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Isolation and Characterization of Native Microalgae from the Peruvian Amazon with Potential for Biodiesel Production

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Abstract: Biodiesel production from microalgae triacylglycerols is growing, because this feedstock is a more sustainable and advantageous alternative. In this study, we isolated and identified fourteen strains of native microalgae from the Peruvian Amazon. These strains showed great heterogeneity in biomass productivity, lipid productivity and lipid content, and thus, three of them (*Acutodesmus obliquus*, *Ankistrodesmus* sp. and *Chlorella lewinii*) were selected for further evaluation under culture of nitrogen-sufficient (+N) and nitrogen-deficient (−N) Chu medium No. 10. These microalgae species showed modifications in biomolecule content (protein, lipid and carbohydrate) with a pronounced increase of lipids and carbohydrate and a decrease of protein content under stress culture. Furthermore, the fatty acid profile was peculiar for each species, and these patterns showed evident changes, particularly in the proportion of saturated and monounsaturated fatty acids. The results of this research suggest that the isolated native microalgae, from the Peruvian Amazon, could be suitable candidates for biodiesel production.

Keywords: biodiesel; fatty acid profiling; lipid content; oleaginous microalgae; Peruvian Amazon

1. Introduction

The global increase of energy demand, the depletion and increasing of the costs of fossil fuels [1], and climate change [2] are problems that require urgent solutions. In order to mitigate these problems, Peru has legal dispositions (Law 28054 of the promotion of the biofuel market, and their regulation with Supreme Decree No. 013-2005-EM), so that the energy matrix of the country depends on renewable resources [3,4]. These legal dispositions have increased the importation of biodiesel, due to insufficient national production, which is currently obtained from plant oils, such as *Jatropha curcas* "white pinion" and *Elaeis guineensis* "palm", both cultured in large areas of the Peruvian Amazon [5].

Biodiesel obtained from crop-based plant oils, however, has major drawbacks. These include low yields, high land and water requirements, detrimental effects on food supplies and associated extensive

deforestation of the rainforest, which pose threats to native biodiversity and ecosystem functions, goods and services [6–8]. These limitations can be overcome by the next generation of microalgae-based biofuels. Compared to terrestrial crops, the main advantages of microalgal systems are that they have a higher photon conversion efficiency, can be harvested batch-wise nearly all-year-round, can utilize salt and waste water streams, can couple CO_2-neutral fuel production with CO_2 sequestration and produce non-toxic and highly biodegradable biofuels [7].

The isolation, selection and culturing of robust oleaginous microalgae with desirable attributes (e.g., high triacylglycerol content, appropriate fatty acid profiles, high growth rate and resistance to invasion of local microorganism), however, are the principal challenges associated with microalgae biofuel production [9,10]. Although such microalgae strains are currently unavailable, optimism persists due to the fact that there is an abundance of species in the wild yet to be isolated [11]. Indeed, only a few thousand (~50,000) are conserved worldwide in microalgal culture collections [9,12], and of those, only a few hundred (<10%) have been investigated for their biochemical composition [13].

Consequently, the isolation, selection, biochemical and molecular characterization of microalgae from aquatic environments must be a continuous effort of screening for strains with the potential for biodiesel production [14]. Therefore, a sustainable development of microalgal-based biodiesel and bioactive chemicals production in Peru requires the bioprospecting and exploitation of native microalgae bioresources as the most viable option. The northeastern region of Peru, particularly the Loreto region, contains a great variety of freshwater aquatic environments (i.e., rivers, lagoons, etc.) that harbor a rich biodiversity of microalgae. In this study, 14 strains of native microalgae from the Peruvian Amazon were isolated, identified and tested for their biomass productivity, lipid productivity and total lipid content. In addition, the growth profile, total carbohydrate, total protein, total lipid content and fatty acids profiles of three selected strains were also investigated and compared under nitrogen-sufficient and -deficient culture conditions.

2. Experimental Section

2.1. Sample Collection and Isolation of Microalgae

The freshwater samples (100 mL) were collected from three river basins in the Loreto Region of the Peruvian Amazon: Amazon River (03°41′0.6″ S, 73°14′8.9″ W), Itaya River (03°43′1.4″ S, 73°14′17.8″ W) and Nanay River (03°42′0.2″ S, 73°15′32″ W). Microalgae samples (50 mL) were enriched with 50 mL of Chu medium No. 10 [15]. The pH was adjusted to 7.5 with 1 M NaOH before autoclaving and the addition of microalgae. The microalgal cells were maintained in Chu medium No. 10 for four weeks in a controlled culture room at 25 ± 1 °C with 12:12-h light-dark cycles using 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity of cool-white fluorescent light and continuous agitation at 110 rpm. After initial cultivation of the mixed cultures, unicellular microalgae were subjected to isolation by the cell washing method [12]. Briefly, a 20-µL aliquot of the mixed microalgae culture was placed on a sterile glass slide under an inverted microscope to pick up a single microalgae cell with a Pasteur pipette containing a cotton-wool filter and connected to a flexible plastic hose. Each microalgae cell was then sequentially washed in six 20-µL drops of Chu medium No. 10. Subsequently, the drop with a single microalgae cell was transferred to a test tube with 1 mL of Chu medium No.10 medium, followed by inoculation onto Petri plates containing Chu medium No. 10 supplemented with 1.5% (*w*/*v*) of agar. Repeated streaking on the nutrient agar plate and routine microscopic examination ensured the purity of the cultures. Single individual colonies appearing on plates (~7 days) were inoculated into Chu liquid medium No. 10 and grown for 14 days at the described conditions.

2.2. Morphological Identification of Microalgae

Isolated strains were preliminarily identified using standard morphological features [16]. Intracellular lipid droplets of isolated microalgae were detected by the fluorescence emitted by interacting with Nile Red [17]. Briefly, 1.5 mL of microalgae cells culture were harvested by

centrifugation at 1900× *g* for 10 min and washed two times with 1 mL of physiological saline solution. Microalgal cell pellets were dissolved in Nile Red acetone solution (2 µg/mL) and incubated for 15 min at 37 °C. After washing once, stained microalgal cells were observed by Carl Zeiss fluorescent microscopy and photographed with a digital camera AxioLab.A1 AxioCamERc real time 5 s. Images were obtained at a magnification of 1000× with visible light and epifluorescence (excitation 510–560, emission 590).

