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Fatty Acids, Hydrocarbons and Terpenes of *Nannochloropsis* and *Nannochloris* Isolates with Potential for Biofuel Production

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Received: 29 October 2018; Accepted: 25 December 2018; Published: 31 December 2018

Abstract: Marine microalgae are a promising feedstock for biofuel production given their high growth rates and biomass production together with cost reductions due to the use of seawater for culture preparation. However, different microalgae species produce different families of compounds. Some compounds could be used directly as fuels, while others require thermochemical processing to obtain quality biofuels. This work focuses on the characterization of three marine microalgae strains native in Mexico and reported for the first time. Ultrastructure and phylogenetic analysis, suggested that they belong to *Nannochloropsis* sp. (NSRE-1 and NSRE-2) and *Nannochloris* sp. (NRRE-1). The composition of their lipid fractions included hydrocarbons, triacylglycerides (TAGs), free fatty acids (FFAs) and terpenes. Based on theoretical estimations from TAG and FFA composition, the potential biodiesels were found to comply with six of the seven estimated properties (ASTM D6751 and EN 14214). On the other hand, hydrocarbons and terpenes synthesized by the strains have outstanding potential as precursors for the production of other renewable fuels, mainly green diesel and bio-jet fuel, which are "drop-in" fuels with quality properties similar to fossil fuels. The validity of this theoretical analysis was demonstrated for the oxygenates of strain NSRE-2, which were experimentally hydrodeoxygenated, obtaining a high-quality renewable diesel as the reaction product.

Keywords: biodiesel; bio-jet fuel; triacylglycerides; Fatty Acid Methyl Ester; lipids; hydrodeoxygenation; drop-in fuel

1. Introduction

Microalgae offer an attractive way of generating renewable and sustainable biofuels [1] capable of helping to meet the global demand for transport fuels. However, a screening procedure to assess microalgae potential is a highly recommended prerequisite for biofuel feedstock production. Marine microalgae, which are abundant in seawater, are a particularly promising raw material due to the cost reductions resulting from using seawater for cultivation. The additional salinity level is also one of the methods of reducing contamination issues [2] in open-pond cultivation. For this reason, a number of companies currently operate commercial-scale mass cultivation of marine microalgae [3].

Proper characterization and adaptation to culture conditions of isolated native strains are the first steps in establishing any biotechnological process, including biofuel production. Strategies for improving microalgae growth include the variation of inoculum size, pH and culture medium composition. Inoculum size significantly affects microalgae cell growth characteristics, such as lag phase duration, maximal specific growth rate, biomass accumulation and metabolites production at the end of culturing [4]. An appropriate inoculum size can reduce cell mortality and increase biomass production and nutrient recovery [5]. With a proper culture pH, high cell densities and lipids accumulation can be obtained [6]. Culture medium composition affects the specific growth rate, biomass production and the biochemical composition of the resulting biomass and lipids [7].

Characterization of marine microalgae must include ultrastructure analysis of organelles and their disposition inside the cell, as well as light microscopy to elucidate reproduction mode, determination of the phylogenetic position of the strains under study, and analysis of their carotenoid and chlorophyll content in order to determine the taxonomic identity of each strain [8,9]. All of these analyses are particularly important in taxa such as *Nannochloropsis* sp. (Eustigmatophyceae) and *Nannochloris* sp. (Chlorophyceae), both extremely small unicellular marine eukaryotic algae with easily confused characteristics, which can even be impossible to distinguish due to their tiny cell size and simple and smooth cell wall morphology [10]. Among the most important marine microalgae for obtaining biofuels, precisely *Nannochloropsis* spp. stand out due to their high growth rates and lipid contents in the form of TAGs [11]. *Nannochloris*, meanwhile, is a species that has received less study for biofuel purposes, but has high lipid productivity [12]. To our knowledge, this is the first report on the isolation and characterization of *Nannochloropsis* and *Nannochloris* species in Mexico.

The lipidic fraction of the microalgal biomass is used for biofuel production. Triacylglycerides are converted to biodiesel, and this procedure has been extensively studied. However, other compounds produced by microalgae such as hydrocarbons and terpenes can also be used for the production of bio-jet fuel and green diesel. For example, phytol stands out as a natural diterpene alcohol that can be used as biofuel in diesel engines [13] or converted to gasoline by catalytic cracking [14]. Another terpene, neophytadiene, is one of the dominant ones in green microalgae [15] and it could also be converted to biofuel. Terpenes are hydrocarbons that are already important as bioactive compounds, with applications as functional compounds due to their broad spectrum of biological activity, such as antitumor and antiviral activity [16], among others. However, from more than 40,000 reported structures, specific terpenes (cyclic and acyclic forms) are recognized as special biofuels that can be used directly or blended with a range of fossil fuels, such as jet fuel, missile propellant, gasoline, or diesel fuels [17]. On the other hand, hydrocarbons produced from microalgae can be directly used in mixtures with the diesel fraction (C_{12}–C_{22}), or they can be subjected to a catalytic improvement stage (hydroisomerization or hydroisomerization plus hydrocracking process), through which paraffins with better fuel properties can be obtained [18]. The selection of the process will depend on the composition of the raw material and the type of fuel that is to be produced. For example, if the hydrocarbon fraction has a high content of paraffins with more than 21 carbon atoms, a hydroisomerization process with simultaneous hydrocracking would be recommended. This process would permit the formation of branched hydrocarbons with shorter carbon chains and products in the range of gasoline (bio-gasoline), jet fuel (bio-jet fuel) and diesel (green diesel).

The vast biodiversity available in Yucatan, Mexico, provides a valuable resource for the development of indigenous microalgal strains as biofuel feedstock. The objective of this research was to collect marine strains from coastal waters and analyze morphology, reproduction mode and organelle disposition by light microscopy and ultrastructure analysis to support phylogenetic studies and determine taxonomic identity. Additionally, the fatty acids and organic compounds synthesized by the strains under study were analyzed to determine their potential for biofuel production, either through catalytic processes for obtaining drop-in fuels (bio-jet fuel and renewable diesel) or biodiesel, or even using them without chemical modification in mixtures with fossil fuels. Finally, a catalytic hydrodeoxigenation reaction was carried out to experimentally demonstrate the potential of the extracted lipids as raw material to obtain high-quality renewable fuels.

2. Materials and Methods

2.1. Isolation and Cultivation of Microalgae

Marine microalgae were collected from the water column at three different sites in Progreso, Yucatan, located at 21°19′01.33′′ N, 89°40′50.05′′ W; 21°17′09.38′′ N, 89°41′50.05′′ W and 21°17′09.36′′ N, 89°41′50.07′′ W. Samples were collected at three different depths (0.0, 0.5 and 1 m) from water bodies during the mornings of May 2014 using a Van Dorn bottle (1120-G45, Wildco, Yulee, FL, USA). The pH, temperature and salinity values (Extech Stick EC500, Extech instruments, Boston, MA, USA) were registered [19]. The collected samples were allowed to settle for 3 to 4 h at 4 °C. The sedimented biomass was analyzed to proceed with the isolation of the microalgae species.

2.2. Obtaining Axenic Cultures

Microalgae cells were observed by inverted microscope (Axio Observer-5 ZEISS, Baden-Württemberg, Oberkoche, Germany) to select individual cells and inoculate in Guillard's f/2 (G9903, Sigma-Aldrich, San Luis, MO, USA) and saline BG-11 medium (2% NaCl) (C3061, Sigma). Then microalgae colonies (500 µL plated in solid Bold's basal medium (BBM) were transferred to both media for decontamination: *Centrifugation.* Microalgae cultures were centrifuged, pellets were washed in sterile seawater and centrifuged again, and were then inoculated [19]. *Serial dilution.* Dilutions of microalgae culture (1:10, 1:100 and 1:1000 (v/v)) were employed. *Antibiotic treatment.* After proliferation of bacteria in Müeller-Hinton media, antibiotic susceptibility testing disks (50295; Bio-Rad, Hercules, CA, USA) were used, giving maximum susceptibility to chloramphenicol, amikacin and streptomycin (100 mg/L) [20]. *Acid shock method*. Cultures in exponential phase were centrifuged at 2291× g for 7 min at 10 °C (Digicen 21R, OrtoAlresa, Madrid, Spain). The pellet was resuspended three times in 1 mL HCl (1N) by manual agitation [21] and re-suspended in both media and incubated. The axenic microalgal strains were cultivated (10 mL) in Guillard's f/2 (G9903, Sigma) and BG-11 (2% NaCl) (C3061, Sigma) under a 12:12 photoperiod at 25 ± 2 °C, 110 µmol $m^{-2}s^{-1}$ light intensity and 120 rpm agitation. To improve cell densities, nutrient content (Guillard's f and f/2 medium) was evaluated at different pH (6, 7 and 8) and inoculum size (10, 20 and 30%). Cell concentration was determined every 48 h using a Neubauer hemocytometer [22].

2.3. Morphological Analysis. Light Microscopy and Transmission Electron Microscopy (TEM)

Morphological features (cell shape and reproductive features) observed through optical microscopy (Eclipse E200, Nikon instruments, Melville, NY, USA) were recorded. For TEM, the strains were preserved in 2% glutaraldehyde for 4 h and then post-fixed in 1% OsO_4 for 30 min, followed by progressive dehydration in 30 to 100% ethanol at 4 °C. The samples were then transferred to 100% propylene oxide and infiltrated into epon-propylene oxide (1-1) for 48 h, followed by embedding in epon and polymerization at 60 °C for 48 h [23]. Cross sections were performed with a Leica ultramicrotome (Leica, Wetzlar, Germany) and observed with the 1200-EX II TEM (JEOL, Peabody, MA, USA) located at the Institute of Cellular Physiology, UNAM.

