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Full Length Research Paper

Improve lipid production by pH shifted-strategy in batch culture of *Chlorella protothecoides*

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Effects of culture broth pH values on cells growth and lipid production were investigated in heterotrophic cultivation of *Chlorella protothecoides*. Based on the phenomenon that lower pH value favors cells growth but retards lipid synthesis and vice versa for higher pH value, a novel pH-shift strategy, optimized via simulating Gauss function, was developed. By controlling pH at 5.0 for first 93 h and switching to 6.5 afterwards, final lipid yield and productivity reached 3.75 g/L and 20.8 mg/L/h, increased by 24.2 and 38.7%, respectively as compared to constant pH 6.0 operations. Moreover, by feeding glucose and aqueous ammonia replacing sulphuric acid (H_2SO4) and NaOH solutions to control pH, maximal lipid yield of 3.96 g/L was achieved, suggesting application of the novel pH-shift strategy for lipid overproduction as being feasible.

Key words: Heterotrophic cultivation, lipid, ph-shift strategy, glucose and *Chlorella protothecoides.*

INTRODUCTION

The depletion of known petroleum reserves makes renewable energy sources more attractive. Biodiesel, as one of promising renewable transportation fuels, was considered as an ideal candidate for fossil fuel (Antolin et al., 2002; Vicente et al., 2004). Bio-diesel is produced mainly from vegetable oils, animal fats and microalgae lipid (Andrade et al., 2011). Compared to biodiesel production from vegetable oils and animal fats, a renewed interest has arisen in recent years towards producing biodiesel from microalgae due to its easy cultivation and relatively lower production cost (Sharma et al., 2008; Bastianoni et al., 2008). Microalgae of *Chlorella vulgaris* and *Chlorella protothecoides*, with the characteristics being cultured, photoautotrophically and heterotrophically are currently used to accumulate substantial intracellular lipids (Chisti, 2007; Chih and Wen, 2009). Compared to autotrophic way, heterotrophic cultivation of microalgae was considered an efficient alternative due to higher biomass and productivity (Han et al., 2006; Miao and Wu, 2006).

Being an intracellular product in microalgae, a combination of high biomass and high lipid content can lead to maximal lipid production. During the past decades, certain methods or strategies were adopted to enhance lipid production (Courchesne et al., 2009; Stephenson et al., 2010). However, main researches were focused on enhancing intracellular lipid content by biochemical engineering approaches, creating physiological stresses to channel metabolic fluxes to lipid accumulation. For instance, nitrogen deprivation (Tornabene et al., 1983), silicon deficiency (Lynn et al., 2000), phosphate and iron limitation (Khozin-Goldberg and Cohen 2006; Liu et al., 2008) and even high salinity (Rao et al., 2007) were used to enhance intracellular lipid content in algae.

It was evident that lipid production cannot be maximized by improving intracellular lipid content alone. So how to enhance cells concentration and lipid content simultaneously is the key to maximizing lipid production

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in heterotrophic culture of microalgae. As it is known, besides nutrition conditions, environmental factors (temperature, pH, and dissolved oxygen) also significantly affect cells growth and lipid synthesis.

For batch lipid production by microalgae, the optimal conditions for cells growth and lipid formation may be quite different. Moreover, lipid formation rate, yield and productivity varied significantly with medium compositions and environmental parameters of pH, temperature and dissolved oxygen (DO). To obtain high lipid yield and productivity, it is vital importance to optimizing environmental conditions for cells growth and lipid formation during batch cultivation besides providing abundant nutrition needed for cell growing and lipid synthesis. Unfortunately, to date, little was known about the influences of environmental factors (temperature, pH, and dissolved oxygen) on cells growth and lipid formation in heterotrophic cultivation of microalgae for lipid production.

Accordingly, effects of broth pH values on cells growth and lipid formation in batch culture of *C. protothecoides* were explored in this work. Based on the results that the optimal pH values for cells growth and lipid formation differed, a novel two-stage pH-shift strategy, optimized by simulating Gauss function, was developed to enhance lipid production. Furthermore, by feeding glucose and aqueous ammonia replacing sulphuric acid (H_2SO_4) and NaOH solutions to control pH, a maximal lipid production was achieved.

