Journal of PopulationTherapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.69980/q0jfwn04

IDENTIFICATION, BIOCHEMICAL CHARACTERIZATION AND BIOFUEL POTENTIALS IN ALGAE IN THE SELECTED REGION OF MALAKAND, PAKSITAN

Bibi Maryam[1*], Dr. Fazli Malik Sarim[2], Dr. Fida Hussain[3]

[1*]PhD Scholar, Department of Botany, Qurtuba University of Science and Information Technology, Peshawar, 25130. maryamghaffar62744@gmail.com
[2]Professor in Botany, Qurtuba University of Science and Information Technology, Peshawar, 25130. fazlemalik19@gmail.com
[3]Lecturer in Botany, Islamia College University, Peshawar, 25132. fidacp@gmail.com

***Correspondence author:** Bibi Maryam
PhD Scholar, Department of Botany, Qurtuba University of Science and Information Technology, Peshawar, 25130. maryamghaffar62744@gmail.com

Abstract
This study examined the viability of algae as a sustainable feedstock for biofuels and the presence of indigenous algal diversity, chemical classification and suitability for biofuel development. Conducted in the Malakand region of Pakistan, the study found 240 algal species among six separate taxonomic divisions. Two algae classification techniques were used; light microscopy and Scanning Electron Microscopy (SEM); both were effective for species classification. Of the species identified, Spirogyra crassa demonstrated the most promising overall potential with high lipid content and favorable growth in BG-11 culture media; these components show good potential for biofuels. Biochemical analysis indicated high concentrations of lipids, proteins and carbohydrates by Fourier-Transform Infrared Spectroscopy (FTIR) and X-ray Diffraction (XRD). Gas Chromatography-Mass Spectrometry (GC-MS) confirmed the presence of palmitic, oleic and linoleic acids provide the fuel stability and calorific value. Spirogyra crassa had superior oxidative stability and energy density indicating its potential for production as biofuel. The study promotes the use of algae-based biofuels within the renewable energy sector because of their ability to lower carbon emissions.

Keywords: Spirogyra crassa, Biofuels, GC-MS, Microscopy

Introduction

Ecological & Geographical Importance of Malakand

Geographically, Malakand is situated between 71.37° to 72.14° East longitude and 34.22° to 34.41° North latitude (Hussain et al., 2019) and is bordered on its north east by mountainous territory separating it from Swat, and on its west by Bajaur and Mohmand. Within Malakand there are two important rivers; Swat River and Panjkora River. These two rivers are an important part of the physical and socio-economic position of Malakand as a district (Hussain et al., 2019). Swat River is the most important river in Malakand. It starts from the confluence of Ushu and Gabral tributaries at Kalam (Swat Kohistan) and after flowing 160 km downstream through Swat valley, it passes Chakdara (Malakand) and diverges into Panjkora River at Bosaq before draining into the Kabul River in

Charsadda district (Khan et al., 2023; Ullah, 2023). The total length of Swat River is 240 km with a basin area of 14,000 km². Swat River is important for irrigation, hydroelectricity, agriculture, and biodiversity (Khan et al., 2023; Ullah, 2023). Panjkora River also plays an important role in terms of irrigated land and freshwater resources in Dir upper and lower districts. Both rivers contribute resources for irrigation of agriculture (crops such as grains, sugarcane, maize, fruits), energy generation, and local industries. In Malakand river water is commercially diverted for irrigation use from Banwan Headworks (near Batkhela), which diverts river water for irrigating land throughout Malakand and into Swabi, Mardan & Charsadda districts.

Apart from agriculture, the rivers sustain fisheries, eco-tourism, and a range of viable aquatic to terrestrial species, including migratory birds, otters, and endemic invertebrates (Aziz et al., 2021; Hussain et al., 2019). Their role in ecology makes them necessary for environmental health and sustainable development (Yaseen et al., 2022). This study investigates the algal diversity of these rivers and the relation between phytoplankton and environmental conditions in the Malakand region.

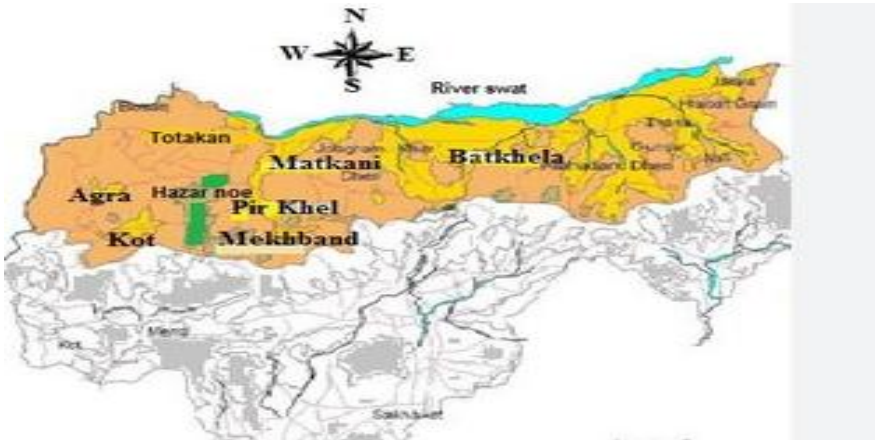


Fig 1.1 Map of the Study Area

Classification and Diversity of Algae

Algae are a heterogeneous group of organisms that live in freshwater, water, and surprisingly, terrestrial habitats, including soil and snow. Algae are primarily photosynthetic; they do not have complex organs organized into roots and stems like higher plants. Algae are important producers of O_2, are part of food webs, and contribute to the stability of ecosystem functions (Rehman et al., 2024; Khan et al., 2023). Algae exist as single cells, multicellular and colonial and reproduce through vegetative means or by spores. Most organisms called algae are, in fact, autotrophic organisms that use light, CO_2, and nutrients (e.g., nitrogen & phosphorous) to fix biomass but some are mixotrophic (Aliyu et al., 2023). Taxonomically, algae are often classified as belonging to the kingdom Protista or Plantae based on similarities in morphology, pigments, reproductive methods, etc. Algae can be classified as microalgae or macroalgae, with macroalgae's a dominance in marine environments (Arsalan et al., 2023; Ijaz et al., 2023). In general, taxonomic studies of algae (i.e., Dufour et al., 2022) try to describe the structural, functional and/or reproductive features of algae based on studies like those made foundational by Fritsch and Papenfus; although, as we continue to study algae and collect data, new categories of classification may arise (e.g., pigmentation and cellular organization).

Roles of Algae in Region

Algae serve many ecological functions and have different forms that include unicellular, multicellular, colonial, and filamentous. Algae represents plants that lack true roots, stems, and leaves and are found in wide variety of habitats from freshwater to marine and even in the deep sea. Algae can be planktonic (free-floating downstream) and use chlorophyll a for photosynthesis. Algae includes both prokaryotes like cyanobacteria and eukaryotic forms such as green algae, brown algae, and red algae. Algae are able to photosynthesize because they have chlorophyll pigments a, b, c, and phycobilins as well as

several non-chlorophyll analogous pigments. Algae can reproduce through processes such as binary fission, budding, and spores. Algae may have sustainable nutritional benefits nutritionally or economically, such as spoils or ingredients (agar or carrageenan, etc.). However, even beneficial algae can form harmful blooms because they can lead to eutrophication or the recall of contaminated waters (Meeranayak et al., 2020; Rao et al., 2024; Ding et al., 2022).

