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Optimization of Simultaneous Biomass Production and Nutrient Removal by Mixotrophic *Chodatella* sp. Using Box-Behnken Experimental Design

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Abstract. The effects of aeration rate, piggery wastewater content, cultivation temperature, and light intensity on the growth rate, lipid content, and nitrogen and phosphorus removal rates are investigated using a Box-Behnken experimental design under full factorial methodology. The aeration rate is the most important factor influencing cell growth and nutrient removal. However, the lipid content is mainly controlled by the piggery wastewater content. The mixotrophic cultivation simultaneously assimilated up to 95.6% ammonia nitrogen, 74.2% total phosphorus, and 80.9% organic carbon from piggery wastewater, which reduced the required nutrient for the culture of microalge, therefore produced biodiesel practically and economically.

Keywords: Microalgae, biomass, wastewater, Box-Behnken experimental design.

1. Introduction

Many microalgae are exceedingly rich in lipid content, which is converted into biodiesel [1]. One of the methods used for reducing the cost of algal biomass is to utilize wastewater for culturing algae [2]. Some microalgae are able to effectively grow in wastewater conditions through their ability to utilize abundant organic carbon and inorganic nitrogen and phosphorous in the wastewater. For example, Lau *et al*. [3] reported that over 90% of N content and 80% of P content were removed by *Chlorella vulgaris* from primary treated sewage. A similar microalgal strain, *Chlorella pyrenoidosa*, in soybean processing wastewater was demonstrated not only to remove 78% of soluble organic carbon, 89% of total nitrogen (TN), and 70% of total phosphate (TP), but also attained an average biomass productivity of 0.64 g/L.d with an average lipid content of 37% [4]. Sydney *et al*. [5] found that *Botryococcus braunii* is able to remove N and P nutrients (79.63%) from treated domestic wastewater, and accumulate oil with a dry biomass of up to 36%. The lipid profiles of this extracted oil were similar to those of other oilseed feedstocks.

Most microalgae have been isolated from local water and methods for acclimating them to a piggery wastewater environment are limited. Few systematic studies have been conducted on the effects of factors affecting lipid production and nutrient removal. The present study examines the correlations among piggery wastewater content, aeration rate, temperature, and light intensity using a full factorial design method to determine the growth rate, nutrient removal efficiency, and biodiesel production for microalgae grown in piggery wastewater.

2. Materials and Methods

2.1. Microalgal Culture

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One of the dominant green microalgal species, *Chodatella* sp., was isolated from local source water and cultured in a medium according to the method presented by Norris *et al*. [6]. Axenic cultures of *Chodatella* sp. were grown in batch mode in a 5-L modified serum bottle containing 4 L of sterilized algal medium. The bottles were placed in an incubator maintained at 20-30 ℃, continuously provided with 3-7 Klux of illumination, and were continuously stirred with air aeration. The piggery wastewater was filtered through a 0.45-μm membrane and sterilized before experiments. Cultures were harvested in the log growth phase for experiments.

2.2. Statistical Analysis and Experimental Design Analysis

A Box-Behnken experimental design was used to optimize the conditions for lipid production using piggery wastewater as the substrate [7]. The experimental design consists of four factors, namely piggery wastewater content (X1), aeration rate (X2), temperature (X3), and light intensity (X4). Three levels for each variable were included. This experimental design was also used to determine the joint effects of several factors on a response and the mutual interactions between them. The complete design consisted of 27 runs (Table 1), which were performed in duplicate to optimize the levels of selected variables. Data processing and calculations were carried out using a commercial statistical package, STATISTICA 9.0 (Statsoft, USA), to estimate the coefficients of the regression equation. The equations were validated using analysis of variance to determine the significance of each term in the fitted equations and to estimate the goodness of fit in each case.

