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Optimization of *Chlorella pyrenoidosa* Y3 biomass production in poultry waste anaerobic-digested effluents using a response surface methodology

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ABSTRACT

Coupling microalgae cultivation with wastewater treatment may provide an economically and environmental friendly way for the production of algae-based biofuel or other value-added products. The nutrient supplement to poultry waste anaerobic-digested effluent (PWADE) as the substrates for *Chlorella pyrenoidosa* Y3 (isolated from Tai Lake) cultivation was investigated and optimized using a response surface methodology (RSM). Based on the Plackett–Burman design, $NaHCO_3$ and $MgSO_4{\cdot}7H_2O$ were selected as the most crucial supplemental nutrients for enhancing the biomass yield and chlorophyll-a content of *C. pyrenoidosa* Y3. A central composite design was employed to determine the optimal concentration of the two selected supplemental nutrients. With the canonical and ridge max analysis method, the maximum biomass yield of 0.819 g/L was obtained from algae cultivated in the 2% (v/v) PWADE substrate with addition of 4.81 g/L $NaHCO_3$ and 92.9 mg/L $MgSO_4{\cdot}7H_2O$ after 20 d of the cultivation, which was 1.5 times higher than that of algae cultured in the non-optimized PWADE medium (0.559 g/L). The chlorophyll-a content of algae reached to 27.51 mg/L, and about 100.0% NH_4-N and 59.5% total phosphorus were removed from the PWADE with the optimized medium condition. Results indicate that RSM is a reliable method in proposing models for optimizing the algae growth in PWADE.

Keywords: Poultry waste anaerobic-digested effluent; *Chlorella pyrenoidosa* Y3; Biomass; Plackett–Burman design; Central composite design

1. Introduction

Anaerobic digestion (AD) has been widely applied to decompose livestock waste, and produce biogas using mesophilic or thermophilic bacteria. But the anaerobic-digested effluent usually contains high amounts of nitrogen, phosphate, organic matter, and suspended solids that cause eutrophication when discharged without proper treatment [1,2]. Therefore, recovery of nutrients in the anaerobic-digested effluents (ADE) from livestock waste is becoming a major concern due to the environmental issues.

Microalgae have been getting a lot of attention as a promising feedstock for the biofuel production, due to rapid biomass growth rates and the ability to extract the nutrients from waste streams. The great potential of the biomass production of microalgae cultivation

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on waste streams for simultaneous wastewater treatment and biofuel has been illustrated [3–5]. Wastewater provides not only water medium, but also most of the necessary nutrients suitable for algae. The feasibility of ADE as medium for microalgae cultivation has been investigated [4–6]. But the ADE from livestock waste are usually opaqueness and biorefractory. Additionally, ADE directly used as a medium for algae is unfavorable due to the unbalanced concentrations of inorganic nutrients and the presence or absence of some micronutrients which can inhibit or promote algal growth [6,7]. The microalgae growth and biomass production mainly depends on the medium nutrients sources and levels under the same environmental conditions (light, temperature, pH, initial inoculation, etc.) [8]. Thus, the nutritional component and level in the ADE need to be optimized for enhancing the algal growth.

The feasibility of poultry waste anaerobic-digested effluent (PWADE) as a medium for cultivating *Chlorella pyrenoidosa* Y3 isolated from Tai Lake in Jiangsu province, China has been conducted [9]. The optimum growth was obtained from 2% (v/v) PWADE with $NaHCO_3$ as an inorganic carbon additive which significantly enhanced the growth of *C. pyrenoidosa* Y3. In the present work, the statistical optimization of medium components in PWADE by response surface methodology (RSM) was performed for enhancing the biomass production of algae. The Plackett–Burman design (PBD) and central composite design (CCD) have been widely applied for optimization of biomass or biological by-products production, which can reduce the quantity of the tests and minimize the error with the reliable results [10–12].

The objective of this work was to optimize the biomass production of *C. pyrenoidosa* Y3 cultured in PWADE using a RSM. The most critical supplemental nutrients added to the PWADE for algae growth were determined by PBD. A CCD was used to optimize the screened components to maximize the biomass yield of *C. pyrenoidosa* Y3.

2. Materials and methods

2.1. Poultry waste anaerobic digested effluent

The anaerobic-digested effluent was collected from a large-scale biogas plant located in a poultry farm in Beijing, China, transported to the lab, and stored at 4˚C until used. The PWADE samples were centrifuged for 15 min at 8,000 rpm at least twice to remove the total suspended solid (TSS) completely. The PWADE (after removing TSS) was characterized by a total nitrogen of 3,565 mg/L, ammonia nitrogen (NH_4-N) of 3,275 mg/L, total phosphorus (TP) of 283 mg/L, total organic carbon of 4,090 mg/L, COD_{cr} of 8,000 mg/L, potassium of 1876 mg/L, sodium of 446 mg/L, magnesium of 59.3 mg/L, and calcium of 152 mg/L. The supernatant was autoclaved (121˚C for 20 min) and used for cultivating microalgae.

