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Photosynthetic pigments and fatty acid composition of four marine green algae from the coastal zones of Goa

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ABSTRACT

Pigments and fatty acid composition of four algal species, *Caulerpa sertulariodies, Chaetomorpha media, Enteromorpha intestinalis* and *Ulva fasciata* belonging to the Chlorophyceae, isolated from marine ecosystems were investigated for their pigments and fatty acid composition. Results revealed a pigment pattern similar to that of higher plants. Chlorophyll a, chlorophyll b, chlorophyllide-B, neoxanthin, violaxanthin and lutein were detected in all the four alga studied, while phaeophytin B and phaeophorbide B were detected only in *Enteromorpha*. Loroxanthin, a carotenoid which has been reported from various species of *Chaetomorpha* and *Ulva* was not detected in the present study. Antheraxanthin was also not found in *Ulva* but present in all other algae studied. Fatty acid profile showed the presence of saturated fatty acids such as lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) and unsaturated fatty acids such as oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) in all investigated species. The Chlorophyta comprise the most modern group and this is supported primarily by occurrence of C18 fatty acids typical of the vegetative tissue of higher plants. Presence of large number of pigment and fatty acid may have possible value for nutritional purpose.

Introduction

Algae are a group of aquatic photosynthetic, eukaryotic organisms ranging from unicellular to multicellular forms and having distinctive characters of chlorophyll bearing organisms, with a thallus like plant body. All algae primarily contain pigments, proteins, carbohydrates, fats and nucleic acids. The amount varies with the type of algae (Boyd 2003).

Pigments present are characteristic of certain algal groups. Each phylum has its own particular combination of pigments and characteristic color. Three major classes of pigments occur among algae: chlorophylls, carotenoids and phycobilins. Chlorophyta is a group of green pigmented algae. Chlorophyll a and b are present giving the green algae their typical grass green coloration. In addition, both α and β-carotenes, lutein and zeaxanthin are present as in higher plants. Siphonoxanthin, a special xanthophyll, is also present and is characteristic of coenocytic (siphonaceous) green algae. Phycobilin is not present in green algae. The majority of green algae are small, unicellular, or filamentous. The primary reserve food is starch; oil may also be found as a reserve food and is more common in older and resting cells (Boyd 2003).

Lipids are esters of fatty acids and alcohols that comprise a large group of structurally distinct organic compounds including fats, waxes, phospholipids, glycolipids etc. Lipids are the most effective source of storage energy and function as insulators of delicate internal organs and hormones and play an important role as the structural constituents of most of the cellular membranes. The acyl lipids owe their

characteristic properties to the fact that their molecules are composed of long chains of carbon atoms; these properties may be modified by the presence of a small number of more reactive and polar groups in the molecule. The hydrocarbon chains are provided by the higher saturated and unsaturated monocarboxylic aliphatic acids, and some similar substituted acids, all of which are termed fatty acids. These acids are chemically combined, usually as esters, but occasionally as amides or ethers, to form complex lipids which may also contain alcohols (especially glycerol), bases, phosphate esters, sugars or sterols. There are different algal types that comprise up to 40% of their overall mass of fatty acids as this fatty acids can be extracted for various uses. Algal lipids usually contain ordinary fatty acids of the major and minor classes, and are sometimes a particularly good source of minor polyunsaturated acids (Fleurance 1994). Algal lipids and pigments are commercially very valuable. Chlorophyta generally contain a small quantity of fatty oils with myristic, palmitic, lauric, stearic acid and a number of unsaturated fatty acid.

