Optimization of Culture Conditions for *Chlorella Vulgaris* Using Corresponding Normal Surface and Determination of Coefficients for Batch Kinetics of Nitrogen Removal Rate

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Abstract. This study investigated the effects of CO_2, light intensity, and phosphorus concentration on rate of NH_4-N removal by *Chlorella vulgaris* and determined kinetic coefficients, such as biokinetic coefficients (k) and reaction rate constant (K_m), using Michaelis–Menten rate expression under optimal conditions. By using Box–Behnken design under conditions of light intensity at 7446 lx, 2% CO_2 content, and phosphorus concentration at 40 mg/L, the model predicted 38.56% maximum NH_4-N removal rate. Maximum NH_4-N removal rate of 18.7% at light intensity of 11216 lx and CO_2 content of 4.4% was further tested by using central composite rotatable design. Batch experimental results confirmed kinetic equations of removal rate by *C. vulgaris*at optimal conditions and determined kinetic coefficients of $k = 11.49\ d^{-1}$, and K_m =64.49mg/L.

1. Introduction

Recently, global energy demand drastically increased because of worldwide population growth and increase in quality of human life. Microalgal are considered as one of the most important sources of renewable biodiesel, which can meet global demands for transport fuel, because of their high biomass productivity [1]. Algae also received growing attention given their potential as source for removing nutrients, such as nitrogen and phosphorus, and are considered as one of the best measures for reducing pollutants. Different reference variables participate in algal removal of nitrogen and phosphorus. Light intensity is an important property influencing microalgal growth, controlling photosynthetic growth in almost all microalgal systems. Light intensity can also affect photosynthesis, biomass concentration, and CO_2 and nitrogen removal rates [2–4]. Hulatt (2011) [5] researched 4% CO_2 concentration at different levels of light intensity (10, 20, and 50 Wm^{-3}) and showed significant differences in growth of *Chlorella vulgaris* and *Dunaliella tertiolecta*. Light intensity also participates in shading between microalgal, increasing penetration depth of incident light and remarkably influencing removal of nitrogen and phosphorus [6–8]. Extremely strong light intensity can restrain microalgal growth; optimal light intensity for removal of nitrogen and phosphorus measures 135.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$[9]. As a carbon substrate, CO_2 plays an important role in microalgal metabolism, participating in formation of microalgal cells while working in synergy with other gases, including other CO_2 molecules [10]. For algae cultures, an efficient system for removal of nitrogen and phosphorus must ensure appropriate CO_2 concentration [11]; 5% CO_2 concentration in air is

Zou, Y., Xu, X., Wang, X., Yang, F. and Zhang, S.
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In *Proceedings of the International Workshop on Environmental Management, Science and Engineering (IWEMSE 2018)*, pages 517-523
ISBN: 978-989-758-344-5

considered the most suitable concentration for organism growth, with maximum biomass dry weight (DW) of 4.4 g/L [12]. At low concentrations of 1% (v/v) [13–14] and 2% to 3% (v/v), CO_2 is widely applied in actual wastewater treatment [15]. Further research should then investigate cross-pollination of factors, such as CO_2, light intensity, and phosphorus concentration and their effects on nitrogen and phosphorus removal rate by microalgal. Thus, the present work investigates different factors influencing conditions of single and multiple effects during nitrogen and phosphorus removal by microalgal and calculates nitrogen dynamics equations under optimal conditions.

2. Methods

2.1. Microalgal strain and culture condition

C. vulgaris was obtained from microalgal culture collection in Wuhan Botanical Garden, Chinese Academy of Sciences. Microalgal were then cultured in 5 L photobioreactor composed of transparent polyvinyl chloride pipe under indoor conditions at 25°C until late-log phase. During cultivation, 3500 lx cool-white fluorescent light illumination and 2% v/v CO_2 content were provided at the top and bottom of the photobioreactor, respectively. All analytical grade chemicals were procured from China. All experiments were performed in triplicate.

