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Research Article

In Vitro Antibacterial Screening of Fatty Acid Fractions from Three Different Microalgae

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ABSTRACT
The spectrum of antibacterial activities shown by fatty acid fractions of different microalgae, depending on the unsaturation pattern and chain length are still need to be explored. This study provides information on the fatty acid composition of three microalgae, *Chlorella marina*, *Nannochloropsis occulata* and *Chaetoceros affinis* along with their antibacterial activities against selected clinical and fish pathogens. PUFA content of fatty acid fractions followed the order *N. occulata* > *C. affinis* > *C.* marina. Greater abundance of eicosapentanoic acid found in *Nannochloropsis occulata* (27.39 ± 0.15%) and *Chaetoceros affinis* (25.34 ± 0.23%) might be responsible for higher bacterial inhibition shown by these fractions.

Key words: Microalgae, Fatty acid, Eicosapentanoic acid, Antibacterial activity.

INTRODUCTION

Fatty acids represent an important class of metabolites due to their multifarious applications based on the variations in structure and relative abundance[1,2]. Fatty acid profiling provides a tool for studying chemotaxonomic features in various species of microalgae[3]. Variations in relative abundance of fatty acids in order with environmental factors described their application as stress responsive biomarkers of microalgae[4,5]. Microalgae are considered as important producers of poly unsaturated fatty acids (PUFA) because a major portion of PUFA in aquatic organisms, including fish, is accumulated from their microalgal diet. Also fatty acid (FA) profile in microalgae is less susceptible to seasonal variations and chemical contaminations[6].

Understanding the distribution of FAs in microalgae is essential owing to numerous health benefits they possess[7]. Long chain poly unsaturated FAs (LC-PUFA) were reported to have significant anti- bacterial activity compared to saturated FAs[8]. But, studies exhibiting the spectrum of activities shown by microalgal FA fractions are limited. Therefore, this study focuses on the fatty acid composition of four microalgae and their spectrum of activities against selected clinical and fish pathogens. We have tried to analyse the activity variations of each fraction in connection with their unsaturation patterns.

MATERIALS AND METHODS

Algal growth and experiment design

Microalgae, *Chlorella marina, Chaetoceros affinis* and *Nannochloropsis occulata* were isolated from Cochin Estuary, a tropical ecosystem, South west coast of India. Isolated microalgal strains were cultured in 2L of sterilized water of desirable salinity, enriched with suitable nutrient media. Details of media and respective conditions for each species are presented in Table 1. The microalgae were harvested in the stationary phase.

Extraction of fatty acids

Fatty acid composition of dried algal biomass was determined and expressed as FA methyl esters (FAME)[11,12]. Lyophilized microalgal samples were ultrasonicated with DCM : Methanol (2:1) and the solvent was removed under reduced pressure. The lipid extract thus obtained was hydrolysed using 6% methanolic KOH to convert fatty acids into their potassium salts. Neutral lipids were separated by sovent extraction. Polar fraction was acidified with 6 N HCl and the resulting free fatty acids were extracted with CH_2Cl_2. Crude fatty acid samples obtained from *Chlorella marina*, *Nannochloropsis occulata* and *Chaetoceros affinis*, were named as FA1, FA2 and FA3 respectively.

Gas chromatographic–mass spectrometric analysis

Dried fatty acid samples were converted into fatty acid methyl esters (FAME) using BF_3/methanol and used for GC-MS analysis. GC-MS analysis was performed on gas chromaotography (Perikin Elmymer Clarus 680) coupled with mass spectrometer (Perikin Elmymer Clarus 600). 1µL of sample was injected to GC equipped with non-polar HP ultra-double fused silica capillary column. Initially, the temperature was increased from 50 °C to 200 °C at a rate of 2 °C per min and held at 200 °C for 5 min. Then, the temperature was again increased from 200 °C to

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Table 1: Growth conditions of selected microalgae.

