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Research Article

STUDIES ON OMEGA 3 FATTY ACIDS PRODUCTION FROM SELECTED MICRO AND MACROALGAE

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ABSTRACT

In today's scenario, algae are widely recognized as a promising source for biofuel production as a renewable source of energy. They are exceedingly rich in oil, which can be converted to biofuel. This paper deals with one of the method to enhance the lipid content in microalgae. The macroalgae *Padina boergesenii* was collected from Rameswaram, Dist. Tamil Nadu, India and *Chlorella vulgaris* was collected from Presidency College (Autonomous), Chennai, Tamil Nadu, India. Chlorophyll a, Chlorophyll b and β Carotene were extracted from the selected macro and microalgae which showed the variation among both the species. High amount of lipid was extracted from *Chlorella vulgaris* when compared to *Padina boergesenii.* These lipids were analyzed for Transesterification and determined the Omega -3 Fatty acids by GC-MS and FT –IR analysis from the selected Macro and Micro algae.

INTRODUCTION

Omega-3 fatty acids refer to the chemical structure of the fatty acid. There are three main omega-3 fatty acids. *Eicosanoids* (EPA) *Eikosa* means 'twenty' in Greek, and denotes the number of carbon atoms in the PUFAs that act as precursors of Eicosanoids. These signaling molecules are called leukotrienes, prostaglandins, thromboxanes, prostacyclins, lipoxins and hydroperoxy fatty acids. Eicosanoids are important for several cellular functions such as platelet agreeability (ability to clump and fuse), chemo taxis (movement of blood cells) and cell growth. Eicosanoids are rapidly produced and degraded in cells where they execute their effects. *Docosahexaenoicacid (DHA)* is a membrane phospholipids. An increased amount of ϕ-3 Poly Unsaturated Fatty Acids (PUFA) may change the physical characteristics of the membranes. Dietary marine ϕ-3 fatty acids (EPA and DHA) decrease plasma triacylglycerol levels by reducing production and enhancing clearance of triacylglycerol rich lipoproteins (Arild Rustan *et al*., 2005). The most common saturated fatty acid in animals, plants and microorganisms is *palmitic acid; Stearic acid* is a major fatty acid in animals and some fungi, algae and a minor component in most plants. α- Linoleic acid (ϕ-3) is found in higher plants (soya bean oil and rape seed oils) and algae. Eicosapentaenoic acid (EPA; ϕ-3) and *docosahexaenoic acid (DHA; ϕ-3)* are major fatty acids of marine algae.

The macro algal species are multicellular and possess plant like characteristics. They are typically comprised of a blade or lamina, the stipe, and holdfast for anchoring the entire structure to hard substrates in marine environments. Some forms possess air bladders that act as flotation devices that enable some species to stand upright or to occur as free-floating on ocean surfaces. (Tseng, 1987; Choi *et al*., 2002). Microalgae are also called as phytoplanktons. They are very small plant like organism between 1-50 mm in diameter where they form the basis for most food chains. Most species contain chlorophyll, using sunlight as energy source and convert carbon dioxide (Co_2) into biomass.

The genus *padina*, brown algae has a worldwide distribution in tropical and subtropical climate zones. Species of the marine brown algal genus *Padina* are widely distributed throughout the tropics and are very easy to recognize in the field. The "ear-like" blades have a circinnately in rolled apical margin (Womersley 1987; Lee and Kamura 1991; Huisman 2000), where a row of meristematic cells produces a thallus that is parenchymatous. Its frond typically consists of two or more layers of cells while the stipe is composed of four or more layers. The upper surface is calcified to varying degrees (Trono 1969), and the reproductive structures occur in bands on the upper and, sometimes, on the lower surface. *Padina sp* .are erect, flattened, fan shaped and parenchymatous. They attach by rhizoidal holdfast. In submerged plants, the "fan" is often

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curved into a funnel shape and is composed of tufts of many overlapping lobes. Concentric lines, formed by hairs or hair scars, mark the front.

Chlorella are single celled, fresh water alga is one of the earliest forms of life, participating in photosynthesis, the process responsible for removing poisonous Carbondioxide from the atmosphere and producing the massive amounts of oxygen that are required by all animal life. *Chlorella sp*. is so small that it cannot be seen in a naked eye, a single cell is 6/1000 mm across. It reproduces at a rapid rate. A single cell can divide and subdivide into four cells every 16 to 20 hours.