2.2. Microalgae strain and cultivation

The green microalgae *C. pyrenoidosa* Y3 was obtained from College of Biological Sciences of China Agricultural University, which was isolated from Tai Lake in Jiangsu province of China [13]. The microalga was chosen in the present study due to its high biomass concentration and mixotrophic characteristic. The algae were maintained in BG-11 medium containing 1,500 mg/L $NaNO_3$, 40 mg/L K_2HPO_4, 6 mg/L citric acid, 6 mg/L ferric ammonium citrate, 75 mg/L $MgSO_4{\cdot}7H_2O$, 36 mg/L $CaCl_2{\cdot}2H_2O$, 20 mg/L Na_2CO_3, 1 mg/L EDTA (ethylene diamine tetraacetic acid), and 1 mL/L A5 solution that contained 2,860 mg/L H_3BO_3, 1,810 mg/L $MnCl_2{\cdot}H_2O$, 222 mg/L $ZnSO_4{\cdot}7H_2O$, 79 mg/L $CuSO_4{\cdot}5H_2O$, 390 mg/L $Na_2MoO_4{\cdot}2H_2O$, and 49 mg/L $Co(NO_3)_2{\cdot}6H_2O$.

2.3. Experimental design and optimization

2.3.1. Microalgae cultivation in PWADE

The *C. pyrenoidosa* Y3 were incubated at 20% (v/v) in 500 mL Erlenmeyer flasks containing 200 mL of 2% (v/v) PWADE under a static condition. The flasks were placed in an illuminating growth chamber (GXZ, Dongqi, China) at 28 ± 2˚C and 250 μmol/m^2/s (Li-250A, Li-COR Lightmeter) light intensity with a 15:9 h of light:dark cycle. Inorganic salts were added to 2% (v/v) PWADE to optimize the medium composition for the biomass production and chlorophyll-a content of *C. pyrenoidosa* Y3. The initial microalgae cell density of each experiment was approximately 0.1 g/L. All experiments were carried out for 20 days and the pH value was not adjusted during the whole cultivation.

2.3.2. Plackett–Burman design

The PBD presented by Plackett and Burman [14] is a two-factorial design based on the balanced incomplete block designs [15]. It is an effective method for screening out the significant medium components according to the main effects [10]. The method is based on the first-order polynomial model [11]:

$$Y = \beta_0 + \sum \beta_i X_i \qquad (1)$$

where Y is the response (biomass yield or chlorophyll-a content), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. In this study, eight nutrients ($NaHCO_3$, K_2HPO_4, KH_2PO_4, $NaNO_3$, $FeSO_4$, $MnCl_2$, $MgSO_4 \cdot 7H_2O$ and, $ZnSO_4$) including three dummy variables were evaluated in 12 experiments. Each factor was prepared in two levels with −1 for the low level and +1 for the high level (Tables 1 and 2). The statistical software design expert (version 8.0.6.1, STAT-EASE Inc., Minneapolis, USA) was used to design the experiment, assess the adequacy of the first-order model equation via the coefficient R^2, and determine its statistical significance by F-test.

2.3.3. Central composite design

Based on the results identified by the PBD, the two most significant nutrients were selected to determine the optimum nutritional condition for *C. pyrenoidosa* Y3 cultivated in 2% (v/v) PWADE. The selected variables were $NaHCO_3$ (X_1) and $MgSO_4 \cdot 7H_2O$ (X_7). A 2^2 CCD of RSM including four cube points, four axial points, and five replicates at the center point was employed in a set of 13 experiments. Each variable in the design was conducted at five different levels ($-\alpha$, −1, 0, +1, $+\alpha$) as shown in Table 3. For statistical analysis, the independent variables are coded as follows:

$$x_i = (X_i - X_0)/\delta X \qquad (2)$$

where X_i is the actual value of variable, x_i is the dimensionless coded value for X_i, X_0 is the value of the X_i at the central point, and δX is the step change.

The SAS software (version 9.0, SAS Institute, Cary, NC, USA) was used for the experimental design and the data analysis. According to the quadratic equation model generated by SAS, the biomass yield (response Y_1) is expressed as:

$$Y_1 = \beta_0 + \beta_1 X_1 + \beta_7 X_7 + \beta_{11} X_1^2 + \beta_{77} X_7^2 + \beta_{17} X_1 X_7 \qquad (3)$$

in which β_0 is the constant coefficient; β_1, β_7, β_{11}, and β_{77} are the linear and quadratic coefficients of X_1 and X_7, respectively; β_{17} is the interactive coefficients between X_1 and X_7 for the production of biomass.

