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Estimation of Pigments from Seaweeds by Using Acetone and DMSO

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Abstract: ***Seaweeds are macroalgae inhabiting marine water with 50% major productivity using pigments. These pigments are important both in classification and biodiversity studies. Solvents play a major role in the process of extracting the pigments. In our present study, the pigments of the algal species were determined using acetone and DMSO for extracting various pigments and were examined comparatively. Acetone is an efficient solvent for pigment extraction but the study showed that DMSO was an equally good solvent for extraction of pigments especially in case of brown algae. However, acetone extraction of pigments was more efficient in green and red algae than DMSO extraction. The better of the two solvents used in this study, appears to be DMSO, because it does not require maceration, centrifugation or filtration.***

Keywords: Acetone, DMSO, pigments, chlorophyta, phaeophyta, rhodophyta.

1. Introduction

Seaweeds are the most abundant photosynthetic species that contain different groups of light harvesting and photo-protective pigments. The pigments are important in classification and biodiversity studies and hence impart their value as reserve food and other valuable products. There are three groups of photosynthetic pigments in macroalgae. They are chlorophylls, carotenoids and phycobiliproteins (Rowan 1989). Based on pigments the macroalgae are classified into three major groups viz., Green algae (Chlorophyta), Brown algae (Phaeophyta) and Red algae (Rhodophyta).

The lipid-soluble Chlorophyll *a* is found in all photosynthetic macroalgae, while chlorophyll *b* is found in Chlorophytes, and the chlorophylls *c1* and *c2* are found in Phaeophytes (Rowan 1989). Fucoxanthin absorbs blue-green light between 400-560 nm in the spectrum (Rowan 1989). Carotenoids are separated into carotenes and xanthophylls (Rowan 1989). The xanthophylls -fucoxanthin, violaxanthin, antheraxanthin and zeaxanthin are found in the Phaeophytes, zeaxanthin is also found in the Rhodophytes, while lutein, neoxanthin, violaxanthin and zeaxanthin are found in the Chlorophytes (Rowan 1989). The carotene β, β-carotene is found in Rhodophytes (and to a certain extent in Phaeophytes and Chlorophytes) (Rowan 1989). Phycobiliproteins are water-soluble pigment-proteins and is characteristic of red macroalgae (Rowan 1989; Zhao *et al.* 2011). They absorb efficiently in the green to red part of the light spectrum (500-650 nm *in vitro*) (Rowan 1989). There are three major phycobiliprotein groups; allophycocyanins, phycocyanins, phycoerythrins (Rowan 1989; Jeffrey *et al.* 2005; Zhao *et al.* 2011).

Recently, the importance of diverse bioactive compounds found in seaweeds is growing rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities (Barrow & Shahidi, 2008; Wijesekara, Yoon, & Kim, 2010). Among functional ingredients identified from marine algae, natural pigments have received particular attention as they have been found to exhibit various beneficial biological activities such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective activities. Therefore, various natural pigments isolated from marine algae have attracted much attention in the fields of food, cosmetic and pharmacology (Pangestuti and Kim, 2011). From this perspective, investigations on carotenoids and chlorophylls are an important part of studies focused on economic applications and in research more particularly directed to ecological issues. However, these studies require the extraction and analysis of pigments, which are quite unstable not only at high temperatures, but also when exposed to light and to oxygen (Borsarelli; Mercadante, 2010), an obstacle to the development of reliable, reproducible analysis protocols. In our present study, the pigments of the algal species were determined using acetone and DMSO for extracting various pigments and were examined comparatively.

2. Materials and Methods

2.1 Sample Collection

Live and healthy specimens were collected along the Rameshwaram coast of Tamilnadu during low tides in the month of April, 2015. The seaweeds were then washed thoroughly with seawater to remove extraneous materials and brought to the laboratory in plastic bags containing water to prevent evaporation.

2.2 Sample Preparation

The samples were gently rinsed with fresh water to remove salt, sand and epiphytes, epizoones, animal castings, calcareous and other adhering detritus matters. The samples were identified following the standard books. The seaweeds collected were identified as Chlorophyceae- *Ulva reticulata* Forsskål; Phaeophyceae- *Sargassum ilicifolium* (Turner) C.Agardh 1820, *Sargassum polycystum* C.Agardh , *Turbinaria conoides* (J.Agardh) Kützing 1860 , *Hydroclathrus clathratus* (C.Agardh) M.Howe ;

Rhodophyceae- *Amphiroa sp.* J.V. Lamouroux, 1812, *Gracilaria salicornia* (C. Agardh) Dawson 1954, *Champia parvula* (C.Agardh) Harvey, *Portieria hornemannii* (Lyngbye) P.C.Silva. The seaweeds were then cut into small pieces, weighed and subjected to pigment analysis.