Algae are considered to be an important nutrient source of diet and food additives because of their high content of essential and free amino acids (Heiba *et al.*, 1993; Naidu *et al.*, 1993). The greatest use of marine algae worldwide is for food. All the food algae are rich in non-digestible fibres, mineral salts, vitamins and proteins, but low in fat content (Fleurance and Kass 1995). Chlorophyta constitute a vast potential resource in varied applications such as mariculture, food, feed, fuel, fertilizer, medicine, industry and in combating pollution. A green microalga, *Dunaliella* provides the basics for the industrial production of trans cis-β-carotene, which also acts as a free radical scavenger and a potential food additive for enhancing the color (Singh *et al.*, 2005). The freshwater microalga, *Haematococcus pluvialis* is one of the best sources of astaxanthin, a pigment with anticancer and immuno-modulation effects that is also used as natural colorant in aquaculture and food industry (Boussiba 2000). The biomass of the unicellular green alga, *Chlorella* has been commercially produced and consumed as a dietary supplement, due to its relatively high levels of proteins, essential amino acids, chlorophylls, ω-3 and ω-6 polyunsaturated fatty acids (PUFA) (Ramazanov and Ramazanov 2006). *Ulva* and *Caulerpa* species are used widely in medicines (Ravikumar *et al.*, 2010). There is currently considerable interest in pigments, lipids and fatty acid profile of marine algae. Thus this work was carried out to characterize the pigments and lipids present in four selected algae belonging to chlorophyceae from coastal zones of Goa.

Materials and methods

Materials

The materials chosen for study were green macroalgae belonging to class Chlorophyceae isolated from marine ecosystem of the Goa coast. The four algae namely; *Caulerpa sertulariodies, Chaetomorpha media, Ulva fasciata* and *Enteromorpha intestinalis* were randomly selected for their easy accessibility.

Extraction of photosynthetic pigments

Algae were collected from marine environment and were washed using distilled water. 0.1g of algal tissue were extracted in 1ml of 80% (v/v) acetone in a glass homogenizer at 4°C under dim light, followed by centrifugation at 6000 *g* for 10 min at 4°C. The samples were filtered through 0.2 μm filter prior to use in HPLC.

Analysis of photosynthetic pigment

The pigments were separated by

HPLC according to method described by Sharma and Hall (1996) using reverse phase column (Waters Spherisorb ODS 25 µm x 4.6mm x 250 mm) and a PDA detector (Waters 2996). 20 µl of filtered sample was injected into the HPLC. The gradient for separation was 0-100% ethyl acetate in acetonitrile/water (9:1) over 25 min with flow rate of 1.2 ml/min. The quantity of pigments was calculated from peak area value using β-carotene as external standard. Identification of pigments was carried out using retention time against standards and using spectral profile of individual peaks using PDA detector in the range of 400-700 nm.

Extraction of total lipids

Total lipids were extracted according to Turnham and Northcote (1984). Freshly harvested algal tissue was boiled in 5 ml of isopropanol for 2 min to inhibit the lipase activity and then dried under nitrogen gas. The dried pellet was homogenized in chloroform: methanol (1:2 v/v) to make the final volume 15 ml with 0.01% BHT added as an antioxidant in the lipid extraction solvent system. Lipid extract was centrifuged for 5 min at 2000 *g* to remove cell debris and to the supernatant, 0.8 ml of distilled water was added followed by 5 ml of chloroform and 5 ml of 0.88% potassium chloride in a separating funnel to make the ratio of chloroform:methanol:water (1:1:0.9). The mixture was shaken vigorously for 5 min and allowed to separate for 30 min. The solvent phase was collected and concentrated under nitrogen gas. The dried lipid extract was redissolved in 5 ml of chloroform and was used for quantitative determination of different classes of lipids.

