Rapid Optimization of Chlorella protothecoides Biomass Production from Low-Cost Biowaste Under Mixotrophic Conditions

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Article

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Abstract

Mixotrophic cultivation of *Chlorella protothecoides* was carried out in this study with an artificial light source. The culturing medium was supplemented with crude glycerol by-product from a biodiesel facility as an organic carbon source, and the spent yeast from a local brewery was used as an organic nitrogen source. Experiments were performed based on a 3×3 factorial design, with algal biomass (g/L) as the response. Data was fitted into a response surface model to investigate the impact on biomass of 3 factors: light intensity, carbon concentration and nitrogen concentration. According to the model, a maximal biomass of 20.94 g/L could be reached at a light intensity of 100 μmol $m^{-2}s^{-1}$, carbon concentration of 24.1 g/L and nitrogen concentration of 0.7 g/L.

Introduction

The human society has been utilizing microalgae since ancient times (Spolaore et al., 2006). Yet mass cultivation of microalgae in the industrial scale only began after World War II (Spolaore et al., 2006). Several algae species are currently mass produced for various purposes, such as food, nutritional supplements, valuable chemicals, etc. (Pulz and Gross, 2004; Spolaore et al., 2006). With the idea of sustainability gaining popularity among modern societies, substantial efforts have been devoted to the development of biofuels, especially microalgae based biofuels (Chisti, 2007; Chen and Walker, 2011; Huang et al., 2010; Xu, Miao and Wu, 2006). Algal species are fast growing and have the ability to rapidly capture carbon and store this within it's biomass until used for energy or food in potentially carbon neutral (e.g. renewable energy) or carbon negative (e.g. biochar) forms. While great challenges are encountered to lower the cost of microalgae-based products, biodiesel in particular, diversifying the product line to overcome this issue has received greater focus. For instance, Campenni et al. (2013) cultivated *Chlorella protothecoides* to produce both carotenoids and lipids. Others are incorporating waste-water treatment with microalgae cultivation, as was accomplished by the Offshore Membrane Enclosures for Growing Algae (OMEGA) project (Wiley, 2013).

Chlorella protothecoides has attracted considerable attention in the research field for its capability of accumulating lipids under certain metabolic conditions, which could reach a lipid content as high as 55.2 wt% of the algal biomass (Miao and Wu, 2004). While *Chlorella protothecoides* can grow in autotrophic, heterotrophic mode, or mixotrophic modes, researchers tend to prefer heterotrophic or mixotrophic cultivation because organic carbon or nitrogen sources that are added to the medium significantly enhance the algae biomass production when compared to the autotrophic growth that relies solely on carbon dioxide as the carbon source (Miao and Wu, 2004; Xu, Miao and Wu, 2006; Chen and Walker, 2011).

Recently mixotrophic growth, where both light and organic carbon source are provided to the microalgal cells, has been extensively investigated for various algae strains; some algae strains achieved higher biomass accumulation in mixotrophic mode than purely heterotrophic growth (Heredia-Arroyo et al., 2010; Liang, Sarkany and Cui, 2009; Mitra et al., 2012).

For mass cultivation of any microalgae to provide feedstock for biofuels, the high cost of algal biomass has been a problem, and a constant target among scientists and engineers. Therefore, development of a process that can maximize the algal biomass yield while keeping the cost at a minimum is necessary. One solution is to use a carbon and/or nitrogen source that is of low or no cost to the algae industry. Crude glycerol has been generated by large quantities with the current biodiesel production. Since it is not cost efficient to refine much of this biodiesel by-product to produce pure glycerol, crude glycerol could serve as a substitute carbon source for more expensive glucose, thus converting a waste into a potentially valuable resource, which has been demonstrated by previous researches (Chen and Walker, 2011; Feng et al., 2014). Similarly, finding a cheaper alternative for the yeast extract typically used as the nitrogen source in heterotrophic cultivation is desired. Spent yeast from brewery waste could serve this purpose. During the beer production process, spent yeast is generated as a by-product after the fermentation is complete, which may make up 1.5-3% of the total volume of the beer produced (Fillaudeau et al., 2006). Common recycling practices, if any, include selling the spent yeast as animal feed or generating methane through anaerobic digestion (dos Santos Mathias et al., 2014; Mussatto, 2009; Neira and Jeison, 2010). Yet since spent yeast is rich in protein and vitamins, it can also become a suitable nitrogen source in heterotrophic or mixotrophic algae cultivation after appropriate pretreatments (dos Santos Mathias et al., 2014).