2.4. DNA Extraction and 18S rRNA Gene Amplification

The microalgae cells were harvested at the stationary growth phase by centrifugation (30 mL) and washed three times in bidistilled sterile water. The cell (c.a. 200 mg) was stored at −20 °C until use. For genomic DNA extraction, the Youssef protocol was used with minor modifications [24]. 18S rRNA gene amplification was implemented using forward primer A (5′-AACCTGGTTGATCCTGCCAG-3′) and reverse primer SSU-inR1 (5′-CACCAGACTTGCCCTCCA-3′) based on the conserved domain region of 18S rDNA [25].

18S rRNA region amplification was carried out with a 10 µL reaction mixture containing 1× PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 mM primer, 1 U *Taq* DNA polymerase (Invitrogen, Waltman, MA, USA) and 10 ng of DNA. PCR conditions included 1 cycle of 1 min at 94 °C for initial denaturation, followed by 40 cycles of 30 s at 94 °C, 30 s at 57.5 °C, 1 min at 72 °C, and finally 7 min at 72 °C. Amplicons were checked by electrophoresis on 1% agarose gel in 1 × TAE buffer. The amplification products of approximately 500 pb were purified using NucleoSpin® Gel and PCR Clean-up commercial kit (Macherey-Nagel, Düren, Germany) to remove primer dimers according to the manufacturer's instructions. Direct sequencing of the amplification products was carried out by a two-directional (reverse and forward) procedure (Clemson Sequencing Service, Clemson, South Caroline, USA).

The 18S rRNA region sequences from DNA of NSRE-1, NRRE-1 and NSRE-2 of 540 pb (approx.) were used as "queries" in nucleotide Blast tools (NCBI website) to recover the ten best matches for each input sequence, based on percentage of sequence coverage, E-value and percentage of identity of 100%. After manual adjustment with MEGA version 2.1, sequences with their homologies in GenBank were deleted to a final sequence of 536 pb and aligned using CLUSTALX (EMBL, Heidelberg, Germany). Molecular phylogenetic analyses by the maximum likelihood method based on the Kimura 2-parameter model were conducted using MEGA version 2.1 [26]. One thousand replica samplings were analyzed for percent bootstrap values in a neighbor-joining tree. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted in MEGA 7 [26]. Reference sequences retrieved from GenBank of 18S rRNA region sequences were: NSRE-1, MH921192; NRRE-1, MH921193; and NSRE-2, MH921194.

2.5. Biomass Production

Algal biomass was produced in a culture system consisting of three serially connected 250 mL Erlenmeyer flasks with 200 mL of culture medium and a 250 mL Erlenmeyer flask with sterile distilled water (with the purpose of acting as an air humidifier). The culture system was maintained under the following conditions: an aeration rate of 3.6 vvm, temperature of 24 ± 2 °C, and light intensity of 81 µmol m^{-2} s^{-1} with a 16:8 (light:dark) photoperiod [27]. The biomass was recovered at the beginning of the stationary phase (15 days of cultivation) by centrifugation at 5057× *g* for 7 min at 25 °C [28]. The pellet was washed with bidistilled sterile water to eliminate minerals and salts from the culture media. The total biomass was maintained at −80 °C for 24 h and freeze dried at 0.1 bar and −53 °C for 72 h [29].

2.6. Total Lipid Extraction

One g of dried biomass was subjected to solvent extraction with 15 mL of a 1:2 (*v*/*v*) chloroform-methanol mixture [30–33] for 3 h at 40 °C and 150 rpm (3×). The solvents were then eliminated by vacuum evaporation.

2.7. Thin Layer Chromatography

It was carried out on TLC Silica gel 60 F_{254} plates (Merck KGaA, New York, NY, USA). A 9:1:0.1 (*v*/*v*) mixture of hexane, ethyl acetate and acetic acid was used as the mobile phase. The plates were developed using a 10% (*w*/*v*) solution of phosphomolybdic acid in sulfuric acid [34].

2.8. Column Chromatography

The lipid extract was fractionated by column chromatography using silica gel (Kieselgel, 70–230 mesh) as the stationary phase. A solvent gradient elution was used to obtain five fractions from the lipid extract. The solvent system was hexane, then 9:1, 8:2 and 7:3 mixtures of hexane-ethyl acetate, and finally methanol [29,34,35].

2.9. Transesterification

The TAG fraction was transesterified with sodium metoxide (6% v/w) in a 25 mL vial with moderate agitation at 60 °C for 90 min. The reaction mixture was passed through silica gel to eliminate excess catalyst and glycerol. The solvent was then evaporated in a fume hood.

2.10. Lipid Profile Analysis (GC-MS)

The fractions were analyzed by gas chromatography–mass spectrometry (7890B and 5977A series GC/MSD, Agilent Technologies, Santa Clara, CA, USA), using a DB5-HT column (30 m × 250 µm ID × 0.25 µm film width) under the following conditions: 3 mL/min of helium flux as carrier gas. The detector and injector temperatures were set at 300 °C. The GC temperature program started at 70 °C/5 min, and was then raised to 200 °C using a rate of 10 °C/min with a 5 min hold at 200 °C and 20 °C/min until 290 °C, which was held for 22.5 min. The injection volume was 1 µL. The NIST 05 (National Institute of Standards and Technology, Gaithersburg, MD, USA) library was used for identification of the main peaks [36]. Six main groups of compounds were identified: hydrocarbons, FAMEs, organic acids, terpenes, sterols and amines.

2.11. Catalytic Hydrodeoxygenation of Oxygenates from Strain NSRE-2

The fraction of oxygenated compounds from strain NSRE-2 was subjected to a catalytic hydrodeoxygenation treatment (HDO) in a stainless steel (SS-316) fixed bed reactor (Microactivity Reference, Madrid, Sapin), with a length of 30.5 cm and a 0.9 cm internal diameter. The reaction mixture (31 mg lipid extract dissolved in 120 mL nonane) was fed using an HPLC pump (model 307 V 3.00, Gilson, Villiers-le-Bel, France) at a constant flow rate of 0.061 mL min-1 to attain a WHSV of 0.1 h^{-1}. Pt/HZSM-22-γ-Al_2O_3 synthesized in-house was used as catalyst (0.046 g) and activated in-situ under H_2 flow (50 mL min^{-1}) at 400 °C for 2 h with a heating rate of 5 °C min^{-1} and 20 bar with a pressure rate of 5 bar min^{-1}. The reaction conditions were 300 °C at a heating rate of 5 °C min^{-1} and a H_2 pressure of 40 bar. H_2 was fed at a gas flow rate of 55 mL min^{-1} and the total reaction time was 4 h. Samples of the reaction product were collected every hour and analyzed by GC-MS.

2.12. Statistical Analysis

Results of biomass and lipid production are presented as means ± standard deviations (SD) from three replicates. The statistical analysis was carried out using OriginPro (v 9.0, OriginLab, Northampton, MA, USA) to determine the degree of significance using one-way analysis of variance (ANOVA) at a probability level of $P < 0.05$.

3. Results and Discussion

3.1. Obtaining Axenic Cultures

The three isolated microalgae were named NSRE-1, NRRE-1 and NSRE-2, and placed under the conditions mentioned. Application of HCl proved to be an effective decontamination technique. This pH-shock treatment has been applied before for effective control of *V. chlorellavorus*, a predatory bacterium that can destroy a *Chlorella* culture in just a few days [37]. This effective treatment with high selectivity and low cost can be considered throughout the scale-up and production process [37]. The pH-shock treatment applied in this study is simpler than the one executed by Ganuza et al. [37]

and patented by Ganuza and Tonkovich. Application of antibiotics has been used by other authors to obtain axenic cultures from freshwater bodies [38]. However, bacterial contamination persisted in all cultures when amikacin, active against aerobic gram-negative bacilli [19], was applied in this work.

The culturing of NSRE-1, NRRE-1 and NSRE-2 in Guillard's f/2 resulted in improved growth compared to BG-11 medium, as indicated by the cell densities (cell mL^{-1}) of NSRE-1, NRRE-1 and NSRE-2. In Guillard's f/2 they were 9.03×10^6, 5.46×10^6 and 6.28×10^6, while in BG-11 they were 1.48×10^6, 2.56×10^5 and 4.50×10^5 respectively. This result could be explained by the fact that Guillard's f/2 medium has a ratio of 16N:1P, which promotes higher cell density in shorter cultivation times [39]. Additionally, vitamins in Guillard's f/2 medium (cyanocobalamin, thiamine and biotin), absent in BG-11, contribute to the initial growth of microalgae species [40].

Improving microalgae culture parameters to increase biomass and growth is considered the most important factor in sustainable product development [41]. This includes the improvement of the medium composition and physical parameters [42]. In this study, modifications to pH were carried out to increase cell density (Figure 1). Inoculum size of microalgae is another important factor that significantly affects microalgae growth, specifically, the lag phase, maximum specific growth rate, biomass accumulation and the metabolite production at the end of culturing (Richmond 2004). In this study, an inoculum size of 10% v/v, yielded a higher cell density than 20% or 30%, probably because a greater availability of nutrients occurs at lower cell densities [43]. In this regard, inoculum size had an effect on final cell density. Selection of the appropriate inoculum size can help reduce the cell mortality ratio in microalgae and increase the biomass production and nutrient recovery [5].

