ORIGINAL ARTICLE

Changes in environmental conditions are critical factors for optimum biomass, lipid pattern and biodiesel production in algal biomass

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Abstract

Microalgae and their metabolites can be influenced by their environment, effects that can be investigated in a laboratory by modifying the composition of the cultivation medium. In this study, pH was varied around (4–10), temperature (10–45 °C), and sodium nitrate (0–5 g/L) concentration to assess the performance of *Chlorella* sp. and *Oedogonium* sp., which were isolated from water bodies from the Lahore district of Pakistan. A decreased algal growth rate was paralleled by an increase in the lipid levels. In dried samples of *Chlorella* sp. the lipid increased from 42% under optimal growth conditions to 73% in samples grown under nitrogen deficiency at 20 °C in a cultivation medium adjusted to pH 4. A similar result was found in cultures of *Oedogonium* sp. while the lipid content increased from 40 to 69%. These two algal strains were further esterified for biodiesel production and fuel properties were analyzed. In both algae, C16 and C18 fatty acids increased preferentially under stress. This was paralleled by improved fuel properties of the produced biodiesel. It can be suggested that microalgal lipid compositions enhanced by acclimatization to sub-optimal environmental conditions opens a new opportunity for the production of cost-effective biodiesel.

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Graphical Abstract
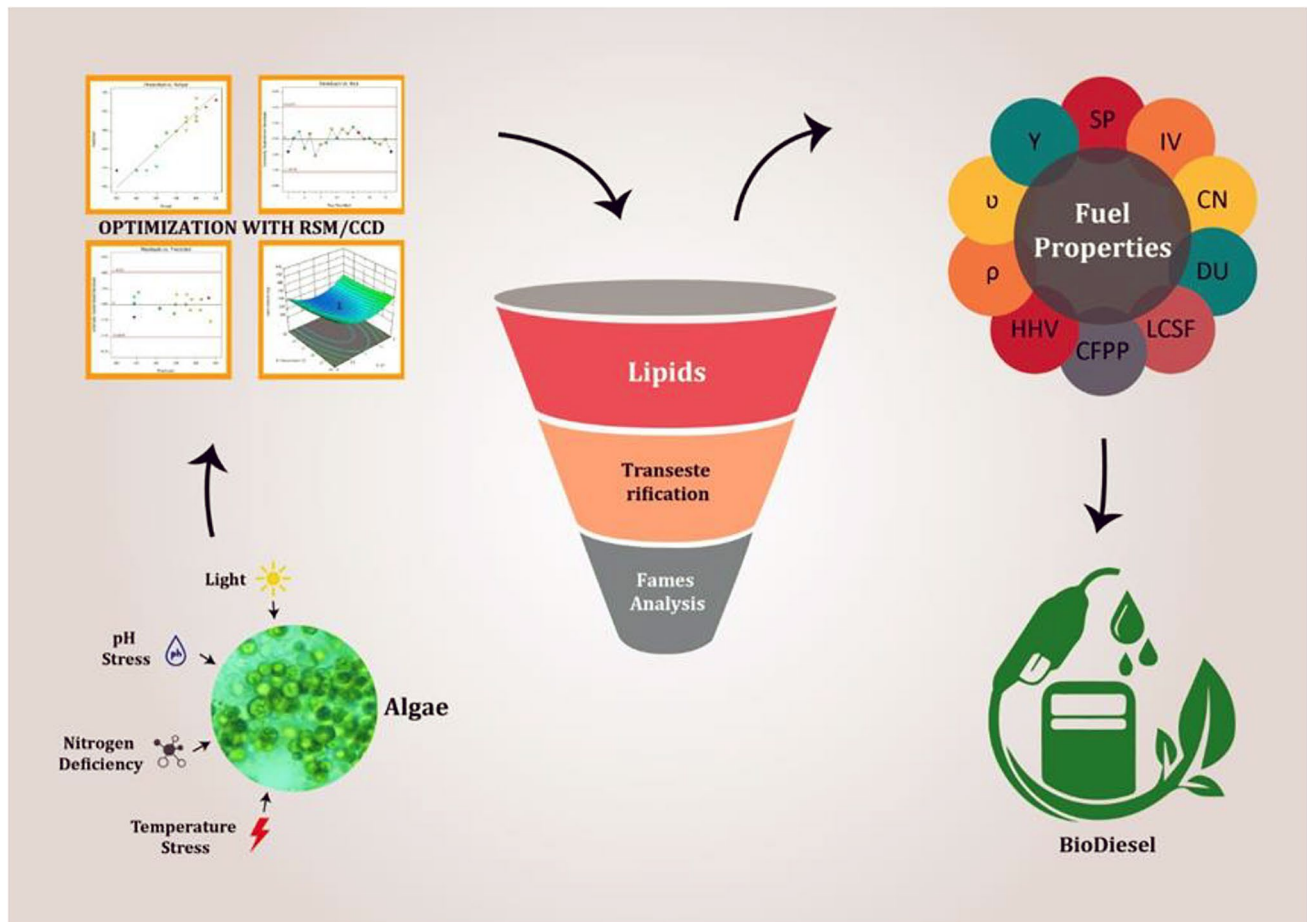
Abbreviations

ASTM	American Society for Testing and Materials
CCD	Central Composite Design
CFPP	Cold filter plugging point
CN	Cetane number
DU	Degree of unsaturation
FAME	Fatty acid methyl ester
GCMS	Gas chromatography-mass spectrometry
HHV	Higher heating value
IV	Iodine value
KV	Kinematic viscosity (υ)
LCSF	Long chain saturated factor
MUFA	Mono-unsaturated fatty acid
PAR	Photosynthetically active radiation
PUFA	Poly-unsaturated fatty acid
RSM	Response surface methodology
SFAs	saturated fatty acids
SV	Saponification value
TAG	Triacylglyceride
USFAs	Unsaturated fatty acids
ρ	Density

Introduction

Depleting energy resources, increase in population and global warming are the major ecological problems for sustainable environmental protection and development (Sharif et al. 2021; Kanwal et al. 2022). To address climate change energy sources other than those based on fossil fuels should be incorporated to meet increasing energy demands (Farooq and Gheewala 2018; Betz 2022). Biofuels are a renewable, non-polluting and sustainable energy sources that are mainly produced from plant materials (Abideen et al. 2015; Sirigeri et al. 2019). Currently various plant feedstock are used for biofuel production (Elkelawy et al.

2020; Chen et al. 2021). In addition to the high cost of bioenergy production from edible resources, the competition for arable land and water between bioenergy production and food production is a major concern (Abideen et al. 2012; Hasnain et al. 2021). For the production of sustainable and economical biofuels, abundant and cost-effective renewable resources are needed without competing with conventional agriculture This challenge can be overcome by using algae as feedstock for third generation biofuel production (Gul et al. 2013; Sekar et al. 2021). Utilization of algae in green energy research has garnered tremendous interest in the current scenario to meet the challenges faced by existing fuel sources (Banerjee et al. 2022). This exploitation of the algae appears to be feasible in warm climates, where they can be cultivated in open ponds throughout the year. A competition for fresh water can be resolved if algae are grown on waste water, brackish water, or alkaline and seawater systems (Hasnain et al. 2022).

It has been shown that the oil yield of algal biomass is 40 fold higher as compared to soybean and canola seeds (Petrie et al. 2020). This makes algae an attractive source for fuel generation (Kandasamy et al. 2022; Siddiki et al. 2022). Algal bioenergy feedstock has serval advantages over the use of terrestrial crops due to their higher growth rate, multiple harvest potential and mitigating CO_2 emission (Adeniyi et al. 2018; Thanigaivel et al. 2022). Dried algal biomass produces a diverse range of carbohydrate, that varies between (20–40%), which is valuable for the use of algae biomass for bio-ethanol production (Jakhar et al. 2022). Algal sp. could be used as a protein source (up to 60% dry weight) subsequent to extraction of lipids and carbohydrates the residual algal cake can be used for additives (Sun et al. 2020; Roman et al. 2021). Twenty four million tons of micro- and macro algae are currently farmed globally, with the majority being used in cosmetics and animal fodder (Munir et al. 2022). It has been demonstrated that the biochemical composition of algal cells and their photosynthetic performance are significantly affected by various environmental, operational factors, cultivation conditions and addition of nitrogen (Yang et al. 2020a, b; Li et al. 2021). Nitrogen is the most essential nutrient related to lipid metabolism in algae and improves the accumulation of lipids in *Nannochloropsis oculata* (Droop) D.J.Hibberd, 1981 and *Chlorella vulgaris* (Martinus) M.W.Beijerinck, 1890 under 25% nitrogen containing medium (Cao et al. 2014; Ru et al. 2020; Qiu et al. 2020). Nutrient deficiency, change of pH, temperature and light can all modify the rate of photosynthesis and metabolite composition (carbohydrates, proteins, chlorophyll and lipid) of algal cells (Barros et al. 2019; Andreotti et al. 2020). The growth rate of many algae is optimal at neutral pH, but it was reported that the TAG content of algal

biomass increased with higher pH of the growth medium (Zaher and Helal 2020). Algae can grow in the temperature range from 15 to 40 °C, depending on species, area and season (Hasnain et al. 2021; Karm and Dwaish 2021).

Algal blooms such asthose from *Chlorella* and *Oedogonium* sp. K. E. Hirn, 1900 could be a potential source of good quality biodiesel with little operational cost or enrgy consumption. *Chlorella*, a unicellular microalga, can be cultivated in waste and saline water. *Oedogonium* belongs to the family Oedogoniaceae and includes three genera: *Hulbochaete* C. A. Agardh, *Oedogonium* Link and *Oedocladium* Stahl. *Oedogonium* species with simple and unbranched filaments are mainly found on moist soil surfaces. Both these algal species have high lipid productivity but their physiology can be changed by their envonment (Yin et al. 2020b, a). Consequently, these genera of microalgae strains have gained the attention of many researchers and were used in the current study of biodiesel production. Many reports have mentioned that more than 10% by weight of the crude oil derived from *Chlorella vulgaris*, was composed of free fatty acids.

Our experimental approach used Response Surface Methodology (RSM) (Design Expert® software, version 11), a statistical tool to analyze multifactorial effects on several parameters (Hasnain et al. 2021; Pereira et al. 2021). The central composite design (CCD) can assess a single variable or many variables in combination with the particle response (Biomass and lipids) (Hasnain et al. 2021). Central composite design can reduced the duration of experimental runs as compared to other full factorial methods (Laid et al. 2021). We aimed to use this method as an optimizing strategy to improve the quality parameters of algae biomass (Behera et al. 2018). Several environmental stresses was varied in this study to assess the growth rate of algal cultures as well as on their lipid content and the suitability for biodiesel production of extracted lipids was observed. Our study focused on optimizing the pH, temperature and nutrients acquisition (nitrogen) in an open pond system. In order to protect the environment, we chose to use algal species native to our region of Pakistan. The present research extends the role of the environmental factors in growth and metabolite stimulation of local algal species *Chlorella* and *Oedogonium* for the first time. It was assuming that native strains from nature always grow better than non-native strains for industrial cultivation in changing environmental conditions, which suggests that native strains could be more profitable to the cost-effective algal biomass production. Thus, screening of suitable strains directly from nature was performed with the use of change in environmental factors in this article to reduce the cost of algal biomass production. To the best of our knowledge, little information was known to illustrate the isolating of Pakistani native microalgae strains that can grow for biomass production.

Materials and methods

Cultivation of microalgae

Samples of algae were collected from home waters in Lahore, Pakistan. Initially, algal samples were identified by microscopic analysis. Subsequently the species we cultivated were identified by partial 18S rDNA sequence analysis and the internal transcribed Spacer (ITS) region. DNA was extracted from collected samples using the CTAB method as described by Lipp et al. (1999). The 18S gene of both, *Chlorella* sp. and *Oedogonium* sp., was amplified by PCR (Meradd ICCC–MPTC02077) using the primers listed in Supplementary Table 1. PCR products were analyzed by agarose gel electrophoresis and bands were visualized under an UV trans-illuminator. PCR products were sequenced by the service provider Macrogen. The resultant sequences were compared to sequences stored in the Nucleotide database. Multiple sequences alignment was done using the Clustal Omega tool that constructed a phylogenetic tree as well (http://www.clustal.org/omega/#Download).

