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Bioprospecting autochthonous marine microalgae strain from the Arabian Gulf Seawater, Kuwait for biofuel feedstocks

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Abstract

Bioprospecting programs are the key to increasing the current portfolio of indigenous microalgal strains accessible for different applications in microalgal biotechnology. In this work, nine fast-growing microalgal strains isolated from Kuwait's Arabian/Persian Gulf coastal waters were evaluated for their potential as biofuel feedstocks. 18S rRNA gene sequencing showed that the strains belong to five different genera: *Chlorella*, *Nannochloris*, *Scenedesmus*, *Tetraselmis*, and *Nannochloropsis*. In terms of the total lipid content, in comparison to the other strains, *Tetraselmis* sp. KUBS13G and *Tetraselmis* sp. KUBS16G displayed higher lipid contents of 29.56% dry weight (DW) and 26.13% DW, respectively, dominated by palmitic and oleic acids. Fuel properties calculated from the fatty acid methyl esters (FAMEs) by empirical equations were compared with EN14214 (European) and ASTM D6751-−02 (American) biodiesel standards. Multicriteria decision analysis (MCDA) methods, such as the Preference Ranking Organization Method for Enrichment Evaluation (PROMETHEE) and Graphical Analysis for Interactive Assistance (GAIA), were used to select suitable microalgae for biofuel feedstock based on their biodiesel fuel properties. Overall, the results suggested that the indigenous microalgal strain *Tetraselmis*, particularly *Tetraselmis* sp. KUBS37G, and *Scenedesmus* sp. KUBS17R are the most suitable strains for biofuel feedstock owing to their improved fuel properties, such as density (ρ) (0.88 g cm−3), low kinematic viscosity (3.1 mm2 s−1), high cetane number (54 and 56, respectively), high oxidation stability (14.6 hr and 14.8 hr), and cold filter plugging point (1.0°C and -6.1°C).

Keywords: Biofuel; bioprospection; fatty acid methyl ester; microalgae.

1. Introduction

Bioprospecting autochthonous microalgae for various applications has been gaining global attention in recent years. These screening programs are crucial for increasing the portfolio of strains available for renewable energy production, food, cosmetics, and drug discovery. Biodiesel upgraded with microalgae lipids is considered a promising alternative for replacing traditional fossil fuels. It reduces greenhouse gas (GHG) emissions which play an essential role in global climate change (Chisti, 2007). The important fuels synthesized from algae include vegetable oil (Huang *et al*., 2016), biogas, biodiesel (Mata *et al*., 2010, Mondal *et al*., 2017; Mureed *et al*., 2018), biomethanol (Perazzoli *et al*., 2016), bioethanol (Shokrkar *et al*., 2017), biobutanol (El-Dalatony *et al*., 2017), and dry fuel 'coalgae' produced from a combination of waste coal dust and algae (Kucukvar & Tatari, 2011).

Marine microalgae-based biofuel production has critical advantages over terrestrial plant feedstocks since microalgae can be grown on non-arable lands as well as in saltwater and wastewater effluents (Chandra *et al*., 2017, Matsumoto *et al*., 2017). The most challenging step in any microalgae-based biofuel production is the identification and selection of lipid-rich microalgae strains with desirable fatty acid profiles, in addition to good biomass yields. Moreover, the ideal microalgae selected for biofuel production should possess characteristic features such as large cells with thin membranes that excrete oils outside cells; insensitive to high oxygen concentrations; able to produce high lipid yield in high light insensitivity; ability to withstand adverse or stress conditions used to improve the lipid production by induction through altering the external parameters such as salinity, pH or nutrient starvation in terms of phosphates, nitrates limitation condition; settle quickly enabling an easy harvest; and most importantly a robust and stable, resistance to infection from non-algal cross- contaminants (Wijffels & Barbosa, 2010).

In microalgae, lipids are one of the primary metabolites constituting between 5 and 50% of cell dry weight, enriching their utility both for nutritional (polyunsaturated fatty acids -PUFA) and fuel applications (saturated-SFAs, and monounsaturated fatty acids-MUFAs) (Griffiths & Harrison, 2009, Chisti, 2007). In particular, triacylglycerides are the desired component within algal lipids for biodiesel stocks (Hu *et al*., 2008).

With its vast non-irrigated arable lands and surrounding coastlines with highly favorable climatic conditions, Kuwait has limited overcast days, and cold weather offers a variety of niches for biotic communities. These factors indicate the existence of hyper-lipid-producing microalgae

species in Kuwait's coastal waters. However, details about the hyper-lipid-producing microalgae strains that can be used as sustainable lipid feedstock from Kuwait's seawater are still non-existent.

Therefore, in the present work, we isolated dominant indigenous marine microalgae strains from seawater samples collected from different locations in the Arabian Gulf in Kuwait, and genetic identification was determined by partial 18S rRNA gene sequencing. Moreover, the novel isolates' biomass production, total lipid content, and fatty acid methyl ester (FAME) profiles were determined, and the potential biotechnological applications were discussed. To our knowledge, the present study is the first report to address the isolation, identification, and selection of autochthonous hyper lipid microalgae strain from Arabian seawater in Kuwait for biofuel feedstocks through transesterification.

2. Methods

2.1. Environmental sampling and microalgae isolation

Seawater samples (3 km offshore) were collected from 4 m below the water surface at different sampling sites along Kuwait's Arabian Gulf region. Before sampling, the physical parameters (pH, salinity, temperature, conductivity, turbidity, and dissolved oxygen) were recorded at the sampling sites using a water quality checker (U-10, Horiba, Japan). Visible intertidal algal biofilm samples were also collected, added directly to the seawater from the site, and placed into the sample container, and the mixture was sealed. All the collected samples were kept in a cool box, transported to the laboratory, and processed on the same day. Three growth media, F/2 (Guillard's), artificial seawater (ASN), and mineral nutrients (MN), were used (Rippka *et al*., 1979). The seawater samples were filtered using glass filters and 5.0- and 1.0-µm cellulose nitrate membranes. Primary cultures were grown by inoculating 10 ml of both non-filtered and filtered aliquots into 100 ml of liquid media and incubating them at 25±1°C for 12 hr. light (150 µmol photons m^{-2} s^{-1}): 12 h. dark cycles for 4-5 weeks. One milliliter aliquot from the enriched cultures was spread over the F/2, ASN, and MN agar (1.5% w/v) plates and incubated under the same growth conditions mentioned. Upon growth, visible microalgae colonies were picked and sub-cultured three to five times on the same media to obtain pure microalgae strains.

2.2 Genetic identification

According to the manufacturer's instructions, genomic DNA was extracted from the mono microalgae cultures using the DNeasy Plant Mini Kit (Qiagen, Cat# 69104). Eukaryotic 18S rRNA

gene regions were amplified by PCR using universal 18S F and R primers (Pereira *et al*., 2013); the PCR thermal profile consisted of an initial denaturation for 2 min at 95°C and 35 cycles of 30 sec at 95°C (denaturation), 30 sec at 55°C (annealing) and 2 min at 72°C extensions, followed by a final extension of 10 min at 72°C. Portions with 3 µl of the amplification products mixed with 3 µl 6X DNA loading dye were examined by electrophoresis using a 1.5% agarose gel with an addition of 1 µl ethidium bromide (50 µg ml−1). Subsequently, the PCR amplicon (~1650 bp) was purified using the QIAquick PCR purification kit (Qiagen, Cat# 28104) following the manufacturer's instructions. The purified PCR DNA concentration was determined with a NanoDrop 8000. Sequencing cycle reactions were carried out with BigDye® Terminator Cycle Sequencing kit v3.1 (Applied Biosystems Foster City, CA; REF: 4337455) using ¼ X standard reaction mixture followed by sequence product clean-up with BigDye XTerminator™ Purification Kit (REF: 4376486) instructions. Post-sequencing reaction samples were analysed on an Applied Biosystems capillary electrophoresis-based genetic analyser (3130 XL Genetic Analyser, USA).

