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Mixotrophic cultivation of *Chlorella vulgaris* for efficient wastewater treatment: optimization using response surface methodology (RSM)

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ABSTRACT

Microalgae cultivation using wastewater as the substrate is a sustainable technology for simultaneously microalgae biomass production and pollutant removal. To enhance the overall performance, response surface methodology with a Box–Behnken design was applied to optimize wastewater composition, including chemical oxygen demand (COD), ammonia (NH_4^+–N) and total phosphorus (TP), in batch photobioreactors for mixotrophic cultivation of *Chlorella vulgaris*. Experimental results showed that under the optimal condition of wastewater composition (COD = 1271 mg/L, NH_4^+–N = 20 mg/L and TP = 18 mg/L), the maximum biomass concentration of *C. vulgaris* reached 0.52 g/L, while COD and TP were observed to exert more important influence than NH_4^+–N on microalgae growth. Moreover, the growth cycle of *C. vulgaris* was in the range of 3–5 d. After 5 d batch cultivation, the removal of COD, NH_4^+–N and TP were in the ranges of 70%–83%, 51%–91% and 30%–94%, respectively, depending on initial nutrients composition in wastewater. Positive correlations among *C. vulgaris* biomass and removed pollutants (especially for COD and TP) were observed. The obtained results were useful for guiding the practical application of microalgae mixotrophic cultivation based processes for wastewater treatment and biomass production.

Keywords: Wastewater treatment; Response surface methodology; Microalgae; Biomass production; Mixotrophic cultivation

1. Introduction

Nowadays, with the increasing urbanization rate and quick economic development, the supply of water resources and the discharge of wastewater show increasing growth. Conventional activated sludge processes are characterized by high energy consumption, large footprint, excess production of surplus sludge and low efficiency for resource recovery [1–4]. More importantly, residual pollutants (mainly nutrients) within a large amount of treated water can cause serious environmental issues (such as eutrophication phenomenon and destruction of water ecological system) when directly discharged into receiving water bodies. Microalgae biotechnology shows the advantages of excellent environmental adaptation, high production rate, CO_2 emission reduction by carbon uptake and generation of high-value products (such as biomass, lipids, and biofuel) [5–9]. When using wastewaters for microalgae cultivation, the pollutants in wastewater can be utilized as nutrients for microalgae growth, thus making the wastewater-microalgae integrated system cost-effective for simultaneous pollutants removal and useful bioresource production.

In recent decades, municipal wastewater, agricultural wastewater, and industrial wastewater all have been successfully adopted for microalgae cultivation [10–13], as cultivating

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microalgae with wastewater is an emerging and sustainable strategy verified by various applications [14]. By comparing the cultivation performance, it was noted that *Chlorella* and *Scenedesmus* were the dominant species surviving well in domestic wastewater for efficient pollutant removal [14–16]. However large scale cultivation of microalgae was limited by insufficient and unbalanced nutrient conditions of individual wastewater, thus commonly causing a long growth cycle and low biomass productivity [17,18]. As for efficient microalgae growth, there should be adequate amounts of carbon (organic or inorganic carbon), N (urea, ammonium or nitrate), and P as well as other trace elements present, even though microalgae biomass production can be performed through autotrophic cultivation, heterotrophic cultivation or mixotrophic cultivation.

Thus to improve the substrate conditions for microalgae growth, researchers tried to supplement carbon source, nutrients to practical wastewater. At the auto/mixotrophic growth of *Chlorella vulgaris* and *Botryococcus terribilis*, the supplementation of 50 mM glycerol to domestic wastewater promoted microalgae biomass production with the biomass productivity of 118 and 282 mg/L d [19]. Another study assessed cheap nutrient-rich waste substrates (such as urea, potassium nitrate, sodium nitrate, and ammonium nitrate) as supplementation to wastewater for enhancing biomass and lipid production of *Chlorella sorokiniana* [20]. It was found that the most appropriate nitrogen source and concentration were 1.5 g/L of urea with the biomass production of 0.218 g/L. It was indicated that adding various nutrients (organics and nutrients) to domestic wastewater was useful for increasing biomass yield. However, it was at the cost of consuming a large number of chemicals, and also the effects on pollutant removal were largely unknown. Thus, the understanding and optimization of chemical oxygen demand (COD), N and P content in wastewater for specific microalgae cultivation need further investigation to obtain efficient overall performance. As nutrient limitation is one of the key challenges for microalgal cultivation various wastewaters, alternate sources such as waste nutrient-rich materials as an additional substrate or utilizing blended wastewaters from different streams to alter nutrients composition were also reported previously [8].

Therefore, the objective of this study is to optimize the composition of synthetic wastewater for *C. vulgaris* cultivation under mixotrophic conditions using response surface methodology (RSM). The optimum substrate composition was investigated and verified, which might provide some useful information for guiding the optimization of nutrients composition in wastewater towards cost-effective microalgae production and pollutant removal. In this work, the algal density and dry weight were detected to reflect microalgae biomass, while various water quality indices, including COD, ammonium, and phosphate, were monitored to show the wastewater treatment performance.