Esterification of fatty acids

The fatty acid methyl esters were prepared for GC analysis according to Christie (1982). The internal standard (ImM heptadeconoic acid) was added to lipid sample and was subjected to methanolysis in the presence of methanolic-HCl at 68-70°C for 2h. The methyl esters were extracted with three successive portions of hexane and was treated with 5 ml of saturated solution of sodium bicarbonate and washed with 5 ml of distilled water and upper solution was evaporated to dryness in a water bath at 35-40°C with the help of nitrogen gas. Briefly the methyl esters were taken in small volume of fresh hexane and 2 µl of sample was injected to the injector port of gas chromatography. Methyl esters of fatty acids were run on a Nucon gas chromatograph equipped with flame ionization detector and chromatopack data processor. The column (6 mm x 2 mm i.d, stainless steel) was packed with DEGS 10% on 80-100 mesh chromosorb W-HP (Chemlabs, Bangalore). Column temperature was 180°C and injector temperature was 220°C and nitrogen was used as carrier gas (flow rate 30 ml/min). Fatty acid methyl esters peaks were identified by comparing their retention times with methyl esters of pure fatty acid standards and were quantified by using the peak areas of individual fatty acids calculated using the program given by the manufacturers of the instrument. The instrument was programmed to give the Mole % of different fatty acid directly.

Results

Characterization of photosynthetic pigments

Pigments were identified based on their absorption maxima and retention time compared with respective standards. In green algae, the pigments detected are chlorophyll a and b, chlorophyllide-B, violaxanthin, neoxanthin, lutein, antheraxanthin, phaeophytin B, phaeophorbide B, β-carotene and α-carotene. Chlorophyll b and chlorophyllide-B were detected in all the 4

members of chlorophytes studied. Carotenoids such as neoxanthin, violaxanthin, lutein and carotene were also present in all four investigated species, while antheraxanthin was present in only *Enteromorpha* but was absent in *Chaetomorpha, Caulerpa* and *Ulva.* Phaeophorbide B were detected only in *Enteromorpha* (Fig. 1 & 2).