Forward and reverse FASTA query sequences obtained from the electropherograms from the genetic analyser were aligned in NCBI BLASTn using the 'align two or more sequences' option. The top hit sequences showing 95%-99% similarity alignment against the query sequence were obtained in FASTA format from Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) GenBank. The Phylogeny.fr online tool was used to analyse the phylogenetic relationships between molecular sequences by adapting the "A la carte" mode (Dereeper *et al*., 2008). The final tree was drawn in FigTree v1.4.3.

2.3 Microalgae cultivation and biomass production

Monoalgal cultures of the selected microalgae strains were grown for 7-8 days in MN medium until reaching the exponential phase. To obtain the microalgae biomass, the exponential phase culture was mixed with the MN medium at a ratio of 2:10 (v/v) to a final volume of 500 ml in 1 L flasks and incubated under a light intensity of 150 µmol photons m^{-2} s^{-1} for 12:12 hr light-dark cycles at 25±1°C for a week. Then, the volume of the culture was doubled with the addition of a medium every four days up to 21 days.

Biomass productivity was assessed by duplicate gravimetric determination of algal biomass dry weight (DW). Microalgae biomass was harvested from the late exponential growth phase by centrifugation (5,000 rpm; 10 min; 21°C). To remove the salt content from the biomass, the pellet

collected was washed with 0.5 M ammonium formate and subsequently in Milli-Q water before freeze-drying. The freeze-dried biomass samples were weighed using a semi-micro analytical balance to the nearest ± 0.001 (Sartorius, USA), and the values were expressed as g. L^{-1}.

2.4 Total lipid content

The total lipid content was extracted from the freeze-dried biomass as per Pereira *et al*. (2013). The complete lipid content (% dry weight) was calculated as follows:

Total lipid (% dry weight) = dry weight of total lipid/ dry weight of microalgae biomass x100

2.5 FAME sample preparation

FAME samples were prepared from the lipid samples as previously published in Hempel *et al*. (2012).

2.5.1 FAME profile analysis

FAME samples were analysed with high-resolution gas chromatography coupled with a mass spectrometer – double-focusing sector (GC-MS DFS), Thermo (USA) with an RTX-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). High purity (99.9%) helium gas was used as the carrier gas (column flow rate of 1 ml min^{-1}) with a precolumn pressure of 49.7 kPa. The initial column temperature conditions were set 40°C for 10 min, followed by a 5°C ramp per min up to 260°C, followed by 55 min at 250°C. The injection volume and temperature were 0.2 µl and 260°C, respectively, and the split ratio was 1/30. The mass spectrometer was operated (in compact electron mode) at electron energy = 70 eV. The temperature for both the ion source and the interface was set at 175°C. The chromatograms were analysed through Thermo X-Calibur software, and each respective peak was identified by comparing the maximum probability against the NIST library program (MS Search 2.0).

2.6 Biodiesel properties calculation using FAME profiles.

The ten essential biodiesel properties were predicted using the following equations mentioned in Islam *et al*. (2013).

- Degree of unsaturation (DU) = Σ MUFA + (2 × PUFA)
- Long-chain saturated factor (LCSF) = (0.1 × C16:0) + (0.5 × C18:0) + (1 × 20:0) + (2 × C24:0)
- Cold filter plugging point (CFPP) = (3.1417 × LCSF) – 16.477

- Iodine value (IV) g I_2 100 g^{-1} = Σ (254 × Di × Ni) / Mi)
- Saponification Value (SV) mg KOH g^{-1} = (Σ (560 × Ni) / Mi)
- Cetane number (CN) = 46.3 + (5458/SV) – (0.225 × IV)
- Kinetic viscosity (υi) at 40 °C in mm^2 s^{-1} = -12.503 + 2.496 × ln (Mi) – 0.178 × Di
- Density (ρ) at 20 °C in g cm^{-3} = 0.8463 + (4.9 / Mi) + 0.0118 × Di
- Higher heating value (HHV) MJ kg^{-1} = 46.19 – (1794/ Mi) – 0.21 × Di
- Predictive oxidation stability Y= (117.9295/X) + 2.5905 (0 < 100)

where, Ni is the percentage of each FAME; Mi is the molecular weight of the ith FAME, and Di is the number of double bonds in the FAME; X is the content of linoleic and linolenic acids (wt %) (0 < X < 100), and Y is the oxidation stability in h.

2.7 Multicriteria decision analysis (MCDA)

MCDA methods, namely PROMETHEE and GAIA, were carried out to select the indigenous microalgae based on their biodiesel properties (Visual PROMETHEE 1.5 Vn). PROMETHEE I is a partial ranking that calculates two preference flows (Phi+ and Phi -), while PROMETHEE II is a complete ranking that uses the net preference flow (Phi).

A GAIA plot that represents actions (turquoise squares), criteria (blue axes), and decision axis (red lines) was generated using the principal component analysis method. The plot was drawn by weighing the parameters using the PROMETHEE II ranking. Fifteen biodiesel parameters were used as criteria, and nine microalgae strains were chosen for action. The preference function was set as linear, and the threshold preference was set as 5, 120, 47, 0.90, 12, and 1 for the biodiesel criteria CFPP, IV, CN, ρ, C18:3, and Db ≥4, respectively. According to the biodiesel standards EN14214 and ASTM D6751-02, the biofuel property values preferred at a lower value for good biodiesel were set as a minimum for SFA, CN, HHV, and the properties selected at higher values for DU, LCSF, CFPP, IV, SV, MUFA, PUFA, C18:3, Db ≥ 4, and oxidation stability were set as a maximum.

2.8 Statistics

Data in this study are expressed as the mean value ± the standard deviation. The mean values of the biofuel FAMEs, SFAs, MUFAs, and PUFAs of the nine microalgae strains were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test (IBM®, Vn 27.0). Significant levels were recorded at $p < .05$.

3. Results

3.1 Microalgae strains isolation and identification

A total of 71 monoalgal strains, consisting of 19 diatoms (Bacillariophyceae), 21 cyanobacteria (Cyanophyceae), and 31 green algae (Chlorophyceae), were isolated from 109 enriched cultures using 156 environmental samples from different sample stations along the Kuwait coastline.

Table 1. Sources, GenBank Accessions, and Biomass productivity of microalgae used in the present study.

