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RESEARCH ARTICLE

Mixed culture of *Chlorella* sp. and wastewater wild algae for enhanced biomass and lipid accumulation in artificial wastewater medium

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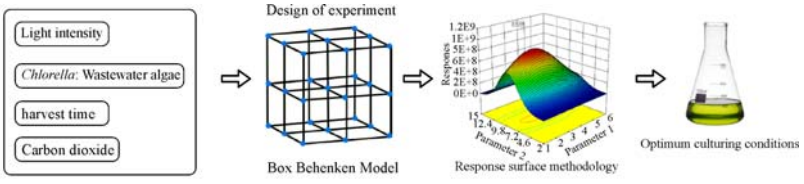
HIGHLIGHTS

- RSM is used to explore the impact of different parameter on algal growth response.
- Mixed algal culture promotes algal biomass and lipid accumulation.
- Optimized conditions achieve maximum productivity of algal biomass and lipid.

GRAPHIC ABSTRACT



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ABSTRACT

The purpose of this work is to study the co-cultivation of *Chlorella* sp. and wastewater wild algae under different cultivation conditions (i.e. CO_2, light intensity, cultivation time, and inoculation ratio) for enhanced algal biomass and lipid productivity in wastewater medium using Response Surface Methodology (RSM). The results show that mixed cultures of *Chlorella* sp. and wastewater wild algae increase biomass and lipid yield. Additionally, findings indicate that CO_2, light intensity and cultivation time significantly affect algal productivity. Furthermore, CO_2 concentration and light intensity, and CO_2 concentration and algal composition, have an interactive effect on biomass productivity. Under different cultivation conditions, the response of algal biomass, cell count, and lipid productivity ranges from 2.5 to 10.2 mg/mL, 1.1×10^6 to 8.2×10^8 cells/mL, and 1.1×10^{12} to 6.8×10^{12} total fluorescent units/mL, respectively. The optimum conditions for simultaneous biomass and lipid accumulation are 3.6% of CO_2 (v/v), 160 μmol/m^2/s of light intensity, 1.6/2.4 of inoculation ratio (wastewater-algae/*Chlorella*), and 8.3 days of cultivation time. The optimal productivity is 9.8 (g/L) for dry biomass, 8.6 E + 08 (cells/mL) for cell count, and 6.8 E + 12 (Total FL units per mL) for lipid yield, achieving up to four times, eight times, and seven times higher productivity compared to non-optimized conditions. Provided is a supportive methodology to improve mixed algal culture for bioenergy feedstock generation and to optimize cultivation conditions in complex wastewater environments. This work is an important step forward in the development of sustainable large-scale algae cultivation for cost-efficient generation of biofuel.

1 Introduction

Current views on environmental pollution, climate change, and resource depletion are focused on fossil fuel consumption and have brought the development of renewable and environmentally benign energy resources to the forefront of scientific inquiry and technical innovation. Herein, biofuels are considered the most promising replacement for non-renewable energy sources, including coal, hydrocarbons, and natural gas (Danielsen et al., 2009; Scott et al., 2010). Microalgae have unique advantages over other biofuel resources, such as animal fat and cultivars (Demirbas and Fatih Demirbas, 2011; Park et al., 2011). Among the advantages of microalgae cultivation, a few that stand out are its ability to make massive quantities of algal biomass rapidly available, it does not diminish arable lands and, importantly, its farming and downstream processes are simple (Chisti, 2007). Most significantly, the level of energy yield in algae is high (Clarens et al., 2010), with a 20%–50% higher oil content compared to other biofuel feedstocks (Chisti, 2007).

Despite the benefits inherent to algae as a source of

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biofuel, algal biofuel has not reached commercial proportions due to enduring technical hurdles (Leite et al., 2013). One significant challenge is the high cost of algae cultivation, particularly the cost associated with nutrients and water supply (Liu et al., 2011; Colosi et al., 2012). As a solution, the integration of algae cultivation with wastewater treatment is a lucrative cost-efficient and sustainable approach to algal biofuel production (Roostaei and Zhang, 2017). Wastewater can provide an ample supply of nutrients and water to support industrial-scale biofuel production, reducing 30%–50% of algal biofuel costs (Clarens et al., 2010; Colosi et al., 2012).

One promising algae candidate for large-scale wastewater-based cultivation is *Chlorella* due to its high lipid content, rapid growth rate, and a capacity to thrive in the wastewater environment (Kobayashi et al., 2013a). Pilot-scale cultivation of chlorella has been carried out in many countries (Borowitzka, 1999; Kobayashi et al., 2013b). Additionally, studies have demonstrated that *Chlorella* grow well in wastewater (Kobayashi et al., 2013a; 2013b). However, contamination by and competition from other microorganisms is a hurdle to scale up this type of exogenous monocultures in wastewater (Johnson and Admassu, 2013; Chen et al., 2015). It is worthwhile to mention that indigenous wastewater wild algae, which have adapted to wastewater conditions, have comparable advantages for a rapid growth rate compared to exogenous monocultures. Nevertheless, while wild wastewater algae have a higher biomass yield, their lipid contents are relatively low (Chen et al., 2011; Chen et al., 2015). This contrast demonstrates how the simultaneous optimization of algal biomass and oil production is difficult to achieve (Scott et al., 2010). This roadblock to progress in the field is especially evident in the wastewater environment where the lipid content of algal biomass is often compromised (Mona, 2013). In address of this obstacle, the optimization of conditions for cultivation of both exogenous and indigenous algae in wastewater presents itself as the logical means to increase algal biomass and lipid productivity simultaneously.

Various parameters affect algal growth and lipid production. In particular, evidence has shown that the level of CO_2 concentration (Huntley and Redalje, 2007; Francisco et al., 2010), light intensity (Ho et al., 2012), and media composition (Georgianna and Mayfield, 2012) have a significant effect on algal growth and lipid production. As of today, existing work has largely studied different cultivation parameters individually, and only investigated the impact of each one on a single output, either biomass or lipid yield. Studies on the interactions of these parameters and their synergized impact on both algal biomass and lipid production are rare, which accounts for the dearth of systematic information for the optimization of wastewater-based algae cultivation.

To overcome the aforementioned challenges, this work uses Response Surface Methodology (RSM) to concurrently investigate the composition of exogenous and indigenous algae and three important cultivation parameters, specifically carbon dioxide ($CO_{2)}$, light intensity, and harvested time, for wastewater-based algal biofuel production. RSM is a polynomial equation with a combination of statistical and mathematical data, which are constructed on the fit of experimental data. It also describes the behavior of the data set to make statistical predictions. Notably, this methodology can evaluate the interactions among up to 50 input variables and optimize them, and it is a proven approach for engineering design and optimization (Zheng et al., 2012; Hallenbeck et al., 2015). Three key objectives comprise this study to 1) understand the interactions of cultivation parameters and their synergized impact on algal biomass and oil productivity; 2) determine the competitive advantages of exogenous and indigenous algae in wastewater-based cultivation; and 3) establish a systematic methodology for the optimization of algal biofuel production within wastewater. The results of this work are integral to a holistic understanding of the design and optimization necessary for the development of integrated wastewater-algae systems in a cost-efficient and sustainable process suitable for algal biofuel production.

2 Materials and methods

2.1 Algal strains and cultivation conditions

Two types of algae were included in this study. One was the pure strain *Chlorella vulgaris* (UTEX 2219, purchased from UTEX's culture collection of algae at the University of Texas, Austin). The other was heterogeneous wastewater wild algae, commonly referred to as mixed green algae, isolated from primary and secondary wastewater effluents from the Detroit Wastewater Treatment Plant. This facility uses a high purity oxygen activated sludge process followed by clarification for secondary treatment in its operation. During the Summer of 2016, primary and secondary wastewater samples with visible presence of algae were collected in sterile falcon tubes. The samples were stored at 4°C during the transportation from the plant to the laboratory. The samples (150 mL each) were transferred to three sterile shake flasks supplied with filter-sterilized carbon dioxide (with air) (v/v: 3%, supplied at 0.25 vvm, volume/volume/min). The flasks were left for shaking (30 r/min) under light (12:12 dark/light cycle) to promote the growth of algae. The algae in the flasks were examined visually and further examined using a flow cytometer (BD Biosciences, California, US). Next, 5 mL of algae culture with the best growth activity was transferred to a sterile 500 mL Erlenmeyer flask containing 200 mL of wastewater medium for another week of cultivation. Enriched algae in the culture were separated by a 1.2 um (pore size) filter paper. The separated algae seed

was cultivated in 200 mL of BG-11 media (Rippka et al., 1979) and used for the study. The *Chlorella vulgaris* culture and the wastewater-borne algae were grown in BG-11 media for seed culture maintenance. To eliminate inconsistency in wastewater characteristics and for a better understanding of the interactions between different parameters, artificial wastewater (AW) was used for experimental trials with the recipe: peptone (160 mg/L), meat extract (110 mg/L), urea (30 mg/L), K_2HPO_4 (28 mg/L), NaCl (7 mg/L), $CaCl_2 \cdot 2H_2O$ (4 mg/L), and $H_{14}MgO_{11}S$ (2 mg/L) (Guideline and Guideline, 2001). The prepared AW has total nitrogen content of 39.6 mg/L and PO_4–P 6.62 mg/L. Algae cultures from the BG-11 medium collected during the exponential-growth phase were used as the seed inoculum for the experimental trials.