Table 1
Variables, levels, and statistical analysis of PBD (biomass and chlorophyll-a as responses) for selection of nutrients added in the PWADE

Treatment	Varibles	Unit	Low level (−1)	High level (+1)	Effects (E_{xi})	F value	Prob > F	Confidence level (%)
Biomass								
Model	–	–	–	–	–	69.13	0.0026	99.74
X_1	$NaHCO_3$	g/L	2	5	0.21	512.33	0.0002*	99.98
X_2	K_2HPO_4	mg/L	0	20	0.0037	0.16	0.7179	28.21
X_3	KH_2PO_4	mg/L	2	20	0.013	1.98	0.2539	74.61
X_4	$NaNO_3$	g/L	0.5	1	0.0057	0.38	0.5828	41.72
X_5	$FeSO_4$	mg/L	3	6	−0.014	2.30	0.2267	77.33
X_6	$MnCl_2$	mg/L	0	2.26	−0.034	13.83	0.0338*	96.62
X_7	$MgSO_4 \cdot 7H_2O$	mg/L	0	50	0.038	17.24	0.0254*	97.46
X_8	$ZnSO_4$	mg/L	0	0.22	0.020	4.85	0.1149	88.51
Chlorophyll-a								
Model	–	–	–	–	–	25.90	0.0109	98.91
X_1	$NaHCO_3$	g/L	2	5	9.90	181.58	0.0009*	99.91
X_2	K_2HPO_4	mg/L	0	20	0.11	0.022	0.8903	10.97
X_3	KH_2PO_4	mg/L	2	20	0.13	0.030	0.8730	12.70
X_4	$NaNO_3$	g/L	0.5	1	0.12	0.028	0.8775	12.25
X_5	$FeSO_4$	mg/L	3	6	−0.89	1.45	0.3144	68.56
X_6	$MnCl_2$	mg/L	0	2.26	−1.94	7.00	0.0773	92.27
X_7	$MgSO_4 \cdot 7H_2O$	mg/L	0	50	2.74	13.95	0.0335*	96.65
X_8	$ZnSO_4$	mg/L	0	0.22	1.30	3.13	0.1748	82.52

Notes: For biomass: R^2 (predict) = 99.46%; R^2 (adjust) = 98.02%. For chlorophyll-a: R^2 (predict) = 0.99; R^2 (adjust) = 0.95.
*5% Significance level.

The chlorophyll-a content (Y_2) was also optimized and expressed as the above quadratic equation using CCD. The purpose of Y_2 optimization was to double validate the model for predicting maximal biomass yield by *C. pyrenoidosa* Y3 with routine validation procedure.

2.4. Measurement

The NH_4-N and TP of the effluents were analyzed by an ultraviolet spectrophotometer (TU-1810, PGENERAL, Beijing, China).

The dry weight (DW) and chlorophyll-a content were used to evaluate the biomass yield of *C. pyrenoidosa* Y3. Algal biomass DW was measured by correlating DW to the optimal density (OD) of the inoculated samples at 680 nm using a spectrophotometer (TU-1810, PGENERAL, Beijing, China). The linear relationship between DW (g/L) and OD_{680} was determined previously for this strain:

$$\mathrm{DW}(\mathrm{g/L}) = 0.24 \times \mathrm{OD}_{680} - 0.074, \quad R^2 = 0.999 \tag{4}$$

The chlorophyll-a content was measured using the Oncel and Sukan method [16].

2.5. Statistical analysis

All experiments had three replications for each treatment and measurement. Values were reported as the means of triplicate measurements plus standard deviation. Statistical analysis was performed using ANOVA with significant differences of $p < 0.05$. The statistical STATISTICA program (version 10.0, StatSoft Inc., Tulsa, Oklahoma, USA) was used for plotting graphs. The option of RIDGE MAX was applied to compute the estimated ridge of maximum response for increasing radii from the center of the original design [17].

3. Results and discussion

3.1. Selection of crucial nutrients added in the PWADE for C. pyrenoidosa Y3 growth by PBD

The single factor tests have been performed prior to PBD [9]. An 8-factor-12-run experiment for *C. pyrenoidosa* Y3 cultured in 2% (v/v) PWADE with addition of $NaHCO_3$ (X_1), K_2HPO_4 (X_2), KH_2PO_4 (X_3), $NaNO_3$ (X_4), $FeSO_4$ (X_5), $MnCl_2$ (X_6), $MgSO_4{\cdot}7H_2O$ (X_7), and $ZnSO_4$ (X_8) was conducted (Table 1). The biomass yield and chlorophyll-a content of *C. pyrenoidosa* Y3 were used as the responses. Table 2 represents the PBD for 12 trials with two levels of each variable and the corresponding response. The variables X_1–X_8 denote the added inorganic salts and X_9–X_{11} denotes the dummy variables, respectively. As the results shown in Table 1, the model *F*-value of the two responses is 69.13 and 25.90, respectively, which implies that both models are significant. In the PBD experiments, the confidence levels of the variables X_1 ($NaHCO_3$, 99.91–99.98%) and X_7 ($MgSO_4{\cdot}7H_2O$, 96.65–97.46%) are greater than 95% and considered to be significant. Therefore, the X_1 and X_7 had a remarkable positive effect on the biomass production and chlorophyll-a amount accumulation of *C. pyrenoidosa* Y3. Previous studies have also demonstrated that the addition of $NaHCO_3$ to the ammonium-rich ADE enhanced the autotrophic microalgae growth and ammonium removal [1,7]. As the central atom of chlorophyll molecule and the second most abundant cation in cells [7,18], magnesium plays a critical role in microalgae metabolism, CO_2 fixation, and long-term ammonia removal during ADE of livestock waste treatment by microalgae [1,19].