Sampling Sites	GPS	Isolate code	GenBank Accession No.	Genera (Phylum)	Biomass productivity (g.L-1)
A abd Allah	N 29°11.207' E 48°07.105'	KUBS35G	MG663221.1	*Chlorella* sp. (Chlorophyta)	0.633±0.11
Al-Sabiyyah	N 29°39 063' E 48° 08 633'	KUSW52G	MG663225.1	*Nannochloris* sp. (Chlorophyta)	0.410±0.09
Al-Sabiyyah	N 29°39 063' E 48° 08 633'	KUSW34G	MG663220.1	*Nannochloropsis* sp. (Ochrophyta)	0.894±0.08
Al-Fintas	N 29°10 477' E 48° 08 249'	KUBS17R	MG761720.1	*Scenedesmus* sp. (Chlorophyta)	1.148±0.08
Al-Shuaybah	N 29°20.275' E 48°05.776'	KUBS13G	MH509958.1	*Tetraselmis* sp. (Chlorophyta)	0.781±0.06
Hawalli	N 29°19.600' E 48°01.600'	KUBS15G	MH509959.1	*Tetraselmis* sp. (Chlorophyta)	0.595±0.09
Hawalli	N 29°19.600' E 48°01.600'	KUBS16G	MG663223.1	*Tetraselmis* sp. (Chlorophyta)	0.790±0.13
Mina Abd Allah	N 29°11.207' E 48°07.105'	KUBS37G	MG663224.1	*Tetraselmis sp.* (Chlorophyta)	0.572±0.03
Mina Abd Allah	N 29°11.207' E 48°07.105'	KUBS38G	MH509960.1	*Tetraselmis* sp. (Chlorophyta)	0.699±0.01

Among the isolates, nine strains surpassed the others regarding their growth performance under non-optimized culture conditions (Table.1), and their micrographs are depicted in Figure 1.

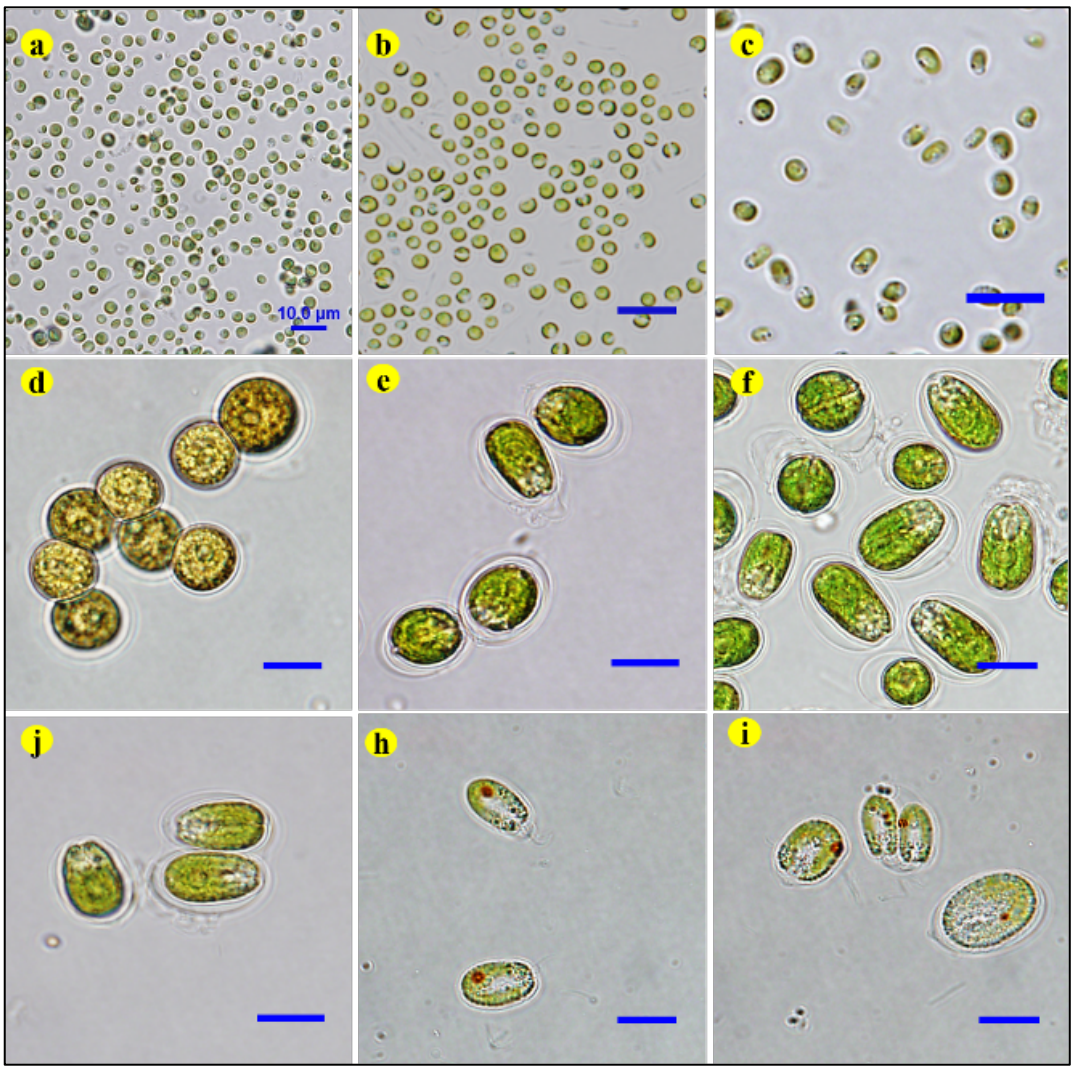

Fig. 1. Selected microalgae strain isolated from Arabian/Persian Gulf, Kuwait (**a**) *Chlorella* sp. KUBS35 (**b**) *Nannochloris* sp, KUSW52G, (**c**) *Nannochloropsis* sp., KUSW34G, (**d**) *Scenedesmus* sp. KUBS17R, (**e**)–(**i**) *Tetraselmis* sp. KUBS13G, KUBS15G, KUBS16G, KUBS37G, and KUBS38G, respectively. Scale bar = 10 µm.

3.2 Genetic identification

Partial 18S rRNA gene sequencing for the native monoalgal strains revealed that the selected microalgae strains belonging to the phylum Chlorophyta included five different genera, namely, *Chlorella*, *Nannochloris*, *Scenedesmus,* and *Tetraselmis*. The only exception was one isolate belonging to Ochrophyta and the genus *Nannochloropsis*. Furthermore, all the sequences were deposited in NCBI GenBank and published in the NCBI databases, and the corresponding GenBank accession numbers are shown in Table 1. The similarity sequences were collected from NCBI BLAST and used to root the tree. The phylogeny was inferred from the 18S rRNA partial gene

sequences determined with the Bayesian inference (BI) method (MrBayes 3.2.6). The maximum likelihood method displayed the evolutionary distances using the number of base substitutions per site as the units. The analysis involved a total of 30 nucleotide sequences with the query nucleotides. In the phylogenetic tree, node labels indicated the posterior probabilities calculated by Bayesian analysis. The identified sequences determined in this study are highlighted in blue font. All five *Tetraselmis* form a single cluster with a BI probability value of 1.0/1.0.

Similarly, *Nannochloropsis* sp. KUSW34G shared a BI probability of 1.0/1.0 with *Nannochloropsis gaditana* KC0129441.1, while *Scenedesmus* sp. KUBS17R, and *Chlorella* sp. KUBS35G was calculated to be 0.87/1.0 and 0.88/1.0, respectively (Figure 2).

