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OPTIMISATION OF BIOMASS, LIPID AND CARBOHYDRATE PRODUCTIVITIES IN *Chlorella vulgaris* FOR BIOFUEL PRODUCTION

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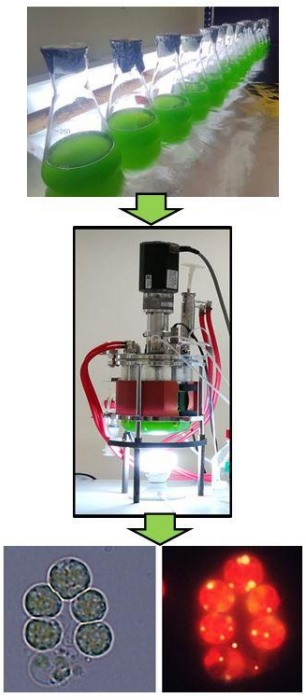
Graphical abstract



Abstract

Synthesis of lipid and carbohydrate in microalgae simultaneously is essential for cost-effective microalgae-based biofuel production. Statistical optimisation approaches of the Plackett–Burman design (PBD) and central composite design (CCD) were applied to optimise the biomass, lipid, and carbohydrate productivities of the *C. vulgaris* UPSI-JRM01. The results obtained from PBD shown that NO_3^- concentration, light intensity and $NaHCO_3$ concentration were the significant factors that affecting biomass productivity. Through CCD, optimum biomass, lipid, and carbohydrate productivities were obtained at 401.81 mg/L NO_3^-, 11238.20 lux light intensity, and 0.30 g/L $NaHCO_3$, achieving the highest biomass productivity of 404.24 mg/L/day, highest lipid productivity of 65.3 mg/L/day, and highest carbohydrate productivity of 165.43 mg/L/day. The major fatty acid methyl esters (FAMEs) produced were palmitic acid (33.54%) and linoleic acid (30.29%), thus producing microalgae-based biodiesel with properties complying with international biodiesel standards.

Keywords: *Chlorella vulgaris*, Optimisation, Lipid, Carbohydrate, Biofuel

Abstrak

Sintesis lipid dan karbohidrat serentak oleh mikroalga adalah penting bagi penghasilan bahan bakar bio berasaskan-mikrolaga yang efektif kos. Pendekatan pengoptimuman statistik *Plackett–Burman design* (PBD) dan *central composite design* (CCD) digunakan untuk mengoptimumkan produktiviti biomas, lipid, dan karbohidrat dari C. *vulgaris* UPSI-JRM01. Hasil yang diperoleh dari PBD menunjukkan bahawa kepekatan NO_3^-, intensiti cahaya dan kepekatan $NaHCO_3$ adalah faktor penting yang mempengaruhi produktiviti biomas. Melalui CCD, produktiviti biomas, lipid, dan karbohidrat optimum diperoleh pada 401.81 mg/L NO_3^-, 11238.20 lux intensiti cahaya dan 0.30 g/L $NaHCO_3$, mencapai produktiviti biomas tertinggi 404.24 mg/L/hari, produktiviti lipid tertinggi 65.3 mg/L/hari, dan produktiviti karbohidrat tertinggi 165.43 mg/ L/hari. Asid lemak metil ester (FAMEs) utama yang dihasilkan adalah asid palmitik (33.54%) dan asid linoleik (30.29%), dengan itu menghasilkan biodiesel berasaskan mikroalga dengan ciri-ciri yang mematuhi piawaian biodiesel antarabangsa.

Kata kunci: *Chlorella vulgaris*, Pengoptimuman, Lipid, Karbohidrat, Bahan Bakar Bio

1.0 INTRODUCTION

The increase in worldwide energy demands creates extreme competition for the world's diminishing petroleum reserves. Nowadays, microalgae biomass-based fuels, such as biodiesel and bioethanol, are among the alternatives to petroleum-based fuels. Microalgae biomass contains lipids and carbohydrates in varying proportions. Lipids from microalgae can be converted to biodiesel, while carbohydrates can be hydrolysed to produce bioethanol [1]. Microalgae based biofuel is a promising replacement for fossil fuels since it is biodegradable, renewable, non-toxic, and it reduces greenhouse gas emissions. Therefore, its utilisation has drawn the attention of researchers in recent years [2]. In addition, Chlorella is a potential alternative to biofuel feedstock due to its simple cultivation requirements, its high photosynthetic capacity as compared to other terrestrial crops and its ability to be cultured on non-arable land including in variety of water sources such as freshwater, brackish, seawater, and wastewater [3].

Microalgae species, especially *C. vulgaris*, possess the ability to accumulate high energy-rich molecules (starch and triacylglycerol) when grown under certain culture conditions [4]. Nitrogen stress, for example, could improve the lipid accumulation of microalgae, which favours biodiesel production. However, the biomass production in nitrogen-deficient cultures was often inhibited and lower than that in nitrogen-sufficient cultures, which effect the economic feasibility of biomass usage [5]. In addition, the nitrogen shortage in the culture medium reduces the carbohydrate and protein content of microalgae [4]. Therefore, optimisation of biomass, lipid and carbohydrate productivity are necessary to develop cost effective microalgae cultivation strategy for biofuel production.

The optimum growth and biomass content of microalgae are species specific and highly dependent on the culture conditions [6]. In addition, determining significant factors for microalgae culture conditions and analysing the complex relationship between them is laborious. Thus, optimisation of these factors, especially environmental and nutritional factors, by statistical methods is necessary. The Plackett–Burman design (PBD) is a well-known statistical method that is trusted to be successful, productive, and time saving for evaluating and screening key factors from a multivariable system [7]. Additionally, response surface methodology (RSM) incorporates mathematical and statistical techniques for modelling and analysing diverse processes. Through RSM, the responses of interest that are influenced by many factors can be optimised. Implementation of the central composite design (CCD) in RSM for the optimisation process is more advantageous over the classical approach due to the increased validity and reliability of the experiment conducted. Moreover, it also provides more comprehensive results [8]. The regression model obtained provides a reasonable clarification for the effect of each particular factor and all potential interactions between the variables.