MATERIALS AND METHODS

Microorganism and culture medium

Microalgae strain used in this study was *C. protothecoides* provided by Charles University (Prague, Czech Republic). Both seed and batch culture mediums were consisted of Bold's basal media (BBM) supplemented with (g/l): glucose 10, yeast extract 2.0, glycine 1.0 at pH 6.0. Main components used in BBM were (g/l): $NaNO_3$ 0.25, $MgSO_4.7H_2O$ 0.075, NaCl 0.025, $K_2HPO_4.3H_2O$ 0.075, KH_2PO_4 0.175, $CaCl_2.2H_2O$ 0.025, and trace elements were (10^{-3}g/l): $ZnSO_4.7H_2O$ 8.82, $MnCl_2.4H_2O$ 1.44, MoO_3 0.71, $CuSO_4.5H_2O$ 1.57, $Co(NO_3)_2.6H_2O$ 0.49, H_3BO_3 11.42, EDTA 50.0, KOH 31.0 and $FeSO_4.7H_2O$ 4.98.

Batch cultivation in 5 L fermentor

Batch fermentation of lipid in 5 L fermentor by BBM supplemented with (g/L) glucose 10, yeast extract 2.0, and glycine 1.0 in 5 L fermentor was carried out. 10% (v/v) seed culture was inoculated into a 5 L fermentor with a working volume of 3 L. The pH was controlled automatically by adding 3 mol/l H_2SO_4 or 3 mol/L NaOH solutions. Agitation speed and temperature were controlled at 200 rpm and 30°C, respectively.

Feeding glucose and aqueous ammonia to control medium pH

Based on the two-stage pH-shift strategy, glucose solution of 500 g/l and aqueous ammonia (5 M) were used to replace H_2SO_4 and NaOH solutions for automatically controlling pH at 5.0 for first 93 h and 6.5 afterwards.

Calculation of specific rates of cells growth, glucose consumption and lipid production

Specific cell growth rate (μ_{cell}) was determined from the slope of semilogarithmic plot of cell density versus cultivation time.

$$\mu_{cell} = \frac{1}{x}\frac{dx}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta x}{\Delta t}$$

Specific glucose consumption rate (q_s) was determined from the slope of semilogarithmic plot of residue glucose concentration versus cultivation time.

$$q_S = \frac{1}{s}\frac{ds}{dt} = \frac{1}{s}\lim_{\Delta t \to 0}\frac{\Delta s}{\Delta t}$$

Specific lipid production rate (q_p) was determined from the reciprocal of cell density and lipid yield versus cultivation time.

$$q_p = \frac{1}{x}\frac{dp}{dt} = \frac{1}{x}\lim_{\Delta t \to 0}\frac{\Delta p}{\Delta t}$$

Analytical methods

Cell concentration was determined after drying cells at 65°C to a constant weight. Culture broths were centrifuged at 8000 rpm for 10 min and cells were washed twice with distilled water and freeze dried. Dried cells were then pulverized into powder in the mortar and lipid was extracted by soxhlet method using n-hexane. Intracellular lipid content was obtained by the ratio of total lipid yield to cell concentration. Total nitrogen in medium was analyzed using High Temperature TOC/TNb Analyzer LiquiTOC II (Elementar Analysensysteme GmBH, Hanau, Germany). Glucose residue in medium was determined according to Miller (1959).

All experiments were repeated twice and average results were used for analysis.

RESULTS

Cells growth and lipid production without pH control

To ascertain suitable pH scopes for cells growth and lipid synthesis, heterotrophic cultivation of *C. protothecoides* by BBM supplemented with (g/l) glucose 10, yeast extract 2.0, and glycine 1.0 without pH control was carried out in 5 L fermentor. Status of cells growth and lipid production at the natural pH condition (initial broth pH was adjusted to 6.0 and not controlled afterwards) was shown in Figure 1. According to biomass increment and lipid accumulation, the whole process was separated into two phases of cells growth and lipid synthesis. Cells growth almost stopped at 120 h and biomass of 5.06 g/L was obtained and intracellular lipid yield was 2.03 g/L at this time point. Afterwards, lipid yield increased slowly and a maximal