2.3. Genomic DNA Isolation

Microalgae cells were harvested from 100 mL of liquid culture by centrifugation at 1900× *g* for 10 min. Genomic DNA was extracted using a modified version of the CTAB method as described by Doyle and Doyle [18]. Briefly, algal cells were completely ground in a mortar containing 50 mg of sterilized sand and 3 mL of extraction buffer (300 mM Tris-HCl pH 8.0, 50 mM ethylenediamine tetraacetic acid (EDTA), 2 M NaCl, 2% cetyltrimethylammonium bromide, 3% polyvinylpyrrolidone (MW 40,000) and 2% β-mercaptoethanol). Homogenized cells were incubated at 70 °C for 30 min with gentle inversion every 2 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, *v*/*v*) was then added followed by centrifugation at 20,000× *g*. The aqueous supernatant was transferred to a new microtube, and an equal volume of chloroform/isoamyl alcohol (24:1, *v*/*v*) was added followed by centrifugation at 20,000× *g*. The aqueous supernatant was transferred to a new microtube and mixed with 0.1 volumes of 3 M sodium acetate (pH 5.2), and an equal volume of chilled isopropanol was added to precipitate the DNA at 15,000× *g*. Washing was done with 70% alcohol, and the air-dried DNA pellet was dissolved in 100 µL of sterilized water treated with RNase A at 40 °C for 30 min and then extracted with chloroform/isoamyl alcohol and DNA precipitation with absolute ethanol. Finally, the air-dried DNA pellet was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at −20 °C. Genomic DNA quality and quantity were assessed by standard OD measurement [19] using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). In order to verify DNA integrity, samples were resolved by standard gel electrophoresis in 1.2% agarose gels [19] stained with ethidium bromide and visualized under ultraviolet light (Supplementary Materials Figure S1).

2.4. PCR Amplification and Sequencing of ITS2-rDNA Region

The ITS-2 rDNA gene was amplified according to Kaur et al. [20] with minor modifications; in 20-µL reactions containing 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.025 U/µL Amplitaq Gold, 1.0 µM each of the forward primer SQITS1 (5′-GAGCATGTCTGCCTCAGC-3′) and reverse primer SQITS2 (5′-GGTAGCCTTGCCTGAGC-3′) and 20 ng of genomic DNA. The general conditions for PCR in a thermal cycler Realplex S4 (Eppendorf, Hamburg, Germany) were 5 min at 94 °C for the first cycle followed by 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C for 35 cycles and a final 10-min extension step at 72 °C. The PCR gene products were separated on 2% agarose gels using 0.5 × TBE buffer at 85 volts for 60 min and detected by staining with ethidium bromide (Figure S2). To purify, 4 µL of the PCR gene product were mixed with 2 µL of ExoSAP-IT (Affymetrix, Santa Clara CA, USA), incubated at 37 °C for 30 min and 80 °C for 15 min and directly sequenced in both directions with primers SQITS1 and SQITS2 following the instructions of the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Foster City, CA, USA). The sequencing products were purified by precipitation with ethanol/EDTA, denatured and resolved on a Genetic Analyzer 3130XL (Applied Biosystems™, Foster City, CA, USA). All sequences presented here were submitted to GenBank [21].

2.5. Phylogenetic Analysis

The ITS-2 rDNA gene sequences of the isolates were searched for homology in the National Center for Biotechnology Information database [22] using BLAST [23]. Sequences were edited in BioEdit 7.2.5 software [24] to trim the non-overlapping 5′ and 3′ regions for the purpose of restricting the multiple sequence alignment within the minimum common region. Those edited sequences were aligned using

Clustal omega [25]. The unrooted phylogenetic tree was constructed using the neighbor-joining (NJ) method [26]] and Kimura's two-parameter algorithm [27], as implemented within the MEGA 6.06 software [28]. The bootstrap analysis [29] was used with 1000 replications to test the relative support for the branches produced by NJ analysis.

2.6. Microalgae Cultivation, Biomass and Total Lipids Measurements

Two hundred milliliters Chu medium No. 10 in a 500-mL Erlenmeyer flask were inoculated with the microalgae cells (OD_{680} 0.05) and incubated at 25 ± 1 °C using 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity of cool-white fluorescent light and continuous agitation at 150 rpm for four weeks. In this culture stage, all microalgae strains were found in the stationary growth phase and centrifuged at 1920× *g* for 10 min to harvest the microalgae cells. The cell pellets were rinsed with 50 mL deionized water, centrifuged again, dried in an oven at 70 °C to a constant weight, and the dry weight was determined gravimetrically with an analytical balance Kern ABJ 220-4NM (Kern & Sohn GmbH, Balingen, Germany). The algal powders were stored at −20 °C for total lipid analysis.

Total lipid was extracted using a modified protocol of Bligh and Dyer [30]. For each sample, approximately 50 mg of microalgal powder were mixed with 3 mL of chloroform/methanol (2:1, *v*/*v*) and homogenized in an ultrasonic Branson 2510 (Branson: Danbury, CT, USA) for 5 min. The mixture was then centrifuged at 20,000× *g* for 5 min, and the supernatant was transferred into a pre-weighed vial. Cellular debris was mixed with another 2 mL of chloroform/methanol (2:1, *v*/*v*) by vortex and then centrifuged as described above. After that, the pooled organic phases were filtered through syringe filters of 0.45 µm, evaporated with nitrogen gas and dried at 80 °C in a vacuum oven Vacucell 55—Comfort (MMM, München, Germany) to a constant weight. The lipid content and productivity of each sample were measured gravimetrically using a semi-micro analytical balance (Sartorius, MSU225S-000-DU, Foster City, CA, USA) and calculated as follows [31]:

$$\text{Total lipid (\% dry weight)} = W_L / W_A \times 100.$$

where W_L (g) is the weight of the total lipids extracted and W_A (g) is the weight of the dry microalgae biomass.

$$P_{\text{Lipid}}\ (\text{mg} \cdot \text{L}^{-1} \cdot \text{d}^{-1}) = W_A\ (\text{g}) \times C_{\text{Lipid}}\ (\%) / \text{V(L)} \times \text{T(d)}$$

where P_{Lipid} is the lipid productivity, C_{Lipid} is the lipid content, V is the working volume and T is the cultivation time.