2. Material and methods

2.1. Algal strain and culture medium

A kind of green microalgae, *C. vulgaris* (FACHB-31) was used during the experimental tests, which was purchased from the Institute of Hydrobiology (Chinese Academy of Sciences, China). The strain was preserved in the BG11 medium as commonly used [17]. Before inoculated, the initial pH of the BG11 medium was adjusted to 7.0–8.0, and sterilized at 121°C for 30 min [17].

The synthetic wastewater was prepared by using various chemicals as follows: glucose (150 mg/L), peptone (150 mg/L), CH_3OONa (80 mg/L), NH_4Cl (80 mg/L), KH_2PO_4 (26 mg/L), $MgSO_4·7H_2O$ (180 mg/L), $CaCl_2$ (10.6 mg/L), $NaHCO_3$ (80 mg/L), EDTA (3 mg/L), $FeCl_3·6H_2O$ (0.45 mg/L), $MnCl_2·6H_2O$ (0.036 mg/L), H_3BO_3 (0.045 mg/L), $ZnSO_4·7H_2O$ (0.036 mg/L), $CuSO_4.5H_2O$ (0.054 mg/L), KI (0.054 mg/L). The pretreatment method for the synthetic wastewater was the same as that of BG11 medium (including pH adjustment and sterilization).

2.2. Culture conditions

Acclimatized by the synthetic wastewater culture medium, the logarithmic phase of *C. vulgaris* was cultivated with 500 mL of sterilized synthetic wastewater in 1 L flasks, inoculated density 3×10^5 cells/mL. The flasks were placed in a light incubator (TQHZ-2002A, Changzhou Jingda Instrument manufacturing, Changzhou, China). The conditions for *C. vulgaris* cultivation were showing below: temperature = 25°C ± 1°C, light intensity = 2,500–3,000 lx, light/dark ratio = 12 h: 12 h, oscillation speed = 80 r/min. Artificial intermittent shaking in a frequency of three times per day was conducted for 6 d, and randomly exchanging the position of the conical flask to make the light evenly. Sterile conditions should be maintained during inoculation and transfer.

2.3. Analytical methods

Two methods, namely dry biomass weight and algal cell density, are adopted to indicate the biomass concentration, thus resulting in concentrations expressed by g/L and Cells/mL, respectively. 30 mL of the algae suspension was collected and centrifuged at 8,000 rpm, 4°C for 10 min, and then the precipitate was dried to constant weight at 105°C. Basing on the average dry weight of 30 mL of microalgae culture, the total *C. vulgaris* biomass production was obtained. Triplicate tests were conducted with the average values reported [21]. During the culture of *C. vulgaris*, sampling started one day after inoculation. Samples for algal cell density were taken in triplicate daily, counted using Cellometer Auto M10 (Lawrence, MA01843, USA). The average value of algal cell density is used for plotting the growth curve.

The pretreatment procedure for water quality measurement was shown as follows: firstly 10 mL of the algae culture was collected and centrifuged at 8,000 rpm, 4°C for 10 min, and then the supernatant was filtered through a 0.45 μm filter to obtain the filtrate for further analysis. Due to the high concentration of various pollutants, the required volume of water samples was 2–3, 1–2 and 1–2 mL for COD, NH_4^+–N and total phosphorus (TP) detection, respectively, thus the samples should be further diluted before detection. COD, NH_4^+–N, and TP were tested according to standard methods [22].

2.4. Experimental design

2.4.1. Single factor optimal steep slope test

$C_6H_{12}O_6$, NH_4Cl, and KH_2PO_4 were selected as the carbon source, nitrogen source, and phosphorus source and the other components concentration were unchanged (based on synthetic wastewater mentioned in Section 2.1). The single-factor steepest climbing tests were performed under different gradients to determine the appropriate concentrations of carbon, nitrogen, and phosphorus for cultivating the microalgae biomass. Base on reported nutrients condition for mixotrophic microalgae cultivation, the designed concentrations for COD, NH_4^+–N and TP were in the ranges of 0.1–10 g/L, 10–100 mg/L and 0.5–40 mg/L, respectively. Triplicate tests were conducted with the average values reported.

2.4.2. RSM design with a Box–Behnken design

Design-Expert software version 8.0.6 (STAT-EASE Inc., USA) with a Box–Behnken design (BBD) was used to design an RSM experiment for further optimizing the concentrations of organic carbon, nitrogen, and phosphorus. As a design tool, BBD effectively fits a second-order response surface model with three or more factors [23]. During the tests, synthetic wastewater as mentioned in Section 2.1 was used with modification, $C_6H_{12}O_6$, NH_4Cl, and KH_2PO_4 were supplemented to change the concentrations of COD, NH_4^+–N, and TP according to designed values. Replicate tests were done with the average values reported. The relationship between the coded and actual values of the variables is shown in Table 1, and the experimental conditions (nutrients composition) and their responses (*C. vulgaris* biomass production) are presented in Table 2. According to the experimental response value, Design-Expert software was used to predict the optimal response value and condition.

3. Results and discussion

3.1. Effects of individual nutrient on microalgae biomass production

COD, ammonia, and phosphorus were regarded as the most important nutrients for microalgae growth under heterotrophic or mixotrophic cultivation conditions [24]. For the latter, organic substances can be used as energy and carbon source in addition to inorganics carbon (such as CO_2). In order to evaluate the individual effect of various nutrients (COD, ammonia, and phosphorus) on *C. vulgaris* production, batch tests were implemented by adjusting nutrients concentration in the ranges of 0.1–10 g/L for COD, 10–100 mg/L for ammonia and 0.5–40 mg/L for phosphorus, which was chose to cover the potential fluctuations in water quality of domestic wastewater from low to high strength [25].

