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ISOLATION AND EVALUATION OF MICROALGAL SPECIES FROM LOCAL HABITATS OF KHYBER PAKHTUNKHWA FOR BIOFUEL PRODUCTION

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Abstract
Microalgae plays a key role in the pursuit of sustainable energy as a promising source of biofuel, offering potential solutions to environmental challenges and contributing to the transition towards cleaner and renewable energy. This study was carried out to explore the biofuel potential of microalgal species isolated from different ecological regions of Khyber Pakhtunkhwa. Twenty-two different microalgal species were isolated from the Peshawar, Charsadda, Nowshehra, Dir, Buner and Swat regions. Among the isolated species *Chlorella vulgaris, Oedogonium capillare, Scenedesmus dimorphus, Desmodesmus communis* and *Nanochloropsis oculata* were selected due to their high prevalence in these regions. The isolated species were grown on different growth media i.e. Bold Basal Media (BBM), Blue Green-11 (BG-11), Bristol and Guillard's F/2 having varied concentrations (10, 20, 30, 40 and 50%) and chemically analyzed for various biochemical traits through advance biochemical techniques. Media optimization for algal biomass showed that microalgal species grown on BBM had maximum biomass (51.77g), while minimum biomass (44.76g) was attained on BG media. Among the selected microalgal species, *Chlorella vulgaris* produced the maximum biomass (50.95g) however least biomass (44.81g) was produced by *Scenedesmus dimorphus*. In the case of media concentrations, maximum biomass (60.30g) was attained on 50% media, while lower biomass (32.98g) was attained on 10% media. The Physico-chemical properties of the selected species depicted that *Nanochloropsis oculata* from Buner area had a higher moisture content (30.3%) while *Oedogonium capillare* from the same area was high in ash content (27.5%), *Chlorella vulgaris* from Charsadda have high crude protein content (31%) while *Nanochloropsis oculata* from Swat had higher crude fiber (27.04%), *Scenedesmus dimorphus* from Dir have higher crude fat (17.8%) and the same species from Nowshehra revealed maximum NFE content (71.3%). The GC-MS analysis of selected microalgal species shows that these species contained appreciable amount of saturated and unsaturated fatty acids including Hexadecanoic acid (6.52%), Heptadecanoic acid (0.11%), Octadecanoic acid (41.87%), Docosanoic acid (0.72%), Tetracosanoic acid (1.01%) and 9, 12-Octadecadienoic acid (0.01%). Among the species, the highest biofuels were produced from the crude fat of *Oedogonium capillare* and *Scenedesmus dimorphus* therefore these species are recommended for commercial biofuel production.

Keywords: Algae, Isolation, GCMS, Biofuel, KPK

Introduction

The energy crises arose from diminishing unsustainable resources such as fossil fuels, which concurrently contribute to greenhouse gases emission. Renewable energy sources are pivotal in decreasing these emissions (Mishra *et al*., 2019). Biofuel, due to its biodegradable nature and low toxicity, has garnered significant attention among researchers (Menezes *et al*., 2016). However, for biofuel to be sustainable, the development of alternative non-feed biomass sources is imperative. Additionally, algal species must exhibit high biomass production and lipid accumulation for effective biofuel production; not all microalgae species meet these criteria (Menezes *et al*., 2016). Biodiesel from microalgae presents a sustainable alternative to fossil fuels and can be obtained through the transesterification process (Daneshvar *et al*., 2018). Algae, a category within natural resources, has garnered significant attention due to its photosynthetic nature and diverse forms, spanning from microscopic unicellular (microalgae) to multicellular (macroalgae) structures. Microalgae, particularly renowned for their heightened photosynthetic efficiency compared to plants, excel in biomass and oil production. Remarkably, depending on the specific species, microalgae can generate oil at rates 10 to 100 times greater than terrestrial plants. Like many freshwater and marine organisms, algae display adaptability to adverse environmental conditions through the synthesis of various secondary metabolites, a rarity among other life forms. The diverse fatty acid composition within microalgae contributes to the production of biodiesel with distinct characteristics (Pratoomyot *et al*., 2015).

Microalgae demonstrate rapid biomass production compared to other macroalgae, with reported doubling times as short as 6 hours for some species (Collet *et al*., 2011). Nearly all algae can accumulate energy-rich lipids, and several microalgae species naturally accumulate high oil content. For instance, certain *Botryococcus* species were found to store up to 50% of long-chain hydrocarbons in their dry mass (Metzger and Largeau, 2005). Research indicates that these microalgae species possess broader ancestral relationships compared to any known potential biofuel crops (Deschamps and Moreira, 2009). Algae represent one of the promising carbon sources present in both fossil-derived crude oil and natural gas. Microalgae are considered a promising source for biofuel production due to their superior photosynthetic efficiency, greater biomass yield, and faster growth compared to other energy crops (Dong *et al*., 2013). With their high oil content, microalgal cells are well-suited for use as a material source in biodiesel production. Cultivating the unicellular species *Chlorella* has been identified as having the potential to yield high biomass and substantial lipid content within the cell, as outlined by (Xu *et al*., 2006).

In recent research advancements, efforts have been made to pinpoint various types of algae for high-quality biomass and potent biofuel with elevated energy yields, aiming to supplant traditional fossil fuels (Miao *et al*., 2004). Limited initiatives have focused on generating biofuel from non-edible sources like Jatropha and Mahua tree oils (Alcantara *et al*., 2000). However, the substantial expense of large-scale biofuel production remains a significant concern due to the high cost of feed in vegetable oils (Lang *et al*., 2001). Microalgae have emerged as a promising and advantageous biofuel source compared to other energy-yielding crops due to their efficiencies, including superior photosynthetic performance, heightened biomass generation and accelerated growth rates (Huang *et al*., 2010).

Microalgae species are being explored for their substantial capacity to generate oil for biodiesel more efficiently than traditional energy crops in terms of land use (Hu *et al*., 2008). The substantial oil production of these microalgae is highly recommended for the production of biofuel as a sustainable energy source. Additionally, microalgae contribute to the production of diverse lipid types, including hydrocarbons and other intricate oils (Lin *et al*., 2011).

The primary objective of this research was to assess the potential of microalgal species collected from different ecological regions of Khyber Pakhtunkhwa for biomass and biofuel production on different growth media having different concentrations. The study aimed to Identify and isolate microalgal species

from selected areas of Khyber Pakhtunkhwa, and to optimize their growth and to analyze their biochemical composition to characterize for biofuel production.

Materials and Methods

Description of the study area

Samples of microalgae were collected from Peshawar, Charsadda, Nowshehra, Dir, Buner and Swat ecological regions of Khyber Pakhtunkhwa, Pakistan.

Collection of Samples

Microalgal samples were collected from diverse water bodies, comprising ponds, lakes, and even open-environment water tanks. Clean and dry, medium-sized plastic bottles were used for sample collection, which were subsequently transported to the Biofuel Research Laboratory (BRL) at the Department of Agricultural Chemistry & Biochemistry for further analysis. Upon arrival at the lab, the samples were meticulously washed with sterilized water and transferred to large bottles containing a suitable growth medium.