Optimization of parameters by response surface methodology (RSM)

In separate glass jars, 1 ml of fresh inoculum of *Chlorella* sp. and 1 g fresh weight of *Oedogonium* sp. were cultivated in Blue Green medium, consisting of: 17.6 mM $NaNO_3$, 0.23 mM K_2HPO_4, 0.3 mM $MgSO_4$, 0.24 mM $CaCl_2$, 0.031 mM citric acid, 0.021 mM ferric ammonium citrate, 0.0027 mM Na_2EDTA, 0.19 mM Na_2CO_3, and 1 mM $Na_2S_2O_3$. In our experiments this medium was used in all control samples, where the pH was adjusted to 6.5. When indicated, $NaNO_3$ was omitted from the medium. For optimal lipid production the cultivation parameters such as pH, temperature and nutrients availability (nitrogen) were optimized by CCD (RSM). Algae were cultured in a 16:8 h light: dark regime with white LEDs (light emitting diodes) of 80 $\mu mol/m^2$ photo-synthetically active radiation (PAR). Glass jars has been used for cultivation of algae species. Biomass and lipid content were evaluated by RSM analysis in 20 runs with varying combination of the three parameters, pH, temperature and nitrogen supply for 14 days and growth of the cultures was monitored daily.

Lipids extraction

A microwave extraction method was used for the lipid extraction. Dried algal sample (1 g) was suspended in 50 ml of methanol: chloroform (1:2) mixture. The mixture was heated by microwave (power set to 800 W) for 120 s. According to the method of Munir et al. (2021) extracted lipids were filtered by filter paper and then dried.

Biodiesel analysis by GCMS

Each 1 g of extracted lipid of all algal strains studied was base trans-esterified with 1.5% (by weight) potassium hydroxide (KOH) and a lipid: methanol ratio (vol/vol) of 1:6 in a 25 mL round bottom flask placed on a hot plate stirrer (AREX-6 Digital PRO) using a reflux condenser system for 120 min. After completion of trans-esterification, the reaction mixture was cooled to room temperature and centrifuged at 3000 rpm to obtain upper layer of fatty acid methyl ester (biodiesel) and lower layer (glycerol). The biodiesel upper layer was washed with warm distilled water at 50 °C to remove traces of catalyst and methanol. Recovered FAMEs were analyzed by gas chromatography mass spectrometry (GCMS) performed using a DB-5 column (length = 3 cm and thickness = 0.5 μm) with an injector temperature of 220 °C; the oven temperature was raised from 110 to 280 °C at a rate of 10 ºC/minute with a flow rate of 1 ml/minute. Helium gas was used as a carrier gas. Ionizing voltage was of 70 eV. Mass spectrometer was scanned at a range of 40 to 950 m/z. 1 μl of each sample was injected (Sharmila and Rebecca 2012).

Mass balance of the biodiesel produced

The mass balance of the biodiesel produced was calculated using Eq. (1), which showed the ratio between mass of biodiesel produced and summation of mass of catalyst, methanol, and lipids used in the trans-esterification reaction (Velásquez et al. 2007);

Mass balance of biodiesel produced(%)

$$= \frac{Mass\ of\ biodiesel\ produced}{Mass\ of\ KOH + Mass\ of\ methanol + Mass\ of\ lipids} \tag{1}$$

Fuel properties

Fuel properties-energy content, cetane number, iodine value, saponification valve, oxidation stability, cold filter plugging point, density and kinematic viscosity (Table 1) were calculated as described by Islam et al. (2013). The following equations were used:

Statistical analysis

Analysis of variance (ANOVA) and least significant difference were performed to analyze the data of central composite design (DESIGN-EXPERT 11).

Table 1 Fuel parameters with their equations

Parameter	Equations
Iodine value (IV)	$IV = \sum(254 \times Di \times Ni/Molecular\ weight\ of\ the\ ith\ fatty\ acid)$ D_i = the number of double bonds of the ith FAME N_i = the percentage of each FAME
Saponification value (SV)	$SV = \sum(560 \times Ni/Molecular\ weight\ of\ the\ ith\ fatty\ acid)$ N_i = percentage of each FAME
Cetane number (CN)	$CN = 46.3 + (5458/saponification\ value) - (0.225 \times Iodine\ value)$
Degree of unsaturation (DU)	$DU = \sum MUFA + (2 \times PUFA)$ MUFA = mass fraction of mono-unsaturated fatty acids PUFA = mass fraction of poly-unsaturated fatty acids
Long chain saturation factor (LCSF)	$LCSF = (0.1 \times C16:0) + (0.5 \times C18:0) + (1 \times C20:0) + (2 \times C24:0)$
Cold filter plugging point (CFPP)	$CFPP = (3.1417 \times LCSF) - 16.477$
Gross energy = Higher heating value (HHV)	$HHVi = 46.19 - 1794/Mi - 0.21 \times N$ Mi = molecular weight of the ith FAME N = number of double bond of the ith FAME
Density (ρ)	$\rho = 0.8463 + 4.9/Mi + 0.0118 \times N$ Mi = molecular weight of the ith FAME N = number of double bond of the ith FAME
Kinematic viscosity (υ)	$ln(\upsilon i) = -12.503 + 2.496 \times ln(Mi) - 0.178 \times N$ Mi = molecular weight of ith FAME N = number of double bond of ith FAME
Predictive Oxidation stability (Y)	$Y = 117.9295/X + 2.5905(0 < 100)$ X = percentage of linoleic and linolenic acids $(0 < X < 100)$ Y = Oxidation stability in hours

Results

Identification of algae

Chlorella sp. and *Oedogonium* sp. were identified on the basis of size and shape under microscope as shown in Fig. 1. This was followed by DNA extraction and subsequent molecular identification based on18S rDNA. Figure 2 shows PCR products having clear bands on 1.5% agarose gel resembling a product size of 400 bp, thus allowing molecular verification of *Chlorella* sp. and *Oedogonium* sp. The sequences were assembled and compared using the tool CLUSTALW and phylogenetic tree was constructed in MegaX. The phylogenetic tree showed the resemblance and relation of different algal species of *Chlorophyceae* (Fig. 3). Phylogenetic tree showed query sequence KU865576 has 100% similarly with *Oedogonium* sp. and query sequence KU563009 has 80% similarly with *Chlorella* sp.

Effect of cultivation parameters on algae biomass

Both algae species, *Chlorella* sp. and *Oedogonium* sp. showed a maximal biomass production of 5 g dry mass in BG medium supplied with extra sodium nitrate to reach a final concentration of 30 mM. In this medium the pH value was adjusted to pH 6.5 and algae were cultivated at a temperature of 30 °C. In the following tests this growth condition was used as a control. Biomass production was significantly reduced to 2.2 g and 2.0 g in cultures of *Chlorella* sp. and *Oedogonium* sp. respectively, if a nitrate deficient (no added nitrate) BG medium was inoculated, and cultivation took place at 20 °C and the pH of the medium had been adjusted to pH 4.0. In a series of experiments, nitrate supply, temperature and pH was modified gradually. All pretreated conditions of environmental stress resulted in reduced biomass production as compared to the control experiment. Data of Tables 2 and 3 were used for modeling analysis.

Effect of cultivation parameters on lipid content

Lipid production was monitored in the experimental set up in parallel to measuring biomass production. In the control experiment, dried algae biomass of 62% and 60% lipids in *Chlorella* sp. and *Oedogonium* sp., respectively, was used. The lipid contents of *Chlorella* sp. and of *Oedogonium* sp. were 62% and 60%, respectively. There was a strict negative correlation between algal biomass production and build-up of lipid content (see Tables 2 and 3; Supplementary Tables S1 and S2 show the statistical analysis of the

Fig. 1 Microscopic image of *Chlorella* sp. and *Oedogonium* sp. at 10× and 40× respectively. **a**) *Chlorella* sp. **b**) *Oedogonium* sp.
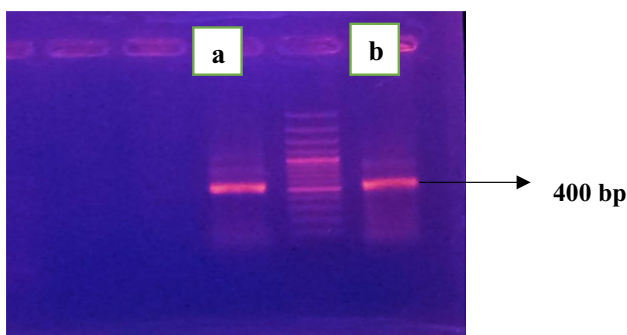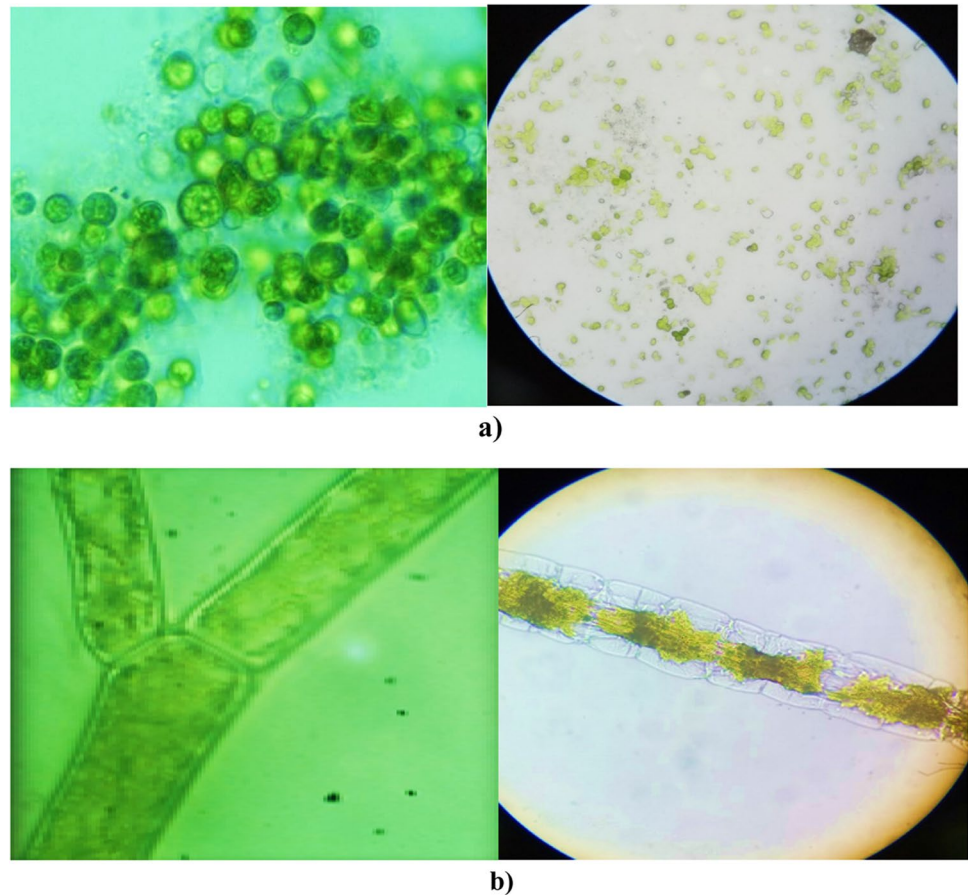

a)

b)

Fig. 2 1 kb DNA Ladder and PCR Amplification of 18S rDNA gene in Algal Strains **a**) *Chlorella* sp. **b**) *Oedogonium* sp.
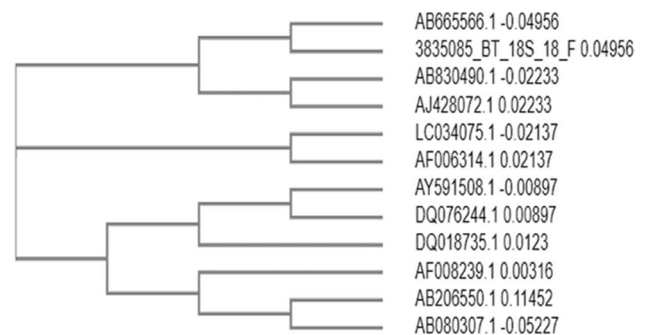

Fig. 3 Phylogenetic tree

response surface quadratic model for *Chlorella* sp. and *Oedogonium* sp. respectively.

