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Prospects for Biodiesel Production from Emerging Algal Resource: Process Optimization and Characterization of Biodiesel Properties

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Abstract: The present work focuses on the optimization of the energy conversion process and the use of algal resources for biodiesel production with ultrasound and microwave techniques in *Oedogonium*, *Oscillatoria*, *Ulothrix*, *Chlorella*, *Cladophora*, and *Spirogyra* for the first time. The fuel properties are investigated to optimize the efficiency of the newly emerging algal energy feedstock. The study indicates that the optimized microwave technique improves the lipid extraction efficiency in *Oedogonium*, *Oscillatoria*, *Ulothrix*, *Chlorella*, *Cladophora*, and *Spirogyra* (38.5, 34, 55, 48, 40, and 33%, respectively). Moreover, the ultrasonic technique was also effective in extracting more lipids from *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella*, *Cladophora* sp., and *Spirogyra* sp. (32, 21, 51, 40, and 36%, respectively) than from controls, using an ultra-sonication power of 80 kHz with an 8-min extraction time. The fatty acid composition, especially the contents of C16:0 and C18:1, were also enhanced after the microwave and sonication pretreatments in algal species. Enhancement of the lipids extracted from algal species improved the cetane number, high heating value, cold filter plugging point, and oxidative stability as compared to controls. Our results indicate that the conversion of biofuels from algae could be increased by the ultrasound and microwave techniques, to develop an eco-green and sustainable environment.

Keywords: microalgae; pretreatment; ultra-sonication; microwave extraction; lipids; biodiesel

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1. Introduction

Many countries are expected to undergo rapid urbanization over the next 25 years, which will influence their food, water, and land demands [1]. Only 2.8% of available water is suitable for human consumption [2], whereas, the remaining 97.2% is in the oceans and is too salty to use. Due to high salinity levels, approximately 1.5 million hectares of land are unfertile now for plant cultivation. These global megatrends of climate change and the growing production of energy from edible crops have created a significant food crisis [3].

The contribution of renewable energy is predicted to increase up to 63% by 2050. Biodiesels are renewable, highly biodegradable, and non-toxic, and thus are considered as favorable alternative fuels [4]. Edible crops such as coconut, soybean oil palm, and

sun-flower, and non-edible feedstocks such as *Jatropha*, *Miscanthus*, rubber seed, waste frying oil, and *Pongamia* can be used for biodiesel production [5]. Compared to these traditional feedstocks, microalgae are considered as an alternative feedstock for third generation biofuels [6]. They have high photosynthetic capacities, able to trap 1.83 kg of CO_2 per kg of biomass [7] and convert it into glucose and oxygen by photosynthesis. As compared to terrestrial crops, algae can produce 30–100 times more energy/hectare, with a potential biodiesel yield of 12,000 L/h [8]. Intracellular lipids are the main metabolites of microalgae which can be used as a feedstock for biodiesel production. Some microalgae contain up to 70% lipids, compared to 20–40% for typical oilseeds. The extracted lipids are converted into biodiesel by trans-esterification. Lipid extraction is the major step in biodiesel production [9], but it is subject to many challenges related to energy conversion and conservation and the optimal use of energy resources due to the structure of the algal cell wall. This is composed of hemi-cellulose and cellulose, together with glycoproteins, that possess high mechanical and chemical resistances that hamper the release of intracellular lipids [10,11]. The oil extraction methods for energy conversion from oil-bearing seeds are quite different to those required for algae, and hence, a fundamental understanding of lipid enhancement needs to be established to develop efficient and cost-effective strategies for oil extraction.

Lipid conversion to biodiesel from algal feedstocks depends upon an effective biomass disruption method to improve oil recovery and optimization of the energy processes [12]. Chemical-mediated extraction can cause bio-toxicity, lipid degradation, and device corrosion. A large quantity of energy is consumed by thermal disruption, while enzymatic degradation is very expensive. Mechanical methods (microwave, sonication, and hydrodynamic cavitation) are eco-friendly and non-toxic to the environment, and can disrupt the algal cells efficiently. Microwave assisted solvent extraction has shown significantly higher lipid recovery than solvent extraction alone [13]. Microwave assistance increases the lipid recovery from all solvent extraction methods (Hara and Radin's [14], Folch's [15], Chen's [16], and Bligh and Dyer's [17]). Direct lipid extraction is time consuming, whereas MAE takes a few minutes and uses ten times less solvent. In ultrasound-assisted extraction (UAE), the sample's cells are ruptured by using ultrasound of frequency > 20 kHz in the culture medium, which generates repetitive regions of high pressure (compressions) and low pressure (rarefactions) [18].

The conversion of the extracted lipids to biofuels is affected by temperature, pressure, and extraction time. To optimize the extraction process for energy conversion, a good understanding of the complex relationship between the factors affecting lipid extraction is crucial [19]. Response surface methodology (RSM) is a powerful statistical technique for simultaneous consideration of independent variables and their interactions that affect an objective function [20]. Response Surface Methodology (RSM) based on Central Composite Design (CCD) has been used to design experiments and develop quadratic equation models to predict the optimum conditions for desirable responses [21]. In the current study, RSM with CCD has been applied to optimize various factors (power, heating time, and extraction time) for lipid extraction (response) from *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Spirogyra* sp., and *Cladophora* sp., using MAE and UAE pretreatments, to evaluate whether these parameters have any significant effect on the percentage of lipid extracted.

In our previous study, 24% lipid was extracted from *Oedogonium* sp., 21% from *Oscillatoria* sp., 48% from *Ulothrix* sp., 33% from *Chlorella* sp., 23% from *Cladophora* sp., and 14% from *Spirogyra* sp., without pretreatment [22]. In the current study it was demonstrated that the extraction of microalga lipids, and their conversion to biodiesel, were significantly higher following pretreatment using MAE and UAE. No study has been attempted to use microwave and sonication pre-treatment techniques on *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. to enhance the extraction of lipids and their conversion to biodiesel with a high heating value, cetane number, and oxidative stability [23]. The results of the present study demonstrate that MAE and UAE

are an effective pretreatment to improve lipid recovery and fuel properties from algae, as compared with a previous study with a relatively short extraction time, and are essential for maintaining a good quality of lipid conversion to biofuel production [22,24]. Therefore, it is important to characterize algal resources for bioenergy productions by using their fatty acid profile and fuel values and their extraction methods. Therefore, the objectives of the study include: (1) Modelling and optimization of microwave and sonication pre-treatment parameters using the response surface method; (2) Evaluation of the effects of pretreatment on the fatty acid composition and on the fuel properties of algal biomass.

2. Materials and Methods

2.1. Algae Collections

Oedogonium sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. were collected at different localities of Lahore, Pakistan. Supplementary Table S1 describes the algal characteristics. All these six algal strains were selected on the basis of abundance, high lipid contents, growth rate, adaptation to the environmental conditions, and high biomass productivity [24].

2.2. Molecular Identification

Further species of algae were collected from different areas of Punjab, Pakistan. Eight algal strains were selected on the basis of morphology and identified by matching 18SrDNA and ITS region. After DNA extraction the 18SrDNA gene was amplified and sequenced by Macrogen, then the sequences were used to draw a phylogenetic tree using the MEGA6 software (Molecular Evolutionary Genetics Analysis, Version 6, Tokyo, Japan) [25].

2.3. Cultivation and Harvesting

The algal species were cultivated in Blue Green medium with pH 7, at 25 °C, and a 16 h:8 h light dark cycle. Algal biomass was harvested by centrifugation at 1800× g for 5 min and the pellets were used for pretreatment [10]. The experimental workflow is illustrated in Figure 1.

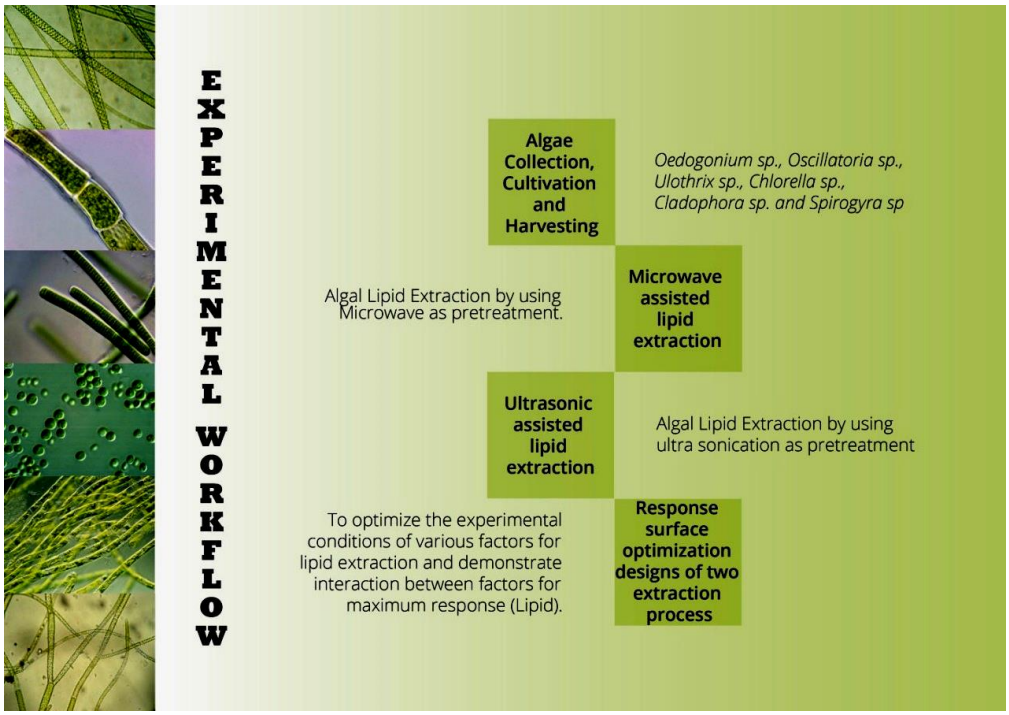


Figure 1. Experimental workflow of screening and pretreatments of algal biomass for biodiesel production.

2.4. Microwave Assisted Lipid Extraction

A weight of 0.2 g of the dried algal samples (*Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp.) was suspended in 30 mL of chloroform/methanol (2:1, *v*/*v*). The samples were treated by microwave with 180–600 watts power, 2–8 min heating, and 3–4 h extraction time. The mixture was centrifuged at 8000 rpm for 10 min, the supernatant was filtered, and then the filtrate was dried in the oven at 40 °C. The lipid contents were calculated as a percentage of the dry weight of the sample.

$$Lipid\ content\ (\%) = (weight\ of\ lipids/weight\ of\ samples) \times 100 \quad (1)$$

2.5. Ultrasonic Assisted Lipid Extraction

Dried algal sample (0.2 g) was added to 30 mL of chloroform/methanol (2:1, *v*/*v*). The sample was treated by ultrasound at 40–80 kHz with 4–8 min extraction time. The mixture was centrifuged at 8000 rpm for 10 min, the supernatant was filtered, and the filtrate was dried in the oven at 40 °C. The lipid contents, as a percentage, were calculated from Equation (1).

2.6. Response Surface Optimization Designs of Two Extraction Processes

The response surface methodology with Central Composite Design (Design Expert® software, version 11, Stat-Ease, Inc. Godward, Minneapolis, MN) was employed to optimize the lipid extraction. The experimental design consisted of power, heating time, and extraction time as design variables for MAE, and lipid extraction and power or heating time for UAE lipid extraction, while considering the lipid content as a response. Tables 1–6, detail the experimental design of the microwave assisted lipid extraction from *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. with 20 experiments. Table 7 shows the experimental design of the sonication assisted lipid extraction from *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. with 13 experiments.

Table 1. Central composite design results of the combined effect of factors of MAE in *Oedogonium* sp.

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	**A:Power**	**B:Heating Times**	**C:Extraction Time**	**Lipid Content**
		Watts	Mins	Hr	%
13	1	390	5	3.5	31
2	2	180	8	3	26
9	3	390	5	3.5	31
19	4	390	5	2.5	30
5	5	390	5	4.5	32
3	6	390	5	3.5	31
17	7	600	8	4	38.5
18	8	600	2	4	28
11	9	390	5	3.5	31
16	10	390	1	3.5	22
7	11	390	5	3.5	31
4	12	180	2	3	19
6	13	390	10	3.5	35
14	14	180	2	4	20
8	15	600	8	3	38
12	16	750	5	3.5	37
10	17	600	2	3	27.5
15	18	180	8	4	27
1	19	390	5	3.5	31
20	20	360	5	3.5	31

Table 2. Central composite design results of the combined effect of factors of MAE in *Oscillatoria* sp.

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	**A:Power**	**B:Heating Times**	**C:Extraction Time**	**Lipid Content**
		Watts	Mins	Hrs	%
7	1	180	8	4	28
3	2	180	8	3	26
16	3	390	5	3.5	29
14	4	390	5	4.5	32
9	5	180	5	3.5	24
8	6	600	8	4	32
1	7	180	2	3	17
15	8	390	5	3.5	29
11	9	390	1	3.5	16
2	10	600	2	3	31
5	11	180	2	4	20
10	12	600	5	3.5	34
20	13	390	5	3.5	29
12	14	390	10	3.5	33.5
19	15	390	5	3.5	29
4	16	600	8	3	32
13	17	390	5	3	31
6	18	600	2	4	32
17	19	390	5	3.5	29
18	20	390	5	3.5	29

Table 3. Central composite design results of the combined effect of MAE in *Ulothrix* sp.

