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Isolation and Characterization of Microalgae from Diverse Pakistani Habitats: Exploring Third-Generation Biofuel Potential

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Abstract: Production of microalgae as feedstock for biofuels must deal with a number of challenges including constraints imposed by local conditions. One solution is to use indigenous strains adapted to local climatic conditions. The present report describes the isolation, identification, and characterization of 32 microalgal strains from different ecological habitats: desert freshwater channels, northern region, and saline regions of Pakistan. The effects of temperature on algal growth rates, biomass productivity, and lipid content were determined through growth at 12, 20, and 35 °C for 15 days under 2% CO_2 Responses to temperature varied among species with 20 °C being the optimum temperature in general, although, exceptionally, the best overall growth rate was found for strain S29 (0.311 d^{-1}) at 12 °C. In some cases high biomass productivity was observed at 35 °C, and, depending upon the strain, the maximum lipid content was obtained at different temperatures, including 12 °C. Fatty acid methyl ester (FAME) analysis showed that the major fatty acids present were palmitic, stearic, oleic, linoleic, and linolenic. Oleic acid (C18:1) was the predominant fatty acid, with the specific FAME profile varying with strain. Thus, there is a rich diversity of microalgal strains native to Pakistan, some of which, characterized here, could be suitable for biodiesel production or other biotechnological applications.

Keywords: microalgae; indigenous Pakistani flora; temperature; growth rate; biomass productivity; lipid content; biofuel; biodiesel; FAME composition

1. Introduction

Microalgae are being intensively investigated due to their rapid growth and variety of potential applications, in particular as promising feedstocks for biofuel production [1–4], biogas production [5,6], wastewater purification [7,8], and animal feed [9], as well as for human food and nutraceutical products [10].

The necessity for developing sustainable sources of energy has become obvious as non-renewable conventional energy extraction and use (petroleum, oil, natural gas, and coal) have already caused worldwide climatic changes [1,11]. One possibility is to derive a third-generation biofuel from microalgae [1,12], which appear to have great potential for biofuel production with a reasonable footprint if a number of significant challenges can be overcome [2,5].

Microalgal characteristics that are favorable for this type of process include: short generation times, high lipid content, cost effective nutrient sources, the capacity to develop in assorted natural surroundings with different types of wastewater, and higher photosynthetic capability compared to terrestrial energy crop plants [7,13,14].

Microalgal growth and productivity are strongly influenced by biotic and abiotic environmental factors. Algal biomass productivity is the net consequence of photosynthesis, which is strongly influenced by both temperature and light [15,16]. Microalgae have the potential to accumulate as much as 30%–70% of their cellular dry weight as lipid under various conditions [7]. One of the key factors that can potentially influence cellular lipid accumulation is temperature [16,17]. However, the adaptability of microalgae to different temperature regimes is species dependent and the prospective biotechnological applications of thermophilic (>50 °C) or even mesophilic (30–50 °C) microalgae are only poorly developed.

Microalgae are a particularly diverse group of organisms found in almost all ecosystems. There are a number of reasons that suggest that bioprospecting for indigenous microalgae would be of great benefit in developing local microalgal production for biotechnological ends, including biofuel production. In addition, the use of locally-sourced strains would avoid restrictions that might be applied to imported strains, and would provide strains that are adapted to local conditions [18–22]. This approach seems especially apt for developing countries that have significant interests in renewable energy production for energy security as well as developing local resources, whereas algal biotechnology application in OECD (Organisation for Economic Co-operation and Development) countries is largely in the hands of private enterprise. For example, one recent work examined some of the characteristics of local green algae isolated in Kerala, India with these considerations in mind [23].

This is the case for Pakistan, which imported 25% of its energy in 2015 [24], and which has a call for development of sustainable energy generation from indigenous renewable sources in its overall development plan [25]. Pakistan possesses distinctive geographical, geological, and environmental properties which promote great biodiversity. In order to more fully address issues related to energy shortages, climate change, and sustainable development in Pakistan, the adoption of clean and renewable energy is essential [26]. Extensive strain surveys have been conducted globally, but inadequate research in this field has been conducted in many energy deficient nations such as Pakistan. Pakistan's diverse habitats with unique geologic and climatic conditions suggest the potential for varied algae diversity in Pakistan, particularly Chlorophyceae [27–29]. Wastewater has already been proposed as substrate for the development of microalgae and their utilization for the biodiesel generation [8] and an initial attempt at algal-based biodiesel production using *Cladophora* sp biomass has already been made [29]. However, in general, microalgae have been little examined in Pakistan, especially in regards to their biofuel or biotechnological potential [26,29,30], and thus additional exploration is warranted.

Here we report on an initial study on native microalgal strains isolated from various ecological zones and their partial characterization. Microalgae were isolated from the Cholistan desert, a cold area (Northern Khyber Pakhtunkhwa Province), and saline ranges, and were used to investigate biomass productivity, as an indicator of suitable characteristics for biotechnological use, and neutral lipid accumulation, to specifically examine their potential for biodiesel production. This is one of the first descriptions of the isolation, purification, and characterization in this manner of microalgae from local habitats in Pakistan. This allowed a comparative study of freshwater green algae of diverse origins (desert, cold, and salt range) and the effects of temperature on their biomass production, growth rate, and neutral lipid content.

While isolation and characterization, in some form, of microalgae from diverse environments is not new, this is the first time that such a collection has been systematically exposed to three different temperatures and important parameters, such as growth rate, biomass, and lipid yield, determined. In addition to providing useful information on individual strains, our data set also provides the opportunity to make several generalizations that might be applied to other collections/groups of algae

and tested. Thus, there is additional utility in carrying out such a study which also increases general knowledge about microalgae diversity and physiology.

2. Materials and Methods

2.1. Sampling and Isolation

Water samples (50–100 mL) were collected from different areas of the Cholistan desert (hot area), northern parts of Khyber Pakhtunkhwa Province viz. Upper-dir, Jaz-Banda (cold area), Katora Lake, Swat valley, Mansehra (cold regions), Islamabad, and Khewra region (salt ponds) (Figure 1). Samples were collected from lakes, running streams, canals, and saline water ponds. Coarse materials and zooplankton were^{-1e} directly removed by vacuum filtration through a 50 µm mesh net, and samples were labelled and stored in sterile falcon tubes (50 mL) for transportation to the laboratory (Plant Genetics and Genomics; Quaid-e-Azam University, Pakistan). Algal water samples were vacuum filtered through a series of membranes. Initially small planktonic nets (50 µm and 20 µm) were used, and then samples were filtered through Whatman 10 µm PC (polycarbonate) filters with a final passage of the filtrate through a Whatmane 2 µm PC filter. Samples were then serially diluted and directly plated on Bold´s basal medium (BBM) agar plates [31] for colony purification and incubated at 24 ± 2 °C without supplemental CO_2 and 25–30 W·m^{-2} (126 µEm^{-2}s^{-1}) light intensity. After seven days of growth, algal colonies were inoculated into 250 mL autoclaved Erlenmeyer flasks containing 150 mL of BBM medium and incubated in a light-mounted shaker at 22 ± 2 °C, at 120 RPM (stroke length 20 mm) employing a light:dark photo-cycle of 16:8 h (126 µEm^{-2}s^{-1} (25–30 W·m^{-2}) light intensity).