Scientific name /Class	Media	Temperature (ºC)	Salinity(psu.)	Light:Dark (hrs.)	Illustration
Chorella marina (Chlorophyceae)	Walne's [9]	22	30	12:12	
Chaetoceros affinis (Bacillariophyceae)	F/2 [10]	22	30	12:12	
Nannochloropsis occulata (Eustigmatophyceae)	F/2 [10]	22	30	12:12	

Table 2: Fatty acid composition of selected microalgae.

Fatty acid	Relative %		
	Chlorella marina FA1	*Nannochloropsis occulata* FA2	*Chaetoceros affinis* FA3
12:0	0.21	0.21	0.27
14:0	15.16	5.54	5.15
16:0	27.66	30.22	31.47
18:0	1.85	2.14	3.27
14 : 1 n-7	0.13	0.12	0.31
16 :1 n-7	1.33	18.23	18.91
18 :1 n-9	21.66	6.58	5.94
18:1 n-11	0.89	n.d	n.d
18:2 n-6	20.48	3.63	4.48
18:3 n-3	2.45	1.12	2.31
20:4 n-6	0.21	4.9	1.81
20:5 n-3	5.23	27.39	23.54
22:6 n-3	3.63	0.63	0.87
ΣSFA	44.88	38.11	40.16
ΣMUFA	24.01	24.93	24.85
ΣPUFA	32	37.67	33.01
ΣEPA + DHA	8.86	28.02	24.41
Σ3-Hydroxy Fatty acids	n.d	n.d	n.d
U.I.[A]	120.32	195.88	171.21

[A]U.I. (unsaturation index) was calculated by multiplying the percentage of each fatty acid by the number of double bonds followed by summing up these contributions.

280 °C at a rate of 10 °C per min and held at 280 °C for 10 min. MS operating parameters were as follows: ionization energy - 70 eV; ion source temperature - 200 °C, solvent delay - 4 min and scan range 40–600 u. Identification of the components was based on direct comparison of mass spectral data with NIST library version 2.1

Assay for antimicrobial effect of fatty acid fractions through disc diffusion

Bacterial culture

Clinical pathogens such as *Enterobacter spp, Escherichia coli, Klebsiella pneumonia, Vibrio cholera, Streptococcus, Proteus* spp., *Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Shigella sonnaie* and fish pathogens such as *Vibrio alginolyticus, Vibrio parahemolyticus* and *Aeromonas* sp. were selected for antibacterial activity studies. Collections were reconstituted in Muller-Hinton's broth and cultured under aerobic conditions at 37 °C and 200 rpm to reach exponential growth. The concentration (107 colony-forming units (CFU)/mL) was routinely estimated by spectrophotometric turbidity measurement at 600 nm on a UV/Vis spectrophotometer (Spectro 200 Plus) and by CFU counts on tryptic soy agar (TSA). Microorganism suspension was smeared thoroughly on the test media before assaying the activity of fatty acid samples.

Disc diffusion assay

Samples were dissolved in DMSO and evaluvated for its antimicrobial activity. Bacterial cultures in exponential growth phase (108 cells mL^{-1}) were used for cultivation in Muller- Hinton agar plates. 20 µL each of the fatty acid fractions (250 µg fatty acid/disc), were applied in sterile filter paper discs of 6 mm diameter and then placed in the agar medium of bacterial culture. All the plates were incubated at 37 °C for 24 h. Antibacterial activities of the test fatty acids were observed through zone of inhibition (in mm) on the plates. Assays were performed in duplicate with two repetitions.

RESULTS

Fatty acid profiling

Fatty acid compositions of the microalgal species are shown in Table 2. Palmittic acid (C16:0) was found to be the major component in all the three crude fatty acid fractions. In *Chlorella marina* (FA1), the major fatty acids like palmitic acid (C16:0), oleic acid (C18:1 n9), linoleic acid (C18:2 n6) and myristic acid (C14:0) were estimated to be in the range of 27.66%, 21.66%, 20.48%, 15.16% of total fatty acid content respectively. In *Chlorella marina*, both oleic and linoleic acids were found to have similar abundance. Eicosapentaenoic acid (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3) were also oserved in small amounts, 5.23 % and 3.63% of total FA content respectively. α-linolenic (18:3 n3), palmitoleic (16:1 n7) and stearic (18:0) acids were also observed in minor amounts viz. 2.45%, 1.33% and 1.85% of total fatty acid content respectively.