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	**A:Power**	**B:Heating Times**	**C:Extraction Time**	**Lipid Content**
		Watts	Mins	Hr	%
12	1	600	2	4	38
13	2	390	5	3.5	47
1	3	180	2	3	24
15	4	390	10	3.5	49
16	5	390	5	2.5	47
5	6	390	5	4.5	48
20	7	180	2	4	30
9	8	180	5	3.5	35
3	9	390	5	3.5	47
10	10	180	8	3	38
11	11	390	1	3.5	29
8	12	600	8	4	45
4	13	750	5	3.5	47
17	14	180	8	4	39
2	15	600	8	3	55
7	16	390	5	3.5	47
6	17	390	5	3.5	47
14	18	600	2	3	37
18	19	390	5	3.5	47
19	20	390	5	3.5	47

Table 4. Central composite design results of the combine effect of factors of MAE in *Chlorella* sp.

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	**A:Power**	**B:Heating Times**	**C:Extraction Time**	**Lipid Content**
		Watts	Mins	Hr	%
16	1	390	5	3.5	40
17	2	390	5	4.5	39
14	3	390	5	3.5	40
15	4	600	8	3	47
2	5	600	2	4	37
7	6	180	2	3	26
1	7	180	8	3	39
6	8	390	5	3.5	40
11	9	390	5	3.5	40
4	10	600	8	4	48
12	11	390	5	2.5	40
20	12	390	1	3.5	27
8	13	390	8	3.5	42
18	14	390	5	3.5	40
19	15	600	2	3	37
3	16	700	5	3.5	41
5	17	180	2	4	26
10	18	390	5	3.5	40
9	19	600	5	3.5	45
13	20	180	8	4	39

Table 5. Central composite design results of the combined effect of factors of MAE in *Cladophora* sp.

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	**A:Power**	**B:Heating Times**	**C:Extraction Time**	**Lipid Content**
		Watts	mins	Hrs	%
14	1	390	5	3.5	32
5	2	180	2	4	22
6	3	600	2	4	34
2	4	600	2	3	33
7	5	180	8	4	35
3	6	180	8	3	33
9	7	365	5	3.5	30
10	8	415	5	3.5	33
8	9	600	8	4	40
1	10	180	2	3	21
11	11	390	5	3.5	32
4	12	600	8	3	37
12	13	390	5	3.5	32
13	14	390	5	3.5	32
19	15	390	5	4.5	34
16	16	390	5	2.5	32
20	17	390	1	3.5	24
15	18	390	8	3.5	37
18	19	700	5	3.5	37.5
17	20	600	5	3.5	38

Table 6. Central composite design results of the combined effect of factors of MAE in *Spirogyra* sp.

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	**A:Power**	**B:Heating Times**	**C:Extraction Time**	**Lipid Content**
		Watts	Mins	Hrs	%
9	1	365	5	3.5	23
1	2	180	2	3	17
10	3	415	5	3.5	26
13	4	390	5	3.5	25
12	5	390	5	3.5	25
6	6	600	2	4	27
7	7	180	8	4	28
14	8	390	5	3.5	25
4	9	600	8	3	30
2	10	600	2	3	26
3	11	180	8	3	26
5	12	180	2	4	18
8	13	600	8	4	33
11	14	390	5	3.5	25
20	15	390	5	4.5	26
17	16	390	5	2.5	24
19	17	390	1	3.5	18
15	18	390	8	3.5	27
18	19	700	5	3.5	32
16	20	600	5	3.5	31.5

Table 7. Central composite design results of the combined effect of factors of UAE in algal species.

		Factor 1	Factor 2	Lipid Content					
Std	**Run**	**A:Power**	**B:Extraction Time**	***Oedogonium* sp.**	***Oscillatoria* sp.**	***Ulothrix* sp.**	***Chlorella* sp.**	***Cladophora* sp.**	***Spirogyra* sp.**
		kHz	Mins	%	%	%	%	%	%
8	1	60	8	25	20	45	35	29	19
12	2	60	6	24	17	44	32	26	16
9	3	60	6	24	17	44	32	26	16
3	4	40	8	17	16	36	26	21	15
1	5	40	4	16	14	35	24	15	13
4	6	80	8	32	21	51	40	36	20
10	7	60	6	24	17	44	32	26	16
7	8	60	3	21	16	38	30	25	15
2	9	80	4	27	18	46	36	29	17
11	10	60	6	24	17	44	32	26	16
13	11	60	6	24	17	44	32	26	16
6	12	80	6	31.5	19	49	38	31.5	18
5	13	30	6	14	14	32	20	14	13

2.7. Statistical Analysis

Analysis of variance (ANOVA) and least significant difference was performed to analyze the data of the central composite design in the Design Expert® software (Stat-Ease, Inc. Godward, version 11, Minneapolis, MN, USA).

2.8. Transesterification and Fatty Acid Methyl Esters (FAMEs) Analysis

The extracted lipids, after pretreatment of all studied algal strains, were transesterified by the protocol described in Munir et al. [10]. After completion of trans-esterification, the reaction mixture was cooled to room temperature and centrifuged at 3000 rpm to obtain an

upper layer, of fatty acid methyl ester (biodiesel), and a lower layer (glycerol). The biodiesel upper layer was washed with warm distilled water at 50 °C to remove traces of catalyst and methanol. After transesterification, recovered FAMEs were injected to GCMS. [26].

2.9. Fuel Properties

The fuels' properties, such as higher heating value, cetane number, iodine value, saponification valve, oxidation stability, cold filter plugging point, density, long chain saturation factor, and kinematic viscosity were calculated using the formula described in [26].

3. Results and Discussions

3.1. Molecular Identification of Algae

The analyzed 18S rRNA or ITS sequences of algae species were assembled and compared by BLAST and CLUSTALW, and a phylogenetic tree was constructed. Figure 2 shows that the accession no KU563009 had 95% identification with *Chlorella sorokiniana* (KJ173792.1), while KU865579 had the closest similarity (91%) to *Chlorella vulgaris*. KU865580, showed the closest similarity (84%) to *Cladophora* sp. (KF318887.1), KU865577 showed 90% homology with *Hydrodictyon reticulatum* (KM676903), KU865576 had 94% similarity to *Oedogonium* sp. (DQ413053), KU865578 showed 93% homology with *Spirogyra* sp. (KM677012.1), KM676563.1 has 90% similarity to *Stigeoclonium* sp., and KU865575 showed 92% similarity to *Ulothrix* sp. (JX491152.1).

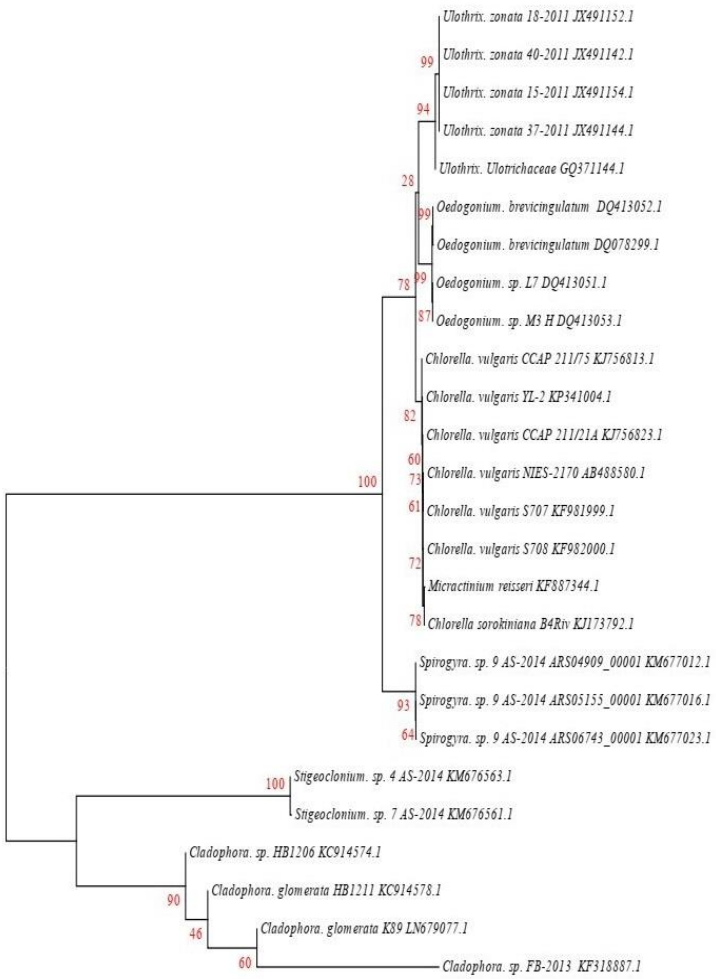


Figure 2. Phylogenetic tree of identified and closely related sequences, constructed using a nucleotide substitution model, 1000 rounds of bootstrap resampling, and the Kimura 2-parameter through-neighbor joining method.

3.2. Experimental Design and Statistical Analysis

The effect of power, heating, and extraction time in lipid extraction with microwave assistance, and power and extraction time in lipid extraction with sonication assistance, was evaluated in the algal species for the lipid content extraction. This included 20 repeats in each experiment of MAE and 13 runs in UAE (Tables 1–7). The impact of each factor on the lipid extraction with microwave assistance is shown in Tables 2–7. From *Oedogonium* sp., 38.5% lipids was obtained, 40% from *Cladophora* sp., and 33% from *Spirogyra* sp., under the optimized conditions: microwave power 600 watts, 8 min heating time, and 4 h extraction time. From *Oscillatoria* sp., 34% lipids was extracted at microwave power 600 watts, 5 min heating time, and 3.5 h extraction time. From *Ulothrix* sp., 55% lipids, and 48% from *Chlorella* sp., were extracted at microwave power 600 watts, 8 min heating time, and 3 h extraction time, and microwave power 600 watts, 8 min heating time, and 4 h extraction time, respectively. Increasing the microwave power from 180 to 600 W resulted in an increase in the lipid extraction efficiency. When the power exceeded 600 W, the extraction rates decreased. Increasing the microwave heating time from 1 to 8 min significantly increased the lipid content. Extending the extraction time after microwaving from 2.5 to 3.5 h significantly increased the lipid content, but a slight reduction of lipid content was found in all observed species after the extraction time was prolonged beyond 3.5 h (Tables 1–6).

During the microwave-assisted lipid extraction, algal cell membranes are weakened by the oscillation of polar substances generated by the heating effects of the microwaves, making it suitable for the extraction of intracellular metabolites. Earlier studies also indicate that MAE heats the extracts quickly and accelerates the extraction process [27,28]. *Ulothrix* sp. is a significant species for oil production, containing up to 60% lipid contents [24], whereas the lipid contents in *Chlorella* sp. have been measured as being up to 30% [24,29]. Gupta et al. [28] extracted 30% lipid from *Oedogonium* sp. by MAE. The lipid content was increased 20-fold, as compared to the controls, by using MAE in *Chlorella* sp. [30]. Ali and Watson [18] used 1021 W microwave power for 5 min to increase the lipid content 3-fold as compared to the control in *Chlorella* sp. From *Dunaliella tertiolecta*, 57.02% lipids was obtained at 160 sec extraction time and 490 W microwave power [31]. Many other researchers have used the MAE technique for algal lipid extraction, obtaining 28.6% lipid from *Chlorella* sp. [32], 32.8% from *Nannochloropsis* sp. [33], 39% from *N. gaditana* [34], and 28.9% from *Chlorella vulgaris* [35]. This oil extraction research suggests that these algal species can compete favorably with other conventional sources for biofuel production, if applied with the proper methods and extraction time. The oil content in algal species has been reported to vary from 14–63% of the dry weight [36]. The algal species studied in this research produced higher yields than those reported above, ranging from 40–50%, with the optimization of different methods.

As listed in Table 7, using the ultrasonic technique, 32% lipid was obtained from *Oedogonium* sp., 21% from *Oscillatoria* sp., 51% from *Ulothrix* sp., 40% from *Chlorella* sp., 36% from *Cladophora* sp., and 20% from *Spirogyra* sp., under an ultra-sonication power of 80 kHz and with an 8 min extraction time. Increasing the sonication power from 30 to 80 kHz, and the extraction time from 3 to 8 min, resulted in an enhanced lipid extraction efficiency in all observed species. In this technique, ultrasonic waves create cavitation bubbles in a solvent and these bubbles collapse to generate shock waves near the algae cells, causing disruptions of cellular walls and the release of the lipids into the solvent [37]. Abd El Fatah et al. [38] extracted 15% lipids from *Oscillatoria* sp. by solvent extraction that could be improved by MAE or UAE. Sonication with higher frequencies caused more effective cell disruption. Many other scientists have used the UAE technique for algal lipid extraction, obtaining 18.9% lipid from *Nanno chloropsis* [33], 8.8% from *Scenedesmus* sp. [39], 36% from *N. gaditana* [34], and 26.4% from *Chlorella vulgaris* [35]. Ferreira et al. [40] extracted 19% of total lipids with ultra-sonication from *Chlorella* sp. Silva et al. [41] conducted a study to find out the best algal lipid extraction method from a mixed algal culture. Zheng et al. [42] extracted 6, 15, 10, and 18% lipids from *Chlorella vulgaris* by grinding, ultrasound, bead milling and microwave, respectively. Approximately

24% lipid was extracted from *Spirogyra* sp. using ultra-sonication at 40 KHz for 30 min–2 h at 30 °C [23].