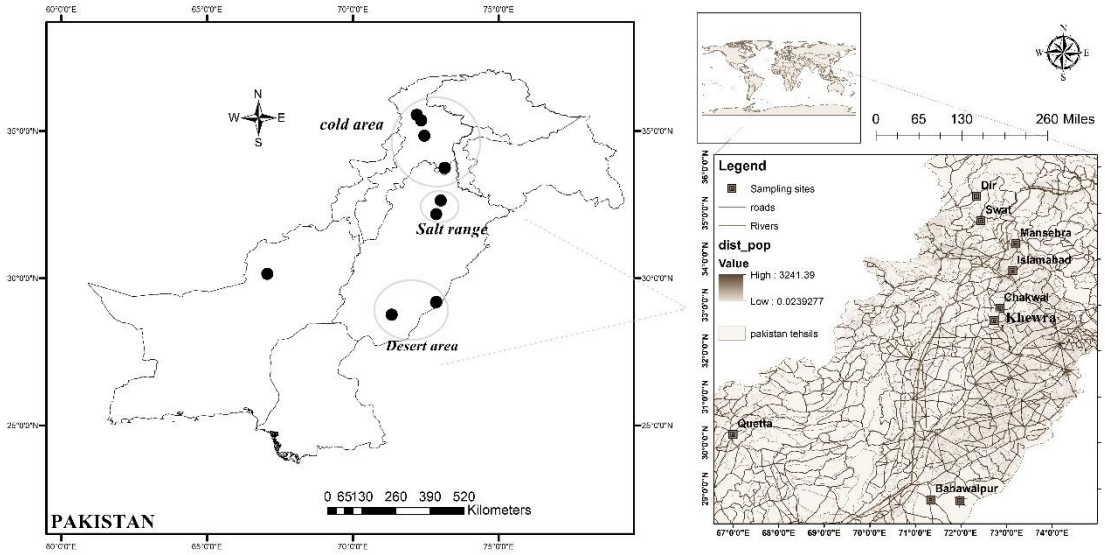

Figure 1. Map of Pakistan showing locations of sampling sites. ARC-GIS version 10.1 software (ESRI, Headlands, California, USA) was used to depict different sampling sites on a map.

2.2. Strain Identification

Algal cultures were examined morphologically under light microscope for preliminary identification and to confirm unialgal purity. A drop of algal-cell-containing medium was obtained using a 20 µL micropipette, and placed on a clean microscopic slide, and covered with a cover-slip, and observed under a microscope. Observations were made as to morphology, appearance of chloroplasts, if the cells are solitary or in small groups, the shape and size of cells, presence/absence of spines, and sheath and spine arrangement. These observations were then used with the aid of standard taxonomic manuals (detailed in [27]). The Plant Genetics and Genomics Laboratory, Department of Plant Sciences, Quaid-i-Azam University, Islamabad in conjunction with the Pakistan Museum of Natural History,

Botanical Sciences Division, Islamabad is setting up a phycological hebarium for experimental analysis, identification, and strain preservation.

2.3. Cultivation of Algal Strains

The 32 isolated strains of microalgae were freshly subcultured in BBM medium prior to experiments. The growth, lipid, and biomass production of the isolated strains were investigated to evaluate the impact of temperature (12, 20, and 35 °C) using triplicated cultures for each temperature. This range was designed with the aim of permitting strains to potentially experience temperatures at the low and high extremes of their thermal niches. A total of 3.5 ± 0.1 mL of each sterile (using 0.45 µm Millipore™ filter, Millipore Sigma, MA, USA) culture medium and individual strains were placed in 12-well transparent microplates (Falcon tissue culture plates, Corning, New York, NY, USA) covered with transparent lids to decrease evaporation. Microplates were placed separately in an photo-incubator at three different temperatures; 12 ± 2 °C, 20 ± 2 °C, and 35 ± 2 °C using warm white fluorescent lights at an intensity of 35 $W \cdot m^{-2}$ (approximately 166 $\mu E \cdot m^{-2} \cdot s^{-1}$) (measured with a Delta OHM HD2102.1 photo-radiometer equipped with a LP-471-RAD probe using a 14:10 h light/dark cycle (Delta Ohm, Padova-Italy). The CO_2 concentration in the photo-incubator was set at ~2% using a CO_2 controller (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All experiments were performed in triplicate and the mean and standard deviation calculated.

2.4. Analytical Methods

Algae strains growth was measured throughout cultivation through measuring the absorbance (OD) at 630 nm using a EL800 plate reader (Biotek, Winooski, VT, USA). The 12-well microplates were agitated for 25 min on a mini-orbital shaker prior to optical density readings. After 15 days of incubation the cellular neutral lipid content of cultures was quantitated by measurement of the fluorescence intensity of Nile red (NR)-stained cultures [16,32,33]. Nile red, a fluorescent dye capable of staining neutral lipids [3,34,35], was used to quantify intracellular lipids using a slight modification of previously used approaches [3,14,36]. Before the NR assay, algal samples were diluted with medium to achieve an OD630 of 0.06 for each sample and 143 µL of the solution was transferred into black flat-bottom 96-well plates [3,14]. To this, 50 µL of dimethyl sulfoxide (DMSO) was added followed by the addition of 6 µL of Nile red solution (15 $\mu g \cdot mL^{-1}$ Nile red in acetone). The plates were incubated for 10 min at room temperature on a microtiter plate shaker (DSG Titertek/4 Flow Laboratories, Meckenheim, Germany) in dim light. Fluorescence measurements were performed using a spectrofluorometer (SpectraMax Gemini readers; Molecular Devices, Sunnyvale, CA, USA) with excitation at 525 nm and emission at 580 nm. A standard curve was generated with triolein. Similarly, biomass and lipid content on a dry weight basis for examined strains were calculated using the relationship $OD_{630} \cdot g^{-1}$) = 1.055 ± 0.12, as previously determined for quantifying growth of 100 different strains of similar microalgae (Chlorophyta) in experiments carried out previously on the same instrument [14]. All measurements were carried out in triplicate.

2.5. Growth Rate, Lipid Productivity, and Lipid Percentage

Growth rates were calculated according to the standard formula [3,14,36]:

$$\mu = (\text{In } (Xt_1) - \text{In } (Xt_2))/t_1 - t_2$$

where µ is the specific growth rate in d^{-1}, Xt_1 and Xt_2 are the optical densities (OD) at time t_1 and t_2. For calculation of the maximal growth rates, Xt_1 and Xt_2 were taken as the points at which cultures entered into and completed exponential growth.

Biomass production (BP) was calculated as follows using the net dry weights obtained at the end of the incubation (15 d):

The cellular content of lipid (CL1) was calculated using:

$$\text{CL1 } (gg^{-1}) = WL/WB$$
$$\text{Percentage Lipid/dry weight biomass } (\%) = (WL/WB \times 100)$$

where WL (g) is lipid weight and WB (g) the algal biomass dry weight.

Lipid productivity (LP) was calculated using the following equation:

$$LP\ (g{\cdot}L^{-1}\ d^{-1}) = (C1B1 - C0B0)/t$$

where C0 (gg^{-1}) is microalgae lipid content at the beginning and C1 (gg^{-1}) is that at the completion of experiment, B0 and B1 ($g{\cdot}L^{-1}$) are the biomass concentrations at the start and end, and "t" is the time period of the experiment.