The total algal cell number of initial inoculum for all experimental trials was 4×10^5 cells/mL. All experiments were carried out in flasks with 200 mL of artificial wastewater. Algal cultures were grown under 12 h light/12 h dark cycles. Cultivation conditions were adjusted under four input parameters, including the inoculation ratio of wastewater-borne algae to *Chlorella* (1:3, 2:2, 3:1), CO_2 mixed with air (volume/volume percent (v/v,1%–6%) supplied at the same flow rate for all experimental flasks (0.25 vvm, volume/volume/min), light intensity (50–250 μmol/m^2/s), and harvesting time (3–15 days). This choice of inoculation ratio was developed by de Wit (1965) and has since been the most widely used experimental design for analysis of the relationships among multi-cultures. Values for other parameters were set based on preliminary results and other studies (Wahidin et al., 2013; Singh and Singh, 2014). During the experiments, 500 mL baffled Elden Mayer flasks closed with a double holed rubber cork for aeration and pressure release were used. The flasks were mounted on the MaxQ™ HP table-top shaker (Thermo Scientific™ USA) that rotates at the speed of 100 r/min.

2.2 Design of experiments

For the experimental design and post-run statistical analysis, Design Expert® Software Version 10 (Stat-Ease Inc., Minneapolis, USA) was employed. Three factorial Box-Behnken design was generated for the four parameters, including inoculation ratio of wastewater-borne algae to *Chlorella*, CO_2 concentration, light intensity, and harvesting time. The parameters were coded as Eq. (1) as follows:

$$x_i = \frac{X_i - X_i^*}{\Delta X_i} \tag{1}$$

where x_i is the coded value of the i^{th} independent variable; X_i is the un-coded value of the i^{th} independent variable; X_i^* is the un-coded value of the i^{th} independent variable at the center point, and ΔX_i is the step change value.

A total of 29 experimental runs determined by the three factorial Box–Behnken design were carried out. The center point condition was replicated five times to estimate experimental errors. The outputs were algal dry biomass, algal cell count, and algal lipid content. The levels of different variables and the experimental design are shown in Table 1(a) and the experimental raw data is displayed in Table 1(b). Here, the quadratic polynomial equation was fitted to correlate the relationship between independent variables and responses with Eq. (2):

$$y = \beta_0 + \Sigma_{j=1}^{k}\beta_j X_j + \Sigma_{j=1}^{k}\beta_{jj}X^2{}_j + \Sigma_{i=1}^{j-1}\Sigma_{j=2}^{k}\beta_{ij}X_i X_j + \varepsilon \tag{2}$$

Table 1(a) Experimental design by using three factorial Box-Behnken model

Run	Factors (Coded)				Factors (Un-coded levels)			
	a	b	c	d	A	B	C	D
1	0	0	0	0	3.5	150	2:2	9
2	+ 1	+ 1	0	0	6	250	2:2	9
3	+ 1	0	0	+ 1	6	150	2:2	15
4	+ 1	+ 1	0	0	1	250	2:2	9
5	0	−1	0	+ 1	3.5	50	2:2	15
6	+ 1	−1	0	0	1	50	2:2	9
7	+ 1	0	−1	0	6	150	1:3	9
8	0	0	−1	−1	3.5	150	1:3	3
9	0	−1	−1	0	3.5	50	1:3	9
10	+ 1	0	0	+ 1	1	150	2:2	15
11	0	+ 1	0	+ 1	3.5	250	2:2	15
12	0	−1	+ 1	0	3.5	50	3:1	9
13	+ 1	−1	0	0	6	50	2:2	9
14	0	+ 1	−1	0	3.5	250	1:3	9
15	0	0	0	0	3.5	150	2:2	9
16	0	+ 1	+ 1	0	3.5	250	3:1	9
17	0	−1	0	−1	3.5	50	2:2	3
18	0	0	+ 1	−1	3.5	150	3:1	3
19	0	+ 1	0	−1	3.5	250	2:2	3
20	+ 1	0	+ 1	0	6	150	3:1	9
21	0	0	0	0	3.5	150	2:2	9
22	+ 1	0	0	−1	6	150	2:2	3
23	0	0	0	0	3.5	150	2:2	9
24	0	0	−1	+ 1	3.5	150	1:3	15
25	0	0	0	0	3.5	150	2:2	9
26	0	0	+ 1	+ 1	3.5	150	3:1	15
27	+ 1	0	−1	0	1	150	1:3	9
28	+ 1	0	0	−1	1	150	2:2	3
29	+ 1	0	+ 1	0	1	150	3:1	9

Notes: a, A- CO_2 concentration (1%–6%, v/v); b, B- Light intensity (50–50 μmol/m^2/s); c, C- Inoculation ratio of wastewater algae to *Chlorella vulgaris* (1:3, 2;2, 3;1); d, D- Harvesting time (3–15 days)

Table 1(b) Level of different factors maintained and the response of cell count, lipid content and dry weight

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response1	Response2	Response3
	CO_2 concentration(%)	Light intensity (μmol/m^2/s)	Inoculum ratio	Harvest time (days)	Cell count (Cells/mL)	Lipid (FL units)	Dry weight (g/L)
1	3.5	150	2:2	9	8.18E + 08	5.85E + 12	10.1
2	6	250	2:2	9	8.34E + 07	5.89E + 12	9.9
3	6	150	2:2	15	1.23E + 06	2.34E + 12	4.4
4	1	250	2:2	9	3.03E + 08	4.99E + 12	9.2
5	3.5	50	2:2	15	4.17E + 06	1.74E + 12	3.9
6	1	50	2:2	9	5.27E + 08	6.05E + 12	10.2
7	6	150	1:3	9	2.44E + 08	5.38E + 12	10.9
8	3.5	150	1:3	3	3.63E + 06	3.73E + 12	4.4
9	3.5	50	1:3	9	8.36E + 08	6.60E + 12	10.2
10	1	150	2:2	15	1.07E + 07	2.14E + 12	3.3
11	3.5	250	2:2	15	2.37E + 06	1.67E + 12	3.1
12	3.5	50	3:1	9	7.54E + 08	6.23E + 12	9.4
13	6	50	2:2	9	1.01E + 08	4.96E + 12	8.3
14	3.5	250	1:3	9	6.12E + 08	5.08E + 12	8.6
15	3.5	150	2:2	9	8.18E + 08	5.85E + 12	10.1
16	3.5	250	3:1	9	5.30E + 08	5.17E + 12	8.7
17	3.5	50	2:2	3	1.66E + 06	3.46E + 12	3.9
18	3.5	150	3:1	3	3.46E + 06	3.46E + 12	2.8
19	3.5	250	2:2	3	1.35E + 06	3.39E + 12	3.3
20	6	150	3:1	9	2.06E + 08	5.02E + 12	10.6
21	3.5	150	2:2	9	8.18E + 08	5.85E + 12	10
22	6	150	2:2	3	1.07E + 06	4.37E + 12	5
23	3.5	150	2:2	9	8.18E + 08	5.85E + 12	9.7
24	3.5	150	1:3	15	2.70E + 06	2.27E + 12	3.1
25	3.5	150	2:2	9	8.18E + 08	5.85E + 12	9
26	3.5	150	3:1	15	2.02E + 06	1.96E + 12	2.5
27	1	150	1:3	9	4.14E + 08	5.48E + 12	10.2
28	1	150	2:2	3	1.40E + 06	3.36E + 12	3.1
29	1	150	3:1	9	3.32E + 08	5.11E + 12	10.1

where y = predicted response, β_0 = a constant, β_j = linear coefficient, β_{jj} = squared coefficient, and β_{ij} = interaction coefficient, X_i and X_j are the independent variables and ε is noise or error.