Accordingly, this study aims to determine and optimise important culture condition (NO_3^- concentration, pH, light intensity, temperature, and $NaHCO_3$ concentration) using statistical techniques of PBD and CCD for achieving optimum biomass, lipid, and carbohydrate productivities simultaneously and to further validate the predicted response with actual experimentation.

2.0 METHODOLOGY

2.1 Microalgae Inoculum

The microalgae strain used in this study (*C. vulgaris* UPSI-JRM01) was previously isolated from Jeram Sanitary Landfill, Selangor [9]. The stock culture was photo-autotrophically grown in BG11 medium under controlled conditions [9]. The culture was performed at 27±2 °C at pH 7, illuminated with 12:12 light/dark cycles on one side with a cool white fluorescent lamp at 4000 lux, and was under constant aeration with filtered atmospheric air (0.03% CO_2) by using an air pump (AP005 Xi Long, China). About 0.05 g/L microalgae culture was used as the initial inoculum.

2.2 Experimental Design

Culture parameters (NO_3^- concentration, pH, light intensity, temperature, and $NaHCO_3$ concentration) were optimised using Design Expert Software Version 7. A two-step approach was taken in this optimisation process. In the first step, the effect of five factors influencing the biomass productivity was investigated using the PBD. After ascertaining the significant factors, the CCD was employed in the second stage to optimise the biomass, lipid, and carbohydrate productivities of *C. vulgaris* UPSI-JRM01. The surface and contour plots were then acquired, and analysis of variance (ANOVA) was performed to examine the interaction between the analysed factors and the significance of the quadratic model obtained.

2.3 Screening Factors using PBD

The screening of significant factors was performed through PBD to decrease the quantity of experimental runs in RSM. The PBD is a fraction of two-level factorial designs (–1 and +1) that allows the analysis of n−1 independent variables with at least n experiments. The PBD with five factors were selected with the following –1 and +1 levels: A) NO_3^- (250, 1000 mg/L), B) pH (7, 8.5), C) light intensity (4000, 17000 lux), D) temperature (25, 35 °C), and E) $NaHCO_3$ (0.1, 1 g/L). The range used for each factor was based on preliminary experimental data and literature reviews [4;10;11]. Each experiment was conducted in triplicate.

The -1 and +1 levels for NO_3^- concentration were selected because even though NO_3^- starvation induced lipid dan carbohydrate accumulation [10], NO_3^- concentration lower than 250 mg/L inhibited the growth of *C. vulgaris* UPSI-JRM01 [4], which resulted in low overall productivity. Hence, this kind of biomass may become unsuitable and not ideal for microalgae-based biodiesel production [11]. Meanwhile NO_3^- concentration higher than 1000 mg/L did not contribute to a further increase in both biomass and carbohydrate productivities, but caused a decrease in lipid productivity [4]. For pH and temperature, preliminary studies on the effect of photo-autotrophic cultural conditions on the productivity of *C. vulgaris* UPSI-JRM01 revealed that any value lower than the selected -1 level and higher than +1 level will inhibit its growth. A pH higher than 8.5 caused an auto-flocculation of microalgae cells and precipitation of salt in the culture medium. Meanwhile light intensity lower than 4000 lux caused light to become a limiting factor, while light intensity higher than 17000 lux caused photooxidation to happen which led to microalgae death. The range selected for $NaHCO_3$ considered the impact of its addition to microalgae productivity and the changes it caused to the pH culture media. Excess $NaHCO_3$ resulted in raised pH, hence reducing the affinity of microalgae to CO_2 and affecting efficiency of photosynthesis [4, 10].

For each PBD experiment, 150 mL of BG11 medium was prepared in a 250 mL Erlenmeyer flask. The factors A (NO_3^- [mg/L]) and E ($NaHCO_3$ [g/L]) were adjusted by adding the calculated amount of $NaNO_3$ and $NaHCO_3$ into the BG11 medium. Factor B (pH) was controlled by measuring the pH of the culture medium using a pH meter (Sartorius PB10, US) every 12 h and adjusting accordingly with NaOH and HCl. Factor C (light intensity [lux]) was controlled by illuminating the culture set-up with an appropriate fluorescent lamp, with the light intensity measured using a digital lux meter (LX-1010B, China). Factor D (temperature [°C]) was controlled by conducting the experiment inside a temperature-controlled incubator (LABWIT ZHWY-200D, Australia). Analysis of cell growth and biomass content was performed every two days for two weeks.

2.4 Optimisation using CCD

RSM was employed through the establishment of the CCD for optimising the biomass, lipid, and carbohydrate productivities of *C. vulgaris* UPSI-JRM01. The PBD experiments showed that three factors (A, NO_3^-; C, light intensity; and E, $NaHCO_3$) were significant for improving the biomass productivity of the microalgae. Therefore, a CCD with three factors (n=3) evaluated at five levels (–a, –1, 0, +1, and +a) was employed. The levels and ranges of the three factors were 0.00, 202.70, 500.00, 797.30, and 1000.00 mg/L for factor A (NO_3^-); 4000, 6635.08, 10500.00, 14364.93, and 17000 lux for factor B (light intensity); and 0.10, 0.28, 0.50, 0.82, and 1.00 g/L for factor C ($NaHCO_3$) The α-value was set at 1.68179 (rotatable), which means that all the points in the design area were at an equal distance from the centre. The CCD generated an experiment set with 20 run numbers with 8 factorials, 6 axials, and 6 centre points.

