Cultivation of Scenedesmus dimorphus for C/N/P removal and lipid production

Xinmiao Xu, Ying Shen⁎, Jiacheng Chen

College of Mechanical Engineering and Automation, Fuzhou University, Fuzhou, Fujian 350108, China

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A B S T R A C T

Background: CO2 emission, water pollution and petroleum shortage are the issues coming with the development of industry. A cost effective system was constructed to fix the CO2 in flue gas (15% CO2), remove nitrogen and phosphorus from manure wastewater and produce biofuels at the same time. The significant cultivation conditions were selected by Plackett–Burman design, and then optimized with central composite design. Results: Optimum culture condition was predicted at light intensity of 238 μmol·m−2·s−1, TN of 152 mg·L−1, inoculum density of 0.3 g·L−1, under which the measured CO2 fixation rate, total nitrogen and phosphorus removing rate, and lipid content were 638.13 mg·L−1·d−1, 88.16%, 73.98% and 11.9%, respectively. The lipid content was then enhanced to 24.2% by a nitrogen starvation strategy. Conclusion: A cultivation strategy was suggested to achieve effective C/N/P removal from flue gas and manure wastewater, and meanwhile obtained high lipid content from microalgal biomass.

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1. Introduction

Microalgae, a group of organisms that can achieve 3–5% photosynthetic rate, are one of the most promising pathways to ease the greenhouse effect [1]. In general, photosynthetic microalgae have 50% of its weight as carbon and hence 1.83 kg of CO2 will be needed theoretically to produce a kilogram of biomass [2]. Utilization of concentrated carbon sources such as industrial emissions and manure wastewater to grow microalgae is a cost-effective concept to achieve concentrated carbon sources such as industrial emissions and manure wastewater (around 106:16:1), the addition of CO2 is one of the effective methods to stimulate algae growth and nutrient uptake. Woertz et al. [4] reported an enhancement of biomass concentration from 317 to 812 mg·L−1 when CO2 was added to a semi-continuous culture of a microalgae consortium in a preliminarily clarified wastewater. Several studies also proved the feasibility of remediating power plants with microalgae using either direct industrial flue gas or by using pure gas mixtures that mimic the flue gas composition [5,6,7].

Microalgal species, such as Scenedesmus sp., Chlorococcum littorale, and Chlorella sp. were reported to have great tolerance of high concentration CO2 [8,9]. For instance, Maeda et al. [6] found a strain of Chlorella sp. T-1 which could grow under 100% CO2, although the maximum growth rate occurred under a 10% concentration [6]. Generally, the CO2 fixation rate (CFR) is affected by many factors, such as light intensity, nitrogen concentration, aeration rate, culture period, pH, inoculum density, and so on [10,11,12]. For instance, Ho et al. [12] found that the specific growth rate increased dramatically with rising light intensity at the beginning of the process (i.e., in the light limited region), and then it gradually leveled off as the light intensity continued to rise (i.e., in the light saturation region). The maximum biomass productivity of 840.56 mg·L−1·d−1 was observed at a light intensity of 420 μmol·m−2·s−1, with the highest CFR of 1435.90 mg·L−1·d−1 [12].

The objective of this study was to optimize the culture conditions of freshwater alga Scenedesmus dimorphus to achieve C/N/P removal from flue gas and manure wastewater, and stimulate lipid accumulation with nitrogen deficiency strategy. Plackett–Burman design and central composite design were applied to screen and optimize the culture conditions, including: light intensity, wastewater concentration, aeration period, inoculum density, pH, and magnesium (Mg) concentration.

2. Materials and methods

2.1. Algal strain and subculture

The alga S. dimorphus (FACHB-496) was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (Wuhan, China). The alga was maintained in Modified Basal medium [13]. The inocula were grown in 250 mL Erlenmeyer flasks, each containing 120 mL medium, and incubated at 26 ± 2°C in an orbital shaker set to 125 rpm. The illumination was provided by 18 W cool white fluorescent light at 60–80 μmol·m−2·s−1. The cell concentration
of the inocula was monitored by Hemocytometer (Cat. No. 0650030, Marienfeld, Germany). Once it reached $10^6$ cells·mL$^{-1}$, the biomass dry weight was determined by gravimetric method. Subsequently, aliquot inocula were centrifuged to make 75 mL high concentrated algal medium, and then the high concentrated algal medium was added into 1.425 L wastewater to achieve the objective inoculum density.