The nutrient concentration of Guillard's medium employed in this study were half strength and full strength, denoted as f/2 and f respectively. The cell density double increased in all strains under study by using f Guillard´s f media (Figure 1). Notably in NRRE-1, cell density was ten times greater than NSRE-1 and NSRE-2. These results are consistent with those obtained by Malakootian et al. (2015), in *Nannochloropsis oculata* with Walne medium, the composition of which is similar to Guillard´s f medium, resulting in a highest cell density than with Guillard's f/2, Sato and TMRL media.

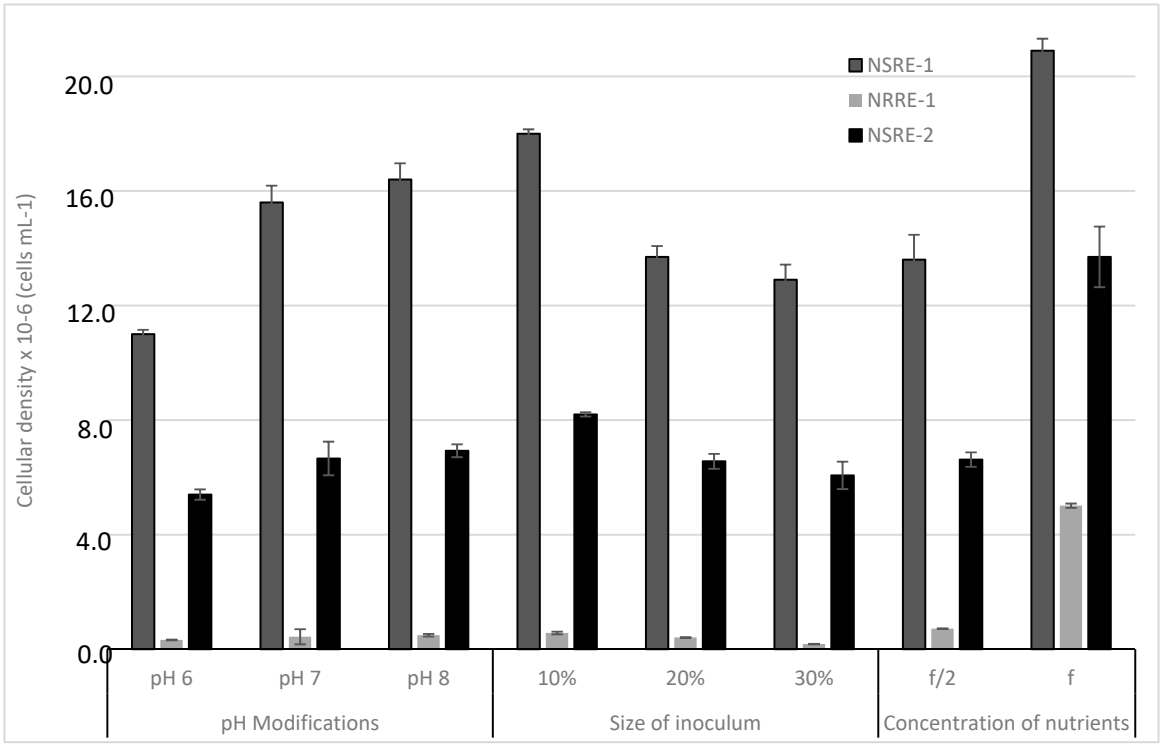

Figure 1. Mean cell density ± SE of NSRE-1, NRRE-1 and NSRE-2 strains at three different pH levels and inoculum sizes and two nutrient concentrations.

Based on the results obtained, stabilized microalgae strain culture conditions were as follows: pH 8, inoculum size 10% *v*/*v* and Guillard's f medium.

The three strains showed a short lag phase (2–4 days), followed by an exponential growth phase of 6 days. The early stationary phase began on day 18 of cultivation (Figure 2). The highest cell density was registered almost at the end of the stationary phase in NSRE-1 and NRRE-1. In NRRE-1 cell concentration decreased on day 20 of culture. Cell density was higher in NSRE-1 (2.16×10^7 cells mL^{-1}) than NSRE-2 (1.89×10^7 cells mL^{-1}), whereas NRRE-1 showed the lowest density (1.21×10^7 cells mL^{-1}).

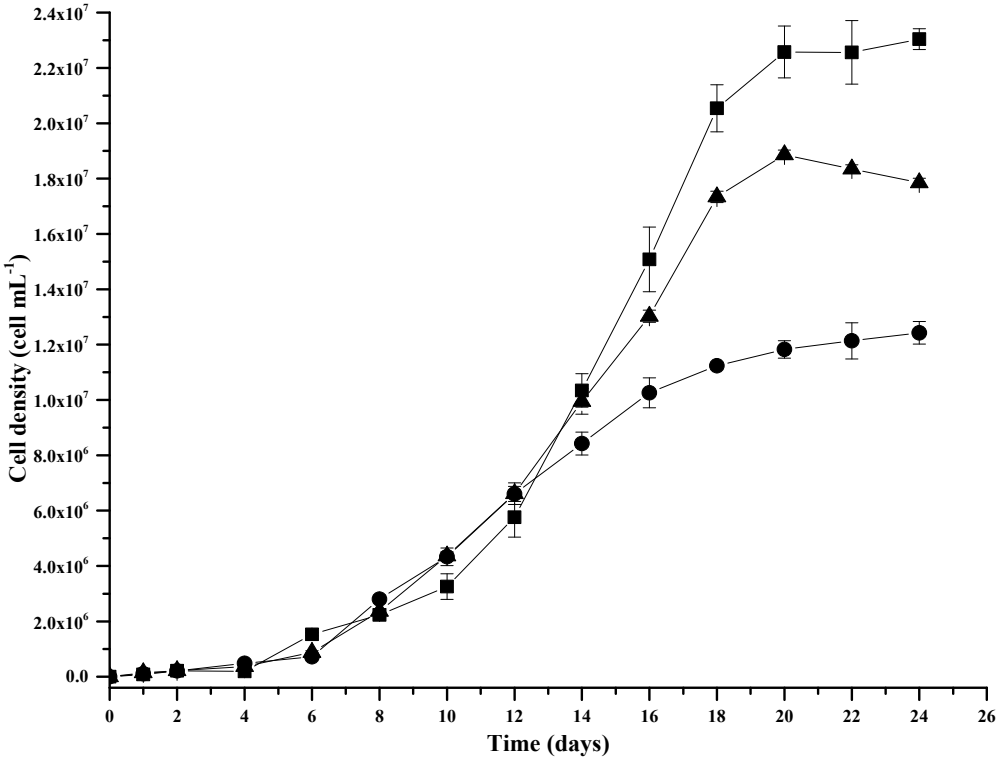

Figure 2. Growth curve of strains NSRE-1 (■), NRRE-1 (●) and NSRE-2 (▲).

3.2. Morphological Analysis (Light Microscopy and TEM)

A suite of analyses (morphological, ultrastructural, biochemical and molecular) may be required for the determination of some taxa [44]. Visualization by light microscopy mainly determines reproduction mode and other characteristics, while ultrastructure analysis determines the disposition of organelles and cellular details for taxa differentiation purposes. Under light microscopy (LM), NSRE-1 showed spherical or oval cells (1.0 to 3.0 µm in diameter) with rounded lobes and a very thin hyaline mucilage. In adult and young cells, a parietal chloroplast without pyrenoid covered three quarters of the cells. An eyespot was observed in some cells. Reproduction was asexual by 2 to 4 autospores, released by rupture and gelatinization of the mother cell wall. Mother cell wall remnants were observed in cell groups. The cells were observed mainly to be solitary vegetative cells or autospores clustered together. Our ultrastructure study through TEM analysis (Figure 3) showed lenticular ornamentations on the cell wall, some lipid bodies and the endoplasmic reticulum attached to the plastid and nuclear membrane. Four membranes were observed covering the plastid, characteristic of *Nannochloropsis* sp. (Eustigmatophyceae) [45].

NRRE-1 showed spherical or oval cells (2.0 to 4.0 µm in diameter), most of them clustered together (many dispersed solitary vegetative cells) or solitary cells of different size and phases. A cup-shaped parietal chloroplast with pyrenoid was observed. Under TEM, the cells were observed to be rounded with a multilayer cell wall (Figure 4). The plastid exhibited thylakoid membranes penetrating into the pyrenoid. Cells in division exhibited an evident cleavage furrow, characteristic of *Nannochloris* sp.

(Chlorophyta). Under LM, reproduction was observed mainly by the formation of two autospores and tetrads of aplanospores, which are converted into colonies of sister cells.