Algae Habitat

Algae are present all around the world, typically in many habitats: freshwater lakes, rivers, and ponds, as well as in marine conditions like shallow seas and the depths of the ocean. Conditions in which algae are found include depth, temperature, and the presence of nutrients, and algae are grouped in terms of abundance. In freshwater, green algae (e.g., Chlorophyceae) are the most abundant group of algae and play crucial ecological roles such as removing nutrients and taking up metals. The group Chlorophyceae contains about 2650 species across 350 genera, some of which are unicellular, some are colonial, and some are filamentous, and they reproduce by means of a haploid life cycle. Examples are *Oedogonium* and *Chlorococcales,* which grow in moist soils and *on* a substrate like the trunks of trees and the surfaces of bricks, wall, rocks, etc. Multicellular fossil algae have been found that date back to the Proterozoic eon, which verifies algae have been a major ecological player for some time (Des et al., 2020; Waqas et al., 2024; Hasnain et al., 2024).

Literature Review

A study was conducted by Kothaman et al. (2022) that investigated the potential of microalgae biomass, with consideration to growth, isolation, cultivation, oil extraction, and production of biodiesel. The authors reviewed harvesting techniques for microalgae, reviewed oil extraction techniques, and compared pond systems with photo-bioreactors. Kothaman et al., (2022) noted the high lipid content, ease of cultivation, and easy separation of microalgae biomass. In summary, as regards alternative sources of biodiesel, microalgae biomass is a potential alternative to edible oils.

Najeeb et al. (2022) explored the lab-scale biodiesel and biogas production from Scenedesmus algae growth in BG-11 medium under a 16:8 light-dark cycle. Lipids were extracted after 16 days incubation using chloroform-methanol-NaCl (NaCl added after extraction) to produce 5 ml of biodiesel and 4 ml of glycerol via NaOH-catalyzed transesterification. The resulting biodiesel was characterized using gas chromatography-mass spectrometry (GC-MS) and was found to contain significant amounts of methyl linoleate and other fatty acids as the predominant methyl esters. Anaerobic digestion of the total biomass produced a total of 34.27 ml of biogas and anaerobic digestion of the biomass from which lipids had been extracted produced a total of 19 ml of biogas. Although the study conclusions are positive on following the footsteps for opportunity, the study does mention the complexities in optimizing biofuel processing and production systems.

Najeeb et al. (2022) showed lab-scale biodiesel and biogas production using Scenedesmus algae grown in BG-11 medium under a 16:8 light-dark cycle. Lipids were extracted after sixteen days of growth with a chloroform-methanol-NaCl solution to be transesterified within NaOH to result in 5 ml of biodiesel and 4 ml of glycerol. Gas chromatography-mass spectrometry (GC-MS) analysis of the biodiesel sample showed predominance of methyl linoleate, and various other fatty acids were present as well. Anaerobic digestion was conducted and 34.27 ml of biogas was quantified from total biomass and 19 ml of biogas from lipid extracted biomass. While the approach shows promise for development, the study recognized barriers to optimizing biofuel processing and production systems.

Agha et al. (2020) investigated the effect of algal vegetation on soil characteristics in fruit farms located in Baluchistan, Pakistan. They examined 10 fields and found 109 algal species (as mostly blue green algae.) from 54 genera. The number of algal species was highest in loamy soil, and highest from Khanozai, Ziarat and Akhtar Abad. Algal species presence correlated with increased soil texture, moisture status, organic matter content, and increased nitrogen and potassium content, indicating a positive effect on soil fertility.

Khanna et al. (2019) noted the move toward eco-friendly methods to synthesize nanoparticles due to the distressing environmental effects of traditional nanomaterials. Algae and seaweed species with the division Cyanophyceae, Chlorophyceae, Phaeophyceae, and Rhodophyceae were used for production of gold, silver, and other metallic nanoparticles. Strong potential for antibacterially synthesized nanoparticles was observed through species like Chlorella and Sargassum to create viable alternatives to traditional antibiotics. There were a number of advanced techniques employed (for example, UV, FT-IR, SEM, TEM) so there was proper characterization of nanoparticles.

Materials and Methods

1.1 Identification of algae using Light and Electron Microscopy

A survey of microalgal diversity was conducted at 12 sites located across District Malakand, Pakistan—Piran, Dheri, Khar, Julagram, Trai, Totakan, Matkanai, Mekhband, Qalangai, Bosaq, Agra, Karkanai (Minhas et al., 2023). This is the first known survey on microalgal diversity from the above sites. During both the autumn and spring bloom seasons in 2022–2023, 240 algal samples were collected from each site, within a 100-meter radius, using forceps. The samples were stored in 100 ml plastic bottles, by scraping each substrate, and subsequently transferred to the Department of Chemical and Life Sciences for analysis ((Chong et al., 2023).

Algae were assessed using the wet-mount (Edler and Elbrachter, 2010) approach. Samples were taken on glass slides using a sterile micromanipulator, stained with lactophenol blue, and covered using coverslips. They were assessed using a Nikon Eclipse E200 (Mott & Ahad bahth, 2011) at 10× , 20× , 40× , 100× and images were taken using a digital microscope (Desikachary, 1959; Wehr et al., 2016; Prescott, 1962; Tiffany, 1955; Ullah et al., 2019). New algal samples were prepared for SEM by washing in ethanol to remove debris and improve visual clarity. The prepared samples were glued onto aluminum stubs with adhesive coated to the surface, then sputter-coated with gold-palladium to increase conductivity and observe details under the SEM microscope. Using a JEOL-5910 SEM found at the Centralized Resource Laboratory, Department of Physics, groups were taken on Polaroid P/N 665 film, looking at the cell wall, surface morphology and ultrastructural aspects (Ullah et al., 2022; Antreich et al., 2024).



Fig 2. Algae in the area of Julagram

Microalgae were sampled from Julagram, Trai, and Matkanai in Malakand, all locations in the Scottish Haplogroup. Samples were placed into sterile labeled bottles and delivered to the lab within one day. Species were isolated using the unialgal method, propagated in BG-11, and ultimately harvested and prepared for biochemical and biofuel analyses at Qurtuba University's Department of Chemical and Life Sciences (Hussain et al., 2023; Arsalan & Zubair, 2024).

To derive a pure algal strain, the algal suspension was centrifuged to isolate the desired group, and re-diluted in sterile water (10g biomass/100ml). Zooplankton was filtered out using 60 µm and 0.45 µm filters, respectively, and cells washed in marine salt, which is less friendly to bacteria. Cultures were grown in BG-11 medium in sterilized 12-well plates (Rippka et al., 1979) and observed for 10

days. All potential cultures for the collection were transferred to agar plates or flasks containing fresh BG-11 media, and were maintained on an orbital shaker at 150 RPM for the purpose of continuous growth (Guleria et al., 2024; Din et al., 2024).