To maximize the biomass yield, culturing conditions should be optimized. Traditional optimization methods, no matter for the best growing conditions of algae or for the optimal trans-esterification parameters, tend to tackle one single factor at a time, while keeping all other factors constant. However, this one-factor approach does not take into account the interactions among different factors by splitting individual factors (Mopkar Anand, Sankar and Daniel, 2013). Ward and Rehmann (2019) used response surface methodology (RSM) to optimize media components for *C. vulgaris* biomass and lipid production. In this study, RSM was incorporated to obtain the best combination of the following 3 factors for *C. protethecoides*: light intensity, carbon concentration and nitrogen concentration in the medium. Also the feasibility of incorporating both crude glycerol and spent yeast in the culturing medium was explored.

Materials & Methods

Materials and chemicals. All chemicals in this project were obtained from commercial sources and of analytical grade. Crude glycerol was obtained from Clemson University Sustainable Biodiesel Lab (Clemson, SC). The spent yeast was kindly provided by Thomas Creek Brewery (Greenville, SC).

Microorganism and culture maintenance. *Chlorella protothecoides* UTEX 256 was originally purchased from the algae collection at the University of Texas at Austin (Austin, TX), and maintained on 1.5% agar plates of proteose medium under ambient light. Single algal colonies are streaked onto fresh agar plates on a regular basis. The components of proteose medium (per liter) are as follows: 1g proteose peptone, 0.25g $NaNO_3$, 0.025g $CaCl_2 \cdot 2H_2O$, 0.075g $MgSO_4 \cdot 7H_2O$, 0.075g K_2HPO_4, 0.175g KH_2PO_4, 0.025g NaCl. Sterile operations were practiced in culture maintenance, inoculum preparation and batch cultivation.

Inoculum preparation. Seed culture or inoculum was prepared by picking single colonies from agar plates, which were then used to inoculate 200 ml of basal medium contained in 500 ml shake flasks. The basal medium was supplemented with 30 g/L of pure glycerol (Fisher Scientific) and 4 g/L of yeast extract (Alfa Aesar). The components of basal medium (per liter) are as follows: 0.7g KH_2PO_4, 0.3g K_2HPO_4, 0.3g $MgSO_4 \cdot 7H_2O$, 25mg $CaCl_2 \cdot 2H_2O$, 25mg NaCl, 3mg $FeSO_4 \cdot 7H_2O$, 0.01 mg vitamin B_1, and 1ml of A5 solution. The seed culture was grown mixotrophically for 8 days on a shaking incubator under the same light intensities as the culture inoculated by it. The incubator was set at 200 rpm and kept in a dark room with constant temperature of 28ºC, and the only light source was a LED light with adjustable light intensities. A light regime of 12-hour light, 12-hour darkness was used.

Mixotrophic batch growth of C. protothecoides. The batch culture was carried out in 500 ml shake flasks. 10 ml of seed culture was inoculated into 190 ml of basal medium supplemented with partially refined crude glycerol and treated spent yeast to achieve an inoculation ratio of 1/20. Then the culture was grown under the same light intensity and light regime as the seed culture. The temperature was maintained at 28 ºC and the shaking incubator was set at 200 rpm. A 5 ml sample was drawn on a daily basis. Samples were centrifuged at 3000 rpm for 15 min, washed with distilled water, and freeze-dried overnight for dry weight biomass (g/L) measurement.

Pretreatment of crude glycerol and spent yeast. The crude glycerol was obtained from Clemson University Sustainable Biodiesel Lab as a by-product of biodiesel production. Biodiesel was produced through the alkali-catalyzed trans-esterification process, in which waste-cooking oil was catalyzed by KOH to react with excessive amount of methanol. Therefore the by-product glycerol would usually have a pH range of about 9–10, and contain methanol as impurity.