Nannochloropsis occulata (FA2) had high amount of C16:0 (30.22 %), C20:5 n-3 (27.39), C16:1 n-7 (18.23%) and C20:4 n-6 (4.9 %). The relative abundance of fatty acids in this species showed that EPA content is fairly high such that it is near to the most abundant palmitic acid. The major fatty acids in FA3 obtained from the diatom *Chaetoceros affinis*, were C16:0 (31.47%), C20:5 n-3 (23.54) and C16:1 n-7 (18.91%). Small quantities of C18:2 n-6 (4.48%), C14:0 (5.15%), 18:1 n-9 (5.94%) and 18:3 n-3 (2.31%) were also found in FA3 fraction. Higher abundance of EPA was also observed in FA3 fraction but

Table 3: Antimicrobial activity of fatty acid fractions against Clinical and fish pathogens.

S.No	Pathogens	Inhibition zone diameter in mm		
		Chlorella marina FA1	*Nannochloropsis occulata* FA2	*Chaetoceros affinis* FA3
1	*Enterobacter spp*	8.2	14.8	14.4
2	*Escherichia coli*	9.3	16.4	15.8
3	*Klebsiella pneumoniae*	9.1	15.7	14.9
4	*Vibrio cholera*	10.9	21.8	20.9
5	*Streptococcus*	11.2	20.4	18.7
6	*Pseudomonas aeruginosa*	12.4	21.5	21.1
7	*Salmonella typhi*	8.5	14.2	13.6
8	*Staphylococcus aureus*	9.1	20.8	18.4
9	*Shigella sonnei*	8.4	16.3	15.7
10	*Proteus spp*	8.7	14.0	13.7
11	*Aeromonas*	12.5	21.5	20.6
12	*Vibrio alginolyticus*	11.6	22.3	21.2
13	*Vibrio parahaemolyticus*	10.8	23.1	20.7

it less than that of FA2. The mass spectra comprising a unique combination of m/z peaks such as m/z 79 (base peak) along with m/z 93, and 107 gave inference to the poly unsaturated fatty acids, particularly a series of methylene interrupted polyenes (Figure 1)[13].

Antibacterial assay

Inhibition zone diameter (mm) of fatty acid fractions against selected bacteria are given in Table 3. Highest activity was shown by fatty acid fraction FA2 derived from *Nannochoropsis occulata* against all the pathogens selected as shown in Figure 2. Higher zone inhibitions (21.5-23.1 mm) were detected for the fish pathogens (*Vibrio alginolyticus*, *Vibrio parahemolyticus* and *Aeromonas* sp). Among the clinical pathogens, greater inhibitions were observed towards the gram negative, *Vibrio cholerae* (21.8 mm) and *Pseudomonas aeruginosa* (21.5 mm), and also towards the gram positive *Streptococcus* sp. (20.4 mm), and *Staphylococcus aureus* (19.8 mm).

Fally acid fraction FA3 from *Chaetoceros affinis* also showed comparable but slightly lesser inhibition than that of FA2 fraction. FA3 also showed higher zone of inhibition towards the fish pathogens (20.6-21.2 mm). Among clinical pathogens, FA3 showed considerable inhibition towards gram negative, *Vibrio cholerae* (20.9 mm) and *Pseudomonas aeruginosa* (21.1 mm), and the gram positive *Streptococcus* sp. (18.7 mm) and *Staphylococcus aureus* (18.7 mm).