Fig. 1 shows the relationships among *C. vulgaris* biomass production and COD, ammonia and TP. Obviously, within a wide range of nutrients content, generally, the tendency

Table 1
Experimental range and levels of independent test variables

Level	Variable		
	COD (mg/L): A	NH_4^+–N (mg/L): B	TP (mg/L): C
–1	500	20	8
0	1,000	30	14
1	1,500	40	20

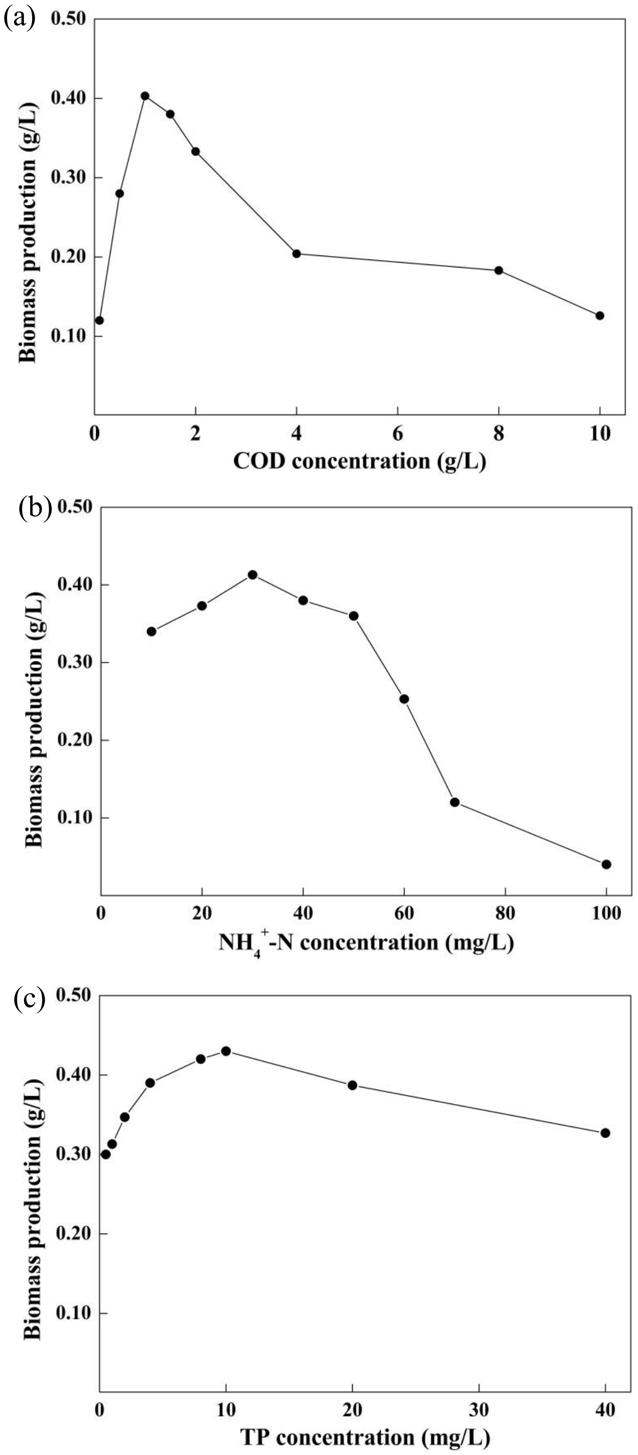

Fig. 1. Relationships among *Chlorella vulgaris* biomass production and individual nutrient: (a) COD, (b) NH_4^+–N, and (c) TP.

Table 2
Experimental plan and results of Box–Behnken design

Run	COD (mg/L)	NH_4^+–N (mg/L)	TP (mg/L)	Experimental biomass (g/L)	Predicted biomass (g/L)
1	0	–1	1	0.5000	0.5008
2	1	0	1	0.4067	0.4138
3	–1	–1	0	0.2800	0.2950
4	1	1	0	0.3700	0.3610
5	0	0	0	0.4100	0.4200
6	0	1	1	0.4033	0.4132
7	–1	0	1	0.2867	0.2802
8	0	0	0	0.4267	0.4200
9	0	0	0	0.4267	0.4200
10	0	–1	–1	0.4367	0.4332
11	0	1	–1	0.3633	0.3688
12	–1	0	–1	0.2167	0.2158
13	1	0	–1	0.3533	0.3662
14	0	0	0	0.4267	0.4200
15	1	–1	0	0.4967	0.4970
16	0	0	0	0.3967	0.4200
17	–1	1	0	0.2733	0.2790

was that an increase in biomass with nutrients concentration was followed by biomass decreasing at higher nutrients concentrations. It was because the presence of nutrients at limiting concentrations can result in reduced growth rates and biomass productivities, however much higher concentrations may also inhibit microalgae growth due to the fast proliferation of various bacteria [13,25], which may compete with microalgae for nutrients [21].