Isolation of microalgae

Upon arrival at the laboratory, the samples were allowed to settle undisturbed. As the mud settled, diatoms floated to the surface, facilitating their collection. The bottles were then illuminated with a flashlight. Light-loving microalgae were selectively collected using a pipette from the illuminated area, while shade-preferring species were collected from the darker regions of the bottles. Serial dilution, as described by Aneja (2005), was then performed to obtain suitable microalgal concentrations for identification and isolation. A series of five 250 ml conical flasks were filled with 100 ml of distilled water each. A 5 ml aliquot of the microalgal suspension was transferred to the first flask and diluted to 250 ml. Subsequent dilutions were prepared by transferring 5 ml aliquots from the previous flask to subsequent flasks, resulting in a five-fold dilution series. Samples from the final flask, with the highest dilution factor, were used for further processing.

Morphological Investigations

Microalgal species were identified using a light microscope equipped with a high-resolution camera and display screen, following the methodology described by Alam *et al*. (2019). Individual specimens were prepared on glass slides by adding a drop of glycerin to slow motility and facilitate identification. Subsequently, a coverslip was carefully placed to seal the preparation. Microscopic examination began at low magnification to provide an overview, followed by gradual increases to capture detailed morphological features. Physical differences observed under the microscope served as preliminary indicators of distinct species. Based on these observations, suspected unique species were carefully isolated using sterilized forceps and needles.

Growth media

Solutions of Bold Basal Medium (BBM), Blue Green (BG-11), Bristol medium, and F/2 were prepared at five different concentrations (10%, 20%, 30%, 40%, and 50%) to optimize the growth of selected microalgal species.

Proximate analysis

The proximate analysis was performed following the AOAC methods (2016). The moisture content was determined using the oven drying method, crude protein was assessed through the Kjeldahl method, crude fat was determined using the Soxhlet extraction method, and crude fiber was analyzed through acid and base digestion methods. The Subtraction formula was employed for calculating Nitrogen Free Extract as digestible carbohydrates.

Fatty acids methyl esters (FAME) analysis

Sample Preparation:

The algal biomass was thoroughly dried. A 24-hour extraction of the dried biomass was performed with n-hexane using a rotary evaporator to recover the extract.

Methylation:

NaOH, BF_3, NaCl, and methanol solutions were prepared using standard procedure. 25-40 mg of the extract was weighed and added it into a tube containing 1.5 ml NaOH and 5 ml methanol solution. The mixture was heated at 100°C for 5 minutes, then cooled. BF_3 and 2.5 ml hexane, mix thoroughly added to the mixture, and heated again at 100°C for 30 minutes. For FAMEs separation, 1 ml hexane and 5 ml saturated NaCl solution was added to the mixture, mixed well, and the upper layer was transferred to a new tube. Repeated with another 1 ml hexane. The combined hexane extracts was filtered through a 0.45 µm membrane filter.

GC-MS Analysis

A GC-MS system (QP-2010 Shimadzu Japan) equipped with a TRB-FFAP column (30 m, 0.25 mm ID, 0.32 film thickness) was used for FAM analysis. The mobile phase was to helium, neon, argon, and nitrogen (all polar). The column oven temperature was increased from 50°C to 150°C, then to 175°C, and finally reached to 220°C. A splitless injection was used for sample injection at 240°C with a sampling hold time of 1-5 minutes. A pressure of 70 kPa with a column flow rate of 1.22 mL min^{-1} achieving a linear velocity of 40.1 cm sec^{-1} was maintained. The purge flow was set to 8.0 mL min^{-1}. Mass scan was started at 85 m/z and end at 380 m/z. The analysis was run from 2.40 to 45.60 minutes.

Statistical analysis

The data obtained from the studied parameters of the selected microalgal species underwent analysis of variance suitable for Completely Randomized Design (CRD) employing a factorial arrangement through the statistical software Statistix 8.1. Parameters displaying significant F-values ($P < 0.05$) underwent a post-hoc analysis using the LSD test to identify pairwise differences among the means.

Results and Discussion

Prevalence of algal species in various areas of Khyber Pakhtunkhwa

Table 1 presents the microalgal species samples collected from diverse ecological regions within Khyber Pakhtunkhwa province, Pakistan, including Peshawar, Charsadda, Nowshehra, Dir, Buner, and Swat. These areas harbour a wide variety of microalgal species, such as *Closterium*, *Gonium*, *diatoms* (a diverse group), *Oocystis*, *Euglena*, *Coelastrum*, *Cladophora*, *Spirogyra*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Desmodesmus communis*, *Scenedesmus dimorphus*, *Oedogonium capillare*, *Tetraselmis alacris*, *Cosmarium grantum*, *Nanochloropsis oculata*, and *Isocrysis galbana*.

Notably, a high degree of commonality exists among these species across the selected ecological regions. Based on their prevalence and potential for biofuel production, we selected five microalgal species for further biochemical analysis. The prevalence of algal species in different ecological regions of Khyber Pakhtunkhwa, including Peshawar, Charsadda, Nowshehra, Dir, Buner, and Swat, was investigated. The diversity of these species was found to be variable, likely due to differences in soil or water conservation, humidity, and temperature. This selection suggests that these species may play a significant role in the ecological dynamics of the region. The relevance of water quality and physicochemical parameters on algal diversity was also highlighted in the context of the study. Similarly, Khuram *et al*., (2014) and Shah *et al*., (2018) found that water quality, including factors such as water temperature, conductivity, Total Suspended Solids (TSS), and pH, significantly influenced the stimulation and diversity of algal species. This emphasizes the importance of considering seasonal variations when studying algal prevalence and diversity.

Table 1. Prevalence (%) of algal species in various areas of Khyber Pakhtunkhwa

Districts	Tehsil	Algae Genus	Algae Specie	Prevalence %
Peshawar	Peshawar	Chlorella	*Chlorella vulgaris*	21
		Chlamydomonas	*Chlamydomonas reinhardtii*	19
		Desmodesmus	*Desmodesmus communis*	21
		Scenedesmus	*Scenedesmus dimorphus*	11
		Oedogonium	*Oedogonium capillare*	9
		Tetraselmis	*Tetraselmis alacris*	5
		Cosmarium	*Cosmarium grantum*	10
		Nanochloropsis	*Nanochloropsis oculata*	4
Charsadda	Charsadda	Chlorella	*Chlorella vulgaris*	19
		Chlamydomonas	*Chlamydomonas reinhardtii*	24
		Desmodesmus	*Desmodesmus communis*	31
		Scenedesmus	*Scenedesmus dimorphus*	12
		Navicula	*Navicula tripunctata*	6
		Isocrysis	*Isocrysis litoralis*	8
	Tangi	Scenedesmus	*Scenedesmus dimorphus*	29
		Oedogonium	*Oedogonium capillare*	9
		Tetraselmis	*Tetraselmis alacris*	3
		Nannochloropsis	*Nannochloropsis oculata*	18
		Navicula	*Navicula tripunctata*	22
		Isocrysis	*Isocrysis litoralis*	12
		Cosmarium	*Cosmarium grantum*	7
Nowshehra	Nowshehra	Chlorella	*Chlorella vulgaris*	29
		Chlamydomonas	*Chlamydomonas reinhardtii*	24
		Desmodesmus	*Desmodesmus communis*	10
		Scenedesmus	*Scenedesmus dimorphus*	13
		Oedogonium	*Oedogonium capillare*	5
		Tetraselmis	*Tetraselmis alacris*	3
		Nannochloropsis	*Nannochloropsis oculata*	11
		Cosmarium	*Cosmarium grantum*	5
Dir	Timergara	Chlorella	*Chlorella vulgaris*	28
		Chlamydomonas	*Chlamydomonas reinhardtii*	12
			Chlamydomonas elegans	8
		Desmodesmus	*Desmodesmus communis*	11
		Scenedesmus	*Scenedesmus dimorphus*	9
		Oedogonium	*Oedogonium capillare*	6
			Oedogonium ackleyae	5
			Oedogonium aculeatum	1
		Tetraselmis	*Tetraselmis alacris*	4
			Tetraselmis suecica	2
		Nannochloropsis	*Nannochloropsis oculata*	1
		Navicula	*Navicula tripunctata*	4
		Isocrysis	*Isocrysis litoralis*	2
		Cosmarium	*Cosmarium grantum*	7
	Adenzai	Chlorella	*Chlorella vulgaris*	51
		Chlamydomonas	*Chlamydomonas caudata*	24
			Chlamydomonas elegans	17
		Desmodesmus	*Desmodesmus granulatus*	8
	Daggar	Chlorella	*Chlorella vulgaris*	15
			Chlorella autotrophica	11
			Chlorella colonials	15
		Chlamydomonas	*Chlamydomonas reinhardtii*	32
			Scenedesmus dimorphus	7