For the analysis of fatty acids, the dried algal samples were freeze-dried and then underwent 2 stages, placing them in a fatty acid methyl ester (FAME) preparation stage, and then a GC–MS analysis stage. Approximately 100 mg of sample was Transesterified in a methanol-sulfuric acid-chloroform solution (Chen et al., 2018; Ammar et al., 2022) for 90 minutes in a water bath at 30°C. The FAME was extracted from the sample and analyzed using a GC–MS system with a DB-5-MS column and a flame ionization detector. The ovens were set between 80°C and 290°C. Compounds were identified through matched spectra in the NIST database and quantitated using external standards (Hasnain et al., 2024; Bibi et al., 2022).

Characterization Techniques:

FTIR Analysis

Samples of spirogyra (Spirogyra crassa and Spirogyra subsalsa) were collected from lift freshwater bodies in Malakand, thoroughly washed and suspended in distilled water. Each spirogyra sample was freeze dried to remove microorganisms and was thoroughly ground to a powder with a mortar and pestle for FTIR analysis (Zamanileha et al., 2024; Akhtar et al., 2024).

FTIR spectra were conducted with a Nicolet 6700 ATR-FTIR spectrometer at room temperature. The spectral range was 4000–400 cm^{-1}, with a resolution of 4 cm^{-1}, measured with 128 scans per sample to account for spectral quality (Deore et al., 2021).

The powdered algae were combined with potassium bromide (KBr) and pressed into pellets for spectral analysis. By following this method, the absorption of water is reduced and infrared light transmittance is improved (Ishfaq et al., 2024).

Scans included both the fingerprint and functional group spectral regions (4000-400 cm^{-1}), allowing for the identification of inorganic and organic compounds. The spectral data displayed important biochemical lipids, proteins, and carbohydrates indicative of biofuel production (Sukhikh et al., 2022).

X-ray Diffraction (XRD) Analysis

The algal biomass of Spirogyra crassa and Spirogyra insignis was rinsed in distilled water to remove impurities such as sand and debris. After 24 hours of drying, the samples were dried in an oven at 60°C until they achieved a constant weight and mechanically ground to a powder for subsequent XRD analysis (Suciyati et al, 2021; Akhtar et al., 2024).

XRD patterns were obtained using a PANalytical X'Pert PRO diffractometer equipped with Cu Kα radiation (λ = 1.54060 Å) operated at 45 kV and 40 mA. The scans were performed using a step size of 0.017° (2θ), over a scan range of 5° to 40°, with a count time of 50.1650 seconds per step (Ismail et al., 2023).

The relative crystallinity (Ka) was calculated using the equation:

$$\mathbf{Ka = \sum Scr / (\sum Scr + \sum Sam)}$$

Where ∑Scr is the total area under crystalline peaks, and ∑Sam is the area under amorphous peaks. These values were obtained using Panalytical X'Pert HighScore software. Determining the crystalline structure and degree of crystallinity is essential for evaluating the suitability of algal biomass in biofuel production (Suciyati et al., 2021; Del Buey et al., 2023).

Biofuel Analysis

Microalgae were collected from freshwater sources in Julagram, Trai, and Matkanai (Malakand region), carried in sterile labeled containers, and shipped to the laboratory for further process. Samples were purified using unialgal methods and cultured in BG-11 medium. Once sufficient growth was achieved, they were harvested for biochemical and biofuel analysis at Qurtaba University (Hussain et al., 2023; Arsalan & Zubair, 2024)

To establish pure strains of algae, the algal suspensions were centrifuged, diluted and filtered for contaminants. The cells were washed in marine salt to inhibit bacterial growth and were then cultured

and sought as sterile BG-11 medium in sterile 12-well plates. Bacterial growth was monitored over a 10-day period and the cultures were then sub-cultured onto solid and liquid BG-11 media with the use of orbital shakers (Rippka et al., 1979; Guleria et al., 2024).

To find strains that can mixotrophically grow using a four-step process:

1. Grown in BG-11 medium supplemented with glucose;
2. Growth in light (autotrophic) and dark (heterotrophic) conditions;
3. Mixotrophic growth (light + glucose);
4. Continued growth for three generations to verify stability. Batch cultures were held under light intensity of 50 μmol m^{-2} s^{-1}, and growth data were collected (Guleria et al., 2024; Hasnain et al., 2024).

The fatty acids were first extracted by freeze-drying, then transesterification with methanol, sulfuric acid, and chloroform was used to make Fatty Acid Methyl Esters (FAMEs). Separation of the FAMEs were completed with by GC-MS (DB-5-MS column, FID detector). Temperature was ramped from 80°C to 290°C, using helium as a carrier gas (1.2 ml/min). The FAMEs were identified and quantified using the chromatographic data from the NIST spectral database and standards (Hasnain et al., 2024; Bibi et al., 2022).

Results and Discussions

Identification of Algae using Light Microscope:

Overall, 240 algal species were noted from specific sites in District Malakand consisting of 6 divisions, 8 classes, 58 orders, 61 families, and 82 genera. The determination of species involved the investigation of chloroplast characteristics, presence of pyrenoid, and cell structure. Bacillariophyta was economically dominant, with Bacillariophyceae accounting for 30% (72 species), Charophyta (Zygnematophyceae) 33.3% (80 species), and Ochrophyta 6% (15 species). Chlorophyta consisted of 38 species (15.6%) that included the classes Chlorophyceae (10%), Cladophoraceae (3%), and Trebouxiophyceae (1.6%) across 17 orders, 17 families, and 19 genera. Cyanophyta consisted of 13% (32 species), with 1 class, 8 orders, 9 families, and 13 genera. Heterokontophyta contributed 1.2% (3 species) with only 1 class, order, family, and genus (Appendix 1).

The class Bacillariophyceae contributed 2% of species, with much of the contribution made up by the species N. vermicularis, N. acicularis and N. palea. Denticula and Stauroneis totaled 1.6%, Amphora and Synedra each 4%, and Diatoma, Meridion, Fragilaria, and Navicula each 3%. There were several other genera: Caloneis, Gyrosigma, Diploneis, Gomphonema, Achnanthes, Cocconeis, Epithemia, and Eunotia that made up 7.9% together. Cymatopleura, Surirella, Neidium, and Cymbella again each added a total of 7%. The last identified contributions from Fragilaria, Nitzschia, Craticula each providing 3% of the total of the identified species. The division of Ochrophyta had a total of Pinnularia, Diatoma, Tabellaria, Aulacoseira, Melosira each providing 2.9%, with genera Cocconeis, Tabellaria, Surirella, Rhopalodia, Gomphonema, and Achnanthes each with a total of 3% of the total identified species (Appendix 2,3).

In the Zygnematophyceae, Closterium contributed about 3.3% of the species (including C. acerosum, C. strigosum, C. littorale, etc.). Cosmarium, Netrium, and Staurastrum made up 4%. Spirogyra was the dominant genus at 13% with species such as S. porticalis, S. crassa, S. setiformis, and S. nitidia. The genera Zygnemopsis, Zygonium, Mougeotia, Pleurotaenium, Gonatozygon, and Hyalotheca contributed back 3.3%, with species such as Z. desmidioides, M. scalaris, and H. mucosa. Cosmarium, Closterium, and Penium totaled 6.6% of the species with some of the other genera also at 3.3% respectively, such as Mougeotia and Zygnemopsis with species including M. americana, M. viridis, and Z. transeauana (Appendix 4).