As the data shown in Table 1, the variable X_6 ($MnCl_2$) also had a significant effect with a 96.62% confidence level but the sign of the effect E_{xi} is negative, which means the influence of the variable X_6 on the biomass yield of algae is greater at a low level [20]. For the present work, the low level (−1) of X_6 is 0 mg/L, thus there is no need to add $MnCl_2$ into the 2% (v/v) PWADE. Therefore, X_1 ($NaHCO_3$) and X_7 ($MgSO_4{\cdot}7H_2O$) were selected for further optimization by a CCD.

3.2. Optimization of the selected supplemental nutrients added to the PWADE for enhancing biomass production of C. pyrenoidosa Y3 by a CCD

With the crucial supplemental nutrients screened by the PBD, two independent variables ($NaHCO_3$, X_1 and $MgSO_4{\cdot}7H_2O$, X_7) were further explored using a CCD of the RSM. Experimental design matrix of coded and actual variables with corresponding results is shown in Table 3. According to SAS user guide [17], the response surface regression (RSREG) procedure was conducted to fit quadratic RSREG models by least squares. The following second-order polynomial equation in the coded form was obtained, which could describe the predicted value of biomass yield (Y_1):

$$\begin{aligned} Y_1 = 0.80 - 0.0098\,X_1 + 0.0076X_7 - 0.016X_1^2 \\ + 0.0043X_1X_7 + 0.0096X_7^2 \end{aligned} \tag{5}$$

Table 2
PBD matrix for evaluating factors influencing biomass content and chlorophyll-a amount from *C. pyrenoidosa* Y3

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Biomass content (g/L)	Chlorophyll-a amount (mg/L)
1	−1	1	1	−1	1	1	1	−1	−1	−1	1	0.320 ± 0.027	3.01 ± 0.29
2	1	1	−1	1	1	1	−1	−1	−1	1	−1	0.490 ± 0.012	10.57 ± 0.88
3	−1	−1	−1	1	−1	1	1	−1	1	1	1	0.336 ± 0.014	5.10 ± 0.70
4	1	−1	1	1	−1	1	1	1	−1	−1	−1	0.573 ± 0.007	15.43 ± 3.72
5	1	−1	−1	−1	1	−1	1	1	−1	1	1	0.566 ± 0.005	16.01 ± 0.63
6	1	−1	1	1	1	−1	−1	−1	1	−1	1	0.541 ± 0.010	13.35 ± 0.21
7	1	1	−1	−1	−1	1	−1	1	1	−1	1	0.526 ± 0.005	13.45 ± 0.52
8	−1	1	1	1	−1	−1	−1	1	−1	1	1	0.355 ± 0.015	4.61 ± 0.03
9	−1	1	−1	1	1	−1	1	1	1	−1	−1	0.389 ± 0.014	7.79 ± 0.91
10	1	1	1	−1	−1	−1	1	−1	1	1	−1	0.598 ± 0.031	17.38 ± 0.50
11	−1	−1	1	−1	1	1	−1	1	1	1	−1	0.319 ± 0.010	3.09 ± 0.59
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.321 ± 0.009	3.17 ± 0.13

Notes: X_9, X_{10}, and X_{11} are the dummy variables.

Table 3
CCD for the biomass production and chlorophyll-a amount from *C. pyrenoidosa* Y3 of two independent variables

	$NaHCO_3$ (g/L)		$MgSO_4·7H_2O$ (mg/L)		Biomass content (g/L)		Chlorophyll-a amount (mg/L)	
Run	X_1	Code X_1	X_7	Code X_7	Actual value[a]	Predicted value	Actual value[a]	Predicted value
1	3	−1	57	−1	0.797 ± 0.029	0.798	27.45 ± 0.73	27.52
2	3	−1	93	+1	0.809 ± 0.040	0.802	27.46 ± 0.79	27.35
3	7	+1	57	−1	0.767 ± 0.044	0.770	27.65 ± 0.75	27.70
4	7	+1	93	+1	0.796 ± 0.011	0.791	28.53 ± 0.52	28.40
5	2	−α	75	0	0.780 ± 0.024	0.778	26.14 ± 0.67	26.15
6	8	+α	75	0	0.755 ± 0.070	0.750	26.97 ± 0.50	27.02
7	5	0	50	−α	0.812 ± 0.074	0.807	28.82 ± 1.44	28.72
8	5	0	100	+α	0.826 ± 0.070	0.824	28.93 ± 1.21	29.08
9	5	0	75	0	0.796 ± 0.002	0.797	27.88 ± 0.24	28.04
10	5	0	75	0	0.799 ± 0.012	0.797	28.12 ± 0.48	28.04
11	5	0	75	0	0.801 ± 0.016	0.797	28.18 ± 0.63	28.04
12	5	0	75	0	0.802 ± 0.001	0.797	28.11 ± 0.44	28.04
13	5	0	75	0	0.799 ± 0.007	0.797	27.94 ± 0.20	28.04

[a] The results are presented as the mean of duplicates.