Chlorella marina fatty acid fraction FA1 showed much less inhibition, while comparing antibacterial activities of other two fractions. The inhibition diameters of FA1 against the fish pathogens range from 10.8 to 12.5 mm. FA1 also showed minor activity towards *clinical pathogens* (8.2-12.4 mm).

DISCUSSION

Extensive studies were carried out for the fatty acid composition of microalga, but studies exhibiting their spectrum of activities are still limited. It was suggested that mixture of fatty acids act synergistically to inhibit bacterial growth and thus more numbers of bacterial strains might be susceptible to fatty acid mixtures compared to individual fatty acid[14]. Microalgae can be used as potential tool for examining antibacterial potential of mixture of fatty acids as the individual members possess a very unique fatty acid composition depending on taxonomic category and growth conditions[15]. In the present study *Nannochloropsis occulata* and *Chaetoceros affinis* were found as eminent producers of EPA. Stationary phase of microalgal cultures were known to be associated with high amount PUFA[16] compared with other growth phases. Therefore, all the fatty acid fractions were taken from stationary phase of algae culture. Hence, percentage abundance of PUFA obtained is assumed as the maximum for those species under the selected growth conditions.

It has been reported that antibacterial and antifungal effect of long-chain saturated fatty acids like 16:0 and 18:0 acids is less than that of long-chain unsaturated fatty acids[17,18,19]. This might be the reason for highest antibacterial activity shown by FA2 fraction derived from *Nannochoropsis occulata*, which is characterised by highest unsaturation index (195.88) compared to other species under study. The class *Eustigmatophyceae*, in which *Nannochloropsis occulata* belongs, are known as good source of EPA[20]. The greater percentage of EPA (20:5 n-3) along with other long chain FAs (18:1 n-9, 18:2 n-6, 18:3 n-3) might be responsible for the increased rate of inhibition. n-3 PUFAs especially EPA and DHA are known to possess biological activities such as antibacterial, antiviral and antifungal effects[21,22]. Higher zone of inhibition shown by FA2 fraction towards the fish pathogens is in accordance with the finding that unsaturated long chain fatty acids possess antibacterial activity against aquaculture bacteria such as *Listonella anguillarum* and other *Vibrio spp*.[14]. FA3 fraction from the diatom *Chaetoceros affinis* also showed significant activity which might be due to the high EPA content and unsaturation index of 171.21 in the fatty acid profile. The diatoms *Phaeodactylum tricornutum* and *Chaetoceros calcitrans* are known as eminent producers of EPA[16,23,24]. *Chlorella marina* fatty acid fraction FA1 showed least activity while comparing the antibacterial inhibition of crude fatty acid fractions. The unsaturation index of FA1