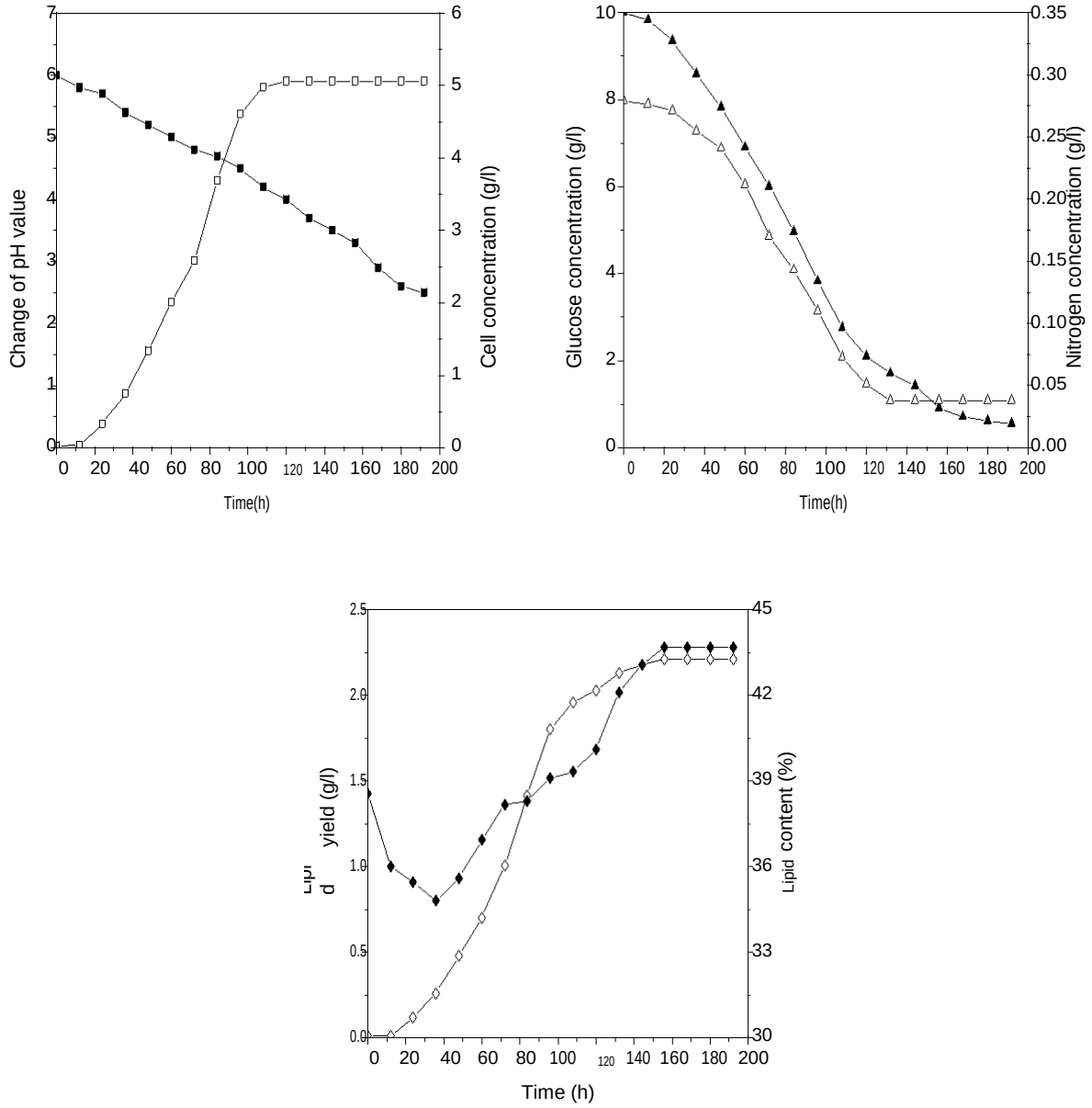

Figure 1. Time course of cells growth and lipid production without pH control. ■, changes of pH; □, dry cell weight; ▲, glucose concentration; △, nitrogen concentration; ◇, lipid yield; ◆, lipid content.

yield of 2.21 g/L was achieved at 200 h. Meanwhile, results indicated that glucose and nitrogen were not consumed completely at the end of cultivation, suggesting cessation of cells growth and lipid synthesis was not caused by nutrients deficiency. Apparently, the pH value in the broth decreased gradually and reached 2.5 or so after 200 h cultivation. It was noted that cells growth became slowly as pH decreased to about 4 at 120 h cultivation, and only 0.18 g/L lipid was synthesized during 120 to 200 h, indicating inhibition of cells growth and lipid production can be attributed to the lowered pH value but not nutrients deficiency. Accordingly, we further investigated the effects of differed pH values on cells growth and lipid production.