2.2. Measurement of temperature, light intensity, dissolved oxygen (DO), and pH

Light intensity was recorded by a photometer (GLZ-C, China), and temperature of algal culture was measured using a thermometer (Brannan, England) at 5 min intervals. Temperature and pH were monitored daily using a pH meter (WTW, Germany) at 3 pm, at which pH was at its daily peak. DO concentration was measured using a DO meter (WTW, Germany). Nitrogen and phosphorusremovalwas evaluated in accordance with the Standard Methods for the Examinations of Water and Wastewater. Optical density (OD) of algal cells was used to determine OD at 680 nm (OD680) using a spectrophotometer (UV-1000) as indicator. DW was calculated in Eq. (1):

$$DW\ (g/L)=2.6828OD_{680nm}+0.0677 \tag{1}$$

2.3. Experimental design and analysis of response surface through Box–Behnken design (BBD)

In this experiment, using Design Expert software version 8.0.6, BBD was used for statistical analysis of experimental data based on three factors, namely, CO_2, phosphorus concentration, and light intensity, which were selected to evaluate optimum conditions and as indicators. Experimental results were calculated in Eq. (2):

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2 \tag{2}$$

where Y, b_0, b_1, b_2, b_3, X_1, X_2,and X_3 represent the response variable, interception coefficients, regression coefficients, and independent variables. Responses were analyzed by analysis of variance (ANOVA), whereas quality of fit of the polynomial model equation was expressed by coefficient of determination (R^2). Interaction of independent variables was investigated by constructing response surface and optimizing the model through exclusion of confidence level of 0.10.

2.4. Experimental design and analysis of response surface through central composite rotatable design (CCRD)

CCRD is based on two factors, namely, CO_2 and light intensity, which serve as indicators and can minimize variable limitations and encompass the largest possible design space. Experimentation was designed as a classic response surface design by a 2^2 full-factorial central composite design. Effects of CO_2 and light intensity were calculated in Eq. (3).

$$x_i = (X_i - X_0)/\Delta X_i \tag{3}$$

where xi, X_i, X_0, and ΔX_i represent coded value, real value, real value at center point, and step change value, respectively. Results were analyzed through ANOVA. These variables were selected based on results of preliminary experimentation. CO_2 concentration and light intensity were used to prepare 13 experimental formulations.

3. Results and discussion

3.1. Effect of initial NH4-N concentration

Figure 1.a depicts the curves of NH_4-N removal rate at different initial NH_4-N concentrations for seven days of batch operation. NH_4-N was removed above 50% from the media when initial concentration was below 79.8 mg/L. Increasing initial NH_4-N concentration resulted in decreased NH_4-N removal rate (23.2%) at NH_4-N concentration of 332 mg/L. These results indicate that *C. vulgaris* can effectively decrease NH_4-N concentrations to below 111 mg/L. Initial substrate removal rate is calculated in Eq. (4).

$$P_i = -\frac{q_0 - q_t}{t_0 - t_t} \tag{4}$$

where P_i, q_0, and q_t represent substrate removal rate, initial NH_4-N concentration, and corresponding NH_4-N concentration, respectively at t_t, which is the time at which biomass DW did not change significantly. Specific rate of substrate removal (P_{bi}) is determined in Eq. (5).

$$P_{bi} = P_i / DW_0 \tag{5}$$

As depicted in Figure 1.b, specific rate of substrate removal decreased with increasing initial NH_4-N concentration. Maximum and minimum specific removal rates reached 11.21 and 3.65 d^{-1}, respectively, when specific rate of substrate removal and initial NH_4-N concentration showed a linear relationship

3.2. BBD statistical analysis on effects of CO_2 concentration, light intensity, and phosphorus concentration on nitrogen removal rate

A BBD experiment was performed using Design-Expert 8.0.6, in which removal rate corresponds to a specified range for optimal culture condition, to investigate three factors (CO_2, light intensity, and phosphorus concentration). As suggested by Statistical Model Fit Summary, a mathematical regression model was fitted as the best model, as indicated by Eq. (6):

$$\text{Removal rate} = 32.93 - 2.57 \times A - 0.40 \times B + 1.78 \times C + 2.25 \times A \times B - 2.81 \times A \times C - 5.35 \times B \times C - 7.76 \times A^2 - 2.79 \times B^2 - 1.12 \times C^2 \tag{6}$$

