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Communication

Isolation and Fatty Acid Profile of Selected Microalgae Strains from the Red Sea for Biofuel Production

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Abstract: The isolation of lipid-rich autochthonous strains of microalgae is a crucial stage for the development of a microalgae-based biofuel production plant, as these microalgae already have the necessary adaptations to withstand competition, predation and the temperatures observed at each production site. This is particularly important in extreme climates such as in Saudi Arabia. Resorting to fluorescence activated cell sorting (FACS) we screened for and isolated several microalgal strains from samples collected from the Red Sea. Relying on the fluorescence of BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazasindacene) and growth performance, four promising candidates were identified and the total lipid content and fatty acid profile was assessed for biofuels production. Selected isolates were classified as chlorophytes, belonging to three different genera: *Picochlorum*, *Nannochloris* and *Desmochloris*. The lipid contents were assessed microscopically by means of BODIPY 505/515-associated fluorescence to detect

intracellular lipid bodies, which revealed several lipid drops in all selected strains. This result was confirmed by lipid gravimetric determination, which demonstrated that all strains under study presented inner cell lipid contents ranging from 20% to 25% of the biomass dry weight. Furthermore, the fatty acid methyl esters profile of all strains seems ideal for biodiesel production due to a low degree of polyunsaturated fatty acid methyl esters and high amount of palmitic and oleic acids.

Keywords: biofuels; BODIPY; FACS; FAME; microalgae; Red Sea; Saudi Arabia

1. Introduction

The continuous instability observed in the price of crude oil and the global dependence of civilization on electricity production and generation of liquid fuels has triggered the search for renewable sources of energy. Liquid fuels obtained from biomass, such as biodiesel obtained from oleaginous terrestrial plants and bioethanol converted from sugars of corn, starch or sugarcane are considered efficient at a small scale. However, large scale biofuel production from edible feedstocks able to compete with and eventually replace fossil fuels, would require a vast area of agricultural land, which is unsustainable due to its negative impact on food and feed supply [1].

Lately, the Gulf Cooperation Council (GCC) countries and especially the Kingdom of Saudi Arabia (KSA) engaged in a sustained effort to diversify its sources of energies, increasing the share of renewable energy in their energetic envelope (*i.e.*, solar, wind and renewable feedstock). These initiatives cover the entire chain of development and implementation of renewable energies in the kingdom through incentives for research, demonstration and transfer of developed technologies to industries [2].

The KSA, with its large area of non-arable land and extensive coastline, coupled to the numerous energy plants (as points of CO_2 capture), desalination plants (for salt reuse) and the extremely favorable climatic conditions (both limited overcast days and cold weather), is proposing to develop an attractive Research, Development and Market Deployment (R&D&D) plan for a complete microalgal feedstock-based biorefinery approach for biofuel production in the Kingdom.

Indeed, microalgae are considered one of the most promising feedstocks of biomass for large scale production of biofuels [3,4]. Microalgae are a group of usually unicellular eukaryotic organisms highly biodiverse, containing a wide array of biochemicals that can be used in biotechnological applications [5,6]. Although the total number of strains is unknown, more than 30,000 species have already been identified [7]. Several strains of these unicellular organisms are known to accumulate a significant amount of lipids, mainly in the form of triacylglycerol [8]. It has been estimated that microalgal lipid productivity can be significantly higher than that of the most productive terrestrial plant, oil palm [3,9]. In addition, microalgae can grow on seawater or wastewater and production facilities may be installed on coastal waters or on non-arable land (including deserts), decreasing the demand for freshwater and agricultural land [3].

Screening for novel strains of microalgae with features ideal for biofuel production, namely cells able to accumulate significant lipid contents, with high growth rates and capable of withstand diverse

environmental conditions [10], is an important step for the successful production of microalgae-based biofuels. Moreover, the isolation of autochthonous strains of microalgae is key for large scale biomass production ventures, since strains isolated close to the area of production are already adapted to the surrounding environment, reducing also any environmental impact that could occur if allochthonous strains were introduced for mass cultivation either in closed or open growth systems.