Buner			*Chlamydomonas caudata*	5
		Desmodesmus	*Desmodesmus communis*	1
		Nannochloropsis	*Nannochloropsis oculata*	14
	Pir Baba	Chlamydomonas	*Chlamydomonas caudate*	31
			Chlamydomonas elegans	6
		Desmodesmus	*Desmodesmus armatus*	6
			Desmodesmus communis	9
			Desmodesmus bicellularis	5
		Scenedesmus	*Scenedesmus dimorphus*	17
		Oedogonium	*Oedogonium capillare*	9
			Oedogonium aculeatum	5
		Cosmarium	*Cosmarium grantum*	12
Swat	Barikot	Chlorella	*Chlorella autotrophica*	28
			Chlorella vulgaris	11
		Chlamydomonas	*Chlamydomonas reinhardtii*	17
			Chlamydomonas ovoidae	9
			Chlamydomonas nivalis	8
		Desmodesmus	*Desmodesmus armatus*	7
			Desmodesmus communis	2
		Scenedesmus	*Scenedesmus dimorphus*	3
		Oedogonium	*Oedogonium capillare*	8
		Tetraselmis	*Tetraselmis alacris*	1
			Tetraselmis apiculate	1
		Nannochloropsis	*Nannochloropsis oculata*	1
		Navicula	*Navicula tripunctata*	1
		Isocrysis	*Isocrysis litoralis*	3
	Matta	Chlorella	*Chlorella autotrophica*	18
			Chlorella vulgaris	14
		Chlamydomonas	*Chlamydomonas reinhardtii*	18
			Chlamydomonas elegans	10
		Desmodesmus	*Desmodesmus armatus*	11
		Scenedesmus	*Scenedesmus dimorphus*	5
		Oedogonium	*Oedogonium aculeatum*	6
		Tetraselmis	*Tetraselmis alacris*	3
		Nannochloropsis	*Nannochloropsis oculata*	2
		Navicula	*Navicula tripunctata*	2
		Cosmarium	*Cosmarium grantum*	3
		Isocrysis	*Isocrysis litoralis*	6
			Isocrysis galbana	2

Media optimization for microalgae growth

Data regarding the biomass of different microalgal species grown on different growth media under different concentrations is given in Table 2. The statistical analysis of the data shows that biomass was significantly ($P<0.05$) different among the selected algal species grown on different media under different concentrations, however, the interaction among the algal species, media and concentration was also exhibited significant ($P<0.05$) impacts on the total biomass. The comparison of mean data shows that algal species grown on Bold Basal Media (BBM) had maximum biomass (51.77g), followed by Bristol media, while minimum biomass (44.76g) was attained by algal species grown on Blue Green 11 (BG-11) media. Among the selected algal species, *Chlorella vulgaris* produced the maximum biomass (50.95g), followed by *Nannochloropsis oculata* (49.35g), however, the least biomass (44.81g) was produced by *Scenedesmus dimorphus*. In case of media concentrations, maximum biomass (60.30g) was attained on 50% media, followed by 40% media (52.86g), while lower biomass (32.98g) was attained

on 10% media. The interactive study of algal species, growth media and concentration shows that maximum biomass (82.83g) was attained in the interaction of *Chlorella vulgaris* grown on BBM under 50% concentration (Fig. 1). Among the interaction of algal species and growth media concentrations, the algal specie *Chlorella vulgaris* grown at 50% growth media had maximum biomass (68.83 g) (Fig. 2). In case of media and concentration interaction, the algal species exerted higher biomass (65.20 g) on BBM under 50% concentration (Fig. 3). Among the interaction of growth media and algal species, the *Chlorella vulgaris* grown on BBM produced higher biomass (61.06 g) respectively (Fig. 4). Our results are in agreement with Rushan *et al*., (2021) who used different culture media with varying chemical compositions for microalgae cultivation. The experiment was carried out under constant conditions. The microalgal species showed various patterns of cell growth throughout the cycle. Their results show the growth performance of microalgal species during cultivation.

Table 2. Means of microalgal species biomass as influenced by different growth media under different concentrations

Media types	**Algal Sp.**	**Media concentrations (%)**					**Means**
		10	**20**	**30**	**40**	**50**	
Bold Basal Media	*Chlorella*	42.0	52.0	64.7	64.3	82.3	
	Scenedesmus	37.3	43.7	49.3	53.7	59.7	
	Desmodesmus	39.3	45.0	50.0	55.0	62.3	
	Oedogonium	38.3	47.0	51.7	56.7	64.0	
	Nannochloropsis	28.3	45.0	50.0	55.0	57.7	
BG-11	*Chlorella*	37.3	42.3	44.3	49.3	65.3	
	Scenedesmus	28.7	38.7	43.7	48.7	60.0	
	Desmodesmus	27.7	37.7	42.7	47.7	62.0	
	Oedogonium	29.3	39.3	44.3	49.3	61.7	
	Nannochloropsis	26.3	39.7	44.7	49.7	58.7	
Bristol Media	*Chlorella*	36.3	44.7	49.7	54.7	66.7	
	Scenedesmus	29.7	43.3	48.3	53.3	55.3	
	Desmodesmus	31.3	45.3	50.3	55.3	54.3	
	Oedogonium	32.7	44.3	49.3	54.3	56.0	
	Nannochloropsis	31.0	46.3	51.3	56.3	52.0	
F/2 Media	*Chlorella*	33.3	37.7	43.0	48.0	61.0	
	Scenedesmus	27.7	36.7	39.7	47.0	52.0	
	Desmodesmus	28.0	36.3	38.3	45.0	49.0	
	Oedogonium	30.3	41.7	44.3	49.3	54.7	
	Nannochloropsis	44.7	54.7	59.7	64.7	71.3	
Means		33.0 e	43.1 d	48.0 c	52.9 b	60.3 a	
Microalgal species							
Chlorella		37.3	41.6	50.4	54.1	68.8	
Scenedesmus		30.8	39.6	45.3	50.7	56.8	
Desmodesmus		31.6	39.8	45.3	50.8	56.9	
Oedogonium		32.7	41.8	47.4	52.4	59.1	
Nannochloropsis		32.6	46.9	51.4	56.4	59.9	
Media × Concentrations							
Bold Basal Media		37.1	46.5	53.1	56.9	65.2	
BG-11		29.9	39.5	39.5	48.9	61.5	
Bristol Media		32.2	44.8	49.8	54.8	56.9	
F/2 Media		32.8	41.4	45.0	50.8	57.6	
Media × Algae		**CV**	**SD**	**DC**	**OC**	**NO**	**Media**
Bold Basal Media		61.1	48.7	50.3	51.5	47.2	51.8 a
BG-11		47.7	43.9	43.5	44.8	43.8	44.8 b
Bristol Media		50.4	46.0	47.3	47.3	47.4	47.7 c
F/2 Media		44.6	40.6	39.3	44.1	59.0	45.5 c
Means		51.0 a	44.8 d	45.1 d	46.9 c	49.4 b	