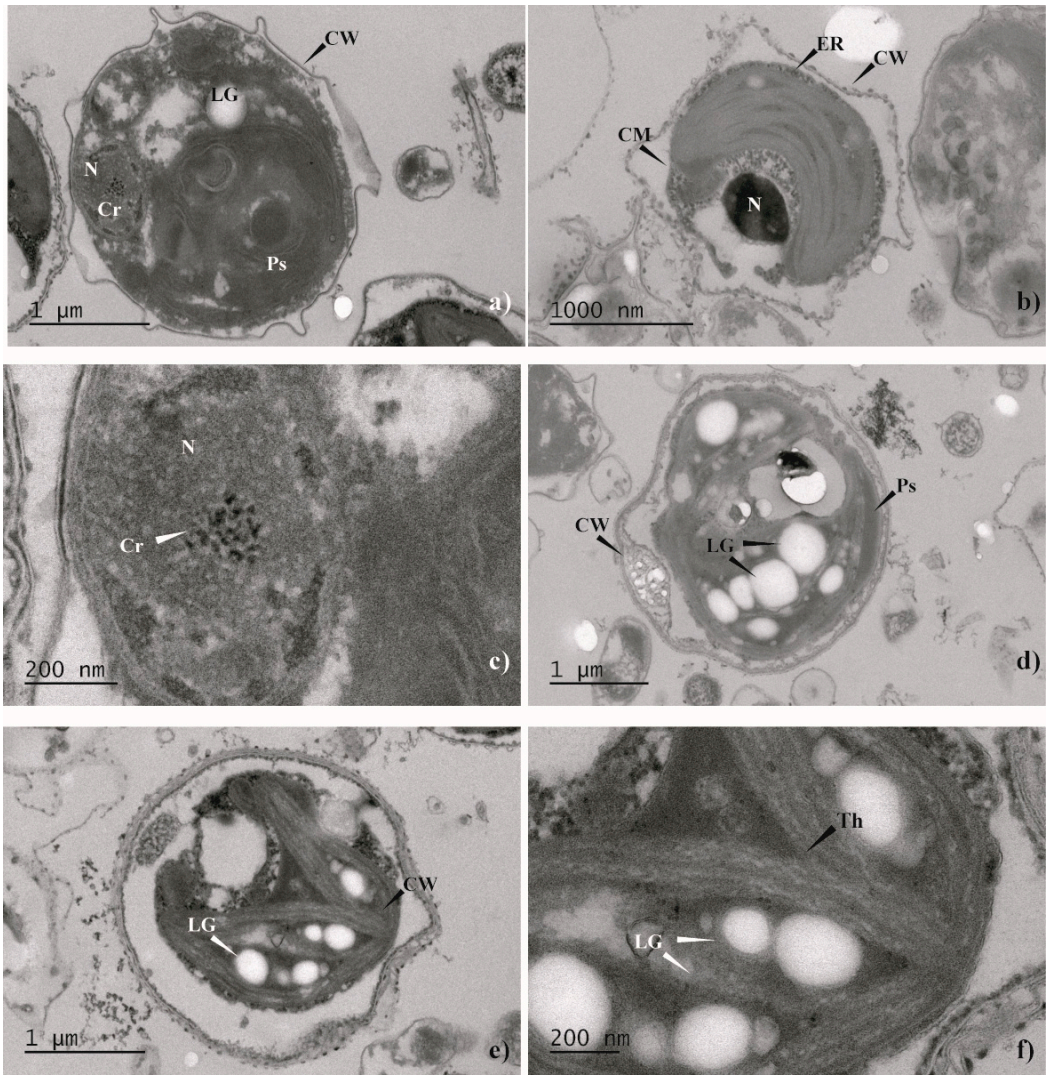

Figure 3. TEM of NSRE-1. (**a**) General appearance of *Nannochloropsis* sp. (**b**) Cell with detail of endoplasmic reticulum adjacent to the plastid, nucleus, membrane and ornamented wall. (**c**) Details of nucleus with chromatin. (**d**) Cell with lipid accumulation. (**e**) General aspect of cell in division. (**f**) Detail of plastids, thylakoids and lipids in dividing cells. N, nucleus; CM, cell membrane; CW, cell wall; ER, endoplasmic reticulum; Ps, plastid; Cr, chromatin; LG, lipid granules; Th, thylakoid.

Finally, NSRE-2 exhibited spherical or oval cells (1.4 to 2.8 µm in diameter), with a single parietal chloroplast, an eyespot, and with the pyrenoid surrounded by reserve substance. Solitary vegetative cells or cells clustered together were also observed in division into two with the formation of tetrads of aplanospores. Reproduction mode is mainly by autospores in two tetrads and by rupture of the autosporangium with the release of aplanospores. NSRE-2 showed large lipid bodies, a smooth cell wall, and the plastid attached to the endoplasmic reticulum and covered by four membranes, according to TEM (Figure 5). This characteristic evident in NSRE-1 and NSRE-2 strongly suggests that they belong to *Nannochloropsis* sp. (Eustigmatophyceae), in which the chloroplasts occupy most of the cell and contain a series of parallel lamellae composed of three thylakoids each, without lamella sheath [45]. Of the four membranes covering the chloroplast, the two external ones correspond to the endoplasmic reticulum, which apparently connects to the nuclear membrane [8].

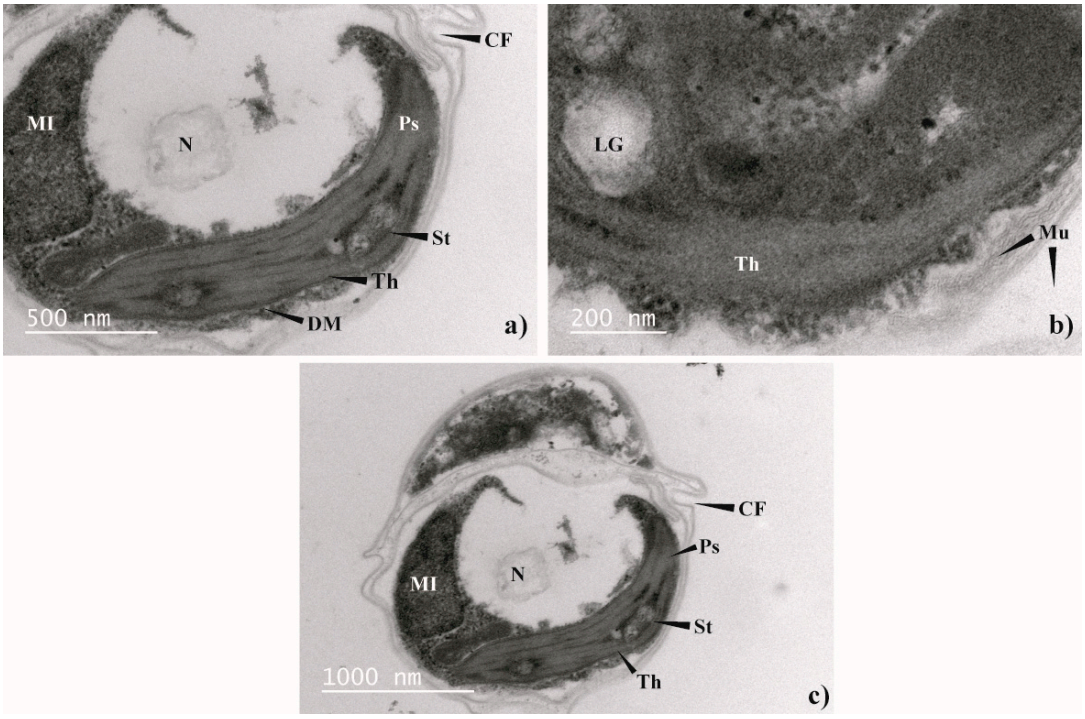

Figure 4. TEM of NRRE-1. (**a**) Cup-shaped plastid with starch granules and very broad, multilayer cell wall. The thylakoids penetrate the starch furrow and cup-shaped plastids with two membranes. (**b**) Detail of cell with nucleus and plastids with starch. (**c**) Cell in division with an evident furrow and cup-shaped chloroplast. MI, mitochondria; N, nucleus; Ps, plastid; Th, thylakoid; St, starch; CF, cleavage furrow; DM, double membrane; Mu, mucilage.

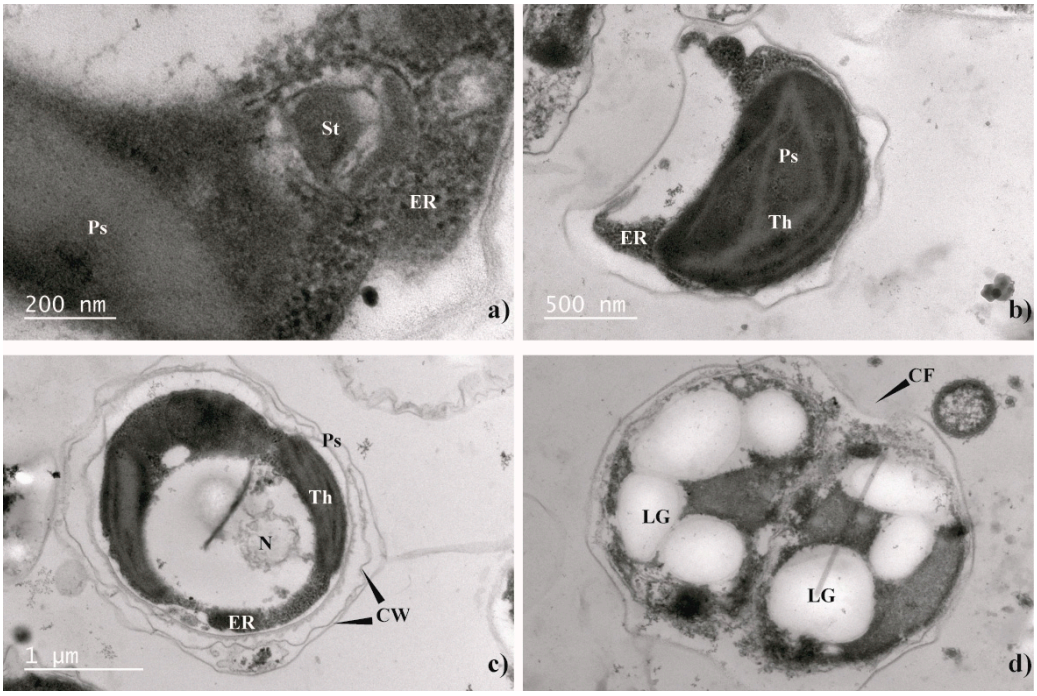

Figure 5. TEM of NSRE-2. (**a**) Detail of cells with reserve granules without the thylakoid membrane. (**b**) Cell with plastids, thylakoids, smooth-walled endoplasmic reticulum and chloroplast bounded by four envelopes. (**c**) Cell with nucleus, membrane and multilayer cell wall. (**d**) Dividing cell with large lipid granules. N, nucleus; Ps, plastid; Th, thylakoid; St, starch; CF, cleavage furrow; ER, endoplasmic reticulum; LG, lipid granules; CW, cell wall.