To pretreat the crude glycerol, 12N of hydrochloric acid was added to adjust the pH to around 7.0. Then the glycerol was centrifuged at 3000 rpm for 15 min and 3 layers were formed that respectively corresponded to biodiesel, glycerol and soap from top to bottom. The top two layers that formed the supernatant were transferred to a separatory funnel, and glycerol was then collected from the bottom once the two layers were formed again. Finally, the collected glycerol was heated to 65ºC with stirring in the fume hood so remaining methanol was evaporated. The pretreated glycerol was autoclaved and refrigerated. A Shimadzu HPLC system and a Shimadzu TOC-V / TMN-1 system were used to analyze the glycerol concentration and total organic carbon / total nitrogen (TOC / TN) in the stock.

Spent yeast was collected at the Thomas Creek Brewery (Greenville, SC). Sodium chloride was added to the slurry of spent yeast to achieve a final concentration of 2% (W/V) (Sugimoto, Takeuchi and Yokotsuka, 1976). The slurry was heated with stirring for 72 hours while the temperature was maintained at 40ºC. The mixture was then centrifuged at 3000 rpm for 30 min and the supernatant was collected, autoclaved and refrigerated as the pretreated spent yeast stock. A Shimadzu TOC-V / TMN-1 system was used to analyze the total organic carbon/total nitrogen (TOC/TN) of the stock.

Experiment design and data analysis. Using a full factorial design as shown in Table 1 and Table 2, we would study the effects of light intensity (L), carbon concentration (C) and nitrogen concentration (N) on biomass production. The biomass data is collected as the response, then fitted to a second-order polynomial model

Table 1
Coded and uncoded levels of variables used in the RSM design.

Variables	**Coded-variables levels (x_j)**			**Δj**
	-1	**0**	**1**	
Light(μmol m^{-2} s^{-1})	100	200	300	100
Carbon (g/L)	14	28	42	14
Nitrogen (g/L)	0.7	1.4	2.1	0.7

Table 2
Factorial design of experiment runs. (n = 3)

Coded levels of factors			
Treatment	light(X_1)	carbon(X_2)	nitrogen(X_3)
1	0	1	1
2	-1	1	-1
3	1	0	0
4	-1	0	1
5	1	-1	-1
6	1	-1	1
7	1	-1	0
8	-1	1	1
9	1	0	-1
10	0	1	0
11	-1	-1	-1
12	0	-1	0
13	-1	0	-1
14	0	1	-1
15	0	0	1
16	-1	1	0
17	1	1	0
18	-1	0	0
19	1	1	-1
20	0	0	-1
21	1	0	1
22	-1	-1	0
23	0	-1	1
24	0	-1	-1
25	0	0	0

Coded levels of factors			
26	1	1	1
27	-1	-1	1

$$Y = \epsilon + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

.

where Y is the predicted response, i.e. the algal biomass (g/L), and X_1, X_2 and X_3 are the coded values of 3 factors, light intensity, carbon and nitrogen concentrations, respectively. A response surface was then generated with its contour plots to find out the factor combination that yields the optimal response. All data were subjected to the least squares technique using the software package SAS JMP11.

Results & Discussion

In this study, all experiments were based on a 3×3 full factorial design to investigate the individual impact and interaction of light intensity, carbon concentration and nitrogen concentration on the biomass yield of *Chlorella protothecoides*. The coded levels and corresponding actual values are explained in Table 1. Each factor was designed with 3 levels, coded as -1, 0, 1, for the low, medium and high respectively. In total 27 experiment runs were performed, with each run being represented by the average of 3 replicates. The response of the predicted model was the algal biomass (g/L). After fitting the data using a response surface method, the following model was obtained:

$$Y = 13.01 - 3.15X_1 - 4.31X_2 - 1.06X_3 - 1.84X_1X_2 + 1.44X_1X_3 - 5.02X_2^2 + 1.40X_3^2$$

Where Y was the predicted response, i.e. the algal biomass (g/L) and X_1, X_2, X_3 were light intensity (μmole m^{-2} s^{-1}), carbon concentration (g/L), nitrogen concentration (g/L) respectively. The fitness of the model was examined by the analysis of variance (ANOVA) as shown in Table 3. The model had an F ratio of 36.97, which was quite significant and indicated a good fitness of the model. Among all terms in the model equation, only 2 were insignificant with a p-value larger than 0.05. They were the quadratic term of light intensity (X_1^2), and the interaction between carbon and nitrogen concentrations (X_2X_3).