2.3. Analytical Methods

2.3.1. Biomass concentration and growth rate

The dry weight of the microalgal biomass was determined gravimetrically. A known volume of microalgal culture was collected and dried at 90 °C for 3 hours. The growth rate (μ) was calculated according to the equation $\mu = (\ln A1 - \ln A0) / (T1 - T0)$, where A1 and A0 are the dry weights of the microalgal biomass at times T1 and T0, respectively.

2.3.2. Lipid content

A stock culture of *Chodatella* sp. cells was collected by centrifugation at 5000 × g for 5 min. The precipitated algal cells were then washed and resuspended in deionized water in triplicate. Cells were collected by centrifugation again and then dried by a freeze dryer. The microalgal total lipids were extracted with n-hexane/methanol (2/1, v/v) in a Soxhlet extractor and quantified gravimetrically. The biomass concentration (mg/L) is expressed as the dry weight of the microalgal biomass.

2.3.3. Nitrogen and phosphorus analysis

The samples were first filtered through a 0.45-μm membrane and then the filtrate was properly diluted and analysed for ammonia nitrogen (NH_3-N), total phosphorous (TP), and nonpurgeable dissolved organic carbon (NPDOC). NH_3-N, TP, and NPDOC concentrations were determined according to Standard Methods [8].

3. Results and Discussion

3.1. Effect of Culture and Optimization

In order to find the best conditions for lipid production using piggery wastewater as a substrate, an experimental Box-Behnken design was used. The coded values of the test variables were piggery wastewater content (X1), aeration rate (X2), temperature (X3), and light intensity (X4). The experimental results of the lipid content of biomass in each case are presented in Table 1. The results show individual effects of the combinations of the test variables, with significant variation between combinations. An analysis of these results shows that high lipid content was obtained for 40% piggery wastewater content, an aeration rate of 40 L/L.h, a temperature of 30 ℃, and a light intensity of 5 Klux (Run 18). The significance of each coefficient for growth rate and lipid content, determined by T-values and P-values, is listed in Table 2 and Table 3, respectively.

The larger the magnitude of the T-value and smaller the P-value, the more significant is the corresponding coefficient. This indicates that the variable with the largest effect on both the growth rate and lipid content was the aeration rate. The interactive effect of piggery wastewater and aeration rate was significant (X1X2, $P<0.05$). Both the aeration rate and piggery wastewater content are highly significant in their quadratic level for the growth rate and lipid content, respectively.

3.2. Effects of Interaction on Biomass Production and Nutrient Removal

Figure 1 shows the interactive effects between piggery wastewater content and the aeration rate on the growth rate and lipid content. Each response surface plot represents a combination of two test variables, with all other variables at zero levels. The growth rate increased with increasing aeration rate in the range of 0 to 40 L/h.L. The maximum growth rate was found at an aeration rate of 40 L/h.L for all piggery wastewater content tested. There is almost no interaction between the aeration rate and piggery wastewater content. This indicates that the aeration rate plays an important role in the growth rate with piggery wastewater used as a substrate. However, lipid content was controlled mainly by piggery wastewater content, rather than the aeration rate. The lipid content increased slightly with increasing aeration rate and increased with increasing piggery wastewater content up to 40%.

Figure 3 compare the effect of the aeration rate on nitrogen, phosphorous, and dissolved carbon removal. The average nutrient removal efficiency were 47.3% of NH_3-N, 53.9% of TP, and 47.3% of NPDOC when culture was aerated at 20 L/h.L, whereas that with no aeration were 37.5%, 46.5%, and 37.5%, respectively. Under the testing conditions, the nutrient removal efficiency obtained with providing aeration was higher than those obtained with no aeration. It is speculated that aeration increased the mixing of culture, thereby improving the cell growth. This result is also in agreement with the growth rate, which was controlled mainly by the aeration rate.