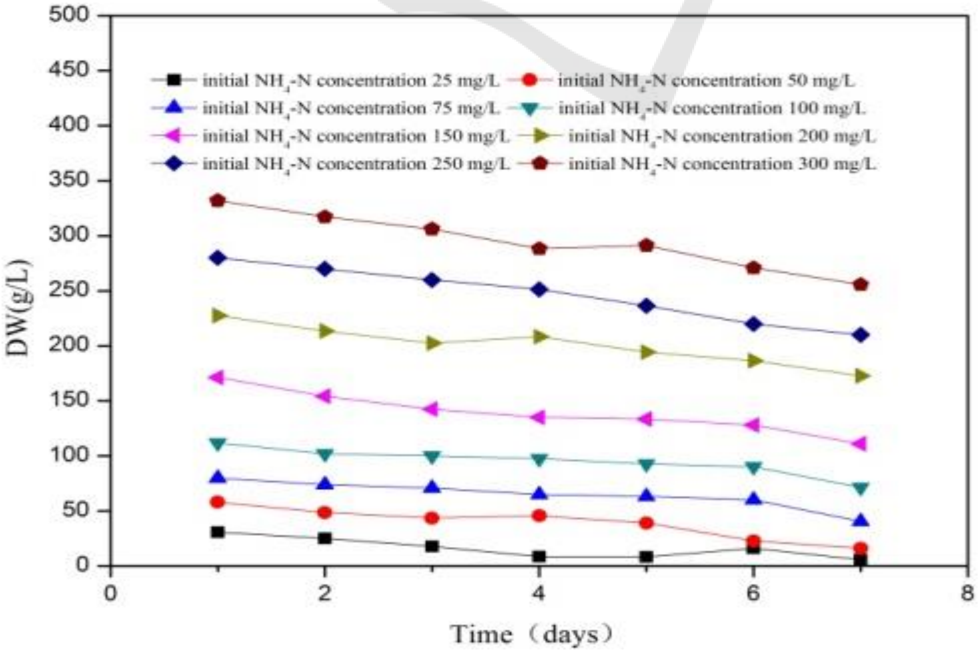
ANOVA results showed that among the parameters, light intensity presents the highest F-value of 1.34 and lowest p-value of 0.2854, revealing that this factor more significantly influence NH_4-N removal in model diesel. Parameters could not be analyzed independently because of interaction effects between variables. Figure 2 provides the contour plots of NH_4-N removal conversion; the plots indicate interaction effects using a regression equation. When phosphorus concentration remained unchanged, light intensity and CO_2 showed slightly remarkable interaction effect on the removal rate of NH_4-N. When CO_2 concentration decreased (6%), NH_4-N removal rate increased with increasing light intensity. When CO_2 concentration increased from 6% to 10%, removal rate and light intensity increased with direct proportions. The same situation was also noted for light intensity. NH_4-N removal rate was inversely and directly proportional to low and high light intensity and CO_2 concentrations, respectively. Raised surfaces were detected at CO_2 concentration ranges and light intensities of 2% to 4% and 5000–7500 lx and 8% to 10% and 12500–15000 lx, respectively, indicating that light intensity and CO_2 are essential for photosynthesis. Extremely high or low levels of these factors can inhibit photosynthesis. Figure 2.b and Figure 2.c respectively show remarkable surface changes and interaction gradient between the two factors and unclear effect of phosphorus on NH_4-N removal rate. As shown in the figures, phosphorus presented a weaker influence than CO_2 concentration and light intensity, which played an important role on growth of algae but was not apparent enough to distinguish it from that of strength. The adjusted coefficient of determination accounted for model significance, which revealed suitability of the equation to describe responses during experiment. All contour plots demonstrated considerable interaction effects of CO_2, light intensity, and phosphorus concentration. Interaction between CO_2 and light intensity was the highest among the three factors of CO_2, light intensity and phosphorus concentration.