Analysis of Variance

The ANOVA analysis resulted a F-value of 6.99 and a P-value less than 0.0500 for the lipid content of *Chlorella* sp. This indicated that model terms were significant. There was only 0.27% chance of noise. A F-value of 26.04 and

a P-values less than 0.0500 found in the ANOVA analysis of *Oedogonium* sp. biomass showed that model terms were also significant. There was only 0.01% chance of noise. ANOVA analysis of results of the lipid content of *Oedogonium* sp. resulted a F-value of 6.84. There is only a 0.30% chance of noise in an F-value. The P-values is less than 0.0500. In case of *Oedogonium* sp. the F-value and degree of freedom of biomass response was 26.04

Table 2 Statistical analysis of response surface quadratic model for *Chlorella* sp

Std	Run	Factor 1 A:pH	Factor 2 B:Temperature	Factor 3 C:Nitrogen Concentration	Response 1 Biomass	Response 2 Lipid Content
			°C	g/L	g	%
20	1	9	40	5	2.8	52
16	2	6.5	45	2.5	4.2	54
4	3	4	20	0	2.2	73
7	4	6.5	30	2.5	5	62
14	5	2.2	30	2.5	3	42
3	6	4	20	5	3.4	58
1	7	9	20	0	2.4	68
5	8	4	40	5	2.8	45
12	9	6.5	10	2.5	3.7	60
19	10	6.5	30	2.5	5	62
13	11	6.5	30	2.5	5	62
2	12	6.5	30	2.5	5	62
11	13	6.5	30	0	3.6	63
10	14	6.5	30	2.5	5	62
15	15	10	30	2.5	2.8	49
17	16	4	40	0	2.3	66
18	17	9	40	0	2.4	61
6	18	6.5	30	2.5	5	62
8	19	9	20	5	3.2	50
9	20	6.5	30	5	4.5	61

Table 3 Statistical analysis of Response Surface Quadratic Model for *Oedogonium* sp

Std	Run	Factor 1 A:pH	Factor 2 B:Temperature	Factor 3 C:Nitrogen Concentration	Response 1 Biomass	Response 2 Lipid Content
			°C	g/L	g	%
8	1	9	40	5	2.5	50
12	2	6.5	45	2.5	4	52
1	3	4	20	0	2	69
19	4	6.5	30	2.5	5	60
9	5	2.2	30	2.5	2.8	40
5	6	4	20	5	3.2	56
2	7	9	20	0	2.2	66
7	8	4	40	5	2.6	43
11	9	6.5	10	2.5	3.5	58
18	10	6.5	30	2.5	5	60
20	11	6.5	30	2.5	5	60
16	12	6.5	30	2.5	5	60
13	13	6.5	30	0	3.3	61
15	14	6.5	30	2.5	5	60
10	15	10	30	2.5	2.4	47
3	16	4	40	0	2.1	64
4	17	9	40	0	2.2	59
17	18	6.5	30	2.5	5	60
6	19	9	20	5	3	48
14	20	6.5	30	5	4.5	59

Table 4 Equations of the quadratic models in terms of coded factors

Algae	Response	Equations of the quadratic model
Chlorella sp.	Biomass	$+5.05 - 0.1257 * A - 0.0901 * B + 0.4300 * C + 0.0125 * AB - 0.0625 * AC - 0.1375 * BC - 0.8950 * A^2 - 0.3905 * B^2 - 1.01 * C^2$
	Lipid content	$+61.38 - 0.7192 * A - 2.78 * B - 6.40 * C - 1.88 * AB + 1.13 * AC + 0.3750 * BC - 6.10 * A^2 - 1.35 * B^2 + 4.38 * C^2$
Oedogonium sp.	Biomass	$+5.02 - 0.1684 * A - 0.1070 * B + 0.4500 * C + 0.0000 * AB - 0.0750 * AC - 0.1500 * BC - 0.9924 * A^2 - 0.4403 * B^2 - 1.07 * C^2$
	Lipid content	$+59.39 - 0.5654 * A - 2.63 * B - 6.20 * C + 1.63 * AB + 0.8750 * AC + 0.1250 BC - 6.10 * A^2 - 1.34 * B^2 + 4.17 * C^2$

Table 5 Statistical analysis of quadratic model

Factors	*Chlorella* sp.		*Oedogonium* sp.	
	Biomass	Lipid Content	Biomass	Lipid Content
R^2	0.9646	0.8628	0.9591	0.8603
Adjusted R^2	0.9327	0.7393	0.9222	0.7346
Adequate Precision	14.1252	9.8179	13.0197	9.9179
Std. Dev	0.2849	3.9700	0.3351	3.9100
Mean	3.6900	58.7500	3.5400	56.6500
C.V %	7.7200	6.7600	9.4700	6.9100

and 9; the F-value and degree of freedom of lipid content response were 6.84 and 9, respectively. For *Chlorella* sp. the F-value and degree of freedom of biomass response was 30.28 and 9, while the F-value and degree of freedom of lipid content response were 6.99 and 9, respectively.

Equations

RSM suggest a quadratic model predicted the response parameters biomass and the lipid contents (Table 4). A, B, C represent the independent variables pH, temperature and Nitrogen deficiency, respectively. The positive Sign in the equation symbol indicates an increased interaction of parameters (pH, temperature and Nitrogen deficiency) towards the responses (biomass and lipid content), while the negative symbol indicates less interaction. In this experiment all the investigated parameters have positive or increasing effect on biomass and lipid content.

Fit statistics

R^2 should be greater than 0.8 for the data to fit the model: Table 5 showed all the values of R^2 were greater than this value, indicating, a fit of the experimental data to the model. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 was desirable. In the present study all values were greater than 4 so, these model can be used to navigate the design space. C.V % should be less than 10. In the given study all the C.V % values were less than 10 confirming good witness of all models of *Chlorella* sp. and *Oedogonium* sp.

The general quadratic equation for response is:

$$Y = \beta 0 + \sum_{i=1}^{N} \beta i x i + \sum_{i=1}^{N} \beta i i x i i + \sum_{i<j}^{N} \beta i j x i x j + \varepsilon$$

In the above given equation, β_0 indicates the constant, while β_{ii} was coefficient of quadratic parameter, β_{ij} represents the coefficient of interaction parameters. The X_i and X_j represents the factors.

Table 6 Optimal conditions and model validation

Factors	*Oedogonium* & *Chlorella* sp.	pH	Temp °C	Nitrogen g/L	Biomass (g)		Lipids (%)	
					Experimental	Predicted	Experimental	Predicted
Optimum condition	*Oedogonium*	4	20	0	2.12	2	68.13	69
	Chlorella	4	20	0	2.35	2.2	71.58	73

Calculated and Predicted value of biomass and lipid by using quadratic equations for *Oedogonium* sp. were as follows:

Equation for biomass response (Y_1) is:

$$Y_1 = 5.02253 - 0.168394X_1 - 0.107022X_2$$
$$+ 0.45X_3 - 4.47332e^{-16}X_1X_2 - 0.075X_1X_3$$
$$- 0.15X_2X_3 - 0.992385X_1{}^2 - 0.440287X_2{}^2 - 1.0664X_3{}^2$$

Equation for lipid content response (Y_2) is:

$$Y_2 = 59.3906 - 0.565403X_1 - 2.63219X_2$$
$$- 6.2X_3 + 1.625X_1X_2 + 0.875X1X_3$$
$$+ 0.125X_2X_3 - 6.10339X_1{}^2 - 1.3414X_2{}^2$$
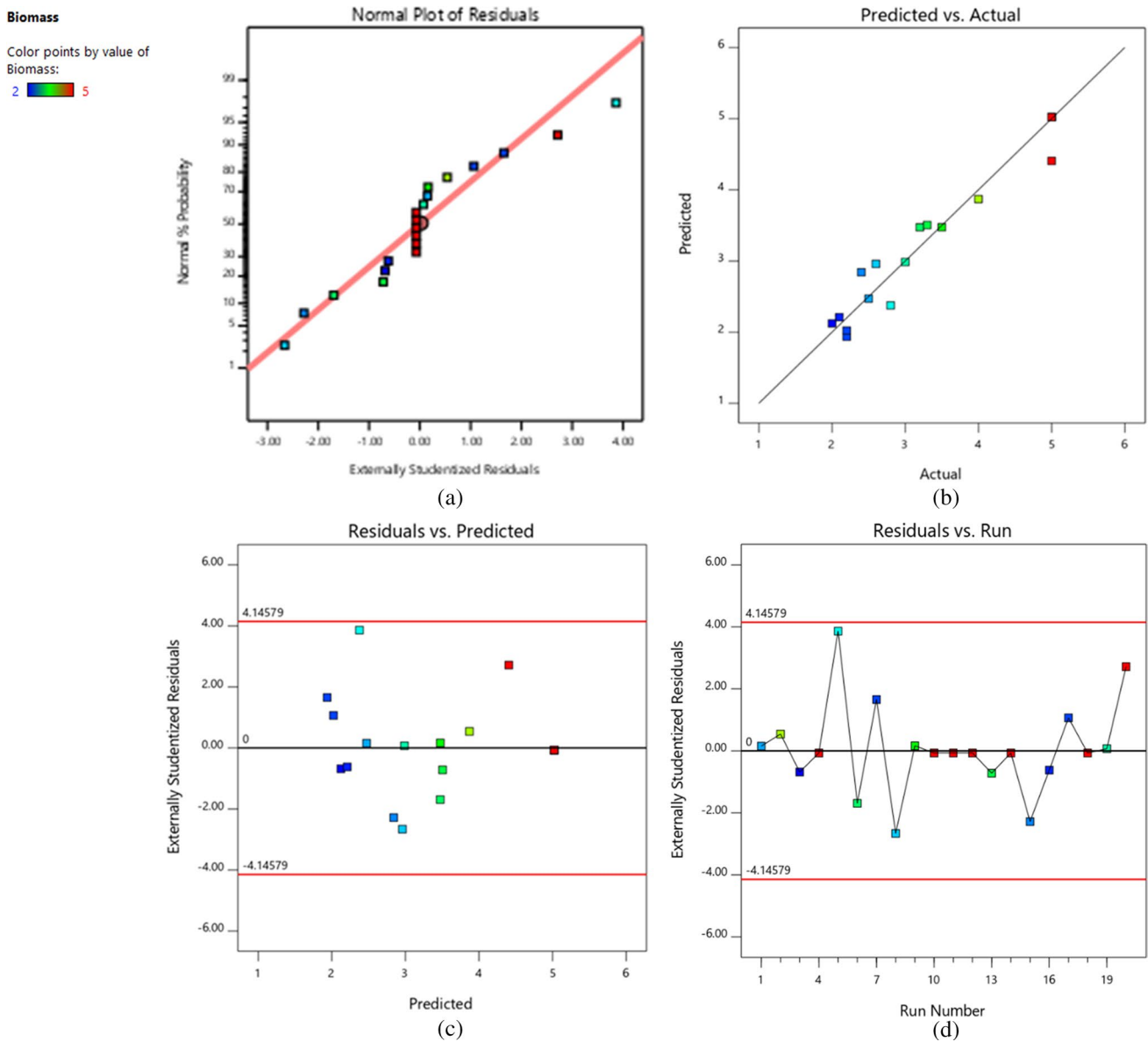$$+ 4.16527X_3{}^2$$
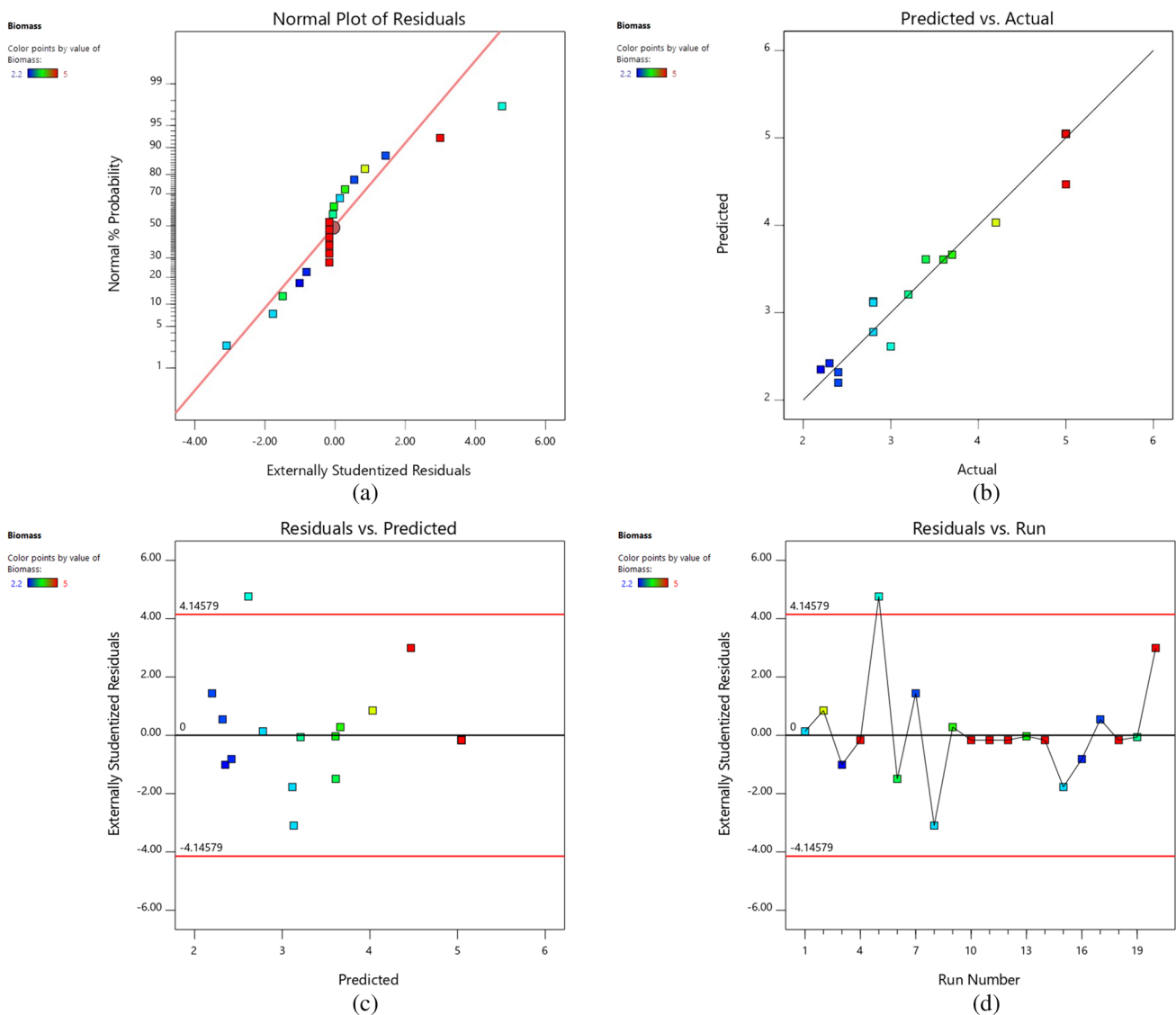
Fig. 4 Diagnostic plots for Biomass of *Chlorella* sp. (**a**) Normal probability plot (**b**) Predicted versus actual values plot (**c**) Studentized residuals versus predicted values plot (**d**) Studentized residuals versus run number plot
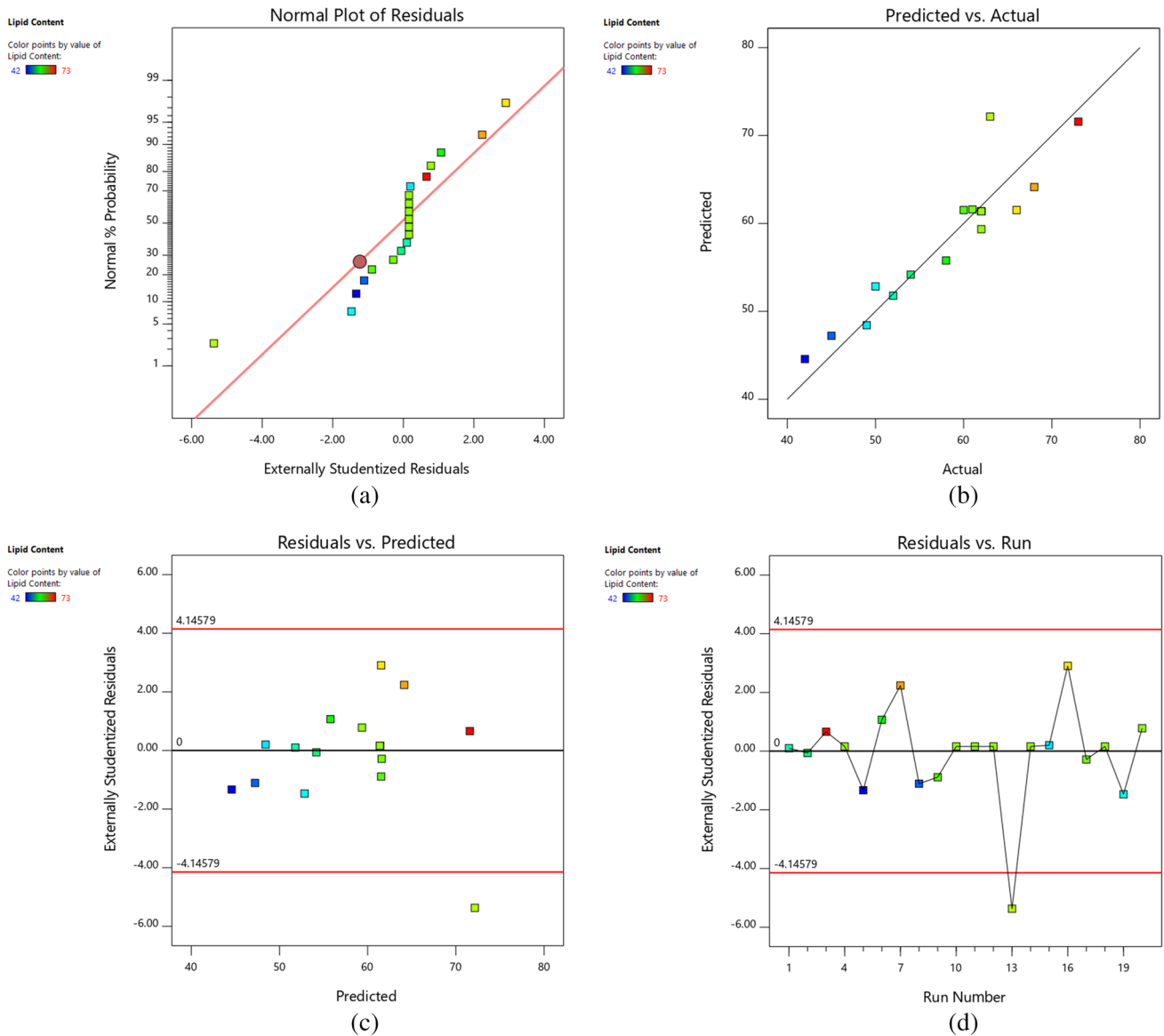
Fig. 5 Diagnostic plots for Lipid Content of *Chlorella* sp. (**a**) Normal probability plot (**b**) Predicted versus actual values plot (**c**) Studentized residuals versus predicted values plot (**d**) Studentized residuals versus run number plot

Calculated and Predicted value of biomass and lipid by using quadratic equations for *Chlorella* sp. were as follows:

Equation for biomass response (Y_1) is:

$$Y_1 = 5.04577 - 0.125688X_1 - 0.0900596X_2$$
$$+ 0.43X_3 + 0.0125X_1X_2 - 0.0625X_1X_3$$
$$- 0.1375X_2X_3 - 0.895023X_1{}^2$$
$$- 0.300537X_2{}^2 - 1.00732X_3{}^2$$

Equation for lipid content response (Y_2) is:

$$Y_2 = 61.3809 - 0.71915X_1 - 2.77597X_2$$
$$- 6.4X_3 + 1.875X_1X_2 + 1.125X_1X_3$$
$$+ 0.375X_2X_3 - 6.09617X_1{}^2$$
$$- 1.35093X_2{}^2 + 4.37682X_3{}^2$$

Fig. 6 Diagnostic plots for Biomass of *Oedogonium* sp. (**a**) Normal probability plot (**b**) Predicted versus actual values plot (**c**) Studentized residuals versus predicted values plot (**d**) Studentized residuals versus run number plot

Calculated and predicted value of biomass and lipid by using quadratic equations

The optimized condition for maximum lipids were pH 4, 20 °C and no nitrogen added. The experimental and predicted values for *Oedogonium* sp. biomass were 2.12 g and 2.00 g while for *Chlorella* sp. biomass were 2.35 g and 2.20 g. The experimental and predicted values for lipid extracted from *Oedogonium* sp. were 68.13% and 69.00% while for *Chlorella* sp. were 71.58 g and 73.00%. From the results, the calculated values were close

to predicted values and we concluded that developed model could predict the biomass and lipid content accurately (Table 6).

Diagnostic of models

The diagnostic analysis of data showed that the model fits well to *o*ptimize the independent variables (pH, temperature and nitrogen deficiency). A standard probability plot indicates whether the residuals follow the normal probability distribution by following a straight line. Figures 4
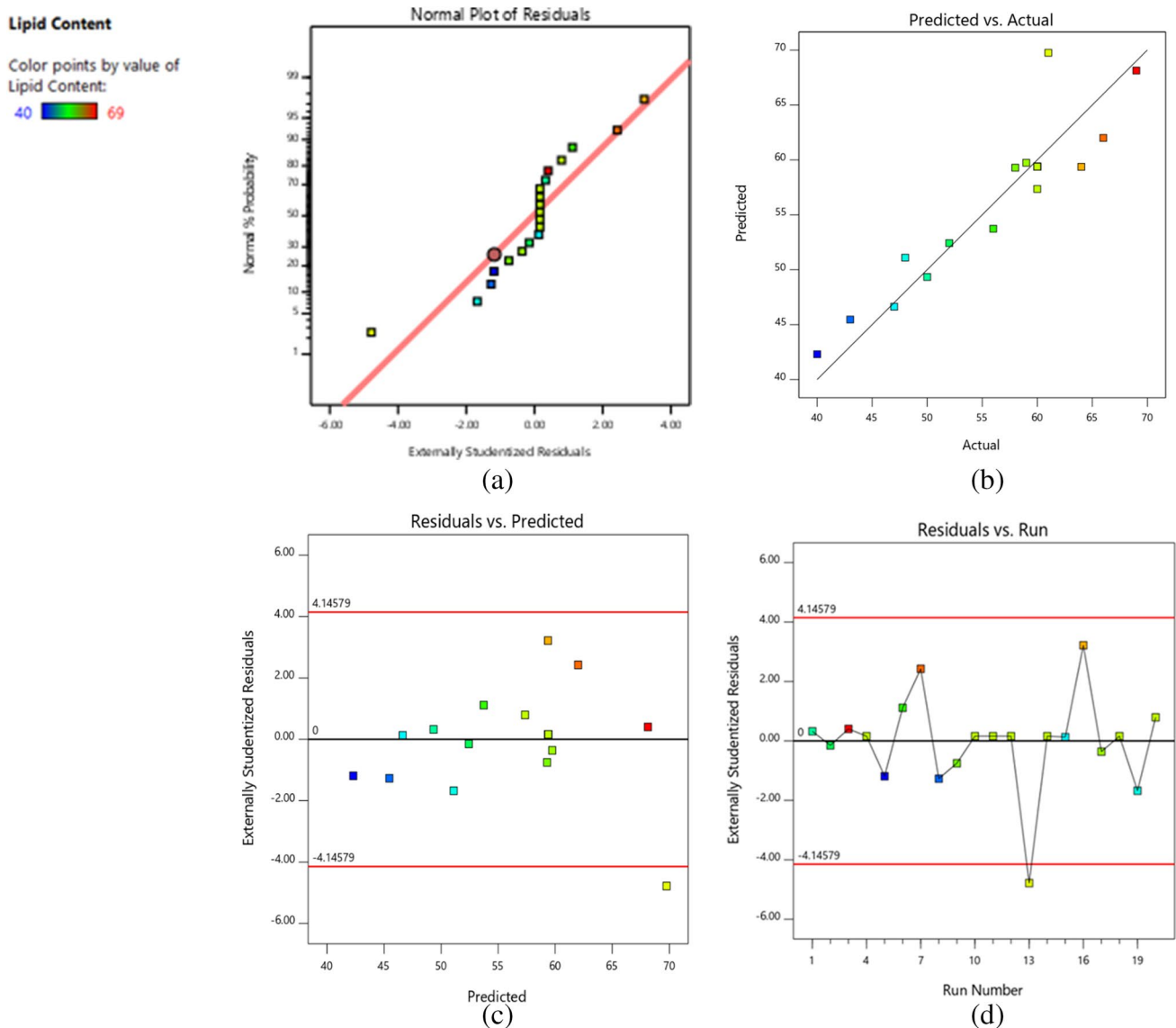
Fig. 7 Diagnostic plots for Lipid Content of *Oedogonium* sp. (**a**) Normal probability plot (**b**) Predicted versus actual values plot (**c**) Studentized residuals versus predicted values plot (**d**) Studentized residuals versus run number plot