CV: *Chlorella Vulgaris*, SD: *Scenedesmus dimorphus*, DC: *Desmodesmus communis* OC: *Oedogonium Capillare*, NO: *Nannochloropsis oculata*

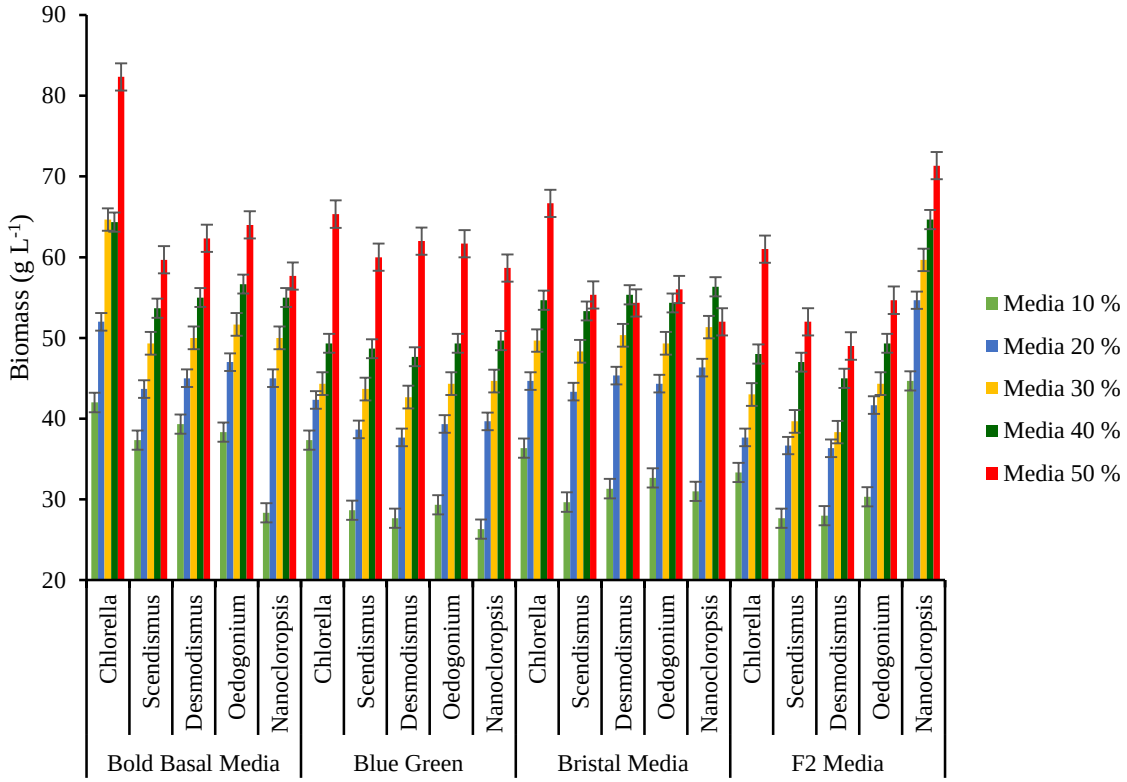

Fig. 1. The interactive effects of microalgal species, growth media and concentrations on total biomass

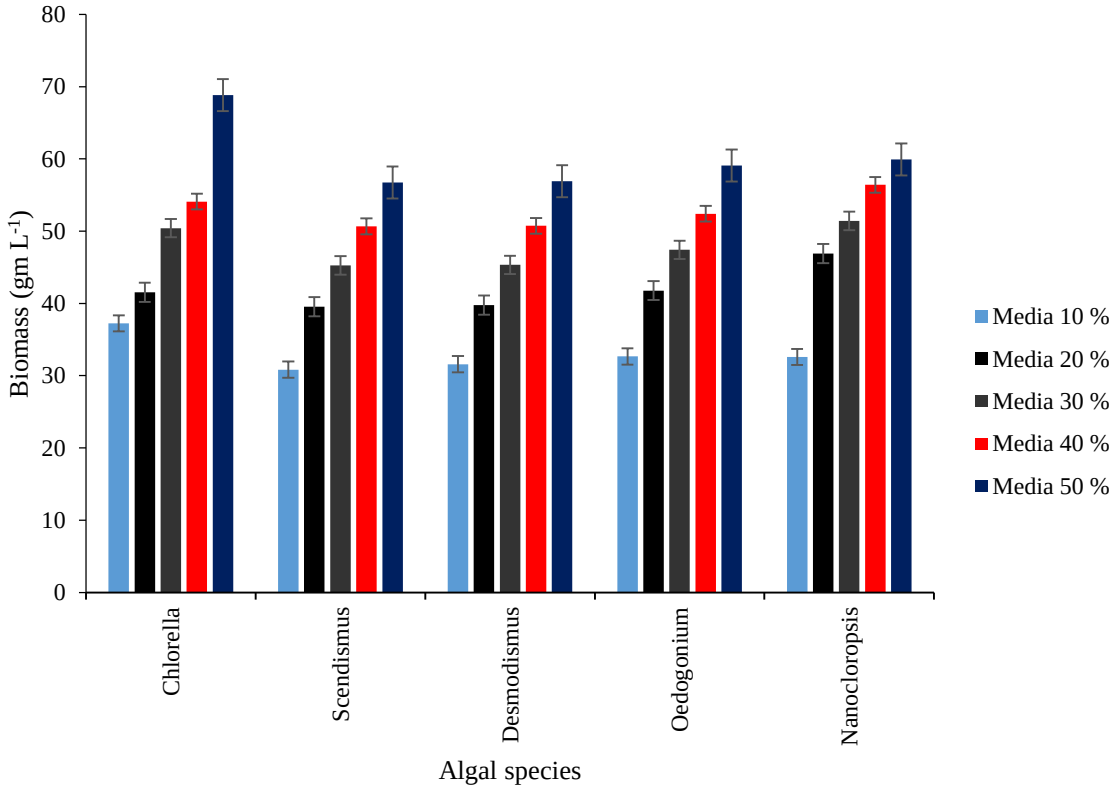

Fig. 2. The interactive effects of microalgal species and concentrations on total biomass