However, UAE is difficult to scale up and uses large amounts of power. Microwaves are a more suitable choice of technique for algal lipid extraction, having numerous benefits such as, a reduced extraction time and solvent usage, an improved extraction yield, a lower response period and operational expenses, as well as being environmentally friendly.

3.3. Equations in Terms of Coded Factors of Quadratic Models

The RSM suggested a quadratic model that relates the lipid content to the independent variables (Equations (2)–(13) in Table 8). The lipid content was the response and A, B, C were the coded terms used for the investigated parameters. A is microwave power, B is microwave heating time, and C is extraction time. These equations can accurately describe the interaction between the interactions, factors, and response.

Table 8. Equations in terms of coded factors of quadratic model of MAE.

Lipid Parameter Contents	Correlation	
***Oedogonium* sp.**	$+31.09 + 5.12 * A + 4.56 * B + 0.4375 * C + 0.8750 * AB - 0.1250 * AC + 0.0000 * BC - 1.18 * A^2 - 1.60 * B^2 - 0.0781 * C^2$	(2)
***Oscillatoria* sp.**	$+32.51 + 3.80 * A + 2.27 * B + 0.5193 * C - 1.0000 * AB - 0.2500 * AC + 0.0000 * BC - 2.66 * A^2 - 1.49 * B^2 - 0.3234 * C^2$	(3)
***Ulothrix* sp.**	$+46.70 + 6.08 * A + 6.73 * B + 0.0000 * C + 0.2500 * AB - 2.00 * AC - 2.00 * BC - 4.05 * A^2 - 4.04 * B^2 + 0.0858 * C^2$	(4)
***Chlorella* sp.**	$+39.79 + 4.66 * A + 6.01 * B - 0.1250 * C - 0.5000 * AB + 0.0000 * AC + 0.0000 * BC - 1.20 * A^2 - 1.78 * B^2 + 0.0802 * C^2$	(5)
***Cladophora* sp.**	$+31.86 + 4.15 * A + 4.37 * B + 0.8750 * C - 1.87 * AB + 0.1250 * AC + 0.3750 * BC - 2.68 * A^2 - 2.60 * B^2 - 0.0991 * C^2$	(6)
***Spirogyra* sp.**	$+25.29 + 3.41 * A + 3.62 * B + 0.8750 * C - 1.12 * AB + 0.1250 * AC + 0.3750 * BC - 1.10 * A^2 - 1.30 * B^2 - 0.0341 * C^2$	(7)
Equations in terms of coded factors of quadratic model of SAE		
***Oedogonium* sp.**	$+24.09 + 6.66 * A + 1.38 * B + 1.00 * AB - 0.1193 * A^2 - 0.6625 * B^2$	(8)
***Oscillatoria* sp.**	$+16.98 + 2.13 * A + 1.29 * B + 0.2500 * AB - 0.0161 * A^2 + 0.4066 * B^2$	(9)
***Ulothrix* sp.**	$+44.01 + 6.44 * A + 1.80 * B + 1.00 * AB - 0.9815 * A^2 - 1.23 * B^2$	(10)
***Chlorella* sp.**	$+32.11 + 6.59 * A + 1.79 * B + 0.5000 * AB - 0.9575 * A^2 + 0.3769 * B^2$	(11)
***Cladophora* sp.**	$+25.88 + 6.87 * A + 2.71 * B + 0.2500 * AB - 1.10 * A^2 + 0.9397 * B^2$	(12)
***Spirogyra* sp.**	$+15.98 + 2.13 * A + 1.29 * B + 0.2500 * AB - 0.0161 * A^2 + 0.4066 * B^2$	(13)

3.4. Analysis of Variance (ANOVA)

To analyze the significance, reliability, and fitness of the model, a lack of fit and an ANOVA test was applied. The results are shown in Supplementary Tables S1–S12. All the models show a p-value < 0.0001, which indicates that all the models are highly significant in the response except the quadratic model in *Oscillatoria* sp. (MAE), having a p-value < 0.0002. Nevertheless, in this study, all the p-values are < 0.0500, which indicates that the model is significant. In the case of MAE, the p value of coefficients of power and heating time were < 0.0001, while the p value for extraction time was 0.0053 in *Oedogonium* sp. In *Oscillatoria* sp., the p value of power was < 0.0006, heating time < 0.0074, and extraction time, 0.6153. In *Ulothrix* sp., the p values of power and heating time were <0.0001, while the p value of the extraction time was 1. In *Chlorella* sp., the p values of power and heating time were < 0.0001, while the p value of the extraction time was 0.7547. In *Cladophora* sp., the p values of power and heating time were < 0.0001, while the p value of extraction time was 0.0043. In *Spirogyra* sp., the p values of power and heating time were < 0.0001, while the p value of extraction time was 0.0222. In the case of UAE, the p value of coefficients power was < 0.0001, and extraction time was 0.0002 in *Oedogonium* sp. The p value of power and extraction time were < 0.0001 in *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. The model F-values in all the models were significant. There is only a 0.01% chance in all models, expect *Oscillatoria* sp. (MAE) which has 0.02% chance, that an F-value this large could occur by chance. The lack of fit value was > 0.05 (insignificant) in all models, indicating that the models are reliable and a good fit to the actual data.

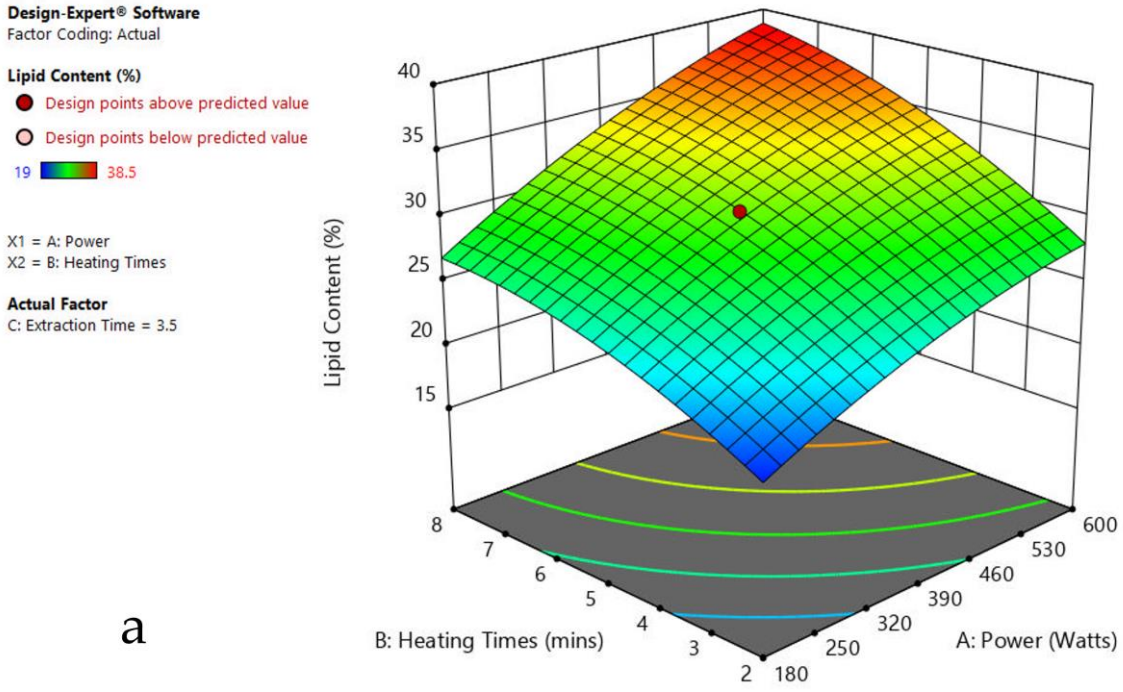
3.5. Validation of Models

R^2 is the correlation coefficient which indicates whether or not the experimental data fit the model. R^2 must be at least 0.90. In all the models, the R^2 values were greater than 0.90 (Supplementary Tables S13 and S14), thus indicating a good compatibility between the actual and calculated results within the wide range of the experiments. C.V% in all models was less than 10, indicating a good fit of all models to the experimental data. In all the models, adequate precision was more than four (4), this indicates that the model noise ratio is located in the satisfactory range. So, all the models were valid and can be used to navigate the design space.

3.6. Interaction of Factors

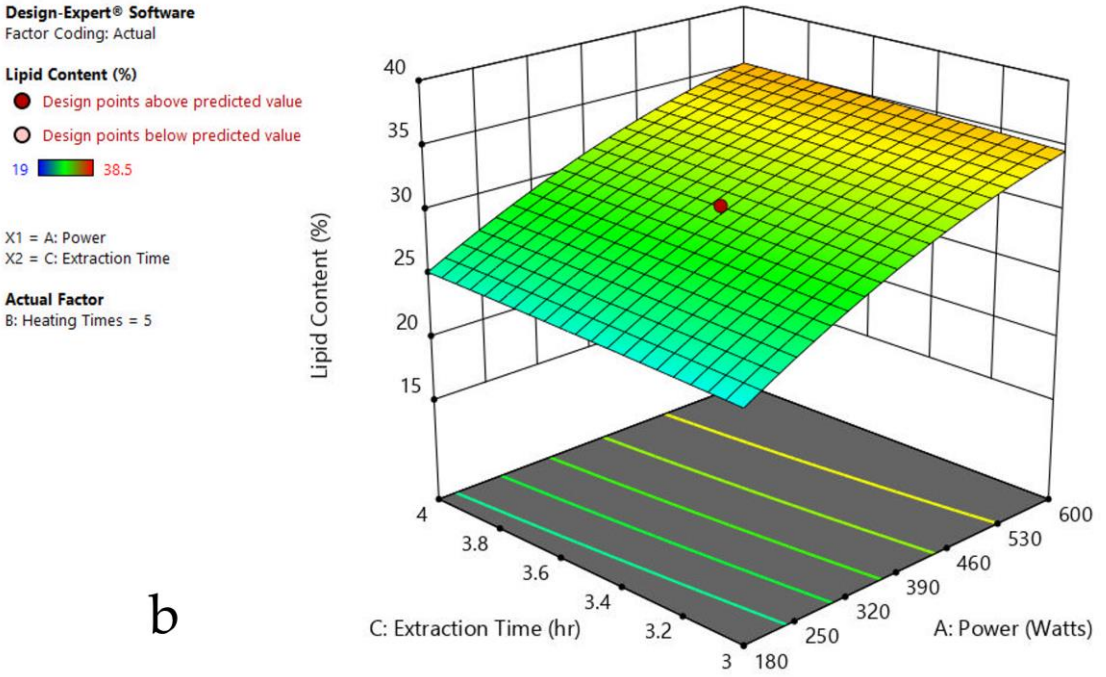
The 3-D response surface graphs show the effect of factors on a particular response, and also demonstrate the interaction between factors, to locate the best level of each factor for the maximum response. The curved slope on the three-dimensional response surface (Figures 3–8) displays the influence of microwave power, heating time, and extraction time on the lipids extracted from particular species. As the figures show, microwave power and heating time significantly influenced the lipid content.

The plots indicate that increasing the microwave power from 180 to 600 W resulted in the increase of the lipid extraction efficiency. When the power exceeded 600 W, the extraction rates were decreased. Increasing the microwave heating time from 1 to 8 min significantly increased the lipid content, but there was a slight reduction of the lipid content if the heating time was longer than 8 min. Extending the extraction time after microwaving from 2.5 to 3.5 h significantly increased the lipid content, but a slight reduction of lipid content was found in all observed species when extraction time was prolonged by more than 3.5 h. The red color indicates the points above the predicted value, while the pink color indicates the points below the predicted value.



