. and Ismail, A. (2011) Carotenoids and their isomers: color pigments in fruits and vegetables. *Molecules***16**(2): 1710-1738.

Lichtenthaler, H.K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. *Methods in Enzymology***148**: 350-382.

Milledge, J., Smith, B., Dyer, P. and Harvey, P. (2014) Macroalgae-derived biofuel: a review of methods of energy extraction from seaweed biomass. *Energies* **7**(11): 7194-7222.

Molnár, É., Rippel-Pethő, D. and Bocsi, R. (2013) Solid-liquid extraction of chlorophyll from microalgae from photoautotroph open-air cultivation. *Hungarian Journal of Industry and Chemistry***41**(2): 119-122.

Osterrothova, K., Culka, A., Nemeckova, K., Kaftan, D., Nedbalova, L., Prochazkova, L. and Jehlicka, J. (2019) Analyzing carotenoids of snow algae by Raman microspectroscopy and high-performance liquid chromatography.*Spectrochimica Acta A: Molecular and Biomolecular Spectroscopy***5**(212): 262-271.

Poojary, M.M., Barba, F.J., Aliakbarian, B., Donsì, F., Pataro, G., Dias, D.A. and Juliano, P. (2016) Innovative alternative technologies to extract carotenoids from microalgae and seaweeds.*Marine Drugs***14**(214): 1-34.

Saini, R.K. and Keum, Y-S. (2018) Carotenoid extraction methods: A review of recent developments. *Food Chemistry* **204**: 90-103.

Sumanta, N., Haque, C.I., Nishika, J. and Suprakash, R. (2014) Spectrophotometric analysis of chlorophylls and carotenoids from commonly grown fern species by using various extracting solvents.*Research Journal of Chemical Sciences***4**(9): 63-69.

Vimala, T. and Poonghuzhali, T.V. (2015) Estimation of pigments from seaweeds by using acetone and DMSO.*International Journal of Science and Research***4**: 1850-1854.

Welfle, A., Gilbert, P. and Thornley, P. (2014) Increasing biomass resource availability through supply chain analysis.*Biomass and Bioenergy***70**: 249-266.