To accurately evaluate the significance and reliability of the mathematical model for predicting the biomass yield, the analysis of variance (ANOVA) was employed to generate the sum of squares, degrees of freedom (DF), mean squares, *F* values, and *p* values by fitting the experimental data (Table 3) to the second-order polynomial equation (Eq. (5)) [12]. As the ANOVA analysis results shown in Table 4, the regression quadratic model, the linear and quadratic effect of $NaHCO_3$ and $MgSO_4·7H_2O$ were highly significant ($p < 0.01$), and the interaction effect between $NaHCO_3$ and $MgSO_4·7H_2O$ was also significant ($p < 0.05$). The experimental model was considered to be adequate attributing to non-significant lack of fit ($p = 0.1025$), satisfactory R^2 (0.9752), R^2_{adj} (0.9644), and the coefficient of variation (CV, 0.44%). The R^2 and R^2_{adj} indicate the fraction of the variation of the response explained by the model and by the model adjusted for DF, respectively [21]. The CV indicates the degree of precision with which the treatments are compared [11]. In the present study, there are two aspects for further demonstrating the high level of significance, accuracy, and the reliability of the fitted model [22]: one is that the R^2_{adj} value (0.9644) is close to the R^2 value (0.9752), and the other is the lower CV value of 0.44%.

The response contour plot was employed to elucidate the interaction effect of two independent variables on the biomass production and to understand

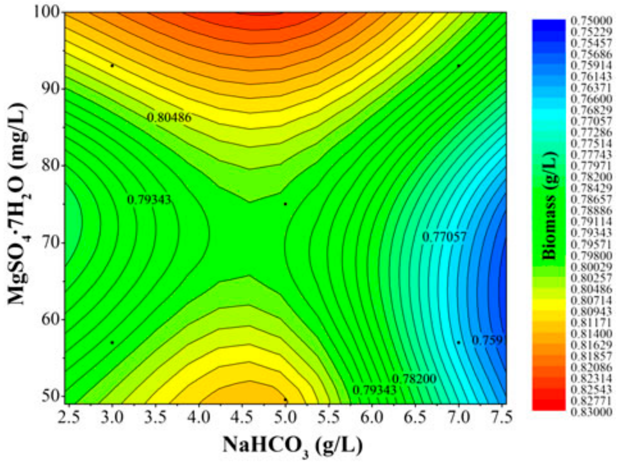

Fig. 1. Response contour plots showing the effect of $NaHCO_3$ (X_1) and $MgSO_4·7H_2O$ (X_7) on the biomass production.

the optimum level of each variable (Fig. 1). The optimum biomass production was determined by the canonical analysis and the ridge maximum analysis. In statistics, canonical analysis belongs to the family of regression methods. This multivariate technique is used for locating the stationary point of the response surface and determining whether it represents a maximum, minimum, or saddle point [23–25]. The fitting model of the canonical analysis is expressed as:

$$Y_1 = 0.80 + 0.009724 W_1^2 - 0.016374 W_7^2 \qquad (6)$$

where W_1 and W_7 are the axes of the response surface. The predicted response surface of the stationary point was shaped like a saddle due to mixed positive and negative eigenvalues [23]. So the estimated surface did not have a unique optimum. The eigenvalue of X_1 (0.009724) indicates that the valley orientation of the saddle is less curved than the hill orientation with the eigenvalue of X_7 (−0.016374). The coefficients of the associated eigenvectors (X_1 and X_7) show that the valley is more aligned with X_1 ($MgSO_4·7H_2O$) and the hill with X_7 ($NaHCO_3$). When the level of $MgSO_4·7H_2O$ was below the critical value of 69.22 mg/L, the biomass production was negatively correlated with the level of $MgSO_4·7H_2O$, even increasing the level of $NaHCO_3$ (Fig. 1). Nevertheless, the correlation was positive when the level of $MgSO_4·7H_2O$ exceeded 69.22 mg/L. As seen in the contour plot, the maximum biomass yield could be obtained under the condition of $NaHCO_3$ concentration ranging from 3 to 5 g/L. Overdose of $NaHCO_3$ could decrease the biomass production because the CO_2 tolerance of microalgae was limited.

A ridge analysis was used to compute and determine the estimated ridge of maximum response by increasing radii from the center of original design. The ridge analysis reveals that the maximum biomass yield could be resulted in a relatively high level of $MgSO_4·7H_2O$ and low level of $NaHCO_3$ (Table 5), which was similar to the contour plot. Thus, the predicted maximum biomass yield of 0.813 g/L was obtained at a $NaHCO_3$ concentration of 4.81 g/L and

Table 4
ANOVA of the quadratic model for biomass production (Y_1) and chlorophyll-a amount (Y_2)

Sources	DF	Sum of squares	Mean square	*F*-value	*p* value
Biomass					
Model	5	0.0041	0.00082	66.05	0.0001**
Linear	2	0.0012	0.00062	49.64	0.0001**
Quadratic	2	0.0028	0.0014	112.57	0.0001**
Cross product	1	0.000072	0.000072	5.84	0.046*
Error	7	0.000087	0.000012		
Lack of fit	3	0.000065	0.000022	4.12	0.10
Pure error	4	5.3E-6			
Total	12	0.0042			
Chlorophyll-a					
Model	5	6.72	1.34	68.76	0.0001**
Error	7	0.14	0.020	–	–
Lack of fit	3	0.073	0.024	1.51	0.34
Pure error	4	0.064	0.016		–
Total	12	6.86	–	–	–

Notes: In the predicted model for Y_1: CV = 0.44%, R^2 = 0.98, R^2_{adj} = 0.96; In the predicted model for Y_2: R^2 = 0.98, R^2_{adj} = 0.97.
*Significant at 95% confidence level ($p < 0.05$).
**Highly significant at 99% confidence level ($p < 0.01$).