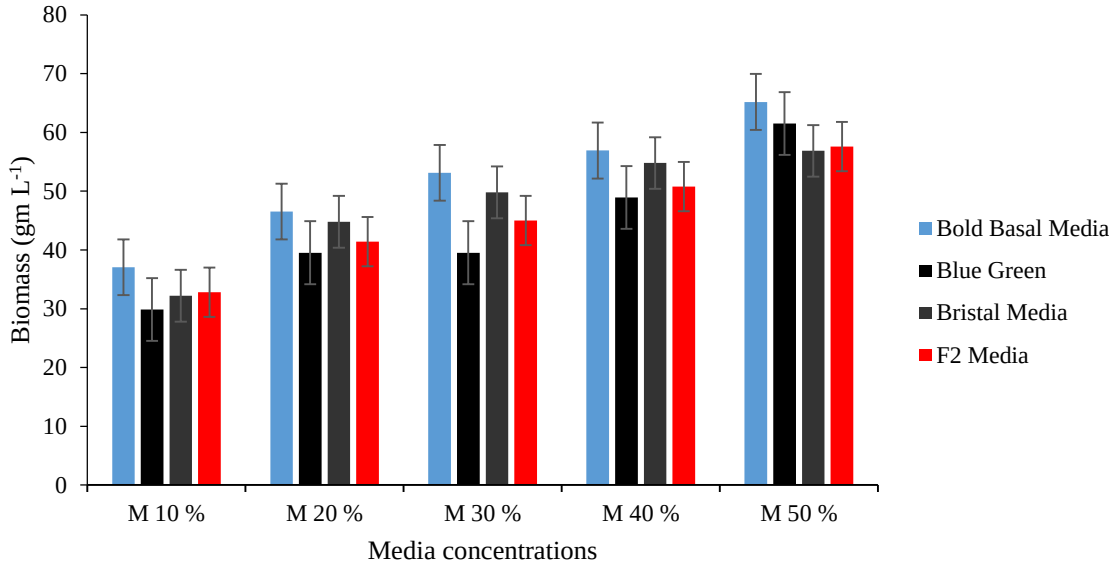

Fig.3. The interactive effects of microalgal species and concentrations on total biomass

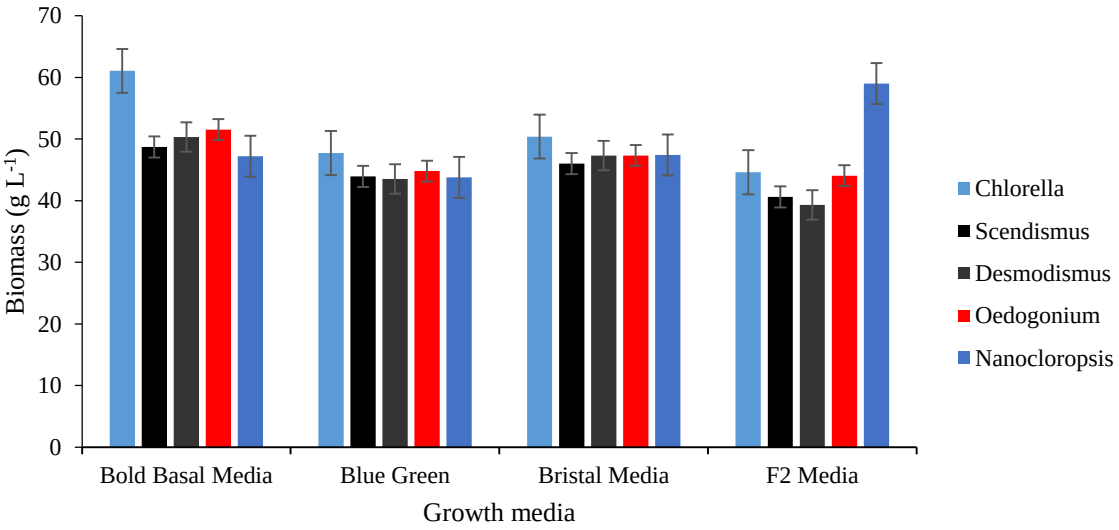

Fig. 4. The interactive effects of microalgal species and different growth media on total biomass

Proximate Analysis

Moisture content (%) of different algal species commonly found in the selected areas of Khyber Pakhtunkhwa is presented in Figure 5. The statistical analysis of the data exhibited that moisture content was significantly varied among the algal species isolated from the different regions of Khyber Pakhtunkhwa. A mean comparison of the data showed that *D. Communis* exhibited higher moisture content (18.08%) among the species, while *O. Capillare* showed minimum moisture content (8.32%). In case of ecological regions, the species isolated from Dir had moisture content (18.93%) while the species collected from Charsadda had lower moisture content (10.11%). The interactive analysis depicted that *Nanochloropsis oculata* collected from Buner showed maximum content of moisture (30.26%). Jiang *et al*. (2012) reported that algal biomass was typically high moisture content while working with algae grown in high saline marine water or brackish environments. Han *et al*., (2014) used different methods for the determination of moisture content of algal species and reported moisture content (39.4 to 96.2 %) in various algal species where the data was slightly high as compared to the present study, the reason was that the present data was recorded after sun dried biomass for 5 days.

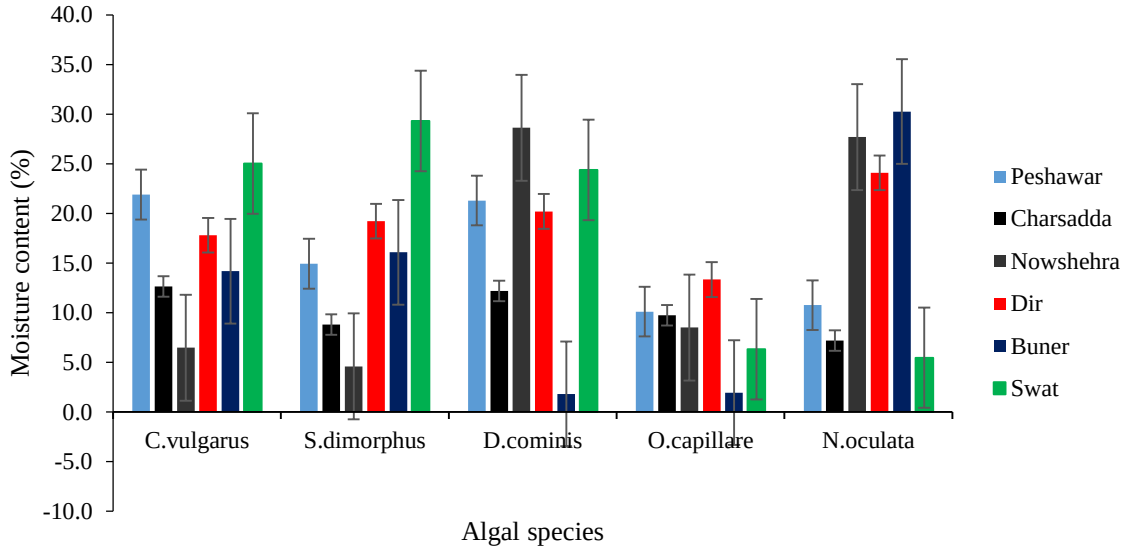

Fig. 5. Moisture content of microalgal species collected from various areas of Khyber Pakhtunkhwa

Ash content

Ash content (%) of different algal species commonly found in the selected areas of Khyber Pakhtunkhwa is presented in Figure 6. Statistical analysis of ash data showed that there was a significant difference in ash content according to area and species and also species and area interaction ($P \leq 0.05$). The data revealed that among all the selected species *Oedogonium capillare* contains the maximum amount of ash (20%), while *D. communis* had the lowest ash content (12.29%). Similarly, the algae species collected from the district Swat showed the maximum content of ash (20.57%) while the species isolated from Nowshehra exhibited lower ash content (10.84%). In the interaction of species and area, the specie *Oedogonium capillare* collected from the swat area has shown the highest amount of ash content (27.50%). Ash represents the minerals matter of algae biomass high ash content is an indication of high mineral content (Milledge *et al*., 2019). Minerals are also responsible for various physiological functions in living organisms the species showing high mineral would be suitable for a few formulations. Fuentes (2000) worked on the ash content of algae species and reported high ash content. Liu et al. (2020) conducted a study on the ash content of some algal species and reported ash content in the range of 1.9 to 37.4 % which was in good agreement with the present data.