In the tested conditions, the optimal concentrations for biomass growth were 1 g/L of COD, 30 mg/L of ammonia and 10 mg/L of TP. Furthermore, the suitable ranges of these nutrients were 0.5–1.5 g/L for COD, 20–40 mg/L for ammonia and 8–20 mg/L for TP, due to the high biomass concentration achieved ranging from 0.28–0.43 g/L. Generally, the results were in agreement with previous studies indicating that domestic wastewater presenting considerable concentrations of these nutrients was suitable for *C. vulgaris* growth [13], and high strength wastewater would be more competitive as higher biomass concentration could be achieved. Thus, the investigation on the effect of an individual nutrient on *C. vulgaris* biomass production illustrated the optimal concentration and suitable concentration ranges, which could guide further experimental design on the influence of interactions among three nutrients on biomass production as well as determining the optimal nutrients composition.

3.2. Statistical analysis

Based on the results from the above tests, RSM with BBD was applied to a three-factor three-level experiment. Table 1 lists the ranges and levels of the independent variables (COD, NH_4^+–N, and TP) and the response variable (*C. vulgaris* biomass production). To implement the minimization influence of uncontrolled variables on the responses, the BBD design consisted of 17 experiments randomly manner (including five times replications the center point), the conditions and response values of which are shown in Table 2. From statistical tests of the experimental response, the RSM with BBD built the reduced second-order models for biomass production. Table 3 summarizes the analysis of variance (ANOVA) results and correlation parameters.

The statistical results showed that the model was highly significant, because the *p*-values were less than 0.05, indicating a 95% confidence interval could be explained by the model. Combining the *F*-values greater than 0.001, the lack of fit is insignificant as the *p*-values are >0.05, which could be successfully used for prediction the response value. The ratio of 26.688 for biomass production was more than 4, indicating an adequate precision to predict the range of response values [26]. Fig. 2 illustrates that the predicted and actual values of the biomass production were distributed around the diagonal, forming a good convergence curve. Moreover, the value of R^2 was 0.9861, which also evidenced that the model predicted values have a good fit with the experimental data. Compared to the value of R^2, the value of R^2_{adj} (0.9682) decreased less than 2%, indicating that the model could more accuracy to predict the range of response values. The value of the coefficient of variation (C.V.) (3.67%) was less than 10%, further indicating good precision and experimental reliability [27]. Overall, it meant that the model was reasonably reliable.

Based on the experimentally measured values, the Design Expert Software Statistics and fits the expression of the reduced quadratic model for biomass production, which is shown as follow:

$$\text{Biomass production} = 0.42 + 0.071 \times A - 0.038 \times B + 0.028 \times C - 0.030 \times A \times B - 0.086 \times (A)^2 + 0.024 \times (B)^2$$

3.3. Interactions among various factors

From the experiment data in Table 2, Fig. 3 reflects the interactions of the independent variable to the response

Table 3
Analysis of variance (ANOVA) of the model

Source	Degree of freedom	Sum of square	Mean square	*F*-value	*p*-value Prob > *F*
Model	9	0.097	0.011	55.22	<0.0001[a]
A	1	0.041	0.041	208.78	<0.0001
B	1	0.012	0.012	59.13	0.0001
C	1	6.422×10^{-3}	6.422×10^{-3}	33.02	0.0007
AB	1	3.600×10^{-3}	3.600×10^{-3}	18.51	0.0036
AC	1	6.944×10^{-5}	6.944×10^{-5}	0.36	0.5690[b]
BC	1	1.361×10^{-4}	1.361×10^{-4}	0.70	0.4305
A^2	1	0.031	0.031	160.71	<0.0001
B^2	1	2.392×10^{-3}	2.392×10^{-3}	12.30	0.0099
C^2	1	9.899×10^{-4}	9.899×10^{-4}	5.09	0.0587
Residual	7	1.362×10^{-3}	1.945×10^{-4}		
Lack of fit	3	6.194×10^{-4}	2.065×10^{-4}	1.11	0.4423
Pure error	4	7.422×10^{-4}	1.856×10^{-4}		
Core total	16	0.098			
($R^2 = 0.9861$, $R^2_{adj} = 0.9682$, adequate precision = 26.688, Std. Dev. = 0.014, C.V.% = 3.67, PRESS = 0.011)					

[a] Significant at $p < 0.05$.
[b] No significant at $p > 0.05$.

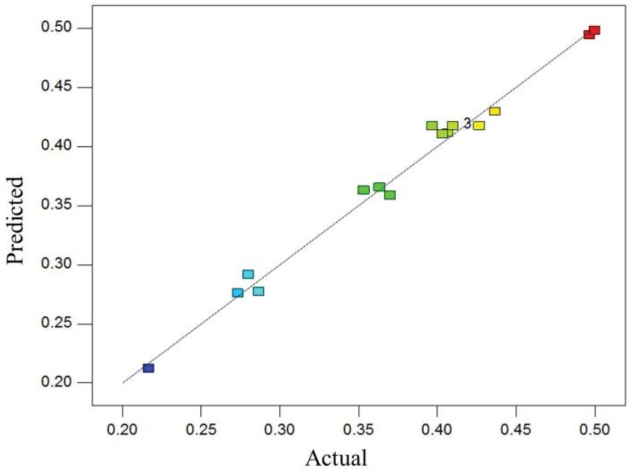

Fig. 2. Predicted and actual values of biomass production.

variable (biomass production), including the three-dimensional response surface and the two-dimensional contour plots. In Figs. 3a and b, a combined effect of COD and ammonia on biomass production at a constant TP concentration (18 mg/L) were shown, and the response variable changed with COD concentration and ammonia concentration. A rapidly increasing trend in biomass production with COD concentration (below 1,300 mg/L) was noted at a constant ammonia concentration.