In this work, we report the isolation of microalgal strains from environmental water samples collected off Al-Lith in the Red Sea (west coast of Saudi Arabia) by flow cytometry with activated cell sorting (FACS). Moreover, we performed the identification of four selected strains by rDNA sequencing and assessed the total lipid content and potential of the FAME profile for biofuel production.

2. Results and Discussion

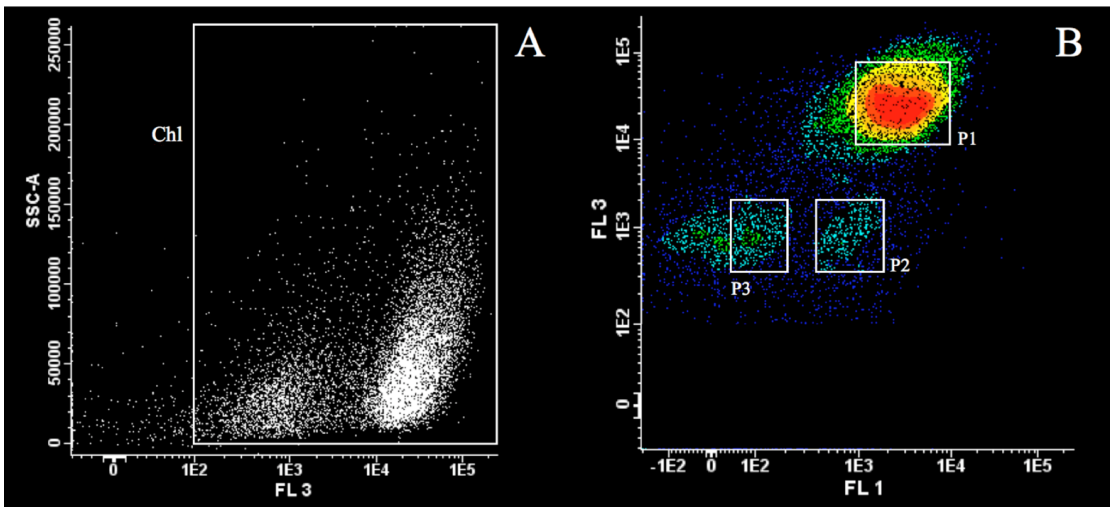
2.1. Pre-enrichment of Cultures

The pre-enrichment step allowed strains with higher growth rates to out-compete other strains of less interest. Therefore, this enrichment step is crucial to screen a taxonomically diverse pool of cells present in the original sample for a limited number of strains which in fact hold promise for large scale production of microalgal biomass for biofuel production.

2.2. Isolation of Strains by FACS

As previously demonstrated [11–13], FACS is an efficient and practical tool for the isolation of marine microalgae from an environmental sample. After the enrichment step, samples were stained with BODIPY 505/515 dye and acquired by fluorescence activated cell sorting (FACS). During sample acquisition several two-dimensional plots registered the distribution of cells among the forward scatter (FSC), side scatter (SSC) and all fluorescence channels (FL1, FL2 and FL3). In all samples the first sorting trait applied was performed using a dot plot combining inner cell complexity (SSC) and the endogenous fluorescence of cells with chlorophyll (FL3). Separation of photosynthetic cells from bacteria and debris was achieved by means of the Chl gate (Figure 1A). The combination of variables displaying the highest cluster separation, coupled with the BODIPY fluorescence signal, was used. In the example given (Figure 1B), we used the combination of FL3 and FL1 channels, which relates the fluorescence of chlorophyll and the maximum emission of BODIPY fluorescence. Clearly, three different clusters/populations were separated from each other by this combination of signals. Therefore, three gates were drawn (P1, P2 and P3) and the corresponding cells were sorted onto solid and liquid media. Upon incubation for two weeks the cultures were later scaled up into higher volumes for further analysis.