### 2.2. Cultivation system

To reduce the capital cost, polyethylene bags with diameter of 7 cm and volume of 2 L (each containing 1.5 L medium) were used as cultivation system, as shown in Fig. 1. Solenoid valves were applied to intermittently aerate simulated flue gas (15% CO$_2$). Pre-treated manure wastewater was used as culture medium. The manure wastewater was obtained from a piggery farm close to Fuzhou University (Fuzhou, Fujian, China). The wastewater was filtered to remove any large solid particles and then diluted with tap water before feeding it to microalgae. The TN and total phosphorus (TP) in filtered wastewater were 306.15 ± 3.90 mg·L$^{-1}$ and 115.08 ± 0.82 mg·L$^{-1}$, respectively. The same batch of wastewater was used throughout the experiments. Based on the nutrient removing rate, each batch of the culture lasted 7-d.

### 2.3. Experimental design

Based on the experience from previous experiments [14] and references [15,16], seven variables were determined to perform Plackett–Burman experiments, including: light intensity, wastewater concentration (described with TN), aeration rate, aeration period, inoculum density, pH, and Mg concentration (MgSO$_4$ × 7H$_2$O), as shown in Table 1. Each taking two levels, in such a way as to minimize the variance of the estimates of these dependencies using a limited number of experiments.

Based on the estimation of Plackett–Burman design, light intensity, TN and inoculum density were determined to be the significant factors ($p$-value ≤ 0.05). The optimal response recommended for central composite design (CCD) was: light intensity of 240 mol·m$^{-2}$·s$^{-1}$, TN of 150 mg·L$^{-1}$, inoculum density of 0.3 g·L$^{-1}$, aeration rate of 0.1vvm, aeration period of 15:45:05 (on:off), and pH of 8. To avoid heavy metal pollution, MgSO$_4$ × 7H$_2$O, which was not significant on CFR in this case, was not added into the second step of the experiments. As shown in Table 2, 5 coded levels (-α, -1, 0, +1, and +α) of light intensity, TN and inoculum density were assessed (the other factors were fixed at the recommended level). A total of 18 experiments were conducted, including 8 fractional factorial designs (2$^3$), 8 star points (2 × 3), and 4 replicates at center points. According to rotatable design of the response surface, the parameter of α was chosen to be 1.681. The treatments were carried out in duplicate as independent experiments to take into account the non-adjustable data and the analysis of variance. The results of the CCD were fit with a second-order polynomial equation using a multiple regression technique in [Equation 1]:

$$Y = X_0 + \sum_{i=1}^{4} a_i X_i + \sum_{i=1}^{4} a_{ij} X_i^2 + \sum_{i=1}^{4} \sum_{j=1}^{3} a_{ij} X_i X_j$$

[Equation 1]

where $Y$ is CFR (mg·L$^{-1}$·d$^{-1}$); $X_0$ stands for the model intercept; $X_1$, $X_2$, and $X_3$, are the levels of light intensity, TN and inoculum density, respectively; $a_{i...a_{ij}}$ are the regression coefficients. The $p$-value of each term was determined to remove insignificant terms. The analysis of variance was carried out through the Design Expert software version 7.0 (Statease, Minneapolis, MN).

The optimal culture condition identified by CCD was validated by duplicate experiments. To increase the lipid content, the validated experiments were continued with a 5-d nitrogen starvation cultivation by replacing 20%, 40%, 60% and 80% of the culture solution with tap water.

### 2.4. Analysis

Biomass dry weight (DW) was measured by filtering aliquot algal sample on pre-weighed glass-fiber filter paper with pore size of 0.45 μm (Q/IEPJ01-1997, Xinya purification material factory, Shanghai, China). The filters were then dried at 105°C in an oven for 12 h. Algal biomass DW was determined by the difference of two weights, pH was measured using pH meter (PB-10, Sartorius, Co., Ltd, China) once every day and adjusted with 0.5 M HCl or NaOH solution to maintain the objective pH value. The TN and TP were measured once every day by using Mi-parameter meter (5H-3BA, Lian-hua Tech. Co., Ltd, China) with standard procedure. Lipid extraction was carried out using a BioSpec Model 3110 BX bead-beater (Bartlesville, Oklahoma) for cell disruption (3 min) followed by solvent extraction with n-hexane. The oils collected after evaporation was dried at 95°C for 1.5 h before weighing. Lipid content was the percentage of lipid weight to disrupted biomass. To obtain the carbon content, part of the algal biomass was freeze-dried (LGJ-10, Songyuanhuaxing Tech. Co., Ltd, China) for 48 h, and then tested by elemental analysis (vario EL cube, Elementar Analysensysteme GmbH, Germany). The CFR was calculated according to [Equation 2] [17]:

$$CFR = \frac{C_i M_{CO_2}}{M_C (DW_2 - DW_1)}$$

[Equation 2]

where $C_i$ is the carbon content of S. dimorphus (44.83 ± 0.39%), $M_{CO_2}$ is the molecular weight of CO$_2$ (44 g·mol$^{-1}$), $M_C$ is the molecular weight of C (12 g·mol$^{-1}$), $DW_2$ is the biomass DW achieved in the end of the cultivation (g·L$^{-1}$), $DW_1$ is the inoculum density (g·L$^{-1}$), and it is the culture time (d).

### 3. Results and discussion

#### 3.1. Optimizing the culture conditions to increase C/N/P removing rate

As shown in Table 1, the CFR, TN and TP removing rate varied from 303.75 ± 10.47 to 610.13 ± 13.09 mg·L$^{-1}$·d$^{-1}$, 68.47 ± 0.54 to 92.13 ± 0.29%, and 58.82 ± 0.33 to 76.85 ± 0.44% in Plackett–Burman design, respectively. Since the TN and TP removing rates were relatively high, the CFR was chosen for further optimization. As shown in Table 3, the significances of the seven factors on CFR was analyzed via F-test. Factors evidencing $p$-values of less than 0.05 were considered to have significant effects on the response, and therefore...
light intensity, TN and inoculum density were selected for further optimization studies.

The optimal control of light intensity, TN and inoculum density was conducted by CCD. The design matrix and corresponding experimental data are given in Table 2. The experimental results were fit to a second-order polynomial in [Equation 3]:

\[
Y = 610.89-27.56 \times X_1 + 12.52 \times X_2 + 19.24 \times X_3 - 27.01 \times X_1 \times X_2 - 35.52 \times X_1 \times X_3 - 30.83 \times X_2 \times X_3 - 64.43 \times X_1^2 - 52.81 \times X_2^2 - 38.48 \times X_3^2
\]

[Equation 3]

The significance of the regression model and individual variables were determined at 95% confidence level. As shown in Table 4, the p-value of second-order polynomial model is lower than 0.05, which indicates that the model is statistically significant. Based on the p-values, the variables of light intensity, inoculum density, as well as the interactions between light intensity and TN, light intensity and inoculum density, and TN and inoculum density were significant on CFR.

### 3.2. Interactions between light intensity and TN

The interactions between light intensity and TN on CFR when inoculum density was constant at the center point were shown in Fig. 2. The contour lines indicate that CFR favored medium light intensity and medium TN. The designed point was predicted at light intensity of 238 μmol·m⁻²·s⁻¹ and TN of 152 mg·L⁻¹. Basically, light intensity, TN and inoculum density were determined at 95% confidence level. As shown in Table 4, the p-value of second-order polynomial model is lower than 0.05, which indicates that the model is statistically significant. Based on the p-values, the variables of light intensity, inoculum density, as well as the interactions between light intensity and TN, light intensity and inoculum density, and TN and inoculum density were significant on CFR.

### 3.3. Interactions between light intensity and inoculum density

The interactions between light intensity and inoculum density on CFR when TN was constant at the center point were shown in Fig. 3. The contour lines indicate that CFR favored medium light intensity.

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**Table 1** Design and responses of Plackett–Burman design.