3.3. 18S rRNA Analysis

Morphological traits suggested the strains under study belong to *Nannochloropsis* (NSRE-1 NSRE-2) and *Nannochloris* species (NRRE-1). Molecular characterization was executed to support this classification based on morphological characteristics. The accession numbers, whose sequences showed homologies with 18S rDNA sequences of the strains under study, correspond to microorganisms with tiny cell sizes and from coastal marine environments. This confirms that the 18S rDNA lineages are consistent with the habitat [46]. The phylogenetic tree constructed with 18S rRNA region sequences supported splitting the strains into two groups (Figure 6), separating NSRE-1 and NRRE-1 from NSRE-2.

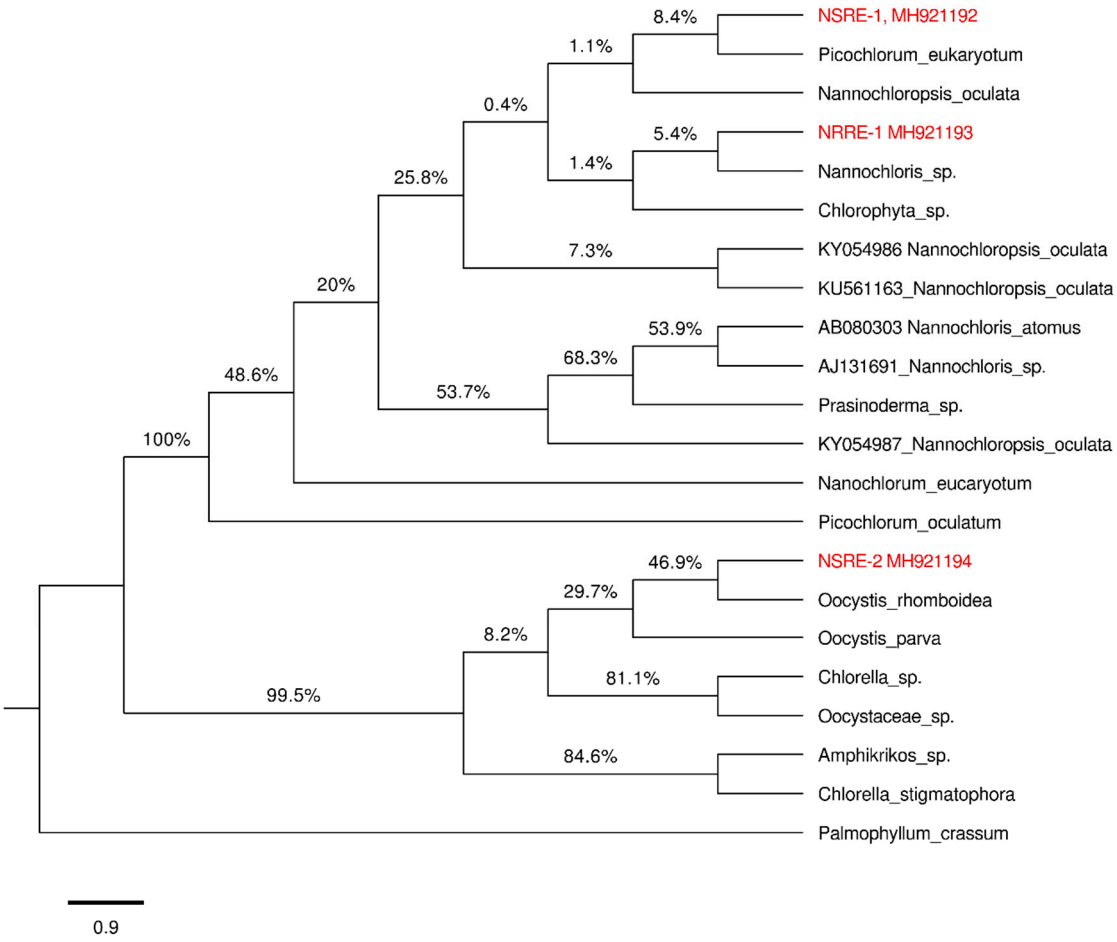

Figure 6. Molecular phylogenetic analysis by the maximum likelihood method inferred from 18S rDNA sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

Interestingly, NSRE-1 and NRRE-1 showed genetic relations with *Nannochloropsis oculata*, *Nannochloris* sp. and *Picochlorum eukaryotum*, all marine species [47] from coastal zones. The phylogenetic nearness of NSRE-1 and NRRE-1 to *Nannochloropsis* and *Nannochloris* must be revised in light of pigment composition as a taxonomic marker for the Eustigmatophyceae; e.g., astaxanthin and canthaxanthin present in authentic species of *Nannochloropsis* [48] and chlorophyll-*b*, to distinguish *Nannochloris* from *Nannochloropsis* [49]. Additionally, a larger fragment of the 18S rDNA, rbcL gene and ITS region must be considered to confirm their identity [8]. For now, NSRE-1 must be considered to belong to *Nannochloropsis* sp. and NRRE-1 to *Nannochloris* sp. pending further analysis.

On the other hand, NSRE-2 was clustered distantly from the rest of the strains under study. It falls into a freshwater clade dominated by *Oocystis* sp., characterized by synthesis of FAMEs

and essential fatty acids. However, there are many morphologic details to reject this possibility. Oocystaceae (Trebouxiophyceae) have a characteristic cell wall substructure that is composed of several cellulose layers with perpendicular fibril orientations [50]. In fact, since the work of Komárek (1979) the definition of the family has been based upon the ultrastructure of the multilayered cell wall [51]. The phylogenetic position of the NSRE-2 strain must be revised, including a longer 18S rDNA fragment, although the morphologic details strongly indicate that it must be considered to be *Nannochloropsis* sp. In this context, it is worth mentioning that there are few *Nannochloropsis* spp. entry sequences in GenBank.

3.4. Biomass Concentration, Lipid Extraction and Analysis

The dry biomass weight for NSRE-1, NRRE-1 and NSRE-2 strains was 0.82 ± 0.03, 0.58 ± 0.01 and 0.56 ± 0.02 g L^{-1} respectively (Figure 7), with no significant differences (ANOVA, $P < 0.05$, $n = 3$) between NRRE-1 and NSRE-2 strains, in contrast with the NSRE-1 strain.

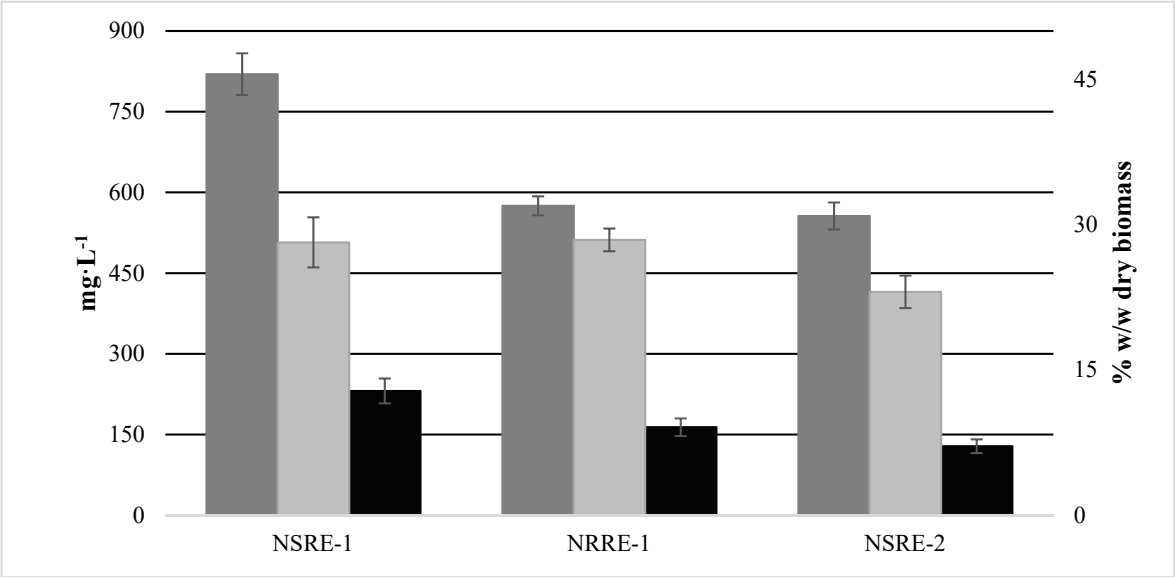

Figure 7. Dry biomass weight (dark gray bars), lipid extract (light gray bars) and lipid content (dark bars) of microalgae NSRE-1, NRRE-1 and NSRE-2.

Similar results to NSRE-1 were obtained in *Nannochloropsis salina* (0.9 g L^{-1}), *Nannochloropsis oleoabundans* (1.1 g L^{-1}) and *Dunaliella primolecta* (0.9 g L^{-1}), all marine microalgae. Meanwhile, for the NRRE-1 and NSRE-2 strains, dry biomass values were similar to those obtained in *Synechocystis salina* (0.6 g L^{-1}), *Microcystis aeroginosa* (0.8 g L^{-1}) and *Pseudokirchneriella subcapitata* (0.4 g L^{-1}) [52,53].

