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Enhanced Biofuel Production from High-Concentration Bioethanol Wastewater by a Newly Isolated Heterotrophic Microalga, *Chlorella vulgaris* LAM-Q

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Microalgal biofuel production from wastewater has economic and environmental advantages. This article investigates the lipid production from high chemical oxygen demand (COD) bioethanol wastewater without dilution or additional nutrients, using a newly isolated heterotrophic microalga, *Chlorella vulgaris* LAM-Q. To enhance lipid accumulation, the combined effects of important operational parameters were studied *via* response surface methodology. The optimal conditions were found to be temperature of 22.8°C, initial pH of 6.7, and inoculum density of 1.2×10^8 cells/ml. Under these conditions, the lipid productivity reached 195.96 mg/l/d, which was markedly higher than previously reported values in similar systems. According to the fatty acid composition, the obtained lipids were suitable feedstock for biodiesel production. Meanwhile, 61.40% of COD, 51.24% of total nitrogen, and 58.76% of total phosphorus were removed from the bioethanol wastewater during microalgal growth. In addition, 19.17% of the energy contained in the wastewater was transferred to the microalgal biomass in the fermentation process. These findings suggest that *C. vulgaris* LAM-Q can efficiently produce lipids from high-concentration bioethanol wastewater, and simultaneously performs wastewater treatment.

Keywords: Microalgae, lipid production, bioethanol wastewater, optimization, energy efficiency

Introduction

Petroleum fuels are considered unsustainable owing to the declining supply of fossil oils and the associated environmental pollution. It is essential to develop eco-friendly renewable energy resources for the current society. In this context, biofuels from microalgae have attracted extensive attention because of their high productivity, low land-area requirement, biodegradability, and capacity to satisfy the existing demands for petroleum fuels. However, economic barriers limit the industrialization of microalgal biofuel technology. For instance, the production of 1 kg of microalgal biodiesel needs to consume approximately 3,726 kg of freshwater, 0.33 kg of nitrogen, and 0.71 kg of phosphate [45]. Therefore, low-cost cultivation techniques are highly needed.

In order to reduce costs, the coupling of microalgal cultivation and wastewater treatment has been proposed as a promising approach for biofuel production. The strategy not only cuts down on nutrient expenses, but also conserves precious water resources. To date, a few researches have been conducted on the integrated system [12, 40]. These studies mainly used municipal wastewater that had a relatively low chemical oxygen demand (COD < 2,500 mg/l). However, energy-intensive and costly pretreatments, such as dilution and extra nutrient addition, were usually required to meet the needs for microalgal growth. Moreover, these reports just focused on the feasibility of

the combined technology, but ignored bioprocess optimization. Hence, it is meaningful to explore microalgal cultivation in high-concentration wastewater without additional nutrients and to seek optimal culture conditions, in view of the practical and economic benefits.

Bioethanol wastewater, the effluent from alcohol manufacturing processes, is rich in organic matter (COD >25,000 mg/l), nitrogen, and phosphorus. Currently, the production of alcohol is increasing rapidly owing to its wide industrial applications, thereby resulting in large amounts of wastewater. For each liter of alcohol produced, 9 to 14 liters of wastewater is generated in a traditional factory [36]. Since the free disposal of bioethanol wastewater into water bodies or soils poses a threat to natural ecosystems and can cause considerable environmental problems, an efficient treatment is required. At present, several treatment methods for bioethanol wastewater have been reported, including both biological and physicochemical methods (anaerobic and aerobic treatments, adsorption, coagulation-flocculation, ozonation, *etc.*) [9]. However, a common feature of these methods is their relatively high operating costs. Although the classical biological treatment could remove most of the COD, it not only requires long start-up periods and hydraulic retention times, but also produces excess sludge [16]. Thus, it is necessary to develop more economical and effective treatment approaches. Considering its abundant nutrients, the use of bioethanol wastewater for microalgal lipid production might be an attractive strategy, which converts wastewater pollutants to useful biomass without additional pollution.

The selection of suitable strains is crucial for wastewater-based microalgal cultivation. According to Zhou *et al.* [46], natural algal species isolated from local habitats could adapt better to the wastewater environments than other species from algae banks. Since microalgae are unlikely to survive in the high-temperature (approx. 90°C) wastewater from alcohol distillation, downstream sewage is supposed to be an ideal source to isolate robust microalgal strains for the bioethanol wastewater system.

In order to maximize the production capacity, the wastewater-to-lipid process needs to be optimized. Several operational parameters have great impacts on microalgal growth and lipid accumulation, such as temperature, pH, and inoculum density. However, the traditional single-factor optimization is time-consuming and even leads to misinterpretation, as the interactions between factors are not considered in such complex biological systems. Response surface methodology (RSM), a collection of mathematical and statistical techniques, takes account of these potential interactions with a minimum number of experimental runs [10]. In addition, the optimal regions of three-dimensional (3D) shapes could provide more valuable guidelines and options for industrial applications, compared with the optimal points obtained by single-factor optimization. In these regards, RSM coupled with Box-Behnken design (BBD) was used to assess the effects of multiple factors for the optimization of the wastewater-based microalgal cultivation system.

In this work, we screened out a heterotrophic oleaginous microalga and optimized its lipid production in bioethanol wastewater medium by RSM. In addition, the fatty acid profile and the efficiencies of wastewater pollutant removal and energy utilization were evaluated.

Materials and Methods

Wastewater

The bioethanol wastewater was provided by Zizhong Hongzhan Industry Co., Ltd. (Sichuan, China), which produces alcohol from cassava. The wastewater was collected at the outlet from the bottom of the distillation column. After centrifugation at 3,400 ×*g* for 5 min and filtration through filter paper, the liquid was autoclaved at 121°C for 15 min and stored at 4°C for later use.