2.7. Strains Culture under Nitrogen-Sufficient and Nitrogen-Deficient Medium

The selected microalgae species *Acutodesmus obliquus* (KP878505.1), *Ankistrodesmus* sp. (KP878500.1) and *Chlorella lewinii* (KP878501.1) (Figure S3) were grown in 500-mL Erlenmeyer flasks containing 250 mL of liquid Chu medium No. 10 with nitrogen sufficient (57.56 µg/mL of $Ca(NO_3)_2 \cdot 4H_2O$) or liquid Chu medium No. 10 without a nitrogen source (deficient) for seven days in a controlled culture room at 25 ± 1 °C with 12:12-h light-dark cycles using 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity of cool-white fluorescent light and continuous agitation at 150 rpm.

For growth profile analysis, the maximum absorption peak for each microalgae strain was determined in the 600–800 nm range by spectrophotometric analysis using a Nanodrop 2000 UV-Vis Spectrophotometer (Figure S4). Then, aliquots of the microalgal cultures were evaluated every 24 h (between 8 and 9 h) at 680 nm. The specific growth rate (µ) was calculated during the exponential growth phase (the first three days of culture) according to the equation $\mu = (\ln N_f - \ln N_i)/(t_f - t_i)$, where N is the optical density (OD_{680}) at final (f) or initial (i) time (t). Previously, it was determined that a high (R^2 from 0.992 to 0.998) and positive correlation exists between optical density and cell densities; this variable was calculated using a hemocytometer. The experiments were carried out in triplicate, and the data were expressed as the mean ± standard deviation (±SD).

2.8. Determination of Total Protein, Total Carbohydrate, Ash Content, Esterification and Fatty Acids Profiling

The microalgae cultures maintained for seven days under +N and −N Chu medium No. 10 were harvested by centrifugation, rinsed and dried as previously described (Section 2.5). From the microalgae powders, total protein content was determined according to the Hartree–Lowry method [32]. To 5 mg of dry microalgal biomass was added 5 mL of 0.5 M NaOH and incubated for 10 min at 80 °C, then centrifuged for 5 min at 14,000× *g*. Protein content in 1 mL of the supernatant was determined by adding 0.5 mL of 0.5 M sodium carbonate, 0.5 mL of 0.5 M sodium potassium tartrate, 0.5 M of copper sulfate and 2 mL of Folin-Ciocalteu reagent. The blue complex was analyzed in a Varian Cary 50 Bio UV-Visible Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) set at 650 nm against a calibration curve based on a known concentration of BSA as the standard.

Total carbohydrate content of the microalgal biomass was determined with the phenol-sulfuric acid method [32,33]. For this purpose, 5 mg of dry microalgae biomass were added to 5 mL of water. After this, a 1-mL aliquot of the sample was added to 3 mL of sulfuric acid and 1 mL of 5% aqueous solution of phenol, and the mixture was stirred and incubated for 5 min at 90 °C. The yellow-brown complex was analyzed by spectrophotometric analysis at 488 nm against a calibration curve based on a known concentration of glucose.

Ash content was done according to the AOAC procedure [34]. One hundred milligrams of dry material were ashed in a muffle furnace Thermolyne™ F6010 (Thermo Fisher Scientific, Walthman, MA, USA) at 550 °C for 16 h, and the content was determined gravimetrically. All analyses were carried out in triplicate, and the data are expressed as the mean ± SD.

Fatty acids methyl esters (FAME) were prepared by acid transesterification according to Ichihara and Fukubayashi [35]. The crude lipid extract (1 mg) was dissolved in 0.2 mL of toluene, 1.5 mL of methanol and 0.3 mL of 0.8% HCl (prepared in methanol:water 85:15 *v*/*v*), transferred into a capped test tube, mixed for 5 min and then incubated at 45 °C for 12 h for derivatization reaction. Then, 2 mL of n-hexane and 2 mL of distilled water were added to the tube. After vortexing, the hexane phases containing FAMEs were transferred into a 1.5-mL tube and dried under a stream of nitrogen. Finally, the FAMEs were redissolved in 10 µL acetonitrile and analyzed by using a gas chromatograph Varian CP-3800 GC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated sampler and injector, a flame ionization detector and using a 30 m × 0.32 mm × 0.25 µm Stabilwax® capillary column (Restek, Bellefonte, PA, USA). The GC conditions were as follows: injector temperature: 250 °C; the column temperature gradient was: 120 °C for 1 min, followed by an increase to 160 °C at the rate of 30 °C/min, 160 °C for 1 min, followed by an increase to 240 °C at the rate of 4 °C/min and 240 °C for 7 min; detector temperature: 260 °C. Gas pressures for He, H_2 and synthetic air were maintained at 40, 80 and 60 psi, respectively. The carrier gas (He) flow was maintained at 1 mL/min. The fatty acids were analyzed by comparing their retention time of the corresponding peaks with a known standard mixture of FAMEs (Nu-Check Prep, Elysian, MN, USA), and methyl tricosanoate (Sigma-Aldrich, Saint Louis, MO, USA) was added to each sample as the internal standard. All chromatograms of the microalgal samples were analyzed by using the Galaxie™ Chromatography Data System Version 1.9.3.2 (Agilent Technologies, Santa Clara, CA, USA). All peaks spanning a peak area of more than 50 units were integrated. All analyses were carried out in triplicate, and the data are expressed as the mean ± SD.

2.9. Statistical Analysis

Statistical analyses were performed using the IBM SPSS Statistics 21.0 software (IBM: Armonk, NY, USA). The growth rate, biomass and lipid content data for microalgal strains were analyzed using a one-way analysis of variance (ANOVA) and the HSD Tukey test. The correlation of biomass productivity and lipid content was analyzed using bivariate correlations, and Pearson's correlation coefficients are given with their significance levels. Differences were considered significant at $p < 0.05$. All data presented here are given as the mean ± SD.