3.3. Statistical analysis using CCRD on effects of CO2 and light intensity on NH4-N removal rate

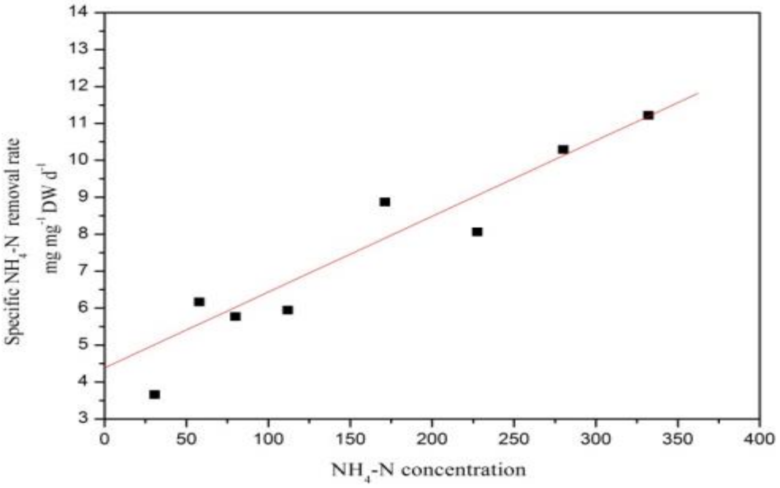
As a result of the slight influence of phosphorus on the three factors, another statistical analysis using CCRD was conducted to identify independent variables that significantly influence NH_4-N removal rate between CO_2 concentration and light intensity. Figure 3 displays mutual influences of light intensity and CO_2 on NH_4-N removal rate. As shown in the figures, removal rate fitted a second-order equation, as shown below. Despite the non-statistical significance of terms B_2 and AB at $p < 0.05$, they were maintained in the model equation to avoid decreasing the value of R^2, as indicated by Eq. (7).

$$\text{Removal rate} = 17.28 + 2.75 \times A - 3.39 \times B - 1.04 \times A \times B - 4.82 \times A^2 - 3.38 \times B^2 \tag{7}$$

where A represents CO_2 concentration in air, and B represents light intensity. Some response data are not available. Possibly, reaction conditions of light intensity at 5000 lx and 6% CO_2 concentration were too mild to remove NH_4-N in Run 3, and removal rate was significantly affected by low CO_2concentration and high light intensity in wastewater . Graphs demonstrated that at a certain constant, very slight and moderate influences occur at low light intensities and high light intensity, respectively. With changing CO_2 concentration in wastewater, removal rate first increased and then decreased. CO_2 concentration increased from 4.65% to 18% and finally decreased to 10%. After crossing the saddle point, final DW increased with decreasing CO_2, and light intensity increased. Maximum NH_4-N removal rate was 18.7% at light intensity of 11216 lx and CO_2 concentration of 4.44%. Under these conditions, the model predicted biomass DW of 1.04 g L^{-1} d^{-1}.



(a)



(b)

Figure 1. Variation of DW with initial NH4-N concentration (a) and effect of initial NH4-N on NH4-N removal rate (b).