2.6. FAME Analysis

Fatty acid methyl esters (FAMEs) were formed by in situ transesterification and extraction as previously described [35] and analyzed using a Shimadzu GC (gas chromatograph)2014 (Shimadzu Scientific Instruments, Columbia, MD, USA) with a Phenomenex Zeron ZB-WAX GC capillary column (Phenomenex, Columbia, MD, USA). GC conditions were: Hold at 160 °C for 1 min, ramp 10 °C/min, hold at 185 °C for 10 min, ramp 1 °C/min, hold at 200 °C for 5 min, ramp 5 °C/min, hold 230 °C for 15.5 min. Transesterification and extraction were carried out as follows. At least 1.5 mL of sample algal culture was centrifuged (10,000 RPM, 5 min, Beckman Microfuge 14™ (Beckman Coulter, Torrance, CA, USA)) and the supernatant removed by pipetting. Samples were dried overnight at 40 °C under vacuum using 1.5 mL centrifuge tubes in an Eppendorf Vacufuge™ (Hamburg, Germany). The dried samples were placed in 2.0 mL GC vials with solid caps and PTFE (polytetrafluoroethylene) liners (Daigger catalog # EF2858A, Daigger Scientific, Vernon Hills, IL, USA) and their weight determined. Transesterification was carried out using a dry heat block set to 85 °C (±3 °C). 200 µL of chloroform:methanol (2:1 v:v), prepared fresh each extraction, was added to samples followed by the addition of 300 µL of 0.6M HCl:methanol, prepared by adding 5 mL of concentrated HCl to 95 mL of methanol. Samples were incubated at 85 °C heat block for 1 h and then allowed to cool at room temperature for at least 15 min but no longer than 1 h. FAMEs were then extracted by adding 1.0 mL hexane, vortexed, and allowed to sit at room temperature for 1 to 2 h to allow phase separation. Samples were then transferred to 1.5 mL GC glass vials (Shimadzu 1.5 mL snap cap vials, catalog # 220-91498-00).

2.7. Statistics

Detailed statistics summaries were obtained through the IBM SPSS Statistics (version 21) software package (IBM, Armonk, NY, USA). The biomass, specific growth rate and lipid content data were analyzed using a one-way analysis of variance (ANOVA), correlations were used to test variation using multivariate correlations, and Pearson's correlation coefficients were determined with their significance levels (Supplementary Materials). Standard error of means values were calculated at $p < 0.05$ level of significance. All data are presented as the mean ± SD. For source identification, Principal component analysis (PCA) was applied to data sets to sort the total variability of the data into factors, which are orthogonal to each other and represent low possible covariance. ARC-GIS version 10.1 software (ESRI, Redlands, CA, USA) was used to depict different sampling sites on a map.

3. Results and Discussion

3.1. Isolation of Indigenous Strains

The sampling protocol used here was effective in establishing a culture collection of thirty two native microalgae (Table 1). Strains were identified using microscopic examination and were members of either Class Chlorophyceae (genera *Coelastrella*, *Desmodesmus*, *Tetraspora*, *Tetradesmus*, *Scenedesmus*, *Asterarcys*, *Chlorosarcinopsis*, *Neochloris*, *Chlamydomonas*) or Class Trebouxiophyceae (genera *Botrycoccus*, *Auxenochlorella*, *Chlorella*, *Nannochloris*, *Coccomyxa*). Thus a wide diversity of Chlorophyta was obtained.

Table 1. Source and Identification of isolates.

Strains	Identification	Site	Location	pH	Source	Coordinates
1.	*Botryococcus sp*	Drawer Fort	Desert/Cholistan	7.6–8.6	Rain-water Pond	28.76°N, 71.33°E
2.	*Coelastrella sp*	Jazzbanda	Dir (cold area)	7.6	Katoora Lake	35.36°N, 72.34°E
3.	*Scenedesmus sp*	Rumli	Islamabad	6.8	Rumli stream	33.75°N, 73.15°E
4.	*Desmodesmus sp.*	Rumli	Islamabad	6.8	Rumli stream	33.75°N, 73.14°E
5.	*Tetraspora sp*	Rawal-Lake	Islamabad	7.3	Lake	33.70°N, 73.12°E
6.	*Tetradesmus sp.*	Cholistan	Desert Bwp	7.9	Small water pond	29.17°N, 72.43°E
7.	*Auxenochlorella sp.*	Hanna	Quetta	8.2	Hanna lake	30.15°N, 67.06°E
8.	*Desmodesmus sp.*	Kaly-Pahar	Desert	–	Mud/soil	29.176°N, 72.06°E
9.	*Scenedesmus/Desmodesmus*	River Swat	Swat (cold area)	7.4	River	34.84°N, 72.45°E
10.	*Chlorella sp*	Jazz Banda	upper Dir (cold)	7.9	Water stream	35.36°N, 72.34°E
11.	*Scenedesmus sp*	Labarkot	Mansehra (cold)	6.7	Stone near water	34.34°N, 73.23°E
12.	*Chlorococcum sp*	Mine	Khewra-mine	8.2	Natural Pond	32.64°N, 73.01°E
13.	*Desmodesmus sp*	Mine	Khewra-mine	–	Muddy	32.64°N, 73.01°E
14.	*Chlorococcum sp*	Cholistan	Desert/Cholistan	7.5	Standing water	28.76°N, 71.33°E
15.	*Desmodesmus bicellularis*	Near mine	Khewra-mine	8.9	Pond	32.64°N, 73.01°E
16.	*Flechtneria/Coelastrella*	Salt range	Khewra-mine	8.4	Water pond	29.19°N, 72.85°E
17.	*Acutodesmus sp*	Fort Abbas	Desert/Cholistan	6.9	Standing water	29. 125°N, 72.5133°E
18.	*Nannochloris sp*	Fort Abbas	Desert/Cholistan	7.4	Standing water	29.1560°N, 72.420°E
19.	*Coelastrella sp*	River	Swat (cold area)	6.9	Swat river	34.837°N, 72.451°E
20.	*Asterarcys sp*	lake	Khewra-mine	7.2	Lake	32.64°N, 73.01°E
21.	*Chlorosarcinopsis sp*	Rumli	Islamabad	7.3	Jinnah Stream	33.748°N, 73.128°E
22.	*Coccomyxa sp*	Salt range	Khewra-mine	–	Soil	32.81°N, 72.92°E
23.	*Chlorella sp*	Labarkot	Manshra (cold)	8.3	Stagnant water	34.34°N, 73.23°E
24.	*Neochloris/Chlorococcum*	Sheringal Panjkora	upper Dir (cold)	6.2	Running water	35.296°N, 72.001°E
25.	*Acutodesmus*	Sheringal	upper Dir (cold)	5.9	Running water	35.285°N, 72.01°E
26.	*Chlorella sp*	Shahi wala	Desert/Cholistan	7.6	Natural pond	29.034°N, 71.29°E
27.	*Desmodesmus sps*	Kalar Kahar	Khewra-mine	8.4	Lake	32.771°N 72.715°E
28.	*Chlorella sp*	Kumrat valley	upper Dir (cold)	6.7	Running water	35.55°N, 72.19°E
29.	*Scenedesmus sp*	Kaly pahar1	Desert/Cholistan	7.3	Natural pond 1	29.187°N, 72.15°E
30.	*Chlamydomonas/Neochloris*	Kaly pahar2	Desert/Cholistan	7.5	Natural pond 2	29.196°N, 72.106°E
31.	*Chlamydomonas sp*	Katora Lake	upper Dir (cold)	6.8	Small Pond	35.374°N, 72.344°E
32.	*Scendusmus/Acutodesmus*	Fort Abbas	Desert/Cholistan	–	Soil	29.18°N, 72.85°E

The primary objective of the present study was not to establish a definitive taxonomy, which is presently dependent upon morphological characterization. This approach, although commonly used, is of limited use in the present case since indigenous algae, although perhaps classed as the same species as those defined in a culture collection, are likely to differ from these well described strains in a number of ways, as great genetic variability has been described in a number of systems at the subspecies, "cryptic" species, and ecotype levels [37–39]. However, on the biotechnological and, perhaps even the ecological levels, what is more interesting and important is the functional diversity (i.e., physiological and metabolic robustness that is present), something that is surprisingly seldom addressed, even with large culture collections, but also even with many small regional collections (for an exception see [40]. Thus, the collection of 32 strains were identified down to the genus level morphologically and characterized functionally.