The experiments were conducted using a 2 L bioreactor (Sartorius BIOSTAT A-plus, Germany) containing 1 L of BG11 medium. The temperature was regulated with a heat blanket and was set at 28 °C [4]. The pH was measured using a pH probe and was automatically adjusted to pH 8 by the addition of NaOH and HCl. Furthermore, the light was provided at the bottom of the bioreactor by a fluorescent lamp. The aeration was provided by an air pump (AP005 Xi Long, China). Meanwhile, the factors A (NO_3^-), B (light intensity), and C ($NaHCO_3$) were manipulated and controlled as stated previously. Finally, the response variables (biomass, lipid, and carbohydrate productivities) were determined using a second-degree polynomial equation [12]. The regression model was generated by evaluating the ANOVA results, the *p*-value, and the *F*-value. The model represented the interactions between the parameters that influenced the responses, and the fitness of the model was conveyed by the coefficient of determination (R^2). The statistical model was subsequently validated using the optimised factors obtained for maximum biomass, lipid, and carbohydrate productivities. The validation experiments were conducted in triplicates.

2.5 Growth and Biomass Analyses

Microalgae growth was measured by dry weight analysis. The dry weight was determined by centrifugation of microalgae cells at 8000 rpm for 10 min, followed by oven drying at 60 °C until a constant weight was achieved. The dry weight was then measured gravimetrically. The biomass productivity was calculated using Equation 1 below, where X_1 and X_2 represent the biomass dry weight on days t_1 and t_2, respectively [13].

$$\text{Biomass productivity (g/L/day)} = (X_2 - X_1) / t_2 - t_1 \quad (1)$$

2.6 Lipid and Carbohydrate Analyses

The compositions of the microalgae biomass (lipid and carbohydrate) were determined at the end of the exponential phase. The lipid analysis was performed using a modified Bligh and Dyer method [14]. Meanwhile, the carbohydrate content was analysed using a modified phenol-sulphuric acid method [15]. The total yields and lipid and carbohydrate productivities were calculated using Equations 2 and 3, respectively.

$$\text{Yield (\%)} = (\text{lipid or carbohydrate weight/biomass weight}) \times 100 \quad (2)$$

$$\text{Productivity (mg/L/day)} = \text{biomass productivity} \times \text{yield} \quad (3)$$

2.7 Analysis of Fatty Acid Methyl Ester (FAME) and Biodiesel Properties

The FAME analysis was performed on the microalgae biomass obtained from cultivation under optimised conditions. The FAME was prepared through the direct transesterification method [16]. About 20 mg of lyophilised microalgae biomass was trans-methylated with 2.5 mL of methanol containing 2% (v/v) H_2SO_4 at 80 °C for 2.5 hours. After the suspension cooled, 1 mL of hexane and 1 mL of saturated NaCl solution were added to the tube to form three separated layers. The upper hexane layer containing FAMEs was removed for analysis. FAME analysis was performed by gas chromatography (7890A, Agilent) equipped with a DB-5MS UI column and directly coupled to a mass spectrometer system (Agilent 5975C inert mass spectrometer design) with a triple-axis detector. Each FAME component was identified and quantified using the Supelco 37 Component FAME mix standard (Sigma, USA).

To determine the properties of biodiesel, the density, kinematic velocity (KV) at 40 °C, iodine value (IV), saponification value (SV), cetane number (CN), high heating value (HHV), degree of unsaturation (DU), long chain saturated factor (LCSF), cold filter plugging point (CFPP), and flash point (FP) were calculated according to empirical equations [17;18;19]. The density, KV, IV, SV, CN, and HHV were calculated in accordance with Equations 4–9, where FP is the percentage of each fatty acid, MW is the molecular weight of the corresponding fatty acid, and DB is the number of double bonds.

$$\text{Density} = 0.8463 + (4.9 / \sum \text{MW}) + (0.0118 \times \sum \text{DB}) \quad (4)$$

$$\ln(\text{KV}) = -12.503 + (2.496 \times \ln \sum \text{MW}) - (0.178 \times \sum \text{DB}) \quad (5)$$

$$\text{IV} = \sum (560 \times \text{FP}) / \text{MW} \quad (6)$$

$$\text{SV} = \sum (254 \times \text{DB}) / \text{MW} \quad (7)$$

$$\text{CN} = 46.3 + (5.458/\text{SV}) - (0.225 \times \text{IV}) \quad (8)$$

$$\text{HHV} = 49.43 - (0.041 \times \text{SV}) - (0.015 \times \text{IV}) \quad (9)$$

The DU was calculated, based on Equation 10, as the amount of monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) present in the microalgae oil.

$$\text{DU} = \text{MUFA wt\%} + (2 \times \text{PUFA wt\%}) \quad (10)$$

The LCSF was estimated through Equation 11. This factor was directly used to calculate the CFPP in Equation 12. These two factors are both related to chain saturation and the length of FAMEs.

$$\text{LCSF} = (0.1 \times \text{C16}) + (0.5 \times \text{C18}) + (1 \times \text{C20}) + (1.5 \times \text{C20}) + (2 \times \text{C24}) \quad (11)$$

$$\text{CFPP} = (3.1417 \times \text{LCSF}) - 16.477 \quad (12)$$

The FP was estimated through Equation 13, where WC is the weighted-average number of carbon atoms and WDB is the weighted-average number of double bonds.

$$\text{FP} = (23.362 \times \text{WC}) + (4.854 \times \text{WDB}) \quad (13)$$

3.0 RESULTS AND DISCUSSION

3.1 Screening of Growth Factors Affecting Biomass Productivity by PBD

The PBD was employed to screen the significant factors affecting biomass productivity. This design does not contemplate the relationship between the parameters analysed [20]. The effect of the selected five factors considered in this study on the biomass productivity was statistically analysed and is summarised in Table 1. Based on these results, the highest biomass productivity of 387.90 mg/L/day was achieved by PBD run no 5. Moreover, the ANOVA results for the selected factorial model (Table 2) clearly indicated that among the five variables, three had a significant influence on the biomass productivity. The *p*-value of the model was less than 0.05 (<0.0001), implying that the model is significant. The predicted R^2 of 0.993 is in rational agreement with the adjusted R^2 of 0.997, which indicated that 99.7% of the variability in the response could be explained by the model [21]. In this case, factors A (NO_3 [mg/L]), C (light intensity [lux]), and E ($NaHCO_3$ [g/L]) were significant and thus included in the CCD.