Table 5
Ridge max analysis and routine verification for the biomass production model

			Real values of independent variables	
Coded radius	Predicted response (g/L)	Experimental response[a] (g/L)	$NaHCO_3$, X_1 (g/L)	$MgSO_4{\cdot}7H_2O$, X_7 (mg/L)
0.2	0.799	0.770 ± 0.0005	4.81	78.1
0.4	0.801	0.778 ± 0.001	4.77	81.9
0.6	0.804	0.789 ± 0.002	4.77	85.6
0.8	0.808	0.794 ± 0.003	4.79	89.3
1.0	0.813	0.819 ± 0.003	4.81	92.9

[a]The results are presented as the mean of duplicates.

$MgSO_4{\cdot}7H_2O$ of 92.9 mg/L with a distance of the coded radius of 1.0.

3.3. Experimental validation of the model

In order to verify the optimized PWADE medium for predicting the maximum biomass production of *C. pyrenoidosa* Y3, five sets of additional experiments were executed based on the ridge max analysis in the routine validation (Table 5). Fig. 2 shows the comparison of the observed and predicted values of the biomass yield in the routine validation. The correlation between the experimental and predicted values of the biomass yield were satisfactory ($R^2 = 0.942$). The actual maximum biomass yield of 0.819 g/L was obtained from *C. pyrenoidosa* Y3 cultivated in the 2% (v/v) PWADE substrate with addition of 4.81 g/L $NaHCO_3$ and 92.9 mg/L $MgSO_4{\cdot}7H_2O$ after 20 d of cultivation. The biomass yield was 1.5 times higher than that of *C. pyrenoidosa* Y3 cultured in the non-optimized 2% (v/v) PWADE medium (0.559 g/L). It can be estimated from Fig. 3 that a total biomass concentration of 0.658 g/L was attained on the 12th d, which was 1.7 times higher than the result (0.387 g/L) reported by Singh et al. [2]. Additionally, previous researchers have used *Spongiochloris* sp. grown in the abattoir digestate supplemented with seven different nutrients but only 2.0×10^6 cell/mL of biomass and 0.706 g/L of TSS were produced after 20 d, respectively [7].

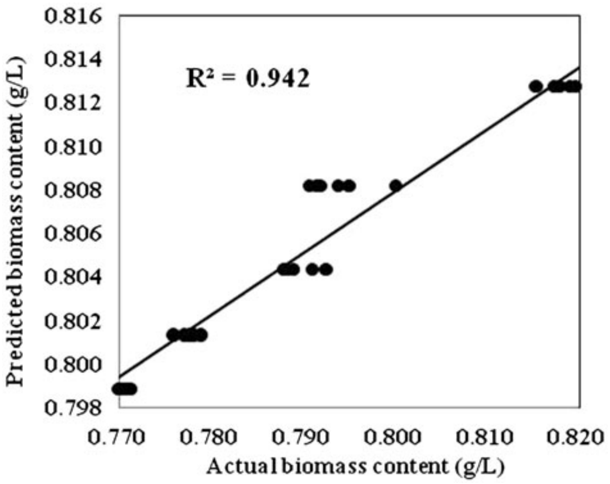

Fig. 2. Comparison between the predicted and actual values of the biomass production in the routine validation.

The optimization of chlorophyll-a content was carried out simultaneously for double verifying the reliability of the biomass yield model. Chlorophyll-a, a proxy measurement of phytoplankton biomass [26], was used as the response Y_2 in the CCD. Experimental design matrix and corresponding results of the response Y_2 are shown in Table 3. A second-order polynomial equation was obtained to describe the predicted value of chlorophyll-a content (Y_2):

$$Y_2 = 28.05 + 0.31X_1 + 0.13X_7 - 0.73X_1^2 + 0.22X_1X_7 + 0.43X_7^2 \tag{7}$$

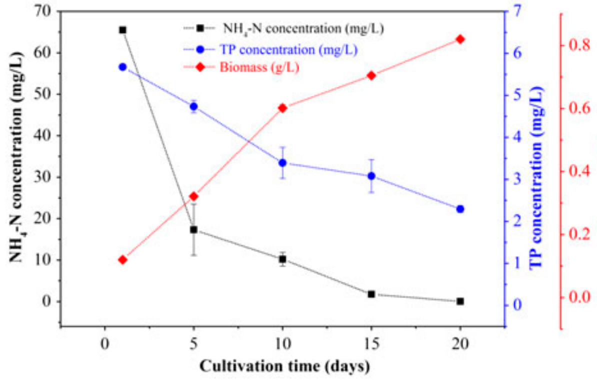

Fig. 3. Relationship between NH_4-N, TP concentration, and biomass production under the optimized medium condition.