2.3 Analytical methods

All samples were analyzed for algal biomass and lipid productivity, including algal cell count, algal dry biomass, and algal lipid content. Flow cytometry analysis was used for algal cell count and lipid analysis by using a BD Accuri™ C6 Flow Cytometer. Specifically, algal cell count was measured by calculation of the fluorescence of chlorophyll *a* with the result of cells/mL. For algal lipid analysis, lipid binding dye, BODIPY 505/515, was used. 10 μL of 1.25×10^{-3} mol/L BODIPY dye was added to 990 μL algal sample. The mixture was mixed well before analysis. A 515 filter in channel 1 (FL1) was used for the detection of lipid binding dye signals (Rumin et al., 2015). The lipid content is estimated by multiplying the cell count containing lipid with the mean florescent intensity detected and the results are expressed as total fluorescent units (Total FL) per mL sample. Note that, in lipid analysis, three outputs of the 29 trials did not fit into the model and are considered statistical errors (Carley et al., 2004). Algal dry biomass was determined by the oven-drying method. Algae were collected using filters (1.2 μm of pore size) and then dried using a hot-air oven until no weight change was

observed. Algal dry biomass was calculated as g/L by measuring the weight of algal culture before and after drying. Please refer to Table 1(b) summarizes the different runs, different factors and algal responses of cell count, lipid content and dry weight.

3 Results and discussion

3.1 Predicted model and statistical analysis

In most studies, as previously alluded, cultivation parameters were individually studied and the interactive effects were not taken into consideration (Gopalakrishnan and Detchanamurthy, 2011; Simionato et al., 2013). These limitations are overcome by RSM design in this study. Herein, the interactions between CO_2 concentration, light intensity, inoculation ratio of wastewater-borne algae to *Chlorella*, and cultivation time, as well as their synergic impact on algal biomass and lipid productivity, are investigated using the Box Behnken statistical model of RSM design. Our findings indicate that the response of algal dry biomass, cell count, and lipid content ranges from 2.5 to 10.2 g/L, 1.069×10^6 to 8.185×10^8 cells/mL, and 1.67×10^{12} to 6.60×10^{12} Total FL/mL, respectively. The ratio of maximum to minimum response is 4.08, 765.96, and 6 for algal dry biomass, cell count, and lipid content, respectively. The ratio of maximum to minimum output of algal cell count is more than 10, which indicates that a power transformation is required. Therefore, a natural log-transformation was performed for the analysis of algal cell count, with the typical response $y' = \ln(y + k)$ being used. In contrast, the typical non-transformed response $y' = y$ is used for the analysis of algal dry biomass and lipid content, since the ratio of maximum to minimum output for these two responses is less than 10. By applying multiple regression analyses of the experimental results, Eqs. (3), (4), and (5) were developed to represent the second order polynomial responses of algal dry biomass, cell count and lipid content, respectively. These equations appear sequentially below:

$$\begin{aligned}\text{Dry Biomass} = {} & +9.78 + 0.000\text{A} + 0.24\text{B} - 0.19\text{C} \\ & - 0.017\text{D} - 1.60\text{AB} - 0.55\text{AC} + 0.25\text{CD} \\ & - 0.74\text{A}^2 - 1.18\text{B}^2 - 0.21\text{C}^2 - 5.67\text{D}^2 \end{aligned} \quad (3)$$

$$\begin{aligned}\text{Cell Count} = {} & +20.52 - 0.53\text{A} - 0.18\text{B} - 0.081\text{C} + 0.24\text{D} \\ & + 0.090\text{AB} - 0.013\text{AC} - 0.47\text{AD} - 0.010\text{BC} \\ & - 0.088\text{BD} - 0.061\text{CD} - 0.86\text{A}^2 - 0.42\text{B}^2 \\ & - 0.073\text{C}^2 - 5.40\text{D}^2 \end{aligned} \quad (4)$$

$$\begin{aligned}\text{Lipid Content} = {} & +6.587 \times 1012 + 2.928 \times 1011\text{A} \\ & + 1.036 \times 1011\text{B} - 6.583 \times 1011\text{C} \\ & - 9.539 \times 1011\text{D} - 2.5 \times 1011\text{AB} \\ & + 7.5 \times 1010 - 2.0333 \times 1011\text{AD} \\ & + 1.083 \times 1011\text{BC} + 1.5 \times 1011 \\ & - 1.167 \times 1010\text{CD} - 6.96 \times 1011\text{A}^2 \\ & - 9.297 \times 1011\text{B}^2 - 4.847 \times 1011\text{C}^2 \\ & - 3.009 \times 1012\text{D}^2 \end{aligned} \quad (5)$$

where A is CO_2 concentration, B is light intensity, C is inoculation ratio of wastewater-borne algae to *Chlorella*, and D is harvesting time.

These equations have been checked for their statistical significance using the F-test. Using Design Expert™ ANOVA analysis, each response (dry biomass, cell count, and lipid content) has been analyzed individually. The results are presented in Tables 2(a), 2(b) and 2(c). Note that the F-values of dry biomass, cell count and lipid content are high: 12.05, 72.11 and 9.05, respectively. The fitness of the model is significant when the F-value is high. Additionally, the P-values of all three responses are low (less than 0.0001), which further confirms that the fitness of the model is highly significant.

The closeness of the fitted regression line with the modeled data is determined by the co-efficient of determination (R^2). The statistical value of the co-efficient of determination is the ratio between the sum of the squares of regression and the total sum of the squares. The R^2 value will be 1, if the regression line fits the data perfectly. Hence, a decline in the level of fit leads to a corresponding decrease in R^2 value. As such, the adjusted R^2 value becomes a statistical measure that illustrates the proportion of deviation from 1 as explained by the estimated regression line. In this study, the R^2 and the adjusted R^2 values are 0.9234 and 0.8468, 0.9863 and 0.9726, and 0.905 and 0.8010 for the algal dry biomass, cell count, and lipid content, respectively. These results clearly show that the model fits closely with the data for all three responses. In Fig. 1, the relationship between the real and the predicted response values is apparent. The points in the graphs of Fig. 1 illustrate the deviation of actual values from predicted values. In all the cases, the constituted model is satisfactory because residuals in the prediction at each response value are low since the points remain close to the diagonal line.

P-values corresponding to each linear co-efficient (β_j in Eq. (2)), cross-parameter coefficient (β_{ij} in Eq. (2)), and quadratic term coefficient (β_{jj} in Eq. (2)) are used to determine the significance of the corresponding input

Table 2 Variance analysis of response surface quadratic model for algal dry biomass (2(a)), cell count (2(b)), and lipid content (2(c)). The sum of squares signifies the deviation of the experimental data from the mean value. Degree of freedom is represented by df, which is the number of freedom independent ways the dynamic system can be moved. The mean square shows the degree of freedom divided by the df

Table 2(a) Variance analysis of response surface quadratic model for algal dry biomass

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob>F	Significance
Model	228.61	14	16.33	12.05	< 0.0001	Significant
A-CO_2	2.842E-014	1	2.842E-014	2.098E-014	1.0000	
B-Light	0.70	1	0.70	0.52	0.4838	
C-Inoculum	0.44	1	0.44	0.33	0.5774	
D-time	3.333E-003	1	3.333E-003	2.461E-003	0.9611	
AB	10.24	1	10.24	7.56	0.0157	Significant
AC	1.21	1	1.21	0.89	0.3606	
AD	0.36	1	0.36	0.27	0.6142	
BC	1.10	1	1.10	0.81	0.3822	
BD	1.000E-002	1	1.000E-002	7.382E-003	0.9327	
CD	0.25	1	0.25	0.18	0.6740	
A^2	3.59	1	3.59	2.65	0.1257	
B^2	9.06	1	9.06	6.69	0.0216	Significant
C^2	0.28	1	0.28	0.20	0.6580	
D^2	208.47	1	208.47	153.90	< 0.0001	Significant
Residual	18.96	14	1.35			
Lack of Fit	18.10	10	1.81	8.34	0.0278	Significant
Pure Error	0.87	4	0.22			
Corrected Total	247.57	28				