2.3 Extraction Using 80% Acetone (Arnon1949)

500 mg of seaweed was kept in a pestle and mortar with 10 ml of 80% acetone and it was ground well and the homogenate was centrifuged at 3000 rpm for 15 minutes and the supernatant was stored. The pellet was re-extracted by repeated washing with 5 ml of 80 % acetone till it became colourless. All the extracts were pooled and utilized for pigment quantification. The process was followed for all the samples.

2.4 Extraction of pigments using DMSO (J. D. Hiscox & G. F. Israelstam, 1979)

500 mg of dry sample was taken, cut into small pieces and suspended in test tubes containing 2 mL of dimethyl sulphoxide (DMSO). Test tubes were incubated at 60° C for 20 min in a water bath. The supernatant was decanted and another 3 mL of DMSO was added to the residue and incubated at 60° C for 20 min. The supernatants were pooled and the volume was made up to 10 mL by adding DMSO. The chlorophyll extract was transferred to a cuvette and the absorbance was read in a Spectrophotometer at 645 and 663 nm against DMSO blank Chlorophyll *a*, *b*, total chlorophyll and chlorophyll *a/b* ratio were calculated.

2.5 Estimation of Chlorophyll

The amount of chlorophyll present in the algae was estimated by the method of Arnon (1949). Absorbance was measured at 645 nm and 663 nm in a spectrophotometer. The chlorophyll content was determined by using the following formula
Arnon's (1949) equations:
Chlorophyll *a* (µg/ml) = 12.7 (A_{663}) - 2.69 (A_{645})
Chlorophyll *b* (µg/ml) = 22.9 (A_{645}) - 4.68 (A_{663})
Total chlorophyll (µg/ml) = 20.2 (A_{645}) + 8.02 (A_{663})

Where, A = Absorbance at respective wave length = Volume of extract (ml), W = Fresh weight of the sample (g).

2.6 Estimation of Carotenoid

The amount of Carotenoid was estimated by the method of Kirk and Allen, 1965.The same chlorophyll extract was measured at 480 nm in spectrophotometer to estimate the carotenoid content.

Carotenoids (µg/g.fr.wt.) = A_{480} + (0.114 × A_{663}) — (0.638 × A_{645})
Where, A = Absorbance at respective wave length

2.7 Estimation of Fucoxanthin

The amount of fucoxanthin was estimated by the method of Seely et al, 1972. The same chlorophyll extract was measured in spectrophotometer to estimate the fucoxanthin content.
Fucoxanthin (mg g-1) = [A_{470} −1.239 (A_{631}+A_{581}−0.3 ×A_{664}) − 0.0275(A_{664})] /141
Where, A = Absorbance at particular wavelength
V = Total volume of the pigment extract
W = Weight of the sample used for extraction

3. Results and Discussion

The major photosynthetic pigments, total chlorophyll and carotenoid content were estimated from fresh seaweeds. The chl *a* content (Table.1) extracted using 80% acetone ranged from 4.29 to 21.99 µg/ml with minimum in the red seaweed *Amphiroa sp.* and maximum in the green seaweed *U.reticulata*, whereas , chl *a* content extracted using DMSO ranged from 0.96 to 21.44 µg/ml minimum in the red seaweed *Amphiroa sp* and maximum in the brown algae *S.ilicifolium.*

Table 1: Chl *a* (µg/ml) content extracted using 80% Acetone and DMSO

Sample	80% Acetone	DMSO
U.reticulata	21.99±4.01	3.17±1.03
H. clathratus	12.52±3.02	18.12±2.03
S.ilicifolium	19.38±3.03	21.44±3.01
S.polycystum	9.93±2.03	17.20±2.02
Turbinaria sp	5.61±1.02	20.94±3.01
Amphiroa sp	4.29±1.02	0.96±0.82
G.salicornia	7.03±2.02	1.60±0.38
C.parvula	6.94±0.78	5.29±1.02
P. hornemonii	12.66±3.05	4.66±0.56