a

Figure 3. *Cont.*



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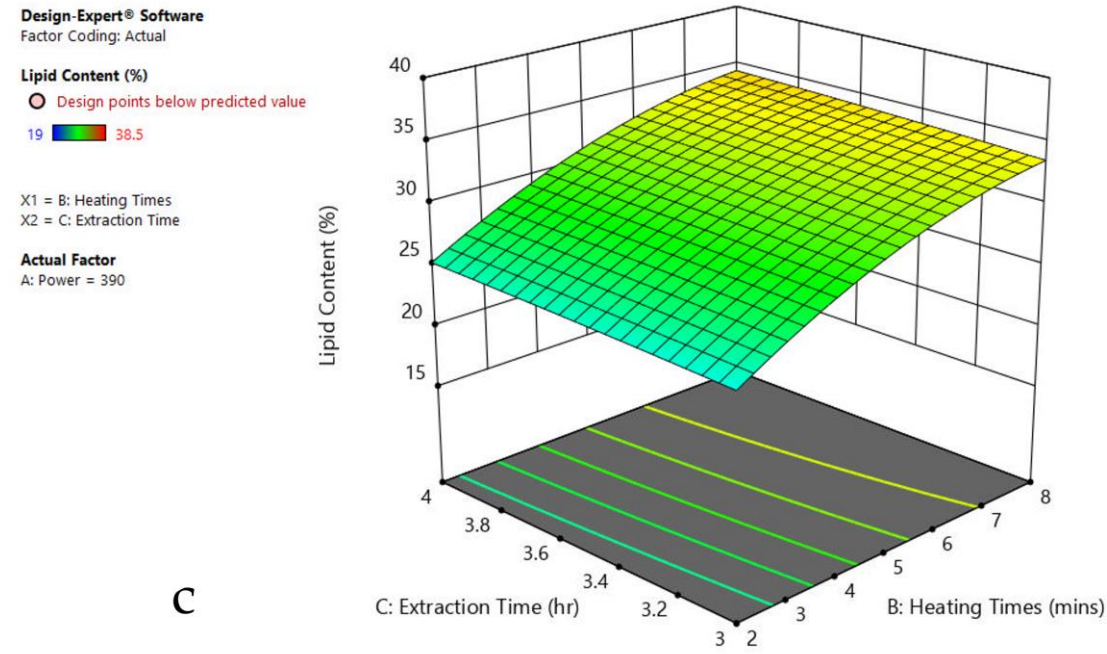
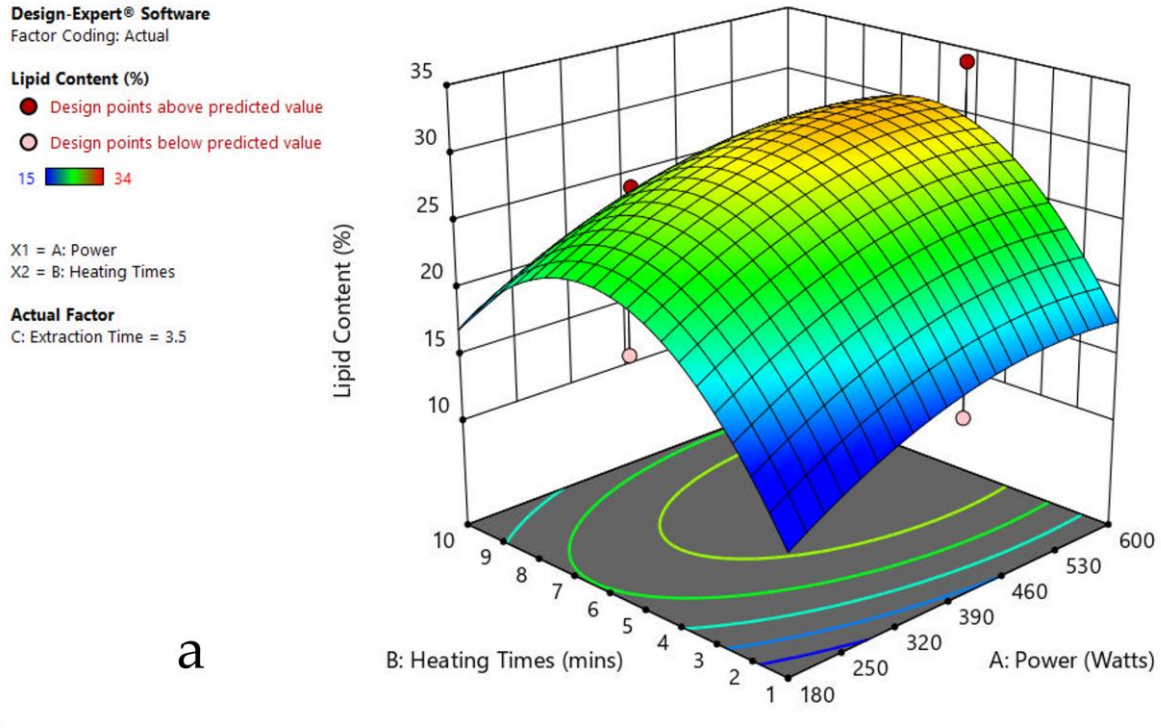
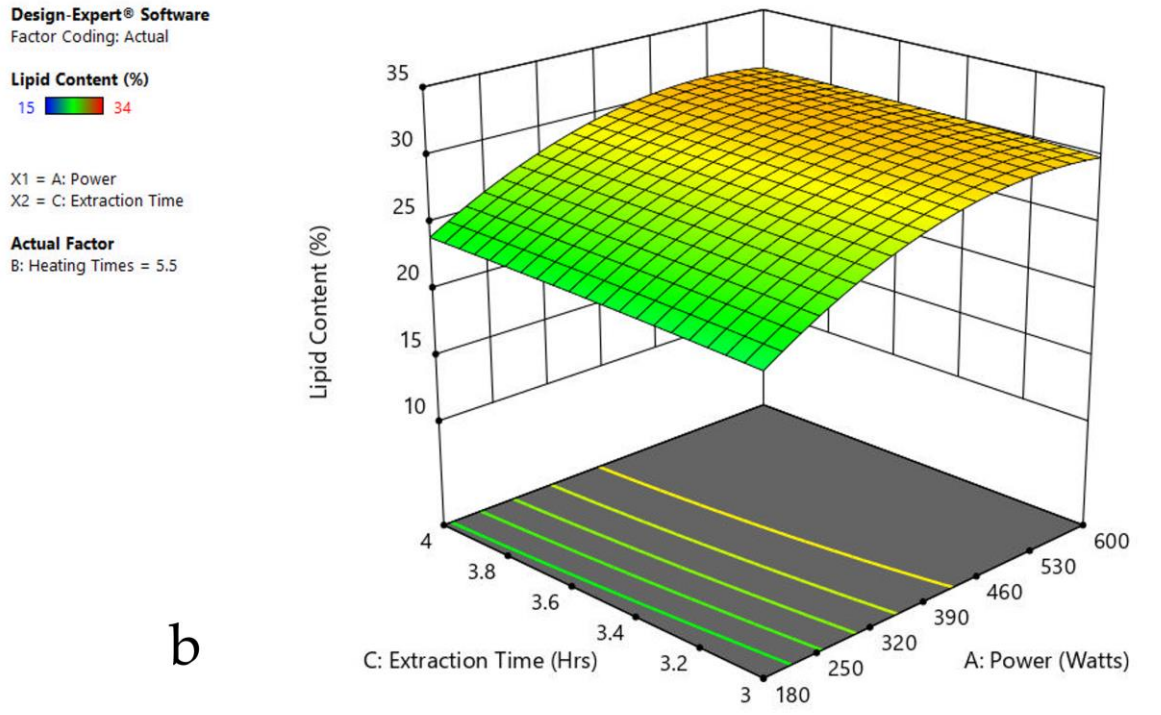


c

Figure 3. Response surface plots of the combined effect of MAE parameters on lipid content of *Oedogonium* sp. (**a**) Heating time (h) and Power (Watts) (**b**) Extraction time (h) and Power (Watts) (**c**) Extraction time (h) and Heating time (Mins). Color based on response value: blue color shows the minimum points, red color shows the maximum points, pink color indicates points below the predicted value.

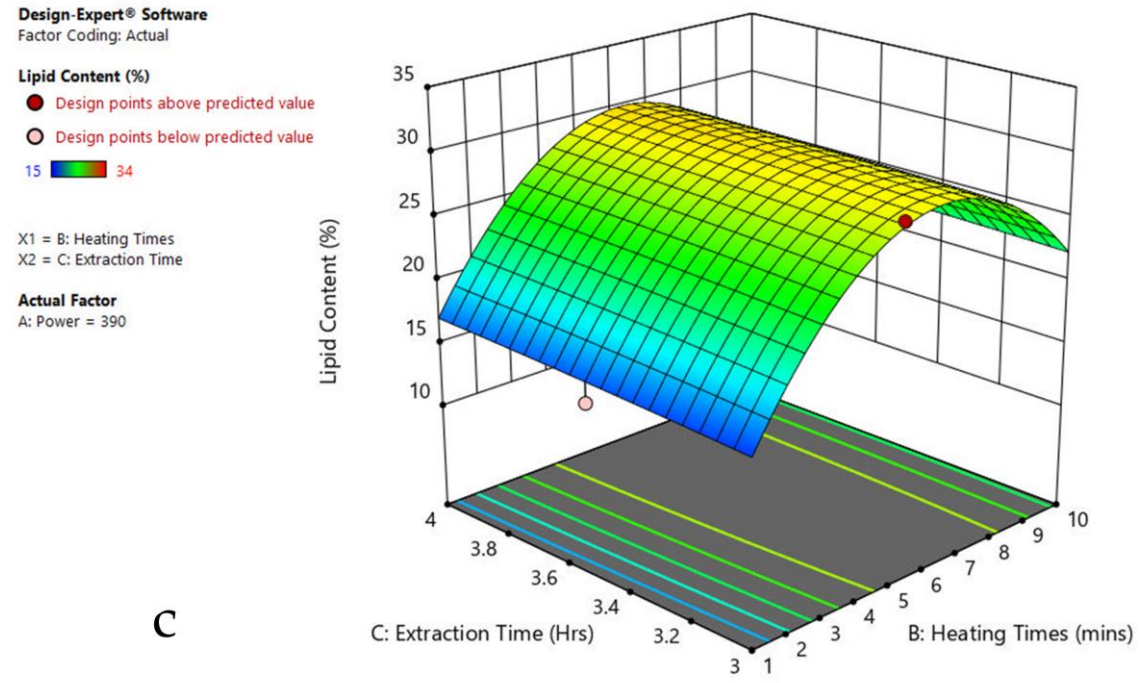


a



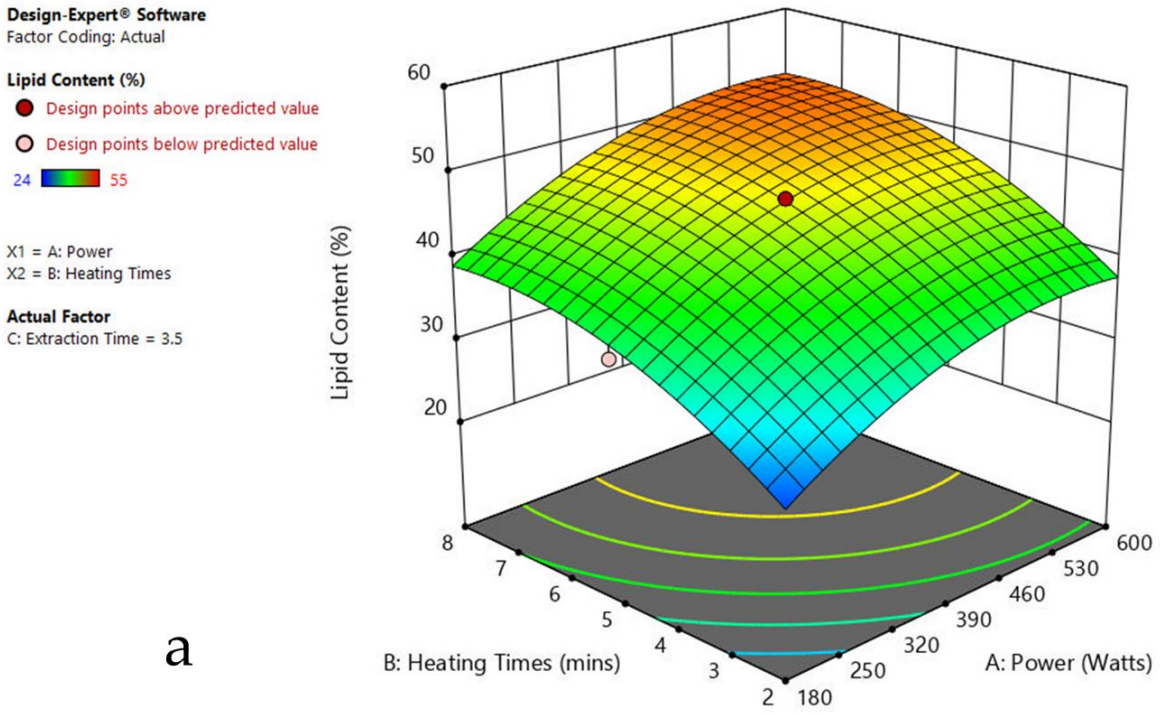
b

Figure 4. *Cont.*



c

Figure 4. Response surface plots of the combined effect of MAE parameters on lipid content of *Oscillatoria* sp. (**a**) Heating time (hr) and Power (Watts) (**b**) Extraction time (hr) and Power (Watts) (**c**) Extraction time (hr) and Heating time (Mins). Color based on response value: blue color shows the minimum points, red color shows the maximum points, pink color indicates points below the predicted value.