Effects of varied pH values on cells growth and lipid production

Based on above results that inhibition of cells growth and lipid synthesis occurred as pH lowered than 4.0, the pH values at the range of 4.0 to 7.0 were adopted. As shown in Figure 2, cells growth was notably affected by pH values. For instance, at pH 5.0, cells grew very quickly and final biomass reached 6.26 g/l at 100 h, which was higher than that at other pH values. Meanwhile, profiles

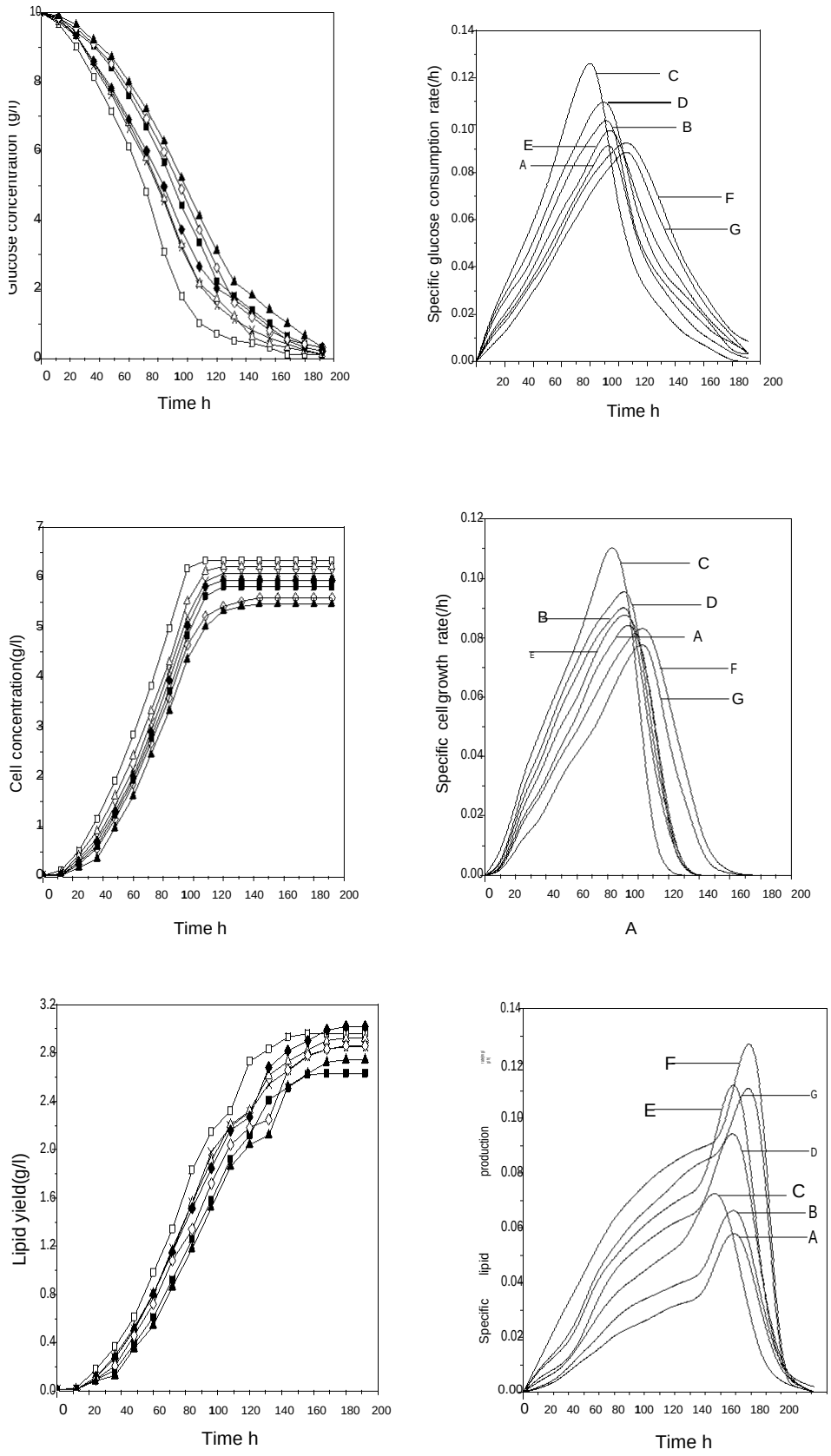

Figure 2. Time course of glucose consumption, cells growth and lipid production at different pH values ■ and A, pH 4.0; × and B, pH 4.5; □ and C, pH 5.0; △ and D, pH 5.5; ◆and E, pH 6.0; ◇ and F, pH 6.5; ▲ and G, pH 7.0.

Table 1. Effect of different pH shift time on lipid production.

Shift time (h)	Lipid concentration (g/l)
0	2.86
20	2.94
40	3.06
60	3.23
80	3.46
100	3.68
120	3.41
140	3.04
160	2.96

Note: The shift time means the point at which pH value changes from 5 to 6.5.

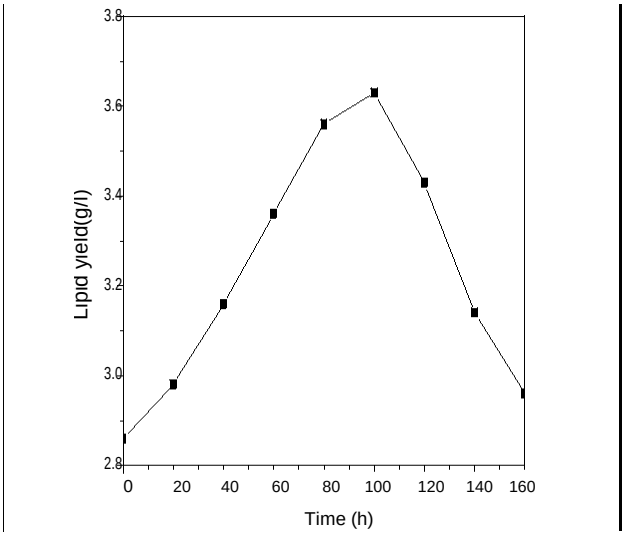

Figure. 3. Lipid production with differed pH shift time ■, lipid concentration.

of μ_{cell} at different pH values showed that μ_{cell} at pH 5.0 was higher than at other pH values before 96 h, documenting the highest biomass being achieved with pH 5.0. Moreover, at pH 5.0, residue glucose was very low at 100 h and its specific consumption rate (q_s) reached the highest at 70 h, also demonstrating the maximal dry cell weight (DCW) being achieved. In brief, it was suggested that the optimal pH for cells growth was 5.0.