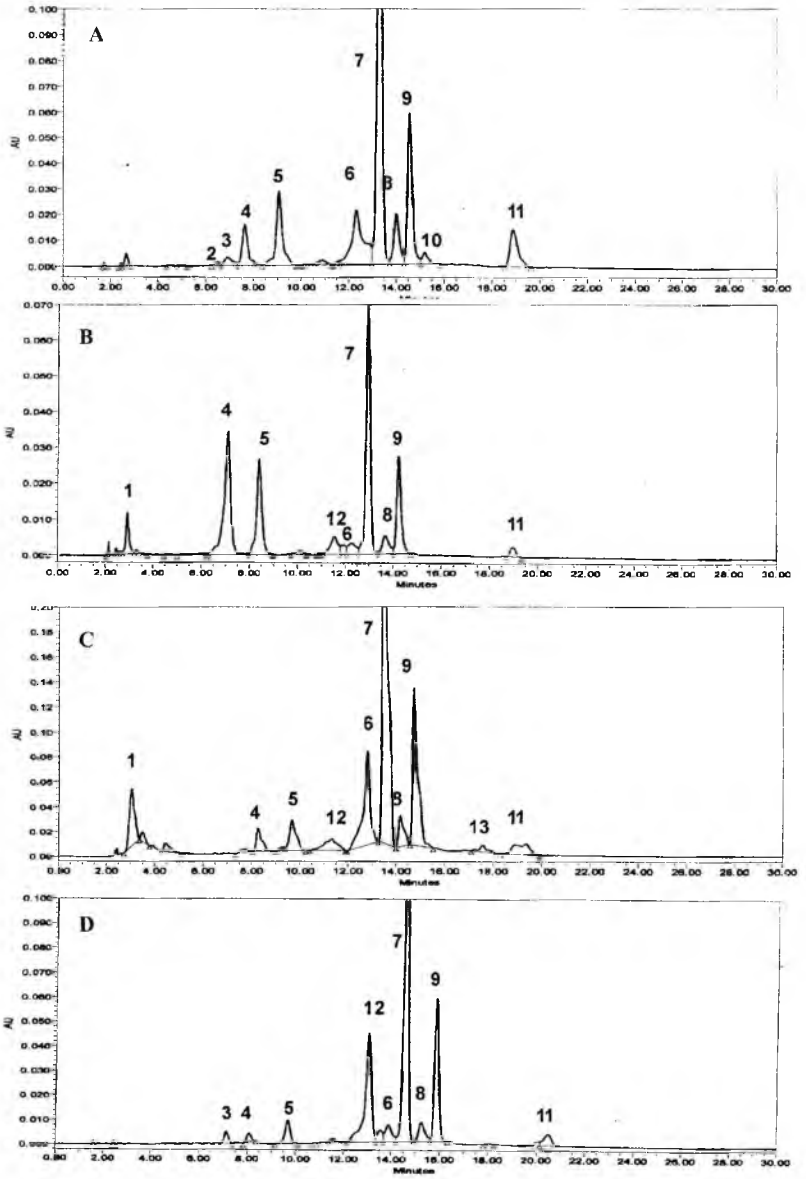

Fig. 1. HPLC chromatograms of the photosynthetic pigments extracted from four green algae (a) *Caulerpa sertulariodies*, (b) *Chaetomorpha media,* (c) *Enteromorpha intestinalis* and (d) *Ulva fasciata.* Peak numbers according to table 1. Chromatograms were taken at different scale

In *Caulerpa sertulariodies* and *Chaetomorpha media,* the pigments that were identified are neoxanthin, violaxanthin, lutein, chlorophyll b, chlorophyll a, chlorophyllide-B, carotene and some unidentified carotenoids. In *Enteromorpha intestinalis,*