MATERIALS AND METHODS

Sample collection

The healthy and mature specimen of different species of macro algae were collected from sandy bays, large and shallow sand bottom flats in various areas of Olaikuda and Pudhumadam in Rameswaram, Ramanathapuram district, Tamil Nadu, India region during February 2016. The collected macro algae transported to the laboratory, the coarser material was removed by filtration through a mesh net. The algal samples were preserved in 4% formalin (aqueous solution of formaldehyde) and the macro algae were brought to the laboratory where they were washed immediately with running water to remove foreign particles, sand, epiphytes and attached debris and later by distilled water.

The microalgae sample was collected from Department of Plant Biology and Plant Biotechnology laboratory, Presidency College (Autonomous), Chennai – 600 005, Tamil Nadu, India. The sample was incubated in the full strength Bold Basal Medium at 22 ± 1˚C and constant light at 50 µmol photons m^{-2} s^{-1} under the photoperiod of 12:12 hour's light/Dark condition (Fig. 1).

At this stage 25 ml culture in Erlenmeyer Flasks was shaken manually twice a day in a full strength medium where sub cultured for further analysis. Most samples were enriched by this way for dominant strains until the samples taken for serial dilution or streaking on to solid media used for strain isolation.

In vitro mass cultivation

The microalga *chlorella vulgaris* was then subjected to *in vitro* mass cultivation in a 5 L Erlenmeyer flask with 2L of BBM broth and incubated under 12:12 hrs of light illumination (Crompton 40 W, Cool day light 6500 K at an intensity of 2000 lux.) and dark respectively at room temperature (28^{o}C) for 16 days.

Harvest and estimation of dry biomass

The macro algae was dried under shade at room temperature and later on in an oven at 30°C to get dried. Later, the dried sample was powdered through pestle and mortar. The biomass after *in vitro* mass cultivation of microalgae was harvested by centrifugation at 8000 rpm for 5 min. at 20^{o}C. The concentrated biomass in the pellet was obtained and mixed using a cyclomixer and transferred to a watch glass, which was then incubated in an incubator at 35^{o}C for overnight. The dry biomass was wiped using a sharp sterilized surgical blade and weighed gravimetrically using an electronic weighing balance.

Biochemical constituents

Estimation of total Chlorophyll a and Chlorophyll b

The green photosynthetic pigment of the both macroalgae and microalgae were determined for Chlorophyll *a* and Chlorophyll *b* were estimated by Jeffrey and Humphery (1975) method and its equation given below.

Chlorophyll a = 11.93E664 - 1.93E647 µgml^{-1}
Chlorophyll b = 20.36E647 - 5.50E664 µgml^{-1}

The absorbance values at 664 and 647 nm were recorded daily for about 16 days using a UV-Vis. Spectrophotometer.

Estimation of total carotene

The total carotene content of the both macroalgae and microalgae was determined by Prieto *et al*. (2011) method and the formula is given below.

Carotene (µg/ml) = A_{450} nm × 25.2

Fig 1 Micro algal Culture maintenance in the Plant Biotechnology Department Laboratory, Presidency College (Autonomous), Chennai

Extraction and estimation of carbohydrate

Padina boergesenii crude was extracted by using ethanol as a solvent. About 400 ml of the *Chlorella vulgaris* culture was centrifuged at 8000 rpm for 5 min. around 20°C and the obtained pellet with biomass was conserved. Then the biomass was allowed to sonicate (Equitron Ultrasonic Cleaner) at 53 KHz for 20 min. at room temperature (28°C) to lyse the cells. The condensate was then centrifuged at 8000 rpm for 10 min. at 20 °C the obtained supernatant was used as a crude extract for the estimation of carbohydrate.

The total carbohydrate was determined based on the method well defined by Dubois *et al*. in the year 1956 (Phenol Sulphuric acid method). About 100 µl of the crude sample was subjected to treat with 5% phenol and 2.5 ml of Conc. Sulphuric acid. Then the whole content was incubated for 10-15 min. at room temperature. Then the optical absorbance values were measured at 490 nm using a UV-Vis. Spectrophotometer. The total carbohydrate content was determined by comparing the absorbance values with the standard graph already constructed using a known carbohydrate sample. The standard graph was constructed using a known carbohydrate D-glucose.