Notes: $R^2 = 0.9234$, Adjusted $R^2 = 0.8468$

Table 2(b) Variance analysis of response surface quadratic model for algal cell count

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob>F	Significance
Model	202.16	14	14.44	72.11	< 0.0001	Significant
A-CO_2	3.39	1	3.39	16.91	0.0011	Significant
B-Light	0.40	1	0.40	1.98	0.1811	
C-Inoculum	0.080	1	0.080	0.40	0.5384	
D-time	0.67	1	0.67	3.33	0.0894	
AB	0.032	1	0.032	0.16	0.6932	
AC	7.180E-004	1	7.180E-004	3.585E-003	0.9531	
AD	0.89	1	0.89	4.45	0.0534	
BC	4.111E-004	1	4.111E-004	2.053E-003	0.9645	
BD	0.031	1	0.031	0.16	0.6989	
CD	0.015	1	0.015	0.074	0.7894	
A^2	4.75	1	4.75	23.74	0.0002	Significant
B^2	1.13	1	1.13	5.65	0.0323	Significant
C^2	0.034	1	0.034	0.17	0.6856	
D^2	189.17	1	189.17	944.69	< 0.0001	Significant
Residual	2.80	14	0.20			
Lack of Fit	2.80	10	0.28			

(*Continued*)

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob>F	Significance
Pure Error	0.000	4	0.000			
Corrected Total	204.96	28				

Notes: $R^2 = 0.9863$, Adjusted $R^2 = 0.9726$

Table 2(c) Variance analysis of response surface quadratic model for algal lipid content

Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob>F	Significance
M odel	7.247E + 025	14	5.176E + 024	9.05	< 0.0001	Significant
A-CO_2	1.029E + 024	1	1.029E + 024	1.80	0.2012	
B-Light	1.288E + 023	1	1.288E + 023	0.23	0.6424	
C-Inoculum	5.201E + 022	1	5.201E + 022	0.091	0.7674	
D-time	1.092E + 025	1	1.092E + 025	19.09	0.0006	Significant
AB	2.500E + 023	1	2.500E + 023	0.44	0.5192	
AC	2.250E + 022	1	2.250E + 022	0.039	0.8456	
AD	1.654E + 023	1	1.654E + 023	0.29	0.5992	
BC	4.694E + 020	1	4.694E + 020	8.208E-004	0.9775	
BD	9.000E + 022	1	9.000E + 022	0.16	0.6976	
CD	5.444E + 020	1	5.444E + 020	9.520E-004	0.9758	
A^2	3.142E + 024	1	3.142E + 024	5.49	0.0344	Significant
B^2	5.607E + 024	1	5.607E + 024	9.80	0.0074	Significant
C^2	1.524E + 024	1	1.524E + 024	2.66	0.1249	
D^2	5.874E + 025	1	5.874E + 025	102.71	< 0.0001	Significant
Residual	8.007E + 024	14	5.719E + 023			
Lack of Fit	7.495E + 024	10	7.495E + 023	5.86	0.0516	
Pure Error	5.120E + 023	4	1.280E + 023			
Corrected Total	8.047E + 025	28				

Notes: $R^2 = 0.9005$, Adjusted $R^2 = 0.8010$

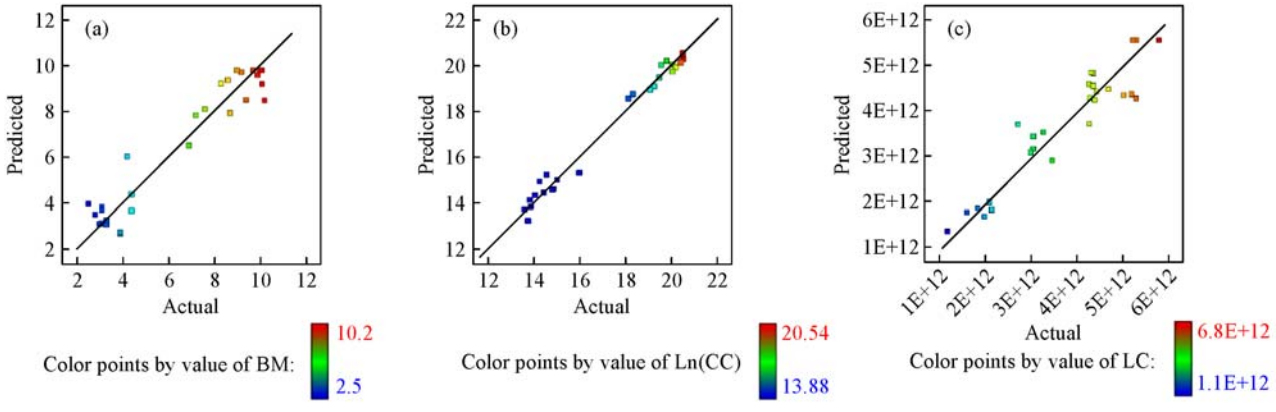

Fig. 1 The fitness of the actual and predicted results. These graphs show the high closeness of the fitted regression between the actual and predicted biomass (BM), cell count (CC) and lipid content (LC). (a) the fitness of the actual and predicted algal dry biomass; (b) the fitness of the actual and predicted algal cell count; (c) the fitness of the actual and predicted algal lipid content

variables. *P*-values less than 0.05 are considered statistically significant because the lower the *p*-value, then the higher the significance of the input variable to the response output (Chen et al., 2010). The significant variables in this data set are AB, B^2 and D^2 for dry biomass (Table 2(a)), A, A^2, B^2 and D^2 for cell count (Table 2(b)), and D, A^2, B^2 and D^2 for lipid content (Table 2(c)). These results show that the impact of CO_2 concentration, light intensity, inoculation ratio, and cultivation time are not equal in all three responses.

3.2 Algal dry biomass

In this study, the optimum conditions for maximum biomass productivity have been determined as 3.4% of CO_2 concentration (v/v), 180 μmol/m^2/s of light intensity, 1.2: 2.8 of inoculation ratio (wastewater-borne algae: *Chlorella*), and 8.4 days of cultivation time (Table 3). The maximum dry biomass attained is 9.9 g/L and the desirability value is 0.961. In optimization studies, desirability is an objective function that determines optimum conditions, and is a multiple response method defined by Montgomery and Myers (1995). A point that maximizes the desirability function is determined by numerical optimization. The 3-D response plots based on the predicted models are shown in Fig. 2(a)–2(f). These figures illustrate the effect of each individual parameter, the interactions between different parameters, and their synergic impact on algal biomass yield. Significant parameters affecting biomass productivity are the light intensity, cultivation time, and the interactive effect of CO_2 and light intensity ($p < 0.05$).

Interestingly, CO_2 concentration and light intensity have a significant synergistic impact on biomass productivity ($p < 0.05$, Table 2(a), Fig. 3(a)). As shown in Fig. 3(a), at low light intensity (50 μmol/m^2/s) algal biomass increases with elevated CO_2 concentration. However, when the light intensity is high (250 μmol/m^2/s), there is a decline in biomass along with an increase in CO_2 concentration. Also, at low CO_2 concentrations, accumulated biomass yield steadily rises in tandem with the increase of light intensity. However, when CO_2 concentration is high, low light intensity is more favorable for biomass accumulation (Fig. 2(a)). The increase in light intensity promotes photosynthesis until the cell reaches its photo inhibition stage (Roach and Krieger-Liszkay, 2014). The results of this study clearly indicate that there is a strong reverse cross-interaction between light intensity and CO_2 concentration in biomass production.

Although not statistically significant, it was evident in this study that CO_2 concentration and inoculation ratio have a cross interactive effect on biomass productivity (Figs. 3(a) and 3(b)). The increase of inoculation from *Chlorella* to wastewater borne algae tends to increase biomass productivity at low CO_2 concentrations. However, when CO_2 concentration is high, the elevation of CO_2 concentration has a negative impact on biomass accumulation with the increased inoculum of wastewater algae. This indicates that wastewater-borne algae could utilize CO_2 more efficiently and have a higher growth rate than that of *Chlorella* when CO_2 concentration is limited. However, with sufficient CO_2 supply, *Chlorella* has a better growth rate than wastewater algae and the reason could be that wastewater-algae are more adapted to harsh cultivation conditions.