Since they were isolated from disparate habitats with different physico-chemical characteristics, in particular temperature, a comparison study of the effects of different temperatures on growth, biomass production, and lipid production was carried out. To ensure adequate carbon dioxide supply at all temperature regimes (CO_2 solubility decreases by almost 50%, going from 12 to 35 °C), growth was carried out in an atmosphere enriched to 2% CO_2 Prominent differences in biomass production parameters and lipid content were exhibited by individual strains during the 15-day growth period.

3.2. Growth Rate

Maximum growth rates were determined by following growth at 12, 20, and 35 °C over 15 days, as described in Materials and Methods using triplicates, The results are presented as bar charts with rates at 12, 20, and 35 °C. The means with standard deviations are shown for each strain. The standard deviation includes both the variation introduced by the variation in the actual measurement introduced by the instrumentation used as well as that due to biological variation.

An examination of Figure 2 shows, perhaps not surprisingly, that the majority of the isolated strains had higher growth rates at 20 °C than at 12 °C. The few exceptions were strains S13, S27, and S29, with growth rates at 12 °C that were 23%, 27%, and 53% higher, respectively, than those at 20 °C. Conversely, some strains showed much higher growth rates at 20 °C as compared to 12 °C, with strains S11, S14, S23, and S30 giving growth rates that were 52%, 48%, 52%, and 93% higher, respectively, at the higher (20 °C) temperature. Finally, the fastest growing strain, S1, gave a significantly higher specific growth rate as compared to other strains and its specific growth rate showed little impact of temperature, as growth rates at 12, 20, and 35 °C were nearly identical (0.29 d^{-1}, 0.31 d^{-1} and 0.30 d^{-1}, respectively).

When the differences in growth at 20 °C were compared with those at 35 °C, a different picture emerges (Figure 2). In general, growth rates showed relatively little effect of increased temperature, with the vast majority showing, at most, a ~50% change, positive or negative, and ~50% of the strains giving a ~25% or less variation. One obvious exception was strain S22, with a lower growth at 35 °C (0.14 d^{-1} (35 °C) compared with0.26 d^{-1} (20 °C)). A 52% reduction in specific growth rate suggests thermal intolerance. The fastest growth was noted for strain S1 at 20 (0.31 d^{-1})and 35 °C (0.30 d^{-1})) and strain S29 at 12 °C (0.31 d^{-1}). In addition to characterizing individual strains as to their growth response to different temperatures, these results suggest that there is a great deal of diversity of responses, even within a relatively small sample of microalgal strains.

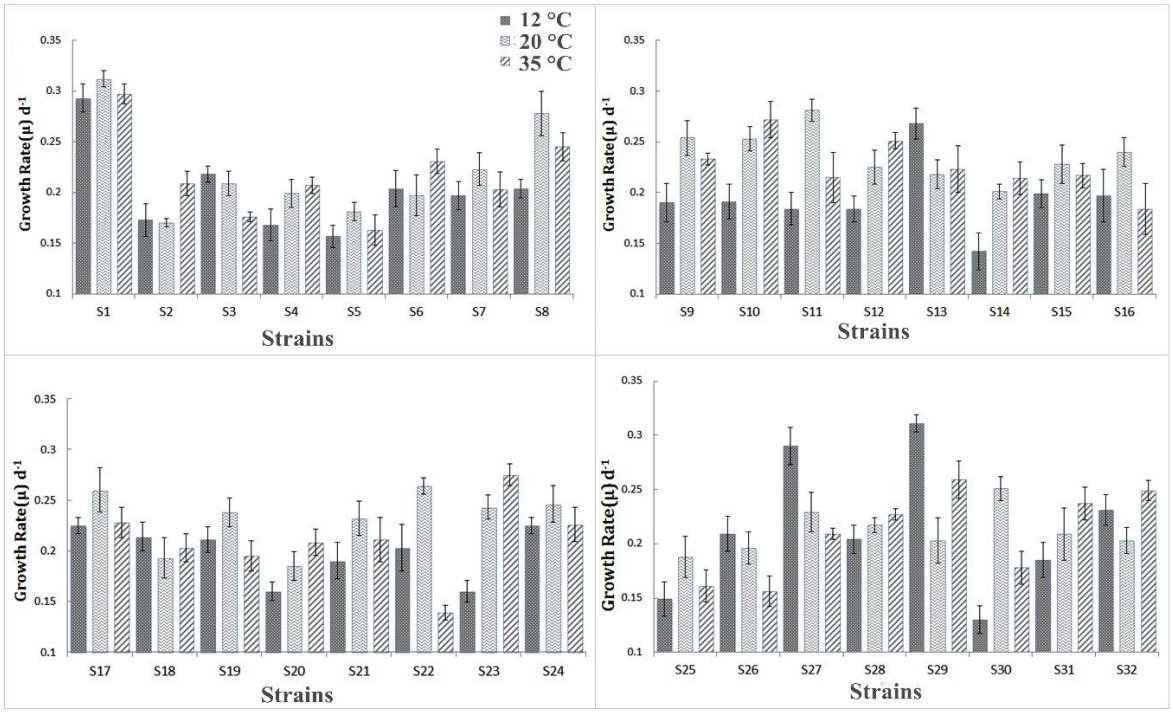

Figure 2. Effects of growth temperature on growth rates of the various strains. The **top left** panel shows the results for strains S1–S8, **top right** panel, results for strains S9–S16, **bottom left** panel, results for strains S17–S24, **bottom right** panel, results for strains S25–S32.

3.3. Biomass Production

Biomass production and lipid content are the two most studied parameters in searches for isolates for large-scale cultivation [41]. Lipids and other key microalgal components can be enhanced by various means including both biotic and abiotic stresses [42]. The importance of temperature for microalgae cultivation for biodiesel production has previously been noted [16,43]. Finding strains that can withstand a range of temperatures is indispensable for large-scale outdoor cultivation of microalgae [44]. Presently, among 32 microalgal strains, about 11 grew well at all three temperatures.

Most practical processes for exploitation of microalgae are more concerned with biomass production or total lipid accumulation and not maximal growth rates in particular, so it was important to compare the biomass production of the strain collection at the three temperatures (Figure 3).

Biomass production was determined at the end of growth (15 days) at 12, 20, and 35 °C, as described in Materials and Methods, using triplicates. The results are presented as bar charts with production at 12, 20, and 35 °C given for each strain The means with standard deviation are shown. The determined standard deviation of a measured biological variable includes both the variation introduced by the variation in the actual measurement introduced by the instrumentation as well as that due to biological variation.