a

Figure 5. *Cont.*

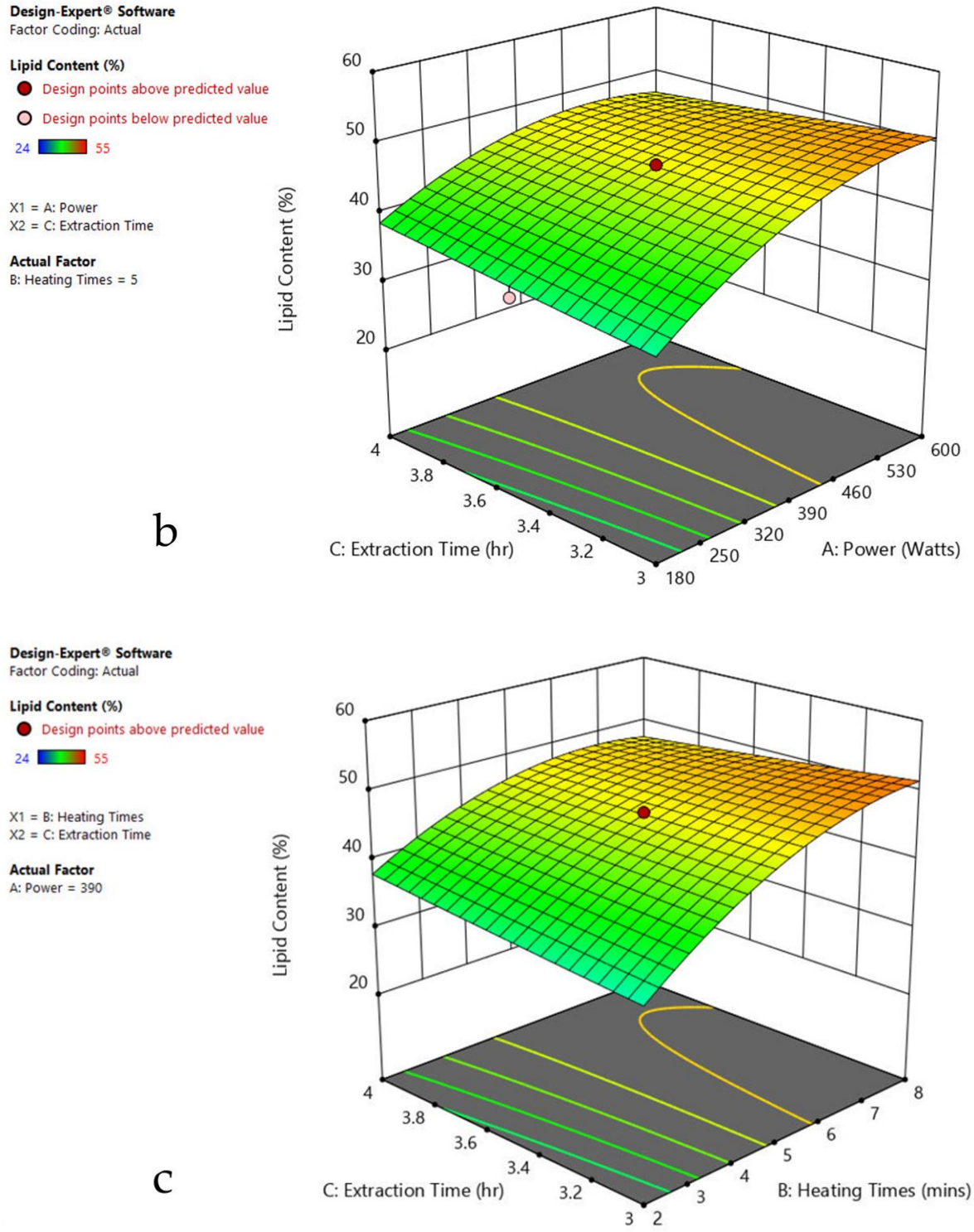
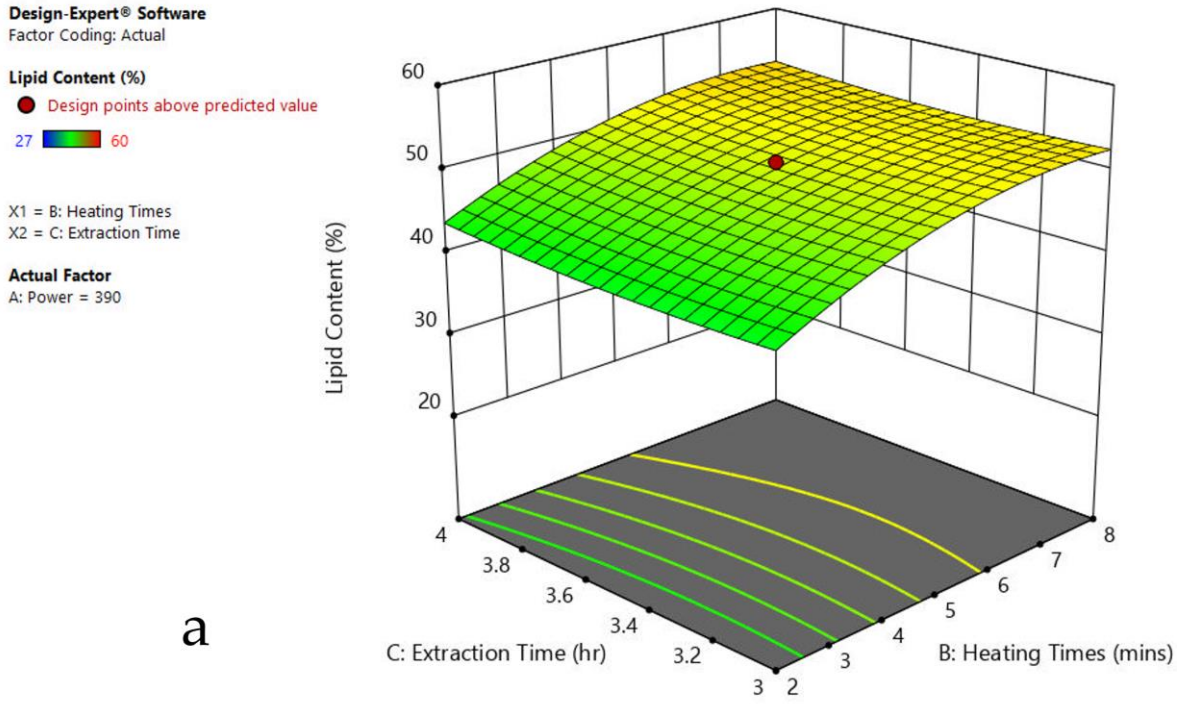
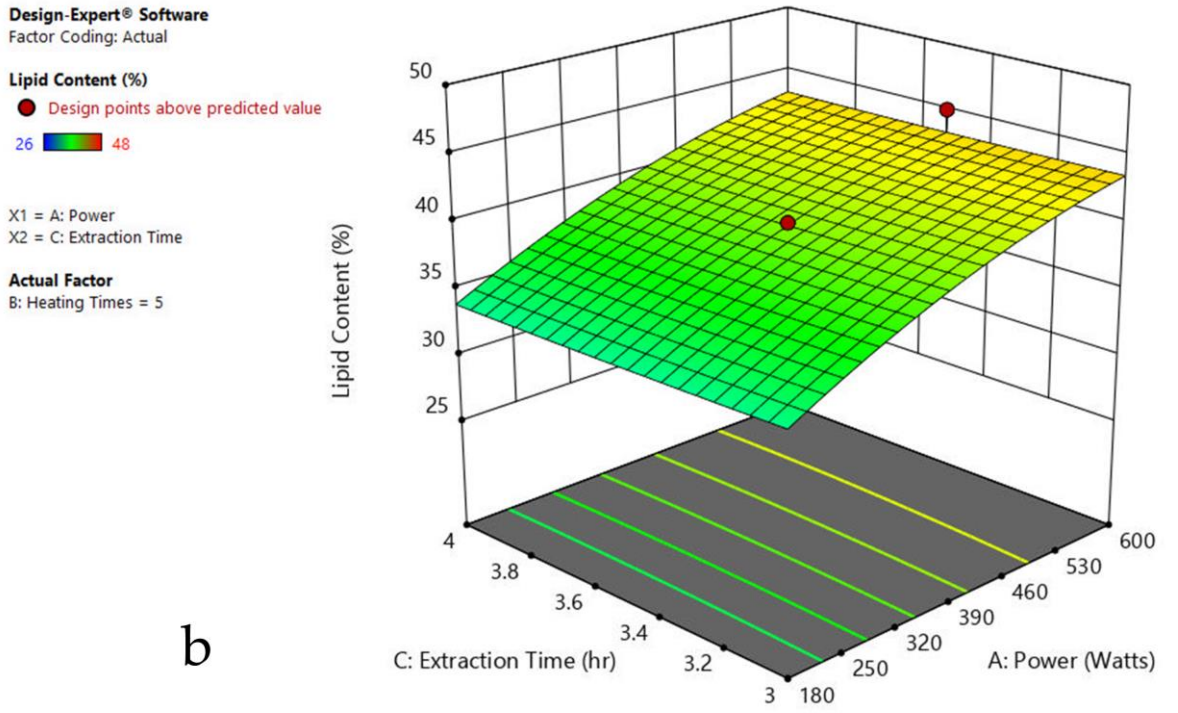


Figure 5. Response surface plots of the combined effect of MAE parameters on lipid content of *Ulothrix* sp. (**a**) Heating time (hr) and Power (Watts) (**b**) Extraction time (hr) and Power (Watts) (**c**) Extraction time (hr) and Heating time (Mins). Color based on response value: blue color shows the minimum points, red color shows the maximum points, pink color indicates points below the predicted value.

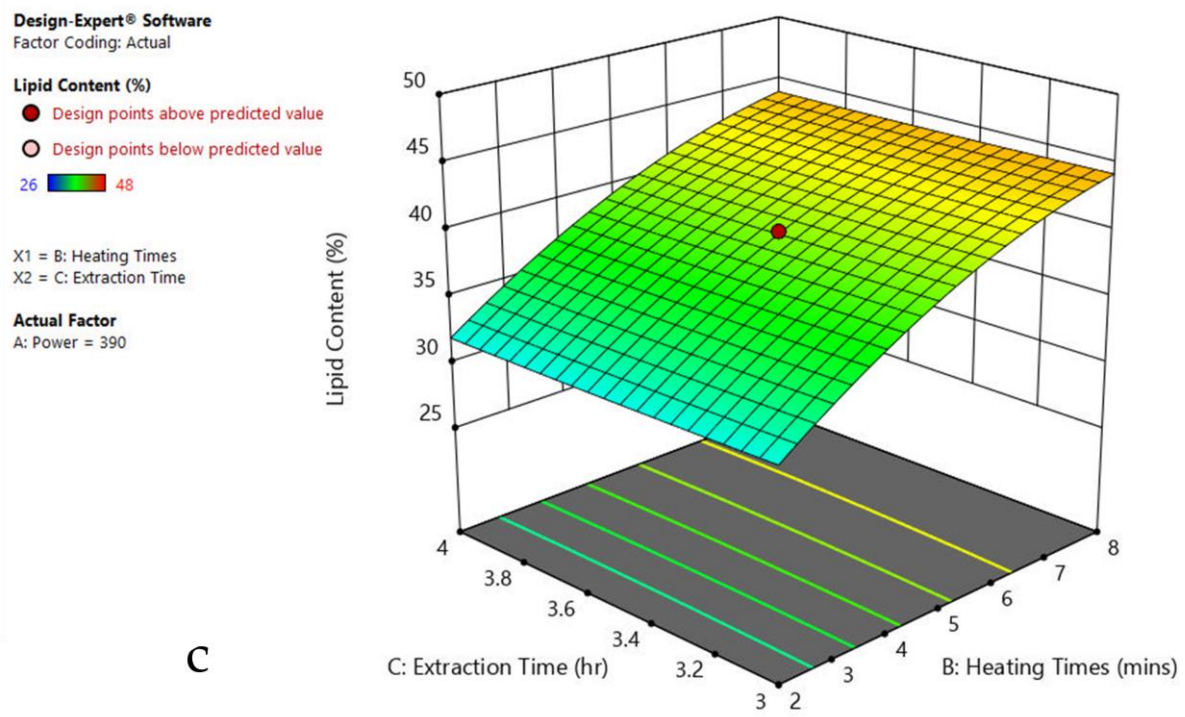


a



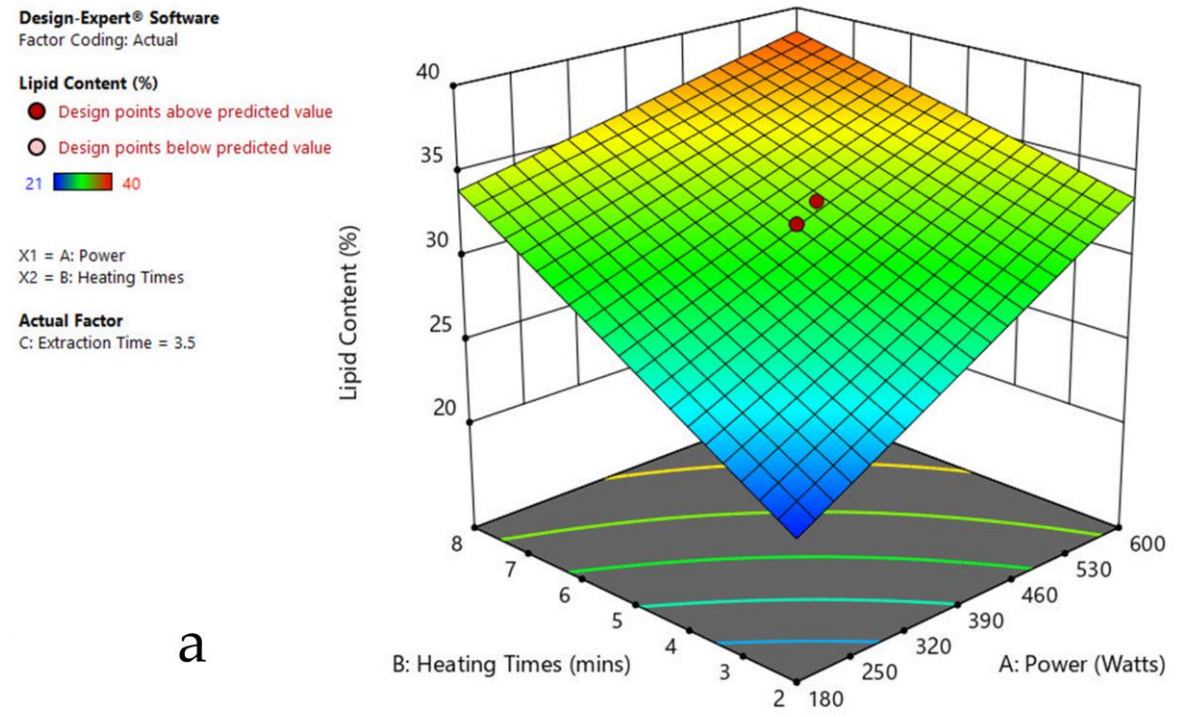
b

Figure 6. *Cont*.