In the class Chlorophyceae, Hydrodictyon and Chaetophora had 4% of the total species, while Stigeoclonium had a few species. The remaining species from Microspora accounted for 2% of the total amount. The same was true with the genera Hormidium, Oedogonium, Chlorococcum, and Palmella, each representing 2.9% species comprising H. klebsii, O. taphrosporum, C. humicola, and P. mucosa. Meanwhile, Microspora, Chlamydomonas, and Chaetomorpha represented 2%. Species

from Cladophora, Pithophora, Binuclearia, and Microspora in the Cladophoraceae together accounted for 3.3% of the species identified (Appendix 5).

In the class Cyanophyceae, the genera Anabaena, Oscillatoria, Merismopedia, Schizothrix, and Chroococcus totaled 2% of the total species. For example, Oscillatoria, Microcoleus, Lyngbya, Phormidium, Anabaena, Gloeocapsopsis, Nodularia, Symploca, and Schizothrix totaled 11%. In Xanthophyceae, Tribonema made up 1%, with several examples including T. bombycine and T. minus. Algae are preserved, primitive organisms that grow widely in a number of different circumstances, and are very important components of aquatic environments as they are primary producers. Although it is known that algae have an important ecological role and are commercially important, particularly as food sources, documentation is scarce, particularly in Pakistan, including the District Malakand (Munir et al., 2020; Kowthaman et al., 2022; Khan et al., 2023; Hasnain et al., 2023).

We recorded a notable algal diversity from freshwater habitats. The most dominant genus was Spirogyra (13%), comparable to Yaseen et al. (2016), who reported 11 species from Peshawar Valley. The leading genus in Cyanophyceae (10 species) was Oscillatoria, also comparable to Zahir et al. (2024). Cymbella (9 species, 4%) corroborated the finding by Khalil et al (2021).

Other important genera were Closterium, Navicula, and Cosmarium (8 species each, 10%), while Minhas et al. (2023) supported these findings; Gomphonema and Mougeotia (6 species, 5%) have already been mentioned in the study by Nazir et al. (2024); Microspora and Chroococcus (5 species, 4%), supported also by Ullah (2023). The rest of the genera, representing 4 species each included Synedra, Fragilaria, Epithemia, Stigeoclonium, Surirella, Nitzschia, and Cladophora (12% together) reflecting high diversity of Bacilliariophyta in Rawal Dam according to Shah et al. (

2019). Genera with three species each (12.5% combined) were Nitzschia, Neidium, Pinnularia, Microcoleus, Lyngbya, Tribonema, Chaetophora, Hormidium, Stauroneis, and Diatoma. Ali et al. (2016) reported the presence of diverse algae in Lund Khwar, Mardan as well. Genera with two species each (8.33%) were Amphora, Caloneis, Cocconeis, Achnanthes, Gonatozygon, Oedogonium, Microspora, Chlorella, Melosira, and Aulacoseira, reflecting the findings of Ullah et al. (2021). The lowest abundances were published in genera with one species each (14.6% together) which in some cases created a pattern. Truly, Khurram et al. (2021) also reports similar species diversity between genera in the Peshawar Valley.