Especially, COD concentration of approximate 1,300 mg/L devoted to the maximum biomass production. A maximum biomass production biomass reached 0.5123 g/L at ammonia concentration of 20 mg/L and a COD concentration of around 1,300 mg/L. The reason was that the carbon source provided nutrients for the mixotrophic cultivation of microalgae to produce energy and cell biosynthesis [28,29]. However, excess COD would inhibit cell growth and then compromise the biomass production [30]. Also, a slight decrease in biomass production was found when the ammonia concentration increased under a constant COD concentration. It was reported that when ammonia concentration was greater than approximately 25 mg/L, excessive absorption of nitrogen by *C. vulgaris* restrained cell division and reduced algal cell density [31]. As predicted by the variance analysis, COD concentration and ammonia concentration had a great effect on the response variable (biomass production). Moreover, the contour plot of *A* and *B* displayed elliptical morphology, indicating significant interactions between the two independent variables as previously reported [32].

Figs. 3c and d show the combined effects of COD and TP on biomass production at a constant ammonia concentration. For a constant ammonia concentration of 20 mg/L, the biomass production improved slightly as the TP concentration increased. And the contour trend demonstrates that COD has a greater effect on the response than TP. However, TP concentration in the range of 14–20 mg/L had no significant effect on the biomass production for a constant COD concentration (1,100–1,500 mg/L), which may be due to the fact that under mixotrophic cultivation condition organic carbon source were more important than TP for the growth of microalgae when TP seemed to be not the limiting factor (8–20 mg/L). It was pointed out that the interactions of COD concentration and TP concentration had no significant effect in predicting biomass production. In addition, the contour plot of *A* and *C* exhibited a circular morphology meaning no significant interactions between COD and TP [32], which further verified the results presented in Table 3.

The effects of ammonia and TP on biomass production at a constant COD concentration (1,271 mg/L) are shown in Figs. 3e and f. Nitrogen source and phosphorus source are