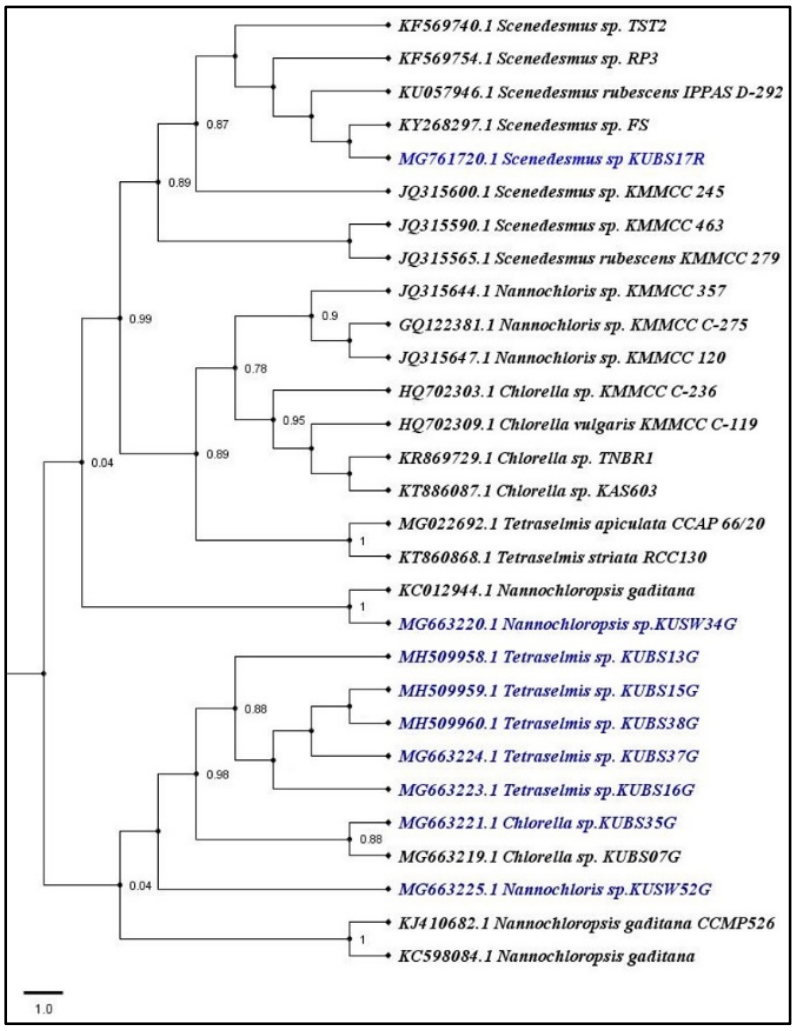

Fig. 2. The maximum likelihood tree shows the phylogenetic relationship of the nine microalgae strains based on partial universal 18S rRNA gene sequences. The node label shows the posterior probabilities calculated by Bayesian inference analysis (BI).

3.3 Productivity of biomass and lipid determination

The biomass productivity of the selected isolates was determined in terms of DWg.L-^{1} and is presented in Table 1. Among the nine isolates, *Scenedesmus* sp. KUBS17R had the highest biomass productivity (1.148 ± 0.08 g.L^{-1}), and *Nannochloris* sp. KUSW52G had the lowest productivity (0.410±0.09 g.L^{-1}) in addition to *Nannochloropsis* sp. KUSW34G and *Chlorella* sp. KUBS35G presented biomass productivity values of 0.894±0.08 g.L^{-1} and 0.633±0.11 g.L^{-1}, respectively. *Tetraselmis* sp. biomass productivity ranged from 0.572±0.03 g.L^{-1} to 0.790±0.13 g.L^{-1}.

The total lipid content of the isolates evaluated in this work varied from 18.9% DW to 29.6% DW. The highest lipid content occurred in *Tetraselmis* sp. KUBS16G and the lowest content was observed in *Nannochloropsis* sp. KUSW34G. The lipid contents of the five *Tetraselmis* strains ranged from 23.46% DW to 29.56% DW. *Chlorella* sp., KUBS35G, *Scenedesmus* sp. KUBS17R and *Nannochloris* sp. KUSW52G had 23.4% DW, 25.8% DW, and 28.7% DW, respectively. (Figure 3).

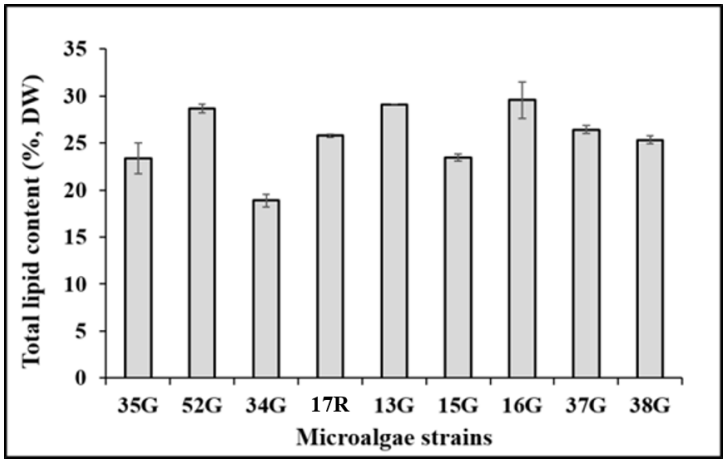

Fig. 3. Total lipid content of selected microalgae strains, dry weight %, shown as mean ± SD (n=3).

3.4 Fatty acid methyl ester (FAME) profiling

The characterization of the fatty acid profiles of the microalgae strains was performed by screening long-chain FAs (C14 - C24) within lipids. A total of 19 different methyl esters were identified by mass spectrometry, as shown in Table 2. ANOVA post hoc Tukey's test Tukey's post hoc test showed a significant difference at p <.05 for the FAME profile and percent among the five species studied. More than 50% of the total FAME profile was composed of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), of which the central FAs identified in the profile were

palmitic (16:0) and oleic (C18:1) acids. Palmitic, stearic (C18:0), palmitoleic, and linoleic (C18:2n6) acids were standard FAs present in all the isolates, whereas eicoseanoic acid (C20:1n9) was detected in *Tetraselmis* sp. KUBS13G and KUBS16G. Myristic acid (C14:0) was exclusively detected in *Nannochloropsis* sp. KUSW34G. Similarly, 7, 10, 13-hexadecatrienoic acid (C16:3n3) was present in both *Nannochloris* sp. KUBS52G and *Scenedesmus* sp. KUBS17R. Likewise, 5, 8, 11, 14-eicosatetraenoic acid (C20:4n6) and cis-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) (C20:5n3) were presented only among *Tetraselmis* sp. and *Nannochloropsis* sp. KUSW34G. Arachidonic acid (C20:4n6) was present only among *Tetraselmis* sp. and *Nannochloropsis* sp. KUSW34G. Oleic acid (C18:1n9c) was the most abundant FA found in 29.9-48.6% of *Tetraselmis* sp. and *Nannochloris* sp. KUSW52G, whereas it was not detected in *Chlorella* sp.KUBS35G, *Nannochloropsis* sp. KUSW34G and *Scenedesmus* sp. KUBS17R. Except for *Nannochloropsis* sp. KUSW34G, and *Chlorella* sp. KUBS35G all strains presented 7, 10-hexadecadienoic acid (C16:2n6). Total SFAs, MUFAs, and PUFAs ranged from 22.5% to 41.0%, 20.6% to 59.8%, and 15.4 % to 39.6 % of total FAMEs. The highest contents of SFAs, MUFA, and PUFAs were present in *Tetraselmis* sp. KUBS15G, *Scenedesmus* sp. KUBS17R and *Chlorella* sp. KUBS35G, respectively.