The influence of CO_2 and cultivation time, which is also known as harvesting time or HT, is in Fig. 2(c). As aforementioned, cultivation time has an enormous impact on biomass productivity. Due to the pronounced impact of cultivation time on biomass accumulation, the effect of CO_2 has a downgraded impact. Moreover, there is no significant interactive effect between CO_2 and cultivation time. The reason could be that the response change is dominated by the harvesting time. Nonetheless, results show that maximum biomass is attained at an intermediate level of CO_2 (3.4% of CO_2, v/v). A too low or too high CO_2 concentration has the potential to undermine algal growth. Carbon dioxide is the source of carbon for algal growth. However, increasing carbon dioxide above the required limit can change the pH of the medium, which will restrict or suppress algal growth.

Figure 2(d) illustrates the effect of light intensity and inoculation ratio. An increase of light intensity from 50 to 250 μmol/m^2/s shows an invert parabolic change in biomass accumulation. Note that when the mixed culture shifts from *Chlorella* to wastewater-algae, the curvature of

Table 3 Optimal conditions for three outputs (biomass, cell count and lipid content) individually and together with desirability values

Optimal condition				Response	Optimum outcome	Desirability
CO_2	LI	IR	HT			
3.4	180	1.2:2.8	8.4	Biomass (g/L)	9.9	0.961
2.2	187	1.5:2.5	8.7	Cell count (Cells/mL)	8.4×10^8	0.921
4.1	153	2.0:2.0	7.7	Lipid content (Total FL)	6.9×10^{12}	1.000
3.6	159	1.6:2.4	8.3	Overall biomass (g/L)	9.9	0.985
				Overall cell count (Cells/mL)	8.6×10^8	
				Overall lipid content (Total FL/mL)	6.8×10^{12}	

Notes: CO_2 - CO_2 concentration (1%–6%, v/v); LI- Light intensity (50–250 μmol/m^2/s); IR- Inoculation ratio of wastewater algae to *Chlorella vulgaris* (1:3, 2;2, 3;1); HT- Harvesting time (3–15 days)

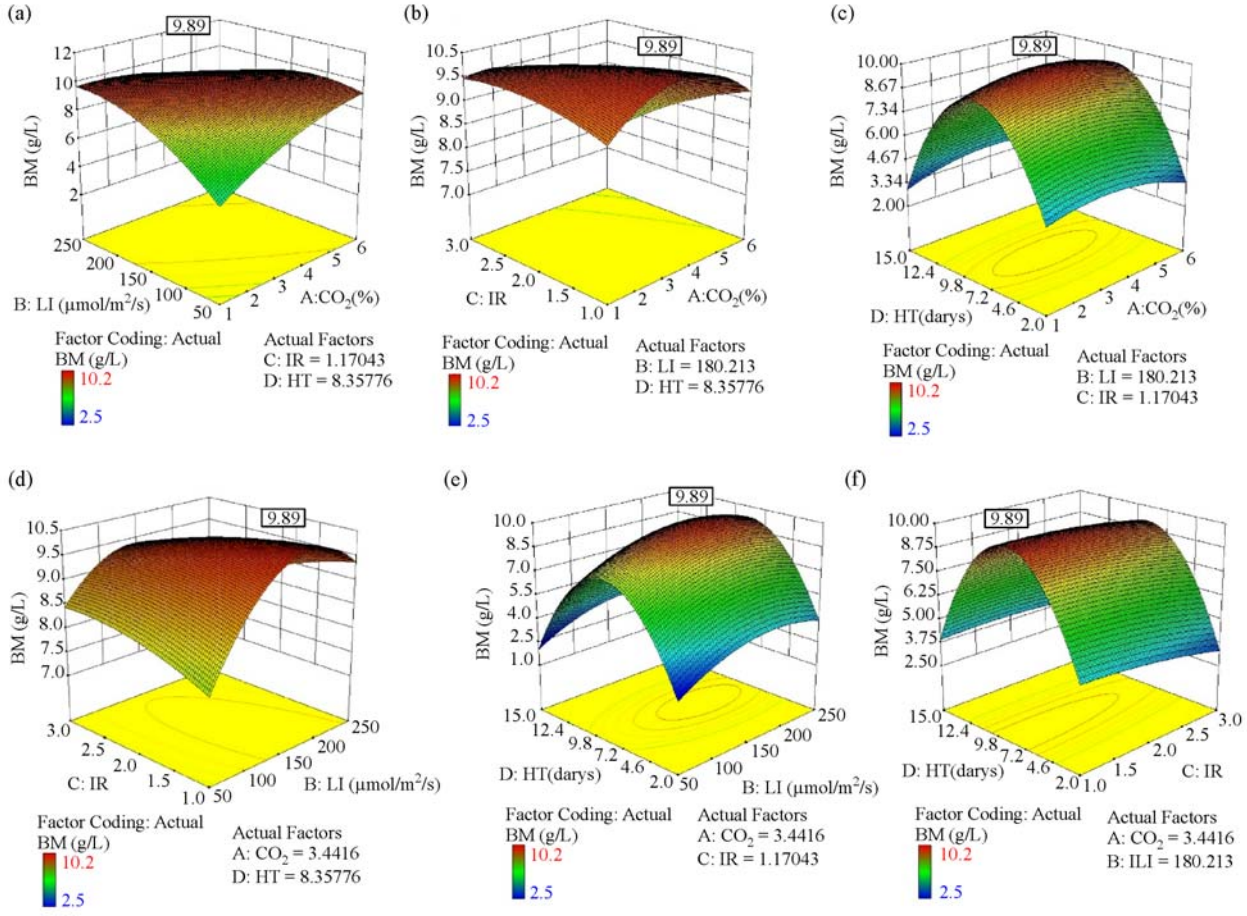

Fig. 2 The response of algal dry biomass to different cultivation parameters. 3D surface response and contour line of Box-Behnken Design showing the mutual effect of different parameters on algal biomass (BM) with maximum response value in boxes. **IR**, inoculation ratio of wastewater algae to *Chlorella* (1:3, 2:2, 3:1), with the higher number indicating a high ratio of wastewater algae; **LI**, light intensity, 50– 50 µmol/m^2/s; **HT**, harvesting time, 3–15 days; **CO_2**, CO_2 concentration, 1%–6% (v/v). (a)the response of algal dry biomass to LI and CO_2; (b) the response of algal dry biomass to IR and CO_2; (c) the response of algal dry biomass to HT and CO_2; (d) the response of algal dry biomass to IR and LI; (e) the response of algal dry biomass to HT and LI; (f) the response of algal dry biomass to HT and IR

the output becomes less significant with respect to light intensity change. This indicates that, compared to *Chlorella*, wastewater-borne algae is less sensitive to changes in light intensity and has a more stable growth performance under variable light.

The effects of cultivation time, light intensity and inoculation ratio appear in Figs. 2(e) and 2(f), respectively. It is shown that, for cultivation time, algal biomass at the lag phase is low. The maximum level is reached at the stationary phase of 8.4 days, and longer cultivation time results in biomass reduction because algal culture enters into a phase of decline. The increase in inoculation ratio of wastewater algae at a short cultivation time reveals a minor decline in biomass yield, but as the cultivation time extends there is no significant impact on the inoculation ratio. This indicates that the growth rate of wastewater algae at the lag phase is slower than that of *Chlorella*. The increase in light intensity shows sufficient BM accumulation as biomass reaches the maximum output at the midway (180 µmol/m^2/s) and then slightly declines as the light intensity further increases.