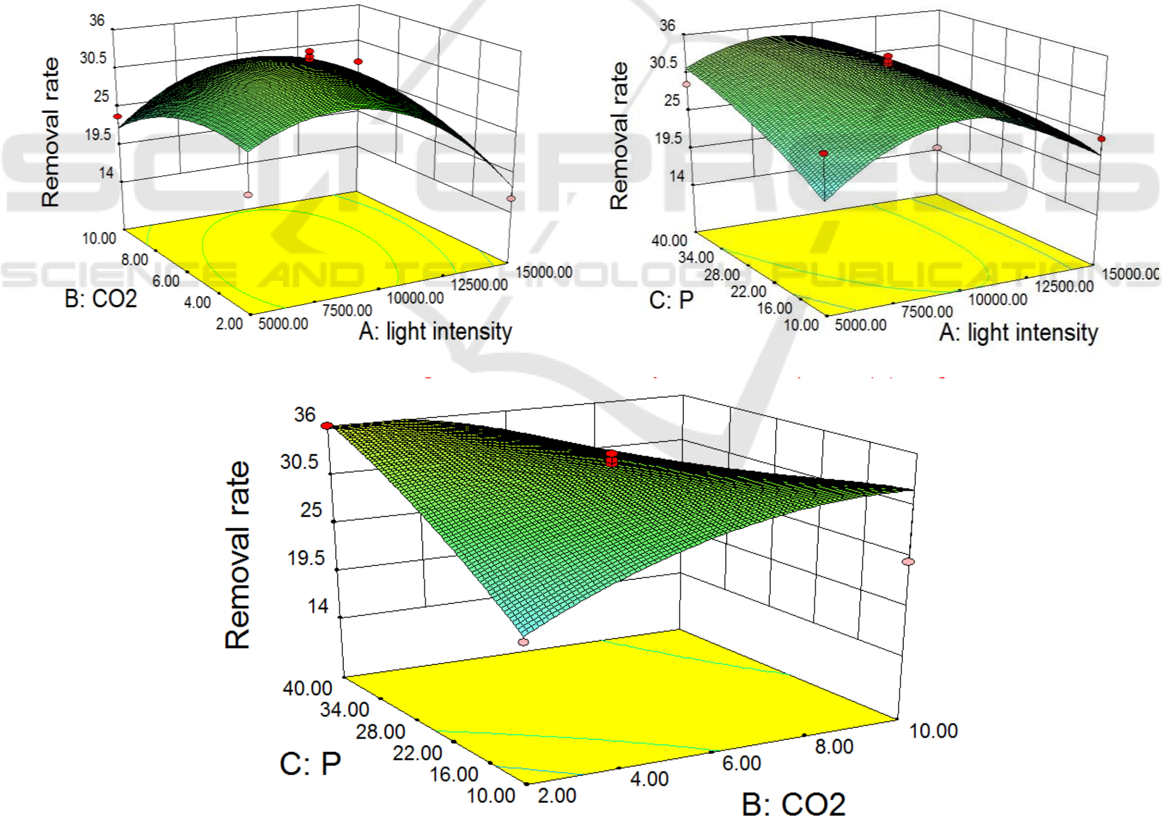


Figure 2. Response surface and contour plots for removal rate; effects of (a) CO_2 and light intensity, (b) phosphorus concentration and light intensity, and (c) CO_2 and phosphorus concentration.

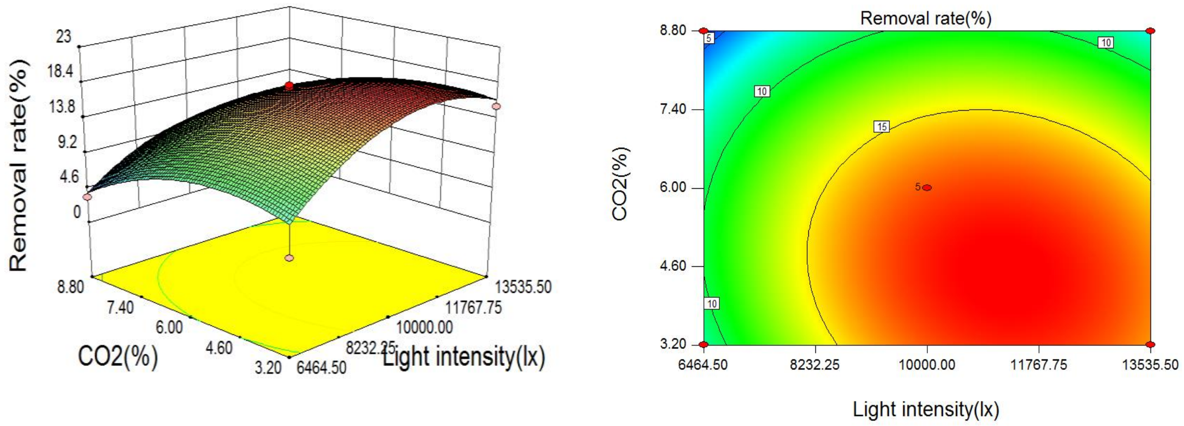


Figure 3. Response surface and contour plots for removal rate on the effects of CO_2 and light intensity.