To estimate the wastewater characteristics, various physical and chemical parameters were examined. The pH was measured using a pH meter (pHS-25; Rex, China). The COD, 5-day biological oxygen demand (BOD_5), total solids (TS), and total suspended solids (TSS) were analyzed in accordance with APHA standard methods [2]. Total nitrogen (TN) was quantified by the alkaline potassium persulfate digestion-UV spectrophotometric method [24]. Ammonia nitrogen (AN) was measured by the nesslerization method [2]. Total phosphorus (TP) was digested with potassium persulfate and determined by ammonium molybdate spectrophotometry [2]. Reducing sugars were analyzed using the 3,5-dinitrosalycylic acid (DNS) method [21]. Sulfates and chlorides were detected by ion chromatography (ICS-1500; Dionex, USA), using an IonPac AG11-HC (4 × 50 mm) guard column and an IonPac AS11-HC (4 × 250 mm) analytical column with 25 mmol/l KOH as eluent. All parameters were measured in triplicate and averaged. The results are summarized in Table 1, and compared with those of reported bioethanol wastewater from different raw materials.

Strain Isolation and Identification

Algal samples were collected from the sewage treatment ponds at the bioethanol plant (Zizhong Hongzhan Industry Co., Ltd.). The unialgal strain LAM-Q was screened out by the pour plate method using SE medium [42]. The isolate was identified by phylogenetic analysis of 18S rRNA gene sequences (Supplementary Appendix A).

To detect intracellular lipids, microalgal cells were washed twice with phosphate-buffered saline (PBS), suspended in 20%

Table 1. Characteristics of bioethanol wastewater from different raw materials.

Parameter[a] (mg/l)	Bioethanol wastewater from various sugar sources						
	Cassava[b] (this study)	Corn stalk and corncob [33]	Sugarcane molasses [32]	Rice straw [6]	Beet molasses [20]	Whisky [1]	n.a. [22]
Color	Brown	n.a.	Brown	n.a.	n.a.	n.a.	Deep brown
pH	2.5-3.0	4.3 ± 0.20	3.5 ± 0.5	n.a.	4.97 ± 0.01	3.8	3.0-4.5
COD	27,930 ± 884	32,700 ± 1,120	52,380 ± 55	23,200	57,390 ± 350	16,600-58,000	110,000-190,000
BOD_5	15,870 ± 1,035	n.a.	37,100 ± 110	n.a.	36,400 ± 2,140	8,900-30,000	50,000-60,000
TS	27,778 ± 593	32,300 ± 970	n.a.	n.a.	n.a.	6,312-25,560	110,000-190,000
TSS	1,780 ± 86	n.a.	n.a.	760	4,640 ± 440	232-7,810	13,000-15,000
TN	688.50 ± 26.10	1,500 ± 50	368 ± 10[c]	n.a.	4,004 ± 165	500-1,200[c]	5,000-7,000
AN	263.87 ± 12.53	n.a.	n.a.	28	187 ±15	100-200	n.a.
TP	81.34 ± 2.46	n.a.	1,905 ± 20[d]	n.a.	56 ± 7	150-600	2,500-2,700[d]
Reducing sugars	1,600 ± 100	15,300 ± 650[e]	n.a.	1,310[f]	3,350 ± 71[g]	n.a.	n.a.
Sulfates	196.46 ± 9.08	n.a.	n.a.	n.a.	n.a.	40-130	7,500-9,000
Chlorides	85.60 ± 2.20	n.a.	n.a.	n.a.	n.a.	n.a.	8,000-8,500

COD, chemical oxygen demand; BOD_5, 5-day biological oxygen demand; TS, total solids; TSS, total suspended solids; TN, total nitrogen; AN, ammonia nitrogen; TP, total phosphorus; n.a., data not available.

[a]All values are in mg/l, except color and pH.

[b]Beside color and pH, data are reported as the mean ± standard deviation (n = 3).

[c]Total Kjeldahl nitrogen.

[d]Phosphate.

[e]Oligomeric glucose, xylose, and oligomeric xylose.

[f]Xylose.

[g]Reducing substances.

dimethyl sulfoxide (DMSO), and incubated at 35°C for 20 min. Then, 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile red; Sigma, USA) solution (10 μg/ml in acetone) was added to the cell suspensions (1:100 (v/v)), and followed by a 10-min incubation in the dark at room temperature. Finally, cell fluorescence was observed under a confocal laser scanning microscope (TCS SP5-II; Leica, Germany). Nile red fluorescence of neutral lipids (yellow) was recorded at the 488 nm laser excitation line, and the emission was captured between 560 and 615 nm; chlorophyll autofluorescence (red) was recorded at the 633 nm laser excitation line, and the emission was captured between 633 and 708 nm. Samples were viewed with a 40× oil immersion lens objective. Post-acquisition image handling was treated with Leica microsystems LAS AF software (Version 2.3.1).

Cultivation

The inoculum was prepared in SE medium supplemented with 10 g/l glucose at 30°C and 130 rpm. After centrifugation and washing, microalgal cells in the late exponential phase were inoculated into the wastewater medium.

In the evaluation experiments of microalgal adaptability, the wastewater was diluted with sterile distilled water to five levels (*i.e.*, 20%, 40%, 60%, 80%, and 100% (v/v)). After the pH value was adjusted to 6.5 by the addition of sterile NaOH solution (1 mol/l), the wastewater at different dilution levels was used as the culture medium. The isolate was cultivated in 250 ml Erlenmeyer flasks containing 100 ml of medium, and the flasks were kept on a shaking incubator at 30°C and 130 rpm without light exposure. The inoculum density was 1.2×10^7 cells/ml. The cultures were harvested after reaching the stationary phase. Each experiment was carried out in triplicate and average values are reported.