3. Results and Discussion

3.1. Isolation and Identification of the Microalgae Strains

Bioprospecting of tropical ecosystems can provide exceptional native microalgae strains with the potential for the production of biodiesel, nutraceuticals, bioactive chemicals and other high value products [36–38]. Furthermore, native microalgae strains can have potential applications for efficient biological sequestration of CO_2, wastewater treatment and other environmentally-friendly applications [38–40]. Consequently, isolation is a fundamental process to obtain pure cultures and is the first phase towards the screening and selection of microalgae strains with the potential for biodiesel production and the aforementioned applications.

According to these demands, in this research, samples were collected from three different types of water bodies, the Amazon River (white-water type), the Itaya and Nanay Rivers, both of the black-water type. The white-water rivers originate from the Andean catchments bringing high amounts of nutrient-rich sediments. In contrast, the black-water rivers drain geochemically-poor watersheds and are characterized by their high content in humic acids, low pH and poor nutrient status [41]. A total of 14 microalgal strains were isolated using the standard cell washing technique, and based on distinguishable morphological characters under light microscopic examination, these strains were preliminary ascribed to the genera: *Acutodesmus* (Hegewald) Tsarenko, 2001; *Ankistrodesmus* Corda, 1838; *Chlorella* Beyerinck Beijerinck, 1890; *Desmodesmus* (Chodat) S.S. An, T. Friedl, and E. Hegewald, 1999; and *Tetradesmus* G.M. Smith, 1913 (Table 1). These genera are green algae that belong to division Chlorophyta, class Chlorophyceae and orders Chlorellales and Sphaeropleales. Although morphological analysis is frequently used to identify microalgae, it is inaccurate and very difficult for the identification at the species level, because the relationship between diagnostic morphology and biological species boundaries are largely unknown in many micro-eukaryotes [42]. Furthermore, according to Yu et al. [31], the microalgae morphology for the same strain varies in relation to age and culture conditions. Consequently, in microalgae, the classic taxonomy is supported by molecular tools [42]. For example, the ITS2 rDNA region is thought to be an excellent marker for molecular phylogenetics studies in lower taxonomic levels, due to being highly divergent and fast-evolving, which can discriminate among closely-related organisms, which otherwise display almost identical sequences [43,44]. For this reason, the molecular identification based in phylogenetic analysis of ITS2 rDNA gene sequences is commonly used to identify and/or delineate species [20,45,46].

Table 1. Genera, GenBank accession number of ITS2 sequences, biomass productivity, lipid productivity and lipid content of isolated microalgae strains.

Source of Collection	Strain	Genera/Species	GenBank Accession Number	Biomass Productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	Lipid Productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	Lipid Content (% dw)
Amazon River (03°41′0.6″ S, 73°14′8.9″ W)	AMA001	*Ankistrodesmus* sp.	KP878496.1	12.2 ± 0.3	3.7 ± 0.1	30.7 ± 1.5
	AMA002	*Ankistrodesmus* sp.	KP878497.1	3.6 ± 0.4	1.0 ± 0.2	27.7 ± 2.1
	AMA003	*Chlorella lewinii*	KP878501.1	22.3 ± 0.7	5.1 ± 0.5	22.7 ± 1.5
	AMA004	*Acutodesmus abliquus*	KP878505.1	31.6 ± 0.4	6.4 ± 0.7	20.3 ± 2.1
Itaya River (03°43′1.4″ S, 73°14′17.8″ W)	ITA001	*Ankistrodesmus* sp.	KP878499.1	5.9 ± 0.2	4.0 ± 0.3	43.7 ± 1.7
	ITA002	*Ankistrodesmus* sp.	KP878500.1	13.6 ± 0.4	5.7 ± 0.5	30.6 ± 2.0
	ITA003	*Chlorella lewinii*	KP878503.1	12.9 ± 0.3	4.0 ± 0.5	22.4 ± 2.7
	ITA004	*Chlorella lewinii*	KP878504.1	22.1 ± 0.7	3.1 ± 0.4	21.5 ± 2.3
	ITA005	*Scenedesmus regularis*	KP878508.1	9.3 ± 0.4	5.6 ± 0.4	24.2 ± 1.4
	ITA006	*Scenedesmus dimorphus*	KP878509.1	18.6 ± 0.6	3.2 ± 0.3	26.0 ± 1.7
	ITA007	*Acutodesmus abliquus*	KP878510.1	17.9 ± 0.4	2.2 ± 0.3	15.6 ± 1.9
Nanay River (03°42′0.2″ S, 73°15′32″ W)	NAN001	*Ankistrodesmus* sp.	KP878498.1	14.3 ± 0.6	2.8 ± 0.2	46.7 ± 1.5
	NAN002	*Desmodesmus tropicus*	KP878506.1	12.2 ± 0.4	2.3 ± 0.2	17.5 ± 1.1
	NAN003	*Tetradesmus* sp.	KP878507.1	13.9 ± 0.4	3.0 ± 0.3	13.5 ± 1.2

To realize phylogenetic analysis, genomic DNA of each isolate strain was purified and used for PCR amplification of ITS2 rDNA. The universal primers used to amplify the ITS2 rDNA successfully amplified the desired DNA fragments (~300 bp) from all microalgal strains (Figure S2). Further, these amplicons were sequenced, published in the NCBI databases, and the corresponding GenBank accession numbers are presented in Table 1. Figure 1 shows a neighbor-joining phylogenetic tree depicting the relatedness of the native microalgae strain isolates with selected sequences from the public GenBank database. This phylogram contained three clusters, each of which included different microalgae genera. For example, the first cluster consisted of microalgae of the genera *Acutodesmus*, *Scenedesmus*, *Tetradesmus* and *Desmodesmus* and six isolated strains (ITA007, AMA004, ITA006, NAN003, ITA005 and NAN002). The second cluster consisted of a microalgae of the genus *Ankistrodesmus* and the isolated strains AMA001, ITA001, ITA002, NAN001 and AMA002. The third one consisted of a microalgae of the genus *Chlorella* and three isolated strains (ITA004, AMA003 and ITA003). Overall, the identification of native microalgae strains presented a good correspondence between morphological characters and sequence-based phylogenetic analysis. Microalgae species of these genera were also isolated from different countries of the world, such as Brazil [47], Argentina [46], United States [48], India [20], Egypt [49], Taiwan [50], South Korea [45] and China [51]. Some of these microalgae genera, such as *Ankistrodesmus*, *Chlorella*, *Desmodesmus* and *Scenedesmus*, were used in large-scale biodiesel production using photobioreactors and raceway pond systems [52,53].