Table 1 Effect of the Selected Factors on the Biomass Productivity of *C. vulgaris*

Run No.	A: NO_3^- (mg/L)	B: pH	C: Light Intensity (lux)	D: T (°C)	E: $NaHCO_3$ (g/L)	Biomass Productivity (mg/L/day)
1	250	8.5	17000	25	1	143.03 ± 1.21
2	1000	8.5	4000	25	0.1	314.11 ± 2.32
3	1000	8.5	17000	25	0.1	376.91 ± 1.23
4	250	7	17000	25	1	128.03 ± 0.53
5	1000	7	17000	35	0.1	387.90 ± 0.72
6	250	8.5	4000	35	1	67.23 ± 0.13
7	250	7	4000	35	0.1	81.75 ± 0.43
8	250	8.5	17000	35	0.1	164.55 ± 0.23
9	1000	8.5	4000	35	1	288.59 ± 1.23
10	1000	7	4000	25	1	273.59 ± 0.54
11	1000	7	17000	35	1	356.39 ± 2.43
12	250	7	4000	25	0.1	91.74 ± 0.34

Note. Data shown are the average of three runs ± the standard deviation (SD)

Table 2 Analysis of Variance (ANOVA) for the Selected Factorial Model

Source	*F*-value	*p*-value probability > *F*	
Model	730.45	<0.0001	significant
A: NO_3^- (mg/L)	3242.40	<0.0001	significant
B: pH	2.28	0.1822	not significant
C: Light Intensity (lux)	359.30	<0.0001	significant
D: Temperature (°C)	0.67	0.4441	not significant
E: $NaHCO_3$ (g/L)	47.62	0.0005	significant

Notes. Predicted R^2 = 0.993 and R^2 = 0.997 and adjusted R^2 = 0.997

3.2 Optimisation of Biomass, Lipid, and Carbohydrate Productivities by RSM and Regression Analysis)

A total of 20 experiments with 3 selected factors (A, NO_3^-; B, light intensity; C, $NaHCO_3$) according to CCD were conducted, and the experimental and predicted values (by RSM) of biomass, lipid, and carbohydrate productivities are summarised in Table 3. The data obtained were subjected to multiple regression analysis, which provides an empirical model that connects the evaluated response to the independent factor.

Table 3 Experimental and Predicted Values of Biomass, Lipid, and Carbohydrate Productivities *in C. vulgaris*

Run No.	Factor			Productivity (mg/L/day)					
				Biomass		Lipid		Carbohydrate	
	A: NO_3^- (mg/L)	B: Light Intensity (lux)	C: $NaHCO_3$ (g/L)	Experimental Values	Predicted Values	Experimental Values	Predicted Values	Experimental Values	Predicted Values
1	202.70	6635.08	0.28	322.80 ± 0.53	322.59	80.70 ± 1.33	82.87	108.98 ± 0.83	110.02
2	797.30	6635.08	0.28	384.60 ± 0.82	383.94	42.31 ± 1.13	40.58	168.64 ± 1.93	167.42
3	202.70	14364.92	0.28	310.20 ± 1.04	309.76	108.57 ± 1.23	109.21	78.46 ± 1.12	81.14
4	797.30	14364.92	0.28	367.90 ± 0.83	368.48	51.51 ± 1.43	49.09	149.30 ± 1.48	149.15
5	202.70	6635.08	0.82	273.50 ± 0.72	272.78	68.38 ± 1.22	64.44	87.85 ± 1.12	87.62
6	797.30	6635.08	0.82	328.15 ± 0.98	328.45	49.22 ± 1.29	42.22	140.15 ± 1.22	137.09
7	202.70	14364.92	0.82	259.76 ± 1.23	260.28	83.12 ± 1.34	78.49	70.13 ± 1.31	73.98
8	797.30	14364.92	0.82	313.26 ± 0.73	313.33	46.98 ± 0.98	38.44	135.46 ± 1.21	134.05
9	0.00	10500.00	0.55	238.30 ± 0.52	238.74	114.38 ± 0.84	114.74	46.65 ± 0.93	43.89
10	1000.00	10500.00	0.55	335.18 ± 0.32	334.94	36.86 ± 0.84	45.50	139.39 ± 0.96	142.68
11	500.00	4000.00	0.55	346.42 ± 1.22	347.12	48.49 ± 1.56	51.67	131.35 ± 1.27	133.23
12	500.00	17000.00	0.55	320.12 ± 0.92	323.62	64.82 ± 1.25	70.64	107.75 ± 1.23	106.39
13	500.00	10500.00	0.10	398.32 ± 1.22	398.69	59.74 ± 1.27	57.47	168.41 ± 1.24	166.83
14	500.00	10500.00	1.00	310.61 ± 0.42	310.44	21.74 ± 0.73	33.01	133.20 ± 0.79	135.30
15	500.00	10500.00	0.55	418.70 ± 1.22	418.55	46.05 ± 1.54	45.78	184.56 ± 1.34	184.48
16	500.00	10500.00	0.55	417.34 ± 1.42	418.55	45.90 ± 1.83	45.78	183.96 ± 1.67	184.48
17	500.00	10500.00	0.55	419.20 ± 1.32	418.55	46.11 ± 1.43	45.78	184.78 ± 1.44	184.48
18	500.00	10500.00	0.55	419.34 ± 1.22	418.55	46.12 ± 1.43	45.78	184.85 ± 1.42	184.48
19	500.00	10500.00	0.55	418.53 ± 1.52	418.55	46.03 ± 1.73	45.78	184.49 ± 1.64	184.48
20	500.00	10500.00	0.55	418.20 ± 1.24	418.55	46.00 ± 1.43	45.78	184.34 ± 1.28	184.48

Note. Data shown for experimental values are the average of three runs ± the SD.