Figure 1. Gating procedure used for the isolation of microalgal strains from one of the environmental samples collected in the Red Sea by fluorescence activated cell sorting. (**A**) Dot plot relating the inner cell complexity (SSC-A) of cells and chlorophyll auto-fluorescence (FL3) and the Chl gate selected to maximize the isolation of photosynthetic cells; (**B**) Density plot combining the FL3 channel with BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene) dye maximum fluorescence emission (FL1), which was used to gate and successfully separate three different populations/strains (P1, P2 and P3).



2.3. Identification of Strains by 18S rDNA Sequencing

The 18S rDNA gene is commonly used for the identification of micro-eukaryotes providing accurate results [14,15]. The internal transcribed spacer 2 (ITS2) and the adjacent partial sequences of the 5.8S and 28S improved the final genetic analysis, providing additional confirmatory data. Sequencing results revealed that all strains isolated are chlorophytes, belonging to three different genera: *Picochlorum*, *Nannochloris* and *Desmochloris* (Table 1). Two *Nannochloris* strains presented very similar 18S rDNA sequences. However, the latter strains displayed distinct cell morphologies: *Nannochloris* sp. SBL1 cells were rod-shaped, showing some similarity to *Nannochloris bacillaris* [16], whereas *Nannochloris* sp. SBL4 presented rounded or slightly oval cells, as commonly found in this genus. The pre-enrichment step using ALGAL medium appeared to favour the isolation of green microalgae. Chlorophyta is considered to be a promising phylum for biofuel production, due to the high growth rates and ease of cultivation. In order to isolate strains from other phyla a different enrichment and gating procedure would be required [13].

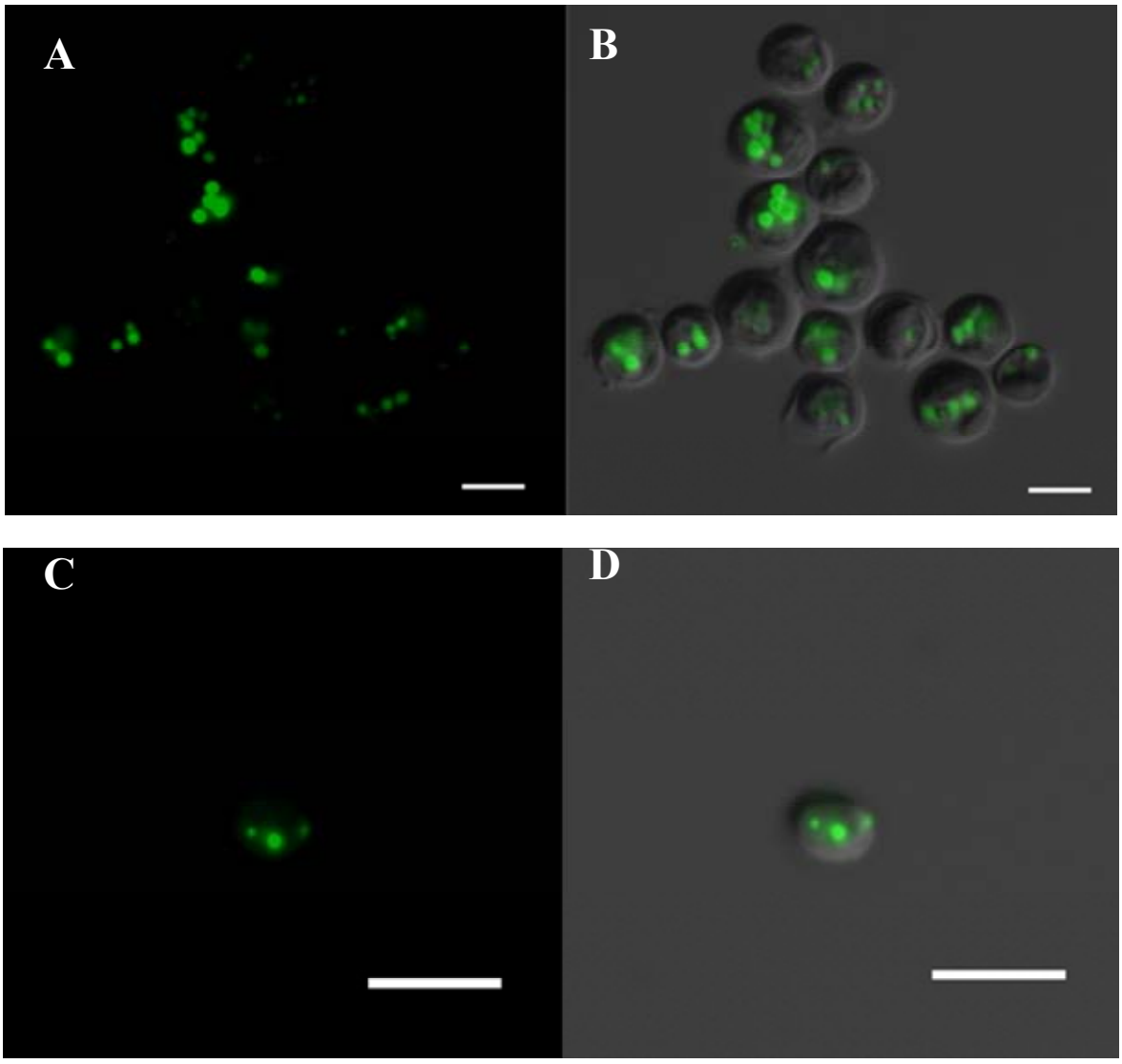
Table 1. Obtained strains, corresponding phylum and the rDNA marker used for identification of cultures acquired by FACS. PS: partial sequence.