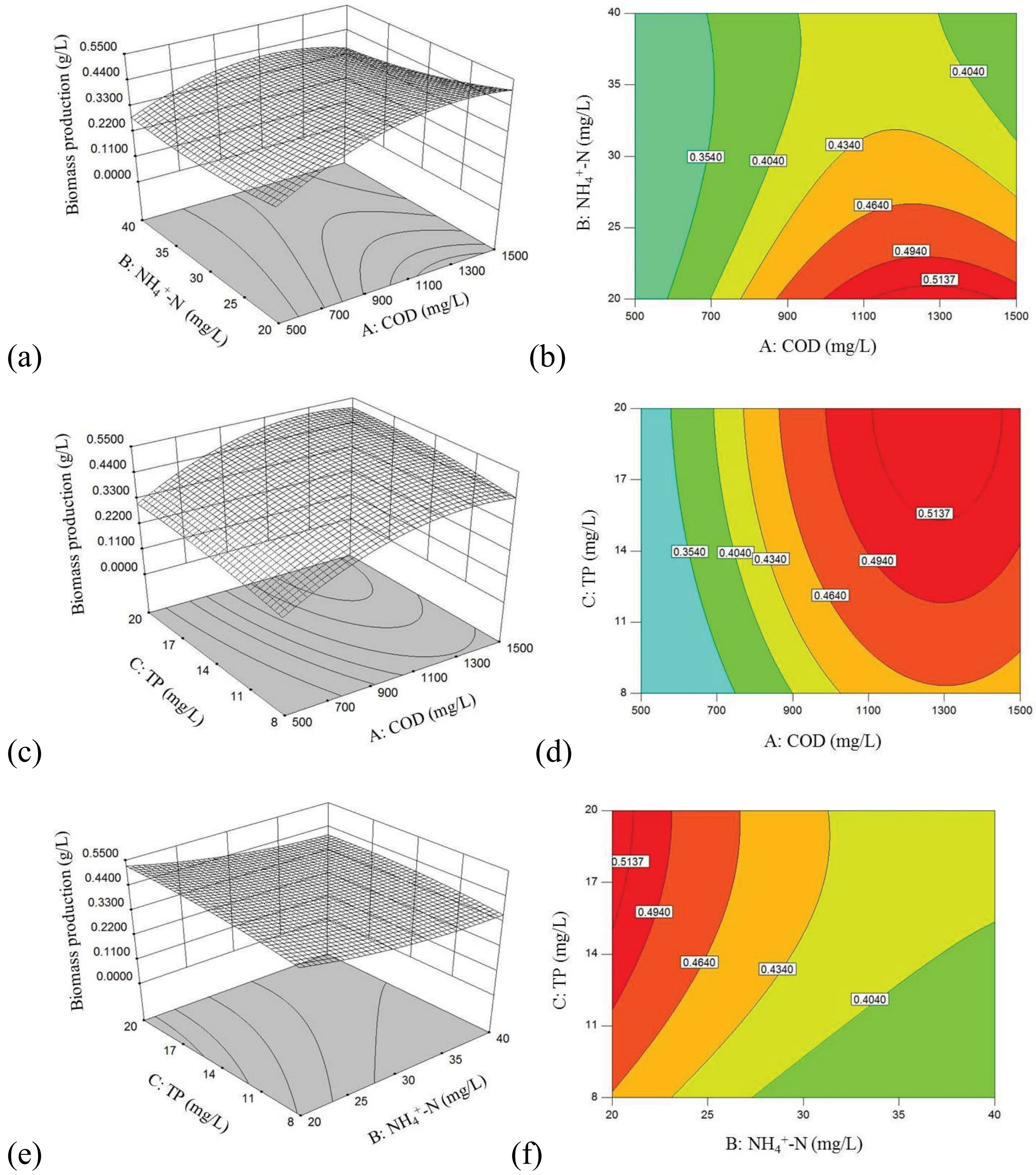

Fig. 3. Three-dimensional (a,c,e) and contour plots (b,d,f) for the response variable (Biomass production) with respect to different experimental factors. Notes: (1) b: COD vs. NH_4^+–N at constant TP = 18 mg/L; (2) d: COD vs. TP at constant NH_4^+–N = 20 mg/L; (3) f: NH_4^+–N vs. TP at constant COD = 1,271 mg/L.

vital for promoting microalgae growth, while the interactions of them with biomass production may be different. For a constant ammonia concentration (25–40 mg/L), the biomass production slowly decreased as the TP concentration increased from 8 to 20 mg/L. However, when ammonia concentration was lower than 25 mg/L, the biomass production was slightly enhanced and then stabilized with the TP concentration increase. It was reported that microalgae growth was negatively affected at high ammonia levels, moreover, the inhibitory effect of ammonia was highly pH depended and unionized ammonia produced at high pH was much more (over 100 times) toxic than its ionized form [14]. This may be one of the reasons to explain the effects of ammonia noted in this work. Moreover, according to the gentle slope in the contour plot and the ANOVA, it was indicated that the interactions between ammonia and TP on biomass production was not so significant.

3.4. Confirmation experiments and adequacy of the models

To confirm the effectiveness of the statistical experimental methods and to gain the maximum biomass production of

C. vulgaris, three sets of verification experimental were conducted, the optimal conditions of which were predicted by statistical models. Comparing the experimental and predicted values in Table 4, it was noted that they matched well with each other. Moreover, the average biomass yield of 0.52 g/L was achieved, much higher than that achieved before optimization (0.15 g/L of control substrate). Thus the results indicated that the applied RSM analysis was suitable for optimizing nutrients composition for efficient microalgae biomass production.

3.5. Nutrients removal performance

Fig. 4a shows the growth profiles of *C. vulgaris* with cultivation time at different substrate conditions. As shown, all the microalgae growth curves confirm to "S" profiles. Specifically, after one day's cultivation, the growth of *C. vulgaris* entered the logarithmic phase (lasting for 2–4 d) and followed by a stable period or decline phase. In this case, during the first 3 d, the rapid increase in biomass content was noted due to the sufficient nutrients provided by the added substrates. However, for the next 4–6 d, the maximal biomass content was achieved with the growth rate near zero or negative values, indicating the biomass growth was inhibited by insufficient substrates or adverse environmental conditions (such as limited light illumination and living space) [33]. Overall, *C. vulgaris* could be cultivated at varied wastewater compositions under mixotrophic conditions, and 3–5 d was the proper cultivation cycle for nutrients removal due to the high biomass concentration and metabolic activity.

Fig. 4b shows COD removal with cultivation time at different substrate conditions. As known, under mixotrophic cultivation conditions, both organic and inorganic carbons are utilized by *C. vulgaris*. In this study, the removals of COD in different tests showed a similar trend; that was a quick increase during the first 4 d with sufficient organic carbon and leveled off afterward with the limitation of organics due to biomass utilization. Thus, the COD removal at the 4th day seemed to be the highest with removal efficiencies ranging from 70%–83% and the COD utilization rates of 52.6–122.2 mg/L d. It should be noted that in some cases (such as Runs 1, 7, 10 and 17) the COD removal efficiencies decreased to some extent after the 4th day, possibly caused by the release of organic biopolymers by *C. vulgaris* biomass under the adverse substrate or environmental conditions [34,35].

Fig. 4c shows ammonia removal with cultivation time at different substrate conditions. The available nitrogen sources of microalgae growth comprise organic nitrogen and inorganic nitrogen [18]. Corresponding to the biomass growth profiles, the removal of ammonia increased rapidly with cultivation time (0–4 d) and still improved slowly afterward (5–6 d) in most runs, due to the fact that nitrogen was dominantly removed by biomass assimilation during the growth period. As such, the ammonia removal efficiencies at the end of the cultivation period ranged from 51%–91% and the utilization rates were 1.9–3.9 mg/L d. It was noted that in some cases (such as Runs 1, and 5) the ammonia removal efficiencies were as high as 90% and 89%, with the effluent ammonia less than 5 mg/L.