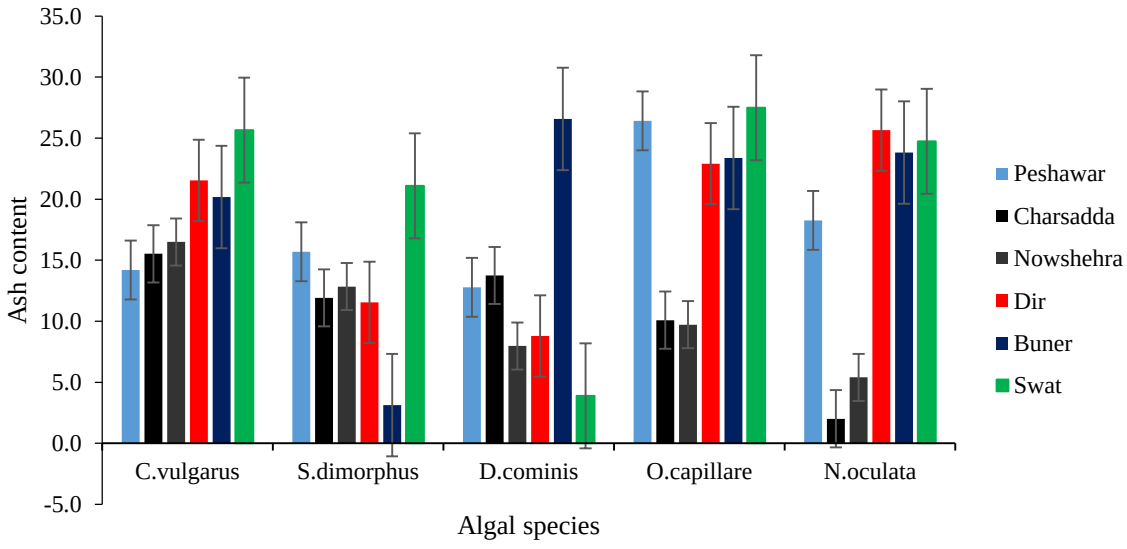

Fig. 6. Ash content of microalgal species collected from various areas of Khyber Pakhtunkhwa

Crude protein

The crude protein content (%) of different algae species commonly found in the selected areas of Khyber Pakhtunkhwa is presented in Figure 7. Statistical analysis of crude protein data showed that CP content was significantly different among the species and regions and their interaction was also significant ($P \leq 0.05$). The mean data comparison showed that *Chlorella vulgaris* contains a maximum amount of crude protein ranging from (16.46%), while lower CP content was exhibited by N. oculata (9%). Among the algal species collected from Peshawar had maximum CP content (32.35%), while the species collected from Buner had minimum CP (5.30%). Interaction data analysis of the species and area shows that *Chlorella vulgaris* collected from Charsadda contained the highest crude protein (31%). The CP amount reported by them favours the present data. They reported 0.38 % crude protein same as detected in the present study. They found that the variation in the CP is due to differences in the areas and may be due to the composition of soil and water resources. These variations in CP can be successfully targeted for a large variety of food, feed and pharmaceutical applications. Crude proteins found in microalgae are increasingly being consumed for functional, nutritional and health purposes.

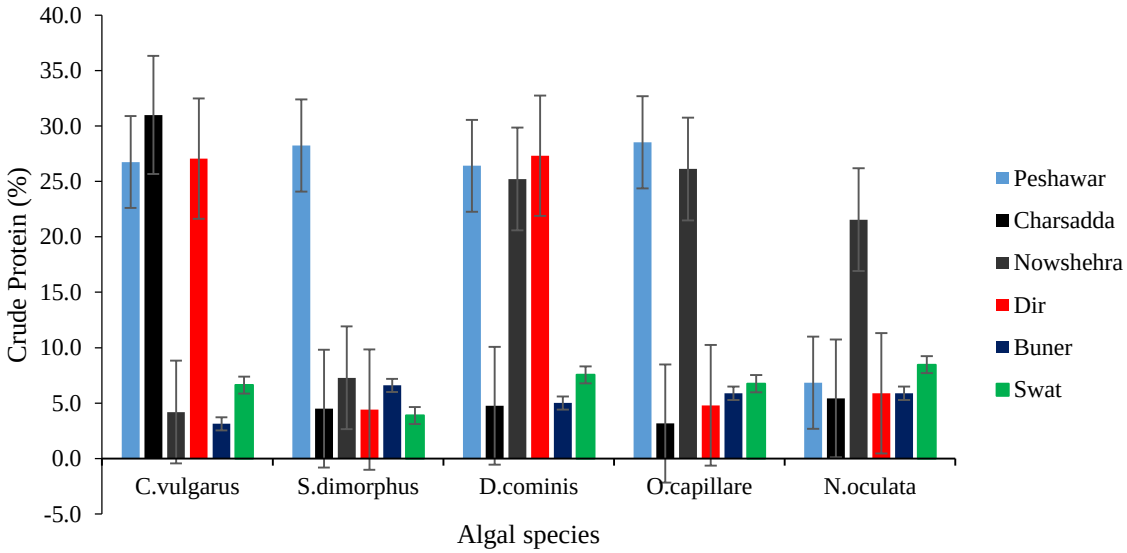

Fig. 7. Crude protein content of microalgal species collected from various areas of Khyber Pakhtunkhwa

Crude fiber

Crude fiber content (%) of selected algal species commonly found in different areas of Khyber Pakhtunkhwa is presented in Figure 8. Statistical analysis of crude fiber data depicted that crude fiber was significantly varied among the species and ecological regions and their interaction was also significant ($P \leq 0.05$). The mean data comparison showed that *Nanochloropsis oculata* contains a maximum amount of crude fiber (15.18%), while *Oedogonium capillare* had lower crude fiber (10.53%). Similarly, the microalgal species collected from Swat showed maximum content of crude fiber (17.69%), while minimum crude fiber (8.2%) was noted in species isolated from Charsadda. The interaction of species and area revealed that *Nanochloropsis oculata* isolated from Swat showed maximum crude fiber content (27.04%). Crude fiber is an important nutritional component because the dietary fibers bind the toxic compounds and eliminate them from the body and the fiber also provides bulk to the food during digestion. Misurcova *et al*., (2010) conducted a study on the crude fiber contents in different algal species which ranged from 0.18 to 12.55 % of dry biomass which supports the present study. Misurcova *et al*., (2010) reported that red and brown algae showed higher amounts of dietary fiber than freshwater algae which means that the crude fiber also depends on variability of the species.