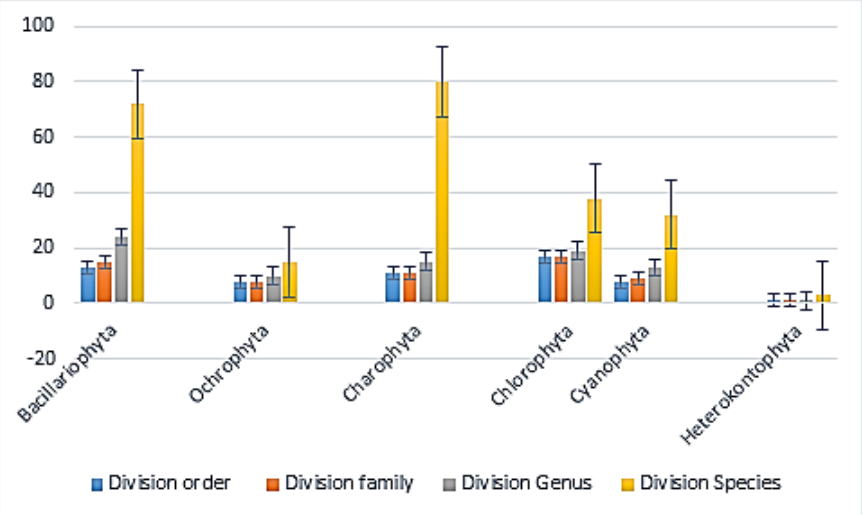


Table 1: Comparison of Income Sources Between MHP and Non-MHP Households

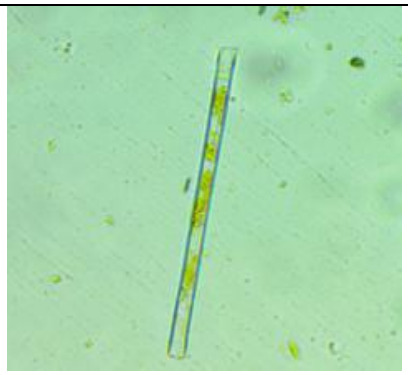


Fig.1. **Nitzschia vermicularis**

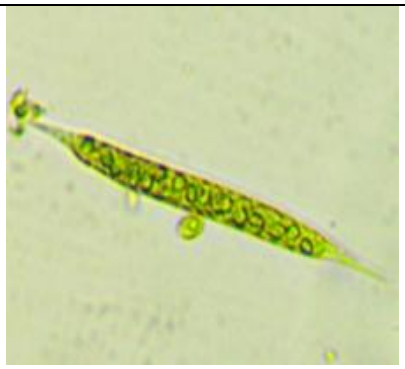


Fig.2 **Nitzschia acicularis**



Fig.3 **Nitzschia palae**

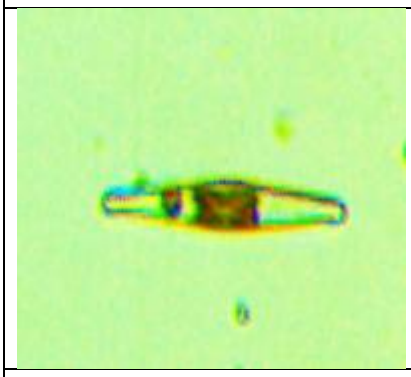


Fig. 4. Denticula tenuis

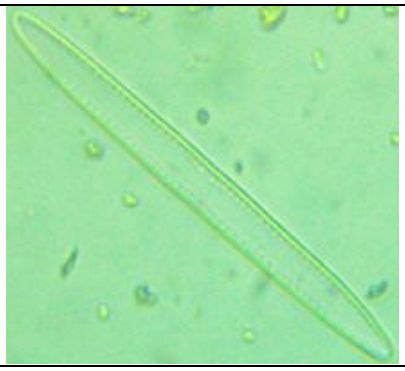


Fig.5. Stauroneis gracilis



Fig.6. Stauroneis producta Grunow



Fig.7. **Stauroneis phoenicentron var. amphilepta**

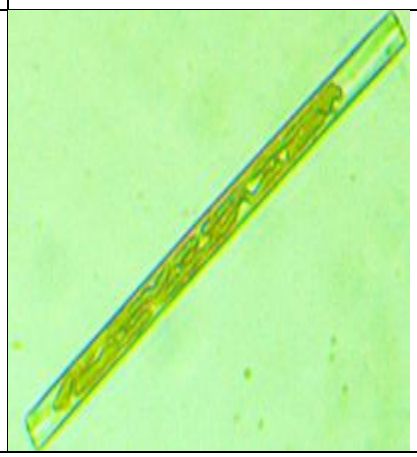


Fig. 8 **Stauroneis phoenicentron**



Fig.9 **Amphora Ovalis**





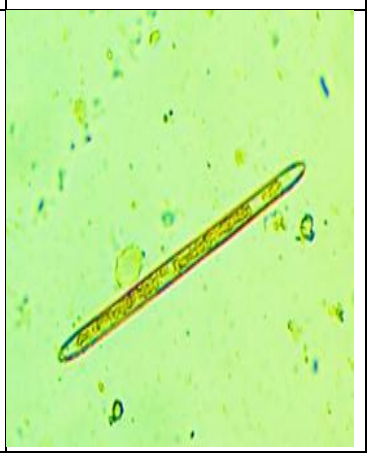
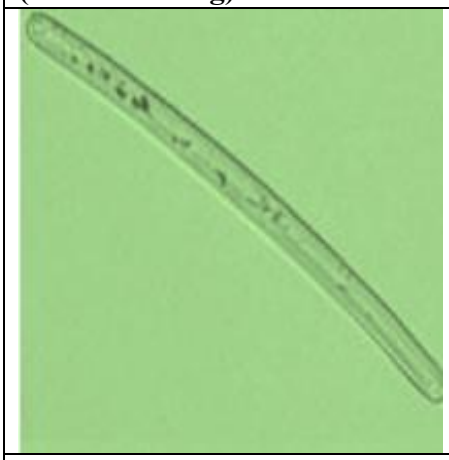
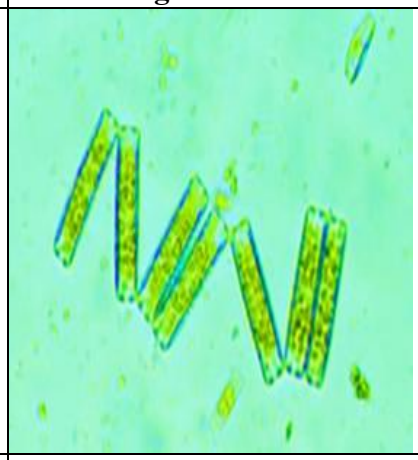
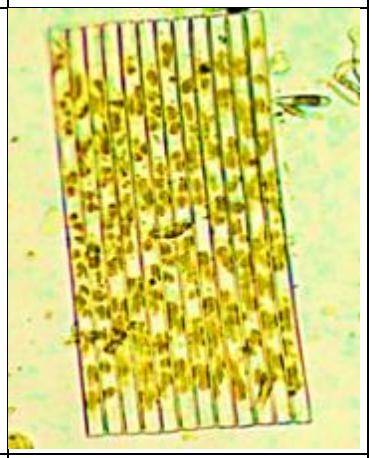
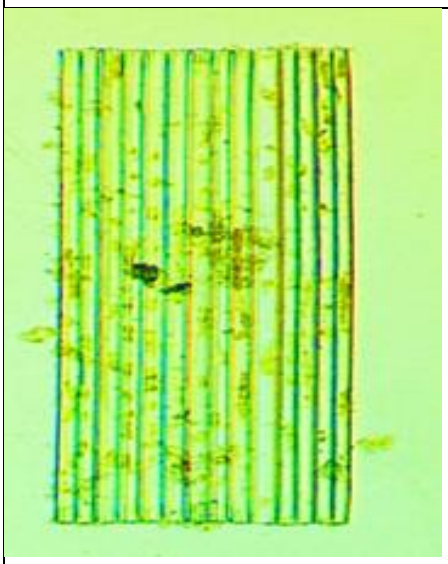
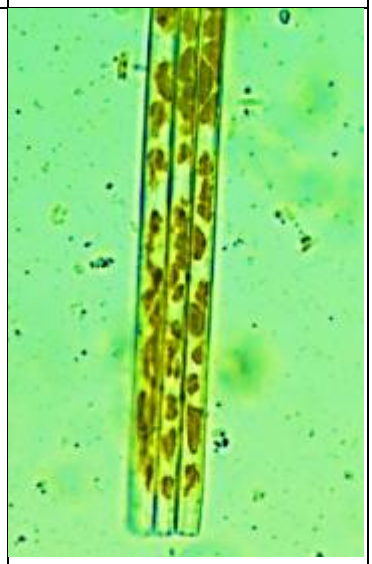


Fig.10. Synedra pulchella (Ralfs kuetzing)	Fig. 11. Synedra ulna (nitzch) Ehrenberg	Fig. 12. Synedra tenera Wm. Smith
		
Fig.13. Synedra acus Kuetzing	**Fig.14. Diatoma vulgare Bory, Colony**	**Fig.15. Diatoma hiemale**
		
Fig.16. Daitoma anceps (Ehrenberg) Kirehner	**Fig. 17. Meridion circulare**	**Fig. 18. Fragilaria capucina**

1. *Nitschschia vermicularis*
2. *Nitschschia acicularis* Smith, Wm.
3. *Nitzschia palae* (Keutzing)
4. *Denticula tenuis* kuetzing
5. *Stauroneis gracilis* Ehrenberg
6. *Stauroneis producta* Grunow
7. *Stauroneis phoenicentron* var. amphilepta (Ehrenberg) Cleve
8. *Stauroneis phoenicentron* var. amphilepta (Ehrenberg) Cleve.
9. *Amphora ovalis* Kuetzing
10. *Synedra pulchella* (Ralfs kuetzing)
11. *Synedra ulna* (Nitzch) Ehrenberg
12. *Synedra tenera* Wm. Smith
13. *Synedra acus*
14. *Diatoma vulgare* Bory, Colony
15. *Diatoma hiemale* (Lynbye) Heiberg
16. *Daitoma anceps* (Ehrenberg) Kirehner