The five common biofuel FAs include palmitic (hexadecanoic), stearic (octadecanoic), oleic (9(Z) - octadecenoic), linoleic (9(Z), 12(Z)-octadecadienoic) and α-linolenic (9(Z), 12(Z), 15(Z)-octadecatrienoic) acids. All five FAs were detected among the *Tetraselmis* strains, except *Tetraselmis* sp. KUBS38G. The summarized contents of the five biofuel FAMEs namely, C16:0, C18:0 (Stearic acid), C18:1n9c, C18:2n6, and C18:3n3 was accounted for almost 34.4% – 89.9% of the total FAMEs with the highest % in *Tetraselmis* sp. KUBS15G. The lowest biofuel relative contents (%) were estimated in *Nannochloropsis* sp. KUSW34G (34.4%) and *Scenedesmus* sp. KUBS17R (34.5%). *Chlorella* sp. KUBS35G accounted for 62.9% of the relative biofuel contents, while *Nannochloris* sp. KUSW52G accounted for 71.5%. All five strains of *Tetraselmis* were detected with a good biofuel relative content % within the range of 67.6% to 89.9%, whereas the minimum was recorded in *Tetraselmis* sp. KUBS13G. The maximum and highest C16-C18 FAMEs of the total FAMEs were 100% in both *Nannochloris* sp. KUSW52G and *Scenedesmus* sp. KUBS17R, and 84.6% - 97.7% among the *Tetraselmis* strains, while the lowest value was recorded in *Nannochloropsis* sp. KUSW34G (73.9%) due to more C20:5n3 content.

3.6 Biofuel properties of the microalgae

The eleven biodiesel properties calculated from the FAME profiles of the nine microalgal strains used in the present study are listed in Table 3. In terms of the combustion behavior of the biofuel, fuel performance is determined by CN. The limit ranges of the CN described in EN 14214, and ASTM D6751−02 are ≥51 and ≥47, respectively. In the present work, all five *Tetraselmis* sp, and *Scenedesmus* sp. KUBS17R isolates showed CN values higher than 51 by both standards. The lowest CN value calculated was 44 in *Nannochloropsis* sp. KUSW34G and the highest was 57 in *Tetraselmis* sp. KUBS15G. Interestingly, the calculated density (ρ) value of all the microalgae strains was more than 0.87 g cm^{-3}, which was within the biodiesel standard EN 14214 (0.86–0.90 g cm^{-3}) limit. The higher heating value (HHV; MJ kg^{-1}) of the isolates ranged from 39.5 (*Nannochloris* sp. KUSW52G) to 39.7 MJ kg^{-1} (*Tetraselmis* strains), which was 6-18% lower than that of petroleum-derived diesel. All isolates displayed IVs below 120 g I_2 100 g^{-1}, which was by the EN 14214 limit (≤120 g 100 g^{-1}). The lowest value, 78 g I_2 100 g^{-1}, was found in *Tetraselmis* sp. KUBS15G followed by that in *Scenedesmus* sp. KUBS17R with 83 g I_2 100 $g^{-1.}$ The CFPP values for all the isolates, except *Tetraselmis* sp.KUBS15G was observed within the range of standard EN 14214 (i.e., ≤5/≤−20). Oxidation stability is also one of the main factors contributing to biodiesel properties. The oxidation stability values of the estimated strains were higher than 6 hrs.

Table 2. FAMEs profiles of the screened native microalgae strains (% of total fatty acids, DW basis).

Fatty acids	35G	52G	34G	17G	13G	15G	16G	37G	38G
C14:0	0.00±0.00	0.00±0.00	3.38±1.33	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C16:0	30.35 ± 0.28^{c}	21.57±0.08^{a}	26.90±1.18^{b}	22.83±1.61^{a}	21.42±0.04^{a}	33.38±0.24^{d}	27.27±0.04^{b}	34.78±0.23^{d}	26.61±1.73^{b}
C16:1n7	3.18 ± 0.03	1.87±0.06	31.04±0.25	1.00±0.30	5.55±0.13	2.56±0.34	4.43±0.31	3.51±0.54	4.31±0.35
C16:1n9	0.00±0.00	4.17±0.55	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C16:2 n6	0.00±0.00	5.63±0.34	0.00±0.00	3.11±0.37	1.90±0.05	1.51±0.20	1.62±0.15	0.96±0.53	1.44±0.08
C16:2n4	8.31 ± 0.37	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C16:3 n3	1.77 ± 0.09	12.66±0.46	0.00±0.00	1.04±0.63	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C16:3 n6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	5.11±0.08	1.28±0.28	3.03±0.44	0.80±0.08	1.02±0.17
C16:4n3	0.00±0.00	0.00±0.00	0.00±0.00	1.16±0.18	0.00±0.00	1.52±0.16	0.00±0.00	4.59±0.34	4.18±0.50
C18:0	3.04 ± 0.12^{c,d}	1.26±0.28^{a,b}	4.21±0.53^{d}	2.01±0.24^{b,c}	1.10±0.23^{a,b}	7.65±0.79^{e}	0.61±0.28^{a}	4.20±0.55^{d}	0.98±0.14^{a,b}
C18:1n7	17.43 ± 0.17	4.17±0.16	8.52±0.25	58.8±2.54	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C18:1n9c	0.00±0.00^{a}	34.38±0.23^{b,c}	0.00±0.00^{a}	0.00±0.00^{a}	29.93±0.51^{b}	35.66±2.08^{c}	38.67±4.57^{c}	36.80±0.94^{c}	48.60±1.55^{d}
C18:2n6	29.56 ± 0.39^{e}	14.3±0.51^{d}	3.32±0.16^{a}	9.68±0.45^{b,c}	13.25±0.33^{d}	12.89±1.70^{d}	10.78±0.70^{c}	9.39±0.71^{b,c}	7.87±0.12^{b}
C18:3n3	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	1.90±0.12^{c}	0.41±0.00^{b}	0.54±0.16^{b}	0.39±0.06^{b}	0.00±0.00^{a}
C18:4n3	0.00±0.00	0.00±0.00	0.00±0.00	0.38±0.22	4.44±0.13	0.84±0.33	1.40±0.25	1.21±0.09	1.36±0.11
C20:0	6.37 ± 0.42	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
C20:1n9	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	9.93±0.22	0.97±0.07	8.68±2.02	1.27±0.29	0.97±0.25
C20:4n6	0.00±0.00	0.00±0.00	3.03±0.14	0.00±0.00	2.11±0.01	0.35±0.05	0.74±0.08	0.43±0.07	0.56±0.01
C20:5n3	0.00±0.00	0.00±0.00	19.62±0.42	0.00±0.00	3.39±0.06	0.99±0.07	2.27±0.78	1.72±0.27	2.12±0.32
ΣSFA	39.76±0.82^{b,c}	22.83±0.37^{a}	34.49±0.38^{b}	24.84±1.85^{a}	22.52±0.09^{a}	41.03±5.03^{d}	27.88±0.32^{a}	38.98±0.32^{b,c}	27.59±1.59^{a}
ΣMUFA	20.61±0.20^{a}	44.59±1.00^{c}	39.56±0.01^{b}	59.80±2.84^{e}	45.41±0.42^{c}	39.19±2.49^{b}	51.78±2.25^{d}	41.58±0.11^{c}	53.88±1.65^{d}
ΣPUFA	39.64±2.53^{e}	32.59±0.63^{d}	25.97±0.00^{c}	15.37±1.65^{a}	32.10±0.25^{d}	19.79±2.53^{a,b}	20.38±2.57^{b}	19.49±0.42^{a,b}	18.55±0.78^{a,b}