Strain	Phylum	rDNA Marker
Nannochloris sp. SBL1	Chlorophyta	18S (PS)
Picochlorum sp. SBL2	Chlorophyta	18S (PS), 5.8S (PS), ITS2, 28S (PS)
Desmochloris sp. SBL3	Chlorophyta	18S (PS), 5.8S (PS), ITS2, 28S (PS)
Nannochloris sp. SBL4	Chlorophyta	18S (PS)

2.4. Fluorescence Microscopy of Cells Stained with BODIPY 505/515

BODIPY effectively stains the lipid bodies in cells and has been successfully used in microalgae [13,17]. Therefore, to address the lipid-producing potential of the isolated strains and confirm the results obtained by FACS, fluorescence microscopy upon BODIPY staining was performed (Figure 2). The obtained images using the FITC filter revealed that all isolated strains internalized BODIPY dye in distinct lipid bodies. The *Desmochloris* sp. (SBL3) strain, shown in Figure 2A,B, presented a high amount of lipid droplets in several observed cells. *Picochlorum* sp. SBL2 also displayed several lipid bodies in all observed cells (Figure 2C,D).

Figure 2. *Desmochloris* sp. SBL3 (**A** and **B**) and *Picochlorum* sp. SBL2 (**C** and **D**) internalized BODIPY dye in distinct lipid bodies. BODIPY dots could be observed with the FITC filter. Panels B and D were obtained by means of differential interference contrast (DIC). All images were acquired using the 100× objective and treated with Image J software (DIC in grey, BODIPY in green; Scale bar = 5 μm).