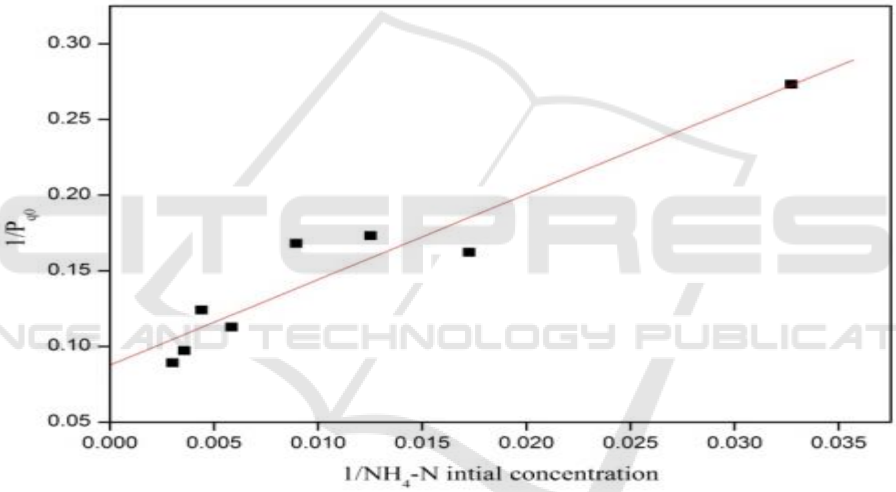


Figure 4. Determination of yield coefficient for NH4-N removal by C. vulgaris.

3.4. Determination of batch kinetic coefficients

For the determination of kinetic coefficients, K_m, saturation constants, k, the Michaelis–Menten kinetic relationship was used in Eq. (8).

$$P = \frac{P_{MAX}\,q}{K_m + q} \tag{8}$$

P_{max} and q represent maximum nitrogen removal rate and effluent nitrogen concentration, respectively. Eq. (8) can be converted into Eq. (9):

$$P_{q_0} = \frac{k b_0 q_0}{K_m + q_0} \tag{9}$$

$P_{max} = k \cdot b_0$ corresponds to the maximum initial rate of substrate removal. k and b_0 represent reaction rate constant ($time^{-1}$) and initial DW of microalgae, respectively. Specific rate of substrate removal (P_{bi}) was determined by dividing the initial rates to DW in Eq. (10):

$$P_{b_i} = \frac{P_{q_0}}{b_0} = \frac{k\,q_0}{K_m+q_0} \tag{10}$$

Eq. (10) can then be converted to Eq. (11):

$$\frac{1}{P_{q_0}} = \frac{1}{k} + \frac{1}{k}\frac{K_m}{q_0} \tag{11}$$

Figure 4 shows $1/P_{q0}$ versus 1/ $(NH_4\text{-}N)_0$. Kinetic equation of biomass DW growth rate by *C. vulgaris* can be obtained from the slope and intercept of the best-fit line of this episode. Thus, kinetic coefficient of ammonia nitrogen removal was determined, as shown in Eq. (12). Kinetic coefficients of NH_4-N removal by *C. vulgaris* are as follows: k=11.49 d^{-1} and K_m=64.49mg/L (R^2 = 0.911).

$$\frac{1}{P_{q_0}} = 0.087 + 5.65\ \frac{1}{q_0} \tag{12}$$

4. Conclusions

CO_2 concentration, phosphorus concentration, and light intensity play vital roles in NH_4-N removal rate, and effects CO_2 and light intensity are more important than those of phosphorus concentration. This study investigated the cross effects of three factors on removal rate by *C. vulgaris* in batch cultivation. Experimental results indicated the optimal conditions for photosynthesis: light intensity at 7446 lx, 2% (v/v) CO_2 concentration, and phosphorus concentration at 40 mg/L. Based on experimental data, batch kinetic coefficients of NH_4-N removal by *C.vulgaris* were determined as follows :k = 11.49 d^{-1}, and K_m = 64.94mg/L. This study constitutes an important step in the development of strategies for rapid cultivation of microalgae using biological approaches. Future research will be conducted to optimize several other cultural parameters, and fractional factorial design experiments will be used widely.

Acknowledgement

The authors wish to thank Dr. Xiaojing Wang and for the help on this study. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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