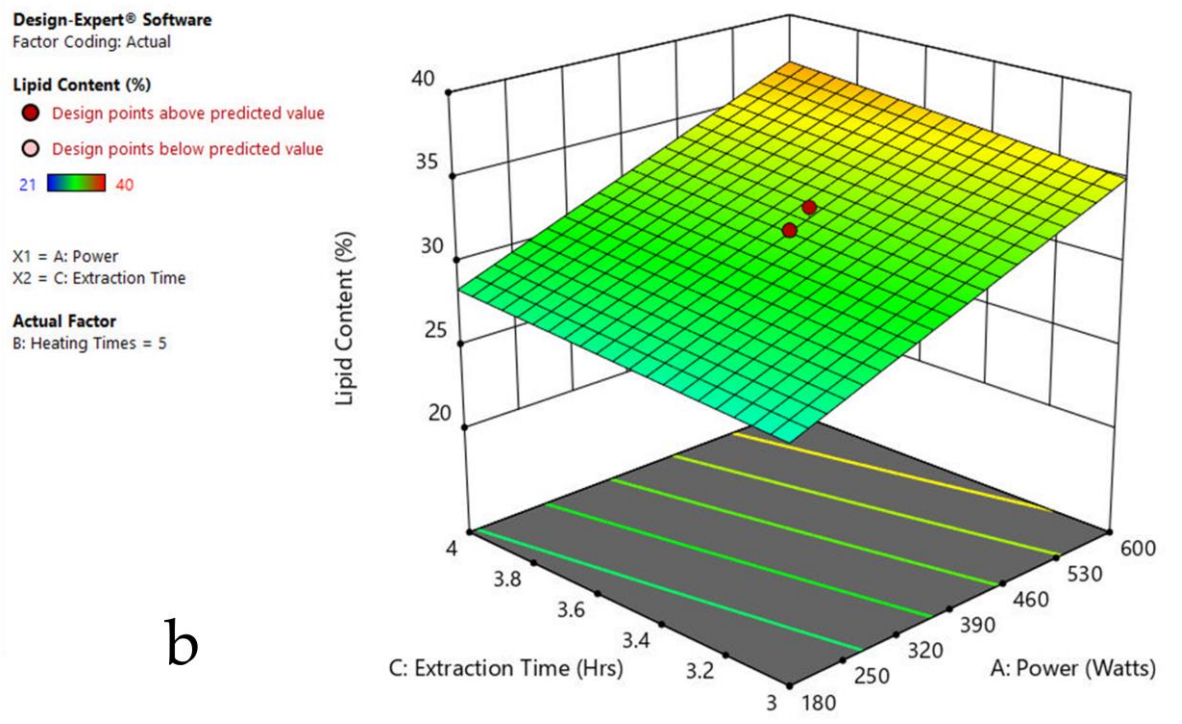
c

Figure 6. Response surface plots of the combined effect of MAE parameters on lipid content of *Chlorella* sp. (**a**) Heating time (hr) and Power (Watts) (**b**) Extraction time (hr) and Power (Watts) (**c**) Extraction time (hr) and Heating time (Mins). Color based on response value: blue color shows the minimum points, red color shows the maximum points, pink color indicates points below the predicted value.



a

Figure 7. *Cont.*



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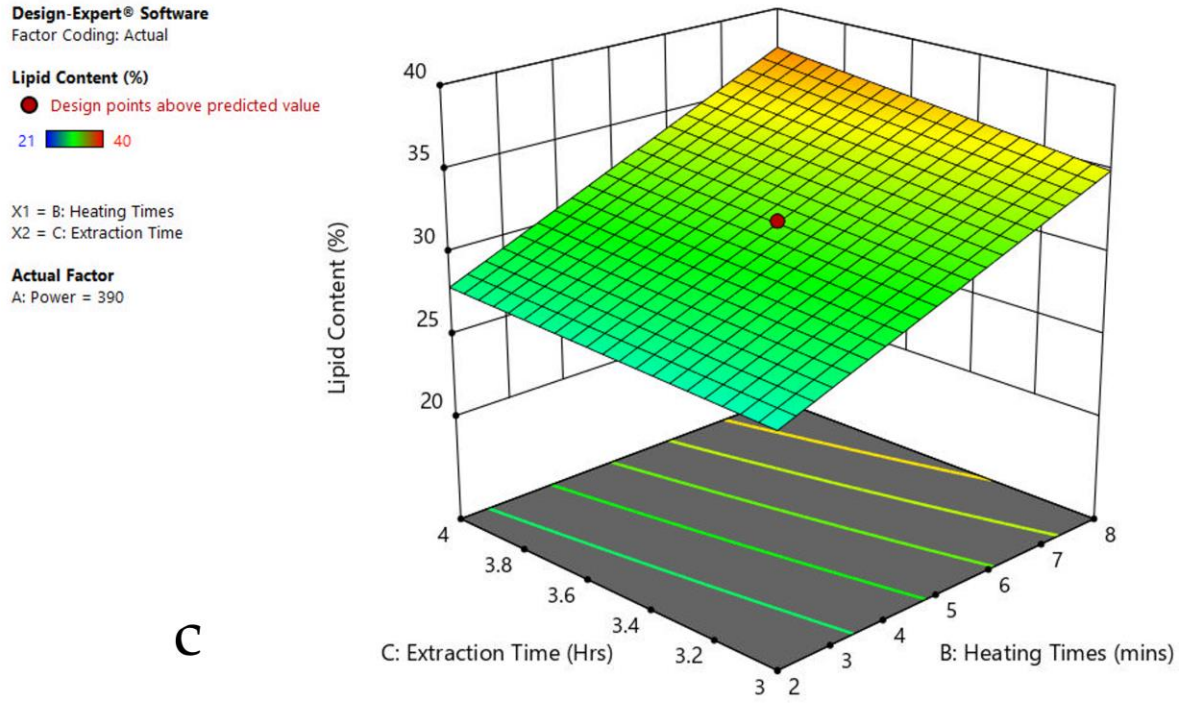


c

Figure 7. Response surface plots of the combined effect of MAE parameters on lipid content of *Cladophora* sp. (**a**) Heating time (hr) and Power (Watts) (**b**) Extraction time (hr) and Power (Watts) (**c**) Extraction time (hr) and Heating time (Mins). Color based on response value: blue color shows the minimum points, red color shows the maximum points, pink color indicates points below the predicted value.

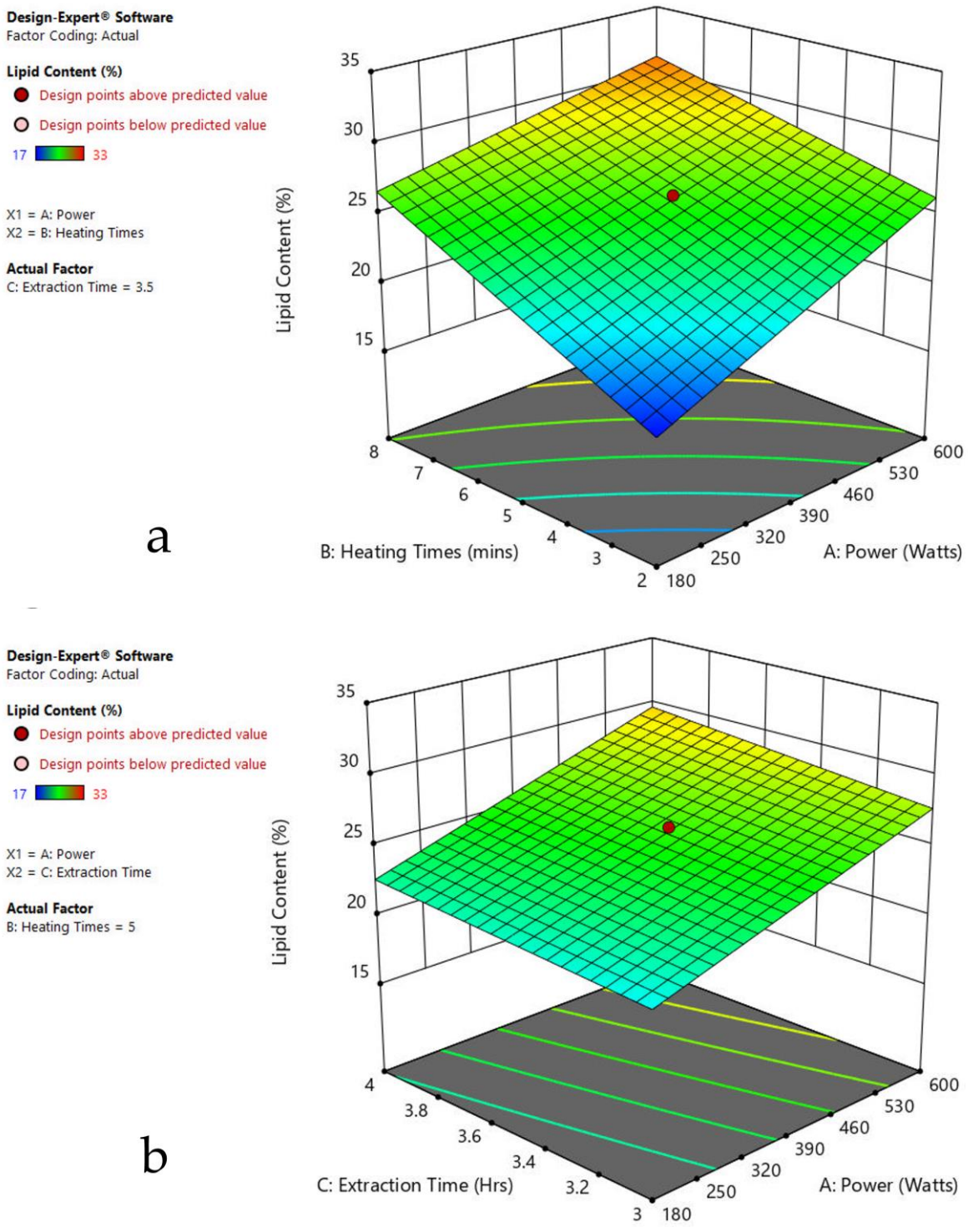


Figure 8. *Cont.*

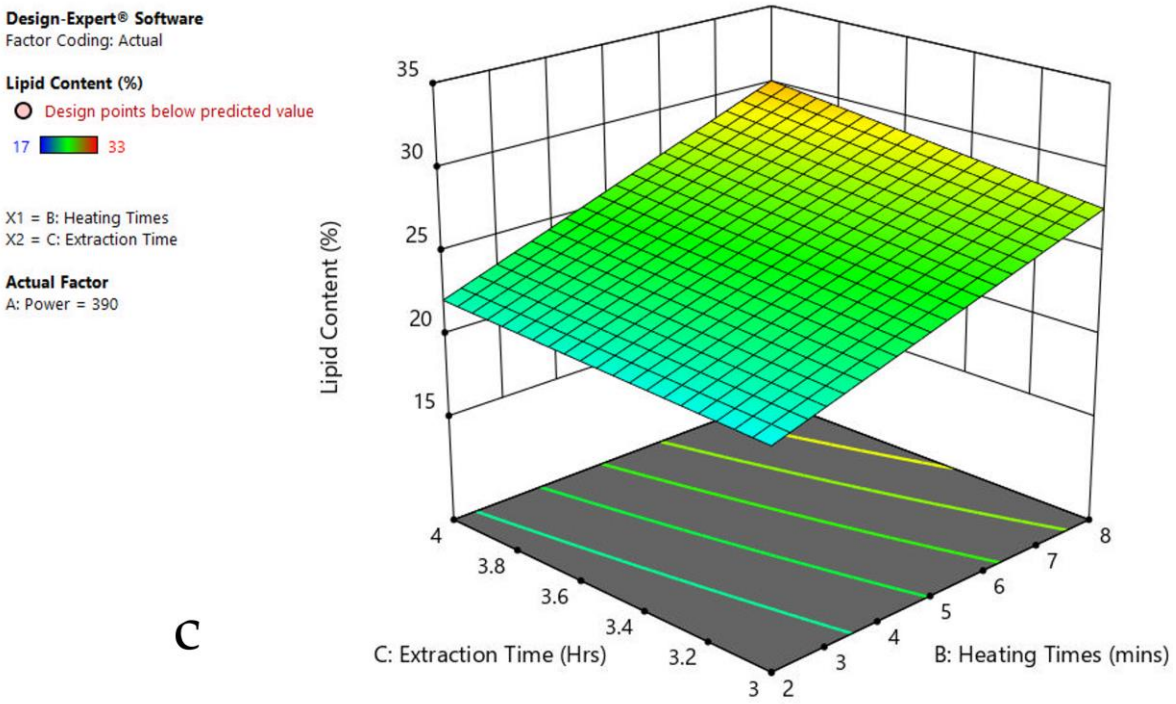
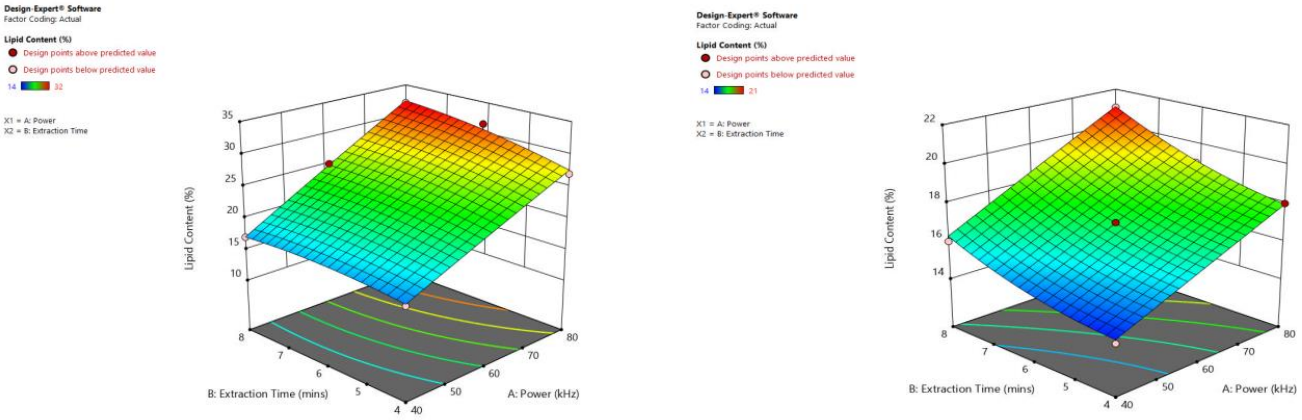


Figure 8. Response surface plots of combined effect of the MAE parameters on lipid content of *Spirogyra* sp. (**a**) Heating time (hr) and Power (Watts) (**b**) Extraction time (hr) and Power (Watts) (**c**) Extraction time (hr) and Heating time (Mins). Color based on response value: blue color shows the minimum points, red color shows the maximum points, pink color indicates points below the predicted value.