2.5. Total Lipid Content and Fatty Acid Profile

The total lipid content was determined gravimetrically and ranged from 20% to 25% of the biomass dry weight (Figure 3). *Picochlorum* sp. SBL2 registered the highest lipid content (25.28% ± 2.38%) and *Nannochloris* sp. SBL1 (19.69% ± 2.19%) showed the lowest content among all strains; *Nannochloris* sp. SBL4 and *Desmochloris* sp. SBL3 displayed 22.65% ± 2.21% and 23.16% ± 3.00%, respectively. The total lipid content of *Nannochloris* strains are in agreement with the range of values reported by Park *et al.* [18] for *Nannochloris oculata,* but lower than the values reported by Takagi *et al.* [19] and Chisti [3] for *Nannochloris* sp. The isolated strain of *Picochlorum* sp. showed a mean lipid content of 25%, slightly higher than the values reported by De la Vega *et al.* [20], who reported values between 20% to 23% of biomass dry weight. Oleaginous chlorophytes are known to accumulate significant amounts of lipids showing an average lipid content of 25% of dry weight [8]. Furthermore, culture conditions can increase the lipid content significantly in many microalgae strains since stress conditions are known to favour lipid induction.

Microalgal oil can be converted by transesterification into biofuel, which corresponds to a mixture of fatty acid methyl esters (FAME). Saturated (SFA) and monounsaturated fatty acids (MUFA) were the main FAME detected in the profile of isolates, predominantly palmitic (C16:0) and oleic (C18:1) acids, which accounted for more than 50% of the total FAME profile (Table 2). *Picochlorum* sp. and *Desmochloris* sp. also presented linoleic acid (C18:2) as a major FAME, representing 24 and 29% of the total FAME, respectively. Both *Nannochloris* strains registered a low percentage of polyunsaturated fatty acids (PUFA), 20% in *Nannochloris* sp. SBL1 and 15% in *Nannochloris* sp. SBL4. The low degree of unsaturation found in these strains is an encouraging feature for biofuel production. The unsaturation of the FAME profile is crucial for the overall performance of the final produced biofuel. For instance, biodiesel is mainly constituted of SFA and MUFA, since PUFA decrease the final stability of biodiesel [21]. α-Linolenic acid (C18:3) was only present in *Desmochloris* sp. SBL3 at a minor concentration (2%); interestingly, highly unsaturated PUFA with ≥4 double bonds were completely absent in the FAME profile of all isolates. These values are in accordance with the European standard EN14214 [22], which states a maximum of 12% for α-linolenic acid and 1% for PUFA with ≥4 double bonds. Regarding the size of the carbon chain, isolates displayed a FAME profile ranging from C_{15} to C_{18}, dominated by C_{16} and C_{18} FAME. These main FAME are the same that compose soy biodiesel [23], proving the suitability of the FAME profile of these isolates for biodiesel production. The calculated iodine values are also in accordance with European standards presenting values lower than 120 g I_2/100 g. Indeed, *Picochlorum* sp. and *Nannochloris* sp. were considered previously as promising feedstocks for large scale production of biofuels [18,20].

Figure 3. Gravimetric determination of total lipid content (%) determined with the Bligh & Dyer method in all isolated strains. Error bars represent the standard deviation obtained from four replicates.

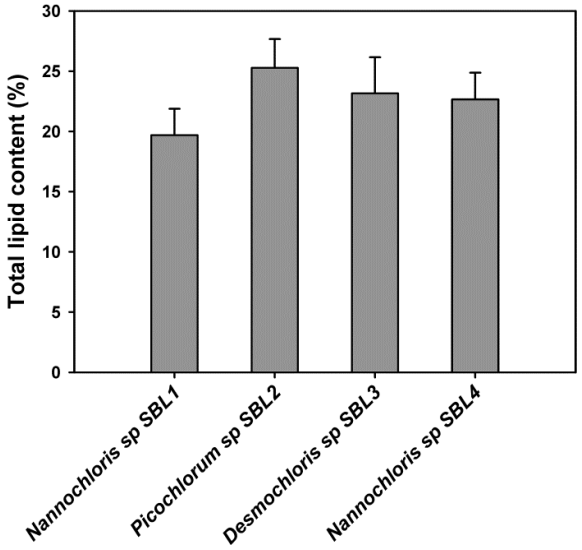


Table 2. Fatty acid profile and iodine value of the strains studied throughout this work. Given values are expressed as mean ± standard deviation. n.d.: not detected (n = 4).