Table 4 Regression Analysis of the CCD

Source	Biomass Productivity				Lipid Productivity				Carbohydrate Productivity			
	Coeff.	Sum of Squares	*F*-value	*p*-value	Coeff.	Sum of Squares	*F*-value	*p*-value	Coeff.	Sum of Squares	*F*-value	*p*-value
Model	418.55	65092.78	12616.56	<0.0001*	45.78	9810.75	25.69	<0.0001*	184.48	35348.42	739.44	<0.0001*
A	28.60	11170.54	19486.09	<0.0001*	–20.58	5786.83	136.36	<0.0001*	29.37	11780.21	2217.83	<0.0001*
B	–6.99	666.89	1163.34	<0.0001*	5.64	434.52	10.24	0.0095*	–7.98	869.34	163.67	<0.0001*
C	–26.24	9402.43	16401.77	<0.0001*	–7.27	721.99	17.01	0.0021*	–9.37	1199.81	225.88	<0.0001*
AB	–0.66	3.45	6.01	0.0342*	–4.46	158.87	3.74	0.0818	2.65	56.23	10.59	0.0087*
AC	–1.42	16.10	28.09	0.0003*	5.02	201.50	4.75	0.0543	–1.98	31.48	5.93	0.0352*
BC	0.084	0.06	0.10	0.7608	–3.07	75.46	1.78	0.2120	3.81	115.90	21.82	0.0009*
A^2	–46.57	31248.81	54511.01	<0.0001*	12.14	2124.63	50.06	<0.0001*	–32.24	14982.75	2820.77	<0.0001*
B^2	–29.41	12463.04	21740.76	<0.0001*	5.44	425.99	10.04	0.0100*	–22.86	7533.61	1418.34	<0.0001*
C^2	–22.62	7374.53	12864.27	<0.0001*	–0.19	0.52	0.01	0.9141	–11.81	2011.25	378.65	<0.0001*

Note. *p-value less than 0.0500 indicate that the model terms are significant.

The regression analysis of the model is represented in Table 4. The regression analysis showed that the three models were highly significant based on the low *p*-value obtained (<0.0001). The *p*-value depicted the significance of the variables, where the lower the *p*-value the stronger the significance of the variables [10]. According to the *p*-value obtained, it can be concluded that A, B, C, AB, AC, A^2, B^2, and C^2 were significant model terms for biomass productivity. The interaction between B and C (light intensity and $NaHCO_3$) was not significant to the biomass productivity. On the other hand, A, B, C, A^2, and B^2 were significant model terms for lipid productivity. It was observed that the interaction between the factor (AB, AC, BC) and C^2 was not significant to the lipid productivity. Meanwhile, for carbohydrate productivity, all model terms (A, B, C, AB, AC, BC, A^2, B^2, C^2) were significant.

The NO_3^- concentration was found to be the most important factor affecting the biomass and

carbohydrate productivities, with the highest coefficient values of 28.6 and 29.37, respectively. However, there was a negative relationship between the NO_3^- concentration and the lipid productivity, with a coefficient value of –20.58. Nitrogen is a vital macronutrient that is compulsory for the synthesis of many microalgae biomolecules, such as amino acids, nucleic acids, and photosynthetic pigments [22]. In the absence of nitrogen, these biomolecules cannot be synthesised and cause the cells to be unable to propagate. Therefore, biomass production declined under this condition. Even though cell growth was ceased, nitrogen stress triggered high lipid accumulation in the microalgae cells, as evidenced in a previous study [23].

The estimated response surface models in the form of second-order regression equations for biomass, lipid, and carbohydrate productivities are shown in the Equations 14–16, as follows:

$$\text{Biomass productivity} = 418.55 + 28.60A - 6.99B - 26.24C - 0.66AB - 1.42AC + 0.084BC - 46.57A^2 - 29.41B^2 - 22.62C^2 \quad (14)$$

$$\text{Lipid productivity} = 45.78 - 20.58A + 5.64B - 7.27C - 4.46AB + 5.02AC - 3.07BC + 12.14A^2 + 5.44B^2 - 0.19C^2 \quad (15)$$

$$\text{Carbohydrate productivity} = 184.48 + 29.37A - 7.98B - 9.37C + 2.65AB - 1.98AC + 3.81BC - 32.24A^2 - 22.86B^2 - 11.81C^2 \quad (16)$$

The three-dimensional surface plots involving the effect of the three factors (NO_3^-, light intensity, and $NaHCO_3$) on the biomass, lipid, and carbohydrate productivities are shown in Figure 1. The shape of the equivalent contour plots stipulated whether the mutual relationship between the independent variables was significant or not. The contour plot with an elliptical shape suggested that the interactions between the independent variables were significant [24]. Based on the three-dimensional response surface, the interactions between each independent pair of variables were interpreted, and the optimal values of the independent variables could be discovered. The predicted biomass, lipid, and carbohydrate productivities by RSM under various conditions are summarised in Table 5.