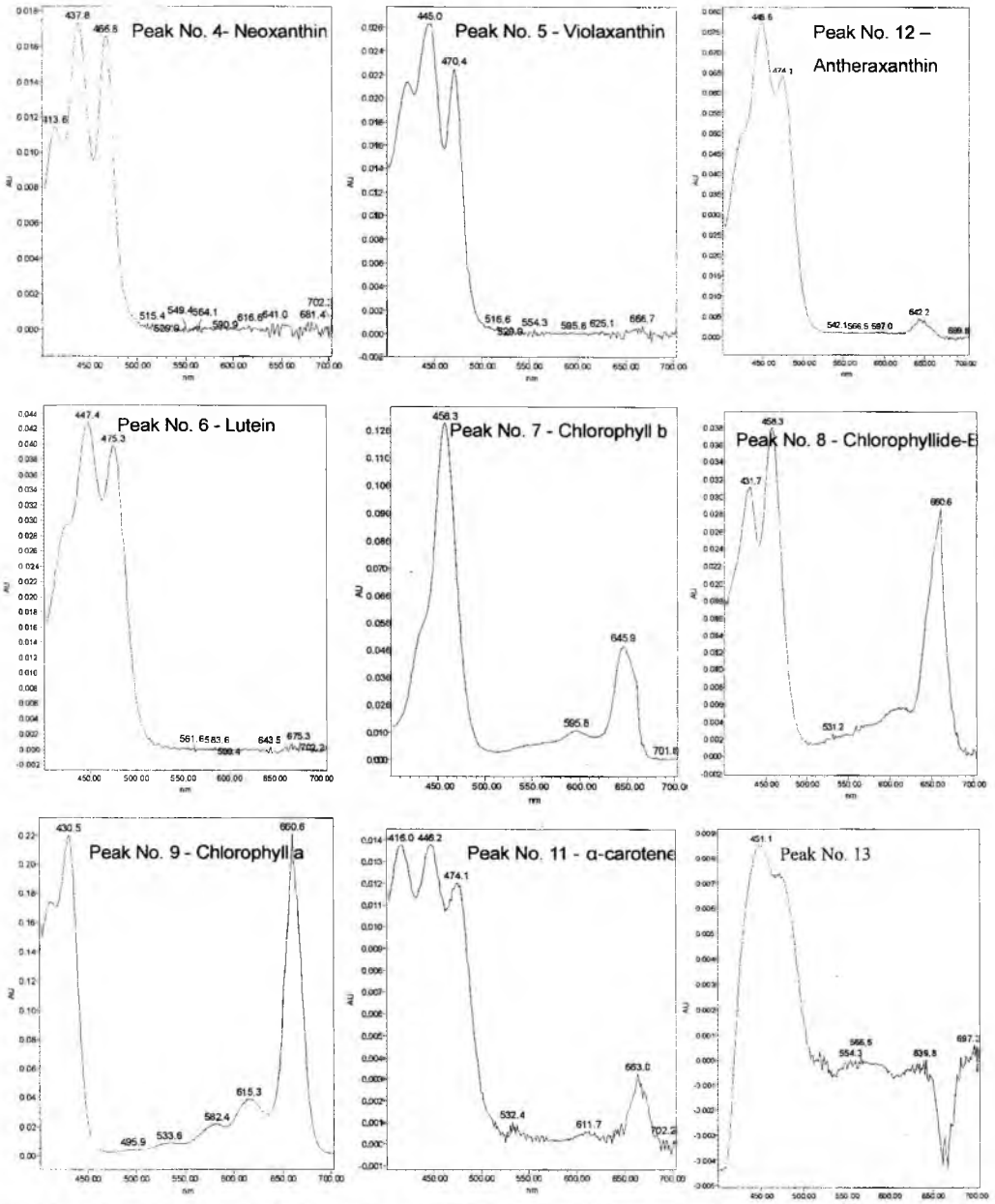

Fig. 2. Absorption spectra of various peaks of HPLC profile of photosynthetic pigments (fig. 1) extracted from four green algae

antheraxanthin and phaeophorbide B were observed in addition to above pigments. In *Ulva fasciata*, the pigments detected were neoxanthin, violaxanthin, lutein, chlorophyll b, chlorophyll a, chlorophyllide-B, β-carotene and unidentified carotenoid (Fig. 1, 2 and Table 1).

Table 1 Photosynthetic pigments, their absorption maxima and amount (μg) extracted from four green algae. * As per the HPLC profile of Fig. 1

Peak No.	Name of the pigments	Retention Time	Absorption maxima (nm)	Amount (μg)
	Caulerpa sertulariodies			
2	Carotenoid	6.7	460	0.0091
3	Carotenoid	7.11	447	0.0087
4	Neoxanthin	7.61	411, 437, 466	0.1182
5	Violaxanthin	9.05	416, 441, 470	0.2309
6	Lutein	12.32	422, 447, 475	0.3041
7	Chlorophyll b	13.29	458, 596, 645	1.1267
8	Chlorophyllide-B	14.00	429, 461, 650	0.1407
9	Chlorophyll a	14.56	408, 428, 617, 660	0.3499
10	Chlorophyll a	15.17	408, 428, 617, 660	0.0353
11	α-carotene	18.88	421, 447, 474	0.1386
	Chaetomorpha media			
1	Chlorophyllide-B	2.1	429, 461, 650	0.0480
4	Neoxanthin	7.06	411, 437, 466	0.2852
5	Violaxanthin	8.40	416, 441, 470	0.1760
6	Lutein	11.51	422, 447, 475	0.0481
7	Chlorophyll b	12.25	458, 596, 645	0.0319
8	Chlorophyllide-B	13.29	458, 596, 645	0.3894
9	Chlorophyll a	14.22	408, 428, 617, 660	0.1512
11	β-carotene	18.96	428, 453, 477	0.0176
	Enteromorpha intestinalis			
4	Neoxanthin	8.27	411, 437, 466	0.1275
5	Violaxanthin	11.32	416, 441, 470	0.1561
12	Antheraxanthin	12.80	419, 447, 476	0.6604
6	Lutein	12.58	422, 447, 475	0.2314
7	Chlorophyll b	13.48	458, 596, 645	2.2283
8	Chlorophyllide-B	14.17	429, 461, 650	0.1640
9	Chlorophyll a	14.72	408, 428, 617, 660	0.7908
13	Phaeophorbide B	17.57	413, 437, 598, 653	0.0506
11	β-carotene	19.35	428, 453, 477	0.1706
	Ulva fasciata			
3	Carotenoids	7.11	447	0.0253
4	Neoxanthin	8.27	411, 437, 466	0.0191
5	Violaxanthin	11.32	416, 441, 470	0.0049
6	Lutein	12.60	422, 447, 475	10
7	Chlorophyll b	13.48	458, 596, 645	0.7477
8	Chlorophyllide-B	14.54	429, 461, 650	0.1640
9	Chlorophyll a	15.22	408, 428, 617, 660	0.0632
10	Chlorophyll a	15.83	408, 428, 617, 660	0.3192
11	β-carotene	20.48	428, 453, 477	0.0458