Fatty acid (%)	***Nannochloris* sp. SBL1**	***Picochlorum* sp. SBL2**	***Desmochloris* sp. SBL3**	***Nannochloris* sp. SBL4**
C15:0	n.d.	n.d.	4.19 ± 0.20	n.d.
C16:0	32.48 ± 3.24	26.16 ± 2.03	33.63 ± 1.65	28.52 ± 1.18
C18:0	2.28 ± 0.30	3.22 ± 0.60	1.94 ± 0.59	n.d.
∑ SFA	34.77 ± 3.55	29.37 ± 2.62	39.76 ± 2.44	28.52 ± 1.18
C16:1	12.19 ± 1.63	12.05 ± 1.26	n.d.	17.10 ± 1.15
C18:1	33.82 ± 2.28	21.96 ± 1.39	28.17 ± 1.22	40.41 ± 4.02
∑ MUFA	46.00 ± 3.91	34.01 ± 2.65	28.17 ± 1.22	57.51 ± 5.16
C16:2	n.d.	7.18 ± 0.20	n.d.	n.d.
C16:3	3.80 ± 0.48	5.68 ± 0.37	1.32 ± 0.07	4.46 ± 0.64
C18:2	15.44 ± 2.12	23.76 ± 0.36	28.55 ± 2.23	9.50 ± 0.59
C18:3	n.d.	n.d.	2.21 ± 0.08	n.d.
∑ PUFA	19.23 ± 2.60	36.62 ± 0.93	32.07 ± 2.38	13.97 ± 1.23
Iodine value	86	99	84	88

3. Experimental Section

3.1. Sampling and Storage

Several water samples were collected during May 2012 in the Red Sea, near the coast off Al-Lith, south of Jeddah, and stored in specialized axenic bottles. Upon collection of samples and in order to isolate strains with potential for large-scale production, a modified ALGAL culture medium (1 mM $ZnCl_2$; 1 mM $ZnSO_4.H_2O$; 1 mM $MnCl_2.4H_2O$; 0.1 mM $Na_2MoO_4.2H_2O$; 0.1 mM $CoCl_2.6H_2O$; 0.1 mM $CuSO_4.5H_2O$; 20 mM EDTA-Na; 2 mM $MgSO_4.7H_2O$; 20 mM $FeCl_3$; 2 M $NaNO_3$; 100 mM

KH_2PO_4) [24] was added to each bottle using a 1:1000 dilution. Tubes were maintained at ambient conditions for two weeks until the isolation procedure (pre-enrichment step).

3.2. Isolation of Microalgae Strains by Fluorescence Activated Cell Sorting

After the enrichment with ALGAL culture medium, different strains were isolated as described in Pereira *et al.* [13]. Briefly, the water sample (5 mL) was stained with BODIPY 505/515 (Life Technologies Europe BV, Porto, Portugal) as described by Cooper *et al.* [17] to attain a final concentration of 1 μM of BODIPY dye. After the addition of the dye, tubes were vortexed for 1 minute and were subsequently incubated in the dark for 10 minutes.

Upon staining, samples were acquired in a BD FACS Aria II (BD Biosciences, Erembodegem, Belgium) using FACS Diva software (Version 6.1.3) and treated with Infinicyt 1.5.0 (Cytognos S.L., Santa Marta de Tormes, Spain). Excitation of cells was performed using the blue laser (488 nm), whereas the emission signal was registered in two channels: FL1 channel centred at 530/30 nm and FL3 channel centered at 695/40 nm. The sheath fluid used in all sorting procedures was filter-sterilized seawater.