Table 3
Analysis of variance for the RSM model.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	2294.0212	254.891	36.9677	< 0.0001
X_1	1	557.2866	557.287	80.8250	< 0.0001
X_2	1	1107.9630	1107.963	160.6913	< 0.0001
X_3	1	60.2281	60.228	8.7351	0.0043
X_1*X_2	1	122.5639	122.564	17.7758	< 0.0001
X_1*X_3	1	74.5920	74.592	10.8183	0.0016
X_2*X_3	1	3.2882	3.288	0.4769	0.4922
X_1*X_1	1	3.9960	3.996	0.5796	0.4491
X_2*X_2	1	443.1800	443.180	64.2758	< 0.0001
X_3*X_3	1	31.9961	31.996	4.6405	0.0348
Error	68	468.8586	6.895		
Total	77	2762.8798			

However, the lack of fit of this model is quite significant, with an F ratio of 47.23. Therefore, more interaction terms were added to the model, as shown in Table 4. After 10 of these interaction terms were added, the lack of fit was rendered insignificant, as shown in Table 5, the ANOVA table of the modified model. Compared to the regular RSM model, the modified model also had a higher R-square at 0.99, while it was only 0.83 in the first RSM model with fewer interaction terms. This could also be demonstrated by the plot of the actual response against the predicted response. As shown in Fig. 1, the predicted responses generated by the modified model were more closely correlated with the actual responses, in comparison with responses generated by the regular RSM model.

Table 4
Estimates of parameters in the modified model.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	13.006353	0.219352	59.29	< 0.0001*
X_1	0.4866667	0.30462	1.60	0.1153
X_2	-4.069917	0.13623	-29.88	< 0.0001*
X_3	-1.57	0.175873	-8.93	< 0.0001*
X_1*X_1	0.4937111	0.175873	2.81	0.0067*
X_1*X_2	-0.768762	0.215399	-3.57	0.0007*
X_2*X_2	-5.023379	0.175873	-28.56	< 0.0001*
X_1*X_3	3.3916667	0.215399	15.75	< 0.0001*
X_2*X_3	1.52	0.215399	7.06	< 0.0001*
X_3*X_3	1.3978698	0.175873	7.95	< 0.0001*
$X_1*X_1*X_3$	2.915	0.278079	10.48	< 0.0001*
$X_2*X_2*X_1$	-3.577905	0.373082	-9.59	< 0.0001*
$X_1*X_3*X_3$	-6.038333	0.373082	-16.19	< 0.0001*
$X_1*X_2*X_3$	1.7433333	0.15231	11.45	< 0.0001*
$X_1* X_1*X_2*X_3$	-1.826667	0.263809	-6.92	< 0.0001*
$X_1*X_2* X_2*X_3$	-2.928333	0.263809	-11.10	< 0.0001*
$X_1*X_2*X_3*X_3$	-1.614571	0.263809	-6.12	< 0.0001*
$X_1* X_1*X_2*X_2*X_3$	-3.245	0.263809	-12.30	< 0.0001*
$X_1*X_2* X_2*X_3*X_3$	6.2529048	0.45693	13.68	< 0.0001*
$X_1* X_1*X_2*X_3*X_3$	-0.573416	0.204345	-2.81	0.0067*

Table 5
Analysis of variance of the modified model.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	19	2734.956533	143.945081	258.5402	< 0.0001
Lack Of Fit	7	4.416872	0.630982	1.1532	0.3448
Pure Error	54	29.545543	0.54714		
Total Error	61	33.962416			
C. Total	80	2768.918949			