GC analysis of lipids

The fatty acids of the four algal species were analyzed using gas chromatography. The fatty acid groups present were Lauric acid C_{12}, myristic acid C_{14}, palmitic acid C_{16}, stearic acid C_{18}, oleic acid $C_{18:1}$, linoleic acid $C_{18:2}$ and linolenic acid $C_{18:3}$ (Table 1a & b). Lauric acid C_{12}, myristic acid C_{14}, palmitic acid C_{16}, stearic acid C_{18} were the saturated fatty acids while oleic acid $C_{18:1}$, linoleic acid $C_{18:2}$ and Linolenic acid $C_{18:3}$ were the unsaturated fatty acids. Lauric acid, myristic acid and palmitic acid were the most commonly occurring fatty acids detected in all of the four analyzed algal species (Fig. 3).

The fatty acid profile of all the green algae studied showed the presence of all seven fatty acids lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic acid. The fatty acids ranged from C12:0, to C18:3. The C14:0, C16:0, C18:0 were dominant. These fatty acids accounted for more than 50-76% of the total fatty acids observed. Palmitic acid (C16:0) was the major fatty acid present, comprising 19-49% of the total fatty acid content. The other fatty acids were present in relatively small quantities (Fig. 3, Table 2).

Discussion

Characterization of photosynthetic pigments

Besides the main pigments, which are the characteristic of higher plants, some class-specific pigments such as prasinoxanthin or siphonoxanthin or loroxanthin in some orders of Prasinophyceae and Ulvophyceae were observed in previous studies in members of Chlorophyta (Schagerl *et al.*, 2003; Yoshii *et al.*, 2004), which was not detected in this study. In the present investigation, secondary carotenoids such as the ketocarotenoids, canthaxanthin or astaxanthin (Lorquin 1997; Fabregas 1998) were also not encountered. Synthesis of secondary carotenoids is