In the optimization experiments of lipid production, the undiluted bioethanol wastewater was used as the culture medium. The temperature, initial pH, and inoculum density were set according to the experimental design (BBD). Other conditions were the same as described above.

Cell Growth Determination

Algal growth was monitored by optical density (OD) measurement with a UV-Visible spectrophotometer (UV-1800PC; Mapada, China). According to the full-wavelength spectra of the bioethanol wastewater and microalgal suspension (Supplementary Appendix B), the cell concentration in the bioethanol wastewater medium could be determined by the difference in the OD values at 680 nm between wastewater culture broth and blank medium (free of cell) [4, 23]. Samples were diluted by appropriate ratios to ensure that the measured OD values were in the range of 0.2 to 0.8, if applicable. Then, the OD values were converted to microalgal

cell concentration *via* the following calibration curve based on Beer's law.

$$\rho = 28.8979 \times \Delta OD_{680} - 1.8152 \quad (R^2 = 0.9915) \tag{1}$$

where ρ represents the cell density (× 10^6 cells/ml), and ΔOD_{680} is the absorbance of fermentation broth against a blank of wastewater medium supernatant at 680 nm. Finally, the growth curve was plotted with the cell concentration *vs.* culture time.

The biomass was determined gravimetrically as dry weight at the end of culture. Samples were centrifuged at 3,400 ×*g* for 5 min, washed twice with distilled water, and dried at 105°C to constant weight.

Lipid Extraction and Fatty Acid Analysis

Microalgal lipids were extracted with chloroform-methanol solution (1:1 (v/v)) and quantified gravimetrically [3]. Lipid productivity was expressed as milligram net lipids per liter medium per day (mg/l/d) after excluding the amount of initial inoculum.

After methyl esterification, the fatty acid profile was analyzed by gas chromatography and mass spectrometry (GC/MS, QP2010; Shimadzu, Japan), following the conditions described by Liu *et al.* [17].

Experimental Design and Statistical Analysis

Design Expert software (trail Version 8.0.6) was used to design the optimization experiments and conduct data processing. On the basis of the single-factor experiment results (data not shown), the key factors were decided and prescribed into three levels (Supplementary Appendix C). The BBD matrix (Table 2) consisted of 12 different level combinations and 5 center point runs. Experiments were randomized in order to minimize the effects of unexplained variability in the observed response due to extraneous factors. The experimental data were fitted to the following quadratic polynomial model.

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i,j=1}^{3} \alpha_{ij} X_i X_j + \varepsilon \tag{2}$$

where Y represents the predicted response; α_0 is the model constant; α_i, α_{ii}, and α_{ij} are the linear, quadratic, and interaction coefficients, respectively; X_i and X_j are the coded variables; and ε is the statistical error. Statistical analysis of the model was performed in the form of analysis of variance (ANOVA).

Energy Efficiency Calculation

The biomass energetic yield (η) denotes the ratio of the heat produced by complete oxidation of biomass to that of consumed organic substrates in a fermentation process. According to Eroshin and Krylova [8], the biomass energetic yield (%) was calculated using Eq. (3).

$$\eta = \gamma_b \sigma_b B / \gamma_S \sigma_S S \times 100 \tag{3}$$

where γ_b and γ_s are the reductance degrees of biomass and

Table 2. Box-Behnken design arrangement and responses.

Run	Factors			Lipid productivity (mg/l/d)	
	Temperature[a] (coded)	Initial pH (coded)	Inoculum density[b] (coded)	Experimental[c]	Predicted
1	28 (0)	7 (0)	6.6 (0)	157.07 ± 7.85	153.52
2	28 (0)	7 (0)	6.6 (0)	146.17 ± 5.31	153.52
3	28 (0)	9 (+1)	1.2 (−1)	64.74 ± 3.07	66.05
4	28 (0)	5 (−1)	1.2 (−1)	71.19 ± 5.21	70.10
5	28 (0)	9 (+1)	12 (+1)	113.85 ± 6.69	114.89
6	36 (+1)	7 (0)	12 (+1)	87.62 ± 4.38	90.89
7	20 (−1)	9 (+1)	6.6 (0)	87.50 ± 7.74	89.46
8	36 (+1)	9 (+1)	6.6 (0)	31.22 ± 1.98	26.87
9	28 (0)	7 (0)	6.6 (0)	154.21 ± 7.71	153.52
10	28 (0)	7 (0)	6.6 (0)	155.48 ± 10.54	153.52
11	36 (+1)	5 (−1)	6.6 (0)	42.25 ± 2.22	40.29
12	36 (+1)	7 (0)	1.2 (−1)	56.00 ± 4.86	59.06
13	20 (−1)	5 (−1)	6.6 (0)	106.48 ± 5.32	110.84
14	28 (0)	7 (0)	6.6 (0)	154.64 ± 6.73	153.52
15	20 (−1)	7 (0)	1.2 (−1)	98.47 ± 4.84	95.20
16	20 (0)	5 (−1)	12 (+1)	147.00 ± 6.35	145.70
17	20 (−1)	7 (0)	12 (+1)	190.91 ± 8.57	187.87

[a]Values are in °C.

[b]Values are in 10^7 cells/ml.

[c]Data are reported as the mean ± standard deviation (n = 3).

substrates, respectively; σ_b and σ_s are the mass fractions of carbon in biomass and substrates (%), respectively; and B and S are the concentrations of produced biomass and consumed substrates (g/l), respectively. In particular, reductance degree represents the amount of available electrons in organics. Hence, biomass energetic yield means the ratio of the available electron concentration in the biomass to that in consumed substrates.