Total lipid extracts for NSRE-1, NRRE-1 and NSRE-2 were 307.91 ± 36, 218.02 ± 21 and 171.02 ± 16 mg L^{-1} (Figure 7) and lipid contents were 37.56, 37.91 and 30.75%, similar to the 25–56% lipid content found in several marine microalgae [52]. Meanwhile, lipid yields in seven marine isolated cultures of *Nannochloropsis* sp. (42.7–23.5% dry weight) [28] were similar to those obtained in this study from NSRE-1 and NSRE-2 (28.17 and 23.06% dry weight respectively).

Thin layer chromatography analysis indicated the presence of non-polar (terpenes and TAGs) and polar (fatty acids and phospholipids) lipids in the extracts. Therefore, to carry out their identification, the partitioning of the extract was executed by polarity gradient using column chromatography to obtain fractions of the extracts, which are summarized in Table 1.

Table 1. Fractions of the lipid extracts.

Fraction	% of Total Extract		
	NSRE-1	**NRRE-1**	**NSRE-2**
1	5.8	8.1	20.1
2	8.9	10.7	7.1
3	8.5	6	23.1
4	5.9	16.4	19.6
5	-	10	8.4
6	71	48.8	21.7

3.5. Lipid Profile Analysis (GC-MS)

The identification of the fractions by GC-MS generated numerous compounds grouped into six main types: hydrocarbons, FAMEs (after transesterification of TAGs), organic acids, terpenes, sterols and amines (Table 2).

Table 2. Compounds identified by GC-MS in strains NSRE-1, NRRE-1 and NSRE-2.

Compound (IUPAC)	Molecular Formula	% of Total Peak Area (wt % Fractions)		
		NSRE-1	**NRRE-1**	**NSRE-2**
Hydrocarbons				
Tetradecane	$C_{14}H_{36}$		5.64 (3.6)	
Pentadecane	$C_{15}H_{32}$	4.89 (8.3)		4.35 (4.5)
1-Heptadecene	$C_{17}H_{34}$	23.75 (59.2)		
Heptadecane	$C_{17}H_{36}$	6.91 (11.8)	8.48 (5.5)	5.97 (6.1)
Octadecane	$C_{18}H_{38}$	4.86 (8.3)		5.72 (5.9)
Nonadecane	$C_{19}H_{40}$		4.66 (3.0)	4.3 (1.1)
Eicosane	$C_{20}H_{42}$		7.8 (5.0)	8.78
Heneicosane	$C_{21}H_{44}$		7.28 (4.7)	8.31 (2.1)
Tetracosane	$C_{24}H_{50}$	3.95 (6.7)		7.76 (2.0)
Hexacosane	$C_{26}H_{54}$			4.86 (5.0)
Heptacosane	$C_{27}H_{56}$	3.37 (5.7)		
Neophytadiene	$C_{20}H_{38}$		64.22 (78.2)	64.98 (61.8)
Total (wt %)		33.8	59.9	46.9
FAMEs (TAG and FFA fractions)				
Hexadecanoic acid, methyl ester (Methyl palmitate, C16:0)	$C_{17}H_{34}O_2$	13.58 (4.16)	13.85 (28.48)	7.82 (10.82)
9,12-Octadecadienoic acid (Z,Z)-, methyl ester (Methyl linoleate, C18:2)	$C_{19}H_{34}O_2$		4.92 (10.11)	12.34 (26.35)
Octadecenoic acid. Methyl stearate (C18:0)			5.4 (10.4)	
(Z)-9-Octadecenoic acid, methyl ester (Methyl oleate, C18:1)	$C_{19}H_{36}O_2$		7.37 (15.15)	
Total (wt %)		9.5	24.7	23
Oxygenated compounds				
3-Hydroxy-butanoic acid	$C_4H_8O_3$	10.78 (2.2)		
Bis (2-ethylhexyl) hexadecanoic acid ester	$C_{22}H_{42}O_4$	45.19 (12.9)	4.93 (9.14)	39.03 (5.6)
Phytol	$C_{20}H_{40}O$		3.02 (10.0)	
6,10,14-Trimethyl-2-pentadecanone	$C_{18}H_{36}O$	10.54 (2.7)		
2-Palmitoylglycerol	$C_{19}H_{38}O_4$	9.94 (10.19)		
Campesterol	$C_{28}H_{48}O$		6.58 (12.20)	
Clionasterol	$C_{29}H_{50}O$		26.79 (49.68)	8.25 (2.4)
(Z)-13-Docosenamide	$C_{22}H_{43}NO$			61.35 (40.88)
2,2,4-Trimethyl-1,3-pentanediol 1-isobutyrate	$C_{12}H_{24}O_3$			5.01 (9.24)
2,4-Di-t-butylphenol	$C_{14}H_{22}O$		5.73 (18.97)	
Total (wt %)		56.8	15.3	30.1
% oxygen		19.2	6	12.5
H/C atomic ratio		1.9	1.7	1.9

3.5.1. Hydrocarbons

The hydrocarbons identified in the strains under study contained 14 to 27 carbon atoms (Table 2). Most common were even numbers (C_{12}, C_{16}, C_{18}, C_{22} and C_{24}), both saturated (alkanes) and unsaturated (alkenes). In all three strains, a higher percentage of saturated straight-chain hydrocarbons was detected (40, 80 and 90%, respectively). This result agrees with those reported in the literature,

where e.g., heptadecane represented more than 80% of total hydrocarbons. Heptadecane, pentadecane, and 7- and 8-heptadecene were detected in several *Nannochloropsis* species (Eustigmatophyceae). In marine microalgae, odd-numbered hydrocarbons account for more than 60%, typically C15, C17 or C21, and are often saturated.

Dry weight percentages of hydrocarbons in all strains yielded 0.055, 0.041 and 0.044% for NSRE-1, NRRE-1 and NSRE-2 respectively. This result is consistent because the hydrocarbon content in microalgae is generally low (less than 0.07% in dry weight) and mostly straight-chain alkanes and alkenes. Alka(e)nes of various chain lengths are important targets for biofuel production because they are major components of gasoline (mainly C5–C9 hydrocarbons), jet fuels (C5–C16) and diesel fuels (C12–C20). Microalgae hydrocarbons represent a potential feedstock for fuel development, although their use is still under development because knowledge of physiological pathways of hydrocarbon synthesis in microalgae is necessary to improve synthesis yields. Additionally, knowledge is also required to attain an economically viable scale-up process.

3.5.2. FAMEs

The fatty acid methyl esters (FAMEs) identified correspond to saturated C_{16} and unsaturated, mono- and di-, C_{18} (Table 2). The NSRE-1 extract contained 10% more C16:0 than C18:1. The ratio of saturated/unsaturated FAMEs was 1:0.89. The NRRE-1 extract contained 11.2% more C16:0 than unsaturated C18:1 and C18:2, resulting in a saturated/unsaturated FAMEs ratio of 1:0.88. In contrast, the NSRE-2 extract showed 57.8% greater production of C18:2 than C16:0, with a saturated/unsaturated ratio of 1:1.57. Hexadecanoic fatty acid methyl ester (C16:0) was identified in all extracts at percentages higher than 15% compared to other fatty acids in marine and freshwater microalgae strains. In *Nannochloropsis* strains, C_{16} to C_{20} fatty acid chains were identified with a variable level of unsaturation: e.g., palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and eicosanoic acid (20:0) [53]. The same compounds were identified in NSRE-1 and NSRE-2, with additional compounds such as 9,12-octadecadienoic acid methyl ester and 9-octadecenoic acid. For biodiesel production, a low ratio of saturated to unsaturated fatty acids is preferred (less than 22% FAMEs by weight), because for high concentrations of saturated FAMEs, the CFPP increases above 0°C, and the biodiesel obtained cannot be used in cold climates [54].

3.5.3. Organic acids

Free fatty acids and unsaponifiable organic acids were identified in the strain extracts under study (Table 2). The 3-hydroxy butanoic acid in NSRE-1 is a precursor in the formation of homopolymers in marine cyanobacteria [55]. *n*-Hexadecanoic acid (palmitic acid) was more abundant in NSRE-2 (26.01%) than in NSRE-1 (7.98%). In marine microalgae species, hexadecanoic acid has been identified among major saturated fatty acids. Hexadecanoic acid bis(2-ethylhexyl) ester or di(2-ethylhexyl)adipate (DEHA), identified at a high percentage in NSRE-1 and NSRE-2, is a constituent of polymeric flexible materials (polyvinyl chloride, PVC) and films used in the flexible vinyl industry [53]. Commonly, this compound is used as a solvent and a component of aviation lubricants, among others uses [56]. DEHA is relatively insoluble in water and therefore it is likely to be distributed in sediment through biota of aquatic ecosystems or by effluents from plastic-producing plants [53]. Possible contamination of the sampling site is discarded due to the constant changes of culture and purification treatments applied in this study. As far as the authors know, this is the first study that reports its identification in the lipid composition of green microalgae.

3.5.4. Terpenes

Bis(2-ethylhexyl) phthalate (DEHP) detected in NSRE-2 (Table 2) is a plasticizer or solvent in a variety of industrial products. It is an environmental pollutant in marine sediments, although it has been identified in lipid extracts of *Tetraselmis suecica* and *Chlorella sorokiniana*, *Synechocystis* sp. and mixed cultures of *Streptomyces* sp. and *Chlorella sorokiniana* [32]. In NSRE-1,

6,10,14-trimethyl-2-pentadecanone (hexahydrofarnesyl acetone) or HHA was identified, derived from the diterpene phytol, a secondary metabolite synthesized by higher plants with the ability to esterify chlorophyll-*a* [53]. It is also characterized as being a natural fragrance in higher plants [57] and as a lipid component in green microalgae, such as *Mougeotia viridis*, *Cladophora rivularis* [58], *Botryococcus braunii* [52] and the marine brown macroalgae *Padina pavonia* [59].