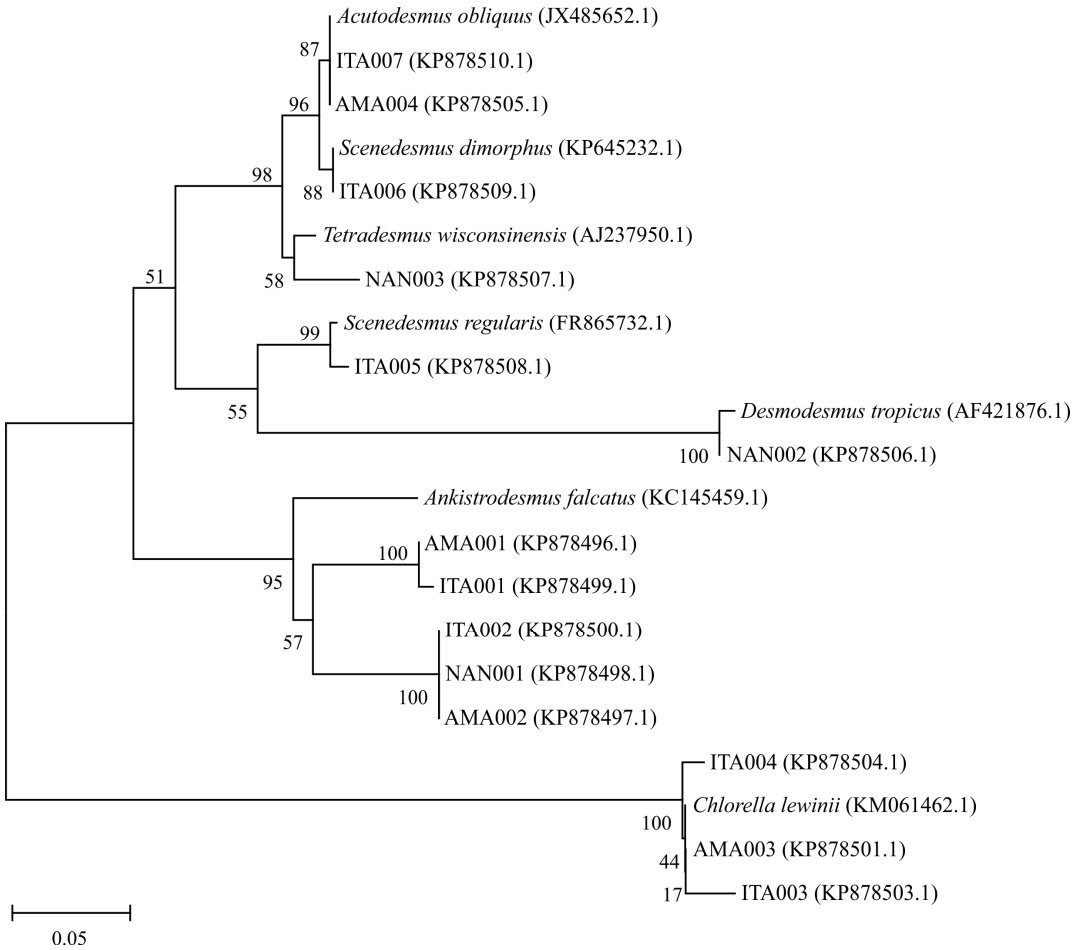

Figure 1. Unrooted phylogenetic tree of isolated microalgae strains using the ITS2 sequences. The tree was constructed by the neighbor-joining method and Kimura's two-parameter algorithm in MEGA 6.0 software based on the multiple sequence alignment by Clustal Omega. Bootstrap values of 1000 replicates (%) are shown at the branches.

3.2. Microalgae Cultivation, Biomass and Total Lipids Measurements

The isolated microalgae strains exhibited remarkable differences in productivity parameters and lipid content among individual strains in the stationary growth phase (Table 1). With regard to the biomass productivity, the 14 strains showed statistically-significant differences (F = 719.11, df = 13, $p < 0.001$) and ranged from 3.6 ± 0.4 mg·L^{-1}·d^{-1} (*Ankistrodesmus* sp. strain AMA002) to 31.6 ± 0.4 mg·L^{-1}·d^{-1} (*Acutodesmus obliquus* strain AMA004). Similar biomass productivities were reported for other marine microalgae species [54]. However, other investigations show major biomass productivities (ranging from 30 to 120 mg·L^{-1}·d^{-1}) in the same and other microalgae genera [45,55,56]. These discrepancies may be attributed to differences in the culture conditions, in the culture media composition and in the microalgae genotypes. Lipid productivity also exhibited statistically-significant differences (F = 47.74, df = 13, $p < 0.001$) and ranged from 1.0 ± 0.4 mg·L^{-1}·d^{-1} to 6.4 ± 0.7 mg·L^{-1}·d^{-1} with strains AMA002 and AMA004, respectively. These lipid productivities were low in comparison to other reports, which reported values from 19.0 to 43.3 mg·L^{-1}·d^{-1} [45,55]. Furthermore, lipid content as a percentage of the microalgal biomass dry weight showed statistically-significant differences (F = 85.09, df = 13, $p < 0.001$) and ranged from 13.5 ± 1.2 (*Tetradesmus* sp. strain NAN003) to 46.7 ± 1.5 (*Ankistrodesmus* sp. strain NAN001). These percentage values in total lipid content are similar to several microalgae species of these genera isolated and characterized by other researchers worldwide [20,45,46,48–50,57]. Furthermore, it is important to emphasize that lipid content is dependent on the different growth phases. Commonly, in several microalgae taxa, the lipid content increases gradually from logarithmic to stationary growth phases [58–60]. For example, Kaur et al. [20] reported that the total lipid content of *Scenedesmus* sp. DRLMA9 demonstrated values of 20.7% in the late exponential phase and 39.7% in the stationary phase; and *Desmodesmus elegans* DRLMA13 displayed values of 10.3% in the late exponential phase and 16.9% in the stationary phase. Likewise, Chiu et al. [61] found that the total lipid content of *Nannochloropsis oculata* was 30.8% in the logarithmic phase, 39.7% in the early stationary phase and 50.4% in the stationary phase. These changes in lipid content with culture age have been associated with nitrate depletion [61], which causes a channeling of photosynthetic ATP to carbohydrate and lipid biosynthesis [60]. Finally, Pearson correlation analysis of biomass productivity and lipid content was inversely related ($r = -0.37$, $p < 0.01$), which is similar to the results obtained by Liang et al. [62] and Rodolfi et al. [52]. In contrast, Li et al. [51] and Hempel et al. [63] found no relationship between these parameters. The negative correlation obtained is likely due to the high metabolic cost of lipid biosynthesis [62].