In general, biomass production was only somewhat sensitive to temperature, with 0.69 + 0.017, 0.87 + 0.21, and 0.88 + 0.0.30 $g{\cdot}L^{-1}$ at 12, 20, and 35 °C when averaged over the 32 strains. On the other hand, for some strains, biomass production at 35 °C was appreciably increased over that of cultures grown at 12 °C; for example, from 0.435 to 1.19 $g{\cdot}L^{-1}$ for strain S4, 0.345 to 0.855 $g{\cdot}L^{-1}$ for strain S10, 0.585 to 1.29 $g{\cdot}L^{-1}$ for strain S12, 0.39 to 1.10 $g{\cdot}L^{-1}$ for strain S20, 0.0.77 to 1.37 $g{\cdot}L^{-1}$ for strain S23, and from 0.71 to 1.40 $g{\cdot}L^{-1}$ for strain S32.

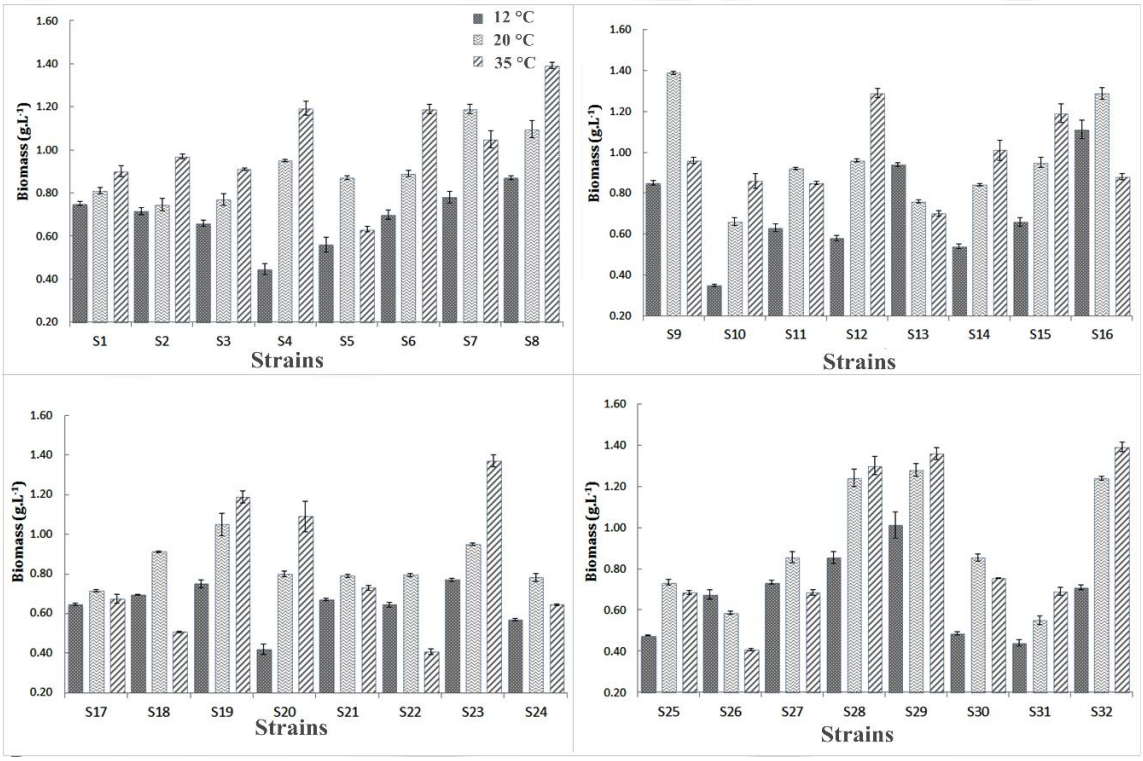

Figure 3. Effects of growth temperature on biomass production. The **top left** panel shows the results for strains S1–S8, **top right** panel, results for strains S9–S16, **bottom left** panel, results for strains S17–S24, **bottom right** panel, results for strains S25–S32.

Interestingly, the bar charts comparing production at 12 °C with that at 20 °C show a much more marked temperature effect than was seen with growth rate, with the vast majority of the strains showing increased production at the higher temperature (20 °C) (Figure 3). Biomass production by strains S4, S10, and S20 showed a marked two-fold increase at 20 °C compared with 12 °C (Figure 3). The highest production at 20 °C was noted with strains S9, (1.35 g·L^{-1}) S16 (1.245 g·L^{-1}), S28 (1.2 g·L^{-1}), and S29 (1.215 g·L^{-1}).

When production at 20 °C was compared with that at 35 °C, in general biomass production in many strains was relatively unaffected (Figure 3). In fact, averaged over the entire collection of 32 strains, no effect was seen (1.02 ± 0.27). This suggests that many of these strains can tolerate temperature fluctuations without drastic effects on biomass production. However, 10 strains showed a 25% or more increase in biomass at the higher temperature (35 °C), while six strains showed a 25% or more decrease at the higher temperature (35 °C). Exceptionally, biomass production by strains S11, S18, S20, and S24 were much lower at the higher temperature; S11, 32%; S18, 33%; S20, 47%; S24, 50% (Figure 3). The highest rates at 35 °C were given by strains S8, S23, S29, and S32 (1.34, 1.35, 1.31, and 1.34 g·L^{-1}, respectively).

In general, Scenedesmaceae strains exhibited good biomass production under the laboratory conditions of the study, with most giving production ranging from 0.072 to 1.01 g·L^{-1}. At 20 °C, the highest production was found for S9 (0.93 g·L^{-1}) followed by S16 (1.2 g·L^{-1}), whereas little biomass production was observed for strains S26 and S31 (0.59 and 0.56 g·L^{-1}, respectively). As compared to the Trebouxiophyceae, members of the Chlorophyceae gave higher biomass production, with a great variation seen at 35 °C (0.71 to 1.37 g·L^{-1}). This attests to the extensive physiological diversity in the strain collection.

3.4. Percent Lipid and Total Lipid Content

For biofuel production, in particular biodiesel production, it is important to ascertain the ability of various strains to accumulate neutral lipids. This is usually a combination of both the percent lipid by dry weight attainable [41,44] and the ability to accumulate biomass.

The percent lipid content of microalgae depends upon a number of genetic and physiological factors. Lipid content is usually higher when growth is restricted, usually at the end of growth, in particular when growth is restricted by nitrogen availability. Although a detailed study of the lipid content of the different strains under different conditions was beyond the scope of this initial study, the lipid content of the 32 strains at the end of 15 days of growth at the three different temperatures was determined. When percent lipid obtained at 12 °C was compared with that at 20 °C, a wide variety of responses could be observed (Figure 4).