3.5.5. Sterols

In NSRE-2, 25-oxo-25-norcholesterol and clionasterol were identified. 25-Oxo-25-norcholesterol has been identified in extracts of marine microalgae (*Skeletonema costatum*) [2], in cyanobacteria subjected to hydrocarbon biodegradation processes, and in the control of the invasive growth of diatom species [32]. The detection of campesterol and clionasterol in NRRE-1 and NSRE-2 can be explained because both derive from the conversion of 24-methylene cholesterol to fucosterol in the synthesis of isoprenoids in green microalgae (Chlorophyceae) [60]. Sterols, especially from green microalgae, differ from those of higher plants in the configuration of the C24 alkyl groups, these being 24*S*, in contrast to the 24*R* of sterols in higher plants. These sterols from marine microalgae have been used as nutrients for the growth of aquaculture organisms [61]. Alternatively, the use of sterols has been proposed as substitutes in edible oils [57]. On the other hand, 2-palmitoylglycerol was reported as a substrate of chloroplast (omega)-3 fatty acid desaturases isolated from plant cells [57].

3.5.6. Amines

(*Z*)-13-Docosenamide was identified in the NSRE-2 strain with 61.35% total peak area (Table 2) is an allelopathic substance in lipid extracts of *Microcystis aeruginosa* (Cyanobacteria) and *Quadrigula closterioides* (Chlorophyceae) [53].

3.6. Potential of Lipids as Feedstock for Biofuel Production

The products extracted from the three strains were grouped according to their nature: hydrocarbons, TAGs and FFAs, and oxygenated compounds (Table 2). The composition of each of these fractions is analyzed in the following sections with a view to their possible direct use as fuel or as a raw material to be chemically processed and obtain high-quality renewable fuels. The quality of the biodiesel that can be obtained from compounds from the three strains was estimated through the use of experimental data and the application of selected correlations taken from the literature, according to the procedures described in reference [62] with the following two exceptions:

i) The equation reported by [63] was used in this work for the calculation of the cold filter plugging point (CFPP):

$$\text{CFPP } (^{\circ}\text{C}) = -26 + [\![5.76([\![\text{SAT}]\!]\_(\text{C16-C24}))]\!]\hat{}0.5 \quad (1)$$

where SATC16–C24 is the content (wt %) of saturated FAMEs having chains with 16 to 24 C atoms.

ii) Density at 15 °C, kinematic viscosity, higher heating value and oxidative stability of methyl oleate were taken from reference [64].

It is important to mention that no equation was found to estimate the CFPP for biodiesels with a content of saturated FAMEs as high as those of the three fractions of this work. However, Equation (1) was chosen because it is one of the most accurate equations found in the literature for estimating this property.

The potential of the hydrocarbon fractions to be used directly in mixtures with fossil fuel or to be fed to a catalytic improvement process for obtaining higher quality biofuels was also analyzed qualitatively, based on the lipid profiles obtained by GC-MS analyses. Finally, the fraction of oxygenated compounds was analyzed in a similar way to the hydrocarbon fraction, with the difference that in this case the composition of *Jatropha curcas* oil (JCO) was taken as a reference raw material for the oxygen elimination processes in the production of transportation biofuels.

3.6.1. Analysis of the Hydrocarbon, FAME and Oxygenated Fractions as Fuel Precursor

The composition of the hydrocarbon fractions obtained from the strains is shown in Table 2. Due to the high content (>59%) of mono-unsaturated compounds in each fraction, their direct use as diesel in mixtures with hydrocarbons of fossil origin is not recommended. This is because the presence of unsaturated molecules causes instability in fuels during storage [65]. For all strains, the shorter hydrocarbons are 14 or 15 C atoms. On the other hand, the fractions from NSRE-1 and NSRE-2 have hydrocarbons of more than 24 C atoms, although the concentration of these is relatively low (5.7 and 5% for strains NSRE-1 and NSRE-2 respectively). According to the above, the hydrocarbons of strain NRRE-1 could be improved through a single hydrogenation stage to saturate the double bonds in neophytadiene. This would produce diesel fuel with a high cetane number, although it would not have very good cold flow properties. It could, however, be mixed with diesel of fossil origin. Another alternative for the hydrocarbon fraction of the NRRE-1 strain would be to process it through a hydroisomerization stage. This could be more convenient, because the conditions of both processes are similar, with the isomerization process having the advantage of generating a given content of branched structures, which is necessary to improve the cold flow properties of the diesel obtained. In both cases, a diesel without the presence of heteroatoms would be obtained, which, when mixed with fossil diesel, would increase the yield of ultra-low-sulfur diesel.

On the other hand, the long hydrocarbons, which exceed the diesel range (>24 C atoms) of strains NSRE-1 and NSRE-2 could be separated by distillation and the remaining fraction could be processed in the manner described for strain NRRE-1. Another alternative for fractions one and three would be to transform the hydrocarbons without prior distillation in a hydroisomerization stage with simultaneous hydrocracking. This would make it possible to obtain high quality saturated and branched hydrocarbons as fuel, with chain sizes in the interval of diesel and bio-jet fuel.

For the analysis of oxygenated fractions, consider the following characteristic data of *Jatropha curcas* as a reference. According to in-house analysis, *J. curcas* oil (JCO), one of the raw materials that has been used to produce bio-jet fuel and green diesel through the HEFA process (HDO), has 11% by weight of oxygen and a H/C atomic ratio of 1.8. These values are close to others for different vegetable oils. Table 2 shows also the composition of the oxygenate fraction for the three strains, discarding TAGs and FFAs. The percentage of oxygen and the H/C atomic ratio for each fraction is also indicated.

As can be seen in Table 2, the oxygenated fraction of NSRE-1 has a component that has only four C atoms, which, when subjected to an HDO/DO process, will give rise to paraffin of three or four C atoms. While propane and butane are useful hydrocarbons as fuel or precursors for obtaining other compounds with higher added value, they are not part of liquid transportation fuels. The rest of the oxygenated components of NSRE-1 correspond to the diesel fraction according to the number of C atoms present in their structures. A simple HDO/DO stage would eliminate the oxygen heteroatoms and obtain linear paraffins with high cetane number. Alternatively, if the process is oriented towards the formation of linear and branched structures (hydrodeoxygenation plus hydroisomerization), a higher quality diesel could be obtained. Additionally, there is the option of favoring a moderate level of breakage of long molecules (hydrocracking), with which a certain yield of bio-jet fuel can also be obtained. However, it must be considered that the presence of cracking reactions and the consequent saturation of the resulting structures implies a higher net consumption of hydrogen in the process. The fractions from strains NRRE-1 and NSRE-2 can also be processed by HDO or DO to obtain renewable diesel. These fractions have the advantage of not having compounds with less than twelve carbon atoms. Therefore, a higher liquid yield would be expected in their processing. On the other hand, these fractions have structures with more than 22 C atoms, which would require the application of a certain level of hydrocracking in order to obtain hydrocarbons in the diesel range or even in the range of bio-jet fuel.

On the other hand, the oxygenated fractions of the three strains possess adequate characteristics to be transformed through the HEFA process to obtain bio-jet fuel as well as renewable diesel. When comparing the percentage of oxygen of the three fractions with that of JCO, that of NSRE-1

is observed to be considerably higher, while that of NRRE-1 corresponds to approximately half that of JCO. Finally, the oxygen content of NSRE-2 is similar to that of JCO. A greater or lesser amount of oxygen in the feed may imply the greater or lesser formation of CO and CO_2 byproducts, which, besides being polluting gases, are inhibiting agents of the bifunctional catalysts used in these processes. Moreover, the formation of CO and CO_2 is also directly related to the favored reaction routes which depend on the catalyst and the reaction conditions applied, since the hydrodeoxygenation route gives rise to the elimination of oxygen as water, while the decarbonylation/decarboxylation routes result in the formation of CO and CO_2. Whatever the catalytic route, the higher the oxygen content in the feed, the higher the catalytic capacity required to remove all oxygen present. Thus, hydroprocessing of the fraction from NSRE-1 may involve a slightly higher effort.

On the other hand, although the fraction from NRRE-1 has lower oxygen content, and this is convenient from the point of view of the removal of this heteroatom, this fraction has the highest content of structures with more than 24 C, which requires higher breaking levels to obtain equivalent yields of biofuels. A more significant presence of cracking reactions is also associated with a higher net consumption of hydrogen, as well as higher exposure to coke precursors, and a potential decrease in the lifetime of the catalysts used in the process. On the other hand, NSRE-1 requires a greater elimination of oxygen, but does not require favoring the breaking reactions. Regarding NSRE-2, it has an oxygen level equivalent to that of feeds processed by HEFA, and it has a low concentration of carbon chains with more than 24 C atoms, so its hydroprocessing would also be similar. As regards the H/C ratio of the three strains, the values are similar to the one present in JCO or other vegetable oils, so the net consumption of hydrogen would be comparable in orders of magnitude to those used in the processing of triglycerides by HEFA.