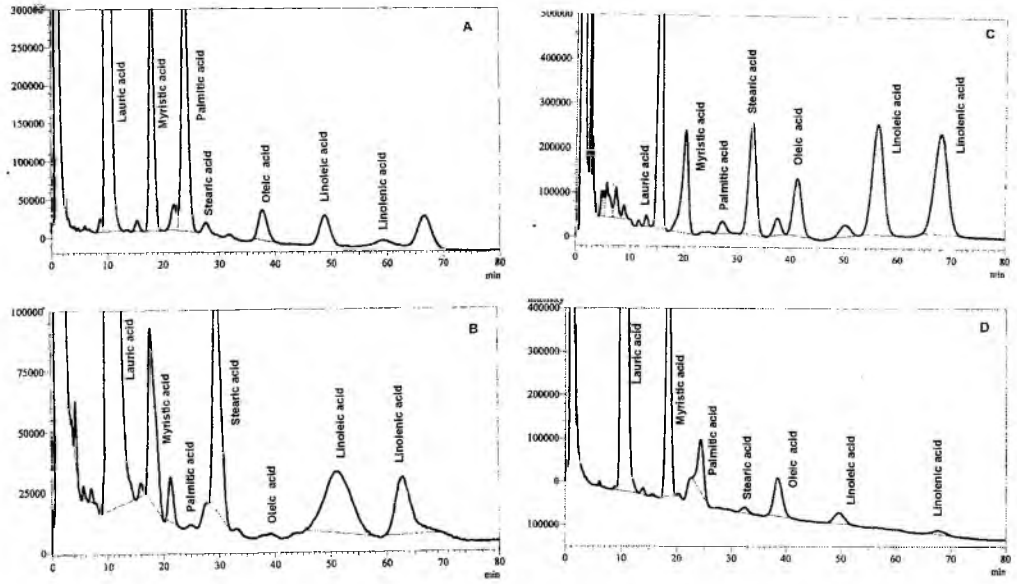

Fig. 3. GC chromatograms of fatty acids extracted from four green algae (a) *Caulerpa sertulariodies*, (b) *Chaetomorpha media,* (c) *Enteromorpha intestinalis* and (d) *Ulva fasciata.* Peak numbers according to table 2. Chromatograms weretaken at different scale

Table 2. Fatty acid composition (%) of four green algal species studied

Fatty acids	*Caulerpa sertulariodies*	*Chaetomorpha media*	*Enteromorpha intestinalis*	*Ulva fasciata*
Lauric acid	9.40	10.25	11.22	8.34
Myristic acid	35.22	28.11	25.18	27.31
Palmitic acid	24.63	7.45	20.88	26.78
Stearic acid	11.23	8.66	12.33	15.22
Oleic acid	20.53	6.43	17.54	18.45
Linoleic acid	10.2	8.89	31.66	23.63
Linolenic acid	26.56	7.52	35.55	21.46

apparently stimulated by a number of factors, such as nitrogen depletion, excessive supply of light and high salinity (Schagerl *et al.,* 2003; Fabregas 1998).

Members of the order Bryopsidales (*Caulerpa*) show peculiar pigment characteristic when compared to other algal groups. Siphonoxanthin and siphonein is indicative of this family as is the relative abundance of α-carotene compared to β-carotene (Benson and Cobb 1981; Hegazi *et al.,* 1998). Absence of siphonoxanthin and siphonein in *Caulerpa filiformis* indicates it is not a definitive character (Strain 1965). In the study conducted by Hegazi *et al.,* (1998) chlorophyll b, micronone, microxanthin, neoxanthin, siphonein and siphonoxanthin were found to be the characteristic pigments of *Caulerpa prolifera.* The present analysis of the pigment composition of *Caulerpa sertulariodes* showed the presence of neoxanthin, lutein, violaxanthin, antheraxanthin, chlorophyll b, chlorophyll a, chlorophyllide-B and α-carotene.

Lutein and loroxanthin are the key caotenoids present in *Chaetomorpha okamurae* along with 9'-cis neoxanthin, violaxanthin, antheraxanthin and β-carotene (Yoshi *et al.,* 2004). Shie *et al.* (2005) first reported the presence of zeaxanthin in *Chaetomorpha basiretrosa.* Lutein, neoxanthin, violaxanthin, antheraxanthin, chlorophyll b, chlorophyll a, chlorophyllide-B and β-carotene were the pigments detected in the presently studied sample of *Chaetomorpha media,* while loroxanthin and 9'-cis neoxanthin were absent.

Enteromorpha intestinalis analyzed showed pigments characteristic of higher plants namely: neoxanthin, violaxanthin, antheraxanthin, lutein, chlorophyll a, chlorophyll b, chlorophyllide-B, phaeophytin B, phaeophorbide B and β-carotene. Some species of *Ulva* were reported to contain loroxanthin. But this pigment was absent in the studied species, *Ulva fasciata.* Takaichi and Mimuro (1998) detected the presence of violaxanthin, lutein, chlorophyll a, chlorophyll b and β-carotene in *Ulva* and *Spirogyra.* The present study confirmed the presence of neoxanthin, violaxanthin, antheraxanthin lutein, chlorophyll b, chlorophyll a and β-carotene (Benson and Cobb 1981).