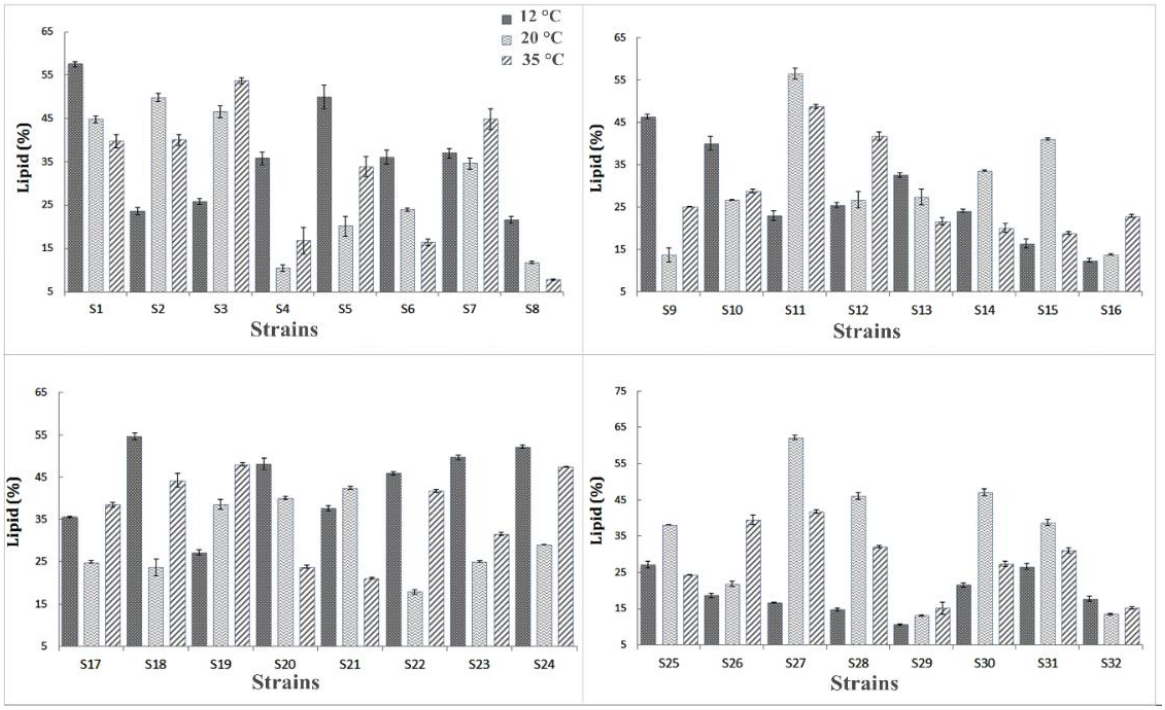

Figure 4. Effects of the growth temperature on the lipid content. The **top left** panel shows the results for strains S1–S8, **top right** panel, results for strains S9–S16, **bottom left** panel, results for strains S17–S24, **bottom right** panel, results for strains S25–S32.

The lipid content of the strains was determined at the end of growth (15 days) at 12, 20, and 35 °C, as described in Materials and Methods, using triplicates. The results are presented as bar charts with values at 12, 20, and 35 °C. The means with standard deviation are shown for each strain. The determined standard deviation includes both the variation introduced by the variation in the actual measurement introduced by the instrumentation as well as that due to biological variation.

The lipid content was markedly higher for some strains at 20 °C, with strains S27, S11, S28, S2, S30, and S15 showing 375%, 245%, 313%, 211%, 220%, and 250% increases, respectively. On the other hand, for some strains the lipid content at 20 °C was lower than that at 12 °C. The lipid content of strains S18, S5, S22, S9, and S4 at 20 °C was only 43%, 40%, 39%, 30%, and 29%, respectively, of that at 12 °C.

Higher temperatures could enhance lipid accumulation [45,46], shown here for a number of the strains examined. However, as previously suggested by Bellou et al. [46], lipid production in the present study was strain dependent. This can be seen in the present study by regarding the lipid content at 12 °C of three *Chlorella* variants, S23 (Manshera), S10 (Jazz Banda), and S28 (Kumrat), which gave 50%, 40%, and 15% lipid by dry weight, respectively. The highest lipid content, 62%, was observed for

strain S27 (isolated from a salt ridge) at 20 °C. In general, cold region strains (S2, S7, S9-11, S23-25, S28, and S31appeared to be relatively good candidates for lipid production, as previously discussed [14], giving on average, 0.21 ± 0.11 (12 °C), 0.32 ± 0.15 (20 °C), and 0.33 ± 0.09 (35 °C) $g{\cdot}L^{-1}$.

As yet there are no standard criteria for the selection of algae for biofuels production. Nevertheless, it would seem that a suitable algal candidate should be a good to excellent neutral lipid producer that satisfies additional requirements, such as the ability to tolerate harsh environmental conditions (temperature), good growth rates, maximal cell density at the end of stationary phase of growth, and possessing potential for high lipid content [46,47].

When lipid content at 35 °C was compared to that at 20 °C, on average for the 32 strains there was little change (1.13 ± 0.50; Figure 4). However, as indicated by the large standard deviation, there was a great deal of variability in responses that was strain dependent, with twelve strains showing a 25% or more increase and 10 strains giving a 25% or more decrease. Remarkably, strain S22 gave 230% more lipids at 35 °C. The lipid content of some other strains (strains S16, S17, S18, and S26) showed appreciable increases at the higher temperature (166%, 155%, 187%, and 181%, respectively). The lipid content of six strains examined decreased by 40% or more at the higher temperature (S13, 37%; S15, 54%; S21, 55%; S25, 42%: S27, 48%: S30, 50%). This suggests that, in general, in a given collection of microalgae a variety of responses can be found and in many cases, as previously suggested, temperature can play a significant role in modulatin g·L ipid content. Of course, for the purposes of biodiesel production, it is not only important to use strains capable of achieving relatively high lipid content, but the strain should also be capable of substantial growth (biomass production), thus maximizing total volumetric lipid production. The amount of neutral lipids that was accumulated after 15 days of growth were assessed for the strains in the collection at 12, 20, and 35 °C (Figure 5).

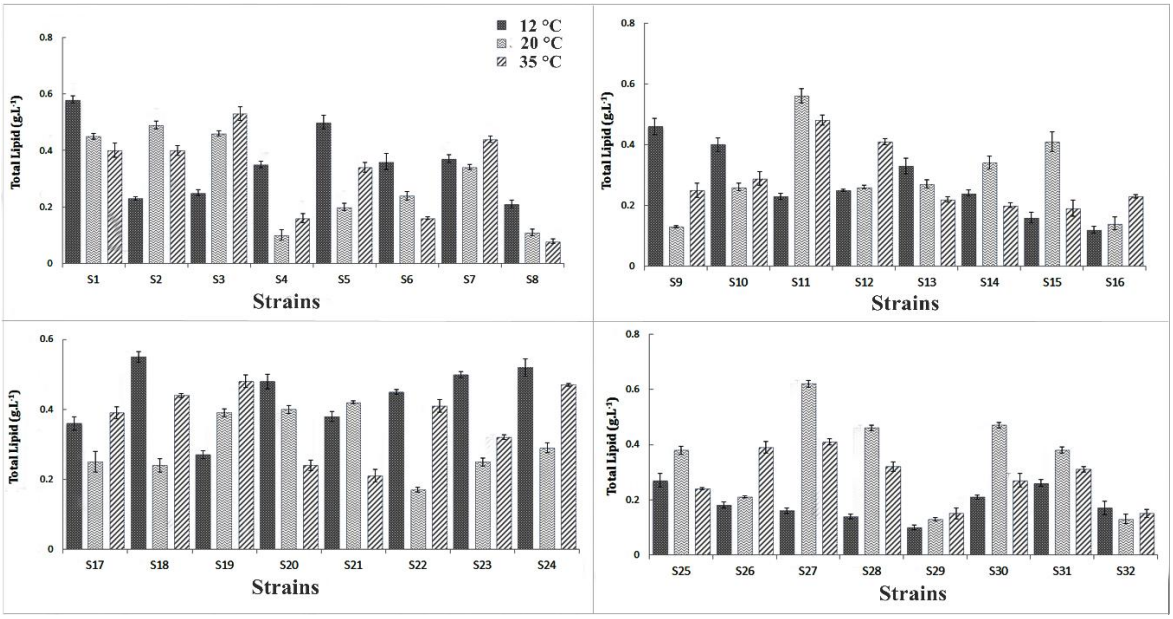

Figure 5. Effects of growth temperature on total lipid production. The **top left** panel shows the results for strains S1–S8, **top right** panel, results for strains S9–S16, **bottom left** panel, results for strains S17–S24, **bottom right** panel, results for strains S25–S32.