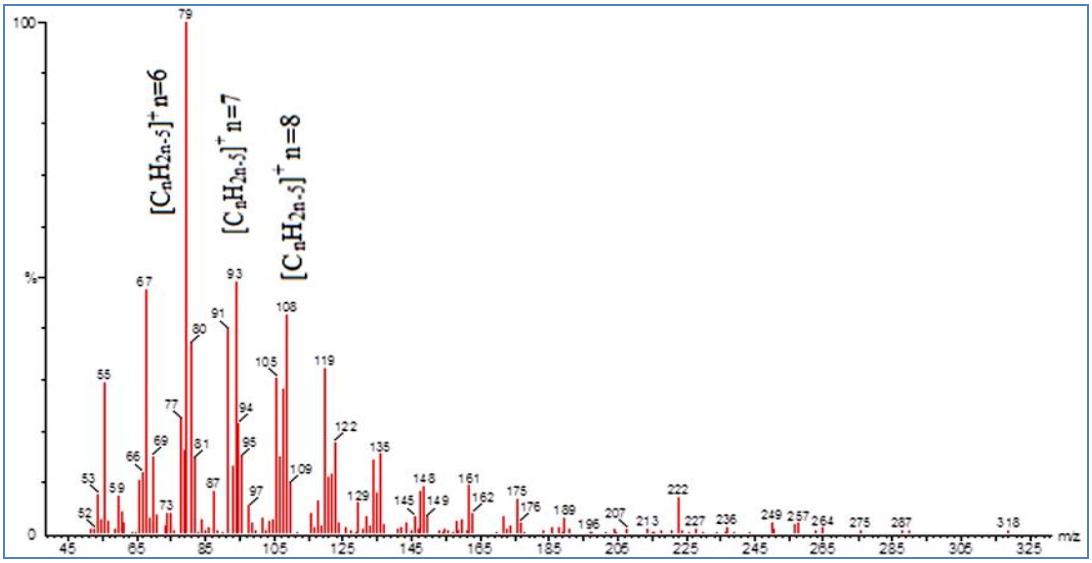

Figure 1: A representative mass fragmentation pattern of polyunsaturated fatty acid, methyl 5,18, 11, 14, 17 eicosapentaenoate, having base peak at *m/z* =79.

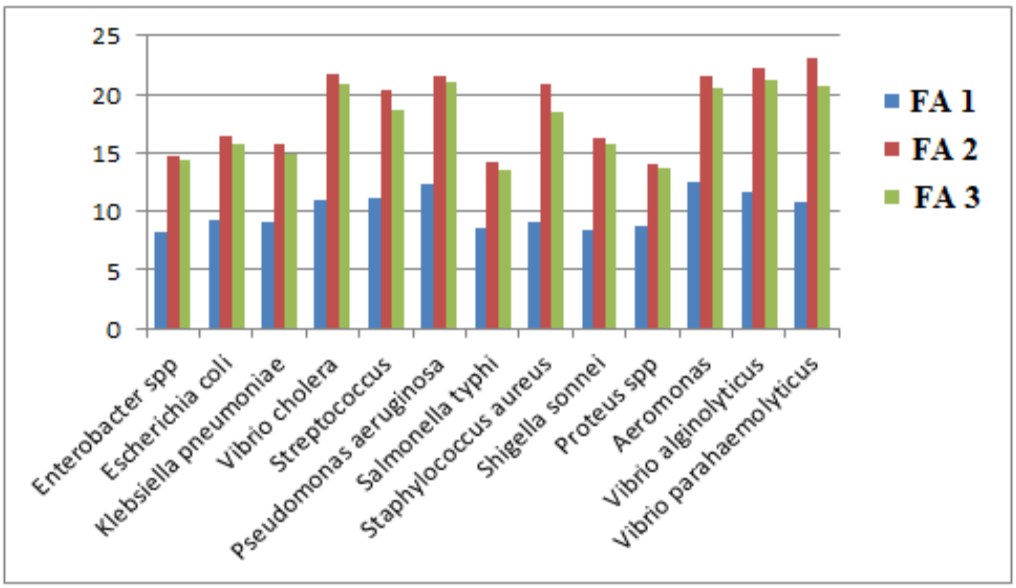

Figure 2: Antibacterial activity of fatty acid fractions from *Chlorella marina* (FA1), *Nannochloropsis occulata* (FA2) and *Chaetoceros affinis* (FA3).

(120.32) fraction is less compared with FA2 and FA3, which is due to the less EPA abundance in that fraction. Linoleic acid (18:2 n6), the major contributor for PUFA content in *Chlorella marina* along with small quantities of EPA (20:5 n3), DHA (22:6 n3) and ALA (18:3 n3) might be responsible for the minor antibacterial activity shown by FA1. Linoleic acid is reported to have activity against a few bacterial pathogens in the free form and also in combinations[25,26].

CONCLUSION

In conclusion, the fatty acid fraction separated from *Nannochloropsis occulata*, possessing high concentration of EPA was found to have the highest antibacterial activity and can be suggested as an important source for the development of new antibacterial drugs. Bactericidal activities shown by the fatty acid fractions were dependent on the abundance of long chain unsaturated fatty acids. Activities may vary depending on the matrix associated with the fatty acids and hence the antibacterial activities of fatty acid fractions from different microalgae under different growth conditions are still need to be explored.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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