± Standard Error

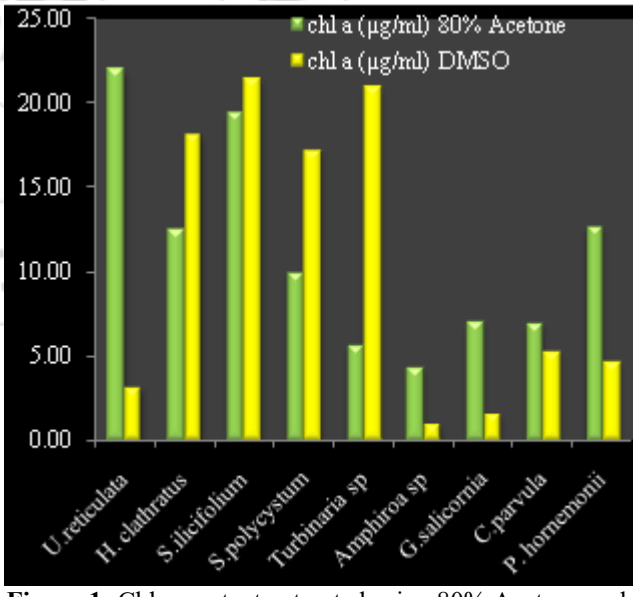

Figure 1: Chl *a* content extracted using 80% Acetone and DMSO

The chl *b* content (Table.2) extracted using 80% acetone ranged from 0.55 to 19.75 µg/ml with minimum in the red seaweed *Amphiroa sp.* and maximum in the brown seaweed *H. clathratus*, whereas, chl *b* content extracted using DMSO ranged from 2.25 to 12.40 µg/ml with minimum in

Amphiroa sp and maximum in the brown algae *Turbinaria sp*.

Table 2: Chl *b* (µg/ml) content extracted using 80% Acetone and DMSO

Sample	80% Acetone	DMSO
U.reticulata	18.01±3.02	3.76±2.03
H. clathratus	19.75±3.01	7.46±3.01
S.ilicifolium	13.63±2.03	3.86±1.02
S.polycystum	6.48±1.03	5.18±1.02
Turbinaria sp	3.82±1.04	12.40±2.03
Amphiroa sp	0.55±0.62	2.25±0.52
G.salicornia	4.09±1.12	2.96±1.02
C.parvula	1.54±0.44	3.31±0.22
P. hornemonii	2.94±0.32	3.08±0.73

± Standard Error

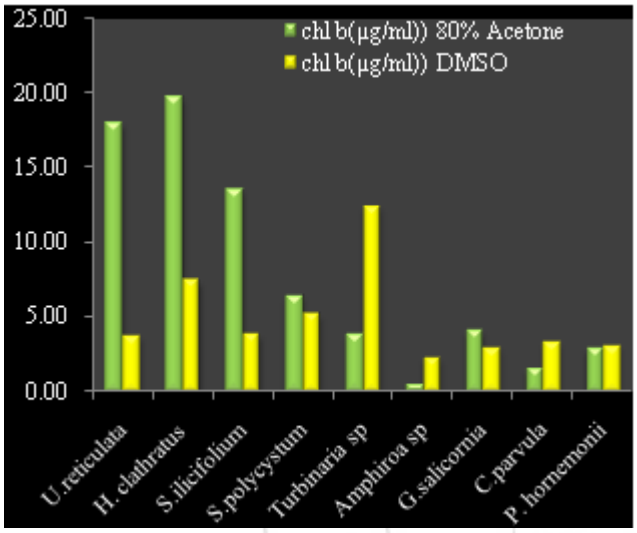

Figure 2: Chl *b* content extracted using 80% Acetone and DMSO

Total chlorophyll content (Table.3) extracted using 80% acetone ranged from 4.83 to 39.99µg/ml with minimum in *Amphiroa sp* and maximum in *U.reticulata* whereas, total chlorophyll extracted using DMSO ranged from 3.21 to 33.32 µg/ml with minimum in *Amphiroa sp* and maximum in *Turbinaria sp.*

N. Kumar J.I. *et.al.*, (2009) reported that Chlorophyll (Chl *a, b* and total) content extracted using acetone shot up in species of Chlorophyta followed by Phaeophyta and Rhodophyta. Carotenoid content was recorded greater in the members of Phaeophyta than Rhodophyta and Chlorophyta. The present study shows similar findings.