Table 6
Experimental and predicted values of chlorophyll-a amount in the five routine validated experiments

			Real values of independent variables	
Coded Radius	Experimental value (mg/L)	Predicted value[a] (mg/L)	$NaHCO_3$, X_1 (g/L)	$MgSO_4·7H_2O$, X_7 (mg/L)
0.2	25.17 ± 0.11	28.04	4.81	78.1
0.4	25.19 ± 0.18	28.01	4.77	81.9
0.6	25.90 ± 0.31	28.21	4.77	85.6
0.8	26.08 ± 0.18	28.36	4.79	89.3
1.0	27.51 ± 0.18	28.54	4.81	92.9

Note: R^2 (the correlation coefficient between experimental value and predicted value) = 0.91.

[a] The predicted value of chlorophyll-a amount was calculated based on Eq. (7).

ANOVA of response surface indicates that the predicted mathematical model of Y_2 was significant and reliable (Table 4).

Table 6 illustrates the experimental and predicted chlorophyll-a content of *C. pyrenoidosa* Y3. The predicted values of chlorophyll-a contents were calculated based on Eq. (7). There was a satisfactory correlation ($R^2 = 0.91$) between the observed and predicted values of chlorophyll-a contents, which can prove the adequacy of the chlorophyll-a content model and further demonstrate the reliability of the biomass yield model.

The experimental chlorophyll-a content (27.51 mg/L) was slightly lower than that of the predicted values (28.54 mg/L) due to the slight variation in experimental conditions [11]. In the predicted model of chlorophyll-a content, the response surface of the stationary point was also shaped like a saddle. If a ridge analysis was used to determine the estimated ridge of maximum chlorophyll-a content, the predicted maximum chlorophyll-a content of 28.66 mg/L was obtained at a $NaHCO_3$ concentration of 5.42 g/L and $MgSO_4·7H_2O$ of 92.6 mg/L with the distance of the coded radius of 1.0 based on Eq. (7). The optimum concentration of $NaHCO_3$ was higher than the one shown in Table 6 (4.81 g/L) which will increase the cost of cultivating microalgae. Therefore, the predicted model for biomass yield of *C. pyrenoidosa* Y3 grown in PWADE is reasonably accurate.

3.4. *Nutrients removal by* C. pyrenoidosa *Y3 cultivated in the optimized PWADE medium*

Fig. 3 shows the NH_4-N and TP removal by *C. pyrenoidosa* Y3 with biomass production in the optimized PWADE medium during 20 days cultivation. On the 10th day of cultivation, NH_4-N content of PWADE was greatly reduced by 84.4%, and TP concentration dropped from initial 5.67–3.39 mg/L. The biomass concentration of algae was 0.602 g/L. After cultivation of 20 d, 100% of NH_4-N and 59.5% of TP were removed from the effluents by algae. From day 10 to day 20, the nutrients removal efficiency and biomass accumulation gradually decreased because microalgae removed nutrients from effluents in a fixed ratio (named the "Redfield ratio") [27]. In the present study, the ratio between nitrogen and phosphorus concentrations deviated from the Redfield ratio, which limited the microalgal growth rate. During the whole cultivation, the growth of *C. pyrenoidosa* Y3 has maintained in the exponential phase and did not enter into the stationary phase. The maximum biomass yield of 0.819 g/L for algae was obtained on day 20.

4. Conclusions

The nutrient supplement to PWADE as a substrate for enhancing biomass production of *C. pyrenoidosa* Y3 (isolated from Tai Lake) was optimized using a RSM. The $NaHCO_3$ and $MgSO_4·7H_2O$ were selected as the most crucial supplemental nutrients to the 2% (v/v) PWADE. The maximum biomass yield of 0.819 g/L was obtained from the PWADE with 4.81 g/L $NaHCO_3$ and 92.9 mg/L $MgSO_4·7H_2O$ after 20 d of cultivation. The chlorophyll-a content reached 27.51 mg/L under the optimized medium condition. About 100.0% NH_4-N and 59.5% TP were removed from the effluent. Moreover, RSM is an effective and reliable approach in proposing models for optimizing and predicting the algae growth in PWADE. The biomass can be applied as a profitable animal feed supplement or the feedstock for biofuel production.

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References

[1] J. Park, H.F. Jin, B.R. Lim, K.Y. Park, K. Lee, Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp., Bioresour. Technol. 101 (2010) 8649–8657.

[2] M. Singh, D.L. Reynolds, K.C. Das, Microalgal system for treatment of effluent from poultry litter anaerobic digestion, Bioresour. Technol. 102 (2011) 10841–10848.

[3] J.K. Pittman, A.P. Dean, O. Osundeko, The potential of sustainable algal biofuel production using wastewater resources, Bioresour. Technol. 102 (2011) 17–25.

[4] Y. Yang, J.F. Xu, D. Vail, P. Weathers, *Ettlia oleoabundans* growth and oil production on agricultural anaerobic waste effluents, Bioresour. Technol. 102 (2011) 5076–5082.

[5] B. Hu, M. Min, W.G. Zhou, Z.Y. Du, M. Mohr, P. Chen, J. Zhu, Y.L. Cheng, Y.H. Liu, R. Ruan, Enhanced mixotrophic growth of microalga *Chlorella* sp. on pretreated swine manure for simultaneous biofuel feedstock production and nutrient removal, Bioresour. Technol. 126 (2012) 71–79.