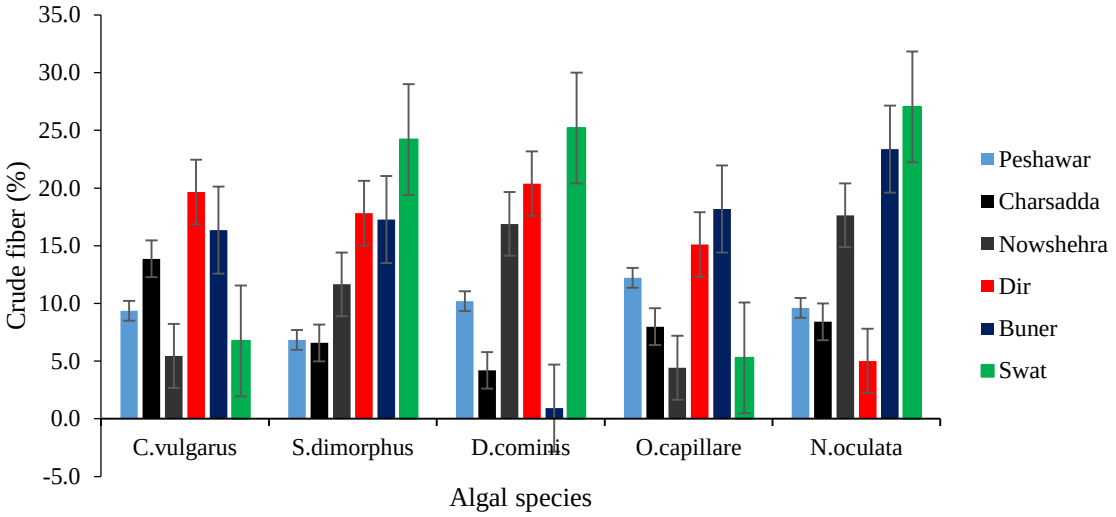

Fig. 8. Crude fiber content of microalgal species collected from various areas of Khyber Pakhtunkhwa

Crude fat

Data on crude fat (%) of algal species collected from selected areas of Khyber Pakhtunkhwa is displayed in Figure 9. Statistical analysis of crude fat data showed significant differences among the algal species and ecological regions and their interaction was also significant ($P \leq 0.05$). The mean data of algal species shows that *Scenedesmus dimorphus* contains the maximum amount of crude fats (7.51%), while lower crude fat was exhibited by *Desmodesmus communis*. Among the regions, the algal species collected from Dir area showed a maximum content of crude fats (17.82%). The interaction of species and area shows that *Scenedesmus dimorphus* collected from Dir showed the highest crude fat. Kris-Etherton *et al*., (2002) reviewed that crude fat represents mainly fatty acid content which is very crucial for growth and sometimes for energy production in the living organism. Adams (2011) worked on the crude fat content of algae species and reported crude fat content that ranged from 0.5 % to 31.9 % and the data was in close agreement with the present study. Mata *et al*., (2010) also conducted a study on the lipid contents in various algal species which ranged from 20-50 % based on dry biomass which was slightly higher than the present study which might be due to differences in species or the variation effect of the area where variation in locations also affect the composition of algal species. The crude fat content of algal species is being tried for use in biofuel production (Krohn *et al*., 2011).

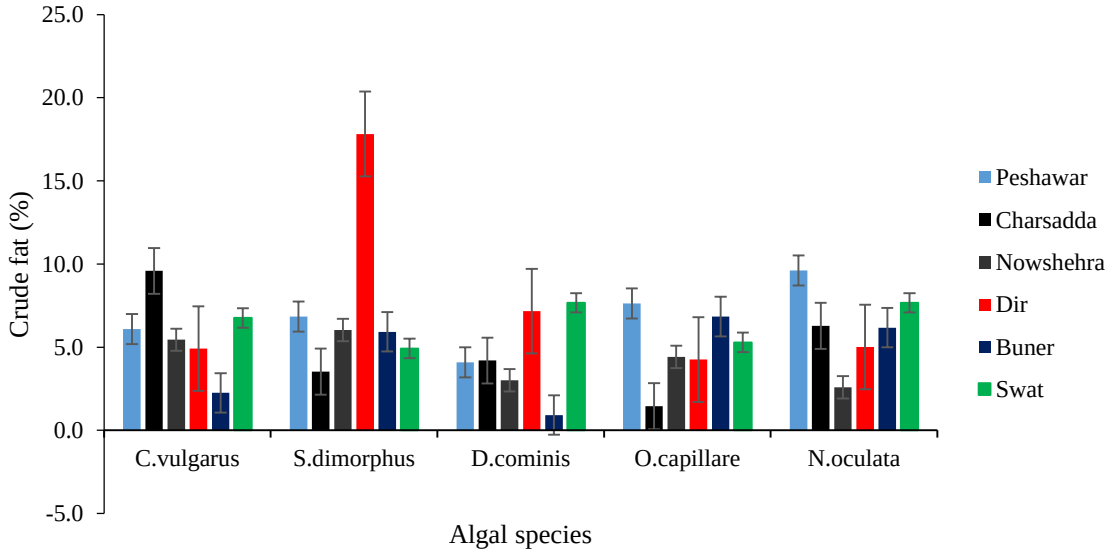

Fig. 9. Crude fat content of microalgal species collected from various areas of Khyber Pakhtunkhwa

Nitrogen Free Extract

NFE content (%) of microalgae species isolated from different ecological regions of Khyber Pakhtunkhwa is visualized in Figure 10. Statistical analysis of the data showed that NFE was significantly varied among the isolated species and selected regions of Khyber Pakhtunkhwa and their interaction was also found significant ($P \leq 0.05$). A comparison of the mean data shows that *Scenedesmus dimorphus* contains maximum NFE content (51.42%), however, lower NFE content was exhibited by *Chlorella vulgaris.* Among the locations, algal species collected from the Charsadda exhibited higher NFE (71.30%), while lower NFE was determined in species isolated from Swat. Interaction among the species and ecological regions shows that *Scenedesmus dimorphus* collected from Nowshehra had the highest NFE. The findings from our study are in agreement with Chew *et al*., (2018) who reported that the carbohydrate production ability varies among the microalgal species and can be altered by using different cultivation conditions. Furthermore, the production of carbohydrates from microalgae biomass is also considered a value-added product as it can be used as a feedstock for energy and biofuel production such as bioethanol, biohydrogen and biogas production.