17. *Meridion circulare*
18. *Fragilaria capucina* Desmazieres
19. *Fragilaria crotonensis* var Prolongate Grunow
20. *Fragilaria construens* var. Venter (Ehrenberg) Grunow
21. *Fragilaria pinnata* Ehrenberg
22. *Navicula protracta* (Grunow) Cleve
23. *Navicula radiosa* var. Tenella (Brebisson) Grunow
24. *Navicula oblangata*. Var. sub-Capitata Pantocsek
25. *Navicula salinarum* Grunow
26. *Nvicula exigua* (Gregory) Mueller
27. *Navicula anglica* Ralfs
28. *Navicula cryptocephala* Kuetzing
29. *Caloneis bacillum* (Grunow) Meresch Kowsky
30. *Caloneis alpestris* (Grunow)
31. *Gyrosigma accuminatum* (Kuetzing) Cleve
32. *Diploneis elliptica* (kuetzing cleve) Wm. Smith
33. *Gomphonema sphaerophorum* Ehrenberg
34. *Gomphonema olivaceum* (Lyngbye) Kuetzing
35. *Gomphonema constrictum* var. Capitatum (Ehrenberg) Grunow
36. *Gomphonema acuminatum* Var. trigonocephalum
37. *Gomphonema angustatum* (Keutzing) Grunow
38. *Gomphonema intricatum* keutzing
39. *Achnanthes minutissima* (Kuetzing)
40. *Achnanthes exilis* Kuetzing
41. *Cocconeis flexella* (kuetzing) Cleve
42. *Cocconeis pediculus* Ehrenberg
43. *Epithemia turgida* var. Wester-manii (Ehrenberg) Grunow
44. *Epithemia argus* var. Alpestris (Wm.smith) Grunow
45. *Epithemia ocellata* (Ehrenberg) Kuetzing
46. *Epithemia zebra* Keutzing
47. *Eunotia lunaris* (Ehrenberg) Grunow
48. *Cymatopleura solea* (Berbisson)
49. *Surirella angusta* Kuetzing
50. *Surirella apiculate*
51. *Surirella robusta*
52. *Neidium iridis* (Ehrenberg) pfitzer
53. *Neidium debium* (Ehrenberg) pfitzer
54. *Neidium amphigomphus* (Ehrenberg) Pfitzer
55. *Cymbella tumida* (Brebisson) van Heurck
56. *Cymbella amphicephala* Naegeli
57. *Cymbella cymbiformis* (Kuetzing) Brebisson
58. *Cymbella cistula* (Hemaprich)
59. *Cymbella affinis* Keutzing
60. *Cymbella lanceolate* (Ehrenberg) Van Heurck
61. *Cymbella leptoceros* (Ehrenberg) Grunow
62. *Cymbella parva* (Wm. Smith) Cleve
63. *Cymbella laevis* Naegeli
64. *Fragilaria vaucheriae* Kutzing J. B. Pretersen
65. *Fragilaria Crotonensis* Kitton 1869
66. *Nitzschia amphibia* (Grunow)
67. *Nitzschia compressa* (Bailey) C.S. Boyer

68. *Nitzschia paleacea Grunow*
69. *Nitzschia solita Hustedt*
70. *Craticula perrotetii* Grunow

Division of Ochrophyta
1. *Pinnularia braunii* (Grunow) Cleve
2. *Pinnularia divergens*
3. *Pinnularia viridis* (Nitzsch) Ehrenberg
4. *Diatoma tenuis C.Agardh*
5. *Tabellaria flocculosa* (Roth) kuetzing
6. *Aulacoseira granulate* (Ehrenberg)
7. *Melosira varians* C. Agardh
8. *Melosira crenulata* (Ehrenberg) Kutzing 1844
9. *Cocconeis placentula*
10. *Tabellaria Fenestrata* Var. geniculate A. Cleve
11. *Surirella guatemalensis* Ehrenberg
12. *Rhopalodia gibba Traugott Kutzing*
13. *Gomphonema augur* Frederick Grunow
14. *Gomphonema micropus* Kutzing
15. *Closterium strigosum* Brebison

Division of Charophyta
1. *Closterium acerosum* (schrank) Ehrenberg
2. *Closterium ehrenbergii* (Meneghini)
3. *Closterium littorale* Gay
4. *Closterium ehrenbergii* Ralfs
5. *Closterium acerosum* var. Elongatum Brebisson
6. *Closterium leibleinii* kuetzing
7. *Closterium turgidum* Kuetzing
8. *Closterium lunula* (Mueller) Nitzsch
9. *Cosmerium supraspeciosum* wolle
10. *Cosmerium formossulum*
11. *Cosmarium quinarium* lundell
12. *Cosmerium pachydermu*
13. Cosmarium botrytis *Meneghini*
14. *Comarium subcrenatum* Hantzsch
15. *Cosmarium moniliforme* (Turpin) Ralfs
16. *Cosmarium nitidulum* De Notaris
17. *Netrium digitus* (ehrenbergii) Itsigsohn and Roth
18. *Staurastrum oxycanthum* archer
19. *Spirogyra porticalis*
20. *Spirogyra subsalsa* Kuetzing
21. *Spirogyra crassa* Kuetzing
22. *Spirogyra scorbiculata* (stock.) Czurda
23. *Spirogyra rhizobrachialis* Jao
24. *Spirogyra daedaloeoides* czurda
25. *Spirogyra setiformis* (Roth) Keutzing
26. *Spirogyra gratiana* Transverse
27. *Spirogyra novae*- angliae Transverse
28. *Spirogyra fuelleborni* Schmidle
29. *Spirogyra liana* (Transeau) Yamagishi

30. *Spirogyra ellipsospora* transeau
31. *Spirogyra equinoctials*
32. *Spirogyra webri* Kuetzing X 350
33. *Spirogyra rectangularis* transeau
34. *Spirogyra pratensis* Transeau
35. *Spirogyra condensata* (Vauch.) Kuetzing
36. *Spirogyra fluviatilis* Hilse
37. *Spirogyra narcissiana* (Transeau)
38. *Spirogyra nitidia* (Dillwyn) Link
39. *Spirogyra juergensii* Kuetzing
40. *Spirogyra porangabae* Transeau
41. *Spirogyra punctiformis* Transeau
42. *Spirogyra silvicola* M. E. Britton
43. *Spirogyra circumlineata* Transeau
44. *Spirogyra majuscula* Kuetzing
45. *Spirogyra insignis Hassall (Kutzing)*
46. *Spirogyra micropunctata Hassall (Kutzing)*
47. *Spirogyra spreeiana* Rabenhorst
48. *Spirogyra protecta*
49. *Spirogyra Ternata var. sphaerica j. N. Misra*
50. *Zygnemopsis desmidioides* (West & West) Transeau, in Transeau
51. *Zygogonium ericetorum* (Kuetzing)
52. *Mougeotia scalaris* (Hassall, X 240)
53. *Mougeotia robusta* (Debary) Wittrock
54. *Mougeostiopsis calospora* Palla
55. *Gonatozygon kinahani* De Dary
56. *Gonatozygon brebissonii* Debary
57. *Hyalotheca mucosa*
58. *Cosmarium angulosum* Brébisson
59. *Cosmarium bioculatum* Brébisson ex Ralfs 1848
60. *Cosmarium crenatum* Ralfs ex Ralfs
61. *Cosmarium granatum*
62. *Cosmarium impressulum* Elfving
63. *Cosmarium laeve*
64. *Cosmarium subcrenatum* Hantzsch
65. *Cosmarium subtumidum* Nordstedt
66. *Cosmarium turpinii* Hantzsch
67. *Closterium attenuatum* Ralfs
68. *Closterium var ehrenbergii*
69. *Closterium incurvum* Brébisson
70. *Closterium moniliferum*
71. *Closterium Strioaltum Ehrenberg ex Ralf*
72. *Closterium desmid Ehrenberg ex Ralf*
73. *Penium exiguum*
74. *Mougeotia Americana*
75. *Mougeotia floridana* Transeau
76. *Mougeotia maltae* Skuja
77. *Mougeotia micropor* Taft in Transeau
78. *Mougeotia nummuloides*
79. *Mougeotia viridis*
80. *Zygnemopsis transeauana*