In addition, maximal lipid yield (g/L) reached 2.61, 2.85, 2.96, 2.92, 3.02, 2.86 and 2.75 with pH at 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, respectively. It was indicated that the impact of medium pH values on total lipid yield was insignificant. Furthermore, it was shown that profiles of q_p at varied pH values had similar tendencies. For example, with cultivation extending, q_p increased gradually exhibiting a climacteric peak after cells growth stopped and then decreased to a lower level. However, the maximal q_p, duration of reaching peak and decreasing rate, varied at different pH values. It was observed that at first 120 h and after 140 h, q_p at pH 6.5 was the highest. At pH 6.0, nitrogen was consumed at 120 h, and a rapid increase in q_p was observed. As a result, lowered q_p at pH 6.5 during 120 to 140 h was observed as compared to that at pH 6.0. Accordingly, by comprehensively analyzing q_p at various pH values, we can reasonably believe that the optimal pH for lipid formation should be 6.5. As the optimal pH valves for cells growth and lipid formation differed, cell concentration and intracellular lipid content cannot reached the highest point simultaneously with single pH value operation, thus leading to insignificant change in total lipid yield.

Concisely, as the optimal pH for cells growth (pH at 5.0) and lipid formation (pH at 6.5) varied, at early cultivation phase, it is appropriate to control pH at lower value to favor cells growth, and at stationary phase, higher pH was suitable to maintain high q_p for lipid overproduction. Consequently, a maximal lipid yield will be obtained provided that control pH at 5.0 for cells growth and 6.5 for lipid synthesis.