Fig. 4d shows TP removal with cultivation time at different substrate conditions. In previous studies, phosphorous was recognized as the limiting factor during microalgae growth, and the utilization rate of phosphorous with different states in wastewater was different [25,35]. In this study, KH_2PO_4 was used as the phosphorous source, which could provide preferential utilization of phosphorus (PO_4^{3-}) for *C. vulgaris* production. Similar curves of TP removal were observed for different runs compared to those of COD and ammonia removal, but great variations in TP removal for different runs were also noted. The highest TP removal efficiencies of different runs ranged from 30%–94% and the utilization rates were 0.6–1.3 mg/L d. Moreover, Runs 10, 11 and 13 showed a high TP removal of approximately 94%. It was reported that microalgae assimilation accounted for more than 90% of total removals of P in photo-bioreactors [14], thus the great fluctuation in TP removal in this study may be due to the limitation of other nutrients (i.e. organic carbon and ammonia) rather than TP during microalgae growth.

The above analysis indicated that *C. vulgaris* biomass content and nutrients removal efficiencies were affected by varied nutrients composition. However, little attention was paid to the relationship between microalgae biomass content and nutrients removal. Thus, the linear correlations among *C. vulgaris* biomass and pollutants removed after the 4 d cultivation period were performed as shown in Fig. 5. It was noted that *C. vulgaris* biomass improved with the increase in removed pollutants concentrations, especially for COD and TP. During the experiment period, *C. vulgaris* biomass leveled off under mixotrophic cultivation conditions at tested substrate conditions. At the maximum biomass of *C. vulgaris* (around 0.50 g/L), the removed amounts of COD, ammonia, and TP were approximately 1,200, 20, and 10 mg/L. In this monoculture microalgae system, microalgae biomass production was considered to be the main factor contributing to pollutant removal; however, other microorganisms such as coexisted bacteria also accounted for organics degradation and nitrification processes as previously reported [21,36], to which more attention should be paid in the future.

In all, the *C. vulgaris* biomass content, growth cycle and nutrients removal efficiency were affected by initial nutrients composition. Moreover, the nutrients in wastewater greatly

Table 4
Optimum conditions of nutrients composition found by the model and its verification

Run	COD (mg/L)	NH_4^+–N (mg/L)	TP (mg/L)	Predicted values (g/L)	Experimental values (g/L)
1	1,271	20	18	0.5220	0.5197
2	1,285	20	17	0.5215	0.5200
3	1,332	20	10	0.4773	0.4735

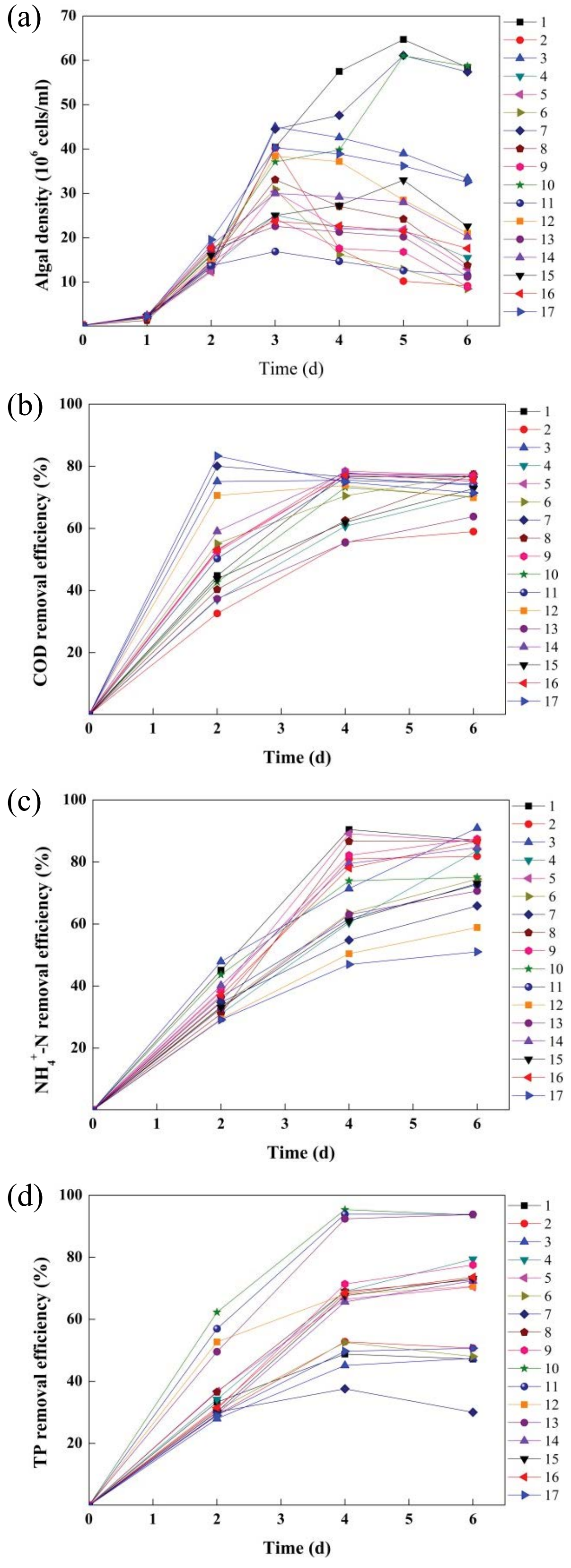

Fig. 4. (a) Growth profiles of *Chlorella vulgaris*, (b) COD removal, (c) NH$_4^+$–N removal, and (d) TP removal with time at different substrate conditions.