and 5 illustrate the normal plot of externally studentized residuals on a probit scale. The maximum number of colors depicted biomass and lipid content of *Chlorella* sp., located in a narrow range on a normal probability line: minor significant points deviated from the normal line. Predicted versus actual plot as a parity plot of the reduced model was depicted between predicted and actual values of average biomass and lipid content of *Chlorella* sp., (Fig. 4 and 5). The dots with various colors showed the experimental values while straight line showed the predicted values. A fairly uniform random scatter of points gathered at a diagonal line. This graph was linear passing through the origin, which

signified the experimentally obtained values of biomass and lipid content with a slight deviation were in close agreement with predicted values using optimization methodology.

In Fig. 4 and 5 a plot of externally studentized residuals against the predicted values revealed that all color points exhibiting the average biomass and lipid content of *Chlorella* sp. Figures 4 and 5 graphically represented the residual versus order of the experimental run, indicating a random uniform scatter of points and absence of obvious pattern. Therefore, the model was adequate and we have no reason to suspect any violation of independence in all runs of *Chlorella* sp.
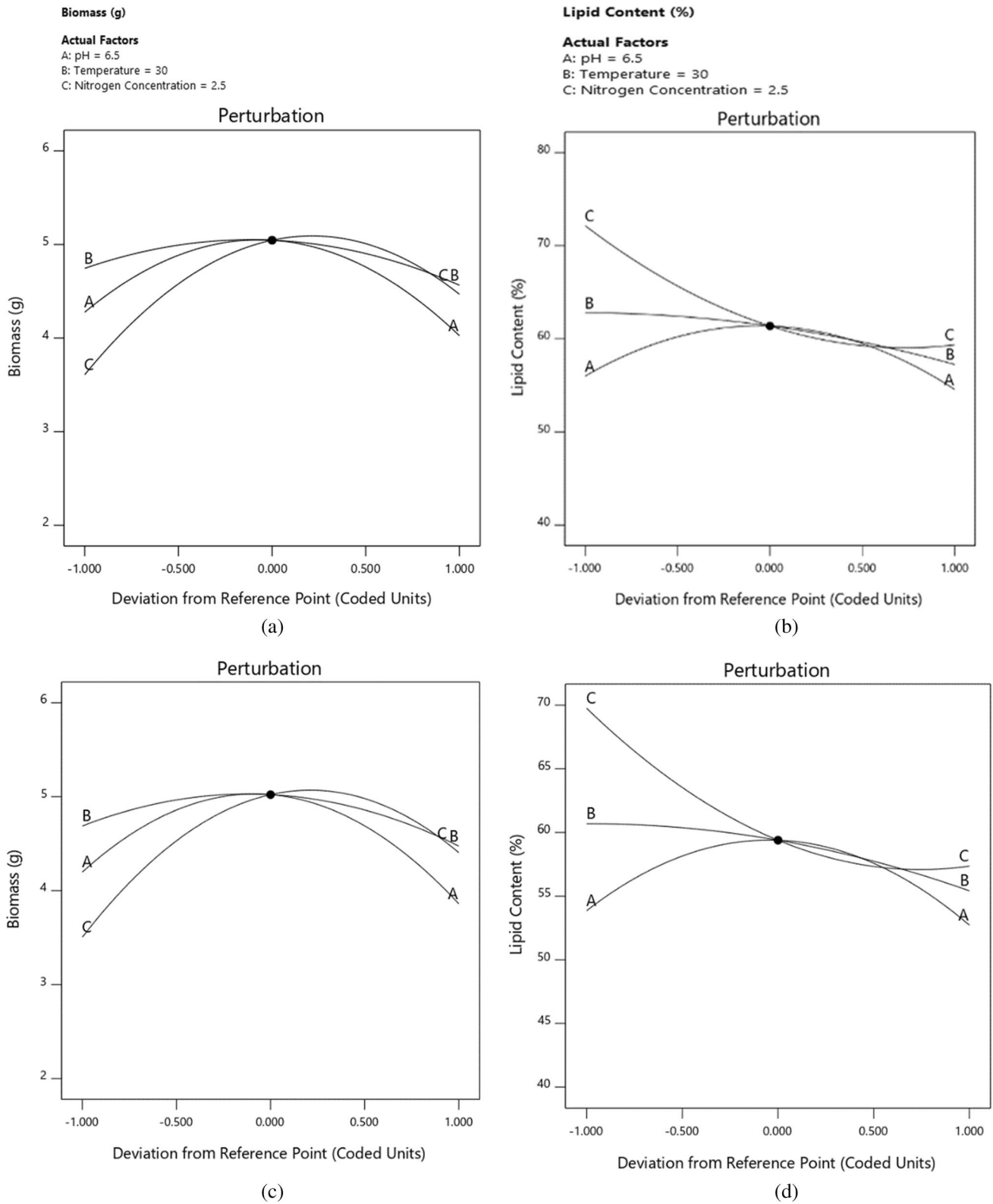
Biomass (g)

Actual Factors
A: pH = 6.5
B: Temperature = 30
C: Nitrogen Concentration = 2.5

Lipid Content (%)

Actual Factors
A: pH = 6.5
B: Temperature = 30
C: Nitrogen Concentration = 2.5

(a)

(b)

(c)

(d)

Fig. 8 Perturbation plots. (**a**) Biomass of *Chlorella* sp. (**b**) Lipid Content of *Chlorella* sp. (**c**) Biomass of *Oedogonium* sp. (**d**) Lipid Content of *Oedogonium* sp

Figures 6 and 7 illustrated the normal plot of externally studentized biomass and lipid content of *Oedogonium* sp. minor significant points deviated from the normal line. A uniform random scatter of points gathered at a diagonal line (Figs. 6 and 7). The plot of externally studentized residuals against the predicted values reveals that all color points exhibiting the average biomass and lipid content of *Oedogonium* sp. (Figs. 6 and 7). Figures 6 and 7 graphically represented the residual versus order of the experimental run, indicating a random uniform scatter of points and absence of obvious pattern. Therefore, the model was adequate and have no reason to suspect any violation of independence in all runs of *Oedogonium* sp.

Interaction of operating factors

Perturbation plot

The Perturbation plot of biomass and lipid yield of *Chlorella* sp. demonstrates that the nitrogen content has the steeper slope compared to the pH and temperature (Fig. 8). We infer that the response biomass and lipid yield was more sensitive to nitrogen content than that of pH and temperature. The Perturbation plot of biomass and lipid yield of *Oedogonium* sp. demonstrated that the factor C (nitrogen content) has the steeper slope compared to the factor A and factor B, (pH and temperature respectively) (Fig. 8). It implicated that the response biomass and lipid yield was more sensitive to nitrogen content than that of pH and temperature.

Response surface plots

Contour and three-dimensional plots provided a visualization of the relationship between the response and interaction between operating variables. The effect of pH, temperature and nitrogen concentration on algal biomass and lipid quantity is shown in Fig. 9. The biomass was observed decreasing in both tested algal strain with decreasing pH, temperature and in the absence of nitrogen as well as by increasing pH, temperature and nitrogen concentration as compared to controls. The trends were the same in both algal strain. Initially, the lipid quantity increased with decreasing pH from 6.5 to 4, temperature 30 °C to 20 °C and nitrogen concentration from 2.5 g/L to 0 g/L while further decreasing the pH (2) lipid content was start to decrease. Moreover, after increasing the pH from 6.5 to 9, temperature 30 °C to 40 °C and nitrogen concentration from 2.5 g/L to 5 g/L pH, lipid content started to decrease. Figure 9. illustrates

two variables one of which was zero at a time. The maximum predicted value relies on two variables at a time. The trends were same in both algal strain.

The relative effect of pH, temperature, and nitrogen deficiency on Biomass of *Oedogonium* sp. is given in Fig. 9. It was evident from the 3D plot, that the maximum biomass in *Oedogonium* sp. was recorded at pH 6.5, temperature 30 °C and nitrogen content of 2.5 g/L. The 2D contour response surface plot of biomass of *Oedogonium* sp. with varying pH, temperature and nitrogen concentration showed the concentration of red color increases at pH 6.5, temperature 30 °C and nitrogen content of 2.5 g/L. In the 3D plot, the maximum lipid Content in *Oedogonium* sp. was recorded at pH 4, temperature 20 °C and nitrogen content of 0 g/L (Fig. 9). The 2D contour response surface plot of lipid content of *Oedogonium* sp. with varying pH, temperature, and nitrogen concentration showed the concentration of red color increased at 6.5 pH. In contrast, the lipid content decreased at this pH value. The concentration of red color increased at temperature 30 °C. Nitrogen content caused a decrease in the concentration of red color at 2.5 g/L. By further decreasing the amount of nitrogen to 0 g/L caused an increase in the concentration of red color. The lipid content of *Chlorella* sp. was more than that of *Oedogonium* sp.