Enhancing lipid production by two stages pH-shift strategy

Based on the results, we reasonably speculate that broth pH can be controlled at 5.0 to favor cells growth and 6.5 benefit lipid formation. However, as it is known that the ultimate aim for lipid production in heterotrophic growth of *C. protothecoides* was to maximize lipid yield and minimize cultivation times simultaneously. So, how to determine the shift time for pH values changing from 5.0 to 6.5 is great importance to achieving maximal lipid yield and productivity. In general, shift time for pH value changing was empirically determined (Zheng et al., 2002). Accordingly, to achieve the highest lipid yield, a peak function of Gauss was adopted in this study. Initially, lipid yields with different shift time for pH values changing from 5.0 to 6.5 were determined and shown in Table 1. And then based on the experiments data, lipid yield versus shift time was plotted. As indicated in Figure 3,

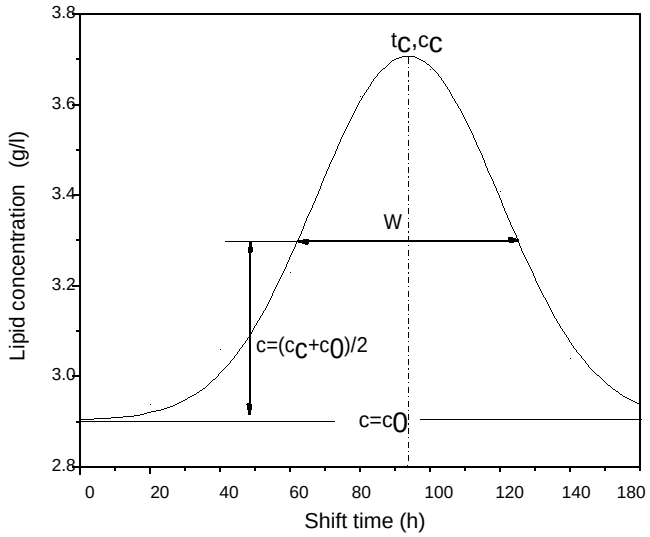

Figure 4. Optimization of lipid production by simulating Gauss function.

Table 2. Regression coefficients for lipid yield by Gauss function.

Parameter	Value	Error
c_0	2.90418	0.02835
t_c	93.51097	1.25719
W	52.89523	3.58544
A	53.33111	4.51204
R^2	0.98142	

lipid yield improved gradually with an increase in shift time and reached the highest point at 100 h followed by a gradual decrease. As profile tendency of lipid yield in Figure 3 was very similar to that of Gauss function, it was further simulated by Gauss function using software origin 8.0 and the simulated one was indicated in Figure 4.

The formula for Gauss (t, c_0, t_c, W, A) function is shown in Equation (1):

$$c = c_0 + \frac{A}{W\sqrt{\pi/2}} e^{-2\frac{(t-t)^2}{}}$$

Where: c_0 is the baseline offset; t_c is the center of the peak; W equals 0.849 the width of the peak at half height; A is the area under the peak. Regression coefficients for lipid yield were indicated in Table 2, and lipid yield can be obtained according to these coefficients. Correlation coefficient of regression models of lipid yields was 98.14%, which means a high fitting accuracy.

In above equation, c represented lipid yield and its maximal yield of 3.71 g/L was calculated according to the parameters in Table 2. To test the accuracy of maximal lipid yield achieved by simulated Gauss function, the pH-shift experiment, in which pH was controlled at 5.0 for first 93 h and switched to 6.5 afterwards, was further operated in 5 L fermentor. As indicated in Figure 5, after 180 h cultivation, final lipid yield reached 3.75 g/L, which was about 1% higher than the simulated one, indicating the effectiveness of optimized strategy. More importantly, by adopting this strategy, lipid productivity was also significantly improved.

In short, by controlling pH at 5.0 for first 93 h and switching to 6.5 afterwards, final lipid yield and productivity reached 3.75 g/L and 20.8 mg/L/h, increased by 24.2 and 38.7%, respectively as compared to constant pH 6.0 operation.