| No | X1 | X2 | X3 | X4 | X5 | X6 | CFR (mg·L⁻¹·d⁻¹) | TN removing rate (%) | TP removing rate (%) |
|----|----|----|----|----|----|----|-----------------|------------------|-------------------|
| A1 | 240| 150| 0.1| 15:450| 0.3| 9 | 602.27 ± 2.09      | 82.03 ± 1.05      | 58.82 ± 0.33       |
| A2 | 160| 150| 0.3| 15:300| 0.3| 9 | 570.85 ± 5.24      | 84.67 ± 0.56      | 73.85 ± 0.56       |
| A3 | 240| 100| 0.3| 15:450| 0.1| 9 | 432.06 ± 23.57     | 76.67 ± 0.88      | 69.64 ± 0.78       |
| A4 | 240| 150| 0.1| 15:450| 0.3| 7 | 610.13 ± 13.09     | 86.87 ± 0.91      | 67.37 ± 0.56       |
| A5 | 240| 100| 0.3| 15:300| 0.9 | 5 | 455.63 ± 10.47     | 69.45 ± 0.34      | 62.01 ± 0.23       |
| A6 | 240| 100| 0.1| 15:450| 0.1| 9 | 303.75 ± 10.47     | 68.47 ± 0.54      | 67.18 ± 0.45       |
| A7 | 240| 100| 0.1| 15:300| 0.1| 7 | 479.20 ± 13.09     | 92.13 ± 0.29      | 67.18 ± 0.56       |
| A8 | 240| 150| 0.1| 15:300| 0.9 | 9 | 555.14 ± 20.95     | 88.27 ± 0.58      | 72.17 ± 0.65       |
| A9 | 240| 150| 0.3| 15:300| 0.3| 7 | 573.47 ± 18.33     | 89.93 ± 0.90      | 76.85 ± 0.44       |
| A10| 240| 150| 0.3| 15:300| 0.7 | 5 | 447.78 ± 18.33     | 82.03 ± 1.09      | 63.44 ± 0.63       |
| A11| 240| 100| 0.3| 15:300| 0.1| 7 | 597.03 ± 5.24      | 85.55 ± 1.00      | 65.62 ± 0.67       |
| A12| 240| 150| 0.3| 15:300| 0.3| 7 | 439.92 ± 15.71     | 70.78 ± 0.98      | 62.37 ± 0.89       |

X1: light intensity, μmol·m⁻²·s⁻¹; X2: TN, mg·L⁻¹; X3: Aeration rate, vvm; X4: Aeration period, on/off s·s⁻¹; X5: inoculum density, g·L⁻¹; X6: pH; X7: Mg concentration, mM.

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**Table 2** Design and responses of CCD.

| No | X1 | X2 | X3 | Experimental data | CN removing rate (%) | TP removing rate (%) |
|----|----|----|----|-------------------|------------------|-------------------|
| B1 | -1 | -1 | -1 | 387.51 ± 17.61    | 87.35 ± 1.03      | 68.44 ± 0.47       |
| B2 |  1 | -1 | -1 | 446.22 ± 7.07     | 86.65 ± 1.12      | 67.50 ± 0.07       |
| B3 | -1 |  1 | -1 | 502.59 ± 4.72     | 90.01 ± 1.09      | 81.16 ± 0.65       |
| B4 |  1 |  1 | -1 | 476.76 ± 21.21    | 83.85 ± 0.98      | 85.54 ± 0.38       |
| B5 | -1 |  1 | -1 | 527.25 ± 22.39    | 90.02 ± 0.37      | 56.77 ± 0.67       |
| B6 |  1 | -1 |  1 | 467.37 ± 11.78    | 84.97 ± 0.56      | 49.27 ± 0.54       |
| B7 | -1 |  1 | -1 | 542.52 ± 20.64    | 85.58 ± 0.76      | 84.50 ± 0.19       |
| B8 |  1 | -1 |  1 | 351.11 ± 4.72     | 86.95 ± 1.05      | 84.85 ± 0.73       |
| B9 | -α | 0  | 0  | 465.02 ± 2.34     | 86.95 ± 0.65      | 69.48 ± 0.65       |
| B10| α  | 0  | 0  | 371.07 ± 5.85     | 85.82 ± 0.45      | 74.17 ± 0.67       |
| B11| 0  | -α | 0  | 413.35 ± 10.6     | 94.04 ± 0.89      | 49.15 ± 0.56       |
| B12| 0  | α  | 0  | 488.50 ± 23.35    | 81.32 ± 0.76      | 76.02 ± 0.48       |
| B13| 0  | 0  | -α | 435.66 ± 20.03    | 86.80 ± 0.56      | 79.22 ± 0.68       |
| B14| 0  | 0  | α  | 547.22 ± 2.34     | 86.90 ± 0.98      | 64.96 ± 0.49       |
| B15| 0  | 0  | 0  | 612.98 ± 9.39     | 86.63 ± 0.47      | 64.42 ± 0.45       |
| B16| 0  | 0  | 0  | 598.89 ± 2.34     | 85.63 ± 0.79      | 62.75 ± 0.70       |
| B17| 0  | 0  | 0  | 631.77 ± 9.39     | 86.63 ± 0.47      | 64.42 ± 0.45       |
| B18| 0  | 0  | 0  | 603.58 ± 2.34     | 85.98 ± 0.89      | 62.38 ± 0.89       |