Biodiesel analysis

In the present study, highest extracted lipid content from *Chlorella* sp. (73%) and *Oedogonium* sp. (69%) at pH 4, 20 °C and 0 g/L nitrogen concentration was further transesterified into biodiesel. GC–MS analysis of biodiesel from *Chlorella* sp. and *Oedogonium* sp. before and after stresses is given in Table 7. A total 15 to 17 fatty acids were detected in both *Chlorella* sp. and *Oedogonium* sp. under optimum condition while 13 to 16 were detected in stress conditions. In the present study, contents of C16:0, C16:1 and C18:1 fatty acids increased under stress (4 pH, 20 °C and 0 g/L nitrogen) in both *Chlorella* sp. and *Oedogonium* sp., while contents of other fatty acids decreased, with the exception of C21:0 and C22:1, which were detected only in *Chlorella* sp. under optimal conditions. In *Chlorella* sp. C16:2, C14:1 and C18:0 were not detected. In the current study, the percentage of saturated fatty acids and poly-unsaturated fatty acids increased by lowering pH, temperature and nitrogen while mono-unsaturated fatty acids decreased in both *Chlorella* sp. and *Oedogonium* sp.
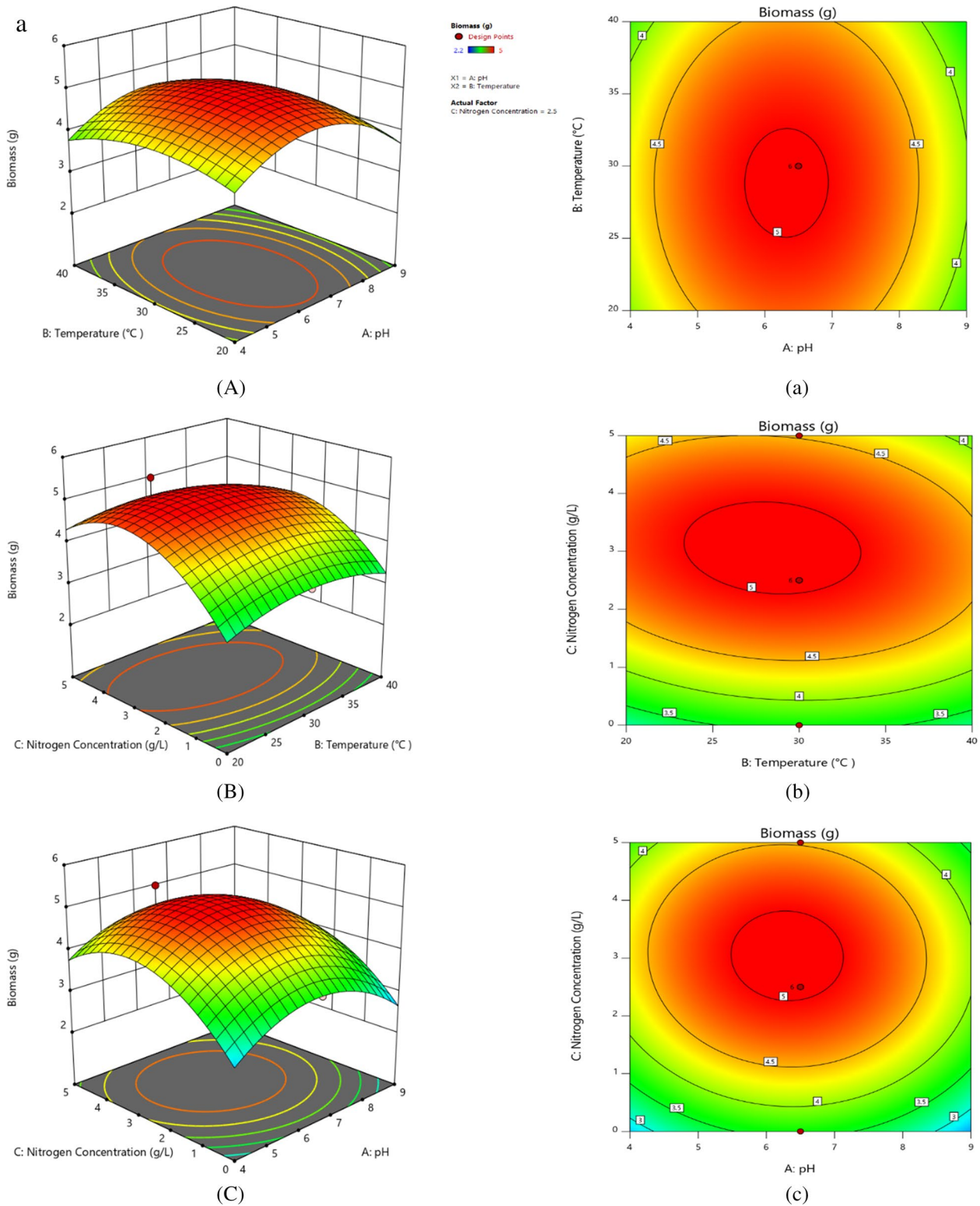
(A)

(a)

(B)

(b)

(C)

(c)

Fig. 9 **a**. 3D and 2D Response Surface Plots of Biomass of *Chlorella* sp. **b**. 3D and 2D Response Surface Plots of Lipid Content of Chlorella *sp.* **c**. 3D and 2D Response Surface Plots of Biomass of *Oedogonium* sp. **d**. 3D and 2D Response Surface Plots of Lipid Content of *Oedogonium* sp

(A)

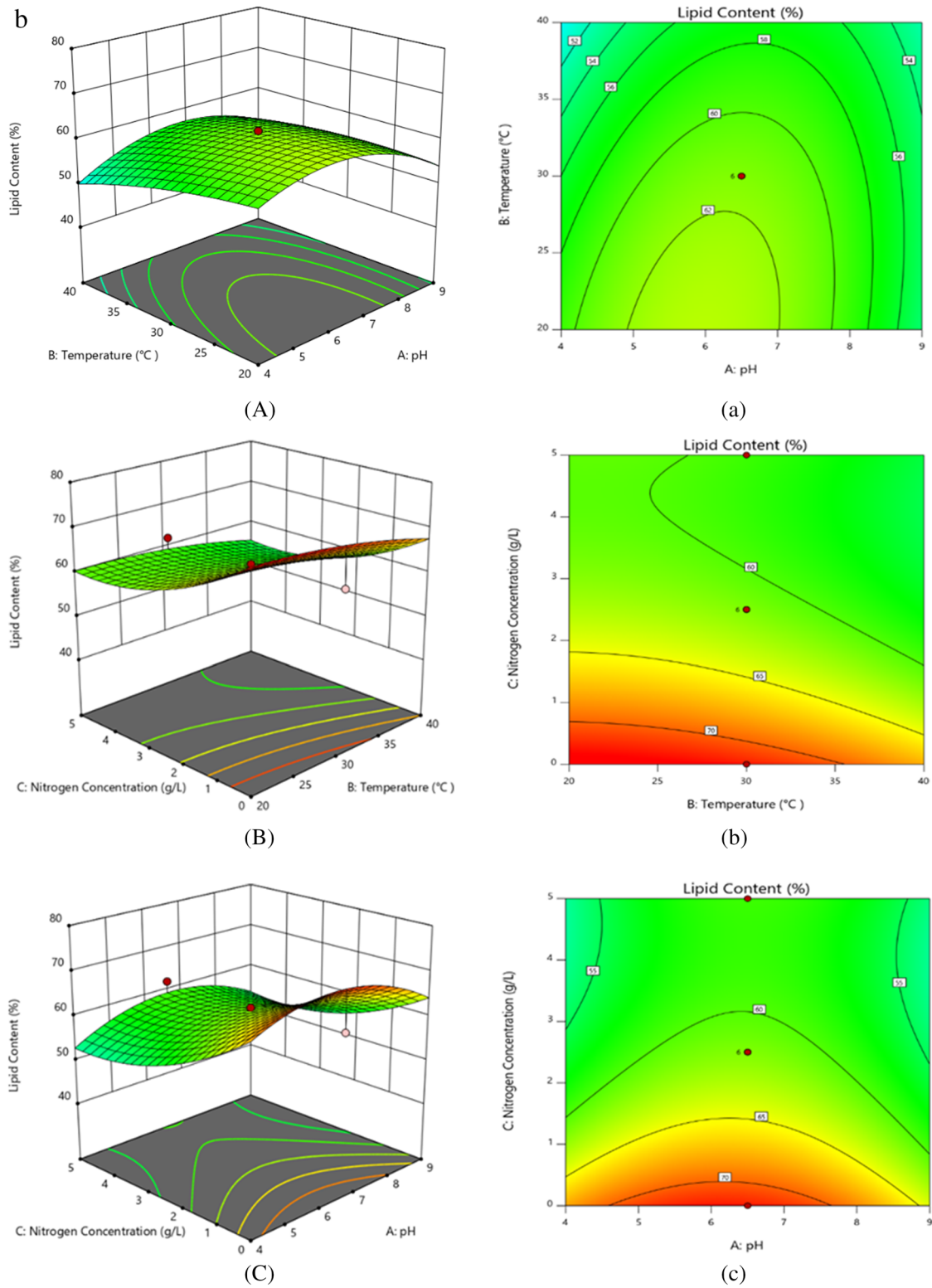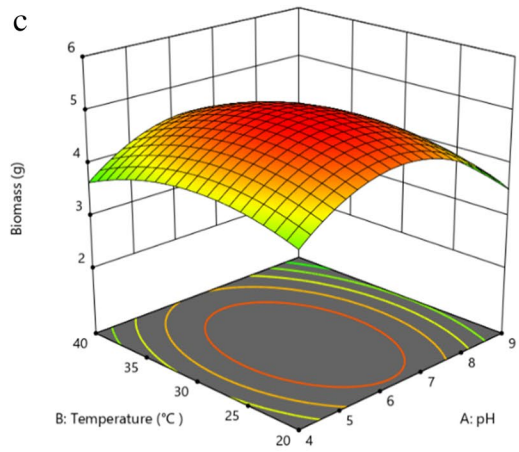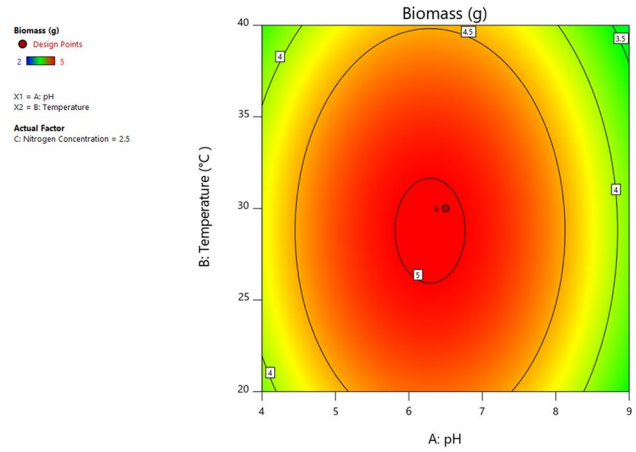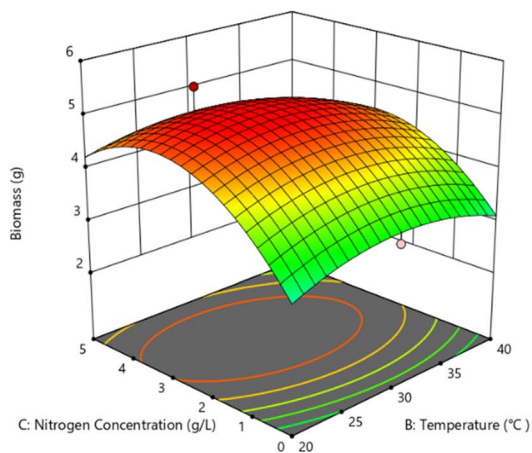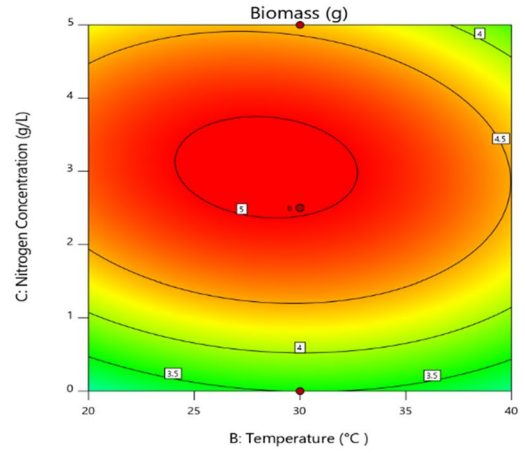(a)

(B)

(b)

(C)

(c)

Fig. 9 (continued)