Extraction and estimation of protein

Padina boergesenii crude extract and about 400 ml of the *Chlorella vulgaris* culture was centrifuged at 8000 rpm for 5 min. around 20°C and the obtained pellet with biomass was conserved. Then the biomass was allowed to sonicate (Equitron Ultrasonic Cleaner) at 53 KHz for 20 min. at room temperature (28°C) to lyse the cells. The condensate was then centrifuged at 8000 rpm for 10 min. at 20°C; the obtained supernatant was used as a crude extract for the estimation of protein.

The estimation of protein in this present study was done based on the method described by Bradford (1976). The protein content of the unknown sample was determined in comparison with the standard graph of the known sample. The standard graph for protein was constructed using Bovine serum albumin as a standard known protein. About 1 ml of the micro and macro algal crude sample used to determine the total protein content with 5 ml of the Bradford reagent. After incubation for 15 min. the content was subjected to determine the absorbance values at 595 nm in a UV-Vis. Spectrophotometer.

Extraction and estimation of lipid

The microalgae *Chlorella vulgaris* biomass was harvested using a centrifuge at 8000 rpm for 5 min. The biomass was allowed to dry and powdered both macro and micro algae using a mortar and pestle. The total lipid constituent was determined based on the method described by Bligh and Dyer (1959). A 6 ml of chloroform and methanol (2:1 ratio) was mixed with the extract and vortexed for 3 min. at room temperature. The extract was then centrifuged at 8000 rpm for 5 min. The resultant liquid phase was washed with 6 ml of 0.9% of NaCl and vortexed for 5 min. The entire content was then centrifuged at 2000 rpm for 5 min. which resulted to form two distinct phases. The upper phase conserved due to its richness in lipid and the lower aqueous phase was discarded. The total lipid content was determined gravimetrically after the complete evaporation of the solvent (Upper phase). After evaporation the total lipid content was weighed.

Gas Chromatographic (GC) analysis

The FAME Samples were processed by Gas Chromatography (GC) analysis was performed on an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID). For separation of FAMEs, a DB225 capillary column (30 m x 0.25 mm I.D.; 0.25 µm) was used. The initial oven temperature was maintained at 160°C for 2 min with a sequential increase to 180°C at 6°C min^{-1} for 2 min and 230°C at 4°C min^{-1}. The final oven temperature was maintained at 230°C for 10 min. Nitrogen was used as the carrier gas with a flow rate of 1.5 ml min^{-1}. The injector and FID temperatures were set at 230°C and 250°C respectively, while a split ratio of 50:1 was maintained for the analysis. The flame ionization detector allows for a large dynamic range and provides good sensitivity. Hydrogen is the carrier gas, nitrogen is the "makeup" gas, and air is used to support the flame.

The electronic signal from the GC detector is passed to the computer where the integration of peaks is performed. The electronic data is stored on the hard disk and the fatty acid methyl ester composition of the sample is compared to a stored database using the Sherlock pattern recognition software. The standard is a mixture of the straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxyl acids. All compounds are added quantitatively so that the gas chromatography performance may be evaluated by the software each time the calibration mixture is analyzed.

Fourier transforms infra-red spectrometric (FT-IR) analysis of FAME

The FAME sample were analyzed under infra-red (Perkin Elmer model spectrum – I PC). The FT-IR spectra with the resolution of 4 cm^{-1}, Scan Number: 3 were performed after the evaporation of the lipid fraction on Thalium bromide tablets. The FT-IR spectrums of all the FAME samples were obtained as a percentage of transmission ranged from 450 cm^{-1} to 4000 cm^{-1}.

RESULTS

Sampling Sites

The abiotic factors variations in the major and minor constituents of various areas of Olaikuda and Pudhumadam, Rameswaram, Ramanathapuram district, Tamil Nadu, India during February 2016. The pH ranges from 7.8 – 8.5 during the collection of macro algae. The collected and identified microalgae and macro algae species were *Padina boergesenii* and *Chlorella vulgaris*.