Note: *Chlorella* sp, *Nannochloris* sp, *Nannochloropsis* sp, *Scenedesmus* sp, *Tetraselmis* sp. are represented by 35G, 52G, 34G, 17G, 13G, 15G, 16G, 37G, and 38G. Data have shown mean ± SD (n=3). In the same row, the means of percentage (%) with different superscript letters ($^{a, b, c, d, e}$) are remarkably other (ANOVA, post hoc Tukey's test p <.05).

Table 3. Biodiesel properties of nine native microalgae strains of Arabian Gulf, Kuwait.

Source	DU	LCSF	CFPP (°C)	IV (g I$_2$100g^{-1})	SV (mg KOHg^{-1})	CN	Kinematic viscosity (υ) (mm^2 s^{-1})	Density (ρ) (g cm^{-3})	HIV (MJ kg^{-1})	C18:3 (wt %)	Db ≥4 (wt %)	Oxidation Stability (OS) (h)	References
Biodiesel Standard EN 14214	–	–	**≤5/≤−20**	**≤120**	–	**≥51**	**3.5–5.0**	**0.86–0.90**	–	**≤12**	**≤1**	**≥ 6**	Islam *et al*. (2013)
Biodiesel Standard ASTM D6751−02	–	–	**NA**	**NA**	–	**≥47**	**1.9–6.0**	**NA**	–	–	–	–	Islam *et al*. (2013)
Chlorella sp. KUBS35G	100	4.6	-2.2	90	196	54	3.5	0.87	39.6	0.0	0.0	6.6	This study
Chlorella Vulgaris	56	8.0	8.8	50	189	64	4.3	0.84	38.1	1.57	0.0	14.3	Islam *et al*. (2013)
Nannochloris sp. KUSW52G	110	2.8	-7.7	111	198	49	3.1	0.88	39.5	0.0	0.0	10.8	This study
Nannochloris sp. SBL1	85	4.4	-2.7	88	198	54	2.9	0.88	39.6	0.0	0.0	10.2	Pereira *et al*. (2013)
Nannochloropsis sp. KUSW34G	92	4.8	-1.4	131	199	44	3.3	0.88	39.6	0.0	22.7	38.1	This study
Nannochloropsis oculata	69	3.7	-4.8	81	203	55	4.2	0.88	39.8	0.0	8.3	95.7	Islam *et al*. (2013)
Scenedesmus sp. KUBS17R	91	3.3	-6.1	83	195	56	3.1	0.88	39.5	0.0	1.5	14.8	This study
Scenedesmus dimorphus	145	3.8	-4.6	184	196	33	3.6	0.91	40.2	26.0	19.1	5.6	Islam *et al*. (2013)
Tetraselmis sp.KUBS13G	110	2.7	-8.0	99	194	52	3.2	0.87	39.7	1.9	15.1	10.4	This study
Tetraselmis sp.KUBS15G	79	7.2	6.0	78	196	57	3.1	0.88	39.7	0.4	3.7	11.5	This study
Tetraselmis sp.KUBS16G	93	3	-7.0	93	195	53	3.2	0.88	39.7	0.5	4.4	13.0	This study
Tetraselmis sp.KUBS37G	81	5.6	1.0	87	197	54	3.1	0.88	39.7	0.4	8.0	14.6	This study
Tetraselmis sp. KUBS38G	91	3.2	-6.6	97	196	52	3.2	0.88	39.7	0.0	8.2	17.6	This study
Tetraselmis sp.CTP4	NA	NA	NA	111	NA	51	3.6	0.85	NA	1.2	NA	4.7	Pereira *et al*. (2016)

Note the degree of unsaturation (DU), long-chain saturated factor (LCSF), cold filter plugging point (CFPP), iodine value (IV), saponification value (SV), cetane number (CN), density (ρ), Kinetic viscosity (υ), higher heating value (HHV), stability (Stb), hour (h), not available (–).

3.7 ROMETHEE and GAIA

The PROMETHEE ranking for the nine microalgae strains based on the biofuel with an aggregated score of 100 is presented in Table 4. *Nannochloropsis* sp. KUSW34G ranked 1st with a maximum aggregated score of 100 and a phi value of 0.162. *Nannochloris* sp. KUSW52G had a phi value of 0.129 with an aggregated score of 93.6%, ranking 2nd, followed by *Tetraselmis* sp. KUBS37G with a phi value of 0.020 and an aggregated score of 75.1%. The other four strains of *Tetraselmis* sp. were ranked 4th, 5th, 7th, and 8th, respectively. *Scenedesmus* sp. KUBS17R and *Chlorella* sp.KUBS35G ranked 6th and 7th, respectively. The quality of the GAIA vector analysis for the biofuel parameters was 82.2% (Figure 4), which was greater than the Visual PROMETHEE software's recommended % of >75.

Table 4. PROMETHEE table showing phi (ϕ) values, ranks, and aggregated scores.

Rank	Microalgae strains	Phi (ϕ)	Aggregated score
1	*Nannochloropsis* sp. KUSW34G	0.162	100.0
2	*Nannochloris* sp. KUSW52G	0.129	93.6
3	*Tetraselmis* sp. KUBS37G	0.020	75.1
4	*Tetraselmis* sp. KUBS38G	-0.007	71.2
5	*Tetraselmis* sp. KUBS13G	-0.026	68.6
6	*Scenedesmus* sp. KUBS17R	-0.049	65.4
7	*Chlorella* sp. KUBS35G	-0.055	64.6
8	*Tetraselmis* sp. KUBS16G	-0.057	64.4
9	*Tetraselmis* sp. KUBS15G	-0.116	57.2