Characterization of Algae using FTIR Spectroscopy and XRD Analysis

FTIR spectroscopy results of Spirogyra crassa and Spirogyra insignis from Malakand confirmed the presence of important biochemical components associated with biofuel development. The presence of a pronounced lipid peak at 1745 cm^{-1} (15% spectral area) indicated a largespannin of fats which are favorable for biodiesel. The presence of peaks related to proteins at 1650 cm^{-1} and 1540 cm^{-1} with a combined spectral area of 20%, suggests there is a high availability of proteins in these samples, which also presents a potential biotechnological value. There was also an indication of glycogen via a peak at 1150 cm^{-1} (C-O-C stretch) that displayed a relationship to carbohydrate contents - all three areas noted are significant when considering biofuels. Statement of formulation of both algae displayed significant distribution of the important biomolecules required (lipids, proteins, and carbohydrates) for biodiesel and bioethanol production.

Microalgae are being explored as renewable biofuel sources because of their high oil and carbohydrate content that has the potential to help reduce carbon emissions. The use of FTIR analysis is essential for determining molecular structures within algal biomass, which will provide better characterization and subsequently increase biofuel extraction and yield (Moraes et al., 2014; Khanna et al., 2019).

The findings from FTIR analyses of Spirogyra crassa and Spirogyra insignis in this study had very strong biochemical indicators of lipids (1745 cm^{-1}), lipids (1650 cm^{-1} and 1540 cm^{-1}) and carbohydrates (1150 cm^{-1}). These are of importance for biofuel production. These results are consistent with other studies (Sohail et al.2020; Suciyati et al.2021; Ozer et al., 2016; Deore et al., 2021), so it appears that microalgae sourced from a wide range of regions often share many similar biochemical characteristics. As a whole, the findings indicate that the Malakand microalgae may be seen as well-balanced feedstocks for biofuel production. High lipid levels can provide good indications of a strong potential for biodiesel production, while the associated protein and carbohydrate levels can also lend support for additional biofuel production and biotechnological applications.

The high lipid content identified using FTIR proposes their possibilities of getting Malakand microalgae as promising biodiesel feedstocks due to their high energy yield (Sweiss et al., 2024). The presence of proteins and carbohydrates aligns with the integrated biorefinery model defining that other products made from the biomass, such as animal feed and bioethanol, will also be manufactured. This approach, stated by Zamanileha et al., (2024), improves economic viability by utilizing every part of the microalgae crop, reducing all extraction costs, and yielding maximum amounts of biofuels.

The X-Ray Diffraction (XRD) analysis results of Spirogyra crassa and Spirogyra insignis indicated the two types contained both crystalline and amorphous phases. The peaks at $2\theta = 29.4°$ and 36.0° indicated the material contained calcite ($CaCO_3$) which resulted from either the biomineralization of calcium in the Spirogyra or can come from calcium from the environment. There were also silica peaks at 20.8° and 26.5°, which may have formed as a result of diatom deposition or accumulation from the surface. There were more minor peaks which may have contained other minerals in small amounts such as magnesium or phosphates. The broad halo given between 15°–30° indicated the amorphous nature of organic materials such as cellulose and potentially other biopolymers which lack a long-range order structure.

XRD analysis of Spirogyra crassa and Spirogyra insignis showed crystalline and amorphous structures, with calcite peaks at 29.4° and 36.0° that suggest biomineralization or calcium uptake, and silica peaks at 20.8° and 26.5° which imply diatoms or surface deposition (Tripathi et al., 2015). These structural features change the mechanical strength of algal cells and can vary the kind of pre-treatment for lipid extractions (Zhao et al., 2024). Furthermore, calcite (~30%) and silica (~10%) can make it difficult to disrupt the cells if the extraction process has high crystallinity, so methods such as bead milling or high-pressure homogenization must be used (Shi et al., 2023). Even though higher silica levels can impact direct combustion due to ash composition, silica can encourage biochar use, soil enrichment, and carbon sequestration alternatives (Amin et al., 2024; Valerio et al., 2023; Paniz et al.,

2020). Overall, these results highlight the importance of recognizing the algal composition when proposing appropriate biofuel and by-products applications.

GC-MS Analysis

GC-MS was employed to characterize FAMEs present in Spirogyra, with parameters explicitly (injected at 1µL) optimized for accurate identification of all fatty acids of interest. The method temperatures in the gas chromatography mass spectrometry set-up were also optimized to gain sufficient ionization and transfer of the analyte into the mass spectrometer, with ion source temperature set to 250°C and the interface temperature set to 240°C. A solvent cut time of 2.20 minutes was included to remove early eluters for greater clarity and more reliable results.

GC-MS analysis identified palmitic (C16:0), stearic (C18:0) and oleic (C18:1) and linoleic acid (C18:2), each which are vital fatty acid precursors for biodiesel production from Spirogyra. These saturated and unsaturated fatty acids provide value to the quality and stability of the fuel and are important components for biodiesel production. The analysis of key fatty acids from the algae supports the notion that it can be a renewable biofuel source, confirming an objective of the study.

GC-MS allowed for confirmation of significant fatty acids identified in Spirogyra's crude oil, further analysis of its biofuel potential (Waqas et al., 2024). The biodiesel obtained contained notable amount of palmitic acid, oleic acid, and linoleic acid which, according to the same reference, are essential for biodiesel quality and performance. This analysis in composition is important for biofuel classification and provides data to assess opportunities for commercialization (Shahbaz et al., 2022)