Feeding glucose and aqueous ammonia to adjust pH for lipid overproduction

As it is known, in heterotrophic growth of *C. protothecoides* for lipid production, at cells growth phase, glucose was degraded to pyruvate followed by citric acid cycle to carbon dioxide (CO_2). As a result, only NaOH

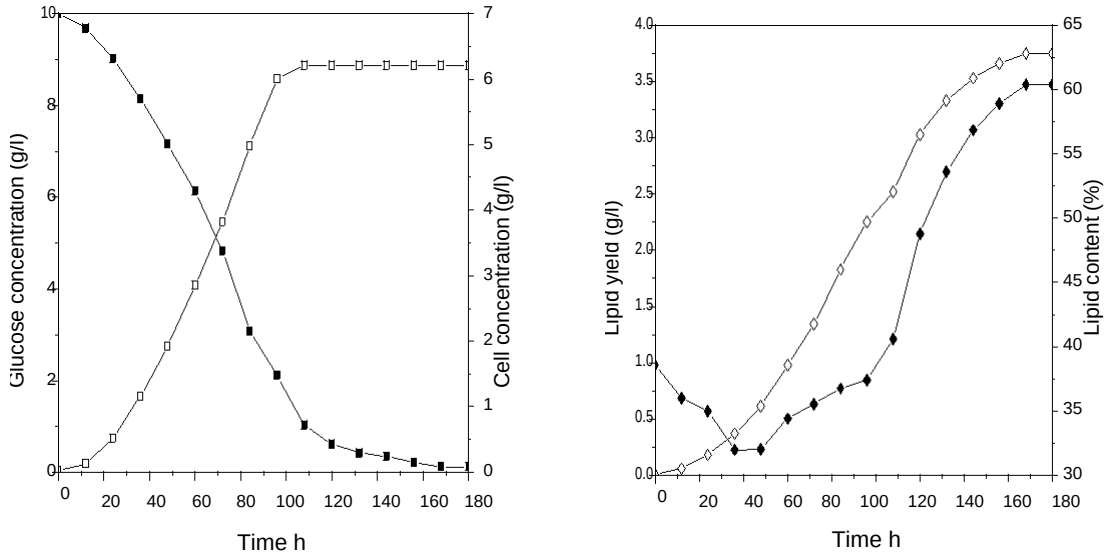

Figure 5. Enhanced lipid production with two-stage pH-shift strategy ■, glucose concentration; □, cell concentration; ◇, lipid yield; ◆, lipid content.

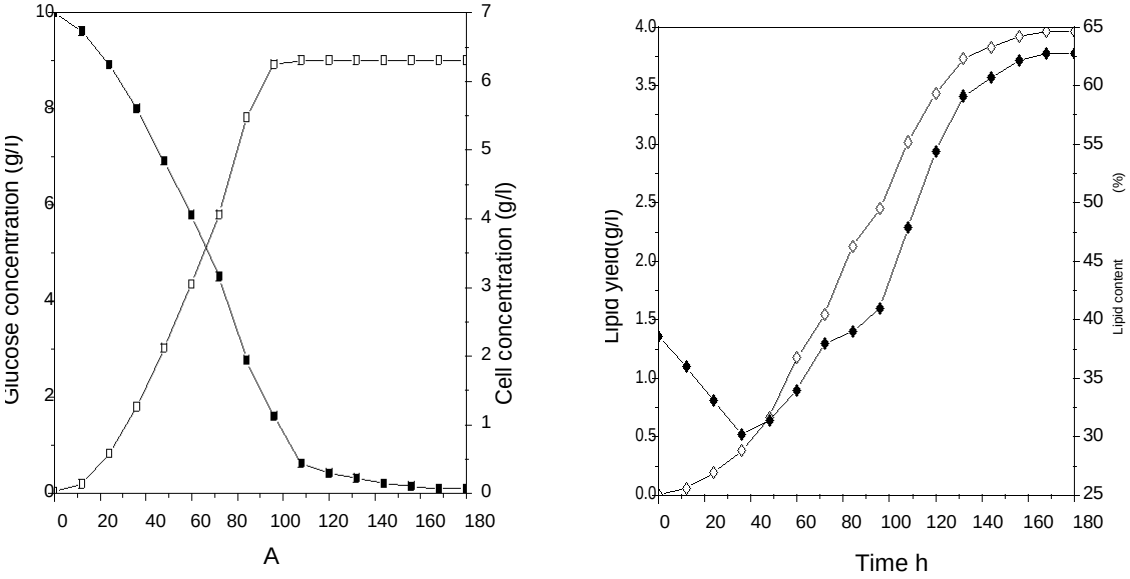

Figure 6. Lipid overproduction by feeding glucose and aqueous ammonia in pH-shift strategy ■, glucose concentration; □, cell concentration; ◇, lipid yield; ◆, lipid content.