According to Zhou *et al.* [47], the relationship between $\gamma_b\sigma_b$ and microbial lipid content (C_L, %) is as follows:

$$\gamma_b\sigma_b = 0.0255 \times C_L + 1.6653 \quad (4)$$

Thus, the molar concentration of available electrons in the biomass (m_b, mol/l) could be obtained by Eq. (5).

$$m_b = (0.0255 \times C_L + 1.6653) \times B/12 \quad (5)$$

Based on the definition of COD, the available electrons in wastewater are equivalent to the obtained electrons by oxygen in the oxidation reaction. Therefore, the molar concentration of available electrons in wastewater substrates (m_s, mol/l) could be calculated by Eq. (6).

$$m_s = C_{COD}/8000 \quad (6)$$

where C_{COD} is the consumed COD in wastewater substrates (mg/l).

Combining Eqs. (3), (5), and (6), the biomass energetic yield of organic wastewater fermentation was determined by Eq. (7).

$$\eta = \frac{(0.0255 \times C_L + 1.6653) \times B/12}{C_{COD}/8000} \times 100 \quad (7)$$

Results and Discussion

Evaluation of the Isolated Microalga

The strain LAM-Q was isolated from the local sewage treatment site and successfully cultivated in pure culture. Under the microscope, the microalgal cells were unicellular, spherical in shape, and about 5 μm in size. Moreover, clear intracellular lipid droplets (yellow fluorescence) were observed by Nile red staining (Figs. 1A-1D). On agar-solidified SE medium supplemented with 10 g/l glucose, the microalgal cells were grown into green, circular, and convex colonies with entire margins (Fig. 1E). In order to clarify the taxonomic position of the isolate, a 685 bp fragment was amplified by a pair of primers for the 18S rRNA gene, and the obtained sequence was submitted to GenBank (Accession No. KC253000). On the basis of BLAST searches, the sequence of strain LAM-Q exhibited over 99% homology to *Chlorella vulgaris*. Furthermore, the neighbor-joining phylogenetic tree confirmed the reliability of the branching order based on bootstrap analysis (Supplementary Appendix A). Hence, the isolated oleaginous microalga was identified as *C. vulgaris* LAM-Q.

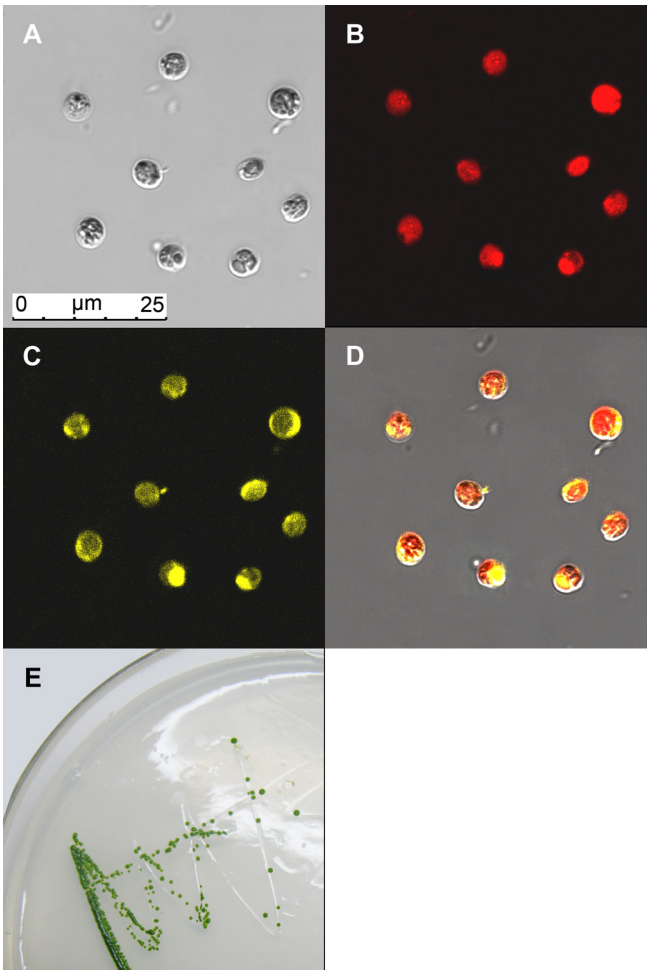

Fig. 1. Morphological characteristics of strain LAM-Q.
Confocal microscopy images of Nile red-stained cells: (**A**) differential interference contrast, (**B**) chlorophyll autofluorescence, (**C**) Nile red fluorescence of neutral lipids, and (**D**) all three images merged. (**E**) colonies grown on agar-solidified SE medium supplemented with 10 g/l glucose.

Chlorella is capable of producing lipids under three typical trophic regimes: autotrophy, heterotrophy, and mixotrophy [29]. Organic substrate supply allows microalgae to produce large amounts of biomass and lipids in a relatively short time through heterotrophic metabolism. Our preliminary experiments showed that the organic bioethanol wastewater could support the heterotrophic and mixotrophic growth of *C. vulgaris* LAM-Q. Nevertheless, light had no significant effect on microalgal biomass in the wastewater medium (data not shown). Two main reasons were supposed to explain the observation. First, the

Table 3. Effect of the bioethanol wastewater on microalgal biomass and lipid production, as well as wastewater pollutant removal.[a]