Systematic position of Padina (Lee, 1999)

Kingdom : Protista
Division : Heterokontophyta
Class : Phaeophyceae
Order : Dictyotales
Family : Dictyotaceae
Genus : *Padina*
Species : boergesenii

Systematic position of Chlorella (Beijerinck, 1890)

Empire : Eukaryota
Kingdom : Plantae
Phylum : Chlorophyta

Class : Trebouxiophyceae
Order : Chlorellales
Family : Chlorellaceae
Genus : *Chlorella*
Species : *vulgaris*

Sample preparation

The sample *Padina boergesenii* and *Chlorella vulgaris* were kept in hot air oven for a period of two weeks and was allowed to dry. The dried sample was weighed initially and then it was coarsely powdered with the help of mortar and pestle. The *Chlorella vulgaris* sample was centrifuged and the biomass was shade dried and blended using mortar and pestle.

Pigment studies of both macro and micro algae

Among all the three pigments β -carotene plays a major role in domination. Chlorophyll a is the predominant type of chlorophyll pigments found in Green algae followed by Chlorophyll b. From this investigation, *Chlorella vulgaris* found dominated among *Padina boergesenii* by scoring high amount in β- carotene and chlorophyll b except chlorophyll a of all the pigments (Fig. 2) *Chlorella vulgaris* (171.1mg ml-1 of β-carotene, 13.199µg ml-1 of Chl. a and 20.465µg ml-1 of Chl. b) having almost moderate amount of all the pigments but, *Padina boergesenii* (120.1mg ml-1 of β-carotene, 32.97mg ml-1 of Chlorophyll a and 0.76mg ml-1 of Chlorophyll b.

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 (Ilavarasi, 2012). Pigments such as chlorophylls and carotenoids were quantified from *Padina sp*. shows Chlorophyll a, chlorophyll b, and total chlorophyll contents were found to be high in *Padina sp*. compared to other seaweeds (Sudhakar, 2013).

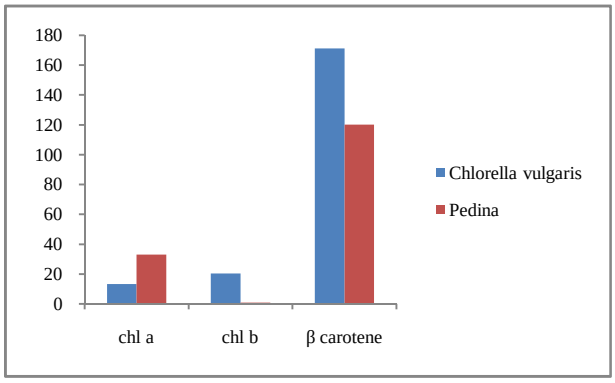

Fig. 2 Pigment constituents of *Padina boergesenii* and *Chlorella Vulgaris*

Biochemical constituents of Padina boergesenii and Chlorella Vulgaris

The pigments and biochemical parameters (Carbohydrate, Protein and Lipid) were analysed from both the macroalgal and microalgal biomass. Protein content varied among different species ranged between *Chlorella vulgaris* (12.6µg/100µl) and *Padina boergesenii* (31µg/100µl). Carbohydrate content ranged between 32µg/100µl and 50.9µg/100µl as in*Chlorella vulgaris* (50.9µg/100µl) and (32 µg/100µl) *Padina boergesenii* respectively. However, high lipid content was recorded in *Chlorella vulgaris* (22.2µg/100µl) and less in *Padina boergesenii* (12µg/100 µl) (Fig. 3).

The rapidly growing microalgae *chlorella* cells were exhibited by a higher protein and low carbohydrate content, so the rapidly growing cells of natural day light at 25-30°C showed higher amount of protein (Sayegh, 2011). Chemical studies of different *Padina sp*. showed a low and variable protein content and it has been observed that the protein content of macro algae is dependent on season and environmental growth conditions (Dawczynski *et al*., 2007). *Padina sp*. showed high contents of non-structural carbohydrates ranging from 30% to 44% dry weight. According to earlier works, mannitol constitutes the major carbohydrate of low molecular weight in *Padina sp*. *(*Mian & Percival, 1973). Macroalgae have been reported to have low lipid contents (Mabeau & Fleurence, 1993), their polyunsaturated fatty acid (PUFA) composition is superior to those of terrestrial vegetables in regard to the human diet (Goecke *et al*., 2010; Kumari *et al*., 2010). Some of those essential fatty acids like omega-3 and omega-6 PUFAs must be consumed by humans and animals in their normal diet (MacArtain *et al*., 2007).