3.3. Strains Culture under Nitrogen-Sufficient and Nitrogen-Deficient Medium

The microalgae species *Acutodesmus obliquus* (KP878505.1), *Ankistrodesmus* sp. (KP878500.1) and *Chlorella lewinii* (KP878501.1) were selected and further characterized, because they displayed the highest biomass and lipid productivities (Table 1). These microalgae showed noticeable variations in the growth profile when cultured in Chu medium No. 10 with nitrogen (+N) or without a nitrogen source (−N). In +N medium, all strains showed exponential growth, but *Chlorella lewinii* showed the highest average specific growth rates (μ = 0.458 ± 0.098 d^{-1}), followed by *Acutodesmus obliquus* (μ = 0.399 ± 0.112 d^{-1}), and the lowest value was produced by *Ankistrodesmus* sp. (μ = 0.265 ± 0.013 d^{-1}). In contrast, all cultures displayed a lag phase pattern in their growth profile under −N medium (Figure 2). These findings corroborate the results obtained by other researchers, who demonstrate that the specific growth rate is directly proportional to the initial nitrate concentration [46,59,64,65], because the reduction or cessation of microalgae cell division is due to nitrogen limitation [66], which is a key nutrient for protein and nucleic acids de novo biosynthesis; consequently, causing an arrest in the early part of the microalgae cell cycle [67,68]. In accordance with these reports, Li et al. [69] showed that nitrogen deficiency induced, in the green microalgae *Micractinium pusillum*, a downregulation in the expression of genes involved in cell growth and cell cycle regulation. Additionally, differences in growth profile and specific growth rates are dependent on several factors, such as microalgae genotypes, the culture medium composition, pH,

nutrients concentration, the cell culture phase (i.e., log phase, stationary phase) and environmental factors [45,49,54,57,70–72].

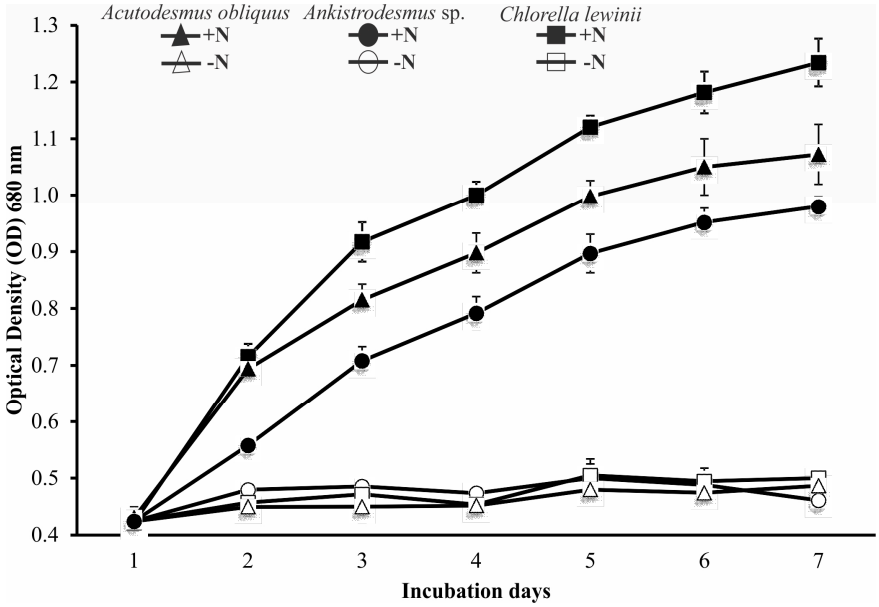

Figure 2. Growth profiles of three microalgal strains cultivated under +N (filled boxes) and −N (empty boxes) Chu medium No. 10. The culture was incubated in a controlled culture room at 25 ± 1 °C with 12:12-h light-dark cycles using 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity of cool-white fluorescent light and continuous agitation at 150 rpm. Experiments were carried out in triplicate.

3.4. Total Protein, Total Lipid and Total Carbohydrate Content

Biochemical parameters of the microalgae species, such as total protein, total lipid and total carbohydrate content, exhibited differences and changes under both culture conditions (Figure 3). For example, *Chlorella lewinii* showed the highest protein content; *Ankistrodesmus* sp. had the highest total lipid content; but *Acutodesmus obliquus* presented the highest levels of total carbohydrate. Therefore, these strains can be valuable stocks for the industry, because total protein and total carbohydrate are potentially byproducts of microalgal biomass processing [73,74]. Additionally, the three strains cultured under −N conditions showed a significant increase in total lipid content (from 23.7% in *Acutodesmus obliquus* to 66.7% in *Ankistrodesmus* sp.) and total carbohydrate content (from 11.9% in *Acutodesmus obliquus* to 96.3% in *Chlorella lewinii*), but showed a decreased in total protein levels (from 24.2% in *Acutodesmus obliquus* to 54.5% in *Chlorella lewinii*) in comparison to +N cultures (Figure 3). These results are in accordance with well-documented results obtained by other researchers with several microalgae species of the genera *Acutodesmus*, *Ankistrodesmus*, *Botryococcus*, Chlamydomonas, *Chlorella*, *Desmodesmus*, *Dunaliella*, *Haematococcus*, *Neochloris*, *Phaeodactylum*, *Pseudokirchneriella* and *Scenedesmus* [46,50,58,59,64,75–80]. Nevertheless, the accumulation of total lipid induced by nitrogen deficiency is not correlated with lipid productivity, but rather is due to a decline and stopping of the microalgae specific growth rate [75]. The changes in biomolecule contents suggest redirection of microalgae metabolism during nitrogen starvation, such as a decrease in biosynthesis/increase in protein degradation and an increase in the biosynthesis of storage biomolecules, such as triacylglycerols and starch. The increase in total lipid content could be partially explained for a boost in transcript levels of genes encoding enzymes of the lipid biosynthesis pathways. According to this assumption, recent investigations showed that genes encoding the enzymes that catalyze the last step in the Kennedy pathway of triacylglycerol biosynthesis [81], such as glycerol-3-phosphate acyltransferase and acyl-glycerol-3-phosphate acyltransferase, are upregulated