Figure 9 displays the significant influence of sonication power and heating time on the lipid content of particular species. Increasing the sonication power from 30 to 80 kHz, and the extraction time from 3 to 8 min, resulted in an enhanced lipid extraction efficiency in all observed species.

According to the three-dimensional response surface (Figures 3–9), there is a strong relationship between microwave power, heating time, and sonication power on the lipid content.



(**a**) (**b**)

Figure 9. *Cont.*

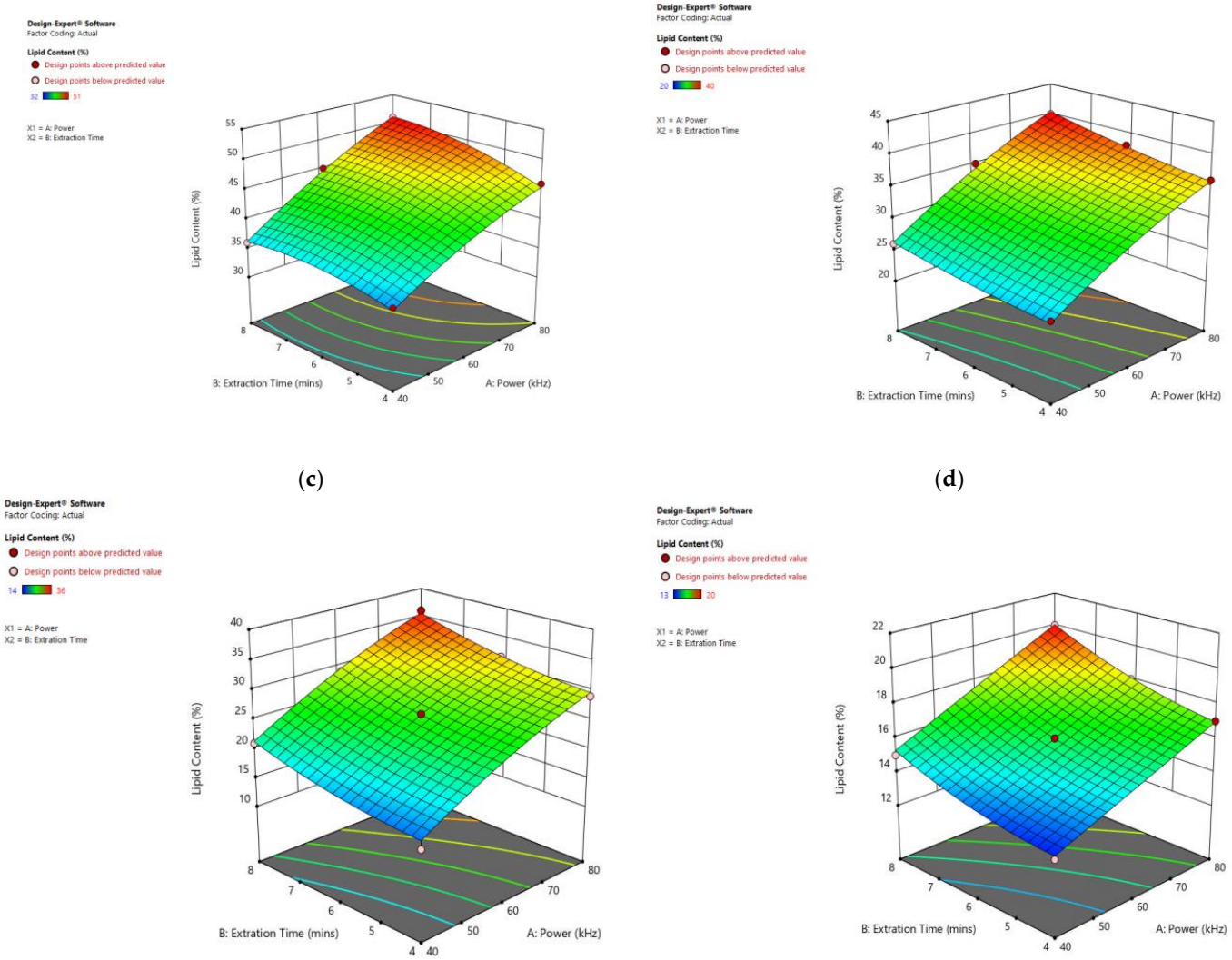


Figure 9. Response surface plots of the combined effect of UAE parameters on lipid content of (**a**) *Oedogonium* sp. (**b**) *Oscillatoria* sp. (**c**) *Ulothrix* sp. (**d**) *Chlorella* sp. (**e**) *Cladophora* sp. (**f**) *Spirogyra* sp. Color based on response value: blue color shows the minimum points, red color shows the maximum points, pink color indicates points below the predicted value.

3.7. Fatty Acid Composition

Fatty acids are a major constituent of microalgae biomass. Fatty acids present in triacylglycerol are of commercial interest, because they can be used for the production of transportation fuels, bulk chemicals, nutraceuticals, and food commodities. To determine the quality of biodiesel, the fatty acid compositions of *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. before and after pretreatment were analyzed, as listed in Table 9. The algae which were not treated with pretreatment methods are termed as the controls. The experiment runs having the highest lipid content (MAE runs number 7, 12, 15, 10, 9, 13; and UAE runs number 6, 6, 13, 2, 6, 2 of *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp. and *Spirogyra* sp., respectively) were further analyzed by GCMS. Palmitoleic acid, palmatic acid, oleic acid, myristic acid, linolenic acid, linoleic acid, and stearic acid are the major fatty acids detected in all six algal strains. Palmitoleic acid, palmatic acid, and oleic acid increased after pretreatment in all algal species, while myristic acid, linolenic acid, linoleic acid, and stearic acid decreased. The results indicate that *Oedogonium* sp. has a relatively high oleic acid as compared to

other tested species. Gondoic acid, myristoleic acid, and arachidic acid were detected in *Spirogyra* sp., while traces of myristoleic acid were detected in *Cladophora* sp. Arachidate (21:0) was found in all tested algal strains except *Oedogonium* sp. and *Cladophora* sp. Erucate (22:1) was found in *Ulothrix* sp., *Chlorella* sp., and *Spirogyra* sp. Behenic acid was detected in *Oedogonium* sp., *Ulothrix* sp., *Chlorella* sp., and *Cladophora* sp. after microwave pretreatment. Arachidate, erucate, behenic acid, gondoic acid, and arachidic acid are only present in algal lipids and not in seed oils. Algal fatty acids range from 12 to 22 carbons in length [43], while 50–65% UFAs were found in *Nannochloropsis oleabundans*, *Dunaliella tertiolecta*, *S. maxima*, *S. obliquus* and C. *vulgaris* [44]. Palmitic acid was detected as the major component in *Chlorella* sp., *Scenedesmus* sp. and *Botryococu* sp., at 35.3%, 33.3%, and 31.7%, respectively, during cultivation in sewage water [45]. Studied algal oils consists of a combination of archidonic, eicosapentaenoic, docosahexaenoic, gamma-linolenic, and linoleic acids [46]. Methyl palmitate, methyl stearate, methyloleate, and methyl linoleate are the main components of *Tolypothryx* [47], *Pithophora* [48], *Spirogyra*, *Hydrodictyon*, *Rhizoclonium* [24] and *Cladophora*, while *Chlorella vulgaris* has mostly methyl linoleate and methyl palmitate [49]. MAE enhanced by 12% MUFAs, and by 1.5-fold PUFAs, in *C. minutissima*. Similar results were reported for *T. cutaneum*, where C16:0 and C18:1 comprise 60% of the total FAMEs [30]. The same trend is shown in the present study, where C16:0, C16:1, C18:1, C18:2, and C20:5 comprise 84.76% of the total fatty acids. In the current study, although both pretreatments induce these valuable fatty acids, microwave treatment induces more than sonication. The same was reported in *Chlorella* sp., where 61% fatty acids were extracted using UAE and 75% after MAE. The present study suggests that pretreatment is very important before lipid extraction, especially microwave treatment rather than sonication, because microwaves selectively release lipids from the algal matrix by causing local superheating of the lipid compounds to selectively extract them [50]. In contrast, ultrasound destroys the algal cell wall and releases unnecessary products, such as aroma compounds and other secondary metabolites, as well as lipids [51].

Table 9. Lipid composition of algal strains.

Fatty Acids	***Oedogonium* sp.**			***Oscillatoria* sp.**			***Ulothrix* sp.**			***Chlorella* sp.**			***Cladophora* sp.**			***Spirogyra* sp.**		
	Control	MAE	UAE	Control	MAE	UAE	Control	MAE	UAE	Control	MAE	UAE	Control	MAE	UAE	Control	MAE	UAE
Myristoleic acid (14:1)	0.15	-	-	-	-	-	-	-	-	-	-	-	0.32	-	-	0.18	0.12	0.15
Myristic acid (14:0)	0.37	0.25	0.28	0.34	0.26	0.30	0.19	0.12	0.16	0.20	0.10	0.15	0.18	0.11	0.15	0.05	-	0.01
Hexadecadienoic (16:2)	0.46	0.29	0.34	-	-	-	-	-	-	-	-	-	-	-	-	2.28	2.11	2.18
Palmitoleic acid (16:1)	2.92	3.5	3.2	0.38	1.12	0.81	-	-	-	0.10	0.31	0.22	-	-	-	0.37	0.56	0.41
Palmatic acid (16:0)	1.19	1.38	1.26	3.12	3.21	3.19	0.28	1.10	0.57	1.0	1.28	1.19	0.44	1.22	0.98	0.19	0.31	0.29
Linolenic acid (18:3)	0.44	0.39	0.40	-	-	-	-	-	-	-	-	-	-	-	-	0.68	0.45	0.51
Linoleic acid (18:2)	0.38	0.23	0.28	2.18	1.91	2.1	0.6	0.4	0.5	0.50	0.41	0.46	2.13	1.80	1.90	1.56	1.11	1.28
Oleic acid (18:1)	3.12	3.52	3.31	2.15	3.11	2.25	0.46	1.91	1.51	0.10	0.25	0.15	0.15	0.19	0.17	0.35	1.23	1.17
Stearic acid (18:0)	1.32	1.23	1.27	3.14	2.72	2.98	-	-	-	-	-	-	-	-	-	0.06	-	-
Gondoic acid (20:1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.18	-	-
Arachidic acid (20:0)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.14	-	-
Erucic acid (22:1)	0.46	0.29	0.31	-	-	-	-	-	-	0.12	0.11	0.10	0.12	0.12	0.12	-	-	-
Caprylate (8:0)	-	-	-	0.72	0.71	0.70	0.34	0.33	0.34	-	-	-	0.34	0.33	0.34	-	-	-
Caprate (10:0)	-	-	-	1.38	1.36	1.38	0.67	0.51	0.61	-	-	-	0.13	0.13	0.12	-	-	-
Laurate (12:0)	-	-	-	8.57	8.51	8.52	0.28	0.28	0.28	-	-	-	0.62	0.60	0.61	-	-	-
Methyl myristate (14:0)	4.51	3.52	3.81	-	-	-	3.35	3.21	3.29	2.21	1.91	1.89	3.34	3.29	3.31	2.16	1.90	1.95
Methyl palmitoleate (16:1)	3.16	3.01	3.11	10.4	9.51	9.81	5.13	4.13	5.10	3.13	2.7	2.9	3.27	2.93	3.12	5.90	4.21	4.34
Methyl palmitate (16:0)	46.41	39.10	40.1	12.5	10.01	11.1	24.41	21.14	22.2	40.0	34	37	34.3	29.1	30.0	49.7	43.2	44.5
Linolenate (18:3)	2.35	2.12	2.24	9.17	8.51	8.81	2.64	2.26	2.43	0.81	0.49	0.62	7.62	6.21	6.91	1.43	1.21	1.32
Linoleate (18:2)	2.58	2.23	2.4	3.13	2.69	2.81	9.16	7.81	8.12	2.12	1.72	1.91	5.46	4.41	4.90	0.54	0.35	0.43
Methyl oleate (18:1)	28.06	25.24	26.1	16.19	14.01	15.6	44.24	40.12	41.1	42.5	39.1	41.8	42.8	38.1	39.0	29.1	27.0	28.3
Methyl stearate (18:0)	2.17	2.25	2.18	27.14	27.21	27.1	3.87	4.15	4.10	3.92	3.25	3.51	-	-	-	5.2	5.8	5.5
Arachidate (21:0)	-	-	-	0.65	0.62	0.64	2.44	2.21	2.36	1.0	-	-	-	-	-	1.1	1.1	1.1
Erucate (22:1)	-	-	-	-			2.23	2.19	2.21	1.0	-	-	-	-	-	0.5	0.5	0.5
Behenic acid (22:0)	-	0.10	-	-	-	-	-	0.15	-	-	0.12	-	-	0.13	-	-	-	-