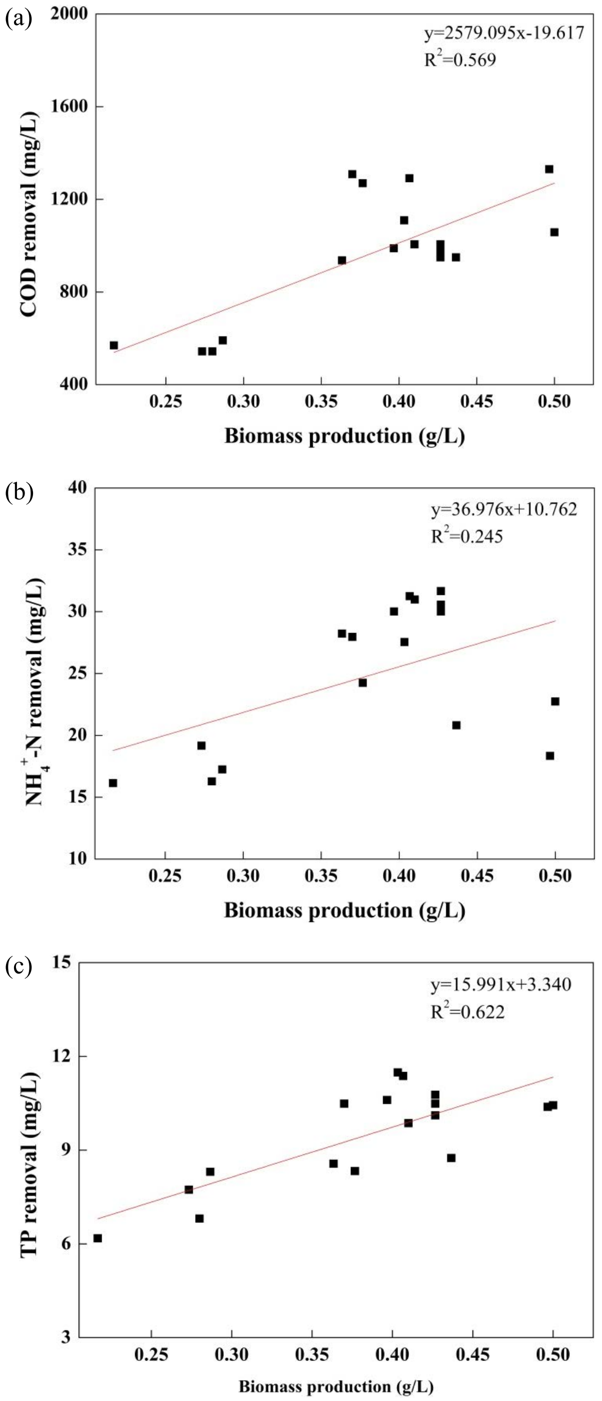

Fig. 5. Linear relationship among *Chlorella vulgaris* biomass and pollutant removal: (a) COD removal, (b) ammonia removal and (c) TP removal.

affected the microalgae biomass, showing a significant positive correlation. Taken all the factors into comprehensive consideration, a cultivation time of 3–5 d at an optimized substrate condition was suggested for simultaneous biomass production and nutrients removal. Moreover, it should be noted that other important factors, such as utilization of real wastewater, light/night cycle, temperature, recycle use of the wastewater, biomass harvesting, etc. [14,37], are also critical for promoting the practical application microalgae-based wastewater treatment technology, thus more efforts should be made to optimize these parameters.

4. Conclusions

Nutrients composition in synthetic wastewater was optimized using RSM for efficient *C. vulgaris* biomass production and nutrients removal under mixotrophic cultivation conditions. The main conclusions are drawn as follows.

- The effects of individual nutrients on microalgae biomass production were investigated and the suitable concentration ranges lied in 500–1,500 mg/L for COD, 20–40 mg/L for ammonia, and 8–20 mg/L for TP, respectively.
- Based on the RSM designed batch tests, a statistic model regarding the influence of initial nutrients concentration on biomass production was developed. Under the optimized nutrients composition (1,271, 20, and 18 mg/L for COD, ammonia, and TP), the highest *C. vulgaris* biomass of 0.52 g/L was predicted, which was enhanced by more than two times as compared to that of the control substrate (0.15 g/L).
- *C. vulgaris* could be cultivated at varied wastewater compositions, and 3–5 d was the proper cultivation cycle for nutrients removal. The removal efficiencies of COD, ammonia and TP were 70.2%–83.3%, 51.0%–91.0% and 30.0%–93.9%. The lowest concentrations of effluent COD, ammonia, and TP were 184.0, 2.1, and 0.56 mg/L. Moreover, the nutrients removed from wastewater had a positive correlation with *C. vulgaris* biomass production. It was indicated that the microalgae-wastewater treatment process is highly promising for efficient nutrients removal accompanied by microalgae biomass accumulation and bioresource recovery.

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