Biomass productivity is critical for algal biofuels production. Furthermore, it is well understood that cultivation conditions have a significant impact on biomass productivity. Moreover, it is extensively documented that different algal species have varied growth rates and optimal conditions for cultivation. Therefore, it is warranted to optimize the conditions for algal cultivation and promote algal culture composition for maximum biomass yield. The results of this study indicate that *Chlorella* and wastewater-algae have different preferences for cultivation parameters. Compared to *Chlorella*, wastewater algae evidence better growth at lower CO_2 concentration, as well as with light change. These are

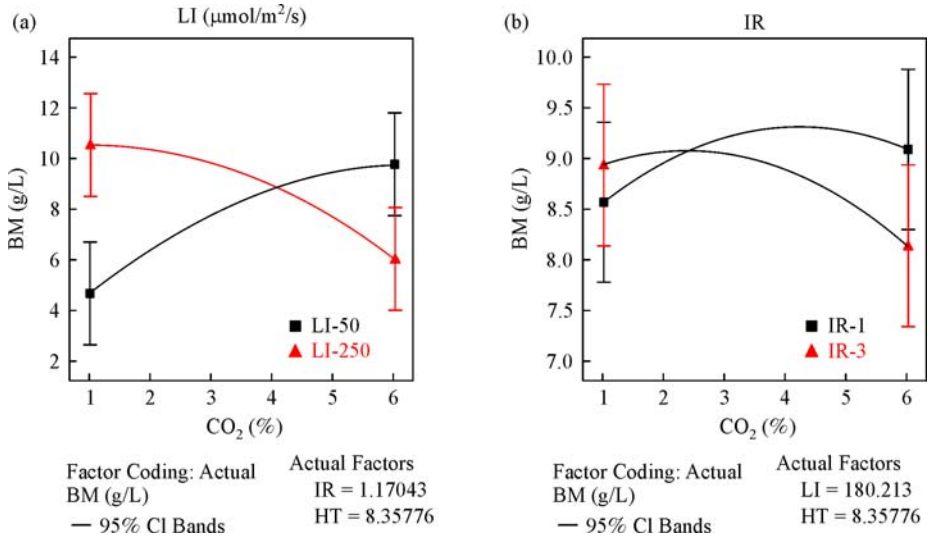

Fig. 3 The cross lines of CO_2 and light intensity, and CO_2 and inoculation ratio demonstrate the cross-interactive impact of these parameters on algal biomass. (a) Interaction plot of CO_2 concentration with respect to light intensity on biomass response. (b) Interaction plot of CO_2 concentration with respect to inoculation ration on biomass response

desirable features when cultivating algae in harsh conditions, such as under the effects of a limited CO_2 supply and drastic light variations. However, *Chlorella* has a shorter lag-phase for initial growth, which indicates that *Chlorella* may require less cultivation time to achieve proper biomass accumulation. Taken together, these factors establish the criticality of biomass productivity to sustainable and profitable algal biofuels production.

3.3 Algal cell count

The optimum conditions for maximum cell count are 2.2% of CO_2, 187 μmol/m^2/s of light intensity, 1.5:2.5 of inoculation ratio (wastewater algae: *Chlorella*), and 8.7 days of harvesting time (Table 3). The maximum algal cell count attained is 8.4×10^8 cells/mL and the desirability value is 0.921. Figures 4(a)–4(f) show 3D response surface plots based on the predicted models. These figures illustrate the effect of each individual parameter, the interactions between different parameters, and their synergic impact on algal cell count. The significant parameters identified during this study that affect algal cell count are CO_2, light intensity, and cultivation time ($p < 0.05$).

Figure 4(a) shows the impact of light intensity and CO_2 on cell count. For both parameters, maximum cell count is attained at the intermediate stage. It was shown that higher or lower values than those at the optimal point lead to a decline in algal cell count. Figure 4(b) illustrates the effect of CO_2 and inoculation ratio. Although the change of inoculation ratio does not have a significant impact on the response of algal cell count, the inoculation ratio of 1.7: 2.3 (wastewater algae: *Chlorella*) yields the maximum cell count. In Fig. 4(c), the effect of harvesting time and CO_2 is shown, which illustrates how algal cell count boosts-off from the exponential phase, reaches its maximum at 8.7 days, and then declines. Note that the impact of CO_2 concentration is significant in the exponential and stationary stages, where the maximum cell count is attained at middle level of CO_2 (2.2%, v/v). However, CO_2 concentration has a minor impact in the lag- and decline- phases, which is reasonable given much lower growth activity at these two stages. For the effect of inoculation ratio and light intensity (Fig. 4(d)), the impact of an increase in light intensity is displayed as an inverted parabolic pattern where the maximum cell count is observed at mode light intensity (187 μmol/m^2/s). In contrast, inoculation ratio has little influence. The effect of harvesting time with respect to light intensity and inoculation ratio is shown in Figs. 4(e) and 4(f), respectively. The impact of light intensity is shown as an inverted parabola, with the most significant impact being observed in the exponential and stationary stages rather than in the lag- and decline-phases. Although not statistically significant, at optimum harvest time, the increased ratio of wastewater algae in the inoculum (up to 1.5: 2.5 of wastewater algae: *Chlorella*) enhances cell count to a small extent. However, any further increase in wastewater algae gradually reduces cell count.

Cell count is another important indicator for algal growth rate. The response patterns of algal cell count to the four parameters identified in the figure above are similar to those of algal dry biomass. The optimal conditions for light intensity and cultivation time are nearly the same as those for algal cell count and biomass productivity. However, the optimal conditions for CO_2 concentration and inoculation ratio (wastewater algae: *Chlorella*) are different (Table 3): 2.2% (v/v) and 1.5: 2.5 for algal cell count versus 3.4% (v/

v) and 1.2: 2.8 for algal biomass. The reason could be the synergic impact of different nutrient supplies and algal species composition. Algal cell count is merely determined by cell proliferation, while algal biomass is affected by both cell number and cell size. Therefore, the preferred nutrient conditions for algal cell proliferation and cell size could be different. For instance, Garcia et al., reported that the cell size of marine phytoplankton depends on nutrient conditions (Garcia et al., 2016). Moreover, different algal species have various cell sizes and proliferation rate. From this study, a higher wastewater-algae ratio and lower CO_2 concentration under optimal conditions for algal cell count reveal that wastewater-algae proliferate more rapidly with a lower nutrient supply.

3.4 Lipid productivity

In Table 3, the optimum conditions for maximum algal lipid content are 4.1% of CO_2 concentration, 153 μmol/m^2/s of light intensity, 2.0: 2.0 of inoculation ratio (wastewater-algae: *Chlorella*), and 7.7 days of harvesting time. Overall lipid productivity is determined by the sum of fluorescent intensity in the algal cells determined by lipid fluorescence reading and the results are expressed as total florescent units per mL sample (total FL/mL). This method can generate information of total lipid productivity and allow the examination of lipid concentration on a cellular level of changes (Rumin et al., 2015). The maximum lipid content attained is 6.9×10^{12} (Total FL/mL) and its desirability value is 1. As shown in Table 2(c), the significant parameters affecting algal lipid content are CO_2, light intensity, and cultivation time ($p < 0.05$).

The 3D response plots based on the predicted models are shown in Fig. 5(a)–5(f). Herein, Fig. 5(a) shows the effect of light intensity and CO_2 concentration. It was found that, for both parameters, lipid content reaches the maximum at the middle range. Figure 5(b) shows the impact of CO_2 and inoculation ratio. While not statistically significant, the maximum lipid content is attained when the inoculation ratio is at the intermediate range and there is no cross interaction between these two parameters. In Fig. 5(c) is shown the impact of harvesting time and CO_2 concentration. Note that lipid content is high at the exponential and stationary phases; it increases with elevated CO_2 concentration in all growth stages of harvesting time and reaches maximum at 4.2% of CO_2 (v/v). Withal, a further increase of CO_2 (up to 6%) reduces lipid content. Figure 5(d) shows the impact of light intensity and inoculation ratio on lipid productivity. For both parameters, maximum lipid content is observed in the middle range. Figure 5(e) shows the impact of light intensity and harvesting time. The lipid content is low at the lag- and decline- phases of algae cultivation. Light intensity in the middle ranges shows a good accumulation of lipids. Finally, Fig. 5(f) shows the impact of inoculation ratio and harvesting time. Since the impact of harvest time is high, the effect of the inoculation ratio is not excessively projected.