3.8. Fuel Properties

The fatty acid compositions and fuel properties of oils are important for the evaluation of biodiesel quality (Table 10). Fatty Acid Methyl Esters (FAME) are esters of fatty acids. The physical characteristics of fatty acid esters are closer to those of fossil diesel fuels than pure vegetable oils, but their properties depend on the type of vegetable oil. FAMEs analysis defines the quality of biodiesel. In the present study, the fuel properties were identified from fatty acid composition by using different formulas. The iodine value correlates with the degree of oil unsaturation in these studied algal strains. A low iodine value is very important for the oxidative stability of biodiesel produced from these new algal resources, because a high level of unsaturation cause glyceride polymerization and deposit formation, which ultimately deceases engine performance [52]. The iodine value of each algal strain used here was in the range of 77–93 g I_2/100 g. According to the EN 14214 standard, biodiesels with an IV less than 120 g I_2/100 g can improve engine performance. Both applied pretreatments on algal oil reduced IV compared to untreated controls. The saponification values were 170–206 mg KOH/g in all six studied algal strains. High SV levels mean a high acid percentage in biodiesel, that causes soap formation, which is not recommended [53]. The results indicated that SV levels reduced after the application of sonication and microwave pretreatments in algal species, especially in *Chlorella* sp. The reduction of the SV values by applying these pretreatment techniques promotes the production of biodiesel from these algal strains on a commercial scale. In the present study, the cetane numbers (CN) in all algal strains were in the range of 49–59, which seems an acceptable range compared to the current standards for biodiesel according to ASTM D6751 standard CN (minimum 47), while European standard EN 14214 (minimum 51) is recommended for better engine performance. A high CN relates to a good engine performance, which has been achieved in *Chlorella* sp. after microwave pretreatment [54]. The high heating values (HHV) in all species tested were in the range of 38.1 to 41 MJ/kg, while the highest HHV was observed in *Chlorella* sp. after microwave pretreatment. According to ASTM standard, the HHV for biodiesel should be more than 35 MJ/kg [55,56]. The cold filter plugging point determines the flow performance of biodiesel [57]. The CFPPs of the biodiesel from the six algae species were in the range of −10.6 to −16.3 °C. According to EN 14214, the CFPP of biodiesel should be 5–20 °C. Biodiesel rich in stearic and palmitic acid has a poor CFPP. In the current study, stearic acid reduced after pretreatment. The CFPPs of different algal strains have been reported to be between −12.3 to 20.8 °C [58], while peanut has a 19 °C CFPP, which is the highest among the seed oils [59].

Kinematic viscosity should be 1.9–6.0 mm^2 s^{-1} (ASTM 6751) and 3.5–5.0 mm^2 s^{-1} (EN 14214). Here, the overall kinematic viscosities of the biodiesels from all six algae strains were in the range of 3.6 to 4.1 mm^2 s^{-1}. According to EN 14214, the density of biodiesel should be 0.86–0.9 g cm^{-3}, while there is no specification for density in ASTM 6751. Here, the densities of the biodiesels from the six algae strains were in the range of 0.87 to 0.88 g cm^{-3}. In order to store biodiesel for a long time without autoxidation, biodiesel must have a suitable oxidation stability, which is $\geq$ 6 h at 110 °C (EN 14214), while no specification was recorded in ASTM 6751. In the current study, the oxidation stabilities of all six algae species were in the range of 56 to 237 h at 110 °C. In the present study, the highest oxidation stability was 237 h at 110 °C, in *Chlorella* sp. A good quality biodiesel has a high cetane number, low iodine value, high heating value, good cold flow properties, and high oxidation stability [60]. Oleic acid is a vital component of biodiesel due to its low meting point, high cetane number, and sufficient oxidative stability [61]. In the present study, when the pretreatment was applied to the algal strains, oleic acid increased in all six species. In the present study, all the studied fuel properties of the biodiesels from *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. were within the range of the ASTM 6751 and EN 14214 standards. Furthermore, pretreatments not only enhance lipid recovery from algal biomass, but also improve lipid composition and fuel properties.

Table 10. Fuel properties of the six algal strains studied here.

Algae		IV (g $I_2$100/g fat)	SV (mg KOH/g)	CN	LCSF	CFPP (°C)	HHV*i* (MJ/kg)	ρ (g cm^{-3})	υ (mm^2/s)	Y (Hour) at 110 °C
Biodiesel Standard EN 14214		≤120	-	≥51	-	≤5/≤−20	NA	0.86–0.9	3.5–5.0	≥6
Biodiesel Standard ASTM D6751-02		NA	-	≥47	-	NA	≥35	NA	1.9–6.0	-
Oedogonium sp.	Control	91.6	202	50	0.85	−13.8	38.18	0.87	4.0	171
	MAE	81	178	56	0.75	−14.7	38.5	0.87	3.8	192
	UAE	84	182	54	0.76	−14.0	38.4	0.87	3.6	176
Oscillatoria sp.	Control	93	206	49	1.60	−11.4	38	0.88	3.9	56
	MAE	88.3	194	52	1.4	−11.8	38.3	0.87	4.0	64
	UAE	90.7	199	50	1.8	−10.7	38.1	0.87	4.1	58
Ulothrix sp.	Control	88.8	195	51	0.028	−16.3	38.12	0.88	3.6	199
	MAE	81.6	180	54	0.11	−16.1	38.6	0.87	3.7	297
	UAE	85	185	53	0.057	−16.2	38.3	0.87	3.6	238
Chlorella sp.	Control	88	195	52	2.05	−14.21	39.1	0.87	3.7	237
	MAE	77	170	59	1.7	−11.13	41	0.87	3.9	290
	UAE	82	182	55	1.8	−10.61	39.1	0.87	3.8	258
Cladophora sp.	Control	91	201	50	0.04	−16.3	38.40	0.87	3.6	58.6
	MAE	80	176	57	0.12	−16.0	39.5	0.87	3.7	68
	UAE	82	182	56	0.09	−16.1	39	0.87	3.7	62
Spirogyra sp.	Control	92	204	50	0.09	−13.6	40	0.88	3.9	55
	MAE	82	182	55	0.03	−16.3	39.5	0.87	4	78
	UAE	85	188	53	0.02	−16.3	39	0.87	3.9	58

3.9. Comparative Analysis of Lipid Extraction Pretreatment

Lipid extraction is a primary bottleneck for commercial algal biodiesel production. Solvent extraction (chloroform, methanol, and hexane) is an extensively applied method for lipid recovery, but the algal cell walls act as a barrier between the lipids and the solvent. To overcome this problem, numerous mechanical, chemical, and biological cell disruption methods have been extensively studied [13,62]. Figure 10 demonstrates that mechanical methods, such as MAE and UAE, are an effective pretreatment to improve lipid recovery from algae as compared with previous studies [22,24].

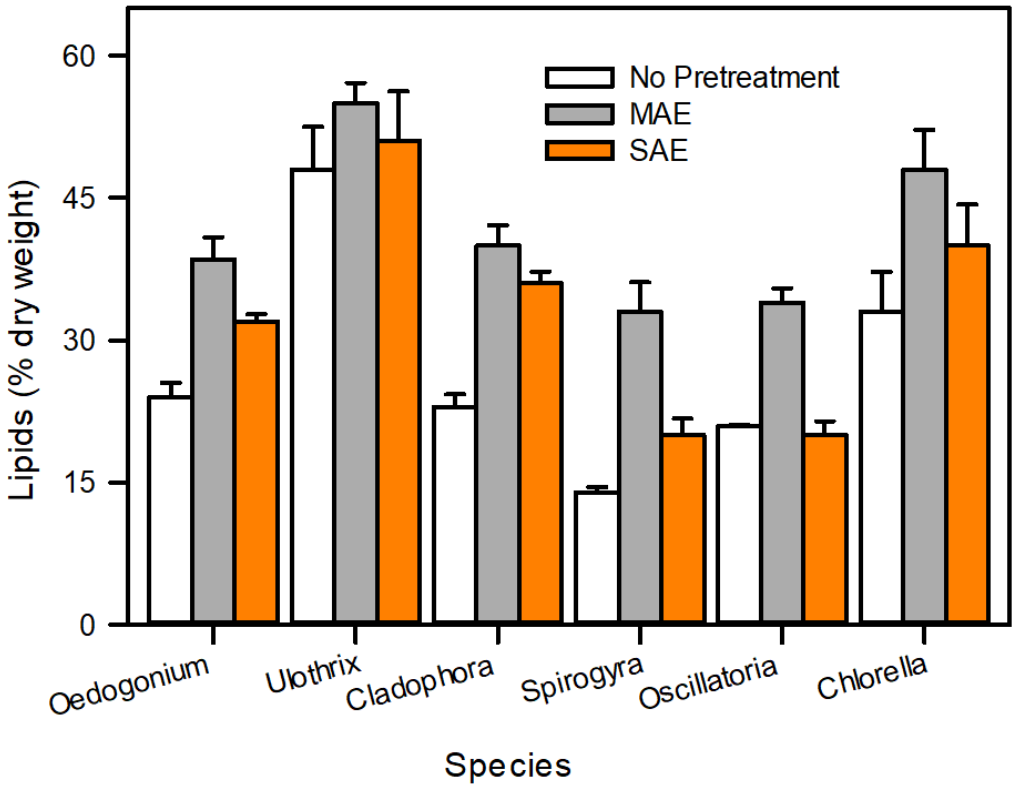


Figure 10. Comparison of algal lipids (%) after pretreatment with conventional lipid extraction method.

4. Conclusions

Pretreatment of microalgal biomass is essential for the maximal recovery of biofuel precursors from complex microalgal cell walls. In the present work, a microwave method was exploited to enhance the biodiesel yield from microalgal biomass. This study suggested that the applied pretreatment protocols are able to enhance the lipid contents, particularly in Ulothrix, for biofuel production. The application of MAE is concluded to be an efficient technique for lipid extraction under the given conditions, due to a higher oil yield, lower solvent consumption, and lower time duration of *Oedogonium* sp., *Oscillatoria* sp., *Ulothrix* sp., *Chlorella* sp., *Cladophora* sp., and *Spirogyra* sp. We conclude that the statistical design methodology offers an efficient and feasible approach for the optimization of lipid extraction parameters in the studied algal strains. An improved fatty acid composition due to pretreatment offers the production of biodiesel at a low cost, which can enhance the energy resources and address the energy crisis at a mass scale in Pakistan. Overall, the current research facilitates a cost-effective, green methodology for microalgal biomass pretreatment in a short duration. Further, scale-up studies and in-depth technological interventions, such as using microwave absorbers, are required to establish the process's credibility on a large scale.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020407/s1, Table S1: ANOVA results for developed quadratic model in Oedogonium (MAE); Table S2: ANOVA results for developed quadratic model in Oscillatoria (MAE); Table S3: ANOVA results for developed quadratic model in Ulothrix (MAE); Table S4: ANOVA results for developed quadratic model in Chlorella (MAE); Table S5: ANOVA results for developed quadratic model in Cladophora (MAE); Table S6: ANOVA results for developed quadratic model in Spirogyra (MAE); Table S7: ANOVA for developed quadratic model in Oedogonium (SAE); Table S8: ANOVA for developed quadratic model in Oscillatoria (SAE); Table S9: ANOVA for developed quadratic model in Ulothrix (SAE); Table S10: ANOVA for developed quadratic model in Chlorella (SAE); Table S11: ANOVA for developed quadratic model in Cladophora (SAE); Table S12: ANOVA for developed quadratic model in Spirogyra (SAE); Table S13: Fit Statistics for Response Surface Quadratic Model (MAE); Table S14: Fit Statistics for response Surface Quadratic Model (SAE).

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Abbreviations

Analysis of variance (ANOVA); Microwave assisted extraction (MAE); Ultrasound-assisted extraction (UAE); Response surface methodology (RSM); Central Composite Design (CCD); Triacylglyceride (TAG); FAME Fatty acid methyl ester; ASTM American Society for Testing and Materials; CN Cetane number; IV Iodine value; CFPP Cold filter plugging point; GCMS Gas chromatography-mass spectrometry; SV Saponification value; DU Degree of unsaturation; LCSF Long chain saturated factor; HHV Higher heating value; KV Kinematic viscosity, υ; ρ Density; MUFA Mono unsaturated fatty acid; PUFA Poly unsaturated fatty acid.

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