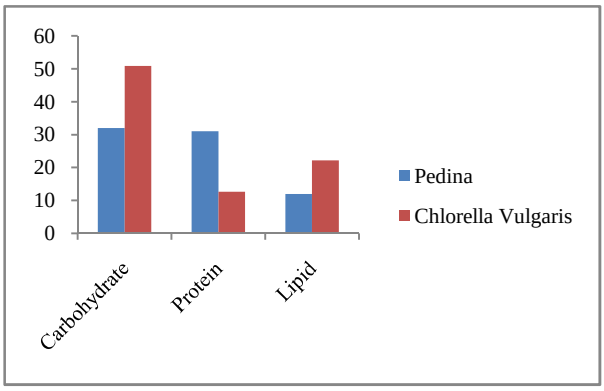

Fig. 3 Biochemical constituents of *Padina boergesenii* and *Chlorella Vulgaris*

Lipid Extration

In lipid extraction both micro and macro algae showed good results. However, high lipid content was recorded in *Chlorella vulgaris* (22.2µg/100µl) and less in *Padina boergesenii* (12µg/100 µl). Overall comparison microalgae contain high lipid content when compare to macro algae.

Saturated fatty acids (SFA) were major components accounting from 33.81% for *Padina pavonica*. The total sum monounsaturated fatty acids (MUFAs) ranged from 39.81% to 42.89%, whereas total sum of PUFAs were 12.86–31.65%. Palmitic acid was the major fatty acid. Fatty acids composition of algal lipids varies widely with species, habitat, light, salinity, pollution and environmental conditions. (Ratana-Arporn and Chirapart, 2006) but in most studies palmitic acid (C16:0) is predominant (Gressler *et al*., 2010). The second major fatty acid were oleic acid (C18:1 n-9). Oleic acid (C18:1 n-9) was the most abundant MUFA in species analyzed, followed by palmitoleic acid (C16:1). C14:1 was detected only in *T. atomaria* and C22:1n-9 only in *Taonia atomaria* and *Padina pavonica*.

These results allow to conclude that the largest lipid recovery 14.9 % of dry matter of biomass is observed when using a mixture of ethanol – petroleum ether 2:1 (vol.) as a solvent. This result can be explained by the fact that some neutral lipids are located in the cytoplasm not only in the form of lipid globules, but also as complexes with polar lipids. Van der Waals forces arising between non-polar organic solvent and the neutral lipids, which are composed of protein-lipid complexes, are not sufficient to destroy the attraction between lipids and proteins. On the other hand, a polar organic solvent (such as ethanol, isopropanol, etc.) is capable of disrupting the lipid-protein associations by forming hydrogen bonds with the polar lipids in the complex (Kates, 1986). However, along with the neutral lipids which are present in the cells in the form of globules and are included in membrane-associated complexes, polar lipids (phospholipids and glycolipids) are extracted as well.

Analysis of fatty acid methyl esters (FAME) by gas chromatography and mass spectrometry (GC-MS)

The GC-MS analysis of the microalgal sample *Chlorella vulgaris* showed the presence of Z,E-2-Methyl-3,13-octadecadien-1-ol, cyclopentanecarboxylic acid, octadecyl ester, Docosanedioic acid dimethyl ester, 7,10,13-hexadecatrienoic acid which confirms the presence of omega 3 fatty acids in these compounds (Table 1).

Eight fatty acids were identified ranging between C13 to C24, in which Oleic acid, Stearic Acid and Linoleic Acid were found to be the main constituent for biodiesel production. Palmitic acid (C16:0) is the most abundant fatty acid in nature and was investigated as a model compound for microalgal lipids. Using palmitic acid is an oversimplification as the fatty acid profiles of microalgal lipids are much more complex than a single fatty acid. The majority of lipids in microalgae are typically present as triglycerides and some free fatty acids stored in the cell chloroplasts, but fatty acids are also present as membrane lipids such as glycolipids and phospholipids (Wang, 2012).