Table 1: Box-Behnken experimental design matrix for biomass production

Run	**Independent variables**				**Response**	
	X1	**X2**	**X3**	**X4**	**Growth rate (day^{-1})**	**Lipid (%)**
1	20	0	20	3	0.08	15.2
2	20	0	25	7	0.08	15.8
3	20	0	30	5	0.04	15.1
4	20	20	20	7	0.31	11.0
5	20	20	25	5	0.29	12.5
6	20	20	30	3	0.24	15.3
7	20	40	20	5	0.32	14.1
8	20	40	25	3	0.29	16.8
9	20	40	30	7	0.28	17.6
10	40	0	20	7	0.07	12.4
11	40	0	25	5	0.06	12.5
12	40	0	30	3	0.13	19.1
13	40	20	20	5	0.32	19.4
14	40	20	25	3	0.30	20.1
15	40	20	30	7	0.29	19.4
16	40	40	20	3	0.24	19.6
17	40	40	25	7	0.31	19.3
18	40	40	30	5	0.27	23.4
19	60	0	20	5	0.14	6.9
20	60	0	25	3	0.15	6.8
21	60	0	30	7	0.18	7.5
22	60	20	20	3	0.25	6.3
23	60	20	25	7	0.25	7.0
24	60	20	30	5	0.31	19.3
25	60	40	20	7	0.34	10.1
26	60	40	25	5	0.29	19.1
27	60	40	30	3	0.24	19.7

X1: Piggery wastewater content (% vol.), X2: Aeration rate (L/h.L), X3: Temperature (℃), X4: Light intensity (Klux)

Table 2: Analysis of variance for cell growth rate (d^{-1})

Source	Sum of Squares	Df	Mean Square	F	*P*
X1	0.0030	2	0.0015	1.567	0.248
X2	0.1985	2	0.0993	103.277	0.000
X3	0.0005	2	0.0002	0.255	0.779
X4	0.0019	2	0.0009	0.979	0.404
X1X2	0.0075	1	0.0075	7.779	0.016
X1X3	0.0034	1	0.0034	3.521	0.085
X1X4	0.0001	1	0.0001	0.112	0.744
X2X3	0.0019	1	0.0019	1.979	0.185
X2X4	0.0042	1	0.0042	4.387	0.058
X3X4	0.0004	1	0.0004	0.422	0.528
Total Error	0.0115	12	0.0010		
Total Sum of Squares	0.2314	26			

Table 3: Analysis of variance for lipid content (%)

Source	Sum of Squares	Df	Mean Square	F	*P*
X1	216.68	2	108.34	15.046	0.0005
X2	131.57	2	65.78	9.136	0.0039
X3	96.62	2	48.31	6.709	0.0111
X4	31.84	2	15.92	2.211	0.1523
X1X2	54.43	1	54.43	7.559	0.0176
X1X3	18.51	1	18.51	2.570	0.1349
X1X4	0.71	1	0.71	0.098	0.7597
X2X3	6.39	1	6.39	0.887	0.3648
X2X4	0.01	1	0.01	0.001	0.9786
X3X4	1.64	1	1.64	0.228	0.6419
Total Error	86.41	12	7.20		
Total Sum of Squares	646.16	26			