in response to nitrogen starvation [64,82,83]. Furthermore, the increase in total carbohydrate content can be explained by changes in the expression levels of genes involved in carbohydrate metabolism. For example, when the oleaginous microalgae *Neochloris oleoabundans* is cultured under nitrogen starvation, the catabolic phase of starch, specifically α-amylase, which hydrolyzes starch to glucose, is strongly repressed [83].

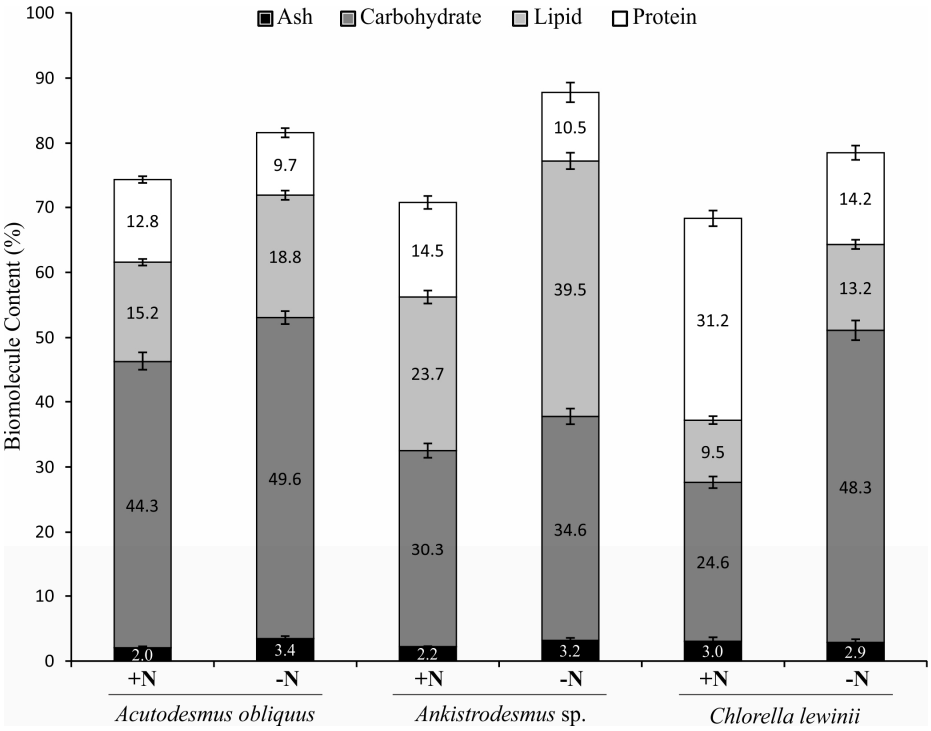

Figure 3. Biomolecule content (ash, carbohydrate, total lipid and protein) of three microalgal strains cultivated under +N and −N Chu medium No. 10. The culture was incubated in a controlled culture room at 25 ± 1 °C with 12:12-h light-dark cycles using 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity of cool-white fluorescent light and continuous agitation at 150 rpm. Experiments were carried out in triplicate.

3.5. Fatty Acids Profiling

The three microalgae strains in the stationary growth phase were further characterized with respect to fatty acids profiles under +N and −N culture conditions. Results in Table 2 show that each microalgae strain has a peculiar fatty acids profile. For instance, in *Ankistrodesmus* sp., the majority of fatty acids were oleic (18:1n-9), palmitic (16:0) and α-linolenic (18:3n-3) acids, which comprised 70.53% and 74.81% of the total FAME in +N and −N conditions, respectively. In *Chlorella lewinii*, the majority of fatty acids were linoleic (18:2n-6), palmitic (16:0), α-linolenic (18:3n-3) and oleic (18:1n-9) acids, which constituted 71.84% and 77.77% of the total FAME in +N and −N conditions, respectively. Finally, in *Acutodesmus obliquus*, the bulk of fatty acids were oleic (18:1n-9), palmitic (16:0), linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, which constituted 87.99% and 89.80% of the total FAME in +N and −N conditions, respectively. Our results agree with multiple previously-published reports in that fatty acids with a length of sixteen (C16) and eighteen (C18) carbon atoms are very frequent in many green microalgae species [45,46,48–50,55]. It is notable that under nitrogen starvation culture, all microalgae strains showed an increase in monounsaturated fatty acids (MUFA) from 7.3% in *Ankistrodesmus* sp. to 55.2% in *Chlorella lewinii*. This increase was attributable commonly to oleic acid (18:1n-9). Furthermore, an increase in saturated fatty acids (SFA) + MUFA was evident. These findings support the results obtained by Ho et al. [50], who revealed in *Desmodesmus* spp. a rise in its content of SFA + MUFA (reaching about 75%) during nitrogen starvation. Similar patterns were observed by other

researchers with *Ankistrodesmus* sp., *Chlorella sorokiniana* and *Scenedesmus obliquus* [46]. Nevertheless, there are some exceptions to that general rule. As an illustration, *Chlorella* sp. strain SP2-1 showed a decrease in SFA + MUFA [46]. The main SFA was palmitic acid (16:0), which experimentally increased between 5.2% and 21.0% in *Acutodesmus obliquus* and *Ankistrodesmus* sp., respectively. However, in the same culture condition, the three microalgae species showed a decrease in polyunsaturated fatty acids (PUFA). This trend was also documented in others identical studies with *Desmodesmus* spp. and *Scenedesmus* spp. [46,50]. Consequently, under nitrogen starvation culture, the microalgae strains shifted their fatty acids profiles with an improvement in the composition of SFA + MUFA, which improves the quality properties of biodiesel, such as an increase in cetane number [84–86]. Considering the SFA and unsaturated fatty acids (UFA) components, approximately 20%–31% are SFA and 69%–80% are UFA. Hence, the SFA/UFA ratio in the microalgae strains evaluated ranges between 0.25 and 0.45, which is similar to the reports of several microalgae species isolated in different latitudes worldwide [20,31,48,49,70,72].