3.3. Identification of Strains by rDNA Sequencing

Identification was performed using molecular tools, resorting to the amplification by PCR of ribosomal DNA. For the identification of all isolates two different markers located in the ribosomal DNA transcribed genomic region were used, by means of 18S, 5.8S, ITS2 and 28S rDNA sequencing as described by Pereira *et al.* [13]. The DNA was extracted using the manufacturer's procedure with a commercial kit supplied by E.Z.N.A. (DNA plant extraction kit). The isolated DNA was amplified by PCR using specific primers for each rDNA marker (Table 3). Sequencing was performed at Macrogen Europe.

Table 3. Primer name, sequence and corresponding gene locus used in the identification of strains.

Gene locus	Primer name	Sequence (5' to 3')
18S	18SUnivFor	ACCTGGTTGATCCTGCCAGT
18S	18SUnivRev	TCAGCCTTGCGACCATAC
5.8S, ITS2, 28S	5.8SFor	AAGAACGCAGCGAAATGC
5.8S, ITS2, 28S	28SRev	GACTCCTTGGTCCGTGTTTC

3.4. Microscopy

The staining procedure used for microscopy was the same as described for the isolation by FACS. The microscope used was a Carl Zeiss AXIOMAGER Z2, equipped with a coollSNApHQ2 camera and AxioVision software (Version 4.8). Images were acquired with the 100x lens, using differential interference contrast (DIC) for the transmitted light images. Fluorescein isothiocyanate (FITC; Zeiss 38 He filter set) filter was used for the acquisition of fluorescent images. Treatment of images was performed using Image J software.

3.5. Assessment of Total Lipid Content and Fatty Acid Profile by Gas Chromatography Coupled with Mass Spectrometry

The total lipid content was determined using a modified protocol of the highly cited Bligh and Dyer method [25]. Concisely, a mixture of methanol, chloroform and water (2:2:1) was added to 100 mg of algae biomass and ground with an IKA Ultra-Turrax disperser. Upon homogenization, the phase separation was achieved by centrifugation and a known volume of the organic phase was transferred into new pre-weighed tubes. Finally, the solvent was evaporated in a gentle nitrogen flow and the tube was weighed again to estimate the lipid content.

The FAME profile was determined using a modified protocol from Lepage and Roy [26], described in Pereira *et al.* [27]. Briefly, 0.1 g of culture was homogenized with an IKA Ultra-Turrax disperser in a mixture of acetyl chloride and methanol (20:1, v/v) in reaction vessels. Subsequently, 1 mL of hexane was added to the mixture and heated to 100 °C for 1 hour. After the derivatization, 1 mL of distilled water was pipetted into the mixture and the organic phase was separated by centrifugation and dried with anhydrous sodium sulphate.

Upon preparation, the extracts were injected in a Varian 450-GC/240-MS (Varian 450-Gas Chromatograph/240-MS IT Mass Spectrometer, Varian Inc., Palo Alto, CA, USA), equipped with a BR-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness, Bruker). The injection temperature was set to 300 °C, while the trap, manifold and transfer line were set to 22, 50 and 250 ºC, respectively. Helium was the carrier gas and an optimized temperature program for the GC oven was used as follows, 60 °C (1 min), 30 °C·min^{-1} to 120 °C, 4 °C·min^{-1} to 250 °C, 20 °C·min^{-1} to 270 °C, and 2.5 °C·min^{-1} to 300 °C.

4. Conclusions

Four microalgal strains were successfully isolated from the Red Sea, identified and selected for scale-up on the basis of lipid-associated BODIPY fluorescence. Fluorescence microscopy and gravimetric measurement exhibited significant content of lipids in all isolates (20%–25% DW). It is noteworthy that the presented lipid contents were established under optimal growth conditions; optimization of stress conditions for lipid induction may further increase the lipid content of cultures. All strains presented a low degree of unsaturation with palmitic and oleic acids as their main fatty acids. Hence, regarding their lipid content and fatty acid profile, all isolates can be considered as suitable feedstocks for the production of lipid-based biofuels, namely biodiesel.

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Conflict of Interest

The authors declare no conflict of interest

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