Proportion of wastewater (%, v/v)	Biomass yield (g/l)	Lipid content (%)	Lipid yield (g/l)	COD removal amount (mg/l)	COD degradation ratio (%)	TN removal amount (mg/l)	TN degradation ratio (%)	TP removal amount (mg/l)	TP degradation ratio (%)
20	1.13 ± 0.10	9.95 ± 0.48	11.24 ± 1.15	1,766 ± 117	31.62 ± 2.09	102.43 ± 1.65	74.38 ± 1.20	13.00 ± 0.19	79.94 ± 1.15
40	1.37 ± 0.14	14.64 ± 0.98	20.06 ± 2.42	4,986 ± 182	44.63 ± 1.63	156.03 ± 5.38	56.65 ± 1.95	17.05 ± 0.86	52.39 ± 2.65
60	1.93 ± 0.16	18.55 ± 1.22	35.80 ± 3.82	9,769 ± 286	58.30 ± 1.71	230.21 ± 8.07	55.73 ± 1.95	25.85 ± 1.17	52.96 ± 2.40
80	2.68 ± 0.22	16.63 ± 0.80	44.57 ± 4.29	12,643 ± 394	56.58 ± 1.76	301.50 ± 8.67	54.74 ± 1.57	34.22 ± 1.51	52.58 ± 2.32
100	3.41 ± 0.22	16.27 ± 0.99	55.48 ± 4.96	15,602 ± 535	55.86 ± 1.92	375.83 ± 10.74	54.59 ± 1.56	43.39 ± 1.73	53.35 ± 2.12

[a]Data are reported as the mean ± standard deviation (n = 3).

opaqueness of the wastewater caused by the strong color, would impair the photosynthetic activity of microalgae [38]. Second, there is mutual inhibition between organic carbon assimilation and photosynthesis metabolism in mixotrophic culture [15, 25]. Therefore, the wastewater-based microalgal cultivation was investigated under heterotrophic condition in this study.

In order to evaluate the adaptability of the isolate to the bioethanol wastewater, *C. vulgaris* LAM-Q was cultivated in varying wastewater dilutions (Table 3). The undiluted wastewater promoted the highest biomass production. With decreasing proportion of wastewater, the biomass accumulation was severely reduced. The biomass yield at 20% wastewater was less than a third of that at 100% wastewater. Moreover, the lipid content reached the highest at 60% wastewater, and thereafter decreased slightly with a further increase in proportion of wastewater. Considering both biomass yield and lipid content, the maximum lipid yield was obtained at 100% wastewater. Furthermore, microalgal growth was accompanied with wastewater pollution removal. The removal amounts of COD, TN, and TP increased continuously along with the increase of proportion of wastewater. Although the degradation ratios of these wastewater parameters at 100% wastewater did not reach the maximum, the undiluted bioethanol wastewater was chosen as the optimum owing to the best lipid production performance. These results demonstrated that the high-concentration bioethanol wastewater had no negative influence on the growth of the microalga, similar with our previous study on yeast [48]. Kothari *et al.* [14] also reported that *Chlorella* could grow well in dairy wastewater with high COD.

The bioethanol wastewater provided suitable nutrients for microalgae. For example, the nitrogen to phosphorus molar ratio of 18.74 (Table 1) was in reasonable agreement with the microalgal molecular formula ($CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$) [26], and trace amounts of growth-promoting elements (such as K, Mg, Cu, Fe, *etc.*) existed in bioethanol wastewater [9]. For these reasons, the undiluted bioethanol wastewater was capable of supporting microalgal heterotrophic growth without the external addition of nutrients, which significantly simplified the operation process and reduced running costs. In this regard, *C. vulgaris* LAM-Q cultured in high-concentration bioethanol wastewater has great potential for biofuel production.

Optimization of Lipid Production

Lipid productivity is regarded as the primary concern for biofuel commercialization. To improve microalgal lipid productivity in the wastewater-based microalgal system, the combined effects of temperature (X_1), initial pH (X_2), and inoculum density (X_3) were investigated by RSM.

Regression model and statistical analysis. By applying multiple regression analysis on the experimental data (Table 2), a second-order polynomial model was established as follows:

$$Y = 153.51 - 33.28X_1 - 8.70X_2 + 31.12X_3 + 1.99X_1X_2 - 15.21X_1X_3 - 6.67X_2X_3 - 38.80X_1^2 - 47.85X_2^2 - 6.46X_3^2 \quad (8)$$

where Y is the response denoted as lipid productivity (mg/l/d), and X_1, X_2, and X_3 are the coded terms for the independent variables. By means of Eq. (8), the predicted lipid productivities matched well with the corresponding experimental values, and exhibited a wide variation ranging from 26.87 to 187.87 mg/l/d (Table 2). It was found that the response was closely related to the mathematical model.

For further explanation in statistics, ANOVA was applied to test the reliability of the regression equation

Table 4. ANOVA results of the regression model.[a]

Source	Sum of squares	df	Mean square	*F*-Value	*p*-Value
Model	35,724.59	9	3,969.40	169.86	<0.0001
X_1	8,863.01	1	8,863.01	379.28	<0.0001
X_2	605.69	1	605.69	25.92	0.0014
X_3	7,748.46	1	7,748.46	331.58	<0.0001
X_1X_2	15.81	1	15.81	0.68	0.4378
X_1X_3	925.06	1	925.06	39.59	0.0004
X_2X_3	178.19	1	178.19	7.63	0.0280
X_1^2	6,338.36	1	6,338.36	271.24	<0.0001
X_2^2	9,641.86	1	9,641.86	412.60	<0.0001
X_3^2	175.85	1	175.85	7.53	0.0288
Residual	163.58	7	23.37		
Lack of fit	91.36	3	30.45	1.69	0.3062
Pure error	72.22	4	18.05		
Total	35,888.17	16			

[a]R^2 = 0.9954, Adj. R^2 = 0.9896, Pred. R^2 = 0.9561, Adeq Precision = 43.425, CV = 4.41%.

(Table 4). The model was highly significant, on account of the high *F*-value (F = 169.86) and the low *p*-value ($p < 0.0001$). The goodness of fit was confirmed by the satisfactory coefficient of determination (R^2 = 0.9954). The Pred. R^2 of 0.9561 agreed with the Adj. R^2 of 0.9896, which implied that the model provided an appropriate explanation for the relationship between the variables and the response. The low coefficient of variation (CV = 4.41%) demonstrated the precision of the optimization experiments. Moreover, the small "lack-of-fit *F*-value" (F = 1.69) and the large "lack-of-fit *p*-value" (p = 0.3062) suggested the absence of significant lack-of-fit relative to the pure error. No abnormality was observed from the diagnoses of residuals (Supplementary Appendix D). Thus, the regression model was statistically sound.