Lipid content is a key factor in algal biofuel production because a higher lipid content is optimal for biofuel productivity and downstream conversion processes. The overall response patterns of algal lipid content to the four parameters are similar to those of algal dry biomass and cell count. However, the optimal conditions for CO_2 concentration, light intensity and inoculation ratio of wastewater algae versus *Chlorella* are different, as shown in Table 3. Higher CO_2 concentration and lower light intensity tend to promote lipid accumulation. This result is consistent with other reports. According to Sun et al. (2016) the increase in carbon metabolism boosts lipid accumulation. In that study it was shown that carbon metabolism is elevated when the concentration of CO_2 is increased. In other findings, lipid production is induced at low light intensity, and decreased at high light intensity (Nogueira et al., 2015). In addition, the ratio of wastewater algae for optimum lipid productivity is higher than that for biomass yield. This observation is very interesting compared to previous studies where lower lipid productivity was usually reported for wastewater algae (Chen et al., 2011; Chen et al., 2015). The total lipid productivity in algae depends on both algal biomass yield and lipid concentration in cells. Currently, the most common approach to improve lipid accumulation is through nutrient starvation. However, this strategy is complicated and unpredictable because it is very challenging to balance the trade-off between biomass yield and lipid concentrations in algal cells under these stress conditions (Chen et al., 2015). To meet this challenge, this work provides comprehensive information in terms of how to optimize cultivation parameters and algal species for improved lipid productivity. In this study, the overall lipid productivity is dependent on the number of algal cells with lipid-bonded fluorescence signal and the mean fluorescence intensity obtained. When the cell count rises along with higher wastewater algae inoculum (up to 2: 2), inevitably the lipid productivity increases. Moreover, the wastewater algae used in this study is a heterogeneous culture containing different algal species. As such, some algal species could have higher lipid content. However, the profile of algal species under the cultivation is not within the scope of this investigation. Looking forward, this work offers the foundation for further study focused on the identification of algal species that have a rapid growth rate as well as high lipid content.

There are a number of cultivation strategies to promote lipid accumulation. Previously, studies have indicated that lipid content could be enhanced under cultivation stresses. For example, nitrogen depletion is one approach for increasing the lipid content of algal cells. However, this strategy may result in an overall reduction of lipid productivity due to low growth rate. This delicate balance between overall lipid productivity and lipid content

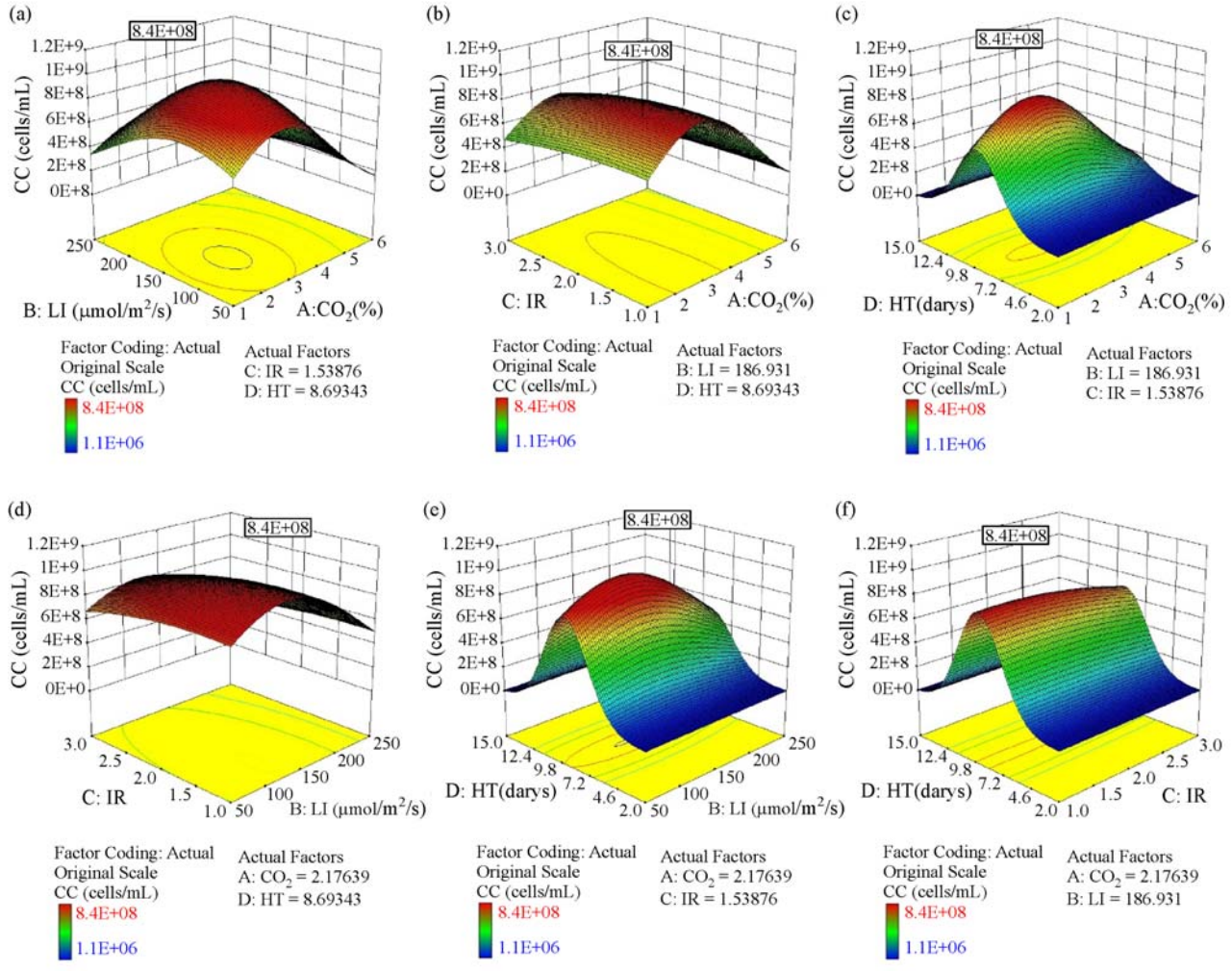

Fig. 4 The response of algal cell count to different cultivation parameters.3D surface response and contour line of Box-Behnken Design showing the mutual effect of different parameters on algal cell count (CC) with maximum response value in the boxes above each parabola. **IR**, the inoculation ratio of wastewater algae to *Chlorella* (1:3, 2:2, 3:1), with the higher number indicating a high ratio of wastewater algae; **LI**, light intensity, 50–250 μmol/m^2/s; **HT**, harvesting time, 3–15 days; **CO_2**, CO_2 concentration, 1%–6% (v/v). (a) the response of algal cell count to LI and CO_2; (b) the response of algal cell count to IR and CO_2; (c) the response of algal cell count to HT and CO_2; (d) the response of algal cell count to IR and LI; (d) the response of algal cell count to IR and LI; (e) the response of algal cell count to HT and LI; (f) the response of algal cell count to HT and IR

demonstrates yet another confounding factor in the optimization of lipid accumulation highlighting present challenges in the field. Some studies have reported that, for a few algal species, *Chlorella* sp., *Dunaliella* sp., *Nannochloris* sp., *Parietochloris incisa*, *Neochloris oleoabundans* and *Botryococcous braunii*, the growth rate and lipid content can be simultaneously enhanced under favorable conditions (Illman et al., 2000; Takagi et al., 2000; Bigogno et al., 2002; Li and Qin, 2005; Takagi et al., 2006; Liu et al., 2008; Xiong et al., 2008). Our results show that, although the optimal conditions for biomass and lipid content differ in some parameters, the response patterns to these parameters are very similar. These findings indicate that simultaneous accumulation of biomass and lipid content are achievable by setting cultivation conditions through an address of system design and optimization with a supportive and systematic methodology.