Table 1 GC-MS analysis of Fatty acid compounds of *Chlorella vulgaris*

Retention Time	Fatty Acids	Molecular formula	Molecular weight g/mol
15. 98	Z,E-2-Methyl-3,13-octadecadien-1-ol	$C_{19}H_{36}$	280.488
17.62	Cyclopentane carboxylic acid, octadecyl ester	$C_{24}H_{46}O_2$	366.621
19.45	Docosanedioic acid dimethyl ester	$C_{24}H_{46}O_4$	398.620
19.45	7,10,13-hexadecatrienoic acid	$C_{16}H_{26}O_2$	250.376

The GC-MS analysis of the macroalgae sample *Padina boergesenii* showed the presence of acid Eicosapentaenoic acid and Docosahexaenoic acid which confirms the presence of omega 3 fatty acids in these compounds and also other common fatty acids found, such as cis-9, 12-Octadecadienoic acid and hexadecanoic acid (Table 2).

Table 2 GC-MS analysis of Fatty acid compounds of *Padina* Sp

Retention Time	Fatty Acids	Molecular formula	Molecular weight
14. 12	Eicosapentaenoic acid	$C_{20}H_{30}O_2$	302.451
16.34	cis-9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280.4455
17.45	Docosahexaenoic acid	$C_{22}H_{32}O_2$	328.488
19.78	hexadecanoic acid	$C_{16}H_{32}O_2$	256.4241

Fourier transforms infra red spectrometric (FT-IR) analysis of FAME

In the macroalgae sample *Padina boergesenii* contain different functional groups such as alcohols, Phenols, Carboxylic acid, alkanes, α, β- unsaturated aldehydes, ketones, Nitro compounds, alkanes, alcohols, carboxylic acids, esters, ethers, alkenes, alkyl halides. These are depends on the stretch bonds orders and frequencies (Table 3). Characteristic functional groups contributing to the formation of absorption bands at specific wave-numbers. In response to Cd stress, there was a general decrease in the protein and carbohydrate content indicated by a decrease in the intensity of absorption bands especially in the 1800 to 800 cm^{-1} region. (Dumas and Miller, 2003).

Table 3 FT-IR functional group of *Padina boergesenii*

Frequency	Bond	Functional Group
3424	O-H stretch, H-bonded	Alcohols, Phenols
3173	O-H stretch	Carboxylic acid
2920	C-H stretch	Alkanes
1694	C=O stretch	α, β- unsaturated aldehydes, ketones
1480	N-O asymmetric stretch	Nitro compounds
1351	C-H rock	Alkanes
1101	C-O stretch	Alcohols, carboxylic acids, esters, ethers
1032	C-O stretch	Alcohols, carboxylic acids, esters, ethers
653	=C-H bend	Alkenes
602	C-Br stretch	Alkyl halides

The microalgae sample *Chlorella vulgaris* contain different functional groups such as Carboxylic acid, alkanes, Esters, saturated aliphatic, Nitro compounds, alkanes, Aromatic amines, Alkyl halides, Aliphatic amines, aromatics, Aromatics, 1^0, 2^0 amines. These are depends on the stretch bonds orders and frequencies (Table 4). Individuals of Chlorella generated FTIR spectra with clear bands. The molecular assignments of bands are based on published data on phytoplankton, bacteria and the other biological materials. In this study, for both algae, the average positions belong to protein (amides I and II), lipid and carbohydrate absorption bands confirmed by the literature. In some spectra of both algae, bands were also seen at ≈1050 and ≈1012 cm^{-1}. These band positions match those attributed to the ν(C-O-C) stretching of polysaccharides (Brandenburg and Seydel, 1996).

Table 4 FT-IR functional group of *chlorella vulgaris*

Frequency	Bond	Functional Group
3174	O-H stretch	Carboxylic acid
2924	C-H stretch	Alkanes
2858	C-H stretch	Alkanes
1743	C=O stretch	Esters, saturated aliphatic
1545	N-O asymmetric stretch	Nitro compounds
1441	C-C stretch (in-ring)	Aromatics
1366	C-H rock	Alkanes
1267	C-N stretch	Aromatic amines
1154	C-H wag –CH2 X1	Alkyl halides
1021	C-N stretch	Aliphatic amines
889	C-H "oop"	aromatics
808	C-H "oop", N-H wag	Aromatics, 1^0, 2^0 amines

CONCLUSION

The macroalgae and microalgae species studied exhibited different profiles important fatty acids in important amounts. Considering PUFA of the ω-3 series, EPA was determined in almost all species and some of them also contained measurable amounts of DHA. In the selected micro and macro algae are obtained results can be regarded as good sources of PUFA, either for direct human nutrition, in food supplements or in feeding materials. These algae are cost effective and eco-friendly for their usage.

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