Effects of factors on lipid productivity. The standardized effects of these individual variables and their interactions were illustrated using a Pareto chart. As seen in Fig. 2, these linear, quadratic, and interaction terms, except for X_1X_2, were significant, which was consistent with the results of their *p*-values ($p < 0.05$) (Table 4). The most prominent components in determining lipid productivity were the quadratic terms of initial pH, temperature, inoculum density, and the quadratic term of temperature. Moreover, lipid productivity was greatly influenced by the interactions (X_1X_3 and X_2X_3). In other words, temperature and initial pH had varying influences on microalgal lipid production when using different inoculum densities. In addition, X_3 and X_1X_2 had synergistic effects on lipid productivity, whereas the other model components had antagonistic effects on lipid productivity.

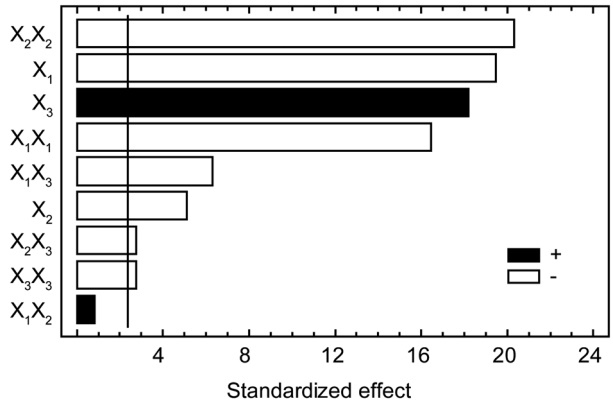

Fig. 2. Pareto chart of the standardized effects for lipid production.
The length of each bar is proportional to the absolute value of the estimated effect. The vertical line indicates the 95% confidence level. If the bar crosses this line, the corresponding term is regarded as a statistically significant parameter.

The visual inspection of 3D surfaces provided direct information about the optimal conditions. Fig. 3A reveals the change of lipid productivity with varying temperature and initial pH at a given inoculum density. Temperature plays a vital role in affecting enzymatic reaction rates and preserving cellular structural integrity, thereby impacting microalgal growth and cellular chemical composition [39]. According to Queiroz *et al.* [34], a relatively high temperature favored microalgal biomass production, whereas it compromised lipid accumulation. It is difficult to achieve high cell concentration and high lipid content simultaneously, because the physiological stress for accumulating vast intracellular lipids always results in the reduction of cell division. However, lipid productivity is determined by both biomass accumulation and lipid content. As indicated in Fig. 3A, the lipid productivity reached the maximum at 22.8°C, and hence the optimal temperature was 22.8°C. Besides this, the pH value of the wastewater medium was also a major factor. Although *C. vulgaris* LAM-Q could survive in a wide range of initial pH from 5 to 9, the best performance in terms of lipid production was observed at the initial pH of 6.7. The reduction in yield at suboptimal pH might result from an energy-dependent system for maintaining suitable intracellular pH [27]. The more energy the pH-maintenance system consumed, the less the available energy for lipid production was. Consequently, the optimal

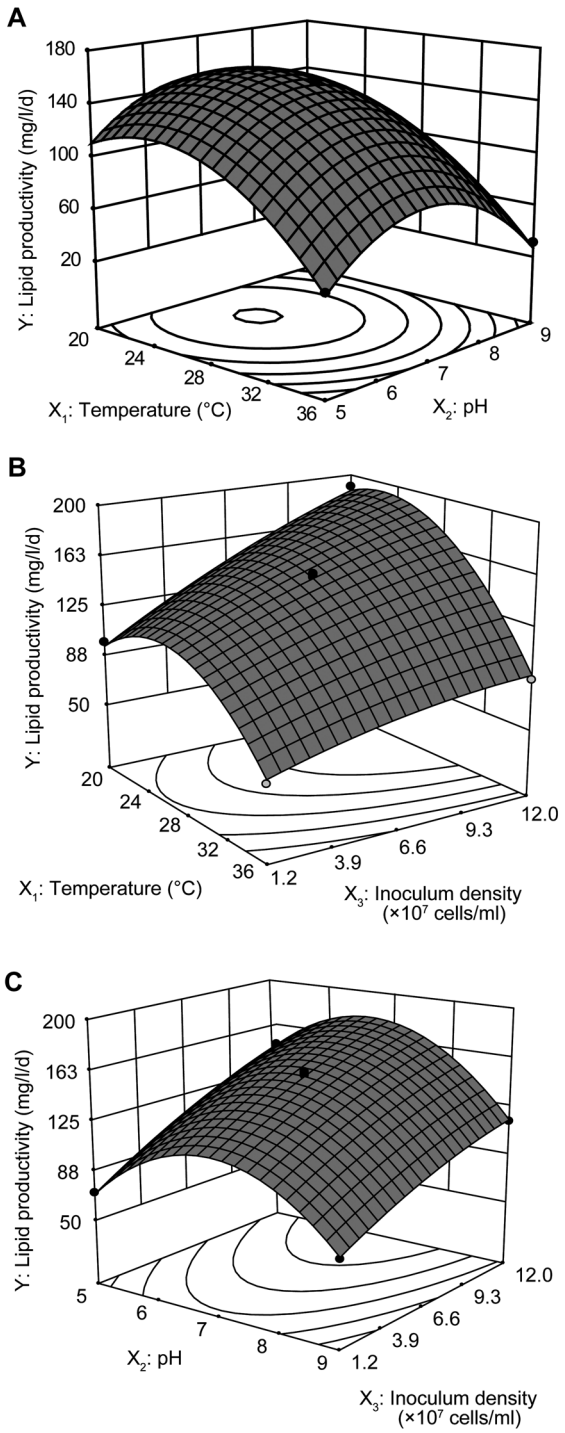

Fig. 3. 3D response surfaces for: (**A**) effects of temperature and initial pH on lipid productivity at a constant inoculum density (6.6×10^7 cells/ml), (**B**) effects of temperature and inoculum density on lipid productivity at a constant initial pH (7), and (**C**) effects of initial pH and inoculum density on lipid productivity at a constant temperature (28°C).