Fig. 9 (continued)

d
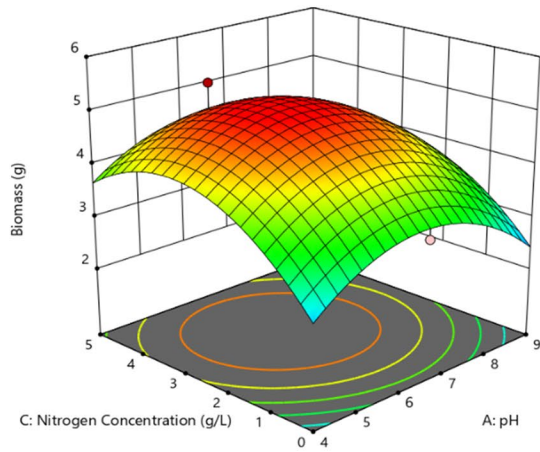

(A)
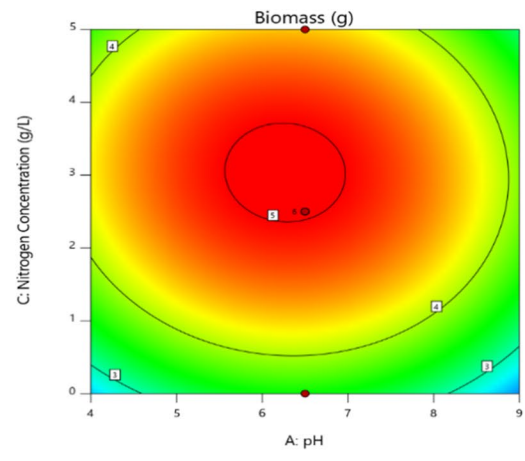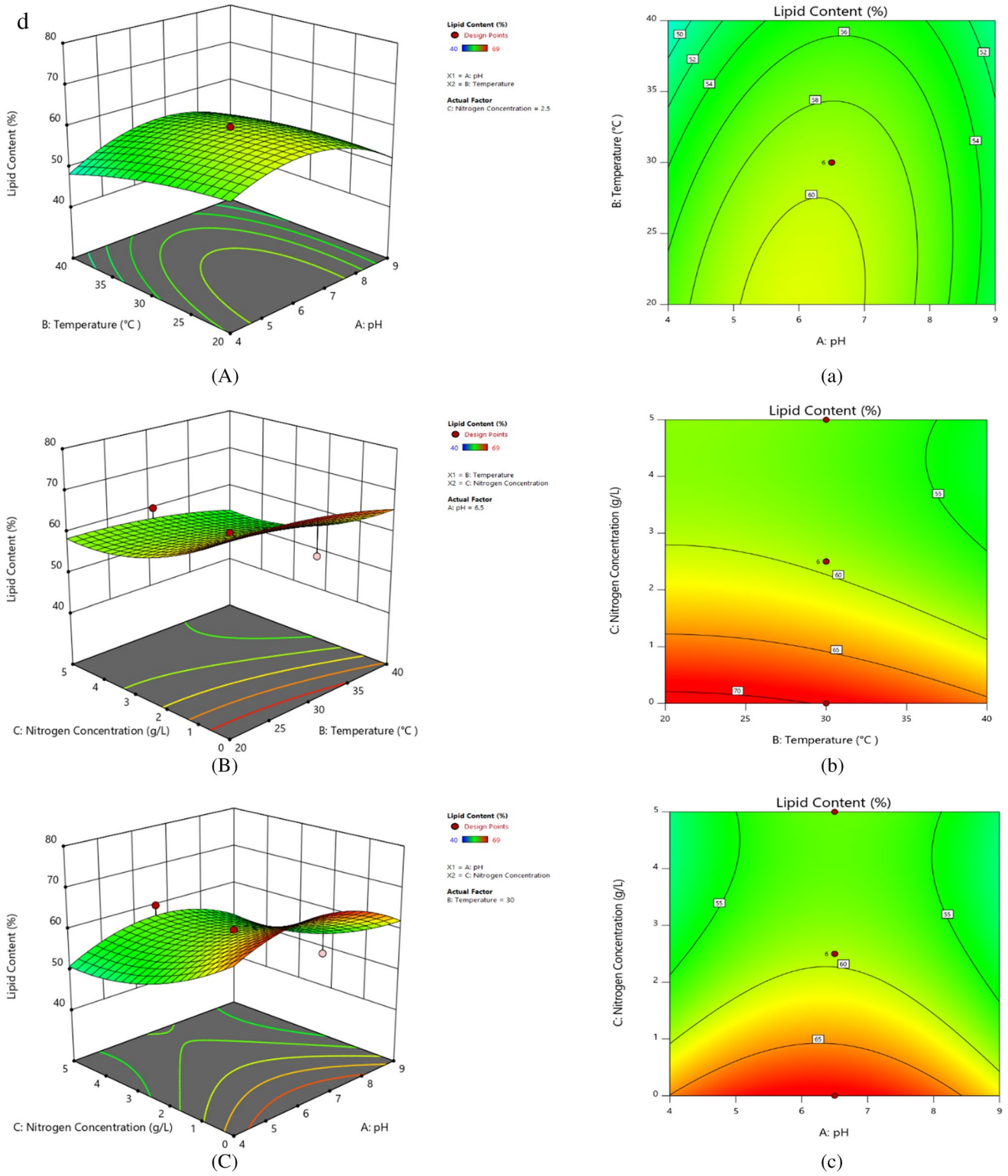

(a)

(B)

(b)

(C)

(c)

Fig. 9 (continued)

Table 7 Compounds (%) detected in biodiesel by gas chromatography–mass spectrometry (GC–MS) analysis in *Chlorella* sp. (1 g DW) and *Oedogonium* sp. (1 g DW)

Fatty acids	C:D	*Chlorella* (Control at 6.5 pH, 30 °C & 2.5 g/L nitrogen)	*Chlorella* sp. (at 4 pH, 20 °C & 0 g/L nitrogen)	*Oedogonium* sp. (Control at 6.5 pH, 30 °C & 2.5 g/L nitrogen)	*Oedogonium* sp. (at 4 pH, 20 °C & 0 g/L nitrogen)
Myristoleic acid	14:1	-	-	0.1	-
Myristic acid	14:0	0.2	0.1	0.3	0.2
Hexadecadienoic	16:2	-	-	0.4	0.3
Palmitoleic acid	16:1	0.1	0.2	2.9	3.0
Palmatic acid	16:0	1.0	1.2	1.1	1.3
Linolenic acid	18:3	0.2	0.1	0.3	0.2
Linoleic acid	18:2	0.5	0.4	0.3	0.2
Oleic acid	18:1	0.1	0.2	3.1	3.2
Stearic acid	18:0	-	-	1.3	1.2
Erucic acid	22:1	0.1	0.1	0.4	0.2
Methyl Myristate	14:0	2.2	2.0	4.5	3.5
Methyl palmitoleate	16:1	3.1	2.9	3.1	3.0
Methyl palmitate	16:0	40.0	35	46.4	39
Linolenate	18:3	0.8	0.5	2.3	2.1
Linoleate	18:2	2.1	1.8	2.5	2.2
Methyl Oleate	18:1	42.5	40	28.0	25.2
Methyl Stearate	18:0	3.9	3	2.1	2.2
Arachidate	21:0	1.0	-	-	-
Erucate	22:1	1.0	-	-	-

Mass balance analysis of biodiesel produced

The mass balance of biodiesel produced by microalgal lipids by base trans-esterification showed 98% (Control) 88% (under stress) in *Chlorella* sp. while 99% (Control) 87% (under stress) in *Oedogonium* sp. showing a positive mass balance with more biodiesel yield as compared to the masses of catalysts, methanol and microalgae lipids consumed for its production.

Fuel properties

In our current study, the data in Table 8 shows the calculated biodiesel properties. CN, DU, SV, CFPP, LCSF, IV, Y, υ, ρ and HHV*i*: all these properties were calculated by using formulas from fatty acid profile of maximum lipid content of experimental run 3 under conditions at 4 pH, 20 °C and 0 g/L nitrogen concentration. In the present study, iodine value of both species was in the range of 83.2

Table 8 Fuel properties of *Chlorella* sp. and *Oedogonium* sp

Fuel properties	Biodiesel standard EN 14,214	Biodiesel standard ASTM D6751-02	*Chlorella* sp. (Control)	*Chlorella* sp. (at 4 pH, 20 °C and 0 g/L nitrogen)	*Oedogonium* sp. (Control)	*Oedogonium* sp. (at 4 pH, 20 °C and 0 g/L nitrogen)
IV (gI$_2$100/g fat)	≤120	NA	95.55	82.8	107	93.3
SV (mg KOH/g)	–	–	196	173	197	174
CN	≥51	≥47	52.6	59.2	51.8	56.6
DU	–	–	55.9	59	51.6	52
LCSF	–	–	2.05	1.62	5.2	0.73
CFPP (°C)	−20 < CFPP < 5	NA	−14.21	−11.38	0.1	−14.18
HHV*i* (MJ/kg)	NA	≥35	38.14	38.18	38.18	38.18
ρ (g cm^{-3})	0.86–0.9	NA	0.87	0.87	0.87	0.87
υ (mm^2/s)	3.5–5.0	1.9–6.0	3.7	3.8	4	4.1
Y at 110 °C (hour)	≥6	NA	237	297	199	238

to 107 and DU from 50 to 56. According to EN 14,214, the maximum levels of Iodine (IV) ranged up to 120 g I_2/100 g, whereas ASTM D6751 does not specify any value for this parameter. Similarly, the standards did not report any specification in respect of DU. The SV were within the limits of 173 to 197 mg KOH/g in both algal strains. No specification for SV has been reported in standards. After that pH, temperature and nitrogen stress, SV reduced in both *Chlorella* sp. and *Oedogonium* sp. as per Standards (ASTM D6751), the minimum value of CN should be 47, and 51 as provided by the EN 14,214. The results indicated that the cetane number of the entire algal strains in this research were higher than 47. CN increased from 52.6 to 59.2 in *Chlorella* sp. and 51.8 to 52 in *Oedogonium* sp. after stress conditions.

The ASTM standard recommends that the heating value should not be less than 35 MJ/kg for biodiesels. Conversely, the EN 14,214 standards have not reported any such specifications. Results indicate that HHV in both algal strains *Chlorella* sp. and *Oedogonium* sp. were higher than 35 MJ/kg. According to EN 14,214, CFPP of biodiesel should be between the limits of -20 and 5 °C. The ASTM 6751 has not prescribed any specifications for CFPP. Results indicated that CFPP of the biodiesel produced from both algae strains were 0.1 to -14.21 °C. Kinematic viscosity should be between 1.9 and 6.0 mm^2 s^{-1} (as per ASTM 6751) and between 3.5 and 5.0 mm^2 s^{-1} (as per EN 14,214). The results of the present study provided that the overall kinematic viscosity of the biodiesel fuel for both algae were within the range of 3.7 to 4.1 mm^2 s^{-1}. As per EN 14,214, the density of biodiesel should be between 0.86 and 0.9 g cm^{-3}. However, ASTM 6751 does not prescribe any specifications for density. So, the density of the biodiesel produced from both algae strains were within the prescribed range. The EN 14,214 prescribed that Oxidation stability must be ≥ 6 h at 110 °C) while no specification has been provided by in ASTM 6751. Hence, it can be concluded that the oxidation stability of the biodiesel produced from both algae strains was within the given range. Results indicated that pH, temperature and nitrogen stress enhance oxidative stability than controls. So, results of this study indicated that pH, temperature and nitrogen stress enhance lipid accumulation, improved FAMEs composition and ultimately biodiesel properties.