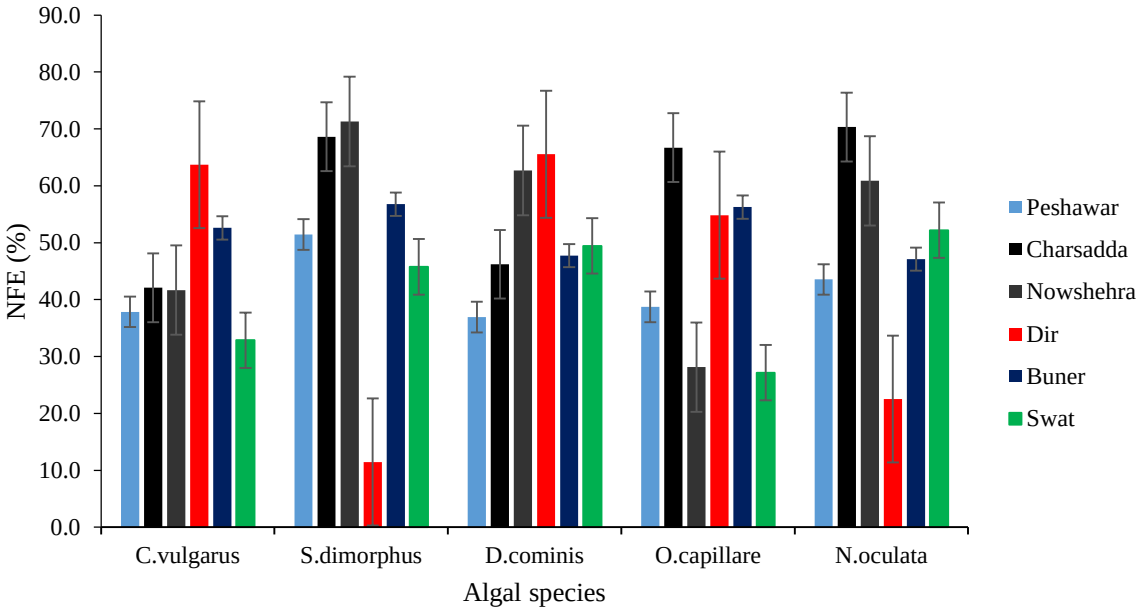

Fig. 10. NFE content of microalgal species collected from various areas of Khyber Pakhtunkhwa

GAS Chromatography-Mass Spectroscopy (GC-MS)

The results determined by GC-MS analysis revealed a number of different compounds, majority of the detected compounds were fatty acids. These fatty acids were classified into Saturated fatty acids and Unsaturated fatty acids. The microalgae specie *Chlorella vulgaris* (Table 3) showed the presence of various fatty acids including, Hexadecanoic acid ($C_{16}H_{32}O_2$), Heptadecanoic acid ($C_{17}H_{34}O_2$), Octadecanoic acid ($C_{18}H_{36}O_2$), Docosanoic acid ($C_{22}H_{44}O_2$), Tetracosanoic acid ($C_{24}H_{48}O_2$) and 9, 12-Octadecadienoic acid ($C_{18}H_{32}O_2$). Totally six different fatty acids were detected with different retention time peaks in the chromatogram. 9, 12-Octadecadienoic acid has the highest retention time of 27.99 minutes with an area of 0.01% and has wide applications in the biofuel industry whereas Hexadecanoic acid has the least retention time of 18.48 minutes with an area of 6.52%. Similarly, three fatty acids were detected in *Oedogonium capillare* as presented in the table. The highest retention time of 19.13 minutes with an area of 0.11% was recorded for Eicosanoic acid followed by 9-Octadecenoic acid of 17.53 minutes with an area of 3.31% and Hexadecanoic acid had 15.90 minutes retention time with an area of 0.63%. The specie *Scenedesmus dimorphus* showed four different types of fatty acids with different retention times and percent area composition. The highest retention time of 17.53 minutes with an area of 1.87% was recorded for 9-octadecenoic acid followed by Hexadecanoic acid having 15.90 minutes with an area of 0.54%, whereas the least retention time of 10.58 minutes with an area of 0.01% was

recorded for 9-Hydroxy-decanoic acid and Nonanoic acid respectively. The table represents four fatty acids detected in *Desmodesmus communis* as well. The highest retention time of 18.27 minutes with an area of 0.99% was recorded for 9-octadecenoic acid (Z) and 9-octadecenoic acid (E) while the least retention time of 14.91 minutes with an area of 0.23% was recorded for Hexadecanoic acid. The specie *Nannochloropsis oculata* revealed the presence of four different fatty acids. The highest retention time of 17.74 minutes with an area of 2.96% was recorded for Octadecenoic acid followed by 9-Octadecenoic acid (Z) having a 16.52 minutes retention time with an area of 1.48% and Hexadecanoic acid having 15.89 minutes with an area of 6.06% while least retention time of 11.63 minutes with an area of 0.05% was recorded for Dodecanoic acid. Similar work on the fatty acid profile of microalgal species was also carried out by Pantami *et al*., (2020) suggesting that microalgal species are good candidates for biodiesel production. Same as Bhuyar *et al*., (2019) reported that the typical analysis of fatty acids must be done by analyzing methyl esters of fatty acids by GC-MS. The fatty acid must be converted to methyl esters (FAME) to be volatile enough for GC-MS analysis. The results show that microalgal species have a high profile of fatty acids and are one of the primary metabolites of microalgae, which are enriched in both forms of food and biofuels.

Table 3. Fatty acid methyl esters (FAME) profile and GC-MS outcomes of different microalgal species

No.	Name of compound	*Chlorella vulgaris*		*Oedogonium capillare*		*Scenedesmus dimorphus*		*Desmodesmus communis*		*Nanochloropsis aculata*	
		RT	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**
1	Hexadecanoic acid	18.48	6.52	15.90	0.63	15.90	0.54	12.69	0.23	15.89	6.06
2	Heptadecanoic acid	19.13	0.11	--	--	--	--	--	--		
3	Octadecanoic acid	20.31	41.87	--	--	--	--	--	--	17.74	2.96
4	Docosanoic acid	21.25	0.72	--	--	--	--	--	--	--	--
5	Tetracosanoic acid	23.24	1.01	--	--	--	--	--	--	--	--
6	9,12-Octadecadienoic acid	27.99	0.01	17.53	3.31	17.53	1.87	--	--	--	--
7	Eicosanoic acid	--	--	21.22	0.11			--	--	--	--
8	Decanoic acid	--	--	--	--	9.08	0.01	--	--	--	--
9	Nonanoic acid	--	--	--	--	10.89	0.01	--	--	--	--
10	8-Octadecadienoic acid	--	--	--	--	--	--	16.52	0.99	--	--
11	9-Octadecadienoic acid (E)	--	--	--	--	--	--	16.54	0.99	--	--
12	9-Octadecadienoic acid (Z)	--	--	--	--	--	--	18.27	0.99	16.52	1.48
13	Dodecanoic acid	--	--	--	--	--	--	--	--	12.43	0.05

Conclusion

Diverse microalgal species were isolated from various ecological zones in Khyber Pakhtunkhwa, Pakistan, with potential for biofuel production. Five species - *Chlorella vulgaris*, *Oedogonium capillare*, *Scenedesmus dimorphus*, *Desmodesmus communis*, and *Nanochloropsis oculata* exhibited significant variation in biochemical composition (minerals, protein, crud fat, carbohydrates and fiber) following growth on different media having different concentrations. Highest biomass production was observed for *Chlorella vulgaris* grown on 50% BBM. Species from Charsadda showed higher protein, Nowshehra displayed higher carbohydrates and Dir species contained richer crude fat. GC-MS analysis revealed the presence of diverse saturated and unsaturated fatty acids, including palmitic, stearic, linoleic, oleic and elaidic acids. *Oedogonium capillare* and *Scenedesmus dimorphus* yielded the highest FAMEs from crude fats.

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