Response surface methodology has been widely used in microalgae researches. Chen and Wang (2013) used RSM design to optimize the concentrations of glucose, $NaNO_3$, and $MgSO_4 \cdot 7H_2O$ in the culturing medium of *Chlorella zofingiensis* (Chen and Wang, 2013). Muge I et al. (2012) applied RSM design to optimize glucose, glycerol and peptone in the *Chlorella saccharophila* cultures for biomass and lipid production (Isleten-Hosoglu et al., 2012). Medium ingredients could be conveniently manipulated through the RSM design. Meanwhile, in this research, a full factorial design was used instead of the response surface method design that would have fewer experiment trials. This was due to the fact that only one LED lamp is available to the researchers, thus only one light intensity level could be applied during one single batch. Therefore the light intensity would become a blocking factor. And more information could be obtained by using a full factorial design than a RSM design that only involved a fraction of the former one.

When all data were collected after the experiment, a regular RSM model was fitted to the data at first, resulting in a significant lack of fit. To eliminate the lack of fit, all 26 possible terms of $X_1^a * X_2^b * X_3^c$ (a, b, c are integers that can only be 0, 1, 2) were added to the model. Then insignificant terms were removed to generate a less complicated model while the lack of fit was kept insignificant. Finally a model with 19 terms was obtained, including those 9 terms as in a regular RSM model. The X_1 linear term, though insignificant, was still kept in the model because the current range of light intensity might not be high enough to trigger the photoinhibition.

Conclusion

Chlorella protothecoides was cultivated mixotrophically in this study. To investigate the impact of light intensity, carbon and nitrogen on algal biomass yield, a full factorial design was used and data was analyzed through response surface methodology. The obtained model, which was modified from a regular RSM model, had better fit with the actual data and therefore could better predict the response.

Declarations

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Additional Information

Availability of Data and Materials: The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Figures

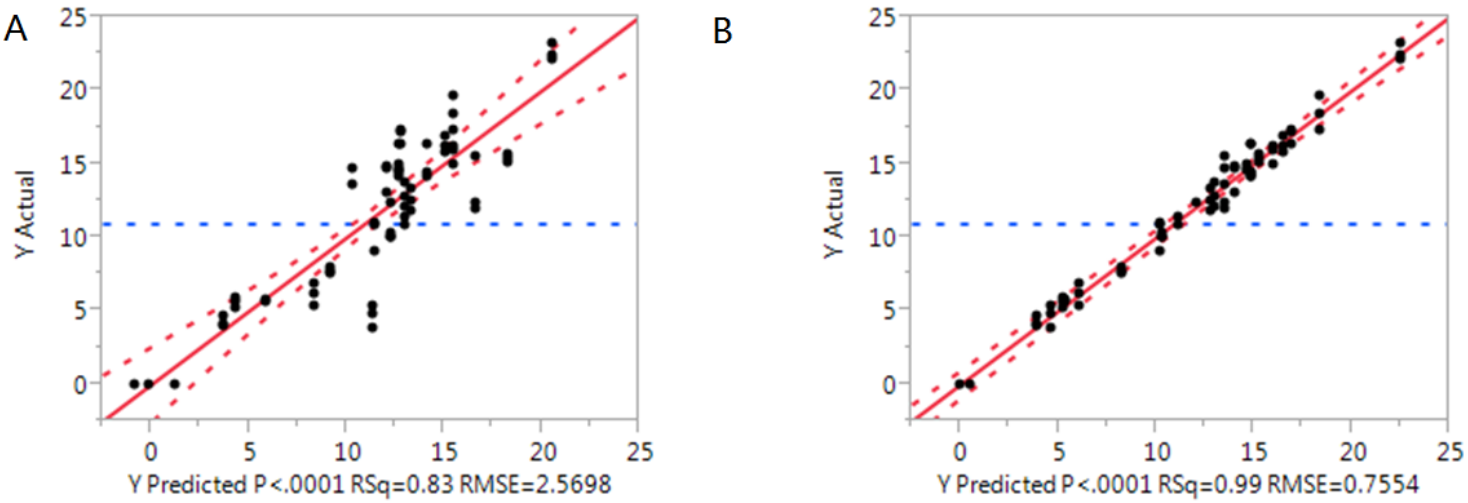

Figure 1

Plot of the actual response against the predicted response