Discussions

In this study, the synergetic effect of nitrogen starvation, pH and temperature on lipid induction in two algal species was investigated. Results of this research indicated that nitrogen starvation, pH and temperature stress reduce algal biomass but increase lipid accumulation. The reduction in algal growth due to increase in pH from the optimal range was linked with the lack of carbon because at high pH carbon was available in the form of carbonates (Murprayana et al. 2021): a change in pH significantly altered photosynthetic performance related to chlorophyll quenching and electron transport chain in algae (Severes et al. 2017). Also an increase in pH changes algal cell wall flexibility, delaying the cell cycle and inhibiting growth (Hamed et al. 2021). The optimal pH of *Thalassiosira pseudonana* Cleve, 1873 was 8.8 with low photosynthetic and growth rates at 6.5 or 9.0 pH (Valenzuela et al. 2021). *Ceratium* sp. Shrank, F. von Paula, 1793 (Saifullah et al. 2019), *Heterocapsa* sp. (Ehrenberg) E.F.Stein, 1883 (Mikhail et al. 2020) and *Prorocentrum* sp. J.Schiller, 1918 (Kim et al. 2021) showed maximum growth at pH 7.0–7.6 while Abu-Ghosh et al. (2020) showed no growth at pH 1.5 to 3.5, highest growth at 7.4 and exponential growth at 5.4 to 8.4 in *Chlamydomonas applanata*. Ettl H & Schloesser UG, 1992 At pH 1.2 to 1.5 death of cells in *Caladenia applanata* Stephen Hopper and Andrew Brown, 2001(Karm and Dwaish 2021), *Chlamydomonas* sp. (Escudero et al. 2020) and *Eugena mutabilis* (Christian) C.G.Ehrenberg, 1830 (Yanagawa et al. 2021) was observed. Changes in temperature also reduces algal biomass, as reported in our results, through photo-inhibition which negatively affects growth rate of algae reducing size, proficiency of carbon/nitrogen consumption and changing cytoplasmic viscosity of algae (Laws et al. 2020). Along with temperature or pH stress, nitrogen deficiency decreased photosynthesis and protein synthesis which reduced algal growth (Yang et al. 2020a, b). Results of the present study indicated that there in direct relationship between nitrogen and the growth of algae. As nitrogen acquisition decreased in the growth medium, the algal growth also decreased in *Chlorella* sp. and *Oedogonium* sp.

In the present study, lipid content in both algal strains was enhanced after nitrogen starvation, pH and temperature stress presumably through altered carbon fixation and allocation into different macromolecules (Almutairi and Toulibah 2017). In a study of *D. acidophila* E. C. Teodoresco, 1905 glycerol accumulated to avoid the osmotic discrepancy caused by low pH in the growth medium (Brindhadevi et al. 2021). *Chlamydomonas* sp. (Abomohra et al. 2020), *Pinnilaria braunii* Ehrenberg, 1843 (Zaher and Helal 2020) and *Pinnilaria amplicephala* Ehrenberg, 1843 (Melo 2017) accumulated TAG under pH 1. An additional defense mechanism was shown in *Chlamydomonas* sp. When the pH was reduced from 7 to 2.7, SFA increased from 2 to 2.4% which decreased membrane fluidity and stops large proton quantity (Morales-Sánchez et al. 2020). Fluctuations in temperature trigger the accumulation of fatty acids in the algal cell membrane (Ma et al. 2020). Results showed that highest lipid was produced at 20 °C, because low temperature decreased cell membrane fluidity and increased unsaturated fatty acids to maintain membrane fluidity (He et al. 2018). Optimum

temperature was 25 °C for lipid accumulation in *N. oculata* (Wang et al. 2021) and *C. vulgaris* (Krishnan et al. 2020). When increasing the temperature up to 30 °C, lipid content increased from 7.90 to 14.92% in *Nannochloropsis oculata* (Peng et al. 2020) while decreased from 14.9 to 7.9% in *Chlorella vulgaris* (Lee et al. 2017). A temperature increment from 10 to 30 °C during the cultivation of *Thalassiosira pseudonana* (Sheehan et al. 2020), *Phaeodactylum tricornutum* Bohlin, 1897 (Cui et al. 2019), and *Pavlova lutheri* (Droop) J.C.Green, 1975, was accompanied by an increment of 4–20% has been observed in SFAs and PUFAs 10 °C (Thompson et al. 1992). Accumulation of unsaturated fatty acids increased in *Dunaliella salina* (Michel) M.F.Dunal, 1838 by dropping the temperature from 30 to 12 °C (Yin et al. 2020b, a).

The present study indicated an inverse relationship between nitrogen and lipid accumulation as deficiency of nitrogen in the growth medium enhanced the lipids in *Chlorella* sp. and *Oedogonium* sp. In the nitrogen deficient condition, high energy loads of adenosine triphosphate and adenosine monophosphate both trigger conversion of glucose into lipids (Dammak et al. 2016). This can shift the carbon flux from other metabolic pathways such as protein synthesis to lipid production as reported in in *N. oculata* (up to 8% Sabzi et al. 2018) and in *C. vulgaris* (6% Kaosol and Keo 2020). Both pH, temperature also had noteworthy effect on lipid with lower pH (4), temperature (20 °C) but followed by reduced biomass productivity. Similar lipid increased were reported in 31 *Chlorella* strains treated with two-fold lower nitrogen concentration (Andeden et al. 2021). *C. minutissima* (Martinus) M.W.Beijerinck, 1890 increased lipid up to 60 mg/L/day with 3% nitrogen at optimum temperature (30 °C) suggesting algal response towards stresses are specie specific (Amaral et al. 2020). The present study also demonstrated that changes of lipid of *Chlorella* sp. and *Oedogonium* sp., associated with pH, temperature and nitrogen levels in the growing media can be effective in enhancing the production of biofuels in a sustainable manner.

Results indicated that fatty acid composition were significantly influenced by lowering the pH to 4, temperature 20 °C and removing nitrogen. By increasing pH, SFAs and MUFAs levels were reduced while PUFAs increased significantly. Alkaline pH stress inhibited algal growth and divert energy towards TAG accumulation (Hossain et al. 2020). *T. suecica* F.Stein, 1878 produced linolelaidic acid with increasing pH from 7 to 9 (Almutairi and Toulibah 2017). Nitrogen availability has great impact on fatty acid composition because of changed metabolic pathways towards hydrocarbon production (Mirizadeh et al. 2020). The increase in saturated fatty acid and instantaneous reduction in PUFA in nitrogen deficient conditions might be due to the oxidative destruction of unsaturated fatty acids (Amiraux et al. 2020). In the present study, Nitrogen, pH and temperature stress enhanced

C16:0, C18:1, C18:2; similar results were reported in *Ankistrodesmus falcatus* (Corda) C.R, 1848 (Singh et al. 2015).

In the current study, C16:0, C16:1 and C18:1 increased in the biomass of *Chlorella* sp. and *Oedogonium* sp. under 4 pH, 20 °C and the absence of nitrogen, perhaps be due to the conversion of glucose into lipids triggered by ATP and AMP which move the carbon flux from other metabolic paths to lipid production (Morales-Sánchez et al. 2020). In *C. pyrenoidosa*, unsaturated fatty acids (USFA) mainly C18:2 and C18:3 increased up to 74%, while SFAs increased from 58 to 77% by increasing pH from 8.3 to 8.5 (Elshobary et al. 2019). In *Chlorella sorokiniana* Shihira and R.W.Krauss, 1965 the PUFAs fraction enhanced from 40 to 57% by increasing the pH from 6.5 to 8.5 at 25 °C and C18:3 improved 3 times at 8.5 pH (Cheng et al. 2017). But in the present study, C18:3 decreased in both *Chlorella* sp. and *Oedogonium* sp. when cultivated at 4 pH, 20 °C and zero nitrogen. Overproduction of PUFAs might be a defense strategy of algae to increase the fluidity of algal membrane under stress. Numerous studies reported that temperature has a considerable effect on the lipid profile of algae. At low temperature, algae trend to accumulate USFAs, while at high temperature accumulation of SFAs were found (Bazarnova et al. 2019; Ciliberti et al. 2019; Diprat et al. 2020; Koutra et al. 2019; Menegazzo et al. 2020; Petruk et al. 2018). In winter at 7–12 °C, *C. pyrenoidosa* has 21% SFAs which increased up to 46% in summer (Zhang et al. 2019). In the present study, under nitrogen limitation, oleic acid increased and linolenic acid decreased in *Chlorella* sp. and *Oedogonium* sp. A similar results was reported in *Neochloris oleoabundans* S. Chantanachat and H.C.Bold 1962, *Chlorella vulgaris* and *Scenedesmus obliquus* Meyen, 1829 (Avila-León et al. 2020; Du et al. 2018, 2017; Hong et al. 2019). Temperature is a parameter which can be regulated to improve the PUFAs content in algae. *L. danicus* Cleve, 1889 grown at 14 °C yielded higher PUFAs (40%) than those grown at 26 °C (PUFAs 20%) (Aussant et al. 2018). Similarly, *Scenedesmus* sp. at 10 °C had much higher percentage of PUFAs than those grown at 20 °C and 30 °C (Lu et al. 2017). Generally, fatty acid composition of algae depends on nitrogen availability. In *Stigeoclonium* sp. Kutzing, 1843 a decrease in the proportion of SFAs in total fatty acids (TFAs) and a concomitant increase in PUFAs were observed in nitrogen-deplete conditions (Pereira et al. 2012). In another study, nitrogen starvation induced an increase unsaturated fatty acids from 40 to 52% of TFAs in *Stigeoclonium* sp. However, this was in contrast to the fatty acid profile changes of the *Nannochloropsis* sp. Hibberd, 1981 and *P. tricornutum* where nitrogen limitation increased the portion of SFAs and a decline in PUFAs (Griffiths et al. 2012).

In addition to satisfactory level of lipid contents in algae, it was imperative to evaluate the quality of biodiesel which was closely related to FAMEs profile. In the present study,

C16 and C18 increased in both algal strains which can potentially influence the biodiesel quality and fuel properties. Iodine value and degree of unsaturation determine oxidation and thermal stability. Biodiesel having lower values of Iodine (IV) as well as lower degree of unsaturation have healthier oxidation stability (Saengsawang et al. 2020). Results indicated that both IV and SV reduced in both *Chlorella* sp. and *Oedogonium* sp. after cultivation at 4 pH, 20 °C and zero nitrogen. Similar results were reported in *C. pyrenoidosa* cultivated in nutrients deficiency medium having IV values lower than controls (Zhang et al. 2019).

Cetane number is an indicator of the ignition quality of the biodiesel (Patel et al. 2017). The higher the CN the better combustion quality of that biodiesel. Low CN caused ignition delay (Yaşar 2020). Our results indicated that the cetane number of the entire algal strains in this research were higher than 47. Results also indicated that pH, temperature and nitrogen stress increased the CN and DU in both *Chlorella* sp. and *Oedogonium* sp. CN was inversely related to SFAs content. High heating value defined the appropriateness of biodiesel (Younis 2020). Results indicated that HHVs in both algal strains *Chlorella* sp. and *Oedogonium* sp. were higher than 35 MJ/kg. Biodiesel flow performance is predicted by cold filter plugging point (Mohamed Saberi 2020). pH, temperature and nitrogen stress decreased the CFPP in both *Chlorella* sp. and *Oedogonium* sp. CFPP was inversely proportional relationship with LCSF. pH, temperature and nitrogen stress reduces LCSF values reduced in *Chlorella* sp. and *Oedogonium* sp. Biodiesel having high content of C18:0 and C16:0 attained greater CFPP (Akhihiero and Ebhodaghe 2020). Results indicated that cultivation of *Chlorella* sp. and *Oedogonium* sp. at 4 pH, 20 °C and 0 g/L nitrogen concentration reduced fraction of C18:0 and C16:0 than controls, lowering the LCSF and CFPP. The results of the present study provided that the overall kinematic viscosity and density of the biodiesel fuel for both algae were within the range of standards. It was also imperative that the biodiesel should depict suitable oxidation and thermal stability at 110 °C. Oxidation stability of the biodiesel produced from both *Chlorella* sp. and *Oedogonium* sp. were within the given range. Hence, results indicated that pH, temperature and nitrogen stress enhanced lipid content along with improve biodiesel quality.

Conclusions and future prospective

This study showed that algal species *Chlorella* sp. and *Oedogonium* sp. both can alter their physiological metabolism in response to changes of pH, temperature and nitrogen supply in the growth media. An optimization of pH, temperature and nitrogen concentration were crucial factors for oil production, good fatty acid composition and growth

performance of the algae. Any deviance from optimal cultivation conditions would reduce algal growth rate and productivity, which in turn caused a significant loss in total lipid yield. Therefore, on the basis of the experiment on *Chlorella* sp. and *Oedogonium* species, a two-step algal cultivation method can be recommended to enhance both, growth rate and total lipid yield. The stress imposition such as temperature, pH and nitrogen limitation should be applied only after sufficient biomass production. In a subsequent cultivation phase stress should be applied to direct produced assimilates to maximal lipid recovery under stress.

Declarations

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