pH of 6.7 was conducive to high lipid productivity. Moreover, Fig. 3B illustrates the effects of temperature and inoculum density on lipid productivity, when the initial pH was fixed at the central level, and Fig. 3C shows the influences of initial pH and inoculum density at a constant temperature. Obviously, the microalga exhibited inoculum-dependent growth in the bioethanol wastewater medium. A larger inoculum density is devoted to achieve higher lipid productivity, as reported by Lu *et al.* [19] and Lopez-Elias *et al.* [18]. There were two reasons for the phenomenon. First, microalgal growth at a low inoculum level was insufficient in the organic-rich wastewater medium [38]. High inoculum density would shorten the lag period and reduce the generation time. Second, microalgae at high inoculum density had superior acclimation ability to the wastewater environment [18]. Similarly, a relatively high inoculum density was adopted in soybean processing wastewater-based microalgal cultivation [37].

Verification of optimization. Under the optimal conditions (temperature 22.8°C, initial pH 6.7, and inoculum density 1.2×10^8 cells/ml), *C. vulgaris* LAM-Q grew well in the bioethanol wastewater, and no obvious lag phase was observed (Fig. 4). After a 3-day culture, the lipid productivity reached 195.96 mg/l/d, which was nearly three times as high as those in municipal wastewater medium (74.5 to 77.8 mg/l/d) [46], and even higher than that in artificial medium (139 mg/l/d) [11].

The optimal surface regions provided by RSM are also interesting, as it is usually difficult to implement a certain

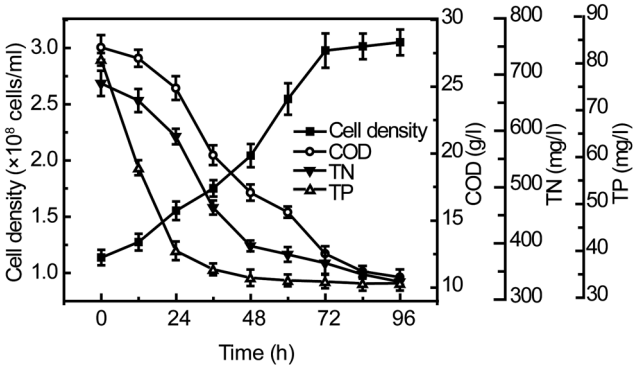

Fig. 4. Microalgal growth curve, and COD, TN, and TP evolutions in the bioethanol wastewater.

The culture conditions were as follows: temperature 22.8°C, initial pH 6.7, inoculum density 1.2×10^8 cells/ml, and rotational speed 130 rpm. Error bars indicate the standard deviation of the mean ($n = 3$).

Table 5. Optimum conditions and verification experiments.

Run	Temperature (°C)	Initial pH	Inoculum density ($\times 10^7$ cells/ml)	Lipid productivity (mg/l/d)	
				Experimental[a]	Predicted
1	22.80	6.70	12.00	195.96 ± 8.12	194.72
2	20.11	6.81	11.67	185.79 ± 7.89	187.38
3	24.83	6.97	9.32	175.23 ± 8.61	177.87
4	26.02	6.20	10.38	177.85 ± 5.76	178.47
5	20.00	7.00	12.00	190.42 ± 9.28	187.87

[a]Data are reported as the mean ± standard deviation (n = 3).

optimal condition in industrial practice. Herein, the lipid productivity of 175 mg/l/d was chosen as the given limit for meaningful application. A series of experiments in the corresponding optimal regions were performed to validate the optimization procedure. As shown in Table 5, these experimental values achieved the desired standard (above 175 mg/l/d), and were quite close to their predicted counterparts. It was concluded that the statistical method of RSM was successfully applied to optimize the lipid production process. After optimization, *C. vulgaris* LAM-Q showed a high production capacity in the bioethanol wastewater.

Fatty Acid Profile

To evaluate the bio-oil quality, the microalgal lipids produced from the bioethanol wastewater were analyzed by GC/MS after methyl esterification (Fig. 5). A predominance of octadecenoic acid (C18:1) and hexadecanoic acid (C16:0) was observed, along with considerable amounts of octadecanoic acid (C18:0) and octadecadienoic acid (C18:2). The four components, which are common fatty acids for biodiesel production, represented over 90% of total fatty acids. Moreover, the fatty acid profile was similar to that of oils obtained from heterotrophic *Chlorella* using glucose feeding, which were able to produce high-quality biodiesel meeting the ASTM biodiesel standard [43]. Accordingly, it was implied that biodiesel with high quality could be produced from the bioethanol wastewater. Furthermore, octadecatrienoic acid (C18:3) accounted for only 4.43%, satisfying the European standard (EN 14214), which specifies a limit of 12%. By using the empirical formula, the lipids could produce biodiesel with a cetane number of 64.92, which was higher than the minimum value of 51 given by the EN 14214 standard [5]. According to Petursson [31], the iodine value of the corresponding biodiesel products (79.89 g I_2/100 g) was well below the upper limit (120 g I_2/100 g) in the EN 14214 standard. These results demonstrated that *C. vulgaris* LAM-Q in the bioethanol wastewater was an ideal candidate for biodiesel production.