Table 3: Total Chlorophyll (µg/ml) content extracted using 80% Acetone and DMSO

Sample	80% Acetone	DMSO
U.reticulata	39.99±3.02	6.93±1.02
H. clathratus	32.25±2.03	25.56±3.03
S.ilicifolium	33.00±3.01	25.30±2.02
S.polycystum	16.41±2.02	22.38±4.03
Turbinaria sp	9.44±1.04	33.32±3.03
Amphiroa sp	4.83±0.94	3.21±0.63
G.salicornia	11.12±1.03	4.56±0.62
C.parvula	8.48±1.22	8.59±1.11
P. hornemonii	15.59±3.02	7.74±2.02

± Standard Error

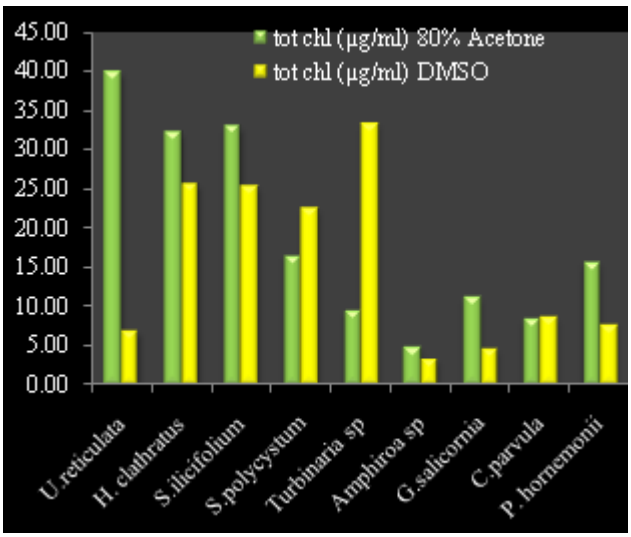

Figure 3: Total Chlorophyll content extracted using 80% Acetone and DMSO

Carotenoid content (Table.4) extracted using 80% acetone ranged from 0.13 µg/g (*Amphiroa sp*) to 1.71µg/g (*S.polycystum*). However, DMSO extraction showed a range between 0.02 µg/g (*Amphiroa sp*) and 1.75µg/g (*S.ilicifolium).* Similar findings have been reported by N. Kumar J.I. *et.al.*, (2009) . According to them the highest carotenoids were present in *S. Polycystum* belonging to the Phaeophyta group while the lowest carotenoid content was observed in Chlorophyta group

Table 4: Carotenoid (µg/g) content extracted using 80% Acetone and DMSO

Sample	**80% Acetone**	**DMSO**
U.reticulata	1.38±0.54	0.14±1.02
H. clathratus	1.35±1.02	0.93±0.33
S.ilicifolium	1.18±1.03	1.75±1.04
S.polycystum	1.71±1.01	0.93±0.52
Turbinaria sp	1.69±1.03	1.55±1.01
Amphiroa sp	0.13±0.71	0.02±0.02
G.salicornia	0.38±0.42	0.09±0.03
C.parvula	0.35±0.02	0.09±0.34
P. hornemonii	0.35±0.72	0.11±0.13

± Standard Error

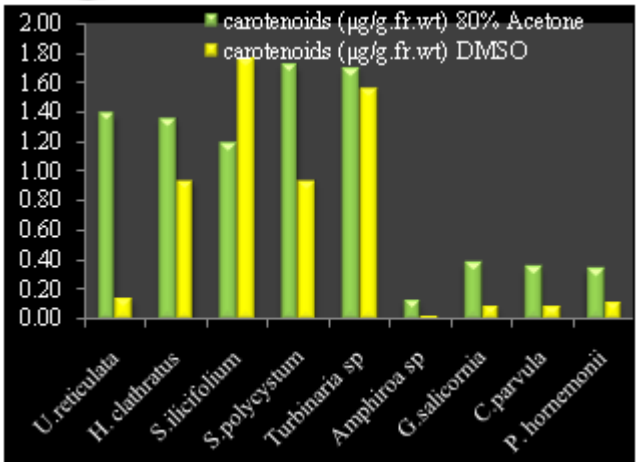

Figure 4: Carotenoid content extracted using 80% Acetone and DMSO

Amphiroa sp also showed a minimum quantity of Fucoxanthin extracted by acetone (0.05 µg/g) and DMSO (0.41 µg/g) whereas *S.ilicifolium* showed maximum fucoxanthin content extracted both by acetone (13.8 µg/g) and DMSO (12.90 µg/g) respectively (Table.5).