Total lipid production of the strains was determined at the end of growth (15 days) at 12, 20, and 35 °C by multiplying the lipid content (%) by the biomass produced ($g{\cdot}L^{-1}$). The results are presented as bar charts with values at 12, 20, and 35 °C given for each strain using the means and standard deviations obtained with triplicate samples. The standard deviation includes both the variation introduced by the variation in the actual measurement introduced by the instrumentation as well as that due to biological variation.

In general, total lipid production was sensitive to temperature. Although there were a few strains whose volumetric lipid production did not change appreciably in response to different temperatures (e.g., strain S16 which had 0.13, 0.17, and 0.19 g·L^{-1} at 12, 20, and 35 °C, respectively), most showed significant changes with: Strain S2—0.16, 0.36, and 0.37 g·L^{-1}; strain S3—0.16, 0.34, and 0.46 g·L^{-1}: strain S12—0.13, 0.24, and 0.51 g·L^{-1}; and strain S28—0.12, 0.55, and 0.40 g·L^{-1} (at 12, 20, and 35 °C, respectively). When compared to 12 °C, total lipid production of 22 strains was increased by at least 25% at 20 °C, whereas lipid production by six strains was decreased by 25% or more at the higher temperature. Strains S11, S15, S27, S28, and S30 showed a very marked increases in total lipid production at 20 °C with 364%, 366%, 431%, 470%, and 406% increase, respectively, over production at 12 °C (Figure 5). Such large increases are due to the fact that, for these strains, both percent lipid content and total biomass production increased at the higher temperature (20 °C). Lipid production of strains S2, S3, S19, S25, and S31 increased ~200% at 20 °C when compared to that at 12 °C. On the other hand, a few strains (S9, S18, S22, and S23) showed significantly less total lipid production at the higher temperature, with lipid production decreasing by ~50% at 20 °C (50%, 43%, 51%, and 40%, respectively.

When total lipid production at 20 and 35 °C was compared, it can be seen that, in general, lipid production was relatively indifferent to the two temperatures. (Figure 5). Total lipid production was increased by 25% or more in six strains and decreased by 25% or more in six strains when production at 35 °C was compared to that at 20 °C. Three strains showed significantly higher total lipid at 35 °C, with total lipid ~200% of that at 20 °C; S4, S12, S23, and four strains, S15, S21, S27, and S30, displayed significant inhibition of lipid production at 35 °C, with their total lipid content only ~50% of that at 20 °C (43%, 55%, 48%, and 50%, respectively (Figure 5). Thus a wide range of responses can be demonstrated with a relatively small group of microalgae.

3.5. Comparison of the Fatty Acid Composition of the Neutral Lipids Produced by the Different Strains

One of the primary objectives of the present study was to develop a collection of indigenous microalgal strains for potential application in biofuels, in particular biodiesel, production. Biodiesel is produced by transesterification of the fatty acids (FAs) contained in the cellular neutral lipid fraction, producing fatty acid methyl esters (FAMEs). Microalgae are capable of synthesizing a variety of saturated, mono-unsaturated, and poly-unsaturated fatty acids. Since important biodiesel properties, such as cetane number, viscosity, and cold filter plugging point, depend upon the fatty acid composition in the FAMEs, it is important to target strains that produce the appropriate fatty acids, or mixture of fatty acids [48–50]. Moreover, it appears that it is necessary, and worthwhile, to determine the composition of individual strains, regardless of their taxonomic relatedness. A study of more than 2000 microalgal isolates in the SAG collection (Sammlung von Algenkulturen der Universität Göttingen (Culture Collection of Algae at Göttingen University) concluded that, although trends could be observed at the phyla and class level, composition is unpredictable at the level of genera and species [48]. In addition, characterization of the fatty acid content of the strain collection established here could open the door to other biotechnological applications in the future.

The composition of the fatty acids of the neutral lipid fraction of the 32 strains was determined by GC-FID (gas chromatograph with flame ionizatikon detector) (Figure 6).

The relative FA composition of the various strains was determined through extraction and trans-esterification followed by GC-FID analysis, as described in Materials and Methods.

The major fatty acids present were; C16:0 palmitic, C18:0 steric, C18:1 (9) oleic, C18:2 (9,12) linoleic, 18:3 (9,12,15) linolenic. Depending upon the strain, these accounted for 85%–100% of the FAMEs recovered. It is evident from a comparison of the FAME composition of the strains that indeed there is great variation in the exact composition, with no two strains showing very similar profiles. Oleic acid (C18:1) was the predominant fatty acid in all the strains, but one, S32 (Figure 6). Nevertheless, there were considerable differences in C18:1 among the strains in terms of percentage of the total. In nine of the strains, this fraction accounted for more than 75% (S1, S6, S7, S9, S13, S17, S18, S23, S25) and in six

less than 50% (S4 S14, S24, S26, S29, S31). In all of these strains, palmitic acid was the second most abundant fatty acid, ranging from 12% to 27% of the total.