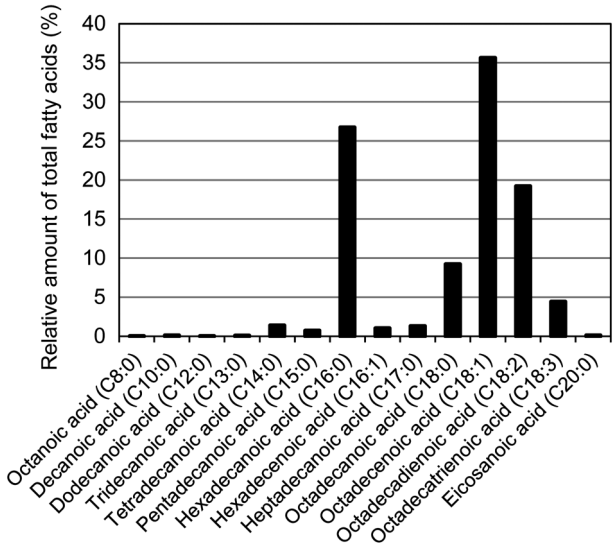

Fig. 5. Fatty acid composition of the microalgal lipids produced from the bioethanol wastewater.

Wastewater Nutrient Removal

Wastewater treatment and lipid production proceeded simultaneously in the integrated system. Wastewater nutrients were assimilated by microalgal cells, thereby reducing the pollutant load of the wastewater. Fig. 4 displays the variations of COD, TN, and TP concentrations in the bioethanol wastewater over microalgal culture time.

The COD concentration was greatly reduced from 27,930 to 10,780 mg/l, implying that the heterotrophic microalga had the ability to utilize organic compounds in the wastewater. Bioethanol wastewater contains reducing sugars, glycerol, organic acids, *etc.*, which are available carbon sources for heterotrophic microalgae [7, 30]. For example, reducing sugars are assimilated through an inducible active hexose/H^+ symport system; glycerol enters

Chlorella cells *via* simple diffusion and then participates in oxidative metabolism; and organic acid molecules (such as acetic acid, citric acid, glycolic acid, and lactic acid) are oxidized inside microalgal cells with the aid of the monocarboxylic/proton transporter protein [30]. At the end of the fermentation, the COD degradation ratio was 61.40%, similar to that in fish processing wastewater with microalgae-containing microbiota (49.1% to 71.1%) [35]. Additionally, the absolute amount of the degraded COD was 17,150 mg/l, much higher than that of the above system (400 to 610 mg/l). However, almost 80% COD of bioethanol wastewater could be biodegraded by methanogens in the anaerobic digestion process, indicating that the conditions of microalgal treatment required further optimization [36].

TN and TP in the bioethanol wastewater were also dramatically consumed by *C. vulgaris* LAM-Q. Microalgae can utilize a variety of nitrogen sources (mainly ammonia, nitrite, nitrate, and simple organic nitrogen compounds) to compose the nitrogen fraction of biomass, accounting for from 1% to more than 10% [35]. These nitrogen sources are usually transformed to ammonium *in vivo*, and then assimilated by the glutamine synthetase enzyme system [30]. Through microalgal treatment, the TN removal efficiency was 51.24%. In addition, phosphorus is another essential element for microbial metabolism, such as energy transfer and biosynthesis. It was observed that the phosphorus uptake rate was higher than the cell growth rate in the first 2 days, and thereafter the phosphorus concentration in the wastewater maintained at a low level (Fig. 4). This was because microalgae are capable of rapidly absorbing excess phosphorus, which is stored as polyphosphate reserves and used later for growth [28]. During microalgal growth, 58.76% of TP was removed from the bioethanol wastewater. Furthermore, the ratio of the removed nitrogen to the removed phosphorus was 7.38, which was in agreement with the optimal nitrogen to phosphorus ratio (6.8 to 10) for freshwater algal growth [41]. In consequence, microalgal cultivation provides an effective approach for bioethanol wastewater treatment and wastewater resource utilization.

Energy Efficiency Evaluation

In order to evaluate the energy efficiency of organic wastewater fermentation, an equation was established to calculate the biomass energetic yield, as a function of biomass yield, lipid content, and substrate COD. In the bioethanol wastewater system, 1.58 g/l of microalgal cells with 10.00% lipids were inoculated, and 3.64 g/l of microalgal biomass with 20.50% lipids was harvested. According to Eq. (7), 19.17% of the energy contained in the bioethanol wastewater was converted into microalgal biomass. In contrast, the biomass energetic yield was only 6.82% when microbial cells grew in organic-rich monosodium glutamate wastewater for lipid production [44]. Additionally, biohydrogen production by anaerobic digestion, another bioethanol wastewater treatment method, had a similar energy conversion efficiency of approximately 20% [13]. Thus, microalgal fermentation had a strong ability to recover waste energy from the bioethanol wastewater with high COD.

In conclusion, high-concentration bioethanol wastewater was successfully used to culture the isolated heterotrophic microalga *C. vulgaris* LAM-Q for biofuel production. In the wastewater-based microalgal system, important operational factors (temperature, initial pH, and inoculum density) were optimized by RSM to enhance lipid production. The highest lipid productivity reached 195.96 mg/l/d at 22.8°C and initial pH of 6.7 with an inoculum density of 1.2×10^8 cells/ml, along with the dramatic reduction of wastewater pollutants (COD, TN, and TP). Moreover, the wastewater-to-lipid fermentation had a high energy efficiency, and the obtained lipids were suitable for biodiesel production. Our work provides valuable insights for industrial designs aiming at combining biofuel production and organic wastewater treatment by microalgal fermentation.

GenBank Accession Number

The 18S rRNA gene sequence (685 bp) of strain LAM-Q was registered in GenBank under the accession number KC253000.

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