In previous studies (Dere *et al.,* 1998) it was determined that there were changes at the pigment level of algal species that live in an environment where light stratification is seen. The presently investigated marine chlorophytes (*Caulerpa, Chaetomorpha, Enteromorpha* and *Ulva*) as a result of their characteristic intertidal zone habitat is subjected to wide daily and seasonal variation

in light quality and quantity. Pigment spectrum outlined above may therefore be adapted for optimum light harvesting in this environment. It is quite possible that some of the pigment such as 9'Cis etc. reported could also be due to epoxidation product as a result of handling the tissue or tissue experiencing oxidative stress in nature.

Characterization of fatty acids

The fatty acid composition of the four species of green algae studied possessed similar fatty acid composition with palmitic acid, oleic acid and linoleic acid clearly predominating. Palmitic acid was the most abundant fatty acid, amounting to 20% of all fatty acids. These data are in agreement with earlier conclusions that a dominance of C16 and C18 is in green algae (Janieson and Reid 1972; Aknin *et al.,* 1992; Khotimchenko 1993). The majority of green algal species studied up to now have 16:4n-3 acid as their characteristic component (Janieson and Reid 1972; Aknin *et al.,* 1992; Khotimchenko 1993). Only green algae from the genera *Bryopsis and Caulerpa* contain hexadecatrienoic acid as their main C16 PUFA (Aknin *et al.,* 1992; Khotimchenko 1995; Vaskovsky *et al.,* 1996). Different green algal species vary in the ratio of individual C18 PUFA components – linoleic, α-linolenic acid and octadecatetraenoic acids. Fatty acid composition of twelve algal species from two different classes were determined (Heiba *et al.,* 1997). In this study all the green algae showed the presence of seven different fatty acids such as lauric acid, myristic acid, palmitic acid, stearic, oleic, linoleic and linolenic acid. In previous studies (Khotimchenko 1993) it was reported that *Chaetomorpha linum* contained significant amounts of C16 and C18 PUFAs. Several studies on the fatty acid composition of algae have been carried out by Heiba *et al.* (1997); Colombo *et al.* (2006); Aknin *et al.* (1992); Khotimchenko (1991); Takagi *et al.* (1985).

Ulva being a source of food has been widely investigated for its fatty acid content. Colombo *et al.* (2006) reported the presence of palmitic, palmitoleic, oleic, linoleic and α-linolenic acids in *Ulva. Ulva pertusa* and *Ulva fenestra* contains high levels of C16 (palmitic acid) and C18 (oleic, linoleic and α-linoleic acids) (Floreto *et al.,* 1993; Sanina *et al.,* 2004). Work done by Ghazala and Shameel (2005) and Cojocaru (2005) on fatty acids of freshwater algae showed occurrence of palmitic, oleic, linoleic and linolenic acids in fresh water members of chlorophyceae.

Colombo *et al.* (2006) studied the fatty acid profile of algae from cold and warm water and reported that fatty acids from cold water (Canadian algae) are generally richer in polyunsaturated fatty acid (PUFA), with a higher n-3/n-6 FA ratio, and a higher degree of total unsaturation. Khotimchenko (2003) reported that green algae investigated for their fatty acid composition possessed similar profiles of fatty acids. The major constituents were C12:0 which together totalled more than 65% of all fatty acids in all species.

The results obtained during the present study also show that fatty acids of Chlorophyceae contain both saturated and unsaturated fatty acids ranging from Lauric acid (C12) to Linoleic acid (C18:3) (Demort 1972; Fleurence 1994; Helmi 1997). The Chlorophyceae comprise the most modern group and this is supported primarily by occurrence of C18 fatty acids typical of the vegetative tissue of higher plants. Members of the Chlorophyceae probably lost the general ability to convert C18 PUFAs to C20 PUFAs during evolution. This reduction may be regarded as an advanced phylogentic character (Graeve *et al.,* 2002).

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