solution was needed to adjust broth pH at 5.0. In comparison, at the stationary phase, with cessation of cells growth and exhaustion of glucose, broth pH value will be inevitably increased. So H_2SO_4 solution needed to be added to control broth pH value at 6.5. Accordingly, to promote cells growth and lipid production as well as control medium pH value, glucose and aqueous ammonia were used to replace H_2SO_4 and NaOH solutions in the whole cultivation. As indicated in Figure 6, cells growth stopped increasing at 96 h and maximal cells concentration of 6.32 g/L was achieved. Afterwards, lipid continued to increase and final lipid yield reached 3.96 g/l after 180 h cultivation. Evidently, by feeding glucose and aqueous ammonia to adjust pH, lipid production increased by 31.3 and 5.6% as compared to constant pH 6.0 operations and pH shift strategy (without glucose and aqueous ammonia addition). It was indicated that glucose and aqueous ammonia feeding at least shows two functions

of promoting lipid production and adjusting pH.

DISCCUSSION

Microalgae have been suggested as very good candidates for biodiesel production because of rapid growth and higher lipid yield (Dote et al., 1994; Minowa et al., 1995). Some microalgae, such as *C. vulgaris* and *C. protothecoides*, are exceedingly rich in lipid and currently used for lipid production on larger scale. Lipid yield can be improved by increasing cell concentration and lipid content. However, increased biomass can inevitably result in lowered intracellular lipid content. Alternatively, some methods or strategies, such as nitrogen deprivation (Tornabene et al., 1983) and iron limitation (Liu et al., 2008), were adopted to enhance lipid synthesis. Besides status of nutrition abundance, environmental conditions, such as dissolved oxygen, temperature and pH, are also very important for cells growth and lipid production. It was known that the major functions of plasma membrane were to regulate what comes in and goes out of cells. Status of external pH can determine complex physiological parameters such as membrane permeability and cell morphology. So a change in broth pH will affect membrane osmosis to certain ions and thus substances absorption. Many researches have investigated the effects of broth pH values on growth kinetics of microorganisms and concluded that pH was the important environmental factors affecting cells growth and products formation (Amanullah et al., 2001). For example, the activities of enzymes catalyzing all metabolic reactions were significantly affected by broth pH, thus further influencing cells growth and products synthesis (Elibol, 2002). Unfortunately, effects of broth pH values on cells growth and lipid production in heterotrophic cultivation of microalgae were little known.

As an intracellular product, lipid production was closely related to biomass and lipid content. Broth pH, as a vital environmental factor, significantly affects cells growth and lipid formation. Accordingly, how to maximize lipid production by optimizing broth pH is the point we tried to explore in this research. Based on the results that the optimal pH values for cells growth and lipid formation differed, a pH-shift strategy, optimized via simulating Gauss function, was developed. As a result, lipid yield was significantly enhanced by this novel strategy.

Furthermore, as it is known, for heterotrophic lipid production, the energy Adenosine Tri-Phosphate (ATP) and precursor substances of acetyl-CoA and glycerol are greatly needed. It was suggested that cells growth and lipid production are related to glucose availability. In this study, the whole cultivation process was divided into two phases of cells growth and lipid synthesis. At cell growth phase, these precursors and ATP can satisfy cells growth and lipid formation. However, with glucose degradation and generation CO_2, broth pH will decrease correspondingly and NaOH must be added. In comparison, at stationary stage, with glucose exhaustion, generation of precursors and ATP will be ceased. Meanwhile, broth pH will increase inevitably. Accordingly, to adjust broth pH as well as generate ATP and precursors for lipid production, glucose and aqueous ammonia were fed instead of sulphuric acid (H_2SO_4) and NaOH solutions to maximize lipid production. As a result, lipid production was significantly enhanced, suggesting application of the novel pH-shift strategy for lipid overproduction as being feasible in batch culture of *C. protothecoides*.

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