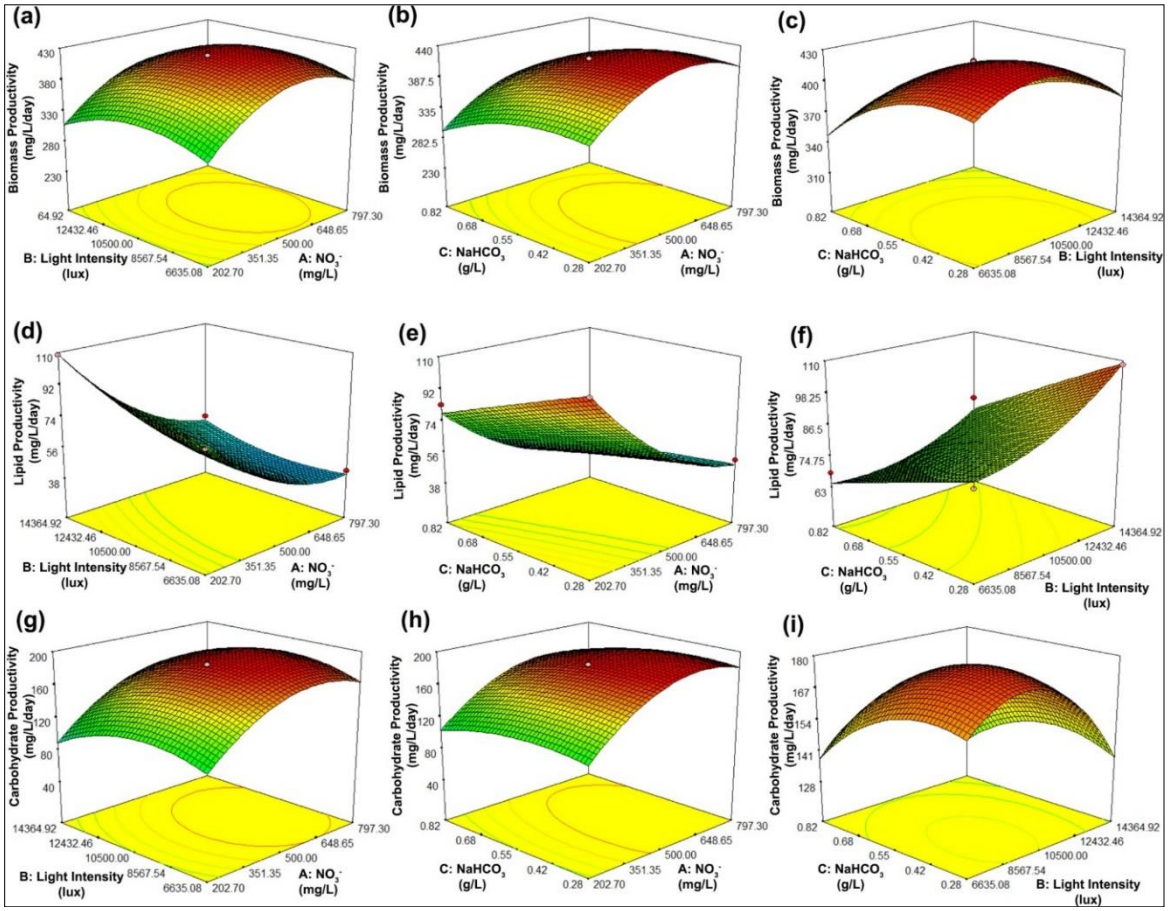


Figure 1 Three-dimensional surface plot of the effect of the three factors (NO_3^-, light intensity, and $NaHCO_3$) on the biomass productivity (a–c), lipid productivity (d–f), and carbohydrate productivity (g–i)

Table 5 Predicted Biomass, Lipid, and Carbohydrate Productivities in *C. vulgaris* by Response Surface Methodology (RSM)

Culture Condition	Factor			Predicted Productivity (mg/L/day)		
	NO_3^- (mg/L)	Light Intensity (lux)	$NaHCO_3$ (g/L)	Biomass	Lipid	Carbohydrate
Highest Biomass Productivity	594.00	10023.24	0.39	431.25	43.10	193.14
Highest Lipid Productivity	3.54	11238.20	0.12	203.262	153.74	1.00
Highest Carbohydrate Productivity	637.64	9778.70	0.43	429.66	40.29	194.19
Optimised Condition	401.81	11238.20	0.30	406.00	64.17	165.17

According to Figure 1 (a–c), the highest biomass productivity could be achieved in a culture condition with 594 mg/L NO_3^-, 10023.24 lux light intensity, and 0.39 g/L $NaHCO_3$. The predicted biomass productivity was 431.25 mg/L/day (Table 5). On the other hand, non-elliptical contour plots were obtained for Figure 1 (d–f) because the interactions between these factors were not significant to the lipid productivity. However, the highest lipid productivity could be achieved in the culture condition with 3.54 mg/L NO_3^-, 11238.20 lux light intensity, and 0.12 g/L $NaHCO_3$ (Table 5). The predicted lipid productivity was 153.74 mg/L/day (Table 5), with an estimated lipid yield of 75.64%. Moreover, based on Figure 1 (g–i), the highest carbohydrate productivity could be achieved in a culture condition with 637.64 mg/L NO_3^-, 9778.70 lux light intensity, and 0.43 g/L $NaHCO_3$. The predicted carbohydrate productivity under this condition was 94.189 mg/L/day (Table 5), with an estimated carbohydrate yield of 45.20%.

Furthermore, based on Table 5, it was observed that the cultivation at the highest biomass productivity condition produced biomass with high carbohydrate productivity, and vice versa. However, cultivation at the highest lipid productivity condition caused the biomass productivity to decrease by almost half of the value obtained from the highest biomass and carbohydrate productivities. Accumulation of lipid in *C. vulgaris* was associated with high light intensity and low NO_3^- and $NaHCO_3$ concentrations. Limited nutrient availability inhibited cell growth and triggered the microalgae survival response by switching their normal energy storage from carbohydrates (starch) to lipids (triacylglycerol). However, low biomass accumulation under this cultivation was not favourable for commercialisation. In order to fully benefit from microalgae biomass, the lipid and carbohydrate productivities should both be increased without severely affecting the biomass productivity. Thus, the RSM predicted that the optimum biomass, lipid, and carbohydrate productivities could be achieved with 401.81 mg/L NO_3^-, 11238.20 lux light intensity, and 0.30 g/L $NaHCO_3$ (Table 5). Under this condition, a notable amount of biomass will be produced with high lipid and carbohydrate content. The predicted optimised condition was subsequently validated through experimentation.

3.3 Experimental Validation of the Optimised Condition

The validation of the RSM model was performed under the optimised condition. The result of the validation is shown in Table 6.