3.6.2. Catalytic Hydrodeoxygenation of Oxygenates from Strain NSRE-2

The reaction products obtained are listed in Table 3, and they were similar for the 4 h of time on stream, indicating a good stability of the catalyst used. The fact that the feed was composed of oxygenated C_{16} to C_{29} molecules, having 12.5 wt % oxygen and some unsaturations in their molecules (Table 2), indicates that certain level of hydrocracking occurred in the reaction, giving rise to hydrocarbons of smaller chain lengths. This, in turn, may imply the formation of some compounds shorter than C_{12}, that could not have been identified and quantified due to the large proportion of the C_9 used as solvent in the catalytic tests. However, these unidentified compounds would correspond to the gasoline fraction and would be free of oxygen, considering that the removal of this element is more difficult to achieve for larger compounds. In accordance with the above, it can be concluded that a complete elimination of the oxygen present in the feed was achieved in the HDO reaction. Likewise, a complete saturation of the identified reaction products was obtained, giving rise to a mixture of saturated hydrocarbons in the range of C_{12}-C_{20}.

Table 3. Compounds identified as HDO reaction products.

Compound (IUPAC)	Molecular Formula	% of Total Peak Area
Dodecane	$C_{12}H_{26}$	82.9
2,6,10-Trimethyldodecane	$C_{15}H_{32}$	1.8
Hexadecane	$C_{16}H_{34}$	1.8
Heptadecane	$C_{17}H_{36}$	5.9
Octadecane	$C_{18}H_{38}$	4.8
10-Methylnonadecane	$C_{20}H_{46}$	2.7

The product obtained is then an ultra-low sulfur renewable diesel with a high cetane number (conferred by the linear molecules of intermediate carbon chains lengths [C_{12}, C_{16}-C_{18}]), and good cold flow properties, due to the significant presence (4.5%) of structures with one or three branches. A diesel with this composition could probably be used in high proportions in mixtures with fossil diesel.

3.6.3. Estimation of Biodiesel Properties

Table 2 shows the composition of the TAG and FFA fraction expressed in terms of FAMEs resulting from their esterification and transesterification. The estimated properties of the biodiesels from the three strains studied are reported in Table 4. The following main fuel properties were considered in this analysis: density at 15 °C and 40 °C (ρ), cetane number (CN), kinematic viscosity at 40 °C (ν), higher heating value (HHV), iodine value (IV), cold filter plugging point (CFPP) and oxidative stability (OSI). Also, the reference values of the estimated properties are indicated in the table when they are considered in the American (ASTM D6751) and European (EN 14214) biodiesel standards.

Table 4. Predicted biodiesel properties and specifications in U.S. and European standards.

Property (Units)	NSRE-1	NRRE-1	NSRE-2	ASTM D6751	EN 14214
ρ @15°C (kg/m^3)	886 [a]	880.4	883.0		860–900
ρ @40°C (kg/m^3)	850.8	855.5	856.3		
CN	74.5	66.6	65.9	47 min	51 min
ν @40°C (mm^2/s)	4.7 [a]	4.5	4.2	1.9–6.0	3.5–5.0
HHV (MJ/kg)	38.4 [a]	39.7	39.5		
IV (g I/100 g biodiesel)	0.0	44.6	45.4		120 max
CFPP (°C)	31.6	17.9	23.4		
OSI (h)	27.4 [a]	5.4	7.1	3 h	6 h

[a] taken from [64].

It can be seen that the estimated densities of the three biodiesels at 15 °C fall within the limits established in EN 14214, while the U.S. biodiesel standard does not specify limits for this property.

Additional considerations on the density and the rest of the evaluated properties can be consulted elsewhere [62]. The CN of the three biodiesels is well above the minimum limit of 51 prescribed by the more stringent European standard. Moreover, the kinematic viscosity at 40 °C of the three biodiesels is also within the limits established by both standards. Furthermore, the higher heating value (HHV) estimated for biodiesels from the three strains lies between 38.4 and 39.7 MJ/kg, approximating the average value of 40 MJ/kg usually found for biodiesel. On the other hand, the estimated iodine values (IV), which are a measure of the unsaturation present in biodiesel, are well below the maximum limit of 120 g I/100 g biodiesel prescribed in the EN 14214 standard. Nevertheless, the estimated CFPP values for the three biodiesels are too high. The American and European standards for biodiesel do not consider defined limits for this property. However, a report is required depending on the season. Typical values of CFPP required in European countries are below 0 °C. For example, in Spain, the maximum limit of CFPP in summer is 0 °C, while the maximum in winter is −10 °C [66]. It is clear that the estimated CFPP values for the three biodiesels are very poor. This is explained by the high percentages of saturated species present in them, which range from 58 to 100%. For this reason, to be able to use these biodiesels it would be necessary to mix them with FAMEs having a higher content of unsaturated species to overcome the limitation on their applicability. Additionally, a warm climate would make their application more feasible. Contrary to what occurs for the CFPP parameter, oxidative stability (OSI) is favored by the presence of saturated fatty esters. For this reason, the biodiesels from the NSRE-1 and NSRE-2 strains, which possess higher percentages of saturated species, present OSI values higher than the minimum limit of 6 h indicated by the more stringent European standard. On the other hand, the OSI estimated for biodiesel from the NRRE-1 strain indicates that it complies with the American standard but not with the European one.

4. Conclusions

The collected strains were identified as belonging to *Nannochloropsis* (NSRE-1 and NSRE-2) and *Nannochloris* sp. (NRRE-1). To our knowledge, this is the first time that these species have been found in Mexico. The hydrocarbons extracted from the three strains studied fall within the range of diesel. However, their direct use is not recommended in pure form or mixed with fossil hydrocarbons due to the presence of

mono-unsaturated components, as these confer instability to the fuel during storage. For hydrocarbons from NSRE-1 and NSRE-2, it would be advisable to apply a process of hydroisomerization plus simultaneous hydrocracking to obtain molecules with shorter and more branched carbon chains, useful as high-quality renewable diesel and even as bio-jet fuel. On the other hand, to properly take advantage of the hydrocarbon fraction from NRRE-1, a single catalytic improvement step would be adequate to saturate the double bonds and obtain a high cetane number diesel.

Among the oxygenated fractions, the one corresponding to NSRE-1 showed the highest oxygen content (19.2 wt %), which could represent a more significant challenge for its processing to obtain renewable transportation fuels. Nevertheless, the three fractions could be processed through a single HDO or DO stage to obtain high-quality renewable diesel as the main product. This was experimentally demonstrated by subjecting the fraction of oxygenated compounds of the NSRE-2 strain to an HDO reaction, which produced a high-quality, renewable diesel that was completely free of oxygen and unsaturated compounds.

On the other hand, if bio-jet fuel is the desired product, it would be more convenient to process the fractions through the HEFA process. The oxygenated fractions from strains NRRE-1 and NSRE-2 are of the higher quality required for processing by HEFA because, their oxygen content is lower than or similar to that of JCO taken as the reference feed for this process.

Regarding the analysis of FAMEs from the three strains studied, it was found that the three biodiesels satisfactorily comply with the majority of the evaluated properties according to the limits established in the American and European standards. The property that showed the poorest results was the cold filter plugging point (CFPP), with values of 17.9–31.6 °C. This is explained by the high content of saturated FAMEs, which ranges from 58 to 100%. For this reason, these biodiesels could only be used in warm climates and mixed with FAMEs with a higher content of unsaturated species.

According to this analysis, the lipids of the strains studied are very valuable precursors for obtaining high quality renewable fuels, since these can be processed through different catalytic routes that involve not only transesterification, but also (hydro)deoxygenation reactions, which have great potential to transform varied feeds and obtain fuels with properties similar or even superior to those of fossil fuels.

Author Contributions: Data curation, A.R.L.-R., J.C.C.-H., E.P.-L. and B.C.-C.; Formal analysis, A.R.L.-R., J.C.C.-H., F.B.-P. and S.L.-A.; Funding acquisition, R.V.-O.; Investigation, A.R.L.-R., G.G.-S. and R.V.-O.; Methodology, A.R.L.-R. an Data curation, A.R.L.-R., J.C.C.-H., E.P.-L. and B.C.-C.; Formal analysis, A.R.L.-R., J.C.C.-H., F.B.-P. and S.L.-A.; Funding acquisition, R.V.-O.; Investigation, A.R.L.-R., G.G.-S. and R.V.-O.; Methodology, A.R.L.-R. and Katia Ancona-Canché; Project administration, A.R.L.-R. and R.V.-O.; Resources, R.V.-O.; Supervision, T.T.-T. and R.V.-O.; Validation, A.R.L.-R., J.C.C.-H., F.B.-P., G.G.-S., E.P.-L., B.C.-C. and R.V.-O.; Visualization, A.R.L.-R. and S.L.-A.; Writing—original draft, A.R.L.-R., G.G.-S. and S.L.-A.; Writing—review & editing, A.R.L.-R., J.C.C.-H., F.B.-P. and R.V.-O. d Katia Ancona-Canché; Project administration, A.R.L.-R. and R.V.-O.; Resources, R.V.-O.; Supervision, T.T.-T. and R.V.-O.; Validation, A.R.L.-R., J.C.C.-H., F.B.-P., G.G.-S., E.P.-L., B.C.-C. and R.V.-O.; Visualization, A.R.L.-R. and S.L.-A.; Writing—original draft, A.R.L.-R., G.G.-S. and S.L.-A.; Writing—review & editing, A.R.L.-R., J.C.C.-H., F.B.-P. and R.V.-O.

Funding: The authors gratefully acknowledge the financial support granted by the Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico) through projects No. 166371 and No. 254667 (SENER –CONACYT), as well as the graduate grant for the first author No. 522627.

Acknowledgments: The authors also wish to thank José Luis Paredes (Instituto de Fisiología Celular, UNAM) for technical Support on TEM and Carlos López Salas for TEM figure editions.

Conflicts of Interest: The authors declare no conflict of interest.

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