Table 5: Fucoxanthin (µg/g) content extracted using 80% Acetone and DMSO

	80% Acetone	DMSO
H. clathratus	6.81±1.01	5.87±1.02
S.ilicifolium	13.8±1.74	12.90±1.02
S.polycystum	7.62±1.02	7.10±1.62
Turbinaria sp	5.16±1.03	8.44±1.32
Amphiroa sp	0.98±0.13	0.54±0.42
G.salicornia	0.05±0.61	0.41±0.32
C.parvula	2.25±0.63	0.71±0.02
P. hornemonii	1.73±1.02	0.98±0.34

± Standard Error

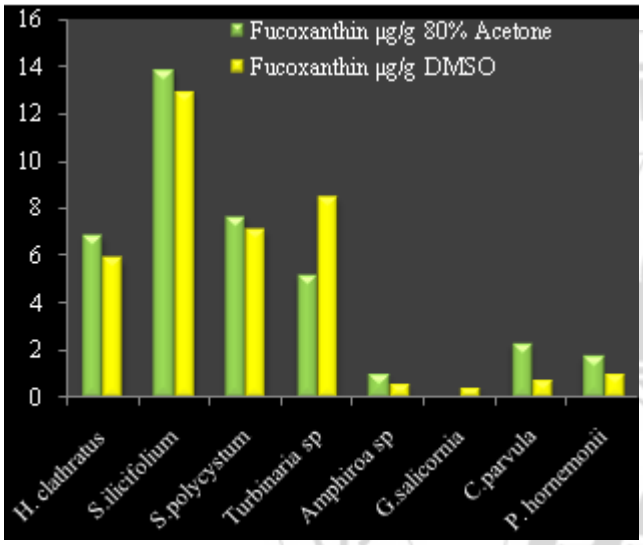

Figure 5: Fucoxanthin content extracted using 80% Acetone and DMSO

Analysis of photosynthetic pigments depends on the appropriate extraction process (Leeuwe *et.al.*, 2006). Even though acetone and methanol are widely used solvents for extraction, DMSO is also used for pigment extraction analysis. Acetone is an efficient solvent for pigment extraction but the study showed that DMSO was an equally good solvent for extraction of pigments especially in case of brown algae. However, acetone extraction of pigments was more efficient in green and red algae than DMSO extraction.

Variation in pigment concentrations are significantly evident for different solvents for every studied species, which can be explained by difference in solubility of pigments (or affinity of bio-molecules) towards different chemical solvents.

Several studies have described methodologies to analyze these pigments. These investigative efforts are based on the evaluation of parameters such as solvents, number of extraction steps and required biomass, all of which are aspects that vary across different organisms. Solvents play a major role in the process of extracting the pigments. Methanol and ethanol are often more efficient extractants and are much easier to transport, and also easier to handle in the field. Unfortunately, the Chl red peaks are generally broader and lower in methanol and ethanol. The peaks for Chl *b*, Chl *c*1+*c*2, and Chl *c*2 and Chl *d* are not only lower and broader in methanol and ethanol, the widened peak of Chl *a* in these solvents tends to interfere more strongly with the absorbance of the other Chlorophylls (Ritchie 2006).

Acetone solvent gives very sharp Chlorophyll absorption peaks but acetone is sometimes a poor extractant of Chlorophyll from some algae, particularly green algae. Acetone is known to have a lower extractability of chlorophylls from the protein matrix (Nakamura and Watanabe, 2001). Acetone, on the other hand, provides a stable environment. Porra *et al.* (1991) and Wright *et al.* (1997) have discussed the merits of dimethyl sulphoxide (DMSO) used for chlorophyll extraction and assay, and reported as efficient when pigment concentrations are low.

Conclusion

Results from this experiment clearly indicate that extraction of photosynthetic pigments by different solvents depends on chemical nature of bio-molecules (cholorophyll-a, chlorophyll-b, carotenoids and fucoxanthin). Investigation revealed DMSO as best extracting solvent of chlorophyll-*a* and *b* for brown algae. The better of the two solvents used in this study, appears to be DMSO, because it does not require maceration, centrifugation or filtration. DMSO is solid at temperatures below 18 ºC and re-crystallizes slowly, but is good for dealing with delicate tissues, such as those found in seaweeds. Care should be taken when it is heated during extraction. The choice of solvent for extracting pigments from algae samples must take several factors into account: toxicity, cost, the number of extractions and efficiency.

Though slight variations persists among the experimented species even for same extractant solvent which can be attribute to inherent physiological characteristics of individual species. Temporal and seasonal changes and local geological condition can also be the reason for variations in pigment concentrations in seaweeds, therefore further study in this context is recommended.

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