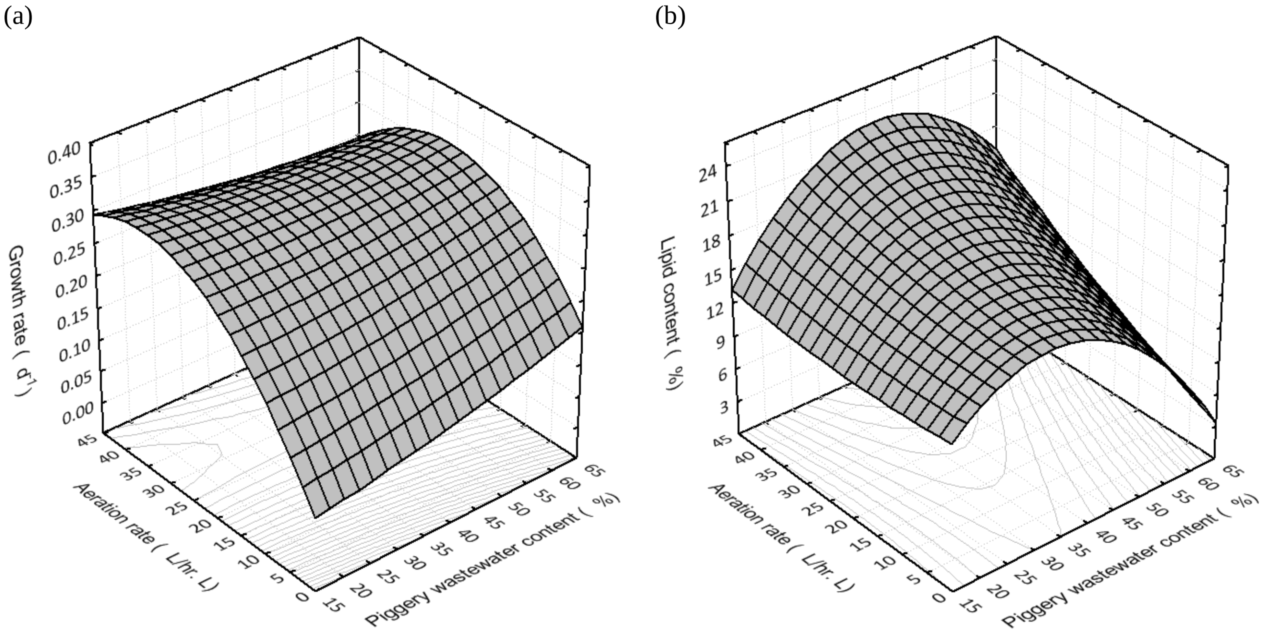

Fig. 1: Response surface plots of (A) growth rate, (B) lipid content as functions of piggery wastewater content and aeration rate.

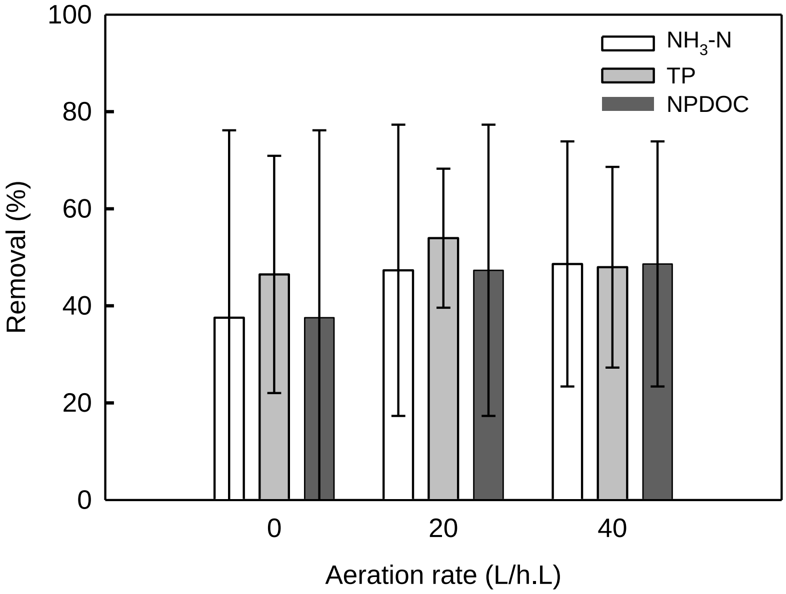

Fig. 2: Effect of aeration rate on NH_3-N, TP, and NPDOC removal efficiency

4. Conclusions

Piggery wastewater was favourable for the growth rate of microalgal strain *Chodatella* sp. Ammonium, total phosphorous and, organic carbon in piggery wastewater were removed. For four factors including piggery wastewater content, aeration rate, temperature, and light intensity, the aeration rate has the largest effect on cell growth. However, lipid content was controlled mainly by piggery wastewater content. The average nutrient removal efficiency obtained with aeration was higher than that of no aeration.

5. Acknowledgements

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