X1: light intensity, μmol·m⁻²·s⁻¹; X2: total nitrogen concentration, mg·L⁻¹; X3: inoculum density, g·L⁻¹.
and medium inoculum density. The designed point was predicted at light intensity of 238 μmol·m⁻²·s⁻¹ and initial concentration of 0.3 g·L⁻¹. According to the analysis of variance shown in Table 4, inoculum density was one of the key factors that affected the CFR. When light intensity was constant, increasing inoculum density increased CFR to some extent. The result was in consistency with several researches [22,23,24]. For instance, Lau et al. [22] found that the efficiency of reducing wastewater-borne nutrients by an algal system was directly related to the physiological activity and growth of the Chlorella cells which in turn were affected by the inoculum density. It is expected that the higher the algal density, the better the growth and the higher the nutrient removal efficiency. However, high algal density would lead to self-shading, an accumulation of auto-inhibitors, and a reduction in photosynthetic efficiency [23,24]. Therefore, the inoculum density was corresponding to light intensity for optimum CFR. Excessive light may cause light saturation, while excessive inoculum density may increase the cell density, leading to high CFR.

Fig. 3. Interaction of light intensity and inoculum density on CFR.

3.4. Interactions between TN and inoculum density

The interactions between TN and inoculum density on CFR when light intensity was constant at the center point were shown in Fig. 4. The contour lines show that either increasing inoculum density or TN increased CFR. The designed point was predicted at TN of 152 mg·L⁻¹, inoculum density of 0.3 g·L⁻¹, under which, the predicted CFR is 639.64 mg·L⁻¹·d⁻¹. The predicted condition was validated and the measured CFR, TN and TP removing rate, and lipid content were 638.13 ± 11.7 mg·L⁻¹·d⁻¹, 88.16 ± 0.98%, 73.98 ± 0.56% and 11.9 ± 0.68%, respectively. The prediction was close to validation experimental results, indicating that the model can be used to guide and optimize the CO₂ fixation of S. dimorphus.

The validated experiments were continued with a nitrogen starvation strategy to increase the lipid content. The maximum lipid content of 24.2 ± 0.13% was achieved by replacing 80% of the culture solution with tap water, followed by 18.2 ± 0.60%, 17.7 ± 0.15% and 14.3 ± 0.34% by replacing 60%, 40% and 20% of the culture solution, respectively. Nutrient (especially nitrogen) starvation has so far been the most commonly employed approach for directing metabolic fluxes to lipid biosynthesis of microalgae. In this scenario, microalgae accumulate lipids as a means of storage under nutrient limitation when energy source (i.e., light) and carbon source (i.e., CO₂) are abundantly available and when the cellular mechanisms for the photo biosynthesis are active [25]. By replacing the culture solution with tap water, both nitrogen concentration and algal density in the medium decreased, which increased the light penetration rate. The stress estimated as follow: light intensity of 238 μmol·m⁻²·s⁻¹, TN of 152 mg·L⁻¹, inoculum density of 0.3 g·L⁻¹, under which, the predicted CFR is 639.64 mg·L⁻¹·d⁻¹. The predicted condition was validated and the measured CFR, TN and TP removing rate, and lipid content were 638.13 ± 11.7 mg·L⁻¹·d⁻¹, 88.16 ± 0.98%, 73.98 ± 0.56% and 11.9 ± 0.68%, respectively. The prediction was close to validation experimental results, indicating that the model can be used to guide and optimize the CO₂ fixation of S. dimorphus.

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culture condition of saturate light, sufficient carbon content and deficient nitrogen doubled the lipid content.

4. Conclusions

To increase the C/N/P removal from flue gas and manure wastewater, Plackett–Burman design and central composite design were applied to optimize the culture conditions for S. dimorphus. The optimized light intensity, TN and inoculum density were identified as 238 μmol·m⁻²·s⁻¹, 152 mg·L⁻¹, and 0.3 g·L⁻¹, respectively. Operated under the optimized conditions, the highest CFR was improved to 638.13 mg·L⁻¹·d⁻¹, and TN and TP removing rate were 88.16% and 73.98%. A 5-d nitrogen starvation strategy with 80% culture medium replacement by tap water enhanced the lipid content from 11.9% to 24.2%.

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