[6] R.B. Levine, M.S. Costanza-Robinson, G.A. Spatafora, *Neochloris oleoabundans* grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production, Biomass Bioenergy 35 (2011) 40–49.

[7] F.S. Bchir, H. Gannoun, S.E. Herry, M. Hamdi, Optimization of *Spongiochloris* sp. biomass production in the abattoir digestate, Bioresour. Technol. 102 (2011) 3869–3876.

[8] G. Chen, F. Chen, Growing phototrophic cells without light, Biotechnol. Lett. 28 (2006) 607–616.

[9] Y. Wu, W. Cao, M.Z. Wang, B.M. Li, Y.H. Zhang, A Bench-scale study of biomass production by *Chlorella pyrenoidosa* cultivated in anaerobic poultry waste effluent, ASABE Annual International Meeting, Kansas City, MO, 2013. Available from: <http://dx.doi.org/10.13031/aim.20131590220>.

[10] W.Q. Guo, N.Q. Ren, X.J. Wang, W.S. Xiang, J. Ding, Y. You, B.F. Liu, Optimization of culture conditions for hydrogen production by *Ethanoligenens harbinense* B49 using response surface methodology, Bioresour. Technol. 100 (2009) 1192–1196.

[11] W.B. Kong, S.F. Hua, H. Cao, Y.W. Mu, H. Yang, H. Song, C.G. Xia, Optimization of mixotrophic medium components for biomass production and biochemical composition biosynthesis by *Chlorella vulgaris* using response surface methodology, J. Taiwan Inst. Chem. Eng. 43 (2012) 360–367.

[12] T.H. Xie, Y. Sun, K.F. Du, B. Liang, R. Cheng, Y.K. Zhang, Optimization of heterotrophic cultivation of *Chlorella* sp. for oil production, Bioresour. Technol. 118 (2012) 235–242.

[13] G.Q. He, Z.P. Deng, L. Tao, S.F. Chen, Screen and fermentation optimization of microalgaes with high lipid productivity, J. Agr. Biotechnol. 18(6) (2010) 1046–1053 (in Chinese).

[14] R.L. Plackett, J.P. Burman, The design of optimum multifactorial experiments, Biometrika 33 (1946) 305–325.

[15] R.C. Bose, On the construction of balanced incomplete block designs, Ann. Eugenics 9 (1939) 353–399.

[16] S. Oncel, F.V. Sukan, Comparison of two different pneumatically mixed column photobioreactors for the cultivation of *Artrospira platensis* (*Spirulina platensis*), Bioresour. Technol. 99 (2008) 4755–4760.

[17] SAS/SAS, User Guide, SAS Institute Inc, Cary, North Carolina, 1990.

[18] G.M. Walker, The role of magnesium in biotechnology, Crit. Rev. Biotechnol. 14 (1994) 311–354.

[19] E.B. Sydney, W. Sturm, J.C. de Carvalho, V. Thomaz-Soccol, C. Larroche, A. Pandey, C.R. Soccol, Potential carbon dioxide fixation by industrially important microalgae, Bioresour. Technol. 101 (2010) 5892–5896.

[20] C.M. Pan, Y.T. Fan, Y. Xing, H.W. Hou, M.L. Zhang, Statistical optimization of process parameters on biohydrogen production from glucose by *Clostridium* sp. Fanp2, Bioresour. Technol. 99 (2008) 3146–3154.

[21] V. García, J. Landaburu-Aguirre, E. Pongrácz, P. Perämäki, R.L. Keiski, Dehydration of water/dichloromethane/n-butanol mixtures by pervaporation; Optimisation and modelling by response surface methodology, J. Membr. Sci. 338 (2009) 111–118.

[22] Y.M. Wang, C.H. Huang, T.W. Xu, Optimization of electrodialysis with bipolar membranes by using response surface methodology, J. Membr. Sci. 362 (2010) 249–254.

[23] D.C. Montgomery, Design and Analysis of Experiments, sixth ed., Wiley, New York, NY, 2005.

[24] M.A. Alim, J.H. Lee, C.C. Akoh, M.S. Choi, M.S. Jeon, J.A. Shin, K.T. Lee, Enzymatic transesterification of fractionated rice bran oil with conjugated linoleic acid: Optimization by response surface methodology, LWT—Food. Sci. Technol. 41 (2008) 764–770.

[25] J.N. Hu, J.H. Lee, X.M. Zhu, J.A. Shin, P. Adhikari, J.K. Kim, K.T. Lee, Optimization of lipase-catalyzed synthesis of ginsenoside rb1 esters using response surface methodology, J. Agric. Food. Chem. 56 (2008) 10988–10993.

[26] A. Abdul-Hadi, S. Mansor, B. Pradhan, C.K. Tan, Seasonal variability of chlorophyll-a and oceanographic conditions in Sabah waters in relation to Asian monsoon-a remote sensing study, Environ. Monit. Assess. 185 (2013) 3977–3991.

[27] A.C. Redfield, The biological control of chemical factors in the environment, Am. Sci. 46 (1958) 205–221.