Table 6 Validation of the Optimised Condition Predicted by RSM

Values	Biomass Productivity (mg/L/day)	Lipid Productivity (mg/L/day)	Carbohydrate Productivity (mg/L/day)
Predicted Values	406.00	64.17	165.17
Experimental Values	404.24 ± 2.10	65.30 ± 4.21	165.43 ± 1.32

Notes. Data shown for experimental values are the average of three runs ± the SD. SDs for the predicted values are not provided because they were obtained from the RSM

Based on these results, it was found that the experimental values from the validation experiment were close to the biomass, lipid, and carbohydrate productivity values predicted by the RSM, with a high coefficient of determination (R^2) of 0.982, indicating that 98.2% of the predicted results were in accordance with experimental values, therefore validating the model. The results recognised the function of RSM obtained as an accepted design that could contribute substantial information for culture condition manipulation to obtain the desired biomass, lipid, and carbohydrate productivities in *C. vulgaris*. In this study, the culture conditions for the highest lipid and carbohydrate productivity could favour the production of biodiesel and bioethanol, respectively (Table 5). Furthermore, under the optimised culture condition, the utilisation of microalgae biomass can be maximised for biofuel feedstock production. The statistical model obtained in this study can be used to predict the productivity of feedstock, thus assisting researchers with the appropriate selection of culture conditions.

In this study, *C. vulgaris* UPSI-JRM01 demonstrates capability as biomass-producing organism for biofuel feedstock. This microalgae strain has the ability to supply several bioenergy carriers, including biodiesel, from accumulated lipids, as well as bioethanol from stored carbohydrates. Moreover, the remaining balance of biomass after biofuel production can be used for soil nourishment and animal feed. Therefore,

this study provides an integrated approach for full utilisation of microalgae biomass for the simultaneous production of biodiesel and bioethanol feedstocks, thus making its application economical.

The results of this study were compared to other studies to demonstrate the ability of *C. vulgaris* to accumulate lipids and carbohydrates under various environmental conditions [25;26;27;28;23;5]. The biomass, lipid, and carbohydrate productivities of *Chlorella* sp. cultured under various culture conditions are presented in Table 7. Nitrogen stress has been used in research to increase the lipid and/or carbohydrate productivities of microalgae [25]. To overcome poor biomass productivity obtained during nitrogen stress, previous researchers have utilised a two-stage cultivation system: stage 1 for biomass accumulation and stage 2 for energy-rich molecule (lipid/carbohydrate) enhancement [5]. However, this approach was time consuming and required more energy for harvesting and transferring microalgae cells from stage 1 to stage 2. In addition, microalgae cells cultured in the stress condition often develop thicker cell walls to protect themselves from harsh conditions, which may increase the cost of cell wall's breakage and reduce the efficiency of lipid extraction [3]. The optimisation of the cultivation process using RSM in the current study enabled the establishment of the culture condition with the maximum biomass, lipid, and carbohydrate productivities without the hassle of a two-stage culturing system.

Most of the studies in Table 7 utilised a temperature in the range of 25−28 °C and a pH of 6.8−8 to culture the microalgae, suggesting that these temperatures and pH values were the optimum ranges for microalgae growth. An increase in temperature of greater than 40 °C inhibited cell growth and caused damage to cell structures and functions. In general, temperature affects enzymatic reactions, the cell membrane system, and metabolite synthesis [20]. Moreover, a pH lower than 5 and higher than 9 may affect the efficiency of photosynthesis due to the changes in the CO_2 concentration in the medium and a reduction in the microalgae affinity to free CO_2 [7].

Table 7 Biomass, Lipid, and Carbohydrate Productivities of *Chlorella* sp. Cultured under Various Culture Conditions

Species	Culture Condition						Productivity (mg/L/day)			Ref.
	Nitrogen Source	Carbon Source	T (°C)	pH	Light Intensity	Cultivation Period (day)	Biomass	Lipid	Carbohydrate	
Chlorella vulgaris UPSI-JRM01	401.81 mg/L NO_3^- ($NaNO_3$)	0.3 g/L $NaHCO_3$	28	8	11238.20 lux 12L:12D	8	404.24	65.30	165.17	This study
Chlorella sp. F&M-M49	–	0.5 L/L/min CO_2	40 (8 h) 25 (16 h)	8 ± 0.5	14000 lux8L:16D	7	430.00	120.00	190.00	[25]
Chlorella sp. NBRI015	1000 mg/L NH_4Cl	acetate	27	7	3000 lux 14L:10D	14	190.00	20.21	41.96	[26]
Chlorella sorokiniana	1500 mg/L Urea	–	25 ± 1	nc	8880 lux 16L:8D	30	218.00	134.11	nd	[27]
Chlorella sp.	1000 mg/L NO_3^- ($NaNO_3$)	0.04% CO_2	25 ± 2	7.1	6000 lux 12L:12D	13	92.31	46.20	nd	[28]
Chlorella vulgaris	25 mg/L NO_3^- (KNO_3)	–	25 ± 2	6.8	5550 lux 14L:10D	13	23.8	13.18	nd	[23]
Chlorella sp. AE10*	1000 mg/L NO_3^-[a], 250 mg/L NO_3^-[b] ($NaNO_3$)	1%[a], 10%[b] CO_2	28	7.5	5200 lux[a], 52000 lux[b] 12L:12D	11	546.00	nd	421.00	[5]

Notes. nc, not controlled; nd, not determined; L:D, light:dark cycle; *cultured under two stages; [a]stage 1, 3 days; [b]stage 2, 8 days

In this study, CO_2 was supplied in the form of $NaHCO_3$ since it is more economic than supplying CO_2 gas. The C:N ratio in the culture media was 25:1. It is believed that 0.3 g/L $NaHCO_3$ is the optimum level to support the photosynthesis reaction and starch and lipid synthesis. According to Cheng *et al*. (2017) [5], increasing the C:N ratio from 48:1 to 192:1 triggered high biomass and carbohydrate accumulation in *Chlorella* sp. AE10. Meanwhile, the optimum light intensity of 11238.20 lux is also important because inadequate illumination causes slower cell growth. Conversely, excessive illumination exceeding the maximum light saturation value will cause photo-inhibition, which consequently damages the photosystem and decreases the biomass, lipid, and carbohydrate productivities.