Table 2. Effect of nitrogen deficiency on fatty acid profiling in three native microalgal species from the Peruvian Amazon.

Fatty Acids	*Acutodesmus obliquus*		*Ankistrodesmus* sp.		*Chlorella lewinii*	
	+N	−N	+N	−N	+N	−N
14:0	-	-	0.52 ± 0.02	0.88 ± 0.02	-	0.58 ± 0.01
16:0	27.24 ± 0.07	28.67 ± 0.17	18.68 ± 0.19	22.61 ± 0.06	19.64 ± 0.21	21.23 ± 0.10
18:0	1.84 ± 0.05	2.55 ± 0.51	1.01 ± 0.02	1.81 ± 0.02	3.71 ± 0.26	1.53 ± 0.15
16:1n-7	0.23 ± 0.00	0.25 ± 0.01	0.71 ± 0.03	0.55 ± 0.01	0.85 ± 0.43	0.38 ± 0.00
18:1n-7	1.31 ± 0.02	1.31 ± 0.00	1.41 ± 0.05	0.94 ± 0.03	1.46 ± 0.06	1.23 ± 0.02
18:1n-9	32.13 ± 0.21	36.08 ± 0.21	36.99 ± 0.04	40.47 ± 0.38	13.71 ± 0.15	23.26 ± 0.41
18:2n-6	17.32 ± 0.04	16.51 ± 0.16	7.47 ± 0.18	6.56 ± 0.07	20.34 ± 0.39	19.57 ± 0.01
18:3n-3	11.30 ± 0.17	8.54 ± 0.31	14.86 ± 0.04	11.73 ± 0.12	18.15 ± 0.22	13.71 ± 0.16
18:3n-6	1.01 ± 0.01	0.78 ± 0.02	0.55 ± 0.00	0.54 ± 0.02	1.24 ± 0.06	1.00 ± 0.20
18:4n-3	2.50 ± 0.05	1.77 ± 0.08	6.31 ± 0.02	6.01 ± 0.05	4.91 ± 0.25	4.03 ± 0.20
20:4n-6	-	-	-	0.84 ± 0.08	-	-
22:5n-3	-	-	-	0.62 ± 0.02	-	-
Unidentified	5.12 ± 0.03	3.55 ± 0.06	11.49 ± 0.04	6.45 ± 0.03	15.99 ± 0.34	13.48 ± 0.06
SFA	29.07	31.22	20.22	25.31	23.35	23.34
UFA	70.93	68.78	79.78	74.69	76.65	76.66
MUFA	33.67	37.64	39.11	41.95	16.02	24.87
PUFA	32.13	27.59	29.18	26.29	44.64	38.31
SFA + MUFA	62.74	68.86	59.33	67.26	39.36	48.21

It is important to highlight that the fatty acid profile determination is an essential step in the process of the characterization of microalgae strains, because it ultimately affects the quality of the biodiesel product [84,85]. The quality properties of biodiesel, such as high cetane number, oxidative stability and cold-flow, are dependent on the hydrocarbonated chain length of saturated and unsaturated fatty acids [85,87]. For example, biodiesel composed of a mixture of a high percentage of SFA and MUFA is preferred for increased energy yield, superior oxidative stability and higher cetane numbers, but is prone to solidify at low temperature. In contrast, biodiesel with high levels of PUFA has decreased energy yield, low cetane numbers and excellent cold-flow properties, but insufficient oxidative stability [54,85,88]. Therefore, to solve these performance problems, five approaches have been developed, such as changing the fatty acids profile by physical means, genetic modification of the feedstock or the use of alternative feedstocks with different fatty acids profiles [89].

4. Conclusions

Fourteen microalgae were isolated from three river basins from the Peruvian Amazon and were identified by morphological features and molecular phylogeny approaches. These strains were initially

characterized in the stationary growth phase and exhibited significant heterogeneity in key parameters, such as biomass productivity (3.6–31.6 $mg \cdot L^{-1} \cdot d^{-1}$), lipid productivity (1.0–6.4 $mg \cdot L^{-1} \cdot d^{-1}$) and total lipid content (13.5%–46.7%). Furthermore, three selected strains, based on elevated biomass and lipid productivities, were cultured under nitrogen-sufficient and nitrogen-deficient medium to boost lipid accumulation. Under these culture conditions, all strains showed noticeable decreases in total protein content and increases in total lipid and total carbohydrate contents. In addition, these strains presented modifications in the fatty acids profiles with improvement in biodiesel properties, due to a predominance of SFA and MUFA, which corresponds to an increase in cetane number. The results of this research suggest that the isolated native microalgae, from the Peruvian Amazon, could be suitable renewable feedstocks for the production of both biodiesel and bioethanol, which could contribute to sustainable development at the local, regional and national levels as a viable alternative to petroleum exploitation. However, it will be necessary to develop open, closed or hybrid systems for large-scale biomass production of selected native microalgae strains and/or de novo microalgae strains designed to produce biofuels and with the abilities to synthesize useful bioactive compounds to be efficiently exploited using biorefinery approaches.

Supplementary Materials:
The following are available online at www.mdpi.com/1996-1073/10/2/224/s1, Figure S1: Results of electrophoretic and spectrophotometric analysis of the genomic DNA isolated from the fourteen microalgae, Figure S2: ITS2-rDNA structure (**A**) and amplicons obtained (**B**) from the microalgae isolated, Figure S3: Microphotographic images of the three microalgae strains analyzed under light and fluorescence stained with Nile Red, Figure S4: Spectrophotometric scanning for the determination of the maximum absorption peak for each microalgae strain.

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

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