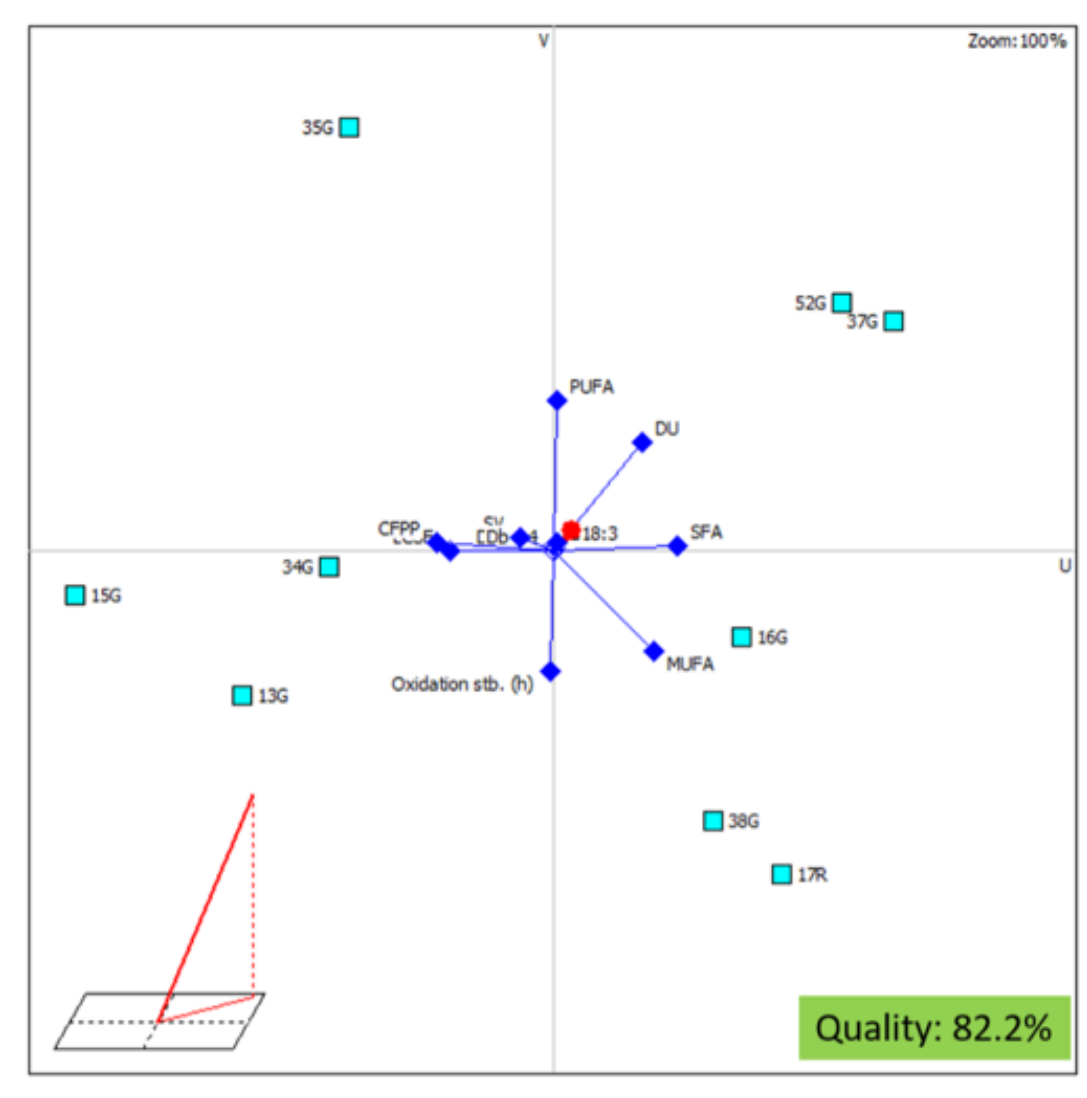

Fig. 4. PROMETHEE -Graphical Analysis for Interactive Assistance (GAIA) plot decision vector showing the 15 biodiesel criteria of the nine microalgae strains.

4. Discussion

Successful biofuel production relies on selecting suitable autochthonous strains with high adaptability to their local environmental conditions. Therefore, under optimal conditions, before focusing on optimization or induction techniques to increase lipid productivity, it is mandatory to investigate critical parameters, such as biomass production, total lipids, and FAME profiles (Wijffels & Barbosa, 2010).

The initial screening program to identify microalgae strains from Kuwait's coastal waters resulted in the isolation of 71 microalgae strains. Nine strains were further evaluated from these isolates due to their improved growth performance, as high growth rates are crucial to scaling up microalgae cultures to higher volumes (pilot and industrial scale). Although knowledge of hyper-lipid producing microalgae strains in Kuwait seawater was previously mostly non-existent, some studies on phytoplankton abundance have been published (Fathi *et al*., 2009; Al-Bader *et al*., 2011). The ROPME sea area and the Arabian Gulf have recorded significant lots of *Lauderia, Rhizosolenia, Chaetoceros, Bacteriastrum, Navicula, Nitzschia, Protoperidinium, Gonyaulax, and Ceratium*. Several species belonging to these genera have been previously identified as good candidates for biodiesel production, such as *Chaetoceros, Navicula,* and *Nitzschia* (Dorgham, 2013; Matsumoto *et al*., 2017). *In addition, Picochlorum*, *Nannochloris,* and *Desmochloris*, isolated from the Red Sea of Saudi Arabia, and thermo and halotolerant *Nannochloris* isolated from the Arabian Sea of Qatar has also been evaluated for biofuel production (Pereira *et al*., 2013; Saadaoui *et al*. 2016).

The most widely used tool for identifying autochthonous microalgae is morphology examination by microscopy, but it is inefficient and difficult to use for species identification. Phycologists use 16S rDNA, 18S rDNA, and ITS sequencing to resolve identification ambiguities caused by morphology alone since sequences are readily accessible in gene databases (e.g., GenBank). These methods have been used to analyse the phylogeny of microalgae taxa (Leliaert *et al*., 2012). Furthermore, 18S rRNA gene sequence analysis was used to classify microalgae strains based on their taxonomic position. All nine isolates were occupied either Chlorophyceae or Trebouxiophyceae. The availability of abundant data inaccessible public repository sources (e.g., GenBank, DDJ, and EMBL) has allowed the determination of the phylogenetic positions of closely related taxa (Zhu *et al*., 2018). Morphological identification of the microalgae strains (see Figure 1) matched the corresponding nucleotide sequence from the BLASTn sequence database, also reflected in the phylogenetic analysis. The dominant microalgae strains identified in this study belong to five different species, namely *Chlorella*, *Nannochloris, Nannochloropsis, Scenedesmus,* and *Tetraselmis*, that have been explored as biofuel and nutraceutical feedstocks. For example, *Nannochloropsis* sp. has been cultured in indoor open ponds for large-scale biodiesel production (Moazami *et al*., 2012). *Tetraselmis* sp. CTP4 has been effectively scaled up for large-scale production using tubular photobioreactors (PBR) (Pereira *et al*., 2018)*; Scenedesmus obliquus* has also been cultured (Abomohra *et al*., 2014). Low-cost, high-value microalgal products have been

produced from *Chlorella Vulgaris* H1993 and *Desmodesmus communis* H522 (Radkova *et al*., 2019), and *Nannochloris* sp. was cultured in large outdoor tanks for aquafeed in marine aquaculture (Witt *et al*., 1981).

Major Biomolecules, such as lipids and carbohydrates, are responsible for energy storage in algae. The total lipid content of the microalgae strains presented in our study ranged between 18.9% and 29.6% DW. Of which *Nannochloropsis* sp. KUSW34G showed a full lipid content of 18.9%DW, similar to that of *Nannochloropsis salina* reported as 18.8% (Bartley *et al*., 2013). Similarly, the lipid content of *Nannochloris* sp. KUSW52G was 28.7% DW by that of *Nannochloris* sp. SBL4 22.7%, isolated from the Red Sea region of Saudi Arabia (Pereira *et al*., 2013). *Scenedesmus* sp. KUBS17R had a total lipid content of 25.8% DW, similar to that of *Scenedesmus* sp. LX1 with full lipid content of 20–25% (Xin *et al*., 2010). The five *Tetraselmis* sp. strains had a total lipid content range of 23.3% to 29.4%, consistent with values previously recorded in the literature (Fon-Sing & Borowitzka, 2016, Pereira *et al*., 2016). It has been reported that lipid content accumulation in lipid bodies depends on algae's growth and nutrient status; in particular, under stress conditions, microalgae tend to produce more lipids (Xin *et al*., 2010).