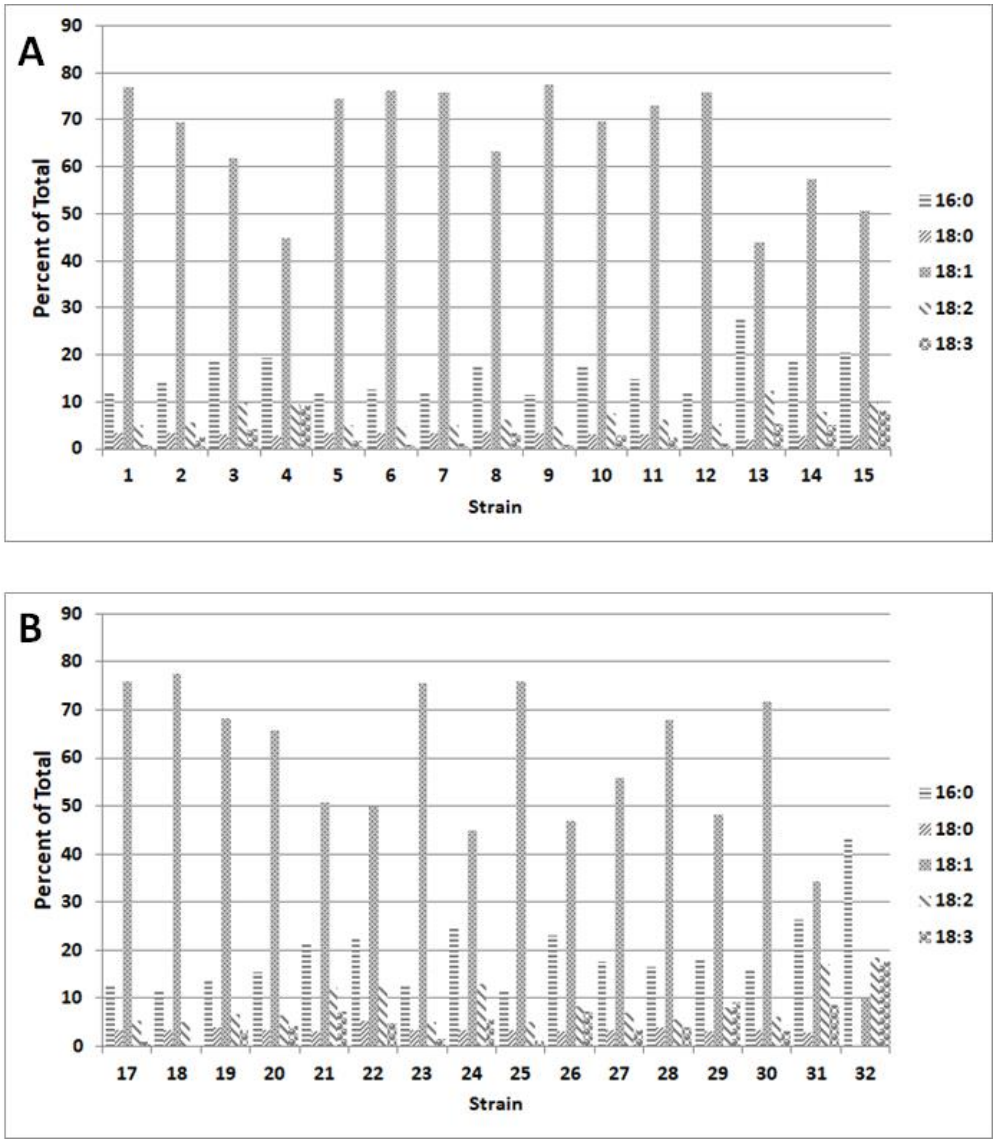

Figure 6. FAME analysis of the various strains. Panel (**A**) shows the results for strains S1–S16, panel (**B**) shows the results for strains S17–S32.

As mentioned before, one strain, S32, gave a FAME profile that was significantly different from the other isolates. Unlike the others, its content of saturated fatty acids (C16:0, C18:0) was greater than 50%, with a very high amount of palmitic acid (44%). In addition, its percentage content of PUFAs C18:2 and C18:3 (36%) was higher than all the other strains, suggesting its possible application in the production of essential fatty acids. This demonstrates the potential application of a local strain collection beyond biofuels production.

3.6. PCA Analysis

Principal component analysis (PCA) is an orthogonal tool mainly applied to identifying patterns in data sets and to further exploring similarities and differences. The association of different parameters (biomass production, growth rates, lipid content) at different temperatures (12, 20, and 35 °C) is shown by a PCA biplot (Figure 7).

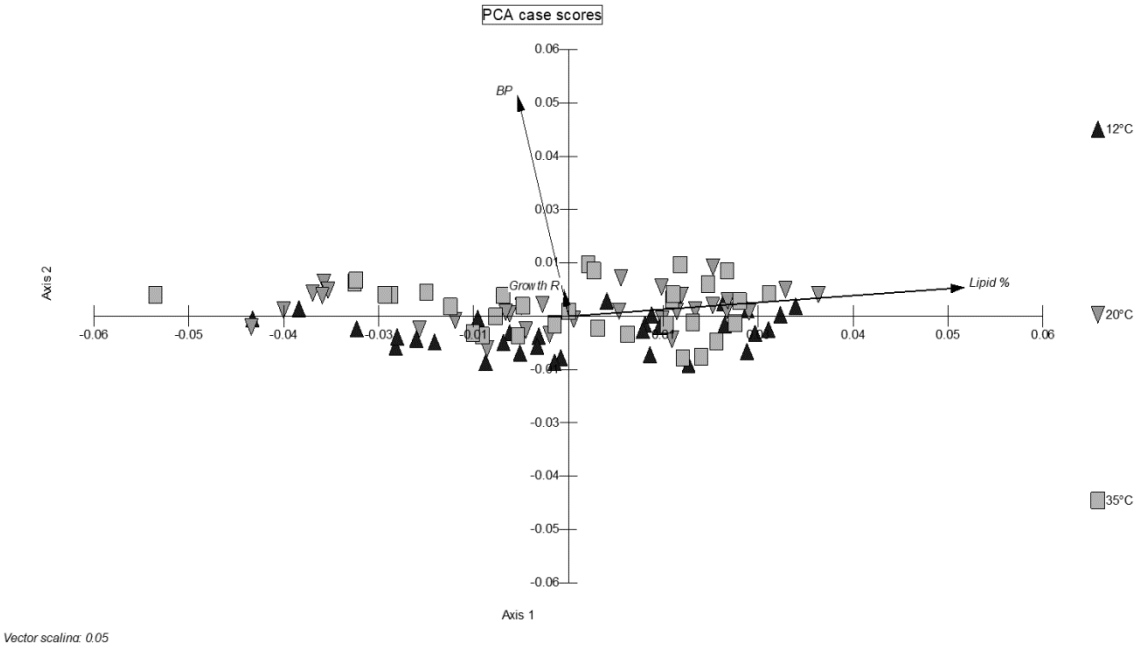

Figure 7. PCA analysis.

Principal component analysis (PCA) was applied to data sets to sort the total variability of the data into factors, which are orthogonal to each other and represent low possible covariance.

In the presented biplot each parameter is represented by an arrow whose direction indicates the maximum change and whose length is associated with the variation of each parameter among the analyzed samples. The PCA biplot associates a total variance of about 92.1% and 7.4% in the algal sample with the first and second component, respectively. Overall, a strong association (linear positive correlation) of lipid content was observed in most of the algal samples with respect to temperature (PC1), while adequate association was found for biomass production, followed by scant association for growth rate. Not surprisingly, biomass production and specific growth rate show a positive correlation with each other at the different temperatures. The different correlation variances were 56.1% for biomass production, 28.7% for lipid content, and 15.1% for growth rate. Moreover, KMO (Kaiser-Meyer-Olkin) and Bartlett's test showed highly significant values at $p < 0.001$. The two principal components, PC1 and PC2, partition the samples collected from different sites along the X-axis (PC1). Overall, the biplot indicates that samples from the various sites have different responses to temperature.

4. Conclusions

Thirty-two indigenous strains of green algae were isolated from disparate areas of Pakistan, identified, and characterized. They were examined for growth rate, biomass production, and lipid content when grown at 12, 20, and 35 °C, with different responses at the different temperatures depending upon the strain. The neutral lipid production of 19 strains is promising for development of biofuel production. It was shown here that relatively unexplored different areas of Pakistan contain novel microalgal strains with potential for different applications. Future R&D could give even greater growth rates and higher lipid productivity, potentially reducing costs of algal biofuel production.

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Author Contributions: Conception and design, data analysis and interpretation, collection and assembly of data, rafting of the article, critical revision of the article for important intellectual content, final approval of the article, M.M.A.; conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding, administrative support A.S.M.; collection and assembly of data, analysis and interpretation of the data, final approval of the article, M.R.; analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, M.G.; drafting of the article, critical revision of the

article for important intellectual content, final approval of the article, obtaining of funding, administrative support, D.V.; conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding, administrative and technical support, P.C.H.

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

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