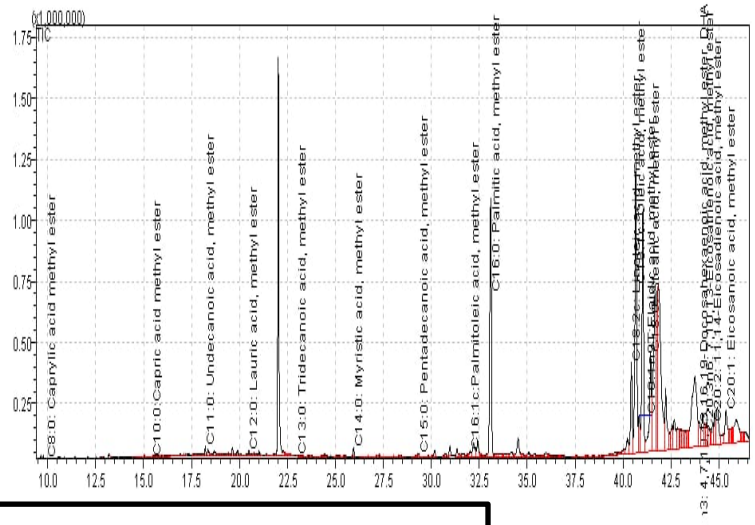


Figure 1.1 GC-MS Analysis

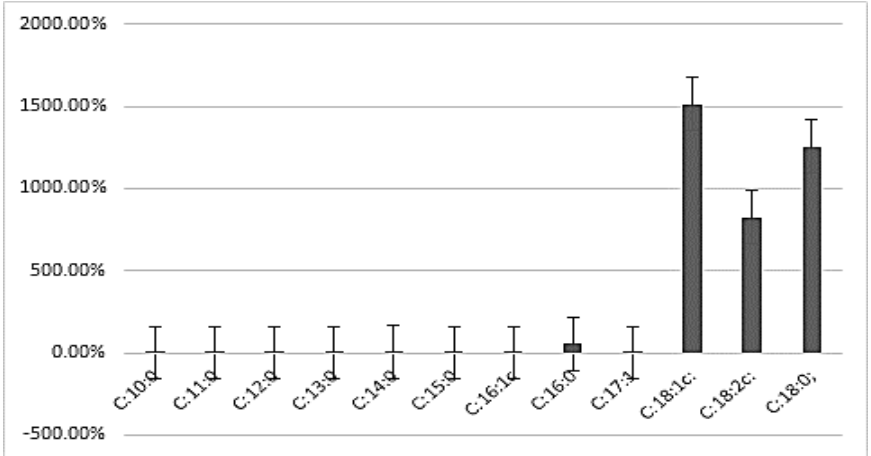
Fatty acid Analysis

GC-MS analysis identified Spirogyra with high concentrations (percent by weight): palmitic acid (54.81%), oleic acid (15.14%), and linoleic acid (8.25%), indicating potential for biodiesel. These are significant fatty acids related to biodiesel quality due to their high energy content, oxidative stability, and combustion characteristics. In comparison to other species analyzed, Spirogyra exhibited fairly

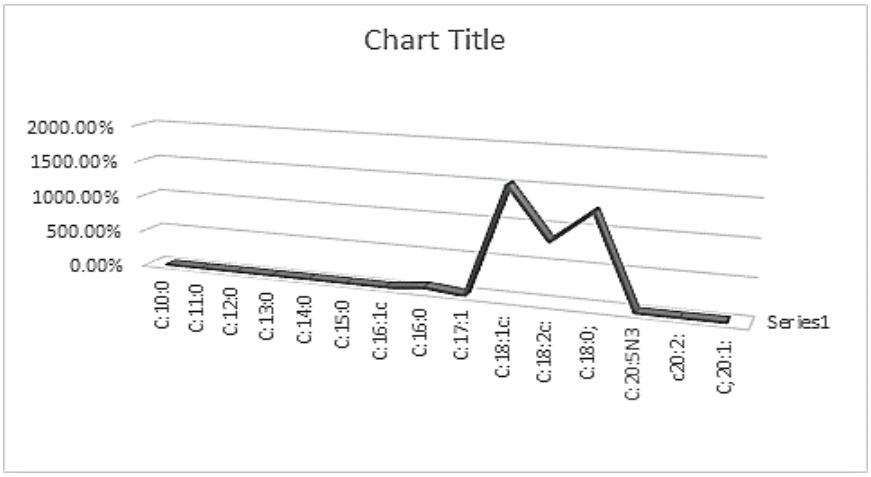
high amounts of lipids overall, creating much more interest as a potential renewable energy feedstock. Palmitic acid and oleic acid contribute to biodiesel cetane number and cold flow performance respectively, and linoleic acid contributes to fluidity in colder environments. Additionally, several other fatty acids (including stearic (12.55%) and eicosanoic (2.98%) acids) along with trace amounts of fatty acids such as EPA, capric, and lauric acids resulted in a good balance of lipid profile well suited for fuel production. The variation in the fatty acid profile enhances the viability of Spirogyra as a biofuel source, and future studies may involve genetic optimization or alteration of the growing conditions for enhancement. The results confirm Spirogyra as a promising candidate for developing sustainable bioenergy as well as some commercial viability given that methods for cultivation and lipid extraction can be improved.

GC-MS analysis confirmed that Spirogyra crassa has significant amounts of key fatty acids, especially palmitic acid (54.81%), oleic acid (15.14%), and linoleic acid (8.25%), which are all highly functional for high-quality biodiesel. These fatty acids provide good oxidative stability, high energy density and good cold flow properties. All of which make Spirogyra a viable option for biofuel production (Ramaraj et al., 2022; Guleria et al., 2024; Shah et al., 2022). While palmitic aids fuel stability, it may require blending due to high cloud points in colder areas (Husnain et al., 2024). Oleic and linoleic acids help improve low-combustion fuels and an important factor is the viscosity—but linoleic acid may require antioxidants due to its high sensitivity to heat for long-term storage (Saeed et al., 2021; Sohail et al., 2020).

The presence of other fatty acids—like stearic, myristic, and EPA—enhances the variability of the fuel profile to reach specialized applications (Khalaji et al., 2022). The tooled ratio of saturated versus unsaturated provided evidence that Spirogyra satisfied biofuel parameters, and could possibly be optimized through cultivation and biotechnology (Erkaya & Yalcin, 2021; Kalyani & Kolakoti, 2023). Further research and testing, especially life cycle assessments (LCA), are suggested to gain a more accurate understand of the largescale viability, environmental footprint, and potential cost of Spirogyra biofuels. Also, the potential contribution to water treatment is indispensible in creating synergies to generate renewable energy or low-impact biofuels while continuing to remediate the environment (Shahbaz et al, 2022; Koley et al., 2024).



Fatty Acid Profile Analysis with Data Variability



Fatty acid Distribution in Algae Samples with Data Range

CONCLUSION AND RECOMMENDATION

In our research, we examined algal species from several water bodies in Malakand and studied their taxonomy, morphology, ecological interactions, and biofuel prospects. Algae were classified through light microscopy, consisting of six divisions; degree of details of the cellular structures of twelve selected species was further revealed through scanning electronic microscopy (SEM). The environmental data obtained such as ambient temperature, pH, conductivity, etc. were gathered for assessing the effects of the environment on the distribution of algae and their productivity. Integration of both morphological data and environmental data revealed that, among the identified algal species, Spirogyra was a superior candidate for biofuel capacity since it had significant lipid accumulation potential and environmental adaptability. The SEM revealed structural characteristics that may affect the lipids and the environmental parameters had a direct effect on growth and metabolism of the algae. Overall, it can be concluded that water quality management is very critical to maximize algal biomass utilization for biofuel applications.

In addition to energy, the study focused on algae's potential for carbon capture and wastewater treatment. Algae capture CO_2 through photosynthesis and use excess nitrogen and phosphorus (nutrients) from wastewater, forming a closed-loop system that is beneficial for both energy generation and environmental cleaning. Both studies explored Spirogyra's potential as biofuel feedstock and created possibilities for future research, such as the exploration of species composition, genetic modification to enhance lipid biosynthesis, or developing a fully integrated system that combines algal biofuel and wastewater treatment. This exploration embodies larger notions regarding renewable energy and sustainable environmental systems, possibly allowing algae to be a key player in the long-term transition to greener technologies.

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