Ideal microalgae candidates for biodiesel production should possess high lipid content in addition to adequate fatty acid composition. Palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids have previously been reported as typical FAs commonly found in microalgae biodiesel (Knothe, 2006). The fatty acid composition has an impact on biodiesel properties. L- linoleic and linolenic acid (wt %) influence oxidative stability. The FAME profile of *Nannochloropsis* sp. KUSW34G closely matched the profiles of *N. salina* CCMP537 and *N. oculata* CS179 (Ma *et al*., 2016). The unsaturation of the FAME profile is also an essential factor that determines the overall performance of the microalgae-based- biofuel. For example, biodiesel is mainly composed of SFAs and MUFAs, since PUFAs decrease the final stability of biodiesel (Knothe, 2009*,* Cobos *et al.,* 2017). The *Tetraselmis* sp. KUBS16G, KUBS37G, and KUBS38G; *Nannochloropsis* sp. KUSW34G and *Scenedesmus* sp. KUBS17R strains from this study showed an increasing pattern of FAs in the sequence order of PUFAs>SFAs>MUFAs, whereas *Nannochloris* sp. KUSW52G and *Tetraselmis* sp. KUBS13G displayed the order of SFAs > PUFAs>MUFAs; in addition, and *Tetraselmis* sp. KUBS15G showed the order of PUFAs > MUFAs > SFAs.

The fuel properties, namely, CN, CFPP, VK, and HHV, were calculated from the FAME profiles of the microalgal strains described in the present work and were in the desired range of biodiesel standards based on those previous reports (Knothe, 2009; Islam *et al*., 2013; Talebi *et al*., 2013; Pereira *et al*., 2016). The carbon chain sizes and the amount and position of double bonds determine the molecular structure of FAMEs. Additionally, these molecular characteristics influence biodiesel quality parameters, such as CN, CFPP, IV, and OS (Knothe, 2009). Biodiesel standards EN 14214 (European) and ASTM D6751−02 (American) were used as references to compare the fuel properties of the selected microalgal species. CN is one of the most critical indicators of fuel performance, determining the combustion behavior of biofuel. The limit ranges of the CN described in EN 14214 and ASTM D6751-02 are ≥51 and ≥47, respectively. In the present work, all five *Tetraselmis* strains, *Chlorella* KUBS35G and *Scenedesmus* sp. KUBS17R displayed CN values higher than 51, consistent with the biodiesel standards. This result might have been due to the absence of very long-chain polyunsaturated fatty acids (C22:5) and (C22:6). The lowest CN value calculated was 44 for *Nannochloropsis* sp. KUSW34G may have been due to a relatively higher C20:5 (19.6%). Among those of five *Tetraselmis* strains, the CN value was estimated to be in the range of 52-57, which was higher than the previously reported CNs of 47.3 (Pereira *et al*., 2016) and 50.3 (Sarpal *et al*., 2016), which might have been due to the corresponding higher amount of SFAs.

An MCDA (PROMETHEE and GAIA) was performed to select and evaluate the robust microalgae strains from the available microalgae strains for biofuel feedstock. In recent years, MCDA methods have been widely used to choose robust microalgae strains for biodiesel feedstock (Islam *et al*., 2013; Nwokoagbara *et al*., 2015; Musa *et al*., 2019).

Life cycle analysis (LCA) is a tool for evaluating the environmental impact of goods, technologies, and services over their entire life cycle. LCA allows for a long-term evaluation of microalgae biodiesel conversion. A recent LCA of the environmental impact assessment of large-scale microalgae biodiesel showed the following environmental consequences: abiotic depletion (depletion of non-renewable natural resources), acidification (loss of base nutrients: Mg, Ca, and K), eutrophication (nutrient enrichment due to fertilizer addition, sediment updraft, and nitrogen deposition), algal toxicity (in a particular phase of the life cycle, algae tend to release toxins from simple ammonia to toxic polypeptides, and polysaccharides), and photochemical oxidation (due to

the emission of the volatile organic compound, carbon monoxide, sulfur dioxide, and ammonium), which ultimately ended up in pollution (Saranya & Ramachandra 2020). A comparative LCA for microalgae fuel production using fertilizer or nitrogen starvation culture conditions and dry or wet extraction for bio-oil conversion recommended nitrogen stress during culturing and wet extraction as the valuable options in microalgae biofuel production (Lardon *et al*., 2009).

Currently, combustion engines power more than 99% of the transportation sector, accounting for approximately 14% of greenhouse gas emissions (GGE). The commercialization of algal fuel is still a challenge. Large-scale production is technically feasible, but it is not currently economically efficient. Biodiesel made from waste frying oil, jatropha, and soybean was projected to sell at $0.73/L, $1.4/L, and $1.35/L, respectively, higher than petrol–diesel ($0.7/L) but lower than algal biodiesel. The cost of producing 1 L of oil ranges from $0.43 to $24.60, making potential biodiesel prices difficult to forecast (Bošnjaković & Sinaga 2020).

Although existing biofuels cannot completely replace fossil fuels, the demand for bio-fossil fuel blends has been motivated mainly by fossil fuel scarcity and the need to minimize carbon dioxide emissions, which lead to climate change. (Kalghatgi *et al*., 2018). Therefore, governments and industries are looking to shift from the complete reliance on fossil fuels towards renewable energy sources, such as biofuels. A recent study recommended two bio-fossil fuel blends for atomization, namely, B5 -biodiesel fuel and diesel fuel at a ratio of 5:95 v/v and E5 -ethanol fuel and gasoline fuel at a ratio of 5:95 v/v. The droplet lifetimes of these mixture blends are comparable to those of complete diesel and gasoline fuels. Furthermore, these mixtures can be used in conventional engines without engine modifications (Al-Qubeissi *et al*., 2018).

5. Conclusion

Nine microalgal strains were isolated from different sampling points around Kuwait's Persian/Arabian Gulf. A molecular technique used partial 18S rRNA gene sequencing and phylogenetic approaches to identify the microalgae strains. Initially, such strains were characterized in the stationary growth phase and exhibited significant heterogeneity in critical parameters such as biomass, lipid productivity, and fatty acid profile. Biofuel properties, calculated from the fatty acid methyl esters of *Scenedesmus* sp. KUBS17R presented good fatty acid profiles with a predominance of MUFAs. These MUFAs were dominated by high levels of oleic acid

(58.8%), which correspond to an increase in cetane number. Additionally, a low amount of polyunsaturated fatty acids <1.5 wt % with ≥4 double bonds act by most of the European (EN14214) and American (ASTM D6751−02) biodiesel standard specifications. Hence, our findings suggest that the indigenous microalgae strain, *Scenedesmus* sp. KUBS17R and *Tetraselmis* sp. KUBS13G is the appropriate renewable feedstock to produce biodiesel.

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