3.4 FAME Profile

The FAME profile of *C. vulgaris* UPSI-JRM01 cultured under the optimised condition is presented in Table 8. The most abundant fatty acids found were palmitic acid C18:0 (33.54%), linoleic acid C18:2 (30.29%), cis-13-octadecenoic acid C18:1 (15.90%), and 7,10-hexadecadienoic acid C16:2 (11.07%). The FAME consisted of a similar percentage of PUFA (41.57%)

and saturated fatty acid (SFA, 40.86%). The composition of the FAME determines the properties of the biodiesel. A high percentage of PUFA results in the best performance in the cold, as denoted by the CFPP value [29]. However, high PUFA content adversely affects the biodiesel's CN and oxidation stability [18]. On the other hand, a high quantity of long-chain SFAs produces biodiesel with a high CN. This kind of biodiesel had a shorter ignition delay and excellent combustion quality [30].

Table 8 Fatty Acid Methyl Esters (FAME) Composition Profile of *C. vulgaris* Cultured under the Optimised Condition

Fatty Acids	Molecular Formula	Carbon No.	Percentage (%) ± SD
Palmitic acid	$C_{16}H_{32}O_2$	C16:0	33.54 ± 0.82
Linoleic acid	$C_{18}H_{32}O_2$	C18:2	30.29 ± 1.21
Cis-13-octadecenoic acid	$C_{18}H_{34}O_2$	C18:1	15.90 ± 0.72
7,10-Hexadecadienoic acid	$C_{16}H_{28}O_2$	C16:2	11.07 ± 0.87
Octadecanoic acid	$C_{18}H_{36}O_2$	C18:0	5.38 ± 0.62
Palmitoleic acid	$C_{16}H_{30}O_2$	C16:1	1.43 ± 0.05
Myristic acid	$C_{14}H_{28}O_2$	C14:0	1.21 ± 0.02
18-Methylnonadecanoic acid	$C_{20}H_{40}O_2$	C20:0	0.26 ± 0.01
10,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	C18:2	0.21 ± 0.01
13-Methyltetradecanoic acid	$C_{15}H_{30}O_2$	C15:0	0.18 ± 0.02
Nonanoic acid	$C_9H_{18}O_2$	C9:0	0.18 ± 0.01
Oleic acid	$C_{18}H_{34}O_2$	C18:1	0.17 ± 0.03
Docosanoic acid	$C_{22}H_{44}O_2$	C22:0	0.10 ± 0.01
Cis-13-octadecenoic acid	$C_{18}H_{34}O_2$	C18:1	0.08 ± 0.00
Total saturated fatty acid (SFA)			40.86 ± 1.51
Total monounsaturated fatty acid (MUFA)			17.57 ± 0.80
Total polyunsaturated fatty acid (PUFA)			41.57 ± 2.09
Total unsaturated fatty acid (UFA)			59.14 ± 2.89

Furthermore, evaluation of the properties of the microalgae-based biodiesel obtained in this study further strengthens the potential of the simultaneous biofuel feedstock production described. The biodiesel properties of *C. vulgaris* UPSI-JRM01 cultured under the optimum condition in comparison to petroleum diesel and biodiesel standards are summarised in Table 9. Based on this table, it can be seen that the properties of the microalgae-based biodiesel obtained in this study complied with the value and limit imposed by the European biodiesel standard EN14214 and US biodiesel standard ASTM D6751 in terms of its density, KV, IV, CN, and FP [29]. Moreover, the properties of the biodiesel are more closely related to petroleum diesel than biodiesel from plant oil [31;32]. Therefore, these findings demonstrate the promising potential of the biodiesel from *C. vulgaris* UPSI-JRM01 biomass as a fossil fuel (petroleum diesel) alternative.

Table 9 Comparison of the Properties of *C. vulgaris* UPSI-JRM01 Biodiesel to the Petroleum Diesel and International Biodiesel Standards

Biodiesel Properties	International Biodiesel Standards		Petroleum Diesel	*C. vulgaris* UPSI-JRM01 Biodiesel
	EN 14214	ASTM D6751		
Density at 15°C (kg/m_3)	860 – 900	-	840 – 855[abc]	876.42 ± 5.41
KV at 40 °C (mm^2/s)	3.5 – 5.0	1.9 – 6.0	1.34 – 4.1[ab]	3.59 ± 0.94
IV (g I_2 /100 g fat)	<120	<120	1.35[b]	93.47 ± 4.69
SV	-	-		209.12 ± 9.14
CN	>51	>47	50 – 53.3[c]	51.37 ± 2.20
DU (%)	-	-		100.71 ± 4.98
LCSF (%)	-	-		6.46 ± 0.42
CFPP (°C)	≤ 5/ ≤ -20	-	-6[b] -14[a]	3.82 ± 1.31
HHV (MJ/kg)	-	-	43.8[c]	39.45 ± 2.83
FP (°C)	>120	>93	60.5 – 76[ac]	129.31 ± 3.56

Notes. [a] [29]; [b][31]; [c] [32]. Data shown for *C. vulgaris* UPSI-JRM01 biodiesel are mean of three runs ± SD.

The limit imposed for CFPP of biodiesel depends on the regional climate. The microalgae-based biodiesel produced in this study can be used in cold climates by improving the biodiesel's cold performance through i) mixing with petroleum diesel in the appropriate proportion, ii) transesterification with a branched-chain alcohol, iii) the winterisation process, iv) utilisation of chemical additives, and v) alteration of the FAME profiles of biodiesel [33]. Therefore, these research findings could be applied to develop an efficient microalgae cultivation strategy for high biofuel feedstock generation.

4.0 CONCLUSION

By utilising the PBD and CCD, the optimum condition to obtain maximum biomass, lipid, and carbohydrate productivities was successfully determined in this study. More importantly, simultaneous production of carbohydrates and lipids was attained under this optimum condition, which could lead to a lower cost of biofuel production. Although the carbohydrate yield was higher than the lipid yield, the high total biomass produced demonstrates the potential for up-scaling production. In addition, the presence of comparable percentage of PUFA and SFA in the FAME composition able to widen the application of microalgae-based biodiesel in both temperate and tropical countries, respectively.

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