3.5 Optimization for algal dry biomass, cell count, and lipid content

Given the equal importance of algal biomass, cell proliferation (cell count), and lipid content for algal biofuel production, simultaneous optimization of all these desired outputs is the answer to longstanding challenges. In this study, the RSM model projects that the optimum conditions for maximum biomass, cell count, and lipid

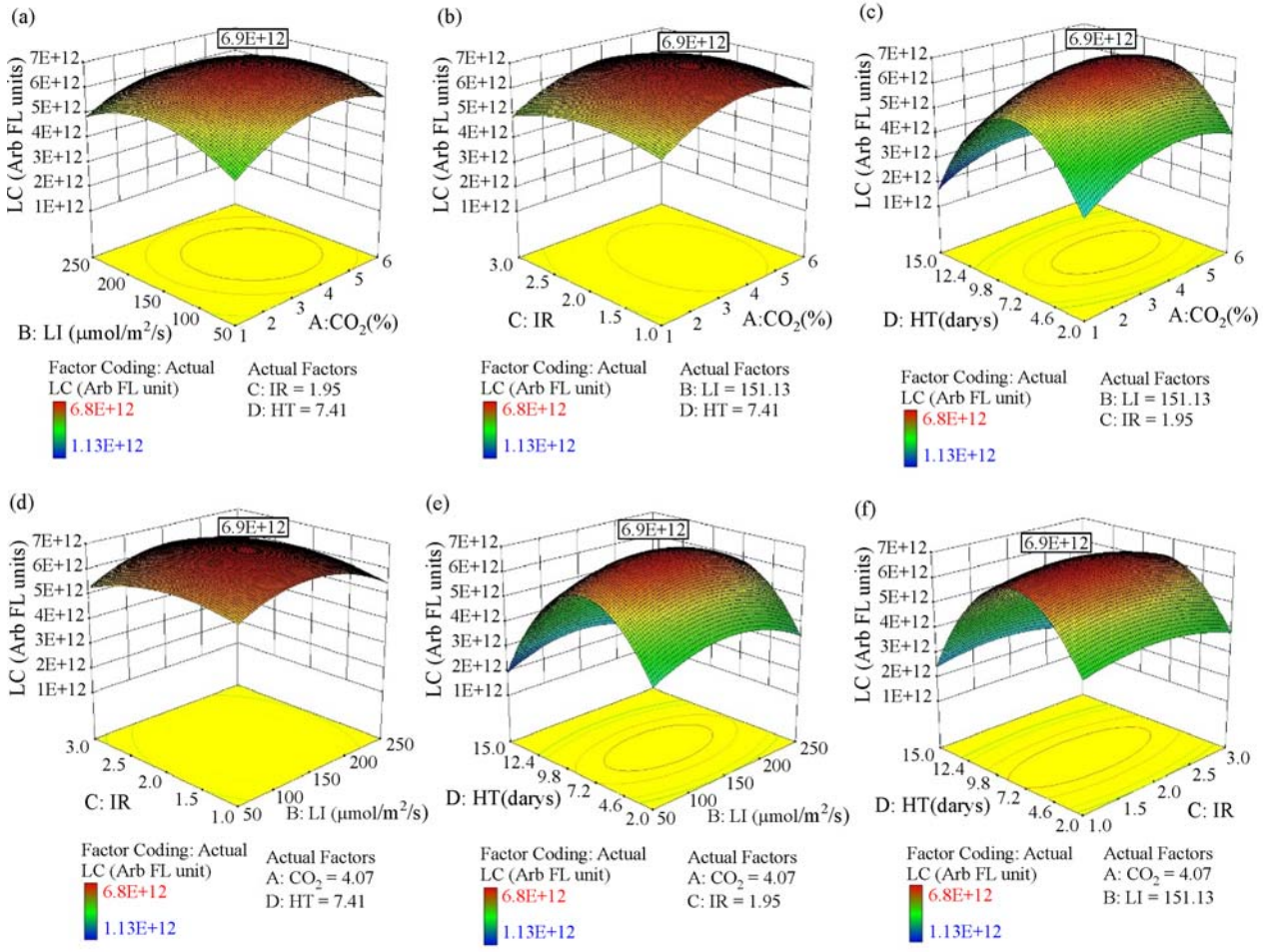

Fig. 5 The response of algal lipid productivity to different cultivation parameters. 3D surface response and contour line of Box-Behnken Design showing the mutual effect of different parameters on algal lipid content (LC) with the maximum response value in the box above eachfigure. **IR**, the inoculation ratio of wastewater algae to *Chlorella* (1:3, 2:2, 3:1), with the higher number indicating high ratio of wastewater algae; **LI**, light intensity, 50–250 μmol/m^2/s; **HT**, harvesting time, 3–15 days; **CO_2**, CO_2 concentration, 1%–6% (v/v). (a) the response of algal lipid productivity to LI and CO_2; (b) the response of algal lipid productivity to IR and CO_2; (c) the response of algal lipid productivity to HT and CO_2; (d) the response of algal lipid productivity to IR and LI; (e) the response of algal lipid productivity to HT and LI; (f) the response of algal lipid productivity to HT and IR

content are 3.6% of CO_2, 160 μmol/m^2/s of light intensity, 1.6:2.4 of inoculation ratio (wastewater-algae: *Chlorella*), and 8.3 days of cultivation time. The attained level is 9.8 (g/L) for dry biomass, 8.6 E + 08 (cells/mL) for cell count, and 6.8 E + 12 (Total FL units per mL) for lipid productivity (Table 3). Note that the optimum conditions to obtain the maximum overall outcome and maximum individual outcomes are not the same. The percentage of carbon dioxide required to obtain a maximum overall response is 3.6%, which is found between the optimum condition for maximum biomass and lipid response. Similarly, optimum inoculation ratio for overall outcome is between those for biomass/cell count and lipid content. Likewise, conditions in light intensity optimized to generate an overall maximum outcome are nearly the same as those for lipid productivity. Lastly, the cultivation time for overall maximum output is about one day shorter than that for the accumulation of maximum biomass and cell count.

It is well known that various nutrient and environmental factors affect algal biomass productivity and lipid accumulation. As it stands today, the optimization of algal cultivation has been studied for a few decades. However, most of these studies have investigated each cultivation parameter individually. Rather than isolating these factors individually, the interaction of different parameters and their synergic effect demands their study as a whole. For instance, the rise of temperature can lead to the diminution of nutrient availability for lipid accumulation (Sterner and Grover, 1998). This leads to speculation

that low temperature tends to promote favorable nutrient conditions for lipid yield. Morris et al. (1974) reported high lipid content in *P. tricornutum* at low temperatures. In contrast, Smith and Morris (1980) described microalgae in the cold environment in the Antarctic ocean incorporated more carbon into the protein fraction, resulting in lipid reduction. These disparate observations indicate that similar nutrient conditions under different environmental conditions generated opposite results. These conflicting results could be attributed to the interactive impact of other environmental parameters that had not been considered in those studies.

There are a few limited studies that have proven the interactive impact of different cultivation parameters. For example, Cloern et al. (1996) investigated carbon conversion efficiency in microalgae as a function of light and nutrients. They reported that, at low light intensity, the nutrients' availability was high and, as such, the growth efficiency was increased. Likewise, Morgan and Kalff (1979) studied the interactive effect of light and temperature on *Cryptomonas erosa* at standard nutrient composition. They discovered that the decline of carbon conversion capacity along with the reduction of light intensity was more significant at a higher temperature.

In our study, the effect of different cultivation parameters, the interaction of these parameters, and their synergic impact on algal biomass and oil productivity were simultaneously studied and optimized. Although the mechanisms underlying the change of output responses were not determined in this work, the effect of each factor, the interactions of these different factors and their synergic impact have been thoroughly investigated. This is essential for the identification of significant cultivation parameters that underpin the mechanisms that determine algal growth responses. Furthermore, the RSM simulation developed in this work provides a supportive methodology to understand the effects of different parameters on algal growth. Most importantly, our work allows for the comprehensive optimization of cultivation conditions in complex wastewater environments, which is a progress toward large-scale algae cultivation in wastewater for biofuel production.

4 Conclusions

This work provides a supportive and systematic methodology for wastewater-based algae cultivation to enhance bioenergy feedstock production. For the first time, a RSM study was carried out to investigate the impact of algal composition, CO_2, light intensity, and harvesting time on algal growth in wastewater media, and to optimize cultivation conditions for simultaneous provision of algal biomass and lipid accumulation. The results of our study demonstrate that algal biomass and lipid productivity are significantly affected by these parameters individual and collectively. In addition, our results show that the co-cultivation of mixed algal cultures and rationally designed optimization of cultivation conditions increase both valuable biomass yield and energy-rich lipid accumulation. This is an important step forward in large-scale algae cultivation under complex wastewater environments for sustainable and cost-efficient algal biofuel generation. Future work should focus on investigating the effects of other cultivation parameters, identifying algal profile in mixed cultures, and elucidating the mechanisms underlying different algal growth responses to provide more detailed information for practical applications of integrating algae cultivation with wastewater.

Abbreviations

AW, artificial wastewater; **BM**, biomass; **CC**, cell count; **FL1**, filter in channel 1; **HT**, harvesting time; **IR**, inoculation ratio of wastewater algae to Chlorella vulgaris; **LI**, Light intensity; **LC**, lipid content**; RSM**, response surface methodology; **Total FL**, total fluorescent units; **v/v**: volume/volume percent.

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